Beverages Applications Notebook

Milk

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Introduction to Beverages

The global beverage industry is growing each year with the introduction of new products, such as vitamin-fortified water, energy drinks, anti-aging water, and herbal nutritional supplements. With this growth, come many more analytical challenges. These challenges are compounded by the continuing and new needs to analyze classic favorites such as sodas, fruit juices, milk drinks, alcoholic beverages, and bottled water. One such example would be the melamine contamination in milk and infant milk formula.

For all beverages, the compositional quality and safety must be monitored to help track contamination, adulteration, product consistency, and to ensure regulatory compliance from raw ingredients (water, additives, and fruits) to the final product.

Thermo Fisher Scientific is a recognized leader in providing analytical solutions for sample preparation, liquid chromatography for compositional testing, and chromatography data management for compliance and quality testing of beverages. From inorganic ions, organic acids, biogenic amines, glycols and alcohols, carbohydrates and sugar alcohols, to vitamins, additives, and sugar substitutes, we are unique in our commitment to provide fast, accurate testing and labeling information for all applications in this industry.

Thermo Scientific and Dionex Integrated Systems

Dionex Products are now a part of the Thermo Scientific brand, creating exciting new possibilities for scientific analysis. Now, leading capabilities in liquid chromatography (LC), ion chromatography (IC), and sample preparation are together in one portfolio with those in mass spectrometry (MS). Combining Dionex's leadership in chromatography with Thermo Scientific's leadership position in mass spec, a new range of powerful and simplified workflow solutions now becomes possible.

For more information on how the new lineup of Thermo Scientific products can expand your capabilities and provide the tools for new possibilities, choose one of our integrated solutions:

- Ion Chromatography and Mass Spectrometry
- Liquid Chromatography and Mass Spectrometry
- Sample Preparation and Mass Spectrometry

UltiMate 3000 UHPLC+ Systems

Best-in-class HPLC systems for all your chromatography needs

Thermo Scientific Dionex UltiMate 3000 UHPLC+ Systems provide excellent chromatographic performance while maintaining easy, reliable operation. The basic and standard analytical systems offer ultra HPLC (UHPLC) compatibility across all modules, ensuring maximum performance for all users and all laboratories. Covering flow rates from 20 nL/min to 10 mL/min with an industry-leading range of pumping, sampling, and detection modules, UltiMate™ 3000 UHPLC+ Systems provide solutions from nano to semipreparative, from conventional LC to UHPLC.

- Superior chromatographic performance
- UHPLC design philosophy throughout nano, standard analytical, and rapid separation liquid chromotography (RSLC)
- 620 bar (9,000 psi) and 100 Hz data rate set a new benchmark for basic and standard analytical systems
- RSLC systems go up to 1000 bar and data rates up to 200 Hz
- ×2 Dual System for increased productivity solutions in routine analysis
- Fully UHPLC compatible advanced chromatographic techniques
- Thermo Scientific Dionex Viper and nanoViper–the first truly universal, fingertight fitting system even at UHPLC pressures

Thermo Fisher Scientific is the only HPLC company uniquely focused on making UHPLC technology available to all users, all laboratories, and for all analytes.

Rapid Separation LC Systems: The extended flow-pressure footprint of the RSLC system provides the performance for ultrafast high-resolution and conventional LC applications.

RSLCnano Systems: The Rapid Separation nano LC System (RSLCnano) provides the power for high-resolution and fast chromatography in nano, capillary, and micro LC.

Standard LC Systems: Choose from a wide variety of standard LC systems for demanding LC applications at nano, capillary, micro, analytical, and semipreparative flow rates.

Basic LC Systems: UltiMate 3000 Basic LC Systems are UHPLC compatible and provide reliable, high-performance solutions to fit your bench space and your budget.

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A complete range of ion chromatography solutions for all customer performance and price requirements

For ion analysis, nothing compares to a Thermo Fisher Scientific ion chromatography system. Whether you have just a few samples or a heavy workload, whether your analytical task is simple or challenging, we have a solution to match your needs and budget. And with your IC purchase, you get more than just an instrument—you get a complete solution based on modern technology and world-class support.

- Thermo Scientific Dionex ICS-5000: The world's first capillary IC system
- Dionex ICS-2100: Award-winning integrated Reagent-Free™ IC system
- Dionex ICS-1600: Standard integrated IC system
- Dionex ICS-1100: Basic integrated IC system
- Dionex ICS-900: Starter line IC system

Ranging from the Dionex ICS-900 to the ICS-5000, these IC systems cover the entire range of IC needs and budgets and come with superior support and service worldwide.

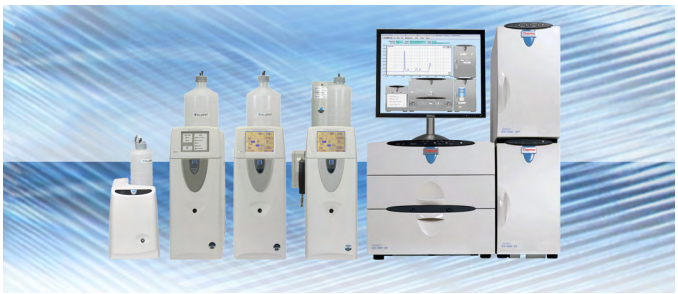
Dionex ICS-5000: Developed with flexibility, modularity, and ease-of-use in mind, the Dionex ICS-5000 combines the highest sensitivity with convenience

Dionex ICS-2100: An integrated Reagent-Free IC (RFIC™) system for electrolytically generated isocratic and gradient separations with conductivity detection, now with electrolytic sample preparation.

Dionex ICS-1600: The Dionex ICS-1600 combines high sensitivity with convenience. Now ready for eluent regeneration, with available dual-valve configuration for automated sample preparation.

Dionex ICS-1100: With dual-piston pumping and electrolytic suppression. Now ready for eluent regeneration, with available dual-valve configuration for automated sample preparation.

Dionex ICS-900: Can routinely analyze multiple anions and cations in 10–15 min—fully automated with Displacement Chemical Regeneration (DCR).



MS Instruments

Single-point control and automation for improved ease-of-use in LC/MS and IC/MS

Thermo Fisher Scientific provides advanced integrated IC/MS and LC/MS solutions with superior ease-of-use and modest price and space requirements. UltiMate 3000 System Wellness technology and automatic MS calibration allow continuous operation with minimal maintenance. The Dionex ICS-5000 instrument and the family of RFIC systems automatically remove mobile phase ions for effort-free transition to MS detection.

- Thermo Scientific MSQ Plus mass spectrometer, the smallest and most sensitive single quadrupole on the market for LC and IC
- Self-cleaning ion source for low-maintenance operation
- Thermo Scientific Dionex Chromeleon Chromatography Data System software for single-point method setup, instrument control, and data management
- Compatible with existing IC and LC methods
- The complete system includes the MSQ Plus™ mass spectrometer, PC datasystem, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) probe inlets, and vaccum system

You no longer need two software packages to operate your LC/MS system. Chromeleon™ LC/MS software provides single-software method setup and instrument control; powerful UV, conductivity, and MS data analysis; and fully integrated reporting.

MS Systems and Modules: MSQ Plus Mass Spectrometer; MSQ18LA nitrogen gas generator; Thermo Scientific Dionex AXP-MS digital auxiliary pump

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Chromeleon 7 Chromatography Data System Software

The fastest way to get from samples to results

Discover Chromeleon software version 7, the chromatography software that streamlines your path from samples to results. Get rich, intelligent functionality and outstanding usability at the same time with Chromeleon software version 7—the Simply Intelligent™ chromatography software.

- Enjoy a modern, intuitive user interface designed around the principle of operational simplicity
- Streamline laboratory processes and eliminate errors with eWorkflows, which enable anyone to perform a complete analysis perfectly with just a few clicks
- Access your instruments, data, and eWorkflows instantly in the Chromeleon Console
- Locate and collate results quickly and easily using powerful built-in database query features
- Interpret multiple chromatograms at a glance using MiniPlots
- Find everything you need to view, analyze, and report data in the Chromatography Studio
- Accelerate analyses and learn more from your data through dynamic, interactive displays
- Deliver customized reports using the built-in Excel-compatible speadsheet

Chromeleon software version 7 is a forward-looking solution to your long-term chromatography data needs. It is developed using the most modern software tools and technologies, and innovative features will continue to be added for many years to come.

The Cobra™ integration wizard uses an advanced mathematical algorithm to define peaks. This ensures that noise and shifting baselines are no longer a challenge in difficult chromatograms. When peaks are not fully resolved, the SmartPeaks™ integration assistant visually displays integration options. Once a treatment is selected, the appropriate parameters are automatically included in the processing method.

Chromeleon software version 7 ensures data integrity and reliability with a suite of compliance tools. Compliance tools provide sophisticated user management, protected database stuctures, and a detailed interactive audit trail and versioning system.

Process Analytical Systems and Software

Improve your process by improving your process monitoring with a Thermo Scientific Dionex on-line IC or HPLC system

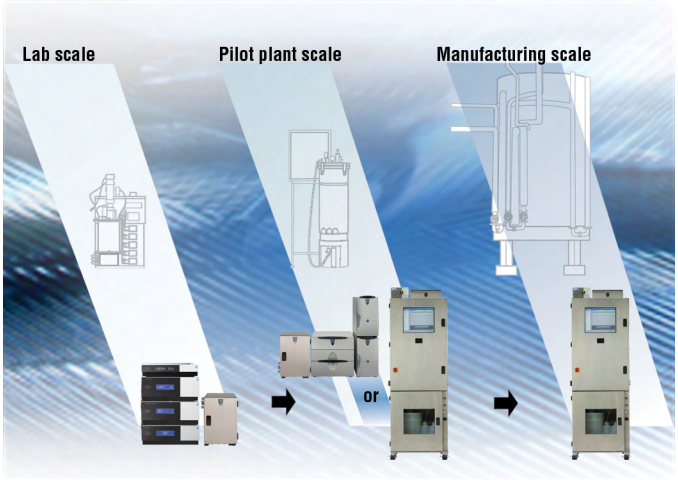
Our process analytical systems provide timely results by moving liquid chromatography-based measurements on-line. Information from the Thermo Scientific Dionex Integral process analyzer can help reduce process variability, improve efficiency, and reduce downtime. These systems provide comprehensive, precise, accurate information faster than is possible with laboratory-based results. From the lab to the factory floor, your plant's performance will benefit from the information provided by on-line LC.

- Characterize your samples completely with multicomponent analysis
- Reduce sample collection time and resources with automated multipoint sampling
- Improve your process control with more timely results
- See more analytes with unique detection capabilities
- 25 years of experience providing on-line IC and HPLC capabilities to a wide range of industries
- The Thermo Scientific Integral Migration Path approach lets you choose the systems that best meets your needs

The Integral Migration Path™ approach enables on-line IC/HPLC to generate timely, high-resolution information when monitoring a small-scale reactor in a process R&D lab, in a pilot plant, or improving current manufacturing plant processes. No matter what the application, the Integral™ process analyzer has the versatility to place a solution using on-line IC/HPLC, whenever and wherever it is needed.

Integral: The Integral Migration Path approach: System solutions wherever you need them: lab, pilot plant, or manufacturing

Chromeleon Process Analytical (PA) Software: Chromeleon PA software provides unique capabilities to support on-line IC or HPLC analysis



Automated Sample Preparation

ACCELERATED SOLVENT EXTRACTORS

Two new solvent extraction systems with pH-hardened Dionium components

We offer two solvent extraction systems. The Thermo Scientific Dionex ASE 150 Accelerated Solvent Extractor is an entry-level system with a single extraction cell, for laboratories with modest throughput. The Dionex ASE™ 350 system is a sequential extraction system capable of automated extraction of up to 24 samples. Both systems feature chemically inert Dionium components that allow the extraction of acid- or base-pretreated samples.

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SOLID-PHASE EXTRACTION SYSTEMS

Faster, more reliable solid-phase extraction while using less solvent

The Thermo Scientific Dionex AutoTrace 280 Solid-Phase Extraction (SPE) instrument unit can process six samples simultaneously with minimal intervention. The instrument uses powerful pumps and positive pressure with constant flow-rate technology. Current analytical methods that require SPE sample preparation include gas chromatography (GC), GC-MS, LC, and LC-MS, IC and IC-MS. The Dionex AutoTrace™ 280 instrument is approved or adapted for U.S. EPA clean water methods and safe drinking water methods (600 and 500 series) and can extract the following analytes:

- PCBs (polychlorinated biphenyls)
- OPPs (organophosphorus pesticides), OCPs (organochlorine pesticides), and chlorinated herbicides
- BNAs (base, neutral, acid semivolatiles)
- Dioxins and furans
- PAHs (polyaromatic hydrocarbons)
- Oil and grease or hexane extractable material

With SPE, large volumes of liquid sample are passed through the system and the compounds of interest are trapped on SPE adsorbents (cartridge or disk format), then eluted with strong solvents to generate an extract ready for analysis. Automated SPE saves time, solvent, and labor for analytical laboratories.

Dionex AutoTrace Systems: The new Dionex AutoTrace 280 system provides fast and reliable automated solid phase extraction for organic pollutants from liquid samples

Dionex AutoTrace Accessories: High-quality parts and accessories are available for Dionex AutoTrace 280 instruments

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Analysis of Milk

Determination of Iodide in Milk Products

INTRODUCTION

Trace levels of iodide are necessary for normal physical and mental development; however, excess iodide can lead to thyroid disorders. Common sources of iodide include iodized table salt and seafood, but other food products also contain iodide. Within the dairy industry, iodophors are used as disinfectants, which can also lead to increased iodide consumption by the public.[1] A concern over high iodide levels in the diet has led to a nutritional labeling requirement for iodide/iodine.

In this application note, ion chromatography coupled with pulsed amperometric detection is used to determine iodide in milk products. This method is specific, sensitive, and rapid. Iodide is separated on the IonPac® AS11 column, which contains a hydrophilic, anion-exchange resin that is well suited to the chromatography of the relatively hydrophobic iodide anion. Using a nitric acid eluent, the iodide ion elutes from the column in less than 5 minutes. Although iodide can be detected by direct current (dc) amperometry on a silver working electrode, a pulsed amperometric waveform is used in this application note to improve the reproducibility of iodide analysis.[2] Like dc amperometry, the detection limit of iodide using pulsed amperometric detection is in the low µg/L range.

EQUIPMENT

Dionex DX-500 Chromatography system consisting of:
- GP40 Gradient Pump with vacuum degas option
- LC25 or LC30 Liquid Chromatography Module
- ED40 Electrochemical Detector
- EO1 Eluent Organizer
- AS3500 Autosampler

Dionex PeakNet Chromatography Workstation
Whatman 2V Filters, 185 mm (Whatman)
OnGuard® II RP Sample Pretreatment Cartridges (Dionex P/N 057083)

REAGENTS AND STANDARDS

Deionized water, 17.8 MΩ-cm resistivity or better
Concentrated nitric acid, ultrapure (J. T. Baker)
Glacial acetic acid (J. T. Baker)
Potassium iodide (Fisher Scientific)

CONDITIONS

Columns:	IonPac AS11 Analytical, 4 × 250 mm (P/N 44076) IonPac AG11 Guard, 4 × 50 mm (P/N 44078)
Expected Operating Pressure:	6.5 MPa (950 psi)
Degas Interval:	10 min
Injection Volume:	50 µL
Injection Loop:	100 µL
Eluent:	50 mM nitric acid
Flow Rate:	1.5 mL/min
Detection:	Pulsed amperometry, silver working electrode, Ag/AgCl reference

Waveform for the ED40 Detector:

Time (sec)	*Potential (V)*	*Integration*
0.00	+0.1	
0.20	+0.1	Begin
0.90	+0.1	End
0.91	–0.8	
0.93	–0.3	
1.00	–0.3	

Collection Rate:	1 Hz
Background:	7–20 nC (typical)
Temperature:	30 °C
Autosampler:	11-min cycle time
Injection Mode:	Pull
Needle Height:	2 mm
Flush Volume:	400 µL

PREPARATION OF SOLUTIONS AND REAGENTS

50 mM Nitric Acid

Add 6.25 mL of concentrated nitric acid to approximately 1000 mL of degassed 17.8 MΩ-cm deionized water in a 2-L volumetric flask. Dilute to the mark with degassed deionized water.

Iodide Standards

Prepare a 1000-mg/L standard by dissolving 1.31 g of potassium iodide in 1000 mL of deionized water. This primary standard was used to prepare a 10 mg/L secondary standard, which was appropriately diluted for linearity studies. Both the primary and secondary standards were stored frozen. Because iodide is light-sensitive, exposure to light should be minimized. All standards prepared from the 10 mg/L stock solution should be used on the day they are prepared.

Electrode Preparation

Polish the silver electrode with the white fine polishing compound. Rinse the electrode well with deionized water and wipe with a damp paper towel. After this initial polish, the electrode should only be polished if it becomes discolored or if it has not been used for a month or longer.

SAMPLE PREPARATION

OnGuard RP Preparation

Pass 5 mL of methanol, followed by 10 mL of deionized water, through the cartridge at 4 mL/min. To save time, up to 12 cartridges can be prepared at one time using the OnGuard Sample Prep Station (P/N 39599).

Milk Sample Preparation

Prepare the infant formula as suggested for feeding. Prepare the nonfat dried milk as recommended for serving (10 mL of water for every 0.95 g of milk powder).

Pipet 10 mL of milk product into a 100-mL polypropylene beaker. Add 2 mL of 3% acetic acid and mix. Add 8 mL of deionized water and mix. Pass the sample through a Whatman 2V filter. Measure the filtrate volume and pass 5 mL of sample through the OnGuard RP cartridge at4 mL/min, discarding the first 3 mL of sample. Collect the remaining filtrate and inject an aliquot into the chromatograph. If the filtrate is cloudy, it should not be used.A cloudy filtrate suggests that a different sample preparation method is necessary.

To determine recovery, add 1 mL of 1 mg/L iodide to the sample prior to the addition of acetic acid and add only 7 mL of water prior to filtration. Calibration standards were prepared by subjecting them to the sample preparation procedure. 10 mL of 0.1 mg/L iodide was prepared in duplicate for each experiment.

RESULTS AND DISCUSSION

Chromatography of Iodide

Figure 1 shows the separation of 1 mg/L iodide on the IonPac AS11 column set using a 50 mM nitric acid eluent. Iodide elutes in less than 4 min and is well separated from the void volume. Compared to other ion-exchange columns, the IonPac AS11 contains a very hydrophilic pellicular resin that improves the peak shape of the hydrophobic iodide ion. The nitric acid eluent also improves peak shape.

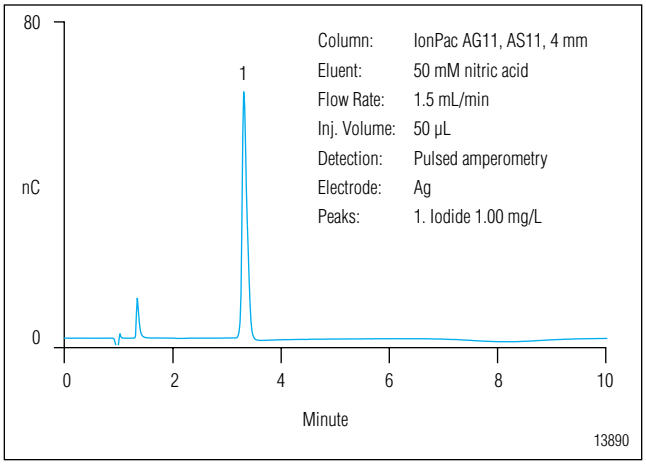

Figure 1. Determination of iodide by ion chromatography with pulsed amperometric detection

Fluoride, chloride, bromide, and iodate elute well before iodide. Chloride elutes at approximately 1.5 minutes. The dip in the baseline at approximately 8 minutes is due to dissolved oxygen. This dip is from the previous injection (elution time of approximately 19 min) and varies from column to column. An 11-min injection-to-injection time (autosampler cycle time) places the dip where it does not interfere with iodide chromatography on either of the

two column sets tested. When installing a new column, the dissolved oxygen elution time should be determined to ensure that 11 min is an appropriate cycle time. Although the iodide peak elutes earlier using higher eluent concentrations, the separation is subject to interferences from early eluting compounds and consequently is not as reproducible as separations using lower eluent concentrations.

Amperometric detection with a silver working electrode is highly specific for iodide, and does not respond to most matrix components when analyzing milk products by ion chromatography. Potential interferences are therefore largely eliminated. The iodide from the sample combines with the silver of the working electrode surface to form silver iodide precipitate, oxidizing silver in the process. Pulsed amperometric detection allows for detection in the µg/L range and has high specificity for the iodide ion. Other halides are detected in the same manner, but less efficiently.

Because the formation of the AgI precipitate is reversible, a small dip is observed after iodide elution due to the dissolution of the AgI remaining on the electrode and concomitant reduction of silver. This dip is much smaller when using pulsed amperometry rather than dc amperometry. The dip should not be integrated as part of the iodide peak. Most importantly, standards and samples should be integrated in the same manner.

Figure 2 shows that the detection of iodide is linear over the concentration range of 25 to 10,000 µg/L ($r^2 = 0.9999$). Figure 3 shows a chromatogram of 10 µg/L iodide, which is greater than 10 times the signal to noise. When analyzing lower concentrations, be sure to check a blank injection, because as much as 1-µg/L carryover has been observed. Greater autosampler rinse volumes may reduce carryover. Lower concentrations can also be analyzed by increasing the injection volume.

Separation and detection reproducibilities were determined by repetitive analyses of 1 mg/L and 0.1 mg/L iodide standards. Figure 4 shows every injection, over a 41-h period, of a 1 mg/L iodide standard (the 8 injection gap was due to an empty vial). The peak area RSD of this analysis was 2.5% and the retention time RSD was 0.5%.

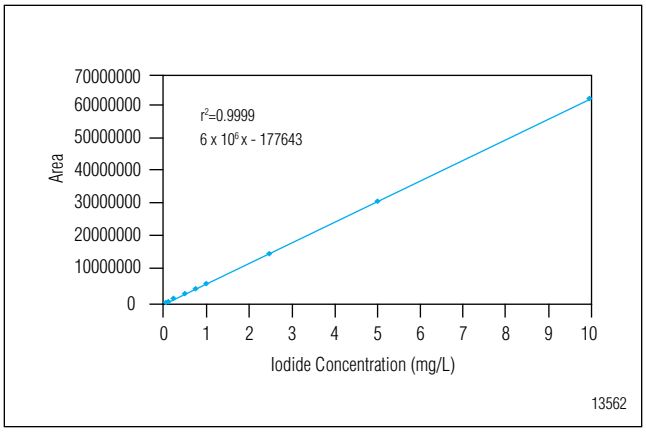

Figure 2. Iodide linearity: 0.025–10 mg/L.

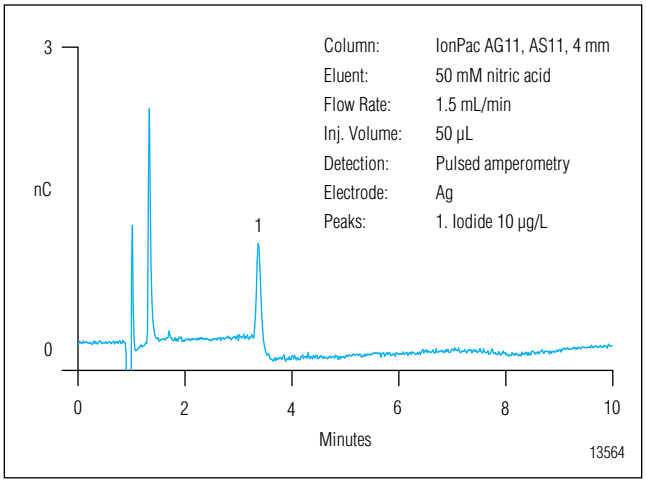

Figure 3. Low-level determination of iodide by pulsed amperometry.

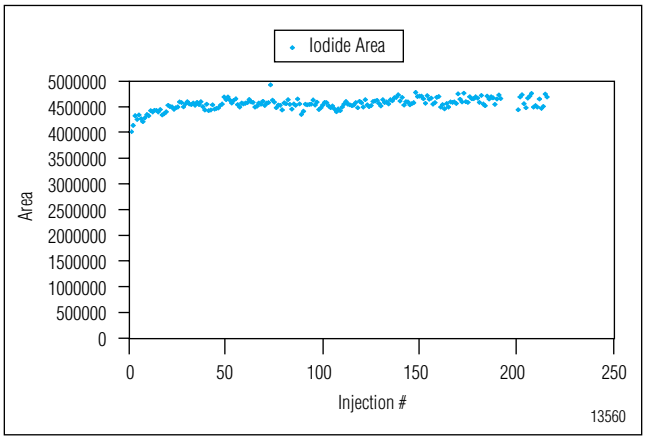

Figure 4. 41-h reproducibility of iodide analysis by pulsed amperometry.

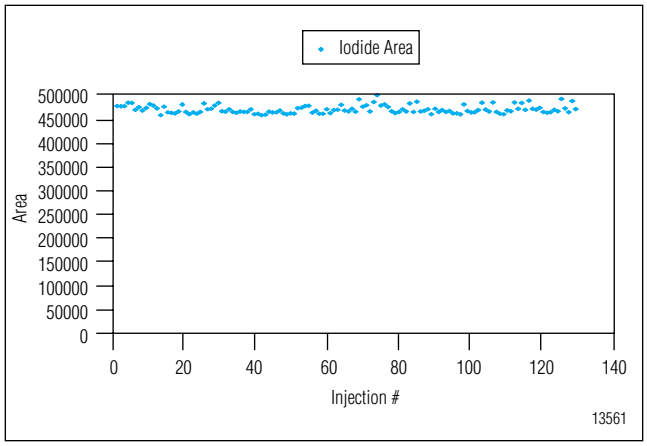

Figure 5. 24-hour reproducibility of iodide analysis, 100 ppb injected.

Figure 5 shows every injection of a 24-h analysis of a 0.1 mg/L iodide standard. In this experiment the peak area and retention time RSDs were 1.8 and 0.3%, respectively. Temperature control of the electrochemical cell and on-line degassing were critical to obtaining these low peak area RSDs.

Sample Preparation

Sample preparation should involve minimal dilution because the concentration of iodide in milk can be near the method detection limit (i.e., in the low- to mid-µg/L range). Here, 2 mL of 3% acetic acid is added to 10 mL of sample to precipitate protein, which is then removed by filtration. After filtration, sample volumes range from 11 to 14 mL. The volume of a standard treated in the same manner ranges from 16 to 17 mL. To remove fat, 5 mL of the filtrate is passed through an OnGuard RP cartridge. Failure to remove fat will lead to greater column back-pressure, loss of column capacity, and eventual column failure. The chromatographic method in this application note should be applicable to any sample preparation method that yields a clear filtrate from which fat has been removed.

Sample Analysis

Figures 6 and 7 show typical chromatograms of milk samples and a 100-µg/L standard prepared with the sample preparation method described above. Chromatograms A and B in Figure 6 and chromatogram A in Figure 7 show milk (2% milkfat), infant formula, and nonfat dried milk, respectively. The identity of iodide was confirmed by adding 10 µL of 0.1 M silver nitrate to

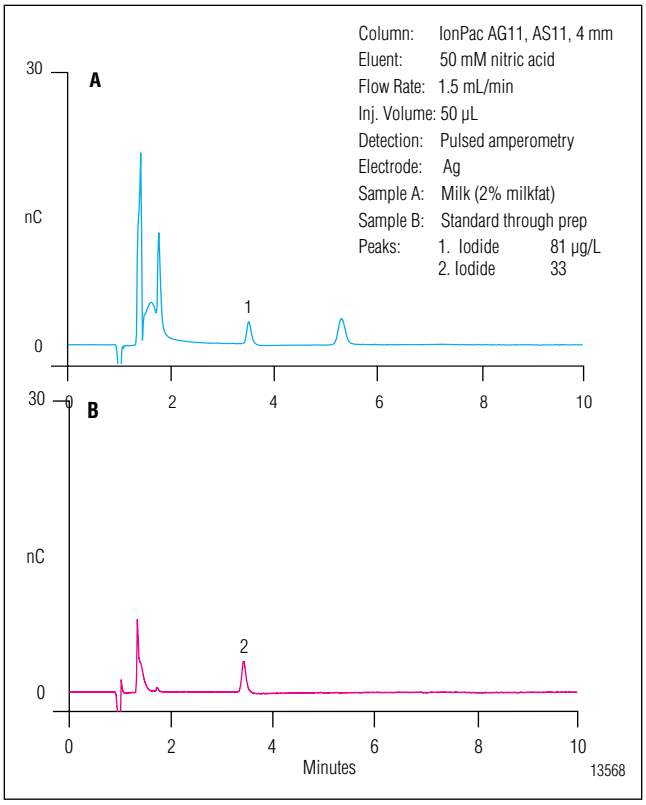

Figure 6. Analysis of iodide in milk samples by IC with pulsed amperometric detection.

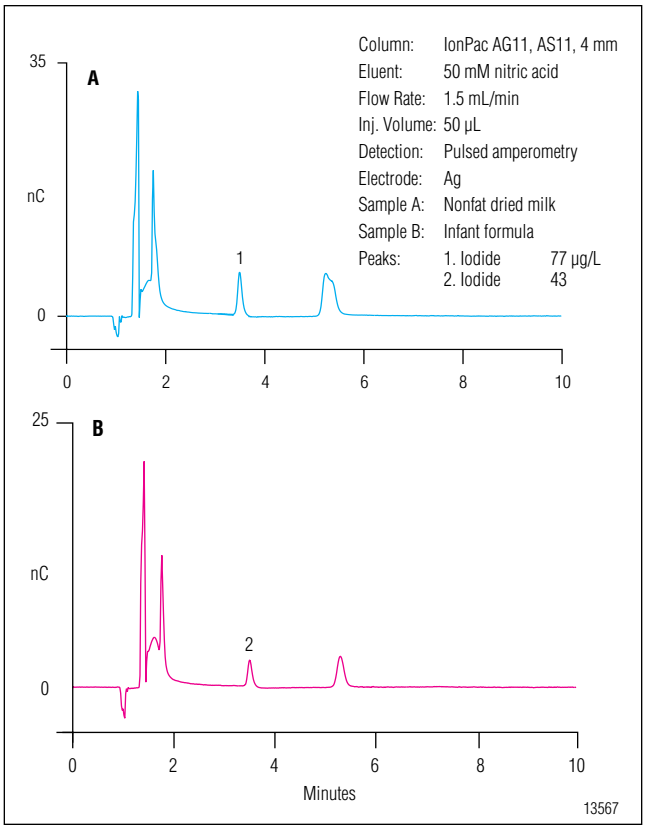

Figure 7. Analysis of iodide in milk samples by IC with pulsed amperometric detection.

Table 1. Iodide in Milk Products				
Sample	# of Samples	Conc. (µg/L)	RSD	% Recovery
Milk (2%) #1	2	152	7.9	82
Milk (2%) #2	2	134	1.1	ND
Nonfat Dried Milk	4	154	5.8	81
Infant Formula	6	66	1.3	85

200 µL of sample and analyzing for the disappearance of the iodide peak.[3] The identity of the peak at 5.2 min, present in all milk samples analyzed, is unknown. The reported concentrations are relative to an external 100-µg/L standard and have not been adjusted for sample dilution. The concentrations of all analyzed samples were between 10 and 100 µg/L. The iodide concentrations in the milk samples are reported in Table 1. These values were calculated using the average of two 100-µg/L standards prepared in the same manner as the samples and then adjusted for sample dilution.

Recovery was determined by preparing four samples and adding standard to two of the samples prior to sample preparation. Recovery was greater than 80% for all samples. Milk (2% milkfat) numbers 1 and 2 represent two different bottles of milk. The labeled value for the iodide in the powdered infant formula is equal to 61 µg/L.

For each analysis, 8 injections of each sample were analyzed. The area RSD for 8 sample injections was typically under 5%. When 50 µL of 0.1 mg/L iodide was added to 200 µL of a milk (2% milkfat) sample that had been prepared for analysis, and then analyzed, the recovery was 100%. This suggests that after preparation, the matrix does not inhibit iodide detection. Figure 8 shows that the analysis of milk samples (the blank injections) does not alter the detection of the 1-mg/L iodide standard. The iodide peak area and retention time RSDs are 1.4% and 0.4%, respectively.

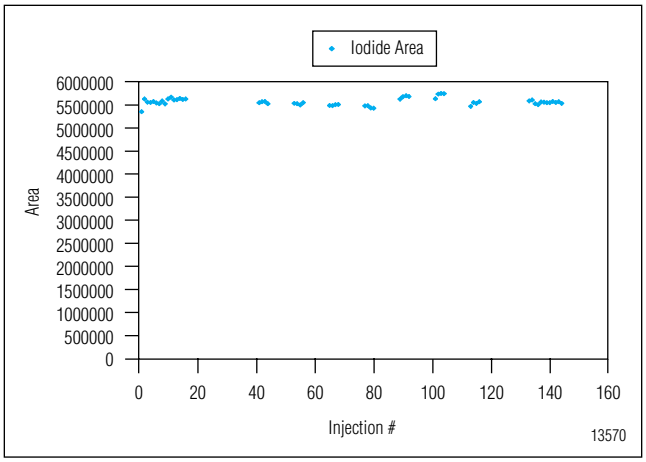

Figure 8. Reproducibility of an iodide standard (1 mg/L) during sample analysis.

PRECAUTIONS AND RECOMMENDATIONS

The IonPac AS11 column is packed in sodium hydroxide solution, so the column should be flushed with water for at least 30 min before equilibrating with the nitric acid eluent. If iodide retention time and peak efficiency start to decrease, the column can be washed with a stronger nitric acid eluent. The AS11 column is stable in the 0–14 pH range, so strong base eluents can also be used for column cleaning. It is best to disconnect the column set from the detector during column cleaning. Changing the inlet column frit or the guard column may be a faster way to restore retention time and efficiency. Installation of a 4-L eluent bottle (P/N 39164) maximizes unattended operation. For best results, the Ag/AgCl reference electrode should be replaced every 6 months.

REFERENCES

1. Hurst, W.J.; Snyder, K.P.; Martin, R.A., Jr. *J. Liq.Chromatogr.* **1983**, *6, (11),* 2067–2077.
2. Rocklin, R.D. and Johnson, E.L. *Anal. Chem*. **1982**, *55,* 4–7.
3. Chadha, W.J. and Lawrence, J.F. *J. Chromatogr*. **1990**, *518,* 268–272.

LIST OF SUPPLIERS

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J.T. Baker, Incorporated, 222 Red School Lane, Phillipsburg, New Jersey, 08865, USA.
Tel.: 800-582-2537.

Whatman LabSales, Inc., 5285 NE Elam Young Parkway, Suite A400, Hillsboro, Oregon, 97124, USA.
Tel.: 800-942-8626.

Determination of Choline in Dry Milk and Infant Formula

INTRODUCTION

Recent research indicates that choline plays an important role in cardiovascular and liver health and in reproduction and development. Choline may even help improve memory and physical performance. Milk, eggs, organ meats, and other meats are good sources of choline, whereas grains, fruits, and vegetables are poor sources. Choline is essential to proper metabolism, and is therefore often added to vitamin formulations, animal feeds, infant formulas, and sports drinks. It is usually added to these products as the bitartrate or chloride salt and supplied as a solution for oral administration.[1]

This Application Note describes methods for extraction of free and bound choline from dry milk and infant formula and its determination in the mg/L range by ion chromatography. The method also allows mineral ions (Na^+, K^+, Mg^{2+}, Ca^{2+}) to be determined simultaneously with choline.

EQUIPMENT

Dionex DX-500 Ion Chromatography System consisting of:

- GP40 Gradient Pump
- CD20 Conductivity Detector
- AS40 Automated Sampler
  - LC20 Chromatography Enclosure with rear-loading valve.
- PeakNet Chromatography Workstation

REAGENTS AND STANDARDS

Deionized water (DI H_2O), Type I reagent grade, 18 MΩ-cm resistance or better

Concentrated Sulfuric acid (18.0 M), ACS reagent grade (95–98%) (J.T. Baker or other)

Hydrochloric acid, ultrapure reagent, ULTREX® II, 36.9% (J.T. Baker or equivalent)

Choline bitartrate, 99% reagent grade or better (Aldrich or other)

BRL (Bethesda Research Laboratories) TRIS (Tris(hydroxymethyl)aminomethane)

Filter Paper, quantitative grade 494 (VWR)

CONDITIONS

Columns:	IonPac® CS12A Analytical, 4 × 250 mm (P/N 46073) IonPac CG12A Guard, 4 × 50 mm (P/N 46074)
Eluent:	18 mN Sulfuric acid
Flow Rate:	1.0 mL/min
Sample Volume:	10 µL
Detection:	Suppressed Conductivity, CSRS®-II AutoSuppression® recycle water mode
System Backpressure:	7.90–11.37 MPa (1200–1650 psi)
Background Conductance:	0.3–3 µS

PREPARATION OF SOLUTIONS AND REAGENTS

Standard Solutions

Stock choline hydroxide solution (1000 mg/L)

Dry approximately 7–10 g of choline bitartrate (MW 253.25) at 102 ˚C to a constant weight. Dissolve 2.092 g of dry choline bitartrate in 1000 mL of deionized water to prepare 1000 mg/L standard. The standard is stable for one week when stored at 4 ˚C.

Stock choline hydroxide solution for the recovery experiment (10000 mg/L)

Dissolve 2.092 g of dry choline bitartrate in 100 mL of deionized water.

Working Standards Solution

Dilute 1000 mg/L standard solution as required with deionized water to prepare the appropriate working standards.

Stock Eluent Solution

1.0 N Sulfuric acid

Weigh 972.80 g of deionized water into an eluent bottle. Degas water for approximately 5 minutes. Carefully add 27.20 mL of concentrated sulfuric acid directly to the bottle.

Working Eluent

18 mN Sulfuric acid

Weigh 982 g of deionized water into an eluent bottle. Degas water for approximately 5 minutes. Carefully add 18 mL of 1.0 N sulfuric acid solution directly to the bottle. Mix and then quickly transfer the eluent bottle to the instrument and pressurize the bottle with helium at 0.055 MPa (8 psi).

