Determination of Choline in Infant Formula and Other Food Samples by IC

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Key Words

Egg Powder, Soy Flour, Dionex IonPac CS19 Column, Suppressed Conductivity

Introduction

Choline is a water-soluble micronutrient vital to cell membrane integrity, support of methyl group metabolism, and nervous system activity.[1] It is present as free choline in small quantities in a wide variety of foods and frequently found in its esterified forms.[2] Choline can also be found in fortified foods and dietary supplements; e.g., choline is a required additive in many infant formulas. It is therefore important to determine the choline content of common foods.

AOAC Official Method 999.14 describes the determination of choline in infant formula and milk using an enzymatic colorimetric method.[3,4] Dionex (now part of Thermo Scientific) Application Note (AN) 124 demonstrates the determination of choline in infant formula by ion chromatography (IC).[5]

This work presents an improved method using a slightly modified sample preparation procedure to increase sample throughput. This approach also replaces the 4 mm Thermo Scientific Dionex IonPac CS12A Analytical and Guard Column set with the 2 mm Dionex IonPac™ CS19 Analytical and Guard Columns, thereby achieving higher peak efficiency for choline, shorter analysis time for the measurement of choline in infant formula, and reduced eluent consumption/waste generation. Eluent generation enhances automation and operation of the IC system.

This method was also successfully applied to defatted soy flour and whole egg powder samples provided by the National Institute of Standards and Technology (NIST) as part of a multilaboratory study in the Dietary Supplement Quality Assurance Program (DSQAP) exercise. This new approach provides a sensitive, selective, and reproducible alternative to AOAC Method 999.14 for choline determination in food samples.

Goal

To develop an improved IC method for determination of choline in infant formula and other food samples (e.g., egg powder and soy flour) using a Reagent-Free™ IC (RFIC™) system with suppressed conductivity detection

Equipment

- Thermo Scientific Dionex ICS-5000 system,* including:
    - DP Dual Pump
    - EG Eluent Generator
    - DC Detector/Chromatography Compartment
    - AS-AP Autosampler
- Thermo Scientific Dionex EGC III MSA Cartridge (P/N 074535)
- Thermo Scientific Dionex CR-CTC II Continuously-Regenerated Cation Trap Column II (P/N 066262)
- Vial kit, Polystyrene with Caps and Blue Septa, 10 mL (P/N 074228)
- Thermo Scientific Dionex Chromeleon Chromatography Data System (CDS) software version 7.1

* This application can also be performed using any Dionex ICS system capable of eluent generation or any Dionex ICS system with manually prepared eluent.

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Reagents and Standards

- Deionized water (DI), Type I reagent grade, 18 MΩ-cm resistance or better
- Hydrochloric acid, Optima (Fisher Scientific P/N A466)
- Choline bitartrate (Sigma-Aldrich® P/N C1629)
- Tris(hydroxymethyl)aminomethane (Tris), Ultra Pure (MPBiomedicals P/N 819620)
- Phospholipase D from Arachis hypogaea (peanut), Type II (Sigma-Aldrich P/N P0515)
- Thermo Scientific Nalgene polyethersulfone (PES) syringe filters, 5 mL, Luer Slip (centric), sterile (Fisher Scientific P/N 14-817-28)
- Fisherbrand™ Easy Reader™ Plastic Centrifuge Tubes, polypropyene, 15 mL (Fisher Scientific P/N 07-200-886)
- SuperClear Centrifuge Tubes, polypropylene, 50 mL (VWR P/N 21008-177)

Samples

Enfamil® PREMIUM® Infant Formula

Defatted soy flour and whole egg powder (provided by NIST)

Conditions

Columns:	Dionex IonPac CS19 Analytical, 2 × 250 mm (P/N 076028) Dionex IonPac CG19 Guard, 2 × 50 mm (P/N 076029)
Eluent:	Methanesulfonic Acid (MSA), 6.4 mM
Eluent Source:	Dionex EGC III MSA Cartridge with CR-CTC II Trap Column
Flow Rate:	0.25 mL/min
Inj. Volume:	5 µL
Sample Tray Temperature:	10 °C
Detection:	Suppressed Conductivity, Thermo Scientific Dionex CSRS 300 Cation Self-Regenerating Suppressor, 2 mm
System Backpressure:	~2350 psi
Background Conductance:	~0.100 µS
Noise:	~0.1 nS/min peak-to-peak

