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(Framework for) Steviol glycosides

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(Framework for) Steviol glycosides

Revised at 96th JECFA (2023) and published in FAO Monographs 31 (2023), superseding specifications prepared at the 91st JECFA (2021) and published in FAO Monographs 26 (2021). An ADI of 0 – 4 mg/kg bw (expressed as steviol) was established at the 69th JECFA (2008).

Introduction

Steviol glycosides are constituents of the leaves of the plant, *Stevia rebaudiana* Bertoni and have a sweet taste. Steviol glycosides have the same steviol aglycone bound to different types and numbers of glycoside units (e.g., glucose, rhamnose, xylose, fructose, or deoxyglucose). More than forty steviol glycosides have been identified to date (see Appendix A). The functional use of steviol glycosides in food is as a sweetener. They are 100 to 300 times sweeter than sucrose.

Background

Steviol glycosides produced by extraction from the leaves of *Stevia rebaudiana* Bertoni were reviewed by the Committee at its fifty-first, sixty-third, sixty-eighth, eighty-second, eighty-fourth, eighty-seventh and ninety-first meetings. At the sixty-eighth meeting the Committee extended the previously designated temporary ADI of 0–2 mg/kg bw for steviol glycosides, expressed as steviol, pending submission of the results of then-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 received additional data and reevaluated steviol glycosides from *S. rebaudiana* Bertoni. The Committee at that meeting 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 for *Steviol glycosides* was prepared.

At the eighty-second meeting, the Committee evaluated steviol glycosides produced by fermentation of a strain of *Yarrowia lipolytica*, genetically modified to simulate the *S. 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 *S. rebaudiana* Bertoni; an ADI of 0–4 mg/kg bw, expressed as steviol equivalents was applied. A new specifications monograph was prepared for *Rebaudioside A from multiple gene donors expressed in Yarrowia lipolytica* to reflect considerations resulting from this method of manufacture. The existing specifications monograph for *Steviol glycosides* was revised with new tentative specifications and the new title of *Steviol glycosides from Stevia rebaudiana Bertoni*. The Definition and Assay were expanded from nine named leaf-derived steviol glycosides to include additional steviol glycoside compounds derived from *S. rebaudiana* Bertoni, provided that the total percentage of steviol glycosides is not less than 95%. The specifications for *Steviol glycosides from S. rebaudiana Bertoni* were established as tentative, pending receipt of a method of assay that was also capable of measuring minor steviol glycosides. At the eighty-fourth meeting, the Committee revised the specifications for *Steviol glycosides from Stevia rebaudiana Bertoni* and removed the tentative status.

At the eighty-seventh meeting, the Committee reviewed data on the methods of manufacture, identity, and purity of steviol glycosides. The Committee noted that the reviewed products consist of > 95% steviol glycosides on the dried basis; the remainder consists of residues of starting material and food-grade processing aids depending on the method of production. The Committee recognized that the < 5% residues may contain impurities other than those listed above, and it is the responsibility of manufacturers to address these issues. The *(Framework*

for) steviol glycosides combined specifications monograph was prepared with four Annexes describing steviol glycosides based on the method of manufacture.

At the present meeting, the Committee revised chemical information for steviol glycosides (Appendix A), the steviol glycosides assay method (Appendix B), the glucosylated steviol glycosides assay method (Annex 4) and the solubility for all steviol glycosides (Annexes 1-3) in the (Framework for) Steviol Glycosides. The Committee also removed the tentative status from Enzyme modified glucosylated steviol glycosides (Annex 4).

Explanation for the framework approach

The two previously existing specification monographs for steviol glycosides required 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 *S. rebaudiana* Bertoni plant are stevioside and rebaudioside A, and the minor glycosides include rebaudioside M and rebaudioside D. Several minor glycosides have been determined to have more favourable sensory characteristics than the major glycosides. This has prompted development of new technologies to produce steviol glycosides with higher proportions of minor glycosides to modify the sensory profile of the articles of commerce. The current framework was developed to address steviol glycosides manufactured using the four existing methods.

The Annexes include the method of production as well as assay and impurity specifications and shall be used in conjunction with information contained elsewhere in the framework including Appendix A and Appendix B. To meet the requirements of the present monograph, steviol glycosides should be produced as described in one of the Annexes (described below) and meet the corresponding specifications. Modifications in the production method will require revisions to an existing Annex or the development of a new Annex. An Annex could have a tentative status if more information is required to complete it. The tentative status of one Annex does not affect the status of the other Annexes.

- Annex 1 - Steviol glycosides from *Stevia rebaudiana* Bertoni: extraction of the leaves of *Stevia rebaudiana* Bertoni.
- Annex 2 - Steviol glycosides from fermentation: a process in which a genetically modified microorganism is used to produce specific steviol glycosides.
- Annex 3 - Enzyme modified steviol glycosides: 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.
- Annex 4 - Enzyme modified glucosylated steviol glycosides: a process in which steviol glycosides that have been extracted from the leaves of *Stevia rebaudiana* Bertoni undergo enzyme catalyzed reactions to add glucose units to the steviol glycosides via α-(1-4) linkages.

