
**Infant formula and adult
nutritionals — Determination of
fructans — High performance anion
exchange chromatography with pulsed
amperometric detection (HPAEC-PAD)
after enzymatic treatment**

*Préparations pour nourrissons et produits nutritionnels pour
adultes — Dosage des fructanes — Chromatographie échangeuse
d'anions haute performance couplée à la détection par ampérométrie
pulsée (CEAHP-DAP) après traitement enzymatique*



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ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11

Email: copyright@iso.org
Website: www.iso.org

Published in Switzerland

International Dairy Federation
Silver Building • Bd Auguste Reyers 70/B
B-1030 Brussels
Phone: +32 2 325 67 40
Fax: +32 2 325 67 41
Email: info@fil-idf.org
Website: www.fil-idf.org

Contents

Page

Foreword	iv
1 Scope	1
2 Normative references	1
3 Terms and definitions	1
4 Principle	2
5 Chemicals and reagents	2
5.1 List of chemicals and reagents	2
5.2 Preparation of reagents	3
5.3 Preparation of mobile phases using column A (6.13.1) or equivalent	5
5.4 Preparation of mobile phases using columns B (6.13.2) or equivalent	5
5.5 Preparation of standard solutions	6
6 Apparatus	6
7 Procedure	8
7.1 Sample preparation	8
7.1.1 Powdered or concentrated products on a ready-to feed (RTF) basis and powder products inhomogeneous at the subgram level	8
7.1.2 Reconstituted products as prepared in 7.1.1 or products sold as RTF	8
7.1.3 Homogeneous powdered products without prior reconstitution	8
7.1.4 Dilution	8
7.1.5 Hydrolysis of sucrose and α -glucans	8
7.1.6 Carrez clarification (optional, use in case of difficulties passing sample through SPE)	8
7.1.7 Removal of monosaccharides	9
7.1.8 Hydrolysis of fructans	9
7.2 Chromatographic conditions using column A (6.13.1)	9
7.3 Chromatographic conditions using columns B (6.13.2)	10
7.4 System suitability test	11
7.5 Calibration	11
8 Calculation	11
9 Reporting	13
10 Precision data	13
10.1 General	13
10.2 Repeatability	13
10.3 Reproducibility	13
11 Test report	14
Annex A (informative) Example chromatograms and calibration curves	15
Annex B (informative) Precision data	18
Annex C (informative) Check test enzyme mixture of sucrase, β-amylase, pullulanase and maltase	19
Bibliography	22

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF), in collaboration with AOAC INTERNATIONAL, and in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 302, *Milk and milk products — Methods of sampling and analysis*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement). It is being published jointly by ISO and IDF and separately by AOAC INTERNATIONAL. The method described in this document is equivalent to the AOAC Official Method 2016.14: *Fructans in Infant Formula and Adult Nutrition*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

IDF (the International Dairy Federation) is a non-profit private sector organization representing the interests of various stakeholders in dairying at the global level. IDF members are organized in National Committees, which are national associations composed of representatives of dairy-related national interest groups including dairy farmers, dairy processing industry, dairy suppliers, academics and governments/food control authorities.

ISO and IDF collaborate closely on all matters of standardization relating to methods of analysis and sampling for milk and milk products. Since 2001, ISO and IDF jointly publish their International Standards using the logos and reference numbers of both organizations.

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This document was prepared by the IDF *Standing Committee on Analytical Methods for Composition* and ISO Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*. It is being published jointly by ISO and IDF.

The work was carried out by the IDF-ISO Action Team on C41 of the *Standing Committee on Analytical Methods for Composition* under the aegis of its project leader Mr S. Austin (CH).

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Infant formula and adult nutritionals — Determination of fructans — High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) after enzymatic treatment

WARNING — The method described in this document employs corrosive (sodium hydroxide, acetic acid) and toxic (sodium azide) chemicals. Refer to the materials safety data sheets and take appropriate additional safety precautions for handling and waste disposal.

1 Scope

This document specifies a method for the determination of inulin-type fructans (including oligofructose, fructooligosaccharides) in infant formula and adult nutritionals (both powder and liquid) containing 0,03 g/100 g to 5,0 g/100 g of fructans in the product as prepared ready for consumption.

The method has been validated in a multi laboratory study^[1] with reconstituted standard reference material (SRM), infant/adult nutritional formula at a level of 0,204 g/100 g, adult nutritionals ready-to-feed (RTF) at levels of 1,28 g/100 g and 2,67 g/100 g, infant formula RTF at a level of 0,300 g/100 g, reconstituted follow-up formula at levels of 0,209 g/100 g to 0,275 g/100 g, reconstituted infant formula at levels from 0,030 g/100 g to 0,264 g/100 g. During the single laboratory validation study^[2], spike-recovery experiments were performed up to 5 g/100 g in reconstituted infant formula powders (milk-based, partially hydrolysed milk-based and soy-based), adult nutritional RTF and reconstituted adult nutritional powders.

2 Normative references

There are no normative references in this document.

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 addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <http://www.electropedia.org/>

3.1 adult nutritional

nutritionally complete, specially formulated food, consumed in liquid form, which may constitute the sole source of nourishment, made from any combination of milk, soy, rice, whey, hydrolysed protein, starch and amino acids, with or without intact protein

3.2 infant formula

breast-milk substitute specially manufactured to satisfy, by itself, the nutritional requirements of infants during the first months of life up to the introduction of appropriate complementary feeding

[SOURCE: Codex Standard 72-1981]

3.3 follow-up formula

food intended for use as a liquid part of the weaning diet for an infant from the age of six months, and for young children up to the age of three years

Note 1 to entry: Follow-up formula includes child formula and toddler formula.

[SOURCE: Codex Standard 156-1987]

4 Principle

Samples are reconstituted in water (if required) and further diluted until the concentration of fructans in solution is such that after hydrolysis the fructose and glucose concentration will be within the range covered by the standard curve. The diluted sample is treated with a mixture of a highly specific sucrase and α -glucanases to hydrolyse sucrose and α -glucooligosaccharides to their constituent monosaccharides. The sample is passed through a solid phase extraction (SPE) column packed with graphitized carbon. Salts and monosaccharides pass through and are washed away, while the fructans are retained. Fructans are released from the column using an acetonitrile solution. The released fructans are hydrolysed with a fructanase mixture, and the released glucose and fructose are analysed by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The fructan content is calculated by summing the glucose and 0,9 times the fructose contents measured. In some matrices, a blank correction may be necessary and can be applied.

