



International Standard

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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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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 (laminaritrise) 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 $\geq 18 \text{ M}\Omega$.

5.1.2 Maltotriose, with accurately known purity $\geq 99,0 \%$, e.g. ultrapure, Carbosynth, Newbury, UK¹⁾. 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 001¹⁾).

5.1.11 β -galactosidase (*Aspergillus niger*), $4\,000 \text{ U/ml}$ (e.g. Megazyme, Bray, Ireland E-BGLAN¹⁾).

5.1.12 Formic acid, 100% .

5.1.13 Ammonium hydroxide solution, 25% to 30% .

5.1.14 Dextran, with average molecular weight of $1\,000 \text{ Da}$.

5.1.15 Isomaltose.

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5.2 Preparation of reagents

5.2.1 Maltotriose (malto-3) stock solution, substance concentration $c = 10 \mu\text{mol/ml}$.

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, $c = 2 \mu\text{mol/ml}$.

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, $c = 1 \text{ mol/l}$.

Dissolve $10 \text{ g} \pm 0,2 \text{ g}$ 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, $c = 0,2 \text{ mol/l}$, $\text{pH} = 4,5$.

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 $50 \text{ ml} \pm 1 \text{ ml}$ of water (5.1.1) to $150 \text{ ml} \pm 1 \text{ ml}$ of acetonitrile (5.1.6) in a glass bottle and mix.

5.2.6 2AB labelling reagent, $c = 2\text{AB}$ ($0,35 \text{ mol/l}$) + 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 ($0,35 \text{ mol/l}$) + 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	$1\ 284 \pm 10$
250	23,3	10,0	30,0	$1\ 430 \pm 15$	$3\ 209 \pm 15$

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

Weigh an amount of amyloglucosidase (5.1.10) corresponding to $600 \text{ U} \pm 20 \text{ U}$ 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 Diagnostics², 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 $60 \text{ U/ml} \pm 2 \text{ U/ml}$. Another amyloglucosidase (Product

N° 10102857001) also available from Roche Diagnostics²⁾ 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 International², 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 $4,6 \pm 0,1$ 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 $4,40 \pm 0,05$ 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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- 6.1 **Analytical balance**, capable of weighing to the nearest 0,1 mg.
- 6.2 **Weighing boats**.
- 6.3 **Volumetric flasks**, 10 ml to 1 000 ml.
- 6.4 **Glass tubes**, 10 ml or 20 ml.
- 6.5 **pH-meter**, readability of pH = 0,1.
- 6.6 **Microcentrifuge tubes**, volume of 1,5 ml and 2 ml, with locking cap or screw cap.
- 6.7 **Floating rack for microtubes**.
- 6.8 **Water bath**, at $70\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$, with magnetic stirring if available.
- 6.9 **Water bath**, at $65\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$.
- 6.10 **Microcentrifuge**, 10 000g.
- 6.11 **Micropipettes with tips**, volume of 0,02 ml to 10 ml.
- 6.12 **Vortex mixer**.
- 6.13 **Ultrasonic bath**.
- 6.14 **Amide LC column**, 1,7 μm , 2,1 mm $\times$ 150 mm (e.g. Waters BEH Amide Glycan³⁾, or equivalent)
- 6.15 **Liquid chromatography instrument**, consisting of:
- pump able to deliver a binary gradient at a flow of up to 0,6 ml/min and a backpressure of 103 420 kPa (15 000 psi);
 - autosampler, able to maintain a temperature around $10\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$, and deliver an injection volume of 2 μl ;
 - column compartment able to maintain a temperature of $25\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$;
 - fluorescence detector able to operate at an excitation wavelength of 330 nm and an emission wavelength of 420 nm;
 - data acquisition system.

