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SCIENTIFIC OPINION

Scientific Opinion on the re-evaluation of aspartame (E 951) as a food additive[1]

EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS)[2, 3]

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

The EFSA ANS Panel provides a scientific opinion on the safety of aspartame (E 951). Aspartame is a sweetener authorised as a food additive in the EU. In previous evaluations by JECFA and the SCF, an ADI of 40 mg/kg bw/day was established based on chronic toxicity in animals. Original reports, previous evaluations, additional literature and data made available following a public call were evaluated. Aspartame is rapidly and completely hydrolysed in the gastrointestinal tract to phenylalanine, aspartic acid and methanol. Chronic and developmental toxicities were relevant endpoints in the animal database. From chronic toxicity studies in animals, a NOAEL of 4000 mg/kg bw/day was identified. The possibility of developmental toxicity occurring at lower doses than 4000 mg/kg in animals could not be excluded. Based on MoA and weight-of-evidence analysis, the Panel concluded that developmental toxicity in animals was attributable to phenylalanine. Phenylalanine at high plasma levels is known to cause developmental toxicity in humans. The Panel concluded that human data on developmental toxicity were more appropriate for the risk assessment. Concentration-response modelling was used to determine the effects of aspartame administration on plasma phenylalanine using human data after phenylalanine administration to normal, PKU heterozygote or PKU homozygote individuals. In normal and PKU heterozygotes, aspartame intakes up to the ADI of 40 mg/kg bw/day, in addition to dietary phenylalanine, would not lead to peak plasma phenylalanine concentrations above the current clinical guideline for the prevention of adverse effects in fetuses. The Panel concluded that aspartame was not of safety concern at the current aspartame exposure estimates or at the ADI of 40 mg/kg bw/day. Therefore, there was no reason to revise the ADI of aspartame. Current exposures to aspartame - and its degradation product DKP - were below their respective ADIs. The ADI is not applicable to PKU patients.

KEY WORDS

aspartame, E 951, methanol, sweetener, EINECS number 245-261-3

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[2] Panel members: Fernando Aguilar, Riccardo Crebelli, Birgit Dusemund, Pierre Galtier, David Gott, Ursula Gundert-Remy, Jürgen König, Claude Lambré, Jean-Charles Leblanc, Alicja Mortensen, Pasquale Mosesso, Agneta Oskarsson, Dominique Parent-Massin, Martin Rose, Ivan Stankovic, Paul Tobback, Ine Waalkens-Berendsen, Rudolf Antonius Woutersen and Matthew Wright. Correspondence: ans@efsa.europa.eu

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SUMMARY

Following a request from the European Commission, the Panel on Food Additives and Nutrient Sources added to Food (ANS) of the European Food Safety Authority (EFSA) was asked to deliver a scientific opinion on the re-evaluation of aspartame (E 951) as a food additive.

Aspartame (E 951) is a sweetener authorised as a food additive in the EU that was previously evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), the EU Scientific Committee for Food (SCF) and the European Food Safety Authority (EFSA). Both JECFA and SCF established an Acceptable Daily Intake (ADI) of 40 mg/kg body weight (bw)/day.

The Panel based its evaluation on original study reports and information submitted following public calls for data, previous evaluations, and additional literature that has become available until the end of the public consultation on the draft Scientific Opinion on the re-evaluation of aspartame (E 951) as a food additive (15th February 2013). The Panel also evaluated literature published after the end of the public consultation, until 15th November 2013 (EFSA ANS Panel, 2013). The Panel noted that although many of the studies were old and were not performed according to current standards (e.g. Good Laboratory Practice (GLP) and Organisation for Economic Co-operation and Development (OECD) guidelines), they should be considered in the re-evaluation of the sweetener as long as the design of such studies and the reporting of the data were considered appropriate. In its re-evaluation of aspartame, the Panel also considered the safety of its gut hydrolysis metabolites methanol, phenylalanine and aspartic acid and of its degradation products 5-benzyl-3,6-dioxo-2-piperazine acetic acid (DKP) and β-aspartame, which may be present in the sweetener as impurities.

Aspartame (E 951) is a dipeptide of L-phenylalanine methyl ester and L-aspartic acid bearing an amino group at the α-position from the carbon of the peptide bond (α-aspartame). The major hydrolysis and degradation products of aspartame are L-phenylalanine, aspartic acid, methanol and DKP. DKP is formed through the intramolecular reaction of the primary amine with the methyl ester group of aspartame. β-Aspartame is a non-sweet isomer of α-aspartame.

Specifications have been defined in the European Commission Regulation (EU) No 231/2012 and by JECFA.

Studies in experimental animals and humans have shown that after oral ingestion, aspartame is fully hydrolysed within the gastro-intestinal tract. The products resulting from these reactions are methanol and the amino acids aspartic acid and phenylalanine. Hydrolysis of aspartame releases a corresponding 10 % by weight of methanol. Due to the very efficient hydrolysis in the gastro-intestinal tract the amount of intact aspartame that enters the bloodstream has been reported as undetectable in several studies conducted in various species, including rats, dogs, monkeys and humans. Further studies conducted in monkeys and pigs have also shown that the potential intermediate metabolite, phenylalanine methyl ester, is rapidly broken down to phenylalanine and methanol in the gastro-intestinal tract. Therefore, the Panel considered that phenylalanine, aspartic acid and methanol are absorbed and enter normal endogenous metabolic pathways.

The acute toxicity of aspartame was tested in mice, rats, rabbits and dogs and was found to be very low. Similarly, sub-acute and sub-chronic studies did not indicate any significant toxic effects in rats, mice or dogs.

Aspartame has been tested for genotoxicity in a number of *in vitro* and *in vivo* studies. The Panel concluded that in mammalian systems, apart from a valid UDS study, which was negative, no conclusion could be drawn at the gene and chromosomal level, as no studies dealing with these endpoints were available. However, the Panel considered that the weight-of-evidence was sufficient to conclude that aspartame was not mutagenic in bacterial systems. *In vivo*, the majority of investigations on genotoxicity reported negative findings. Equivocal findings were only described in one NTP (US National Toxicology Program) study, positive in female but not in male p53 haploinsufficient mice. In

two other transgenic mouse strains the genotoxicity results were negative. The available *in vitro* data did not indicate a direct genotoxic activity of aspartame that might predispose to a site of first contact effect *in vivo*. Overall, the Panel concluded that the available data do not indicate a genotoxic concern for aspartame.

The results from three chronic toxicity and carcinogenicity studies in rats and one in mice revealed no aspartame-related increase in any type of neoplasms at all doses tested. The incidences of intracranial neoplasms observed in some studies were within the range of spontaneous brain tumours observed in the strain of rats used. In the rat studies, the highest doses tested (4000 or 8000 mg aspartame/kg bw/day) produced minor renal changes, considered by the Panel to be of minimal toxicological significance. A dose-dependent depression of body weight gain at 2000 and 4000 mg/kg bw/day correlating with decreased feed consumption was reported in one study. Overall, the Panel derived a no observable adverse effect level (NOAEL) of 4000 mg/kg bw/day from the four studies.

Furthermore, the NTP carried out several 9-month carcinogenicity studies with aspartame in genetically modified Tg.AC hemizygous, p53 haploinsufficient and Cdkn2a deficient mice. The Panel agreed that there was no evidence of treatment-related neoplastic or non-neoplastic lesions in any of these studies.

Since the last evaluation of aspartame by the SCF in 2002, two new long term carcinogenicity studies on aspartame in rats and one in mice were published by the European Ramazzini Foundation. The two rat studies have already been evaluated by the former Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) and the ANS Panel and were considered to have methodological flaws. In addition to a high background incidence of chronic inflammatory changes in the lungs and other vital organs and tissues there is uncertainty about the diagnoses of some tumour types, which rendered the validity of the findings questionable. Moreover, EPA has recently concluded that many of the malignant neoplasms and the lymphoid dysplasias diagnosed in the studies from the European Ramazzini Foundation were hyperplasias related to unknown chronic infection in the animals and not related to aspartame intake. Furthermore, in the mouse study, the ANS Panel noted that the hepatic and pulmonary tumour incidences reported fell within the institute's own historical control ranges for spontaneous tumours.

The available reproductive and developmental toxicity studies on aspartame comprised nine studies: an embryotoxicity and teratogenicity study performed in the mouse, a two-generation reproduction toxicity study in the rat, five peri- and postnatal developmental studies in the rat, a reproductive performance and developmental study in the rat and an embryotoxicity and teratogenicity study in the rat. In addition, eight embryotoxicity and teratogenicity studies were performed in the rabbit, four with administration of aspartame by diet and four by gavage.

From the rodent studies, the Panel identified a NOAEL of 5700 mg/kg bw/day, the highest dose level tested, in a developmental toxicity study in the mouse. The results of the reproductive and developmental toxicity studies in rats indicated NOAELs that ranged from 2000 to 4000 mg aspartame/kg bw/day. The Panel noted that developmental changes in pup body weight were observed at birth in studies at the dose of 4000 mg aspartame/kg bw/day and considered, these could be attributed to a combination of malnutrition and nutritional imbalance due to excessive exposure to phenylalanine derived from aspartame. In support of this hypothesis, the Panel noted that administration of a dose of L-phenylalanine equimolar to aspartame led to a similar decrease in maternal and pup body weight, as observed in a concurrent aspartame group.

Several reproductive and developmental toxicity studies performed in rabbits, where aspartame was administered via the diet or by gavage, were available to the Panel. Overall, the Panel considered that the data from these studies were confounded both by the decrease in feed intake (when aspartame was administered via the diet or by gavage), or the poor health of the animals, and, in many cases, by the number of deaths of pregnant rabbits in the treated groups possibly related to misdosing during gavage treatment. In one particular study with aspartame, pregnant rabbits were also dosed by gavage

with L-phenylalanine and L-aspartic acid at dose levels equimolar to the top dose of 2000 mg aspartame/kg bw tested. A decrease in feed consumption was observed in the high aspartame dose and the L-phenylalanine-treated animals in this study and a significant body weight loss in the high aspartame dose was reported. Maternal toxicity and growth reduction were observed in the high dose aspartame group and to a lesser extent in the L-phenylalanine group compared to the controls. Mean fetal body weight and length were significantly reduced in both the high aspartame group and the L-phenylalanine group animals and a significantly higher rate of total (major and minor) malformations in the 2000 mg aspartame/kg bw/day group animals as compared to the concurrent control group was reported. The Panel considered the possibility that, in addition to a reduced feed intake by the mothers and gastrointestinal disturbances, exposure to high levels of aspartame-derived phenylalanine may be in part responsible for these effects in the high dose aspartame group because similar effects, though less severe, were seen in the phenylalanine group. Based on the above considerations the Panel identified a NOAEL of 1000 mg aspartame/kg bw/day for maternal (weight loss) and developmental toxicity (weight loss and malformations).

The Panel noted there was no epidemiological evidence for possible associations of aspartame with various cancers in the human population.

A large prospective cohort study in Denmark found no consistent association between the consumption of artificially sweetened beverages (but not with aspartame specifically) during pregnancy and the diagnosis of asthma or allergic rhinitis in children.

Another analysis of the same cohort showed a small but significantly elevated risk of medically induced pre-term delivery in women with higher reported consumption of artificially sweetened drinks. However, another prospective study in Norway found that the association of pre-term delivery with artificially sweetened soft drinks was much weaker and barely discernible, and applied more to spontaneous than medically induced deliveries and was exceeded by an association with consumption of sugar-sweetened soft drinks.

Methanol is a metabolite of aspartame and is subject to significant first pass metabolism. The main route of metabolism of methanol proceeds by stepwise oxidation via formaldehyde to formate and then to carbon dioxide. Formate can also enter the one-carbon metabolic pool. Since some authors have suggested that methanol is responsible for the potential carcinogenicity and toxicity of aspartame, the Panel evaluated the available toxicological information on methanol.

The Panel considered the database on the genotoxicity of methanol and concluded that the data set was limited but that the available reliable *in vitro* and *in vivo* data did not indicate a genotoxic potential for methanol.

The oral studies on chronic toxicity and carcinogenicity of methanol are limited to a mouse study and a rat study. Overall, the Panel concluded that the mouse study was inadequate for the assessment of the carcinogenic potential of methanol and that the rat study was not suitable for the cancer risk assessment of methanol.

The reproductive and developmental toxicity database of methanol is limited. As oral studies available on methanol performed at high dose levels (4000 or 5000 mg/kg bw/day) did not allow the Panel to identify a NOAEL for reproductive and developmental toxicity for methanol by oral exposure, the Panel calculated the oral NOAEL using available data from animals exposed to methanol via inhalation. The Panel identified NOAECs of 1300 mg methanol/m^3 in mice and 6500 mg methanol/m^3 in rats that were exposed to methanol via the inhalation route. Based on these NOAECs, the Panel calculated oral NOAELs for mice and rats of approximately 560 and 2070 mg/kg bw/day, respectively.

The Panel considered the NOAEL of 560 mg/kg bw/day as the most conservative taking into account that the developmental effects observed in the mouse study tend to disappear as the pups grow. The

Panel noted that the calculated NOAELs for methanol by oral exposure are 140 and 515-fold higher than the maximum amount of methanol that could be released when aspartame is consumed at the ADI.

The Panel concluded that the data on reproductive and developmental toxicity did not suggest that there was a risk from methanol derived from aspartame at the current exposure estimates or at the ADI of 40 mg/kg bw/day.

In addition, the Panel concluded that, based on recent measurements of basal levels of formaldehyde in blood and on the modelling of its biological turnover and steady state concentration in cells, formaldehyde formed from aspartame-derived methanol was not of safety concern at the current exposure estimates or at the ADI of 40 mg/kg bw/day.

Another aspartame metabolite, aspartic acid is itself a neurotransmitter and can be converted to the more potent excitatory neurotransmitter glutamate. The Panel noted that there was no evidence *in vivo* for neurotoxicity associated with aspartame exposure. The Panel concluded, however, that aspartic acid generated from aspartame was not of safety concern at the current exposure estimates or at the ADI of 40 mg/kg bw/day.

Concerning the third aspartame metabolite, phenylalanine, the Panel concluded that it is the main metabolite of concern in terms of potential developmental effects in humans. The Panel considered that it was plausible that phenylalanine could be responsible for some or all of the adverse effects reported for aspartame in rat and rabbit developmental toxicity studies.

Humans heterozygous for phenylalanine hydroxylase mutations, show a slightly reduced capacity to metabolise phenylalanine compared to normal individuals. Individuals homozygous for phenylalanine hydroxylase mutations (phenylketonuria (PKU) patients) have a markedly reduced capacity for phenylalanine metabolism. After birth, homozygous PKU babies show severe impairment in development and cognition if the phenylalanine intake via the diet is not strictly controlled.

PKU mothers with poorly controlled phenylalanine intake in their diet during pregnancy may give birth to babies with congenital heart diseases, microcephalus and impaired neurological function.

The Panel considered that adverse effects reported for aspartame in animal studies could be attributed to the metabolite phenylalanine, which was particularly the case for the rat and rabbit developmental toxicity studies. The Panel noted that adverse developmental effects were seen in children born to PKU patients and that these effects appeared to be related to maternal phenylalanine levels. The Panel was aware that the knowledge on effects of phenylalanine in PKU mothers and their children both before and after birth had developed considerably since the initial evaluation of aspartame.

The Panel undertook a formal Mode of Action (MoA) analysis of the putative role of phenylalanine in the developmental toxicity seen in animal studies. This MoA analysis is described in Section 11.

The Panel considered that the proposed MoA is plausible and relevant based on the weight-of-evidence of the available data, summarised in the opinion. There are uncertainties from the limited kinetic data in animals and in the human aspartame dose-phenylalanine concentration response data. The Panel decided to base the risk characterisation on comparison of plasma phenylalanine levels following aspartame administration with plasma phenylalanine levels associated with developmental effects in children born from mothers with PKU. The Panel decided these human data were more appropriate than the results of animal studies of reproductive and developmental toxicity for the risk characterization of aspartame.

Having established that the MoA was plausible and relevant, the Panel reviewed the information on plasma levels of phenylalanine associated with adverse effects on the fetuses of mothers with PKU. The Panel noted that current clinical guidelines recommend that plasma levels of phenylalanine

should be maintained below 360 μM. In calculating a safe level of aspartame exposure (based on plasma phenylalanine concentrations), the Panel assumed the worst-case scenario that intake of aspartame occurs in combination with a meal which leads to circulating plasma phenylalanine concentrations of 120 μM (the maximum plasma concentration based on conservative assumptions of dietary exposure to phenylalanine). The concentration of plasma phenylalanine derived from aspartame was therefore set to 240 μM (i.e. 360 μM minus 120 μM) by the Panel. Based on the modelling, a plasma phenylalanine concentration of 240 μM would result from the administration of a bolus dose of 103 mg aspartame/kg bw (lower bound distributions: 88 mg aspartame/kg bw; 95th percentile, CI 59-125) to a normal subject. For a PKU heterozygous individual the concentration of 240 μM would be reached by the administration of a bolus dose of 59 mg aspartame/kg bw (lower bound distributions: 50 mg aspartame/kg bw; 95th percentile, CI 28-69). The Panel considered that given the conservative assumptions and the confidence intervals provided by the modelling, for realistic (i.e. non-bolus) dietary intake of aspartame, the peak plasma phenylalanine levels would not exceed 240 μM.

The Panel noted that in the normal population the 95th percentile confidence interval of the lower bound estimate of the dose resulting in a peak plasma level of 240 μM following a bolus administration of aspartame was greater than 40 mg/kg bw (which is equivalent to the current ADI). In the PKU heterozygous population the 95th percentile confidence interval for the lower bound of the dose resulting in a peak plasma level of 240 μM following a bolus administration of aspartame was greater than 40 mg/kg bw (which is equivalent to the current ADI) in 82 % of the simulations. The Panel considered that following bolus administration of aspartame of 40 mg/kg bw (which is equivalent to the current ADI) the PKU heterozygous population would not exceed the current clinical guideline of 360 μM.

The Panel also noted that in order to exceed the phenylalanine plasma concentration of 240 μM following repeated administration of aspartame in normal individuals, a bolus administration at 40 mg/kg bw (which is equivalent to the current ADI) would need to be given every hour.

The Panel considered the following:

- the conservative assumptions used in the modelling, which all overestimate peak plasma concentrations
- the available information on adverse effects on development in humans with PKU
- the allocation of 2/3 of the current clinical guideline level of 360 μM phenylalanine in plasma to phenylalanine from ingested aspartame, in order to account for simultaneous ingestion of phenylalanine from other components of the diet results of the modelling
- kinetic data from repeated oral administration of aspartame in humans
- bolus intakes based on consumption of one litre of soft drink containing aspartame at the maximum permitted level (MPL) of 600 mg/L by a child of 20-30 kg will not exceed 30 mg aspartame/kg bw.

Based on these considerations and evaluations, the Panel concluded that under realistic conditions of aspartame intake, phenylalanine plasma levels would not exceed 240 μM in normal or PKU heterozygous individuals. The Panel noted that this was well below the concentrations at which adverse effects in the fetus are reported and is also below the current clinical guideline (360 μM) for prevention of effects in the fetuses of pregnant PKU patients. The Panel noted that in young children who did not suffer from PKU, plasma levels of phenylalanine resulting from aspartame ingestion at or below the ADI (as either a bolus or other aspartame consumption patterns) were likely to remain below 240 μM. For pregnant women, the Panel noted that there was no risk to the fetus from

phenylalanine derived from aspartame at the current ADI (40 mg/kg bw/day) in normal or PKU heterozygous individuals.

The Panel noted that it was currently not possible to include chronic endpoints in the postulated MoA. The Panel noted that the ADI previously derived by JECFA and SCF of 40 mg/kg bw/day was established based on the long-term animal studies using the default uncertainty factor of 100. The Panel considered that this remained appropriate for the evaluation of long-term effects of aspartame.

The current evaluation was based on analysis of human reproductive and developmental effects of phenylalanine in PKU patients, who are more susceptible than the general and PKU heterozygous population. Therefore, no additional allowance for toxicodynamic variability was required. The modelling of the aspartame dose-phenylalanine concentration response was based on data from PKU heterozygous individuals who at any dose would have a higher plasma phenylalanine concentration than the normal population; therefore, no additional allowance for toxicokinetic variability was required for the general population. The Panel concluded that exposures at or below the current ADI were not of safety concern for reproductive and developmental toxicity in humans excluding PKU homozygous individuals.

The Panel also conducted an uncertainty analysis of the assumptions used in the postulated MoA. While the Panel was not able to place a specific numerical value on the uncertainties related to these assumptions, the Panel considered that these assumptions would be more likely to overestimate than underestimate any potential developmental risk. Therefore, the Panel considered that the results of the uncertainty analysis further support its conclusion, that there is no safety concern for aspartame at the current ADI in normal and heterozygous subjects.

Overall, the Panel concluded from the present assessment of aspartame that there were no safety concerns at the current ADI of 40 mg/kg bw/day. Therefore, there was no reason to revise the ADI for aspartame.

The Panel emphasised that its evaluation of phenylalanine plasma levels from a dose of aspartame at the ensuing ADI is not applicable to PKU patients. These individuals require total control of dietary phenylalanine intake to manage the risk from elevated phenylalanine plasma levels. The Panel noted it is a requirement of EU legislation that products containing aspartame indicate through labelling that they contain a source of phenylalanine.

Conservative estimates of exposure to aspartame made by the Panel for the general population were up to 36 mg/kg bw/day at the 95th percentile. These were below the ADI. The current ADI for DKP is 7.5 mg/kg bw/day. Estimates of DKP exposure at the ADI for aspartame are below this ADI based on the current specification for DKP in aspartame (1.5 %) and up to the highest value (24 % for soft drinks) used from the whole database; this latter percentage was taken into account for all food categories where no degradation percentage of DKP was available. The Panel noted that high-level exposure estimates for the general population are up to 5.5 mg/kg bw/day at the 95th percentile, which is below the ADI. Finally, conservative estimates of exposure to methanol showed that aspartame-derived methanol contributed to less than 10 % of the total mean anticipated exposure to methanol from all sources.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

Regulation (EC) No 1333/2008 of the European Parliament and of the Council on food additives requires that food additives are subject to a safety evaluation by the European Food Safety Authority (EFSA) before they are permitted for use in the European Union. In addition, it is foreseen that food additives must be kept under continuous observation and must be re-evaluated by EFSA.

For this purpose, a programme for the re-evaluation of food additives that were already permitted in the European Union before 20 January 2009 has been set up under the Regulation (EU) No 257/2010[4]. This Regulation also foresees that food additives are re-evaluated whenever necessary in light of changing conditions of use and new scientific information. For efficiency and practical purposes, the re-evaluation should, as far as possible, be conducted by group of food additives according to the main functional class to which they belong.

The order of priorities for the re-evaluation of the currently approved food additives should be set on the basis of the following criteria: the time since the last evaluation of a food additive by the Scientific Committee on Food (SCF) or by EFSA, the availability of new scientific evidence, the extent of use of a food additive in food and the human exposure to the food additive taking also into account the outcome of the Report from the Commission on Dietary Food Additive Intake in the EU[5] of 2001. The report 'Food additives in Europe 2000[6]' submitted by the Nordic Council of Ministers to the Commission, provides additional information for the prioritisation of additives for re-evaluation. As colours were among the first additives to be evaluated, these food additives should be re-evaluated with a highest priority.

In 2003, the Commission already requested EFSA to start a systematic re-evaluation of authorised food additives. However, as a result of adoption of Regulation (EU) 257/2010 the 2003 Terms of References are replaced by those below.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

The Commission asks the European Food Safety Authority to re-evaluate the safety of food additives already permitted in the Union before 2009 and to issue scientific opinions on these additives, taking especially into account the priorities, procedures and deadlines that are enshrined in the Regulation (EU) No 257/2010 of 25 March 2010 setting up a programme for the re-evaluation of approved food additives in accordance with the Regulation (EC) No 1333/2008 of the European Parliament and of the Council on food additives.

[4] Commission Regulation (EU) No 257/2010 of 25 March 2010 setting up a program for the re-evaluation of approved food additives in accordance with Regulation (EC) No 1333/2008 of the European Parliament and of the Council on food additives. OJ L 80, 26.3.2010, p. 19-27.

[5] Report from the Commission on dietary food additive intake in the European Union. Commission of the European Communities, COM (2001) 542 final.

[6] Food Additives in Europe 2000. Status of safety assessments of food additives presently permitted in the EU. Nordic Council of Ministers, TemaNord 2002:560.

ASSESSMENT

1. Introduction

The present opinion deals with the re-evaluation of the safety of aspartame (E 951) when used as a sweetener added to foods, and including its use as a tabletop sweetener.

Aspartame is a sweetener authorised as a food additive in the EU that was previously evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), the latest in 1981 (JECFA, 1975, 1980, 1981), the EU Scientific Committee for Food (SCF), the latest in 2002 (SCF 1985, 1989, 1997, 2002), the European Food Safety Authority (EFSA), the latest in 2011 (EFSA, 2006, 2009a 2009b, 2011a; EFSA ANS Panel, 2011).

The Panel based its evaluation on original study reports and information submitted following public calls for data[7], previous evaluations, and additional literature that has become available until the end of the public consultation on the draft Scientific Opinion on the re-evaluation of aspartame (E 951) as a food additive (15th February 2013). The Panel also evaluated literature published after the end of the public consultation, until 15th November 2013 (EFSA ANS Panel, 2013).

The selection criteria for scientific data consideration for the re-evaluation of aspartame described in this opinion applied to both the existing published and unpublished scientific literature. These criteria were agreed at the 28th Scientific Panel on Food Additives and Nutrient Sources added to Food (ANS) Plenary meeting on 25-27 October 2011 and were published with the minutes of that meeting. These criteria are reproduced in Appendix A.

In its re-evaluation of aspartame, the ANS also considered the safety of its gut derived metabolites, methanol, phenylalanine and aspartic acid and its degradation products 5-benzyl-3,6-dioxo-2-piperazine acetic acid (DKP) and β-aspartame, which also may be present in the sweetener as an impurity.

The Panel noted that in the early studies, the stereochemical forms of the amino acids and other metabolites formed from aspartame were not specified.

2. Technical data

2.1. Identity of the substances

Aspartame is a dipeptide of L-phenylalanine methyl ester and L-aspartic acid bearing an amino group at the α-position from the carbon of the peptide bond (Figure 1).

Aspartame has a molecular formula of $C_{14}H_{18}N_2O_5$. It has a molecular weight of 294.31 g/mol, the CAS Registry Number is 22839-47-0 and the EINECS number 245-261-3. The chemical name for aspartame is (*S*)-3-amino-*N*-[(*S*)-1-methoxycarbonyl-2-phenylethyl] succinamic acid. The structural formula of aspartame is presented in Figure 1.

Aspartame is a white odourless, crystalline powder having a sweet taste.

At pH 7 and room temperature, aspartame has a solubility in water of approximately 10 g/L (Homler et al., 1991). According to data from a producer, aspartame has a solubility in ethanol of 0.37 % and is insoluble in oil.[8] The theoretical log $P_{o/w}$ value calculated with Advanced Chemistry Development Labs software (ACD Labs) is 0.542 ± 0.626 (at 25 °C). Aspartame has two melting points, melting initially at

[7] 'Call for scientific data on Aspartame (E951)' (published: 1 June 2011). Available online: http://www.efsa.europa.eu/en/dataclosed/call/110601.htm
'Call for scientific data on aspartame (E 951) related to 5-benzyl-3,6-dioxo-2-piperazine acetic acid (DKP) and other primary or secondary degradation products from aspartame' (published: 26 July 2012). Available online: http://www.efsa.europa.eu/en/dataclosed/call/120726.htm

[8] http://www.nutrasweet.com/articles/sendfile.asp?Id=130&filename=AG%2DTB%2D03%2D001%2Epdf

190 °C, solidifying and re-melting at 246-247 °C. A possible explanation for this behaviour is that the intramolecular reaction of the primary amine with the methyl ester takes place at the initial melting point to generate 5-benzyl-3,6-dioxo-2-piperazine acetic acid (DKP) and methanol (Prankerd, 2002).

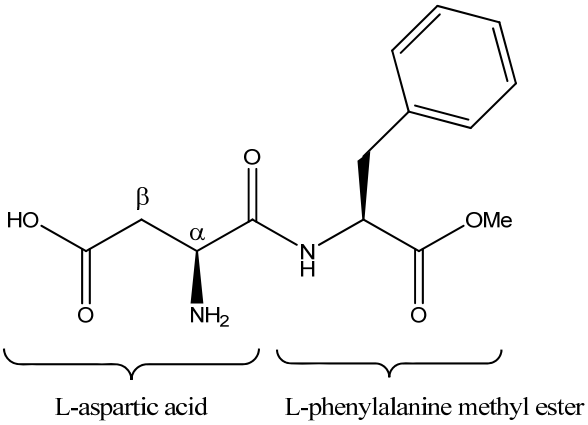


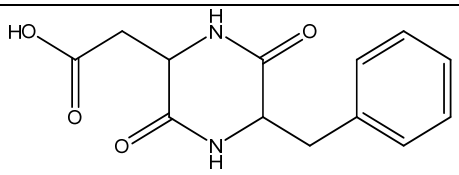
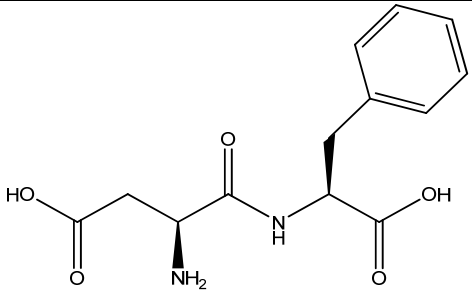
Figure 1: Structural formula of aspartame E951 (α-aspartame).

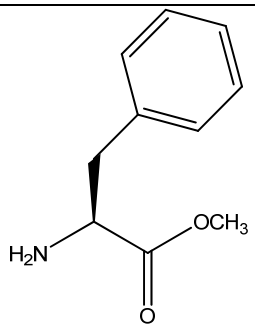
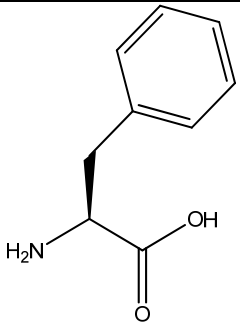
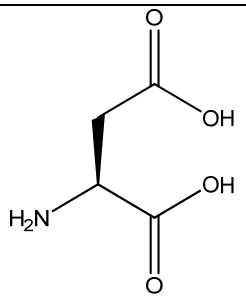
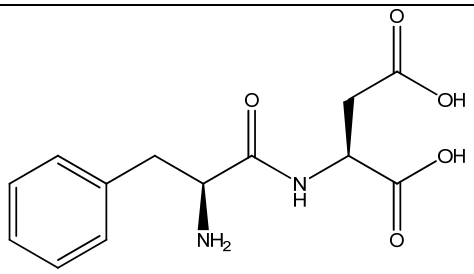
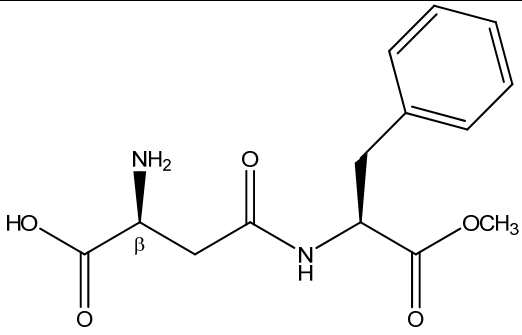
Some of the synonyms of aspartame include α-aspartame, α-APM, N-L-alpha-aspartyl-L-phenylalanine-1-methyl ester, aspartyl phenylalanine methyl ester, L-aspartyl-L-phenylalanyl methyl ester, L-aspartyl-L-3-phenylalanine methyl ester, L-alpha-aspartyl-L-phenylalanine methyl ester, alpha-L-aspartyl-L-phenylalanine methyl ester, methyl aspartylphenylalanate, L-aspartame, alpha-aspartame.

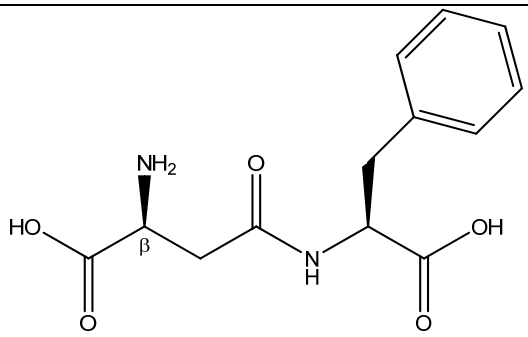
Identity of degradation products of aspartame

The major degradation products of aspartame are listed in Table 1.

Table 1: Major degradation products of aspartame

Name	***CAS Registry Number***	***Structural formula***
5-benzyl-3,6-dioxo-2-piperazine acetic acid (DKP)	55102-13-1 (stereochemistry not specified) 5262-10-2 (configuration 2*S*,5*S*-)	
Methanol	67-56-1	CH_3OH
L-Aspartyl-L-phenylalanine (α-AP)	13433-09-5	

Name	***CAS Registry Number***	***Structural formula***
L-Phenylalanine methyl ester	2577-90-4	
L-Phenylalanine (Phe)	63-91-2	
L-Aspartic acid (Asp)	56-84-8	
L-Phenylalanine-L-aspartic acid		
β-Aspartame (β-APM)	2577-90-4	

Name	*CAS Registry Number*	*Structural formula*
N-L-β-Aspartyl-L-phenylalanine (β-AP)		

5-benzyl-3,6-dioxo-2-piperazine acetic acid (DKP)

Via the intramolecular reaction of the primary amine in α-aspartame with its methyl ester group, DKP and methanol are formed. The molecular formula of DKP is $C_{13}H_{14}N_2O_4$. Its molecular weight is 262.26 g/mol.

The log $P_{o/w}$ value is -0.269 ± 0.515 at 25 °C, the pK_{a1} is 4.02 ± 0.10 and pK_{a2} is -1.55 ± 0.60 both at 25 °C, values calculated with Advanced Chemistry Development (ACD/Labs).

The synonyms for DKP include 3-benzyl-6-(carboxymethyl)-2,5-diketopiperazine, 3-benzyl-6-carboxymethyl-2,5-dioxopiperazine, 3-carboxymethyl-6-benzyl-2,5-diketopiperazine, cyclo(aspartyl-phenylalanyl) and 5-benzyl-3,6-dioxo-2-piperazineacetic acid (5BZ).

Hydrolysis of one of the amides in the diketopiperazine may occur leading to the formation of phenylalanine aspartic acid or α-aspartylphenylalanine (see Table 1). The stereochemistry of the compounds is not given due to possible racemisation of aspartic acid and phenylalanine moieties in DKP (Boehm and Bada, 1984). Both intermediates hydrolyse to yield two amino acids: aspartic acid and phenylalanine[9] (Prodolliet and Bruelhart, 1993; Langguth et al., 1991).

β-Aspartame

β-Aspartame has as chemical name *N*-L-β-aspartyl-L-phenylalanine 1-methyl ester. Unlike α-aspartame, β-Aspartame has no sweet taste.

2.2. Specifications

Specifications for aspartame according to Commission Regulation (EU) No 231/2012[10] and to JECFA (2006) are listed in Table 2.

Table 2: Specifications for aspartame according to Commission Regulation (EU) No 231/2012 and JECFA (2006)

	Commission Regulation (EU) 231/2012	**JECFA (2006)**
Assay	Not less than 98 % and not more than 102% of $C_{14}H_{18}N_2O_5$ on the anhydrous basis	Not less than 98 % and not more than 102 % on the dried basis

[9] The term phenylalanine and phenyalanine methyl ester are used when the stereochemistry of the compounds is not specified in the original reference.

[10] Commission Regulation (EU) No 231/2012 of 9 March 2012 laying down specifications for food additives listed in Annexes II and III to Regulation (EC) No 1333/2008 of the European Parliament and of the Council OJ L 83, 22.3.2012 p 1-295.

Description	White odourless, crystalline powder having a sweet taste. Approximately 200 times as sweet as sucrose	White, odourless, crystalline powder, having a strong sweet taste
Identification		
Solubility	Slightly soluble in water and in ethanol	Slightly soluble in water and in ethanol
Test for amine group	-	Dissolve 2 g of ninhydrin in 75 ml of dimethyl sulphoxide, add 62 mg of hydrindantin, dilute to 100 ml with 4 M lithium acetate buffer solution (pH 9) and filter. Transfer about 10 mg of the sample to a test tube, add 2 ml of the reagent solution, and heat. A dark purple colour is formed.
Test for ester	-	Dissolve about 20 mg in 1 ml of methanol, add 0.5 ml of methanol saturated with hydroxylamine hydrochloride, mix, and then add 0.3 ml of 5 *N* potassium hydroxide in methanol. Heat the mixture to boiling, then cool, adjust the pH to between 1 and 1.5 with hydrochloric acid TS, and add 0.1 ml of ferric chloride TS. A burgundy colour is produced.
pH (1 in 125 solution)	4.5 and 6.0	4.5-6.0
Specific rotation (Determine in a 4:100, 15 *N* formic acid solution within 30 minutes after preparation of the sample solution)	$[\alpha]_D^{20}$: + 14.5 to + 16.5 °	$[\alpha]_D^{20}$: + 14.5 to + 16.5 °
Purity		
Loss on drying (105 °C, 4 hours)	Not more than 4.5 %	Not more than 4.5 %
Sulphated ash	Not more than 0.2 % (expressed on a dry weight basis)	Not more than 0.2 % Test 1 g of the sample (Method I)
Transmittance/ Spectrophotometry	The transmittance of a 1 % solution in 2 *N* hydrochloric acid, determined in a 1-cm cell at 430 nm with a suitable spectrophotometer, using 2 *N* hydrochloric acid as reference, is not less than 0.95, equivalent to an absorbance of not more than approximately 0.022	The transmittance of a 1 in 100, 2 *N* hydrochloric acid solution, determined in a 1 cm cell at 430 nm with a suitable spectrophotometer, using 2 *N* hydrochloric acid as a reference, is not less than 0.95, equivalent to an absorbance of not more than approximately 0.022
Arsenic	Not more than 3 mg/kg	
Lead	Not more than 1 mg/kg	Not more than 1 mg/kg
5-Benzyl-3,6-dioxo-2-piperazineacetic acid (DKP)	Not more than 1.5 %	Not more than 1.5 %
Other optical isomers	-	Passes test

In the JECFA specifications, aspartame is identified by an amine test and an ester test, whilst the EC specifications do not include these tests. JECFA specifications analyse aspartame for the presence of other optical isomers whilst the EC specifications do not include this test.

The Panel noted that the EC specifications for aspartame do not include a maximum limit for β-aspartame, which may be formed as a by-product of the chemical synthesis of aspartame or from aspartame degradation.

The Panel noted that the EC specifications for aspartame may need to be amended to include a restriction for protein content due to the potential use of enzymes in the production of aspartame.

The Panel noted that both EC and JECFA specifications for aspartame are dated in terms of the tests proposed for identification. These specifications should be updated to include characterisation of aspartame by chromatographic and mass spectrometric methodologies.

2.3. Manufacturing process

There are several methods to produce aspartame on a commercial scale, either through chemical or enzymatic synthesis (Prankerd, 2002; Yagasaki and Hashimoto, 2008).

According to the information provided by a producer, the chemical synthesis of aspartame involves the reaction of L-aspartic acid and L-phenylalanine methyl ester (Burdock Group, 2006). L-phenylalanine methyl ester reacts with *N*-protected L-aspartic anhydride to form *N*-protected aspartame. After re-crystallisation, the protecting group is removed to yield aspartame. Both α-aspartame (the sweet isomer) and β-aspartame (the non-sweet isomer) are produced, requiring separation step to obtain only α-aspartame. A similar description of the manufacturing process was provided by another manufacturer that uses L-phenylalanine and *N*-protected L-aspartic anhydride (Nutrasweet, 2012). Two major producers have indicated that the chemical synthesis is the process used for their aspartame production (Ajinomoto, 2012a; Nutrasweet 2012).

One of the difficulties encountered in the chemical synthesis of aspartame is avoiding the formation of the non-sweet isomer (β-aspartame) as one of the by-products.

Enzymatic methods allow the use of racemic starting materials, and the process has the advantage of producing only α-aspartame. The enzyme typically used is thermolysin, derived from *Bacillus thermoproteolyticus.* Enzymatic synthesis using protected and unprotected aspartic acid and phenylalanine methyl ester are described in the literature (Yagasaki and Hashimoto, 2008). The Panel noted that *Bacillus thermoproteolyticus* is not included in the list of qualified presumption of safety (QPS) biological agents intentionally added to food and feed (EFSA BIOHAZ Panel, 2012).

2.4. Methods of analysis in food

There is a number of published methods for the determination of aspartame alone or in combination with other sweeteners (Zygler et al., 2009). Most methods are based on extraction, with or without subsequent clean-up and high performance liquid chromatographic (HPLC) analysis using various detectors (Lino et al., 2008; Ma et al., 2012; Cheng and Wu, 2011; Kobayashi et al., 1999; Ji et al., 2009; Kang et al., 2012; Serdar and Knezevic, 2011; Sik 2012) or ion chromatography (Chen and Wang, 2001). There are three EU standardised inter-laboratory validated methods for determination of aspartame in foods. Methods EN 12856:1999[11] and CEN/TS 15606:2009[12] employ HPLC with UV detection and EN 15911:2010[13] uses HPLC with evaporative light scattering detector (ELSD). A validated HPLC-ELSD method has been reported for aspartame in carbonated and non-carbonated beverages, canned and bottled fruits and yoghurt (Wasik et al., 2007). For samples fortified with aspartame, at the maximum permitted limits for aspartame according to the EU regulation, the method gave recoveries ranging from 90 to 100 % and RSD_R values < 7 %. HorRat values of < 1.7 indicated satisfactory performance of the method for determination of aspartame in all matrices tested (Buchgraber and Wasik, 2007). Others have employed various forms of combined liquid chromatography-mass spectrometry (LC-MS) for determining aspartame in Chinese liquors (Xia et al., 2011), in beverages, dairy and fish products (Zygler et al., 2011), juices and syrups (Ferrer and Thurman, 2010), wine (Cheng and Wu, 2011) and other foods (Yang et al., 2008, Koyama et al., 2005, Liu et al., 2010). LC-MS methods are rapid, do not require extensive clean-up and offer low limits of

[11] http://webstore.ansi.org/RecordDetail.aspx?sku=BS+EN+12856%3A1999
[12] https://www.astandis.at/shopV5/preview.action;jsessionid=B20DC77D150ABD17C37DD9EEF850B4EB?preview=&dokkey=354291&selectedlocale=en
[13] http://webstore.ansi.org/RecordDetail.aspx?sku=BS+EN+15911:2010

detection with high specificity, but have, up to now, not been validated by any inter-laboratory studies. Other successful approaches for direct analysis of aspartame in commercial sweeteners have employed Fourier transform infrared spectroscopy (FT-IR) (Mazurek and Szostak, 2011), in soft drinks and tabletop sweeteners capillary electrophoresis with capacitively coupled contactless conductivity detection (CE-C^4D) (Bergamo et al., 2011) and in carbonated beverages proton nuclear magnetic resonance (^{1}H-NMR) (Maes et al., 2012).

2.5. Stability, reaction and fate in food

The stability of aspartame is affected by moisture, pH, temperature and storage time.

Figure 2 shows the degradation pathways (Bell and Labuza, 1991; Prodolliet and Bruelhart, 1993). At neutral and alkaline pH, aspartame releases methanol and forms α-aspartylphenylalanine or undergoes cyclisation to form DKP. DKP and α-aspartylphenylalanine can interconvert, but do not revert to aspartame to any significant rate. Conversion of aspartame into DKP or α-aspartylphenylalanine results in the loss of the sweet taste. At acidic pH below 4.5, in addition to the two main reaction pathways occurring at neutral and alkaline pH, aspartame can undergo structural rearrangement to produce β-aspartame. Also the peptide bond of aspartame can be cleaved, producing L-phenylalanine methyl ester and L-aspartic acid. β-aspartylphenylalanine can be produced via structural rearrangement of α-aspartylphenylalanine. Phenylalanine is produced by hydrolysis of phenylalanine methyl ester. Finally, α-aspartylphenylalanine can hydrolyse to the individual amino acids.

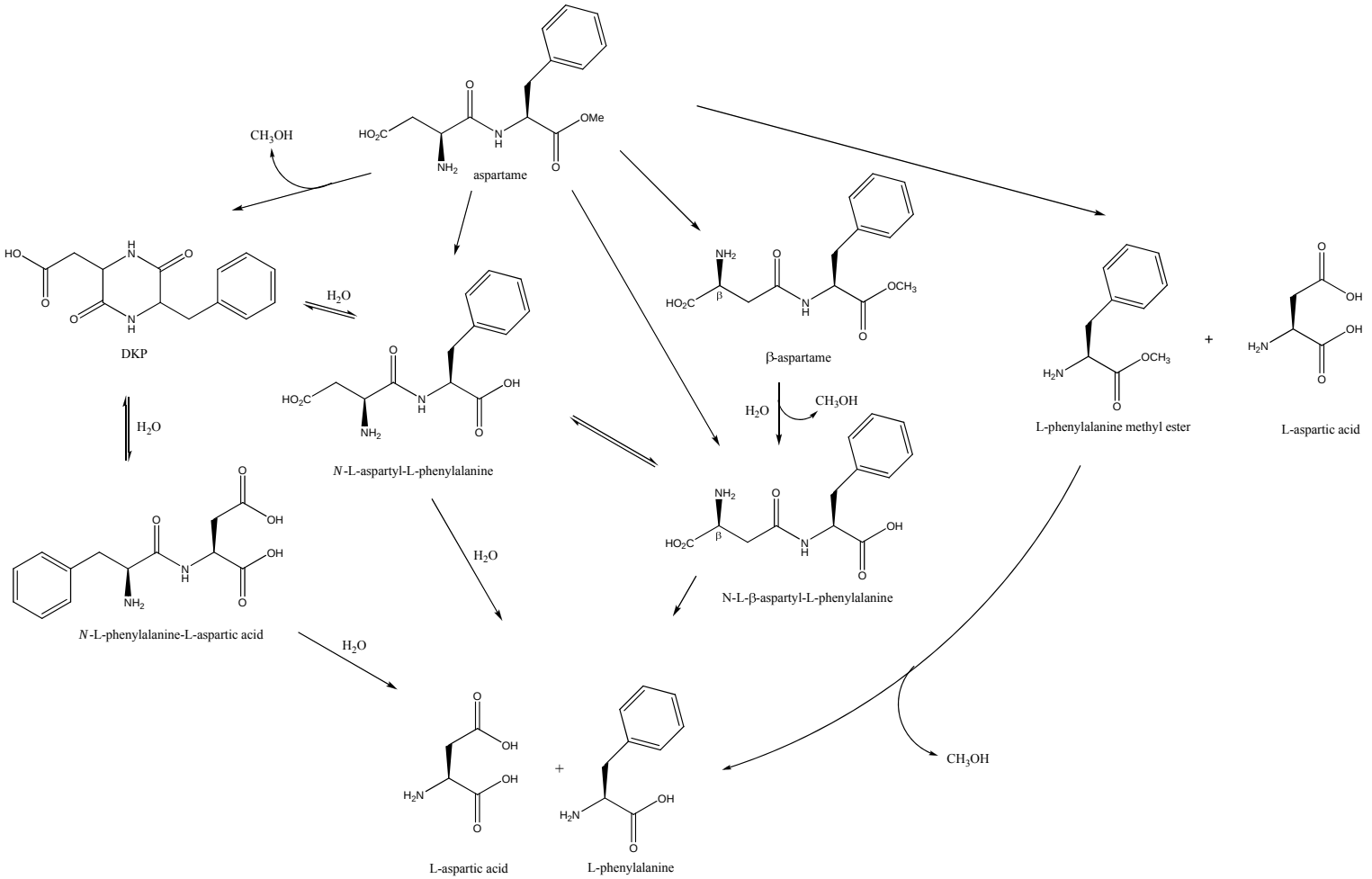


Figure 2: Degradation pathways for aspartame

Stability of aspartame in solid state

According to industry at moisture contents of less than 8 %, aspartame in its pure form is stable with a minimum shelf life of 5 years (storage temperature not indicated) (Ajinomoto, 2012). Solid-state stability studies showed that at temperatures higher than 80 °C, aspartame cyclises with the release of methanol to form DKP (Leung and Grant 1997; Lin and Cheng 2000; Conceicao et al., 2005). Homler reported that under dry conditions, conversion of aspartame into DKP is slow (5 % per 100 hours at 105 °C). At higher temperatures, the rate of conversion to DKP increases (around 50 % per 80 hours at 120 °C and 100 % per 30 hours at 150 °C) (Homler, 1984). Graves and Luo (1987) reported that when heated in an acidified and dried state at 110 °C for 24 hours in vacuo, in addition to the degradation products, aspartame could dehydrate to form an anhydro derivative. In addition, oligopeptides may be generated containing ratios of L-aspartic acid to L-phenylalanine that are higher than the starting material.

Decomposition of aspartame in lyophilised instant coffee with a residual moisture content of 3-4 % was less than 10 % after 260 days of storage (Jost et al., 1982).

The degradation kinetics of aspartame as a function of the water activity (relative vapour pressure, a_w) in low and intermediate moisture food systems showed that an increase in a_w for each 0.1 units in the 0.3-0.7 range, resulted in about 30-80 % increase in the degradation rate (Bell and Labuza, 1991).

Stability of aspartame in solution

Aspartame has limited stability in solutions and undergoes pH, temperature and time-dependent degradation (Pariza et al., 1998). Extensive studies on the kinetics of aspartame degradation in solution have been performed and the degradation has been reported to follow first order kinetics (Langguth et al., 1991).

Aspartame is most stable between pH 4-5 (Homler, 1984; Neirynck and Nollet, 1988; Bell and Labuza, 1991; Bell and Labuza, 1994; Sabah and Scriba, 1998; Rowe et al., 2003). Rowe et al. (2003) showed that within this range of pH, aspartame dissolved in aqueous buffers at 25 °C has a half-life > 250 days. When the pH was decreased to 3, the half-life was shortened to 100-125 days and further decreased to below 25 days at pH 1. When the pH was increased from 5 to 6, the half-life was reduced to 50-100 days and further decreased to below 25 days at pH 7. Pattanaargson et al. (2001) investigated the degradation products of aspartame at different pH ranges. The major degradation product found at pH 2-6 was L-phenylalanine methyl ester, in the range of pH 7-10 the major degradation product was DKP and at pH 12, it was L-aspartyl-phenylalanine.

In addition to the pathways discussed above, due to its free amino group, aspartame can react with reducing sugars in the presence of water via the Maillard reaction. This was determined through measurement of brown colour formation that resulted from mixing aspartame and glucose in solution at 70, 80, 90 and 100 °C at a_w of 0.8. Browning of aspartame followed zero order kinetics; the respective time to reach a browning absorbance of 0.1 absorbance units at 420 nm was 11.4, 5.3, 2.15 and 1.0 hour at the given temperatures (Stamp and Labuza 1983; Huang et al., 1987). The formation of a Schiff base, a step in the Maillard degradation pathway, was also noted between aspartame and vanillin in methanol/water solutions (Cha and Ho, 1988).

Aspartame, at room temperature, is stable with respect to racemisation at mildly acidic pH values, but at 100 °C, racemisation of the DKP generated from aspartame takes place (Boehm and Bada 1984; Gaines and Bada 1988; Gund and Veber, 1979). The authors' explanation was that DKP formation is accelerated by electrostatic repulsion between the two carboxy groups which force the dipeptide into the *cis* conformation. At neutral pH the relative racemisation rates of aspartame dipeptides are much slower than of DKP, and amino acids at the *N*-terminal position racemise more rapidly than *C*-terminal amino acids (Gaines and Bada, 1988).

The analytical results of the electro-thermal and microwave induced hydrolysis of aspartame in cola samples imply that the hydrolysis of aspartame increases with an increase in temperature, microwave power and hydrolysis time. D-aspartic acid and D-phenylalanine can be observed with the electro-thermal racemisation at the hydrolysis temperature of 120 °C for 1 day and only D-aspartic acid can be observed at the hydrolysis temperature of 90 °C for 2 and 3 days. For microwave-induced hydrolysis, only L-aspartic acid was detected at a power of 56 W for 1 minute and 320 W for 3 minutes (Cheng and Wu, 2011).

The study by Lawrence and Yuan (1996) showed that aspartame, in aqueous acidic solutions in the presence of ascorbic acid and a transition metal catalyst, such as copper (II) or iron (III), under aerobic conditions can produce benzaldehyde via a free radical attack on aspartame. Benzaldehyde production was dependent on ascorbic acid concentration, but the yield of benzaldehyde decreased as the concentration of ascorbic acid exceeded that of aspartame.

In lime-lemon carbonated beverages with pH 3.14-3.21 stored for 60 days at temperatures of 4 °C and 37 °C, the loss of aspartame was 4.5 % and 29.5 % respectively (Malik et al., 2002). In lime-lemon and diet cola drinks stored at 22 °C, after 6 months, 28 and 37 % of the label claim of aspartame was detected, respectively, and after 36 months, the detected level was reduced to 6 and 4 % (Tsang et al., 1985).

According to Jost et al. (1982), the half-life of aspartame in cola is approximately 150 days at 23 °C and pasteurization of orange juice results in immediate loss of approximately half of the initial aspartame content.

Stability tests in sterilised flavoured dairy beverages with pH 6.7 showed that aspartame had a half-life of 1-4 days at 30 °C and 24-58 days at 4 °C. Decreasing the pH from 6.7 to 6.4 doubles the half-life of aspartame (Bell and Labuza, 1994).

Noda et al. (1991) demonstrated the instability of aspartame in fresh juice solutions of particular kinds of fruit such as kiwi, pineapple, papaya and melon, probably by the action of proteolytic enzymes. Yogurt cultures (strains of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*) degrade aspartame during fermentation; however, losses of aspartame appeared related to the rate of the metabolism of the cultured microorganisms. Aspartame degradation was highest during culture growth and became negligible once the pH of yogurt dropped below 5.0 and when the culture reached a stationary phase (Keller et al., 1991; Prodolliet and Bruelhart 1993).

Review of DKP concentration in food from aspartame degradation

The content of aspartame and its degradation products, DKP, aspartyl-phenylalanine and phenylalanine, were analysed in 24 different commercial foods by Prodolliet and Bruelhart (1993). The decomposition of aspartame was particularly high in fruit cream (40 %), milk chocolate (26 %), malted beverage (22 %) and fruit yogurt (15 %). It has been determined that up to 21 % and 12 % of the aspartame in cola drink and fruit cream, respectively, is in the form of DKP.

The degradation of aspartame to DKP in soft drinks depends on the storage temperature. DKP concentration in soft drinks after two months storage was about four times higher at a temperature range of 25-27 °C compared to 4-5 °C (Saito et al., 1989).

Grosse et al. (1991) determined the level of degradation of aspartame and the extent of the formation of DKP in heat-treated dessert products. In acidified (pH 3) gelatin-based desserts, no formation of DKP was observed, whereas in dessert products containing milk or cacao having a pH of 6, the degradation of aspartame rose to 20-35 %. According to the authors in heat-treated cacao containing desserts with starch and milk as basic ingredients, at pH 6 a degradation of about 34 % of the aspartame to DKP can occur, whereas in heat-treated vanilla containing desserts at pH 6, about 21-25 % is degraded to DKP.

Cyprus submitted analytical data on levels of DKP in soft drinks sold in the Cyprus market. Levels found range between 3.4 and 19.4 mg/L. No information on the packaging or storage conditions (duration, temperature) was given (Cyprus, 2012).

The Panel was provided with data on the determination of aspartame and its main degradation products in carbonated beverages (UN11, 1986), in baked goods (UN12, 1990), in beer (UN13), instant and quick cooked oatmeal (UN14, 1987), in soft candy (UN15, 1987). Table 3 shows the concentration of DKP in food from aspartame degradation observed in the above-mentioned studies as well as in the available literature.

Table 3: Concentration of 5-benzyl-3,6-dioxo-2-piperazine acetic acid (DKP) in food from aspartame (APM) degradation

Food	Conditions	APM degraded (%)	Degradation of APM to DKP (%)	Reference
Carbonated Beverages APM initial: 0.5 g/L	pH 2.5 Time: 52 weeks T 20 °C	75	24	UN11, 1986
	pH 2.5 Time: 39 weeks T 30 °C	95	31	
	pH 4.4 Time: 38 weeks T 20 °C	21	8.3	
	pH 4.4 Time: 52 weeks T 30 °C	62	26.6	
Cheese cake (APM encapsulated) APM: 1.5 g/kg	Time: 50 min T 121 °C	16	6.4	UN12, 1990
Chocolate cake (APM encapsulated) APM: 4.5 g/kg	Time: 35 min T 177 °C	24	15	
Beer APM: 20 mg/kg	pH 4.3 Time: 90 days T 22 °C Ethanol 2.7 %	8-10	5	UN13
Instant cereals APM: 1400-2000 mg/kg	Boiling water added, time in cooling water: 17 min	6	3	UN14, 1987
Instant cereals APM: 1400-2000 mg/kg	Cooked in microwave, time in cooling water: 16 min	2.5	2.3	
Gelled candy APM: 0.84 g/kg	21.1 °C Time 22 weeks	21	17	UN15, 1987
	26.6 °C Time 17 weeks	41	25	
Lime-lemon	Time 6 months T : 22 °C	43-63	17-22	Tsang et al., 1985
	Time 36 months T : 22 °C	86-94	32-34	
Diet Cola	Time 6 months T : 22 °C	62-72	24-28	
	Time 36 months T : 22 °C	96	32-35	

Food	Conditions	APM degraded (%)	Degradation of APM to DKP (%)	Reference
Diet cola APM: 20-100 µg/mL	All samples analysed were all commercial products. The samples were dissolved to yield an aspartame concentration of 20-100 µg/mL, taking into account the declared values.	40	21	Prodolliet and Bruelhart, 1993
Malted beverage APM: 20-100 µg/mL		22	3	
Fruit (prune) yogurt APM: 20-100 µg/mL		15	3	
Vanilla cream APM: 20-100 µg/mL		8	7	
Fruit cream APM: 20-100 µg/mL		40	12	
Milk chocolate APM: 20-100 µg/mL		26	7	

The Panel noted that in the carbonated beverage study (UN11, 1986) it was observed that aspartame was also degraded to β–aspartame (β-APM) and β–aspartyl-L-phenylalanine (β-AP). At the beginning (4-12 weeks), aspartame was degraded into β–APM and with time (> 12 weeks), β–APM was hydrolysed to β-AP. Table 4 shows the concentration of β– APM and β-AP in food from aspartame degradation.

Table 4: Concentration of β–aspartame (β-APM) and β–aspartyl-L-phenylalanine (β-AP) in food from aspartame degradation

Food	Conditions	APM degraded (%)	Degradation of APM to β-AP (%)	Degradation of APM to β-APM (%)	Reference
Carbonated Beverages APM initial: 0.5 g/L	pH 2.5 Time: 52 weeks T 20 °C	75	9	3.4	UN11, 1986
	pH 2.5 Time: 39 weeks T 30 °C	95	18.3	1.8	
	pH 4.4 Time: 38 weeks T 20 °C	21	1	1.4	
	pH 4.4 Time: 52 weeks T 30 °C	62	10.5	3.8	

2.6. Case of need and proposed uses

Aspartame (E 951) is authorised in the EU as a sweetener. The maximum permitted levels (MPLs) range from 25 to 6000 mg/kg in foods, except for tabletop sweeteners for which it is authorised *quantum satis*.

Table 5 summarises foods that are permitted to contain aspartame (E 951) and the corresponding MPLs as set by Commission Regulation (EU) No 1129/2011[14] on food additives for use in foodstuffs.

[14] Commission regulation (EU) No 1129/2011 of 11 November 2011 amending Annex II to Regulation (EC) N°1333/2008 of the European Parliament and of the Council establishing a Union list of food additives. The Panel noted that the Commission Regulation (EC) No. 1129/2011 of 11 November 2011 will enter into force on June, 1st 2013 but confirm the approved uses of aspartame as a food additives as described in previous directive: Council Directive No. 94/35/EC of 30 June 1994 on sweeteners for use in foodstuffs.

Table 5: MPLs of aspartame (E 951) in foods according to the Commission Regulation (EU) No 1129/2011

Category number	Foods	restrictions/exception	Maximum level (mg/L or mg/kg as appropriate)
1.4	**Flavoured fermented milk products including heat-treated products**	only energy-reduced products or with no added sugar	**1000**
3	**Edible ices**	only energy-reduced or with no added sugar	**800**
4.2.2	**Fruit and vegetables in vinegar, oil, or brine**	only sweet-sour preserves of fruit and vegetables	**300**
4.2.3	**Canned or bottled fruit and vegetables**	only fruit energy-reduced or with no added sugar	**1000**
4.2.4.1	**Fruit and vegetable preparations excluding compote**	only energy-reduced	**1000**
4.2.5.1	**Extra jam and extra jelly as defined by Directive 2001/113/EEC**	only energy-reduced jams jellies and marmalades	**1000**
4.2.5.2	**Jam, jellies and marmalades and sweetened chestnut puree as defined by Directive 2001/113/EEC**	only energy-reduced jams, jellies and *marmalades*	**1000**
4.2.5.3	**Other similar fruit or vegetable spreads**	only dried-fruit-based sandwich spreads, energy-reduced or with no added sugar	**1000**
5.1	**Cocoa and Chocolate products as covered by Directive 2000/36/EC**	only energy-reduced or with no added sugars	**2000**
5.2	**Other confectionery including breath refreshing microsweets**	only cocoa or dried fruit based, energy reduced or with no added sugar	**2000**
5.2	**Other confectionery including breath refreshing microsweets**	only cocoa, milk, dried fruit or fat based sandwich spreads, energy-reduced or with no added sugar	**1000**
5.2	**Other confectionery including breath refreshing microsweets**	only starch based confectionary energy reduced or with no added sugar	**2000**
5.2	**Other confectionery including breath refreshing microsweets**	only confectionary with no added sugar	**1000**
5.2	**Other confectionery including breath refreshing microsweets**	only breath-freshening micro-sweets, with no added sugar	**6000**
5.2	**Other confectionery including breath refreshing microsweets**	only strongly flavoured freshening throat pastilles with no added sugar	**2000**
5.3	**Chewing gum**	only with added sugars or polyols, as flavour enhancer	**2500**
5.3	**Chewing gum**	only with no added sugar	**5500**
5.4	**Decorations, coatings and fillings, except fruit based fillings covered by category 4.2.4**	only starch based confectionary energy reduced or with no added sugar	**2000**
5.4	**Decorations, coatings and fillings, except fruit based fillings covered by category 4.2.4**	only confectionary with no added sugar	**1000**
5.4	**Decorations, coatings and fillings, except fruit based fillings covered by category 4.2.4**	only cocoa or dried fruit based, energy reduced or with no added sugar	**2000**
5.4	**Decorations, coatings and fillings, except fruit based fillings covered by category 4.2.4**	only sauces	**350**
6.3	**Breakfast cereals**	only breakfast cereals with a fibre content of more than 15 %, and containing at least 20 % bran, energy reduced or with no added sugar	**1000**
7.2	**Fine bakery wares**	only essoblaten - wafer paper	**1000**
7.2	**Fine bakery wares**	only fine bakery products for special nutritional uses	**1700**
9.2.	**Processed fish and fishery products including molluscs and crustaceans**	only sweet-sour semi-preserves of fish and marinades of fish, crustaceans and molluscs	**300**
11.4.1	**Table-top Sweeteners in liquid form**		***quantum satis***
11.4.2	**Table-top Sweeteners in powder form**		***quantum satis***
11.4.3	**Table-top Sweeteners in tablets**		***quantum satis***
12.4	**Mustard**		**350**
12.5	**Soups and broths**	only energy-reduced soups	**110**
12.6	**Sauces**		**350**
12.7	**Salads and savoury based sandwich spreads**	only *Feinkostsalat*	**350**

Category number	Foods	restrictions/exception	Maximum level (mg/L or mg/kg as appropriate)
13.2	**Dietary foods for special medical purposes defined in Directive 1999/21/EC (excluding products from food category 13.1.5)**	Products in this category can also use additives that are allowed in the corresponding food counterparts categories	**1000**
13.3	**Dietary foods for weight control diets intended to replace total daily food intake or an individual meal (the whole or part of the total daily diet)**		**800**
14.1.3	**Fruit nectars as defined by Council Directive 2001/112/EC and vegetable nectars and similar products**	only energy-reduced or with no added sugar	**600**
14.1.4	**Flavoured drinks**	only energy reduced or with no added sugar	**600**
14.2.1	**Beer and malt beverages**	only alcohol-free beer or with an alcohol content not exceeding 1.2 % vol; 'Bière de table/Tafelbier/Table beer' (original wort content less than 6 %) except for 'Obergäriges Einfachbier'; Beers with a minimum acidity of 30 milli-equivalents expressed as NaOH; Brown beers of the 'oud bruin' type	**600**
14.2.1	**Beer and malt beverages**	only energy-reduced beer	**25**
14.2.3	**Cider and perry**		**600**
14.2.8	**Other alcoholic drinks including mixtures of alcoholic drinks with non-alcoholic drinks and spirits with less than 15 % of alcohol**		**600**
15.1	**Potato-, cereal-, flour- or starch-based snacks**		**500**
15.2	**Processed nuts**		**500**
16	**Desserts excluding products covered in category 1, 3 and 4**	only energy-reduced or with no added sugar	**1000**
17.1	**Food supplements supplied in a solid form including capsules and tablets and similar forms excluding chewable forms**		**2000**
17.2	**Food supplements supplied in a liquid form**		**600**
17.3	**Food supplements supplied in a syrup-type or chewable form**		**5500**

Considering the very specific foods in which aspartame is allowed and the restrictions within each food group, it was not possible to disaggregate the foods in the food consumption database used for this estimate. Therefore, approximations have been made and are explained in detail later in this section.

2.6.1. Actual level of use of aspartame

2.6.1.1. Reported use levels or data on analytical levels of aspartame (E 951)

Most food additives in the EU are authorised at a specific MPL. However, a food additive may be used at a lower level than the MPL. For those additives where no MPL is set and which are authorised as *quantum satis*, information on actual use levels is required. In the framework of Regulation (EC) No 1333/2008 on food additives and of Regulation (EC) No 257/2010 regarding the re-evaluation of approved food additives, EFSA issued a public call for scientific data on 1 June 2011 for aspartame (E 951) including current use and use patterns (i.e. which food categories and subcategories, proportion of food within categories/subcategories in which it is used, actual use levels (typical and maximum use levels), especially for those uses which are only limited by *quantum satis*).

Following the EFSA call, data from industry and others sources were received:

- from FoodDrinkEurope (FDE, 2011): usage data for several food uses were provided by its memberships

- from the International Sweetener's Association (ISA) for table-top sweetener products in the form of tablet and powder (ISA, 2011, 2012)

- from the International chewing gum association (ICGA, 2011): data on the uses of aspartame as a sugar substitute in chewing gum and as flavour enhancer in chewing gum with added sugars

- from the Spanish association on sweets (ProDulce, 2012): data on the uses of aspartame in sweets and flavoured drinks (only cocoa-based)

- from national authorities:

    - the Austrian Agency for Health and Food Safety (AGES)

    - the Dutch Food and Consumer Product Safety Authority (NVWA)

    - The Slovakian State Veterinary and Food Institute of Bratislava

- data from an Italian publication (Arcella et al., 2004) were also made available to EFSA.

2.6.1.2. Summarised data on reported use levels in foods from industries and other sources

Table 6 provides data on the use levels of aspartame in foods as reported by industry and as analysed by national agencies. This table also shows the levels used for the refined exposure assessment identified by the Panel based on data for several food categories in finished products reported by industry or analytical data from other sources (Member States, scientific literature).

In the EU regulation, tabletop sweeteners are allowed to contain aspartame at *quantum satis* level. Industrial data provided for this food group come from the International Sweetener's Association only, which did not consider them as being representative for the European market because these data were received from only two tabletop sweeteners companies. However, in order to take into account this food group in the refined exposure assessment, the Panel decided to use available information from the other sources (analytical and published data). Therefore, in place of *quantum satis* (QS) data, a maximum estimated usage level of 500 000 mg/kg was used by the Panel in its refined exposure assessment for tabletop sweeteners. This estimated usage level appears to be consistent with the upper end of the range reported by industry (ISA) for the tablet form.

The Panel noted that FoodDrinkEurope's membership and ISA do not cover all manufacturers in a particular sector or country and that the values reported for each individual food category are a general output, i.e. a compilation of the individual contributions provided by their members. Due to the limited representativeness of some of the data received for the European market, the Panel decided not to take these data into account for use in the refined exposure assessment with the exception of data received from the International chewing gum association (ICGA, 2011) on the uses of aspartame as a sugar substitute in chewing gum and as flavour enhancer in chewing gum with added sugars.

Table 6: Summary of levels used in the refined exposure assessment

Matching Foodex Food codes	Food items	MPL (mg/kg or mg/L)	Use levels reported by industry (mg/kg or mg/L)						Analytical data reported by food agencies/literature range min-max in mg/kg or mg/L (number of samples)				Levels used for calculation (mg/kg or mg/L)
			FDE		ISA	ICGA	ProDulce	Comments from industry	Austria	The Netherlands	Slovakia	Italy	
			typical	max	typical-max	typical-max	typical (range)						
1.4 - Flavoured fermented milk products including heat-treated products	Water-based flavoured drinks, energy-reduced or with no added sugar	1000	50-350	50-1000				Partly representative of the European market		54-132 (n=12)	93.5 (n=1)		1000
3 - Edible ices	Edible ices energy reduced or with no added sugar	800	40	50				Limited representation of the European market					800
4.2 - Processed fruit and vegetables	Energy-reduced jams, jellies and marmalades/energy-reduced fruit and vegetable preparation	1000	350-800	1000				Partly representative of the European market	107-695 (n=137)		74.5 (n=1)		1000
5.1 - Cocoa and Chocolate products as covered by Directive 2000/36/EC		2000					500-1000	No information on the representativeness, most probably only representative of Spanish products					2000
5.2.2 - Other confectionery without added sugar		1000	500-800	500-1000			100-1000	Partly representative of the European market	68.3 (n=7)	12 (n=4)	151.1-911.7 (n=6)		1000
5.3.1 - Chewing gum with added sugar		2500				600-1450		Maximum levels up to 7 % of chewing-gums and 50 % of chewing-gum contains aspartame as flavour enhancer at a typical level.					1450

Matching Foodex Food codes	Food items	MPL (mg/kg or mg/L)	Use levels reported by industry (mg/kg or mg/L)						Analytical data reported by food agencies/literature range min-max in mg/kg or mg/L (number of samples)				Levels used for calculation (mg/kg or mg/L)
			FDE		ISA	ICGA	ProDulce	Comments from industry	Austria	The Netherlands	Slovakia	Italy	
			typical	max	typical-max	typical-max	typical (range)						
5.3.2 - Chewing gum without added sugar		5500				3650-5420		Maximum levels for 4 % of the products and up to 70 % of sugar-free chewing gum contain aspartame at a typical level.	40-1747 (n=5)		560.8 (n=1)		5420
6.3 - Breakfast cereals		1000											1000
9.2 - Processed fish and fishery products including molluscs and crustaceans		300											300
11.4 - Table-top Sweeteners		QS			8700-36,000 (Powder form) 100,000 - 360,000 (tablet form)			Limited representation of the European market. Response from 2 table-top sweeteners	< 0.25-238,600 * (n=19)	230 647 (n=1, tablet form)		0-500,000 (n=10) tablet form)	500,000
12.4 - Mustard		350											350
12.5 - Soups and broths		110											110
12.6 - Sauces		350	200	200				Limited representation of the European market. Levels refer to ketchup	< 4 (n=2)				350
12.7 - Salads and savoury based sandwich spreads	only Feinkostsalat	350							< 20 (n=6)				350
13.2 - Dietary foods for special medical purposes defined in Directive 1999/21/EC		1000	180	180				Limited representation of the European market					1000

Matching Foodex Food codes	Food items	MPL (mg/kg or mg/L)	Use levels reported by industry (mg/kg or mg/L)						Analytical data reported by food agencies/literature range min-max in mg/kg or mg/L (number of samples)				Levels used for calculation (mg/kg or mg/L)
			FDE		ISA	ICGA	ProDulce	Comments from industry	Austria	The Netherlands	Slovakia	Italy	
			typical	max	typical-max	typical-max	typical (range)						
(excluding 13.1.5)													
13.3 - Dietary foods for weight control diets intended to replace total daily food intake or an individual meal		800											800
14.1.3 - Fruit nectars as defined by Council Directive 2001/112/EC and vegetable nectars and similar products		600							< 20 -71 (n=19)				75[1]
14.1.4.2 - Flavoured drinks with sweeteners		600	100-350	600			110	FDE: Representative for the European market. 600 mg/L only apply in 1 product, rest of the applications reported usage levels below 430 mg/L ProDulce: refer to powder, only for cocoa-based soft drinks	< 1-496 (n=272)	< 3-470 (n=40)	3.5-2559.1** (n=60)		600
14.2 - Alcoholic beverages, including alcohol-free and low-alcohol counterparts	Drinks consisting of a mixture of a non-alcoholic drink and beer, cider, perry, spirits or wine. Spirits drinks containing less than 15% alcohol by volume	600	100-350	600				Partly representative of the European market	< 1-104 (n=47)		7.4-25.6 (n=4)		600

Matching Foodex Food codes	Food items	MPL (mg/kg or mg/L)	Use levels reported by industry (mg/kg or mg/L)						Analytical data reported by food agencies/literature range min-max in mg/kg or mg/L (number of samples)				Levels used for calculation (mg/kg or mg/L)
			FDE		ISA	ICGA	ProDulce	Comments from industry	Austria	The Netherlands	Slovakia	Italy	
			typical	max	typical-max	typical-max	typical (range)						
15.1 - Potato-, cereal-, flour- or starch-based snacks		500											500
15.2 - Processed nuts		500											500
16 - Desserts excluding products covered in category 1, 3 and 4		1000											1000
17 - Food supplements as defined in Directive 2002/46/EC[5] excluding food supplements for infants and young children		5500							< 20-1845.4 (n=3)	< 20-2245 (n=17)			2245

[1] rounded figure

*no clear information on the form of the tabletop sweeteners but the Panel assumes that values refer to tablet forms.

** 2 values out of the 60 exceed the MPL of 600 mg/kg (one on 'herbal tea with honey, cinnamon and orange'; another on 'milk chocolate with added sweeteners')

Following rules defined by the Panel (Appendix B.I), maximum values between use levels and analytical data were used for the refined exposure. Data from industry could be considered as non-representative due to the comments provided. Analytical data were considered as representative when the number of data reported was above or equal to 10 samples. When no data were suitable, MPLs were used to estimate the exposure.

2.7. Information on existing authorisations and evaluations of aspartame, methanol and DKP

2.7.1. Existing authorisations and evaluations of aspartame

Aspartame has been authorized in the EU for use in foods and as a tabletop sweetener by several Member States since the 1980s.

The SCF evaluated aspartame in 1984 (SCF, 1985) and in 1988 (SCF, 1989). The SCF established an acceptable daily intake (ADI) of 40 mg/kg body weight (bw)/day. Further reviews of data on aspartame were carried out by the SCF in 2002 (SCF, 2002). In the 2002 evaluation, the SCF concluded that there was no evidence to suggest a need to revise the ADI previously established for aspartame.

The European legislation harmonized its use in foodstuffs in 1994 through the European Parliament[15] and Council Directive 94/35/EC, following safety evaluations by the SCF (SCF, 1985; SCF 1989).

Aspartame was evaluated by the Joint FAO/WHO Expert Committee on Food Additives on several occasions (JECFA, 1975, 1976, 1980, 1981). JECFA established an ADI of 40 mg/kg bw/day (1980, 1981).

In 1974, US FDA (US Food and Drug Administration) approved aspartame for restricted use in dry foods in the United States. This was followed by its full approval for use as an artificial sweetener in 1981. The FDA has set the ADI for aspartame at 50 mg/kg bw.

In 1996, a report suggesting a connection between aspartame and an increase in the incidence of brain tumours in the USA was published (Olney et al., 1996). The conclusions of this epidemiological study have been criticised by a number of scientists who questioned the methodology used and the interpretation of the data (Levy and Heideker, 1996; Ross, 1998; Smith et al., 1998; Seife, 1999). The SCF considered this report at its 107th meeting in June 1997 and concluded that the data did not support the proposed biphasic increase in the incidence of brain tumours (SCF, 1997). The issue has also been considered by the FDA and by the UK Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment (COC, 1996). The FDA stated that analysis of the National Cancer Institute database on cancer incidence in the USA did not support an association between the use of aspartame and increased incidence of brain tumours (FDA, 1996). The COC agreed that the findings provided no evidence for the proposed biphasic increase in the incidence of either all brain tumours or selected tumour types in the USA during the 1980's and concluded that the data published by Olney et al. (1996) did not raise any concerns about the use of aspartame in the UK (COC, 1996).

In 2002, a review of aspartame safety was published by AFSSA (Agence française de sécurité sanitaire des aliments). AFSSA (2002) concluded that aspartame was not genotoxic and that the previously conducted carcinogenicity tests in rodents did not indicate a relationship between treatment with aspartame and the appearance of brain tumours:

'Taking into account all the studies that have been conducted, the frequency of spontaneous tumours in laboratory rats, the types of tumours observed and the absence of a dose-response relationship, it

[15] Directive 94/35/EC of the European Parliament and of the Council of 30 June 1994 on sweeteners for use in foodstuffs. OJ L 237, 10.9.1994, p. 3-12.

was concluded that aspartame had no carcinogenic potential on the brain in experimental animals (FDA FR, 1981-1984; Koestner, 1984; Cornell et al., 1984; Flamm, 1997)'.

AFSSA concluded that the epidemiological study by Olney et al. (1996) did not provide any scientific evidence of a relationship between exposure to aspartame and the appearance of brain tumours. AFSSA (2002) also reported on the study by Gurney et al. (1997) who published the results of a case-control study on the relationship between the consumption of aspartame and the frequency of brain tumours. AFSSA agreed with the authors' conclusion that no relationship could be established between the consumption of aspartame and the frequency of brain tumours.

Furthermore AFSSA noted that the epidemiological data from the cancer registers in France did not permit a possible aspartame-brain tumour relationship to be clearly determined, but they did show that, at the time, the sale of this food additive in France was not accompanied by an increase in the frequency of brain tumours or increased mortality from this disease in the general population.

The SCF in its opinion of 2002 agreed with the AFSSA evaluation of data on a relationship between exposure to aspartame and brain tumours in humans. The SCF conclusions in the opinion from 2002 were supported by the UK Food Standards Agency (FSA, 2002).

In 2006, at the request from the European Commission, the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) assessed a long-term carcinogenicity study in rats exposed to aspartame performed by European Ramazzini Foundation (ERF) (Soffritti et al., 2006). On the basis of all the evidence available from the Soffritti et al. (2006) study, other recent studies and previous evaluations, the AFC Panel concluded that there was no reason to revise the previously established ADI for aspartame of 40 mg/kg bw/day, as established by the SCF in 1984 (SCF, 2002; EFSA, 2006). The UK Committee on Carcinogenicity of Chemicals in Food Consumer Products and the Environment (COC) also evaluated the Soffritti et al. (2006) study on aspartame following the evaluation by EFSA (COC, 2006). In light of the limitations in the design of this study and the use of animals with a high infection rate, the COC considered that no valid conclusions could be drawn from this study. Therefore, the COC agreed that the Soffritti et al. (2006) study did not indicate a need for a review of the ADI for aspartame (COC, 2006).

In 2009, following a request from the European Commission, the ANS Panel delivered a scientific opinion on the results of a second long-term carcinogenicity study in rats starting with prenatal exposure to aspartame, performed by the ERF (Soffritti et al., 2007). The ANS Panel concluded that there was no indication of any genotoxic or carcinogenic potential of aspartame and no reason to revise the previously established ADI for aspartame of 40 mg/kg bw/day (EFSA 2009a, b).

In 2009 a series of meetings of National Experts nominated by the EU Member States was organized, with the support of the EFSA Advisory Forum, to review the scientific literature on aspartame, including non-peer-reviewed and anecdotal evidence that had become available since 2002. The report of these meetings was published in 2010, and included a re-evaluation of the data on the potential carcinogenicity (including cancer epidemiology), genotoxicity and neurotoxicity of aspartame (EFSA, 2010). The conclusion by the National Expert meeting of the EFSA Advisory Forum was that there was no new evidence that required a recommendation to EFSA that the previous opinions on aspartame adopted by the AFC and ANS Panels and the SCF should be reconsidered.

In 2011 the ANS Panel and EFSA evaluated a new long-term carcinogenicity study in mice exposed to aspartame from the 12th day of fetal life until death (Soffritti et al., 2010) and a prospective cohort study on the association between intakes of artificially sweetened soft drinks and pre-term delivery (Halldorsson et al., 2010). The authors of the first study concluded that aspartame induced cancer in the livers and lungs of male Swiss mice. EFSA and the ANS Panel observed that the hepatic and pulmonary tumour incidences reported by Soffritti et al. (2010) all fell within their own historical control ranges for spontaneous tumours and noted that Swiss mice are known to have a high background incidence of spontaneous hepatic and pulmonary tumours. The overall conclusion by the

ANS Panel and EFSA was that the information available from the Soffritti et al. (2010) publication did not give reason to reconsider the previous evaluations of aspartame (EFSA 2011a; EFSA ANS Panel, 2011). The authors of the Halldorsson et al. (2010) study concluded that there was an association between intake of artificially sweetened soft drinks and pre-term delivery in the cohort; however, additional studies were required to reject or confirm the association. The ANS Panel advised EFSA on the need for epidemiological expertise to provide additional insights on the methodology and statistical aspects of this study, taking into account confounding factors (EFSA ANS Panel, 2011). EFSA concluded that there was no evidence available to support a causal relationship between the consumption of artificially sweetened soft drinks and preterm delivery and that additional studies were required to reject or confirm the association (EFSA, 2011a). In 2011, the French Agency for Food, Environmental, and Occupational Health and Safety (ANSES) also concluded that no causal relationship was established between the consumption of artificially sweetened beverages and the risk of pre-term delivery, and, as the authors of the Halldorsson study mentioned, there was a need to perform further studies to negate or confirm the results (ANSES, 2011).

2.7.2. Existing authorisations and evaluations of methanol

In 1997, WHO (World Health Organization) stated that both methanol and its metabolite formate occur naturally in humans and that normal background levels should not pose any risk to health and consequently that levels of human exposure that do not result in levels of blood formate above background levels could be considered to pose insignificant risk. Based on information from limited studies in humans, WHO concluded that occupational exposure to current exposure limits (around 260 mg/m^3) or single oral exposure to approximately 20 mg/kg bw would fall into this category.

In 2003, the Center for the Evaluation of Risks to Human Reproduction (CERHR) evaluated the potential effects of methanol on human reproduction and development and concluded that there was minimal concern for adverse developmental effects when humans are exposed to methanol levels that result in low blood methanol concentrations, i.e., < 10 mg/L blood (NTP-CERHR, 2003). The CERHR Panel also concluded that there was negligible concern for adverse male reproductive effects when exposed to methanol levels that result in a low blood methanol level (< 10 mg/L blood) but there was insufficient evidence to assess the effects of methanol on female reproduction.

The Subcommittee on the Classification of Carcinogenic Substances of the Dutch Expert Committee on Occupational Exposure Safety (DECOS), a committee of the Health Council, concluded that methanol cannot be classified with respect to its carcinogenicity (comparable with EU class 'not classifiable') (Health Council of the Netherlands, 2010). The subcommittee was further of the opinion that results from genotoxicity tests indicate that methanol is not likely to have a genotoxic potential.

In March 2011, the UK COT (UK Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment) issued a statement on effects of chronic dietary exposure to methanol. The COT was asked to consider the effects of chronic oral methanol exposure in the light of consumer concerns that methanol arising from the breakdown of the sweetener aspartame could be harmful (COT, 2011). In its statement, the COT concluded that exposure to methanol at the levels found in the diet (dietary methanol has been estimated to be up to 1 g/day), both naturally occurring and from currently permitted levels of aspartame, would not be expected to result in adverse effects.

The US Environmental Protection Agency (EPA) recently conducted a toxicological review of methanol for all non-cancer endpoints. The report is expected to be published by the end of 2013.

2.7.3. Existing authorisations and evaluations of DKP

JECFA and SCF established an ADI for DKP of 7.5 mg/kg bw/day (JECFA, 1980; SCF, 1989). According to Commission Regulation (EU) No 231/2012, aspartame can contain DKP up to 1.5 % (expressed on a dry weight basis). In 1983, the US FDA established an ADI for DKP of 30 mg/kg bw/day (FDA, 1983).

2.8. Exposure assessment

Anticipated exposures to aspartame from its use as a food additive and its related by-product compounds, methanol, DKP, phenylalanine and aspartic acid, have been estimated in this section of the opinion. β-aspartame exposure was not considered due to its low presence, as aspartame is not degraded to this product.

The estimated exposures are based on the degradation process of aspartame as presented in Figure 2. Exposure calculations are based on the complete metabolism of aspartame to phenylalanine, aspartic acid and methanol. The calculation of exposure to DKP is based on the occurrence data received by EFSA and summarised in Table 3.

2.8.1. Food consumption data used for exposure assessment

In 2010, the EFSA Comprehensive European Food Consumption Database (Comprehensive Database) was built from existing national information on food consumption at a detailed level. Competent authorities in European countries provided EFSA with data on the level of food consumption by the individual consumer from the most recent national dietary survey in their country (cf. Guidance of EFSA 'Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment' (EFSA, 2011b)).

The food consumption data gathered at EFSA were collected by different methodologies and thus direct country-to-country comparison should be made with caution.

Anticipated exposures to aspartame from its use as a food additive and its related by-product compounds (methanol, DKP, phenylalanine and aspartic acid) have been calculated (mean and 95th percentile of consumers only) using the food consumption data at the individual level (e.g. raw data on food consumption by the individual consumer).

