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(Framework for) STEVIOL GLYCOSIDES

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(Framework for) STEVIOL GLYCOSIDES

(TENTATIVE)

Prepared at the 87th JECFA (2019) and published in FAO JECFA Monograph 23 (2019), superseding specifications included in "Steviol glycoside from Stevia rebaudiana Bertoni" prepared at the 84th JECFA (2017), published in FAO JECFA Monographs 20(2017).

Introduction

The functional use of steviol glycosides in food is as a general purpose sweetener. They are 100 to 300 times sweeter than sucrose. Steviol glycosides are the constituents of the leaves of the plant, Stevia rebaudiana Bertoni and are responsible for the sweet taste. They share a similar molecular structure in that the same steviol aglycone is bound to different type and number of glycoside units (e.g., glucose, rhamnose, xylose, fructose, or deoxyglucose). More than forty steviol glycosides have been identified to date (see Annex A).

Background

Steviol glycosides produced by the extraction from the leaves of Stevia rebaudiana Bertoni were reviewed by the Committee at its fifty-first, sixty-third, and sixty-eighth meetings. At the sixty eighth meeting the Committee extended the temporary ADI of 0–2 mg/kg bw for steviol glycosides, expressed as steviol, pending submission of the results of the ongoing studies by the end of 2008. The sixty-eighth meeting also removed the 'tentative' designation on the specifications for steviol glycosides. At the sixty-ninth meeting, the Committee evaluated steviol glycosides extracted from the leaves of the plant Stevia rebaudiana Bertoni for use as a sweetener. After reviewing all the information, the Committee concluded that the data was sufficient to establish an ADI for steviol glycosides of 0–4 mg/kg bw, expressed as steviol equivalents. A specifications monograph was prepared.

At the eighty-second meeting, the Committee evaluated steviol glycosides produced by fermentation of a strain of Yarrowia lipolytica, genetically modified to express the Stevia rebaudiana metabolic pathway. The primary steviol glycoside from this process is rebaudioside A. Based on its chemical structure and toxicological studies, the Committee considered it to be as safe as steviol glycosides extracted from the leaves of the plant Stevia rebaudiana Bertoni; an ADI of 0–4 mg/kg bw, expressed as steviol equivalents was applied to this ingredient as well. A new specifications monograph was prepared to reflect additional considerations resulting from the new source material. (Steviol Glycosides from *Stevia rebaudiana* Bertoni).

The Definition and Assay specification was expanded from nine named leaf-derived steviol glycosides to include any mixture of steviol glycoside compounds derived from Stevia rebaudiana Bertoni, provided that the total percentage of steviol glycosides is not less than 95%. The specifications for steviol glycosides were established as tentative pending method of assay to replace the existing method and including as many steviol glycosides as possible (at least those listed in Appendix 1 of the specifications) in steviol glycoside mixtures.

At the eighty-fourth meeting, the Committee revised the specifications for steviol glycosides from *Stevia rebaudiana* Bertoni and removed the tentative status.

Explanation for the framework approach

The two existing specification monographs for steviol glycosides produced either from leaf extracts or by fermentation require that the products consist of at least 95% steviol glycosides on a dried basis. The major glycosides present in the extract of the leaves from the Stevia rebaudiana Bertoni

plant are stevioside and rebaudioside A, and the minor glycosides include rebaudioside M and rebaudioside D. According to industry, several minor glycosides have more favourable sensory characteristics than the major glycosides. Consequently, technologies were developed to produce steviol glycosides, which enhance the proportion of minor glycosides to modify the sensory profile of steviol glycosides, the article of commerce.

Apart from the extraction of the leaves (see annex 1 for further information and specifications), three other technologies were presented by industry for evaluation:

a) Fermentation; a process in which a genetically engineered microorganism is used to produce specific steviol glycosides from simple sugars (see annex 2 for further specifications and information).

b) Bioconversion; a process in which steviol glycosides that have been extracted from the leaves of Stevia rebaudiana Bertoni undergo enzymatic conversion of major steviol glycosides to minor ones (see annex 3 for further information and specifications).

c) Glucosylation; a process in which steviol glycosides that have been extracted from the leaves of *Stevia rebaudiana* Bertonti undergo enzymatically catalyzed reactions to add glucose units to the steviol glycosides via α-linkages (see annex 4 for further information and specifications).

After reviewing the data 87th JECFA consented that as long as the reviewed products consist of 95% steviol glycosides and the remaining 5% can be explained by varying amounts of starting material, dietary sugars, water and residues of food-grade processing aids there is the possibility to include all four different ways of production into this presented framework document. For this reason, the annexes, each a specification monograph for one production pathway, were included in this document. Although the annexes are including redundant information about specifications or methods that the different production pathways share, it was the opinion of 87th JECFA to keep the annexes apart for clarity.

