

Reporter

Volume 27.2

 **SUPELCO**
Analytical

Profiling of Stevia Extract by HPLC



The US FDA has issued letters of non-objection for the use of a natural, zero-calorie sweetener.

- Liquid Chromatography
- Sample Handling
- Gas Chromatography
- Standards
- Accessories

SIGMA-ALDRICH[®]

Table of Contents

Liquid Chromatography

Profiling of Stevia rebaudiana Extract by Accurate Mass Using HILIC and Reversed-Phase Chromatography 3

Discovery® BIO GFC Separation Volume and Peak Capacity 6

Astec CYCLOBOND™ I 2000 HP-RSP – Unique CSP for Chiral HPLC and LC-MS Separations..... 7

Fast LC-MS on Astec CHIROBIOTIC™ CSPs..... 8

High Purity LC-MS Mobile Phase Additives and Water..... 9

Sample Handling

Troubleshooting Analyte Recovery when Using HybridSPE-Precipitation Technology..... 17

SPME Analysis of Drugs of Abuse..... 20

Pipette Tips for Micro-Purification 22

Gas Chromatography

Optimize GC Separations with the Proper Column Choice..... 12

GC Analysis of Glycols and Diols..... 16

Standards

New Standards Kit for ASTM D5501: Determination of Ethanol Content of Denatured Fuel Ethanol..... 10

Standards Kit for Monitoring Food Antioxidants..... 11

Accessories

Certified Vials – Every Vial is Autosampler Compatible..... 23

Dear Colleague,

Here at Sigma-Aldrich, we are committed to accelerating your success through leadership in separation science, high technology and service. Over 7000 employees around the globe are working for one common goal: solving the issues of today and providing solutions for tomorrow. Supelco's Technical Service Scientists represent decades of expertise and can assist you in finding the right product or solution for your most difficult analysis.



*Janice Burger, Linda Koch, Dave Williams
Rayden Weber, Gary Oishi, Barbara Vogler*

Our experts can help you in a variety of different ways such as:

- Assisting in developing an analytical method
- Reducing acetonitrile usage during HPLC applications
- Recommending the best product before you buy
- Providing knowledgeable advice and support
- Troubleshooting product performance issues

For solutions to any of your analysis and purification needs, Supelco's Technical Service is the answer. If you cannot find the analytical product you are looking for, call or drop us a note and we will be glad to assist you. We have one purpose and it is putting the needs of our customers first. For expert assistance with all of your analytical or chromatography needs, contact Supelco Technical Service at:

Phone: 800-359-3041 (US & Canada only) or 814-359-3041

Fax: 800-359-3044 (US & Canada only) or 814-359-5468

E-mail: techservice@sial.com

Regards,

Technical Service

techservice@sial.com

Profiling of *Stevia rebaudiana* Extract by Accurate Mass Using HILIC and Reversed-Phase Chromatography

Craig Aurand

craig.aurand@sial.com

Introduction

There is growing public interest in low-calorie alternatives to carbohydrate-based sweeteners. Synthetic sweeteners are often regarded as having an undesirable aftertaste. Recent publications have shown a dramatic increase in attention toward natural extracts including the *Stevia rebaudiana* plant, not only for its sweetening effect but also for additional health benefits attributed to the plant. The major sweetening components are stevioside, rebaudioside A, rebaudioside C, and dulcoside A, each of which is over 300 times sweeter than sucrose-based sweeteners. The concern with the human consumption of the stevia leaf had been attributed to the possible mutagenic properties of steviol, but more recent studies conducted by the World Health Organization have established the safety for steviol and its glycosides.

In this study, an evaluation of the *Stevia rebaudiana* plant extract was conducted using modern chromatographic and mass spectrometry techniques for the determination of extracted components. The purpose was to evaluate the utility of performing two different modes of chromatographic separation for component identification. An accurate mass time of flight (TOF) mass spectrometer was used in the detection and identification of components. A novel software package was then utilized for the determination of common components between the two chromatographic modes and to depict the impact of chromatographic selectivity.

