Beverages Applications Notebook

Additives in Beverages

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Introduction to Beverages

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

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

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

Thermo Scientific and Dionex Integrated Systems

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

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

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

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

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

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

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

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

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IC and RFIC Systems

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

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

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

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

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

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

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

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

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

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MS Instruments

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

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

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

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

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

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Chromeleon 7 Chromatography Data System Software

The fastest way to get from samples to results

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

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

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

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

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

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Process Analytical Systems and Software

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

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

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

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

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

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

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Automated Sample Preparation

ACCELERATED SOLVENT EXTRACTORS

Two new solvent extraction systems with pH-hardened Dionium components

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

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SOLID-PHASE EXTRACTION SYSTEMS

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

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

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

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

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

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

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Analysis of Additives in Beverages

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.

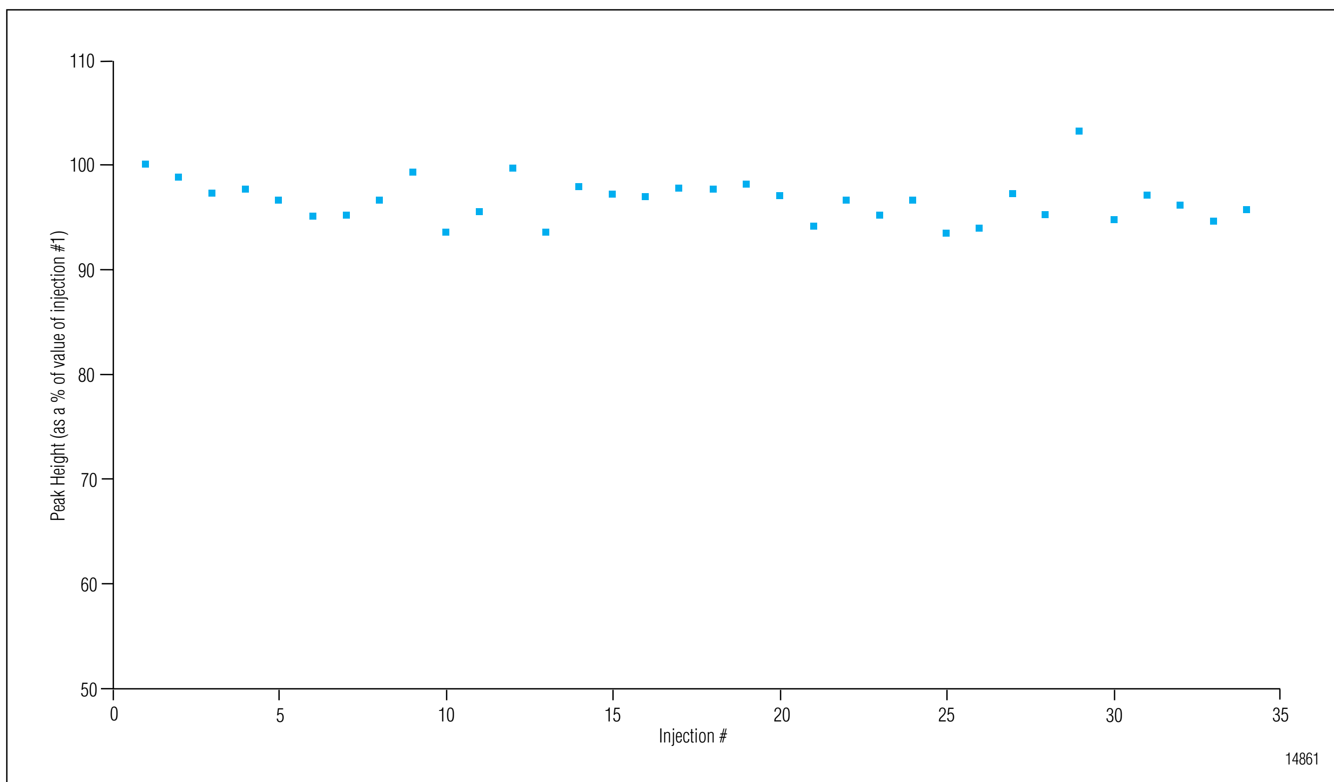

Figure 1. Consecutive injections (34) of a 14-mg/L sulfite standard. Peak heights were plotted as a percentage of the peak height from injection #1. Each analysis required approximately 15 min.

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	

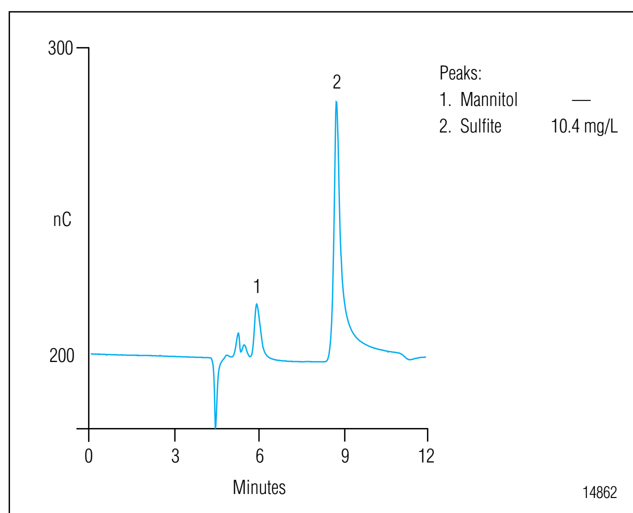

Figure 2. Dried apricot homogenate.

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.

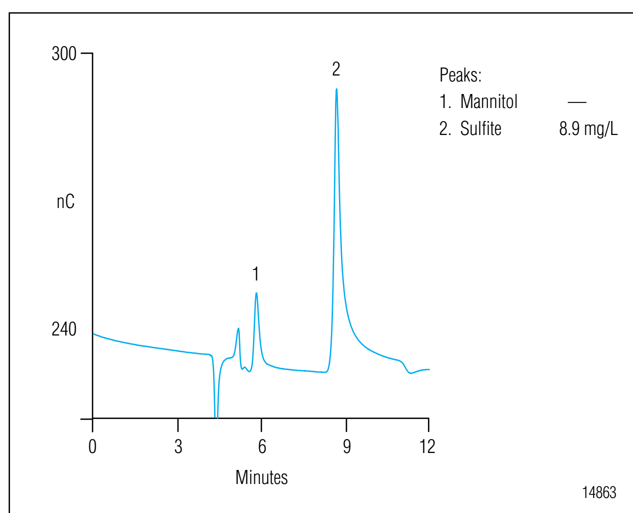

Figure 3. Lime juice, 1/36 dilution.

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.

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

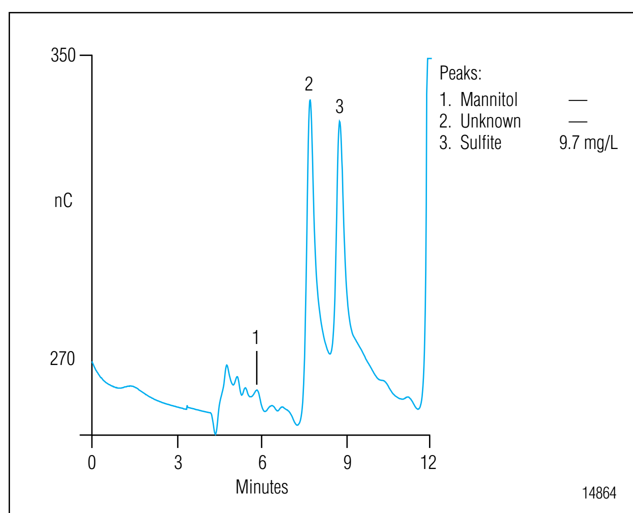

Figure 4. White wine.

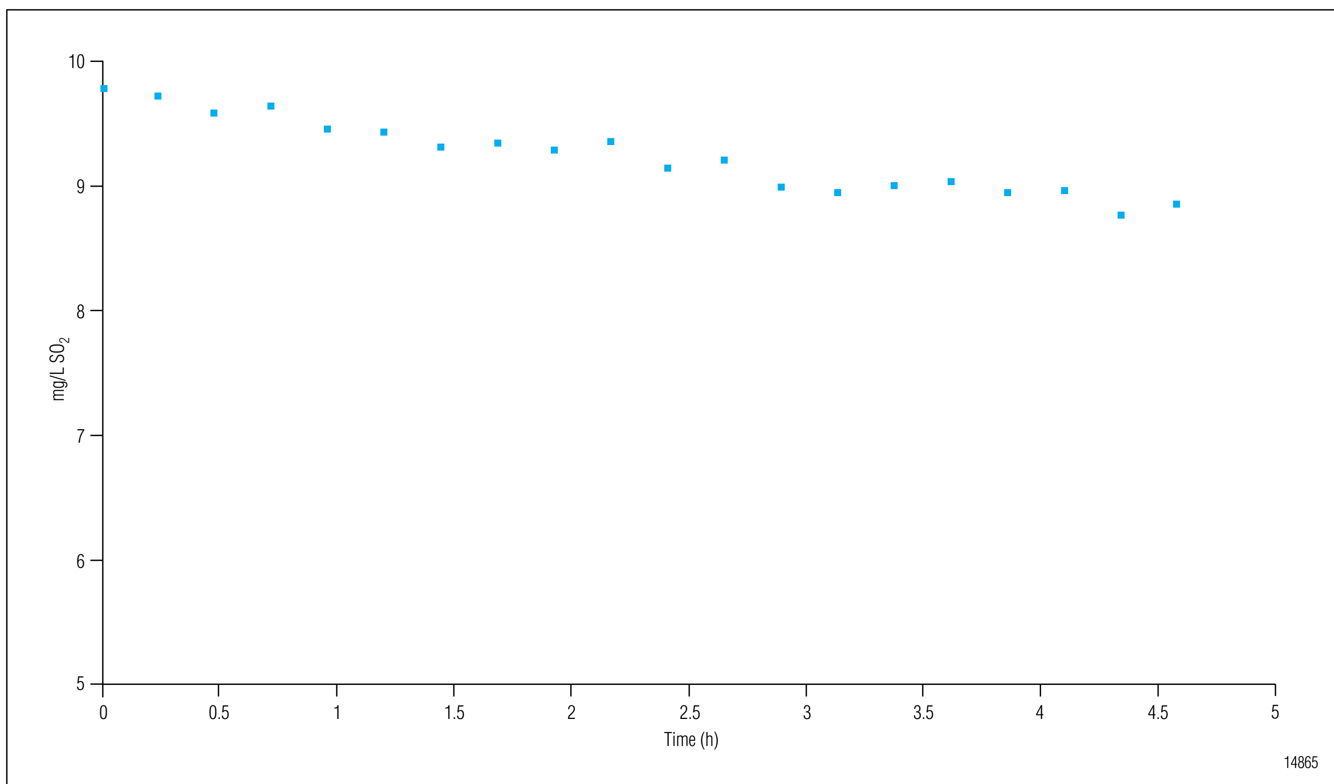

Figure 5. Oxidation of sulfite in white wine over time. Original concentration of SO_2 in fresh sample was 9.7 mg/L.

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.

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).

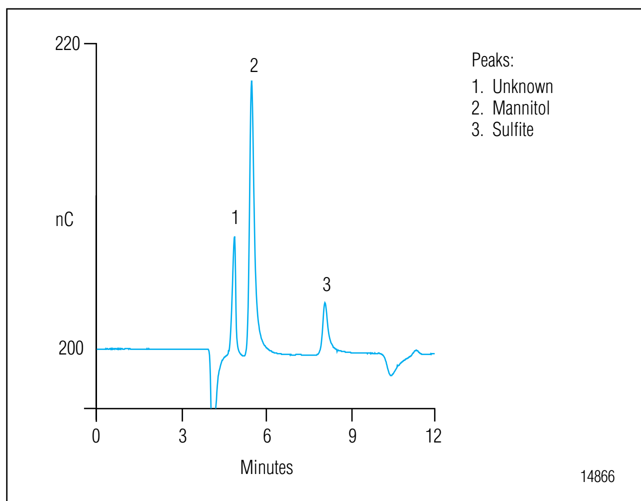

Figure 6. Sulfite standard, 530 µg/L.

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.

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) and is not recommended for use at higher pH.[1]

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

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)

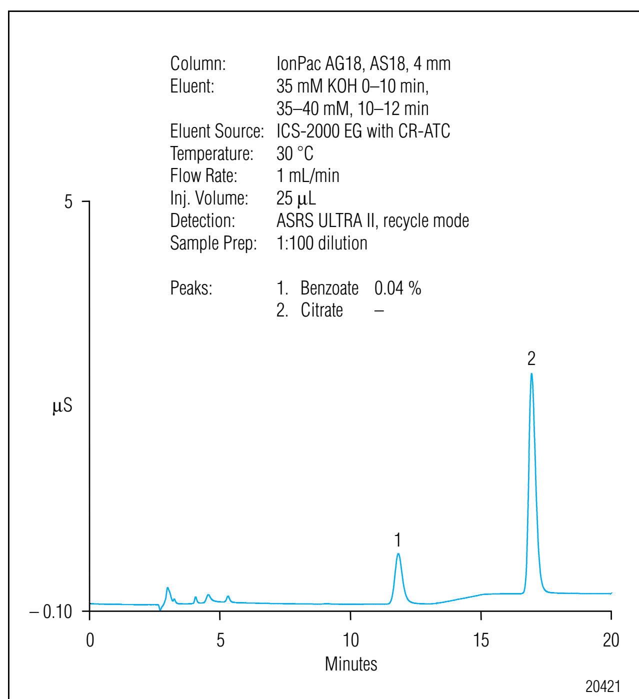

Figure 1. Determination of benzoate in flavored soda.

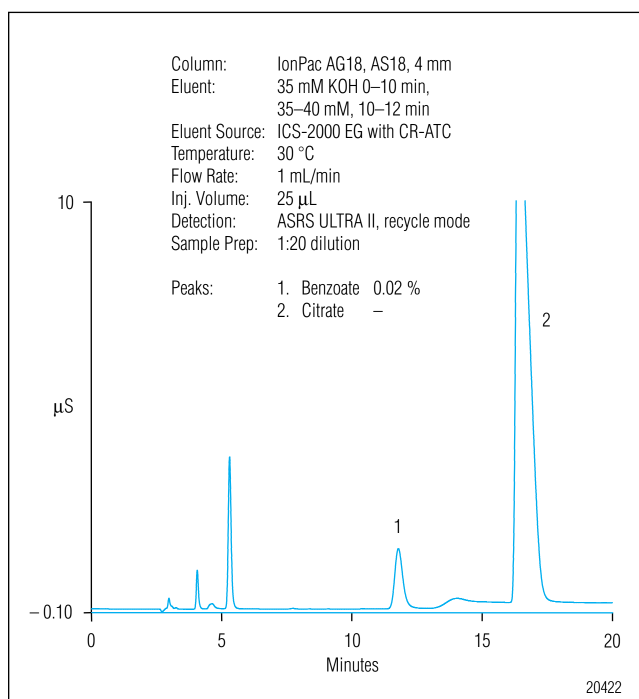

Figure 2. Determination of benzoate in diet soda.

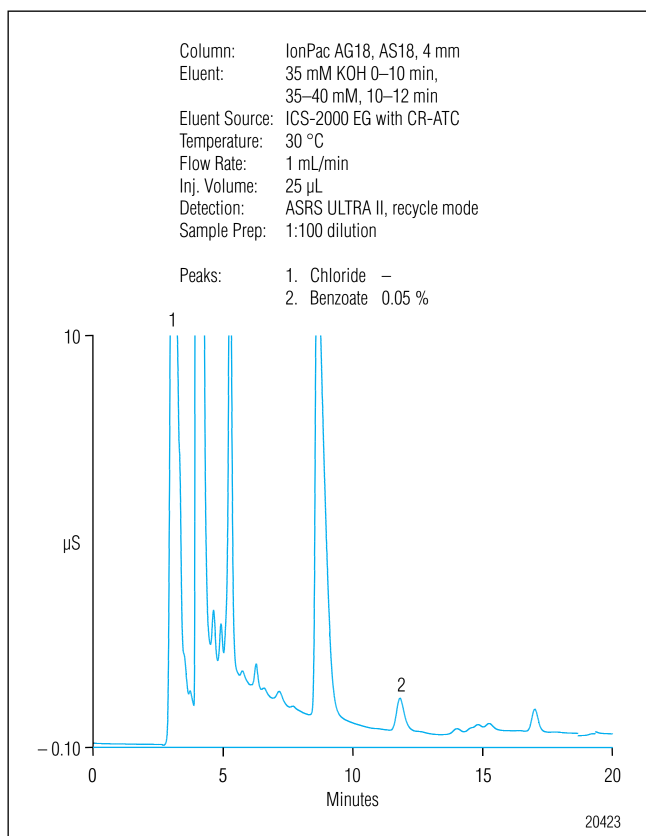

Figure 3. Determination of benzoate in soy sauce.

