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(Framework for) STEVIOL GLYCOSIDES

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(Framework for) Steviol Glycosides

Prepared at the 91st JECFA (2021) and published in *FAO JECFA Monographs 26 (2021)*, superseding specifications prepared at the 87th JECFA (2019) and published in *FAO JECFA Monograph 23 (2019)*.

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.

Appendix A: Chemical Information for Major and Minor Steviol Glycosides

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(1-2)]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-	Ohya et al. (2010)
1.15	Rebaudioside L		SVG5	1129	0.28	Glcβ1-	Glcβ(1-6)Glcβ(1-2)Glcβ(1-3)Glcβ1-	Ohya 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-	Ohya 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)Glcβ(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
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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[Adapted from Purkayastha & Kwok (2020)]

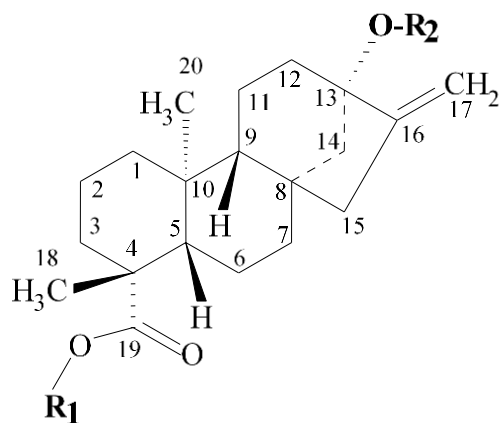
#	Common Name	CAS Number	Trivial Formula	Mol. Wt	Steviol Equivalent	R ₁	R ₂	Reference
2.16	Rebaudioside O6		SVR1G7	1600	0.20	Glcβ(1-3)Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-6)Glcβ(1-3)[Glcβ(1-2)]Glcβ1-	Prakash & Ma (2018)
2.17	Rebaudioside O7		SVR2G6	1584	0.20	Glcβ(1-3)Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-3)Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Prakash & Ma (2018)
3. Steviol + Xylose + Glucose (SVX1Gn)								
3.01	Stevioside F		SVX1G2	775	0.41	Glcβ1-	Xy β(1-2)Glcβ1-	Chaturvedula & Prakash (2011c)
3.02	Rebaudioside F	438045-89-7	SVX1G3	937	0.34	Glcβ1-	Xy β(1-2)[Glcβ(1-3)]Glcβ1-	Starratt et al. (2002)
3.03	Rebaudioside F2		SVX1G3	937	0.34	Glcβ1-	Glcβ(1-2)[Xy β(1-3)]Glcβ1-	Chaturvedula & Prakash (2011c)
3.04	Rebaudioside F3		SVX1G3	937	0.34	Xy β(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)]Xy β1-	Ibrahim et al (2016)
3.06	Rebaudioside U		SVX1G4	1099	0.29	Xy β(1-2)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Purkayastha et al. (2019)
3.07	Rebaudioside U2		SVX1G4	1099	0.29	Xy β(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)Glcβ1-	Purkayastha et al. (2016)
3.08	Rebaudioside U3		SVX1G4	1099	0.29	Xy β(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)[Xy β(1-3)]Glcβ1-	Purkayastha et al. (2019)
3.10	Rebaudioside V2		SVX1G5	1261	0.25	Xy β(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- μ m filter, with 0.01% 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- μ m 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. An unopened reference standard with moisture listed on a Certificate of Analysis may be used without equilibrating. 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). Karl Fischer titration may be used as an alternative to loss on drying for determining moisture of equilibrated samples and standards when performing the assay.

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 μ g/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 ($\mu\text{g/mL}$).

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:

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

where:

C_{SG} is the average concentration of the steviol glycoside in the Sample Solution, as determined from the relevant standard curve ($\mu\text{g/mL}$)

C_{sample} is the concentration of the Sample Solution ($\mu\text{g/ml}$)

2) Using a rebaudioside A standard and relative response factors (RRF)

Calculate the percentage of each steviol glycoside in the Sample Solution:

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

where:

C_{X} is the average concentration of the steviol glycoside as rebaudioside A, as determined from the rebaudioside A standard curve ($\mu\text{g/mL}$)

F is the UV RRF for the steviol glycoside at 210 nm, from Table 2

C_{sample} is the concentration of the Sample Solution ($\mu\text{g/mL}$)

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

Compounds	RRF against rebaudioside A	
	Experimental *	Molecular Weight
Rebaudioside A	---	1
Rebaudioside B	0.82	0.83
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	0.66
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
Molecular mass ion [M-H] ⁻	Identity
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:

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

where:

C_X is the average concentration of the minor steviol glycoside as rebaudioside A, as determined from the rebaudioside A standard curve ($\mu\text{g/mL}$)

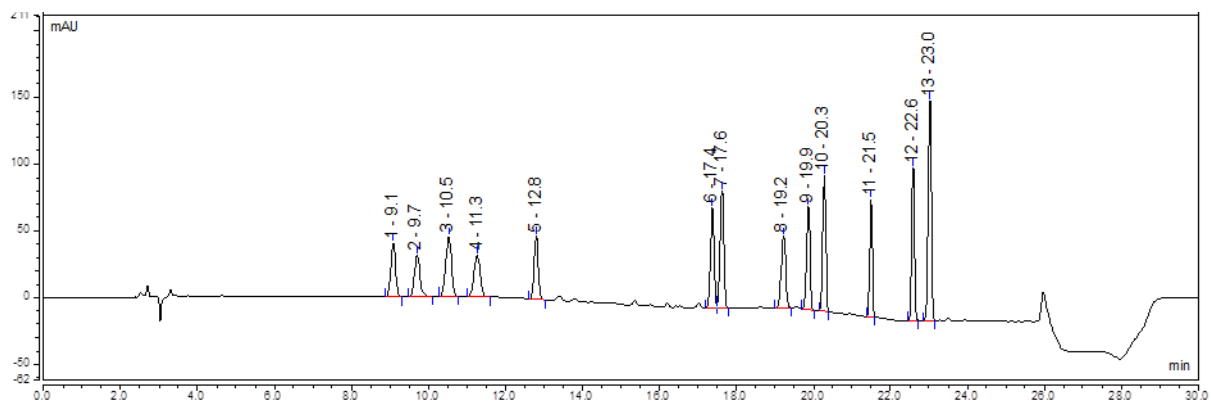
M_X is the molecular weight of the minor steviol glycoside obtained by mass spectrometry

M_A is the molecular weight of rebaudioside A

C_{sample} is the concentration of the Sample Solution ($\mu\text{g/mL}$)