Methods section

Method of assay

METHOD OF ASSAY (for annexes 1-4)

Determine the percentages of major steviol glycosides (those with analytical standards) using Method A (HPLC, Vol. 4). Confirm the presence of each minor steviol glycoside (compounds where analytical standards are not available) using Method B (HPLC-MS). Calculate the concentration of the minor compounds using respective molecular mass corrected UV peak area against the rebaudioside A UV standard curve. Calculate their sum and express the content on the dried basis.

Method A: Determination of Major Steviol Glycosides by HPLC:

Reagents:

- Acetonitrile: HPLC grade with transmittance more than 95% at 210 nm.
- Deionized water: HPLC grade
- Standards (Reference and Quality Control Standards): Stevioside, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside E, rebaudioside F, rebaudioside M, rebaudioside N, rebaudioside O, dulcoside A, rubusoside and steviolbioside. Chromadex, USA; Wako Pure Chemical Industries Ltd., Japan; Sigma-Aldrich; US Pharmacopeia or equivalent.

Note: Standards of other steviol glycosides, which may become commercially available in the future, may also be included. The analyst

should consider that the inclusion of additional standards will lower the concentration of the mixed standards described below.

Preparation of Steviol Glycosides Standard Solutions:
Prepare individual stock standard solutions (1.5 mg/mL) in water:acetonitrile (7:3).

Prepare mixed standard solution (115 µg/mL) by mixing 1.0 mL each individual stock standard solutions.

Prepare Peak Identification Standard Solutions (0.1 mg/mL) from individual stock standard solutions in water:acetonitrile (7:3).

Prepare mixed working standard solutions in the range of 20 – 100 µg/mL by following appropriate dilution of mixed standard solution (b) with water:acetonitrile (7:3).

Prepare quality control and system suitability individual stock standard solutions (1.5 mg/mL) as well as mixed standard solution (115 µg/mL) using standards from a different batch /manufacturer (if available).

Prepare quality control mixed working standard solutions (40 and 80 µg/mL) and system suitability standard (52 µg/mL) by following appropriate dilutions of mixed standard solution.

Preparation Sample Solution:
Accurately weigh 50 mg of sample and quantitatively transfer into a 50-mL volumetric flask. Add about 20 mL of water:acetonitrile (7:3), sonicate and shake well to dissolve the sample and make up to volume.

Procedure:
Use a HPLC consisting of a high precision binary pump and an auto sampler (capable of operating at 2 -8°); Diode-Array detector @ UV at 210 nm; and Mass Spectrometric Detector (Electrospray Negative Ionisation over a mass range from 50 to 1500 m/z using a unit mass resolution, for use in Method B below) connected in series. Agilent 1200 with Waters Quattro or equivalent:

- Column: Luna 5µ C18(2), 100A, (150 mm x 4.6 mm, 5µm, Phenomenex) or Capcell pak C18 MG II (250 mm x 4.6 mm, 5µm, Shiseido Co. Ltd) or equiv.
- Column temperature: 50°

- Autosampler temperature: 2 – 8°
- Injection volume: 10 µl
- Mobile phase A: Deionised or LC-MS grade water (0.2 µm filtered)
- Mobile phase B: LC-MS grade Acetonitrile (0.2 µm filtered)

HPLC Gradient Time table:

Time (min)	%Solvent A	%Solvent B	Flow Rate (mL/min)
0.00	85.0	15.0	0.3
40.0	70.0	30.0	0.3
60.0	55.0	45.0	0.3
70.0	55.0	45.0	0.3
70.1	85.0	15.0	0.3
80.0	85.0	15.0	0.3

Inject peak identification standard solutions (c), identify peaks and calculate relative retention times (RRT) with respect to rebaudioside A (Typical RRT values and an example chromatogram are provided below the method).

Inject working mixed standard solutions (d) and construct standard curves for each steviol glycoside. Inject quality control and system suitability standard solutions (f) to ensure a satisfactory working system.

Inject prepared samples. Dilute sample solution, if required, to bring the concentration of each analyte within the standard curve range. Make duplicate injections. Deduce concentration of each steviol glycoside from its corresponding standard curve and obtain average concentration in sample solution (µg/mL).

Calculation of major steviol glycosides content:
Calculate the concentration of each steviol glycoside in the sample solution using the following formula:

$$\text{Conc (\%w/w)} = C_{sample} \times 100 / W_{sample}$$

Where:
- C_{sample} is the average concentration (µg/mL) in the sample solution
- W_{sample} is the weight of sample (µg) in 1 mL of sample solution (~1000 µg/mL)

Note: Above calculation will change if additional dilutions were done prior to LC injection. Analyst shall account such dilutions in the calculation.

Calculate the percentage of major steviol glycosides in the sample by summation of percentages of individual steviol glycosides in the sample (A).