Table A.
Summary of Formula and R-Groups of Identified Steviol Glycosides from the Leaves of *Stevia rebaudiana* Bertoni (see Figure A for Backbone Structure)
[Adapted from Purkayastha & Kwok (2020)]

#	Common Name	CAS Number	Trivial Formula	Mol. Wt	Steviol Equivalent	R_1	R_2	Reference
1. Steviol + Glucose (SvGn)								
1.01	Steviolmonoside		SvG1	481	0.66	H	Glcβ1-	Ohta et al. (2010)
1.02	Steviolmonoside A		SvG1	481	0.66	Glcβ1-	H	Gardana et al. (2010)
1.03	Rubusoside	64849-39-4	SvG2	643	0.49	Glcβ1-	Glcβ1-	Ohta et al. (2010)
1.04	Steviolbioside	41093-60-1	SvG2	643	0.49	H	Glcβ(1-2)Glcβ1-	Kohda et al. (1976)
1.05	Stevioside	57817-89-7	SvG3	805	0.40	Glcβ1-	Glcβ(1-2)Glcβ1-	Bridel & Lavielle (1931)
1.06	Stevioside A		SvG3	805	0.40	Glcβ(1-2)Glcβ1-	Glcβ1-	Wu et al. (2012)
1.07	Rebaudioside B	58543-17-2	SvG3	805	0.40	H	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Kohda et al. (1976)
1.08	Rebaudioside G		SvG3	805	0.40	Glcβ1-	Glcβ(1-3)Glcβ1-	Ohta et al. (2010)
1.09	Stevioside B		SvG3	805	0.40	Glcβ(1-3)Glcβ1-	Glcβ1-	Chaturvedula & Zamora (2014)
1.10	Rebaudioside E	63279-14-1	SvG4	967	0.33	Glcβ(1-2)Glcβ1-	Glcβ(1-2)Glcβ1-	Sakamoto et al. (1977a)
1.11	Rebaudioside A	58543-16-1	SvG4	967	0.33	Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Kohda et al. (1976)
1.12	Rebaudioside A2		SvG4	967	0.33	Glcβ1-	Glcβ(1-6)Glcβ(1-2)Glcβ1-	Chaturvedula & Prakash (2011a)
1.13	Rebaudioside D	63279-13-0	SvG5	1129	0.28	Glcβ(1-2)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Sakamoto et al. (1977a)

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#	Common Name	CAS Number	Trivial Formula	Mol. Wt	Steviol Equivalent	R_1	R_2	Reference
1.14	Rebaudioside I		SvG5	1129	0.28	Glcβ(1-3)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Ohta et al. (2010)
1.15	Rebaudioside L		SvG5	1129	0.28	Glcβ1-	Glcβ(1-6)Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Ohta et al. (2010)
1.16	Rebaudioside Q2		SvG5	1129	0.28	Glcα(1-2)Glcα(1-4)Glcβ1-	Glcβ(1-2)Glcβ1-	Chaturvedula & Prakash (2011b)
1.17	Rebaudioside Q		SvG5	1129	0.28	Glcβ1-	Glcα(1-4)Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	-
1.18	Rebaudioside I2		SvG5	1129	0.28	Glcβ1-	Glcα(1-3)Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Chaturvedula et al. (2011a)
1.19	Rebaudioside Q3		SvG5	1129	0.28	Glcβ1-	Glcα(1-4)Glcβ(1-3)[Glcβ(1-2)]Glcβ1-	Chaturvedula et al. (2011a)
1.20	Rebaudioside I3		SvG5	1129	0.28	Glcβ(1-2)[Glcβ(1-6)]Glcβ1-	Glcβ(1-2)Glcβ1-	Chaturvedula et al. (2011a)
1.21	Rebaudioside AM		SvG5	1129	0.28	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)Glcβ1-	Prakash & Ma (2018)
1.22	Rebaudioside M	1220616-44-3	SvG6	1291	0.25	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Ohta et al. (2010)
1.23	Rebaudioside 1h		SvG7	1453	0.22	Glcβ(1-3)Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Prakash & Ma (2018)
1.24	Rebaudioside IX		SvG9	1778	0.18	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-3){Glcβ(1-3)[Glcβ(1-2)]Glcα(1-6)Glcβ(1-2)}Glcβ1-	Prakash & Ma (2018)

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#	Common Name	CAS Number	Trivial Formula	Mol. Wt	Steviol Equivalent	R_1	R_2	Reference
1.14	Rebaudioside I		SvG5	1129	0.28	Glcβ(1-3)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Ohta et al. (2010)
1.15	Rebaudioside L		SvG5	1129	0.28	Glcβ1-	Glcβ(1-6)Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Ohta et al. (2010)
1.16	Rebaudioside Q2		SvG5	1129	0.28	Glcα(1-2)Glcα(1-4)Glcβ1-	Glcβ(1-2)Glcβ1-	Chaturvedula & Prakash (2011b)
1.17	Rebaudioside Q		SvG5	1129	0.28	Glcβ1-	Glcα(1-4)Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	-
1.18	Rebaudioside I2		SvG5	1129	0.28	Glcβ1-	Glcα(1-3)Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Chaturvedula et al. (2011a)
1.19	Rebaudioside Q3		SvG5	1129	0.28	Glcβ1-	Glcα(1-4)Glcβ(1-3)[Glcβ(1-2)]Glcβ1-	Chaturvedula et al. (2011a)
1.20	Rebaudioside I3		SvG5	1129	0.28	Glcβ(1-2)[Glcβ(1-6)]Glcβ1-	Glcβ(1-2)Glcβ1-	Chaturvedula et al. (2011a)
1.21	Rebaudioside AM		SvG5	1129	0.28	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)Glcβ1-	Prakash & Ma (2018)
1.22	Rebaudioside M	1220616-44-3	SvG6	1291	0.25	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Ohta et al. (2010)
1.23	Rebaudioside 1h		SvG7	1453	0.22	Glcβ(1-3)Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Prakash & Ma (2018)
1.24	Rebaudioside IX		SvG9	1778	0.18	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-3){Glcβ(1-3)[Glcβ(1-2)]Glcα(1-6)Glcβ(1-2)}Glcβ1-	Prakash & Ma (2018)