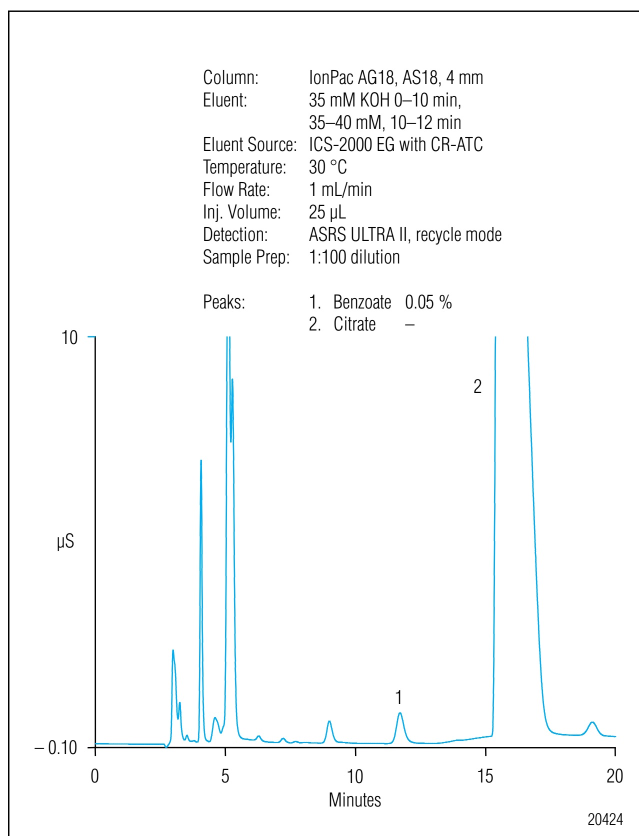

Figure 4. Determination of benzoate in lemon juice.

soda, diet soda, soy sauce, and lemon juice, respectively. 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.

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SUPPLIER

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

Determination of Steviol Glycosides by HPLC with UV and ELS Detections

INTRODUCTION

The stevia plant and extracts from stevia leaves have long been used as sweeteners in Asia and Latin America. The U.S. Food and Drug Administration (US FDA), however, has not approved stevia leaf for use in food and therefore, stevia extracts in the U.S. have been limited to dietary supplements.[1] The sweet components in the leaf extract, known as steviol glycosides, are closely related in structure and have both sweet and bitter flavor profiles.

Two steviol glycosides present in plant tissue, stevioside and rebaudioside A, are largely responsible for the sweet flavor of stevia leaves (Figure 1). Both compounds are approximately 300 times sweeter than sucrose. Additionally, rebaudioside A exhibits reduced bitterness compared to stevioside when used at low to medium sweetening levels.[2] In December 2008, the US FDA approved the request to grant rebaudioside A (also known as rebiana), purified from stevia rebaudiana (Bertoni), Generally Recognized as Safe (GRAS) status for use as a sugar substitute in foods.[3,4] This recognition allows the use of rebiana as a commercial sweetener. Multiple other combinations of steviol glycosides may also be allowed in the future under GRAS status.

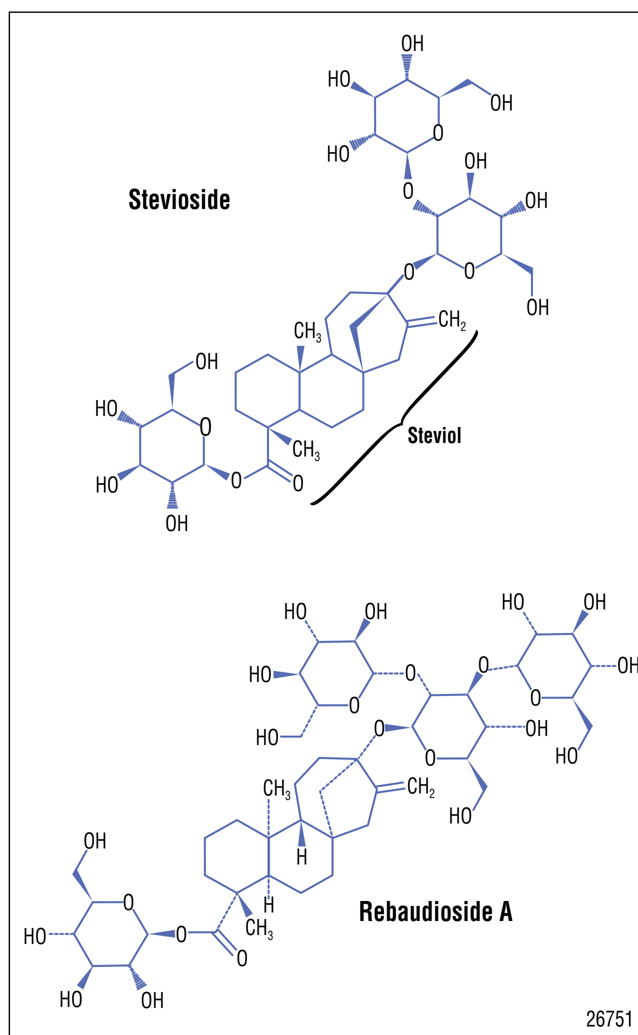

Figure 1. Chemical structures of stevioside and rebaudioside A.

Typically, individual steviol glycosides are determined by liquid chromatography methods that require a polar amine column. The Joint European (FAO/WHO) Commission on Food Additives (JECFA) published a monograph for the determination of steviol glycosides by UV detection at 210 nm using 80/20 acetonitrile/water at pH = 3.0, adjusted with phosphoric acid.[5] This method determines the weight percentage of seven glycosides: stevioside, rubusoside, dulcoside A, steviolbioside, rebaudioside C, rebaudioside B, and rebaudioside A. The percentages (w/w) of glycosides present in the sample are normalized to a 0.5 mg/mL stevioside standard. This method specifies a 3.9–4.6 mm i.d. column format and can use flow rates of up to 2.0 mL/min to meet a retention time requirement of 10 min for stevioside. This high flow rate coupled with a 25 min run time results in 25–50 mL of mobile phase used per sample.

The USP rebaudioside A monograph in the food codex is similar to the JECFA monograph. The rebaudioside A monograph uses an acetate buffer to adjust the pH rather than phosphoric acid and a propyl-amine silane phase bonded to a silica gel column.[6] Because this method is intended to determine rebaudioside A, it is the only glycoside quantified. The method uses UV detection and a flow rate of 1.5 mL/min with a run time of 40 min resulting in 60 mL of mobile phase used per sample.

Analytical methods to determine these glycosides are challenging because the compounds do not absorb strongly in the UV region. Other detection methods, such as evaporative light scattering (ELS) can be used to improve steviol glycoside quantification. Here, steviol glycosides are determined in consumer sweeteners by UV and ELS detections. The glycosides are separated on the Acclaim® Mixed-Mode WAX-1 column using 80/20 acetonitrile/ammonium formate buffer at pH = 3.0. This method uses the HILIC mode of the mixed-mode column, thereby allowing separation of multiple steviol glycosides. By using a volatile mobile phase, ELS detection is feasible, which adds further flexibility to the method for detection of glycosides that do not have strong UV extinction coefficients. In addition, the 2.1 mm i.d. column format reduces solvent use to 12.5 mL/sample compared to the range of 25–60 mL/sample for the JECFA and proposed USP methods.

EQUIPMENT

Dionex UltiMate® 3000 Intelligent LC system:
- SRD-3200 Solvent rack (Dionex P/N 5035.9250)
- HPG-3200M Pump (Dionex P/N 5035.0018)
- WPS-3000TSL Micro autosampler (Dionex P/N 5822.0025)
  - Sample loop, 25 µL (Dionex P/N 6820.2415)
- TCC-3200 Column compartment (Dionex P/N 5722.0025)
- VWD-3400 Detector (Dionex P/N 5074.0010)
  - Semi-Micro PEEK™ flow cell, 2.5 µL (Dionex P/N 6074.0300)

Varian 380-LC ELS detector

Chromeleon® 6.8 Chromatography Data System

Glass injection vials with caps and septa, 1.5 mL (Dionex P/N 055427) or

Polypropylene injection vials with caps and septa, 300 µL (Dionex P/N 055428)

Nalgene® Filter Unit, 0.2 µm nylon membrane, 1 L capacity (Nalgene P/N 164-0020)

REAGENTS AND STANDARDS

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

pH buffer, 4.00 (VWR P/N BDH4018-500ML)

pH buffer, 2.00 (VWR P/N BDH5010-500ML)

Stevia standards kit, (ChromaDex P/N KIT-00019566-0HK) containing:
- Isosteviol
- Rebaudioside A
- Stevioside

Rebaudioside B (ChromaDex P/N 00018227)

Formic acid (Sigma-Aldrich P/N 06440)

Ammonium formate (Sigma-Aldrich P/N 51691)

Acetonitrile (Honeywell P/N 015-4)

Samples

Brand A: sweetener containing stevia leaf extract and inulin

Brand B: sweetener containing rebiana (rebaudioside A) and erythritol

CONDITIONS

Column:	Acclaim Mixed-Mode WAX-1 (5 µm), 2.1 × 150 mm (Dionex P/N 067084)
	Acclaim Mixed-Mode WAX-1 (5 µm), 2.1 x 10 mm guard column (Dionex P/N 069686) with guard holder (Dionex P/N 069580)
Mobile Phase:	80/20 acetonitrile/10 mM ammonium formate, pH = 3.0
Flow Rate:	0.5 mL/min
Temperature:	40 °C
Inj. Volume:	5 µL
Detection:	Variable Wavelength UV-vis Detector, 210 nm
	Evaporative Light Scattering Detector,
	N_2 flow, 1.2 SLM
	Nebulizer temperature, 35 °C
	Evaporator temperature, 60 °C
	PMT Gain, 2
System Backpressure:	~1100 psi
Noise:	~ 0.09 mAU (UV)
	~ 0.7 mV (ELS)
Run Time:	25 min

PREPARATIONS OF SOLUTIONS AND REAGENTS

Mobile Phase Preparation:

Transfer 0.63 g ammonium formate to a 1 L bottle and add 1000 g DI water. Adjust the pH of the resulting 10 mM ammonium formate solution to 3.00 ± 0.05 by adding 1700 µL formic acid. Filter the buffer through a 0.2 µm nylon filter unit to remove any insoluble particles that may be present.

Using the ammonium formate buffer described above, prepare a solution of 80/20 (v/v) acetonitrile/ammonium formate by transferring 200 g of the ammonium formate solution to a 1 L glass volumetric flask and bringing to volume with acetonitrile. This will yield 634 g of acetonitrile. Invert the flask to mix well. Do not top off the flask after mixing. Mixing formate and acetonitrile causes an endothermic reaction and the solution will cool substantially, resulting in a visible reduction in volume. Allow the solution to return to ambient temperature before using as a mobile phase.

Autosampler Syringe Wash Solution:

To prevent carryover from the autosampler, a DI water wash solution was used.

Standards and Sample Solutions

Standards

Standard solutions of stevioside, rebaudioside A, rebaudioside B, and isosteviol were prepared by adding 2.0 mg to 1.0 mL of mobile phase to prepare a 2.0 mg/mL standard. This stock standard was then used to prepare standards of 0.06 mg/mL to 0.5 mg/mL of stevioside and rebaudioside A by appropriate dilution in the mobile phase.

Samples

Samples were prepared for analysis by extracting 0.25 g of sweetener with 10 mL of mobile phase. Brand A contains inulin, which is minimally soluble in organic solvents. The samples were mixed using a vortex mixer for a minimum of 20 s at least 4 times to dissolve the glycosides. Brand B fully dissolved followed by separation of erythritol, which was saturated in the mobile phase.

Precautions

Care must be taken to prepare the mobile phase consistently. Changes in either the ionic strength or pH of the mobile phase can lead to shifts in analyte retention times. If a decrease in resolution is observed, prepare the ammonium formate buffer again, paying close attention to the amount of ammonium formate added and to the final pH. If the pH is below 2.95, discard the solution and prepare another batch of buffer.

Using a premixed mobile phase will substantially decrease noise in the UV channel. Increased noise will result from online mixing of acetonitrile and the formate buffer that do not absorb equally in the UV range.

RESULTS AND DISCUSSION

Separation

Figure 2 shows the separation of isosteviol, stevioside, and rebaudioside A on the Acclaim Mixed-Mode WAX-1 column. In Figure 2A, stevioside and rebaudioside A are easily observed by UV detection. Isosteviol does not have a strong UV extinction coefficient and is not detected. As seen in Figure 2B, isosteviol is easily detected by ELS. However, the concentration of isosteviol in the standard leads to detector saturation in the ELS channel. To calibrate isosteviol by ELS, 1 µL injections were used, reducing the amount on the column by a factor of five. Figure 3

shows the ELS detector response from a 1 µL injection of the same standard shown in Figure 2, which permits the quantification of isosteviol. In UV and ELS detection, the stevioside and rebaudioside A peaks are well-resolved, with no interferences.

Quantification Assay Linearity

Table 1 shows the linear relationship of peak area to concentration for rebaudioside A and stevioside using UV detection. The correlation coefficients for rebaudioside A and stevioside are 0.9974 and 0.9977 respectively, by UV detection.

Calibration curves using ELS detection are inherently nonlinear and were fit using exponential curves. This nonlinearity is the result of physical interactions that contribute to ELS detection; light scattering, reflection, and refraction by the particles each depend on the radius of the particle compared to the wavelength used for detection. Small particles predominately scatter but as the particles increase in size, reflection and refraction begin to impact the particle detection. Due to this, the peak area and concentration are related by the following exponential expression:

$$\text{Peak Area} = am^b$$

where m is the sample mass and a and b are coefficients related to droplet size, concentration, and detection parameters. By plotting the concentration against the peak area, a curved line is observed. To obtain a straight line, a logarithmic plot is required. To fit the calibration curves for ELS detection, use the exponential fitting option available within the Chromeleon software. This calibration option best fits the data obtained in lieu of a log-log fit. Correlation coefficients reported within Chromeleon are from linear fits of converted data. These values are reported in Table 1.

Table 1. Calibration of Steviol Glycosides by UV and ELS Detections

Analyte	Detector	Range (mg/mL)	Linearity[a]	Calibration Type
Rebaudioside A	UV	0.06 - 0.50	0.9974	Linear Offset
	ELS	0.06 - 0.50	0.9920	Exponential
Stevioside	UV	0.06 - 0.50	0.9977	Linear Offset
	ELS	0.06 - 0.50	0.9930	Exponential
Isosteviol	UV	ND*	—	—
	ELS (1 µL injection)	0.03 - 0.25	0.9720	Exponential

* ND = not determined
[a] linearity reported for exponential calibration refers to the exponential plots as described in the text.

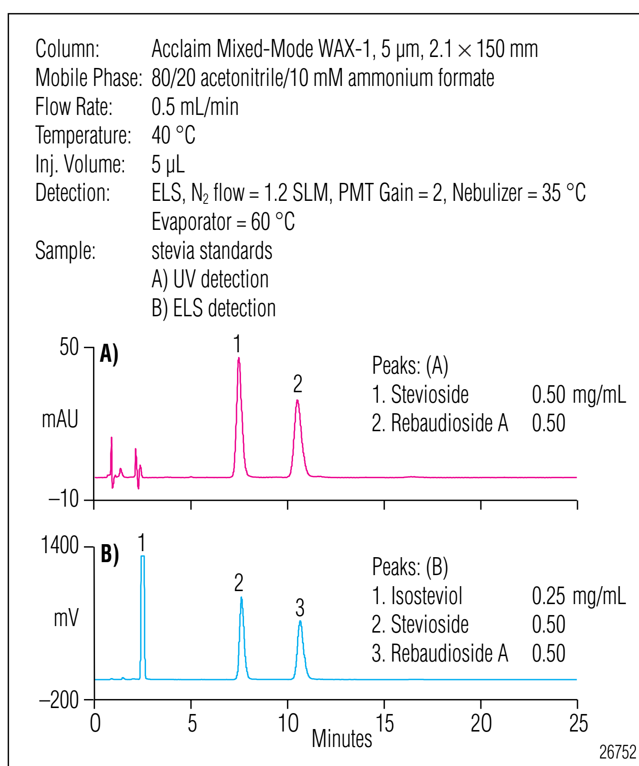

Figure 2. Separation of steviol glycoside standards on the Acclaim Mixed-Mode WAX-1 column.

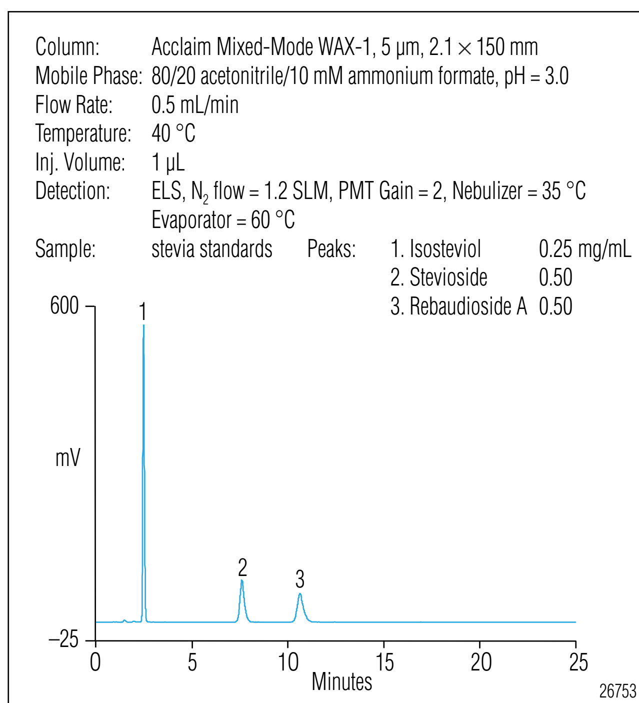

Figure 3. Isosteviol separated on the Mixed-Mode WAX-1 column and detected by ELS.