5 Chemicals and reagents

5.1 List of chemicals and reagents

Use only reagents of recognized analytical grade, unless otherwise specified. Solvents shall be of quality for HPLC analysis, unless otherwise specified.

5.1.1 Deionized water, purified with resistivity $\geq 18 \text{ M}\Omega$.

5.1.2 Maleic acid, purity $\geq 99,0 \%$.

5.1.3 Acetonitrile.

5.1.4 Acetic acid, glacial 100 %, anhydrous.

5.1.5 Potassium hexacyanoferrate(II) trihydrate, optional.

5.1.6 Zinc acetate, optional.

5.1.7 Trifluoroacetic acid (TFA).

5.1.8 Hydrochloric acid, substance concentration $c = 1 \text{ mol/l}$.

5.1.9 Sodium acetate anhydrous, purity $\geq 99,0 \%$, only when using columns B ([6.13.2](#)) for HPAEC-PAD.

5.1.10 Sodium hydroxide solution, a mass fraction of 50 %.

5.1.11 Sodium hydroxide pellets.

5.1.12 Sodium chloride.

5.1.13 Sodium azide, optional.

5.1.14 D-(-)-fructose, purity $\geq 99,0$ % (dry weight basis).

5.1.15 D-(+)-glucose, purity $\geq 99,5$ % (dry weight basis).

5.1.16 N,N'-diacetylchitobiose, purity > 90 %.

5.1.17 Mixture of highly purified sucrase, β -amylase, pullulanase and maltase, from Fructan Assay Kit K-FRUC¹ (Megazyme International Ireland Ltd or equivalent). 200 μ l of enzyme mixture working solution (5.2.10) should be able to completely hydrolyse 2 mg of sucrose under the conditions described in the method (90 min at 40 °C) without hydrolysing any fructans.

5.1.18 Mixture of highly purified recombinant exo- and endo-inulinases and recombinant endo-levanase, from Fructan Assay Kit K-FRUC¹ (Megazyme International Ireland Ltd or equivalent). 100 μ l of enzyme mixture working solution (5.2.11) should be able to hydrolyse 70 μ g of fructans under the conditions described in the method (40 min at 40 °C).

5.2 Preparation of reagents

5.2.1 Sodium hydroxide solution, $c = 2$ mol/l.

Dissolve 40 g $\pm$ 1 g of sodium hydroxide pellets in 250 ml of deionized water in a 500 ml volumetric flask. After cooling down to room temperature, make up to the mark with deionized water and mix well. This solution is stable for six months at room temperature.

5.2.2 Sodium maleate buffer solution, $c = 0,100$ mol/l, pH = 6,5.

Into a large beaker (>500 ml), weigh 5,8 g of maleic acid and dissolve with 450 ml of deionized water using a magnetic stirrer. Adjust to pH = 6,5 with sodium hydroxide solution (5.2.1). Transfer the solution to a 500 ml volumetric flask and make up to the mark with deionized water. This solution is stable for three months at 4 °C.

5.2.3 Sodium acetate buffer solution, $c = 0,100$ mol/l, pH = 4,5.

Into a large beaker (>500 ml) containing 450 ml of deionized water, pipette 2,9 ml of glacial acetic acid. Adjust to pH = 4,5 with sodium hydroxide solution (5.2.1). Transfer the solution to a 500 ml volumetric flask and make up to the mark with deionized water. This solution is stable for three months at 4 °C.

5.2.4 N,N'-diacetylchitobiose internal standard solution, mass concentration $\rho = 600$ μ g/ml.

Into a 25 ml volumetric flask, weigh 15 mg of N,N'-diacetylchitobiose and make up to the mark with deionized water. This solution is stable for one year at -20 °C.

5.2.5 Glucose stock solution, $\rho = 5$ mg/ml.

Into a 25 ml volumetric flask, weigh 125 mg of glucose and make up to the mark with deionized water. This solution is stable for one year at -20 °C.

1) This is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of the product named. Equivalent products may be used if they can be shown to lead to the same results.

5.2.6 Fructose stock solution, $\rho = 10$ mg/ml.

Into a 25 ml volumetric flask, weigh 250 mg of fructose and make up to the mark with deionized water. This solution is stable for one year at -20°C .

5.2.7 Carrez I solution

Dissolve 10,6 g of potassium hexacyanoferrate(II) trihydrate in 100 ml of deionized water and store in a brown bottle. This solution is stable for six months at room temperature (optional reagent).

5.2.8 Carrez II solution

In a 100 ml volumetric flask, dissolve 22,0 g of zinc acetate in 90 ml of deionized water and add 2,9 ml of glacial acetic acid. Make up to the mark with deionized water and homogenize. This solution is stable for six months at room temperature (optional reagent).

5.2.9 Sodium azide solution, $\rho = 5$ g/l.

Dissolve 1 g of sodium azide in 200 ml of deionized water (optional reagent).

5.2.10 Mixture of sucrase, β -amylase, pullulanase and maltase solution.

Dissolve the contents of the bottle containing freeze-dried powdered sucrase, β -amylase, pullulanase and maltase in 22,0 ml of sodium maleate buffer solution (5.2.2). Mix well and divide into aliquots of 2 ml each and store frozen at -20°C in polypropylene tubes until use. This solution is stable for five years at -20°C .

IMPORTANT — For the development and validation of this method, a commercially available pre-prepared enzyme mixture from Megazyme²⁾ (5.1.17) was used. When enzymes from another source are used, the buffer composition (type, concentration and pH) may need to be adapted to the recommendations of the supplier. It is also imperative to ensure the enzyme mixture employed will completely hydrolyse any sucrose in the product without hydrolysing the fructan. This can be checked by performing an analysis with sucrose as an analyte and with a pure fructan as an analyte. No fructan should be measured when sucrose is analysed, and more than 90 % recovery should be achieved when a fructan of known purity is analysed (recommended to check using both long-chain inulin and short-chain fructooligosaccharides). An alternative test for checking the suitability and performance of the enzyme mixture is described in Annex C.