7 Procedure

7.1 Sample preparation

7.1.1 Powdered or concentrated products on a ready-to feed (RTF) basis

Reconstitute powder or liquid concentrates according to instructions. For example, accurately weigh 25 g of infant formula powder into an appropriate bottle, add 200 g of deionized water ([5.1.1](#)) and mix well at room

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temperature to form a homogeneous suspension. Place the mixture in a water bath at 70 °C for 25 min under constant stirring. Cool the solution to room temperature. 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 an amount of sample (*m*) containing a maximum of 80 mg of GOS but not more than 5 g of sample, in to a 25 ml volumetric flask (*V*) and make up to the mark with water ([5.1.1](#)).

7.1.3 Homogeneous powdered products without prior reconstitution

Weigh an amount of sample (*m*) containing a maximum of 80 mg of GOS but not more than 1,1 g of powder, into a 50 ml volumetric flask (*V*). Add 30 ml of deionized water ([5.1.1](#)) and heat at 70 °C with constant agitation for 25 min. Cool to room temperature and make up to the mark with deionized water ([5.1.1](#)).

7.1.4 Calibration curve

With each series of analyses, prepare a maltotriose calibration curve (6 level, [Table 2](#)). For each of six calibration standards transfer 500 µl into a microtube (1,5 ml). Add 250 µl of water ([5.1.1](#)). Mix (vortex) and place in a water bath at 60 °C for 2 hours. At the end of the incubation time, mix (vortex) and place at 4 °C for 5 min to 10 min and then continue with the standards from step [7.1.8](#).

7.1.5 Dextran ladder

With each series of analyses, prepare a dextran ladder. Into a microtube (1,5 ml), transfer 500 µl of dextran solution ([5.2.9](#)). Add 250 µl of water ([5.1.1](#)), mix (vortex) and then continue with the dextran ladder from step [7.1.8](#).

7.1.6 Hydrolysis of maltodextrins and GOS

Into 2 microtubes (1,5 ml) marked A1 (assay 1) and A2 (assay 2), transfer 500 µl of sample solution ([7.1.2](#) or [7.1.3](#)). Add 200 µl of amyloglucosidase solution ([5.2.7](#)) to both tubes. Add 50 µl of water ([5.1.1](#)) in tube marked A1 and 50 µl of β-galactosidase solution ([5.2.8](#)) in the tube marked A2. Mix (vortex) and place in a water bath at 60 °C for 2 h ± 5 min. At the end of the incubation time, put all tubes with β-galactosidase (A2) in a boiling water bath for 5 min to 6 min to stop the reaction. Then mix (vortex) and place all tubes at 4 °C for 5 min to 10 min.

7.1.7 Reagent blank

With each series of analyses, prepare a reagent blank by performing the whole procedure on water ([5.1.1](#)) instead of the sample solution (for both A1 and A2 procedures).

7.1.8 Internal standard addition

Centrifuge all tubes (Standard curve, Assay 1, Assay 2, blank 1, blank 2 and dextran ladder) for 10 s to 20 s at 10 000*g* to remove drops from the lid. Add 100 µl of laminaritriose internal standard working solution ([5.2.2](#)) in all tubes and mix well (vortex).

7.1.9 Derivatization

Transfer 20 µl of solutions containing internal standard ([7.1.8](#)) into 2 ml microtubes (safe lock or screw cap) and add 100 µl of water ([7.1.1](#)) and 100 µl of 2AB labelling reagent ([5.2.6](#)) to each tube. Mix (vortex) and place the tubes in a water bath at 65 °C ± 1 °C for 1 h ± 5 min. After 1 h mix the tubes (vortex) then place at 4 °C for 5 min to 10 min.

7.1.10 Dilution

Once cooled, centrifuge for 10 s to 20 s at 10 000*g* to remove drops from the lid. Carefully open the microtubes and dilute by addition of 1 ml of a mixture of acetonitrile/water solution (5.2.5). Mix well (vortex) then centrifuge for 5 min at 10 000*g* before transferring 1 ml of supernatant to an injection vial.

