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**Steviol glycosides — Specification**

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## Foreword

Uganda National Bureau of Standards (UNBS) is a parastatal under the Ministry of Trade, Industry and Cooperatives established under Cap 327, of the Laws of Uganda, as amended. UNBS is mandated to co-ordinate the elaboration of standards and is

- (a) a member of International Organisation for Standardisation (ISO) and
- (b) a contact point for the WHO/FAO Codex Alimentarius Commission on Food Standards, and
- (c) the National Enquiry Point on TBT Agreement of the World Trade Organisation (WTO).

The work of preparing Uganda Standards is carried out through Technical Committees. A Technical Committee is established to deliberate on standards in a given field or area and consists of key stakeholders including government, academia, consumer groups, private sector and other interested parties.

Draft Uganda Standards adopted by the Technical Committee are widely circulated to stakeholders and the general public for comments. The committee reviews the comments before recommending the draft standards for approval and declaration as Uganda Standards by the National Standards Council.

The committee responsible for this document is UNBS/TC 2 [*Food and Agriculture standards*], Subcommittee SC 6, [*Food Additives and contaminants*].

## Introduction

*Stevia rebaudiana* Bertoni is a perennial shrub in South America and Asia. In the two regions, water extracts of crushed leaves of the stevia plant are widely used as a sweetener/sugar replacer. Their use is expanding in water-based beverages and dietetic food products as a natural sugar replacer.

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, from which they are obtained. 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 and the final product may be spray-dried.

Stevial glycosides or extracts are reported to be 200 – 300 times sweeter than sucrose. Codex established the Acceptable Daily Intake for steviol glycosides at 0 – 4 mg/kg body weight and according to CODEX STAN 192-1995 – General Standard for Food Additives, they are approved for use in a variety of food products as INS 960.

This standard has been developed to regulate the trade and use of steviol glycosides in food products.



# Steviol glycosides — Specification

## 1 Scope

This Draft Uganda Standard specifies requirements, sampling and test methods for steviol glycosides from *Stevia rebaudiana* Bertoni intended for human consumption.

## 2 Normative references

The following referenced documents are referred to in the text in such a way that some or all their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

AOAC 972.25, *Lead in food. Atomic absorption spectrophotometric method*

AOAC 952.13, *Arsenic in food. Silver diethyldithiocarbamate*

US 277, *General standard for the labelling of food additives when sold as such*

US CAC/GL 50, *General guidelines on sampling*

US ISO 4833-1, *Microbiology of the food chain — Horizontal method for the enumeration of microorganisms — Part 1: Colony count at 30 °C by the pour plate technique*

US ISO 4833-2, *Microbiology of the food chain — Horizontal method for the enumeration of microorganisms — Part 2: Colony count at 30 °C by the surface plating technique*

ISO 7251, *Microbiology of food and animal feeding stuffs — Horizontal method for the detection and enumeration of presumptive Escherichia coli — Most probable number technique*

US ISO 6579-1, *Microbiology of the food chain — Horizontal method for the detection, enumeration and serotyping of Salmonella — Part 1: Detection of Salmonella spp.*

US ISO/TS 6579-2, *Microbiology of food and animal feed — Horizontal method for the detection, enumeration and serotyping of Salmonella — Part 2: Enumeration by a miniaturized most probable number technique*

US ISO 21527-2, *Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of yeasts and moulds — Part 2: Colony count technique in products with water activity less than or equal to 0.95*

## 3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following address:

— ISO Online browsing platform: available at <http://www.iso.org/obp>

### **food grade material**

material made of substances which are safe and suitable for their intended use and which will not impart any toxic substance or undesirable odour or flavour to the product

## 4 Requirements

### 4.1 General requirements

Steviol glycosides shall be:

- a) white to light yellow, odourless or having a slight odour characteristic of the stevia extracts; and
- b) freely soluble in a mixture of water and ethanol (50:50) when tested in accordance with the procedure elaborated in Annex A.

### 4.2 Specific requirements

#### 4.2.1 Physicochemical requirements

Steviol glycosides shall comply with the physicochemical requirements given in Table 1 when tested in accordance with the test methods specified therein.

