
**Infant formula and adult
nutritional — Determination of myo-
inositol by liquid chromatography and
pulsed amperometry**

*Formules infantiles et produits nutritionnels pour adultes —
Détermination de la teneur en myo-inositol par chromatographie
liquide et ampérométrie pulsée*

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Contents

Page

Foreword	iv
1 Scope	1
2 Terms and definitions	1
3 Principle	1
4 Reagents and materials	1
5 Apparatus	3
6 Procedure	4
6.1 Free myo-inositol	4
6.1.1 Sample preparation	4
6.1.2 Extraction	5
6.2 Myo-inositol bound as phosphatidylinositol	5
6.2.1 Sample preparation	5
6.2.2 Extraction	5
6.2.3 Cleanup	6
6.2.4 Hydrolysis	6
6.3 HPLC analysis	6
6.3.1 Instrument operating conditions	6
6.3.2 PAD settings with gold electrode	8
6.3.3 Instrument startup	8
6.3.4 Standard and sample analysis	8
6.3.5 System shutdown	8
7 Calculations	8
7.1 General	8
7.2 Concentration of calibration standards	9
7.3 Preparation of standard curve	9
7.4 Calculation of free or free plus bound myo-inositol in samples	9
7.4.1 Calculation of free myo-inositol	9
7.4.2 Calculation of bound myo-inositol	10
7.4.3 Calculation of free plus bound myo-inositol	10
Annex A (informative) Examples of chromatograms	11
Annex B (informative) Precision data	12
Bibliography	15

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. www.iso.org/directives

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For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: Foreword - Supplementary information

The committee responsible for this document is ISO/TC 34, *Food products* in collaboration with AOAC INTERNATIONAL. It is being published by ISO and separately by AOAC INTERNATIONAL. The method described in this International Standard is equivalent to the AOAC Official Method 2011.18: *Myo-inositol (free and bound as phosphatidyl inositol) in infant and pediatric formula and adult nutritional*.

Infant formula and adult nutritionals — Determination of myo-inositol by liquid chromatography and pulsed amperometry

WARNING — The use of this International Standard can involve hazardous materials, operations and equipment. This International Standard does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this International Standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

1 Scope

This International Standard specifies a method for the determination of myo-inositol (free or free plus bound as phosphatidylinositol) in infant formula and adult nutritionals using liquid chromatography and pulsed amperometry with column switching.

2 Terms and definitions

For the purposes of this document the following terms and definitions apply.

2.1

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 and without intact protein

2.2

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 Principle

Free myo-inositol and phosphatidyl bound myo-inositol are extracted using two different sample preparation procedures. Free myo-inositol is extracted from samples with dilute hydrochloric acid and water. Phosphatidylinositol is extracted from samples with chloroform and separated from other fats with silica solid phase extraction cartridges. Myo-inositol is then released from the glycerol backbone with concentrated acetic and hydrochloric acid at 120°C. The ion chromatographic method uses a combination of two different ion exchange columns with column switching and pulsed amperometric detection (PAD). The concentration of myo-inositol is calculated by comparison with external standards of known concentration.

4 Reagents and materials

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and distilled or demineralized water or water of equivalent purity.

4.1 Chemicals and solvents

4.1.1 **Acetic acid**, glacial, ACS.

4.1.2 **Chloroform**, high-purity, HPLC grade.

4.1.3 **Diethyl ether**, anhydrous, HPLC grade.

4.1.4 **Drierite**, (desiccant), anhydrous calcium sulfate, 8 mesh.

4.1.5 **Helium**, zero grade or equivalent.

4.1.6 **Hexane**, HPLC grade.

4.1.7 **Hydrochloric acid**, concentrated (36 % to 38 %), ACS.

4.1.8 **Metaphosphoric acid**, ACS.

4.1.9 **Methanol**, HPLC grade.

4.1.10 **Myo-inositol**, primary reference standard, official lot, store desiccated. See standard label for purity.