7.2 Chromatographic conditions

The UHPLC system is equipped with an amide column (2,1 mm x 150 mm, 1,7 µm) (6.14). The column is held at 25 °C ± 2 °C and the injection volume is 2 µl. The analytes are separated using the gradient described in Table 3 and they are detected by means of a fluorescence detector tuned at the following wavelengths: excitation λ = 330 nm and emission λ = 420 nm.

Table 3 — UHPLC gradient

Time min	Flow ml/min	% A acetonitrile (5.3.1)	% B ammonium formate (5.3.2)
0,0	0,6	88,0	12,0
7,0	0,6	88,0	12,0
17,0	0,6	85,0	15,0
21,0	0,6	85,0	15,0
36,0	0,6	72,6	27,4
44,0	0,6	54,0	46,0
44,1	0,3	54,0	46,0
44,5	0,3	30,0	70,0
49,5	0,3	30,0	70,0
52,0	0,3	88,0	12,0
54,0	0,6	88,0	12,0
60,0	0,6	88,0	12,0

7.3 System suitability test

Before starting an analysis allow the chromatographic system to equilibrate and the fluorescence detector warm up (if necessary) under the initial conditions, for at least 15 min. Let the derivatized standard and sample solutions equilibrate to the autosampler temperature before making any injections. Ensure the system pressure and baseline are stable and there are no leaks. Before starting a series of analysis, make at least one injection of the dextran ladder, and check the relative retention times (RRT) of the oligosaccharides against the laminaritriose internal standard. See Annex D for an example chromatogram in Figure D.2. The RRTs should be in the following range: isomaltose 0,56 – 0,60, isomaltotriose 1,50 – 1,57, isomaltotetraose 1,92 – 2,12, isomaltopentaose 2,16 – 2,40, isomaltohexaose 2,29 – 2,58.

7.4 Calibration

It is recommended to use bracketed calibration, injecting 3 standards followed by a maximum of 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.

Use the instrument software to plot a six-point standard curve of “Instrument response for maltotriose/ Instrument response for laminaritriose” against the “concentration of maltotriose” in the standard (in µmol/ml). Fit a linear model to the data including the origin as a point (but not forced through the origin).

When integrating the peaks in the chromatogram it is important to make an estimate of the signal to noise (S/N) ratio of the smaller peaks. Only include peaks with a S/N ratio of 10 or greater in the calculations. Smaller peaks cannot be quantified accurately and introduce inaccuracies in the measurement if included. Do not integrate any peaks smaller than disaccharides. Do not integrate the lactose peak or the maltose peak (if present). In Assay 2, a peak may appear in the region which was covered by the lactose signal in

assay 1, do not integrate this peak. Beware that some human milk oligosaccharides (HMOs) that contain a terminal galactose at the non-reducing end (e.g. lacto-N-tetraose, lacto-N-neotetraose, lacto-N-hexaose, etc) can be partially hydrolysed by the β -galactosidase treatment. See [Annex D](#) for an example chromatogram in [Figure D.1](#). If present, they will appear to have a smaller molecular weight in Assay 2 compared to assay 1. Signals from such HMOs should be identified correctly and assigned the same molecular weight in both assays. In case of doubt run the HMO through the method and the concerned peaks can be identified.

Use the standard curve to calculate the molar concentration (in $\mu\text{mol/ml}$) of each oligosaccharide in the chromatograms in Assay 1 and Assay 2.

8 Calculation

Use the standard curve to calculate the molar concentration (c_m , in $\mu\text{mol/ml}$) of each oligosaccharide in the chromatogram without β -galactosidase treatment (Assay 1), and calculate the total oligosaccharides in that sample using [Formula \(1\)](#). Then use the standard curve to calculate the molar concentration (c_m in $\mu\text{mol/ml}$) of each oligosaccharide in the chromatogram after β -galactosidase treatment (Assay 2), and calculate the total oligosaccharides in that sample according to [Formula \(2\)](#). Then calculate the GOS content of sample using [Formula \(3\)](#). If it is desired to calculate the dietary fibre content of the GOS, exclude the disaccharides from the calculations in [Formulae \(1\)](#) and [\(2\)](#).