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[Adapted from Purkayastha & Kwok (2020)]

#	Common Name	CAS Number	Trivial Formula	Mol. Wt	Steviol Equivalent	R_1	R_2	Reference
2.02	Dulcoside B		SvR1G2	789	0.40	H	Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Ohta et al. (2010)
2.03	Rebaudioside C	63550-99-2	SvR1G3	951	0.33	Glcβ1-	Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Sakamoto et al. (1977b)
2.04	Rebaudioside C2		SvR1G3	951	0.33	Rhaα(1-2)Glcβ1-	Glcβ(1-2)Glcβ1-	Purkayastha et al. (2019)
2.05	Rebaudioside S		SvR1G3	951	0.33	Rhaα(1-2)Glcβ1-	Glcα(1-2)Glcβ1-	Ibrahim et al (2016)
2.06	Rebaudioside H		SvR1G4	1113	0.29	Glcβ1-	Glcβ(1-3)Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Ohta et al. (2010)
2.07	Rebaudioside K		SvR1G4	1113	0.29	Glcβ(1-2)Glcβ1-	Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Ohta et al. (2010)
2.08	Rebaudioside K2		SvR1G4	1113	0.29	Glcβ(1-6)Glcβ1-	Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Purkayastha et al. (2019)
2.09	Rebaudioside J		SvR1G4	1113	0.29	Rhaα(1-2)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Ohta et al. (2010)
2.10	Rebaudioside N	1220616-46-5	SvR1G5	1275	0.25	Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Ohta et al. (2010)
2.11	Rebaudioside N2		SvR1G5	1275	0.25	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Prakash & Ma (2018)
2.12	Rebaudioside N6		SvR1G5	1275	0.25	Glcβ(1-3)Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)Glcβ1-	Prakash & Ma (2018)
2.13	Rebaudioside O	1220616-48-7	SvR1G6	1437	0.22	Glcβ(1-3)Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Ohta et al. (2010)
2.14	Rebaudioside O2		SvR1G6	1437	0.22	Glcβ(1-4)Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Purkayastha et al. (2016)

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#	Common Name	CAS Number	Trivial Formula	Mol. Wt	Steviol Equivalent	R_1	R_2	Reference
2.16	Rebaudioside O6		SvR1G7	1600	0.20	Glcβ(1-3)Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-6)Glcβ(1-3)[Glcβ(1-2)]Glcβ1-	Prakash & Ma (2018)
2.17	Rebaudioside O7		SvR2G6	1584	0.20	Glcβ(1-3)Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-3)Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Prakash & Ma (2018)
3. Steviol + Xylose + Glucose (SvX1Gn)								
3.01	Stevioside F		SvX1G2	775	0.41	Glcβ1-	Xylβ(1-2)Glcβ1-	Chaturvedula & Prakash (2011c)
3.02	Rebaudioside F	438045-89-7	SvX1G3	937	0.34	Glcβ1-	Xylβ(1-2)[Glcβ(1-3)]Glcβ1-	Starratt et al. (2002)
3.03	Rebaudioside F2		SvX1G3	937	0.34	Glcβ1-	Glcβ(1-2)[Xylβ(1-3)]Glcβ1-	Chaturvedula & Prakash (2011c)
3.04	Rebaudioside F3		SvX1G3	937	0.34	Xylβ(1-6)Glcβ1-	Glcβ(1-2)Glcβ1-	Chaturvedula et al. (2011b)
3.05	Rebaudioside R		SvX1G3	937	0.34	Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Xylβ1-	Ibrahim et al (2016)
3.06	Rebaudioside U		SvX1G4	1099	0.29	Xylβ(1-2)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Purkayastha et al. (2019)
3.07	Rebaudioside U2		SvX1G4	1099	0.29	Xylβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)Glcβ1-	Purkayastha et al. (2016)
3.08	Rebaudioside U3		SvX1G4	1099	0.29	Xylβ(1-2)[Glcβ(1-4)]Glcβ1-	Glcβ(1-2)Glcβ1-	Purkayastha et al. (2019)
3.09	Rebaudioside V		SvX1G5	1261	0.25	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Xylβ(1-3)]Glcβ1-	Purkayastha et al. (2019)
3.10	Rebaudioside V2		SvX1G5	1261	0.25	Xylβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Prakash & Chaturvedula (2013)

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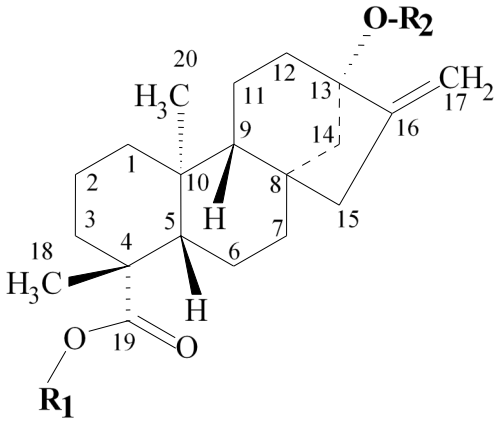
#	Common Name	CAS Number	Trivial Formula	Mol. Wt	Steviol Equivalent	R_1	R_2	Reference
4.02	Rebaudioside W2		SvA1G4	1099	0.29	Araα(1-2)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Purkayastha et al. (2016)
4.03	Rebaudioside W3		SvA1G4	1099	0.29	Araα(1-6)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Purkayastha et al. (2019)
4.04	Rebaudioside Y		SvA1G5	1261	0.25	Glcβ(1-2)[Araα(1-6)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Purkayastha et al. (2019)
5. Steviol + Fructose + Glucose (SvF1Gn)								
5.01	Rebaudioside A3		SvF1G3	967	0.33	Glcβ1-	Glcβ(1-2)[Fruβ(2-3)]Glcβ1-	Chaturvedula et al. (2011c)
6. Steviol + Galactose + Glucose (SvGa1Gn)								
6.01	Rebaudioside T		SvGa1G4	1129	0.28	Glcβ(1-2)Glcβ1-	Galβ(1-2)[Glcβ(1-3)]Glcβ1-	Purkayastha et al. (2016)
7. Steviol + Deoxyglucose + Glucose (SvdG1Gn)								
7.01	Stevioside D		SvdG1G2	789	0.40	Glcβ1-	6-deoxyGlcβ(1-2)Glcβ1-	Chaturvedula & Prakash (2011d)
7.02	Stevioside E		SvdG1G3	951	0.33	Glcβ1-	6-deoxyGlcβ(1-2)[Glcβ(1-3)]Glcβ1-	Chaturvedula & Prakash (2011d)
7.03	Stevioside E2		SvdG1G3	951	0.33	6-deoxyGlcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Chaturvedula et al. (2011d)