Sample Analysis

Figure 4 shows the separation of an extract of a sweetener derived from extracted stevia leaves and dispersed in inulin. Several steviol glycosides are detected in this product. When analyzed by UV, which is a standard by the JECFA and USP monographs, four steviol glycosides are easily determined and baseline resolved: dulcoside A, stevioside, rebaudioside C, and rebaudioside A. When the sample is analyzed using ELS detection, there are a number of additional peaks not observed by UV (Figure 4B). The early eluting peaks, 1 and 2, are partially due to small amounts of inulin that dissolve in the mobile phase. This has been confirmed by an injection of an inulin sample. Stevioside and rebaudioside A are resolved from other peaks, and rebaudioside B is more easily detected than by UV detection. Both detection methods yield equivalent quantification of stevioside and rebaudioside A.

Figure 5 shows the separation of a 25 mg/mL solution of a commercial sweetener composed of rebiana. In this sweetener, the rebaudioside A is mixed with erythritol to dilute the glycoside and improve flavor. As seen in Figure 5A, when analyzing samples using UV detection, only rebaudioside A is observed. There is no evidence that this sweetener contains additional steviol glycosides. When using ELS detection (Figure 5B), both erythritol and rebaudioside A are observed, although the amount of erythritol present exceeds the dynamic range of the detector. The quantification of rebaudioside A is identical by both detection schemes.

Precision and Accuracy

Method precision was first tested by replicate injections of standards using two different batches of mobile phase. Table 2 summarizes the precision data. In both cases, the standards were properly quantified. Peak area RSDs range from 0.74 to 2.43, with the ELS peak area precision consistently higher than the UV peak area precision. This is a result of the nonlinear response in ELS that exaggerates small changes compared to UV. Retention time precision is excellent with retention time RSDs ranging from 0.05 to 0.14. Retention time variability between batches of mobile phase was within 1.1% for rebaudioside A. These data confirm that the method is reproducible with consistent mobile phase preparation.

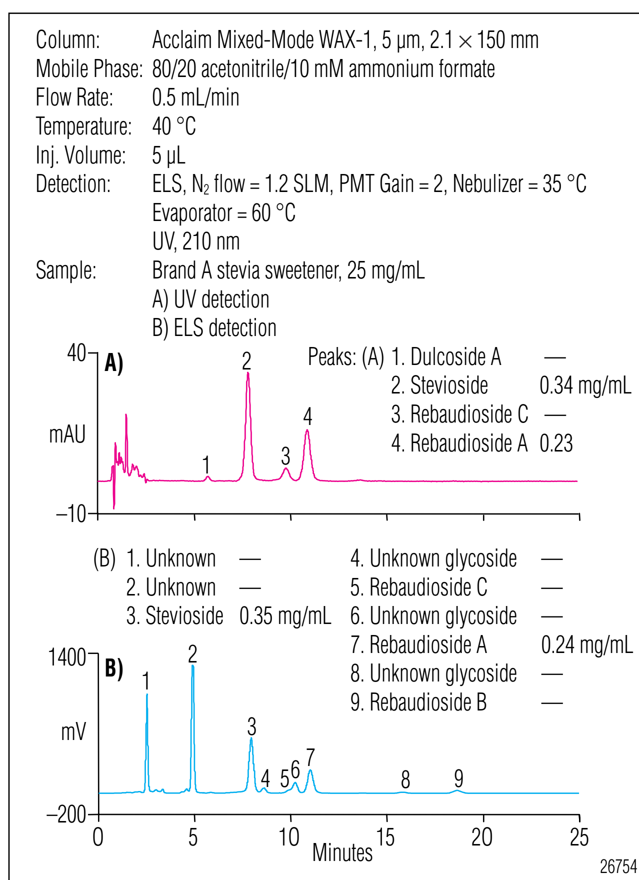

Figure 4. Separation of a stevia sweetener on the Acclaim Mixed-Mode WAX-1 column.

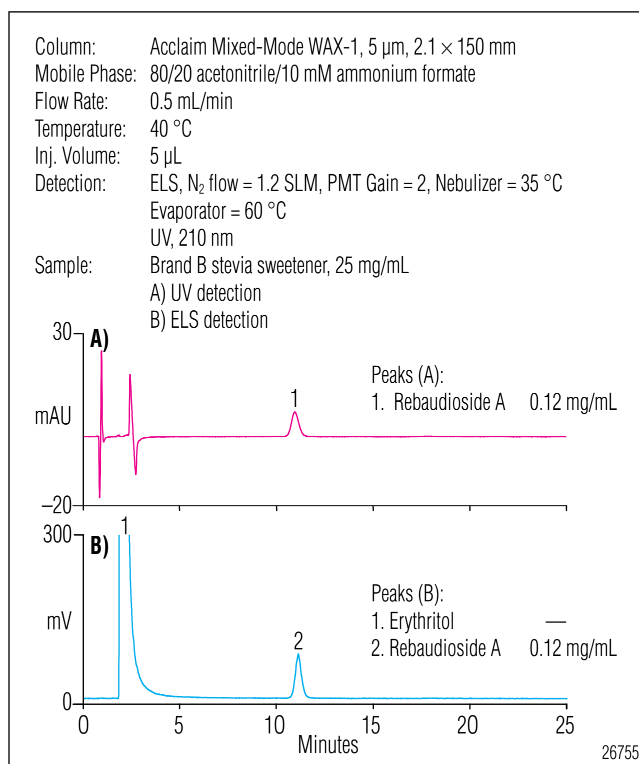

Figure 5. Determination of rebaudioside A in a commercial sweetener.

Samples of the two sweeteners were analyzed in triplicate over three days. Table 3 shows analyte quantification, retention time precision, and peak area precision data for Day 1 of sample analysis. The determined concentrations of glycosides in the samples are consistent by both UV and ELS detections. Table 4 shows the calculated mass of stevioside and rebaudioside A in a 1 g portion of the sweeteners along with intraday and between-day precision. During three days of analysis, Brand B was found to contain 4.0–4.3 mg/g of rebaudioside A. This corresponds to the expected amount of ~4 mg/g specified for use as a sweetener.[2]

Because it is an extract of stevia leaves without further purification to isolate an individual compound, Brand A contains higher concentrations of steviol glycosides compared to the rebiana sweetener. Table 3 shows determined concentrations in the dissolved sample. Similar to Brand B, the concentrations determined by UV and ELS detection methods are well-correlated. Table 4 shows the intraday precision for Brands A and B, which range from 0.5–6.3% from triplicate sample preparations.

Accuracy of the method was tested by spiking standards at known concentration into samples of Brand A and B sweeteners. The recoveries of stevioside and rebaudioside A ranged from 94.2–104%, suggesting that the method is accurate. The isosteviol recovery was lower at 90.6% due to the elution on the tail of erythritol in Brand A. However, isosteviol was not detected in any of the samples. Recovery of isosteviol in Brand B was evaluated, but close elution with a peak from the inulin interfered.

Table 2. Precision of Standard Injections (n=7, each standard at 0.20 mg/mL)

Analyte	Detection	Retention Time (min)	Retention Time Precision (RSD)	Peak Area Precision (RSD)	Amount (mg/mL)
Mobile Phase Batch 1					
Rebaudioside A	UV	11.08	0.05	0.79	0.18
	ELS	11.19	0.10	1.86	0.18
Stevioside	UV	7.81	0.03	0.74	0.18
	ELS	7.94	0.10	1.13	0.19
Isosteviol	UV	ND*	—	—	—
	ELS	2.61	0.08	1.47	0.22
Mobile Phase Batch 2					
Rebaudioside A	UV	10.96	0.09	1.54	0.18
	ELS	11.08	0.12	1.47	0.18
Stevioside	UV	7.75	0.12	1.09	0.18
	ELS	7.88	0.14	1.98	0.18
Isosteviol	UV	ND*	—	—	—
	ELS	2.60	0.07	2.43	0.23

* ND = not determined.

Table 3. Precision Values of Triplicate Analysis of Commercially Available Sweeteners, Day 1						
Sample	**Analyte**	**Detection**	**Retention Time (min)**	**Retention Time Precision (RSD)**	**Peak Area Precision (RSD)**	**Amount (mg/mL)**
Brand A, Replicate 1	Rebaudioside A	UV	10.96	0.04	1.10	0.23
		ELS	11.08	0.07	2.00	0.25
	Stevioside	UV	7.74	0.05	0.36	0.32
		ELS	7.86	0.11	1.18	0.34
Brand A, Replicate 2	Rebaudioside A	UV	10.97	0.04	1.15	0.24
		ELS	11.10	0.07	1.19	0.25
	Stevioside	UV	7.74	0.05	0.91	0.32
		ELS	7.87	0.15	2.40	0.35
Brand A, Replicate 3	Rebaudioside A	UV	10.97	0.04	0.74	0.26
		ELS	11.11	0.04	0.04	0.28
Brand B, Replicate 1	Rebaudioside A	UV	10.95	0.06	0.94	0.10
		ELS	11.06	0.24	0.23	0.10
Brand B, Replicate 2	Rebaudioside A	UV	10.96	0.06	0.06	0.12
		ELS	11.09	0.07	1.17	0.12
Brand B, Replicate 3	Rebaudioside A	UV	10.96	0.04	0.81	0.11
		ELS	11.08	0.19	0.57	0.11

Table 4. Intra- and Interday Precision of Sweetener Analysis					
Day/Sample	**Analyte**	**Detection**	**Average mg Analyte/g Sweetener**	**Intraday Precision* (RSD)**	**Between-day Precision RSD**
Day 1 Brand A	Rebaudioside A	UV	9.8	5.6	3.2
		ELS	10.4	6.3	3.5
	Stevioside	UV	13.3	6.2	4.0
		ELS	14.4	6.3	2.2
Day 2 Brand A	Rebaudioside A	UV	9.8	0.7	
		ELS	9.7	2.8	
	Stevioside	UV	13.5	0.5	
		ELS	13.8	1.0	
Day 3 Brand A	Rebaudioside A	UV	10.4	4.7	
		ELS	10.3	3.2	
	Stevioside	UV	14.3	4.4	
		ELS	14.1	1.5	
Day 1 Brand B	Rebaudioside A	UV	4.3	10.2	1.6
		ELS	4.3	9.0	3.4
Day 2 Brand B	Rebaudioside A	UV	4.2	1.5	
		ELS	4.1	2.0	
Day 3 Brand B	Rebaudioside A	UV	4.2	8.6	
		ELS	4.0	6.2	

* Precision values are normalized to the amount of steviol glycoside determined per 1 g of sweetener.

Table 5. Recovery of Standards Added to Stevia Sweetener Analysis					
Sample	Analyte	Detection	Determined Amount with Spiking (mg/mL)	Amount Spiked (mg/mL)	Recovery (%)
Brand A	Rebaudioside A	UV	0.46	0.30	97.6
		ELS	0.48	0.30	104
	Stevioside	UV	0.52	0.30	95.3
		ELS	0.53	0.30	98.2
	Isosteviol	UV	ND	0.05	ND
		ELS	0.07	0.05	51.8*
Brand B	Rebaudioside A	UV	0.24	0.15	101
		ELS	0.25	0.15	103
	Stevioside	UV	0.14	0.15	94.9
		ELS	0.14	0.15	94.2
	Isosteviol	UV	ND	0.05	ND
		ELS	0.04	0.05	90.6

* Close elution of isosteviol with another component of the sweetener.

CONCLUSION

In this method, steviol glycosides are determined in consumer sweeteners by UV and ELS detections. The Acclaim Mixed-Mode WAX-1 column is used to resolve the steviol glycosides of interest. The method proposed requires only 12.5 mL of mobile phase sample compared to 25 to 60 mL/sample in the JECFA and proposed USP methods, respectively. This saves time and resources during mobile phase preparation, and reduces waste. Quantification of the two principal glycosides, stevioside and rebaudioside A, was precise and accurate by both detection methods. In addition, ELS detection has the added advantage of distinguishing additional components in the sample that are not UV absorbing, thus allowing for an additional method to check sweetener purity.

LIST OF SUPPLIERS

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

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

ChromaDex, 10005 Muirlands Blvd, Suite G, First Floor, Irvine, CA 92618 USA. Tel: 949-419-0288, www.chromadex.com

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6. Rebaudioside A USPC, Inc. Dec 2008, http://forum.foodchemicaslcodex.org/online/pub/data/v200812/mon_pca_200812_f101747.xml

DIONEX

Application Note 245

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 ($C_{20}H_2Br_4C_{14}Na_2O_5$), 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.

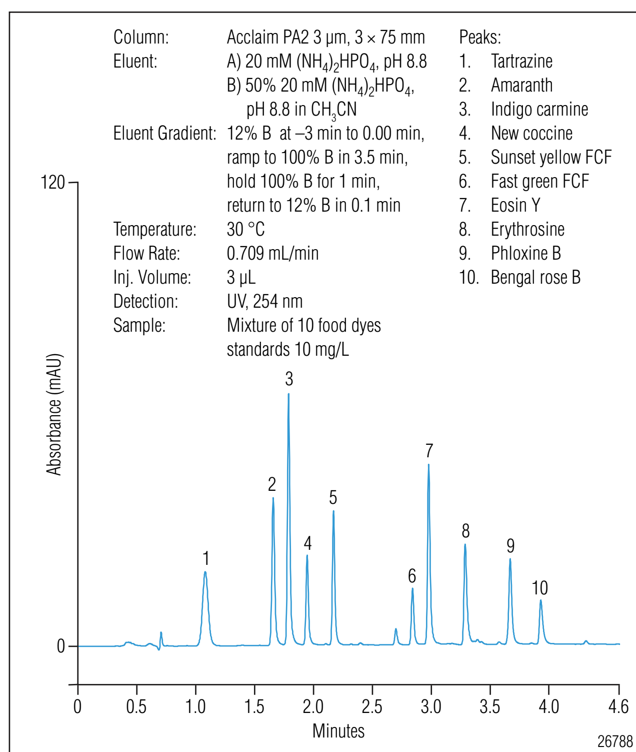

Figure 1. Chromatogram of the standard mixture of 10 dyes.

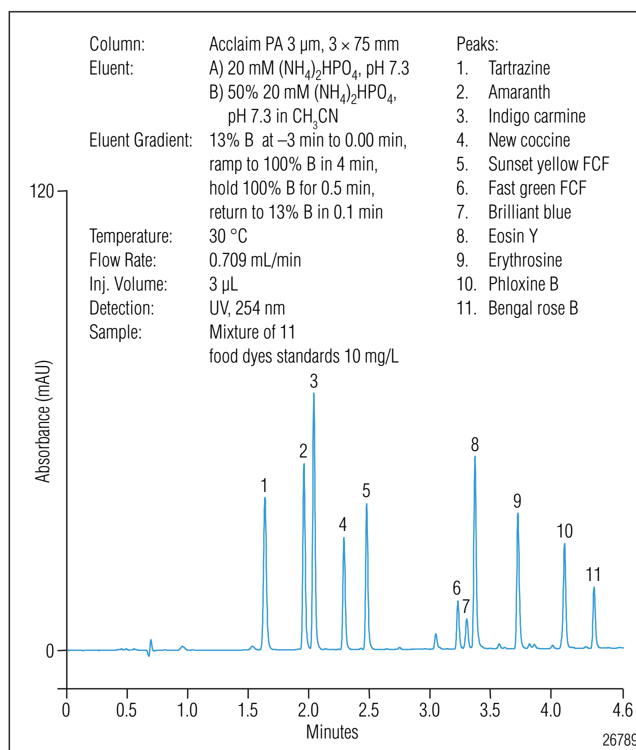

Figure 2. Chromatogram of the standard mixture of 11 dyes.

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.

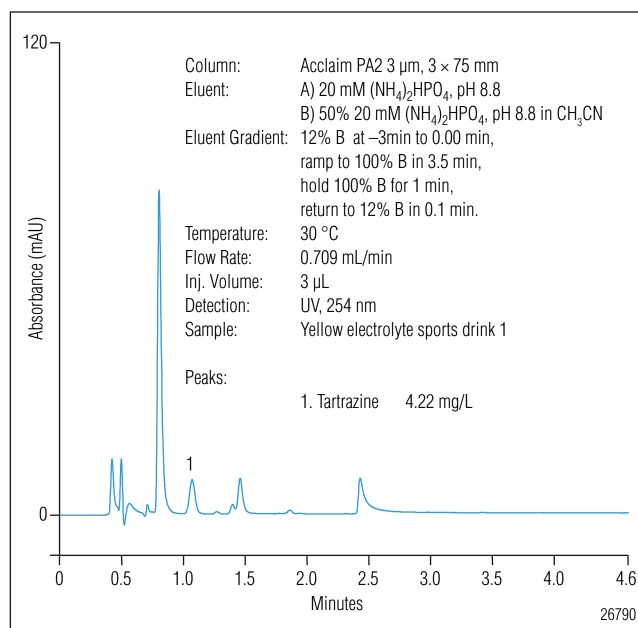

Figure 3. Chromatogram of electrolyte sports drink 1.