Preparation of Solutions and Reagents

Eluent Solution

Generate 6.4 mM of MSA eluent by pumping DI water through the Dionex EGC III MSA cartridge. Chromeleon™ CDS software tracks the amount of MSA used and calculates the remaining lifetime. The EG degasser requires at least 2000 psi of system backpressure to ensure optimal removal of electrolysis gas from the eluent.

As an alternative, manually prepared MSA may be used. Prepare a 1.0 N stock solution by adding 96.10 g of MSA to a 1 L volumetric flask containing approximately 500 mL of DI water. Bring to volume with DI water and mix thoroughly. Prepare 6.4 mM MSA by diluting 6.4 mL of the 1 N MSA stock solution to 1 L with degassed DI water.

Standard Solutions

Stock choline hydroxide solution (1000 mg/L)

Dry ~6–10 g of choline bitartrate (MW 235.25 Da) at 102 °C to a constant weight. To prepare the 1000 mg/L standard, accurately weigh 1.046 g of dry choline bitartrate and dissolve in 500 mL of DI water. The standard is stable for one week when stored in the dark at 4 °C.

Stock choline hydroxide solution for the recovery experiment (20,000 mg/L)

Dissolve 4.184 g of dry choline bitartrate in 100 mL of DI water.

Working Standard Solutions

Prepare working solutions daily by diluting the 1000 mg/L standard solution as required with DI water.

Hydrochloric acid, 1 M

Weigh 909.70 g of DI water into an eluent bottle. Tare the bottle weight and carefully add 90.3 mL of ultrapure reagent-grade hydrochloric acid directly to the bottle.

Tris buffer, 50 mM

Weigh 6.057 g of tris(hydroxymethyl)aminomethane (Tris) into a 1 L volumetric flask containing ~900 mL of DI water. Dissolve the Tris and adjust the pH to 8.0 by adding ~28 mL of 1.0 M hydrochloric acid. Add DI water to bring to a final volume of 1.00 L.

Sample Preparation and Extraction

Infant Formula

- Accurately weigh 5 g of infant formula powder into a 50 mL centrifuge tube with a cap.
- Add 30 mL of 1 M hydrochloric acid, cap, and mix by shaking until well dispersed.
- Place the tube in a water bath at 70 °C for 3 h, shaking every hour. Occasionally loosen or temporarily remove the cap during the early heating stage to avoid the buildup of excess pressure.
- After 3 h, cool to room temperature.
- Filter the hydrolysate through a 0.2 µm PES syringe filter.
- Transfer 3 mL of the filtrate to a 100 mL volumetric flask and dilute to volume with DI water. This filtrate is ready for injection and may be stored in the dark at 4 °C for three days.

Egg Powder

- Accurately weigh 200 mg of egg powder into a 15 mL centrifuge tube with a cap.
- Add 10 mL of 1 M hydrochloric acid, cap, and mix by shaking until well dispersed.
- The remainder of the sample preparation is the same as for the infant formula, except only 2 mL of the filtrate is transferred to a 100 mL volumetric flask.

Soy Flour

- Accurately weigh 400 mg of soy flour into a 15 mL centrifuge with a cap.

- The remainder of the sample preparation is the same as for the egg powder, except only 0.5 mL of the filtrate is transferred to a 100 mL volumetric flask.

Preparation for Phospholipase D Treatment

- Dissolve 150 U of phospholipase D in 200 mL of 50 mM Tris [one unit (U) will liberate 1.0 µmol of choline from L-α-phosphatidylcholine (egg yolk) per hr at pH 5.6 at 30 °C].

