Food and Agriculture Organization
of the United Nations

82nd JECFA - Chemical and Technical Assessment (CTA), 2016

STEVIOL GLYCOSIDES

Chemical and Technical Assessment

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1. Summary

Steviol glycosides are a group of compounds naturally occurring in the plant *Stevia rebaudiana* Bertoni sharing a similar molecular structure where different sugar moieties are attached to the aglycone steviol (an *ent*-kaurene-type diterpene). They include any compound containing a steviol backbone conjugated to any number or combination of the principal sugar moieties in any of the orientations occurring in the leaves of *Stevia rebaudiana* Bertoni including, glucose, rhamnose, xylose, fructose, and deoxyglucose. Newly revised specification for Steviol Glycosides are divided into two monographs by source:

> **Steviol Glycosides from *Stevia rebaudiana* Bertoni** are produced from the crushed leaves of the the stevia plant, *Stevia rebaudiana* (Bertoni) by extraction with hot water and recoveryy from the aqueous extract using only alcohols and ion-exchange resins for the isolation and purification of the desired product. The commercial material contains at least 95% of total steviol glycosides (dried basis) with a variable composition depending upon the composition within the leaves of the *Stevia rebaudiana* plant, which is influenced by both soil and climate, as well as manufacturing techniques (adsorption column) and conditions (temperature, pH)
>
> **Rebaudioside A from Multiple Gene Donors Expressed in *Yarrowia lipolytica*** is produced by fermentation of a genetically modified strain of *Yarrowia lipolytica* to express the *Stevia rebaudiana* metabolic pathway. It is at least 95% rebaudioside A (anhydrous basis).

Commercial products containing steviol glycosides are white to light yellow powders that are freely soluble in mixtures of water and ethanol (50:50). The powders can be odourless or have a slight characteristic odour. Water solutions are 200 to 300 times sweeter than sucrose. On the basis of results from thermal and hydrolytic stability studies on steviol glycosides and consideration of several summaries from the literature on the stability of stevioside and rebaudioside A, the 68th

JECFA agreed that steviol glycosides is sufficiently thermally and hydrolytically stable for use in foods, including acidic beverages, under normal conditions of processing and storage. Preparations of steviol glycosides are reported to be used principally as sweeteners in fruit and milk-based drinks, desserts, yoghurts, confectionaries, delicacies, and pickles. The use of a steviol glycosides as a table-top sweetener is also well known.

2. Structures of steviol glycosides

Steviol glycosides can be categorised into separate groups based on the type of glycosidic residues linked to the steviol backbone: glucosyl steviol (only glucose residues); rhamnosyl steviol (rhamnose and glucose residues); xylosyl steviol (xylose and glucose residues); fructosyl steviol (fructose and glucose residues); and deoxyglucose steviol (deoxyglucose and glucose residues), see Figure 1. The chemical information for steviol glycosides identified commercial products are given in Table 1.

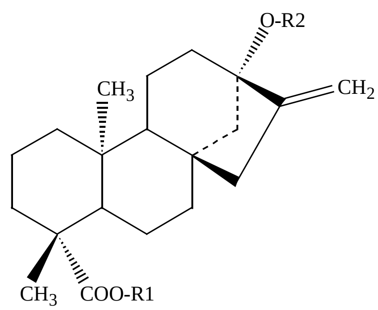

Figure 1. Structures of steviol and related glycosides. Steviol (R1 = R2 = H) is the aglycone of the steviol glycosides. Glc, Rha, Fru, deoxyGlc and Xyl represent, respectively, glucose, rhamnose, fructose, deoxyglucose and xylose sugar moieties.

Table 1: Steviol Glycosides Identified in Commercial Products

Common Name	R_1	R_2	Chemical Name	CAS Number	Chemical Formula	Formula Weight
Group 1: Steviol + Glucose (SvGn)						
Rubusoside	Glcβ1-	Glcβ1-	13-[(β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	64849-39-4	$C_{32}H_{50}O_{13}$	642.73
Steviolbioside	H	Glcβ(1-2)Glcβ1-	13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid	41093-60-1	$C_{32}H_{50}O_{13}$	642.73
Stevioside	Glcβ1-	Glcβ(1-2)Glcβ1-	13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	57817-89-7	$C_{38}H_{60}O_{18}$	804.87
Rebaudioside *B*	H	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid	58543-17-2	$C_{38}H_{60}O_{18}$	804.87
Rebaudioside *E*	Glcβ(1-2)Glcβ1-	Glcβ(1-2)Glcβ1-	13-[(*O*-β- D-Glucoopyranosyl-(1,2)-*O*-[β- D-glucopyranosyl)-oxy]-kaur-16-en-18-oic acid (4')-*O*-β-D-glucopyranosyl-deoxy-(1,2)-*O*-[β-D-glucopyranosyl ester	63279-14-1	$C_{44}H_{70}O_{23}$	967.01
Rebaudioside *A*	Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	58543-16-1	$C_{44}H_{70}O_{23}$	967.01