The concept behind the study was to utilize both reversed-phase chromatography and HILIC chromatography for the determination of extract components. By using two different modes of selectivity, components that co-retain, do not retain, or do not elute under one chromatographic mode may be resolved under a separate mode. By resolving a component chromatographically, a more accurate assessment of the component can be made without relying specifically on accurate mass data.

With traditional reversed-phase chromatography, analytes are primarily retained on an alkyl based stationary phase by partitioning interaction between the non polar stationary phase and the analyte. Though this

mode of chromatography is widely accepted for separation of moderately polar to non-polar compounds, highly polar analytes often have minimal or no retention on these phases. More popular polar embedded stationary phases address this issue with the addition of a polar functional group within the alkyl chain. Polar embedded phases can enhance retention of polar compounds, but it is not a solution for all applications. Often highly polar analytes require alternative modes of chromatographic retention. In particular, HILIC chromatography allows for alternative selectivity by utilizing a highly polar stationary phase with a relatively non polar mobile phase. Under HILIC conditions, the partitioning of analytes is achieved through a preferential solvation of an aqueous environment on the polar surface. More polar analytes will partition more into the surface solvent and thus be retained longer than a less polar analyte. In addition to the partitioning, the polar surface of the stationary phase allows for adsorptive interactions via hydrogen bonding, dipole, etc. When ionic samples are separated, the potential for ion-exchange interactions also exists and in many cases becomes the dominant retention mechanism. Using silica-based stationary phases, ionized surface silanol groups may interact via ion-exchange with positively charged analytes.

Experimental

In this study, both reversed-phase and HILIC separations were conducted using the Ascentis® Express RP-Amide and Ascentis Express HILIC. The polar embedded group of the Amide was chosen over traditional C18 phases to increase the retention of the polar analytes in the stevia

(continued on page 4)



Stevia rebaudiana

(continued from page 3)

extract. The Ascentis HILIC allowed for alternative selectivities for polar analytes. Because of the large amount of unknown components in the stevia extract, using both reversed-phase and HILIC modes enabled orthogonal selectivity to resolve co-retained components and enable better determination of components in the extract with confirmation between the two modes.

Stevia leaves were obtained from Sigma Aldrich (S5381). Sample extraction of the stevia leaves was performed by weighing 400 mg of crushed stevia leaves into a 7 mL amber vial. A total of 4 mL of 50:50 acetonitrile:water was added and the sample was vortexed and sonicated for 3 minutes. The sample was then centrifuged for 2 minutes at 15000 rpm. The supernatant was then collected and analyzed directly.

The sample extract was analyzed using a gradient elution profile for both HILIC and reversed-phase chromatographic modes. Analysis was conducted using an Agilent® 1200SL Rapid Resolution system in sequence with an Agilent 6210 TOF mass spectrometer. The TOF enabled the use of accurate mass for determination of components. The acquired data was processed using the Mass Hunter software package. The data was pushed to the Mass Profiler package for statistical comparison of the two chromatographic modes. This software package enabled the identification of common components between the two chromatographic separations of the stevia extract. By performing this type of statistical

comparison, the components attributed to the stevia extract were differentiated from components attributed to chromatographic anomalies. From this comparison the major components of the stevia extract were determined. Available standards were then used to confirm the identification of several of the components.

