EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF); Scientific Opinion on Flavouring Group Evaluation 310 (FGE.310): Rebaudioside A from chemical group 30

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SCIENTIFIC OPINION

Scientific Opinion on Flavouring Group Evaluation 310 (FGE.310):

Rebaudioside A from chemical group 30[1]

EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF)[2, 3]

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

The Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids of the European Food Safety Authority was requested to evaluate rebaudioside A [FL-no: 16.113], a steviol glycoside. The substance was not considered to have genotoxic potential. Since a comprehensive and adequate toxicological database, including human studies, is available for steviol glycosides, the Panel based its evaluation of rebaudioside A on a comparison of the ADI of 4 mg/kg bw, expressed as steviol, established by EFSA, with the estimated dietary exposure figures based on the MSDI and mTAMDI approaches. The Panel concluded that rebaudioside A [FL-no: 16.113] would not give rise to safety concerns at the estimated level of intake arising from its use as flavouring substance.

1 On request from the Commission, Question No EFSA-Q-2009-00571, adopted on 19 May 2011.

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SUMMARY

The European Food Safety Authority (EFSA) asked the Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (the Panel) to provide scientific advice to the Commission on the implications for human health of chemically defined flavouring substances used in or on foodstuffs in the Member States. In particular, the Panel was requested to evaluate one flavouring substance in the Flavouring Group Evaluation 310, using the Procedure as referred to in the Commission Regulation (EC) No 1565/2000. This flavouring substance belongs to chemical group 30, Annex I of the Commission Regulation (EC) No 1565/2000.

The present Flavouring Group Evaluation deals with the steviol glycoside rebaudioside A [FL-no: 16.113].

It has been reported that the flavouring substance occurs naturally in Stevia rebaudiana, up to 16200 – 72700 mg/kg, depending on plant species.

The flavouring substance is included in the specifications for the food additive (sweetener) steviol glycosides which has recently been evaluated by both the JECFA (JECFA, 2009a) and EFSA (EFSA, 2010k). The flavouring substance rebaudioside A [FL-no: 16.113] is closely related structurally to stevioside. Both compounds contain the same aglycon steviol, the only difference being that rebaudioside A contains an extra glucose molecule in the glycoside moiety. Steviol glycosides are chemically defined mixtures that comprise not less than 95 % stevioside and/or rebaudioside A. Both the JECFA and EFSA established an ADI for steviol glycosides (including rebaudioside A), expressed as steviol equivalents, of 4 mg/kg bw/day.

In its evaluation, the Panel as a default used the "Maximised Survey-derived Daily Intake" (MSDI) approach to estimate the *per capita* intakes of the flavouring substances in Europe. However, when the Panel examined the information provided by the European Flavouring Industry on the use levels in various foods, it appeared obvious that the MSDI approach in a number of cases would grossly underestimate the intake by regular consumers of products flavoured at the use level reported by the Industry, especially in those cases where the annual production values were reported to be small. In consequence, the Panel had reservations about the data on use and use levels provided and the intake estimates obtained by the MSDI approach.

In the absence of more precise information that would enable the Panel to make a more realistic estimate of the intakes of the flavouring substances, the Panel has decided also to perform an estimate of the daily intakes per person using a "modified Theoretical Added Maximum Daily Intake" (mTAMDI) approach based on the normal use levels reported by Industry. In those cases where the mTAMDI approach indicated that the intake of a flavouring substance might exceed its corresponding threshold of concern, the Panel decided not to carry out a formal safety assessment using the Procedure. In these cases the Panel requires more precise data on use and use levels.

Overall, stevioside and rebaudioside A do not show evidence of genotoxicity *in vitro* or *in vivo*.

Because a comprehensive and adequate toxicological database, including human studies, is available for steviol glycosides the candidate substance rebaudioside A [FL-no: 16.113] should not be evaluated using the Procedure as referred to in the Commission Regulation EC No 1565/2000 (EC, 2000a). Instead the Panel based its evaluation on a comparison of the ADI of 4 mg/kg bw, expressed as steviol, established by EFSA (EFSA, 2010k) with the estimated MSDI and mTAMDI values.

According to the default MSDI approach, the substance has a daily per capita intake as a flavouring of 1200 microgram/*capita*/day. The MSDI of 1200 microgram rebaudioside A/*capita*/day, equivalent to 20 microgram rebaudioside A/kg bw/day, for a person weighing 60 kg, corresponding to a daily intake of 6.6 microgram steviol/kg bw/day, using a conversion factor of 0.33 (EFSA, 2010k) for converting the amount of rebaudioside A into steviol equivalents. This intake, as a flavouring substance, amounts

to 0.17 % of the ADI of 4 mg/kg bw, expressed as steviol, established by EFSA (EFSA, 2010k). The Panel concluded that rebaudioside A [FL-no: 16.113] does not pose a safety concern when used as flavouring substance at the estimated level of intake, based on the MSDI approach.

The estimated intake of the candidate substance rebaudioside A [FL-no: 16.113] based on the mTAMDI is 10888 microgram/person/day, is equivalent to 181 microgram rebaudioside A/kg bw/day, for a person weighing 60 kg. This correspond to a daily intake of 60 microgram steviol/kg bw/day, using a conversion factor of 0.33 (EFSA, 2010k) for converting the amount of rebaudioside A into steviol equivalents. This intake amounts to 1.5 % of the ADI of 4 mg/kg bw, expressed as steviol, established by EFSA (EFSA, 2010k). The Panel concluded that rebaudioside A [FL-no: 16.113] does not pose a safety concern when used as flavouring substance at the estimated level of intake, based on the mTAMDI approach.

Steviol glycosides are (expected to be) authorised as food additives in the EU. The Panel noted that in its recent evaluation the EFSA ANS Panel estimated the potential exposure to steviol glycosides from use as food additives. When considering the proposed maximum use levels as food additive, the mean dietary exposure to steviol glycosides expressed as steviol equivalents in European children might exceed the ADI of 4 mg/kg bw/day. Thus, the estimated intake of rebaudioside A [FL-no: 16.113] from its use as a flavouring substance contributes to the total intake of steviol glycosides.

In order to determine whether the conclusion for the flavouring substance can be applied to the materials of commerce, it is necessary to consider the available specifications. Adequate specifications including complete purity criteria and identity for the materials of commerce have been provided for the flavouring substance rebaudioside A [FL-no: 16.113].

The Panel concluded that on the basis of the default MSDI approach and the mTAMDI approach, rebaudioside A [FL-no: 16.113] would not give rise to safety concerns at the estimated level of intake arising from its use as flavouring substance.

KEYWORDS

Rebaudioside A, steviol glycosides, diterpene, flavouring, safety, FGE.310.

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BACKGROUND

Regulation (EC) No 2232/96 of the European Parliament and the Council (EC, 1996a) lays down a Procedure for the establishment of a list of flavouring substances the use of which will be authorised to the exclusion of all other substances in the EU. In application of that Regulation, a Register of flavouring substances used in or on foodstuffs in the Member States was adopted by Commission Decision 1999/217/EC (EC, 1999a), as last amended by Commission Decision 2009/163/EC (EC, 2009a). Each flavouring substance is attributed a FLAVIS-number (FL-number) and all substances are divided into 34 chemical groups. Substances within a group should have some metabolic and biological behaviour in common.

Substances which are listed in the Register are to be evaluated according to the evaluation programme laid down in Commission Regulation (EC) No 1565/2000 (EC, 2000a), which is broadly based on the Opinion of the Scientific Committee on Food (SCF, 1999a). For the submission of data by the manufacturer, deadlines have been established by Commission Regulation (EC) No 622/2002 (EC, 2002b).

After the completion of the evaluation programme the Union List of flavouring substances for use in or on foods in the EU shall be adopted (Article 5 (1) of Regulation (EC) No 2232/96) (EC, 1996a).

TERMS OF REFERENCE

The European Food Safety Authority (EFSA) is requested to carry out a risk assessment on flavouring substances in the Register prior to their authorisation and inclusion in a Community List according to Commission Regulation (EC) No 1565/2000 (EC, 2000a). In addition, the Commission requested EFSA to evaluate newly notified flavouring substances, where possible, before finalising the evaluation programme.

In addition, in letter of 28 January 2010 the Commission requested EFSA to carry out a risk assessment on rebaudioside A [FL-no: 16.113] in accordance with Commission Regulation (EC) No 1565/2000 (EC, 2000a):

"The European Commission requests the European Food Safety Authority to carry out a safety assessment on eighteen new flavouring substances in accordance with Commission Regulation (EC) No 1565/2000 (EC, 2000a) by end 2010", if possible by the end of the authorisation programme, if not within nine months from the finalisation of the programme."

The deadline of the Terms of Reference for rebaudioside A [FL-no: 16.113] was negotiated to 31 May 2011.

The remaining substances of this request were evaluated in other FGEs.