High-level consumption was only calculated for those foods and population groups where the sample size was sufficiently large to allow calculation of the 95th percentile (EFSA, 2011b). The Panel estimated chronic exposure for the following population groups: toddlers, children, adolescents, adults and the elderly. Calculations were performed using individual body weights.

Thus, for the present assessment, food consumption data were available from 26 different dietary surveys carried out in 17 different European countries, as mentioned in Table 7.

Table 7: Population groups considered for the exposure estimates of aspartame (E 951)

Population	Age range	Countries with food consumption surveys covering more than one day
Toddlers	from 12 up to and including 35 months of age	Belgium, Bulgaria, Finland, Germany, Italy, Netherlands, Spain
Children[a]	from 36 months up to and including 9 years of age	Belgium, Bulgaria, Czech Republic, Denmark, Finland, France, Germany, Greece, Italy, Latvia, Netherlands, Spain, Sweden
Adolescents	from 10 up to and including 17 years of age	Belgium, Cyprus, Czech Republic, Denmark, France, Germany, Italy, Latvia, Spain, Sweden
Adults	from 18 up to and including 64 years of age	Belgium, Czech Republic, Denmark, Finland, France, Germany, Hungary, Ireland, Italy, Latvia, Netherlands, Spain, Sweden, UK
The elderly[a]	Older than 65 years	Belgium, Denmark, Finland, France, Germany, Hungary, Italy

a: The terms 'children' and 'the elderly' correspond respectively to 'other children' and the merge of 'elderly' and 'very elderly' in the Guidance of EFSA on the 'Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment' (EFSA, 2011b).

Consumption records were codified according to the FoodEx classification system (EFSA, 2011b). Nomenclature from FoodEx classification system has been linked to the Food Classification System as presented in the Commission Regulation (EU) No 1129/2011, part D, to perform exposure estimates.

In order to have an accurate selection of foods in which aspartame is authorised and thus an accurate exposure estimate of aspartame, in addition to the FoodEx nomenclature, the original national food names (i.e. original food descriptors as written in the national surveys) were used as much as possible.

2.8.2. Exposure to aspartame from its use as a food additive

Exposure to aspartame (E 951) from its use as a food additive has been calculated by using (1) MPLs as listed in Table 5 and (2) data on reported use levels, or data reported on analytical levels as listed in Table 6, both combined with national consumption data for the five population groups. Detail summary of total estimated exposure (using MPLs and use levels) per age class and survey is presented in Appendix C.

No specific food consumption data for diabetics were available, therefore this sub-group of the population was not considered in this exposure estimate. However, aspartame intakes by diabetics have been estimated in several publications based on conservative dietary exposure to aspartame (using individual consumption data from food dairy method and MPLs). The exposure ranges from 1.2-5.3 mg/kg bw/day at the mean, to 1.9-15.6 mg/kg bw/day at the high level (Garnier-Sagne et al., 2001; Magnusson et al., 2007; Huvaere et al., 2012).

Most of the food items in which aspartame is authorised are subject to limitations (Table 5), therefore, specific foods within the Comprehensive Database were selected. The Panel noted that its estimates should be considered as being conservative as it is assumed that all processed foods contain the sweetener aspartame (E 951) added at the MPL or the maximum reported use levels.

However, due to the very detailed limitations laid down in the legislation, it was not always possible to match food categories in the food consumption database.

- Fine bakery wares for special nutritional purposes are not defined by EU legislation. Some Member States have their own definition, such as bakery ware that is targeted at diabetics. As it is not the intention of the Commission to define this category under 'foods intended for particular nutritional uses' (PARNUTS), category 13, it is referred to under category 7.2, fine

bakery ware in the food categorisation system[16]. Indeed, these products are not targeted at the general population; therefore, the Panel decided not to consider this category in the dietary exposure estimate for aspartame in the general population.

- Essoblaten-wafer paper is a specific food item in the fine bakery ware category that is not present in the FoodEx nomenclature. Since its weight is very low and its consumption most probably marginal, this food was not taken into account.

- Breakfast cereals with a fibre content of more than 15 % and containing at least 20 % bran, energy-reduced or with no added sugar are a very specific kind of breakfast cereals, not consumed by children and not described as such in the consumption data nomenclature. Therefore, this food was not taken into account in the present estimate.

- The distinction between products with and without added sugar was only possible for the food groups: flavoured drinks, confectionery and chewing gums, although this was unclear for some surveys. This may result in an over/under-estimation depending on the reporting of these products in the surveys. Therefore, the whole food groups were taken into account for flavoured fermented milk products including heat-treated products, edible ices, soups and broths, fruit nectars, desserts, jams and other fruit and vegetable preparations. This may result in an over-estimation depending of the real consumption of these products within the food groups.

- The food group decorations, coatings, fillings are not described and available in the nomenclature of the consumption database; thus, this food group could not be taken into account. This resulted in a minor underestimation.

- The distinction between the form of tabletop sweeteners (tablet, powder, liquid) was not available in the EFSA Comprehensive Database. Therefore, these three food groups were considered as a whole and the highest reported use level (from tablets) was taken into account. This would result in an overestimation.

- The same applies to the food supplements: no distinction between the forms of the food supplements (liquid, solid, chewable forms) is possible within the FoodEx nomenclature, therefore these three food groups were considered as a whole and the highest levels (MPLs and reported use levels) were taken into account. This represents a minor overestimation.

Table 8 summarises the estimated exposure to aspartame (E 951) from its use as a food additive in all five population groups.

[16] Part D (food categories) of the Commission Regulation (EU) N°1129/2011 of 11 November 2011.

Table 8: Summary of anticipated exposure to aspartame (E 951) from its use as a food additive using MPLs and reported use levels or analytical data on use levels in five population groups (min-max across the dietary surveys in mg/kg bw/day)

	Toddlers (12-35 months)	Children (3-9 years)	Adolescents (10-17 years)	Adults (18-64 years)	The elderly (> 65 years)
Estimated exposure using MPLs					
• Mean	3.2-16.3	2.3-12.8	0.8-4.0	0.8-8.6	0.5-4.4
• High level[a]	11.8-36.9	7.1-32.9	2.3-13.3	2.5-27.5	1.5-23.5
Estimated exposure using reported use levels or analytical data					
• Mean	1.6-16.3	1.8-12.6	0.8-4.0	0.7-8.5	0.4-4.4
• High level[a]	7.5-36.0	6.3-32.4	2.3-13.2	2.4-27.5	1.4-23.5

a: 95th percentile of consumers only

Despite being presented as the estimated exposure based on MPLs, the reported use levels for tabletop sweeteners were used for this exposure estimate. Indeed, tabletop sweeteners do not have MPLs but their conditions of use are defined in the legislation at *Quantum Satis* (QS).

The Panel noted that no major changes were seen in the anticipated exposure to aspartame using the reported use levels in comparison to the use of MPLs due to a very small difference observed between reported use levels and MPLs (Table 8).

2.8.3. Main food categories contributing to exposure of aspartame using MPLs

Table 9: Main food categories contributing to the total anticipated mean dietary exposure to aspartame (E 951) (> 5 % of total exposure) using MPLs and number of surveys in which each food category is contributing over 5 %.

	Toddlers	Children	Adolescents	Adults	The elderly
Food Categories	**% contribution to total exposure (Number of surveys)***				
1.4 - Flavoured fermented milk products including heat-treated products	15-72 (7)	13-52 (13)	8-47 (10)	6-16 (13)	6-33 (7)
5.1 - Cocoa and chocolate products as covered by Directive 2000/36/EC	10-17 (6)	8-35 (15)	8-52 (12)	5-25 (14)	12-22 (2)
3 - Edible ices	5-22 (4)	5-21 (14)	5-29 (11)	7-17 (6)	8 (2)
14.1.4 - Flavoured drinks with sweeteners	6-25 (2)	7-15 (8)	8-44 (6)	7-35 (11)	11 (1)
4.2 - Processed fruit and vegetables**	13 (2)	5-10 (9)	6-8 (9)	5-26 (11)	6-46 (7)
14.1.3 - Fruit nectars as defined by Council Directive 2001/112/EC and vegetable nectars and similar products	10-56 (2)	44 (1)		20-33 (2)	13 (1)
16 - Desserts excluding products covered in category 1, 3 and 4	6.5-19 (3)	5.1-17.6 (7)	7-14 (4)	7-10 (3)	7 (1)
11.4 - Table-top sweeteners***	-	6-14.7 (2)	9-16 (2)	5-55 (11)	36-66 (5)
12.5 - Soups and broths	-	14 (1)	13 (1)		5 (1)
12.7 - Salads and savoury based sandwich spreads	-	9-12 (2)	14 (1)	7-25 (2)	

	Toddlers	Children	Adolescents	Adults	The elderly
Food Categories	**% contribution to total exposure (Number of surveys)***				
14.2 - Alcoholic beverages, including alcohol-free and low-alcohol counterparts	-	-		6-7 (5)	
15.1 - Potato-, cereal-, flour- or starch- based snacks	7 (1)	5 (1)	6-11 (3)		
12.6 - Sauces	-	5.8-8.2 (2)	5-9 (6)	8 (1)	
17 - Food supplements					10 (1)

* Total number of surveys may be greater than total number of countries as listed in Table 7, as some countries submitted more than one survey for a specific age range.

** this category fruits and vegetables encompasses all the foods under the 4.2 category (Table 5): fruit and vegetables in vinegar, oil, or brine, canned or bottled fruit and vegetables, fruit and vegetable preparations excluding compote, extra jam and extra jelly as defined by Directive 2001/113/EEC, jam, jellies and marmalades and sweetened chestnut puree as defined by Directive 2001/113/EEC, other similar fruit or vegetable spreads.

*** by default, reported use levels for tabletop sweeteners were used for this estimate in order to take them into account even if QS.

As expressed previously, the Panel noted that the exposure estimate calculated with reported use levels/analytical levels was comparable to that calculated with MPLs. For this reason, a separate table on the contributions of food groups to the total exposure calculated with the reported use levels and analytical levels is not presented.

2.8.4. Exposure to methanol

The Panel noted that exposure to methanol is not only from aspartame as a food additive and from natural sources through the diet where it is a normal constituent, but also to a large extent from endogenous sources (basal endogenous pathway, endogenously metabolised pectin).

Methanol exposure from all sources is presented in Table 12. A detailed summary of total estimated exposure to methanol from all sources per age class and survey is presented in Appendix D.

2.8.4.1. Methanol from anticipated exposure to aspartame from its use as a food additive

Consumers may be exposed to methanol from consumption of aspartame containing products as methanol is released from aspartame in the gastrointestinal tract. Approximately 10 % methanol is released by weight of aspartame. Based on the anticipated exposure estimates for aspartame (Table 5) using MPLs or reported use levels or analytical data the estimated exposure to methanol from all food and beverage applications of the sweetener for the five population groups would range from 0.05 to 1.6 mg/kg bw/day at the mean and from 0.2 to 3.7 mg/kg bw/day at the high level (95^{th} percentile consumers only) (Table 10).

Table 10: Summary of anticipated exposure to methanol from anticipated exposure to aspartame (E 951) as a food additive using MPLs and reported use levels or analytical data on use levels in five population groups (min-max across the dietary surveys in mg/kg bw/day)

	Toddlers (12-35 months)	Children (3-9 years)	Adolescents (10-17 years)	Adults (18-64 years)	The elderly (> 65 years)
Estimated exposure using MPLs					
• Mean	0.3-1.6	0.2-1.3	0.1-0.4	0.1-0.9	0.05-0.4
• High level[a]	1.2-3.7	0.7-3.3	0.2-1.3	0.3-2.7	0.2-2.4
Estimated exposure using reported use levels or analytical data					
• Mean	0.2-1.6	0.2-1.3	0.1-0.4	0.1-0.8	0.04-0.4
• High level[a]	0.8-3.6	0.6-3.2	0.2-1.3	0.2-2.7	0.1-2.4

a: 95th percentile of consumers only

2.8.4.2. Methanol from natural food occurrence

Methanol may be present *per se* or can be released from foods including fruits, fruit juices, vegetables, roasted coffee, honey and alcoholic beverages. In fruit juices, concentrations vary widely (1-640 mg methanol/L) with an average of 140 mg/L (WHO, 1997). More recent measurements have confirmed that juices from fruits such as apples, bananas, grapes and oranges may contain methanol at levels ranging from 8-148 mg/L (Wu et al., 2007; Hou et al., 2008; Hammerle et al., 2011). In the case of tomatoes, levels of methanol up to 240 mg/L juice and 200 mg/kg diced and canned tomatoes have been reported (Hou et al., 2008; Anthon and Barrett, 2010). Likewise, vegetable juices (e.g. from alfalfa sprouts, carrots and spinach) were found to contain methanol levels ranging from 53-194 mg/L (Hou et al., 2008). The highly variable methanol content not only depends on the type of fruit and vegetable, i.e. methanol present before processing, but also on methanol released from pectin through the action of pectinesterase during processing and storage (Massiot et al., 1997).

Methanol is also found in alcoholic drinks. It is a residue of the manufacturing process of alcoholic beverages and maximum levels of methanol have been defined for spirit drinks in the Regulation (EC) No 110/2008. WHO reported methanol concentrations of 6-27 mg/L in beer, 96-321 mg/L in wine and 10-220 mg/L in distilled spirits (WHO, 1997). Depending on the cultivars, apple ciders have been reported to contain 300-700 mg methanol/L, whereas a methanol content of up to 1.6 g/L has been reported in apple spirit (Hang and Woodams, 2010).

To estimate exposure to methanol due to its natural presence in foods, the Panel used data from VCF (Volatile Compounds in Food) a TNO database (2012) compiled from 340 literature references for methanol in foods from papers dating from 1965 to 2006. The Panel noted that occurrence of methanol predominantly in fruit and fruit-based beverages is consistent and in line with current knowledge (Appendix B-II) and that methanol levels for individual products vary considerably.

In its review, the Panel considered that quantitative measurements of methanol in foods were generally reliable and that data generated using what would now be considered out-dated methodologies could be considered reasonably accurate (the current analytical method used for the determination of methanol in foods is based on headspace GC/MS).

Exposure to methanol has been estimated by the Panel using the raw individual food consumption data at the most detailed level from the Comprehensive Database (EFSA, 2011b) and using the mean methanol concentration, calculated from the natural occurrence data range reported in Appendix B-II. Mean values have been calculated by the Panel for the purpose of estimating background chronic intake of methanol from natural food occurrences which were based on the assumption that the

distribution of composition data per category (only when range is expressed) followed a uniform distribution.

Considering all the dietary surveys and populations groups, the exposure estimates for methanol from natural food occurrence range from 0.2-1.4 mg/kg bw/day at the mean, to 0.6-3.8 mg/kg bw/day for high level consumers (Table 11).

Table 11: Summary of anticipated exposure to methanol from natural food occurrence in five population groups (min-max across the dietary surveys in mg/kg bw/day)

	Toddlers (12-35 months)	Children (3-9 years)	Adolescents (10-17 years)	Adults (18-64 years)	The elderly (> 65 years)
Estimated exposure					
• Mean	0.2-1.4	0.3-1.4	0.2-0.6	0.3-0.6	0.2-0.6
• High level[a]	0.9-3.8	0.9-3.3	0.6-1.4	0.7-1.6	0.6-1.4

a: 95th percentile of consumers only

Mean and 95th percentile total intakes of methanol from foods naturally containing methanol have been reported to be 10.7 and 33.3 mg methanol/kg bw/day for US consumers among the general population using Daily Intake via Natural Food Occurrence (DINFO) analysis (Magnuson et al., 2007). However, this analysis did not include intake from processed foods or from food sources such as potatoes or onions, or the methanol released by pectin breakdown in fruits and vegetables during processing and storage, and as such will be an underestimation of the true dietary exposure to methanol.

The Panel noted that exposure estimates for methanol from natural sources reported in the above mentioned literature though lower, were globally within the same order of magnitude as the estimates derived by the Panel (assuming a 70 kg body weight, the mean and high consumers would have an exposure range of 0.2 to 0.5 mg/kg bw/day, respectively, in comparison to 0.2-1.6 mg/kg bw/day in the adult population).

2.8.4.3. Methanol from endogenous pathways

In addition to the direct contribution of methanol from fruits and vegetables and their juices, there is clear evidence for the production of methanol in the gastrointestinal tract from ingested pectins and endogenous production in other parts of the body, such as liver and brain (WHO, 1997).

In two papers (Taucher et al., 1995; Lindinger et al., 1997), methanol concentration was measured in the breath of human volunteers using proton-transfer-reaction mass spectrometry in a selected ion flow drift tube. The volunteers were male, in a fasted state to minimize the contribution of dietary pectin to the measured methanol and had received ethanol at time zero and at 2.5 hours in order to maintain blood ethanol concentration in excess of 150 mg/L. The latter approach was used to competitively inhibit methanol metabolism by alcohol dehydrogenase and CYP2E1, thereby enabling the measurement of methanol production by the body. The authors showed that basal endogenous methanol production by the human body, measured over a period of 6-12 hours, amounted to between 300 and 600 mg methanol/day (Lindinger et al., 1997). The same authors showed that the ingestion of 10 to 15 g of pectin (corresponding to 1 kg apples) resulted in the additional production of 400-1400 mg methanol/person/day.

Dhareshwar and Stella (2008) estimated that exposure to methanol from combined endogenous and exogenous sources was between 0.13 and 1.03 g/day.

In another study performed in six healthy male subjects, basal endogenous methanol production was assessed over a period of 5 hours. The subjects were given an infusion of ethanol to inhibit methanol metabolism after a period of fasting and abstinence from alcohol (Haffner et al., 1998). Methanol was

measured in blood using gas chromatography. The observed rate of increase in blood methanol concentration was linear between 30 min and 5 hours and ranged from 0.08–0.35 mg/L/hour. The Panel noted that when converting the blood levels to whole body production using a volume of distribution (V_D) for methanol of 0.77 l/kg (Graw et al., 2000), the daily endogenous production of methanol could be approximated to 1.5-6.5 mg/kg bw/day, or 107-453 mg/person/day.

Basal endogenous methanol production was measured in 22 female and 13 male volunteers given 4-methylpyrazole to inhibit ADH in a cross-over study (Sarkola and Eriksson, 2001). Compared to the placebo control, a linear increase in blood methanol levels was measured of up to 3.6 µM/hour, after 4-methylpyrazole application. The Panel noted that when converting the blood levels to whole body production using a volume of distribution (V_D) for methanol of 0.77 L/kg (Graw et al., 2000), a daily endogenous production of methanol could be estimated to be 2.1 mg/kg bw/day or 149 mg/person/day.

Ernstgård et al. (2005) investigated uptake and disposition of inhaled methanol in four female and four male subjects who had not consumed any alcohol, drugs or fruits for at least 48 hours prior to the beginning of the measurements. From the absorption, half-life and area under the concentration-time curve (AUC) values reported by the authors for methanol, the Panel derived a value for endogenous production of 9.1 mg/kg bw/day or 640 mg methanol/person/day.

The Panel concluded that basal endogenous methanol production ranges from 2 to 9 mg/kg bw/day (minimum to maximum) in adults. In the elderly, the endogenous methanol production may be up to 15 % less, as the resting metabolic rate is decreasing with age (Speakman and Westerterp, 2010). In infants and children less than 18 years of age there is a higher metabolic rate per body mass as compared to the adult (Wang, 2012). Hence, use of the endogenous production rate of the adult (expressed in mg/kg) in lieu of experimentally measured methanol production rates in toddlers may underestimate the endogenous production rate in this age by a factor of two. In addition, the body increases methanol production following consumption of pectin-containing foods such as apples by an additional 6-20 mg/kg bw/day (Lindinger et al., 1997).

To estimate exposure to methanol due to pectin degradation, the Panel used data on pectin content from Baker (1997), Mahattanotawee et al. (2006) and Holloway et al. (1983).

The exposure to methanol from pectins has been estimated by the Panel using the raw individual food consumption data at the most detailed level from the Comprehensive Database (EFSA, 2011b) and using the occurrence levels of methanol derived from pectin contents reported. Values are detailed in Appendix B-III. The Panel noted that occurrence of pectin is predominantly in fruit and vegetables. The degradation factor from pectin to methanol was derived from Lindinger et al. (1997) i.e. 10 g pectins resulted in a mean additional production of methanol of 900 mg.

2.8.4.4. Combined exposure to methanol from endogenous and exogenous sources

The Panel estimated the anticipated combined exposure to methanol from endogenous sources (basal endogenous pathway, endogenously metabolised pectin) and exogenous sources (aspartame as a food additive and natural food occurrence). A summary of the estimated exposures is given in Table 12.

The exposure to methanol has been estimated by the Panel using the raw individual food consumption data at the most detailed level from the Comprehensive Database (EFSA, 2011b) and using the occurrence data (Table 5 and Appendixes B-II; B-III).

The Panel estimated that the exposure to methanol from all sources (basal endogenous pathway and endogenously metabolised pectin, natural food occurrence through the diet and aspartame as a food additive) in five population groups would range from 8.4 to 18.9 mg/kg bw/day at the mean and from 15.1 to 35.1 mg/kg bw/day for high-level consumers.

Table 12: Total estimated exposure of methanol from all sources (food additive, endogenous pathways and natural food occurrence) in five population groups (min-max across the dietary surveys in mg/kg bw/day)

	Toddlers		Children		Adolescents		Adults		The elderly	
	Mean	**High level**	**Mean**	**High level**	**Mean**	**High level**	**Mean**	**High level**	**Mean**	**High level**
	mg/kg bw/day									
Exposure range of methanol from basal endogenous pathway	5.5[a]	9	5.5[a]	9	5.5[a]	9	5.5[a]	9	5.5[a]	9
Exposure range of methanol from endogenously metabolised pectin	7.9-12.5	15.2-24.1	3.9-10.5	7.7-20.0	2.1-5.0	5.3-10.5	2.4-4.2	5.1-7.9	3.0-4.5	6.0-8.1
Exposure range of methanol from natural food occurrence	0.2-1.4	0.9-3.8	0.3-1.4	0.9-3.3	0.2-0.6	0.6-1.4	0.3-0.6	0.7-1.6	0.2-0.6	0.6-1.4
Exposure range of methanol from all endogenous pathways (basal, endogenously metabolised pectin) and natural food occurrence	**13.8-18.4**	**25.2-34.2**	**9.8-17.4**	**17.2-31.5**	**8.0-10.7**	**15.0-19.9**	**8.2-10.3**	**14.7-18.1**	**8.9-10.6**	**15.7-17.7**
Exposure range of methanol from aspartame as a food additive (using MPLs)	0.3-1.6	1.2-3.7	0.2-1.3	0.7-3.3	0.1-0.4	0.2-1.3	0.1-0.9	0.3-2.7	0.05-0.4	0.2-2.4
Total anticipated exposure ranges for methanol from all sources (endogenous pathways (basal, endogenously metabolised pectin) and natural food occurrence and aspartame as a food additive)[b]	**14.7-18.9**	**26.3-35.1**	**10.4-17.6**	**18.1-31.8**	**8.4-11.0**	**15.5-20.2**	**8.4-10.3**	**15.1-18.2**	**9.0-10.7**	**15.8-17.9**

a: average of 2-9 mg/kg bw/day (assuming uniform distribution)

b: the exposure ranges were calculated by the Panel using individual data from each survey (figures listed in Appendix D). Note: it is not scientifically valid to simply sum all the high percentile values for each methanol source to derive the total high level exposure to methanol from all sources. Individuals who are high level consumers in one category are unlikely to be high level consumers in the other categories (EFSA, 2011b).

The contribution of aspartame-derived methanol is shown in Table 13.

Table 13: Total contribution of aspartame–derived methanol to the exposure from all sources (aspartame as a food additive, endogenous (basal and pectin-derived) and natural food occurrence) in five population groups (min-max in %)

	Toddlers Mean	Children Mean	Adolescents Mean	Adults Mean	The elderly Mean
			%		
Contribution of aspartame-derived methanol (using MPLs) for average consumers of aspartame to total mean anticipated exposure to methanol from all sources	1.7-9.2	1.6-9.7	0.8-4.8	0.7-9.4	0.5-4.7
Contribution of methanol from endogenous source (basal + pectin derived) to total mean anticipated exposure to methanol from all sources	84-95	86-94	91-96	87-96	91-97
Contribution of methanol from natural food occurrence to total mean anticipated exposure to methanol from all sources	1.2-7.6	2.1-7.8	1.9-5.5	2.7-6.2	2.4-5.3

The Panel noted that for average consumers of aspartame, the contribution from aspartame to the overall exposure to methanol ranged from 1 % to 10 % in the general population.

In this estimate, the Panel also noted that exposure to methanol from natural food occurrence is a minor contributing source (< 10 %) compared to exposure from endogenous pathways (> 80 % in all population groups), and also that the exposure from aspartame-derived methanol is similar to methanol exposure from natural food occurrence.

In addition to aspartame, there are other food additives that release methanol as a result of their metabolism. Examples of methanol releasing food additives include dimethyldicarbonate (E 242, DMDC), which is used in beverages and breaks down to CO_2 and methanol; pectins (E 440) that are subject to the methyl ester linkage cleavage in the upper gastrointestinal tract as described above; methyl p-hydroxybenzoate and sodium methyl p-hydroxybenzoate (E 218, E 219); hexamethylene tetramine (E 239). Therefore, while not considering the methanol release from other food additives the present estimation is considered as being an under-estimation of the total exposure to methanol.

An additional source of exposure to methanol is from extraction solvents. The Panel noted that in the EU the maximum residue limits for methanol in extracted foodstuff or food ingredients is set at 10 mg/kg.[17]

2.8.5. Exposure to 5-benzyl-3,6-dioxo-2-piperazine acetic acid (DKP) from anticipated exposure to aspartame from its source as a food additive

5-Benzyl-3,6-dioxo-2-piperazine acetic acid (DKP) exposure intakes were estimated from anticipated exposure to aspartame (E 951) from its use as a food additive. DKP is a degradation product of aspartame. The degradation from aspartame to DKP ranges from 1.5 % (EU specifications) up to 24 % based on the analytical data reviewed by the Panel and reported in Table 3. The maximum of 24 % is for soft drinks and is estimated from an average degradation of 80 % of aspartame of which DKP accounts for 30 %. The concentrations of DKP in foods taken into account by the Panel in estimating exposure were assigned to the relevant food categories in which aspartame is authorised according to MPLs and use levels scenarios as described in Appendix B-IV. For foods categories where data were

[17] Directive 2009/32/EC of the European Parliament and of the Council of 23 April 2009.

available, the highest value was taken into account; for the ones where no degradation percentage of DKP was available, the highest value (24 %) from the whole database was used. In a few food categories (processed fish and fishery products, tabletop sweeteners, food supplements) depending on the food type (solid vs. liquid), other degradation percentages of DKP were used.

Anticipated exposures to DKP from aspartame consumption are described in the Table 14. Detail summary of total estimated exposure (from the use of aspartame MPLs) per age class and survey is presented in Appendix E.

Table 14: Summary of anticipated exposure to DKP from anticipated exposure to aspartame (E 951) as a food additives using MPLs and reported use levels or analytical data on use levels in five population groups (min-max across the dietary surveys in mg/kg bw/day)

	Toddlers (12-35 months)	Children (3-9 years)	Adolescents (10-17 years)	Adults (18-64 years)	The elderly (> 65 years)
Estimated exposure using MPLs					
• Mean	0.3-1.9	0.2-1.3	0.1-0.7	0.1-1.6	0.1-0.8
• High level[a]	1.2-4.1	0.5-3.2	0.3-2.5	0.3-5.5	0.2-4.8
Estimated exposure using reported use levels or analytical data					
• Mean	0.2-1.9	0.2-1.3	0.1-0.7	0.1-1.6	0.1-0.8
• High level[a]	0.7-3.9	0.5-3.1	0.3-2.5	0.3-5.5	0.2-4.8

a: 95th percentile of consumers only

Based on the anticipated exposure estimates for aspartame using MPLs (Table 5), the estimated exposure to DKP from all food and beverage use of the sweetener would approximately, 0.1 to 1.9 mg/kg bw/day at the mean and 0.2 to 5.5 mg/kg bw/day for the 95th percentile consumer across the dietary surveys and populations groups.

2.8.6. Exposure to phenylalanine

The Panel noted that exposure to phenylalanine is not only from aspartame as a food additive but also largely from natural food occurrence through the diet.

Phenylalanine exposure from both sources is presented in Table 16. Detail summary of total estimated exposure (from the use of aspartame MPLs) per age class and survey is presented in Appendix E.

2.8.6.1. Phenylalanine from natural food occurrence

Phenylalanine is an essential amino acid and therefore must be supplied by the diet. The recommended aromatic amino acid requirement (phenylalanine or tyrosine) is set at 25 mg/kg bw/day (WHO, 2007). In the US, the Institute of Medicine sets estimated average requirement (EAR) for amino acids; phenylalanine + tyrosine EAR for adults are estimated at 27 mg/kg bw/day (IOM, 2005). The mean daily consumption of phenylalanine is estimated in the US at 3.4 g/day (IOM, 2005). A French study (Rousseau et al., 2006) reported in an AFSSA report on protein intake (AFSSA, 2007) estimated the daily intakes of different populations,: from sedentary people to sportspersons (several levels of expenditure of physical energy). The mean phenylalanine intake for sedentary people was estimated to be 3.8 g/day.

Additional information on phenylalanine intake from natural food occurrence through the diet and phenylalanine content per food category of the EFSA Comprehensive Database was provided to EFSA (König et al., (2012) unpublished data). Concentration data of phenylalanine in foods came from the German food composition database (Bundeslebensmittelschlüssel). The levels of phenylalanine

content per food category (according to level 2 of the food additives nomenclature from Regulation No 1129/2011) range from 0.01 mg/g in wine to 31.05 mg/g in meat substitutes; some food groups do not contain any phenylalanine. These levels linked to the EFSA Comprehensive Database allow calculation of phenylalanine intake from the diet. These intakes range from 0.9 g/day (corresponding to 93.0 mg/kg bw/day for toddlers) up to 4.1 g/day (corresponding to 58.7 mg/kg bw/day for adults) at the mean within the EU population (see Table 16).

2.8.6.2. Phenylalanine from anticipated exposure to aspartame from its source as a food additive

Phenylalanine exposure intakes were estimated from anticipated exposure to aspartame (E 951) from its use as a food additive. Aspartame is fully hydrolysed in the gastrointestinal tract to L-phenylalanine, L-aspartic acid and methanol. Taking the molecular weight of phenylalanine into account, the factor for aspartame exposure to phenylalanine exposure is 56 %. Phenylalanine intakes from aspartame consumption are described in Table 15.

Table 15: Summary of anticipated exposure to phenylalanine from estimated exposure to aspartame (E 951) as a food additive using MPLs and reported use levels or analytical data on use levels in five population groups (min-max across the dietary surveys in mg/kg bw/day)

	Toddlers (12-35 months)	Children (3-9 years)	Adolescents (10-17 years)	Adults (18-64 years)	The elderly (> 65 years)
Estimated exposure using MPLs					
• Mean	1.8-9.2	1.3-7.2	0.4-2.3	0.4-4.8	0.3-2.5
• High level[a]	6.6-20.7	4.0-18.4	1.3-7.5	1.4-15.4	0.9-13.2
Estimated exposure using reported use levels or analytical data					
• Mean	0.9-9.2	1.0-7.1	0.4-2.3	0.4-4.8	0.2-2.5
• High level[a]	4.2-20.2	3.5-18.2	1.3-7.4	1.3-15.4	0.8-13.2

a: 95th percentile of consumers only

2.8.6.3. Combined exposure to phenylalanine from the use of aspartame as a food additive and from natural food occurrence through the diet

In this estimate, the Panel noted that exposure to phenylalanine from aspartame-derived phenylalanine for all population groups ranged from 1 % to 8 % at the mean (compared to the total intake of phenylalanine from aspartame and natural food occurrence).

Table 16: Summary of anticipated combined exposure to phenylalanine from exposure to aspartame (E 951) as a food additive using MPLs and from natural food occurrence in five population groups (min-max across the dietary surveys in mg/kg bw/day)

	Toddlers (12-35 months)	Children (3-9 years)	Adolescents (10-17 years)	Adults (18-64 years)	The elderly (> 65 years)
Estimated exposure from natural food occurrence					
• Mean	93.0-167.1	72.2-136.7	39.1-79.0	35.3-58.7	32.5-41.6
• High level[a]	144.2-322.2	126.5-214.3	73.1-130.3	60.2-88.7	54.6-64.4
Estimated exposure using MPLs					
• Mean	1.8-9.2	1.3-7.2	0.4-2.3	0.4-4.8	0.3-2.5
• High level[a]	6.6-20.7	4.0-18.4	1.3-7.5	1.4-15.4	0.9-13.2
Combined exposure to PHE					
• Mean	95.4-176.3	74.0-140.5	41.3-80.7	37.9-59.1	34.8-41.9
• High level[a]	146.5-331.1	130.9-224.1	78.4-136.9	64.8-89.1	58.9-68.2

a: 95th percentile of consumers only

2.8.7. Exposure to aspartic acid from anticipated exposure to aspartame from its source as a food additive

Aspartic acid exposure intakes were estimated from anticipated exposure to aspartame (E 951) from its use as a food additive. Taking the molecular weight into account, the factor to derive aspartic acid exposure from aspartame exposure is 45 %. Aspartic acid intakes from aspartame consumption are described in the Table 17. Detail summary of total estimated exposure (from the use of aspartame MPLs) per age class and survey is presented in Appendix E.

Table 17: Summary of anticipated exposure to aspartic acid from estimated exposure to aspartame (E 951) as a food additives using MPLs and reported use levels or analytical data on use levels in five population groups (min-max across the dietary surveys in mg/kg bw/day)

	Toddlers (12-35 months)	Children (3-9 years)	Adolescents (10-17 years)	Adults (18-64 years)	The elderly (> 65 years)
Estimated exposure using MPLs					
• Mean	1.5-7.4	1.0-5.8	0.4-1.8	0.3-3.9	0.2-2.0
• High level[a]	5.3-16.7	3.2-14.9	1.0-6.0	1.1-12.4	0.7-10.6
Estimated exposure using reported use levels or analytical data					
• Mean	0.7-7.4	0.8-5.7	0.4-1.8	0.3-3.8	0.2-2.0
• High level[a]	3.4-16.3	2.8-14.7	1.0-6.0	1.1-12.4	0.6-10.6

a: 95th percentile of consumers only

In the US, the Third National Health and Nutrition Examination Survey (1988-1994) estimated the mean intake of aspartic acid of 6.5 g/day (IOM, 2005). High levels (99th percentile) were estimated at 15.4 g/day for the 31-50 year old male population.

2.9. Uncertainty analysis

According to the guidance provided by the EFSA opinion (EFSA, 2006), the following sources of uncertainties have been considered. These have been presented and discussed in detail in the sections above:

- ***Input data:***

  - Food consumption data: the data used for the exposure estimates were data reported at the individual level in several different dietary surveys that applied different methodologies. Under-reporting and/or misreporting often represent a bias in dietary surveys.

  - Food nomenclature: the food nomenclatures used in the dietary surveys considered and then the FoodEx nomenclature used in the Comprehensive database are different from the Food Classification System developed for the definition of food additives uses in the Commission Regulation (EU) No 1129/2011. Therefore, linking between different food nomenclatures leads to uncertainties.

  - Reported use levels: the estimates calculated are considered as being conservative because they are based on the assumption that all products within a given food category contain aspartame at the maximum reported use level.

Table 18: Qualitative evaluation of influence of uncertainties

Sources of uncertainties	**Direction** [a]
Consumption data: different methodologies / representativeness / under reporting / misreporting / no portion size standard	+/-
Extrapolation from food consumption survey of few days to estimate chronic exposure	+
Linkage between reported use levels and food items in the consumption database: uncertainties on which precise types of food the use levels refer.	+/-
Occurrence data: maximum reported use levels within a food category	+
Exposure model: uncertainty in possible national differences in use levels of food categories, data set not fully representative of foods on the EU market, exposure calculations based on the maximum reported use levels (no use of typical use levels when available)	+

a: +: uncertainty with potential to cause over-estimation of exposure;
-: uncertainty with potential to cause under-estimation of exposure.

As a whole, the total estimated uncertainty from all sources should generally lead to an overestimation of the calculated exposures (for aspartame and its related by-products: DKP, methanol, aspartic acid, phenylalanine), thus providing conservative estimates.

3. Biological and toxicological data of aspartame

A public call was launched by EFSA in 2011 following which original reports of unpublished studies were received (http://www.efsa.europa.eu/en/dataclosed/call/110601.htm). The Panel was aware that many of these reports would have been available at the time of the JECFA and SCF evaluations. The present evaluation by the Panel of the biological and toxicological data includes the assessment of all these unpublished studies, together with the published literature identified until the end of November 2012.

The Panel noted that the majority of these unpublished studies were performed in the period 1970-1978 and as such had not been performed according to Good Laboratory Practice (GLP) and Organisation for Economic Co-operation and Development (OECD) test guidelines (http://www.oecd.org/chemicalsafety/testingofchemicals/oecdseriesonprinciplesofgoodlaboratorypracticeglpandcompliancemonitoring.htm). However, according to the Panel, the fact that the studies were old and were not performed according to current standards should not *per se* disqualify them from being included in the risk assessment of aspartame. As long as the design of any such study and the reporting of the data were appropriate, the Panel agreed that the study should be considered in the re-evaluation of the sweetener.

3.1. Absorption, distribution, metabolism and excretion of aspartame

Throughout the following sections, the term aspartame refers to the α-form.

3.1.1. Studies on absorption, distribution, metabolism and excretion received following a public call for data which were available at the time of the previous JECFA and SCF evaluations

The Panel noted studies conducted in the 1970s using methods that were available and appropriate at that time. Although advances in analytical methodologies have occurred since, the Panel considered the results suitable for assessment despite the methodological limitations of these studies.

In various animal species, including mouse, rat, rabbit, dog and monkey, the absorption, distribution, metabolism and excretion of aspartame have been investigated using different radiolabelled forms of ^{14}C- aspartame (E15, 1972; E17, 1972; E18, 1972; E80, 1974). Radiolabelling was located either on the phenylalanine or aspartic acid moiety, or on the methyl group. In some studies (in rats and dogs) data have been compared with corresponding animals receiving ^{14}C(U)-phenylalanine, ^{14}C(U)-aspartic acid or ^{14}C-methanol.

In mice given 20 mg/kg bw of [^{14}C(U)-phe]-aspartame by gavage, the radioactivity found in plasma, urine, faeces and expired air clearly demonstrated that aspartame was hydrolysed in the gut of the mouse before absorption of its phenylalanine moiety occurred (E18, 1972). In plasma, the major fractions of ^{14}C-materials corresponded to phenylalanine or tyrosine, both of which were incorporated in plasma proteins.

Several studies have been carried out in rats, primates and man receiving oral ^{14}C-aspartame (20-30 mg/kg bw) radiolabelled at the phenylalanine or aspartic acid moiety or on the methyl group (E15, 1972). Comparisons were made with rats given ^{14}C(U)-phenylalanine, ^{14}C(U)-aspartic acid, ^{14}C-methylphenylalanine or ^{14}C-methanol. In all studies, results clearly indicated that aspartame was hydrolysed in the gut.

When aspartame was radiolabelled at the phenylalanine moiety, ([^{14}C(U)-phe]-aspartame), it was handled in a fashion similar to that of the naturally occurring amino acid phenylalanine.

When aspartame was radiolabelled at the aspartic acid moiety, [^{14}C(U)-asp]-aspartame, the compound was metabolised by experimental animals to free ^{14}C-L-aspartic acid, which was subsequently metabolised in the same way as aspartic acid in the diet. The major fraction (60-70 % of the dose) of the ^{14}C was excreted as $^{14}CO_2$ in the expired air, while the remainder was incorporated into body proteins.

When aspartame was radiolabelled at the methyl group, like ^{14}C-methylphenylalanine, the compound was rapidly hydrolysed by esterases in the gastrointestinal tract, and the methyl group was handled by the body in a manner equivalent to the methyl group of methanol.

In rabbits, unlabelled aspartame was administered daily for 4 days (20 mg/kg bw/day) and a 8.2 mg/kg bw dose of [^{14}C(U)-phe]-aspartame was given the following day (E18, 1972). As in mice and rats, aspartame was hydrolysed in the gut before absorption of phenylalanine. A biphasic mean plasma radioactivity curve was observed, the authors suggested a rapid appearance of the phenylalanine moiety of aspartame as a plasma peptide fraction with a short half-life. Aspartame was metabolised in a fashion qualitatively similar to the other species. However, the cumulative excretion of $^{14}CO_2$ by the rabbit (7 %) was lower than in rat or monkey (17 %).

In a further study (E80, 1974), plasma metabolites were isolated and identified in rabbits given [^{14}C(U)-phe]-aspartame. Since the plasma metabolic profile was similar to that identified in other species, the authors concluded that the differences observed in plasma kinetics would be due to differences in the rates of digestion and absorption in rabbits.

In dogs, unlabelled aspartame (20 mg/kg bw/day) or phenylalanine (11.2 mg/kg bw/day) were administered for 5 days; on the following day, animals received 20 mg of [^{14}C(U)-phe]-aspartame or 11.2 mg of ^{14}C(U)-phenylalanine by gavage (E17, 1972). The administered aspartame was hydrolysed in the gut (extent of hydrolysis not quantified) and the resulting radiolabelled amino acid (phenylalanine) was handled by the body identically to phenylalanine administered alone or ingested in the diet. No unchanged aspartame was detected in plasma of dogs receiving aspartame. The biological half-life of plasma ^{14}C after oral administration of [^{14}C(U)-phe]-aspartame was about 12 days. The authors state that this value would be consistent with reported half-lives for various plasma proteins in experimental animals and in human volunteers.

In rhesus monkeys, [^{14}C(U)-phe]-aspartame, [^{14}C(U)-CH_3]-aspartame, ^{14}C(U)-phenylalanine or ^{14}C-methanol were given as separate single oral doses of 0.068 mmol/kg (20 mg/kg for aspartame) (E15, 1972). These data demonstrated that monkeys metabolised phenylalanine via the same metabolic route as rats. Little phenylalanine was excreted in the urine or as $^{14}CO_2$ in the expired air and the absorbed labelled material entered the normal metabolic pathways for phenylalanine. In monkeys receiving [^{14}C(U)-CH_3]-aspartame or ^{14}C-methanol, the kinetics of the methyl group metabolism illustrated the similarity with which monkeys and rats metabolise this moiety of aspartame. The methyl moiety was hydrolysed by esterases in the intestine, absorbed, and metabolised in the carbon pool of the body. The only difference appeared to be a slower hydrolysis in the gut of the monkey compared to the rat.

In a further study, monkeys were administered unlabelled aspartame (20 mg/kg bw/day) or aspartic acid (9 mg/kg bw) orally for 5 days; on the following day, the animals received 20 mg/kg of [^{14}C(U)-asp]-aspartame or 9 mg/kg of ^{14}C(U)-aspartic acid by gavage (E17, 1972). Results clearly indicated that aspartate from aspartame was released by hydrolysis of the parent compound in the gut and that the free amino acid was metabolised in the same way as the dietary aspartic acid.

The data of these early reports on pharmacokinetics of aspartame (E15, 1972; E17, 1972; E18, 1972; E80, 1974) showed that radioactivity associated with unchanged aspartame was not detectable in plasma of experimental animals. Based on the sensitivity of the detection of radioactivity following labelling of the phenylalanine, aspartate and methyl moieties of aspartame, it can be concluded that aspartame is completely hydrolysed in the gut to yield aspartate, phenylalanine and methanol. These metabolites are then absorbed and enter normal endogenous metabolic pathways.

3.1.2. Additional studies on absorption, distribution, metabolism and excretion

In addition to these basic kinetic studies, further studies (summarised below) have been performed elucidating specific aspects of absorption, distribution, metabolism and excretion.

Studies in rats, dogs and monkeys have shown that after oral ingestion, aspartame is hydrolysed, either within the lumen of the gastro-intestinal (GI) tract, or within the mucosal cells lining the GI-tract. While aspartame in acidic aqueous solutions under *ex vivo* conditions has a half-life of several days (Prankerd et al., 1992), its half-life in the GI tract is in the order of minutes. The accelerated hydrolysis of aspartame in the GI-tract is mediated by specialised enzymes in the intestine, including esterases and peptidases (especially aminopeptidase A) (Oppermann, 1984; Hooper et al., 1994). The products that result from these reactions are methanol and the amino acids, aspartic acid and phenylalanine. Hydrolysis of aspartame releases a maximum of 10 % methanol by weight (Magnuson et al., 2007). Hydrolysis is very efficient and the amount of aspartame that enters the bloodstream has been reported as undetectable in several studies (Oppermann, 1984; Burgert et al., 1991).

Burton et al. (1984) investigated the fate of phenylalanine methyl ester in two female rhesus monkeys fitted with hepatic portal vein cannulae. Following intragastric and intraduodenal administration of 20 mg doses of [^{14}C(U)-phe]phenylalanine methyl ester hydrochloride, small amounts of unchanged phenylalanine methyl ester were detected during the first 1-2 hour after administration, but none was detectable (< 0.001 µg/mL) at later times. When comparing the areas under the phenylalanine methyl ester and total radioactivity blood concentration-time curves, the authors concluded that only 0.2 % of

the administered phenylalanine methyl ester reached the portal blood unchanged and ≤ 0.1 % reached the peripheral blood unchanged. Additional *in vitro* work showed that intestinal mucosa homogenates, blood and plasma rapidly metabolise phenylalanine methyl ester to phenylalanine and methanol (Burton et al., 1984).

The intestinal metabolism of aspartame and L-phenylalanine methyl ester in the gut lumen was further studied in a young pig model *in vitro* and *in vivo* (Burgert et al*.,* 1991). In the *in vivo* studies, 6 young female Chester white pigs (10-15 kg) were catheterised and had a 10-French double lumen tube with a proximal port opening in the proximal jejunum and the distal port 30 cm further down the intestine. At least three days were allowed for recovery after surgery. In bolus dosing studies, compounds (L-phenylalanine methyl ester, aspartame or L-phenylalanine) were administered at a dose of 2.5 mmol/kg in a Latin square design. For aspartame, this corresponded to 735 mg/kg bw. Portal vein and vena cava blood samples were taken over a period of 4 hours following dosing, and each animal received all three compounds with three days for recovery between doses. In steady state perfusion studies, equimolar solutions (25 mM) of each compound were administered directly into the jejunum at a rate of 5 ml/min and the perfusate was analysed.

In the bolus dosing studies, no intact L-phenylalanine methyl ester, aspartame or aspartylphenylalanine were detected in blood samples from either the portal vein or the inferior vena cava after dosing with L-phenylalanine methyl ester, aspartame or L-phenylalanine. No significant differences were observed with regard to AUC in the concentration of phenylalanine in portal blood after dosing with L-phenylalanine methyl ester, aspartame or L-phenylalanine. A similar pattern was observed in vena caval blood although the absolute phenylalanine concentration detected and corresponding AUCs were lower by 15 %. Portal blood tyrosine concentration was similar after all three compounds but lower than phenylalanine concentration (mean peak values of 167 µM for tyrosine vs. 2510 µM for phenylalanine).

Neither portal nor vena caval methanol levels changed after bolus administration of phenylalanine, but values in portal blood increased from a baseline of 0 mM to 3.50 ± 0.75 mM after aspartame and 3.25 ± 0.65 mM after phenylalanine methyl ester, while vena caval levels peaked at 3.26 ± 0.65 mM after aspartame and 3.13 ± 0.75 mM after phenylalanine methyl ester. Thus, the vena caval values were slightly, but not significantly, lower than those in the portal circulation.

In steady state perfusion studies, direct infusion of aspartame into the jejunum resulted in the appearance of methanol, aspartic acid, phenylalanine and aspartylphenylalanine in the perfusate, with the concomitant disappearance of aspartame from the perfusate (1.46 µmol/min/cm gut). L-Phenylalanine methyl ester was also present at detectable levels. Perfusion with L-phenylalanine methyl ester itself resulted in the appearance of phenylalanine and methanol in the perfusate. Perfusion with L-phenylalanine resulted in rapid disappearance of phenylalanine but not any of the other compounds in the perfusate. From the steady state perfusion data the authors concluded that aspartame was hydrolysed to methanol and aspartylphenylalanine in the intestinal lumen (Burgert et al*.,* 1991).

In a study by Trocho et al. (1998), [^{14}C-methyl]-aspartame was administered to male Wistar rats (1-5 animals per time point investigated) at a single oral dose level of 68 µmol/kg bw (20 mg/kg bw) and 122 µCi/kg bw by gavage. The authors reported that in liver, kidney and plasma about 0.1-0.4 % of the dose of radioactivity/g tissue was detected at 1 hour and persisted between 6-24 hours. In particular, in blood (0.1-0.2 % of the dose of radioactivity/g tissue) approximately 98 % of the radioactivity was incorporated into protein and in the liver about 78 % was incorporated to protein or nucleic acids at 6 hours. In an additional study, two rats were administered radiolabelled [^{14}C-methyl]-aspartame (37 MBq; 85.5 µmoles) orally after pre-treatment for 2 days with unlabelled aspartame. At an unspecified time point, plasma was collected and subjected to acid precipitation followed by hydrolysis (6N HCl, 48 hours), liberating amino acids from proteins. The hydrolysate was then filtered to remove 'Maillard adducts'. Maillard adducts are produced through the reaction of sugar carbonyl groups with amino groups (in, for example, amino acids) and would have been produced during the hydrolysis procedure and therefore as artefacts associated with sample processing. The authors

reported that the filtered Maillard adducts were radioactive. The filtered amino acids, which were assumed not to contain any Maillard adducts, were then derivatised with dinitrofluorobenzene and analysed by 2D thin layer chromatography.

The authors present data to show that a single radiolabelled species was observed in plasma after hydrolysis and derivatisation. The authors speculated that the bound radioactivity resulted from the formation of formaldehyde from aspartame-derived ^{14}C-methanol (Trocho et al., 1998).

The Panel noted that the methodology used by Trocho was not able to differentiate between ^{14}C incorporated into proteins through the metabolic one carbon tetrahydrofolate pathway and direct covalent reaction (e.g. through Schiff base formation) of formaldehyde with proteins. The Panel noted that methanol liberated from aspartame in the gut is rapidly metabolised to formaldehyde, formic acid and CO_2 and that the radioactivity that the authors have identified to be present in nucleic acids and proteins in liver and serum (protein only) could be attributed to other metabolic pathways (e.g. creatinine synthesis, DNA and protein methylation, 5' cap mRNA methylation and phosphatidylcholine synthesis) that have not been fully investigated.

The authors failed to compare the single radioactive species obtained after hydrolysis of plasma protein and derivatisation of amino acids, with several of the amino acids susceptible to reaction with formaldehyde. This did not exclude the possibility that the single radioactive species in hydrolysates was not a trace level of a Maillard adduct (and therefore an artefact from sample processing) as well as omitting other controls.

The Panel noted that the study of Trocho et al. (1998) has also been criticised by Tephly (1999) arguing that the adducts were not identified with certainty and adduct formation with ^{14}C-methanol or ^{14}C-formaldehyde were not studied *in vivo* to allow adequate comparison.

Overall the Panel concluded that the work of Trocho et al. (1998) does not demonstrate unequivocally that formaldehyde from aspartame-derived methanol reacts with tissue macromolecules.

3.1.3. Human Studies

3.1.3.1. Single Dose Administration Studies

The pharmacokinetics and metabolism of [^{14}C(U)-phe]-aspartame were determined in three healthy male adults receiving a single dose of 500 mg aspartame/person in water (E15, 1972). Results demonstrated that the radiolabel from aspartame was handled in a fashion similar to that of the natural amino acid phenylalanine. As in mice, rats, rabbits, dogs and monkeys, aspartame was hydrolysed in the intestine (extent of hydrolysis not quantified), and the resulting ^{14}C-phenylalanine was rapidly absorbed from the gut and was detected in the plasma as naturally occurring polar components comprising a mixture of proteins, peptides and free amino acids. Both qualitatively and quantitatively the metabolites of [^{14}C(U)-phe]-aspartame found in the plasma appeared to be the same as those detected in the other animal species studied.

When human volunteers were given a single oral dose of 34 mg aspartame/kg bw, no detectable increase in the blood methanol concentration was observed (limit of detection: 3.5-4 mg/L) (Tephly and McMartin, 1984). When adults were given a dose of 50 mg aspartame/kg bw, peak blood levels were around the limit of detection. With doses of 100, 150 or 200 mg aspartame/kg bw, peak blood levels of 13, 21 and 26 mg methanol/L blood were observed between 1-2 hours post dosing. Following a dose of 100 mg/kg bw, the blood methanol returned to undetectable levels at 8 hours post dosing. With the 150 and 200 mg/kg bw doses, methanol persisted longer in the blood, but at 24 hours post dosing, blood methanol was below the limit of detection. Both blood peak levels and blood AUCs were proportional to the dose. The values reported are consistent with the known kinetics of methanol (Horton et al., 1992).

Plasma levels of formate and the (reactive) formaldehyde intermediate metabolite have also been examined (Stegink, 1984). After giving aspartame to human volunteers at oral dose levels up to 200 mg/kg bw, no clear increase in the blood concentration of formic acid could be observed because of the high background before dosing and the high variability of the value 3 hours and 24 hours after dosing. At this dose (200 mg/kg bw), the background level of blood formic acid was 18 ± 5.3 mg/L, 22 ± 11 mg/L 3 hours after dosing and 11 ± 8.4 mg/L 24 hours after dosing (values derived from Fig 28, Stegink, 1984). Renal excretion of formic acid was higher by 2 to 3-fold over basal levels (34 ± 22 µg/mg creatinine) during the 0-8 hour period following aspartame intake.

The same author (Stegink, 1984) reported a series of experiments in humans to investigate the dose relationships between oral intake of aspartame and systemic plasma levels of aspartic acid, phenylalanine and methanol. The relationships were studied for doses ranging from 0-200 mg aspartame/kg bw/day, and the studies focused on blood levels that occurred following one single dose of 34 mg/kg bw/day, because at that time, this was the 99th percentile of the 'projected' daily ingestion[18]. Plasma phenylalanine, aspartate and methanol concentrations were measured in normal subjects ingesting aspartame at 34, 50, 100, 150 and 200 mg/kg bw in a randomised crossover study.

Following administration of aspartame at a dose level of 34 mg/kg bw, plasma phenylalanine levels increased significantly ($p \leq 0.001$) from a normal baseline level of 50-60 µM to 110 ± 25 µM. At a dose of 50 mg/kg bw of aspartame, plasma phenylalanine levels increased significantly ($p \leq 0.001$) from baseline values reaching a mean (± SD) peak value of 162 ± 49 µM. Mean (± SD) peak plasma phenylalanine levels were 203 ± 20.5, 351 ± 113 and 487 ± 151 µM following administration of aspartame at 100, 150 and 200 mg/kg bw, respectively, and were statistically higher than baseline levels. All peak values were reached between 30 min and 2 hours following dosing.

Following administration of aspartame at a dose of 34 mg/kg bw and 50 mg/kg bw, no significant differences from baseline values of 2.2 ± 0.8 µM were noted in plasma aspartate. The mean (± SEM) peak plasma aspartate levels (after 0.5 hours from aspartame administration) were 4.3 ± 2.3 µM ($p < 0.02$) (at 100 mg/kg bw dose), 10.0 ± 7.0 µM (at 150 mg/kg bw dose) (not statistically significant) and 7.6 ± 5.7 µM (at 200 mg/kg bw dose) (not statistically significant), showing that aspartate levels did not appear to be dose-dependently increased. The authors concluded that these data indicate that rapid metabolism of the aspartate portion of aspartame occurred at all doses studied (Stegink, 1984).

Peak plasma levels of amino acids after aspartame intake have been compared to peak plasma levels after ingestion of dietary proteins. It was noted that amino acids from proteins in meals were liberated in the gut and absorbed more slowly than the amino acids in small peptides such as aspartame (EFSA 2006).

Stegink et al. (1979a) measured plasma, erythrocyte and milk levels of free amino acids in six normal female subjects with established lactation after oral administration of either aspartame or lactose at 50 mg/kg body weight in a cross-over study. No significant change in plasma or erythrocyte aspartate levels was noted following aspartame or lactose administration. Plasma phenylalanine levels increased approximately four-fold over fasting values 45 minutes after aspartame loading ($P < 0.001$), and returned to baseline by 4 hours. Milk phenylalanine, but not aspartate, levels were increased between 1 and 12 hours after aspartame intake.

[18] Projected levels of aspartame ingestion have been calculated by the FDA, the Market Research Corporation of America (MRCA), and by Stegink research group. If aspartame totally replaces estimated mean daily sucrose intake, aspartame intake would range between 3 and 11 mg/kg bw with the highest daily aspartame ingestion would vary from 22 to 34 mg/kg bw. The latter is equivalent to ingesting 12-19 mg/kg bw phenylalanine, 9.8-15.2 mg/kg bw aspartate and 2.4-3.7 mg/kg bw methanol. According to the MRCA, an aspartame intake of 34 mg/kg bw represented the 99th percentile of projected daily ingestion (calculation carried out before approval of aspartame for use in carbonated beverages).

3.1.3.2. Studies in adults heterozygous for phenylketonuria and other sub-populations

In addition to the aforementioned studies investigating the metabolism of aspartame in healthy adult volunteers, there are a number of additional absorption, distribution, metabolism and excretion (ADME) studies on aspartame that were conducted in one-year-old infants, lactating women, in individuals who respond adversely to monosodium glutamate (MSG; so-called 'Chinese Restaurant Syndrome'), in adults heterozygous for phenylketonuria (PKU) and the elderly.

The study by Filer et al. (1983) shows that the metabolism of a single dose of aspartame (up to 100 mg/kg bw, measured as peak plasma levels and AUC for phenylalanine and aspartate) given orally to one-year-old infants was not different from that measured in adults. Likewise, the metabolism of a single oral dose of aspartame of 50 mg/kg bw in lactating women (measured as plasma phenylalanine and aspartate levels) was not different from other adults (Stegink et al., 1979a; E93, 1977). There was also no apparent difference in aspartame metabolism in individuals who respond adversely to monosodium glutamate (E110, 1979).

Several studies on the metabolism of aspartame in adults heterozygous for PKU were conducted using single doses ranging from 10 to 100 mg aspartame/kg bw (E108, 1978; E109, 1978; Stegink et al., 1979b; Stegink et al., 1980; Caballero et al., 1986; Stegink et al., 1987b; Filer and Stegink, 1989, Curtius et al., 1994). In two studies (Stegink et al., 1979b, 1981a) fasted subjects heterozygous for PKU were compared with fasted normal subjects following a dose of 34 mg aspartame/kg bw (dissolved in orange juice). In heterozygotes, basal plasma phenylalanine levels and the phenylalanine levels following administration of aspartame were significantly higher and the plasma concentration-time curve broader than those recorded in normal subjects. While maximal plasma phenylalanine levels in healthy normal adults (111 $\pm$ 24.9 µM) were in the range observed postprandially (Stegink, 1984), maximal plasma phenylalanine levels in subjects heterozygous for PKU were 160 $\pm$ 22.5 µM, and therefore were slightly above the normal postprandial level. The plasma phenylalanine concentration-time curve was also significantly greater in heterozygous female subjects (21.36 ± 5.10 IU) than in normal female subjects (10.84 ± 2.32 IU). In a further study (Stegink et al., 1980) subjects known to be heterozygous for PKU and subjects assumed to be normal were administered a dose of aspartame of 100 mg/kg bw (dissolved in orange juice). Plasma phenylalanine levels in heterozygous subjects after aspartame loading were significantly higher than values in normal subjects from 30 to 180 min, and the area under the plasma concentration-time curve greater. The maximal plasma phenylalanine level in heterozygous subjects after aspartame administration was 417 $\pm$ 23.5 µM vs. 202 $\pm$ 67.7 µM in normal subjects. The authors concluded that fasted heterozygotes given an acute dose of aspartame metabolised the phenylalanine part of aspartame approximately half as fast as normal subjects. In another study (Curtius et al., 1994), the effect of a protein-rich meal alone or in combination with 25 mg aspartame/kg bw on plasma phenylalanine levels in normal subjects and subjects known to be heterozygous for PKU was investigated. In meals supplemented with aspartame (25 mg/kg bw), the highest mean plasma phenylalanine levels were only slightly higher than the postprandial range for normal subjects (values for normal subjects were not reported but the Panel estimated mean values from the graph in the publication to be 80 µM vs. 110 µM). In the case of subjects known to be heterozygous for PKU, the increase was from a postprandial level of 126 $\pm$ 21 µM to 153 $\pm$ 21 µM.

A study that compared young with elderly adult volunteers revealed that following a single dose of 40 mg aspartame/kg bw, C_{max} and AUC values for plasma phenylalanine were modestly but significantly higher in the elderly due to a rise in the elimination half-life of phenylalanine from 3.5 to 3.9 hours (Puthrasingam et al., 1996).

3.1.3.3. Repeat Dose Administration Studies

Several studies were conducted to address the effects of repeated ingestion of aspartame on its metabolism, and plasma phenylalanine and aspartate levels, and are summarised as follows. The ingestion by volunteers of a 600 mg aspartame dose every hour for eight hours resulted in significant increases in plasma phenylalanine and tyrosine levels, reaching a plateau after several doses (five

doses for phenylalanine levels, three to six doses for tyrosine levels) (UN05, 1987; Stegink et al., 1989). However, the plateau levels did not exceed the normal postprandial range in plasma and returned to baseline values by 24 hours. The peak ratios of phenylalanine levels to large neutral amino acids (LNAA) levels were increased compared to the placebo in the subjects, similar to that observed following a single 34 mg/kg bw dose of aspartame. Similarly, the peak tyrosine/LNAA ratio was increased. The Panel noted that in this study, volunteers received hourly doses of 600 mg aspartame for 8 hours each day, a daily dose of 4800 mg or 80 mg/kg bw/day. This total dose corresponded to the administration of a dose equivalent to twice the ADI each day. Since the plateau levels of phenylalanine did not exceed the normal postprandial range, the Panel considered that there is no accumulation of phenylalanine resulting from this high dose of aspartame.

Healthy adults (108 volunteers) were given either placebo or 75 mg/kg bw/day aspartame in three divided doses for 6 months (Leon et al., 1989). Throughout the treatment period, there were no changes in phenylalanine, aspartate or methanol plasma concentrations, in phenylalanine/LNAA ratio and in urinary formate excretion. The Panel noted that in this study healthy adults received almost twice the ADI for 6 months without any consequences in terms of phenylalanine or methanol accumulation. In further studies, the effect of repeated ingestion of aspartame (in increasing doses from 600 up to 8100 mg per person per day) over periods of up to 27 weeks on plasma amino acid levels were also investigated in obese subjects, insulin-dependent and -independent diabetic subjects and in children (E23, 1972; E24, 1972; E60, 1973; E61, 1972; E64, 1972). None of the studies showed a significant difference in plasma phenylalanine or aspartate levels compared to controls.

Stegink et al. (1990) showed that the hourly ingestion of 600 mg aspartame by adults heterozygous for PKU over a period of 8 hours significantly increased plasma phenylalanine levels, reaching a plateau after five doses (see also UN05, 1987). The highest mean value was 165 ± 34.4 μM at 7.5 hours, which remains within the normal postprandial range. Phenylalanine plasma levels returned to baseline values by 24 hours. The peak ratio of phenylalanine levels to LNAA levels, but not the peak tyrosine/LNAA ratio, was increased compared to the placebo. In another study (E25, 1972), aspartame was administered in increasing doses from 600 up to 8100 mg person per day to adults heterozygous for PKU for six weeks. No statistically significant difference in plasma phenylalanine levels were observed between placebo controls and aspartame-exposed subjects. In a follow-up to this study, adults heterozygous for PKU were given 1800 mg aspartame per day (600 mg doses three times daily) over 21 weeks. Again, there was no difference between placebo and aspartame-exposed individuals in terms of plasma phenylalanine levels except for a small but statistically significant difference at week 16 (107 vs. 126 μM for control and aspartame, respectively).

Overall, the Panel noted that in all the repeat dose administration studies, healthy adults, children or patients suffering various diseases including heterozygous for PKU received daily dose of aspartame above the current ADI of 40 mg/kg bw/day for periods up to 6 months. Considering the absence of a significant increase in plasma phenylalanine concentration from the normal postprandial range, the Panel concluded that in line with normal uses and use levels there is no risk of phenylalanine accumulation in such human populations.

3.1.4. β-Aspartame

β-Aspartame (β-L-aspartyl-L-phenylalanine methyl ester) is formed to a minor extent during the manufacturing process and in storage of aspartame-containing beverages. In a series of studies, the pharmacokinetics and metabolism of orally administered ^{14}C-β-aspartame were determined in humans and in various animal species. In all these studies, ^{14}C-β-aspartame was radiolabelled on the phenylalanine moiety.

In humans, six healthy subjects received a single oral dose of ^{14}C-β-aspartame dissolved in water (31.9 or 40 mg, corresponding to 0.5-0.7 mg/kg bw) and were observed for 7 days (E172, 1987; E173, 1988). The use of HPLC enabled quantification of both the unchanged β-aspartame and its major metabolites in plasma, urine and faeces. The authors reported that more than 90 % of the orally

administered radioactivity was absorbed based on the low recovery of radioactivity excreted in faeces for 7 days (9.6 % of the radioactive dose). Unchanged ^{14}C-β-aspartame was not detected in plasma and less than 0.15 % of the dose was excreted unchanged in the urine after 7 days. The demethylated metabolite (^{14}C-β-L-aspartyl-L-phenylalanine) was the major metabolite observed in plasma (T_{max} = 1.5 hours, plasma half-life = 1.1 hours) and was an important urinary metabolite (7 % of the administered radioactivity). In plasma, phenyl-acetyl-glutamine was detected as another major circulating metabolite whereas free ^{14}C-phenylalanine appeared as a minor one. The major route of excretion of radioactivity was urine (42 % of the radioactive dose). The major urinary metabolite was ^{14}C-phenyl acetyl glutamine, its excretion accounted for 30.8 % of the radioactive dose. ^{14}C-phenylalanine was the only metabolite recovered in faeces. There were no clinical effects related to ^{14}C-β-aspartame administration at these dose levels in healthy subjects.

In rabbits (E170, 1986), dogs (E169, 1987) and monkeys (E171, 1985) similar data were obtained including the absence of unchanged ^{14}C-β-aspartame in plasma and the percentages of excretion in urine and faeces. In these animal species, 4 to 5 % of the radioactive dose was excreted in the breath.

The Panel noted that these studies indicated that there is a negligible systemic bioavailability of the unchanged β-aspartame. The Panel also noted the low plasma level of free phenylalanine subsequent to the administration of β-aspartame and that the major urinary metabolites are phenyl-acetyl-glutamine and β-L-aspartyl-L-phenylalanine, a normal constituent of human urine (Burton et al., 1989).

3.1.5. Overall summary of the ADME data

Overall, the Panel noted that no unchanged aspartame was identified in body fluids or in tissues from experimental animals or humans in any of the studies. Therefore, the Panel concluded that after oral ingestion, aspartame was hydrolysed in the gastrointestinal tract to yield aspartic acid, phenylalanine and methanol. These metabolites are then absorbed and enter normal endogenous metabolic pathways. In humans, only subjects heterozygous for PKU showed a somewhat reduced capacity to metabolise the phenylalanine moiety of the aspartame molecule.

3.2. Toxicological data of aspartame

The content of DKP in the aspartame used in these studies, as reported by the authors of the original reports, varied from 0.1 % to 4.0 % per batch of aspartame; the aspartame dose was not corrected for this. In some of the unpublished reports, the DKP content is not reported.

3.2.1. Acute toxicity of aspartame

3.2.1.1. Acute toxicity studies received following a public call for data which were available at the time of the previous JECFA and SCF evaluations

The acute toxicity of aspartame was studied in three species, mice, rats, and rabbits (E46, 1973). Aspartame was administred by gavage (as 15-25 % suspension in 1 % Tween 80) at doses up to 5000 mg/kg bw (E46, 1973) or by i.p. injection (in mice and rats only). Male Schmidt Ha/ICR mice (n = 6) were dosed by gavage at 1000 or 5000 mg/kg bw or by i.p. injection at 200 or 450 mg/kg bw (n = 2) or 1000 mg/kg bw (n = 6) (E46, 1973). Male Sprague Dawley rats (n = 6) were dosed by gavage at 5000 mg/kg bw or by i.p. injection at 2033 mg/kg bw. Male New Zealand White Luenberg rabbits were dosed by gavage at 2000, 2500 or 3200 mg/kg bw (n = 1) and 4000 or 5000 mg/kg bw (n = 3) (E46, 1973). The animals were observed intermittently during the 7 day post-treatment period and no remarkable motor or behavioural activites were noted. No mortalities were observed in the experimental period. According to the authors, the LD_{50} values were in excess of the highest doses administered to each species.

Male Charles River CD rats (10 weeks of age; 6 animals/group) received 100 mg/kg of aspartame by intravenous injection (5 ml/kg bw of 2 % aspartame in 0.9 % aqueous sodium chloride solution administered into the jugular vein over a period of 10 minutes); control animals received an equivalent

volume of sodium chloride (E84, 1974). The animals were monitored for a 72-hour period post-treatment, then sacrificed and representative tissues examined microscopically. Survival was 100 % in both control and treated animals. Physical, ophthalmoscopic and body weight changes were unremarkable. No compound-related changes were observed at the gross or microscopic level, organ weights and organ to body weight ratios were unchanged. Moderate to severe phlebitis was observed at the site of implantation of the cannula in all rats; the authors concluded that the inflammation observed in the kidneys and endocardium could be a result of an infection.

Two groups of four male Beagle dogs received either 0 or 100 mg/kg of aspartame by intravenous injection (5 ml/kg injections of 0.5 % aqueous sodium chloride solutions administered over a period of 10 minutes) (E85, 1974). The dogs were monitored for 72 hours post-injection before sacrifice. Physical appearance, feed consumption and body weight were recorded. All animals survived during the observation period and no remarkable changes were observed. Electrocardiograms were made at 0, 0.5, 3, 6, 24, 48 and 72 hours, patterns were unremarkable pre and post-injection in both placebo and treated dogs. No statistically significant changes were observed in the haematology, clinical chemistry and urinalysis parameters evaluated. Post-mortem gross and microscopic changes were unremarkable.

The overview of these studies is presented in Appendix F.

3.2.2. Short-term and sub-chronic toxicity of aspartame

3.2.2.1. Studies of short-term and sub-chronic toxicity received following a public call for data which were available at the time of the previous JECFA and SCF evaluations

Mice of the Ha/ICR strain (8 weeks of age) were randomly assigned, five per group to study groups (E2, 1972). It was planned to administer aspartame for 4 weeks at dose levels of 0, 2000, 4000 and 10 000 mg/kg bw/day, but due to an unforeseen increase in feed consumption, the actual intake of the test compound exceeded this by 25-30 % in all the groups. The actual intake was calculated to be 3000, 5000 and 13 000 mg/kg bw/day in the test groups. No statistically significant difference in body weight was observed between treated and control animals. No adverse clinical conditions were noted and 100 % survival was reported. Upon sacrifice, no treatment-related pathological changes were reported except that the intestinal mucosa from the high dose treated mice was heavily coated with a clear, moderately viscous fluid. Only the control animals and those from the high dose group were examined.

In a sub-acute study, aspartame was given in the diet for 4 weeks to male and female Charles River CD rats (E3, 1972). Seven-week-old rats (5 animals per dose group) were administered aspartame incorporated in the diet on a w/w basis, resulting in an exposure of 0, 2000, 4000 or 10 000 mg/kg bw/day. Actual consumption was estimated to be within 10 % of the proposed dose. No consistent statistically significant effect in body weight was observed, though a significant decrease in feed consumption was reported in the high dose females at weeks 2 and 3; however, this was not reflected in body weight gain, which increased during the treatment-period. No adverse clinical conditions were reported during the study period and 100 % survival was reported in both control and treated groups. Only five control animals (3 males and 2 females) and the animals from the high dose groups were examined histopathologically. No treatment-related changes were reported except that the intestinal mucosa from the treated rats was heavily coated with a clear, moderately viscous fluid.

Groups of 10 male and 10 female sexually mature Charles River CD rats were dosed for 8 weeks with aspartame incorporated into the diet, resulting in a dose of 5 or 125 mg/kg bw/day, with further groups of 10 animals acting as controls (E20, 1969). Dosages were adjusted weekly based on weight of animals and feed consumption for each sex at the end of the preceding week. Survival was reported to be 100 %. No effect on body weight and feed consumption was reported. No effect on physical appearance or behaviour was observed at either test level. At sacrifice, biochemistry and pathological examination was conducted on five animals from each dose and the control group. No treatment-related changes in haematology or urinanalysis were reported, organ weights were unaltered except in

the high dose males where a significantly higher liver to body weight ratio was observed for the high dose group males compared to controls. There were no remarkable histopathological findings; bile duct hyperplasia and pericholangitis were present in the livers of animals of all three groups, with no apparent increase in severity in the treated animals.

Groups of 5 male and 5 female weanling Charles River CD rats (23 days of age at commencement of study) were randomly assigned to groups receiving either basal diet or diet containing aspartame (9 % w/w, average dosage of 12.2 mg/kg day), or phenylalanine (5 % w/w, average dosage 6.5 mg/kg day) for 9 weeks (E4, year not reported). Phenylalanine and aspartame were consumed at progressively decreasing mean daily dosage levels of 13 to 4 mg/kg and 24 to 7 mg/kg. Two deaths occurred during the study period, one control male and one aspartame-treated male, but no physical or behavioural abnormalities were observed in the surviving animals. No significant treatment-related changes in haematology, clinical chemistry, urinalysis were recorded and no treatment-related changes in relative organ weight or pathology were observed. Reduced body weight gain was noted for both treatments and both sexes (though less marked in females); mean relative feed consumption (g/kg bw/day) was equivalent to control values; absolute (g/rat/day) feed consumption was reduced proportionally to the depressed body weight gain.

Aspartame was administered in the form of gelatine capsules to groups of male and female Beagle dogs (n = 2) daily for a period of 8 weeks (E21, 1969). The dogs are described as 'young' and weighed 7.2-13.2 kg at initiation. In order to administer an appropriate dose of aspartame, capsules were prepared specifically for the individual animals calculated upon their body weight at the end of the previous week. Animals received either 5 or 125 mg aspartame/kg bw/day, further groups of male and female dogs (n = 2) served as controls and received empty capsules. The dogs were described as being generally normal in appearance and behaviour throughout the study, with a variable pattern of weight loss/gain/maintenance reported. All dogs survived the study period; animals were then sacrificed at the end of the 8-week dosing regime. Upon sacrifice, no consistent effects upon organ weights were recorded and no consistent changes in haematological and clinical chemistry parameters, gross pathology and histopathology were observed.

The overview of these studies is presented in Appendix G.

3.2.2.2. Additional studies on short-term and sub-chronic toxicity

In two consecutive reports, the effects of aspartame on liver (Abhilash et al., 2011) and brain (Abhilash et al., 2013) antioxidant systems and tissue injury were studied in male Wistar rats. The animals (6/group) were dosed daily by gavage for 6 months with 3 ml of aspartame (500 or 1000 mg/kg bw/day) dissolved in water; control rats received the same volume of water. A range of enzyme activities was assayed in serum, liver homogenates and brain homogenates. Histological examination was also performed on tissue from the liver and brain (left half). The authors reported that rats that had received aspartame (1000 mg/kg bw/day) showed a significant serum increases in activities of alanine aminotransferase, aspartate amino-transferase, alkaline phosphatase and gammaglutamyl transferase. The concentration of reduced glutathione (GSH) and the activities of glutathione peroxidase and glutathione reductase (GR) were significantly reduced in the liver of rats that had received aspartame (1000 mg/kg bw/day). The levels of GSH were significantly decreased in the two experimental groups. Histopathological examination revealed leukocyte infiltration in the livers of aspartame-treated rats (1000 mg/kg bw/day). As regards the brain, the authors reported that the concentration of GSH and GR activity were significantly reduced in rats that had received the dose of 1000 mg/kg bw/day of aspartame, whereas only a significant reduction in GSH concentration was observed in the 500 mg/kg bw/day aspartame-treated group. Histopathological examination revealed mild vascular congestion in the 1000 mg/kg bw/day group of aspartame-treated rats. The authors concluded from these observations that 'long-term consumption of aspartame leads to hepatocellular injury' and that 'an alteration in the glutathione-dependent system in the brain by long-term intake of aspartame is dose dependent' suggesting a relationship between aspartame toxicity and GSH.

The Panel noted that only six rats were used per group and that the exposure was not long-term but only 6 months. The Panel noted that in the article by Abhilash et al., 2011, the abstract and main text described the method of dose administration differently (drinking water and gavage respectively). Only two doses were tested and the dose at which some statistically significant effects were reported by the authors was 25 times greater than the ADI. In addition, the changes were mild and limited both qualitatively (many markers of antioxidant system were unchanged) and quantitatively and cannot be interpreted in the absence of historical values from the institute for these rats. The Panel also noted that the method used by the authors for the determination of GSH is not specific for GSH (since it detects a variety of reduced cellular thiols). Finally, focal inflammatory infiltration of the liver is a variable (even within the same liver) but a common occurrence in rodents, particularly in the portal tract regions of the liver. This phenomenon is often observed in liver sections from control populations of rodents. According to the authors, a detailed investigation with a large sample size is necessary to substantiate the findings and to understand the exact mechanism behind the vascular congestion. The Panel considered that the histopathological and biochemical findings presented in the reports did not convincingly support the conclusion of the authors.

3.2.3. Genotoxicity of aspartame

3.2.3.1. Studies on genotoxicity received following a public call for data which were available at the time of the previous JECFA and SCF evaluations

In vitro studies

Aspartame was tested for mutagenicity in *Salmonella typhimurium* strains TA1535, TA1537, TA1538, TA98 and TA100 both in the absence and the presence of a rat liver metabolic activation system at dose levels from 10 up to 5000 µg/plate (E97, 1978; E101, 1978) (Appendix H). The Panel considered that, for both studies, the methods implemented appeared to be sufficiently robust to support the results reported. Aspartame was not mutagenic in this test system, either in the absence or in the presence of the metabolic activation system.

In vivo studies

Mutagenicity of aspartame was studied using a host-mediated assay in mice (E81, 1974). Mice were treated by gavage with 0 (control), 1000, 2000, 4000, 8000 mg aspartame/kg bw/day (as three separate doses given at two hour intervals for five consecutive days) (E81, 1974). Thirty minutes after the final dose, the animals were inoculated with *Salmonella typhimurium*, G-46, by intraperitoneal injection. Three hours later the bacteria were recovered and the peritoneal washing was evaluated for the presence of mutants. In mice, the host-mediated assay revealed no evidence for mutagenicity of aspartame. The Panel noted some discrepancies in description of doses in different sections of the report and that the test system employed has not received further validation and is presently considered obsolete and therefore, the results of the study were not included in the assessment.

Two dominant lethal tests in rats (15 males/group) were reported (E40, 1973; E41, 1973). Aspartame was dosed by gavage to 21-week-old male albino rats of the Charles River CD strain at a dose level of 2000 mg/kg bw given in two equally divided doses administered on the same day. Immediately following treatment, each male was mated with two sexually mature virgin females weekly for eight consecutive weeks. On gestation day (GD) 14, the mated females were sacrificed for ovarian and uterine examinations. The Panel considered that the methods implemented were thought to be sufficiently robust to support the results reported. The following parameters were analysed: paternal growth, maternal pregnancy rate, uterine and ovary examination data and incidence of fetal deaths. None of these parameters was affected by aspartame treatment.

Aspartame was administered by gavage to five groups of 10 male albino rats for five consecutive days, at dose levels of 0 (control), 500, 1000, 2000 and 4000 mg/kg bw/day (E43, 1972). Twenty-four hours after the last dose, each animal was administered colcemid to arrest mitosis, and sacrificed. Bone marrow cells were prepared and evaluated for chromosome aberrations. Aspartame did not increase

the normal aberration frequencies compared to the control rats. The authors concluded that aspartame was not mutagenic. The Panel considered that the methods implemented were sufficiently robust to support the results reported, but considered the study limited since mitotic indices were not reported (Appendix H).

Aspartame was reported not to induce chromosome aberrations in bone marrow or spermatogonial cells after administration by gavage to rats for five days (E12, 1970). However, the dose levels applied were reported inconsistently. On page 2 of the relevant Study Report (E12, 1970) it is stated that animals received aspartame at dose-levels of 400, 800, 1200 and 1600 mg/kg bw/day. In contrast, in the results section, it is reported that aspartame was administered at dose-levels of 2000, 4000, 6000, 8000 mg/kg. The Panel considered that the reported results of this study were not supported by the outcome of the methods applied (Appendix H).

Aspartame was tested in a host-mediated assay with rats (E44, 1972) and was administered by gavage to five groups of 10 male albino rats for five consecutive days, at dose levels of 0 (control), 500, 1000, 2000 and 4000 mg/kg bw/day, given in three equally divided doses. Following the final dose, the animals were inoculated with *Salmonella typhimurium*, G-46, by intraperitoneal injection. Three hours later the bacteria were recovered, and the peritoneal washing was analysed for the presence of mutants. No statistically significant effects on mutation frequency were noted in the treatment groups, as compared to the control. The Panel noted that the test system employed has not received further validation and it is presently considered obsolete, and therefore the results of the study were not included in the assessment.

3.2.3.2. Additional studies on genotoxicity

In vitro studies

Aspartame was studied in *Salmonella* mutagenicity tests in the absence and in the presence of metabolic activation (Appendix H). No mutagenicity was detected in strains TA98, TA100, TA1535, TA1537 or TA97 for doses up to 10 000 µg/plate (NTP, 2005). The Panel considered that the methods implemented were to be sufficiently robust to support the results reported (Appendix H). However, the Panel noted a deviation from OECD 471 (i.e. tester strains TA102 or WP2uvrA bearing AT mutation were not used). The authors of the study judged the small increase in mutant colonies with 30 % rat liver S9 as equivocal.

Rencuzogullari et al. (2004) tested aspartame in strains TA98 and TA100 at doses ranging from 50 to 2000 µg/plate in the presence and in the absence of metabolic activation. No mutagenicity was observed. However, the Panel considered that the methods implemented were not sufficiently robust to support the results reported, due to major deviations from the OECD guideline (i.e. only two tester strains were used; the highest dose-level employed was lower than 5 mg/mL).

Bandyopadhyay et al. (2008) reported aspartame to be negative in a test with *Salmonella typhimurium* TA97 and TA100 strains (plate incorporation, with and without metabolic activation; 10, 100, 250, 500, 1000 or 10 000 µg/plate). The Panel considered that the methods implemented were not sufficiently robust to support the results reported, due to major deviations from the OECD guideline (i.e. only two tester strains were used; the concentration intervals were too wide; no replicate experiment were performed).

Aspartame was tested for DNA damaging activity in the *in vitro* primary rat hepatocyte/DNA repair assay at concentrations of 5 and 10 mM (corresponding to 1.47 and 2.94 mg/mL, respectively) (Jeffrey and Williams, 2000). The Panel considered that the methods implemented were sufficiently robust to support the results reported. Aspartame was found to be negative in this assay.

Rencuzogullari et al. (2004) tested aspartame *in vitro* in a sister chromatid exchange (SCE) assay, a chromosomal aberration test and a micronucleus test on human lymphocytes. Dose-related and statistically significant increases were observed for chromosomal aberration at both 24 and 48 hours

and for induction of micronuclei only at the highest dose-levels employed (2000 µg/mL). Negative findings were observed for SCEs. The Panel noted that the pattern of results obtained suggests an indirect clastogenic effect of aspartame, e.g. secondary to cytotoxicity, not related to primary DNA damage. The latter is usually efficiently flagged by an increased incidence of SCEs, which was not observed in aspartame-treated cells at any dose. The involvement of an indirect mechanism in the reported clastogenic effect is also suggested by the absence of electrophilic centres, potentially reactive toward DNA, in the molecule of aspartame. The Panel noted several flaws in this study (see Appendix H) which was only performed in the absence of exogenous metabolism, while aspartame undergoes extensive biotransformation after ingestion resulting in no significant systemic exposure to intact aspartame. Overall, the Panel considered the experimental findings reported in this study of limited relevance for aspartame risk assessment.

In vivo studies

In the study by Durnev et al. (1995) which aimed at evaluating the genotoxicity of five sugar substitutes in mice, aspartame was investigated for induction of chromosomal aberrations in bone marrow cells of C57Bl/6 mice administered aspartame by oral gavage for five days at dose-levels of 40 and 400 mg/kg bw to groups of five animals. A concurrent negative control group was also included. The animals were sacrificed 6 hours after the last administration of test compound. In the final two hours, colchicine was administered by intraperitoneal injection to accumulate cells in metaphase. A minimum of 100 metaphases per animal were scored. The results obtained indicate that aspartame did not induce any increase in the incidence of chromosomal aberrations compared with negative control values. However, the Panel noted that the study was poorly reported and that a concurrent positive control animal group to show whether the test system was functioning correctly had not been included. Furthermore, the sampling of bone marrow cells 6 hours after the last administration of test compound was not adequate for chromosomal aberration analysis and dose-levels administered appear to be very low. On these grounds, the Panel considered that the methods implemented were not sufficiently robust to support the results reported.

In a study by Mukhopadhyay et al. (2000), male Swiss Albino mice were exposed by oral gavage to a blend of aspartame and acesulfame K (ratio 3.5:1.5) at doses up to 350 mg aspartame/kg bw. The blend of the two sweeteners showed a negative outcome for chromosomal aberrations. The Panel noted that no evaluation of cell cycle progression (e.g. mitotic index) was performed. Given the limitations of the study (blend of sweeteners and no mitotic index determination), the Panel considered it to be of limited relevance for the evaluation of the genotoxicity of aspartame.

