Determination of Myo-Inositol (Free and Bound as Phosphatidylinositol) in Infant Formula and Adult Nutritionals

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Key Words
Dionex CarboPac MA1 Column, Dionex CarboPac PA1 Column, Column Switching, Au Disposable Working Electrode, Dionex OnGuard II RP Cartridge

Introduction

Myo-inositol is one of the most abundant sugars in the body, where it occurs in its free form and as a component of phosphoinositides in cell membranes. It plays an important role in various biological functions, including the regulation of cell osmolality,[1] phosphoinositide-mediated processes of cell signaling,[2] formation of the neural system,[3] and pulmonary surfactant phospholipid production.[4]

Studies indicate relatively high concentrations of myo-inositol in human breast milk (~1200 µmol/L),[5] suggesting that exogenous myo-inositol is required for postnatal development of formula-fed infants. Unless fortified with myo-inositol, milk-based infant formula can provide the infant with only 20% as much inositol as from an equal amount of human breast milk.[6] Therefore, it is necessary to add myo-inositol to infant formula to prevent nutritional deficiency. The U.S. Food and Drug Administration requires myo-inositol to be added to nonmilk-based formula at a minimum concentration of 4 mg/100 kcal.[7] The Life Sciences Research Office Expert Panel of the American Society for Nutritional Sciences recommends 4–40 mg/100 kcal of myo-inositol in all types of infant formulas.[6] To ensure that infant formula and adult nutritionals meet the minimum requirements for myo-inositol, simple and fast methods to determine myo-inositol content in these food products are needed.

The existing methods for the determination of myo-inositol include enzymatic analysis,[8,9] gas chromatography (GC),[10,11] high-performance liquid chromatography (HPLC),[12–15] and anion-exchange chromatography methods.[16] However, sample derivatization of myo-inositol is required for separation by GC[10,11] and HPLC when optical detection is used.[12,13] In contrast, high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) requires no derivatization and offers high sensitivity and selectivity for the determination of myo-inositol. AOAC Official Method 2011.18 describes an HPAE-PAD method with column switching to determine

myo-inositol (free and bound as phosphatidylinositol) in infant formula and adult nutritionals.[17,18] AOAC Official Method 2012.12 describes an HPAE-PAD method coupled with microwave-assisted acid hydrolysis and enzymatic treatment for total myo-inositol determination.[19,20]

This study evaluates and describes execution of the HPAE-PAD method combined with the column-switching technique described in AOAC Official Method 2011.18.[17] For the determination of myo-inositol bound as phosphatidylinositol (hereinafter referred to as bound myo-inositol), myo-inositol is released from its bound form using acid hydrolysis prior to chromatographic analysis.

Goal

To evaluate an HPAE-PAD method for the determination of free and bound myo-inositol in infant formula and adult nutritionals

Thermo SCIENTIFIC

Equipment

- Thermo Scientific™ Dionex™ ICS-5000+ HPIC™ ion chromatography (IC) system, capable of supporting high-pressure IC, including:
  - DP Dual Pump
  - EG Eluent Generator
  - DC Detector/Chromatography Compartment
- Thermo Scientific Dionex AS-AP Autosampler with Sample Syringe, 250 µL (P/N 074306) and 1200 µL Buffer Line Assembly (P/N 074989)
- ED Electrochemical Detector (without Cell, P/N 072042)
- Electrochemical Cell (no Reference Electrode or Working Electrode, P/N 072044)
- pH, Ag/AgCl Reference Electrode (P/N 061879)
- Gold on Polytetrafluoroethylene (Au on PTFE) Disposable Electrode (P/N 066480)
- Thermo Scientific Dionex EGC 500 Potassium Hydroxide (KOH) Eluent Generator Cartridges (P/N 075778)
- Thermo Scientific Dionex CR-ATC 500 Continuously Regenerated Anion Trap Column (P/N 075550)
- Thermo Scientific Dionex ICS-3000/5000 EG Vacuum Degas Conversion Kit (P/N 063353)
- Vial Kit, 10 mL, Polystyrene with Caps and Blue Septa (P/N 074228)
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software, version 7.2

Reagents and Standards

- Deionized water (DI), Type I Reagent Grade, 18 MΩ-cm resistance or better
- Hydrochloric Acid (HCl), Fisher Scientific™ Optima™ Ultrapure Reagent, 32–35% (Fisher Scientific P/N A466)
- Acetic Acid, Glacial, Certified ACS, ≥99.7 w/w % (Fisher Scientific P/N A38S)
- Sodium Hydroxide Solution, 50% w/w (Fisher Scientific P/N SS254)
- Methanol (CH_3OH), Certified ACS, ≥99.8% (Fisher Scientific P/N A412)
- U.S. Pharmacopeia (USP) Inositol Reference Standard (Fisher Scientific P/N NC0189755)
- L-α-Phosphatidylinositol from *Glycine max* (soybean), ~50% by Thin-Layer Chromatography (TLC), (Sigma-Aldrich® P/N P6636)
- Metaphosphoric Acid, Stabilized (Fisher Scientific P/N AC21922)
- Chloroform, Ethanol as Preservative, Certified ACS, ≥99.8% (Fisher Scientific P/N C298)
- Hexane, Certified ACS, ≥98.5% (Fisher Scientific P/N H292)
- Diethyl Ether, 99+%, Stabilized with Ethanol (Fisher Scientific P/N AC41005