$$w_t = \sum (c_m \times M) \times \frac{V}{m} \times 0,000\ 1 \quad (1)$$

$$w_b = \sum (c_m \times M) \times \frac{V}{m} \times 0,000\ 1 \quad (2)$$

$$w_g = w_t - w_b \quad (3)$$

where

- w_t is the total mass fraction of oligosaccharides in the untreated sample (in g/100 g);
- w_b is the total mass fraction of oligosaccharides in the enzyme-treated sample (in g/100 g);
- w_g is the total mass fraction of GOS in the sample (in g/100 g);
- c_m is the molar concentration of each individual oligosaccharide in the sample (in $\mu\text{mol/ml}$);
- M is the molecular weight of each individual oligosaccharide in the sample, which is either estimated from the glucose unit (GU) value (see [Annex B](#)) or measured by mass spectrometry (see [Annex C](#));
- V is the volume to which the original mass of sample was diluted (in ml);
- m is the mass of sample diluted to volume (V) (in g);
- 0,000 1 is the factor to convert result from $\mu\text{g/g}$ to g/100 g.

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

9 Precision data

9.1 General

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

9.2 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 13,2 % in not more than 5 % of the cases.

The values are:

$\bar{x} = 0,594 \text{ g/100 g}$	$r = 0,043 \text{ 2 g/100 g}$	Infant formula powder with HMO (1)
$\bar{x} = 0,688 \text{ g/100 g}$	$r = 0,043 \text{ 2 g/100 g}$	Infant formula powder (2)
$\bar{x} = 0,616 \text{ g/100 g}$	$r = 0,039 \text{ 1 g/100 g}$	Infant formula powder (3)
$\bar{x} = 0,858 \text{ g/100 g}$	$r = 0,051 \text{ 3 g/100 g}$	Infant formula RTF (1)
$\bar{x} = 0,316 \text{ g/100 g}$	$r = 0,041 \text{ 9 g/100 g}$	Infant formula RTF (2)
$\bar{x} = 0,236 \text{ g/100 g}$	$r = 0,009 \text{ 0 g/100 g}$	Infant formula powder (4)

9.3 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 32,4 % in not more than 5 % of the cases.

The values are:

$\bar{x} = 0,594 \text{ g/100 g}$	$R = 0,170 \text{ 8 g/100 g}$	Infant formula powder with HMO (1)
$\bar{x} = 0,688 \text{ g/100 g}$	$R = 0,186 \text{ 1 g/100 g}$	Infant formula powder (2)
$\bar{x} = 0,616 \text{ g/100 g}$	$R = 0,139 \text{ g/100 g}$	Infant formula powder (3)
$\bar{x} = 0,858 \text{ g/100 g}$	$R = 0,228 \text{ 7 g/100 g}$	Infant formula RTF (1)
$\bar{x} = 0,316 \text{ g/100 g}$	$R = 0,096 \text{ 6 g/100 g}$	Infant formula RTF (2)
$\bar{x} = 0,236 \text{ g/100 g}$	$R = 0,076 \text{ 4 g/100 g}$	Infant formula powder (4)

10 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 7102 | IDF 257;
- the date and type of sampling procedure (if known);
- the date of receipt;
- the date of the 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 can have affected the results.

Annex A (normative)

Assessment of maltotriose concentration

A.1 General

In general, the manufacturer's certificate of analysis (CoA) provides sufficient information to accurately calculate the concentration of the maltotriose stock solution (having corrected for both the moisture content and the purity of the standard). However, in some cases the CoA may not be available or the data provided may be inaccurate. In these cases, the purity and moisture content of the maltotriose standard needs to be assessed.

A.2 Moisture determination

A.2.1 General

The moisture content is assessed by Karl-Fischer using a system adapted to measuring small quantities of water.