Common Name	R_1	R_2	Chemical Name	CAS Number	Chemical Formula	Formula Weight
Rebaudioside *D*	Glcβ(1-2)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl ester	63279-13-0	$C_{50}H_{80}O_{28}$	1129.15
Rebaudioside *M*	Glcβ(1-2)[Glcβ (1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	13-[(*O*-β- D-Glucopyranosyl-(1,2)-*O*-[β- D-glucopyranosyl-(1,3)]-β- D-glucopyranosyl)oxy]-kaur-16-en-18-oic acid (4')-*O*-β- D-glucopyranosyl-(1,2)-*O*-[β- D-glucopyranosyl-(1,3)]-β- D-glucopyranosyl ester	1220616-44-3	$C_{56}H_{90}O_{33}$	1291.29
Group 2:Steviol + Rhamnose + Glucose (SvR1Gn)						
Dulcoside *A*	Glcβ1-	Rhaα(1-2)Glcβ1-	13-[(2-*O*-β-D-rhamnopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	64432-06-0	$C_{38}H_{60}O_{17}$	788.87
Rebaudioside *C*	Glcβ1-	Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	13-[(2-*O*-β-D-rhamnopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	63550-99-2	$C_{44}H_{70}O_{22}$	951.01
Rebaudioside *N*	Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	13-[(*O*-β- D-Glucopyranosyl-(1,2)-*O*-[β- D-glucopyranosyl-(1,3)]-β- D-glucopyranosyl)oxy]-kaur-16-en-18-oic acid (4')-*O*-6-deoxy-L-mannopyranosyl-(1,2)-*O*-[β- D-glucopyranosyl-(1,3)]-β- D-glucopyranosyl ester	1220616-46-5	$C_{56}H_{90}O_{32}$	1275.29
Rebaudioside *O*	Glcβ(1-3)Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	13-[(*O*-β-D-Glucopyranosyl-(1,2)-*O*-[β-D-glucopyranosyl-(1,3)]-β-D-glucopyranosyl)oxy]-kaur-16-en-18-oic acid (4')-*O*-β-D-glucopyranosyl-(1,3)-*O*-6- deoxy-L-mannopyranosyl-(1,2)-*O*-[β-D-glucopyranosyl-(1,3)]-β-D-glucopyranosyl ester	1220616-48-7	$C_{62}H_{100}O_{37}$	1437.44

Common Name	R_1	R_2	Chemical Name	CAS Number	Chemical Formula	Formula Weight
Group 3: Steviol + Xylose + Glucose (SvX1Gn)						
Rebaudioside *F*	Glcβ1-	Xylβ(1-2)[Glcβ(1-3)]Glcβ1-	13-[(2-*O*-β-D-xylopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	438045-89-7	$C_{43}H_{68}O_{22}$	936.99

3. Description

Commercial steviol glycoside preparations are white to light yellow powders that are freely soluble in ethanol and water (50:50) mixtures. The powders can be odourless or have a slight characteristic odour. Water solutions are 200 to 300 times sweeter than sucrose.

4. Manufacturing

Steviol Glycosides from *Stevia rebaudiana* Bertoni is obtained from the leaves of *Stevia rebaudiana* Bertoni. The leaves are extracted with hot water and the aqueous extract is passed through an adsorption resin to trap and concentrate the component steviol glycosides. The resin is washed with a solvent alcohol to release the glycosides and the product is recrystallized from methanol or aqueous ethanol. Ion exchange resins may be used in the purification process. The final product may be spray-dried. The Committee, at its 68th and 69th meetings reviewed information on additional purification steps included in the manufacturing process of steviol glycosides considered by the 63rd JECFA. These steps included further recrystallization and separation steps; and the introduction of ethanol as a new solvent.

Rebaudioside A from Genetically Modified *Yarrowia lipolytica* is is obtained from the fermentation of a non-toxigenic non-pathogenic strain of *Yarrowia lipolytica* that is genetically modified with heterologous genes from multiple donor organisms to overexpress steviol glycosides. After removal of the biomass by solid-liquid separation and heat treatment, the process involves concentration of the steviol glycosides (e.g. by resin adsorption), followed by purification of the rebaudioside A by crystallization and drying. Ion exchange resins may be used in the purification process. The final product may be spray-dried.

