

SCIENTIFIC OPINION

Scientific Opinion on the safety of steviol glycosides for the proposed uses as a food additive¹

EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS)^{2,3}

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ABSTRACT

Steviol glycosides in the present evaluation are mixtures of steviol glycosides that comprise not less than 95% of stevioside and/or rebaudioside A. Stevioside as a sweetener was evaluated by the SCF in 1984, 1989 and 1999. JECFA reviewed the safety of steviol glycosides in 2000, 2005, 2006, 2007, and 2009 and established an ADI for steviol glycosides (expressed as steviol equivalents) of 4 mg/kg bw/day. The Panel considers that the results of toxicology studies on either stevioside or rebaudioside A are applicable for the safety assessment of steviol glycosides as both rebaudioside A and stevioside are metabolised and excreted by similar pathways, with steviol being the common metabolite for both. Considering the available toxicity data (*in vitro* and *in vivo* animal studies and some human tolerance studies), the Panel concludes that steviol glycosides, complying with JECFA specifications, are not carcinogenic, genotoxic or associated with any reproductive/developmental toxicity. The Panel establishes an ADI for steviol glycosides, expressed as steviol equivalents, of 4 mg/kg bw/day based on application of a 100-fold uncertainty factor to the NOAEL in the 2-year carcinogenicity study in the rat of 2.5% stevioside in the diet. This is equal to 967 mg stevioside/kg bw/day (corresponding to approximately 388 mg steviol equivalents/kg bw/day). Conservative estimates of steviol glycosides exposures both in adults and in children suggest that it is likely that the ADI would be exceeded at the maximum proposed use levels.

KEY WORDS

Steviol glycosides, stevioside, rebaudioside A, Stevia.

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SUMMARY

Following a request from the European Commission, the Panel on Food Additives and Nutrient Sources added to Food (ANS) was asked to deliver a scientific opinion on the safety of steviol glycosides as a sweetener for use in the food categories specified in the dossiers from the three petitioners.

The steviol glycosides produced by the three petitioners are chemically defined mixtures that comprise not less than 95% stevioside and/or rebaudioside A. Stevioside and/or rebaudioside A are more than 95% of the mixture in two of the products. In the third product, rebaudioside A is the major component of the mixture ($\geq 95\%$) together with other glycosides. In addition, smaller amounts of rebaudiosides B, C, D, E and F, steviolbioside, rubusoside and dulcoside A are present in the final mixtures, as indicated by the petitioners.

The three petitioners proposed that the specifications for the steviol glycosides should comply with the specifications adopted by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) at the 69th meeting.

The petitioners indicated that all manufacturers use the same basic steps to extract steviol glycosides from the leaves of the *Stevia rebaudiana* Bertoni plant, although there is some variation in the later stages of purification and separation of the glycosides. Steviol glycosides can be identified in foods and beverages by High Performance Liquid Chromatography (HPLC) methods.

Different studies assessed the bulk stability of dry steviol glycosides under various storage conditions and in food matrices over a range of pH values, processing conditions, at both room temperature and elevated temperatures. The photostability of the preparation was examined under dry and aqueous conditions. The Panel notes that in these experiments the extent of degradation of the tested steviol glycoside (rebaudioside A) that occurred ranged from a few percent up to 63% under different storage (pH and temperature) and food production conditions. The Panel notes that in presence of high temperatures (e.g. heating, baking) substantial degradation of steviol glycosides might take place.

Stevioside as a sweetener was evaluated by the Scientific Committee for Food (SCF) in 1984, 1989 and 1999. The SCF concluded that the use of stevioside was “toxicologically not acceptable” due to insufficient available data to assess its safety.

JECFA reviewed the safety of steviol glycosides (in 2000, 2005, 2006, 2007, and 2009) and established an ADI for steviol glycosides (expressed as steviol equivalents) of 4 mg/kg bw/day.

The Panel evaluated oral animal studies of metabolism and toxicokinetics, laboratory animal toxicity studies, *in vitro* and *in vivo* genotoxicity studies, and human studies with single or repeated administration of steviol glycosides.

Metabolic studies with steviol glycosides in animals and humans demonstrated that intact steviol glycosides are poorly absorbed after oral exposure but that they are hydrolysed by the microflora in the colon to steviol. A large amount of steviol is absorbed; the rest is excreted in the faeces. In the liver, steviol undergoes conjugation with glucuronic acid to form steviol glucuronide. The only interspecies difference is that the glucuronide is excreted primarily *via* the urine in humans and *via* the bile in rats. No accumulation of steviol glycoside derivatives occurs in the body. Besides steviol glucuronide, no other derivatives could be detected in the urine of humans exposed orally to steviol glycosides.

Rebaudioside A and stevioside both show similar pharmacokinetics in the rat. In humans, rebaudioside A and stevioside are also metabolised and excreted by similar pathways. Therefore, the Panel considers the results of toxicological studies on either stevioside or rebaudioside A applicable for the safety assessment of steviol glycosides in general.

In some subchronic and carcinogenicity studies, and also in the 2-generation reproductive toxicity study body weight gains were lower in the treated groups compared to the controls. In these studies decreases in feed consumption and in feed conversion efficiency were recorded. The Panel considers the effects on body weight as not adverse or indicative of toxicity but related to lower palatability and lower nutritional value of feed containing the steviol glycosides. Therefore body weight parameters are not considered to be appropriate endpoints for setting No-Observed-Adverse-Effect Levels (NOAELs) for these studies.

Overall, stevioside and rebaudioside A do not show evidence of genotoxicity *in vitro* or *in vivo*. Although a single Comet assay was reported to show effects indicative of DNA damage, the Panel considers that this study does not provide substantive evidence of a genotoxic potential for stevioside, given methodological concerns and also the fact that similar findings were not seen in earlier studies in mice using steviosides of higher or lower purities. The Panel notes that steviol and some of its oxidative derivatives show clear evidence of genotoxicity *in vitro*, particularly in the presence of a metabolic activation system. However, studies of DNA damage and micronucleus formation in rats, mice and hamsters have shown that the genotoxicity of steviol is not expressed *in vivo* at doses of up to 8000 mg/kg bw. Given that the available toxicokinetic data indicate that free steviol is absent from the systemic circulation in humans or, at worst, present at very low (negligible) levels, any concern raised by the *in vitro* genotoxicity profile of steviol is fully addressed by the fact that the genotoxic potential of steviol is not expressed *in vivo*, and by the negative genotoxicity findings for steviol glycosides *in vitro* and *in vivo*.

The results of toxicological testing indicated that steviol glycosides are not genotoxic, carcinogenic, nor associated with any reproductive/developmental toxicity. The NOAEL in the 2-year carcinogenicity study in the rat was 2.5% stevioside (95.6% purity) equal to 967 mg stevioside/kg bw/day (corresponding to approximately 388 mg steviol equivalents/kg bw/day).

Single doses of 1000 mg steviol glycosides/person/day (97% rebaudioside A) (corresponding to approximately 330 mg steviol equivalents/day) did not affect glucose homeostasis and did not affect blood pressure in individuals with normal glucose tolerance or type-2 *diabetes mellitus*. Also repeated use for 16 weeks of 1000 mg rebaudioside A/person/day did not alter glucose homeostasis in individuals with type-2 *diabetes mellitus*. Blood pressure parameters were not significantly affected by oral intake of 1000 mg rebaudioside A/person/day for 4 weeks in individuals with normal and low systolic blood pressure. This daily dose corresponds to 16.6 mg of rebaudioside A/kg bw for a person weighing 60 kg and to approximately 5.5 mg steviol equivalents/kg bw/day.

Available data concerning anaphylaxis-like reactions by stevioside in children with atopic eczema do not, according to the Panel, raise concern regarding the potential for oral exposure to steviol glycosides to trigger anaphylactic reactions. Sparse *in vitro* and *in vivo* data indicate that stevioside may have immunostimulating effects and modulatory activities on inflammation. The Panel considered that immunostimulating and immunomodulating effects of steviol glycosides in cell lines and rodent models have not been demonstrated in a robust and reproducible way, which could enable them to be used as pivotal studies for risk assessment. However, these observations deserve more in-depth examination as, if they are confirmed, they may raise concern regarding the use of steviosides in some sub-groups of the population, particularly for individuals suffering from auto-immune diseases or inflammation of the gastrointestinal tract.

When considering the proposed maximum use levels (Tier 2), the mean dietary exposure to steviol glycosides expressed as steviol equivalents in European children (aged 1-14 years) ranged from 0.7 to 7.2 mg/kg bw/day, and from 3.3 to 17.2 mg/kg bw/day at the 95th percentile. The main contributors (>10% in all countries) to the total anticipated exposure to steviol glycosides, expressed as steviol equivalents, are soft drinks (11 to 58%) and desserts, including flavoured milk products (14 to 71%). Confectionery accounted for 11% of exposure in two countries. Dried potato granules and flakes and candied fruits and vegetables, mostardo di frutta accounted for 17 and 18% of exposure in one country.

Estimates reported for the UK adult population give a mean dietary exposure to steviol glycosides, expressed as steviol equivalents of 2.2-2.7 mg/kg bw/day and of 8.0-9.7 mg/kg bw/day for high level exposures (97.5th percentile). The main contributors to the total anticipated exposure to steviol glycosides expressed as steviol equivalents (>10 %) are soft drinks (37%) and beer, cider and perry (33%).

After considering all the data on stability, degradation products, metabolism and toxicology, the Panel establishes an Acceptable Daily Intake (ADI) for steviol glycosides, expressed as steviol equivalents, of 4 mg/kg bw/day based on application of a 100-fold uncertainty factor to the NOAEL for stevioside of 967 mg stevioside/kg bw/day (corresponding to approximately 388 mg steviol equivalents/kg bw/day) from a 2-year carcinogenicity study in the rat.

Conservative estimates of steviol glycosides exposures both in adults and in children suggest that it is likely that the ADI would be exceeded at the maximum use levels proposed by the petitioners.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

The authorisation and conditions of use of sweeteners are regulated in the Annex of Directive 94/35/EC of the European Parliament and the Council on sweeteners for use in foodstuffs.

The Scientific Committee of Food (SCF) has considered the safety of stevioside as a sweetener in 1984 and the review was updated in 1988 and in 1999. The most recent opinion of the Scientific Committee dates from June 1999⁴. In its latest opinion, the Committee concluded that “the substance is not acceptable as a sweetener on the presently available data”. Consequently, the Commission did not propose the authorization of this substance under Directive 94/35/EC.

In 1998 another request was made for *Stevia rebaudiana* Bertoni plants and leaves to be marketed in the EU as a novel food under the novel food Regulation (EC) No 258/97. With regard to the plant products, the Committee concluded that the information submitted was insufficient with respect to specification of the commercial product and contained no safety studies⁵. Consequently, the Commission refused the placing on the market of *Stevia rebaudiana* Bertoni plants and dried leaves as food or food ingredient⁶.

In 2007, two applicants requested the authorization of steviol glycosides under Directive 94/35/EC for its use as a sweetener in foodstuffs such as drinks, desserts, yogurts, confectionary, cakes, biscuits and pastries, sauces, toppings, spread, cereals, canned fruits, jams etc.

In 2008, a third applicant requested the authorization for the use of steviol glycosides, as a sweetener, for uses and use-levels reflecting basically the current authorisation of aspartame under the Directive 94/35/EC.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

In accordance with Article 29 (1) (a) of Regulation (EC) No 178/2002, the European Commission asks the European Food Safety Authority to provide a scientific opinion on the safety of steviol glycosides as a food additive for use in the food categories specified in the dossiers.

⁴ SCF Opinion on STEVIOSIDE AS A SWEETENER (adopted on 17/6/99):
http://europa.eu.int/comm/food/fs/sc/scf/out34_en.pdf

⁵ SCF Opinion on *Stevia Rebaudiana* Bertoni plants and leaves (adopted on 17/6/99):
http://europa.eu.int/comm/food/fs/sc/scf/out36_en.pdf

⁶ Commission Decision of 22 February 2000 refusing the placing on the market of *Stevia Rebaudiana* Bertoni: plants and dried leaves as a novel food or novel food ingredient under Regulation (EC) No 258/97 of the European Parliament and of the Council, OJ L 61, 8.3.2000, p.14

ASSESSMENT

1. Introduction

The present opinion deals with the safety of steviol glycosides as a food additive for use as a sweetener in food categories specified in the dossiers from the three petitioners.

Steviol glycosides used as a sweetener are extracted from the leaves of the plant *Stevia rebaudiana* Bertoni of the family Asteraceae (Compositae), native to Paraguay.

Steviol glycosides, in the present evaluation contain stevioside and/or rebaudioside A as the principal sweetening components.

Stevioside as a sweetener was evaluated by the Scientific Committee for Food (SCF) in 1984 (SCF, 1985), in 1988 (SCF, 1989) and in 1999 (SCF, 1999).

Several questions of concern were raised by the SCF regarding the specifications of the extracts that had been tested (for the majority of toxicological studies a precise composition of the extract was not adequately defined), metabolism, questionable chronic toxicity and carcinogenicity studies, the possible effects on the male reproductive system that could affect fertility, and the effects of steviol glycosides on renal and cardiovascular function and on carbohydrate metabolism. Furthermore, steviol, one of the main metabolites of stevioside, was found to be genotoxic and to induce developmental toxicity. Therefore, due to limited data the SCF concluded that the use of stevioside was “toxicologically not acceptable” and considered the data available at that time to be insufficient to adequately assess the safety of stevioside (SCF, 1985; 1999).

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) (2000, 2004, 2005, 2007, and 2009) reviewed the safety of steviol glycosides and established an Acceptable Daily Intake (ADI) for steviol glycosides (expressed as steviol equivalents) of 4 mg/kg bw/day.

In addition, the SCF evaluated the safety of *Stevia rebaudiana* Bertoni plants and leaves as a novel food (SCF, 1999) and concluded that no appropriate data were presented to enable the safety of the commercial plant product to be evaluated.

The Panel on Food Additives and Nutrient Sources added to Food (ANS) in assessing the safety of steviol glycosides considers whether the data submitted by the three petitioners support the safety of steviol glycosides as a sweetener and answer the safety concerns expressed in the past by the SCF.

2. Technical data

2.1. Identity of the substance

The steviol glycosides produced by the three petitioners are chemically defined mixtures that comprise not less than 95% stevioside and/or rebaudioside A. Stevioside and/or rebaudioside A are more than 95% of the mixture in two of the products. In the third product, rebaudioside A is the major component of the mixture ($\geq 95\%$) together with other glycosides. In addition, smaller amounts of rebaudiosides B, C, D, E and F, steviolbioside, rubusoside and dulcoside A are present in the compositions of the final mixtures, as indicated by the petitioners.

The mixtures from the three petitioners are described as white to light yellow powders, odourless or having a slight characteristic odour, about 200-300 times sweeter than sucrose.

The structural formulas of steviol and its glycosides extracted from the leaves of the *Stevia rebaudiana* Bertoni plant are presented below (Figure 1 and Table 1).

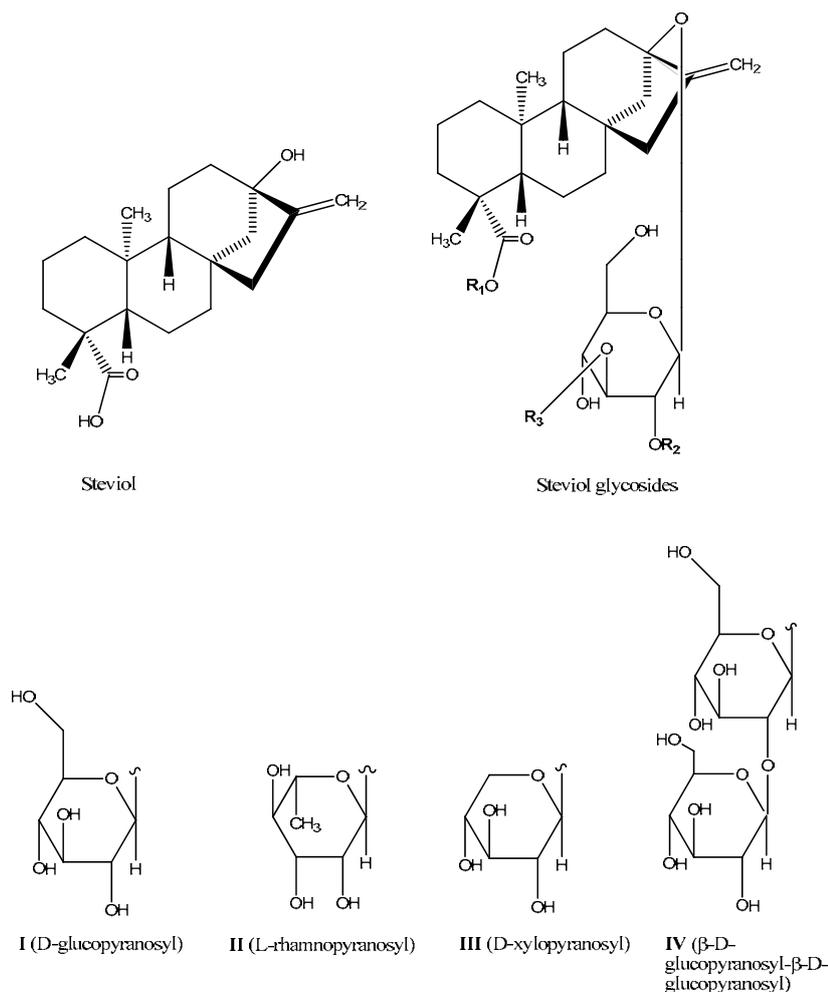


Figure 1: Structural formula of steviol and steviol glycosides

The trivial names, CAS numbers, molecular formulas, molecular weights and conversion factors for calculation of the steviol equivalents⁷ of the different steviol glycosides are presented in Table 1.

Table 1: Trivial names of steviol and its glycosides, their CAS Numbers, molecular formulas, molecular weights (MW) and conversion factors (X) for calculation of steviol equivalents.

Trivial name	R ₁	R ₂	R ₃	CAS Number	Formula	MW (g/mol)	Conversion factor X
Steviol				471-80-7	C ₂₀ H ₃₀ O ₃	318.45	1.00
Stevioside	I	I	H	57817-89-7	C ₃₈ H ₆₀ O ₁₈	804.87	0.40
Rebaudioside A	I	I	I	58543-16-1	C ₄₄ H ₇₀ O ₂₃	967.01	0.33
Rebaudioside C	I	II	I	63550-99-2	C ₄₄ H ₇₀ O ₂₂	951.01	0.34
Dulcoside A	I	II	H	64432-06-0	C ₃₈ H ₆₀ O ₁₇	788.87	0.40
Rubusoside	I	H	H	63849-39-4	C ₃₂ H ₅₀ O ₁₃	642.73	0.50
Steviolbioside	H	I	H	41093-60-1	C ₃₂ H ₅₀ O ₁₃	642.73	0.50

⁷ All the exposure results in this opinion are presented in terms of steviol equivalents, based on a conversion of 40% from stevioside (MW: steviol, 318.45 g/mol; stevioside, 804.87 g/mol), or 33% from rebaudioside A (MW: steviol, 318.45 g/mol; rebaudioside A, 967.01 g/mol).

Rebaudioside B	H	I	I	58543-17-2	C ₃₈ H ₆₀ O ₁₈	804.87	0.40
Rebaudioside D	IV	I	I	63279-13-0	C ₅₀ H ₈₀ O ₂₈	1129.15	0.29
Rebaudioside E	IV	H	I	63279-14-1	C ₄₄ H ₇₀ O ₂₃	967.01	0.33
Rebaudioside F	I	III	I	438045-89-7	C ₄₃ H ₆₈ O ₂₂	936.99	0.34

2.2. Specifications

The three petitioners proposed that the specifications for the steviol glycosides should comply with the specifications adopted by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) at the 69th meeting (JECFA, 2008).

The JECFA specifications outline that the purity of steviol glycosides should not be less than 95% of the total amount of the seven named glycosides (stevioside, rebaudioside A, rebaudioside C, dulcoside A, rubusoside, steviolbioside and rebaudioside B) on the dried basis with the major glycosides in the product being stevioside and rebaudioside A. The Panel notes that the steviol glycosides produced by the three petitioners are comprised of not less than 95% of stevioside and/or rebaudioside A.

Furthermore, the three petitioners proposed to include in the EU specifications rebaudioside D and rebaudioside F, two minor steviol glycosides, which may also be present in the final mixture.

Current chemical specifications for steviol glycosides as adopted by JECFA at the 69th meeting are presented in Table 2.

Table 2: Chemical specifications for steviol glycosides as adopted by JECFA at the 69th meeting.

Parameter	Limit
Assay	Not less than 95% of the total of the seven named steviol glycosides ¹ on the dried basis
<u>Identification</u>	
Solubility	Freely soluble in water
Stevioside and rebaudioside A	The main peak in the chromatogram obtained by the following procedure in Method of Assay corresponds to either stevioside or rebaudioside A
pH	Between 4.5 and 7.0 (1 in 100 solution) ²
<u>Purity</u>	
Total ash	Not more than 1%
Loss on drying	Not more than 6% (105 °c, 2h)
Residual solvents	Not more than 200 mg/kg methanol and not more than 5000 mg/kg ethanol
Arsenic	Not more than 1 mg/kg
Lead	Not more than 1 mg/kg

¹ Stevioside, rebaudioside A, rebaudioside C, dulcoside A, rubusoside, steviolbioside, and rebaudioside B.

² 1 part of solute in 100 parts of solvent

According to the information provided by the petitioners regarding the physicochemical data, only the product from one petitioner was in full compliance with JECFA specifications, while for the others the solubility in water did not comply with JECFA specifications.

According to the petitioners, the composition of the steviol glycoside mixtures may differ according to the cultivar from which the steviol glycosides are extracted, and differences in the manufacturing

process. For this reason, the Panel considers that the specifications should be detailed and should indicate the percentage of each majority component. Likewise, the Panel notes that in the JECFA specifications the impurities are not detailed and 5% of the material is not specified.

According to JECFA (2007), impurities occurring in extracts of *Stevia* leaves are typical plant materials, such as pigments and saccharides. JECFA (2007) quoted one literature study that reports identification of the following substances in the non-glycosidic fractions of extracts of *Stevia* leaves, obtained using Supercritical Carbon-dioxide Fluid Extraction (SCFE): spathulenol; decanoic acid; 8,11,14-ecosatrienoic acid; 2-methyloctadecane; pentacosane; octacosane; stigmasterol; β -sitosterol; α - and β -amyrine; lupeol; β -amyrin acetate; and pentacyclic triterpene. These substances (corresponding to approximately 5% of the steviol glycosides preparation) represent 56% of the total non-glycosidic extracts, while 44% remain unidentified.

The specified additive (>95% total steviol glycosides) will contain, in addition to saccharides other than those associated with the individual steviol glycosides, residual extraction/recrystallisation solvent and possibly also residues of ion-exchange resins used in the manufacturing process. According to JECFA, the level of the non-glycosidic fraction, because of its highly non-polar character can be considered insignificant in the additive.

According to one petitioner several other related steviol glycosides that may be generated as a result of the production process, but do not occur naturally in the leaves of *Stevia rebaudiana* plant, have been identified in small amounts (0.10 to 0.37%, w/w) by High Performance Liquid Chromatography (HPLC) in the steviol glycoside bulk material. Some of them share the same steviol aglycone backbone structure as rebaudioside A and differ only with respect to the number of glucose units, while the remaining compounds have slight structural differences in the aglycone backbone like an endocyclic double bond, an additional hydroxyl group or isosteviol instead of steviol aglycone.

2.3. Manufacturing process

Steviol glycosides are reported by the petitioners to be produced in accordance with current Good Manufacturing Practices (GMPs).

Steviol glycosides manufacturing methodology is unique to each petitioner, but the overall process scheme is similar.

The manufacturing process comprises two main phases: the first involving water extraction of the leaves of the *Stevia rebaudiana* Bertoni plant and preliminary purification of the extract by employing ion exchange chromatography to yield a steviol glycoside primary extract, and the second involving recrystallisation of the steviol glycosides from methanol or aqueous ethanol resulting in a final product of not less than 95% stevioside and/or rebaudioside A according to two petitioners or not less than 95% rebaudioside A, according to the other petitioner.

2.4. Methods of analysis in foods

All petitioners indicated that in-house HPLC methods were developed and validated for the identification of stevioside, rebaudioside A and other related steviol glycosides (minor steviol glycoside constituents and degradation products) in food and beverage matrices, including beverages with and without alcohol. Validated HPLC methods have been recently published (Geuns *et al.*, 2008a; Gardana *et al.*, 2010).

2.5. Stability, reaction and fate in food

According to all three petitioners, different studies have assessed the bulk stability of dry steviol glycosides under normal and accelerated storage conditions and in food matrices over a range of pH

values, processing conditions, at both room temperature and at elevated temperatures. The photostability of the preparations have been examined under dry and aqueous conditions. Two of the petitioners concluded that their products were stable, mainly based upon information from the literature, and the other petitioner conducted stability studies with rebaudioside A.

According to data presented by the latter petitioner, the steviol glycoside (rebaudioside A) was more stable at a pH between 4 and 6 and at temperatures between 5 and 25°C than under other pH and temperature conditions. Concerning photostability, no differences were observed in the composition of the rebaudioside A powder between light-exposed and un-exposed samples stored under similar conditions.

A model was developed to evaluate the stability of a steviol glycoside (rebaudioside A) preparation for all proposed food uses. This model was previously validated for aspartame stability determinations (Pariza *et al.*, 1998). The stability studies included a table-top product (low moisture, low heat, neutral pH), mock beverages (high moisture, low heat, low pH), thermally processed beverages (high moisture, medium to high heat, low pH), yogurt (high moisture, medium heat, low pH), and white cake (moderate moisture, high heat, neutral pH). The results demonstrated that the stability of the steviol glycoside preparation is pH-, temperature- and time-dependent. The Panel notes that in these experiments the extent of degradation that occurred in the tested steviol glycoside (rebaudioside A) ranged from a few percent up to 63% under different storage (pH, temperature and period of time) and food production conditions.

The petitioner characterised minor degradation products by Liquid Chromatography-Mass Spectrometry (LC-MS). Detected degradation products were related steviol glycosides that do not occur naturally in the leaves of the *Stevia rebaudiana* Bertoni plant. Isosteviol, the structural isomer of steviol was also identified as a degradation product. Based on the results of the stability studies, seven identified compounds are related steviol glycosides that increase over time under certain storage conditions, while three degradation products have not been identified in the starting bulk material by either HPLC or LC-MS. Two degradation products share the same steviol aglycone backbone structure as rebaudioside A and differ only with respect to the number of glucose units, while the third possesses a hydroxyl group at C-16 and one less glucose unit than rebaudioside A.

According to the literature (Chang and Cook, 1983) only 68.5% of the original content of rebaudioside A was detected after heating this steviol glycoside (purity not reported) in an aqueous solution (6.5 mg/mL) at 100°C for 48 hours. Rebaudioside B and glucose were identified degradation products, indicating that the C-19 ester linkage appeared to be the most heat-labile bond in rebaudioside A. Heating of acidic solutions of rebaudioside A (6.5 mg/mL) at 60°C for up to 137 hours did not cause any appreciable degradation of this steviol glycoside. After heating at 100°C for 13 hours only 18.3% and 24.1% of rebaudioside A were present in phosphoric and in citric acid solutions, respectively. Degradation products observed by Thin-Layer Chromatography (TLC) were rebaudioside B, glucose and two unknown components. Degradation reactions were reported by the authors of the study to be somewhat more pronounced in the phosphoric acid system, in comparison to the citric acid system, as indicated by higher concentrations of one of the unknown degradation products. Rebaudioside A showed no significant changes in HPLC and TLC analyses during 4 months of storage at 4°C, 3 months at room temperature, or 1 month at 37°C in either citric or phosphoric beverages, but again the authors noted that rebaudioside A exhibited greater stability in the citric acid system than in the phosphoric acid system. Exposure to 1 week of sunlight resulted in 22% and 18% degradation of rebaudioside A in phosphoric (cola-like) and citric (citrus-like) beverages, respectively (Chang and Cook, 1983). The Panel notes that these results could not be reproduced by the stability studies carried out by the petitioner for rebaudioside A.