ASSESSMENT

1. Presentation of the Substances in Flavouring Group Evaluation 310

1.1. Description

The present Flavouring Group Evaluation 310, deals with one steviol glycoside from chemical group 30, Annex I of Commission Regulation (EC) No 1565/2000 (EC, 2000a). The flavouring substance rebaudioside A [FL-no: 16.113] (candidate substance) is covered by the specifications for the food additive (sweetener) steviol glycosides which has recently been evaluated by both JECFA (2009a) and EFSA (2010k). Because a comprehensive and adequate toxicological database is available for steviol glycosides the candidate substance rebaudioside A [FL-no: 16.113] should not be evaluated using the

Procedure as referred to in the Commission Regulation EC No 1565/2000 (EC, 2000a). The flavouring substance under consideration, as well as its chemical Register name, FLAVIS- (FL-), Chemical Abstract Service- (CAS-), Council of Europe- (CoE-) and Flavor and Extract Manufacturers Association- (FEMA-) number, structure and specifications, are listed in Table 1.

The flavouring substance rebaudioside A (13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl) oxy]kaur-16-en-8-oic acid, β-D-glucopyranosyl ester) [FL-no: 16.113] (candidate substance) is closely related structurally to stevioside (13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl) oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester). Both compounds contain the same aglycon steviol, the only difference being that rebaudioside A contains an extra glucose molecule in the glycoside moiety.

Steviol glycosides are chemically defined mixtures that comprise not less than 95 % stevioside and/or rebaudioside A. Smaller amounts of rebaudiosides B, C, D, E and F, steviolbioside, rubusoside and dulcoside A are present in the final mixtures. In one product evaluated as a sweetener, rebaudioside A is the major component of the mixture (≥ 95%).

The outcome of the Safety Evaluation is summarised in Table 2a.

The hydrolysis products of rebaudioside A are shown in Table 2b and the closely related substances, stevioside and steviol (supporting substances), are listed in Table 3.

1.2. Stereoisomers

It is recognised that geometrical and optical isomers of substances may have different properties. Their flavour may be different, they may have different chemical properties resulting in possible variability in their absorption, distribution, metabolism, elimination and toxicity. Thus information must be provided on the configuration of the flavouring substance, i.e. whether it is one of the geometrical/optical isomers, or a defined mixture of stereoisomers. The available specifications of purity will be considered in order to determine whether the safety evaluation carried out for candidate substances for which stereoisomers may exist can be applied to the material of commerce. Flavouring substances with different configurations should have individual chemical names and codes (CAS number, FLAVIS number etc.).

The candidate substance possesses several chiral centres. The geometrical stereoisomer is as designated in the structural formula (See Table 1).

1.3. Natural Occurrence in Food

According to the Industry, rebaudioside A [FL-no: 16.113] has been found in Stevia rebaudiana, up to 16200 – 72700 mg/kg, depending on plant species (Flavour Industry, 2009q).

1.4. Evaluations From Other Expert Groups

Rebaudioside A [FL-no: 16.113] (together with stevioside) was latest evaluated as a sweetener by the JECFA in 2008 (JECFA 2009a) and by EFSA in 2010 (EFSA 2010k). Both established an Acceptable Daily Intake (ADI) for steviol glycosides, expressed as steviol, of 0-4 mg/kg body weight (bw)/day.

At its meeting in 1998 (JECFA, 1999a) the JECFA requested further toxicological data on stevioside and the aglycone steviol. In 2004, based on new data, the JECFA established a temporary ADI of 0–2 mg/kg bw for steviol glycosides, expressed as steviol, on the basis of the NOEL for stevioside of 970 mg/kg bw per day (or 383 mg/kg bw per day expressed as steviol) in a 2-year study in rats and a safety factor of 200 (JECFA, 2006a). The ADI was made temporary and the additional safety factor applied was due to lack of data on pharmacological effects of steviol glycosides in humans. The JECFA noted

that stevioside was being investigated as a potential treatment for hypertension and diabetes, with some evidence of pharmacological effects at higher doses. There was inadequate data to assess whether these pharmacological effects would also occur at lower levels of dietary exposure, which could lead to adverse effects in some individuals. Therefore, the JECFA requested additional human studies to address effects in diabetics, as well as normotensive and hypotensive individuals. It was reconsidered again at the JECFA meeting in 2007 at which meeting the temporary ADI of 0 - 2 mg steviol glycosides/kg bw was confirmed (JECFA, 2008b). In 2008, the JECFA removed the temporary designation and established an ADI for steviol glycosides of 0 - 4 mg/kg bw expressed as steviol based on new studies, showing no adverse effects of steviol glycosides when taken at doses of about 4 mg/kg bw per day (as steviol), for up to 16 weeks by individuals with type 2 diabetes mellitus and individuals with normal or low-normal blood pressure for 4 weeks. Steviol glycosides are a mixture of compounds with different molecular weights. Since the actual active ingredient is the steviol part of the different molecules, the 0 - 4 mg refers only to the molecular weight of total steviol in the mixture. The JECFA further stated in its evaluation that "The Committee noted that some estimates of high-percentile dietary exposure to steviol glycosides exceeded the ADI, particularly when assuming complete replacement of caloric sweeteners with steviol glycosides, but recognized that these estimates were highly conservative and that actual intakes were likely to be within the ADI range." (JECFA, 2009a).

The SCF latest evaluated stevioside as a potential sweetener in 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 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, 1999).

However, EFSA has recently, following a request from the European Commission and based on a number of new studies, published a scientific Opinion on the safety of steviol glycosides (including rebaudioside A) as a sweetener for use in food (EFSA, 2010k). The new studies removed the concerns expressed by the SCF (1999) and EFSA established an ADI for steviol glycosides of well-characterised composition, expressed as steviol equivalents, of 4 mg/kg bw/day based on a 2-year carcinogenicity study in the rat given 2.5 % stevioside in the diet. This is equal to 967 mg/kg bw/day.

In the present scientific Opinion, the evaluation of rebaudioside A [FL-no: 16.113] as a flavouring substance, is based on the data in the EFSA Opinion on the use of steviol glycosides as a food additive (sweetener) (EFSA, 2010k) and on the review by Roberts & Renwick (2008).

2. Specifications

Purity criteria for the substance have been provided by the Flavour Industry (Flavour Industry, 2009q) (Table 1).

Judged against the requirements in Annex II of Commission Regulation (EC) No 1565/2000 (EC, 2000a), this information is adequate for the candidate substance (See Section 1.2 and Table 1).

3. Intake Data

Annual production volumes of the flavouring substances as surveyed by the Industry can be used to calculate the "Maximised Survey-derived Daily Intake" (MSDI) by assuming that the production figure only represents 60 % of the use in food due to underreporting and that 10 % of the total EU population are consumers (SCF, 1999a).

However, the Panel noted that due to year-to-year variability in production volumes, to uncertainties in the underreporting correction factor and to uncertainties in the percentage of consumers, the reliability of intake estimates on the basis of the MSDI approach is difficult to assess.

The Panel also noted that in contrast to the generally low *per capita* intake figures estimated on the basis of this MSDI approach, in some cases the regular consumption of products flavoured at use levels reported by the Flavour Industry in the submissions would result in much higher intakes. In such cases, the human exposure thresholds below which exposures are not considered to present a safety concern might be exceeded.

Considering that the MSDI model may underestimate the intake of flavouring substances by certain groups of consumers, the SCF recommended also taking into account the results of other intake assessments (SCF, 1999a).

One of the alternatives is the "Theoretical Added Maximum Daily Intake" (TAMDI) approach, which is calculated on the basis of standard portions and upper use levels (SCF, 1995) for flavourable beverages and foods in general, with exceptional levels for particular foods. This method is regarded as a conservative estimate of the actual intake by most consumers because it is based on the assumption that the consumer regularly eats and drinks several food products containing the same flavouring substance at the upper use level.

One option to modify the TAMDI approach is to base the calculation on normal rather than upper use levels of the flavouring substances. This modified approach is less conservative (e.g., it may underestimate the intake of consumers being loyal to products flavoured at the maximum use levels reported) (EC, 2000a). However, it is considered as a suitable tool to screen and prioritise the flavouring substances according to the need for refined intake data (EFSA, 2004a).

3.1. Estimated Daily *per Capita* Intake (MSDI Approach)

The intake estimation is based on the Maximised Survey-derived Daily Intake (MSDI) approach, which involves the acquisition of data on the amounts used in food as flavourings (SCF, 1999a). These data are derived from surveys on annual production volumes in Europe. These surveys were conducted in 1995 by the International Organization of the Flavour Industry, in which flavour manufacturers reported the total amount of each flavouring substance incorporated into food sold in the EU during the previous year (IOFI, 1995). The intake approach does not consider the possible natural occurrence in food.

Average *per capita* intake (MSDI) is estimated on the assumption that the amount added to food is consumed by 10 % of the population[4] (Eurostat, 1998). This is derived for candidate substances from estimates of annual volume of production provided by Industry and incorporates a correction factor of 0.6 to allow for incomplete reporting (60 %) in the Industry surveys (SCF, 1999a).