An acute bone marrow micronucleus test was conducted with aspartame administered orally to male Fisher 344 rats at three daily doses of 0 (control), 500, 1000 or 2000 mg/kg bw. No increase in the number of micronucleated polychromatic erythrocytes was observed at any of the tested dose levels (NTP, 2005). The Panel considered that the methods implemented were sufficiently robust to support the results reported.

Peripheral blood micronucleus tests were conducted in male and female transgenic mice (Tg.AC hemizygous, p53 haploinsufficient or Cdkn2a deficient) after 9 months of exposure to aspartame at doses ranging from 3.1 to 50 g/kg diet. The highest dose tested was equivalent to 7660 and 8180 mg aspartame/kg bw/day in males and in females, respectively (NTP, 2005). The Panel considered that the methods implemented were sufficiently robust to support the results reported (Appendix H; NTP, 2005). Negative results, indicative of an absence of clastogenic activity of aspartame, were obtained in male and female Tg.AC hemizygous and Cdkn2a deficient mice and in male p53 haploinsufficient mice. In female p53 haploinsufficient mice, the results of the test were judged to be positive by the authors of the study, based on a trend test revealing a statistically significant 2.3-fold increased frequency of micronucleated erythrocytes seen in the 50 g/kg diet group (NTP, 2005). However, the Panel noted that the incidence of micronucleated erythrocytes in female controls was the lowest among the historical control values of the same laboratory; this rendered the outcome of the trend analysis positive. Nevertheless, the observed incidence of micronucleated erythrocytes in the highest

dose group fell outside the range of the historical controls. However, the Panel also noted that the reported increase in micronucleated erythrocytes was observed in one gender only. Furthermore, the effect described was observed after 9 months of administration of a daily dose, which exceeded approximately 8-fold, the highest recommended dose level for genotoxicity testing according to OECD guideline 474. The Panel concluded that the findings were equivocal in the p53 transgenic strain (positive in female but not in male p53 haploinsufficient mice) but negative in the other two strains, and, overall, did not indicate a genotoxic potential for aspartame.

Two studies addressing DNA damage as detectable by Comet assay are available, both following administration of aspartame by gavage.

Sasaki et al. (2002) administered a single dose of aspartame (2000 mg aspartame/kg bw) to mice (four male/group) and analysed the stomach, colon, liver, kidney, bladder, lung, brain, bone marrow. Aspartame did not induce any significant increases in DNA migration. Based on these results, the Panel considered that aspartame was not genotoxic in the organs assayed. However, the Panel noted that for the 24-hour sampling time, a reduction in DNA migration (a marker for DNA cross-linking agents) was observed in all organs analysed, but the reduction was not significant. The Panel considered that the methods implemented were sufficiently robust to support the results reported.

Bandyopadhyay et al. (2008) administered aspartame as a single dose of 0 (control), 7, 14, 28 and 35 mg aspartame/kg bw to mice (four males/group) by oral gavage, and at the highest dose-level, aspartame was reported to induce DNA damage in bone-marrow cells. However, the Panel noted that an insufficient number of cells was scored (total of 50 cells/animal) and the evaluation of cytotoxicity performed (trypan blue exclusion method) is inadequate to exclude an artifactual outcome. In addition, sampling of bone-marrow cells performed 18 hours after administration of the test compound and not at the recommended time for the *in vivo* comet assay (2-6 hours) cannot support permanence of residual DNA damage due to fast DNA repair activity. Furthermore, increases of DNA damage evaluated (% tail DNA and tail extent) were small and no historical control values were reported and used to exclude possible spontaneous biological fluctuations.. Therefore, the Panel considered that the methods implemented were not sufficiently robust to support the results reported in the study, and that no conclusion could be drawn from it.

Kamath et al. (2010) used three endpoints to assess the genotoxic potential of aspartame following administration of 0 (control), 250, 455, 500 and 1000 mg aspartame/kg bw by gavage to mice. The authors concluded that aspartame induced (a) micronuclei in bone marrow erythrocytes, (b) micronuclei in peripheral blood and (c) chromosome aberrations in bone marrow erythrocytes. However, the number of animals and the ratio of polychromatic erythrocytes to normochromatic erythrocytes (PCE/NCE) ratio (the authors only stated that it was changed) are not given. The Panel considered that the methods implemented were thought not to be sufficiently robust to support the results reported, and that no conclusion could be drawn from it.

AlSuhaibani (2010) tested aspartame for its ability to induce chromosome aberrations (CA) and SCE in bone marrow cells of mice (five males/group; by gavage) at dose levels of 0 (control), 3.5, 35 and 350 mg aspartame/kg bw. Based on the increase in the incidence of chromatid breaks, the authors concluded that aspartame induced CA at 35 and 350 mg/kg bw, but neither dose level induced SCE nor decreased the mitotic index (MI). The Panel noted that an insufficient number of cells were scored (total of 50 metaphase cells/animal for CA) and that the incidence of chromosome breaks observed in treated mice (4.4 % at the highest dose, compared to 2.0 % in concurrent controls) is comparable to the incidence found in control animals from other studies for this type of assay; therefore the increase of CA observed in this study was not considered of biological relevance. Furthermore, no positive control was included in the study to ensure that experimental system was working correctly. The Panel considered that the methods implemented were not sufficiently robust to support the results reported, and that no conclusion could be drawn from the study.

Other studies

Karikas et al. (1998) reported a non-covalent interaction of excess aspartame, aspartic acid and phenylalanine with calf thymus DNA, inferred from the altered chromatographic profile of DNA. This effect was attributed to the electrostatic interaction of amino groups and the negatively charged phosphate in naked DNA. The Panel considered these findings, obtained in an acellular system in presence of excess aspartame, of minimal relevance for the evaluation of the genotoxic potential of aspartame.

Meier et al. (1990) investigated the kinetics of formation, stability and reactivity of nitrosation products of aspartic acid, aspartame, and glycine ethyl ester. Nitrosation products were obtained *in vitro*, with incubation of 40 mM substrate and nitrite at pH 2.5 for varying times. The nitrosation products displayed an 'alkylating' activity *in vitro*, as indicated by the reactivity with the nucleophilic scavenger 4-(4-nitrobenzyl)pyridine, measured with a colorimetric method. In the same study, co-administration of glycine ethyl ester and nitrite to rats did not result in the formation of detectable DNA adducts in rat stomach.

The issue of nitrosation was also addressed by Shephard et al. (1993). Aspartame and several naturally occurring dipeptides were nitrosated *in vitro* at low pH (3.5) in the presence of 40 mM nitrite and tested for mutagenicity in *Salmonella typhimurium* TA100. The nitrosation products of some dipeptides (Trp-Trp, Trp-Gly) and aspartame exhibited a direct mutagenic activity, which was related by the study authors to the nitrosation of their primary amino groups.

Concerning the studies of Meier et al. (1990) and Shephard et al. (1993), the Panel noted the harsh conditions utilised for the *in vitro* nitrosation of substrates and considered the results of doubtful relevance for the assessment of the genotoxic risk posed by the dietary intake of aspartame or other natural amino acids and dipeptides.

3.2.3.3. Conclusion on the genotoxicity of aspartame

The Panel concluded that the *in vitro* genotoxicity data on bacterial reverse mutation exhibited some limitations (e.g. absence of TA102 and WP2 uvrA *Escherichia coli*). However, the Panel considered the weight-of-evidence was sufficient to conclude that aspartame was not mutagenic in bacterial systems. Concerning mammalian systems *in vitro*, the Panel concluded that, apart from the valid UDS study that was negative, no conclusion could be drawn at the gene and chromosomal level because no studies dealing with these endpoints were available.

In vivo, the majority of investigations on systemic genotoxicity reported negative findings. Equivocal positive findings were only described in a NTP study, positive in female but not in male p53 haploinsufficient mice; in two other transgenic mouse strains the results were negative.

Concerning the possible site of first contact effects *in vivo*, limited data are available. However, the available *in vitro* data do not indicate a direct genotoxic activity of aspartame that might predispose to a site of first contact effect *in vivo*.

Overall, the Panel concluded that the available data do not indicate a genotoxic concern for aspartame.

Summary tables on the genotoxicity of aspartame are presented in Appendix H.

3.2.4. Chronic toxicity and carcinogenicity of aspartame

3.2.4.1. Studies on chronic toxicity and carcinogenicity received following a public call for data which were available at the time of the previous JECFA and SCF evaluations

In these chronic toxicity and carcinogenicity studies, aspartame was administered in the diet. Although the purity of aspartame used in these studies was considered to be 100 %, it was reported in the studies that the content of DKP varied from 0.2 % to 1.5 % per batch.

Mice

Aspartame (DKP content varied from 0.8 % to 1.2 % per batch) was administered in the diet to groups of 36 male and 36 female ICR Swiss mice (approximately 28 days of age) at dose levels amounting to 0 (control), 1000, 2000 and 4000 mg/kg bw/day for 104 weeks (E75, 1974). Criteria evaluated were physical appearance, behaviour, body weight gain, feed consumption, survival, clinical chemistry, eye examination, organ weights, tumour incidence and gross and histopathology. There was no effect on the physical appearance and behaviour of the animals. Body weight gain for the male mice at all dose levels was significantly lower than that for the male controls but this was accompanied by reduced total feed consumption during the first year for the males at the low and high dose levels. At week 52 differences between control and treated male group body weight values did not exceed 3 % and these differences were not considered biologically meaningful. Mean terminal body weights were unremarkable for each of the male and female treatment groups. Survival in all treated groups was comparable to that of the controls. Haematological and blood chemistry analyses revealed no compound-related alterations at any exposure level compared to the control. Random fluctuations reaching statistical significance were observed occasionally but, according to the authors of the report, the values were within the range of historical control data. Gross observations at necropsy did not reveal compound-related changes in any organs or tissues. Relative organ weights were unaffected except for the relative heart weight in females that was increased at the high dose level and the relative thyroid weight that was increased in females at the low dose level only. In males, the relative thyroid weight was increased at the low dose level only and the relative prostate weight was decreased at the low and mid dose but not at the high dose. Histopathological examination was performed on all gross lesions from all animals at each treatment level, and on 20-27 grossly unremarkable organs from control and high dose level animals, as well as from roughly two-thirds and one-third of the animals in the mid and low dose level. Detailed histopathological evaluations were performed on brain and urinary bladder from all mice in the control and the three treatment groups. The incidence of tumours was not reported to be significantly higher than that in controls for any type of tumour in either sex at any dose. Histopathological examination revealed no evidence for any treatment-related non-neoplastic changes in any organ or tissue. The authors concluded that aspartame, administered to the mouse for 104 weeks in the diet at dose levels of 1000, 2000 and 4000 mg/kg bw/day exhibited no adverse effects regarding survival rate, and that there was no evidence of an effect with respect to the incidence of neoplasms or with regard to non-neoplastic changes in any organ or tissue. The Panel agreed with this evaluation and identified a NOAEL for this study of 4000 mg/kg bw/day, the highest dose level tested.

Rats

Aspartame (DKP content varied from 0 % to 1.5 % per batch) was administered in the diet to groups of 40 male (initial body weight of 75-108 g) and 40 female (initial body weight of 80-102 g) Charles River Albino rats at dose levels of 0 (control), 1000, 2000, 4000, and 8000 mg/kg bw/day for 104 weeks (E33-34, 1973). The highest dose was 6000 mg/kg bw/day from week 0 to week 16, 7000 mg/kg bw/day from week 16 to week 44, and 8000 mg/kg bw/day in week 44 through week 104. There was no evidence that the administration of aspartame resulted in any effect on the physical appearance and behaviour of the animals. Growth rates at 8000 mg/kg bw/day were significantly lower than that of controls, but this could be related to the significantly lower values for feed consumption for the first 52 weeks for the male and female rats in the 8000 mg/kg bw/day group and

for the females in the 4000 mg/kg bw/day group. Survival after 104 weeks was comparable to controls for all treated groups except for the females of the 8000 mg/kg bw/day group that showed statistically significant lower survival rates. Evaluation of the clinical chemistry data revealed no consistent statistically significant alterations outside the normal range of values. The results from ophthalmoscopic examinations revealed no indication of a compound-related effect. Changes in relative organ weights were not accompanied by gross nor histopathological changes and were therefore considered by the authors to be without biological significance. Histopathological examination was performed on all gross lesions from all animals at all treatment levels and on 20-25 grossly unremarkable organs from the groups at 0, 4000 and 8000 mg/kg bw/day and from roughly 25 % of the animals at the 1000 and 2000 mg/kg bw/day groups. Urinary bladder received more extensive examination since four intermittent transverse bladder sections were examined microscopically from each rat in the study. Tumour incidence for the types of tumours analysed was not statistically significantly higher than in the controls in any of the dose groups of either sex. Histopathological examination revealed no treatment-related changes in brain, pituitary, spinal cord, peripheral nerve, thyroid, adrenal, salivary gland, skin, skeletal muscle, heart, liver, mesenteric lymph node, bone marrow, stomach, small intestine, large intestine, uterus, vagina, mammary gland, testis and urinary bladder. In lungs, pneumocyte hyperplasia was slightly increased in the 8000 mg/kg bw/day group females. Effects on ovaries were unremarkable except for an increased incidence of cystic follicles at all treatment levels, probably related to an unusually low incidence in the controls. Seminal vesicle atrophy of slight to moderate degree was inconsistently present, with a slightly increased incidence observed in the 4000 and 8000 mg/kg bw/day groups (2/23, 4/23 and 6/21 in control, 4000 and 8000 mg/kg bw/day groups, respectively). Acute and chronic prostatitis was also observed somewhat more frequently in treated animals but no dose-relationship was observed. An increased incidence of focal pancreatic fibrosis and mild atrophy was inconsistently observed in treated groups, affecting primarily the animals in the 8000 mg/kg bw/day group. Nodular hyperplasia of the pancreas was also observed more often in females at this high dose level (3/60 in controls versus 11/40 in the high dose groups) than in males (1/60 in controls versus 1/40 in the high dose group). Since there was no increased incidence of pancreatic tumours, the Panel considered the toxicological relevance of the slight increase in incidence of nodular hyperplasia in the pancreas of the female high dose group unremarkable. The authors indicated that all the changes in lungs, ovaries, seminal vesicles, prostate, pancreas were inconsistently present and lacked clear dose-response relationships. A renal pigment deposit (identified as iron-containing haemosiderin) was present in tubular and pelvic epithelial cells with increased incidence in male rats at 8000 mg/kg bw/day. Females and low dose males were not affected. Focal hyperplasia of the renal pelvic epithelium was also present with increased incidence in the 8000 mg/kg bw/day group in males. Tubular degeneration was increased in the 8000 mg/kg bw/day group in males. The authors concluded that administration of aspartame to rats for two years did not result in adverse effects or an increased incidence of neoplasms, and produced no evidence of treatment-related non-neoplastic lesions in any organ examined except for the renal changes in males at 8000 mg/kg bw/day. Based on these observations the Panel identified a NOAEL of 4000 mg aspartame/kg bw/day in this study.

In a chronic toxicity study in the rat, aspartame (DKP content varied from 0.8 % to 1.2 % per batch) was administered in the diet to groups of male and female Charles River albino rats at dose levels of 0 (control; 60 males/60 females), 2000 (40 males/40 females) and 4000 (40 males/40 females) mg/kg bw/day for 104 weeks post-weaning (E70, 1974). These treated rats were obtained as F_1 weanlings from parental animals which had been pre-treated with aspartame at the same levels for 60 days prior to mating and continued to receive the test material during mating and throughout the gestation and nursing periods. Endpoints evaluated were physical appearance, behaviour, eye examination, growth, feed consumption, survival, clinical chemistry, organ weights, tumour incidence and gross and microscopic pathology. There was no effect on the physical appearance and behaviour of the animals. Growth rates for the exposed animals were comparable to growth rates of the controls except for the high dose in males for which growth rates were significantly lower than for controls, this was accompanied by a significantly lower feed consumption. Survival at 104 weeks was comparable to controls for both treatment groups. Clinical chemistry data revealed no consistent statistically significant alterations outside the normal range. The results from ophthalmoscopic examinations

revealed no indication of a compound-related effect. Gross observations at necropsy revealed no compound-related changes in any organs or tissues. Some statistically significant changes in relative organ weights were reported but these were not accompanied by histopathological alterations and these were therefore considered by the authors to be of no biological significance. Histopathological examination was performed on all gross lesions from all animals at all treatment levels and on 20-25 grossly unremarkable organs (see below) from all control and treated animals. Microscopic evaluation of eight coronal sections representing all major neuroanatomic areas of the brain was performed for each control and treated rat. Urinary bladder also received more extended examination, since four intermittent transverse bladder sections were examined microscopically from each rat in the study. Incidences for the types of tumours analysed was not statistically significantly higher in any dose group compared to the control group. Histopathology revealed that at all levels administered, there were no effects on brain, spinal cord, eye, salivary gland, thyroid, lung, small intestine, large intestine, lymph nodes, peripheral nerve, skin, testis, prostate, seminal vesicle, vagina, bone, bone marrow, and mammary gland. For the stomach, the incidence of focal gastritis, mucosal ulceration, and pigmentation was slightly increased in the treated females, but these effects were considered by the authors to be of little biological relevance. Pancreatic focal acinar hypertrophy occurred more frequently among low dose male rats than among control animals but was considered by the authors to be spontaneous in nature. Likewise, an increased incidence of ovary atrophy among the high dose animals and a higher incidence of endometritis in low dose survivors and high dose non-survivors were considered by the authors to be unrelated to treatment. Pituitary chromophobe hyperplasia and cysts involving the pars distalis were observed in higher numbers of treated animals, but because of the overall low incidence of these findings, the authors did not consider them compound-related. The incidence of nodular hyperplasia of the adrenal cortex was somewhat higher for the treated males and angiectasis of the adrenal was more frequently noted for the treated females than for the respective controls. However, the authors indicated that these findings were not common to both sexes and the differences were not considered significant. The incidence of renal cortical pigmentation among treated male and female survivors and focal mineralisation among only the treated females was slightly increased as compared to the controls but the authors indicated that there was no meaningful difference between findings for the control and treated groups. The incidence of parenchymal hyperplastic nodules was higher in the livers of treated females (3/23 in the 2000 mg/kg bw/day and 1/20 in the 4000 mg/kg bw/day group) when compared with controls (0/28), whereas the incidence was similar between control (2/35) and treated males (1/20 in the 2000 mg/kg bw/day and 1/18 in the 4000 mg/kg bw/day group). This finding among the treated females was not considered by the authors as compound-related since the incidence for the control females was low compared to the historical control data of this strain, for studies conducted in their laboratories and since there was no evidence of a dose-response relationship. The only other notable finding for this organ was a slightly increased incidence of pericholangitis among the high dose male non-survivors. The authors concluded that rats treated with 2000 or 4000 mg aspartame/kg bw/day throughout the prenatal period, during weaning and post-weaning for 104 weeks by direct dietary administration, exhibited no adverse effects regarding survival rates or incidence of neoplasms, nor did they exhibit evidence of treatment- related non-neoplastic changes in any organ or tissue of either sex. The Panel agreed with this evaluation and identified a NOAEL for this study of 4000 mg/kg bw/day, the highest dose level tested.

In an additional study (E87, 1973), a supplemental histopathological examination of all brains from these two chronic rat carcinogenicity studies (E33-34, 1973 and E70, 1974) was performed (since the initial post-mortem assessment only involved two coronal sections of brains from each rat in the high dose and concurrent control groups with additional examination restricted to rats that exhibited gross evidence of brain lesion(s)). In the E33-34 studies, 440 animals were examined from the 104-week study (120 control and 320 treated animals), 12 neoplasms involving the brain were discovered in rats from treated groups. Their type and distribution are presented in Table 19.

Table 19: Intracranial neoplasms as reported in rats in the 104 week oral toxicity study on aspartame (E33-34; E87)

Intracranial neoplasms	Male (Dose mg aspartame/kg bw/day)					Female (Dose mg aspartame/kg bw/day)				
	Cont	**1000**	**2000**	**4000**	**8000**	**Cont**	**1000**	**2000**	**4000**	**8000**
Survivors	23/60	18/40	21/40	23/40	21/40	26/60	23/40	20/40	14/40	10/40
Astrocytoma	0/60*	1/40	0/40	0/40	0/40	0/60	1/40	0/40	0/40	0/40
Astrocytoma + Ependymal components	0/60	1/40	1/40	1/40	0/40	0/60	1/40	0/40	0/40	0/40
Oligodendroglioma	0/60	0/40	0/40	1/40	0/40	0/60	0/40	0/40	0/40	0/40
Ependymoma	0/60	0/40	0/40	2/40	0/40	0/60	0/40	0/40	1/40	0/40
Meningeal Sarcoma	0/60	0/40	0/40	0/40	0/40	0/60	0/40	0/40	0/40	1/40
Glioma unclassified	0/60	0/40	0/40	0/40	0/40	0/60	0/40	0/40	0/40	1/40

* In the authentication review of selected materials submitted to the Food and Drug Administration relative to application of Searle Laboratories to market aspartame (E102a, b, c) the number of animals with neoplasm (intracranial tumour) in the control group was reported as 1 instead of 0 (as reported in E33-34 and E87).

In the second rat study (E70, 1974), the tumourigenic potential of aspartame was assessed after treatment throughout the prenatal period, during weaning and post-weaning for 104 weeks by dietary administration. The parents of the rats in this study were also pre-treated with aspartame for 60 days prior to mating. Nine neoplasms involving the brain were identified in the 280 animals in this study. Their type and distribution are presented in Table 20.

Table 20: Intracranial neoplasms in rats exposed to aspartame in a lifetime toxicity study (E70, 1974; E87, 1973)

Histopathological lesions	Male (Dose mg aspartame/kg bw/day)			Female (Dose mg aspartame/kg bw/day)		
	Control	**2000**	**4000**	**Control**	**2000**	**4000**
Astrocytoma	3/60	1/40	1/40	1/60	1/40	0/40
Ependymoma	0/60	1/40	0/40	0/60	0/40	0/40
Meningeoma	0/60	0/40	0/40	0/60	0/40	1/40

These results indicated a random occurrence of intracranial neoplasms, unrelated to treatment.

Based on the results of these two studies, the Panel concluded that evidence of an intracranial tumourigenic effect was not demonstrated. The Panel noted that in treated animals of the first 104-week study, more brain tumours originating from different tissues (astrocytoma, oligodendroglioma, ependymoma and meningeoma) were observed. However, there was no dose-relationship and males of the high dose group did not show any brain tumours. Moreover, with the exception of males in the 4000 mg/kg bw/day group, only one or two animals per group exhibited a brain tumour and the overall incidence of intracranial neoplasms in the 104-week toxicity study (2.7 %) was lower than the incidence in the control group of the lifetime carcinogenicity study (3.3 %). The incidence of spontaneous brain tumours in Sprague-Dawley-derived rats has been reported by Ward and Rice (1982) to vary from 0 % up to 3.3 %. A similar incidence was reported by Weisburger et al. (1981).

JECFA also evaluated these data and concluded that neither the overall incidence nor the appearance of any particular neoplasm was associated with aspartame exposure. Therefore, JECFA concluded that aspartame did not cause brain tumours in rats (JECFA, 1980).

An additional 2-year chronic toxicity and carcinogenicity study was conducted in Wistar rats (Ishii et al., 1981; Ishii, 1981). Wistar rats (86 males and 86 females) were administered aspartame (DKP content up to 1 %) at dose levels of 0 (control), 1000, 2000 and 4000 mg/kg bw/day in the diet for up to 104 weeks; an additional group was fed 4000 mg/kg bw/day aspartame plus DKP (3:1) for up to 104 weeks. There was a dose-dependent depression of body weight gain at 2000 and 4000 mg/kg bw/day and at 4000 mg/kg aspartame plus DKP (3:1) in males, and at all dose levels in females. This effect was associated with decreased feed consumption. Survival was not consistently affected. In males, but not females, there was a trend for decreasing body weight in all groups including controls after one year. There were no effects on liver weight throughout the study. Clinical biochemistry and organ pathology were unremarkable and any significant changes observed were not treatment-related. Subsequent detailed analysis for brain tumours showed no evidence for increased incidence of brain tumours (Ishii, 1981). One atypical astrocytoma was found in a female control rat, and two astrocytomas, two oligodendrogliomas and one ependymoma were found in the treated groups, with no statistical difference in incidence between the groups. There was no evidence of a treatment-related effect on the incidence of non-neoplastic changes other than a dose-related increase in focal mineralisation of the renal pelvis in both males and females (incidences in males: control, 1/57; 1000 mg aspartame/kg bw/day, 5/55; 2000 mg aspartame/kg bw/day, 10/60; 4000 mg aspartame/kg bw/day, 15/59; incidences in females: control, 16/59; 1000 mg aspartame/kg bw/day, 23/59; 2000 mg aspartame/kg bw/day, 30/59; 4000 mg aspartame/kg bw/day, 46/60) associated with a dose-related increase in urinary calcium (Ishii et al., 1981; JECFA, 1981; EFSA, 2006). The authors attributed this lesion to irritation caused by the mineral deposition and considered it to be of minimal toxicological significance. The Panel noted that mineralisation in the kidney is a common finding in rats, the aetiology being associated with mineral imbalance (Lord and Newberne, 1990). Thus, there was no evidence for toxicity of aspartame alone or in combination with its degradation product DKP, over 2 years in rats. The Panel identified from this study a NOAEL of 4000 mg aspartame/kg bw/day. In addition, the Panel noted that the study provided information on the lack of toxicity of aspartame when administered in conjunction with DKP.

A re-evaluation of all neoplastic and non-neoplastic lesions of the Ishii et al. (1981) study was reported (Iwata, 2006). No significant differences between controls and treated animals were identified and it was concluded that all the tumours observed could have occurred spontaneously. Increased mineralisation of the renal pelvis (as reported in Ishii et al., 1981) was observed in males at 2000 and 4000 mg/kg bw/day and in females at 4000 mg/kg bw/day. The original study report (Ishii et al., 1981) stated that this was not related to pelvic epithelial hyperplasia or pelvic inflammatory reactions, although the follow-up study reported an increase in transitional-cell hyperplasia in males at 4000 mg/kg bw/day. This was attributed to irritation caused by the mineral deposition and was considered by the authors of the re-evaluation to be of negligible, if any, toxicological relevance. The Panel agreed with this conclusion.

Hamsters

The JECFA evaluation (JECFA, 1980) also described a chronic toxicity study in hamsters. In this study groups consisting of five male and five female hamsters were fed aspartame (containing < 1% DKP) at dietary levels of 1000, 2000, 4000 and 12 000 mg/kg bw/day for 46 weeks (E27, 1972; E35-36; 1972).

The Panel noted the inadequacies of this study due to the short duration of the study, the limited number of animals per group and the presence of infection in the animals. The Panel agreed that this study cannot be used for the present evaluation.

Dogs

5-month_old Beagle dogs (5 animals/sex/group) were exposed to aspartame (a content of DKP up to 1 %) via dietary administration for 106 weeks at dose levels that amounted to 0 (control), 1000, 2000 and 4000 mg/kg bw/day (E28, 1972). Physical examinations were performed at 4-week intervals and ophthalmoscopic evaluations at 24, 52, 78 and 104 weeks of treatment. Haematology, clinical chemistry and urinalysis were performed periodically for all dogs. After necropsy, representative tissues from control and all treated animals were processed for microscopic examination. Survival rates were 100 % in all groups. No compound-related variations in feed consumption and body weight gain were observed. Physical examination findings were unremarkable. No changes in motor behaviour activity or in general physical appearance were noted. Periodic ophthalmoscopic examination did not reveal any treatment-related changes. Haematology and clinical chemistry findings revealed no biologically meaningful treatment-related alterations, although transient sporadic significant differences were reported in several of the parameters. However, most values remained within physiological limits and no dose-related pattern was evident. A significant increase in excretion of urinary phenylketones was observed in some high dose dogs at week 2, 4 and 26 of treatment, but not at all other time intervals. There was no increase in plasma L-phenylalanine levels during the 106-week treatment period. Gross and microscopic findings and organ weight data were unremarkable. An extended histopathological examination of the brain tissue did not reveal any lesions (E86, 1973). The authors concluded that continuous dietary administration of aspartame at daily intake levels of 1000, 2000 and 4000 mg/kg bw/day in Beagle dogs of both sexes for 106 weeks causes no biologically meaningful alterations in body weight, feed consumption, physical examination, clinical chemistry examinations or gross and microscopic findings. The Panel agreed with this evaluation. This study has the advantage of providing data on systemic toxicity in a non-rodent species. However, the duration of the study is only 2 years and the number of animals involved is only 5/sex/group, which limits the power of the statistical analysis. The Panel concluded that the NOAEL in this study was 4000 mg/kg bw/day.

Monkeys

The only study addressing the chronic effects of aspartame on primates is a 52-week oral toxicity study in neonatal rhesus monkeys (*Macaca mulatta*) (E32, 1972). Starting in infancy, rhesus monkeys were dosed with aspartame (containing 0.2-1.0 % DKP), which was mixed with Similac milk formula and administered four times daily. Following gradual increases in the doses administered, mean daily doses of aspartame of 970 mg/kg bw/day (planned dose 1000 mg/kg bw/day; 1 male/1 female), 3010 mg/kg bw/day (planned dose 3000 mg/kg bw/day; 2 males/1 female) and 3620 mg/kg bw/day (planned dose 4000-6000 mg/kg bw/day; 2 males) were attained. The actual duration of dosing ranged from 210-363 days.

Survival was 100 % in all except the high dose group; one monkey in this group died after 300 days of treatment; the cause of death was unknown. Feed intake and growth rate were mildly reduced by treatment with aspartame. One low dose monkey, which had an apparent congenital abnormality, was kept in the study but had reduced weight gain compared with other monkeys in the study. Haematology and clinical chemistry parameters were generally unremarkable in treated animals except for increased plasma phenylalanine and tyrosine levels, associated with increased urinary phenylketone levels, in medium and high dose groups after six months.

The main adverse effect observed in this study was that animals in both the mid and high dose groups started to experience grand mal seizures, the first such event occurring on day 218. Occurrence of seizures was associated with high plasma phenylalanine levels (Median dose: mean peak of 420 µmol/100 ml at 28 weeks; high dose: mean peak of 480 µmol/100 ml at 36 weeks); similar convulsions occurred in monkeys fed L-phenylalanine in equimolar quantities. In the low dose group no increase in plasma phenylalanine levels was observed and no seizures occurred. Intermittent seizures occurred thereafter for the remainder of the study, usually occurring during handling.

Treatment was withdrawn for the last three months of the study; no convulsions were observed during this period.

All the mid dose and high dose monkeys in this study developed *Shigella* infections during this study, and the data reassurance programme conducted by Searle rejected the study on this basis (Rust, 1976). It should also be noted that the study included no control group; historical control data were relied upon for comparison.

In summary, treatment of rhesus monkeys with high doses of aspartame for the first year of life did not have any significant effect on biochemical parameters, but animals treated with aspartame at doses above 3000 mg/kg bw/day experienced grand mal seizures, beginning after about 30 weeks of treatment. The significance of this finding is not clear, because all the monkeys manifesting grand mal seizures were also affected by a *Shigella* infection during the study. The Panel concluded that the study provided insufficient information to conclude on chronic effects of aspartame in monkeys.

3.2.4.2. Additional long-term carcinogenicity studies

Since the last evaluation of aspartame by the SCF, two new long-term carcinogenicity studies on aspartame in rats (Soffritti et al., 2006, 2007; Chiozzotto et al., 2011) and one in mice (Soffritti et al., 2010) have been reported. All three studies were performed by the European Ramazzini Foundation (ERF).

In the Soffriti et al. (2006) study, Sprague Dawley rats (100-150 animals/sex/group) from the in-bred colony of the ERF were exposed to 0, 80, 400, 2000, 10 000, 50 000 or 100 000 mg aspartame/kg in the diet, equivalent to 0, 4, 20, 100, 500, 2500 or 5000 mg aspartame/kg bw/day, from eight weeks of age until natural death. A range of inflammatory changes was observed in a variety of organs and tissues particularly in the lungs and kidneys, in both males and females. Furthermore, an increased incidence of lymphomas/leukaemias in female rats, with a positive significant trend in both male and female rats was reported by the authors. Additional effects included an increased incidence of transitional-cell carcinomas of the renal pelvis and ureter and their precursors (dysplasias), with a positive significant trend in female rats, an increased incidence of malignant Schwannomas of peripheral nerves, with a positive significant trend in male rats and an increased incidence of malignant tumour-bearing animals, with a positive significant trend in both sexes.

In 2007, Soffriti et al. (2007) published a follow-up study in which groups of 70–95 male and female Sprague-Dawley rats were exposed to 0, 400 or 2000 mg aspartame/kg in the diet, equivalent to 0, 20 or 100 mg aspartame/kg bw/day from the 12th day of fetal life until natural death. The authors of the study reported a significant, dose-related increase in the incidence of malignant tumour-bearing animals in males and a dose-related increased incidence in lymphomas/leukaemias in females, and an increased incidence of lymphomas/leukaemias in treated males and females at the high dose group. Furthermore, a dose-related increased incidence of mammary gland carcinomas, in females and a significantly higher incidence of these carcinomas in females exposed to the high dose was reported (Soffritti et al., 2007; Chiozzotto et al., 2011).

The two rat studies have already been evaluated by the former AFC Panel (EFSA, 2006) and the ANS Panel (EFSA, 2009a, 2009b), respectively. Both Panels considered that the two ERF rat studies had flaws that bring into question the validity of the findings, as interpreted by the ERF. In particular, the high background incidence of chronic inflammatory changes in the lungs and other vital organs and tissues, and the uncertainty regarding the correctness of the diagnoses of some tumour types were major confounding factors in the interpretation of the findings of the study. The ANS Panel also noted that the increase in incidence of mammary carcinomas was not considered indicative of a carcinogenic potential of aspartame since the incidence of mammary tumours in female rats is rather high and varies considerably between carcinogenicity studies. The ANS Panel noted that the only consistent findings reported by the authors in the two rat studies were an increased incidence of lymphomas/leukaemias in female rats and an increased incidence of lymphomas/leukaemias in treated males and females of the

high dose group (EFSA, 2009a). A full evaluation of the ERF studies, including an assessment of the malignant schwannomas and the hyperplastic and neoplastic lesions of the epithelium of the renal pelvis, ureter and urinary bladder, has been published elsewhere (EFSA, 2006; EFSA, 2009a, 2009b). In the present evaluation, the ANS Panel noted that an NTP review of selected ERF studies (conducted between 1974 and 1993) had found evidence of high rates of infection in the animals at the ERF institute (NTP-EPA, 2011). Furthermore, the NTP evaluation included a review of original histopathological slides. The review noted that there was no formal quality assessment process for pathology diagnoses, and that in a significant number of cases the NTP reviewers did not confirm malignancy as diagnosed by ERF pathologists. In view of these methodological concerns, given the consistency of findings in the studies reviewed, the Panel considered that these would apply to other studies carried out by Soffritti and co-authors at the ERF including the aspartame studies. The COC in 2006 also evaluated the Soffritti et al. (2006) study on aspartame (COC, 2006). In light of the limitation in the design of this study and the use of animals with a high infection rate, the COC considered that no valid conclusions could be derived from it (COC, 2006).

In the mouse study (Soffritti et al., 2010) groups of 62–122 male and female Swiss mice were treated with aspartame in the feed at doses of 0, 2000, 8000, 16 000 or 32 000 mg/kg feed, equivalent to 0, 250, 1000, 2000 or 4000 mg/kg bw/day, from prenatal life (12 days of gestation) until death. The authors of the study reported a dose-related increase in hepatocellular carcinomas in the two highest dose male mice groups, and an increase in the incidence of alveolar/bronchiolar carcinomas in males of the high dose group, whereas no compound-attributed carcinogenic effects were reported in female mice at any of the doses tested.

The ANS Panel (EFSA ANS Panel, 2011) and EFSA (EFSA, 2011a) concluded that the hepatic and pulmonary tumour incidences reported by Soffritti et al. (2010) all fall within their own historical control ranges for spontaneous tumours. It was also noted that Swiss mice are known to have a high background incidence of spontaneous hepatic and pulmonary tumours (Prejean et al., 1973; Fox et al., 2006).

Based on these data, the Panel concluded that the results of the studies performed by Soffritti et al. (2010) do not provide evidence for a carcinogenic effect of aspartame in mice.

Since the evaluations by the SCF and JECFA, the US National Toxicology Program (NTP) has carried out several 9-month carcinogenicity studies with aspartame in genetically modified mouse models (NTP, 2005). Fifteen male and fifteen female transgenic mice/group of three strains (Tg.AC hemizygous, p53 haploinsufficient and Cdkn2a deficient) were exposed to aspartame in the diet at doses ranging from 0, 3.1 to 50 g/kg diet for 9 months. The high dose tested was equivalent to 7660 and 8180 mg aspartame/kg bw/day in males and in females, respectively (Tg.AC hemizygous); 7280 and 9620 mg aspartame/kg bw/day in males and in females, respectively (p53 haploinsufficient); 7400 and 9560 mg aspartame/kg bw/day in males and in females, respectively (Cdkn2a deficient) (NTP 2005). According to the NTP, there was no evidence of treatment-related neoplastic or non-neoplastic lesions in any of these studies. The former AFC Panel evaluated these studies and agreed with this conclusion. The overall number of animals with primary neoplasms, with benign neoplasms or with malignant neoplasms in aspartame-exposed groups of both sexes was not significantly increased compared to the respective controls (EFSA, 2006). The Panel agreed with these conclusions.

3.2.5. Reproductive and developmental toxicity of aspartame

3.2.5.1. Studies on reproductive and developmental toxicity received following a public call for data which were available at the time of the previous JECFA and SCF evaluations

In the reproductive and developmental toxicity studies evaluated, aspartame was either administered in the diet or given by gavage (rabbit studies only).

3.2.5.1.1. Reproductive studies

Rats

A two-generation reproduction toxicity study was performed in Charles River albino rats (12 male and 24 female animals per group) that received aspartame (assumed to be 100 % pure) in the diet at dose levels of 0 (control), 2000 and 4000 mg/kg bw/day (E11, 1971). The results of this study were not reported in detail. Dietary administration was conducted through two parental generations and one-litter per generation. Parameters evaluated included survival, body weights, feed consumption, physical appearance, and behaviour of the parental generations; indices of fertility, gestation, live birth and lactation; the litter size, appearance, behaviour, body weights and growth of offspring; and results of gross necropsy on selected weanlings. All parameters were comparable between the control and test groups with the exception of body weight suppression at weaning among the high dose pups which was evident during both the first (F_1) and second (F_2) generation litters. Statistical analysis performed on the F_1 and F_2 litters revealed the body weights at the end of weaning of both sexes at the high dose level to be statistically significantly lower (F_1: 41 g and 38 g in males and females, respectively; F_2: 43 g and 41 g, in males and females, respectively) than those of the controls (F_1: 48 g for both sexes; F_2: 52 g for both sexes). Growth among the low dose pups was comparable to the controls during both reproduction phases. The high dose pups were noted to be generally smaller than the control and low-dose pups. The effect on haematological and biochemical parameters and on tissue morphology was examined in F_2 pups from 1 up to 21 days of age. The results of this part of the study were described in more detail in another report below (E9, 1972).

Forty pups, 20 males and 20 females (of the F_2 generation from the study described above), offspring from the maternal groups that received aspartame at dose levels of 0 (control), 2000, or 4000 mg/kg bw/day, were used (E9, 1972). At 24 hours and at 5, 15, and 21 days postpartum 10 pups from each group (5 pups/sex) were sacrificed and haematology, clinical chemistry and histopathology of selected tissues were performed. The appearance and behaviour of the pups during the study was comparable between control and test groups. Results of haematology and clinical chemistry (measured at days 5, 15 and 21 postpartum) were unchanged at both dose levels. Microscopic examination of sections of heart, liver, stomach, kidney and urinary bladder revealed no apparent compound-related effects. In the kidney, minimal to slight hypertrophy and vesiculation of nuclei in cells of tubules in the inner cortex was noted primarily in the 15 and 21-day 4000 mg/kg bw/day males and females. Minimal changes were also seen in two 21-day 2000 mg/kg bw/day females. The authors noted that features of this same condition were also observed in a 15-day control male, but microscopic examination of renal tissue from weanlings aged 28-30 days revealed that such changes were not present in either control or treated rats of this age. The authors concluded that these changes were treatment-related but of a transient nature. A treatment-related effect at 2000 mg/kg bw/day at 21 days was equivocal, since the control group was not negative throughout. Such changes were not present in the treated rats at 28-30 days of age. The Panel noted that the histological changes observed in the kidney seemed to be consistent with normal histological processes during postnatal development. There was evidence of a treatment-related effect at 15 and 21 days of age, but this was transient and appeared to have resolved by 28-30 days of age. The Panel identified a NOAEL from these studies of 2000 mg/kg bw/day, based on the lower pup weights at weaning in both generations (E9, 1972; E11, 1971).

In study E10 (1972), aspartame (DKP content 0.3 %) (0, 2000 or 4000 mg/kg bw/day) was administered in the diet to Charles River rats (12 males and 48 females in the control group and 40 females in the aspartame groups) for 64 days (males) or 14 days (females) prior to mating, followed

by a further dosing period until the end of lactation (female rats). During the pre-mating period, rats in the male groups ingested a mean daily dose of 1950 and 3867 mg/kg bw, respectively. In females the mean daily doses were 2017 and 4430 mg/kg bw during the pre-mating period, 1988 and 4100 mg/kg bw/ during gestation, 2375 and 4925 mg/kg bw during the first 14 days of lactation and 2700 and 5900 mg/kg bw during the last 14 days of lactation. Aspartame had no significant effect on maternal sexual behaviour or fertility. Female body weights were slightly but significantly decreased in the high dose group at pre-mating day 7, GD 18 and postpartum day 7. The authors also reported that the pups from aspartame-treated groups did not differ from controls in growth, survival or subsequent reproductive performance. The Panel agreed with this conclusion and identified a NOAEL of 4000 mg/kg bw/day (actual ingested doses ranged from 4100 mg/kg bw/day during gestation to 5900 mg/kg bw/day during the last 14 days of lactation), this being the highest dose level tested.

In study E39 (1973), the effect of aspartame on peri and postnatal development was studied in female rats (30 animals per dose group). Aspartame (with a content of DKP of 0.5 %) was administered in the diet at intended dose levels of 0 (control), 2000 or 4000 mg/kg bw/day. The recorded dose levels were 0, 2000 or 4000 mg/kg bw/day during gestation, increasing to 0, 3500 and 6800 mg/kg bw/day during lactation. The number of viable pups per litter at birth and pup survival until weaning was significantly decreased in the high dose group. Despite the reduced number of pups no effect on pup body weight (through to postpartum day 21) was recorded. There was no difference in feed consumption between the groups throughout the experiment. Maternal body weights were comparable between control and treated groups during gestation, except for GD 21, where the low and high dose groups showed a 5 and 9 % reduction in body weight, respectively. During lactation, maternal body weights were comparable between the control and low dose groups, whereas in the high dose group mean body weight was significantly lower at postpartum day 21 (approximately 12 %). According to the authors, the NOAEL in this study was 2000 mg/kg bw/day (recorded doses were 2000 mg/kg bw/day during gestation and 3500 mg/kg bw/day during lactation). The Panel agreed with the author's conclusion on the NOAEL but noted that, considering the poor survival of control pups, the health status of all the animals in the study might have been compromised.

Monkeys

A report summarising fragmentary data from a monkey study with aspartame was also submitted to EFSA following the public call for data (E88, 1975). The incompleteness of the data, however, severely limited the usefulness of this study. In summary, administration of aspartame to eight pregnant monkeys at dose levels up to 3800 mg/kg bw/day did not alter maternal appetite or body weight and did not induce seizure. There were no malformations observed in the fetuses at term. In addition, plasma phenylalanine levels in pregnant monkeys were not significantly increased, and the two abortions reported were spontaneous. However, no information on the reproductive capabilities, the reproductive status at the time of mating, the identity and reproductive history of the mate, evidence of actual establishment of the pregnancy or assessment of the gestational status of pregnant animals or experimental conditions of the animals were provided. The Panel concluded that the study provided insufficient information to evaluate reproductive and developmental effects of aspartame in monkeys.

3.2.5.1.2. Developmental studies

Mice

The developmental toxicity of aspartame was investigated in Charles River CD-1 mice (E89, 1975) (Appendix I). Aspartame (DKP content 0.29 %) was administered in the diet to mated mice (four groups of 36 pregnant female) for 10 days from GD 6 to 15 at the intended dose levels equating to 0 (control), 1000 (1400)[19], 2000 (2700), 4000 (5700) mg/kg bw/day. On GD 18, all females were sacrificed and necropsied. Fetuses were examined for external, soft tissue and skeletal abnormalities.

[19] The doses in brackets refer to the actual average doses to which the animals were exposed to based on feed intake.

The treatment had no effect on maternal survival and conception rates, maternal body weight changes, feed consumption, incidence of abortions and premature deliveries, proportion of litters that consisted of fetuses only, of both fetuses and resorption sites, or of resorption sites only, mean litter size, mean number of resorptions per litter, mean male and female fetal body weights and crown-rump distances, and the number of major malformations and skeletal variants, when compared to controls on a fetal incidence and a litter incidence basis. According to the Panel, the NOAEL for developmental toxicity in this study was 5700 mg/kg bw/day, the highest dose level tested.

Rats

Three segment III studies were performed in rats (peri- and postnatal development: E47, 1973; E48, 1973; E 49, 1973).

In two studies on peri- and postnatal development (E47, 1973 and E48, 1973), aspartame (DKP content 0.2 and 0.5 %, respectively) was administered to rats (24 female rats per group in E47 and 30 female rats per group in E48) at the intended doses of 0, 2000 or 4000 mg/kg bw/day. In E47, the rats received 2500 and 4400 mg/kg bw/day during gestation and 3600 and 6800 mg/kg bw/day during lactation, whereas in E48, the actual doses were 1800 and 4000 mg/kg bw/day during gestation and 3700 and 7000 mg/kg bw/day during lactation. The authors reported significant body weight suppression of the pups at birth at the high dose (E47, E48) and weaning (at postpartum day 21) in females at both low and high doses (E47, E48) and in males at the high dose (E48). In the high dose group a significant decrease in survival at weaning was observed (E47, E48) and incomplete eyelid opening in 5/164 pups (E47) and 2/79 pups (E48). The mothers exposed to the high dose showed decreased feed intake at postpartum day 21 and reduced body weight at postpartum day 21 and during lactation (5-16 %). Based on the results reported in these studies, the Panel identified a NOAEL of 2000 mg/kg bw/day (E47:2500 mg/kg bw/day during gestation, 3600 mg/kg bw/day during lactation; E48: 1800 mg/kg bw/day during gestation, 3700 mg/kg bw/day during lactation).

In a further segment III study (E49, 1973) in rats (30 females per group), the effects of aspartame (DKP content 0.5 %) (0 or 4000 mg/kg bw/day in the diet) on peri and postnatal development were compared with those of L-phenylalanine (1800 mg/kg bw/day) or L-aspartic acid (1700 mg/kg bw/day) or the combination of L-phenylalanine and L-aspartic acid (2100 and 1800 mg/kg bw/day). The actual doses that the rats received were 4000 mg aspartame/kg bw/day, 1800 mg L-phenylalanine/kg bw/day, 1700 mg L-aspartic acid/kg bw/day or 2100 + 1800 mg L-phenylalanine + L-aspartic acid/kg bw/day during gestation and 7800 mg aspartame/kg bw/day, 4200 mg L-phenylalanine/kg bw/day, 4000 mg L-aspartic acid/kg bw/day or 4600 + 3900 mg L-phenylalanine + L-aspartic acid/kg bw/day during lactation. The authors of the study reported that feed consumption was comparable between all groups but noted a significant decrease in body weight in the aspartame, L-phenylalanine and L-phenylalanine + L-aspartic acid groups as compared to the control group at postpartum days 7, 14 and 21. For example, at postpartum day 21, mean maternal weights recorded were 369.2 $\pm$ 6.5 g for controls, 327.7 $\pm$ 5.8 g for the aspartame group, 318.4 $\pm$ 10.8 g for the L-phenylalanine group and 316.4 $\pm$ 9.6 g for the L-phenylalanine + L-aspartic acid group. No differences in litter size were found, but at postpartum day 21, L-phenylalanine and L-phenylalanine + L-aspartic acid group pup weights were notably lower (28.4 $\pm$ 3.3 g and 28.0 $\pm$ 2.3 g for male pups and 26.9 $\pm$ 3.1 g and 28.5 $\pm$ 2.3 g for female pups) (statistically not significant) and significantly decreased in the aspartame group (25.9 $\pm$ 1.5 g for male pups and 25.9 $\pm$ 1.3 g for female pups) compared to controls (34.2 $\pm$ 2.5 g for male pups and 33.4 $\pm$ 2.0 g for female pups). Pup survival was comparable between all the groups except for the L-phenylalanine + L-aspartic acid group (30.7 % versus 49.5 % in controls), where it was significantly lower. The authors of the study concluded that L-phenylalanine on its own or in combination with aspartic acid decreased maternal and pup body weight, which reproduced the observed effects of aspartame on these endpoints. The Panel agreed with the author's conclusion but noted the poor survival of control pups.

The developmental toxicity of aspartame (was evaluated in a segment II study using dietary administration to pregnant albino rats (30 animals per group) from GD 6 to 15 inclusive (E5, 1970).

Dose levels were 0 (control), 2000 and 4000 mg/kg bw/day. Parameters evaluated included maternal mortality, body weight, feed consumption, necropsy findings of the reproductive organs, in utero litter size (viable fetuses), resorptions, non-viable fetuses and fetal sex distribution; fetal size, gross appearance and examination of the fetal visceral and skeletal systems. Maternal mortality, body weight changes and necropsy findings were comparable between both aspartame-treated groups and the control. Mean feed consumption throughout gestation was comparable in the control and low dose groups, but was decreased by 15 % at the beginning of the treatment period at the high dose level. Feed consumption in the latter group gradually recovered and reached control values by the time compound administration was discontinued. No evidence of treatment induced fetopathological effects was observed in the 47 treated litters (589 term fetuses) examined. The Panel identified a NOAEL of 4000 mg aspartame/kg bw/day for developmental and maternal toxicity.

The Panel considered that, the two-generation reproductive toxicity study (E11, 1971) was not reported according to current standards but taken together with the segment I study (E10, 1972) and segment III studies (E47, 1973; E48, 1973) the available data were sufficient to conclude on reproductive toxicity. In summary, the results of the reproductive and developmental toxicity studies in rats indicate NOAELs that ranged from 2000 mg aspartame/kg bw/day (E11, 1971; E39, 1973; E47, 1973; E48, 1973) to 4000 mg/kg bw/day (E5, 1970; E9, 1972; E10, 1972).

Rabbits

In several studies aspartame was administered in the diet at dose levels of 0 (control), 2000, or 4000 mg/kg bw/day (E53, 1973; E54, 1974; E55, 1973) or 0 (control), 1000, or 2000 mg/kg bw/day (E62, 1973) from GD 6 to GD 17-19, or 0 (control), 1400, or 2400 mg/kg bw/day (E63, 1973) from GD 6 to GD 18. The Panel noted that the actual dose to which the rabbits were exposed did not exceed 1880 mg/kg bw/day (range 1160-1880 mg/kg bw/day) when the intended dose was 4000 mg/kg bw/day (administered as 4.8 % (w/w) aspartame in the diet). This was a result of the considerable decrease in feed intake observed in the pregnant rabbits (32-62 % decrease) in this dose group. The decrease in feed intake was much less pronounced in the 2000 mg/kg bw/day groups than in the 4000 mg/kg bw/day groups; as such the Panel noted that the actual aspartame doses in the low and high aspartame dose groups were often comparable.

For example in E54 (1974), aspartame (DKP content 0.1 %) was administered in the diet from GD 6 to 19 to 16 pregnant female rabbits per dose group. The actual aspartame doses were reported to be 1880 and 1870 mg/kg bw/day for the intended 2000 and 4000 mg/kg bw/day groups, respectively. The Panel noted that a significant decrease in fetal body weight and skeletal anomalies were reported for the 4000 mg/kg bw/day group but not for the 2000 mg/kg bw/day group even though both groups received the same dose of aspartame based on feed intake. Thus, the Panel concluded from these observations that the developmental effects on body weight and skeletal development reported in the aspartame feeding studies may be caused by the significant depression of feed consumption in the high dose group.

In study E63 (1973) artificially inseminated rabbits (n = 26 per group) received aspartame (with a DKP content of 1-4 % as analysed in the diets) in a commercially-prepared pellet diet at a level of 3.4 or 6.7 % w/w from GD 6 to 18 resulting in a dose of 1400 and 2400 mg/kg bw/day. The control group was pair-fed (GD 6 to 24). There were no deaths in the pair-fed control group, two deaths in the 1400 mg/kg bw group and three deaths in the 2400 mg/kg bw group. One female in the high dose group had an early delivery. Conception rates were 96, 81 and 77 % in the control, 1400 and 2400 mg/kg bw groups, respectively. During gestation (GD 6 to 24), there were similar increases in body weight (9 and 7 %) in both aspartame-treated groups and an increase of 3 % in the pair-fed controls. Increases in body weight during the entire gestation period were 5, 2, and 2 % in these same groups. The feed consumption during treatment and post-treatment as compared to pre-treatment, decreased by 6 and 19 %, respectively for the 1400 mg/kg bw group, increased by 3 and 2 %, respectively for the 2400 mg/kg group and decreased by 21 and 33 %, respectively for the pair-fed control. The Panel noted that the feed consumption was lower in the pair-fed controls than in the

treated animals. Uterine and litter data for the pregnant females sacrificed at term showed no evidence of compound-related effect. All fetuses were examined for external abnormalities and half of the fetuses were examined for skeletal or visceral examination. The total incidence of major malformations was 5 of 153 fetuses (3 of 21 litters) in the pair-fed control group, 2 of 100 fetuses (2 of 13 litters) in the 1400 mg/kg bw group and 4 of 121 fetuses (3 of 17 litters) in the 2400 mg/kg group.

In another series of prenatal developmental studies, rabbits were exposed to aspartame (at doses up to 2000 mg/kg bw/day) using administration by gavage from GD 6 to 18. Most of these studies were confounded by poor health of the animals, and, in many cases, by a number of deaths in the treated groups, possibly related to the misdosing (E51, 1973; E52, 1973; E79, 1974).

In study E51 (1973), artificially inseminated rabbits (36) received a daily dose of 2000 mg aspartame/kg bw/day (with a content of DKP of 1 %) suspended in an aqueous solution of Tween-80 and methylcellulose by gavage twice daily as equal doses from GD 6 through 18. Twelve control rabbits received the equivalent volume of vehicle as the treated animals. The study was confounded by poor health of the animals and the gavage technique issues. As a result, maternal mortality was high. The administration of aspartame was associated with depression of feed consumption by up to 40 %. The controls were pair-fed to match the aspartame-treated animals with the lowest feed intake, but feed intake was noted to be lower still in control animals. The authors concluded that no embryotoxic or teratogenic effects were observed.

Study E52 (1973) also suffered from maternal mortality due to poor health and the gavage technique issues. The study involved 24 control and 72 treated animals receiving a daily dose of 2000 mg aspartame/kg bw/day (DKP content up to 2 %) suspended in an aqueous solution of Tween-80 and methylcellulose that was administered twice daily as equal doses. The control rabbits received the equivalent volume of vehicle. The control animals were pair-fed to compensate for the decrease in feed intake caused by aspartame. The control animals were pair-fed to match the aspartame-treated animals with the lowest feed intake, but feed intake was noted to be lower still in control animals. The authors concluded that no embryotoxic or teratogenic effects were observed.

In study E79 (1974) artificially inseminated rabbits received a daily dose of 750 (37 animals) or 2000 mg aspartame/kg bw/day (with a content of DKP up to 2 %) (95 animals) in suspension in an aqueous solution of Tween-80 and methylcellulose and administered by gavage twice daily as equal doses to artificially inseminated rabbits from post-insemination day 6 through day 18. Control rabbits (32 animals) received the same volume of vehicle as the treated animals. The study was confounded by poor health of the animals and the misdosing. As a result, maternal mortality was high. The administration of aspartame was associated with decreased feed consumption by up to 36 %. The control animals were pair-fed to match the aspartame-treated animals with the lowest feed intake. However, especially aspartame-treated animals with a very restricted feed consumption died and the aspartame-treated animals with a more normal food consumption survived. As a result, the food consumption in the pair-fed control animals was noted to be lower than the food consumption of the aspartame-treated animals. The authors concluded that no embryotoxic or teratogenic effects were observed. However, the Panel considered the study not to be adequate to reach such a conclusion.

Overall, the Panel considered that the data from the studies described above were confounded by the decrease in feed intake (when aspartame was administered via the diet or by gavage), the poor health of the animals, and, in many cases, by a number of deaths of pregnant rabbits in the treated groups, most possibly related to misdosing.

In a separate study in pregnant rabbits (E90, 1975), which contained a sufficient number of animals for the evaluation of developmental toxicity, aspartame (DKP content 0.29 %) suspended in an aqueous solution of Tween-80 and methylcellulose was administered by gavage twice daily to artificially inseminated rabbits from GD 6 up to 18. The doses of aspartame were 0 (control), 500, 1000 and 2000 mg/kg bw/day. The controls received the same volume of vehicle per kg bw as the treated animals. The study included two additional groups, one that received L-phenylalanine (820 mg/kg

bw/day) and one that received L-aspartic acid (1100 mg/kg bw/day), which, on a molar basis, were levels equivalent to 75 % and 134 % of the amount of the same amino acids theoretically available from 2000 mg/kg bw aspartame. Each treatment group consisted of 50 inseminated rabbits. On GD 28 all surviving females were sacrificed and necropsied. Fetuses were removed by hysterectomy and examined, weighed, measured and preserved for either soft tissue or skeletal examination. Parameters evaluated included: maternal survival rates; maternal conception rates; incidences of abortion or of premature delivery; maternal body weights, feed consumption incidences of litters consisting of fetuses only, of both fetuses and resorptions, or of resorptions only; mean litter sizes; mean number of resorption sites per litter; mean fetal body weights and crown-rump distances; incidences of major and minor malformations on a per litter or per fetus basis. A number of animals died spontaneously during the study (4/control; 4/500 mg/kg bw/day; 1/1000 mg/kg bw/day; 4/2000 mg/kg bw/day; 5/phenylalanine; 2/aspartic acid), mainly due to misdosing. Eight control females, 4 low and mid dose females and 13 high dose females were non-pregnant. No abortions were detected in the control, mid dose and L-aspartic acid groups, two abortions in the low dose aspartame group and 24 abortions were observed in the high dose aspartame group (a significant increase compared to controls) and four in the L-phenylalanine group. An approximately 65 % decrease in mean feed consumption was observed in the 2000 mg/kg bw/day group animals, beginning on the first day of treatment (GD 6) and continuing throughout the treatment period (GD 18). Similarly, feed consumption for the L-phenylalanine group females from gestation days 6 through 18 was significantly decreased by approximately 40 % as compared to the controls. The decrease in feed consumption in the high dose aspartame group was followed by body weight loss, first apparent on day 10. These body weight losses were statistically significant ($P < 0.05$) in the high dose group beginning on GD 13, and were more marked in those females that ultimately aborted, which comprised well over one-half of the pregnant high dose females. Feed consumption was lower in those high dose animals that subsequently aborted than in those that maintained their pregnancies to GD 28. In view of the statistically significant decrease in feed consumption and the high-incidence of abortions, the authors of the report subdivided the data from the high dose animals into two groups for further analysis: those from animals surviving until sacrificed on gestation day 28 and retaining their litter (no abortions); and those from animals surviving until day 28 but aborting their litter. This analysis showed that females in the high dose group having aborted had statistically significantly lower body weights ($p < 0.05$) compared to controls and to non-aborting females, starting at gestation days 13 and 18, respectively. Females in the high dose group having retained their litter (non-aborting) did not show any statistically significant difference in body weight as compared to controls or to aborting females during the entire duration of the study (28 days). Since the decrease in body weight started several days before (13 and 18 days) the abortions (28 days), the authors concluded that abortion was a consequence of significant and rapid body weight loss caused by decreased feed consumption.

At Caesarean section, only 11 pregnant females of the high dose group were available for evaluation versus 36 control, 41 low dose and 44 mid dose pregnant females. The mean number of resorptions in the high dose group was increased although not statistically significant due to the low numbers and large variability between these litters. However, the mean number of resorptions was significantly ($P < 0.05$) increased in the L-phenylalanine group. Mean fetal body weight and length were significantly reduced in both the 2000 mg/kg bw/day group and the L-phenylalanine group (mean body weights (ig) for male fetuses 23.5 (aspartame) and 28.2 (L-phenylalanine) vs. 33.2 in controls; for female fetuses 23.3 (aspartame) and 27.1 (phenylalanine) vs. 32.0 in controls). The decrease was approximately proportional to the reduction in feed consumption. The number of tarsal and metacarpal ossification centres, an indicator of normal fetal growth, was significantly ($p < 0.05$) reduced in the rabbit fetuses of the 2000 mg/kg bw/day and phenylalanine groups. Additionally, a significant increase in the incidence of an extra (13^{th}) pair of ribs and a reduction in sternebral ossification centres were observed in high dose aspartame group. Comparisons based on either fetal incidence or litter incidence revealed a significantly higher rate of total (major and minor) malformations in the 2000 mg/kg bw/day group animals, as compared to the concurrent control group. The increases in major malformations were due primarily to three instances of cleft palate in two litters in the high dose aspartame group and five instances of umbilical hernia or omphalocele in five separate litters in the L-phenylalanine group. The increases in minor malformations were due, in large part, to various

vertebral defects and fused or split sternebrae. The authors indicated that all the above reported effects, observed at 2000 mg/kg bw/day and to a lesser extent in the L-phenylalanine group, appeared to be produced by a severe reduction of nutrient intake.

Aborting female rabbits from the high dose group in study E90 (1975) manifested body weight loss and decreased to negligible feed consumption compared to those that maintained their pregnancies. The authors suggested that the effects on dams and fetus were due to a reduced nutrient intake.

In summary, the results of the reproductive and developmental toxicity studies in rats indicate NOAELs that ranged from 2000 mg aspartame/kg bw/day (E11, 1971; E39, 1973; E47, 1973; E48, 1973) to 4000 mg/kg bw/day (E5, 1970; E9, 1972; E10, 1972). The highest NOAEL for developmental toxicity in rabbits of 2400 mg aspartame/kg bw/day was identified by the Panel in study E63 (1973), in which the test compound was administered in the diet. The NOAEL in the study in rabbits dosed by gavage (E90) was 1000 mg aspartame/kg bw/day, based on maternal toxicity in the 2000 mg/kg bw/day group, which was accompanied by a severe decrease in mean feed intake. The Panel noted that the severely decreased feed intake could have a negative effect on the nutritional status of these females and the observed adverse effects could result from gastrointestinal disturbances as well as from the potential effect of phenylalanine.

The overview of the reproduction and developmental studies is presented in Appendix I.

3.2.5.2. Additional studies on reproductive and developmental toxicity

3.2.5.2.1. Reproductive studies

Rats

The effect of aspartame on reproduction was investigated in Sprague Dawley rats by Brunner et al. (1979). Rats (total number not stated) were fed diets containing 0, 2, 4 or 6 % aspartame (w/w) during pre-breeding, gestation and lactation and post-weaning. Feed consumption measurements suggest that the animals were exposed to doses of aspartame of approximately 0, 1600, 3500 and 5000 mg/kg bw/day during pre-breeding and gestation, 0, 4000, 7000 and 9600 mg/kg bw/day during lactation and 0, 3000, 6000 and 9000 mg/kg bw/day post-weaning. Another group of rats received phenylalanine in the diet (3 %) (approximately 2500 mg/kg bw/day during pre-breeding and gestation, 4600 mg/kg bw/day during lactation). Breeding pairs were enrolled in the study until 18 litters were available for each group. There was no effect on pre-breeding body weights or on maternal weight gain during gestation, but rats exposed to the highest aspartame dose during lactation lost more weight than the other dietary groups (9 % of decrease from Day 1 to Day 21). Increased offspring mortality was observed in rats fed the highest aspartame dose and in the phenylalanine-exposed animals. Gestation length and litter size were not affected by aspartame or phenylalanine; however, pups fed 6 % aspartame weighed significantly less than controls after 30 days and remained lighter throughout the study. Eye opening was delayed by one day in pups in the 6 % aspartame and 3 % phenylalanine diet groups, but timing of pinnae detachment and incisor eruption were not affected. The Panel agreed with the conclusion of the authors.

Potential post-coital fertility was assessed in Charles River rats (6 animals/group) following administration of 300 mg aspartame/kg bw/day in corn oil by gavage for seven days after mating (Lennon et al., 1980). Controls received corn oil. There were no differences noted in the number of rats that became pregnant. The Panel noted that the number of animals used in these studies was small.

Hamsters

Potential post-coital fertility was also assessed in a similar experiment in female hamsters with a control group of 15 animals treated with corn oil and a group of five animals given daily doses of 300 mg aspartame/kg bw in corn oil by gavage for seven days after mating (Lennon et al., 1980). No

differences were reported in implantation and regression of corpora lutea. The Panel noted that the number of animals used in these studies was small.

3.2.5.2.2. Developmental studies

Mice

Mahalik and Gautieri (1984) reported a study in pregnant CF-1 mice (number not reported) which were administered aspartame by gavage at doses of 1000 mg/kg bw/day and 4000 mg/kg bw/day from GD 15 to 18. 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 (22 ± 0.7 at 1000 mg/kg bw/day and 24.4 ± 0.7 at 4000 mg/kg bw/day vs. 20 ± 0.9 in controls). The Panel noted that uncontrolled litter size and pup weight were not taken into consideration and performance of the offspring was assessed only the last day of achievement for the entire litter.

In a similar study by McAnulty et al. (1989), CF-1 pregnant mice (20/group) were administered aspartame by gavage at doses of 0, 500, 1000, 2000, and 4000 mg/kg bw/day from GD 15 to 18. Maternal body weight, feed consumption, gestation length, reproductive indices, and litter size were not affected by the treatment. In the pups, there were no changes in body weights, negative geotaxis, surface and midair righting reflexes. In addition, reflex pupil closure, and ophthalmoscopic examination in the offspring were not altered by the treatment. Time of eye opening was statistically significantly later than control at the lowest and highest doses (14.3 ± 0.15 for both doses vs. 14.8 ± 0.15 in controls), as well as the development of the visual placing which was statistically significantly lower than control at the lowest dose of 500 mg aspartame/kg bw/day (18.8 0.28 vs. 20.5 ± 0.40 in controls). The authors considered these findings as isolated points that may vary in either direction. They were not dose-related and as such, the results were not considered to be biologically meaningful. The authors concluded that *in utero* exposure to aspartame in CF-1 mice did not affect the physical and functional development of the visual system of the pups. The Panel agreed with the conclusion of the authors.

Rats

Holder (1989) studied the effects of aspartame administered in the drinking water to rats from 12 days prior to conception until the pups were 38 days old. Adult Sprague-Dawley rats (10/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). The authors reported that adult rats consumed an average of 14, 68, 347, and 1614 mg/kg bw/day of aspartame respectively, and 835 mg/kg bw/day of phenylalanine. After weaning, the pups (4/sex/litter) were given the same treatment as the adult rats, and consumed an average of 32, 154, 836, and 3566 mg/kg bw/day of aspartame respectively, and 1795 mg/kg bw/day of phenylalanine. The two parameters of morphological development, latency of pinnae detachment and eye opening, as well as the two parameters of reflex development, latencies for surface righting reflex (which decreased in a dose-dependent manner with aspartame treatment) and negative geotaxis were not statistically significantly affected by aspartame or phenylalanine. There were no differences on the performance of spatial memory in the radial-arm maze or in the milk maze. Moreover, the latency of mothers to retrieve their pups was not affected by either treatment. The authors concluded that exposure in utero and later directly of the pups did not affect reflex development, morphological development and spatial memory. The Panel agreed with the conclusion of the authors.

Monkeys

In a postnatal developmental study, monkeys were fed with 0, 1000, 2000, or 2500-2700 mg aspartame/kg bw/day for 9 months. No effects on body weight gain, crown-heel length, teething, vocalization, alertness, or general behaviour were observed. No effects on haematological, plasma chemistry and urinalysis parameters or on ECG were observed. Aspartame exposure had no effects on

learning performance and hearing ability. The NOAEL was 2500-2700 mg aspartame/kg bw/day, the highest dose of aspartame tested (NTP-CERHR Report, 2003).

3.2.5.2.3. Other studies

Mice

Collison et al. (2012a) investigated the effects of neonatal exposure to monosodium glutamate (MSG), aspartame or a combination of both, on glucose and insulin homeostasis in C57BL/6J mice chronically exposed to these food additives starting *in utero*. Mouse offspring (12 to 18/sex/group) were bred, weaned and maintained on the following diet groups for 17 weeks: standard diet (control), standard diet with MSG (120 mg/kg bw/day) alone, standard diet with aspartame (approximately 50 mg/kg bw/day) alone, standard diet with MSG and aspartame (at the same doses as when the compounds were administered separately). Aspartame alone caused a 1.6-fold increase in fasting blood glucose and reduced insulin sensitivity. MSG alone decreased blood triglyceride and total cholesterol levels. The combination of MSG and aspartame increased body weight, and caused a further 2.3-fold increase in fasting blood glucose compared to control diets. Total cholesterol levels were reduced in both aspartame-containing diets in both sexes. A positive correlation between aspartame intake and body weight at 6 weeks and 17 weeks, and aspartame intake and fasting blood glucose levels at 17 weeks was also noted. The authors concluded that aspartame exposure might promote hyperglycaemia and insulin intolerance, and MSG might interact with aspartame to impair further glucose homeostasis.

The authors attributed indirectly the increases in fasting blood glucose and reduced insulin sensitivity observed in their study to an elevated level of aspartame-derived phenylalanine that could potentially accumulate in the brain leading to changes in the regional brain concentrations of neurotransmitters and a dose-dependent reduction in dopamine, serotonin and noradrenalin levels. The Panel noted that the mouse strain used in this study is known to harbour a dominant trait showing a high susceptibility to diet-induced type-2 diabetes, as well as obesity and atherosclerosis (JAX Mice Database). The Panel also noted that no dose-response was assessed in this study and that other authors (Berglund et al., 2008) have reported higher insulin and glucose basal levels in the C57BL/6J mice strain suggesting that those parameters vary in that mice strain. The Panel also noted that phenylalanine and glutamate can also arise from the protein content of the diet and thus the actual contribution of aspartame to the total phenylalanine and glutamate amino acids pool should be clearly defined to be able to attribute exclusively an increment in fetal amino acid pool to aspartame intake. Finally, the Panel also observed that short term preliminary interventional trials undertaken in human volunteers suggest that test meals containing aspartame significantly reduce postprandial glucose levels and insulin levels compared to a test meal containing sucrose (Anton et al., 2010).

In another study Collison et al. (2012b) investigated the effects of neonatal exposure to aspartame, commencing *in utero*, on changes in blood glucose parameters, spatial learning and memory in C57BL/6J mice (numbers of animals not clearly reported but according to the tables published they are between 12 and 18 per group depending on the parameter measured) that were administered standard diet (control) or standard diet with aspartame (approximately 50 mg/kg bw/day) for 17 weeks. Increased weight gain, increased fasting glucose levels and decreased insulin sensitivity was observed in treated male mice compared to controls. These effects were less apparent in females, which did however show significantly raised fasting glucose levels. The escape latencies used in the Morris Water Maze to test spatial learning and memory were consistently higher in treated male mice than in controls, as well as the thigmotactic behaviour and time spent floating directionless. Spatial learning of female-treated mice was not significantly different from controls. Reference memory was changed in both sexes, and the aspartame-fed mice spent significantly less time searching for the former location of the platform. Visceral fat deposition positively correlated with non-spatial search strategies (i.e. floating and thigmotaxis), and negatively with time spent in the target quadrant and swimming across the location of the escape platform. Overall, the authors of the study concluded that lifetime exposure to aspartame, commencing *in utero*, might affect spatial cognition and glucose homeostasis in C57BL/6J mice, particularly in males.

The Panel noted that the selection method of pups for several tests was not clearly reported by Collison et al. and that only one dose was used thus rendering any assessment of dose-response relationship impossible.

The Panel noted that the findings in mice reported by Collison et al. (2012 b) might not apply to other species, since in a large study on Sprague-Dawley rats (Holder, 1989) performances on radial-maze and milk maze was similar for rat pups given aspartame at doses from 14 to 1614 mg/kg bw/day or phenylalanine at a dose of 835 mg/kg bw/day compared to controls. These findings indicated to the authors that spatial memories, as well as motor and visual components of these tasks were not affected by perinatal exposure to aspartame.

Although the study by Collison et al. (2012b) is an *in-utero*-derived study in mice, the authors do not report the initial number of parent females, the number of litters used for each group, nor any litter data such as litter size, birth weight and male/female ratio. As these parameters may influence nutritional and metabolic parameters associated with fat accretion the observed changes cannot be unequivocally attributed to aspartame feeding. The Panel was concerned about the statistical procedures used. Based on the study description in Collison et al. (2012a) more than one pup/sex/litter was apparently used. In this case a nested evaluation should have been done, calculating group means from within-litter-means, instead of treating all pups as unrelated entities.

In contrast, in the rat study by Holder (1989) the litter data were provided and also the correct statistical procedures were applied to account for testing of more than one pup/sex/litter. Thus the rat study was considered by the Panel to be more reliable than the mouse study. The Panel concluded that the rat study also overruled the results of the mouse study because the rat study had multiple dose groups whereas the mouse study had one dose group and a control group only. The Panel considered that studies which employ multiple doses and show a trend in response are intrinsically more powerful.

Rats

Lennon et al. (1980) conducted a larger experiment with 60 female Charles River rats to assess the effect of aspartame in the diet on lactation. After mating and delivery of pups, the dams were allocated in body weight matched pairs to treatment groups for a pair-feeding experiment. Groups of six dams were fed diets containing l, 2, 4, 7.5, or 14 % aspartame by weight for 21 days (during the lactation period). Pair-matched controls were fed the same amount of feed as their match consumed *ad libitum* the previous day, but without added aspartame. Feed consumption and body weights were significantly lower in dams fed 7.5 and 14 % aspartame diets on day one and throughout the experiment (e.g. body weight reduction by up to 65 %). Similar body weight losses were observed in the pair-fed controls; therefore, the loss of body weight was considered by the Panel to be due to the lower feed consumption. Based on feed consumption data, the actual doses of aspartame by the different groups were 0, 1870, 3680, 7120, 9110 and 8830 mg/kg bw/day. Pup body weight was also significantly reduced in the 9110 and 8830 mg/kg bw/day groups. Pup survival was significantly reduced in the highest dose group and pair-matched controls. Dams fed the highest dose of aspartame and pair-matched controls had a higher incidence of resting or inactive mammary glands, which were attributed by the authors of the study to a lack of suckling pups and severe feed restriction. The Panel noted that there were no effects of aspartame at doses up to 7120 mg/kg bw/day on feed consumption, dam and pup body weight, pup survival and mammary gland histology. Therefore, the Panel identified a NOAEL of 7120 mg/kg bw/day.

Rabbits

Ranney et al. (1975) investigated the phenylalanine and tyrosine content of maternal and fetal body fluid in rabbits following dietary exposure to aspartame. Thirty pregnant New Zealand female rabbits were fed with either a standard diet (control) or a diet containing aspartame at a level of 6 %, beginning from GD 6. Maternal and fetal blood samples and fetal amniotic fluid were taken up to

GD 20. Maternal plasma phenylalanine and tyrosine levels significantly increased in aspartame-fed animals compared to controls, reaching a peak of 4.9 ± 0.3 mg/dL (Phe, 296.6 µM) and 9.9 ± 0.6 mg/dL (Tyr, 546 µM) on GD 9. These values returned to normal on GD 20. Fetal plasma tyrosine was significantly higher in aspartame-fed animals compared to controls, reaching a peak of 7.4 ± 0.2 mg/dL (408 µM) on GD 20, whereas phenylalanine was unchanged (2.5 ± 0.2 mg/dL (151.3 µM) in controls vs. 2.3 ± 0.2 mg/dL (139.2 µM) in aspartame-treated rabbits). This resulted in a significant drop in the Phe/Tyr ratio in both maternal (up to 58 %) and fetal plasma (53 %). Phenylalanine and tyrosine concentrations in the amniotic fluid were consistently higher in the aspartame -treated animals compare to controls at GD 16 and 20, with peaks at 20.4 ± 2.8 mg/dL (Phe, 1235 µM) and 33.4 ± 2.0 mg/dL (Tyr, 1843 µM) at GD 20. The ratios of amniotic fluid/maternal and fetal plasma phenylalanine and tyrosine concentrations at GD 20 were higher in treated-animals compared to controls. Both phenylalanine and tyrosine concentrations of the supernatants from whole fetal homogenates at GD 16 and 20 were higher in treated-animals than in control, and, as a consequence, the ratio of Phe/Tyr was consistently lower than control values. The authors concluded that the treatment of pregnant rabbits with a high dose of aspartame did not affect the transport of phenylalanine and tyrosine across the placental membrane since the ratios of fetal/maternal plasma amino acid concentrations were unaffected by the treatment.

3.2.6. Other studies on aspartame

3.2.6.1. Neurotoxicity

In order to investigate whether aspartic acid generated from aspartame might induce neurotoxicity, a study in neonatal mice was performed. Neonatal hybrid A/JAX-ICR mice aged 6-10 days (12-18 animals of both sexes per dose group, sex ratio not specified) were dosed between PND 6 and 10 by gavage with aspartame at single doses of 250 (n = 12 out of 3 litters), 500 (n = 19 out of 5 litters), 1000 (n = 21 out of 3 litters), 1500 (n = 17 out of 4 litters) or 2000 (n = 5 out of 18 litters) mg/kg as a 10 % slurry (volume, vehicle and duration of exposure not stated) (E94, year not reported). Upon sacrifice, brains were subjected to histopathological examination. The Panel however noted that the design of the study was not adequately described and no control group was included, and, therefore, this study was considered not relevant for the overall risk assessment.

In another study using mice aged 6-12 days (sex and strain not specified) aspartame was dosed orally at 500 (27 animals), 1000 (15 animals), 1500 (17 animals) or 2000 (18 animals) mg aspartame/kg bw in a 10 % (w/v) slurry (volume and vehicle not stated) (Reynolds et al., 1976). The Panel noted the absence of control animals in the study and therefore did not take this study into consideration.

Long Evans weanling rats (21-day-old, 32/sex/group) were given a standard diet (control) or standard diet supplemented with L-phenylalanine (2.5 or 5 %), or aspartame (4.5 or 9 %) for 13 weeks (E14, 1972). For aspartame in the diet, the calculated mean daily intakes of L-phenylalanine were: 3000 mg/kg bw/day (4.5 % aspartame) and 6100 mg/kg bw/day (9 % aspartame) in males, and 3100 mg/kg bw/day (4.5 % aspartame) and 6800 mg/kg bw/day (9 % aspartame) in females. For L-phenylalanine in the diet, the calculated mean daily intakes of L-phenylalanine were: 3000 mg/kg bw/day (2.5 % L-phenylalanine) and 6700 mg/kg bw/day (5 % L-phenylalanine) in males, and 3200 mg/kg bw/day (2.5 % L-phenylalanine) and 6500 mg/kg bw/day (5 % L-phenylalanine) in females. No differences in physical condition were noted between the groups. For the low dose groups (2.5 % L-phenylalanine and 4.5 % aspartame), some effects were reported on occasions but they were transient and not dose-related. For the high dose groups (5 % phenylalanine and 9 % aspartame), statistically significantly impaired learning performances were reported (i.e. conditioned or nondiscriminated avoidance responses). The Panel concluded that L-phenylalanine administered at doses of 6000 mg/kg bw/day for 90 days to rats, impaired learning performances. Doses of aspartame providing the same dose of L-phenylalanine induced similar effects. At a lower dose (3000 mg L-phenylalanine/kg bw/day), the effects were not reported.

Male weanling Long-Evans rats (8 animals) were dosed with aspartame (1000 mg/L in drinking water; actual intake not specified) for 14 weeks, after which terminal blood samples were taken for measurement of plasma glucose, immunoreactive insulin and leptin (Beck et al., 2002). The study contained a control group of 16 rats that received tap water. Epididymal, perirenal and subcutaneous fat pads were dissected and the liver, spleen and heart were also taken. Frozen sections of brain (300 µm) were cut and individual brain regions were sampled by micropunching for the analysis of neuropeptide Y. The authors of the study reported that feed and fluid intakes were similar between treated and control groups, but aspartame treated rats gained less body weight than controls, with fat pads weighing less than controls (34.86 ± 1.39 g vs. 27.59 ± 1.89 g; $p < 0.02$). Whereas terminal plasma insulin and glucose levels did not differ between groups, the level of hypothalamic neuropeptide Y in the arcuate nucleus (but not in other parts of the brain) was significantly lower in aspartame-treated rats than in controls by 23.2 % ($p < 0.02$). The author reported that the reasons for the neuropeptide Y decrease were not clear and further analysis of other neuropeptides in the arcuate nucleus plus further histological controls will contribute to elucidate if the observed effects are physiological or treatment-related. The panel agreed with these observations.

Christian et al. (2004) reported effects of chronic aspartame treatment on the T-maze performance in rats and on brain muscarinic cholinergic receptors and Na^+,K^+-ATPase, potentially related to learning and memory processes. Twelve male rats were exposed to aspartame (250 mg/kg bw/day) for 120 days through drinking water and compared to a control group of the same size which received regular tap water. Learning was unimpaired and during the first 60 days both groups exhibited similar latencies to get the reward, a piece of chocolate placed in the same arm of the maze for all trials. From day 90 onward, latencies of the aspartame group increased which the authors interpreted as a sign of memory loss. The aspartame group also showed an increase of midbrain Na^+,K^+-ATPase activity and of muscarinic receptors in various brain regions. However, no association with regions specifically involved with memory functions was apparent. Moreover, the change in brain Na^+,K^+-ATPase activity restricted to midbrain would indicate a difference in motivation rather than a memory problem (Shen and Johnson, 1998). The Panel noted that a sweet reward may have held a much higher attractiveness for control animals given unsweetened drinking water than for animals given aspartame in drinking water. The Panel concluded that this study is insufficient to ascertain an effect of aspartame on memory functions.

Rabbits (3 months old) and Wistar rats (30 days old) were dosed with aspartame (2000 mg/kg bw/day in diet slurry) for 30 days (Puica et al., 2008, 2009). No vehicle or sham-gavaged control group is mentioned and the frequency of dosing is not stated. At the end of 30 days of dosing, animals were decapitated and the brains prepared for EM. Ultrastructural damage, described as 'selective degeneration of all subcellular neurons ultrastructures both in CA1 pyramidal neurons of the hippocampus and ventromedial area of the hypothalamus' was reported in both species, and the authors conclude that juvenile rats and rabbits are particularly susceptible to neurotoxic effects induced by aspartame. However, the Panel noted that the interpretation of these studies was not possible because of the lack of experimental details, the absence of appropriate control animals and of statistical analysis of the data.

The potential neurotoxic effects of high doses of aspartame were also examined in monkeys (E104, 1979; Reynolds et al., 1980; E105, year not reported). Macaques aged 1-22 days (species *Macaca mulatta*, *Macaca fascicularis* and *Macaca arctoides*) weighing 280-820 g were administered by gavage a single dose of either vehicle (2-5 ml water/animal; 2 males and 3 females), aspartame (2000 mg/kg bw; 2 males and 6 females) or a combination of aspartame and monosodium glutamate (MSG) (2000 + 1000 mg/kg bw; 3 males and 3 females) as a 10 % (w/v) slurry. For aspartame, the dose was calculated to correspond to 6-11 ml administered/animal. The Panel noted that this dosing volume was higher than that received by controls. The study also included an untreated control group of five males and one female. Blood samples were taken up to 4-5 hours after dosing and the animals were then sacrificed. Light and electron microscopic examination of the brain sections revealed no significant abnormalities in response to aspartame or aspartame + MSG treatments. Measurements of plasma amino acid levels showed that aspartic acid reached a peak concentration of 230 ± 330 µM at

60 min ($p < 0.0001$) and that phenylalanine reached a peak concentration of 950 ± 590 µM at 90 min. Basal levels (0 minute time point) for aspartic acid and phenylalanine were 0.69 ± 0.43 µM and 5.93 ± 2.81 µM, respectively. The Panel noted that the design of the study is inappropriate to evaluate effectively any potential neurotoxic effects of aspartame.

The Panel is aware that the neurotoxicity of aspartame was also reviewed by Magnuson et al. (2007) and subsequently by the National Expert meeting of the EFSA Advisory Forum (EFSA, 2010). Magnuson et al. (2007) concluded that the data obtained from the extensive investigations of the potential neurotoxic effects due to aspartame consumption, did not support the hypothesis that aspartame present in the human diet would cause any impairment of the neuronal function, learning or behaviour. Likewise, the National Expert Meeting of the EFSA Advisory Forum (EFSA, 2010) concluded that no new data were identified which reported a link between aspartame intake and enhanced susceptibility to seizures, behaviour, mood and cognitive function (EFSA, 2010). These conclusions were in line with the SCF conclusions in 2002 (SCF, 2002). The Panel agreed with their conclusions.