4.1.11 **Sodium chloride**, ACS.

4.1.12 **Sodium hydroxide**, 50 % (m/m), low carbonate form.

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4.2 Preparation of reagents and standard solutions

4.2.1 **General.** All solutions can be scaled up or down for convenience provided good laboratory practices are observed. Solutions can be stored at refrigerated or at ambient temperature in tight, inert containers unless otherwise specified.

4.2.2 **Myo-inositol stock standard solution** (approximately 2 000 mg/l). Accurately weigh approximately 0,100 g myo-inositol and quantitatively transfer to a 50 ml volumetric flask. Dilute to volume with water. Mix well. Store refrigerated. Expiration: 3 months.

4.2.3 **Myo-inositol intermediate standard solution** (approximately 200 mg/l). Dilute 10,0 ml stock standard (4.2.2) to 100 ml with water and mix well. Discard after use.

4.2.4 Preparation of calibration standard solutions

4.2.4.1 **Myo-inositol calibration standard solutions high**, (approximately 4 mg/l, 2 mg/l, 1 mg/l, 0,5 mg/l).

Into separate volumetric flasks, dilute 2,0 ml, 1,0 ml and 0,5 ml myo-inositol intermediate standard (4.2.3) to 100 ml with water. Dilute 0,5 ml myo-inositol intermediate standard (4.2.3) to 200 ml with water. Expiration: 2 weeks

4.2.4.2 **Myo-inositol calibration standard solutions low**, (approximately 0,2 mg/l and 0,05 mg/l).

Into separate volumetric flasks, dilute 4 ml and 1 ml of the 0,5 mg/l myo-inositol calibration standard to 10 ml with water. Expiration: 2 weeks.

4.2.5 Hydrochloric acid, 0,5 %. Add 1,25 ml concentrated hydrochloric acid to approximately 200 ml water in a 250 ml volumetric flask. Dilute to volume with water and mix well. Expiration: 6 months.

4.2.6 Sodium chloride, 1 mol/l. Dissolve 5,8 g sodium chloride and dilute to 100 ml with water. Expiration: 1 month.

4.2.7 Sodium hydroxide, 0,12 % or 30 mmol (Pump 1). Quickly weigh ($4,8 \pm 0,1$) g of 50 % sodium hydroxide into a 2 000 ml volumetric flask containing approximately 1 900 ml water. It is important that the sodium hydroxide does not absorb carbon dioxide from the air. Swirl to mix well. Dilute to volume with water and mix well. Expiration: 1 month.

4.2.8 Sodium hydroxide, 4,0 % or 1 mol/l (Pump 2). Quickly weigh (160 ± 3) g of 50 % sodium hydroxide into a 2 000 ml volumetric flask containing approximately 1 900 ml water. It is important that the sodium hydroxide does not absorb carbon dioxide from the air. Swirl to mix well. Dilute to volume with water and mix well. Expiration: 1 month.

4.2.9 Metaphosphoric acid, 6 %. Weigh 6,0 g metaphosphoric acid into a 100 ml volumetric flask. Dissolve and dilute to volume with water. Mix well. Store refrigerated. Expiration: 1 week.

4.2.10 Phosphatidylinositol extraction solutions. Prepare fresh on day of use.

4.2.10.1 Chloroform:methanol (2:1). Mix 60 ml chloroform and 30 ml methanol.

4.2.10.2 Hexane:diethyl ether (80:20). Mix 80 ml hexane and 20 ml diethyl ether.

4.2.10.3 Hexane:diethyl ether (50:50). Mix 50 ml hexane and 50 ml diethyl ether.

4.2.10.4 Methanol:chloroform:water (75:15:10). Mix 75 ml methanol, 15 ml chloroform and 10 ml water.

5 Apparatus

Usual laboratory glassware and equipment and, in particular, the following.