Results and Discussion

Figure 1 and Figure 2 represent the total ion chromatogram for the stevia extract under both HILIC and reversed-phase conditions. Both of these chromatographic separations demonstrate the complexity of the stevia extract. Table 1 depicts the major components that were common in both the reversed-phase and HILIC separations of the stevia extract. More than 250 components were identified with this comparison, but only the major components were targeted in this study. The highlighted components in Table 1 depict co-retention of analytes under reversed-phase conditions. A good example of using this orthogonal approach is observed in the case of steviobioside and ducloside A. Under the reversed-phase separation, these components were co-retained. By performing the separation under HILIC conditions, steviobioside and ducloside A were well separated. Other unidentified major components that were unresolved under the reversed-phase conditions were also separated under the HILIC conditions. The data in Table 1 also depicts the selectivity difference between the two chromatographic modes. Polar components that were poorly retained in the reversed-phase conditions were

Figure 1. Component Chromatogram of Stevia Extract on Ascentis Express HILIC

column: Ascentis Express HILIC, 15 cm x 2.1 mm I.D., 2.7 µm with upchurch inline filter
 flow: 0.2 mL/min.
 mobile phase A: 2 mM ammonium formate (98:2 acetonitrile:water)
 mobile phase B: 2 mM ammonium formate (80:20 acetonitrile:water)
 temp.: 35 °C
 inj. vol.: 1 µL
 system: Agilent 1200SL 6210 TOF, ESI(+)
 datafile: 1228082.d

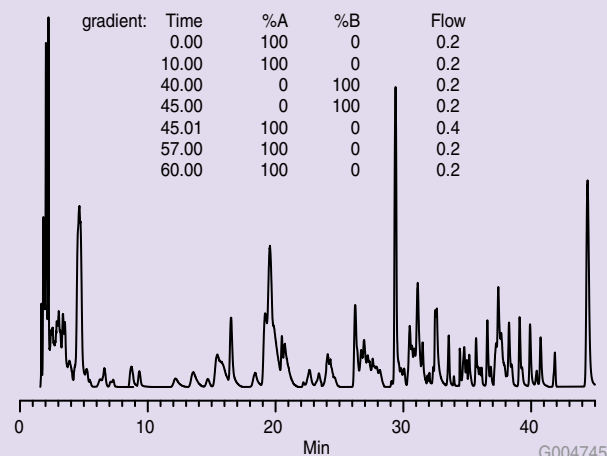


Figure 2. Component Chromatogram of Stevia Extract on Ascentis Express RP-Amide

column: Ascentis Express RP-Amide, 15 cm x 2.1 mm I.D., 2.7 µm with Upchurch inline filter
 flow: 0.2 mL/min.
 mobile phase A: 10 mM ammonium formate water
 mobile phase B: 10 mM ammonium formate (95:5 acetonitrile:water)
 temp.: 35 °C
 inj. vol.: 1 µL
 system: Agilent 1200SL 6210 TOF, ESI(+)
 datafile: 012009002.d

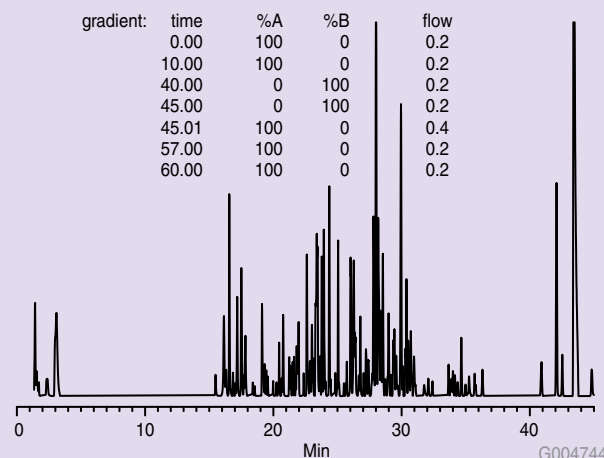


Table 1. Major Component Retention Comparison Between HILIC and Reversed-Phase Modes