Extracting Solution

1 M Hydrochloric acid

Weigh 909.70 g of deionized water into an eluent bottle. Degas for approximately 5 minutes. Tare the bottle and carefully add 90.3 mL of ultrapure reagent-grade hydrochloric acid directly to the bottle.

Preparation of 50 mM TRIS buffer

Weigh 6.057 tris(hydroxymethyl)aminomethane into a 1-L flask containing approximately 500 mL of water. Dissolve the TRIS and adjust the pH to 8.0 by the addition of 1.0 M hydrochloric acid. Add water to a final volume of 1.00 L.

SAMPLE PREPARATION AND EXTRACTION

- Accurately weigh 5 g of dry milk sample into a 50-mL conical plastic tube with a cap (VWR).
- Add 30 mL of 1 M hydrochloric acid, cap, and mix by shaking until well dispersed.
- Place the flasks in a water bath at 70 ˚C for 3 hours, shaking every hour. Occasionally loosen or temporarily remove stoppers during the early heating stage to avoid excessive pressure build-up.
- After 3 hours, cool to room temperature.
- Filter hydrolysate through a filter paper (VWR, Quantitative Filter Paper) into a 100-mL volumetric flask. Rinse the filter with water.
- Adjust the total volume of the filtrate in a volumetric flask (± .08) to 100-mL with water.* This filtrate may be stored at 4 ˚C for three days.
- Prior to analysis, a final dilution with water should be made so that the amount of choline is in the calibrated linear range (10–200 mg/L). A 1 to 5 dilution is often appropriate.

* Note: As a precaution, phospholipase D may be added to the filtrate to release choline that may still be present as phosphatidylcholine. Choline recoveries were found to be the same with or without phospholipase D treatment for the samples analyzed in this Application Note.

Preparation of Phospholipase D Solution

1. Dissolve 150 U of phospholipase D in 200 mL of 50 mM TRIS.
2. Add 1.0 mL of enzyme solution to 1.0 mL of sample extract. Incubate at 37 ˚C for 15 minutes and cool to room temperature prior to analysis.

Determination of Choline Recovery from the Milk Samples

Add 400 µL of 10000 mg/L choline standard to 5 g of each dry milk or infant formula sample, and follow the extraction procedure described above. The final spike concentration is 10 mg/L if the final sample filtrate volume (after dilution) is 400 mL.

REPRODUCIBILITY OF CHOLINE RECOVERY FROM MILK SAMPLES

Choline was extracted from duplicates of eight milk samples and analyzed by IC.

RESULTS AND DISCUSSION

Acid digestion releases bound choline from milk samples. In this Application Note we used hydrochloric acid to extract choline from infant formula samples prior to IC analysis.

An equilibrated IC system will demonstrate a background conductance between 0.3 and 3.0 µS. Peak-to-peak noise is typically 2 nS and system backpressure is between 7.90 and 11.37 MPa (1200 and 1650 psi). A system blank is determined by using deionized water as the sample. This blank establishes the baseline and confirms the lack of contamination in the system.

Figure 1A shows that a typical chromatogram of a 100 ppm choline standard and Figure 1B an infant formula sample (Similac). The choline peak is well resolved from other peaks in the preparation. The concentrations of inorganic cations, ammonium and choline can be determined in the same run.

Figure 2 shows that the detection of choline is linear in the mg/L (ppm) range. The choice of the calibration range is based on the amount of choline usually found in milk samples.

To evaluate recovery, a known amount of choline standard was added to each of the dry milk samples. The total amount of choline was then determined following the same hydrolysis, filtration, and separation processes. Table 1 shows the recovery results. Over 95% recoveries of choline from the nonfat dry milk and low-iron infant formula were obtained.

Table 2 shows the results of an experiment in which eight infant formula and milk powder samples and their blind duplicates were analyzed. These data demonstrate good agreement for the duplicate samples that were run as part of a collaborative study[2]. Sample pairs were revealed upon completion of the study.

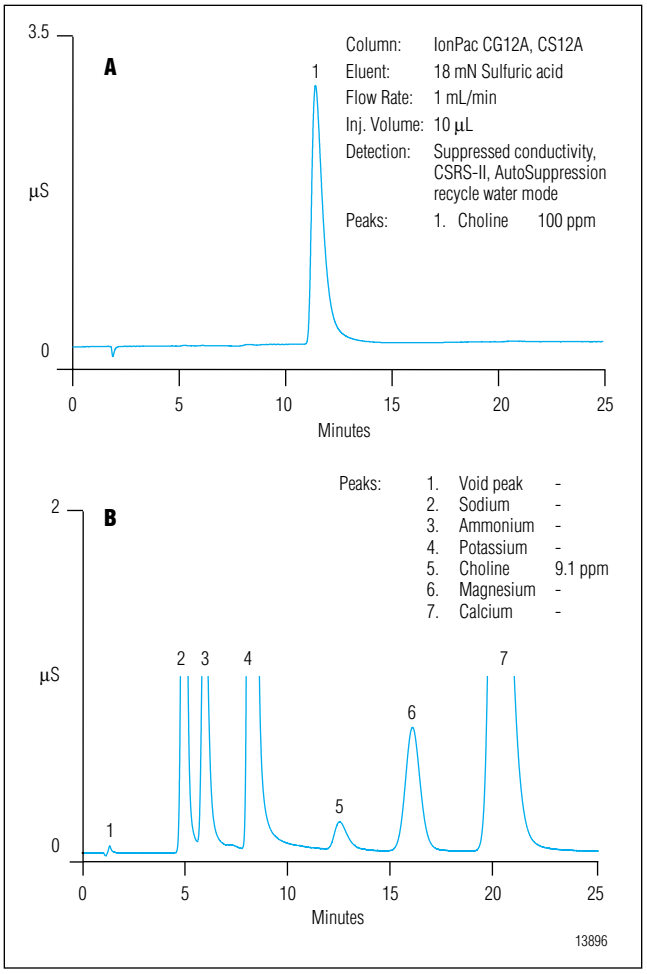

Figure 1. A) 100 ppm choline standard;B)infant formula sample.

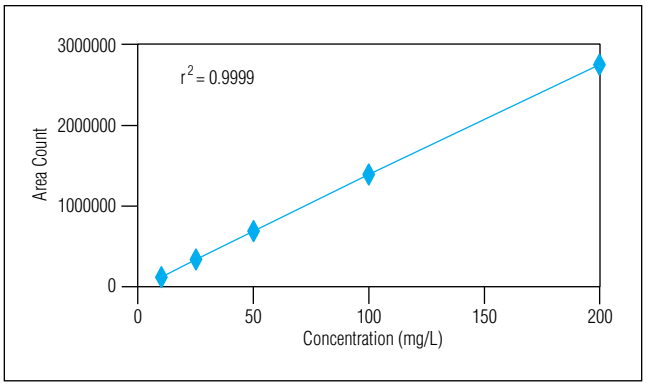

Figure 2. Choline calibration.

Table 1 Choline recoveries in milk samples

Sample Name	Amount Present (mg/L)	Amount Added (mg/L)	Total Recovered (mg/L)	Recovery (%)
Similac	9.1	10.0	18.5	94
Dry milk	14.4	10.0	23.9	95

Table 2 Precision of the choline analysis in blind duplicate infant formula and milk powder samples

Sample	Sample Concentration (mg/100 g)		% Difference (A-B)/Larger Value
	Sample A	Sample B	
1	75.0	75.3	0.40
2	60.5	62.1	2.58
3	152.7	152.0	0.46
4	39.3	39.4	0.25
5	84.1	78.6	6.54
6	111.1	103.6	6.75
7	92.2	89.2	3.25
8	109.6	110.2	0.54

SUMMARY

The method outlined in this Application Note accurately quantifies mg/L (ppm) amounts of choline in milk samples.

REFERENCES

1. Canty, D., Lecithin and Choline Redeemed. Nutrition Science News. October 1997, 1–6.
2. Wollard, D.C. and Indyk, H.E., *J. AOAC Int.* (2000) 83, 131-138.

LIST OF SUPPLIERS

Sigma Chemical Company, P.O. Box 14508, St. Louis, Missouri, 63178, USA, Tel:1-800-325-3010

Fisher Scientific, 711 Forbes Ave., Pittsburgh, Pennsylvania, 15219-4785, USA, Tel: 1-800-766-7000

VWR Scientific, P.O. Box 7900, San Francisco, California 94120, USA, Tel: 1-800-932-5000

Rapid Determination of Melamine in Liquid Milk and Milk Powder by HPLC on the Acclaim Mixed-Mode WCX-1 Column with UV Detection

INTRODUCTION

Melamine (2,4,6-triamino-1,3,5-triazine, structure shown in Figure 1) is a chemical used in some plastics and fertilizer products. Recent investigations of death and health problems of babies in China have revealed that some baby foods (milk powder) have been contaminated by melamine. Some manufacturers illegally used melamine as an adulterant to increase the apparent protein content. Melamine was also used as an adulterant to increase the apparent protein content of animal feeds and there were news reports that melamine was found in eggs obtained from some markets.

The reported methods for quantitative determination of melamine include enzyme immunoassay (EIA), gas chromatography mass spectrometry (GC-MS), liquid chromatography mass spectrometry (LC-MS), and high-performance liquid chromatography (HPLC) with UV detection.[1–4] Standard methods enacted by the Chinese government for determining melamine in raw milk and dairy products included HPLC-UV, LC-MS, and GC-MS methods.[5] However, the high cost of operation and maintenance of GC/LC-MS systems as well as the labor intensive derivatization that GC-MS requires limits their use in milk product factories. The HPLC-UV method therefore is presently the popular choice for most factories. Because numerous batches of raw milk had to be monitored, another standard HPLC-UV method for rapidly determining melamine in raw milk was soon recommended;[6] however, its application is limited in the analysis of liquid milk products.

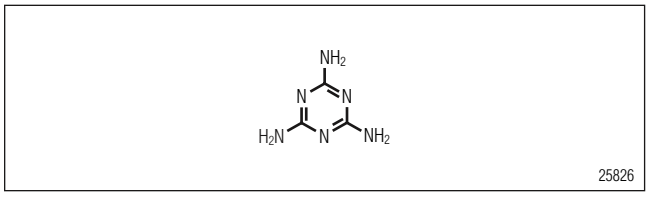

Figure 1. Structure of melamine.

In this Application Note (AN), we developed a simple HPLC method for rapid analysis of melamine in both liquid milk and milk powder samples. The separation was performed on the Acclaim® Mixed-Mode WCX-1 column[7] and UltiMate® 3000 HPLC system with UV detection using an acetate buffer and acetonitrile mobile phase. A sample analysis is completed within 10 min. The Acclaim Mixed-Mode WCX-1 column features a new mixed-mode silica-based packing material that incorporates both hydrophobic and weak cation-exchange properties, and demonstrates great potential for separating samples that contain a mixture of ionic and neutral compounds. Using an Acclaim Mixed Mode WCX-1 column with an UltiMate 3000 system allows a fast analysis of both liquid and powdered milk for melamine.

EQUIPMENT

Dionex UltiMate 3000 HPLC system consisting of:

- HPG 3400A pump
- WPS 3000TSL autosampler
- TCC-3000 thermostatted column compartment
- VWD-3400RS UV-vis Detector

Dionex Summit® UVD-340U Photodiode Array Detector

Chromeleon® 6.80 SP5 Chromatography Management Software

Kudos® SK3200LH Ultrasonic generator, Kudos Ultrasonic Instrumental Co., Shanghai, China

Mettler Toledo AL-204 Electoral o balance, Mettler-Toledo (Shanghai) Co., Shanghai, China

Anke® TGL-16B centrifuge, Anting Scientific Instrumental Factory, Shanghai, China

IKA® MS1 Minishaker, IKA Works, Guangzhou, China

REGENTS AND STANDARDS

Water, from Milli-Q® Gradient A 10

Methanol (CH_3OH), HPLC grade, Fisher

Acetonitrile (CH_3CN), HPLC grade, Fisher

Ammonium acetate (NH_4Ac), analytical grade, SCRC, China

Acetic acid (HAc), analytical grade, SCRC, China

Sodium 1-octane sulfate (98%), Baker Analyzed HPLC Reagent, USA

Melamine (99.0%), HPLC grade, Fluka

CHROMATOGRAPHIC CONDITIONS

Guard Column:	Acclaim® Mixed-Mode WCX-1, 5 µm, 4.3 × 10 mm, P/N 068354, with guard column holder, P/N 59526
Analytical Column:	Acclaim Mixed-Mode WCX-1, 5 µm, 4.6 × 250 mm, P/N 068352
Column Temp.:	30 °C
Mobile Phase:	Acetate buffer (mixture of 700 mL of 10 mM HAc and 300 mL of 10 mM NH_4Ac, ~ pH 4.3) – CH_3CN (8 : 2, v/v)
Flow Rate:	1.0 mL/min
Inj. Volume:	20 µL
UV detection:	Absorbance at 240 nm

PREPARATION OF STANDARDS

Stock Standard Solution

Accurately weigh ~100 mg of melamine, dissolve in a 100 mL volumetric flask with aqueous methanol (50%, v/v). The melamine concentration is 1000 µg/mL

Working Standard Solutions

Prepare seven working standard solutions for calibration by adding defined volumes of the stock standard solution and diluting with the acetate buffer used in the mobile phase. The concentrations of melamine are 0.05, 0.1, 0.2, 1.0, 5.0, 20, and 40 µg/mL, respectively.

PREPARATION OF SAMPLES

Milk Powder Sample

Put an accurately weighed ~1 g of dried sample into a 15 mL centrifuge tube, and then add 10 mL water. After 1 min of vortex shaking, put in an ultrasonic bath for 30 min. Add 1 mL dilute HAc (3%, v/v) and store the solution at 4 °C for at least 30 min. After 15 min of centrifugation (setting = rpm ≥ 10000), move the supernatant to a 10-mL volumetric flask, and add water to the mark. Prior to injection, filter the solution through a 0.2 µm filter (Millex-HV).

Liquid Milk Sample

Directly add 1 mL dilute HAc (3%, v/v) to an accurately measured 10 mL of liquid milk sample in a 15 mL centrifuge tube and store the solution at 4 °C for at least 30 min. The remainder of the procedure is the same as that for milk powder.

Spiked Milk Powder and Liquid Milk Samples

Add 40 µL of the stock standard solution of melamine to the 15 mL centrifuge tubes together with the accurately weighed ~1 g of dried sample, and together with the accurately measured 10 mL of liquid milk sample, respectively. The remainder of the sample preparation procedure is the same as that for the milk powder and liquid milk samples.

RESULTS AND DISCUSSION

Optimized Procedure for Preparing Milk and Milk Powder Samples

The common procedure for preparing milk and milk powder samples for melamine consists of two steps, sample extraction and cleaning the sample extract on an activated SPE column; and then drying the cleaned extract with N2 at 50 °C.[5] A simpler preparation procedure for use with an ion-exchange (IEX) analysis method was reported;[6] however, it is only for liquid milk samples. Therefore it is necessary to find an efficient and simple way to prepare both milk and milk powder samples.

The optimized procedure in this AN is simple and efficient, and does not require clean up by SPE and sample drying. It requires only precipitation with dilute acetic acid, subsequent centrifugation and filtration, and is suitable for both liquid milk and milk powder products. Additionally, this procedure is also suitable for the reversed-phase ion-pair chromatography method (RP-PIC). Figure 2 shows that this sample preparation method yields good chromatography for melamine using either the Acclaim Mixed-Mode WCX-1 column method or the RP-PIC method with the Acclaim 120 C18 column, as no interfering matrix peaks elute in the retention time range of melamine.

Optimized Chromatographic Conditions

Melamine is a hydrophilic compound that is poorly retained on a typical RP column (e.g. C18 or C8 column). Most RP methods for melamine use an ion-pairing reagent. With an ion-pairing reagent in the mobile phase, e.g. octane sulfate, melamine is well retained. However, the ion-pairing reagent may coat the RP stationary phase, changing the retention property of RP column, which may not be desired if the column is used for other methods. The RP-PIC method is also not compatible with MS detection. We therefore attempted to separate the cationic melamine on the Acclaim Mixed-Mode WCX-1 column using an ammonium acetate buffer as the eluent.

The Acclaim Mixed-Mode WCX-1 column features a mixed mode silica-based packing material that incorporates both hydrophobic and weak cation-exchange properties. Mobile phase pH affects the charge and hydrophobicity of the stationary phase. At a pH below the pKa of the stationary phase carboxylate group, the cation-exchange functionality is OFF so that hydrophobic interaction is the primary retention mechanism. At a pH above the pKa of the stationary phase carboxylate group, the cation-exchange functionality is ON so that both cation-exchange and hydrophobic interaction contribute to retention depending on the structures of analytes. Our experiments revealed that melamine does not have good retention when the pH is lower than 3.5 and higher than 5.0. Ionic strength is crucial for changing retention of charged molecules. An increase in ionic strength results in a retention decrease for melamine based on its basicity. Hydrophobic retention is markedly affected by the organic modifier composition of the mobile phase. In general, all types of molecules (acids, bases, and neutrals) are less

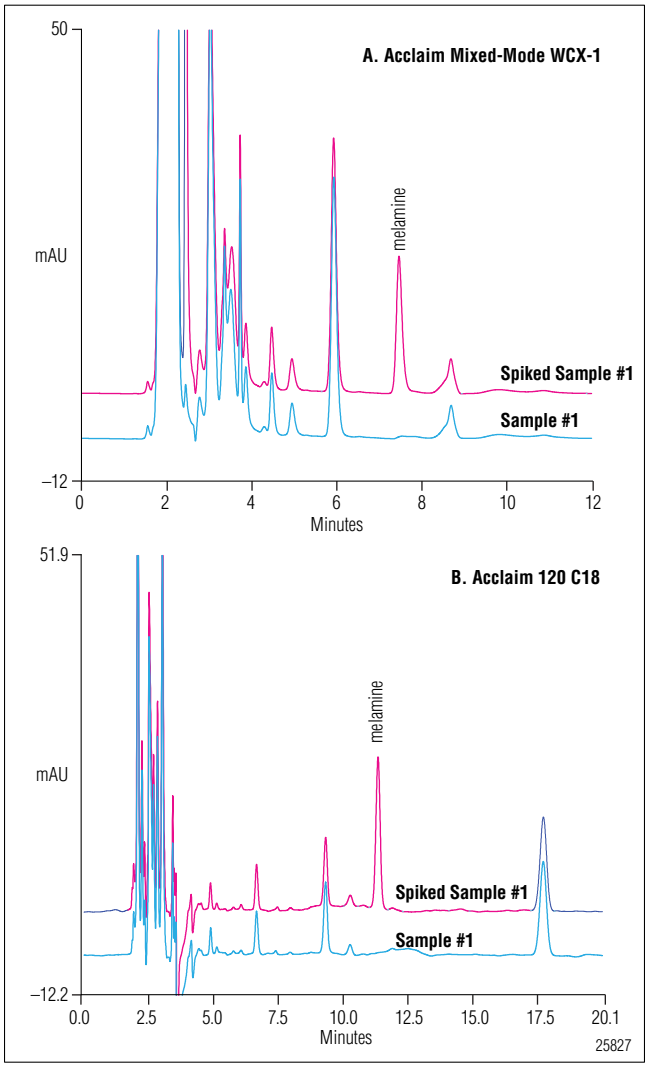

Figure 2. Chromatograms of a milk sample and the same sample spiked with melamine on (A) an Acclaim Mixed-Mode WCX-1 column, and (B) an Acclaim 120 C18 column. Chromatograms: 1, sample #1; and 2, sample #1 spiked with 4 µg/mL melamine. Melamine is detected by absorbance at 240 nm.

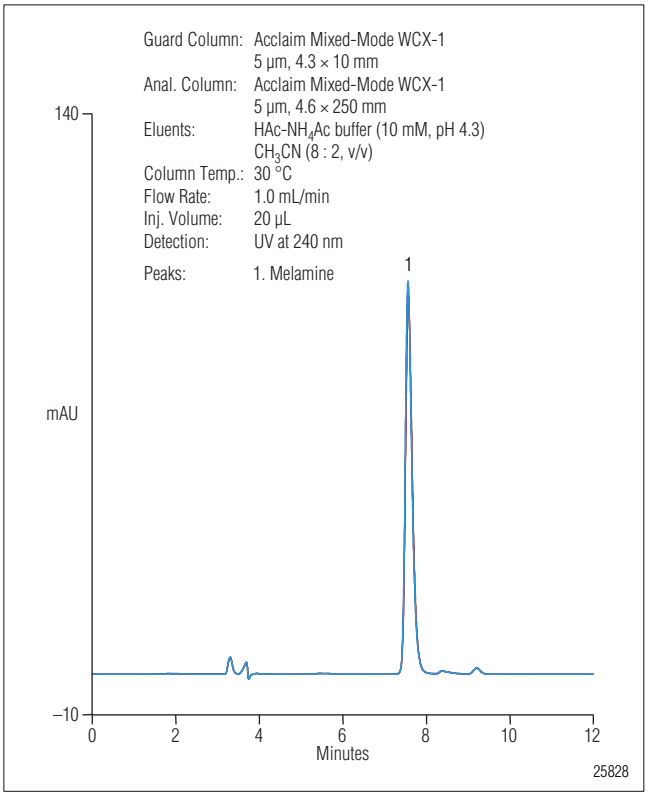

Figure 3. Overlay of chromatograms of five consecutive injections of a 20 µg/mL melamine standard.

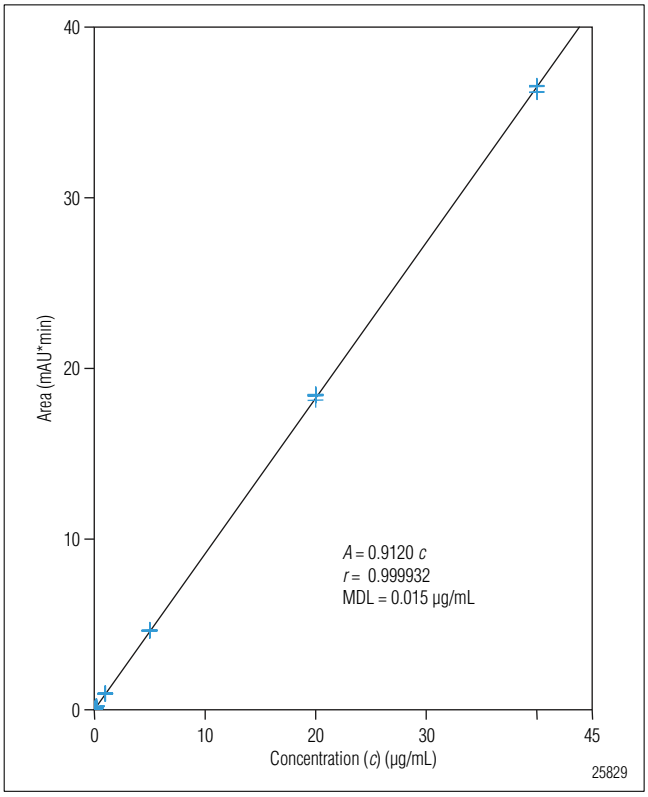

Figure 4. Calibration curve for melamine.

retained with an increase in the organic content of the mobile phase, though to different extents, when other conditions (e.g. ionic strength, pH, temperature, etc.) remain constant.7

The optimized mobile phase for these samples is 10 mM ammonium acetate pH 4.3, 20% CH_3CN. For more complex samples, a higher buffer capacity may be required. In these situations, increase the concentration of ammonium acetate. This may result in a decrease in melamine retention time, but it may be possible to restore it by decreasing the percent of CH_3CN in the mobile phase.

Chromatographic Performance

Figure 3 shows overlay chromatograms of five consecutive injections of a 20 µg/mL melamine standard. Note the good reproducibility of retention time and peak area. Calibration linearity for melamine was investigated by making five replicate injections of each standard prepared at seven different concentrations. The external standard method was used to establish the calibration curve and to quantify melamine in samples. As shown in Figure 4, excellent linearity was achieved throughout the range from 0.05 to 40 µg/mL. The linearity equation of melamine is as follows, with the curve forced through the origin.

$$A = 0.9120\ c$$

Here, A stands peak area, and c stands for melamine concentration (µg/mL). The correlation coefficient (r) is 0.9999.

Method reproducibility was estimated by making ten consecutive injections of a 1 µg/mL standard. The RSD for retention time was 0.037, and the RSD for peak area was 0.472.

The detection limit of melamine was calculated using the equation:

$$\text{Detection limit} = S t_{(n-1,\ 1-\alpha=0.99)}$$

S = standard deviation of replicate analyses

n = number of replicates

$t_{(n-1,\ 1-\alpha=0.99)}$ = Student's value for the 99% confidence level with n–1 degrees of freedom

Using the same 10 injections of 1 µg/mL standard the calculated MDL was 0.015 µg/mL.

Sample Analysis

Five samples, including three milk powder samples (#1 - #3) and two liquid milk samples (#4 and #5), were analyzed using the sample preparation method and WCX chromatography method described in this AN. No melamine was detected in #1. Melamine was found in the other milk and milk powder samples. The results were summarized in Table 1 and Figure 5 shows the chromatograms of these samples. We also spiked samples #1 and #4 with melamine before sample preparation and found that melamine was sufficiently recovered from both.

Comparison of Sample Preparation and Analysis Methods

The three milk powder samples, #1–#3, and three additional milk powder samples, #6– #8, were prepared with the sample preparation method presented in the application note and the procedure for milk powder samples that uses an activated SPE column (detailed in reference 5). These samples were then analyzed using an Acclaim 120 C18 column under the chromatographic conditions in Ref. 5 and on the Acclaim Mixed-Mode WCX-1 column under the chromatographic conditions described in this AN. This allowed an evaluation of both the simplified sample preparation procedure and the chromatography procedure in this AN. Table 2 shows the results for all six samples prepared using both sample preparation procedures and analyzed by both chromatography methods. The results show good agreement for all six samples using either of the two sample preparation methods or chromatography procedures.

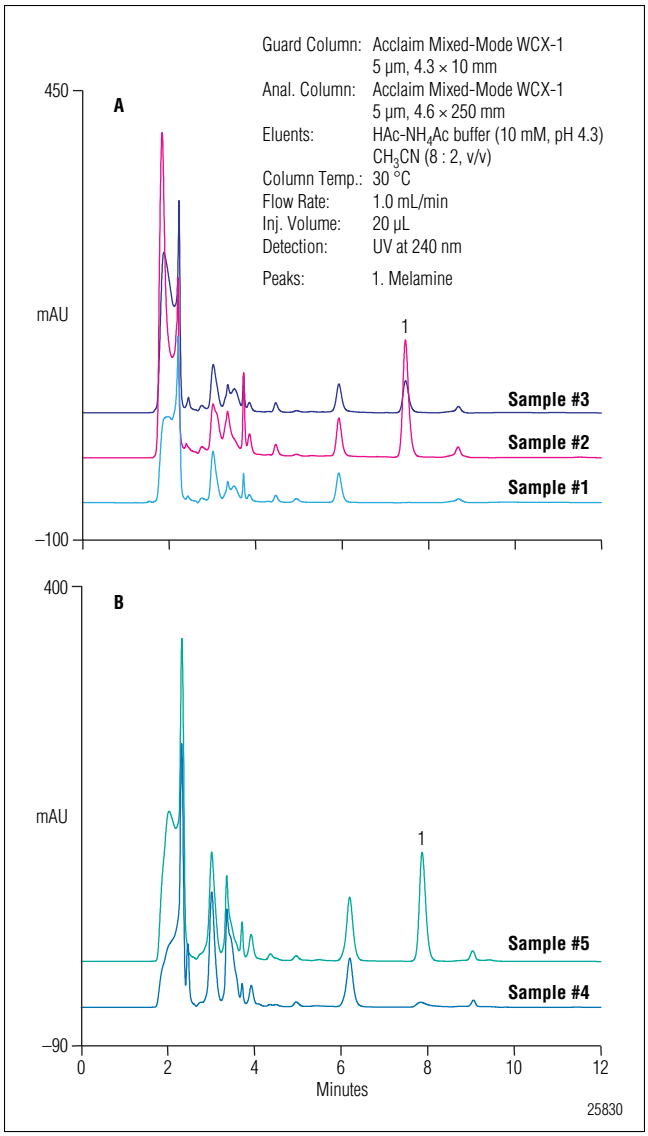

Figure 5. Chromatograms of (A) milk powder samples #1– #3, and (B) liquid milk samples #4 and #5.

Table 1.Sample Analysis Results

	Milk Powder						Liquid Milk				
	#1				#2	#3	#4				#5
	Detected (mg/Kg)	Added (mg/Kg)	Found (mg/Kg)	Recovery (%)	Detected (mg/Kg)	Detected (mg/Kg)	Detected (mg/L)	Added (mg/L)	Found (mg/L)	Recovery (%)	(mg/L)
Melamine	n.a.	40	35	88	262	67	1.8	4.0	3.6	90	22

Note: * Injections were made for each sample

** Found = Measured value of spiked sample - Measured value of sample

The complicated matrix of milk products may sometimes yield in a false positive for melamine. An efficient way to determine if the peak is melamine is by comparison of the peak's UV spectrum to that of melamine. When we analyzed sample #8, using the sample preparation method described in Ref. 5 and the Mixed-Mode WCX-1 column method, a small peak with retention time near that of melamine was found, and labeled as melamine with a concentration was 3.3 mg/Kg. The other sample preparation method and other chromatography method did not detect melamine in this sample. We reanalyzed the sample after substituting the VWD UV-Vis detector with a photodiode array detector. Comparison of the UV spectra, shown in Figure 6 revealed that the peak was not melamine. Using a photodiode array detector for this analysis will help reduce the possibility of false positives for melamine.

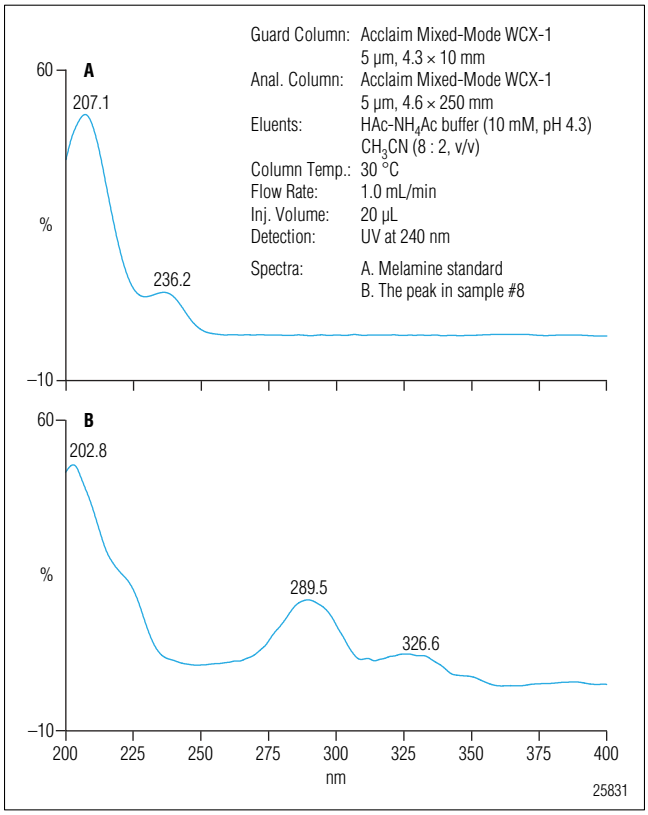

Figure 6. UV-spectra of (A) melamine standard and (B) the putative melamine peak in sample #8.

CONCLUSION

This application note describes an efficient and simple method for preparing liquid milk and milk powder samples coupled to an HPLC method for rapid analysis of melamine in these samples. The Acclaim Mixed-mode WCX-1 column exhibits good retention of melamine, using ammonium acetate buffer and acetonitrile as the mobile phase. This mobile phase should make this method compatible with MS detection.

Table 2. Comparison of Milk Powder Sample Analysis Results Using Different Sample Preparation Methods and HPLC Analysis Methods

	Melamine Concentration using the RP-PIC method (mg/Kg)[1]		Melamine Concentration using the Acclaim Mixed-Mode WCX-1 Column (mg/Kg)[2]	
Sample #	Prepared following the description in this AN	Prepared following the description in Ref. 5	Prepared following the description in this AN	Prepared following the description in Ref. 5
-	n.a.	n.a.	n.a.	n.a.
-	274	229	262	229
-	67	68	67	68
-	2.2	2.3	2.4	2.8
-	n.a.	n.a.	n.a.	n.a.
-	n.a.	n.a.	n.a.	n.a.

Note: 1. Using the chromatographic conditions described in Ref. 5.
2. Using the chromatographic conditions described in this AN.

REFERENCES

1. Filigenzi M.S., Tor E.R, Poppenga R.H., Aston L.A. and Puschner B., The determination of melamine in muscle tissue by liquid chromatography/tandem mass spectrometry. Rapid Commun. *Mass Spectrom.* **2007,** *21(24),* 4027–4032.
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3. Kim B, Perkins LB, Bushway RJ, Nesbit S, Fan T, Sheridan R, Greene V., Determination of melamine in pet food by enzyme immunoassay, high-performance liquid chromatography with diode array detection, and ultra-performance liquid chromatography with tandem mass spectrometry. *J. AOAC Int.* **2008,** *91(2),* 408-413.
4. Leo Wang, Stacy M. Henday, Xiaodong Liu, Mark Tracy and William C. Schnute, Simultaneous Determination of Melamine and Cyanuric Acid Using LC-MS with the Acclaim Mixed-Mode WAX-1 Column and Mass Spectrometric Detection. Dionex Corporation LPN 1991-01 10/07.
5. Determination of melamine in raw milk and dairy products, GB/T 22388 2008.
6. Rapid determination of melamine in raw milk (High performance liquid chromatography method), GB/T 22400 2008.
7. Dionex Corporation. *Product Manual for the Acclaim Mixed-Mode WCX-1 Column.*

Determination of Melamine in Milk Powder by Reversed-Phase HPLC with UV Detection

INTRODUCTION

Recent investigations of death and health problems of babies in China have revealed that some baby foods (milk powder) have been contaminated by melamine (structure shown in Figure 1). Some manufacturers illegally used melamine as an adulterant to increase the apparent protein content. Standard methods enacted by the Chinese government for determining melamine in raw milk and dairy products included HPLC-UV, LC-MS, and GC-MS methods.[1] However, the high cost of operation and maintenance of GC/LC-MS systems as well as the labor intensive derivatization that GC-MS requires limits their use in the milk product factories. The HPLC-UV method therefore is presently the popular choice for most factories. In this method, melamine is separated on a C8 or C18 column using an ion pair buffer (mixture of citric acid and sodium 1-octane sulfonate) and acetonitrile mobile phase.

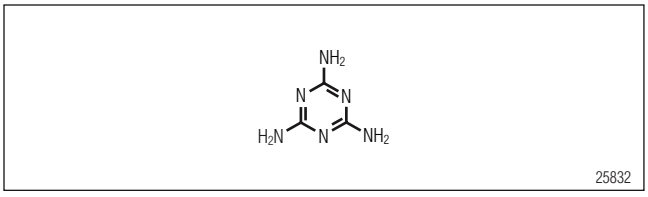

Figure 1. Structure of melamine.

In this Application Note (AN), we determined melamine in milk powder samples following the regulated method.[1] Melamine was separated from other components in the powdered milk samples using an Acclaim® 120 C18 column and an UltiMate® 3000 HPLC system with UV detection. The results of method detection limits (MDL), recovery, and permitted detection deviation match the requirements in the regulated method.

EQUIPMENT

Dionex UltiMate 3000 HPLC system consisting of:
- HPG 3400A pump
- WPS 3000TSL autosampler
- TCC-3000 thermostatted column compartment
- VWD-3400 UV-vis detector

Chromeleon® 6.80 SP5 Chromatography Data System

Kudos® SK3200LH ultrasonic generator, Kudos Ultrasonic Instrumental Co., Shanghai, China

Mettler Toledo AL-204 Electoral o balance, Mettler (Shanghai) Co., Shanghai, China

Anke® TGL-16B centrifuge, Anting Scientific Instrumental Factory, Shanghai, China

IKA® MS1 Minishaker, IKA Works, Guangzhou, China

Strata™-x-c SCX SPE (Phenomenex) column

SE-506 Nitrogen Purge Instrument, Shine Tech., Beijing, China

REAGENTS AND STANDARDS

Water, from Milli-Q® Gradient A 10

Methanol (CH_3OH), HPLC grade, Fisher

Acetonitrile (CH_3CN), HPLC grade, Fisher

Trichloroacetic acid, analytical grade, SCRC, China

Citric acid ($C_6H_8O_7 \cdot H_2O$), analytical grade, SCRC, China

Sodium 1-octane sulfonate (98%), Baker Analyzed @ HPLC Reagent, USA

Melamine (99.0%), HPLC grade, Fluka

Nitrogen (N_2, 99.999%), Lumin Gas Works, Shanghai, China

Ammonia solution (25%–28%), analytical grade, SCRC, China

CHROMATOGRAPHIC CONDITIONS

Guard Column:	Acclaim 120 C18, 5 µm, 4.3 × 10 mm, P/N 059446, with guard column holder, P/N 59526
Analytical Column:	Acclaim 120 C18, 5 µm, 4.6 × 250 mm, P/N 059149
Mobile Phase:	Buffer (dissolve 2.10 g citric acid and 2.16 g sodium 1-octane sulfonate in 980 mL H_2O, adjust pH value to 3.0 with 1 M NaOH solution, add water to the mark of 1000-mL volumetric flask)—CH_3CN (92 : 8, v/v)
Column Temp.:	40 °C
Flow Rate:	1.0 mL/min
Inj. Volume:	20 µL
UV Detection:	Absorbance at 240 nm

PREPARATION OF STANDARDS

Stock Standard Solution

Accurately weigh ~100 mg of melamine, dissolve in a 100-mL volumetric flask with aqueous methanol (50%, v/v). The concentration of melamine is 1000 µg/mL.

Working Standard Solutions

Prepare seven working standard solutions for calibration by adding defined volumes of the stock standard solution and diluting with mobile phase. The concentrations of melamine are 0.2, 0.5, 2.0, 20, 25, 50 and 100 µg/mL, respectively.