(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

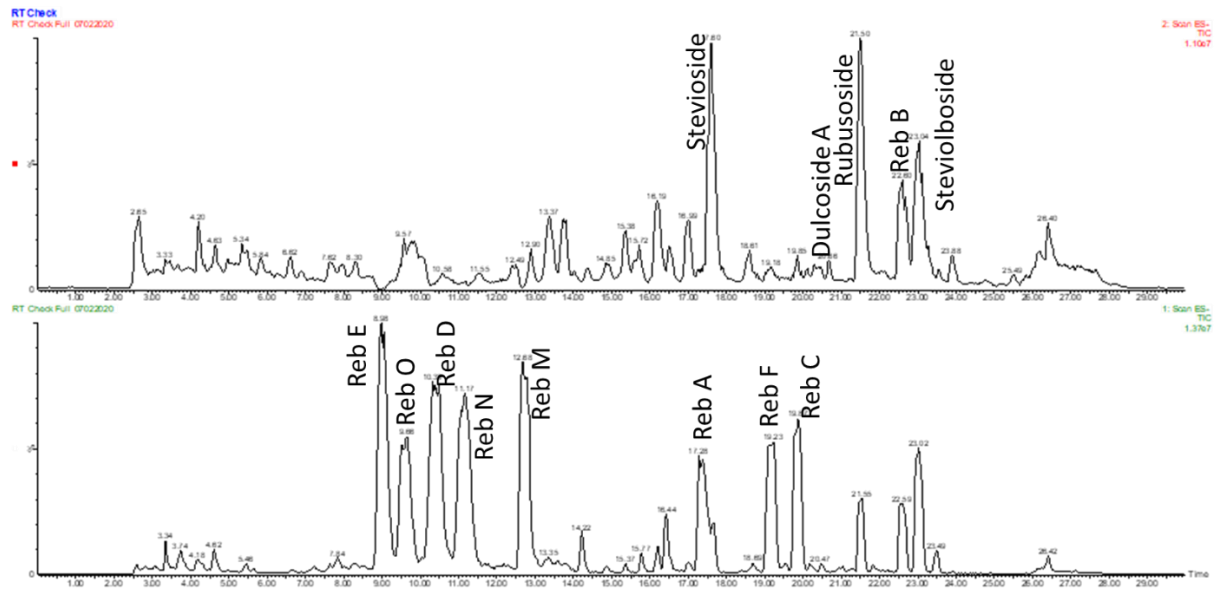
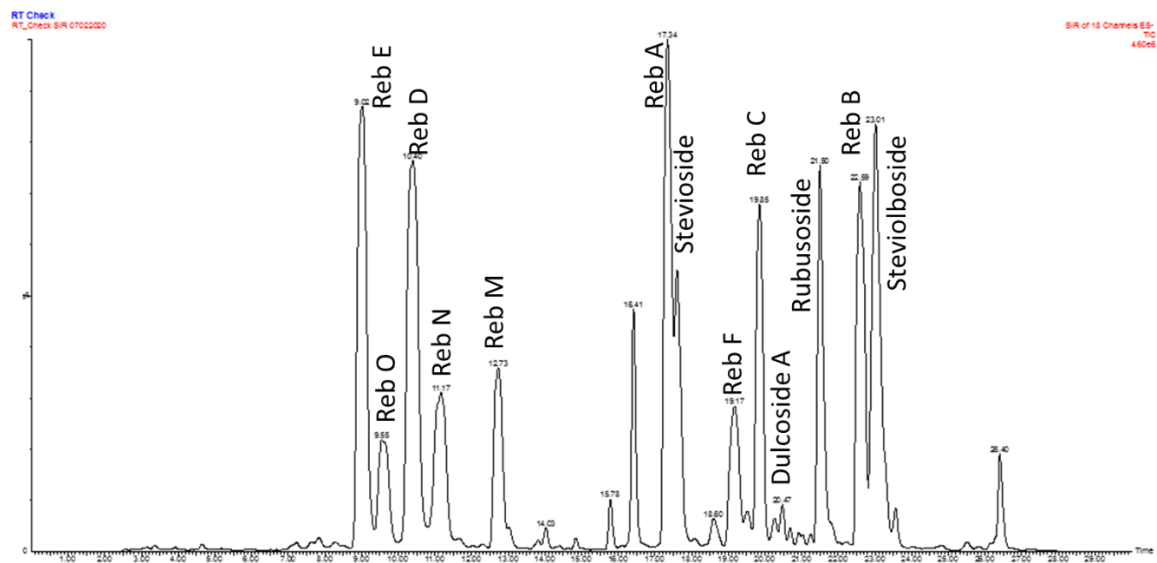


Figure 3. Example SIM scan using the m/z values listed in Table 3



Annex 1: Steviol Glycosides from *Stevia Rebaudiana* Bertoni

Prepared at the 91st JECFA (2021) and published in FAO Monographs 26 (2021), superseding specifications prepared at the 87th JECFA (2019) and published in FAO Monographs 23 (2019). 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 <i>Stevia rebaudiana</i> Bertoni. The product is obtained from the leaves of <i>Stevia rebaudiana</i> 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 <i>Stevia rebaudiana</i> 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

Prepared at the 91st JECFA (2021) and published in FAO Monographs 26 (2021), superseding specifications prepared at the 87th JECFA (2019) and published in FAO Monographs 23 (2019). 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-toxicogenic 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

Prepared at the 91th JECFA (2021) and published in FAO Monographs 26 (2021), superseding specifications prepared at the 87th JECFA (2019) and published in FAO Monographs 23 (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-toxicogenic 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

Pichia pastoris

Escherichia coli

Gene source

Horedum vulgare L
Stevia rebaudiana Bertoni
Vigna radiate

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

Prepared at 91st JECFA (2021) and published in FAO Monographs 26 (2021), superceding specifications prepared at the 87th JECFA (2019) and published in FAO Monographs 23 (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-toxicogenic 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 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):

$$\text{Dry weight initial sample (g)} = \text{wet weight initial sample (g)} \times \left[\frac{100 - \text{loss on drying (\%)}}{100} \right]$$

$$\text{Dextrin (\%)} = \left[\frac{\text{weight of dried aqueous fraction (g)}}{\text{dry weight of initial sample (g)}} \right] \times 100$$

$$\text{TSG (\%)} = \left[\frac{\text{weight of dried ethanol fraction (g)}}{\text{dry weight of initial sample (g)}} \right] \times 100$$

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

$$\text{Adjusted TSG (\%)} = \text{TSG (\%)} \times \frac{\text{dry weight initial sample (g)}}{\text{dry weight initial sample (g)} - \text{weight of dextrin (g)}}$$

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- μ m
- Column temperature: 40°
- Flow rate: 1.0 ml/minute
- Injection volume: 12 μ l
- Detection: UV at 210 nm (4 nm bw); Reference: 360 nm (100 nm bw)
- Run time: 90 minutes
- Post time: 10 minutes

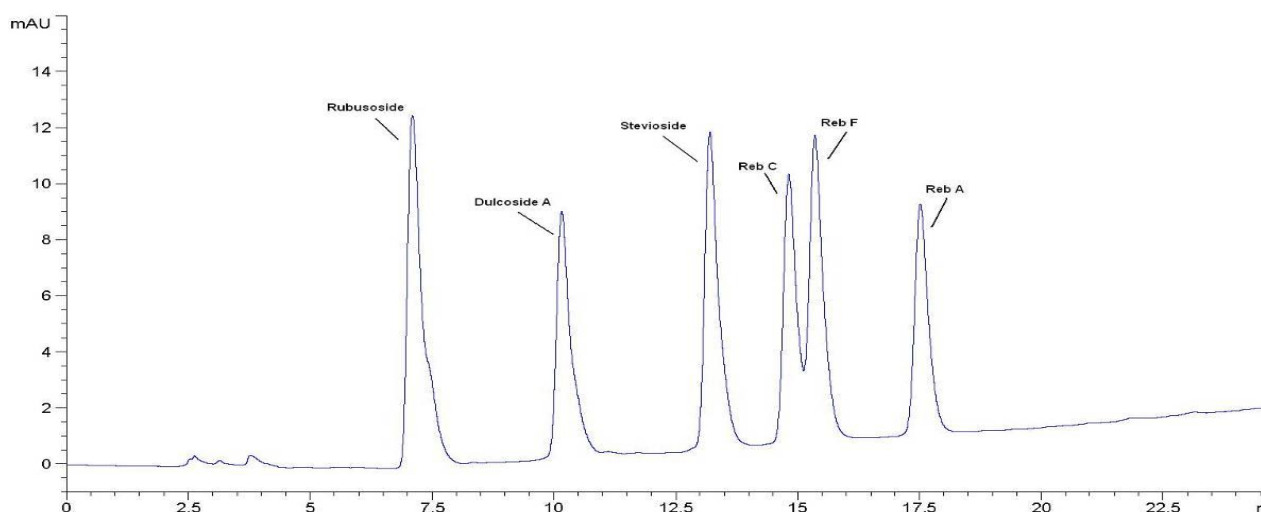
Table 1. HPLC Gradient Timetable

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- μ l 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 μ l 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 μ l 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:

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

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:

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

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%).

