Pharmaceutical Applications Notebook

Over-the-Counter Drugs

Thermo SCIENTIFIC

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Introduction to Pharmaceuticals

The pharmaceutical industry is the largest consumer of high-performance liquid chromatography (HPLC) instrumentation. In drug discovery, HPLC and ion chromatography (IC) systems are used both as stand-alone tools and as front ends for mass spectrometers to screen drug candidates. In pre-clinical development, they are used for analyzing in-vitro and in-vivo samples. In clinical trials, they are used to gather data on a potential drug's safety and efficacy. They are used in manufacturing for many tasks including quality assurance/quality control (QA/QC), and the validation of cleaning procedures.

This applications notebook has been compiled to help the pharmaceutical scientist by providing a wide range of application examples relevant to the pharmaceutical market.

Thermo Fisher Scientific understands the demands of chemical analysis in the pharmaceutical industry. Our separation and detection technologies, combined with experience and applications competence, provide solutions for the analysis of inorganic ions, small drug molecules, and large components such as biologics and polysaccharides. Your laboratory now has a partner who can help you conduct reliable, accurate, and fast analyses. This notebook contains a wide range of pharmaceutical-related application notes and relevant information that will help address your challenges in drug discovery, development, and manufacturing.

Although, some of the applications published in this notebook were created some time ago, they are still relevant today. In the event that specific models of systems or modules used in these applications are no longer available, their methods may still be used on current instrumentation with similar performance.

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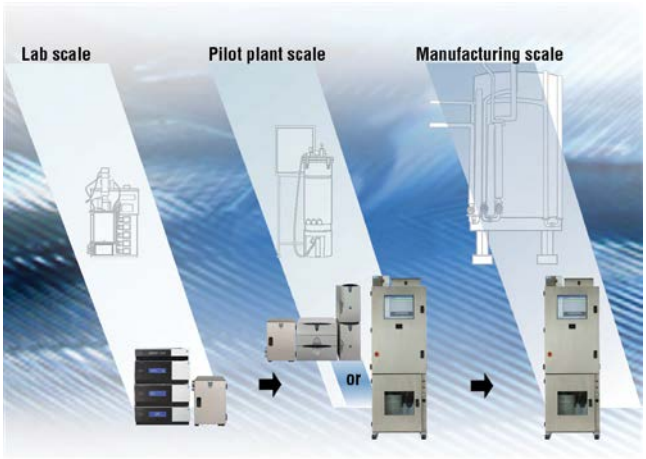
Integral: The Integral Migration Path approach: System solutions wherever you need them: lab, pilot plant, or manufacturing

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



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- CLP SOW OLM 0.42
- ASTM Standard Practice D7210
- Chinese Method GB/T 19649-2005
- German Method L00.00-34

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Analysis of Over-the-Counter Drugs

Pharmaceutical Applications Notebook

Accelerated USP Assay of Aspirin on the Acclaim 120 C18 RSLC Column

INTRODUCTION

USP and EP monographs are available to pharmaceutical manufacturers as records of standard procedures for the analysis of drug substances and drug products. The current USP monograph given for both coated and uncoated aspirin tablets[1] is based on conventional HPLC instrumentation and indicates a 4 × 300 mm column operated at a flow rate of 2.0 mL/min. Although the monograph's system suitability test calls for a resolution between the salicylic acid and aspiring peaks of no less than 2.0, when used with the Acclaim 120 C18 column it delivers a resolution of 5.7. However the analysis time is 15 min and thus is an excellent candidate for acceleration using a UHPLC method. Simply recomputing the operating parameters for a 2.1 × 50 mm column leads to a 10-fold increase in throughput which translates into a 96% savings in mobile phase. The accelerated method still produces baseline resolution of the two components that is greater than the required 2.0 stated in the monograph.

RESULTS

As shown in Figure 1, the original HPLC method produces a chromatogram with a salicylic acid/aspirin peak resolution of 5.7, far more than the monograph's stated specification of 2.0. By transferring the method to a UHPLC method using a shorter, narrower column with smaller particle diameter the analysis time is reduced to less than 2 min. The resulting resolution is still sufficient at a value of 2.9.

Reference

1. USP-31 NF-26, page 1450.

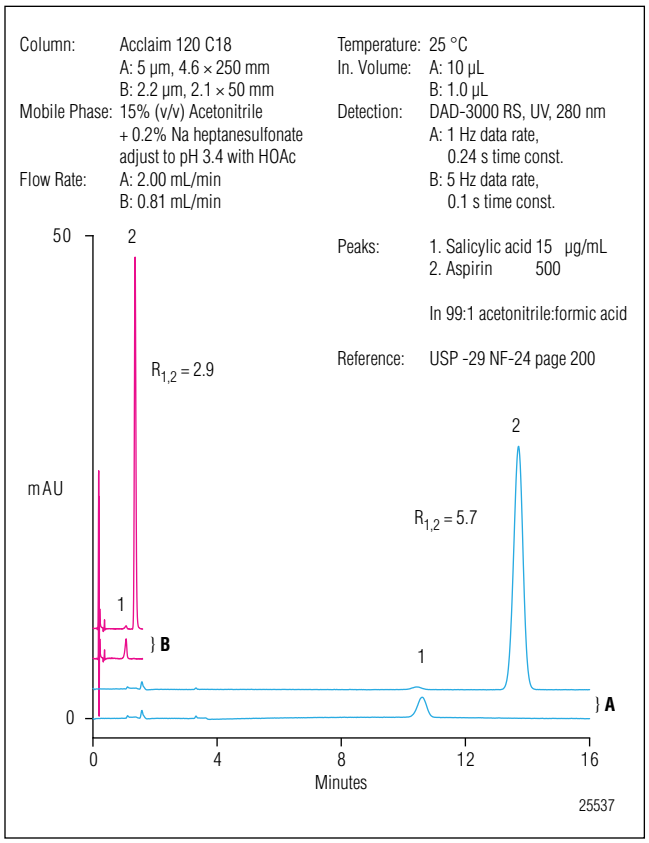

Figure 1. Injections of salicylic acid (peak 1) and aspirin (peak 2) under HPLC conditions (lower chromatograms) and UHPLC conditions (upper chromatograms).

Determination of Four Active Ingredients in a Multi-symptom Cold Remedy Using Acclaim PA

INTRODUCTION

This simple application of the Acclaim PA resolves all the active ingredients and most of the inactive ones in cough syrup. At pH 3, three of the active ingredients are only weakly hydrophobic and one is moderately hydrophobic. The Acclaim PA is well suited to the low-organic conditions needed for this separation. Note that the Acclaim PA delivers a sharp peak for pseudoephedrine, a hydrophilic, basic drug that is difficult for columns with high silanol activity.

SAMPLE PREPARATION

For the two chromatograms in Figure 1, the sample is prepared by a simple dilution of the cold remedy with mobile phase B at a rate of 20:1 and 400:1, respectively.

METHOD

A 3 µm Acclaim PA column with the dimensions of 4.6 × 150 mm is used to separate a 10 µL injection of the diluted sample into the 4 major components of the multi-symptom cold remedy. The gradient separation starts with a 2 min composition hold (8% mobile phase A) followed by an 8 min ramp from 8% to 20% A. The gradient then ramps up to 50% A over the next 7 min. The flow rate is 1.0 mL/min. yielding a run time of 17 min. Detection is accomplished using UV absorbance at 210 nm.

RESULTS

Trace A shows the acetaminophen peak at a 400:1 dilution. Trace B is a chromatogram of the sample diluted 20:1. At this concentration you can clearly see the other 3 active ingredients as well as the non-active components. As shown in the figure, the Acclaim PA column provides sufficient separation efficiency to baseline resolve the four active ingredients. It also provides baseline resolution of 5 other non-active ingredients.

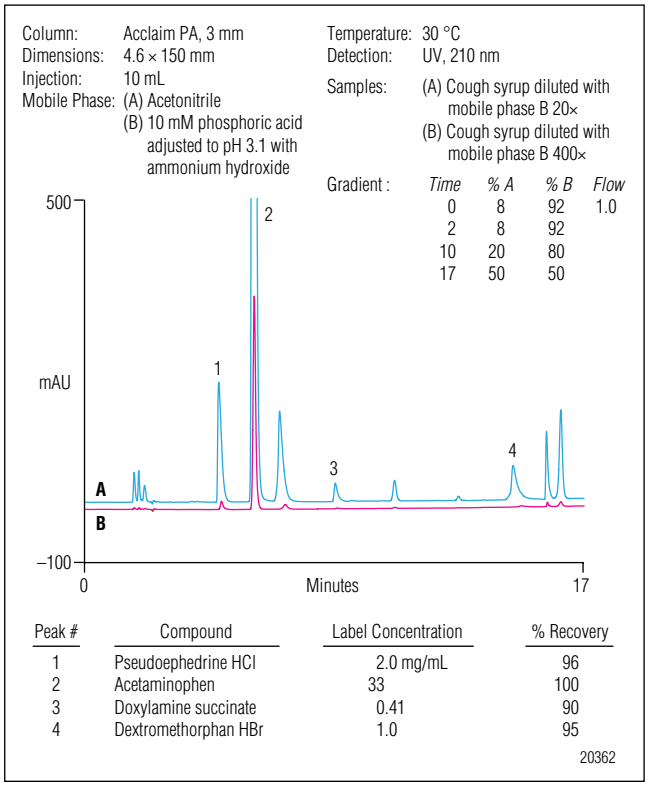

Figure 1. Determination of four active ingredients in a multi-symptom cold remedy using Acclaim PA.

Fast Analysis for Loratidine and Pseudo-ephedrine in a Time-Release Tablet on Acclaim PA

INTRODUCTION

Loratidine is a second-generation, non-sedating antihistamine. Pseudo-ephedrine is a decongestant. This combination is a popular treatment for relief of allergy symptoms. The analysis is difficult because the two drugs have very different hydrophobicities. Also, both are strong basic amines with a tendency to tail. The Acclaim PolarAdvantage (PA) column is well suited to this challenge as it delivers good base symmetry over a wide range of mobile phase compositions. The short, 50 mm column enables this analysis to be completed in less than 4 min.

SAMPLE PREPARATION

For this analysis, the sample is prepared by dissolving a time-release tablet in a solution of roughly 10% methanol, 3% 1N HCl and 87% water. After filtration, the 10 µL of the sample is injected.

METHOD

A 5 µm Acclaim PA column with the dimensions of 4.6 × 50 mm is used to separate the 2 active ingredients. The column was thermostatically maintained at 35 °C. The separation starts with a 0.41 min composition hold at 0% mobile phase B. Next there is essentially a gradient step up to a final composition of 100% B which is then held to the end of the run. Mobile phase A is an acetonitrile/water (15:85) solution containing 0.05% TFA. Mobile phase B is also an acetonitrile/water solution but at a ratio of 40:60 and it also contains 0.05% TFA. The flow rate is 1.25 mL/min. Detection is accomplished using UV absorbance at 257 nm.

RESULTS

As shown in Figure 1, this HPLC method using the Acclaim PA column produces a chromatogram showing more than adequate resolution of pseudo-ephedrine and loratidine. Furthermore, the separation is accomplished in under 4 min. At a flow rate of 1.25 mL/min. the analysis consumes less than 5 mL of mobile phase.

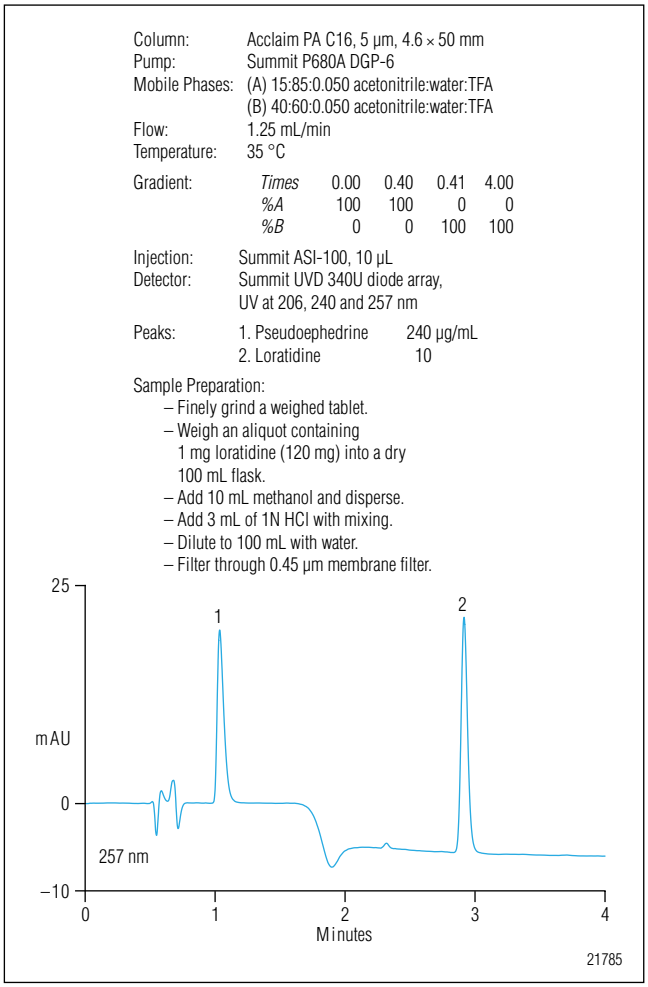

Figure 1. Fast Analysis for loratidine and pseudo-ephedrine in a time-release tablet on Acclaim PA.

Miconazole in Athlete's Foot Ointment on Acclaim 120 C18

INTRODUCTION

Miconazole is a popular antifungal agent used in various forms to treat athlete's foot and related conditions. Here an Acclaim C18 column is used to determine Miconazole content in a topical ointment. The label listed the active ingredient concentration as 2.0%. The analysis measured it at 2.1%.

SAMPLE PREPARATION

In this application, the sample is prepared using liquid-liquid extraction to remove the miconazole from the ointment. The ointment is first dissolved in a solution of ethanol and heptane. Water is then added to the ethanol/heptane solution, the mixture is centrifuged and the miconazole is extracted in the aqueous layer. 4 µL of the aqueous extract is injected on column.

METHOD

A 3 µm Acclaim 120 C18 column with the dimensions of 4.6 × 150 mm is used to separate miconazole from the other aqueous-soluble ingredients. The separation is performed under isocratic conditions using 80% methanol and 20% buffer. The buffer is composed of 10 mM TEA adjusted to pH 2.7 with phosphoric acid. The flow rate is 1.0 mL/min. which produces a run time of less than 6 min. Detection is accomplished using UV absorbance at 225 nm.

RESULTS

As shown in Figure 1, the miconazole is very well retained and easily distinguished from the other components. This separation is clearly suitable for quantitatively assaying of miconazole in difficult samples such as creams and ointments.

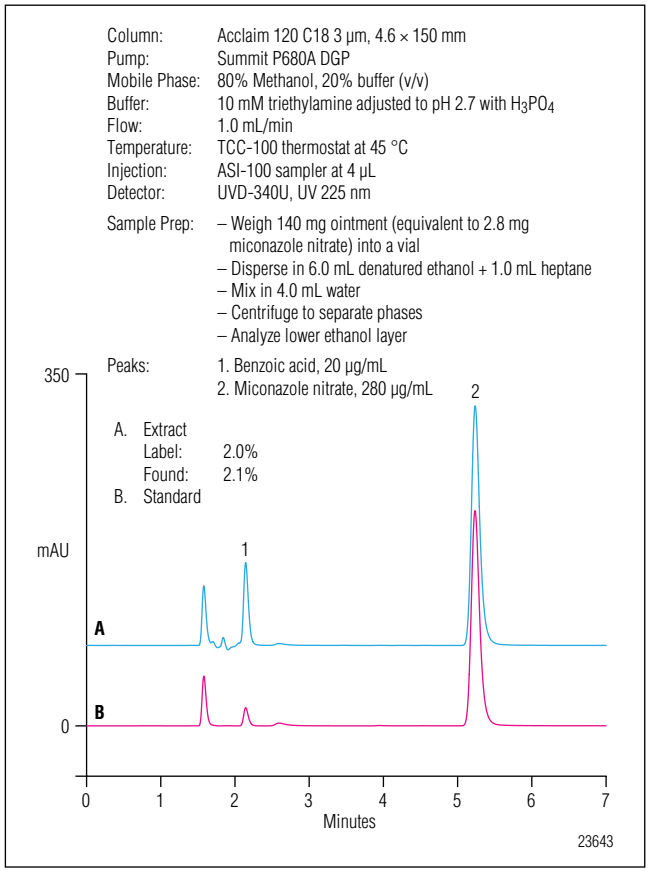

Figure 1. Miconazole in athlete's foot ointment on Acclaim 120 C18.

Ketoconazole in Anti-Dandruff Shampoo on the Acclaim 120 C18 Column

INTRODUCTION

Ketoconazole is an antifungal agent used both internally and as a topical treatment for dandruff. Here an Acclaim C18 column easily resolves this active ingredient from shampoo, a matrix that is complex and can be difficult to analyze.

The current USP monograph given for ketoconazole[1] is based on a 4.6 × 250 mm column packed with 5 µm C18 stationary phase. However the analysis time can be easily shortened by substituting a shorter (150 mm) column packed with smaller (3 mm) particles to maintain adequate resolution.

SAMPLE PREPARATION

For this analysis, the sample is prepared by dissolving 400 mg of shampoo in 3 mL of water to which is added 10 mL of methanol. After filtration, 4 µL of the sample solution is injected.

METHOD

A 3µm Acclaim 120 C18 column, with the dimensions of 4.6 × 150 mm and maintained at 30 °C, is used to resolve ketoconazole from other formulation ingredients. The separation is performed under isocratic conditions using a mobile phase consisting of 2.55 g tetrabutylammonium hydrogen sulfate dissolved in 750 mL of water and 250 mL of acetonitrile. A flow rate of 1.0 mL/min. produces a run time of less than 6 min. Detection is accomplished using UV absorbance at 223nm.

RESULTS

As shown in Figure 1, the ketoconazole is very well retained and easily distinguished from the other components. This separation is clearly suitable for quantitatively assaying of ketoconazole in formulations such as creams and shampoos.

Column:	Acclaim 120 C18 3 µm, 4.6 × 150 mm
Pump:	Summit P680A DGP
Mobile Phase:	2.55 g $Bu_4N^+HSO_4^-$, 750 mL water, 250 mL acetonitrile
Flow:	1.0 mL/min
Temperature:	TCC-100 thermostat at 30°C
Injection:	ASI-100 sampler at 4 µL
Detector:	UVD-340U, UV 223 nm
Sample Prep:	– Weigh 400 mg shampoo (equivalent to 4 mg ketoconazole) into a flask – Disperse in 3.0 mL water – Dilute to 10.0 mL with MeOH – Filter
Peaks:	1. Ketoconazole, approx. 400 µg/mL
Reference:	U.S. Pharmacopeia 29th Ed., p.1216 (2006).

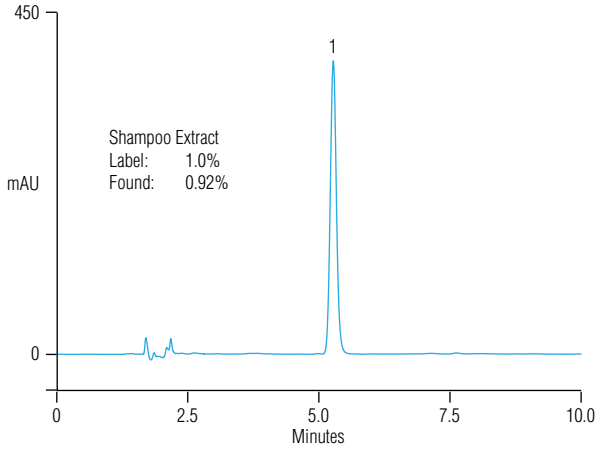

Figure 1.Ketoconazole in anti-dandruff shampoo on Acclaim 120 C18.

REFERENCE

1. USP-31 NF 36, page 2488 (2008).

Assay for Water-Soluble Vitamins

INTRODUCTION

Vitamin supplement tablets are complex formulations with many ingredients. Some vitamins are strongly hydrophilic, so the column needs to operate in 100% aqueous buffer to gain sufficient retention to resolve them from the matrix. The Acclaim PA can do this reliably whereas a hydrophobic C18 column would likely suffer dewetting. The multiple wavelength capability of the diode array detector provides primary wavelengths for quantitation and alternate wavelengths for confirmation.

SAMPLE PREPARATION

For this analysis, the vitamin tablet was crushed and dissolved in 100 mL of mobile phase buffer. After sonication followed by filtration, 20 µL of the sample solution was injected.

METHOD

A 3 µm Acclaim PA column, with the dimensions of 4.6 × 150 mm is used to resolve 8 vitamins. The gradient separation ramps from 0% to 20% mobile phase B in 15 min. Mobile phase A is phosphate buffer at pH 3.4. Mobile phase B is acetonitrile. A flow rate of 1.0 mL/min. produces a run time of less than 17 min. Detection is accomplished using UV absorbance at 210, 246, 265 and 285 nm.

RESULTS

As shown in Figure 1, the 8 vitamins contained in the vitamin tablet are all well resolved using the Acclaim PA column. By selectively monitoring multiple wavelengths, sensitivity can be maximized while avoiding potential interferences. This separation is clearly suitable for quantitatively assaying water soluble vitamin tablets.

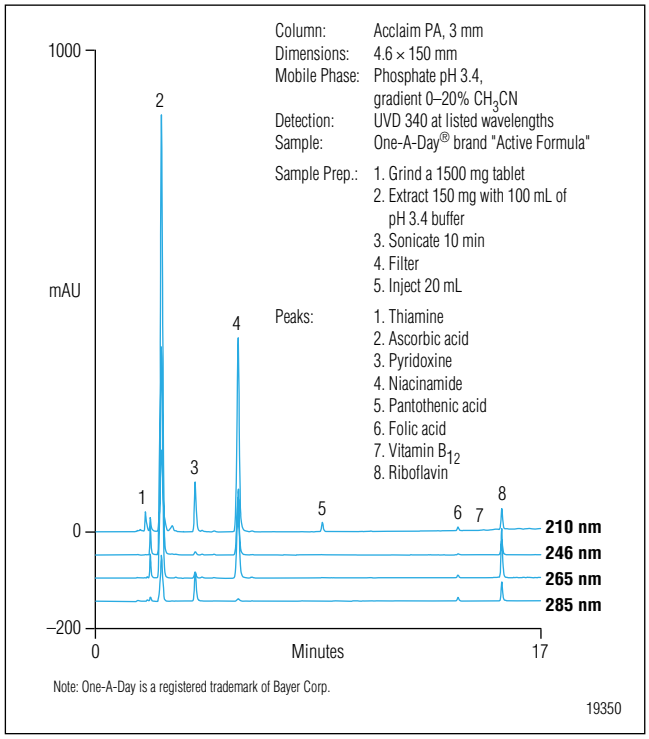

Figure 1. Assay for water-soluble vitamins in vitamin tablets using Acclaim PA.

Fat-Soluble Vitamins and Carotenoids in a Vitamin Tablet on Acclaim PA

INTRODUCTION

Vitamin supplements often include carotenoids together with fat-soluble vitamins in their formulations. This convenient analysis measures vitamins A and E and a complex of carotenoids in a single run. The Acclaim PA column shows a different selectivity under low-aqueous conditions than conventional reversed-phase columns. The conditions above have been used with a variety of sample types.

SAMPLE PREPARATION

In this application, the sample is prepared using liquid-liquid extraction to remove extract the vitamins from the tablet. A portion of the crushed tablet is taken up in a mixture of water and ethyl acetate. The mixture is sonicated and 2 mL of the ethyl acetate layer is removed and evaporated to dryness. The residue is then reconstituted in 1mL of mobile phase B. 10 µL of the reconstituted sample is injected on column.

METHOD

A 3 µm Acclaim PA column, with the dimensions of 4.6 × 150 mm and maintained at 30 °C, is used to resolve 7 fat-soluble vitamins and carotenoids. The separation starts with a 8 min composition hold at 95% mobile phase B. Next, there is essentially a gradient step up to a final composition of 100% B which is then held to the end of the run. Mobile phase A is water and mobile phase B is a solution of methanol, acetonitrile and isopropanol at a ratio of 54:44:2. A flow rate of 1.25 mL/min. produces a run time of less than 18 min. Detection is accomplished using multiple wavelength UV absorbance at 285 and 450 nm.

RESULTS

As shown in Figure 1, the 7 vitamins and carotenoids contained in the vitamin tablet are all resolved using the Acclaim PA column. By selectively monitoring two wavelengths, sensitivity can be maximized while avoiding potential interferences. This separation is clearly suitable for quantitatively assaying fat-soluble vitamin tablets.

Column:	Acclaim® PA C16, 3 µm, 4.6 × 150 mm
Pump:	Summit P580 HPG/4
Mobile Phase:	(A) Water (B) 54:44:2 methanol:acetonitrile:isopropanol

Gradient:	Time	0	8.0	8.1	25
	%A	5	5	0	0
	%B	95	95	100	100

Flow:	1.25 mL/min
Temperature:	30 °C
Injection:	Summit ASI-100, 10 µL
Detector:	Summit UVD 340U diode array, 450 and 285 nm, and spectra 200–595 nm

Sample Prep.:
- Finely grind a weighed tablet
- Take an aliquot of about 1/10 tablet (150 mg)
- Partition between 2.0 mL water and 6.0 mL ethyl acetate with sonication
- Evaporate 2.0 mL of ethyl acetate phase and reconstitute in 1.0 mL mobile phase B

Peaks:
1. *trans*-Retinol acetate
2. *trans*-Lutein
3. α-Tocopherol acetate
4. *trans*-Lycopene
5. *cis*-Lycopene
6. *trans*-β-Carotene
7. *cis*-β-Carotene

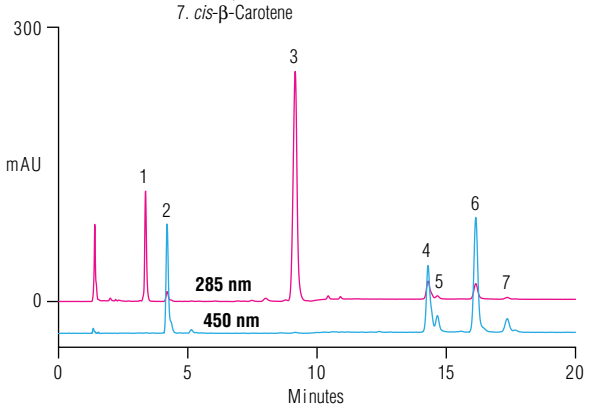

Figure 1. Fat-soluble vitamins and carotenoids in a vitamin tablet.

Analysis of APIs in a Pain-Relief Medicine on Acclaim Mixed-Mode WAX-1

INTRODUCTION

Pain relief medications often contain a combination of ingredients that can be challenging to separate. Acclaim Mixed-Mode WAX-1 resolves all the active pharmaceutical ingredients (APIs) in this product.

SAMPLE PREPARATION

For this analysis, the sample is prepared by dissolving a ground up time-release tablet in water. After sonication, followed by filtration, 1 µL of the sample is injected.

METHOD

In this application, a 5 µm Acclaim Mixed-Mode WAX-1 column with the dimensions of 4.6 × 150 mm is used to separate a 5 active pharmaceutical ingredients found in a pain-relief medication. The column was maintained at 30 °C. The separation is performed under isocratic conditions at a flow rate of 1.0 mL/min. The mobile phase consisted of 40% acetonitrile and 60% buffer. The buffer was composed of 6.8 g KH_2PO_4 and 0.5 g NaP_2O_7*10 H_2O in 1000 mL water. The pH of the buffer was adjusted to 6.0 with NaOH. The method yielded a total run time under 15 min. Detection is accomplished using UV absorbance at 220 nm.

RESULTS

As shown in Figure 1, this method provides baseline separation of all 5 components in the mixture. Clearly this separation is suitable for the quantitative assaying of a multi-component pain-relief medication.

Note: Pyrophosphate in the buffer suppresses interference from metal ions.

Column:	Acclaim Mixed-Mode WAX-1, 5 µm, 4.6 × 150 mm
Mobile Phase:	40/60 v/v Acetonitrile/buffer (6.8 g KH_2PO_4 and 0.5 g $NaP_2O_7 \bullet 10H_2O$ in 1000 g D.I. H_2O, pH is adjusted to 6.0 with NaOH)
Temperature:	30 °C
Flow Rate:	1 mL/min
Inj. Volume:	1 µL
Detection:	UV @ 220 nm
Note:	Pyrophosphate in the buffer suppresses interference from metal ions
Peaks:	1. Caffeine 2. Acetaminophen 3. Salicylamide 4. Acetyl salicylic acid (Aspirin) 5. Salicylic acid

Sample Preparation:
1. A 500 mg tablet is ground into fine powder.
2. Weigh 400 mg of the powder and dissolve with 40 g of D.I. water.
3. After sonicating for 5 min, the resulting suspension was filtered with 0.45 µm membrane filter.
4. Dilute the filtrate 4 times with D.I. water

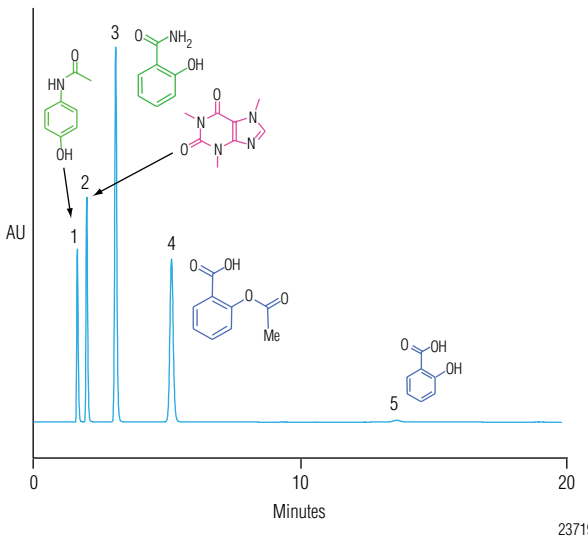

Figure 1. Analysis of APIs in a pain-relief medicine on Acclaim Mixed-Mode WAX-1.

Nonsteroidal Anti-inflammatory Drugs on Acclaim PA2 C18

INTRODUCTION

Nonsteroidal Anti-inflammatory Drugs (SAIDs) are a widely-used class of drugs used for pain relief in joints and muscles. These acidic drugs may be separated with excellent performance using the Acclaim PA2 column at low pH

METHOD

In this application, a 5 µm Acclaim PA2 column with the dimensions of 4.6 × 150 mm is used to separate a 3 active pharmaceutical ingredients found in an anti-inflammatory medication. During the analysis, the column was maintained at 30 °C. The separation is performed under isocratic conditions at a flow rate of 1.0 mL/min. The mobile phase consisted of 55% acetonitrile and 45% phosphoric acid (0.1%). The method yielded a total run time of approximately 10 min and the compounds were detected using UV absorbance at 265 nm.

RESULTS

As shown in Figure 1, this method provides baseline separation of ketoprofen and naproxen and both elute much earlier than ibuprofen. Clearly this separation is suitable for the quantitative assaying of a multi-component nonsteroidal anti-inflammatory medication.