Figure A

Backbone structure for steviol glycosides



Appendix B: Method of Assay Details

Method of assay

METHOD OF ASSAY (for annexes 1-4)

Determine the percentages of major steviol glycosides (those with analytical standards, e.g. rebaudioside A, B, C, D, E, F, M, N, O; dulcoside A; rubusoside; stevioside; and steviolbioside) on the dry basis using an HPLC-UV technique (see Part 1 and HPLC, Vol. 4). If the sum of the major steviol glycosides in the sample is <95%, an optional HPLC-UV-MS based technique may be utilized to identify the minor steviol glycosides (see Part 2) and obtain their corresponding molecular weights. The minor steviol glycosides are quantified using either the standard curve of the commercially available minor steviol glycoside reference standards or the respective molecular weight-corrected UV peak area and the rebaudioside A standard curve (obtained using Part 1). Calculate the sum of the major and minor (if applicable) steviol glycosides and express the total glycoside content on the dried basis.

Reagents:

- Mobile phase A: Deionized water, HPLC or LC-MS grade, filtered using a 0.2-µm filter, with 0.1% formic acid or acetic acid. (Note: If only UV detection will be used, 20 mM sodium phosphate buffer at pH 2.6 or 0.01% trifluoroacetic acid may be used.)
- Mobile phase B: Acetonitrile, HPLC or LC-MS grade, filtered using a 0.2-µm filter
- Diluent: Water:acetonitrile (7:3)
- 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 may be included when they become commercially available.)

Equilibration:

Powdered samples should be equilibrated in the lab not less than 12 hours before assaying. Spread 1–2 g of sample into a thin layer not more than 6 mm thick in an open container, stirring occasionally to ensure uniform moisture absorption. The loss on drying of the equilibrated sample should be determined concurrently with performing the assay using the conditions in Annexes 1-4 (Vol. 4).

Preparation of Steviol Glycoside Standard Solutions:

If multiple commercially available reference standards are being used, prepare individual or mixed five point working standard solutions in the range of 5–500 µg/mL. Prepare all solutions in the Diluent.

If the only commercially available reference standard is rebaudioside A, prepare five working standard solutions of rebaudioside A in the range of 5–500 µg/mL. Prepare all solutions in the Diluent.

Prepare quality control solution(s) with concentrations that fall within the calibration range.

Preparation of Sample Solution:
Accurately weigh 40-50 mg of the equilibrated sample and quantitatively transfer into a 100-mL volumetric flask. Add about 80 mL of Diluent, sonicate and shake well to dissolve the sample. Allow to return to room temperature and dilute to volume with Diluent.

Part 1: Determination of Major Steviol Glycosides by HPLC-UV

Procedure:

- Column: C18 column (150 mm x 4.6 mm, 2.7µm), for example Agilent Poroshell 120 SB-C18, or equivalent.
- Column temperature: 45°
- Autosampler temperature:10 – 20°
- Detector: UV-Vis or DAD at 210 nm
- Flow rate: 0.5 mL/min
- Injection volume: 5 µl

Table 1. HPLC Gradient Timetable

Time (min)	%Solvent A	%Solvent B
0.0	75.0	25.0
8.0	75.0	25.0
13.0	68.0	32.0
16.0	68.0	32.0
19.0	60.0	40.0
23.0	60.0	40.0
23.5	40.0	60.0
25.0	40.0	60.0
25.5	75.0	25.0
35	75.0	25.0

Inject blank(s) and peak identification standard solutions.

Inject working mixed Standard Solutions and create standard curves for each steviol glycoside. If rebaudioside A is the only commercially available reference standard, derive a standard curve for rebaudioside A from the Standard Solutions. (Note: Use of 1/x weighting and not forcing the curve through 0 are recommended.)

Inject quality control and system suitability standard solutions to ensure the system performance is acceptable.

Inject prepared samples. Dilute the Sample Solution, if required, to bring the concentration of each analyte within the standard curve range. Make at least duplicate injections. Determine the concentration of each steviol glycoside from its corresponding standard curve and determine the average concentration in the Sample Solution (µg/mL).

Identification:
The retention times of each major peak in the chromatogram of the Sample Solution should be determined using primary reference standards. Example HPLC-UV chromatogram of major steviol glycosides obtained using commercially available quantitative reference standards, *Figure 1*.