5.2.11 Fructanase solution (exo-/endo-inulinases and endo-levanase).

Dissolve the contents of the bottle containing freeze-dried powdered exo- and endo-inulinases and endo-levanase in 22,0 ml of sodium acetate buffer solution (5.2.3). Mix well and divide into aliquots of 2 ml each and store frozen at -20°C in polypropylene tubes until use. This solution is stable for five years at -20°C .

IMPORTANT — For the development and validation of this method, a commercially available pre-prepared enzyme mixture from Megazyme²⁾ (5.1.18) was used. When enzymes from another source are used, buffer composition (type, concentration and pH) may need to be adapted to the recommendations of the supplier. It is also imperative to ensure the enzyme mixture employed will completely hydrolyse the fructan without hydrolysing any other glucose or fructose containing oligo- or polysaccharide that may be present after treatment with the sucrase mixture above. This can be checked by performing an analysis with a pure fructan as an analyte. More than 90 % recovery should be achieved when a fructan of known purity is measured.

2) This is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of the product named. Equivalent products may be used if they can be shown to lead to the same results.

5.2.12 Wash solution for graphitized carbon solid phase extraction (SPE) column, TFA 0,1 % in acetonitrile, a volume fraction of 80 %.

Into a 100 ml volumetric flask, add 80 ml of acetonitrile and 100 µl of TFA. Make up to the mark with deionized water. This solution is stable for six months at room temperature.

5.2.13 Sodium chloride solution, $c = 1$ mol/l, for graphitized carbon SPE column.

Into a 100 ml volumetric flask, weigh 5,8 g of sodium chloride and dissolve with 90 ml of deionized water. Make up to the mark with deionized water. This solution is stable for six months at room temperature.

5.2.14 Elute solution for graphitized carbon SPE column, TFA 0,05 % in acetonitrile, a volume fraction of 25 %.

Into a 100 ml volumetric flask, add 25 ml of acetonitrile and 50 µl of TFA. Make up to the mark with deionized water. This solution is stable for six months at room temperature.

5.3 Preparation of mobile phases using column A (6.13.1) or equivalent

If using columns B (6.13.2), skip this clause and go directly to 5.4.

5.3.1 Eluent A for column A (6.13.1), sodium hydroxide solution, $c = 0,600$ mol/l.

Into an eluent bottle, introduce 970 ml of deionized water and degas with helium for 20 min. Add 31,2 ml of sodium hydroxide solution (5.1.10) (using a single-use plastic pipette). Degas with helium for 20 min and protect the eluent from exposure to carbon dioxide until, and during, use by suitable means (e.g. by keeping under a blanket of helium). This solution is stable for one week at room temperature.

5.3.2 Eluent B for column A (6.13.1), deionised water with resistivity ≥ 18 MΩ.

Into an eluent bottle, introduce 2 000 ml of deionized water (5.1.1) and degas with helium for 20 min. Protect the eluent from exposure to carbon dioxide until, and during, use by suitable means (e.g. by keeping under a blanket of helium). This solution is stable for four days at room temperature.

5.3.3 Eluent C for column A (6.13.1), sodium hydroxide solution, $c = 0,030$ mol/l.

Into an eluent bottle, introduce 1 000 ml of deionized water and degas with helium for 20 min. Add 1,6 ml of sodium hydroxide solution (5.1.10) (using a single-use plastic pipette). Degas with helium for 20 min and protect the eluent from exposure to carbon dioxide until, and during, use by suitable means (e.g. by keeping under a blanket of helium). This solution is stable for one week at room temperature.

5.3.4 Post-column addition reagent, sodium hydroxide, $c = 0,300$ mol/l.

Into an eluent bottle, introduce 985 ml of deionized water and add 15,6 ml of sodium hydroxide solution (5.1.10) (using a single-use plastic pipette). Swirl the solution gently to mix. Degas with helium for 20 min. This solution is stable for one month at room temperature. Degas on the day of use.

5.4 Preparation of mobile phases using columns B (6.13.2) or equivalent

If using column A (6.13.1), skip this clause.

5.4.1 Eluent A for columns B (6.13.2), sodium hydroxide solution, $c = 0,200$ mol/l.

Weigh $1\,923\text{ g} \pm 2\text{ g}$ of deionized water into an eluent bottle and degas with helium for 20 min. Add 20 ml of sodium hydroxide solution (5.1.10) (using a single-use plastic pipette). Degas with helium for 20 min and protect the eluent from exposure to carbon dioxide until, and during, use by suitable means (e.g. by keeping under a blanket of helium). This solution is stable for one week at room temperature.

5.4.2 Eluent B for columns B (6.13.2), deionized water with resistivity $\geq 18 \text{ M}\Omega$.

Fill a 2 l eluent bottle with 2 000 ml of deionized water. Degas with helium for 20 min and protect the eluent from exposure to carbon dioxide until, and during, use by suitable means (e.g. by keeping under a blanket of helium). This solution is stable for four days.

Optionally, 5 g/l sodium azide solution (5.2.9) can be added to a final concentration of 0,125 g/l to mobile phase B. This extends the shelf life of eluent to about two weeks and can improve chromatographic resolution around chitobiose in case of problems.

5.4.3 Eluent C for columns B (6.13.2), sodium acetate solution, $c = 1 \text{ mol/l}$.

Into a 1 000 ml volumetric flask, weigh 82,0 g of anhydrous sodium acetate (5.1.9) and dissolve with 800 ml of deionized water by mixing. Make up to the mark with deionized water and filter on a 0,20 μm nylon membrane filter into an eluent bottle. Degas with helium for 20 min and protect the eluent from exposure to carbon dioxide until, and during, use by suitable means (e.g. by keeping under a blanket of helium). This solution is stable for one week at room temperature.

5.4.4 Post-column addition reagent, sodium hydroxide solution, $c = 0,300 \text{ mol/l}$.

Into an eluent bottle, introduce 985 ml of deionized water and add 15,6 ml of sodium hydroxide solution (5.1.10) (using a single-use plastic pipette). Swirl the solution gently to mix. Degas with helium for 20 min. This solution is stable for one month at room temperature. Degas on the day of use.

5.5 Preparation of standard solutions

Using volumetric flasks, prepare a six-level standard curve by diluting the glucose stock solution (5.2.5) and the fructose stock solution (5.2.6) made up to the final volume with deionized water, as described in Table 1.