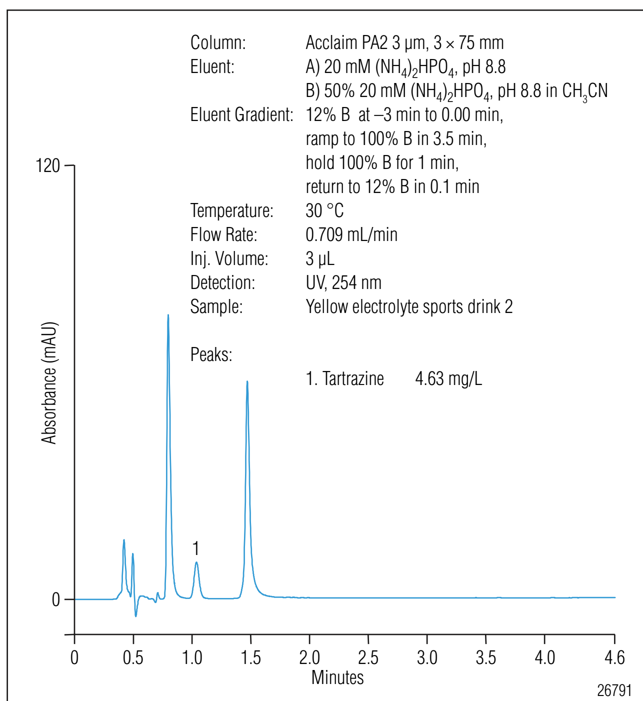

Figure 4. Chromatogram of electrolyte sports drink 2.

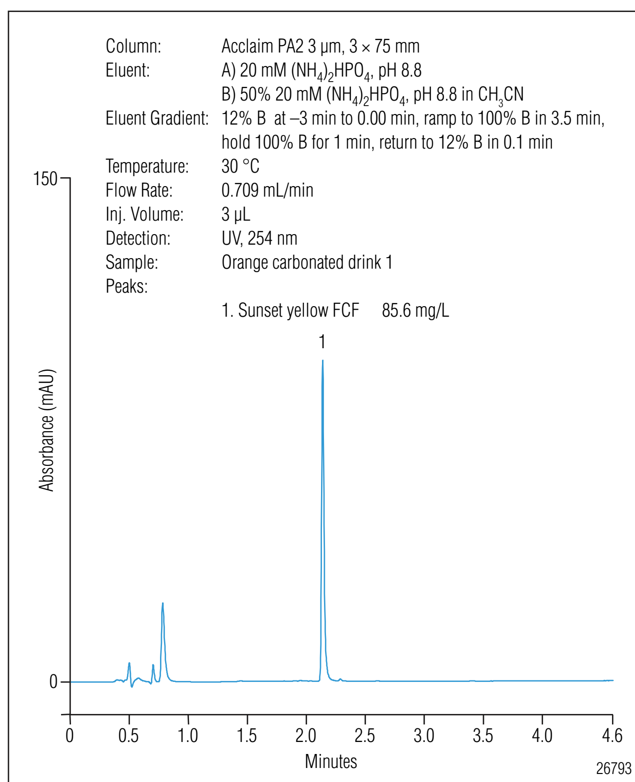

Figure 6. Chromatogram of carbonated drink 1.

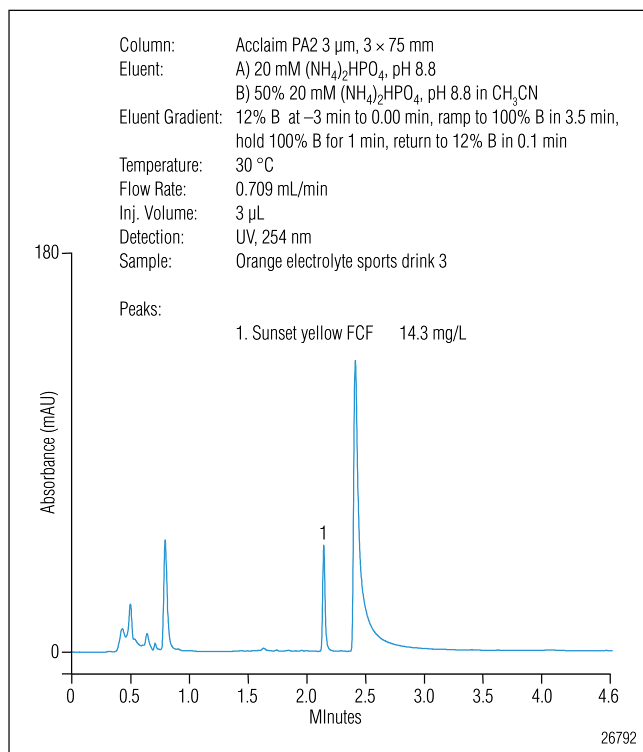

Figure 5. Chromatogram of electrolyte sports drink 3.

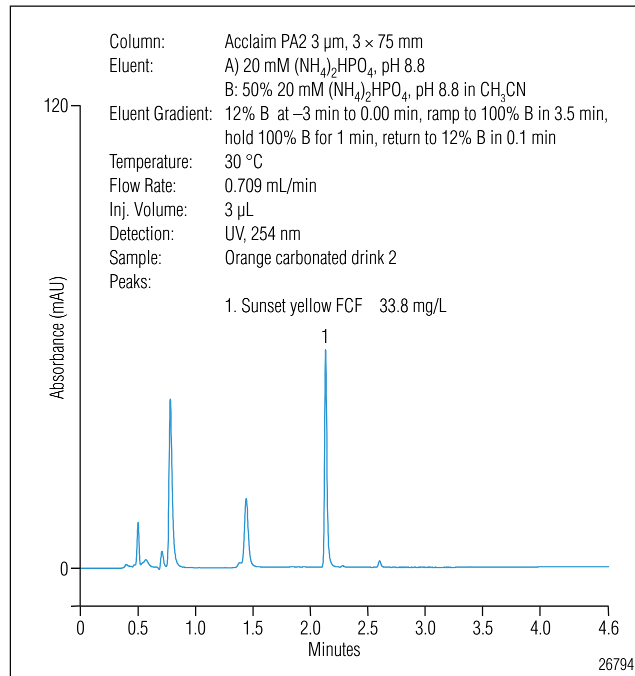

Figure 7. Chromatogram of carbonated drink 2.

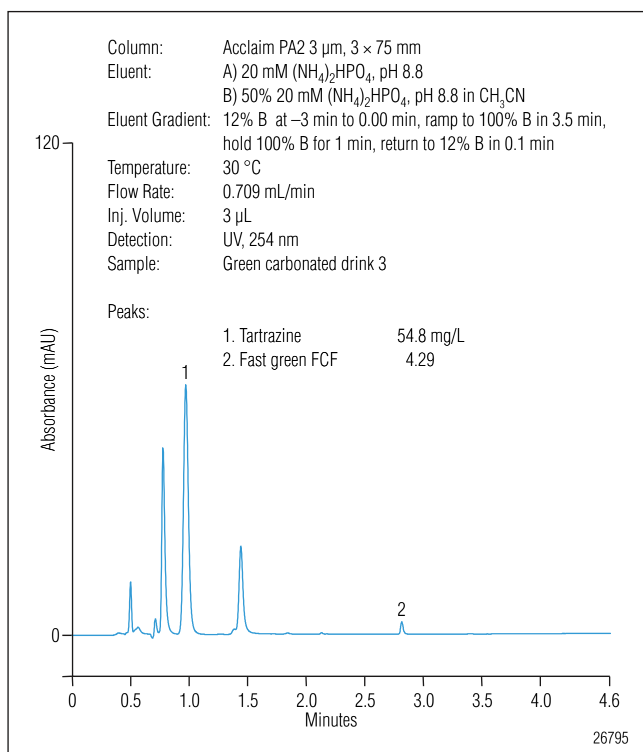

Figure 8. Chromatogram of carbonated drink 3.

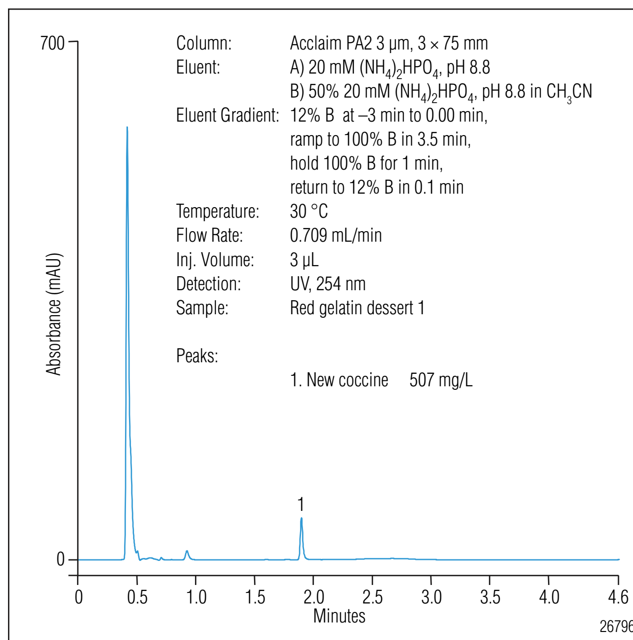

Figure 9. Chromatogram of the red gelatin dessert sample.

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

Sample	Color	Dyes Found in Samples and Spiked Samples	Concentrations Found in Samples and Spiked Samples (mg/L) Injection #					RSD
			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

Table 5. Reproducibility of Five Injections of Samples and Spiked Samples

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

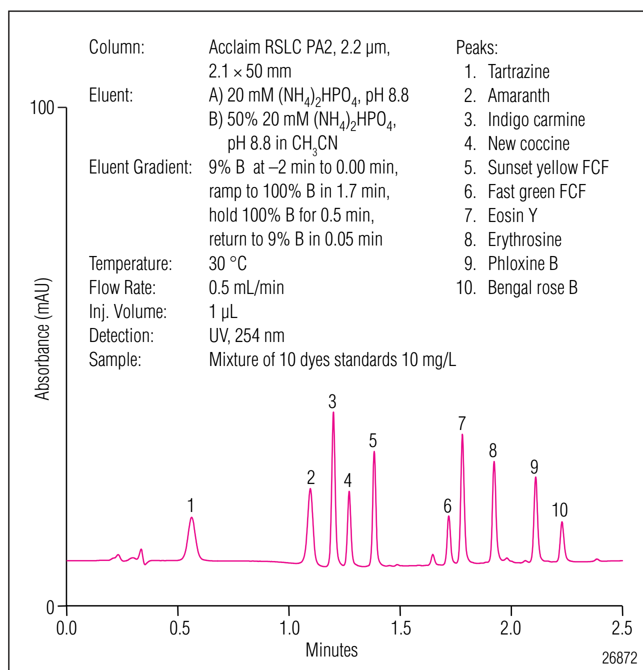

Figure 10. Faster separation of the 10 dyes standard.

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.

Application Note: 293

Steviol Glycoside Determination by HPLC with Charged Aerosol and UV Detections Using the Acclaim Trinity P1 Column

Deanna Hurum and Jeffrey Rohrer; Sunnyvale, CA USA

Key Words

- Stevia
- Rebaudioside A
- HILIC
- Natural Sweeteners

Introduction

In December 2008, the U.S. FDA recognized rebaudioside A purified from *Stevia rebaudiana* (Bertoni) as Generally Recognized as Safe (GRAS) for use as a sugar substitute in foods.[1,2] Since this recognition, stevia products have become popular as table-top and beverage sweeteners. Although the stevia plant and extracts from stevia leaves have long been used as sweeteners in Asia and Latin America, the terpene glycosides have different flavor profiles with both sweet and unpleasant bitter flavors.[3] Two steviol glycosides, stevioside and rebaudioside A, are largely responsible for the desired sweet flavor of the leaves (Figure 1), with rebaudioside A preferred for sweeteners.[4]

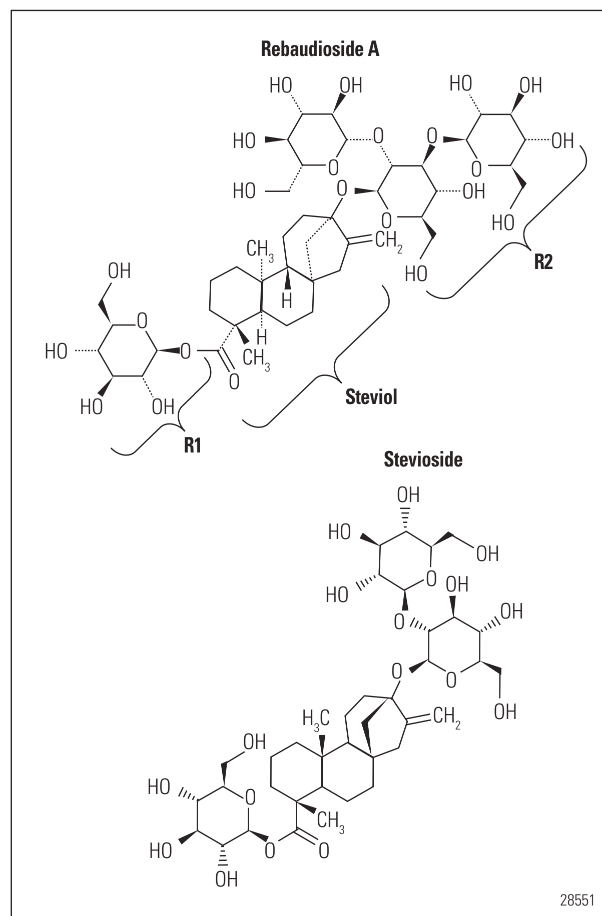

Figure 1: Chemical structures of the steviol glycosides stevioside and rebaudioside A.

Steviol glycoside determination is challenging for multiple reasons. The structures of the steviol glycosides are quite similar, differing in small changes in glycosylation. For example, rebaudioside B, an impurity that can be formed during processing of the leaves, differs in structure from rebaudioside A primarily by the presence or absence of a glucose residue at the R1 position on the terpene (Figure 2). These structural similarities make chromatographic separation difficult. In addition to the separation challenges, sensitive detection of these compounds also can be difficult. They do not absorb strongly in the UV, and typical detection wavelengths for steviol glycosides, such as 210 nm, are nonspecific. Other detection methods, such as charged aerosol, can be used in addition to UV detection to improve steviol glycoside quantification.

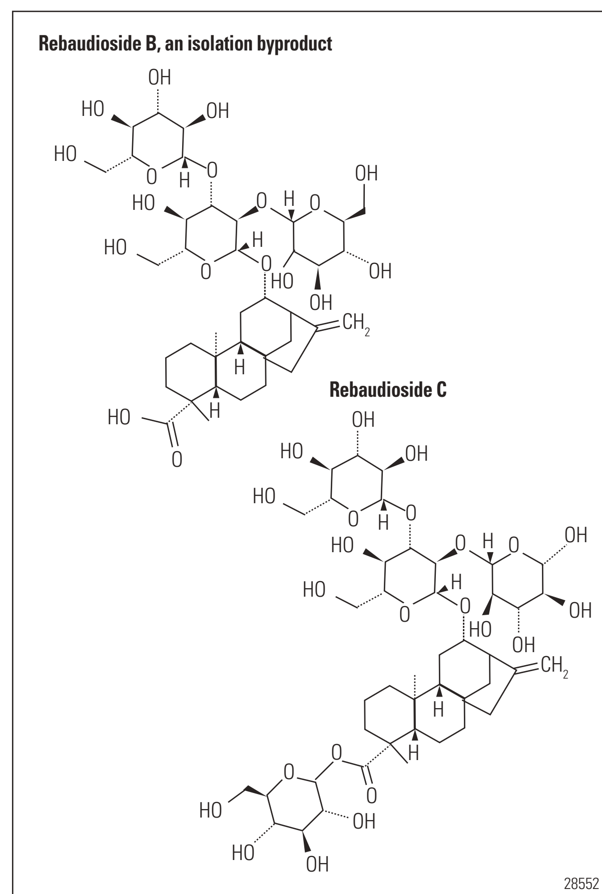

Figure 2: Chemical structures of rebaudioside B and rebaudioside C.

Thermo
SCIENTIFIC

Typically, individual steviol glycosides are determined by liquid chromatography methods using a polar amine column. The Joint FAO/WHO Commission on Food Additives (JECFA) has published a monograph for the determination of steviol glycosides by UV detection at 210 nm using 80/20 acetonitrile/water at pH = 3.0, adjusted with phosphoric acid.[5] This method specifies a 3.9–4.6 mm i.d. format column and can use flow rates of up to 2.0 mL/min to meet a retention time requirement of 10 min for stevioside. This high flow rate, coupled with a 25 min run time, results in 25–50 mL of mobile phase used per sample. The U.S. Pharmacopeia (USP) rebaudioside A monograph in the Food Codex is similar to the JECFA monograph. It uses an acetate buffer to adjust the pH rather than phosphoric acid, and a propylamine silane phase bonded to a silica gel column.[6] This method uses UV detection and a flow rate of 1.5 mL/min with a run time of up to 70 min, thereby requiring 105 mL of mobile phase per sample.

Here, steviol glycosides are determined by charged aerosol and UV detections in consumer sweeteners. The glycosides are separated on the Thermo Scientific Acclaim Trinity P1 column using 81/19 acetonitrile/ammonium formate buffer at pH = 3.0. This method uses the hydrophilic interaction liquid chromatography (HILIC) mode of the trimode column allowing separation of multiple steviol glycosides. By using a volatile mobile phase, charged aerosol detection is feasible, which adds further flexibility to the method for detection of glycosides that do not have strong UV extinction coefficients. Furthermore, the 2.1 mm i.d. column format reduces solvent use to 3–9 mL/sample compared to the range of 25–105 mL/sample for the JECFA and USP methods.

