Determination of Anions in Breast Milk

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Key Words
Chloride, Nitrate, Phosphate, Citrate, Conductivity Detection, Mass Spectrometry (MS) Detection, Ion Chromatography (IC), Food Analysis

Goal
To develop an efficient IC method for sensitive determination of anions in breast milk

Introduction
Breast milk is the primary source of nutrition for newborns before they are able to eat and digest other foods, so there is a close correlation between breast milk quality and infant health. In addition to proteins and vitamins, the ionic elements such as sodium, potassium, calcium, magnesium, chloride, and nitrate in breast milk also are important for infant health. However, consumption of breast milk that contains a high concentration of these ions may upset the desired balance in the infant's blood stream, potentially resulting in damage to the kidneys and cardiovascular system.[1] Therefore, by monitoring the concentrations of these ions in breast milk, the maternal diet can be adjusted to improve milk quality. IC is an efficient means to determine anions, even in samples with complex matrixes.

Equipment
- Thermo Scientific™ Dionex™ ICS-5000+ HPIC™ system,* capable of supporting high-pressure IC, including:
  – DP Dual Pump
  – EG Eluent Generator
  – DC Detector/Chromatography Module
- Thermo Scientific Dionex AS-AP Autosampler with 25 µL sample loop (P/N 042857)
- Thermo Scientific™ MSQ Plus™ Mass Spectrometer (P/N 063116)
- Thermo Scientific™ Sorvall™ ST16 Centrifuge (P/N 75004240)
- Thermo Scientific™ Dionex™ AERS™ 500 Anion Electrolytically Regenerated Suppressor, 4 mm (P/N 082540)
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) software, version 7.2

*This method can be performed using any Dionex ICS system capable of eluent generation.

Consumables
- Thermo Scientific™ Target2™ Nylon Syringe Filters, 0.45 µm, 30 mm (P/N F2500-1)
- Thermo Scientific™ Dionex™ OnGuard™ II RP Cartridge (P/N 057083)
- Thermo Scientific Dionex EGC-500 Potassium Hydroxide (KOH) Eluent Generator Cartridge (P/N 075778)
- Thermo Scientific™ Sun-SRi™ Luer-Lock Syringe (Fisher Scientific P/N 14-823-261)

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Reagents and Standards

- Deionized (DI) water, 18.2 MΩ-cm resistivity
- Sodium Fluoride (Fisher Scientific P/N S299-100)
- Potassium Bromide (Fisher Scientific P/N P205-500)
- Potassium Iodide (Fisher Scientific P/N P410-100)
- Sodium Chloride (Fisher Scientific P/N S671-500)
- Sodium Nitrate (Fisher Scientific P/N BP360-500)
- Sodium Nitrite (Fisher Scientific P/N S347-250)
- Sodium Sulfate Anhydrous (Fisher Scientific P/N S429-500)
- Sodium Phosphate Tribasic Dodecahydrate (Fisher Scientific P/N S377-500)
- Potassium Citrate Monohydrate (Fisher Scientific P/N P218-500)

Standard Solutions

Stock Standard Solutions

To prepare the stock standard solutions, weigh 221 mg of sodium fluoride, 149 mg of potassium bromide, 131 mg of potassium iodide, 165 mg of sodium chloride, 137 mg of sodium nitrate, 150 mg of sodium nitrite, 148 mg of sodium sulfate, 400 mg of sodium phosphate tribasic dodecahydrate, and 171 mg of potassium citrate into nine separate volumetric flasks and dilute each to 100 mL with DI water. The concentration of each stock standard solution will be 1000 mg/L.

Standard Mix for Method Development

For method development, prepare a mixed standard solution containing nine analytes with different concentrations that will yield a similar peak height. Prepare this standard by diluting proper amounts of the stock standard solutions with DI water. The volume of each stock standard solution needed and the final concentration of each analyte in the standard mix are shown in Table 1.

Table 1. Preparation of a mixed standard for method development.