The total annual volume of production of the candidate substance in the present Flavouring Group Evaluation (FGE.310) from (the anticipated) use as flavouring substance in Europe has been reported to be approximately 10000 kg (Flavour Industry, 2009q).

On the basis of the annual volume of production reported for the candidate substance, the daily *per capita* intake for the flavouring has been estimated. The estimated daily *per capita* intake of rebaudioside A from use as a flavouring substance is 1200 microgram (Table 2a).

[4] EU figure 375 millions. This figure relates to EU population at the time for which production data are available, and is consistent (comparable) with evaluations conducted prior to the enlargement of the EU. No production data are available for the enlarged EU.

3.2. Intake Estimated on the Basis of the Modified TAMDI (mTAMDI)

The method for calculation of modified Theoretical Added Maximum Daily Intake (mTAMDI) values is based on the approach used by SCF up to 1995 (SCF, 1995).

The assumption is that a person may consume a certain amount of flavourable foods and beverages per day.

For the candidate substance information on food categories and normal and maximum use levels[5,6] were submitted by the Flavour Industry (Flavour Industry, 2009q). The candidate substance is used in flavoured food products divided into the food categories, outlined in Annex III of the Commission Regulation (EC) No 1565/2000 (EC, 2000a), as shown in Table 3.1. For the present calculation of mTAMDI, the reported normal use levels were used. In the case where different use levels were reported for different food categories the highest reported normal use level was used.

Table 3.1 Use of Candidate Substance*

Food category	Description	Flavouring used
01.0	Dairy products, excluding products of category 2	Yes
02.0	Fats and oils, and fat emulsions (type water-in-oil)	No
03.0	Edible ices, including sherbet and sorbet	Yes
04.1	Processed fruits	Yes
04.2	Processed vegetables (incl. mushrooms & fungi, roots & tubers, pulses and legumes), and nuts & seeds	No
05.0	Confectionery	Yes
06.0	Cereals and cereal products, incl. flours & starches from roots & tubers, pulses & legumes, excluding bakery	Yes
07.0	Bakery wares	No
08.0	Meat and meat products, including poultry and game	No
09.0	Fish and fish products, including molluscs, crustaceans and echinoderms	No
10.0	Eggs and egg products	No
11.0	Sweeteners, including honey	No
12.0	Salts, spices, soups, sauces, salads, protein products etc.	Yes
13.0	Foodstuffs intended for particular nutritional uses	No
14.1	Non-alcoholic ("soft") beverages, excl. dairy products	Yes
14.2	Alcoholic beverages, incl. alcohol-free and low-alcoholic counterparts	Yes
15.0	Ready-to-eat savouries	No
16.0	Composite foods (e.g. casseroles, meat pies, mincemeat) - foods that could not be placed in categories 1 – 15	No

*The flavouring substance is also anticipated to be used in chewing gum, which is not included in any of the categories in the above table.

According to the Flavour Industry the normal use levels for the candidate substance are 20 mg/kg food and the maximum use levels are 30 mg/kg (Flavour Industry, 2009q).

The mTAMDI value is 10,888 microgram/person/day for the candidate substance from structural class III (see Section 5).

The use levels as a sweetener range from 110 to 10,000 mg/kg food (EFSA, 2010k).

[5] "Normal use" is defined as the average of reported usages and "maximum use" is defined as the 95th percentile of reported usages (EFFA, 2002i).
[6] The normal and maximum use levels in different food categories (EC, 2000) have been extrapolated from figures derived from 12 model flavouring substances (EFFA, 2004e).

For detailed information on use levels and intake estimations based on the mTAMDI approach, see Section 6 and Annex II.

4. Absorption, Distribution, Metabolism and Elimination

In 2008 Roberts & Renwick reviewed the available literature on the role of the gut microflora in the metabolism of steviol glycosides, stevioside and rebaudioside A to establish that the safety data on stevioside can be extrapolated to the structurally related glycoside rebaudioside A (Roberts & Renwick, 2008). Roberts & Renwick (2008) considered that:

- Both stevioside and rebaudioside A undergo hydrolysis by mixed intestinal flora to steviol and the rate of hydrolysis of stevioside is slightly greater than that of rebaudioside A.
- Hydrolysis proceeds via initial formation of steviolbioside with steviol as the final product of hydrolysis.
- Steviol is not metabolized by the intestinal flora and is absorbed from the intestine.
- Steviol-16,17-epoxide was not detected as a microbial metabolite of steviol glycosides.
- Fecal incubation studies with both human and animal mixed flora provide similar results and hence the rat represents a suitable model for human metabolism of stevioside and rebaudioside A.

Based on these findings Roberts & Renwick (2008) concluded that the data on the toxicological effects of stevioside can be extrapolated to rebaudioside A because of the overall similarities in the metabolic fates of stevioside and rebaudioside A on incubation with the intestinal microflora with essentially quantitative formation of steviol, the comparable rates of hydrolysis, and the negligible changes in flora produced by prolonged incubation with these glycosides.

In 2010 the EFSA ANS Panel in its Opinion on steviol glycosides considered that: “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 ANS Panel considered the results of toxicological studies on either stevioside or rebaudioside A applicable for the safety assessment of steviol glycosides in general.“

The CEF Panel concurs with the conclusions, that rebaudioside A and stevioside both show similar pharmacokinetics in the rat and humans, that they are both metabolised to the same active metabolite steviol, and the results of toxicological studies on either stevioside or rebaudioside A are applicable for the safety assessment of steviol glycosides in general.

For more detailed information on absorption, distribution, metabolism and excretion, see Annex III.

5. Safety Evaluation of the Flavouring Substance

The application of the Procedure as referred to in the Commission Regulation EC No 1565/2000 (EC, 2000a) is based on intakes estimated on the basis of the MSDI approach. Where the mTAMDI approach indicates that the intake of a flavouring substance might exceed its corresponding threshold of concern, a formal safety assessment is not carried out using the Procedure. In these cases the Panel requires more precise data on use and use levels. For comparison of the intake estimations based on the MSDI approach and the mTAMDI approach, see Section 6.

For the safety evaluation of the candidate substance rebaudioside A [FL-no: 16.113] from chemical group 30 the Panel decided that the Procedure should not be applied. Instead the Panel based its evaluation on a comparison of the ADI of 4 mg/kg bw for steviol glycosides, expressed as steviol, established by EFSA (EFSA, 2010k) with the estimated MSDI (and mTAMDI). The ADI includes rebaudioside A which is also converted to steviol following ingestion.

The MSDI of 1200 microgram rebaudioside A/*capita*/day, equivalent to 20 microgram rebaudioside A/kg bw/day, for a person weighing 60 kg, corresponds to a daily intake of 6.6 microgram steviol/kg bw/day, using a conversion factor of 0.33 (EFSA, 2010k) for converting the amount of rebaudioside A into steviol equivalents. This intake as a flavouring substance amounts to 0.17 % of the ADI of 4 mg/kg bw, expressed as steviol, established by EFSA (2010k).

The Panel concluded that rebaudioside A [FL-no: 16.113] does not pose a safety concern when used as flavouring substance at the estimated level of intake, based on the MSDI approach.

6. Comparison of the Intake Estimations Based on the MSDI Approach and the mTAMDI Approach

The estimated intake of the candidate substance rebaudioside A [FL-no: 16.113] based on the mTAMDI is 11088 microgram/person/day, which is equivalent to 181 microgram rebaudioside A/kg bw/day, for a person weighing 60 kg. This correspond to a daily intake of 60 microgram steviol/kg bw/day, using a conversion factor of 0.33 (EFSA, 2010k) for converting the amount of rebaudioside A into steviol equivalents. This intake as a flavouring substance amounts to 1.5 % of the ADI of 4 mg/kg bw, expressed as steviol, established by EFSA (2010k).

The Panel concluded that rebaudioside A [FL-no: 16.113] does not pose a safety concern when used as flavouring substance at the estimated level of intake, based on the mTAMDI approach.

For comparison of the MSDI and mTAMDI values, see Table 6.1

Table 6.1 Estimated intakes based on the MSDI approach and the mTAMDI approach

FL-no	EU Register name	MSDI (µg/*capita*/day)	mTAMDI (µg/person/day)	Structural class	ADI (µg/person/day)
16.113	Rebaudioside A	1200	11088	Class III	240000

The intake as a flavouring substance amounting to 1.5 % of the ADI of 4 mg/kg bw, is far lower than the estimated intake as a sweetener. When considering the proposed maximum use levels for rebaudioside A as a sweetener, the mean dietary exposure to steviol glycosides expressed as steviol equivalents in European children and adults might in some cases exceed the ADI of 4 mg/kg bw/day (EFSA, 2011l).