SAMPLE PREPARATION

Sample Extraction

Put an accurately weighed ~2 g of dried sample to a 50-mL centrifuge tube, and then add 15 mL aqueous trichloroacetic acid (1%, v/v) and 5 mL acetonitrile. After 1 min of vortex shaking, put in an ultrasonic bath for 30 min, and then shake for 10 min. After 10 min of centrifugation (setting = rpm ≥ 10,000), move the supernatant to a 25-mL volumetric flask while passing through filter paper, and add the 1% aqueous trichloroacetic acid to the mark.

Cleaning Sample Extract on an SCX SPE Column

Prior to use, the SPE column should be activated by passing 3 mL CH_3OH and 5 mL H_2O in turn.

Mix 5 mL of the sample extract and 5 mL water, move them to the activated SCX SPE column. Wash the SPE column with 3 mL methanol and 3 mL water, respectively, then elute with 6 mL of aminated methanol solution (mixture of 5 mL ammonia solution and 95 mL methanol). Dry the collected eluent with N_2 at 50 °C, dissolve the residue in 1-mL mobile phase, and then vortex the solution for 1 min. Prior to injection, filter the solution through a 0.2-µm filter (Millex®-HV).

Spiked Milk Powder Samples

Add 20 µL of stock standard solution of melamine to the 50-mL centrifuge tubes together with the accurately weighed ~2 g of dried sample. The remainder of the sample preparation procedure is the same as that for the milk powder sample.

RESULTS

Method Reproducibility

Figure 2 shows an overlay of chromatograms of seven melamine standards with different concentrations. The RSD for retention time is 0.143%.

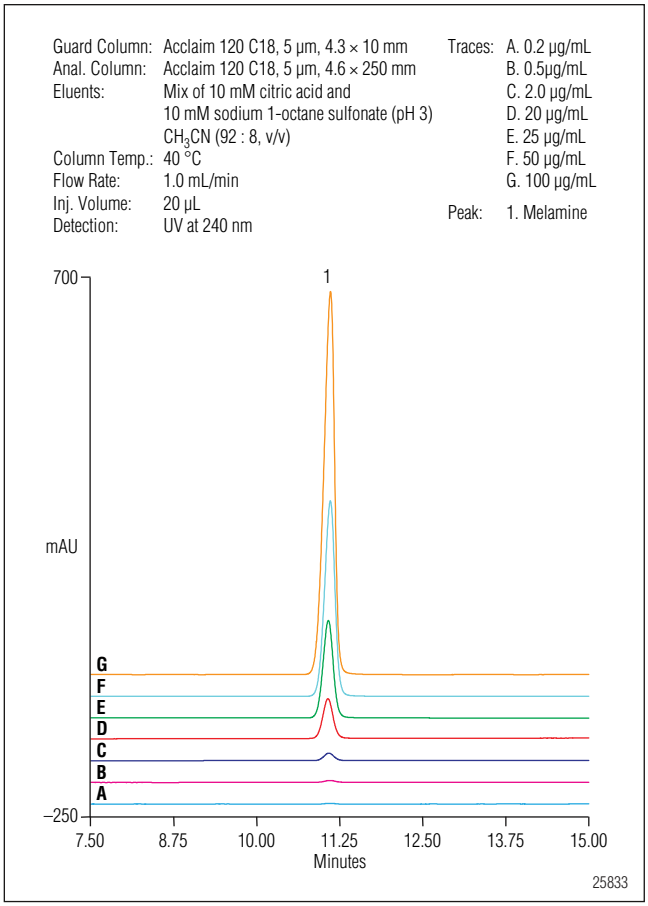

Figure 2. Overlay of chromatograms of seven melamine standards with different concentrations.

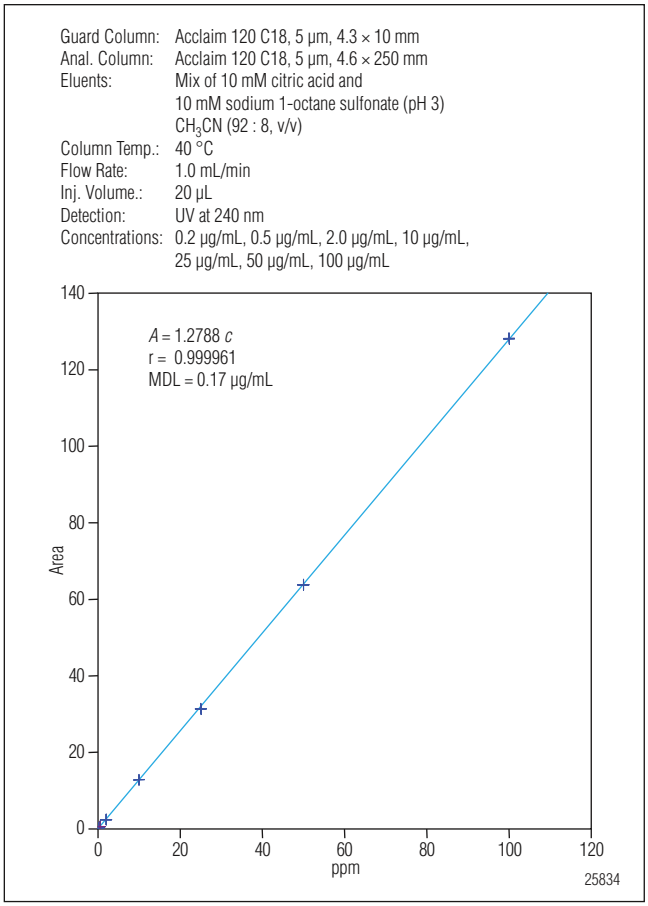

Figure 3. Calibration curve for melamine.

Linearity and Detection Limit

Calibration linearity for melamine was investigated by making three replicate injections of each standard prepared at seven different concentrations. The external standard method was used to establish the calibration curve and to quantify melamine in samples. As shown in Figure 3, excellent linearity was achieved throughout the range from 0.2 to 100 µg/mL. The linearity equation of melamine is as follows with the curve forced the through origin.

$$A = 1.2788\ c$$

Where, A stands peak area, and c stands melamine concentration (µg/mL). The correlation coefficient (r) is 0.999961.

Method detection limit (MDL) of melamine was calculated by using S/N = 3, where S = signal, N = noise. The calculated value of MDL is 0.17 µg/mL.

Sample Analysis

Five milk powder samples (1–5) obtained from a manufacturer were analyzed. Melamine was found in samples #1 through #4. An amount of melamine less than the MDL was detected (0.12 µg/mL) in sample 5 and therefore can not be reliably identified as melamine. The results are summarized in Table 1. Figure 4 is an overlay chromatograms of these samples.

Table 1. Sample Analysis Data								
Sample #	**1**				**2**	**3**	**4**	**5**
Melamine	**Detected (µg/mL)**	**Added (µg/mL)**	**Found (µg/mL)**	**Recovery (%)**	**Detected (µg/mL)**	**Detected (µg/mL)**	**Detected (µg/mL)**	**Detected (µg/mL)**
	0.83	4.0	3.2	80	0.58	0.61	3.6	0.12

Notes: 1. 3 injections were made for each sample
2. Spiked sample was prepared according to the description in the sample section.
3. The detected amount in the list is for the prepared sample (diluted sample).
4. The amount in original sample should be calculated by multiplying "Diluted fold", which is 2.5 in this Application Note.
5. Found = Measured Value of spiked sample - Measured Value of sample.

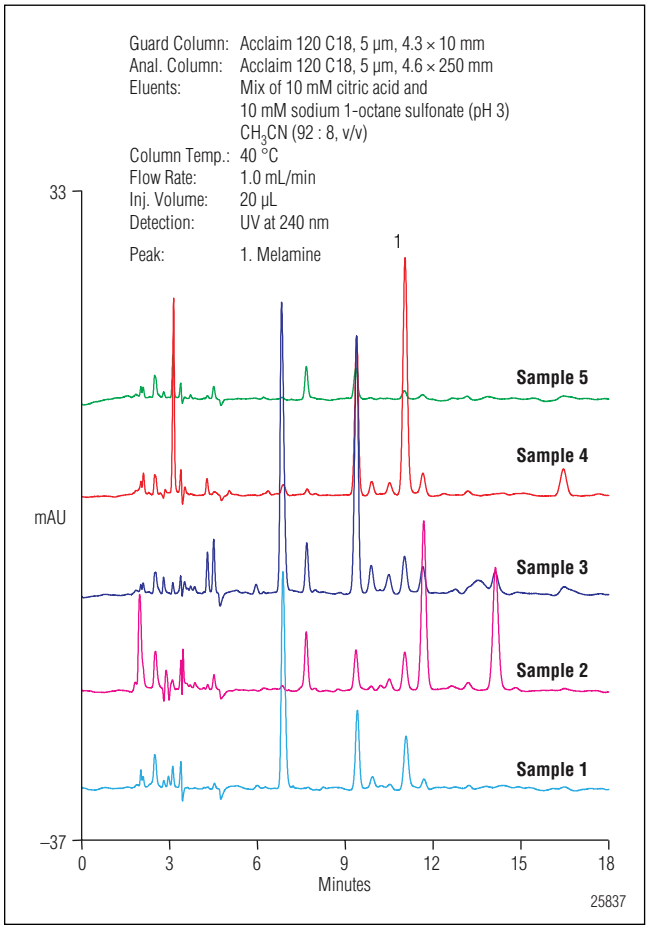

Figure 4. Chromatograms of five milk powder samples.

CONCLUSION

The determination of melamine in milk powder was performed on the Acclaim 120 C18 column and UltiMate 3000 HPLC system with UV detection following the regulated method.1 Table 2 lists the comparison of chromatographic performance data obtained in this experiment to the requirements in the standard method, which demonstrates that the results match the requirements in the regulated method.

Table 2. Method Performance		
	Method in this Application Note	**Requirement in the Regulated Method**
Correlation coefficient (r)	0.999961	\
Linearity range (µg/mL)	0.2 ~ 100	0.8 ~ 80
Recovery (%)	80	80 ~ 110

Note: Regulated method, see Ref. 1.
\ Not specified.

REFERENCE

1. Determination of Melamine in Raw Milk and Dairy Products, *GB/T 22388* **2008.**

Determination of Melamine in Milk by Ion Chromatography with UV Detection

INTRODUCTION

In 2008, melamine was found as a contaminant of milk and milk-containing products after the discovery of melamine contamination of pet food. These contaminations resulted in infant and pet deaths. Both deliberate contaminations originated in China, with some contaminated products exported to neighboring and more distant countries. Melamine was added to both products to increase their apparent protein content, as it was determined by a nonspecific total nitrogen test and melamine has a large amount of nitrogen per unit mass.

Dionex has designed two reversed-phase HPLC methods to determine the melamine adulteration of liquid and powdered milk.[1, 2] One method is an ion-pairing HPLC method using an Acclaim®120 C18 column and the other method uses the Acclaim Mixed-Mode WCX column, where both the hydrophobic and cationic properties of melamine are used to affect the separation. As a cation, melamine can be separated by cation-exchange chromatography and therefore be determined by Ion Chromatography (IC). This Application Note (AN) shows how melamine can be determined in milk, powdered milk, and a milk-containing candy by IC using an IonPac® CS17 column and UV detection at 240 nm. This gives the analyst another chromatographic option, providing a selectivity that may be needed for some samples, or a way to increase sample throughput with existing laboratory instrumentation.

EQUIPMENT

Dionex ICS-3000 consisting of:

- DP Dual Pump
- DC Detector/Chromatography module with dual temperature zone equipped with
- 6-port valve (injection valve)
- AM Automation Manager equipped with,
- 10-port valve (high pressure valve)
- EG Eluent Generator
- AS Autosampler
- PDA-3000 Photodiode Array Detector*

Chromeleon® 6.8 Chromatography Data System

* The Dionex VWD detector can also be used for this application. The photodiode array detector is required to confirm the melamine peak identity with the peak purity option.

Conditions

Column:	IonPac CS17 Analytical, 4 × 250 mm (P/N 060557)
Guard:	IonPac CG17 Guard, 4 × 50 mm (P/N 060560)
Concentrator:	IonPac TCC-LP1, 4 × 35 mm (P/N 046027)
Eluent Source:	EGC II MSA (P/N 058902) with CR-CTC (P/N 066262)
Gradient:	See chromatogram
Flow Rate:	Pump 1:1.0 mL/min Pump 2:1.0 mL/min
Inj. Volume:	See chromatogram
Pressure:	~2100 psi
Detection:	UV at 240 nm

REAGENT AND STANDARDS

Deionized water (DI), Type I reagent grade, 18 MΩ-cm resistivity or better

Melamine (Sigma-Aldrich)

Acetic acid (Labscan)

PREPARATION OF SOLUTIONS AND REAGENTS

Eluent Solution

The eluent generator produces the eluent using the EluGen EGC II MSA cartridge and deionized water supplied by the pump, with the eluent concentration controlled by the Chromeleon software. Backpressure tubing must be added to achieve 2000–2500 psi backpressure that will allow the EG degasser to function properly. See the *ICS-3000 Ion Chromatography System Operator's Manual,* (P/N 065031-03) for instructions on adding backpressure.[3]

Standard Solutions

Stock Standard Solutions (1000 mg/L)

To prepare the 1000 mg/L melamine standard, dissolve 0.1 g of melamine in 100 mL of deionized water.

Secondary Standards

Prepare a 1 mg/L secondary standard from the stock standard. From this secondary standard, prepare the standard calibrations and MDL standards as follows.

1. Calibration standards. To prepare melamine standards at concentrations of 25, 50, 100, 200, 400, and 800 µg/L, add the appropriate volumes of 1 mg/mL standard to separate 100 mL volumetric flasks. For example, add 5.0 mL of 1 mg/L standard for the 50 µg/L standard. Bring to volume with deionized water. These six standards were used to calibrate one of the IC methods presented here, and for the second method, we prepared an additional 12.5 µg/L standard and calibrated with seven standards.

2. MDL standard. Prepare a 25 µg/L melamine standard by adding 2.5 mL of 1 mg/L melamine standard to a 100 mL volumetric flask and bring to volume with deionized water.

SAMPLE PREPARATION

OnGuard RP Preparation

Flush the OnGuard RP cartridge with 5 mL of methanol and then with 10 mL of deionized water at about 4 mL/min.

Liquid Milk Preparation

1. Mix 10 mL of liquid milk and 8 mL of deionized water.
2. Add 2 mL of 3% acetic acid and mix.
3. Pass the sample through a Whatman 2V filter.
4. Pass 5 mL of the filtered sample through a prepared OnGuard RP cartridge, discarding the first 3 mL and collecting the remaining sample into an AS vial.

Milk Powder and Candy Sample Preparation

Add about 5 g of sample to a 50 mL volumetric flask, dissolve, and bring to volume with deionized water. Take 10 mL of this sample and prepare by the same method as the liquid milk sample.

Samples Spiked with Melamine

To prepare a 50 µg/L melamine spiked sample, in step 1 of the sample preparation instead of adding 8 mL of deionized water to the sample, add 1 mL of 1 mg/L melamine secondary standard and 7 mL of deionized water. For the 100 µg/L standard, use 2 mL of 1 mg/L melamine and 6 mL deionized water.

Calculating Amount of Melamine in the Milk-Containing Candy Sample

The sample preparation involved dissolving 5.079 g of candy in 50 mL DI water and diluted 1:1.

Amount of melamine in 100 µL of the prepared candy sample:

$= (13.78\ \mu g/L) \times (L/1{,}000{,}000\ \mu L) \times 100\ \mu L$

$= 13.78 \times 10^{-4}\ \mu g$

Amount of candy in the 100 µL injection:

$5.079 g/50\ mL \times ((mL/1000\ \mu L)/2) \times 100\ \mu L$

$= 5.079 \times 10^{-3}\ g$

Amount of melamine per g of candy:

$= 13.78 \times 10^{-4}\ \mu g / 5.079 \times 10^{-3}\ g$

$= 0.27\ \mu g/g$

RESULTS AND DISCUSSION

Melamine is a cation and, therefore, can be separated from other compounds by cation-exchange chromatography. The IonPac CS17 column was designed for the separation of hydrophobic amines like melamine. While melamine is a cation at neutral pH, it is not fully ionized at pH 7 and therefore, suppressed conductivity does not provide a sensitive detection method for this compound. Sensitivity can be increased using the salt converter cation self-regenerating suppressor. However, more sensitivity and selectivity for melamine was found by using absorbance detection at 240 nm.

Milk and milk-based products can be difficult for chromatographic methods due to the large variety of compounds present that can interfere with the analytes of interest. To determine melamine, we used a sample preparation technique first developed for the IC determination of iodide in milk.4 Despite this sample preparation, we found it difficult to determine melamine in the sample. Therefore, we first loaded the sample loop installed on the AM-HP1 with the prepared sample. Then, using deionized water, we moved the sample onto the cation-exchange concentrator installed on the injection valve, and then eluted from the concentrator, directly onto the IonPac CS17 column set. Figure 1 shows the schematic of this system configuration and Table 1 shows the valve programming that allows the sample delivery to the concentrator while washing unbound compounds to waste, and subsequent chromatography. This method ultimately proved more successful for melamine determination.

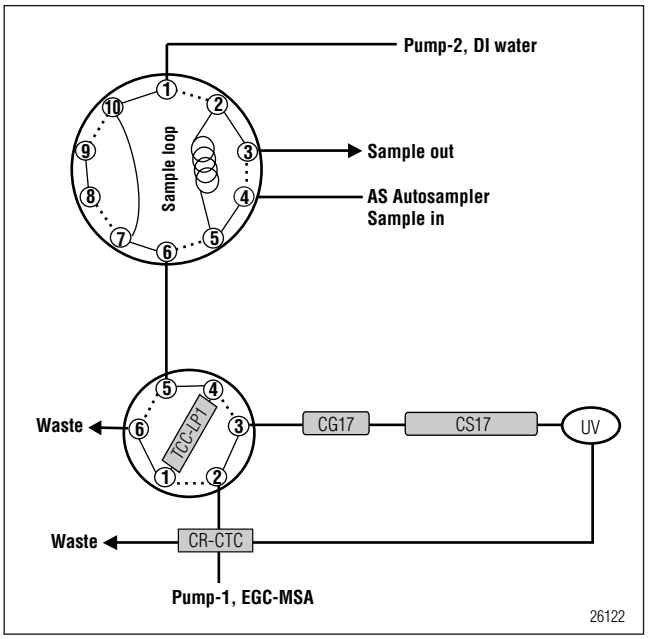

Figure 1. System configuration schematic.

Table 1. Valve Switching Program

Retention Time (min)	AM_HP1	Injection Valve	Note
–5.0	A	Load	
–3.0	B	Load	AS loads the sample to sample loop before the AM_HP1 switches from A to B.
0.0	A	Inject	
End run	A	Inject	The end of runtime depends on the gradient.

Prior to quantitative sample analysis, we calibrated the method as described in the section *Secondary Standards,* earlier in this application note. Figure 2 shows the chromatography from the calibration, which was linear with a correlation coefficient of 0.9998. To estimate the minimum detection limit, we made seven injections of the 25 µg/L standard. Figure 3 shows the seven injections along with the blank, an injection of water. The blank shows that there are no peaks from the water or chromatography system interfering with melamine determination. Table 2 shows the data from the MDL experiment and that the MDL estimate was 4.4 µg/L.

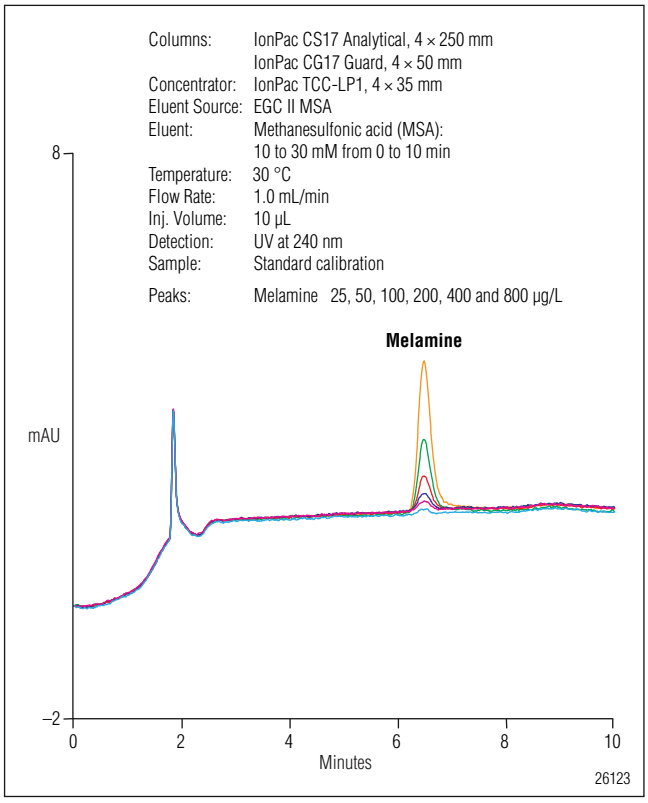

Figure 2 Chromatograms of six melamine standards used for calibration.

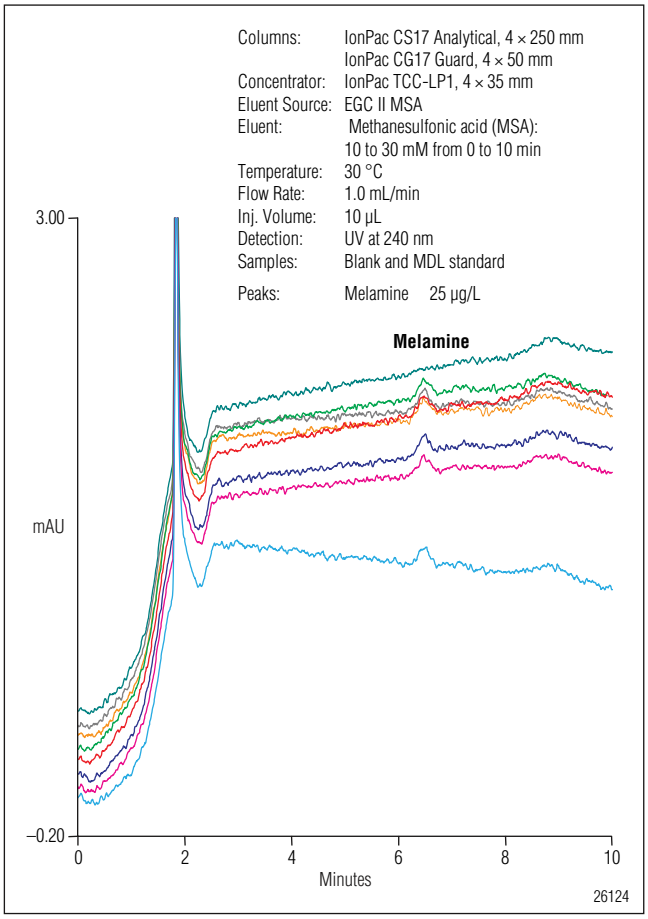

Figure 3. Chromatograms of a water injection (blank) and seven consecutive injections of 25 µg/L melamine.

After method qualification, we evaluated the melamine content of milk, milk powder, and a milk-containing candy. Only the candy was known to contain melamine. Figures 4 and 5 together with Tables 3 and 4 show the results of the determinations of melamine in milk and milk powder. The analysis shows that neither sample contained melamine.

To demonstrate that melamine was not lost during sample preparation, melamine was added to each sample prior to sample preparation. One portion of each sample was spiked with 50 µg/L melamine and a second portion was spiked with 100 µg/L melamine. The chromatography in Figures 4 and 5 and the quantitative results in Tables 3 and 4 show that melamine was recovered from both samples with recoveries greater than 90%.

Table 2. Data from Seven Consecutive Injections of 25 µg/L Melamine

Injection No.	Height (mAU)
1	0.0906
2	0.1071
3	0.0959
4	0.0924
5	0.0948
6	0.0969
7	0.0999
Average:	0.0968
RSD:	5.63
MDL (µg/L):	4.4

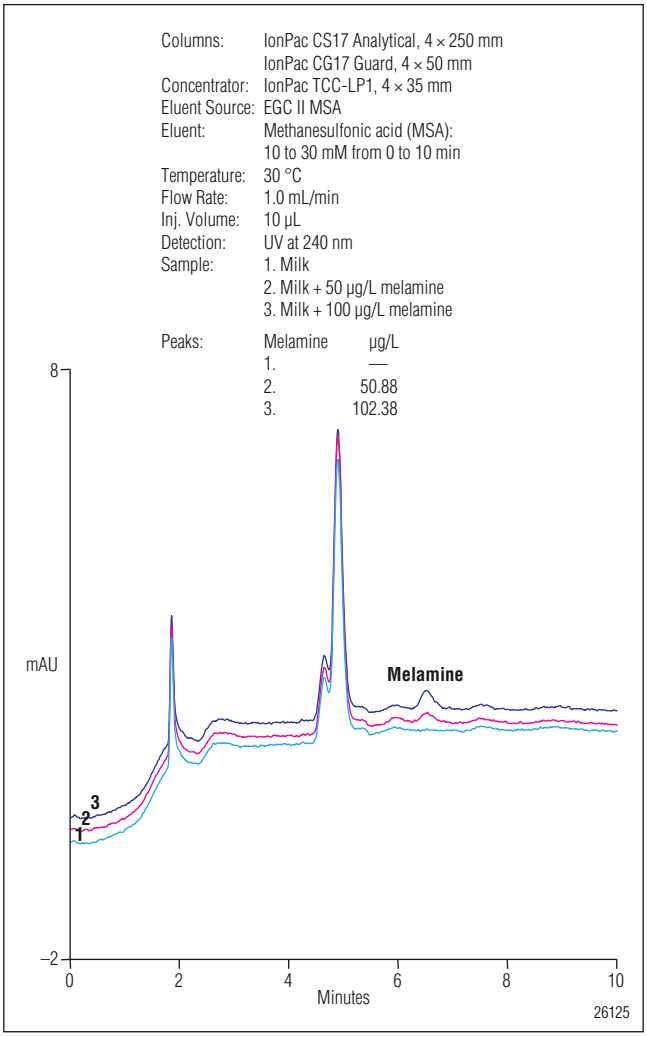

Figure 4. Chromatograms of milk and milk spiked with melamine. Milk (1); milk + 50 µg/L melamine (2) ; and milk + 100 µg/L melamine (3).

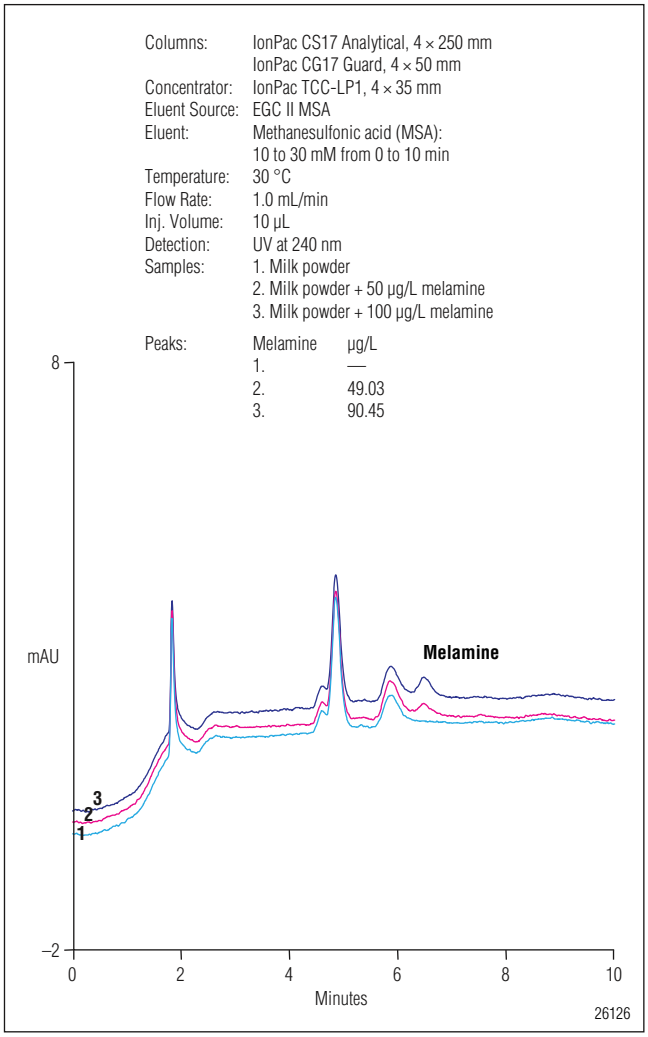

Figure 5. Chromatograms of milk powder and milk powder spiked with melamine. Milk (1); milk + 50 µg/L melamine (2); and milk + 100 µg/L melamine (3).

Table 3. Recovery of Melamine in the Milk Sample

Injection #	Amount (µg/L)		
	Liquid Milk	Liquid Milk + 50 µg/L Melamine	Liquid Milk + 100 µg/L Melamine
1	ND	53.58	96.20
2	ND	53.12	110.31
3	ND	44.42	93.54
4	ND	52.00	100.33
5	ND	51.27	111.51
Average:	NA	50.88	102.38
RSD:	NA	7.32	7.98
% Recovery:	NA	101.8	102.4

Table 4. Recovery of Melamine in the Milk Powder Sample

Injection #	Amount (µg/L)		
	Milk Powder	Milk Powder + 50 µg/L Melamine	Milk Powder + 100 µg/L Melamine
1	ND	49.56	92.30
2	ND	56.27	89.74
3	ND	45.07	88.04
4	ND	49.16	88.74
5	ND	45.07	93.44
Average:	NA	49.03	90.45
RSD:	NA	9.35	2.57
% Recovery:	NA	98.06	90.45

The analysis of the melamine-containing candy sample proved more difficult. Melamine was not completely resolved from another peak. This was not observed in the milk and milk powder samples. To resolve these two peaks, we changed the mobile phase composition from a 10 min 10–30 mM MSA gradient to 5 mM MSA for 20 min. Due to the use of a RFIC system, this mobile phase change and other changes made to arrive at the final method did not require the preparation of new eluents. We simply used the Chromeleon chromatography workstation to instruct the eluent generator to prepare a new mobile phase.

Our initial chromatography of the candy sample also suggested that there was only a small amount of melamine in the sample. Therefore, when we calibrated the system for the new separation method, we added a lower concentration standard (12.5 µg/L) to the calibration and increased the injection volume from 10 to 100 µL. The calibration was linear with a correlation coefficient of 0.9997.

Figure 6 and Table 5 show the results of the analysis of the candy sample for melamine. The candy sample contained melamine with a concentration of about 14 µg/L in the prepared sample, or 0.27 µg/g in the candy. To assess the accuracy of this determination, we prepared two spiked candy samples with (a) a 10 µg/L spike, and (b) a 20 µg/L spike. Melamine was recovered from both samples suggesting that the method is accurate. After installing the photodiode array detector on our system, we also confirmed that the melamine peak in the candy sample was a spectral match to the melamine standard.

This IC method accurately determined melamine in milk, milk powder, and a milk-containing candy after a simple sample preparation. As this method uses a RFIC system, the analyst does not have to prepare eluents and can easily change the mobile phase for samples where unknown peaks coelute with melamine.

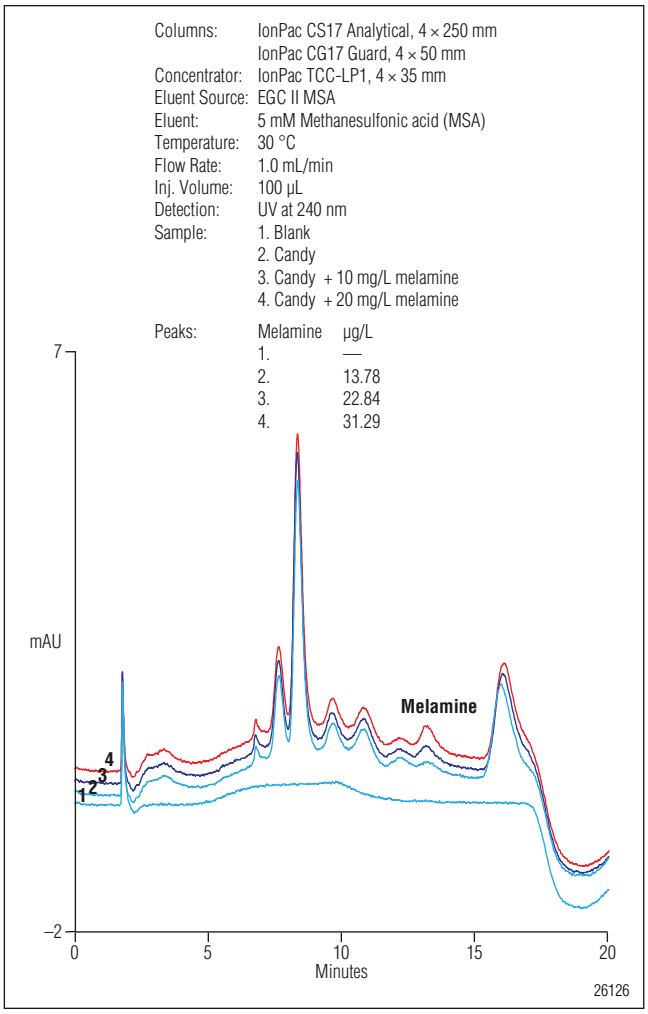

Figure 6. Chromatograms of milk-containing candy and candy spiked with melamine. Blank (1); candy (2); candy + 10 µg/L melamine (3); and candy + 20 µg/L melamine (4).

Table 5. Recovery of Melamine in the Candy Sample

Injection #	Amount (µg/L)		
	Candy	Candy + 10 µg/L Melamine	Candy + 20 µg/L Melamine
1	14.27	23.16	28.86
2	15.31	25.46	31.47
3	12.70	21.96	31.23
4	12.49	21.59	32.56
5	14.10	22.05	32.32
Average:	13.78	22.84	31.29
RSD:	8.51	6.91	4.69
% Recovery:		90.66	87.55

REFERENCES

1. *Rapid Determination of Melamine in Liquid Milk and Milk Powder by HPLC on the Acclaim Mixed-Mode WCX-1 Column with UV Detection.* Application Note 221 (LPN 2181, March 2009), Dionex Corporation, Sunnyvale, CA.
2. *Determination of Melamine in Milk Powder by Reversed-Phase HPLC with UV Detection.* Application Note 224 (LPN 2184, March 2009), Dionex Corporation, Sunnyvale, CA.
3. *ICS-3000 Ion Chromatography System Operator's Manual,* Document No. 065031-03. Dionex Corporation, Sunnyvale, CA
4. *Determination of Iodide in Milk Products.* Application Note 37 (LPN 0702-03, October, 2004), Dionex Corporation, Sunnyvale, CA.

Determination of Total Fat in Powdered Infant Formula Using Accelerated Solvent Extraction (ASE)

INTRODUCTION

New government regulations, such as the U.S. Nutritional Labeling and Education Act of 1990, require food products to carry labels that list the content of saturated and unsaturated fats. Accurate determination of fat in certain foods is difficult due to the binding or entrapment of the fat by the matrix. Most methods used to determine fat in these difficult matrices include a pretreatment step to denature or destroy the physical structure of the matrix and allow greater accessibility to the fat (e.g., AOAC Intl. Method 933.05, Fat in Cheese, calls for sample treatment with ammonium hydroxide followed by hydrochloric acid). Ammonium hydroxide is a common reagent used to pretreat dairy-based products before extraction because it dissolves casein. Common fat determination methods used by the dairy industry are the Roese-Gottlieb and Modified Mojonnier methods (AOAC Intl. Methods 905.02 and 989.05, respectively). These methods specify the use of ammonium hydroxide to dissolve casein and liberate the fat.

Accelerated Solvent Extraction (ASE) is a new automated extraction technique that can significantly reduce extraction time and solvent consumption and yields equivalent results, without requiring a pretreatment step. Samples are loaded onto the ASE system and solvent is pumped into an extraction cell, which is then pressurized and heated for several minutes. The increased temperature allows extractions to be done in a fraction of the time required for traditional extractions perfomed at room temperature or with warm solvents. Extraction under pressure allows the solvents to be heated while maintaining their liquid state. Following the extraction step, the ASE system transfers the solvent, along with the dissolved components of interest, into a collection vial (within the instrument) for further cleanup or analysis. The extraction process is automated and up to 24 samples can be extracted sequentially.

ASE has shown significant advantages over competing techniques with regard to time savings, solvent use, automation, and efficiency. In this application note, fat is extracted from infant formulas by ASE without aggressive pretreatments. The results are equivalent to traditional pretreatment/extraction methods. The procedures described in this application note apply to powdered infant formulas and may be suitable for similar matrices. The fat content in these examples is determined by collecting the extract in preweighed vials, evaporating the solvent and any extracted water and reweighing the vials. The extraction efficiency was verified by fatty acid methyl ester (FAME) analysis of the dried extract after the gravimetric recovery had been determined.

EQUIPMENT

ASE 200 Accelerated Solvent Extractor equipped with 11-mL stainless steel extraction cells

Cellulose filter disks (P/N 49458)

Analytical balance (0.001 g or better)

Mortar and pestle

Solvent evaporator (TurboVap®, Caliper Life Sciences)

Forced air oven

Sand (Ottawa Standard, Fisher Scientific, Cat. No. S23-3)

REAGENTS

ASE Prep DE (diatomaceous earth) (P/N 062819)

SOLVENTS

Acetone

Hexane

Water

All solvents are pesticide-grade or equivalent and available from Fisher Scientific.

EXTRACTION CONDITIONS

Solvent:	Hexane acetone, 4:1 volume
Temperature:	125 °C
Pressure:	10.3 MPa (1500 psi)
Cell Heatup:	6 min
Static Time:	5 min
Flush Volume:	100%
Purge Time:	60 s
Cycles:	3
Total Time:	24 min
Total Solvent:	20–25 mL

SAMPLE PREPARATION

Without Water (Similac®, Enfamil®, SRM 1846)

Place a cellulose filter in each extraction cell before loading the sample. Samples are mixed with ASE Prep DE to prevent sample compaction and ensure efficient solvent contact. Weigh approximately 1 g of sample, to the nearest 0.1 mg. Grind with 3 g of ASE Prep in a mortar and pestle and place it in an 11-mL cell. After the sample is loaded, fill any void volume in the extraction cell with sand.

