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Steviol Glycosides from *Stevia rebaudiana* Bertoni

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STEVIOL GLYCOSIDES FROM *STEVIA REBAUDIANA* BERTONI

Prepared at the 84th JECFA (2017) and published in FAO JECFA Monographs 20 (2017), superseding tentative 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. 960

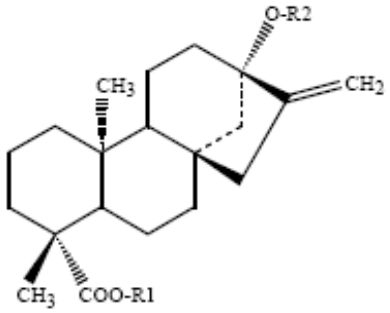
DEFINITION Steviol glycosides consist of a mixture of compounds containing a steviol backbone conjugated to any number or combination of the principal sugar moieties (glucose, rhamnose, xylose, fructose, arabinose, galactose and deoxyglucose) in any of the orientations occurring in the leaves of *Stevia 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 name See Appendix 1

C.A.S. number See Appendix 1

Chemical formula See Appendix 1

Structural formula



Steviol (R1 = R2 = H) is the aglycone of the steviol glycosides.

	Glc, Rha, Fru, deoxyGlc, Gal, Ara and Xyl represent, respectively, glucose, rhamnose, fructose, deoxyglucose xylose, galactose, arabinose and xylose sugar moieties.
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 Vol. 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 CFCU/g
E. coli: Negative in 1 g
Salmonella: Negative in 25 g

METHOD OF ASSAY

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^{o}); 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 C_{18} MG II (250 mm x 4.6 mm, 5µm, Shiseido Co. Ltd) or equiv.
- Column temperature: 50°
- Autosampler temperature: 2 – 8^{o}
- 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 are given in Appendix-3). See Appendix 2 for an example of a chromatogram obtained using 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:

$$Conc\ (\%w/w)\ = c_{sample}\ \times \frac{100}{W_{sample}}$$

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 in the Annexure.

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 given in Appendix-3) 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 in Appendix 2 is 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:

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

Where:

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

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:

$$\textbf{Minor Steviol Glycoside Conc.}\ (\%\mathbf{w/w}) = \frac{\text{Conc}_{\text{ sample}} \times 100}{\text{Weight}_{\text{ Sample}}}$$

Where

- $\text{Conc}_{\text{sample}}$ is the assayed concentration (µg/mL) in the test sample
- $\text{Weight}_{\text{sample}}$ is the sample weight in 1 mL 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:

$$TSG = \frac{(A + B) \times 100}{(100 - M)}$$

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

Annex 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

Appendix 1: Chemical Information of Some Steviol Glycosides

Common Name	Trivial Name	R_1	R_2	Chemical Name	CAS Number	Chemical Formula	Formula Weight
Group 1: Steviol + Glucose (SvGn)							
Steviolmonoside	SvG1	H	Glcβ1-	13-[(β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid	60129-60-4	$C_{26}H_{40}O_8$	481
Steviolmonoside A	SvG1	Glcβ1-	H	13-[(hydroxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	64977-89-5	$C_{26}H_{40}O_8$	481
Rubusoside	SvG2	Glcβ1-	Glcβ1-	13-[(β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	64849-39-4	$C_{32}H_{50}O_{13}$	643
Steviolbioside	SvG2	H	Glcβ(1-2)Glcβ1-	13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid	41093-60-1	$C_{32}H_{50}O_{13}$	643
Stevioside	SvG3	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	$C_{38}H_{60}O_{18}$	805
Stevioside A Or Rebaudioside KA	SvG3	Glcβ(1-2)Glcβ1-	Glcβ1-	13-[(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	127345-20-4	$C_{38}H_{60}O_{18}$	805
Stevioside B	SvG3	Glcβ(1-3)Glcβ1-	Glcβ1-	13-[(2-O-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, *O*-β-D-glucopyranosyl-deoxy-(1,3)-*O*-[β-D-glucopyranosyl ester	-	$C_{38}H_{60}O_{18}$	805
Rebaudioside *B*	SvG3	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	$C_{38}H_{60}O_{18}$	805