Equipment

Thermo Scientific Dionex UltiMate Rapid Separation LC (RSLC) system including:

- SRD-3600 Integrated Solvent and Degasser (P/N 5035.9230)
- HPG-3400RS Binary Pump with Solvent Selector Valves (P/N 5040.0046)
- WPS-3000TRS Analytical Autosampler (P/N 5840.0020)
- TCC-3000RS Thermostatted Column Compartment (P/N 5730.0000)
- DAD-3000RS Diode Array Detector (P/N 5082.0020)
- Thermo Scientific Dionex Corona *ultra* Charged Aerosol Detector (P/N 70-9298)

Polypropylene injection vials with caps and septa, 300 µL (Thermo Scientific Dionex P/N 055428)

Nalgene™ Filter Unit, 0.2 µm nylon membrane, 1 L capacity (Thermo Scientific Nalgene P/N 164-0020)

Reagents and Standards

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

pH buffer, 4.00 (VWR P/N BDH4018-500ML)

pH buffer, 2.00 (VWR P/N BDH5010-500ML)

Stevia Standards Kit, (ChromaDex P/N KIT-00019566-005) containing:

- Rebaudioside A
- Stevioside
- Rebaudioside B
- Rebaudioside C
- Dulcoside A
- Steviolbioside
- Rebaudioside D

Formic Acid (Sigma-Aldrich P/N 06440)

Ammonium Formate (Sigma-Aldrich P/N 51691)

Acetonitrile (Honeywell P/N 015-4)

Samples

Brand A: sweetener containing stevia leaf extract and inulin

Brand B: sweetener containing rebaudioside A and erythritol

Conditions

Column: Acclaim™ Trinity™ P1 (3 µm), 2.1 × 100 mm (P/N 071389)
Acclaim Trinity P1 (3 µm), 2.1 × 10 mm guard column (P/N 071391) with Guard Holder (P/N 069580)

Mobile Phase: 81/19 acetonitrile/10 mM ammonium formate, pH = 3.0

Flow Rate: 0.3 mL/min

Inj. Volume: 5 µL

Temperature: 20 °C

Detection: Diode Array UV-vis detector, 210 nm
Charged aerosol detector, nebulizer temperature, 10 °C

System Backpressure: ~1500 psi

Noise: ~0.15 mAU (UV)
~0.07 pA (charged aerosol)

Run Time: 10 or 30 min

Preparation of Solutions and Reagents

Mobile Phase Preparation

Transfer 0.63 g of ammonium formate to a 1 L bottle and add 1000 g (1000 mL) of DI water. Adjust the pH of the resulting 10 mM ammonium formate solution to 3.00 ± 0.05 by adding 1700 µL of formic acid. Using a precleaned (with DI water) 0.2 µm nylon filter unit, filter the stock buffer to remove insoluble particles.

Transfer 192.5 mL (192.5 g) 10 mM ammonium formate solution to a 1 L volumetric glass flask and add acetonitrile to fill the flask to the mark. The resulting solution will be approximately 644 g acetonitrile. Mix well. After converting the mass of acetonitrile to a volume, based on density, this method prepares a solution of 81/19 (v/v) acetonitrile/ammonium formate. Mixing aqueous ammonium formate and acetonitrile is endothermic and the solution will cool, resulting in a substantial reduction in volume. This volume change may cause variability in the actual mobile phase composition. These changes in the mobile phase composition will change analyte retention times, and for this reason, gravimetric preparation of the mobile phase will provide the most consistent retention times between mobile phase preparations. Allow the solution to return to ambient temperature before use.

Standards and Sample Solutions

Standards

Prepare individual 2.0 mg/mL steviol glycoside stock standards as shown in Table 1. Additionally, prepare a retention time standard of rebaudioside C by adding approximately 0.5 mg to 500 µL of mobile phase. Combine 50 µL of each solution (dulcoside A, stevioside, rebaudioside A, rebaudioside B, steviolbioside, and rebaudioside C) for a total of 350 µL of solution. Use this combined stock standard of 0.280 mg/mL steviol glycosides to prepare standards of 0.007 mg/mL to 0.190 mg/mL of dulcoside A, stevioside, rebaudioside A, steviolbioside, and rebaudioside B.

Table 1: Stock standards preparation.

Analyte	Amount (mg)	Volume Mobile Phase (µL)	Stock Concentration (mg/mL)
Dulcoside A	1.1	550	2.0
Stevioside	1.8	900	2.0
Rebaudioside A	1.6	800	2.0
Steviolbioside	1.4	700	2.0
Rebaudioside B	1.0	500	2.0

Samples

Prepare samples for analysis by extracting 0.10 g of sweetener with 10 mL of mobile phase. Brand A contains inulin, which is minimally soluble in organic solvents. Vortex the samples for a minimum of 20 s at least four times to dissolve the glycosides. Remove insoluble inulin by filtration through a 0.2 µm Acrodisc® IC syringe filter. Fully dissolve Brand B. Due to the higher concentrations of steviol glycosides in Brand A samples, dilute them fourfold with mobile phase before injection.

Precautions

Take care to consistently prepare the mobile phase. Changes in the ionic strength, pH, or organic content of the mobile phase can lead to shifts in analyte retention times. If chromatographic resolution decreases without a change in overall peak shape, reprepare the ammonium formate buffer, paying close attention to the amount of ammonium formate and the final pH. Increasing the amount of acetonitrile by up to 5% in the mobile phase will increase retention times, which may improve the resolution for complex samples; however the late-eluting peak sensitivity will decrease due to peak broadening from dispersion during the isocratic elution.

Metal contamination of the column will reduce both column efficiency and capacity. If reduced retention times coupled with poor peak shape are observed, remove the Corona™ *ultra*™ Charged Aerosol Detector from the flow path and follow the column wash procedure in Section 4 of the Acclaim Trinity P1 column manual.[7] Be sure to thoroughly equilibrate the column with the ammonium formate mobile phase before reconnecting the Corona *ultra* Charged Aerosol Detector.

For this work, a column temperature of 20 °C was chosen to maximize resolution between dulcoside A and components within stevia leaf extracts. Temperatures between 15–35 °C were evaluated. For the mobile phase conditions, 20 °C was determined to provide the best separation between the early eluting components. For samples which have been purified and do not contain raw leaf extracts, column temperatures between 20–30 °C may be used; however, a temperature-controlled column compartment is recommended to ensure consistent retention times of all components within a sample.

Table 2: Calibration (0.007–0.28 mg/mL for each steviol glycoside) and precision (n = 7) of standard injections. Precision values calculated for 7 injections of a 70 µg/mL standard of each of the glycosides.

Analyte	Detector	RT (min)	RT RSD (%)	Peak Area (mAU*min) or (pA*min)	Peak Area RSD (%)	Coeff. of Deter.	Calibration Model
Dulcoside A	UV: 210 nm	3.40	0.08	2.78	1.40	0.9997	linear
Dulcoside A	Charged Aerosol	3.44	0.25	1.10	1.03	0.9983	quadratic
Stevioside	UV: 210 nm	3.51	0.11	5.28	0.47	0.9996	linear
Stevioside	Charged Aerosol	3.54	0.24	2.15	0.70	0.9969	quadratic
Rebaudioside A	UV: 210 nm	4.42	0.14	2.90	0.58	0.9995	linear
Rebaudioside A	Charged Aerosol	4.46	0.18	1.62	0.53	0.9984	quadratic
Steviolbioside	UV: 210 nm	5.38	0.07	4.24	0.86	0.9996	linear
Steviolbioside	Charged Aerosol	5.42	0.15	1.70	0.35	0.9990	quadratic
Rebaudioside B	UV: 210 nm	6.30	0.09	4.62	0.87	0.9995	linear
Rebaudioside B	Charged Aerosol	6.33	0.21	2.30	0.75	0.9987	quadratic

Results and Discussion

Separation

Figure 3 shows the separation of steviol glycosides on the Acclaim Trinity P1 column. In Figure 3A, stevioside and rebaudioside A are easily observed by UV detection. In UV and charged aerosol detections, the rebaudioside A peak is well resolved with no interferences. Stevioside may closely elute with dulcoside A; however, the two peaks are clearly identifiable. Mogroside V was also added to the standard mixture, but is discussed separately.[8]

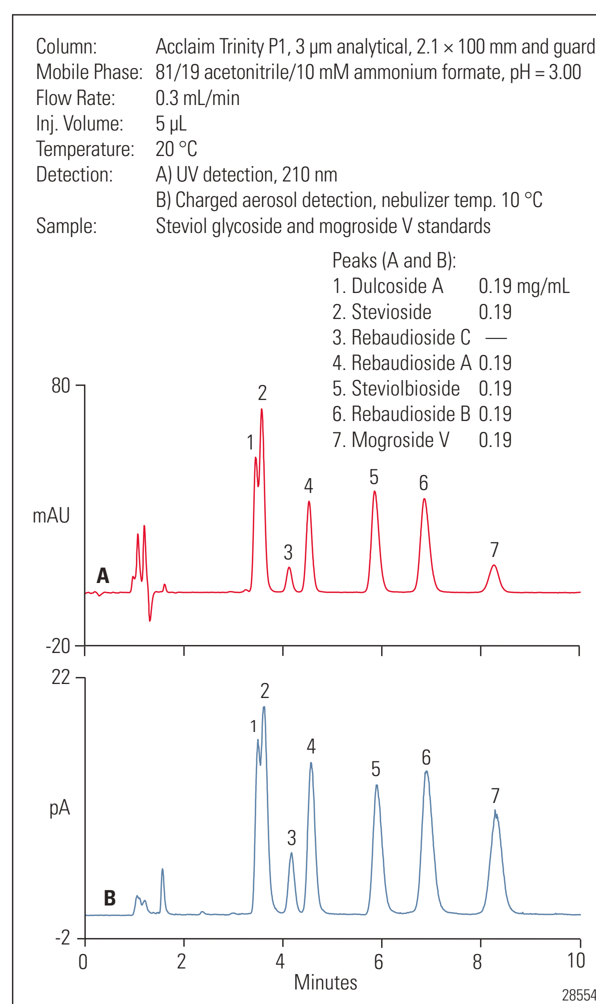

Figure 3: Separation of steviol glycoside standards on the Acclaim Trinity P1 column.

Quantification Assay Linearity, LOD, and LOQ

Table 2 shows the linearity for several steviol glycosides determined using UV detection. As shown, the coefficients of determination for UV detection are 0.9996 and 0.9995 for rebaudioside A and stevioside, respectively. The coefficients of determination for rebaudioside A and stevioside when detected by charged aerosol detection were 0.9984 and 0.9969, respectively. Calibration curves using charged aerosol detection are inherently nonlinear and were fit with quadratic curves. This nonlinearity is the result of physical interactions that contribute to charged aerosol detection. To fit the calibration curves for charged aerosol detection, use the quadratic fitting option within the Thermo Scientific Dionex Chromeleon Chromatography Data System software. Coefficient of determination values reported within Chromeleon™ software are from linear fits of converted data. These values are reported in Table 2 for each of the steviol glycosides investigated.

The limits of quantification (LOQs) and limits of detection (LODs) for the steviol glycosides are different for the two detection methods, and charged aerosol detection detects the steviol glycosides with greater sensitivity than UV at 210 nm (Table 3). However, the improvement in sensitivity varies by the individual compound. For example, the LOQ for rebaudioside A is improved by a factor of 3 from 7.0 µg/mL to 2.3 µg/mL by using charged aerosol detection compared to UV at 210 nm. For comparison, the sensitivity increases a factor of 2, from 2.3 µg/mL to 4.6 µg/mL, for steviolbioside.

Table 3: LOQ and LOD based on injections of standards.

	LOQ (µg/mL)		LOD (µg/mL)	
Analyte	**UV: 210 nm**	**Charged Aerosol**	**UV: 210 nm**	**Charged Aerosol**
Dulcoside A	4.6	2.3	1.4	0.7
Stevioside	4.5	1.4	1.2	0.4
Rebaudioside A	7.0	2.3	2.3	0.7
Steviolbioside	4.6	2.3	1.4	0.7
Rebaudioside B	7.0	2.3	2.3	0.7

Sample Analysis

Figure 4 shows the separation of a sweetener extract that is derived from extracted stevia leaves and dispersed in inulin. There are several steviol glycosides detected in this product. When analyzed by UV, which is standard by the JECFA and USP monographs, three steviol glycosides are easily determined. Stevioside, rebaudioside C, and rebaudioside A are each present and baseline resolved. When the sample is analyzed using charged aerosol detection, there are a number of additional peaks that are not observed by UV (Figure 4B). The early eluting unlabeled peaks are from unglycosylated terpenes. Rebaudioside A is resolved from other peaks, stevioside is separated from inulin present in this sample, and a small amount of steviobioside that is not obvious by UV is detected by charged aerosol detection. Quantification of stevioside and rebaudioside A is equivalent by both detection methods. Figure 5 shows an extended chromatogram of the sample in Figure 4. Because the Corona *ultra* Charged Aerosol Detector is a near-universal detector, the presence of sodium in a sample, which is likely in natural products, can be detected. Under these conditions, sodium elutes at 20.4 min and the run time must be extended to avoid coelution of the sodium with analyte peaks in following injections.

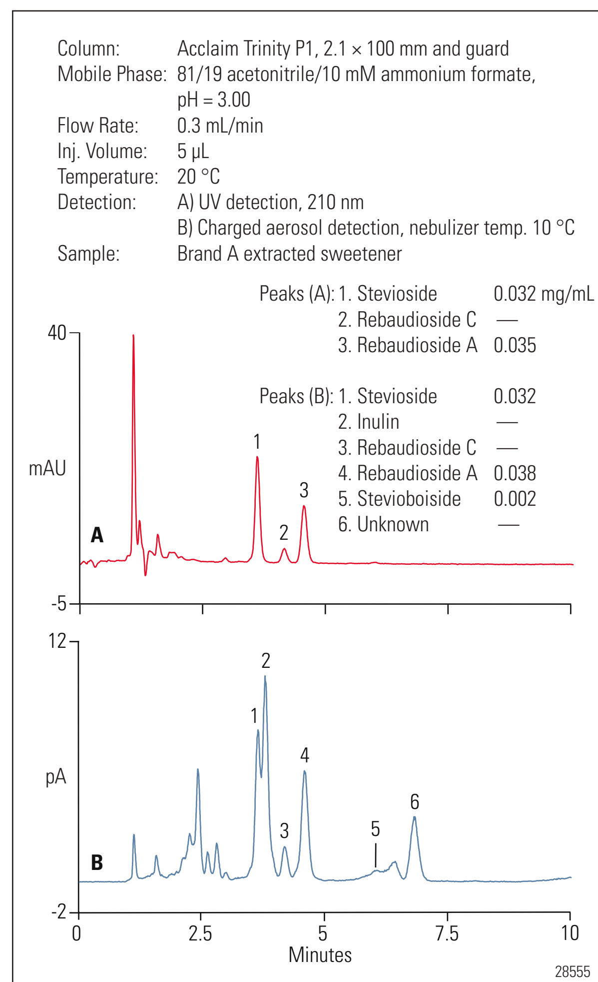

Figure 4: Separation of Brand A sweetener on the Acclaim Trinity P1 column and detected by A) UV and B) charged aerosol detections.

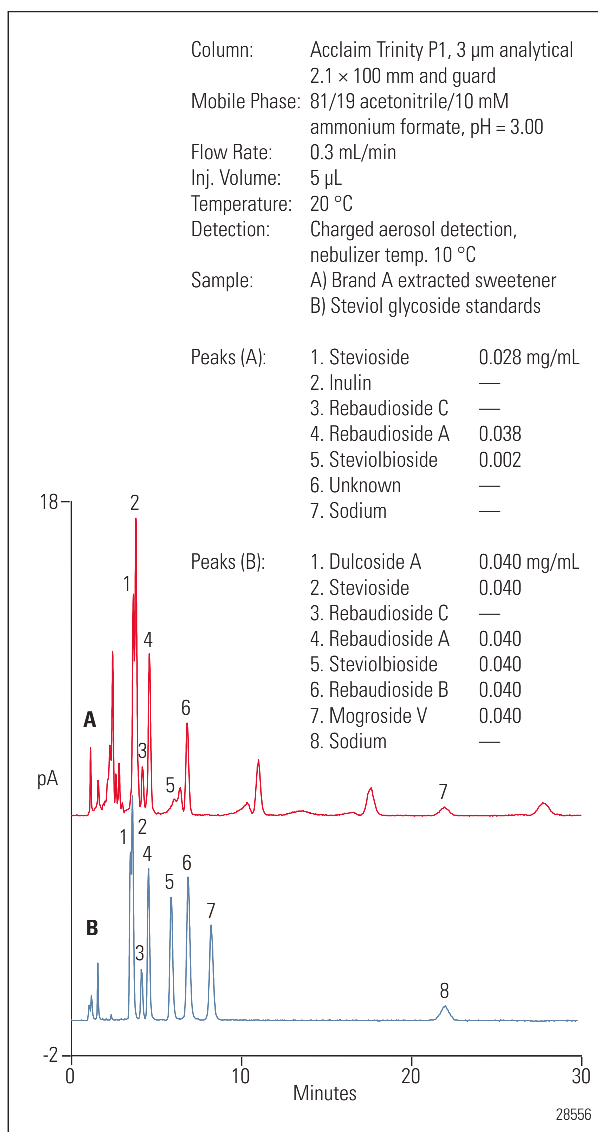

Figure 5: Separation of a A) stevia sweetener Brand A in comparison to B) standards on the Acclaim Trinity P1 column detected by charged aerosol detection.