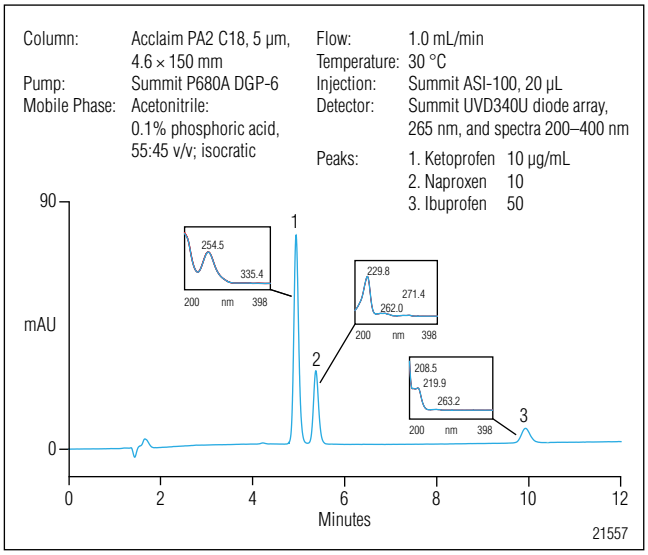

Figure 1. Nonsteroidal anti-inflammatory arugs on Acclaim PA2 C18.

Hydrocortisone in Skin Ointment

INTRODUCTION

Hydrocortisone is the active ingredient in a number of topical formulations used to treat many types of skin rashes. The product formulations often have an assortment of other ingredients making the analysis complex and demanding. Here, we have employed a straightforward liquid-liquid extraction and simple isocratic analysis for quick, clean, and accurate results.

SAMPLE PREPARATION

In this application, sample preparation is accomplished by dissolving the sample ointment in a mixture of ethanol and heptane followed by an aqueous extraction of interfering compounds. The ethanol layer is then filtered and 6 µL injected.

METHOD

A 3 µm Acclaim C18 column with the dimensions of 3.0 × 75 mm is used to separate hydrocortisone from two preservatives in a topical ointment. The total run time is only four min. The Ultimate 3000 system provides high resolution and fast analysis without producing UHPLC pressures. Detection is accomplished using UV absorbance at 245 nm.

RESULTS

As shown in Figure 1, the method provides more than adequate resolution of all 3 components in a short period of time. This separation is clearly suitable for assaying this formulation. Additionally, because of the short analysis time and 3.0 mm diameter column, consumption of mobile phase is only 4 mL per analysis. This reduces cost of solvent, cost of waste disposal and cost of operation.

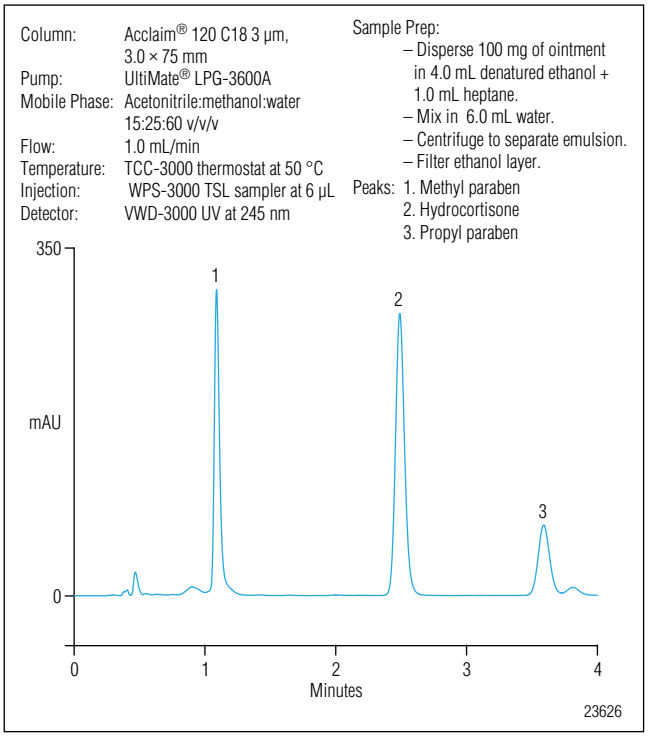

Figure 1. Hydrocortisone in skin ointment on Acclaim 120 C18.

Omeprazole in Anti-Heartburn Tablet on the Acclaim PA2 Column

INTRODUCTION

Omeprazole is an inhibitor of gastric acid secretion and is widely used to treat chronic heartburn. It is commonly available under its original brand names as well as a number of generic medicines. This application brief demonstrates the use of the Acclaim PA2 column which gives excellent performance for this assay. The method described in this applications brief is suitable for performing the USP assay[1] and chromatographic purity.

SAMPLE PREPARATION

The sample is prepared by dissolving an omeprazole tablet in a solution of 20% ethanol and 80% buffer (10 mM borate, 1.5 mM EDTA, pH 11.0). the sample is filtered prior to injecting 10 µL on column.

METHOD

This method uses a 4.5 × 150 mm column packed with 5 µm Acclaim PA2 stationary phase maintained thermostatically at 30 °C to separate omeprazole from its related substances.

The separation is achieved using a 20 min binary gradient going from 12% to 60% mobile phase B at a flow rate of 1.2 mL/min. Mobile phase A is also a solution of 85% acetonitrile and 15% methanol and mobile phase B is a 40 mM glycine solution adjusted to pH 9.0. Detection is by UV absorbance at 224 nm.

RESULTS

As shown in Figure 1, the HPLC method produces a chromatogram showing good retention of the omeprazole peak (trace A). The recovery of omeprazole was calculated as 19.5 mg or 97.5% of the labeled amount.

REFERENCE

1. USP-31 NF-26, page 2850, (2008).

Column:	Acclaim PA2, 5 µm, 4.6 × 150 mm
Pump:	UltiMate® LPG-3400
Mobile Phases:	(A) Acetonitrile:methanol 85:15 (v/v) (B) 40 mM glycine, pH 9.0

Gradient Time:	–7	0	20
%A	12	12	60
%B	88	88	40

Flow:	1.2 mL/min
Temperature:	TCC-3100 thermostat at 30 °C
Injection:	WPS-3000 SL sampler at 10 µL
Detector:	VWD-3400, UV 305 nm
Sample Prep:	– Weight and finely grind tablets. – Extraction solvent: 20% ethanol, 80% (10 mM borate, 1.5 mM EDTA, pH 11.0) (v/v) – Extract with sonication an aliquot equiv. to 20 mg omeprazole in 100 mL solvent. – Filter
Peak:	1. Omeprazole, approx. 225 µg/mL
Reference:	U.S. Pharmacopeia, 29th Ed., p.1578, (2006).

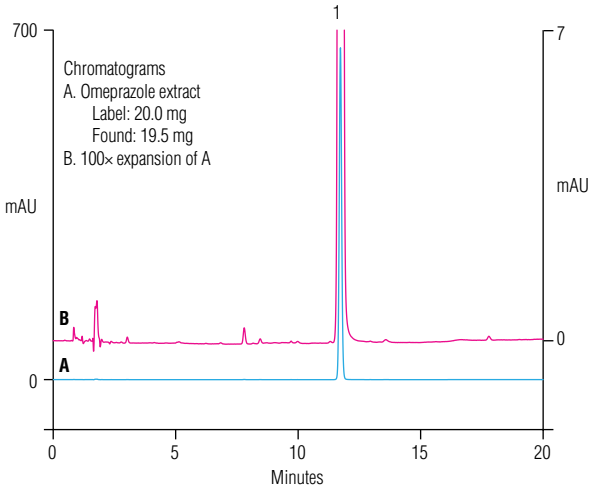

Figure 1. Omeprazole in anti-heartburn tablet on Acclaim PA2.

Alkaloids in Bitter Orange on Acclaim 120 C18

INTRODUCTION

Combinatorial libraries are not the only source for pharmacologically active compounds. For thousands of years, civilizations around the world have employed naturally occurring ingredients to treat human maladies. Currently there is much research is taking place to study areas such as Traditional Chinese Medicines (TCM). Bitter orange, the immature fruit of Citrus Aurantium, is an ingredient in traditional Chinese medicines. It contains tyramine alkaloids, with synephrine as a major component.

METHOD

The method described in this application is suitable for assaying ingredients and formulations for bitter orange and its active constituents. The method uses a 3 × 150 mm column packed with 3 µm Acclaim 120 C18 stationary phase. The column is maintained thermostatically at 35 °C.

A buffer composed of 20 mM boric acid adjusted to pH 8.2 with KOH was first created for use in sample dilution and mobile phase modification. Mobile phase A is 10 mM sodium hexanesulfonate solution made up with buffer. Mobile phase B is also a 10 mM sodium hexanesulfonate solution but made up with a mixture of 20% acetonitrile and 80% buffer. The separation is achieved using a 30 min binary gradient going from 100% mobile phase A to 100% mobile phase B. Detection is by UV absorbance at 224 nm.

RESULTS

As shown in Figure 1, the HPLC method produces a chromatogram showing good separation of 6 potential components of Bitter Orange (trace A). The separation of the bitter orange extract (trace B) clearly indicates the presence of Synephrine demonstrating its usefulness in assaying components of bitter orange extracts.

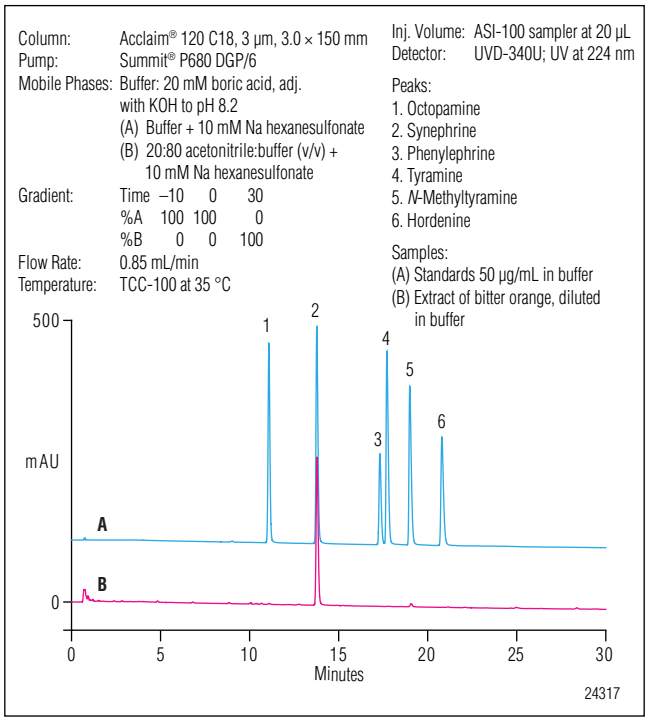

Figure 1. Alkaloids in bitter orange extract on Acclaim 120 C18.

Determination of Ten Active Ingredients in Sunscreen-Containing Products in a Single Injection

INTRODUCTION

To prevent skin damage from the sun's radiation, many skin care products, such as lipstick, makeup, and lotions contain one or more compounds to block UV radiation. The lotions containing these compounds are commonly referred to as sunscreens and other products that contain one or more of these compounds are said to contain sunscreen. The active ingredients in sunscreens are usually aromatic compounds conjugated with carbonyl groups (e.g. homosalate) and rather than literally blocking the UV radiation, they absorb it and release it as lower-energy UV radiation. The FDA allows over 15 different compounds to be used as the active ingredient in sunscreens and sunscreen-containing products. Additional compounds have been approved for use in the European Union and other parts of the world.

In this Application Note (AN) we developed a separation of the following 10 compounds used in sunscreen: 2-phenyl-benzimidazole-5-sulfonic acid, benzophenone-3, diethylamino-hydroxybenzoylhexyl benzoate, 4-methylbenzylidine-camphor, octocrylene, methylanthranilate, octyl-methoxycinnamate, butyl-methoxydibenzoylmethane, octyl-salicylate, and homosalate. A manufacturer of sunscreen products chose these 10 compounds and requested baseline resolution of all 10 in a single injection.

Using a 3-µm Acclaim® 120 C18 column with an ethanol-containing mobile phase we were able to baseline resolve all 10 compounds in less than 12 min. This method successfully determined subsets of these 10 compounds in a lipstick, a cosmetic powder, and a lotion provided by the manufacturer. The Acclaim 120 C18 column paired with a Dionex UltiMate® 3000 system is an ideal platform for developing methods to determine sunscreen ingredients in a variety of products.

EQUIPMENT

Dionex UltiMate 3000RS chromatography system consisting of:

- SRD-3600 Solvent Rack with integrated vacuum degasser
- HPG-3400RS Binary gradient pump with 200 µL static mixer kit (P/N 6040.5150)
- WPS-3000RS split loop sampler with 100 µL sample loop
- TCC-3000RS Thermostatted column compartment
- DAD-3000RS Diode array detector

Chromeleon® Chromatography Data System, Version 6.80 SP5

REAGENTS AND STANDARDS

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

Absolute ethanol ($C2H_5OH$), AR grade (LAB-SCAN)

Methanol (CH_3OH), HPLC grade (LAB-SCAN)

Glacial acetic acid (CH_3COOH), AR grade (LAB-SCAN)

2-Phenyl-Benzimidazole-5-sulfonic acid (PHS)

Benzophenone-3 (B-3)

Diethylamino-hydroxybenzoylhexyl benzoate (DHHB)

4-Methylbenzylidine-camphor (4-MBC)

Octocrylene (OCR)

Methylanthranilate (MA)

Octyl-methoxycinnamate (OMC)

Butyl-methoxydibenzoylmethane (BMDM)

Octyl-salicylate (OS)

Homosalate (HMS)

CHROMATOGRAPHIC CONDITIONS

Column:	Acclaim 120 C18 3 µm, 4.6 × 100 mm (P/N 059132)
Eluent:	A: 0.8% Acetic acid B: Ethanol
Eluent Gradient:	25% B from -5 to 1 min, 25 to 80% from 1 to 1.5 min, and 80% B from 1.5 to 11.5 min
Flow rate:	0.7 mL/min
Column Temp.:	25 ºC
Inj. Volume:	5 µL
Detection:	UV, 310 and 354 nm, Wavelength scanning 250–600 nm
Backpressure:	2600–2900 psi

PREPARATION OF SOLUTIONS AND REAGENTS

Eluent A

0.8% Acetic acid

Add approximately 100 mL deionized water to a 1000 mL volumetric flask, pipet 8 mL of glacial acetic acid to the same volumetric flask, bring to volume with deionized water, and mix.

Standards

Stock Standard Solutions

To prepare a 1000 mg/L stock standard for each of ten compounds, weigh 0.1 g of the compound, add to a 100 mL breaker, add 70 mL of methanol, and place in an ultrasonic bath for 10 min to ensure dissolution. Move this solution to a 100 mL volumetric flask and bring it to volume with methanol.

Working Standard Solutions

To prepare the five mixed standard solutions with analyte concentrations of 10, 25, 35, 50, and 75 mg/L, pipet 100, 250, 350, 500, and 750 µL of the individual stock standards into 100 mL volumetric flasks and bring to volume with methanol. Filter each standard with a 0.2 µm nylon filter prior to analysis.

Sample Preparation

Three products containing sunscreen compounds were provided by a customer. These products were a lotion, a lip balm, and a cosmetic powder. The customer also provided versions of these products without sunscreen compounds and, in this note, we refer to these as placebo products. Accurately weigh 0.1 g of sample and place in a 100 mL breaker. Add 70 mL of methanol, and place in an ultrasonic bath for 10 min to ensure dissolution. Move the sample solution to a 100 mL volumetric flask and bring to volume with methanol. Filter this sample solution with a 0.2 µm nylon filter prior to analysis.

RESULTS AND DISCUSSION

Separation

The 10 compounds in this study are all ideal candidates for reversed-phase chromatography with UV detection. A spectral scan of the ten compounds revealed that eight of them would be ideally detected at 310 nm and the other two at 354 nm. We chose the Acclaim 120 C18 column because it contains small-pore, high-purity, low-metal content silica with high C18 surface coverage (i.e. high carbon load), ideal for developing high resolution separations of compounds typically determined by reversed-phase chromatography. Using a methanol/acetic acid mobile phase we were unable to achieve a separation with all resolution factors 2.0 or greater.Switching to an ethanol/acetic acid mobile phase yielded the required separation (Figure 1). The

Table 1. Resolution and Peak Purity of the Ten Sunscreen Ingredient Standards in an Injection of a Mixed Standard (35 mg/L) with Detection at 310 nm (Wavelength Scanning 250–600 nm for Peak Purity)

Compound	Resolution* (USP)	Match	RSD Match	PPI (nm)	RSD PPI
2-Phenyl-benzimidazole -5-sulfonic acid (PHS)	20.53	1000	0.02	279.6	0.01
Benzophenone-3 (B-3)	7.17	1000	0.11	284.4	0.04
Diethylamino-hydroxybenzoylhexyl-benzoate (DHHB)	3.64	1000	0.39	335.6	0.11
4-Methylbenzylidine-camphor (4-MBC)	3.07	1000	0.21	281.0	0.07
Octocrylene (OCR)	2.57	999	0.74	285.0	0.25
Methylanthranilate (MA)	2.93	976	5.56	313.5	0.88
Octyl-methoxycinnamate (OMC)	2.52	999	0.38	284.7	0.12
Butyl-methoxydibenzoylmethane (BMDM)	4.38	1000	0.20	332.9	0.06
Octyl-salicylate (OS)	3.32	986	3.37	283.8	0.59
Homosalate (HMS)	n.a.	995	1.22	285.5	0.20

* All values in this table were calculated by Chromeleon.

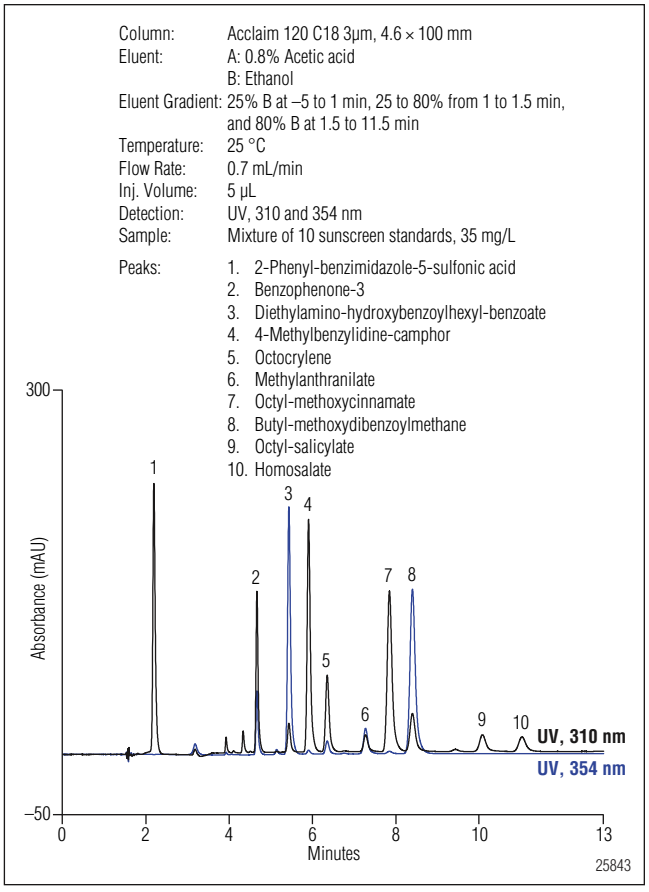

Figure 1. Chromatogram of a mix of 10 sunscreen ingredient standards with detection at 310 nm (Black) and 354 nm (Blue).

resolution of all 10 components is greater than 2.5. Spectral matching of each peak compared to the spectral library (loaded by making single injections of a each of the standards) showed high purity of all ten peaks (Table 1). The low RSD of the peak purity index (PPI) of all ten peaks also indicates peak purity.

Table 2. Calibration Data from Chromeleon for 10 Sunscreen Ingredient Standards at 310 nm, Unless Otherwise Noted

Compound	Cal.Type	Points	R-Squared	Slope
PHS	Lin	5	0.9994	0.4408
B-3	Lin	5	0.9994	0.2347
DHHB	Lin	5	0.9983	0.0578
DHHB – 354 nm	Lin	5	0.9993	0.4991
4-MBC	Lin	5	0.9992	0.5007
OCR	Lin	5	0.9992	0.1876
MA	Lin	5	0.9994	0.0517
OMC	Lin	5	0.9992	0.5248
BMDM	Lin	5	0.9989	0.1352
BMDM- 354 nm	Lin	5	0.9993	0.6049
OS	Lin	5	0.9986	0.0678
HMS	Lin	5	0.9989	0.0681

Method Calibration

Before sample analysis, the 10 sunscreen compounds was separated at 5 concentrations: 10, 25, 35, 50, and 75 mg/L, and the data used to prepare a calibration curve that was forced through the origin. Table 2 displays the calibration data and shows a good linear fit for all ten compounds between 0 and 75 mg/L.

Sample Analysis

The manufacturer provided three products containing sunscreen compounds, a lipstick, a cosmetic powder, and a lotion. They also supplied the same products without added sunscreen compounds, referred to here as placebo products. We analyzed each of the placebo products after sample preparation to determine if there were any peaks from the sample that would interfere with sample analysis. None of the three products contained interfering compounds. Figure 2 shows the chromatogram of the cosmetic powder placebo. Chromatography of the lipstick and lotion placebos was indistinguishable from Figure 2.

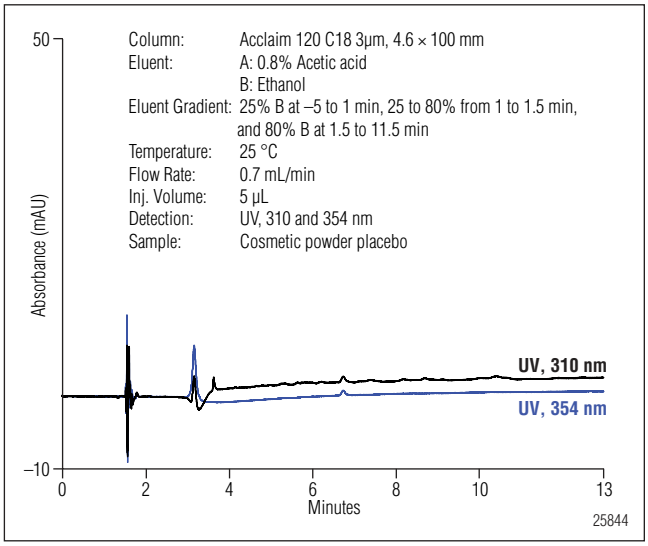

Figure 2. Chromatography of a cosmetic powder placebo with detection at 310 nm (Black) and 354 nm (Blue). The other 2 placebos yielded the same result.

To evaluate recovery, we spiked each placebo product with the 35 mg/L mixed standard. Table 3 shows that there was excellent recovery of all 10 compounds from each of the 3 samples, suggesting that this method is accurate for the determination of these compounds in the three products. Figure 3 shows chromatography of the lotion placebo product spiked with the 35 mg/L mixed standard. Chromatography of the spiked lipstick and cosmetic powder placebos was nearly identical to Figure 3.

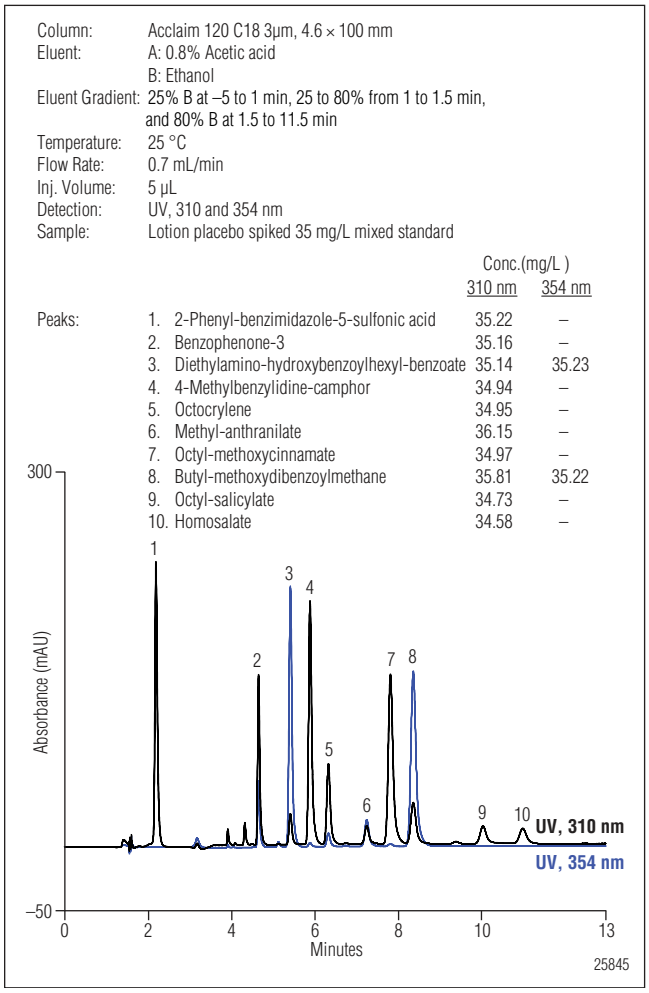

Figure 3. Overlay of three injections of a lotion placebo spiked with a 35 mg/L mixed standard with detection at 310 nm (Black) and 354 nm (Blue)

Finally we determined the amounts of the 10 sunscreen ingredients in 3 injections of each of the 3 products. The cosmetic powder sample was found to contain MA, OMC, and OS (Figure 4), the lotion sample contained PHS, B-3, 4-MBC, OMC, OS, and HMS (Figure 5), and the lipstick sample contained PHS, B-3 DHHB, 4-MBC, OMC, BMDM, OS, and HMS (Figure 6). Table 4 summarizes the amount of each sunscreen compound found in each sample.

Table 3. Recovery Results for the Spiked (35 mg/L) Cosmetic Powder, Lotion, and Lipstick Placebo Samples													
		Concentration (mg/L) Determined at 310 nm and 354 nm When Noted											
		PHS	B-3	DHHB	DHHB (354 nm)	4-MBC	OCR	MA	OMC	BMDM	BMDM (354 nm)	OS	HMS
Cosmetic Powder Placebo	Average*	35.07	36.05	35.80	36.00	35.76	35.82	37.25	35.89	34.52	33.94	37.45	35.46
	RSD	0.27	0.23	0.74	0.17	0.23	0.37	1.06	0.24	0.25	0.34	0.39	0.34
	%Recovery	100.2	103.0	102.3	102.9	102.2	102.3	106.4	102.5	98.60	97.00	107.0	101.3
Lotion Placebo	Average*	35.22	35.16	35.14	35.23	34.94	34.95	36.15	34.97	35.81	35.22	34.73	34.58
	RSD	0.11	0.02	0.12	0.12	0.01	0.03	0.60	0.03	0.09	0.02	0.22	0.14
	%Recovery	100.6	100.5	100.4	100.7	99.83	99.86	103.3	99.90	102.3	100.63	99.23	98.80
Lipstick Placebo	Average*	37.09	35.55	35.71	35.54	35.22	35.30	35.63	35.27	35.95	35.45	35.89	34.91
	RSD	0.07	0.08	0.12	0.13	0.12	0.12	0.74	0.14	0.26	0.30	0.04	0.39
	%Recovery	106.0	101.6	102.0	101.5	100.6	100.9	101.8	100.8	102.7	101.3	102.5	99.74

*Three injections were made of each sample.

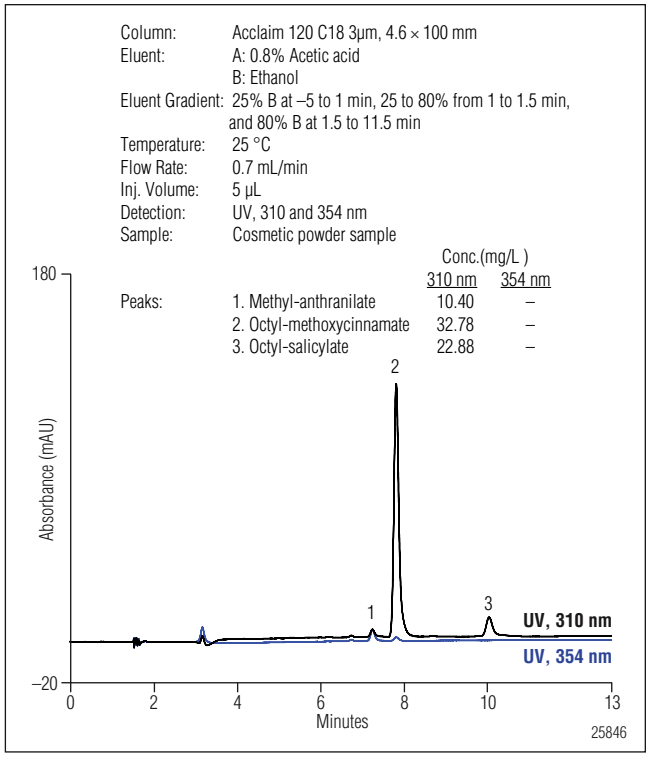

Figure 4. Overlay of three injections of the cosmetic powder sample with detection at 310 nm (Black) and 354 nm (Blue).

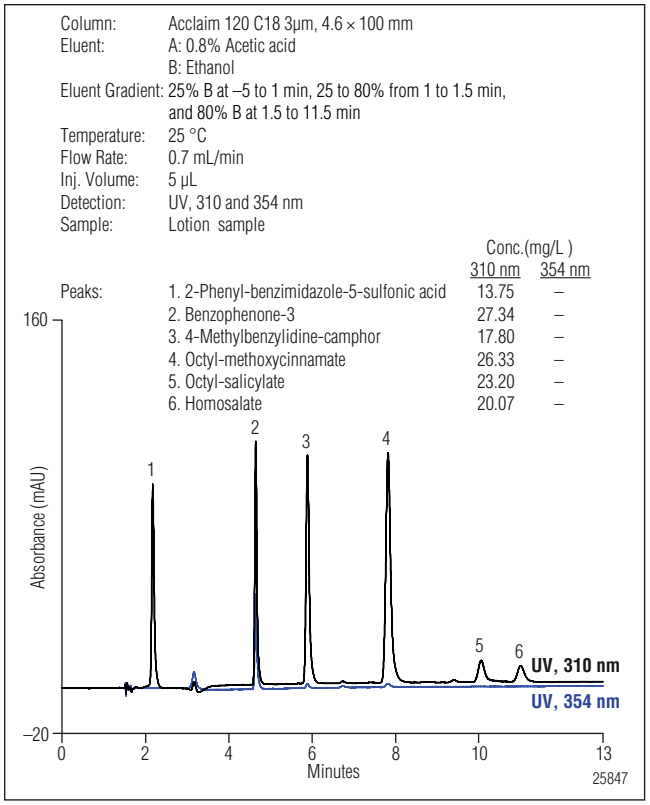

Figure 5. Overlay of three injections of the lotion sample with detection at 310 nm (Black) and 354 nm (Blue).