Common Name	Trivial Name	R_1	R_2	Chemical Name	CAS Number	Chemical Formula	Formula Weight
Rebaudioside G	SvG3	Glcβ1-	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(4')-*O*-β-D-glucopyranosyl ester	127345-21-5	$C_{38}H_{60}O_{18}$	805
Rebaudioside *E*	SvG4	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
Rebaudioside *A*	SvG4	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
Rebaudioside A2	SvG4	Glcβ1-	Glcβ(1-6)[Glcβ(1-2)]Glcβ1-	13-[(6-O-β-D-glucopyranosyl-2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl ester	1326217-29-1	$C_{44}H_{70}O_{23}$	967
Rebaudioside *D*	SvG5	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
Rebaudioside L	SvG5	Glcβ1-	Glcβ(1-6) Glcβ(1-2) [Glcβ(1-3)]Glcβ1-	13-[(6-O-β-D-glucopyranosyl-2-O-β-D-glucopyranosyl-3-β-D-glucopyranosyl- β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl ester	1220616-38-5	$C_{50}H_{80}O_{28}$	1129

Common Name	Trivial Name	R_1	R_2	Chemical Name	CAS Number	Chemical Formula	Formula Weight
Steviolmonoside	SvG1	H	Glcβ1-	13-[(β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid	60129-60-4	$C_{26}H_{40}O_8$	481
Steviolmonoside A	SvG1	Glcβ1-	H	13-[(hydroxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	64977-89-5	$C_{26}H_{40}O_8$	481
Rubusoside	SvG2	Glcβ1-	Glcβ1-	13-[(β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	64849-39-4	$C_{32}H_{50}O_{13}$	643
Steviolbioside	SvG2	H	Glcβ(1-2)Glcβ1-	13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid	41093-60-1	$C_{32}H_{50}O_{13}$	643
Stevioside	SvG3	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	$C_{38}H_{60}O_{18}$	805
Stevioside A Or Rebaudioside KA	SvG3	Glcβ(1-2)Glcβ1-	Glcβ1-	13-[(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	127345-20-4	$C_{38}H_{60}O_{18}$	805
Stevioside B	SvG3	Glcβ(1-3)Glcβ1-	Glcβ1-	13-[(2-O-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, *O*-β-D-glucopyranosyl-deoxy-(1,3)-*O*-[β-D-glucopyranosyl ester	-	$C_{38}H_{60}O_{18}$	805
Rebaudioside *B*	SvG3	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	$C_{38}H_{60}O_{18}$	805

Common Name	Trivial Name	R_1	R_2	Chemical Name	CAS Number	Chemical Formula	Formula Weight
Rebaudioside I	SvG5	Glcβ(1-3) 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, 3-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester	-	$C_{50}H_{80}O_{28}$	1129
Rebaudioside I2	SvG5	Glcβ1-	Glcα(1-3) Glcβ(1-2) [Glcβ(1-3)]Glcβ1-	13-[(3-O-β-D-glucopyranosyl-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 ester	-	$C_{50}H_{80}O_{28}$	1129
Rebaudioside I3	SvG5	[Glcβ(1-2) Glcβ(1-6)]Glcβ1-	Glcβ(1-2)Glcβ1-	13-[(2-O-β-D-glucopyranosyl-O-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl-6-O- β-D-glucopyranosyl-β-D-glucopyranosyl ester	-	$C_{50}H_{80}O_{28}$	1129
Rebaudioside Q	SvG5	Glcβ1-	Glcα(1-4)Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	13-[(4-O-β-D-glucopyranosyl-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 ester	-	$C_{50}H_{80}O_{28}$	1129
Rebaudioside Q2	SvG5	[Glcα(1-2) Glcα(1-4)] Glcβ1-	Glcβ(1-2)Glcβ1-	13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl-4-O-β-D-glucopyranosyl- β-D-glucopyranosyl ester	-	$C_{50}H_{80}O_{28}$	1129
Rebaudioside Q3	SvG5	Glcβ1-	Glcα(1-4) Glcβ(1-3) [Glcβ(1-2)]Glcβ1-	13-[(4-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-2-O-β-D-glucopyranosyl- β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl ester	-	$C_{50}H_{80}O_{28}$	1129