3.2.6.2. Studies on the effect of aspartame administration on xenobiotic metabolising enzymes

An early study (E15, 1972) indicated that the oral administration of aspartame to male Charles River rats (2000-4000 mg/kg) for four days had no acute effect on hepatic cytochrome P450 (CYP)-mediated xenobiotic metabolism, as measured as aminopyrine N-demethylation, p-nitroanisole N-demethylation, hexobarbital sleeping time or zoxazolamine paralysis time. Two studies subsequently showed that aspartame administration (≤ 4000 mg/kg bw/day for up to 90 days) had small effects on hepatic Phase 1 and Phase 2 metabolism in rodents after 45 days treatment but not after 90 days treatment (Tutelyan et al., 1990). In a more recent study (Vences-Mejia et al., 2006), male Wistar rats were dosed with aspartame (75 or 125 mg/kg bw/day) daily for 30 days. Using hepatic microsomal samples from liver, cerebrum (pooled) and cerebellum (pooled), the authors investigated CYPs by immunoblotting (CYPs 1A1, 1A2, 2B, 3A2 and 2E1) and enzymatic activity measurement (ethoxyresorufin O-deethylase, methoxyresorufin O-demethylase, pentoxyresorufin O-dependentylase, benzyloxyresorufin O-debenzylase, 4-nitrophenol hydroxylase and erythromycin-N-demethylase). No effect of aspartame treatment was observed in the liver samples. However, bands corresponding to CYPs 1A1, 1A2, 2B, 3A2 were detectable in the cerebrum and cerebellum samples brain samples after aspartame treatment (but not in the control samples) and increases in all the enzyme activities were reported. The Panel noted that the findings have minimal relevance for human risk assessment.

3.2.6.3. Miscellaneous studies on aspartame

Alleva et al. (2011) evaluated the ability of aspartame to induce angiogenesis in an *in vitro* model which used human endothelial cells (HUVEC) co-cultured in a matrix of human fibroblasts (IMR-90) following treatment with aspartame at concentrations of 20-100 µM in culture medium. The formation of blood vessels was associated with a transient release of the inflammatory cytokine IL-6 and the growth factor VEGF-A into the medium. Transient increases in the inflammatory cytokine IL-6 and the growth factor VEGF-A were also observed in the HUVEC cells following treatment with aspartame. This increase coincided with a temporary induction of ROS in HUVEC cells but not in IMR-90 fibroblasts thus concluding that generation of ROS is related to the target. The Panel noted that production of ROS could not be attributed to specific cell types but rather a general phenomenon. Furthermore, the authors did not evaluate the fate of aspartame in culture medium ascertaining whether it was hydrolysed to its usual metabolites or remained intact. For induction of angiogenesis, no positive and negative controls were reported. Therefore, the findings reported might be ascribed specifically to the conditions of the study. For these reasons, the Panel considered that this study was not relevant for the risk assessment of aspartame.

Haque and Mozaffar (1993) reported the effect of aspartame on acetylcholinesterase activity in the brain of Swiss Wistar mice. Forty-two males and forty-two females at the age of 22, 32 and 52 days, were fed with a formulated diet containing 1 % aspartame for 30 days. Brain filtrates were analysed for acetylcholine (ACh) levels and acetylcholine esterase (AChE) activity. No effect on ACh protein

was reported. Aspartame did not influence AChE activity except in the oldest female group where a 10 % decrease of the activity was observed. The authors calculated that for a mean body weight of 35 g for female animals, the daily consumption of aspartame was 286 mg/kg bw. The study authors concluded that aspartame does not affect AChE activity. The Panel noted that there was an error in authors' calculation of aspartame daily consumption dose, because applying EFSA's conversion factor (EFSA Scientific Committee, 2012) gives a daily dose of 2150 and 1690 mg/kg bw for females and for males respectively.

The effect of aspartame on AChE activity was investigated in an *in vitro* system by Simintzi et al. (2007a, b). Aspartame metabolites were tested on AChE pure enzyme or on AChE extracted from the frontal cortex (Simintzi et al., 2007a) or hippocampus (Simintzi et al., 2007b) of albino Wistar rats (21 days of age). Four mixes of methanol, aspartic acid and phenylalanine were tested, simulating the assumed concentrations of these metabolites in the cerebrospinal fluid (CSF) of humans after ingestion of aspartame at different doses: 10 mg/kg bw (mix 1), 34 mg/kg bw (mix 2), 150 mg/kg bw (mix 3) and 200 mg/kg bw (mix 4). According to the authors, mixes 3 and 4 represented doses that could be ingested by accident. The results showed a significant reduction of AChE activity from the frontal cortex and of AChE pure enzyme after incubation with mix 3 and 4. Mix 2 caused a significant reduction only of the AChE activity from the frontal cortex (Simintzi et al., 2007a). Mix 3 and 4 induced a similar reduction of AChE activity from hippocampal protein extract (Simintzi et al., 2007b).

The authors of the study also described the effect of each aspartame metabolite on the activity of both AChE pure enzyme and AChE from frontal cortex homogenates (Simintzi et al., 2007a) or from hippocampal homogenates (Simintzi et al., 2007b). The enzyme activity was measured after a 1-hour incubation with each aspartame metabolite, separately, at the concentration expected in CSF corresponding to the aspartame intake of 10, 34, 150 or 200 mg/kg. The results indicated that the incubation with metabolites corresponding to 10 mg aspartame/kg bw (methanol 0, aspartic acid 0.05 mM, phenylalanine 0.012 mM) did not affect AChE activity (both with pure enzyme and with enzyme present in frontal cortex homogenates). At the aspartame dose of 34 mg/kg bw, only aspartic acid (0.16 mM) reduced the activity of both AChE from frontal cortex and AChE pure enzyme. Methanol (0.14 mM) reduced the activity only of AChE from frontal cortex while phenylalanine (0.023 mM) did not affect the enzyme activity (Simintzi et al., 2007a). The incubation with methanol, phenylalanine or aspartic acid at concentrations corresponding to an aspartame intake of 150 or 200 mg/kg bw reduced the activity of both AChE pure enzyme and AChE from frontal cortex (Simintzi et al., 2007a) or from hippocampal homogenates (Simintzi et al., 2007b).

According to the authors, aspartame metabolites can reach the cerebrospinal fluid and brain tissues. The authors suggest that phenylalanine could inhibit AChE activity through a direct interaction with the positively charged sites of the enzyme or with an oxidative effect. Another mechanism is the saturation of the blood-brain barrier transport system by a large excess of phenylalanine. As hypothesised for PKU patients, a high phenylalanine concentration could saturate the transport system excluding other amino acids, e.g. tyrosine and tryptophan from entry into the brain. As a consequence, phenylalanine would interfere with the conversion of tyrosine to biogenic amines (dopamine, adrenaline, serotonin) and possibly affecting AChE activity. Aspartic acid could reduce AChE activity inducing a membrane lipid peroxidation affecting the function of membrane AChE. Methanol could affect AChE activity through its interaction with lipids or proteins.

From these *in vitro* experiments, the authors concluded that AChE activity could be reduced by aspartame metabolites after ingestion of very high amounts of aspartame. Metabolites derived from a realistic beverage consumption of aspartame does not affect AChE activity. The Panel, noting the unrealistic high doses of aspartame metabolites assumed to be present in CSF and several assumptions and speculations that were applied to explain the effect on AChE activity, considered that no conclusions can be drawn from these studies. The Panel concluded that these studies are of no relevance for aspartame risk assessment under realistic use conditions.

Aspartame was administered to zebrafish (70 per dose group) in the diet (50 g/kg diet) for 14 days (Kim et al., 2011). No effect of aspartame on survival was observed.

Following a public call for data, preliminary investigations on a wide range of potential pharmacological and endocrine effects of aspartame, summarised in two data compendia (E1, 1972; E19, year not provided), were received. The compendia did not report any adverse effects.

3.2.7. Human studies of aspartame

3.2.7.1. Epidemiological studies

Epidemiological data on aspartame were previously reviewed by SCF (2002). The Panel considered and agreed with the conclusions of SCF that there was no evidence for adverse effects of aspartame in the human population. New epidemiology studies, published since the SCF opinion, are described below.

3.2.7.1.1. Recent epidemiological studies on artificially sweetened drinks and pre-term delivery

A large prospective cohort study (Halldorsson et al., 2010) based on data from the Danish National Birth Cohort investigated associations between consumption of artificially sweetened and sugar-sweetened soft drinks during pregnancy and subsequent pre-term delivery. Recruitment of the cohort of participants occurred between January 1996 and October 2002.

The reasons cited by the authors for undertaking the analysis were:

a) Both artificially and sugar-sweetened soft drinks might be associated with hypertension which is a known risk factor for pre-term delivery.

b) Intake of sugar-sweetened soft drinks has been related to the metabolic syndrome and type 2 diabetes mellitus. High blood glucose concentrations have been linked to a shorter duration of gestation.

c) Methanol is a metabolite of aspartame. Low dose methanol exposure has been linked to a shorter duration of gestation.

The study population included 91 827 pregnant women from all over Denmark, equating to approximately 35 % of all deliveries in Denmark during the study period. Of these, 59 334 (64.6 %) women were analysed in this study, the main reason for attrition being failure to provide the required dietary information during pregnancy. Since the events leading to exclusion occurred before the outcome of pregnancy was known, they would not be expected to have given rise to bias. Analysis was restricted to each woman's first singleton pregnancy registered during the study period. This restriction appears appropriate, since multiple pregnancies are a significant factor in pre-term birth. It would not be expected to produce bias.

Dietary exposures were obtained at about week 25 of gestation by a food frequency questionnaire (FFQ) covering the past month. Among other things, questions were asked about frequency of consumption (in servings) of each of: sugar-sweetened carbonated drinks; sugar-sweetened uncarbonated drinks; sugar-free carbonated drinks; and sugar-free uncarbonated drinks. It appears that no attempt was made to analyse exposures to other more important dietary sources of methanol such as fruit and vegetables. A subsample of 103 women completed a repeat FFQ at 33-35 weeks gestation. Spearman's correlation coefficients for the soft drink exposure variables in paired questionnaires were about 0.7. Due to the prospective design, any misclassification of exposures would be expected to bias risk estimates towards the null rather than to produce spurious associations between exposure and health outcome. It should be noted that the exposure assessment was for sweeteners in general and not specific for aspartame. Furthermore, the exposure assessment did not consider other dietary sources of sweeteners (e.g. sweeteners added to coffee, tea or other food products).

Pre-term delivery was defined as delivery occurring before week 37 of gestation. Data on date of delivery and type of delivery (spontaneous or induced) were obtained from the Medical Birth Registry. Data on expected date of delivery were obtained from mothers at approximately week 12 of gestation (43 % of pregnancies) or week 30 of gestation (56 %), or from the Medical Birth Registry (1 %).

Statistical analysis was by logistic regression and apparently did not adjust for year of delivery. This could have produced bias if both consumption of soft drinks and thresholds for induction of delivery pre-term changed over the course of the study period. However, as the study period was only seven years, it is highly unlikely that any such bias would have been large. Analyses were adjusted for seven non-dietary factors that were known to predispose to pre-term delivery: maternal age, maternal height, pre-pregnancy BMI, cohabitant status, parity, smoking during pregnancy, and familial socioeconomic status. This information was ascertained in prenatal telephone interviews conducted at around weeks 12 and 30 of gestation.

Statistically significant trends were found in the risk of pre-term delivery with increasing consumption of artificially sweetened drinks (both carbonated and non-carbonated), but not for sugar-sweetened drinks. In the highest exposure groups (≥ 4 servings/day) the odds ratios relative to non-consumption were 1.78 (95 % CI 1.19-2.66) and 1.29 (95 % CI 1.05-1.59) respectively for carbonated and non-carbonated artificially sweetened drinks. The associations with different categories of soft drinks were not mutually confounded, and the associations with consumption of artificially sweetened carbonated drinks did not differ demonstrably according to whether delivery was very early (< 32 weeks) or only moderately or late pre-term.

A notable finding was that the trend in risk for artificially sweetened carbonated drinks was strongest for medically induced pre-term deliveries, and was not clearly apparent for spontaneous pre-term deliveries. No analysis was made by reason for pre-term delivery (data on which may not have been available).

The Panel noted that the prospective design and large size of the study sample were major strengths, and there were no important flaws in the methods used. However, risk estimates may have been inflated by residual confounding (including by year of delivery). No account was taken of other dietary sources of methanol, and use of aspartame specifically was not distinguished from that of other artificial sweeteners). Therefore, given these limitations, the Panel agreed with the authors who concluded that replication of their findings in another setting was warranted.

Another study (Englund-Ögge et al., 2012) investigated the relation between consumption of artificially sweetened and sugar-sweetened soft drinks during the first 4-5 months of pregnancy and subsequent pre-term delivery in a large cohort of Norwegian women.

The authors used data from a prospective cohort study of 90 700 pregnant women recruited during 1999-2008 (comprising 38.5 % of those invited to participate), which they analysed retrospectively. After exclusions for various specified reasons (e.g. not a singleton pregnancy, incomplete data on covariates, baby not born alive between gestational weeks 22 and 41), analysis was based on 60 761 women. The exclusions are unlikely to have been an important source of bias. In particular, where data were missing on covariates (6630 women), this occurred before the outcome of pregnancy was known.

Dietary exposures were ascertained at about week 22 of gestation by a semi-quantitative food frequency questionnaire covering the first 4-5 months of pregnancy. Among other things, six questions were asked about frequency of consumption (in 250 ml servings) of artificially and sugar-sweetened carbonated drinks (carbonated cola and other carbonated drinks) and non-carbonated drinks. From this information, total intakes of artificially sweetened and sugar-sweetened soft drinks were calculated.

Pre-term delivery was defined as occurring before week 37 of gestation. Gestational age was determined by ultrasonography at gestational weeks 17-18. This information, and that about pregnancy outcome, was obtained from a national birth registry.

Statistical analysis was by logistic regression. Analyses were carried out with and without adjustment for eight co-variates known to be associated with pre-term delivery: maternal history of previous pre-term delivery, maternal age at delivery, maternal pre-pregnancy BMI (calculated from self-report of weight and height), marital status, parity, smoking during pregnancy, maternal education, and total energy intake during the first half of pregnancy. The result of adjusting for co-variates was generally a reduction of the risk estimates. Some analyses also adjusted for consumption of the other type of beverage (sugar-sweetened or artificially sweetened), and this increased risk estimates slightly.

No significant trends were found in the risk of pre-term delivery with increasing consumption either of artificially sweetened drinks or of sugar-sweetened drinks. Small elevations of risk were observed with higher consumption of artificially sweetened soft drinks, but after adjustment for covariates, these reached statistical significance only when categories of consumption were aggregated to four levels, and then the odds ratio for the highest category (≥ 1 serving/day) was only 1.11 (95 % CI 1.00-1.24) in comparison with non-consumption. This was driven by an increase in spontaneous but not medically-induced pre-term delivery. Associations with sugar-sweetened soft drinks tended to be somewhat stronger, with an adjusted odds ratio of 1.25 (95 % CI 1.08-1.45) for consumption of at least one serving per day.

This pattern of results contrasts with that reported by Halldorsson et al. The association of pre-term delivery with artificially sweetened soft drinks was much weaker and barely discernible, applied more to spontaneous than medically induced deliveries, and was exceeded by an association with consumption of sugar-sweetened soft drinks. The Panel noted that effects may have been underestimated because of non-differential inaccuracies in the assessment of dietary exposures, but the method was similar to that used by Halldorsson et al., and the same for sugar-sweetened as for artificially sweetened soft drinks.

Both Halldorsson et al. (2010) and Englund-Ögge et al. (2012) studies appear to have been well designed and conducted. Noting this, the Panel concluded that even at high levels of exposure to artificially sweetened soft drinks the risk of pre-term delivery is likely to be small, if any. The observed associations could be a consequence of uncontrolled residual confounding, and the inconsistencies in the patterns of association reinforce this uncertainty.

La Vecchia (2013) performed a meta-analysis of findings from the studies by Halldorsson et al. (2010) and Englund-Ögge et al. (2012). The methods used were described as 'standard', but were not reported in detail. The analysis indicated similarly elevated risks of pre-term delivery with higher consumption both of sugar-sweetened and of artificially sweetened drinks. This lack of specificity in the associations again points to possible residual confounding.

Overall, currently available epidemiological data do not suggest that consumption of artificially sweetened soft drinks is a cause of pre-term delivery.

3.2.7.1.2 Epidemiological study on artificially sweetened drinks and allergy

Maslova et al. (2013) explored how intake of artificially sweetened beverages during pregnancy related to asthma and allergic rhinitis in children at 18 months and 7 years of age. Analysis was based on 60 466 pregnant women who enrolled in the prospective longitudinal Danish National Birth Cohort between 1996 and 2002.

Maternal diet was assessed by a food frequency questionnaire completed at around gestation week 25. Mothers were subsequently interviewed about their child's health and medical diagnoses since birth when the child was 18 months old, and 7 years of age. At the 7 year follow-up, the study authors also ascertained diagnoses of asthma and treatment for asthma through two national registries: the Danish National Patient Registry (DNPR) and Register of Medicinal Product Statistics (RMPS).

Logistic regression was used to assess an association between mother's intake of artificially-sweetened drinks during pregnancy and allergic disease in the children. Various potential confounding factors

were taken into account: maternal age, smoking, parity, pre-pregnancy BMI, physical activity, breastfeeding, socioeconomic status, child' sex, maternal and paternal history of asthma and allergies, and maternal energy intake during pregnancy. Cases of asthma in the children were identified using four alternative definitions (mother reported doctor's diagnosis at age 18 months; a positive response at age 7 years to the standardised International Study of Asthma and Allergies in Childhood questions on doctor-diagnosed asthma and wheezing symptoms; ever hospital admission for asthma by age 7 years (through the Danish National Patient Registry); and at least two prescriptions of certain drugs for obstructive airways disease by age 7 years (through the Register of Medicinal Product Statistics)). The study authors indicated that data from both registries were limited to children with self-reported diagnosis, in order to avoid discordance in findings due to differences in study populations. In addition, previous occurrence of allergic rhinitis was defined by a reported doctor's diagnosis of hay fever by age 7 years. This method to identify allergic rhinitis would not include all cases of allergic rhinitis, but it would reduce the chance of false positive diagnosis.

In comparison with no consumption of artificially sweetened non-carbonated soft drinks during pregnancy, consumption of at least one serving per day was associated with an increased risk of asthma by three of the four case definitions (odds ratios up to 1.23, 95% CI 1.13-1.33 for asthma at 18 months), but there was no consistent exposure-response relationship across lower frequencies of consumption. In a corresponding analysis for artificially sweetened carbonated soft drinks, elevated odds ratios were observed for all four case definitions with the highest odds ratios of 1.30, 95% CI 1.01 – 1.66 for asthma at 7 years of age identified through the Danish National Patient Registry, but again without clear exposure-response relationships. Allergic rhinitis was non-significantly associated with daily consumption of artificially sweetened carbonated soft drinks (OR 1.31, 95% CI 0.98-1.74); no association was observed with daily consumption of artificially sweetened non-carbonated drinks (odds ratios of 1.03, 95% CI 0.86-1.24).

Although the study distinguished between carbonated and non-carbonated soft drinks, the exposure assessment was for sweeteners in general and not specific for aspartame.

Because in epidemiological terms, the elevations of risk were only small and inconsistent, the findings from this study can only be considered weakly suggestive of hazard i.e. an association between the consumption of artificially sweetened beverages during pregnancy and the diagnosis of asthma or allergic rhinitis in children. Before a final conclusion can be reached with regard to aspartame, the findings need to be explored further with more detailed assessment of exposure to specific artificial sweeteners.

3.2.7.1.3. Epidemiological studies of aspartame and cancer

In a Swedish case-control study 209 patients with brain tumour were compared with 425 controls, who were selected from the Swedish Population Register (Hardell et al., 2001) and matched to the cases for sex, age and region of residence. The focus of the study was exposure to ionising radiation and cellular telephones, but information was also collected about consumption of low-calorie drinks, most of which contained aspartame. Non-significant elevations of risk were observed for consumption of such drinks in relation to brain tumours overall (OR 1.24, 95 % CI 0.72-2.14) and malignant brain tumours specifically (OR 1.70, 95 % CI 0.84-3.44).

The study had a high response rate, but was limited by its relatively small size, the basic assessment of exposure (low-calorie drinks were the only source of aspartame investigated in this study), and the potential for recall bias (because cases knew that they had a brain tumour) all of which could have led to spurious inflation of risk estimates.

A US case-control study included 315 children with medulloblastoma/primitive neuroectodermal tumour diagnosed before the age of 6 years, and 315 control children (selected from the general population by random digit dialling) (Bunin et al., 2005). Their mothers were interviewed retrospectively (on the telephone by trained interviewers) about their diet before (to ascertain the

periconception period) and during pregnancy (second trimester). This was assessed by a modified food frequency questionnaire covering 112 items.

In an unadjusted analysis, a significant trend of increasing risk was observed with more frequent consumption of low-calorie carbonated drinks (diet soda) in the pre-conception period. However, this was attenuated after adjustment for potential confounders, with an adjusted odds ratio of 1.3 (95 % CI 0.7-2.5 for ≥ 2/day versus < 1/month). There were no significant associations with reported frequency of consuming diet soda during midpregnancy.

Response rates were reasonable, but the assessment of exposure to diet soda required recall after an interval of several years and therefore may not have been reliable. Furthermore, it served only as a proxy for exposure to aspartame (which at the time was the most widely used sweetener in soft drinks), and other possible sources of aspartame were not evaluated.

The authors concluded that their results generally did not support an association with aspartame, but the above limitations, and also the low statistical power, restrict the conclusions that can be drawn from this study.

A paper by Gallus et al. (2006) reports a linked set of case-control studies carried out in Italy between 1991 and 2004 to assess the association of artificial sweeteners with nine types of cancer. Patients with incident, histologically confirmed cancers of the oral cavity and pharynx (598), oesophagus (304), colon (1225), rectum (728), larynx (460), breast (2569), ovary (1031), prostate (1294) and kidney (767) were compared with 7028 controls admitted to the same hospitals for acute, non-neoplastic disorders. Odds ratios for consumption of sweeteners other than saccharin (mainly aspartame) ranged from 0.71 to 1.62, and their upper 95 % confidence limits from 1.00 to 3.14. The highest lower 95 % confidence limit was 0.86 (prostate cancer).

Dietary exposures were ascertained in an interview in hospital, which included a food frequency questionnaire with 78 items. Assessment of dietary exposure was restricted to usual diet in only the past two years, and did not cover all possible sources of artificial sweeteners (only sachets or tablets). However, the authors state that dietetic soft drinks had only recently been introduced in Italy, and that they were therefore unlikely to have contributed to the cancers in the age groups investigated.

It seems likely that any misclassification of exposures will have been non-differential (i.e. similar for cases and controls), in which case the effect will have been to bias risk estimates towards the null. Thus, while the results do not suggest a hazard for the cancers studied, on their own they provide only limited reassurance of safety.

In an extension to this series of studies, the possible associations of artificial sweeteners with three additional types of cancer were further explored (Bosetti et al. 2009).

The cases were 230 patients with histologically confirmed stomach cancer, 326 patients with pancreatic cancer and 454 patients with endometrial cancer. These were compared with 547, 652 and 908 controls, frequency matched by age, sex and study centre. Cases and controls were interviewed during their hospital stay. A food frequency questionnaire was used to investigate the patient's usual diet in the 2 years before diagnosis (or hospital admission for controls), and included questions on weekly consumption of sugar, saccharin and other low-calorie sweeteners (mainly aspartame). Odds ratios (ORs) and corresponding 95 % CIs for consumption of sweeteners and of sugar were derived by logistic regression with adjustment for age, sex, study centre, year of interview, education, body max index, tobacco smoking, history of diabetes, consumption of hot beverages and total energy intake.

ORs for ever use of low-calorie sweeteners versus non-use were 0.80 (95 % CI, 0.45-1.43) for gastric cancer, 0.62 (95 % CI, 0.37-1.04) for pancreatic cancer, and 0.96 (95% CI, 0.67-1.40) for endometrial cancer. Corresponding ORs for saccharin were 0.65 (95 % CI, 0.25-1.68), 0.19 (95 % CI, 0.08-0.46),

and 0.71 (95 % CI, 0.36-1.38), and for other sweeteners were 0.86 (95 % CI, 0.45-1.67), 1.16 (95 % CI, 0.66-2.04), and 1.07 (95 % CI, 0.71-1.61).

Again, the findings do not suggest a hazard, but because exposures to aspartame specifically were not distinguished, and because of possible non-differential misclassification of exposures, on their own they provide only limited evidence of safety.

A case-control study (Andreatta et al., 2008) was carried out between 1999 and 2006 in Còrdoba (Argentina) to investigate the relation between urinary tract tumours (UTT) and the use of artificial sweeteners as sugar substitutes in infusions (e.g. tea, coffee, matè). A cohort of 197 patients with incident histopathologically confirmed transitional-cell UTTs were recruited (response rate almost 80 %), along with 397 controls from the same area who had no history of cancer, and had been admitted to hospital with acute non-urinary tract diseases. It is unclear from the report how potential controls were identified, or what response rate was obtained form those invited to take part.
A food frequency questionnaire was administered at interview to assess the intake of artificial sweeteners before diagnosis or hospitalisation. Subjects were classified according to their duration of exposure to artificial sweeteners as non-consumers, short-term consumers (1 to 9 years) or long-term consumers (10 or more years). In addition, subjects were further classified according to the types of artificial sweeteners they had used: saccharin/cyclamate or aspartame/acesulfame-K.

The use of artificial sweeteners was more frequent in cases than in controls. For short-term consumers, no association with UTT was observed, but a positive association was found between long-term use of artificial sweeteners and risk of UTT (OR 2.18, 95 % CI: 1.22 – 3.89, adjusted for age, sex, body max index, social status, and years of tobacco use).
The Panel noted that the analysis of exposure data was limited to the use of artificial sweeteners in infusions, and that other dietary sources of sweeteners were not considered. Moreover, approximately 80 % of both cases and controls who consumed artificial sweeteners, used saccharin and/or cyclamate. Thus, the study provided little information about possible risks from aspartame.

A prospective cohort study by Lim et al. (2006) was based on data from the NIH-AARP Diet and Health Study. The analysis included 285 079 men and 188 905 women aged 50 to 71 years at entry to the cohort, which was drawn from members of the American Association of Retired Persons in 8 study areas in the US. In this cohort, risk of haematopoietic cancer (1888 cases) and malignant glioma (315 cases) during five years of follow-up (1995-2000) was examined in relation to daily intake of aspartame assessed at baseline. For haematopoietic cancer, intake of $\geq$ 600 mg aspartame per day carried no elevation of risk (RR 0.98, 95 % CI 0.76-1.27), and there was no association between glioma and consumption of aspartame (RR 0.73, 95 % CI 0.46-1.10). Assessment of exposure to aspartame was through a food frequency questionnaire. The authors concluded that their prospective study suggested that aspartame consumption derived from its main source, aspartame-containing beverages, does not raise the risk of haematopoietic or brain malignancies.

The Panel noted that major strengths of this investigation were its prospective longitudinal design, the large number of cases, and that exposures were assessed at baseline and therefore unbiased by knowledge of disease outcome. Ascertainment of cancers was reliable. Confounding is unlikely to have been a major problem, although there was no adjustment for socio-economic status (which has shown some relation with brain cancer). Assessment of exposure to aspartame covered aspartame-containing drinks as well as addition of aspartame to coffee and tea, but it was limited to one point in time, and usage in the years before the study may have been lower. This would have limited the ability of the study to detect effects that occurred with a long latency.

In a small pilot case-control study of brain cancer conducted in southern France, 122 incident adult cases were compared to 122 controls with other neurological diagnoses (Cabaniols et al., 2011). There was no association with aspartame consumption during the past five years of at least once per week (OR 1.02, 95 % CI 0.57-1.85). However, information on the method of dietary assessment is limited, and there was no attempt to control for potential confounding factors other than sex and age. In view

of this, and the low statistical power of the study (reflected in the confidence interval), the non-positive finding provides little reassurance of an absence of hazard.

The risk of lymphatic and haematopoietic cancers in relation to consumption of diet soda and aspartame sweeteners added at the table was examined in two US cohorts (one of 77 218 female registered nurses and one of 47 810 male health professionals) followed prospectively from 1984 and 1986 to 2006 (Schernhammer et al., 2012).

Exposure to aspartame through consumption of diet soda and packets used at table was assessed by semi-quantitative food frequency questionnaires repeated at four-yearly intervals, and at each point in follow-up was characterised by the average from all questionnaires that the participant had completed up to that time. Various potential confounding factors (e.g. weight, smoking habits, physical activity) were also assessed through repeated questionnaires. Cancer outcomes were ascertained from biennial questionnaires, state tumour registries and death records, and where possible, diagnoses were verified using medical records. Analysis was by Cox regression.

During follow-up, 1,324 subjects developed non-Hodgkin lymphoma (NHL), 285 multiple myeloma, and 339 leukaemia (mostly myeloid leukaemia). After adjustment for potential confounders, the highest category of aspartame intake defined by the authors (≥ 143 mg/day) was associated with a significantly elevated relative risk of NHL (1.64, 95 % CI 1.17-2.29) and of multiple myeloma (3.36, 95 % CI 1.38-8.19) in men. However, there was no consistent trend in risk with increasing exposure, and there were no corresponding elevations in risk in women. No clear association with leukaemia was apparent in either men or women. The authors speculated that the differential findings for men and women might reflect differences in the activity of alcohol dehydrogenase type 1, which converts methanol (a metabolite of aspartame) to formaldehyde.

Major strengths of this study are its prospective design, the substantial number of cancer cases, the repeated assessment of dietary intake and the fact that various potential confounders were addressed. However, the positive findings can be given little weight, given their limitation to men, the small relative risks observed, and the lack of clear dose-response relationships. The authors' proposed explanation for the differential associations in men and women is unconvincing in the absence of data on other more important dietary sources of methanol.

Overall, the Panel considered that the results of these epidemiological studies do not suggest an increased risk associated with aspartame consumption for the types of cancer examined.

3.2.7.2. Single dose studies in humans

A number of single dose studies on the kinetics of aspartame in human subjects have been carried out; these are described in Section 3.1.

In one study, single loading doses of aspartame (34 mg/kg bw) and, two weeks later, of L-phenylalanine (19 mg/kg bw) were administered in orange juice to two female adolescents (E66, 1973). The authors of the study reported that the two subjects tolerated the doses well. In another study (E110, 1979), six subjects who claimed to be sensitive to monosodium glutamate (Chinese Restaurant Syndrome) were given a loading dose of 34 mg aspartame/kg bw or 1000 mg sucrose/kg bw in a cross-over study. The test doses were dissolved in orange juice. Although one subject reported slight nausea after aspartame, no symptoms of Chinese Restaurant Syndrome were reported.

3.2.7.3. Repeat dose studies in humans

A number of repeat dose studies on the kinetics of aspartame in human subjects have been carried out, and some of them are described in Section 3.1.

Several repeat dose studies in healthy non-obese human subjects have been performed with aspartame. In preliminary studies (E23, 1972) 31 men and 38 non-pregnant women (age 21-45 years) were

administered aspartame under double blind conditions in capsules containing placebo, 200 mg aspartame or 300 mg aspartame. The subjects took an appropriate number of capsules per day to deliver increasing doses of aspartame (or placebo) over a six-week period (600, 1200, 2400, 4500, 6300 and 8100 mg/day in a weekly dose-escalation protocol) followed by a one week period with no treatment. Standard clinical chemistry profiles were analysed together with methanol, determined by gas chromatography in serum and urine at weeks 4 and 6. All 69 subjects completed the study, and there were no obvious physical or biochemical differences between the aspartame and placebo groups before, during or at the end of the study. No significant differences in glucose and insulin homeostasis were identified at weeks 0 or 6, or during follow up (week 7). There was no accumulation of phenylalanine or tyrosine in subjects taking aspartame; phenylalanine was not detected in urine in weeks 0, 3, 6 or 7. The only symptoms reported were mild and often contradictory (loose stools/constipation, increased/decreased appetite and headache).

A similar short-term (6-week) study was conducted in obese adults (ages 21-70 years; weight exceeding more than 20 % of the mean normal weight for height, sex, frame and age) (E24, 1972). Of the 95 subjects entering the study, 84 successfully completed the 6 weeks of treatment (44 on aspartame and 40 on placebo). The dose regime was the same as that described in E23 (1972), as were the biochemical analyses. As with the normal adults, no obvious physical or biochemical differences between the aspartame and placebo group were reported before, during or at the end of the study. According to the authors, few complaints were reported during the study and none were serious (e.g. loose stools, constipation, decreased appetite, increased appetite, headache). The incidence of these complaints was slightly greater among females than males, and among the aspartame users than placebo subjects. Subjects in both groups lost a significant amount of weight between the initial weighing and the end of the study, but the difference in weight loss between two groups was not significant.

A follow up study (E60, 1973) was conducted in subjects who agreed to continue from the study described above (E23, 1972) together with a number of new recruits. This follow up study ran for 21 weeks, so continuing subjects received aspartame for a total of 27 weeks and the new recruits were dosed for 21 weeks. Subjects from the previous study stayed on the same preparation (either aspartame or placebo) as they had received during the short-term study, but all the new recruits received aspartame. All subjects were dosed at 1800 mg aspartame/day (2 x 300 mg capsules, 3 times daily). Laboratory tests conducted during weeks 6, 12, 20 and 21 revealed some variability in results, but this was not associated with consumption of aspartame. There were no consistent differences in glucose and insulin homeostasis between aspartame and placebo groups and, despite fluctuations in plasma phenylalanine and tyrosine levels, there was no accumulation of either amino acid in male or female subjects. No phenylpyruvic acid was detected in urine. No changes in weight and no product-related side effects were reported; the symptoms that were reported were of a similar nature to those in the previous study.

Aspartame tolerance was also evaluated in a 13-week double-blind study in children and adolescents, aged 2-20 years (E61, 1972). Five groups of children (ages 2-3, 4-6, 7-9, 10-12 and 13-20 years) were randomly assigned to receive sucrose or aspartame at doses of 39.5–58.1 mg/kg/day, and older subjects (ages 13-20 years) also took aspartame capsules (200 mg, 3 times daily). Subjects returned for evaluation at the end of weeks 1, 3, 5, 7, 9, 11 and 13. Clinical chemistry profiles indicated some minor differences between aspartame and placebo groups, but all were considered clinically trivial and none persisted. No compound-related differences in profiles were detected at the end of the study. Both groups exhibited a slight decline in plasma phenylalanine levels during the study but tyrosine levels remained unchanged and there were no trends in phenylalanine/tyrosine ratios. Urinary phenylpyruvic acid measurements were negative and both serum and urinary methanol levels (in 33 subjects selected at random) were below the limit of detection at all times. There was slight weight gain during the study in all groups, consistent with the age of the participants. No severe adverse effects were reported and the minor complaints received were similar for aspartame and placebo groups. Hives and rashes were observed in four individuals but these were not related to aspartame consumption. No eye abnormalities or changes in acne incidence or severity were observed.

A metabolic study was designed to evaluate the effect of added monosodium glutamate (MSG) plus aspartame upon plasma amino acid levels (E95, 1977). Three male and three female subjects were given, in a cross-over manner, a test meal or the same test meal containing aspartame at 34 mg/kg and MSG at 34 mg/kg. Blood samples were collected up to 8 hours. The addition of aspartame (34 mg/kg) to the meal increased the phenylalanine content from 43 to 64 mg/kg. The addition of aspartame plus MSG had no effects upon the plasma glutamate curves, the plasma amino acid levels, and the plasma aspartate levels. The plasma levels of phenylalanine and tyrosine after ingestion of MSG and aspartame were only slightly higher than those noted after the ingestion of the test meal alone, and all levels were within the normal postprandial limits.

Leon et al. (1989) conducted a study of the effects of aspartame involving 101 subjects (ages 18-62 years). This was a randomised, double-blind, placebo-controlled, parallel-group design. Participants included 57 women and 51 men, randomly assigned to either the aspartame (n = 53) or placebo (n = 55) groups. Exclusion criteria were well-defined and included body weight greater than 20 % above normal range. Subjects consumed three capsules per day containing either 300 mg aspartame or 300 mg microcrystalline cellulose plus 0.9 mg silicon dioxide for 24 weeks. Compliance was verified by counting capsules and subjects were asked to avoid additional sources of aspartame. The approximate dose of aspartame was 75 mg/kg bw/day. No treatment-related haematological changes, consistent alterations in clinical chemistry and analyses or urinary abnormalities were observed and no significant differences in vital signs or body weight were noted. In addition, no differences were observed in relation to blood formate or methanol; urinary Ca^{2+} or formate; serum folate; or serum lipids. There were no significant changes in amino acid profiles and no evidence of accumulation of phenylalanine or tyrosine. Various adverse events were reported which were all considered by the authors as frequently occurring minor ailments and mild in nature.

In a controlled diet study (Porikos and Van Italie, 1983) in which 21 men (aged 24-45; 15 obese, 6 non-obese) were given a baseline diet (25-30 % of calories from sucrose) alternating with a calorie-restricted diet (25-30 % of calories from sucrose (days 1-6 and 19-30, sweetness being replaced with aspartame on days 7-18, so volunteers ate significantly less calories on days of aspartame consumption) for 12 days at a time, the ALT and AST levels in serum were elevated (but remained within the normal range) on the baseline diet, fell on the aspartame-containing diet then rose again when the baseline diet was reintroduced. There were also small increases in blood urea nitrogen (BUN) levels in subjects on the aspartame containing diet, but renal function remained within normal parameters. Serum triglycerides decreased by 33 % on the aspartame diet (from 130 mg/dL to 87 mg/dL). The Panel noted that the dose of aspartame was not specified and that there were other changes in the diet, including changes in its caloric content.

A short-term tolerance study was conducted in adult PKU heterozygotes (parents of homozygote children) in three different laboratories (E25, 1972). A total of 65 subjects (men and women between the ages of 21 and 45) were administered under double-blind conditions capsules containing placebo, 200 mg aspartame or 300 mg aspartame. The subjects took an appropriate number of capsules per day to deliver increasing doses of aspartame (or placebo) over a six-week period (600, 1200, 2400, 4500, 6300 and 8100 mg/day in a weekly dose escalation protocol) followed by one week with no treatment. Total daily phenylalanine from aspartame was 340, 670, 1350, 2530, 3540, 4550 mg/day, respectively. All 65 subjects completed the study and there were no biochemical differences between the aspartame and placebo group either initially or at the end of the study in two out of the three groups. However, in one of the laboratories, a significantly higher mean initial value on BUN was reported in the placebo group, which was not detected at the end of the study. This laboratory also reported a significantly higher mean value in haematocrit in the aspartame group at the end of the study; this value was however within the normal range. No significant differences in glucose and insulin levels were identified at weeks 0 or 6, or during follow-up (week 7). There were no changes in serum phenylalanine, tyrosine levels (mean levels were below the 8 mg set as the upper limit for women) or in the ratio phenylalanine/tyrosine in subjects taking aspartame after fasting overnight and without preceding aspartame intake for more than 12 hours. No abnormal findings were reported on the

urinary phenylalanine metabolites. The only complaints reported were a feeling of fullness and some unusual bowel movements.

Long-term tolerance studies were also conducted in adult PKU heterozygotes. In a double-blind study a total of 52 subjects (men and women between the ages of 21 and 45) were randomly assigned to be dosed at 1.8 g aspartame/day (2 x 300 mg capsules, 3 times daily) or placebo for 21 weeks (E67, 1973). Laboratory tests (complete blood count, urinanalysis, partial thromboplastin, prothrombin time, BUN, thyroxine, bilirubin, SGOT, alkaline phosphatise, uric acid, creatinine, cholesterol, triglycerides) conducted during weeks 6, 12, 16, 20 and 21 revealed no differences between the groups in the final laboratory values as well as in the final serum glucose and insulin levels. The only significant difference between the treatment groups occurred in the phenylalanine levels at week 16. No abnormal findings on the phenylalanine metabolism were observed in any subject.

In another study, six healthy adult subjects (3 males and 3 females) and five females clinically identified by the authors as PKU heterozygous subjects were administered aspartame at 100 mg/kg bw dissolved in orange juice (E109, 1978). The PKU heterozygous subjects were selected after detection of a significant difference in plasma phenylalanine levels following loading with phenylalanine. Plasma and erythrocyte samples were collected up to 8 hours after the load. Plasma aspartate levels were not significantly affected by aspartame administration in either normal subjects or presumed PKU heterozygous subjects. Levels for glutamate, asparagine and glutamine were also unchanged after aspartame loading in the two groups. Plasma phenylalanine levels increased significantly after aspartame loading in both normal subjects and PKU heterozygous subjects. The plasma phenylalanine levels in PKU heterozygous subjects ingesting aspartame at 100 mg/kg bw were similar to those observed in normal subjects ingesting aspartame at 200 mg/kg bw. In the normal subjects plasma phenylalanine levels increased from fasting levels of 5.6 ± 1.2 µM to values in the normal postprandial range of 11.1 ± 2.49 µM and returned to baseline by 8 hours. In heterozygous subjects, the postprandial levels were higher; 16.0 ± 2.25 µM. Plasma tyrosine levels increased after aspartame loading in both groups, with higher levels in normal subjects. Levels of aspartate in erythrocytes were unchanged after aspartame administration in both groups, whereas phenylalanine and tyrosine levels in erythrocytes increased significantly over baseline.

Another study (E26, 1972) assessed the tolerance of loading doses of aspartame by PKU homozygous children. Two 14-year-old PKU homozygous boys were given a loading dose of 34 mg/kg bw aspartame in orange juice and 2 weeks later they were given a molecular equivalent amount of phenylalanine (19 mg/kg bw) in orange juice. One of the boys was on a liberalized Lofenalac diet (infant powder formula to replace milk in the diet of PKU suffers) with an allowable phenylalanine dietary intake of 70 mg/kg/day; the other boy was on a well-controlled Lofenalac diet (phenylalanine intake of 17 mg/kg/day). The subject on a liberalised diet received a total amount of 3228 mg of phenylalanine (total dietary intake/occasion: 2539 mg plus phenylalanine added from loading dose: 689 mg), which was well within his permitted phenylalanine range during the loading study. The subject on a restricted diet received a total amount of 2037 mg of phenylalanine (total dietary intake/occasion: 965 mg plus phenylalanine added from loading dose: 1072 mg), which far exceeded his dietary limitation. The patient on a liberalised diet was excreting phenylalanine metabolites in large quantities, whereas the patient on a restricted diet did not excrete phenylpyruvic acid after either loading. The serum analysis indicated that the loading dosages of aspartame and phenylalanine did not cause any significant increase in either phenylalanine or tyrosine levels.

Overall, the Panel concluded that no significant adverse effects were observed following repeated administration of aspartame for different durations and at different dose levels of aspartame either in healthy (adults, adolescents and infants) or in PKU heterozygous subjects.

3.2.7.4. Effect of Aspartame on behaviour and cognition

Children

Kruesi et al. (1987) investigated the effect of sugar and aspartame consumption on behaviour in 30 preschool boys (ages 2 to 6 years), of which 18 were described as 'sugar responders' because their parents reported a history of behavioural change following ingestion of sugar. The study was a double-blind cross-over challenge with aspartame (30 mg/kg bw), sucrose (1.75 g/kg bw), saccharin (amount not specified) and glucose (1.75 g/kg bw). The sweeteners were given in lemon-flavoured carbonated drinks on four separate challenge days, with a 5-7-day washout period between challenges. The subjects were scored for behavioural effects, including aggression and activity. There was no significant difference in scores of aggression or observers' ratings of behaviour between the sugar-responsive and age-matched control boys following any of the four treatments. Lower but not significantly different activity was scored during the aspartame challenges in both sugar-responsive and the age-matched control children (recorded by means of an actometer) but the differences were not apparent to the observers.

In a double-blind controlled cross-over study (Wolraich et al., 1994), 25 normal preschool children (3 to 5 years of age) and 23 primary school-age children (6 to 10 years of age) described by their parents to respond adversely to sugar were recruited. Over three consecutive 3-week periods, they received in a randomised distribution the following diets:

- diets containing aspartame (38 $\pm$ 13 mg/kg bw/day for the preschool children and 32 $\pm$ 8.9 mg/kg bw/day for the school-age children) or,
- diets containing sucrose (5600 $\pm$ 2100 mg/kg bw/day for the preschool children and 4500 $\pm$ 1200 mg/kg bw/day for the school-age children) or,
- diets containing saccharin (as placebo) (12 $\pm$ 4.5 mg/kg bw/day for the preschool children and 9.9 $\pm$ 3.9 mg/kg bw/day for the school-age children).

The children were scored for 31 (preschool children) or 39 (school-age children) behavioural and cognitive variables. There were no significant differences in any of the 39 behavioural and cognitive variables among the school-age children receiving any of the three diets. For the preschool children statistically significant differences were measured in 4 (Parents' ratings of cognition, grooved pegboard, dominant hand, nondominant hand) of the 31 measures; however, no consistent pattern in behavioural and cognitive differences was observed amongst the three diets. Wolraich et al. (1994) concluded that 'neither dietary sucrose nor aspartame affects children's behaviour or cognitive function'.

In another randomised, double blind, and placebo-controlled crossover trial (Shaywitz et al., 1994a), the effect of large doses of aspartame on behaviour, cognitive function and monoamine metabolism in 15 children with attention deficit disorder (11 boys and 4 girls, 5-13 years of age) was investigated. The trial consisted of two 2-week periods that were identical except for the administration of either aspartame (34 mg/kg bw/day) or placebo (microcrystalline cellulose). Aspartame or placebo were administered. each morning in a single capsule. All children were on an aspartame-free diet throughout the trial. The children were scored in behavioural and cognitive tests. Blood samples were collected for biochemical and haematological tests and urine was analysed for catecholamine and monoamine metabolite excretion. The authors did not observe any significant effect of aspartame administration on cognitive, attentive or behavioural testing. Likewise, the biochemical and haematological parameters were not altered by aspartame ingestion except that plasma phenylalanine levels increased by approximately 40 % two hours following aspartame administration. The authors considered this increase as within the normal postprandial range. The Panel noted that the changes in plasma phenylalanine levels were consistent with the toxicokinetic studies.

The study of Roshon and Hagen (1989) examined the effect of sucrose consumption on the behaviour of 12 preschool children (6 boys and 6 girls, age 3-5) and did not find any significant difference in

locomotion, task orientation and learning in participants exposed to either sucrose or placebo (9 mg aspartame/kg bw).

The study by Saravis et al. (1990) showed that a single dose of aspartame (9.7 or 34 mg/kg bw) did not have a detrimental effect on learning, behaviour and mood in children.

The Panel noted that no effects of aspartame on behaviour and cognition were observed in children in these studies.

Adults

Lapierre et al. (1990) reported a double-blind randomised crossover study on 10 healthy adult volunteers (6 men, 4 women, aged 21–36 years) who received a single dose of aspartame (15 mg/kg bw) or placebo capsules. No significant differences between aspartame and placebo were found in measures of sedation, hunger, headache, reaction-time, cognition or memory during the study. The authors stated that plasma phenylalanine levels rose within thirty minutes of administration of aspartame.

In another study (Ryan-Harshman et al., 1987), healthy males age 20 to 35 years (n = 13/group) were given capsules containing phenylalanine (0.8, 2.5, 5 and 10 g) or aspartame (5 or 10 g) as a single dose in a randomised crossover design to investigate their neurobehavioral effects on energy and macronutrient selection and on subjective feelings of hunger, mood and arousal. Neither phenylalanine nor aspartame altered mean energy intakes or macronutrient selection nor caused any behavioural effects.

Pivonka and Grunewald (1990) studied the effect on mood and well-being in 120 young women of 18 to 30 years of age receiving water, aspartame-sweetened or sugar-sweetened beverages. The only observed effect was increased sleepiness following the consumption of sugar-sweetened beverages as compared to the consumption of aspartame or water.

In a double-blind study (Stokes et al., 1991), 12 healthy certified active pilots (four females and eight males) were given trial placebo capsules, aspartame (50 mg/kg bw) or ethanol (positive control, dose not reported but estimated to raise plasma alcohol to 0.1 %). Each subject performed the SPARTANS cognitive test battery of aviation-relevant information-processing tasks on 5 occasions, with at least 1 week between treatments that were given in random order among the 12 participants. The tests included an initial pre-test at week 1 and a final post-test at week 5. The authors reported that no detectable performance decrements were associated with the exposure to aspartame, but decrements in psychomotor and spatial abilities were detected following ethanol administration.

The follow up study (Stokes et al., 1994) was undertaken in 12 subjects (college students, sex not reported) in order to examine the effects of double-blind repeated dosing of aspartame on performance in aviation-relevant cognitive tasks. The subjects received placebo capsules or aspartame capsules (50 mg/kg bw/day) for 9 days, or an acute dose of ethanol to achieve 0.1 % blood ethanol levels. On the last day of treatment periods and 90 min after administration of the capsules, plasma phenylalanine levels averaged 59 µM following placebo treatments and 121.5 µM following aspartame consumption. Forty-seven task variables were measured using the SPARTANS 2.0 cognitive test battery and no significantly impaired performance on flight-relevant cognitive tasks were observed by the authors. Instead, in the case of three tasks, the consumption of aspartame was associated with an apparent improvement in performance and a significant correlation between blood phenylalanine levels and performance on the scheduled task was noted.

The study by Walton et al. (1993) was designed to test whether subjects with mood disorders were sensitive to adverse effects caused by aspartame. The initial study design required the recruitment of 40 adult patients with unipolar depression and a similar number of adult subjects without a psychiatric history. Some of the latter group considered themselves to be sensitive to aspartame; however, no

information on possible intolerance to aspartame among the patients with depression was provided by the authors. The participants were given aspartame (30 mg/kg bw/day) or placebo (sucrose) in capsules for a period of 7 days with two 3-day washout periods using a double-blind cross-over study design. Subjects monitored themselves and self-scored the severity of a list of symptoms, including headache, nervousness, dizziness, nausea, feeling 'blue' or depressed, temper outbursts. After eight adult patients (five women and three men; age range, 24-60 years) undergoing treatment for depression and five non-depressed controls (three men and two women; age range, 24-56 years; three self-assessed as sensitive to aspartame (headaches)) had completed the study. The project was stopped because of reactions experienced by three subjects within the group of patients. One patient suffered retinal detachment during the placebo week and another patient experienced a conjunctival haemorrhage during the aspartame week. It was not clear what kind of reaction the third subject experienced and whether it occurred during the placebo or the aspartame phase. The authors reported that despite the small number of subjects, there was a significant difference in the number and severity of self-scored symptoms between aspartame and placebo in the patient group while there was no noted difference in the non-depressed volunteer group.

Using a randomised double-blind placebo-controlled 3-way crossover study design, a group of 48 healthy volunteers (24 men, 24 women, ages 18–34 years) were exposed after an initial one-month aspartame-free period, to aspartame, sucrose or placebo administered for 20 days each (Spiers et al., 1998). Twenty-four participants were given a high dose of aspartame (45 mg/kg bw/day) and the remaining received a low dose of aspartame (15 mg/kg bw/day). The dose of sucrose was 90 g/day for all subjects. Administration of aspartame or placebo (microcrystalline cellulose) was in the form of capsules and for sucrose, a beverage. Effects were evaluated on day 10 and 20 of each treatment, with testing starting 90 minutes after consumption of test material. Plasma phenylalanine levels increased dose-dependently with aspartame consumption from 56 µM (placebo) to 79 µM (high dose), but no neuropsychologic, neurophysiologic and behavioural effects linked to aspartame consumption were observed.

The Panel noted the limited number of participants, the short duration and the inconsistency of the reporting of the results in all these adult human studies. These limitations apply to both positive and negative studies.

Overall, the Panel concluded that there was no evidence that aspartame affects behaviour or cognitive function in children or adults.

3.2.7.5. Effects of aspartame on seizures

A double-blind study was undertaken in eight girls and two boys (age range, 5.1 to 14.6 years) recently diagnosed with generalised absence seizures or also called petit mal seizures to investigate whether aspartame, might exacerbate the occurrence of such seizures (Camfield et al., 1992). The day before the study and during the study, children were allowed to eat as they wished, except any food or beverages containing aspartame. No information was provided regarding the diet. The children ate their normal breakfast on the morning prior to starting the study. Following 1-hour baseline recordings of the number and length of spike-wave bursts (determined using an ambulatory cassette EEG recorder), the children were given 250 ml orange juice sweetened with either aspartame (40 mg/kg bw) or sucrose (1 g sucrose for every 25 mg aspartame to achieve similar sweetness) (assigned in a random way) and the EEG recordings were continued for a further six hours. Each child was tested once with each substance. The authors reported that following the consumption of aspartame but not of sucrose, the total duration of spike-wave discharge per hour was significantly increased and concluded that aspartame appeared to exacerbate the amount of EEG spike wave in children with absence seizures. The Panel noted that the combination of the two parameters (number and length of spike-wave bursts) into a single measure was not adequately explained, and lack of control of food and drink intake before and after dosing may have affected the results. The Panel further noted that aspartame was given in a single dose at the ADI.

In a randomised double-blind placebo-controlled, crossover study (Shaywitz et al., 1994b) aspartame (34 mg/kg bw) was administered to epileptic children (5 boys, 5 girls, ages 5-13 years) for two weeks to investigate the induction of seizures following aspartame consumption. Measurements and parameters tested included EEG, Subjects Treatment Emergent Symptoms Scale (STESS), routine haematology, plasma levels of amino acids, methanol, formate and glucose, liver function tests, urine analysis, and plasma and urinary monoamines and their metabolites. Children were requested to eat a normal diet that was free of aspartame. Nine children completed the study and it was reported that there was no difference in the occurrence of seizures between aspartame and placebo exposure. The plasma levels of phenylalanine increased from 60 µM to 82 µM by one hour post aspartame administration. The possibility that aspartame provokes seizures in self-reported aspartame-sensitive individuals was further investigated in a randomised double-blind placebo-controlled crossover study by Rowan et al. (1995). In this trial, subjects (sixteen adults and two children) who claimed to have experienced epileptic seizures reportedly due to aspartame were given capsules either containing microcrystalline cellulose (placebo) or aspartame (total dose of 50 mg/kg bw). This dose was divided into three portions and administered in the morning at two-hour intervals. All meals were uniformly standardised on treatment days. The authors reported no seizures or other adverse effects from aspartame ingestion. Mean plasma phenylalanine levels increased from 52 µM (after placebo) to 84 µM two hours after the first two doses aspartame.

The Panel noted that the changes in the plasma phenylalanine levels in the studies described above (Shaywitz et al., 1994b; Rowan et al., 1995) were consistent with those in the toxicokinetic studies.

Overall the Panel concluded that the available data do not provide evidence for a relationship between aspartame consumption and seizures.

3.2.7.6. Effect of aspartame on headaches

Schiffman et al. (1987) reported a randomised double-blind crossover trial with aspartame on 40 subjects with a history of headache and related neurologic symptoms within 24h of aspartame consumption. The subjects (12 males and 28 females; age range, 19–69 years) were given aspartame (30 mg/kg bw) or placebo (microcrystalline cellulose) in capsules; the dose was divided into three doses administered in the morning at two-hour intervals. The treatment days were interposed by a washout day. The authors reported that the incidence rate of headache after consumption of aspartame (35 %) was not significantly different from that after placebo (45 %).

In a controlled thirteen-week, double-blind, randomised cross-over study, Koehler and Glaros (1988) comparing the effect of aspartame to that of a matched placebo on the frequency and intensity of migraine headache. The subjects (two males, eight females; ages 18 to 47 years) who had medical diagnosis of migraine, consumed aspartame (1200 mg/person) or placebo (microcrystalline cellulose) in capsules and during two four-week experimental phases (separated by a 1-week washout phase) and submitted self-their diet and headache charts each week during the study. According to the study authors, the statistical analysis indicated a significant increase in the frequency of migraine headaches from the placebo to the aspartame treatment (mean number of migraines per subject: 1.72 (baseline phase), 1.55 (placebo phase), and 3.55 (aspartame phase)). No differences were reported in the intensity or duration of migraine headaches. The high drop-out rate, from 25 to 11 participants in this study was not due to increased frequency or intensity of migraines. The Panel noted that the high inter individual variability in the response of the remaining volunteers makes interpretation unreliable.

In the study by Lipton et al. (1989) 171 patients at a headache unit completed a survey in which alcohol, aspartame, or carbohydrates intake were felt to be triggers of their headaches. About 8 % reported aspartame as a trigger of headaches compared to 2.3 % for carbohydrates, and to about 50 % for alcohol. The Panel considered that having only listed possible triggers of headaches, was a major limitation of this study.

Van den Eeden et al. (1994) conducted a double-blind randomised cross-over trial with subjects self-diagnosed as sensitive to aspartame. Of the 32 subjects recruited and randomised to receive aspartame (in capsules given three times/day to give a daily dose of 30 mg/kg bw/day for seven days) and placebo (microcrystalline cellulose in capsules given three times/day), only 18 participants completed the full study. The authors stated several reasons for the dropouts, including adverse effects. The participants reported headaches on 33 % of the days during aspartame intake, compared with 24 % on placebo treatment ($p = 0.04$). However, no significant difference in the length or intensity of headaches or in the occurrence of side effects associated with the headaches was observed between treatments. The authors concluded that a small subset of the population may be susceptible to headaches induced by aspartame.

The possible effect of aspartame on headaches has been investigated in various studies, which reported conflicting results, ranging from no effect to the suggestion that a small subset of the population may be susceptible to aspartame-induced headaches. The number of existing studies was small, and several had high participant drop-out rates, both under placebo and aspartame treatment. Overall, the Panel noted that because of the limitations of the studies it is not possible to conclude on a relationship between aspartame consumption and headaches.

3.2.7.7. Effects of aspartame on eating behaviour

The Panel is aware that a number of studies have focused on the effects of aspartame on appetite, hunger and food intake.

The Panel considered that these studies of the effect of aspartame (or other low calorie sweeteners) on eating behaviour were not relevant for the assessment of the safety of aspartame and that risk benefit assessment of aspartame are outwith the term of reference and the remit of the Panel.

3.2.7.8. Allergenicity of aspartame

In studies reported by Szucs et al. (1986) aspartame did not affect IgE-mediated histamine release from mast cells *in vitro*. However, mast cells cultured in the presence of aspartame for periods of up to 9 days showed enhanced proliferation and decreased responsiveness to releasing stimuli. The authors concluded that the effect of aspartame on proliferation of cells in culture could be ascribed to a non-specific enhancing effect of its constituent amino acids. Aspartame did not stimulate mast cell or basophil *in vivo* as assessed by skin testing.

Kulczycki (1986) reported a case of aspartame induced urticaria confirmed by double blind challenge in a 23 year old woman with no history of allergic disease. A second case in a 42 year old woman was also briefly reported in the same paper.

Garriga et al. (1991) attempted to identify subjects with hypersensitivity reactions to aspartame with blinded challenge procedures. A total of 61 self-referrals and physician referrals were screened, with 20 referrals evaluated in the clinic. Twelve patients underwent single- and double-blind challenge with up to 2000 mg of aspartame. At the end of the study, no subject with a clearly reproducible adverse reaction to aspartame was identified. The authors concluded that subjects who believed themselves to be allergic to aspartame did not have reproducible reactions.

Geha et al. (1993) conducted a multi-centre placebo-controlled clinical study to evaluate individuals who had experienced urticaria and/or angioedema associated with ingestion of food containing aspartame. In a double-blind crossover study, 21 recruited subjects with a self reported history of hypersensitivity to aspartame were exposed to aspartame and placebo. Conversion products of aspartame, aspartyl-phenylalanine diketopiperazine and beta-aspartame, were also included in the study. Patients received, on different days, increasing doses of aspartame (50, 300, 600 mg) and placebo. Four urticaria reactions were observed, two followed aspartame ingestion and two followed placebo ingestion. The authors concluded that aspartame and its conversion products were no more

likely than placebo to cause allergic symptoms in subjects with a history consistent with hypersensitivity to aspartame.

In a letter to the editor, Kulczycki (1995) considered that in the Geha et al. (1993) study, several aspects in subject recruitment method, convenience, compensation and safety for subjects, as well as inclusion and exclusion criteria and challenge may limit the conclusions of their study.

Butchko et al. (2002) reviewed all published papers from 1980 onwards reporting allergic-type reactions and attributed to aspartame exposure. In an evaluation of consumer complaints related to aspartame by the Centers for Disease Control and Prevention (CDC, 1984) approximately 15 % of the anecdotal complaints were assigned to allergic-dermatologic reactions attributed to aspartame ingestion, such as rashes, sore throat/mouth, swelling and itching.

Cases of urticaria (Kulczycki, 1986) and granulomatous panniculitis (Novick, 1985, McCauliffe and Poitras, 1991) thought to be related to aspartame were reported. The SCF (2002) noted that studies on allergic-like reactions in individuals who themselves reported such reactions to aspartame have not confirmed the occurrence when later studied under control conditions.

Very few cases of presumed systemic allergic dermatitis in patients with contact sensitivity to formaldehyde, apparently caused by the intake of aspartame in artificial sweeteners, have been described. The four patients described in the literature all had eyelid dermatitis (as cited in Veien et al, 2012).

The Panel noted that the studies available were performed on a limited number of participants.

Overall, taking into account the limited data currently available, the Panel considered that the weight-of-evidence does not suggest that aspartame is associated with allergic-type reactions in experimental models or in humans. However, the Panel cannot exclude the possibility that in rare instances individuals could be susceptible to allergic reactions following aspartame ingestion.

3.2.7.9. Anecdotal reports on aspartame

Whilst acknowledging that these data exist, the Panel noted that the data do not meet the pre-specified inclusion/exclusion criteria. To ensure a comprehensive risk assessment the Panel examined the recent assessment of anecdotal reports by the EFSA National Expert Group (EFSA, 2010).

The EFSA National Expert Group assessed anecdotal data of spontaneous reports of cases with symptoms imputed to be related to aspartame (EFSA, 2010). The anecdotal reports explored were largely self-reported, and the information was not structured and was without medical confirmation. This resulted in an incomplete database with limited possibilities to carry out meaningful analyses. The total number of cases considered was 1135. The number of cases where symptoms were classified was 1059. The case reports consisted of reports published in peer reviewed journals and reports compiled by Dr H.J. Roberts and published under the title ‘Aspartame Disease – An ignored Epidemic’ (Roberts, 2001). Of the total number of reports from the latter book entered in the database, 689 were female, 299 were male and 77 were unclassified (the gender was not specified). Forty-three cases were children and an additional 154 cases were documented with the index case (i.e. friends and relatives).

The total number of symptoms reported from all sources was 4281, as most cases reported more than one symptom. Headache was the most frequently reported adverse effect (28.5 %), followed by dizziness and giddiness (19.2 %). Although the results of a questionnaire-based study (Lipton et al., 1989) and two double-blind out-patient investigations (Koehler and Glaros, 1988; Van den Eeden et al., 1994) employing daily doses of up to 30 mg/kg bw/day indicated a potential association between aspartame intakes and headache, it is still not possible to deduce causality, as the effect of diet has not been adequately controlled for and the interpretation of the data was complicated by a high dropout rate and a limited experimental design.

The Panel noted that the number of cases is low when compared with the widespread use and that the effects were mild to moderate.

4. Discussion of aspartame toxicity database

This section discusses the toxicity database on aspartame and undertakes a hazard identification and characterisation of key end points, critical studies and points of departure for derivation of the ADI. It also provides a rationale for the description of the toxicity databases on methanol and DKP, which summarise relevant data and a hazard identification, and characterisation for their integration into the risk characterisation in Section 13.

Aspartame is the methyl ester of the dipeptide of the amino acids aspartic acid and the essential amino acid phenylalanine. After oral ingestion, aspartame is fully hydrolysed, either within the lumen of the gastro-intestinal (GI) tract, or within the mucosal cells lining the inside of the GI-tract. The products that result from these reactions are methanol and the amino acids aspartic acid and phenylalanine. Hydrolysis of aspartame releases a corresponding 10 % by weight of methanol. Due to the very efficient hydrolysis in the gastro-intestinal tract the amount of aspartame that enters the bloodstream has been reported as undetectable in several studies conducted among others in rats, dogs, monkeys and humans (Oppermann, 1984; Burgert et al., 1991). Further studies conducted in monkeys and pigs have shown that also the potential intermediate metabolite phenylalanine methyl ester is rapidly broken down to phenylalanine and methanol in the intestinal lumen (Burton et al., 1984; Burgert et al., 1991).

The Panel concluded that the products formed following hydrolysis of aspartame are normal constituents of the diet and are metabolised by endogenous metabolic pathways. The Panel noted that there were acute intravenous toxicity studies in rat and dog at doses of 100 mg aspartame (equivalent to approximately 500 mg/kg bw in a 200 g rat and 10 mg/kg in a 10 kg beagle dog) with no effects. The Panel concluded that these data would provide reassurance over potential systemic exposure to aspartame if it was possible to administer doses of aspartame that were not fully hydrolysed in the gastrointestinal tract (complete breakdown has been observed with no detectable plasma aspartame at doses up to the highest tested of 200 mg/kg bw).

Overall, the Panel noted that no unchanged aspartame was identified in body fluids or in tissues from experimental animals or humans in any of the studies. Therefore, the Panel concluded that after oral ingestion, aspartame was hydrolysed in the gastrointestinal tract to yield aspartic acid, phenylalanine and methanol. These metabolites are then absorbed and enter normal endogenous metabolic pathways. In humans, individuals heterozygous for phenylalanine hydroxylase mutations showed a somewhat reduced capacity compared to normal individuals to metabolise phenylalanine including that derived from the aspartame molecule. Individuals homozygous for phenylalanine hydroxylase mutations (PKU patients) have a markedly reduced capacity for phenylalanine metabolism.

Several acute and sub-chronic toxicity, genetic toxicity, chronic toxicity and carcinogenicity and reproductive and developmental toxicity studies were made available for re-evaluation to EFSA following a public call for data that was launched in 2011 (see http://www.efsa.europa.eu/en/dataclosed/call/110601.htm). The Panel was aware that many of these reports would have been available at the time of the JECFA and SCF evaluations. The present evaluation by the Panel of the biological and toxicological data includes the assessment of all these unpublished studies, together with the published literature identified up to the end of November 2012. The Panel noted that the majority of these studies were performed in the period 1970-1978 and thus not performed according to Good Laboratory Practice (GLP) and OECD guidelines (http://www.oecd.org/chemicalsafety/testingofchemicals/oecdseriesonprinciplesofgoodlaboratorypracticeglpandcompliancemonitoring.htm). However, the Panel concluded that although the studies were old and were not performed according to current standards, they should not *per se* be disqualified and should be included in the risk assessment of aspartame. As long as the design of such studies and the report of the data were appropriate, the Panel agreed that the studies should be considered in the re-

evaluation of the sweetener. These studies appeared to be the basis for deriving the ADI by JECFA and SCF, using the NOAELs of 4000 mg/kg bw/day identified in the chronic and carcinogenicity studies in rats and applying an uncertainty factor of 100. Due to perceived concerns over the safety of aspartame, the Panel regarded aspartame as a special case and looked again at the studies available to JECFA and the SCF.

The acute toxicity of aspartame was tested in mice, rats, rabbits and dogs and was found to be very low. Similarly, sub-acute and sub-chronic studies did not indicate any significant toxic effects in rats, mice or dogs over dosing periods up to 9 weeks.

Abhilash et al., (2011, 2013) treated male Wistar rats with aspartame in water by gavage, daily for 6 months. They found slight increases (1.50, 1.84, 1.11 and 4.03-fold increase, respectively) in serum liver enzyme (ALAT, ASAT, ALP and γGT) levels in animals exposed to 1000 mg/kg bw/day. The Panel considered these increases as relatively low and likely to be within the normal historical range seen in many laboratories. Therefore, they were not considered to be of biological relevance. Furthermore, the Panel considered the focal infiltration of the liver by inflammatory cells, which is generally also present in the liver of control animals, as normal background pathology. Moreover, these effects were not reported in the chronic toxicity and carcinogenicity studies in both rats and mice, which were performed with much higher doses (up to 8000 mg/kg bw/day).

Aspartame has been tested for genotoxicity in a number of *in vitro* and *in vivo* studies. The Panel concluded that the *in vitro* genotoxicity data on bacterial reverse mutation exhibited some limitations (e.g. absence of TA102 and WP2 uvrA *Escherichia coli*). However, the Panel considered the weight-of-evidence was sufficient to conclude that aspartame was not mutagenic in bacterial systems. Concerning mammalian systems *in vitro*, the Panel concluded that, apart from the valid UDS study that was negative, no conclusion could be drawn at the gene and chromosomal level because no studies dealing with these endpoints were available.

In vivo, the majority of investigations on systemic genotoxicity reported negative findings. Equivocal findings were described only in one NTP study, positive in female but not in male p53 haploinsufficient mice; in two other transgenic mouse strains the results were negative.

Concerning the possible site of first contact effects *in vivo*, limited data are available. However, the available *in vitro* data do not indicate a direct genotoxic activity of aspartame that might predispose to a site of first contact effect *in vivo*.

Overall, the Panel concluded that the available data do not indicate a genotoxic concern for aspartame.

The results from three chronic toxicity and carcinogenicity studies in rats (E33-34, 1973; E70, 1974; Ishii et al., 1981) and in mice (E75, 1974) revealed no compound-related increase in neoplasms at all doses. In the rat study E33-34 (1973), in which the highest dose tested was 8000 mg/kg bw/day renal changes (pigment deposits, focal tubular degeneration and focal tubular hyperplasia) were recorded in males. In an additional two-year chronic toxicity and carcinogenicity study in Wistar rats (Ishii et al., 1981; Ishii, 1981) there was a dose-dependent depression of body weight gain at 2000 and 4000 mg/kg bw/day, and this effect was correlated with decreased feed consumption. Furthermore a dose-related increase in focal mineralisation of the renal pelvis in both males and females, associated with a dose-related increase in urinary calcium was recorded but this common lesion in rats attributable to mineral imbalance was considered by the Panel to be of minimal toxicological significance. The Panel derived a NOAEL of 4000 mg/kg bw/day in these four studies (E33-34, 1973; E70, 1974; E75, 1974; Ishii et al., 1981), which was the highest dose level tested in three out of the four studies (E70, 1974; E75, 1974; Ishii et al., 1981).

The results of the three studies in rats did not provide any consistent evidence of an intracranial tumourigenic effect, but showed a study-to-study variability in the incidence of such neoplasms in controls. The incidences of spontaneous brain tumours in Sprague-Dawley derived rats have been

reported by Ward and Rice (1982) to vary from 0 % up to 3.3 %. A similar incidence was reported by Weisburger et al. (1981). The Panel noted that the incidences observed in the studies with aspartame were within the range of spontaneous brain tumours observed in CD-rats. JECFA also evaluated these data and reported that no particular type of neoplasm or the general incidence of intracranial neoplasms seemed to predominate in any treated group. Therefore, JECFA concluded that aspartame did not cause brain tumours in rats (JECFA, 1980). The Panel agreed with this conclusion.

The NTP carried out several 9-month carcinogenicity studies with aspartame in genetically modified Tg.AC hemizygous, p53 haploinsufficient and Cdkn2a deficient mice (NTP, 2005). According to NTP, there was no evidence of treatment-related neoplastic or non-neoplastic lesions in any of these studies. The former AFC Panel independently evaluated these studies and agreed with this conclusion (EFSA, 2006). The Panel also agreed with these conclusions but noted that the three mouse models remain at an experimental stage and had not been formally validated as experimental models for carcinogenicity testing. Therefore, the Panel did not use the results from the NTP study to derive a NOAEL.

Since the last evaluation of aspartame by the SCF, Soffritti and co-workers reported new long-term carcinogenicity studies on aspartame in both rats (Soffritti et al., 2006, 2007) and mice (Soffritti et al., 2010). The two rat studies have already been evaluated by the former AFC Panel (EFSA, 2006) and the ANS Panel (EFSA, 2009a), respectively. Both Panels considered that the two ERF rat studies had flaws that brought into question the validity of the findings, as interpreted by the ERF. In particular, the high background incidence of chronic inflammatory changes in the lungs and other vital organs and tissues and the uncertainty regarding diagnosis of some tumour types were major confounding factors in the interpretation of the study. The ANS Panel also noted that the increase in incidence of mammary carcinoma was not considered indicative of a carcinogenic potential of aspartame since the incidence of mammary tumours in female rats is rather high and varies considerably between carcinogenicity studies. The ANS Panel noted that the only consistent findings reported by the authors in the two rat studies were an increased incidence of lymphomas/leukaemias in female rats and an increased trend or incidence of lymphomas/leukaemias in treated males and females at the high dose group (EFSA, 2009a).

In the mouse study (Soffritti et al., 2010) Swiss mice were given aspartame (up to 4000 mg/kg bw/day) in the feed from prenatal life (12 days of gestation) until death. The authors of the study reported a dose-related increase in the incidence of hepatocellular carcinomas in male mice, and an increase in the incidence of alveolar/bronchiolar carcinomas in males of the highest dose group, whereas no compound-attributed carcinogenic effects were reported in female mice. The former ANS Panel (EFSA ANS Panel, 2011) and EFSA (EFSA, 2011a) observed that the hepatic and pulmonary tumour incidences reported by Soffritti et al. (2010) all fall within their own historical control ranges for spontaneous tumours. It was also noted that Swiss mice are known to have a high background incidence of spontaneous hepatic and pulmonary tumours (Prejean et al., 1973; Fox et al., 2006).

The validity of the ERF studies has been questioned by several investigators, and more recently in an US EPA (US Environmental Protection Agency) commissioned NTP report (NTP-EPA, 2011). For instance, Cruzan (2009) argued that lung infections probably played a role in the development of what Soffritti et al. (2002) referred to as lympho-immunoblastic lymphomas. The Panel noted that the same arguments are applicable to the Soffritti et al. (2006) and Soffritti et al. (2007) studies and considered it plausible that lung infections might have played a role in the development of neoplastic changes that originated from mediastinal lymph nodes. Similarly, Schoeb et al. (2009) and Schoeb and McConnell (2011) assessed the likelihood that lifetime cancer bioassays of aspartame, methanol, and methyl tertiary butyl ether conducted with conventional (not specific pathogen-free) Sprague-Dawley rats were compromised by *Mycoplasma pulmonis* disease. The authors concluded, based on published reproductions of the lesions (Belpoggi et al., 1999, Soffritti et al., 2005), that the accumulation of lymphocytes, plasma cells and neutrophils in the lungs of the rats used in these studies was more plausibly due to *M. pulmonis*, possibly with contributions by other pathogens, than to a rare cellular type of lymphoma having an organ distribution uncharacteristic of recognised forms of lymphoma in

rats (Schoeb et al., 2009). The Committee on Carcinogenicity of Chemicals in Food Consumer Products and the Environment (COC) also evaluated the Soffritti et al. (2006) study on aspartame (COC, 2006). In light of the limitations in the design of this study and the use of animals with a high infection rate, the COC considered that no valid conclusions could be drawn from this study. Schoeb and McConnell (2011) subsequently confirmed the occurrence of inflammatory lesions in not only the respiratory tract but also lymph nodes, thymus, pleura and brain, suggesting the involvement of other pathogens in addition to *M. pulmonis.* They furthermore stated that upon screening of the ERF animal colony, antibodies to 'mycoplasma' had been found. Overall, Schoeb and McConnell (2011) concluded that the reported development of lymphomas in the ERF studies performed with Sprague-Dawley rats should not be taken into account in cancer risk assessments. The Panel agreed with the above conclusions.

Finally, the ANS Panel noted that an NTP review of ERF studies on methanol, methyl-*t*-butyl-ether, ethyl-*t*-butyl-ether, acrylonitrile and vinyl chloride had been performed following a request by US EPA (NTP-EPA, 2011). In the case of the methanol study (Soffritti et al., 2002), the NTP pathologists did not confirm the diagnosis of malignancy and reported fewer lymphoid neoplasms, mainly of the respiratory tract. They also observed fewer neoplasms of the inner ear and cranium and noted that there was chronic inflammation of the nasal cavity, ear canal, trachea, and lung, indicating infection of the animals by one or more respiratory pathogens. EPA concluded that many of the malignant neoplasms and the lymphoid dysplasias diagnosed by the ERF pathologists were cases of hyperplasia related to chronic infection (NTP-EPA, 2011).

The reproductive and developmental toxicity studies on aspartame comprised an embryotoxicity and teratogenicity study performed in the mouse, a two-generation reproduction toxicity study in the rat, five peri- and postnatal developmental studies in the rat, a reproductive performance and developmental study in the rat and an embryotoxicity and teratogenicity study in the rat. In addition, eight embryotoxicity and teratogenicity studies were performed in the rabbit, four with administration of aspartame by diet and four by gavage.

The Panel identified a NOAEL for developmental toxicity in the mouse (E89, 1975) of 5700 mg/kg bw/day, the highest dose level tested. In the case of the rat, the two-generation reproduction toxicity study was reported with limited details. However, the Panel considered this study (E11, 1971) together with other segment I and segment III studies that included a full examination of the F2A pups from the two-generation reproduction toxicity study (E9, 1972) sufficient to fulfil the requirements of a two-generation reproduction toxicity study. The animals received aspartame in the diet at dose levels of 0 (control), 2000 and 4000 mg/kg bw/day. Statistically significant pup body weight suppression and smaller size at weaning were recorded in the 4000 mg/kg bw/day. The Panel identified a NOAEL from these studies of 2000 mg/kg bw/day based on the lower pup weights at weaning in both generations (E9, 1972; E11, 1971).

Further rat studies included a male and female fertility study (E10, 1972) and three segment peri and postnatal developmental studies (E39, 1973; E47, 1973; E48, 1973). In all these studies, the route of aspartame administration was via the diet at dose levels of 0 (control), 2000, or 4000 mg/kg bw/day. The effects on the pups were depressed body weights at birth and a decreased survival rate, which were observed at the high dose only. For all these studies the Panel derived a NOAEL of 2000 mg/kg bw/day. In an embryotoxicity and teratogenicity study (E5, 1970), no evidence of treatment-induced fetopathological effects was observed, and the Panel derived from this study a NOAEL of 4000 mg aspartame/kg bw/day for developmental and maternal toxicity, the highest dose level tested.

In a peri- and postnatal development study in rats (E49, 1973), the effects of aspartame (4000 mg/kg bw/day) were compared with those of L-phenylalanine (1800 mg/kg bw/day) or L-aspartic acid (1700 mg/kg bw/day) or the combination of L-phenylalanine and L-aspartic acid (2100 and 1800 mg/kg bw/day). The intention of the study was to have equimolar doses of each substance in the diet. The authors of the study reported a significant depression in body weight in the aspartame, L-phenylalanine and L-phenylalanine + L-aspartic acid groups as compared to the control group and

concluded that L-phenylalanine on its own or in combination with aspartic acid decreased maternal and pup body weight and therefore duplicated the observed effects of aspartame on these endpoints. The Panel agreed with the author's conclusion but noted the poor survival of control pups.

The Panel noted that the two-generation reproduction toxicity study (E11, 1971) was not reported according to current standards. However, when taken together with the segment I study (E10, 1972) and segment III studies (E47, 1973; E48, 1973) the Panel considered the available data sufficient to conclude on reproductive toxicity. In summary, the results of the reproductive and developmental toxicity studies in rats indicate NOAELs that ranged from 2000 mg aspartame/kg bw/day (E11, 1971; E39, 1973; E47, 1973; E48, 1973) to 4000 mg/kg bw/day (E5, 1970; E9, 1972; E10, 1972). The Panel noted that where developmental changes in pup body weight were observed at birth in studies at the dose of 4000 mg/kg bw/day, these could be attributed to a combination of malnutrition and nutritional imbalance due to excessive exposure to phenylalanine from metabolism of aspartame. In support of this hypothesis, the Panel noted that administration of a dose of L-phenylalanine (1800 mg/kg bw/day) that was equimolar to 4000 mg aspartame/kg bw/day led to a similar decrease in maternal and pup body weight as observed in a concurrent aspartame group (E49, 1973). Furthermore, supplementation of the diet with L-phenylalanine (5-7 %) (Kerr and Waisman, 1967) or the combination of 3 % L-phenylalanine and 0.5 % α-methylphenylalanine (Brass et al., 1982) has been shown to result in severe nutritional imbalance in rats (Kerr and Waisman, 1967) and to lead to a reduction in pup body weight at birth (Brass et al., 1982). In addition, milk production in mothers exposed to excessive L-phenylalanine intake has been reported to be decreased, which could further contribute to malnutrition of the pups during weaning and result in pup mortality (Boggs and Waisman, 1962; Kerr and Waisman, 1967).

The Panel considered that the depression in pup body weight in the high dose groups might be accounted for by the effect of high exposure to phenylalanine from metabolism of aspartame and it should be regarded as an (indirect) effect related to the test compound. The Panel noted that milk quality in the rat could be adversely affected by high levels of plasma phenylalanine and that body weights of pups could have been adversely affected (decreased) by decreased milk quality. This might provide a plausible explanation for the effects of aspartame on pup body weight at weaning.

In several reproductive and developmental toxicity studies, aspartame was administered to rabbits via the diet (E53, 1973; E54, 1974; E55, 1973; E62, 1973; E63, 1973). The Panel noted that the actual doses of aspartame to which the rabbits were exposed did not exceed 1880 mg/kg bw/day (range 1160–1870 mg/kg bw/day when the intended dose was 4000 mg aspartame/kg bw/day; 670–1880 mg/kg bw/day when the intended dose was 2000 mg aspartame/kg bw/day) due to the considerable decrease in feed intake observed in the does (by 32-62 %) in this dose group. In another series of prenatal developmental studies, rabbits were exposed to aspartame (at doses up to 2000 mg/kg bw/day) by gavage (E51, 1973; E52, 1973; E79, 1974). Overall, the Panel considered that the data from the studies described above were confounded both by the decrease in feed intake (when aspartame was administered via the diet or by gavage), the poor health of the animals, and, in many cases, by a number of deaths of pregnant rabbits in the treated groups, possibly related to misdosing via the gavage technique.

In a separate study, pregnant rabbits were dosed by gavage at levels of 0 (control), 500, 1000 and 2000 mg/kg bw/day (E90, 1975). This study also included L-phenylalanine and L-aspartic acid groups at dose levels equimolar to the top dose of 2000 mg aspartame/kg bw. An approximately 65 % and 40 % decrease in feed consumption was observed in the 2000 mg aspartame/kg bw/day group and the L-phenylalanine treated animals, respectively, which in the high dose aspartame group was accompanied by a significant body weight loss. Twenty-four abortions were observed in the high dose aspartame group compared to no abortions in controls and four in the L-phenylalanine group. Mean fetal body weight and length were significantly reduced in both the 2000 mg/kg bw/day group and the L-phenylalanine group animals and a significantly higher rate of total (major and minor) malformations in the 2000 mg aspartame/kg bw/day group animals compared to the concurrent control group was reported. The authors of the study suggested that the effects observed at 2000 mg/kg

bw/day and to a lesser extent in the L-phenylalanine group appeared to be produced by a severe reduction of nutrient intake.

The Panel concluded that despite this plausible explanation, a direct toxic effect of treatment could not be ruled out. Although pregnant female rabbits from the high dose group in study E90 (1975) manifested body weight loss and decreased to negligible feed consumption, the Panel was unable to determine that the reported developmental effects on fetus were due to a reduced nutrient intake by the mother, as suggested by the authors. The Panel also considered the possibility that exposure to high levels of phenylalanine resulting from metabolism of aspartame may be in part or wholly responsible for these effects in the high dose aspartame group because similar effects, although less severe, were seen in the phenylalanine group. However, the Panel noted that the dose of L-phenylalanine administered was 25 % lower than the animals would have received as phenylalanine from the high dose of 2000 mg aspartame/kg bw/day.

The Panel agreed that the results in developmental rabbit studies with aspartame may have been influenced by the nutritional status of the female rabbits as a result of gastrointestinal disturbances and the potential effect of phenylalanine from metabolism of the test compound. Gastrointestinal disturbances have also been reported in pregnant rabbits following administration of sucralose (SCF, 2000), steviol glycosides (EFSA ANS Panel, 2010) and neotame (EFSA, 2007).

Rabbits are known to be susceptible to disturbances of the gastrointestinal tract (Otabe et al., 2011). Gastrointestinal disturbances and lower feed intake in the rabbits may result from administering high levels of test preparation by gavage and may lead to disturbed clinical appearance, death or abortions.

Matsuzawa et al. (1981) reported that pregnant rabbits restricted to 20 or 60 g feed/day from gestation days 6 to 20 showed statistical significantly higher fetal losses than pregnant rabbits fed 150 g feed/day and control group.

Cappon et al. (2005) studied the effects of feed restriction on reproductive performance and development of the fetuses in rabbits. Groups of 15 New Zealand White rabbits were offered 150 (control), 110, 75, 55, 35, and 15 g feed/day from GD 7-19. An increased number of abortions associated with an impairment of body weight gain were observed after feed restriction to 15 g feed/day. Feed restriction to 75 g feed/day was associated with reduced ossification of the fetal skeleton whereas reduced fetal weight and increased incidence of fetuses with reduced ossification of the skeleton were noted at feed consumption of less 75 g feed/day. Cappon reported no association of fetal malformation with up to 90 % feed restriction. The author concluded that the weight-of-evidence suggested that restricting rabbit feed during pregnancy, and the associated impairment of maternal gestational body weight gain could cause abortion, and fetal growth retardation exhibited by reduced fetal body weight and reduced skeletal ossification, but induced, remarkably, few (or no) malformations. Based on the available descriptions of the developmental rabbit studies the Panel was unable to ascertain whether the gastrointestinal disturbances were a cause of or a consequence of the reproductive and developmental effects seen. The Panel further noted that the gastrointestinal effects in the rabbit studies may be species specific and would therefore not be relevant for humans. Overall, the Panel concluded that the rabbit studies should not be considered as pivotal in the safety evaluation of aspartame because of the confounding factors affecting these studies.

The Panel noted that there were limitations and deficiencies in the reproductive and developmental toxicity database in rats and rabbits. In many cases, these were likely to be attributable to study methodology and reporting requirements at the time when the studies were conducted. The Panel recognised the difficulty to evaluate retrospectively whether the reported effects would account for the toxicity of aspartame and thus be of toxicological relevance. The Panel considered whether this could be resolved by performing reproductive and developmental toxicity studies according to current guidelines. The Panel noted that this would require doses equivalent to, if not higher than, those used in the earlier studies. For animal welfare reasons, modern studies would not generally be conducted at doses greater than the lower end of the dose range identified to cause maternal toxicity, and/or

associated with a severe decrease in feed consumption; the Panel therefore concluded that it was unlikely that a definitive modern study would be possible or ethical.