Consumables

- Thermo Scientific™ Nalgene™ Syringe Filters, Polyethersulfone (PES), 0.2 µm (Fisher Scientific P/N 09-740-61A)
- AirTite™ All-Plastic Norm-Ject™ Syringes, 5 mL, Sterile (Fisher Scientific P/N 14-817-28)
- Thermo Scientific™ Dionex™ OnGuard™ II RP Cartridges, 1 cc (P/N 057083)
- Thermo Scientific™ HyperSep™ Silica Solid-Phase Extraction (SPE) Cartridge, 500 mg Bed Weight, 3 mL Column Volume (Thermo Scientific P/N 60108-315)
- Fisher Scientific™ Fisherbrand™ Easy Reader™ Centrifuge Tubes, Sterile, Polypropylene, 15 mL Flat Cap (Fisher Scientific P/N 07-200-886)
- LC Pump Priming Syringe, 5 mL, Glass Syringe, Fixed Luer Lock (SGE Analytical Science P/N 008762)
- Stainless Steel 304 Syringe Needle, Noncoring Point, Gauge 16, L 6 in. (Sigma-Aldrich P/N Z108782)
- Fisherbrand Magnetic Micro Stirring Bars, 10 × 3 mm (Fisher Scientific P/N 14-513-65)
- Nalgene Narrow-Mouth Bottles, HDPE, 125 mL (Fisher Scientific P/N 03-313-87A)

Samples

- Standard Reference Material (SRM) 1849, National Institute of Standards and Technology (NIST), discontinued
- Milk-based infant formula (from a local store)
- Soy-based infant formula (from a local store)
- Adult nutritional liquid (from a local store)

Conditions (Applicable to Figures 2–7)

Dimension 1	
Column:	Thermo Scientific™ Dionex™ CarboPac™ PA1 Guard, 4 × 50 mm (P/N 043096)
Eluent:	750 mM Sodium Hydroxide (NaOH)
Flow Rate:	0.4 mL/min
Injection Volume:	20 µL
System Backpressure:	800–900 psi

Dimension 2	
Column:	Dionex CarboPac MA1 Guard, 4 × 50 mm (P/N 044067) Dionex CarboPac MA1 Analytical, 4 × 250 mm (P/N 044066)
Eluent:	15 mM KOH
Eluent Source:	Dionex EGC 500 KOH Cartridge with Dionex CR-ATC 500 Trap Column
Flow Rate:	0.4 mL/min
Injection Volume:	20 µL
Temperature:	30 °C
Detection:	PAD, Au on PTFE Disposable Working Electrode
System Backpressure:	2800–2900 psi
Background Conductance:	28–41 nC
Noise:	~16 pC/min peak-to-peak
Run Time:	25 min

Waveform for the ED:

Time (s)	Potential (V)	Last Step*	Ramp*	Gain Region*	Integration
0.00	0.1	Off	On	Off	Off
0.20	0.1	Off	On	On	On
0.40	0.1	Off	On	Off	Off
0.41	–2	Off	On	Off	Off
0.42	–2	Off	On	Off	Off
0.43	0.6	Off	On	Off	Off
0.44	–0.1	Off	On	Off	Off
0.50	–0.1	On	On	Off	Off

* Setting required on Dionex ICS-3000/5000/5000+ systems but not used in older Dionex IC systems; reference electrode in AgCl mode

Preparation of Solutions and Reagents

750 mM NaOH

Weigh 120 g of 50% NaOH into a 2 L polypropylene volumetric flask containing ~1.9 L degassed DI water. Dilute to volume with degassed DI water and mix well. Transfer to a 2 L plastic eluent bottle pressurized with helium or nitrogen to prevent intrusion of carbon dioxide from the air.

1000 mg/L Myo-Inositol Stock Solution

Accurately weigh ~0.100 g myo-inositol and quantitatively transfer to a 50 mL volumetric flask. Dilute to volume with DI water and mix well. Store at 4 °C for up to 3 months.

100 mg/L Myo-Inositol Secondary Stock Solution

Transfer 5 mL of 1000 mg/L myo-inositol stock solution to a 50 mL volumetric flask. Dilute to volume with DI water and mix well. Prepare this solution daily.

Myo-Inositol Working Standard Solutions

Dilute 100 mg/L myo-inositol secondary stock solution with the appropriate amount of DI water to prepare 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, and 4 mg/L standards.

0.5% HCl Solution

In a well-ventilated hood, add 1.25 mL concentrated HCl to ~200 mL DI water in a 250 mL volumetric flask. Dilute to volume with DI water and mix well.

6% Metaphosphoric Acid Solution

Weigh 6.0 g metaphosphoric acid into a 100 mL volumetric flask. Dissolve and dilute to volume with DI water. Mix well. Store at 4 °C.

1 N Sodium Chloride

Weigh 5.8 g sodium chloride into a 100 mL volumetric flask. Dissolve and dilute to 100 mL with DI water.

Chloroform:Methanol (2:1)

In a well-ventilated hood, measure 60 mL chloroform and 30 mL CH_3OH separately in a glass graduated cylinder, then mix well in a 250 mL glass bottle.

Hexane:Diethyl Ether (80:20)

In a well-ventilated hood, measure 80 mL hexane and 20 mL diethyl ether separately in a glass graduated cylinder, then mix well in a 250 mL glass bottle.

Hexane:Diethyl Ether (50:50)

In a well-ventilated hood, measure 50 mL hexane and 50 mL diethyl ether separately in a glass graduated cylinder, then mix well in a 250 mL glass bottle.

Methanol:Chloroform:Water (75:15:10)

In a well-ventilated hood, measure 75 mL CH_3OH, 15 mL chloroform and 10 mL DI water separately in a glass graduated cylinder, then mix well in a 250 mL glass bottle.

Sample Preparation

Dionex OnGuard II RP Cartridge Preparation

Pass 5 mL CH_3OH followed by 10 mL DI water through the 1 cc cartridge at a maximum flow rate of 4 mL/min. To increase throughput, up to 12 cartridges can be prepared simultaneously using the Dionex OnGuard Sample Prep Workstation (P/N 039599).