**Table 1 — Physicochemical requirements for steviol glycosides**

S/N	Characteristic	Requirement	Test method
i)	Total steviol glycoside content (dry basis), min.	95	Annex B
ii)	pH (1% solution)	4.5 – 7.0	Annex C
iii)	Loss on drying, % m/m, max.	6	Annex D
iv)	Total ash, % (dry basis), max.	1	AOAC 923.03
Residual solvents			
v)	Methanol (mg/kg), max.	200	Annex E
vi)	Ethanol (mg/kg), max.	5 000	

#### 4.2.2 Microbiological requirements

Steviol glycosides shall conform to the microbiological requirements given in Table 2 when tested in accordance with the methods specified therein.

**Table 2 — Microbiological requirements for steviol glycosides**

S/N	Characteristic	Requirement	Test method
i)	Total (aerobic) plate count, CFU/g, max.	1 000	US ISO 4833-1 / US ISO 4833-2
ii)	<i>Escherichia coli</i> , CFU/g	Not detected	ISO 7251
iii)	<i>Salmonella</i> , CFU/25 g	Not detected	US ISO 6579-1 / US ISO/TS 6579-2
iv)	Yeasts and moulds, CFU/g, max.	200	US ISO 21527-2

## 5 Contaminants

Steviol glycosides shall conform to the contaminant limits specified in Table 3 when tested in accordance with the methods specified therein.



Table 3 — Limits for contaminants for steviol glycosides

S/N	Characteristic	Requirement	Test method
i)	Arsenic (mg/kg), max.	1	AOAC 952.13
ii)	Lead (mg/kg), max.	1	AOAC 972.25

## 6 Pesticide residues

Steviol glycosides shall comply with the maximum pesticide residue limits established by the Codex Alimentarius Commission.

## 7 Packaging

The product shall be securely packaged in containers made of food grade materials. The packaging shall be able to safeguard the product from contamination and protect its properties throughout the shelf life period.

## 8 Weights and Measures

The weight of the packages shall comply with the Weights and Measures (Sales and Labelling of goods) Regulations of Uganda.

## 9 Labelling

The product shall be labelled in accordance with US 277.

## 10 Sampling

Representative samples of the product shall be drawn in accordance with US CAC/GL 50.

**Annex A**  
(normative)

**Test for solubility**

**A.1 Procedure**

Transfer 1 g of sample into a calibrated 10-ml glass-stoppered cylinder graduated in 0.1-ml subdivisions. Slowly and in small portions, add a 50:50 mixture of distilled water and ethanol (95%). Maintain the temperature at 20 °C.

A clear solution should be obtained.

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## Annex B (normative)

### Total steviol glycoside content – HPLC chromatographic profile

#### B.1 Principle

Determine the percentages of major steviol glycosides (those with analytical standards) using HPLC (Method A). Confirm the presence of each minor steviol glycoside (compounds where analytical standards are not available) using HPLC-MS (Method B). Calculate the concentration of the minor compounds using respective molecular mass corrected UV peak area against the rebaudioside A UV standard curve. Calculate their sum and express the content on the dried basis.

#### B.2 Method A: Determination of major steviol glycosides by HPLC

##### B.2.1 Reagents

Acetonitrile: HPLC grade with transmittance more than 95% at 210 nm.

- Deionized water: HPLC grade
- Standards (Reference and Quality Control Standards): Stevioside, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside E, rebaudioside F, rebaudioside M, rebaudioside N, rebaudioside O, dulcoside A, rubusoside and steviolbioside.

Note: Standards of other steviol glycosides may also be included.

##### B.2.2 Apparatus

HPLC consisting of: a high precision binary pump and an auto sampler (capable of operating at 2 °C – 8 °C); Diode-Array detector @ UV at 210 nm; and Mass Spectrometric Detector (Electrospray Negative Ionisation over a mass range from 50 to 1500 m/z using a unit mass resolution, For use in Method B below) connected in series. Agilent 1200 with Waters Quattro or equivalent:

- Column: Luna 5 $\mu$  C18 (2), 100A, (150 mm x 4.6 mm, 5 $\mu$ m, Phenomenex) or Capcell pak C18 MG II (250 mm x 4.6 mm, 5 $\mu$ m, Shiseido Co. Ltd) or equiv.
- Column temperature: 50 °C
- Auto sampler temperature: 2 °C – 8 °C
- Injection volume: 10  $\mu$ l
- Mobile phase A: Deionised or LC-MS grade water (0.2  $\mu$ m filtered)
- Mobile phase B: LC-MS grade Acetonitrile (0.2  $\mu$ m filtered)

##### B.2.3 Preparation of steviol glycosides standard solutions

- Prepare individual stock standard solutions (1.5 mg/ml) in water:acetonitrile (7:3);
- Prepare mixed standard solution (115  $\mu$ g/ml) by mixing 1.0 ml each individual stock standard solutions;
- Prepare Peak Identification Standard Solutions (0.1 mg/ml) from individual stock standard solutions in water:acetonitrile (7:3).