7. Considerations of Combined Intakes from Use as Flavouring Substances

Because of structural similarities of candidate and supporting substances, it can be anticipated that many of the flavourings are metabolised through the same metabolic pathways and that the metabolites may affect the same target organs. Further, in case of combined exposure to structurally related flavourings, the pathways could be overloaded. Therefore, combined intake should be considered. As flavourings not included in this FGE may also be metabolised through the same pathways, the combined intake estimates presented here are only preliminary. Currently, the combined intake estimates are only based on MSDI exposure estimates, although it is recognised that this may lead to underestimation of exposure. After completion of all FGEs, this issue should be readdressed.

The total estimated combined daily *per capita* intake of structurally related flavourings is estimated by summing the MSDI for individual substances.

On the basis of the reported annual production volumes in Europe (Flavour Industry, 2009q), the estimated daily *per capita* intake as flavourings of the candidate substance belonging to structural class III is 1200 microgram (No structurally related flavourings).

Steviol glycosides may also be authorised as food additives in the EU. In its recent revised exposure assessment the EFSA ANS Panel estimated the potential exposure to steviol glycosides from use as food additives (EFSA, 2011l). 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 0.4 to 6.4 mg/kg bw/day, and from 1.0 to 12.7 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.

8. Toxicity

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 results of toxicological studies on either stevioside or rebaudioside A are applicable for the safety assessment of rebaudioside A.

All the available toxicological studies on stevioside and rebaudioside A were recently evaluated by the EFSA ANS Panel (EFSA, 2010k).

8.1. Acute Toxicity

Acute oral toxicity studies with stevioside (purity 96 %) indicated an LD_{50} of more than 15 g/kg body weight (bw) in the mouse, rat and hamster (EFSA, 2010k).

More information can be found in EFSA (2010k).

8.2. Subacute, Subchronic, Chronic and Carcinogenicity Studies

The subacute, subchronic, chronic and carcinogenicity studies on stevioside and rebaudioside A were recently evaluated by the EFSA ANS Panel (EFSA, 2010k):

In some of the subchronic and the 2-generation reproductive toxicity studies in rats with rebaudioside A, body weight gains were slightly lower in the treated groups compared to the controls. In these studies, decreases in feed consumption and in feed conversion efficiency were also recorded. The ANS Panel considered 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 ANS Panel considered 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.

"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). Since negative carcinogenicity data were consistently observed in three studies in the rat and steviol glycosides do not exert tumour promoting

activity in various experimental models the Panel considers that there is no need to further test the potential carcinogenicity of steviol glycosides in other species (i.e. mouse).

More detailed information can be found in EFSA (2010k).

Other studies

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 had shown that steviol glycosides interfered with the transport of anions in the renal tubules, inhibited vasoconstriction, and stimulated insulin secretion from isolated pancreatic islet cells. Most of these studies did not provide data that can be extrapolated to the *in vivo* situation. However, *in vivo* studies in normal, diabetic or obese rats also indicated that steviol glycosides may impact blood glucose homeostasis parameters and lower blood pressure. 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.

More detailed information can be found in EFSA (2010k).

8.3. Developmental / Reproductive Toxicity Studies

The developmental/reproductive toxicity studies on stevioside and rebaudioside A were recently evaluated by the EFSA ANS Panel (EFSA, 2010k):

Earlier concerns by the SCF (SCF, 1999b) about potential adverse effects on the male reproductive system raised by the findings in a chronic toxicity study in F344 rats with Stevia extract (74.54% stevioside and 16.27 % rebaudioside A) were considered by the Panel to have been adequately clarified by the results of later reproductive toxicity studies with test materials of known composition and high purity. The testicular changes reported in the chronic toxicity study were unlikely to have been caused by steviol glycosides. The ANS Panel noted 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 and that adverse male reproductive effects were observed in rats following administration of Stevia rebaudiana leaf extracts. Since publication of these studies, several reproductive (multigenerational studies) and developmental (teratology) studies were conducted with stevioside and steviol glycosides and the studies with steviol glycosides complying with the JECFA specifications did not affect reproduction or the developing fetus. 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. 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. 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, it was concluded 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. The ANS Panel noted that any studies conducted on steviol at high doses are of little relevance to the safety assessment of the steviol glycoside preparations used as food additives. This is because steviol is absorbed immediately in the gastrointestinal tract following oral administration, 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.

More detailed information can be found in EFSA (2010k).

8.4. Genotoxicity Studies

The gentoxicity studies on stevioside and rebaudioside A were recently evaluated by the EFSA ANS Panel (EFSA, 2010k):

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 steviol glycosides of higher or lower purities. The Panel notes that steviol and some of its oxidative derivates 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*.

9. Human Studies

The available studies in humans on stevioside and rebaudioside A were recently evaluated by the EFSA ANS Panel (EFSA, 2010k):

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. Thus, no adverse effects of steviol glycosides were observed when taken at doses of about the ADI of 4 mg/kg bw per day (as steviol), by individuals with type 2 diabetes mellitus and individuals with normal or low-normal blood pressure.

10. Conclusions

The present Flavouring Group Evaluation deals with one newly notified substance, the steviol glycoside rebaudioside A [FL-no: 16.113] from chemical group 30, Annex I of Commission Regulation (EC) No 1565/2000 (EC, 2000a).

It has been reported that the flavouring substance occurs naturally in Stevia rebaudiana, up to 16200 – 72700 mg/kg, depending on plant species.

The flavouring substance is included in the specifications for the food additive (sweetener) steviol glycosides which has recently been evaluated by both the JECFA (JECFA, 2009a) and EFSA (EFSA, 2010k). The flavouring substance rebaudioside A [FL-no: 16.113] is closely related structurally to stevioside. Both compounds contain the same aglycon steviol, the only difference being that rebaudioside A contains an extra glucose molecule in the glycoside moiety. Steviol glycosides are chemically defined mixtures that comprise not less than 95 % stevioside and/or rebaudioside A. Both

the JECFA and EFSA established an ADI for steviol glycosides (including rebaudioside A), expressed as steviol equivalents, of 4 mg/kg bw/day.

Overall, stevioside and rebaudioside A do not show evidence of genotoxicity *in vitro* or *in vivo*.

Because a comprehensive and adequate toxicological database, including human studies, is available for steviol glycosides the candidate substance rebaudioside A [FL-no: 16.113] should not be evaluated using the Procedure as referred to in the Commission Regulation EC No 1565/2000 (EC, 2000a). Instead the Panel based its evaluation on a comparison of the ADI of 4 mg/kg bw, expressed as steviol, established by EFSA (EFSA, 2010k) with the estimated MSDI and mTAMDI values.

According to the default MSDI approach, the substance has a daily per capita intake as a flavouring of 1200 microgram/*capita*/day. The MSDI of 1200 microgram rebaudioside A/*capita*/day, equivalent to 20 microgram rebaudioside A/kg bw/day, for a person weighing 60 kg, corresponding to a daily intake of 6.6 microgram steviol/kg bw/day, using a conversion factor of 0.33 (EFSA, 2010k) for converting the amount of rebaudioside A into steviol equivalents. This intake, as a flavouring substance, amounts to 0.17 % of the ADI of 4 mg/kg bw, expressed as steviol, established by EFSA (EFSA, 2010k). The Panel concluded that rebaudioside A [FL-no: 16.113] does not pose a safety concern when used as flavouring substance at the estimated level of intake, based on the MSDI approach.

The estimated intake of the candidate substance rebaudioside A [FL-no: 16.113] based on the mTAMDI is 10888 microgram/person/day, is equivalent to 181 microgram rebaudioside A/kg bw/day, for a person weighing 60 kg. This correspond to a daily intake of 60 microgram steviol/kg bw/day, using a conversion factor of 0.33 (EFSA, 2010k) for converting the amount of rebaudioside A into steviol equivalents. This intake amounts to 1.5 % of the ADI of 4 mg/kg bw, expressed as steviol, established by EFSA (EFSA, 2010k). The Panel concluded that rebaudioside A [FL-no: 16.113] does not pose a safety concern when used as flavouring substance at the estimated level of intake, based on the mTAMDI approach.

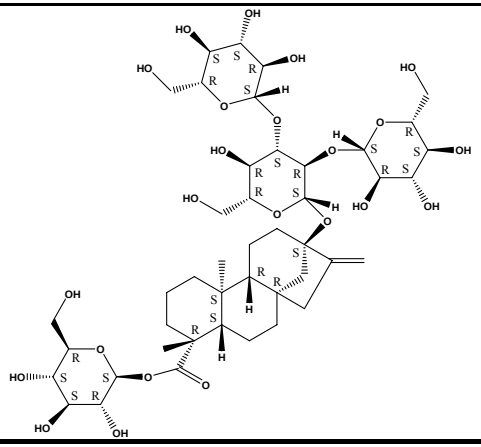
Steviol glycosides are (expected to be) authorised as food additives in the EU. The Panel noted that in its recent evaluation the EFSA ANS Panel estimated the potential exposure to steviol glycosides from use as food additives. When considering the proposed maximum use levels as food additive, the mean dietary exposure to steviol glycosides expressed as steviol equivalents in European children might exceed the ADI of 4 mg/kg bw/day. Thus, the estimated intake of rebaudioside A [FL-no: 16.113] from its use as a flavouring substance contributes to the total intake of steviol glycosides.