The same authors (Chang and Cook, 1983) reported data on the stability of stevioside. Prolonged heating at 100°C of stevioside (purity not reported) in an aqueous solution (6.5 mg/mL) resulted in a decrease in the stevioside concentration. Retention of pure stevioside was 66% and 38.2% after 48 and 66 hours, respectively. Steviolbioside and glucose were identified degradation products and the C-19

ester linkage appeared to be the most heat-labile bond in stevioside. Heating of acidic solutions of stevioside (6.5 mg/mL) at 60°C for up to 137 hours did not cause any appreciable degradation of this steviol glycoside. Following heating at 100°C for 13 hours 18.7% and 32.2% of stevioside were present in phosphoric and in citric acid solutions, respectively. By TLC were detected as degradation products steviolbioside, glucose and two unknown components. Stevioside showed no significant changes in HPLC and TLC analyses at 4°C or at room temperature for at least 5 months when formulated in carbonated beverage containing 0.1% stevioside with phosphoric or citric acid as the acidulant. Thirty-six percent and 17% degradation of stevioside in phosphoric (cola type) and citric acid (citrus type) beverages was reported after 4 months of storage at 37°C. Exposure to 1 week of sunlight did not affect stevioside in either the phosphoric or citric acid beverages (Chang and Cook, 1983).

Other published data (Kroyer, 1999) on the stability of stevioside (purity not reported) demonstrated that solid stevioside at elevated temperatures for 1 hour showed good stability up to 120°C, whilst at temperatures exceeding 140°C forced decomposition was seen which resulted in total decomposition by heating to 200°C. The same author reported that stevioside in an aqueous solution (0.5 g/L) was stable within a pH range of 2 - 10 over 2 hours at 60°C and losses that occurred on heating to a temperature of 80°C were up to 5%. Similarly, losses up to 5% of stevioside were seen after 4 hours incubation of tea or coffee at 80°C. Under strong acidic conditions (pH 1), forced decomposition of stevioside was observed which resulted in total decomposition after incubation at a temperature of 80°C for 2 hours. After 4 months of storage at room temperature no evidence was found of degradation of stevioside in 1 g/L solutions of acetic acid (pH 3.1), citric acid (pH 2.6), and tartaric acid (pH 2.6), but losses of 30% occurred in equivalent solutions of phosphoric acid (pH 2.2). In 10 g/L stevioside solutions of acetic acid (pH 2.6), citric acid (pH 2.1), tartaric acid (pH 2.1) and phosphoric acid (pH 1.6) losses in stevioside concentration of 2, 22, 33 and 75% were observed after 4 months of storage, respectively, and steviolbioside and glucose were detected as degradation products (Kroyer, 1999). The Panel notes that in presence of high temperatures (e.g. heating, baking), substantial degradation of steviol glycosides might take place.

Based on information from the published literature (Kroyer, 1999) provided by one of the petitioners, incubation of stevioside with individual water soluble B vitamins and vitamin C showed no significant change in nutrient levels resulting from the presence of the sweetener. With regard to the practical application of low-calorie sweeteners in synergistic mixtures, the stability of the sweeteners in binary aqueous solutions of stevioside with other individual low-calorie sweeteners, saccharin, cyclamate, aspartame, acesulfame and neohesperidin dihydrochalcone, was investigated. No interactions between the individual sweeteners were found in the course of thermal treatment at 80°C for up to 4 hours as well as for over 4 months of incubation at room temperature (Kroyer, 1999).

The same petitioner further argued that no indications of any influence of steviol glycosides on the bioavailability of nutrients, or on physiological parameters were found in different experiments with animals fed with steviol glycosides (Geuns *et al.*, 2003b, Yamada *et al.*, 1985; Yodyingyud and Bunyawong, 1991).

The French Food Safety Agency (AFFSA) addressed thermal stability of rebaudioside A, with a purity higher than 97%, in an opinion published in December 2009 (AFSSA, 2009). In this opinion it was observed that its authorisation as table-top sweetener could lead regular consumers to use rebaudioside A as sweetener to prepare foodstuffs such as cakes and biscuits. A stability test performed on rebaudioside A tested as an ingredient of a white cake model was regarded as only indicative of thermal stability at heating conditions up to 182 °C for 20-25 minutes. The AFSSA opinion noted that most heating conditions used to prepare common types of bakeries apply higher temperatures (up to 220 °C) and/or longer baking times (up to 45 minutes). Therefore taking also into consideration that the thermal instability of steviol glycosides has been reported previously (Kroyer, 1999), as a precaution AFSSA recommended to avoid heating rebaudioside A at temperatures over 100 - 120 °C.

Enzymatic degradation of steviol glycosides was reported by Tomoyoshi *et al.* (1991). Stevioside was completely degraded at 45°C overnight in raw soy sauce, but it was not degraded in heat-treated soy sauce. Rebaudioside A and C were not degraded under the same conditions. The degradation products were separated and identified as the β-D-glycosyl ester of 13-O-β-D-glycosylsteviol (rubusoside) and 13-O-β-D-glycosyl steviol (steviol monoside). Authors concluded that stevioside in raw soy sauce was hydrolysed by β-glucosidase, and that enzyme was inactivated after heating. The stability of rebaudiosides A and C was probably due to steric hindrance around the β-1,2-glycosyl linkage and lack of the β-1,2-glycosyl linkage, respectively (Tomoyoshi *et al.*, 1991).

2.6. Case of need and proposed uses

The three petitioners indicated that steviol glycosides are low-calorie, high-intensity sweeteners (~200-300 times sweeter than sucrose) of similar taste quality as sucrose that provide an alternative to the already approved high-intensity sweeteners.

Steviol glycosides are proposed to be used in a variety of foods and beverages, such as soft drinks, canned fruit and jams, ice creams and other dairy products, cakes and desserts and alcoholic beverages, etc.

To aid the Panel's assessment of steviol glycosides, the three petitioners submitted to EFSA on 28 April 2009, a cross-reference document ("roadmap") which intended to harmonise their proposed uses and use-levels for steviol glycoside preparations in food categories.

From all three petitioners, the uses and use levels for steviol glycosides intended to be used in food categories reflect those currently permitted for aspartame in the EU (Directive 94/35/EC⁸ on sweeteners for use in foodstuffs) with major exceptions for milk and milk derivative-based or fruit juice-based drinks, energy-reduced or with no added sugar (1 000 mg steviol glycosides/kg product instead of 600 mg aspartame/kg product) and other minor exceptions for chewing gums with no added sugar (10 000 mg steviol glycosides/kg product instead of 5 500 mg aspartame/kg product), fine bakery products for special nutritional uses (1 000 mg steviol glycosides/kg product instead of 1 700 mg aspartame/kg product), breath-freshening micro-sweets with no added sugar (10 000 mg steviol glycosides/kg product instead of 6 000 mg aspartame/kg product), confectionery with no added sugar (soft candy, nougats, and marzipans; 1 500 mg steviol glycosides/kg product instead of 1 000 mg steviol glycosides/kg product), soy-based beverages for which a use of steviol glycosides is proposed (600 mg steviol glycosides/kg product) and for energy-reduced beer for which the use of steviol glycosides was not proposed.

Table 3 summarises the individual proposed food-uses and use-levels for steviol glycosides as proposed by the petitioners.

Table 3: Summary of food uses and use levels for aspartame and steviol glycosides as proposed by the petitioners.

Foodstuffs	Maximum Use Levels		
	Aspartame	Steviol Glycosides ¹	Steviol Equivalents ²
Non-alcoholic drinks			

⁸ European Parliament and Council Directive 94/35/EC of 30 June 1994 on sweeteners for use in foodstuffs. OJ L 237, 10.0.1994, 3-12

Foodstuffs	Maximum Use Levels		
	Aspartame	Steviol Glycosides ¹	Steviol Equivalents ²
-Water-based flavoured drinks, energy-reduced or with no added sugar	600 mg/L	600 mg/L	198 mg/L
-Milk- and milk-derivative-based or fruit juice-based drinks, energy-reduced or with no added sugar	600 mg/L	1000 mg/L	330 mg/L
Desserts and similar products			
-Water-based flavoured desserts, energy-reduced or with no added sugar	1000 mg/kg	1000 mg/kg	330 mg/kg
-Milk- and milk-derivate-based preparations, energy-reduced or with no added sugar	1000 mg/kg	1000 mg/kg	330 mg/kg
-Fruit- and vegetable-based desserts, energy-reduced or with no added sugar	1000 mg/kg	1000 mg/kg	330 mg/kg
-Egg-based desserts, energy-reduced or with no added sugar	1000 mg/kg	1000 mg/kg	330 mg/kg
-Cereal-based desserts, energy-reduced or with no added sugar	1000 mg/kg	1000 mg/kg	330 mg/kg
-Fat-based desserts, energy-reduced or with no added sugar	1000 mg/kg	1000 mg/kg	330 mg/kg
-‘snacks’: certain flavours of ready to eat, prepacked, dry, savoury starch products and coated nuts	500 mg/kg	500 mg/kg	165 mg/kg
Confectionery			
-Confectionery with no added sugar	1000 mg/kg	1000 mg/kg (hard candy)	330 mg/kg (hard candy)
		1500 mg/kg (soft candy, nougats, and marzipans)	495 mg/kg (soft candy, nougats, and marzipans)
-Cocoa- or dried-fruit-based confectionery, energy-reduced or with no added sugar	2000 mg/kg	2000 mg/kg	660 mg/kg
-Starch-based confectionery, energy-reduced or with no added sugar	2000 mg/kg	2000 mg/kg	660 mg/kg
-Cocoa-, milk-, dried-fruit-, or fat-based sandwich spreads, energy-reduced or with no added sugar	1000 mg/kg	1000 mg/kg	330 mg/kg
-Chewing gum with no added sugar	5500 mg/kg	10 000 mg/kg	3300 mg/kg
-Cider and perry	600 mg/L	600 mg/L	198 mg/L
-Alcohol-free beer or with an alcohol content not exceeding 1,2% vol	600 mg/L	600 mg/L	198 mg/L
-‘Bière de table/tafelbier/Table beer’ (original wort content less than 6%) except for ‘Obergäriges Einfachbier’	600 mg/L	600 mg/L	198 mg/L
-Beers with a minimum acidity of 30 milli-equivalents expressed as NaOH	600 mg/L	600 mg/L	198 mg/L
-Brown beers of the ‘oud bruin’ type	600 mg/L	600 mg/L	198 mg/L
-Edible ices, energy-reduced or with no added sugar	800 mg/L	800 mg/L	264 mg/L
-Canned or bottled fruit, energy-reduced or with no added sugar	1000 mg/kg	1000 mg/kg	330 mg/kg

Foodstuffs	Maximum Use Levels		
	Aspartame	Steviol Glycosides ¹	Steviol Equivalents ²
-Energy-reduced jams, jellies, and marmalades	1000 mg/kg	1000 mg/kg	330 mg/kg
-Energy-reduced fruit and vegetable preparations	1000 mg/kg	1000 mg/kg	330 mg/kg
-Sweet-sour preserves of fruit and vegetables	300 mg/kg	600 mg/kg	198 mg/kg
-Sweet-sour preserves and semi-preserves of fish and marinades of fish, crustaceans, and molluscs	300 mg/kg	600 mg/kg	198 mg/kg
-Sauces	350 mg/kg	350 mg/kg	115.5 mg/kg
-Mustard	350 mg/kg	350 mg/kg	115.5 mg/kg
-Fine bakery products for special nutritional uses	1700 mg/kg	1000 mg/kg	330 mg/kg
-Complete formulae for weight control intended to replace total daily food intake or an individual meal	800 mg/kg	800 mg/kg	264 mg/kg
-Complete formulae and nutritional supplements for use under medical supervision	1000 mg/kg	1000 mg/kg	330 mg/kg
-Liquid food supplements/dietary integrators	600 mg/kg	600 mg/kg	198 mg/kg
-Solid food supplements/dietary integrators	2000 mg/kg	2000 mg/kg	660 mg/kg
-Essoblaten (a type of wafer)	1000 mg/kg	1000 mg/kg	330 mg/kg
Food supplements/diet integrators based on vitamin and/or mineral elements, syrup-type or chewable	5500 mg/kg	5500 mg/kg	1815 mg/kg
-Breakfast cereals with a fibre content >15% and containing at least 20% bran, energy-reduced or with no added sugar	1000 mg/kg	1000 mg/kg	330 mg/kg
Energy-reduced soups	110 mg/L	110 mg/L	36.3 mg/L
Breath-freshening micro-sweets with no added sugar	6000 mg/kg	10 000 mg/kg	3300 mg/kg
Strongly flavoured freshening throat pastilles with no added sugar	2000 mg/kg	2000 mg/kg	660 mg/kg
Energy-reduced beer	25 mg/L		
Drinks consisting of a mixture of a non-alcoholic drink and beer, cider, perry, spirits or wine	600 mg/L	600 mg/L	198 mg/L
Spirit drinks containing <15% alcohol by volume	600 mg/kg	600 mg/L	198 mg/L
Feinkostsalat (delicatessen salads)	350 mg/kg	350 mg/kg	115.5 mg/kg
Soy-based beverages		600 mg/L	198 mg/L

¹ Use levels are represented as “steviol glycosides” based on the similarity of sweetness potency between stevioside and rebaudioside A, which are the major constituent glycosides of steviol glycoside sweetness.

² The maximum use levels expressed as steviol equivalents provided by the petitioners were based on a conversion factor of 0.33 of the maximum use levels of steviol glycosides.

2.7. Information on existing authorisations and evaluations

As informed by the petitioners steviol glycosides are permitted for use in foods and beverages in South Korea, Japan, Argentina, Paraguay and Brazil (Marie, 1991; Das *et al.*, 1992; Kinghorn *et al.*, 1998; Chung *et al.*, 2005; Ferlow, 2005). According to one petitioner, other countries permitting the use of steviol glycosides include China, Russia, Indonesia, Mexico (since 2005), Senegal (since 2006), Thailand and Israel.

In the US, steviol glycosides are allowed as a dietary supplement since 1995. Furthermore, a specific steviol glycoside (rebaudioside A, purity higher than 97%) received no objection letters from the US

Food and Drug Administration (US FDA) (December 2008) in response to two independent self-conducted Generally Recognized as Safe (GRAS) determinations (FDA, 2008).

The SCF evaluated stevioside as a potential sweetener in 1985 (SCF, 1985), 1989 (SCF, 1989) and 1999 (SCF, 1999). The SCF considered the data available at the time of the evaluation to be insufficient to adequately assess the safety of stevioside (SCF, 1985, 1989 1999) and concluded that the use of stevioside was “toxicologically not acceptable”. Several concerns were raised by the SCF regarding the specifications of the extracts that had been tested (for the majority of toxicological studies a precise composition of the extract was not adequately defined), metabolism, chronic toxicity and carcinogenicity studies, possible effects on the male reproductive system, on renal and cardiovascular function and on carbohydrate metabolism. Furthermore, steviol, the main metabolite of stevioside, was found to be genotoxic and to induce developmental toxicity (SCF, 1985 1999). The SCF evaluated the safety of *Stevia rebaudiana* Bertoni plants and leaves as a novel food (SCF, 1999) and concluded that no appropriate data were presented to enable the safety of the commercial plant product to be evaluated.

JECFA reviewed the safety of steviol glycosides as a sweetener on several occasions (in 2000, 2005, 2006, 2007 and 2009) and established an ADI for steviol glycosides of 4 mg/kg bw/day (expressed as steviol equivalents).

In 2007, AFSSA (2007) issued an opinion on steviol glycosides in which it was considered that current scientific knowledge indicated that the health risk of using steviol glycosides, extracts of *Stevia rebaudiana*, as a food sweetener, could not be assessed because of observed pharmacological effects in humans and animals associated with the oral consumption of these substances. Following new information provided concerning only the use of rebaudioside A with a purity level higher than 97%, in September 2008 AFSSA considered that the use of rebaudioside A, extracted from *Stevia rebaudiana*, with a purity level higher than 97%, did not present a health risk for consumers (AFSSA, 2008). This conclusion only concerned rebaudioside A, with a purity level higher than 97%, which was the focus of the most recent studies published. The use of rebaudioside A has been authorised in France since August 2009 (JORF, 2009). More recently AFSSA issued a new opinion on rebaudioside A in December 2009 (AFSSA, 2009). In this opinion modifications on purity criteria and modifications on definitions of two food categories introduced by the French administration were considered; the use of rebaudioside A, with a purity higher than 97%, as table-top sweetener was considered and new exposure calculations were done taking into consideration table-top use. Exposure calculations based on an assumption of similar sweetening power between rebaudioside A and aspartame did not exceed the health based value (toxicological reference value) identified previously by AFSSA (1000 mg rebaudioside A/day) which was derived from the studies done in humans by Maki *et al.* (2007, 2008a).

2.8. Dietary Exposure

The petitioners only provided estimates of dietary exposure based on international consumption data. As these are not directly applicable to the European situation, the Panel decided to perform its own assessment.

In order to perform the exposure assessment, the Panel took into account the table of harmonised maximum proposed use levels provided by all three petitioners (Table 3) and followed the principles of the stepwise approach, which were used in the report of the Scientific Co-operation (SCOOP) Task 4.2, to estimate intakes of additives (EC, 1998). In the tiered approach, the First Tier is based on theoretical food consumption data and Maximum Permitted use Levels (MPLs) for additives as permitted by relevant Community legislation. The Second and Third Tiers refer to assessment at the level of individual Member States, combining national data on food consumption with the MPLs for the additive (Tier 2) and its actual usage patterns (Tier 3). As no MPLs exist for steviol glycosides at present, only Tier 1 for the Budget method and Tier 2 using maximum proposed use levels for both exposure estimates have been made by the Panel.

2.8.1. Crude estimates (Budget method)

The dietary exposure to steviol glycosides, expressed as steviol equivalents from the maximum proposed use levels was estimated using the Budget method (Tier 1) according to the report of the SCOOP Task 4.2 (EC, 1998) while assuming that 25% of solid foods and all beverages for adults and children contain the sweetener. This assumes that a typical adult, weighing 60 kg, consumes daily 1.5 L of beverages and 375 g of solid foods, containing the steviol glycosides and that a typical 3-year old child, weighing 15 kg, consumes daily 1.5 L of beverages and 94 g of solid foods, containing the steviol glycosides. The theoretical maximum daily exposure would be 37.1 mg/kg bw/day for children and adults.

2.8.2. Refined estimates

Refined exposure estimates have been performed for Tier 2 using maximum proposed use levels from the three petitioners, presented in Table 3, with individual food consumption data for child and adult populations.

Exposure estimates for children (aged 1-10 years old except for Cyprus: 12-14 years old) have been performed by the Panel using the detailed individual food consumption data from 11 European countries (Belgium, France, the Netherlands, Spain, Czech Republic, Italy, Finland, Germany, Greece, Cyprus, Sweden) of the EXPOCHI (“Individual food consumption data and exposure assessment studies for children”) consortium (Huybrechts *et al.*, in press).

Additionally, as the UK is not part of the EXPOCHI consortium, data for children from the UK National Dietary and Nutrition Survey (NDNS) programme were used for pre-school children aged 1.5-4.5 years (Gregory *et al.*, 1995). Detailed individual food consumption data, obtained from a 4-day weighed dietary record method and categorised according to the use applications in Directive 94/36/EC⁹ on colours for use in foodstuffs, were available from an earlier report provided by the Union of European Beverages Associations (UNESDA) (Tennant, 2006).

Since the UK population is considered to be the one with the highest consumption of soft drinks in Europe and as estimates were calculated from more refined adult food consumption data than those currently available to EFSA Panels (e.g. EFSA Concise European Food Consumption Database, which gives aggregate food categories consumed in 19 European countries (EFSA, 2008)) it was decided to select the UK population as representative of EU consumers for the steviol glycosides exposure estimates for adults. Detailed individual food consumption data, obtained from a 7-day weighed dietary record method of subjects aged 19-64 years (Henderson *et al.*, 2002) and categorised via the use applications listed in Directive 94/36/EC were available from an earlier report provided by UNESDA (Tennant, 2006).

For children, the data from the EXPOCHI countries and UK data were used to calculate the mean and high level exposure to steviol glycosides using proposed maximum use levels. High level exposure (95th percentile of consumers only) was based on the assumption that an individual might be a high level consumer of one food category and would be an average consumer of the others. This approach has been tested several times by the Panel for food colour opinions, which were in agreement with the intake figures obtained by computer analysis. Therefore, this approach was preferred for the calculations based on the maximum proposed use levels in order to avoid excessively conservative estimates. Table 4 summarises the ranges of those results and the results of the Budget method.

When considering the proposed maximum use levels (Tier 2), the mean dietary exposure to steviol glycosides expressed as steviol equivalents in European children (aged 1-14 years) ranged from 0.7 to 7.2 mg/kg bw/day and from 3.3 to 17.2 mg/kg bw/day at the 95th percentile. The main contributors

⁹ European Parliament and Council Directive 94/36/EC of 30 June 1994 on colours for use in foodstuffs. Official Journal of the European Communities, L 237 10.9.94.

(>10% in all countries) to the total anticipated exposure to steviol glycosides, expressed as steviol equivalents are soft drinks (11 to 58%) and desserts, including flavoured milk products (14 to 71%). Confectionery accounted for 11% of exposure in two countries. Dried potato granules and flakes and candied fruits and vegetables, mostardo di frutta accounted for 17 and 18% of exposure in one country.

Exposure estimates reported for the UK adult population give a mean dietary exposure to steviol glycosides, expressed as steviol equivalents of 2.2-2.7 mg/kg bw/day, and of 8.0-9.7 mg/kg bw/day for high level consumers (97.5th percentile). The main contributors to the total anticipated exposure to steviol glycosides, expressed as steviol equivalents (>10 %) are soft drinks (37%) and beer, cider and perry (33%).

Table 4: Summary of anticipated exposure to steviol glycosides expressed as steviol equivalents in children and adult populations.

	Adult UK population (>18 years old)	Children population (1-14 years old)
	mg/kg bw/day	
Tier 1. Budget method	37.1	37.1
Tier 2. Maximum Proposed use Level*		
• Mean exposure	2.2-2.7	0.7-7.2
• Exposure 95 th or 97.5 th percentile	8.0-9.7	3.3-17.2

* Estimates were made by the Panel with a conversion factor of 0.33 (lower range corresponding to the proposed use levels on steviol equivalents provided by the petitioner and summarised in Table 3) and 0.4 (upper range corresponding to the conversion factor agreed by the Panel and summarised in Table 1) for calculation of anticipated exposure to steviol glycosides expressed as steviol equivalents.

The Panel compared its exposure assessments to those derived from Renwick (2008) which represent predicted exposure data for European consumers to steviol equivalents based on the observed exposure data for aspartame. Renwick’s (2008) calculations assumed relative sweetness potencies in relation to sucrose, of 180 for aspartame and 200 for rebaudioside A and divided by a factor of three to obtain steviol equivalents. The observed exposure data for aspartame were derived from national individual intake surveys which included diabetics (Denmark, France, Germany, Netherlands and UK).

The mean dietary exposure to steviol glycosides, expressed as steviol equivalents for children (aged 1-14 years), including diabetics, ranged from 0.4 to 1.3 mg/kg bw/day, and from 1.5 to 4.2 mg/kg bw/day at the high percentile (90th/97.5th). For adults, the mean dietary exposure to steviol glycosides, expressed as steviol equivalents, including diabetics, ranged from 0.3 to 0.7 mg/kg bw/day, and from 1.5 to 3.1 mg/kg bw/day at the high percentile (90th/97.5th).

The Panel notes that its estimates for upper range of high percentile are three (for children) to four (for adults) times higher than those based on Renwick (2008) because the Panel assumed that all processed foods and beverages contain the sweetener steviol glycosides added at the maximum proposed use levels (i.e. not only energy-reduced beverages as proposed for the use of steviol equivalents). But the Panel notes that the exposure data from Renwick (2008) uses older consumption figures, a conversion factor of three for calculation of predicted exposure to steviol glycosides expressed as steviol equivalents and that the food category “Milk and milk derivative-based or fruit juice-based drinks, energy-reduced or with no added sugar”, which is one of the main contributors to the overall exposure in the assessment made by the Panel has higher proposed use levels (1000 mg steviol glycosides/kg instead of 600 mg aspartame/kg).

Despite the limitations of the present assessment, the Panel notes that its assessment presents a good geographical spread of the food consumption of children in Europe, a standardised approach in food

categorisation via use applications in Directive 94/36/EC and methodology to calculate anticipated dietary exposure assessments and concluded that its estimates are considered to be the most up to date anticipated dietary exposure to a steviol glycosides preparation expressed as steviol equivalents.

3. Biological and toxicological data

3.1. Absorption, distribution, metabolism and excretion

3.1.1. *In vitro* studies

The metabolism of stevioside (purity not reported) was studied by using various digestive enzymes or fluids like salivary α -amylase, pancreatic α -amylase, saliva, pepsin, gastric secretion, pancreatin and intestinal brush border membrane enzymes of rodents as well as by the intestinal microflora of various species including humans (Hutapea *et al.*, 1997). None of these enzymes digested stevioside. However, the caecal microflora of all species tested was able to metabolise stevioside to steviol. A transient formation of steviol-16,17 α -epoxide was observed in mouse caecal contents and human faeces. The authors suggested that steviol is the major metabolite produced by caecal microflora from various animal species and humans.

Regarding human intestinal metabolism of steviol glycosides, a study was undertaken by Koyama *et al.* (2003a) to investigate human intestinal metabolism of a *Stevia* mixture (28.8% rebaudioside A, 17% stevioside, 25.2% rebaudioside C, 10.2% dulcoside) and its α -glucose derivative by LC-MS analysis. The Panel notes that the authors had also studied α -glucose derivatives of the *Stevia* mixture, the precise composition of this is unclear from the published information and the Panel considers that these data are irrelevant to the steviol glycosides considered in this opinion. Metabolism was examined by incubating the *Stevia* mixture, its α -glucose derivative, stevioside, rebaudioside A, α -monoglucosylstevioside, α -monoglucosylrebaudioside A and the aglycone steviol with pooled human faecal homogenates (obtained from five healthy volunteers, no age indicated) for 0, 8 and 24 hours under anaerobic conditions. The *Stevia* mixture, its α -glucose derivative, stevioside and rebaudioside A (0.2 mg/mL) were completely metabolised to steviol within 24 hours, whereas no metabolism of steviol (0.08 and 0.2 mg/mL) appeared to be found during the incubation period. The *Stevia* mixture, stevioside and rebaudioside A appeared to be hydrolysed to steviol by human intestinal microflora. This observation is consistent with previous rat metabolism studies. Similarly, the α -glucose derivative appeared to be finally metabolised to steviol.

Gardana *et al.* (2003) investigated the *in vitro* transformation of stevioside and rebaudioside A (*Stevia* extract containing either 85% stevioside or 90% rebaudioside A, respectively) after incubation with human intestinal microflora, the influence of these sweeteners on the human microbial faecal composition and which specific microbial species metabolise preferentially stevioside and rebaudioside A. The experiments were carried out under strict anaerobic conditions in batch cultures inoculated with mixed faecal bacteria from volunteers (6 males and 5 females aged between 20 and 50 years old). The hydrolysis was monitored by LC-MS analysis. Isolated bacterial strains from faecal materials incubated in selective broths were added to stevioside and rebaudioside A; these sweeteners were completely hydrolysed to their aglycone steviol within 10 and 24 hours, respectively. Interestingly, the human intestinal microflora was not able to degrade steviol. Furthermore, stevioside and rebaudioside A did not significantly influence the composition of faecal cultures. Among the selected species, bacteroides were the most efficient in converting steviol glycosides to steviol.

The intestinal transport characteristics of stevioside, rebaudioside A (purity not reported) and steviol (purity not reported) were studied in Caco-2 cells (Geuns *et al.* 2003b). In comparison to steviol (apparent permeability value of 31.9×10^{-6} cm/s), only a minor fraction of stevioside and rebaudioside A was transported through the Caco-2 cell layer giving apparent permeability values of 0.16×10^{-6} and 0.11×10^{-6} cm/s, respectively. In addition, the apparent permeability value for the absorptive transport

of steviol was about 7 times higher than that for the secretory transport of steviol, suggesting a carrier-mediated transport. The Panel notes that in this intestinal model, the apparent permeability value for steviol is 200 to 300-times higher than that for stevioside or rebaudioside A.