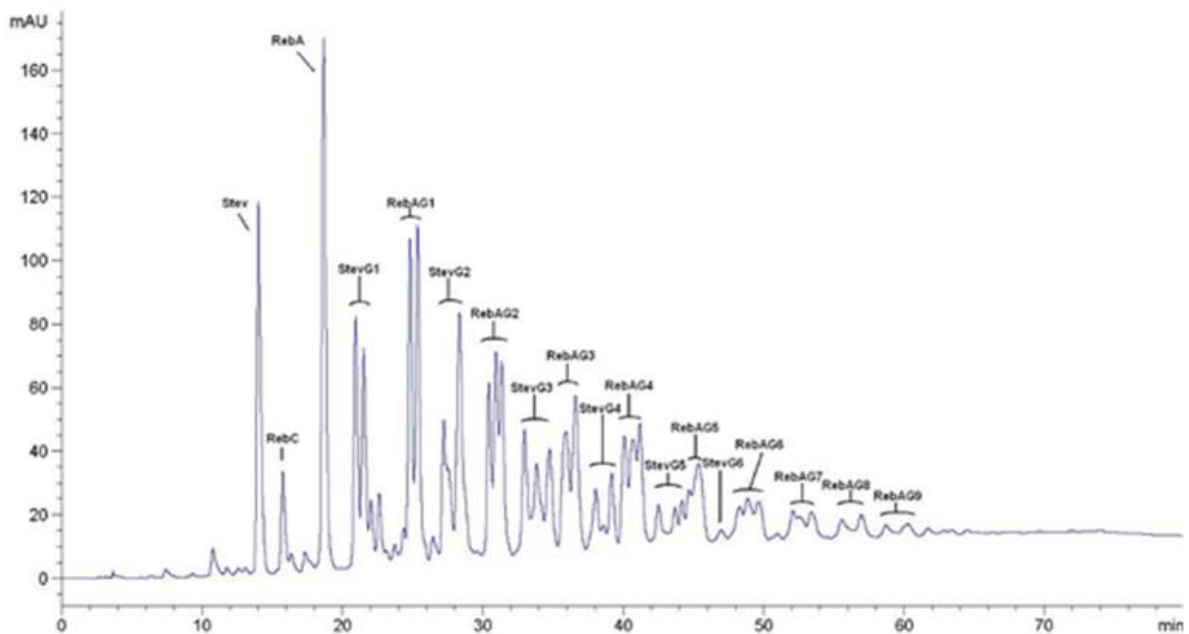
Analysis of the α -Glucosyl Steviol Glycosides:

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:

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

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



Summary of recommendations from the 91st JECFA



JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES

Ninety-first meeting (Safety evaluation of certain food additives and contaminants)

Virtual meeting, 1–12 February 2021

SUMMARY AND CONCLUSIONS

Issued on 5 March 2021

A meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) was held on a virtual online platform from 1 to 12 February 2021, with an additional day for the report adoption on 25 February. The purpose of the meeting was to evaluate the acceptability of certain substances as previous cargoes and the safety of certain food contaminants, as well as to revise the specifications on steviol glycosides. The present meeting was the ninety-first in a series of similar meetings.

If conditions had permitted, the ninety-first meeting of JECFA would have been held at FAO headquarters in Rome, Italy. Because of the travel restrictions and lock-downs due to the COVID-19 pandemic in many countries, the joint FAO/WHO JECFA secretariat was unable to convene a physical meeting. Therefore, the meeting was held as a video-conference.

In view of the time differences in the countries of origin of the invited experts, the only possible time for a video-conference was restricted to a 4-hour time slot (12:00–16:00 CEST) each day. This allowed only 40% of the usual daily length (8–10 hours) of a JECFA meeting. In an effort to regain some additional meeting time, the ninety-first JECFA meeting was extended by 3 days, adding Monday 1 February, Friday 12 February and Thursday 25 February 2021.

Dr R. Cantrill served as Chairperson and Dr D. Benford served as Vice-Chairperson.

Ms K.B. Laurvick and Dr U. Mueller served as joint rapporteurs.

The Committee evaluated the contaminants cadmium and ergot alkaloids, and 5 substances that may occur as previous cargoes, as well as revising the specifications for steviol glycosides. The tasks before the Committee were (a) to undertake

toxicological evaluations and dietary exposure assessments in relation to certain contaminants in food and (b) to revise the specifications for certain food additives.

This document summarizes the conclusions of the ninety-first meeting of JECFA. More details than are normally made available in a summary report are included on cadmium. This decision was made on an exceptional basis to facilitate the deliberations of the upcoming Codex Committee on Contaminants in Food.

The report of the meeting will be published in the WHO Technical Report Series. The report will summarize the main conclusions of the Committee. Its presentation will be similar to that of previous reports – namely, comments on specific contaminants in food including previous cargoes, and on specific food additives, followed by recommendations. An annex will include a summary (similar to the summary in this report) of the main conclusions of the Committee in terms of toxicological and safety recommendations.

The participants are listed in Annex 1 to this summary document. Future work and recommendations arising from the meeting are summarized in Annex 2. Annex 3 summarizes observations by experts with regard to the practicability of holding these expert meetings online rather than in-person.

Toxicological and dietary exposure monographs on the contaminants and additives considered will be published in WHO Food Additives Series No. 82.

More information on the work of JECFA is available at:

<http://www.fao.org/food-safety/scientific-advice/jecfa/en/> and

[https://www.who.int/groups/joint-fao-who-expert-committee-on-food-additives-\(jecfa\)/](https://www.who.int/groups/joint-fao-who-expert-committee-on-food-additives-(jecfa)/)

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Toxicological and dietary exposure information and conclusions

Contaminants evaluated

Cadmium (exposure assessment from all food sources)¹

Explanation

Cadmium was evaluated by the Committee at its sixteenth, thirty-third, forty-first, fifty-fifth, sixty-first, sixty-fourth, seventy-third and seventy-seventh meetings. At the sixty-first and sixty-fourth meetings, the Committee noted that the estimated total mean dietary exposure to cadmium from all foods, derived from per capita data from the five GEMS/Food regional diets, ranged from 40% to 60% of the provisional tolerable weekly intake (PTWI) applicable at that time of 7 µg/kg bw. The seven commodity groups that contributed significantly to total mean dietary exposure to cadmium were rice, wheat, root vegetables, tuber vegetables, leafy vegetables, other vegetables and molluscs (40–85% of the total mean dietary exposure to cadmium across the five regional diets).