- Prepare mixed Committee standard solutions in the range of 20 – 100 µg/ml by following appropriate dilution of mixed standard solution (b) with water:acetonitrile (7:3).
- Prepare quality control and system suitability individual stock standard solutions (1.5 mg/ml) as well as mixed standard solution (115 µg/ml) using standards from a different batch /manufacturer (if available).
- Prepare quality control mixed Committee standard solutions (40 and 80 µg/ml) and system suitability standard (52 µg/ml) by following appropriate dilutions of mixed standard solution

#### B.2.4 Preparation of sample solution

Accurately weigh 50 mg ( $w_{sample}$ ) of sample and quantitatively transfer into a 50-ml volumetric flask. Add about 20 ml of water:acetonitrile (7:3), sonicate and shake well to dissolve the sample and make up to volume.

#### B.2.5 Procedure

Inject peak identification standard solutions, identify peaks and calculate relative retention times (RRT) with respect to rebaudioside A.

Inject committee mixed standard solutions and construct standard curves for each steviol glycoside. Inject quality control and system suitability standard solutions to ensure a satisfactory committee system.

Inject prepared samples. Dilute sample solution, if required, to bring the concentration of each analyte within the standard curve range. Make duplicate injections. Deduce concentration of each steviol glycoside from its corresponding standard curve and obtain average concentration in sample solution as  $C_{sample}$  (µg/ml).

Calculate the concentration of each steviol glycoside in the sample solution using the following formula:

$$Concentration (\% w/w) = C_{sample} \times \frac{100}{w_{sample}}$$

where,

$C_{sample}$  = average concentration in micrograms per millilitre of steviol glycosides in the sample solution

$w_{sample}$  = mass of sample in micrograms per millilitre in 1 ml of sample solution (~1000 µg/ml)

Note: Above calculation will change if additional dilutions were done prior to LC injection. Analyst shall account such dilutions in the calculation.

Calculate the percentage of major steviol glycosides in the sample by summation of percentage concentrations of individual steviol glycosides in the sample (A).

Note: If the concentration of major steviol glycosides in the sample is less than 95%, the analyst should perform Method B.

### B.3 Method B: Determination of minor steviol glycosides by HPLC-MS

#### B.3.1 Apparatus

HPLC-MS conditions may vary based on the manufacturer and model of the system used. Analyst should set the conditions following the manufacturer's instructions.

The mass spectrometer is connected to the HPLC-UV system used in method A. Analyse the mass spectral data of the minor peaks (major steviol glycoside peaks are identified from RRT in method A). Confirm the presence of each minor steviol glycoside from the observed molecular mass ion and one or more of the following mass spectral diagnostic ions in Table 5 below.

**Table 5 — Mass spectral diagnostic ions observed during in-source fragmentation of steviol glycosides.**

[Fragment-H] -m/z	Identity	
317	Steviol	
427	Related Steviol glycoside #3	
479	Steviol-GLC	
625	Steviol-2GLC [M-16]	
641	Steviol-2GLC	
787	Steviol-3GLC deoxyglucose [M-16]	
803	Steviol-3GLC	
819	—	
965	Steviol-4GLC	

After confirming the presence of a minor steviol glycoside, correct its mean peak area (obtained from the UV chromatogram) as described below.