5.Chemical characterization

Composition of Steviol Glycosides from *Stevia rebaudiana* Bertoni

In response to the call for data on "stevioside" for the 63rd meeting of the Committee, submissions from several countries showed that the main components of the commercially available extracts of stevia are stevioside and rebaudioside A, in various amounts ranging from about 10-70% stevioside and 20-70% rebaudioside A. The information indicated that most commercial products contained more than 90% steviol glycosides with the two main steviol glycosides comprising about 80% of the material. The 63rd JECFA required that the summed content of stevioside and rebaudioside A was not less than 70% and established a minimum purity of 95% total steviol glycosides defined as the sum of rebaudioside A, stevioside, rebaudioside C and dulcoside A. Analytical data showed that most of the remaining 5% could be accounted for by saccharides other than those associated with the individual steviol glycosides.

Noting that the additive could be produced with high purity (at least 95%) and that all the steviol glycosides hydrolyze upon ingestion to steviol, on which the temporary ADI is based, the 68th JECFA decided it was unnecessary to maintain a limit for the sum of stevioside and rebaudioside content and established an identification specification that rebaudioside A or stevioside be the main components of the product. A purity specification of 95% of seven named steviol glycosides (rebaudioside A,stevioside, rebaudioside C, dulcoside A, rubusoside, steviolbioside, and rebaudioside B) was also established . The Committee recognized that the newly revised specifications would cover a range of compositions that could include, on the dried basis, product that was at least 95% stevioside or at least 95% rebaudioside A.

At the 73rd JECFA the definition of steviol glycosides and purity specification of 95% was further expanded to include an additional two steviol glycosides for a total of nine (rebaudioside A, stevioside, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside F, dulcoside A, rubusoside and steviolbioside)

At the 82nd JECFA the definition was expanded from nine named compounds to any mixture of compounds derived from leaves of *Stevia rebaudiana* Bertoni containing a steviol backbone conjugated to any number or combination of the principal sugar moieties in any of the orientations occurring in the leaves of *Stevia rebaudiana* Bertoni including, glucose, rhamnose, xylose, fructose, and deoxyglucose.

Composition of Rebaudioside A from Multiple Gene Donors Expressed in *Yarrowia lipolytica*

At the 82nd JECFA a new monograph for Rebaudioside A from Multiple Gene Donors Expressed in Yarrowia lipolytica was established. It is composed of at least 95% (on the anhydrous basis) of rebaudioside A (13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester, CAS 58543-16-1, chemical formula $C_{44}H_{70}O_{23}$) with minor amounts of other steviol glycosides.

5.1 Possible impurities

Products containing steviol glycosides will contain, in addition to saccharides other than those associated with the individual steviol glycosides, residual extraction/recrystallization solvent and possibly residues of ion-exchange resins used in the manufacturing process. The level of the non-glycosidic fraction, because of its highly non-polar character, can be considered insignificant in the additive.The limit of not more than 4% loss-on-drying established by the 63rd JECFA was increased to not more than 6% by the 68th JECFA and the maximum limit for residual methanol of 100 mg/kg also established by the 63rd JECFA was raised to 200 mg/kg. The 69th JECFA introduced a maximum limit for residual ethanol of 5000 mg/kg. Maximum limits of 1 mg/kg for both arsenic and lead were maintained.

Rebaudioside A from Multiple Gene Donors Expressed in *Yarrowia lipolytica* will contain minor amounts of rebaudioside D, rebaudioside B, rebaudioside M, and stevioside and may contain minor amounts of steviol and kaurenoic acid which are intermediates in the biosynthetic pathway of steviol glycosides.

5.2 Analytical methods

Different methods, mainly IR, and liquid chromatographic methods, are currently available for the identification and determination of the principal steviol glycosides.

6. Functional uses and reactions/fate in foods

Preparations of steviol glycosides have been reported to be used principally as sweeteners in fruit and milk-based drinks, table-top sweeteners, desserts, yoghurts, confectionaries, delicacies, and pickles.