A.2.2 Additional apparatus

- Karl-Fischer apparatus for measuring small amounts of water (e.g. Metrohm 899 Coulometer⁴).
- Glass tubes (10 ml) with rubber stoppers for air-tight sealing.
- Gas-tight syringe (1 ml).

A.2.3 Additional chemicals

- Hydranal™ Formamide⁴ (Honeywell Research Chemicals, Charlotte, North Carolina, USA or equivalent).
- Hydranal™ Coulomat AD⁴ (Honeywell Research Chemicals, or equivalent).

A.2.4 Procedure

- Prepare a solvent mixture of one part per volume of Hydranal™ Formamide and one part per volume of Hydranal™ Coulomat AD. Sufficient for the number of analyses to be made (~ 5 g per analysis + 2 g).
- Accurately weigh the maltotriose sample (200 mg ± 20 mg) into a glass tube, and seal with the rubber stopper.
- Add 5 g of the solvent mixture and dissolve the sample (use a vortex mixer and/or sonic bath).
- Weigh 500 mg of the solvent mixture (without sample) in a syringe and inject into the coulometer to determine the moisture content of the solvent.
- Repeat the analysis of the blank solvent mixture two more times, to have a total of three measurements.
- Weigh 500 mg of the sample solution in a syringe and inject into the coulometer to determine the moisture content of the sample and solvent.
- Repeat the analysis of the sample solution.

4) 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.

- Calculate the moisture content of the sample by subtracting the average moisture content of the solvent mixture from the average moisture content of the sample and solvent.

A.3 Purity determination

The purity of the maltotriose is assessed by analysing the standard prepared at level 6 (expected concentration approximately 1 600 nmol/ml). The standard is prepared and injected on the chromatographic system as described in the main method. The chromatogram is then assessed for signals in-addition to the ones expected (maltotriose and the internal standard, laminaritriose). The peak areas are measured for all peaks except for laminaritriose and assigned a corresponding molecular weight depending on the identity of the signal (180 for glucose, 342 for Hex 2, 504 for Hex 3 (including maltotriose), 666 for Hex 4, 828 for Hex 5, 990 for Hex 6, 1 152 for Hex 7. The peak area of maltotriose multiplied by its molecular weight (504) is then divided by the sum of all the peak areas multiplied by their corresponding molecular weights to calculate the purity of maltotriose. This is best illustrated by an example.

An example data set is shown in [Table A.1](#). Taking these data, the purity of the maltotriose is calculated as shown in [Formula \(A.1\)](#):

$$P_{M3} = \frac{A_{M3} \times M_{M3}}{\sum_{i=1}^n A_{Mi} \times M_{Mi}} \times 100 = \frac{40\,320}{43\,362} \times 100 = 93,0 \quad (\text{A.1})$$

where

P_{M3} is the purity of maltotriose, in %;

A_{M3} is the peak area of maltotriose;

M_{M3} is the molecular weight of maltotriose, in amu,

A_{Mi} is the peak area of signal with i hexose units;

M_{Mi} is the molecular weight of oligosaccharide with i hexose units, in amu.

Table A.1 — Example data set for illustration of calculation of maltotriose purity

Identity	Molecular weight amu	Peak area	Peak area × molecular weight
Glucose	180	2	360
Maltose	342	3	1 026
Maltotriose	504	80	40 320
Hex4	666	1	666
Hex5	990	1	990
SUM	—	—	43 362

If the measured moisture and purity are in good agreement with the manufacturer's CoA (each ± 2 g/100 g), it is recommended to use the data provided on the manufacturer's CoA. If the difference is large, it is recommended to use the measured moisture and purity or to use a different batch of maltotriose.

Annex B
(informative)

Determination of molecular weight using dextran ladder

B.1 Principle

To determine the molecular weight of each signal it is possible to calibrate the column using the dextran ladder. The dextran oligosaccharides elute from the smallest to the largest. Isomaltose is the first signal and is composed of 2 glucose units (GU) it is therefore assigned a GU value of 2, the next oligosaccharide of the dextran ladder has a GU of 3, and so on. See [Annex D](#) for an example chromatogram in [Figure D.2](#).