Note: If the concentration of major steviol glycosides in the sample is <95%, then analyst should perform Method B.

Method B: Determination of Minor Steviol Glycosides by HPLC-MS:

HPLC-MS conditions may vary based on the manufacturer and model of the system used. Analyst should set the conditions following the

manufacturer's instructions. Typical HPLC-MS Conditions for Waters Quattro Micro mass spectrometer are shown at the end of this document.

The mass spectrometer is connected to the HPLC-UV system used in method A. Analyse the mass spectral data of the minor peaks (major steviol glycoside peaks are identified from RRT in method A). Confirm the presence of each minor steviol glycoside from the observed molecular mass ion (Typical molecular mass ions of steviol glycosides are provided at the end of this document) and one or more of the following mass spectral diagnostic ions:

Mass spectral diagnostic ions observed during in-source fragmentation of steviol glycosides:

[Fragment-H] m/z	**Identity**
317	Steviol
427	Related steviol glycoside #3
479	Steviol-GLC
625	Steviol-2GLC [M-16]
641	Steviol-2GLC
787	Steviol-3GLC deoxyglucose [M-16]
803	Steviol-3GLC
819	-
965	Steviol-4GLC

Note: The example chromatogram of minor steviol glycosides shown at the end of this document was obtained from the purified in-house standards.

After confirming the presence of a minor steviol glycoside, correct its mean peak area (obtained from the UV chromatogram) as described below.

Calculation of minor steviol glycosides content:
Calculate the molecular mass corrected peak area abundance for each minor steviol glycoside using the formula:

Molecular mass corrected peak area = $M_x \times MPA\ M_{RebA}$

Where:
- M_x is the molecular mass of the minor steviol glycoside
- MPA is the mean peak area
- M_{RebA} is the molecular mass of Rebaudioside A (967 amu)

Deduce the concentration (µg/mL) of each minor steviol glycoside using from the UV standard curve of rebaudioside A. Calculate the concentration of each minor steviol glycoside in the sample solution using the following formula:

Minor steviol glycosides conc. (%w/w) = $C_{sample} \times 100 / W_{sample}$

Where:
- C_{sample} is the assayed concentration (µg/mL) in the test sample
- W_{sample} is the sample weight in 1 mL of solution (µg/mL)

Note: Above calculation will change if additional dilutions were done prior to LC injection. Analyst shall account such dilutions in the calculation.
Calculate the percentage of minor steviol glycosides in the sample by summation of percentages of individual minor steviol glycosides in the sample (B).

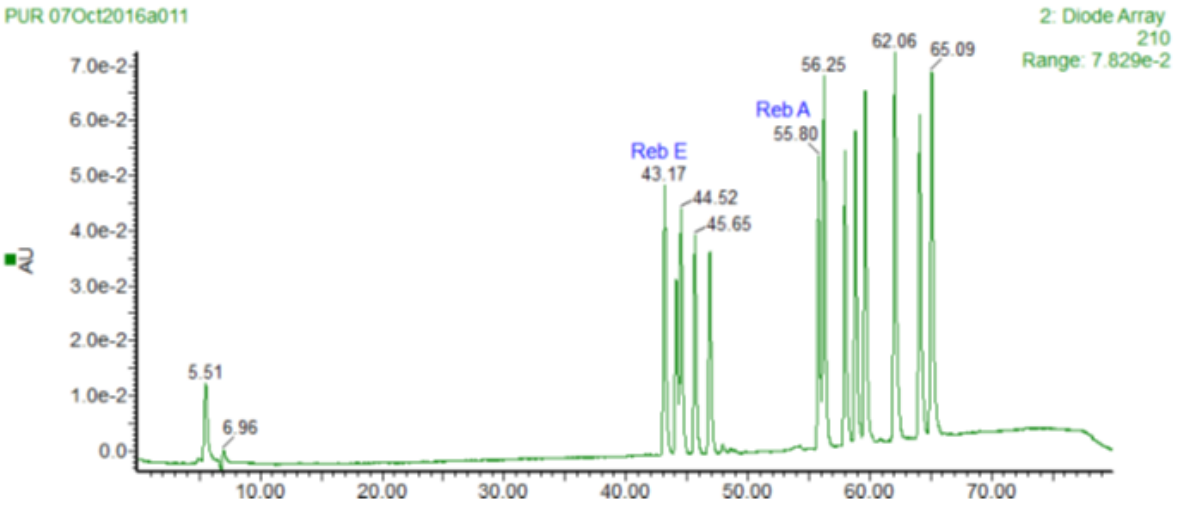
Determine the total amount of steviol glycoside content using the following formula:

$TSG = (A + B) \times 100 / (100 – M)$

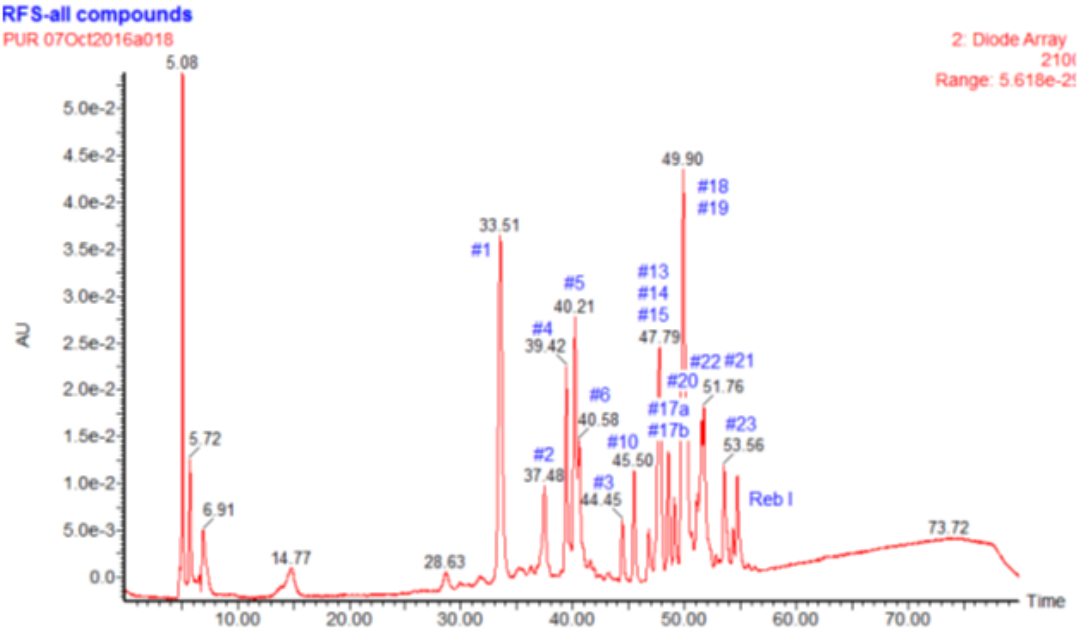
Where:
- TSG is the Total steviol glycosides content (%w/w, on the dried basis)
- A is the percent major steviol glycosides
- B is the percent minor steviol glycosides
- M is the percent loss on drying

Typical LCMS Conditions

Instrumentation:	Waters Quattro Micro mass spectrometer
Ionization:	Electrospray negative polarity
Capillary voltage:	4.0 kV
Cone voltage:	35 V (low) and 60 V (high)
Extractor voltage:	5.0 V
RF lens voltage:	1.0 V
Source temperature:	90 °
Desolvation temperature:	350 °
Desolvation flow rate:	400 L/h
Collisional pressure:	Not applicable
Collisional voltage:	Not applicable
Collision gas:	Not applicable
Resolution:	1 amu
Data acquisition:	Scanning from 50 to 1500 m/z using Mass Lynx



Example Chromatogram of Representative Steviol Glycoside Standards from a Phenomenex Luna C18 (150 mm x 4.6 mm, 5µm). Order of retention times from left to right: rebaudioside E, rebaudioside O, rebaudioside D, rebaudioside N, rebaudioside M, rebaudioside A, stevioside, rebaudioside F, rebaudioside C, dulcoside A, rubusoside, rebaudioside B and steviolbioside.



Example Chromatogram from a Phenomenex Luna C18 (150 mm x 4.6 mm, 5µm) of Minor Steviol Glycosides using in-house purified referencce standards.

Compound Name	Typical Retention Time (RT)*	Relative Retention Time to Rebaudioside A (RRT)*	Molecular Mass Ion [M-H]
Related steviol glycoside #1	32.6	0.58	517 or 427
Related steviol glycoside #2	33.6	0.60	981
Related steviol glycoside #3	34.3	0.61	427 or 735
Related steviol glycoside #4	38.1	0.68	675 or1127
Related steviol glycoside #5	40.8	0.73	981
Rebaudioside V	43.0	0.77	1259
Rebaudioside T	42.0	0.75	1127
Rebaudioside E	43.7	0.78	965
Rebaudioside O	44.6	0.79	1435
Rebaudioside D	45.1	0.80	1127
Rebaudioside K	45.8	0.81	1111
Rebaudioside N	46.1	0.82	1273
Rebaudioside M	47.5	0.84	1289
Rebaudioside S	48.3	0.86	949
Rebaudioside J	48.4	0.86	1111
Rebaudioside W	49.1	0.87	1097
Rebaudioside U2	49.1	0.87	1097
Rebaudioside W2	49.7	0.88	1097
Rebaudioside W3	50.3	0.89	1097
Rebaudioside U	50.7	0.90	1097
Rebaudioside O2	50.6	0.90	965
Rebaudioside Y	50.8	0.90	1259
Rebaudioside I	50.7	0.90	1127
Rebaudioside V2	52.2	0.93	1259
Rebaudioside K2	51.7	0.93	1111
Rebaudioside H	53.7	0.96	1111
Rebaudioside A	56.2	1.00	965
Stevioside	56.6	1.01	803
Rebaudioside F	58.3	1.04	935
Rebaudioside C	59.2	1.05	949
Dulcoside A	60.0	1.07	787
Rubusoside	62.4	1.11	641
Rebaudioside B	64.5	1.15	803
Steviolbioside	65.5	1.17	641