Regarding steviol hepatic metabolism, Compadre *et al.* (1988) demonstrated the very low conversion of steviol (purity not reported) into oxidative metabolites by microsomal fractions from Aroclor 1254-pretreated rats. However, the authors noted the possible mutagenic activity of 15-oxosteviol, a metabolite which could be formed after a further oxidation of 15-hydroxysteviol.

Koyama *et al.* (2003b) incubated steviol (purity not reported) with rat (no pretreatment mentioned) or human (pooled from ten healthy donors, 5 male and 5 female) liver microsomes. In rats, monohydroxy- and dihydroxy-metabolites of steviol were observed by Liquid Chromatography Electrospray Ionisation Tandem Mass Spectrometry (LC-ESI-MS) after incubation with human liver microsomes. The intrinsic clearance of steviol in human liver microsomes was 4-times lower than that found in rat liver microsomes. However, this study suggested that there are no major species differences in steviol metabolites between rats and humans. The Panel notes that the authors concluded that extrapolation of toxicity data on steviol glycosides from rats to humans would therefore be valid.

3.1.2. *In vivo* studies

Animals

By using intact, ligated oral or bile duct cannulated rats, Wingard *et al.* (1980) demonstrated that ^{14}C -steviol (purity not reported) was almost totally absorbed from the rat lower bowel following intracaecal administration.

Uniformly labelled ^3H -stevioside (95% purity) prepared by gas tritiation was administered orally at a dose of 125 mg/kg bw to Wistar rats, and its disposition and metabolism were studied (Nakayama *et al.*, 1986). The level of radioactivity in the blood increased slowly to a maximum of 4.83 μg stevioside equivalents/mL at 8 hours, exhibiting a biological half-life of 24 hours. At 1 hour, the highest concentration was observed in the small intestine, followed by the stomach and caecum in that order. At 4 hours, the concentration in the caecum was markedly higher than that in other tissues. Radioactivity remaining in the body at 45 hours was 30.7% of the original dose. At 120 hours, the percentages of radioactivity excreted into the faeces and expired air were 68.4% and 23.9%, respectively, while radioactivity excreted into the urine was only 2.3%. Radioactivity excreted into the bile at 72 hours was 40.9 % of the original dose. From the results of biliary and faecal excretion, it was concluded that enterohepatic circulation occurs in the body. TLC analysis of the intestinal contents, faeces and bile showed that stevioside is metabolised by caecal flora to steviol and sugars, and indicated that steviol and these sugars are absorbed from the caecum, distributed throughout the whole body, and excreted mainly into faeces and expired air.

Koyama *et al.* (2003b) investigated the absorption and the hepatic metabolism of both a *Stevia* mixture (main components: rebaudioside A, stevioside, rebaudioside C, dulcoside A) and steviol (purity not reported) in rats. Absorption was investigated both *ex vivo* and *in vivo*. In *ex vivo* experiments using the rat everted sac method, no absorption of the *Stevia* mixture was observed, but significant absorption of steviol was noted. In the *in vivo* experiment, rats received a single oral administration of either steviol or the *Stevia* mixture. A steviol peak concentration of 18 $\mu\text{g}/\text{mL}$ in plasma was observed 15 minutes after oral administration, demonstrating rapid absorption. However, after oral administration of the *Stevia* mixture, the steviol concentration in plasma increased steadily over 8 hours, suggesting that the *Stevia* mixture components are first metabolised and then absorbed as steviol in the rat intestine.

Recently, the toxicokinetics and metabolism of rebaudioside A, stevioside, and steviol (purity $\geq 97\%$) were compared in rats to determine whether toxicological studies conducted previously with stevioside would be applicable to the structurally-related glycoside, rebaudioside A (Roberts and Renwick,

2008). Single oral doses of the ^{14}C -compounds radiolabelled in the methylene group ($=\text{CH}_2$) of the steviol moiety were extensively and rapidly absorbed with plasma concentration–time profiles following similar patterns for stevioside and rebaudioside A. Peak concentrations of radioactivity were at approximately 8 and 4 hours following doses of ^{14}C -stevioside and ^{14}C -rebaudioside A, respectively. Elimination of radioactivity from plasma was essentially complete within 72 hours. All plasma samples had similar proportions of radioactive derivatives; the predominant radioactive component in all samples was steviol, with 5 to 17-times lower amounts of steviol glucuronide. One or two other unidentified metabolites were also present in plasma. Rebaudioside A, stevioside, and steviol were metabolised and excreted rapidly, since 83 to 98% of the radioactivity was eliminated in the faeces within 48 hours. Urinary excretion accounted for less than 2% of the administered dose for all compounds in both intact and bile duct-cannulated rats, and 69 to 98% of the absorbed dose was excreted via the bile. After administration of the compounds to intact and bile duct-cannulated rats, radioactivity in the faeces was present primarily as steviol. The predominant radioactive compound detected in the bile of all cannulated rats was steviol glucuronide, indicating de-conjugation of steviol glucuronide and rebaudioside A in the lower intestine. Overall, the data on toxicokinetics and metabolism indicate that stevioside and rebaudioside A are handled in a similar manner. The authors considered that these studies support the use of toxicological safety studies conducted with stevioside for the safety assessment of rebaudioside A.

In pigs (6 females/group, body weight 26 kg) fed stevioside (purity $\geq 96\%$) at a dose of 1.67 g/kg feed (equivalent to approximately 0.13g stevioside/kg bw/day), stevioside was completely converted into steviol by the bacteria of the colon (Geuns *et al.*, 2003a). However, no stevioside or steviol could be detected in the blood of the animals, by using a very sensitive fluorescent method of analysis (detection limits of 0.5 ng/mL and 0.5 pg/mL for stevioside and steviol respectively).

The petitioner also indicated a study on broiler chickens administered either a single-dose or repeated-doses of stevioside by gavage in which the glycoside was reported to be recovered largely unchanged within the excreta (Geuns *et al.*, 2003b).

Humans

In a briefly described study, Kraemer and Maurer (1994) investigated the fate of stevioside (purity and dose not reported) in humans (gender and number not reported). After ingestion of stevioside, urine and faeces were collected over one week. The samples were analysed with or without enzymatic cleavage of conjugates after liquid-liquid extraction or solid phase extraction using HPLC and Gas chromatography-Mass Spectrometry (GC-MS). The structures of the metabolic products were determined using Mass Spectrometry (MS), Nuclear Magnetic Resonance (NMR) spectroscopy and chemical synthesis. Only small amounts of unchanged stevioside were excreted in faeces. Stevioside was readily metabolised to its aglycone steviol by human intestinal flora. The absorbed steviol was conjugated in the liver to an acyl-glucuronide which was excreted via bile and urine. Sixty percent of the applied amount of stevioside was recovered from urine as steviol glucuronide over a period of about 100 hours. This metabolite was also detected in the faeces during this period. Part of the glucuronide was metabolised by the intestinal flora to steviol, which can be reabsorbed and undergo an entero-hepatic circulation. Further phase I or phase II metabolites were not found in urine or faeces (Kraemer and Maurer, 1994).

Simonetti *et al.* (2004) investigated stevioside bioavailability and metabolic fate in human healthy volunteers (9 males aged between 25 and 50 years old) receiving 375 mg stevioside (from a *Stevia* extract containing 85% stevioside) as a single oral dose. At the beginning and at different time-points after stevioside administration, plasma (0-5 hours post-dose), urine and faecal samples were collected, extracted and analysed for the presence of stevioside or its possible metabolites such as steviol, steviol-16,17- α -epoxide and 15- α -hydroxysteviol by means of a LC-MS method. In plasma, two peaks of steviol glucuronide occurred at 1-2 and 4 hours post-dose. The results obtained proved that stevioside is converted to steviol, which is subsequently absorbed and that steviol glucuronide is only

found in plasma whilst steviol is only found in faeces. In addition, steviol-16,17- α -epoxide and 15- α -hydroxy-steviol were not found in plasma, urine and faecal samples.

In a further study (Geuns *et al.*, 2007), stevioside (250 mg capsules; 97% purity, impurities were 2.7% steviolbioside and 0.3% rebaudioside A) was given thrice daily for 3 days to 10 healthy subjects (5 females and 5 males aged between 21 and 29 years old). Blood samples were collected, before and at different time-points during the third day of stevioside administration. Stevioside, free steviol, and steviol metabolites were analysed in blood, faeces, and urine after 3 days of stevioside administration. No uptake of stevioside was found by the gastrointestinal tract and the amounts taken up were below the detection limit of the analytical method (200 ng/mL). In plasma, no stevioside, no free steviol nor other free steviol metabolites were found. Steviol glucuronide was found at a maximum concentration of 33 $\mu\text{g/mL}$ (21.3 μg steviol equivalents/mL). On the third day of the experiment, two plasma peaks occurred at 0.5-1 hour and 5-7 hours post-dose. In urine, no stevioside or free steviol were present, but steviol glucuronide was unambiguously identified (Geuns *et al.*, 2006). Steviol glucuronide in human urine was found in amounts of up to 318 mg/24-hour urine (205 mg steviol equivalents/24 hours). No other steviol derivatives were detected. In faeces, besides free steviol, no other steviol metabolites or conjugates were detected.

Recently, a double-blind, cross-over study assessed the comparative pharmacokinetics of steviol and steviol glucuronide following single oral doses of rebaudioside A (98.7% purity) and stevioside (96.6% purity) in healthy adult male subjects (8 males aged between 18 and 45 years old) (Wheeler *et al.*, 2008). Steviol glucuronide appeared in the plasma of all subjects after administration of rebaudioside A or stevioside, with median plasma peak time values of 12 and 8 hours post-dose, respectively. In both cases, two plasma peaks occurred at 6-12 and 24 hours post-dose. Steviol glucuronide was eliminated from the plasma, with similar half-life values of approximately 14 hours for both compounds. No steviol epoxide, which may be mutagenic, was detected in plasma. Administration of rebaudioside A resulted in a significantly lower steviol glucuronide maximal plasma concentration (1472 ng/mL) than after administration of stevioside (1886 ng/mL). However, there was no significant difference between the geometric mean AUC_{0-t} values found for steviol glucuronide after administration of rebaudioside A (30.8 ng hour/mL) or after administration of stevioside (34.1 ng hour/mL). Steviol glucuronide was excreted primarily in the urine of the subjects during the 72 hours collection period, accounting for 59% and 62% of the rebaudioside A and stevioside doses, respectively. No steviol glucuronide was detected in faeces (Wheeler *et al.*, 2008). This pharmacokinetic analysis indicated that rebaudioside A and stevioside underwent similar metabolic and elimination pathways in humans with steviol glucuronide excreted primarily in the urine and steviol in the faeces.

In summary, the Panel notes that *in vitro* studies demonstrated that human digestive enzymes are not capable of hydrolysing β -glycosidic bonds of steviol glycosides. However, the intestinal microflora of humans (and rats) is able to convert steviol glycosides to steviol. In addition, in the Caco-2 cell model the apparent permeability value of steviol was found to be 200 to 300-times higher than that of stevioside or rebaudioside A. Other *in vitro* studies assessing the metabolic transformation of steviol showed a similar formation of hydroxy-metabolites of steviol in the presence of rat or human liver microsomes.

In vivo studies in rats receiving stevioside demonstrated that free steviol was the main metabolite present in plasma and it reached maximum plasma concentration 24 hours after administration. In animal liver, steviol was shown to primarily undergo conjugation with glucuronic acid to form steviol glucuronide, identified as the major metabolite in bile. From the results of biliary and faecal excretion, it can be concluded that in rats, enterohepatic circulation occurs. In rats, steviol has been shown to be primarily excreted in the faeces via the bile, and in smaller amounts in the urine.

In human volunteers exposed orally to stevioside or rebaudioside A, no free steviol was detected in the blood but steviol glucuronide was found to be the main metabolite in plasma. No steviol epoxide, which may be mutagenic, was detected in human plasma. The presence of multiple peaks in time of

plasma concentrations of steviol glucuronide indicates enterohepatic circulation of steviol in humans as experimentally demonstrated in rats. Steviol glucuronide was also reported to be the main metabolite found in the urine of subjects receiving stevioside or rebaudioside A; this elimination pathway accounted for about 60% of the dose. Steviol was reported to be the main metabolite found in the faeces of humans receiving oral stevioside or rebaudioside A. The Panel considers that these toxicokinetic analyses indicated that rebaudioside A and stevioside underwent similar metabolic and elimination pathways in humans. Therefore, the Panel considers that the results of toxicology studies on either stevioside or rebaudioside A can be applicable for the safety evaluation of steviol glycosides in general.

The main metabolite in plasma is steviol glucuronide in humans and free steviol in rats; no steviol epoxide, which may be mutagenic, was detected in human plasma. Steviol glucuronide is excreted primarily via the urine in humans and via the bile in rats due to known species differences in the molecular weight threshold for biliary elimination.

3.2. Toxicological data

This section summarises the relevant studies which used steviol glycosides (i.e. purified stevioside, or purified rebaudioside A, or mixtures) complying with the specifications proposed by the petitioners and in laboratory animal species which are considered relevant models for extrapolation to the human situation.

3.2.1. Acute oral toxicity

Acute oral toxicity studies with stevioside (purity 96%) indicated an LD₅₀ of >15 g/kg bw in the mouse, rat and hamster (Toskulkao, 1997).

Other acute oral toxicity studies with steviol glycosides not complying with JECFA specifications are listed in Appendix I.

3.2.2. Short-term and subchronic toxicity

Several short-term and subchronic toxicity studies have been provided by the petitioners. The studies with the test compounds meeting JECFA specifications are reported and also listed in Appendix II.

Steviol glycoside (97% rebaudioside A) was administered to Wistar rats at concentrations up to 100 000 mg/kg diet in a 4-week dose-range finding study (10 animals/sex/group) and at concentrations of 0, 12 500, 25 000 or 50 000 mg/kg diet in a 90-day toxicity study (20 animals/sex/group) (Curry and Roberts, 2008). In these studies no deaths, clinical signs of toxicity, changes in clinical chemistry and haematology parameters, and no pathological findings related to treatment were reported except for effects on body weight, feed intake and feed conversion efficiency.

In the 90-day study, body weight gains were statistically significantly lower in males and females receiving 25 000 and 50 000 mg rebaudioside A/kg diet. After the first 4 days of treatment to termination, the body weight gains were statistically significantly lower in all male test groups compared to the controls. Overall body weight gains were slightly but statistically significantly lower in females treated with 25 000 and 50 000 mg rebaudioside A/kg diet, compared with controls. During the first 3 days of the study, feed intake was statistically significantly lower in all groups of treated males and in mid- and high-dose female groups as compared to controls.

Feed conversion efficiency was statistically significantly decreased at various times in treated males early in the study (days 1 to 14) and sporadically later in the study. In females, feed conversion efficiency was unaffected by treatment except for a significant decrease during days 1 to 3 in the high-dose group and a statistically significant increase in all dose groups during days 8 to 10. Over the

course of the entire study, statistically significant decreases in feed conversion efficiency values were limited to the high-dose male group (Curry and Roberts, 2008).

Similar effects on body weight gains were also observed in the dose-range finding study with rebaudioside A. However, in this study no statistically significant differences in the feed conversion factor were seen between the treated and control groups and the feed intake for the entire study period was comparable to controls in all treated groups except for males from the 100 000 mg rebaudioside A/kg diet (corresponding to approximately 31 912 mg steviol equivalents/kg diet) group for which this parameter was statistically significantly increased (Curry and Roberts, 2008).

With regard to interpretation of the effects of the test preparation on body weight and feed intake, the Panel notes that similar effects have been observed in studies with other intense sweeteners (Flamm *et al.*, 1993). In the absence of other effects, the Panel considers the effects on body weight and on feed conversion efficiency observed in the dose-range finding study and in the 90-day study not as an adverse effect of the preparation but due to the consumption of a test material with known low nutritive value and palatability effects. In light of this consideration, the Panel notes that the NOAELs are 100 000 mg/kg diet equal to 9938 and 11 728 mg rebaudioside A/kg bw/day for males and females, respectively, (approximately 3280 and 3870 mg steviol equivalents/kg bw/day for males and females, respectively) in the 4-week dose-range finding study or 50 000 mg/kg diet equal to 4161 and 4645 mg/kg bw/day for males and females, respectively (corresponding to approximately 1373 and 1533 mg steviol equivalents/kg bw/day for males and females, respectively) in the 90-day study (WHO, 1987).

In another study, rebaudioside A (97% purity) fed to Sprague Dawley rats (20 animals/sex/group) in the diet *ad libitum* for 90 days at doses of 0 (control), 500, 1000, or 2000 mg/kg bw/day (corresponding to approximately 0, 165, 330, and 660 mg steviol equivalents/kg bw/day, respectively) was without adverse effects on body weight gains, terminal body weights, clinical and functional observations, or on the results of the haematology, serum chemistry, or urinalysis (Nikiforov and Eapen, 2008). Treatment was not associated with any organ weight variations or macroscopic or microscopic tissue changes. Similar to Curry and Roberts (2008), a slight decrease in food conversion efficiency was noted in the high-dose males, with decreased body weights and body weight gains. According to the authors, this effect resulted from the inclusion of a high concentration of a non-nutritive substance in the diet and therefore the NOAEL was 2000 mg rebaudioside A/kg bw/day corresponding to approximately 660 mg steviol equivalents/kg bw/day), the highest dose tested (Nikiforov and Eapen, 2008).

Stevioside (95.6% purity) was administered to Fischer 344 rats (10 animals/sex/group) at dietary concentrations of 0, 0.31, 0.62, 1.25, 2.5, or 5% (equivalent to 0, 155, 310, 625, 1250, and 2500 mg/kg bw/day) for 90 days. No deaths occurred during the treatment and there was no statistically significant difference in bodyweight gain or food intake between the control and treated groups. However, the terminal body weights were statistically significantly decreased in the female 2.5%-dose group and male and female 5%-dose group in comparison to the controls. Blood Urea Nitrogen (BUN) and Lactate Dehydrogenase (LDH) in biochemical investigations and single cell necrosis in the liver revealed by microscopy were statistically significantly increased in all treated male groups. These findings are not considered by the Panel as attributable to the treatment, because of the lack of any clear dose-response, the relatively low severity and the limitation of effects to only one sex. Other clinical biochemistry or haematology parameters found in males and/or females to demonstrate statistically significant differences with the controls were sporadic and considered by the Panel to be of no toxicological significance. Statistically significant increases in absolute and relative liver weights in the three highest dose groups, increases in relative brain and spleen weights in the highest dose group, and in absolute and relative kidneys weights in the two highest dose groups were recorded for females only and were not connected with any histopathological changes (Aze *et al.*, 1991). The Panel considers these differences to be non adverse and therefore considers that 5% stevioside in the diet, equivalent to 2500 mg/kg bw/day (corresponding to approximately 942 mg steviol equivalents/kg bw/day) is the NOAEL for this study.

The studies submitted by the petitioners with the compounds not meeting JECFA specifications (i.e. Xili *et al.*, 1992; Mitsuhashi, 1981; Akashi and Yokoyama, 1975; Lee *et al.*, 1979; Wood *et al.*, 1996; Pomaret and Lavielle, 1931) were considered by the Panel not to be directly applicable for the safety evaluation of the steviol glycoside under evaluation, but a valuable supplementation to the toxicological database. These studies were reviewed by the Panel and are listed in Appendix III.

3.2.3. Genotoxicity

In evaluating the safety of steviol glycosides in 1999, the SCF requested additional *in vivo* mutagenicity studies with steviol due to the mutagenic effect observed *in vitro* in the presence of metabolic activation and the paucity of *in vivo* data available at that time. Subsequently, several *in vitro* and *in vivo* studies examining the genotoxic potential of steviol glycosides, steviol, and oxidative derivatives of steviol have been conducted. A review of the genetic toxicity of steviol glycosides and steviol has been published in the open literature (Brusick, 2008).

The *in vitro* and *in vivo* studies on the genotoxicity of steviol glycosides, steviol and steviol metabolites, provided by the petitioners are listed in Appendix IV, Tables 1-2. Some of these studies were previously evaluated by the SCF (1999) and JECFA (1999, 2004, 2006).

3.2.3.1. *In vitro* genotoxicity studies with steviol glycosides

Stevioside and rebaudioside A have not shown evidence of genotoxicity in a range of gene mutation assays *in vitro*. As shown in Appendix IV, Table 1, these studies have been carried out with a range of stevioside and rebaudioside A preparations, with some complying with JECFA specifications and others of lower purity. Negative results have been obtained in forward or reverse mutation assays in *S. typhimurium* at levels of up to 10 to 50 mg/plate of rebaudioside A (Pezzuto *et al.*, 1985; purity not reported) or stevioside (Okumura *et al.*, 1978 (18-98% purity); Suttajit *et al.*, 1993 (99% purity); Matsui *et al.*, 1996 (83% purity); Klongpanichpak *et al.*, 1997 (purity not reported)) respectively, with and without metabolic activation, in the *rec* assay using *B. subtilis* at levels of up to 10 mg stevioside/disk (Okumura *et al.*, 1978 (18-98% purity); Matsui *et al.*, 1996a (83% purity)) or in the *umu* gene mutation assay using *S. typhimurium* at levels up to 5 mg stevioside/mL (Matsui *et al.*, 1996a; 83% purity). The steviol glycosides rebaudiosides A, B, C, dulcoside A and steviolbioside (purity of the test compounds not reported) (Medon *et al.*, 1982; Pezzuto *et al.*, 1985) did not induce mutations in *S. typhimurium* TM677, in contrast to the positive response obtained with the aglycone steviol in the same strain (Pezzuto *et al.*, 1985, 1986; see below). Negative results have also been obtained with stevioside (96.8% purity) in a mammalian cell mutation assay using mouse lymphoma L5178Y, Tk[±] cells (Oh *et al.*, 1999a,b).

Rebaudioside A and stevioside did not induce chromosome aberrations *in vitro* in Chinese Hamster Lung fibroblasts (CHL/IU) with or without metabolic activation at levels of up to 5 mg/mL (rebaudioside A) (Nakajima, 2002a, purity not reported) or 12 mg/mL (stevioside) (Ishidate *et al.*, 1984 (85% purity); Matsui *et al.*, 1996; (83% purity)). Stevioside (purity not reported) also showed no evidence of clastogenicity in three separate studies using human lymphocytes (Flores *et al.*, 1987; Höhn and Zankl, 1990; Suttajit *et al.*, 1993); these studies were however judged by the Panel to be of limited validity. In a study in Chinese hamster D-6 cells, reported in abstract form only, stevioside (purity not reported) at the two highest concentration of 2 or 4%, (20 or 40 mg/mL, based on an assumed density of 1 g/mL), produced a higher proportion of total chromosomal aberrations as compared to untreated cells, and increased sister chromatid exchange frequencies (Nadamitsu *et al.*, 1985). No increase in chromosomal aberrations was reported at lower concentrations of stevioside (1 or 2%) (Nadamitsu *et al.*, 1985). The Panel considers that this study does not provide substantive evidence of a clastogenic potential of stevioside, given the lack of experimental detail provided in the abstract, the high concentrations of stevioside used in the study, and the negative results obtained in other chromosomal aberration tests with stevioside. Similarly, a positive response was reported for stevioside (purity not reported) in an *in vitro* study investigating micronuclei formation in human

lymphocytes and in exfoliated human buccal mucosal cells (Höhn and Zankl, 1990). However, for this study only an abstract was available which provided limited experimental details and the Panel also considers that this study does not provide substantive evidence for a clastogenic potential of stevioside.

3.2.3.2. *In vitro* genotoxicity studies with steviol and steviol metabolites

Steviol and some of its oxidative derivatives show clear evidence of genotoxicity *in vitro*, particularly in the presence of a metabolic activation system, as summarised in Appendix IV, Table 1. It is unknown which metabolite of steviol is responsible for the *in vitro* genotoxicity of steviol. The major metabolite of steviol *in vitro*, 15- α -hydroxysteviol, was inactive at doses up to 7.5 mg/mL in the forward mutation assay in *S. typhimurium* strain TM677 with metabolic activation (Pezzuto *et al.*, 1986; Compadre *et al.*, 1988; Terai *et al.*, 2002). Steviol itself gave a positive response in this assay system (Pezzuto *et al.*, 1983, 1985, 1986, Terai *et al.*, 2002). The Panel notes that, according to Pezzuto *et al.* (1985) the strain TM677 is uniquely sensitive to steviol when it is incubated in the presence of S9 from rats induced by polychlorinated biphenyls only, and therefore this is unlikely to be relevant for the situation *in vivo*. 15-Oxosteviol, a product of the oxidation of 15- α -hydroxysteviol, was a directly acting mutagen at 25-200 μ g/mL and was highly toxic to bacteria (Pezzuto *et al.*, 1986; Compadre *et al.*, 1988). Moreover, the expression of mutagenicity required the presence of the 13-hydroxy group and the C-16 exomethylene group (Compadre *et al.*, 1988). 15-Oxosteviol was not mutagenic in various other test systems (Procinska *et al.*, 1991). Repetition of the experiment with *S. typhimurium* TM677 failed to show statistically significant induction of 8-azaguanine-resistant mutants by 15-oxosteviol, even when the number of bacteria tested was greatly increased. The authors concluded that the earlier positive results reported with 15-oxosteviol were due to the particular sensitivity of the TM677 system and that 15-oxosteviol is unlikely to be the active metabolite responsible for the mutagenicity of steviol (Procinska *et al.*, 1991).

3.2.3.3. *In vivo* studies on genotoxicity of steviol glycosides

A mouse micronucleus test has been carried out with Rebaudioside A (purity not specified) at dose levels of 500-2000 mg/kg bw, administered once daily for 2 days by gavage, with negative results (Nakajima, 2000b). In a micronucleus study with stevioside (purity not specified) in rats, using a single i.p. dose of 150 mg/kg bw, with examination of bone marrow for micronucleus formation 24, 48 and 72 hours after dosing, negative results were reported in a short communication (Flores *et al.*, 1987). In the same publication it was reported that stevioside did not induce chromosome aberrations *in vivo*, in Wistar rats given 7.2 mg/kg bw stevioside/day for 60 days in the drinking water (Flores *et al.*, 1987). It was reported in an abstract that stevioside was non-genotoxic in the bone marrow of ddYb mice and in regenerating hepatocytes (Oh *et al.*, 1999). No details on the experimental design or results were provided by the authors. The potential for *Stevia* extracts to produce DNA damage has also been investigated *in vivo* in the Comet assay, again with negative results (Sekihashi *et al.*, 2002; Sasaki *et al.*, 2002). Finally, a negative result has also been obtained in *Drosophila* given 2% stevioside in the feed (Kerr *et al.*, 1983).

In contrast to these studies indicating that steviol glycosides are not genotoxic *in vivo*, a more recent study has reported Comet assay results indicative of DNA damage (Nunes *et al.*, 2007). Stevioside (purity 88.6%) was administered in the drinking water at concentrations of 0 (control) or 4000 mg/l for 45 days (approximately equivalent to 400 to 500 mg/kg bw/day) to groups of 5 male Wistar rats. In this Comet assay, increased numbers of cells, including blood, liver, brain, and spleen cells, with “tails” and statistically significantly higher total “tail” scores (measure of tail length and overall size) compared to untreated rats were recorded. The Panel notes a number of factors that limit the interpretability and utility of the study in assessment of the safety of stevioside: the use of a single dose group, the relatively small group size (N=5), no measure of the amount of water consumed by the rats (*i.e.*, no precise measure of dose), poor characterisation of the apparently manual method of cell/tail scoring (*i.e.*, no computer image analyses), no provision of representative photographs, and no

information on the cell densities in the agarose solutions used to prepare the slides). The Panel considers that this single positive study with stevioside is of little biological relevance and does not provide substantive evidence of a genotoxic potential for stevioside, given the methodological concerns and also the fact that similar findings were not seen in earlier studies in mice using stevioside of higher or lower purities (Sasaki *et al.*, 2002; Sekihashi *et al.*, 2002).