- Add 1.0 mL of enzyme solution to 0.5 mL of sample extract. Incubate at 37 °C for 15 min and cool to room temperature prior to analysis.

Note: Phospholipase D treatment releases choline that may still be present as phosphatidylcholine after the acid hydrolysis. Choline content was determined to be the same with or without phospholipase D treatment for the samples analyzed in this study.

Determination of Choline Recovery

For infant formula samples, add 450 µL of 20,000 mg/L choline standard to 5 g of each infant formula sample and follow the extraction procedure described here. The spike concentration is 9 mg/L after the final dilution.

For egg powder samples, add 150 µL of 20,000 mg/L choline standard to 200 mg of each egg powder sample and follow the extraction procedure described here. The spike concentration is 6 mg/L after the final dilution.

For soy flour samples, prepare the 10,000 mg/L choline standard by a 1:2 dilution of 20,000 mg/L choline standard. Then add 100 µL of 10,000 mg/L choline standard to 400 mg of each soy flour sample and follow the extraction procedure described here. The spike concentration is 0.5 mg/L after the final dilution.

Reproducibility of Choline Analysis from Different Samples

The intraday peak area precisions were investigated based on the extraction of choline from three replicates of the selected food samples followed by five successive injections of each replicate into the IC system. The between-day peak area precisions were examined by analyzing one sample with five successive injections each day over three consecutive days.

Results and Discussion

Sample Preparation

Free choline is water soluble and can be easily extracted from foods. However, total choline is usually determined by hydrolysis of esters to release the bound choline to its free form.

In this study, hydrochloric acid was used to extract choline from the food samples prior to IC analysis. After acid digestion, the hydrolysate was filtered through a 0.2 µm PES syringe filter. It was determined that the PES syringe filter did not bind choline and therefore was suitable for efficient filtration of these samples. This also yielded a significant time-saving advantage when compared to using the filter papers described in AN 124.

Because choline is sensitive to light exposure and heat,[3,4] shortening sample preparation time helps ensure the accuracy and reproducibility of the analysis.

Phospholipase D may be added to the filtrate to release choline that may still be present as phosphatidylcholine. In this study, the influence of the enzyme concentration and pH of the filtrate was examined and the choline recoveries were found to be the same with or without phospholipase D treatment for the samples analyzed.

Separation

The manually prepared sulfuric acid eluent used in AN 124 was replaced with an electrolytically generated methanesulfonic acid eluent to eliminate the labor, possible contamination, and possible error associated with manual preparation. An equilibrated IC system will demonstrate a background conductance of ~0.1 µS and typically a peak-to-peak noise of 0.1 nS. An injection of a DI water blank confirmed the absence of other cations that may interfere with the determination of choline.

Compared to the Dionex IonPac CS12A column used in AN 124, the Dionex IonPac CS19 column used here has optimized selectivity for small hydrophilic amines and therefore provides a much higher efficiency for choline. The Dionex IonPac CS19 column also achieved an analysis time within 20 min compared to 25 min in AN 124. Figure 1 shows a chromatogram of a 10 mg/L choline standard and six common cations, demonstrating a good separation of the seven compounds and an excellent choline peak shape.

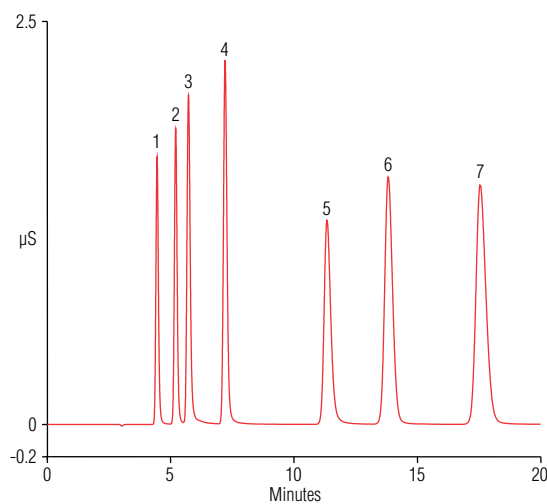|		Peaks:		
Columns:	Dionex IonPac CG19 and CS19, 2 mm		1. Lithium	0.25 mg/L
			2. Sodium	1.00
Eluent Source:	Dionex EGC III MSA cartridge with CR-CTC II trap column		3. Ammonium	1.25
			4. Potassium	2.50
Eluent:	6.4 mM MSA		5. Choline	10.00
Flow Rate:	0.25 mL/min		6. Magnesium	1.25
Inj. Volume:	5 µL		7. Calcium	2.50
Detection:	Suppressed conductivity, Dionex CSRS™ 300 suppressor, 2 mm, 4 mA, recycle mode			

