Residue Monograph prepared by the meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), 82nd meeting 2016

**Steviol Glycosides From *Stevia Rebaudiana* Bertoni**
(Tentative)

This monograph was also published in: *Compendium of Food Additive Specifications. Joint FAO/WHO Expert Committee on Food Additives (JECFA), 82nd meeting 2016. FAO JECFA Monographs 19*

© FAO/WHO 2016
STEVIOL GLYCOSIDES FROM STEVIA REBAUDIANA BERTONI
(TENTATIVE)

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

Information required by December 2017:
- Method of Assay to replace the existing method and including as many steviol glycosides as possible (at least those listed in Appendix 1) in steviol glycoside mixtures, along with supporting validation information and chromatograms
- Analysis results from a minimum of 5 batches for commercial samples including supporting chromatograms

SYNONYMS
INS no. 960

DEFINITION
Steviol glycosides consists of a mixture of compounds containing a steviol backbone conjugated to any number or combination of the principal sugar moieties in any of the orientations occurring in the leaves of Stevia rebaudiana Bertoni including, glucose, rhamnose, xylose, fructose, and deoxyglucose. The product is obtained from the leaves of Stevia rebaudiana Bertoni. The leaves are extracted with hot water and the aqueous extract is passed through an adsorption resin to trap and concentrate the component steviol glycosides. The resin is washed with a solvent alcohol to release the glycosides and the product is recrystallized from methanol or aqueous ethanol. Ion exchange resins may be used in the purification process. The final product may be spray-dried.

Chemical name, C.A.S. number, Chemical formula
See Appendix 1

Structural formula

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

© FAO/WHO 2016
Assay  
Not less than 95% of total of steviol glycosides on the dried basis.

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

FUNCTIONAL USES  
Sweetener

CHARACTERISTICS

IDENTIFICATION

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

HPLC chromatographic profile  
The main peaks in the sample chromatogram obtained by following the procedure in METHOD OF ASSAY correspond to steviol glycoside compounds.

pH (Vol. 4)  
Between 4.5 and 7.0 (1 in 100 solution)

PURITY

Total ash (Vol. 4)  
Not more than 1%

Loss on drying (Vol. 4)  
Not more than 6% (105°, 2h)

Residual solvents (Vol. 4)  
Not more than 200 mg/kg methanol and not more than 5000 mg/kg ethanol (Method I, General Methods, Organic Components, Residual Solvents)

Arsenic (Vol. 4)  
Not more than 1 mg/kg  
Determine using a method appropriate to the specified level (Use Method II to prepare the test (sample) solution). The selection of sample size and method of sample preparation may be based on the principles of the methods described in Vol. 4 (under “General Methods, Metallic Impurities”).

Lead (Vol. 4)  
Not more than 1 mg/kg  
Determine using a method appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Vol. 4 (under “General Methods, Metallic Impurities”).

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

Reagents
Acetonitrile: more than 95% transmittance at 210 nm.

Standards
Stevioside: more than 99.0% purity on the dried basis.
Rebaudioside A: more than 99.0% purity on the dried basis.
Mixture of nine steviol glycosides standard solution: Containing stevioside, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside F, dulcoside A, rubusoside and steviolbioside. This solution is diluted with water-acetonitrile (7:3) accordingly and is used for the confirmation of retention times. Standards are available from Wako Pure Chemical Industries, Ltd. Japan and ChromaDex, USA.

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

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

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

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

\[%X = \frac{W_S}{W} \times \frac{f_X A_X}{A_S} \times 100\]

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

\[% \text{Rebaudioside A} = \frac{W_R}{W} \times \frac{A_R}{A_S} \times 100\]

where
- X is each steviol glycoside;
- \(W_S\) is the amount (mg) calculated on the dried basis of stevioside in the standard solution;
- \(W_R\) is the amount (mg) calculated on the dried basis of rebaudioside A in the standard solution;
- W is the amount (mg) calculated on the dried basis of sample in the sample solution;
- \(A_S\) is the peak area for stevioside from the standard solution;
A_R is the peak area for rebaudioside from the standard solution; A_X is the peak area of X for the sample solution; and f_X is the ratio of the formula weight of X to the formula weight of stevioside: 1.00 (stevioside), 1.20 (rebaudioside A), 1.00 (rebaudioside B), 1.18 (rebaudioside C), 1.40 (rebaudioside D), 1.16 (rebaudioside F), 0.98 (dulcoside A), 0.80 (rubusoside) and 0.80 (steviolbioside).

