Food Safety Applications Notebook

Food Additive Contaminants

Thermo SCIENTIFIC

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ANALYTES

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Introduction to Food Safety

Food contamination stories in the news media have raised awareness of the fact that we live with a global food supply chain, and food safety is increasingly becoming an important concern. All types of fruits, vegetables, seafood, and meat can be purchased year round independent of the local growing season. For example, in many countries, well-stocked grocery stores carry cantaloupes from Guatemala, cucumbers from Mexico, shrimp from Vietnam, and fish from China. With fruit, vegetables, seafood, and meat traveling thousands of miles to reach far-flung destinations, and with poor or no knowledge of the agricultural practices, the need for food testing is increasingly important.

Thermo Fisher Scientific understands the demands of food safety related testing. Our separation and detection technologies, combined with experienced applications competence, and our best suited chemistries provide solutions for the analysis of inorganic ions, small drug molecules, pesticides to large components, such as polysaccharides. Your laboratory can now conduct reliable, accurate, and fast testing of food. This notebook contains a wide range of food safety related application notes that will help address your food safety issues.

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- Dionex ICS-1100: Basic integrated IC system
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Dionex ICS-5000: Developed with flexibility, modularity, and ease-of-use in mind, the Dionex ICS-5000 combines the highest sensitivity with convenience

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- 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

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.

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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.

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- 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

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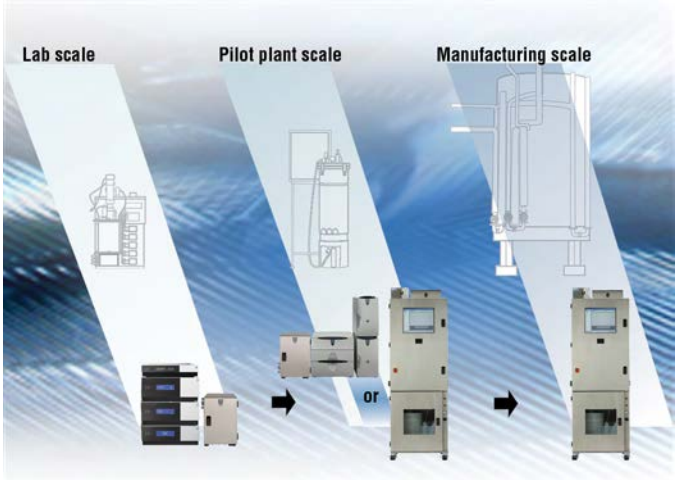
Integral: The Integral Migration Path approach: System solutions wherever you need them: lab, pilot plant, or manufacturing

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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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

DIONEX

Analysis of Food Additive Contaminants

Determination of Benzoate in Liquid Food Products by Reagent-Free™ Ion Chromatography

INTRODUCTION

Preservatives are commonly added to many food products, such as soda, fruit juice, soy sauce, jams and jellies, and other condiments, to inhibit decay. Since the early 1900s, benzoate has been widely used worldwide as a preservative due to its antimicrobial properties combined with its low toxicity and taste. Benzoate is most effective in an acidic environment (pH ≤4.5)

Benzoic acid is an effective antimicrobial agent for the purpose of preservation. However, sodium benzoate is more effective and preferred because it is approximately 200 times more soluble than benzoic acid. The soft drink industry is the largest user of benzoate as a preservative due to the amount of high fructose corn syrup in many carbonated beverages. Soft drinks account for the largest human consumption of benzoate in the USA, Australia/ New Zealand, France, and the United Kingdom.[2] Although soft drinks do not normally spoil due to their acidity and carbonation, preservatives are required to prevent changes during long-term storage.[3]

The Food and Drug Administration (FDA) regulates the uses of benzoate as a preservative in the USA. The FDA lists benzoate as a substance that is generally recognized as safe (GRAS) with a maximum permitted concentration of 0.1% in accordance with good manufacturing or feeding practices.[4] Similarly, benzoate is regulated in Europe by the European Union Legislation (Directive 95/2/EC) with a limit of 0.015% in soft drinks and up to 0.2% in other food products.[5] If higher concentrations of benzoate are used (~0.1%), then alterations in taste may occur in soft drinks.[1]

On the other hand, concentrations less than 0.010% will have little inhibitory effect.[2,6] Therefore, a reliable testing method is required to assure that the concentration of benzoate is within product and regulatory specifications.

Methods used to determine benzoic acid or its corresponding salt in foods, beverages, and other matrices include titrimetry, ion-selective electrodes, gas chromatography (GC), thin-layer chromatography, and high-performance liquid chromatography (HPLC). Many of these methods have significant disadvantages and are therefore not preferred for use in a quality control environment if a large number of samples are to be analyzed. For example, the GC method proposed by the Association of Official Analytical Chemists for the determination of benzoic acid and sorbic acid requires solvent extractions and derivatization techniques. This process involves complex procedures and is exceptionally time-consuming.[7] From the previously listed techniques, HPLC (including reversed phase, ion exchange, and ion exclusion) is used most often for the determination of benzoic acid. With this technique, many samples can be simply diluted and injected directly into the chromatography system without any complex sample preparation.

In this application note, we describe a simple ion chromatography method for the direct determination of benzoate in liquid food products. This method incorporates a Reagent-Free Ion Chromatography (RFIC™) System, requiring only deionized water to electrolytically produce a potassium hydroxide eluent, thus further simplifying user operation.

EQUIPMENT

A Dionex ICS-2000 RFIC System was used in this work. The ICS-2000 is an integrated ion chromatograph that includes:

- Eluent generator
- Column heater
- Pump degas
- EluGen® EGC II KOH Cartridge (Dionex P/N 058900)
- CR-ATC (P/N 060477)

AS50 Autosampler

Chromeleon® Chromatography Workstation

REAGENTS AND STANDARDS

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

Sodium benzoate, 99% (Sigma-Aldrich P/N 10,916-9)

CONDITIONS

Columns:	IonPac® AS18 Analytical, 4 × 250 mm (P/N 060549) IonPac AG18 Guard, 4 × 50 mm (P/N 060551)
Eluent:	35 mM KOH from 0–10 min, 35–40 mM from 10–12 min
Eluent Source:	ICS-2000 EG with CR-ATC
Flow Rate:	1.0 mL/min
Temperature:	30 °C
Injection:	25 µL
Detection:	Suppressed conductivity, ASRS® ULTRA II, 4 mm (P/N 061561) AutoSuppression® recycle mode 112 mA current
Background Conductance:	1 µS
System Backpressure:	~2400 psi
Run Time:	20 min

PREPARATION OF SOLUTIONS AND REAGENTS

1000 mg/L Benzoate Standard Solution

Dissolve 0.119 g sodium benzoate in 100 mL of deionized water. Working standards were prepared by serial dilutions from the 1000-mg/L concentrate.

SAMPLE PREPARATION

Carbonated samples should be degassed in an ultrasonic bath prior to dilution. All samples were diluted with deionized water by 1:100 prior to analysis, except the diet soda that was diluted 1:20.

RESULTS AND DISCUSSION

If a product contains a preservative, such as benzoate, then the chemical must be declared on the label according to U.S. FDA regulation. The U.S. FDA permits the use of up to 0.1% benzoate. In this study, four samples were analyzed for the presence of benzoate. Each product declared the use of benzoate on their respective labels. In addition to benzoate, many diet sodas and other types of soft drinks contain appreciable amounts of citrate. Citrate is commonly added to soft drinks as a food acidulant and flavor enhancer.[3]

In this application note, the IonPac AS18 was found to be the most suitable column for the separation of benzoate in food products. The AS18 is a high-capacity, hydroxide-selective, anion-exchange column, enabling it to tolerate the high-ionic-strength samples sometimes encountered in the food and beverage industry. In addition, the column provides an optimum selectivity for benzoate, resulting in excellent resolution between anions present at higher concentrations—such as chloride and phosphate—while still eluting anions with a higher affinity for the anion-exchange resin—such as citrate—within a reasonable time period (<20 min).

The system was calibrated from 1–20 mg/L to cover the expected range of benzoate in the diluted samples. In this application note, citrate was not of interest and was therefore not included in the calibration. Table 1 summarizes the calibration data and limit of detection for benzoate. The response for benzoate was linear over the concentration range investigated with an r^2 value of 0.9998. The method detection limit (MDL) was determined by performing seven replicate injections of a 20-µg/L benzoate standard and calculating the MDL based on the standard deviation of the mean multiplied by 3.143 (Student's *t* value for a 99% confidence level for n = 7). The calculated MDL, based on the replicate injections, was 4.9 µg/L.

Table 1. Linearity and Method Detection Limits for Benzoate

Analyte	Range (mg/L)	Linearity (r^2)	Calculated MDL (µg/L)	MDL standard (µg/L)
Benzoate	1–20	0.9998	4.9	20

Table 2 summarizes the data obtained from the analysis of four samples for benzoate. As shown, most samples contained approximately 0.05% (500 ppm) of benzoate as a preservative, which is well below the 0.1% regulation specified by the FDA. However, the diet soda contained about half the benzoate (~0.02%) compared to the other samples analyzed. Figures 1–4 show chromatograms of benzoate determinations for flavored soda, diet soda, soy sauce, and lemon juice, respectively.

Table 2. Concentrations and Retention Time and Peak Area Precisions of Benzoate in Food Products

Sample	Concentration Found (%)[a]	Retention Time Precision (%RSD[a])	Peak Area Precision (%RSD)
Flavored soda	0.043	0.020	0.49
Diet soda	0.019	0.021	0.44
Soy sauce	0.051	0.055	0.47
Lemon juice	0.048	0.019	0.40

[a] The concentrations and relative standard deviations (RSDs) were calculated from 10 replicate injections (n = 10)

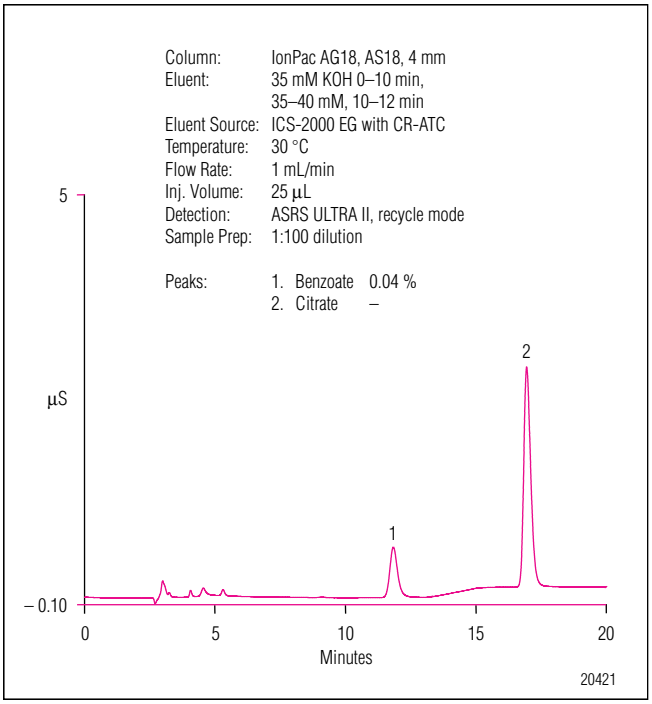

Figure 1. Determination of benzoate in flavored soda.

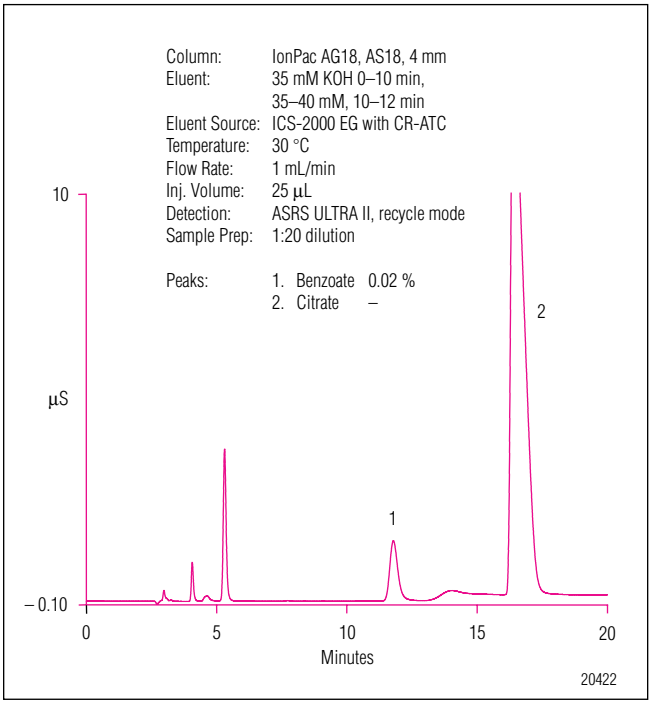

Figure 2. Determination of benzoate in diet soda.

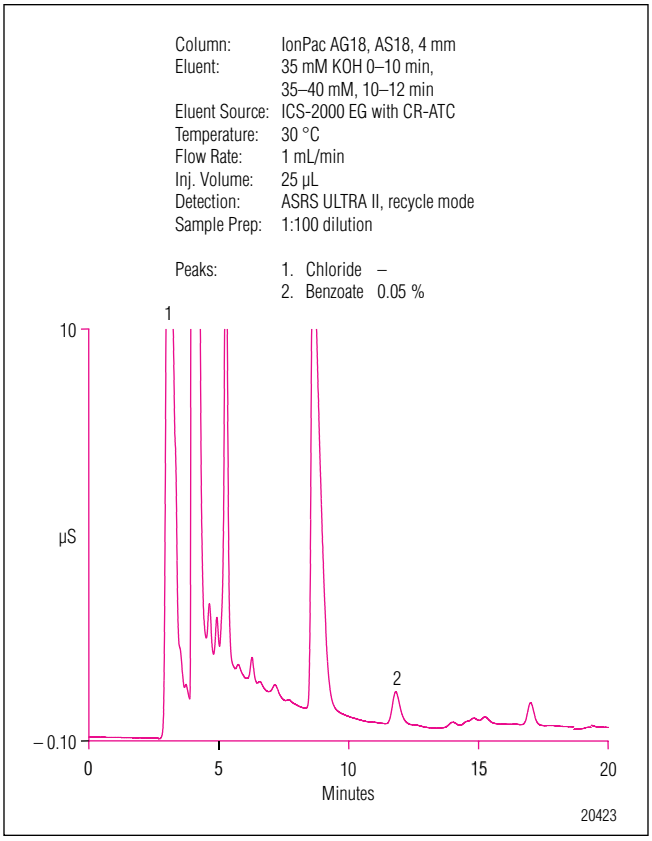

Figure 3. Determination of benzoate in soy sauce.

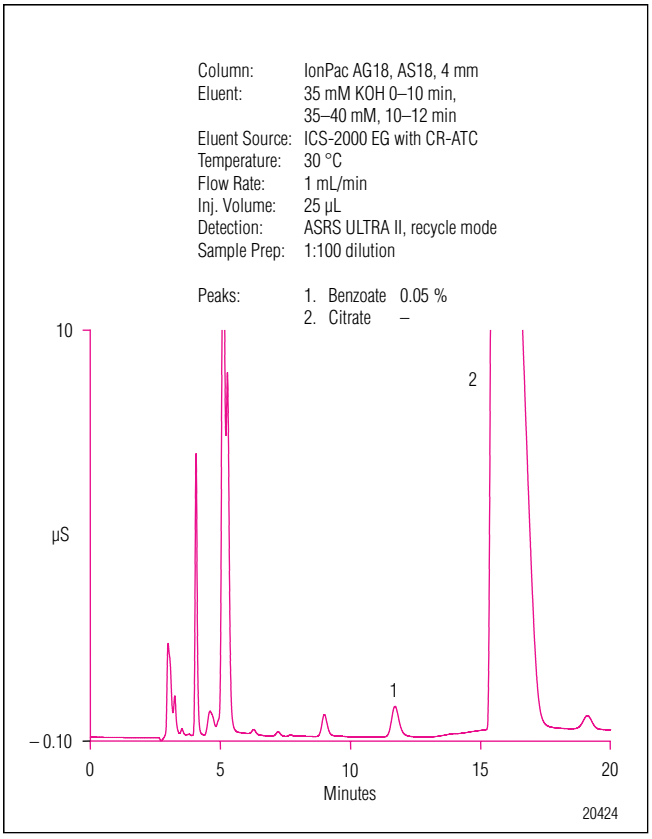

Figure 4. Determination of benzoate in lemon juice.

The high capacity of the AS18 column enabled it to tolerate the high amounts of chloride in soy sauce and citrate in lemon juice, while still providing a good selectivity for benzoate. The precision of ten replicate sample injections resulted in retention time and peak area RSD values of <0.06% and <0.50%, respectively. The high repeatability of the method reflects results typically found when using an RFIC system. Each sample was spiked with benzoate at approximately the same amount of benzoate found in the diluted samples. The average spiked recoveries, based on triplicate injections, yielded recoveries in a range of 90–101% (Table 3).