Sample Preparation for Free Myo-Inositol Determination

For the powdered products (i.e., SRM 1849 and milk- and soy-based infant formulas), accurately weigh 2.5 g (±10%) of each powder in separate 20 mL polypropylene bottles, then add 20 mL DI water to dissolve the samples. Transfer 1 mL of reconstituted liquid to a 50 mL beaker.

For liquid products (i.e., the adult nutritional liquid), shake well before use, then transfer 1 mL of the product to a 50 mL beaker. Add 30 mL of DI water to the beaker containing the reconstituted liquid or liquid products.

Add the appropriate amount (~0.6–2 mL, depends on the sample type) of 0.5% HCl to each sample to adjust the pH to 4.5 ± 0.2. Quantitatively transfer the sample to a 50 mL volumetric flask, dilute to volume with DI water, and mix well. Apply ~6 mL of the solution to a 1 cc Dionex OnGuard II RP cartridge, discarding the first 3 mL of the effluent. Then filter the remaining cartridge effluent through a 0.2 µm PES syringe filter prior to injection.

Sample Preparation for Phosphatidylinositol Determination

Method 1: Cleanup using the HyperSep Silica SPE Column

Extraction

For powdered products (i.e., SRM 1849 and milk- and soy-based infant formulas), accurately weigh 2.5 g (± 10%) of each powder in separate 125 mL polyethylene bottles, then add 20 mL DI water to dissolve the samples. Transfer 1 mL of the reconstituted liquid to a 15 mL centrifuge tube.

For the adult nutritional liquid, transfer 1 mL of the product to a 15 mL centrifuge tube. Add 2.5 mL CH_3OH and a stir bar to each centrifuge tube and stir for at least 20 min. Add 5 mL chloroform and stir for at least 5 min. Remove the stir bar with a stir bar retriever. Add 1.25 mL of 6% metaphosphoric acid and 0.25 mL of 1 N NaCl, then mix well. Centrifuge at 6200 rpm at 2 °C for 15 min.

After centrifugation, in a well-ventilated hood, carefully insert a long wide-bore flat-end stainless steel needle (i.e., with a noncoring point where the sharp end has been removed by machine-cut) attached to a 5 mL glass gas-tight syringe into the chloroform layer near the bottom of the centrifuge tube. Be careful not to disturb the upper aqueous CH_3OH layer and protein layer (In this study, when the protein was not on the walls of the tube, it was contained in a thin layer between the chloroform and methanol phases.). Slowly draw out as much of the chloroform layer as possible. Transfer the chloroform-containing phosphatidylinositol to a clean 15 mL centrifuge tube. Place the centrifuge tube in a 60 °C water bath and evaporate the chloroform with nitrogen in the solvent evaporator.

Sample cleanup

Due to the use of hazardous volatile elution solvents, this step must be conducted in a well-ventilated hood. Configure a sample preparation station in the hood. Condition a 500 mg silica SPE cartridge with 3 mL hexane. Add 0.5 mL chloroform:methanol (2:1) to dissolve the residue in the bottom of centrifuge tube left from the solvent evaporation. Quantitatively transfer the dissolved residue to the conditioned silica SPE cartridge, then rinse the SPE cartridge with 1.5 mL hexane:diethyl ether (80:20) and discard the eluent. Rinse the SPE cartridge with 1.5 mL hexane:diethyl ether (50:50), 2 mL methanol, and 2 mL methanol:chloroform:water (75:15:10); collect all effluents in a single 15 mL centrifuge tube. Evaporate the collected effluents with nitrogen in a 60 °C water bath in the solvent evaporator.

Hydrolysis

Add 20 µL concentrated acetic acid and 1 mL concentrated HCl to the residue left in the centrifuge tube from the sample cleanup step and tightly cap the tube. Heat the tube in a 110 °C oven for 2 h. Cool to room temperature. Dilute the sample with ~10 mL DI water. Slowly add 0.625 mL 50% (w/w) sodium hydroxide. Transfer the sample to a 25 mL volumetric flask and dilute to volume with DI water. Filter an aliquot of sample through a 0.2 µm PES syringe filter prior to injection.

Precautions: During the first solvent evaporation, it is important to evaporate as much of the chloroform as possible so that the composition of the solvent used to dissolve the residue for the SPE cleanup will not be changed significantly. This will maximize the amount of phosphatidylinositol retained by the SPE column during the loading. To ensure the quantitative transfer of residue from the centrifuge tube to the SPE column, use the elution solvents to rinse the tube, then transfer the elution solvents to the SPE column.

Method 2: Cleanup using the Dionex OnGuard II RP Cartridge

Extraction

Use the same extraction procedure described in Method 1.

Hydrolysis

Use the same hydrolysis procedure described in Method 1, except do not filter through the syringe filter after the sample dilution.

Sample cleanup

Apply ~6 mL of the sample solution to a 1 cc Dionex OnGuard II RP cartridge, discarding the first 3 mL of the effluent. Then filter the remaining effluent through a 0.2 µm PES syringe filter prior to injection.

Sample Preparation for Recovery Study

Free myo-inositol

For the powdered infant formula products, spike known volumes from the 1000 mg/L myo-inositol stock that are equivalent to 50, 100, and 150% of the native amount in the 2.5 g powder before reconstitution. For the adult nutritional liquid, spike known volumes from the 1000 mg/L myo-inositol stock that are equivalent to 50, 100, and 150% of the native amount in the 1 g of liquid in the centrifuge tube.

Bound myo-inositol

The phosphatidylinositol from *Glycine max* was used as the spiking material in this study, but this reagent only has an estimated purity of ~50% by TLC from the supplier. Therefore, the material was analyzed using acid hydrolysis to determine an actual purity of myo-inositol.