Component	Accurate Mass	Ascentis Express HILIC RT	Ascentis Express RP-Amide RT
	137.0476	44.397	1.429
	120.0574	34.695	3.075
	102.0473	29.394	3.077
	368.1698	18.414	15.502
	120.0574	31.082	17.166
	402.1518	15.471	17.196
	162.1405	8.727	19.128
	378.2242	8.736	19.13
Rebaudioside A/E	966.4281	34.439	21.755
	516.1254	20.784	23.016
	498.1154	20.714	23.016
Stevioside	804.3743	26.52	23.63
Steviolbioside	642.3234	19.213	24.356
Dulcoside A	788.3817	26.247	24.475
	338.2448	2.248	25.737
	176.1555	2.049	26.026
	284.2134	3.372	26.028
	246.1977	3.359	26.06
	380.255	2.002	26.672
	284.2131	2.811	27.881
	360.083	1.867	28.187
	444.2002	1.825	30.391
Steviol	318.2186	1.91	32.19
	592.2655	2.239	42.539

Figure 3. Stevia Extract on Ascentis Express RP-Amide, Extracted Ion Chromatogram for Steviol

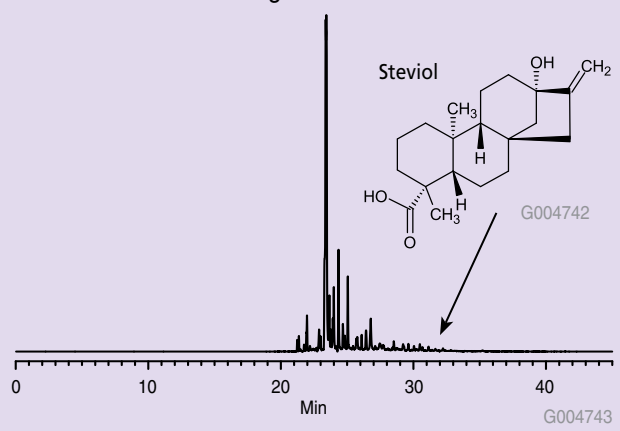
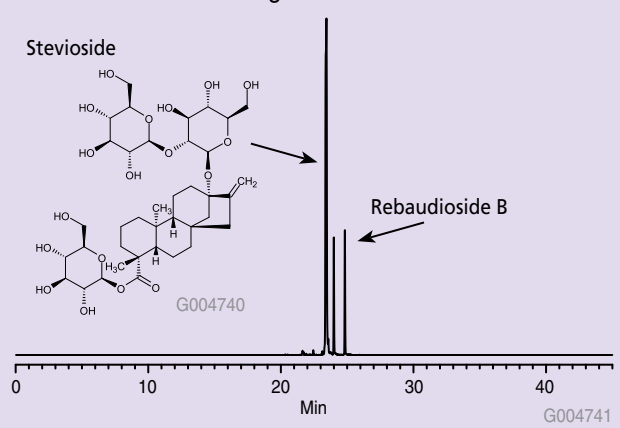


Figure 4. Stevia Extract on Ascentis Express RP-Amide, Extracted Ion Chromatogram for Stevioside



strongly retained under HILIC conditions. In two cases, where known components were identified, it was necessary to use standards to confirm their retention. Figures 3 and 4 depict the extracted ion chromatogram for the accurate mass of steviol and stevioside. As can be seen in both chromatograms, multiple peaks are observed for each of the accurate masses. In the case of stevioside, it is isobaric with rebaudioside B making identification difficult. A stevioside standard (Sigma Aldrich) was used for positive identification. In addition, the reversed-phase separation of the extract resulted in multiple peaks observed for the accurate mass of steviol. This was due to fragments from additional glycosides that resulted in a steviol fragment ion, again it was necessary to confirm the steviol retention with a standard.

Conclusions

The profiling of the *Stevia rebaudiana* extract demonstrates the utility of performing orthogonal chromatographic modes when handling complex samples. The two modes of chromatography were complimentary for the determination of major components from the stevia extract. In most cases where coelution occurred in one chromatographic mode, the components were separated under the orthogonal mode. Though component identification was made easier through the accurate mass of the TOF, it was still necessary to have good chromatographic resolution to confirm component identity. In both cases, the Fused-Core™ particle demonstrated the ability to perform complex matrix analysis in both HILIC and reversed-phase separations.

