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Infant formula — Determination of β-galactooligosaccharides — Ultra high performance liquid chromatography (UHPLC) with fluorescence detection after pre-column derivatization

Préparation pour nourrissons — Détermination de la teneur en β-galacto-oligosaccharides — Chromatographie liquide à ultra-haute performance (CLUHP) couplée à une détection par fluorescence après dérivation précolonne

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Forewords

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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 document 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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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. 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 2021.01.

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.

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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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Infant formula — Determination of β-galactooligosaccharides — Ultra high performance liquid chromatography (UHPLC) with fluorescence detection after pre-column derivatization

1 Scope

This document specifies a method for the determination of β-galactooligosaccharides (GOS) in infant formula (both powder and liquid) containing 0,2 g/100 g to 3,0 g/100 g of GOS in the product as prepared ready for consumption.

The method has been validated in a multi-laboratory study with reconstituted infant formula at levels of 0,236 g/100 g, 0,594 g/100 g, 0,616 g/100 g and 0,688 g/100 g and infant formula RTF at levels of 0,316 g/100 g and 0,858 g/100 g. During the single laboratory validation study[1] spike-recovery experiments were performed up to 3 g/100 g in reconstituted infant formula powders (milk-based, partially hydrolysed milk-based and soy-based), 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 terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
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]

4 Principle

Powdered or concentrated samples are reconstituted in water and oligosaccharides present in samples are extracted at 70 °C. The ready-to-feed products (including reconstituted concentrates or powders) are diluted and two aliquots of the diluted sample are taken and both are treated with amyloglucosidase to hydrolyse any maltooligosaccharides present (Assay 1), one of the two aliquots is additionally treated with β-galactosidase (Assay 2) to hydrolyse all the GOS present. An internal standard (laminaritriose) is added to both aliquots and the oligosaccharides are fluorescently labelled with 2-aminobenzamide (2AB). Labelled extracts are diluted with acetonitrile prior to injection on an UHPLC-FLD system equipped with a hydrophilic interaction liquid chromatography (HILIC) analytical column. The analytes are separated using a gradient of aqueous ammonium formate in acetonitrile and detected with a fluorescence detector.

An external maltotriose calibration curve is prepared in the same way as the samples but without enzymatic treatment. Since it is the 2AB label that is detected, each oligosaccharide has an equivalent molar response. The maltotriose calibration curve can thus be used to determine the molar concentrations of the

oligosaccharides in the two assays. It is then necessary to know the molecular weight of each signal in the chromatogram to convert the molar concentrations to mass concentrations. This can be done by coupling a mass spectrometer. The molecular weight of each oligosaccharide may also be estimated by comparing the relative retention time of the oligosaccharide against that of a dextran ladder. The GOS content is obtained by subtracting the OS content obtained in Assay 2 from the OS content obtained in Assay 1.

5 Chemicals and reagents

5.1 List of chemicals and reagents

Use only reagents of recognized analytical/HPLC grade, unless otherwise specified.

5.1.1 Deionized water, purified with resistivity ≥ 18 MΩ.

5.1.2 Maltotriose, with accurately known purity ≥ 99,0 %, e.g. ultrapure, Carbosynth, Newbury, UK[1)]. In case of issues, check the moisture content and purity following the procedure described in Annex A.

5.1.3 Laminaritriose (50 mg), purity > 90 % (e.g. Megazyme, Bray, Ireland)[1].

5.1.4 Acetic acid, glacial 100 %.

5.1.5 Sodium hydroxide pellets.

5.1.6 Acetonitrile, HPLC grade.

5.1.7 Dimethylsulfoxide.

5.1.8 2-aminobenzamide (2AB, anthranilic acid amide)

The 2AB should be a white to off-white crystalline powder. If the 2 AB is not white it is recommended to recrystallize twice from ethanol (95 %) to obtain a white crystalline powder before use.

5.1.9 2-methylpyridine borane complex (2-picoline borane), purity 95 %.

5.1.10 Amyloglucosidase (Aspergillus niger), 9 U/mg (e.g. Roche Diagnostics: 11 202 367 001[1]).

5.1.11 β-galactosidase (Aspergillus niger), 4 000 U/ml (e.g. Megazyme, Bray, Ireland E-BGLAN[1]).

5.1.12 Formic acid, 100 %.

5.1.13 Ammonium hydroxide solution, 25 % to 30 %.

5.1.14 Dextran, with average molecular weight of 1 000 Da.

5.1.15 Isomaltose.

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 Preparation of reagents

5.2.1 Maltotriose (malto-3) stock solution, substance concentration c = 10 µmol/ml.

Weigh 50 mg of maltotriose (5.1.2) into a weighing boat and record the mass to 0,1 mg. Transfer quantitatively into a 10 ml volumetric flask with water (5.1.1) and dilute to the volume with the same solvent.

5.2.2 Laminaritriose internal standard working solution, c = 2 µmol/ml.

Weigh the whole content of a 50 mg laminaritriose (5.1.3) vial into a weighing boat and record the mass to 0,1 mg. Transfer quantitatively into a 50 ml volumetric flask and complete to the mark with water (5.1.1).

5.2.3 Sodium hydroxide solution, c = 1 mol/l.

Dissolve 10 g ± 0,2 g of sodium hydroxide pellets (5.1.5) in 200 ml of water (5.1.1) in a 250 ml volumetric flask. After cooling down to room temperature, make up to the mark with demineralised water and mix well.