In order to determine whether the conclusion for the flavouring substance can be applied to the materials of commerce, it is necessary to consider the available specifications. Adequate specifications including complete purity criteria and identity for the materials of commerce have been provided for the flavouring substance rebaudioside A [FL-no: 16.113].

The Panel concluded that on the basis of the default MSDI approach and the mTAMDI approach, rebaudioside A [FL-no: 16.113] would not give rise to safety concerns at the estimated level of intake arising from its use as flavouring substance.

TABLE 1: SPECIFICATION SUMMARY OF THE SUBSTANCES IN THE FLAVOURING GROUP EVALUATION 310

Table 1: Specification Summary of the Substances in the Flavouring Group Evaluation 310

FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)	Specification comments
16.113 INS960	Rebaudioside A		4601 58543-16-1	Solid $C_{44}H_{70}O_{23}$ 967.01	Very soluble Very soluble	 210-215 NMR MS 99 %	n.a. n.a.	

1) *Solubility in water, if not otherwise stated.*

2) *Solubility in 95 % ethanol, if not otherwise stated.*

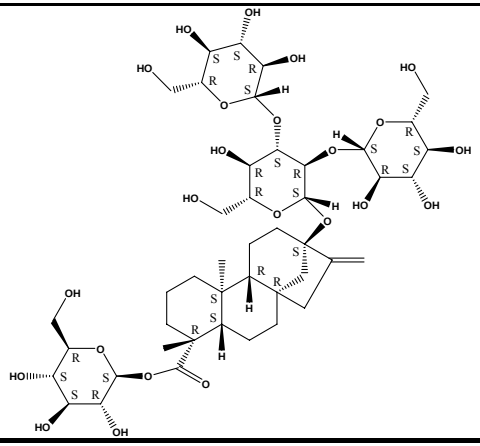
3) *At 1013.25 hPa, if not otherwise stated.*

4) *At 20°C, if not otherwise stated.*

5) *At 25°C, if not otherwise stated.*

TABLE 2A: SUMMARY OF SAFETY EVALUATION APPLYING THE PROCEDURE (BASED ON INTAKES CALCULATED BY THE MSDI APPROACH)

Table 2a: Summary of Safety Evaluation Applying the Procedure (based on intakes calculated by the MSDI approach)

FL-no	EU Register name	Structural formula	MSDI 1) (µg/capita/day)	Class 2) Evaluation procedure path 3)	Outcome on the named compound [4) or 5]	Outcome on the material of commerce [6), 7), or 8)]	Evaluation remarks
16.113	Rebaudioside A		1200	Class III No evaluation via the Procedure	4	6)	

1) EU MSDI: Amount added to food as flavour in (kg / year) x 10E9 / (0.1 x population in Europe (= 375 x 10E6) x 0.6 x 365) = µg/capita/day.

2) Thresholds of concern: Class I = 1800, Class II = 540, Class III = 90 µg/person/day.

3) Procedure path A substances can be predicted to be metabolised to innocuous products. Procedure path B substances cannot.

4) No safety concern based on intake calculated by the MSDI approach of the named compound.

5) Data must be available on the substance or closely related substances to perform a safety evaluation.

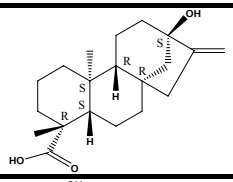
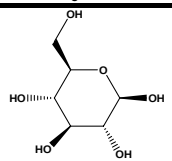
6) No safety concern at estimated level of intake of the material of commerce meeting the specification of Table 1 (based on intake calculated by the MSDI approach).

7) Tentatively regarded as presenting no safety concern (based on intake calculated by the MSDI approach) pending further information on the purity of the material of commerce and/or information on stereoisomerism.

8) No conclusion can be drawn due to lack of information on the purity of the material of commerce.

TABLE 2B: EVALUATION STATUS OF HYDROLYSIS PRODUCTS OF CANDIDATE ESTERS

Table 2b: Evaluation Status of Hydrolysis Products of Candidate Esters

FL-no	Name	Structural formula	SCF status 1) JECFA status 2) CoE status 3) EFSA status	Structural class 4) Procedure path (JECFA) 5)	Comments
-	Steviol		Evaluated as a sweetener Evaluated as a sweetener - Evaluated as a sweetener (ADI of 4 mg/kg bw) as steviol (EFSA, 2010k)	ADI of 0-4 mg/kg bw as steviol (JECFA, 2009a)	Not in Register
-	Glucose		-	-	Not in Register

1) *Category 1: Considered safe in use Category 2: Temporarily considered safe in use Category 3: Insufficient data to provide assurance of safety in use Category 4): Not acceptable due to evidence of toxicity.*

2) *No safety concern at estimated levels of intake.*

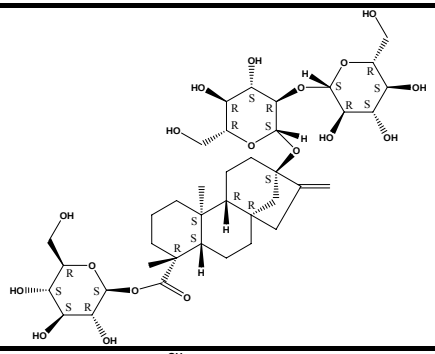
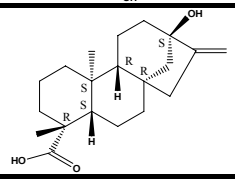
3) *Category A: Flavouring substance, which may be used in foodstuffs Category B: Flavouring substance which can be used provisionally in foodstuffs.*

4) *Threshold of concern: Class I = 1800 µg/person/day, Class II = 540 µg/person/day, Class III = 90 µg/person/day.*

5) *Procedure path A substances can be predicted to be metabolised to innocuous products. Procedure path B substances cannot.*

TABLE 3: SUPPORTING SUBSTANCES SUMMARY

Table 3: Supporting Substances Summary

FL-no	EU Register name/ Name of structurally related substance	Structural formula	FEMA no CoE no CAS no	JECFA no Specification available	MSDI (EU) 1) (µg/capita/day)	SCF status 2) JECFA status 3) CoE status 4)	Comments
-	Stevioside		57817-89-7				Not in Register. Evaluated as a sweetener (ADI of 4 mg/kg bw) as steviol (EFSA, 2010k).
-	Steviol		471-80-7				Not in Register. Evaluated as a sweetener (ADI 4 mg/kg).

1) *EU MSDI: Amount added to food as flavouring substance in (kg / year) x 10E9 / (0.1 x population in Europe (= 375 x 10E6) x 0.6 x 365) = µg/capita/day.*

2) *Category 1: Considered safe in use, Category 2: Temporarily considered safe in use, Category 3: Insufficient data to provide assurance of safety in use, Category 4: Not acceptable due to evidence of toxicity.*

3) *No safety concern at estimated levels of intake.*

4) *Category A: Flavouring substance, which may be used in foodstuffs, Category B: Flavouring substance which can be used provisionally in foodstuffs.*

ANNEX I: PROCEDURE FOR THE SAFETY EVALUATION

The approach for a safety evaluation of chemically defined flavouring substances as referred to in Commission Regulation (EC) No 1565/2000 (EC, 2000a), named the "Procedure", is shown in schematic form in Figure I.1. The Procedure is based on the Opinion of the Scientific Committee on Food expressed on 2 December 1999 (SCF, 1999a), which is derived from the evaluation Procedure developed by the Joint FAO/WHO Expert Committee on Food Additives at its 44th, 46th and 49th meetings (JECFA, 1995; JECFA, 1996a; JECFA, 1997a; JECFA, 1999b).

The Procedure is a stepwise approach that integrates information on intake from current uses, structure-activity relationships, metabolism and, when needed, toxicity. One of the key elements in the Procedure is the subdivision of flavourings into three structural classes (I, II, III) for which thresholds of concern (human exposure thresholds) have been specified. Exposures below these thresholds are not considered to present a safety concern.

Class I contains flavourings that have simple chemical structures and efficient modes of metabolism, which would suggest a low order of oral toxicity. Class II contains flavourings that have structural features that are less innocuous, but are not suggestive of toxicity. Class III comprises flavourings that have structural features that permit no strong initial presumption of safety, or may even suggest significant toxicity (Cramer et al., 1978). The thresholds of concern for these structural classes of 1800, 540 or 90 microgram/person/day, respectively, are derived from a large database containing data on subchronic and chronic animal studies (JECFA, 1996a).

In Step 1 of the Procedure, the flavourings are assigned to one of the structural classes. The further steps address the following questions:

- can the flavourings be predicted to be metabolised to innocuous products[7] (Step 2)?
- do their exposures exceed the threshold of concern for the structural class (Step A3 and B3)?
- are the flavourings or their metabolites endogenous[8] (Step A4)?
- does a NOAEL exist on the flavourings or on structurally related substances (Step A5 and B4)?