Analyte	1000 mg/L Stock Std Solution Vol (mL)	DI Water Vol (mL)	Std Mix Final Vol (mL)	Final Concn of Each Analyte (mg/L)
Fluoride	0.65	87.35	100	6.5
Chloride	0.60			6.0
Nitrite	1.00			10
Bromide	1.60			16
Nitrate	1.60			16
Sulfate	0.40			4.0
Iodide	3.00			30
Phosphate	1.20			12
Citrate	2.60			26

Working Mixed Standard Solutions for Calibration

Prepare a standard solution with a 20 mg/L concentration of chloride by diluting 2 mL of the 1000 mg/L chloride stock standard solution to 100 mL with DI water. Follow the same procedure using nitrite to prepare a 20 mg/L nitrate standard solution. These two solutions will be used to prepare the working mixed standard solutions for calibration.

For calibration, prepare eight working mixed standard solutions with different concentrations by diluting proper amounts of the 20 mg/L chloride and 20 mg/L nitrate standard solutions with DI water. The volumes of the 20 mg/L standard solutions needed and the final concentrations of chloride and nitrate in the working mixed standard solutions are shown in Table 2.

Table 2. Preparation of working mixed chloride and nitrate standard solutions for calibration.

20 mg/L Chloride and 20 mg/L Nitrate Std Solution Vol (mL)	DI Water Vol (mL)	Working Mixed Std Solution Final Vol (mL)	Calibration Std Final Concn (mg/L)
0.010	9.98	10	0.02
0.025	9.95		0.05
0.050	9.90		0.10
0.100	9.80		0.20
0.250	9.50		0.50
0.50	9.00		1.0
1.000	8.00		2.0
2.500	5.00		5.0

Sample Preparation

Cartridge Activation

Use the Dionex OnGuard II RP cartridge for sample pretreatment to remove hydrophobic organic compounds. The cartridge requires activation before use. Pass 10 mL of methanol through the cartridge, followed by 10 mL of DI water, and then allow it to stand for 15 min before use.

Sample Pretreatment

Add 1 g of the breast milk sample and 14 mL of DI water to a 25 mL centrifuge tube. Mix for 5 min to extract, add 4 mL of trichloromethane, then let the solution stand for 20 min. Centrifuge the extract for 10 min at 10,000 rpm, then pass the supernatant through an activated Dionex OnGuard II RP cartridge. Prior to injection, filter the solution through a 0.45 µm syringe filter. The treated sample may be diluted using DI water, if necessary.

To prepare the spiked samples, add 0.9 mL of 1000 mg/L stock standard solution of chloride, 0.19 mL of 1000 mg/L stock standard solution of phosphate, 0.42 mL of 20 mg/L stock standard solution of nitrate, 12.49 mL of DI water, and 1 g of breast milk sample to a 25 mL centrifuge tube. Other procedures are the same as described above. The spike concentrations in the breast milk sample are 60 mg/L for chloride, 13 mg/L for phosphate, and 0.56 mg/L for nitrate.

Chromatographic Conditions	
Columns:	Thermo Scientific™ Dionex™ IonPac™ AG19 Guard, 4 × 50 mm (P/N 062887) Dionex IonPac AS19 Analytical, 4 × 250 mm (P/N 062885)
Eluent:	KOH
Eluent Source:	Dionex EGC KOH Cartridge
Gradient:	0–12 min, 2 mM; 12–35 min, 2–18 mM; 35–40 min, 18–50 mM; 40–50 min, 50 mM; 50.1–60 min, 2 mM
Flow Rate:	1.2 mL/min
Injection Volume:	25 µL (full-loop injection mode)
Temperature:	30 °C
Detection:	Suppressed conductivity, Dionex AERS 500 Suppressor, recycle mode, 149 mA
System Backpressure:	2152 psi
Background Conductance:	0.3 µS

Mass Spectrometric Conditions	
Ionization Mode:	ESI
Operating Mode:	Negative scan
Probe Temperature:	450 °C
Needle Voltage:	4000 V
Selected Ion Monitoring Mode:	35 *m/z* for chloride 97 *m/z* for phosphate 191 *m/z* for citrate 62 *m/z* for nitrate
Dwell Time:	0.5 sec
Cone Voltage:	50 V
Nebulizer Gas:	Nitrogen at 75 psi

Results and Discussion

Separation of Nine Anions

The Dionex IonPac AS19 column with hydrophilic quaternary ammonium groups provides excellent separation of a variety of anions including inorganic anions, oxyhalides, oxyanions, and organic acids.[2] Figure 1 shows a chromatogram of a mixed standard containing nine anions separated by a Dionex IonPac AS19 column under the previously described chromatographic conditions.