+ Related Products

I.D. (mm)	Length (cm)	Cat. No.
Ascentis Express C18		
2.1	5	53822-U
2.1	10	53823-U
2.1	15	53825-U
4.6	5	53826-U
4.6	10	53827-U
4.6	15	53829-U
Ascentis Express C8		
2.1	5	53831-U
2.1	10	53832-U
4.6	15	53838-U
Ascentis Express RP-Amide		
2.1	5	53911-U
2.1	10	59313-U
4.6	15	53931-U
Ascentis Express HILIC Silica		
2.1	5	53934-U
2.1	10	53939-U
4.6	15	53981-U

! Related Information

For more information on Ascentis Express HPLC Columns, visit sigma-aldrich.com/express

ordering: 800-247-6628 (us only) / 814-359-3441 technical service: 800-359-3041 (us and Canada only) / 814-359-3041

Discovery BIO GFC Separation Volume and Peak Capacity

Hillel Brandes

hillel.brandes@sial.com

Two measures of the effectiveness of a gel filtration HPLC (GFC) column are separation volume and peak capacity. Separation volume (V_s) is the difference in the retention volume between a fully retained analyte and a completely excluded analyte. It is, in effect, the total accessible pore volume of the column. A larger separation volume provides a greater volume in which chromatographic separation takes place and potentially greater peak resolution. Separation volume is calculated using the following equation:

$$V_s = V_t - V_0 \quad \text{Eq. 1}$$

Where:

V_s = separation volume

V_t = total column volume; V_t = elution volume (V_e) of fully retained analyte (analyte not adsorbed)

V_0 = void volume of column; $V_0 = V_e$ of excluded analyte

Peak capacity (P_c) is the number of peaks that are resolved within a given retention time window. There are both isocratic and gradient forms of the equation.

Isocratic peak capacity is determined using this equation:

$$P_c = (t_m - t_{r1})/W \quad \text{Eq. 2}$$

Where:

P_c = peak capacity

t_m and t_{r1} = retention time of last and first eluting peaks, respectively

W = average peak width at baseline

Peak capacity is directly proportional to $t_m - t_{r1}$, which is related to $V_t - V_0$ in equation 1 by the flow rate, and inversely proportional to peak width (W). Peak width is, in turn, related to column efficiency in that the higher the efficiency, the narrower the peaks. Therefore, the ideal GFC column exhibits high separation volume and high efficiency, which together provide high peak capacity for maximum analyte resolution.

Figure 1. Peptide Mix 1 - Separation Volume Comparison on Discovery BIO GFC and Competitive GFC Column

column: 30 cm x 7.8 mm ID., 5 μ m particles
mobile phase: 150 mM phosphate buffered saline, pH 7
flow rate: 1 mL/min.
temp.: ambient (~23° C)
det.: 214 nm
injection: 10 μ L