Table 3. Recovery of Benzoate in Food Products

Sample	Amount Added (mg/L)	Recovery[a] (%)
Flavored soda	4.3	101.2
Diet soda	9.6	90.2
Soy sauce	4.8	94.5
Lemon juice	4.7	97.7

[a] The average recovery was calculated from triplicate injections.

CONCLUSION

This application note demonstrates a simple and reliable RFIC method for the determination of benzoate in food products using a high-capacity, hydroxide-selective, anion-exchange column. In addition to benzoate, the method can determine other anions that are often present in many food products, such as chloride, phosphate, and citrate. In comparison to previously reported methods for benzoate, RFIC provides added convenience and simplicity for the user by enabling full control of the hydroxide eluent concentration through Chromeleon software. In addition, samples only require a simple dilution prior to injection. Furthermore, the precision is significantly improved by avoiding manual preparation of eluents.

REFERENCES

1. Velsicol Chemical Corporation, *Product Information Bulletin: Sodium Benzoate Food Grade, FCC*; http://www.velsicol.com/asps/documents/pibs/pdfpib42.pdf.
2. World Health Organization. *Concise International Chemical Assessment Document No. 26 on Benzoic Acid and Sodium Benzoate*; Geneva, Switzerland, 2000; http://www.inchem.org/documents/cicads/cicad26.htm
3. National Soft Drink Association. *About Soft Drinks*; updated 10/3/03; http://www.ndsa.org/softdrinks/History/whatsin.html
4. U.S. Food and Drug Administration. Code of Federal Regulations; Title 21 Volume 6, Section 582.3733 sodium benzoate; U.S. Government Printing Office, Washington, DC, Revised April 1, 2003.
5. European Parliament and Council Directive No. 95/2/EC. *Food Additives Other Than Colours and Sweeteners*; February, 1995.
6. Pezza, L.; Santini, A. O.; Pezza, H. R.; Melios, C. B.; Ferreira, V. J. F.; Nasser, A. L. M. *Anal. Chim. Acta* **2001**, *433*, 281–288.
7. Association of Official Analytical Chemists (AOAC). *Benzoic Acid and Sorbic Acid in Food*; Method 983.16; Gaithersburg, MD, 1995.

SUPPLIER

Sigma-Aldrich Chemical Co., P.O. Box 14508, St. Louis, MO 63178 USA, Tel: 800-325-3010, www.sigmaaldrich.com.

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 m0ilk 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 at 4 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.

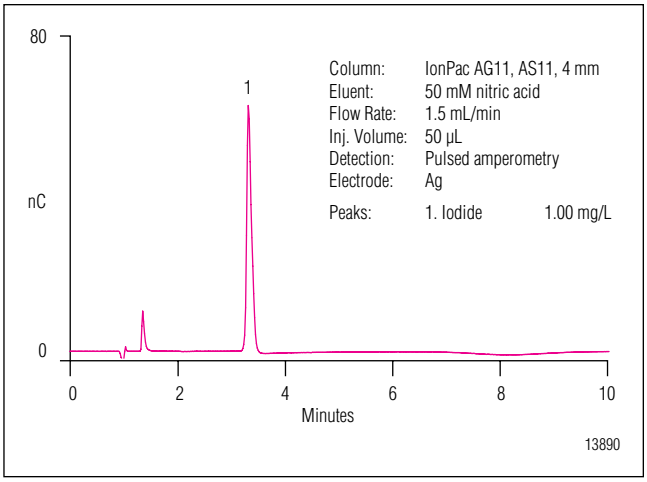

Figure 1. Determination of iodide by ion chromatography with pulsed amperometric detection

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.

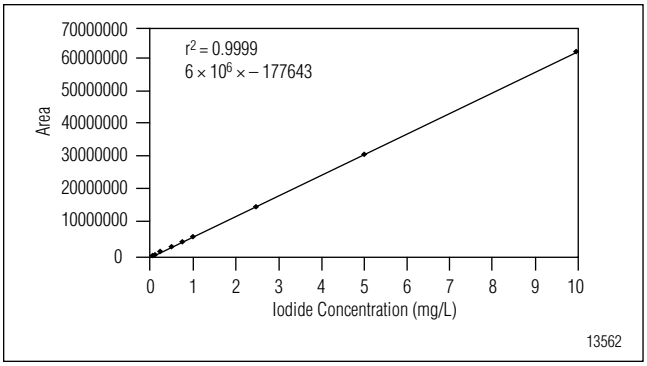

Figure 2. Iodide linearity: 0.025–10 mg/L.

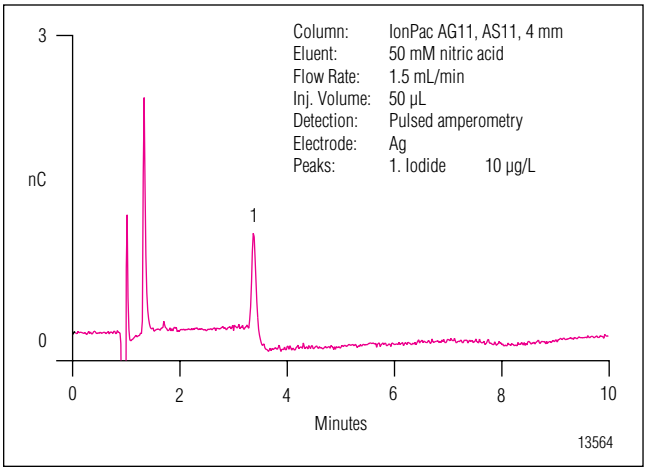

Figure 3. Low-level determination of iodide by pulsed amperometry.

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.

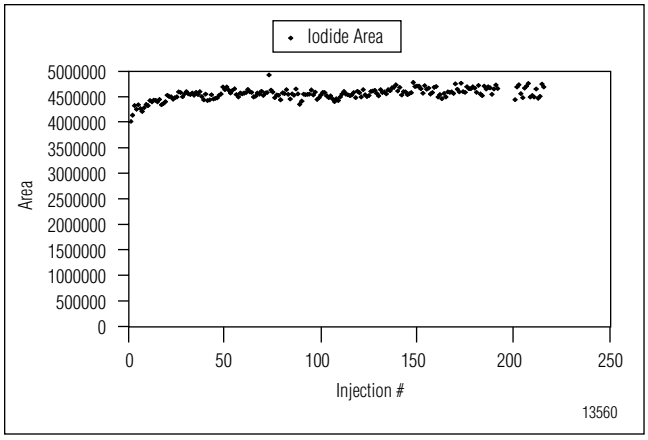

Figure 4. 41-h reproducibility of iodide analysis by pulsed amperometry.

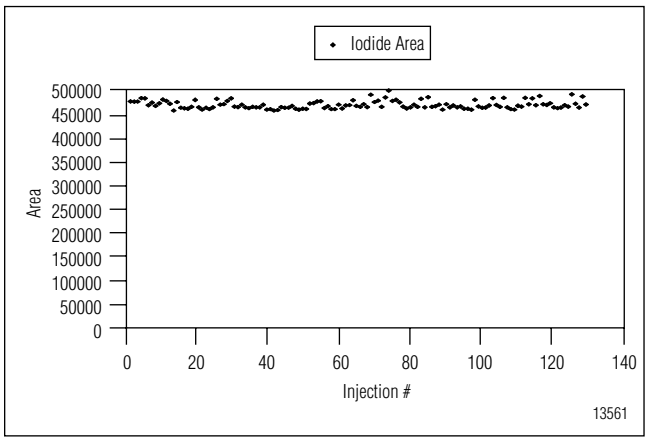

Figure 5. 24-hour reproducibility of iodide analysis, 100 ppb injected.

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 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.

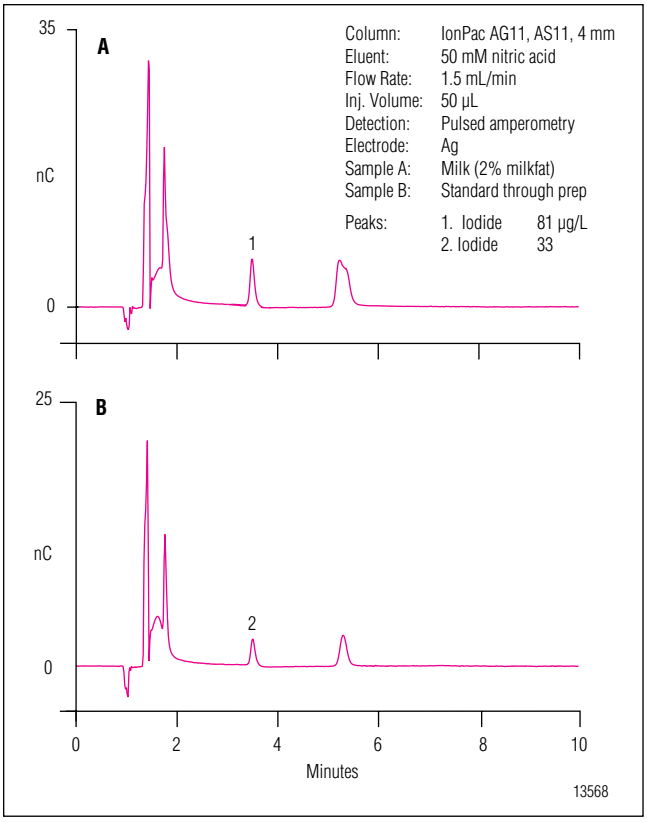

Figure 6. Analysis of iodide in milk samples by IC with pulsed amperometric detection.

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 backpressure, 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.

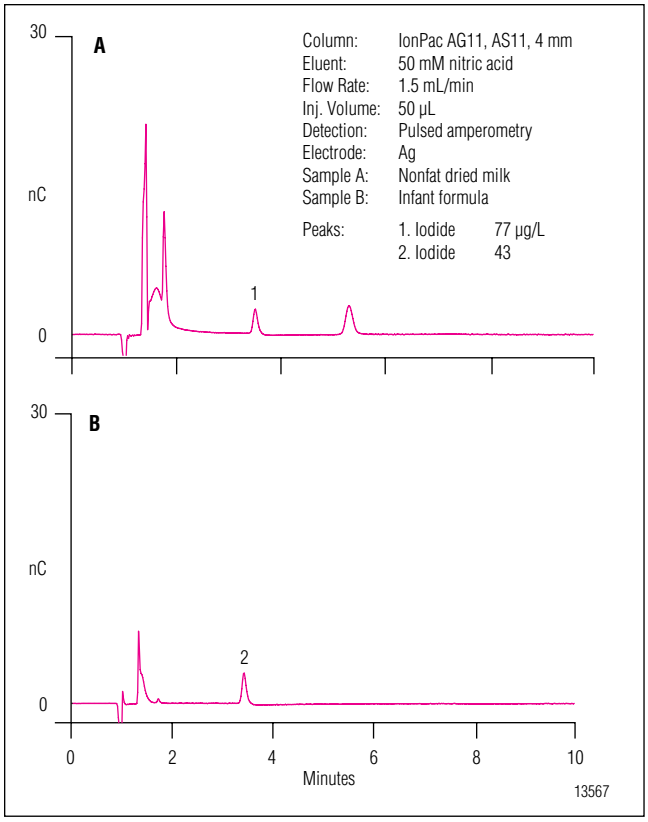

Figure 7. Analysis of iodide in milk samples by IC with pulsed amperometric detection

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 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

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	8
Infant Formula	6	66	1.3	85

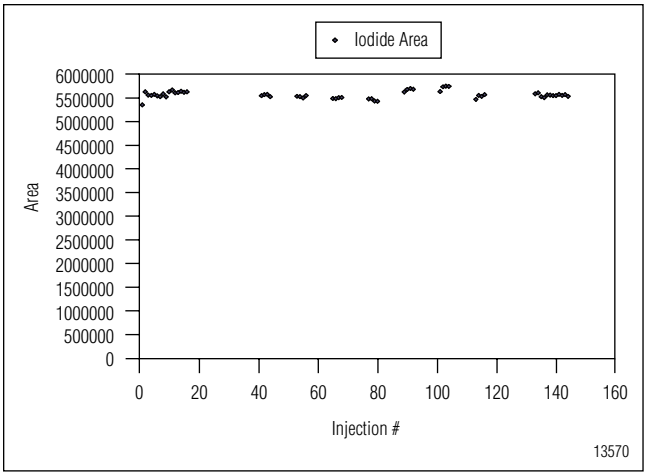

Figure 8. Reproducibility of an iodide standard (1 mg/L) during sample analysis

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.

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 Nitrate and Nitrite in Meat Using High-Performance Anion Exchange Chromatography

INTRODUCTION

Nitrate and nitrite are usually added to processed meat products to protect against microorganisms that can cause food poisoning, such as Clostridium botulinum.[1–3] However, nitrite can react with secondary amines to form nitrosoamines, a class of carcinogenic compounds, in food products or in the digestive system. Nitrate, although more stable than nitrite, can act as a reservoir for nitrite. Also, nitrate can readily be converted into nitrite by microbial reduction.4,5 Thus, both nitrate and nitrite must be monitored to ensure the quality and safety of meat products. This application note describes an accurate and sensitive method in which nitrate and nitrite are extracted from meat products and then determined directly using anion exchange chromatography with UV detection. Commercially available ham and salami were used as model samples.

Several HPLC methods have been developed to analyze for nitrate and nitrite in meat. However, these methods require lengthy sample processing or pretreatment steps, such as adding protein precipitation procedures after extraction or using reversed-phase cartridges to remove sample matrix interferences. 6-9 Unlike most other HPLC techniques, the method described in this note does not require the use of protein precipitating reagents. In addition, a reversed-phase or ion-exchange pretreatment cartridge is not needed because a five-minute, 100 mM sodium hydroxide wash step in the chromatographic procedure is sufficient to remove bound proteins and other sample matrix interferences.

EQUIPMENT

Dionex DX 500 chromatography system consisting of:
- GP40 Gradient Pump
- AD20 UV/Visible Absorbance Detector
- LC20 Enclosure
- AS40 Autosampler
- PeakNet Chromatography Workstation

Scovill Hamilton Beach Blender

Beckman Spinchron R Centrifuge

MATERIALS

Sodium hydroxide, 50% w/w (Fisher Scientific)

Sodium nitrate (Fisher Scientific)

Sodium nitrite (Fisher Scientific)

Whatman Filters (Whatman)

Sterile Acrodisc Syringe Filters, 1.2 and 0.2 mm (Gelman Sciences)

CONDITIONS

Column:	IonPac® AS11 (4 × 250 mm) and AG11 guard (4 × 50 mm)
Inj.Volume:	25 µL
Flow Rate:	1 mL/min
Detection:	UV, 225 nm
Eluent A:	50 mM Sodium hydroxide
Eluent B:	Deionized water
Eluent C:	100 mM Sodium hydroxide

Method

Time (min)	Eluent A (%)	Eluent B (%)	Eluent C (%)
0.0	10	90	0
10.0	10	90	0
10.1	0	0	100
15.0	0	0	100
15.1	10	90	0
25.0	10	90	0

Calibration Curves

Calibration curves for nitrate and nitrite were generated by plotting the peak areas against the concentrations of the standards injected. For nitrate, nine different con-centrations between 50 µg/L (500 µg/kg) and 375 mg/L (3.75 g/kg) were used. For nitrite, nine different concen-trations between 30 µg/L (300 µg/kg) and 300 mg/L (3.00 g/kg) were used. At least two peak area data points were collected per order of magnitude. Each data point was an average of duplicate injections.

PREPARATION OF SAMPLES AND SOLUTIONS

Eluent A: 50 mM Sodium hydroxide

Filter 1.0 L of deionized water through a 0.2 µm filter. Then vacuum degas the deionized water for 5 minutes. Add 2.5 mL of 50% w/w sodium hydroxide to the 1.0 L of degassed water.

Eluent B: Deionized water

Filter 1.0 L of deionized water through a 0.2 µm filter.

Eluent C: 100 mM Sodium hydroxide

Filter 1.0 L of deionized water through a 0.2 µm filter. Then vacuum degas the deionized water for 5 minutes. Add 5.0 mL of 50% w/w sodium hydroxide to the 1.0 L of degassed water.

Nitrate Stock Solution

Dissolve 137 mg of sodium nitrate in 100 mL of deionized water to make up a 1 g/L stock solution.

Nitrite Stock Solution

Dissolve 150 mg of sodium nitrite in 100 mL of deionized water to make up a 1 g/L stock solution.

Extraction Procedure

Weigh 10.0 g of ham or salami and transfer to a blender. Add 100 mL of deionized water to the meat sample. Liquify the meat sample in the blender for 1 minute. Heat the liquified sample and maintain the temperature of the sample between 70 °C and 80 °C for 15 minutes[6-9]. Allow the sample to cool to room temperature. Centrifuge the sample at 4960 x g (6000 rpm in a Beckman GA-10 rotor) for 10 minutes. Remove the supernatant. Successively filter the supernatant through the following filters: Whatman No. 2 and GF/A filters and 1.2 µm and 0.2 µm Acrodisc filters. Collect the filtrate for HPLC analysis.