In addition to the data provided for the flavouring substances to be evaluated (candidate substances), toxicological background information available for compounds structurally related to the candidate substances is considered (supporting substances), in order to assure that these data are consistent with the results obtained after application of the Procedure.

The Procedure is not to be applied to flavourings with existing unresolved problems of toxicity. Therefore, the right is reserved to use alternative approaches if data on specific flavourings warranted such actions.

[7] *"Innocuous metabolic products": Products that are known or readily predicted to be harmless to humans at the estimated intakes of the flavouring agent" (JECFA, 1997a).*

[8] *"Endogenous substances": Intermediary metabolites normally present in human tissues and fluids, whether free or conjugated; hormones and other substances with biochemical or physiological regulatory functions are not included (JECFA, 1997a).*

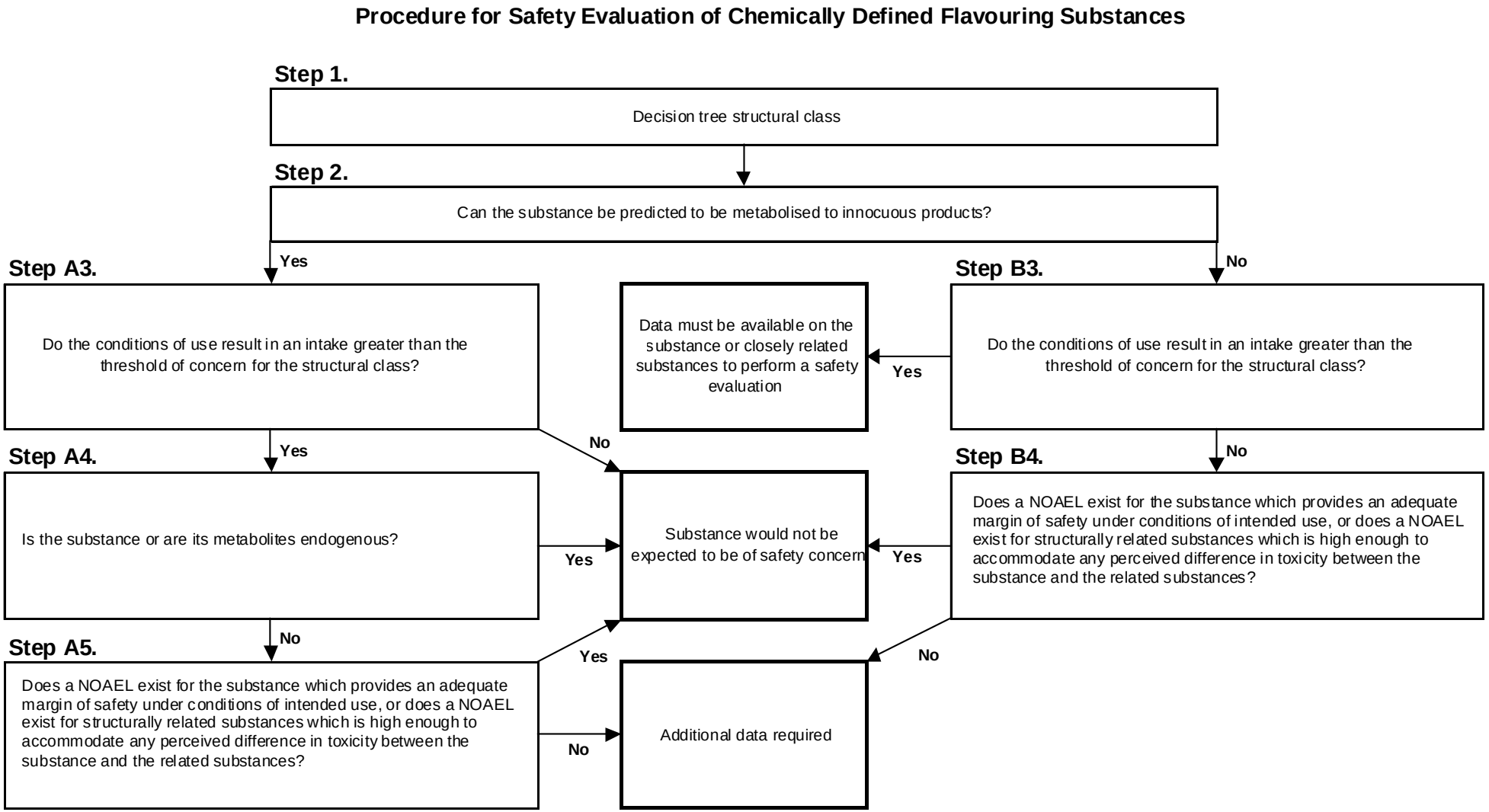


Figure I.1 *Procedure for Safety Evaluation of Chemically Defined Flavouring Substances*

ANNEX II: USE LEVELS / MTAMDI

II.1 Normal and Maximum Use Levels

For each of the 18 Food categories (Table II.1.1) in which the candidate substances are used, Flavour Industry reports a "normal use level" and a "maximum use level" (EC, 2000a). According to the Industry the "normal use" is defined as the average of reported usages and "maximum use" is defined as the 95th percentile of reported usages (EFFA, 2002i). The normal and maximum use levels in different food categories have been extrapolated from figures derived from 12 model flavouring substances (EFFA, 2004e).

Table II.1.1 Food categories according to Commission Regulation (EC) No 1565/2000 (EC, 2000a)

Food category	Description
01.0	Dairy products, excluding products of category 02.0
02.0	Fats and oils, and fat emulsions (type water-in-oil)
03.0	Edible ices, including sherbet and sorbet
04.1	Processed fruit
04.2	Processed vegetables (incl. mushrooms & fungi, roots & tubers, pulses and legumes), and nuts & seeds
05.0	Confectionery
06.0	Cereals and cereal products, incl. flours & starches from roots & tubers, pulses & legumes, excluding bakery
07.0	Bakery wares
08.0	Meat and meat products, including poultry and game
09.0	Fish and fish products, including molluscs, crustaceans and echinoderms
10.0	Eggs and egg products
11.0	Sweeteners, including honey
12.0	Salts, spices, soups, sauces, salads, protein products, etc.
13.0	Foodstuffs intended for particular nutritional uses
14.1	Non-alcoholic ("soft") beverages, excl. dairy products
14.2	Alcoholic beverages, incl. alcohol-free and low-alcoholic counterparts
15.0	Ready-to-eat savouries
16.0	Composite foods (e.g. casseroles, meat pies, mincemeat) - foods that could not be placed in categories 01.0 - 15.0

The "normal and maximum use levels" are provided by Industry for the candidate substance in the present flavouring group (Table II.1.2).

Table II.1.2 Normal and Maximum use levels (mg/kg) for the candidate substance in FGE.310 (Flavour Industry, 2009q).

FL-no	Food Categories (a)																	
	Normal use levels (mg/kg) Maximum use levels (mg/kg)																	
	01.0	02.0	03.0	04.1	04.2	05.0	06.0	07.0	08.0	09.0	10.0	11.0	12.0	13.0	14.1	14.2	15.0	16.0
16.113	20	-	20	20	-	20	20	-	-	-	-	-	20	-	20	20	-	-
	30	-	30	30	-	30	30	-	-	-	-	-	30	-	30	30	-	-

(a) The candidate substance [FL-no. 16.113] is also anticipated to be used in chewing gum, which is not covered by any of the food categories. Normal/maximum use levels for chewing gum are reported to be 200 mg/kg for [FL-no: 16.113]. For chewing gum, the intake estimate is 2 g/day. It is anticipated that all of the flavouring substance is released from the chewing gum. In the calculation of the mTAMDI of the candidate substance, use level figures in Table II.1.2 and the use level of chewing gum (use level of chewing gum reported to be 200 mg/kg) x 2 (g daily intake of chewing gum) = mg/person/day) is summed up with the other food categories to a total mTAMDI value of 10888 µg/person/day presented in table II.2.3 and 6.1.

II.2 mTAMDI Calculations

The method for calculation of modified Theoretical Added Maximum Daily Intake (mTAMDI) values is based on the approach used by SCF up to 1995 (SCF, 1995). The assumption is that a person may consume the amount of flavourable foods and beverages listed in Table II.2.1. These consumption estimates are then multiplied by the reported use levels in the different food categories and summed up.