Table 4. Determination of Sunscreen Ingredients in Cosmetic Powder, Lotion, and Lipstick Samples													
		Concentration (mg/L) Determined at 310 nm and 354 nm When Noted											
		PHS	B-3	DHHB	DHHB (354 nm)	4-MBC	OCR	MA	OMC	BMDM	BMDM (354 nm)	OS	HMS
Cosmetic Powder Sample	Average*	N.A	N.A	N.A	N.A	N.A	N.A	10.40	32.78	N.A	N.A	22.88	N.A
	RSD	N.A	N.A	N.A	N.A	N.A	N.A	3.30	0.12	N.A	N.A	0.21	N.A
	%W/W	N.A	N.A	N.A	N.A	N.A	N.A	1.04	3.28	N.A	N.A	2.29	N.A
Lotion Sample	Average*	13.75	27.34	N.A	N.A	17.80	N.A	N.A	26.33	N.A	N.A	23.20	20.07
	RSD	0.74	0.20	N.A	N.A	0.08	N.A	N.A	0.08	N.A	N.A	0.17	0.19
	%W/W	1.38	2.73	N.A	N.A	1.78	N.A	N.A	2.63	N.A	N.A	2.32	2.01
Lipstick Sample	Average*	26.87	27.37	N.A	0.32	26.99	N.A	N.A	24.11	27.39	26.99	34.00	30.39
	RSD	0.33	0.32	N.A	2.15	0.15	N.A	N.A	0.31	0.50	0.28	0.68	0.56
	%W/W	2.69	2.74	N.A	0.03	2.70	N.A	N.A	2.41	2.74	2.70	3.40	3.04

*Three injections were made of each sample.

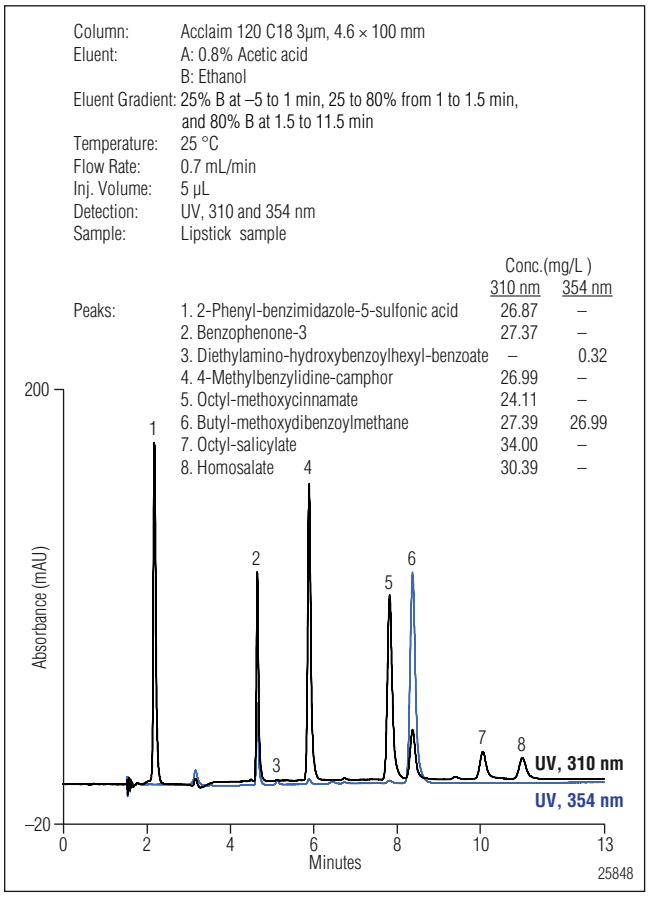

Figure 6. Overlay of three injections of the lipstick sample with detection at 310 nm (Black) and 354 nm (Blue).

CONCLUSION

This application note shows that 10 sunscreen compounds are baseline resolved in less than 12 min using an Acclaim 120 C18 column on an UltiMate 3000 system. This method accurately determines these compounds in a cosmetic power, a lotion and a lipstick.

Isocratic Separation of Basic, Neutral, and Acidic Molecules on Acclaim Mixed-Mode WAX-1

INTRODUCTION

The separation of different types of molecules in mixtures containing bases, neutrals and acids can prove to be challenging. The novel column chemistry of the Acclaim Mixed-Mode WAX-1 results in a multi-mode separation mechanism that includes hydrophobic, anion-exchange and cation-exclusion interactions. This column can separate all these molecules with baseline resolution.

METHOD

In this application, a 5 µm Acclaim Mixed-Mode WAX-1 column with the dimensions of 4.6 × 150 mm is used to separate a mixture of 11 basic, neutral and acidic molecules. The column was maintained at 30 °C. The separation is performed under isocratic conditions using an acetonitrile/buffer (1:1) mobile phase at a flow rate of 1.0 mL/min. The buffer was composed of 6.8 g KH_2PO_4 and 0.5 g NaP_2O_7*10 H_2O in 1000 mL water, adjusted to pH 6.0 with NaOH. A 5 µL injection was made and the method yielded a total run time under 20 min. Detection is accomplished using UV absorbance at 220 nm.

RESULTS

As shown in Figure 1, this method provides baseline separation of all 11 components in the mixture. This separation is clearly suitable for quantitatively assaying a wide variety of molecules in a single analysis.

Note: Pyrophosphate in the buffer suppresses interference from metal ions.

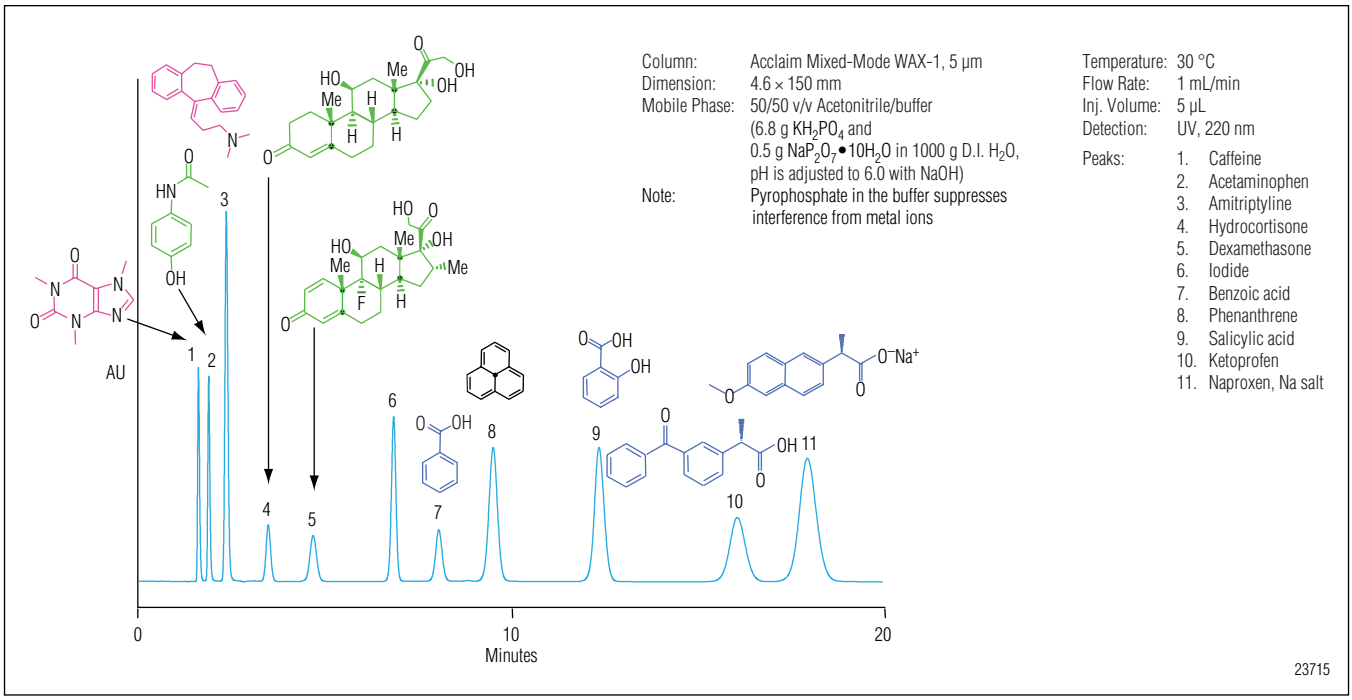

Figure 1. Isocratic separation of basic, neutral, and acidic molecules on Acclaim Mixed-Mode WAX-1.

Gradient Separation of Basic, Neutral, and Acidic Pharmaceuticals on Acclaim Mixed-Mode WAX-1

INTRODUCTION

The separation of different types of molecules in mixtures containing bases, neutrals and acids can prove to be challenging. The novel column chemistry of the Acclaim Mixed-Mode WAX-1 results in a multi-mode separation mechanism that includes hydrophobic, anion-exchange and cation-exclusion interactions. This column can separate all these molecules with baseline resolution.

METHOD

In this application, a 5 µm Acclaim Mixed-Mode WAX-1 column with the dimensions of 4.6 × 150 mm is used to separate a mixture of 16 basic, neutral and acidic molecules. The column was maintained at 30 °C. The separation is performed under ternary gradient conditions. Mobile phase A is acetonitrile, mobile phase B is water and mobile phase C is 150 mM phosphate buffer adjusted to pH 6.0 with NaOH. The 1.0 mL/min. flow rate yielded a total run time under 40 min. Detection is accomplished using UV absorbance at 220 nm.

RESULTS

As shown in Figure 1, this ternary gradient method provides baseline separation of all 16 components in the mixture. This separation is clearly suitable for quantitatively assaying a wide variety of molecules in a single analysis.

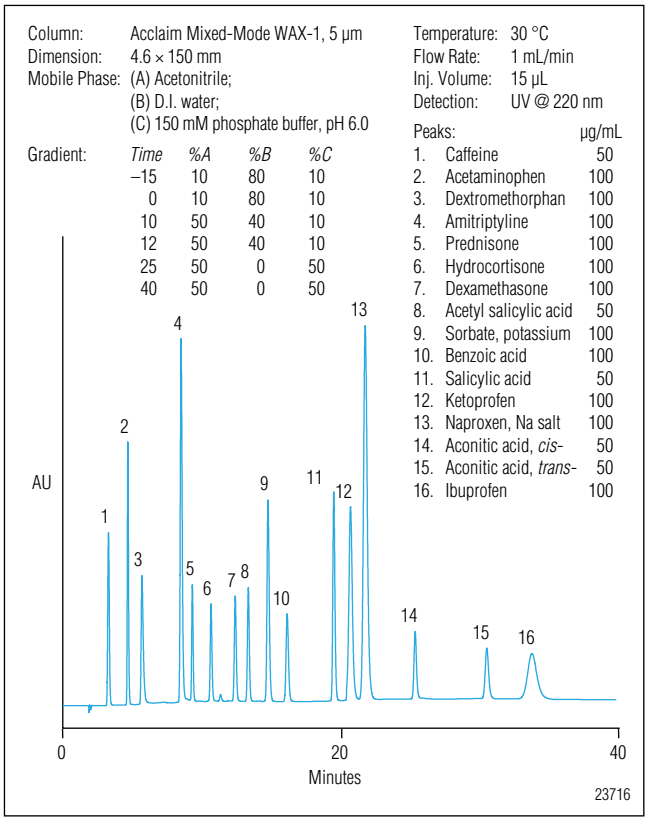

Figure 1. Gradient separation of basic, neutral, and acidic pharmaceuticals on Acclaim Mixed-Mode WAX-1.

Determination of Aucubin, Genipoide, and Pinoresinol Diglucoside in *Cortex Eucommiae* Using ASE and HPLC

This Application Brief (AB) describes an efficient method for the determination of aucubin, genipoide, and pinoresinol diglucoside in *Cortex eucommiae* using Accelerated Solvent Extraction (ASE®) and an UltiMate® 3000 HPLC system. The ASE method for the extraction of *C. eucommiae* replaced the soxhlet extraction method in the Chinese Pharmacopeia Edition, 2005.1 The Soxhlet method requires large volumes of two organic solvents and at least 12 h, whereas the ASE method required only 15 mL of methanol and 15 min per extraction. The extract was separated on an Acclaim® PA column. As shown in Figure 1, aucubin, genipoide, and pinoresinol diglucoside were found in a C. eucommiae sample, with the extraction and separation being completed within 1 h.

INSTRUMENTATION

Dionex UltiMate 3000 HPLC system consisting of:
- HPG 3400A pump
- WPS 3000TSL autosampler
- TCC-3200 thermostatted column compartment
- VWD3400 UV-vis Detector

Chromeleon® 6.80 SP5 Chromatography Data System
ASE 200 Accelerated Solvent Extractor

SAMPLE PREPARATION USING ASE

Add an accurately weighed 2 g of powdered (through a 250 µm sifter) *C. eucommiae* sample to an 11 mL stainless steel extraction cell equipped with two cellulose filters on the bottom. The samples should nearly fill the cell. Then extract with the ASE method, using the conditions described in Figure 1, and transfer the extracts into separate 50 mL volumetric flasks. Bring the flasks to volume with water.

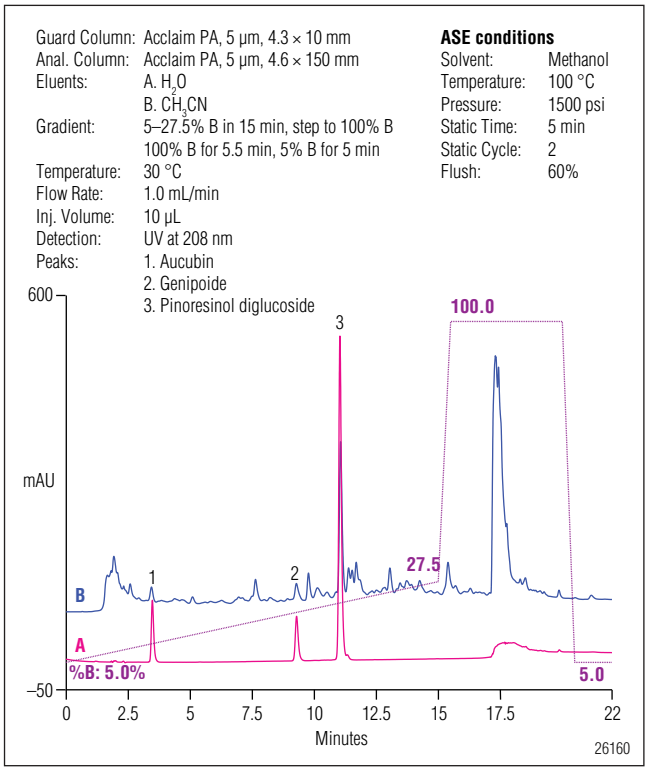

Figure 1. Chromatograms of A) mixed standards, and B) a c.eucommiae *sample.*

REFERENCE

1. Chinese Pharmacopoeia (Vol. 1) 2005, p114.

Separation of Sinapine Thiocyanate in Semen Raphani Using an Acclaim Phenyl-1 Column

Semen Raphani (Figure 1), the seed of *Raphanus sativus L.,* is a Chinese medicinal plant commonly used for treatment of dyspepsia and hypertension. Its anti-hypertension properties are attributed to the presence of sinapine thiocyanate.[1] The Chinese Pharmacopoeia (CP) monitors the quality control of Semen Raphani with a reversed-phase (RP) high-performance liquid chromatography (HPLC) method for the determination of sinapine thiocyanate (structure shown in Figure 2).[2] The method specifies a stationary phase with phenyl groups bonded to silica.

This work describes a separation of sinapine thiocyanate using an Acclaim® Phenyl-1 column, which is based on covalent modification of high-purity, spherical, and porous silica particles with a specially designed silane ligand bearing a proprietary alkyl aromatic functionality.[3] Figure 2 shows the separation of sinapine thiocyanate extracted from Semen Raphani following the CP method. The analyte of interest eluted as a symmetrical peak with adequate retention. The UV spectra of sinapine thiocyanate collected in the standard, Semen Raphani sample, and spiked Semen Raphani sample are highly consistent. The purity of sinapine thiocyanate may be estimated using the peak purity match factor, which can be calculated by Chromeleon® Chromatography Data System (CDS) software.

The calculated peak purity match factor for sinapine thiocyanate separated from the Semen Raphani extract is 950 (the corresponding value for 100% purity is 1000). These results demonstrate that the Acclaim Phenyl-1 column provides good selectivity and is suitable for analysis of sinapine thiocyanate.

Figure 1. Semen Raphani.

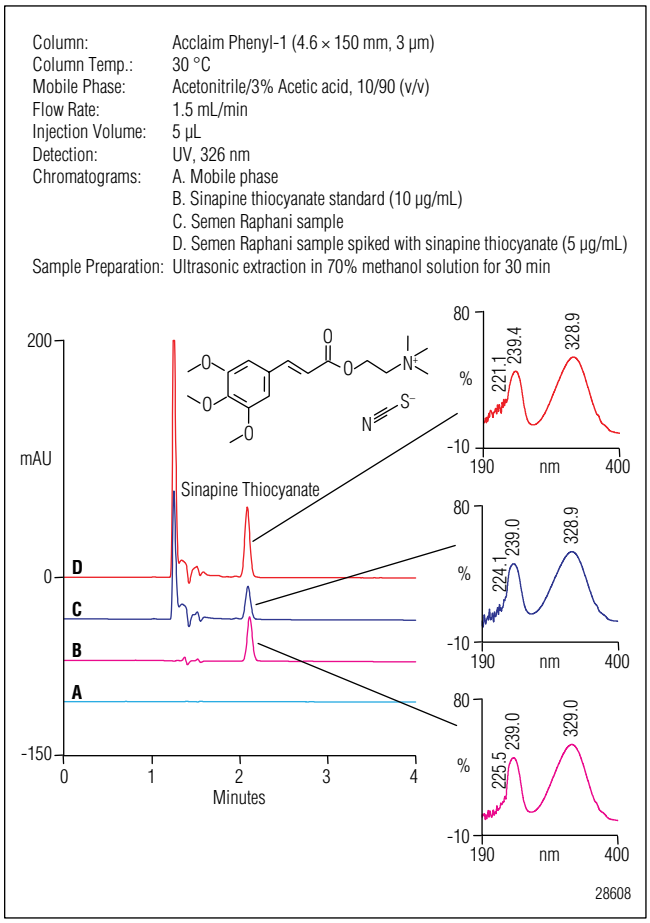

Figure 2. Chromatograms of sinapine thiocyanate on the Acclaim Phenyl-1 column following the CP method.

EQUIPMENT

Dionex UltiMate® 3000 RSLC system, including:

- LPG 3400RS pump
- WPS 3000RS autosampler
- TCC 3000RS thermostatted column compartment
- DAD 3000RS UV-vis detector
- Chromeleon 6.80 SR9 software

REFERENCES

1. Li, T.Y.; Li, T.G.; Zhang, G.X.; Guo, H.Y.; Liu, J.; Li, B.G.; Piao Z.Y.; Gai G.G. Experimental Study of Hypotensive Effect of Soluble Semen Raphani Alkaloids in Spontaneous Hypertensive Rats. *World J. Integrated Traditional and Western Med.* **2007,** *2* (1), 25.
2. Chinese Pharmacopoeia (Vol. 1), 2010, p 255.
3. Dionex Corporation, *Acclaim Phenyl-1 Unique Reversed-Phase Column with High Aromatic Selectivity,* Sunnyvale, CA. http://www.dionex.com/en-us/products/columns/lc/reversed-phase/acclaim-phenyl/lp-87227.html (accessed April 4, 2011).

Determination of Anthraquinones and Stilbenes in Giant Knotweed Rhizome by HPLC with UV Detection

INTRODUCTION

Giant knotweed rhizome, the dried rhizome and root of *Polygonum cuspidatum* Sieb. et Zucc. is a common medicinal plant in China (Figure 1). Chinese Pharmacopeia Edition 2005 (Ch. P 2005) regulates its use as an herbal medicine.[1] It is used with the belief that it cures angiocardiopathy, skin inflammations, liver diseases, reduces fever, and relieves arthritis pain etc.

Figure 1. Giant Knotweed Rhizome.

There are many compounds that are considered active components in giant knotweed rhizome, including anthraquinones (for example, anthraglycoside A, anthraglycoside B, emodin, physcion, rhein, and chrysophanol) and stilbenes (for example, resveratrol and polydatin). Their chemical structures are shown in Figure 2, and their UV absorption spectrums are shown in Figure 3. Ch. P 2005 regulates giant knotweed rhizome with two different HPLC methods for the determination of emodin and polydatin, respectively.[2] The quality control for giant knotweed rhizome, therefore, is inconvenient (that is, two methods) and inadequate, as the methods only determine two of the putative active components. Besides determining emodin and polydatin, some reported

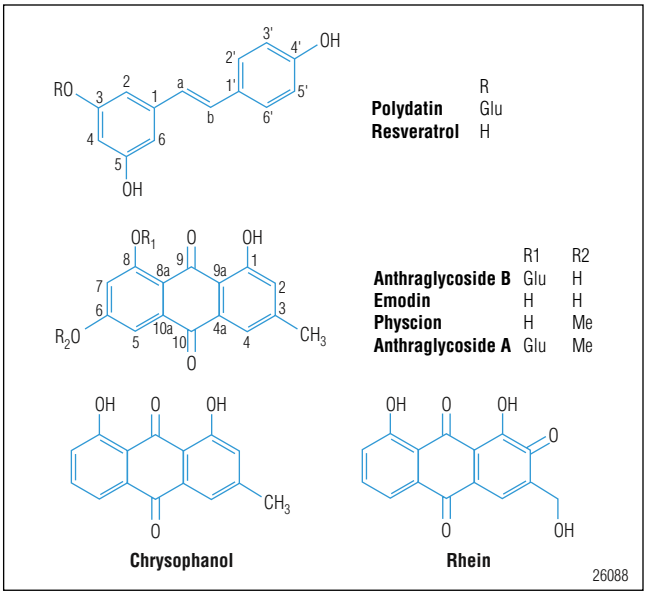

Figure 2. Structures of some compounds in giant knotweed rhizome.

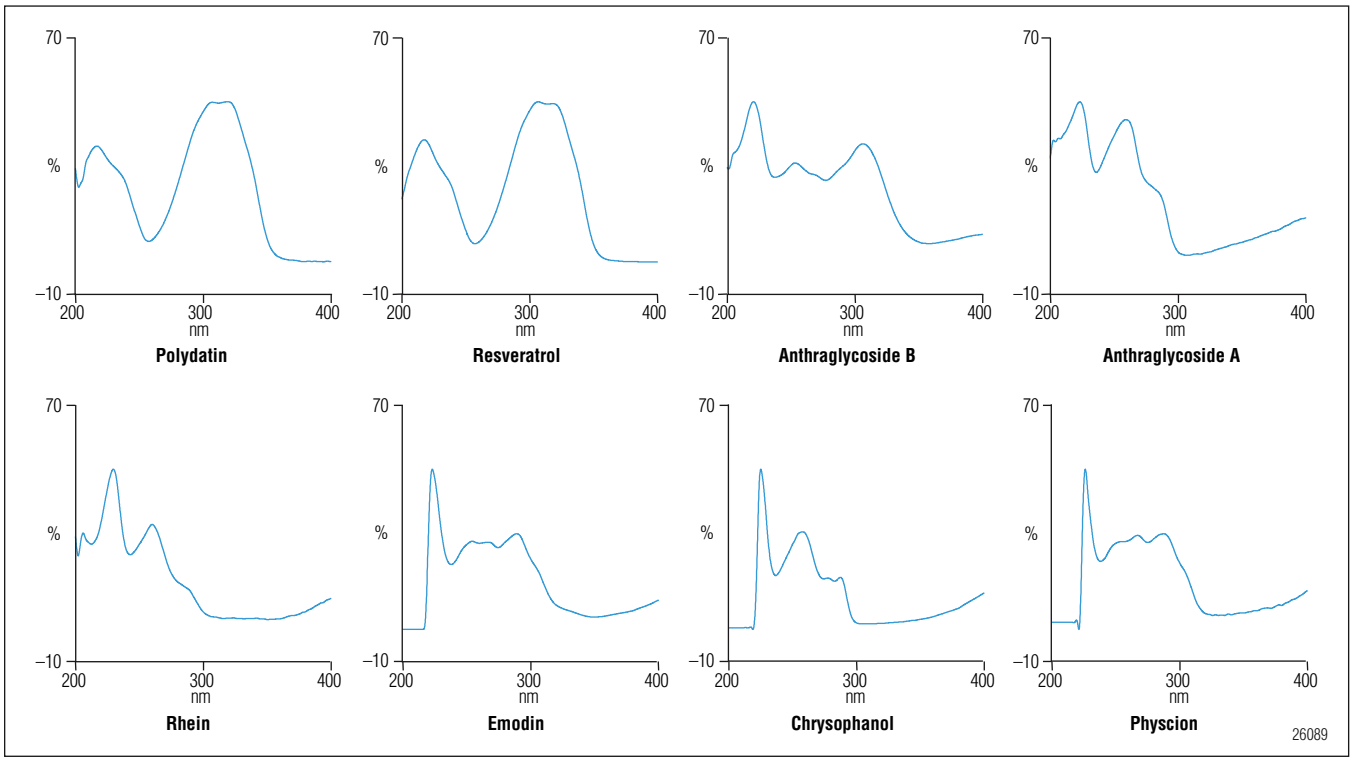

Figure 3. UV absorption spectra (obtained by auto background subtraction using Chromeleon software).

evaluation methods for the quality control of giant knotweed rhizome use the HPLC method to determine anthraglycoside A, anthraglycoside B, resveratrol, emodin, and physcion.[3,4] However, these methods are still inadequate for evaluating the quality of giant knotweed rhizome as one method determines the first three compounds listed previously and the second method determines the last four compounds.

In this application note (AN), we report an efficient HPLC method that in a single injection determines the eight main active components of giant knotweed rhizome: anthraglycoside A, anthraglycoside B, emodin, physcion, rhein, chrysophanol, resveratrol, and polydatin. Samples were extracted using ultrasonic extraction and the ASE® 200 Accelerated Solvent Extractor respectively, with satisfactory results obtained for both extraction methods.

EQUIPMENT

Dionex UltiMate® 3000 HPLC system consisting of:
- HPG 3400A pump
- WPS 3000TSL autosampler
- TCC-3200 thermostatted column compartment
- VWD-3400 UV-vis detector

Dionex Summit® 680 HPLC system consisting of:
- HPG 680 pump with SOR 100 Air Solvent Rack
- ASI-100™ Automated Sample Injector
- TCC-100 thermostatted column compartment
- PDA 100 UV-vis detector

Chromeleon® 6.80 SP5 chromatography data system

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

ASE 200 Accelerated Solvent Extractor

REGENTS AND STANDARDS

Water (Milli-Q® Gradient A10)

Methanol (CH_3OH) (HPLC grade, Fisher Scientific)

Acetonitrile (CH_3CN) (HPLC grade, Fisher Scientific)

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

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

Anthraglycoside A, anthraglycoside B, and polydatin, ≥98% (Kaimi Chem. Co., Shanghai, China)

Resveratrol, emodin, rhein, chrysophanol, and physcion, ≥98%, (Research Center of Standardization of CTM, Shanghai, China)

CONDITIONS

ASE Conditions

Solvent:	Methanol
Temperature:	100 °C
Pressure:	1500 psi
Static Time:	5 min
Static Cycles:	2
Flush:	20%
Purge:	60 s

Chromatographic Conditions

Guard Column:	Acclaim®120 C18 column, 5 µm, 4.3 × 10 mm, P/N 059446 with guard column holder, P/N 59526
Analytical Column:	Acclaim 120 C18 column, 5 µm, 4.6 × 250 mm, P/N 002762
Column Temp.:	30 °C
Mobile Phase:	(A) CH_3CN
	(B) 20 mM NH_4Ac
Gradient:	See Table 1
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
UV Detection:	Absorbance at 254 nm

Table 1. Gradient Elution Program

Time (min)	25 mM NH_4Ac (%)	CH_3CN (%)	Curve
0.0	75	25	
10.0	60	40	5
15.0	0	100	5
20.0	0	100	5
20.1	75	25	5
23.0	75	25	5

PREPARATION OF STANDARDS AND SAMPLES

Stock Standard Solution

Accurately weigh 100 mg of resveratrol and polydatin, 40 mg of anthraglycoside B, physcion and chrysophanol, and 20 mg of emodin, rhein, and anthraglycoside A, respectively; add them to individual 10 mL volumetric flasks and dilute to the mark with methanol. The concentration is 1000 µg/mL for resveratrol and polydatin, 400 µg/mL for anthraglycoside B, physcion, and chrysophanol, and 200 µg/mL for emodin, rhein, and anthraglycoside A.

Sample Preparation

Five giant knotweed rhizome samples from different regions in China were purchased for this study. Sample details are shown in Table 2.

Table 2. Five Purchased Giant Knotweed Rhizome Samples

Sample	Source
1	Zhejiang
2	Hebei
3	Hubei
4	Beijing Tongrentang 1
5	Beijing Tongrentang 2

Accurately weigh 0.25 g of each giant knotweed rhizome sample, dried and powdered, through a 250 µm sifter.

Ultrasonic method: Add the weighed sample to a 25 mL volumetric flask, and then add methanol to the mark. After 30 min of ultrasonic extraction of the uncovered flask, supplement the methanol to the mark to replace liquid lost by evaporation.

ASE method: Add the weighed sample to a 5 mL stainless steel extraction cell equipped with two cellulose filters on the bottom. Sample should nearly fill the cell. Extract with ASE using the conditions above and transfer each extract into a 25 mL volumetric flask. Bring to volume with methanol.

RESULTS AND DISCUSSION

Effects of Buffer pH Value

Giant knotweed rhizome is the dried rhizome and root of *Polygonum cuspidatum*. Like many Chinese medicines, it is a complex mixture of compounds. Although extraction is performed before analysis, the sample extract still contains many polar and nonpolar compounds that can interfere with determination of the target analytes. Given the complexity of the sample, an acetonitrile-ammonium acetate buffer prepared at different pH values is a good HPLC mobile phase choice to separate the target analytes from the other sample components.

To determine the optimum mobile phase pH value we evaluated the separation of the sample 4 extract at different pH values (Figure 4). Retention of both peaks I

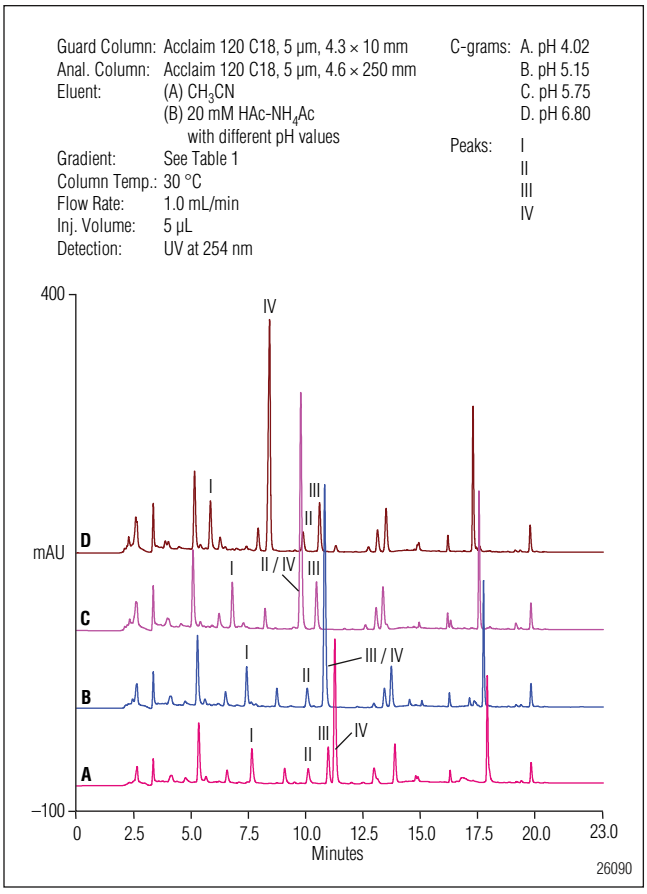

Figure 4. Chromatograms of sample 4 at different pH values of acetate buffer. The sample was extracted using the ultrasonic method.