With Water (Isomil®, Alsoy®, Good Start®)

Place a cellulose filter in each extraction cell before loading the sample. Weigh 3 g of ASE Prep DE into a suitable weighing dish. Add, by pipette, 0.4 g water to the ASE Prep DE and mix to disperse the water. Weigh approximately 1 g of sample, to the nearest 0.1 mg. Grind the sample with the prepared wet ASE Prep DE in a mortar and pestle and place in an 11-mL cell. After the sample is loaded, fill any void volume in the extraction cell with sand.

Note: With the addition of water to the samples, the optimum extraction temperature is changed to 100 °C.

ASE EXTRACTION PROCEDURE

Place the loaded cells into the upper carousel and the appropriate number of clean, preweighed collection vials in the lower carousel. Set up the methods to be used and start the extraction. After the extractions are complete, remove the collection vials from the lower carousel. Evaporate the extraction solvent with a nitrogen stream and then dry each extract in an oven at 100 °C until a constant weight is achieved. The extract weight is obtained by subtracting the vial tare weight from the total weight.

TRADITIONAL MOJONNIER PROCEDURE

To compare the values of fat obtained from an ASE extraction with a traditional extraction, the infant formulas were extracted by the AOAC Intl. Method 932.06, Fat in Dried Milk. This method requires alkaline pretreatment with ammonium hydroxide, heating, and liquid–liquid extraction with a mixture of petroleum ether, diethyl ether, and ethanol. Following the extraction, the combined organic solvent (~125 mL) is evaporated and the residue is dried to a constant weight.

FAME ANALYSIS

Fatty acid methyl ester analysis was performed on the extracts to verify the extraction efficiency. The method followed was AOAC Intl. Method 991.39, modified by substituting nonadecanoic acid methyl ester for tricosanoic acid methyl ester as the internal standard. Nonadecanoic acid methyl ester was found to be more suitable because it is more representative of the fatty acids in the samples studied here. The gas chromatographic conditions were: split injection, injector temperature 225 °C, oven program 70 °C to 240 °C at 8 °C min^{-1} hold for 4 min at 240 °C, FID detector temperature 280 °C. The column used was 30 m × 0.32 mm × 0.25 µm SUPELCOWAX 10™.

DISCUSSION AND RESULTS

Many laboratories use standard reference materials to verify their procedures and methods. The U.S. National Institute of Standards and Technology (NIST) has made available an SRM with a reported value of fat content. SRM 1846 is a milk-based powdered infant formula. The fat content as listed in the certificate of analysis was determined by nine collaborating labs using three

different methods: alkaline pretreatment followed by Roese-Gottlieb extraction, alkaline pretreatment followed by Mojonnier extraction, and acid digestion followed by ether extraction. Alkaline pretreatment followed by Mojonnier extraction (AOAC Intl. Method 932.06, Fat in Dried Milk) was used to confirm accurate results from SRM 1846. The same method was used to extract the other powdered infant formulas and the results are shown in Table 1.

ASE is capable of extracting fat from powdered infant formulas without the use of aggressive pretreatments. Using the extraction conditions previously listed, most of the samples were extracted easily. Nevertheless, a few samples did not give the desired results. It was found that for these samples the addition of water to the ASE Prep DE before the samples were dispersed greatly assisted the extraction of fat. This sequence of adding the water to the dispersant worked better than adding the water directly to the sample or adding water to the extraction solvent. The samples that benefited from the addition of water were the soy protein-based formulas (Alsoy and Isomil) and the hydrolyzed milk protein-based formula (Good Start). The results of the ASE extractions are also shown in Table 1. For each sample, there is close agreement between the traditional Mojonnier method and the ASE methods.

A potential concern, when using gravimetric analysis with any extraction method, is skewing of the results due to coextraction of nonfat materials. One procedure to ascertain the validity of the values is to determine the sample's fat content by some other technique. Analysis of the fatty acids by gas chromatography is an accepted technique to determine the amount of fat in a sample. The fatty acids in the sample are converted to their methyl esters (FAMEs) and are then quantified by gas chromatography. Following each extraction and gravimetric analysis of the commercial infant formulas, FAMEs were generated from the residue and analyzed by gas chromatography. The results of the FAMEs analyses are shown in Table 2. Again, there is close agreement between the samples extracted by the traditional Mojonnier method and the ASE methods. The composition of the fatty acids can be determined from the GC chromatograms. Examples of chromatograms from both extraction techniques are shown in Figures 1 and 2. The comparison of the fatty acid profile is shown in Table 3. No significant differences are observed in the fatty acid profile obtained from either the ASE or Mojonnier extractions.

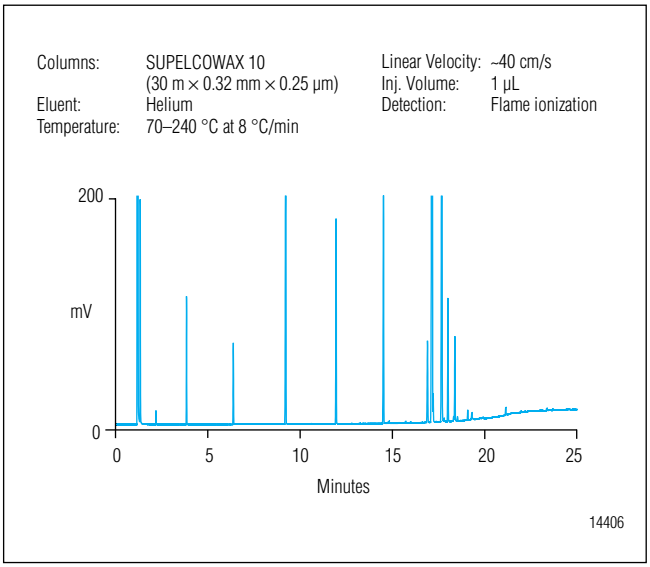

Figure 1. Chromatogram of FAMEs from ASE extraction of Isomil.

Table 1. Percent Fat in Powdered Infant Formulas Gravimetric Results from ASE and Mojonnier Extractions

	ASE Extraction (n = 3)			Mojonnier Extraction (n = 3)		
Sample	**Method**	**% Fat**	**% RSD**	**Method**	**% Fat**	**% RSD**
Isomil	With water	27.86	0.53	932.06	28.71	0.62
Alsoy	With water	25.35	1.10	932.06	25.58	0.82
Good Start	With water	26.21	1.67	932.06	26.72	0.06
Similac	Without water	29.06	0.94	932.06	28.84	0.07
Enfamil	Without water	28.89	0.72	932.06	28.83	0.24
SRM 1846	Without water	27.51	0.70	932.06	26.68	0.41

CONCLUSION

ASE can extract total fat from infant formulas with recoveries comparable to traditional hydrolysis/extraction methods. The amount of fat obtained from infant formula using ASE represents the total fat in the sample as confirmed by good agreement between the gravimetric and FAME analysis methods. Furthermore, ASE provides a method that is faster and requires far less solvent and labor than traditional methods. The automation features of ASE allow up to 24 samples to be run sequentially and unattended, unlike the labor-intensive Mojonnier method. In addition, ASE requires only 20 mL of solvent compared to 125 mL for the Mojonnier method.

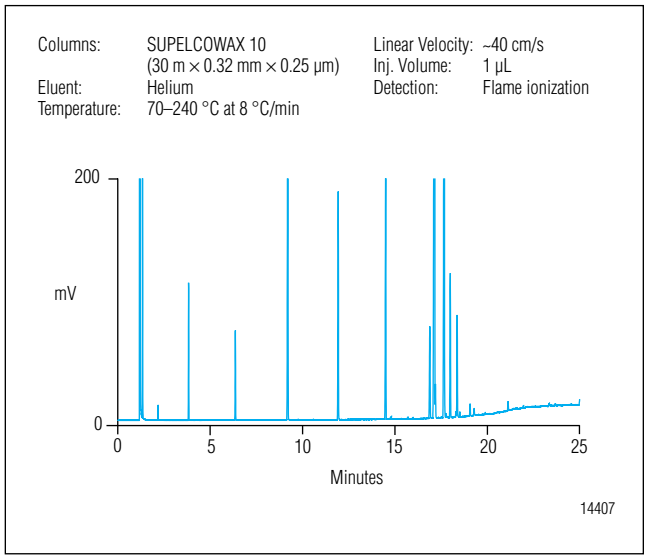

Figure 2. Chromatogram of FAMEs from Mojonnier extraction of Isomil.

Table 2. FAME Analysis of ASE and Mojonnier Extractions

ASE FAME results (n = 1)		Mojonnier FAME results (n = 1)	
Sample	**% Fat**	**Sample**	**% Fat**
Isomil	27.12	Isomil	27.13
Alsoy	25.13	Alsoy	24.03
Good Start	25.27	Good Start	25.38
Similac	27.52	Similac	27.57
Enfamil	26.52	Enfamil	26.06
SRM 1846	27.24	SRM 1846	26.73

Table 3. Fatty Acid Comparison (Sample: Isomil) Normalized Weight Percent

ASE Extraction		Mojonnier Extraction	
Fatty Acid	**% Fat**	**Fatty Acid**	**% Fat**
Saturated fatty acids	34.9	Saturated fatty acids	34.5
Monounsaturated	37.2	Monounsaturated	37.5
Polyunsaturated	27.8	Polyunsaturated	28.0

SUPPLIERS

Fisher Scientific, 2000 Park Lane, Pittsburgh, PA 15275-1126 USA, Tel: 800-766-7000, www.fishersci.com.

Caliper Life Sciences, 68 Elm Street, Hopkinton, MA 01748 USA, Tel: 508-435-9500, www.caliperls.com.

Determination of Fat in Dried Milk Products Using Accelerated Solvent Extraction (ASE)

INTRODUCTION

Many extraction techniques for the determination of fat in food are labor-intensive or require long extraction times. The Roese-Gottlieb (RG) method requires alkaline pretreatment of the sample before a labor intensive liquid-liquid extraction. The Schmidt-Bondzynski-Ratzlaff (SBR) method calls for acid digestion before liquid-liquid extraction of the sample. Some Soxhlet methods require extraction times from 4 to 24 hours in duration.

Because of the labor and time requirements of these fat extraction methods, many labs are investigating automated techniques that allow more efficient operation. Accelerated Solvent Extraction (ASE) is an automated technique that can be used to replace the traditional extraction techniques. ASE uses common liquid solvents at elevated temperatures and pressures to extract analytes from solid or semisolid samples. The higher temperature increases the solubility of the analytes and the kinetics of the extraction. The higher pressure keeps the solvents in their liquid state throughout the extraction process.

Samples are placed into the extraction cells and then loaded onto the carousel. The corresponding number of collection vials are loaded onto the vial tray. The extraction cell is transferred into the oven and solvent is pumped into the cell. The sample in the cell is held at the extraction temperature and pressure for a specified amount of time. After the extraction time, fresh solvent is rinsed through the cell to transfer the extraction solvent into the collection vial. The fresh solvent is then flushed from the cell with nitrogen gas. The extraction solvent and the dissolved analytes end up in the collection vial and are ready for analysis or further clean-up.

This Application Note describes the extraction of fat from a variety of dried milk products. The samples range from very low-fat products such as skim milk powder to very high-fat products such as cream powder. The extraction of fat from these matrices is rapid and the results are equivalent to the referenced traditional methods.

EQUIPMENT

Dionex ASE 200 Accelerated Solvent Extractor equipped with 11-mL stainless steel extraction cells

Cellulose filter disks (P/N 49458)

Analytical balance (0.0001 g or better)

Solvent evaporator (TurboVap®, Zymark Corp., or dry block heater or equivalent)

Forced air oven

SOLVENTS

Hexane

Dichloromethane

Methanol

Petroleum ether (40–60 ˚C boiling range)

EXTRACTION CONDITIONS

Temperature: 80 ˚C

Pressure: 1500 psi

Cell Heat-up: 5 min
Static Time: 1 min
Flush Volume: 100%
Purge Time: 40 s
Static Cycles: 3
Total Time: 11 min
Total Solvent: <30 mL
Solvent: Mixtures of hexane, dichloromethane, and methanol in various volume ratios. See the "Samples" section below for extraction solvent ratios.

SAMPLES

	Extraction Solvent Ratio (hexane: dichloromethane: methanol)
Whole milk powder (WMP)	5:2:1
Cream powder	5:2:1
Skim milk powder (SMP)	3:2:1
Whey protein concentrate (WPC)	2:3:3
Whey protein isolate	2:3:3
Sodium caseinate	2:3:3

ASE METHODS

A cellulose filter is placed in the bottom of each extraction cell before loading the samples. Samples of 2 g ± 0.2 g are placed directly into 11-mL extraction cells, which are then placed in the upper carousel. The same number of preweighed collection vials are placed in the lower carousel. The methods are entered into the ASE 200 and the extractions are started. The samples are extracted with a mixture of hexane, dichloromethane, and methanol. The extraction temperature is 80 ˚C; the extraction time is 3 cycles at 1 min each. After extraction, the solvent is evaporated at 80 ˚C under a nitrogen purge and the fat is dried according to the reference gravimetric method to which the ASE method is being compared.

REFERENCE EXTRACTION METHODS

ASE extraction results are compared to reference methods of extraction. For the cream powder, whole milk powder, and skim milk powder the reference method is Roese-Gottlieb (RG) AOAC Official Method 932.06, Fat in Dried Milk (International IDF Standard 9C:1987). For the whey protein concentrate and caseinate powders, the reference method is SBR (International IDF Standard 127A:1988) or a 24-h Soxhlet method with chloroform-methanol 2:1.[1]

ANALYSIS METHODS

Gas Chromatography

Fatty acid methyl esters (FAMEs) were prepared from the extracted fat by an in-house method based on the work of Christopherson and Glass.[2]

High Performance Liquid Chromatography (HPLC)

HPLC of lipids were analyzed in the normal phase using a Lichrosorb Diol column with an evaporative light scattering detector (ELSD).[3]

Table 1 Gravimetric Comparison of ASE and RG Methods (typical single point data)

Sample	% Fat	
	ASE	RG
Cream powder	54.88	54.96
Whole milk powder[a]	29.41	29.45
Skim milk powder	0.96	0.95

[a]WMP recovery by ASE = 99.48% ± 0.15%, (n=8)

Table 2 Gravimetric Comparison of ASE with SBR and Soxhlet Methods (typical single point data)

Sample	% Fat		
	ASE	SBR	Soxhlet
Lactic WPC powder	5.47	4.95	5.50
Acid WPC powder	5.71	5.66	6.38
Cheese WPC powder	6.93	6.75	7.32
Whey protein isolate	0.45	0.58	0.50
Sodium caseinate	0.66	0.65	0.55

RESULTS AND DISCUSSION

The most rapid and complete extractions to yield a fat sample practically free of nonlipid were obtained using the solvent mixtures of hexane, dichloromethane, and methanol listed in the "Samples" section above. Extractions of milk products using ASE are compared with traditional techniques in Tables 1 and 2. Table 1 shows that ASE and Roese-Gottlieb extractions of milk and cream powders yield identical results. Table 2 shows that ASE generates results similar to acid hydrolysis and Soxhlet methods. ASE solvent use per sample was less than 30 mL. For most products, fat was measured gravimetrically directly from ASE extractions. For skim milk powder and caseinate, a solvent mixture could not be found that resulted in quantitative yield of fat without significant nonlipid in the extract (evidenced by nonsoluble solids in the extract.) Fat from these dried extracts of the products was therefore redissolved in petroleum ether and determined by weight loss, similar to the Roese-Gottlieb procedure.

Figure 1 shows the correlation of percent fat values obtained from ASE extractions to accepted values within the New Zealand dairy industry. Roese-Gottlieb is the referenced method used for WMP and SMP. SBR is the referenced method used for WPC and caseinate. Confidence limits shown in Figure 1 represent the limits of acceptability set by New Zealand's Inter-Laboratory Comparison Program, which are approximately ± 0.2% absolute for SMP and caseinate and ± 0.5% absolute for WPC and WMP. The higher ASE results for WPC were associated with a high proportion of phospholipid in the fat. The fate of dairy phospholipids is dependent upon the extraction technique. Phospholipids are hydrolyzed by acid digestion in the SBR method[4], whereas ASE and Soxhlet methods are able to extract the phospholipids intact. The presence or absence of phospholipids in the extracts was confirmed by HPLC and is shown in Figure 2. ASE and Soxhlet extracts contain non-hydrolyzed phospholipids and represent a more accurate value of the fat.

The FAME data (Table 3) revealed no significant differences in saturated, monounsaturated, and polyunsaturated ratios from ASE extraction compared with conventional extraction methods.

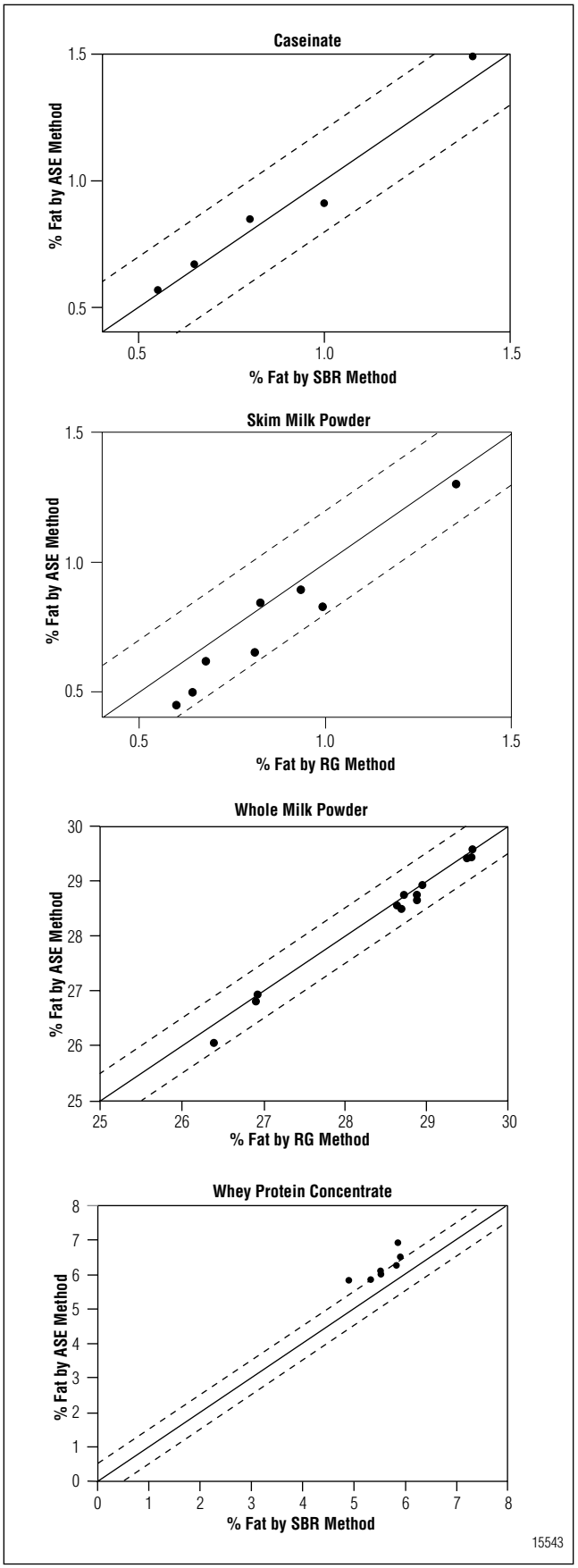

Figure 1. Correlation of extraction methods: ASE vs. RG and ASE vs. SBR.

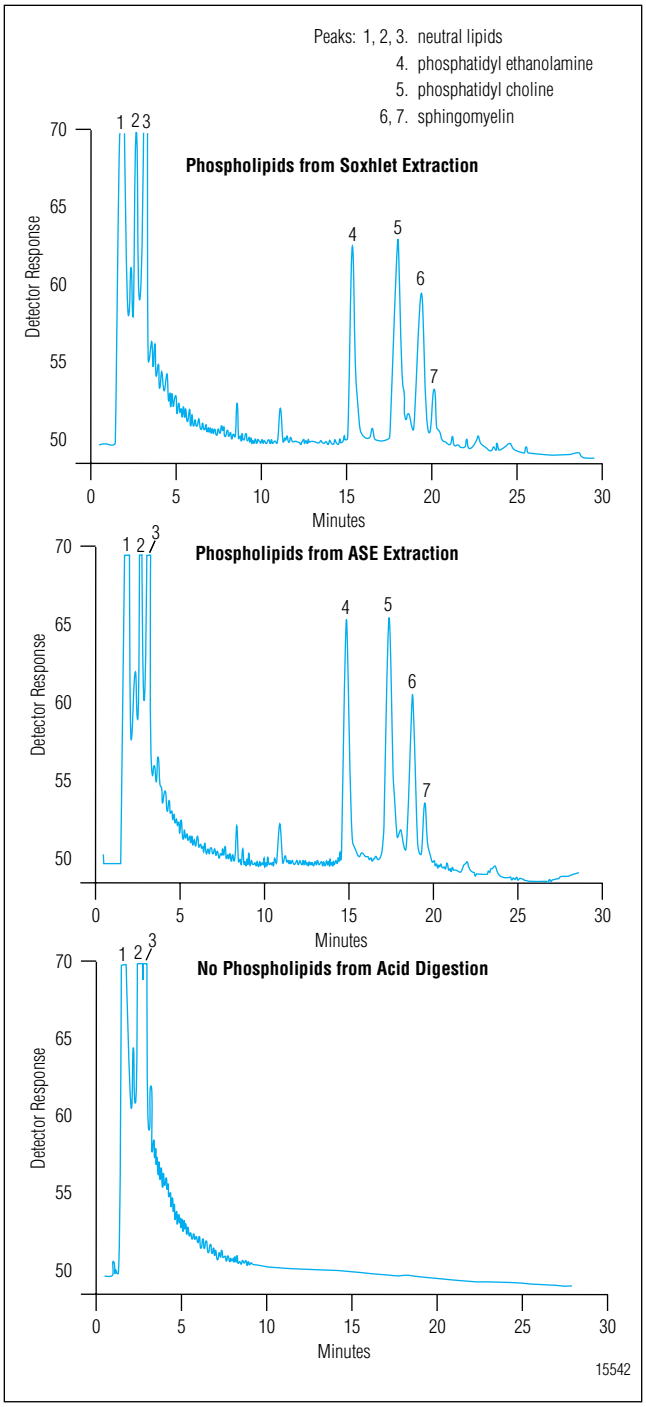

Figure 2. HPLC with ELSD traces of lipids extracted from whey protein concentrate.

Table 3 FAME Analysis of Extracted Fat

Fatty Acid Class	Percent of Total Fatty Acids					
	WMP		SMP		WPC[a]	
	ASE	RG	ASE	RG	ASE	Soxhlet
Saturated	74.1	74.1	68.8	69.2	64.4	64.4
Monounsaturated	22.8	22.9	27.4	26.7	28.8	28.8
Polyunsaturated	3.1	3.0	3.8	4.1	2.3	2.2

[a]Lactic acid WPC

CONCLUSIONS

ASE can rapidly extract fat from dried milk products containing <1% to >50% fat with accuracy and precision equivalent to or better than manual gravimetric reference methods. ASE extractions are completed in 11 minutes and use only 30 mL of solvent. Extractions performed by ASE do not hydrolyze phospholipids, resulting in a more accurate value of fat than the SBR method. Because no degradation of fat results from the ASE extraction process, extracts are suitable for further lipid analyses.

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2. Christopherson, S. W. and Glass, R. L. *Journal of Dairy Science* **1969** *52,* 1289–1290.
3. Arnoldson, K. C. and Kaufmann, *P. Chromatographia* **1994** *38,* 317–324.
4. Walstra, P. and Mulder, H. *Netherlands Milk & Dairy Journal* **1963** *17,* 334–346.

Based on data from Russell K. Richardson and Colin G. Hughes, New Zealand Dairy Research Institute, presented at PittCon 98.

Extraction of Fat from Dairy Products (Cheese, Butter, and Liquid Milks) Using Accelerated Solvent Extraction (ASE)

INTRODUCTION

The current methods for determining fat in dairy products, though acceptable, have several drawbacks. Many dairy-based products require a pretreatment prior to extraction. This denatures the casein, allowing greater exposure of the fat to the solvents. For example, a 1-g sample of cheese must be pretreated with ammonium hydroxide followed by hydrochloric acid and boiled for several minutes. The standard fat extraction methods, including the pretreatment, are very manual and thus very time consuming. Large amounts of solvents are required to remove the fat from each sample matrix, which can be quite costly. For example, manual extraction of pretreated cheese usually requires 2–3 h and more than 110 mL of solvent per sample. Therefore, the standard fat extraction methods are not time- or cost-efficient and can expose laboratory technicians to potentially dangerous solvents.

Accelerated Solvent Extraction (ASE) is an automated extraction technique that uses the same solvents as current extraction methods but in significantly smaller amounts and with minimal analyst exposure. ASE achieves equivalent results in a fraction of the time by using increased temperature and pressure to enhance the kinetics of the extraction process. For example, the 1-g cheese sample mentioned above can be extracted by ASE in only 8–10 min without any pretreatment using less than 30 mL of solvent. And because ASE is automated, it allows unattended extraction of up to 24 samples.

In this application note, fat is extracted from cheese, butter, and liquid milks using the ASE 200 Accelerated Solvent Extractor. The results are shown to be equivalent to those of traditional fat extraction methods. ASE results reported here are compared to Rose-Gottlieb (RG), Schmid-Bondsynski-Ratzlaff (SBR), and International Dairy Federation (IDF) tests that were conducted by 20 laboratories participating in the Interlaboratory Comparison (ILC).

EQUIPMENT

ASE 200 Accelerated Solvent Extractor with 11- and 33-mL stainless steel extraction cells (P/N 048765 and 048764)
Cellulose Filters (P/N 049458)
Collection Vials, 40 mL (P/N 048783) and 60 mL (P/N 048784)
Analytical balance (to read to nearest 0.0001 g or better)
Mortar and pestle (Fisher Scientific or equivalent)
Solvent evaporator (N-EVAP®, Organomation Associates, Inc.; heated block, Equatherm® CMS, Inc. or equivalent)
Forced air oven
Sand (Ottawa Standard, Fisher Scientific, Cat. No. S23-3)

REAGENTS

ASE Prep DE (diatomaceous earth) (P/N 062819)

SOLVENTS

Petroleum ether
Acetone
Isopropanol
Hexane
All solvents are pesticide-grade or equivalent and are available from Fisher Scientific.

EXTRACTION CONDITIONS

Cheese

Solvent:	Hexane:isopropanol (3:2)
Temperature:	110 °C
Pressure:	1500 psi
Cell Heatup Time:	6 min
Static Time:	2 min
Flush Volume:	100%
Purge Time:	60 s
Cycles:	3
Total Time:	10 min
Total Solvent:	<30 mL

Butter

Solvent:	Petroleum ether:acetone (3:2)
Temperature:	100 °C
Pressure:	1500 psi
Cell Heatup Time:	5 min
Static Time:	2 min
Flush Volume:	60%
Purge Time:	60 s
Cycles:	1
Total Time:	8 min
Total Solvent:	<30 mL

Milk and Cream

Sample:	Cream (40%)
Solvent:	Petroleum ether:acetone:isopropanol (3:2:1)
Sample:	Whole milk (4–6%)
Solvent:	Petroleum ether:isopropanol (2:1)
Sample:	Homogenized/UHT milk (3%)
Solvent:	Petroleum ether:isopropanol (3:2)
Sample:	Skim milk (0.1%)
Solvent:	Petroleum ether:isopropanol (3:2)
Temperature:	120 °C
Pressure:	1500 psi
Cell Heatup Time:	6 min
Static Time:	1 min
Flush Volume:	100%
Purge Time:	60 s
Cycles:	3
Total Time:	10 min
Total Solvent:	<30 mL

SAMPLE PREPARATION

Cheese

Place a cellulose filter into an 11-mL extraction cell before loading the sample. Finely grate the sample cheese. Weigh out 1 g of the grated cheese and grind with 2 g of ASE Prep DE using a mortar and pestle. Grind thoroughly and completely, as this can affect the fat recovery. (ASE Prep DE prevents the sample from compacting and absorbs moisture). Transfer the sample/Hydromatrix mixture to the 11-mL cell containing the cellulose filter. Fill any void volume in the extraction cell with sand. Repeat these steps with any other cheese samples to be extracted.

Butter

Place a cellulose filter into an 11-mL extraction cell before loading the sample. Weigh out 0.25 g of ASE Prep DE and add to the cell. *Note: Be careful not to use more than 0.25 g of ASE Prep DE, as this may cause inaccurate fat recovery.* Carefully mix the butter sample at 32 ºC and load into a plastic syringe. Deliver 1 g of butter from the syringe onto the bed of ASE Prep DE in the extraction cell. Fill any void volume in the extraction cell with sand. Repeat these steps with any other butter samples to be extracted.

Cream

Place a cellulose filter into an 11-mL extraction cell before loading the sample. Weigh out 0.9 g of ASE Prep DE and add to the cell. Deliver 1 g of cream from a plastic syringe onto the bed of ASE Prep DE.

Whole Milk

Place a cellulose filter into an 11-mL extraction cell before loading the sample. Weigh out 2 g of ASE Prep DE and add to the cell. Deliver 1 g of whole milk from a plastic syringe onto the bed of ASE Prep DE.

Homogenized/UHT Milk

Place a cellulose filter into an 11-mL extraction cell before loading the sample. Weigh out 2 g of ASE Prep DE and add to the cell. Deliver 1 g of low-fat milk from a plastic syringe onto the bed of ASE Prep DE.

Skim Milk

Place a cellulose filter into a 33-mL extraction cell before loading the sample. Weigh out 5.5 g of ASE Prep DE and add to the cell. Deliver 3–5 g of skim milk from a plastic syringe onto the bed of ASE Prep DE.

EXTRACTION PROCEDURE

Place the loaded cells into the ASE 200 Extractor. Weigh and label the appropriate number of collection vials and place these into the extractor. Set up the method that is suggested for fat extraction of the dairy product used and begin the extraction. When the extractions are complete, remove the collection vials and evaporate the solvent with a nitrogen stream. Dry each sample in an oven at 102 °C for 1 h. Let these vials reach room temperature and reweigh. The total solids extracted are regarded as "crude" fats; dissolving the dried extract with petroleum ether, evaporating the solvent, and then weighing again will provide a "true" fat result. It has been found that if a heated block with a nitrogen purge is used on the butter extracts, no additional oven drying is needed. Heat samples in the heat block at 1 °C per min from 80 to 105 °C. Samples should be dry in 35–40 min.

RESULTS AND DISCUSSION

ASE results were compared with routine ILC data received from 20 participating laboratories using standard methods (RG, SBR, EU880, and IDF80).

Cheese

The ASE method was compared to the SBR method by looking at the fat recovery data from ILC. Table 1 shows the fat recovery from eight different cheeses relative to the ILC median value and shows that the chosen solvent provided a relatively pure fat extract. It was found that complete dispersion of the sample within the ASE Prep DE during sample preparation was essential for the greatest fat recovery.

Butter

The ASE method was compared to the IDF80 method (equivalent to AOAC Method 938.06) and the EU880 method. Table 2 shows the percent fat recovery from each of these methods using three butter samples. It was found that larger amounts of ASE Prep DE used during the sample preparation retained a small amount of the fat. To obtain accurate results required, add no more than 0.25 g of ASE Prep DE to each sample.

Table 1. ASE Recovery[a] of Fat from Eight Cheeses

	% Fat Recovery	Non-lipid Extracted (% of Sample)
Mean	98.4	0.08
Range	97.1–99.7	0.04–0.11

[a] Relative to the ILC median value using the SBR method

Table 2. Butter % Fat Recovery: ASE vs IDF80 and EU880 Methods (ILC)

	ASE[a]	IDF80[b]	EU880[c]
Sample 1	81.49 ± 0.09	81.66 ± 0.06	81.68 ± 0.04
Sample 2	83.49 ± 0.05	83.49 ± 0.19	83.54 ± 0.02
Sample 3	81.26 ± 0.06	81.27 ± 0.06	81.48 ± 0.08

[a] Mean ± SD of four replicates
[b] Mean ± SD of three replicates
[c] Mean ± SD of two replicates

Table 3. Milk and Cream % Fat Recovery: ASE vs. RG Method

Sample	ASE Mean ± SD (n)	RG Method
Cream	40.62 ± 0.06 (3)	40.58
Whole Milk	4.42 ± 0.02 (4)	4.50
Homogenized Milk	3.39 ± 0.03 (6)	3.39
Skim Milk	0.053 ± 0.010 (7)	0.053

Milk and Cream

The ASE method was compared to the RG method. Table 3 shows the percent fat recovery for both of these methods using different liquid milks. Table 4 shows the repeatability for UHT milk using the ASE 200. As with butter, the ASE Prep DE had a tendency to retain a small portion of the fat in the cream samples. With milk, too little ASE Prep DE resulted in a breakthrough of water and nonlipid material. An optimum sample/ASE Prep DE mixture, giving a high fat recovery with minimal nonlipid, was found to be 1:1 for cream and 1:2 for milks. It was also found that varying the ratio of petroleum ether to isopropanol for each sample resulted in better fat recoveries. See the "Extraction Conditions" section for milk.

Table 4. ASE Repeatability for UHT Milk

Fat Content %	3.84 3.46 3.45 3.53 3.48 3.49
Mean	3.48
SD	0.03
RSD	0.86

CONCLUSION

ASE can quickly and efficiently extract fat from cheese, butter, and milk with a precision that is equivalent to the standard methods used currently by the dairy industry. ASE has also been shown to reduce extraction times of these dairy products and to use significantly less solvent than these methods. Furthermore, ASE eliminates any pretreatment steps, saving additional labor.

SUPPLIERS

Fisher Scientific, 2000 Park Lane, Pittsburg, PA 15275-1126 USA, Tel: 800-766-7000, www.fishersci.com.

Organomation Associates, Inc., 266 River Road West, Berlin, MA 01503, USA. Tel: 888-838-7300, www.organomation.com.

ACKNOWLEDGEMENT

Thanks to the New Zealand Dairy Research Institute (Palmerston North, New Zealand) for providing the data.

Determination of Lactose in Lactose-Free Milk Products by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection

INTRODUCTION

Lactose is the major disaccharide found in milk products and is catabolized into glucose and galactose by the enzyme lactase. Lactose-intolerant individuals have a lactase deficiency; therefore, lactose is not completely catabolized. While lactose intolerance is not a dangerous condition, its global prevalence has created a large market for lactose-free products. Commercially available lactose-free products are produced by breaking down lactose into glucose and galactose by enzymatic hydrolysis. However, the resulting milk products contain varying amounts of residual lactose. This has created the need for simple, reliable, and accurate analytical methods to quantify lactose.

Milk changes structurally and chemically when heat-treated, but the extent of the change depends on the temperature and duration of the heating. Lactulose is a disaccharide containing galactose and fructose that is not naturally found in raw milk but is formed during the heat treatment of milk by the isomerization of lactose. Lactulose levels in milk can be used to determine the method that was used to sterilize the milk. The average lactulose content when using in-container sterilization is 744 mg/L, but only 3.5 mg/L in milk treated by low-temperature pasteurization methods.[1]

Currently available analytical methods for the detection of lactose include mid-infrared detection, fluorometry, photometric methods, polarimetry, gravimetric detection, differential pH techniques, and enzymatic assays.[2–5] These methods are time consuming because of extensive sample preparation. They cannot differentiate individual carbohydrates, and polarimetry measurements have interferences from other optically active components.

The Association of Official Analytical Chemists (AOAC) Method 984.15 uses enzymatic hydrolysis of lactose to glucose and galactose at pH 6.6 by β-galactosidase. This method is time consuming, however, and needs extensive reagent preparations. The reported detection limits of this assay may not allow for the determination of lactose in lactose-free samples.[6]

The work shown here describes a sensitive and accurate method to determine lactose and lactulose in dairy products, including lactose-free products, using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) in six different commercial products. HPAE-PAD is a widely used technique for the determination of monosaccharides, disaccharides, oligosaccharides, smaller polysaccharides, sialic acids, and other sugar acids. Being a direct-detection technique, HPAE-PAD eliminates errors associated with analyte derivatization. The CarboPac® PA20 column in combination with PAD provides high-resolution separations of small and larger carbohydrates with sensitive detection. The method described here was used to determine low levels of lactose in several commercially available products, four of which were lactose-free. Lactose-free Gouda and Havarti cheeses had no detectable lactose, whereas lactose-free cottage cheese and lactose-free milk had 2.17 mg/mL and 0.6 mg/mL lactose, respectively.

EQUIPMENT

Dionex ICS-3000 system including:

- SP Single Pump (P/N 061707) or DP Dual Pump (P/N 061712) with degas option
- DC Detector compartment (P/N 061767) single- or dual-temperature zone
- Electrochemical detector (P/N 061719)
- Disposable Au on PTFE working electrode, pack of 6 (with 2 mil gaskets included) (P/N 066480)
- pH-Ag/AgCl reference electrode (P/N 061879)
- AS Autosampler (P/N 061289) with cooling tray option (recommended)

Chromeleon® Chromatography Data System software

CONSUMABLES

CarboPac PA20 Analytical Column, 3 × 150 mm (Dionex P/N 060142)

CarboPac PA20 Guard Column, 3 × 30 mm (Dionex P/N 060144)

OnGuard® IIA, 2.5 cc cartridge (Dionex P/N 057092)

Syringe filters (Gelman IC Acrodisc® 0.2 µm, P/N 4483)

Disposable filtration units, 0.20 µm nylon membrane (Nalgene®, P/N 164-0020)

Centrifuge equipped with a ten-place, aluminum fixed-angle rotor (Beckman)

Spinchron R, GS-6R Series (Beckman Coulter, P/N 358702) or equivalent

REAGENTS AND STANDARDS

Reagent-grade water, Type I, 18 MΩ-cm resistance or better, filtered through a 0.2 µm filter immediately before use

Sodium acetate, anhydrous (Fluka P/N 71183)

Sodium hydroxide, 50% (Fischer P/N SS254-500)

Potassium hexacyanoferrate(III), ACS reagent, ≥ 99%, powder (Sigma-Aldrich P/N 393517)

Zinc sulfate, monohydrate (Sigma-Aldrich P/N 96495)

α–Lactose, monohydrate (Sigma P/N L-3625)

β–D-Glucose (Sigma-Aldrich P/N G-5250)

D–Galactose (Sigma-Aldrich P/N G-0625)

Lactulose, 4-O-β Galactopyranosyl-D fructofuranose (Sigma-Aldrich P/N L-7877)

Sucrose, α–D-Glucopyranosyl-β-D-fructofuranoside (Sigma-Aldrich P/N S-9378)

Nitrogen; 4.8-grade, 99.998%

SAMPLES

Low-fat cottage cheese
Lactose-free Havarti cheese
Lactose-free Gouda cheese
Light nonfat yogurt
Whole milk
Lactose-free 1% milk

CONDITIONS

Columns:	CarboPac PA20 Analytical Column, 3 × 150 mm (Dionex P/N 060142) CarboPac PA20 Guard Column, 3 × 30 mm (Dionex P/N 060144)
Flow Rate:	0.4 mL/min
Inj. Volume:	10 µL
Tray Temp:	4 ºC
Detection:	Integrated pulsed amperometry, Au on PTFE disposable or conventional Au working electrodes
Waveform:	Carbohydrate (standard quad)
Background:	<20 nC
Noise:	30 to 80 pC
Temperature:	30 ºC
Eluents:	A) Deionized water B) 200 mM NaOH C) 200 mM NaOH, 100 mM sodium acetate D) 200 mM NaOH, 1 M sodium acetate

Time (s)	Potential (V)	Gain Region*	Ramp*	Integration
0.00	+0.1	Off	On	Off
0.20	+0.1	On	On	On
0.40	+0.1	Off	On	Off
0.41	–2.0	Off	On	Off
0.42	–2.0	Off	On	Off
0.43	+0.6	Off	On	Off
0.44	–0.1	Off	On	Off
0.50	–0.1	Off	On	Off

*Settings required when using the ICS-3000 or ICS-5000 but not for older Dionex systems.