RESULTS AND DISCUSSION

Figure 1 shows a separation of the nitrate and nitrite standards. Figure 2 shows a separation of nitrate and nitrite from the ham extract. The amounts of nitrate and nitrite, as shown in Table 1, were determined to be 5.37 and 11.6 mg/kg, respectively. The dip before the nitrite peak apparently is due to the elution of chloride (approximately 400 mg/L). The amount of chloride can be determined using a suppressed conductivity detector in series with the UV detector (data not shown).

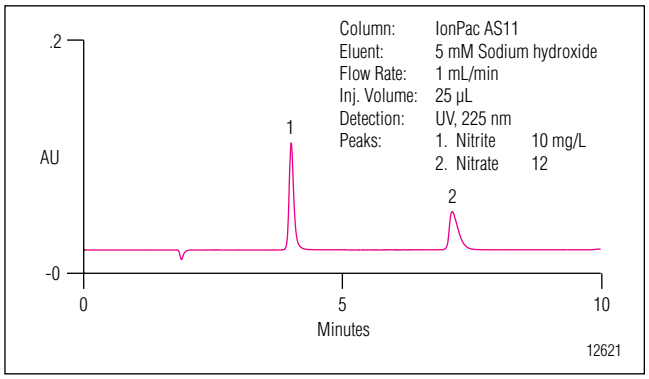

Figure 1. Separation of nitrate and nitrite standards. Amount Injected: 12 mg/L nitrate and 10 mg/L nitrite; Injection Volume: 25 µL.

Table 1. Concentration of Nitrate and Nitrite in the Ham and Salami Samples

	Amount of Nitrite (mg/kg)	Amount of Nitrite (mg/kg)	Concentration of Nitrite in 100 mL of Extract (mg/L)	Concentration of Nitrite in 100 mL of Extract (mg/L)
Salami	108	98.5	10.8	9.85
Ham	11.6	5.37	1.16	0.54

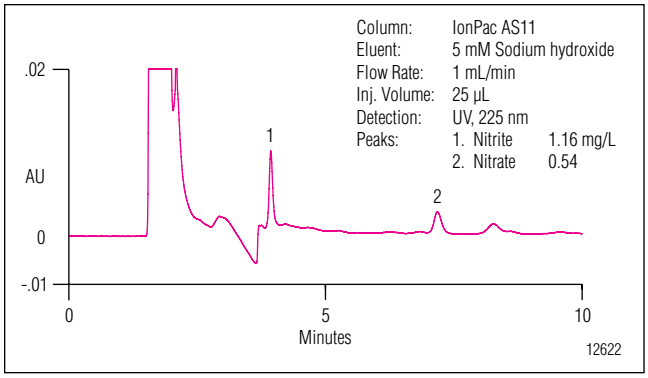

Figure 2. Separation of nitrate and nitrite from ham. Injection Volume: 25 µL.

Figure 3 shows a separation of nitrate and nitrite from the salami extract. As shown in Table 1, the amounts of nitrate and nitrite in salami were determined to be 98.5 and 108 mg/kg, respectively. Similar to the ham sample, a dip immediately before the nitrite peak due to the elution of chloride is also observed.

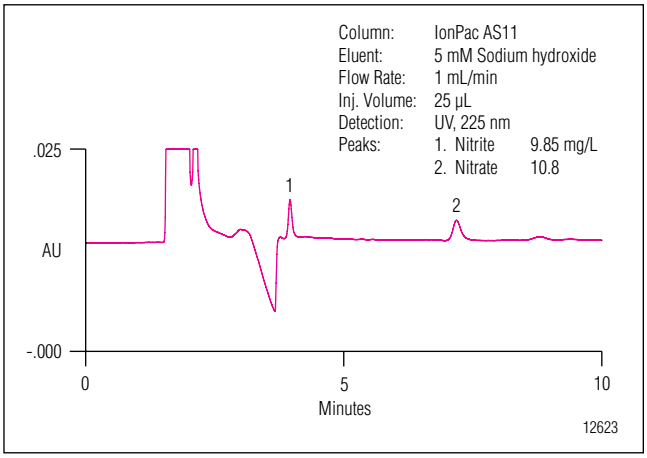

Figure 3. Separation of nitrate and nitrite from salami. The extract was diluted fourfold before injection; Injection Volume: 25 µL.

Recovery

As shown in Table 2, predetermined amounts of nitrate or nitrite standards were added to each of the meat samples and allowed to be absorbed into the meat samples for 10 minutes. The amounts of nitrate and nitrite were then determined following the same extraction and separation processes. Table 2 shows the recovery results. Over 90% recoveries of nitrate and nitrite standards from both the ham and salami samples were obtained.

Table 2. Spike Recovery Data

	Amount Present (mg/kg)	Amount Added (mg/kg)	Total Recovered (mg/kg)	Recovery (%)
Nitrite in Salami	108	120	218	92
Nitrate in Salami	98.5	100	195	97
Nitrite in Ham	11.6	15.0	27.0	103
Nitrate in Ham	5.37	6.50	11.4	92

Precision

The degree of agreement among individual test results was determined and expressed as RSDs (Relative Standard Deviations). Table 3 shows the RSDs of retention time and peak areas of nitrate and nitrite. For both ham and salami, peak area RSDs were below 3% and retention time RSDs were less than 0.5%. No detectable changes in retention time were noticed after 117 injections of salami and ham samples.

Table 3. Peak Area and Retention Time RSDs

	RSDs of Nitrate Peak Area (%)	RSDs of Nitrate Peak Area (%)	RSDs of Nitrate Retention Time (%)	RSDs of Nitrate Retention Time (%)
Salami, n = 5	2.7	2.9	0.2	0.3
Ham, n = 5	2.3	1.0	0.2	0.2

Linearity and Limit of Detection

Detection limits and linearity data are shown in Table 4. The coefficients of determination for nitrate and nitrite were 0.9991 and 0.9995, respectively. These values were calculated over three orders of magnitude. Detection limits for nitrate and nitrite, determined at three times the noise, were 50 µg/L (500 µg/kg) and 30 µg/L (300 µg/kg), respectively.

Table 4. Detection Limits and Linearity Data

	Concentration Range	r^2	Detection Limit
Nitrate	500 µg/kg–3.75 g/kg	0.9991	500 µg/kg
Nitrite	300 µg/kg–3.00 g/kg	0.9995	300 µg/kg

CONCLUSION

- The method described in this note is a simple and accurate analytical method for determining nitrate and nitrite in meat samples. The IonPac AS11 column provides ideal selectivity not only for the separation ofnitrate and nitrite, but also for the separation of the analytes from matrix components, which are eluted mostly in the void.
- With the five-minute, 100-mM sodium hydroxide wash step, retained ions and organic species are eluted. Thus, the method eliminates the need for time consuming and costly sample pretreatment using reversed-phase or ion-exchange cartridges and protein precipitating reagents.
- The DX 500 system (PEEK system) is designed to be compatible with high pH eluents such as sodium hydroxide. Combined with the AS40 autosampler, the analytical system provides an ion-free environment for the determination of nitrate and nitrite at the sub-mg/L (sub-ppm) levels.

PRECAUTIONS

Detectable changes (more than 10%) of nitrate and nitrite concentrations from the meat extracts were observed after the extracts were kept at room temperature for more than 24 hours. Analysis should be completed within 24 hours after extraction.

Chloride was present at concentrations of approximately 400 mg/L and 1600 mg/L in the ham and salami samples, respectively. It is important to dilute the extracts so that no more that 400 mg/L of chloride is loaded onto the column. If too much chloride is injected, the nitrite peak may elute earlier and be poorly resolved from the chloride dip, causing difficulty in peak area determination.

REFERENCES

1. Swann, P. F. *Proc. Roy. Soc. Med.* **1977**, *70*, 113.
2. Roberts, T.A.; Ingram, M.C. *Proceedings of the Second International Symposium in Nitrite on Meat Products.* eds. Tinbergen, B.J.; Krol, B., Wageningen: PUDOC **1977**, 29–38.
3. Olsman, W. J. P*roceedings of the Second International Symposium in Nitrite on Meat Products.* Eds: Tinbergen, B.J.; Krol, B., Wageningen: PUDOC, **1977**, 101–110.
4. Dennis, M.J.; Key, P.E.; Papworth, T.; Pointer, M.; Massey, R.C. *Food Additives and Contaminants.* **1990**, *7*, 455–461.
5. Cassens, R.G. *Food Technology.* **1995**, 72–115.
6. de Kleijin, J. P.; Hoven, K. *Analyst.* **1984**, *109*, 527–528.
7. Jackson, P. E.; Haddad, P.R; Dilli, S. *J. Chromatogr.* **1984**, *295*, 471–478.
8. Alonso, A.; Etxaniz, B.; Martinez, M.D. *Food Additives and Contaminants.* **1992**, *9*, 111-117.
9. Eggers, N.J.; Cattle, D.L. *J. Chromatogr.* **1986**, *354*, 490–494.

LIST OF SUPPLIERS

1. Fisher Scientific, 711 Forbes Ave., Pittsburgh, Pennsylvania 15219-4785, USA.
   Tel.: 800-766-7000
2. Whatman LabSales, Inc., 5285 NE Elam Young Parkway, Suite A400, Hillsboro, Oregon 97124, USA.
   Tel.: 800-942-8626
3. Gelman Sciences, Inc., 600 S. Wagner Road, Ann Arbor, Michigan 48106-1448, USA.
   Tel.: 800-521-1520

Determination of Sulfite in Food and Beverages by Ion Exclusion Chromatography with Pulsed Amperometric Detection

INTRODUCTION

Sulfite is a widely used food preservative and whitening agent that received GRAS (generally recognized as safe) status from 1959 until 1986. In 1986, the U.S. Food and Drug Administration (FDA) revoked GRAS status when adverse reactions in sulfite-sensitive individuals were reported. Since then, the FDA has required warning labels on any food containing more than 10 mg/kg of sulfite or beverage containing more than 10 mg/L. Six sulfiting agents are currently approved by the FDA for use as food additives: sulfur dioxide, sodium sulfite, sodium and potassium bisulfite, and sodium and potassium metabisulfite.

The Modified Monier-Williams method[1] is the most widely used method for analyzing the amount of sulfite in various food matrices. However, this method is time-consuming and quite labor-intensive. More recently, the Association of Official Analytical Chemists (AOAC) International adopted a method developed by Kim and Kim that uses ion exclusion chromatography with direct current (dc) amperometric detection.[2] This method (AOAC Method 990.31) is selective enough that samples need only be homogenized in buffer, filtered, and injected for analysis.

One drawback to the Kim and Kim method is that fouling of the platinum working electrode occurs rather quickly, leading to a significant decrease in detector response over time. As much as a 40% loss of the detector response to sulfite over an 8-h period has been reported.[3] Not only does this necessitate frequent polishing of the working electrode, but accurate quantification requires injecting a standard after every sample injection.

The method described in this Application Note is a modification of the Kim and Kim method. It uses the same sample preparation and chromatographic procedures, but solves the detection problems by using pulsed amperometry instead of dc amperometry. The pulse sequence constantly cleans the working electrode, thus preventing fouling. Detector response remains stable, as shown in Figure 1, resulting in more reliable quantification. In addition, standards can now be injected much less frequently, resulting in higher sample throughput.

Sample Preparation and Preservation

The sample preparation buffer, adopted from AOAC Method 990.31, is alkaline so that both free and bound sulfite can be extracted. Mannitol is included to slow the oxidation of sulfite to sulfate. Food samples are prepared by homogenization in the buffer, followed by filtration. Liquid samples are diluted in the buffer prior to injection.

Summary of Analytical Method

Sulfite is separated from other matrix components by ion exclusion chrmatography using a sulfuric acid eluent and detected by pulsed amperometry using a platinum working electrode. Amperometry is a highly sensitive and specific detection method for oxidizable species such as sulfite. The waveform includes oxidizing and reducing potentials, which are constantly cycled to maintain a reproducible working electrode surface.

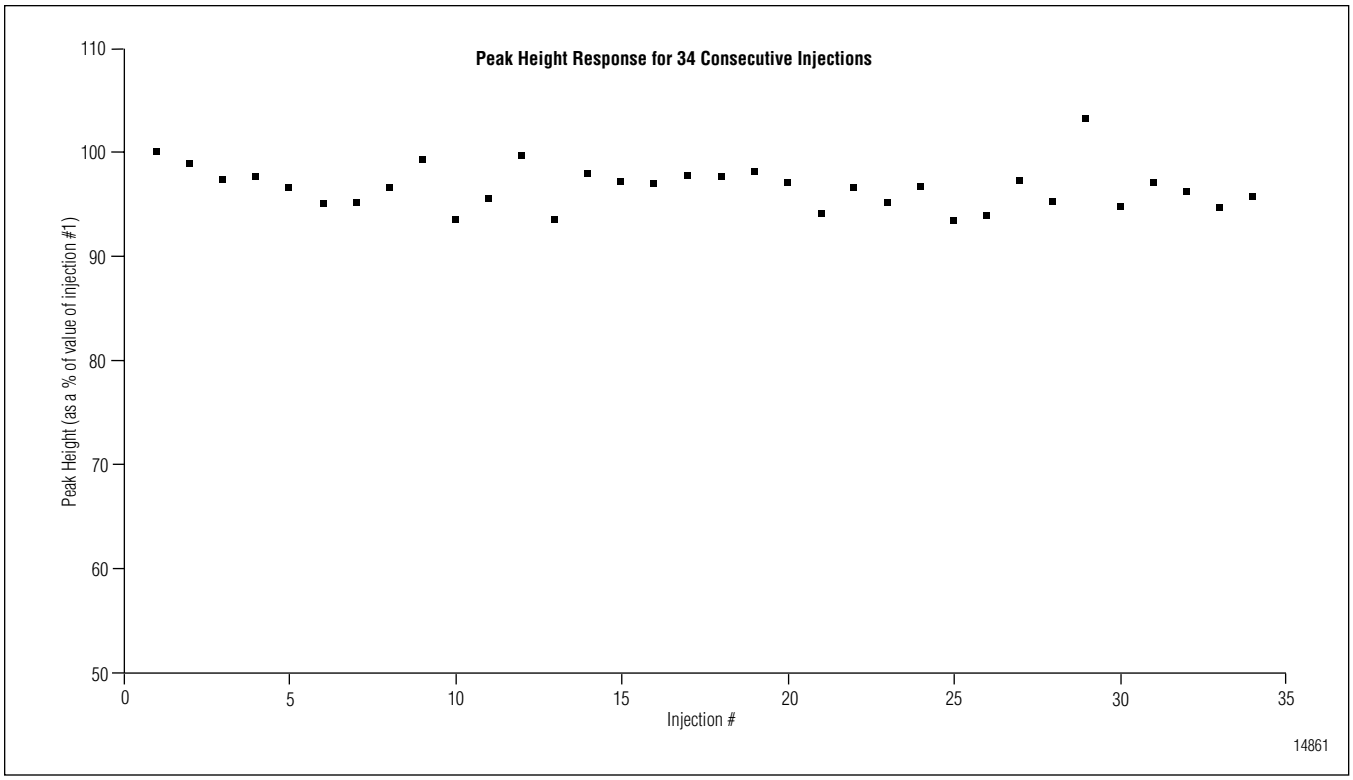

Figure 1. Consecutive injections (34) of a 14-mg/L sulfite standard. Peak heights were plotted as a percentage of the peak height from injection #1. Each analysis required approximately 15 min.

EQUIPMENT

Dionex DX-500 HPLC system consisting of:

- High Performance Pump (IP25 or GP50) with vacuum degas
- ED40 Electrochemical Detector equipped with a platinum working electrode
- LC20 Chromatography Module
- EO1 Eluent Organizer

PeakNet Chromatography Workstation

REAGENTS

Concentrated sulfuric acid, ACS Reagent Grade (Fisher Scientific, Fair Lawn, NJ)

Deionized water, 18 MΩ-cm

Sodium sulfite, anhydrous, ACS Reagent Grade (Sigma Chemical Co., St. Louis, MO)

Sodium phosphate dibasic heptahydrate ($Na_2HPO_4 \cdot 7H_2O$) (Sigma Chemical Co., St. Louis, MO)

D-Mannitol (J.T. Baker Chemical Co., Phillipsburg, NJ)

REAGENT PREPARATION

20 mN H_2SO_4

Dilute 0.55 mL of concentrated sulfuric acid to 1.0 L with deionized water. Pressurize with helium.

Sample Buffer (20 mM Na_2HPO_4/10 mM Mannitol, pH 9)

Dissolve 5.36 g of sodium phosphate dibasic heptahydrate and 1.82 g of D-mannitol in 1.0 L of water. Filter through a 0.45-µm filter.

STANDARD PREPARATION

Stock Solutions

Prepare a stock solution of sulfite (approximately 1000 mg/L) by accurately weighing approximately 195 mg of Na_2SO_3. Transfer to a 100-mL volumetric flask and dilute to volume with buffer.

Working Standards

Make appropriate dilutions in buffer to bracket expected sample concentrations.