Common Name	Trivial Name	R_1	R_2	Chemical Name	CAS Number	Chemical Formula	Formula Weight
Rebaudioside *M*	SvG6	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	$C_{58}H_{90}O_{33}$	1291
Related SvGn#1		-	-	-	-	$C_{21}H_{30}O_{11}$	458
Related SvGn#2		-	-	-	-	$C_{40}H_{70}O_{24}$	982
Related SvGn#3		-	-	-	-	$C_{32}H_{52}O_{15}$	676
Related SvGn#4		-	-	-	-	$C_{50}H_{80}O_{28}$	1129
Related SvGn#5		-	-	-	-	$C_{40}H_{70}O_{24}$	982
Group 2: Steviol + Rhamnose + Glucose (SvR1Gn)							
Dulcoside *A*	SvR1G2	Glcβ1-	Rhaα(1-2)Glcβ1-	13-[(2-O-α–L-rhamnopyranosyl-β–D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	64432-06-0	$C_{38}H_{60}O_{17}$	789
Dulcoside C	SvR1G2	H	Rhaα(1-2)[Glcβ(1-3)] Glcβ1-	13-[(2-O-β-D-rhamnopyranosyl-3-β-D-glucopyranosyl- β-D-glucopyranosyl -oxy]kaur-16-en-18-oic acid		$C_{38}H_{60}O_{17}$	789
Rebaudioside *C*	SvR1G3	Glcβ1-	Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	13-[(2-O-α–L-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

Common Name	Trivial Name	R_1	R_2	Chemical Name	CAS Number	Chemical Formula	Formula Weight
Rebaudioside C2	SvR1G3	Rhaα(1-2)Glcβ1	Glcβ(1-2)Glcβ1-	13-[(2-O-β-D-glucopyranosyl- β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-rhamnopyranosyl- β-D-glucopyranosyl ester	-	$C_{44}H_{70}O_{22}$	951
Rebaudioside *N*	SvR1G5	Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	13-[(2-*O*-β- D-glucopyranosyl-(1,2)-*O*-[β- D-glucopyranosyl-(1,3)]-β- D-glucopyranosyl)oxy]-kaur-16-en-18-oic acid (4')-*O*-2-deoxy-L-rhmanopyranosyl-3-*O*-β- D-glucopyranosyl-β- D-glucopyranosyl ester	1220616-46-5	$C_{56}H_{90}O_{32}$	1274
Rebaudioside *O*	SvR1G6	Glcβ(1-3)Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] ent-kaur-16-en-19-oic acid-[(2-O-(3-O-β-D-glucopyranosyl-α-L-rhamnopyranosyl)-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl) ester]	1220616-48-7	$C_{62}H_{100}O_{37}$	1436
Rebaudioside *O2*	SvR1G6	Glcβ(1-4*)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-(1,4)-*O*-6- deoxy-L-rhmnopyranosyl-(1,2)-*O*-[β-D-glucopyranosyl-(1,3)]-β-D-glucopyranosyl ester	-	$C_{62}H_{100}O_{37}$	1436

Common Name	Trivial Name	R_1	R_2	Chemical Name	CAS Number	Chemical Formula	Formula Weight
Rebaudioside K	SvR1G4	Glcβ(1-2)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, 2-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester	1220616-40-9	$C_{50}H_{80}O_{27}$	1112
Rebaudioside *S*	SvR1G3	Rhaα(1-2)Glcβ1-	Glcα (1-2)Glcβ1-	13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, O-2-deoxy-L-rhamnopyranosyl β-D-glucopyranosyl ester	1931085-11-8	$C_{44}H_{70}O_{22}$	951
Rebaudioside *K2*	SvR1G4	Glcβ(1-6)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, 6-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester	-	$C_{50}H_{80}O_{27}$	1112
Rebaudioside *H*	SvR1G4	Glcβ1-	Glcβ(1-3)Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	13-[(3-*O*-β-D-glucopyranosyl-2-O-β-D-rhamnopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	1220616-36-3	$C_{50}H_{80}O_{27}$	1112
Rebaudioside *J*	SvR1G4	Rhaα(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-6- deoxy-L-rhmnopyranosyl-β-D-glucopyranosyl ester	1313049-59-0	$C_{50}H_{80}O_{27}$	1112
Group 3: Steviol + Xylose + Glucose (SvX1Gn)							
Stevioside F	SvX1G2	Glcβ1-	Xylβ(1-2)Glcβ1-	13-[(2-O-β-D-xylopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	-	$C_{37}H_{59}O_{17}$	775