*RT and RRT values given in the above table are for information purpose only. They may vary based on the chromatographic system and conditions used. Analyst needs to establish during method validation.

Appendix A: Chemical Information for Some Steviol Glycosides

Note: This list is not exhaustive - at least 30 steviol glycosides have been identified in stevia leaf extracts in literature.

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
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

Common Name	R_1	R_2	Chemical Name	CAS Number	Chemical Formula	Formula Weight
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
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

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.

ANNEX 1: STEVIOL GLYCOSIDES FROM STEVIA REBAUDIANA BERTONI

Prepared at the 87th JECFA (2019) and published in FAO Monographs XX (2019), superseding specifications prepared at the 84th JECFA (2017) and published in FAO JECFA Monographs 20 (2017). An ADI of 0 - 4 mg/kg bw (expressed as steviol) was established at the 69th JECFA (2008).

SYNONYMS INS No. 960a

DEFINITION Steviol glycosides consist of a mixture of compounds containing a steviol backbone conjugated to any number or combination of the principal sugar moieties (glucose, rhamnose, xylose, fructose, arabinose, galactose and deoxyglucose) in any of the orientations occurring in the leaves of *Stevia rebaudiana* Bertoni. The product 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.

Chemical names See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK

C.A.S number See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK

Chemical formula See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK

Assay Not less than 95% of total of steviol glycosides, on the dried basis, determined as the sum of all compounds containing a steviol backbone conjugated to any number, combination or orientation of saccharides (glucose, rhamnose, fructose, deoxyglucose xylose, galactose, arabinose and xylose) occurring in the leaves of *Stevia rebaudiana* Bertoni.

DESCRIPTION White to light yellow powder, odourless or having a slight characteristic odour. About 200 - 300 times sweeter than sucrose.

FUNCTIONAL USES Sweetener

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Freely soluble in a mixture of ethanol and water (50:50)

HPLC chromatographic profile	The main peaks in a chromatogram obtained by analysing a sample following the procedure in METHOD OF ASSAY correspond to steviol glycosides
pH (Vol. 4)	Between 4.5 and 7.0 (1 in 100 solution)
PURITY	
Total Ash (Vol. 4)	Not more than 1%
Loss on drying (Vol. 4)	Not more than 6% (105°, 2 h)
Residual solvents (Vol. 4)	Not more than 200 mg/kg methanol and not more than 5000 mg/kg ethanol (Method I, General Methods, Organic Components, Residual Solvents)
Arsenic (Vol. 4)	Not more than 1 mg/kg Determine using a method appropriate to the specified level (Use Method II to prepare sample solution). The selection of sample size and method of sample preparation may be based on the principles of the methods described in Vol. 4 (under “General Methods, Metallic Impurities”).
Lead (Vol. 4)	Not more than 1 mg/kg. Determine using a method appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4, under “General Methods, Metallic Impurities”.
Microbiological criteria (Vol. 4)	Total (aerobic) plate count: Not more than 1,000 CFU/g Yeasts and moulds: Not more than 200 CFU/g *E. coli*: Negative in 1 g *Salmonella*: Negative in 25 g
METHOD OF ASSAY	See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK

ANNEX 2: STEVIOL GLYCOSIDES FROM FERMENTATION

Prepared at the 87th JECFA (2019) and published in FAO Monographs XX (2019), superseding specifications prepared at the 82nd JECFA (2016) and published in FAO JECFA Monographs 19 (2016). An ADI of 0 - 4 mg/kg bw (expressed as steviol) was established at the 69th JECFA (2008).

SYNONYMS INS No. 960b

DEFINITION Steviol glycosides from fermentation consist of a mixture of compounds containing a steviol backbone conjugated to various sugar moieties (e.g. glucose or sucrose) depending on the specific production organism and fermentation conditions used.

Steviol glycosides from fermentation are obtained from the fermentation of non-toxigenic non-pathogenic strains of *Yarrowia lipolytica* and *Saccharomyces cerevisiae* that have been 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 desired steviol glycosides by crystallization and drying. Ion exchange resins may be used in the purification process. The final product may be spray-dried. Commercial products are primarily composed of either rebaudioside A, rebaudioside M, or a combination of rebaudioside M and rebaudioside D; additional minor steviol glycosides may be present.

