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Steviol Glycosides From *Stevia Rebaudiana* Bertoni
(Tentative)

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STEVIOL GLYCOSIDES FROM *STEVIA REBAUDIANA* BERTONI (TENTATIVE)

Tentative specifications prepared at the 82nd JECFA (2016) and published in FAO JECFA Monograph 19 (2016) superseding specifications prepared at the 73rd JECFA (2010) and published in FAO JECFA Monographs 10 (2010). An ADI of 0 - 4 mg/kg bw (expressed as steviol) was established at the 69th JECFA (2008).

Information required by December 2017:

- *Method of Assay to replace the existing method and including as many steviol glycosides as possible (at least those listed in Appendix 1) in steviol glycoside mixtures, along with supporting validation information and chromatograms*
- *Analysis results from a minimum of 5 batches for commercial samples including supporting chromatograms*

SYNONYMS

INS no. 960

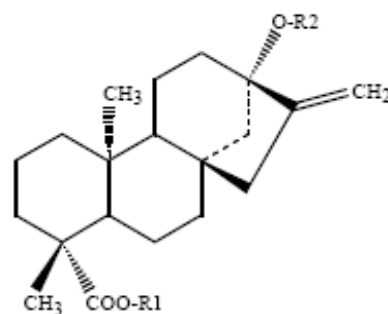
DEFINITION

Steviol glycosides consists of a mixture of compounds 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. 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 name, C.A.S. number, Chemical formula

See Appendix 1

Structural formula



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.

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 the sample chromatogram obtained by following the procedure in METHOD OF ASSAY correspond to steviol glycoside compounds.

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°, 2h)

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 the test (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 Vol. 4 (under "General Methods, Metallic Impurities").

METHOD OF ASSAY Determine the percentages of the individual steviol glycosides by HPLC (Vol. 4) under the following conditions.

Reagents

Acetonitrile: more than 95% transmittance at 210 nm.

Standards

Stevioside: more than 99.0% purity on the dried basis.

Rebaudioside A: more than 99.0% purity on the dried basis.

Mixture of nine steviol glycosides standard solution: Containing stevioside, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside F, dulcoside A, rubusoside and steviolbioside. This solution is diluted with water-acetonitrile (7:3) accordingly and is used for the confirmation of retention times. Standards are available from Wako Pure Chemical Industries, Ltd. Japan and ChromaDex, USA.

Standard solution

Accurately weigh 50 mg of stevioside and rebaudioside A standard into each of two 50-ml volumetric flasks. Dissolve and make up to volume with water-acetonitrile (7:3).

Sample solution

Accurately weigh 50-100 mg of sample into a 50-ml volumetric flask. Dissolve and make up to volume with water-acetonitrile (7:3).

Procedure

Inject 5 µl of sample solution under the following conditions.
 Column: Capcell pak C₁₈ MG II (Shiseido Co.Ltd) or Luna 5µ C18(2) 100A (Phenomenex) or equivalent (length: 250 mm; inner diameter: 4.6 mm, particle size: 5µm)
 Mobile phase: 32:68 mixture of acetonitrile and 10 mmol/L sodium phosphate buffer (pH 2.6)
 Flow rate: 1.0 ml/min
 Detector: UV at 210 nm
 Column temperature: 40°
 Record the chromatogram for about 30 min.

Identification of the peaks and Calculation

Identify the peaks from the sample solution by comparing the retention time with the peaks from the mixture of nine steviol glycosides standard solution (see under figure). Measure the peak areas for the nine steviol glycosides from the sample solution. Measure the peak area for stevioside and rebaudioside A from their standard solutions. Calculate the percentage of each of the eight steviol glycosides except rebaudioside A in the sample from the formula:

$$\%X = [W_S/W] \times [f_X A_X/A_S] \times 100$$

Calculate the percentage of rebaudioside A in the sample from the formula:

$$\% \text{Rebaudioside A} = [W_R/W] \times [A_X/A_R] \times 100$$

where

X is each steviol glycoside;

W_S is the amount (mg) calculated on the dried basis of stevioside in the standard solution;

W_R is the amount (mg) calculated on the dried basis of rebaudioside A in the standard solution;

W is the amount (mg) calculated on the dried basis of sample in the sample solution;

A_S is the peak area for stevioside from the standard solution;

A_R is the peak area for rebaudioside from the standard solution;
 A_X is the peak area of X for the sample solution; and
 f_X is the ratio of the formula weight of X to the formula weight of stevioside: 1.00 (stevioside), 1.20 (rebaudioside A), 1.00 (rebaudioside B), 1.18 (rebaudioside C), 1.40 (rebaudioside D), 1.16 (rebaudioside F), 0.98 (dulcoside A), 0.80 (rubusoside) and 0.80 (steviolbioside).

Calculate the percentage of total steviol glycosides (sum the nine percentages).

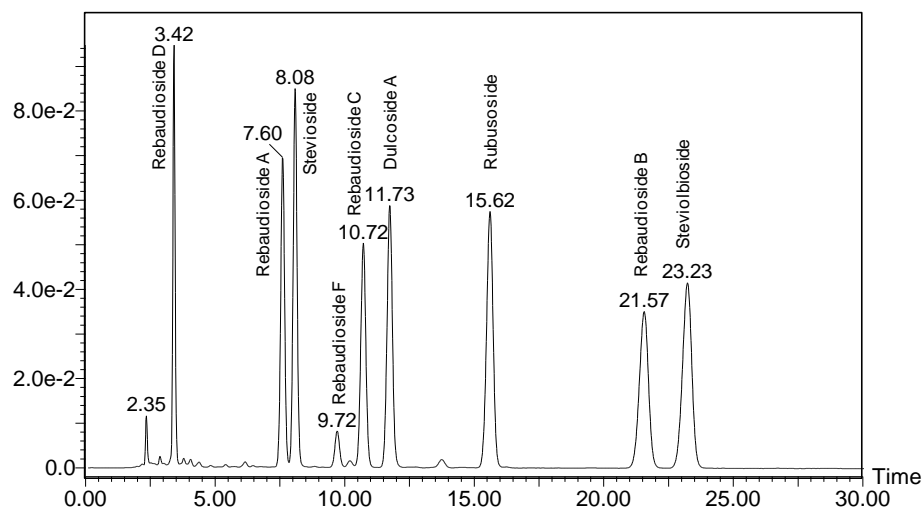


Figure. Chromatogram of mixture of nine steviol glycosides standard solution

Column: Capcell pak C₁₈ MG II

Concentration: 0.5 mg/ml each except rebaudioside F (about 0.1 mg/ml)

Appendix 1: 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-Glucopyranosyl-(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

Common Name	R ₁	R ₂	Chemical Name	CAS Number	Chemical Formula	Formula Weight
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 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