Calculation:

1) Using individual steviol glycoside reference standards
Calculate the weight percentage of each steviol glycoside in the Sample Solution:

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

where:
C_{SG} is the average concentration of the steviol glycoside in the Sample Solution, as determined from the relevant standard curve (µg/mL)
C_{sample} is the concentration of the Sample Solution (µg/ml)

2) Using a rebaudioside A standard and relative response factors (RRF)
Calculate the percentage of each steviol glycoside in the Sample Solution:

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

where:
C_X is the average concentration of the steviol glycoside as rebaudioside A, as determined from the rebaudioside A standard curve (µg/mL)
F is the UV RRF for the steviol glycoside at 210 nm, from Table 2
C_{sample} is the concentration of the Sample Solution (µg/mL)

The RRF of a given steviol glycoside (reb X) to rebaudioside A may alternatively be calculated using experimental data obtained at 210 nm with reference standards.

Table 2. Relative Response Factors (RRFs) at 210 nm

	RRF against rebaudioside A	
Compounds	**Experimental ***	**Molecular Weight**
Rebaudioside A	---	1

Rebaudioside B	0.82	0.82
Rebaudioside C	1.03	0.98
Rebaudioside D	1.16	1.17
Rebaudioside E	0.98	1.00
Rebaudioside F	0.97	0.97
Rebaudioside M	1.36	1.34
Rebaudioside N	1.25	1.32
Rebaudioside O	1.56	1.49
Stevioside	0.80	0.83
Dulcoside A	0.83	0.82
Steviolbioside	0.76	0.83
Rubusoside	0.65	0.66

* All experimental RRFs were obtained using a 10 mm long UV/PDA flow cell. The RRF is calculated using the assigned purity values as provided by the reference standard manufacturer, including their corrections for moisture and solvents. Independent confirmation of the RRFs of major glycosides is suggested when adopting the method or when changing any operational parameters.

Calculate the percentage of major steviol glycosides in the sample by summation of percentages of individual steviol glycosides in the sample. If the sum of the concentrations of the major steviol glycosides in the sample is <95%, then proceed to Part 2.

Part 2: Determination of Minor Steviol Glycosides by LC-UV-MS

The mass spectrometer is connected to the LC-UV system used in Part 1. It is used to identify the minor peaks that do not match the retention times of the major steviol glycosides identified in Part 1. Quantification of minor glycosides is based on comparison to standard curves for the minor glycosides, where pure reference standards are available (see calculation 1, described in Part 1). If pure reference standards for the minor glycosides are not available, quantification is based on comparison to the rebaudioside A standard curve and the molecular weight-based relative response factor (see calculation 2, described in Part 1).

LC-UV-MS operating conditions may vary based on the manufacturer and model of the system used; conditions should be set following the manufacturer's instructions. Example MS conditions are provided below for a Waters Acquity SQD mass spectrometer. The MS can be operated in full scan mode or in selected ion monitoring mode (SIM) using the m/z values listed in Table 3. (Note: Table 3 m/z values encompass identified major and minor steviol glycosides from the leaves of *Stevia rebaudiana* Bertoni. See Appendix A, Table A for chemical information related to these identified steviol glycosides.)

Table 3. Example SIM m/z values and their possible identification if peak retention time differs from major steviol glycosides.

Molecular mass ion $[M-H]^-$	Identity
317	Steviol or its isomers
479	Steviol +1 glucoside
625	Dulcoside A1
641	Isomers of steviolbioside or rubusoside
774	Stevioside F or isomers
787	Isomers of dulcoside A
803	Isomers of reb B or stevioside
935	Isomers of reb F
949	Isomers of reb C
965	Isomers of reb A
1097	Steviol + 4 Glc + 1 Arabinose or isomers
1111	Steviol + 4 Glc +1 Rhamnose or isomers
1127	Isomers of reb D
1259	Steviol + 5 Glc + Xyl or isomers
1273	Isomers of reb N
1289	Isomers of reb M
1435	Isomers of reb O

This list does not include every possible steviol glycoside; additional m/z may be evaluated.

In the absence of available reference standards for the minor steviol glycosides, one or more of the in-source fragmentation ions in Table 4 should be used along with the molecular ions for identification purposes:

Table 4. Example diagnostic fragmentation ions

Fragmentation ion $[M-H]^-$	Identity
317	Steviol
479	Steviol+1 glucoside
625	Steviol+2 glucoside-oxygen [M-16]
641	Steviol +2 glucoside
787	Steviol +2 glucoside +1 rhamnoside
803	Steviol +3 glucoside
949	(Deoxy)-Steviol +4 glucoside
965	Steviol+4 glucoside

This list does not include every possible diagnostic ion; additional m/z may be evaluated.

Example MS Conditions

Instrumentation:	Waters Acuity SQD MS
Ionization:	Electrospray negative polarity
Cone voltage:	35 V (low – m/z ≤ 900 amu) and 80 V (high – m/z >900 amu)
Resolution:	1 amu
Data acquisition:	Scanning 50 to 2000 m/z

(Note: See example scans in *Figures 2* and *3*.)

Calculation:

Calculate the concentration of minor glycosides using the rebaudioside A standard curve:

$$\text{Conc. (\% w/w)} = C_X \times M_X \times 100 / M_A \times C_{sample}$$

where:

C_X is the average concentration of the minor steviol glycoside as rebaudioside A, as determined from the rebaudioside A standard curve (µg/mL)