Gradient Conditions:

Time (min)	Flow (mL/min)	% A	% B	% C	% D
0.0	0.40	94.0	6.0	0.0	0.0
10.0	0.40	94.0	6.0	0.0	0.0
20.0	0.40	90.0	7.5	2.5	0.0
25.0	0.40	90.0	7.5	2.5	0.0
31.0	0.40	92.5	7.5	0.0	0.0
33.0	0.40	0.0	25.0	0.0	75.0
43.0	0.40	0.0	25.0	0.0	75.0
43.1	0.40	0.0	100.0	0.0	0.0
49.0	0.40	0.0	100.0	0.0	0.0
49.1	0.40	94.0	6.0	0.0	0.0
65.0	0.40	94.0	6.0	0.0	0.0

PREPARATION OF SOLUTIONS AND REAGENTS

200 mM NaOH

Transfer 10.4 mL of 50% sodium hydroxide into a polypropylene 1 L volumetric flask containing approximately 800 mL degassed and filtered deionized water using a plastic serological pipette. Mix by inverting the volumetric flask and bring to volume with degassed and filtered deionized water. Refer to Dionex TN 71 for a more detailed discussion on proper preparation, storage and use of eluents for HPAE-PAD.[7] Keep the eluent blanketed under 5 to 8 psi of nitrogen (or any other inert gas) at all times to reduce carbonate contamination.

200 mM NaOH, 100 mM Sodium Acetate

Dissolve 8.204 g high-purity anhydrous sodium acetate into approximately 800 mL deionized water. Vacuum filter the solution through a 0.20 µm filter to remove any particulates. Transfer the filtered solution into a 1 L plastic volumetric flask, and add 10.4 mL of 50% sodium hydroxide using a plastic serological pipette. Bring to volume with degassed and filtered deionized water. Keep the eluent blanketed under 5 to 8 psi of nitrogen (or any other inert gas) at all times to reduce carbonate contamination.

200 mM NaOH, 1 M Sodium Acetate

Dissolve 82.04 g high-purity anhydrous sodium acetate into approximately 800 mL of deionized water. Vacuum filter the solution through a 0.20 µm filter to remove any particulates. Transfer the filtered solution into a 1 L plastic volumetric flask and add 10.4 mL of 50% sodium hydroxide using a plastic serological pipette. Bring to volume with degassed and filtered deionized water. Keep the eluent blanketed under 5 to 8 psi of nitrogen (or any other inert gas) at all times to reduce carbonate contamination.

Carrez I Solution

Dissolve 15.0 g potassium hexacyanoferrate(III) in 75 mL DI water and filter through a 0.20 µm filter. Transfer to a 100 mL volumetric flask and bring to volume.

Carrez II Solution

Dissolve 30.0 g zinc sulfate monohydrate in 75 mL DI water and filter through a 0.20 µm filter. Transfer to a 100 mL volumetric flask and bring to volume.[8]

Standards

All standard concentrates can be stored for up to 6 months at -40 °C. Diluted intermediate standards are stable for 3 months at -40 °C, and working and mixed standards are stable for two weeks at 2 to 4 °C.

1000 mg/L Standard Concentrates

Prepare the stock standard solution by dissolving the appropriate amount of carbohydrate (Table 1) in approximately 75 mL DI water and diluting to 100 mL in a volumetric flask. Store the stock solution in a high-density polyethylene or polypropylene bottle at 4 °C.

Table 1. Amounts for the Preparation of Standard Concentrates (100 mL)

Carbohydrate	Weight (g)
α–Lactose	0.360
β–D-Glucose	0.180
D–Galactose	0.182
Lactulose	0.342
Sucrose	0.342

Working Standards and Standards for Method Linearity

To prepare working standards, use a calibrated pipette to deliver the appropriate volume of 1000 mg/L stock standard into a volumetric flask and dilute to volume with DI water. For method linearity studies, the following standards of lactose and lactulose were used: 100, 50, 25, 15, 10, 7.5, 5, 3, 2, 1, 0.5, and 0.25 mg/L. The exception was the linearity studies for lactulose for which the minimum concentration was 0.5 mg/L.

Mixed Standards

To prepare mixed carbohydrate standards, combine appropriate volumes of individual stock carbohydrate standards into a volumetric flask and dilute to volume with DI water.

SAMPLE PREPARATION

Weigh 1 g of sample and add 10 mL DI water to the sample. Add 200 µL Carrez I solution and 200 µL Carrez II solution to the mixture, shaking after each addition. Transfer the mixture to a 100 mL volumetric flask and bring the volume to 100 mL. Centrifuge a portion of this sample at 3000 RPM. Aspirate the supernatant and filter through a 0.20 µm filter. Prepare an OnGuard IIA, 2.5 cc cartridge by flushing it with 15 mL
DI water at a flow rate of less than 2 mL/min, then discarding the effluent. Load 8 mL of sample, discard the first 6 mL into a waste container, and collect the next 2 mL for analysis. Samples can be stored at -4 ºC for up to 2 weeks. Filter the sample through an IC-grade syringe filter prior to injection.

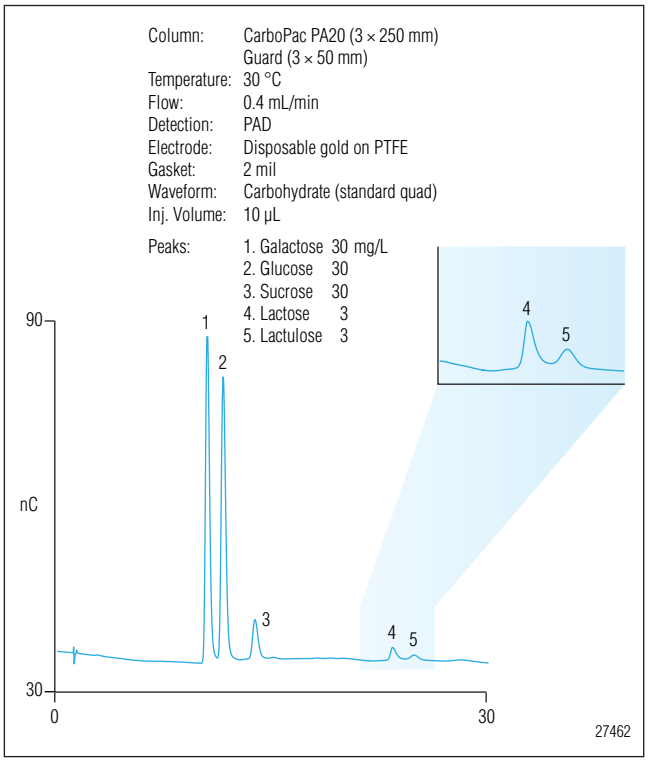

Figure 1. Separation of a mixed-carbohydrate standard, including lactose and lactulose.

RESULTS AND DISCUSSION

Chromatography and Interference Studies

To optimize the separation of lactose and lactulose in the presence of expected sample carbohydrates, a mixed carbohydrate standard was prepared. Figure 1 shows a chromatogram of a mixed carbohydrate standard with an optimized gradient for the separation of lactose and lactulose. The retention times of galactose, glucose, sucrose, lactose, and lactulose are 9.63, 10.65, 13.79, 22.98, and 24.36 min, respectively. All the carbohydrates are well separated from each other, including lactose and lactulose.

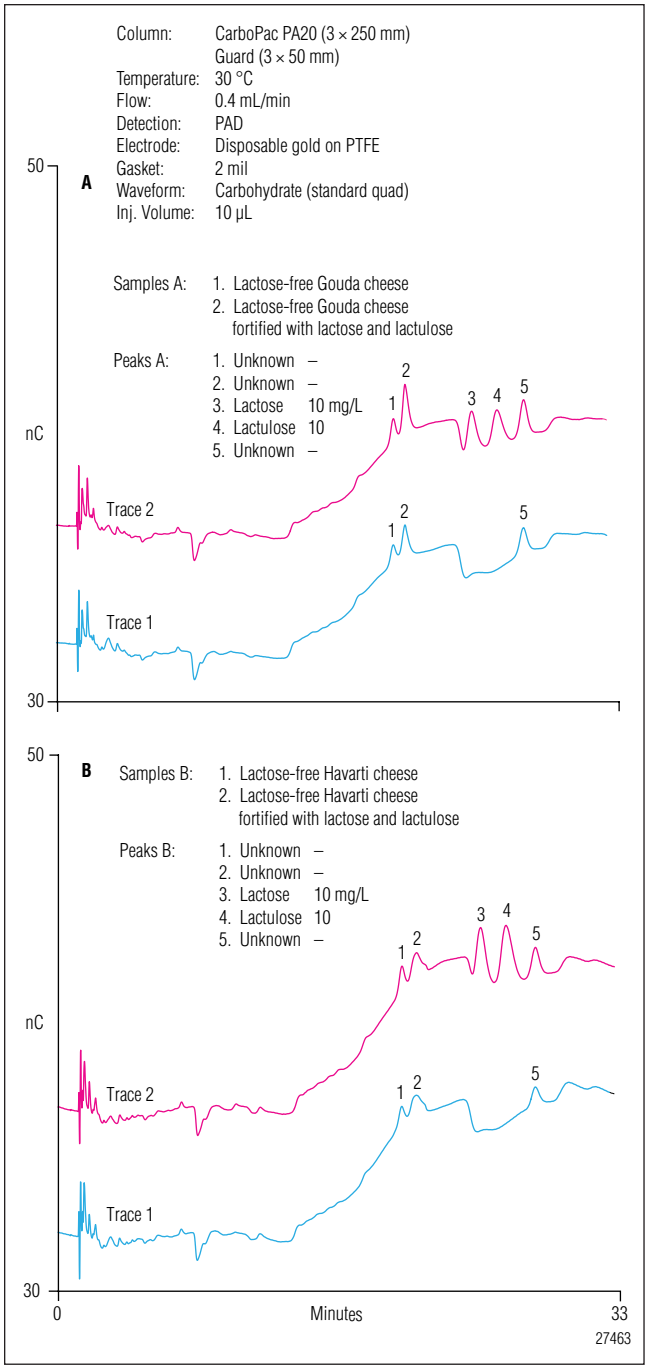

Figure 2. A) Separation of carbohydrates in fortified and unfortified lactose-free Gouda cheese samples. B) Separation of carbohydrates in fortified and unfortified lactose-free Havarti cheese samples.

Preliminary Sample Analysis

The optimized separation was applied to two samples matrices, lactose-free Gouda cheese and lactose-free Havarti cheese. Figure 2A shows overlayed chromatograms of fortified and unfortified Gouda cheese. Trace 1 shows a separation of unfortified cheese with no detectable lactose. Trace 2 shows the separation of a Gouda cheese sample fortified with 10 mg/L each of lactose and lactulose. This chromatogram shows that lactose and lactulose are well separated from each other and matrix-related interferants.

Figure 2B shows overlayed chromatograms of fortified and unfortified Havarti cheese. Trace 1 shows a separation of unfortified Havarti cheese with no lactose detected. Trace 2 shows the separation of an Havarti cheese sample fortified with 10 mg/L each of lactose and lactulose. The chromatography is similar to Figure 2A.

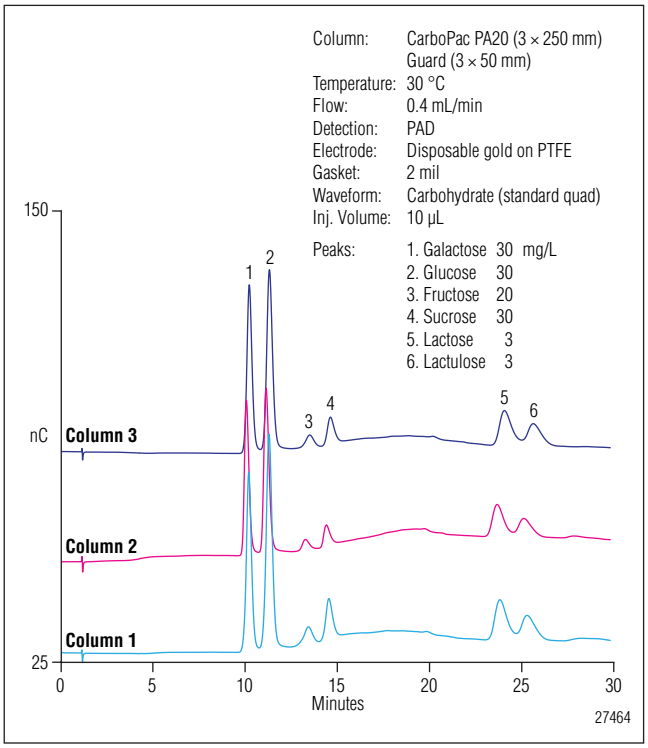

Figure 3. Overlay of the lactose and lactulose separation on columns from three different lots.

Table 2. Intraday Reproducibility

Analyte	Conc. (mg/L)	RSD		
		Ret. Time	Peak Area	Peak Height
Galactose	30	0.15	1.03	1.01
Glucose	30	0.14	1.30	1.08
Sucrose	30	0.16	1.51	1.37
Lactose	3	0.13	3.70	5.74
Lactulose	3	0.12	5.06	5.42

n = 30 injections

Column-to-Column Reproducibility

Due to the close elution of lactose and lactulose, the method ruggedness was evaluated by checking the separation on three columns from three different lots. Figure 3 shows that lactose and lactulose are well separated on each column.

Short-Term Reproducibility

Table 2 shows intraday reproducibility measured by making 30 consecutive injections of a mixed-carbohydrate standard containing 30 mg/L each of galactose, glucose, and sucrose; 3 mg/L each of lactose and lactulose; and 20 mg/L fructose. The method exhibited good short-term reproducibility; the intraday retention time RSDs ranged from 0.12 for lactulose to 0.16 for sucrose, and the peak area RSDs ranged from 1.03 for galactose to 5.07 for lactulose.

Table 3. Linearity and MDL for Lactose and Lactulose

Carbohydrate	Range mg/L	r^2	MDL Standard (mg/L)	*Calculated MDL (mg/L)
Lactose	0.25–100	0.9966	0.5	0.12
Lactulose	0.5–100	0.9942	1.0	0.23

*The method detection limits (MDL) for lactose and lactulose were determined by making seven injections of a low-level solution fortified with lactose and lactulose at 3 to 5 times the estimated MDL.

Determination of Linearity for Lactose and Lactulose

Calibration standards were prepared in DI water. Table 3 summarizes the calibration data for a calibration curve obtained by injecting calibration standards between 0.25 to 100 mg/L lactose. Table 3 also summarizes the calibration data for lactulose using the same calibration standards with the exception of the 0.25 mg/L standard. The calibration curve for both compounds was linear with a correlation coefficient (r^2) of 0.9966 for lactose and 0.9942 for lactulose, respectively.

MDL for Lactose and Lactulose

The MDL is defined as the minimum concentration of an analyte that can be identified, measured, and reported with 99% confidence that the analyte concentration is greater than zero. It is basically a measure of the precision of preparing and analyzing low-level standards according to the method. The MDLs for lactose and lactulose were determined by making seven injections of a low-level solution fortified with lactose and lactulose at 3 to 5 times the estimated MDL. MDLs were calculated using the calibration curve.

The calculated MDLs in DI water obtained by this method are 0.12 mg/L for lactose and 0.23 mg/L for lactulose. Table 3 summarizes the data for this determination.

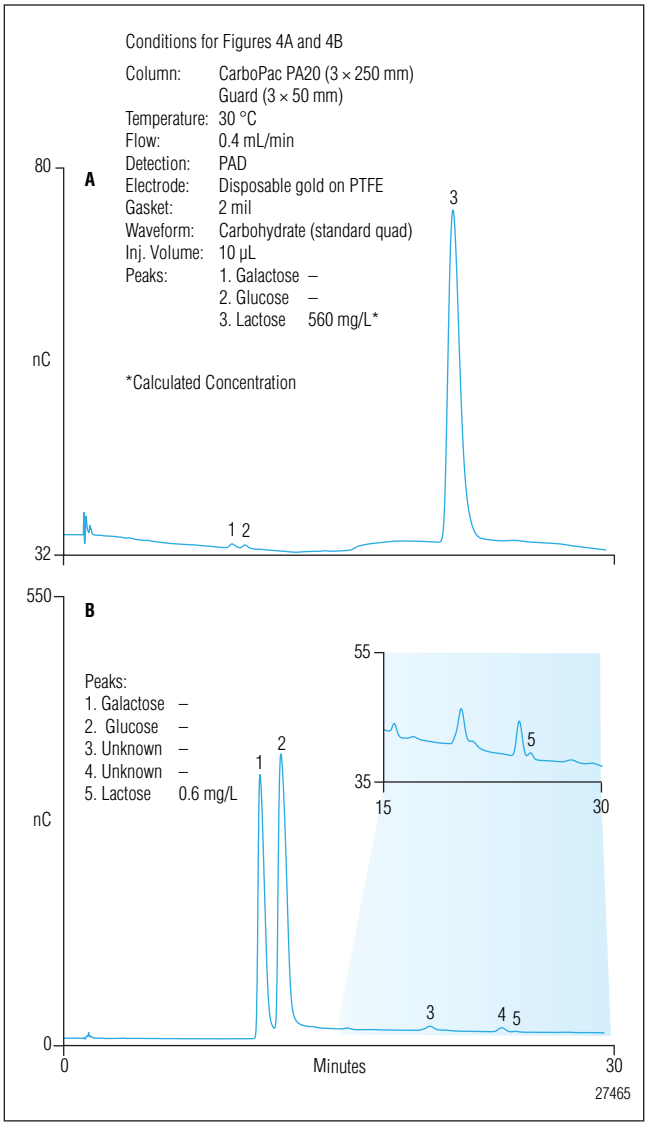

Figure 4. A bi-panel showing the following chromatograms: A) separation of carbohydrates in whole milk, and B) separation of carbohydrates in lactose-free low-fat milk.

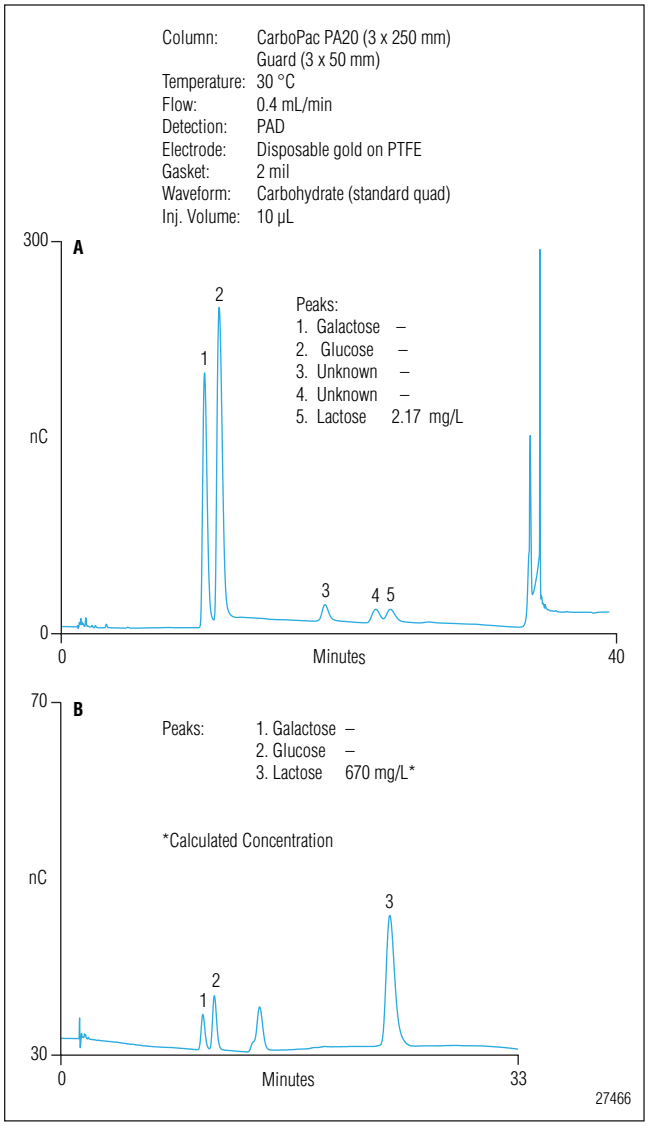

Figure 5. A bi-panel showing the following chromatograms: A) separation of carbohydrates in lactose-free low-fat cottage cheese, and B) separation of carbohydrates in 1:20 diluted low-fat yogurt.

Sample Analysis

Several milk-based products were evaluated for their lactose and lactulose content. Figure 4A shows the separation of carbohydrates in whole milk. The prepared milk samples were diluted 1:10 to prevent overloading with lactose. The diluted milk sample showed some galactose and glucose and large amounts of lactose. Figure 4B shows the separation of carbohydrates in lactose-free milk. The chromatogram shows that lactose-free milk contains high concentrations of galactose and glucose and trace amounts of lactose (0.6 mg/L, 0.00006%). This product showed the lowest detected lactose concentration among the products evaluated.

Figure 5A shows the separation of carbohydrates in lactose-free cottage cheese. This sample contained high concentrations of galactose and glucose, 21.7 mg/L (0.00217%) of lactose, and unknown peaks. Figure 5B shows the separation of carbohydrates in low-fat yogurt.

Table 4. Recovery of Lactose and Lactulose in Various Matrices			
Matrix	**Amount Added (mg/L)**	**Lactose Recovery (%) n=3**	**Lactulose Recovery (%) n=3**
Whole milk (1:10 diluted)	10	85.3	98.1
Lactose-free low-fat milk	10	97.6	94.5
Lactose-free Gouda cheese	10	90.1	100.8
Lactose-free Havarti cheese	10	99.7	93.2
Lactose-free cottage cheese	10	102.0	86.0
Low-fat yogurt (1:20 diluted)	10	89.9	97.0

The chromatogram shows that low-fat yogurt contains galactose, glucose, and 33.5 mg/L (0.00335%) lactose.

A duplicate of each of the samples was fortified with known amounts of lactose and lactulose prior to sample preparation. Recoveries were calculated following analysis of both native and spiked samples. Recoveries of lactose and lactulose for all matrices were 86 to 100% (Table 4).

CONCLUSION

This work describes a sensitive and accurate method to extract, separate, and quantify lactose and lactulose in milk-based products. The method uses a CarboPac PA20 column with PAD to quantify lactose and lactulose in a separation time of less than 30 min. The use of disposable gold electrodes provides the benefit of high electrode-to-electrode reproducibility and rapid equilibration upon installation.

PRECAUTIONS

Potassium hexacyanoferrate is a red crystalline solid that may be harmful. It may cause respiratory tract irritation if inhaled. In addition, it can be harmful if absorbed through the skin or swallowed, and it can cause irritation to the eyes. Consult the potassium hexacyanoferrate MSDS for additional information. Potassium hexacyanoferrate should be stored tightly closed in a dry and well-ventilated place and should not be stored near acids. Potassium hexacyanoferrate should not be disposed into drains. Contact a licensed waste disposal organization to ensure all disposals are in accordance with existing federal, state, and local environmental regulations.

The reference electrode must be hydrated at all times and should not be allowed to dry out, especially when the cell is on. Reduce the eluent flow to 0.25 mL/min if you anticipate not using the system. For long-term storage of more than a week, disassemble the cell, remove the reference electrode, and store it in saturated KCl.

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7. 7. Dionex Corporation. *Eluent Preparation for High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection.* Technical Note 71, LPN 1932, 2009, Sunnyvale, CA.
8. 8. *AOAC. Int.,* 481 North Frederick Avenue, Suite 500, Gaithersburg, Maryland 20877-2417 USA, **1984, 67,** 637.

SUPPLIERS

Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO 63103, U.S.A. Tel: 800-521-8956. www.sigmaaldrich.com

Thermo Fisher Scientific (Pierce Biotechnology, Nalgene, Mallinckrodt Baker, J.T. Baker, and Savant Instruments), 308 Ridgefield Court, Asheville, NC 28806-2210, U.S.A. Tel: 866- 984-3766. www.thermo.com

Sarstedt Inc., 1025, St. James Church Road, P.O. Box 468, Newton NC 28658-0468, U.S.A. Tel.: 828 465 4000. www.sarstedt.com

Praxair Specialty Gases and Equipment, 39 Old Ridge bury Road, Dansbury, CT 06810-5113 U.S.A. Tel: 877-772-9247. www.praxair.com

HPAE-PAD Determination of Infant Formula Sialic Acids

INTRODUCTION

Dietary sialic acids are important for infant development, serving both immune system and cognitive development roles.[1] Although these functionalized neuraminic acids are present in all mammalian milk, the proportions vary significantly according to the species. Even though many neuraminic acids have been identified in human milk, sialyl-conjugates contain *N*-acetylneuraminic acid (Neu5Ac) but not *N*-glycolylneuraminic acid (Neu5Gc). In comparison, bovine milk has primarily Neu5Ac, but also a small proportion of oligosaccharides possessing Neu5Gc.[1]

In addition to containing different forms of sialic acids, bovine milk has been shown to contain less than 25% of the total sialic acid content of human milk.[2] Therefore, unfortified infant formulas made from bovine milk have a lower sialic acid content than human milk. Because of the critical role these carbohydrates play in infant development, manufacturers have begun enriching infant formulas with sialic acids to supplement the base of the formula and more closely mimic human milk.

Determination of sialic acids in a complex matrix, such as a dairy product, presents many challenges. The majority of sialic acids are found as part of a glycoconjugate rather than in the free form. In human milk, ~ 73% of sialic acids are bound to oligosaccharides, while some infant formulas have been shown to contain sialic acids primarily bound to glycoproteins.[2] In order to determine the sialic acids, they must first be released from the glycoproteins, glycolipids, and oligosaccharides. In dairy products, this is typically accomplished by a dilute (25 to 100 mM) acid digestion at 80 °C.[3] Many acid hydrolysis methods have been published. While sulfuric acid is commonly used, other acids have been evaluated including acetic acid, TFA, and HCl.[3,4] TFA and HCl have the advantage of being volatile and easily removed by lyophilization, depending on the needs of further sample-preparation steps.

Following sample hydrolysis, many sialic acid determination methods exist. Numerous spectroscopic methods have been previously reviewed.[3] Interferences in these methods can overestimate the concentration of sialic acids in complex samples; therefore, chromatographic methods that separate the sialic acids from potentially interfering compounds are preferred. Among the chromatographic methods, some require further sample derivatization for analyte detection, such as fluorescent labeling, followed by high-performance liquid chromatography (HPLC). Direct detection methods, such as high-performance anion-exchange with pulsed amperometric detection (HPAE-PAD), offer the advantage of direct analysis without sample derivatization.

In the work shown here, sialic acids are determined in infant formulas following acid hydrolysis. Two sample preparation methods are presented: one uses ion-exchange and the other uses enzyme digestion. Each method has

advantages for a specific type of sample, allowing options for sample-preparation optimization. Both methods remove many potentially interfering compounds present in a complex matrix such as infant formula. Subsequent sialic acid determination by HPAE-PAD on a CarboPac® PA20 column is specific and direct, eliminating the need for sample derivatization after sample preparation.

EQUIPMENT

Dionex ICS-3000 Ion Chromatography System including:
- SP Single Pump or DP Dual Pump module
- DC Detector/Chromatography module (single- or dual-temperature zone configuration)
- AS Autosampler

Electrochemical Detector (Dionex P/N 061719)

Electrochemical Cell (Dionex P/N 061757)

Disposable Gold Electrode, Carbohydrate Certified (Dionex P/N 060139)

Reference Electrode (Dionex P/N 061879)

10 µL PEEK™ Sample Injection Loop (Dionex P/N 042949)

Chromeleon® 7 Chromatography Workstation

Polypropylene injection vials with caps, 0.3 mL (Dionex P/N 055428)

Polypropylene injection vials with caps, 1.5 mL (Dionex P/N 079812)

Nalgene® 1000 mL 0.2 µm nylon filter units (VWR P/N 28198-514)

Polypropylene screw-cap tubes, 7 mL (Sarstedt P/N 60.550)

IC Acrodisc® syringe filters, 0.2 µm, 25 mm (Gelman Sciences P/N 4583T)

OnGuard® IIA, 2.5 cc cartridges (Dionex P/N 057092)

OnGuard Sample Prep Station (Dionex P/N 039599)

Polymethylpentene (PMP) volumetric flasks, 500 mL, Class A (Vitlab P/N 67504)

Dry block heater (VWR P/N 13259-005)

REAGENTS AND STANDARDS

Deionized (DI) water, Type I reagent-grade, 18 MΩ-cm resistivity or better

Sodium hydroxide, 50% (w/w) (Fisher P/N SS254-500)

Sodium acetate, anhydrous (Fluka P/N 71183)

Sulfuric acid (JT Baker P/N 9673-00)

N-Acetylneuraminic acid (Neu5Ac, NANA) (Ferro Pfanstiehl)

N-Glycolylneuraminic acid (Neu5Gc, NGNA) (Ferro Pfanstiehl)

Amyloglucosidase (Sigma P/N 10115)

SAMPLES

Three brands of infant formula were purchased for analysis. A soy-based formula was chosen for use as a matrix blank. Because this formula is dairy-free, it is expected to contain no sialic acids.

Brand A: Dairy-based infant formula.

Brand B: Dairy-based infant formula containing added maltodextrins.

Brand C: Soy-based infant formula.

CONDITIONS

Columns:	CarboPac PA20, 3 × 150 mm (P/N 060142) CarboPac PA20 Guard, 3 × 30 mm (P/N 060144)
Eluent A:	100 mM NaOH
Eluent B:	400 mM sodium acetate in 100 mM NaOH
Eluent Gradient:	10 to 200 mM acetate in 100 mM NaOH from 0 to 15 min, 200 mM acetate in 100 mM NaOH from 15 to 20 min, 10 mM acetate in 100 mM NaOH from 20 to 25 min
Flow Rate:	0.5 mL/min
Temperature:	30 °C (column and detector compartments)
Inj. Volume:	10 µL
Detection:	Pulsed amperometric, disposable carbohydrate certified gold working electrode
Background:	16–25 nC (using the carbohydrate waveform)
Noise:	~20 to 50 pC
System Backpressure:	~2900 psi

Carbohydrate 4-potential waveform for the ED:

Time(s)	Potential (V)	Gain Region*	Ramp*	Integration
0.00	+0.1	Off	On	Off
0.20	+0.1	On	On	On
0.40	+0.1	Off	On	Off
0.41	-2.0	Off	On	Off
0.42	-2.0	Off	On	Off
0.43	+0.6	Off	On	Off
0.44	-0.1	Off	On	Off
0.50	-0.1	Off	On	Off

*Settings required in the ICS-3000 and -5000 systems but not used in older Dionex systems.

Reference electrode in Ag mode (Ag/AgCl reference). See Dionex AU 141 for more information.[5]

PREPARATION OF REAGENTS AND STANDARDS

Eluent Solution

Prepare 1 L of 100 mM sodium hydroxide by adding 5.2 mL of 50% NaOH to 994.8 mL degassed DI water.

Prepare 1 L of 400 mM sodium acetate in 100 mM sodium hydroxide by dissolving 32.8 g anhydrous sodium acetate in ~ 800 mL DI water. Filter and degas the acetate solution through a 0.2 µm nylon filter unit. Transfer the solution to a 1 L volumetric flask, add 5.2 mL of 50% NaOH, and bring to volume with degassed DI water.

See Dionex TN 71 for detailed information on manual eluent preparation for HPAE-PAD applications.[6]

50 mM Sulfuric Acid for Sample Digestion

Prepare 1 L of 50 mM sulfuric acid by adding 2.8 mL concentrated sulfuric acid to a 1 L polypropylene volumetric flask that contains ~ 500 mL DI water. Bring to volume with DI water and mix thoroughly.

1000 U/mL Amyloglucosidase Stock Solution

On the day of analysis, prepare a stock solution of 1000 U/mL amyloglucosidase. The exact weight of amyloglucosidase will vary by the activity of the lot of enzyme purchased. For example, if the enzyme contains 57.7 U/mg, add 86.7 mg amyloglucosidase to 5.0 mL DI water and gently swirl to dissolve the enzyme. This solution will contain 1000 U/mL enzyme.

Table 1. Sialic Acid Standards Used for Sample Analysis

Stock Standard Volume Diluted to 1000 (µL)	Neu5Ac Conc. (nM)	Neu5Gc Conc. (nM)	Neu5Ac Amount (pmol/10 µL)	Neu5Gc Amount (pmol/10 µL)
1	100	6.8	1	<LOD*
2.5	250	17	2.5	<LOD*
5	500	34	5	0.34
10	1000	68	10	0.68
25	2500	170	25	1.7
50	5000	340	50	3.4
75	7500	510	75	5.1
100	10000	680	100	6.8

*Not used for Neu5Gc calibration.

Standard Stock Solutions

Prepare sialic acid stock solutions by dissolving Neu5Ac (149.8 mg in 50 mL DI water) and Neu5Gc (41.0 mg in 50 mL DI water). This results in 9.68 mM and 2.52 mM stock solutions, respectively. In dairy samples, ~ 95% of sialic acids are Neu5Ac. For this reason, prepare a mixed stock of 0.10 mM Neu5Ac and 6.8 µM Neu5Gc by diluting 500 µL of 9.68 mM Neu5Ac and 130 µL of 2.52 mM Neu5Gc to 48.4 mL total. Place aliquots of this solution into 1.5 mL cryogenic storage vials and store at -40 °C.

Standard Solutions

Calibration standards are prepared by diluting the stock standard solution as detailed in Table 1. For example, 10 µL of stock solution are added to 990 µL DI water to prepare a calibration standard of 1.0 µM Neu5Ac, or 10 pmol/10 µL injection. Prepare standards daily from stocks stored at -40 °C.

Ion-Exchange Cartridge Preparation

For best recoveries, convert a 2.5 cc OnGuard IIA cartridge from carbonate to chloride form by washing it with 15 mL DI water and then 15 mL of 100 mM NaCl. Even hydration of the resin is necessary and can be done using a slow and controlled flow of the initial water wash and the subsequent NaCl wash. Recommended cartridge washing steps and methods are further described in the OnGuard II cartridges product manual.[7] An OnGuard workstation can be used to control flow rate through the cartridges when simultaneously preparing multiple samples.

Powdered Infant Preparation, Acid Hydrolysis, and Maltodextrin Removal

Prepare powdered infant formulas by suspending 0.750 g of formula in 10.0 mL DI water. Use a vortexing mixer to ensure even mixing of the samples. Hydrolyze this solution by adding 900 µL formula to 5.0 mL of 50 mM sulfuric acid in a 7 mL polypropylene screw-cap vial. Heat the capped vial in a heat-block maintained at 80 °C for 1 h. After 1 h, remove samples and allow to cool to room temperature (~ 10 min). Before further treatment, centrifuge samples to separate fats and proteins suspended in the sample. To remove maltodextrins by anion exchange, prepare an OnGuard IIA cartridge. Skim off fat from the centrifuged sample with a pipet tip and pour the acid-hydrolyzed sample directly into the cartridge reservoir, taking care to leave precipitated proteins in the digestion tube. After loading the sample on the cartridge, wash the cartridge with 10 mL DI water to remove any residual uncharged compounds from the resin. Elute the bound sialicacids with 25 mL of 50 mM NaCl. Before the samples are injected, filter them through an IC syringe filter (0.2 µm, 25 mm) and dilute 1:2.5 with DI water to minimize retention-time shifting of Neu5Gc due to chloride.

Maltodextrins in hydrolyzed samples were removed by two independent methods. The first method tested was anion exchange and the second was enzymatic digestion. To remove maltodextrins by enzyme digestion, dilute the acid-hydrolyzed sample with DI water to nearly 500 mL in a 500 mL PMP volumetric flask. Add 500 µL of amyloglucosidase to the solution and dilute to 500 mL. Mix gently and allow the sample to digest for a minimum of 1 h at ambient temperature.

PRECAUTIONS AND CONSIDERATIONS

Labware: Avoid using glass volumetric flasks for dilution of samples and standards. Class A PMP flasks are recommended, although polypropylene is acceptable. Similarly, use polypropylene (rather than glass) digestion vials and injection vials.

When filling PMP or polypropylene labware, remove bubbles from the surface by gently swirling the solution in the volumetric flask while it is approximately three-quarters full. Bubbles on the walls of the flask cause dilution errors. Make the final dilution by gently adding water down the side of the flask. Similarly, bubbles in injection vials can lead to inconsistent injections and must be removed.