3.2.3.4. *In vivo* studies on genotoxicity of steviol

No genotoxic effect of steviol (90 to 99% purity) has been reported *in vivo* in the Swiss mouse and Wistar rat, micronucleus assays with bone marrow cells following oral treatment of a dose level up to 8000 mg/kg bw or in Syrian golden hamster bone marrow cells following oral treatment with a dose of 4000 mg/kg bw (Temcharoen *et al.*, 2000). Steviol (purity not specified) was also reported to be non-genotoxic in a ddYb mouse micronucleus assay with hepatocytes following treatment of up to 200 mg/kg bw as a single oral dose (Oh *et al.*, 1999ab). However the Panel considers this study as insufficient, as it was reported in abstract form only and no details on the experimental design or results were provided. Furthermore, no genotoxic effect of steviol (99% purity) was reported *in vivo* in a MS/Ae mouse micronucleus assay with bone marrow cells following i.p. single doses up to 1 000 mg/kg bw (Matsui *et al.*, 1996).

In the Comet assay, 3 and 24 hours after administration of steviol (>99% purity) in single oral doses up to 2000 mg/kg bw, no effects were observed on the length of DNA migration of DNA isolated from the stomach, colon, or liver cells of BDF1 mice or from the liver, kidney, colon and testes cells of CRJ mice (Sekihashi *et al.*, 2002).

Overall stevioside and rebaudioside A do not show evidence of genotoxicity *in vitro* and *in vivo*. Although a single Comet assay was reported to show effects indicative of DNA damage (Nunes *et al.*, 2007), the Panel considers that this study does not provide substantive evidence of a genotoxic potential of stevioside, given methodological concerns and the fact that similar findings were not seen in earlier studies in mice using stevioside of higher or lower purities (Sasaki *et al.*, 2002; Sekihashi *et al.*, 2002). Steviol and some of its oxidative derivatives showed clear evidence of genotoxicity *in vitro*, particularly in the presence of a metabolic activation system. However, studies of DNA damage and micronucleus formation in rats, mice and hamsters *in vivo* showed that the genotoxicity of steviol was not expressed at doses of up to 8000 mg/kg bw.

3.2.4. Chronic toxicity and carcinogenicity

No new chronic toxicity or carcinogenicity studies with steviol glycosides since the evaluation of stevioside by the SCF in 1999 were provided by the petitioners. The SCF evaluated three studies with the test material of the first two studies not complying with JECFA specifications:

- a chronic toxicity study in F344 rats by Yamada *et al.* (1985), which was negative with regard to carcinogenicity, but raised the SCF's concerns about potential adverse effects on the male reproductive system. This study was available to the SCF only as an abstract and a partial English translation of the Japanese paper and this summary information was considered by the SCF insufficient to assess if the study adequately investigated carcinogenic aspects.
- a combined chronic toxicity and carcinogenicity study in Wistar rats by Xili *et al.* (1992), which was negative with regard to carcinogenicity. However, in view of a lack of toxic effects, an inadequately described chemical composition of the test compound, and the relatively low purity and low doses of the stevioside that were used in the study, it was not possible for the SCF to evaluate if this study adequately investigated carcinogenic aspects.
- a carcinogenicity study in F344 rats by Toyoda *et al.* (1997) which was negative with regard to carcinogenicity. However, the SCF found this study inadequate to address its concerns about potential adverse effects on the male reproductive system raised by the findings in the

study by Yamada *et al.* (1985). According to the SCF, a possible treatment-related effect on the testicular system could not be evaluated in a strain of rats that normally seem to develop testicular changes.

These studies were provided by the petitioners and were reviewed by the Panel.

Groups of male and female 4-week old F344/Du Crj rats were administered 0 (control; 70 animals/sex), 0.1 (30 animals/sex), 0.3 (70 animals/sex), or 1% (70 animals/sex) *Stevia* extract (comprised of 74.54% stevioside and 16.27% rebaudioside A) in the diet up for to 2 years of age (Yamada *et al.*, 1985). The dietary concentrations in the low- and mid-dose groups were equivalent to 50 and 150 mg/kg bw/day *Stevia* extract and corresponded to approximately 20 and 60 mg steviol equivalents/kg bw/day, respectively. The dietary concentration in the high-dose group was equal to 550 mg *Stevia* extract/kg bw/day based on food consumption and body weight, and corresponded to approximately 200 mg steviol equivalents/kg bw/day. Interim sacrifices on 10 animals of each sex per group were performed at 6 and 12 months and a terminal sacrifice was performed at 22 months for males and 24 months for females. The mid-dose induced slight growth retardation in both sexes, but at the high-dose the growth was reduced only transiently. General appearance and behaviour were the same in all groups. Survival at the end of the study in the treated rats was not statistically significantly different from that in the controls. At 6 months, a variety of changes were found in the urinary, haematological and blood chemistry parameters and in organ weights but there were no such differences at 12 months or at termination. Macroscopic findings at interim and terminal sacrifices revealed a range of abnormalities considered by the Panel to be typical of pathological changes in older rats. Enlargement of the spleen in male rats was however seen only in those given the *Stevia* extract. On microscopic examination, the non-neoplastic changes seen in most cases were similar in the groups fed *Stevia* extract compared to the controls. The more common non-neoplastic changes in rats given *Stevia* extract were decreased spermatogenesis (control: 34/50, low-dose: 7/10, middle-dose: 39/50, high-dose: 37/50), interstitial cell proliferation in the testes (control: 16/50, low-dose: 5/10, middle-dose: 21/50, high-dose: 21/50), medullary cell proliferation in the adrenal glands of males, inflammatory lesions in the trachea and in the lungs, changes in the kidneys, such as degeneration of tubular epithelium, hyaline casts and glomerular sclerosis pigmentation, and increased haematopoiesis in the spleen. The Panel notes however that the increases in the incidences of these lesions were slight and without statistical significance, with no apparent dose dependence, and of common pathology in aging rats. The Panel therefore considered these changes as being of no toxicological significance. The incidences of neoplastic changes in the treated groups were similar to those in the controls. The most common neoplasms were interstitial cell adenoma of the testes and pheochromocytoma of the adrenal glands in the males, and interstitial polyps of the endometrium and adenoma of the anterior lobe of the pituitary in females (Yamada *et al.*, 1985).

The Panel notes that the NOAEL in this study was the highest dose tested of 1% in the diet equal to 550 mg *Stevia* extract/kg bw/day and corresponding to 220 mg steviol equivalents/kg bw/day. The Panel further notes that the steviol preparations in this study do not meet the specifications proposed by the petitioners for the steviol glycosides under evaluation.

Stevioside (85% purity) was administered to weanling Wistar rats (45 animals/sex/group) at concentrations of 0 (control), 0.2, 0.6, or 1.2% in the diet for a period of 24 months (Xili *et al.*, 1992). These concentrations were reported by the authors to result in respective stevioside intakes of 0, 128.5, 367.6, and 748.6 mg/kg bw/day in males, (equivalent to approximately 0, 51.4, 147.0, and 299.4 mg steviol equivalents/kg bw/day, respectively), and 0, 146.3, 416.2, and 838.9 mg stevioside/kg bw/day in females, respectively (equivalent to approximately 0, 58.5, 166.5, and 335.6 mg steviol equivalents/kg bw/day, respectively). Sporadic statistically significant differences between treatment and control groups were noted in haematology and clinical chemistry parameters, but were within historical control values and thus, considered to be toxicologically irrelevant. No statistically significant differences in relative organ weights were reported. The incidences of non-neoplastic and neoplastic lesions were unrelated to the level of the stevioside in the diet (Xili *et al.*, 1992). The Panel notes that the NOAEL is 1.2% in the diet, reported by the authors to correspond to 748.6 and 838.9

mg stevioside/kg bw/day in males and females, respectively (equivalent to approximately 300 and 336 mg steviol equivalents/kg bw/day in males and females, respectively). The Panel further notes that the steviol preparation in this study does not meet JECFA specifications for the steviol glycosides.

The only chronic toxicity and carcinogenicity study considered in the SCF evaluation where the stevioside preparation met JECFA specifications was reported by Toyoda *et al.* (1997). Stevioside (95.6% purity) was administered to F344 rats (50 animals/sex/group) at dietary concentrations of 0, (control), 2.5 and 5% for 104 weeks. These dietary doses corresponded to approximately 0, 1.25 and 2.5 g stevioside/kg bw/day. At the end of the study period, all surviving rats received the control diet for 4 weeks and were euthanised at week 108. Body weight gains were slightly depressed, in line with the dose of stevioside with mean depression rates for males and females during the study being, respectively, 2.3% and 2.4% in the 2.5% group, and 4.4% and 9.2% in the 5% group, in comparison to the control groups. No statistically significant difference in mean survival times were observed among the groups during the treatment period but the final survival rate for the 5% treated males was statistically significantly lower than that of the controls. Haematological parameters examined in week 108 did not reveal any statistically significant differences between the stevioside-treated groups and the control group in either sex. Absolute kidney weights were statistically significantly decreased in the 5% group of both sexes, while absolute left ovary weights were statistically significantly decreased, and relative brain weights were statistically significantly increased in the 5% group females compared to controls. There were no significant differences between the stevioside-treated and control groups of either sex in the incidences of any non-neoplastic lesions but the severity of chronic nephropathy was statistically significantly lower in 2.5% and 5% treated males compared to the controls. No statistically significant differences in the incidences of neoplastic changes, with the exception of a statistically significantly decreased incidence of mammary adenomas in both groups of treated females were reported compared to controls. In males, tumours of the testes were the most frequent, followed by tumours of the thyroid, adrenal glands, haematopoietic system, mammary glands and pituitary. In females, tumours of the pituitary, haematopoietic system, uterus and mammary glands were common (Toyoda *et al.*, 1997). The Panel notes that the NOAEL is 2.5% of stevioside in the diet, equal to 967 and 1120 mg stevioside/kg bw/day in males and females, respectively (corresponding to approximately 388 mg steviol equivalents/kg bw/day).

The Panel notes that the tumour occurrence observed in the three studies, of which two were performed in F344 and one in Wistar rats, was typical for these species and strains (e.g. testicular tumours in F344 rats for which spontaneous incidence is very high and variable). Since no additional cancer incidence related to the treatment was observed, overall the Panel considers that the three studies are negative with respect to carcinogenicity.

3.2.4.1. Other studies related to carcinogenicity

One of the petitioners submitted *in vitro* and *in vivo* studies investigating a modulating effect of stevioside, steviol glycoside preparations or *Stevia* extracts on tumour development in animals initiated with carcinogens, which are described briefly below.

In vitro screening studies that assessed Epstein-Barr virus activation by treatment of Raji cells with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) demonstrated no tumour promoting effects of stevioside (Okamoto *et al.*, 1983; Konoshima and Takasaki, 2002) or steviol (Okamoto *et al.*, 1983).

Rebaudioside A orally administered (20 mg/kg bw/day) (6.58 mg steviol equivalents/kg bw/day) had no effect on development of colonic aberrant crypt foci in male F344 rats initiated with azoxymethane (Kawamori *et al.*, 1995).

Stevioside at a dietary concentration of 5% (equivalent to 2 500 mg/kg bw) had no effect on urinary bladder tumour development in male F344 rats initiated with *N*-butyl-*N*-(hydroxybutyl)nitrosamine (Hagiwara *et al.*, 1984; Ito *et al.*, 1984).

Stevioside or *Stevia* extract administered topically decreased skin tumour formation in female ICR mice initiated with 7,12-dimethylbenz[a]anthracene and 12-*O*-tetradecanoylphorbol-13-acetate (Konoshima and Takasaki, 2002; Yasukawa *et al.*, 2002).

3.2.5. Reproductive and developmental toxicity

Several reproduction and developmental toxicity studies were conducted with steviol glycoside preparations meeting JECFA specifications (i.e. highly purified rebaudioside A, and purified stevioside). These studies are summarised below and listed in Appendix II.

In a preliminary reproductive dose-range finding and palatability study, a steviol glycoside preparation (>97% rebaudioside A) was administered to adult F₀ female Wistar rats (6 animals/group) from the 14th to the 21st day of lactation at concentrations of 0 (control), 25 000, 37 500, or 50 000 mg/kg diet (equivalent to 0, 1250, 1875, or 2500 mg rebaudioside A/kg bw/day). The F₁ juveniles were treated from day 14 to 21 of age. Thereafter, 10 male and female F₁ juveniles (maximum of 2/sex selected from each litter) from each dose group continued on the treatment until the 35th day post-partum, at concentrations of 0 (control), 25 000, 37 500, or 50 000 mg/kg diet (Curry *et al.*, 2008). No toxic effects were reported in the F₀ generation. In light of the specific concerns on the male reproductive system raised by the SCF (1999), the male reproductive organs were examined and no effects were reported on testicular morphology by gross and histological examination of the testes in the F₀ high-dose group. Compared to controls, the F₁ generation had statistically significantly decreased body weight gains and slightly decreased food consumption at concentrations higher than 25 000 mg/kg diet (corresponding to approximately 7978 mg steviol equivalents/kg diet) accompanied by a concomitant decrease in food consumption. The authors attributed the decreased food consumption to the palatability of the diet (Curry *et al.*, 2008).

In a subsequent 2-generation study, a steviol glycoside preparation (97% rebaudioside A) was administered via the diet to male and female Wistar rats at concentrations of 0 (control), 7500, 12 500, or 25 000 mg/kg diet for 2 generations to determine its potential reproductive and developmental effects (Curry *et al.*, 2008). Male and female F₀ rats (30 animals/sex/group) received the respective test diets for a period of 10 weeks prior to mating. Dams continued to receive the test diets for the duration of gestation and lactation. The selected F₁ generation (30 animals/sex/group) was allocated to its specific treatment group at the age of 25 days. The scheduled sacrifice of F₁ offspring not selected for parental generation and F₂ offspring was performed on post-natal day 30. No compound-related deaths or clinical signs of toxicity were reported among the F₀ or F₁ rats. Compared to controls, decreased body weights and body weight gains were reported in all generations of Wistar rats administered the preparation in the diet at concentrations of 12 500 and 25 000 mg/kg diet. However, terminal body weights of all parental animals in all test groups did not differ statistically significantly from controls. No adverse effects on reproductive function or reproductive organs (mating performance, fertility, oestrus cycles, or sperm motility, concentration, or morphology) at dietary concentrations of up to 25 000 mg/kg were reported in either the F₀ or F₁ generations. No treatment-related clinical signs were observed in the F₁ and F₂ offspring (Curry *et al.*, 2008). The NOAEL is considered by the Panel to be 25 000 mg/kg diet for reproductive performance in the F₀ and F₁ adult rats, for survival, growth, and general condition of the F₁ and F₂ offspring, and for sexual maturation of the F₂ offspring. Based on feed intake this dietary concentration corresponds to approximately 2048 and 2567 mg steviol glycoside preparation/kg bw/day for males from F₀ and F₁ generations, respectively. The mean dose levels of the steviol glycoside preparation achieved in females at this dietary concentration were 2273 mg/kg bw/day (F₀) and 2768 mg/kg bw/day (F₁) during the pre-mating period, 2322 mg/kg bw/day (F₀) and 2124 mg/kg bw/day (F₁) during gestation, and 3811 mg/kg bw/day (F₀) and 4091 mg/kg bw/day (F₁) during lactation.

In a teratology study with a steviol glycoside preparation (97% rebaudioside A), submitted by one of the petitioners, New Zealand White (NZW) rabbits (20 animals/group) were administered the test compound via oral gavage through Gestation Days (GDs) 6 to 28 in doses of 0 (vehicle control), 350,

700 and 1400 mg/kg bw/day. The animals were assessed for clinical signs, and body weight and feed consumption were recorded daily. Surviving animals were sacrificed on GD 29 and examined for numbers of corpora lutea, pregnancy, number and distribution of implantation sites and numbers of live fetuses, and early and late resorptions. All fetuses were weighed and examined for their sex and any gross external alterations. The standard fetal organs and visceral and skeletal tissues were examined for potential alterations. Owing to maternal toxicity encountered within the high-dose group, the dose administration schedule and vehicle was changed during the study in an attempt to reduce the severe decrease in food intake resulting in a decrease of the body weight gain in the 1400 mg/kg bw/day dose group. The NZW rabbits were administered daily doses of the steviol glycoside preparation in sterile water on GDs 6-14 and then twice daily in 0.5% carboxymethylcellulose to provide the same dosage level on GDs 15 to 28. Compared to controls, the body weights were statistically significantly reduced in all the steviol glycoside preparation treatment groups. The body weights of surviving animals were 96.5, 94.1, and 91.7% of the control group value on GD 29 in the low-, mid- and high-dose groups, respectively. The reductions in body weight gain were closely linked to statistically significant reductions in feed consumption. No adverse clinical signs were noted in the low- and mid- dose groups while does from the high-dose group manifested disturbed clinical appearance. The numbers of does surviving on GD 29 were 19, 20, 19, and 5 within the control, 350, 700, and 1400 mg/kg bw/day dose groups respectively. Several does were either found dead (6), aborted (3) or were sacrificed prior to scheduled termination (6) (Charles River Laboratories, 2008). The authors of the study attributed the disturbed clinical appearance and the deaths that occurred in the high-dose group to the severe reductions in food intake that resulted from gastrointestinal effects of administering high levels of the steviol glycoside preparation and not to the toxicity of the test compound. The Panel concurs with the view of the authors. Rabbits are well known to be susceptible to disturbances of the alimentary tract. Similar effects have been reported in pregnant rabbits following the administration of other intense sweeteners, sucralose (SCF, 2000) and neotame (EFSA, 2007) and the SCF (2000) and EFSA (2007) considered the maternal toxicity to be a secondary effect following the gavage administration of the sweetener. As a consequence, the developmental NOAEL for this stevioside preparation is considered by the Panel to be 1400 mg of steviol glycosides/kg bw/day (corresponding to 447 mg steviol equivalents/kg bw/day), the highest dose tested.

In a reproduction study, Wistar rats (22 animals/sex/group) were administered diets containing 0 (control), 0.15, 0.75, or 3% stevioside (95.98% purity). These dietary concentrations were equivalent to 0, 100, 480, and 2100 mg/kg bw/day (corresponding to approximately 0, 40, 192, and 840 mg steviol equivalents/kg bw/day) for males and to 0, 120, 530, and 2100 mg/kg bw/day (corresponding to approximately 0, 48, 212, or 840 mg steviol equivalents/kg bw/day) for females. Males received stevioside before and during mating for a total period of 60 days and females received stevioside for a period of 14 days before mating and for 7 days during gestation. On GD 20, pregnant females were killed and their fetuses were examined for abnormalities. No significant differences were reported in food and water consumption in both sexes of all treated groups compared with controls; however, a delayed increase in body weight in the early period of administration was reported in both sexes of the high-dose group (i.e., 3%) compared with the control group. The authors reported that stevioside treatment had no significant effects on mating performance and fertility, and external, internal, and skeletal examinations of the fetuses revealed no significant changes. The authors concluded that stevioside administration before and during the early gestation period had no adverse effect on fertility and the development of fetuses (Mori *et al.*, 1981). The NOAEL is considered by the Panel to be 3% stevioside in the diet, the highest dose tested, equivalent to 2100 mg/kg bw/day (corresponding to approximately 794 mg steviol equivalents/kg bw/day).

Female Wistar rats (21-24 animals/group) were administered stevioside (95.6% purity) at doses of 0, 250, 500, or 1000 mg/kg bw/day in drinking water between GDs 6 to 15. No adverse effects on body weight, food consumption, fetal malformation, or toxicity signs were reported for pregnant rats and fetuses (Usami *et al.*, 1995, article in Japanese, information based on English abstract and tables, Tanaka *et al.*, 1991 unpublished report). The NOAEL is considered by the Panel to be 1000 mg/kg bw/day (approximately 400 mg steviol equivalents/kg bw/day), the highest dose tested, for both pregnant rats and the fetuses.

Furthermore, the petitioners provided studies with a test material not meeting the specifications proposed by the petitioners. Different steviol glycoside preparations such as 0.69% crude *Stevia* extract (20% stevioside), 0.35% refined *Stevia* extract (40 to 55% stevioside), or 0.15% crystallised stevioside (93 to 95% purity) in the diet did not affect reproductive performance or the general condition of rats (Akashi and Yokoyama, 1975). Stevioside (90% purity) administered by gavage did not affect reproductive performance or clinical condition of three generations of Golden Syrian hamsters in doses up to 2500 mg/kg bw/day (Yodyingyud and Bunyawong, 1991) (Appendix III).

3.2.5.1. Other studies related to reproductive and developmental toxicity with steviol or *Stevia* extracts not complying with the specifications proposed by the petitioners

A study with steviol (approximately 90% purity) in female Golden Syrian hamsters (20 animals/group, doses 0, 250, 500, 750, or 1000 mg/kg bw/day by gavage on GDs 6 to 10) demonstrated that the compound at the three highest doses induced maternal toxicity (statistically significant decrease in maternal body weight gain, high percentage of mortality in the two highest doses: 7/20 = 35% and 5/12 = 45%, microscopical changes in kidneys, dilation of proximal and distal convoluted tubules in dose levels of 500 mg/kg bw/day to 1 000 mg/kg bw/day, hyaline content in tubules, desquamation of the epithelial cells of some proximal convoluted tubules at two highest dose levels) and developmental toxicity (statistically significantly increased mortality rate and statistically significantly decreased fetal weight in the three highest dose levels and statistically significantly lower number of fetuses per litter in the two highest dose levels) (Wasuntarawat *et al.*, 1998). The Panel concurs with the SCF (1999) opinion that the NOAEL for both effects was 250 mg/kg bw/day.

The potential anti-androgenic effects of an aqueous extract of *Stevia rebaudiana* leaves were examined in male Wistar rats (5 animals/group) administered 0 (control), 5, 25, or 100% of the aqueous extract of *Stevia rebaudiana* leaves (10 mL/kg), containing 2.6% (m/V) stevioside, by oral gavage for 31 days. No signs of intolerance or toxicity were observed in any of the treatment groups and no significant differences in body or absolute organ weights were noted. Upon microscopic examination of the 100% treatment group, no atrophy of the somniferous tubules was observed. Furthermore, interstitial Leydig cells were not hyperplastic and did not display any degenerative changes. No evidence of infiltrative inflammation of the testicles was observed. The authors concluded that *Stevia* leaves do not cause anti-androgenic effects (Sincholle and Marcocelles, 1989).

Several studies on the impact of crude *Stevia* extracts on reproduction in laboratory animals reported contradictory results. Some studies reported adverse reproductive effects in female mice and rats (Mazzei-Planas and Kuc, 1968; Nunes and Pereira, 1988) or on reproductive organs (Oliveira-Filho *et al.*, 1989; Melis, 1999) while others reported no adverse effects related to reproduction in rats of either sex (Shiotsu, 1996), on male reproductive organs or sperm (Sinchomi and Marcocities, 1989) or on female rat reproduction or fetal development (Saenphet *et al.*, 2006).

The Panel considers the studies with crude *Stevia* extracts of little relevance for the safety assessment of the steviol glycosides under evaluation. In these studies, the description of the chemical composition of the test extracts was sparse, the extracts were of relatively low purity and there were limitations in the study design, which altogether brings into question the adequacy of these bioassays for assessment of reproductive toxicity of the steviol glycosides under evaluation. Therefore, these studies are not further discussed.

3.2.6. Special studies on the pharmacological effects of steviol glycosides

The SCF noted in 1999 that the effects of stevioside and steviol on carbohydrate metabolism were not entirely clear and that the data reviewed by the SCF (Ishii *et al.*, 1987; SCF 1999) indicated effects on blood glucose levels and liver glycogen content. The SCF further noted that in rats there seemed to be a vasodilator effect of stevioside resulting in decreased mean arterial pressure and lowering of renal vascular resistance (Melis and Sainati, 1991a) and concluded that definitive clinical studies were

needed before a final conclusion of possible effects of stevioside on renal and cardiovascular function could be reached. Since then a number of *in vitro* and *in vivo* studies (both in humans and in laboratory animals) on the possible effects of steviol glycosides on blood glucose homeostasis, insulin secretion, clinical chemistry parameters, and on the cardiovascular and renal systems have been published and also submitted by the petitioners.

On the other hand, the Panel notes that several of these studies used non-standard protocols and non-oral routes of exposure. The Panel further notes that most of these studies may be of limited relevance to the safety assessment of steviol glycosides as the studies were conducted with preparations that do not meet JECFA specifications.

The Panel also considers that the human studies investigating such effects that have been carried out in recent years are of most relevance for the safety assessment of steviol glycosides and that studies in animals have yielded conflicting results.

For this reason, only brief summaries of the results of *in vitro* studies and *in vivo* studies in laboratory animals have been provided in the following sections, as supporting information for the interpretation of findings in the available human studies. Fuller descriptions of the *in vitro* studies are provided in Appendix V, descriptions of *in vivo* studies in laboratory animals with steviol glycosides meeting JECFA specifications are provided in Appendix VI, while Appendix VII presents the results of *in vivo* studies in laboratory animals with steviol glycosides that do not meet JECFA specifications.

3.2.6.1. Studies on glucose metabolism and insulin sensitivity

In vitro studies

Several *in vitro* studies showed that steviol glycosides can stimulate insulin secretion from isolated pancreatic islet cells, up-regulate key genes controlling insulin secretion and have effects on insulin signalling and release (Jeppesen *et al.*, 1996, 2000, 2003; Costa *et al.*, 2003a; Abudula *et al.*, 2004; Xiao and Hermansen, 2005; Xiao *et al.*, 2005; Chen *et al.*, 2006a, 2006b, 2006c; Nakamura *et al.*, 2003; Yamamoto *et al.*, 1985) as summarised in Appendix V-I.

In vivo studies in animals

A number of studies using steviol glycoside preparations (i.e. purified stevioside, or purified rebaudioside A) complying with the specifications proposed by the petitioners have shown effects on insulin sensitivity and plasma glucose levels in normal, type-2 diabetic or obese rats (Suanarunsawat and Chaiyabutr, 1997; Jeppesen *et al.*, 2003; Dyrskog *et al.*, 2005a; Dyrskog *et al.*, 2005b; Chen *et al.*, 2005; Chang *et al.*, 2005). While overall these studies have indicated that administration of steviol glycosides results in lowering of blood glucose levels, possibly by enhancing insulin secretion and regulating gluconeogenesis, the results have not been totally consistent, showing increases, decreases, or no change in blood glucose homeostasis parameters. More detailed descriptions of those *in vivo* studies using steviol glycoside preparations complying with JECFA specifications are summarised in Appendix VI, while studies on steviol glycosides that do not meet JECFA specifications (Jeppesen *et al.*, 2006a; Lailerd *et al.*, 2004; von Schmeling *et al.*, 1977; Suzuki *et al.*, 1977; Hübler *et al.*, 1994) and/or using routes of administration other than the oral route (Jeppesen *et al.*, 2002; 2003; Ma *et al.*, 2007; Raskovic *et al.*, 2004a,b, 2005; Suanarunsawat and Chaiyabutr, 1997) are summarised in Appendix VII. These studies were considered by the Panel to be a valuable supplementation to the toxicological database but not directly applicable for the safety evaluation of the steviol glycoside preparations described by the petitioners. The Panel notes that the results of studies employing non-oral routes or not describing routes of exposure are of limited relevance to the safety of orally administered steviol glycoside under evaluation.

3.2.6.2. Studies on blood pressure and cardiac function

In vitro studies

Stevioside has been the subject of several *in vitro* studies designed to evaluate possible mechanisms by which these compounds may reduce blood pressure or show vasorelaxation in *in vivo* studies (Lee *et al.*, 2001; Liu *et al.*, 2003). These studies suggest that stevioside inhibits vasoconstriction caused by vasopressin in the presence of calcium.

The Panel notes that these studies are of limited relevance for the present evaluation because stevioside administered *in vivo*, following ingestion, is hydrolysed to steviol prior to absorption. As a result, there is limited, if any, exposure of the smooth muscle cells to stevioside *in vivo*. Therefore, the *in vitro* studies of the effects of stevioside and its metabolites on vasoconstriction/vasorelaxation do not provide results that can be directly extrapolated, or interpreted, in terms of the effects of stevioside on blood pressure reported in the animal and human studies.

In vivo studies in animals

The only available oral study with stevioside which met JECFA specifications (i.e. 99.6% purity) indicated a mild antihypertensive effect in adult Goto-Kazaki (GK) rats. The Systolic Blood Pressure (SBP) and Diastolic Blood Pressure (DBP) of adult GK male rats (10 animals/group) orally administered 25 mg stevioside/kg bw/day (approximately 10 mg steviol equivalents/kg bw/day) or 16.7 mg glucose/kg bw/day via the drinking water for 6 weeks was measured weekly. Stevioside was reported to cause a progressive lowering of SBP and DBP throughout the study period. After the 6-week treatment period, the stevioside group was reported to have statistically significantly decreased SBP/DBP compared to the control. Stevioside was reported to have no statistically significant effects on body weight (Jeppesen *et al.*, 2003).