1. Thyroglobulin
2. BSA dimer
3. BSA monomer
4. Ribonuclease A
5. Poly-DL-alanine (1-5 kDa)
6. Uracil

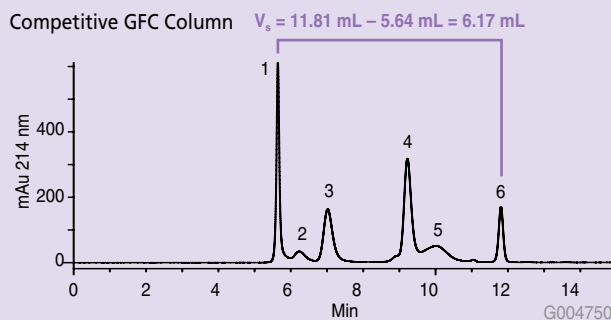
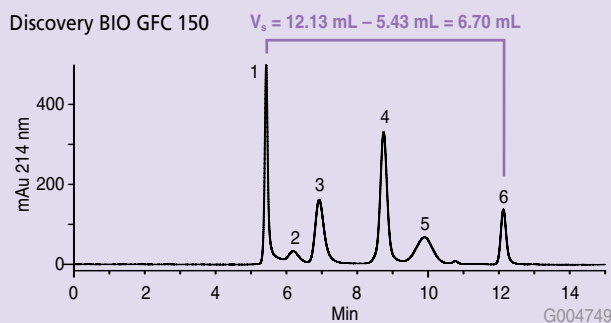
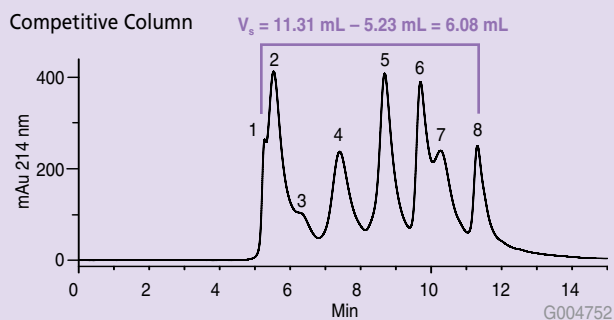
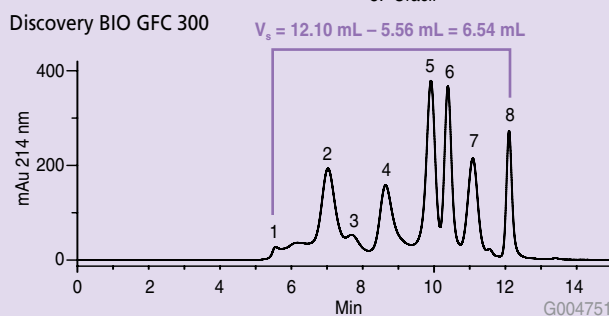


Figure 2. Peptide Mix 2 - Separation Volume and Peak Capacity Improvements on Discovery BIO GFC Compared to Competitive GFC Column

Same conditions as Figure 1, except for sample mix

1. Thyroglobulin aggregate
2. Thyroglobulin
3. γ -Globulin dimer
4. γ -Globulin
5. Ovalbumin
6. Myoglobin
7. Poly-DL-alanine (1-5 kDa)
8. Uracil



These properties are demonstrated on Discovery BIO GFC columns in Figures 1 and 2. In this study, we compared Discovery BIO GFC columns to competitive silica-based GFC columns of comparable particle and column dimensions in the isocratic separation of two sets of peptides. In both examples, Discovery BIO GFC was shown to have ~0.5 mL higher separation volume than the competitive column. In Figure 2, Discovery BIO GFC also provided much narrower peaks and significantly higher resolution of the peptides than the competitive GFC column. Both of these attributes, combined with excellent column stability, long lifetime and the wide

molecular weight separation range of individual columns and the entire line, make Discovery BIO GFC the columns of choice for gel filtration HPLC separations.

+ Featured Products

Description	Pore Diameter (Å)	mw (min.)	mw (max.)	Cat. No.
Discovery BIO GFC 150, 30 cm x 7.8 mm, 5 µm particles	150	500	150,000	567300-U
Discovery BIO GFC 300, 30 cm x 7.8 mm, 5 µm particles	300	5,000	1,250,000	567304-U