5.2.4 Sodium acetate buffer solution, c = 0,2 mol/l, pH = 4,5.

Into a large beaker (>500 ml) containing 400 ml of demineralised water (5.1.1), pipette 5,8 ml of glacial acetic acid (5.1.4). Adjust to a pH of 4,5 with sodium hydroxide solution (5.2.3). Transfer the solution to a 500 ml volumetric flask and make up to the mark with water (5.1.1).

5.2.5 Mixture of 25 parts per volume of water and 75 parts per volume of acetonitrile.

Add 50 ml ± 1 ml of water (5.1.1) to 150 ml ± 1 ml of acetonitrile (5.1.6) in a glass bottle and mix.

5.2.6 2AB labelling reagent, c = 2AB (0,35 mol/l) + 2-picoline borane (1 mol/l) in a mixture of 70 parts per volume of dimethylsulfoxide (DMSO) and 30 parts per volume of acetic acid.

Pipette the volume of DMSO (5.1.7) and glacial acetic acid (5.1.4) in a 20 ml glass tube according to the number of tests to perform (see Table 1 for quantities). Mix the solution using a vortex mixer. Weigh the amount of 2AB (5.1.8) and 2-picoline borane (5.1.9) in another 20 ml glass tube (see Table 1), then add the corresponding volume of a mixture of 30 parts per volume of acetic acid and 70 parts per volume of DMSO. Mix (vortex) and use an ultrasonic bath for complete dissolution if necessary.

Table 1 — Example of quantities for 2AB reagent preparation

Max. number of tests	DMSO - Acetic acid (70 + 30)		2AB (0,35 mol/l) + 2-picoline borane (1 mol/l) in DMSO - Acetic acid (70 + 30)		
	DMSO ml	Acetic acid ml	DMSO - Acetic acid ml	2AB mg	2-picoline borane mg
50	4,7	2,0	6,00	286 ± 5	642 ± 5
100	9,3	4,0	12,0	572 ± 10	1 284 ± 10
250	23,3	10,0	30,0	1 430 ± 15	3 209 ± 15

5.2.7 Amyloglucosidase solution, (60 U/ml in 0,2 mol/l sodium acetate buffer pH = 4,5).

Weigh an amount of amyloglucosidase (5.1.10) corresponding to 600 U ± 20 U and dissolve with 10,0 ml of sodium acetate buffer (5.2.4). This solution is prepared on the day of use and kept at 4 °C until use.

IMPORTANT — For the development and validation of this method, the amyloglucosidase (Product N° 11202367001) available from Roche Diagnostics[2], was used. Enzyme activities may vary slightly from one batch to the other (units per mg are mentioned on the label). Adapt the mass of enzyme in order to reach a concentration of 60 U/ml ± 2 U/ml. Another amyloglucosidase (Product

N° 10102857001) also available from Roche Diagnostics[2)] has also been tested and found to be suitable. This enzyme is already in suspension (140 U/ml) and can be diluted with 0,2 mol/l sodium acetate buffer pH = 4,5 in order to be in working concentration (60 U/ml). When enzymes from another source are used it is imperative to ensure the enzyme employed will completely hydrolyse any maltodextrins in the product without hydrolysing any analytes, as well as not showing any interference in the chromatogram. This can be checked by performing an analysis with maltodextrin as a sample, a GOS ingredient as a sample, and running a blank with the amyloglucosidase only.

5.2.8 β-Galactosidase solution (4 000 U/ml).

Use the solution as is.

IMPORTANT — For the development and validation of this method, the β-galactosidase E-BGLAN available from Megazyme International[2], was used. When enzyme from another source is used it is imperative to ensure the enzyme employed will completely hydrolyse the galacto-oligosaccharides without hydrolysing any other oligosaccharides that may be present in the sample.

5.2.9 Dextran solution.

Weigh about 20 mg of isomaltose (5.1.15) and about 50 mg of dextran 1 000 (5.1.14) into a weighing boat. Transfer into a 50 ml volumetric flask with water (5.1.1) and dilute up to the mark.

5.3 Preparation of mobile phases

5.3.1 Eluent A, acetonitrile.

5.3.2 Eluent B, ammonium formate solution, (c = 0,1 mol/l, pH = 4,4).

Add 4,6 g ± 0,1 g (3,78 ml) of formic acid (5.1.12) in a beaker containing 800 ml of water (5.1.1). Adjust the pH to 4,40 ± 0,05 with ammonium hydroxide solution (5.1.13). Transfer quantitatively to a 1 000 ml volumetric flask and dilute to the volume with water (5.1.1). This solution is stable for seven days at room temperature.

5.4 Preparation of standard solutions

Prepare a six-level calibration curve by diluting the maltotriose stock solution (5.2.1) as described in Table 2 using volumetric flasks made up to the final volume with water (5.1.1).

Table 2 — Dilution scheme for the preparation of the standard curve

Standard solution	Volume of maltotriose stock solution (5.2.1) µl	Final volume ml	Maltotriose concentration nmol/ml
Level 1	80	20	40
Level 2	200	10	200
Level 3	400	10	400
Level 4	800	10	800
Level 5	1 200	10	1 200
Level 6	1 600	10	1 600

6 Apparatus

The usual laboratory apparatus and, in particular, the following shall be used.

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