Three published studies with stevioside preparations not meeting JECFA specifications were submitted by the petitioners. These studies were reviewed by the Panel and are described in Appendix VII. In these studies stevioside was administered orally to spontaneously hypertensive (SH) rats (Hsu *et al.*, 2002; De-Yi *et al.*, 1990) or healthy mongrel dogs (Liu *et al.*, 2003). Stevioside was reported to prevent the development of hypertension in young SH rats, or to reduce blood pressure in healthy mongrel dogs. These studies were considered by the Panel to be a valuable supplementation to the toxicological database but not directly applicable for the safety evaluation of the steviol glycosides described by the petitioners.

Several studies that used i.p. or i.v. injections to examine the potential effects of stevioside on blood pressure parameters demonstrated that stevioside may decrease blood pressure values at doses between 25 and 400 mg/kg bw/day (approximately 10 and 158 mg steviol equivalents/kg bw/day) (De-Yi *et al.*, 1990; Chan *et al.*, 1998; Lee *et al.*, 2001; Hsu *et al.*, 2002; Liu *et al.*, 2003).

The Panel notes that the results of studies employing non-oral routes of exposure are of limited relevance to the safety evaluation of oral steviol glycoside preparations as steviol glycosides are not detected to any appreciable degree in the systemic circulation following oral administration.

3.2.6.3. Studies on renal function

In the past, several *in vitro* studies that assessed the potential effects of stevioside and steviol on renal function, particularly renal transport mechanisms, reported that stevioside and steviol both interfere with the transport of anions in the renal tubules (Melis and Sainati, 1991b; Melis, 1992a,b,c, 1995, 1996; Jutabha *et al.*, 2000; Toskulkao *et al.*, 1994a; Chatsudithipong and Jutabha, 2001; Chatsudithipong *et al.*, 2003; Srimaroeng *et al.*, 2005a,b). Moreover a number of *in vivo* studies using i.v. injection as a route of exposure to stevioside and *Stevia* extract were conducted to assess effects on blood pressure in relation to renal function (Chagas *et al.*, 1990; Melis, 1992a,b,c; Melis and Sainati, 1991a,b; Sainati *et al.*, 1986; Chatsudithipong and Thongouppakarn, 1995; Melis, 1995, 1996).

The Panel notes that the findings from the *in vitro* assays and *in vivo* studies by the non-oral route are not relevant to the situation in humans as stevioside was not detected in the systemic circulation following oral administration to any appreciable degree. Furthermore, steviol has been shown to be glucuronidated in humans prior to excretion in the urine and is not present in the plasma to any significant degree in its free form (Wheeler *et al.*, 2008).

One of the petitioners identified in the literature a study which elucidated the effects of a *Stevia* extract and stevioside (purity not reported) on kidney function in dogs of both sexes exposed by an oral route (Chagas *et al.*, 1990). The authors reported that both *Stevia* and stevioside were well-tolerated and displayed no treatment-related effects on kidney function in dogs with urinary osmolality above 1100 mOsm/kg water or in dogs with water overload (Chagas *et al.*, 1990). The study is described in more detail in Appendix VII-III.

The Panel notes that the results of this study are in accordance with a lack of any adverse effects on renal function of the steviol glycoside preparation (>97% rebaudioside A) in the recently published 4-week dose range finding and 90-day studies in rats (Curry and Roberts, 2008).

3.2.7. Human studies

Several studies were conducted in humans to evaluate metabolism and pharmacokinetics (see Section 3.1), the safety and tolerability of purified steviol glycosides and *Stevia* extracts on glucose homeostasis following single or repeated administrations to healthy subjects and those with type-2 *diabetes mellitus*. Many of the studies also included endpoints to assess effects on blood pressure, and some of the studies were specifically performed in hypertensive subjects.

3.2.7.1. Studies on carbohydrate metabolism

Since the purported mechanism of action for steviol glycosides, including rebaudioside A, involves enhanced secretion of insulin from the pancreas when there is impaired response to glucose stimulation, subjects with type-2 diabetes (characterised by insulin resistance) were considered appropriate to provide definitive data on the effects, if any, of steviol glycoside on glucose homeostasis.

Studies with steviol glycoside preparations meeting the specifications proposed by the petitioners are summarised below.

In a randomised, double-blind, placebo-controlled crossover trial, the glycaemic responses during meal tolerance tests to a steviol glycoside preparation (>97% rebaudioside A) consumed at three different dose levels (500 mg, 750 mg, and 1000 mg) by men and women with normal glucose tolerance (45 individuals) or with type-2 *diabetes mellitus* (48 individuals) were examined. Blood pressure responses were also investigated during the test. Incremental Areas Under the concentration Curves (IAUC) (pre-meal to 240 minutes) for glucose, insulin, C-peptide, and glucagon did not differ statistically significantly for any dose of the steviol glycoside preparation tested versus placebo or for all the steviol glycoside preparation doses combined versus placebo. No statistically significant differences between placebo and the steviol glycoside preparation treatments in the effects on postprandial SBP or DBP were observed. According to the authors, these results indicated that consumption of a single dose of up to 1 000 mg of the steviol glycoside preparation (corresponding to 319 mg steviol equivalents) does not affect glucose homeostasis and blood pressure in individuals with normal glucose tolerance or type-2 *diabetes mellitus* (Maki *et al.*, 2007). The Panel agrees with this conclusion.

To examine the effects of longer-term consumption of a steviol glycoside preparation (>97% rebaudioside A) in subjects with type-2 *diabetes mellitus*, a total of 122 male and female subjects

(from 33 to 75 years of age) were randomised to receive 4 capsules daily each containing 250 mg of the preparation, for a total dose of 1000 mg/day (approximately 330 mg steviol equivalents/day; 32 males and 28 females, mean age 59 ± 1 years) or placebo (powdered cellulose; 30 males and 32 females, mean age 61 ± 1) for 16 weeks in a double-blind clinical trial (Maki *et al.*, 2008a). Changes in glycosylated haemoglobin levels did not differ statistically significantly between the groups receiving the steviol glycoside preparation ($0.11 \pm 0.06\%$) or placebo ($0.09 \pm 0.05\%$). Changes from baseline for the group receiving the steviol glycoside preparation and placebo, respectively, in fasting glucose (7.5 ± 3.7 mg/dL and 11.2 ± 4.5 mg/dL), insulin (1.0 ± 0.64 μ U/ml and 3.3 ± 1.5 μ U/mL), and C-peptide (0.13 ± 0.09 ng/mL and 0.42 ± 0.14 ng/mL) did not differ statistically significantly. The steviol glycoside preparation was well-tolerated and the number of hypoglycaemic episodes showed no excess versus placebo. Changes in blood pressure, body weight, fasting blood lipids, haematology and urinalysis indicated no differences by treatment versus placebo. These results suggest according to the authors, that repeated use of 1 000 mg of the steviol glycoside preparation (>97% rebaudioside A) (319 mg steviol equivalents) does not alter glucose homeostasis in individuals with type-2 *diabetes mellitus* (Maki *et al.*, 2008a). The Panel agrees with this conclusion.

In an acute, paired, cross-over study, 12 subjects with type-2 *diabetes mellitus* (4 females and 8 males, with an average age of 65.8 ± 1.6 years) and a mean HbA1c of $7.4 \pm 0.4\%$ were provided capsules of 1 g of stevioside extracted from *Stevia rebaudiana* leaves (91% stevioside, 4% rebaudioside A, and 5% other derivatives) or maize starch (placebo) in a standard diet of 412 kcal. Blood samples were collected at 30 minutes prior to and up to 240 minutes after ingestion of the test meal for the evaluation of haematological and biochemical parameters. Urine samples were collected during the experiment for the determination of urine volume, excretion of albumin, glucose, sodium, and potassium. Blood pressure also was monitored during the test meal. Postprandial blood glucose levels were statistically significantly decreased by 18% after stevioside treatment compared to treatment with the placebo. The authors reported a statistically significant increase of 40% in the insulinogenic index after stevioside ingestion compared to after placebo ingestion. According to the authors ingestion of stevioside tended to decrease glucagon levels while it did not statistically significantly alter the levels of glucagon-like peptide, gastric inhibitory polypeptide, DBP or SBP, triglycerides, or free fatty acids, or excretion of urinary glucose, sodium, or potassium versus placebo. Furthermore, the authors reported that stevioside did not statistically significantly change the AUCs for glucose or glucagon response or urine output. The authors concluded that stevioside may potentiate the insulin secretion in subjects with type-2 *diabetes mellitus* (Gregersen *et al.*, 2001; 2004).

Stevioside (97% stevioside, 2.7% steviolbioside, and 0.3% rebaudioside A) in doses of 250 mg in capsules administrated 3 times per day for a total of 3 days to 9 healthy males had no effect on plasma glucose or insulin levels or on DBP or SBP compared to baseline or control values examined 30 or 60 minutes after stevioside consumption. Analysis of blood samples at 60, 180, and 420 minutes following stevioside ingestion on the 3rd day revealed no differences in Alkaline Phosphatase (ALP), Alanine Transaminase (ALT), Creatine Kinase (CK), or Lactate Dehydrogenase (LDH) levels compared to pre-treatment values. In comparison to control levels, the volume of urine collected over the 24 hours after stevioside consumption on the 3rd day was 36% greater. The difference, however, did not reach statistical significance (Temme *et al.*, 2004).

Apart from the studies mentioned above, studies with stevioside of a purity not specified and with *Stevia* extracts of unknown or not described contents of steviol glycosides were provided by the petitioners. These studies were considered by the Panel to be not directly applicable for the safety evaluation of the steviol glycoside preparations described by the petitioners, but provide valuable supplementary data. These studies were reviewed by the Panel and are summarised below.

Stevioside in daily doses of 1500 mg/person for 3 months did not affect glucose metabolism, blood lipid levels and blood pressure in 55 subjects with type-2 *diabetes mellitus* (Jeppesen *et al.*, 2006b), while treatment of healthy subjects with one daily dose of 1 g stevioside/person for 4 days was shown to increase glucose tolerance (Alvarez *et al.*, 1981).

In 25 healthy volunteers 4 doses of 200 mg of the *Stevia* extract (110 mg stevioside) consumed at 8-hour interval decreased plasma glucose levels compared to a placebo control (15 healthy volunteers). However, this treatment had no effect on other metabolic parameters measured or on cardiovascular parameters (Boeckh-Haebisch, 1992).

In 16 healthy volunteers ingestion of aqueous extract of 65 g of dry leaves of *Stevia rebaudiana* in 13 equal doses taken at 6-hour intervals decreased plasma glucose levels compared to the control group of 6 people who received placebo on the same schedule (Curi *et al.*, 1986).

A 4-week treatment (8 males and 12 females, aged 20-40 years) with an infusion from 100 g of dried *Stevia rebaudiana* Bertoni leaves, containing 6.5% stevioside (providing daily doses of stevioside ranging from 200 to 220 mg) increased blood glucose in subjects without a family history of diabetes, but not in patients with a known family history of diabetes. No changes in blood pressure were observed in any of the subjects following consumption of the *Stevia rebaudiana* infusion (Boeckh and Humboldt, 1981).

A single oral dose (level not specified) of dried aqueous extract of *Stevia rebaudiana* (glycoside composition not reported) was associated with an average reduction of 35.2% in blood glucose levels from baseline values within 6 to 8 hours in 25 healthy subjects (sex and age not stated) (Oviedo *et al.*, 1970).

3.2.7.2. Studies on renal and cardiovascular effects

Only one study with a test preparation meeting the specifications proposed by the petitioners was submitted.

The haemodynamic effects of a 4-week consumption of a 1000 mg/day steviol glycoside preparation (>97% rebaudioside A) (250 mg, 4 times daily) versus placebo was studied in a randomised double-blind trial with 100 individuals (predominantly females, age ~41 years, range 18-73 years) with normal and low-normal SBP and DBP. At baseline, mean resting, seated SBP/DBP was 110.0/70.3 mm Hg and 110.7/71.2 mm Hg for the steviol glycoside preparation and placebo groups, respectively. Compared with placebo, the steviol glycoside preparation did not statistically significantly alter resting, seated SBP, DBP, Mean Arterial Pressure (MAP), Heart Rate (HR) or 24-hour ambulatory blood pressures responses. These results indicate that consumption of as much as 1000 mg/day of the steviol glycoside preparation produced no clinically important changes in blood pressure in healthy adults with normal and low-normal blood pressure. Results in clinical chemistry, haematology, and urinalysis (prior to the start of the study and at week 4) were unremarkable and no statistically significant differences were recorded between the preparation-treated and placebo groups. The preparation was well-tolerated and side effects reported by the study participants were equally distributed between the test and placebo groups (Maki *et al.*, 2008b).

Furthermore, four other studies on renal function and cardiovascular effects with preparations not meeting JECFA specifications were submitted. These studies were considered by the Panel to be a valuable supplementation to the toxicological database but not directly applicable for the safety evaluation of the steviol glycosides described by the petitioners. These studies were reviewed by the Panel and are briefly mentioned below.

The treatment with 750 mg of a steviol glycoside mixture ($\geq 92\%$ steviol glycosides; exact composition not reported), or placebo in 3 equally divided doses for 3 months was well-tolerated and did not produce any pharmacological effects in 16 subjects with type-1 (age 20-60 years), or 30 subjects with type-2 diabetes (age 40-70 years) or in 30 non-diabetics (age 40-70 years) (Barriocanal *et al.*, 2008).

Administration of a crude steviol glycoside extract at doses up to 15 mg/kg bw/day for 24 weeks had no effect on blood pressure in patients with mild essential hypertension (12 males and 2 females divided equally between the placebo and treatment groups) (Ferri *et al.*, 2006).

After 3 months of treatment with stevioside (purity not reported, 3 times daily of 250 mg) in Chinese subjects with mild to moderate hypertension, SBP and DBP values were statistically significantly decreased in the stevioside-treated group (34 males and 26 females, age 54.1 years \pm 3.8 (SD)) in comparison to baseline values and placebo group (19 males and 27 female, age 54.1 years \pm 4.1 (SD)) values (Chan *et al.*, 2000).

After 2 years of treatment with stevioside (purity not reported, 3 times daily of 500 mg) in Chinese subjects with mild to moderate hypertension, SBP and DBP values were statistically significantly decreased in the stevioside-treated group (42 males and 40 females, age 52 years \pm 7 (SD)) in comparison to baseline values and placebo group values (42 males and 44 females, age 53 years \pm 7 (SD)) (Hsieh *et al.*, 2003).

3.2.7.3. Studies of hypolipidaemic and hepatotoxic potential of steviol glycosides

Only one study with a test preparation not meeting with JECFA specifications has been submitted. This study was considered by the Panel to be a valuable supplement to the toxicological database as the preparation was well characterised and close in its composition to the proposed specifications.

Forty-nine male and female hyperlipidaemic subjects were provided either 200 mg per day of a *Stevia* extract from *Stevia rebaudiana* leaves (70% stevioside, 20% rebaudioside A, 2% other rebaudiosides) (25 subjects total; number/sex and age not reported) or talcum capsules (24 subjects total; number/sex and age not reported) in 4 equally divided doses taken twice daily (i.e. 2 capsules taken at lunch and 2 capsules taken at supper) for 90 days in a randomised, double-blind, placebo-controlled clinical trial designed to evaluate the oral tolerability of stevioside. After 90 days of treatment, no statistically significant differences in body mass index, or serum Aspartate Transaminase (AST), ALT, Gamma-glutamyl Transferase (GGT), glucose, High Density Lipoprotein (HDL) cholesterol, Very-Low-Density Lipoprotein cholesterol (VLDL-C), or triglyceride levels were reported in either group compared to baseline values. Statistically significant decreases in total cholesterol and LDL cholesterol were observed in both the stevioside and placebo groups compared to baseline values. The authors concluded that these decreases were compatible with a modification in the subjects' lifestyles during the study. No adverse effects were reported by the authors (Cavalcante da Silva *et al.*, 2006).

3.2.8. Allergenicity of steviol glycosides

A literature search performed by the petitioners revealed a single paper concerning "Anaphylaxis by stevioside in children with atopic eczema" (Kimata, 2007). Two cases were reported: one in a 7-month old female after chewing *Stevia* leaves and the second in a 2-year old male upon drinking warm water containing stevioside powder. A reaction to *Stevia* leaves or stevioside was confirmed by a Skin Prick Test (SPT) in both cases. Both subjects suffered poorly controlled atopic eczema, were allergic to egg and the younger infant was allergic to cow's milk. The underlying presumed immunological mechanism has not been described further. Upon avoiding stevioside-containing foods, the atopic eczema significantly improved and no further acute allergic reactions were reported in both subjects. The SPT in both subjects was performed after 2 months of avoiding stevioside- and *Stevia*-containing foods. Both children had a negative skin test response to stevioside and *Stevia* leaves. The children were not orally re-exposed to *Stevia* compounds and no further immunological investigations have been reported. Following the above clinical observations, 200 male and female children between the ages of 4 months and 2 years were subjected to a SPT using *Stevia* leaves or 10% stevioside (*Stevia* extract powder; purity not reported) before and after a 2-month avoidance period of stevioside-containing foods. Of the infants, 50 were healthy without prior history of allergies, 50 had allergic rhinitis, 50 had bronchial asthma, and 50 had atopic eczema. None of the healthy children responded positively to the SPT; 26, 34 and 64% of the children with allergic rhinitis, bronchial asthma, or atopic eczema respectively were found to react to *Stevia* leaves or stevioside. Avoidance of stevioside-containing foods for 2 months decreased the reactivity to *Stevia* and stevioside in children with allergic rhinitis (4/50 to *Stevia* leaves and 3/50 to 10% stevioside), bronchial asthma (3/50 to *Stevia*

leaves and 2/50 to 10% stevioside), or atopic eczema (7/50 to *Stevia* leaves and 5/50 to 10% stevioside), respectively. Alterations of the symptoms of rhinitis, asthma or eczema after the 2-month avoidance of stevioside-containing food were not documented in the report (Kimata, 2007). Considering the high numbers of positive SPTs in these atopic patient groups, *Stevia* leaves and extracts could contain some common (cross-reacting) plant allergens of the Asteraceae family, although no reports to this effect have been identified. The Panel noted that the reduction in sensitivities after elimination of stevioside containing foods is an interesting observation but does not provide a strong indication for an IgE mediated process. Whether this effect could be due to a possible immune-stimulatory function of steviosides (Sehar *et al.*, 2008) is unresolved.

3.2.9. Other studies

3.2.9.1. Cariogenicity

Stevioside and rebaudioside A were tested for cariogenicity in albino Sprague-Dawley rats (Das *et al.*, 1992). Sixty rat pups colonised with *Streptococcus sobrinus* were divided into 4 groups and fed stevioside, rebaudioside A or sucrose added to the cariogenic diet as follows: group 1: 30% sucrose; group 2: 0.5% stevioside; group 3: 0.5% rebaudioside A and group 4 no addition. All pups received fresh cultures of *S. sobrinus* in the drinking water on days 18, 19 and 20. All 4 groups were sacrificed after 5 weeks. *S. sobrinus* counts were made on plaque samples collected from all the molars and cavities were evaluated. There were no differences in food and water intake and body weight gains between the 4 groups. Group 1 (30% sucrose) had statistically significantly higher caries score and *S. sobrinus* counts than the other three groups. There were no statistically significant differences between the stevioside, rebaudioside A and no-addition groups. This indicated that neither stevioside nor rebaudioside A are cariogenic.

3.2.9.2. Effect on monosaccharide transport

Several *in vitro* studies have examined the potential effects of steviol glycosides on monosaccharide transport (Ishii *et al.*, 1987; Toskulkao *et al.*, 1995a,b; Costa *et al.*, 2003b; Maier *et al.*, 2003). In isolated livers from male albino Wistar rats, stevioside, isosteviol, and steviolbioside were reported to significantly inhibit the transport of D-glucose and D-fructose across cell membranes (Ishii *et al.*, 1987). Using everted intestinal sacs or jejunal rings, stevioside did not have any statistically significant effects on glucose transport, whereas steviol was reported to inhibit glucose absorption at high concentrations (Toskulkao *et al.*, 1995a,b; Maier *et al.*, 2003). Furthermore, rebaudioside A was reported to inhibit insulin-stimulated glucose transport and basal glucose transport in isolated adipocytes from the periepididymal fat of rats (Costa *et al.*, 2003b).

No statistically significant differences in weight or length of the small intestine, or in activity of lactase or maltase compared to the control group was reported in groups of male Golden Syrian hamsters (12 to 15/group) administered a stevioside test material (approximately 90% purity) at doses of 0 (water control), 0.5, 1, or 2.5 g/kg bw/day (approximately 0, 0.2, 0.4, and 1 g steviol equivalents/kg bw/day, respectively) by gavage for a period of 12 weeks. A statistically significant increase in sucrase activity was reported in the mid- or high-dose stevioside-treated groups (i.e., 1 or 2.5 g stevioside/kg bw/day) compared to the control group. Furthermore, the body weights of the high-dose stevioside-treated hamsters were statistically significantly lower than those of the control animals, beginning on the first week of the study and continuing thereafter. The authors attributed the decrease in body weight gain to the observed diarrhoea at the beginning of the study, the purity of the stevioside used, the strain of animals used (as hamsters have been reported to be more sensitive to stevioside than other laboratory animals), the route of administration, and the age of the animals (Toskulkao and Sutheerawattananon, 1994b).

3.2.9.3. Effects on immunological function

At a non cytotoxic concentration (1 mM), stevioside (98% purity) extracted from dried *Stevia rebaudiana* leaves, statistically significantly suppressed the release of Tumour Necrosis Factor- α (TNF- α) and interleukin-1 β (IL-1 β) as well as slightly suppressed Nitric Oxide (NO) release by lipopolysaccharide (LPS)-stimulated human monocytic THP-1 cells (Boonkaewwan *et al.*, 2006). At lower concentrations, these effects were not observed. Furthermore, stevioside (1 mM) could directly activate unstimulated THP-1 cells to release TNF- α , IL-1 β and NO; the magnitude of induction by stevioside (1 mM) being consistently less than that of LPS (1 μ g/ml). Steviol (90% purity) had no effect in the same assay system. The authors interpreted these data as demonstrative of anti-inflammatory and immunomodulatory activities of stevioside; steviol being ineffective. Using a different cell culture system, namely human colon carcinoma cell lines (T84, Caco-2 and HT29), it was shown (Boonkaewwan *et al.*, 2008) that at non-cytotoxic, low concentrations (0.01-0.2 mM), steviol suppressed TNF-alpha induced IL-8 release in all three cell lines. The immunomodulatory effects of steviol appear to involve NF-Kappa B signalling.

The immunomodulatory effects of stevioside (purity not reported) were also investigated *in vivo* (Sehar *et al.*, 2008), in Balb/c mice of either sex (6 animals/group; number per sex not reported) orally administered (presumably by gavage) 0 (vehicle control: gum acacia), 6.25, 12.5, or 25 mg/kg bw/day of a fresh suspension of stevioside (purity not specified) for 7 days and immunised with Sheep Red Blood Cells (SRBCs) (0.2 mL of 5 x 10⁹ SRBCs/mL i.p.). Statistically significantly increased antibody synthesis, delayed type hypersensitivity response, and proliferative response in LPS-stimulated B-lymphocytes were reported following administration of 12.5 mg stevioside/kg bw/day. Statistically significant increases in the percentage of phagocytosis were reported after *in vitro* exposure of peritoneal macrophages at a concentration of 12.5 μ g stevioside/mL and *ex vivo* as well, in phagocytic cells from the blood of mice treated with 12.5mg stevioside/kg bw/day.

3.2.9.4. Effects on enzymes and mitochondria

Several *in vitro* studies have examined the potential effects of steviol glycosides on enzymes and mitochondria (Vignais *et al.*, 1966; Kelmer Bracht *et al.*, 1985; Yamamoto *et al.*, 1985; Ishii and Bracht, 1986; Constantin *et al.*, 1991; Levy *et al.*, 1994).

The Panel notes that the relevance of the effects noted in isolated mitochondria is of questionable relevance for intact systems because the steviol glycosides, stevioside and rebaudioside A do not appear to permeate cell membranes (Huxtable, 2002). Furthermore, the significance of these studies to the safety evaluation of orally dosed steviol glycosides is limited given the high concentration of stevioside and steviol in the assays. These high concentrations of stevioside are not expected to be reached *in vivo*, as stevioside would be hydrolysed to steviol prior to absorption after oral exposure. Similarly, high concentrations of steviol are not expected in plasma after oral exposure to stevioside.

3.2.10. Toxicity of related steviol glycosides and degradation products

One of the petitioners discussed the potential toxicity of related steviol glycosides and degradation products in a preparation containing not less than 97% rebaudioside A.

Several steviol glycosides have been identified in a preparation originating from the leaves of the plant *Stevia rebaudiana* Bertoni (Table 1). Other related glycosides that may occur in the final preparation as a result of the production process have been identified in small amounts (0.10 to 0.37%, w/w) by HPLC in the steviol glycoside bulk material. Some of them share the same steviol aglycone backbone structure as rebaudioside A and differ only with respect to the number of glucose units, while the remaining compounds have slight structural differences in the aglycone backbone like an endocyclic double bond, an additional hydroxyl group or an isosteviol aglycone instead of steviol.

Based on the results of the stability studies, seven identified compounds are related steviol glycosides initially identified in the steviol glycoside preparation, that increase over time under certain conditions, while 3 degradation products have not been identified in the starting bulk material by either HPLC or LC-MS/MS. Two degradation products share the same steviol aglycone backbone structure as rebaudioside A and differ only with respect to the number of glucose units, while the third degradation product possesses a hydroxyl group at C-16 and one less glucose unit than rebaudioside A.

According to the petitioner, the safety assessment of related steviol glycosides and the degradation products identified in the steviol glycoside preparation is based on their presence in the test materials used in studies conducted to assess the safety of the preparation: the 90-day study (Curry and Roberts, 2008), the 2-generation reproductive toxicity studies (Curry *et al.*, 2008), the human trials (Maki *et al.*, 2007, 2008a,b), and/or on their expected metabolic fate.

4. Discussion

The steviol glycosides considered in this opinion are sweeteners extracted from the leaves of the plant *Stevia rebaudiana* Bertoni. The steviol glycosides produced by the three petitioners are chemically defined mixtures that comprise not less than 95% of the following steviol glycosides: stevioside, rebaudiosides A, B, C, D, E and F, steviolbioside, rubusoside and dulcoside A. Stevioside and rebaudioside A are the dominant components in two out of the three preparations, whereas rebaudioside A is the major glycoside present in the third preparation. Rebaudioside B and F are present in very small percentages (around 1.5% and 0.15%, respectively).

Steviol glycosides, in the present evaluation, contain stevioside and/or rebaudioside A as main active ingredients.

Different studies conducted by one of the petitioners or available in the open literature (Chang and Cook, 1983; Kroyer, 1999) assessed the bulk stability of steviol glycosides under various storage conditions and in food matrices over a range of pH values, processing conditions, at both room temperature and elevated temperatures. The photostability of the preparation was examined under dry and aqueous conditions. The Panel notes that in these experiments the extent of degradation of the tested steviol glycoside (rebaudioside A) that occurred ranged from a few percent up to 63% under different storage (pH, temperature and period time) and food production conditions. The Panel notes that in presence of high temperatures (e.g. heating, baking), substantial degradation of steviol glycosides might take place.

The Panel evaluated oral animal studies of metabolism and toxicokinetics, animal toxicological studies and human studies with single or repeated administration of steviol glycosides.

Metabolic studies with steviol glycosides in rats (Nakayama *et al.*, 1986; Koyama *et al.*, 2003b), pigs (Geuns, 2003a) and humans (Kraemer and Mauer, 1994; Simonetti *et al.*, 2004; Geuns *et al.*, 2006, 2007a) have demonstrated that intact steviol glycosides are poorly absorbed after oral exposure but that they are hydrolysed by the microflora in the gut to steviol (Hutapea *et al.*, 1997). A large portion of this steviol is absorbed and is conjugated to steviol glucuronide which undergoes enterohepatic circulation. The remaining steviol is excreted in the faeces. In the liver, steviol undergoes conjugation with glucuronic acid to form steviol glucuronide in both humans and in rats. As demonstrated by Roberts and Renwick (2008), rebaudioside A and stevioside are metabolised and excreted by similar pathways, steviol being the common metabolite for both. Therefore, the Panel considers the results of toxicology studies on either stevioside or rebaudioside A applicable for the safety assessment of steviol glycosides in general.