EXPERIMENTAL CONDITIONS

Column: IonPac® ICE-AS1

Eluent: 20 mN H_2SO_4

Flow Rate: 1.0 mL/min

Inj. Vol.: 50 µL

Detection: Pulsed amperometry, Pt electrode;
Waveform:

Time (s)	Voltage (V)	Integration
0.00	0.80	
0.40	0.80	begin
0.60	0.80	end
0.61	1.20	
0.70	1.20	
0.71	0.10	
1.00	0.10	

DISCUSSION AND RESULTS

Food Analysis

Sulfite is found in large quantities as a preservative in dried fruits. To prepare a sample of dried apricots for the chromatogram shown in Figure 2, 100 mL of the mannitol buffer was added to 20 g of sample. The mixture was blended at high speed for about 1 min. After homogenization, the sample was centrifuged for 15 min at 2200 × g. The resulting supernatant was diluted 20-fold in mannitol buffer and 50 µL were injected onto the column. Quantification of the sulfite peak showed that the original dried apricot sample contained 0.8 mg of sulfite per gram of fruit.

Liquid Sample Analysis

For the analysis of lime juice, shown in Figure 3, the lime juice sample was diluted 36-fold in mannitol buffer and injected. Lime juice was found to contain 260 mg/L of sulfite.

Sample Stability

Because sulfite readily oxidizes to sulfate, samples and standards should be analyzed in a timely fashion. Standards should be made fresh daily, and reasonable care should be taken to reduce air exposure of both standards and samples.

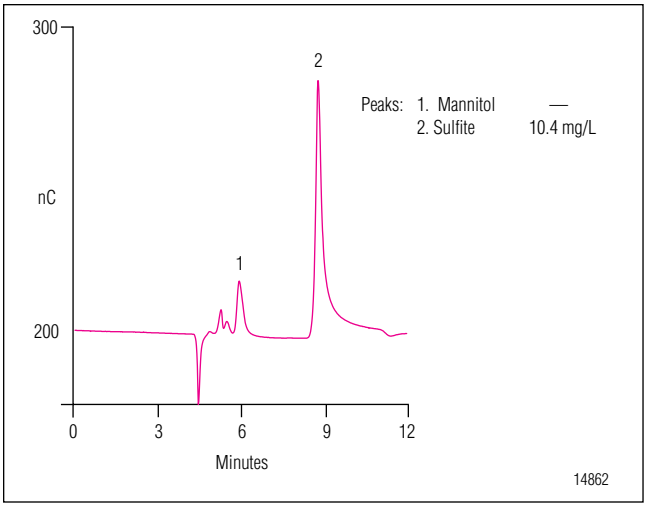

Figure 2. Dried apricot homogenate.

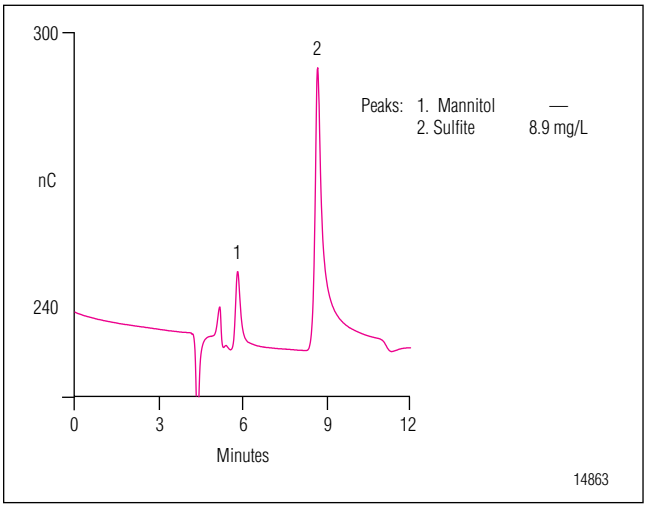

Figure 3. Lime juice, 1/36 dilution.

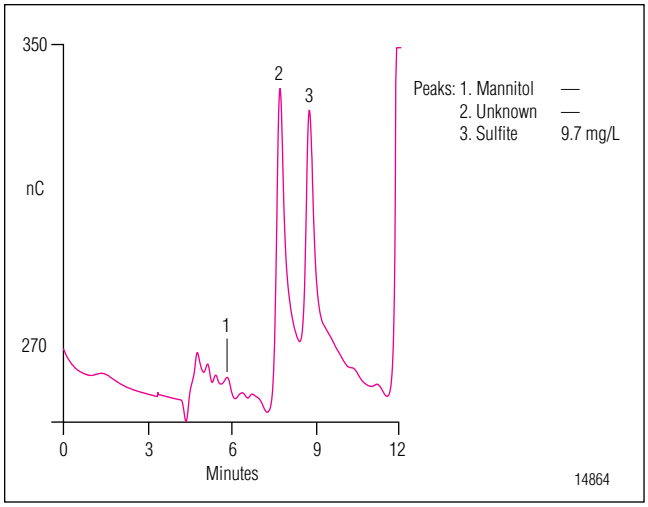

Figure 4. White wine.

Studies indicate that standards and samples prepared in the mannitol buffer should be stable for 24 hours. However, unpreserved samples should be analyzed as soon as possible after opening the sample container. In one study, untreated white wine was injected repeatedly (an example chromatogram is shown in Figure 4) over a period of about 4 h. A plot of sulfite concentration in the wine versus time is shown in Figure 5. A linear fit of this plot yields a line with the equation $y = -0.2x + 9.7$, indicating that sulfite is oxidizing at a rate of approximately 2% per hour.

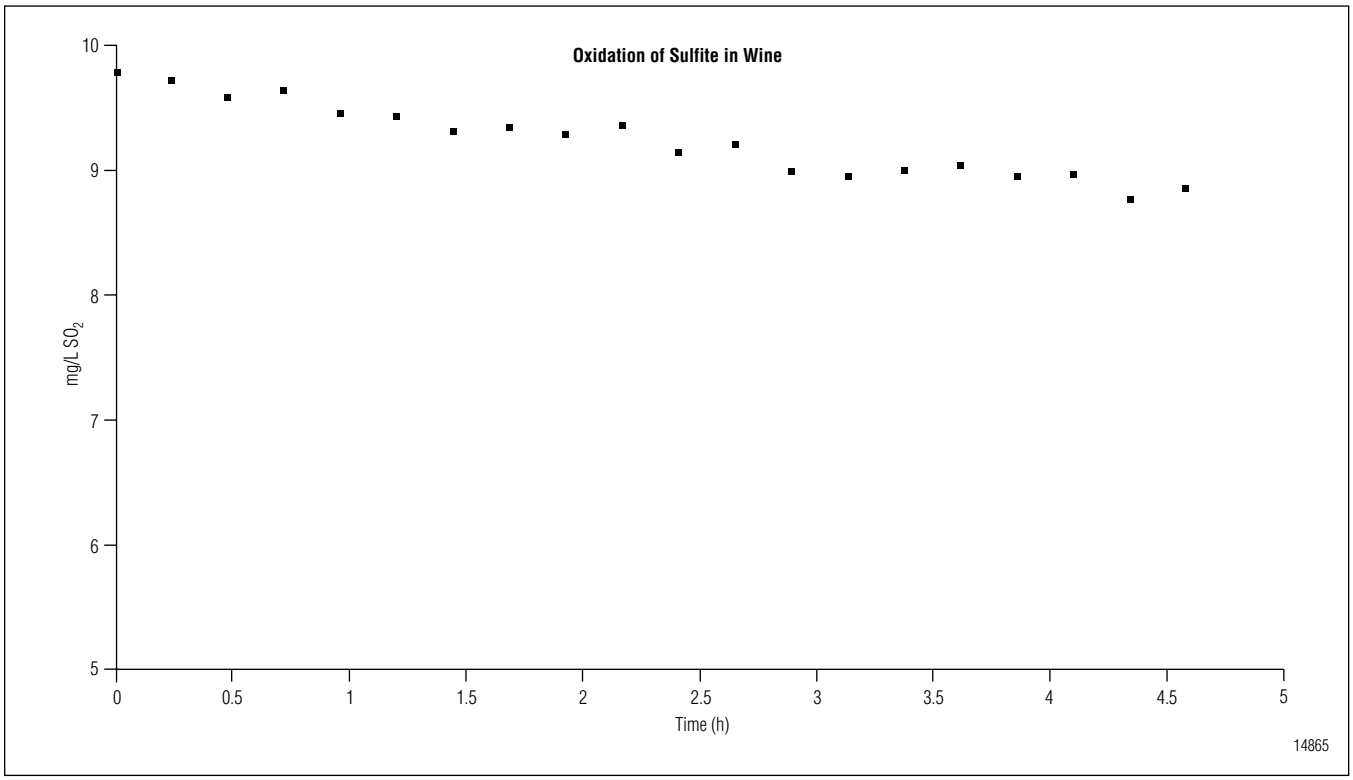

Figure 5. Oxidation of sulfite in white wine over time. Original concentration of SO_2 in fresh sample was 9.7 mg/L.

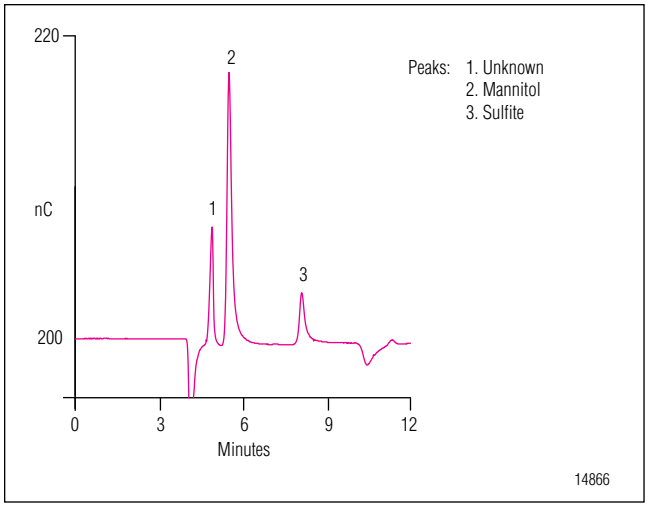

Figure 6. Sulfite standard, 530 µg/L.

Method Performance

Quantification by peak height, which will give more reliable data for this method, is recommended. All method performance data shown below were calculated using peak height data.

Method Detection Limits (MDL)

A volume of 50 µL of a 530-µg/L standard (shown in Figure 6) was injected repeatedly. Using the student's t calculation (99% confidence level, 19 degrees of freedom), the MDL was found to be approximately 40 µg/L SO_2.

Linearity

Detection of sulfite was found to be linear over the range of 0.9 to 90 mg/L ($r^2 = 0.998$).

Repeatability

Injection-to-injection repeatability was measured by calculating the relative standard deviation (RSD) of the data shown in Figure 1. A 14-mg/L sulfite standard, which had been prepared in mannitol buffer, was injected 34 times. The relative standard deviation for resulting peak heights was 2.2%.

Recovery

Recovery of sulfite from white wine, which as packaged contained 9.7 mg/L of sulfite, was studied. A 20-mL aliquot of wine was spiked with 20 µL of a 530-mg/L stock solution of sulfite in mannitol buffer. Analysis of the spiked sample showed 104% recovery of the added sulfite (n=3).

PRECAUTIONS

Maintaining Working Electrode

Depending on the cleanliness and complexity of samples analyzed, the working electrode should remain stable for several weeks to months. Typical background is around 100 to 200 nC.

When the background starts to rise and baseline noise increases, it may be necessary to polish the working electrode. Follow the electrode polishing procedure outlined in the ED40 manual. After polishing, it is good practice to condition the electrode for 24 hours (i.e., run eluent at a low flow rate with the cell on) before resuming sample analysis.

On-Line Vacuum Degas

Because dissolved oxygen in the eluent can affect the performance of an amperometric detector, on-line degassing is recommended to maintain an oxygen-free environment. Degassing the eluent and pressurizing it with helium is only partly effective because the Teflon® tubing usually used to carry eluent from the reservoir to the pump is oxygen-permeable.

Instrument Shutdown

For short-term instrument shutdown (less than a week), it is good practice to keep eluent flowing through the system at 0.1 to 0.2 mL/min. The amperometric cell should be left on. Following this recommendation will prevent the need for frequent reconditioning of the working electrode.

For long-term shutdown, the cell should be disassembled and the reference electrode stored in saturated KCl.

CONCLUSION

The method outlined in this Application Note offers a substantial improvement in the detection of sulfite. Using pulsed amperometry, the working electrode surface is continuously cleaned, resulting in a more stable detector response. Good method performance was shown, with injection-to-injection repeatability less than 3%. Using this method, it is possible to achieve more accurate sulfite quantification using fewer standard injections than required for dc amperometric methods.

REFERENCES

1. AOAC Official Method 962.16 in Official Methods of Analysis of AOAC International, 16th ed., Vol. II; Cunniff, P., Ed.; 1995.
2. AOAC Official Method 990.31 in Official Methods of Analysis of AOAC International, 16th ed., Vol. II; Cunniff, P., Ed.; 1995.
3. Wagner, H.P.; McGarrity, M.J. *J. Chrom.* **1991**, *546*, 119–124.

Fast HPLC Analysis of Dyes in Foods and Beverages

INTRODUCTION

Dyes have many applications in the food and beverage industries, such as being used to make food more appealing, hide defects, or to strengthen consumer perception of the association between color and flavor. For example, lime flavor is associated with the color green and thus, lime soft drinks are often colored with green food dye. Despite the existence of many dyes, only a few have been approved for use in foods and beverages. The U.S. FDA permits seven artificial colorings in food: Brilliant Blue FCF (FD&C Blue 1), Indigotine (FD&C Blue 2), Sunset Yellow FCF (FD&C Yellow 6), Tartrazine (FD&C Yellow 5), Allura Red AC (FD&C Red 40), Fast Green FCF (FD&C Green 3), and Erythrosine (FD&C Red 3).

Reversed-phase chromatography is an excellent technique for the analysis of dyes. Many dyes are hydrophobic, readily soluble in reversed-phase eluents, and have strong visible and UV absorbance properties. This application note (AN) demonstrates fast separation of 10 dyes in less than 5 min using an Acclaim® PA2 (3 µm) column in a 3 × 75 mm format. The Acclaim PA2 column is ideal for resolving mixtures of compounds with a wide range of hydrophobicities, including very polar compounds. This method was used to determine the quantity of food dyes in six soft drinks and a gelatin dessert. The combination of an UltiMate® 3000 Rapid Separation LC (RSLC) system and an Acclaim PA2 column is suitable for the fast analysis of food and beverage samples that have both approved and illegal dyes.

EQUIPMENT

Dionex UltiMate 3000 RSLC system consisting of:

- SRD-3600 Solvent rack with integrated vacuum degasser
- HPG-3400RS Binary gradient pump with 400 µL static mixer kit
- WPS-3000RS Split loop sampler with 100 µL sample loop
- TCC-3000RS Thermostatted column compartment
- DAD-3000RS Diode array detector, 5 µL flow cell

Chromeleon® Chromatography Data System, Version 6.80 SR7

REAGENTS AND STANDARDS

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

Acetonitrile (CH_3CN), LAB-SCAN

Di-ammonium hydrogen phosphate ($(NH_4)_2HPO_4$), Ajax

8 N Potassium hydroxide solution (KOH), KANTO

Tartrazine ($C_{16}H_9N_4Na_3O_9S_2$), Fluka

Amaranth ($C_{20}H_{11}N_2Na_3O_{10}S_3$), Fluka

Indigo Carmine ($C_{16}H_8N_2Na_2O_8S_2$), Fluka

New Coccine ($C_{20}H_{11}N_2Na_3O_{10}S_3$), Fluka

Sunset Yellow FCF ($C_{16}H_{10}N_2Na_2O_7S_2$), Fluka

Fast Green FCF ($C_{37}H_{34}N_2Na_2O_{10}S_3$), Fluka

Eosin Y ($C_{20}H_6Br_4Na_2O_5$), Fluka

Erythrosin B ($C_{20}H_6I_4Na_2O_5$), Fluka

Phloxine B ($C_{20}H_2Br_4C_{l4}Na_2O_5$), Fluka

Bengal Rose B ($C_{20}H_2Cl_4I_4Na_2O_5$), Fluka

Brilliant Blue ($C_{37}H_{34}N_2Na_2O_9S_3$), Fluka

CHROMATOGRAPHIC CONDITIONS

Column:	Acclaim PA2, 3 µm, 3 × 75 mm (P/N 066277)
Mobile Phase:	A) 20 mM $(NH_4)_2HPO_4$ pH 8.8 B) 50% 20 mM $(NH_4)_2HPO_4$ pH 8.8 in CH_3CN (v/v)
Flow Rate:	0.709 mL/min
Gradient:	12% B from –3 to 0.00 min, ramp to 100% B in 3.5 min, hold 100% B for 1.0 min and return to 12% B in 0.1 min.
Column Temp.:	30 °C
Inj. Volume:	3 µL
Detection:	UV 254 nm and wavelength scanning 200–800 nm
System Backpressure:	2100 psi

PREPARATION OF SOLUTIONS AND REAGENTS

Eluent A [20 mM $(NH_4)_2HPO_4$ pH 8.8]

Weigh 2.64 g di-ammonium hydrogen orthophosphate in a 250 mL beaker. After dissolving with deionized water (used for all eluent and sample preparation), transfer to a 1 L volumetric flask. Add 850 µL of 8 N sodium hydroxide, dilute to 1 L with water, and mix. Filter with a 0.45 µm filter before use.