To prepare 1000 mg/L of phosphatidylinositol solution, accurately weigh 50 mg of phosphatidylinositol, transfer it to a 50 mL volumetric flask, dissolve, and dilute to volume with chloroform. Transfer 0.1 and 0.2 mL of the 1000 mg/L phosphatidylinositol into two separate centrifuge tubes. Add 20 µL concentrated acetic acid and 2 mL concentrated HCl to each tube. Place the tubes in a 110 °C oven for 2 h. Cool to room temperature. Quantitatively transfer the hydrolysate to a 50 mL volumetric flask and dilute to volume with DI water. Apply ~6 mL of the solution first through a 1 cc Dionex OnGuard II RP cartridge, discarding the first 3 mL of the effluent. Then filter the remaining effluent through a 0.2 µm PES syringe filter prior to injection.

The Certificate of Analysis of phosphatidylinositol from the supplier indicates two fatty acid groups (18:0) in the structure of this molecule (MW 867.14). The purity of the phosphatidylinositol material was calculated based on the amount of myo-inositol measured against the amount of myo-inositol calculated from the MW.

In this study, the purity of the phosphatidylinositol spiking material was 18.5%. To prepare 1000 mg/L of myo-inositol (phosphatidylinositol solution), accurately weigh 0.260 g of phosphatidylinositol dissolved in 10 mL of chloroform. For SRM 1849, spike known volumes of 100 mg/L of myo-inositol (phosphatidylinositol solution) equivalent to ~50, 100, and 150% of the native level in 2.5 g powder before reconstitution. For powdered milk- and soy-based infant formula samples, spike known amounts of 1000 mg/L of myo-inositol (phosphatidylinositol solution) at ~50, 100, and 150% of the native level to 2.5 g powder before reconstitution. For the adult nutritional liquid, spike known amounts of 1000 mg/L of myo-inositol (phosphatidylinositol solution) at ~50, 100, and 150% of the native level to 1 g liquid in the centrifuge tube, then proceed to the remaining sample preparation steps to determine the bound myo-inositol.

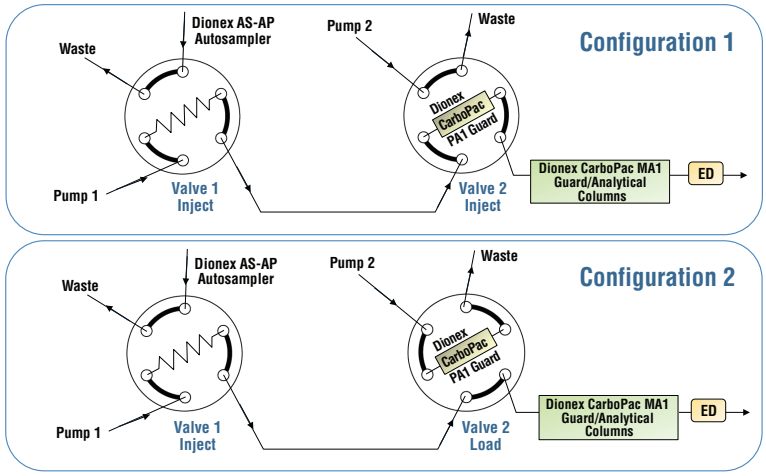
System Preparation and Configuration

Install and configure the Dionex AS-AP Autosampler in Push Full mode. Follow the instructions in the Dionex AS-AP Autosampler Operator's Manual (Document No. 065361-07) to calibrate the sample transfer line to ensure accurate and precise sample injections.

Condition the Dionex EGC 500 KOH cartridge before first use by running 50 mM KOH at 1 mL/min for 45 min. For more information on installation and operation of the Dionex EGC 500 KOH cartridge, consult the product manual (Document No. 065018-04). To obtain optimal cartridge performance, install 0.003 in. (i.d.) PEEK backpressure tubing between the outlet of the high-pressure degas tubing assembly and the inlet of the sample injector to achieve a total system backpressure greater than 2000 psi.

Install the vacuum degas conversion kit in the EG module to allow sufficient removal of the hydrogen gas formed with KOH eluent generated by the EG to help maintain a stable baseline. Refer to the Dionex ICS-3000 EG Vacuum Degas Conversion Kit Installation Instructions (Document No. 065067) for more information. Note that the eluent degasser in the Dionex ICS-5000⁺ HPIC IC system has one Vent in place of the Regen In and Regen Out of the Dionex ICS-3000 system. Therefore, connect the tube from the Degasser Port on the bottle cap to the Vent on the eluent degasser (refer to Figures 8 and 9 in Document No. 065067). Ensure that all connections and fittings for the vacuum degasser are vacuum tight. If there is a leak in the vacuum system, the baseline will be unstable.

Each Dionex CarboPac column is shipped in a specific solution and requires a unique startup procedure before use. Refer to the column manual (Document No. 031824) for the startup procedure for these columns. After finishing the column startup, configure the injection valve plumbing for the column switching in the lower compartment of the DC (Figure 1). Use the 6-port Valve 1 (on the left) for sample loading from the autosampler. Install the Dionex CarboPac PA1 Guard and MA1 Guard and Analytical columns on the 6-port Valve 2 (on the right). After connecting the column inlet, connect the Dionex CarboPac MA1 column outlet to the ED cell. Keep the lengths of the connecting tubing to a minimum.



Valve Switching Configuration Time Table	
Time (min)	**Configuration**
0.00	1
1.50	2
11.2	1

Figure 1. Valve-switching configurations with time table.