At its seventy-third meeting in 2011, the Committee re-evaluated cadmium and established a provisional tolerable monthly intake (PTMI) of 25 µg/kg bw, reflecting the long half-life of cadmium in humans. Reported national estimates of mean dietary exposure to cadmium from all foods for adults ranged from 2.2 to 12 µg/kg bw per month, or 9–48% of the PTMI. For European children up to 12 years of age, this estimate was 11.9 µg/kg bw per month or 47% of the PTMI. High percentile dietary exposures to cadmium for adults from Europe, Lebanon and the USA were reported to range from 6.9 to 12.1 µg/kg bw per month (28–48% of the PTMI), and from 20.4 to 22.0 µg/kg bw per month (82–88% of the PTMI) for children aged 0.5–12 years from Australia and the United States of America (USA). Data on cadmium occurrence and consumption of foods containing cocoa and its derivatives were included in all 2011 estimates. Although not all estimates of dietary cadmium exposure evaluated at the seventy-third meeting reported the major contributing foods, for those estimates that did report this information, cereals and cereal products and vegetables were consistently reported as major contributors, with seafood and meat, including offal, also reported in some studies. None of the studies reported cocoa products as major contributors to dietary cadmium exposure.

At its seventy-seventh meeting in 2013, the Committee conducted an assessment of dietary exposure to cadmium from cocoa and cocoa products at the request of the sixth session of the Codex Committee on Contaminants in Foods (CCCF). The Committee considered the exposure to cadmium from foods containing cocoa and its derivatives in the context of overall dietary exposure. The estimates of mean dietary exposure to cadmium from foods containing cocoa and its derivatives ranged from 0.005 to 0.39 µg/kg bw per month or 0.2–1.6% of the PTMI across the 17 GEMS/Food cluster diets, assuming a body weight of 60 kg. Mean dietary exposure estimates for individual cocoa products based on national food consumption data ranged from 0.001 to 0.46 µg/kg bw per month or 0.004–1.8% of the PTMI. The cocoa products included were cocoa beverages, cocoa powder and other cocoa products. The highest high exposure (P97.5) was estimated at 12 µg/kg bw per month for European children 7–11 years of age solely due to the consumption of cocoa powder. Combining the highest P97.5 dietary exposure estimate for adults and children out of the three cocoa products with the mean dietary exposure estimates for both age groups from the whole diet, the Committee estimated a total dietary exposure of 7.4–17.2 µg/kg bw per month or 30–69% of the PTMI for adults and 23.9 µg/kg bw per month or 96% of the PTMI for children aged 0.5–12 years. The Committee noted that these estimates of total dietary cadmium exposure very likely overestimated the exposure, because the estimates from the whole diet also included a contribution from cocoa and cocoa products.

At the request of the thirteenth session of CCCF for more comprehensive occurrence data for

¹ More details than are normally made available in a summary report are included on cadmium. This decision was made on an exceptional basis to facilitate the deliberations of the upcoming Codex Committee on Contaminants in Food.

cadmium in food, the JECFA Secretariat issued a call for data on cadmium in chocolate and cocoa-derived products in 2019. The submitted data included a wider geographical range of occurrence data for cadmium in cocoa products than considered at the seventy-seventh meeting of the Committee. The occurrence data also showed a higher mean concentration for cadmium in cocoa products than previously noted by the Committee. As a result, the JECFA Secretariat considered it appropriate to revise the dietary exposure assessment of cadmium to include not only chocolate and cocoa products but the contribution from all food sources. At the present meeting the Committee reassessed cadmium exposure to include the contribution of all food sources, particularly cocoa products.

Data submitted or available to the Committee

The GEMS/Food contaminants database was queried for records relating to cadmium in any food. The database query was restricted to records submitted since the previous assessment of dietary cadmium exposure from the whole diet by the Committee in 2011. Data submitted since 1 January 2011 originated from 27 countries or country groups (WHO European Region, WHO African Region), representing 10 of the 17 GEMS/Food cluster diets. It should be noted that for several of the countries or clusters the available data were limited in quantity or restricted to a narrow range of foods. For example, the sole country providing data from cluster G09 (Indonesia) submitted analytical results for 30 samples of cocoa products only. Five clusters (G07, G08, G10, G11 and G15) cover the countries of Europe; however, most of the contaminant concentration data available for these countries were only identified at the level of the WHO European Region and it was not possible to examine differences in contamination profile between these clusters using these data.

The final data set contained 277 292 records, of which 216 373 (78%) were from the WHO European Region. A considerable body of non-European data was available for cluster G10, submitted by Canada (n = 21 501), Japan (n = 5332) and the USA (n = 5887). Records were widely spread across different food types, with the most commonly analysed food types being edible pig offal (7.3%), marine fish (6.9%) and cattle meat (3.7%).

Given the focus of the current assessment on cadmium in cocoa and cocoa products, an overview of these data as included in the dietary exposure assessment was prepared. In total, 6957 records for cocoa and cocoa products were available, representing 2.5% of all records in the final data set. These records related to five groups of cocoa products: cocoa beans (n = 108), cocoa beverage (n = 20), cocoa butter (n = 20), cocoa mass (n = 218), cocoa powder (n = 2583) and chocolate (n = 4008). As for the whole database, the main single source of records for cocoa products was the WHO European Region, accounting for 2293 records (33%).

The Committee additionally evaluated published data on dietary exposure to cadmium at a national level. Since the evaluation of cadmium at the seventy-third meeting of the Committee in 2011, a number of national evaluations of chronic dietary exposure have been published. The Committee evaluated 44 national studies conducted worldwide in 32 countries and a country grouping, as reported in the literature. Studies evaluated were from Australia, Bangladesh, Benin, Brazil, Cameroon, Canada, Chile, China, Denmark, Europe, France, French Polynesia, Germany, Hong Kong Special Administrative Region of China, Ireland, Islamic Republic of Iran, Italy, Japan, Republic of Korea, Mali, the Netherlands, New Zealand, Nigeria, Poland, Serbia, Spain, Sri Lanka, Sweden, Thailand, the United Kingdom, the United States of America and Viet Nam. Evaluation was restricted to studies that included most of the foods commonly eaten in the country.

Given the large number of national estimates of dietary cadmium exposure available from the literature, their coverage of countries across the world, and their consistency, the Committee considered that deriving less refined international and national estimates of dietary exposure was inappropriate. The GEMS/Food cluster diets were used only to examine the contribution of cocoa products to dietary cadmium exposure.

National estimates of dietary exposure

The mean dietary exposure to cadmium from the total diet at a national level ranged from 0.6 µg/kg bw per month for adults in the Sikasso region of Mali (2.4% of the PTMI) up to 24 µg/kg bw per month in children aged 4–11 years in China (96% of the PTMI). The maximum reported high percentile estimate of dietary cadmium exposure was 66 µg/kg bw per month in boys aged 8 years from Australia (260% of the PTMI). However, this estimate was based on a one-day 24-hour dietary recall (24HDR), which may have inflated the high percentile estimate. The highest high percentile estimate of dietary cadmium exposure based on multiple-day dietary records was for children aged

4–11 years in China (48.2 µg/kg bw per month; 190% of the PTMI). High percentile estimates of adult dietary cadmium exposure were only occasionally above the PTMI and were typically 20–60% of the PTMI. The main sources of cadmium exposure were grain and grain-based products, vegetables, and fish and seafood.

Temporal trends in dietary cadmium exposure

Owing to differences in study design and study location, it is not possible to identify any trends in dietary exposure to cadmium across the Committee evaluations (sixty-first, sixty-fourth, seventy-third and current). Most studies continue to report estimated mean dietary exposure to cadmium approximately in the range of 10–40% of the health-based guidance value, and sometimes higher. Similarly, the major foods contributing to dietary cadmium exposure have not changed, with cereals, vegetables and seafood, especially molluscs being consistent major contributors across evaluations. None of the Committee evaluations have identified cocoa products as major contributors to dietary cadmium exposure.

Contribution of cocoa products to dietary exposure

Where relevant information was included in the published national estimates of dietary exposure, the contribution of cocoa products to the total mean dietary exposure to cadmium ranged from 0.2 to 9%.