The Panel considered that the attribution of effects to phenylalanine was both plausible and supported by the data on metabolism and kinetics of aspartame. Developmental toxicity studies, in which phenylalanine instead of aspartame was administered to rabbits, showed that phenylalanine induced maternal toxicity and effects in pups that were similar to those observed with aspartame. Therefore, the Panel considered that data on adverse effects of elevated plasma phenylalanine on pregnancy outcomes in phenylketonuria sufferers could be helpful. The Panel considered that combining these data with information on toxicokinetics of phenylalanine derived from aspartame might allow a mode of action approach to assess the reproductive and developmental effects of aspartame.

Epidemiological studies have explored possible associations of aspartame, or artificial sweeteners more broadly, with pre-term delivery and various cancers. The epidemiological data on aspartame were previously reviewed by SCF (2002). The Panel considered and agreed with the conclusions of SCF that there was no evidence for adverse effects of aspartame in the human population. The new epidemiology studies, published since the SCF opinion, are described below.

A large and well conducted prospective cohort study in Denmark found a significantly elevated risk of pre-term delivery (particularly medically induced pre-term delivery) in women with higher reported consumption of artificially sweetened drinks (but not aspartame specifically) at about 25 weeks gestation (Halldorsson et al., 2010). However, even in the highest exposure category (≥ 4 servings per day), odds ratios were less than two, and in the absence of any identified plausible underlying toxic mechanism or of independent replication in other studies, it is possible that the finding resulted from a combination of uncontrolled residual confounding and chance.

To explore whether the findings of the Danish study could be replicated, another prospective study in Norway investigated the relation between consumption of artificially sweetened and sugar-sweetened soft drinks during the first 4-5 months of pregnancy and subsequent pre-term delivery in a large cohort of Norwegian women (Englund-Ögge et al., 2012). No significant trends were found in the risk of pre-term delivery with increasing consumption either of artificially sweetened drinks or of sugar-sweetened drinks. Small elevations of risk were observed with higher consumption of artificially sweetened soft drinks, but after adjustment for covariates, these reached statistical significance only when categories of consumption were aggregated to four levels, and then the odds ratio for the highest category (≥ 1 serving/day) was only 1.11 (95 % CI 1.00-1.24) in comparison with never consumption. This was driven by an increase in spontaneous but not medically induced pre-term delivery. Associations with sugar-sweetened soft drinks tended to be somewhat stronger, with an adjusted odds ratio of 1.25 (95 % CI 1.08-1.45) for consumption of at least one serving per day.

This pattern of results contrasts with that reported by the Danish study. The association of pre-term delivery with artificially sweetened soft drinks was much weaker and barely discernible, applied more to spontaneous than medically induced deliveries, and was exceeded by an association with consumption of sugar-sweetened soft drinks. The Panel noted that effects may have been underestimated because of non-differential inaccuracies in the assessment of dietary exposures, but the method was similar to that used by the Danish study, and the same for sugar-sweetened as for artificially sweetened soft drinks.

When findings from the two studies are considered together, as recently done in a meta-analysis of the data from both studies (La Vecchia, 2013), they do not point clearly to a hazard. In summary, both studies appear to have been well designed and conducted. Noting this, the Panel concluded that even at high level of exposure to artificially sweetened soft drinks the risk of pre-term delivery is likely to be small if it exists at all. This small potential risk could be a consequence of uncontrolled residual confounding, and the inconsistencies in the patterns of association reinforce this uncertainty. The Panel noted that the studies only allowed conclusion on artificially sweetened soft drinks and not on aspartame specifically.

The study by Schernhammer et al. (2012) examined the risk of lymphatic and haematopoietic cancers in relation to consumption of diet soda and aspartame sweeteners added at the table was examined, in two US cohorts. The authors reported that the category of highest aspartame intake (≥ 143 mg/day) was associated with a significantly elevated relative risk of non-Hodgkin Lymphoma (NHL) (1.64, 95 % CI 1.17-2.29) and of multiple myeloma (3.36, 95 % CI 1.38-8.19) in men. However, there was no consistent trend in risk with increasing exposure, and there were no corresponding elevations in risk in women. No clear association with leukaemia was apparent in either men or women. The Panel noted that the positive findings could be given little weight, given their limitation to men, the small relative risks observed, and the lack of clear dose-response relationships. The authors' proposed explanation for the differential associations in men and women is unconvincing in the absence of data on other more important dietary sources of methanol.

A Swedish case-control study of malignant brain tumours suggested a possible link with consumption of low-calorie drinks, most of which contained aspartame (Hardell et al., 2001). However, the association was not statistically significant, and a more rigorous prospective cohort study in the United States found that risk of malignant glioma was significantly lower in subjects with higher intakes of aspartame at baseline. Moreover, a case-control study of children with medulloblastoma/primitive neuroectodermal tumour, also in the United States, found only a weak and statistically non-significant association with mothers' recalled consumption of low-calorie carbonated drinks during pregnancy (Bunin et al., 2005).

A cohort study of haematopoietic cancer in the United States showed no relation to consumption of aspartame, and in a set of linked case-control studies in Italy (Gallus et al., 2006), associations between consumption of sweeteners other than saccharin (mostly aspartame) and cancers of the oral cavity and pharynx, oesophagus, colon, rectum, larynx, breast, ovary, prostate and kidney were unremarkable.

In a small pilot case-control study on brain cancer in adults conducted in southern France, 122 cases were compared to 122 controls with other neurological diagnoses (Cabaniols et al., 2011). There was no association with aspartame consumption during the past five years of at least once per week (OR 1.02, 95 % CI 0.57-1.85). However, the Panel noted that, due to different study limitations, the non-positive finding provides little reassurance of an absence of hazard.

Overall, the reassurance of safety that is provided by non-positive findings in these epidemiological investigations is limited by (sometimes unavoidable) shortcomings in their design, and particularly in their assessment of exposures. At the same time, the studies do not point strongly to any hazard, and do not provide a suitable point of departure for derivation of a toxicological reference value.

Magnuson et al. (2007) and Weihrauch and Diehl (2004) reviewed strengths and weaknesses of these four cancer epidemiological studies and concluded that there was no evidence that aspartame posed a carcinogenic risk. The National Expert meeting of the EFSA Advisory Forum (EFSA, 2010) also concluded that there was no evidence to support an association between aspartame and the prevalence of brain, haematopoietic or other tumours. The Panel agreed with these conclusions.

5. Biological and toxicological data on methanol

The section contains summarised information on methanol metabolism and kinetics and a few detailed descriptions of the studies on release of methanol from aspartame.

5.1. Absorption, distribution, metabolism and excretion of methanol

Methanol is converted to formaldehyde through one of at least four pathways, involving alcohol dehydrogenase, catalase, CYP2E1 or a Fenton-like system ($Fe(II)/H_2O_2$) (Dikalova et al., 2001; Sweeting et al., 2010; MacAllister et al., 2011). Primates, including humans, metabolise methanol primarily using the alcohol dehydrogenase pathway whereas rodents appear to use all four pathways. In monkeys, about 25 % of the ^{14}C from [^{14}C-methyl]-aspartame enters into transmethylation or

formylation biosynthetic reactions (E92, 1976). Formaldehyde is converted to formate by a specific formaldehyde dehydrogenase, and formate is converted to carbon dioxide. In monkeys, about 70 % of the ^{14}C from [^{14}C-methyl]-aspartame follows this path to carbon dioxide (E92, 1976). Formate can enter the one-carbon metabolic pool by formylating tetrahydrofolic acid to N5,N10-methylene-tetrahydrofolate.

In mammals, methanol is readily absorbed following ingestion, inhalation and dermal exposure (COT, 2011). Methanol can readily enter the total body water and has a volume of distribution of 0.6-0.7 l/kg bw. Methanol is subject to a significant first pass metabolism. The overall metabolism of methanol proceeds by stepwise oxidation via formaldehyde to formate and then to carbon dioxide. The metabolism of formaldehyde is very efficient. Even after intravenous infusion, it was difficult to detect formaldehyde in blood, in which formaldehyde has been reported to display a half-life of about 1 minute (McMartin et al., 1979; Tephly and McMartin, 1984). The oxidation of formate to carbon dioxide varies between species, the rate of formate elimination in humans and non-human primates being half of that in rats (Kavet and Nauss, 1990). In rodents, formate is converted to carbon dioxide through a folate-dependent enzyme system and a catalase-dependent pathway (Dikalova et al., 2001) whereas in humans metabolism occurs exclusively through the folate-dependent pathway (Hanzlik et al., 2005). Studies in humans have shown that approximately 80 % of an oral [^{14}C]formate dose (administered as a single dose of 3.9 g calcium formate) is exhaled as $^{14}CO_2$, 2-7 % excreted in urine and approximately 10 % is metabolically incorporated (Hanzlik et al., 2005).

Formaldehyde is an intermediate in the formation of formic acid from methanol. Even after intravenous infusion of formaldehyde in dogs, cats, rabbits, guinea pigs and rats, it was difficult to find this substance in the blood, because of a very short half-life (~ 1 minute). Formaldehyde formation was studied in older publications on acute toxicity of methanol, but this intermediate could not be detected in animals or in humans (Tephly and McMartin, 1984). In monkeys given methanol orally via a nasogastric tube [3 g/kg bw as a 20 % (w/v) solution of [C^{14}]methanol (1300 dpm/µmol of methanol)], formaldehyde could be detected in the blood at a level of 27-45 µM over the period up to 18 hours following dosing with methanol. No formaldehyde could be found in liver, kidney, urine or several neural tissues (LOD 25 µM; ~ 0.75 mg/L or 0.75 mg/kg tissue). Formic acid was readily detected in all tissues and body fluids studied, in particular in urine, liver, blood and kidney (McMartin et al., 1979).

When ^{14}C-methanol or [^{14}C-methyl]-aspartame were given orally to rats or monkeys in equimolar doses, in both species, the radioactivity was rapidly eliminated via exhaled air (~ 60 % of the dose in rats; ~ 70 % in monkeys) within 8 hours post dosing. With methanol, a slightly higher rate of exhalation was observed, probably because hydrolysis and absorption are required when the radioactivity was administered as labelled aspartame. Virtually no radioactivity appeared in the faeces and 2-5 % of the dose was eliminated via urine. In rats, about 40 % and in monkeys about 30 % of the dose was not accounted for, but it is noted that residual radioactivity in the carcass from incorporation through the 1-carbon cycle was not studied. These studies were undertaken with dose levels of 10 to 20 mg aspartame/kg bw (Oppermann, 1984).

In a recent study (Lu et al., 2012) formaldehyde hydroxymethyl DNA adducts have been measured after administration of labelled [$^{13}CD4$]-methanol to rats (500 and 2000 mg/kg bw/day for 5 days) in multiple tissues in a dose dependent manner. This finding is in line with the known metabolism of methanol.

The half-life for the systemic clearance of methanol has been reported to be 2.5-3 hours following oral doses of less than 100 mg/kg bw methanol given to human volunteers, increasing to 24 hours or more for doses greater than 1000 mg/kg (WHO, 1997). Stegink et al. (1981b) reported a human volunteer study in which methanol cleared with a half life of 2.5 to 3 hours after administration for a dose of 80 mg aspartame/kg bw.

5.2. Toxicological data of methanol

5.2.1. Acute oral toxicity

A limited number of studies have assessed the acute oral toxicity of methanol. The LD_{50} reported were 7300 mg/kg bw in mice, 5628 mg/kg bw in rats and 7000 mg/kg bw in monkeys (Lewis, 1992).

5.2.2. Short-term and sub-chronic toxicity

In a 90-day study, Sprague-Dawley rats (30/sex/group) were administered 0, 100, 500, or 2500 mg methanol/kg bw/day by gavage for 6 weeks until the day of the interim necroscopy. After the interim sacrifice, all surviving rats were dosed daily until the end of the experiment (EPA, 1986). No differences in body weight gain, food consumption, gross or microscopic examinations were reported between treated animals and controls. Serum alanine transaminase (ALT) and serum alkaline phosphatase (ALP) of the high dose group's males ($p \leq 0.05$) and females were approximately 30 % and 40 % higher respectively than those of the control group at the end of the experiment. Blood Urea Nitrogen (BUN) averages in the high dose group were 17 % and 12 %, respectively lower than control groups ($p \leq 0.05$). Mean Corpuscular Haemoglobin (MCH) averages in the high dose group females were also significantly lower than controls ($p \leq 0.001$) as well as Mean Cell Volume (MCV) averages were lower in the mid dose and low dose females groups ($p \leq 0.05$). Brain weights of high dose group males and females were significantly decreased compared to the control group at the end of the treatment. The authors considered these differences to be treatment-related but very small, and not associated with any histological lesion. A higher incidence of colloid in the hypophyseal cleft of the pituitary gland was also observed in the high dose group (24/40) compared to the controls (3/40). The authors identified a NOAEL from this study of 500 mg/kg day.

5.2.3. Genotoxicity of methanol

The genotoxicity of methanol has been studied in bacterial and mammalian *in vitro* tests and in some *in vivo* tests. A brief description of the studies found in the literature, is given below and summarised in Appendix H.

In vitro studies

De Flora et al. (1984) published a survey of 71 drinking water contaminants and their capacity to induce gene reversion in the Ames test with *Salmonella typhimurium* strains TA98, 100, 1535, 1537, and 1538 and found methanol to be negative. De Flora et al. (1984) also reported methanol to be negative for induction of DNA repair in *Escherichia coli* strains WP2, WP2 (*uvrA-, polA-*), and CM871 (*uvrA-, recA-, lexA-*), both when tested in the presence or absence of metabolic activation. The Panel considered these studies as providing insufficient information and therefore, were not taken into further consideration.

Griffiths (1981) reported a genotoxicity study of methanol using fungi, and reported methanol to be negative for the induction of aneuploidy in *Neurospora Crassa*. The Panel considered that the methods implemented were thought not to be sufficiently robust to support the results reported.

Simmon et al. (1977) listed methanol as one of 45 chemicals that gave negative results with *Salmonella typhimurium* strains TA98, 100, 1535, 1537 and 1538, tested with or without metabolic activation. However, for the reasons explained in detail in Appendix H, the Panel considered this study provided insufficient details and therefore, was not taken into further consideration.

Abbondandolo et al. (1980) used a *ade6-60/rad10-198,h*$^{-}$ strain of *Schizosaccharomyces pombe* (P1 strain) to determine the capacity of methanol to induce forward mutations. Negative results were obtained for methanol, both without and with metabolic activation. In contrast to this finding, weakly positive results for methanol were obtained for the induction of chromosomal malsegregation in the diploid strain P1 of *Aspergillus nidulans (*Crebelli et al., 1989). The disturbance of chromosome segregation by methanol was attributed by the study authors to the non-specific damage of cell

membranes induced at high doses by methanol and other aliphatic alcohols. The Panel noted that the fungal systems are not considered relevant for chromosomal malsegregation induction in mammalian cells. The Panel considered that the methods implemented were sufficiently robust to support the results reported.

Methanol was studied in a *Salmonella* mutagenicity test in the presence or in the absence of metabolic activation. No mutagenicity was detected in strains TA98, 100, 1535, 1537, 1538 in 5 doses up to 3.6 mg/plate (Gocke et al., 1981). The bacterial reverse mutation assay followed a standard protocol of that time. However, no detailed results for methanol were reported. Furthermore, TA102 and *Escherichia coli* were not included in the study and the maximal concentration tested (3.6 mg/plate) was lower than recommended in current guidelines. The Panel considered the bacterial reverse mutation assays, which were part of the screening study by Gocke et al. (1981), as providing insufficient information for further consideration.

NEDO (1987) reported methanol to be negative for gene mutation in *Salmonella typhimurium* TA98, 100, 1535, 1537, 1538; *Escherichia coli* WP2 uvvA (Pre-incubation, absence and presence of S9; 10, 50, 100, 500, 1000, 5000 µg/plate). The Panel considered that the methods implemented were sufficiently robust to support the results reported.

NEDO (1987) also used Chinese hamster lung cells to monitor the capacity of methanol to induce (a) forward mutations to azaguanine, 6-thioguanine, and ouabain resistance (15.8, 31.7, 47.4, 63.3 mg/mL), (b) chromosomal aberrations (7.1, 14.3, 28.5 mg/mL) and (c) SCEs (7.1, 14.3, 28.5 mg/mL). Methanol was negative in tests for gene mutation and CA, but displayed some capacity to induce SCE in Chinese hamster lung cells, since the incidence of these lesions at the highest concentration (28.5 mg/mL) was significantly greater than in controls (9.41 ± 0.416 vs. 6.42 ± 0.227) expressed as mean ± SE per 100 cells. The Panel noted that this concentration of methanol exceeded the maximum concentration recommended for *in vitro* studies (i.e. 5 mg/mL or 5 µL/mLor 10 mM, whichever is the lowest) (OECD test guidelines 473 and 487.) The Panel considered that the methods implemented were not sufficiently robust to support the results reported.

In a test for forward mutation in *Escherichia coli* SA500, methanol concentrations above 23 % were cytotoxic, reducing survival of the cells to less than 40 %. A 23 % methanol solution produced no mutagenic effects; cell survival was 53 % (Hayes et al., 1990). However, the assay was performed with one *Escherichia coli* strain (SA500) as well as in the absence of metabolic activation only. The Panel considered that the methods implemented were not sufficiently robust to support the results reported.

Ohno et al. (2005) developed a genotoxicity test system based on p53R2 gene expression in human cells. The assay was based on examination of the chemical activation of the p53R2 gene, which was assessed by the incorporation of a p53R2-dependent luciferase reporter gene into two human cell lines, MCF-7 and HepG2. The Panel considered that the methods implemented were sufficiently robust to support the results reported, but not adequate for the present evaluation, because the endpoint used was not relevant for the mechanism of action of non-radiomimetic chemical compounds.

McGregor et al. (1988) aimed to define the optimal conditions for the metabolic activation of chemicals to mutagens in the L5178Y/TK+/- Mouse Lymphoma Assay using different pro-mutagenic compounds. Methanol was assessed for its mutagenicity at 5-50 µL/mL (approximately 3.95-39.5 mg/mL). In the presence of 2.5 mg S9/mL, 10 µL methanol/mL exhibited a mutagenic effect, relative total growth (RTG) was reduced to less than 30 %. Furthermore, increasing the concentration of the S9 mix to 7.5 mg/mL reduced RTG to about 15 % but increased the mutant fraction from 60 in the control to 650 mutants/10^6 at 5 µL methanol/mL. However, the Panel noted that the lowest concentration tested (5 µL/mL) exceeded in terms of molarity (13.4 M) the maximum concentration recommended in OECD guideline 476 (5 mg/mL, 5 µL/mL, or 0.01 M, whichever is the lowest). Therefore, indirect effects due to non-physiological culture conditions at all concentrations tested

cannot be excluded. The Panel considered that the methods implemented were not sufficiently robust to support the results reported.

DeMarini et al. (1991) examined methanol in two independent microscreen prophage-induction assays with *Escherichia coli* WP2 (λ). Five methanol concentrations ranging from 0.15 up to 5 % (i.e. from 37 mM to 1.23 M; assuming the concentrations are expressed as %, v/v), which included toxic levels, did not result in prophage-induction, without and with metabolic activation. The Panel considered that the methods implemented were sufficiently robust to support the results reported.

Hamada et al. (1988) examined methanol (2-8 %*;* 0.49-1.97 M)) for induction of gene mutations in *Saccharomyces cerevisiae* ATCC26422 as a control culture within a study which aimed at elucidating the effects of ethidium bromide. Methanol did not induce gene mutations, but was only tested without a metabolic activation system. The Panel considered that the methods implemented were not sufficiently robust to support the results reported.

In a survey by Shimizu et al. (1985) which aimed to investigate the genotoxicity of forty-three industrial chemicals, methanol was assessed for its mutagenic potential in the Ames test with the *Salmonella typhimurium* tester strains TA1535, TA1537, TA1538, TA98, TA100 and *Escherichia coli* WP2 uvr A, using the preincubation method, both in the absence and presence of rat liver S9 metabolism. Dose-levels of 5, 10, 50, 100, 500, 1000, 5000 µg/plate were used. The Panel considered that the methods implemented were sufficiently robust to support the results reported. Methanol was not mutagenic in this test system either in the absence or in the presence of S9 metabolic activation system.

In the study by Lasne et al. (1984) which aimed to compare the sensitivity of the SCEs and *in vitro* micronucleus test assays to detect cytogenetic effects induced by different chemical agents, methanol was assessed for its potential clastogenicity for the induction of micronuclei in Chinese hamster V-79 cells at a single dose-level of 50 µL/mL for 48 hours in the absence of S9 metabolism only. Results obtained indicate that methanol did not induce micronuclei, under the reported experimental condition. However, the Panel noted that the treatment time used was exceedingly long compared to the 1.5-2 cell-cycle length recommended by the OECD test guideline 487 for the testing of chemicals. Furthermore, the dose-level administered was 10 times greater than the maximum recommended dose-level to be used in *in vitro* studies (i.e. 5 µL/mL) to keep culture physiological conditions. On these grounds, the Panel considered that the methods implemented were not sufficiently robust to support the results reported.

In vivo studies

Pregnant CD-mice were administered by gavage twice daily with 2500 mg methanol/kg bw on GD 6-GD 10 (Fu et al*.*, 1996). No evidence of methanol-induced formation of micronuclei in the blood of fetuses or mothers was found. However, the Panel noted that blood samples were collected eight days after last administration of the compound. Normal procedures indicate sampling not later than 72 hours, Therefore this study was not taken into further consideration (Appendix H).

NEDO (1987) carried out an *in vivo* micronucleus test in 6 male SPF mice/group who received a single gavage dose of 1050, 2110, 4210, and 8410 mg methanol/kg bw. Twenty-four hours later, 1000 cells were counted for micronuclei in bone marrow smears. The Panel considered that the methods implemented were sufficiently robust to support the results reported. At any dose level, methanol did not induce micronuclei significantly compared with the vehicle control (i.e. sterilized distilled water).

In a recent study, DNA adducts were measured in several organs after oral administration to rats by gavage of the stable isotope methanol ([$^{13}CD_4$]-methanol) in doses of 500 mg/kg bw and 2000 mg/kg bw per day for 5 days (Lu et al., 2012). The method can distinguish between adducts formed by endogenous substances and exogenous agents. It has already been used to study formaldehyde adducts in the nose and at distant sites after inhalation exposure to rats (Lu et al., 2010; Lu et al., 2011) and to

cynomolgus macaques (Moeller et al., 2011). The data of the study (Lu et al., 2012) show that labelled formaldehyde arising from [$^{13}CD_4$]-methanol induced N2-hydroxymethyl-dG DNA adducts in increasing numbers with increasing dose in all tissues, including liver, but in particular in the bone-marrow. The number of exogenous DNA adducts was lower than the number of endogenous hydroxymethyl-dG adducts in all tissues of rats. The ratio of exogenous/endogenous dG adducts was 0.18 and 0.45 in bone-marrow and 0.056 and 0.2 in the liver after doses of 500 mg/kg bw and 2000 mg/kg bw of stable isotope methanol respectively after adjusting for isotopic effects in the metabolism of [$^{13}CD_4$]-methanol.

The Panel identified some concerns about the lower number of exogenous hydroxymethyl DNA adducts measured in the analysed tissues of rats following administration of [$^{13}CD_4$]-methanol at 500 mg/kg bw for 5 days compared to the number of endogenous hydroxymethyl DNA adducts. A previous study has shown a slower metabolic conversion of deuterium-labelled methanol compared to the unlabelled one (Brooks and Shore, 1971; Kraus and Simon, 1975). The Panel noted that the isotope effect factor employed was the mean of several widely varied published values. Furthermore, the measurements of N-hydroxymethyl-dG and dA adducts was not direct but required an *in vitro* reduction process with $NaCNBH_3$ to the corresponding N-methyl derivative. Based on the authors' comments, this procedure appeared not to be quantitative as the rate of reduction was in the range of 65-85 %. Furthermore, deuterium could have been exchanged by the hydride during the reduction steps, which would have allowed for depletion of the labelled material and therefore led to an underestimation of the number of exogenous DNA adducts. The Panel noted that the measurements of endogenous N-hydroxymethyl-dA adducts in the untreated animal group and in methanol treated group at 500 mg/kg bw for 5 days were rather variable especially in the case of white blood cells, bone-marrow and brain cells.

Furthermore, the Panel noted that adduct formation is a biomarker of exposure of organs and tissues to methanol and that a second step is necessary between DNA adduct formation and mutagenic events (Swenberg et al., 2008; Jarabek et al., 2009). Moreover, the Panel noted that doses applied in the study were in the lethal range for humans. Therefore, the Panel considered that the methods implemented were not sufficiently robust to support the results reported, and that no conclusion could be drawn from the study.

No increase in sex-linked lethal mutations in either the wild type or the Basc strain of *Drosophila melanogaster* was found after methanol feeding (1000 mM) (Gocke et al., 1981).

Pereira et al. (1982) reported that 'the oral administration in mice of methanol (1000 mg/kg), increased the incidence of chromosomal aberrations particularly aneuploidy and exchanges and the micronuclei of polychromatic erythrocytes'.The study was available as an abstract only, without data to support the statement of the authors. Therefore the Panel was not able to reach a conclusion.

Ward et al. (1983) reported an increase in chromosome aberrations (exchanges (Robertsonian translocation), aneuploidy) in male B6C3F1 mice upon a single dose of 1000 mg methanol/kg bw). However, the Panel noted that the concurrent vehicle control showed 10 exchanges out 665 cells scored. Since chromatid and chromosome exchanges are not expected in healthy animals from this strain the Panel considered that the methods implemented were not sufficiently robust to support the results reported.

In vivo studies by other routes of exposure (inhalation and intraperitoneal administration)

Although the Panel noted that inhalation studies and intraperitoneal administration provide routes of exposure that are different from oral exposure, once methanol is absorbed, it is rapidly distributed to all organs and the tissue/blood concentration ratios should be similar (Cruzan, 2009). Therefore, similar effects from all three routes of exposure can be expected. Consequently, four *in vivo* reports using inhalation or the intraperitoneal route of administration (Gocke et al., 1981; Campbell et al.,

1991; McCallum et al., 2011a; 2011b) were considered by the Panel as valid studies and useful for a weight-of-evidence (WOE) approach in the assessment of the genotoxic potential of methanol.

Gocke et al. (1981) examined the potency of methanol to induce micronuclei formation in bone marrow upon intraperitoneal injection. Two male and two female NMRI mice were given 1920, 3200 or 4480 mg methanol/kg bw as single intraperitoneal injections. No significant increase in the number of micronucleated polychromatic erythrocytes was observed. The Panel considered that the methods implemented were sufficiently robust to support the results reported.

In the study by Campbell et al. (1991), ten male C57BL/6J mice/group were exposed by inhalation to 0, 800, or 4000 ppm (0, 1048, and 5242 mg/m^3) methanol, six hours/day, for five days. At sacrifice, blood cells were examined for the formation of micronuclei. In addition, primary lung cells cultures established following perfusion of lung with 0.13 % trypsin, 0.01 % EDTA and 50 U of collagenase/mL, were examined for SCEs, CA and micronuclei. Furthermore, the synaptonemal complex damage in meiotic prophase nuclei obtained from teasing seminiferous tubules and subsequent fixation of cells was analysed. All endpoints studied gave negative results. However, the Panel noted that cytogenetic analyses in primary lung cultures are not validated procedures and, more specifically, the spontaneous incidences of micronuclei, SCEs and chromosomal aberrations were unexpectedly very high for primary cell cultures (23.2 %, 11.2 % and 6.9 % respectively in experiment 1). Furthermore, the absence of a concurrent positive control strongly biased the negative outcome of the study. The Panel considered that the methods implemented were sufficiently robust to support the results reported.

Groups of 10 male Swiss-Webster mice which had been kept on either a normal or a folate-deficient diet for 9 weeks before dosing, were given intraperitoneal methanol doses of 300, 600, 1200 or 2500 mg/kg bw on 4 consecutive days. 24 hours after the last dose, the incidence of micronuclei in the RNA-positive erythrocytes of the animals was determined. The treatment with methanol did not significantly increase the incidence of micronuclei above the control values (O'Loughlin et al., 1992).

McCallum et al. (2011a; 2011b) investigated the potential indirect DNA-damaging activity of methanol by a reactive oxygen species-mediated mechanism. Male CD-1 mice (9-13 weeks old), New Zealand white rabbits (5 months old) and cynomolgus monkeys (3.4-5.7 years) received 2000 mg methanol/kg bw by intraperitoneal injection. Tissue oxidative DNA damage was assessed 6 hours post-dose, measured as 8-hydroxy-2'-deoxyguanosine (8-oxodG) by HPLC with electrochemical detection. The results obtained showed no methanol-dependent increases in 8-oxodG in bone marrow or spleen (McCallum et al., 2011a) or lung, liver or kidney (McCallum et al., 2011b) in any species. Chronic treatment of CD-1 male mice with 2000 mg/kg bw of methanol daily by intraperitoneal injection for 15 days also did not increase 8-oxodG levels in bone marrow or spleen (McCallum et al., 2011a) or lung, liver or kidney (McCallum et al., 2011b). To further assess the possibility that the formation of 8-oxodG could have been enhanced by methanol, but rapidly removed via excision repair, DNA repair-deficient mice lacking oxoguanine glycosylase 1 (Ogg1-deficient mice) were treated with methanol at 2000 mg/kg once by intraperitoneal injection and tissues and organs collected 6 and 24 hours later and analysed for oxidative DNA damage. The results obtained indicated an accumulation of 8-oxodG levels in the untreated Ogg1-deficient mice compared to the wild type mice, but no evidence for methanol-induced oxidative DNA damage was observed in lung, liver or kidney despite increases in 8-oxodG levels by the positive control potassium bromate (McCallum et al., 2011b) or in bone marrow or spleen (McCallum et al., 2011a). In the study by McCallum et al. (2011b), the potential induction of 8-oxodG formation by methanol was further assessed in fibroblasts from Ogg1-deficient mice treated with methanol *in vitro*. The results obtained indicated an accumulation of 8-oxodG levels following a 3-hour treatment with the renal carcinogen potassium bromate (2.0 mM); however, the fibroblasts did not accumulate 8-oxodG following exposure to 125 mM methanol for 6 hours. The Panel noted that in this *in vitro* assessment, methanol was administered at a dose-level far exceeding the recommended maximum dose-level of 10 mM to keep physiological treatment conditions (OECD test guidelines 483 and 487) In addition, free radical-mediated hydroxynonenal-histidine protein adducts were reported not to be enhanced by methanol in

primate bone marrow or spleen, or in rabbit bone marrow or mouse spleen, although modest increases were observed in rabbit spleen and mouse bone marrow. The authors concluded that taken together these observations suggest that methanol exposure did not promote the accumulation of oxidative DNA damage in lung, kidney or liver (McCallum et al., 2011a) or in bone marrow and spleen (McCallum et al., 2011b) in any species investigated including Ogg1–deficient mice. The Panel considered that the methods implemented were sufficiently robust to support the results reported and agreed with the conclusions of the authors.

5.2.3.1. Conclusion on the genotoxicity of methanol

The *in vitro* genotoxicity data on methanol which were considered as relevant for the evaluation comprise a negative bacterial reverse mutation test, a negative gene mutation test with *Schizosaccharomyces pombe* and a negative prophage induction test with *Escherichia coli* WP2(λ).

The *in vivo* genotoxicity data upon oral uptake on methanol considered as relevant for the evaluation comprise a negative mouse micronucleus test in bone marrow. In addition, in studies following intra peritoneal application or inhalation exposure of mice, rabbits or monkeys, no evidence of induction of DNA-damage (8-oxodG) formation, micronuclei and SCE was found.

In a weight-of-evidence approach, the Panel also considered *in vivo* studies with inhalation and intraperitoneal applications. Taken together the Panel concluded that the data set is limited but that the available reliable *in vitro* and *in vivo* data did not indicate a genotoxic potential of methanol.

Overall, the Panel concluded that available data do not indicate a genotoxic concern for methanol.

Summary tables on the genotoxicity of methanol are presented in the Appendix H.

5.2.4. Chronic toxicity and carcinogenicity of methanol

Methanol is a breakdown product of aspartame and was considered by the SCF in its safety assessment of aspartame (SCF 1985; 2002). The SCF concluded that there was no reason of concern regarding the chronic toxicity and carcinogenicity over the amounts of methanol likely to be produced by the metabolism of aspartame when compared with those present naturally in food (SCF, 1985). However, Soffritti and co-workers in their study recently proposed a role for methanol in the potential carcinogenic effects of aspartame (Soffritti et al., 2010). Although the ANS Panel had previously concluded that the results of Soffritti did not provide evidence for a carcinogenic effect of aspartame in mice, the Panel decided to present a more detailed analysis of the suggested chronic toxicity and carcinogenicity of methanol (EFSA ANS Panel, 2011). Only oral chronic and carcinogenicity studies of methanol have been considered for this evaluation.

The oral studies on chronic toxicity and carcinogenicity of methanol are limited to a mouse study reported by Apaja (1980) and a rat study reported by Soffritti et al. (2002).

The study by Apaja (1980) is part of a PhD thesis that investigated the effect of malonaldehyde bis-dimethylacetal that decomposes to malonaldehyde and methanol and aimed at studying the carcinogenic effect of malonaldehyde. The study design contained groups dosed with methanol but no concurrent unexposed controls. In the study, male and female Eppley Swiss Webster mice (25/sex/dose group; 8 weeks old at study initiation) were exposed 6 days per week until natural death to 0.222 %, 0.444 % and 0.889 % methanol in drinking water (Apaja, 1980). Although no unexposed control group was included in the study, the author provided pathology data from historical records of untreated Swiss mice of the Eppley colony used in two separate chronic studies, one involving 100 untreated males and 100 untreated females (Toth et al., 1977) and the other involving 100 untreated females histopathological analyzed by Apaja (Apaja, 1980). Mice were housed five per plastic cage and fed Wayne Lab-Blox pelleted diet. Water was available *ad libitum* throughout life. Liquid consumption per animal was measured 3 times a week. The methanol doses in the drinking water study were reported as 22.6, 40.8 and 84.5 mg/day (560, 1000 and 2100 mg/kg bw/day) for females,

and 24.6, 43.5 and 82.7 mg/day (550, 970, and 1800 mg/kg bw/day) for males, 6 days/week. The animals were checked daily and body weights were monitored weekly. Test animals were sacrificed and necropsied when moribund. The experiment ended at 120 weeks with the death of the last animal.

The author reported that survival of the methanol-exposed females in the drinking water study was lower than that of untreated historical controls ($p < 0.05$), but no significant differences in survival were noted for males. An increase in liver parenchymal cell necrosis was reported in the male and female high dose groups, with the incidence in females (8 %) being significant ($p < 0.01$) relative to untreated historical controls. Incidence of acute pancreatitis was higher in high dose males ($p < 0.001$), but did not appear to be dose-related in females, increasing at the mid ($p < 0.0001$) and low doses ($p < 0.01$) when compared to historical controls but not appearing at all in the high dose females. Significant increases relative to untreated historical controls were noted in amyloidosis of the spleen, nephropathy and pneumonia, but the increases did not appear to be dose-related.

The author reported incidences of malignant lymphomas in females of 560, 1000, and 2100 mg/kg bw/day of 4/25 (16 %), 9/25 (36 %), and 10/25 (40 %), respectively. Males from the drinking water study had incidences of malignant lymphoma of 1/25 (4 %), 6/25 (24 %), and 4/25 (16 %) at 550, 970, and 1800 mg/kg bw/day respectively. The lymphomas were classified according to Rappaport's classification (Rappaport, 1966), but it was not clear from the paper whether the lymphomas were generalised or whether they were restricted to one organ. The author indicated that the incidences in both males and females were 'within the normal range of occurrence of malignant lymphomas in Eppley Swiss mice'. However, elsewhere in the document, Apaja states that 'there was an increased ($p < 0.05$ in both) occurrence of malignant lymphomas (40.0 % and 24.0 % of the effective animals respectively) compared to historical data of untreated controls (Table 9)'. The latter table reports the historical control values of Toth et al. (1977).

The Panel noted the lack of a concurrent control group and the small number of animals used per dose group. In particular, the Panel noted that even though the incidence of malignant lymphomas in the high dose group appears higher than the historical data reported by Apaja (1980), a comparison with other contemporary control groups from the same institute and from the same colony of mice (e.g. Cabral et al., 1979) shows that the background lymphoma incidence in untreated animals can be as high as 26 % for male and 43 % for female mice. Furthermore, the study was inadequately described in that tumour nomenclature and description were poorly defined, and there was a lack of individual animal results regarding tumour incidence and time of death. Finally, the Panel noted that the animals were not maintained under pathogen-free conditions and this is reflected by an overall incidence rate of pneumonia of approximately 22 % (Apaja, 1980). Overall, the Panel concluded that the study by Apaja is inadequate for the assessment of the carcinogenic potential of methanol.

Soffritti et al. (2002) reported a chronic study in which methanol was given to 100 Sprague-Dawley rats/sex/group *ad libitum* in drinking water at concentrations of 0, 500, 5000, and 20 000 ppm (v/v). The study was published as a non-peer-reviewed paper. The animals were 8 weeks old at the onset of the study. All rats were exposed for up to 104 weeks, and after that maintained until they died naturally. The experiment ended at 153 weeks with the death of the last animal. Mean daily drinking water, feed consumption, and body weights were monitored weekly for the first 13 weeks, every 2 weeks thereafter for 104 weeks, then every 8 weeks until the end of the experiment. Clinical signs were monitored 3 times/day, and the occurrence of gross changes was evaluated every 2 weeks. All rats were necropsied at death and underwent histopathological examination of organs and tissues.

No substantial dose-related differences in survival were observed, although the data were not provided. Body weight, water and feed consumption were monitored in the study, but the data were not documented in the published report. However, average doses of 0, 55, 542 and 1840 mg/kg bw/day in males and 0, 67, 630 and 2250 mg/kg bw/day in females were calculated by Cruzan (2009) from drinking water concentrations of 0, 500, 5000, and 20 000 ppm and data made public by the ERF.

Water consumption in high dose females was reduced compared to controls between 8 and 56 weeks and the mean body weight in high dose males tended to be higher than that of control males. Overall, there was no pattern of compound-related clinical signs of toxicity, and the available data did not provide any indication that the control group was not concurrent with the treated group (Cruzan, 2009). Soffritti et al. (2002) further reported that there were no compound-related signs of gross pathology or histopathological lesions indicative of non-cancer toxicological effects in response to methanol.

The study reported a number of oncogenic responses to methanol including haemolymphoreticular neoplasms, the majority of which were reported to be lympho-immunoblastic lymphomas (Table 21). In the ERF bioassays, including this methanol study, haemolymphoreticular neoplasms are generally divided into specific histological types (lymphoblastic lymphoma, lymphoblastic leukaemia, lymphocytic lymphoma, lympho-immunoblastic lymphoma, myeloid leukaemia, histocytic sarcoma, and monocytic leukaemia) for identification purposes. According to Soffritti et al. (2007), the overall incidence of haemolymphoreticular tumours (lymphomas/leukaemias) in ERF studies is 13.3 % (range, 4.0–25.0 %) in female historical controls (2274 rats) and 20.6 % (range, 8.0–30.9 %) in male historical controls (2265 rats). The high dose responses, reported in the methanol study amounted to 28 % ($p < 0.05$) and 40 % (not significant) for females and males, respectively. The study also suggested a significant increase in the incidence of ear duct carcinoma.

In 2010, US Environmental Protection Agency (EPA) proposed that the chronic cancer study on methanol performed in rats by Soffritti et al. (2010) should be subjected to an independent pathology review following discrepancies in the diagnosis identified during a site visit to ERF by pathologists from NTP conducted in 2010. The EPA commissioned the NTP to conduct this pathology review. The NTP pathologists diagnosed fewer lymphoid neoplasms, mainly of the respiratory tract, and fewer neoplasms of the inner ear and cranium and noted that there was chronic inflammation of the nasal cavity, ear canal, trachea, and lung, indicating infection of the animals by one or more respiratory pathogens. EPA concluded that many of the malignant neoplasms and the lymphoid dysplasias diagnosed by the ERF pathologists were cases of hyperplasia related to chronic infection (Table 21, NTP-EPA, 2011).

In view of the concerns identified by EPA, the Panel did not support the validity of the conclusions in studies reported by Soffritti and colleagues.

Table 21: Summary Incidences of Malignant Lymphoma or Leukaemia in Male and Female Rats

	0 ppm[c]		500 ppm		5000 ppm		20 000 ppm	
MALES								
Number of animals per group	100		100		100		98-100	
	ERF	PWG	ERF	PWG	ERF	PWG	ERF	PWG
Lung only	7	0	10	0	13	1	13	0
Multiple tissues	19	13	21	11	22	12	26	8
Total rats with Lymphoma[a]/Leukaemia[b]	26	13	31	11	35	13	39	8
FEMALES								
Number of animals per group	100		100		100		99-100	
	ERF	PWG	ERF	PWG	ERF	PWG	ERF	PWG
Lung only	3	3	5	0	6	0	7	0
Multiple tissues	9	5	16	4	16	7	18	6
Total rats with Lymphoma/Leukaemia	12	8	21	4	22	7	25	6

ERF = European Ramazzini Foundation study diagnosis; PWG = Pathology Working Group EPA Panel consensus
[a] 'Lymphoma' includes lymphoblastic lymphoma, lymphocytic lymphoma, lymphoimmunoblastic lymphoma for the ERF diagnoses. For QA pathologist and PWG, all lymphoma sub types were diagnosed as malignant lymphoma.
[b] 'Leukaemia' includes lymphoblastic leukaemia and myeloid leukaemia for SD diagnoses and for QA pathologist and PWG, includes myeloid leukaemia and mononuclear cell leukaemia.
[c] Average doses of 0, 55, 542 and 1840 mg/kg bw/day in males and 0, 67, 630 and 2250 mg/kg bw/day in females were calculated by Cruzan (2009) from drinking water concentrations of 0, 500, 5000, and 20 000 ppm and data made public by the ERF.

5.2.5. Reproductive and developmental toxicity of methanol

A few studies on oral reproductive and developmental toxicity of methanol were reported. In most of the studies, especially in the rat, limitations were observed (maternal toxicity not described, limited number of animals and dose levels tested, time of dosing).

Four studies were identified on the reproductive and developmental toxicity of methanol in mice via the oral route.

5.2.5.1. Reproductive toxicity

Mice

Four month old male $B_6C_3F_1$ mice were dosed by gavage with 0 (n = 5) or 1000 (n = 10) mg/kg bw/day methanol in water for 5 days (Ward et al., 1984). An increase in the number of mice with 'banana-type' sperm morphology was reported. The total number of sperm abnormalities was not significantly increased in the methanol group. The Panel noted that these effects were considered of unknown biological significance as fertility (by mating of the animals) was not tested and general toxicity was not described.

5.2.5.2. Developmental toxicity

Mice

In mice, one prenatal toxicity study by inhalation, in which also one high oral dose was tested, and two oral prenatal developmental studies at high dose levels were identified and are summarised below.

Rogers et al. (1993) dosed pregnant CD-1 mice (n= 70, 26, 41, 40, 15, 11, 6) with 0, 1000, 2000, 5000, 7500, 10 000 and 15 000 ppm (equivalent to approximately 0, 1300, 2600, 6500, 9750, 13 000 and 19

500 mg methanol/m^3) by inhalation on GD 6-15 for 7 hours/day. Increased incidences of exencephaly and cleft palate were found in the offspring at 6500 mg/m^3 (5000 ppm) or higher. There was increased embryo/fetal death at 9750 mg/m^3 (7500 ppm) or higher and an increased incidence of full-litter resorptions. Reduced fetal weight was observed at 13 000 and 19 500 mg/m^3 (10 000 and 15 000 ppm). A dose-related increase in cervical ribs was significant at doses $\geq$ 2600 mg/m^3 (2000 ppm). The no observed adverse effect concentration (NOAEC) for developmental toxicity was 1300 mg/m^3 (1000 ppm) methanol. There was no evidence of maternal toxicity at methanol exposure concentrations below 9750 mg/m^3 (7500 ppm). To evaluate the significance of route of exposure, a study was conducted in which pregnant mice were dosed twice a day with 2 g methanol/kg bw/day (4 g daily) by oral gavage. Plasma methanol levels on GD 6 and GD 15 were comparable in the 10 000 ppm inhalation group and the 4000 mg/kg bw/day oral group. Incidences of resorptions, external defects including cleft palate, and decreases in fetal weight were similar to those found in the 13 000 mg/m^3 (10 000 ppm) inhalation exposure group, presumably due to the greater rate of respiration of the mouse. The Panel considered the NOAEC of 1000 ppm based on the cervical rib effects as conservative as these effects tend to disappear as the pups grow and are often observed in inhalation studies. From this concentration, an oral dose of approximately 560 mg/kg bw/day was calculated by the Panel according to the method of Alexander et al., 2008[20].

Both Fu et al. (1996) and Sakanashi et al. (1996) studied the influence of dietary folic acid on the developmental toxicity of methanol.

In the study of Fu et al. (1996), virgin female CD-1 mice were fed a standard diet (containing 1200 nmol folic acid (FA)/kg) or diet deficient in FA (400 nmol FA/kg referred to as 'marginal'). Both of these diets contained 1 % succinylsulfathiazole (to prevent endogenous synthesis of folate by the intestinal flora). The animals were fed these diets for 5 weeks prior to mating and throughout breeding and gestation. From GD 6-10 dams were given by gavage deionised, distilled water or methanol (MeOH, 15.65 % Optima HPLC grade MeOH) at 2.5 g/kg body weight, twice daily. On GD 18, mice were weighed and killed and the liver, kidneys, and gravid uteri removed and weighed (21-24 litters/group). Implantation sites, live and dead fetuses, and resorptions were counted; fetuses were weighed individually and examined for cleft palate and exencephaly. The marginal FA dietary supply alone resulted in low maternal liver (50 % reduction) and red cell folate (30 % reduction) concentrations, as well as low fetal tissue folate concentrations (60 to 70 % reduction) relative to the standard FA diet groups. In addition, marginal FA supply alone resulted in cleft palate in 13 % of the litters; there were no litters affected with cleft palate in the standard FA diet group. Marginal FA-MeOH treatment resulted in a further increase in the litters affected by cleft palate (72 % of litters affected). The percentage of litters affected by exencephaly was highest in the marginal FA-MeOH group. The authors considered that the results show that marginal folate deficiency in pregnant dams significantly increases the teratogenicity of MeOH.

Sakanashi et al. (1996) fed Crl: CD-1 mice a purified, amino acid-based folic acid-free diet fortified with either 400, 600, or 1200 nmol/kg diet folic acid commencing 5 weeks prior to mating and throughout breeding and gestation. All diets contained 1 % succinylsulfathiazole to prevent endogenous synthesis of folate by intestinal flora. On GD 6-15, mice were gavaged twice daily with water or methanol (purity not specified) in water at 2000 or 2500 mg/kg bw for a total daily dose of 0, 4000 or 5000 mg/kg bw. On GD 18, dams were weighed and killed and the liver, kidneys, and gravid uteri removed and weighed. Implantation sites, live and dead fetuses, and resorptions were counted; fetuses were weighed individually and examined for cleft palate and exencephaly. One third of the fetuses in each litter were examined for skeletal morphology. Twelve to 29 litters were examined per group; in the low folate/2000 mg methanol group only 3 litters were examined. Methanol treatment decreased gestational weight gain in groups fed diets containing 600 or 1200 nmol folic acid/kg diet; these effects were not seen in the 400 nmol/kg group. Methanol did not affect pregnancy or implantation rate. There was no consistent effect of methanol exposure on haematocrit or liver folate

[20] Definition extracted from Alexander et al. (2008). Daily dose [mg/kg bw]= 0.608* Concentration [mg/L]* Duration [minutes]/$BW^{0.148}$ [kg]; 1000 ppm (1.32 g/m^3)= 0.608*1.32*7*60/$0.035^{0.148}$ = 562 mg/kg bw

level; plasma folate was increased in mice from the 1200 nmol/kg group that received 5000 mg/kg/day methanol. Methanol decreased fetal body weight in each of the folic acid dietary groups. An increase in the litter incidence of cleft palate was seen with methanol treatment in all dietary groups; the incidence was exacerbated in the 400 nmol/kg group. The litter incidence of exencephaly was increased by exposure to methanol in the 400 nmol folic acid/kg group. Methanol increased anomalies affecting the cervical region, although the incidence tended to decrease in dietary groups receiving larger amounts of folic acid. The authors concluded that the developmental toxicity of methanol was enhanced when maternal folic acid stores were low.

The Panel concluded from the latter two studies, that the effects found on developmental toxicity were related to methanol. However, it is not proven that the enhancement of the developmental effect was due to the deficiency in folate as the effect described can also be related to malnutrition as also described in the NTP-CERHR 2003.

Rats

In rats, one study by inhalation and three oral prenatal developmental toxicity studies were identified and are summarised below.

In the study by Nelson et al. (1985), the inhalation of methanol by pregnant Sprague-Dawley rats (n=15-16) throughout the period of embryogenesis induced a wide range of concentration-dependent teratogenic and embryo lethal effects. Treatment–related malformations, predominantly extra or rudimentary cervical ribs and urinary or cardiovascular defects, were found in fetuses of rats exposed 7 hours/day to 26 000 mg/m^3 (20 000 ppm) methanol during GD 7-15. Maternal toxicity (decreased weight gain and feed intake) during the first week of exposure was found at this exposure level. No adverse effects to the mother of offspring were found in animals exposed to 6500 mg/m^3 (5000 ppm) 7 hours/day from GD 1-19. The authors considered this dose as the NOAEC in this study (Nelson et al., 1985). The Panel agreed with this conclusion. From this concentration, an oral dose of approximately 2070 mg/kg bw/day was calculated by the Panel according to the method of Alexander et al., 2008.

The reproductive toxicity of methanol was studied by Infurna and Weiss (1986) in an experiment where Long-Evans rats were divided into 3 groups of 10 animals. Two of the groups consumed drinking solutions of 2 % v/v methanol, one from GD 15-17 and the other from GD 17-19. The control group received distilled water. No maternal toxicity was apparent, as measured by weight gain, gestational duration, and daily fluid intake. To assess maternal and fetal toxicity the following variables were recorded during gestation and throughout the pre-weaning period: weight gained during the 3rd week of gestation, daily fluid intake during this period, duration gestation, birth weight and body weight on postnatal days 0, 7, 14, and 20, day of eye opening, maternal behaviour following parturition, and litter size. Postnatal behavioural evaluations began the day after birth by testing the suckling behaviour of rat pups from all 3 groups on day 1 and nest seeking behaviour on day 10. The only observed effect, in methanol-dosed groups, was a significant impairment in suckling behaviour measured as mean latency of attachment to the nipple during a 2-minute test. This was the only observed effect but the authors concluded that these data suggest that prenatal methanol exposure induces behavioural abnormalities early in life, since no other signs of toxicity was apparent either in the mothers or the offspring (Infurna and Weiss, 1986). The Panel did not agree with this conclusion because of the limited usefulness of this test in isolation.

Cummings (1993) demonstrated that in the rat (n = 8), 3.2 g methanol/kg bw/day administered by gavage from day 1 to day 8 of gestation was associated with a range of maternal toxic effects and fetotoxic effects. The maternal toxicity was characterized by a reduction in uterine weight on GD 9 that represents an inhibition of the growth of decidualized tissue in the treated animals. At 3.2 g/kg/day, the number of atypical sites (defined as implantation sites, often small, exhibitimg adjacent extravasation of blood) was significantly increased at GD 9 but not at GD 11 and GD 20. When dams were killed on GD 20, there was no evidence of fetal resorptions and no effects on

maternal ovary weight and corpora lutea were observed. Methanol did not affect either uterine weight or maternal body weight on GD 20.

Long-Evans rats (n = 10-13/group) received by gavage a single dose of methanol of 1.3, 2.6, or 5.2 ml/kg bw (corresponding to 1023, 2045, or 4090 mg/kg bw/day according to CERHR calculation) on GD 10 (Youssef et al., 1997). Signs of maternal toxicity were only seen in the highest dose group and included decreased body weight gain and feed intake. Fetal body weights were significantly reduced in all treatment groups, but not in a dose-dependent manner. Dose related-anomalies were undescended testes and eye defects, which reached statistical significance in the high dose group, and facial haemorrhage and dilated renal pelvis. The Panel considered this study to be of limited relevance due to the timing of the single dose, which was not at what is considered the most sensitive period for developmental toxicity of methanol.

The Panel noted that the design of the oral rat studies was inadequate. In one study (Youssef et al., 1997) rats were only administered once and not during the most sensitive period for induction of developmental effects. In two other studies in rats the duration of the administration period (GD 15-17 and GD 17-19 or GD 1-8) did not cover the period of organogenesis (Infurna and Weiss, 1986 and Cummings, 1993).

Monkeys

In a study by Burbacher et al. (2004), Macaque monkeys (11-12 females per dose group) were exposed to 0, 200, 600 or 1800 ppm methanol vapour for 2.5 hours/day for 7 days a week (corresponding to oral doses of 0, 21, 61 and 185 mg/kg bw/day (Alexander et al., 2008)) prior to breeding and throughout pregnancy (approximately 120 days). The mothers remained healthy during the study and tests to evaluate motor incoordination, vision loss, and/or respiratory effects were negative. Methanol exposure did not affect menstrual cycles, number of matings to conception or conception rate. The mean length of pregnancy was significantly reduced by 6-8 days in treated animals compared to controls but the reduction was not dose-related. In addition, although not statistically significant, five methanol treated females (in different dose groups) were Caesarean-sectioned due to pregnancy complications. No methanol-related effects were apparent on the birth weight or health of the offspring (Burbacher et al., 2004). The Panel considered that it was not possible to identify an effect level from this study.

The Panel concluded that mice were more sensitive than rats to the developmental toxic effects of methanol by inhalation, as was found by Rogers et al. (1993). A contributing factor to this species difference may be the difference in their respiratory frequencies. Blood levels reported in the studies of Nelson et al. (1985) in rats were lower than those reported for mice at the same exposure levels and regimen. Oral studies were only performed at high dose levels of 4000 or 5000 mg/kg bw/day and no NOAELs could be derived from these studies. Therefore, a NOAEL for oral exposure was calculated from the studies as described by Alexander et al. (2008). The Panel considered 1000 ppm as the NOAEC in mice in the study of Rogers et al. (1993), and 5000 ppm as a NOAEC in rats in the study of Nelson et al. (1985). From these exposure concentrations by inhalation, oral dose levels of 560 and 2070 mg/kg bw/day respectively, were calculated by the Panel. The overall NOAEL of 560 mg/kg bw/day is considered as conservative by the Panel.

6. Discussion of methanol toxicity database

When ingested, methanol is subject to a significant first pass metabolism. Principally, methanol (CH_3OH) metabolism proceeds by stepwise oxidation via formaldehyde (HCHO) to formate (HCOOH), and then to carbon dioxide (CO_2)via different metabolic pathways.

Formaldehyde and formate can also enter the one carbon metabolic pool through tetrahydrofolic acid and from there, may contribute to the biosynthesis of purines and pyrimidines (Figures 3 and 4).

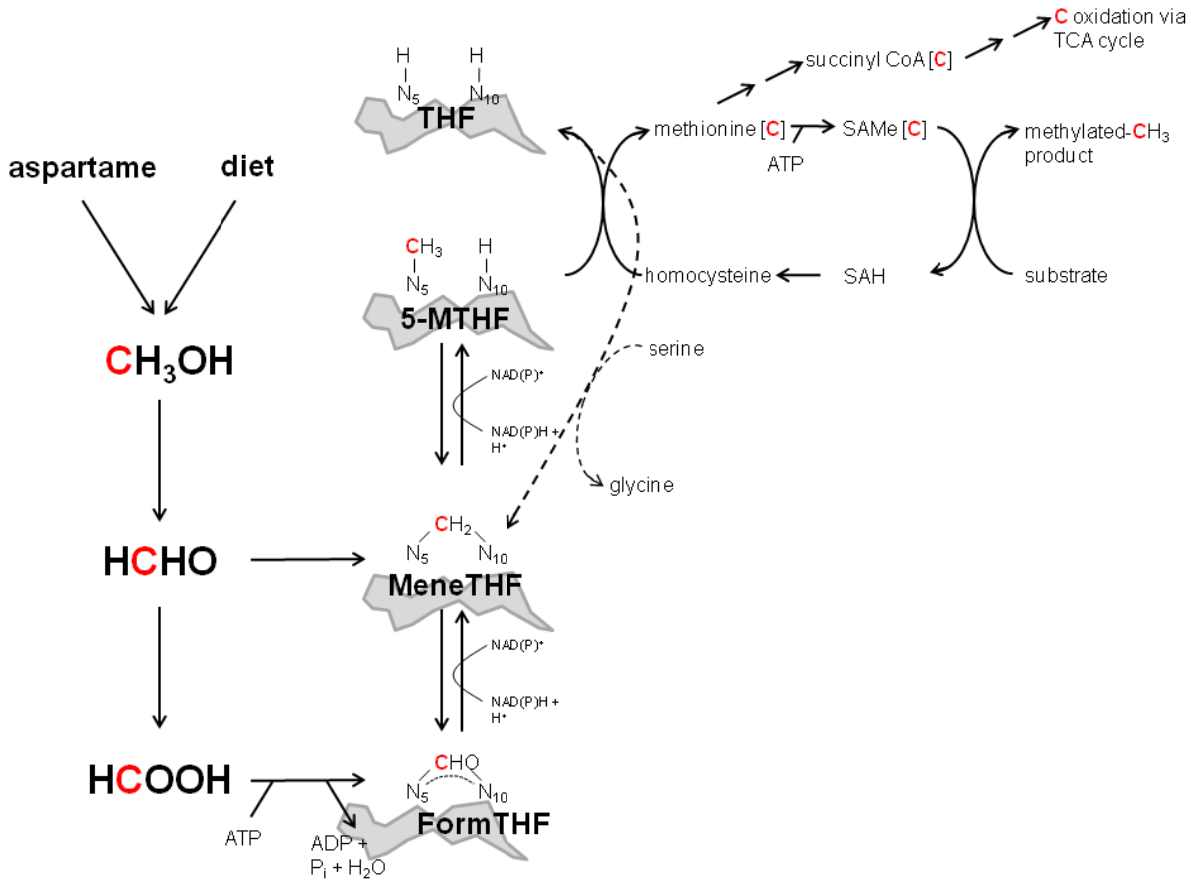


Figure 3: Metabolism of methanol part 1. THF, tetrahydrofolate; 5-MTHF, 5-methyltetrahydrofolate; MeneTHF, 5-10 methylene tetrahydrofolate; FormTHF formyltetrahydrofolate; SAMe, S-adenosylmethionine; SAH, S- adenosylhomocysteine

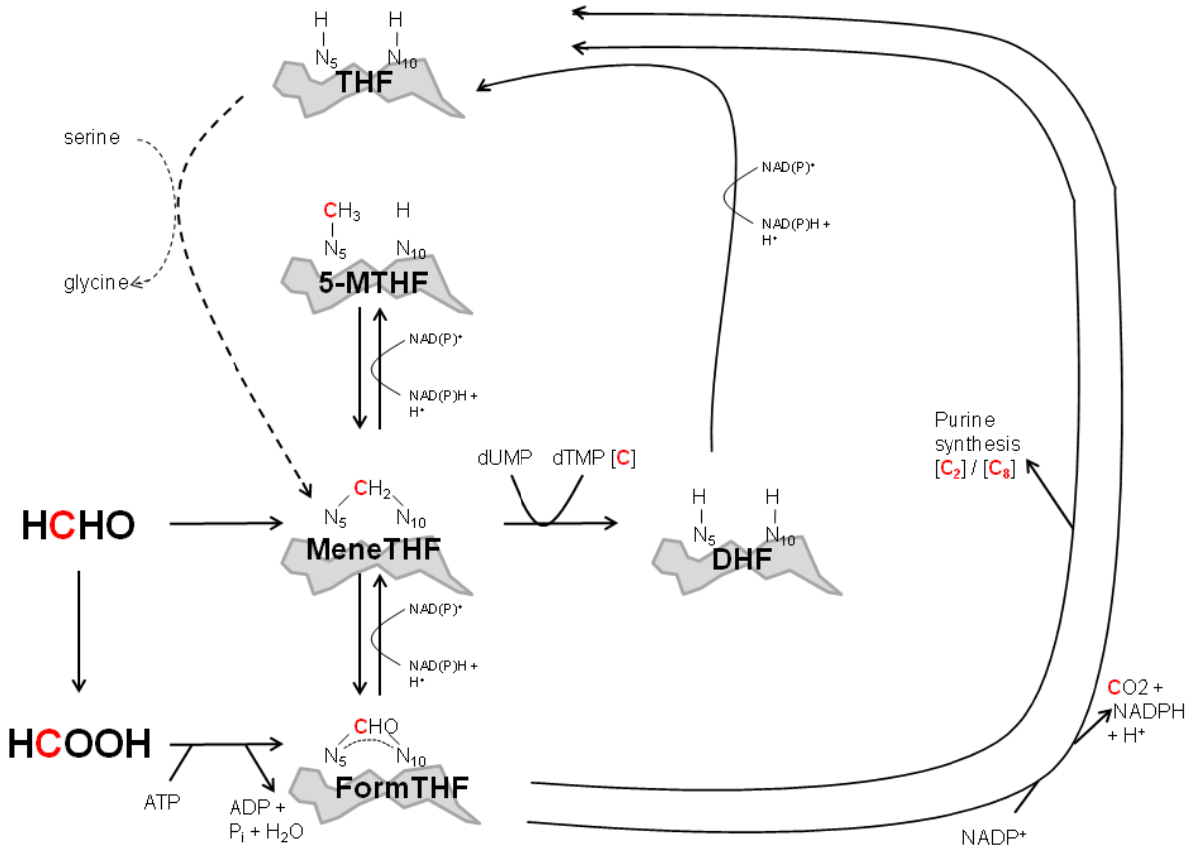


Figure 4: Metabolism of methanol part 2. THF, tetrahydrofolate; 5-MTHF, 5-methyltetrahydrofolate; MeneTHF, 5-10 methylene tetrahydrofolate; FormTHF formyltetrahydrofolate; DHF, dihydrofolate.

It is estimated that 25 % of ^{14}C from ^{14}C-methyl aspartame is utilised in formylation (E92, 1976). The metabolism of formaldehyde to formate is very efficient with a half-life of approximately one minute (McMartin et al., 1979; Tephly and McMartin, 1984). The oxidation of formate to carbon dioxide varies between species, the rate of formate elimination in humans and non-human primates being half of that in rats (Kavet and Nauss, 1990). In rodents, formate is converted to carbon dioxide through a folate-dependent enzyme system and a catalase-dependent pathway (Dikalova et al., 2001) whereas in humans metabolism occurs exclusively through the folate-dependent pathway (Hanzlik et al., 2005).

The adequate *in vitro* genotoxicity data on methanol comprised a negative bacterial reverse mutation test, a negative gene mutation test with *Schizosaccharomyces pombe* and a negative prophage induction test with *Escherichia coli* WP2(λ). The adequate *in vivo* genotoxicity data following oral administration of methanol comprised a negative mouse micronucleus test in bone marrow. In addition, in studies following intraperitoneal administration or inhalation exposure to mice, rabbits or monkeys, no evidence for induction of DNA-damage (8-oxodG), micronuclei or SCE was found.

In a weight-of-evidence approach, the Panel concluded that the data set was limited but that the available reliable *in vitro* and *in vivo* data did not indicate a genotoxic concern for methanol.

Soffritti et al. (2010) suggested that the metabolism of aspartame resulting in the formation of methanol might have played a role in the development of hepatocellular tumours. Therefore, the ANS Panel has undertaken a more detailed evaluation of the suggested role implied for methanol.

The oral studies on chronic toxicity and carcinogenicity of methanol are limited to a mouse study reported by Apaja (1980) and a rat study performed by the ERF and reported by Soffritti et al. (2002).

The Panel noted that the experimental design of the poorly reported study by Apaja (1980) is flawed due to the lack of a concurrent control group, the small number of animals used per dose group that were not maintained under pathogen-free conditions resulting in a high incidence rate of pneumonia. Therefore, the Panel concluded that the study by Apaja (1980) was inadequate for the assessment of the carcinogenic potential of methanol.

The rat study on methanol (Soffritti et al., 2002) demonstrated a statistically significant increased incidence of haemolymphoreticular tumours (lymphomas/leukaemias) but only in females at the highest dose level.

As already discussed above, the validity of the ERF study on methanol has recently been criticised (Cruzan, 2009; Schoeb et al., 2009; Schoeb and McConnell, 2011; NTP-EPA, 2011). The ANS Panel concurred with the concerns identified by EPA and others, and did not support the validity of the conclusions in the study on methanol as reported by Soffritti et al. (2002). The Panel concluded that the ERF rat study was not a suitable for the cancer risk assessment of methanol.

No adequate animal studies on the effects of methanol on fertility and reproductive performance were described.

The database on developmental toxicity of methanol is limited.

In mice, one prenatal developmental toxicity study by inhalation in which one group was also dosed orally and two oral prenatal developmental studies at high dose levels were available. The Panel derived a NOAEC by inhalation for prenatal developmental toxicity in mice of 1000 ppm from the study of Rogers et al. (1993) and calculated a NOAEL for oral exposure of approximately 560 mg/kg bw/day. The Panel considered the NOAEC of 1000 ppm based on the cervical rib effects as conservative as these effects tend to disappear as the pups grow and are often observed in inhalation studies.

The design of oral prenatal studies in rats was inadequate. In one study (Youssef et al., 1997), rats were administered methanol only once and not during the most sensitive period for induction of developmental effects. In two other studies in rats the duration of the administration period (GD 15-17 and GD 17-19 or GD 1-8) did not cover the period of organogenesis (Infurna and Weis, 1986; Cummings, 1993). In the study of Nelson et al. (1985), pregnant females were exposed by inhalation during organogenesis and a NOAEC of 5000 ppm was found. The Panel calculated from this concentration a corresponding oral NOAEL of approximately 2070 mg/kg bw/day.

In a study by Burbacher et al. (2004), Macaque monkeys were exposed to 0, 200, 600 or 1800 ppm methanol vapour for 2.5 hours/day for 7 days a week (estimated doses of 0, 21, 61 and 185 mg/kg bw/day (Alexander et al, 2008)) prior to breeding and throughout pregnancy (approximately 120 days). No methanol-related effects were apparent on the birth weight or health of the offspring.

The Panel noted that there is 140-515-fold difference between the calculated NOAELs in mice and rats, respectively, and the amount of methanol released from aspartame consumed at the ADI.

The Panel noted that for average consumers of aspartame, the contribution to the overall exposure to methanol ranged from 1 % up to 10 % across the EU general population. In this estimate, the Panel also noted that exposure to methanol from natural sources is a minor contributor compared to exposure from endogenous pathways (less than 10 %). The Panel noted that the exposure from aspartame-derived methanol is similar to methanol exposure from natural sources.

The Panel concluded that there is no safety concern from the levels of methanol released from aspartame under the current uses and permitted use levels.

The Panel also considered the metabolite of methanol, formaldehyde, in its risk assessment. IARC classified formaldehyde as a known human and animal carcinogen in 2006 that causes nasopharyngeal cancer in humans and squamous cell carcinomas in the nasal passages of rats (IARC, 2006). The recent evaluation of formaldehyde by NTP (2011) and its re-evaluation by IARC in 2012 confirmed it to be a human and animal nasal carcinogen following exposure by inhalation (NTP, 2011; IARC 2012). Some epidemiological studies have identified an association between formaldehyde exposure by inhalation and the induction of leukaemia in professional and industrial workers, however other studies have not demonstrated this association (NTP, 2011; IARC, 2012). The epidemiological evidence was recently criticised by the US National Academy of Sciences (NRC, 2011). The Panel considered that the epidemiological evidence for leukaemia from occupational exposure was inconsistent. Moreover, a recent analysis of DNA adducts in rats and cynomolgus macaques caused by inhaled formaldehyde revealed not only the existence of high levels of endogenous formaldehyde adducts in all tissues examined, including bone marrow, but also that DNA adducts and DNA-DNA cross-links from inhaled formaldehyde only occurred in the nasal mucosa and not in sites remote from the portal of entry (Lu et al., 2010, 2011; Moeller et al., 2011). The Panel noted that the distribution of DNA adducts from inhaled formaldehyde did not support systemic effects of formaldehyde or a causal association with leukaemia in humans from occupational exposure.

As previously concluded by EFSA (2006), the carcinogenicity of formaldehyde is only observed under occupational exposure by inhalation and oral exposure is not linked to cancer in animals (Til et al., 1989).

To further consider any potential for DNA adduct formation after oral aspartame exposure in humans, the Panel estimated the steady state plasma levels of formaldehyde likely to be generated from aspartame exposure at current ADI. The Panel also assessed the potential for DNA adduct formation at the C_{max} if aspartame were given in 3 equal doses equivalent in total to the ADI (40 mg/kg bw/day) over the day.

Methanol released from aspartame in the gut, is absorbed and enters the body. The released methanol is 10 % (by weight) of aspartame and therefore the amount of methanol formed at an aspartame dose of 40 mg/kg bw/day (current ADI) is 4 mg/kg bw/day. Using this dose, the plasma steady state concentration that could arise maximally from aspartame-derived methanol can be calculated using the following equation:

$$Css = f \times D/Cl \times tau$$

Where:

Css = plasma steady state concentration

f = fraction absorbed (=1.0 for methanol)

D = dose (4 mg/kg bw = 280 mg)

Cl = total body clearance (= 0.500 L/min)

tau = dosing interval (=1 day =1440 min)

Using this approach, the plasma steady state concentration that could arise maximally from aspartame-derived methanol is calculated to be 12 µM. Peak concentrations of methanol after three divided doses of aspartame at the current ADI of 40 mg/kg bw are calculated to be about 60 µM.

According to the COT report (2011), methanol is oxidised sequentially to formaldehyde, then to formic acid or formate (depending on the pH) and finally to carbon dioxide (see above). In humans and non-human primates, the oxidation of methanol to formaldehyde is mediated by alcohol dehydrogenase (ADH). In non-primate mammals, the reaction is primarily mediated by a catalase-

peroxidative system, but the conversion rates are similar. The oxidation of formaldehyde to formate is mediated by several enzyme systems including formaldehyde dehydrogenase. Formate is then oxidised to carbon dioxide through the action of formyl-THF synthetase, whereby formic acid combines with tetrahydrofolic acid (THF) to form 10-formyl-THF which is subsequently converted to carbon dioxide by formyl-THF dehydrogenase (COT, 2011).

At the tissue level, formaldehyde is reported to be present as formaldehyde acetal ($CH_2(OH)_2$):

$HCHO + H_2O \rightarrow CH_2(OH)_2$

before undergoing conjugation with glutathione (GSH):

$CH_2(OH)_2 + GSH \rightarrow HO\text{-}CH_2 - SG + H_2O$

which is a substrate for enzymatic transformation to formic acid by formaldehyde dehydrogenase (Andersen et al., 2010):

$HO\text{-}CH_2 - SG + NAD^+ + H_2O \rightarrow HCOOH + GSH + NADH + H^+$

Thus, a concentration of 12 µM methanol will produce at most, a steady state concentration of 12 µM formaldehyde/formaldehyde acetal. The maximum concentration (C_{max}) of formaldehyde was estimated to be about 60 µM. In nasal tissue, Andersen et al. (2010) have shown that in the nasal cells the amount of GSH will remain stable as long as the tissue concentration of formaldehyde acetal does not increase above 500 µM. The nasal intracellular baseline formaldehyde acetal concentration is 400 µM (Andersen et al., 2010, based on data by Casanova and Heck, 1987 and Casanova et al., 1994). Increase in formaldehyde bound to DNA was not measurable for concentrations below 400 µM in the experiments done by Casanova et al. (1994) used for the modelling by Andersen et al. (2010). It is acknowledged that the nasal tissue and other tissues such as the bone marrow may vary in terms of absolute concentrations of GSH present, however, Esmali et al. (2012) demonstrated that bone marrow cells are well equipped with GSH. The increase in formaldehyde acetal associated with an exposure to aspartame at the maximal ADI (and if ingested as a single dose), even if all the methanol liberated from aspartame was converted into formaldehyde, which then formed a formaldehyde acetal with water, would be less than 3 % (for the steady state level) and less than 15 % (for the peak level) of the normal intracellular endogenous levels. It can be used to make an estimate of the additional burden of formaldehyde acetal associated with the intake of aspartame at the level of the ADI. Such an additional burden should be evaluated in the light of the naturally occurring inter-species and intra-species variation in the internal level of methanol, formaldehyde and formaldehyde acetal which by far exceed the difference between internal concentration of these endogenous substances and the additional exposure by oral intake of aspartame at the current ADI level. Kleinnijenhuis et al. (2013) using a sensitive and specific method have measured a formaldehyde concentration in blood of 2.25 ± 0.67 mg/L in rats. This corresponds to a coefficient of variation of 30 % in endogenous formaldehyde blood levels in rats.

Thus, the Panel concluded the additional methanol and formaldehyde arising from aspartame at the ADI does not constitute a significant additional risk above the risk from naturally occurring endogenous methanol and formaldehyde, given the fact that worst case assumptions have been used.

7. Biological and toxicological data on DKP

A public call was launched by EFSA in 2011 and 2012. The present evaluation of the biological and toxicological data is based on the evaluation of unpublished and published studies following this call together with additional studies identified from the published literature up to the end of November 2012.

7.1. Absorption, distribution, metabolism and excretion of DKP

7.1.1. Studies on absorption, distribution, metabolism and excretion of DKP received following a public call for data and which were available at the time of the previous JECFA and SCF evaluations

The toxicokinetic properties of DKP have been studied extensively and are summarised below. Studies in the rat, rabbit, monkey and man (E15, E18, 1972) have shown that [^{14}C(U)-Phe]-DKP (20-30 mg/kg bw) did not enter the normal metabolic pathways of phenylalanine and was not well absorbed from the gut. Radioactivity was recovered in the faeces, primarily as unchanged DKP. Following oral administration of DKP metabolism to phenylacetic acid occurs in the gastro-intestinal tract, phenylacetic acid can then be absorbed and is rapidly excreted in the urine as phenylacetic acid and following conjugation with glutamate to phenylacetylglutamine (E80, 1974). The urinary excretion of phenylacetic acid and phenylacetylglutamine accounts for 20 % (rat) to 50 % (monkey and man) of the orally administered DKP. Phenylacetylglutamine is a naturally occurring compound that is also generated during the metabolism of phenylalanine. It was not observed following direct intravenous administration of DKP in monkeys, suggesting that it was generated by bacterial metabolism in the gut rather than by endogenous metabolism following absorption.

When [^{14}C(U)-Phe]-DKP was administered orally to monkeys (E80, 1974), the distribution curve was biphasic, with an early phase representing absorption and distribution among tissues while the second phase represented the appearance in the circulation of phenylacetylglutamine. Thus, the major metabolite of DKP, was the major form excreted via the urine in monkeys following oral administration of DKP. It has also been identified in human urine following administration of [^{14}C(U)-Phe]-DKP (E80, 1974).

Additional studies were carried out to characterise the biotransformation of DKP by intestinal flora (E80, 1974). The urinary metabolites of DKP were compared in conventional rats with normal microflora and in germ-free rats following dosing with [^{14}C(U)-Phe]-DKP. In the absence of gut flora, 89 % of urinary ^{14}C was present as DKP itself whereas in conventional rats only 24 % of the urinary label was in this form with most of the urinary radioactivity recovered as phenylacetylglutamine. When the germ-free rats were populated with microflora, the pattern of metabolism was converted to that seen in conventional rats. It appeared, therefore, that the primary metabolites of DKP arose as a result of bacterial biotransformation.

Cho et al. (1987) measured plasma and urinary concentrations of DKP in samples obtained from six normal adult subjects ingesting 2.2 mg DKP/kg bw as part of a dose of 200 mg aspartame/kg bw. DKP concentrations were measured by HPLC in plasma (0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 24 hours) and urine (0-4, 4-8, 8-24 hours) for 24 hours. In plasma, DKP concentrations were always below the detection limit (less than 1 µg/mL). In urine, DKP was measurable in all collected samples. Mean total urinary DKP excreted during the first 24-hour period after dosing was 6.68 $\pm$ 1.30 mg (corresponding to 4.83 % of the ingested DKP dose). Approximately 44 % of the total urinary excretion of DKP occurred in the first 4 hours after dosing.

Another study reported the effect of repeated ingestion of beverages containing DKP on amino acid and DKP concentrations in plasma and urine of normal healthy subjects (UN05, 1987) and in PKU heterozygous subjects (UN06, 1987). Six normal healthy adult and six healthy adult PKU heterozygotes received a dose of 150 mg of DKP every hour for eight hours. Blood and urine samples were collected up to 24 hours. DKP administration produced a small increase in plasma DKP concentration that reached a plateau after four doses in both normal and PKU subjects. DKP was detectable in the urine during the 24-hour period following ingestion, and amounted to 5 % or 2 % of the total dose in normal or PKU heterozygous subjects, respectively. DKP had no significant effect on plasma amino acids concentrations, on blood formate and methanol, on urinary formate concentrations, on the plasma LNAA ratios in both normal and PKU subjects.

7.2. Toxicological data of DKP

7.2.1. Acute oral toxicity of DKP

The acute toxicity of the DKP was studied in mice, rats and rabbits (E45, 1973). DKP was administered by gavage (as 15-17.5 % suspension in 1 % Tween 80, or an aqueous 2 % suspension for low doses) at doses up to 5000 mg/kg bw. Male Sprague Dawley rats (n = 6) were dosed at 2191 or 5000 mg/kg bw and male Schmidt Ha/ICR mice (n = 6) were dosed at 3710 or 5000 mg/kg bw. Male New Zealand White Luenberg rabbits were dosed at 2000, 2500 or 3200 mg/kg bw (n = 1) and 4000 or 5000 mg/kg bw (n = 3). The animals were observed intermittently during the 7 day post-treatment period and no remarkable motor or behavioural activites were noted. No mortalities were observed in the experimental period.

In the same report, the acute toxicity of DKP was also studied by i.p. injection (E45, 1973). Male Sprague Dawley rats (n = 6) were dosed at 1562 mg/kg bw and male Schmidt Ha/ICR mice at 1577 mg/kg bw (n = 6). Motor and behavioural activites were unremarkable and no deaths were recorded during the study period.

As for aspartame, the authors concluded for DKP that 'it can be assumed that the LD_{50} values are in excess of the highest doses administered to each species by the routes employed'.

The overview of these studies is presented in Appendix F.

7.2.2. Short-term and sub-chronic toxicity of DKP

7.2.2.1. Studies on short-term and sub-chronic toxicity received following a public call for data which were available at the time of the previous JECFA and SCF evaluations

DKP was administered daily for 2 weeks to Charles River CD rats by gavage (as 5 % aqueous suspension) (E7, 1971). The daily dosage was 1000 mg/kg bw. The young adult rats (11 weeks) were randomly assigned, 5 males and 5 females to the control and treatment groups. Survival of the animals was 100 %, no effects on body weight, or physical or behavioural changes were noted during the study period. In addition, no treament-related changes in haematoloy, clinical chemistry, urinalysis or histopathology were reported.

Groups of 10 Ha/ICR mice (4 weeks old; sex unspecified) were administered 1000 mg/kg bw DKP or an equal volume of vehicle only by gavage (as 5 % aqueous suspension) daily for 2 weeks (E6, 1971). Survival of the animals was 100 %, no effects on body weight, or physical or behavioural changes were noted during the study period, nor were any treament-related changes in haematology, clinical chemistry, urinalysis or pathological changes reported.

Groups of eight-week-old male and female Charles River CD rats (n = 5) were randomly assigned to study groups (E8, 1972). DKP was administered in diet to result in dose levels of 0, 2000, 4000 and 6000 mg/kg bw/day; actual consumption resulted in dosing within 5 % of these doses. A moderate (statistically significant at sacrifice) decrease was noted in the body weight of the female rats in the highest dose group and was attributed to a proportional decrease in feed intake. All animals survived the experimental period and no physical or behavioural changes were noted. There were no treament-related changes in haematology, clinical chemistry, urinalysis or histopathology, but several incidences of non-treatment-related disease were observed.

The overview of these studies is presented in Appendix G.

7.2.3. Genotoxicity of DKP

7.2.3.1. Studies on genotoxicity of DKP received following a public call for data which were available at the time of the previous JECFA and SCF evaluations

In vitro studies

DKP was tested in two bacterial reverse mutation assays with *Salmonella typhimurium* strains TA1535, TA1537, TA1538, TA98 and TA100, both plate incorporation without and with metabolic activation, (a) 10 to 5000 µg/plate (E98, 1978) and (b) 50 to 10 000 µg/plate (E106, 1978) revealed no evidence for mutagenicity of DKP (Appendix H)**.** The Panel considered that the methods implemented were sufficiently robust to support the results reported.

Negative results were also reported in a study in which DKP was tested in the Ames test with *Salmonella typhimurium* strains TA1535, TA1537, TA1538, TA98 and TA100 at 870 and 4370 µg/mL both in the absence and presence of S9 and in limited test for reversion/mitotic crossing-over in the yeast D7, only without metabolic activation (Tateo et al., 1989). The Panel noted that the study lacks TA102 strain and that only two dose levels were tested.

In vivo studies

DKP was tested for the induction of chromosome aberrations in bone marrow cells in rats (10 males/group) following administration of 0 (control), 250, 500, 1000 and 2000 mg DKP/kg bw/day by gavage for 5 consecutive days, given in three equally divided daily doses (E30, 1972). No evidence for CA-induction was found, the Panel considered that the methods implemented were sufficiently robust to support the results reported (Appendix H).

DKP was studied for mutagenicity by host-mediated assay in rats (E31, 1972). Rats were treated with 0 (control), 250, 500, 1000, 2000 mg DKP/kg bw/day by gavage, divided equally in three doses for five consecutive days (E31, 1972). 30 minutes after the final dose, the animals were inoculated with *Salmonella typhimurium*, G-46, by intraperitoneal injection. Three hours later the bacteria were recovered and the peritoneal washing was evaluated for the presence of mutants. In rats, the host-mediated assay revealed no evidence for mutagenicity of DKP. However, the Panel noted that the test-system employed has not received further validation.

DKP was studied for mutagenicity by a host-mediated assay in mice (E82, 1974). Mice were treated orally with 0 (control), 1000, 2000, 4000 or 8000 mg DKP/kg bw/day, given in three equally divided doses at two-hour intervals for five consecutive days. The authors concluded that the study did not provide evidence for mutagenicity of DKP. However, for the reasons given in Appendix H, the Panel considered that the methods implemented were not sufficiently robust to support the results reported.

For a dominant lethal assay, rats were treated by gavage with 1000 mg DKP/kg bw/day, given in two equally divided doses on a single day (E42, 1973). Each male was mated with two sexually mature virgin females weekly for eight consecutive weeks. On GD 14 these mated females were sacrificed for ovarian and uterine examinations. The Panel considered that the methods implemented were sufficiently robust to support the results reported. DKP did not affect paternal growth, maternal pregnancy rate, corpora lutea and implantation sites.

7.2.3.2. Conclusion on the genotoxicity of DKP

The genotoxicity data on bacterial reverse mutation exhibit some limitations (e.g. absence of TA102 and WP2 uvrA *Escherichia coli*) but were considered sufficient to conclude that DKP was not mutagenic in bacterial systems. The *in vivo* studies on genotoxicity of DKP comprise chromosome aberration in bone marrow erythrocytes and dominant lethal assays, all of which gave negative results. Taken together, the Panel considered that the data available *in vitro* and *in vivo* did not indicate a genotoxic potential of DKP.

Overall, the Panel concluded that available data do not indicate a genotoxic concern for DKP.

Summary tables on the genotoxicity of DKP are presented in the Appendix H.

7.2.4. Chronic toxicity and carcinogenicity of DKP

7.2.4.1. Studies on chronic toxicity and carcinogenicity received following a public call for data which were available at the time of the previous JECFA and SCF evaluations

The chronic toxicity of DKP was investigated in mice (E76, 1974) and rat (E77-78, 1974).

Mice

DKP was administered in the diet to groups of 36 male and 36 female ICR Swiss albino mice at dose levels equating to 0 (control), 250, 500, and 1000 mg/kg bw/day for 110 weeks (E76, 1974). Another group of 72 males and 72 females served as controls. Criteria evaluated were physical appearance, behaviour, body weight gain, feed consumption, survival, clinical chemistry, eye examination, organ weights, tumour incidence and gross and microscopic pathology. Histopathological examination was performed on all gross lesions from all animals at each treatment level, and on 20-27 grossly unremarkable organs from control and high dose level animals, as well as from roughly two-thirds and one-third of the animals in the mid and low dose groups. Detailed histopathological evaluations were performed on brain and urinary bladder from all mice in the control and the three treatment groups. There was no effect on the physical appearance and behaviour of the animals. Mean terminal body weights were significantly increased only for the low dose males. No significant differences in body weight gain during the first year were noted despite significantly reduced feed consumption in males at all three doses of DKP. Mean survival was significantly decreased for the mid dose females (16.7 % ± 6.3 %) compared to the controls (33.9 % ± 5.7 %). However, this finding was not considered treatment-related with respect to mean survival time. Mean survival to week 108 was 20-39 %. No consistent treatment-related haematology/clinical chemistry and urinalysis findings were noted. Random fluctuations reaching statistical significance were occasionally observed but the values remained within historical control data. No compound-related effects in ocular examinations were reported. Gross observations at necropsy did not reveal compound-related changes in any organs or tissues. Relative organ weights were unaffected, except for relative thyroid weight, that was increased in females at the mid and high doses. In no instance was the adjusted tumour incidence of any of the analysed tumour types significantly higher than that of the respective controls. No unusual tumours were encountered. One primary brain tumour (i.e. meningioma) was identified in a control female. Secondary lymphoreticular tumours were detected in brain and urinary bladder from small numbers of mice at all dose levels and controls. No consistent non-neoplastic alterations attributable to compound treatment were observed. The authors concluded that when DKP was administered to the mouse for 110 weeks in the diet at dose levels up to 1000 mg/kg bw/day there was no evidence of an effect, with respect to the incidence of neoplasms, or with regard to non-neoplastic changes in any organ or tissue. The Panel agreed with this evaluation and identified a NOAEL for this study of 1000 mg/kg bw/day, the highest dose level tested.