Common Name	Trivial Name	R_1	R_2	Chemical Name	CAS Number	Chemical Formula	Formula Weight
Rebaudioside *F*	SvX1G3	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	$C_{43}H_{68}O_{22}$	937
Rebaudioside F2	SvX1G3	Glcβ1-	Glcβ (1-2)[Xylβ (1-3)]Glcβ1-	13-[(2-O-β-D-glucopyranosyl-3-O-β-D-xylopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	-	$C_{43}H_{68}O_{22}$	937
Rebaudioside F3	SvX1G3	Xylβ(1-6) Glcβ1-	Glcβ(1-2)Glcβ1-	13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 6-O-β-D-xylopyranosyl-β-D-glucopyranosyl ester	-	$C_{43}H_{68}O_{22}$	937
Rebaudioside R	SvX1G3	Glcβ1-	Glcβ(1-2)[Glcβ1-3] Xylβ1	13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-xylopyranosyl-3)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	1931083-53-2	$C_{43}H_{68}O_{22}$	937
Rebaudioside *U2*	SvX1G4	Xylβ(1-2*)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)Glcβ1-	13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-xylopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester	-	$C_{50}H_{82}O_{26}$	1099
Rebaudioside *T*	SvX1G4	Xylβ(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-xylopyranosyl-β-D-glucopyranosyl ester		$C_{50}H_{82}O_{26}$	1099
Rebaudioside *V2*	SvX1G5	Xylβ (1-2)[Glcβ(1-3)]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-xylopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester	-	$C_{56}H_{92}O_{31}$	1261

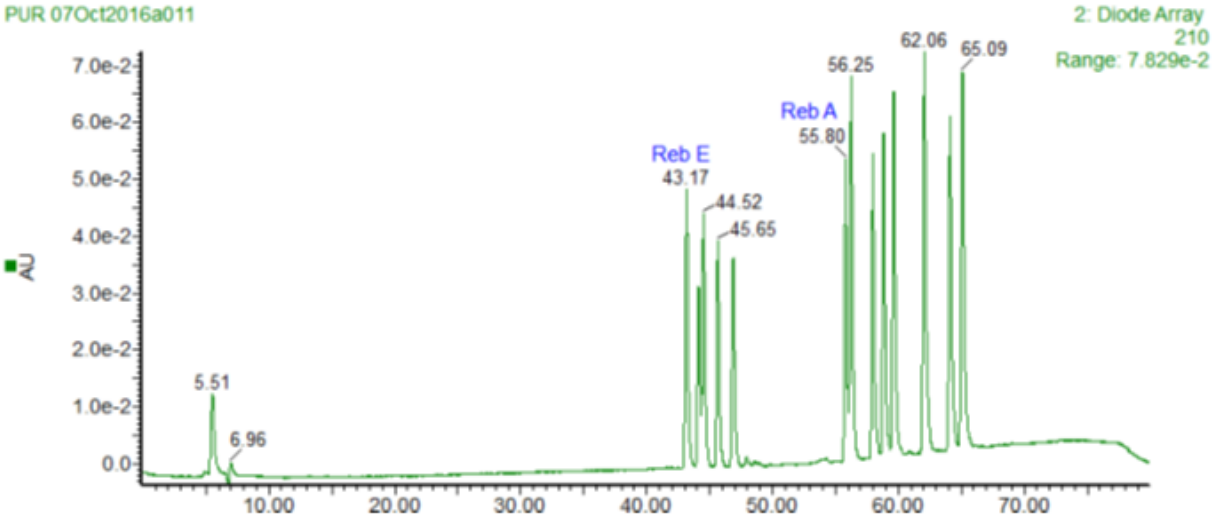
Common Name	Trivial Name	R_1	R_2	Chemical Name	CAS Number	Chemical Formula	Formula Weight
Rebaudioside *V*	SvX1G5	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Xylβ(1-2*)[Glcβ(1-3)]Glcβ1-	13-[(2-O-β-D-xylopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester	-	$C_{56}H_{92}O_{31}$	1261
Group 4: Steviol + Arabinose + Glucose (SvA1Gn)							
Rebaudioside U	SvA1G4	Araα(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]ent-kaur-16-en-19-oic acid-(6-O-α--L-arabinopyranosyl- β -D-glucopyranosyl) ester		$C_{50}H_{82}O_{26}$	1098
Rebaudioside W	SvA1G4	Glcβ(1-2)[Araβ(1-3*)]Glcβ1	Glcβ(1-2)Glcβ1-	13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-Dglucopyranosyl-3-O-β-D-arabinopyranosyl-β-D-glucopyranosyl ester	-	$C_{50}H_{82}O_{26}$	1098
Rebaudioside *W2*	SvA1G4	Araβ(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-arabinopyranosyl-β-D-glucopyranosyl ester	-	$C_{50}H_{82}O_{26}$	1098
Rebaudioside *W3*	SvA1G4	Araβ(1-6)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, 6-O-β-D-arabinopyranosyl-β-D-glucopyranosyl ester	-	$C_{50}H_{82}O_{26}$	1098
Rebaudioside Y	SvA1G5	Glcβ(1-2)[Araβ(1-3*)]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-3-O-β-D-arabinopyranosyl-β-D-glucopyranosyl ester	-	$C_{56}H_{92}O_{31}$	1260