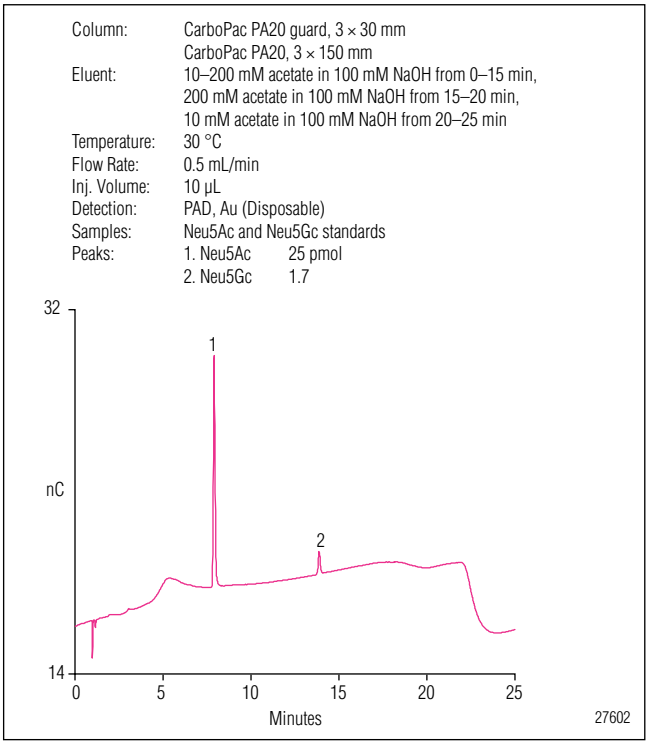

Figure 1. Separation of sialic acids on the CarboPac PA20 column.

RESULTS AND DISCUSSION

Figure 1 shows separation of Neu5Ac and Neu5Gc on the CarboPac PA20 column with a 10 to 200 mM acetate gradient in 100 mM NaOH. The peaks are well separated and easily quantified. For samples that have few interfering compounds, the gradient can easily be shortened by eluting with a gradient of 20–200 mM acetate in 100 mM NaOH and reducing the gradient time. In dairy samples, however, numerous other carbohydrates are present that can potentially interfere with sialic acid quantification. Infant formula, for example, contains added lactose, maltodextrins, and cereal starches. After acid hydrolysis, these carbohydrates interfere with sialic acid determination. While sample-preparation steps minimize these interfering compounds, they may still be present and detected by HPAE-PAD. By using a shallower gradient, other carbohydrates in the sample will be resolved from the sialic acids.

Table 2. Linearity, LOD, LOQ, and Precision of Sialic Acid Determination

Analyte	Range (pmol)	Corr. Coef. (r^2)	RT (min)	RT Precision (RSD)	Peak Area Precision[a] (RSD)	LOQ[b] (pmol)	LOD (pmol)
Neu5Ac	5.0–100	0.9995	7.89	0.05	0.98	0.8	0.24
Neu5Ac	1.0–75	0.9995					
Neu5Gc	0.34–6.8	0.9997	13.86	0.05	1.98	0.7	0.21

[a] Precision is measured by seven injections of 25 pmol Neu5Ac, 1.7 pmol Neu5Gc.

[b] LOD and LOQ are confirmed by injections at the concentrations listed with response measured at 3× and 10× the noise, respectively.

Linear Range, Limits of Quantification and Detection, and Precision

Table 2 shows the calibration results for Neu5Ac and Neu5Gc. In both cases, response is linear for the range studied. Two calibration ranges for Neu5Ac were investigated. The first calibration range covers concentrations of Neu5Ac present when determined by acid hydrolysis and OnGuard IIA sample preparation; the second calibration range is extended to include lower concentrations of Neu5Ac present when enzyme digestion is used after acid hydrolysis. In both cases, the response is linear. Neu5Gc is a minor component of infant formula and is derived from the bovine dairy source. The calibration range used is the same for both sample preparation methods.

The limit of detection (LOD) and limit of quantification (LOQ) were confirmed by standard injections that resulted in a response of 3× and 10× the noise, respectively. Neu5Ac was determined to have an LOD of 0.24 pmol on column and an LOQ of 0.80 pmol. Similarly, Neu5Gc limits were found to be 0.21 pmol and 0.70 pmol.

Retention time and peak area precisions of standards were determined by seven injections of a mid-range standard. In both cases, precision was excellent, with retention time RSD of 0.05 for both sialic acids and peak area RSDs of 0.98 and 1.98 for Neu5Ac and Neu5Gc, respectively.

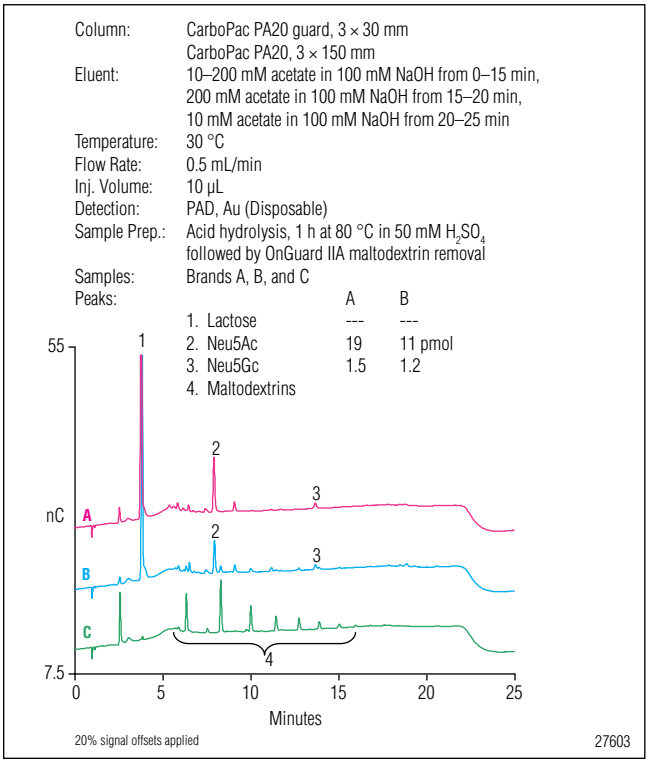

Figure 2. Separation of anion-exchange resin prepared infant formula samples based on A) dairy, B) dairy with added maltodextrins, and C) soy with added maltodextrins.

Sample Analysis

Before choosing a sample-preparation method, a number of methods were investigated to remove interfering compounds. Because sialic acids are charged at neutral pH, ion-exchange resins will trap the sialic acids on the resin and neutral compounds will not be retained.[8] The sulfuric acid matrix of samples loaded onto the cartridge does not affect Neu5Ac and Neu5Gc binding to the resin. This was confirmed experimentally with standards in sulfuric acid. The retained sialic acids may then be eluted using a stronger eluent, such as formate, acetate, or chloride. Each of these eluents was tested to evaluate resin loading and recovery of standards on an OnGuard IIA cartridge. Of the eluents tested, the best condition determined was elution with 50 mM sodium chloride followed by a 1:2.5 dilution of the sample in DI water. This process yields the highest recoveries and offers the simplest preparation before sample injection. Chromatographic results of this preparation are shown in Figure 2. Neu5Ac is well resolved from interfering peaks under these conditions, and peaks from neutral maltodextrins are minimized.

Table 3. Sample Analysis Precision Data, Day 2						
Sample	**Analyte**	**Amount (pmol)**	**mg/100 g of Sample**	**Peak Area Precision (RSD)**	**RT Precision (RSD)**	**Analysis Precision (RSD)**
Brand A, Replicate #1	Neu5Ac	22	85	4.25	0.06	0.59
	Neu5Gc	1.6	6.3	3.51	0.04	8.4
Brand A, Replicate #2	Neu5Ac	23	86	2.96	0.06	
	Neu5Gc	1.5	5.9	2.89	0.04	
Brand A, Replicate #3	Neu5Ac	22	85	2.39	0.12	
	Neu5Gc	1.3	5.4	2.44	0.13	
Brand B, Replicate #1	Neu5Ac	14	54	5.50	<0.01	15.7
	Neu5Gc	1.3	5.0	2.23	0.06	11.0
Brand B, Replicate #2	Neu5Ac	13	48	5.52	<0.01	
	Neu5Gc	1.1	4.4	5.08	<0.01	
Brand B, Replicate #3	Neu5Ac	10	40	8.52	0.11	
	Neu5Gc	1.0	4.0	4.81	0.13	

Table 4. Between-Day Sample Analysis Precision						
Day	**Sample**	**Analyte**	**Average Amount (pmol)**	**mg/100 g of Sample**	**Intraday Precision (RSD)**	**Between-day Precision (RSD)**
1	Brand A	Neu5Ac	16	62	20.6	18
		Neu5Gc	1.2	4.7	23.5	11
	Brand B	Neu5Ac	11	44	6.8	11
		Neu5Gc	1.3	5.2	4.0	8.9
2	Brand A	Neu5Ac	22	86	0.59	
		Neu5Gc	1.4	5.8	8.4	
	Brand B	Neu5Ac	12	47	15.7	
		Neu5Gc	1.1	4.5	11.0	
3	Brand A	Neu5Ac	17	64	5.3	
		Neu5Gc	1.3	5.3	1.4	
	Brand B	Neu5Ac	10	38	13.6	
		Neu5Gc	1.1	4.5	10.3	

Sample Analysis Precision and Accuracy

Precision was evaluated over three days of triplicate sample analysis. Representative results for one day of triplicate sample analysis are presented in Table 3. Table 4 shows data collected after three days of analysis. Sample C, the soy-based infant formula, did not contain detectable Neu5Ac or Neu5Gc and was used as a blank matrix for comparing recovery of spiked sialic acids.

When corrected for dilution during the sample preparation process, the prepared samples of Brands A and B contained 86 and 47 mg Neu5Ac in 100 g of sample, respectively. Retention time precision was similar to that determined by injecting standards, with RSDs ranging from < 0.01 to 0.13. Peak area precision RSDs ranged from 2.23 to 8.52.

Table 5. Recovery Data for Three Types of Infant Formulas						
Day	**Sample**	**Analyte**	**Amount Spiked into 5.9 mL Hydrozylate (nmol)**	**Theoretical Concentration of Spiked Sample (pmol)**	**Measured Concentration (pmol)**	**% Recovery**
1	Brand A	Neu5Ac	75.0	25	27	108
		Neu5Gc	5.1	1.8	2.0	111
	Brand B	Neu5Ac	75.0	20	20	100
		Neu5Gc	5.1	1.9	1.8	94.7
	Brand C	Neu5Ac	75.0	8.9	8.5	95.6
		Neu5Gc	5.1	0.6	0.61	100
2	Brand A	Neu5Ac	75.0	31	28	90.3
		Neu5Gc	5.1	2.0	1.7	85
	Brand B	Neu5Ac	75.0	21	20	96.6
		Neu5Gc	5.1	1.7	1.8	102
	Brand C	Neu5Ac	75.0	8.7	8.1	93.1
		Neu5Gc	5.1	0.59	0.54	91.5
3	Brand A	Neu5Ac	75.0	26	23	88.5
		Neu5Gc	5.1	1.9	1.6	84.2
	Brand B	Neu5Ac	75.0	19	20	105
		Neu5Gc	5.1	1.7	1.6	94.1
	Brand C	Neu5Ac	75.0	8.9	7.1	80.1
		Neu5Gc	5.1	0.6	0.47	78.3

Variability between sample replicates of dairy samples may be large, as shown in Table 5; therefore, optimization of digestion and sample-preparation methods for individual infant formulas is highly recommended.

Method accuracy was investigated by spiking infant formula acid hydrozylates with known amounts of Neu5Ac and Neu5Gc and evaluating recovery of the amended sample through the sample-preparation procedure. Recoveries for Neu5Ac ranged from 80 to 109% for three different formulas treated by anion-exchange sample preparation (Table 5). Recoveries for Neu5Gc were similar, ranging from 78 to 111%.

Maltodextrin Removal by Enzymatic Digestion

Amyloglycosidase was chosen to remove maltodextrins from the samples without the need for ion-exchange sample cleanup. This enzyme was chosen for its broad activity against glycosidic linkages (α1–2, 1–6, and 1–4) as well as its optimal activity at low pH.[9] Because amyloglucosidase is active at pH 3, samples may simply be diluted after acid hydrolysis without the need to further adjust pH prior to adding enzyme. When samples were digested by this method, maltodextrins were significantly reduced (Figure 3B). However, as shown in Figure 3A, in some formulas there are other potential interferences in addition to maltodextrins.

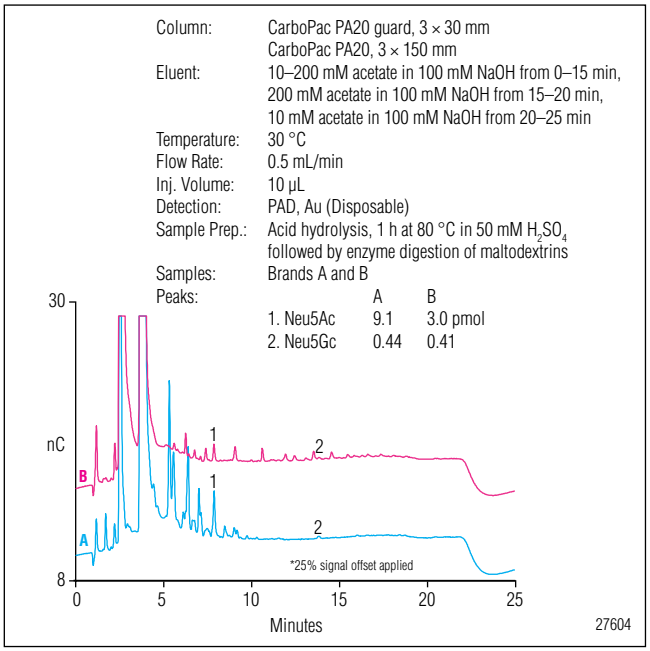

Figure 3: Separation of enzyme digestion prepared infant formula samples based on A) dairy, B) dairy with added maltodextrins. Soy-based formula maltodextrins are not well digested by amyloglycosidase and a chromatogram is not shown.

Table 6. Sialic Acid Content Determined after Enzyme Digestion of Infant Formula Acid Hydrozylates

Sample	Analyte	Average Concentration of Sialic Acid (pmol) (n=3)	Sialic Acid Amount (mg/100 g of Sample)
Brand A	Neu5Ac	5.97	131
	Neu5Gc	0.39	9
Brand B	Neu5Ac	1.96	45
	Neu5Gc	<LOD	

For samples that contain maltodextrins, this sample-preparation method is useful; however, conditions must be customized for each sample type. For samples that do not contain significant amount of maltodextrins, this method will not reduce interfering compounds. For example, results for Brand A are highly elevated compared to the results found when using the anion-exchange sample-preparation method (Tables 5 and 6). This brand does not have additional maltodextrins added to the formula. Results for Brand B are similar for both sample-preparation methods. In both cases, recoveries of standards spiked into the sample digest are good. Recoveries from infant formulas treated by this method range from 89.4 to 95%. For soy formulas, the enzyme did not sufficiently remove the putative maltodextrins after 24 h of digestion; therefore, the method is not recommended for these formulas. The polysaccharides added to the soy-based formula tested are likely linked by different glycosidic linkages that are not easily digested by amyloglycosidase. The enzyme and the conditions for digestion may be improved, depending on additives in a specific infant formula sample.

CONCLUSION

Sialic acids in infant formulas are accurately determined by HPAE-PAD using the CarboPac PA20 column following acid hydrolysis and maltodextrin removal using one of two sample-preparation methods. HPAE-PAD provides reliable determination of sialic acids in acid-hydrolyzed infant formula samples without sample derivatization. This method may be used to quantify sialic acids in formulas that have been enriched with sialic acids.

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SUPPLIERS

VWR, 1310 Goshen Parkway, West Chester, PA 19380, U.S.A. Tel: 800-932-5000. www.vwr.com

Fisher Scientific, One Liberty Lane, Hampton, NH 03842, U.S.A. Tel: 800-766-7000. www.fishersci.com

Sigma-Aldrich, P.O. Box 14508, St. Louis, MO 63178, U.S.A. Tel: 800-325-3010. www.sigma-aldrich.com

Ferro Pfanstiehl, 1219 Glen Rock Avenue, Waukegan, IL, 60085, U.S.A. Tel: 800-383-0126. www.ferro.com

Determination of Sialic Acids Using UHPLC with Fluorescence Detection

INTRODUCTION

Dietary sialic acids play an important role in infant development, serving both immune system and cognitive development roles.[1] Many neuraminic acids have been identified in human milk, however N-acetylneuraminic acid (Neu5Ac) is predominant, and N-glycolylneuraminic acid (Neu5Gc) is generally absent. In comparison, bovine milk contains approximately 5% Neu5Gc.[1] In addition to containing different forms of sialic acids, bovine milk has been shown to contain less than 25% of the total sialic acid content of human milk.[2] The sialic acid content in unfortified infant formulas is dependent on the sialic acids from bovine milk. As such, these formulas have lower sialic acid contents and different sialic acid proportions compared to human milk. Because of the critical role these carbohydrates play in infant development, many manufacturers enrich infant formulas with sialic acids to more closely mimic human milk.

Sialic acid determination in a complex matrix, such as a dairy product, presents many challenges. The majority of milk sialic acids are found as part of a glycoconjugate rather than as the free acid. In human milk, ~73% of sialic acid is bound to oligosaccharides, but infant formulas have been shown to contain sialic acids primarily bound to glycoproteins.[2] In order to determine the sialic acids, they must first be released from the glycoproteins, glycolipids, and oligosaccharides. In dairy products, this is typically accomplished by a dilute (25 to 100 mM) acid digestion at 80 °C.[3] Many acid hydrolysis methods have been published. While sulfuric acid is commonly used, other acids have been evaluated, including acetic acid, TFA, and HCl.[3,4] These acids have the advantage of being volatile and easily removed by lyophilization, which could be important, depending on the needs of further sample preparation steps.

Following sample hydrolysis, many options are available for determination of sialic acids. Numerous spectroscopic methods exist. However, interferences in these methods can overestimate the concentration of sialic acids and, therefore, chromatographic methods that separate the sialic acids from potentially interfering compounds are preferred. Both direct and indirect chromatographic methods such as High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD, direct) or fluorescent labeling followed by HPLC (indirect) have been published.[3,4] One common fluorescent labeling method, using 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (DMB) to label the sialic acids, was first published by Hara, et al.[5-6] This method has previously been modified to determine sialic acids in infant formulas.[7-8] Although the fluorescent labeling method determines sialic acids indirectly, the chromatographic conditions are less likely to change the O-acetylation of the sialic acids, allowing identification of a wider range of sialic acids.[9]

In this work, *N*-acetylated sialic acids are determined and O-acetylated sialic acids are identified by HPLC with fluorescence detection following acid hydrolysis and DMB derivatization of infant formula samples. By using a water:acetonitrile gradient, high resolution of the sialic acids was obtained in a 20 min analysis time, compared to the common 40 min isocratic method. The described assay uses a short format Acclaim® RSLC 120 C18 column that allows fast run times and requires less acetonitrile than other published methods by using a lower flow rate and having a shorter run time. The sensitivity of fluorescence detection easily allows determination of sialic acids in the infant formula which are present in the pmol range. The sensitivity provides for simple determination of Neu5Ac, Neu5Gc, and O-acetylated sialic acids in the derivatized samples.

EQUIPMENT

Dionex UltiMate® 3000 RSLC system including:

- SRD-3600 Solvent Rack and Degasser (Dionex P/N 5035.9230)
- HPG-3400RS Binary Pump with a 350 µL mixer (Dionex P/N 5040.0046)
- WPS-3000TRS Well Plate Sampler, Thermostatted (Dionex P/N 5840.0020)
- Sample loop, 25 µL (Dionex P/N 6820.2415)
- TCC-3000RS Thermostatted Column Compartment (Dionex P/N 5730.0000)
- Precolumn Heater (Dionex P/N 6722.0530)
- Viper UHPLC Fingertight Fitting and Capillary Kit, RSLC Systems, SST (Dionex P/N 6040.2301)
- FLD-3400RS Fluorescence Detector with dual PMT (Dionex P/N 5078.0025)
- Chromeleon® 7.0 Chromatography Workstation
- Polypropylene injection vials with caps and septa, 0.3 mL (Dionex P/N 055428)
- 7 mL Polypropylene screw cap tubes (Sarstedt P/N 60.550)
- IC Acrodisc® syringe filters, 0.2 µm, 25 mm (Pall Corporation P/N 4583T)
- OnGuard® IIA, 2.5 cc Cartridges (Dionex P/N 057092)
- OnGuard Sample Prep Workstation (Dionex P/N 039599)
- 1.5 mL Microcentrifuge tubes (Sarstedt P/N 72.692.005)
- Dry block heater (VWR P/N 13259-005)

REAGENTS AND STANDARDS

Deionized (DI) water, Type I reagent grade, 18 MΩ-cm resistivity or better

Acetonitrile (Honeywell, P/N 015-4)

Formic acid (Fluka P/N 06440)

Sulfuric acid (JT Baker P/N 9673-00)

N-Acetylneuraminic acid (Neu5Ac, NANA) Ferro Pfanstiehl

N-Glycolylneuraminic acid (Neu5Gc, NGNA) Ferro Pfanstiehl

Glyko® Sialic Acid Reference Panel (ProZyme P/N GKRP-2503)

Glacial acetic acid (JT Baker P/N 9515-03)

2-Mercaptoethanol (Aldrich P/N M6250)

Sodium hydrosulfite (Sigma P/N 157953)

1,2-Diamino-4,5-methylenedioxybenzene dihydrochloride (DMB) (Sigma P/N D4784)

SAMPLES

Three brands of commercially available infant formula were purchased for analysis. A soy-based formula was chosen for use as a matrix blank, because sialic acids are not expected in this nondairy product.

Brand A: Dairy-based infant formula
Brand B: Dairy-based infant formula with maltodextrins
Brand C: Soy-based infant formula

CONDITIONS

Column:	Acclaim RSLC 120 C18, 2.2 µm, 2.1 × 100 mm
Gradient:	5% B from 0–5 min, 5%–20% B from 5–13 min, 20–40% B from 13–15 min, 40% B from 15–20 min, 3 min equilibration at 5% B before injection
Flow Rate:	0.42 mL/min
Inj. Volume:	5 µL
Temperature:	45 °C (column compartment)
Detection:	Excitation λ, 373 nm Emission λ, 448 nm
Noise:	~2000 counts
System Backpressure:	~300 bar (~4350 psi)
Run Time:	20 min

Table 1. Sialic Acid Standards Preparation						
Combined Stock Standard (µL)	2 M Formic Acid (µL)	DI Water (µL)	Neu5Ac (µM)	Neu5Gc (µM)	Neu5Ac in 5 µL Injection (pmol)	Neu5Gc in 5 µL Injection (pmol)
10	500	490	1.0	0.78	5.0	0.39
25	388	363	3.2	2.5	16	1.3
50	400	350	6.3	4.9	31	2.4
100	500	400	10.0	7.8	50	3.9
100	375	275	13.0	1.0	67	5.2
100	300	200	17.0	1.3	83	6.5
100	250	150	20.0	1.6	100	7.8
100	200	100	25.0	2.0	130	9.8
200	200	0	50.0	4.0	260	20.0

PREPARATION OF SOLUTIONS AND REAGENTS

Mobile Phases A and B

Mobile Phase A: DI water, Type I reagent grade, 18 MΩ-cm resistivity or better.

Mobile Phase B: Acetonitrile, HPLC grade or better.

Reagents

Formic acid, 1 M

Add 42.5 mL concentrated formic acid to a 1 L volumetric flask containing ~500 mL DI water. Fill the flask to the mark with DI water, cap the flask, and invert to mix the solution.

Formic acid, 2 M

Add 21.25 mL concentrated formic acid to a 250 mL volumetric flack containing ~150 mL DI water. Fill the flask to the mark with DI water, cap the flask, and invert to mix the solution.

Sulfuric acid, 100 mM

Add 540 µL of concentrated sulfuric acid to 99.46 mL (g) of DI water. Mix well.

Standard Stock Solutions

Dissolve 149.8 mg dried Neu5Ac in 50 mL of deionized water to prepare a 9.68 mM stock solution. Similarly, dissolve 41.0 mg dried Neu5Gc in 50 mL of deionized water to prepare a 2.52 mM stock solution. In dairy samples, ~95% of the sialic acids are Neu5Ac. Replicate this proportion of sialic acids in the samples by diluting 250 µL of 9.68 mM Neu5Ac and 75 µL of 2.52 mM Neu5Gc in 24.23 mL total volume.
This combined stock standard solution contains 0.10 mM Neu5Ac and 7.8 µM Neu5Gc. Aliquot this solution into 1.5 mL cryogenic storage vials and store at -40 °C. Avoid repeated freeze–thaw cycles.

Standard Solutions

Both the stock solution described above and a sialic acids standard mixture containing Neu5Gc, Neu5Ac, Neu5,7Ac2, Neu5Gc9Ac, Neu5,9Ac2, and Neu5,7,(8),9Ac3 were used to identify sialic acids in infant formulas. Dissolve the contents of the standard mixture vial in 25 µL DI water to prepare the panel for derivatization.

Prepare calibration standards by diluting the combined stock solution as shown in Table 1. For example: Pipet 100 µL combined stock solution into a 1.5 mL microcentrifuge tube. Pipet an additional 100 µL DI water and 200 µL of 2 M formic acid to prepare a standard of 25 µM Neu5Ac and 2.0 µM Neu5Gc in 1 M formic acid. It is critical that the standards are in the same matrix as the samples. If the standards are not prepared in formic acid, the derivatization reaction efficiency will not be the same for both standards and samples, resulting in a potentially large systematic error in the quantification of the samples.

Powdered Infant Formula Preparation, Acid Hydrolysis, and Maltodextrin Removal

Prepare powdered infant formulas by suspending 0.75 g in 10.0 mL DI water. Mix using a vortexing mixer to ensure an even suspension. Hydrolyze this solution by adding 2.5 mL formula suspension to 2.5 mL of 100 mM sulfuric acid in a 7 mL polypropylene screw cap vial. Heat the capped vial in a heat block maintained at 80 °C for 1 h. After 1 h, remove the samples and cool to room temperature (~10 min). Before further treatment, centrifuge the hydrolysates at 5000 rpm and 5 °C for 10 min to separate the fats and proteins suspended in the sample.

To remove maltodextrins by anion exchange, prepare an OnGuard II A cartridge as described in the manual.[10] Skim the fat off the top of the centrifuged sample with a pipet tip and pour the acid-hydrolyzed sample directly into the cartridge reservoir, taking care to leave the precipitated proteins in the digestion tube. Load the sample onto the anion-exchange cartridge and wash the cartridge with 10 mL DI water. This step washes off the neutral carbohydrates. Elute the sialic acids with 20 mL of 1 M formic acid. After elution, filter the sample with a 0.2 µm IC syringe filter. Promptly derivatize this sample as described below.

DMB Derivatization Reagent

Prepare the DMB reagent in the following order. Add 1.5 mL of DI water to a glass vial. To this solution add 172 µL of glacial acetic acid. Mix well. To this solution add 112 µL of 2-mercaptoethanol. Mix the solution well. Add 4.9 mg of sodium hydrosulfite to the solution and mix. The solution may become cloudy in appearance. Lastly, add 3.5 mg of DMB hydrochloride and 200 µL DI water and mix the solution well. Prepare the reagent fresh each day of analysis. The reagent is light sensitive and should be stored at -20 °C in the dark when not in use. Best results are obtained in this work with fresh derivatization reagent. As the DMB reagent ages, additional peaks that are unrelated to carbohydrate derivatization were observed in reagent blanks.

Derivatization Conditions

Derivatize samples and standards by adding 50 µL of the derivatization reagent to 50 µL of sample in a 1.5 mL screw cap microcentrifuge vial. Transfer the vials to a heating block and incubate for 2.5 h in the dark at 50 ± 2 °C.

Samples, standards, and controls must be derivatized at the same time with the same preparation of derivatization reagent. After 2.5 h of incubation, freeze the solutions at -40 °C to slow the reaction. Thaw the samples and transfer to 0.3 mL injection vials. Best results are obtained within 24 h of derivatization. Derivatized samples degrade with exposure to light and oxygen and should be analyzed as soon as possible.

Precautions

Perform derivatization reagent preparation, sample derivatization, and sample transfers to injection vials in a fume hood. Analyze samples promptly. Derivatized samples will degrade faster on exposure to light. It is strongly recommended that a temperature-controlled autosampler be set to 4 °C and the samples be kept in the dark by use of amber vials or by keeping the autosampler cover closed. When filling low-volume conical vials, it is important to ensure that all air is removed from the cone of the vial. If bubbles are present, peak area precision will be poor.

As noted by Hara et al., the concentration of acid will affect the efficiency of the reaction. It is important for the sample conditions to be mimicked in the standards that are derivatized to avoid systematic error due to different derivatization efficiency. For example, standards that were derivatized in 750 mM acetic acid showed 57% of the peak area for Neu5Ac as compared to the same concentration standards that were derivatized in 750 mM acetic acid in addition to 500 mM formic acid. Furthermore, sodium chloride strongly impacts the derivatization reaction efficiency. Samples containing high amounts of sodium chloride (50 mM) will degrade during the derivatization incubation time, leading to peak areas of <35% of those without the added salt. Standards in formic acid containing 5 mM of sodium chloride exhibited decreased peak areas of 12–13% compared to those without. This effect is reduced compared to 50 mM sodium chloride, and is similar to the between-day variability observed in standards. For best accuracy, the standards should be derivatized in a matrix as similar to the samples as possible, including both the concentration of acid and salts. Optimization of derivatization conditions is highly recommended.

The commonly reported isocratic method using water/methanol/acetonitrile is not recommended for these samples. Backpressure was found to increase after multiple sample injections.

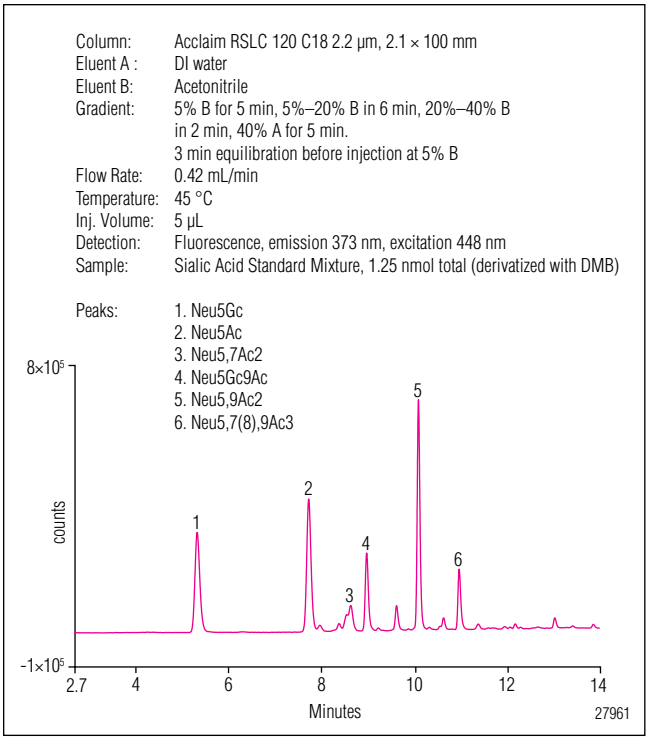

Figure 1. Separation of a derivatized sialic acid standard mixture on the Acclaim RSLC 120 C18 column.

The implication of this is that components of the samples are not eluted from the column. With continued injections, the efficiency of the column will decrease. The gradient method described in this work is recommended for best column performance during routine analysis. Direct injection of sample hydrolysates on to column is not recommended because it may result in lipids and other materials accumulating on the column.

RESULTS AND DISCUSSION

Figure 1 shows the separation of a sialic acid reference standard mixture on the Acclaim RSLC C18 column. As can be seen, Neu5Gc and Neu5Ac are well separated from one another. The O-acetylated sialic acids are also present in this standard with Neu5,7Ac2, Neu5Gc9Ac, Neu5,9Ac2, and Neu5,7(8),9Ac3 identified. In the case of Neu5,7Ac2, a reagent peak can interfere. The intensity of this reagent peak will vary with the derivatization reagent preparation. The sialic acids of interest are separated in under 15 min. However, to maintain column performance, a column wash step is added after each injection. Separation of the reference standards was evaluated on the Acclaim PA and PA2 columns. The shortest run time for standards was obtained with the PA column; however, when injecting samples, the best resolution was found with the C18 column.

Table 2. Linearity, LOD, and LOQ for Sialic Acids

Analyte	Range (pmol)	Correlation Coefficient (r^2)	LOD (pmol)	LOQ (pmol)
Neu5Ac	5–260	0.9952	0.06	0.17
Neu5Gc	0.2–9.8	0.9940	0.08	0.23

Table 3. Peak Area Reproducibility for Multiple Days of Derivatization (n = 3)

Analyte	Day	RT (min)	RT Precision* (RSD)	Peak Area (counts*min)	Peak Area Precision (RSD)
Neu5Ac	1	7.69	0.04	1088000	0.55
Neu5Gc		5.29	0.03	80650	0.76
Neu5Ac	2	7.69	0.02	1229000	0.35
Neu5Gc		5.28	0.05	90430	0.79
Neu5Ac	3	7.69	0.03	1096000	0.96
Neu5Gc		5.28	0.05	81060	1.16
Neu5Ac	4	7.70	0.06	895000	1.45
Neu5Gc		5.29	0.08	66010	1.69

*A standard of 67 pmol Neu5Ac and 5.2 pmol Neu5Gc was used for determination of retention time (RT) and peak area precisions.

The effect of temperature was investigated between 35 and 50 °C. At 50 °C, the peak areas were reduced compared to 40 °C, indicating on-column decomposition. At 45 °C, the overall run time was shortest, with no detectable decomposition of the standards compared to 40 °C.

Linear Range, Limit of Quantification (LOQ), Limit of Detection (LOD), and Precision

Table 2 shows the calibration range, correlation coefficients, and precisions for several days of sialic acid standard preparations. The efficiency of the derivatization reaction impacts the standard peak area from day to day. Preparing standards along with samples limits the effects of this variability; however, between-day peak areas were observed to vary by 13% for both Neu5Ac and Neu5Gc, as detailed in Table 3. Similarly, the LOQ and LOD may vary between analysis days. Using the conditions described, the LOQ and LOD were determined to be 0.17 pmol and 0.06 pmol, respectively, for Neu5Ac. The LOQ and LOD for Neu5Gc were 0.23 and 0.08 pmol, respectively. In addition to variability of the determined

LOQ and LOD based on the derivatization conditions, the detection settings of the fluorescence detector must be considered. In this work, the photomultiplier tube (PMT) was set to the least sensitive collector voltage setting of 1 and the lamp set to the standard flash lamp rate. If greater sensitivity is required, the flash lamp frequency can be increased and sensitivity settings can be changed to further increase the sensitivity. It should be noted that even without optimizing the detector conditions, the method discussed has ample sensitivity to determine sialic acids in infant formulas.

Determination of Sialic Acids in Infant Formulas

The separation of sialic acids in infant formulas is shown in Figure 2. As expected, the dominant sialic acid present in dairy-based formulas is Neu5Ac. Neu5Gc is present to a lesser extent. Brand A also contains minor amounts of Neu5,7Ac2 and both Brands A and B contain a small amount Neu5,9Ac2. Hydrolysis conditions are not optimal for determining these sialic acids; however, they are present. As expected, Brand C, a soy-based formula, does not contain the identified sialic acids. However, it should be noted that under the gradient conditions described here, there is a small unknown peak that elutes near Neu5Gc and could potentially interfere with determination of this sialic acid. Different gradients and use of an isocratic method (8:7:85 CH_3OH:CH_3CN:water) did not fully resolve this peak from Neu5Gc. Previously published work did not observe this peak under isocratic conditions and it is likely dependent on the specific ingredients of the soy infant formula.[7] However, the RT is consistently shorter than Neu5Gc and in spiked samples it is evident there are two components eluting.

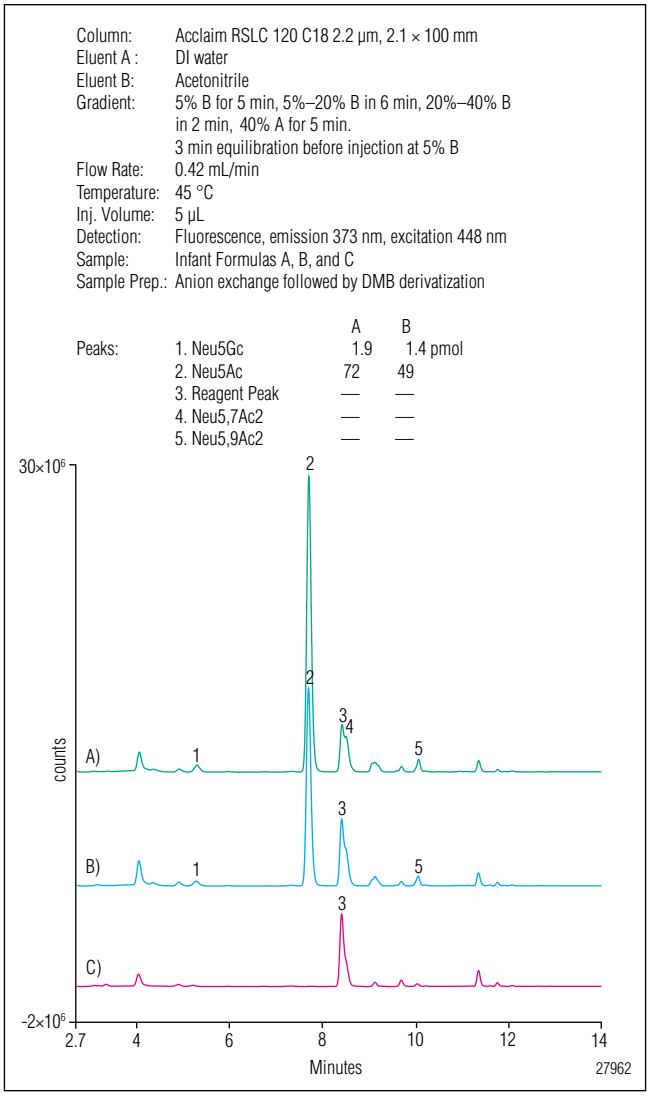

Figure 2. Determination of sialic acids in infant formulas on the Acclaim RSLC 120 C18 column.