Table 1 — Dilution scheme for the preparation of the standard curve

Standard solution	Volume of fructose stock solution (5.2.6) μl	Volume of glucose stock solution (5.2.5) μl	Final volume ml	Fructose concentration $\mu\text{g/ml}$	Glucose concentration $\mu\text{g/ml}$
Level 1	200	40	100	20	2
Level 2	400	200	20	200	50
Level 3	800	400	20	400	100
Level 4	1 200	600	20	600	150
Level 5	1 600	800	20	800	200
Level 6	2 000	1 000	20	1 000	250

Treat each of the six standard solutions as follows: into a 1,5 ml microtube, transfer 200 μl of standard solution (see Table 1), 200 μl of deionized water (5.1.1) and 100 μl of N,N'-diacetylchitobiose internal standard solution (5.2.4), and mix well. Then transfer 400 μl of this solution to a 2 ml microtube, add 1 200 μl of elute solution for SPE (5.2.14) and mix well. To 700 μl of this solution, add 300 μl of sodium acetate buffer solution (5.2.3). Mix well then centrifuge at 10 000g for 5 min. Transfer 900 μl of the supernatant into a vial suitable for the instrument autosampler.

6 Apparatus

Usual laboratory apparatus and, in particular, the following.

6.1 Analytical balance, readability of 0,1 mg.

- 6.2 pH-meter**, readability of pH = 0,1.
- 6.3 Microcentrifuge tubes**, volume of 1,5 ml and 2 ml.
- 6.4 Water bath**, at 80 °C ± 1 °C, with magnetic stirring if available.
- 6.5 Water bath**, at 40 °C ± 1 °C.
- 6.6 Centrifuge**, for 1,5 ml and 2 ml microtubes able to operate at 10 000g.
- 6.7 Micropipettes with tips**, volume of 0,1 ml to 1 ml.
- 6.8 Vortex mixer**.
- 6.9 Single-use plastic pipettes**, graduated, volume of 2,10 ml and 25 ml.
- 6.10 Single-use syringes**, volume of 2 ml.
- 6.11 Graphitized carbon (100 mg, 1 ml) SPE columns** (Supelclean™ ENVI-Carb™³) or equivalent).
- 6.12 Membrane filter nylon**, 0,2 µm and a diameter of 4,7 cm.
- 6.13 Analytical columns.**
- 6.13.1 Column A**, CarboPac™³ PA20, (150 mm × 3 mm, 6,5 µm) or equivalent.
- 6.13.2 Columns B**, CarboPac™³ PA1, (250 mm × 2 mm, 10 µm) with guard (50 mm × 2 mm, 10 µm) or equivalent.
- 6.13.3 Metal free liquid chromatograph**, consisting of:
- pump able to deliver a ternary gradient at a flow of 0,5 ml/min for column A ([6.13.1](#)) or 0,25 ml/min for column B ([6.13.2](#));
 - autosampler (cooling system recommended), able to inject 25 µl for column A ([6.13.1](#)) or 20 µl for column B ([6.13.2](#));
 - column compartment able to maintain a temperature of 30 °C for column A ([6.13.1](#)) or 20 °C for column B ([6.13.2](#));
 - electrochemical detector working in pulsed amperometric detection mode, equipped with a gold working electrode;
 - isocratic pump for post-column delivery, with pulse dampener, 125 µl reaction coil and T-piece;
 - data acquisition system.

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7 Procedure

7.1 Sample preparation

7.1.1 Powdered or concentrated products on a ready-to feed (RTF) basis and powder products inhomogeneous at the subgram level

Reconstitute powder or liquid concentrates according to instructions. For example, accurately weigh 25 g (m_R) of infant formula powder into an appropriate bottle, add 200 g of deionized water (V_R) and mix well at room temperature to form a homogeneous suspension. Proceed as described in 7.1.2.

7.1.2 Reconstituted products as prepared in 7.1.1 or products sold as RTF

Mix well to ensure homogeneity of the test portion. Weigh 9 g (m_A) of sample into a 50 ml volumetric flask (V_A). Add 30 ml of deionized water and confirm the pH is between pH = 5 and pH = 9 (adapt if needed, using hydrochloric acid or sodium hydroxide). Heat at 80 °C with constant agitation for 20 min. Cool to room temperature and make up to the mark with deionized water. This is solution A. Proceed as described in 7.1.4.

7.1.3 Homogeneous powdered products without prior reconstitution

Weigh 1 g (m_A) of powder into a 50 ml volumetric flask (V_A). Add 30 ml of deionized water and confirm the pH is between pH = 5 and pH = 9 (adapt if needed, using hydrochloric acid or sodium hydroxide). Heat at 80 °C with constant agitation for 20 min. Cool to room temperature and make up to the mark with deionized water. This is solution A. Proceed as described in 7.1.4.

7.1.4 Dilution

Dilute the solutions prepared above further depending on the expected fructan content. Suggested dilutions are given in Table 2. The resulting solution is solution B.

Table 2 — Scheme for sample dilution depending on expected fructan content

Expected fructan content g/100 g		Dilution		Dilution factor
Powder	RTF	Volume of solution A (7.1.2 or 7.1.3) ml	Final volume ml	
0,2 to 4,0	0,03 to 0,45	No dilution	No dilution	1
2,5 to 45	0,3 to 4,5	5	50	10
25 to 100	3,0 to 45	0,5	50	100

7.1.5 Hydrolysis of sucrose and α -glucans

Transfer 200 μ l of solution B into a 1,5 ml microtube, add 100 μ l of N,N'-diacetylchitobiose internal standard solution (5.2.4) and 200 μ l of the sucrase, β -amylase, pullulanase and maltase enzyme mixture (5.2.10). Mix well, place the microtubes on a floating rack and incubate at 40 °C for 90 min. Cool down to room temperature.

IMPORTANT — Adapt these conditions (time and temperature) according to enzyme manufacturer recommendations.

7.1.6 Carrez clarification (optional, use in case of difficulties passing sample through SPE)

Add 10 μ l of Carrez I solution (5.2.7) to the sample and mix well. Then add 10 μ l of Carrez II solution (5.2.8) and mix again. Centrifuge at 10 000g for 10 min and use the supernatant for the next step.