Eluent B [50% 20 mM $(NH_4)_2HPO_4$ pH 8.8 in CH_3CN]

Mix eluent A and CH_3CN 1:1. Filter with a 0.45 µm filter before use.

Standard Solutions

All 1000 mg/L stock standard solutions were prepared separately in water and used to prepare four mixtures of the 10 dyes that were the working standards for method calibration. The dye concentrations in the working standard solutions are shown in Table 3.

Sample Preparation

All samples were purchased from a supermarket in Bangkok, Thailand.

Table 1. Samples

Sample	Designation	Color
Electrolyte sports drink	1	Yellow
	2	Yellow
	3	Orange
Carbonated drink	1	Orange
	2	Orange
	3	Green
Gelatin dessert	1	Red

The electrolyte sports drinks were filtered with a 0.45 µm filter before analysis. The carbonated drinks were placed in an ultrasonic bath for 5 min to degas, then diluted with water (1:2 for sample 1 and 1:1 for samples 2 and 3), and filtered with a 0.45 µm filter. Then, half a gram of the gelatin dessert was placed in a 25 mL beaker, mixed with 7 mL water, and placed in hot water for 5 min or until it completely dissolved. After cooling to room temperature, the sample was transferred to a 10 mL volumetric flask, and diluted to 10 mL with water.

RESULTS AND DISCUSSION

Figure 1 shows the separation of a mixture of 10 dyes, including the US FDA-permitted food dyes Tartrazine, Sunset Yellow, Fast Green, and Erythrosine, in less than 5 min. This separation uses an ammonium phosphate/acetonitrile eluent at pH 8.8, a pH value that would pose a problem for most silica-based, reversed-phase columns. The Acclaim PA2 column is tolerant of this high pH. The separation is achieved in less than 5 min by using a 3 µm particle size and a 3 × 75 mm column format.

When the food dye Brilliant Blue was added to the standard mixture, complete resolution between Fast Green and Brilliant Blue was not achieved. Though few food samples will contain both dyes, a method for the fast separation of the 10 dyes (listed in Figure 1) and Brilliant Blue using the Acclaim PA column is presented in Figure 2. Similar to the Acclaim PA2 column, the PA column provides a polar-embedded phase that can be used to separate compounds with a wide range of hydrophobicities. Figure 2 shows that Brilliant Blue is resolved from Fast Green (peaks 6 and 7). Because the Acclaim PA column does not have the high pH tolerance of the PA2 column, the eluent pH was lowered to 7.3.

The separation on the Acclaim PA column is also less than 5 min because it uses the 3 µm particle size resin and the 3 × 75 mm column format. The eluents used in both the PA and PA2 separations are compatible with MS detection. In both figures, the dyes are detected at 254 nm. Given that both these dyes absorb at other wavelengths, a more selective wavelength can be chosen for each dye. The PA2 separation was used for the rest of the analysis but both methods can be used to analyze the samples. Because the last compound elutes at about 0.5 min earlier on the PA2 column and the resolution between peaks 2 and 3 is better, it is possible to make the PA2 separation slightly faster than the PA separation using the 3 x 75 mm column format, but this was not evaluated.

Spectral scanning was used for the analysis of the standard mix (Figure 1). Table 2 displays the match and PPI values from the spectral scanning. The high match values suggest that the peaks are pure and the peak spectra were loaded in the spectral library for use in identifying dye peaks in samples. Table 2 also displays resolution values, with no resolution values less than 2.8.

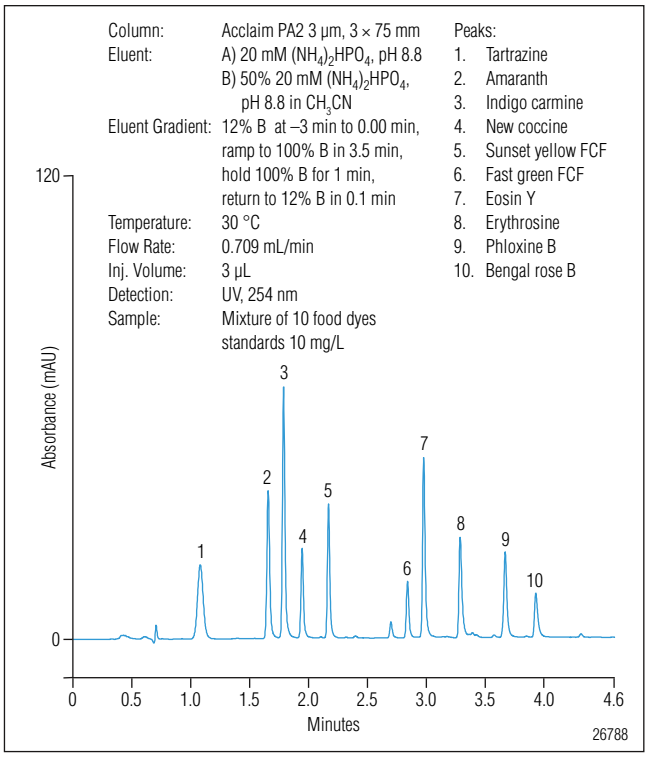

Figure 1. Chromatogram of the standard mixture of 10 dyes.

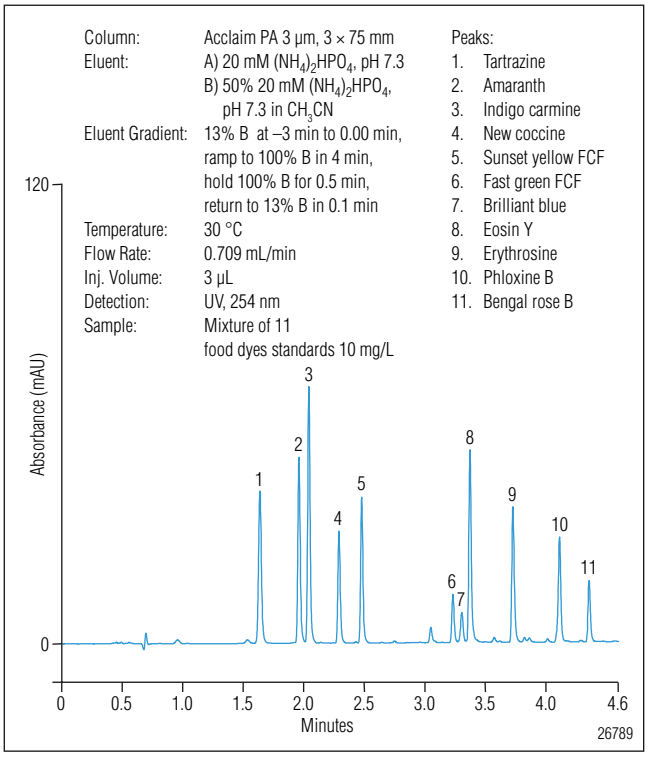

Figure 2. Chromatogram of the standard mixture of 11 dyes.

Table 2. Resolution and Peak Purity Results					
Component Name	Resolution (USP)	Match	%RSD Match	PPI (nm)	%RSD PPI
Tartrazine	8.69	998	3.55	335.8	1.05
Amaranth	3.26	1000	0.10	318.0	0.03
Indigo Carmine	4.18	999	3.19	360.9	0.88
New Coccine	6.12	1000	0.52	324.5	0.16
Sunset Yellow FCF	5.78	1000	0.25	328.7	0.08
Fast Green FCF	3.53	1000	0.56	563.6	0.10
Eosin Y	7.41	1000	0.32	440.7	0.07
Erythrosin	2.83	1000	0.15	441.8	0.03
Phloxine B	5.46	1000	0.74	437.8	0.17
Bengal Rose B	8.05	1000	0.56	439.2	0.13

METHOD CALIBRATION

Prior to sample analysis, the method was calibrated for each of the 10 dyes between 1 and 30 mg/L. Four concentrations, 1, 10, 20, and 30 mg/L, were used and the curves were forced through the origin. Table 3 shows that for this range, calibration for each of the 10 dyes was linear.

Table 3. Standard Calibration Results							
Compound	Calibration Standard Concentration (mg/L)				Cal. Type	Coeff. Det × 100%	Slope
	Level 1	Level 2	Level 3	Level 4			
Tartrazine	1	10	20	30	Lin	99.9970	0.1090
Amaranth	1	10	20	30	Lin	99.9973	0.1067
Indigo Carmine	1	10	20	30	Lin	99.9383	0.1692
New Coccine	1	10	20	30	Lin	99.9969	0.0574
Sunset Yellow FCF	1	10	20	30	Lin	99.9933	0.0811
Fast Green FCF	1	10	20	30	Lin	99.9959	0.0361
Eosin Y	1	10	20	30	Lin	99.9989	0.1197
Erythrosin	1	10	20	30	Lin	99.9991	0.0756
Phloxine B	1	10	20	30	Lin	99.9979	0.0667
Bengal Rose B	1	10	20	30	Lin	99.9921	0.0375

SAMPLE ANALYSIS

Seven samples were purchased from a local supermarket for analysis. Three different electrolyte sports drinks, two yellow and one orange, were analyzed (Table 1). Three carbonated drinks, two orange and one green, were also analyzed. The seventh sample was a red gelatin dessert. All samples were labeled to contain a dye, but none listed the dye or dyes used. Figures 3–9 show the chromatography for each sample. Using the spectral library and retention time, the two yellow sports drinks were found to contain Tartrazine (Figures 3 and 4). The samples were found to have similar concentrations of the dye (Table 4). The same approach identified the allowed food dye Sunset Yellow FCF in the orange sports drink (Figure 5). The dye was found in both orange carbonated drinks (Figures 6 and 7). The green carbonated drink contained two dyes, Tartrazine and Fast Green FCF (Figure 8). More importantly, the red dye New Coccine was found in the red gelatin dessert (Figure 9). This dye is banned for use in foods in the United States and some other countries.

All samples were spiked with the standard or standards identified by spectral matching and retention time to assess the accuracy of the determination. Table 4 shows the concentration of each dye in each sample, the spectral match, and the results of the spiking studies. Good recoveries were observed in each sample, suggesting that the method is accurate. Method reproducibility was evaluated by making five injections of each sample and each spiked sample, and concentrations of dyes in each sample were determined. Table 5 shows that the method has good short-term reproducibility.

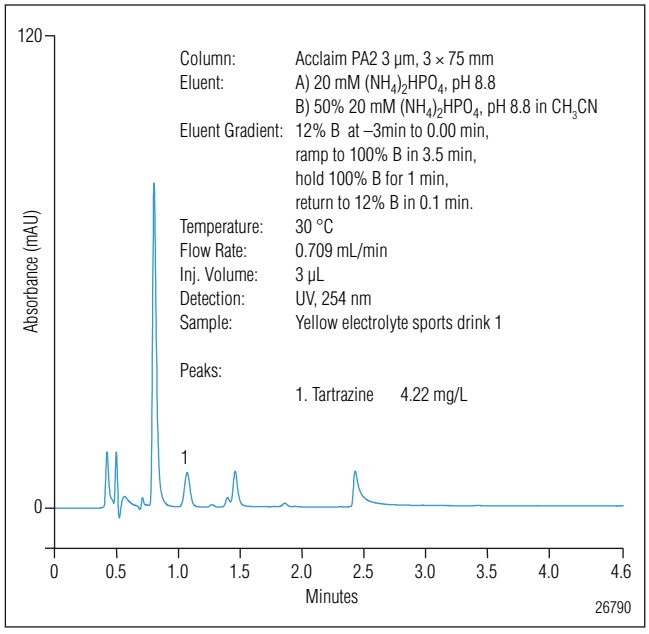

Figure 3. Chromatogram of electrolyte sports drink 1.

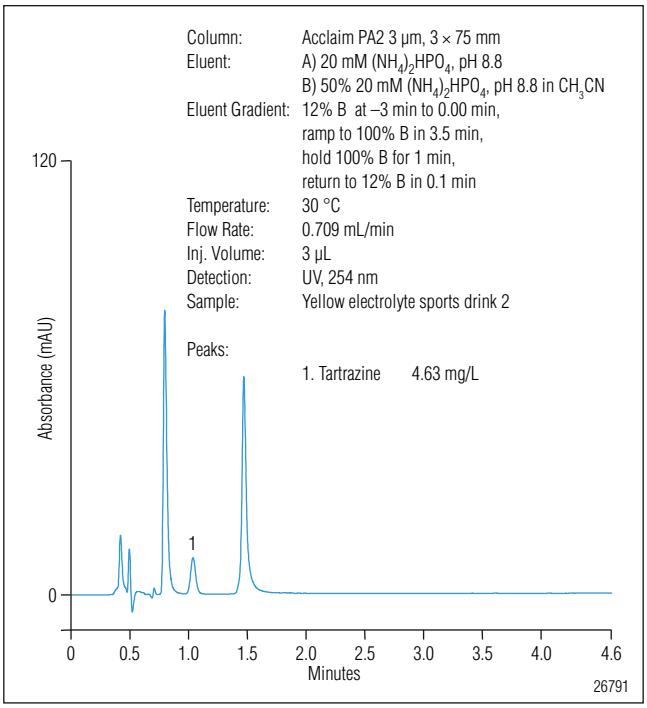

Figure 4. Chromatogram of electrolyte sports drink 2.

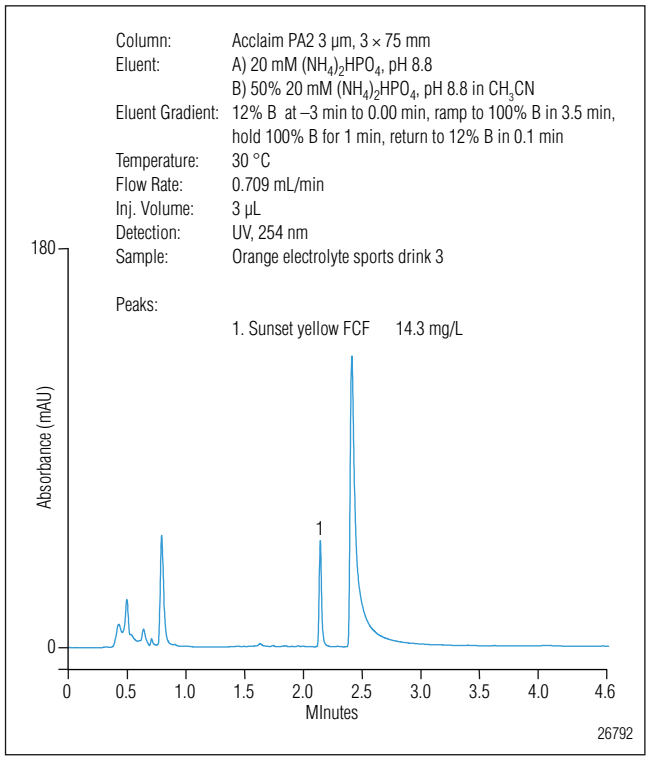

Figure 5. Chromatogram of electrolyte sports drink 3.

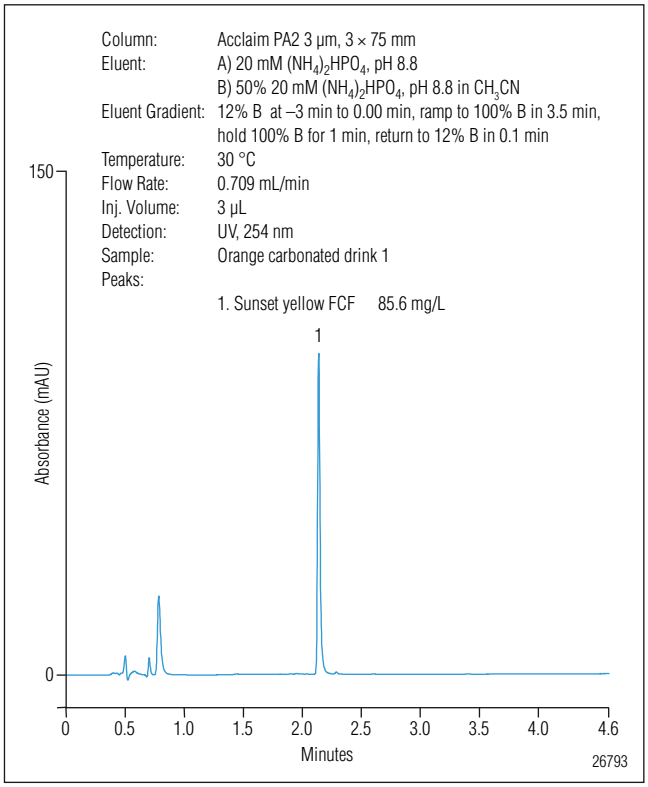

Figure 6. Chromatogram of carbonated drink 1.