Table 4. Sample Analysis Results, Triplicate Infant Formula Sample Preparations								
Sample	**Analyte**	**RT (min)**	**RT Precision (RSD)**	**Peak Area (counts*min)**	**Peak Area Precision (RSD)**	**Measured Concentration (pmol)**	**mg/100 g of Sample**	**Sample Analysis Precision (RSD)**
A replicate #1	Neu5Ac	7.69	0.05	1925000	3.95	70.2	91	7.7
	Neu5Gc	5.29	0.07	55900	3.99	1.86	2.5	8.0
A replicate #2	Neu5Ac	7.69	0.05	1931000	0.40	70.4	92	—
	Neu5Gc	5.28	0.09	57110	1.16	1.9	2.6	—
A replicate #3	Neu5Ac	7.69	0.05	2198000	0.28	80.1	100	—
	Neu5Gc	5.28	0.06	64640	1.67	2.14	2.9	—
B replicate #1	Neu5Ac	7.69	0.08	1060000	1.59	38.7	50	12
	Neu5Gc	5.27	0.14	31590	1.97	1.06	1.4	16
B replicate #2	Neu5Ac	7.69	0.03	1346000	1.47	49.1	63	—
	Neu5Gc	5.27	0.02	40790	1.85	1.36	1.8	—
B replicate #3	Neu5Ac	7.69	0.05	1161000	1.84	42.4	56	—
	Neu5Gc	5.28	0.06	30790	2.25	1.03	1.4	—

Precision and Accuracy of Determination

Samples were analyzed in triplicate to evaluate the precision of the assay. Table 4 details the results for one day of analysis. Peak area RSDs for Neu5Ac are generally <2, with the exception of replicate #1 of Brand A, which had a single injection with consistently lower peak areas than the other injections. RTs were stable, indicating that under these gradient components nonpolar sample components elute from the column and do not impact subsequent analyses. The analysis precision (RSD) for triplicate samples was 7.7 for Neu5Ac and 8.0 for Neu5Gc for Brand A.

Replicates of Brand B were more variable, with RSDs of 12 and 16 for Neu5Ac and Neu5Gc, respectively. Between-day precision was evaluated by repeating sample analysis. When comparing the average determined amounts, between-day precision (RSD) was 1.3 and 1.0 for Neu5Ac and 6.6 and 8.9 for Neu5Gc in infant formula Brands A and B, respectively. This is exceptional, considering the precision when comparing replicates within a day can vary widely. Expecting to routinely achieve such low values for between-day precision is unrealistic.

Table 5. Recoveries of Sialic Acids from Infant Formula Samples						
Sample	**Analyte**	**Amount (Unspiked) (pmol)**	**Amount Spiked into Hydrolysate (pmol)**	**Theoretical Spiked Concentration (after Sample Prep.) (pmol)**	**Measured Amount (Spiked) (pmol)**	**Recovery (%)**
Brand A	Neu5Ac	63.5	225	28.5	92.4	100
	Neu5Gc	0.52	18	2.22	3.20	120
Brand B	Neu5Ac	47.5	170	21.3	72.8	120
	Neu5Gc	1.13	13	1.66	3.20	120
Brand C	Neu5Ac	<LOD	75	9.52	9.00	95
	Neu5Gc	<LOD	5.8	0.74	0.70	95
Blank	Neu5Ac	<LOD	75	9.41	8.62	92
	Neu5Gc	<LOD	5.8	0.73	0.65	89

Accuracy was evaluated by spiking the sample hydrolysates before sample preparation by anion exchange with known amounts of Neu5Ac and Neu5Gc to approximately double the amount present in the samples (Table 5). This spiking was also done in a reagent blank and soy formula for comparison. Recoveries range from 89 to 120%. Recoveries were higher in dairy-based infant formulas compared to the soy infant formula and reagent blank control samples. Accuracy can be highly impacted by the efficiency of the derivatization, which, as noted in the precautions section, can by affected by the matrix of the derivatization reaction.

Sample Preparation Comparison to HPAE-PAD Analysis

Previous work illustrates the application of HPAE-PAD in the analysis of these samples.[11] Some comparisons can be made to this work. HPAE-PAD is a direct method that does not require derivatization; however, typical strong base elution conditions do not allow for determination of *O*-acetylated sialic acids. If only the total amount of Neu5Ac and Neu5Gc are of interest, both methods are appropriate as the O-acetylated sialic acids will degrade in base to the parent Neu5Ac or Neu5G. The time required to prepare samples for the two methods are dramatically different. Both methods require the same sample hydrolysis optimization and anion-exchange sample preparation for consistent sample analysis. These steps will take approximately 4 h in total for a set of three triplicate samples and three controls (12 digestions total). In addition to the sample preparation time, derivatization for fluorescence detection will require 2.5 h for the reaction with an additional 1 h to stop the reaction and prepare the samples for injection after the derivatization is complete.

CONCLUSION

In this work, *N*-acetylated sialic acids were determined and *O*-acetylated sialic acids are identified by HPLC with fluorescence detection following acid hydrolysis and DMB derivatization of infant formula samples. By using a water:acetonitrile gradient, high resolution of the sialic acids was obtained in a 20 min analysis time, including a column-wash step to maintain method performance. The sensitivity of the fluorescence detector easily allows determination of sialic acids in the infant formula that are present in the pmol range.
The sensitivity provides for simple determination of Neu5Ac, Neu5Gc, and *O*-acetylated sialic acids in the derivatized samples.

SUPPLIERS

VWR, 1310 Goshen Parkway, West Chester, PA 19380 U.S.A. Tel: 800-932-5000.
www.vwr.com

Fisher Scientific, One Liberty Lane, Hampton, NH 03842 U.S.A. Tel: 800-766-7000.
www.fishersci.com

Sigma-Aldrich, P.O. Box 14508, St. Louis, MO 63178 U.S.A. Tel: 800-325-3010.
www.sigma-aldrich.com

Ferro Pfanstiehl, 1219 Glen Rock Avenue, Waukegan, IL, 60085, U.S.A. Tel: 800-383-0126.
www.ferro.com

ProZyme, 1933 Davis Street, Suite 207, San Leandro, CA 94577, U.S.A. Tel: 800-457-9444.
www.prozyme.com

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Time Savings and Improved Reproducibility of Nitrate and Nitrite Ion Chromatography Determination in Milk Samples

INTRODUCTION

Cow's milk is of particular dietary value to infants, small children, and expectant mothers as it is an important source of calories, minerals (including calcium), fat-soluble vitamins A, D, E, and K, and protein. Because of its nutritional value, it is imperative that the commercial milk supply be free of contaminants such as nitrate and nitrite. The excessive consumption of nitrate can lead to underoxygenation of the blood and, consequently, underoxygenation of the tissues, which can cause numerous health problems, the most severe of which is death. With a much smaller total blood volume, infants and small children are more severely impacted than adults when consuming the same nitrate-contaminated product.

The most likely source of nitrate in the blood stream is drinking water. Drinking water can become contaminated in areas where there has been excessive application of nitrate-based fertilizers and where sodium or potassium nitrate is used in canisters designed to kill rodents. For an infant, the water used to prepare infant formula (baby food), the water consumed by the nursing mother, or the water consumed by dairy cattle whose milk is used to prepare milk-based infant formulas, are possible sources of nitrate. For most children, infant formula and mother's milk will eventually be replaced by cow's milk.

Nitrite is also a concern because it is easily oxidized to nitrate. Excessive consumption of nitrite and nitrate also has been implicated as a cause of other health problems.[1] For these reasons, the United States Environmental Protection Agency (U.S. EPA) regulates the amount of nitrite and nitrate in drinking water and has published an ion chromatography (IC) method for the determination of these two anions, along with fluoride, chloride, bromide, sulfate, and phosphate.[2] For the same reasons, the concentration of nitrite and nitrate should also be determined in milk.

The IC analytical technique is the most commonly used for simultaneously measuring nitrite and nitrate in samples. These two anions can be detected either by suppressed conductivity detection or by their absorbance at 210 nm.[3] Unfortunately, milk samples cannot be injected directly onto the IC system to measure nitrite and nitrate because the milk fat will foul and eventually poison the column, and milk proteins will interfere with the chromatography and compromise the detection of nitrite and/or nitrate by either suppressed conductivity or absorbance detection. Even after one or more sample preparation steps, the remaining protein or other anionic molecules can interfere with nitrite and nitrate determination, or foul the column. The analyst must remove as many interfering compounds from the milk as possible while still achieving full recovery of nitrite and nitrate.

In this study, a milk sample is subjected to an acid precipitation step prior to loading the sample into an autosampler vial. The remainder of the sample preparation is completed in-line with an InGuard® HRP sample preparation cartridge. This saves the analyst time and reduces the possibility of sample contamination. Nitrite and nitrate are then separated on an IonPac® AS20 column set and detected by suppressed conductivity detection using a Reagent-Free™ IC (RFIC™) system. The RFIC system prepares the hydroxide eluent with high fidelity, which augments method reproducibility. The InGuard cartridge must be changed every 100 injections, which allows the column set to be used for approximately1000 sample injections while still accurately determining the nitrite and nitrate contents of the milk sample with only minimal off-line sample preparation.

EQUIPMENT

Dionex ICS-3000 system* including:

DP Dual Pump

DC Detector/Chromatography module with dual-temperature zone equipped with two 6-port valves and a conductivity detector

EG Eluent Generator

AS Autosampler

EWP Electrolytic Water Purifier (P/N 071553)

AXP Auxiliary Pump (P/N 063973)

Chromeleon® Chromatography Data System (CDS) software Version 6.80 SR9

*This application can also be executed on an ICS-5000 system.

REAGENTS AND STANDARDS

Deionized water (DI), Type I reagent grade, 18 MΩ-cm resistivity or better

Concentrated acetic acid (CH_3COOH, Labscan)

Sodium Nitrite ($NaNO_2$, Fluka)

Sodium Nitrate ($NaNO_3$, Fluka)

Samples

Instant Powder Milk Sample #1 (containing 6.203% fat)

Instant Powder Milk Sample #2 (containing 1.799% fat)

PREPARATION OF SOLUTIONS AND REAGENTS

Eluent Solution

The eluent generator produces the eluent using the EluGen EGC II KOH cartridge and DI water supplied by the pump, with the eluent concentration controlled by the Chromeleon software. Backpressure tubing must be added to achieve 2000–2500 psi backpressure that will allow the EG degasser to function properly. See the ICS-3000 Ion Chromatography System Operator's Manual (P/N 065031-03) for instructions on adding backpressure.

Acetic Acid (3%)

Add 3 mL of concentrated acetic acid to approximately 50 mL of DI water in a 100 mL volumetric flask. Dilute to volume with DI water and mix.

Standard Solutions

Nitrite Stock Standard Solution (1000 mg/L)

Dissolve 0.150 g of sodium nitrite in a 100 mL volumetric flask with DI water.

Nitrate Stock Standard Solution (1000 mg/L)

Dissolve 0.137 g of sodium nitrate in a 100 mL volumetric flask with DI water.

Nitrite Standard Solution (10 mg/L)

Dilute 1 mL of 1000 mg/L nitrite standard in a 100 mL volumetric flask with DI water.

Nitrate Standard Solution (20 mg/L)

Dilute 2 mL of 1000 mg/L nitrate standard in a 100 mL volumetric flask with DI water.

Calibration Standard and Sample Preparation

Prepare calibration standard solutions by adding a known amount of standard solution into the sample during sample preparation. Weigh 1 g of milk powder into a 100 mL bottle, then add the appropriate volumes of 10 mg/L nitrite and 20 mg/L nitrate solutions to produce each calibration standard. Table 1 lists the volumes to be added of each standard and the subsequent concentrations in the sample.

Table 1. Volumes of 10 mg/L Nitrite, 20 mg/L Nitrate, 3% Acetic Acid, and DI Water Used for Each Preparation

Sample	Volume of Added Standard and Resulting Concentration (mL, mg/L)		Volume of 3% Acetic Acid Added (mL)	Volume of DI Water Added (mL)
	10 mg/L Nitrite	20 mg/L Nitrate		
Blank (no milk)	—	—	1	49.0
Unspiked*	—	—	1	49.0
Spiked 1*	0.1, 0.02	0.1, 0.04	1	48.8
Spiked 2*	0.2, 0.04	0.2, 0.08	1	48.6
Spiked 3*	0.4, 0.08	0.4, 0.16	1	48.2
Spiked 4*	0.8, 0.16	0.8, 0.32	1	47.4

*The preparation is for 1 g of milk sample. The total volume of the final samples is 50 mL.

Add the appropriate amount of DI water to bring the volume of each sample to 49 mL (Table 1), shake, and put in an ultrasonic bath for 10 min. Add 1 mL of 3% acetic acid and shake to precipitate protein. Let the sample sit for 20 min. Use a 3 mL syringe to remove 3 mL of sample solution and filter with a 0.45 µm syringe filter washed with DI water before use. Discard the first 1.5 mL of sample and collect the remaining sample into a 1.5 mL glass vial. Rinse the vials with DI water prior to adding sample. The sample solutions to which known amounts of standard are added are referred to as Spiked 1, Spiked 2, Spiked 3, and Spiked 4. Sample solution without added standard solution is referred to as Unspiked.

Spiked Sample Preparation for Recovery and MDL Studies

Prepare spiked samples for recovery and MDL studies in the same manner as described above. For the recovery study, prepare the spiked sample to yield the same concentration as Spiked 1. Due to the nitrate present in the sample, spike only nitrite into the sample for the MDL study. Spike in an amount to yield 0.01 mg/L after preparation.

Table 2. Gradient Program and Valve Switching

Time (min)	Eluent Conc. (mM)	Inject-Valve_1	Inject-Valve_2	Remark
-20.0	50	Inject	Inject	Wash column and concentrator
-7.1	50	Inject	Inject	
-7.0	7	Inject	Load	
-5.0	7	Load, Inject*	Load	Load sample and then begin in-line sample preparation
0.0	7	Inject	Inject	Begin separation
25.0	7	Inject	Inject	
25.1	50	Inject	Inject	

*InjectValve_1 is controlled by the AS so that the program clock will be held during loading of the sample into the sample loop. After loading, InjectValve_1 is immediately switched to the inject position and the program resumes.

CHROMATOGRAPHIC CONDITIONS

Column:	IonPac AS20 Analytical, 4 × 250 mm (P/N 063148) IonPac AG20 Guard, 4 × 50 mm (P/N 063154)
InGuard:	InGuard* HRP, 9 × 24 mm (P/N 074034)
Concentrator:	IonPac UTAC-LP1, 4 × 35 mm (P/N 063079)
Eluent Source:	EGC II KOH (P/N 058900) with CR-ATC (P/N 060477)
Gradient:	See Table 2
Flow Rate:	1.0 mL/min
Sample Volume:	25 µL
Column Temp.:	30 °C (both zones of the DC are set to 30 °)
Detection:	Suppressed conductivity ASRS® 300, 4 mm (P/N 064554), External water mode (AXP flow rate 1 mL/min), 125 mA

*Prewash the InGuard cartridge in the IC system with water for a few minutes before use.

RESULTS AND DISCUSSION

Milk is a challenging sample because it has high concentrations of protein and fat. The protein can consume column capacity and interfere with the detection of nitrite and nitrate either by suppressed conductivity or UV absorbance detection. The fat can damage the column by a number of mechanisms including the generation of excessive backpressure. Therefore, removing the protein and fat from the sample is required for a successful application and for extending column lifetime. In traditional sample treatment, off-line sample treatment with an OnGuard® RP cartridge should be done before sample injection. There are some disadvantages to off-line sample treatment. It requires analyst time and the sample can be contaminated. OnGuard cartridges are designed for a single use and, therefore, each study requires multiple cartridges. InGuard cartridges are designed for on-line sample treatment during which multiple injections can be made on a single InGuard cartridge. With on-line sample preparation, analyst time is reduced, sample contamination is minimized, and the cost of sample analysis is reduced.

In this application, protein was first precipitated using acetic acid and then fat was removed on-line with an InGuard HRP cartridge. The goal was to have the InGuard cartridge last for at least 50 sample injections. In this study, the InGuard cartridge was changed after 100 milk sample injections (previously treated with acetic acid). The InGuard cartridge was changed before failure to ensure that the column was protected. Without protein precipitation prior to sample injection, high backpressure caused the InGuard cartridge to fail after less than 50 injections, and the chromatography was compromised by a noisy baseline. The goal of this challenging application was to have the column set withstand 500 sample injections before failure. With this sample treatment, approximately 1000 milk sample injections were made before nitrite had too great a loss of retention to be resolved from other peaks. Figure 1 shows the loss of retention time of nitrite and nitrate after approximately 800 sample injections, which still yielded acceptable resolution of nitrite from the unknown peak.

Column:	IonPac AS20 Analytical, 4 × 250 mm IonPac AG20 Guard, 4 × 50 mm
InGuard:	HRP, 9 × 24 mm
Concentrator:	IonPac UTAC-LP1, 4 × 35 mm
Eluent Source:	EGC II KOH with CR-ATC
Gradient:	*Time (min)* *Eluent Conc. (mM)* -20 50 -7.1 50 -7.0 7 -5.0 7 0.0 7 25.0 7 25.1 50
Flow Rate:	1.0 mL/min
Inj. Volume:	25 µL
Column Temp.:	30 °C
Detection:	Suppressed conductivity, ASRS 300, 4 mm with external water mode, current 125 mA
Sample:	A. Spiked Milk Sample at start B. Spiked Milk Sample after 800 sample injections

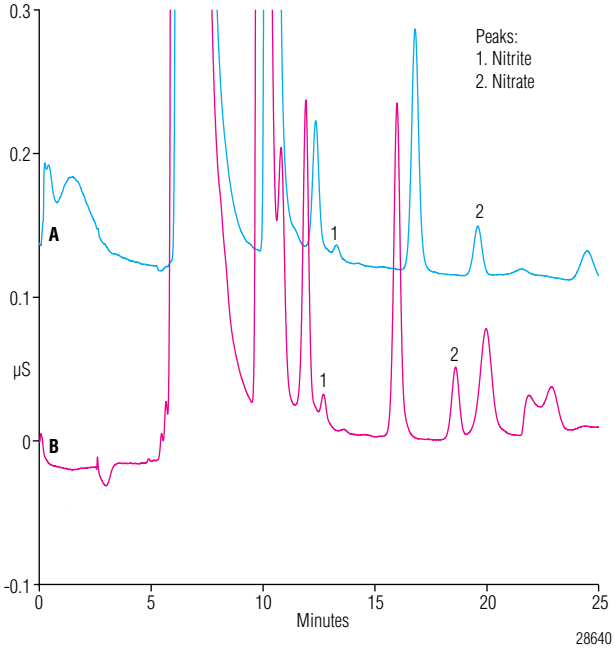

Figure 1. Chromatogram of Spiked Milk Sample #2 at the start and after 800 sample injections.

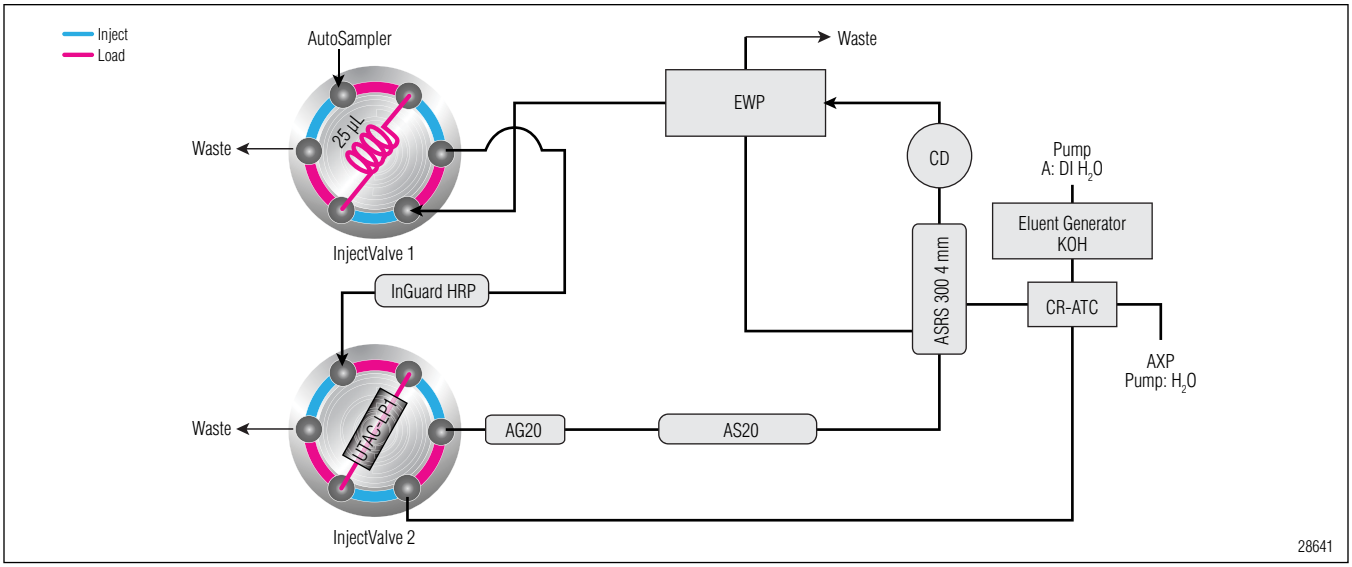

Figure 2. System configuration (both valves are in the load position).

Figure 2 shows the configuration of the system. After protein precipitation with 3% acetic acid, 25 µL of the sample were injected. Water from the outlet of the conductivity cell, purified by the EWP, was used to flush the sample from the sample loop to the InGuard cartridge. The sample compounds not bound by the InGuard cartridge, including nitrite and nitrate, were collected on the concentrator. The concentrator was then eluted onto the IonPac AS20 column set to separate nitrite and nitrate from the other bound sample components. Other hydroxide selective columns—including the IonPac AS11, AS11-HC, AS15, AS18, and AS19 columns—were tested, but the resolution on the AS20 column and its high capacity made it the most suitable column for this application.

To achieve the highest retention time reproducibility, this application was configured on an RFIC system. This system eliminates the labor and possible error of manual hydroxide eluent preparation. After a sample injection, the column and concentrator must be washed with 50 mM KOH for 13 min. This will remove the anionic compounds that were not eluted during the separation. The suppressed conductivity detection was configured with external water mode so that the effluent from the conductivity cell could be a source of water to move the sample from the sample loop to the InGuard cartridge.

When this configuration is used to execute the method in Table 2 on a blank sample (the acetic acid used for protein precipitation), the result is shown in Figure 3. The large peak between approximately 6 and 11 min is

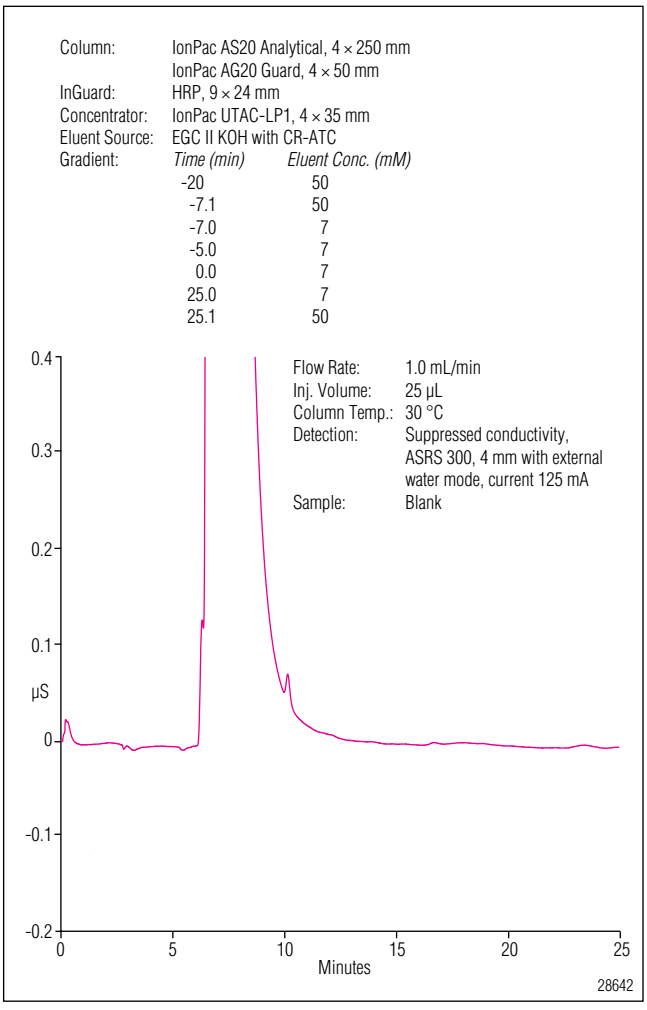

Figure 3. Chromatogram of an acetic acid blank.

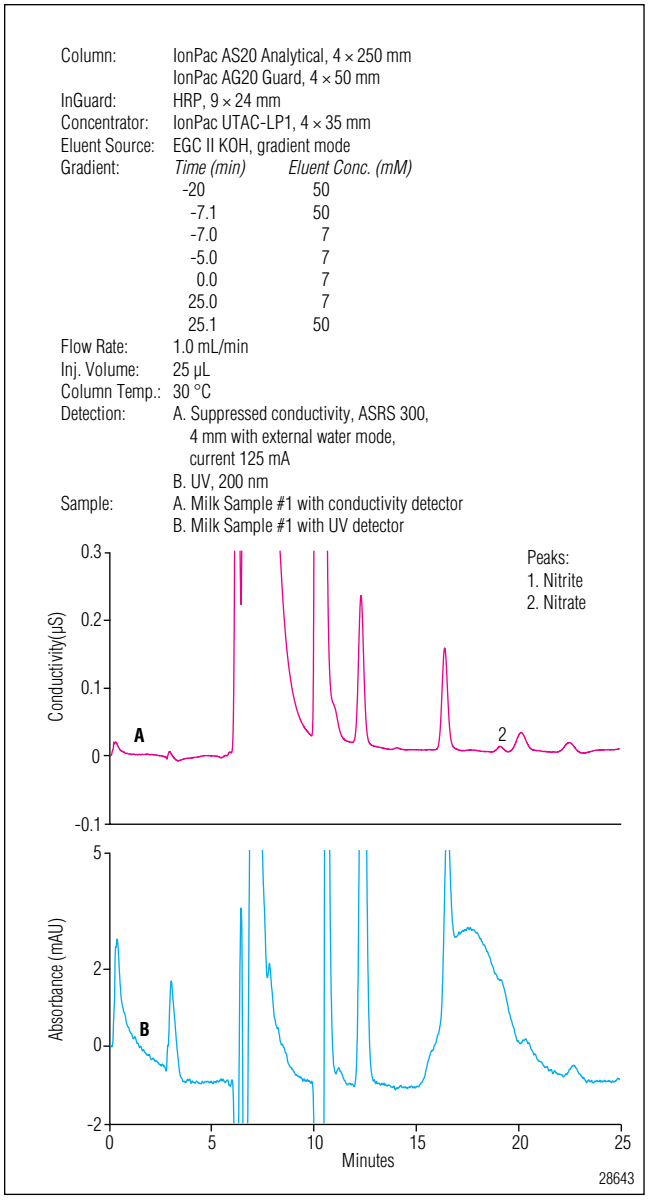

Figure 4. Overlay of chromatograms of Milk Sample #1 with (A) suppressed conductivity detection and (B) UV detection.

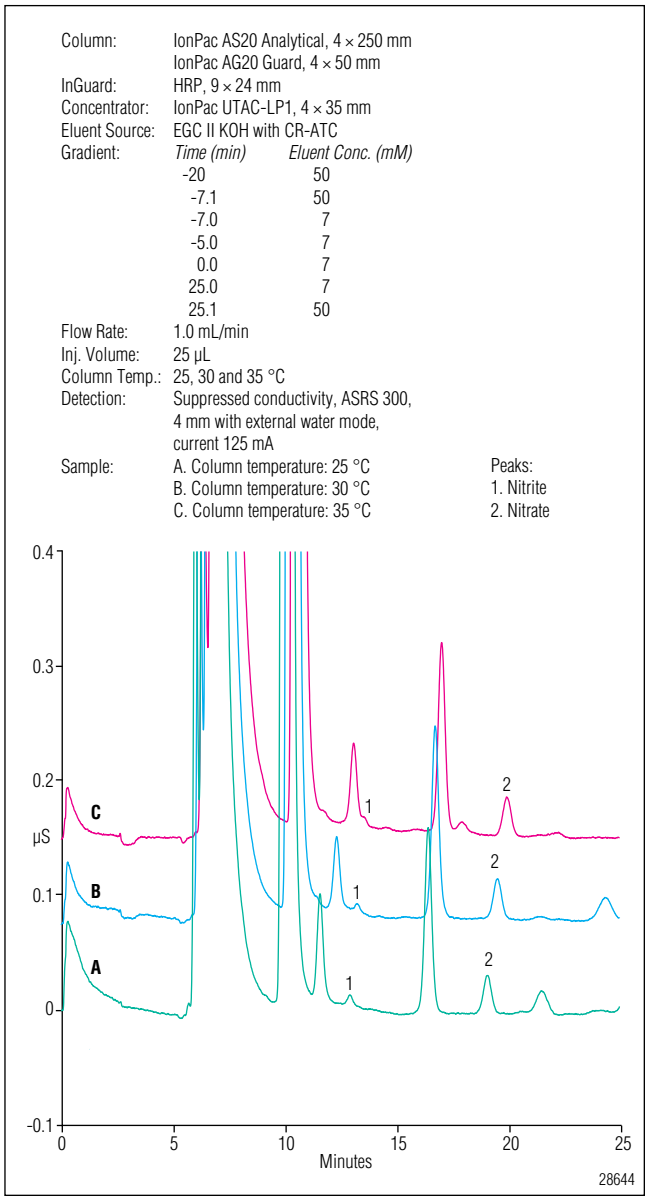

Figure 5. Overlay of chromatograms of prepared Milk Sample #2 analyzed at different column temperatures.

acetate, which does not interfere with nitrite or nitrate detection. A UV detector is placed after the conductivity detector to determine the best mode of detection for this analysis. Figure 4 shows chromatograms of Milk Sample #1 with conductivity and UV detections. The nitrate present in Sample #1 is difficult to determine with the UV detector, whereas it is readily determined by the conductivity detector.

This study showed that column temperature control is important to the success of this application. Specifically, if the column temperature was too high, the desired separation was not achieved. Figure 5 shows the effect of column temperature on the separation of nitrite and nitrate in milk. At 35 °C, nitrite co-elutes with an unknown peak in the milk sample. For the remainder of the study, the column temperature was set at 30 °C, but 25 °C also yielded a good separation. These separation conditions were successful for the two samples studied. Some milk samples may require an adjustment of eluent concentration and/or column temperature to resolve nitrite and/or nitrate from unknown sample components.

Table 3. Calibration Results								
Analyte	**Sample #1**				**Sample #2**			
	Points	**r^2**	**Offset**	**Slope**	**Points**	**r^2**	**Offset**	**Slope**
Nitrite	4	0.9990	-0.0002	0.1463	4	0.9991	-0.0008	0.1224
Nitrate	5	0.9995	0.0023	0.1268	5	0.9993	0.0101	0.1189

The method of standard additions was chosen for this application. Calibration standards were prepared in the sample and the added standard concentration was plotted versus the measured signal. Using this calibration curve, the amount of endogenous analyte in the sample can be determined. Two brands of milk purchased in a local supermarket are referred to as Sample #1 and Sample #2. Figure 6 shows the calibration chromatograms obtained for Sample #1 (chromatography of the calibration for Sample #2 is similar to Sample #1). Table 3 shows calibration results for both samples.

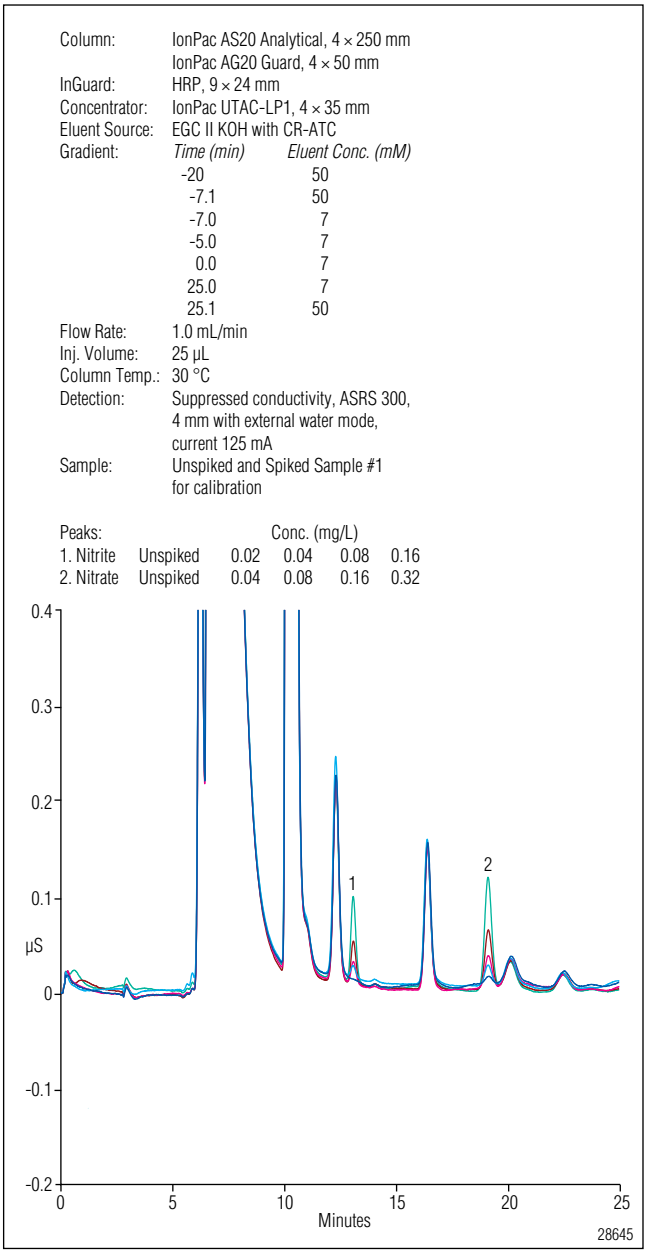

Figure 6. Overlay of five chromatograms of calibration standards of Sample #1 (chromatography of calibration standards for Sample #2 is similar).

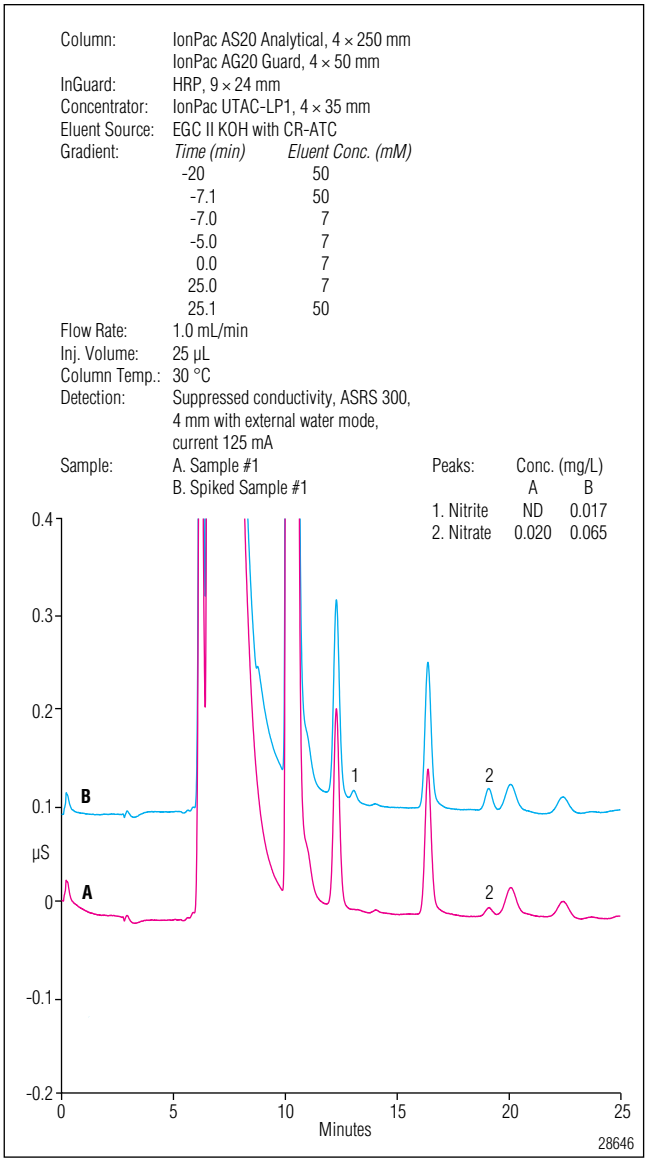

Figure 7. Overlay of chromatograms of Sample #1 and Spiked Sample #1.

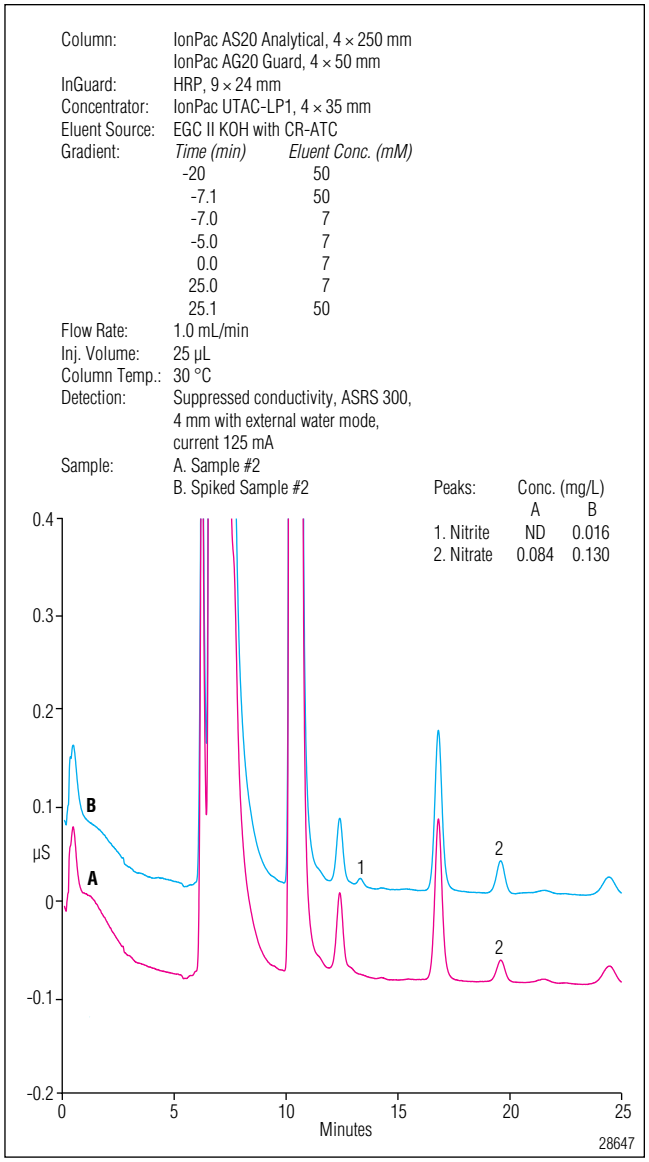

Figure 8. Overlay of chromatograms of Sample #2 and Spiked Sample #2.