7.1.7 Removal of monosaccharides

Prepare the graphitized carbon SPE column (6.11) as follows (try to avoid passing air through the SPE column before the final elution step as this may result in higher blank measurements):

- flush with 3×400 µl of wash solution for SPE (5.2.12);
- flush with 3×400 µl of deionized water.

Then perform the following steps under gravity if possible, or up to an elution flow rate of 2 ml/min with positive pressure:

- apply 400 µl of the enzyme treated solution (7.1.5 or 7.1.6);
- wash with $2 \times 1\,000$ µl of sodium chloride solution ($c = 1$ mol/l) (5.2.13);
- wash with $4 \times 1\,000$ µl of deionized water;
- elute the fructans into a 2 ml microtube using 4×400 µl of elute solution for SPE (5.2.14);
- apply a little positive pressure to eliminate all solution from the column;
- mix eluate from the SPE column well.

7.1.8 Hydrolysis of fructans

Transfer 700 µl of the eluate from the SPE column (see 7.1.7) into a 1,5 ml microtube (marked “Sample”). Add 200 µl of sodium acetate buffer (5.2.3) and 100 µl of the fructanase mixture (5.2.11). Into a second microtube (marked “Blank”), transfer 700 µl of the eluate (see 7.1.7) and add 300 µl of sodium acetate buffer (5.2.3). For all tubes, mix well and incubate at 40 °C for 40 min to 45 min.

The blank is necessary only for some matrices and may be skipped if it has already been established in the laboratory that the blank measurement has a negligible impact on the result of a given sample.

After cooling, centrifuge at 10 000g for 5 min. Then transfer 700 µl of the supernatant into a vial suitable for the instrument autosampler.

7.2 Chromatographic conditions using column A (6.13.1)

If using columns B (6.13.2) (or equivalent), skip this clause and go to 7.3.

The HPAEC-PAD system is equipped with column A (6.13.1), or equivalent. The column is held at 30 °C and the injection volume is 25 µl. Sodium hydroxide solution ($c = 0,300$ mol/l) is added post-column (before the PAD detector) at a flow rate of 0,2 ml/min, using a T-piece and a 125 µl reaction coil (or equivalent). Fructose and glucose are separated using the gradient described in Table 3. Carbohydrates are detected by pulsed amperometry using a gold working electrode, a suitable reference electrode, and an appropriate waveform for the detection of carbohydrates. An example of a suitable waveform is given in Table 4. Other waveforms may be applied depending on instrument requirements, which vary between manufacturers.

Table 3 — HPAEC-PAD gradient for column A (6.13.1) or equivalent

Time min	Flow ml/min	% A $c(\text{NaOH}) = 0,600 \text{ mol/l}$	% B water	% C $c(\text{NaOH}) = 0,030 \text{ mol/l}$
0,0	0,5	0	80	20
17,0	0,5	0	80	20
17,1	0,5	100	0	0
27,0	0,5	100	0	0
27,1	0,5	0	80	20
33,0	0,5	0	80	20

Table 4 — Example PAD waveform for carbohydrate detection

Time s	Voltage V	Gain region
0,00	+0,10	Off
0,20	+0,10	On
0,40	+0,10	Off
0,41	-2,00	Off
0,42	-2,00	Off
0,43	+0,60	Off
0,44	-0,10	Off
0,50	-0,10	Off

7.3 Chromatographic conditions using columns B (6.13.2)

If using column A (6.13.1) (or equivalent), skip this clause.

The HPAEC-PAD system is equipped with columns B (6.13.2) or equivalent, connected in series. The columns are held at 20 °C and the injection-volume is 20 µl. Sodium hydroxide solution ($c = 0,300 \text{ mol/l}$) is added post-column (before the PAD detector) at a flow rate of 0,13 ml/min, using a T-piece and a 125 µl reaction coil (or equivalent). Fructose and glucose are separated using the gradient described in Table 5. Carbohydrates are detected by pulsed amperometry using a gold working electrode, a suitable reference electrode, and an appropriate waveform for the detection of carbohydrates. An example of a suitable waveform is given in Table 4. Other waveforms may be applied depending on instrument requirements, which vary between manufacturers.

Table 5 — HPAEC-PAD gradient for columns B (6.13.2) or equivalent

Time min	Flow ml/min	% A $c(\text{NaOH}) = 0,200 \text{ mol/l}$	% B water	% C $c(\text{NaOAc}) = 1 \text{ mol/l}$
0,0	0,25	7,5	92,5	0,0
13,0	0,25	7,5	92,5	0,0
14,1	0,25	25,0	75,0	0,0
20,0	0,25	25,0	75,0	0,0
21,0	0,25	40,0	30,0	30,0
28,0	0,25	40,0	30,0	30,0
30,0	0,25	40,0	60,0	0,0
31,0	0,25	7,5	92,5	0,0
43,0	0,25	7,5	92,5	0,0

7.4 System suitability test

Allow the chromatographic system to equilibrate for at least 1 h at the initial conditions. Ensure the system pressure and the baseline are stable and there are no leaks. Start the series with one injection of deionized water (to check the baseline), then inject at least three times a working standard solution and check stability of peak retention times and response. The relative standard deviation (RSD, %) of retention times should be $\leq 1\%$, and the RSD (%) of peak areas should be $\leq 3\%$. If these criteria are not met, further system equilibration may be needed. Check the separation by comparison with a previous analysis if available. Examples of suitable chromatograms and calibration curves are given in [Annex A](#).

7.5 Calibration

Bracketed calibration should be used, injecting 3 standards followed by 10 samples then 3 standards, etc. For example, inject standards at levels 1, 3 and 5, then 10 samples, then standards at levels 2, 4 and 6, then 10 samples, then standards 1, 3, 5, etc. For each analyte (glucose and fructose), use the instrument software to plot a six-point standard curve of [Instrument response for analyte (area) / Instrument response for internal standard (area)] against the concentration of the analyte in the standard. Fit a quadratic curve to the data without forcing through zero. Use the calibration curve to calculate the glucose and fructose concentrations in solution B. For the blank measurements, ignore any signals if they are less than 10 times the signal-to-noise ratio.