The legend in Figure 1 shows the time table for valve switching. Valve 1 is controlled by the AS-AP autosampler, while Valve 2 is controlled by the DC. To set the commands for valve switching in the Instrumental Method, go to the Script Editor page and click on Stop Run Stage. Then click on Insert Time on the Home ribbon and type *1.500* in the inserted row under the Time column. Click on Stop Run Stage again, then on Insert Command on the Home ribbon, and type *DC.InjectValve_2.LoadPosition* in the inserted row under the Command column. Click on Stop Run Stage again, then on Insert Time on the Home ribbon, and type *11.200* in the inserted row under the Time column. Finally, click on Stop Run Stage again, then on Insert Command on the Home ribbon, and type *DC.InjectValve_2.InjectPosition* in the inserted row under the Command column.

After configuring the system, set the flow rate of Pump 1 at 0.4 mL/min to deliver DI water through the Dionex EGC 500 KOH cartridge and set the KOH concentration at 15 mM. Set the flow rate of Pump 2 at 0.4 mL/min to deliver 750 mM KOH through Dimension 2. Allow the system to equilibrate for at least 45 min before injecting.

Results and Discussion

Separation and Detection

The samples were first injected onto a Dionex CarboPac PA1 guard column using 15 mM KOH, which resolves myo-inositol from other late-eluting carbohydrates. At 1.5 min after the injection, the 6-port Valve 2 was switched to the Load position and Pump 2 delivered 750 mM NaOH through the Dionex CarboPac PA1 guard column to elute the strongly retained carbohydrates from the guard column to waste. Meanwhile, myo-inositol was further separated on a Dionex CarboPac MA1 column. The high-purity KOH eluent used for separation on the Dionex CarboPac MA1 column was generated by the Dionex EGC 500 cartridge, which eliminates the need for manual preparation of KOH eluent and ensures excellent run-to-run reproducibility. Glycerol and myo-inositol were both present in the infant formula and adult nutritional liquid samples treated for the determination of bound myo-inositol. Using 30 mM KOH eluent as described in AOAC Official Method 2011.18, glycerol and myo-inositol were eluted at 10.80 min and 11.82 min, respectively.

For samples containing high concentrations of glycerol, the resolution was compromised. To achieve a baseline separation between myo-inositol and glycerol, the concentration of the eluent was optimized to 15 mM KOH; the retention times of glycerol and myo-inositol were then 10.85 min and 12.09 min, respectively. The resolution varied with the concentrations of these two analytes in different samples. For a sample containing a relatively high concentration of glycerol, the resolution improved from 1.34 to 1.49 after the optimization. Samples treated with a Dionex OnGuard II RP cartridge tended to have greater amounts of glycerol and myo-inositol than those treated with the SPE cartridge. A chromatogram of a 2 mg/L myo-inositol standard is shown in Figure 2.

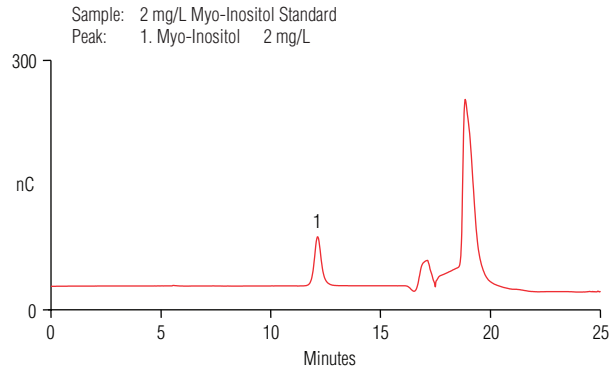


Figure 2. The 2 mg/L myo-inositol standard.

A Au on PTFE disposable electrode that offers the convenience of simple installation and no electrode polishing was used for the detection of myo-inositol. Under the recommended experimental conditions, one disposable electrode can last for ~4 weeks of continuous use. The background response will gradually increase as the electrode ages.

Calibration, Limit of Detection (LOD), and Limit of Quantitation (LOQ)

A calibration curve with eight concentration levels ranging from 0.02 to 4 mg/L was constructed for myo-inositol. The calibration curve of peak area versus concentration was fit using linear least squares regression analysis that yielded a coefficient of determination (r^2) greater than 0.999. To determine the LOD and LOQ, the baseline noise was first determined by measuring the peak-to-peak noise in the 14–15 min segment of the baseline where no peaks elute but close to the peak of interest. The LOD and LOQ were then calculated from the average peak height of seven injections of 0.02 mg/L myo-inositol. The results of the calibration, LOD, and LOQ are summarized in Table 1.

Table 1. Calibration, LOD, and LOQ for myo-inositol.

Analyte	Range (mg/L)	Coefficient of Determination (r^2)[a]	LOD[b] (µg/L)	LOQ[c] (µg/L)
Myo-Inositol	0.02–4	0.9996	1.6	5.4

[a] Linear fit
[b] LOD = 3× signal-to-noise ratio (S/N)
[c] LOQ = 10× S/N

Sample Analysis

Free myo-inositol

Figure 3 shows typical chromatograms of free myo-inositol in infant formula and adult nutritional samples. The NIST SRM 1849 contains 40.3 mg/100 g free myo-inositol, which is within range of the certified value (39.8 ± 2.6 mg/100 g). The milk- and soy-based infant formulas contain relatively similar amounts of free myo-inositol, 35.8 mg/100 g (7.16 mg/100 kcal) and 34.7 mg/100 g (6.94 mg/100 kcal), which are 119 and 116% of the labeled inositol values on the infant formula packages (6 mg/100 Cal), respectively. Compared to the infant formula samples, the adult nutritional liquid sample contains a significantly lower concentration of free myo-inositol. The results of these samples are summarized in Table 2.