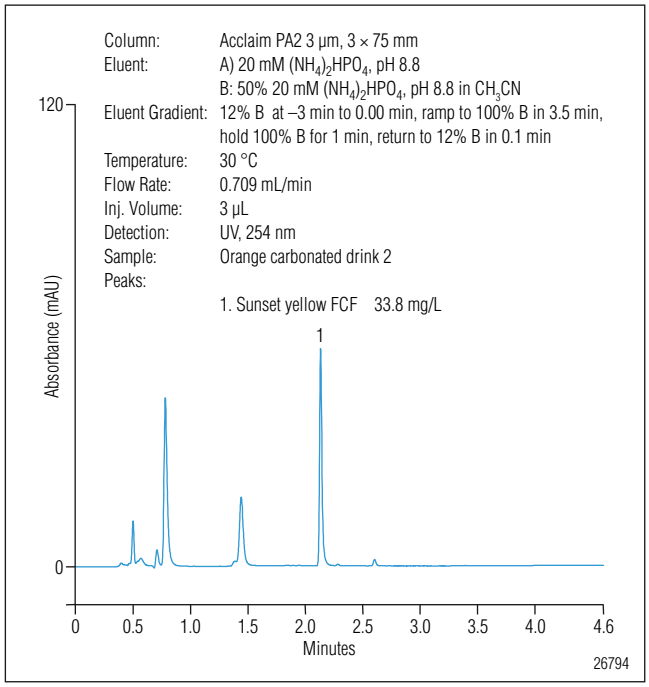

Figure 7. Chromatogram of carbonated drink 2.

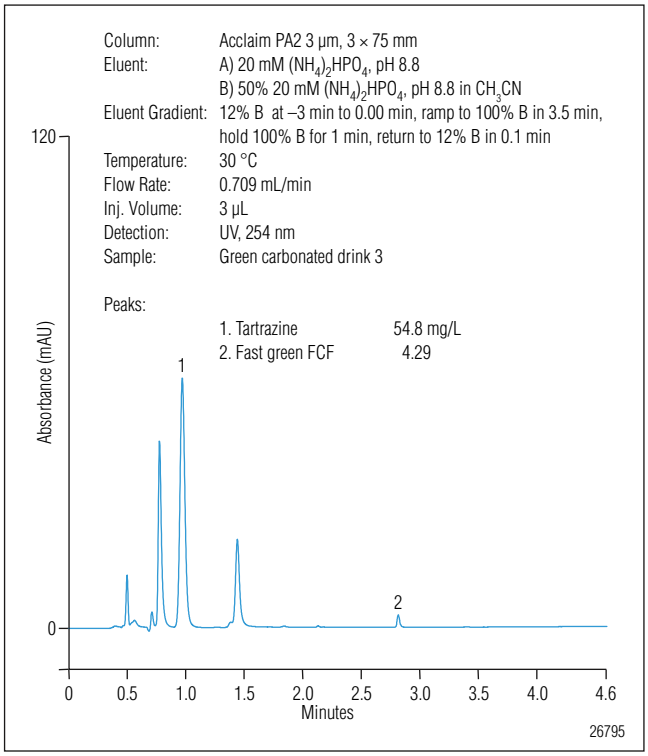

Figure 8. Chromatogram of carbonated drink 3.

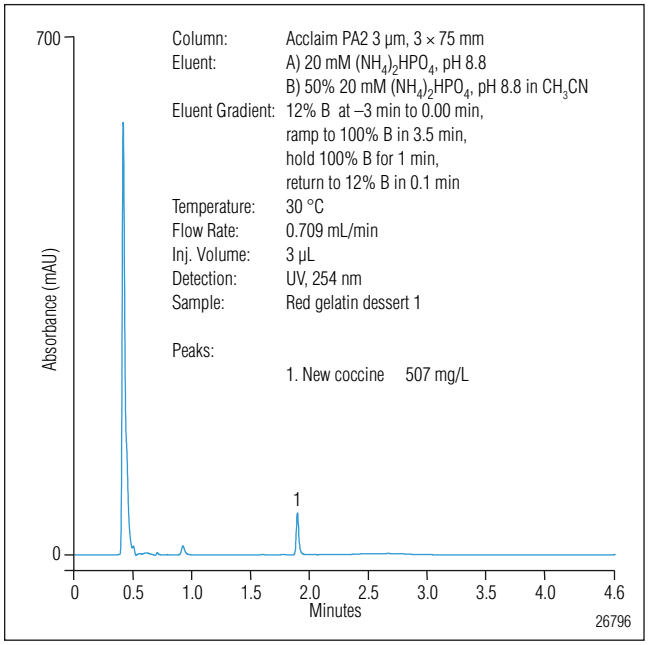

Figure 9. Chromatogram of the red gelatin dessert sample.

FASTER ANALYSIS

While the method presented in this application note is fast, it is possible to make it faster. Figure 10 shows that by switching to a shorter column with a smaller particle size, it is possible to reduce the separation time from 4.5 min to 2.5 min while still resolving all 10 dyes. To accomplish this, the injection volume was reduced to 1 µL, the 400 µL static mixer was replaced with a 200 µL static mixer (P/N 6040.5150), and the flow cell was changed to a semi-micro 2.5 µL flow cell. The data collection rate was also set to 25 Hz and the response time to 0.5 s. The backpressure of this separation was 3150 psi.

Table 4. Sample and Recovery Results

Sample	Color	Dye Spiked into the Sample	Spiked Conc. (mg/L) Added to Sample	Average Dye Concentration in Sample (mg/L)	Average Dye Concentration in Spiked Sample (mg/L)	%Recovery	Peak Purity Match	Match with Spectra Library
Electrolyte sports drink 1	Yellow	Tartrazine	1	4.22	5.18	96.0	997	996
Electrolyte sports drink 2	Yellow	Tartrazine	1	4.63	5.57	94.0	999	996
Electrolyte sports drink 3	Orange	Sunset yellow FCF	4	14.3	18.3	100	1000	1000
Carbonated drink 1	Orange	Sunset yellow FCF	10	85.6	93.8	82	1000	1000
Carbonated drink 2	Orange	Sunset yellow FCF	10	33.8	43.3	95.0	1000	1000
Carbonated drink 3	Green	Tartrazine	10	54.8	63.1	83.0	1000	997
Carbonated drink 3		Fast Green FCF	2	4.29	6.12	91.5	1000	999
Gelatin dessert	Red	New Coccine	40	507	545	95.0	1000	1000

Table 5. Reproducibility of Five Injections of Samples and Spiked Samples								
Sample	Color	Dyes Found in Samples and Spiked Samples	Concentrations Found in Samples and Spiked Samples (mg/L)					RSD
			Injection #					
			1	2	3	4	5	
Electrolyte sports drink 1	Yellow	Tartrazine in sample	4.20	4.27	4.21	4.21	4.21	0.72
		Tartrazine in spiked sample	5.19	5.16	5.20	5.17	5.17	0.31
Electrolyte sports drink 2	Yellow	Tartrazine in sample	4.62	4.64	4.63	4.62	4.62	0.17
		Tartrazine in spiked sample	5.58	5.56	5.56	5.57	5.56	0.17
Electrolyte sports drink 3	Orange	Sunset yellow FCF in sample	14.3	14.3	14.3	14.3	14.3	0.13
		Sunset yellow FCF in spiked sample	18.3	18.3	18.3	18.3	18.3	0.11
Carbonated drink 1	Orange	Sunset yellow FCF in sample	85.6	85.7	85.6	85.7	85.5	0.10
		Sunset yellow FCF in spiked sample	93.7	93.8	94.0	93.7	93.8	0.14
Carbonated drink 2	Orange	Sunset yellow FCF in sample	33.8	33.8	33.7	33.7	33.8	0.13
		Sunset yellow FCF in spiked sample	43.3	43.3	43.3	43.2	43.3	0.10
Carbonated drink 3	Green	Tartrazine in sample	54.8	54.8	54.8	54.7	54.8	0.10
		Tartrazine in spiked sample	63.0	63.1	63.0	63.3	63.1	0.19
		Fast Green FCF in sample	4.29	4.33	4.32	4.27	4.23	0.95
		Fast Green FCF in spiked sample	6.09	6.14	6.12	6.15	6.12	0.39
Gelatin dessert	Red	New Coccine in sample	507	506	507	507	507	0.08
		New Coccine in spiked sample	546	546	544	543	545	0.23

Note: The results were multiplied by the appropriate dilution factor in the Chromeleon software.

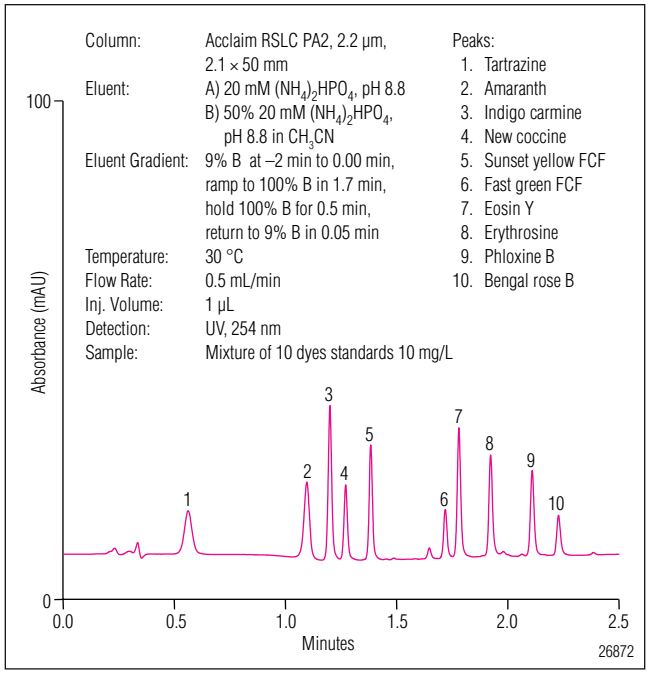

Figure 10. Faster separation of the 10 dyes standard.

CONCLUSION

This application note presents a fast HPLC method (< 5 min) for the accurate determination of dyes in food and beverage samples. This method can be used to quantify permitted dyes and identify illegal dyes in food and beverage samples.

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.

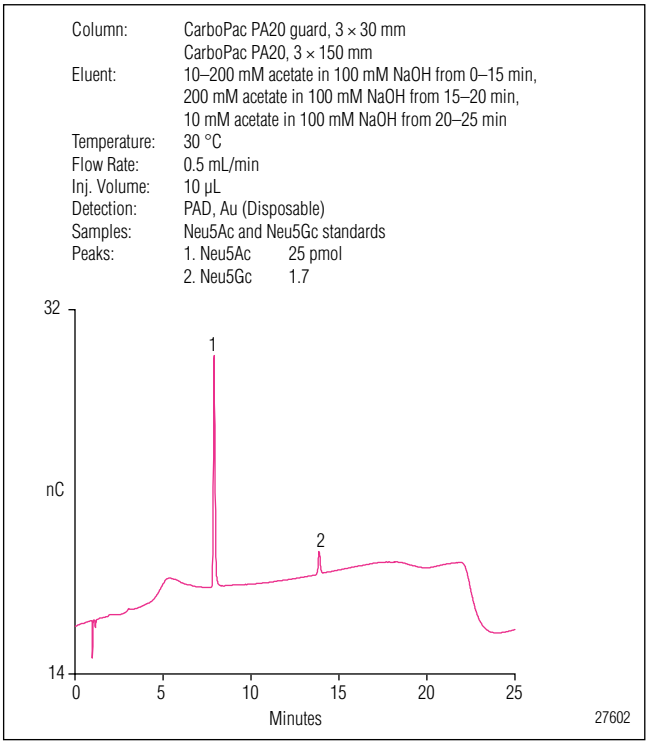

Figure 1. Separation of sialic acids on the CarboPac PA20 column.

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.

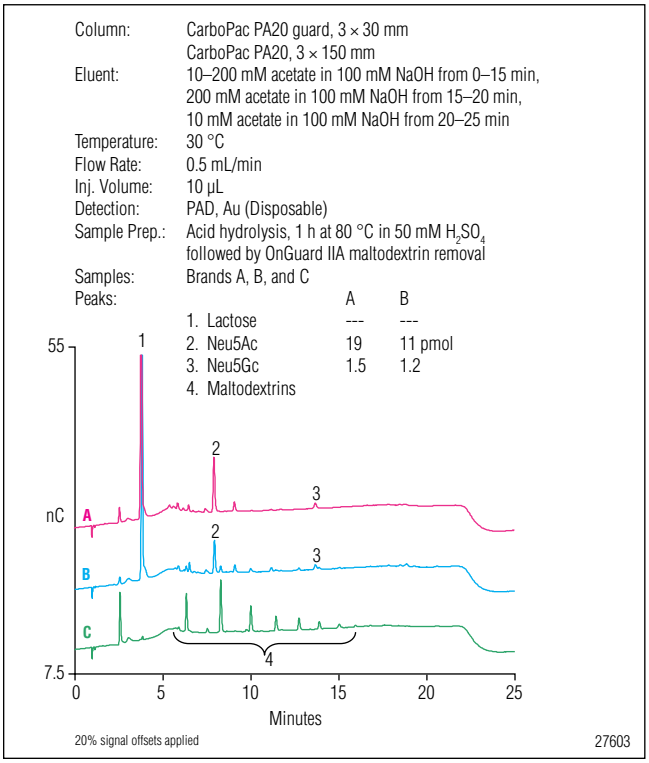

Figure 2. Separation of anion-exchange resin prepared infant formula samples based on A) dairy, B) dairy with added maltodextrins, and C) soy with added maltodextrins.

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.

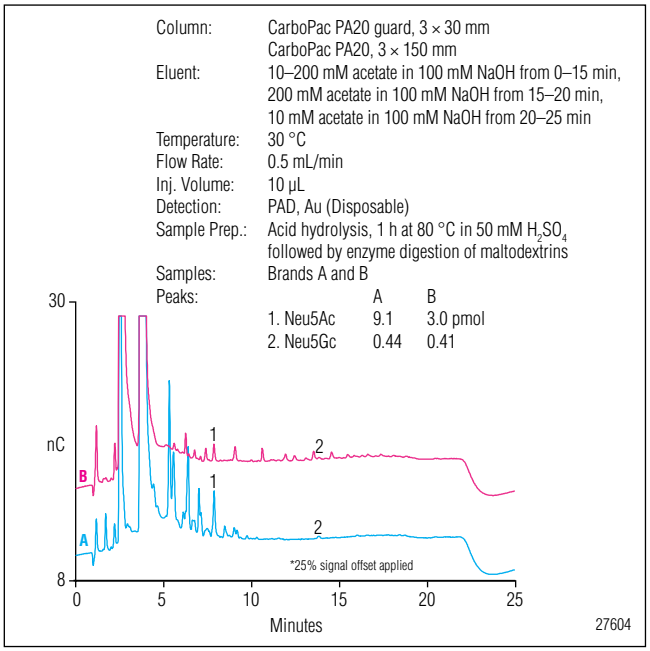

Figure 3: Separation of enzyme digestion prepared infant formula samples based on A) dairy, B) dairy with added maltodextrins. Soy-based formula maltodextrins are not well digested by amyloglycosidase and a chromatogram is not shown.

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.

REFERENCES

Wang, B.; Brand-Miller, J. The Role and Potential of Sialic Acid in Human Nutrition. *European J. of Clin. Nutr.* **2003**, *57*, 1351–1369.

Wang, B.; Brand-Miller, J.; McVeigh, P.; Petocz, P. Concentration and Distribution of Sialic Acid in Human Milk and Infant Formulas. *Am. J. Clin. Nutr.* **2001**, *74*, 510–515.

Lacombe, R.; Salcedo, J.; Algeria, A.; Lagarda, M.J.; Barbera, R.; Mantencio, E. Determination of Sialic Acid and Gangloisides in Biological Samples and Dairy Products. *J. Pharm. and Biomed. Anal.* **2010**, *51*, 346–357.

Rohrer, J.S. Analyzing Sialic Acids Using High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection. *Anal. Biochem.* **2000**, *283*, 3–9.

Dionex Corporation, *Improved Long-Term Stability of N-Acetylneuraminic Acid and N-Glycolylneuraminic Acid Peak Area Responses Using Waveform A, a Quadruple Potential Waveform*. Application Update 141, LPN 125, 2000, Sunnyvale, CA.

Dionex Corporation, *Eluent Preparation for High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection.* Technical Note 71, LPN 1932-01, 2007, Sunnyvale, CA.

Dionex Corporation, Product Manual: *OnGuard II Cartridges.* Document No. 031688-06, 2004, Sunnyvale, CA.

Martín, M.J.; Vázquez, E.; Rueda, R. Application of a Sensitive Fluorometric HPLC Assay to Determine the Sialic Acid Content of Infant Formulas, *Anal. Bioanal. Chem.* **2007**, *387,* 2943–2949.

Oh, J.; Kim, J. Preparation and Properties of Immobilized Amyloglucosidase on Nonporous PS/PNaSS Microspheres. *Enzyme and Microbial Technology* **2000**, *27,* 356–361.

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.