Table II.2.1 Estimated amount of flavourable foods, beverages, and exceptions assumed to be consumed per person per day (SCF, 1995)

Class of product category	Intake estimate (g/day)
Beverages (non-alcoholic)	324.0
Foods	133.4
Exception a: Candy, confectionery	27.0
Exception b: Condiments, seasonings	20.0
Exception c: Alcoholic beverages	20.0
Exception d: Soups, savouries	20.0
Exception e: Others, e.g. chewing gum	e.g. 2.0 (chewing gum)

The mTAMDI calculations are based on the normal use levels reported by Industry. The seven food categories used in the SCF TAMDI approach (SCF, 1995) correspond to the 18 food categories as outlined in Commission Regulation (EC) No 1565/2000 (EC, 2000a) and reported by the Flavour Industry in the following way (see Table II.2.2):

- Beverages (SCF, 1995) correspond to food category 14.1 (EC, 2000a)
- Foods (SCF, 1995) correspond to the food categories 1, 2, 3, 4.1, 4.2, 6, 7, 8, 9, 10, 13, and/or 16 (EC, 2000a)
- Exception a (SCF, 1995) corresponds to food category 5 and 11 (EC, 2000a)
- Exception b (SCF, 1995) corresponds to food category 15 (EC, 2000a)
- Exception c (SCF, 1995) corresponds to food category 14.2 (EC, 2000a)
- Exception d (SCF, 1995) corresponds to food category 12 (EC, 2000a)
- Exception e (SCF, 1995) corresponds to others, e.g. chewing gum.

Table II.2.2 Distribution of the 18 food categories listed in Commission Regulation (EC) No 1565/2000 (EC, 2000a) into the seven SCF food categories used for TAMDI calculation (SCF, 1995)

	Food categories according to Commission Regulation 1565/2000	Distribution of the seven SCF food categories		
Key	Food category	Food	Beverages	Exceptions
01.0	Dairy products, excluding products of category 02.0	Food		
02.0	Fats and oils, and fat emulsions (type water-in-oil)	Food		
03.0	Edible ices, including sherbet and sorbet	Food		
04.1	Processed fruit	Food		
04.2	Processed vegetables (incl. mushrooms & fungi, roots & tubers, pulses and legumes), and nuts & seeds	Food		
05.0	Confectionery			Exception a
06.0	Cereals and cereal products, incl. flours & starches from roots & tubers, pulses & legumes, excluding bakery	Food		
07.0	Bakery wares	Food		
08.0	Meat and meat products, including poultry and game	Food		
09.0	Fish and fish products, including molluscs, crustaceans and echinoderms	Food		
10.0	Eggs and egg products	Food		

Table II.2.2 Distribution of the 18 food categories listed in Commission Regulation (EC) No 1565/2000 (EC, 2000a) into the seven SCF food categories used for TAMDI calculation (SCF, 1995)

	Food categories according to Commission Regulation 1565/2000	Distribution of the seven SCF food categories	
11.0	Sweeteners, including honey		Exception a
12.0	Salts, spices, soups, sauces, salads, protein products, etc.		Exception d
13.0	Foodstuffs intended for particular nutritional uses	Food	
14.1	Non-alcoholic ("soft") beverages, excl. dairy products	Beverages	
14.2	Alcoholic beverages, incl. alcohol-free and low-alcoholic counterparts		Exception c
15.0	Ready-to-eat savouries		Exception b
16.0	Composite foods (e.g. casseroles, meat pies, mincemeat) - foods that could not be placed in categories 01.0 - 15.0	Food	

The mTAMDI values (see Table II.2.3) are presented for each of the candidate substance in the present flavouring group, for which Industry has provided use and use levels (Flavour Industry, 2009q). The mTAMDI values are only given for the highest reported normal use levels.

TableII.2.3 Estimated intakes based on the mTAMDI approach

FL-no	EU Register name	mTAMDI (µg/person/day)	Structural class	Threshold of concern (µg/person/day)
16.113	Rebaudioside A	10888	Class III	90

ANNEX III: ABSORPTION, DISTRIBUTION, METABOLISM AND EXCRETION (ADME)

In the following, the text on ADME from the EFSA ANS Panel evaluation of steviol glycosides (EFSA, 2010k) and additional information provided by the industry (Robers and Renwick 2008) is given in order to provide insight in the background for establishing an ADI covering both stevioside and rebaudioside A.

EFSA ANS Panel evaluation:

***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. (2003) incubated steviol (purity not reported) with rat (no pretreatment mentioned) or human (pooled from ten healthy donors, five male and five 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 (Koyama et al., 2003b). 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.

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 (Wingard et al., 1980).

Uniformly labelled ^{3}H-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 microg 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. 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 microg/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 (Koyama et al., 2003b).

Recently, the toxicokinetics and metabolism of rebaudioside A, stevioside and steviol (purity ≥ 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 & Renwick, 2008). Single oral doses of the ^{14}C-compounds radiolabeled in the methylene group (=CH2) 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 glucoronide 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 ≥ 96 %) at a dose of 1.67 g/kg feed (equivalent to approximately 0.13 g 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 enterohepatic 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 us 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(Simonetti et al., 2004). In a further study by Geuns et al. 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) (Geuns et al., 2007). 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 microg/mL (21.3 microg 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) (Simonetti et al., 2004). 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 AUC0-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 (Simonetti et al., 2004). 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.

Additional studies on the ADME of steviol glycosides provided by industry:

The metabolism of rebaudioside A, stevioside and steviol were examined in Sprague-Dawley rats in order to determine the toxicokinetic and metabolic similarities between stevioside and rebaudioside A (Roberts & Renwick, 2008).

Animals

Method:
Test compounds were radiolabeled with ^{14}C in the =CH2 group of the steviol moiety. For all studies the rats were given a single oral dose of rebaudioside A (5 mg/kg bw), stevioside (4.2 mg/kg bw) and steviol (1.6 mg/kg bw) in the pharmacokinetic, metabolism and excretion portions of the study. The pharmacokinetic study was conducted in 3 experiments. For experiment 1 groups of 3 male and 3 female Sprague-Dawley rats were used and blood samples were taken from the tail vein of each animal at 0.5, 1, 4, 8, 12, and 24 hours after dosing. To obtain plasma metabolite profiles (experiment 2), four animals per sex per test compound were used. Blood samples were collected from two animals per sex at two different times after dosing by cardiac puncture under terminal anaesthesia. Blood was collected from the rats receiving ^{14}C-rebaudioside A at 8 and 14hours after dosing. Blood was collected from the rats receiving ^{14}C-stevioside at 4 and 8 hours after dosing for males and at 8 and 12 hours after dosing for females. Blood was collected from the rats receiving ^{14}C-steviol at 0.5 and 8h after dosing. In the main study on plasma pharmacokinetics (experiment 3) 27 rats per sex per test compound received a single oral dose of the test substance and blood samples were collected from three animals per sex per compound by cardiac puncture under terminal anaesthesia at 0.25, 0.5, 1, 2, 4, 8, 24, 28 and 72 hours after dosing. For the metabolism and excretion study five intact rats per sex and five bile duct cannulated rats per sex received a single dose of each test substance. For each intact animal, urine was collected separately into solid CO_2-cooled containers from 0 to 6 and 6 to 24 hours after dosing and at 24-h intervals thereafter, up to 96 hours. Feces were collected from 0 to 24 h, 24 to 48, 48 to 72, and 72 to 96 hours after dosing. For each cannulated rat, bile was collected at intervals of 0-3, 3-6, 6-9, 9-12, 12-24 and 24 – 48 hours after dosing. At 96 and 48 hours after dosing, the intact and cannulated rats, respectively, were killed by cervical dislocation. The gastrointestinal tract (including contents) was removed from the carcasses of the intact rats and retained with the remaining carcasses. For the cannulated rats, the gastrointestinal tract (including contents) and the livers were removed from the carcasses and retained with the remaining carcasses.

Results:
Comparison of the pharmacokinetic parameters of total radioactivity following administration of ^{14}C-rebaudioside A, ^{14}C-stevioside and ^{14}C-steviol indicate that the pharmacokinetics of ^{14}C-rebaudioside A, ^{14}C-stevioside are similar, while the pharmacokinetics of ^{14}C-steviol differ, especially in the rate of absorption. Qualitatively all plasma samples had similar metabolite profiles with steviol being the main radioactive component in the plasma. Lower amounts of steviol glucuronide and still lower amounts of one or two unidentified metabolites were also recorded. The observed pharmacokinetic parameters indicate that the peak plasma concentration (C_{max}) and the Area Under Curve (AUC) for stevioside were similar but slightly higher than those of rebaudioside A. The plasma kinetic data indicate a sex difference in the C_{max} and AUC for the total radioactivity, which was less apparent for steviol and was not reflected in the excretion data or the metabolism data (Table A).

Table A: Pharmacokinetic parameters derived from mean total radioactivity concentrations in plasma following the administration of single oral doses of 14C-rebaudioside A, ^{14}C-stevioside, and ^{14}C-steviol (Roberts & Renwick, 2008).