Further estimates of the contribution of cocoa products to dietary cadmium exposure were derived using the GEMS/Food cluster diets and global estimates of mean concentrations of cadmium derived from all extracted data in the GEMS/Food contaminants database (277 292 records). Across cluster diets, cocoa products contributed 0.1–5.9% of dietary cadmium exposure. Clusters with the highest contributions to dietary cadmium exposure from cocoa products were the “westernized” clusters (G07, G08, G10 and G15), including predominantly European and North American countries. Contributions for these clusters ranged from 3.4–5.9%, with the greatest contribution for G07. These contributions reflect the higher consumption of chocolate and, more particularly, cocoa powder in the countries within these clusters, as the cadmium concentrations in foods were assumed not to differ between clusters.

The major producers of cocoa are African countries (Cameroon, Côte d’Ivoire, Ghana and Nigeria), Indonesia and South and Central American countries (Brazil, Colombia, Dominican Republic, Ecuador and Peru). These countries are represented by the clusters G03, G05, G09 and G13. Interestingly, cocoa products were generally very low contributors to dietary cadmium exposure (<1%) in these regions.

The potential impact on the contribution of cocoa products to dietary cadmium exposure of consuming products sourced from a single geographical region (GEMS/Food cluster) was explored for the cluster diet (G07) with the greatest contribution from cocoa products to cadmium exposure. In addition, sufficient information for such an analysis was also available from the European dietary exposure assessment, carried out by the European Food Safety Authority (EFSA).² Based on these data, the Committee conducted a more detailed analysis of the impact of consumption of cocoa products from a single geographical region on dietary cadmium exposure for different age groups in Europe. The results of these analyses are summarized in Table 1. This analysis suggests that there are potential scenarios under which cocoa products would be the main contributor to dietary cadmium exposure.

Table 1

Impact of the source of cocoa products consumed on the contribution of cocoa products to dietary cadmium exposure, GEMS/Food cluster G07 and European countries

Population ^a	Contribution of cocoa products to dietary cadmium exposure (%) dependent on the source of cocoa products consumed			
	All ^b	G03 ^c	G05 ^c	G09 ^c
Literature national estimate	0.1–9.4			
Cluster G07 ^c	5.9	0.9	9.8	3.8
Population ^a	Contribution of cocoa products to dietary cadmium exposure (%) dependent on the source of cocoa products consumed			
European countries				

² EFSA. Cadmium dietary exposure in the European population: European Food Safety Authority. EFSA J. 2012;10:2551.

Infants	0.2	0.1	1.3	0.4
Toddlers	4.2	1.2	19.7	7.0
Other children	9.4	3.9	39.4	17.6
Adolescents	9.4	4.2	39.5	17.9
Adults	4.6	1.4	21.1	7.6
Elderly	2.6	0.7	12.6	4.2
Very elderly	2.8	0.8	13.7	4.7

^a Infants: 12 weeks–11 months; toddlers: 12–35 months; other children: 3–9 years; adolescents: 10–17 years; adults: 18–64 years, elderly: 65–74 years; very elderly: ≥ 75 years

^b For the GEMS/Food cluster G07, “all” refers to the total data set on cadmium concentrations in cocoa products submitted to the GEMS/Food contaminants database. For literature and European estimates (1), “all” refers to the cadmium concentration data used in the original analyses

^c Cluster G03 includes African countries, G05 includes mainly South and Central American countries, G09 includes mainly South-East Asian countries, and G07 includes mainly European countries, Australia, Bermuda and Uruguay

Impact of established and proposed maximum limits for cadmium on cocoa product rejection rates and dietary cadmium exposure

The Codex Alimentarius General Standard for Contaminants and Toxins in Food and Feed includes maximum limits (MLs) for cadmium in:

- chocolate containing or declaring ≥ 50% to < 70% total cocoa solids on a dry matter basis of 800 µg/kg; and
- chocolate containing or declaring ≥ 70% total cocoa solids on a dry matter basis of 900 µg/kg.

At the thirteenth meeting of CCCF in 2019, further MLs were discussed and it was proposed to derive MLs proportional to the cocoa solids content of the cocoa products:

- ML of 300 µg/kg for chocolates containing or declaring <30% total cocoa solids on a dry matter basis;
- ML of 500 µg/kg for chocolates containing or declaring ≥30% to <50% total cocoa solids on a dry matter basis; and
- ML of 1500 µg/kg for cocoa powder (100% total cocoa solids on a dry matter basis, sold for final consumption).

Of the 4008 records in the GEMS/Food contaminants database related to chocolate, it was only possible to establish the percentage of cocoa solids for 638 (15.9%). These records were virtually all from countries in cluster G05 (South/Central America). The proportion of samples that exceeded the established or proposed ML ranged from 2.1% for chocolate with a ≥30 to <50% cocoa solids content to 16.3% for cocoa powder. Virtually all cocoa powder samples with cadmium concentrations above the ML were from countries in cluster G05 (South/Central America), resulting in a substantially higher potential rejection rate for cocoa powder samples from this cluster (405 of 1345 samples, 30.1%).

A summary of potential rejection rates for chocolate and cocoa powder from application of established and proposed MLs and the impact of applying the MLs on mean cadmium concentrations is provided in Table 2.

Table 2

Proportion of chocolate samples in different cocoa solids content classes and cocoa powder from different sources exceeding the established or proposed Codex maximum limit (ML) and the impact on mean cadmium concentration (medium bound)

	Chocolate, classified by cocoa solids content (%) ^a					Cocoa powder			
	<30	≥30 to <50	≥50 to <70	≥70	All	G03	G05	G09	
ML (µg/kg)	300	500	800	900	1500	1500	1500	1500	
Number of samples	114	187	251	86	2583	74	1345	9	
Number of samples with cadmium concentration > ML (%)	3 (2.6)	4 (2.1)	27 (10.7)	4 (4.7)	420 (16.3)	0 (0.0)	405 (30.1)	0 (0.0)	
MB mean, all samples	121	180	474	318	971	141	1600	609	
MB mean, sample ≤ ML only (µg/kg)	110	172	418	255	502	141	814	609	

G03: mainly African countries; G05: mainly South/Central American countries; G09: mainly South-East Asian countries; LOD: limit of detection; MB: medium bound, analytical results below the limit of detection (LOD) are substituted by a value equal to LOD/2; ML: maximum limit

^a Samples for which the cocoa solids content was available were almost all from countries in cluster G05

Using the data across all clusters with sufficient information to allow application of the MLs, the mean contribution of cocoa products to dietary cadmium exposure was 2.2% without application of the MLs and 1.5% with application of MLs (see Table 3). Application of the MLs resulted in a mean reduction in dietary cadmium exposure of 0.7% across all clusters with reductions ranging from 0.0% (cluster G16) to 2.4% (cluster G07).

Application of the MLs had the greatest impact on dietary cadmium exposure when it was assumed that cocoa powder was sourced entirely from countries in cluster G05. This is not surprising as, for clusters G03, G05 and G09, only cocoa powder samples from cluster G05 had cadmium concentrations above the ML (30.1%, see Table 2). For cocoa products sourced from countries in cluster G03 and G09, application of the MLs had a negligible impact on dietary cadmium exposure, as the changes in exposure were only due to changes in the mean cadmium concentration for chocolate. The results of these analyses are summarized in Table 3.

