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Milk and milk products — Determination of the sugar contents — High performance anion exchange chromatography with pulsed amperometric detection method (HPAEC-PAD)

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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).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

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*, 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), and the International Dairy Federation (IDF). It is being published jointly by ISO and IDF.

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 C22 of the *Standing Committee on Analytical Methods for Composition* under the aegis of its project leader Mr H. Cruijsen (NL).

Milk and milk products — Determination of the sugar contents — High performance anion exchange chromatography with pulsed amperometric detection method (HPAEC-PAD)

1 Scope

This document specifies the quantitative liquid chromatographic determination of specific sugars (galactose, glucose, fructose, sucrose, lactose and maltose) in various milk and milk products, applying arabinose as an internal standard.

The method is applicable to the following dairy matrices: milk, sweetened condensed milk, milk powder, cheese, whey powder, infant formula, milk dessert and yoghurt.

The method does not apply to dairy products containing soy or to the determination of the lactose content in low-lactose milk products at levels below 1 mg/g.

A high performance anion exchange chromatography method in combination with pulsed amperometric detection (HPAEC-PAD) method is applied[5][3][4]. With this method, thirteen different monosaccharides, disaccharides and trisaccharides can be separated: fucose, arabinose, galactose, glucose, fructose, sucrose, lactose, lactulose, maltose, melibiose, trehalose, isomaltulose and maltotriose.

The method is applicable to labelling for the six most important sugars that can be present by nature or by addition in milk and milk products. The method does not apply to sugar contents less than 0,1 %.

2 Normative references

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

ISO 3696, *Water for analytical laboratory use — Specification and test methods*

3 Terms and definitions

No terms and definitions are listed in this document.

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/

4 Principle

The sugars present in the sample are extracted with an aqueous ethanol buffer solution in order to inhibit potential probiotic activities. The obtained extract is deproteinized with a Carrez clarification. After clarification, the solution is diluted and the sugars present are separated and quantified by HPAEC. HPAE allows carbohydrates separation at high pH. In order to improve sensitivity and stability, post-column sodium hydroxide solution is added to the HPAEC-PAD. GOS (galacto-oligosaccharides) and fructans do not interfere with the analysis of the sugars[5]. Arabinose is applied as an internal standard for the quantification of the sugars.

5 Reagents

Use only reagents of recognized analytical grade and water in accordance with ISO 3696, unless otherwise specified.

5.1 Water, conforming to ISO 3696, grade 3 and grade 1.

5.2 Sodium hydroxide (NaOH) pellets.

5.3 Aqueous sodium hydroxide solution, substance concentration c = 1 mol/l.

Add to a 1 000 ml volumetric flask 40 g ± 1 g NaOH pellets (5.2), dissolve in about 500 ml of water and, after cooling down, dilute with water to the mark and homogenize.

5.4 Sodium hydroxide solution, mass fraction w(NaOH) = 33 % in water.

5.5 Sodium hydroxide solution, mass fraction w(NaOH) = 50 % in water.

The amount of carbonate and mercury in the reagent should be minimized. Do not shake or stir the solution before use. A suitable commercially available carbonate sodium hydroxide solution may also be used.

5.6 Concentrated hydrochloric acid (HCl), mass fraction of 36 % to 38 % in water.

5.7 Aqueous hydrochloric acid solution, c = 1 mol/l.

Add to a 1 000 ml volumetric flask (6.2) 500 ml of water followed by 83 ml of concentrated HCl (5.6) and, after cooling down, dilute with water to the mark and homogenize.

5.8 Acetonitrile (HPLC quality).

5.9 Acetonitrile in water, a volume fraction of 5 % in water.

Add to a 1 000 ml volumetric flask (6.2) 50 ml of acetonitrile (5.8), dilute with water grade 3 to the mark and homogenize.