Common Name	Trivial Name	R_1	R_2	Chemical Name	CAS Number	Chemical Formula	Formula Weight
Group 5: Steviol + Galactose + Glucose (SvGa1Gn)							
Rebaudioside T1	SvGa1G4	Galβ(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-galactopyranosyl-β-D-glucopyranosyl ester	-	C50H80O28	1128
Group 6: Steviol + Fructose + Glucose (SvFruGn)							
Rebaudioside *A3*	SbF1G3	Glcβ1-	Glcβ(1-2)[Fruβ(1-3)]Glcβ1-	13-[(2-O-β-D-glucopyranosyl-3-O-β-D-fructofuranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	-	$C_{44}H_{70}O_{22}$	951
Group 7: Steviol + -de-oxy glucose + Glucose (SvdG1Gn)							
Stevioside D	SvDg1G2	Glcβ1-	6-deoxy Glcβ(1-2)Glcβ1-	13-[(2-O-β-D-6-deoxyglucopyranosyl-β-D-glucopyranosy)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	-	$C_{38}H_{60}O_{17}$	789
Stevioside E	SvDg1G3	Glcβ1-	6-deoxy Glcβ(1-2)[Glcβ(1-3)] Glcβ1-	13-[(2-O-β-D-6-deoxyglucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	-	$C_{44}H_{70}O_{22}$	951
Stevioside E2	SvDg1G3	6-deoxy 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-6-deoxyglucopyranosyl-ester	-	$C_{44}H_{70}O_{22}$	951

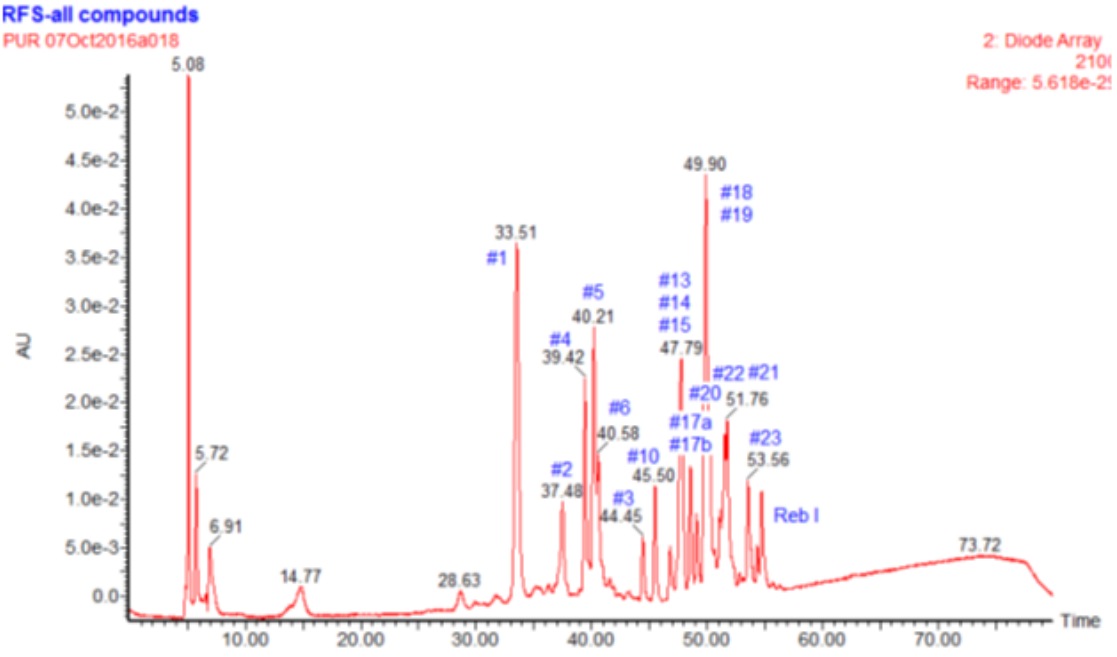
Steviol (R1 = R2 = H) is the aglycone of the steviol glycosides.
Glc, Rha, Fru, deoxyGlc, Gal, Ara and Xyl represent, respectively, glucose, rhamnose, fructose, deoxyglucose, galctose, arabinose and xylose sugar moieties.

Note: This list is not exhaustive. More steviol glycosides may have been identified in stevia leaf extracts in the literature

Appendix- 2: Representative chromatograms for steviol glycosides using Method of Assay



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.

Appendix-3: Typical Retention Time (RT), Relative Retention Time (RRT) and Mass Ions of Steviol Glycosides

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.