
**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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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.

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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, $\rho = 10 \text{ mg/ml}$.

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, $\rho = 5 \text{ g/l}$.

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.