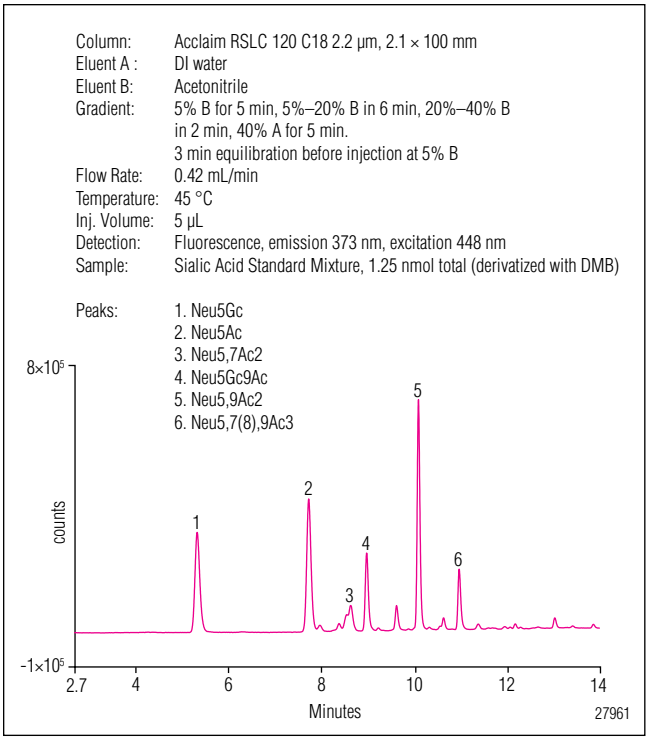

Figure 1. Separation of a derivatized sialic acid standard mixture on the Acclaim RSLC 120 C18 column.

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.

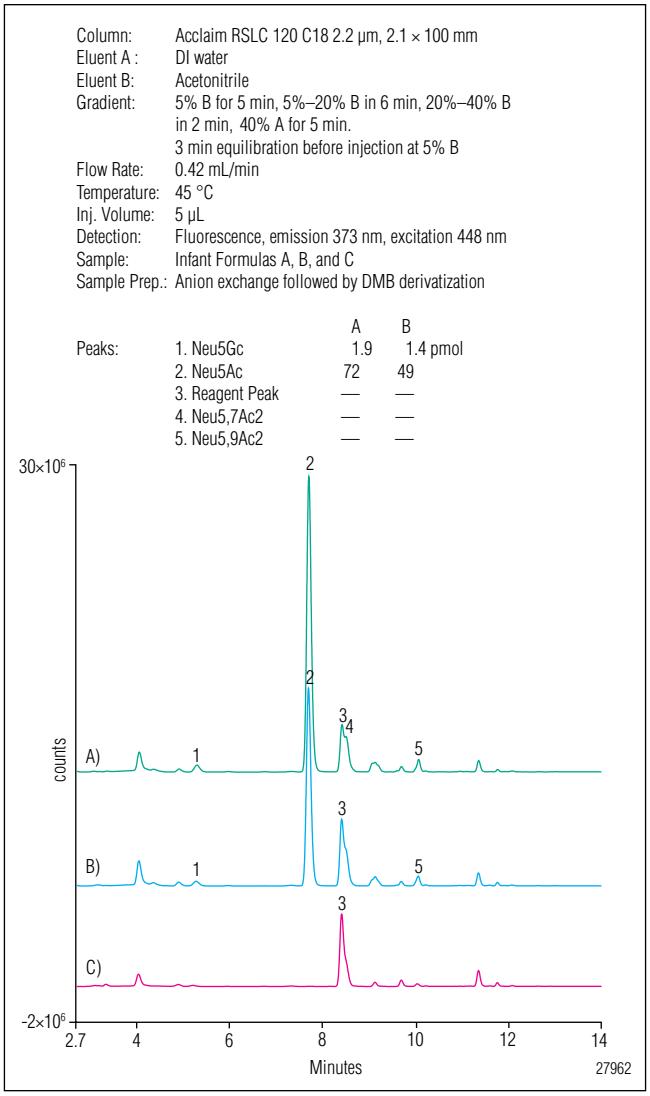

Figure 2. Determination of sialic acids in infant formulas on the Acclaim RSLC 120 C18 column.

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

REFERENCES

Wang, B.; Brand-Miller, J. The Role and Potential of Sialic Acid in Human Nutrition. European *J. of Clin. Nutr.,* **2003,** *57,* 1351–1369.

Wang, B.; Brand-Miller, J.; McVeigh, P.; Petocz, P. Concentration and Distribution of Sialic Acid in Human Milk and Infant Formulas. *Am. J. Clin. Nutr.* **2001,** *74,* 510–515.

Lacombe, R.; Salcedo, J.; Algeria, A.; Lagarda, M.J.; Barbera, R.; Mantencio, E. Determination of Sialic Acid and Gangliosides in Biological Samples and Dairy Products. *J. Pharm. and Biomed. Anal.* **2010,** *51,* 346–357.

Rohrer, J.S. Analyzing Sialic Acids Using High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection. *Anal. Biochem.* **2000,** *283,* 3–9.

Hara, S.; Yamaguchi, M.; Takemori, Y.; Nakamura, M. Highly Sensitive Determination of *N*-Acetyl-and *N*-Glycolylneuraminic Acids in Human Serum and Urine and Rat Serum by Reversed-Phase Liquid Chromatography with Fluorescence Detection. J. *Chromatogr., B: Biomed.* **1989,** *377,* 111–119.

Hara, S.; Yamaguchi, M.; Takemori, Y.; Furuhata, K.; Ogura, H.; Nakamura, M. Determination of Mono-*O*-Acetylated *N*-Acetylneuraminic Acids in Human and Rat Sera by Fluorometric High-Performance Liquid Chromatography. *Anal. Biochem.* **1989,** *179,* 162–166.

Martín, M.J.; Vázquez, E.; Rueda, R. Application of a Sensitive Fluorometric HPLC Assay to Determine the Sialic Acid Content of Infant Formulas. *Anal. Biomed. Chem.* **2007,** *387,* 2943–2949.

Spichtig, V.; Michaud, J.; Austin, S. Determination of Sialic Acids in Milks and Milk-Based Products. *Anal. Biochem.* **2010,** *405,* 28–40.

Klein, A.; Diaz, S.; Ferreira, I.; Lamblin, G.; Roussel, P.; Manzi, A.E. New Sialic Acids from Biological Sources Identified by a Comprehensive and Sensitive Approach: Liquid Chromatography-Electrospray Ionization-Mass Spectrometry (LC-ESI-MS) of SIA Quinoxalinones. *Glycobiology* **1997,** *7,* 421–432.

Dionex Corporation, *Product Manual for OnGuard II Cartridges;* Document No. 031688-06, Sunnyvale, CA, 2004.

Dionex Corporation, *HPAE-PAD Determination of Infant Formula Sialic Acids.* Application Note 253, LPN 2561, Sunnyvale, CA, 2010.

Two-Dimensional HPLC Combined with On-Line SPE for Determination of Sudan Dyes I–IV in Chili Oil

INTRODUCTION

Sudan dyes belong to a family of industrial dyes normally used for coloring plastics and other synthetic materials. Although use of these dyes in food is restricted, they are nevertheless sometimes added to foods to improve the appearance and command a higher price. Because Sudan dyes may create health problems such as genotoxic and carcinogenic effects, concerns over contamination of Sudan dyes in chili oil, powder, other spices, and baked foods have promoted increased awareness and testing for these compounds.[1] The typical adulterants are Sudan dyes I, II, III, and IV (structures shown in Figure 1).

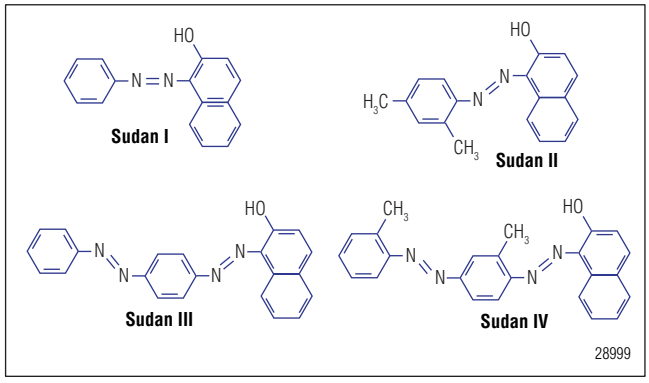

Figure 1. Structures of Sudan dyes I, II, III, and IV.

Reversed-phase high-performance liquid chromatography (RP-HPLC) is one of the preferred methods for separating Sudan dyes, and the analysis of Sudan dyes by RP-HPLC has been published by the European Union (EU) with a detection limit of 13 µg/L for Sudan dye I,[2] and by the Chinese government with a detection limit of 10 µg/L for Sudan dyes I–IV.[3]

The determination of Sudan dyes in complex matrices (e.g., chili oil) often requires extensive sample preparation prior to HPLC analysis. Due to the fat-solubility of Sudan dyes,[4] extraction with organic solvents such as acetonitrile, methanol, n-hexane, cyclohexane, and petroleum ether is typically used. Some supplementary means (e.g., ultrasound-assisted [5] and microwave-assisted [6] extractions) are applied to improve the extraction efficiency. The procedure following extraction is cleanup, which is the bottleneck for the sensitive determination of Sudan dyes in chili oil. Solid-phase extraction (SPE),[3] gel permeation chromatography,[7] thin-layer chromatography,[8] and dispersive solid-phase extraction[9] have been reported for cleanup.

Here, a two-dimensional HPLC with on-line SPE intercolumn trapping method was developed for fast, effective determination of Sudan dyes I–IV in chili oil. Following extraction with methylene dichloride and acetonitrile, the analytes are separated in the first dimension and removed selectively to be absorbed on an SPE column. This eliminates numerous matrix interferences. When analyte trapping is complete on the SPE column, it is switched into the flow path of the second dimension, where the absorbed dyes are eluted from the SPE column, separated, and detected. The 2-D HPLC with on-line SPE intercolumn trapping system runs automatically on the Thermo Scientific Dionex UltiMate™ 3000 HPLC dual-pump system controlled by Thermo Scientific Dionex Chromeleon™ software, and provides the advantages of full automation, absence of operator influence, and strict process control compared to a typical off-line SPE method.[3]

EQUIPMENT

Dionex UltiMate 3000 HPLC system including:

- DGP-3600 RS Pump with SRD 3600 solvent rack with degasser
- WPS-3000T RS Autosampler
- TCC-3000 Thermostatted column compartment equipped with two 2p-10p valves
- DAD-3000 RS UV-vis Detector
- LPG-3400 Pump (for dilution)

Thermo Scientific MSQ Plus™ mass detector with electrospray ionization (ESI) source

Dionex Chromeleon software, Version 6.80, SR9 or higher

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

IKA® MS1 Minishaker, IKA Works, Guangzhou, China

REAGENTS

Deionized (DI) water, Milli-Q® Gradient A10, Millipore Corporation

Acetonitrile (CH_3CN) HPLC grade (Cat.# AC6100-0040) Fisher Chemical

Methanol (CH_3OH), HPLC grade, (Cat.# AC61009-0040) Fisher Chemical

Tetrahydrofuran (THF), HPLC grade, SCRC, China

Formic acid (FA), HPLC grade, SCRC, China

Methylene dichloride (CH_2Cl_2), analytical grade, SCRC, China

STANDARDS

Sudan I (CAS 842-07-9), Sudan II (CAS 3118-97-6), Sudan III (CAS 85-86-9), and Sudan IV (CAS 85-83-6) were purchased from Sigma-Aldrich.

Prepare stock standard solutions with concentrations of 3 µg/mL for Sudan I and IV, and 1 µg/mL for Sudan II and III by dissolving the appropriate amount of standards in acetonitrile. Prepare five working standard solutions ranging from 0.5 to 60 µg/L for the calibration by adding the proper amount of stock standard solution and making dilutions with water.

SAMPLE PREPARATION

The chili oil sample was donated by a customer.

Weigh 1 g of chili oil and place in a 100 mL volumetric flask, then add 20 mL CH_2Cl_2. After 5 min of vortex shaking and 30 min in an ultrasonic bath, bring to volume with acetonitrile. Return the flask to the bath for 15 min, then transfer 10 mL of the mixture to a 10 mL centrifuge tube. Centrifuge for 10 min (rpm ≥10,000), decant the acetonitrile layer, and filter through a 0.45 µm membrane (Millex-LH®) before injection.

CONDITIONS

Chromatographic Conditions

Analytical Column 1:	Thermo Scientific Acclaim™ PolarAdvantage II (PA2), 3 µm, 3.0 × 150 mm (P/N 063705)
Analytical Column 2:	Acclaim RSLC 120 C18 column, 2.2 µm, 2.1 × 100 mm (P/N 068982)
On-Line SPE Column:	Acclaim 120 C18, 5 µm, Guard Cartridge, 4.6 × 10 mm (P/N 069580), use V-2 holder
Mobile Phase:	For the separation on analytical column 1 (the first dimension) A: DI Water B: CH_3CN C: CH_3OH/THF, 1:1 (v/v) In gradient (Table 1) For on-line SPE 0.1% FA in DI water, isocratic For the separation on analytical column 2 (the second dimension) A: DI Water B: CH_3CN C: 0.1% FA in CH_3CN in gradient (Table 1)

Valve-Switching:	Table 1
Flow Rate:	For analytical column 1 0.6 mL/min For on-line SPE column 1.0 mL/min For analytical column 2 0.3 mL/min
Inj. Volume:	20 µL on analytical column 1
Column Temp.:	30 °C
Detection:	Absorbance at 500 nm

MSQ-Plus Mass Detector Conditions

Ionization Mode:	ESI
Operating Mode:	Positive Scan
Probe Temp.:	450 °C
Needle Voltage:	4000 V
SIM Mode:	249 *m/z* for Sudan dye I 277 *m/z* for Sudan dye II 353 *m/z* for Sudan dye III 381 *m/z* for Sudan dye IV
Dwell Time:	0.2 sec
Cone Voltage:	35 V for Sudan dyes I and II 50 V for Sudan dyes III and IV
Nebulizer Gas:	Nitrogen at 75 psi

Table 1. Gradients and Valve Switching for On-Line SPE Two-Dimensional HPLC

Time (min)	Right Pump (for the First Dimension)				Left Pump (for the Second Dimension)				Pump for Dilution (for On-Line SPE)		Valve Switching		Detector	
	Flow Rate (mL/min)	Solvent A H_2O (% vol.)	Solvent B CH_3CN (% vol.)	Solvent C CH_3OH/THF 1:1 v/v (% vol.)	Flow Rate (mL/min)	Solvent A H_2O (% vol.)	Solvent B CH_3CN (% vol.)	Solvent C CH_3CN-0.1%FA (% vol.)	Flow Rate (mL/min)	Solvent 0.1%FA in H_2O (% vol.)	Right	Left	UV-vis	MS
-3.50		30	50	20		40	50	10			10_1	10_1		
-0.50		30	50	20							1_2	10_1		
0.00													AcqOn	
4.72											10_1			
5.00											1_2			
5.50		0	50	50										
5.94											10_1			
6.13											1_2			
6.78											10_1			
6.96											1_2			
7.00						40	50	10						
7.53	0.6				0.3				1.0	100%	10_1			
7.70											1_2			
8.00												1_2	AcqOff	
11.00		0	20	80										
12.00														Start.On Duration =10.00
14.00		0	20	80										
14.10		30	50	20										
16.00						0	90	10						
22.40						0	90	10						
22.50						40	50	10						
23.00		30	50	20		40	50	10						

RESULTS AND DISCUSSION

Evaluation of Sample Preparation

The sample preparation method typically used for HPLC analysis of Sudan dyes in chili oil includes two steps: extraction and cleanup. The official EU Method 03/99 uses acetonitrile to extract Sudan dyes I and II, and CH_2Cl_2 to extract Sudan dyes III and IV from chili oil.[2] Experiments leading to the methods described here also demonstrate that the recoveries of Sudan dyes I and II obtained using CH_3CN as extractant were good, whereas recoveries of Sudan dyes III and IV were poor. Thus, CH_2Cl_2 and CH_3CN were used for the extraction. Vortex shaking and use of an ultrasonic bath can improve the extraction efficiency.

For the cleanup step, the Chinese GB Method recommends using activated alumina (Al_2O_3) the stationary phase to absorb Sudan dyes I–IV;[3] however, the use of activated alumina may result in poor method reproducibility due to the facile reaction of alumina with Sudan dye I, which is usually used as an indicator to evaluate the alumina activity. Therefore, an efficient cleanup method with the advantages of full automation, absence of operator influence, and strict process control is required. This can be achieved using the design shown in Figure 2. The cleanup step using on-line SPE combined with a two-dimensional separation is run automatically on the Dionex UltiMate 3000 HPLC dual-pump system controlled by the Dionex Chromeleon Chromatography Data System software.

Configuration of the Two-Dimensional HPLC with On-Line SPE Intercolumn Trapping System

Figure 2 shows the flow scheme of a two-dimensional HPLC with on-line SPE intercolumn trapping system with two detectors—UV-vis and mass spectrometry (MS)—for method development and determination of Sudan dyes, respectively. This configuration requires two 2-position 10-port valves for column switching and use of the two detectors. Connect column 2 to position 9 on the left valve for the method development. With this connection, the separation of analytes on columns 1 and 2 can be observed on the UV detector.