Rats

DKP was administered in the diet to groups of 36 male and 36 female Charles River CD rats at dose levels of 0 (control), 750, 1500, 3000 mg/kg bw/day for 115 weeks (E77-78. 1974). Another group of 72 males and 72 females served as controls. Histopathological examination was performed on available tissues from all animals in the study; also, all usual and unusual gross lesions and tissue masses were examined microscopically. Microscopic examinations of the brains, including seven coronal sections from each brain representing all major neuroanatomical areas, and bladder were also performed. There was no evidence that the administration of DKP at any level resulted in any effect on the physical appearance and behaviour of the animals. Two transient unidentified infectious diseases occurred during the study; these affected control and treated rats equally. One arose in weeks 12-14 of the study and was diagnosed as a putative viral infection of the salivary and Harderian glands. The

other arose during weeks 48-52 and was assumed to be bacterial; rats were treated with penicillin G in an attempt to control this infection. No compound-related decrease in survival rates was observed in any of the group. Body weights were significantly reduced in the high dose groups of both sexes in later stages, and periodically at lower doses. Feed intake was not consistently affected except for an increase in the mid-study in the high dose male group. Statistically significant changes in haematology or clinical chemistry and urinalysis were limited to a mild but significant decrease in serum cholesterol in high dose animals from day 42 (female) and 92 (male) onwards, and decreased urinary pH in all treated female groups, which was statistically significant at the high dose level. This effect was thought to be associated with the presence of acidic DKP metabolites in urine. No compound-related effects in ocular examination were noted. No biologically meaningful compound-related variation in organ weights was observed. There was no treatment-related non-neoplastic histopathology in any organ or tissue, and no unusual non-neoplastic lesions were observed. The adjusted tumour incidence of the analysed tumour types was not significantly higher than that of the respective controls. No unusual tumours were encountered. The authors concluded that the dietary administration of DKP to rats for 115 weeks at dose levels up to 3000 mg/kg bw/day indicated no evidence of an effect with respect to the survival rate, rate of body weight gain, physical examination and haematology findings, and incidence of neoplasms or non-neoplastic changes in any organ or tissue. DKP produced a significant decrease in urinary pH in the high dose female group, which was attributed to acidic metabolites of DKP in the urine, and a mild but significant decrease in serum cholesterol at the high dose level only. In addition, a significant increase in uterine polyps (benign tumours) was also found in females treated with the mid and high doses of DKP (1/69, 1/34, 4/34, 6/33, in control, 750, 1500, 3000 mg/kg bw/day, respectively). Based on these last observations, and in agreement with the JECFA monograph (JECFA, 1980) and the SCF evaluation (1989), the Panel identified a NOAEL for this study of 750 mg DKP/kg bw/day.

7.2.4.2. Additional chronic toxicity and carcinogenicity studies

An additional 2-year chronic toxicity and carcinogenicity study on aspartame was conducted in Wistar rats (Ishii et al., 1981; Ishii, 1981). This study is described in section 3.2.4.1. This study also included a group of rats that was fed 4000 mg/kg bw/day aspartame + DKP (3:1) for up to 104 weeks. There was a dose-dependent depression of body weight gain at 4000 mg/kg aspartame + DKP in males and females. This effect was correlated with decreased feed consumption. There was no evidence of a treatment-related effect on incidence of neoplastic and non-neoplastic changes other than an increase in focal mineralisation of the renal pelvis in both males and females (incidences in males: control, 1/57; aspartame + DKP, 13/59; incidences in females: control, 16/59; aspartame + DKP, 38/60) associated with a dose-related increase in urinary calcium (Ishii et al., 1981; JECFA, 1981; EFSA, 2006). In addition, medullary mineralisation was observed in females (control, 23/59; aspartame + DKP, 36/60). The authors attributed these effects to irritation caused by the mineral deposition and considered to be of minimal toxicological significance. The Panel noted that mineralisation in the kidney is a common finding in rats, the aetiology being associated with mineral imbalance (Lord and Newberne, 1990). Thus, there was no evidence for toxicity of aspartame with its degradation product DKP over 2 years in rats. The Panel noted that this study provided information on the lack of toxicity of aspartame when administered in conjunction with DKP.

7.2.5. Reproductive and developmental toxicity of DKP

7.2.5.1. Studies on reproductive and developmental toxicity received following a public call for data which were available at the time of the previous JECFA and SCF evaluations

Rats

The available data were a segment I study (male and female fertility study; E37, 1973), a segment II study (embryotoxicity and teratogenicity; E38, 1973) and a segment III study (peri- and postnatal development; E13, 1972) in rats. In addition, there was a segment II study in rats given a mixture of aspartame and DKP (3:1) (E56, 1972). The route of administration was via the diet. Animals were fed the test material from 17 days prior mating through gestation and lactation (E37, 1973), from GD 6 to

15 (E38, 1973), from GD 14 through weaning (E13, 1972), and from GD 6 to 14 (E56, 1972). None of the endpoints studied showed a difference between treated groups and controls and therefore, the lowest NOAEL observed in these studies was 2000 mg DKP/kg bw/day (E37, 1973; E38, 1973), corresponding to the highest dose tested. In the segment II study with a mixture of aspartame and DKP (E56, 1972), the NOAEL was 3040 mg/kg bw/day, the highest dose tested.

Rabbits

The rabbit studies included two developmental toxicity studies, which were confounded by poor health of the animals, and, in many cases, by a number of deaths in the treated groups, possibly related to misdosing due to gavage technique issues. In one study, DKP was administered to albino rabbits at doses of 500, 1000, and 2000 mg/kg bw/day from GD 6 to 18 (E57, 1972), whereas in a second study albino rabbits were administered a mixture of aspartame and DKP (3:1) at doses of 1000, 2000, and 3000 mg/kg bw/day from GD 6 to 18 (E29, 1972). In the first study (E57, 1972), according to the authors the NOAEL for maternal toxicity was 1000 mg/kg bw/day, due to a large gastric bolus comprised of granular white material (presumed DKP) admixed with hair found at necropsy in some animals from the high dose group. In the second study (E29, 1972), there was a statistically significant decrease in live mean fetal weights at the two highest doses, therefore according to the authors the NOAEL for this study was 1000 mg/kg bw/day (E29, 1972).

7.2.6. Other Studies on DKP

In a PhD Thesis (Bolteus, 2002), the effect of DKP treatment (0-10 mM) for 1 or 24 hours on MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction in cultures of neonatal Wistar rat astrocytes and human astrocytes obtained from a biopsy taken during a craniotomy is described. There was no apparent change in MTT reduction when rat astrocytes cultures when exposed to DKP up to 10 mM for 1 hour or 24 hours as compared to culture medium-exposed control cultures. Likewise, there was no apparent change in MTT reduction when human astrocyte cultures when exposed to DKP up to 10 mM for 1 hour or up to 5 mM for 24 hours as compared to culture medium-exposed control cultures. However, the author reported a 30 % increase in mean MTT reduction over control with 10 mM DKP. The Panel noted that the exposure to DKP was performed under serum-free conditions and that a 24-hour serum starvation period can be highly toxic to untransformed cells. No information on the effect of serum removal on MTT reduction was provided by the author except that a gradual removal of the serum from the culture medium over two days was necessary before the experiments with DKP could be initiated. The Panel further noted that MTT reduction is dependent on the reductive capacity of cells and, therefore, is only an indirect indicator of a change in cell viability and cell number, Overall, the Panel considered that based on the methods applied, the results of the study by Bolteus (2002) did not provide evidence for a proliferative effect of DKP in human astrocytes.

8. Discussion of DKP toxicity database

DKP is one of the degradation products of aspartame that is formed under certain processing and storage conditions. The EU and JECFA specifications for aspartame set a limit for DKP levels of 1.5 % (w/w) in aspartame as supplied (Commission Regulation (EU) No 231/2012; JECFA, 2006). The levels of DKP will potentially increase in aspartame-containing food and drinks during processing and storage. For example, it has been determined that up to 24 % of the aspartame is degraded into DKP in carbonate beverages (UN11, 1986; Table 3).

The Panel noted that the content of DKP in aspartame tested in the toxicological studies, including the long-term studies and carcinogenicity studies ranged from 0.1 % up to 4 %.

Some toxicological studies have been conducted on DKP.

Studies in animals and man have shown that DKP was not well absorbed from the gut and was recovered in the faeces, primarily as unchanged DKP. Following oral administration of DKP

metabolism to phenylacetic acid occurs in the gastrointestinal tract, phenylacetic acid can then be absorbed and is rapidly excreted in the urine as phenylacetic acid and following conjugation with glutamate to phenylacetylglutamine. The urinary excretion of phenylacetic acid and phenylacetylglutamine accounts for 20 % (rat) to 50 % (monkey and man) of the orally administered DKP. Phenylacetylglutamine is a naturally occurring compound that is also generated during the metabolism of phenylalanine.

The available *in vitro* genotoxicity data on bacterial reverse mutation have some limitations (e.g. absence of assays with Salmonella strain TA102 and *Escherichia coli* strain WP2 uvrA) but were considered sufficient to conclude that DKP is not mutagenic in bacterial systems. The *in vivo* data on genotoxicity of DKP comprise chromosome aberration in bone-marrow erythrocytes and dominant lethal assay, all of which gave negative results. Taken together, the Panel considered that the data available from *in vitro* and *in vivo* studies did not indicate a genotoxic concern for DKP.

DKP administration to mice for 110 weeks in the diet at dose levels up to 1000 mg/kg bw/day indicated neither a carcinogenic effect nor a treatment-related increase in non-neoplastic lesions at the doses tested (E76, 1974). The Panel considered that the NOAEL was 1000 mg DKP/kg bw/day, the highest dose level tested.

In another study, DKP was administered in the diet to male and female Charles River CD rats at dose levels of 0 (control), 750, 1500, 3000 mg/kg bw/day for 115 weeks (E77-78, 1974). A significant decrease at the high dose level in feed intake (males), body weight (both sexes), urinary pH (females) and a mild but significant decrease in serum cholesterol (both sexes) were reported. In addition, a significant increase in uterine polyps (benign tumours) was also found in females treated with the mid and high doses of DKP. Based on these last observations, and in agreement with the JECFA monograph (JECFA, 1980) and the SCF evaluation (1989), the Panel identified a NOAEL for this study of 750 mg DKP/kg bw/day.

Several studies were performed in rats and rabbits to assess the reproductive and developmental toxicity of DKP. The lowest NOAEL observed in the rat studies was 2000 mg DKP/kg bw/day (E37 and E38), the highest doses tested. For the rabbit studies, the NOAEL was 1000 mg/kg bw/day, for maternal toxicity and the effects observed on fetal weight.

9. Summary of biological and toxicological data on phenylalanine and aspartic acid

Both phenylalanine and aspartic acid occur naturally and are normally consumed as part of the diet.

9.1. Biological and toxicological data on phenylalanine

9.1.1. Absorption, distribution, metabolism and excretion of phenylalanine

Phenylalanine exists as D and L enantiomers, and L-phenylalanine is an essential amino acid required for protein synthesis in humans (Salway, 2012). Dietary intake of L-phenylalanine along with endogenous recycling of amino acid stores are the major sources of L-phenylalanine in the body, while utilisation of L-phenylalanine occurs through integration into proteins, oxidation to tyrosine by phenylalanine hydroxylase (PAH) or, to a minor extent, conversion to other metabolites such as phenylpyruvate.

PAH (EC 1.14.16.1) catalyses the stereospecific hydroxylation of L-phenylalanine to tyrosine in a reaction requiring molecular oxygen and the cofactor tetrahydrobiopterin. PAH activity is mainly associated with the liver, although minor activity has been demonstrated in rat kidney (Richardson et al., 1993). A five-fold greater rate of L-phenylalanine metabolism between rats and humans has been reported (Fernstrom, 1989). The human *PAH* gene is found on the long arm of chromosome 12 and contains 13 exons which encode a polypeptide of 452 amino acids. Mutations at the *PAH* locus result in phenylketonuria (PKU; MIM# 261600), an autosomal recessive inborn error of L-phenylalanine metabolism resulting in hyperphenylalaninaemia (HPA). Untreated PKU is associated with an

abnormal phenotype including growth failure, microcephaly, seizures and intellectual impairment caused by the accumulation of L-phenylalanine and its by-products (see section 10).

9.1.2. Toxicological data on phenylalanine

9.1.2.1. Studies in animals

Many studies have been conducted where rats and other experimental animals have been fed large amounts of L-phenylalanine in an effort to reproduce the biochemical and behavioural effects observed in PKU patients. The rat studies show a decrease in growth rate when fed large amounts of L-phenylalanine (reviewed in Harper, 1984; also Section 3.2.5.). Effects of oral administration of L-phenylalanine in pregnant rabbits are described in Section 3.2.5.

Additional studies on the effect of oral administration of high levels of L-phenylalanine have been conducted in primates. In one study (Waisman and Harlow, 1965), experimental PKU was produced in six infant rhesus monkeys by feeding excessive quantities of L-phenylalanine (3000 mg/kg bw/day) soon after birth and for up to three years. The phenylketonuric monkeys had elevated plasma levels of phenylalanine, and excreted almost the same phenylalanine metabolites in the urine as humans. Grand mal convulsions, observed in some children with phenylketonuria, were also observed in the experimental animals. The observed slowness in adapting to testing procedures, or even failure to adapt, and the inadequate performance was interpreted by the authors as an intellectual deficit induced by the excessive dose of L-phenylalanine. In another study conducted on nine rhesus monkeys (Gibbs and Smith, 1977), an intragastric preload of 1 g L-phenylalanine/kg bw, but not 1 g D-phenylalanine produced large reductions in food consumption. The authors proposed that L-phenylalanine releases a satiety signal.

9.1.2.2. Studies in humans

A large number of papers have investigated the untoward effects of uncontrolled diets in subjects suffering from PKU and have led to a substantial understanding of the toxic effects of L-phenylalanine intake in humans. Some of these are briefly summarised below.

Studies have shown that infants with PKU are particularly vulnerable to adverse effects of L-phenylalanine if their diet is unrestricted (Smith, 1971; Giovannini et al., 2012). High levels of blood phenylalanine during infancy and childhood can lead to severe, permanent brain damage, but the exact mechanism underlying the effects on neuronal function is not yet fully understood. During childhood, adolescence and adulthood tolerance to dietary L-phenylalanine may increase but is often very variable depending on the severity of the form of PKU. Infants born to mothers with PKU have also been found to be very susceptible to the toxic effects of L-phenylalanine presenting a high rate of birth defects and developmental disabilities: congenital heart disease, mental retardation, facial dysmorphism and intra uterine growth retardation.

A detailed discussion on the effect of plasma phenylalanine level management strategies on health is found in Section 10.

9.2. Biological and toxicological data on aspartic acid

9.2.1. Absorption, distribution, metabolism and excretion of aspartic acid

The metabolism of orally administered L-aspartic acid has been studied in several species and is summarised as follows (Oppermann, 1984). The majority of radioactivity following oral administration of [^{14}C(U)]-aspartic acid to rats or monkeys is accounted to 60-70 % by $^{14}CO_2$ expired in the air. This rapid and high level of elimination as CO_2 appears to result from both the decarboxylation of L-aspartate to alanine and CO_2 and the transamination reaction with pyruvate to form alanine and oxaloacetate in the intestinal mucosal cells. The alanine and oxaloacetate formed then enter the tricarboxylic acid cycle to form eventually CO_2. Approximately 25 % of the [^{14}C(U)]-

aspartic acid dose is not accounted for in the expired air, urine or faeces but appears to be incorporated into normal body constituents or used for gluconeogenesis.

9.2.2. Toxicological data on aspartic acid

L-Aspartic acid is among the most common amino acids found in our diet. It is an excitatory neurotransmitter of the central nervous system (brain, spinal cord). In contrast to L-phenylalanine, L-aspartic acid is a nonessential amino acid.

Excitatory amino acids are known to cause neurodegeneration when nerve cells expressing glutamate receptors become overstimulated following exposure to excessive levels of glutamate and L-aspartate (Rothman and Olney, 1995; Choi, 2001). The concentration of extracellular L-aspartate and glutamate in the central nervous system must therefore be controlled to prevent neuronal degeneration.

Even though orally administered L-aspartate is rapidly metabolised and kinetic studies on aspartame have shown that plasma L-aspartate levels do not substantially change following bolus or repeated administration of aspartame (see Section 3.1), some authors (Olney, 1982; Reif-Lehrer, 1976) have speculated that aspartame-derived L-aspartate, especially when consumed with foods containing monosodium glutamate (MSG), would cause an increase in the combined plasma concentrations of L-aspartate and L-glutamate, which might pose a risk of focal neuronal degeneration.

9.2.2.1. Studies in animals

Aspartate and glutamate can cause hypothalamic neuronal death in neonatal rodents, if given orally in large doses (500 mg/kg bw or higher) to infant animals (Olney, 1969; Olney and Ho, 1970; Lemkey-Johnson and Reynolds, 1974; Okaniwa et al., 1979; Finkelstein et al., 1983; Daabees et al., 1985). For instance, in mouse pups, which may be considered the most sensitive of all animal species, single doses of 1000 mg L-aspartate/kg bw caused extensive hypothalamic neuronal necrosis. In another study, eight-day-old mice were administered by gavage aspartate at 0, 1.88, 3.76, 4.89, 5.64 and 7.52 mmol/kg bw (corresponding to 0, 250, 500, 650, 750 and 1000 mg L-aspartate/kg bw) and the degree and extent of neuronal necrosis determined. Dose-dependent hypothalamic neuronal necrosis was found with 4.89 mmol L-aspartate/kg bw and higher doses. The extent of neuronal necrosis was proportional to dose, once a neurotoxic dose of L-aspartate was reached. The authors also reported that the highest concentration of L-aspartate in the plasma not inducing lesions was 870 $\pm$ 230 μM.

Olney and co-workers (Olney and Sharpe, 1969; Olney et al., 1972) also reported neuronal necrosis in neonatal nonhuman primates administered large bolus doses of glutamate (1000-4000 mg/kg bw subcutaneously or orally. This observation, however, could not be reproduced by a number of other scientists with either glutamate or aspartame at high dosages (reviewed by Butchko et al., 2002).

A 90-day aspartate feeding study (Tada et al., 2008) performed in Fischer 344 rats (55 males and 55 females) was recently evaluated by EFSA (EFSA, 2008). The animals were divided into 5 treatment groups and fed diets controlled for L-aspartic content. Dietary L-aspartic acid dosing was 0, 0.05, 1.25, 2.5 and 5.0 %, corresponding to 0, 28.7, 715.2, 1470.2 and 2965.9 mg/kg bw/day for females and to 0, 26.9, 696.6, 1416.6 and 2770.2 mg/kg bw/day for males. No signs or symptoms of neurotoxicity were observed at any of the doses tested. A NOAEL of approximately 700 mg/kg bw/day for males was identified by the ANS Panel based on renal toxicity findings (an apparent dose-related regenerative renal tubules dilation in males accompanied by inflammatory cell infiltration) (EFSA, 2008).

9.2.2.2. Studies in humans

Numerous intervention trials have been performed in adults with different aspartate compounds (sodium, magnesium, potassium-magnesium, buffered aspartic acid, arginine aspartate) in doses ranging from 1 to 10 g/day, for time periods between one single dose and four weeks. These studies have previously been reviewed by the ANS Panel (EFSA, 2008). None of these studies were undertaken to assess toxicity of aspartate intake, however, excluding reports on plasma amino acid

imbalance and soft stools/diarrhoea, no other adverse effects were reported (Colombani et al., 1999; Chouinard et al., 1990).

Consumption of 15 g of arginine aspartate daily, corresponding to 6.3 g L-aspartate, over 14 days in a double-blind, placebo-controlled cross-over trial by 14 healthy individuals resulted in significantly higher plasma levels of arginine, ornithine and urea as compared to placebo. In contrast, plasma levels of most other amino acids, including aspartic acid, and total amino acids were reduced (Colombani et al., 1999).

10. PKU data

PKU is an autosomal recessive disorder that leads to high levels of L-phenylalanine in the plasma and low level of tyrosine in the blood (Widaman, 2009; Zimmermann et al., 2012). High plasma L-phenylalanine levels are neurotoxic, probably through its inhibitory action on the transport of other amino acids (leucine, isoleucine, valine, tyrosine, tryptophan, and lysine), necessary for the protein and neurotransmitter synthesis (e.g. serotonin) (Giovannini et al., 2012). Furthermore, the inhibition of L-phenylalanine metabolism to tyrosine impacts on the formation of melanin and neurotransmitters derived from tyrosine such as dopamine, adrenaline, noradrenaline and thyroid hormones. HPA results due to a number of different mutations within the PAH allele producing a spectrum of phenotypes including classic phenylketonuria (PKU), moderate PKU, mild PKU and mild HPA (Zimmermann et al., 2012). For all these PKU phenotypes, plasma L-phenylalanine levels are above 10 mg/dL (600 μM) when on an unrestricted diet.

The restriction of dietary L-phenylalanine is the mainstay of PKU management. Patients with PKU have to avoid foods rich in protein (meat, fish, eggs, standard bread, dairy products, nuts, and seeds) and foods and drinks containing aspartame, flour, soya, beer or cream liqueurs. Therefore, PKU diets consist mainly of low-protein natural foods (vegetables, fruits and some cereals) that are modest in L-phenylalanine (Giovannini et al., 2012). The aim of the dietary treatment is to avoid acute and chronic increased concentrations of L-phenylalanine in plasma and consequently in cerebral tissue, responsible for an important worsening of the neuronal performance and behaviour in PKU patients.

Growth and brain development are most rapid during the first 6 months, and an infant with PKU is particularly vulnerable to adverse effects of L-phenylalanine if their diet is unrestricted (Smith, 1971; Giovannini et al., 2012). High levels of plasma L-phenylalanine during infancy and childhood lead to severe, permanent brain damage, but the exact mechanism of the effects on neuronal function is not understood.

Inversely, excessive restriction of L-phenylalanine intake during the first year of life is also detrimental to health given that L-phenylalanine is an essential amino acid that cannot be synthesised by the body. Cases of loss of weight, vomiting, listlessness, generalized eczematous rash and mental retardation have been attributed to suboptimal L-phenylalanine intake.

According to the NIH Consensus Statement on phenylketonuria, it is assumed that levels in excess of dietary requirement for this essential amino acid but below 10 mg/dL (600 μM) do not lead to brain damage.

The reference values for plasma L-phenylalanine concentration recommended by NIH (NIH, 2000) are L-phenylalanine levels between 2–6 mg/dL (120-360 μM) during pregnancy and for neonates through 12 years of age, and L-phenylalanine levels between 2–10 mg/dL (120-600 μM) after 12 years of age. This document recommends that L-phenylalanine levels between 120 and 360 μM are achieved at least 3 months before conception and that metabolic control should be achieved as soon as possible. Infants with PKU should start treatment to establish dietary control of their L-phenylalanine levels, ideally within 7–10 days of birth (NIH, 2000).

Waisbren and co-workers (Waisbren et al., 2007) assessed the reliability of plasma phenylalanine levels as a predictive biomarker of clinical outcomes in the development of treatments for PKU by

performing a systematic literature review and meta-analysis of published trials of PKU, which included L-phenylalanine level and neurological and dietary compliance outcome measures. The authors found that each 100 µM increase in L-phenylalanine (assessed by mean Index of Dietary Control during the critical childhood period or lifetime through 18 years) predicted a 1.3 to 3.9 point decrease in IQ for early-treated patients with PKU, over a L-phenylalanine range from 394 to 750 µM. A correlation was also found between concurrent L-phenylalanine level and IQ for early-treated patients with PKU, in which each 100 µM increase in L-phenylalanine predicted a 0.5 to 1.4 point reduction in IQ, over a L-phenylalanine range from 429 to 1664 µM.

Although the maintenance of a plasma L-phenylalanine concentration within the recommended range is clearly important in the treatment of PKU, a recent meta-analysis of 40 studies (Anastasoaie et al., 2008) demonstrated that the stability of plasma L-phenylalanine levels may be more important to cognitive functioning (FSIQ, Full Scale Intelligence Quotient) in PKU children continuously treated from shortly after birth than the overall exposure to L-phenylalanine. The authors of the study concluded that occasional increases ('spikes') in plasma L-phenylalanine levels of approximately 600 µM, which occur in the majority of children with PKU, may be benign, but that it is the long-term variability that appears to be important in the effects of L-phenylalanine on cognitive function (Anastasoaie et al., 2008). Cockburn and Clark (1996) suggested that adolescents and adults should continue the diet with the aim to maintain phenylalanine plasma concentration no higher than 700 µM.

In women with PKU, the most critical phase in controlling the L-phenylalanine plasma concentration is pregnancy. In 1980, Lenke and Levy (1980) reported results of over 500 pregnancies of women with PKU who were not on low-phenylalanine diets during pregnancy. Infants born to mothers with PKU had a high rate of birth defects and developmental disabilities: congenital heart disease, mental retardation, facial dysmorphism and intra uterine growth retardation (IUGR). Although these infants would have carried one functional *PAH* allele from their fathers, the exposure of the fetus to high levels of phenylalanine from the mother's blood via the placenta appeared sufficient to cause prenatal damage. These prenatal exposure effects show a dose-response relation, with higher levels of exposure leading to higher levels of disability.

Data from the prospective international Maternal PKU Collaborative (MPKUC) Study (Koch et al., 2003a), the French survey (Feillet et al., 2004) and the United Kingdom PKU Registry (Lee et al., 2005) have shown that many features of the maternal PKU syndrome were preventable when dietary L-phenylalanine intake was restricted before conception or soon afterward (Maillot et al., 2008).

The MPKUC Study (Koch et al., 2003a) was initiated in 1984 to monitor pregnancies of women with PKU, to maintain mothers on a low L-phenylalanine diet throughout their pregnancies, and to study relations between levels of L-phenylalanine in the mothers' blood during pregnancy and birth and developmental outcomes in their offspring. Women with plasma L-phenylalanine levels > 240 µM were enrolled; 382 women contributed to 572 pregnancies. The 413 offspring were examined at birth and annually. Congenital abnormalities were noted, and the focus was on the consequences of congenital heart disease and microcephaly on developmental outcome. To this effect, the McCarthy General Cognitive Index was administered at age four, followed by the Wechsler Intelligence Scale for Children (Revised) at age six. Microcephaly was noted in 137 offspring (33 %), and 32 (7.7 %) had congenital heart disease. Maternal plasma L-phenylalanine levels were higher for infants with congenital heart disease and microcephaly than for infants with congenital heart disease only ($P = 0.02$). Mean L-phenylalanine levels at weeks four and eight of gestation predicted congenital heart disease ($P < 0.0001$). The McCarthy General Cognitive Index score was lower with congenital heart disease ($P = 0.005$) and microcephaly ($P = 0.0017$), as was the Wechsler Intelligence Scale for Children (Revised) full-scale IQ (intelligence quotient) score ($P = 0.0002$ for congenital heart disease and $P = 0.0001$ for microcephaly). Optimal birth outcomes occurred when maternal plasma L-phenylalanine levels between 120 and 360 µM were achieved by 8 to 10 weeks of gestation and maintained throughout pregnancy.

Widaman and Azen (2003) modelled the relationship between prenatal exposure to L-phenylalanine and measures of offspring intellectual development in the offspring of the MPKUC Study (Koch et al., 2003). From the model, a nonlinear relation between prenatal L-phenylalanine exposure and offspring cognitive outcomes, with damage to the developing fetus if average L-phenylalanine levels are above approximately 360 μM was derived. Moreover, prenatal L-phenylalanine exposure had a strong effect on offspring outcomes at one year of age and was the only one of the background, pregnancy-related, or perinatal variables to directly influence offspring outcomes at 2, 4 and 7 years of age.

Data from a retrospective review of pregnancies from January 1977 to June 2006 showed that an L-phenylalanine-restricted diet should start before conception to avoid malformations and congenital heart disease and to ensure the best possible developmental outcome for the offspring. The study concluded that not only was it important to aim to maintain maternal plasma L-phenylalanine concentrations of < 300 μM, but, also, that variations in L-phenylalanine levels should be minimized (Maillot et al., 2008).

The Panel concluded that a critical threshold for the developmental and cognitive effects of L-phenylalanine is 360 μM, which is consistent with the NIH Consensus Statement on phenylketonuria (NIH, 2000). This document recommends that L-phenylalanine levels between 120 and 360 μM are achieved at least 3 months before conception and that metabolic control should be achieved as soon as possible. After 12 years of age, the range for recommended L-phenylalanine levels may increase to 120-600 μM. During pregnancy, the recommended level is 120-360 μM (NIH, 2000).

11. Mode of action

The basis for the ADI of 40 mg/kg bw previously set by SCF and JECFA was not explicitly stated, but the Panel's understanding of their brief reports was that it was derived from NOAELs of 4000 mg/kg bw/day identified in the chronic and carcinogenicity rat studies and applying an uncertainty factor of 100. Reanalysing the data the Panel noted that the effects reported in the rat and rabbit reproductive and developmental toxicity studies at doses below 4000 mg aspartame/kg bw/day should not be ignored. As described in section 4, the Panel considered that the relationship of these effects to treatment could not be definitively excluded. Therefore the Panel examined the possibilities that aspartame or one (or more) of its metabolites could be responsible for these effects.

The Panel noted that aspartame is the methyl ester of the dipeptide of the amino acids, L-aspartic acid and L-phenylalanine. After oral ingestion, aspartame is hydrolysed, either within the lumen of the gastrointestinal (GI) tract, or within the mucosal cells lining of the GI-tract. Hydrolysis is very efficient and the amount of aspartame that enters the bloodstream has been reported as undetectable in several studies conducted among others in rats, dogs, monkeys and humans (Oppermann, 1984; Burgert et al., 1991). The products that result from the hydrolysis reaction are methanol and the amino acids aspartic acid and phenylalanine. Hydrolysis of aspartame releases equimolar quantities of aspartic acid and phenylalanine, together with methanol corresponding to 10 % by weight of the aspartame dose. Further studies conducted in monkeys and pigs have shown that the potential intermediate metabolite phenylalanine methyl ester was rapidly broken down to phenylalanine and methanol in the intestinal lumen (Burton et al., 1984; Burgert et al., 1991). The Panel concluded that hydrolysis of aspartame in the gastrointestinal tract was essentially complete and that there was no systemic exposure to aspartame but systemic exposure to aspartic acid, phenylalanine and methanol did occur. The Panel considered therefore that systemic exposure to aspartame itself could not be responsible for the reproductive and developmental toxicity.

11.1. Mode of Action Approach

Based upon their framework for assessing chemical carcinogens in experimental animals, the International Programme on Chemical Safety has published frameworks for assessing chemicals with cancer (Boobis et al., 2006) and non-cancer endpoints (Boobis et al., 2008). This type of approach enhances transparency and harmonisation of the risk assessment process.

First, it was necessary to establish whether the weight-of-evidence from experimental observations was sufficient to ascertain a mode of action (MoA). Using the Bradford Hill criteria key events causally related to the toxic effect were identified. These key events were compared qualitatively and then quantitatively between experimental animals and humans. The decision tree from Boobis et al. (2008) was used to determine the human relevance of a MoA for toxicity observed in experimental animals. It must be noted that this Framework was an analytical tool that enabled a structured approach to assess the overall weight-of-evidence and its relevance to humans (mode of action), it was not designed to provide a description of the underlying events at the molecular level (mechanism of action). If the output from applying the Framework identified a credible and relevant MoA, this would become the basis for the risk characterisation.

Application of the Framework was considered appropriate for evaluating the effects observed in reproductive and developmental toxicity studies after careful consideration of the weight-of-evidence for the observed toxicological responses following exposure to aspartame in experimental animals. The Panel chose to use this approach as the application of such a framework results in a transparent evaluation of the data; their application of the IPCS criteria are described in detail below.

11.1.1. Postulated Mode of Action for aspartame

The MoA proposed by the Panel for aspartame was that the toxicological effects observed in rat and rabbit in pregnancy were due to the metabolite phenylalanine.

11.1.2. Key events

The reproductive and developmental toxicity studies on aspartame consisted of an embryotoxicity and developmental study performed in the mouse, a two-generation reproduction toxicity study in the rat, five peri- and postnatal developmental studies in the rat, a reproductive performance and developmental study in the rat and an embryotoxicity and teratogenicity study in the rat. In addition, eight embryotoxicity and teratogenicity studies were performed in the rabbit, four of which were by administration of aspartame in the diet and four by gavage. Some of these studies were also conducted with the aspartame metabolites phenylalanine and aspartic acid. In addition, reproductive and developmental toxicity studies were also available following administration of methanol.

The Panel concluded that the depression in pup body weight in the high dose groups could not be disregarded. The Panel noted that in earlier assessments of aspartame the observed effects were not considered critical for the evaluation. The published evaluations were not explicit on the rationale behind this decision. After the public consultation on this opinion, following a request from a peer the UK Food Standards Agency made available in the House of Lords library the working documents and correspondence from the COT evaluations in the 1990s. These documents demonstrated that detailed evaluation of the reproductive and developmental toxicity studies had been undertaken and the rationale for not considering these studies critical.

The Panel considered the depression in pup body weight in the high dose groups might be due to high exposure to phenylalanine from metabolism of aspartame. This was supported by the findings in rats and rabbits treated with phenylalanine. The Panel further noted that although the data from study (E11, 1975) were sparse this study together with other segment I and segment III studies was considered sufficient to fulfil the requirements of a two-generation reproduction toxicity study in rats and derived from these studies a NOAEL of 2000 mg/kg bw/day based on the lower pup weights at weaning in both generations.

The Panel agreed that observed effects in the developmental rabbit studies may have resulted from the nutritional status as a result of gastrointestinal disturbances and the potential effect of phenylalanine; it was neither possible to discriminate if these acted independently or in combination, nor which was responsible for the effects. Overall, the Panel agreed that the rabbit studies should not be considered key because these confounding factors affecting the studies and the likelihood that the gastrointestinal effects were species specific in rabbits and would be without direct relevance to humans.

11.1.3. Biological Plausibility

Considering the biological plausibility of the MoA, the Panel noted that there was long established evidence for increased severity and frequency of adverse developmental effects with high phenylalanine plasma levels as found in human patients with phenylketonuria (PKU) (Prick et al., 2012). The Panel noted that the database on PKU and particularly on effects during pregnancy had increased since the earlier evaluations of aspartame.

PKU is an autosomal recessive metabolic genetic disorder characterized by mutations in the gene for the hepatic enzyme phenylalanine hydroxylase (PAH), decreasing or inactivating its functionality. This enzyme is necessary to metabolize the amino acid phenylalanine to the amino acid tyrosine. When PAH activity is reduced, circulating phenylalanine levels are increased.

Maternal PKU syndrome refers to the teratogenic effects of PKU during pregnancy. These effects include mental retardation, microcephaly, congenital heart disease, and intrauterine growth retardation. In untreated pregnancies wherein the mother has classic PKU with a plasma phenylalanine level greater than or equivalent to 1200 μM (20 mg/dL), abnormalities in offspring occur at exceedingly high frequencies, approximately 75-90 % for microcephaly and mental retardation, and 15 % for congenital heart disease. There is a dose-response relationship with progressively lower frequencies of these abnormalities at lower phenylalanine levels. The pathogenesis of this syndrome is unknown; it may be related to inhibition by phenylalanine of large neutral amino acid transport across the placenta or to direct toxicity of phenylalanine and/or a phenylalanine metabolite (phenylpyruvic acid) in certain fetal organs. Malformations and mental retardation in the offspring of women with PKU can be prevented by maintaining maternal plasma phenylalanine within a target range (120-360 μM) through a phenylalanine-restricted diet (NIH, 2000).

11.1.4. Concordance of Dose-Response Relationships

Phenylalanine is released in a direct one to one molar ratio from the metabolism of aspartame, on a weight basis this would be 45 % of the aspartame dose. The Panel noted that the effects observed in the reproductive and developmental toxicity studies in animals occurred at equivalent molar doses of phenylalanine following administration of either aspartame or phenylalanine.

As described previously, PKU is an autosomal recessive disorder resulting in high levels of phenylalanine and low levels of tyrosine in the blood (Widaman, 2009; Zimmermann et al., 2012). High blood phenylalanine levels are neurotoxic, mainly due to its inhibitory action on the transport of free L-amino acids (leucine, isoleucine, valine, tyrosine, tryptophan, and lysine), necessary for protein and neurotransmitter synthesis (dopamine and serotonin) (Giovannini et al., 2012). It is recognised that malformations and mental retardation in the offspring of women with PKU can be prevented by maintaining maternal plasma phenylalanine within a target range 120-360 μM (Koch et al., 2003) (or below a threshold thought to be between 330 and 360 μM (Widaman and Azen, 2003)) through a phenylalanine-restricted diet.

The form of the relation between prenatal phenylalanine exposure and offspring cognitive outcomes appears to be nonlinear, with no damage to the developing fetus until exposure passes a critical threshold level. The best estimate of the critical threshold of phenylalanine exposure without damage to the offspring occurs is 330 to 360 μM (Widaman and Azen, 2003). A critical threshold in the range from 330 to 360 μM is consistent with reference values indicated by the NIH Consensus Statement on phenylketonuria (NIH, 2000). However, in contrast to the majority of the literature the data presented by Lenke and Levy (1980), showed elevated rates of mental retardation even in offspring with maternal phenylalanine levels between 180 and 600 μM. The Panel noted that control of phenylalanine plasma levels in PKU patients by dietary restriction can be highly variable and occasional high exposures or prolonged exposures above the target range can occur, which could be responsible for effects observed in PKU patients controlling levels by dietary restriction. The original range between 180 and 600 μM proposed by Lenke and Levy (1980) has been replaced by a range between 120 and

360 μM (Koch et al., 2003) and the best estimate of the threshold has been defined in the range from 330 to 360 μM (Widaman and Azen 2003).

11.1.5. Temporal Association

Apart from the hydrolysis of aspartame in the gastrointestinal tract, resulting in the increased plasma phenylalanine levels that the Panel considered associated with the effects observed in the reproductive and developmental studies in animals, there is currently no further information on a step by step mechanistic explanation. This mechanistic explanation is lacking for animals and humans.

11.1.6. Strength, Consistency, and Specificity of Association of Toxicological Response with Key Events

There was consistency in the observed outcomes of the reproductive and developmental toxicity studies on aspartame in rat and rabbit; including a two-generation reproduction toxicity study in the rat, five peri- and postnatal developmental studies in the rat, a reproductive performance and developmental study in the rat and an embryotoxicity and teratogenicity study in the rat and eight embryotoxicity and teratogenicity studies performed in the rabbit. Consistent observations from a number of studies with differences in experimental design, increases the support for the MoA. As the association was consistent, it was more likely that the relationship was causal. The effects observed in animals following aspartame exposure were reproduced if phenylalanine was given at the equivalent molar dose.

11.1.7. Other Modes of Action

Intact aspartame *per se* cannot be responsible for any adverse effects observed, due to its complete hydrolysis within the GI tract.

The Panel noted that the products formed following hydrolysis of aspartame were normal constituents of the diet (aspartic acid, phenylalanine and methanol) and were metabolised by endogenous metabolic pathways.

Methanol is subject to a significant first pass metabolism. The overall metabolism of methanol proceeds by stepwise oxidation via formaldehyde to formate, and then to carbon dioxide. Formaldehyde and formate enter the one carbon metabolic pool through tetrahydrofolic acid and from there, may contribute to the biosynthesis of purines, pyrimidines and amino acids. The metabolism of formaldehyde to formate is very efficient with a half-life of about one minute (McMartin et al., 1979; Tephly and McMartin, 1984). The oxidation of formate to carbon dioxide varies between species, the rate of formate elimination in humans and non-human primates being half of that in rats (Kavet and Nauss, 1990). In rodents, formate is converted to carbon dioxide through a folate-dependent enzyme system and a catalase-dependent pathway (Dikalova et al., 2001) whereas in humans metabolism occurs exclusively through the folate-dependent pathway (Hanzlik et al., 2005).

The Panel noted that the methanol released from aspartame may enter the general circulation. However, on a weight basis the amount released would be one tenth of the aspartame dose.

In studies on the reproductive and developmental toxicity of methanol marked effects were observed at oral methanol doses greater than 2000 mg/kg bw/day, no lower doses were tested orally. From inhalation studies a NOAEL equivalent to an oral methanol dose of 560 mg/kg bw/day was derived. The Panel noted that a similar exposure to methanol from aspartame ingestion would occur at an oral dose of aspartame of 5600 mg/kg bw/day. In studies on aspartame, effects were seen at doses of 1000-2000 mg aspartame /kg bw/day or higher. At these aspartame doses the maximum amount of methanol would be 100-200 mg methanol /kg bw/day respectively which is below the NOAEL derived by the Panel for reproductive and developmental toxicity of methanol. The Panel therefore considered that the effects observed following aspartame ingestion could not be due to methanol.

The Panel noted that no effects were observed in reproductive and developmental toxicity studies with aspartic acid even when administered at an equivalent molar dose to the dose of aspartame that gave rise to adverse effects in animals (Table 22). The Panel therefore considered that the effects observed following aspartame ingestion could not be due to aspartic acid.

During the public consultation the possibility was raised that rather than a single metabolite being responsible for the reproductive and developmental effects in animals following aspartame exposure, the mixture of some or all of the three metabolites could act together to generate the effects. The Panel considered this is unlikely based on the following observations:

- reproductive and developmental effects observed with either phenylalanine or aspartame were similar
- these effects observed with phenylalanine and aspartame were seen at equivalent molar doses
- no effects were observed with aspartic acid administered alone
- co-administration of aspartic acid with phenylalanine did not add to the effect of phenylalanine alone. The respective administered doses of aspartic acid and phenylalanine were equivalent to an aspartame dose causing reproductive and developmental effects
- although similar reproductive and developmental effects were observed with methanol as with aspartame and phenylalanine, the doses of methanol causing these effects were 10-fold greater than the doses of aspartame or phenylalanine.

If one disregards these observations and assume that methanol and phenylalanine could interact following aspartame ingestion, based on the dose response data for all three compounds (aspartame, phenylalanine, methanol) and using conservative dose addition assumptions, the Panel considered that combined administration of methanol and phenylalanine (either from aspartame or with co-administration at equivalent amounts) could add at most 10% to the activity of phenylalanine alone (Table 22).

Table 22: Doses of aspartame metabolites having molar equivalence to aspartame from the critical studies E90 (rabbit) and Rogers et al., 1993 (mouse)

	Dose (of the substance as used in the study) or *equivalent dose* (mg/kg bw/day)			
Aspartame	**2000***	*1464*	*2444*	*20 000*
Phenylalanine	*1120*	**820***	-	-
Aspartic acid	*900*	-	**1100***	-
Methanol	*200*	-	-	**2000****

Doses in bold in the table were effect levels with the exception of aspartic acid for which 1100 mg/kg bw/day was the highest dose tested

* Data from E90 study

** Data from Rogers et al., 1993

11.2. Assessment of postulated Mode of Action

The Panel evaluated the plausibility and human relevance of the proposed mode of action using the criteria developed by Boobis et al., 2008.

11.2.1. Life stage considerations

The key effects observed in rats and rabbit reproductive studies related to a specific life stage acknowledged to be critical in both species observed and to humans, thus the effects were considered as relevant for risk assessment. A spectrum of effects was observed in the experimental species, particularly maternal toxicity and growth restriction of the offspring. The latter effect was recognised as an important outcome in humans because it was associated with an increased risk of perinatal mortality and morbidity, including perinatal asphyxia (Barker, 2001). Moreover, there was epidemiological evidence that human fetal growth restriction correlates with adverse effects in

adulthood. For example, affected individuals have an increased incidence of metabolic syndrome, manifesting as obesity, hypertension, hypercholesterolemia, cardiovascular disease, and type-2 diabetes (Barker, 1997).

The Panel noted that neurodevelopment endpoints had not been extensively measured in the animal studies, acknowledging that this would have provided highly relevant information on the mental retardation seen in PKU children.

11.2.2. Human relevance

A wealth of evidence exists from the human population (maternal PKU syndrome) showing that increased phenylalanine plasma levels during pregnancy result in adverse effects in the offspring, including mental retardation, microcephaly, congenital heart disease, and intrauterine growth retardation. It was highly plausible that the proposed mode of action for adverse effects observed in animals during pregnancy would be relevant to humans if sufficiently high aspartame exposures occurred.

Lenke and Levy (1980) reported results from over 500 pregnancies of women with PKU who were not on low-phenylalanine diets during pregnancy. Infants born to mothers with maternal PKU syndrome had a high rate of birth defects and developmental disabilities: congenital heart disease, mental retardation, facial dysmorphism and fetal growth restriction.

Data from the prospective international Maternal PKU Collaborative (MPKUC) Study (Koch et al., 2003), the French Survey (Feillet et al., 2004), and the United Kingdom PKU Registry (Lee et al., 2005) showed that many features of the maternal PKU syndrome were preventable when dietary phenylalanine intake was restricted before conception or soon after conception (Maillot et al., 2008; Prick et al., 2012).

In contrast to these results are the findings of Teisser et al. (2012) who reported data from 115 pregnancies in 86 women. Ninety percent of women had been informed of the risk of maternal PKU in the absence of a strict diet during pregnancy, 88 % of women started a phenylalanine restricted diet before conception, and 45 % of infants were born small for gestational age (birth length and/or weight $\leq$ 2 SD). Phenylalanine intakes were lower in the group with fetal growth restriction from the fifth to the eighth month of pregnancy and duration of time spent at plasma phenylalanine levels $\leq$ 120 µM during pregnancy was associated with a higher risk of fetal growth restriction. However, the Panel noted that as phenylalanine is an essential amino acid, it was likely that the increased risk of fetal growth restriction observed at low phenylalanine plasma levels in this study was due to dietary over restriction, i.e. providing not enough phenylalanine to allow normal development of the fetus.

11.2.3. Conclusion on the MoA analysis

The Panel considered that the proposed mode of action was credible and highly likely to explain the results seen in the animal studies with aspartame based on:

- the metabolic data on the complete hydrolysis of aspartame in the gastrointestinal tract
- the known reproductive and developmental toxicity of high phenylalanine blood concentrations in several species
- data demonstrating that administered doses of phenylalanine and aspartame (having molar equivalence) had similar reproductive and developmental toxicity
- that effects were not seen with administered doses (having molar equivalence to aspartame) of either aspartic acid or methanol alone
- the absence of additive effects of aspartame metabolites.

Despite uncertainties from the limited animal kinetic data, and in the human dose response data, the Panel considered that the proposed mode of action was relevant to humans based on the data from PKU patients.

Therefore, for the risk characterization of aspartame for humans, the Panel decided that the information on effects and dose response in PKU patients and human pharmacokinetic data were more appropriate than the results of animal studies of reproductive and developmental toxicity.

12. Dose-response modelling of plasma phenylalanine levels following aspartame administration

Data on the concentrations of phenylalanine in plasma after different doses of aspartame were extracted from various studies, mainly unpublished studies submitted in response to EFSA's call for data. These studies were principally conducted in the 1970s as part of the original evaluations of aspartame; the details of the studies are described in section 3.1. The studies involved a range of dose levels given as a single bolus administration. The time to reach peak plasma phenylalanine concentration (C_{max}) was comparable at all doses and varied from 30 minutes to 1 hour; thereafter levels fell sharply. The number of participants in each study differed, ranging from 6 to 20 (Appendix J).

Studies were conducted in three subsets of the population; individuals with 'normal' phenylalanine metabolism, heterozygous for phenylalanine hydroxylase, i.e. those with one recessive allele and those who are homozygous, i.e. individuals with PKU syndrome. The data (C_{max} values) from studies in normal and heterozygous individuals were used to generate a model that could be used in risk characterization. The data from PKU homozygotes were not used as these individuals are unrepresentative of the general population and specific labelling of aspartame containing products is required to permit these individuals to control dietary phenylalanine intake, which is a risk management issue.

In specifying the modelling parameters, the Panel made various decisions. These decisions are described below together with the rationale for their choice, but were intended to reflect the 'worst case' scenario. Consequently, the model would be expected to incorporate an additional margin of safety.

The first decision was to use data after a single bolus dose to represent the total daily aspartame intake, whilst acknowledging that normal consumption patterns would be in smaller quantities spread throughout the day. The administration of a bolus dose will overestimate the peak plasma concentration resulting from a daily dietary intake at the corresponding dose level. All the administration was as bolus doses which ranged from 4 to 200 mg/kg bw. This range included the current ADI of 40 mg/kg bw/day.

The second decision was to use the measured peak plasma levels (C_{max} levels) following a single bolus administration as a surrogate for the response at the administered dose. Phenylalanine has a short half-life in plasma of approximately 1.7 hours (Filer and Stegink, 1991). The peak plasma levels were transient, and the Panel noted that these would be higher than steady state plasma levels arising from repeated intake at normal pattern of dietary intake of the same total daily dose.

The third decision was to base comparisons on the mean plasma levels, which were assumed clinically safe for the critical endpoint (effects during pregnancy) in PKU patients. This subgroup of the population build up higher phenylalanine plasma levels which makes them more susceptible than the normal and heterozygous subgroups to adverse effects from phenylalanine during pregnancy. The plasma levels upon which the clinical advice is based are regarded as a threshold but the true threshold for adverse effects from phenylalanine would be expected to be higher. It was acknowledged by the Panel that it is not known whether effects seen at levels above the clinical target threshold were associated with the mean or peak plasma levels. The Panel noted that in PKU patients even with good dietary control of phenylalanine intake, plasma levels of phenylalanine can be highly variable and can result in either higher than targeted mean plasma levels or very high spike peak plasma levels. For PKU patients it is recognised that mild effects have been associated with mean plasma levels of 600-

800 µM, whilst significant detrimental effects have been associated with both mean and spike peak plasma levels of 1100-1200 µM phenylalanine (Rouse et al., 2000; Koch et al., 2003).

Current clinical practice guidelines recommend that PKU patients restrict their dietary intake of phenylalanine to keep plasma levels below 360 µM. The Panel noted that intakes of aspartame as a food additive could occur at the same time as other dietary phenylalanine sources. Therefore, the Panel considered that the threshold utilised for comparisons to the modelling should be lowered to allow for simultaneous intake of the food additive with meals. In toddlers it can be assumed that the mean daily exposure to phenylalanine from diet is taken up in five meals, in children in four meals, in adolescence, adults and the elderly in three meals rendering the phenylalanine intake per kg bw and meal into 18.6-33.4 mg/kg bw/meal (toddlers), 18.1-34.2 mg/kg bw/meal (children), 13.0–26.3 mg/kg bw/meal (adolescents), 11.8-19.6 mg/kg bw/meal (adults), and 10.8–13.9 mg/kg bw/meal (elderly). The highest mean dietary phenylalanine exposure per meal is 34.2 mg/kg bw (Table 16) and this corresponds to a phenylalanine plasma concentration of 120 µM (as calculated from the dose-plasma phenylalanine modelling carried out and considering that phenylalanine represents 56 % by weight of aspartame). Thus, the plasma phenylalanine from the diet (120 µM) has been subtracted from the current clinical guideline of 360 µM to determine the maximum safe plasma concentration of phenylalanine that can be derived from aspartame (240 µM). The Panel noted that this plasma concentration was above normal phenylalanine plasma concentrations in man and therefore represents an overestimation of the phenylalanine intake from the regular diet (Levy, 2003).

The Panel initially modelled the data for normal individuals, comparing dose with the highest peak plasma levels of phenylalanine in individuals to determine whether it was feasible to undertake dose-response modelling. Having considered the output from this initial model, the Panel considered that whilst modelling was feasible, a more sophisticated statistical model was appropriate. Subsequent modelling included multiple studies at several dose levels and allowed for the use of data from different studies and different numbers of subjects within the individual studies. The data were used in a model developed by the SAS Unit at EFSA and an internal report was provided to the Panel (Appendix K). The model enabled an estimation to be determined of the plasma levels associated with the mean and 95th percentile confidence limits over the dose range studied.

Based on the model, a plasma phenylalanine concentration of 240 µM would result from the administration of a bolus dose of 103 mg aspartame/kg bw (lower bound distributions: 88 mg aspartame/kg bw (CI 59-125) using a confidence level of 0.95) to a normal subject. For an individual heterozygous for PKU, the concentration would be reached by the administration of a bolus dose of 59 mg aspartame/kg bw (lower bound distributions: 50 mg aspartame/kg bw (CI 28-69) using a confidence level of 0.95. The Panel considered that given the conservative assumptions, realistic dietary intake of aspartame and the confidence intervals provided by the modelling, the peak plasma phenylalanine levels would not exceed the clinical target threshold when a normal individual consumed aspartame at levels below the current ADI of 40 mg/kg bw/day.

The Panel noted that studies on the effect of hourly ingestion of 600 mg aspartame over a period of eight hours by normal individuals have shown that plasma phenylalanine levels did not exceed the normal postprandial range (Stegink et al., 1989). This dose corresponded to approximately 80 mg aspartame/kg bw or twice the current ADI, in one day. Moreover, using a published steady state model to compute the effect of repeated dosing of aspartame on plasma phenylalanine levels (Stegink et al., 1989), the Panel predicted that bolus administration of 40 mg/kg bw (the current ADI) on an hourly basis was required to achieve steady state plasma levels greater than 240 µM.

The Panel therefore concluded that there would not be a risk of adverse effects on pregnancy in the general population including heterozygous individuals at the current ADI. The Panel noted that the use of a sensitive subpopulation (PKU patients) obviated the need for a toxicodynamic uncertainty factor (UF), for inter-individual variability. The Panel concluded that the use of the 95th percentile confidence level of the lower bound estimate in the dose-response modelling of plasma phenylalanine concentrations from heterozygotes replaced the toxicokinetic default UF.

13. Risk characterisation

Based on the discussion of the aspartame toxicity database (Section 4) the Panel concluded that there were two key endpoints in the animal data for characterising the risk from aspartame consumption. These endpoints were: 1) reproductive and developmental toxicity; 2) chronic toxicity and carcinogenicity. The Panel concluded from the animal and human ADME data that aspartame was rapidly and completely hydrolysed in the GI tract to methanol and the amino acids phenylalanine and aspartic acid. The Panel concluded that any systemic effects following aspartame ingestion must be due to effects of these metabolites rather than aspartame *per se*.

When considering all the genotoxicity, chronic toxicity and carcinogenicity studies on aspartame the Panel overall concluded that there was no convincing evidence for genotoxic or carcinogenic potential of aspartame in experimental animals. In the hazard identification and characterisation, the Panel has discussed the ERF studies performed in mice and rats and confirmed the conclusion of previous assessments that there were major deficiencies in these studies and as such, they were not suitable for hazard identification and characterisation. Based on the chronic toxicity and carcinogenicity data, the Panel concurred with the NOAEL of 4000 mg aspartame/kg bw/day previously identified by JECFA, the SCF and other assessors.

In a Weight-of-Evidence approach, the Panel considered the database on the toxicity of methanol, including also *in vivo* studies by inhalation and intraperitoneal administration. Taken together the Panel concluded that the data set was limited but that the available reliable *in vitro* and *in vivo* data did not indicate a genotoxic potential of methanol.

The oral studies on chronic toxicity and carcinogenicity of methanol were limited to a mouse study reported by Apaja (1980) and a rat study performed by the ERF and reported by Soffritti et al. (2002).

Overall, the Panel concluded that the study by Apaja did not contribute to the assessment of the carcinogenic potential of methanol.

As already discussed in section 4, the validity of the ERF study on methanol has recently been criticised (Cruzan, 2009; Schoeb et al., 2009; Schoeb and McConnell, 2011; NTP-EPA, 2011). The Panel concurred with the concerns identified by EPA and others, and did not support the validity of the conclusions in the study on methanol reported by Soffritti et al. (2002). The Panel concluded that the ERF rat study was not a suitable basis for the cancer risk assessment of methanol.

The reproductive and developmental toxicity database on methanol was limited. As described in section 5 and discussed in section 6, the studies in rats and monkeys were not adequate to derive a NOAEL. In mice, one prenatal developmental toxicity study by inhalation (in which a high oral dose was also tested) and two oral prenatal developmental studies (at high dose levels) were identified. Oral studies on methanol performed at high dose levels (4000 or 5000 mg/kg bw/day) did not allow the Panel to identify a NOAEL for reproductive and developmental toxicity. Therefore the Panel calculated a NOAEL of approximately 560 mg/kg bw/day for oral methanol exposure from the NOAEC of 1300 mg methanol/m^3 from inhalation study in mice (Rogers et al., 1993). The Panel considered the NOAEL of 560 mg/kg bw/day as conservative because it was based on the presence of extra cervical ribs which are transient and disappear during further development of the pup.

The Panel noted that the lowest calculated NOAEL for methanol by oral exposure is 140 -fold higher than the maximum amount of methanol that could be released when aspartame is consumed at the ADI.

Overall, the Panel concluded that the data on toxicity, kinetics, reproductive and developmental toxicity, genotoxicity and carcinogenicity of methanol do not suggest that there was a risk from methanol derived from aspartame at the current exposure estimates, or at the ADI of 40 mg/kg bw/day.

The Panel noted that aspartic acid is itself a neurotransmitter and can be converted to the more potent neurotransmitter glutamate. The Panel did not see any convincing evidence of neurotoxicity associated with the consumption of aspartame. The Panel concluded that aspartic acid generated from aspartame was not of safety concern at the current exposure estimates, or at the ADI of 40 mg/kg bw/day.

The Panel considered that adverse effects reported for aspartame in animal studies could be attributed to the metabolite phenylalanine, which was particularly the case for the rat and rabbit developmental toxicity studies. The Panel noted that adverse developmental effects were seen in children born to PKU patients and that these effects appeared to be related to maternal phenylalanine levels. The Panel was aware that the knowledge on effects of phenylalanine in PKU mothers and their children both before and after birth had developed considerably since the initial evaluation of aspartame.

The Panel undertook a formal MoA analysis of the putative role of phenylalanine in the developmental toxicity seen in animal studies. This MoA analysis is described in Section 11.

The Panel considered that the proposed MoA was plausible and relevant based on the weight-of-evidence based on the available data summarised in the opinion. There were uncertainties from the limited kinetic data in animals and in the human aspartame dose-phenylalanine concentration response data. The Panel decided to base the risk characterisation on comparison of plasma phenylalanine levels following aspartame administration with plasma phenylalanine levels associated with developmental effects in children born from PKU mothers. The Panel decided these human data were more appropriate than the results of animal studies of reproductive and developmental toxicity for the risk characterization of aspartame.

Having established that the MoA was plausible and relevant, the Panel reviewed the information on plasma levels of phenylalanine associated with adverse effects on the fetuses of PKU mothers and noted that current clinical guidelines recommend that plasma levels of phenylalanine should be maintained below an average value of 360 µM. The Panel was aware that there continues to be discussion and research about the actual threshold and whether this was associated with average or peak plasma concentrations of phenylalanine.

The Panel modelled peak plasma concentrations of phenylalanine following a bolus administration of aspartame to normal and PKU heterozygous individuals. The Panel chose assumptions, which overestimated the plasma concentrations of phenylalanine associated with administration of aspartame. These are discussed in more detail in Section 12. The Panel considered that comparing peak plasma concentrations derived in this way should be regarded as a conservative/worst case scenario. In comparing these values to the plasma phenylalanine concentrations that have no effect on the fetuses of PKU mothers, the Panel took into account that aspartame could be consumed at the same time, as a meal that would contain proteins which when digested would release phenylalanine. In calculating a safe level of aspartame exposure (based on plasma phenylalanine concentrations), the Panel assumed the worst-case scenario that intake of aspartame occurs in combination with the meal which leads to circulating plasma phenylalanine concentrations of 120 µM (the maximum plasma concentration based on conservative assumptions of dietary exposure to phenylalanine). The concentration of plasma phenylalanine derived from aspartame was therefore set to 240 µM (i.e. 360 µM minus 120 µM) by the Panel.

Based on the modelling, a plasma phenylalanine concentration of 240 µM would result from the administration of a bolus dose of 103 mg aspartame/kg bw (lower bound distributions: 88 mg aspartame/kg bw, 95th percentile, CI 59-125) to a normal subject. For a PKU heterozygous individual the concentration of 240 µM would be reached by the administration of a bolus dose of 59 mg aspartame/kg bw (lower bound distributions: 50 mg aspartame/kg bw, 95th percentile, CI 28-69). The Panel considered that given the conservative assumptions and the confidence intervals provided by the modelling, for realistic dietary intake of aspartame, the peak plasma phenylalanine levels would not exceed 240 µM.

The Panel noted that in the normal population the 95th percentile confidence interval for the lower bound of the dose for resulting in a peak plasma level of 240 µM following a bolus administration of aspartame was greater than 40 mg/kg bw (the current ADI). In the PKU heterozygous population the 95th percentile confidence interval for the lower bound of the dose for resulting in a peak plasma level of 240 µM following a bolus administration of aspartame was greater than 40 mg/kg bw (the current ADI) in 82 % of the simulations. The Panel considered that following bolus administration of aspartame of 40 mg/kg bw (the current ADI) the PKU heterozygous population would not exceed the current clinical guideline of 360 µM for plasma phenylalanine.

The Panel also noted that in order to exceed the phenylalanine plasma concentration of 240 µM following repeated administration of aspartame in normal individuals, a bolus administration at 40 mg/kg bw (equivalent to the current ADI) would need to be given every hour.

The Panel considered the following:

- the conservative assumptions used in the modelling, which would all overestimate peak plasma concentrations
- the available information on adverse effects in development in humans with PKU
- the allocation of 2/3 of the current clinical guideline level of 360 µM phenylalanine in plasma to phenylalanine from ingested aspartame, in order to account for simultaneous ingestion of phenylalanine from other components of the diet
- results of the modelling
- kinetic data from repeated oral administration of aspartame in humans
- bolus intakes based on consumption of one litre of soft drink containing aspartame at the MPL of 600 mg/L by a child of 20-30 kg will not exceed 30 mg aspartame/kg bw.

Based on these considerations and evaluations, the Panel concluded that under realistic conditions (i.e. not a single bolus dose of 40 mg/kg bw), phenylalanine plasma levels would not exceed 240 µM in normal or PKU heterozygous individuals. The Panel noted that this concentration was well below the concentrations at which adverse effects in the fetus were reported and was also below the current clinical guideline (360 µM) for prevention of effects in the fetuses of pregnant PKU patients. The Panel noted that in young children who did not suffer from PKU, plasma levels of phenylalanine resulting from aspartame ingestion at or below the ADI (as either a bolus or other aspartame consumption patterns) were likely to remain below 240 µM. For pregnant women, the Panel noted that there was no risk to the fetus from phenylalanine derived from aspartame at the current ADI (40 mg/kg bw/day) in normal or PKU heterozygous individuals.

The Panel noted that it was currently not possible to include chronic endpoints in the postulated MoA. The Panel noted that the ADI previously derived by JECFA and the SCF of 40 mg/kg bw/day appeared to be based on the long-term animal studies using the default uncertainty factor of 100. The Panel considered that this remained appropriate for the evaluation of long-term effects of aspartame. The current evaluation was based on analysis of human reproductive and developmental effects of phenylalanine in PKU patients, who are more susceptible to dietary phenylalanine than the general and PKU heterozygous population. Therefore, no additional allowance for toxicodynamic variability was required. The modelling of the aspartame dose - phenylalanine concentration response was based on data from PKU heterozygous individuals who at any dose would have a higher plasma phenylalanine concentration than the normal population; therefore no additional allowance for toxicokinetic variability was required for the general population. The Panel concluded that exposures at or below the

current ADI were not of safety concern for reproductive and developmental toxicity in humans excluding PKU homozygous individuals.

The Panel concluded from the present assessment of aspartame that there were no safety concerns at the current ADI of 40 mg/kg bw/day. Therefore, there was no reason to revise the ADI for aspartame.

The Panel emphasised that its evaluation of phenylalanine plasma levels from a dose of aspartame at the ensuing ADI is not applicable to PKU patients. These individuals require total control of dietary phenylalanine intake to manage the risk from elevated phenylalanine plasma levels. The Panel noted it is a requirement of EU legislation that products containing aspartame indicate through labelling that they contain a source of phenylalanine.

Conservative estimates of exposure to aspartame made by the Panel for the general population were up to 36 mg/kg bw/day at the 95th percentile. These were below the ADI. The Panel noted that the percentage of PKU heterozygous individuals with a lower bound below 36 mg would be considerably lower. Due to the conservatism of both the exposure assessment and the aspartame dose-phenylalanine concentration response modelling, the Panel considered that it was highly unlikely that any individual in the normal and PKU heterozygous population would have plasma levels of phenylalanine above 240 μM following aspartame ingestion. Based on the available data, the Panel further considered that even in combination with diet, these aspartame intakes (either as a single or repeated dose) would not lead to peak plasma phenylalanine concentrations above the current clinical guideline for prevention of adverse effects on the fetuses of PKU mothers of 360 μM.

The Panel noted that the current ADI for DKP is 7.5 mg/kg bw/day. Based on the current specification for DKP in aspartame (1.5 %), estimates of DKP exposure at the ADI for aspartame were below the ADI for DKP. Based on available data on DKP levels in food products following breakdown of aspartame (up to 24 % from soft drinks), the Panel estimated high level exposure for the general population as ≤ 5.5 mg DKP/kg bw/day at the 95th percentile, which is still below the ADI for DKP.

14. Uncertainties, inconsistencies and data gaps

In the postulated MoA approach, the developmental effects of aspartame mainly seen in rabbits were attributed to L-phenylalanine. However, the Panel acknowledged that the effects seen in rabbits receiving aspartame may also be due to a significant reduction in feed intake during pregnancy. The aborting rabbits from the high dose group manifested body weight loss and decreased to negligible feed consumption, compared to those that maintained their pregnancies. The Panel noted that such a marked reduction in feed intake may have had a negative effect on the nutritional status of these females as a result of gastrointestinal disturbances and the potential effect of phenylalanine. Because the decreased feed consumption occurred in both aspartame- and phenylalanine-dosed animals, the Panel was unable to determine how much of the reported effects on the maternal organism and fetus were due to a reduced nutrient intake, and therefore to a negative effect on the nutritional status of these females as a result of gastrointestinal disturbances, and how much to a direct effect of the high phenylalanine intake (from metabolism of aspartame).

The Panel concluded that the high phenylalanine intake (from metabolism of aspartame) could at least in part be responsible for these effects in the high dose aspartame group because similar effects, although less severe, were seen in the phenylalanine-dosed animals.

The Panel noted that there were no measures of plasma phenylalanine levels from the rat and rabbit reproductive studies with aspartame. This constituted a data gap in animals as the contemporaneous biochemical measures would increase confidence in the assumption that phenylalanine was responsible for the effects seen. However, the Panel took into consideration available human data on plasma phenylalanine levels from highly susceptible subpopulation showing that phenylalanine has concentration – related developmental effects (see section 11.2.2).

Due to the limitations (in terms of dose and time points) in the kinetic data in animals, phenylalanine levels were not modelled. From the data available following aspartame administration, it was possible to model reliably the peak plasma phenylalanine levels. Although potentially these could have been used to predict steady state concentrations of phenylalanine, had a more comprehensive dataset been available steady state phenylalanine levels could have been modelled more reliably and this would have been undertaken.

The Panel excluded from its evaluation PKU patients, since these individuals require total control of dietary phenylalanine intake to manage the risk from elevated phenylalanine plasma levels.

The following main assumptions were made by the Panel in the present risk characterization (based on the MoA):

- Phenylalanine plasma level of 360 µM is the threshold for developmental effects.
- The diet results in phenylalanine plasma level not exceeding 120 µM.
- Peak plasma phenylalanine concentration can be used in the dose-response modelling as surrogate of steady-state plasma phenylalanine concentration.
- Bolus administration of aspartame can be used in the dose-concentration modelling of plasma phenylalanine to represent a more typical pattern of aspartame intake.
- The 95th percentile confidence interval of the lower bound estimate of the aspartame dose-plasma phenylalanine concentration curve provides a safe limit for plasma phenylalanine for the entire population (with the exception of homozygous PKU patients).
- The increase in plasma phenylalanine concentrations following aspartame administration will be the same in the general population as in individuals heterozygous for PKU.
- Reproductive and developmental toxicity of aspartame is solely dependent on systemic exposure to phenylalanine.
- There is no requirement for a pharmacodynamic uncertainty factor (a sensitive human population (PKU patients) was used to define the threshold).
- There is no requirement for a pharmacokinetic uncertainty factor (the aspartame plasma phenylalanine concentration was based on a more sensitive human sub-population (PKU heterozygous)).

The Panel was not able to place a specific numerical value on the uncertainties related to these assumptions, but the Panel considered that these assumptions would be more likely to overestimate than underestimate any potential developmental risk. Therefore the Panel considered that the results of uncertainty analysis further support its conclusion, that there is no safety concern for aspartame at the current ADI in normal and heterozygous subjects.

CONCLUSIONS

The Panel concluded that chronic toxicity and reproductive and developmental toxicity were the critical endpoints in the animal database. The Panel considered that the evaluation of long-term effects of aspartame should continue to be based on the animal data. Based on a MoA analysis and the weight-of-evidence, the Panel considered that the reproductive and developmental toxicity in animals was due to phenylalanine released from aspartame and concluded that the basis for evaluation of the reproductive and developmental endpoint should be the available data in humans.

Conservative estimates of exposure to aspartame made by the Panel for the general population were ≤ 36 mg/kg bw/day at the 95th percentile.

Due to the conservatism of both the exposure assessment and the aspartame dose-phenylalanine concentration response modelling, the Panel considered that it was highly unlikely that any individual in the normal and PKU heterozygous population would have plasma levels of phenylalanine above 240 µM following oral aspartame exposure up to the ADI of 40mg/kg bw/day. The Panel further considered that even in combination with diet, these aspartame intakes would not lead to peak plasma

phenylalanine concentrations above 360 μM, the current clinical guideline for prevention of adverse effects on the fetuses of PKU mothers.

The Panel concluded from the present assessment of aspartame that there were no safety concerns at the current ADI of 40 mg/kg bw/day. Therefore, there was no reason to revise the ADI for aspartame.

The Panel emphasised that its evaluation of phenylalanine plasma levels from a dose of aspartame at the ensuing ADI is not applicable to PKU patients. These individuals require total control of dietary phenylalanine intake to manage the risk from elevated phenylalanine plasma levels.

DOCUMENTATION PROVIDED TO EFSA

1) List of unpublished study submitted to EFSA following the public call for data.

E1	Sc-18862: A Sweetening Agent	1972	Ajinomoto and Nutrasweet
E2	Sc-18862: Four Week Oral Tolerance Study In The Mouse	1972	Ajinomoto and Nutrasweet
E3	Sc-18862: Four Week Oral Tolerance Study In The Rat	1972	Ajinomoto and Nutrasweet
E4	Sc-18862: Nine Week Oral Toxicity Study In The Rat	Not reported	Ajinomoto and Nutrasweet
E5	Sc-18862: Evaluation Of Embryotoxic And Teratogenic Potential In The Rat	1970	Ajinomoto and Nutrasweet
E6	Sc-19192: Two Week Oral Toxicity Study In The Mouse	1971	Ajinomoto and Nutrasweet
E7	Sc-19192: Two Week Oral Toxicity Study In The Rat	1971	Ajinomoto and Nutrasweet
E8	Sc-19192: Five Week Oral Toxicity Study In The Rat	1972	Ajinomoto and Nutrasweet
E9	Sc-18862: Toxicological Evaluation In The Neonatal Rat (Final Report)	1972	Ajinomoto and Nutrasweet
E10	Toxicological Evaluation Of Sc-18862: Evaluation Of Reproductive Performance. Segment I Of The Teratology-Reproduction Profile	1972	Ajinomoto and Nutrasweet
E11	Sc-18862: Two Generation Reproduction Study - Rats (Final Report)	1971	Ajinomoto and Nutrasweet
E12	Sc-18862: Mutagenic Study In Rats (Final Report)	1970	Ajinomoto and Nutrasweet
E13	Sc-19192: Segment III - Perinatal Weaning Study In The Rat (Final Report)	1972	Ajinomoto and Nutrasweet
E14	Behavioral Effects Of Chronic Feeding Of L-Phenylalanine And Sc-18862 To Weanling Rats	1972	Ajinomoto and Nutrasweet
E15	The Metabolism Of Aspartame - Volume I Parts I-XIV	1972	Ajinomoto and

			Nutrasweet
E16	Sweetening Agents (Bibliography)	Not reported	Ajinomoto and Nutrasweet
E17	The Metabolism of Aspartame - Volume 2 Parts XV-XIX	1972	Ajinomoto and Nutrasweet
E18	The Metabolism of Aspartame - Volume 3 Parts XX-XXIII	1972	Ajinomoto and Nutrasweet
E19	Sc-18862: A Sweetening Agent (Endocrine Studies)	Not reported	Ajinomoto and Nutrasweet
E20	Sc-18862: 2 Month Oral Administration - Rats (Final Report)	1969	Ajinomoto and Nutrasweet
E21	Sc-18862: 2 Month Oral Toxicity - Dogs (Final Report)	1969	Ajinomoto and Nutrasweet
E22	Chicken Embryo Study Sc-18862 Calcium Cyclamate Sucrose (Final Report)		Ajinomoto and Nutrasweet
E23	Short Term Tolerance Of Aspartame By Normal Adults	1972	Ajinomoto and Nutrasweet
E24	Short Term Tolerance Of Aspartame By Obese Adults	1972	Ajinomoto and Nutrasweet
E25	Short Term Tolerance Of Aspartame By Adult Pku Heterozygotes	1972	Ajinomoto and Nutrasweet
E26	Tolerance Of Loading Doses Of Aspartame By Phenylketonuric (Pku) Homozygous Children	1972	Ajinomoto and Nutrasweet
E27	Sc-18862: 46 Week Oral Toxicity Study In The Hamster	1972	Ajinomoto and Nutrasweet
E28	Sc-18862: 106 Week Oral Toxicity Study In The Dog	1972	Ajinomoto and Nutrasweet
E29	Sc-18862 and 19192 (3:1 Ratio): Segment II Teratology Study In The Rabbit (Final Report)	1972	Ajinomoto and Nutrasweet
E30	Sc-19192: An Evaluation Of Mutagenic Potential Employing The In vivo Cytogenetics Method In The Rat (Final Report)	1972	Ajinomoto and Nutrasweet
E31	Sc-19192: An Evaluation Of Mutagenic Potential Employing The Host-Mediated Assay In The Rat (Final Report)	1972	Ajinomoto and Nutrasweet
E32	Sc-18862: 52 Week Oral Toxicity Study In The Infant Monkey	1972	Ajinomoto and Nutrasweet
E33	Sc-18862: Two Year Toxicity Study In The Rat (Annex)	1973	Ajinomoto and Nutrasweet
E34	Sc-18862: Two Year Toxicity Study In The Rat (Final Report)	1973	Ajinomoto and Nutrasweet

E35	Sc-18862: 46 Week Oral Toxicity Study In The Hamster (Supplement N°1 Microscopy For Biological Research, Ltd Histopathology Report - Part I)	1972	Ajinomoto and Nutrasweet
E36	Sc-18862: 46 Week Oral Toxicity Study In The Hamster (Supplement N°1 Microscopy For Biological Research, Ltd Histopathology Report - Part II)	1972	Ajinomoto and Nutrasweet
E37	Sc-19192: Evaluation Of Reproductive Performance In The Rat - Segment I Of The Teratology - Reproduction Profile	1973	Ajinomoto and Nutrasweet
E38	Sc-19192: An Evaluation Of The Embryotoxic And Teratogenic Potential In The Rat - Segment II Of The Teratology-Reproduction Profile	1973	Ajinomoto and Nutrasweet
E39	Sc-18862: A Study Of The Pregnant And Lactating Rat And Of Her Offspring - Segment III Of The Teratology Reproduction Profile	1973	Ajinomoto and Nutrasweet
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UA01	Report Of The Scientific Committee For Food On Sweeteners	1984	Ajinomoto
UA02	Opinion Of An Expert Panel On The Safety Status Of Aspartame As A Non-Nutritive Sweetener (Final)	2006	Ajinomoto
UA03	Food Additive Approval Process By FDA Followed For Aspartame (GAO Report To Senator Metzenbaum)	1987	Ajinomoto

UA04	Histopathological Examination In A Carcinogenecity Study Of Sc-18862 In Slc: Wistar Rats (Final Report)	2006	Ajinomoto
UN01	Final Clinical Research Report. Effects Of Aspartame Ingestion Without Food On Serum Glucose, Insulin, And Glucagon Concentrations In Normal Subjects And Subjects With Diabetes	1988	Nutrasweet
UN02	Aspartame, Brain Amino Acids And Neurochemical Mediators. IlSI International Aspartame Workshop, November 17-21, 1986, Marbella, Spain.	1986	Nutrasweet
UN03	Toxicology Studies Done With Beta-Aspartame (Revised). IlSI International Aspartame Workshop, November 17-21, 1986, Marbella, Spain.	1986	Nutrasweet
UN04	Clinical Studies To Evaluate Seizures In Children And Adults Allegedly Due To Aspartame Consumption (N15-85-02-109 And N16-85-02-110)	1991	Nutrasweet
UN05	Effect Of Repeated Ingestion Of Beverage Containing Aspartame Or Diketopiperazine On Amino Acid Or Diketopiperazine Concentrations In Plasma And Urine Of Normal Healthy Subjects (N02-84-02-075).	1987	Nutrasweet
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UN09	A Study Of The Clinical And Biochemical Effects Of Aspartame In Subjects With Chronic Stable Alcoholic Liver Disease (N23-87-02-001)	1991	Nutrasweet
UN10	A Randomised, Double-Blind, Placebo-Controlled Approach To Evaluate The Potential Of Chronic High Dose Aspartame Consumption To Influence Psychometric Performance And Mood In Normal Healthy Volunteers (Pcr 0195). Vol I, II, III, IV.	1993	Nutrasweet
UN11	Study of aspartame in carbonated beverages	1986	Nutrasweet
UN12	Nutrasweet's response to FDA following a communication dated April 25, 1989 requesting additional information on the use of encapsulated aspartame in baked good	1990	Nutrasweet
UN13	Use of aspartame as flavor enhancer in beer containing less than 3% alcohol by volume		Nutrasweet
UN14	Asparatame in breakfast cereals	1987	Nutrasweet

UN15	Aspartame in soft candy. Appendix III	1987	Nutrasweet

2) The list of published studies submitted to EFSA following the public call for data is published on the EFSA website (http://www.efsa.europa.eu/en/dataclosed/call/110601.htm).

3) Additional information submitted to EFSA following the public call for data:

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APPENDICES

Appendix A. Identification and selection criteria for scientific data consideration for the re-evaluation of aspartame

For the risk assessment of aspartame, the Panel applied the general principles of the risk assessment procedure as follows:

- The potential health effects of aspartame or its metabolites and degradation products identified and characterised on the basis of the available scientific studies and literature (hazard identification and characterisation).
- An exposure assessment for aspartame and its degradation products performed in order to compute the current level of intake of the aspartame, its metabolites and degradation products in the population.

The principles stated in the following EFSA publications were applied in the various steps of the process:

- EFSA, 2009. Guidance of the Scientific Committee on transparency in the scientific aspects of risk assessments carried out by EFSA. Part 2: General principles. The EFSA Journal 1051, 1-22.
- EFSA Scientific Committee, 2011. Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment. EFSA Journal 2011;9(9):2379.
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- EFSA Scientific Committee, 2012. Scientific Opinion on Risk Assessment Terminology. EFSA Journal 2012;10(5):2664.
- EFSA, 2010. Strategy of the ANS Panel for the re-evaluation of food additives. Available online: http://www.efsa.europa.eu/en/events/event/ans100413-m.pdf

The Panel considered the criteria developed by Boobis et al. (2008) in the mode of action approach.

Identification

The collection of the scientific information used as a basis for the aspartame risk assessment followed three main roots:

- Search in scientific databases aimed at identifying studies that have appeared in scientific literature such as journals, conference proceedings, editorials, letters.
- 'Open call for data' procedure adopted as a standard practice by EFSA to collect scientific information either unpublished or published such as study reports or case reports.
- Previous scientific evaluations by national agencies, national and international independent expert advisory committees.

The collection of the scientific information through searching scientific literature databases was performed to cover the period between 1 January 1970 and 15 February 2013. In order to update the database, a search was conducted on a monthly basis until 15 November 2013.

An exhaustive complete search strategy was used with the search terms described below, with no limits applied to the search. Web of Science covers over 46 million records contained in 30 000 scholarly books, 12 000 journals and 148 000 conference proceedings while PubMed covers over 23 million records.

Search term	**Search type**	**To capture:**
aspartame	Major/minor MeSH heading	Publications in which aspartame is a major or minor topic.
	Free text	Publications in which aspartame is mentioned.
aspartam	Major/minor MeSH heading	Publications in which aspartame is a major or minor topic.
	Free text	Publications in which aspartame is mentioned.
L-aspartyl-L-phenylalanine methyl ester	Free text	Publications in which aspartame is identified as L-aspartyl-L-phenylalanine methyl ester.
E951	Free text	Publications in which aspartame is identified as E951.
Sweetening agents	Major/minor MeSH heading	Publications in which artificial sweetening agents are a major or minor topic
(Sweeten*) and (artificial* or intens*)	Free text	Publications mentioning sweeteners, sweetening, sweetened….. etc., together with artificial, artificially….. or intense, intensity, intensified….. etc.

Using this search strategy in Web of Science and PubMed, a total of 2207 and 1149 records relevant to aspartame were identified, respectively.

An open call for data entitled 'Call for scientific data on Aspartame (E 951)' was launched on 1 June 2011 and closed on 30 September 2011. This call resulted in over 600 documents that were submitted to EFSA. These included previously unpublished scientific data, among which the 112 original studies on aspartame which were submitted to support the request for authorisation of aspartame in Europe in the early 1980s and documents identified through the search in scientific databases. The publication details and results of this call can be retrieved under http://www.efsa.europa.eu/en/dataclosed/call/110601.htm. A further call entitled 'Call for scientific data on aspartame (E 951) related to 5-benzyl-3,6-dioxo-2-piperazine acetic acid (DKP) and other primary or secondary degradation products from aspartame' was launched on 26 July 2012 with a closure date of 30 September 2012. This call resulted in the retrieval of an additional 121 documents which can be retrieved under http://www.efsa.europa.eu/en/dataclosed/call/120726.htm.

In addition to the data retrieval described above, a targeted search strategy was applied to the scientific databases to retrieve scientific literature on metabolites and breakdown products of aspartame. The keywords were related to the following subjects: methanol, formaldehyde, aspartic acid, phenylalanine, phenylketonuria and DKP. The following aspects were included in the search: stability, reaction and fate in food, exposure, ADME, acute, sub-chronic and chronic toxicity, genotoxicity, carcinogenicity, reproductive and developmental toxicity, neurotoxicity, hypersensitivity and allergy, clinical and epidemiological data. For the search strategy all synonymous terms and Boolean operators were applied to cover the period between 1960 and 15 February 2013 using Web of Science and

PubMed. These literature keyword searches were narrowed down further by manual review. In order to update the database, a search was conducted on a monthly basis until 15 November 2013.

The study selection process and its results

The selection criteria for scientific data consideration for the re-evaluation of aspartame described in this report were applied to the existing published and unpublished scientific literature. These criteria were defined *a priori* by the Panel and published in June 2010 (EFSA, 2010). The literature database retrieved included scientific peer reviewed papers and relevant non-peer reviewed papers (such as technical reports and published conference proceedings) identified as described above.

Types of studies that were considered within the criteria for inclusion in the selection process

a) Experimental studies
b) Epidemiological studies in humans
c) Case reports supported by medical evidence

Table 1: Source and Type of information available that may fall in these categories:

Peer-reviewed	Not peer-reviewed
Published papers	Unpublished study reports
Meeting abstracts (conference proceedings)	Papers in non-peer reviewed journals or non-peer reviewed e-papers
Published case reports	Meeting abstracts
	Case reports in non-peer reviewed journals

Tiered Approach for the Selection Process

Tier 1. Criteria to be used for the inclusion of scientific papers and reports in the selection process:

1. All studies provided by the applicants (including unpublished study reports non peer-reviewed) with the original application dossier.
2. All studies on the safety and use of aspartame commissioned by national authorities.
3. Papers and reports that have been subject to an independent scientific peer-review process (i.e. process that scientific journals use to ensure that the articles to be published represent the best scholarship available in terms of solid scientific soundness and quality control) and have been subsequently published in a scientific journal.
4. For non independently peer reviewed papers and reports assessment based on the quality control procedures applied and the study designs used with reference to validated standards (e.g. OECD protocols and GLP Guidelines).

Tier 2. Criteria to be used for the rejection of papers and reports in the selection process:

1. Insufficient details provided on the performance or outcome of the studies (EFSA, 2009).
2. Insufficient information to assess the methodological quality of the studies (EFSA, 2009).

The result of the study selection process was as follows.

- 2207 published documents and 173 unpublished documents were retrieved for aspartame.
- The selection of the studies for inclusion in the risk assessment of aspartame was based on consideration of the extent to which the study was informative and relevant to the assessment and general study quality considerations.

- The further application selection process described above resulted in 147 unpublished and 365 published studies to be considered for the final risk assessment. The references selected are cited in the opinion.

- When studies were considered of acceptable quality, irrespective of whether they yielded positive, negative, or null results, they were considered in assessing health effects associated with exposure to aspartame.