8 Calculation

Calculate the mass fraction of glucose released from fructans, w_G , in g/100 g of RTF or reconstituted product (sample preparation according to [7.1.2](#)) or powder product (sample preparation according to [7.1.3](#)), using [Formula \(1\)](#):

$$w_G = \rho_{GB} \times D \times \frac{V_A}{m_A} \times 0,000\,1 \quad (1)$$

where

- ρ_{GB} is the mass concentration of glucose in solution B, in $\mu\text{g/ml}$;
- D is the dilution factor between solution A and solution B (from [Table 2](#));
- V_A is the total volume of solution A, in ml;
- m_A is the mass of the sample weighed to prepare solution A, in g;
- 0,000 1 is the factor to convert the analyte mass concentration in solution (in $\mu\text{g/ml}$) to the analyte mass fraction in the sample (in g/100 g).

Calculate the mass fraction of fructose released from fructans, w_F , in g/100 g RTF or reconstituted product (sample preparation according to [7.1.2](#)) or powder product (sample preparation according to [7.1.3](#)), using [Formula \(2\)](#):

$$w_F = \rho_{FB} \times D \times \frac{V_A}{m_A} \times 0,000\,1 \quad (2)$$

where

- ρ_{FB} is the mass concentration of fructose in solution B, in $\mu\text{g/ml}$;
- D is the dilution factor between solution A and solution B (from [Table 2](#));
- V_A is the total volume of solution A, in ml;
- m_A is the mass of the sample weighed to prepare solution A, in g;
- 0,000 1 is the factor to convert the analyte mass concentration in solution (in $\mu\text{g/ml}$) to the analyte mass fraction in the sample (in g/100 g).

Calculate the mass fraction of total fructans in the sample, w_{TF} , in g/100 g of RTF or reconstituted product (sample preparation according to [7.1.2](#)) or powder product (sample preparation according to [7.1.3](#)), using [Formula \(3\)](#):

$$w_{TF} = (w_F \times 0,9) + w_G \quad (3)$$

where

- w_F is the mass fraction of fructose released from fructans, in g/100 g;
- 0,9 is the factor to correct for uptake of water during fructans hydrolysis;
- w_G is the mass fraction of glucose released from fructans, in g/100 g.

For samples requiring the blank correction, adapt [Formulae \(1\)](#) and [\(2\)](#) as shown by [Formulae \(4\)](#) and [\(5\)](#):

$$w_G = (\rho_{GB} - \rho_{G0}) \times D \times \frac{V_A}{m_A} \times 0,000\,1 \quad (4)$$

$$w_F = (\rho_{FB} - \rho_{F0}) \times D \times \frac{V_A}{m_A} \times 0,000\,1 \quad (5)$$

where

- ρ_{G0} is the mass concentration of glucose in blank solution B, in $\mu\text{g/ml}$;
- ρ_{F0} is the mass concentration of fructose in blank solution B, in $\mu\text{g/ml}$.

For samples requiring a result in g/100 g of original product (powder or liquid concentrate) that have been subjected to reconstitution as described in [7.1.1](#), adapt [Formulae \(1\)](#), [\(2\)](#), [\(4\)](#) and [\(5\)](#) as shown by [Formulae \(6\)](#), [\(7\)](#), [\(8\)](#) and [\(9\)](#):

$$w_G = \rho_{GB} \times D \times \frac{V_A}{m_A} \times \frac{m_R + V_R}{m_R} \times 0,000\,1 \quad (6)$$

$$w_F = \rho_{FB} \times D \times \frac{V_A}{m_A} \times \frac{m_R + V_R}{m_R} \times 0,000\,1 \quad (7)$$

$$w_G = (\rho_{GB} - \rho_{G0}) \times D \times \frac{V_A}{m_A} \times \frac{m_R + V_R}{m_R} \times 0,000\,1 \quad (8)$$

$$w_F = (\rho_{FB} - \rho_{F0}) \times D \times \frac{V_A}{m_A} \times \frac{m_R + V_R}{m_R} \times 0,000\,1 \quad (9)$$

where

m_R is the mass of the sample weighed to prepare reconstitution (e.g. 25 g), in g;

V_R is the mass of the water weighed to prepare reconstitution (e.g. 200 g), in g.

9 Reporting

Report the results in g/100 g of (RTF, reconstituted, liquid concentrate or powder) product, to three significant digits.

10 Precision data

10.1 General

Details of the interlaboratory test of the precision of the method are summarized in [Annex B](#). The values derived from the interlaboratory test may not be applicable to analyte concentration ranges and/or matrices other than those given in [Annex B](#).

10.2 Repeatability

The absolute difference between two single test results found on identical test material by one operator using the same apparatus with the shortest feasible time interval will exceed the repeatability limit r of 12,3 % in not more than 5 % of the cases.

The values are:

$\bar{x} = 0,204$ g/100 g	$r = 0,021$ 6 g/100 g	NIST SRM 1869
$\bar{x} = 1,28$ g/100 g	$r = 0,275$ g/100 g	Adult nutritional RTF (1)
$\bar{x} = 2,67$ g/100 g	$r = 0,276$ g/100 g	Adult nutritional RTF (2)
$\bar{x} = 0,300$ g/100 g	$r = 0,019$ 0 g/100 g	Infant formula RTF
$\bar{x} = 0,275$ g/100 g	$r = 0,029$ 3 g/100 g	Child formula powder
$\bar{x} = 0,209$ g/100 g	$r = 0,024$ 9 g/100 g	Toddler formula powder milk-based
$\bar{x} = 0,030$ 8 g/100 g	$r = 0,003$ 10 g/100 g	Infant formula powder FOS/GOS-based
$\bar{x} = 0,264$ g/100 g	$r = 0,038$ 9 g/100 g	Infant formula powder milk-based

10.3 Reproducibility

The absolute difference between two single test results found on identical test material reported by two laboratories will exceed the reproducibility limit R of 30,7 % in not more than 5 % of the cases.