Stevioside and rebaudioside A are reasonably thermally stable under the elevated temperatures used in food processing and do not undergo browning or caramelization when heated. In response to a request by the 63rd JECFA for information on the hydrolytic stability of steviol glycosides in acidic foods, the Committee received results of thermal and hydrolytic stability studies for the specified material: It appears that citric acid solutions (pH 2-4) of steviol glycosides (ca. 1000 mg/l; ca. 29% stevioside, 62% rebaudioside A) are highly stable for at least 180 days at 20º. At elevated temperatures (80º, in water, 8 h), however, the same samples showed 4% and 8% decomposition at, respectively, pH 4.0 and 3.0. At 100º, decomposition was expectedly higher: 10% and 40% at, respectively, pH 4.0 and pH 3.0. Also, at 100º decomposition was 4% at pH 6.0, but increased to about 16% at pH 8.0. And in an acidic beverage (pH 3.8) held at 24º for one year, essentially no decomposition of steviol glycosides (ca. 94% rebaudioside) was noted. Isosteviol (Figure 2) was identified as a decomposition product in the tested samples:

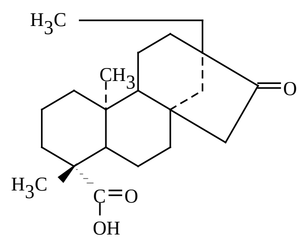

Figure 2. Isosteviol (C.A.S. no. 27975-19-5)

The Committee also had available a summary of literature studies that addressed the stabilities of stevioside and rebaudioside A, which it considered relevant to its evaluation because the specified material includes products that may be 95% stevioside or 95% rebaudioside. Unfortunately, information on the purities of the substances used in these studies was not provided. The studies are summarized as follows:

1) Refluxing stevioside (5 h; 0.4% hydrochloric acid (pH 1.16, aq. methanol)): identifiable hydrolysis products: 49% steviol, 29% steviolbioside, 4% 13-O-ß-D-glucopyranosyl-steviol, and 19% 13-O-ß-D-glucopyranosyl-steviol-ß-D-glucopyranosyl ester.
2) Heating stevioside or rebaudioside A (citric or phosphoric acid solutions; 5.7 days): no decomposition at 60 º; at 100 º, decomposition products noted, but not identified. No steviol was observed.
3) Stevioside (aqueous acid, pH 2.5 and 3.0; 80 º and 100 º): After one hour, maximum decomposition (10%) noted at pH 2.5 and 100 º. No information on the acid used.
4) Stevioside and rebaudioside A (650 mg/l; 100 º): Decomposition in neutral solutions after 13 hours; 40% decomposition in acid solutions (citric acid, pH 2.6; phosphoric acid, pH 2.4) after 4 hours.
5) Stevioside (130 mg/l; citric acid; room temperature; 6 months): 2.5% loss at pH 4.0; 10% loss at pH 3.0.
6) Stevioside (1000 mg/l; pH 2.6 - citric acid solution,; 4º and 22º): stable for up to 4 months. Rebaudioside A (22º; pH 2.6): slightly less stable than stevioside; some decomposition after three months. At 37º, both substances begin to decompose after 2 months; ca. 15% decomposed at 4 months. These data also suggest that, in citric acid solutions, stevioside is more thermally stable than Rebaudioside A.
7) Rebaudioside A (1000 mg/L; phosphoric acid - pH 2.6)) showed slightly greater thermal stability than stevioside (1000 mg/L; phosphoric acid - pH 2.6)).

8) Stevioside (500 mg/l; room temp.; solutions of 10 g/l citric and phosphoric acids (pH: 2.1 and 1.6, respectively): decomposition begins after one month; stable in 5% acetic acid (pH 2.6). In the phosphoric acid solution (4 months), decomposition reached 75%; in citric acid, decomposition was 20%. The greatest stability was observed for the acetic acid solution.

The 68th JECFA concluded that steviol glycosides is sufficiently thermally and hydrolytically stable for food use, including acidic beverages, under normal conditions of processing/storage.

Study results made available to JECFA for the 82nd meeting supported that the stability of steviol glycosides extract preparations established by JECFA at the 68th meeting can be extended to include steviol glycoside extract preparations containing higher levels of new glycosides added to the definition appearing in commercial products, mainly rebaudioside D and rebaudioside M.

7. Use levels in foods

Food use-levels reported to the Committee at the 63rd, 68th and 69th meetings

Food type	Reported maximum use-level (mg/kg)
Beverages (soft drinks, fruit drinks)	600
Desserts	500
Yogurt	500
Cold confectionery	500
Sauces	1000
Pickles	1000
Delicacies	1000
Sweet corn	200
Bread	160
Biscuits	300

Additional uses include use as a table-top sweetener and as a sweetener for ready-to-eat-cereals.

8. References

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