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

Figure. Chromatogram of mixture of nine steviol glycosides standard solution

Column: Capcell pak C_{18} MG II
Concentration: 0.5 mg/ml each except rebaudioside F (about 0.1 mg/ml)
## Chemical Information for Some Steviol Glycosides

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

<table>
<thead>
<tr>
<th>Common Name</th>
<th>( R_1 )</th>
<th>( R_2 )</th>
<th>Chemical Name</th>
<th>CAS Number</th>
<th>Chemical Formula</th>
<th>Formula Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1: Steviol + Glucose (SvGn)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rubusoside</td>
<td>Glcβ1-</td>
<td>Glcβ1-</td>
<td>13-[(β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester</td>
<td>64849-39-4</td>
<td>C(<em>{32})H(</em>{50})O(_{13})</td>
<td>642.73</td>
</tr>
<tr>
<td>Steviolbioside</td>
<td>H</td>
<td>Glcβ(1-2)Glcβ1-</td>
<td>13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid</td>
<td>41093-60-1</td>
<td>C(<em>{32})H(</em>{50})O(_{13})</td>
<td>642.73</td>
</tr>
<tr>
<td>Stevioside</td>
<td>Glcβ1-</td>
<td>Glcβ(1-2)Glcβ1-</td>
<td>13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester</td>
<td>57817-89-7</td>
<td>C(<em>{38})H(</em>{60})O(_{18})</td>
<td>804.87</td>
</tr>
<tr>
<td>Rebaudioside B</td>
<td>H</td>
<td>Glcβ(1-2)[Glcβ(1-3)]Glcβ1-</td>
<td>13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid</td>
<td>58543-17-2</td>
<td>C(<em>{38})H(</em>{60})O(_{18})</td>
<td>804.87</td>
</tr>
<tr>
<td>Rebaudioside E</td>
<td>Glcβ(1-2)Glcβ1-</td>
<td>Glcβ(1-2)Glcβ1-</td>
<td>13-[(O-β-D-Glucopyranosyl-(1,2)-O-β-D-glucopyranosyl)oxy]-kaur-16-en-18-oic acid (4')-O-β-D-glucopyranosyl-deoxy-(1,2)-O-β-D-glucopyranosyl ester</td>
<td>63279-14-1</td>
<td>C(<em>{44})H(</em>{70})O(_{23})</td>
<td>967.01</td>
</tr>
<tr>
<td>Rebaudioside A</td>
<td>Glcβ1-</td>
<td>Glcβ(1-2)[Glcβ(1-3)]Glcβ1-</td>
<td>13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester</td>
<td>58543-16-1</td>
<td>C(<em>{44})H(</em>{70})O(_{23})</td>
<td>967.01</td>
</tr>
<tr>
<td>Rebaudioside D</td>
<td>Glcβ(1-2)Glcβ1-</td>
<td>Glcβ(1-2)[Glcβ(1-3)]Glcβ1-</td>
<td>13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester</td>
<td>63279-13-0</td>
<td>C(<em>{50})H(</em>{80})O(_{28})</td>
<td>1129.15</td>
</tr>
<tr>
<td>Common Name</td>
<td>Chemical Name</td>
<td>CAS Number</td>
<td>Chemical Formula</td>
<td>Formula Weight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>---------------</td>
<td>------------</td>
<td>-----------------</td>
<td>----------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rebaudioside M</td>
<td>13-[(O-β-D-glucopyranosyl-(1,2)-O-[β-D-glucopyranosyl-(1,3)]-β-D-glucopyranosyl-oyl]-kaur-16-en-18-oic acid (4')-O-β-D-glucopyranosyl-(1,2)-O-[β-D-glucopyranosyl-(1,3)]-β-D-glucopyranosyl ester</td>
<td>1220616-44-3</td>
<td>C₅₆H₉₀O₃₃</td>
<td>1291.29</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Group 2: Steviol + Rhamnose + Glucose (SvR1Gn)**

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Chemical Formula</th>
<th>Formula Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulcoside A</td>
<td>13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester</td>
<td>C₅₆H₆₀O₁₇</td>
</tr>
<tr>
<td>Rebaudioside C</td>
<td>13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester</td>
<td>C₄₄H₇₀O₂₂</td>
</tr>
<tr>
<td>Rebaudioside N</td>
<td>13-[(O-β-D-glucopyranosyl-(1,2)-O-[β-D-glucopyranosyl-(1,3)]-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid (4')-O-6-deoxy-L-mannopyranosyl-(1,2)-O-[β-D-glucopyranosyl-(1,3)]-β-D-glucopyranosyl ester</td>
<td>C₅₆H₉₀O₃₂</td>
</tr>
<tr>
<td>Rebaudioside O</td>
<td>13-[(O-β-D-glucopyranosyl-(1,2)-O-[β-D-glucopyranosyl-(1,3)]-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid (4')-O-6-deoxy-L-mannopyranosyl-(1,2)-O-[β-D-glucopyranosyl-(1,3)]-β-D-glucopyranosyl ester</td>
<td>C₆₂H₁₀₀O₃₇</td>
</tr>
</tbody>
</table>

**Group 3: Steviol + Xylose + Glucose (SvX1Gn)**

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Chemical Formula</th>
<th>Formula Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rebaudioside F</td>
<td>13-[(2-O-β-D-xylopyranosyl-3-O-β-D-xylopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester</td>
<td>C₄₃H₆₈O₂₂</td>
</tr>
</tbody>
</table>

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