The main metabolites in plasma is steviol glucuronide in humans (Geuns *et al.*, 2007) and free steviol in rats (Roberts and Renwick, 2008); no steviol epoxide, which may have genotoxic potential, was detected in human plasma (Geuns *et al.*, 2007). Consequently, in rats, the internal dose of steviol

would be higher than in humans. Steviol glucuronide is excreted primarily via the urine in humans and in rats via the bile. The differences in the route of elimination could be due to the lower molecular weight threshold for biliary excretion in rats as compared to humans. This would also result in a difference in the enterohepatic circulation between humans and rats. Indeed, in humans, there is efficient hepatic glucuronidation resulting in the presence of only steviol glucuronide in plasma and a 60% urinary excretion. On the contrary, in rats, there is probably less efficient glucuronidation and also a higher degree of enterohepatic circulation, resulting in a major faecal excretion and in measurable plasma levels of free steviol.

Despite these inter-species differences, the Panel concluded that studies in rats can be used to evaluate the safety of steviol glycosides in humans. In addition, there are extensive studies in humans, thus the safety evaluation is not primarily dependent on studies in rodents.

There is no concern with respect to the acute toxicity of steviol glycosides based on the data available from oral route exposure experiments in rats, mice and hamsters (Toskulkao, 1997).

In some of the subchronic and the 2-generation reproductive toxicity studies with rebaudioside A, body weight gains were slightly lower in the treated groups compared to the controls (Curry and Roberts, 2008; Nikiforov and Eapen, 2008; Curry *et al.*, 2008). In these studies, decreases in feed consumption and in feed conversion efficiency were also recorded. The Panel considers the effects on body weight as not adverse or indicative of toxicity but related to lower palatability and lower nutritional value of feed containing the test steviol glycosides (97% rebaudioside A). Therefore the body weight parameters are not considered appropriate endpoints for setting NOAELs for these studies. Accordingly, the Panel considers that steviol glycosides administered in the diet to rats did not produce adverse effects in subchronic studies at doses up to 4.6 g/kg bw/day. The NOAELs in these studies were the maximum doses tested.

Overall, stevioside and rebaudioside A do not show evidence of genotoxicity *in vitro* or *in vivo*. Although a single Comet assay was reported to show effects indicative of DNA damage (Nunes *et al.*, 2007), the Panel considers that this study does not provide substantive evidence of a genotoxic potential for stevioside, given methodological concerns and also the fact that similar findings were not seen in earlier studies in mice using steviosides of higher or lower purities (Sasaki *et al.* 2002; Sekihashi *et al.*, 2002). The Panel notes that steviol and some of its oxidative derivatives show clear evidence of genotoxicity *in vitro*, particularly in the presence of a metabolic activation system. However, studies of DNA damage and micronucleus formation in rats, mice and hamsters have shown that the genotoxicity of steviol is not expressed *in vivo* at doses of up to 8000 mg/kg bw (Temcharoen *et al.*, 2000). Given that the available toxicokinetic data indicate that free steviol is absent from the systemic circulation in humans or, at worst, present at very low (negligible) levels, any concern raised by the *in vitro* genotoxicity profile of steviol is fully addressed by the fact that the genotoxic potential of steviol is not expressed *in vivo*, and by the negative genotoxicity findings for steviol glycosides *in vitro* and *in vivo*.

No new chronic toxicity or carcinogenicity studies with steviol glycosides since the evaluation of stevioside by the SCF in 1999 were provided by the petitioners. The available long-term toxicity/carcinogenicity studies showed no indication of toxicity associated with prolonged high-dose dietary exposure to steviol glycosides or evidence of carcinogenic potential. The NOAEL in the 2-year carcinogenicity rat study conducted with stevioside (95.6% purity) was 2.5% stevioside in the diet, equal to 967 and 1120 mg/kg bw/day in males and females, respectively (corresponding to approximately 388 mg steviol equivalents/kg bw/day) (Toyoda *et al.*, 1997). Since negative carcinogenicity data were consistently observed in three studies in the rat (Yamada *et al.*, 1985; Xili *et al.*, 1992; Toyoda *et al.*, 1997) and steviol glycosides do not exert tumour promoting activity in various experimental models (Okamoto *et al.*, 1983, Konoshima and Takasaki, 2002; Kawamori *et al.*, 1995; Hagiwara *et al.*, 1984; Ito *et al.*, 1984; Yasukawa *et al.*, 2002) the Panel considers that there is no need to further test the potential carcinogenicity of steviol glycosides in other species (i.e. mouse).

The SCF's concerns about potential adverse effects on the male reproductive system raised by the findings in the chronic toxicity study in F344 rats with *Stevia* extract (74.54% stevioside and 16.27% rebaudioside A) (Yamada *et al.*, 1985) have been considered by the Panel to be adequately clarified by the results of later reproductive toxicity studies with test materials of known composition and high purity (Curry *et al.*, 2008; Mori *et al.*, 1981). The testicular changes in the chronic toxicity study (Yamada *et al.*, 1985) were unlikely to have been caused by steviol glycosides

The Panel notes that in the past, aqueous *Stevia rebaudiana* extracts administered orally to female mice and rats at doses up to 2000 mg/kg bw/day were reported to have contraceptive effects (Mazzei-Planas and Kuc, 1968; Nunes and Pereira, 1988) and that adverse male reproductive effects were observed in rats following administration of *Stevia rebaudiana* leaf extracts (Oliveira-Filho *et al.*, 1989; Melis, 1999). Since publication of these studies, several reproductive (multigenerational studies) and developmental (teratology) studies were conducted with stevioside and steviol glycosides. The Panel notes that the studies with steviol glycosides complying with the specifications proposed by the petitioners did not affect reproduction or the developing fetus (Curry *et al.*, 2008; Charles River Laboratories, 2008; Mori *et al.*, 1981; Usami *et al.*, 1995). Administration of stevioside (purity 90 - 96%) at doses up to 2500 mg/kg bw/day to hamsters and 2100 mg/kg bw/day (3% in the diet) to rats had no adverse effects on fertility and the development of fetuses (Yodyingyud and Bunyawong, 1991; Mori *et al.*, 1981). The 2-generation study in rats with the steviol glycosides (97% rebaudioside A) did not reveal any adverse effects at the highest dietary dose tested of 25 000 mg/kg diet, corresponding to 2048–2273 mg/kg bw/day (Curry *et al.*, 2008). Steviol glycosides (97% rebaudioside A) in doses up to 1400 mg/kg bw/day had no adverse effects on developing fetuses in NZW rabbits (Charles River Laboratories, 2008). Overall, the Panel considers that steviol glycosides complying with JECFA specifications administered orally are unlikely to have adverse reproductive and developmental effects.

Steviol, the metabolite of all the steviol glycosides has been shown to induce maternal and developmental toxicity at high doses (Wasuntarawat *et al.*, 1988). The Panel notes that any studies conducted on steviol at high doses are of little relevance to the safety assessment of the steviol glycoside preparations under evaluation. This is because steviol is absorbed immediately in the gastrointestinal tract following oral administration (see Section 3.1), but steviol glycosides are not readily absorbed in the gastrointestinal tract and are slowly hydrolysed to the aglycone steviol. The plasma levels of steviol after administration of a high-dose of steviol, therefore, would be expected to be much greater than the plasma levels of steviol following administration of a steviol glycoside.

In its evaluation of stevioside as a sweetener, the SCF (1999) expressed a concern regarding the potential effects of steviol glycosides on the renal and cardiovascular function and on carbohydrate metabolism. Several *in vitro* studies showed that steviol glycosides interfered with the transport of anions in the renal tubules (Melis and Sainati, 1991b; Melis, 1992a,b,c; 1995, 1996; Jutabha *et al.* 2000; Toskulkae *et al.*, 1994a; Chatsudithipong and Jutabha, 2001; Chatsudithipong *et al.*, 2003; Srimaroeng *et al.*, 2005a,b), inhibited vasoconstriction (Lee *et al.*, 2001; Liu *et al.*, 2003), stimulated insulin secretion from isolated pancreatic islet cells (Jeppesen *et al.*, 1996, 2000, 2003; Costa *et al.*, 2003a; Abudula *et al.*, 2004; Xiao and Hermansen, 2005; Xiao *et al.*, 2005; Chen *et al.*, 2006a,b,c), but most of these studies did not provide data that can be extrapolated to the *in vivo* situation. Also *in vivo* studies in normal, diabetic or obese rats indicated that steviol glycosides impacted blood glucose homeostasis parameters (Suanarunsawat and Chaiyabutr, 1997; Jeppesen *et al.*, 2003; Dyrskog *et al.*, 2005a; Dyrskog *et al.*, 2005b; Chen *et al.*, 2005; Chang *et al.*, 2005) and may lower blood pressure (Jeppesen *et al.*, 2003; Hsu *et al.*, 2002; De-Yi *et al.*, 1990). An *in vivo* study of potential effects on renal function from *Stevia* extract and stevioside administered orally, demonstrated that both compounds were well-tolerated and displayed no treatment-related effects on kidney function in dogs (Chagas *et al.*, 1990).

The results of human studies demonstrated that single doses of 1000 mg of rebaudioside A/person (corresponding to approximately 330 mg steviol equivalents/day) did not affect glucose homeostasis and blood pressure among individuals with normal glucose tolerance or type-2 *diabetes mellitus* (Maki

et al., 2007). Also, repeated use for 16 weeks of 1000 mg of rebaudioside A/person/day did not alter glucose homeostasis in individuals with type-2 *diabetes mellitus* (Maki *et al.*, 2008a). Blood pressure parameters were not significantly affected in individuals with normal and low SBP by oral intake of a 1 000 mg rebaudioside A/person/day for 4 weeks (Maki *et al.*, 2008b). This daily dose corresponds to 16.6 mg/kg bw/day of rebaudioside A for a person weighing 60 kg (corresponding to approximately 5.5 mg steviol equivalents/kg bw/day).

The Panel notes a report (Kimata 2007) concerning anaphylactic-like reactions associated with stevioside in children with atopic eczema. However, given the limited data provided, the significance of the results presented is unclear. In addition, the purity of the extracts used in this study is far below the required specifications for the steviol glycosides considered in this evaluation. The Panel also notes that Kimata (2007) did not provide any individual histories of food allergies for the groups of children tested for hypersensitivity to stevioside or *Stevia* leaves. The Kimata (2007) study shows that while a number of atopic children reacted positively to *Stevia* leaves or a stevioside preparation in SPTs, oral exposure to the stevioside preparation was not associated with any anaphylactic-like reaction. The petitioners claimed that steviol glycosides are not reactive and are not metabolised to reactive compounds, therefore, it is unlikely that the steviol glycosides under evaluation should cause by themselves allergic reactions when consumed in foods. In addition, *Stevia* leaves have a long history of use as a food ingredient in a number of countries with no other published reports of allergic reactions in either healthy or allergic populations. The Panel notes that this is not a strong argument as vulnerable populations presenting low frequency but still serious effects, may be missed.

Reports by Boonkaewwan *et al.* (2006, 2008) and Sehar *et al.* (2008) have concluded that stevioside may have immunostimulating effects and modulatory activities of inflammation in both *in vitro* and *in vivo* in a mouse model.

The Panel notes that these studies have some methodological limitations. The Panel considers that immunostimulating and immunomodulating effects of steviol glycosides in cell lines and rodent models have not been demonstrated in a robust and reproducible way, such that they could be used as pivotal studies for risk assessment. However, these observations deserve more in-depth examination as, if they are confirmed, they may raise concern regarding the uses of steviosides for some sub-groups of the population. This is especially the case for individuals suffering from auto-immune diseases or inflammation of the gastrointestinal tract.

The potential toxicity of related steviol glycosides and of degradation products which may be present in steviol glycosides used as a sweetener in minor amounts was not studied in laboratory animals. However, the Panel considers that these studies are not needed considering that the safety of these compounds can be extrapolated from the presence of sufficient amounts of the compounds in the test materials used in studies conducted to assess the safety of steviol glycosides complying with proposed specifications and/or having the same general structure as rebaudioside A. Therefore, under the conditions of intended use of steviol glycosides the related steviol glycosides and degradation products are not expected to be associated with any adverse effects following oral consumption of the steviol glycosides by humans.

On order to perform the exposure assessment, the Panel took into account the harmonised table of maximum proposed use levels provided by all three petitioners (Table 3) and followed the principles of the stepwise approach, which were used in the report of the Scientific Co-operation (SCOOP) Task 4.2, to estimate intakes of additives (EC, 1998). In the tiered approach, the Tier 1 is based on theoretical food consumption data and MPLs for additives as permitted by relevant Community legislation. The Second and Third tiers refer to assessment at the level of individual Member States, combining national data on food consumption with the MPLs for the additive (Tier 2) and its actual usage patterns (Tier 3). As no MPLs exist for steviol glycosides, only Tier 1 for the Budget method and Tier 2 using maximum proposed use levels for both exposure estimates have been made by the Panel. The Panel calculated a theoretical maximum daily exposure of 37.1 mg/kg bw/day for an adult and a 3 year-old child.

Refined exposure estimates have been performed for Tier 2 using maximum proposed use levels from the three petitioners with individual food consumption data for child and adult populations. Exposure estimates for children (aged 1-10 years; except for Cyprus: 12-14 years old) have been performed by the Panel using the detailed individual food consumption data from 12 European countries (Belgium, France, the Netherlands, Spain, Czech Republic, Italy, Finland, Germany, Greece, Cyprus, Sweden, UK).

Since the UK population is considered to be one of the highest consumers of soft drinks in Europe and as estimates were calculated from more refined adult food consumption data than those currently available to EFSA Panels (e.g. EFSA Concise European Food Consumption Database, which gives aggregate food categories consumed in 19 European countries (EFSA, 2008) it was decided to select the UK population as representative of the EU consumers for the steviol glycosides estimates for adults.

When considering maximum proposed use levels (Tier 2), the mean dietary exposure of European children (aged 1-14 years) ranged from 0.7 to 7.2 mg/kg bw/day, and from 3.3 to 17.2 mg/kg bw/day at the 95th percentile. The main contributors (>10% in all countries) to the total anticipated exposure to steviol glycosides, expressed as steviol equivalents, are soft drinks (11 to 58%) and desserts, including flavoured milk products (14 to 71%). Confectionery accounted for 11% of exposure in 2 countries. Dried potato granules and flakes and candied fruits and vegetables, mostardo di frutta accounted for 17 and 18% of exposure in one country.

Estimates reported for the UK adult population give a mean dietary exposure to steviol glycosides (as steviol equivalents) of 2.2-2.7 mg/kg bw/day and of 8.0-9.7 mg/kg bw/day for high level consumers (97.5th percentile). The main contributors to the total anticipated exposure to steviol glycosides (as steviol equivalents) (>10%) are soft drinks (37%) and beer, cider and perry (33%).

The Panel compared its exposure assessment to that derived from Renwick (2008) which represent predicted exposure data to steviol equivalents for European consumers based on the observed exposure data for aspartame. Renwick's (2008) calculations assumed a relative sweetness potency to sucrose of 180 for aspartame and 200 for rebaudioside A and divided by a factor of three to obtain steviol equivalents. The observed exposure data for aspartame were derived from national individual intake surveys which included diabetics (Denmark, France, Germany, Netherlands and UK).

The mean dietary exposure of children (aged 1-14 years) including diabetics, to steviol glycosides expressed as steviol equivalents, ranged from 0.4 to 1.3 mg/kg bw/day, and from 1.5 to 4.2 mg/kg bw/day at the high percentile (90th/97.5th). For adults including diabetics, the mean dietary exposure to steviol glycosides expressed as steviol equivalents ranged from 0.3 to 0.7 mg/kg bw/day, and from 1.5 to 3.1 mg/kg bw/day at the high percentile (90th/97.5th).

The Panel notes that its estimates are three times higher than those based on Renwick (2008) because the Panel assumed that all processed foods and beverages contain the sweetener steviol glycosides added at the maximum proposed use levels (i.e. not only energy-reduced beverages as proposed by the petitioners). But the Panel notes that the exposure data from Renwick uses older consumption figures, a conversion factor of three for calculation of predicted exposure to steviol glycosides expressed as steviol equivalents and that the food category "Milk and milk derivative-based or fruit juice-based drinks, energy-reduced or with no added sugar", which is one of the main contributors to the overall exposure in the assessment made by the Panel, has higher proposed use levels (1000 mg steviol glycosides/kg instead of 600 mg aspartame/kg).

Despite the limitations of the present assessment, the Panel notes that its exposure assessment presents a good geographical spread of the food consumption of children in Europe, a standardised approach in food categorisation (by using applications as stated in Directive 94/36/EC) and methodology to calculate anticipated dietary exposure assessments and concludes that its estimates are considered to

be the most up to date anticipated dietary exposure to steviol glycosides (expressed as steviol equivalents).

CONCLUSIONS

There are extensive *in vitro* and *in vivo* animal studies and some human tolerance studies on the steviol glycosides rebaudioside A and stevioside and their metabolite steviol. After considering all the data available, the Panel concludes that steviol glycosides covered by the proposed specifications are not carcinogenic, genotoxic or associated with any reproductive/developmental toxicity. The Panel considers a carcinogenicity study with stevioside of high purity meeting the specifications proposed by the petitioners, as pivotal for the present evaluation. The NOAEL in this study was 2.5% stevioside in the diet, equal to 967 mg stevioside/kg bw/day (approximately 388 mg steviol equivalents/kg bw/day), the highest dose tested.

Studies in humans demonstrated that daily doses of the steviol glycosides up to 1000 mg/person/day, equivalent to 16.6 mg/kg bw/day for a 60 kg person (corresponding to approximately 330 mg steviol equivalents/person/day or to 5.5 mg steviol equivalents/kg bw/day) were well-tolerated by individuals with normal glucose metabolism or type-2 *diabetes mellitus*.

The Panel establishes an ADI for steviol glycosides, expressed as steviol equivalents, of 4 mg/kg bw/day based on application of a 100-fold uncertainty factor to the NOAEL for stevioside of 2.5% in the diet, equal to 967 mg stevioside/kg bw/day (approximately 388 mg steviol equivalents/kg bw/day), from a 2-year carcinogenicity study in the rat.

Conservative estimates of steviol glycosides exposure both in adults and in children suggest that it is likely that the ADI would be exceeded at the maximum proposed use levels.

DOCUMENTATION PROVIDED TO EFSA

1. Technical dossier 2007a. Dossier for registration of Steviol Glycosides (*Stevia*) for use as a commercial food additive. January 2007. Submitted by Morita Kagaku Kogyo Co., Ltd, Japan. Last dossier update: January 2009. Additional data submitted in January 2009, March 2009.
2. Technical dossier 2007b. Dossier for the safety evaluation of Eustas *Stevia* Glycosides for use as a food additive. September 2007. Submitted by Eustas, Barbastro, Huesca, Spain. Last dossier update: February 2009. Additional data submitted in March 2009
3. Technical dossier 2008. Dossier for the safety evaluation of Cargill's Steviol Glycoside preparation for use as a sweetener. May 2008. Submitted by Cargill, Incorporated, Wayzata, USA. November 2009.
4. Steviol Glycoside EU Food Additive Petition Roadmap. Section II and III, Technical and safety data. April 2009. Submitted by Cantox Health Sciences International, Ontario, Canada.

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APPENDICES

APPENDIX I: ACUTE ORAL TOXICITY STUDIES WITH STEVIOL, STEVIOL GLYCOSIDES OR STEVIA EXTRACT(S) NOT COMPLYING WITH THE JECFA SPECIFICATIONS.

Study	Dose	Reference
Stevioside in mice and rats.	LD ₅₀ = 15g/kg bw in mice; 8.2 g/kg in rats	Mitsubishi, 1981
Stevioside, RebA, Reb B and steviolbioside in mice.	2 g/kg bw LD ₅₀ ≥ 2 g/kg bw	Medon <i>et al.</i> , 1982
<i>Stevia</i> extract (20% stevioside) in mice.	LD ₅₀ = 17 g/kg bw	Mitsubishi, 1981
<i>Stevia</i> extracts (20.4% stevioside - 41.4% stevioside) in mice and rats.	LD ₅₀ = 17g/kg bw to > 42 g/kg bw in mice; LD ₅₀ = 17 g/kg bw in rats	Akashi and Yokoyama, 1975; Lee <i>et al.</i> , 1979
Steviol (90%)	LD ₅₀ values of >15 g/kg bw in mice and rats; 5-6 g/kg bw in hamsters	Toskulkao <i>et al.</i> , 1997
Isosteviol to mice, rats or dogs.	LD ₅₀ ≥ 500 mg/kg bw	Bazotte <i>et al.</i> , 1986

APPENDIX II: CORE TOXICOLOGICAL STUDIES IN LABORATORY ANIMALS WITH STEVIOL GLYCOSIDES MEETING THE JECFA SPECIFICATIONS.

Study	Dose	NOAEL	Reported Effects	Reference
Short-term and subchronic				
Dose-range finding study (4 weeks) in rats with steviol glycoside preparation (97% RebA)	0, 12 500, 25 000, 50 000 or 100 000 mg/kg diet (0, 2367, 4842, 7143, and 9938 mg/kg bw/day for males, and 0, 2616, 5 422, 8190, and 11 728 mg/kg bw/day for females)	100 000 mg/kg diet (equal to 9938 and 11 728 mg/kg bw/day for males and females, respectively)	<ul style="list-style-type: none"> ▶ bw gain (only beginning of the studies) ▶ feed intake (only at the beginning of the studies) <p><u>Highest dose NOAEL</u></p>	Curry and Roberts, 2008
13-week study in rats with steviol glycoside preparation (97% RebA)	0, 12 500, 25 000 or 50 000 mg/kg diet ([0, 1506, 3040, and 5828 mg/kg bw/day in week 1 and 0, 698, 1473, and 3147 mg/kg bw/day in week 13 for males, and 0, 1410, 2841, and 5512 mg/kg bw/day in week 1 and 0, 980, 1914, and 3704 mg/kg bw/day in week 13 for females)	50 000 mg/kg diet (equal to 4161 and 4645 mg/kg bw/day for males and females, respectively)	<ul style="list-style-type: none"> ▶ bw gain (at the two highest doses) ▶ feed intake (only at the beginning of the studies) ▶ feed conversion efficiency (at the high-dose in male) <p><u>Highest dose NOAEL</u></p>	Curry and Roberts, 2008
13-week study in rats with steviol glycoside preparation (97% RebA)	0, 500, 1000, or 2000 mg/kg bw/day	2000 mg/kg bw/day	<p>NSD</p> <p><u>Highest dose NOAEL</u></p>	Nikiforov and Eapen, 2008
13-week study in rats with stevioside (95.6%)	0, 155, 310, 625, 1250, and 2500 mg/kg bw/day (equal to 0, 0.31, 0.62, 1.25, 2.5, or 5%)	2500 mg/kg bw/day	<ul style="list-style-type: none"> ▶ terminal body weight (at the two highest doses) ▶ ↑ urea nitrogen and lactate dehydrogenase (in male) <p><u>Highest dose NOAEL</u></p>	Aze <i>et al.</i> , 1991
Chronic and carcinogenicity				
104-week study in rats with stevioside (95.6%)	0, 969, and 1997 mg/kg bw/day in males; 0, 1120, and 2387 mg/kg bw/day in females (0, 2.5, 5%)	967 and 1120 mg/kg bw/day in males and females, respectively (2.5%)	<ul style="list-style-type: none"> ▶ ↓ final survival rate in males at the highest dose ▶ ↓ bw males and females at the highest dose ▶ ↓ absolute kidney weights at the highest dose 	Toyoda <i>et al.</i> , 1997

			<ul style="list-style-type: none"> ▶ ↓ absolute left ovary weights at the highest dose ▶ ↑ relative brain weights in female at the highest dose 	
Reproductive and developmental toxicity				
Preliminary reproductive study in rats with steviol glycoside preparation (RebA ≥ 97%); F ₀ females from 14 th to 21 st day of lactation, F ₁ juveniles from day 14 until day 35 of age.	0, 25 000, 37 500, or 50 000 mg/kg diet (0, 4711, 8021, 9484 mg/kg bw/day during the first 4 days, or 0, 6291, 10 045, 11 386 mg/kg bw/day during day17 to 20 of lactation		<ul style="list-style-type: none"> ▶ ↓ body weight gains and food consumption at conc higher than 25000 mg/Kg in F₁ generation 	Curry <i>et al.</i> , 2008
Two-generation repro/devep study in rats with steviol glycosides preparation (RebA ≥ 97%). F ₀ males: 17 weeks F ₀ females: Pre-mating, 10 weeks; Mating, up to 3 weeks; Gestation, days 1 to 20 after conception; Lactation, days 1 to 21 after parturition (total of approximately 20 weeks)	0, 7500, 12 500, or 25 000 mg/kg diet (0, 586, 975, 2048 mg/kg bw/day, males); 0, 669, 1115, 2273 mg/kg bw/day, pre-mating females; 0, 648-713, 1119-1169, 2263-2381 mg/kg bw/day, gestation; and 0, 715-1379, 1204-2388, and 2602-5019, lactation.	25 000 mg/kg diet (2 048 and 2 567 mg /kg bw/day for males from F ₀ and F ₁ ; 2273 mg/kg bw/day (F ₀) and 2768 mg/kg bw/day (F ₁) during the pre-mating , 2322 mg/kg bw/day (F ₀) and 2124 mg/kg bw/day (F ₁) during the gestation, and 3811 mg/kg bw/day (F ₀) and 4091 mg/kg bw/day (F ₁) during the lactation, for females.	<ul style="list-style-type: none"> ▶ NSD <u>Highest dose NOAEL</u> (for reproductive performance in the F₀ and F₁ adult rats, for survival, growth, and general condition of the F₁ and F₂ offspring, and for sexual maturation of the F₂ offspring). 	Curry <i>et al.</i> , 2008
Teratology study in rabbits with steviol glycosides preparation (RebA ≥ 97%) from day 6 to 28 of gestation.	0 , 350, 700 and 1400 mg/kg bw/day	1400 mg/kg/day	<ul style="list-style-type: none"> ▶ ↓ body weight and food consumption ▶ disturbed clinical appearance and death in the high-dose group. <u>Highest dose NOAEL</u> (rabbits are susceptible to disturbances of the alimentary tract). 	Charles Rivers Laboratories, 2008
Fertility study in rats with stevioside (95.98%) before and during mating for a	0, 100, 480, 2100 mg/kg bw/day for males, and 0, 120, 530, 2100 mg/kg bw/day for females (0, 0.15, 0.75, or 3%)	2100 mg/kg bw/day (3%)	<ul style="list-style-type: none"> ▶ delayed increase in body weight in early period of administration in highest dose. 	Mori <i>et al.</i> , 1981

total period of 60 days (males); for 14 days before mating and 7 days during gestation (females).			<u>Highest dose NOAEL</u> (based on fertility and development of fetuses).	
Repro./Develp. toxicity study in rats with stevioside (95.6%) between day 6 to 15 of gestation.	0, 250, 500, or 1000 mg/kg bw/day	1000 mg/kg bw/day	► NSD <u>Highest dose NOAEL</u> (for both pregnant rats and fetuses).	Usami <i>et al.</i> , 1995; Tanaka <i>et al.</i> , 1991 (unpublished report)

↓ = decreased; ↑ = increased; NOAEL = no observed adverse effect level; NR = not reported; NSD = no significant differences

APPENDIX III: TOXICOLOGICAL STUDIES IN LABORATORY ANIMALS WITH STEVIOL GLYCOSIDES OR *STEVIA* EXTRACT(S) NOT COMPLYING WITH THE JECFA SPECIFICATIONS, AND STEVIOL ADMINISTERED EITHER ORALLY OR BY NON-ORAL ROUTE