Chemical names See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK

C.A.S number See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK

Chemical formula See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK

Assay Not less than 95% of total of steviol glycosides, on the dried basis.

DESCRIPTION White to light yellow powder, odourless or having a slight characteristic odour. About 200 - 300 times sweeter than sucrose.

FUNCTIONAL USES Sweetener

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Freely soluble in a mixture of ethanol and water 50:50, sparingly soluble in water and sparingly soluble in ethanol.

HPLC chromatographic profile	The main peaks in a chromatogram obtained by analysing a sample following the procedure in METHOD OF ASSAY correspond to steviol glycosides
pH (Vol. 4)	Between 4.5 and 7.0 (1 in 100 solution)
PURITY	
Total Ash (Vol. 4)	Not more than 1%
Loss on drying (Vol. 4)	Not more than 6% (105°, 2 h)
Residual solvents (Vol. 4)	Not more than 200 mg/kg methanol and not more than 5000 mg/kg ethanol (Method I, General Methods, Organic Components, Residual Solvents)
Arsenic (Vol. 4)	Not more than 1 mg/kg Determine using a method appropriate to the specified level (Use Method II to prepare sample solution). The selection of sample size and method of sample preparation may be based on the principles of the methods described in Vol. 4 (under “General Methods, Metallic Impurities”).
Lead (Vol. 4)	Not more than 1 mg/kg. Determine using a method appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4, under “General Methods, Metallic Impurities”.
Microbiological criteria (Vol. 4)	Total (aerobic) plate count: Not more than 1,000 CFU/g Yeasts and moulds: Not more than 200 CFU/g *E. coli*: Negative in 1 g *Salmonella*: Negative in 25 g
METHOD OF ASSAY	See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK

ANNEX 3: ENZYME MODIFIED STEVIOL GLYCOSIDES

Prepared at the 87th JECFA (2019) and published in FAO Monographs XX (2019). An ADI of 0 - 4 mg/kg bw (expressed as steviol) was established at the 69th JECFA (2008).

SYNONYMS

DEFINITION

Enzyme modified steviol glycosides consist of a mixture of compounds containing a steviol backbone conjugated to any number or combination of the principal sugar moieties (glucose, rhamnose, xylose, fructose, arabinose, galactose and deoxyglucose) in any of the orientations occurring in the leaves of *Stevia rebaudiana* Bertoni. The product is obtained from the enzymatic treatment of purified steviol glycosides extracted from the leaves of *Stevia rebaudiana* Bertoni. The purified leaf extract is treated with enzymes produced by non-toxigenic non-pathogenic strains of *Pichia pastoris* and *Escherichia coli* that have been genetically modified with genes from multiple donor organisms (listed below) to produce glucosyltransferase (EC 2.4.1.17) and sucrose synthase (EC 2.4.1.13). The resulting material is heated and filtered to denature and remove the enzymes. The raw product is concentrated using resin adsorption/desorption or solid/liquid filtration, followed by purification and preparation of the product of commerce using processes that may include decolourization, crystallization, and spray drying.

This manufacturing technique maximizes the production of specific steviol glycosides that are not naturally present in high concentrations in the leaf extract, primarily rebaudioside M and rebaudioside D with minor amounts of other steviol glycosides.

Enzyme production organism	**Gene source**
Pichia pastoris	*Horedum vulgare* L *Stevia rebaudiana Bertoni* *Vigna radiate*
Escherichia coli	*Acidithiobacillus caldus* *Arapidopsis thaliana* *Solanum tuberosum* *Stevia rebaudiana*

Chemical names

See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK

C.A.S number

See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK

Chemical formula

See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK

Assay

Not less than 95% of total of steviol glycosides, on the dried basis

DESCRIPTION	White to light yellow powder, odourless or having a slight characteristic odour. About 200 - 300 times sweeter than sucrose.
FUNCTIONAL USES	Sweetener
CHARACTERISTICS	
IDENTIFICATION	
Solubility (Vol. 4)	Freely soluble in a mixture of ethanol and water 50:50
HPLC chromatographic profile	The main peaks in a chromatogram obtained by analysing a sample following the procedure in METHOD OF ASSAY correspond to steviol glycosides
pH (Vol. 4)	Between 4.5 and 7.0 (1 in 100 solution)
PURITY	
Total Ash (Vol. 4)	Not more than 1%
Loss on drying (Vol. 4)	Not more than 6% (105°, 2 h)
Residual solvents (Vol. 4)	Not more than 200 mg/kg methanol and not more than 5000 mg/kg ethanol (Method I, General Methods, Organic Components, Residual Solvents)
Arsenic (Vol. 4)	Not more than 1 mg/kg Determine using a method appropriate to the specified level (Use Method II to prepare sample solution). The selection of sample size and method of sample preparation may be based on the principles of the methods described in Vol. 4 (under “General Methods, Metallic Impurities”).
Lead (Vol. 4)	Not more than 1 mg/kg. Determine using a method appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4, under “General Methods, Metallic Impurities”.
Microbiological criteria (Vol. 4)	Total (aerobic) plate count: Not more than 1,000 CFU/g Yeasts and moulds: Not more than 200 CFU/g *E. coli*: Negative in 1 g *Salmonella*: Negative in 25 g
METHOD OF ASSAY	See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK

ANNEX 4: ENZYME MODIFIED GLUCOSYLATED STEVIOL GLYCOSIDES

Prepared at the 87th JECFA (2019) and published in FAO Monographs XX (2019). An ADI of 0 - 4 mg/kg bw (expressed as steviol) was established at the 69th JECFA (2008).

SYNONYMS

DEFINITION

Enzyme modified glucosylated steviol glycosides are steviol glycoside mixtures composed predominantly of glucosylated steviol glycosides (e.g., mono-, di-, and tri-glucosylated glycosides) with small amounts of steviol glycosides from *Stevia rebaudiana* Bertoni. Glucosylated steviol glycosides are obtained through the enzymatic addition of glucose [1–20 additional subunits via α-(1-4) glucosyl linkages] to purified steviol glycosides obtained from the leaves of *Stevia rebaudiana* Bertoni. Cyclomaltodextrin glucanotransferase (EC 2.4.1.19) and α-amylase (EC 3.2.1.1) from non-toxigenic non-pathogenic strains of *Bacillus stearothermophilus*, *Bacillus licheniformis*, and *Bacillus subtilis* are used to facilitate the transfer of glucose to steviol glycosides. The resulting material is heated heating and treated with activated carbon to remove the enzymes. The raw product is concentrated using resin adsorption/desorption, followed by purification and preparation of the product of commerce using processes that may include decolourization, crystallization, and spray drying.

This manufacturing technique maximizes the production of enzyme modified glucosylated steviol glycosides that are not naturally present in the leaf extract.

[Note: Strains of *Bacillus stearothermophilus* and *Bacillus licheniformis* used to produce the enzymes used in this manufacturing technique may be products of genetic modification.]

Chemical names

See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK

C.A.S number

See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK

Chemical formula

See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK

Assay

Not less than 95% of total of steviol glycosides, on the dried , dextrin-free basis, determined as the sum of glucosylated steviol glycosides and steviol glycosides

DESCRIPTION

White to light yellow powder, odourless or having a slight characteristic odour. About 100 - 167 times sweeter than sucrose.

FUNCTIONAL USES

Sweetener

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)	Freely soluble in water
HPLC chromatographic profile	Following treatment with glucoamylase, the main peaks in a chromatogram obtained by analysing a sample following the procedure in METHOD OF ASSAY correspond to steviol glycosides from *Stevia rebaudiana* Bertoni
pH (Vol. 4)	Between 4.5 and 7.0 (1 in 100 solution)

PURITY

Total Ash (Vol. 4)	Not more than 1%
Loss on drying (Vol. 4)	Not more than 6% (105°, 2 h)
Residual solvents (Vol. 4)	Not more than 200 mg/kg methanol and not more than 5000 mg/kg ethanol (Method I, General Methods, Organic Components, Residual Solvents)
Arsenic (Vol. 4)	Not more than 1 mg/kg Determine using a method appropriate to the specified level (Use Method II to prepare sample solution). The selection of sample size and method of sample preparation may be based on the principles of the methods described in Vol. 4 (under “General Methods, Metallic Impurities”).
Lead (Vol. 4)	Not more than 1 mg/kg Determine using a method appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4, under “General Methods, Metallic Impurities”.
Microbiological criteria (Vol. 4)	Total (aerobic) plate count: Not more than 1,000 CFU/g Yeasts and moulds: Not more than 200 CFU/g *E. coli*: Negative in 1 g *Salmonella*: Negative in 25 g
METHOD OF ASSAY	Total steviol glycosides in enzyme modified glucosylated steviol glycosides are measured as the combined percentage of steviol glycosides and glucosylated steviol glycosides on the dried, dextrin-free basis. The percentage of steviol glycosides (those with analytical standards; these are present as unreacted steviol glycoside residues from the manufacturing process) is determined using a chromatographic method (See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK). The percentage of glucosylated steviol glycosides as glucose after

hydrolysis with glucoamylase using the colorimetric method described below. The content of dextrin is gravimetrically determined as indicated following the determination of glucosylated steviol glycosides.