Figure 6 shows the separation of a 10 mg/mL solution of a commercial table top sweetener composed of rebaudioside A, the FDA approved stevia sweetener. In this sweetener, the rebaudioside A is mixed with erythritol. As shown in Figure 6A, primarily rebaudioside A is observed when analyzing samples using UV detection, with a possible identification of rebaudioside B. When using charged aerosol detection (Figure 6B), erythritol, rebaudioside A, and a small amount of rebaudioside B are observed. The quantification of rebaudioside A is equivalent by both detection techniques.

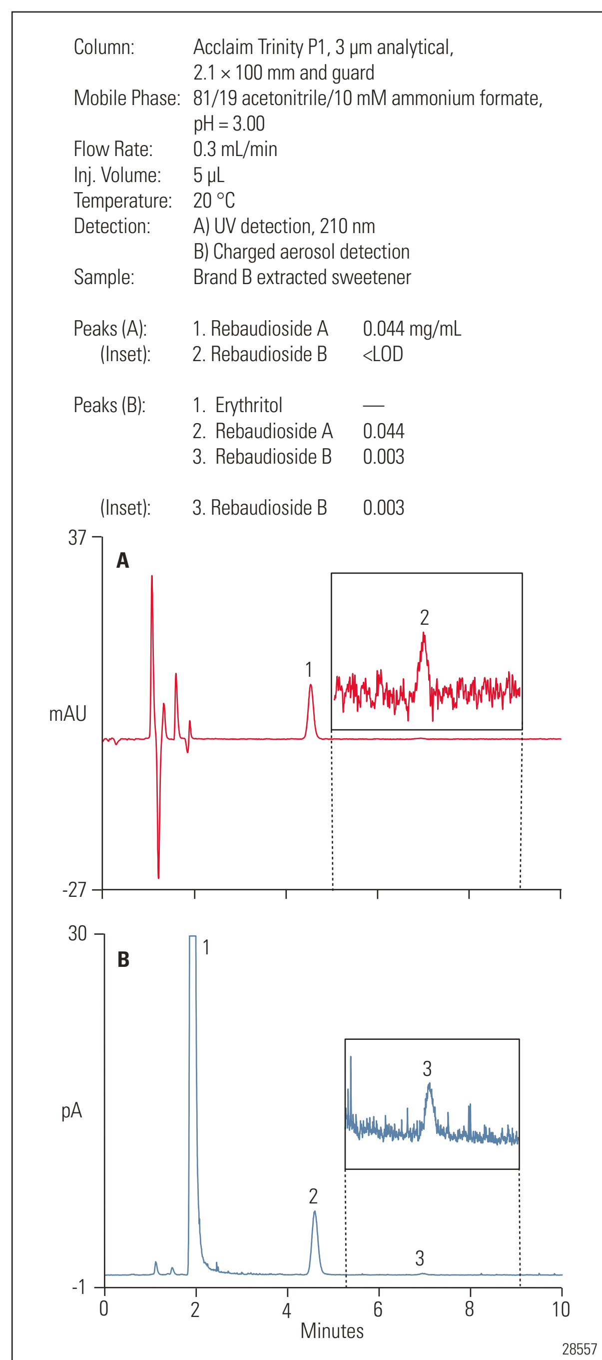

Figure 6: Rebaudioside A determination in Brand B, commercial sweetener, by A) UV and B) charged aerosol detection.

Precision and Accuracy

In addition to assay linearity, Table 2 also summarizes the standard injection precision data for both UV and charged aerosol detection. Peak area RSDs range from 0.47 to 1.40 with the charged aerosol detection peak area precision similar to the UV peak area precision. Retention time precision is excellent with retention time RSDs ranging from 0.07 to 0.25. Retention time variability between batches of mobile phase was within 1.3% for rebaudioside A. These data indicate that with consistent mobile phase preparation, the method is reproducible.

Table 4: Intra- and between-day precision of sweetener analysis.

Day/Sample	Analyte	Detector	Average mg Analyte/g Sweetener	Intraday Precision (RSD)	Interday Precision (RSD)
Day 1 Brand A	Rebaudioside A	UV	13	5.0	2.3
	Rebaudioside A	Charged Aerosol	15	1.5	4.5
	Stevioside	UV	12	5.1	1.6
	Stevioside	Charged Aerosol	10	6.2	10
Day 2 Brand A	Rebaudioside A	UV	14	4.6	—
	Rebaudioside A	Charged Aerosol	15	4.6	—
	Stevioside	UV	12	4.0	—
	Stevioside	Charged Aerosol	11	1.9	—
Day 3 Brand A	Rebaudioside A	UV	14	5.3	—
	Rebaudioside A	Charged Aerosol	16	11	—
	Stevioside	UV	12	3.1	—
	Stevioside	Charged Aerosol	12	10	—
Day 1 Brand B	Rebaudioside A	UV	3.9	4.4	12
	Rebaudioside A	Charged Aerosol	4.2	5.1	13
Day 2 Brand B	Rebaudioside A	UV	3.6	6.9	—
	Rebaudioside A	Charged Aerosol	3.8	6.7	—
Day 3 Brand B	Rebaudioside A	UV	4.8	7.1	—
	Rebaudioside A	Charged Aerosol	5.1	8.5	—

Table 5: Recovery of standards added to Stevia sweetener samples, n = 3.

	Analyte	Detector	Determined Amount with Spiking (mg/mL)	Amount Spiked (mg/mL)	Recovery (%)
Brand A	Rebaudioside A	UV	0.076	0.051	94
	Rebaudioside A	Charged Aerosol	0.080	0.051	85
	Stevioside	UV	0.076	0.051	98
	Stevioside	Charged Aerosol	0.068	0.051	87
Brand B	Rebaudioside A	UV	0.080	0.051	92
	Rebaudioside A	Charged Aerosol	0.079	0.051	88
	Stevioside	UV	0.053	0.051	104
	Stevioside	Charged Aerosol	0.046	0.051	92

Samples of the two sweeteners were analyzed in triplicate over three days. Table 4 shows the calculated mass of stevioside and rebaudioside A in a 1 g portion of the sweeteners along with intraday and interday precision. The determined concentrations of glycosides in the samples are consistent by both UV and charged aerosol detection. During three days of analysis, Brand B was determined to contain 3.6–4.8 mg/g rebaudioside A by UV and 3.8–5.1 mg/g by charged aerosol detection. This corresponds to the expected amount of ~4 mg/g specified for use as a sweetener.[2]

Brand A contains higher concentrations of steviol glycosides in addition to rebaudioside A than the commercial Brand B sweetener because Brand A is an extract of the stevia leaves without further purification to isolate an individual compound. Table 4 shows the calculated masses of stevioside and rebaudioside A in the sample. As with Brand B, the concentrations determined by UV and charged aerosol detection methods are well correlated. The intraday precision for Brands A and B, which range from 1.5–11% from triplicate sample preparations, are similar to the interday precisions of 1.6–10%.

Accuracy of the method was tested by spiking standards at known concentration into samples of Brand A and Brand B sweeteners (Table 5). Rebaudioside A recoveries range from 85–104% by charged aerosol detection and 82–102% by UV detection at 210 nm. These recoveries are quite comparable and suggest overall accuracy of the method. Similarly, stevoiside recoveries range from 81–98% by charged aerosol detection and 84–105% by UV detection.

Conclusion

Here, steviol glycosides are determined by charged aerosol detection and UV detection in consumer sweeteners. Using the Acclaim Trinity P1 column, the proposed method resolves the steviol glycosides of interest. Only 3–9 mL of mobile phase/sample are required compared to 25–60 mL/sample for the JECFA and USP methods, respectively. The reduction in mobile phase use provides savings in both time and resources during mobile phase preparation and reduces waste. Quantification of the two principal glycosides, stevioside and rebaudioside A, was accomplished by both detection methods. In addition, charged aerosol detection has the added advantage of distinguishing additional components in the sample that are not UV absorbing, allowing for a flexible method to check sweetener purity.

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

ChromaDex, 10005 Muirlands Blvd, Suite G, First Floor, Irvine,CA 92168 U.S.A., Tel: 949-419-0288. www.chromadex.com

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Application Note: 294

Simultaneous Analysis of Water-Soluble Vitamins in Vitamin-Enriched Beverages and Multivitamin Dietary Supplements by UHPLC-MS/MS

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Key Words

- TSQ Quantum Access
- WSV
- B Vitamins
- Vitamin C
- Food Analysis

Introduction

Vitamins are nutrients essential to human health, and are present in almost all types of foods. In addition to food sources, vitamin supplements are often consumed to ensure adequate vitamin intake, as a customary diet does not always provide sufficient vitamins due to bias and/or limitation in the choice of foods, or malfunctions in digestion and ingestion. Vitamin supplements are available in various forms such as single- or multivitamin tablets, formula, and vitamin-enriched beverages (VEB). Certain foods are commercially fortified with vitamins and/or other nutritional essentials such as minerals. Based on their solubility, vitamins are divided into two categories: water-soluble vitamins (WSV) and fat-soluble vitamins (FSV). WSVs include vitamin C (ascorbic acid), B_1 (thiamine), B_2 (riboflavin), B_3 (niacin, niacinamide), B_5 (pentothenic acid), B_6 (pyrodoxine), B_7 (biotin), B_9 (folic acid), and B_{12} (cyanocobalamine). Accurate quantitative measurements for vitamins are required to ensure product quality and regulatory compliance as well as to monitor vitamin intake.

Established methods for vitamin analysis include microbiological methods, which are typically designed for single vitamin analysis and are time consuming,[1,2] and chromatographic methods, including gas chromatography,[3,4] capillary electrophoresis,[5,6] and liquid chromatography (LC) with various methods of detection.[7–16]

LC methods are generally used for simultaneous determination of multiple vitamins of interests and for establishing vitamin profiles in a variety of matrices with various modes of detection.[7–10, 13–16] Here we present a high-throughput method for simultaneous determination for the above mentioned ten WSVs using ultrahigh-performance LC and tandem mass spectrometry (UHPLC-MS/MS). Chromatography was optimized for the total resolution of all target analytes on a Thermo Scientific Acclaim C30 reversed-phase (RP) column. An MS/MS instrument was operated in selected reaction monitoring (SRM) mode for the best selectivity and sensitivity, and an isotope labeled internal standard (IStd) was used for accurate quantitation.

Randomly selected VEBs and multivitamin supplement tablets (MVSTs) were assayed by this method for selected vitamins. Much higher values were observed for most vitamins in VEBs than indicated on product labeling. Results also showed close agreement between observed and label values for MVSTs.

Equipment

Thermo Scientific Dionex UltiMate 3000 Rapid Separation LC (RSLC) system including:

- DGP-3600RS Dual Ternary Rapid Separation Pump System
- SRD-3600RS Integrated Solvent and Degasser
- WPS-3000TRS Analytical Autosampler with 130 µL sample loop
- TCC-3000RS Thermostatted Column Compartment
- VWD-3400RS Four-Channel Variable Wavelength Detector

Thermo Scientific TSQ Quantum Access MAX Triple Stage Quadrupole Mass Spectrometer with heated electrospray ionization (HESI II) probe

Thermo Scientific Dionex DCMS^{Link} 2.11 software

Thermo Scientific Xcalibur 2.1 with the Foundation 1.0.2 Framework and TSQ Series 2.3 control software

Reagents

Deionized (DI) water, 18.2M –cm resistivity

Acetonitrile (CH_3CN), HPLC grade or equivalent, Fisher Scientific (AC610010040)

Ammonium formate, ≥99.995% trace metals basis, Sigma-Aldrich Co. LLC (516961)

Formic acid, Sigma-Aldrich Co. LLC (06440)

Thermo SCIENTIFIC

Standards

A set of water-soluble vitamin standards was purchased from AccuStandard, Inc. (VIT-WSK-R1-SET) including:

VIT-001N: Vitamin B1 hydrochloride (B_1)

VIT-002N: Vitamin B_2

VIT-003N: Vitamin B_6

VIT-004N: Vitamin C

VIT-005N: Niacin (B_3)

VIT-006N: Nicotinamide (B_3')

VIT-007N: Vitamin M (B_9)

VIT-008N: D-Pantothenic acid (B_5)

VIT-009N-R1: Vitamin H (B_7)

VIT-010N-R1: Vitamin B_{12}

Isotope labeled IStd: pyrodoxine-d_2, C/D/N Isotopes, Inc. (D-6819)

Prepare individual stock solution by dissolving appropriate amount of pure chemical in 1% formic acid at 1 mg/mL (1000 parts per million, ppm) unless noted. Prepare folic acid and riboflavin in basic solution at 1000 ppm and 100 ppm respectively (basified by ammonia, 4% and 0.7% respectively). Prepare IStd in 1% formic acid at 10 ppm to prepare calibration standards and spike unknown samples.

Prepare calibration standards in 0.1% formic acid from 10 parts per billion (ppb) to 5000 ppb at seven levels: 10 ppb, 50 ppb, 100 ppb, 500 ppb, 1000 ppb, 2000 ppb, and 5000 ppb from stock solution by series dilution.

Divide target analytes into three groups: Group 1 to contain only B_9 (folic acid); Group 2 to contain B_7 (biotin) and B_{12} (cyanocobalamine); and Group 3 to contain B_1, B_3 (niacin and niacinamide), B_5 (pantothenic acid) and B_6 (pyridoxine). Spike IStd in each calibration standard at 500 ppb.

Preparation of Vitamin-Enriched Beverage Samples

Vitamin-enriched beverage (VEB) samples were randomly selected and purchased from a local grocery store and kept at room temperature until analysis. Degas carbonated VEBs using a sonication bath for 30 s. Transfer 1 mL of each sample to a 1.5 mL autosampler vial, spike with IStd at 500 ppb, vortex mix, and analyze for Group 1 and Group 2 vitamins. Pipet 10 µL of each sample to another 1.5 mL autosampler vial, dilute with 990 µL DI H_2O, spike with IStd at 500 ppb, vortex mix, and analyze for Group 3 vitamins.

Preparation of Multivitamin Tablet Samples

Three bottles of multivitamin tablets were purchased from the same grocery store. Weigh 20 tablets from each bottle to calculate the average weight of one tablet. Grind the 20 weighed tablets to fine powder in a coffee grinder (Cuisinart, DCG-12BC) for 1 min (20 s × 3). Weigh three subsamples from each ground sample to 0.1 g in a 15 mL centrifuge tube, recording exact weight. Dissolve each subsample in 10 mL DI H_2O in a sonicator bath for 30 min, spiking the IStd at 500 ppb. Centrifuge the samples for 15 min at 4000 RPM. Pipet 1 mL of the clear supernatant from each sample to a 1.5 mL amber autosampler vial for the analysis of Group 1 and Group 2 vitamins. Pipet 10 µL of supernatant from each sample to another 1.5 mL amber autosampler vial, dilute with 990 µL DI H_2O, spike with IStd to 500 ppb, vortex mix, and analyze for Group 3 vitamins.

UHPLC-MS/MS Conditions

Chromatographic Condition

System: UltiMate™ 3000 RSLC
Column: Acclaim™ C30 (P/N 075725)
Dimensions: 2.1 × 150 mm, 3 µm
Mobile Phases: A) Ammonium formate, pH 4.0
B) Ammonium formate, pH 3.0
C) 90% Acetonitrile/10% NH_4OOCH pH 3.0 Buffer at 10mM in each component
Gradient events listed in Table 1
Flow Rate: 0.6 mL/min
Temperature: 15 °C
Injection: 10 µL, 20 ppm of each vitamin
Detection: UV at 260 nm or TSQ Quantum Access MAX™ Triple Stage Quadrupole MS/MS detection mode

Mass Spectrometric Condition

System: TSQ Quantum Access MAX Triple Stage Quadrupole Mass Spectrometer
Interface: Heated electrospray (HESI II)
Spray Voltage: 4000 V
Vaporizer Temp.: 350 °C
Capillary Temp.: 200 °C
Sheath Gas: 40 arbitrary units
Auxiliary Gas: 60 arbitrary units
Detection Mode: SRM (see Table 2 for details of SRM events)

Table 1: Mobile phase gradient events.

Time (min)	% A	% B	% C
-5.0	100	0	0
0.0	100	0	0
3.5	100	0	0
3.6	0	100	0
12.0	0	70	30
12.1	0	20	80
14.9	0	20	80
15.0	100	0	0

Table 2: SRM MS/MS events and parameters.

Analyte		Retention Time (min)	Scan Time (min)	Precursor (*m/z*)	Product (*m/z*)	Collision Energy (V)
Ascorbic Acid	C	1.2	0–1.4	-175	-87	21
					-115	13
Niacin	B_3	1.7	1.4–2.5	124	80	22
					78	22
Thiamine	B_1	3.0	2.5–4.7	265	122	15
					124	13
Pyridoxine	B_6	5.0	4.7–7.0	170	134	21
					152	13
IStd: Pyrodoxine-d_2	IStd	5.0	4.7–7.0	172	136	21
					154	13
Niacinamide	B_3'	5.8	4.7–7.0	123	80	20
					78	24
Pantothenic Acid	B_5	7.2	7.0–9.0	220	90	14
					184	13
Cyanocobalamine	B_{12}	10.6	9.0–15.0	679	147	37
				1356	1209	53
Folic Acid	B_9	10.9	9.0–15.0	-440	-311	23
					-175	39
Biotin	B_7	11.2	9.0–15.0	245	227	15
					97	33
Riboflavin	B_2	11.7	9.0–15.0	377	295	16
					243	21

Results and Discussion

Chromatography

Although many LC methods have been reported for simultaneous analysis of WSVs, these methods usually suffer from low throughput or incomplete chromatographic resolution, and several highly hydrophilic analytes are poorly retained on the commonly used C18 RP columns. In this study, a C30 column was used to improve the retention of poorly retained analytes, such as vitamin C and thiamine. In addition, ammonium formate was buffered at two pH conditions: pH 3.0 and pH 4.0, with the higher pH buffer used in the early phase of the gradient to further improve the retention for thiamine, and the lower pH buffer used to provide complete resolution for later eluted vitamins.