Study	Dose	NOAEL	Reported Effects	Reference
Short-term and subchronic				
90-day study in rats with stevioside (85%)	0, 1500, or 2500 mg/kg bw/day (0, 3, or 5%)	2500 mg/kg bw/day (5%)	► NSD <u>Highest dose NOAEL</u>	Xili <i>et al.</i> , 1992
1-month study in rats (by gavage) with stevioside (purity not stated)	Up to 2500 mg/kg bw/day	2500 mg/kg bw/day	► NSD <u>Highest dose NOAEL</u>	Mitsuashi, 1981
13-week study in rats with <i>Stevia</i> extract (53.1% stevioside)	0, 112, 590, and 2988 mg/kg bw/day in male, or 0, 115, 629, and 3026 mg/kg bw/day in female (0, 0.28, 1.4, or 7%)	2988 mg/kg bw/day for males, and 3036 mg/kg bw/day for females (7%)	► NSD <u>Highest dose NOAEL</u>	Akashi and Yokoyama, 1975
56-day study in rats with <i>Stevia</i> extract (50% stevioside)	0, 1250, or 2500 mg/kg bw/day	2500 mg/kg bw/day	► NSD <u>Highest dose NOAEL</u>	Lee <i>et al.</i> , 1979
Chicken fed with <i>Stevia</i> extract (content NR) from 1 st day to 42 nd day of age	0.0425 or 0.085%	NR	► ↓ body weight (with the lowest conc of 0.0085%)	Wood <i>et al.</i> , 1996
15-day in rabbits (non-oral routes) with stevioside	2.22 g (total)	NR	► NSD	Pomaret and Lavieille, 1931
Chronic and carcinogenicity				
2-year study in rats with <i>Stevia</i> extract (74% stevioside and 16.27% Reb A)	0, 50, 150, and 550 mg/kg bw/day (0, 0.1, 0.3, 1%)	550 mg/kg bw/day (1%)	► Changes in biochemical parameters (urine, blood) and organ weights (at 6 months)	Yamada <i>et al.</i> , 1985

			<ul style="list-style-type: none"> ▶ Enlargement of spleen in male treated- rats. ▶ Microscopic and macroscopic changes observed in all groups. ▶ Non-neoplastic changes similar to the controls. <p><u>Highest dose NOAEL</u></p>	
2-year study in rats with stevioside (85%)	0, 128.5, 367.6, and 748.6 mg/kg bw/day in males and 0, 146.3, 416.2, and 838.9 mg/kg bw/day in females (0, 0.2, 0.6, or 1.2%)	748.6 and 838.9 mg/kg bw/day in males and females, respectively (1.2%)	<ul style="list-style-type: none"> ▶ <i>NSD</i> <p><u>Highest dose NOAEL</u></p>	<i>Xili et al., 1992</i>
Other studies related to carcinogenicity				
<i>In vitro</i> study with stevioside or steviol in Epstein-Barr virus	NR		<ul style="list-style-type: none"> ▶ No tumor promoting effect 	<i>Okamoto et al., 1983; Konoshima and Takasaki, 2002</i>
34-week study in rats with stevioside	0-2500 mg/kg bw/day (5%)		<ul style="list-style-type: none"> ▶ No effects on urinary bladder tumours in male. 	<i>Hagiwara et al., 1984; Ito et al., 1984</i>
Stevioside or <i>Stevia</i> extract administrated topically in mice	0-68 µg		<ul style="list-style-type: none"> ▶ ↓ skin tumour formation 	<i>Konoshima and Takasaki, 2002; Yasukawa et al., 2002</i>
Reproductive and developmental toxicity				
Repro./develop. study 0.69% crude <i>Stevia</i> extract (20% stevioside), 0.35%, refined <i>Stevia</i> extract (40 to 55% stevioside), or 0.15% crystallized stevioside (93 to 95% purity) in rats for 21 days prior to mating	83.4, 80.0, or 84.9 mg/kg bw/day of stevioside for male rats, and 96.2, 101.7, or 101.2 mg/kg bw/day of stevioside for female rats, respectively	NR	<ul style="list-style-type: none"> ▶ <i>NSD</i> 	<i>Akashi and Yokoyama, 1975</i>
3-generation study in hamster with	0, 500, 1000, or 2500 mg/kg	2500 mg/kg bw/day	<ul style="list-style-type: none"> ▶ <i>NSD</i> 	<i>Yodyingyuad and</i>

stevioside (90%)	bw/day		<u>Highest dose NOAEL</u> (regard to growth and reproduction)	Bunyawong, 1991
Developmental toxicity study in zebrafish with stevioside, rebaudioside A, rubusoside, steviol monoside, and steviol glucuronide.	Up to 200 µM	NR	► NSD, except for steviol: developmental delays, pericardial edema, circulatory defects and lethality at the highest conc.	Crawford <i>et al.</i> , 2008
Developmental toxicity study in hamster with steviol (90%) on days 6 to 10 of gestation	0, 250, 500, 750, or 1000 mg/kg bw/day	250 mg/kg bw/day (for both maternal and developmental toxicity)	<ul style="list-style-type: none"> ► ↑ mortality rate ► ↓ maternal body weight ► ↑ pathological effect in kidneys (dilation and hyaline formation of convoluted tubules) ► ↓ foetal weight and numbers of live foetuses 	Wasuntarawat <i>et al.</i> , 1998
Aqueous <i>S. rebaudiana</i> in mice and rats	up to 2000 mg/kg bw	NR	► ↓ fertility (contraceptive effects)	Mazzei-Planas and Kuc, 1968; Nunes and Pereira, 1988
<i>Stevia</i> extract rats before mating (males and females) and during mating (females)	0-5000 mg/kg bw/day	NR	► NSD	Shiotsu (1996)
<i>Stevia</i> extracts (= 2.6 % stevioside) in rats for 31 days.	10 mL extract/kg bw/day (0, 5, 25 and 100 %) (equal to 0, 13, 65 and 260 mg/kg bw/day of stevioside)	NR	► NSD	Sinchomi and Marcorities (1989)
Aqueous <i>S. rebaudiana</i> in rats for 60 days	0-0.667 g dried leaves/mL, 2 mL/rat twice a day (2.668 g of dry <i>Stevia</i> leaves per day; 14.58 g dried leaves/kg bw/day)	NR	► ↓ absolute and relative seminal vesicle weights	Oliveira-Filho <i>et al.</i> , 1989
<i>Stevia</i> extracts in rats for 60 days	0-6.778 mg/kg bw/day	NR	<ul style="list-style-type: none"> ► ↓ final weight of testes, seminal vesicle and cauda epididymidis ► ↓ fructose content of the accessory sex glands, the epididymal sperm concentration, and testosterone level 	Melis, 1999
<i>Stevia Rebaudiana</i> in combination with an aqueous extract of Aegle marmelos (6%) in rats for 60 days	0, 8.5, 41, and 433 mg <i>Stevia</i> extract/kg bw/day (0, 0.2, 1, or 10%)	433 mg <i>Stevia</i> extract/kg bw/day	NSD	Saenphet <i>et al.</i> , 2006

prior mating			
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↓ = decreased; ↑ = increased; NOAEL = no observed adverse effect level; NR = not reported; NSD = no significant differences

APPENDIX IV: GENOTOXICITY STUDIES

Table 1: List of *in vitro* genotoxicity studies of steviol glycosides, *Stevia* extract, steviol, isosteviol and steviol metabolites

Endpoint	Test object	Test material (purity)	Conc/dose	Results	Reference
Forward mutation	<i>S. typhimurium</i> TM677	Rebaudioside A (NS).	0.1-10.0 mg/plate ^a	Negative	Pezzuto <i>et al.</i> (1985). Purity of materials tested appears likely to be high, from description in publication
Forward mutation	<i>S. typhimurium</i> TM677	Rebaudioside A	NS ^a	Negative	Medon <i>et al.</i> (1982)
Reverse mutation	<i>S. typhimurium</i> TA98, TA100	Stevioside (99%)	12.5-50 mg/ plate ^a	Negative ^b	Suttajit <i>et al.</i> (1993)
Reverse mutation	<i>S. typhimurium</i> TA 1535, 1537, 97, 98, 100, 102, 104 <i>E. coli</i> WP2uvrA/pkM10	Stevioside (83%)	0.05-5 mg/plate ^c 0.05-1 mg/plate ^d	Negative	Matsui <i>et al.</i> (1996)
Reverse mutation	<i>S. typhimurium</i> TA98, TA100	Stevioside (96%)	12.5-50 mg/plate ^a	Negative	Klongpanichpak <i>et al.</i> (1997)
Reverse mutation	<i>S. typhimurium</i> TA98, TA100, TA 1535, 1537, TA 1538, WP2	Stevioside (18%)	0.01-10 mg/plate ^a	Negative	Okumura <i>et al.</i> 1978
Forward mutation	<i>S. typhimurium</i> TM677	Rebaudioside B (NS)	0.1-10.0 mg/mL ^a	Negative	Pezzuto <i>et al.</i> (1985)
Forward mutation	<i>S. typhimurium</i> TM677	Rebaudioside B	NS ^a	Negative	Medon <i>et al.</i> (1982)
Forward mutation	<i>S. typhimurium</i> TM677	Rebaudioside C (NS)	0.1-10.0 mg/mL ^a	Negative	Pezzuto <i>et al.</i> (1985)
Forward mutation	<i>S. typhimurium</i> TM677	Rebaudioside C	NS ^a	Negative	Medon <i>et al.</i> (1982)
Forward	<i>S. typhimurium</i>	Dulcoside A (NS)	0.1-10.0 mg/mL ^a	Negative	Pezzuto <i>et al.</i> (1985)

mutation	TM677				
Forward mutation	<i>S. typhimurium</i> TM677	Dulcoside C	NS ^a	Negative	Medon <i>et al.</i> (1982)
Forward mutation	<i>S. typhimurium</i> TM677	Steviolbioside (NS)	0.1-10.0 mg/mL ^a	Negative	Pezzuto <i>et al.</i> (1985)
Forward mutation	<i>S. typhimurium</i> TM677	Steviolbioside C	NS ^a	Negative	Medon <i>et al.</i> (1982)
Gene mutation	<i>Bacillus subtilis</i> H17 rec+, M45 rec-	Stevioside (83%)	10 mg/disc ^a	Negative	Matsui <i>et al.</i> (1996)
Gene mutation	<i>B. subtilis</i> H17 rec+, M45 rec-	Stevioside (18, 55, >95%)	0.02-2 mg/disc ^c	Negative	Okumura <i>et al.</i> (1978)
Gene mutation	Mouse lymphoma L5178Y Tk+/- locus	Stevioside (96.8%)	1.25-5mg/mL ^a	Negative	Oh <i>et al.</i> (1999a,b)
Chromosome aberrations	Chinese hamster lung fibroblasts (CHL/IU)	Rebaudioside A (NS)	1.25-5 mg/mL ^a	Negative	Nakajima, (2000a)
Chromosome aberrations	Chinese hamster lung fibroblasts (CHL/IU)	Stevioside (85%)	12 mg/mL ^c	Negative	Ishidate <i>et al.</i> (1984)
Chromosome aberrations	Chinese hamster lung fibroblasts (CHL/IU)	Stevioside (83%)	2-12 mg/mL ^a	Negative	Matsui <i>et al.</i> (1996)
Chromosome aberration	Chinese hamster D-6 cells	Stevioside (NS)	2-4% ^e (20-40 mg/mL) ^f	Positive	Nadamitsu <i>et al.</i> (1985)
Chromosome aberration	Human lymphocytes	Stevioside (NS)	10 mg/mL	Negative	Suttajit <i>et al.</i> (1993)
Chromosome aberration	Human lymphocytes	Stevioside (NS)	0.008-8 µg/mL ^e	Negative	Höhn and Zankl, (1990)
Sister chromatid exchange	Human lymphocytes	Stevioside (NS)	7.5 µg/mL	Negative	Flores <i>et al.</i> (1987)
Sister chromatid exchange	Human lymphocytes	Stevioside (NS)	0.008-8 µg/mL ^e	Negative	Höhn and Zankl, (1990)
Micronucleus formation (<i>in vitro</i>)	Human lymphocytes	Stevioside (NS)	0.008-8 µg/mL ^e	Positive	Höhn and Zankl, (1990)
Micronucleus formation (<i>in vitro</i>)	Human buccal mucosa	<i>Stevia extract</i> – <i>Stevia sweet</i>	NS	Positive	Höhn and Zankl, (1990)

<i>vitro</i>)					
Reverse mutation	<i>S. typhimurium</i> TA98, TA100	Steviol (NS)	20 mg/plate ^a	Negative	Suttajit <i>et al.</i> (1993)
Reverse mutation	<i>S. typhimurium</i> , TA97, TA98, TA100, TA102, TA104	Steviol (99%)	0.05-5 mg/plate ^a	Negative	Matsui <i>et al.</i> (1996)
Reverse mutation <i>Escherichia coli</i>	<i>S. typhimurium</i> TA1535, TA1537, WP2, uvrA/pKM101	Steviol (99%)	0.05-5 mg/plate ^c	Negative	Matsui <i>et al.</i> (1996)
Reverse mutation	<i>S. typhimurium</i> , TA98, TA100	Steviol (NS)	0.25-2 mg/plate ^a	Negative	Klongpanichpak <i>et al.</i> (1997)
Forward mutation	<i>S. typhimurium</i> TM677	Steviol (99%)	0.1-10 mg/mL ^c	Negative	Matsui <i>et al.</i> (1996)
			0.1-10 mg/mL ^{d,h}	Positive	
Forward mutation	<i>S. typhimurium</i> , TM677	Steviol (99%)	10 mg/plate ^g	Positive	Pezzuto <i>et al.</i> (1986)
Forward mutation	<i>S. typhimurium</i>	Steviol (NS)	0.1-10 mg/mL ^c	Negative	Pezzuto <i>et al.</i>
			0.1-10 mg/mL ^{d,h}	Positive	
Forward mutation	<i>S. typhimurium</i> , TM677	Steviol (NS)	NS ^d	Positive	Terai <i>et al.</i> (2002)
<i>umu</i> gene mutation	<i>S. typhimurium</i> TA1535/pSK1002	Steviol (99%)	625-1,250 µg/plate ^a	Weakly positive	Matsui <i>et al.</i> (1996)
Gene mutation	<i>Bacillus subtilis</i> H17 rec+, M45 rec-	Steviol (99%)	10 mg/disc ^a	Negative	Matsui <i>et al.</i> (1996)
Gene mutation	Chinese hamster lung fibroblasts	Steviol (99%)	0.4 mg/mL ^d	Positive f	Matsui <i>et al.</i> (1996)
Gene mutation	Mouse lymphoma L5178Y Tk+/- locus	Steviol (NS)	340 µg/mL ^a	Negative	Oh <i>et al.</i> (1999a,b)
Chromosomal aberration	Chinese hamster lung fibroblasts	Steviol (99%)	0.125-5 mg/mL ^c	Negative	Matsui <i>et al.</i> (1996)
			0.5 mg/mL ^d	Positive	
			1.0-1.5 mg/mL ^d		
Chromosomal aberration	Human lymphocytes	Steviol (NS)	0.1-0.2 mg/mL ^a	Negative	Suttajit <i>et al.</i> (1993)
DNA damage (Comet assay)	Human lymphoblastoid TK6 and WTK1 cells	Steviol (NS)	62.5-500 µg/mL ^a	Negative	Sekihashi <i>et al.</i> (2002)
Forward	<i>S. typhimurium</i> , TM677	Isosteviol (NS)	1.0-10 mg/mL ^a	Negative	Pezzuto <i>et al.</i> (1985)

mutation					
Forward mutation	<i>S. typhimurium</i> , TM677	Isosteviol (NS)	NS ^a	Negative	Pezzuto <i>et al.</i> (1983)
Forward mutation	<i>S. typhimurium</i> , TM677	Dihydrosteviol A (NS)	1.0-10 mg/mL ^a	Negative	Pezzuto <i>et al.</i> (1985)
Forward mutation	<i>S. typhimurium</i> , TM677	Dihydrosteviol B (NS)	1.0-10 mg/mL ^a	Negative	Pezzuto <i>et al.</i> (1985)
Forward mutation	<i>S. typhimurium</i> , TM677	Steviol-16 α ,17-epoxide (NS)	1.0-10 mg/mL ^a	Negative	Pezzuto <i>et al.</i> (1985)
Forward mutation	<i>S. typhimurium</i> , TM677	Steviol-16 α ,17-epoxide (NS)	NS ^g	Positive	Terai <i>et al.</i> (2002)
Forward mutation	<i>S. typhimurium</i> , TM677	Steviol-16 β ,17 β -epoxide, trans (NS)	NS ^j	Positive	Pezzuto <i>et al.</i> (1983)
Forward mutation	<i>S. typhimurium</i> , TM677	Steviol-16 β ,17 β -epoxide, cis (NS)	NS ^k	Negative	Pezzuto <i>et al.</i> (1983)
Forward mutation	<i>S. typhimurium</i> , TM677	15 α -hydroxysteviol (NS)	0.31-7.5 mg/mL ^d	Negative	Compadre <i>et al.</i> (1988)
Forward mutation	<i>S. typhimurium</i> , TM677	15 α -hydroxysteviol (NS)	0.31-7.5 mg/mL ^a	Negative	Pezzuto <i>et al.</i> (1986)
Forward mutation	<i>S. typhimurium</i> , TM677	15 α -hydroxysteviol (NS)	NS ^a	Negative	Terai <i>et al.</i> (2002)
Forward mutation	<i>S. typhimurium</i> , TM677	15-oxosteviol (NS)	25-200 μ g/mL ^c	Positive	Compadre <i>et al.</i> (1988)
Forward mutation	<i>S. typhimurium</i> , TM677	15-oxosteviol (NS)	25-200 μ g/mL ^j	Positive	Pezzuto <i>et al.</i> (1986)
Forward mutation	<i>S. typhimurium</i> , TM677	15-oxosteviol (NS)	25-200 μ g/mL ^c	Negative	Procinska <i>et al.</i> (1991)
Forward mutation	<i>S. typhimurium</i> , TM677	15-oxosteviol (NS)	NS ^d	Weakly Positive	Terai <i>et al.</i> (2002)
Forward mutation	<i>S. typhimurium</i> , TM677	Steviol methylester (NS)	NS ^d	Positive	Terai <i>et al.</i> (2002)
Forward mutation	<i>S. typhimurium</i> , TM677	16-oxo-steviol methylester (NS)	NS ^a	Positive	Terai <i>et al.</i> (2002)
Forward mutation	<i>S. typhimurium</i> , TM677	13,16-seco-13-oxo-steviol methylester (NS)	NS ^d	Positive	Terai <i>et al.</i> (2002)

Forward mutation	<i>S. typhimurium</i> , TM677	13,16-seco-13 α -hydroxy-steviol methylester (NS)	NS ^a	Positive	Terai <i>et al.</i> (2002)
Forward mutation	<i>S. typhimurium</i> , TM677	Steviol methylester-8, 13-lactone (NS)	NS ^a	Positive ^l	Terai <i>et al.</i> (2002)
Forward mutation	<i>S. typhimurium</i> , TM677	<i>ent-Kaurenoic acid</i> (NS)	0.625-15 mg/mL ^d	Negative	Pezzuto <i>et al.</i> (1986)
Forward mutation	<i>S. typhimurium</i> , TM677	Steviol acetate (NS)	0.625-15 mg/mL ^d	Negative	Pezzuto <i>et al.</i> (1986)

NS = not specified

^a With and without metabolic activation.

^b A positive response in TA98 without metabolic activation at 50 mg/plate was reported, but not at lower concentrations of up to 20 mg/plate.

^c Without metabolic activation.

^d With metabolic activation.

^e Inadequate detail provided.

^f Based on an assumed density of 1 g/mL.

^g With metabolic activation derived from human liver microsomes.

^h Exhibited a positive dose-response relationship.

ⁱ Diphtheria toxin-resistant colonies.

^j Authors described test material as a direct-acting mutagen, presumed that metabolic activation was not present.

^k Inadequate detail provided.

^l The presence of metabolic activation decreased the mutagenicity.

Table 2: List of *in vivo* genotoxicity studies of steviol glycosides, *Stevia* extract and steviol

Endpoint	Test object	Test material (purity)	Conc/dose	Results	Reference
Micronucleus formation	Male BDF1 mouse bone marrow	Rebaudioside A (NS)	500-2000 mg/kg bw once daily for 2 days by gavage a	Negative	Nakajima, (2000b)
Mutation	<i>Drosophila melanogaster</i> Muller 5 strain	Stevioside (NS)	2% in feed	Negative	Kerr <i>et al.</i> (1983)
Chromosome aberration	Wistar rat bone marrow cells	Stevioside (NS)	7.2 mg/kg bw/day for 60 days in the drinking water	Negative	Flores <i>et al.</i> (1987)
DNA damage (Comet assay)	Male Wistar rat blood cells, liver, brain, spleen cells	Stevioside (88.6%)	4mg/L in drinking water ad libitum for 45 days (~400 to 500 mg/kg bw/day)	Positive	Nunes <i>et al.</i> (2007)
Micronucleus formation	ddYb mouse bone marrow and regenerating liver cells ^c	Stevioside (96.8%)	62.5-250 mg/kg bw as a single oral administration d	Negative	Oh <i>et al.</i> (1999a,b)
Micronucleus formation	Wistar rat ^e	Stevioside (NS)	150 mg/kg bw in the drinking water for 60 days	Negative	Flores <i>et al.</i> (1987)
DNA damage (Comet assay)	Male Wistar rat	Stevioside (88.6%)	4 mg/mL orally in the drinking water (appr.400 to 500 mg/kg bw/day) for 45 days	Positive	Nunes <i>et al.</i> , 2007
DNA damage (Comet assay)	Male BDF1 mouse stomach, liver, colon, kidney, bladder, lung, brain, and bone marrow cells	<i>Stevia extract</i> (stevioside, 52%, rebaudioside A, 22%)	250, 500, 1000 or 2000 mg/kg bw as a single dose by gavage	Negative	Sekihashi <i>et al.</i> (2002)
DNA damage (Comet assay)	Male ddY mouse stomach, colon, liver, kidney, bladder, lung, brain, bone marrow cells	<i>Stevia</i> (NS)	2000 mg/kg bw as a single oral dosed	Negative	Sasaki <i>et al.</i> (2002)

DNA damage (Comet assay) Male CRJ:CD1	Male BDF1 mouse stomach, colon, liver cells; Male CRJ:CD1 mouse liver, kidney, colon, and testes cells	Steviol (>99%)	250-2000 mg/kg bw as a single oral dose ^{g,h}	Negative	Sekihashi <i>et al.</i> (2002)
Micronucleus formation	MS/Ae mice	Steviol (99%)	125-1000 mg/kg bw as a single intraperitoneal injection ⁱ	Negative	Matsui <i>et al.</i> (1996)
Micronucleus formation	Swiss mouse bone marrow cells	Steviol (approximately 90%)	8000 mg/kg bw as a single dose by gavage ^j	Negative	Temcharoen <i>et al.</i> (2000)
Micronucleus formation	ddY mousee regenerating liver cells	Steviol (NS)	50-200 mg/kg bw as a single oral dose ^h	Negative	Oh <i>et al.</i> (1999a,b)
Micronucleus formation	Wistar rat bone marrow cells	Steviol (approximately 90%)	8000 mg/kg bw as a single dose by gavage ^j	Negative	Temcharoen <i>et al.</i> (2000)
Micronucleus formation	Syrian golden hamster bone marrow cells	Steviol (approximately 90%)	4000 mg/kg bw as a single dose by gavage ^j	Negative	Temcharoen <i>et al.</i> (2000)

NS = not specified

^a Killed 30 hours after second administration.

^b From English abstract; ICR mice described in study.

^c Animals killed 24 hours after administration.

^d Route not specified

^e Animals killed 48, 72, and 96 hours after administration.

^f Animals killed 3 and 24 hours after administration.

^g Killed at 3 hours and 24 hours.

^h Exact route not specified.

ⁱ 4/6 mice at highest dose given intraperitoneally died.

^j Killed at 24, 30, 48, or 72 hours. Ratio of polychromatic to normochromatic erythrocytes was decreased at later time-point(s) in females.

APPENDIX V: SUMMARIES OF SPECIAL STUDIES *IN VITRO* WITH STEVIOL GLYCOSIDES

APPENDIX V-I: *IN VITRO* STUDIES ON GLUCOSE METABOLISM AND INSULIN SENSITIVITY

Stevioside and rebaudioside A (at millimolar concentrations) and steviol (at micromolar concentrations) stimulated insulin secretion from isolated pancreatic islet cells (Jeppesen *et al.*, 1996, 2000, 2003; Costa *et al.*, 2003a; Abudula *et al.*, 2004; Xiao and Hermansen, 2005; Xiao *et al.*, 2005; Chen *et al.*, 2006a).

Stevioside and rebaudioside A appeared to have minimal effects on basal insulin secretion (Chen *et al.*, 2006a, b, c), but greater activity with respect to glucose-stimulated insulin secretion (Chen *et al.*, 2006b, c).

Several studies indicated that up-regulation of acetyl-CoA carboxylase-1 (ACC-1) activity (Chen *et al.*, 2007), modulation of the expression of gene sequences controlling insulin secretion (Chen *et al.*, 2006c), modulation of the phosphorylation of insulin receptor substrates (i.e., insulin signalling) (Nakamura *et al.*, 2003), and counteraction of the desensitisation of β -cells to glucose-stimulated insulin release (Chen *et al.*, 2006b,c) may in part explain the effects of stevioside, rebaudioside A, and steviol, on insulin secretion.

An *in vitro* study designed to assess the effects of steviol glycosides and steviol on gluconeogenesis demonstrated that steviol and isosteviol inhibited glucose production or oxygen from L-lactate, pyruvate, fructose, glycerol, and succinate; however, stevioside or steviolbioside had no effect on glucose production or oxygen uptake (Yamamoto *et al.*, 1985).

APPENDIX V-II: *IN VITRO* STUDIES ON BLOOD PRESSURE AND CARDIAC FUNCTION

Stevioside has been the subject of several *in vitro* studies designed to evaluate possible mechanisms by which these compounds may reduce blood pressure or show vasorelaxation in *in vivo* studies (Lee *et al.*, 2001; Liu *et al.*, 2003). These studies assessed the effects of stevioside on intracellular calcium concentrations in rat thoracic aorta preparations following treatment with vasopressin and/or phenylephrine. Inter alia they have demonstrated that stevioside inhibits vasoconstriction caused by vasopressin in the presence of calcium.

APPENDIX V-III: *IN VITRO* STUDIES ON RENAL FUNCTION

In the past several *in vitro* studies that assessed potential stevioside and steviol potential effects on renal function, particularly renal transport mechanisms, have reported that stevioside and steviol both interfere with the transport of anions in the renal tubules (Melis and Sainati, 1991b; Melis, 1992a, b, c; 1995, 1996; Jutabha *et al.*, 2000; Toskulkao *et al.*, 1994a; Chatsusdithipong and Jutabha, 2001; Chatsudthipong *et al.*, 2003; Srimaroeng *et al.*, 2005a, b).

APPENDIX VI: SUMMARIES OF SPECIAL STUDIES IN ANIMALS WITH STEVIOL GLYCOSIDES MEETING JECFA SPECIFICATIONS

APPENDIX VI-I: *IN VIVO* STUDIES ON GLUCOSE METABOLISM AND INSULIN SENSITIVITY

In order to determine the potential effects of stevioside on blood glucose levels, a group of Wistar rats (N=8) were orally administered distilled water or 13.33 mL stevioside (95% purity)/kg bw (approximately 2000 mg stevioside/kg bw or 800 mg steviol equivalents/kg bw/day) twice over 2 consecutive 45-minute periods. Six hours after administration, plasma glucose levels were measured.

No statistically significant effects on blood glucose levels were observed after oral administration of stevioside as compared to the control group (Suanarunsawat and Chaiyabutr, 1997).

Potential antihyperglycaemic and blood pressure lowering effects of stevioside (99% purity) were studied in the diabetic rat (following long-term administration, as well as acute hypoglycaemic effects in a fasting state). Stevioside was administered to male Goto-Kazaki (GK) (N=20) rats (non-obese model of type-2 diabetes) via drinking water at a dose of 25 mg/kg bw/day for a period of 6 weeks (approximately 10 mg steviol equivalents/kg bw/day). Control rats (N=10) received the same molar amount of glucose (16.7 mg/kg bw/day). During week 6, glucose (2.0 g/kg bw) was administered via an intra-arterial catheter and blood samples were collected at different times up to 3 hours. Stevioside significantly suppressed the rise in plasma glucose and decreased the incremental area under the glucose response-curve. Plasma insulin levels measured over the first 30 minutes were significantly increased in the stevioside group compared to the control group; however, the difference in insulin response did not persist over the entire observation period. A concomitant decrease in plasma glucagon also occurred in stevioside-treated rats during the first 30 minutes (Jeppesen *et al.*, 2003).