Preparation of Sample Solution:
Weigh accurately about 1 g of sample and dissolve in 50 mL of water. Pass this solution through a glass column (25-mm internal diameter) packed with 50 mL of acrylic acid ester resin or styrene-divinyl-benzene resin at a rate of less than 3 mL/min. Wash the resin with 250 mL of water; collect the aqueous eluate and evaporate to dryness under vacuum for estimation of dextrin content (if necessary; Sample solution B). Pass 250 mL of 50% (v/v) ethanol through the column at a rate of not more than 3 mL/min. Evaporate the ethanolic eluate to about 100 mL, add 40 mL of acetate buffer (pH 4.5) and water to make about 180 mL. Allow this solution to stand at 55°C for about 5 min; add 20,000 units of glucoamylase, and allow to stand at 55°C for 45 min. Heat at 95°C for about 30 min, cool to room temperature, transfer to a 200-mL volumetric flask and dilute to volume with water (Sample solution A).

Procedure:
Proceed as directed under the METHOD OF ASSAY in the GENERAL SPECIFICATIONS FOR STEVIOL GLYCOSIDES using Sample solution A.

Calculate the percentage of major steviol glycosides in the sample by summation of percentages of individual steviol glycosides in the sample.

Determination of Glucosylated Steviol Glycosides as Glucose:

Prepartion of Standard solutions:
Transfer 1 g of glucose to a 100-mL volumetric flask and dilute to volume with water. Using this stock solution, prepare a series of solutions containing 0.5 – 3.0 mg/mL of glucose in water.

Preparation of Sample solution:
Use Sample solution A as prepared for the Determination of Steviol Glycosides by HPLC.

Blank Sample solution:
Dilute 40 mL of acetate buffer (pH 4.5) to 180 mL with water and hold at 55° for about 5 min. Add 20,000 units of glucoamylase and allow to stand at 55° for 45 min. Heat the solution at 95° for 30 min, cool to room temperature, transfer to a 200-mL volumetric flask and dilute to volume with water.

Color fixing solution:

Dissolve 0.50 g of phenol, 130 units of galactose mutarotase, 9000 units of glucose oxidase, 650 units of peroxidase and 0.1 g of 4-aminoantipyrine in phosphate buffer (pH 7.1) and make to exactly 1L. Store at 2-10° and use within 1 month of preparation.

Procedure:
Add 20 µL of the sample solution to 3.0 mL of color fixing solution and allow to stand at 37° for exactly 5 min. Cool to room temperature and measure the absorbance of the resulting solution at a wavelength of 505 nm against a reference solution similarly prepared using 20 µL of water in place of the sample solution. Perform a blank test by measuring the absorbance of the blank sample solution prepared in the same manner as the sample solution and make any necessary correction.

Prepare a calibration curve by measuring the absorbances of the standard solutions prepared in the same manner as the sample solution.

Determine the concentration of d-glucose in the sample solution (corrected by subtracting the absorbance of the blank test solution) from the calibration curve and calculate the α-glucosyl residue content by the following formula:

$$\text{α-glucosyl residues (\%, w/w)} = C_{glucose} / (C_{sample} \times 0.900 \times 100)$$

Where:
- $C_{glucose}$ is the concentration of d-glucose in the sample solution as determined from the calibration curve (mg/mL)
- C_{sample} is the concentration of enzyme modified glucosylated steviol glycosides in the sample solution (mg/mL)

Total steviol glycosides content
Calculate the total steviol glycosides content on the dried basis by the following formula:

$$TSG = (A + B) \times 100 / (100 – M)$$

Where:
- TSG is the Total steviol glycosides content (%w/w, on the dried basis)
- A is the percent of steviol glycosides
- B is the percent of α-glucosyl residues
- M is the percent loss on drying

Where necessary, the content of residual dextrin can be determined as follows:

Evaporate Sample solution B (as prepared for the Determination of Steviol Glycosides by HPLC) to dryness. Further dry the residue in a vacuum oven at 105° for 2h and record the dry

weight of the fraction. Calculate the content of residual dextrin by the following formula:

Residual dextrin (%, w/w) = $(W_{eluate} / W_{sample}) \times 100$

Where:
- W_{eluate} is the weight of the dried aqueous fraction (Sample solution B, g)
- W_{sample} is the dry weight of the enzyme modified glucosylated steviol glycosides used to prepare Sample solution B (g)

If the content of residual dextrin is more than 3%, the adjusted TSG on the dextrin-free basis is calculated by the following formula:

Adjusted TSG (%, w/w) = $TSG \times (W_{sample}) / (W_{sample} – RD)$

Where:
- W_{sample} is the dry weight of the enzyme modified glucosylated steviol glycosides used to prepare the Sample solutions (g)
- RD is the amount of residual dextrin present in the sample (g)