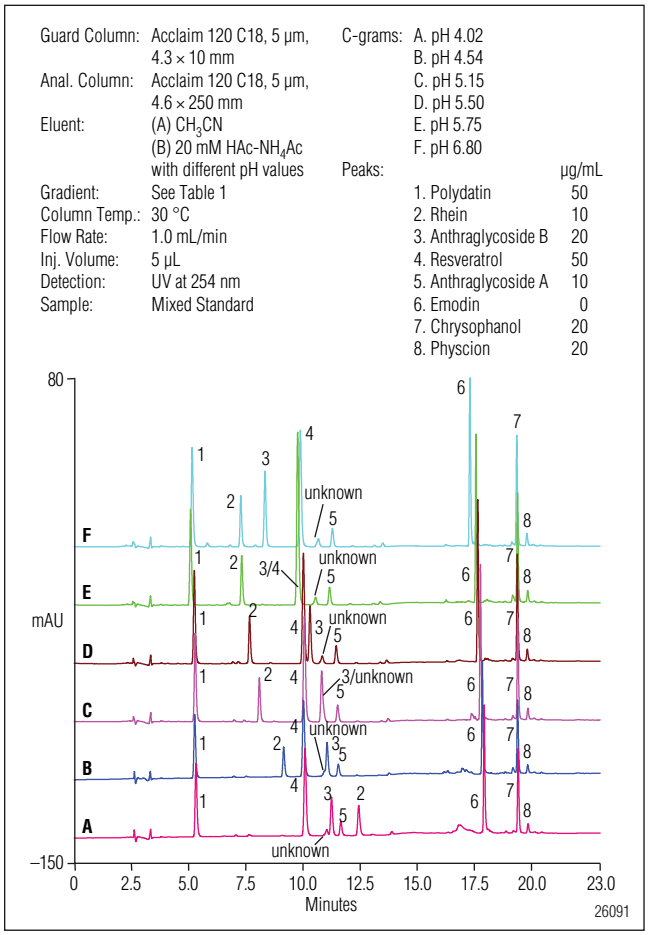

Figure 5. Chromatograms of a mixed standard at different pH values of acetate buffer. Compounds polydatin, rhein, anthraglycoside B, resveratrol, anthraglycoside A, emodin, chrysophanol, and physcion maintain the same peak numbers in Figures 6, 7, and 8. The numbering is not related to their order of elution.

and IV decrease with increasing mobile phase pH. Peak IV coelutes with peak III at pH 5.2, and with peak II at pH 5.8. The best separation of the components in the giant knotweed rhizome sample extract is at pH 6.8.

After investigating the effect of mobile phase pH value on the sample extract separation, we investigated the effect of mobile phase pH on an eight-component standard. As shown in Figure 5, retention of rhein (peak 2) and anthraglycoside B (peak 3) decrease with increasing mobile phase pH. Anthraglycoside B coelutes with resveratrol (peak 4) at pH 5.8. Notably, an impurity (unknown peak) can interfere with the determination of anthraglycoside B between pH 4.0–5.2. The impurity peak is well-separated from other components when pH ≥ 5.5. Similar to the study with the sample extract, this study suggests that 20 mM NH4Ac (pH 6.8) was the optimum mobile phase composition. At pH 6.8, the main components in the giant knotweed rhizome extract and the eight target analytes in the mixed standard are well-resolved, and the impurity found in the mixed standard does not interfere with the analysis. While ammonium acetate has little buffering capacity at pH 6.8, our studies found no reproducibility issues with the analysis of these samples under the conditions presented in this application note.

Reproducibility

Method reproducibility was estimated by making nine consecutive replicate injections of the mixed standard. Figure 6 is the overlay of the nine chromatograms and Table 3 summarizes the retention time and peak area precision data. The figure and table suggest that this method has good intraday reproducibility.

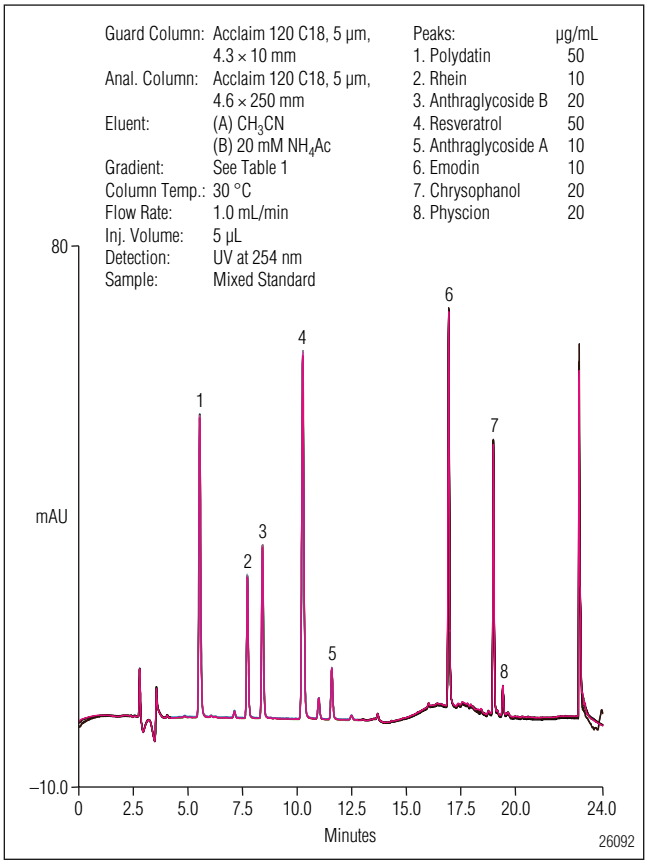

Figure 6. Overlay of chromatograms of nine consecutive injections of a mixed standard.

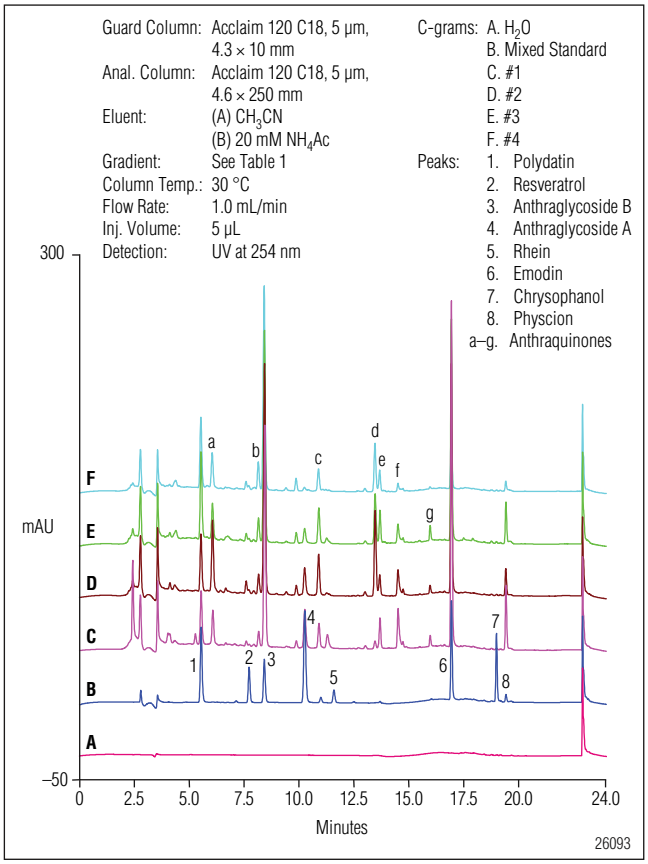

Figure 7. Overlay of chromatograms of water (chromatogram A), the mixed standard (chromatogram B), and samples 1-4 (chromatograms C–F, respectively).

Table 3. Retention Time and Peak Area Reproducibility

Analyte	Retention Time RSD	Peak Area RSD
Polydatin	0.075	0.385
Resveratrol	0.036	0.753
Anthraglycoside B	0.052	0.661
Anthraglycoside A	0.000	0.633
Rhein	0.036	0.953
Emodin	0.022	0.987
Chrysophanol	0.015	0.526
Physcion	0.019	0.727

Discrimination of the Components in Giant Knotweed Rhizome Samples

The putative active components in giant knotweed rhizome are stilbenes and anthraquinones. Stilbenes have three UV absorption maxima: 217, 307, and 318 nm. Anthraquinones have one UV absorption peak (~230 nm) and three UV absorption zones (240-260 nm, and 305–389 nm contributed by the benzene structure; and 262–295 nm contributed by the quinone structure). The absorption peak wavelength and intensity of UV absorption zones are related to the structure, including the number and position of the substituents. Figure 7 shows the chromatograms of the samples and the mixed standards. Six components, polydatin (peak 1), rhein (peak 2), anthraglycoside B (peak 3), resveratrol (peak 4), emodin (peak 6), and physcion (peak 8) were identified in each sample by comparing retention times and UV absorption spectra. Anthraglycoside A (peak 5) and chrysophanol (peak 7) were not found. Another seven peaks (peaks a–g) were found in each sample (peak c is the unknown peak shown in Figure 5). They each have one UV absorption peak (~ 230 nm) and three UV absorption zones between 240 and 295 nm with different absorption peak wavelengths and intensities, similar to the UV absorption spectra of anthraglycoside A, anthraglycoside B, and rhein (Figure 3) suggesting that they are anthraquinones.

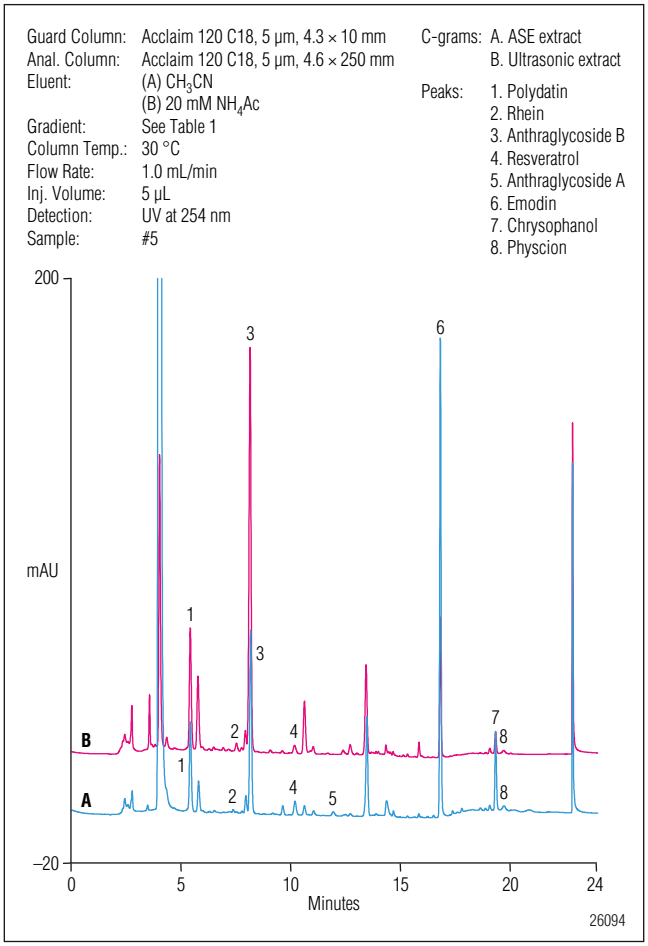

Figure 8. Chromatograms of sample 5 extracted using A) ASE and B) the ultrasonic method.

Using ASE for Sample Preparation and the UltiMate 3000 HPLC System for Chromatography

The giant knotweed rhizome sample 5 was extracted using ASE under different conditions (that is, temperatures and static cycles). The final conditions are as described in this AN. Figure 8 shows the chromatograms of the same sample extracted using ASE and the ultrasonic method. The separations were performed on the UltiMate 3000 HPLC system. Table 4 shows a comparison of the extraction methods using the same extraction solvent (methanol). The biggest difference is anthraglycoside A (peak 5), present in the ASE extract, but not found in the ultrasonic extract. Peak 5 in the chromatogram of the ASE extract matched the UV spectrum of anthraglycoside A. This ASE method shows higher recovery for the later eluting analytes and lower recovery of the early eluting analytes compared to the ultrasonic method, but it is possible that all high results (ASE and ultrasonic extraction) are a result of coextractables. Without a reference standard, it is difficult to compare the quality of the extraction methods for this application, but these results suggest that ASE, which is automated to save time and improve reproducibility, can be used for this application.

Table 4. Comparison of Extraction Efficiency Using ASE and Ultrasonic Extraction

Peak No.	Analyte	Peak Area (mAU*min)	
		Peak Area Obtained by ASE	Peak Area Obtained by Ultrasonic
1	Polydatin	3.45	5.16
2	Rhein	0.07	0.12
3	Anthraglycoside B	7.11	16.6
4	Resveratrol	0.71	0.45
5	Anthraglycoside A	0.21	0.00
6	Emodin	13.4	3.88
7	Chrysophanol	3.27	1.05
8	Physcion	0.88	0.21

CONCLUSION

This AN describes an HPLC method that baseline resolves eight active components in giant knotweed rhizome using 20 mM NH4Ac (pH 6.8) as part of the mobile phase. This method can be used for the quality control of giant knotweed rhizome, a common medicinal plant in China, and is superior to existing methods that measure only a few of the purported active components of giant knotweed rhizome. Sample extractions can be automated with ASE to save time and improve reproducibility.

ACKNOWLEDGEMENT

We would like to thank Ms. Jia Wenjun (Shanghai University of Traditional Chinese Medicine) for her enthusiastic assistance in the completion of this project.

REFERENCES

1. Chinese Pharmacopoeia (Vol. 1), 2005, p 1454.
2. Chinese Pharmacopoeia (Vol. 1), 2005, p 180–181.
3. Yang, F.Q., Zhang, T.Y., Ito, Y. Large-scale separation of resveratrol, anthraglycoside A and anthraglycoside B from *Polygonum cuspidatum* Sieb. et Zucc. by high-speed counter-current chromatography, *J. Chromatogr. A* **2001,** *919,* 443–448.
4. Chu, X., Sun, A.L., Liu, R.M. Preparative isolation and purification of five compounds from the Chinese medicinal herb Polygonum cuspidatum Sieb. et Zucc. by high-speed counter-current chromatography, *J. Chromatogr. A* **2005,** *1097,* 33–39.

Analysis of Benzalkonium Chloride on the Acclaim Surfactant Column by High-Performance Liquid Chromatography

INTRODUCTION

Benzalkonium chloride, a quaternary ammonium salt with antiseptic properties, is often used as an antiseptic, similar to other cationic surfactants. Its general formula is $[C_6H_5CH_2N(CH_3)_2R]Cl$, where R represents n-C_8H_{17} to n-$C_{18}H_{37}$ (Figure 1). The mode of action of quaternary ammonium compounds appears to be associated with their effect on the cytoplasmic membrane, which controls cell permeability. Extensive data are reported for effective concentrations (0.01%–0.1%) and affected bacterial species. The homologs n-C_{12} and n-C_{14} in benzalkonium chloride products comprise a major portion of the alkyl group.[1] In general, the homolog n-C_{12} is most effective against yeast and fungi, and the homolog n-C_{14} against gram-positive bacteria.[2] Benzalkonium chloride is not effective against gram-negative bacteria, and is not recommended for treatment when sanitization is critical.[3]

Although high-performance liquid chromatography (HPLC) has been used to separate and quantify benzalkonium chloride homologs,[2,4-7] the analysis can be challenging because the analyte peaks often tail on silica-based reversed-phase (RP) columns. This is due to the presence of residual silanols on the silica surface and the high degree of hydrophobicity of the surfactant. In this application note (AN), a complete HPLC methodology for determining benzalkonium chloride homologs in real samples, such as a sterile elastic strip and eye drops, is presented. The HPLC analysis was performed on a specialty column, the Acclaim® Surfactant column, with UV detection. The stationary phase features a unique surface chemistry that effectively deactivates the ionic interaction between the silica surface and cationic surfactants, resulting in excellent peak shapes for cationic surfactants.[8] In this AN, method development, linearity, and the limit of detection are also discussed. The total amount of benzalkonium chloride in sterile elastic strips and eye drops was determined and the amounts of homologs n-C_{12} and n-C_{14} were estimated by area normalization.

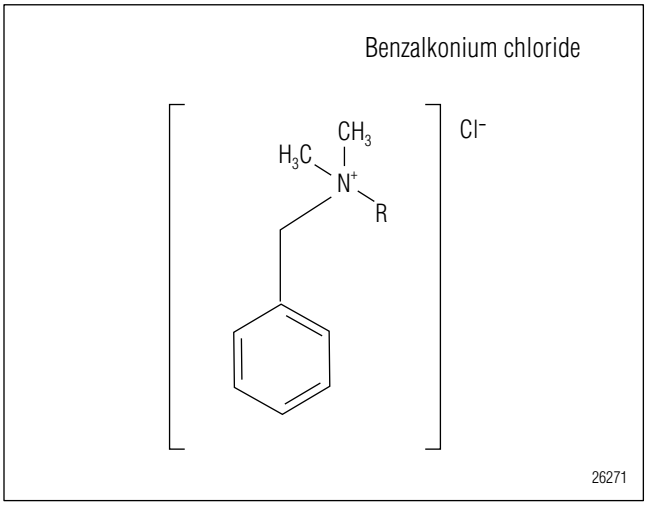

Figure 1. Structure of benzalkonium chloride.

EQUIPMENT

UltiMate® 3000 HPLC system

DGP-3600 pump with SRD-3600 Solvent Rack with degasser

TCC-3200 thermostatted column compartment

WPS-3000TSL autosampler

VWD-3400 UV-vis detector

Chromeleon® 6.80 SP3 Chromatography Data System

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

REAGENTS AND STANDARDS

Deionized water, from Milli-Q® Gradient A10

Acetonitrile (CH_3CN), HPLC grade, Fisher Scientific

Benzalkonium chloride (50% in water, CAS 63449-41-2, PN 09621), Fluka

Formic acid, ≥ 98%, Fluka

SAMPLES

Two sterile elastic strip samples (benzalkonium chloride patches, samples 1 and 2) and two samples of eye drops (samples 3 and 4) were purchased at a local store. Sterile elastic strip samples 1 and 2 were the same brand and product name but purchased from different stores. The two eye drop samples were over-the-counter drugs to which benzalkonium chloride was added as a supplementary component. Sample 3 is sold for anti-eyestrain and sample 4 is sold for allergic conjunctivitis treatment.

CONDITIONS

Chromatographic Conditions

Column:	Acclaim Surfactant column, 5 µm, 4.6 × 250 mm (P/N: 063203)
Column Temp.:	30 °C
Mobile Phase:	A: 100 mM Formic acid (dissolve 1.90 mL formic acid in 500 mL water)
	B: Water
	C: Acetonitrile : H_2O (70 : 30, v/v)
	In gradient (Table 1)
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Detection:	Absorbance at 208 nm (other wavelengths, for example, 225 and 230 nm, may also be used to achieve a better baseline but analyte response is lower)

Table 1. Gradient Elution Program

Time (min)	Formic acid 100 mM (%)	CH_3CN / H_2O (70 / 30, v/v) (%)	H_2O
-5	15	5	80
0	15	5	80
10	15	55	30
15	15	55	30

PREPARATION OF STANDARDS AND SAMPLES

Stock Standard Solution

Measure 2 mL of benzalkonium chloride standard (50% in water) and dilute in a 10 mL volumetric flask with water. The concentration of stock standard solution is 10%.

Working Standard Solutions

Prepare five working standard solutions for calibration by adding defined volumes of the stock standard solution and diluting with water. The concentrations of benzalkonium chloride are 20, 50, 200, 500, and 1000 µg/mL, respectively.

Sample Preparation

Add two patches of sterile elastic strips and 20 mL water to a 50 mL conical flask with stopper, and then use ultrasonic extraction for 15 min. Prior to injection, filter the solution through a 0.22 µm filter (Millex-HV).

The eye drop samples can be injected directly without dilution.

Spiked Brand of Sterile Elastic Strip Samples

Add 200 µL of stock standard solution of benzalkonium chloride, two patches of sterile elastic strips, and 19.8 mL water to a 50 mL conical flask with stopper, and then use ultrasonic extraction for 15 min. Filter the solution through a 0.22 µm filter (Millex®-HV) prior to injection.

RESULTS AND DISCUSSION

Retention Behavior of Benzalkonium Chloride on the Acclaim Surfactant Column

The Acclaim Surfactant column features reversed-phase, anion-exchange, and hydrogen-bonding retention mechanisms.[8] These features allow the column to be used differently than a conventional reversed-phase column. Thus, the retention behavior of benzalkonium chloride on the Acclaim Surfactant column was evaluated by varying the selectivity modifiers, ionic strength, pH value, and the amount and type of organic solvent in the mobile phase. Experiments show that increasing the buffer concentration (ionic strength) and buffer pH value produces an increase in retention time; and increasing the proportion of the organic solvent in mobile phase decreases the retention time. The results are consistent with the reported retention behavior of cationic surfactants on the Acclaim Surfactant column.[9]

Method Reproducibility

In the benzalkonium chloride standard (CAS 63449-41-2, Sigma-Aldrich), homolog n-C_{12} is predominant, with homologs n-C_{14} and n-C_{16} also being present.[10,11] Figure 2 shows an overlay of chromatograms of seven benzalkonium chloride standards with concentrations ranging from 20 to 1000 µg/mL. The peak area proportion of homolog n-C_{16} on 1000 µg/mL level (chromatogram 7) is 0.71%, which was calculated by comparing homolog n-C_{16} area to the total peak area of the three homologs; therefore, the amount of homolog n-C_{16} in the standard was ignored in subsequent calculations.

The method reproducibility was estimated by making seven consecutive injections of a 500 µg/mL benzalkonium chloride standard. The retention time reproducibility for both homologs, n-C_{12} and n-C_{14} was 0.107%; the peak area reproducibility for homolog n-C_{12} and n-C_{14} were 0.656% and 1.809% respectively.

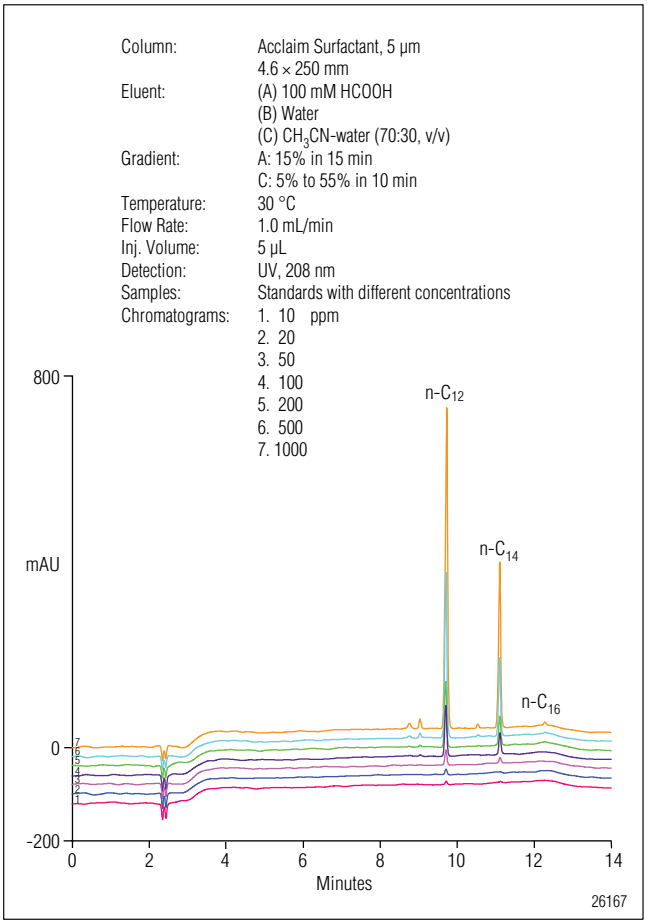

Figure 2. Overlay of chromatograms of seven benzalkonium chloride standards with different concentrations.

Linearity and Method Detection Limit of Benzalkonium Chloride

Calibration linearity for benzalkonium chloride was investigated by making five replicate injections of each of the five standard concentrations. The external standard method was used to establish the calibration curve and to quantify benzalkonium chloride in samples. As shown in Figure 3, excellent linearity was observed from 20 to 1000 µg/mL on plotting the concentration versus the sum of peak areas for homologs n-C_{12} and n-C_{14}. Separate calibration curves for homologs n-C_{12} and n-C_{14} were also prepared. These data are summarized in Table 2.

The detection limits for each of the homologs n-C_{12} and n-C_{14} were calculated using the equation:

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

where S = standard deviation of replicate analyses

n = number of replicates

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

Using eight consecutive injections of 500 µg/mL benzalkonium chloride standard to determine the S value, the estimated method detection limits (MDL) were 15 and 12 µg/mL for n-C_{12} and n-C_{14} respectively.

Table 2. Calibration Data

Analyte	Equation	r
Benzalkonium Chloride	A = 0.0763c–1.2514	0.9993
Homolog n-C_{12}	A = 0.0734c–0.7593	0.9980
Homolog n-C_{14}	A = 0.0817c–0.6133	0.9978

Determination of Total Benzalkonium Chloride in Samples

Two sterile elastic strip (samples 1 and 2) and two eye drop (samples 3 and 4) samples were analyzed. The results are summarized in Table 3. Figure 4(A) shows overlays of chromatograms of sample 1 and the same sample spiked with standard. The determined amount is in agreement with the labeled value. Sample 1 was found to contain homolog n-C_{12} but no homolog n-C_{14}. Sample 2, despite being the same brand and same product type as sample 1, may be a faulty product or at least not a very effective one, as no homolog of benzalkonium chloride was found (Figure 4B). Figure 5 shows chromatograms of the eye drop samples. Homologs n-C_{12} and n-C_{14} were found in sample 3, and only homolog n-C_{12} was found in sample 4.

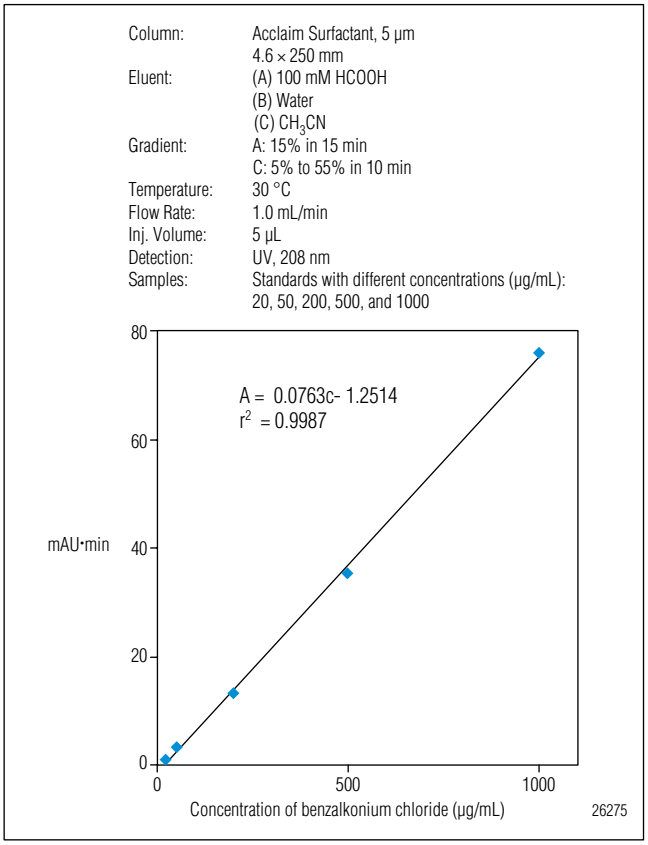

Figure 3. Calibration curve for benzalkonium chloride.

Determination of Homologs n-C_{12} and n-C_{14} of Benzalkonium Chloride in Samples

Because homologs n-C_{12} and n-C_{14} only differ by two units of CH_2, their sensitivity by UV detection may be assumed to be similar. Thus, the proportions of homologs n-C_{12} and n-C_{14} in samples can be estimated using the following formula:[12]

$$Cc_{12} = \frac{Ac_{12}}{Ac_{12} \times Ac_{14}} \times C \quad (1)$$

$$Cc_{14} = \frac{Ac_{14}}{Ac_{12} \times Ac_{14}} \times C \quad (2)$$

where, Ac_{12} and Ac_{14} represent peak areas of homologs n-C_{12} and n-C_{14} respectively; Cc_{12} and Cc_{14} represent concentrations of homologs n-C_{12} and n-C_{14} respectively; and C is the concentration of benzalkonium chloride.

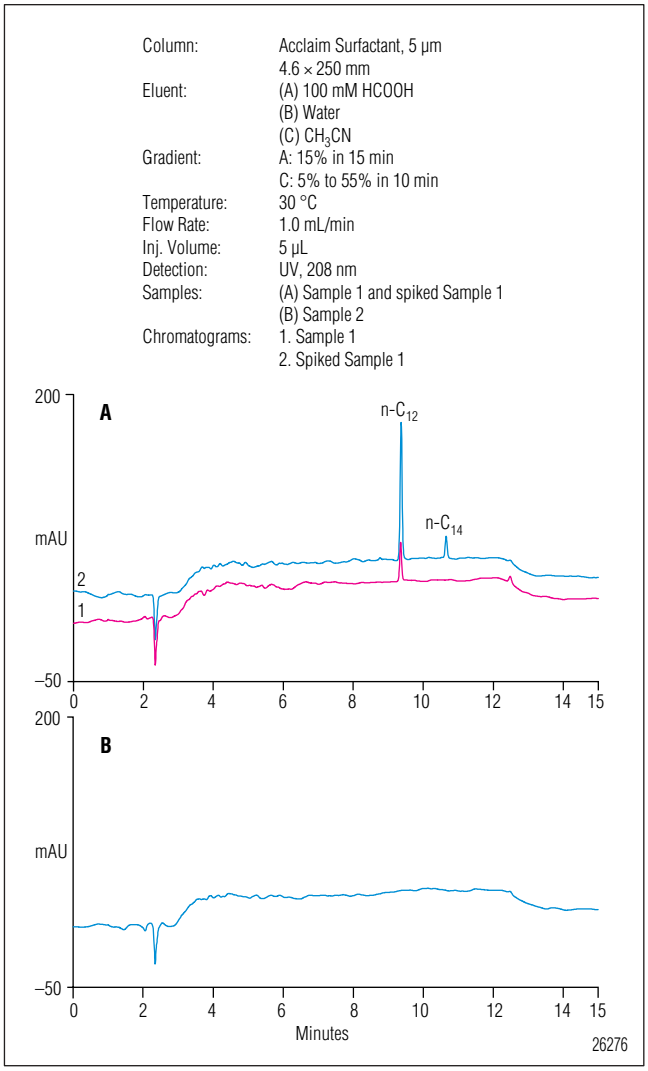

Figure 4. Chromatograms of sterile elastic strips sample (A) 1 and (B) 2.