$\bar{x} = 0,204$ g/100 g	$R = 0,033$ 7 g/100 g	NIST SRM 1869
$\bar{x} = 1,28$ g/100 g	$R = 0,461$ g/100 g	Adult nutritional RTF (1)
$\bar{x} = 2,67$ g/100 g	$R = 0,786$ g/100 g	Adult nutritional RTF (2)
$\bar{x} = 0,300$ g/100 g	$R = 0,127$ g/100 g	Infant formula RTF
$\bar{x} = 0,275$ g/100 g	$R = 0,064$ 4 g/100 g	Child formula powder

$\bar{x} = 0,209 \text{ g/100 g}$	$R = 0,058 \text{ 2 g/100 g}$	Toddler formula powder milk-based
$\bar{x} = 0,030 \text{ 8 g/100 g}$	$R = 0,010 \text{ 1 g/100 g}$	Infant formula powder FOS/GOS-based
$\bar{x} = 0,264 \text{ g/100 g}$	$R = 0,108 \text{ g/100 g}$	Infant formula powder milk-based

11 Test report

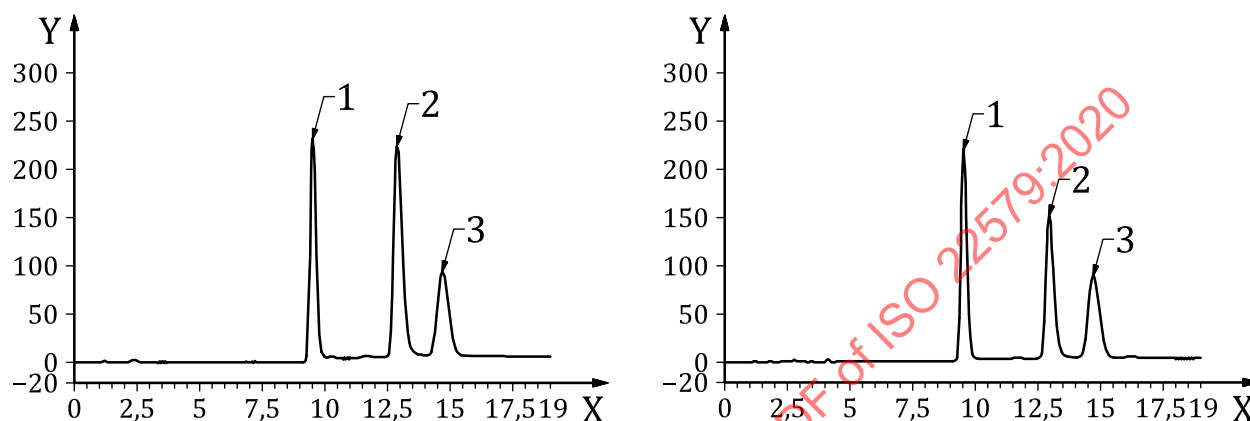
The test report shall contain the following data:

- all information necessary for the identification of the sample (type of sample, origin and designation of the sample);
- a reference to this document, i.e. ISO 22579 | IDF 241;
- the date and type of sampling procedure (if known);
- the date of receipt;
- the date of test;
- the test results and the units in which they have been expressed;
- any operations not specified in the method or regarded as optional, which might have affected the results.

STANDARDSISO.COM : Click to view the full PDF of ISO 22579:2020

Annex A (informative)

Example chromatograms and calibration curves



a) Standard solution level 4
(fructose: 600 µg/ml, glucose: 150 µg/ml)

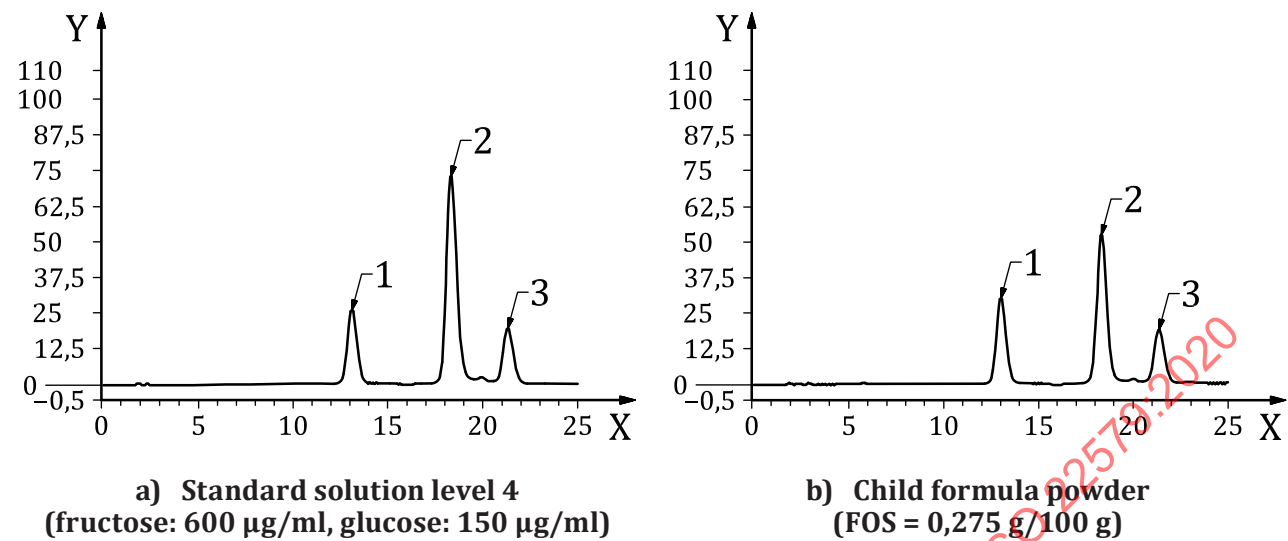
b) Child formula powder
(FOS = 0,275 g/100 g)