Figure 1. Separation of 10 mg/L of choline standard and six common cations.

Linearity, Limit of Quantitation, Limit of Detection

To determine the linearity of the method, calibration standards were injected in five replicates at seven concentration levels in the range of 0.5–50 mg/L of choline. This produced a calibration curve with a coefficient of determination (r^2) value of 0.9999 using a least-squares regression fit. To determine the limit of detection (LOD) and limit of quantification (LOQ), baseline noise was first determined by measuring peak-to-peak noise in a representative 1 min segment of the baseline where no peaks are present. Typical baseline noise for this method was 0.1 nS. The LOD for choline was determined to be ~2.3 µg/L (signal-to-noise [S/N] = 3) and the LOQ was estimated to be 7.7 µg/L (S/N = 10).

Sample Analysis

U. S. FDA regulations require a minimum of 7.0 mg/100 Cal of choline in infant formula.[6] The amount of choline present in the infant formula samples in this study was found to be 35 mg/100 Cal, 146% of the labeled choline value (24 mg/100 Cal) on the infant formula package. [Note: The U.S./Canada food calorie (Calorie or Cal) is equal to 1 kcal or 4.184 kJ.]

In the whole egg powder and defatted soy flour samples, the amounts of choline were found to be 15.3 mg/g and 3.13 mg/g, respectively. The measured values are reasonably close to the reported values of 14.1 mg/g for egg powder and 2.49 mg/g for soy flour, determined by NIST.

Figures 2A–C show the chromatograms of infant formula, egg powder, and soy flour samples. The choline peak is well resolved from other cations, which are also present in each sample. If desired, the concentrations of inorganic cations and choline can be determined in the same run, though a sample dilution may be needed to accurately determine the calcium in infant formula.

Columns:	Dionex IonPac CG19 and CS19, 2 mm
Eluent Source:	Dionex EGC III MSA cartridge with CR-CTC II trap column
Eluent:	6.4 mM MSA
Flow Rate:	0.25 mL/min
Inj. Volume:	5 µL
Detection:	Suppressed conductivity, Dionex CSRS 300 suppressor, 2 mm, 4 mA, recycle mode

*Calculated concentration (total sample dilution is ~200×)

*Calculated concentration (total sample dilution is ~2500×)