### B.3.2 Calculation of minor steviol glycosides content

Calculate the molecular mass corrected peak area abundance for each minor steviol glycoside as:

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

where,

$M_x$  = molecular mass of the minor steviol glycoside

$M_{\text{RebA}}$  = molecular mass of Rebaudioside A (967 amu)

MPA = mean peak area

Deduce the concentration of each minor steviol glycoside in micrograms per millilitre from the UV standard curve of rebaudioside A. Calculate the concentration of each minor steviol glycoside in the sample solution using the following formula:

$$\text{Minor steviol glycoside concentration (\%w/w)} = \frac{\text{Conc}_{\text{sample}} \times 100}{\text{Weight}_{\text{sample}}}$$

Note: The above calculation will change if additional dilutions were done prior to LC injection. Analyst shall account for such dilutions in the calculation.

Calculate the percentage of minor steviol glycosides in the sample by summation of percentages of individual minor steviol glycosides in the sample (B).

Determine the total steviol glycosides content using the following formula:

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

where:

TSG is the total steviol glycosides content in %w/w, on the dried basis;

A is the percentage major steviol glycosides;

B is the percent minor steviol glycosides; and

M is the percentage loss on drying

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## Annex C (normative)

### Determination of pH (Potentiometric Method)

#### C.1 Requirements

- pH meter
- Distilled/deionised water
- Absorbent tissue
- Two standard buffer solutions (High pH and low pH, respectively)

#### C.2 Procedure

Use a calibrated pH meter and follow the manufacturer's instructions. Each time the electrodes are used, rinse them with distilled or deionised water and carefully blot them dry with clean absorbent tissue. Form a fresh reference electrode liquid junction. Rinse the sample vessel three times with each new solution to be introduced.

Choose two standard buffers to bracket, if possible, the anticipated pH of the unknown. Warm or cool these standards as necessary to match within 2 °C the temperature of the unknown, and initially set the temperature compensator to that temperature. Immerse the electrodes in a portion of the first standard buffer, and following the manufacturer's instructions adjust the appropriate standardization control (knob, switch, or button) until the pH reading is that of the buffer. Repeat this procedure with fresh portions of the first standard buffer until two successive readings are within  $\pm 0.02$  pH unit without an adjustment of the standardization control.

Rinse the electrodes, blot dry, and immerse them in a portion of the second standard buffer of lower pH. Do not change the setting of the standardization control. Following the manufacturer's instructions, adjust the slope control (thumbwheel switch, knob, or temperature compensator) until the exact buffer pH is displayed.

Repeat the sequence of standardization with both buffers until the pH readings are within  $\pm 0.02$  pH unit for both buffers without any adjustment of either control (the amount of sample to be used in sample preparation is given where applicable in the individual specification).

The pH of the unknown solution may then be measured. The difference between the results of two pH determinations when carried out simultaneously on in rapid succession by the same analyst, under the same conditions, should not exceed 0.05 pH unit.

Note: Always re-standardize the instrument after even a short period during which the amplifier is turned off.

## Annex D (normative)

### Loss on drying

#### D.1 Requirements

- Weighing bottle, with a stopper
- Air oven
- Desiccator
- Weighing scale

#### D.2 Procedure

##### D.2.1 Sample preparation

Weigh 1 to 2 g of sample ( $M_1$ ). Tare a glass-stoppered, shallow weighing bottle that has been dried for 30 minutes at 105 °C. Transfer the sample into the bottle, replace the cover, and weigh the bottle and the sample ( $M_2$ ). Distribute the sample as evenly as practicable to a depth of about 5 mm, and not over 10 mm.

##### D.2.2 Drying

Place the bottle with its contents in the drying chamber, removing the stopper and leaving it also in the chamber, and dry the sample at the 105 °C for 2 hours. Upon opening the chamber, close the bottle promptly and allow it to come to room temperature in a desiccator. Weigh the cool bottle and its contents ( $M_3$ ).

Calculate the loss on drying from the following equation:

$$\text{Loss on drying (\%w/w)} = \frac{M_2 - M_3}{M_1} \times 100$$

where:

$M_1$  is the mass of sample in grams;

$M_2$  is the mass of sample and weighing bottle in grams before drying; and

$M_3$  is the mass of sample and weighing bottle in grams after drying and cooling in a desiccator.

If the sample melts at a temperature lower than 105 °C, prepare the sample as described above, then place it in a vacuum desiccator containing sulfuric acid. Evacuate the desiccator to 130 Pa (1 mm of mercury), maintain this vacuum for 24 h, and then weigh the dried sample. Calculate the loss on drying using the same equation above.