5.10 Anhydrous sodium acetate (CH_3COONa) (HPLC quality).

5.11 Eluent 1 (E1), aqueous solution of sodium acetate (CH_3COONa), c = 1,0 mol/l.

Add to a 1 000 ml volumetric flask (6.2) about 800 ml of degassed water grade 1 (eluent 3, 5.13) followed by 82,0 g sodium acetate (5.10). Then dilute the aqueous solution with degassed water (eluent 3, 5.13) to the mark and homogenize. Store the eluent under an inert atmosphere.

5.12 Eluent 2 (E2), aqueous solution of carbonate free sodium hydroxide (NaOH), c = 0,2 mol/l.

Add to a 1 000 ml volumetric flask (6.2) about 800 ml of degassed water grade 1 (eluent 3, 5.13) and purge for 15 min with helium. Add 16,0 g of sodium hydroxide solution (5.5). Then quickly dilute the aqueous solution with degassed water grade 1 (eluent 3, 5.13) to the mark, immediately close the bottle and homogenize. Store the eluent under an inert atmosphere.

5.13 Eluent 3 (E3), degassed water grade 1, stored under an inert atmosphere.

5.14 Eluent 4 (E4), aqueous solution of sodium acetate (CH_3COONa), c = 0,025 mol/l.

Add to a 1 000 ml volumetric flask (6.2) about 800 ml of degassed water grade 1 (eluent 3, 5.13) followed by 2,05 g of sodium acetate (5.10). Then dilute the aqueous solution with degassed water (eluent 3, 5.13) to the mark and homogenize. Store the eluent under an inert atmosphere.

5.15 Post column reagent, aqueous solution of sodium hydroxide, c = 0,3 mol/l.

Add to a 1 000 ml volumetric flask (6.2) about 800 ml of degassed water grade 1 (eluent 3, 5.13). Purge for 15 min with helium. Add 24,0 g of sodium hydroxide solution (5.5) and quickly fill up to the mark with the degassed water grade 1 (eluent 3, 5.13). Immediately close the flask and homogenize. Store the post column reagent under an inert atmosphere.

IMPORTANT — It is extremely important to remove dissolved carbon dioxide from the eluents and post column reagent prior to use and during use to avoid fast reduction in detector sensitivity. The eluents and post column reagent are maintained under an inert gas during use.

5.16 Mixture of a volume fraction of 95 % of ethanol (with a volume fraction of 96 % ethanol and 4 % of water) and a volume fraction of 5 % methanol.

5.17 Potassium hexacyanoferrate (II) trihydrate, $K_4Fe(CN)_6{\cdot}3H_2O$.

5.18 Zinc acetate dihydrate, $Zn(CH_3COO)_2{\cdot}2H_2O$.

5.19 Glacial acetic acid.

5.20 Carrez reagent I.

Weigh 106 g of $K_4Fe(CN)_6{\cdot}3H_2O$ (5.17) in a 1 000 ml volumetric flask (6.2), dissolve in 800 ml of water (5.1) and dilute with water grade 3 to the mark. Store the Carrez reagent I in the refrigerator.

5.21 Carrez reagent II.

Weigh 220 g of $Zn(CH_3COO)_2{\cdot}2H_2O$ (5.18) in a 1 000 ml volumetric flask (6.2), dissolve in 800 ml water, add 30 ml of glacial acetic acid (5.19) and dilute with water grade 3 to the mark. Store the Carrez reagent II in the refrigerator.

IMPORTANT — Do not use Carrez reagent II with zinc sulfate.

5.22 Buffer solution of piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) (c = 1,5 mol/l and pH = 6,9).

Add 22,5 g of the PIPES buffer solution to a 100 ml conical flask and add 20 ml of sodium hydroxide solution (5.3). Adjust the pH to pH = 6,9 with NaOH 33 % (5.4) in water. Transfer the PIPES buffer solution quantitatively into a 50 ml calibrated tube and fill up with water grade 3 till 50 ml. The pH of the obtained buffer solution shall be within the range of 6,8 to 7,0.