Under the optimized conditions, all target vitamins were baseline separated within 12 min. The minimum retention factor was observed for vitamin C at 1.3 (retention time 1.2 min), and the retention factor for thiamin was observed at 4.5, which was significantly improved over previously reported methods where thiamin eluted first with a retention factor of less than 1.[14,15] Vitamins B_5 and B_7 lack a UV chromophore and were not visible in the UV chromatogram. However, the peak labels for both analytes are shown in Figure 1A to demonstrate the chromatographic separation. These two analytes were detected by MS/MS with great sensitivity, as seen in Figure 1B and Figure 1C.

Chromatographic Condition

System: UltiMate 3000 RSLC
Column: Acclaim C30
Dimensions: 2.1 × 150 mm, 3 µm
Mobile Phases: A) Ammonium formate, pH 4.0
B) Ammonium formate, pH 3.0
C) 90% Acetonitrile/
10% ammonium formate, pH 3.0
Buffer at 10 mM in each component
Gradient events listed in Table 1
Flow Rate: 0.6 mL/min
Temperature: 15 °C
Inj. Volume: 10 µL
20 ppm of each vitamin
with UV detection
50 ppb of each vitamin
with MS/MS detection
IS pyridoxine-d_2 at 500 ppb
Detection: A. UV at 260 nm
B and C. SRM

Mass Spectrometric Condition

System: TSQ Quantum Access Max
Interface: Heated Electrospray (HESI)
Spray Voltage: 4000 V
Vaporizer Temp.: 350 °C
Capillary Temp.: 200 °C
Sheath Gas: 40 arbitrary units
Auxiliary Gas: 60 arbitrary units
Detection Mode: SRM (see Table 2 for details of SRM events)

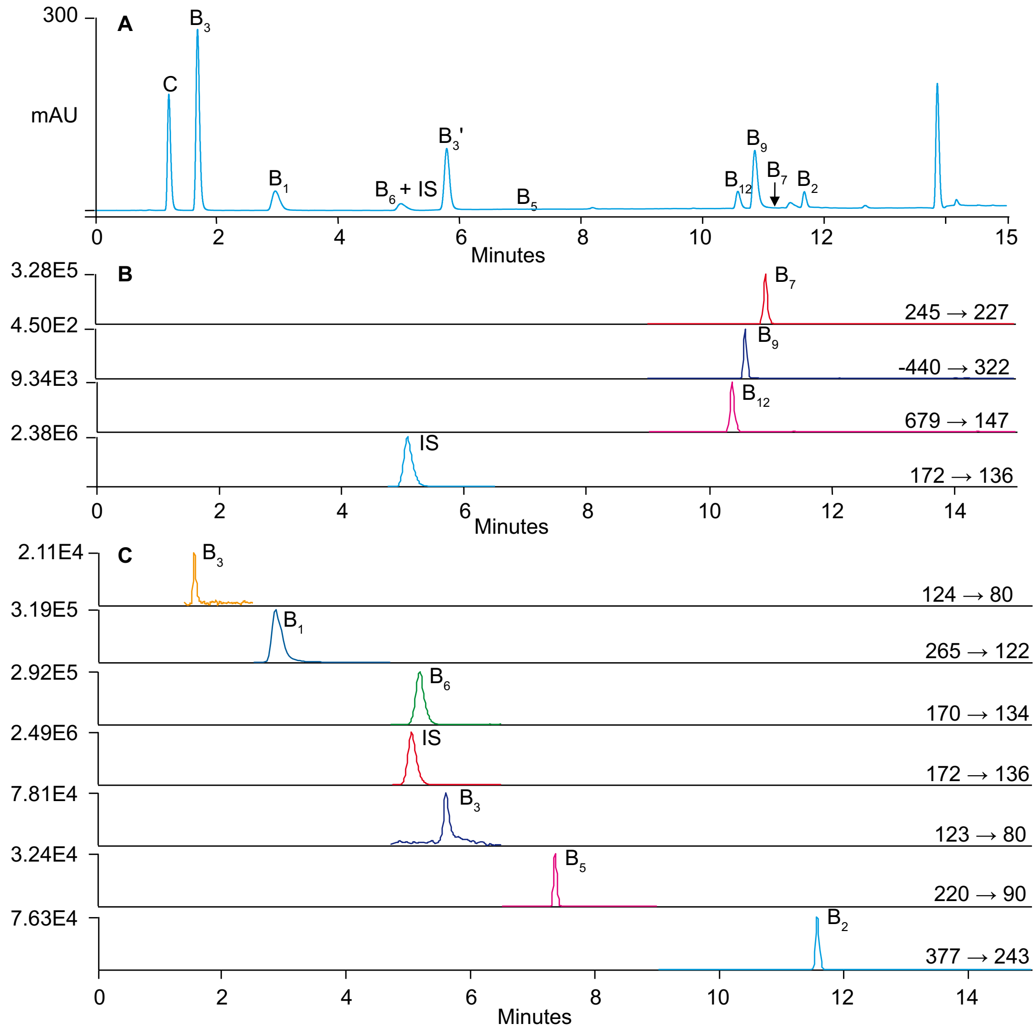

Figure 1: A) UV chromatograms of all target WSVs.
B) Q-SRM chromatograms of Group 1 and 2 vitamins.
C) Q-SRM chromatograms of Group 3 vitamins.

Mass Spectrometry

Electrospray ionization (ESI) was used in this study as the ionization interface due to its suitability and better sensitivity for polar compounds than other atmospheric pressure ionization (API) techniques. Ionization source parameters for the HESI probe used in this study were optimized to provide best sensitivity and were described in the sample preparation section. Under the optimized chromatographic and ionization conditions, most analytes exhibited strong protonated molecular ions (i.e., $[M+H]^+$) except for vitamin C and folic acid where deprotonated molecular ions $[M–H]^-$ were observed as the dominant MS peak. A strong doubly charged MS peak was observed for vitamin B_{12} at 679 *m/z* as well as the $[M+H]^+$ at 1356 *m/z*. For each analyte, the two most intense fragments were selected as the monitored product ions, which are listed in Table 2 along with the optimized collision energies. Thus for each analyte, two SRM transitions were monitored with one being quantitative SRM (Q-SRM), which showed relatively stronger MS response, and the other being confirmative SRM (C-SRM). The Q-SRM chromatograms are shown in Figure 1B and Figure 1C, with each of the vitamins at 50 ppb. The MS/MS detection demonstrated great sensitivity and selectivity for vitamin analysis even at low ppb levels. At 50 ppb, the minimum signal-to-noise ratio (S/N) was observed at 20 for niacin (26 for niacinamide) with the rest of the target analytes showing S/N greater than 100. The great sensitivity provided by MS/MS instrumentation enables the quantitation for low level vitamins such as B_{12} and folic acid in complex matrices, which was not achievable with previously reported methods using only UV detection.

Quantitation

One of the challenges encountered in this study was the large differences in concentration of the vitamins present in beverages or tablets. In the tested samples, the concentrations of Group 3 vitamins (mg levels per serving) were roughly 1000 times the concentrations of Group 1 and 2 vitamins (µg levels per serving). The lowest concentration observed was 0.6 µg per serving (B_{12}) while the highest concentration was at 20 mg per serving (B_3).

A single assay trying to cover the whole concentration range is beyond the linear response range of any mass spectrometer. Two approaches are usually practiced to address this challenge among reported methods covering these wide concentration ranges. Some reported methods use MS for lower concentration analytes and less sensitive detectors such as UV for the quantitation of high concentration vitamins,[14] thus losing the selectivity of MS quantitation and may suffer from interferences and/or lower quantitation accuracy. Another approach performs several assays for each sample with different dilution factors and results are reported with the most appropriate dilution. In this study, the latter approach was used and two assays were performed: the primary one quantitating low concentration vitamins including B_7, B_9, and B_{12}, which were assayed directly after spiking IStd; and the secondary assay quantitating the remaining vitamins at higher concentrations after a 100-fold dilution and respiking the diluted sample with IStd to 500 ppb. This technique took full advantage of the selectivity and specificity provided by MS detection, thus ensuring quantitation accuracy.

Stability of vitamins in solution was another challenge. Vitamin C was extremely unstable in multivitamin solutions, and degradation was observed within 20 min even though the sample was prepared in acidic solution and placed in a thermostatted autosampler at 4 °C. Instability of the analyte itself can cause substantial variance in the quantitative determination of vitamin C, and thus it was not included for quantitation in this study. Instability was also reported for other vitamins, such as riboflavin, pyridoxine, and thiamin, which are light sensitive,[17,18] and thiamin, pentothenic acid (in acid or basic condition), folic acid, and pyridoxine, which are heat labile. To avoid loss of analytes during analysis, samples were prepared in amber autosampler vials and promptly placed in the refrigerated autosampler at 4 °C.

Table 3: Calibration, coefficient of determination, precision, accuracy and detection limits.

Analyte		Calibration Range	r^2	50 ppb*			2000 ppb			LOQ (S/N)
				Mean	% RSD	% Accuracy	Mean	% RSD	% Accuracy	
B_3	Niacin	50–5000	0.9998	43.6	6.14	87.2	1960	3.34	98.0	50 (>10)
B_1	Thiamine	10–5000	0.9999	42.0	3.59	84.0	2029	2.92	101.5	10 (>200)
B_2	Riboflavin	10–5000	0.9996	44.8	3.03	89.6	1864	4.12	93.2	10 (>10000)
B_3	Nicotinamide	50–5000	0.9999	46.0	3.60	91.9	1844	2.93	92.2	50 (>14)
B_5	Pantothenic acid	10–5000	0.9999	44.7	4.57	89.4	1882	3.27	94.1	10 (>100)
B_6	Pyridoxine	10–5000	1.0000	50.4	1.22	101.0	1940	1.66	97.0	10 (>40)
B_7	Biotin	10–5000	0.9985	47.8	4.22	95.6	1910	4.15	95.5	10 (>1000)
B_9	Folic Acid*	100–5000	0.9984	113.0	15.8	113.0	1946	4.73	97.3	100 (>1000)
B_{12}	Cyanocobalamine	10–5000	0.9977	47.7	10.2	95.4	1735	4.61	86.8	10 (>1000)

All precision and accuracy results were summarized from seven replicate assays. *Precision and accuracy results for folic acid obtained from 100 ppb and 2000 ppb standards.

An additional challenge for accurate quantitation was the interactions of vitamins when present together in solution. Interactions between B12, folic acid, and riboflavin have been reported,[19–22] thus targeted vitamins were divided into three groups with additional consideration of their concentrations in samples: Group 3 included higher concentration vitamins (B1, B2, B3, B5, and B6) and Group 2 included lower concentration vitamins (B7 and B12). Although folic acid was also present in lower concentration in samples and could be included in Group 2 vitamins, observations revealed that quantitation of low concentration folic acid could be significantly interfered with by the presence of B7 and B12. Thus three calibration standard sets were prepared for the three groups of vitamins to generate individual calibration curves for quantitation.

Method Performance

Method performance was evaluated against calibrations, coefficients of determination, precision, and accuracy. Calibration curves for each analyte were generated from calibration standards with concentrations from 10 ppb to 5000 ppb at seven levels. Quadratic fits were used to fit the experimental data and 1/x was used as the weighting factor. Detailed results are shown in Table 3. Excellent coefficients of correlation, precision, and accuracy were achieved for each target vitamin. Limits of quantitation (LOQs) were determined as the lowest concentration in calibration standards exhibiting signal-to-noise ratios (S/N) greater than 10. LOQ was observed at 10 ppb for most analytes, except for niacin and niacinamide at 50 ppb, and folic acid at 100 ppb. Although S/N for folic acid was achieved with values much greater than 10 at lower concentrations, poor quantitation accuracy was observed, which was believed to be due to the reduced stability of this analyte when present in solution at low concentration. The instruments used in this study are capable of quantification of target vitamins below the LOQs set in this method, proven by the S/N values observed at LOQ. However, this method was designed and the calibration range set to minimize sample preparation procedures, number of dilutions, and assays to be run in order to maintain a high analytical throughput.

Analysis of Vitamin-Enriched Beverages Samples

As described in the sample preparation section, ten beverage samples were selected and analyzed for their vitamin content. Among the selected beverage samples, five were vitamin-fortified water samples with different flavors, and the other five were vitamin-enriched energy drinks. The results are shown in Table 4. Large differences were observed between measured and labeled values, and this observation agreed with previously conducted studies.[23,24] An explanation for these descrepancies could be that the fortification was performed at levels higher than label claims, deviating in the direction of no harm,[24] to compensate for extrapolated degradations during storage and shelf life.

Analysis of Multivitamin

Three types of MVST samples were randomly selected and analyzed for target vitamins. Contrary to results for VEBs, the measured amounts were within good agreement to their label values, as seen in Table 5. The observed label agreement variance between beverage and tablet samples may suggest differences in vitamin stability when in different formulations, i.e., solution or tablet.

Table 5: Water-soluble vitamins in multivitamin tablets.

Analyte	MVST-1	MVST-2	MVST-3
Thiamine (mg/serving)	1.20 (1.5)	1.3 (1.5)	1.7 (1.5)
Riboflavin (mg/serving)	2.40 (1.7)	2.1 (1.7)	2.0 (1.7)
Nicotinamide (mg/serving)	20.00 (20)	19.0 (20)	20.0 (20)
Pantothenic acid (mg/serving)	11.00 (10)	11.0 (10)	11.0 (10)
Pyridoxine (mg/serving)	2.70 (2)	2.5 (2)	3.8 (3)
Biotin µg/serving)	29.00 (30)	25.0 (30)	25.0 (30)
Folic Acid (µg/serving)	590.00 (400)	173.0 (400)	247.0 (400)
Cyanocobalamine (µg/serving)	7.10 (6)	6.0 (6)	27.0 (25)

Label values of vitamins are included in parentheses.
Duplicate assays were performed for each sample.

Table 4: Water-soluble vitamins in vitamin-enriched beverages.

Analyte	VEB-1	VEB-2	VEB-3	VEB-4	VEB-5	VEB-6	VEB-7	VEB-8	VEB-9	VEB-10
Riboflavin (mg/serving)							2.2 (1.7)		5.3 (3.4)	1.1 (0.7)
Nicotinamide (mg/serving)	9.7 (8)	2.2 (2)	3.9 (4)	2.6 (2)	12.0 (8)	20.0 (20)	24.0 (20)	23.0 (20)	26.0 (20)	20.0 (10)
Pantothenic acid (mg/serving)	4.3 (4)	1.6 (1)	3.0 (2)	3.5 (1)	8.3 (4)	5.3 (5)		24.0 (10)	24.0 (10)	3.9 (2.5)
Pyridoxine (mg/serving)	1.1 (0.8)	0.35 (0.2)	0.75 (0.4)	0.40 (0.2)	1.6 (0.8)	6.3 (5)	2.8 (2)	3.8 (2)	4.1 (2)	6.5 (2.5)
Cyanocobalamine (µg/serving)					4.7 (2.4)	3.6 (4.8)	5.4 (6)	7.9 (6)	8.0 (6)	1.1 (2.4)

Label values of vitamins are included in parentheses. Duplicate assays were performed for each sample.

Conclusion

This study describes a UHPLC-MS/MS method for simultaneous quantitation of WSVs in beverages and supplement tablets. This method demonstrated excellent correlation of determination, precision, accuracy, and selective and sensitive detection with low quantitation limits. This method was successfully applied to the determination of WSVs in beverages and supplement tablets with presented results.

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Application Update: 184

Mogroside V Determination by HPLC with Charged Aerosol and UV Detections

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Key Words

- Glycosides
- HILIC
- Natural Sweetners
- Sugar Substitutes

Introduction

Luo han kuo fruit (*Siraitia grosvenori* Swingle) has long been used in traditional Asian medicine. Recently cucurbitane-type and other triterpene glycosides have been isolated from the fruit and investigated for numerous potential health benefits such as antioxidant activity, anticancer effects, and antihyperglycemic effects.[1] Many of these compounds are intensely sweet and therefore have also been investigated as sugar substitutes and flavor enhancers. Extracts of luo han kuo fruit used as sweeteners were acknowledged as Generally Recognized as Safe (GRAS) based on a GRAS submission to the U.S. FDA in January of 2010.[2]

Typical reversed-phase high-performance liquid chromatography (HPLC) methods to determine these glycosides are challenging due to the lack of a strong, specific chromophore in the compound. Other detection methods, such as charged aerosol detection, can be used to improve triterpene glycoside quantification. In this work, mogroside V (Figure 1) is determined in a luo han kuo beverage by both charged aerosol and UV detections. This triterpene glycoside is separated on the Thermo Scientific Acclaim Trinity P1 column using 81/19 acetonitrile/ammonium formate buffer at pH = 3.0. The developed method uses hydrophilic interaction liquid chromatography (HILIC) conditions suitable for the trimode column, allowing separation of multiple terpene glycosides. The same method has also been used to separate steviol glycosides.[3] The volatile mobile phase makes charged aerosol detection possible, which adds further flexibility to the method for detection of such glycosides.