5.1 Analytical balance, minimum weighing capacity of at least 0,000 1 g.

5.2 Centrifuge.

5.3 Desiccator.

5.4 Nitrogen evaporator, with water bath or equivalent.

5.5 Oven, capable of maintaining 120 °C.

5.6 pH-meter, with pH 4 and 7 buffers.

5.7 Stir plate, multiposition with stir bars.

5.8 Vacuum manifold.

5.9 Vortex mixer.

5.10 System HPLC, with corrosion-resistant components including an autosampler, two isocratic pumps, 6-port switching valve, pulsed amperometry detector with a gold electrode and PEEK or polytetrafluoroethylene (PTFE) 0,18 mm to 0,25 mm (0,007 inch to 0,01 inch) internal diameter tubing. Autosampler capable of injecting 20 µl.

5.11 Columns, Dionex CarboPac¹⁾ MA1 (4 mm × 250 mm) P/N, 44066, MA1 (4 mm × 50 mm) P/N 44067, and PA1 (4 × 50 mm) P/N 43096, or equivalent.

5.12 Beakers, assorted sizes.

5.13 Centrifuge tubes, 50 ml with polytetrafluoroethylene (PTFE)-coated caps.

5.14 Syringe filters, polyamide, 0,45 µm and 0,2 µm.

5.15 Filter paper, Whatman 2V¹⁾ or equivalent.

5.16 Conical flasks, 50 ml or 125 ml or equivalent.

5.17 Volumetric flasks, assorted sizes.

5.18 Funnels, suitable for use with filter paper.

5.19 Pipets, volumetric, assorted sizes.

5.20 Solid-phase extraction (SPE) cartridge, silica, 1 g²⁾.

5.21 Syringes, 1 ml disposable and 25 ml gas-tight glass with 100 mm (4 in) stainless steel needles.

6 Procedure

6.1 Free myo-inositol

6.1.1 Sample preparation

6.1.1.1 General

Prepared samples that are constantly stored at 1 °C to 8 °C in closed containers are stable for up to 5 days. After 5 days, samples shall be prepared again. Mix liquid samples well to ensure homogeneity. If the powder sample homogeneity is unknown, assume that it is non-homogenous and proceed with the preparation of dry blended/non-homogenous powder samples as given in [6.1.1.3](#).

6.1.1.2 Liquid samples

For ready-to-feed liquid samples, accurately weigh (0,5 ± 0,05) g to (5 ± 0,5) g of product into a 100 ml volumetric flask and record the mass to the nearest 0,000 1 g.

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 of the product named. Equivalent products may be used if they can be shown to lead to the same results.

2) J.T. Baker P/N 7086-07 (www.avantormaterials.com) 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 of the product named. Equivalent products may be used if they can be shown to lead to the same results.

6.1.1.3 Dry blended powder samples

For dry blended/non-homogenous powder samples, reconstitute per the product label instructions. Accurately weigh 0,5 g to 5 g reconstituted product into a 100 ml volumetric flask. Record the mass to the nearest 0,000 1 g.

6.1.1.4 Wet blended powder samples

For wet blended/homogenous powder samples, accurately weigh 0,25 g to 1,5 g powder into a 100 ml volumetric flask and record the mass to the nearest 0,000 1 g. Add approximately 10 ml to 15 ml water to the volumetric flask and swirl or stir to completely dissolve the powder.

6.1.2 Extraction

Add enough 0,5 % hydrochloric acid ([4.2.5](#)) to each sample to adjust the sample pH to $4,5 \pm 0,2$ and swirl to mix.

Allow the samples to react with 0,5 % hydrochloric acid for a minimum of 2 min and then dilute to volume with water. Mix well. Filter samples through filter paper ([5.15](#)) into 125 ml conical flasks or appropriate glassware.

NOTE Although some samples will filter cloudy, the filtrates can still be used.