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Appendix B. Appendix to the exposure section

I. Rules defined by the Panel to deal with quantum satis (QS) authorisation, usage data or observed analytical data for all regulated food additives to be re-evaluated

Figure 5: Rules defined by the Panel to deal with usage data or observed analytical data for all regulated food additives to be re-evaluated and procedures for estimating intakes using these rules

II. Composition data for methanol from natural food sources

Category	FoodEx name	min—max	mean value* (mg/kg)
ANISE BRANDY	Herb liqueur	trace—563 ppm	281.5
	Spirits	trace—563 ppm	281.5
ARCTIC BRAMBLE (Rubus arcticus L.)	Blackberries (Rubus fruticosus)	< 0.05 ppm in juice	0.05
ARRACK	Punch	25.6—750 ppm	387.8
BANANA (Musa sapientum L.)	Bananas	15 ppm	15.0
	Dried bananas	15 ppm	15.0
	Candied fruit, Bananas	15 ppm	15.0
	Nectar, Banana	15 ppm	15.0
	Soft drink, banana flavour	15 ppm	15.0
BEANS	Beans, with pods	1.3 ppm	1.3
	Legumes, beans, green, without pods	1.3 ppm	1.3
	Beans, green, without pods	1.3 ppm	1.3
	Legumes, beans, dried	1.3 ppm	1.3
	Beans	1.3 ppm	1.3
	Broad bean	1.3 ppm	1.3
	Lima bean	1.3 ppm	1.3
	Mung bean	1.3 ppm	1.3
	Urd bean	1.3 ppm	1.3
	Black eye bean	1.3 ppm	1.3
	Horse bean	1.3 ppm	1.3
	Scarlet runner bean	1.3 ppm	1.3
	Rice bean	1.3 ppm	1.3
	Yam bean	1.3 ppm	1.3
	Castor bean	1.3 ppm	1.3
	Beans-based meals	1.3 ppm	1.3
	Beans and meat meal	1.3 ppm	1.3
	Beans and vegetables meal	1.3 ppm	1.3
	Beans, meat, and vegetables meal	1.3 ppm	1.3
	Legume (beans) soup	1.3 ppm	1.3
	Prepared legume (beans) salad	1.3 ppm	1.3
BLACK CURRANTS (Ribes nigrum L.)	Currants (red, black and white) (*Ribes nigrum, Ribes rubrum*)	4 ppm	4.0
	Jam, Currants (black) (*Ribes nigrum*)	4 ppm	4.0
	Juice, Blackcurrant	4 ppm	4.0
	Juice concentrate, Currants (black)	4 ppm	4.0
CABBAGE (Brassica oleracea)	Head cabbage (*Brassica oleracea* convar. *capitata*)	0.6 ppm	0.6
	Chinese cabbage	0.6 ppm	0.6
CARROT (Daucus carota L.)	Carrots (*Daucus carota*)	trace—0.05 ppm	0.03
	Juice, Carrot	trace—0.05 ppm	0.03
	Apple/carrot juice	trace—0.05 ppm	0.03
	Multi-fruit-carrot juice	trace—0.05 ppm	0.03
CIDER (APPLE WINE)	Cider	16—248 ppm	132.0
CITRUS FRUITS	Citrus fruits	0—213 ppm	106.5
	Juice, Orange	0—213 ppm	106.5
	Juice, Grapefruit	0—213 ppm	106.5
	Juice, Lemon	0—213 ppm	106.5

Category	FoodEx name	min—max	mean value* (mg/kg)
	Juice, Lime	0—213 ppm	106.5
	Juice, Mixed fruit	0—213 ppm	106.5
	Juice concentrate, Oranges	0—213 ppm	106.5
	Juice concentrate, Mandarins	0—213 ppm	106.5
	Nectar, Orange	0—213 ppm	106.5
	Juice, Orange-Grapefruit	0—213 ppm	106.5
	Juice, Mandarin-Banana	0—213 ppm	106.5
	Juice, Apricot-Orange	0—213 ppm	106.5
	Juice, Orange-Peach	0—213 ppm	106.5
	Juice, multi-fruit	0—213 ppm	106.5
FIG (Ficus carica L.)	Figs (*Ficus carica*)	< 1—5 ppm	3.0
	Dried figs (*Ficus carica*)	< 1—5 ppm	3.0
GIN	Gin	76 ppm	76.0
GRAPE (Vitis species)	Vine leaves (grape leaves) (*Vitis euvitis*)	0.032—0.04 ppm	<0.04
	Table grapes (*Vitis euvitis*)	0.032—0.04 ppm	<0.04
	Wine grapes (*Vitis euvitis*)	0.032—0.04 ppm	<0.04
	Canned fruit, Table grape (Vitis euvitis)	0.032—0.04 ppm	<0.04
	Fruit compote, Table grape (Vitis euvitis)	0.032—0.04 ppm	<0.04
grape juice (Filer and Stegink, 1989)	Juice, Grape	12-680 mg/L	346.0
	Juice concentrate, Grapes	12-680 mg/L	346.0
	Juice, Apple-Grape	12-680 mg/L	346.0
	Juice, Berries-Grapes	12-680 mg/L	346.0
	Fruit soft drink, Grape red	12-680 mg/L	346.0
	Fruit soft drink, Grape white	12-680 mg/L	346.0
	Soft drink, grapefruit flavour	12-680 mg/L	346.0
LENTILS	Lentils, green (*Lens culinaris* syn. *L. esculenta*)	4.4 ppm	4.4
	Lentils (*Lens culinaris* syn. *L. esculenta*)	4.4 ppm	4.4
MILK and MILK PRODUCTS	Milk and dairy products	< 0.009 ppm	<0.005
	Butter	< 0.009 ppm	<0.005
	Butter oil	< 0.009 ppm	<0.005
	Ghee	< 0.009 ppm	<0.005
PASSIFLORA SPECIES	Passion fruit (Passiflora edulis)	4 ppm	4.0
	Juice, Passion fruit	4 ppm	4.0
PEAR BRANDY	Fruit liqueur	4300—9300 ppm	6800.0
	Brandy	4300—9300 ppm	6800.0
	Spirits made from fruits	4300—9300 ppm	6800.0
PEAS (Pisum sativum L.)	Peas, with pods (*Pisum sativum*)	1 ppm	1.0
	Mushy peas (*Pisum sativum*)	1 ppm	1.0
	Peas, green, without pods (Pisum sativum)	1 ppm	1.0
	Peas (*Pisum sativum*)	1 ppm	1.0
RASPBERRY, BLACKBERRY and BOYSENBERRY	Boysenberry (*Rubus ursinus x idaeus*)	7—16 ppm	11.5
	Juice, Blackberry	7—16 ppm	11.5
	Fruit soft drink, Raspberry	7—16 ppm	11.5
	Soft drink, raspberry flavour	7—16 ppm	11.5
RUM	Rum	0.08—36 ppm	18.0

Category	FoodEx name	min—max	mean value* (mg/kg)
SHERRY	Sherry	31—163 ppm	97.0
SOYBEAN (Glycine max. L. merr.)	Soya beans	0.02—6 ppm	3.0
	Soya beans flour	0.02—6 ppm	3.0
STRAWBERRY (Fragaria species)	Strawberries (*Fragaria* × *ananassa*)	0.01—5 ppm	2.5
	Jam, Strawberries	0.01—5 ppm	2.5
	Juice concentrate, Strawberries	0.01—5 ppm	2.5
	Juice, Strawberry-Cherry	0.01—5 ppm	2.5
TEA	Tea (dried leaves and stalks, fermented or otherwise of *Camellia sinensis*)	0.01—1.8 ppm	0.9
	Tea (dried leaves and stalks, fermented or otherwise of *Camellia sinensis*), decaffeinated	0.01—1.8 ppm	0.9
	Tea (Infusion)	0.01—1.8 ppm	0.9
TEQUILA (Agave tequilana)	Spirits made from vegetables (Tequila)	500—1000 ppm	750.0
TOMATO (Lycopersicon esculentum Mill.)	Tomatoes (*Lycopersicum esculentum*)	2.7—560 ppm	281.4
	Tomato purée	2.7—560 ppm	281.4
	Sun-dried tomatoes	2.7—560 ppm	281.4
	Juice, Tomato	2.7—560 ppm	281.4
	Juice, Tomato-Vegetable	2.7—560 ppm	281.4
	Juice, Tomato-Carrot	2.7—560 ppm	281.4
	Tomato ketchup	2.7—560 ppm	281.4
	Tomato chutney	2.7—560 ppm	281.4
WHISKY	Whisky	15—160 ppm	87.5
WINE	Wine	0.086— 30 ppm	115.0

* mean values have been calculated by the Panel for the purpose of estimating background chronic intake of methanol from natural food occurrence which is based on assumption that distribution of composition data per category (only when range is expressed) is following a uniform distribution.

Analytical values from VCF., 28th March 2012 and Filer and Stegink, 1989.

III. Composition data for methanol from pectine degradation

Matrix description	pectin content (g/100g)	methanol (mg/kg)
Vegetables and vegetable products (including fungi)	0.66	594
Root vegetables	0.86	774
Bulb vegetables	0.66	594
Fruiting vegetables	0.67	603
Brassica vegetables	0.66	594
Leaf vegetables	0.66	594
Legume vegetables	0.45	405
Stem vegetables (Fresh)	0.66	594
Vegetable products	0.66	594
Fungi, cultivated	0.45	405
Fungi, wild, edible	0.45	405
Starchy roots and tubers	0.695	625.5
Legumes, nuts and oilseeds	0.45	405
Fruit and fruit products	0.59	531
Citrus fruits	0.59	531
Grapefruit (*Citrus paradisi*)	0.445	400.5
Oranges (*Citrus sinensis*)	0.59	531
Lemons (*Citrus limon*)	0.63	567
Limes (*Citrus aurantifolia*)	0.63	567
Mandarins (*Citrus reticulata*)	0.59	531
Pomelo (*Citrus grandis*)	0.445	400.5
Pome fruits	0.565	508.5
Apple (*Malus domesticus*)	0.565	508.5
Pear (*Pyrus communis*)	0.565	508.5
Quince (*Cydonia oblonga*)	0.565	508.5
Medlar (*Mespilus germanica*)	0.565	508.5
Loquat (*Eriobotrya japonica*)	0.565	508.5
Hawthorn (*Crataegus* and *Rhaphiolepis)*	0.565	508.5
Rowan (*Sorbus*)	0.565	508.5
Service tree (*Sorbus domestica*)	0.565	508.5
Nashi pear (*Pyrus pyrifolia*)	0.565	508.5
Stone fruits	0.635	571.5
Apricots (*Prunus armeniaca*)	0.87	783
Plums (*Prunus domestica*)	0.635	571.5
Damson plum (*Prunus domestica* var *institia*)	0.635	571.5
Mirabelle (*Prunus domestica* var *syriaca*)	0.635	571.5
Greengage (*Prunus domestica* var *italica*)	0.635	571.5
Sweet cherry (*Prunus avium*)	0.4	360
Sour cherry (*Prunus cerasus*)	0.4	360
Chickasaw plum (*Prunus angustifolia*)	0.635	571.5
Susina (*Prunus salicina*)	0.635	571.5
Sloe (*Prunus spinosa*)	0.635	571.5
Peaches (*Prunus persica*)	0.635	571.5
Chokecherry (*Prunus virginiana*)	0.4	360
Berries and small fruits	0.55	495
Table grapes (*Vitis euvitis*)	0.75	675
Wine grapes (*Vitis euvitis*)	0.75	675
Strawberries (*Fragaria* × *ananassa*)	0.47	423
Blackberries (*Rubus fruticosus*)	0.55	495
Dewberries (*Rubus ceasius*)	0.7	630
Raspberries (*Rubus idaeus*)	0.49	441
Boysenberry (*Rubus ursinus x idaeus*)	0.55	495
Blueberries (*Vaccinium corymbosum*)	0.55	495
Cranberries (*Vaccinium macrocarpon*)	0.55	495
Currants (red, black and white) (*Ribes nigrum, Ribes*	0.55	495
Gooseberries (*Ribes uva-crispa*)	0.55	495
Rose hips (*Rosa canina*)	0.55	495

Matrix description	pectin content (g/100g)	methanol (mg/kg)
Mulberries (*Morus spp.*)	0.55	495
Azarole (mediteranean medlar) (*Crataegus azarolus*)	0.55	495
Elderberries (*Sambucus nigra*)	0.55	495
Cloudberry (*Rubus chamaemorus*)	0.55	495
Loganberry (*Rubus loganobaccus*)	0.55	495
Salmonberry (*Rubus spectabilis*)	0.55	495
Thimbleberry (*Rubus parviflorus*)	0.55	495
Wineberry (*Rubus phoenicolasius*)	0.55	495
Bearberry (*Arctostaphylos spp.*)	0.55	495
Bilberry or whortleberry (*Vaccinium spp.*)	0.55	495
Crowberry (*Empetrum spp.*)	0.55	495
Cranberry (*Vaccinium spp.*)	0.55	495
Huckleberry (*Vaccinium spp.*)	0.55	495
Lingonberry (*Vaccinium vitis-idaea*)	0.55	495
Strawberry Tree (*Arbutus unedo*), not to be confused with	0.55	495
Jabuticaba (Brazilian grape tree) (*Myrciaria cauliflora*)	0.55	495
Physalis (*Physalis peruviana*)	0.55	495
Sea buckthorn (*Hippophae rhamnoides*)	0.55	495
Miscellaneous fruits	0.54	486
Dates (*Phoenix dactylifera*)	0.54	486
Figs (*Ficus carica*)	0.54	486
Table olives (*Olea europaea*)	0.54	486
Kumquats (*Fortunella species*)	0.54	486
Carambola (*Averrhoa carambola*)	0.27	243
Persimmon (Sharon fruit) (*Diospyros kaki*)	0.54	486
Jambolan (java plum) (*Syzygium cumini*)	0.54	486
Kiwi (*Actinidia deliciosa* syn. *A. chinensis*)	0.54	486
Lychee (Litchi) (*Litchi chinensis*)	0.48	432
Passion fruit (*Passiflora edulis*)	0.54	486
Prickly pear (cactus fruit) (*Opuntia ficus-indica*)	0.54	486
Star apple (*Chrysophyllum cainito*)	0.54	486
American persimmon (*Virginia kaki*) (*Diospyros virginiana*)	0.54	486
Avocados (*Persea americana*)	0.54	486
Bananas (*Musa × paradisica*)	0.73	657
Mangoes (*Mangifera indica*)	0.48	432
Papaya (*Carica papaya*)	0.6	540
Pomegranate (*Punica granatum*)	0.54	486
Cherimoya (*Annona cherimola*)	0.54	486
Guava (*Psidium guajava*)	1.04	936
Pineapples (*Ananas comosus*)	0.54	486
Bread fruit (jackfruit) (*Artocarpus altilis*)	0.54	486
Durian (*Durio zibethinus*)	0.54	486
Soursop (guanabana) (*Annona muricata*)	0.54	486
Anona (cherimoya) (*Annona cherimola*)	0.54	486
Babaco (*Carica pentagona* Heilb., sin. *Vasconcellea x*	0.54	486
Coco plum (*Chrysobalanus icaco*)	0.54	486
Feijoa, Pineapple Guava (*Feijoa sellowiana*)	0.54	486
Pawpaw (*Asimina*)	0.54	486
Chayote (*Sechium edule*)	0.54	486
Rambutan (*Nephelium lappaceum*)	0.54	486
Carissa (*Carissa* sp.)	0.54	486
Jujube (*Ziziphus zizyphus*)	0.54	486
Longan fruit (*Dimocarpus longan*)	0.2	180
Malabar plum (jambu) (*Syzygium jambos*)	0.54	486

IV. Degradation of DKP from aspartame (from data reported in the technical section and from the MPLs/Use levels of aspartame as reported from table 6)

Cat N°	Foods	restrictions/exception	MPLs (mg/L or mg/kg)	Used levels (mg/L or mg/kg)	% DKP	mg DKP from MPLs	mg DKP from used levels
1.4	**Flavoured fermented milk products including heat treated products**	only energy-reduced products or with no added sugar	1000	1000	5	45	45
3	**Edible ices**	only energy-reduced or with no added sugar	800	800	5	36	36
4.2.2	**Fruit and vegetables in vinegar, oil, or brine**	only sweet-sour preserves of fruit and vegetables	300	300	24	64	64
4.2.3	**Canned or bottled fruit and vegetables**	only fruit energy-reduced or with no added sugar	1000	1000	24	214	214
4.2.4.1	**Fruit and vegetable preparations excluding compote**	only energy-reduced	1000	1000	24	214	214
4.2.5.1	**Extra jam and extra jelly as defined by Directive 2001/113/EEC**	only energy-reduced jams jellies and marmalades	1000	1000	24	214	214
4.2.5.2	**Jam, jellies and marmalades and sweetened chestnut puree as defined by Directive 2001/113/EEC**	only energy-reduced jams, jellies and *marmalades*	1000	1000	24	214	214
4.2.5.3	**Other similar fruit or vegetable spreads**	only dried-fruit-based sandwich spreads, energy-reduced or with no added sugar	1000	1000	24	214	214
5.1	**Cocoa and Chocolate products as covered by Directive 2000/36/EC**	only energy-reduced or with no added sugars	2000	2000	10	178	178
5.2	**Other confectionery including breath refreshening microsweets**	only cocoa or dried fruit based, energy reduced or with no added sugar	2000	1000	10	178	89
5.2	**Other confectionery including breath refreshening microsweets**	only cocoa, milk, dried fruit or fat based sandwich spreads, energy-reduced or with no added sugar	1000		10	89	89
5.2	**Other confectionery including breath refreshening microsweets**	only starch based confectionary energy reduced or with no added sugar	2000		10	178	89
5.2	**Other confectionery including breath refreshening microsweets**	only confectionary with no added sugar	1000		10	89	89
5.2	**Other confectionery including breath refreshening microsweets**	only breath-freshening micro-sweets, with no added sugar	6000		10	535	89
5.2	**Other confectionery including breath refreshening microsweets**	only strongly flavoured freshening throat pastilles with no added sugar	2000		10	178	89
5.3	**Chewing gum**	only with added sugars or polyols, as flavour enhancer	2500	1450	0.1	2	1
5.3	**Chewing gum**	only with no added sugar	5500	5420	0.1	5	5
5.4	**Decorations, coatings and fillings, except fruit based fillings covered by category 4.2.4**	only starch based confcetionary energy reduced or with no added sugar	2000	2000	10	178	178
5.4	**Decorations, coatings and fillings, except fruit based fillings covered by category 4.2.4**	only confectionary with no added sugar	1000	1000	10	89	89
5.4	**Decorations, coatings and fillings, except fruit based fillings covered by category 4.2.4**	only cocoa or dried fruit based, energy reduced or with no added sugar	2000	2000	10	178	178
5.4	**Decorations, coatings and fillings, except fruit based fillings covered by category 4.2.4**	only sauces	350	350	10	31	31

Cat N°	Foods	restrictions/exception	MPLs (mg/L or mg/kg)	Used levels (mg/L or mg/kg)	% DKP	mg DKP from MPLs	mg DKP from used levels
6.3	**Breakfast cereals**	only breakfast cereals with a fibre content of more than 15 %, and containing at least 20 % bran, energy reduced or with no added sugar	1000	1000	0.2	2	2
7.2	**Fine bakery wares**	only essoblaten - wafer paper	1000	1000	0.2	2	2
7.2	**Fine bakery wares**	only fine bakery products for special nutritional uses	1700	1700	0.2	3	3
9.2.	**Processed fish and fishery products including mollusks and crustaceans**	only sweet-sour semi-preserves of fish and marinades of fish, crustaceans and molluscs	300	300	5	13	13
11.4.1	**Table-top Sweeteners in liquid form**		*QS*	500000	24	106932	106932
11.4.2	**Table-top Sweeteners in powder form**		*QS*	500000	0.2	891	891
11.4.3	**Table-top Sweeteners in tablets**		*QS*	500000	0.2	891	891
12.4	**Mustard**		350	350	24	75	75
12.5	**Soups and broths**	only energy-reduced soups	110	110	24	24	24
12.6	**Sauces**		350	350	24	75	75
12.7	**Salads and savoury based sandwich spreads**	only *Feinkostsalat*	350	350	24	75	75
13.2	**Dietary foods for special medical purposes defined in Directive 1999/21/EC (excluding products from food category 13.1.5)**	Products in this category can also use additives that are allowed in the corresponding food counterparts categories	1000	1000	24	214	214
13.3	**Dietary foods for weight control diets intended to replace total daily food intake or an individual meal (the whole or part of the total daily diet)**		800	800	24	171	171
14.1.3	**Fruit nectars as defined by Council Directive 2001/112/EC and vegetable nectars and similar products**	only energy-reduced or with no added sugar	600	75	24	128	16
14.1.4	**Flavoured drinks**	only energy reduced or with no added sugar	600	600	24	128	128
14.2.1	**Beer and malt beverages**	only alcohol-free beer or with an alcohol content not exceeding 1,2 % vol; 'Bière de table/Tafelbier/Table beer' (original wort content less than 6 %) except for 'Obergäriges Einfachbier'; Beers with a minimum acidity of 30 milli-equivalents expressed as NaOH; Brown beers of the 'oud bruin' type	600	600	0.7	4	4
14.2.1	**Beer and malt beverages**	only energy-reduced beer	25	25	0.7	0.2	0.2
14.2.3	**Cider and perry**		600	600	0.7	4	4
14.2.8	**Other alcholic drinks including mixtures of alcoholic drinks with non-alcoholic drinks and spirits with less than 15 % of alcohol**		600	600	0.7	4	4
15.1	**Potato-, cereal-, flour- or starch-based snacks**		500	500	24	107	107
15.2	**Processed nuts**		500	500	24	107	107
16	**Desserts excluding products covered in category 1, 3 and 4**	only energy-reduced or with no added sugar	1000	1000	24	214	214
17.1	**Food supplements supplied in a solid form including capsules and tablets and similar forms excluding chewable forms**		2000	2245	0.2	4	4

Cat N°	Foods	restrictions/exception	MPLs (mg/L or mg/kg)	Used levels (mg/L or mg/kg)	% DKP	mg DKP from MPLs	mg DKP from used levels
17.2	**Food supplements supplied in a liquid form**		600		24	128	480
17.3	**Food supplements supplied in a syrup-type or chewable form**		5500		24	1176	480

Appendix C. Summary of total estimated exposure to aspartame per age class and survey*: mean and high level (mg/kg bw/day)

	MPLs		Use levels	
	Mean	High level	Mean	High level
Toddlers				
Belgium (Regional_Flanders)	16.3		16.3	
Bulgaria (Nutrichild)	3.2	11.8	1.6	7.5
Finland (DIPP)	6.3	21.2	6.3	21.2
Germany (Donald 2006_2008)	4.3	13.2	4.3	13.2
Italy (INRAN_SCAI_2005_06)	3.9		3.5	
The Netherlands (VCP_Kids)	14.2	36.9	14.1	36.0
Spain (enKid)	8.3		8.3	
Children				
Belgium (Regional_Flanders)	11.4	27.1	11.4	27.1
Bulgaria (Nutrichild)	2.9	9.3	1.8	6.3
Czech Republic (SISP04)	5.2	12.1	5.2	12.1
Denmark (Danish Dietary Survey)	3.7	8.7	3.7	8.7
Finland (DIPP)	6.8	17.2	6.8	16.9
Finland (STRIP)	5.3	11.7	5.2	11.7
France (INCA 2)	6.5	15.9	6.4	15.3
Germany (Donald 2006_2008)	6.4	14.9	6.3	14.2
Greece (Regional_Crete)	2.7	8.0	2.7	8.0
Italy (INRAN_SCAI_2005_06)	2.3	7.1	2.2	7.1
Latvia (EFSA_TEST)	3.4	8.3	3.4	8.3
The Netherlands (VCP_Kids)	12.8	32.9	12.6	32.4
Spain (enKid)	6.0	17.3	6.0	17.3
Spain (Nut_Ink05)	5.4	14.2	5.4	14.2
Sweden (NFA)	7.0	16.0	6.9	16.0
Adolescents				
Belgium (Diet_National_2004)	2.7	7.2	2.7	7.2
Cyprus (Childhealth)	0.8	2.3	0.8	2.3
Czech Republic (SISP04)	2.7	6.9	2.7	6.9
Denmark (Danish Dietary Survey)	2.0	5.3	2.0	5.3
France (INCA 2)	3.1	7.6	3.1	7.2
Germany (National_Nutrition_Survey_II)	4.0	13.3	4.0	13.2
Italy (INRAN_SCAI_2005_06)	1.1	4.1	1.1	4.1
Latvia (EFSA_TEST)	2.1	5.6	2.1	5.6
Spain (AESAN_FIAB)	1.2	3.1	1.2	3.1
Spain (enKid)	2.4	6.3	2.4	6.3
Spain (Nut_Ink05)	3.1	8.0	3.1	8.0
Sweden (NFA)	3.5	9.5	3.5	9.5

	MPLs		Use levels	
	Mean	High level	Mean	High level
Adults				
Belgium (Diet_National_2004)	3.8	14.0	3.8	14.0
Czech Republic (SISP04)	1.1	3.4	1.1	3.4
Denmark (Danish_Dietary_Survey)	1.1	3.3	1.1	3.2
Finland (FINDIET_2007)	2.1	6.5	2.1	6.3
France (INCA2)	3.4	8.9	3.4	8.9
Germany (National_Nutrition_Survey_II)	4.6	17.3	4.6	17.1
Hungary (National_Repr_Surv)	0.9	3.1	0.7	2.4
Ireland (NSIFCS)	3.1	8.2	3.1	8.2
Italy (INRAN_SCAI_2005_06)	0.9	3.1	0.9	3.1
Latvia (EFSA_TEST)	1.2	3.2	1.2	3.2
The Netherlands (DNFCS_2003)	8.6	27.5	8.5	27.5
Spain (AESAN)	1.5	5.0	1.4	5.0
Spain (AESAN_FIAB)	0.8	2.5	0.7	2.5
Sweden (Riksmaten_1997_98)	2.3	5.9	1.6	4.1
United Kingdom (NDNS)	4.3	20.6	4.2	20.0
The elderly				
Belgium (Diet_National_2004)	4.4	23.5	4.4	23.5
Denmark (Danish_Dietary_Survey)	0.8	2.6	0.8	2.6
Finland (FINDIET_2007)	1.4	4.7	1.3	4.7
France (INCA2)	2.8	11.3	2.8	11.3
Germany (National_Nutrition_Survey_II)	4.1	18.7	4.1	18.7
Hungary (National_Repr_Surv)	0.5	1.5	0.4	1.4
Italy (INRAN_SCAI_2005_06)	0.8	2.7	0.8	2.7

Appendix D. Summary of total estimated exposure to methanol from all sources per age class and survey*: mean and high level (mg/kg bw/day)

	from endogenous pathways (basal + pectine-derived)		from natural food occurence		from food additive (using aspartame MPLs)		Total exposure from all sources	
	Mean	High level	Mean	High level	Mean	High level	Mean	High level
Toddlers								
Belgium (Regional_Flanders)	15.5		1.4		1.6		18.6	
Bulgaria (Nutrichild)	17.0	31.8	1.4	3.8	0.3	1.2	18.7	34.6
Finland (DIPP)	18.0	33.1	0.2	0.9	0.6	2.1	18.9	35.1
Germany (Donald 2006_2008)	14.7	29.1	0.5	1.9	0.4	1.3	15.6	29.9
Italy (INRAN_SCAI_2005_06)	15.4		1.0		0.4		16.8	
The Netherlands (VCP_Kids)	13.4	24.2	0.6	2.6	1.4	3.7	15.5	26.3
Spain (enKid)	13.7		0.2		0.8		14.7	
Children								
Belgium (Regional_Flanders)	13.5	24.4	1.1	3.1	1.1	2.7	15.8	28.1
Bulgaria (Nutrichild)	16.0	29.0	1.4	3.3	0.3	0.9	17.6	31.8
Czech Republic (SISP04)	13.1	23.2	0.3	0.9	0.5	1.2	13.9	24.5
Denmark (Danish Dietary Survey)	13.1	24.6	0.6	1.4	0.4	0.9	14.0	25.7
Finland (DIPP)	13.0	23.6	0.4	1.4	0.7	1.7	14.1	25.4
Finland (STRIP)	9.4	16.7	0.4	1.1	0.5	1.2	10.4	18.1
France (INCA 2)	11.2	20.1	0.6	1.5	0.6	1.6	12.4	21.8
Germany (Donald 2006_2008)	11.5	21.1	0.5	1.8	0.6	1.5	12.7	22.8
Greece (Regional_Crete)	9.8	18.0	0.8	2.1	0.3	0.8	10.8	19.6
Italy (INRAN_SCAI_2005_06)	13.2	23.5	1.0	2.1	0.2	0.7	14.4	25.4
Latvia (EFSA_TEST)	10.1	20.2	0.3	1.2	0.3	0.8	10.7	21.2
The Netherlands (VCP_Kids)	11.4	21.1	0.6	2.1	1.3	3.3	13.2	23.5
Spain (enKid)	11.5	24.1	0.3	1.1	0.6	1.7	12.5	25.5
Spain (Nut_Ink05)	10.3	19.4	0.5	1.6	0.5	1.4	11.4	21.2
Sweden (NFA)	11.0	20.3	0.3	1.1	0.7	1.6	12.1	21.9

	from endogenous pathways (basal + pectine)		from natural sources		from food additive (using aspartame MPLs)		total exposure to all sources of methanol	
	Mean	High level	Mean	High level	Mean	High level	Mean	High level
Adolescents								
Belgium (Diet_National_2004)	8.1	14.8	0.5	1.2	0.3	0.7	8.8	15.9
Cyprus (Childhealth)	8.6	15.1	0.5	1.4	0.1	0.2	9.2	16.5
Czech Republic (SISP04)	10.5	19.5	0.2	0.7	0.3	0.7	11.0	20.2
Denmark (Danish Dietary Survey)	9.4	16.9	0.4	1.0	0.2	0.5	10.1	17.8
France (INCA 2)	8.5	15.4	0.4	0.9	0.3	0.8	9.2	16.4
Germany (National_Nutrition_Survey_II)	7.6	14.3	0.4	1.3	0.4	1.3	8.4	15.5
Italy (INRAN_SCAI_2005_06)	10.1	17.5	0.6	1.3	0.1	0.4	10.8	18.8
Latvia (EFSA_TEST)	8.8	17.3	0.3	0.8	0.2	0.6	9.3	18.1
Spain (AESAN_FIAB)	9.1	15.6	0.3	0.8	0.1	0.3	9.5	16.4
Spain (enKid)	9.1	16.1	0.2	0.7	0.2	0.6	9.5	17.2
Spain (Nut_Ink05)	8.4	15.3	0.5	1.4	0.3	0.8	9.2	16.7
Sweden (NFA)	8.4	15.1	0.2	0.6	0.3	1.0	8.9	15.8
Adults								
Belgium (Diet_National_2004)	8.2	14.8	0.5	1.4	0.4	1.4	9.1	16.3
Czech Republic (SISP04)	8.2	14.3	0.3	0.8	0.1	0.3	8.5	15.1
Denmark (Danish_Dietary_Survey)	9.0	16.2	0.4	1.0	0.1	0.3	9.6	16.9
Finland (FINDIET_2007)	8.5	15.4	0.6	1.6	0.2	0.7	9.3	16.8
France (INCA2)	8.6	15.3	0.4	1.1	0.3	0.9	9.3	16.5
Germany (National_Nutrition_Survey_II)	8.1	14.9	0.4	1.2	0.5	1.7	8.9	16.4
Hungary (National_Repr_Surv)	9.1	15.8	0.3	0.8	0.1	0.3	9.5	16.3
Ireland (NSIFCS)	9.6	16.7	0.3	0.9	0.3	0.8	10.2	17.9
Italy (INRAN_SCAI_2005_06)	9.6	16.3	0.6	1.1	0.1	0.3	10.2	17.4
Latvia (EFSA_TEST)	7.9	14.9	0.3	0.9	0.1	0.3	8.4	15.7
The Netherlands (DNFCS_2003)	7.9	14.1	0.4	0.9	0.9	2.7	9.1	16.2
Spain (AESAN)	9.1	16.6	0.4	1.2	0.1	0.5	9.7	17.9
Spain (AESAN_FIAB)	9.7	16.9	0.6	1.3	0.1	0.3	10.3	18.2
Sweden (Riksmaten_1997_98)	8.1	14.2	0.3	0.7	0.2	0.6	8.6	15.1
United Kingdom (NDNS)	8.3	14.4	0.4	1.0	0.4	2.1	9.1	16.1

	from endogenous pathways (basal + pectine)		from natural sources		from food additive (using aspartame MPLs)		total exposure to all sources of methanol	
	Mean	High level	Mean	High level	Mean	High level	Mean	High level
The elderly								
Belgium (Diet_National_2004)	8.6	15.3	0.4	1.3	0.4	2.4	9.4	17.2
Denmark (Danish_Dietary_Survey)	9.5	16.7	0.5	1.1	0.1	0.3	10.0	17.5
Finland (FINDIET_2007)	8.5	15.0	0.4	1.4	0.1	0.5	9.0	16.5
France (INCA2)	9.5	16.4	0.4	1.0	0.3	1.1	10.2	17.2
Germany (National_Nutrition_Survey_II)	8.5	15.3	0.3	1.0	0.4	1.9	9.3	16.6
Hungary (National_Repr_Surv)	9.3	15.3	0.2	0.6	0.0	0.2	9.5	15.8
Italy (INRAN_SCAI_2005_06)	10.0	17.1	0.6	1.2	0.1	0.3	10.7	17.9

* The different methodologies of European dietary surveys included in the EFSA Comprehensive Database are fully described in the Guidance on the use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment (EFSA, 2011b). A summary is available p.11, Table 1 of the guidance.

Appendix E. Summary of total estimated exposure to aspartic acid, phenylalanine and DKP (from the use of aspartame MPLs) per age class and survey*: mean and high level ((mg/kg bw/day)

	Aspartic acid		PHE		DKP	
	Mean	High level	Mean	High level	Mean	High level
Toddlers						
Belgium (Regional_Flanders)	7.4		9.2		1.9	
Bulgaria (Nutrichild)	1.5	5.3	1.8	6.6	0.5	2.0
Finland (DIPP)	2.8	9.6	3.5	11.9	0.5	1.5
Germany (Donald 2006_2008)	2.0	5.9	2.4	7.4	0.4	1.2
Italy (INRAN_SCAI_2005_06)	1.8		2.2		0.3	
The Netherlands (VCP_Kids)	6.4	16.7	8.0	20.7	1.5	4.1
Spain (enKid)	3.7		4.6		0.7	
Children						
Belgium (Regional_Flanders)	5.2	12.2	6.4	15.2	1.1	3.2
Bulgaria (Nutrichild)	1.3	4.2	1.6	5.2	0.4	1.7
Czech Republic (SISP04)	2.3	5.5	2.9	6.8	0.4	1.1
Denmark (Danish Dietary Survey)	1.7	3.9	2.1	4.9	0.4	1.0
Finland (DIPP)	3.1	7.8	3.8	9.7	0.7	1.6
Finland (STRIP)	2.4	5.3	3.0	6.6	0.7	1.6
France (INCA 2)	2.9	7.2	3.6	8.9	1.0	2.7
Germany (Donald 2006_2008)	2.9	6.7	3.6	8.4	0.6	1.9
Greece (Regional_Crete)	1.2	3.6	1.5	4.5	0.3	0.8
Italy (INRAN_SCAI_2005_06)	1.0	3.2	1.3	4.0	0.2	0.5
Latvia (EFSA_TEST)	1.5	3.8	1.9	4.7	0.5	1.6
The Netherlands (VCP_Kids)	5.8	14.9	7.2	18.4	1.3	3.2
Spain (enKid)	2.7	7.8	3.4	9.7	0.5	1.6
Spain (Nut_Ink05)	2.4	6.4	3.0	8.0	0.6	1.6
Sweden (NFA)	3.1	7.3	3.9	9.0	0.7	1.5
Adolescents						
Belgium (Diet_National_2004)	2.7	7.2	2.7	7.2	0.4	1.2
Cyprus (Childhealth)	0.8	2.3	0.8	2.3	0.1	0.3
Czech Republic (SISP04)	2.7	6.9	2.7	6.9	0.2	0.6
Denmark (Danish Dietary Survey)	2.0	5.3	2.0	5.3	0.2	0.7
France (INCA 2)	3.1	7.6	3.1	7.2	0.5	1.3
Germany (National_Nutrition_Survey_II)	4.0	13.3	4.0	13.2	0.7	2.5
Italy (INRAN_SCAI_2005_06)	1.1	4.1	1.1	4.1	0.1	0.3
Latvia (EFSA_TEST)	2.1	5.6	2.1	5.6	0.3	0.9
Spain (AESAN_FIAB)	1.2	3.1	1.2	3.1	0.1	0.4
Spain (enKid)	2.4	6.3	2.4	6.3	0.2	0.6
Spain (Nut_Ink05)	3.1	8.0	3.1	8.0	0.5	1.5
Sweden (NFA)	3.5	9.5	3.5	9.5	0.3	0.9

	Aspartic acid		PHE		DKP	
	Mean	High level	Mean	High level	Mean	High level
Adults						
Belgium (Diet_National_2004)	3.8	14.0	3.8	14.0	0.7	2.8
Czech Republic (SISP04)	1.1	3.4	1.1	3.4	0.1	0.3
Denmark (Danish_Dietary_Survey)	1.1	3.3	1.1	3.2	0.1	0.4
Finland (FINDIET_2007)	2.1	6.5	2.1	6.3	0.3	0.9
France (INCA2)	3.4	8.9	3.4	8.9	0.6	1.7
Germany (National_Nutrition_Survey_II)	4.6	17.3	4.6	17.1	0.8	3.4
Hungary (National_Repr_Surv)	0.9	3.1	0.7	2.4	0.1	0.5
Ireland (NSIFCS)	3.1	8.2	3.1	8.2	0.5	1.1
Italy (INRAN_SCAI_2005_06)	0.9	3.1	0.9	3.1	0.1	0.3
Latvia (EFSA_TEST)	1.2	3.2	1.2	3.2	0.2	0.5
The Netherlands (DNFCS_2003)	8.6	27.5	8.5	27.5	1.6	5.5
Spain (AESAN)	1.5	5.0	1.4	5.0	0.2	0.7
Spain (AESAN_FIAB)	0.8	2.5	0.7	2.5	0.1	0.3
Sweden (Riksmaten_1997_98)	2.3	5.9	1.6	4.1	0.4	1.0
United Kingdom (NDNS)	4.3	20.6	4.2	20.0	0.8	4.1
The elderly						
Belgium (Diet_National_2004)	4.4	23.5	4.4	23.5	0.8	4.8
Denmark (Danish_Dietary_Survey)	0.8	2.6	0.8	2.6	0.1	0.2
Finland (FINDIET_2007)	1.4	4.7	1.3	4.7	0.2	0.8
France (INCA2)	2.8	11.3	2.8	11.3	0.5	2.0
Germany (National_Nutrition_Survey_II)	4.1	18.7	4.1	18.7	0.8	3.8
Hungary (National_Repr_Surv)	0.5	1.5	0.4	1.4	0.1	0.2
Italy (INRAN_SCAI_2005_06)	0.8	2.7	0.8	2.7	0.1	0.3

* The different methodologies of European dietary surveys included in the EFSA Comprehensive Database are fully described in the Guidance on the use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment (EFSA, 2011b). A summary is available p.11, Table 1 of the guidance.

Appendix F. Tables on acute toxicity studies on aspartame and DKP

1. Aspartame

	Animals	Route	Dose range	Reported Result	Comments and reference(s)
Oral					
Rat	Male Sprague Dawley, Charles River (n=6/group)	p.o. as 15-25 % suspension in 1 % Tween 80	Up to 5000 mg/kg	No deaths within 7 days at any dose	Motor and behavioural activities unremarkable. Ref: E46, 1973
Mouse	Male Sprague Dawley, Schmidt Ha/ICR (n=2 or 6/group)				
Rabbit	Male New Zealand White Luenberg (n=1 or 3/group)				
Intraperitoneal /Intravenous					
Rat	Male Sprague Dawley, Charles River (n=6/group)	i.p. in aqueous solution	Up to 2033 mg/kg	No deaths within 7 days at any dose	Motor and behavioural activities unremarkable. Ref: E46, 1973
Mouse	Male Sprague Dawley, Schmidt 11a/ICR (2 or 6/group)	i.p. as 2 % aqueous suspension	Up to 1000 mg/kg		
Rat	Male Charles River CD (n=6/group)	i.v. as 5 ml/kg bw of 2 % aspartame in 0.9 % aqueous sodium chloride solution	100 mg	No deaths up to 72 hours post-injection	Physical, opthalmoscopic, gross or microscopic parameters unremarkable. Phlebitis at site of implantation; kidney and endocardium inflammation. Ref: E84, 1974
Dog	Male Beagle (n=4/group)	i.v. as 5 ml injection of 0.5 % aqueous sodium chloride solutions	100 mg	No deaths up to 72 hours post-injection	Physical and biochemical parameters, ECC unremarkable. Ref: E85, 1974

2. DKP

	Strain	Route	Dose range	Reported Result	Comments and reference(s)
Oral					
Rat	Male Sprague Dawley, Charles River (n=6/group)	p.o. as 15-17.5 % suspension in 1 % Tween 80 (aqueous 2 % suspension for low doses)	Up to 5000 mg/kg	No deaths within 7 days at any dose	Motor and behavioural activities unremarkable. Ref: E45, 1973
Mouse	Male Sprague Dawley, Schmidt Ha/ICR (n=2 or 6/group)				
Rabbit	Male New Zealand White Luenberg (n=1 or 3/group)				
Intraperitoneal					
Rat	Male Sprague Dawley, Charles River (n=6/group)	i.p. as 2% aqueous suspension	Up to 1562 mg/kg	No deaths within 7 days at any dose	Motor and behavioural activities unremarkable. Ref: E45, 1973

Appendix G. TABLES ON SHORT TERM AND SUBCHRONIC TOXICITY STUDIES ON ASPARTAME AND DKP

1. Aspartame

Strain	Dose range	Duration	Reported Result	Comments and reference(s)
Oral				
'Young' purebred beagles, 7.2-13.2 kg (2M/2F per group)	5/125 mg/kg bw/day via gelatine capsules	8 weeks	100 % survival; variable pattern of body weight loss/maintenance/gain	No consistent haematology, clinical chemistry or urinalysis findings; no consistent ocular findings; no gross pathology; no consistent effects on organ weights; no cytopathological changes. Refs: E21, 1969.
Via diet				
Charles River CD rats, 7 wk old (5/sex per group)	2000/4000/10 000 mg/kg bw/day via diet; actual consumption within 10 % of planned	4 weeks	100 % survival; no unequivocal effect on body weight or food consumption except decreased consumption in high dose females, wks 2-3.	No adverse physical/behavioural changes; no treatment-related pathological changes except for clear, viscous fluid coating intestinal mucosa in treated rats. N.B. only examined 5 controls and all high dose animals; remaining animals were discarded. Refs: E3, 1972.
Charles River CD rats, sexually mature (10/sex per group). Group allocation by stratified randomisation	5/125 mg/kg bw/day via diet	8 weeks	100 % survival; no unequivocal effect on body weight or food consumption	At sacrifice, biochemistry and pathological examination was conducted on 5 animals from each dose and the control group. No signs of systemic toxicity or pharmacological effects. No treatment related changes in haematology or urinalysis; increased terminal blood sugar at 125 mg/kg bw/day but no other significant changes in clinical chemistry. Organ weights unaffected except for increased liver/body weight ratio in high dose males. No histopathological findings except bile duct hyperplasia/ pericholangitis in all groups. Refs: E20, 1969.
Charles River CD rats, weanlings (5/sex per group)	9:100 ratio in diet (aspartame) 5:100 ratio in diet (Phe)	9 weeks	Two deaths; one control male, one aspartame-treated male. Relative food consumption comparable between groups. No treatment-related effect on terminal body weight.	No physical/behavioural abnormalities; no significant treatment related changes in haematology, clinical chemistry, urinalysis; no treatment-related changes in organ weight or pathological observations. Reduced body weight gain in treated groups, both compounds and both sexes; less marked in females than males. Refs: E4.

Ha/ICR mice, 8 wk old (5/sex per group)	Planned: 2000, 4000, 10 000 mg/kg bw/day via diet; Actual: 3000, 5000, 13 000 mg/kg bw/day	4 weeks	100 % survival; no unequivocal effect on body weight	The actual intake of the test compound exceeded by 25-30 % in all dose groups, due to unforeseen increase in food consumption. No adverse physical/behavioural changes; no treatment-related pathological changes except for clear, viscous fluid coating intestinal mucosa in treated mice. N.B. only examined 5 controls and all high dose animals; remaining animals were discarded. Refs: E2, 1972.

2. DKP

Strain	Dose range	Duration	Reported Result	Comments
Oral				
Charles River CD rats, 11 wk old (10/sex per group)	1000 mg/kg bw/day p.o. as 5 % aqueous suspension	Daily for 2 weeks	100 % survival; no unequivocal effect on body weight	No physical/behavioural changes; no treatment related changes in haematology, clinical chemistry, urinalysis; no treatment-related pathological changes. Refs: E7, 1971.
Ha/ICR mice, 4 wk old (10M per group)	1000 mg/kg bw/day p.o. as 5 % aqueous suspension	Daily for 2 weeks	100 % survival; no unequivocal effect on body weight	No physical/behavioural changes; no treatment related changes in haematology, clinical chemistry, urinalysis; no treatment-related pathological changes. Refs: E6, 1971.
Via diet				
Charles River CD rats, 8 wk old (5/sex per group)	1000/2000/4000/6000 mg/kg bw/day via diet; actual consumption within 5 % of planned	5 weeks	100 % survival; reduced body weight in high dose females due to reduced food consumption.	No physical/behavioural changes; no dose-related changes in haematology, clinical chemistry, urinalysis; no dose-related changes in organ weight or treatment-related pathological changes. Evidence of colonic parasites, mild chronic bronchitis and mild chronic inflammation of liver and bile ducts. Refs: E8, 1972.

Appendix H. TABLES ON GENOTOXICITY STUDIES ON ASPARTAME, METHANOL AND DKP[21]

1. Aspartame genotoxicity studies *in vitro*

Test system	Test Object	Doses	Reported Result	Comments and reference(s)
Salmonella typhimurium Tester strains: TA1535,TA1537, TA1538, TA98, TA100	Gene mutation	[µg/plate] 10, 50, 100, 500, 1000, 5000	Negative [a)]	Reported identity of test compound (SC-18862; Lot no.89300); non-GLP but well conducted and reported study; deviations from current OECD TG 471: Tester strains *S. typhimurium* TA102 or *E. coli* WP2uvrA bearing AT mutation were not used. **Overall judgement:** The methods implemented were thought to be sufficiently robust to support the results reported. Ref: E97, 1978
Salmonella typhimurium Tester strains: TA1535, TA1537, TA1538, TA98, TA100	Gene mutation	[µg/plate] 10, 50, 100, 500, 1000, 5000	Negative [a)]	Reported identity of test compound: SC-18862; Lot no.C-0096-89300; Non-GLP but well conducted and reported study; Deviations from current OECDTG471: Tester strains (TA102 or WP2uvrA) bearing AT mutation were not used. **Overall judgement:** The methods implemented were thought to be sufficiently robust to support the results reported. Ref: E101 (1978)
Salmonella typhimurium Tester strains: TA1535, TA1537, TA97, TA98, TA100	Gene mutation	[µg/plate] 100, 333, 1000, 3333, 10000	Negative [a)] in TA1535, TA98, TA100 Negative [c)] in TA1537; Negative without S9 or with hamster liver S9 in TA97; TA97: small increases in mutant colonies with 30 % rat liver S9	GLP-study; Deviation from OECD 471: Tester strains *S. typhimurium* TA102 or *E. coli* WP2uvrA bearing AT mutation were not used; TA1337 was only assayed in the presence of S9 metabolism; Study authors judged small increase in mutant colonies with 30 % rat liver S9 as equivocal. **Overall judgement:** The methods implemented were thought to be sufficiently robust to support the results reported. Ref: NTP (2005)

[21] **Negative findings:** the substance tested does not produce dose-related genotoxic effects in the test system compared to concurrent and/or historical controls.
Positive findings: the substance tested produces dose-related genotoxic effects in the test system compared to concurrent and/or historical controls.

Test system	Test Object	Doses	Reported Result	Comments and reference(s)
Salmonella typhimurium Tester strains: TA98; TA100	Gene mutation	[µg/plate] 50, 100, 250, 500, 1000, 2000	Negative [a)]	Major deviations from current OECD TG 471: Only two tester strains used; Highest dose-level employed was lower than 5 mg/mL; Second experiment performed was identical to the first one. **Overall judgement:** The methods implemented were thought not to be sufficiently robust to support the results reported. Ref: Rencuzogullari et al. (2004)
Primary rat hepatocytes	Unscheduled DNA Synthesis (UDS)	5 and 10 mM	Negative	The study meets requirements of OECD TG 482 (1986). **Overall judgement:** The methods implemented were thought to be robust to support the results reported. Ref: Jeffrey and Williams (2000)
Human lymphocyte	Chromosome aberrations (CA)	0.5, 1.0 and 2 mg/mLfor 24 and 48 hours performed only in the absence of S9 metabolism	Positive [b)]	Major deviations from current OECD TG 473, 479 and 487 for all studies conducted: Culture initiation and treatment schedule inappropriate; No treatment in the presence of S9 metabolism; No measurement of pH and osmolarity values. In addiction: For CA: Analyses of chromosomal aberrations performed mainly in M2 and M3 cells (Table 2); Method for scoring of aberrations do not refer to any published literature; CA do not exhibit a dose-response. For MN: Historical control range values not reported and used to accept validity of the study; MN formation concurrent with cytotoxicity (suggestive of indirect effects of aspartame); **Overall judgement:** The methods implemented were thought not to be sufficiently robust to support the results reported. Ref: Rencuzogullari et al. (2004)
	Sister chromatid exchanges (SCE)		Negative [b)]	
	Micronuclei (MN)		Positive [b)]	

a) With and without metabolic activation.
b) Without metabolic activation.
c) With metabolic activation.

2. Aspartame genotoxicity studies *in vivo*

Test system	Test Object	Route	Doses	Reported Result	Comments
Rat Charles River CD strain 15 males/group	Dominant lethal assay	Control (vehicle) and SC-18862: intragastric. Positive control (methyl methane sulphonate): i.p.	0 (control), 2000 mg/kg bw, two equally divided doses on single day (2 hour interval)	Negative (paternal growth, maternal pregnancy rate, uterine and ovary examination data and incidence of fetal deaths)	Reported identity of test compound: SC-18862, containing 0.2% DKP (SC-19192) No Guidelines available in 1973 (since 1984: OECD TG 478). Study protocol followed general recommendations **Overall judgement:** The methods implemented were thought to be sufficiently robust to support the results reported. Ref: E40 (1973)
Rat Charles River CD strain 15 males/group	Dominant lethal assay	Control (vehicle) and SC-18862: intragastric.Positive control (methyl methane sulphonate): i.p.	0 (control), 2000 mg/kg bw, two equally divided doses on single day (2 hour interval)	Negative (paternal growth, maternal pregnancy rate, uterine and ovary examination data and incidence of fetal deaths)	Reported identity of test compound: SC-18862 containing 0.7% DKP (SC-19192); No Guidelines available in 1973 (since 1984: OECD TG 478). Study protocol followed general recommendations; **Overall judgement:** The methods implemented were thought to be sufficiently robust to support the results reported. Ref: E41 (1973)
Rat Purina CD strain 10 males /group	Chromosome aberrations in bone-marrow erythrocytes	Control (vehicle) and SC-18862: intragastric. Positive control (triethylene-melamine): i.p.	0 (control), 500, 1000, 2000 and 4000 mg/kg bw/day, given in three equally divided doses every three hours, for five consecutive days	Negative	Reported identity of test compound: SC-18862, Lot#76040C, containing 0.7 % DKP; GLP: no statement, but well documented report; Deviations from current OECD TG 475: 50 spreads/rat instead of 100, but 10 instead of 5 rats. No mitotic index determination: 'The mitotic index should be determined as a measure of cytotoxicity in at least 1000 cells per animal for all treated animals (including positive controls) and untreated negative control animals'. **Overall judgement:** The methods implemented were thought to be sufficiently robust to support the results reported. Ref: E43 (1972)

Test system	Test Object	Route	Doses	Reported Result	Comments
Rat Holtzman strain 10 males/group	Chromosome aberrationsin bone marrow erythrocytes and spermatogonial cells	Oral (intragastric)	0 (control), 400, 800, 1200 and 1600 mg/kg bw/day for five consecutive days	1)Negative in bone marrow erythrocytes; 2)Negative in spermatogonial cells	Reported identity of test compound: SC-18862; Lot# A3427; Major deviations from current OECD TG 475: Sampling time selected 24 hours + 5 hours; Colchicine (total 29 hours). It should have been 1.5 cell cycle (12-18hours; The highest dose may also be defined as a dose that produces some indication of toxicity in the bone marrow (e.g. greater than 50 % reduction in mitotic index). In this study mitotic indices were not even scored; 50 metaphases per animal were scored. Dose-levels administered of test substance 0.4, 0.8, 1.2 and 1.6 g/kg bw/day are different from those reported in the conclusion of report; The second positive control employed (cyclohexylamine) did not show positive results expected. **Overall judgement:** The methods implemented were thought not to be sufficiently robust to support the results reported. Ref: E12 (1970)
Rat Purina CD strain 10 males/group	Host-mediated assay	Control (vehicle) and SC-18862: oral. Positive control (dimethylnitro samine): i.p.	0 (control), 500, 1000, 2000 and 4000 mg/kg bw/day, given in three equally divided doses every three hours, for five consecutive days.	Negative	Reported identity of test compound: SC-18862, Lot#76040C, containing 0.7 % DKP; No Guidelines available; Study protocol followed general recommendations. Single use of G-46 Salmonella typhimurium, tester strain strongly reduce the capability to identify different classes of genotoxins. **Overall judgement:** The methods implemented were thought to be sufficiently robust to support the results reported. Ref: E44 (1972)
Mouse Sprague-Dawley Ha/ICR Swiss strain 10 males/group	Host-mediated assay	Oral (intragastric)	0 (control), 1000, 2000, 4000, 8000 mg/kg bw/day three times daily at two hour intervals, for five consecutive days.	Negative	Reported identity of test compound: SC.18862 Lot#76060R; No Guidelines available; Study protocol followed general recommendations; Single use of G-46 Salmonella typhimurium, tester strain strongly reduce the capability to identify different classes of genotoxins. **Overall judgement:** The methods implemented were thought to be sufficiently robust to support the results reported. Ref: E81 (1974)
Mouse C57/black strain 5 animals/group	Chromosomal aberrations in bone marrow erythrocytes	Oral	0 (control), 40 and 400 mg/kg bw, 5 days.	Negative	Study is poorly reported. Concurrent positive control animal group to show whether the test system was functioning correctly was not included; Concurrent negative control group was included;

Test system	Test Object	Route	Doses	Reported Result	Comments
					A minimum number of 100 metaphases per animal were scored; Dose-levels administered appear to be very low. Sampling of bone marrow cells 6 hours from last administration of test compound is not adequate for chromosomal aberration analysis. **Overall judgement:** The methods implemented were thought not to be sufficiently robust to support the results reported. Ref: Durnev et al. (1995)
Mouse Swiss albino strain 5 males/group	Chromosome aberrations in bone marrow erythrocytes	Oral (intragastric)	ASP+Acesulfame K (3.5+1.5) mg/kg bw ASP+Acesulfame K (35+ 5) mg/kg bw ASP+Acesulfame K (350+15) mg/kg bw	Negative	Study not relevant for evaluation of aspartame because aspartame was blended with acesulfame K. Ref: Mukhopadhyay et al. (2002)
Rat Fisher 344/N strain 5 males / group	Micronuclei in bone marrow erythrocytes	Oral (gavage)	0 (control), 500, 1000 or 2000 mg/kg bw, 3 times at 24 hour intervals.	Negative	Lot 8415-14-02 RTI; Purity: > 98 %; GLP-study; Deviation from OECD TG 475: only male animals were used - Sampling time selected 24 h. It should have been 1.5 cell cycle (12-18 hours). Cytotoxicity: %PCE not greatly altered but in slight decrease (60.8 to 39 %) in 1000 mg/kg group. **Overall judgement:** The methods implemented were thought to be sufficiently robust to support the results reported. Ref: NTP (2005).

Test system	Test Object	Route	Doses	Reported Result	Comments
Transgenic Mouse FVB/N-TgN (v-*Ha-ras*)Led (Tg.AC) hemizygous 15 males and 15 females/group	Micronuclei in peripheral blood combined with carcinogenicity	Oral (via diet)	0, 3125, 6250, 12500, 25 500, 50 000 ppm for 9 months Males: 490, 980, 1960, 3960, 7660 mg/kg bw Females: 550, 1100, 2260, 4420, 8180 mg/kg bw	Negative	Lot 8415-14-02 RTI;Purity: > 98 %; GLP-study; Deviation from OECD 474 (use of transgenic animals covered by 'any appropriate species'): - study does not include positive control - Sampling time: authors refer to McGregor et al. (1990) but did not specify the actual time point. No evidence of carcinogenic activity in TgAc haplo insufficient male or female mice. **Overall judgement:** The methods implemented were thought to be sufficiently robust to support the results reported. Ref: NTP (2005).
Transgenic Mouse B6.129-*Trp53*^{tm1Brd} (N5) haploinsufficient 15 males and 15 females/group	Micronuclei in peripheral blood combined with carcinogenicity	Oral (via diet)	0, 3125, 6250, 12500, 25 500, 50 000 ppm for 9 months Males: 490, 970, 1860, 3800, 7280 mg/kg bw Females: 630, 1210, 2490, 5020, 9620 mg/kg bw	3125 to 25 500 mg/kg diet group: males and females negative 50 000 mg/kg diet group: females judged to be positive based on a significantly increased frequency (x 2.3) of micronucleated erythrocytes	Lot 8415-14-02 RTI; Purity: > 98 %; GLP-study; Deviation from OECD 474 (use of transgenic animals covered by 'any appropriate species'): - study does not include positive control - Sampling time: authors refer to McGregor et al. (1990) but did not specify the actual time point. No evidence of carcinogenic activity in TgAc haploinsufficient male or female mice. **Overall judgement**: The methods implemented were thought to be sufficiently robust to support the results reported. Ref: NTP (2005)
Transgenic Mouse Cdkn2a deficient	Micronuclei in peripheral blood	Oral (via diet)	0, 3125, 6250, 12500, 25500, 50000 ppm for 9 months.	Negative	Lot 8415-14-02 RTI; Purity: > 98 %; GLP-study; Deviation from OECD 474 (use of transgenic animals covered by 'any appropriate species'): study does not include positive control; sampling time: authors refer to McGregor et al. (1990) but did not specify the actual time point. No evidence of carcinogenic activity in

Test system	Test Object	Route	Doses	Reported Result	Comments
15 males and 15 females/group					TgAc haploinsufficient male or female mice (no evidence: no chemical- related increases in malignant or benign neoplasms. **Overall judgement:** The methods implemented were thought to be sufficiently robust to support the results reported. Ref: NTP (2005)
Mouse ddY strain 4 males/group	Comet assay in Stomach, colon, liver, kidney, bladder, lung, brain, bone marrow	Oral (intragastric)	2000 mg/kg bw	Negative in all organs assayed	Deviations from minimum requirements: 1) Four animals used; 2) Only one dose-level employed (2000 mg/kg bw); 3) Two slides analysed (50 nuclei/slide); 4) Manual scoring: DNA migration was evaluated as the difference between length of the tail and diameter of the nucleus. 5) Isolated nuclei instead of whole cells were employed; 6) Cell viability not reported; 7) the study does not include positive control. **Overall judgement:** The methods implemented were thought to be sufficiently robust to support the results reported. Ref: Sasaki et al. (2002)
Mouse Swiss albino strain 4 males/group	Comet assay in bone marrow cells	Oral (intragastric)	0 (control), 7, 14, 28 and 35 mg/kg bw	Positive at higher dose-level	The study is generally poorly reported; Deviations from recommended protocol: 1) Insufficient number of cells scored (total of 50 cells/animal); 2) Historical control values not reported **Overall judgement:** The methods implemented were thought not to be sufficiently robust to support the results reported. Ref: Bandyopadhyay et al. (2008)
Mouse Swiss albino strain (Number of animals not clearly reported)	Micronuclei in bone-marrow erythrocytes	Oral (intragastric)	0 (control), 250, 455, 500 and 1000 mg/kg bw	Positive	The study is generally poorly reported. Deviations from OECD TG 474 guideline: 1) Number of animals employed is not reported; 2) The PCE/NCE ratio is described as 'changed' but no data are given 3) No criteria for selection of higher dose-level. 4) no historical control data.
	Micronuclei in peripheral blood			Positive	

Test system	Test Object	Route	Doses	Reported Result	Comments
	Chromosome aberrations in bone-marrow erythrocytes			Positive	Deviations from OECD TG 475: 1) Number of animals employed is not reported; 2) No mitotic indices scored; 3) Treatment with colcemid too short (1.5 hours instead of 3-5 hours); 4) Sampling times used (e.g. 24, 48 and 72 hous) not appropriate; 5) No criteria for selection of higher dose-level. **Overall judgement:** The methods implemented were thought not to be sufficiently robust to support the results reported. Ref: Kamath et al. (2010)
Mouse Swiss albino strain (5 males/group)	Chromosome aberrations in bone marrow erythrocytes	Oral (intragastric)	0 (control), 3.5, 35 and 350 mg/kg bw	Positive	Deviations from OECD TG 475 : 1) Insufficient number of cells scored; 2) Any supplementary information on cytotoxicity relevant for CA and SCE-analysis in the present experiment not given; 3) No positive control.: No Guidelines available. Number of cells scored (30 cells/animal for SCE) insufficient for statistical analyss. **Overall judgement:** The methods implemented were thought not to be sufficiently robust to support the results reported. Ref. Alsuhaibani et al. (2010)
	SCE in bone-marrow erythrocytes			Negative	

3. Methanol genotoxicity studies *in vitro*

Test system	Test Object	Doses	Reported Result	Comments
Salmonella typhimurium Tester strains: TA1535, TA1537, TA1538, TA 98 and TA100	Gene mutation	No data. Various dilutions performed by geometric ratio starting from solubility or toxicity limit.	Negative [a)]	Not assignable Survey of the capacity of 135 compounds incl. methanol. Concentration of test compound not given. Ref: DeFlora et al. (1984)
E. coli Tester strains: WP2; WP2 (*uvrA-*, *polA-*); CM871 *uvrA-*, *recA-*, *lexA-*)	DNA repair	No data. Various dilutions performed by geometric ratio starting from solubility or toxicity limit.	a) Microtiter plate: negative [a)] b) 2-hour preincubation: without S9: negative; with S9: 1.5 to 2-fold increase: 'weak positive' c) spot-test: negative	Not assignable Survey of the capacity of 135 compounds incl. methanol Concentration of test compound not given. Statement by MAK (1999): 'As it was conceivable that the weak positive results obtained with methanol were caused by impurities, the authors classify methanol as an uncertain or questionable mutagen' Ref: DeFlora et al. (1984)
Neurospora Crassa	Chromosome aberrations	Not reported	Negative	A study which included 57 compounds; No dose-levels for methanol reported; Test system not currently employed to detect aneugens. **Overall judgement:** The methods implemented were thought not to be sufficiently robust to support the results reported. Ref: Griffiths (1981)
Salmonella typhimurium Tester strains: TA1535, TA1537, TA1538, TA98 and TA100	Gene mutation	No data	Negative (not described: with or without S9)	Not assignable Not described: with or without S9as well as MeOH concentration. MeOH listed as one of 45 chemicals that gave negative results. No conclusion can be drawn from the publication. Ref: Simmon et al. (1977)
Salmonella typhimurium Tester strains: TA1535, TA1537, TA1538, TA 98	Gene mutation	5 doses up to 3.6 mg/plate	Negative [a)]	Not assignable Screening study on genotoxicity of 31 chemicals. Bacterial reverse mutation assay followed standard protocol of that time. No detailed results on methanol are reported. Deviations from current OECD Guideline 471: - TA102, E. coli not included in the study - maximal concentration tested (3.6 mg/plate) lower than recommended - range, but not actual

Test system	Test Object	Doses	Reported Result	Comments
and TA100				concentration tested given. The study does not include positive control Ref: Gocke et al. (1981)
Salmonella typhimurium Tester strains: TA1535, TA1537, TA1538, TA 98 and TA100	Gene mutation	2.5 mM/plate (733 µg/plate)	Negative [a)]	DeFlora et al. (1981) cited from MAK-Liste - TA 102, E. coli not included. Ref: DeFlora et al. (1981)
Salmonella typhimurium Tester strains: TA1535, TA1537, TA1538, TA 98 and TA100	Gene mutation	[µg/plate] 5, 10, 50, 100, 500, 1000, 5000	Negative [a)]	Both in the absence and presence of rat liver S9 metabolism. **Overall judgement**: The methods implemented were thought to be sufficiently robust to support the results reported. Ref: Shimizu et al. (1985)
E. coli Tester strain: WP2 uvrA *Schizosaccharomyces pombe ade6-60/rad10-198,h⁻* (P1 strain)	Gene mutation	5 %	Negative [a)]	Non-GLP study; Material and methods described in sufficient details; use of mouse S10 metabolism; maximum concentration for mutagenicity test was based upon cytotoxicity at concentrations of 0.5, 2, 5 and 10 % (v/v); the study does not include positive control **Overall judgement**: The methods implemented were thought to be sufficiently robust to support the results reported. Ref: Abbondandolo et al. (1980)
Aspergillus nidulans (diploid strain P1)	Chromosome aberrations	5.2, 5.6, 6, 7 % (v/v)	Positive [b)] Cross over: negative [b)]	Non-GLP study; Material and methods described in sufficient details; Exposure to test compound during early germination; No use of metabolic activation; Aneuploids (non-disjunctional diploids and haploids) at toxic concentration 5.6 and 6 % MeOH (survival: 19 and 10 %, respectively). **Overall judgement**: The methods implemented were thought to be sufficiently robust to support the results reported. Ref: Crebelli et al. (1989)
Salmonella typhimurium Tester strains: TA1535, TA1537,	Gene mutation (Pre-incubation)	[µg/plate] 10, 50, 100, 500, 1000, 5000	Negative [a)]	No GLP-statement; Deviation from OECD TG 471: - no historical control data **Overall judgement:** The methods implemented were thought to be sufficiently robust to support the results reported. Ref: NEDO (1987)

Test system	Test Object	Doses	Reported Result	Comments
TA1538, TA 98 and TA100 *E. coli* Tester strain: WP2 uvrA				
Chinese Hamster lung fibroblasts (V79 cells)	Gene mutation (azaguanine, 6-thioguanine, ouabain)	15.8, 31.7, 47.4, 63.3 mg/mL exposure: 3 hours	Negative [a)]	63.3 mg/mL inhibit cell colony formation by approximately 70 %; Mutant selection after 6 days; No GLP-statement; Deviation from OECD TG 476: - no historical data - concentration of methanol exceeded the maximum concentration recommended in in vitro studies (i.e. 5 mg/mL); **Overall judgement**: The methods implemented were thought not to be sufficiently robust to support the results reported. Ref: NEDO (1987)
	Chromosomal aberration	7.1, 14.3, 28.5 mg/mL for 2 days	Negative [a)]	28.5 mg/mL inhibit cell growth by approximately 50 %; Chromosome preparation at 6, 24, 48 hours after treatment; 200 cells/dose-level scored; No GLP-statement; Deviations from OECD TG 473: - treatment schedule and sampling times; - no cytotoxicity measured (mitotic index, cell counts or degree of confluence); - no historical data - concentration of methanol exceeded the maximum concentration recommended in in vitro studies (i.e. 5 mg/mL); **Overall judgement**: The methods implemented were thought not to be sufficiently robust to support the results reported. Ref: NEDO (1987)
Chinese Hamster lung fibroblasts (V79 cells)	Sister chromatid exchanges (SCE)	7.1, 14.3, 28.5 mg/mL	7.1, 14.3 mg/mL negative [a)] 28.5 mg/mL: without S9: positive with S9: negative	Highest concentration (28.5 mg/mL) SCE-incidence of 9.41 ± 0.416 significantly greater than controls 6.42 ± 0.227 [mean ± SE per 100 cells]); Sampling: 24-26 after treatment; 100 cells/dose level scored; No GLP-statement; Deviation from OECD TG 479: - no duplicate cultures - no cytotoxicity measured (mitotic index, cell counts or degree of confluence) - concentration of methanol exceeded the maximum concentration recommended in in vitro studies (i.e. 5 mg/mL); **Overall judgement**: The methods implemented were thought not to be sufficiently robust to support the results reported. Ref: NEDO (1987)

Test system	Test Object	Doses	Reported Result	Comments
E. coli (SA500)	Gene mutation (forward mutation)	23-31 %	Negative	Study focussed on ethanol: Methanol was included for comparison. Methanol concentrations above 23 % were cytotoxic, reducing survival of the cells to less than 40 %. A 23 % methanol solution produced no mutagenic effects; cell survival was 53 %. However, assay performed with one E. coli strain E. coli (SA 500) as well as in the absence of metabolic activation system only. **Overall judgement**: The methods implemented were thought not to be sufficiently robust to support the results reported. Ref: Hayes et al. (1990)
MCF-7 and HepG2 cells	Activation of the p53R2 gene		Negative	Assay based on chemical activation of the p53R2 gene as assessed by the incorporation of a p53R2-dependent luciferase reporter gene into human cells (MCF-7 and HepG2). The Panel considered this study 'reliable' but not suitable for the present evaluation, because P53R2 gene is activated by DNA damage induced by ionizing radiation, UV light, including adriamycin but not by general chemical mutagens. **Overall judgement**: The methods implemented were thought to be sufficiently robust to support the results reported. Ref: Ohno et al. (2005)
Mouse lymphoma L5178Y(Tk+/Tk-)	Gene mutation	5-50 µL/mL (approx. 3.95-39.5 mg/mL)	Negative [b)] Positive [c)]	Significant increases in mutation frequency were observed at increased concentrations of S9. However, the lowest concentration tested (5 µL/mL) exceeded in terms of molarity (13.4 M) the maximum concentration recommended in OECD TG 476 (5 mg/mL, 5 µL/mL, or 0.01 M, whichever is the lowest). Therefore, indirect effects due to non-physiological culture conditions cannot be excluded. **Overall judgement**: The methods implemented were thought not to be sufficiently robust to support the results reported. Ref: McGregor et al. (1985, 1988)
Saccharomyces cerevisiae (ATCC26422)	Gene mutation	2-8 %	Negative [b)]	Methanol (2-8 %) was assessed for induction of gene mutations in Saccharomyces cerevisiae ATCC26422 as a control culture within a study aimed at elucidating the effects of ethidium bromide. Methanol did not induce gene mutations, but was tested without metabolic activation system only. The Panel considered the study as 'not reliable' in view of assessing the genotoxic potential of methanol. **Overall judgement**: The methods implemented were thought not to be sufficiently robust to support the results reported. Ref: Hamada et al. (1988)

Test system	Test Object	Doses	Reported Result	Comments
E. coli WP2 (λ)	Prophage-induction	0.15, 0.31, 0.62, 1.25, 2.5, 5 %	Negative [a)]	Materials and methods described in sufficient details; In two independent experiments MeOH was tested up to toxic concentrations. **Overall judgement**: The methods implemented were thought to be sufficiently robust to support the results reported. Ref: De Marini et al. (1991)
Chinese hamster (V79 cells)	Sister chromatid exchanges (SCE) and micronuclei	50 µL/mL	Negative	Treatment: single dose-level (50 µL/mL) which was 10 times greater than the maximum recommended dose-level to be used in in vitro studies (e.g. 5 µL/mL) to keep culture physiological conditions; Assayed in the absence of S9 only; Treatment time: 48 hours, which was exceedingly long compared to 1.5-2 cell cycle length recommended by OECD TG 487. **Overall judgement**: The methods implemented were thought not to be sufficiently robust to support the results reported. Ref: Lasne et al. (1984)

a) With and without metabolic activation.
b) Without metabolic activation.
c) With metabolic activation.

4. Methanol genotoxicity studies *in vivo*

Test system	Test Object	Route	Doses	Reported Result	Comments
Mouse (Pregnant CD-1 females)	Micronuclei in peripheral blood reticulocytes	Oral (intragastric)	2500 mg/kg twice a day from GD 6 to GD 10	Negative both in blood of foetuses or mothers	Only one dose-level assayed; Blood samples were collected eight days after last administration of compound but normal procedure indicate sampling not later than 72 hours; The frequency of MN in maternal and fetal reticulocytes was similar among the groups; the frequency of MN was not associated with either maternal or fetal hepatic folate concentrations. Furthermore, no significant difference in the frequency of MN in reticulocytes of foetuses that were affected by malformations compared to foetuses without malformations. The study does not include positive control. **Overall judgement:** The methods implemented were thought not to be sufficiently robust to support the results reported. Ref: Fu et al. (1996)
Mouse ICR strain 6 males/group	Micronuclei in bone marrow erythrocytes	Oral	1050, 2110, 4210, and 8410 mg/kg bw, single dose	Negative	GLP-study; Deviation from OECD TG 475: - only male animals were used - only one sampling time selected **Overall judgement:** The methods implemented were thought to be sufficiently robust to support the results reported. Ref: NEDO 1987
Rats	Hydroxymethyl DNA adducts	Oral	500 mg/kg per day for 5 days (stable isotope labelled methanol)	Labelled DNA adducts lower than the number of endogenous hydroxymethyl DNA adducts in all tissues	Concerns about the lower number of exogenous hydroxymethyl DNA adducts measured in the analysed tissues of rats following administration of [$^{13}CD_4$]-methanol at 500 mg/kg bw for 5 days compared to the number of endogenous hydroxymethyl DNA adducts. The isotope effect factor employed was the mean of several widely varied published values. Furthermore, the measurements of N-hydroxymethyl-dG and dA adducts was not direct but required an *in vitro* reduction process with $NaCNBH_3$ to the corresponding N-methyl derivative. The measurements of endogenous N-hydroxymethyl-dA adducts in the untreated animal group and in methanol treated group at 500 mg/kg bw for 5 days were rather variable. The study does not include positive control

Test system	Test Object	Route	Doses	Reported Result	Comments
					Overall judgement: The methods implemented were thought not to be sufficiently robust to support the results reported. Ref: Lu et al., 2012
Drosophila melanogaster wild type and Basc strains	Sex-linked lethal mutations		1000 mM	Negative	Ref: Gocke et al. (1981)
Mouse	Chromosomal aberrations	Oral	1000 mg/kg	Positive	Not assignable Increased the incidence of chromosomal aberrations (particularly aneuploidy and exchanges and the micronuclei of polychromatic erythrocytes). Results of the study were available as an abstract only. The study does not include positive control Ref: Pereira et al., 1982
Mouse B6C3F1 strain 15 males/group		Oral (gavage)		Increase in structural chromosome aberrations [exchanges (Robertsonian translocations) and aneuploidy; no breaks]	Deviations from current OECD TG 475: - one dose only - one time point only - mitotic indices not determined. Significant increases in chromosome aberrations [exchanges (Robertsonian translocation), aneuploidy] in male B6C3F1 were reported. However, concurrent vehicle control showed 10 exchanges out 665 cells scored and chromatid and chromosome exchanges are not expected in healthy animals. **Overall judgement:** The methods implemented were thought not to be sufficiently robust to support the results reported. Ref: Ward et al. (1983)

5. *In vivo* genotoxicity studies by other routes of exposure (inhalation and intraperitoneal administration)

Test system	Test Object	Route	Doses	Reported Result	Comments
Mouse C57black/6J 10 males/group	Micronuclei, SCE	Inhalation	0, 800, or 4000 ppm (0, 1048, and 5242 mg/m^3) methanol, 6 hours/day, for 5 days	Endpoints: 1. blood cells: micronuclei (MN) 2. excised lung cells: Chromatid Exchanges (SCEs), Sister chromosomal aberrations (CA) and micronuclei (MN), 3. excised testicular germ: synaptonemal damage. All endpoints studied gave negative results	Inhalation route of administration not relevant for evaluation of aspartame. The study does not include positive control. However, the study may be used for weight-of-evidence approach. **Overall judgement:** The methods implemented were thought to be sufficiently robust to support the results reported. Ref: Campbell et al. (1991)
Mouse male CD-1	DNA damage (8-oxodG)	Intraperitoneal	1 x 2000 mg/kg bw	No increase of 8-oxodG at 6 and 24 h in bone marrow, spleen lung, kidney of any species	Limit dose of 2000 mg/kg bw MeOH based on guidelines established for the comet assay developed in accordance with the *in vivo* genetic toxicology guidelines of (OECD. Valid study but because of i.p. route of administration study considered not relevant for present evaluation of aspartame. However, the study may be used for weight-of-evidence approach. **Overall judgement:** The methods implemented were thought to be sufficiently robust to support the results reported. Ref: McCallum et al. (2011a; 2011b)
Mouse male CD-1			Chronic 15 x 2000 mg/kg (one dose per day)		
Mouse male CD-1 mice OGG1 (-/-)			1 x 2000 mg/kg bw		
Rabbit male New Zealand white (NZW)			1 x 2000 mg/kg bw		
Monkey male(Macaca fascicularis)			1 x 2000 mg/kg bw		
Mouse Swiss-Webster 10 males/group	Micronuclei	Intraperitoneal	300, 600, 1200 or 2500 mg/kg b w on 4 consecutive days	Negative	Ref: US EPA 1991

Test system	Test Object	Route	Doses	Reported Result	Comments
Mouse NMRI strain 2 males and 2 females/group	Micronuclei in Bone-marrow erythrocytes	Single intraperitoneal injection	1920, 3200, 4480 mg/kg bw	Negative	**Overall judgement:** The methods implemented were thought to be sufficiently robust to support the results reported. Ref: Gocke et al. (1981)

6. DKP genotoxicity studies *in vitro*

Test system	Test Object	Doses	Reported Result	Comments
Salmonella typhimurium Tester strains: TA1535 TA1537 TA1538, TA98 and TA100	Gene mutation (plate incorporation)	10 to 5000 µg/plate	Negative	Reported identity of test compound: SC19192; Lot# TJT-12-32; Deviations from current OECD Guideline: 1) Tester strains (TA102 or WP2uvrA) bearing AT mutation were not used; 2) Second experiment performed was identical to the first performed. **Overall judgement:** The methods implemented were thought to be sufficiently robust to support the results reported. Ref: E98 (1978)
Salmonella typhimurium Tester strains: TA1535, TA1537, TA1538, TA98 and TA100	Gene mutation (plate incorporation with and without metabolic activation)	50 to 10 000 µg/plate	Negative	SC-19192, Lot TJT- 12-32. It meets requirements of previous version of OECD Guideline (OECD TG 471, adopted 26 may 1983); Deviations from current OECD TG 471: Tester strains *S. typhimurium* TA102 or E. coli WP2uvrA bearing AT mutation were not used; Second experiment performed was identical to the first performed. **Overall judgement:** The methods implemented were thought to be sufficiently robust to support the results reported. Ref: E106 (1978)

7. DKP genotoxicity studies *in vivo*

Test system	Test Object	Route	Doses	Reported Result	Comments
Rat 10 males /group	Chromosome aberrations in bone marrow erythrocytes	Oral (intragastric) (10% suspension in tween-80/water)	0 (control), 250, 500, 1000 and 2000 mg/kg bw/day for 5 consecutive days, given in three equally divided daily doses	Negative	Reported identity of test compound: SC-19192; Lot# 3R-A-7273; Deviations from current OECD TG 475: Sampling time selected 24 h; The highest dose may also be defined as a dose that produces some indication of toxicity in the bone marrow (e.g. greater than 50 % reduction in mitotic index). In this study mitotic indices were not even scored;50 metaphases per animal were scored; **Overall judgement:** The methods implemented were thought to be sufficiently robust to support the results reported. Ref: E30 (1972)
Rat 10 males/group	Host-mediated assay	Oral (intragastric)	0 (control), 250, 500, 1000, 2000 mg/kg bw/day, three equally divided doses for five consecutive days. 30 minutes after the final dose, the animals were inoculated with *Salmonella typhimurium*, G-46, by intraperitoneal injection. Three hours later the bacteria were recovered and the peritoneal washing was evaluated for the presence of mutants.	Negative	Reported identity of test compound: SC-19192; Lot# 3R-A-7273; No Guidelines available; Study protocol followed general recommendations; Single use of G-46 Salmonella typhimurium, tester strain strongly reduce the capability to identify different classes of genotoxins. **Overall judgement:** The methods implemented were thought to be sufficiently robust to support the results reported. Ref: E31 (1972)

Test system	Test Object	Route	Doses	Reported Result	Comments
Mouse Ha/ICR Swiss strain 10 males/group	Host-mediated assay	Oral	0 (control), 1000, 2000, 4000, 8000 mg/kg bw/day, given in three equally divided doses at two hour intervals for five consecutive days low and mid dose: 10 % DKP suspension (1 % aqueous Tween-80) high and very high dose: 15 % DKP suspension (1 % aqueous Tween-80).	Negative	Reported identity of test compound: SC-19192; Lot#6-R); Approximately 30 min. after the final dose, the animals were inoculated (i.p.) with Salmonella typhimurium (G-46), intraperitoneally. Three hours later the bacteria were recovered and the peritoneal washing was evaluated for the presence of mutants. 2000 mg/kg bw/day: 1 of 10 animals died (compound in lung). 4000 mg/kg bw/day: 2 of 10 animals died: one animal with diarrhoea, dehydration, other animal blood clot (unknown cause). 8000 mg/kg bw/day: diarrhoea, dehydration, 70 % mortality. 1000 mg/kg bw/day: discrepancy between number of survivors given in tab. 1 (n=10) and results shown in tab. 2,3 (n=7). Furthermore, this group differed significant from controls (tab. 2,3). Both points are not addressed by the authors. **Overall judgement:** The methods implemented were thought not to be sufficiently robust to support the results reported. Ref: E82 (1974)
Rat	Dominant lethal assay	Oral (intragastric)	1000 mg/kg bw/day given in two equally divided doses on single day.	Negative (paternal growth, maternal pregnancy rate, corpora lutea and implantation sites). Implantation sites were sub-classified as: 1) Viable fetal swelling; 2) Early fetal deaths; 3) Late fetal deaths.	Reported identity of test compound: SC-19192; Lot# A6906; No Guidelines available; Study protocol followed general recommendations; Overall judgement: The methods implemented were thought to be sufficiently robust to support the results reported. Ref: E42 (1973)

Appendix I. TABLES ON REPRODUCTIVE AND DEVELOPMENTAL TOXICTY STUDIES ON ASPARTAME

Species	Endpoint	Doses tested (mg/kg bw/day)	Animals per group	NOAEL (mg/kg bw/day)	Observed adverse effects at higher dose(s) in pups	Observed adverse effects in mothers (dose)	Comments
Mouse	Embryotoxicity and teratogenicity (Segment 2)	0, 1000 (1400)[a)], 2000 (2700), 4000 (5700) (diet)	F:36,36,36, 36	4000	N/A[b)]	None	Pregnant animals: 27,25,27,21. Study E89, 1975
Rat	Embryotoxicity and teratogenicity (Segment 2)	0, 2000 (2000), 4000 (4100) (diet)	F:30,30,30	4000 (4100)	N/A	Decreased feed consumption (high dose)	Pregnant animals: 24,26,23. Study E5, 1970
Rat	Peri- and postnatal development (Segment 3)	0, 2000 (1800), 4000 (3600) (diet)	M/F:20,20, 20	2000 (1800)	Transient effects on renal tubuli that were not observed in an extra group of pups examined at or shortly after weaning	None	This study is a evaluation of F2A pups of study E11 on PP 5,15, 21. Study E9, 1972
Rat	Reproductive performance and development (Segment 1)	0, 2000 (2400), 4000 (4900) (diet)	F: 48,30,30 M:14,14,14	4000 (4900)	N/A	Mild but statistically significant decrease in bw during gestation but feed consumption unaffected (high dose)	Exposure: M, 64 days PM[d]; F: 14 days PM, gestation, lactation; half F sacrificed GD 13, half allowed to litter. Study E10, 1972
Rat	Two-generation reproductive study	0, 2000 (1800), 4000 (3700) (diet)	P_1: F: 24,24,24 M: 12,12,12 P_2: F: 19,20,20 M: 10,10,10	2000 (1800)	BW suppression and smaller size of pups at weaning (statistically significant)	None	Study E11, 1971
Rat	Peri- and postnatal development	0, 2000 (2000), 4000 (4000) (diet)	F: 30,30,30	2000 (2000)	Number of viable pups per litter at birth and at weaning decreased (statistically	Decreased bw at GD 21 (low and high dose) and PP day 21	GD 14- lactation d21. Study E39, 1973

Species	Endpoint	Doses tested (mg/kg bw/day)	Animals per group	NOAEL (mg/kg bw/day)	Observed adverse effects at higher dose(s) in pups	Observed adverse effects in mothers (dose)	Comments
	(Segment 3)				significant); no effect on bw.	(high dose)	
Rat	Peri- and postnatal development (Segment 3)	0, 2000 (2500), 4000 (4400) (diet)	F:24,24,24	2000 (2500)	BW suppression of pups at birth (statistically significant) and weaning (not significant). Pup number increased but survival significantly decreased. Incomplete eyelid opening in 5/164 pups and lens opacity in 2/164 pups.	Decreased bw during lactation	GD 14- lactation d21. Study E47, 1973
Rat	Peri- and postnatal development (Segment 3)	0, 2000 (1800), 4000 (4000) (diet)	F: 30,30,30	2000 (1800)	BW suppression of pups at birth (statistically significant) and weaning (not significant). Pup survival significantly decreased. Incomplete eyelid opening in 2/79 pups.	Decreased feed intake at PP day 21 (high dose) and reduced bw at PP day 21 and during lactation (high dose).	GD 14- lactation d21. Study E48, 1973
Rabbit	Embryotoxicity and teratogenicity (Segment 2)	0, 2000 (1880), 4000 (1870) (diet)	F: 16,16,16	2000 (1880)	Serious congenital anomalies in the 'high' dose group: Cleft palate, microstomia, open eyes, digital malformations; decreased fetal weight (by 25 %).	Severe depression (> 50 %) in feed consumption	GD 6-19(morning); pregnant animals: P:14,16,16; aborted: 2,1,3. Study E54, 1974
Rabbit	Embryotoxicity and teratogenicity (Segment 2)	0, 1000 (840), 2000 (1450), 4000 (1260) (diet)	F: 14,14, 14,14	2000	N/A	Significant depression of feed consumption (35 %) in high dose. Abortions (17 %) in the high dose group.	GD 6-19(morning); pregnant animal: 12,14,14,13 Deaths: 0,1,1,0 Abortions: 0,0,0,2 Premature deliveries: 1,0,0,0. Study E53, 1973 Due to insufficient amount of test compound not all animals were fed the correct diets.