Parameters for total radioactivity	Administrated substance					
	Rebaudioside A		Stevioside		Steviol	
	Male	Female	Male	Female	Male	Female
C_{max} (ng equiv./g)	90	177	101	279	114	264
t_{Max} (h)	2	8	4	8	0.25	264
AUC_{72} (ng equiv. h/g)	645	3329	1617	4287	1251	1604
AUC^{OC} (ng equiv. h/g)	630	3349	1607[a]	4359	1926[a]	1926[a]
K (hl)	0.1462	0.0821	0.0795[a]	0.0460	0.0437[a]	0.0427[a]
$T_{½}$ (h)	5	10	9[a]	15	16[a]	16

[a]Not all of the criteria for reliability were met (see method section).
C_{max} - maximum observed plasma concentration.
t_{Max} - time of maximum observed plasma concentration.
AUC_{72} - AUC calculated from 0-72 hours after administration.
AUC^{OC} - AUC extrapolated to infinity using the terminal slope.

The absorption through the gut from rebaudioside A treatment was 71 % for males and 82 % for females. The corresponding absorption rates for stevioside was 97 % for males and 99 % for females and the one for steviol was 97 % for males and 99 % for females. Steviol was excreted predominantly in the feces where it was quantitatively the most significant radioactive component of intact rats dosed with ^{14}C-rebaudioside A, ^{14}C-stevioside and ^{14}C-steviol. The parent glycoside and steviol glucuronide were identified as minor components as well. Steviol was also the principle radioactive component in the feces of bile duct-cannulated rats treated with 14C-rebaudioside A, ^{14}C-stevioside. Steviol glucuronide was the predominant radioactive components in the bile of cannulated rats treated with ^{14}C-rebaudioside A, ^{14}C-stevioside and ^{14}C-steviol. The metabolite profile following administration of ^{14}C-rebaudioside A, ^{14}C-stevioside and ^{14}C-steviol is indicative of rapid first pass Phase II metabolism. Steviol glucuronides are subsequently eliminated in the bile and de-conjugated in the gastrointestinal tract prior to excretion in the feces.

Conclusions:
Steviol glycosides and steviol are extensively absorbed after oral dosing. Both, steviol glycoside and rebaudioside A are metabolised to steviol by the gut microflora prior to absorption. After absorption from the gut steviol is metabolized mainly to steviol glucuronide and excreted into the gastrointestinal tract via the bile. Due to the low levels of steviol glucuronide and high level of steviol in the feces of intact rats, it appears that the majority of steviol glucuronide is hydrolyzed back to steviol by the intestinal microflora. In rats rebaudioside A, stevioside and steviol are rapidly excreted as steviol, primarily in the feces with limited urinary elimination. The similarities in the kinetics and metabolism of rebaudioside A and stevioside support the use of previous toxicological studies conducted with stevioside for the human safety evaluation of rebaudioside A.

Humans

The comparative pharmacokinetics of steviol and steviol glucuronide following single oral doses of rebaudioside A and stevioside was studied in healthy adult males (Simonetti et al., 2004).

Method:
In a randomized, double-blind, cross-over study 8 healthy males received single oral doses of 5 mg/kg rebaudioside A and 4.2 mg/kg stevioside, equivalent to approximately 1.6 mg/kg of steviol equivalents. Plasma, urine and fecal samples where collected prior to dosing and up to 72 hours after dosing.

Results:
Steviol glucuronide appeared in the plasma of all subjects after administration of rebaudioside A or stevioside, with median t_{max} values of 12.0 and 8.00 hours post-dose, respectively. Steviol glucuronide was eliminated from the plasma, with similar $t_{1/2}$ values of approximately 14 hours for both compounds. Administration of rebaudioside A resulted in a significantly (approximately 22 %) lower

steviol glucuronide geometric mean C_{max} value (1472 ng/mg) than administration of stevioside (1886 ng/ml). The geometric mean AUC_{0-t} value for steviol glucuronide after administration of rebaudioside A (30,788 ng h/ml) was approximately 10 % lower than after administration of stevioside (34.090 ng h/ml) (Table B and C). 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 feces but steviol in the feces accounted for 4.8 % and 5.2 % of rebaudioside A and stevioside, respectively. Pharmacokinetic analysis indicated that rebaudioside A and stevioside underwent similar metabolic and elimination pathways in humans with steviol glucuronide excreted primarily in the urine ad steviol in the feces.

Table B: Summary of the mean (SD) pharmacokinetic data for steviol (Wheeler et al., 2008)

Parameters (units)	Treatment			
	N	Rebaudioside A	N	Stevioside
C_{max} (ng / ml)	1	227 (NA)	1	121 (NA)
t_{Max} (h)	1	72.0 (NA)	1	6.00 (NA)
AUC_{0-t} (ng h/ml)	0	NA (NA)	0	NA (NA)
AUC_{0-inf} (ng h/ml)	0	NA (NA)	0	NA (NA)
$T_{½}$ (h)	0	NA (NA)	0	NA (NA)
λ_z (l/h)	0	NA (NA)	0	NA (NA)
Ae_u (0-72) (mg)	1	227 (NA)	1	121 (NA)
CL_R (L/h)	0	NA (NA)	0	NA (NA)
Ae_F (0-72) (mg)	6	5.88 (6.95)	7	6.50 (7.08)

NA = not applicable

Table C: Summary of the mean (SD) pharmacokinetic data for steviol glucuronide (Wheeler et al., 2008)

Parameters (units)	Treatment			
	N	Rebaudioside A	N	Stevioside
C_{max} (ng / ml)	8	1588 (700)	8	2222 (1078)
t_{Max} (h)	8	12.0 (6.02, 24.0)	8	8.00 (6.00, 12.0)
AUC_{0-t} (ng h/ml)	8	33904 (15139)	8	39928 (20129)
AUC_{0-inf} (ng h/ml)	4	46197 (18604)	4	53211 (23782)
$T_{½}$ (h)	4	14.8 (3.32)	4	14.0 (5.61)
λ_z (l/h)	4	0.0483 (0.00908)	4	0.0551 (0.0221)
Ae_u (0-72) (mg)	8	106 (24.0)	8	112 (36.87)
CL_R (L/h)	8	3.73 (2.01)	8	3.36 (2.51)
Ae_F (0-72) (mg)	6	0 (0)	7	0 (0)

t_{Max} is presented as the Median (Min, Max)

Conclusions:

Administration of both rebaudioside A and stevioside to healthy human subjects results in substantial formation of steviol glucuronide systemically with very limited amounts of steviol observed. The pharmacokinetic analysis indicated that both rebaudioside A and stevioside were hydrolysed to steviol in the gastrointestinal tract prior to absorption. The majority of circulatory steviol was in the form of steviol glucuronide indicating rapid first-pass conjugation prior to urinary excretion (rebaudioside A: 59%; stevioside: 62%). Only a small amount of steviol was detected in urine (rebaudioside A: 0.04%; stevioside: 0.02 %). The formation of steviol from stevioside was more rapid than that of rebaudioside A, which might be explained by the presence of an additional glucose moiety in rebaudioside A that must be removed prior to absorption as steviol in the colon. No meaningful differences were observed in the urinary recovery after administration of either rebaudioside A or stevioside.

On the basis of the similarity in human metabolism to the primary metabolite steviol glucuronide following administration of rebaudioside A or stevioside through the classical phase II detoxification

mechanism, it can be concluded that previous human studies and rodent toxicological studies conducted with stevioside are relevant for assessing the human safety of rebaudioside A.

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ABBREVIATIONS

ADI	Acceptable Daily Intake
ADME	Absorption, distribution, metabolism and excretion
ANS	Panel on Food Additives and Nutrient Sources Added to Food
AUC	Area Under Curve
BW	Body weight
CAS	Chemical Abstract Service
C_{max}	Maximum observed plasma concentration.
CEF	Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids Chemical Abstract Service
CHO	Chinese hamster ovary (cells)
CoE	Council of Europe
DNA	Deoxyribonucleic acid
EC	European Commission
EFFA	European Flavour and Fragrance Association
EFSA	The European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FEMA	Flavor and Extract Manufacturers Association
FGE	Flavouring Group Evaluation
FLAVIS (FL)	Flavour Information System (database)
HPLC	High Performance Liquid Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
ID	Identity
IOFI	International Organization of the Flavour Industry
IR	Infrared spectroscopy
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
LD_{50}	Lethal Dose, 50%; Median lethal dose
LC-ESI-MS	Liquid Chromatography Electrospray Ionisation Tandem Mass Spectrometry
MS	Mass spectrometry
MSDI	Maximised Survey-derived Daily Intake
mTAMDI	Modified Theoretical Added Maximum Daily Intake
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NMR	Nuclear Magnetic Resonance (NMR)
No	Number
NOAEL	No Observed Adverse Effect Level

1	NOEL	No Observed Effect Level
2	NTP	National Toxicology Program
3	SCE	Sister Chromatid Exchange
4	SCF	Scientific Committee on Food
5	SMART	Somatic Mutation and Recombination Test
6	TAMDI	Theoretical Added Maximum Daily Intake
7	TLC	Thin Layer Chromatography
8	t_{Max}	Time of maximum observed plasma concentration
9	UDS	Unscheduled DNA Synthesis
10	WHO	World Health Organisation