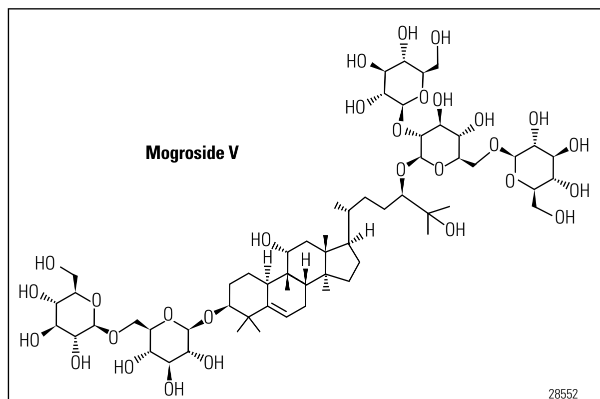

Figure 1: Chemical structure of mogroside V.

Equipment

Thermo Scientific Dionex UltiMate Rapid Separation LC (RSLC) system including:

- SRD-3600 Integrated Solvent and Degasser (P/N 5035.9230)
- HPG-3400RS Binary Pump with Solvent Selector Valves (P/N 5040.0046)
- WPS-3000TRS Analytical Autosampler (P/N 5840.0020)
- TCC-3000RS Thermostatted Column Compartment (P/N 5730.0000)
- DAD-3000RS Diode Array Detector (P/N 5082.0020)

Thermo Scientific Dionex Corona *ultra* Charged Aerosol Detector (P/N 70-9298)

Polypropylene injection vials with caps and septa, 300 µL (Thermo Scientific Dionex P/N 055428)

Nalgene™ Filter Unit, 0.2 µm nylon membrane, 1 L capacity (Thermo Scientific Nalgene P/N 164-0020)

Reagents and Standards

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

pH buffer, 4.00 (VWR P/N BDH4018-500ML)

pH buffer, 2.00 (VWR P/N BDH5010-500ML)

Stevia Standards Kit, (ChromaDex P/N KIT-00019566-005) containing:

- Rebaudioside A
- Stevioside
- Rebaudioside B
- Rebaudioside C
- Dulcoside A
- Steviolbioside
- Rebaudioside D

Mogroside V standard (ChromaDex P/N ASB-00013881)

Formic Acid (Sigma-Aldrich P/N 06440)

Ammonium Formate (Sigma-Aldrich P/N 51691)

Acetonitrile (Honeywell P/N 015-4)

Sample

Brand A: Luo han kuo beverage, supplied as a pair of water-soluble cubes (15.2 g)

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Conditions

Column:	Acclaim™ Trinity™ P1 (3 µm), 2.1 × 100 mm (Thermo Scientific Dionex P/N 071389) Acclaim Trinity P1 (3 µm), 2.1 × 10 mm guard column (P/N 071391) with guard holder (P/N 069580)
Mobile Phase:	81/19 acetonitrile/10 mM ammonium formate, pH = 3.0
Flow Rate:	0.3 mL/min
Inj. Volume:	5 µL
Temperature:	20 °C
Detection:	Diode Array UV-vis detector, 210 nm Charged aerosol detector, nebulizer temperature, 10 °C
System Backpressure:	~1500 psi
Noise:	~0.15 mAU (UV) ~0.07 pA (charged aerosol detection)
Run Time:	10–30 min, depending on sample matrix

Preparation of Solutions and Reagents

Mobile Phase Preparation

Transfer 0.63 g of ammonium formate to a 1 L bottle and add 1000 g (1000 mL) of DI water. Adjust the pH of the resulting 10 mM ammonium formate solution to 3.00 ± 0.05 by adding 1700 µL of formic acid. Using a precleaned (with DI water) 0.2 µm nylon filter unit, filter the stock buffer to remove any insoluble particles that may be present.

Transfer 192.5 mL (192.5 g) 10 mM ammonium formate solution to a 1 L volumetric glass flask and fill to the mark. The resulting solution will be approximately 644 g acetonitrile. Mix well. After converting the mass of acetonitrile to a volume, based on density, this method prepares a solution of 81/19 (v/v) acetonitrile/ammonium formate. Mixing aqueous ammonium formate and acetonitrile is endothermic and the solution will cool, resulting in a substantial reduction in volume. This volume change may cause variability in the actual mobile phase composition. These changes in the mobile phase composition will change analyte retention times, and for this reason, gravimetric preparation of the mobile phase will provide the most consistent retention times between mobile phase preparations. Allow the solution to return to ambient temperature before use.

Standards and Sample Solutions

Standards

Prepare a 2.0 mg/mL stock standard of mogroside V by adding 1.4 mg to 700 µL of mobile phase. Then use this stock standard to prepare standards of 0.06 mg/mL to 0.5 mg/mL of mogroside A by appropriate dilution in mobile phase. Steviol glycoside standards were added to standard solutions, in addition to mogroside V. For further details on these standards, see Application Note 293.[3]

Samples

Dissolve cubes in 100 mL of DI water. Further dilute a 100 µL sample aliquot by a factor of 20 in acetonitrile. Samples that show precipitates should be filtered through a 0.2 µm polyethersulfone (PES) membrane syringe filter.

Precautions

Take care to consistently prepare the mobile phase. Changes in the ionic strength, pH, or organic content of the mobile phase can lead to shifts in analyte retention times. If chromatographic resolution decreases without a change in overall peak shape, reprepare the ammonium formate buffer, paying close attention to the amount of ammonium formate and the final pH. Increasing the amount of acetonitrile by up to 5% in the mobile phase will increase retention times, which may improve the resolution for complex samples; however, the late-eluting peak sensitivity will decrease due to peak broadening from dispersion during the isocratic elution.

Metal contamination of the column will reduce both column efficiency and capacity. If reduced retention times and poor peak shape are observed, remove the Corona™ *ultra*™ Charged Aerosol Detector from the flow path and follow the column wash procedure in Section 4 of the Thermo Fisher Scientific, Inc. (formerly Dionex Corp.) Acclaim Trinity P1 column manual.[4] Be sure to thoroughly equilibrate the column with the ammonium formate mobile phase before reconnecting the Corona *ultra* detector.

For this work, a column temperature of 20 °C was chosen to maximize resolution between the steviol glycoside dulcoside A and components within stevia extracts.[3] For mogroside V, column temperatures between 20–30 °C may be used. The use of a temperature-controlled column compartment is highly recommended to ensure consistent retention times.

Results and Discussion

Separation

Figure 2 shows the separation of a mixed standard containing steviol glycosides and mogroside V within 10 min. In foods there is the potential presence of multiple sweeteners. In this example, steviol glycosides are well resolved from mogroside V, with the later eluting well after the steviol glycosides.

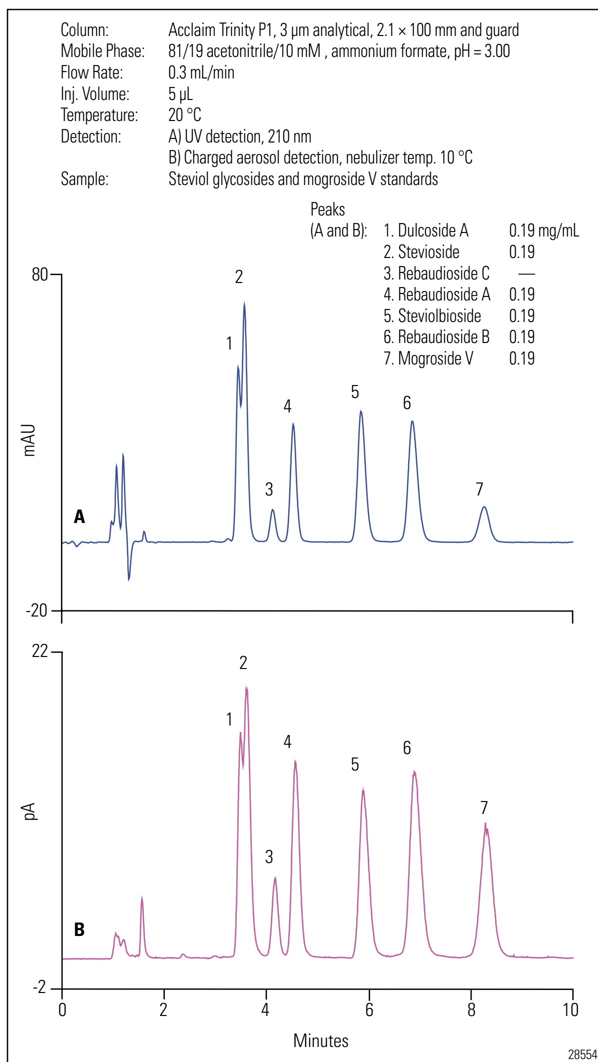

Figure 2: Separation of steviol glycosides and mogroside V standards on the Acclaim Trinity P1 column.

Quantification Assay Linearity and LOD

Table 1 shows the correlation between peak area and concentration for mogroside V determined using UV and charged aerosol detections. As shown, the coefficients of determination are 0.9995 and 0.9991 for mogroside V, by UV (210 nm) and charged aerosol detection, respectively. Calibration curves using charged aerosol detection are inherently nonlinear and were fit with quadratic curves. This nonlinearity is the result of physical interactions that contribute to the detection technique. To fit the calibration curves for charged aerosol detection, use the quadratic fitting option within the Thermo Scientific Dionex Chromeleon Chromatography Data System (CDS) software. Coefficient of determination values reported within Chromeleon CDS software are from linear fits of converted data.

The limit of detection (LOD), determined as 3× the signal-to-noise (S/N) ratio, for mogroside V was 1.4 µg/mL for charged aerosol detection and 7.0 µg/mL for UV detection. This is a fivefold difference in sensitivity between the two detection methods. A similar improvement in detection was determined when evaluating the Limit of Quantification (LOQ) by injecting standards that resulted in a signal which was 10× the S/N ratio.

Sample Analysis

Sucrose, a component of many beverages, including the one analyzed here, can potentially interfere with mogroside V determination. With the proposed method, sucrose elutes early and mogroside V is well retained. Mogroside V can be detected by both UV (210 nm) or charged aerosol detection with equivalent results in this sample (Figure 3.) However, for samples that contain natural products such as fruit extracts, sodium may be present. Under these conditions, sodium elutes at 20.4 min and the run time must be extended to avoid coelution of the sodium with analyte peaks in subsequent injections.

Table 1: Mogroside V calibration (0.007–0.28 mg/mL), LOD, LOQ, and method precision.

Detector	Coeff. of Deter.	Calibration Model	LOD (µg/mL)	LOQ (µg/mL)	RT (min)	RT (RSD)	Peak Area	Peak Area (RSD)
UV: 210 nm	0.9995	Linear	7.0	22.0	8.03	0.16	0.926 mAU*min	1.67
Charged Aerosol Detection	0.9991	Quadratic	1.4	4.6	8.07	0.07	1.72 pA*min	0.75

Precision values calculated for seven injections of a 70 µg/mL standard.

An expansion of the chromatogram shown in Figure 3 is shown in Figure 4. Charged aerosol detection is more sensitive to mogroside V than UV detection. In addition to the luo han kuo beverage, a mixed glycoside standard detected using charged aerosol detection is shown for retention time reference. Mogroside V is easily identified.

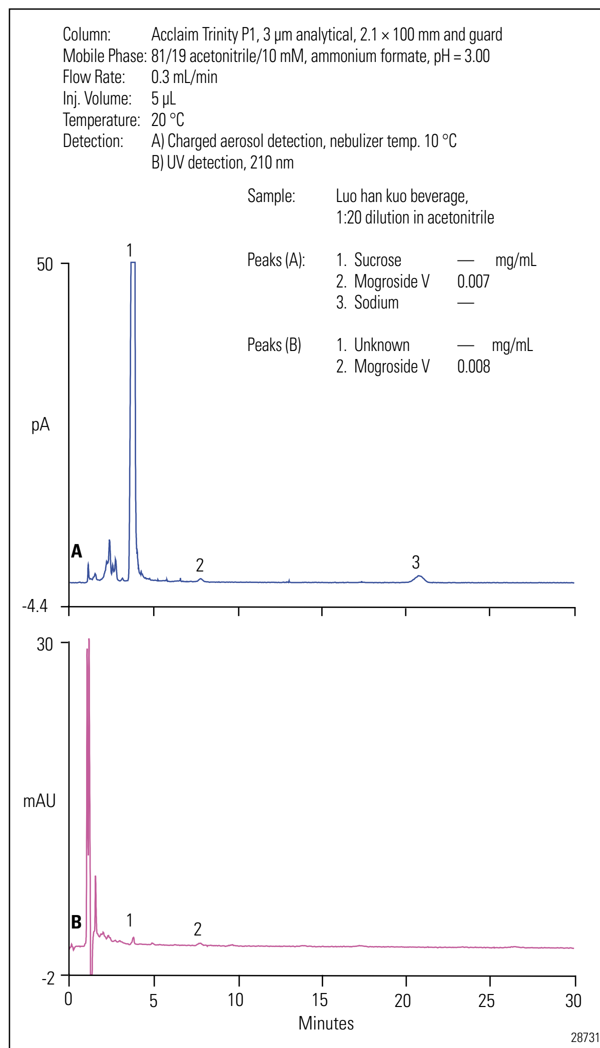

Figure 3: Separation of mogroside V in a luo han kuo beverage as detected by A) charged aerosol detection and B) UV, 210 nm. Note the good separation between sucrose and mogroside V in chromatogram A.

Precision and Accuracy

The chromatographic precision for determination of mogroside V is listed in Table 1. Retention time and peak area precisions are also listed in Table 1. Retention time precision (n = 7), as RSD, was very good at <0.2. Peak area precision (RSD) was <2.0. Mogroside V (0.050 mg/mL) was spiked into table-top sweeteners to evaluate separation from other components as well as recoveries from the sample matrix. Recoveries ranged from 89–105% with charged aerosol detection and 88–103% by UV detection, demonstrating method accuracy.

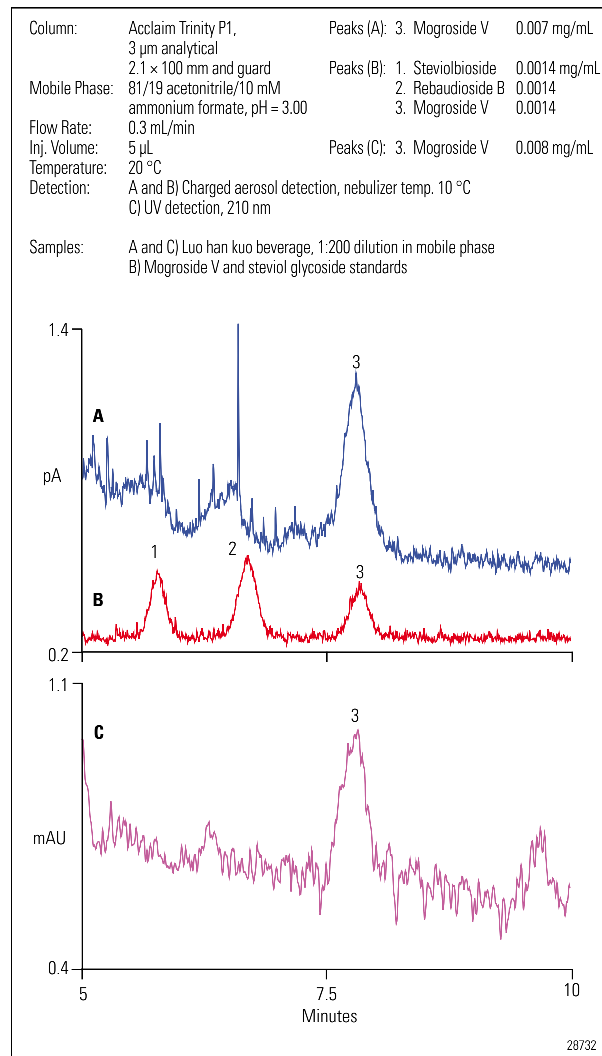

Figure 4: Expanded view of the separation of mogroside V detected by charged aerosol detection (A) and UV, 210 nm (C). Chromatogram B shows the separation of a mixed terpene glycoside standard for comparison.

Conclusion

Here the separation of mogroside V is shown both in a mixture of standards and in a commercially available beverage. The glycoside is separated on the Acclaim Trinity P1 column using 81/19 acetonitrile/ammonium formate buffer at pH = 3.0. This method uses HILIC conditions suitable for the trimode column, allowing separation of multiple terpene glycosides, and has also been used to separate steviol glycosides. The volatile mobile phase makes charged aerosol detection possible, which adds further method flexibility and improved detection sensitivity.

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

ChromaDex, 10005 Muirlands Blvd, Suite G, First Floor, Irvine, CA 92618U.S.A., Tel: 949-419-0288. www.chromadex.com

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Column Selection Guide

Silica Columns

			Reversed-Phase (RP)						Mixed-Mode			HILIC		Application-Specific					
			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	Example Applications
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																								
	Oligosccharides																								
	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												√		√	√	√	√														
	Oligosccharides														√	√			√	√												
	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			5 µm		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	Ultrapure silica	Spherical	3 and 5 µm	<10 ppm	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; polymethacrylate 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; polymethacrylate 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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