Species	Endpoint	Doses tested (mg/kg bw/day)	Animals per group	NOAEL (mg/kg bw/day)	Observed adverse effects at higher dose(s) in pups	Observed adverse effects in mothers (dose)	Comments
							Mid dose decreased number of fetuses
Rabbit	Embryotoxicity and teratogenicity (Segment 2)	0, 2000 (670), 3000 (1350), 4000 (1160) (diet)	F:15,15,15, 15	4000 (1160)	N/A	Feed consumption decreased by up to 29% in the high dose group.	GD 6-19(morning); pregnant animals: 12,13,10, 12 Deaths: 1,0,0,0,0 Abortions: 2,2,1,0 Premature deliveries: 1,1,0,0. Study E55, 1973
Rabbit	Embryotoxicity and teratogenicity (Segment 2)	0, 1000 (1100), 2000 (1900) (diet)	F:30,30,30	2000	N/A	None	GD 6-19(morning); pregnant animals: 26,23,25 Deaths: 0,1,3 Abortions: 0,0,0 Premature deliveries: 1,1,1 Control group was pair-fed; High dose 3 fetuses of one litter open eye (considered by the authors as minor malformations). Study E62, 1973
Rabbit	Embryotoxicity and teratogenicity (Segment 2)	0, 1400 (1380), 2400 (2360) (diet)	F: 26, 26, 26	2400	N/A[b)]		Study E63, 1973
Rabbit	Embryotoxicity and teratogenicity (Segment 2)	0, 2000 (intragastric)	F:12,36	2000	N/A[c)]	Study confounded by poor health and gastric intubation technique issues; high	GD 6-18; pregnant animals: 5,11 Deaths: 4,9 Abortions: 1,2 Feed consumption: control

Species	Endpoint	Doses tested (mg/kg bw/day)	Animals per group	NOAEL (mg/kg bw/day)	Observed adverse effects at higher dose(s) in pups	Observed adverse effects in mothers (dose)	Comments
						maternal mortality; depression of feed consumption by 40% compensated by pair-feeding of controls.	21.2 g/kg; treated group 34.6 g/kg. Very low pregnancy rate. Authors mention infectious pulmonary disease and dosing errors. No malformations. Study E51, 1973
Rabbit	Embryotoxicity and teratogenicity (Segment 2)	0, 2000 (intragastric)	F:24,72	2000	N/A	Study confounded by poor health and gastric intubation technique issues; pregnancy rate decreased in high dose group; depression of feed consumption by up to 62% compensated by pair-feeding of controls.	GD 6-18; NP animals: 3,25 Deaths: 1(NP), 6 (P) + 6 (NP) Abortions: 3,0 Controls had lower bw and fetal weight; increased no. of resorptions: 0.7 vs. 1.6 (not significant); historical control 0-1.1. Study E52, 1973
Rabbit	Embryotoxicity and teratogenicity (Segment 2)	0, 750, 2000 (intragastric)	F: 32,37,95	750	Major malformations in 7 fetuses of high dose group	Study confounded by poor health and gastric intubation technique issues; pregnancy rate decreased in high dose group; depression of feed consumption by up to 37% compensated by pair-feeding of controls. Increased no. of resorptions 0.9 vs 0.5 in controls and decreased no. of live fetuses (high dose)	GD 6-18; pregnant animals: 27,25,61 Deaths: 3,5,9 Abortions: 3,0,0 Premature deliveries:1,1,0 Implantation scars at C-section: 5,1,16. Study E79, 1974

Species	Endpoint	Doses tested (mg/kg bw/day)	Animals per group	NOAEL (mg/kg bw/day)	Observed adverse effects at higher dose(s) in pups	Observed adverse effects in mothers (dose)	Comments
Rabbit	Embryotoxicity and teratogenicity (Segment 2)	0, 500, 1000, 2000 (intragastric)	F: 50,50,50 (52),50	1000	Decreased pup weight and skeletal abnormalities at the high dose only.	A 67% decrease in feed intake and up to a 15% bw decrease (GD 22) at the high dose; high incidence of abortions in the high dose group that correlated with the severity in feed intake decrease in individual animals.	GD 6-18; pregnant animals: 42,46,48,37 Deaths: 4,4,1,4. NP: 8,4,4,13 Abortions: 0,2,0,24 Premature deliveries: 3,0,1,0 High number of abortions; Increased no. of resorptions (not significant). Study E90, 1975

a) In brackets, dose level consumed
b) N/A, Not applicable, (NOAEL was at the highest dose tested)
c) Study based on small number of animals.
d) Abbreviations: PM, pre-mating; F, female; M, male; GD, gestation day; G, gestation; L, lactation, P, pregnant; NP, non-pregnant

Appendix J. Tables on human studies (unpublished and published) reporting phenylalanine and aspartate levels after aspartame dosing

Subjects/Study	Doses	Duration	Plasma Phe	Plasma Aspartate	Comments
HEALTHY					
	SINGLE DOSE				
One 12 year-old healthy male and one 15-year old healthy female	Loading dose of 34 mg/kg bw aspartame; 2 weeks later: equivalent amount of Phe (19 mg Phe/kg).		No significant differences reported.	NR	E66, 1973. Tolerance of loading doses of aspartame by normal adolescent.
6 healthy males and 6 healthy females /cross over	Loading dose of 34 mg/kg bw aspartame; equivalent amount of aspartate (13 mg/kg).		Increased from 5-6 µmol/dL (normal fasting level) to 120 ± 30 µmol/L (after aspartame load), within normal postprandial range. Returned rapidly to normal.	Unchanged (2-8 µmol/L).	
6 healthy lactating females/cross over	Loading dose of 50 mg/kg bw either aspartame or lactose.		Increased to peak value of 162 ± 49 µmol/L (after aspartame load only), only slightly higher than those noted postprandially (120 ± 30 µmol/L).	Unchanged	Small increases in breast milk aspartate and Phe to postprandial range. E93, 1977. Effect of aspartame loading upon plasma and erythrocyte free amino acid levels in normal adult subjects

Subjects/Study	Doses	Duration	Plasma Phe	Plasma Aspartate	Comments
3 healthy males and three females/cross over	Loading dose of 100 mg/kg bw aspartame either dissolved in cold orange juice or as a slurry.		Increased levels to about 200-260 µmol/L with aspartame in solution. Aspartame in a slurry: peak at about 510 µmol/L after 1 hour (2 subjects), at over 300 umol/L after 2 hours (2 other subjects), at about 180 µmol/L after 3 hours (2 other subjects).	Little/no effects with aspartame in solution. Increased with aspartame as a slurry in two out of the 6 subjects: peak of 36 and 58 µmol/L; mean peak about 15 ± 18 µmol/L (4 subjects no changes).	
3 healthy males and three females	Loading dose of 150 mg/kg bw aspartame		Increased level, peak at about 351 ± 113 µmol/L	Very small changes, peak at 10 ± 7 µmol/L	
3 healthy males and three females	Loading dose of 200 mg/kg bw aspartame		Increased level, peak at about 487 ± 15 µmol/L after about 2 hours	Very small changes, peak at 7 ± 3.8 µmol/L at 90 min	
3 healthy males and three females	Loading dose of 200 mg/kg bw aspartame		Increased level, peak at about 487 ± 15 µmol/L after about 2 hours	Very small changes, peak at 7 ± 3.8 µmol/L at 90 min	
3 healthy males and 3 females/cross over	High protein meal containing aspartame plus MSG at 34 mg/kg bw		Slightly increased, peak around 80-90 µmol/dL after 3-4 hours (only slightly higher than those noted postprandially, 120 ± 30 µmol/L)	Unchanged.	The addition of aspartame into the meal increased Phe content from 43 to 64 mg/kg. E95, 1977. Metabolic studies of aspartame and monosodium glutamate (MSG) ingested as a meal component.

Subjects/Study	Doses	Duration	Plasma Phe	Plasma Aspartate	Comments
12 healthy infants (8-12 months)	Loading dose of 34 mg/kg bw aspartame		Slightly increased, from 63 ± 12 µmol/L (zero time) to 97 ± 27 µmol/L after 0.5 hours.	Unchanged or decreased from level observed at zero time (51 ± 33 µmol/L), but higher compared to adults even at zero time (3 µmol/L).	E107, 1977. Effect of aspartame loading upon plasma and erythrocyts, free amino acid levels and blood methanol levels in normal one-year old children.
	Loading dose of 50 mg/kg bw aspartame		Increased after aspartame ingestion, from 57 ± 5 µmol/L (zero time) to 116 ± 44 µmol/L after 1 hour.	Unchanged from level observed at zero time (30.3 ± 15 µmol/L) but higher compared to adults even at zero time (4.2 ± 3.3 µmol/L).	
8 healthy infant s (8-12 months)	Loading dose of 100 mg/kg bw aspartame		Increased after aspartame ingestion, from 48 ± 8 µmol/L (zero time) to 214 ± 56 µmol/L after 45 min.	Unchanged from level observed at zero time (16.3 ± 7.4 µmol/L) but higher compared to adults even at zero time (1.6 ± 0.5 µmol/L)	
6 subjects sensitive to Chinese restaurant syndrome/cross over	Loading dose of 34 mg/kg bw aspartame and sucrose (1000 mg/kg bw)		Increased after aspartame loading from baseline (56.6 ± 8.2 µmol/L) to a peak of 130 ± 25 µmol/L after 30 min. No significant changes after sucrose loading.	No significant differences.	E110, 1979. Effect of aspartame loading in subjects who report symptoms of Chinese restaurant syndrome after glutamate ingestion.
9 healthy subjects (6 males and 3 females)	Soup beverage meal without MSG or aspartame, with MSG (50 mg/kg bw) or with MSG (50 mg/kg bw)		Increased significantly after aspartame ingestion; mean peak level of 133 ± 50 µmol/L after 30 min of ingestion of the meal, returning to baseline after 4 hours.	Small but significant increases after aspartame ingestion; mean peak values of 49 ± 29 µmol/L at 30 min, which rapidly returned to baseline 150 min after aspartame	E111, 1979. Metabolic studies of aspartame and monosodium glutamate (MSG) when ingested together as part of a soup beverage meal.

Subjects/Study	Doses	Duration	Plasma Phe	Plasma Aspartate	Comments
	plus aspartame (34 mg/kg bw)			ingestion.	
6 healthy subjects (3 males and 3 females)	Hamburger-milk shake alone or with MSG (150 mg/kg bw) or with MSG (150 mg/kg bw) plus aspartame (23 mg/kg bw)		Increased after aspartame ingestion compared to MSG alone; significant peak level of 107 ± 23 µmol/L after 3 hours of ingestion of the meal.	Small increases (not significant compared to MSG alone) after aspartame ingestion; peak values of 36 ± 19 µmol/L at 1 hour); 32.5 ± 14 µmol/L after 1.5 hour ($p \leq 0.05$ compared to MSG alone).	E112, 1979. Metabolic studies of aspartame and monosodium glutamate (MSG) ingested as components of a hamburger-milk shake meal system in normal adult subjects.
6 healthy males	560 mg Phe in the form of 1000 mg aspartame or 12 200 mg albumin or water		Increased from 488 ± 96 to 75.8 ± 27.5 µmol/L at 0.25 hours after aspartame ingestion. AUC: 40 % increased (not significantly) after aspartame ingestion: 53 ± 26 µmol/L hr (aspartame); 38 ± 15 µmol/L hr (albumine)	Significantly increased at 0.25 hours after aspartame ingestion. Peak at 6 µmol/L.	Ref: Moller, 1990
8 healthy subjects/randomised cross-over	500 mg aspartame alone or with 100 g of sucrose		Significantly increased after aspartame ingestion from 62.1 ± 12.0 µmol/L (baseline) to 75 ± 13.4 µmol/L (15 min after dosing). Not significantly increased from baseline (60.4 ± 11.3 µmol/L) after aspartame with sucrose.	Not significantly changed.	Ref: Burns et al., 1991
10 healthy subjects/randomised cross-over	3000 mg aspartame in solution or in capsules		Significantly increased: peak = 191 ± 65.4 µmol/L at 35 ± 15 min, AUC = 15.340 ± 4.820 µmol/L/min (solution) vs 117 ± 39.5 µmol/L (capsules) at 123 ± 74 min, AUC = 8465 ± 3356 µmol/L/min.	Significantly increased: peak = 26.2 ± 16.3 µmol/L at 30 ± 14 min (solution) vs. 10.4 ± 5 µmol/L (capsules) at 106 ± 61.3 min.	Ref: Stegink et al., 1987a

Subjects/Study	Doses	Duration	Plasma Phe	Plasma Aspartate	Comments
12 healthy subjects	4 mg/kg bw aspartame		Not significantly increased.	Not significantly increased	Ref: Stegink et al., 1987b
20 healthy subjects/randomised cross-over	20 mg/kg aspartame (capsules or solution)		Significantly increased: Cmax = 103.3 µmol/L at Tmax = 108.6 min (capsule) vs. Cmax 126.6 µmol/L at Tmax = 36.6 min (solution). AUCs not significally different.	Not affected.	Ref: Burns et al., 1990
Healthy subjects	Loading dose of 34, 50, 100, 150, 200 mg aspartame/kg bw		Significantly increased. Peak at 110 ± 25 µmol/L, 162 ± 49 µmol/L, 203 ± 20.5 µmol/L, 351 ± 113 µmol/L and 487 ± 151 µmol/L after 30 min-2 hours	Not significant differences at 34, 50, 150, 200 mg/kg bw. Statistically significant increase with peak at 4.3 ± 2.3 µmol/L at 100 mg/kg bw (after 0.5 hours aspartame ingestion). Increased but less than those observed post-prandially in infants or adults fed a protein containing meal.	Ref: Stegink, 1984
6 healthy subjects	184 mg aspartame (in a diet coke can), providing 104 mg Phe		Not affected over the 3-hours post ingestion period.		Ref: Mackey and Berlin, 1992
6 healthy women/cross over	Aspartame or lactose at 50 mg/kg bw		Significantly increased: 4-fold over fasting values 45 min after aspartame loading. Returned to baseline in 4 hours.	Not significant changes.	Ref: Stegink et al., 1979a.
24 one-year old infants	34, 50 and 100 mg/kg bw aspartame in a beverage		Significantly increased. Mean peak: 93 ± 14, 116 ± 44, 223 ± 115 µmol/L at 34, 50, and 100 mg/kg bw (similar to values	Not significantly changed at 34 and 50 mg/kg bw. Significantly increased at 100 mg/kg bw.	Ref: Filer et al., 1983.

Subjects/Study	Doses	Duration	Plasma Phe	Plasma Aspartate	Comments
			observed in adults).		
	REPEATED DOSES				
31 healthy man and 38 women (21-45 years old)/double blind	Placebo, 200 mg or 300 mg aspartame in capsule. Weekly dose escalation protocol: 0.6, 1.2, 2.4, 4.5, 6.3, 8.1 g aspartame/day.	Six weeks	No significant difference with placebo, fluctuations (mean below 120 µmol/L). Peak = 120 µmol/L after 9 weeks.	NR	E23, 1972. Short term tolerance of aspartame by normal adults.
95 obese men and women (21-70 years old)/double blind	Placebo, 200 mg or 300 mg aspartame in capsule. Weekly dose escalation protocol: 0.6, 1.2, 2.4, 4.5, 6.3, 8.1 g aspartame/day.	Six weeks	No significant difference with placebo, fluctuations (mean below 120 µmol/L). Peak = 120 µmol/L after 3 weeks.	NR	E24, 1972. Short term tolerance of aspartame by obese adults.
Group 1: 18 males and 32 females who participate in E23 study. Group 2: 12 males and 5 females.	1800 mg aspartame/day (2 x 300 mg capsules, 3 x daily).	27 weeks (group 1); 21 weeks (group 2)	No significant difference with placebo, fluctuations (mean below 120 µmol/L). Peak = 110 µmol/L (male), 103 µmol/L (female), after 1 week.	NR	E60, 1973. Long term tolerance of aspartame by normal adults.
62 males and 64 females. Group A: 2-3 year old. Group B: 4-6 year-old. Group C: 7-9 year-old. Group D: 10-12 year-old. Group E:13-20 year-old/double blind	Sucrose or aspartame: 43.7 ± 1.66 (A) 39.5 ± 0.99(B) 58.1 ± 2.83(C) 55 ± 3.57(D) 42.9 ± 1.84	13 weeks	No significant difference with sucrose, fluctuations. Peaks (asp): 110 µmol/L 1 week after dosing (Group A, B), 114 µmol/L 1 week postdosing (Group C), 103 µmol/L 3 weeks after dosing (Group D), 96 µmol/L 1 week after dosing	NR	E61, 1972. Long term tolerance of aspartame by normal children and adolescents.

Subjects/Study	Doses	Duration	Plasma Phe	Plasma Aspartate	Comments
	(E) mg aspartame/kg/day.		(Group E).		
105 obese adults (21-70 years old). Group 1: 69 subjects who participate in E24 study. Double blind. Group 2: 36 subjects.	1800 mg aspartame/day (2 x 300 mg capsules, 3 x daily).	27 weeks (Group 1); 21 weeks (Group 2)	No significant difference with placebo, fluctuations (mean below 120 µmol/L). Peak = 96 µmol/L after 8-11 weeks.	NR	E64, 1972. Long term tolerance of aspartame by obese adults.
52 men and women (21-45 years old). Follow up of the E25 study.	1800 mg aspartame/day (2 x 300 mg capsules, 3 x daily).	21 weeks	No significant difference with placebo, fluctuations (mean around 120 µmol/L). Peak = 132 µmol/L after 1 week. The only significant difference between the treatment groups occurred in the Phe levels at week 16.	NR	E67, 1973. Long term tolerance of aspartame by adults PKU heterozygotes.
6 healthy adult subjects/cross over	Test beverage alone or with aspartame (600 mg/hour or 18 mg/kg bw) or DKP (150 mg/hour or 4.5 mg/kg bw)	8 hours/Wash out of 1 week between treatments	Significantly increased after aspartame ingestion; mean plasma Cmax = 113 µmol/L (aspartame), 58 µmol/L (placebo); Tmax = 5.83 hours (aspartame) and 2.83 hours (placebo); Total AUC (0-9) = 788 µmol/L (aspartame), 444 µmol/L (placebo). Plateau concentration after 5 servings. Small increased after DKP ingestion (not clinically significant changes).	Slightly increased; Mean Cmax = 15 µmol/L (aspartame), 10.8 µmol/L (placebo). No significant difference for Tmax and AUC.	UN05, 1987. Effect of repeated ingestion of beverage containing aspartame or DKP on amino acid and DPK concentrations in plasma and urine of normal healthy subjects. Stegink et al, 1989.
53 healthy adult subjects (23 males and 30 females) for aspartame group; 55 healthy subjects (28	75 mg/kg/day (capsules of 300 mg divided into 3 doses/day)	24 weeks	No statistically significant differences. Mean: 49 ± 18 µmol/L after 24 weeks (aspartame); Mean: 45 ± 6 µmol/L after	No statistically significant changes. Mean: 7.7 ± 5.1 µmol/L after 24 weeks (aspartame); Mean: 7.1 ± 2 µmol/L	UN 08, 1988. Safety of long term aspartame administration in normal subjects.

Subjects/Study	Doses	Duration	Plasma Phe	Plasma Aspartate	Comments
males and 27 females) for placebo group/randomise double-blind, placebo-controlled			24 weeks (placebo). Cmax = 60 µmol/L, Tmax = 9.79 weeks (aspartame); Cmax = 54 µmol/L, Tmax = 8.22 weeks(placebo).	after 24 weeks (placebo). Cmax = 11.1 µmol/L Tmax = 8.85 weeks (aspartame); Cmax = 10.1 µmol/L Tmax = 9.98 weeks (placebo)	
8 healthy adults (4 males and 4 females)/randomised cross-over	10 mg/aspartame bw/serving	3 servings at 2 hours of interval (6 hours)	Significantly increased: 16.4 to 20.5 µmol/L above baseline value (51 ± 8.2 µmol/L) 30 to 45 minutes after each dose (within normal postprandial value at each time).	Unaffected	Ref: Stegink et al., 1988
6 healthy young adults (3 males and 3 females)/cross- over	600 mg aspartame/serving	8 servings at 1 hours of interval (8 hours)	Significantly increased: 14.1 to 23.5 µmol/L above baseline value, 30 min after ingestion (within normal postprandial value at each time). Steady state after 4-5 serving.	Not significantly increased.	Ref: Stegink et al., 1989
PKU HETEROZYGOTE					
	SINGLE DOSE				
12 healthy subjects/4 female PKU Heterozygotes	Loading dose of 34 mg/kg bw aspartame		Slightly increased from 110 to 160 µmol/L after 1 hour but close than those noted postprandially (120 ± 30 µmol/L).	Unchanged.	E93, 1977. Effect of aspartame loading upon plasma and erythrocyte free amino acid levels in normal adult subjects.
4 healthy women (20-28 years) and 4 PKU heterozygotes women.	Loading dose of 34 mg/kg bw aspartame		Remained within the normal range (60-182 µmol/L) in both healthy and PKU women. Peak at about 140-150 µmol/L after 1 hour (PKU heterozygotes) and 50-120 µmol/L after 1 hour (normal subjects).	NR	E108, 1978. Effect of aspartame in plasma and red cell amino acids of apparently healthy female adults and on presumed PKU heterozygotes.
6 healthy men and	Loading dose of		Increased after aspartame ingestion	No significant changes in	E109, 1978. Effect of aspartame

Subjects/Study	Doses	Duration	Plasma Phe	Plasma Aspartate	Comments
women and 5 PKU heterozygotes women.	100 mg/kg bw aspartame		in both normal and PKU subjects; mean maximum levels of 200 ± 60 µmol/L after 30-90 min (normal subjects); ranging from 355 ± 76 µmol/L at 30 min to 417 ± 24 µmol/L at 90 min (in PKU subjects).	both normal and PKU subjects.	loading at 100 mg/kg bw upon plasma and erythrocyte levels of free amino acid in normal subjects and subjects presumed to be heterozygotes for PKU.
8 healthy subjects and 6 PKU heterozygotes/randomised cross-over	10 mg/kg bw aspartame		Increased. Healthy subjects: from baseline of 51 ± 8.2 µmol/L to 67 ± 7.5 µmol/L. PKU: from baseline of 90 ± 17.1 µmol/L to 121 ± 20.8 µmol/L	Not significantly increased.	Ref: Stegink et al., 1987b
12 healthy subjects (6 men and 6 women) and 8 PKU heterozygotes women	Loading dose of 34 mg aspartame/kg bw		Significantly increased. Peak at 110 µmol/L after 1 hour (normal) and 160 µmol/L after 1 hour (PKU).	Not affected.	Ref: Filer and Stegink 1989
13 PKU heterozygotes (7 women and 6 men) and 13 healthy subjects (5 women and 8 men)	A meal providing 303 µmol/kg Phe		Increased. Mean peak around 100 ± 10 µmol/L after 3 hours (PKU); around 80 ± 10 µmol/L after 3 hours (control).	NR	Ref: Curtius et al., 1994
10 PKU heterozygotes (5 women and 5 men) and 10 healthy subjects (5 women and 5 men)	Same meal with addition of 85 µmol/kg aspartame (providing 75 µmol/kg Phe)		Increased. Mean plasma level: 95 ± 7 µmol/L after 1 hour (control); 153 ± 21 µmol/L after 3 hours (PKU heterozugous). Only slightly grater than the usual postprandial range for both controls and PKU heterozygotes.		
10 healthy subjects as control; subjects with classic PKU; subjects	10 mg/kg bw aspartame		Significantly increased from 44.5 ± 12.9 µmol/L (baseline) to 58.0 ± 9.5 µmol/L (1 hour after	NR	Ref: Caballero et al., 1986

Subjects/Study	Doses	Duration	Plasma Phe	Plasma Aspartate	Comments
with atypical hyperphenylalaninemia; subjects PKU heterozygotes			ingestion) in controls (+ 30 %); from 68.8 ± 13.8 µmol/L (baseline) to 82.3 ± 17.3 µmol/L (1 hour after ingestion) in PKU heterozygotes) (+ 20 %)		
6 PKU heterozygotes females and 12 healthy subjects (6 males and 6 females)	34 mg/kg aspartame in orange juice		Increased. Healthy subjects: from 56.6 ± 12.1 µmol/L (baseline) to 111 ± 25 µmol/L (within normal postprandial range); returned to baseline after 8 hours. PKU subjects: mean peak at 160 ± 22.5 µmol/L (only slightly above postprandial values in human infant and adult).	Not affected	Ref: Stegink et al., 1979b.
REPEATED DOSES					
65 men and women PKU heterozygotes (21-45 years old)/double blind	Placebo, 200 mg or 300 mg aspartame in capsule. Weekly dose escalation protocol: 0.6, 1.2, 2.4, 4.5, 6.3, 8.1 g aspartame/day; 0.34, 0.67, 1.35, 2.53, 3.54, 4.55 g Phe/day.	6 weeks	No significant difference with placebo, fluctuations (mean below 120 µmol/L). Peak = 168 µmol/L after 6 weeks.	NR	E25, 1972. Short term tolerance of aspartame by adults PKU heterozygotes.
6 heterozygotes PKU adult subjects/cross over	Test beverage alone or with aspartame (600 mg/hour or 18 mg/kg bw) or DKP (150 mg/hour or 4.5 mg/kg bw)	8 hours/Wash out of 1 week between treatments	Significantly increased after aspartame ingestion; mean plasma Cmax = 167 µmol/L (aspartame), 75.2 µmol/L(placebo); Tmax = 6.83 hours (aspartame) and 3.08 hours (placebo); Total AUC (0-9) = 1162.6 µmol/L (aspartame),	Unaffected	UN06, 1987. Effect of repeated ingestion of beverage containing aspartame or DKP on amino acid and DPK concentrations in subjects heterozygotes for PKU.

Subjects/Study	Doses	Duration	Plasma Phe	Plasma Aspartate	Comments
			599 µmol/L (placebo). Plateau concentration after 6 servings. Small increased after DKP ingestion (not clinically significant changes).		
6 adults PKU heterozygous/randomised cross-over	600 mg aspartame/serving	8 servings at 1 hours of interval (8 hours)	Significantly increased from 23.5 to 40.3 µmol/L above baseline 30 min after ingestion. Steady state after 5 serving (slightly but significantly higher than usual postprandial values for heterozygotes PKU).	Not affected	Ref: Stegink et al., 1990
48 adults (21 men and 27 women) PKU heterozygotes/ randomised, double blind, placebo-controlled, crossover	Placebo or 15 or 45 mg/kg day	12 weeks	Significantly increased at 1 and 3 hours following ingestion of 45 mg/kg day aspartame. Placebo: 90.4 ± 21.23 µmol/L (1 hour post dose) and 88.7 ± 22.36 µmol/L (3 hours post-dose). 45 mg/kg day group: 116.9 ± 29.48 µmol/L (1 hour post-dose) and 112.5 ± 40.30 µmol/L (3 hours post-dose)	NR	Ref: Trefz et al., 1993
PKU HOMOZYGOTE					
	SINGLE DOSE				
2 14 year-old PKU homozygous boys: one on a liberalised Phe diet (70 mg/kg/day), the other on a restricted Phe diet (17 mg/kg/day).	Loading dose of 34 mg/kg bw aspartame; 2 weeks later: equivalent amount of Phe (19 mg Phe/kg). Liberalised diet:		No significant differences reported.	NR	E26, 1972. Tolerance of loading doses of aspartame by PKU Homozygous children.

Subjects/Study	Doses	Duration	Plasma Phe	Plasma Aspartate	Comments
	total amount of 3228 mg of Phe (dietary intake: 2539 mg + Phe added from loading dose: 689 mg); restricted diet: total amount of 2037 mg of Phe (dietary intake: 965 mg + Phe added from loading dose: 1072).				
7 subjects homozygous for PKU and 7 healthy subjects	200 mg aspartame in beverage with or without carbohydrate (CHO)		Not increased over baseline values (55 ± 8.5 μmol/L, healthy; 1500 ± 230 μmol/L, PKU).	NR	Ref: Wolf-Novak et al., 1990
5 subjects homozygous for PKU	184 mg aspartame (in a diet coke can), providing 104 mg Phe		Increased levels vary from 15.6 μmol/L (above Baseline = 302 μmol/L) to 106 μmol/L (above basline = 798 μmol/L)		Not considered clinically significant. Ref: Mackey and Berlin, 1992

NR: not reported

Appendix K. SAS internal report

ESTABLISHING LOWER BOUND ASPARTAME DOSE TO REACH PHENYLALANINE PLASMA THRESHOLD LEVELS

European Food Safety Authority[22]

European Food Safety Authority (EFSA), Parma, Italy

EXECUTIVE SUMMARY

Simulation models can combine several sources of variation that could arise due to diversity in the population, but also on the limited amount of information from which the summary measures are built. Summary data collected on C_{max} phenylalanine plasma concentration for different studies on different doses were used to estimate the minimum (lower bound) dose of aspartame that is obtained for a particular phenylalanine plasma level threshold. The data contain arithmetic means and standard deviation of phenylalanine levels in plasma (μM), the number of individuals involved and the aspartame dose administered (mg/kg of body weight). The arithmetic measures were transformed to obtained geometric measures. In each simulation, individual phenylalanine levels in plasma for each dose were generated according to the number of individuals reported, geometric mean and the geometric standard deviations based on a lognormal distribution. For each simulated dataset a generalized additive model (Wood 2003, 2004, 2006a, 2006b and 2011), considering spline smoothing functions of aspartame doses, was used. The simulation model used allows studying the relationship between aspartame dose (mg/kg bw) and phenylalanine plasma level (μM) for healthy and heterozygote individuals. Flexible models were used to fit the simulated individual data based on geometric transformations of the summary measures drawn from lognormal distribution, the smoothed fitted model was evaluated in term of monotonicity and results shown that the model obtained was strictly increasing monotone, even when the model structure was left flexible, allowing for other type of relationship, but the data at hand proved to be monotonically increasing. The results obtained for the three different threshold used (240, 300 and 360 in μM) in normal individuals consistently produced aspartame dose lower bound that were above the ADI level (40 mg/kg of body weight) regardless of the confidence level (α) used. In the case in which the model was fitted for heterozygote individuals, thresholds of 300 or 360 μM shown consistent aspartame dose lower bounds above the ADI level when the level of confidence considered was 0.05, and only for the threshold of 360 when α was equal to 0.01. If the threshold considered is 240 μM and α is 0.05, the aspartame dose lower bound could be below the ADI level (in about 18 % of the times), for the case in which the confidence level considered was 0.01, then in 46.6 % of the simulations, the aspartame dose lower bound was below the ADI level for the phenylalanine threshold of 240 μM and 5.1 % for the phenylalanine threshold of 300 μM. It is important to highlight that for the heterozygote population, the results obtained should be interpreted with caution due to the limited number of doses found and the amount of individuals used to derive the summary data.

KEY WORDS

spline model, dose-response models

[22] Correspondence: sas@efsa.europa.eu

BACKGROUND

The Commission has asked the European Food Safety Authority to re-evaluate the safety of food additives already permitted in the Union before 2009 and to issue scientific opinions on these additives, taking especially into account the priorities, procedures and deadlines that are enshrined in the Regulation (EU) No 257/2010 of 25 March 2010 setting up a programme for the re-evaluation of approved food additives in accordance with the Regulation (EC) No 1333/2008 of the European Parliament and of the Council on food additives.

OBJECTIVES AND SCOPE OF THE REPORT

The objective of this report is to:

- Establish lower bound doses of aspartame that give rise to different phenylalanine plasma level thresholds.
- Develop a simulation model that uses summary information regarding C_{max} phenylalanine plasma level for certain aspartame bolus doses collected from different studies in humans that account for the variability in each dose groups and the limited number of individuals used to estimate the phenylalanine C_{max} levels to obtain the lower bound aspartame dose for the pre-specified thresholds.

1. Introduction

Simulation models are a powerful tool when dealing with summary data, it can combine several sources of variation that could arise from the outcome of interest in itself, due to diversity in the population under study, but also on the limited or scarce amount of information from which the summary measures are built. Moreover, when the purpose of the study is to establish the kind of relationship between outcomes and covariates of interest, these types of model also offer the advantage of generating (mimicking) the experimental conditions more than once, and thus accounting for possible variations that could occur if the experiment was conducted several times. Spline models (Wood 2003, 2004, 2006a, 2006b and 2011) is a non/semi-parametric flexible tool that could be used to establish the relationship between the outcome variable under consideration and the covariates that could potentially influence the outcome observed, offering the possibility to generate smoothing curves without having to specify any predefined mathematical expression. The SAS unit was asked to derive a simulation model that could be used to predict an estimated aspartame dose and confidence band through modelling the relationship between aspartame bolus doses and C_{max} phenylalanine levels in plasma. For this purpose, simulation models in combination with spline modelling techniques were used.

The objective of this report is to:

- Establish the lower bound doses for different phenylalanine plasma level thresholds
- Develop a simulation model that uses summary information regarding C_{max} phenylalanine plasma level for certain aspartame bolus doses collected from different studies in humans that account for the variability in each dose groups and the limited number of individuals used to estimate the phenylalanine C_{max} levels to estimate the lower bound aspartame dose for the pre-specified thresholds.

2. Methodology Proposed

Summary data collected on C_{max} phenylalanine plasma concentrations from different studies using different doses of aspartame were used to estimate the minimum (lower bound) dose of aspartame giving rise to a particular phenylalanine plasma level threshold concentration. The data contain arithmetic means ($\bar{y}$) and standard deviation (S_y) of phenylalanine levels in plasma (y in μM), number of individuals involved (n) and the aspartame dose administered (d in mg/kg of body weight). The data were taken from the following sources.

Burns ST, Stargel, WW and Horwitz A, 1990. Bioavailability of phenylalanine and aspartate from aspartame (20 mg/kg) in capsules and solution. Metabolism, 39, 1200-1203.

Stegink LD, Wolf-Novak LC, Filer LJ Jr, Bell EF, Ziegler EE, Krause WL and Brummel MC, 1987. Aspartame-sweetened beverage: effect on plasma amino acid concentrations in normal adults and adults heterozygous for phenylketonuria. The Journal of Nutrition, 117, 1989-1995.

E93, 1977. Final Report On Effect Of Aspartame Loading Upon Plasma And Erythrocyte Free Amino Acid Levels In Normal Adult Subjects. Searle.

E108, 1978. Effect of aspartame on plasma and red cell amino acids of apparently healthy female adults and on presumed phenylketonuric heterozygotes. Searle.

E110, 1979. Effect of Aspartame Loading In Subjects Who Report Symptoms Of Chinese Restaurant Syndrome After Glutamate Ingestion. Searle.

The arithmetic measures were transformed to obtain geometric measures ($\bar{z}$ and S_z), given that the plasma concentration of phenylalanine should follow a lognormal distribution ($z = ln(y)$). The transformation was as follows:

$$CV_y = \frac{S_y}{\bar{y}}$$

$$\bar{z} = ln\left[\frac{\bar{y}}{\sqrt{1 + CV_y^2}}\right]$$

$$S_z^2 = ln\left(1 + CV_y^2\right)$$

Simulation models (1000 simulations) were used in order to estimate the lower bound dose for three different phenylalanine plasma level thresholds (240, 300 and 360 µM) and two significance α levels (0.05 and 0.01).

In each simulation, phenylalanine levels in plasma for each aspartame dose were generated according to the number of individuals reported, geometric mean ($\bar{z}$) and the geometric standard deviations (S_z) based on a lognormal distribution with these parameters ($\bar{z}$ and S_z). The number of samples drawn for each aspartame dose from the assumed lognormal distribution was equal to the number of individuals reported. Each simulated dataset containing aspartame dose and individual C_{max} phenylalanine levels in plasma was fitted using a generalized additive model (Wood 2003, 2004, 2006a, 2006b and 2011), considering the smoothing functions to be spline functions of aspartame doses and the confidence band around the fitted model used to estimate the α-percentile lower bound for aspartame dose that can potentially give rise to the three different phenylalanine plasma level thresholds (240, 300 and 360 µM).

A generalized additive model (GAM, Hastie and Tibshirani, 1986, 1990) is a generalized linear model (GLM, McCullagh and Nelder, 1989) where the linear predictor is specified as a sum of smooth functions of some or all of the covariates collected.

$$E(Z_i) \equiv \mu_i, \qquad g(\mu_i) = \eta_i \equiv D_i^* \beta^* + f_1(d_{1i}) + f_2(d_{2i}) + \ldots$$

where Z_i is the response variable; $g(\cdot)$ is a monotonic link function; D_i^* is the ith row of the strictly parametric part of the model (called design matrix) and β^* the parameter vector expressing the parametric association between the response and the covariate of interest, and the $f_j(\cdot)$ are the smooth functions of the covariates d_j. In this report, for all models a thin plate regression spline was used. In order to avoid over fitting, the model parameters are estimated using a penalized maximum likelihood approach and smoothing parameters are chosen based on cross-validation method (Hastie and Tibshirani, 1986, 1990). Ample details on the method used to fit the model are presented in Wood (2004).

The process was repeated 1000 times and the lower bound dose α-percentile (α = 0.05 and α = 0.01) for aspartame was retained to finally construct the distribution of lower bounds for each of the three different phenylalanine plasma level thresholds, which account for the uncertainty on the observed summary levels reported in terms of limited number of subjects for each of the aspartame dose groups.

3. Results

3.1 Normal Population

The simulation model generated individual C_{max} phenylalanine plasma levels for each of the aspartame dose groups collated from the different studies, based on a lognormal distributional assumptions. The spline model was fitted to the individual phenylalanine plasma level data (Figure 1 and Figure 6, considering α being equal to 0.05 and 0.01 respectively). It is important to highlight that the advantage of this model is that the type of relationship between the response (C_{max} phenylalanine plasma level) and the covariate (aspartame dose) is left unspecified, meaning that deterministic/mathematical models are not assumed. The confidence band around the smoothed fitted line was estimated and the upper confidence line was used to estimate the lower bound aspartame dose that could give rise to any particular phenylalanine threshold C_{max}. For example Figure 1 shows that when the threshold phenylalanine C_{max} is 240 µM, the associated aspartame lower bound dose would be 76.4 mg/kg of body weight (when $\alpha = 0.05$). In order to explore monotonicity of the fitted smooth function, first derivatives of the function were estimated for each simulation, which were strictly greater than zero for all doses of aspartame between 0 and 200 mg/kg bw, indicating strictly monotonically increasing smooth functions, result for a single simulation is shown in Figure 2.

Figure 3 to Figure 5 and Figure 7 to Figure 9 shown the aspartame dose lower bound distribution when the phenylalanine plasma threshold levels are 240, 300 and 360 considering different level of confidence (α). It is important to highlight that when the confidence band around the smoothed line is using a confidence level of 0.95 (1- α), then for all threshold levels of phenylalanine the distribution of aspartame dose lower bound is always above the ADI value of 40 mg/kg bw. It should also be noted that even when $\alpha = 0.01$ and the phenylalanine threshold is considered to be 240 µM, the aspartame dose lower bound 95th confidence interval (54.6; 118.1) is above the ADI value of 40 mg/kg bw.

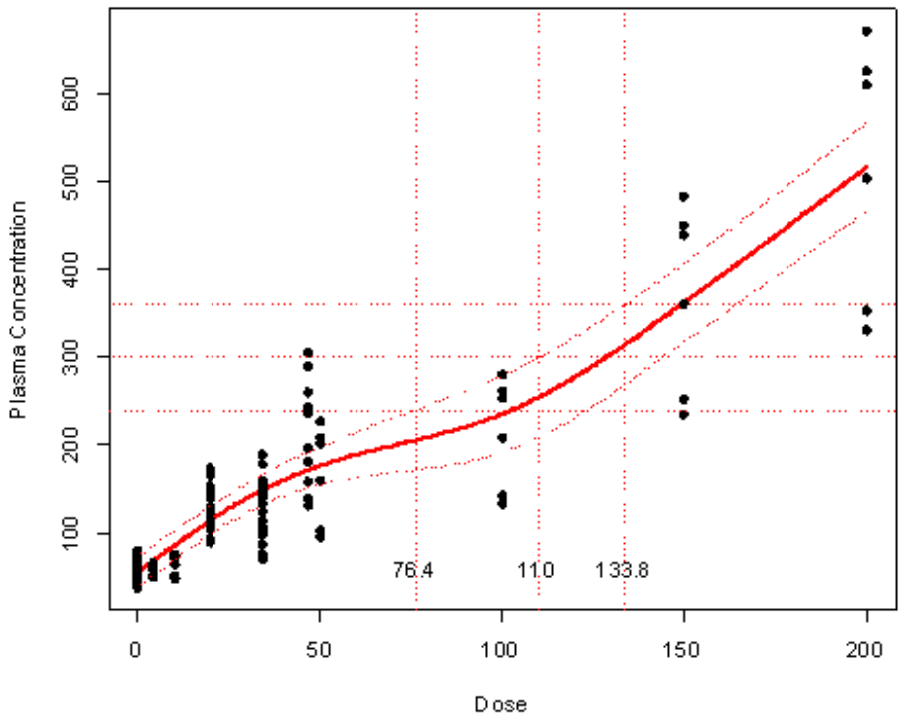

Figure 1. Single simulation example to illustrate dose response model between C_{max} phenylalanine plasma concentration (y-axis in µM) and aspartame dose (x-axis in mg/kg bw) considering $\alpha = 0.05$

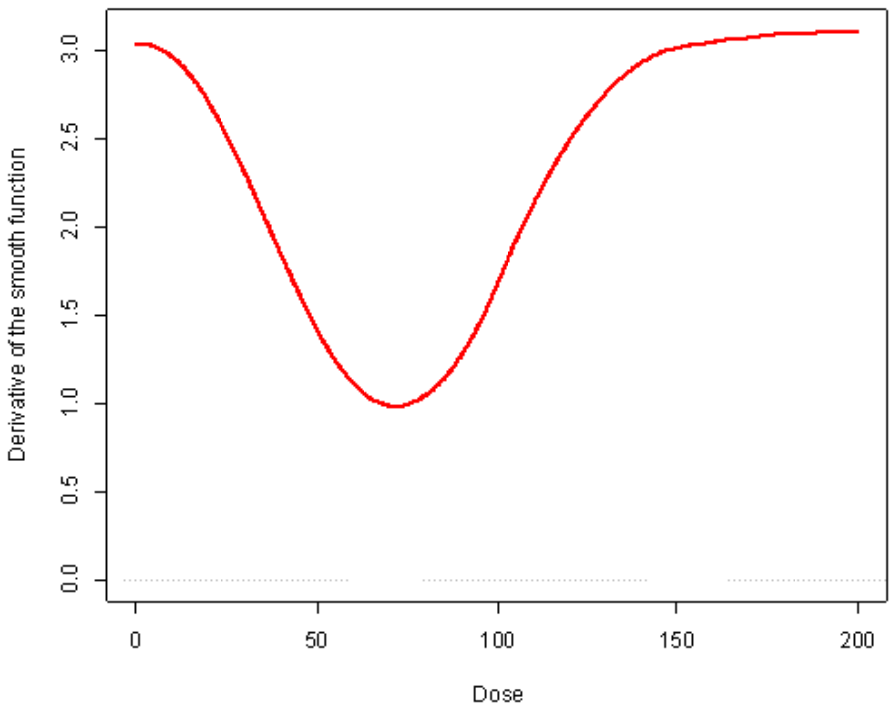

Figure 2. First derivative of the smoothed dose response function from the previous simulation example (x-axis: aspartame dose in mg/kg bw) considering α = 0.05.

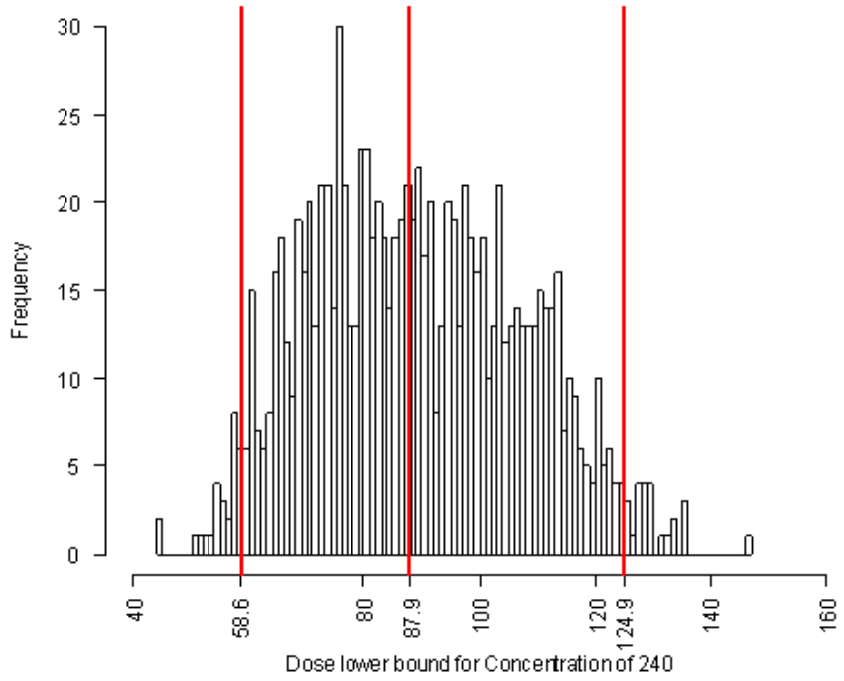

Figure 3. Histogram of aspartame dose lower bound (x-axis in mg/kg bw) considering α = 0.05 for phenylalanine plasma level threshold of 240 μM.

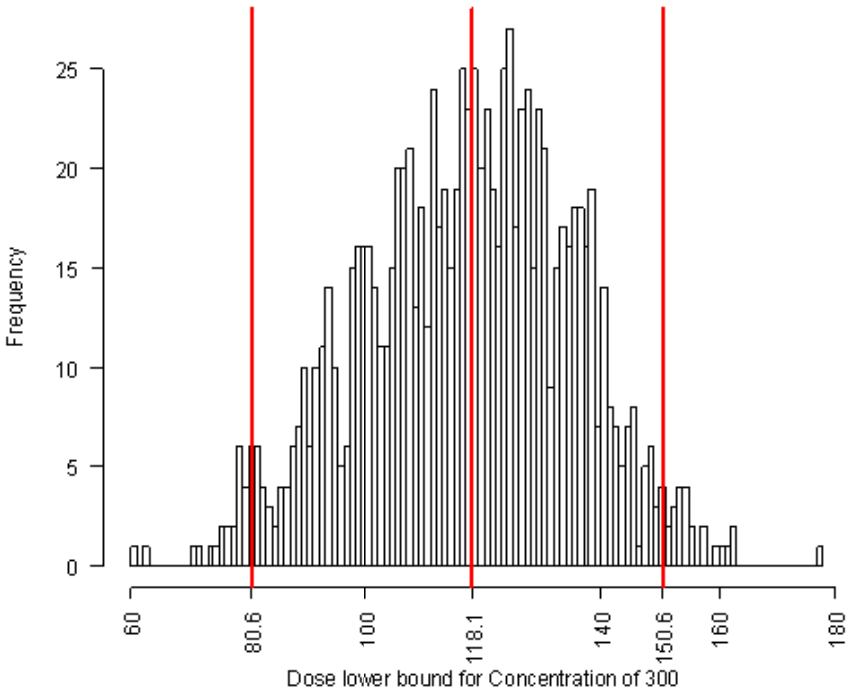

Figure 4. Histogram of aspartame dose lower bound (x-axis in mg/kg bw) considering α = 0.05 for phenylalanine plasma level threshold of 300 μM.

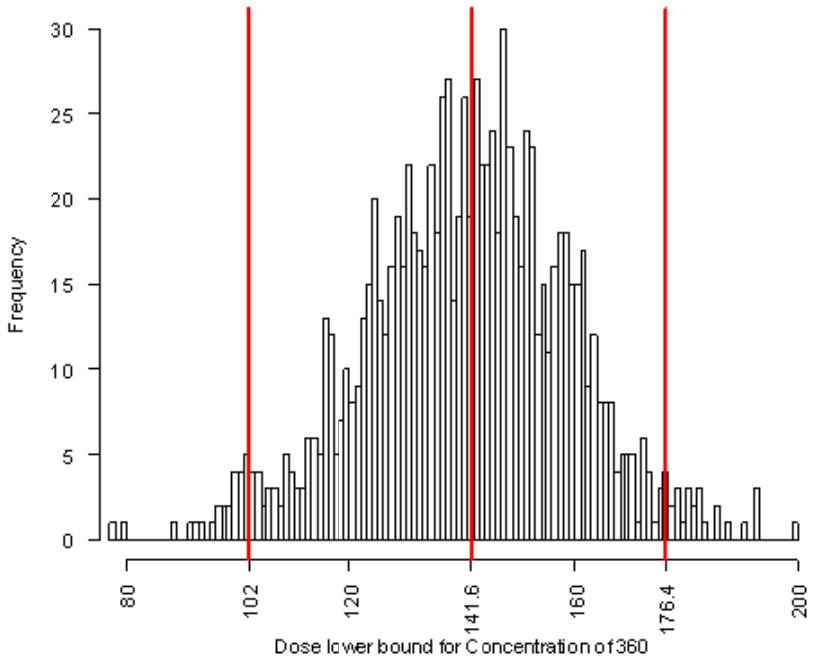

Figure 5. Histogram of aspartame dose lower bound (x-axis in mg/kg bw) considering α = 0.05 for phenylalanine plasma level threshold of 360 μM.

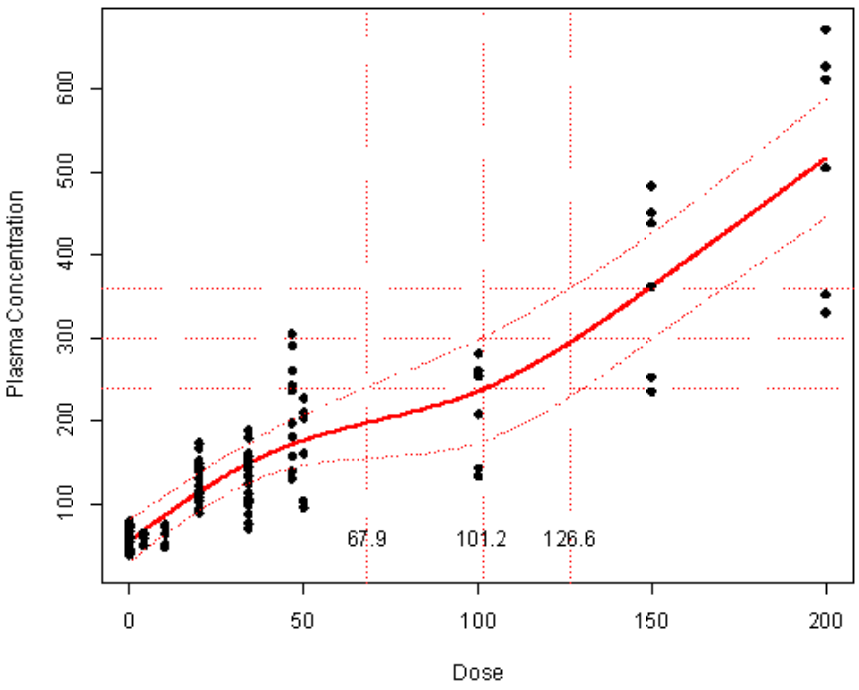

Figure 6. Single simulation example to illustrate dose response model between C_{max} phenylalanine plasma concentration (y-axis) and aspartame dose (x-axis in mg/kg bw) considering $\alpha = 0.01$

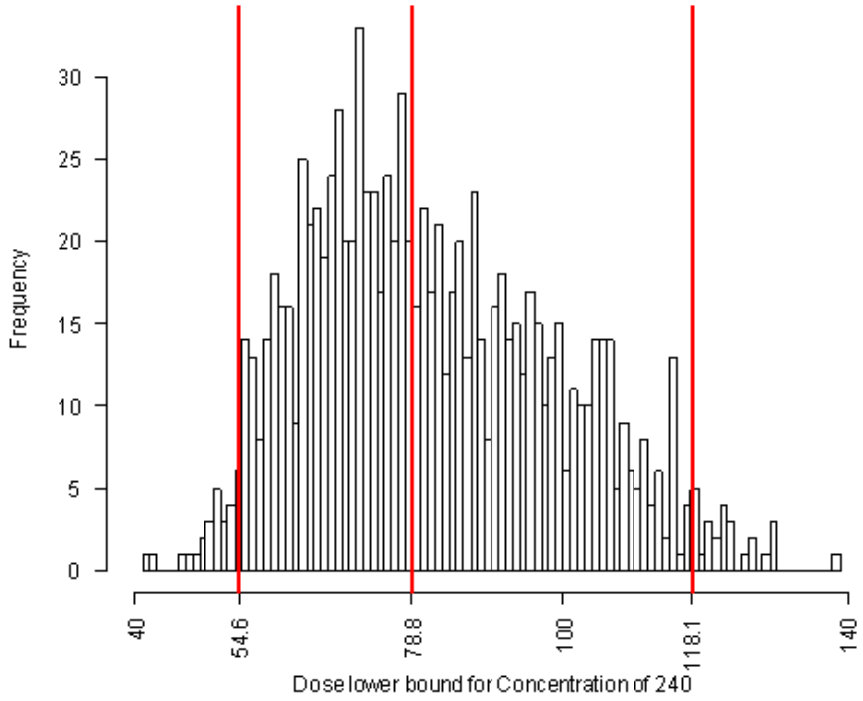

Figure 7. Histogram of aspartame dose lower bound (x-axis in mg/kg bw) considering $\alpha = 0.01$ for phenylalanine plasma level threshold of 240 µM.

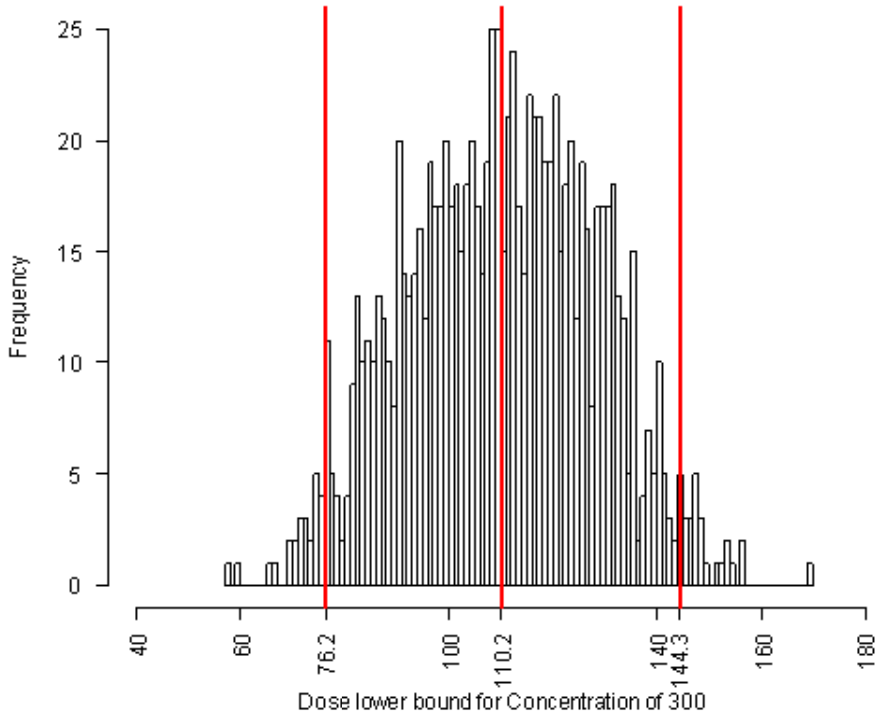

Figure 8. Histogram of aspartame dose lower bound (x-axis in mg/kg bw) considering $\alpha = 0.01$ for phenylalanine plasma level threshold of 300 µM.

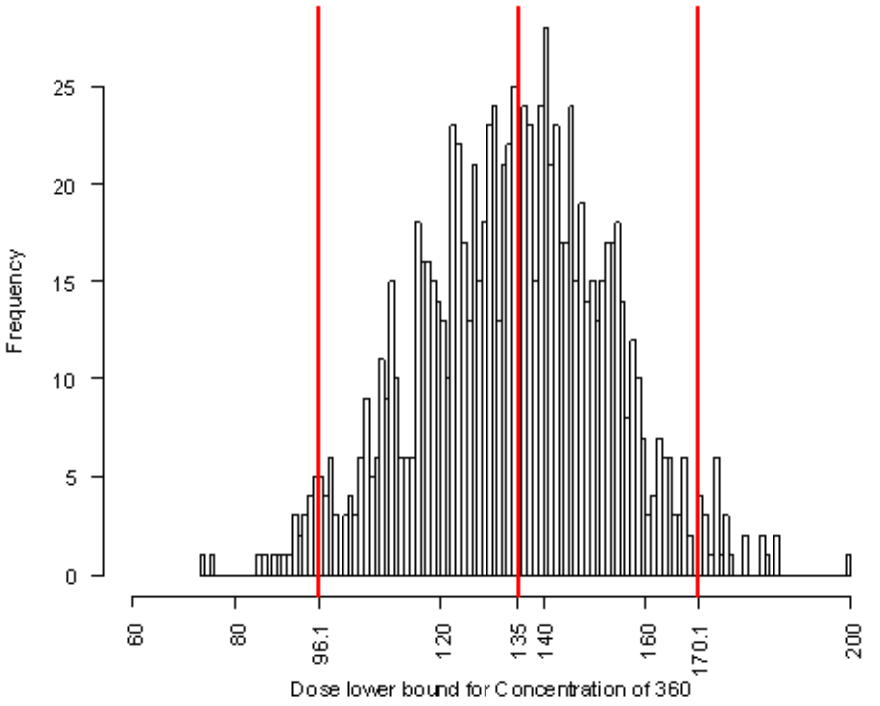

Figure 9. Histogram of aspartame dose lower bound (x-axis in mg/kg bw) considering $\alpha = 0.01$ for phenylalanine plasma level threshold of 360 µM.

3.2. Heterozygote Population

Similarly the spline model was fitted for the heterozygote population; it should be highlighted that only information on doses of aspartame of 0, 10, 34 and 100 mg/kg bw were available for this group, thus results should be interpreted with caution. The results obtained when the level of confidence (α) was 0.05 are shown in Figure 10 to Figure 13. It can be seen that the aspartame dose lower bound limit giving rise to a phenylalanine plasma threshold concentration of 240 µM was estimated to be between 27.9 and 69 mg/kg bw, therefore straddling the current ADI dose level of 40mg/kg bw. Therefore around 18.1% of the simulated data resulted in an aspartame dose lower bound below the ADI level. For other C_{max} phenylalanine thresholds (300 µM and 360 µM) the confidence interval obtained did not contain the current aspartame ADI value. In these cases, representing only 0.8% and 0% respectively of the total number of simulations run. When the simulation was repeated considering the level of confidence (α) to be 0.01 (Figure 14 to Figure 17), then in 46.6 % of the simulations, the aspartame dose lower bound was below the current ADI level for the phenylalanine threshold of 240 µM, 5.1 % for the threshold of 300 µM and 0.5 % for 360 µM.

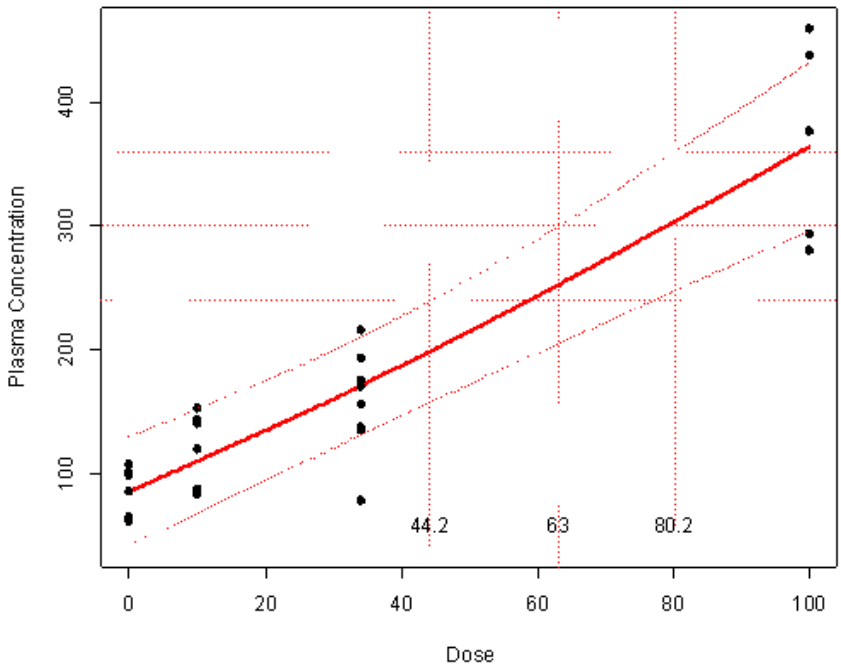

Figure 10. Single simulation example to illustrate dose response model between C_{max} phenylalanine plasma concentration (y-axis in µM) and aspartame dose (x-axis in mg/kg bw) considering α = 0.05

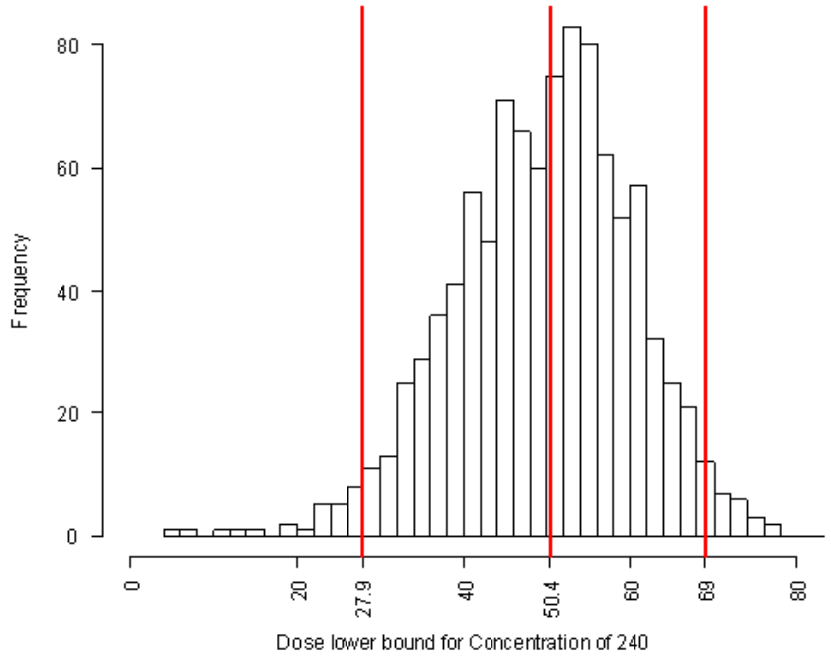

Figure 11. Histogram of aspartame dose lower bound (x-axis in mg/kg bw) considering α = 0.05 for phenylalanine plasma level threshold of 240 µM.

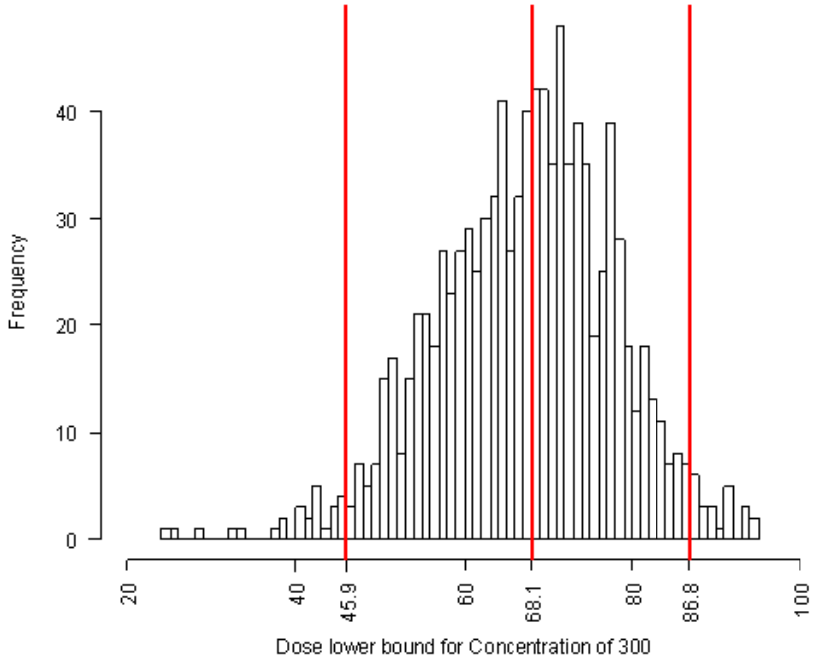

Figure 12. Histogram of aspartame dose lower bound (x-axis in mg/kg bw) considering α = 0.05 for phenylalanine plasma level threshold of 300 µM.

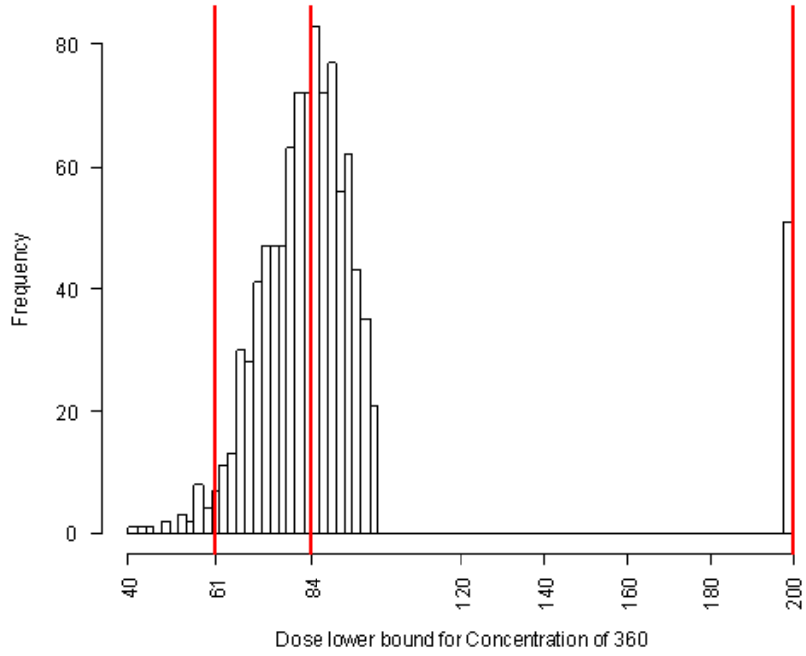

Figure 13. Histogram of aspartame dose lower bound (x-axis in mg/kg bw) considering α = 0.05 for phenylalanine plasma level threshold of 360 µM.

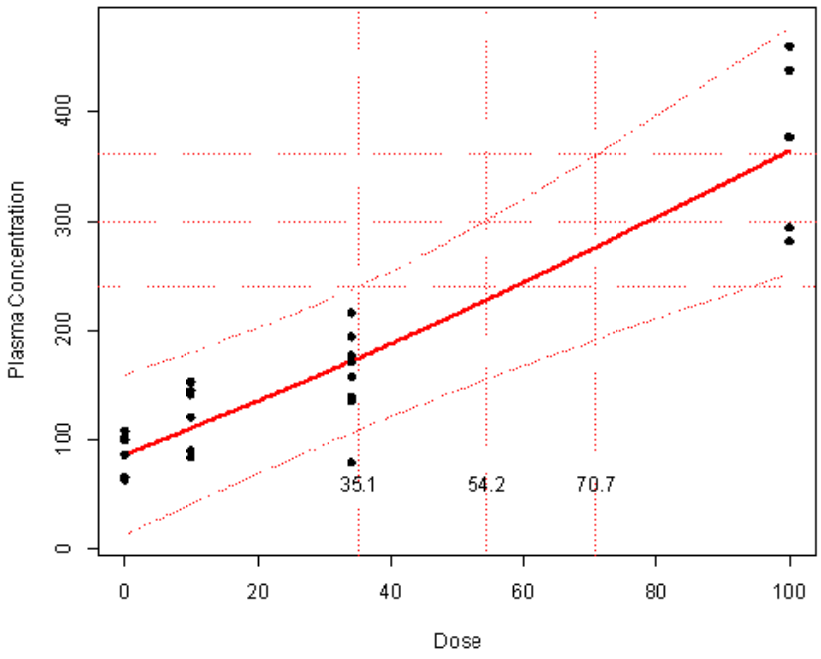

Figure 14. Single simulation example to illustrate dose response model between C_{max} phenylalanine plasma concentration (y-axis in µM) and aspartame dose (x-axis in mg/kg bw) considering α = 0.01

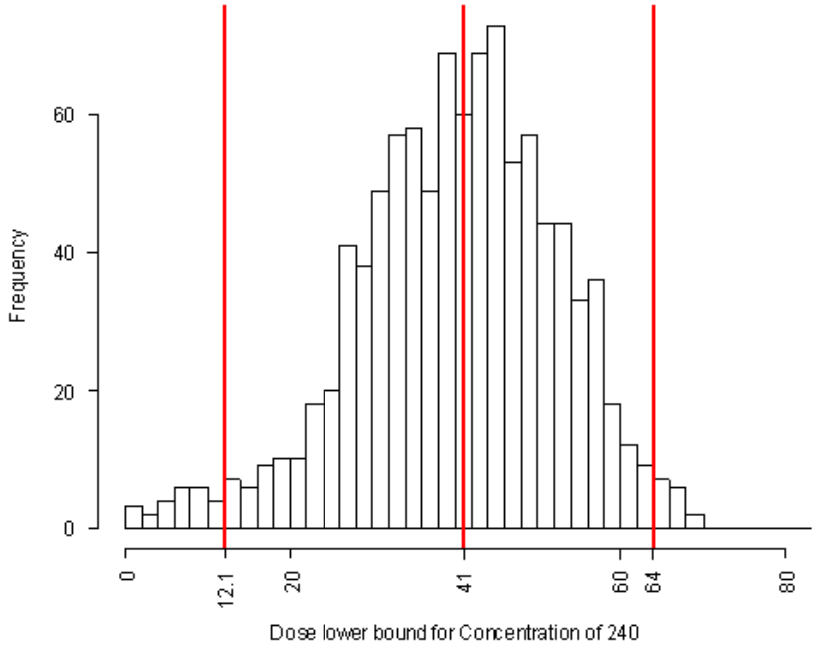

Figure 15. Histogram of aspartame dose lower bound (x-axis in mg/kg bw) considering α = 0.01 for phenylalanine plasma level threshold of 240 μM.

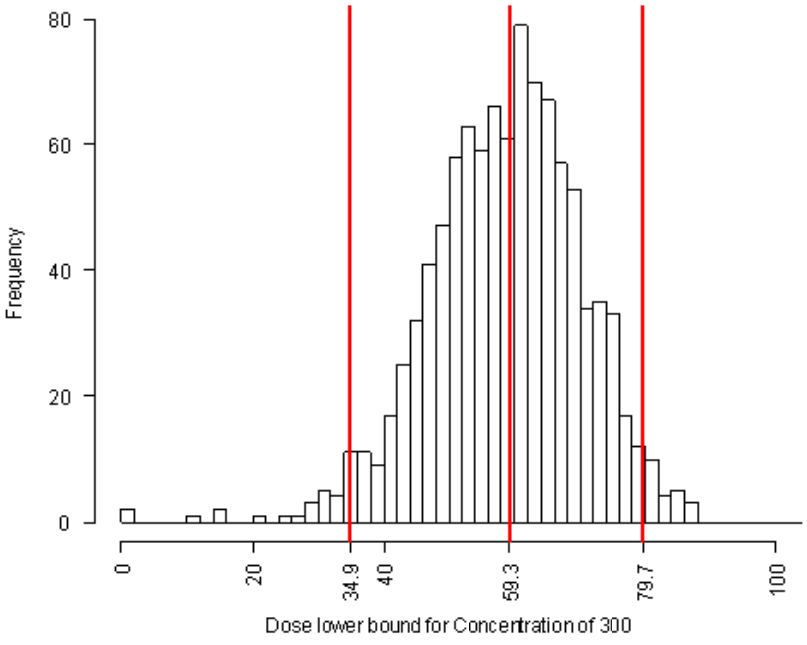

Figure 16. Histogram of aspartame dose lower bound (x-axis in mg/kg bw) considering α = 0.01 for phenylalanine plasma level threshold of 300 μM.

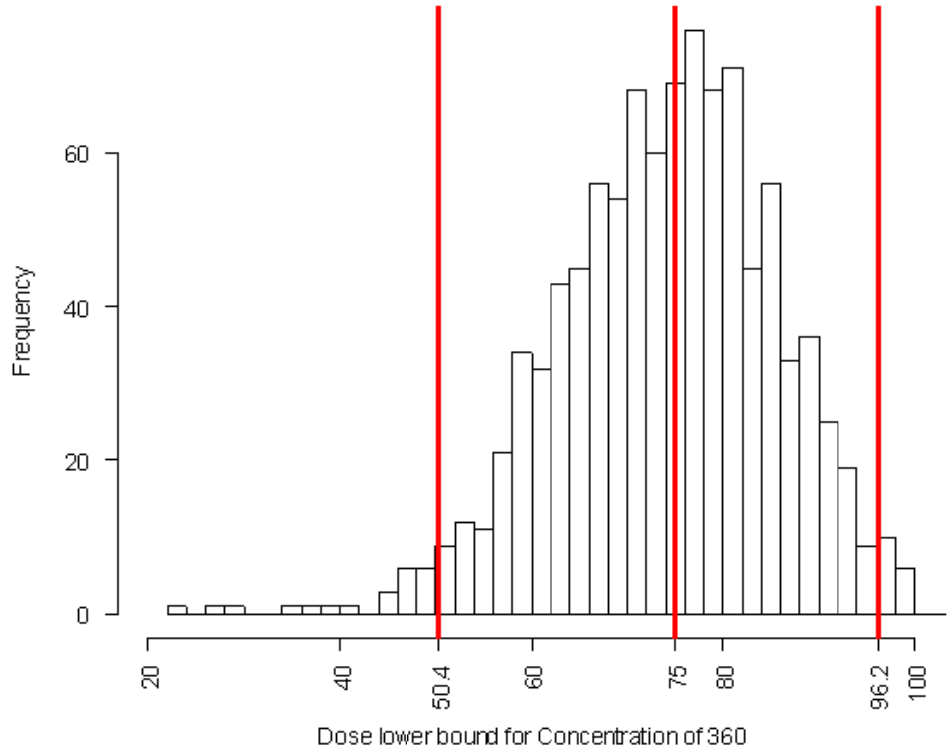

Figure 17. Histogram of aspartame dose lower bound (x-axis in mg/kg bw) considering α = 0.01 for phenylalanine plasma level threshold of 360 µM.

CONCLUSIONS

The simulation model enabled the relationship between aspartame dose (mg/kg bw) and C_{max} phenylalanine plasma level (µM) to be estimated. Flexible models (smoothing spline models) were used to fit the simulated individual data based on geometric transformations of the summary measures drawn from lognormal distribution. The smoothed fitted model was assessed in terms of monotonicity and the results showed that the model obtained was strictly increasing monotonically, even when the model was left flexible (smoothing spline), allowing for other types of relationships. The results obtained for the three different thresholds used (240, 300 and 360 in µM) consistently produced aspartame dose lower bounds that were above the aspartame ADI of 40 mg/kg bw, regardless of the confidence level (α) used. For heterozygous individuals, thresholds of 300 or 360 µM showed consistently aspartame dose lower bounds above the current ADI level, when the level of confidence considered was 0.05, and for the threshold of 360 µM when α was equal to 0.01. For the threshold of 240 µM and α is 0.05 and 0.1, the aspartame dose lower bounds were below the current ADI in about 18% and 46.6% of the simulations run respectively and 5.1% for the phenylalanine threshold of 300 µM. It is important to highlight that for the heterozygote population, the results obtained should be interpreted with caution due to limited data from individuals with this genotype.

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Appendix L. Assessment of both the validity and reliability of the long-term studies on aspartame and di-ketopiperazine based on the summaries of the UAREP reports.

Only scientific issues have been considered in this examination of the UAREP reports (E102a, b, c).

The Universities Associated for Research and Education in Pathology (UAREP) report consisted in an authentication review of selected materials submitted to the FDA relative to the application of Searle laboratories to market aspartame. It was issued on 18th November 1978.

From nearly 100 studies which Searle has presented to the FDA to market aspartame, 12 were selected by FDA and other parties for this review. They included:

- five major studies of long term toxicity effects in the dogs, rats, and mice

- two special neuropathology studies

- the effects of aspartame in newborn rats

- a two-generation reproduction toxicity study in the rat

- a series of screening tests for endocrinological and physiological responses

- a report covering a few inconsequential observations on pregnant monkeys

- a major study of the effects of aspartame and its metabolites in embryogenesis and teratogenesis in rabbits.

E-28, 106 week oral toxicity study in the dog. Forty Beagles (five/sex/treatment goup) were fed aspartame daily in different dosages. Seven haematology, ten clinical chemistry and eight urinalysis parameters were measured periodically throughout the experiment. An additional ten chemical constituents were determined at 78 and/or 106 weeks. All the dogs were killed at 106 weeks and complete necropsies and histopathologic examinations performed.

E-33,34, E-70, E-75 and E-76 share the objective of testing the effects of aspartame in life time toxicity studies. E-33,34 involved 440 rats in five groups with the highest dosage being 8 g/kg bw/day), E-70 involved 280 rats in control and two treatment groups. E-75 and E-76 utilized each 360 mice, E-75 evaluated again aspartame whereas E-76 provided 3 graded dosages of its conversion product, diketopiperazine. The duration of the four experiments ranged from 103 to 108 weeks. Validation of the histopathology diagnosis was carried out on 35 000 tissue sections from these 4 experiments. As expected, some discrepancies did exist. More than 99 % of the slides were available for UAREP review.

The discrepancies in diagnosis and the occasional missing slides had no predilection for any of the animal groups, or organ systems. UAREP' pathologists concluded that: 'certainly there was no evidence that either aspartame or DKP enhanced the production of tumours in these experiments'.

E-86 Supplemental study of dog brains from a 106 week oral toxicity study (E-28) and E-87 Supplemental study of rat brains from 2 tumorigenicity studies (E-33,34 and E-70). Because of the possibility that there might be an increased incidence of brain tumours in dogs in study E-28 and in rats in studies E-33,34 and E-70, additional sections of brain were cut from dogs (E-86) and rats (E-86) and reviewed by Searle's neuropathology consultant Dr J.R.M. Innes. A panel of neuropathology experts convened by UAREP:

- agreed completely with Dr Innes that there were no brain tumours in the dogs (E-86)

- generally agreed well with Dr Innes (E-86) for the rat studies. The 20 brain tumours diagnosed showed no statistically significant increase in any group when the tumours for the two experiments were combined.

E-9 Toxicological evaluation of aspartame in the neonatal rat. Groups of five male and five female newborn rats were sacrificed at 5, 15, and 21 days with analysis of five hematologic parameters and six chemical tests. The white blood cells counts were variable but statistically significantly depressed in the treatment groups. White blood counts were corrected for nucleated blood cells on the lab sheets but the uncorrected figures were used in the Entry report. This did not significantly affect the outcome of the statistical analysis. UAREP agreed with the EPL pathologic diagnostic of nuclear changes in renal tubular cells in the 15 and 21 day old rats, which apparently related to aspartame exposure.

E-11 Two generation reproduction study in rats. This study was designed to characterize the effects of aspartame in the reproductive performance of the same strain of rats as used in other HLA-Searle experiments. UAREP agreed with the findings in E-11 report. Fewer discrepancies or problems were noted in this than in most of the other studies reviewed.

E-19 Endocrine studies

This report covers a battery of screening tests (7 hormone related tests and 6 physiologic responses tests) which were used by Searle for compound clinical testing. Some of these routine tests appeared to be carried out with less precision or documentation than some might desire. UAREP encountered some difficulty in reconstructing and interpreting the results of these routine tests.

E-88 Experiments in mated and pregnant Rhesus monkeys. Searle informed that the E-88 report was based on fragmentary data developed independently of Searle and without Searle's involvement in the design of the study. UAREP felt that this report was without design, inconsequential, and based on woefully inadequate and confusing data from an inadequate number of animals.

E-90 An evaluation of the embryotoxic and teratogenic potential of aspartame in rabbits. These experiments involved 300 female rabbits and studied the effects of administering by gavage, aspartame and its metabolites, L-phenylalanine and L-aspartic acid, during fetal organogenesis in pregnant rabbits. The dosage level of administration was not equivalent to that recorded by the highest aspartame dose group. UAREP authenticated the findings in the skeletal system of the cleared whole fetuses. Seven of the 10 discrepancies that UAREP found were minor malformations occurring in fetuses which were previously reported by Searle to have other malformations. Although it was not possible to examine the body cross-sections because of their altered conditions, UAREP felt that its validation of the skeletal specimens was adequate indication of the accuracy and significance of Searle findings.

Overall, UAREP has interpreted the results only to the experiments as designed, it has addressed itself to the question of whether the experiments were carried out according to protocol plans and the accuracy and reliability with which the experiments were performed and reported to the FDA. At times, UAREP has commented on the interpretation of the significance of the data. Attempt was made to quantitate not only the number but the magnitude and significance of discrepancies and problems noted.

There were many instances in which the earliest recorded data were not available. UAREP failed to agree with Searle and Hazleton on less than 1 % of the computations and in most instances the differences were small in magnitude.

For the histopathology slides, the full information on which the original pathologist based his diagnosis was still available. Autolysis of tissues was present in both the rat and mouse slides from HLA. UAREP pathologists did not feel that it was sufficiently severe to materially interfere with making diagnoses on all but a few slides.

Not only the agreement in diagnosis was generally good in the four larger studies but there was also good agreement in the smaller study in E-9.

There was obviously considerable variation in the results within and between groups when measuring variability and statistical differences. Although a substantial number of minor and inconsequential discrepancies were noted in UAREP's validation studies, there were few if any, discrepancies which would produce a change of greater than 5 % in the final numerical data being compared.

The UAREP report finally stressed that the discrepancies it observed appeared randomly distributed between treated and control groups of animals.

The Panel noted that UAREP and Bressler (1977) reported that in very many instances, the earliest original raw data were not available, some of the protocols appeared to be variable in time and fully documented only at termination of the study. They identified a lot of discrepancies and variability in the results of the studies under investigation. They underlined that:

- in all groups, many of the old animals were obviously in poor health and had tumours or advanced renal or liver diseases which, in the view of some of the UAREP investigators may have make it difficult to recognize a mild carcinogenic action of the study compound. In this connection limiting the experiment in time according to survival percentage was suggested
- the development of standard operating procedures along with the non intentional use of ill animals (in some studies, several animals in both control and treated groups received potassium penicillin G due to unidentified infection diseases outbreaks) and together with the availability of the data and transparency of the methodology are fundamental requirements for a study to be fully reliable. All of these requirements were unfortunately not usual practice when these studies have been carried out.

The Panel noted that, both reports did not conclude that the studies were not useful but that they were rather in line with the current practice in the early '70s before the development of the standardised procedures and Good Laboratory Practice (GLP); therefore their results should be considered. In addition, UAREP did not find evidence that the studies investigated were deliberately treated to produce biased results.

GLOSSARY AND ABBREVIATIONS

ADI	Acceptable Daily Intake
ADME	absorption, distribution, metabolism and excretion
AFC	Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food
AFSSA	Agence française de sécurité sanitaire des aliments
ANS	Scientific Panel on Food Additives and Nutrient Sources added to Food
ANSES	Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail
bw	body weight
β-aspartame (β-APM)	1-methyl N-L-β-aspartyl-3-phenyl-L-alaninate
β-AP	β-aspartylphenylalanine
CAS	Chemical Abstracts Service
CE-C^4D	Capillary electrophoresis (CE) with capacitively coupled contactless conductivity detection (C4D)
C_{max}	Maximum Plasma Concentration
COC	UK Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment
DKP	5-benzyl-3,6-dioxo-2-piperazine acetic acid
EFSA	European Food Safety Authority
EC	European Commission
EINECS	European Inventory of Existing Commercial chemical Substances
ERF	European Ramazzini Foundation
EU	European Union
FSA	UK Food Standards Agency
GD	Gestational Day
GLP	Good Laboratory Practice
GSH	Glutathione
HPLC	High-performance liquid chromatography
ICGA	International Chewing Gum Association
ISA	International Sweeteners' Association
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LD_{50}	Lethal Dose, 50 % i.e. dose that causes death among 50 % of

	treated animals
LNAA	large neutral amino acids
LOAEL	Lowest Observed Adverse Effect Level
MoA	Mode of Action
MPL	Maximum Permitted Level
NOAEC	No Observed Adverse Effect Concentration
NOAEL	No Observed Adverse Effect Level
OECD	Organisation for Economic Co-operation and Development
PARNUTS	Food for Particular Nutritional Uses
PKU	Phenylketonuria
QS	*Quantum Satis*
RSD_R	Relative standard deviation for reproducibility
SCEs	Sister chromatid exchanges
SCF	Scientific Committee on Food
UK COT	UK Committee on toxicity of chemicals in food, consumer products and the environment
US EPA	US Environmental Protection Agency
US FDA	US Food and Drug Administration
WHO	World Health Organization