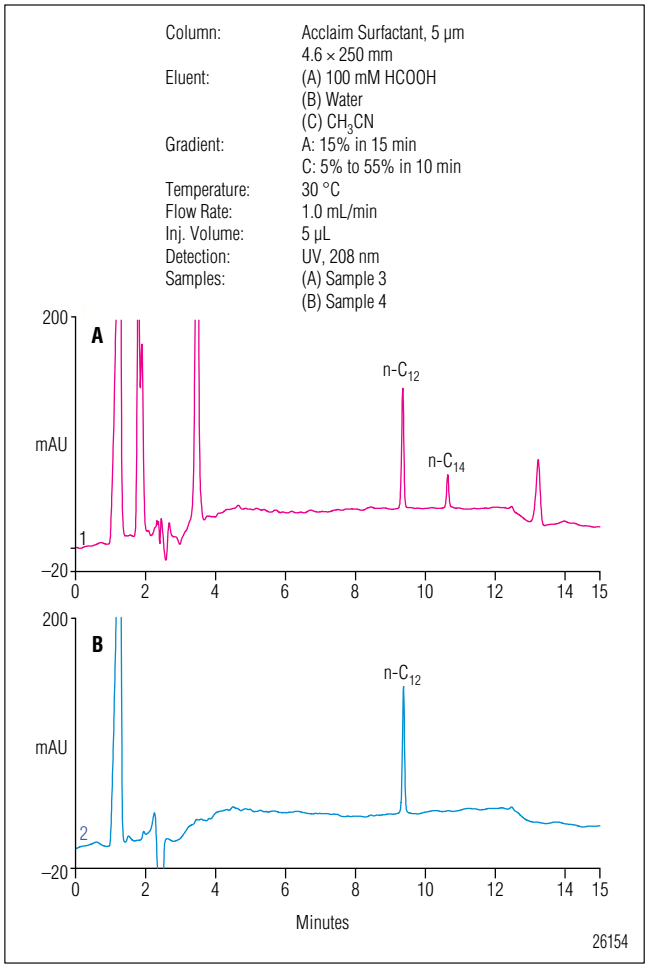

Figure 5. Chromatograms of eye drop sample (A) 3 and (B) 4.

Table 3. Analysis Results for Sterile Elastic Strips and Eye Drops

Analyte	Sterile Elastic Strips						Eye Drops	
	1					2	3	4
	Detected (mg/patch)	Labeled (mg/patch)	Added (µg/mL	Found (µg/mL)	Recovery (%)	Detected (µg/mL)	Detected (µg/mL)	Detected (µg/mL)
Benzalkonium Chloride	0.47	0.5	100	97	97	ND	146	118
Homolog n-C_{12}	0.43		70	83	119	–	116	116
Homolog n-C_{14}	ND		30	25	83	–	32	ND

Table 4. Concentrations of Homologs C_{12} and C_{14} in the Working Standards				
Conc. of Benzalkonium Chloride Standard (µL/mL)	C_{12} Homolog		C_{14} Homolog	
	Proportion	Concentration (µL/mL)	Proportion	Concentration (µL/mL)
20	70%	14	30%	6
50		35		15
200		140		60
500		350		150
1000		700		300

The proportions of homologs n-C_{12} and n-C_{14} in the benzalkonium chloride standard solutions used for calibration were estimated using formulas 1 and 2. The average distribution was 70% for homolog n-C_{12} and 30% for homolog n-C_{14}, based on which concentrations of homologs n-C_{12} and C_{14} in working standards were used to determine calibration linearity (Table 4). The calibration curves for the individual homologs were used to determine the amounts of these homologs in samples 1 through 4 (Table 3).

CONCLUSION

This application note describes an efficient and simple method for the analysis of benzalkonium chloride used as an antiseptic in sterile elastic strips and eye drops. The Acclaim Surfactant column yields efficient and symmetrical peaks for benzalkonium chloride, demonstrating its effectiveness for cationic surfactant analysis.

REFERENCES

1. Yang, Y.W.; Zhang, W.Q.; Zhu, Y. Simultaneous Determination of Quaternary Ammonium Salts in Antiseptics by High-Performance Liquid Chromatography. *Chin. J. Health Lab. Tech.* **2006**, *16* (7), 823–824.
2. Prince, S.J.; McLaury, H.J.; Allen, L.V.; McLaury, P. Analysis of Benzalkonium Chloride and Its Homologs: HPLC versus HPCE. *J. Pharm. & Biomed. Anal.* **1999**, *19*, 877–882.
3. Yuan, Q.X.; Ling, B. Utility Techniques of Antisepsis, Beijing: Chemical Industry Press, 2002.
4. Xue, Y.Y.; Hieda, Y.; Kimura, K.; Nishiyama, T.; Adachi, T. Sensitive Determination of Benzalkonium Chloride in Blood and Tissues Using High-Performance Liquid Chromatography with Solid Phase Extraction. *Legal Medicine* **2002**, *4*, 232–238.
5. Dudkiewicz-Wilczy´nska, J.; Tautt, J.; Roman, I. Application of the HPLC Method for Benzalkonium Chloride Determination in Aerosol Preparations. *J. Pharm. & Biomed. Anal.* **2004**, *34*, 909–920.
6. Radke, M.; Behrends, T.; Forster, J.; Herrmann, R. Analysis of Cationic Surfactants by Microbore High-Performance Liquid Chromatography-Electrospray Mass Spectrometry. *Anal. Chem*. **1999**, *71*, 0005362–5366.
7. Nunez, O.; Moyano, E.; Galceran, M.T. Determination of Quaternary Ammonium Biocides by Liquid Chromatography–Mass Spectrometry. *J. Chromatogr., A* **2004**, *1058*, 89–95.
8. Dionex Corporation. Pohl, C.; Liu, X.; Tracy, M. Analysis of Cationic Surfactants in Formulated Products by HPLC. LPN 1908-04 06/07, Sunnyvale, CA. 2007.
9. Dionex Corporation. Product Manual, Acclaim Surfactant Column. Doc. no. 065087-02, Sunnyvale, CA, 2009.
10. Product Information, Product Number B6295, Sigma Aldrich.
11. Product Information, Product Code 23442-7, Sigma Aldrich.
12. *Instrumental Analysis*. Shanghai Press of East China Normal University, 1986.

Determination of Ethylene Glycol and Diethylene Glycol in a Sorbitol Solution

INTRODUCTION

Glycerin, sorbitol, and propylene glycol (PG) are widely used in pharmaceuticals and personal care products, such as toothpaste, mouth wash, and medicinal syrups.[1–4] Adulteration of products containing glycerin or PG with the less expensive and highly toxic diethylene glycol (DEG) has been reported in several countries.[3,5] DEG and ethylene glycol (EG) are known poisons that are commonly used as industrial solvents and in antifreeze solutions, and therefore should not be found in pharmaceutical formulations. Ingestion or application of DEG-contaminated products, such as toothpaste and medicinal syrups (e.g., cough, teething, and acetaminophen syrups)[1–4, 6] is known to have caused systemic alcohol intoxication, acidosis, and subsequent multiorgan failure, leading to hundreds of fatalities.[5,6]

Glycerin, sorbitol, DEG, and EG have similar physical properties, such as sweetness and viscosity, which facilitates the adulteration process. Additionally, inconsistent or improper labeling of globally manufactured raw materials (DEG labeled as glycerin, industrial-grade glycerin labeled as pharmaceutical-grade glycerin) combined with inadequate international regulations[3,5] and analytical methods[2,3,8] increase the risk of contamination. To help prevent future adulteration, the U.S. FDA has classified sorbitol solution and its related excipients as being at high risk for contamination and requested the United States Pharmacopeia (USP) to revise the USP 32 NF 26–27 monographs to identify and quantify EG and DEG in PG, sorbitol, and glycerin solutions.[8,9] Based on FDA recommendations and CDC estimations of potential DEG contamination of 0.1 to 12.7% in pharmaceutical formulations using liquid excipients, the FDA proposed a limit of 0.1% DEG in sorbitol and other excipient solutions.[1,3,9] Therefore, the USP revised the sorbitol solution monograph to meet this requirement.[9] In this monograph, gas chromatography with flame-ionization detection is used to assay 0.08 mg/mL EG and DEG in 80 mg/mL sorbitol solution, uncorrected for sorbitol assay i.e., 0.1% EG and DEG in sorbitol solution.[1,9] To minimize the possibility of false positives, a second confirmatory method is needed to verify the presence of EG and DEG in the sample.

The application note (AN 246) presented here describes two confirmatory methods to determine 0.1% EG and DEG in sorbitol solutions by liquid chromatography with pulsed amperometric detection (PAD). One method separates EG by high-performance anion-exchange chromatography (HPAE) using sodium hydroxide (NaOH) on a CarboPac® MA1 column set with PAD using a Au on PTFE-working electrode and a four-potential carbohydrate waveform. The second method separates DEG in the sorbitol solution using a mixed-mode separation with an ion-exclusion (ICE) guard column and a cation-exchange analytical column with reversed-phase properties using a methanesulfonic acid (MSA) eluent.[10] The analytes are detected by PAD with a Pt working electrode and glycol waveform. AN 246 presents method qualifications and the analysis of EG and DEG in a sorbitol solution using the described methods. The technique presented in AN 246 takes advantage of the dual-pump capabilities, which enables the sequential operation of the two methods for the accurate, precise, and direct determination of 0.1% DEG and EG in a sorbitol solution.

EQUIPMENT

Dionex ICS-3000 Ion Chromatography system consisting of:

- DP Dual-Gradient Pump module to sequentially run both methods
- DC Detector/Chromatography Module
- AS Autosampler with Diverter Valve option (P/N 063294) to sequentially run both methods and 1.5 mL sample tray
- ED Electrochemical Detector (P/N 079830)
- Electrochemical cell (cell and reference electrode, P/N 061756)
- Combination pH–Ag/AgCl reference electrode (P/N 061879)

Chromeleon® 6.8 Chromatography Data System

Knitted reaction coil, 375 µL (P/N 043700) for each method

Filter unit for vacuum filtration, 0.2 µm nylon (Nalgene® Media-Plus with 90 mm filter, Nalge Nunc International, P/N 164-0020) or equivalent nylon filter

Vortex mixer to mix standard and sample solutions.

Vial Kit, 1.5 mL glass sample vials, with caps and slit septa (vial kit, P/N 055427)

Vacuum pump

PEEK™ Tubing:

- Black (0.25 mm or 0.01 in i.d., P/N 052307 for 5 ft) tubing used for liquid line connections from the pumps to the injection valve.
- Red (0.127 mm or 0.005 in i.d., P/N 052310 for 5 ft) tubing used for liquid line connections from injection valve to the guard and analytical columns, and cell.
- Blue (0.33 mm or 0.013 in i.d., P/N 052303 for 5 ft; in Diverter Valve kit)
- 25 µL PEEK sample loop (P/N 042857) for each method

Method 1: Determination of Ethylene Glycol in Sorbitol

Au on PTFE disposable working electrode (P/N 066480 package of six)

Method 2: Determination of Diethylene Glycol in Sorbitol

Type GL45, 2 L glass eluent bottle (P/N 045901)

Pt disposable working electrode (P/N 064440 package of six)

REAGENTS AND STANDARDS

Deionized water, Type 1 reagent-grade, 18.2 MΩ-cm resistivity, freshly degassed by ultrasonic agitation and applied vacuum

Use only ACS reagent-grade chemicals for all reagents and standards.

pH 7 (yellow) buffer solution (VWR International, P/N BDH5046)

pH 4 (red) buffer solution (VWR International, P/N BDH5018)

Diethylene glycol (Sigma-Aldrich, P/N 32160)

Ethylene glycol (Sigma-Aldrich, P/N E9129)

Method 1: Sodium hydroxide, 50% (w/w) (Fisher Chemicals, P/N SS254-500)

Method 2: Methanesulfonic acid (Fluka P/N 64280)

Standards for retention time studies only:

- Propylene glycol (1, 2 propanediol, Sigma, P/N P6209)
- Glycerin (VWR International, P/N JT2140)

SAMPLE

Sorbitol Solution, 67% assay, USP grade (VWR International, P/N 89050, Spectrum Chemical Mfg. Corporation)

CONDITIONS

Method 1: Ethylene Glycol in Sorbitol

Column:	CarboPac® MA1 Guard, 4 × 50 mm (P/N 044067), CarboPac MA1 Analytical 4 × 250 mm (P/N 044066)
Flow Rate:	0.4 mL/min
Eluent:	200 mM Sodium hydroxide
Source:	60% Eluent A, deionized degassed water; 40% Eluent B, 500 mM NaOH
Column Temp.:	30 °C
Inj. Volume:	25 µL
Detection:	PAD, disposable Au on PTFE
Data Collection Rate:	2.0 Hz
Waveform:	See Table 1
Reference Electrode:	AgCl mode
Typical Background:	40–60 nC
Typical System Backpressure:	1250 psi
Typical Noise:	60–80 pC
Typical pH:	12.5–12.9
Run Time:	60 min

Table 1. Carbohydrate Four-Potential Detection Waveform[11]

Time (s)	Potential vs Ag/AgCl (V)	Gain Region[a]	Integration	Ramp[a]
0.00	+ 0.10	Off	Off	Ramp
0.20	+ 0.10	On	On (Start)	Ramp
0.40	+ 0.10	Off	Off (End)	Ramp
0.41	- 2.00	Off	Off	Ramp
0.42	- 2.00	Off	Off	Ramp
0.43	+ 0.60	Off	Off	Ramp
0.44	- 0.10	Off	Off	Ramp
0.50	- 0.10	Off	Off	Ramp

[a]The gain and ramp are instrument settings for the ICS-3000 IC electrochemical detector.

Method 2: Diethylene Glycol in Sorbitol

Column:	IonPac® ICE-AS1 Guard, 4 × 50 mm (P/N 067842) IonPac CS14 Analytical, 2 × 250 mm (P/N 044121)
Flow Rate:	0.2 mL/min
Eluent:	100 mM MSA
Column Temp.:	30 °C
Inj. Volume:	25 µL
Detection:	PAD, disposable platinum
Data Collection Rate:	0.9 Hz
Waveform:	See Table 2
Reference Electrode:	AgCl mode
Typical Background:	90–110 nC
Typical System Backpressure:	900 psi
Typical Noise:	40–70 pC
Typical pH:	0.9–1.1
Run Time:	17 min

Table 2. PAD Detection Waveform Optimized for Glycols in Acid Eluents[12]

Time (s)	Potential vs Ag/AgCl (V)	Gain Region[a]	Integration	Ramp[a]
0.00	+ 0.30	Off	Off	Ramp
0.31	+ 0.30	On	Off	Ramp
0.32	+ 1.15	On	Off	Ramp
0.64	+ 1.15	On	On (Start)	Ramp
0.66	+ 1.15	On	Off (End)	Ramp
0.67	- 0.30	On	Off	Ramp
1.06	- 0.30	Off	Off	Ramp
1.07	+ 0.30	Off	Off	Ramp

[a]The gain and ramp are instrument settings for the ICS-3000 IC electrochemical detector.

PREPARATION OF SOLUTIONS AND REAGENTS

Eluent Solutions

When preparing eluents, it is essential to use high-quality, Type 1, 18.2 MΩ-cm resistivity deionized water that is free of carbon dioxide. Carbon dioxide dissolved in water produces carbonate, which adversely affects chromatography when using hydroxide eluents. In addition, dissolved gases also cause increased noise. Degas the deionized water before eluent preparation by using vacuum filtration and ultrasonic agitation with applied vacuum for 10 to 20 min.[13,14] Also, prepare 1 L of degassed Type 1 water weekly for the AS Autosampler flush solution.

Method 1: Eluent A Solution (Degassed Deionized Water)

Connect the eluent bottle to the Eluent A line from the pump and apply ~ 4–5 psi of head pressure using nitrogen or another inert gas, and prime the pump with the new eluent.

Method 1: Eluent B Solution, 500 mM NaOH

It is essential that high-purity (Fisher) 50% NaOH is used for eluent preparation. Do not use NaOH pellets because they are coated with sodium carbonate; this carbonate will bind to the column and interfere with the column selectivity, resolution, and efficiency.[13]

To prepare 2 L of 500 mM NaOH, add 1947.6 g of degassed, deionized water into a 2 L precleaned eluent bottle. Do not use glass bottles with hydroxide eluents because this can foul the working electrode. Use a top loader balance that is accurate to ± 0.01 g. Using the 25 mL transfer pipette, position the pipette in the center of the bottle and transfer 80 g (52.4 mL) of 50% NaOH solution to the 2 L eluent bottle. Immediately close and cap the hydroxide bottle and eluent bottle. Connect the eluent bottle to the Eluent B line from the pump and place the bottle under ~ 4–5 psi of head pressure using nitrogen or another inert gas. Swirl the eluent bottle to thoroughly mix the eluent, and prime the pump. For additional information on eluent preparation, please refer to Technical Note 71.[14]

Method 1: 2 M NaOH for System Decontamination

Prepare 1 L of 2 M NaOH similar to the 500 mM NaOH eluent using 160 g (105 mL) of 50% sodium hydroxide solution and 895 g of degassed, deionized water into a 1 L precleaned eluent bottle. Connect the eluent bottle to the Eluent C line from the pump and place the bottle under ~ 4–5 psi of head pressure using nitrogen or another inert gas. Swirl the eluent bottle to thoroughly mix the eluent, and prime the pump.

Method 2: Eluent A, 100 mM MSA

Prepare 2 L of degassed deionized water as previously described. Glass eluent bottles are recommended for this method to minimize noise and contamination from acid-extractable compounds from plastic containers. To prepare 2 L of 100 mM MSA eluent, pipette 13.0 mL (19.2 g) of MSA (FW 96.10) into a 2 L, type GL45 glass eluent bottle containing 1987 g of degassed, deionized water. Immediately cap the bottle, connect it to Pump 2 Eluent A line, place the eluent under ~ 4–5 psi of head pressure using nitrogen or another inert gas, thoroughly mix the eluent solution, and prime the pump.

Stock Solutions

Prepare the stock solutions monthly. Store at 5 °C.

Method 1: 100 mg/mL EG Stock Standard Solution

To prepare the 100 mg/mL EG stock solution from 99% EG solution, weigh 2 g (1.8 mL) of the 99% EG solution in a 20 mL glass scintillation vial containing 20 mL of deionized water. Mix the standard solution thoroughly using a vortex mixer.

Method 2: 100 mg/mL DEG Stock Standard Solution

Prepare the 100 mg/mL DEG stock solution in the same manner as 100 mg/mL EG stock solution using 99% DEG solution (2 g, 1.8 mL).

Stock Standards for Determining Glycol Selectivity

Prepare individual 100 mg/mL stock standards of PG and glycerin by adding 2 g (1.9 mL) of 99% PG and 2 g (1.6 mL) of 99% glycerin to 20 mL glass scintillation vials. Then, add 20 mL of deionized water to each vial.

Intermediate and Working Standard Solutions

Prepare the working standards daily and the intermediate standard weekly. Store at 5 °C.

Method 1: EG Intermediate and Working Standard Solutions

To prepare a 1.0 mg/mL EG intermediate standard solution, pipette 200 µL of 100 mg/mL EG into 19.8 mL of deionized water on an analytical balance and mix the solution with the vortex mixer. Prepare the 1.3, 2.5, 5.0, 10, and 20 µg/mL EG working standards by pipetting 26, 50, 100, 200, and 400 µL, respectively of the 1.0 mg/mL EG intermediate standard solution in deionized water to a total of 20.0 g. Mix with a vortex mixer. Prepare a 0.5 mg/mL EG spiking standard solution by diluting 5 mL of 1 mg/mL EG stock solution with 5 mL of deionized water, and mixing with a vortex mixer. Prepare the working standards and spiking standard solution daily, the intermediate standard weekly, and the stock standard monthly. Store at 5 °C.

Method 2: DEG Intermediate and Working Standard Solutions

To prepare a 1 mg/mL DEG intermediate standard solution, pipette 200 µL of 100 mg/mL DEG into 19.8 mL deionized water on an analytical balance. Mix with a vortex mixer. Prepare the 0.013, 0.025, 0.050, 0.10, and 0.025 mg/mL DEG working standards in a similar way with 260, 500, 1000, 2000, and 5000 µL, respectively of the 1.0 mg/mL DEG intermediate standard solution and deionized water to a total of 20.0 g, and mixing with a vortex mixer. Prepare a 5 mg/mL spiking standard solution by adding 1 mL of 50 mg/mL DEG stock solution into 9 mL deionized water and mixing the standard solution thoroughly.

SAMPLE PREPARATION

To prepare a stock solution containing 50 mg/mL sorbitol from the 67% sorbitol solution, add 746 mg of the 67% sorbitol solution to 10 mL of deionized water on an analytical balance. Use the vortex mixer to mix the solutions.

Method 1

To prepare the 1.3 mg/mL sorbitol sample for EG determinations, dilute the 50 mg/mL sorbitol solution 40-fold by pipetting 500 µL of the 50 mg/mL sorbitol into 19.5 mL of deionized water on an analytical balance. Mix thoroughly using a vortex mixer. To prepare a spike recovery sample of 1.3 µg/mL EG in 1.3 mg/mL sorbitol (0.1% w/w EG in sorbitol), pipette 52 µL of 0.5 mg/mL EG into 20 mL of 1.3 mg/mL sorbitol, and mix the final solution thoroughly with a vortex mixer.

Method 2

To prepare the 13 mg/mL sorbitol sample for DEG determinations, dilute the 50 mg/mL sorbitol solution four-fold by pipetting 5 mL of the 50 mg/mL sorbitol into 15 mL deionized water on an analytical balance and mix the final solution thoroughly with a vortex mixer. To prepare a spike recovery sample of 0.013 mg/mL DEG in 13 mg/mL sorbitol (0.1% (w/w) DEG in sorbitol), pipette 52 µL of 5 mg/mL DEG into 20 mL of 13 mg/mL sorbitol and mix the final solution thoroughly with a vortex mixer.

SYSTEM PREPARATION AND SETUP

Do not remove or install the ED while the DC is turned on as these power surges may cause internal damage to the ED module. To reduce analysis time, configure the ICS-3000 system to run both methods sequentially under separate timebases.

Configuring Virtual Channel to Monitor pH

The continuous monitoring of pH during sample analyses provides details on reference electrode drift and noise, and also confirms proper eluent preparation. To monitor the pH, follow the instructions in AN 188 to create a Virtual Channel using the Server Configuration program.[12] Each Virtual Channel must be assigned a unique name, such as *VirtualChannel_01 and VirtualChannel_02* to prevent confusion in programming. Once configured, the pH virtual channel becomes one of the available signal channels.

Plumbing and Configuring the AS Autosampler

To run both systems sequentially, installation of a diverter valve in the AS Autosampler is required. To ensure accurate injections, calibrate the injection port tubing volume that now includes the diverter valve and the blue PEEK tubing according to the calibration instructions (Calibrate IPTV) under the AS Autosampler tab on the Chromeleon panel and in Section 5.9 in the AS Autosampler Operator's manual.[15] After the calibration is complete, verify that the values for each system have been loaded in the AS Autosampler module. Change the mode to sequential and enter the sample loop size for both systems. The additional commands needed to control the position of the diverter valve, use of the AS Autosampler, and control of temperature are thoroughly discussed in Technical Note 64.[16]

Plumbing the Chromatography System

Method 1: General Tips

As a precaution to minimize microbial contamination, temporarily install a 1000 psi backpressure loop between the pump and the injection valve and pump 2 M NaOH through the pump and eluent lines at 0.5 mL/min for 1 to 2 h, followed by deionized water for another 1 to 2 h.[13] Remove the backpressure loop prior to installing the column.

Method 1: Plumbing System 1

To plumb System 1 for EG determinations, install black PEEK tubing between the pump and injection valve and red PEEK tubing for all other eluent lines after the injection valve to the cell inlet. Follow the instructions in the product manual to install the CarboPac MA1 column set on System 1.[17] Once installed, the expected system backpressure of the CarboPac MA1 column set is typically ~ 1300 psi. Install the 375 µL knitted reaction coil between the outlet of the analytical column and the electrochemical cell as described in AN 188. Allow the column to equilibrate overnight using the method conditions.

Method 2: Plumbing System 2

To plumb System 2 for DEG determinations, install black PEEK and red PEEK tubing, the IonPac ICE-AS1 Guard, IonPac CS14 Analytical column, and 375 µL knitted reaction coil in a similar way as required for System 1. For more information on the IonPac ICE-AS1 Guard and IonPac CS14 Analytical columns, refer to the product manuals.[18,19]

Assembling the Electrochemical Cell

To assemble the electrochemical cell, follow the instructions in AN 188, and install a disposable Au on PTFE-working electrode on System 1, and a disposable Pt working electrode on System 2 for the EG and DEG applications, respectively. The Au on PTFE-working electrode was designed for the high base concentrations used in the EG application.[20] The disposable Au on PTFE-working electrode can be used with NaOH concentrations up to 750 mM without loss in response. The disposable Pt working electrode has a typical lifetime of two weeks in a strong acid eluent using the waveform optimized for glycol determination.

RESULTS AND DISCUSSION

In preliminary experiments, the authors evaluated the separation of 0.1 mg/mL DEG, EG, sorbitol, glycerin, and PG standards by HPAE, ICE, and by a mixed-mode separation using the IonPac ICE-AS1 guard column with the CS14 cation-exchange column and detected by PAD. In HPAE-PAD, EG was well resolved from sorbitol, however, DEG showed a poor peak response and was not resolved from PG and small unknown peaks in the sorbitol solution. In ICE-PAD, sorbitol was well resolved from glycerin but EG, DEG, and PG eluted at approximately the same time. In the mixed-mode separation, sorbitol, glycerin, and EG eluted near the void. However, DEG was well resolved from the void and was nearly baseline-separated from PG. Therefore, to accurately determine 0.1% EG and DEG in a sorbitol solution, the authors optimized HPAE-PAD and the mixed-mode separation with PAD to separately determine EG and DEG, respectively.

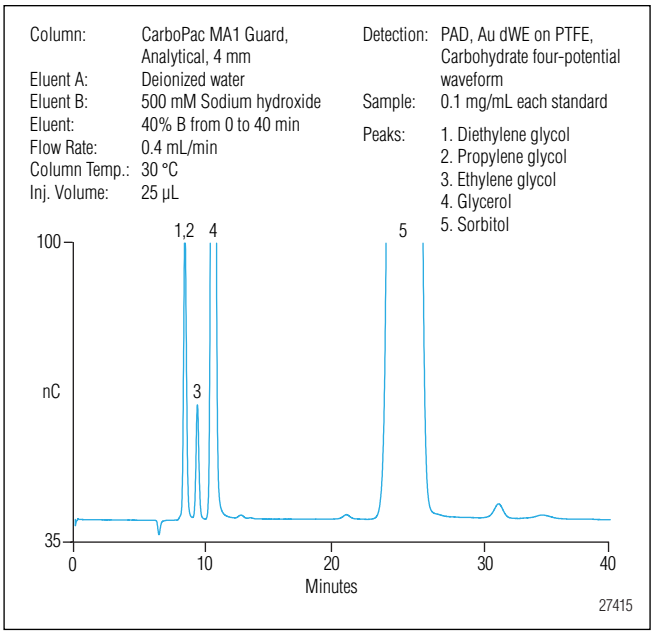

Figure 1. Separation of a 100 mg/mL standard mix by HPAE-PAD.

Method 1: Determination of EG by HPAE-PAD

Figure 1 shows separation of 0.1 mg/mL each of PG, DEG, EG, glycerin, and sorbitol using the CarboPac MA1 column with 200 mM NaOH and detection by HPAE-PAD. The analytes have strong peak responses and good separation from each other with the exception of DEG, which has a poor peak response and coelutes with PG (Peaks 1–2). This column has the high capacity (1450 µEq/column) needed to prevent overloading when injecting high concentrations of sorbitol, and good selectivity for the analytes with the exception of DEG. The large sorbitol peak elutes after the peaks of interest. The separation requires 60 min to elute other late-eluting compounds.

Table 3. Method Results for EG and DEG Determinations

Analyte	Method	LOD (µg/mL)	LOQ (µg/mL)	Linear Range (mg/mL)	Linearity (r^2)
EG	HPAE-PAD	0.25	0.85	0.0013 to 0.010	0.9991
DEG	Mixed Mode and PAD	3.1	10	0.013 to 0.10	0.9993

Limit of Detection, Limit of Quantification, Linear Range, and Precision

To qualify the method, the authors determined the estimated limit of detection (LOD), limit of quantification (LOQ), linear range, and precision. The LOD and LOQ were determined by measuring the peak-to-peak noise in 1-min increments from 20 to 60 min in four replicate runs without a sample injection. Noise averaged 62 ± 0.28 pC for this series of blank runs. The estimated LOD and LOQ were calculated as 0.25 and 0.85 µg/mL EG, respectively, based on the peak response of the standard at 3× and 10× the signal-to-noise (S/N). These values demonstrate the sensitivity of HPAE-PAD for this application. To determine the method linearity, four EG calibration standards from 1.3 to 10 µg/mL in deionized water were injected in triplicate. The results were linear with correlation coefficient (r^2) of 0.9991 (see Table 3). The retention time and peak area precisions—based on seven replicate injections of a 1.3 µg/mL EG standard—produced RSDs of < 0.2 and < 3, respectively.

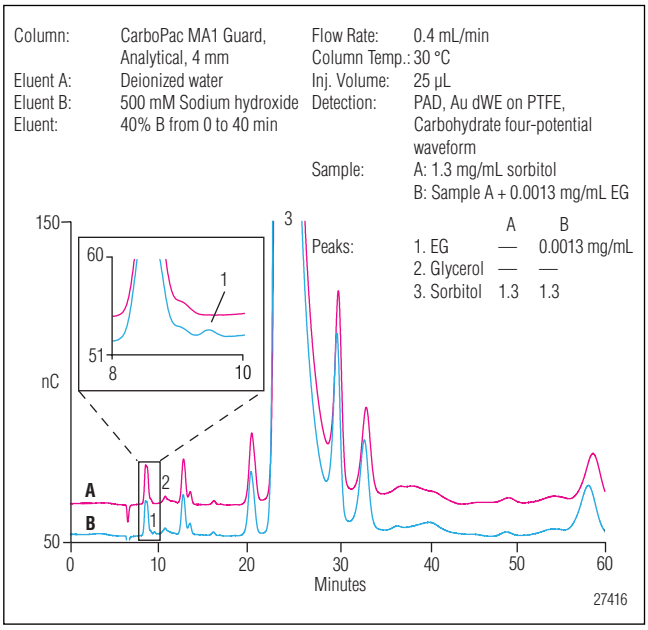

Figure 2. Comparison of 1.3 mg/mL sorbitol A) without and B) with 0.0013 mg/mL ethylene glycol (EG) by HPAE-PAD.