Reproducibility, Linearity, and Detection Limits

Method precision was estimated by making seven consecutive 25 µL injections of a calibration standard with a concentration of 1.0 mg/L for chloride and nitrate. Retention time reproducibilities (RSDs) were 0.00 for chloride and 0.01 for nitrate and peak area RSDs were 0.51 for chloride and 0.64 for nitrate, demonstrating good short-term precision for this method.

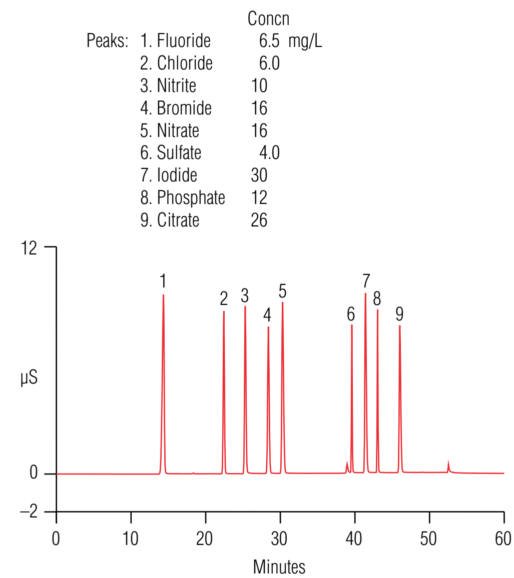

Figure 1. Separation of a mixed standard.

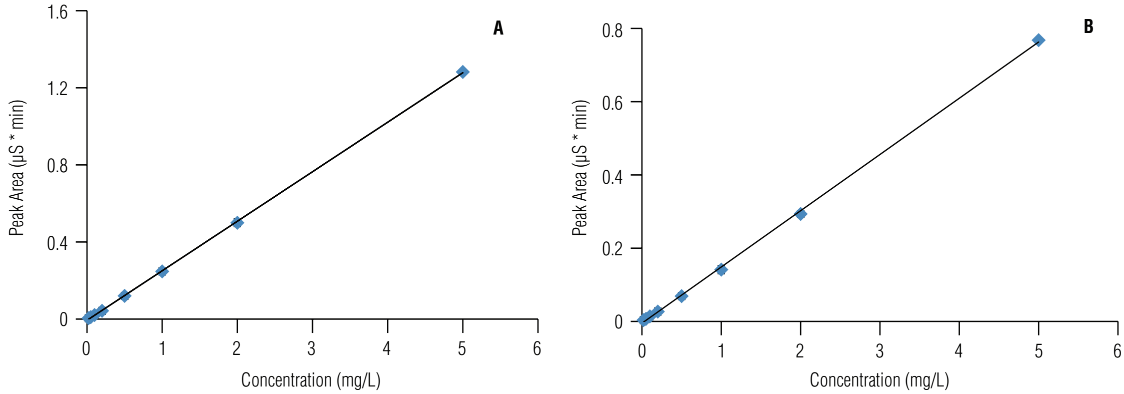

Figure 2. Calibration curves for (A) chloride and (B) nitrate.

Calibration linearity of chloride and nitrate was investigated by making three consecutive 25 µL injections of the working mixed standard solutions prepared at eight different concentrations (i.e., 24 total injections). Linearity was observed when plotting concentration versus suppressed conductivity peak area. Detailed calibration data calculated by Chromeleon CDS software are shown in Table 3. Figure 2 shows the calibration curves for chloride and nitrate.

Five consecutive 25 µL injections of a calibration standard with a concentration of 0.1 mg/L for chloride and nitrate were used for estimating method detection limits (MDLs) using a signal-to-noise ratio = 3. The calculated MDLs are listed in Table 3.

Table 3. Calibration data and MDLs.