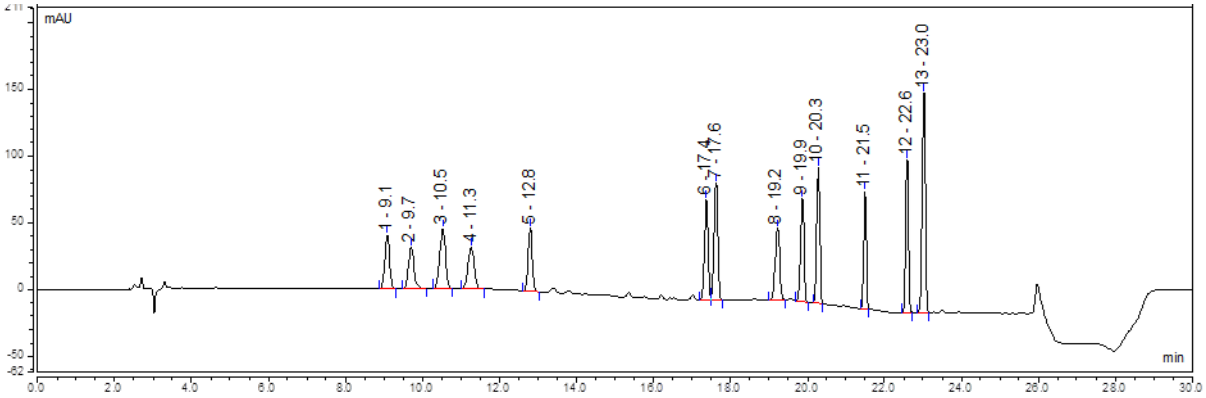
M_X is the molecular weight of the minor steviol glycoside obtained by mass spectrometry

M_A is the molecular weight of rebaudioside A

C_{sample} is the concentration of the Sample Solution (µg/mL)

(Note – Calculate the concentration of minor steviol glycosides using commercially available minor steviol glycoside reference standards when available.)

Figure 1. Example LC-UV chromatogram and retention times of major steviol glycosides with commercially available quantitative reference standards



Peak Number	Analyte	Approximate Retention Time (min)
1	Rebaudioside E	9.1
2	Rebaudioside O	9.7
3	Rebaudioside D	10.5
4	Rebaudioside N	11.3
5	Rebaudioside M	12.8
6	Rebaudioside A	17.4
7	Stevioside	17.6
8	Rebaudioside F	19.2
9	Rebaudioside C	19.9
10	Dulcoside A	20.3
11	Rubusoside	21.5
12	Rebaudioside B	22.6
13	Steviolbioside	23.0

Figure 2. Top trace: Full scan m/z 50 to 900amu; Bottom trace: Full scan m/z 901 to 2000 amu

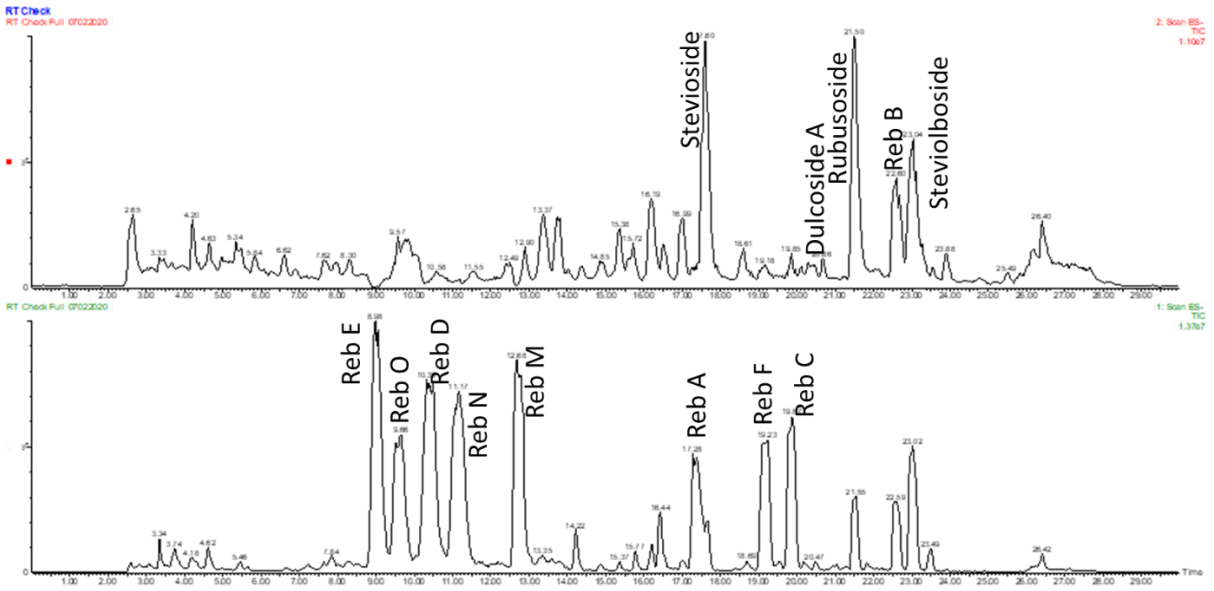
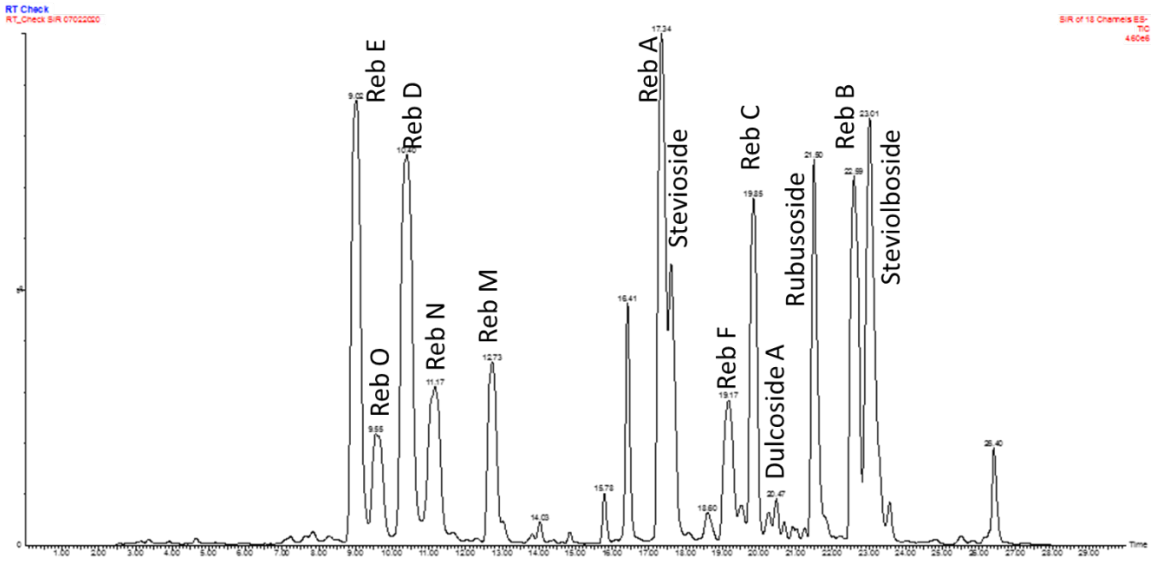