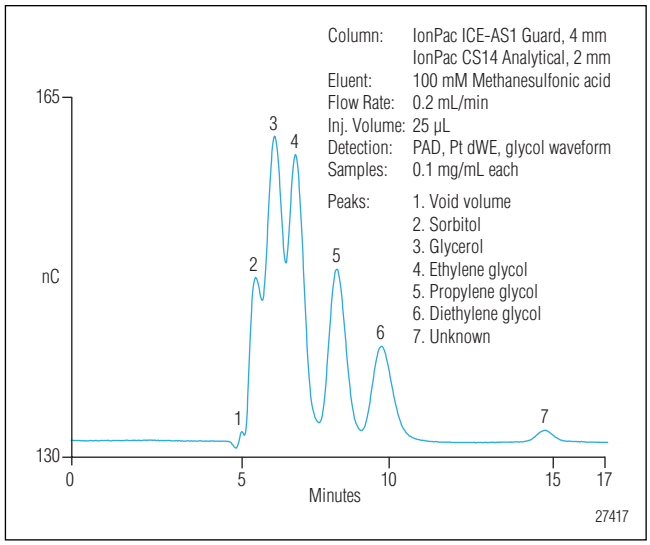

Figure 3. Separation of standards by mixed-mode separation and PAD.

EG in Sorbitol Samples

To determine EG in sorbitol, the authors prepared 0.05 mg/mL EG in 50 mg/mL sorbitol (a 0.1% w/w solution of EG in sorbitol) and found low recovery (< 75%) due to possible column overload. Therefore, to improve the accuracy of the method, a 40-fold sample dilution was used. Figure 2 compares the separation of 1.3 mg/mL sorbitol sample with and without 1.3 µg/mL EG. Replicate injections of the spiked sample produced an average recovery of 106% (n = 7) with retention time and peak area RSDs of 0.11 and 1.4, respectively. To determine the limits of the method, the recovery of 1.3 µg/mL EG was determined in 0, 1.3, 1.6, 1.8, and 2.0 mg/mL sorbitol using triplicate injections. The acceptable recoveries of 106–120% of 1.3 µg/mL EG in the sorbitol solutions demonstrated that sorbitol solutions can be diluted up to 1.8 mg/mL without sacrificing accuracy.

Method 2: Determination of DEG by Mixed-Mode Separation and PAD

Figure 3 shows a chromatogram of 0.1 mg/mL each of sorbitol, glycerin, EG, DEG, and PG using a mixed-mode separation on the high-capacity, fully sulfonated ion-exclusion IonPac ICE-AS1 guard column combined with the moderately hydrophobic IonPac CS14 analytical cation-exchange column using 100 mM MSA and PAD.

In ICE, undissociated glycols and sorbitol are separated using a strong acid eluent by steric exclusion and thus, elute in order of their decreasing hydroxyl groups.[21] The ICE-AS1 guard column separates sorbitol from DEG; however, DEG, EG, and PG having two hydroxyl groups, coelute. Combining the IonPac ICE-AS1 guard with the IonPac CS14 Analytical column takes advantage of the ICE properties of the ICE-AS1 Guard column and uses the hydrophobic interaction properties of the CS14 column to resolve PG from DEG and sorbitol.

LOD, LOQ, Linear Range, and Precision

To qualify the mixed-mode separation and PAD method, the authors evaluated the same parameters as discussed in Method 1 using HPAE-PAD. The estimated LOD and LOQ, based on the average peak-to-peak noise of 59 ± 1.1 pC, were 3.1 and 10 µg/mL DEG, respectively. This is approximately 10× higher than with HPAE-PAD for EG determinations. The calibration from 0.013 to 0.10 mg/mL DEG produced a correlation coefficient (r^2) of 0.9993. The retention time and peak area precision of a 0.013 mg/mL DEG (n = 7) standard produced RSDs of 0.17 and 2.2, respectively.

DEG in Sorbitol Samples

To determine an appropriate sample dilution for this method, 0.05 mg/mL DEG added to 50 mg/mL sorbitol [a 0.1% (w/w) solution of DEG in sorbitol] was diluted two-, four-, and eight-fold. The experiments showed that the four-fold dilution was optimum based on recovery

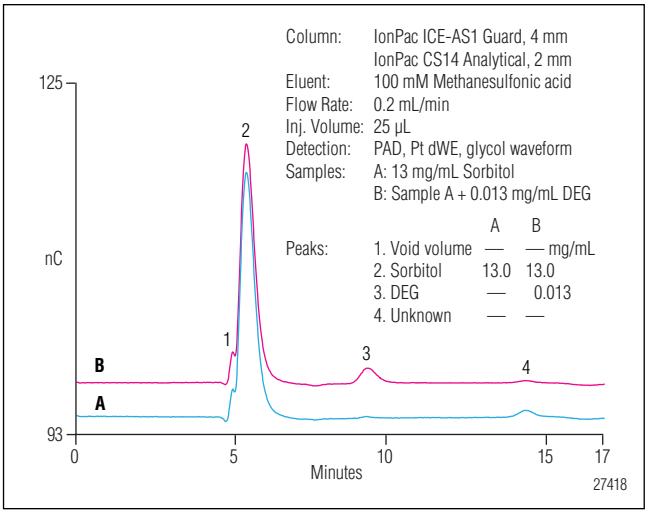

Figure 4. Comparison of 13 mg/mL sorbitol A) without and B) with 0.013 mg/mL diethylene glycol (DEG) by mixed-mode separation and PAD.

of DEG, therefore this dilution was used for the sample analyses. Figure 4 shows a separation of the four-fold diluted sample 0.013 mg/mL DEG resolved from the 13 mg/mL sorbitol. The precision and accuracy were determined by performing seven replicate injections of 0.013 mg/mL DEG added to 13 mg/mL sorbitol. The average recovery was 107% with the retention time and peak area RSDs of 0.23 and 3.9, respectively. To determine the limits of the method, the recovery of 0.013 mg/mL DEG was determined in 0, 6, 8, 13, and 18 mg/mL sorbitol using triplicate injections. Recoveries of 107–112%, of 0.013 mg/mL DEG added to sorbitol concentrations from 0 to 13 mg/mL were achieved. The analysis of sorbitol concentrations > 13 mg/mL by this method is not recommended due to column overload.

CONCLUSION

This work describes two methods to determine 0.1% EG and DEG in diluted sorbitol solutions. EG was determined in 1.3 mg/mL sorbitol on a CarboPac MA1 column by HPAE-PAD with a Au working electrode. DEG was determined in 13 mg/mL sorbitol by mixed-mode separation using an IonPac ICE-AS1 ion-exclusion guard and IonPac CS14 cation-exchange column and detected by PAD and a Pt working electrode. Both methods were run sequentially on an ICS-3000 dual system. These applications provide confirmatory analytical methods to directly, accurately, and selectively determine µg/mL concentrations of EG and DEG in mg/mL concentrations of sorbitol solution, and meet the USP monograph 0.1% of EG and DEG limits.

LIST OF SUPPLIERS

Sigma-Aldrich Chemical Company
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Milwaukee, WA, USA
Tel: 1-800-558-9160
www.sigmaaldrich.com

VWR International, Inc.
1310 Goshen Parkway
West Chester, PA, USA 19380
Tel: 1-800-932-5000
www.vwrsp.com

REFERENCES

1. Holloway, G.; Maheswaran, R.; Leeks, A.; Bradby, S.; Wahab, S. Screening Method for Ethylene Glycol and Diethylene Glycol in Glycerin-Containing Products. *J. Pharm. Biomed. Anal.* **2009,** *51*(2010), 507–511.
2. Centers for Disease Control. Fatalities Associated with Ingestion of Diethylene Glycol-Contaminated Glycerin Used to Manufacture Acetaminophen Syrup–Haiti, November 1995–June 1996, *MMWR Weekly.* **1996,** *45,* 649–650.
3. O'Brien, K. L.; Selanikio, J. D.; Hecdivert, C.; Placide, M-F.; Louis, M.; Barr, D. B.; Barr, J. R.; Hospedales, C. J.; Lewis, M. J.; Schwartz, B.; Philen, R. M.; St. Victor, S.; Espindola, J.; Needham, L. L.; Denerville, K. Acute Renal Failure Investigation Team. Epidemic of Pediatric Deaths From Acute Renal Failure Caused by Diethylene Glycol Poisoning. *JAMA* **1998,** *279* (15)*,* 1175–1180.
4. United States Federal Drug Association (FDA). FDA Advises Manufacturers: Test Glycerin for DEG Contamination, Glycerin Contaminated with Diethylene Glycol (DEG) Remains a Potential Health Hazard to Consumers, FDA News Release, P07-81, May 4, 2007. Last updated 06/18/2009.
5. Bogdanich, W.; Hooker, J. From China to Panama, a Trail of Poisoned Medicine, *NY Times,* May 6, 2007.
6. Increased surveillance of bulk ingredients susceptible to contamination with diethylene glycol. USDA Import Alert, Import Alert 55-02, October 5, 2009.
7. Vale, A. Poisonous Substances, Ethylene Glycol and Diethylene Glycol. *Medicine* **2007,** *35* (11), 617–618.

8. United States Pharmacopeia, Press release. New tests for identifying harmful and potentially deadly adulterants in pharmaceutical ingredients part of revised standards. October 30, 2009.
9. United States Pharmacopeia. Sorbitol Solution, ***Pharmacopeial Revision Bulletin.*** To be printed in USP 33–NF 28 First Supplement, released 1 February 2010, official 1 August 2010.
10. Cheng, J.; Jandik, P.; Liu, X.; Pohl, C. Pulsed Amperometric Detection Waveform with Disposable Thin-Film Platinum Working Electrodes in High Performance Liquid Chromatography. *J. Electroanal. Chem.* **2007,** *608,* 117–124.
11. Dionex Corporation. *Optimal Settings for Pulsed Amperometric Detection of Carbohydrates Using the Dionex ED40 Electrochemical Detector,* Technical Note 21, LPN 034889, Sunnyvale, CA, 1998.
12. Dionex Corporation. *Determination of Glycols and Alcohols in Fermentation Broths by Ion-Exclusion Chromatography and Pulsed Amperometric Detection,* Application Note 188, LPN 1944, Sunnyvale, CA, 2008.
13. Dionex Corporation. *Analysis of Carbohydrates by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD),* Technical Note 20, LPN 032857. Sunnyvale, CA, 2000.
14. Dionex Corporation. *Eluent Preparation for High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection,* Technical Note 71, LPN 1932, Sunnyvale, CA, 2009.
15. Dionex Corporation. Operator's Manual for AS Autosampler, Section B.3.6 Connecting the Diverter Valve (For Sequential Injections), Section 5.9 Calibrating the Inject Port Volume; Document No. 065051-03. Sunnyvale, CA, 2008, 197–200, 155–160.
16. Dionex Corporation. *Using the AS Automated Sampler in the Simultaneous, Sequential, and Concentrate Modes,* Technical Note 64, LPN 1756. Sunnyvale, CA, 2008.
17. Dionex Corporation. Product Manual for CarboPac MA1, PA1, PA10, PA100 Columns, Document No. 031824. Sunnyvale, CA, 2009.
18. Dionex Corporation. Product Manual for IonPac ICE-AS1 Guard and Analytical Columns, Document No. 031181. Sunnyvale, CA, 2006.
19. Dionex Corporation. Product Manual for IonPac CS14 Guard and Analytical Columns, Document No. 034848. Sunnyvale, CA, 2005.
20. Dionex Corporation. Product Manual for Disposable Gold, Disposable Silver, Disposable Platinum, and Disposable Carbon Electrodes, Document No. 065040. Sunnyvale, CA, 2009.
21. Weiss, J. Ion-Exclusion Chromatography (HPICE). In *Handbook of Ion Chromatography;* 3rd ed.; Wiley-VCH, Weinheim, Germany 2004; pp 359–360.

HPLC Assay of Water-Soluble Vitamins, Fat-Soluble Vitamins, and a Preservative in Dry Syrup Multivitamin Formulation

INTRODUCTION

Vitamins are vital to human development and long-term health; therefore, infants are usually prescribed a vitamin supplement to ensure they receive the recommended daily allowance of each vitamin. Children under one year of age are usually given this supplement in liquid form. This supplement can be produced as a dry syrup using a powdered preparation to which the pharmacist adds liquid to produce the dosage form for the patient. The work shown here describes an HPLC method to quantify water- and fat-soluble vitamins in a dry syrup.

Vitamins are a chemically diverse set of compounds varying in size, structure, and other properties. They are generally classified by their water solubility, with the classifications of water-soluble and fat-soluble (water-insoluble). Differences in chemical properties, water solubility, and sample concentrations make it difficult to analyze all vitamins in all samples using a single chromatography method.

In AN 216, both water-soluble vitamins (WSV) and fat-soluble vitamins (FSV) were determined in bottled waters fortified with vitamins.[1] In these products, the FSV stay in solution as a result of other additives. AN 216 showed that the Acclaim® PA2 column, which features a polar-embedded phase, is ideal for vitamin determinations. The Acclaim PA2 column is compatible with fully aqueous eluents (making it ideal for retaining the more polar vitamins such as vitamin B_6) and fully organic mobile phases (ideal for retaining FSV). The column is also compatible with a low-pH mobile phase that allows suppression or partial suppression of ionization, depending on the pH, for vitamins that are anionic at neutral pH (e.g., vitamin C).

AN 216 covers determination of vitamins B_3 (the nicotinamide and nicotinic acid forms), B_5 (pantothenic acid), B_6 (pyridoxine), B_9 (folic acid), B_{12} (cyanocobalamine), A (retinol), C (ascorbic acid), and E (α-tocopherol) in vitamin-fortified bottled waters. This newer work covers determination of the same vitamins studied in AN 216, plus vitamins B_1 (thiamine) and B_2 (riboflavin) in a dry syrup. This determination also uses the Acclaim PA2 column, albeit with a different mobile phase; rather than the formic acid/methanol/acetonitrile mobile phase used in AN 216, the separation reported here uses a methanesulfonic acid/ammonium phosphate/acetonitrile mobile phase.

Vitamins were extracted from the dry syrup prior to analysis. The WSV were extracted with water and a pH adjustment with KOH to dissolve folic acid. The FSV were extracted with either DMSO or ethyl acetate. To include all vitamins in the same chromatogram, the authors used a Chromeleon® Chromatography Data System (CDS) software feature that allows more than one injection for the same analysis. The WSV sample was injected first, then after elution of all WSV, the FSV sample was injected. This application also can be run by UHPLC using a 2.2 µm Acclaim PA2 column in 2.1 × 100 mm format to save time, reduce mobile phase consumption, and reduce waste. Like AN 216, this document shows that the UltiMate® 3000 system with an Acclaim PA2 column is an excellent solution for vitamin determinations.

EQUIPMENT

Dionex UltiMate 3000 system including:

Equipment	Conventional LC	UHPLC
Integrated vacuum degasser solvent rack	SRD-3600	SRD-3600
Pump	DGP-3600A	HPG-3400RS
Split-loop sampler	WPS-3000TSL	WPS-3000TRS
Column compartment	TCC-3200	TCC-3000RS
Diode array detector	PDA-3000	DAD-3000RS
Sample loop size*	100 µL	100 µL
Mixer	Standard	200 µL Static mixer kit
Flow cell	13 µL SST	2.5 µL SST
Chromeleon software version	6.80 SP 6	6.80 SR 7

*The work was done with 100 µL loop but the authors recommend using a 10 µL loop.

REAGENTS AND STANDARDS

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

Acetonitrile (CH3CN), HPLC grade (LAB-SCAN)

Methanesulfonic acid (MSA), puriss. ≥ 99% grade (Fluka)

Ammonium di-hydrogen orthophosphate, AR grade (Ajax)

Ethyl acetate, AR grade (Ajax)

Dimethyl sulfoxide (DMSO), AR grade (Sigma-Aldridge)

Thiamine*

Nicotinamide*

Ascorbic acid*

Pyridoxine hydrochloride*

Calcium pantothenate*

Cyanocobalamine*

Folic acid*

Riboflavin*

Sodium benzoate*

Retinol acetate*

α-Tocopherol acetate*

* These standards were provided by the customer but are available from a number of companies that supply laboratory chemicals.

CONDITIONS

Conventional HPLC

Column: Acclaim PA2, 3 µm, 4.6 × 150 mm (P/N 063191), Acclaim PA2 Guard, 5 µm, 4.3 × 10 mm (P/N 063195) Acclaim Guard Kit (P/N 059526)

Mobile Phase: A: 0.05% MSA

B: CH_3CN

C: 10 mM $NH_4H_2PO_4$ pH 2.5 with MSA

Sampler Temp.: 10 °C

Column Temp.: 35 °C

Injection Volume: 30 µL for water-soluble vitamins at 0.00 min, and 30 µL for fat-soluble vitamins at 18.00 min

Detection: UV-vis at 210 nm, 285 nm, wavelength scanning 200–800 nm, data collection rate 5 Hz, rise time 0.5 sec

Gradient: Table 1

UHPLC

Column: Acclaim RSLC PA2, 2.2 µm, 2.1 × 100 mm (P/N 068990)

Mobile Phase: A: 0.05% MSA

B: CH_3CN

C: 5 mM $NH_4H_2PO_4$ pH 3.0 with MSA

Sampler Temp.: 10 °C

Column Temp.: 35 °C

Injection Volume: 4 µL for WSV at 0.00 min, 0.5 µL for FSV at 7.5 min

Detection: UV-vis at 210 nm, 285 nm, data collection rate 10 Hz, response time 0.5 sec

Gradient: Table 1

Table 1. Gradient Program, Flow Program, Sample Injection Times, and Wavelength Switching Times							
Chromatographic Condition	**Time (min)**	**Flow (mL/ min)**	**% A**	**% B**	**% C**	**Remark**	**UV_VIS_1**
Conventional HPLC	-7.00	1.00	100.0	0.0	0.0		210
	0.00	1.00	100.0	0.0	0.0	Inject WSV (position in the sequence)	
	3.00	1.00	100.0	0.0	0.0		
	3.10	1.00	0.0	0.0	100.0		
	9.00	1.00	0.0	30.0	70.0		
	9.50	1.00	0.0	45.0	55.0		
	13.00	1.00	0.0	45.0	55.0		
	13.10	1.00	55.0	45.0	0.0		
	15.00	1.00	55.0	45.0	0.0		
	16.00	1.50	5.0	95.0	0.0		
	17.00	1.50	5.0	95.0	0.0		*285
	18.00	1.50	5.0	95.0	0.0	*Inject FSV (position in the sequence+1)	
	21.00	1.50	5.0	95.0	0.0		
	22.00	1.50	0.0	100.0	0.0		
	27.00	1.50	0.0	100.0	0.0		
	28.00	1.00	100.0	0.0	0.0		
UHPLC	-5.00	0.40	100.0	0.0	0.0		210
	0.00	0.40	100.0	0.0	0.0	Inject WSV (position in the sequence)	
	1.00	0.40	100.0	0.0	0.0		
	1.00	0.40	0.0	0.0	100.0		
	1.10	0.40	0.0	4.0	96.0		
	2.00	0.40	0.0	4.0	96.0		
	4.70	0.40	0.0	45.0	55.0		
	5.50	0.40	0.0	45.0	55.0		
	5.50	0.40	55.0	45.0	0.0		
	6.50	0.40	55.0	45.0	0.0		
	6.60	0.60	5.0	95.0	0.0		
	7.50	0.60	5.0	95.0	0.0	*Inject FSV (position in the sequence+1)	
	7.60	0.60	5.0	95.0	0.0		*285
	8.00	0.60	5.0	95.0	0.0		
	8.10	0.60	0.0	100.0	0.0		
	11.0	0.60	0.0	100.0	0.0		

*Manually insert the command in the program file; for example, see the commands in red at 17 min in the program in Appendix A.

Table 2. Summary of Calibration Results (DMSO Extraction)								
Vitamin	Standard Conc. (mg/L)			Cal.Type	Points	Coeff.Det. (× 100%)	Offset	Slope
	L1	L2	L3					
Thiamine	1.5	2.0	3.0	LOff	3	99.9872	-0.0310	0.6078
Nicotinamide	15.0	20.0	30.0	LOff	3	99.9977	0.4961	1.6711
Ascorbic acid	60.0	80.0	120.0	LOff	3	99.9862	-0.5339	0.3030
Pyridoxine hydrochloride	1.5	2.0	3.0	LOff	3	99.9995	-0.0489	1.8413
Calcium Pantothenate	6.0	12.0	18.0	LOff	3	99.9989	0.0153	0.1595
Cyanocobalamin	0.1	0.5	1.0	LOff	3	99.9929	-0.0027	1.3739
Folic acid	0.1	0.2	0.3	LOff	3	99.9626	-0.0096	1.6568
Riboflavin	1.5	3.5	5.0	LOff	3	99.8216	-0.0692	0.8223
Benzoate	5.0	10.0	15.0	LOff	3	99.9974	0.1505	0.6664
Retinol acetate	25.0	35.0	50.0	LOff	3	99.9759	0.0151	0.0948
α-Tocopherol acetate	25.0	35.0	50.0	LOff	3	99.9152	-0.0814	0.0905

PREPARATION OF SOLUTIONS AND REAGENTS

Mobile Phases

Mobile Phase A (0.05% MSA)

Weigh 999.5 g water, transfer 0.5 mL MSA to the same bottle, and mix well.

Mobile Phase C (10 mM $NH_4H_2PO_4$ pH 2.5)

Weigh 1.15 g ammonium di-hydrogen orthophosphate into a 250 mL beaker, add 100 mL water, stir until completely dissolved, transfer to a 1 L volumetric flask, and bring to volume with water. Adjust to pH 2.5 with MSA (350 µL).

Standard solutions and sample preparation

1000 mg/L Stock standard solutions

WSV standard solutions

Weigh 0.01 g of each vitamin into separate 10 mL volumetric flasks, add 5 mL water, and swirl the flask until dissolved. Prepare the preservative (sodium benzoate) in the same manner. To dissolve folic acid, add 10 µL of 8 M KOH. Bring to volume with water.

FSV standard solutions in ethyl acetate

Weigh 0.01 g (0.02 g for α-tocopherol) of standard in separate 50 mL glass bottles, add 2 mL water, add 10 mL ethyl acetate, quickly cap the bottle, place in an ultrasonic bath for 10 to 15 min, shake, and wait until the layers are completely separated. Use the top ethyl acetate layer as the stock standard solution.

FSV standard solutions in DMSO

Weigh 0.01 g (0.02 g for α-tocopherol) of standard in separate 50 mL glass bottles, add 10 mL DMSO, and place in an ultrasonic bath for 10 to15 min.

Working standards preparation

For concentrations of working standard solutions, see Table 2. Table 3 shows an example of the volumes of stock standards required to make the level 2 working standard. The WSV and FSV standards were prepared separately. The WSV working standards (each containing the preservative sodium benzoate) were diluted with mobile phase A and the FSV working standards were diluted with mobile phase B.

Table 3. Preparation of the Level 2 Working Standard		
Vitamin	Concentration (mg/L)	Volume of 1000 mg/L Stock Standard Solution in Final 25 mL for WSV and 10 mL for FSV (µL)
Thiamine	2.0	50
Nicotinamide	20.0	500
Ascorbic acid	80.0	2000
Pyridoxine hydrochloride	2.0	50
Pantothenic acid	12.0	300
Cyanocobalamine	0.5	12.5
Folic acid	0.2	5.0
Riboflavin	3.5	87.5
Sodium benzoate	10.0	250
Retinol acetate	35.0	350
α-Tocopherol acetate	35.0	350

Note: Prepare stock standard and working standard solutions just prior to the analysis. Store these solutions in brown bottles and use brown vials for analysis.

Table 4. Comparison of Sample Results between DMSO and Ethyl Acetate Extractions							
Vitamin	**Labeled Content for Each 5 mL (mg)**	**DMSO Extraction**			**Ethyl Acetate Extraction**		
		Average Found Concentration of 3 Preparations (mg per 5 mL)	**RSD**	**Assay (%)**	**Average Found Concentration of 3 Preparations (mg per 5 mL)**	**RSD**	**Assay (%)**
Thiamine	1	1.1	0.62	110	1.1	1.18	110
Nicotinamide	10	10.3	1.19	103	10.3	1.23	103
Ascorbic acid	35	36.1	0.86	103.1	36.6	1.05	105
Pyridoxine hydrochloride	1	1.2	0.86	120	1.2	1.04	120
Calcium pantothenate	5	6.7	1.55	134	6.7	0.66	134
Cyanocobalamin	0.0025	n.a.	—	—	n.a.	—	—
Folic acid	0.1	0.1	2.70	100	0.1	1.40	100
Riboflavin	1	1.1	2.01	110	1.1	0.36	110
Benzoate	—	4.7	1.32	—	4.9	1.15	—
Retinol acetate	0.05 (1990IU)	6.6	2.46	13200	6.9	1.23	13800
α-Tocopherol acetate	7.5	7.0	2.96	93.3	7.4	3.78	98.7

Sample preparation

A dry syrup containing a mixture of vitamins is provided in small bottles with a mark to indicate how much liquid to add to prepare the syrup. Add water to this mark (45 mL) and shake for few minutes. The sample is now ready for further preparation. A placebo consisting of the dry syrup without added vitamins is also used.

Sample Preparation for WSV Analysis

Shake the sample bottle and pipet 0.25 mL of sample, wipe the outside of the pipette, dispense into a 25 mL volumetric flask, rinse the inside of the pipette with 0.25 mL water, add 10 µL of 8 M KOH, swirl the flask, and bring to volume with mobile phase A.

Sample Preparation for FSV Analysis (Ethyl Acetate Extraction)

Shake the sample bottle and pipet 0.5 mL of sample, wipe the outside of the pipette, dispense into a 50 mL glass bottle, rinse the inside of the pipette with 0.5 mL water, add 5 mL ethyl acetate, and then cap the bottle. Place the capped bottle in an ultrasonic bath for 10 min, shake for few minutes, and then wait until the layers are completely separated. Pipet 1 mL of the top layer and dispense into 3 mL CH_3CN.

Sample Preparation for FSV Analysis (DMSO Extraction)

Shake the sample bottle and pipet 0.25 mL of sample, wipe the outside of the pipette, dispense into a 10 mL volumetric flask, rinse the inside of the pipette with 0.25 mL water, add 2 mL DMSO, and place in an ultrasonic bath for 10 min. Bring to volume with CH_3CN.

Note: Prepare samples just prior to analysis. Store these solutions in brown bottles and use brown vials for analysis.

The label states "Add water, shake, and then continue to add water to reach the mark on the side of the bottle." Table 4 shows the composition of 5 mL of a correctly prepared sample.

Table 5. Standard Amounts for Preparation of the Spiked Placebo Sample

Vitamin	Amount Added (mg)
Thiamine	12
Nicotinamide	120
Ascorbic acid	420
Pyridoxine hydrochloride	12
Pantothenic acid	60
Cyanocobalamine	—
Folic acid	—
Riboflavin	12
Sodium benzoate	60
Retinol acetate	100
α-Tocopherol acetate	200

Table 6. Resolution and Peak Purity Results

Vitamin	Resolution* (USP)	Match	% RSD Match	PPI (nm)	% RSD PPI
Thiamine	8.07	999	0.53	229.5	0.21
Nicotinamide	5.97	1000	0.06	214.9	0.03
Ascorbic acid	6.84	1000	0.03	221.8	0.01
Pyridoxine hydrochloride	43.68	999	0.35	240.5	0.14
Calcium pantothenate	20.73	997	2.27	194.3	1.08
Cyanocobalamin	2.78	997	2.61	235.7	1.02
Folic acid	4.02	987	7.75	251.5	2.61
Riboflavin	31.30	1000	1.00	274.0	0.36
Benzoate	72.44	1000	0.03	208.5	0.01
Retinol acetate	36.22	1000	0.65	302.8	0.21
α-Tocopherol acetate	n.a.	999	0.42	196.5	0.20

* All values were calculated by Chromeleon software.

Spiked placebo sample preparation

Weigh 24 g of placebo into an empty bottle and add accurately weighed vitamin standards to the same bottle (except vitamin B_{12} and folic acid, which are added later using the 1000 mg/L stock standard solutions). Add water to reach the mark on the side of the bottle, shake for few minutes, and continue the sample preparation either for WSV or FSV. The amounts of added standards are listed in Table 5. For folic acid and vitamin B_{12}, 5 µL and 2.5 µL of the 1000 mg/L standards, respectively, were added to the 25 mL volumetric flask during the WSV sample preparation.

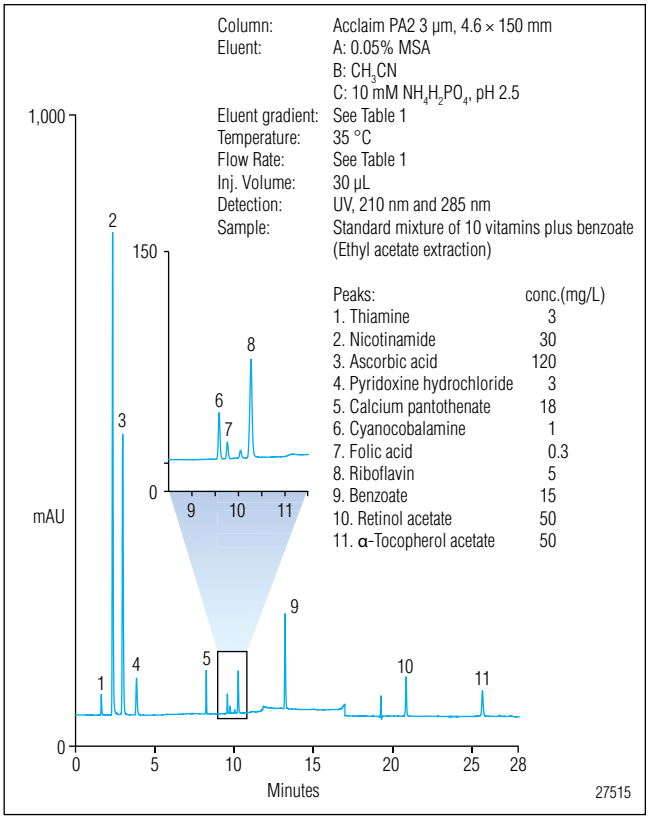


Figure 1. Chromatogram of a standard mixture of 10 vitamins plus benzoate (ethyl acetate extraction).

RESULTS AND DISCUSSION

Separation and Detection

This application uses the Acclaim PA2 column to separate water- and fat-soluble vitamins[1] and features of the Dionex UltiMate 3000 system and Chromeleon software that allow multiple injections during a single separation. The WSV, FSV, and benzoate were separated on Acclaim PA2 column in 28 min using a $CH_3CN/MSA/NH_4H_2PO_4$ mobile phase. The WSV standard containing benzoate was injected at 0.0 minute. After separation, the flow rate was increased to 1.5 mL/min and CH_3CN was increased to 95% for several minutes, then the FSV were injected. Table 6 shows that the resolution of all compounds was greater than 2.78. Spectral-matching data in the same table suggest that each peak represents one compound. Figure 1 shows the separation of both sets of vitamins and benzoate using ethyl acetate for extracting the FSV from the level 3 working standard.