5.23 Arabinose.

5.24 Galactose.

5.25 Glucose.

5.26 Fructose.

5.27 Sucrose.

5.28 Lactose.

5.29 Maltose.

5.30 Internal standard stock solution arabinose.

Weigh, to the nearest mg, approximately 7 g of arabinose (5.23) into a 50 ml volumetric flask (6.2). Add about 30 ml of water grade 3 and dissolve the arabinose. Add 2,5 ml of acetonitrile (5.8), fill up to the mark with water and homogenize the solution.

5.31 Sugar standard stock solution.

Weigh, to the nearest 0,1 mg, approximately 260 mg of the monosaccharides galactose (5.24), glucose (5.25) and fructose (5.26), and approximately 400 mg of the disaccharide sucrose (5.27), lactose (5.28) and maltose (5.29) into a 500 ml volumetric flask (6.2). Add about 200 ml of water grade 3 and dissolve the sugars. Add 25 ml of acetonitrile (5.8), fill up to the mark with water grade 3 and homogenize the solution.

5.32 Sugar standard solutions for calibration.

Prepare the different dilutions of the sugar calibration standards as specified in Table 1. Mix the specified volumes of the internal standard stock solution arabinose (5.30) and sugar standard stock solution (5.31) in a 200 ml volumetric flask, add about 50 ml of water grade 3 and homogenize. Add 10 ml of acetonitrile (5.8), fill up to the mark with water and homogenize.

Table 1 — Preparation of the sugar standard solutions for calibration

Sugar standard solution	Volume of sugar standard stock solution (5.31) ml	Volume of arabinose internal standard stock solution (5.30) ml
1	0,2	0,050
2	1,0	0,050
3	6,0	0,050
4	10,0	0,050
5	20,0	0,050
6	40,0	0,050
7	80,0	0,050
8	100,0	0,050

6 Apparatus

6.1 Analytical balance, capable of weighing to an accuracy of ±0,1 mg.

6.2 Volumetric flask, volume of 50 ml, 500 ml and 1 000 ml.

6.3 pH meter.

6.4 Black band filter paper.

6.5 Centrifugation tubes.

6.6 Homogenizer[1].

6.7 Vortex mixer.

6.8 Screw cap closed graduated tubes, 50 ml volume.

6.9 Positive displacement multipipette.

6.10 Dispensers, resistant to organic solvent and adjusted to 2,5 ml and 25 ml.

6.11 HPLC vials.

6.12 Metal-free liquid chromatographic system, e.g. Thermo Dionex ICS 3000[2], applicable for a (quaternary) gradient elution.

6.13 Column oven, with a temperature stability of ±1 °C with an operating temperature of 20 °C to 35 °C.

6.14 High performance anion exchange analytical column[3], filled with pellicular polystyrene-divinylbenzene resin or filled with an anion exchanger resin enabling the required separation.

6.15 High performance anion exchange guard column[4], filled with pellicular polystyrene-divinylbenzene resin or filled with an anion exchanger resin enabling the required separation.

6.16 Pulsed amperometric detector (PAD)[5] with a stability of ±1 °C and an operating temperature range of 20 °C to 35 °C.

Use pulsed amperometric detection and potential settings and waveforms recommended by the instrument supplier. Example detector potential settings (versus Ag/AgCl reference) are given in Table 2.

6.17 Metal-free post column reagent pump[6].

1) An Ultra turrax with an appropriate probe is an example of a suitable homogenizer 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 this product.

2) Thermo Dionex ICS 3000 is an example of a suitable homogenizer 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 this product.

3) A Thermo-Dionex Carbopac PA1 analytical column (2 mm × 250 mm) is an example of a suitable high performance anion exchange column 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 this product.

4) A Dionex Carbopac PA1 guard column (2 mm × 50 mm) is an example of a suitable high performance anion exchange column 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 this product.

5) A Thermo-Dionex model PAD is an example of a suitable PAD 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 this product.

6) A single piston Thermo Dionex Axp pump is an example of a suitable metal-free post column reagent pump 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 this product.