Figure 3. Example SIM scan using the m/z values listed in Table 3



ANNEX 1: STEVIOL GLYCOSIDES FROM STEVIA REBAUDIANA BERTONI

Revised at 96th JECFA (2023) and published in FAO Monographs 31 (2023), superseding specifications prepared at the 91st JECFA (2021) and published in FAO Monographs 26 (2021). 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 of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES
C.A.S number	See Appendix A of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES
Chemical formula	See Appendix A of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES
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)	Very slightly soluble to freely soluble in water; slightly soluble to freely soluble in a mixture of ethanol and water (50:50 v/v)

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 B of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES

ANNEX 2: STEVIOL GLYCOSIDES FROM FERMENTATION

Revised at 96th JECFA (2023) and published in FAO Monographs 31 (2023), superseding specifications prepared at the 91st JECFA (2021) and published in FAO Monographs 26 (2021). 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 of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES
C.A.S number	See Appendix A of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES
Chemical formula	See Appendix A of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES
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)	Very slightly soluble to freely soluble in water; slightly soluble to freely soluble in a mixture of ethanol and water (50:50 v/v)
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 B of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES

ANNEX 3: ENZYME MODIFIED STEVIOL GLYCOSIDES

Revised at 96th JECFA (2023) and published in FAO Monographs 31 (2023), superseding specifications prepared at the 91st JECFA (2021) and published in FAO Monographs 26 (2021). 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* Bertoni

Chemical names

See Appendix A of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES

C.A.S number

See Appendix A of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES

Chemical formula	See Appendix A of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES
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)	Very slightly soluble to freely soluble in water; slightly soluble to freely soluble in a mixture of ethanol and water (50:50 v/v)
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 B of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES

ANNEX 4: ENZYME MODIFIED GLUCOSYLATED STEVIOL GLYCOSIDES

Revised at 96th JECFA (2023) and published in FAO Monographs 31 (2023), superseding specifications prepared at the 91st JECFA (2021) and published in FAO Monographs 26 (2021). 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 *Anoxybacillus caldiproteoliticus*, *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.
Chemical names	See Appendix A of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES
C.A.S number	See Appendix A of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES
Chemical formula	See Appendix A of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES
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 dextrin and steviol glycoside fractions are separated using an adsorption column and elution with water and ethanol. The two fractions are dried and weighed to obtain the relative percentages of dextrin and total steviol glycosides (step 1). The percentage of glucosylated and unreacted parent steviol glycosides are determined using the HPLC method below (step 2).

Reagents:

- Ethanol
- Mobile phase A: Acetonitrile, HPLC grade
- Mobile phase B: Water, HPLC grade
- Reference Standards: Stevioside, rebaudioside A, rebaudioside C, rebaudioside F, rubusoside, and steviolbioside. Chromadex, USA; Wako Pure Chemical Industries Ltd., Japan; Sigma-Aldrich; US Pharmacopeia or equivalent.

Step 1: Column Adsorption

Weigh accurately 4 g of glucosylated steviol glycosides sample and dissolve with 100 mL water in a graduated cylinder. Record the exact weight and volume; the concentration of the Sample Solution is approximately 4%. Load the solution onto a glass column (25-mm ID) packed with 200 mL of Sigma Amberlite XAD 7 HP resin, or equivalent, at a rate of < 3 ml/min. Elute with 1000 ml of water to remove the dextrin. Next, elute with 1000 mL of 70% ethanol at a rate of 3 ml/min or less to remove the steviol glycosides. Evaporate the two eluted fractions to dryness, then dry in a vacuum oven at 105° for 2 hours. Weigh and record the dry weight of each fraction.

Calculate the percent of dextrin and of total steviol glycosides (TSG):

Dry weight initial sample (g) = wet weight initial sample (g) x [(100 – loss on drying %) / 100]

Dextrin (%) = [weight of dried aqueous fraction (g) / dry weight of initial sample (g)] X 100

TSG (%) = [weight of dried ethanol fraction (g) / dry weight of initial sample (g)] X 100

If the content of residual dextrin is more than 4%, the adjusted TSG on the dextrin-free basis is calculated by the following formula:

Adjusted TSG (%) = TSG (%) x dry weight initial sample (g) / [dry weight initial sample (g) – weight of dextrin (g)]

Step 2: HPLC Assay

Reagents:

- Diluent: 50% (v/v) ethanol in water

- Mixed Marker Solution: Prepare a solution containing 100 mg/l each of rubusoside, dulcoside A, stevioside, rebaudioside C, rebaudioside F and rebaudioside A solution in Diluent.