Table 7. Summary of Calibration Results (Ethyl Acetate Extraction)								
Vitamin	**Standard Conc. (mg/L)**			**Cal.Type**	**Points**	**Coeff.Det. (× 100%)**	**Offset**	**Slope**
	L1	**L2**	**L3**					
Thiamine	1.5	2.0	3.0	LOff	3	99.9993	-0.0439	0.6165
Nicotinamide	15.0	20.0	30.0	LOff	3	100.0000	0.3913	1.6799
Ascorbic acid	60.0	80.0	120.0	LOff	3	99.9983	-0.8858	0.3027
Pyridoxine hydrochloride	1.5	2.0	3.0	LOff	3	99.9858	-0.0636	1.8455
Calcium Pantothenate	6.0	12.0	18.0	LOff	3	99.9994	0.0076	0.1599
Cyanocobalamin	0.1	0.5	1.0	LOff	3	99.9865	0.0010	1.3293
Folic acid	0.1	0.2	0.3	LOff	3	99.9998	-0.0150	1.6203
Riboflavin	1.5	3.5	5.0	LOff	3	99.8485	-0.0868	0.8248
Benzoate	5.0	10.0	15.0	LOff	3	100.0000	0.0928	0.6667
Retinol acetate	25.0	35.0	50.0	LOff	3	99.9989	0.0687	0.0949
α-Tocopherol acetate	25.0	35.0	50.0	LOff	3	99.9154	0.1184	0.0813

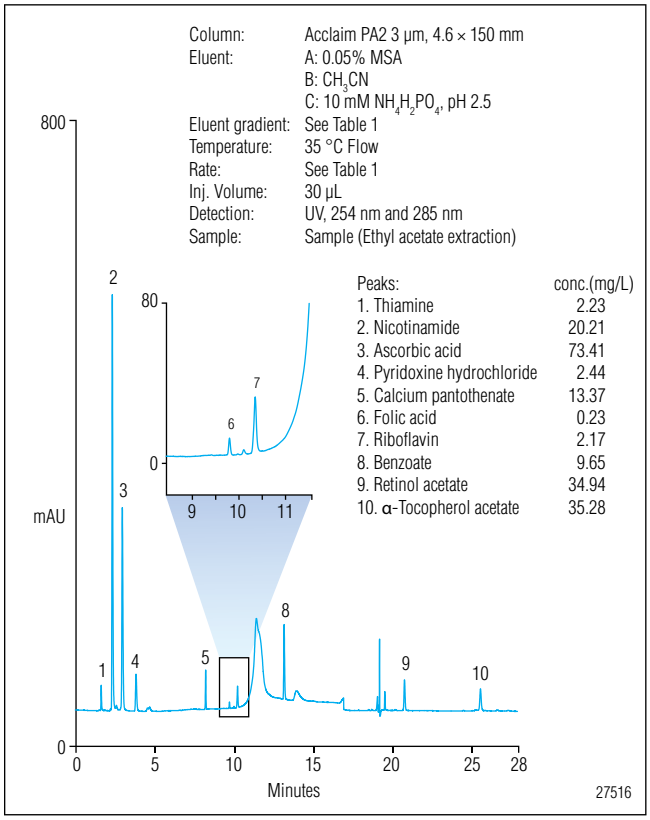


Figure 2. Chromatogram of the dry syrup sample (ethyl acetate extraction).

Method Calibration

Before sample analysis, a three-point calibration was prepared for each vitamin and each extraction method. The concentration range of each vitamin was chosen so that the sample concentration would fall in the middle of that range. The calibration data in Tables 2 and 7 show linear peak area response for each vitamin in the specified concentration range using either extraction method.

Sample Analysis

The multivitamin dry syrup sample and the same product without added vitamins (the placebo) were provided by a customer. Both samples were prepared as described on the label before using the sample preparation described here. The product label showed the amount of each vitamin in 5 mL, and the authors used those values to judge the success of the assay. The authors also compared the extraction of FSV using either DMSO or ethyl acetate. The original work was performed with DMSO, but there was concern that samples extracted using DMSO could damage the column, so extraction with ethyl acetate was also evaluated. Figure 2 shows the chromatogram of the sample extracted with ethyl acetate (chromatograms from the DMSO extraction are equivalent to those obtained for ethyl acetate extraction and, therefore, are not presented). The amounts of WSV determined ranged between 100 to 134%. These values suggest the assay is accurate due to over-fortification. For the FSV, the assay measured 93.3% and 98.7% of the labeled value for vitamin E using DMSO and ethyl acetate extractions, respectively.

Table 8. Vitamin Recovery from the Placebo: Comparison of DMSO and Ethyl Acetate Extractions							
Vitamin	**Spiked Concentration (mg/L)**	**DMSO Extraction**			**Ethyl Acetate Extraction**		
		Average Found Concentration of 3 Preparations (mg/L)	**RSD**	**Recovery (%)**	**Average Found Concentration of 3 Preparations (mg/L)**	**RSD**	**Recovery (%)**
Thiamine	2.0	2.0	1.10	100	2.0	0.63	100
Nicotinamide	20.0	18.5	0.17	92.5	18.5	0.32	92.5
Ascorbic acid	70.0	70.0	0.49	100	71.6	0.29	102
Pyridoxine hydrochloride	2.0	2.1	0.45	105	2.1	0.39	105
Calcium pantothenate	10.0	10.6	0.27	106	10.6	0.42	106
Cyanocobalamin	0.1	0.1	1.99	100	0.1	1.29	100
Folic acid	0.2	0.2	3.58	100	0.2	2.84	100
Riboflavin	2.0	2.0	1.40	100	2.0	1.58	100
Benzoate	10.0	9.8	0.39	98.0	9.9	0.25	99.0
Retinol acetate	41.7	31.1	1.62	74.6	38.1	0.89	91.4
α-Tocopherol acetate	41.7	34.1	2.99	81.8	35.3	2.09	84.7

Table 9. Sample Peak Purity Result and Spectral Matching with the Spectral Library										
Vitamin	**DMSO Extraction**					**Ethyl Acetate Extraction**				
	Match	**% RSD Match**	**PPI**	**% RSD PPI**	**Match with Library**	**Match**	**% RSD Match**	**PPI**	**% RSD PPI**	**Match with Library**
Thiamine	999	1.34	232.6	0.56	999.87	1000	0.24	229.5	0.10	999.87
Nicotinamide	1000	1.02	215.9	0.47	999.71	1000	0.67	215.4	0.31	991.74
Ascorbic acid	1000	0.05	231.1	0.02	999.93	1000	0.02	221.8	0.01	999.94
Pyridoxine hydrochloride	1000	0.56	219.4	0.25	999.95	999	0.63	240.9	0.26	999.97
Calcium pantothenate	1000	0.11	192.9	0.05	999.97	998	1.14	194.0	0.55	999.98
Cyanocobalamin	992	3.76	248.2	1.21	995.95	993	3.84	251.5	1.35	996.59
Folic acid	1000	0.92	281.8	0.32	999.93	1000	1.01	274.0	0.37	999.93
Riboflavin	999	0.37	208.7	0.17	997.13	1000	0.03	208.5	0.01	999.09
Benzoate	999	0.43	312.8	0.12	999.95	999	1.15	302.4	0.37	999.98
Retinol acetate	1000	0.10	196.3	0.05	999.91	999	0.38	196.5	0.18	998.48
α-Tocopherol acetate	999	1.34	232.6	0.56	999.87	1000	0.24	229.5	0.10	999.87

A very large amount of vitamin A was found in this FSV sample, compared to the label value. There were no anomalies in the recovery and peak purity results (Tables 8 and 9), so perhaps a mistake was made during preparation of the original sample. Each sample was prepared three times to evaluate reproducibility. Reproducibility and assay results are shown in Table 4.

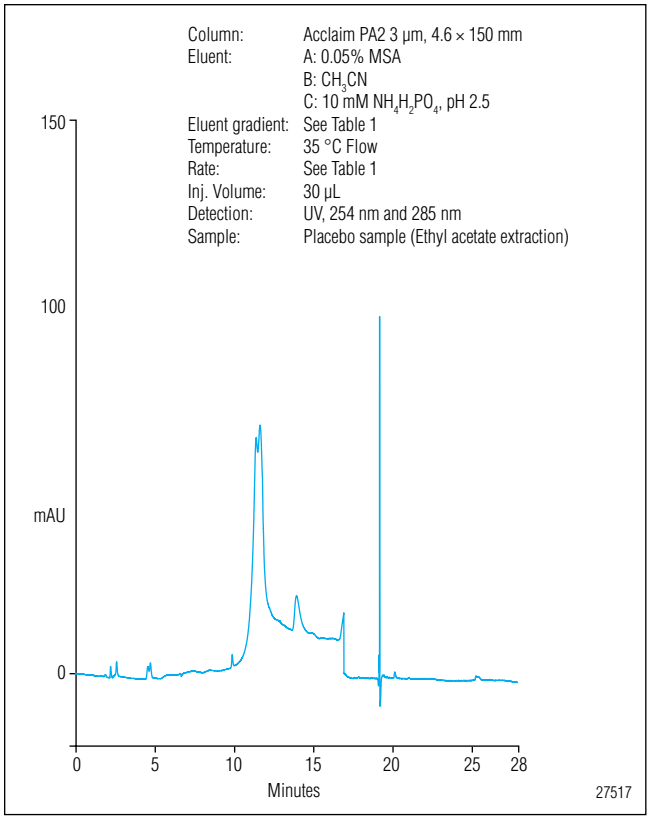


Figure 3. Chromatogram of the placebo sample (ethyl acetate extraction).

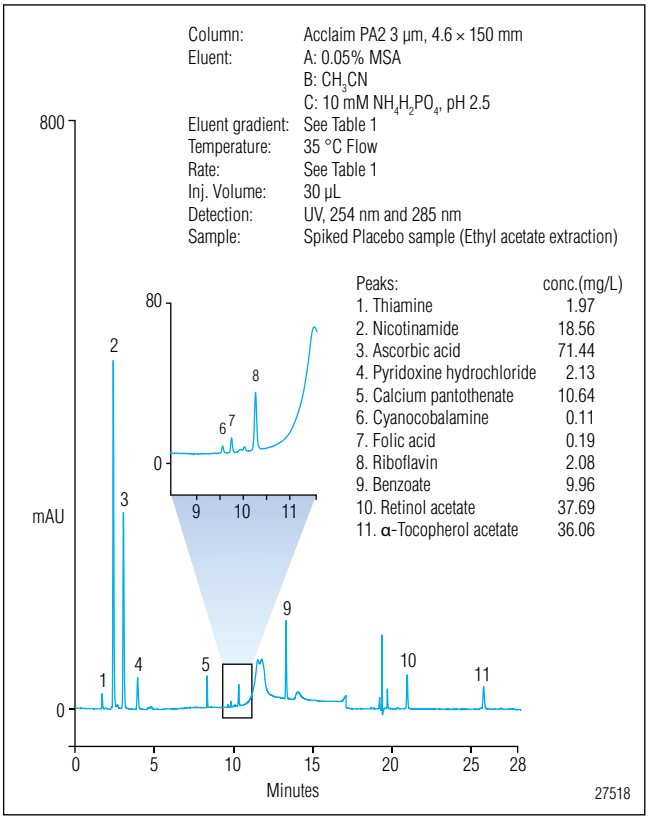


Figure 4. Chromatogram of the spiked placebo sample (ethyl acetate extraction).

To evaluate recovery, individual vitamins were added to the placebo sample prior to sample preparation in order to achieve a final concentration equivalent to the level 2 calibration standard, or the amount expected in the sample (see Spiked Placebo Sample Preparation). Recoveries for both extraction methods ranged from 74.6 to 106%. The recoveries of FSV by DMSO and ethyl acetate extractions were evaluated in triplicate, and the recovery results were between 74.6 to 81.8% and 87.7 to 91.4%, respectively.

Recoveries and reproducibility results are reported in Table 8. Figure 3 shows chromatography of the placebo sample after ethyl acetate extraction, and Figure 4 shows chromatography of the placebo spiked with the mixed vitamin standard. Although results from the two extraction techniques are similar, ethyl acetate is recommended because injecting DMSO on the column may shorten column lifetime, compared to ethyl acetate.

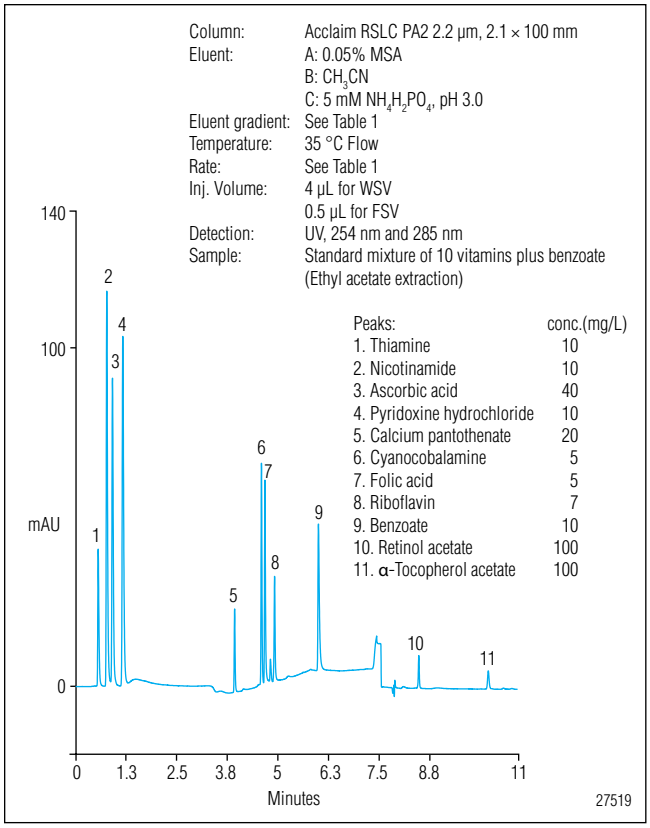


Figure 5. Chromatogram of a mixture of 10 vitamins plus benzoate (ethyl acetate extraction).

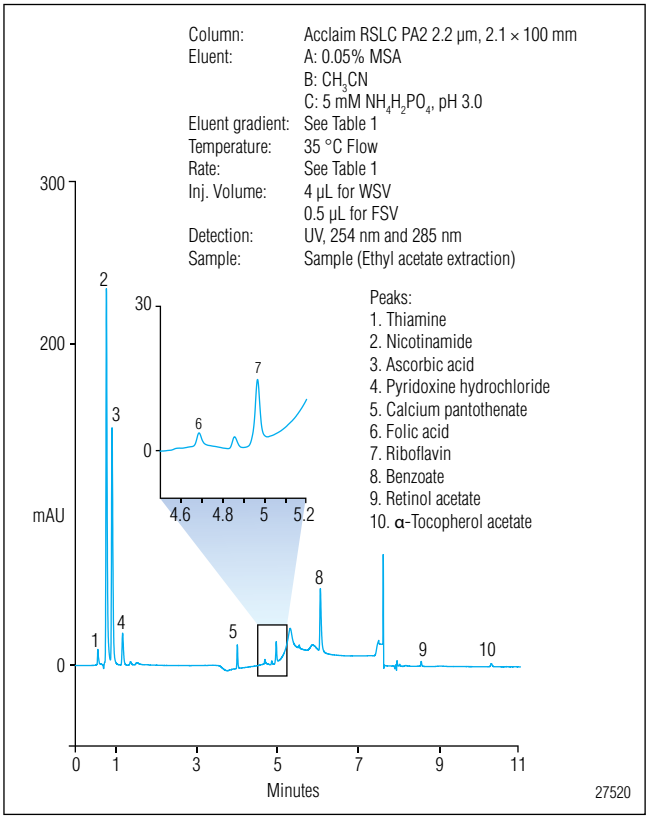


Figure 6. Chromatogram of the dry syrup sample (ethyl acetate extraction).

Faster Analysis

The Acclaim PA2 column is available in a 2.2 µm particle size and a 2.1 × 100 mm format. Therefore, it is possible to accelerate the vitamin separation on an UltiMate 3000 Rapid Separation LC (RSLC) system, saving both analysis time and solvent usage. Figure 5 shows the result of the method acceleration using the standard extracted with ethyl acetate. Run time was reduced from 28 to 11 min, and flow was reduced 60%. The RSLC method uses 5.3 mL of mobile phase over the 11 min run time, compared to 34 mL for the conventional method. This represents a significant savings in solvent use and reduction in waste production. Figure 6 demonstrates that the faster method is also successful for analyzing the dry syrup sample. Because the authors used a smaller column and had more efficient peaks, the sample size was reduced from 30 to 4 µL for WSV and from 30 to 0.5 µL for FSV.

CONCLUSION

The Acclaim PA2 column can successfully analyze a sample from 100% aqueous to 100% organic solvent, thereby allowing water- and fat-soluble vitamins to be separated in a single analysis. The Dionex UltiMate 3000 system and Chromeleon software facilitate this analysis by allowing multiple injections during the same run. This method is judged accurate, based on analysis of multivitamin dry syrup and a spiked placebo product. The Acclaim PA2 column, combined with an UltiMate 3000 system, is an excellent solution for vitamin determinations.

REFERENCE

Dionex Corporation, *Determination of Water- and Fat-Soluble Vitamins in Functional Waters by HPLC with UV-PDA Detection*. Application Note 216, LPN 2145, 2009, Sunnyvale, CA.

APPENDIX A:

Example program file

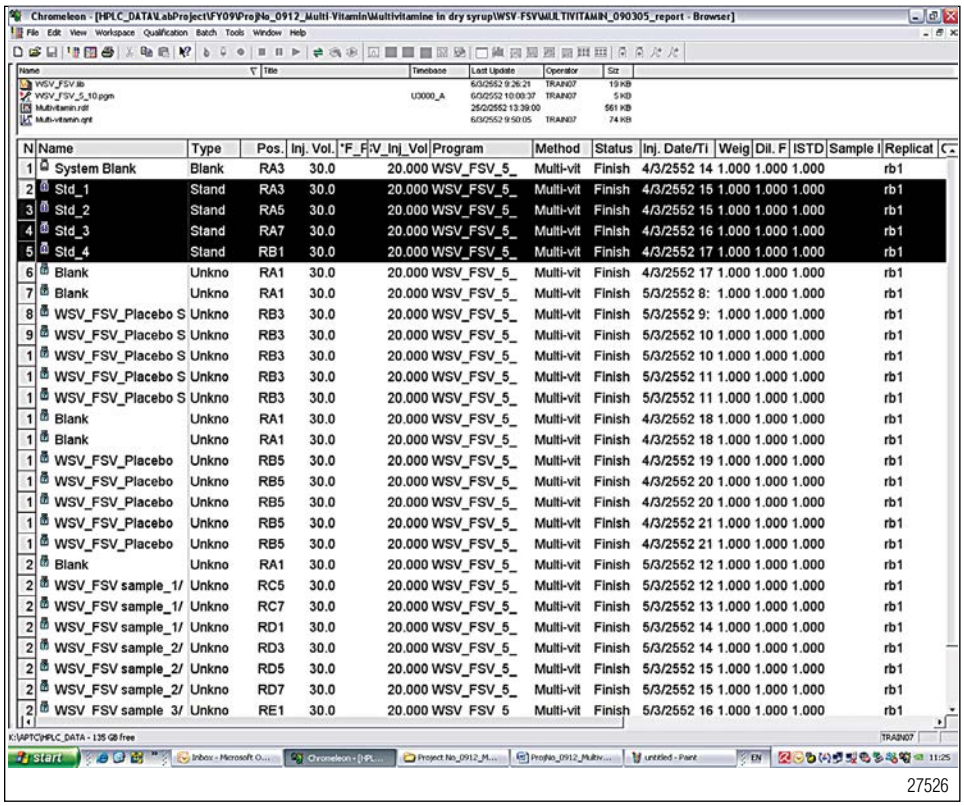
```
Sampler.TempCtrl =                  On
Sampler.Temperature.Nominal =       10.0 [°C]
Sampler.Temperature.LowerLimit =    4.0 [°C]
Sampler.Temperature.UpperLimit =    45.0 [°C]
Sampler.ReadyTempDelta =            1.0 [°C]
ColumnOven.TempCtrl =               On
ColumnOven.Temperature.Nominal =    35.0 [°C]
ColumnOven.Temperature.LowerLimit = 5.0 [°C]
ColumnOven.Temperature.UpperLimit = 85.0 [°C]
EquilibrationTime =                 0.5 [min]
ColumnOven.ReadyTempDelta =         0.5 [°C]
Column_A.ActiveColumn =             No
Column_B.ActiveColumn =             Yes
Column_B.SystemPressure =           "PumpRight"
Column_C.ActiveColumn =             No
Column_D.ActiveColumn =             No
PumpLeft.Pressure.LowerLimit =      0 [bar]
PumpLeft.Pressure.UpperLimit =      345 [bar]
PumpLeft.MaximumFlowRampDown =      3.000 [ml/min²]
PumpLeft.MaximumFlowRampUp =        3.000 [ml/min²]
PumpLeft.%A.Equate =                "%A"
PumpLeft.%B.Equate =                "%B"
PumpLeft.%C.Equate =                "%C"
PumpRight.Pressure.LowerLimit =     0 [psi]
PumpRight.Pressure.UpperLimit =     4000 [psi]
PumpRight.MaximumFlowRampDown =     3.000 [ml/min²]
PumpRight.MaximumFlowRampUp =       3.000 [ml/min²]
PumpRight.%A.Equate =               "0.05%MSA"
PumpRight.%B.Equate =               "ACN"
PumpRight.%C.Equate =               "10mM NH4H2PO4_pH2.5 with MSA"
DrawSpeed =                         3.000 [µl/s]
DrawDelay =                         3000 [ms]
DispSpeed =                         20.000 [µl/s]
DispenseDelay =                     0 [ms]
WasteSpeed =                        20.000 [µl/s]
SampleHeight =                      0.100 [mm]
InjectWash =                        AfterDraw
WashVolume =                        100.000 [µl]
WashSpeed =                         20.000 [µl/s]
PunctureOffset =                    0.0 [mm]
PumpDevice =                        "PumpRight"
InjectMode =                        Normal
SyncWithPump =                      On
PumpRight_Pressure.Step =           Auto
PumpRight_Pressure.Average =        On
Data_Collection_Rate =              5.00 [Hz]
Rise_Time =                         0.50 [s]
UV_VIS_1.Wavelength =               210 [nm]
UV_VIS_1.Bandwidth =                2 [nm]
UV_VIS_1.RefWavelength =            Off
UV_VIS_1.RefBandwidth =             1 [nm]
UV_VIS_2.Wavelength =               270 [nm]
UV_VIS_2.Bandwidth =                2 [nm]
```

```
            UV_VIS_2.RefWavelength =            Off
            UV_VIS_2.RefBandwidth =             1 [nm]
            UV_VIS_3.Wavelength =               280 [nm]
            UV_VIS_3.Bandwidth =                2 [nm]
            UV_VIS_3.RefWavelength =            Off
            UV_VIS_3.RefBandwidth =             1 [nm]
            UV_VIS_4.Wavelength =               360 [nm]
            UV_VIS_4.Bandwidth =                2 [nm]
            UV_VIS_4.RefWavelength =            Off
            UV_VIS_4.RefBandwidth =             50 [nm]
            UV_VIS_5.Wavelength =               380 [nm]
            UV_VIS_5.Bandwidth =                2 [nm]
            UV_VIS_5.RefWavelength =            Off
            UV_VIS_5.RefBandwidth =             50 [nm]
            3DFIELD.RefWavelength =             750 [nm]
            3DFIELD.RefBandwidth =              2 [nm]
            PumpLeft.Flow =                     0.000 [ml/min]
            PumpLeft.%B =                       100.0 [%]
            PumpLeft.%C =                       0.0 [%]
            PumpLeft.Curve =                    5
            3DFIELD.MinWavelength =             190 [nm]
            3DFIELD.MaxWavelength =             800 [nm]
            3DFIELD.BunchWidth =                2 [nm]

-7.000      PumpRight.Flow =                    1.000 [ml/min]
            PumpRight.%B =                      0.0 [%]
            PumpRight.%C =                      0.0 [%]

 0.000      Autozero
            Wait                                AZ_Done
            Wait                                ColumnOven.Ready and Sampler.Ready
            Inject
            PumpRight_Pressure.AcqOn
            UV_VIS_1.AcqOn
            UV_VIS_2.AcqOn
            UV_VIS_3.AcqOn
            UV_VIS_4.AcqOn
            UV_VIS_5.AcqOn
            3DFIELD.AcqOn

 3.000      PumpRight.Flow =                    1.000 [ml/min]
            PumpRight.%B =                      0.0 [%]
            PumpRight.%C =                      0.0 [%]

 3.100      PumpRight.Flow =                    1.000 [ml/min]
            PumpRight.%B =                      0.0 [%]
            PumpRight.%C =                      100.0 [%]

 9.000      PumpRight.Flow =                    1.000 [ml/min]
            PumpRight.%B =                      30.0 [%]
            PumpRight.%C =                      70.0 [%]
```

```
 9.500      PumpRight.Flow =                    1.000 [ml/min]
            PumpRight.%B =                      45.0 [%]
            PumpRight.%C =                      55.0 [%]

13.000      PumpRight.Flow =                    1.000 [ml/min]
            PumpRight.%B =                      45.0 [%]
            PumpRight.%C =                      55.0 [%]

13.100      PumpRight.Flow =                    1.000 [ml/min]
            PumpRight.%B =                      40.0 [%]
            PumpRight.%C =                      0.0 [%]

15.000      PumpRight.Flow =                    1.000 [ml/min]
            PumpRight.%B =                      45.0 [%]
            PumpRight.%C =                      0.0 [%]

16.000      PumpRight.Flow =                    1.500 [ml/min]
            PumpRight.%B =                      95.0 [%]
            PumpRight.%C =                      0.0 [%]

17.000      UV_VIS_1.Wavelength =               285 [nm]

18.000      Position =                          Position+1
            Volume =                            30
            Inject

21.000      PumpRight.Flow =                    1.500 [ml/min]
            PumpRight.%B =                      95.0 [%]
            PumpRight.%C =                      0.0 [%]

22.000      PumpRight.Flow =                    1.500 [ml/min]
            PumpRight.%B =                      100.0 [%]
            PumpRight.%C =                      0.0 [%]

27.000      PumpRight.Flow =                    1.500 [ml/min]
            PumpRight.%B =                      100.0 [%]
            PumpRight.%C =                      0.0 [%]

28.000      PumpRight.Flow =                    1.000 [ml/min]
            PumpRight.%B =                      0.0 [%]
            PumpRight.%C =                      0.0 [%]
            PumpRight_Pressure.AcqOff
            UV_VIS_1.AcqOff
            UV_VIS_2.AcqOff
            UV_VIS_3.AcqOff
            UV_VIS_4.AcqOff
            UV_VIS_5.AcqOff
            3DFIELD.AcqOff
            End
```

Note: The second injection comes from Position=Position+1, Volume=30 and Inject commands. In the command Position=Position+1, Position is the current position in the autosampler (water-soluble vitamins position), so the Position+1 is the next position (fat-soluble vitamins position).

For example, in the figure below, the sequence lines for standard injections are 2 through 5; autosampler positions RA3, RA5, RA7, and RB1 have water-soluble vitamin standards; RA4, RA6, RA8, and RB2 have fat-soluble vitamin standards.



Column Selection Guide

Pharmaceutical Applications Notebook

Thermo Scientific Acclaim Column Selection Guide

Please refer to www.thermoscientific.com/dionex for more information

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

Transferring HPLC Methods to UHPLC

Pharmaceutical Applications Notebook

Easy Method Transfer from HPLC to RSLC with the Dionex Method Speed-Up Calculator

INTRODUCTION

The goal of every chromatographic optimization is a method that sufficiently resolves all peaks of interest in as short a time as possible. The evolution of packing materials and instrument performance has extended chromatographic separations to new limits: ultrahigh-performance liquid chromatography (UHPLC).

The new Dionex UltiMate® 3000 Rapid Separation LC (RSLC) system is ideal for ultrafast, high-resolution LC. The RSLC system was designed for ultrafast separations with flow rates up to 5 mL/min at pressures up to 800 bar (11,600 psi) for the entire flow-rate range. This industry-leading flow-pressure footprint ensures the highest flexibility possible; from conventional to ultrahigh-resolution to ultrahigh-speed methods. The RSLC system, with autosampler cycle times of only 15 seconds, oven temperatures up to 110 °C, and data collection rates up to 100 Hz (even when acquiring UV-Vis spectra), sets the standard for UHPLC performance. Acclaim® RSLC columns with a 2.2 µm particle size complete the RSLC dimension.

A successful transfer from an HPLC method to an RSLC method requires recalculation of the chromatographic parameters. Underlying chromatographic principles have to be considered to find the appropriate parameters for a method transfer. With the Method Speed-up Calculator, Dionex offers an electronic tool that streamlines the process of optimum method transfer. This technical note describes the theory behind the Method Speed-Up Calculator and the application of this interactive, multi-language tool, illustrated with an exemplary method transfer from a conventional LC separation to an RSLC separation. You may obtain a copy of this calculator from your Dionex representative.

METHOD SPEED-UP STRATEGY

The purpose of method speed-up is to achieve sufficient resolution in the shortest possible time. The strategy is to maintain the resolving power of the application by using shorter columns packed with smaller particles. The theory for this approach is based on chromatographic mechanisms, found in almost every chromatography text book. The following fundamental chromatographic equations are applied by the Method Speed-Up Calculator for the method transfer from conventional to ultrafast methods.

The separation efficiency of a method is stated by the peak capacity *P*, which describes the number of peaks that can be resolved in a given time period. The peak capacity is defined by the run time divided by the average peak width. Hence, a small peak width is essential for a fast method with high separation efficiency. The peak width is proportional to the inverse square root of the number of theoretical plates N generated by the column. Taking into account the length of the column, its efficiency can also be expressed by the height equivalent to a theoretical plate *H*. The relationship between plate height H and plate number *N* of a column with the length L is given by Formula 1.

Formula 1: $$N = \frac{L}{H}$$

Low height equivalents will therefore generate a high number of theoretical plates, and hence small peak width for high peak capacity is gained. Which factors define *H*? For an answer, the processes inside the column have to be considered, which are expressed by the Van Deemter equation (Formula 2).

Formula 2: $$H = A + \frac{B}{u} + C \cdot u$$

The Eddy diffusion *A* describes the mobile phase movement along different random paths through the stationary phase, resulting in broadening of the analyte band. The longitudinal diffusion of the analyte against the flow rate is expressed by the term *B*. Term *C* describes the resistance of the analyte to mass transfer into the pores of the stationary phase. This results in higher band broadening with increasing velocity of the mobile phase. The well-known Van Deemter plots of plate height *H* against the linear velocity of the mobile phase are useful in determining the optimum mobile phase flow rate for highest column efficiency with lowest plate heights. A simplification of the Van Deemter equation, according to Halász[1] (Formula 3), describes the relationship between column efficiency (expressed in plate height *H*), particle size d_p (in µm) and velocity of mobile phase *u* (in mm/s):

Formula 3: $$H = 2 \cdot d_p + \frac{6}{u} + \frac{d_p^2 \cdot u}{20}$$

The plots of plate height H against velocity u depending on the particle sizes dp of the stationary phase (see Figure 1, top) demonstrate visually the key function of small particle sizes in the method speed-up strategy: The smaller the particles, the smaller the plate height and therefore the better the separation efficiency. An efficiency equivalent to larger particle columns can be achieved by using shorter columns and therefore shorter run times.

Another benefit with use of smaller particles is shown for the 2 µm particles in Figure 1: Due to improved mass transfer with small particle packings, further acceleration of mobile phases beyond the optimal flow rate with minimal change in the plate height is possible.

Optimum flow rates and minimum achievable plate heights can be calculated by setting the first derivative of the Halász equation to zero. The optimal linear velocity (in mm/s) is then calculated by Formula 4.