Determine the relative retention time (RRT) of each of the dextran signals compared to the internal standard according to [Formula \(B.1\)](#). For GU from 2 to 6, make a plot of GU against RRT and fit a 3rd order polynomial ($y = ax^3+bx^2+cx+d$). For each signal in the sample chromatograms calculate the RRT in the same way as for the dextran ladder, then assign a GU value from the polynomial. The molecular weight of each signal can then be assigned based on the GU value using [Table B.1](#).

$$R_{(Dn)} = \frac{T_{(Dn)}}{T_{(IS)}} \tag{B.1}$$

where

- $R_{(Dn)}$ is the relative retention time of the signal n in the dextran ladder;
- $T_{(Dn)}$ is the retention time of signal n in the dextran ladder;
- $T_{(IS)}$ is the retention time of laminartriose internal standard.

Table B.1 — Assignment of peak molecular weight according to its GU value

GU Range	GOS Type	Molecular weight (amu)
1,6 to 2,5	Hex2	342
2,5 to 2,6	Internal standard	—
2,6 to 3,4	Hex3	504
3,4 to 4,2	Hex4	666
4,2 to 5,2	Hex5	828
5,2 to 6,2	Hex6	990
> 6,2	Hex7	1 152

Annex C
(informative)

Determination of molecular weight using LC-MS

C.1 Sample preparation

The same vial prepared for the quantitative determination of GOS can also be used for the analysis of molecular weight assignment. Alternatively, a sample may be prepared using only the GOS ingredient. In case the mass spectrometer has insufficient sensitivity, it is possible to prepare a sample having 10 times greater GOS concentration for the purposes of peak identification only (in this case, it is recommended to use the GOS ingredient).

C.2 Mass spectrometer set-up

C.2.1 General

In addition to the UHPLC-FLD instrument, a mass spectrometer is required. Use the same chromatography set-up and conditions as described in the main method, but split the flow eluting from the analytical column in a ratio of about 1:1. One half of the flow is passed through the fluorescence detector, the other half is directed to a mass spectrometer.

NOTE If the mass spectrometer is connected in series after the fluorescence detector, there is a high chance that the flow cell will rupture.

This clause describes the setup of the API 4000 QTrap⁵⁾ mass spectrometer in the laboratory. The mass spectrometer settings in other laboratories should be optimized locally.

C.2.2 LC parameters

Use the same LC conditions as described in the quantitative method.

Injection volume can be increased up to 10 µl.

C.2.2.1 MS parameters

Experiment type: Multiple ion monitoring (Q1 or EMS), monitoring the masses listed in [Table C.1](#).

Mode: ESI negative

Curtain gas (CUR): 17

Ion spray voltage (IS): -3 800 V

Gas 1 (GS1): 60

Gas 2 (GS2): 20

Interface heater temperature (IHT): 400 °C

Declustering potential (DP): -60

Entrance potential (EP): -10

5) 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.

In some cases, there can be some overlap of peaks having different molecular weight. In these cases, the analyst should assign the molecular weight which would be expected to result in the least error (e.g. if the MS signal of a trisaccharide and tetrasaccharide overlap, but the signal is stronger for the trisaccharide, then assign the peak the molecular weight of a trisaccharide).

Table C.1 — m/z ratios monitored for the assignment of GOS molecular weights

Q1 m/z	Dwell time (ms)	Corresponding GOS	GOS molecular weight (amu)
461,3	50,0	Hex ₂ (including lactose)	342
623,4	50,0	Hex ₃	504
785,4	50,0	Hex ₄	666
947,4	50,0	Hex ₅	828
1 109,5	50,0	Hex ₆	990
1 271,6	50,0	Hex ₇	1 152
1 433,6	50,0	Hex ₈	1 314

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