Filter an aliquot of sample filtrate through a 0,45 µm syringe filter ([5.14](#)) into an autosampler vial.

6.2 Myo-inositol bound as phosphatidylinositol

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6.2.1 Sample preparation

6.2.1.1 General

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Prepared samples that are constantly stored at 1 °C to 8 °C in closed containers are stable for up to 5 days. After 5 days, samples shall be prepared again. Thoroughly mix or stir products prior to sampling. Mix liquid samples well to ensure homogeneity. If the powder sample homogeneity is unknown, assume that it is non-homogenous and proceed with the preparation of dry blended/non-homogenous powder samples given in [6.2.1.3](#).

6.2.1.2 Liquid samples

For ready-to-feed liquid samples, accurately weigh ($4 \pm 0,4$) g of product into a 50 ml centrifuge tube and record the mass to the nearest 0,000 1 g.

6.2.1.3 Dry blended powder samples

For dry blended/non-homogenous powder samples, reconstitute per the product label instructions. Accurately weigh ($4 \pm 0,4$) g reconstituted sample into a 50 ml centrifuge tube. Record the mass to the nearest 0,000 1 g.

6.2.1.4 Wet blended powder samples

For wet blended homogenous powder samples, accurately weigh ($1 \pm 0,1$) g powder into a 50 ml centrifuge tube and record the mass to the nearest 0,000 1 g. Add 4 ml water centrifuge tube and mix well.

6.2.2 Extraction

In a fume hood, add 10 ml methanol to each sample and stir for at least 20 min or vortex for at least 1 min and allow samples to set for at least 20 min. Add 20 ml chloroform and stir for at least 5 min or vortex for at least 1 min and allow samples to set for at least 5 min. If large clumps form when chloroform is

added, cap tube and shake well for at least 1 min to mix sample. Add 5 ml 6 % metaphosphoric acid (4.2.9) and 1 ml 1 mol/l NaCl (4.2.6) and mix well. Centrifuge until layers separate. Using a 25 ml glass gas-tight syringe with a stainless steel needle (5.21), transfer the bottom chloroform layer to a clean 50 ml centrifuge tube and evaporate the chloroform with nitrogen in a 60 °C water bath.

6.2.3 Cleanup

In a fume hood, condition a 1 g silica SPE cartridge (5.20) with 6 ml hexane. Dissolve residue in the bottom of the centrifuge tube in 1 ml chloroform:methanol (2:1). Quantitatively transfer dissolved residue to the conditioned silica SPE cartridge. Rinse the 50 ml centrifuge tube with 3 ml hexane:diethyl ether (80:20) and then transfer to the SPE cartridge. Discard the eluent. Rinse the 50 ml centrifuge tube with 3 ml hexane:diethyl ether (50:50) and then transfer to the SPE cartridge. Collect eluent in a clean 50 ml centrifuge tube. Rinse 50 ml centrifuge tube with 4 ml methanol and then transfer to the SPE cartridge. Collect eluent in the same 50 ml centrifuge tube. Rinse 50 ml centrifuge tube with 4 ml methanol:chloroform:water (75:15:10) and transfer to the SPE cartridge. Collect eluent in the same 50 ml centrifuge tube. Evaporate eluents collected from SPE cartridge with nitrogen in a 60 °C water bath.

6.2.4 Hydrolysis

In a fume hood, add 40 µl glacial acetic acid (4.1.1) and 2 ml concentrated hydrochloric acid (4.1.7) to the residue in the centrifuge tube from the sample cleanup step. Tightly cap tube. Heat in a 120 °C oven for 2 h. Cool. Add approximately 10 ml of water and swirl to mix. Add 1,25 ml 50 % (m/m) sodium hydroxide (4.1.12). Transfer sample to a 50 ml volumetric flask and dilute to volume with water. Filter an aliquot of sample filtrate through a 0,45 µm syringe filter into an autosampler vial.