Formula 4: $$u_{opt} = \sqrt{\frac{B}{C}} = \frac{10.95}{d_p}$$

The minimum achievable plate height as a function of particle size is calculated by insertion of Formula 4 in Formula 3, resulting in Formula 5.

Formula 5: $$H_{min} \approx 3 \cdot d_p$$

Chromatographers typically prefer resolution over theoretical plates as a measure of the separation quality. The achievable resolution *R* of a method is directly proportional to the square root of the theoretical plate number as can be seen in Formula 6. *k* is the retention factor of the analyte and *k* the selectivity.

Formula 6: $$R = \frac{1}{4} \cdot \sqrt{N} \cdot \frac{k_2}{1 + k_2} \cdot \frac{\alpha - 1}{\alpha}$$

If the column length is kept constant and the particle size is decreased, the resolution of the analytes improves. Figure 1, bottom, demonstrates this effect using 5 µm and 2 µm particles.

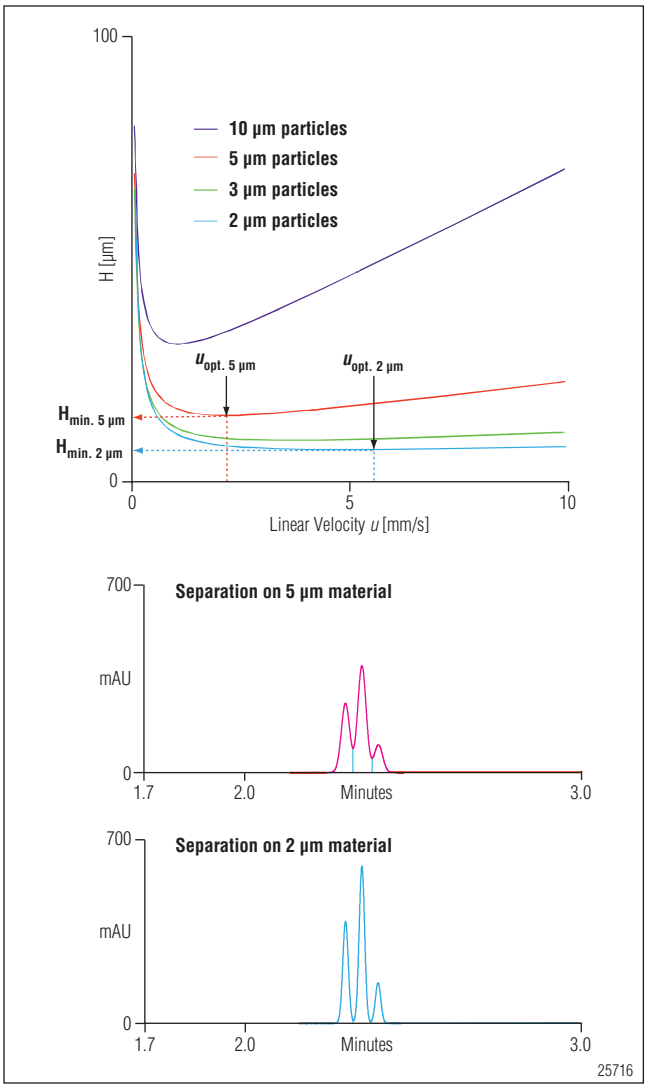


Figure 1. Smaller particles provide more theoretical plates and more resolution, demonstrated by the improved separation of three peaks (bottom) and smaller minimum plate heights H *in the Van Deemter plot (top). At linear velocities higher than uopt,* H *increases more slowly when using smaller particles, allowing higher flow rates and therefore faster separations while keeping separation efficiency almost constant. The speed-up potential of small particles is revealed by the Van Deemter plots (top) of plate height* H *against linear velocity u of mobile phase: Reducing the particle size allows higher flow rates and shorter columns because of the decreased minimum plate height and increased optimum velocity. Consequently, smaller peak width and improved resolution are the result (bottom).*

When transferring a gradient method, the scaling of the gradient profile to the new column format and flow rate has to be considered to maintain the separation performance. The theoretical background was introduced by L. Snyder[2] and is known as the gradient volume principle. The gradient volume is defined as the mobile phase volume that flows through the column at a defined gradient time t_G. Analytes are considered to elute at constant eluent composition. Keeping the ratio between the gradient volume and the column volume constant therefore results in a correct gradient transfer to a different column format.

Taking into account the changed flow rates F and column volume (with diameter d_c and length L), the gradient time intervals t_G of the new methods are calculated with Formula 7.

Formula 7: $$t_{G,new} = t_{G,old} \cdot \frac{F_{old}}{F_{new}} \cdot \frac{L_{new}}{L_{old}} \cdot \left(\frac{d_{c,new}}{d_{c,old}} \right)^2$$

An easy transfer of method parameters can be achieved by using the Dionex Method Speed-Up Calculator (Figure 2), which incorporates all the overwhelming theory and makes manual calculations unnecessary. This technical note describes the easy method transfer of an example separation applying the calculator. Just some prerequisites described in the following section have to be taken into account.

PREREQUISITES

The Method Speed-Up Calculator is a universal tool and not specific for Dionex products. Nevertheless, some prerequisites have to be considered for a successful method transfer, which is demonstrated in this technical note by the separation of seven soft drink additives.

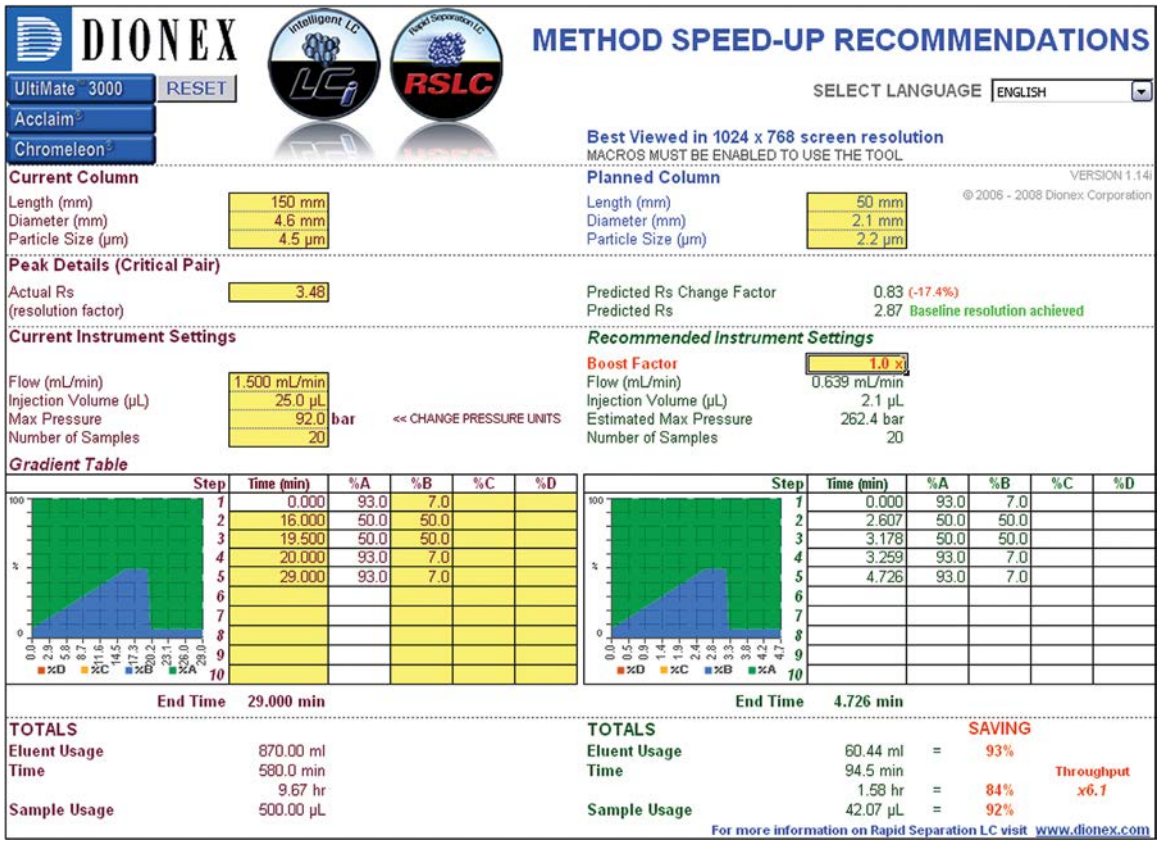


Figure 2. The Dionex Method Speed-Up Calculator transfers a conventional (current) HPLC method to a new (planned) RSLC method.

Column Dimension

First, the transfer of a conventional method to an RSLC method requires the selection of an adequate column filled with smaller particles. The RSLC method is predicted best if the selectivity of the stationary phase is maintained. Therefore, a column from the same manufacturer and with nominally identical surface modification is favoured for an exact method transfer. If this is not possible, a column with the same nominal stationary phase is the best choice. The separation is made faster by using shorter columns, but the column should still offer sufficient column efficiency to allow at least a baseline separation of analytes. Table 1 gives an overview of the theoretical plates expected by different column length and particle diameter size combinations using Dionex Acclaim column particle sizes. Note that column manufacturers typically fill columns designated 5 µm with particle sizes 4–5 µm. Dionex Acclaim 5 µm columns are actually filled with 4.5 µm particles. This is reflected in the table.

Table 1. Theoretical Plates Depending on Column Length and Particle Diameter (Calculated Using Formula 5)

	Theoretical Plates N		
Particle size	4.5 µm	3 µm	2.2 µm
Column length: 250 mm	18518	27778	37879
150 mm	11111	16667	22727
100 mm	7407	11111	15152
75 mm	5555	8333	11364
50 mm	3703	5556	7576

If the resolution of the original separation is higher than required, columns can be shortened. Keeping the column length constant while using smaller particles improves the resolution. Reducing the column diameter does not shorten the analysis time but decreases mobile phase consumption and sample volume. Taking into account an elevated temperature, smaller column inner diameters reduce the risk of thermal mismatch.

System Requirements

Smaller particles generate higher backpressure. The linear velocity of the mobile phase has to be increased while decreasing the particle size to work within the Van Deemter optimum. The UltiMate 3000 RSLC system perfectly supports this approach with its high maximum operation pressure of 800 bar (11,600 psi). This maximum pressure is constant over the entire flow rate range of up to 5 mL/min, providing additional potential to speed up applications even further by increasing the flow rate.

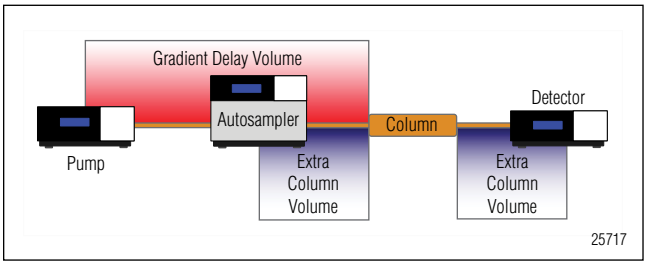


Figure 3. Gradient delay volume and extra column volume of an HPLC system. Both play an important role in method speed-up.

For fast gradient methods, the gradient delay volume (GDV) plays a crucial role. The GDV is defined as the volume between the first point of mixing and the head of the column. The GDV becomes increasingly important with fast, steep gradients and low flow rate applications as it affects the time taken for the gradient to reach the head of the column. The larger the GDV, the longer the initial isocratic hold at the beginning of the separation. Typically, this leads to later peak elution times than calculated. Early eluting peaks are affected most. In addition, the GDV increases the time needed for the equilibration time at the end of a sample and therefore increases the total cycle time. A general rule is to keep the gradient steepness and the ratio of GDV to column volume constant when transferring a standard method into a fast LC method. This will maintain the selectivity of the original method.[3]

The GDV can be adjusted to the column volume by installing appropriate mixer kits to the RSLC pump (see Table 2), which contributes most to the GDV. Typically, 100 µL or 200 µL mixers are good starting points when operating a small volume column in an RSLC system.

Another option is to switch the sample loop of the split-loop autosampler out of the flow path. The GDV is then reduced by the sample loop volume in the so-called bypass mode. The GDV of a standard sample loop of the RSLC autosampler is 150 µL, the micro injection loop has a 50 µL GDV.

Table 2. Mixer Kits Available for UltiMate 3000 RSLC System to Adapt GDV of Pump

Mixer Kit	GDV pump
Mixer kit 6040.5000	35 µL
Static mixer kit 6040.5100	100 µL
Static mixer kit 6040.5150	200 µL

Besides the gradient delay volume, the extra column volume is an important parameter for fast LC methods. The extra column volume is the volume in the system through which the sample passes and hence contributes to the band broadening of the analyte peak (Figure 3). The extra column volume of an optimized LC system should be below $^{1}/_{10}$th of the peak volume. Therefore the length and inner diameter of the tubing connections from injector to column and column to detector should be as small as possible. Special care has to be taken while installing the fittings to avoid dead volumes. In addition, the volume of the flow cell has to be adapted to the peak volumes eluting from the RSLC column. If possible, the flow cell detection volume should not exceed $^{1}/_{10}$th of the peak volume.

Detector Settings

When transferring a conventional method to an RSLC method, the detector settings have a significant impact on the detector performance. The data collection rate and time constant have to be adapted to the narrower peak shapes. In general, each peak should be defined by at least 30 data points. The data collection rate and time constant settings are typically interrelated to optimize the amount of data points per peak and reduce short-term noise while still maintaining peak height, symmetry, and resolution.

The Chromeleon® Chromatography Management Software has a wizard to automatically calculate the best settings, based on the input of the minimum peak width at half height of the chromatogram. This width is best determined by running the application once at maximum data rate and shortest time constant. The obtained peak width may then be entered into the wizard for optimization of the detection settings. Refer to the detector operation manual for further details.

METHOD SPEED-UP USING THE CALCULATOR

Separation Example

Separation was performed on an UltiMate 3000 RSLC system consisting of a HPG-3200RS Binary Rapid Separation Pump, a WPS-3000RS Rapid Separation Well Plate Sampler with analytical sample loop (100 µL), a TCC-3000RS Rapid Separation Thermostatted Column Compartment with precolumn heater (2 µL), and a VWD-3400RS Variable Wavelength Detector with semi-micro flow cell (2.5 µL). Chromeleon Chromatography Management Software (version 6.80, SR5) was used for both controlling the instrument and reporting the data. The modules were connected with stainless steel micro capillaries, 0.01" ID, $^{1}/_{16}$" OD when applying the conventional LC method, 0.007" and 0.005" ID, $^{1}/_{16}$" OD when applying the RSLC methods. A standard mixture of seven common soft drink additives was separated by gradient elution at 45 °C on two different columns:

- Conventional HPLC Column: Acclaim 120, C18, 5 µm, 4.6 × 150 mm column, (P/N 059148)
- Rapid Separation Column: Acclaim RSLC 120, C18, 2.2 µm, 2.1 × 50 mm column (P/N 068981).

The UV absorbance wavelength at 210 nm was recorded at 5 Hz using the 4.6 × 150 mm column and at 25 Hz and 50 Hz using the 2.1 × 50 mm column. Further method details such as flow rate, injection volume, and gradient table of conventional and RSLC methods are described in the following section. The parameters for the method transfer were calculated with the Dionex Method Speed-Up Calculator (version 1.14i).

The conventional separation of seven soft drink additives is shown in Figure 4A. With the Method Speed-Up Calculator, the method was transferred successfully to RSLC methods (Figure 4B and C) at two different flow rates. The easy method transfer with this universal tool is described below.

Column Selection for Appropriate Resolution

The column for method speed-up must provide sufficient efficiency to resolve the most critical pairs. In this example, separating peaks 5 and 6 is most challenging. A first selection of the planned column dimensions can be made by considering the theoretical plates according to Table 1. The 4.6 × 150 mm, 5 µm column is actually filled with 4.5 µm particles. Therefore, it provides 11,111 theoretical plates. On this column, the

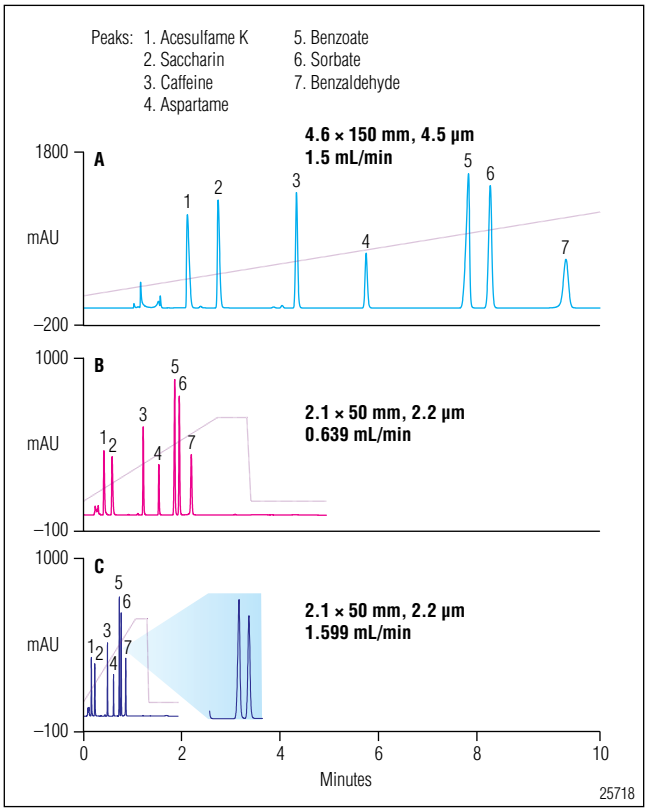


Figure 4. Method transfer with the Method Speed-Up Calculator from A) a conventional LC separation on an Acclaim 5 µm particle column, to B) and C) RSLC separations on an Acclaim 2.2 µm particle column.

resolution is $R_{(5,6)}$=3.48. This resolution is sufficiently high to select a fast LC column with fewer theoretical plates for the speed up. Therefore, a 2.1 × 50 mm, 2.2 µm column with 7579 plates was selected.

The first values to be entered into the yellow field of the Method Speed-Up Calculator are the current column dimension, planned column dimension, and the resolution of the critical pair. To obtain the most accurate method transfer, use the particle sizes listed in the manufacturer's column specifications sheet instead of the nominal size, which may be different. Dionex Acclaim columns with a nominal particle size of 5 µm are actually filled with 4.5 µm particles, and this value should be used to achieve a precise method transfer calculation. This has a positive impact on the performance and pressure predictions for the planned column. Based on the assumption of unchanged stationary phase chemistry, the calculator then predicts the resolution provided by the new method (Figure 5).

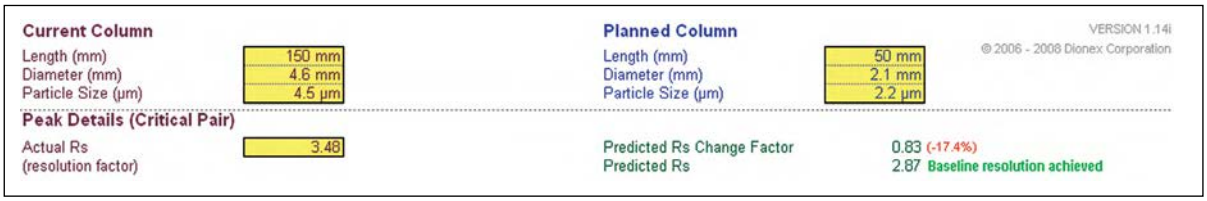


Figure 5. Column selection considering the resolution of the critical pair.

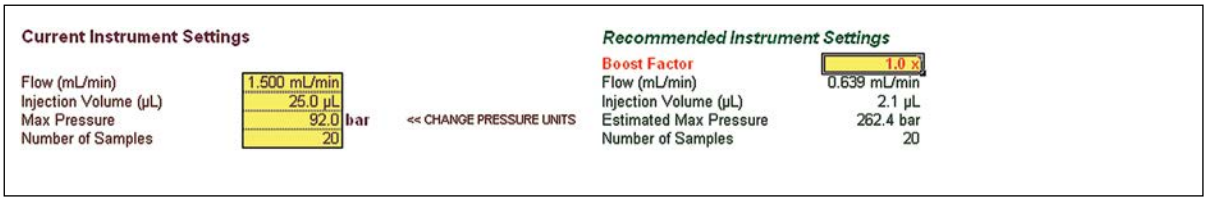


Figure 6. The flow rate, injection volume and backpressure of the current method are scaled to the new column dimension.

In the example in Figure 5, the predicted resolution between benzoate and sorbate is 2.87. With a resolution of $R \geq 1.5$, the message "Baseline resolution achieved" pops up. This indicates that a successful method transfer with enough resolution is possible with the planned column. If R is smaller than 1.5, the red warning "Baseline is not resolved" appears. Note that the resolution calculation is performed only if the boost factor BF is 1, otherwise it is disabled. The function of the boost factor is described in the Adjust Flow Rate section.

Instrument Settings

The next section of the Method Speed-Up Calculator considers basic instrument settings. These are flow rate, injection volume, and system backpressure of the current method (Figure 6). In addition to these values, the detector settings have to be considered as described in the earlier section "Detector Settings". Furthermore, the throughput gain with the new method can be calculated if the number of samples to be run is entered.

Adjust Flow Rate

As explained by Van Deemter theory, smaller particle phases need higher linear velocities to provide optimal separation efficiency. Consequently, the Dionex Method Speed-Up Calculator automatically optimizes the linear velocity by the ratio of particle sizes of the current and planned method. In addition, the new flow rate is scaled to the change of column cross section if the column inner diameter changed. This keeps the linear velocity of the mobile phase constant. A boost factor (*BF*) can be entered to multiply the flow rate for a further decrease in separation time. If the calculated resolution with $BF=1$ predicts sufficient separation, the method can be accelerated by increasing the boost factor and therefore increasing the flow rate. Figure 1 shows that applying linear velocities beyond the optimum is no problem with smaller particle phases, as they do not significantly loose plates in this region. Note that the resolution calculation of the Method Speed-Up Calculator is disabled for $BF \neq 1$.

For the separation at hand, the flow rate is scaled from 1.5 mL/min to 0.639 mL/min when changing from an Acclaim 4.6 × 150 mm, 4.5 µm column to a 2.1 × 50 mm, 2.2 µm column (see Figure 6), adapting the linear velocity to the column dimensions and the particle size. The predicted resolution between peak 5 and 6 for the planned column is $R=2.87$. The actual resolution achieved is $R=2.91$, almost as calculated (chromatogram B in Figure 4).

A Boost Factor of 2.5 was entered for further acceleration of the method (Figure 7). The method was then performed with a flow rate of 1.599 mL/min, and resolution of the critical pair was still sufficient at $R=2.56$ (see zoom in chromatogram C in Figure 4).

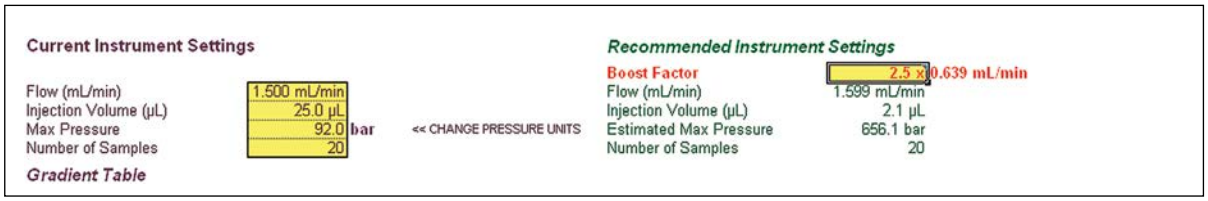


Figure 7. The new flow rate is further accelerated by applying the Boost Factor of 2.5.

Scale Injection Volume

The injection volume has to be adapted to the new column dimension to achieve similar peak heights by equivalent mass loading. Therefore the injection plug has to be scaled to the change of column cross section. In addition, shorter columns with smaller particles cause a reduced zone dilution. Consequently, sharper peaks compared to longer columns are expected. The new injection volume $V_{inj,new}$ is then calculated by Formula 8, taking a changed cross section and reduced band broadening by changed particle diameter into account.

Formula 8: $$V_{inj,new} = V_{inj,old} \cdot \left(\frac{d_{c,new}}{d_{c,old}}\right)^2 \cdot \sqrt{\frac{L_{new} \cdot d_{p,new}}{L_{old} \cdot d_{p,old}}}$$

Generally, it is recommended that a smaller flow cell be used with the RSLC method to minimize the extra column volume. Also, the difference in path length of different flow cell sizes has to be taken into account while scaling the injection volume. In the example of the soft drink analysis, the injection volume is scaled from 25 µL to 2.1 µL when replacing the Acclaim 4.6 × 150 mm, 4.5 µm column with a 2.1 × 50 mm, 2.2 µm column (see Figure 6).

Predicted Backpressure

Speeding-up the current method by decreasing particle size and column diameter and increasing flow rate means elevating the maximum generated backpressure. The pressure drop across a column can be approximated by the Kozeny-Carman formula.[4] The pressure drop of the new method is predicted by the calculator considering changes in column cross section, flow rate, and particle size and is multiplied by the boost factor. The viscosity of mobile phase is considered constant during method transfer. The calculated pressure is only an approximation and does not take into account nominal and actual particle size distribution depending on column manufacturer. If the predicted maximum pressure is above 800 bar (11,600 psi) the warning "Exceeds pressure limit RSLC" is shown, indicating the upper pressure limit of the UltiMate 3000 RSLC system. However, in the case the method is transferred to a third party system, its pressure specification has to be considered.

In the example of the soft drink analysis, the actual pressure increases from 92 bar to 182 bar with *BF*=1 on the 2.1 × 50 mm column, and to 460 bar for the RSLC method with *BF*=2.5. The pressures predicted by the Method Speed-Up Calculator are 262 bar and 656 bar, respectively. The pressure calculation takes into account the change of the size of the column packing material. In a speed up situation, the pressure is also influenced by other factors such as particle size distribution, system fluidics pressure, change of flow cell, etc. When multiplication factors such as the boost factor are used, the difference between calculated and real pressure is pronounced. The pressure calculation is meant to give an orientation, what flow rates might be feasible on the planned column. However, it should be confirmed by applying the flow on the column.

Adapt Gradient Table

The gradient profile has to be adapted to the changed column dimensions and flow rate following the gradient-volume principle. The gradient steps of the current method are entered into the yellow fields of the gradient table. The calculator then scales the gradient step intervals appropriately and creates the gradient table of the new method.

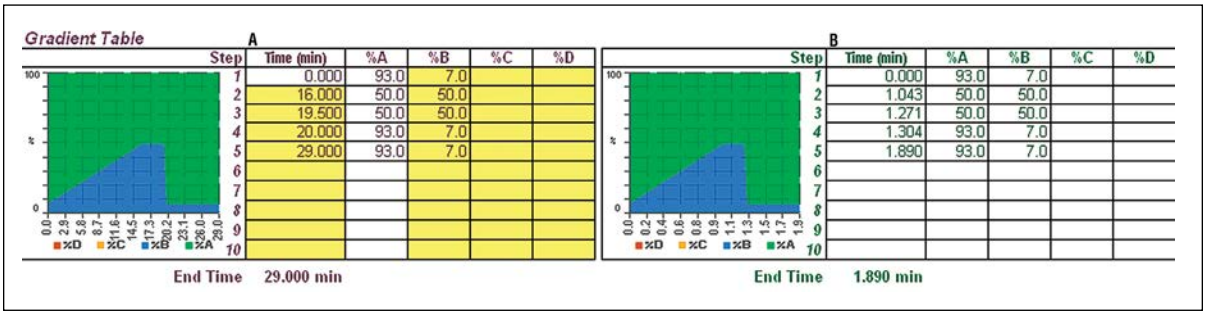


Figure 8. The gradient table of the current method (A) is adapted to the boosted method (B) according to the gradient-volume principle.

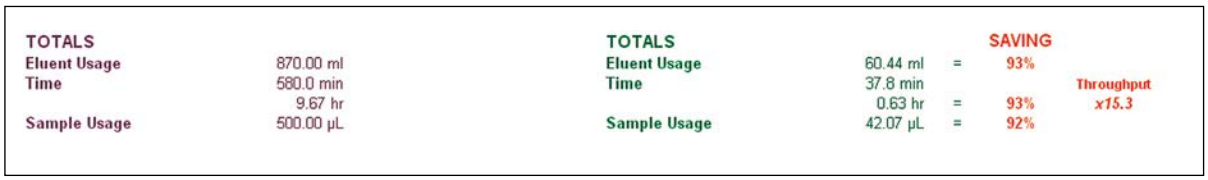


Figure 9. The absolute values for analysis time, eluent usage, and sample usage of the current (purple) and planned (green) method are calculated by the Method Speed-Up Calculator. The savings of eluent, sample, and time due to the method transfer are highlighted.

The adapted gradient table for the soft drink analysis while using a boost factor *BF*=1 is shown in Figure 8. According to the gradient-volume principle, the total run time is reduced from 29.0 min to 4.95 min by taking into account the changed column volume from a 4.6 × 150 mm, 5 µm (4.5 µm particles entered) to a 2.1 × 50 mm, 2.2 µm column and the flow rate reduction from 1.5 mL/min to 0.639 mL/min. The separation time was further reduced to 1.89 min by using boost factor *BF*=2.5. Gradient time steps were adapted accordingly. The comparison of the peak elution order displayed in Figure 4 shows that the separation performance of the gradient was maintained during method transfer.

Consumption and Savings

Why speed-up methods? To separate analyte peaks faster and at the same time reduce the mobile phase and sample volume consumption. Those three advantages of a method speed-up are indicated in the Method Speed-Up Calculator sheet right below the gradient table. The absolute values for the time, eluent, and sample usage are calculated taking the numbers of samples entered into the current instrument settings section of the calculation sheet into account (see Figure 6).

Regarding the soft drink analysis example, geometrical scaling of the method from the conventional column to the RSLC method means saving 93% of eluent and 92% of sample. The sample throughput increases 6.1-fold using *BF*=1. The higher flow rate at *BF*=2.5 results in a 15.3-fold increased throughput compared to the conventional LC method (Figure 9).

CONCLUSION

Fast method development or increased sample throughput are major challenges of most analytical laboratories. A systematic method speed-up is accomplished by reducing the particle size, shortening the column length, and increasing the linear velocity of the mobile phase. The Dionex Method Speed-Up Calculator automatically applies these rules and scales the conventional LC parameters to the conditions of the RSLC method. The interactive electronic tool is universally applicable. New instrument settings are predicted and gradient tables are adapted for optimum performance for the new method. The benefit of the method transfer is summarized by the integrated calculation of savings in time, eluent and sample. In addition, users can benefit from getting results earlier and thereby reducing the time to market. The Dionex Method Speed-Up Calculator is part of Dionex's total RSLC solution, which further consists of the industry leading UltiMate 3000 RSLC system, powerful Chromeleon Chromatography Management Software, and high-efficiency Acclaim RSLC columns.

REFERENCES

1. Halász, I.; Endele, R.; Asshauer, J. *J. Chromatogr., A* **1975,** *112,* 37–60.
2. Snyder, L.R.; Dolan, J.W.; Grant, J.R. *J. Chromatogr., A* **1979,** *165,* 3–30.
3. Schellinger, A.P.; Carr, P.W. *J. Chromatogr., A* **2005,** *1077,* 110-119.
4. Bear, J. *Dynamics of Fluids in Porous Media;* Dover: Mineola, NY, 1988.

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