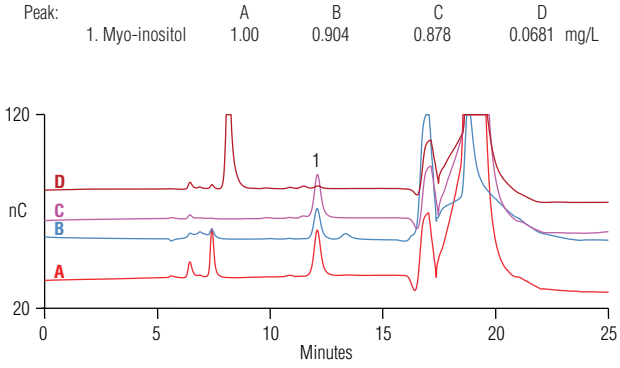


Figure 3. Free myo-inositol in (A) SRM 1849, (B) milk-based powdered infant formula, (C) soy-based powdered infant formula, and (D) adult nutritional liquid. A 15% signal offset has been applied.

Table 2. Summary of myo-inositol (free and bound as phosphatidylinositol) in triplicate infant formula and adult nutritional samples.

Sample	Analyte/Sample Preparation	Amount (mg/100g[a])					Average Amount (mg/100 kcal)
		Replicate 1	Replicate 2	Replicate 3	Average	RSD[b]	
SRM 1849[c]	Free Myo-Inositol/None	40.0	40.6	40.2	40.3	0.73	8.05
	Myo-Inositol Bound as Phosphatidylinositol/SPE Cartridge	0.832	0.867	0.820	0.840	2.84	0.168
	Myo-Inositol Bound as Phosphatidylinositol/RP Cartridge	1.15	1.09	1.17	1.14	3.68	0.227
Milk-Based Powdered Infant Formula[d]	Free Myo-Inositol/None	35.7	35.6	36.2	35.8	1.00	7.16
	Myo-Inositol Bound as Phosphatidylinositol/SPE Cartridge	5.22	5.32	5.32	5.29	1.07	1.06
	Myo-Inositol Bound as Phosphatidylinositol/RP Cartridge	6.06	6.00	5.96	6.01	0.80	1.20
Soy-Based Powdered Infant Formula[d]	Free Myo-Inositol/None	34.7	34.9	34.4	34.7	0.79	6.94
	Myo-Inositol Bound as Phosphatidylinositol/SPE Cartridge	8.48	8.34	8.32	8.38	0.97	1.68
	Myo-Inositol Bound as Phosphatidylinositol/RP Cartridge	10.7	10.6	10.8	10.7	0.80	2.14
Adult Nutritional Liquid	Free Myo-Inositol/None	0.337	0.344	0.332	0.338	1.93	0.0675
	Myo-Inositol Bound as Phosphatidylinositol/SPE Cartridge	2.54	2.66	2.74	2.65	3.82	0.530
	Myo-Inositol Bound as Phosphatidylinositol/RP Cartridge	2.60	2.62	2.68	2.63	1.50	0.527

[a] mg/100 g expressed as myo-inositol in original product
[b] n = 3
[c] NIST-certified value = 398 ± 26 mg/kg
[d] The labeled inositol value for the infant formula package is 6 mg/100 cal. The label states that one scoop of the infant formula powder (8.8 g) provides 44 cal. Therefore, the measured myo-inositol amount in mg/100 g is converted to mg/100 kcal (1 cal = 1 kcal).

Bound myo-inositol

Two sample preparation methods were examined in this study. Method 1 follows AOAC Official Method 2011.18 that contains three steps: extraction of phosphatidylinositol from the sample using chloroform, isolation of phosphatidylinositol from other fats in the sample using a silica SPE column, and hydrolysis of phosphatidylinositol to free myo-inositol using concentrated acid.[17] During SPE cleanup (i.e., the second step), phosphatidylinositol is initially retained on the column while the other fats are removed from the column by nonpolar eluents. As the polarity of the eluents increase, phosphatidylinositol is eluted from the column and collected.

Method 2 uses the same extraction step but proceeds to acid hydrolysis after the extraction without the SPE cleanup step. After myo-inositol is released from phosphatidylinositol, the hydrolysate is passed through a Dionex OnGuard II RP cartridge to remove the hydrophobic substances, including the fats from the sample extract. The concentrations of bound myo-inositol found in the samples using the two methods are summarized in Table 2.

The concentrations determined by Method 1 are ~73–88% of that determined by Method 2. However, both methods generated the same results for the adult nutritional liquid sample. The possible reason for the lower amount determined using Method 1 may be due to loss of phosphatidylinositol during SPE cleanup. Because phosphatidylinositol has hydrophilic and hydrophobic structural characteristics, it is not strongly bound to the silica phase. During the cleanup, a certain amount of phosphatidylinositol can be eluted off the column with other fats. Comparably, the treatment using the Dionex OnGuard II RP cartridge removes the fats after the myo-inositol has been released, thus minimizing loss of the analyte. Method 2 also offers the advantage of shorter sample preparation time by eliminating the solvent evaporation.

Glycerol was found in all the samples prepared for the determination of bound myo-inositol. Glycerol was well resolved from myo-inositol at 10.9 min (see Figure 4, Chromatogram A). Figures 4–7 demonstrate the release of free myo-inositol from the bound form using the two sample preparation methods applied to each sample and spiked sample studied here.

In the milk- and soy-based infant formulas, bound myo-inositol is a small percentage of the total myo-inositol (the sum of free myo-inositol and phosphatidylinositol, as defined in AOAC Standard Method Performance Requirements 2011.007).[21] In contrast, the amount of bound myo-inositol is ~8× the concentration of the free form in the adult nutritional liquid sample.