Astec CYCLOBOND I 2000 HP-RSP – Unique CSP for Chiral HPLC and LC-MS Separations

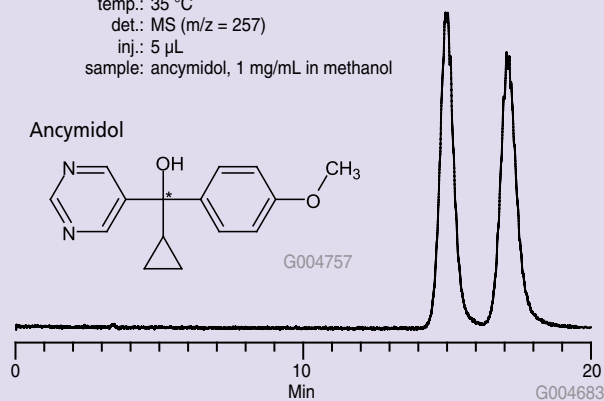
This (R,S)-hydroxypropyl modified beta-cyclodextrin offers the benefits of short retention times and high enantioselectivity. CYCLOBOND I 2000 HP-RSP separates by extended H-bonding capability, and offers broad chiral selectivity for chiral screening. It is most beneficial for basic and neutral compounds found in pharmaceutical, environmental and general organic chemistry applications.

The separation of the enantiomers of ancymidol, a pyrimidine herbicide and plant growth regulator, on CYCLOBOND I 2000 HP-RSP is shown here.

The ability to use aqueous/polar mobile phases with ionic additives makes CYCLOBOND phases ideal for LC-MS applications.

Figure 1. Ancymidol Enantiomers on Astec CYCLOBOND I 2000 HP-RSP

column: CYCLOBOND I 2000 HP-RSP, 25 cm x 4.6 mm I.D., 5 µm particles (24024AST)
mobile phase: (80:20) 5 mM ammonium acetate (pH 6.0):acetonitrile
flow rate: 1 mL/min.
temp.: 35 °C
det.: MS (m/z = 257)
inj.: 5 µL
sample: ancymidol, 1 mg/mL in methanol



+ Featured Products

Description	Cat. No.
Astec CYCLOBOND I 2000 HP-RSP, 25 cm x 4.6 mm I.D., 5 µm particles	24024AST

<http://extech2009.sdstate.edu>

October 4-7, 2009

Rushmore Plaza Holiday Inn,
Rapid City, South Dakota, USA

ExTech® is a symposium series highlighting new extraction technologies for chemical and biochemical analysis in laboratory and on-site settings.



ExTech 2009
11th International Symposium on
Advances in Extraction Technologies

ordering: 800-247-6628 (US only) / 814-359-3441 technical service: 800-359-3041 (US and Canada only) / 814-359-3041

Liquid Chromatography

SUPELCO
Analytical

Fast LC-MS on Astec CHIROBIOTIC CSPs

Craig Aurand
craig.aurand@sial.com

Improve throughput, sensitivity and solvent savings by leveraging column dimensions and flow rate.

Astec CHIROBIOTIC CSPs (chiral stationary phases) offer several different types of molecular interactions and separate enantiomers of many analyte classes. Of particular interest are ionic interactions. These permit operation in the polar ionic mode, which is preferred for LC-MS because its mobile phases typically comprise high percentages of methanol or acetonitrile containing low concentrations (1 to 15 mM) of volatile acids, bases or salts, such as ammonium acetate or ammonium formate. Additionally, the polar ionic mode often yields more efficient analyte ionization and correspondingly improved LC-MS sensitivity compared to reversed-phase systems.

To maximize laboratory throughput, HPLC analyses should provide the needed resolution and sensitivity in the shortest amount of time. Column configuration can be leveraged to achieve this objective. The separation of fluoxetine enantiomers on Astec CHIROBIOTIC V2 in Figure 1 demonstrates the improvement in speed and sensitivity gained by using short columns with narrow internal diameter (I.D.). At constant linear velocity, retention and sensitivity achieved by a traditional 25 cm x 4.6 mm I.D. column (Figure 1A) was dramatically improved by reducing the column length and I.D. (Figure 1B). Because column efficiency is directly proportional to column length, the shorter column provided fewer theoretical plates, which resulted in lower resolution. However, Figure 1C shows that by decreasing the flow rate on the short column, resolution was maintained with increased sensitivity compared to the 4.6 mm I.D. column. The lower flow rates also permit direct connection to the MS without flow splitting.