## Annex E (normative)

### Residual solvents – Methanol and Ethanol

#### E.1 Reagent

- Blank solution: sample with very low solvent content
- Internal standard solution: 3-methyl-2-pentanone
- Methanol
- Distilled water

##### E.1.1 Method I (Determination carried out in water)

**Blank solution:** Weigh accurately 0.20 g of the blank into an injection vial. Add 5.0 ml of distilled water and 1.0 ml of the internal standard solution. Heat at 60 °C for 10 min and shake vigorously for 10 sec.

**Internal standard solution:** Add 50 ml of distilled water to a 50 ml injection vial and seal. Accurately weigh and inject 15 µl 3-methyl-2-pentanone through the septum and reweigh to within 0.01 mg.

**Samples:** Weigh accurately 0.20 g sample into an injection vial. Add 5.0 ml water and add 1.0 ml of the internal standard solution. Heat at 60 °C for 10 min and shake vigorously for 10 sec.

**Calibration solution:** Weigh accurately 0.20 g of the blank into an injection vial. Add 5.0 ml of the water and 1.0 ml of the internal standard solution. Weigh the vial accurately to within 0.01 mg. Inject a known volume of the component of interest through the septum and again reweigh the vial. Heat at 60 °C for 10 min and shake vigorously for 10 sec.

##### E.1.2 Method II (Determination carried out in methanol)

**Internal standard solution:** Add 50.0 ml methanol to a 50.0 ml injection vial and seal. Accurately weigh and inject 15 µl 3-methyl-2-pentanone through the septum and reweigh to within 0.01 mg.

**Blank solution:** Weigh accurately 0.20 g of the blank into an injection vial. Add 5.0 ml of methanol and 1.0 ml of the internal standard solution. Heat at 60° for 10 min and shake vigorously for 10 sec.

**Samples:** Weigh accurately 0.20 g sample into an injection vial. Add 5.0 ml methanol and add 1.0 ml of the internal standard solution. Heat at 60 °C for 10 min and shake vigorously for 10 sec.

**Calibration solution:** Solution A: Add 50.0 ml methanol to a 50.0 ml vial and seal. Accurately weigh, to within 0.01 mg, the vial and inject 50 µl of the component of interest through the septum. Reweigh the vial. Mix well.

Weigh into an injection vial, 0.20 g of blank, add 4.9 ml of methanol and 1.0 ml internal standard solution. Introduce 0.1 ml of Solution A into the injection vial. Mix well and heat at 60 °C for 10 min and shake vigorously for 10 sec.

#### E.2 Procedure

Place the sample, blank and calibration samples in the sample tray of the head-space gas chromatograph – FID system. Analyse using the following analytical conditions.

**Column:** Fused silica, length 0.8 m, i.d. 0.53mm, coated with DB-wax, film thickness 1 µm

**Coupled with:** Fused silica, length 30 m, i.d. 0.53 mm, coated with DB-1, film thickness 5µm

**Conditions:**

- Carrier gas: Helium
- Flow rate: 208 kPa, 5 ml/min
- Detector: FID
- Temperatures
  - Injector: 140 °C
  - Oven conditions: 35 °C for 5 min, then 5 °C /min to 90 °C, then 6 min at 90 °C
  - Detector: 300 °C
- Head space sampler
  - Sample heating temperature: 60 °C
  - Sample heating period: 10 min
  - Syringe temperature: 70 °C
  - Transfer temperature: 80 °C
  - Sample gas injection: 1.0 ml in split mode

**Calculation:**

$$A \times B \times C / 50 = \text{mg component per injection vial}$$

where:

A = relative peak area of the component concerned

B = mass of internal standard in milligrams

C = calibration factor

**Determination of calibration factors**

Method 1:

$$C = D \times 50 / (E \times (F - G))$$

Method 2:

$$C = D / (E \times (F - G) \times 10)$$

where:

D = mass of sample in milligrams

E = mass of internal standard in milligrams

F = relative peak area of component for the calibration solution

G = relative peak area of the same component for the blank solution

## Bibliography

- [1] JECFA (2017), Steviol Glycosides from *Stevia rebaudiana* Bertoni Residue monograph
- [2] JECFA (2006), Analytical methods, test procedures and laboratory solutions used by and referenced in the food additive specifications, Volume 4.

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