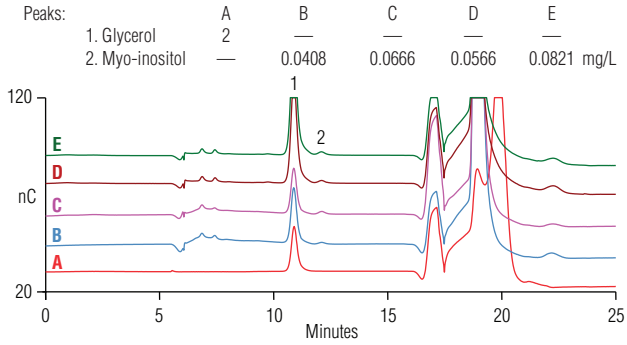


Figure 4. Myo-inositol from phosphatidylinositol in (A) 2 mg/L glycerol, (B) SRM 1849, and (C) SRM 1849 with 100% spike prepared using Method 1; (D) SRM 1849 and (E) SRM 1849 with 50% spike prepared using Method 2. A 15% signal offset has been applied.

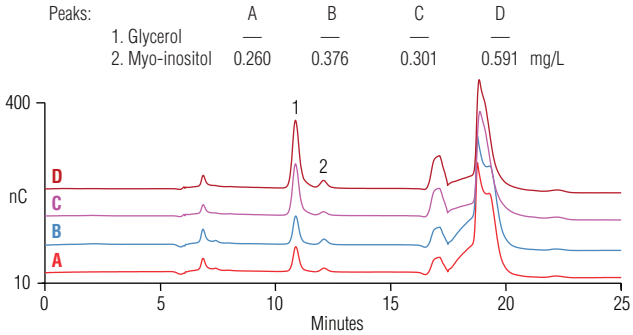


Figure 5. Myo-inositol from phosphatidylinositol in (A) milk-based powdered infant formula and (B) milk-based powdered infant formula with 50% spike prepared using Method 1; (C) milk-based powdered infant formula and (D) milk-based powdered infant formula with 100% spike prepared using Method 2. A 15% signal offset has been applied.

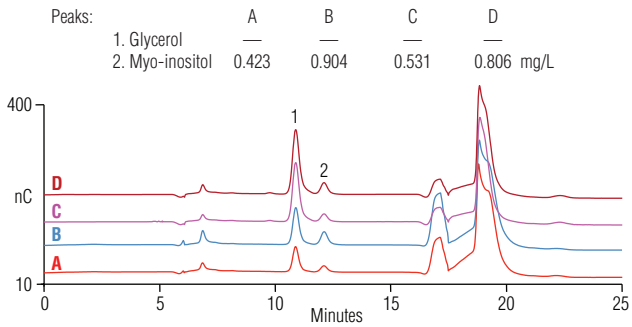


Figure 6. Myo-inositol from phosphatidylinositol in (A) soy-based powdered infant formula and (B) soy-based powdered infant formula with 150% spike prepared using Method 1; (C) soy-based powdered infant formula and (D) soy-based powdered infant formula with 50% spike prepared using Method 2. A 15% signal offset has been applied.

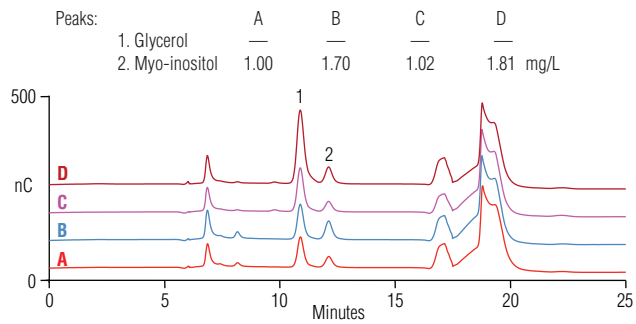


Figure 7. Myo-inositol from phosphatidylinositol in (A) adult nutritional liquid and (B) adult nutritional liquid with 100% spike prepared using Method 1; (C) adult nutritional liquid and (D) adult nutritional liquid with 100% spike prepared using Method 2. A 15% signal offset has been applied.

Sample Accuracy and Precision

The accuracy of the methods was verified by determining the recoveries of free and bound myo-inositol in spiked SRM 1849, milk- and soy-based infant formulas, and adult nutritional liquid samples. For free myo-inositol, USP inositol reference standard was used to prepare the spiking solutions. For bound myo-inositol, the phosphatidylinositol spiking material was analyzed to determine its actual purity. Two different concentrations were prepared from the spiking material and each was analyzed in triplicate. The purity was 18.5% with a RSD (n = 6) of 4.85. Therefore, the phosphatidylinositol spiking solutions were prepared based on this purity. The myo-inositol and phosphatidylinositol reference materials were spiked at 50, 100, and 150% of the native amount of myo-inositol in the samples.

The recoveries of free myo-inositol were in the range of 92.6–109% (Table 3). The recoveries of bound myo-inositol were in the range of 63.0–81.4% using Method 1 and in the range of 80.5–106% using Method 2 (Table 3). The lower recoveries using Method 1 may be due to the loss of phosphatidylinositol during SPE cleanup, as discussed in the Sample Analysis section. Low recoveries of phosphatidylinositol in food samples using a similar SPE cleanup sample preparation were reported in other studies.[22,23]

Table 3. Recoveries of free myo-inositol and myo-inositol from phosphatidylinositol in selected infant formula and adult nutritional samples.