Anion	Regression Equation	r^2	Range (mg/L)	MDL (µg/L)
Chloride	A = 0.2571c – 0.0055	0.9999	0.02–5.0	8
Nitrate	A = 0.1533c – 0.0041	0.9995	0.02–5.0	10

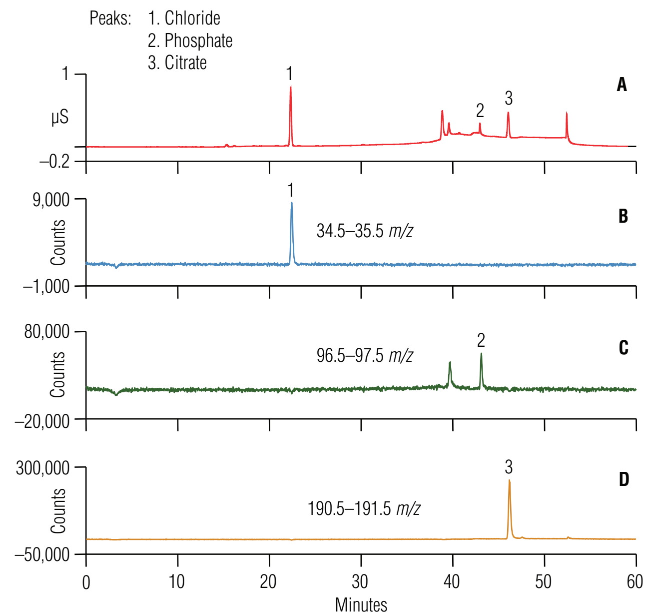

Figure 3. (A) A chromatogram obtained with suppressed conductivity detection of a breast milk sample diluted 100-fold with DI water and SIM chromatograms of the same sample with (B) 35.0 *m/z*, (C) 97.0 *m/z*, and (D) 191 *m/z*.

Sample Analysis

Using IC, the anionic ingredients in the breast milk sample can be identified by comparing their retention time to that of the standards; to confirm these identifications, a mass selective detector can record selected ion monitoring (SIM) channels at significant mass-to-charge ratios. Figure 3 shows the chromatogram and SIM chromatograms of the breast milk sample diluted 100-fold with DI water. The ion in Figure 3B with 35.0 *m/z* and a retention time of 22.5 min was identified as chloride. The ion in Figure 3C with 97.0 *m/z* and a retention time of 43 min was identified as phosphate. The other peak in Figure 3C, also with 97.0 *m/z* but a retention time of 39 min, can be speculated as sulfate; however, as shown in Figure 4A, its presence in blank may affect its identification. The ion in Figure 3D with 191 *m/z* and a retention time of 47 min was identified as citrate. To confirm the identities of chloride—as well as phosphate and citrate, for which calibrations were not performed—the sample was spiked with chloride, phosphate, and citrate standards (Figure 4).

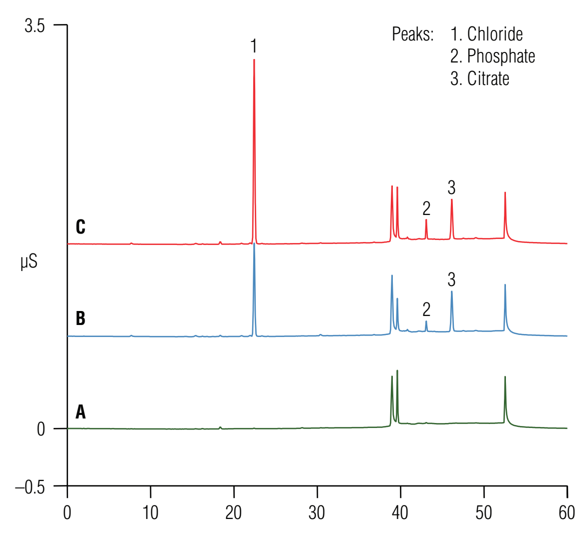

Figure 4. (A) A blank, (B) a prepared breast milk sample diluted 100-fold with DI water, and (C) the same sample spiked with chloride, phosphate, and citrate standards.