Table 3

Impact of maximum limits (MLs) for cadmium in chocolate and cocoa powder and source of cocoa products on potential rejection rates and the contribution of cocoa products to dietary cadmium exposure for GEMS/Food cluster diets

Source of cocoa products ^a	Potential rejection rate (%) for cocoa powder samples from application of ML ^b	Mean contribution (range) of cocoa products to dietary cadmium exposure, GEMS/Food cluster diets (%)		Mean reduction (range) in dietary cadmium exposure due to application of MLs, GEMS/Food cluster diets ^c (%)
		Without MLs applied	With MLs applied	
All ^d	16.3	2.2 (0.1–6.6)	1.5 (0.1–4.3)	0.7 (0.0–2.4)
Cluster G03	0.0	1.1 (0.0–2.9)	1.1 (0.0–2.6)	0.1 (0.0–0.3)
Cluster G05	30.1	2.9 (0.2–9.3)	1.9 (0.1–5.7)	1.1 (0.0–3.8)
Cluster G09	0.0	1.7 (0.1–5.0)	1.6 (0.1–4.8)	0.1 (0.0–0.3)

ML: maximum limit, both proposed and established MLs were applied in this analysis; G03: mainly African countries; G05: mainly South/Central American countries; G09: mainly South-East Asian countries

^a Cocoa products included in the GEMS/Food cluster diets are cocoa beans, cocoa butter, cocoa mass, cocoa powder and chocolate

^b Potential rejection rates for chocolate are not given, as submitted data with sufficient information to allow application of MLs were only received from countries in cluster G05. The total rejection rate for chocolate samples was 4.9%

^c The percentages in this column are the percentage decreases in the estimated dietary cadmium exposure due to application of the MLs, rather than the difference in the contribution from cocoa products

^d "All" refers to the total data set on cadmium concentrations in cocoa products submitted to the GEMS/Food contaminants database with sufficient information to apply the MLs

Evaluation

The Committee assessed information related to exposure to cadmium from all food sources, with a particular focus on cocoa products. Information assessed was restricted to the period since the previous assessment of dietary exposure to cadmium in 2011. The Committee summarized dietary cadmium exposure estimates from 44 national studies conducted worldwide in 32 countries and a country grouping as reported in the literature. The mean dietary exposure to cadmium from the whole diet ranged from 0.6 µg/kg bw per month (2.4% of the PTMI) for adults in the Sikasso region of Mali up to 24 µg/kg bw per month (96% of the PTMI) in children aged 4–11 years in China. These children from China also had the highest high percentile estimate of dietary cadmium of

48.2 µg/kg bw per month (190% of the PTMI). High percentile estimates of adult dietary cadmium exposure were only occasionally above the PTMI and were typically 20–60% of the PTMI. Consistent with the previous evaluations of the Committee, the present evaluation identified the main sources of dietary cadmium exposure in these national studies as cereals and cereal-based products, vegetables, and fish and seafood. Of the 44 studies reviewed, only nine reported the contribution of cocoa products to the total mean dietary exposure to cadmium, which ranged from 0.2 to 9%.

Given the large number of national estimates of dietary cadmium exposure available from the literature, their coverage of countries across the world, and their consistency, the Committee considered that deriving less refined international and national estimates of dietary exposure was unnecessary.

Based on data on the concentration of cadmium in foods submitted to the GEMS/Food contaminants database since 1 January 2011, the Committee examined the contribution of cocoa products to the mean dietary exposure to cadmium using the GEMS/Food cluster diets. Analyses using these data showed that the contribution of cocoa products to the dietary exposure to cadmium was consistent with the estimates based on national dietary exposure studies, ranging from 0.1% to 5.9%. The highest contributions were calculated for European and North American countries, reflecting the higher consumption of chocolate and cocoa powder in these countries.

The potential impact of consumption of cocoa products from a single geographical region, as represented by GEMS/Food clusters was examined. For the cluster with the greatest contribution to dietary cadmium exposure from cocoa products (G07, mainly European countries, 5.9%) this contribution would decrease to 0.9% or increase to almost 10% if cocoa products were sourced only from countries in cluster G03 (Africa) or G05 (South/Central America), respectively. The Committee carried out a similar analysis using data (mean concentrations of cadmium in cocoa products, dietary cadmium exposure estimates and contributions of cocoa products to dietary exposure) for European countries reported by EFSA.¹ In the EFSA study, the age group with the greatest contribution to dietary cadmium exposure from cocoa products was children aged 3–9 years (contribution 9.4%). From the Committee's analysis, if this age group were to consume cocoa products sourced solely from cluster G03 (Africa), dietary cadmium exposure would decrease modestly (16.8 to 15.8 µg/kg bw per month), while the contribution from cocoa products would decrease to 3.9%. If this group were to consume cocoa products sourced solely from cluster G05 (South/Central America), dietary cadmium exposure would increase to 25.1 µg/kg bw per month, with cocoa products contributing 39% of dietary cadmium exposure.

CCCF has proposed MLs for chocolate with proportions of total cocoa solids of <30% and ≥30% to <50% on a dry matter basis and for cocoa powder with 100% total cocoa solids on a dry matter basis. These MLs are proposed in addition to existing MLs for chocolate with ≥50% to <70% and ≥70% total cocoa solids on a dry matter basis. Cocoa solids content information was available for a limited subset (15.9%) of the chocolate records in the GEMS/Food contaminants database. Comparing the cadmium concentrations in chocolate and cocoa powder in the GEMS/Food contaminants database to the existing and proposed MLs showed that 2.1–10.7% of the chocolate samples and 16.3% of the cocoa powder samples had concentrations higher than the MLs and could potentially be rejected by importing countries through application of the MLs. Applying these MLs compared to not applying them resulted in an average decrease in the contribution of cocoa products (including also cocoa beans, cocoa butter and cocoa mass) to the dietary exposure to cadmium of 0.7% across all clusters.

At its seventy-third meeting in 2011, the Committee established a PTMI of 25 µg/kg bw, reflecting the long half-life of cadmium in humans. The PTMI was not reviewed at the current meeting. The national exposure estimates were predominantly below this PTMI, with some exceptions for young children or adults living in China. The Committee noted that the current JECFA PTMI for cadmium is based on long-term bioaccumulation in the kidney, with steady-state not achieved until after 45–60 years of exposure. The Committee concluded that dietary exposure above the PTMI for limited periods may be of lesser concern in younger age groups. However, there may be a health concern in areas where the cadmium exposure during adulthood exceeds the PTMI.

The Committee concluded that major contributors to dietary cadmium exposure were cereals and cereal products, vegetables and seafood. The contribution of cocoa products to dietary cadmium exposure was minor in comparison (0.1–9.4% for national studies and estimates based on GEMS/Food cluster diets), even in countries in which the consumption of cocoa products is relatively high.

Application of both established and proposed MLs for chocolate and cocoa powder may result in substantial rejection rates (up to 30%) for products from some regions, but has only a minor impact

(mean decrease across clusters of 0.7%, range 0.0–2.4%) on total dietary cadmium exposure.

A dietary exposure monograph was prepared.

¹ EFSA. Cadmium dietary exposure in the European population: European Food Safety Authority. EFSA J. 2012;10:2551.

Ergot alkaloids

The Committee identified the pharmacological effect of ergometrine maleate on the uterus, causing uterine contractions in humans during late pregnancy and postpartum, as the critical effect for the evaluation of ergot alkaloids (EAs) in the diet.

The Committee established an acute reference dose (ARfD), based on the following considerations:

1. The lowest oral therapeutic dose of 0.2 mg ergometrine maleate (equivalent to 2.5 µg/kg bw, expressed as ergometrine) is considered a pharmacological effect level in the most sensitive individuals, i.e. those with high absorption.
2. Of the EAs that have been used as drugs, ergometrine is known to have the highest potency for uterine contractions and its uterotonic effect increases towards the end of pregnancy.