Key

X time, min
Y nC
1 glucose

2 fructose
3 chitobiose

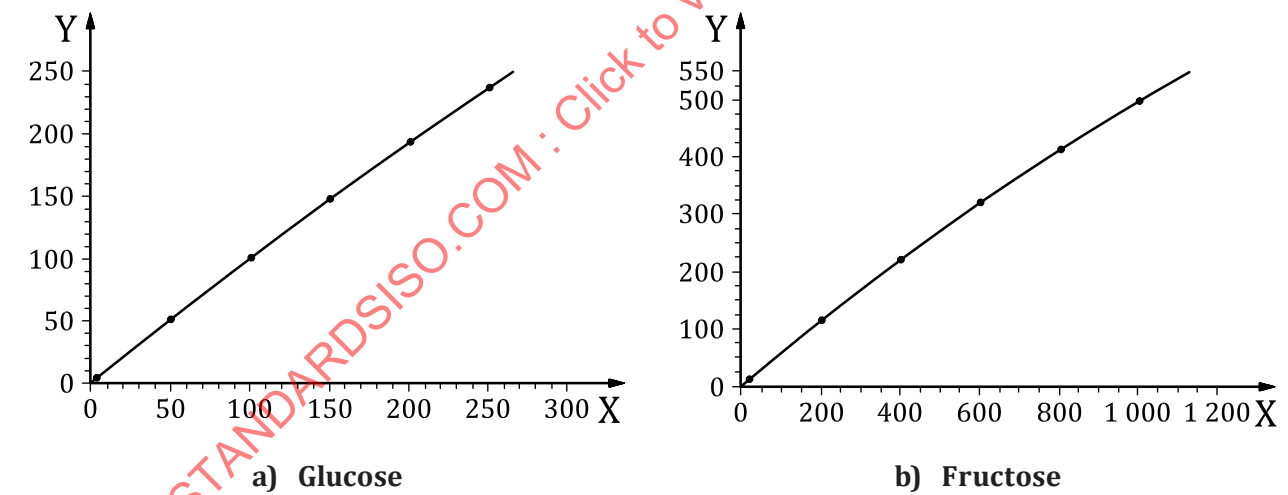
Figure A.1 — Example chromatograms on standard and product solution obtained with CarboPac PA20 column and conditions



Key

X	time, min	2	fructose
Y	nC	3	chitobiose
1	glucose		

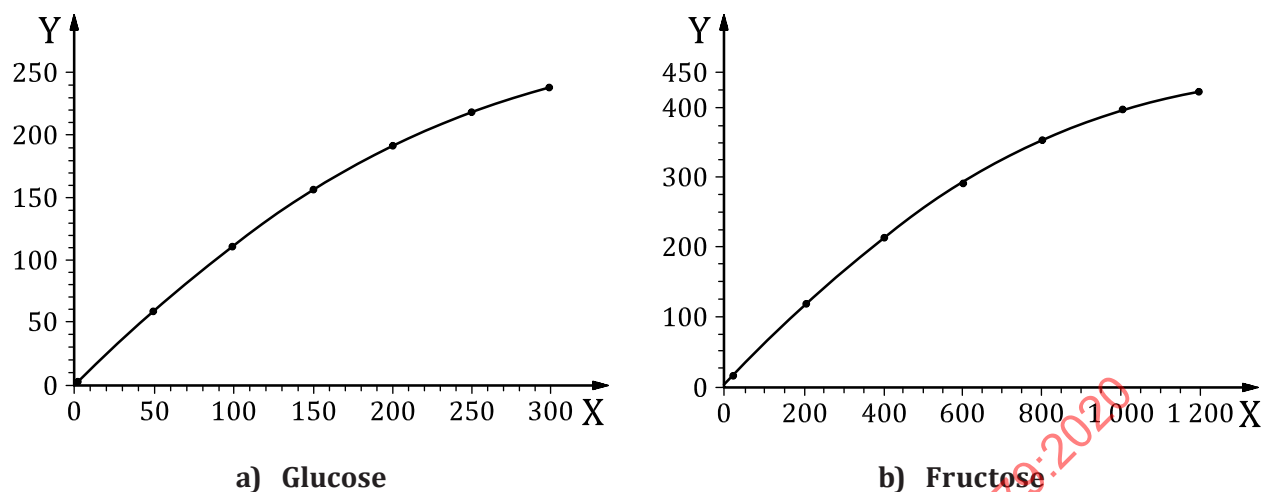
Figure A.2 — Example chromatograms on standard and product solution obtained with CarboPac PA1 column and conditions



Key

X	amount, µg/ml
Y	area, relative to internal standard

Figure A.3 — Example of suitable calibration curves obtained with CarboPac PA20 column with post-column addition



Key

X amount, $\mu\text{g/ml}$

Y area, relative to internal standard

Figure A.4 — Example of calibration curves obtained with CarboPac PA20 column and conditions, without post-column addition resulting in reduced quantitative range

Annex B (informative)

Precision data

The data given in [Table B.1](#) were obtained in an international interlaboratory study^[2] organized in 2018 by Société des produits Nestlé in accordance with ISO 5725-2^[3] and the AOAC-IUPAC Harmonized Protocol for collaborative study procedures to validate characteristics of a method of analysis^[4].

Table B.1 — Precision data for fructans

Sample	1	2	3	4	5	6	7	8
Year of interlaboratory test	2018	2018	2018	2018	2018	2018	2018	2018
Number of laboratories retained after eliminating outliers	11	12	12	12	12	12	10	12
Number of outliers (laboratories)	1	0	0	0	0	0	2	0
Number of accepted results	22	24	24	24	24	24	20	24
Mean value, $\bar{x}$, g/100 g	0,204	1,28	2,67	0,300	0,275	0,209	0,030 8	0,264
Repeatability standard deviation s_r , g/100 g	0,007 73	0,098 2	0,098 7	0,006 79	0,010 5	0,008 89	0,001 11	0,013 9
Reproducibility standard deviation s_R , g/100 g	0,012 0	0,164 6	0,280 7	0,045 3	0,023 0	0,020 8	0,003 61	0,038 6
Coefficient of variation of repeatability, $C_{V,r}$, %	3,78	7,65	3,70	2,27	3,80	4,25	3,60	5,26
Coefficient of variation of reproducibility, $C_{V,R}$, %	5,90	12,8	10,5	15,1	8,35	9,94	11,7	14,6
Repeatability limit, r [$r = 2,8 \times s_r$], g/100 g	0,021 6	0,275	0,276	0,019 0	0,029 3	0,024 9	0,003 10	0,038 9
Reproducibility limit, R [$R = 2,8 \times s_R$], g/100 g	0,033 7	0,461	0,786	0,127	0,064 4	0,058 2	0,010 1	0,108
Recovery, %	96,2	—	—	—	—	—	—	—
HorRat value	1,16	3,33	3,05	3,15	1,72	1,96	1,74	2,99
Key 1 NIST SRM 1869 2 adult nutritional RTF (1) 3 adult nutritional RTF (2) 4 infant formula RTF 5 child formula powder 6 toddler formula powder milk-based 7 infant formula powder FOS/GOS-based 8 infant formula powder milk-based								