Because the chosen breast milk sample was low in nitrate, a direct injection of the pretreated sample was performed to determine it. Figure 5 shows the chromatogram (A) and SIM chromatogram (B). The ion in Figure 5B with 62.0 *m/z* and a retention time of 30.3 min was identified as nitrate. Figure 6 shows the chromatograms of the breast milk sample and the same sample spiked with a nitrate standard.

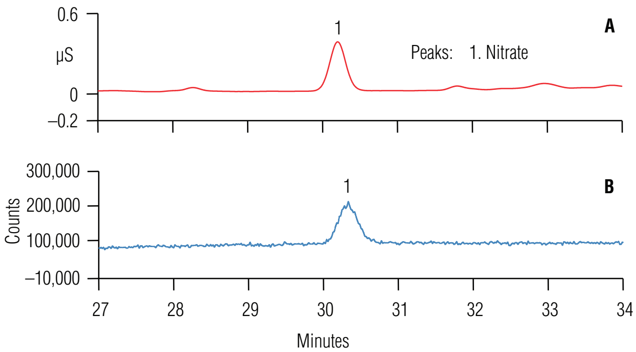

Figure 5. (A) A chromatogram of a prepared breast milk sample without dilution and (B) the SIM chromatogram (61.5–62.5 *m/z*) of the same sample.

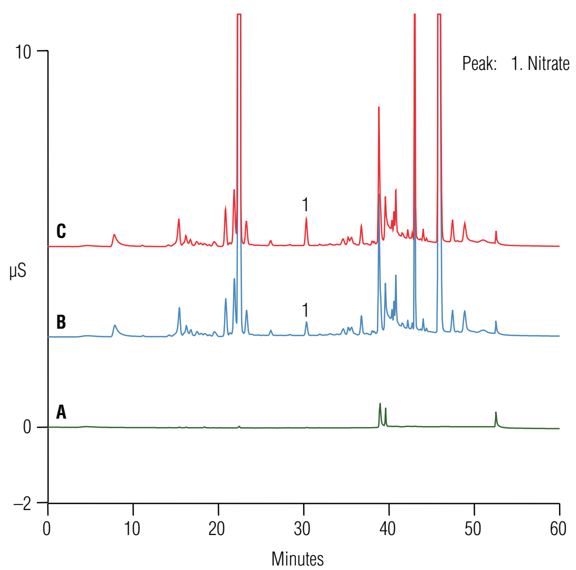

Figure 6. Chromatograms of (A) a blank, (B) a prepared breast milk sample without dilution, and (C) the same sample spiked with nitrate.

Method recovery was investigated by determining the recoveries in the spiked breast milk samples. The recoveries were 97% for chloride and 98% for nitrate, demonstrating that this IC method provides good selectivity and suitability for the determination of chloride and nitrate in breast milk. Table 4 summarizes the analysis results.

Table 4. Sample analysis results.

Analyte	Detected (g/L)	Spiked Sample		
		Added (g/L)	Found (g/L)	Recovery (%)
Chloride†	0.91	0.60	0.58	97
Nitrate	0.0088	0.0084	0.0082	98
Phosphate†	0.15	0.13	0.11	85
Citrate†	1.33	—	—	—

† Calculated using the single-point correction method by MS detection

Conclusion

This work describes an IC method with suppressed conductivity detection that enables sensitive determination of inorganic anions and carboxylic acids in breast milk. The determination is performed using a Dionex ICS-5000+ Reagent-Free™ HPIC system controlled by Chromeleon CDS software and equipped with a Dionex IonPac AS19 column.

References

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2. Dionex (now part of Thermo Scientific) Data Sheet: IonPac AS19 Anion-Exchange Column. Sunnyvale, CA, 2010. [Online] www.thermoscientific.com/content/dam/tfs/ATG/CMD/CMD%20Documents/Product%20Manuals%20&%20Specifications/Chromatography/GC%20HPLC%20and%20UHPLC%20Columns%20and%20Accessories/Chromatography%20Column%20Accessories/7088-DS-IonPac-AS19-17Mar2010-LPN1616-04.pdf (accessed September 12, 2014).

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