Sample	Free Myo-Inositol				Myo-Inositol from Phosphatidylinositol							
					HyperSep Silica SPE Treatment				Dionex OnGuard II RP Treatment			
	Amount Added (mg/L)	Total Found (mg/L)	Peak Area RSD (n = 3)	Recovery (%)	Amount Added (mg/L)	Total Found (mg/L)	Peak Area RSD (n = 3)	Recovery (%)	Amount Added (mg/L)	Total Found (mg/L)	Peak Area RSD (n = 3)	Recovery (%)
SRM 1849	—	1.00	0.20	—	—	0.0411	0.86	—	—	0.0557	0.29	—
SRM 1849 with 50% Spike	0.460	1.44	0.59	94.6	0.0200	0.0537	2.73	63.0	0.0280	0.0814	0.93	91.8
SRM 1849 with 100% Spike	0.980	1.90	0.61	92.1	0.0400	0.0671	2.95	65.0	0.0560	0.112	0.33	101
SRM 1849 with 150% Spike	1.26	2.18	0.90	93.6	0.0600	0.0839	4.35	71.3	0.0840	0.134	1.76	93.7
Milk-Based Powdered Infant Formula	—	0.923	0.43	—	—	0.261	0.89	—	—	0.301	0.35	—
Milk-Based Powdered Infant Formula with 50% Spike	0.419	1.38	0.49	109	0.140	0.375	0.42	81.4	0.150	0.447	1.48	97.2
Milk-Based Powdered Infant Formula with 100% Spike	0.838	1.74	1.07	97.7	0.280	0.452	0.83	68.3	0.300	0.588	1.26	95.7
Milk-Based Powdered Infant Formula with 150% Spike	1.26	2.18	0.23	99.8	0.420	0.565	0.26	72.4	0.450	0.719	2.61	93.0
Soy-Based Powdered Infant Formula	—	0.860	0.14	—	—	0.424	0.32	—	—	0.534	1.32	—
Soy-Based Powdered Infant Formula with 50% Spike	0.434	1.27	0.61	93.6	0.212	0.574	0.28	70.7	0.260	0.810	0.42	106
Soy-Based Powdered Infant Formula with 100% Spike	0.868	1.70	1.53	97.4	0.424	0.709	0.24	67.2	0.520	1.00	0.35	90.8
Soy-Based Powdered Infant Formula with 150% Spike	1.30	2.11	2.75	95.8	0.636	0.902	0.5	75.2	0.780	1.35	0.8	104
Adult Nutritional Liquid	—	0.0689	1.22	—	—	1.03	0.54	—	—	1.01	0.59	—
Adult Nutritional Liquid with 50% Spike	0.0342	0.102	1.58	95.8	0.505	1.40	0.57	73.1	0.500	1.46	0.67	89.9
Adult Nutritional Liquid with 100% Spike	0.0683	0.134	2.14	94.7	1.01	1.69	0.33	65. 4	1.00	1.82	0.36	80.5
Adult Nutritional Liquid with 150% Spike	0.102	0.164	1.99	92.6	1.52	2.05	0.97	67.3	1.50	2.32	0.31	87.4

Table 4. Precision of myo-inositol determination in infant formula and adult nutritional samples over three days.

Sample	Analyte/Sample Preparation	Results (mg/100g)				RSD
		Day 1	Day 2	Day 3	Average	
SRM 1849	Free Myo-Inositol/None	40.4	40.2	39.8	40.1	0.68
	Myo-Inositol Bound as Phosphatidylinositol/SPE Cartridge	0.832	0.808	0.822	0.821	1.46
	Myo-Inositol Bound as Phosphatidylinositol/RP Cartridge	1.16	1.15	1.11	1.14	2.35
Milk-Based Powdered Infant Formula	Free Myo-Inositol/None	36.7	35.7	35.7	36.0	1.62
	Myo-Inositol Bound as Phosphatidylinositol/SPE Cartridge	5.22	5.32	5.25	5.26	1.04
	Myo-Inositol Bound as Phosphatidylinositol/RP Cartridge	6.04	6.02	6.00	6.02	0.30
Soy-Based Powdered Infant Formula	Myo-Inositol Bound as Phosphatidylinositol/SPE Cartridge	8.97	8.34	8.47	8.59	3.88
	Myo-Inositol Bound as Phosphatidylinositol/RP Cartridge	12.0	11.7	10.7	11.5	6.24
Adult Nutritional Liquid	Myo-Inositol Bound as Phosphatidylinositol/SPE Cartridge	2.58	2.54	2.50	2.54	1.47
	Myo-Inositol Bound as Phosphatidylinositol/RP Cartridge	2.53	2.62	2.76	2.64	4.51

Method precision was evaluated with both intraday and between-day analysis. Table 2 shows the results of three replicates analyzed on the same day. The RSDs of myo-inositol content found in the selected samples are in the range of 0.729–3.82%. Table 4 shows the results obtained on three days. The RSDs of the between-day analysis of the selected samples are in the range of 0.300–6.24%.

Conclusion

This study describes an HPAE-PAD method (AOAC Official Method 2011.18) to determine free and bound myo-inositol in infant formula and adult nutritional liquid samples. A column-switching technique is used to effectively remove the strongly retained carbohydrates in the sample matrix, thereby reducing run time. PAD with a Au on PTFE disposable electrode offers high sensitivity while eliminating the need for sample derivatization and electrode polishing.

For the determination of bound myo-inositol, this study compares the results obtained using a silica SPE column as described in AOAC Official Method 2011.18 versus using a Dionex OnGuard II RP cartridge. The results demonstrate that the Dionex OnGuard II RP cartridge produced higher content and provided improved recoveries of bound myo-inositol in the samples. The Dionex OnGuard II RP cartridge also reduces the time-consuming process and hazardous chemicals associated with use of the silica SPE column. The analysis of NIST SRM 1849 produced results within the specified certified value range, thus confirming accurate results for determination of free myo-inositol. Therefore, this work validates AOAC Official Method 2011.18 and offers possible improvements with modifications to the sample preparation and separation conditions for samples containing high concentration of glycerol relative to myo-inositol.

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