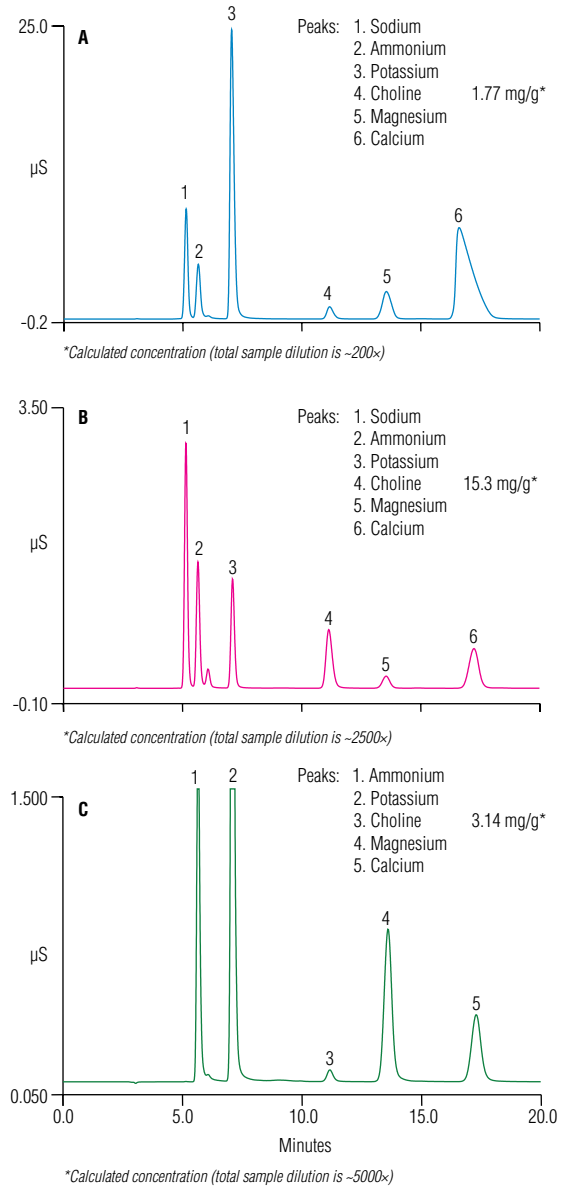*Calculated concentration (total sample dilution is ~5000×)

Figure 2. A) Determination of choline in infant formula, B) determination of choline in egg powder, and C) determination of choline in soy flour.

Sample Accuracy and Precision

As an additional evaluation of accuracy, a known amount of choline standard was added to each solid sample. The total choline amount was then determined following the same hydrolysis, filtration, and separation processes. The recoveries obtained from the infant formula, egg powder, and soy flour samples ranged from 94–98% (Table 1). The between- and intraday peak area precisions were <0.8% for all the tested food samples. Table 2 shows the results of three blind duplicates of all the tested samples. The data demonstrate good accuracy and precision.

Table 1. Choline recoveries in infant formula, egg powder, and soy flour samples.

Sample	Amount Found (mg/L)	Amount Added (mg/L)	Total Recovered (mg/L)	Recovery (%)
Infant Formula	8.82	9.01	17.3	93.7
Egg Powder	6.17	6.01	12.1	98.3
Soy Flour	0.63	0.50	1.12	98.2

Table 2. Precision of the choline determination in blind duplicate infant formula, egg powder, and soy flour samples.

Sample	Sample Concentration (mg/g)				RSD	Average Amount (mg/100 Cal)*	Choline Content Determined by NIST** (mg/g)
	Sample A	Sample B	Sample C	Average			
Infant Formula	1.77	1.74	1.77	1.76	0.76	35.2***	—
Egg Powder	15.1	15.4	15.3	15.3	0.92	—	14.1
Soy Flour	3.11	3.14	3.14	3.13	0.57	—	2.49

*The labeled choline value for the infant formula package is 24 mg/100 Cal. The label states that one scoop of the infant powder (8.8 g) provides 44 Calories. When weighed, 5 g of sample is equivalent to 25 Calories. Therefore, the measured choline amount in mg/g is converted to mg/100 Cal.

**Choline content in Standard Reference Material (SRM) 3234 Soy Flour samples and SRM 1845a Whole Egg Powder are provided by NIST as a result of participation in DSQAP exercises.

***35.2 mg/100 Cal = 0.35 mg/kcal = 0.084 mg/kJ

Conclusion

This study demonstrates an IC method for determining choline in infant formula and other food samples, including several improvements compared to AN 124. A column set with optimized selectivity and a smaller dimension provides much improved efficiency for choline, and therefore better sensitivity. The electrolytically generated eluent replaces the manually prepared eluent, enhancing the level of automation and ease of operating the IC system and achieving a better S/N ratio. A slightly modified sample preparation procedure also increases sample throughput.

The results reported in this study are close to the infant formula label value and the values determined by NIST, thus indicating that IC is a viable alternative to other analytical techniques for choline determination. In addition, IC allows simultaneous determination of sodium, ammonium, potassium, and other cations present in the sample, an efficiency not possible with spectroscopic or biosensory techniques.

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