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6.3 HPLC analysis

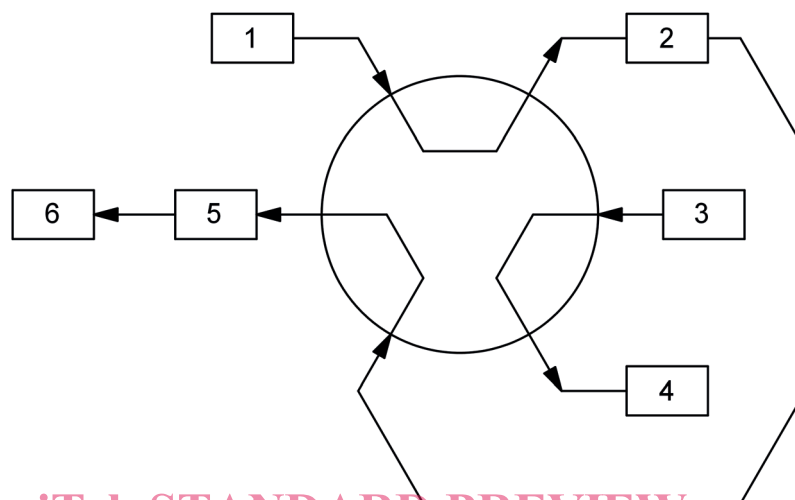
6.3.1 Instrument operating conditions

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Pump 1 pressure limit	13 790 kPa (2 000 psi)
Pump 1 mobile phase	0,12 % (30 mmol/l) NaOH
Pump 1 flow rate	0,40 ml/min
Pump 2 pressure limit	13 790 kPa (2 000 psi)
Pump 2 mobile phase	4 % (1 mol/l) NaOH
Pump 2 flow rate	0,40 ml/min
Injection volume	20 µl
Myo-inositol retention time	11 min to 13 min
Run time	25 min

Switching valve configuration time:

0,00 min	Configuration 1	See Figure 1
1,50 min	Configuration 2	See Figure 2
13,50 min	Configuration 1	See Figure 1

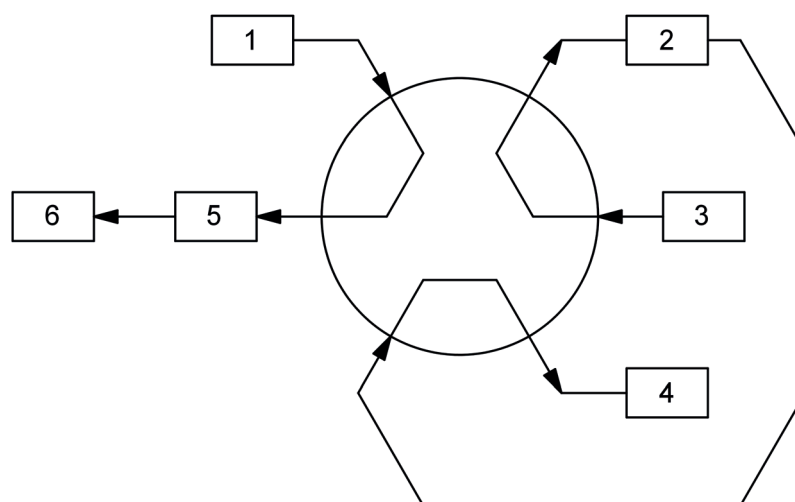


Key

- | | |
|--------------------|------------------------------------|
| 1 Pump 1 | 5 MA1 guard and analytical columns |
| 2 PA1 guard column | 6 electrochemical detector |
| 3 Pump 2 | |

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Figure 1 — Switching valve configuration 1



Key

- | | |
|--------------------|------------------------------------|
| 1 Pump 1 | 4 waste |
| 2 PA1 guard column | 5 MA1 guard and analytical columns |
| 3 Pump 2 | 6 electrochemical detector |

Figure 2 — Switching valve configuration 2