Rebaudioside A (97.8% purity) (25 mg/kg bw/day or 8 mg steviol equivalents/kg bw/day) was administered in a standard laboratory animal chow twice daily to 11-week old male GK rats (12 animals/group) for 8 weeks. After 7 weeks of treatment, the animals were equipped with an intravascular catheter for an Intra-Arterial Glucose Tolerance Test (IAGTT) to be conducted at the end of the 8-week treatment period. Throughout the study, no statistically significant differences in plasma insulin and glucagon levels, food consumption, or body weights were reported between the treated and control rats. Plasma glucose levels were significantly decreased in the rebaudioside A group at weeks 2, 3, and 5; however, the overall AUC (0-9 weeks) for plasma glucose in the rebaudioside A group was not significantly different from the control group. Furthermore, no significant differences in free fatty acid, triglyceride, or total cholesterol levels were reported between the treated and control groups at the end of the treatment period. In response to the glucose challenge during the intra-arterial glucose test, no significant differences in plasma glucose, insulin, or glucagon levels were reported between the test and control animals (Dyrskog *et al.*, 2005a).

In order to study the combination effects of stevioside (91% stevioside, 4% rebaudioside A, 5% other glycosides) and a soy-supplement on glycaemic control and blood pressure, during a 10 week period male obese ZDF rats (8-week old, N=12/group) received respective diets ad libitum: Group A standard rodent chow; Group B standard rodent chow + 0.03 g/kg bw/day stevioside; Group C 50% standard rodent chow + 50% soy protein; and Group D, 50% rodent chow + 50% soy protein + 0.03 g/kg bw/day stevioside. Throughout the study period, no significant differences among groups were reported for fasting plasma glucose, insulin, or glucagon levels. After glucose infusion, plasma glucose levels were lower in Groups B and D in comparison to Groups A and C; however, these differences were not statistically significant. Plasma insulin and glucagon levels were not significantly different in Groups B and D compared to Groups A and C during the IAGTT. In comparison to Groups A and C, blood pressure in Groups B and D was significantly decreased 2 weeks after stevioside treatment was initiated. All groups exhibited comparable Stevioside did not have any independent effects on plasma lipid levels/body weights. (Dyrskog *et al.*, 2005b).

A series of studies to evaluate the potential effects of stevioside (99% purity) on glucose and insulin parameters were conducted in normal male Wistar rats, streptozotocin (STZ)-induced Insulin Dependent Diabetes Mellitus (IDDM) Wistar rats, and Non-Insulin Dependent Diabetes Mellitus (NIDDM) Wistar rats (induced by feeding rats with 60% fructose) (Chen *et al.*, 2005).

Groups of normal fasting Wistar rats and STZ-induced diabetic rats (N=10/group) were administered 0 (vehicle control), 0.5, 1, or 5 mg stevioside/kg bw twice by gavage (approximately 0, 0.2, 0.4, and 2 mg steviol equivalents/kg bw, respectively). Blood glucose and insulin levels (normal rats only) were measured at 60, 90, and 120 minutes after dosing. In the normal rats, stevioside decreased blood glucose levels and increased blood insulin levels in a dose-dependent manner, with levels being significantly different from control values at all dose levels and all time points evaluated. Likewise in

the STZ-induced IDDM rats, blood glucose levels were significantly decreased in comparison to vehicle-treated controls at all time points in a dose-dependent manner (Chen *et al.*, 2005).

In a second experiment, STZ-induced IDDM rats and NIDDM rats (10 animals/group) were administered 0 (vehicle control), 0.5, 1, or 5 mg stevioside/kg bw twice daily (0, 1, 2, and 10 mg/kg bw/day, respectively or approximately 0, 0.4, 0.8, and 4 mg steviol equivalents/kg bw/day, respectively) by gavage for a period of 15 days. In the STZ-induced IDDM rats, all doses of stevioside significantly decreased blood glucose levels within one day in a dose-dependent manner compared to the respective vehicle controls. Blood glucose levels continued to decline over the 15-day period in a dose-dependent fashion, such that by the end of the treatment period blood glucose levels in high-dose animals were approximately 20% lower compared to baseline. In the NIDDM rats, significant reductions in blood glucose levels compared to the vehicle control-treated group were observed beginning on day 1 at the highest dose levels of stevioside and on day 5 in all other stevioside-treated groups of NIDDM rats. After 15 days, the high-dose groups exhibited blood glucose levels that were approximately 52% lower than at baseline. Additionally, insulin resistance was verified in the NIDDM rats by i.p injection of tolbutamide (10 mg/kg bw) to all groups of rats concurrently with the gavage administrations of either the vehicle or stevioside. The plasma glucose lowering activity of tolbutamide was lowest in the control group and increased significantly in a dose-dependent manner when provided together with stevioside (Chen *et al.*, 2005).

In a third experiment, Wistar rats (N=10/group) were administered single gavage doses of 0 (vehicle control), 0.5, 1, or 5 mg stevioside/kg bw (approximately 0, 0.2, 0.4, and 2 mg steviol equivalents/kg bw, respectively) and subjected to a glucose tolerance test by injecting 0.5 g glucose/kg bw into the tail vein 60 minutes following stevioside administration. Blood glucose levels were measured 5, 10, 20, 30, 60, 90, and 120 minutes after glucose injection. In all groups, blood glucose levels peaked 5 minutes after glucose injection; however, glucose levels were significantly lower in all stevioside groups compared to the control group, and the reductions were dose-dependent. Blood glucose levels began to approach normal values 30 minutes after glucose injection, but continued to be significantly lower in the high-dose stevioside group up to 90 minutes after glucose injection (Chen *et al.*, 2005).

In a final investigation, the potential effects of stevioside on phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting enzyme for gluconeogenesis, also were evaluated in STZ rats (10 animals/group) administered stevioside by gavage twice daily at dose levels of 0 (vehicle control), 0.5, 1, or 5 mg/kg bw for 15 days (approximately 0, 0.4, 0.8, and 4 mg steviol equivalents/kg bw/day, respectively). Stevioside was reported to significantly decrease PEPCK mRNA expression and PEPCK protein levels relative to control values. Based on the results of these experiments, the authors concluded that stevioside may regulate blood glucose levels by enhancing insulin secretion and regulating gluconeogenesis through its effects on PEPCK gene expression (Chen *et al.*, 2005).

The effects of stevioside on insulin sensitivity were studied in male Wistar rats. Insulin-resistance was induced in groups of 8 male Wistar rats by providing fructose-rich chow (60% fructose) for a period of 4 weeks. Subsequently, rats were treated orally with a stevioside test material (98.6% purity) at doses of 0 (saline control), 0.5, 1, or 5 mg/kg bw (route of administration not specified, but presumed gavage based on use of vehicle; approximately 0, 0.2, 0.4, and 2 mg steviol equivalents/kg bw, respectively) for determination of plasma glucose levels at 60, 90, and 120 minutes. The plasma glucose levels of the treated groups were reported to decrease in a time-dependent manner and were significantly lower than those of the vehicle control at all time-points. Subsequently, rats were subjected to an i.p glucose tolerance test (1 g glucose/kg bw) 90 minutes after treatment with stevioside at the previously mentioned doses. An additional group of standard chow-fed rats treated with the vehicle was also included. Plasma glucose and insulin levels were recorded 0, 30, 60, 90, and 120 minutes after glucose injection. In comparison to the fructose-chow-fed vehicle control group, plasma glucose at baseline in the high-dose (i.e., 5 mg/kg bw) stevioside group and standard-chow groups and baseline insulin levels in the mid- (i.e., 1 mg/kg bw) and high-dose stevioside groups were significantly decreased. At all times after the glucose injection, plasma glucose levels for the standard chow and for the mid- and high-dose stevioside groups were significantly lower compared to the fructose-chow-fed vehicle

control group. Likewise, plasma insulin levels were significantly lower in all stevioside-treated groups and in the standard chow group compared to the fructose-chow-fed vehicle control group. The glucose insulin index and the AUCs for plasma glucose and insulin levels were reported to be significantly decreased in all stevioside-treated groups and in the standard chow group compared to the fructose-chow-fed vehicle control group. In a further experiment, additional groups of rats adapted to a fructose-chow diet received daily 3 oral administrations of 0 (vehicle control) or 5 mg/kg bw of a stevioside-containing test material (equivalent to 6 mg steviol equivalents/kg bw/day) for a period of 28 days. Another group of rats was provided standard chow and was treated with the vehicle. At baseline, body weights and fasting plasma glucose and insulin levels of rats fed the fructose-chow diets were significantly greater than those of the standard chow fed rats; however, following the 28-day stevioside treatment period, body weights, plasma glucose, and plasma levels of the fructose-chow diet rats control were markedly lower than their baseline values (day 0). The reduction in plasma glucose levels attained statistical significance. The plasma glucose lowering ability of a single dose of tolbutamide (10 mg/kg bw) in rats provided in standard chow, high-fructose-chow, or high-fructose chow providing 15 mg stevioside/kg bw/day for 28 days was also examined. The ability of tolbutamide to decrease plasma glucose was demonstrated as early as 3 days in rats fed the high-fructose diet compared to the normal diet treatment. On day 6, the reduction was statistically significant, and on the last day of treatment the plasma glucose lowering ability of tolbutamide was reduced to 5.4% (compared to approximately 33% at baseline). In rats treated with stevioside, the glucose lowering activity of tolbutamide was retained for the first 9 days, with statistically significant reductions compared to the standard chow-fed rats occurring from day 12 onward; however, on the last day of treatment the glucose-lowering activity of tolbutamide remained at 15% (Chang *et al.*, 2005).

APPENDIX VI-II: *IN VIVO* STUDIES ON BLOOD PRESSURE AND CARDIAC FUNCTION

The available oral study shows that stevioside may have a mild antihypertensive effect in adult adult GK rat. The Systolic Blood Pressure (SBP) and Diastolic Blood Pressure (DBP) of adult GK male rats (10 animals/group) orally administered 25 mg stevioside/kg bw/day (99.6% purity; approximately 10 mg steviol equivalents/kg bw/day) or 16.7 mg glucose/kg bw/day via the drinking water for 6 weeks was measured weekly. Stevioside was reported to cause a progressive lowering of SBP and DBP throughout the study period. After the 6-week treatment period, the stevioside group was reported to have significantly decreased SBP/DBP compared to the control. Stevioside was reported to have no significant effects on body weight (Jeppesen *et al.*, 2003).

APPENDIX VII: SUMMARIES OF SPECIAL STUDIES IN ANIMALS WITH STEVIOL GLYCOSIDE NOT MEETING JECFA SPECIFICATIONS, OR ADMINISTERED BY ROUTES OTHER THAN THE ORAL ROUTE

APPENDIX VII-I: *IN VIVO* STUDIES ON GLUCOSE METABOLISM AND INSULIN SENSITIVITY

The combined effects of stevioside (Wako Pure Chemical Industries; purity not reported) and a soy-supplement on hyperglycaemia, hypertension, dyslipidaemia, and weight gain were studied in male GK rats (12 animals/group) fed different diets ad libitum for up to 5 weeks: Group A, standard high carbohydrate rodent chow; Group B, chow + 0.03 g/kg bw/day stevioside; Group C, 20% chow + 80% soy protein isolate; and Group D, 20% chow + 80% soy protein isolate + 0.03 g/kg bw/day stevioside. After the intra-arterial glucose challenge, the IAUC₀₋₂₄₀ minutes for glucose was significantly decreased by 24 and 46% in Groups B and D respectively compared to Groups A and C, respectively. Stevioside significantly increased the IAUC (0-30 minutes) for first-phase plasma insulin 72 (Group A vs. Group B) and 166% (Group C vs. Group D); however, stevioside had no effect on the IAUC (0-240 minutes) for plasma insulin. Furthermore, stevioside significantly decreased the AUC (0-240 minutes) plasma glucagon by 22 and 33% (Group A vs. Group B and Group C vs. Group D, respectively). After 5 weeks of dietary treatment, stevioside significantly reduced total plasma

cholesterol by 8 and 14% and plasma triglycerides by 31 and 24%, respectively (Group A vs. Group B and Group C vs. Group D, respectively). After 4 weeks of dietary treatment, stevioside significantly decreased systolic blood pressure by 15 and 10% (Group A vs. Group B and Group C vs. Group D, respectively). At the end of the study, body weights were comparable in all groups of rats. The authors noted further that stevioside and soy protein isolate had positive synergistic effects on hyperglycaemia, hypertension, dyslipidaemia, and weight gain (Jeppesen *et al.*, 2006a).

Female lean (insulin sensitive) and obese (insulin resistant) Zucker rats (N=5/group) were administered either stevioside (200 or 500 mg/kg bw; purity not reported) or a vehicle by gavage for an Oral Glucose Tolerance Test (OGTT) to determine insulin and blood glucose response in skeletal muscle. Two hours after the administration of 500 mg stevioside/kg bw, the fasting plasma levels of glucose, insulin, and free fatty acids were reported not to be significantly different from the lean and obese control groups. Treatment with 200 mg stevioside/kg bw was also reported not to significantly alter the plasma glucose and insulin responses during the OGTT in either lean or obese rats. No effects of stevioside (500 mg/kg bw) in comparison to the vehicle controls were noted following treatment of fasted rats. During the OGTT, in lean Zucker rats, stevioside (500 mg/kg bw) had no significant effect on plasma glucose levels, but was reported to significantly reduce insulin levels 15 and 30 minutes after glucose administration in similarly treated vehicle controls. Furthermore, the total and incremental AUCs for insulin and the glucose-insulin index (calculated as the product of the respective glucose and insulin AUCs and is inversely related to whole-body insulin sensitivity) were reported to be significantly decreased compared to the controls. In obese rats, stevioside (500 mg/kg bw) was reported to significantly decrease blood glucose levels 30 minutes after glucose exposure, but no significant effects on insulin levels were reported in comparison to the vehicle controls. The total and incremental AUC for glucose and the glucose-insulin index, as calculated using the incremental AUCs, were reported to be significantly decreased compared to the control group. The authors concluded that acute oral stevioside dosing increased whole-body insulin sensitivity as evidenced by decreased insulin levels in lean rats and decreased blood glucose levels in obese rats (Lailerd *et al.*, 2004).

A 0.5% aqueous extract of *Stevia Rebaudiana* leaves (composition not reported) was administered to 7-month old chinchilla rabbits (sex not reported) to determine the hypoglycaemic effects of *Stevia Rebaudiana*. The rabbits were randomised into 3 groups (N=6/group) as follows: Group 1, alloxan-induced diabetic rabbits administered a single dose of 12 mg *Stevia rebaudiana* extract/kg bw by gavage; Group 2, non-diabetic rabbits administered a single dose of 12 mg *Stevia rebaudiana* extract/kg bw by gavage; and Group 3, non-diabetic rabbits administered water by gavage (control). No significant difference in the plasma glucose levels between Group 2 and Group 3 were observed; however, Group 1 had significantly higher plasma glucose levels in comparison to Group 2. The maximal decrease in plasma glucose levels in Group 1 was observed 60 minutes after *Stevia rebaudiana* administration (von Schmeling *et al.*, 1977).

In order to study the effects of stevioside (purity not reported) extracted from *Stevia rebaudiana* leaves on blood glucose and liver glycogen levels, male rats (number/group and strain not identified) were administered either 0.1% stevioside (approximately 100 mg stevioside/kg bw/day) in a high-carbohydrate or high-fat diet, or 10% powdered *Stevia rebaudiana* leaves (approximately 10 g/kg bw/day) in a high-carbohydrate diet for up to 4 weeks. Control groups were fed high-carbohydrate diet or high-fat diet. The *Stevia rebaudiana* leaves were reported to contain 0.5% stevioside. As such, 10% of *Stevia rebaudiana* leaves in the diet provided approximately 50 mg stevioside/kg bw/day. Among animals fed 0.1% stevioside in the high-carbohydrate or high-fat diets, no significant differences in food efficiency, relative liver, thyroid, or adrenal weights were reported. Animals fed 0.1% stevioside in the high-carbohydrate diet had significantly lower absolute and relative liver glycogen content, but no significant changes in blood glucose levels compared to animals fed a high-carbohydrate diet alone. In comparison to animals fed a high-fat diet alone, no significant changes in absolute or relative liver glycogen content or blood glucose levels were reported among animals fed 0.1% stevioside in a high-fat diet. Likewise, no significant changes in food efficiency, relative liver, thyroid, or adrenal weights were observed among animals administered 10% *Stevia rebaudiana* leaves in a high carbohydrate diet

after 2 weeks of treatment compared to the respective control group (the high-carbohydrate diet group). After 4 weeks of treatment, these results were largely unchanged with the exception of a significant increase in relative thyroid gland weight compared to the control group. Furthermore, after 2 weeks of treatment, absolute liver glycogen content was significantly decreased in animals administered 10% *Stevia rebaudiana* leaves, with no significant changes in relative liver glycogen content or blood glucose levels as compared to the high-carbohydrate diet group. After 4 weeks of treatment, however, relative and absolute liver glycogen content and blood glucose levels were significantly decreased compared to animals fed a high-carbohydrate diet alone (Suzuki *et al.*, 1977).

The influence of stevioside (purity not reported) on the hepatic glycogen levels was studied in fasted male Wistar rats (6 to 9 animals/group). The animals received orally 2 mL of equimolar (200 µmol) fructose (approximately 167.6 mg/kg bw, based on body weight of 215 g), stevioside (approximately 748.7 mg/kg bw, based on body weight of 215 g), steviol (approximately 296.2 mg/kg bw, based on body weight of 215 g), fructose+stevioside, or fructose+steviol (intake amounts were the same as for rats treated with each compound individually). A slight, but non-significant, increase in glycogen accumulation was reported in the rats treated with stevioside or steviol compared to the rats administered fructose. Stevioside and steviol significantly enhanced the glycogen accumulation associated with fructose intake in the fructose+stevioside and fructose+steviol dose groups; however, the increased glycogen content was reported to be more pronounced in the fructose+stevioside group (Hübler *et al.*, 1994).

In another set of experiments by the same author, water, aqueous stevioside (1.0 or 2.0 mmol/L) or aqueous steviol (1.0 mmol/L) were administered ad libitum during a 24-hour or 48-hour fasting period (total intake for time period was between 30 and 80 µmol, approximately 112.3 to 300 mg stevioside/kg bw and 44.4 to 118.5 mg steviol/kg bw) to male Wistar rats. In the groups receiving aqueous stevioside during the fasting period, a significant increase in glycogen levels was observed in the 24-hour, 2.0 mmol/L dose group and in the 48-hour, 1.0 mmol/L dose group in comparison with the water controls. No significant changes in glycogen levels were seen in the aqueous steviol groups in relation to the water controls. The authors hypothesised that stevioside has a stimulatory effect on glycogen deposition due to the 3 molecules of glucose that are produced when stevioside is hydrolysed. Steviol, however, is the aglycone of stevioside (lacks the glucose molecules present in stevioside) and, therefore, the release of glucose molecules does not explain the increased glycogen levels as observed with steviol (Hübler *et al.*, 1994).

Male Wistar rats (number/group not reported) were administered *Stevia* [dried powdered leaves from *Stevia Rebaudiana* Bertoni] at a dose of 20 mg/kg bw/day or stevioside (purity not reported) at a dose of 5.5 mg/kg bw/day by gavage for 15 days to determine the effect of either test material on blood glucose levels. A group of rats administered water served as the control. In comparison to the control group, rats treated with *Stevia* exhibited significantly lower blood glucose levels. Conversely, no effects were observed in rats treated with stevioside. In order to examine effects on gluconeogenesis, isolated livers of rats administered *Stevia* or water were perfused with L-alanine (5 mM), L-lactate (2 mM), L-glutamine (5 mM), and glycerol (2 mM) and the AUC was calculated for glucose (all 3 substrates), urea (L-alanine only), pyruvate (L-alanine and L-lactate), and L-lactate (L-alanine only) production. Compared to controls, the AUC values for glucose were significantly lower when livers from *Stevia*-treated rats were perfused with L-alanine, L-lactate, and L-glutamine, but not with glycerol. AUC test and control values for urea, pyruvate, and L-lactate (indicative of L-alanine catabolism) with L-alanine perfusion were comparable, whereas the AUC for pyruvate was increased following L-lactate perfusion of livers from *Stevia*-treated rats compared to controls. The authors suggested that *Stevia* inhibited pyruvate carboxylase (PC) and PEPCK. When the perfusion experiments were repeated with livers from stevioside-treated rats, no differences in glucose production were observed between livers from rats administered stevioside or water. Additionally, glucose production was also evaluated in isolated hepatocytes from stevioside-treated rats. Like the perfusion assays, no differences were observed between test cells (from stevioside-treated rats) and control cells in glucose production following incubation of cells with no substrate, glycerol, L-alanine, L-glutamine, pyruvate, or L-lactate (Ferreira *et al.*, 2006).

In studies with non-oral routes of administration, plasma glucose and/or insulin levels were reported to increase or decrease at various times following the i.p. or i.v. infusion of stevioside (Jeppesen *et al.*, 2002; 2003; Ma *et al.*, 2007; Raskovic *et al.*, 2004a,b, 2005; Suanarunsawat and Chaiyabutr, 1996). The results of a study in which the route of exposure was not described, demonstrated that pretreatment with a Stevia extract lowered blood glucose levels (Raskovic *et al.*, 2006). Due to the lack of description regarding the route of exposure, the results of this study cannot be associated to the effects of steviol glycosides on glucose homeostasis.

APPENDIX VII-II: STUDIES ON BLOOD PRESSURE AND CARDIAC FUNCTION

Spontaneously Hypertensive (SH) rats (4-week old; N=10/group) received 0.1% stevioside (purity not reported) in their drinking water (equivalent to 100 mg/kg bw/day) for 14 weeks. SBP was significantly decreased in the stevioside-treated group compared to the control group from 8 to 18 weeks of age. From 20 to 28 weeks of age, SBP remained significantly lower in the stevioside-treated group compared to the control group despite the withdrawal of stevioside during that time-period. Heart Rate (HR) values were reported to be not significantly different between treatment groups. Additionally, the SBP of 12-week old male SH rats (N=13/group) administered 0.1% stevioside in their drinking water for 2 weeks (equivalent to 200 to 250 mg/kg bw/day), followed by a 3-day recovery period was significantly decreased throughout the treatment period and during the first 2 days of the recovery period. By the third day of the recovery period, SBP levels were similar to the control group and HR and body weight did not differ significantly from the control group. The authors concluded that 0.1% stevioside provided in the drinking water to immature SH rats prevented the development of hypertension and that 0.1% stevioside provided in the drinking water of mature SH rats may have antihypertensive effects (Hsu *et al.*, 2002).

In SH male rats (N=13), the ad libitum consumption of a 0.1% stevioside (purity not reported) solution in drinking water (47 mL \pm 5 mL per day providing approximately 171 mg stevioside/kg bw/day) for a period of 14 days was associated with 23 \pm 4 to 44 \pm 6 mmHg reductions in blood pressure (presumably SBP (De-Yi *et al.*, 1990).

In another study designed to evaluate the antihypertensive effects of stevioside (purity not reported) healthy mongrel dogs of both sexes (N=8) received a single dose of 200 mg stevioside (purity not reported)/kg bw by nasogastric feeding. The treatment significantly decreased SBP, DBP, and Mean Arterial Blood Pressure (MABP) between 60 and 120 minutes after stevioside administration compared to baseline; however, by 180 minutes after stevioside administration, SBP, DBP, and MAP were reported to return to baseline values (Liu *et al.*, 2003).

Several studies that used i.p. or i.v. injections to examine the potential effects of stevioside on blood pressure parameters demonstrated that stevioside may decrease blood pressure values at doses between 25 and 400 mg/kg bw/day (De-Yi *et al.*, 1990; Chan *et al.*, 1998; Lee *et al.*, 2001; Hsu *et al.*, 2002; Liu *et al.*, 2003).

APPENDIX VII-III: STUDIES ON RENAL FUNCTION

A study which elucidated the effects of a Stevia extract and stevioside (purity not reported) administered orally on kidney function was carried out in 40 adult dogs of both sexes (body weights ranging between 5 to 10 kg). The dogs were divided equally into 2 groups. with one group of dogs comprised animals with a urinary osmolality above 1100 mOsm/kg water. The groups were provided oral doses of 10% Stevia extract per day [approximately 5000 mg/kg bw/day (U.S. FDA, 1993)] or 6 mg stevioside/kg bw/day for 10 days presumably in their drinking water. In the animals with a urinary osmolality above 1100 mOsm/kg water, arterial blood pressure decreased following oral administration of both Stevia and stevioside; however, these values returned to the baseline readings after 10 minutes post-exposure. The decreases in arterial blood pressure were generally more pronounced following exposure to stevioside, but were not statistically significant compared to

controls. Additionally, respiratory function, urinary output, along with changes in various urinary and plasma parameters were unaffected by exposures to Stevia or stevioside. The authors concluded that both Stevia and stevioside were well-tolerated and displayed no treatment-related effects on kidney function in dogs with urinary osmolality above 1100 mOsm/kg water or in dogs with water overload (Chagas *et al.*, 1990).

A number of *in vivo* studies using i.v. injection as a route of exposure to stevioside and Stevia extract were conducted to assess effects on blood pressure in relation to renal function (Chagas *et al.*, 1990; Melis, 1992a,b,c; Melis and Sainati, 1991a,b; Sainati *et al.*, 1986; Chatsudthipong and Thongouppakarn, 1995; Melis, 1995, 1996).

GLOSSARY AND ABBREVIATIONS

ADI	Acceptable Daily Intake
AFC	Scientific Panel on Additives, Flavourings, Processing Aids and Materials in Contact with Food
ALP	Alkaline Phosphatase
ALT	Alanine Transaminase
ANS	Scientific Panel on Food Additives and Nutrient Sources added to Food
AFSSA	Agence Française de Sécurité Sanitaire des Aliments
AST	Aspartate Transaminase
AUC	Area under the curve
BUN	Blood Urea Nitrogen
CAS	Chemical Abstracts Service
CK	Creatine Kinase
DBP	Diastolic Blood Pressure
EC	European Commission
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
EXPOCHI	Individual food consumption data and exposure assessment studies for children
EU	European Union
FDA	US Food and Drug Administration
FAO/WHO	Food and Agriculture Organization/World Health Organization
GC-MS	Gas chromatography-Mass Spectrometry
GD	Gestation Day
GGT	Gamma-glutamyl Transferase
GK	Goto-Kazachi rats
GMP	Good Manufacturing Practice
HPLC	High-performance liquid chromatography
HR	Heart Rate

IAUC	Incremental Areas Under the concentration Curves
IC ₅₀	Median Inhibitory Concentration
i.p.	Intraperitoneal
i.v.	Intravenous
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC-MS	Liquid Chromatography Mass Spectrometry
LC-ESI-MS	Liquid Chromatography Electrospray Ionisation Tandem Mass Spectrometry
LD ₅₀	Lethal Dose, 50 % i.e. dose that causes death among 50 % of treated animals
LDH	Lactate Dehydrogenase
LOAEL	Lowest-Observed-Adverse-Effect-Level
MAP	Mean Arterial Pressure
MS	Mass Spectrometry
MW	Molecular Weights
NO	Nitric Oxide
NMR	Nuclear Magnetic Resonance
NOAEL	No Observed Adverse Effect Level
NZW	New Zealand White rabbits
OECD	Organisation for Economic Co-operation and Development
SBP	Systolic Blood Pressure
s.c.	Subcutaneous
SCF	Scientific Committee on Food
SCFE	Supercritical Carbon-dioxide Fluid Extraction
SH	Spontaneously Hypertensive rats
SPT	Skin Prick Test
TLC	Thin-Layer Chromatography
TNF- α	Tumour Necrosis Factor- α
UNESDA	Union of European Beverages Associations
US FDA	U.S. Food and Drug Administration

VLDL-C	Very-low-density Lipoprotein Cholesterol
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