In selecting an uncertainty factor (UF) for extrapolation from the pharmacological effect level at the therapeutic dose (LOEL) to a NOEL, the Committee took into consideration that the data relate to a short-lived, reversible, pharmacological effect, seen within a very sensitive subpopulation (women in late pregnancy or postpartum). A UF of 2 was considered appropriate for extrapolating from a pharmacological LOEL to a NOEL.

To derive an ARfD from a NOEL based on human data, in the absence of additional information, the default UF would normally be 10. However, for a substance that reversibly interacts with specific receptors, as is the case here, with a pharmacological effect that is predominantly dependent on its maximum plasma concentration (i.e. C_{max}), a UF for toxicokinetic differences is considered unnecessary. The Committee therefore applied the UF of 3.16 to cover possible interindividual toxicodynamic differences.

Applying a composite UF of 6.3 (2×3.16) results in an acute reference dose of 0.4 µg ergometrine/kg bw ($2.5 \div 6.3 = 0.4$). The Committee noted that it is appropriate to establish a group acute reference dose for EAs but concluded that the available data are not sufficient to establish toxic equivalency factors (TEFs) for different EAs. Therefore, the ARfD is established as a group ARfD for the simple sum of total EAs in the diet.

This ARfD would also be protective for other potentially sensitive subgroups in the population, such as children, based on similar calculations in relation to adverse effects (gastrointestinal symptoms) in that group following unintentional exposure to ergometrine maleate.

Limited data from two 4-week studies on ergotamine tartrate and α-ergocryptine in rats allowed the determination of a reference point (BMDL₁₀) of 1.3 mg/kg bw for EAs, based on muscular degeneration in the tail, secondary to vasoconstriction. The Committee noted that the human pharmacological effect level of 2.5 µg/kg bw and its derived NOEL provided a much more sensitive reference point for derivation of an ARfD than the BMDL₁₀ value from a downstream toxic effect in animals.

As a first approach to establishing a TDI, the Committee considered the data from repeated-dose animal studies and selected the lowest BMDL₁₀ value of 0.6 mg/kg bw per day calculated for ergotamine, based on tail muscular

atrophy, secondary to vasoconstriction, observed in the 13-week study in rats as reference point. Applying a default UF of 100 for intra- and inter-species differences, a UF of 2 for extrapolation from a 13-week study to chronic exposure and an additional UF of 3 to take into account the limitations of the available toxicity data would indicate derivation of a TDI of 1 µg/kg bw per day.

The Committee considered that a TDI should not be higher than the ARfD and decided to establish a group TDI for the sum of total EAs in the diet at the same value as the group ARfD of 0.4 µg/kg bw per day.

The Committee noted that some estimates of the mean (0.46–0.47 µg/kg bw per day) and high percentile (0.56–0.86 µg/kg bw per day) chronic dietary exposure in children and some estimates of the high percentile acute dietary exposure in children (0.65–0.98 µg/kg bw per day) and in adults (0.49 µg/kg bw per day) exceeded the EAs group health-based guidance value (HBGV), and that this may indicate a human health concern.

Previous cargoes

Margins of exposure and other conclusions on toxicology and dietary exposure

Solvents/reactants (Group 1)

Acetic anhydride

No information regarding the short-term and long-term toxicity of acetic anhydride was identified. However, upon evaluation of the available information, the Committee noted that it had previously allocated a group acceptable daily intake (ADI) “not specified” to acetic anhydride’s immediate hydrolysis product, i.e. acetic acid and its potassium and sodium salts. Since acetic anhydride is anticipated to be rapidly hydrolysed to acetic acid during tank washing, within the edible oil cargo and after ingestion, the group ADI “not specified” for acetic acid and its potassium and sodium salts is considered directly relevant for this assessment of acetic anhydride. The United States National Research Council estimated that mean exposure to acetic acid from all food sources is 2.1 g/day for persons above 2 years of age, which is equivalent to 35 mg/kg bw per day for adults based on a body weight of 60 kg. It is not expected that exposure to acetic acid present due to hydrolysis of acetic anhydride in carryover from previous cargoes would add significantly to total exposures to acetic acid. Therefore, acetic anhydride at the generic human dietary exposure value for previous cargoes of 0.3 mg/kg bw per day would only contribute marginally to the overall dietary exposure to acetic acid and is not expected to result in adverse effects on human health.

The Committee concluded that considering the widespread presence of acetic acid in the diet, it is unlikely that acetic anhydride present in low concentrations such as when transported as a previous cargo will produce an allergic response.

Acetic anhydride acetylates free hydroxyl groups without a catalyst, but esterification is more complete in the presence of acids, so acetic anhydride and acetic acid could react with alcohols (for example mono- and diglycerides) forming acetates. Reaction rates are likely to be slow at ambient temperature.

Although exposure to acetic anhydride and acetic acid as a result of transporting acetic anhydride as a previous cargo does not appear to be a health concern, there is uncertainty concerning the purity or “grade” of acetic anhydride that is transported as a previous cargo. Since acetic anhydride may contain impurities (e.g. diketene), which are potentially genotoxic, the Committee could not reach a conclusion on the safety of transporting acetic anhydride as a previous cargo for edible fats and oils until the nature and quantities of these impurities have been clarified.

sec-Butyl acetate

No information regarding the short-term and long-term toxicity of sec-butyl acetate was identified; however, for sec-butanol, the Committee identified a BMDL₀₅ of 657 mg/kg bw per day based on reduced offspring body weight from a two-generation reproductive and developmental toxicity study in rats. sec-Butyl acetate is naturally present in vinegar and is approved for use as a flavouring agent in Europe. The Committee estimated that exposure to sec-butyl acetate from vinegar consumption and its use as a flavouring agent is approximately 0.1 mg/kg bw per day. A comparison of the BMDL₀₅ of 657 mg/kg bw per day for sec-butanol with the generic human dietary exposure value for previous cargoes of 0.3 mg/kg bw per day for sec-butyl acetate as a previous cargo plus its presence in the diet (0.1 mg/kg bw per day) yields a margin of exposure (MOE) of 1643, which is considered sufficient to address the uncertainties in the database.

There are no data on allergenicity upon oral exposure to sec-butyl acetate that indicate that it is or it contains a known food allergen.

sec-Butyl acetate hydrolyses to acetic acid and sec-butanol, which in the presence of acid may participate in transesterification with lipids, producing a mixture of fatty acid sec-butyl esters and glycerol. However, the reactions are slow, requiring an excess of alcohol and temperatures above 100 °C.

Therefore, sec-butyl acetate meets the criteria for acceptability as a previous cargo for edible fats and oils.

tert-Butyl acetate

No information regarding the short-term and long-term toxicity of tert-butyl acetate was identified; however, the Committee identified a LOAEL of 180 mg/kg bw per day based on renal effects observed in female rats chronically exposed to a metabolite of tert-butyl acetate (i.e. tert-butanol) in drinking-water. The LOAEL for tert-butanol is lower than the NOAEL of 400 mg/kg bw per day of tert-butyl acetate for developmental toxicity and represents a conservative metric for risk assessment of tert-butyl acetate. No data were found on concentrations of tert-butyl acetate in food from any source. A comparison of the LOAEL of 180 mg/kg bw per day with the generic human dietary exposure value for previous cargoes of 0.3 mg/kg bw per day for tert-butyl acetate as a previous cargo yields a MOE of 600, which is considered sufficient to address the uncertainties in the database.