Equilibration:
Powdered samples should be equilibrated in the lab not less than 12 hours before assaying. Spread 4–5 g of sample into a thin layer not more than 6 mm thick in an open container, stirring occasionally to ensure uniform moisture absorption. The loss on drying of the equilibrated sample should be determined concurrently with the HPLC analysis.

Preparation of Rebaudioside A Standard Solution:
Weigh approximately 125 mg of rebaudioside A reference standard into a 25-ml volumetric flask and dilute to volume with the Diluent. Prepare in duplicate. The approximate concentration of each solution is 5000 mg/l.

Preparation of Sample Solution:
Weigh approximately 2500 mg of the sample into a 50 ml volumetric flask and dilute to volume with the Diluent. Prepare in duplicate. The approximate concentration of each solution is 50 000 mg/l.

Procedure:

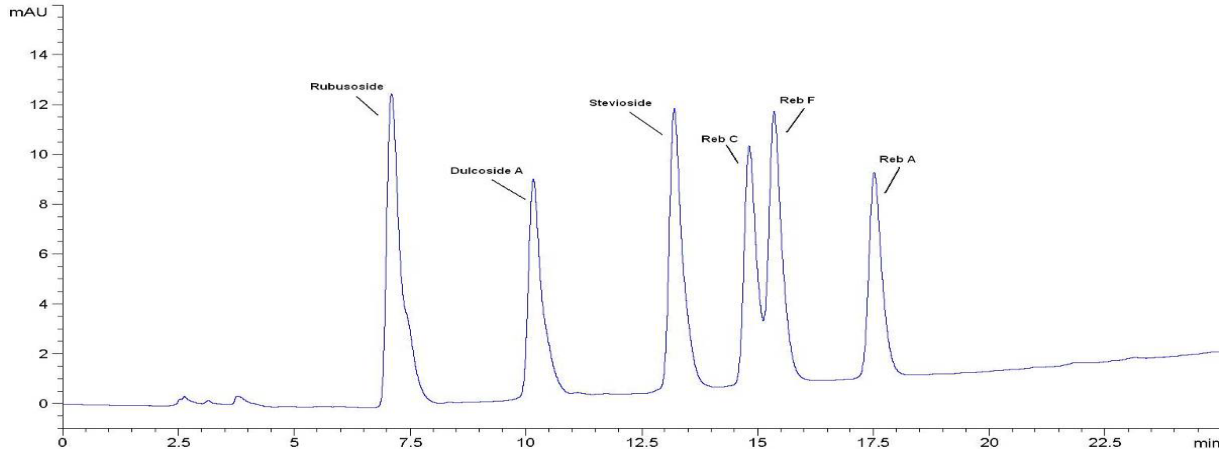
- Column: Zorbax NH2, or equivalent; 250 x 4.6 mm, 5-µm
- Column temperature: 40°
- Flow rate: 1.0 ml/minute
- Injection volume: 12 µl
- Detection: UV at 210 nm (4 nm bw); Reference: 360 nm (100 nm bw)
- Run time: 90 minutes
- Post time: 10 minutes

Table 1. HPLC Gradient Timetable

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	80	20
2	80	20
90	50	50
91	80	20
100	80	20

Analysis of the Parent Steviol Glycosides:
Inject the Mixed Marker Solution into the chromatograph and identify the retention time of each steviol glycoside on the resulting chromatogram by comparison to the following chromatogram (Annex 4 Figure 1):

Annex 4 Figure 1. HPLC chromatogram of Mixed Marker Solution



Inject 8.0-, 10.0- and 12.0-µl aliquots of the first (rebaudioside A) Standard Solution into the chromatograph and record the resulting chromatograms. Prepare a 3-point standard curve of peak area vs. concentration of rebaudioside A (mg/l).

Inject 12 µl of the duplicate (rebaudioside A) Standard Solution; its recovery should be within 98 -102% when the peak area response is compared to the 3-point standard curve.

Inject 12 µl each of the duplicate Sample Solutions and report the average of their responses. The % RSD for rebaudioside A and stevioside content in the duplicate Sample Solutions should be less than 2.0%.

Calculate the concentration of the steviol glycosides in the Sample Solution using following formula:

$$SG\ (mg/l) = A \times m + b$$

where:
A is the peak area of the steviol glycoside
m is the slope of the rebaudioside A standard curve
b is the y-intercept of the rebaudioside A standard curve

Multiply the concentration of other steviol glycosides present by their respective correction factors to correct for the differences in molecular weight. The correction factors for rubusoside, dulcoside A, stevioside, rebaudioside C, rebaudioside F are 0.665, 0.815, 0.832, 0.983, and 0.969, respectively (as compared to rebaudioside A).

Calculate the percentage of each steviol glycoside in the Sample Solution using the following formula:

$$\text{Conc. (w/w) \%} = SG \times 100/C_{Sample}$$

Where:
SG is the concentration of the steviol glycoside determined above (mg/l)
C_{Sample} is the concentration of the Sample Solution (mg/l)

Sum the steviol glycoside components to determine the “Total Parent Steviol Glycosides” (TPSG%).

<u>Analysis of the α-Glucosyl Steviol Glycosides:</u>

Use the “Total Parent Steviol Glycosides” data obtained from the HPLC assay and the following formula to calculate the total content of α-glucosyl steviol glycosides:

$$\text{Total α-glucosyl steviol glycosides \%} = TSG\ \% - TPSG\ \%$$

Each individual α-glucosyl steviol glycoside component is identified by comparison to the following chromatogram (Annex 4 Figure 2):

Annex 4 Figure 2. HPLC chromatogram of an Enzyme Modified Glucosylated Steviol Glycoside sample solution