To evaluate recovery, spiked samples were prepared to yield known concentrations of 0.02 mg/L nitrite and 0.04 mg/L nitrate; recoveries were calculated using the calibration curves prepared for each samples. Nitrate was found at 0.020 and 0.084 mg/L in Samples #1 and #2, respectively, and nitrite was absent in both samples.

Figures 7 and 8 show the chromatography from the spike recovery experiments. Note the difference in the number, size, and retention times of the unknown peaks in Samples #1 and #2. This again suggests that chromatography may need to be optimized for individual milk samples.

The sample analysis and recovery results are shown in Table 4. To assess method sensitivity, the method detection limit (MDL) was determined. Due to the nitrate present in the sample, only nitrite was spiked into the sample to yield a concentration of 0.01 mg/L. The endogenous concentration of nitrate was used to estimate the MDL. Seven injections were made and the single-sided Student's *t* test at a 99% confidence level used to estimate the MDLs. This resulted in MDLs for nitrite and nitrate of 0.002 mg/L and 0.005 mg/L, respectively. During this study, the conductivity background and baseline noise were approximately 0.37 µS and 0.25 nS, respectively. Chromatography of one of the seven injections from the MDL study is shown in Figure 9.

Table 4. Concentrations of Nitrite and Nitrate Determined in Sample #1, Spiked Sample #1, Sample #2, and Spiked Sample #2								
Injection No.	Concentration in Milk Sample #1 (mg/L)				Concentration in Milk Sample #2 (mg/L)			
	Sample		Spiked Sample (Spiked 0.02 mg/L Nitrite and 0.04 mg/L Nitrate)		Sample		Spiked Sample (Spiked 0.02 mg/L Nitrite and 0.04 mg/L Nitrate)	
	Nitrite	Nitrate	Nitrite	Nitrate	Nitrite	Nitrate	Nitrite	Nitrate
1	ND	0.021	0.016	0.065	ND	0.084	0.016	0.126
2	ND	0.022	0.017	0.065	ND	0.085	0.016	0.127
3	ND	0.019	0.017	0.065	ND	0.084	0.016	0.135
Average	ND	0.020	0.017	0.065	ND	0.084	0.016	0.130
RSD f	ND	6.29	2.87	0.18	ND	0.93	2.84	3.81
Recovery (%)	—	—	84.0	111	—	—	80.0	113

CONCLUSION

This application demonstrates the determination of nitrite and nitrate in milk by IC with suppressed conductivity detection using in-line sample preparation. This method uses a simple acid precipitation followed by additional in-line automated sample preparation to prepare the sample prior to chromatography. The prepared sample is separated on the high-capacity IonPac AS20 column to resolve nitrite and nitrate from the remaining sample components. The RFIC system automatically prepares the separation eluent to achieve high separation reproducibility. The automated sample and eluent preparation saves time and improves the reproducibility of the analysis.

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Dionex Corporation, *Determination of Nitrite and Nitrate in Drinking Water Using Ion Chromatography with Direct UV Detection.* Application Update 132, LPN 034527, 1991, Sunnyvale, CA.

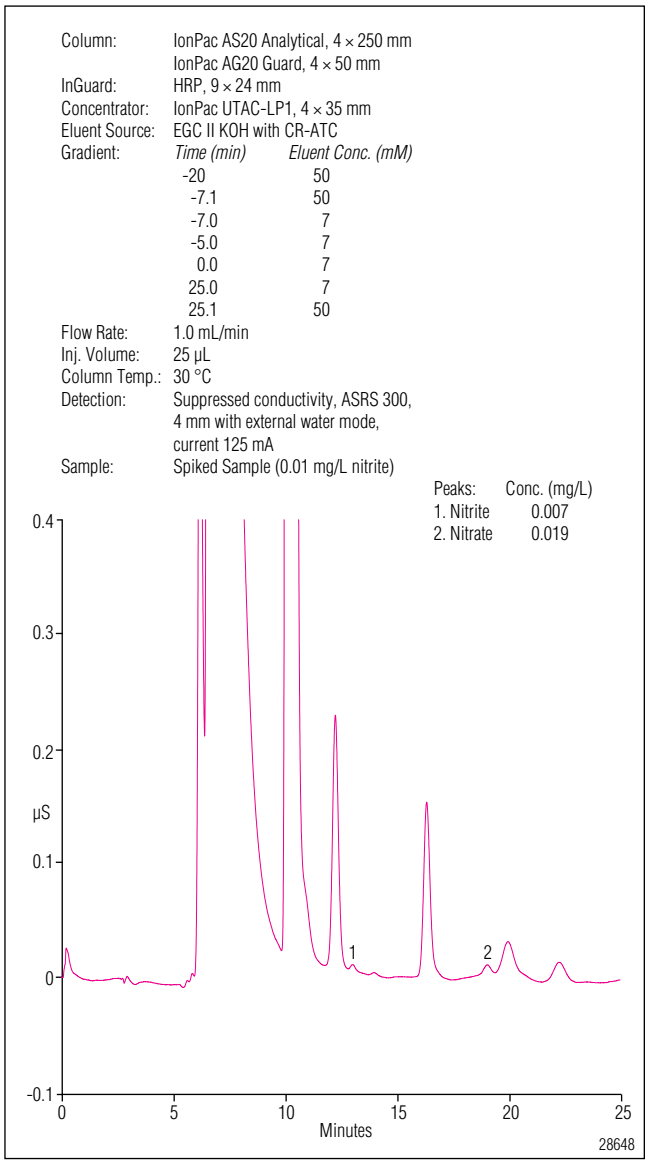

Figure 9. Chromatogram of a spiked sample for the MDL study.

Thermo
SCIENTIFIC

Column Selection Guide

Silica Columns

			Reversed-Phase (RP)						Mixed-Mode			HILIC		Application-Specific					Example Applications
			Acclaim 120 C18	Acclaim 120 C8	Acclaim 300 C18	Acclaim Polar Advantage (PA)	Acclaim Polar Advantage II (PA2)	Acclaim Phenyl-1	Acclaim Trinity P1	Acclaim Mixed-Mode WAX-1	Acclaim Mixed-Mode WCX-1	Acclaim Mixed-Mode HILIC-1	Acclaim HILIC-10	Acclaim Organic Acid	Acclaim Surfactant	Acclaim Explosives E1	Acclaim Explosives E2	Acclaim Carbamate	
General Applications	Neutral Molecules	High hydrophobicity	√	√	√	√	√	√	√	√	√	√	√						Fat-soluble vitamins, PAHs, glycerides
		Intermediate hydrophobicity	√	√	√	√	√	√	√	√	√	√							Steroids, phthalates, phenolics
		Low hydrophobicity	√			√	√					√	√						Acetaminophen, urea, polyethylene glycols
	Anionic Molecules	High hydrophobicity	√	√	√	√	√	√	√	√	√	√							NSAIDs, phospholipids
		Intermediate hydrophobicity	√	√	√	√	√	√	√	√		√							Asprin, alkyl acids, aromatic acids
		Low hydrophobicity				√			√	√		√	√						Small organic acids, e.g. acetic acids
	Cationic Molecules	High hydrophobicity	√	√	√	√	√	√		√	√	√							Antidepressants
		Intermediate hydrophobicity	√	√	√	√	√	√	√		√	√							Beta blockers, benzidines, alkaloids
		Low hydrophobicity	√			√			√		√	√	√						Antacids, pseudoephedrine, amino sugars
	Amphoteric/ Zwitterionic Molecules	High hydrophobicity	√	√	√	√	√	√	√	√	√	√							Phospholipids
		Intermediate hydrophobicity	√	√	√	√	√	√			√								Amphoteric surfactants, peptides
		Low hydrophobicity				√	√		√	√	√	√	√						Amino acids, aspartame, small peptides
	Mixtures of Neutral, Anionic, Cationic Molecules	Neutrals and acids	√			√	√		√	√									Artificial sweeteners
		Neutrals and bases	√			√	√		√		√								Cough syrup
		Acids and bases				√			√										Drug active ingredient with counterion
		Neutrals, acids, and bases				√			√										Combination pain relievers
Specific Applications	Surfactants	Anionic	√	√	√	√	√								√				SDS, LAS, laureth sulfates
		Cationic													√				Quats, benzylalkonium in medicines
		Nonionic	√	√	√	√	√					√			√				Triton X-100 in washing tank
		Amphoteric	√	√	√	√	√								√				Cocoamidopropyl betaine
		Hydrotropes													√				Xylenesulfonates in handsoap
		Surfactant blends													√				Noionic and anionic surfactants
	Organic Acids	Hydrophobic							√	√				√					Aromatic acids, fatty acids
		Hydrophilic							√	√				√					Organic acids in soft drinks, pharmaceuticals
	Environmental Contaminants	Explosives														√	√		U.S. EPA Method 8330, 8330B
		Carbonyl compounds															√		U.S. EPA 1667, 555, OT-11; CA CARB 1004
		Phenols	√			√													Compounds regulated by U.S. EPA 604
		Chlorinated/Phenoxy acids				√													U.S. EPA Method 555
		Triazines	√			√													Compounds regulated by U.S. EPA 619
		Nitrosamines				√													Compounds regulated by U.S. EPA 8270
		Benzidines	√			√													U.S. EPA Method 605
		Perfluorinated acids				√													Dionex TN73
		Microcystins	√																ISO 20179
		Isocyanates					√					√							U.S. OSHA Methods 42, 47
		Carbamate insecticides																√	U.S. EPA Method 531.2
	Vitamins	Water-soluble vitamins				√	√		√										Vitamins in dietary supplements
		Fat-soluble vitamins	√	√	√	√	√	√		√									Vitamin pills
	Pharmaceutical Counterions	Anions							√	√									Inorgaic anions and organic acids in drugs
		Cations							√		√								Inorgaic cations and organic bases in drugs
		Mixture of Anions and Cations							√										Screening of pharmaceutical counterions
		API and counterions							√										Naproxen Na^+ salt, metformin Cl^- salt, etc.

Polymer Columns

		IonPac AS23	IonPac AS22	IonPac AS22-Fast	IonPac AS14/A	IonPac AS12A	IonPac AS9/HC/SC	IonPac AS4A/SC	IonSwift MAX-100	IonPac AS24	IonPac AS21	IonPac AS20	IonPac AS19	IonPac AS18	IonPac AS18-Fast	IonPac AS17-C	IonPac AS16	IonPac AS15	IonPac AS11(-HC)	IonPac AS10	IonPac AS7	IonPac AS5	IonPac Fast Anion IIIA	OmniPac PAX-100	OmniPac PAX-500
ANIONS	Inorganic Anions	√	√	√	√	√	√	√	√	√		√	√	√	√	√		√	√	√					
	Oxyhalides	√				√	√			√			√												
	Bromate	√					√			√			√												
	Perchlorate										√	√					√								
	Organic Acids								√							√		√	√	√					
	Phosphoric/Citric Acids																						√		
	Poly/High-Valence Anions								√			√					√		√		√	√			
	Hydrophobic Anions								√			√					√		√						
	Hydrophobic/Halogenated Anions								√			√							√					√	
	Anionic Neutral Molecules									√	√	√	√												√
CATIONS	Inorganic Cations																								
	Sodium/Ammonium																								
	Amines/Polyvalent Amines																								
	Aliphatic/Aromatic Amines																								
	Alkanol/Ethhanolamines																								
	Biogenic Amines																								
	Transition/Lanthanide Metals																								
	Hydrophobic Cations																								
	Cationic Neutral Molecules																								
BIO-MOLECULES	Amino Acids																								
	Phosphorylated Amino Acids																								
	Amino Sugars																								
	Oligoscharides																								
	Mono-/Di-Saccharides																								
	Glycoproteins																								
	Alditols/Aldoses mono/di Saccharides																								
	ds Nucleic Acids																								
	Single-Stranded Oligonucleotides																								
	Peptides																								
	Proteins																								
	Metal-binding Proteins																								
	Monoclonal antibodies																								
ORGANIC MOLECULES	Aliphatic Organic Acids																								
	Alcohols																								
	Borate																								
	Large Molecules, Anions																								
	Small Molecules																								
	Small Molecules/LC-MS																								
	Polar/Non-Polar Small Molecules																								
	Hydrophobic/Aliphatic Organic Acids																								
	Surfactant Formulations																								
	Explosives/EPA 8330																								
MODE	Anion Exchange / Carbonate	√	√	√	√	√	√	√																	
	Anion Exchange / Hydroxide								√	√	√	√	√	√	√	√	√	√	√	√	√	√			
	Cation Exchange																								
	Multi-Mode																							√	√
	Affinity																								
	Ion Exclusion																								
	Reversed Phase																								
	Anion Exchange/Other																								

		IonPac CS18	IonPac CS17	IonPac CS16	IonPac CS15	IonPac CS14	IonPac CS12A	IonPac CS11	IonPac CS10	IonPac CS5A	OmniPac PCX-100	OmniPac PCX-500	AminoPac PA10	AminoPac PA1	CarboPac PA200	CarboPac PA100	CarboPac PA20	CarboPac PA10	CarboPac PA1	CarboPac MA1	DNAPac PA200	DNAPac PA100	ProPac WAX/SAX	ProPac WCX/SCX	ProPac IMAC	ProPac HIC	ProPac PA1	ProSwift	IonPac ICE-AS6	IonPac ICE-AS1	IonPac ICE-Borate	IonPac NS1
ANIONS	Inorganic Anions																														
	Oxyhalides																														
	Bromate																														
	Perchlorate																														
	Organic Acids																														
	Phosphoric/Citric Acids																														
	Poly/High-Valence Anions																														
	Hydrophobic Anions																														
	Hydrophobic/Halogenated Anions																														
	Anionic Neutral Molecules																														
CATIONS	Inorganic Cations	√	√	√	√	√	√	√	√																						
	Sodium/Ammonium			√	√		√																								
	Amines/Polyvalent Amines	√	√																												
	Aliphatic/Aromatic Amines		√			√	√																								
	Alkanol/Ethhanolamines	√	√		√																										
	Biogenic Amines	√	√																												
	Transition/Lanthanide Metals									√																					
	Hydrophobic Cations	√	√			√					√																				
	Cationic Neutral Molecules											√																			
BIO-MOLECULES	Amino Acids												√	√																	
	Phosphorylated Amino Acids												√																		
	Amino Sugars												√		√	√	√	√													
	Oligoscharides														√	√			√	√											
	Mono-/Di-Saccharides															√	√	√	√												
	Glycoproteins																					√	√	√			√				
	Alditols/Aldoses mono/di Saccharides															√	√	√	√												
	ds Nucleic Acids																			√	√										
	Single-Stranded Oligonucleotides																			√	√							√			
	Peptides																								√	√	√	√			
	Proteins																							√	√	√	√	√	√		
	Metal-binding Proteins																									√					
	Monoclonal antibodies														√	√			√			√	√	√	√	√	√	√			
ORGANIC MOLECULES	Aliphatic Organic Acids																												√	√	√
	Alcohols																												√	√	
	Borate																														√
	Large Molecules, Anions																														√
	Small Molecules																														
	Small Molecules/LC-MS																														
	Polar/Non-Polar Small Molecules																														
	Hydrophobic/Aliphatic Organic Acids																														
	Surfactant Formulations																														√
	Explosives/EPA 8330																														
MODE	Anion Exchange / Carbonate																														
	Anion Exchange / Hydroxide														√	√	√	√	√	√											
	Cation Exchange	√	√	√	√	√	√	√	√	√														√			√	√			
	Multi-Mode										√	√																			
	Affinity																								√						
	Ion Exclusion																												√	√	√
	Reversed Phase																										√	√			√
	Anion Exchange/Other												√	√						√	√	√						√			

Column Specifications

IC Anion Columns

Column	Format	Primary Eluent	Application	Particle Diameter	Substrate Crosslinking	Latex Diameter	Latex Crosslinking	Capacity (per column)	Functional Group	Hydrophobicity
IonPac AS24	2 × 250 mm	Hydroxide	Recommended column for haloacetic acids prior to MS or MS/MS detection	7 µm	55%	-	-	140 µeq	Alkanol quaternary ammonium	Ultralow
IonPac AS23	2 × 250 mm 4 × 250 mm	Carbonate	Recommended column for inorganic anions and oxyhalides. Trace bromate in drinking water.	6 µm	55%	-	-	80 µeq 320 µeq	Alkyl quaternary ammonium	Ultralow
IonPac AS22	2 × 250 mm 4 × 250 mm	Carbonate	Recommended column for fast analysis of common inorganic anions.	6.5 µm	55%	-	-	52.5 µeq 210 µeq	Alkyl quaternary ammonium	Ultralow
IonPac AS21	2 × 250 mm	Hydroxide	Recommended column for trace perchlorate prior to MS or MS/MS detection	7.0 µm	55%	-	-	45 µeq	Alkanol quaternary ammonium	Ultralow
IonPac AS20	2 × 250 mm 4 × 250 mm	Hydroxide	Recommended column for trace perchlorate prior to suppressed conductivity detection.	7.5 µm	55%	-	-	77.5 µeq 310 µeq	Alkanol quaternary ammonium	Ultralow
IonPac AS19	2 × 250 mm 4 × 250 mm	Hydroxide	Recommended column for inorganic anions and oxyhalides. Trace bromate in drinking water.	7.5 µm	55%	-	-	60 µeq 350 µeq	Alkanol quaternary ammonium	Low
IonPac AS18	2 × 250 mm 4 × 250 mm	Hydroxide	Recommended column for the analysis of common inorganic anions.	7.5 µm	55%	65 nm	8%	75 µeq 285 µeq	Alkanol quaternary ammonium	Low
IonPac AS17-C	2 × 250 mm 4 × 250 mm	Hydroxide	Trace anions in HPW matrices. Carboxylated resin, no sulfate blank. Low capacity for fast analysis of common inorganic anions using gradient elution with the Eluent Generator.	10.5 µm	55%	75 nm	6%	7.5 µeq 30 µeq	Alkanol quaternary ammonium	Low
IonPac AS16	2 × 250 mm 4 × 250 mm	Hydroxide	High capacity for hydrophobic anions including iodide, thiocyanate, thiosulfate, and perchlorate. Polyvalent anions including: polyphosphates and polycarboxylates	9 µm	55%	80 nm	1%	42.5 µeq 170 µeq	Alkanol quaternary ammonium	Ultralow
IonPac AS15	2 × 250 mm 4 × 250 mm	Hydroxide	High capacity for trace analysis of inorganic anions and low molecular weight organic acids in high purity water matrices.	9 µm	55%	-	-	56.25 µeq 225 µeq	Alkanol quaternary ammonium	Medium-High
IonPac AS15-5mm	3 × 150 mm	Hydroxide	Fast run, high capacity for trace analysis of inorganic anions and low molecular weight organic acids in high purity water matrices.	5 µm	55%	-	-	70 µeq	Alkanol quaternary ammonium	Medium-High
IonPac AS14A- 5 µm	3 × 150 mm	Carbonate	Recommended column for fast analysis of common inorganic anions.	5 µm	55%	-	-	40 ueq	Alkyl quaternary ammonium	Medium
IonPac AS14A	4 × 250 mm	Carbonate	For analysis of common inorganic anions.	7 µm	55%	-	-	120 µeq	Alkyl quaternary ammonium	Medium
IonPac AS14	2 × 250 mm 4 × 250 mm	Carbonate	Moderate capacity for fast analysis of common inorganic anions.	9 µm	55%	-	-	16 µeq 65 µeq	Alkyl quaternary ammonium	Medium-High

Column	Format	Primary Eluent	Application	Particle Diameter	Substrate Crosslinking	Latex Diameter	Latex Crosslinking	Capacity (per column)	Functional Group	Hydrophobicity
IonPac AS12A	2 × 200 mm 4 × 200 mm	Carbonate	Moderate capacity for analysis of inorganic anions and oxyhalides. Trace chloride and sulfate in high carbonate matrices.	9 µm	55%	140 nm	0.20%	13 µeq 52 µeq	Alkyl quaternary ammonium	Medium
IonPac AS11-HC	2 × 250 mm 4 × 250 mm	Hydroxide	High capacity for the determination of organic acids and inorganic anions in uncharacterized samples.	9 µm	55%	70 nm	6%	72.5 µeq 290 µeq	Alkanol quaternary ammonium	Medium-Low
IonPac AS11	2 × 250 mm 4 × 250 mm	Hydroxide	Low capacity for fast profiling of organic acids and inorganic anions in well-characterized samples.	13 µm	55%	85 nm	6%	11 µeq 45 µeq	Alkanol quaternary ammonium	Very Low
IonPac AS10	2 × 250 mm 4 × 250 mm	Hydroxide	High capacity for the analysis of inorganic anions and organic acids in high nitrate samples.	8.5 µm	55%	65 nm	5%	42.5 µeq 170 µeq	Alkyl quaternary ammonium	Low
IonPac AS9-HC	2 × 250 mm 4 × 250 mm	Carbonate	High-capacity column for inorganic anions and oxyhalides. Trace bromate in drinking water.	9 µm	55%	90 nm	18%	48 µeq 190 µeq	Alkyl quaternary ammonium	Medium-Low
IonPac AS9-SC	4 × 250 mm	Carbonate	Low capacity for fast analysis of inorganic anions and oxyhalides. Specified column in US EPA Method 300.0 (B).	13 µm	55%	110 nm	20%	30-35 µeq	Alkyl quaternary ammonium	Medium-Low
IonPac AS4A-SC	2 × 250 mm 4 × 250 mm	Carbonate	Low capacity for fast analysis of common inorganic anions. Specified column in U.S. EPA Method 300.0 (A).	13 µm	55%	160 nm	0.50%	5 µeq 20 µeq	Alkanol quaternary ammonium	Medium-Low
IonPac Fast Anion IIIA	3 × 250 mm	Hydroxide	Recommended column for phosphoric and citric acids in cola soft drinks.	7.5 µm	55%	-	-	55 µeq	Alkanol quaternary ammonium	Ultralow
IonPac AS7	4 × 250 mm	Specialty Eluents	Polyvalent anions including chelating agents, polyphosphates and polyphosphonates. Cyanide, sulfide, hexavalent chromium, and arsenic speciation.	10 µm	2%	530 nm	5%	100 µeq	Alkyl quaternary ammonium	Medium-High
IonPac AS5A	4 × 150 mm	Hydroxide	Low capacity for fast profiling of organic acids and inorganic anions in well-characterized samples.	5 µm	2%	60 nm	4%	35 µeq	Alkanol quaternary ammonium	Low
IonPac AS5	4 × 250 mm	Hydroxide	Metal-EDTA complexes, metal-cyanide complexes, and oxyanions.	15 µm	2%	120 nm	1%	20 µeq	Alkanol quaternary ammonium	Low

IC Cation Columns

Column	Format	Primary Eluent	Application	Particle Diameter	Substrate Crosslinking	Latex Diameter	Latex Crosslinking	Capacity (per column)	Functional Group	Hydrophobicity
IonPac CS18	2 × 250 mm	MSA	Recommended column for polar amines (alkanolamines and methylamines) and moderately hydrophobic and polyvalent amines (biogenic and diamines). Nonsuppressed mode when extended calibration linearity for ammonium and weak bases is required	6 µm	55%	-	-	0.29 µeq	Carboxylic acid	Medium
IonPac CS17	2 × 250 mm 4 × 250 mm	MSA	Recommended column for hydrophobic and polyvalent amines (biogenic amines and diamines)	7 µm	55%	-	-	0.363 µeq 1.45 µeq	Carboxylic acid	Very Low
IonPac CS16	3 × 250 mm 5 × 250 mm	MSA	Recommended column for disparate concentration ratios of adjacent-eluting cations such as sodium and ammonium. Can be used for alkylamines and alkanolamines.	5 µm	55%	-	-	3.0 µeq 8.4 µeq	Carboxylic acid	Medium
IonPac CS15	2 × 250 mm 4 × 250 mm	MSA	Disparate concentration ratios of ammonium and sodium. Trace ethanolamine in high-ammonium or high- potassium concentrations. Alkanolamines.	8.5 µm	55%	-	-	0.7 µeq 2.8 µeq	Carboxylic acid/ phosphonic acid/ crown ether	Medium
IonPac CS14	2 × 250 mm 4 × 250 mm	MSA	Aliphatic amines, aromatic amines, and polyamines plus mono- and divalent cations.	8.5 µm	55%	-	-	0.325 µeq 1.3 µeq	Carboxylic acid	Low
IonPac CS12A-MS	2 × 100 mm	MSA	IC-MS screening column for fast elution and low flow rates required for interfacing with IC-MS	8.5 µm	55%	-	-	0.28 µeq	Carboxylic acid/ phosphonic acid	Medium
IonPac CS12A-5 µm	3 × 150 mm	MSA	Recommended column for high efficiency and fast analysis (3 min) of mono- and divalent cations.	5 µm	55%	-	-	0.94 µeq	Carboxylic acid/ phosphonic acid	Medium
IonPac CS12A	2 × 250 mm 4 × 250 mm	MSA	Recommended column for the separation of mono- and divalent cations. Manganese morpholine, alkylamines, and aromatic amines.	8.5 µm	55%	-	-	0.7 µeq 2.8 µeq	Carboxylic acid/ phosphonic acid	Medium
IonPac CS11	2 × 250 mm	HCl + DAP	Separation of mono- and divalent cations. Ethanolamines if divalent cations are not present.	8 µm	55%	200 nm	5%	0.035 µeq	Sulfonic acid	Medium
IonPac CS10	4 × 250 mm	HCl + DAP	Separation of mono- and divalent cations.	8.5 µm	55%	200 nm	5%	0.08 µeq	Sulfonic acid	Medium
IonPac CS5A	2 × 250 mm 4 × 250 mm	Pyridine dicarboxylic acid	Recommended column for transition and lanthanide metals analysis. Aluminum analysis.	9 µm	55%	140 nm 75 nm	10% 20%	0.02 µeq/ 0.005 µeq 0.04 µeq/ 0.01 µeq	Sulfonic acid/ alkanol quaternary ammonium	-

Ion-Exclusion Columns

Column	Format	Primary Eluent	Application	Particle Diameter	Substrate Crosslinking	Latex Diameter	Latex Crosslinking	Capacity (per column)	Functional Group	Hydrophobicity
IonPac ICE-AS1	4 × 250 mm 9 × 250 mm	Heptafluorobutyric acid	Organic acids in high ionic strength matrices. Fast separation of organic acids.	7.5 µm	8%	-	-	5.3 µeq 27 µeq	Sulfonic acid	Ultra Low
IonPac ICE-AS6	9 × 250 mm	Heptafluorobutyric acid	Organic acids in complex or high ionic strength matrices.	8 µm	8%	-	-	27 µeq	Sulfonic and carboxylic acid	Moderate
IonPac ICE-Borate	9 × 250 mm	MSA/ Mannitol	Trace concentrations of borate	7.5 µm	8%	-	-	27 µeq	Sulfonic acid	Ultra Low

Acclaim General and Specialty Columns

Column	Bonded Phase	USP Type	Endcapped	Substrate	Particle Shape	Particle Size	Metal Impurity (ppm) Na, Fe, AL	Average Pore Diameter	Surface Area (m^2/g)	Total Carbon Content
Mixed-Mode WAX	Proprietary alkyl amine	na	Proprietary	Ultrapure silica	Spherical	5 µm	<10 ppm	120 Å	300	na
Mixed-Mode HILIC	Proprietary alkyl diol	na	Proprietary			5 µm		120 Å	300	na
Mixed-Mode WCX	Proprietary alkyl carboxyl	na	Proprietary			5 µm		120 Å	300	na
Organic Acid (OA)	Proprietary	na	Yes			5 µm		120 Å	300	17%
Surfactant and Explosives E1/2	Proprietary	na	Yes			5 µm		120 Å	300	na
120 C18	C18	L1	Yes			2, 3 and 5 µm		120 Å	300	18%
120 C8	C8	L7	Yes			3 and 5 µm		120 Å	300	11%
300 C18	C18	L1	Yes			3 µm		300 Å	100	7%
Polar Advantage	Sulfamido C16	na	Yes			3 and 5 µm		120 Å	300	17%
Polar Advantage II	Amide C18	na	Yes			2, 3 and 5 µm		120 Å	300	17%
HILIC	Proprietary hydrophilic		Yes			3 µm		120 Å	300	
Phenyl-1	Proprietary alkyl phenyl		Yes			3 µm		120 Å	300	
Carbamate	Proprietary alkyl group		Yes			3 and 5 µm		120 Å	300	
Trinity			Yes					120 Å	300	

Bio Columns

Protein

Column	Phase	Target Applications	Base Matrix Material	Substrate Crosslinking	Capacity	Recommended Flow Rate	Solvent Compatibility	Maximum Backpressure	pH Range
MAbPac SEC-1									
MAbPac SCX-10									
ProPac WCX-10	Weak Cation Exchange	High resolution and high efficiency separations of proteins and glycoproteins, pI =3-10, MW>10,000 units	10-µm diameter nonporous substrate to which is grafted a polymer chain bearing carboxylate groups.	55%	6 mg/ mL lysozyme	0.2–2 mL/min	80% ACN, acetone. Incompatable with alcohols and MeOH	3000 psi (21 MPa)	2–12.0
ProPac SCX-10	Strong Cation Exchange	High resolution and high efficiency separations of proteins and glycoproteins, pI =3-10, MW>10,000 units	10 µm diameter nonporous substrate to which is grafted a polymer chain bearing sulfonate groups.	55%	3 mg/ mL lysozyme	0.2–2.0 mL/min	80% ACN, acetone, MeOH	3000 psi (21 MPa)	2–12.0
ProPac SCX-20									
ProPac WAX-10	Weak Anion Exchange	High resolution and high efficiency separations of proteins and glycoproteins, pI =3-10, MW>10,000 units	10 µm diameter non-porous substrate to which is grafted a polymer chain bearing tertiary amine groups.	55%	5 mg/ mL BSA/ mL	0.2–2.0 mL/min	80% ACN, acetone, MeOH,	3000 psi (21 MPa)	2–12.0
ProPac SAX-10	Strong Anion Exchange	High resolution and high efficiency separations of proteins and glycoproteins, pI =3-10, MW>10,000 units	10 µm diameter non-porous substrate with grafted polymer chain bearing quaternary ammonium groups.	55%	15 mg/ mL BSA	0.2–2.0 mL/min	80% ACN, acetone, MeOH	3000 psi (21 MPa)	2–12.0
ProSwift RP-1S	Reversed-Phase	Fast protein separation with high capacity using Reversed Phase	Monolith; polystyrene-divinylbenzene with phenyl functional group	Monolith Standard permeability	5.5 mg/mL Insulin	2–4 mL/min	Most common organic solvents	2800 psi (19.2 Mpa)	1–14
ProSwift RP-2H	Reversed-Phase	Fast protein separation with high capacity using Reversed Phase	Monolith; polystyrene-divinylbenzene with phenyl functional group	Monolith High permeability	1.0 mg/mL Lysozyme	1–10 mL/min	Most common organic solvents	2800 psi (19.3 Mpa)	1–14
ProSwift RP-4H									
ProSwift RP-3U	Reversed-Phase	Fast protein separation with high capacity using Reversed Phase	Monolith; polystyrene-divinylbenzene with phenyl functional group	Monolith Ultrahigh permeability	0.5 mg/mL Lysozyme	1– 16 mL/min	Most common organic solvents	2800 psi (19.3 Mpa)	1–14
ProSwift SAX-1S	Strong Anion Exchange	Fast protein separation with good resolution using Anion Exchange	Monolith; polymethac-rylate with quaternary amine functional group	Monolith Standard permeability	18 mg/mL BSA	0.5–1.5 (4.6 mm), 0.05–.25 (1.0 mm)	Most common organic solvents	1000 psi (4.6 mm) 2000 psi (1.0 mm)	2–12.0
ProSwift SCX-1S	Strong Cation Exchange	Fast protein separation with good resolution using Cation Exchange	Monolith; polymethac-rylate with sulfonic acid fuctional group	Monolith Standard permeability	30 mg/mL Lysozyme	0.5–1.5 mL/min (4.6 mm)	Most common organic solvents	1000 psi (4.6 mm)	2–12.0
ProSwift WAX-1S	Weak Anion Exchange	Fast protein separation with good resolution using Anion Exchange	Monolith; polymethacrylate with tertiary amine (DEAE) functional group	Monolith Standard permeability	18 mg/mL BSA	0.5–1.5 mL/min (4.6 mm), 0.05–.25 (1.0 mm)	Most common organic solvents	1000 psi (4.6 mm) 2000 psi (1.0 mm)	2–12.0
ProSwift WCX-1S	Weak Cation Exchange	Fast protein separation with good resolution using Cation Exchange	Monolith; polymethacrylate with carboxylic acid (CM) functional group	Monolith Standard permeability	23 mg/mL Lysozyme	0.5–1.5 mL/min (4.6 mm), 0.05–.20 (1.0 mm)	Most common organic solvents	1000 psi (4.6 mm) 2000 psi (1.0 mm)	2–12.0
ProPac IMAC-10	Immobilized Metal Affinity	High resolution separation of certain metal-binding proteins and peptides	10 µm diameter non-porous polystyrene divinylbenzene substrate with poly (IDA) grafts.	55%	>60 mg lysozyme/ mL gel (4 x 250 mm)	1.0 mL/min	EtOH, urea, NaCl, non- ionic detergents, glycerol, acetic acid, guanidine HCl	3000 psi (21MPa)	2–12
ProSwift ConA-1S									
ProPac HIC-10	Reversed-Phase	Protein separation using hydrophobic interaction with salt gradient elution	Spherical 5 µm, ultrapure silica, 300 A, surface area 100 m^2/ g,	n/a	340 mg lysozyme per 7.8 x 75 mm column	1.0 mL/ min	2M Ammonium sulfate/ phosphate salts, organic solvent for cleanup	4,000 psi	2.5–7.5

Carbohydrate

Column	Target Applications	Base Matrix Material	Substrate Crosslinking	Latex Crosslinking	Capacity	Recommended Eluents	Recommended Flow Rate	Solvent Compatibility	Maximum Backpressure	pH Range
CarboPac MA1	Reduced mono- and disaccharide analysis.	7.5 µm diameter macroporous substrate fully functionalized with an alkyl quaternary ammonium group	15%	No latex	1450 µeq (4 × 250 mm)	Hydroxide	0.4 mL/min	0%	2000 psi (14 MPa)	0–14
CarboPac PA1	General purpose mono-, di-, and oligosaccharide analysis	10 µm diameter nonporous substrate agglomerted with a 500 nm MicroBead quaternary ammonium functionalized latex	2%	5%	100 µeq (4 × 250 mm)	Hydroxide, acetate/ hydroxide	1.0 mL/min	0–5%	4000 psi (28 MPa)	0–14
CarboPac PA10	Monosaccharide compositonal anaylysis	10 µm diameter nonporous substrate agglomerated with a 460 nm MicroBead di-functionalized latex	55%	5%	100 µeq (4 × 250 mm)	Hydroxide, acetate/ hydroxide	1.0 mL/min	0–90%	3500 psi (24.5 MPa)	0–14
CarboPac PA20	Fast mono-, and disaccharide analysis	6.5 µm diameter nonporous substrate agglomerated with a 130 nm MicroBead quaternary ammonium functionalized latex	55%	5%	65 µeq (3 × 150 mm)	Hydroxide, acetate/ hydroxide	0.5 mL/min	0–100%	3000 psi (21 MPa)	0–14
CarboPac PA100	Oligosaccharide mapping and analysis	8.5 µm diameter nonporous substrate agglomerated with a 275 nm MicroBead di-functionalized latex	55%	6%	90 µeq (4 × 250 mm)	Hydroxide, acetate/ hydroxide	1.0 mL/min	0–90%	4000 psi (28 MPa)	0–14
CarboPac PA200	High resolution oligosaccharide mapping and analysis	5.5 µm diameter nonporous substrate agglomerated with a 43 nm MicroBead quaternary ammonium functionalized latex	55%	6%	35 µeq (3 × 250 mm)	Hydroxide, acetate/ hydroxide	0.5 mL/min	0–100%	4000 psi (28 MPa)	0–14

DNA

Column	Target Applications	Base Matrix Material	Substrate Crosslinking	Latex Crosslinking	Capacity	Recommended Eluents	Recommended Flow Rate	Solvent Compatibility	Max. Backpressure	pH Range
DNAPac PA100	Single stranded DNA or RNA oligonucleotides, restriction fragments, glycoprotein isoforms.	13-µm diameter nonporous substrate agglomerated with a 100-nm MicroBead alkyl quaternary ammonium functionalized latex.	55%	5%	40 µeq	Chloride, acetate, bromide, perchlorate: in lithium sodium or ammonium forms	1.5 mL/min	0–100%	4000psi (28MPa)	2–12.5
DNAPac PA200	High resolution single stranded DNA or RNA oligonucleotides, restriction fragments, glycoprotein isoforms.	8-µm diameter nonporous substrate agglomerated with a 130-nm MicroBead alkyl quaternary ammonium functionalized latex.	55%	5%	40 µeq	Chloride, acetate, bromide, perchlorate: in lithium sodium or ammonium forms	1.2 mL/min	0–100%	4000psi (28MPa)	2–12.5
DNASwift										

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