There are no data on allergenicity upon oral exposure to tert-butyl acetate that indicate that it is or it contains a known food allergen.

tert-Butyl acetate hydrolyses to acetic acid and tert-butanol, which in the presence of acid may participate in transesterification with lipids producing a mixture of fatty acid tert-butyl esters and glycerol. However, the reactions are slow, requiring an excess of alcohol and temperatures above 100 °C.

Therefore, tert-butyl acetate meets the criteria for acceptability as a previous cargo for edible fats and oils.

***n*-Pentane**

No reliable information regarding the short-term and long-term toxicity of *n*-pentane was identified; however, the Committee identified a NOAEL of 1000 mg/kg bw per day for *n*-pentane based on developmental toxicity testing in rats. The Committee also identified a NOAEL of 300 mg/kg bw per day for an isomer (isopentane) following short-term oral exposure in a one-generation toxicity test in rats (12 and 10 weeks of exposure in males and females, respectively). A comparison of the NOAEL of 300 mg/kg bw per day for isopentane with the generic human dietary exposure value for previous cargoes of 0.3 mg/kg bw per day yields a MOE of 1000, which is sufficient to address the uncertainties in the database.

There are no data on allergenicity upon oral exposure to *n*-pentane that indicate that it is, or it contains a known food allergen.

n-Pentane as a previous cargo is not expected to react with edible fats and

oils to form any reaction products.

Exposure to impurities in *n*-pentane is not anticipated to contribute significantly to background exposures. Therefore, *n*-pentane meets the criteria for acceptability as a previous cargo for edible fats and oils.

Cyclohexane

No information regarding the short-term and long-term toxicity of cyclohexane was identified; however, cyclohexane exhibits relatively low systemic toxicity following short-term exposure via inhalation. The Committee identified a NOAEL of 62.5 mg/kg bw per day from two short-term oral toxicity studies with the structural analogue methylcyclohexane. Cyclohexane may be used as an extraction solvent for flavouring agents or as a diluent in colour additive mixtures. However, no estimates of cyclohexane concentrations in foods or of exposure from these sources were identified. A comparison of the NOAEL of 62.5 mg/kg bw per day with the estimated generic human dietary exposure value for previous cargoes of 0.3 mg/kg bw per day yields a MOE of 208. The Committee noted that this MOE is based on a potentially more toxic compound and a sensitive critical effect (hyaline droplets in the renal tubules of male rats). In consideration of the conservative nature of both the exposure and hazard metrics used, the Committee concluded that this MOE is sufficient to address the uncertainties in the database.

There are no data on allergenicity upon oral exposure to cyclohexane that indicate that it is or it contains a known food allergen.

Cyclohexane as a previous cargo is not expected to react with edible fats and oils.

Although exposure to cyclohexane as a result of transporting cyclohexane as a previous cargo does not appear to be a health concern, there is uncertainty concerning the purity or "grade" of cyclohexane that will be transported as a previous cargo. Since cyclohexane may contain carcinogenic impurities in amounts that could significantly increase dietary exposure, the Committee could not reach a conclusion on the safety of transporting cyclohexane as a previous cargo for edible fats and oils until the nature and the quantities of these impurities in cyclohexane has been clarified.

Revision of specifications

Steviol glycosides

The Committee replaced the existing assay for steviol glycosides in the (*Framework for*) *steviol glycosides* (Appendix B) with the HPLC-UV-MS technique utilizing external reference standards. The Committee additionally replaced the assay method in Annex 4 (enzyme modified glycosylated steviol glycosides) with the submitted HPLC-UV technique and removed the tentative status of Annex 4. An updated table of chemical information for steviol glycosides from *Stevia rebaudiana* Bertoni replaced Appendix A; and Annexes 1, 2 and 3 were revised to include the harmonized solubility parameters and a reference to Appendix B (the assay for steviol glycosides). The Committee noted that the revised (*Framework for*) *steviol glycosides* specifications monograph, including the appendices and four annexes, replaces the tentative specifications prepared at its eighty-seventh meeting. All specifications for steviol glycoside products evaluated by JECFA are now incorporated in the (*Framework for*) *steviol glycosides* prepared at the present meeting.

Future work and recommendations

Ergot alkaloids

The Committee recommended the following:

- additional data on the EAs to allow for the derivation of toxic equivalency factors (TEFs);
- additional data on the occurrence of EAs (at least for the 12 considered at this meeting) in wheat and wheat-based products and in rye and rye products from WHO regions and clusters where no data were submitted for this evaluation;
- the establishment of sampling plans for EAs.

Previous cargoes

1. The Committee reiterated the recommendations made at the ninetieth meeting that the Codex Committee on Fats and Oils (CCFO) consider revising Criterion no. 2 in RCP-36-1987 as adopted by CAC 34 (2011).
 - Based on the consumption of fats and oils by infants and young children, there is no health concern for the general population from dietary exposure to previous cargo chemical substances if the ADI or TDI is sufficiently protective, for example, the ADI or TDI is greater than, or equal to 0.3 mg/kg bw per day. Substances for which there is no numerical ADI or TDI should be evaluated on a case-by-case basis (e.g. margin of exposure (MOE) approach).
 - Where there are additional sources of dietary exposure to the previous cargo chemical substances, they should be considered in the exposure assessment.
2. The Committee recommended that sufficient chemical information that allows the evaluation of acetic anhydride and cyclohexane transported as previous cargoes be made available prior to the next evaluation. At a minimum this information should address the following:
 - product grade(s) and composition, including characterization and levels of impurities arising from all methods of manufacture.

Procedural Matters

The ninety-first meeting of JECFA was held from 1 to 12 February 2021. Owing to the travel restrictions and lockdowns due to the COVID-19 pandemic in many countries, it was not possible to convene a physical meeting and it was instead decided to hold it online by video-conferencing. In view of the time differences in the countries of origin of the invited experts, the only possible time for a video-conference was restricted to a 4-hour time slot (12:00–16:00 CET) each day. This allowed only 40% of the usual daily length (8–10 hours) of a typical JECFA meeting. Although the experts participated fully, they noted that online meetings do not permit the necessary in-depth, robust scientific discussions that are characteristic of JECFA meetings and are therefore not a suitable substitute for face-to-face JECFA meetings. In particular, the experts felt that the online format did not foster the atmosphere of trust, inclusiveness and openness that has marked physical JECFA meetings. The experts considered that the success of the ninety-first meeting was mainly due to the cohesion between them, which stemmed from the trust built on the relationships they had formed during previous face-to-face meetings. The experts also decried the significant difficulty of holding any informal meetings outside the scheduled meeting times because of the widely differing time zones. They noted that such informal interactions during the physical meetings were instrumental in solving problems and discussing issues in depth, bilaterally or in small groups, and added that such informal settings often gave rise to equitable solutions to stubborn problems.

The experts emphasized further that an invitation to a physical JECFA meeting at the FAO or WHO headquarters gives rise to a more significant recognition by the expert's employer of the weight, reach, responsibility and workload required for full participation in a JECFA meeting. The same degree of acknowledgement was not granted by employers for this online meeting, as the experts remained available locally. This lack of recognition of the workload and significance of participation in a JECFA meeting led to an increase in other demands on the experts, resulting in greater distractions and more frequent scheduling conflicts. The experts concluded that, cumulatively, such factors would be counterproductive for participation in future JECFA meetings if FAO and WHO maintained the online-only format.

In recognition of the difficulties and the tremendous efforts made, the Joint FAO/WHO Secretariat expressed its deep gratitude to all the experts for their commitment and flexibility, not least as the scheduled meeting times were exceedingly inconvenient for many.

The meeting report was adopted on 25 February 2021.