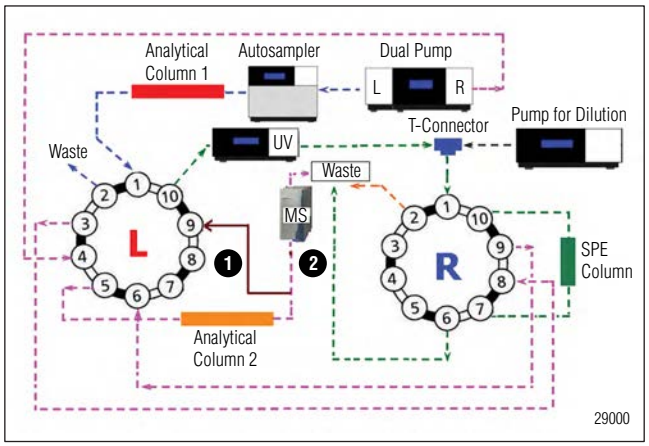

Figure 2. Flow schematics for a two-dimensional HPLC with on-line SPE intercolumn trapping system. Flow path 1 is configured for method development using a UV-vis detector; flow path 2 is configured for the determination of Sudan dyes in chili oil using an MS detector.

After method development, connect Column 2 directly to the MS detector. The two-dimensional HPLC with on-line SPE intercolumn trapping system includes an additional pump for delivering 0.1% FA into the SPE flow path through a T-connector to dilute the acetonitrile from the mobile phase in the first-dimension separation. This ensures absorption of the dyes to the SPE column. The pump runs at a constant flow rate during the entire process. While the processes of first-dimension separation (position 1-10 on left valve, and separation on analytical column 1) and on-line SPE (valve-switching between the positions 1-10 and 1-2 on right valve) are running, the second dimension (analytical column 2) is equilibrating. Before the front portion of the Sudan dye I (first analyte peak) elutes from the first analytical column, switch the SPE column into the first-dimension flow path (position 1-10 on right valve). As soon as dye I elutes from analytical column 1 and is absorbed onto the SPE column completely, switch the SPE column out of the first dimension (position 1-2 on right valve).

Removal and absorption of the other dyes follows the same protocol, so they are captured one by one on the SPE column. When the last analyte (Sudan dye IV) has been absorbed onto the SPE column completely, switch the SPE column into the second-dimension flow path (position 1-2 on both left and right valves) where the absorbed dyes are flushed from the SPE column, separated on analytical column 2, and detected with the MS detector. For method development, use a UV-vis detector connected to column 1 to determine valve-switching times. See Reference 10 for details.

Evaluation of the Two-Dimensional HPLC with On-Line SPE Intercolumn Trapping System

There are many applications of two-dimensional HPLC in the field of proteomics based on its high peak capacity for complex samples. Thermo Scientific Dionex dual-pump technology has many applications in this field.[12] A two-dimensional separation combined with on-line SPE between the two dimensions allows this technique to be applied to samples outside of proteomics. The key for this combination to be successful is to keep the on-line SPE trap working efficiently. Because the organic solvents (CH_3CN and CH_3OH) in the mobile phase from the first dimension make absorbing the Sudan dyes to the SPE column (Acclaim 120 C18) difficult, an additional pump is used to dilute the acetonitrile in the mobile phase from the first dimension with acidic aqueous (0.1% FA) (Figure 2). Use of 0.1% FA matches the mobile phase used in the second dimension, to which 0.1% FA is added as a component. Experiments show that using a dilution flow rate of either 1.0 or 1.2 mL/min yields satisfactory recovery of Sudan dyes spiked in the chili oil sample. Here, a 1.0 mL/min dilution flow rate was used.

A larger Acclaim PA2 column was used in the first dimension due to its ability to handle a large sample injection, and better selectivity for matrix removal. A smaller Acclaim 120 C18 column was used in the second dimension to obtain improved detection sensitivity and good resolution of Sudan dyes I–IV.[13,14] For the SPE column, an Acclaim 120 C18 Guard Cartridge was used, on which Sudan dyes I–IV are easily retained using an acidic aqueous solution, and easily eluted using organic solvent.

Addition of THF to the mobile phase can compress the natural pigments in capsicum products into a sharp band with retention longer than Sudan dyes I–IV;[15] for this reason, the authors suggest using THF for the first dimension separation.

Figure 3 shows chromatograms of Sudan dyes I–IV standards and the same standards spiked into chili oil samples with UV-vis detection under the chromatographic conditions specified above. The second half of the chromatogram (representing the second-dimension separation) shows efficient elimination of interferences by the on-line SPE two-dimensional HPLC system.

Method Precision, Linearity, and Detection Limits

Method precision was estimated using UV-vis detection by making five consecutive 20 µL injections of a chili oil sample spiked with 1.0 mg/L of each Sudan dye standard. The retention time and peak area reproducibilities are summarized in Table 2 and show good precision.

Calibration linearity for MS detection of Sudan dyes I–IV was investigated by making three consecutive injections of a mixed standard prepared at five different concentrations. The external standard method was used to establish the calibration curve and to quantify these dyes in the sample. Excellent linearity was observed from 0.5 to 60 µg/L when plotting the concentration versus the peak area, and the correlation coefficient was ≥ 0.9958 for each plot. The method detection limits of each Sudan dye for MS detection calculated by using S/N = 3 (signal to noise) were all ≤ 0.2 µg/L.

Sample Analysis

The customer who supplied a chili oil sample that tested positive for Sudan dyes requested confirmation of the types of dyes in the sample and their contents. Figure 4 shows total ion current (TIC) chromatograms of the chili oil sample and the same sample spiked with a mixed Sudan dye standard. Sudan dyes I, II, and III were identified in the chili sample. By comparing the calculated values of molecular weights with the theoretical values—249, 277, and 353, respectively—the identity of the three dyes was confirmed. Recoveries for each dye standard in the sample ranged from 67–97%. Table 3 reports the data for quantitative sample analysis as automatically calculated by the Dionex Chromeleon software.

CONCLUSION

The work shown here describes a two-dimensional HPLC method with on-line SPE intercolumn trapping for determination of Sudan dyes I, II, III, and IV in chili oil, a complex sample. This design eliminates the need for off-line sample preparation, uses the separation power of the first column to efficiently eliminate interferences, and uses the second column to separate the analytes. The method reduces the labor required for the analysis of edible oil for Sudan dyes. The Dionex UltiMate 3000 ×2 Dual HPLC system provides an efficient platform for this method design, with detection limits exceeding the requirements of the EU and GB.

Column:	Analytical column 1 (for the first dimension) Acclaim PA2, 3 µm, 3.0 × 150 mm (P/N 063705) On-line SPE Acclaim 120 C18, 5 µm, Guard Cartridge, 4.6 × 10 mm (P/N 069580) Analytical column 2 (for the second dimension) Acclaim RSLC 120 C18, 2.2 µm, 2.1 × 100 mm (P/N 068982)
Mobile Phase:	For analytical column 1 (the first dimension) A: Water B: CH_3CN C: CH_3OH/THF, 1:1 (v/v) In gradient: -3.5 ~ -0.5 min, B 50%, C 20%; 5.5 min, B 50%, C 50%; 11.0 ~ 14.0 min, B 20%, C 80%; 14.1 ~ 23 min, B 50%, C 20% For on-line SPE 0.1% FA in water, isocratic For analytical column 2 (the second dimension): A: Water B: CH_3CN C: 0.1% FA in CH_3CN In gradient: -3.5 ~ 7.0 min, B 50%; 16.0 ~ 22.4 min, B 90%; 22.5 ~ 23.0 min, B 50%. A and C maintain 40% and 10%, respectively.
Flow Rate:	For analytical column 1 (the first dimension), 0.6 mL/min For on-line SPE, 1.0 mL/min For analytical column 2 (the second dimension), 0.3 mL/min
Inj. Volume:	20 µL on the analytical column 1
Column Temp.:	30 °C
UV-vis Detection:	Absorbance at 500 nm
Chromatograms:	(A) Sudan dye standards (2 mg/L for each Sudan dye) (B) Chili oil sample, spiked with 6 mg/L of each Sudan dye standard
Peaks:	1. Sudan dye I 2. Sudan dye II 3. Sudan dye III 4. Sudan dye IV

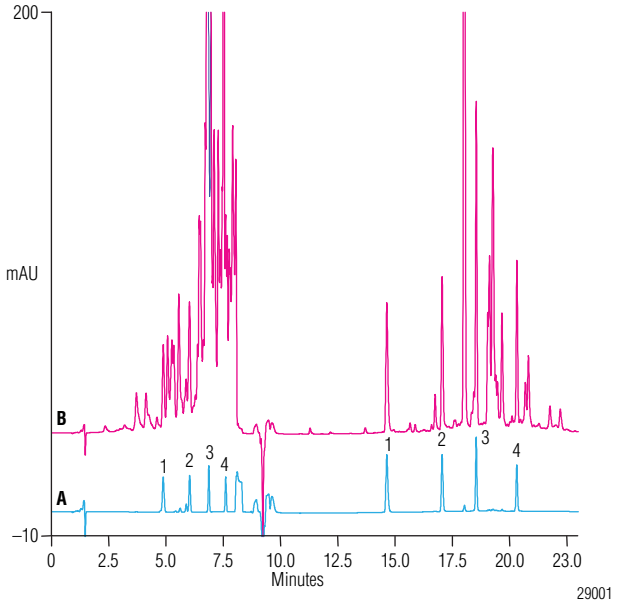

Figure 3. Chromatograms of (A) Sudan dye standards (2 mg/L for each Sudan dye), and (B) chili oil sample, spiked with 6 mg/L of each dye standard using the two-dimensional HPLC with on-line SPE intercolumn trapping system with UV-vis detection. The second dimension separation starts at 10 min.

Column:	Analytical column 1 (for the first dimension) Acclaim PA2, 3 µm, 3.0 × 150 mm (P/N 063705) On-line SPE Acclaim 120 C18, 5 µm, Guard Cartridge, 4.6 × 10 mm (P/N 069580) Analytical column 2 (for the second dimension) Acclaim RSLC 120 C18, 2.2 µm, 2.1 × 100 mm (P/N 068982)
Mobile Phase:	For analytical column 1 (the first dimension) A: Water B: CH_3CN C: CH_3OH/THF, 1:1 (v/v) In gradient: -3.5 ~ -0.5 min, B 50%, C 20%; 5.5 min, B 50%, C 50%; 11.0 ~ 14.0 min, B 20%, C 80%; 14.1 ~ 23 min, B 50%, C 20% For on-line SPE 0.1% FA in water, isocratic For analytical column 2 (the second dimension): A: Water B: CH_3CN C: 0.1% FA in CH_3CN In gradient: -3.5 ~ 7.0 min, B 50%; 16.0 ~ 22.4 min, B 90%; 22.5 ~ 23.0 min, B 50%. A and C maintain 40% and 10%, respectively.
Flow Rate:	For analytical column 1 (the first dimension), 0.6 mL/min For on-line SPE, 1.0 mL/min For analytical column 2 (the second dimension), 0.3 mL/min
Inj. Volume:	20 µL on the analytical column 1
Column Temp.:	30 °C
MS Detection:	ESI mode, positive scan, probe temp at 450 °C, needle voltage at 4000 V, SIM at 249 *m/z* for Sudan dye I, 277 m/z for Sudan dye II, 353 *m/z* for Sudan dye III, and 381 *m/z* for Sudan dye IV; cone voltage at 35 V for Sudan dye I and II, 50 V for Sudan dye III and IV; nebulizer gas, Nitrogen at 75 psi
Chromatograms:	(A) blank (B) Sudan dye standards (5 µg/L for Sudan dyes II and III, and 15 µg/L for Sudan dyes I and IV) (C) chili oil sample (D) Chili oil sample, spiked with 10 µg/L of Sudan dye II and III, and 30 µg/L of Sudan dye I and IV standards
Peaks:	1. Sudan dye I 2. Sudan dye II 3. Sudan dye III 4. Sudan dye IV

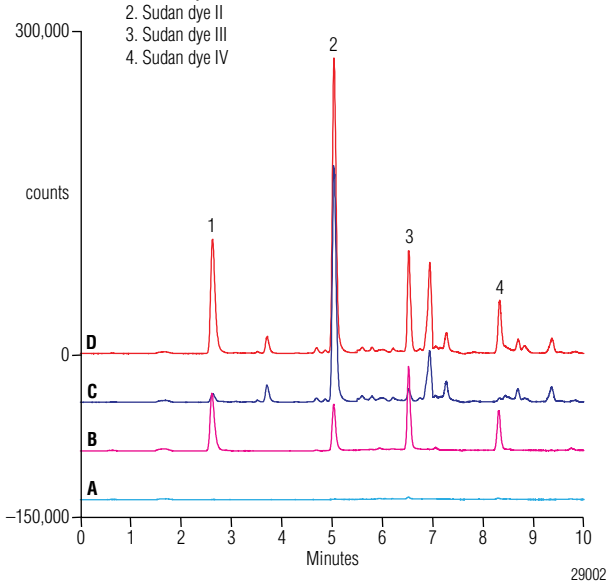

Figure 4. MS TIC chromatograms of (A) blank (CH_3CN), (B) mixed Sudan dye standards (5 µg/L for Sudan dye II and III, and 15 µg/L for Sudan dye I and IV), (C) chili oil sample, and (D) the same sample spiked with 10 µg/L of Sudan dyes II and III, and 30 µg/L of Sudan dyes I and IV using the two-dimensional HPLC with on-line SPE intercolumn trapping system with MS detection.

Table 2. Precision for Peak Retention Time and Area

Sudan Dye	Retention Time RSD	Peak Area RSD	Concentration of Standard (mg/L)
I	0.090	1.211	1.0
II	0.060	0.871	1.0
III	0.052	1.350	1.0
IV	0.064	1.933	1.0

Table 3. Analysis Results of Sudan Dyes I, II, III, and IV in Chili Oil*

Sudan Dye	Detected (µg/L)	Added (µg/L)	Found (µg/L)	Recovery (%)
I	2.0	30	29	97
II	29	10	8.0	80
III	0.6	10	67	67
IV	ND**	30	24	80

*Average of three determinations

**Not detected

REFERENCES

1. *The Risk of Sudan Dyes in Food,* Assessment Report, No. 5; Ministry of Health of the People's Republic of China: Beijing, China, 2005.
2. Vérétout, O.; Demesse, L.; Szymanski, L. *Analysis and Dosage of the Colorants Sudan and Bixin in Chili Powder and Pepper-Based Products;* Health & Consumer Protection Directorate–General, European Commission, NEWS notification: 03/99.
3. *The Method for the Determination of Sudan Dyes in Foods–High Performance Liquid Chromatography,* GB/T19681, China, 2005.
4. Pang, Y.L. Development in the Analysis Methods of Sudan Red in Food. *Food and Fermentation Industries (Chin.)* **2008,** *34,* 114.
5. Zacharis, C.K.; Kika, F.S.; Tzanavaras, P.D.; Rigas, P.K.; Efstratios, R. Development and Validation of a Rapid HPLC Method for the Determination of Five Banned Fat-Soluble Colorants in Spices Using a Narrow-Bore Monolithic Column. *Talanta,* In Press.
6. Ridgway, K.; Lalljie, Sam P.D.; Smith, R.M. Sample Preparation Techniques for the Determination of Trace Residues and Contaminants in Foods. *J. Chromatogr. A* **2007,** *1153,* 36.
7. Pardo, O.; Yusà, V.; León, N.; Pastor, A. Development of a Method for the Analysis of Seven Banned Azo-Dyes in Chili and Hot Chili Food Samples by Pressurised Liquid Extraction and Liquid Chromatography with Electrospray Ionization-Tandem Mass Spectrometry. *Talanta* **2009,** *78,* 178.
8. Rebane, R.; Leito, I.; Yurchenko, S.; Herodes, K. A Review of Analytical Techniques for Determination of Sudan I–IV Dyes in Food Matrixes. *J.Chromatogr. A* **2010,** *1217,* 2747.
9. Liu, H.C.; Tang, L.B.; Li, Q.W.; Liu, J.F.; Zou, Y.H. Identification of Sudan in Pepper Oil by HPLC with Two Columns and Matrix Solid Phase Dispersion. *Chin. J. Anal. Lab.* **2007,** *26* (6), 37.
10. Dionex Corporation. *Determination of Vitamin B_{12} in Beverages Using Online Solid-Phase Extraction Followed by HPLC with UV Detection,* Application Note 256, LPN 2574, Sunnyvale, CA, 2010.
11. Dionex Corporation. *Sensitive Determination of Microcystins in Drinking and Environmental Waters.* Application Note 261, LPN 2607, Sunnyvale, CA, 2010.
12. Dionex Corporation. *Intelligent LC Solutions, UltiMate 3000 Proteomics MDLC System.* LPN 2013, Sunnyvale, CA, 2008.
13. Dionex Corporation. *Sudan Dyes on Acclaim 120 C18.* Ref. No. 23630, Sunnyvale, CA, 2009.
14. Dionex Corporation. *Oil-Soluble Dyes on Acclaim 120 C18 RSLC.* Ref No. 25641, Sunnyvale, CA, 2009.
15. Dionex Corporation. *Sudan Dyes in Paprika.* Ref No. 21787, Sunnyvale, CA, 2009.

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	Hydro-phobicity
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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