Featured Products

Description	Cat. No.
Astec CHIROBIOTIC V2	
10 cm x 2.1 mm I.D., 5 µm particles	15018AST
25 cm x 4.6 mm I.D., 5 µm particles	15024AST

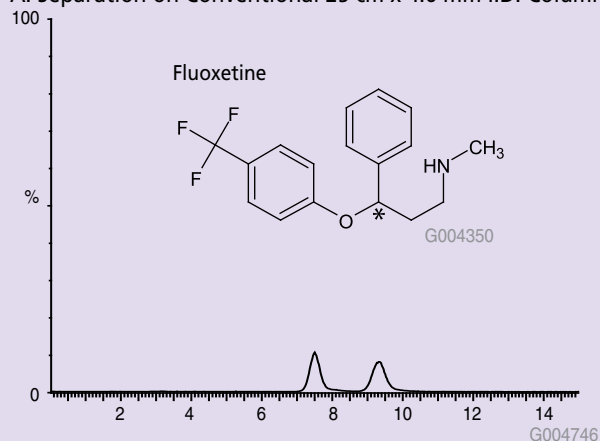
Related Information

For the complete Astec CHIROBIOTIC product line, please visit sigma-aldrich.com/astec

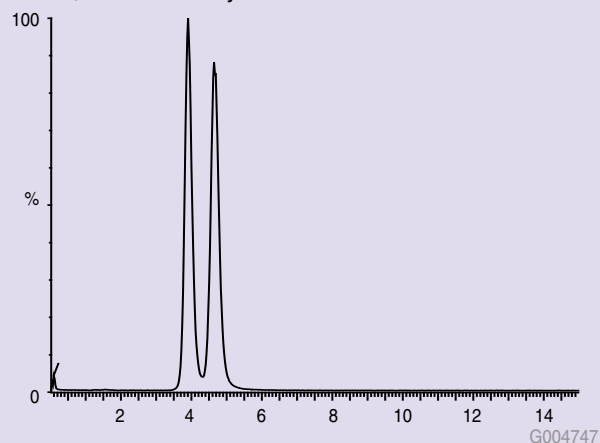
Figure 1. LC-MS of Fluoxetine Enantiomers on Astec CHIROBIOTIC V2

column: Astec CHIROBIOTIC V2, 25 cm x 4.6 mm (A)
or 10 cm x 2.1 mm I.D. (B and C), 5 µm particles
mobile phase: 10 mM ammonium formate in 10:90 v/v water:methanol
flow rate: 1.0 (A), 0.2 (B) or 0.1 (C) mL/min.
temp.: ambient
det.: +ESI, m/z 310
injection: 2 µL
sample: 5 µg/mL in methanol

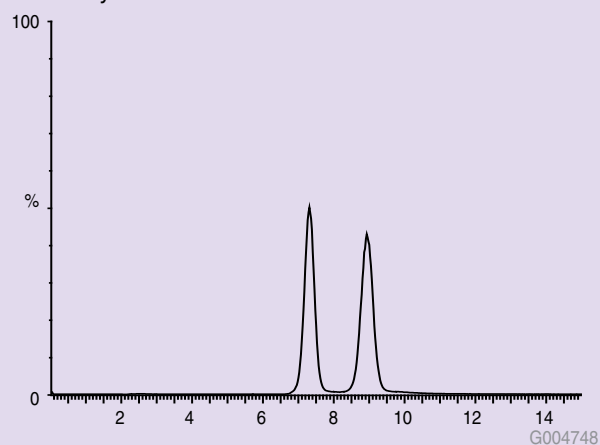
A. Separation on Conventional 25 cm x 4.6 mm I.D. Column



B. Fast, Sensitive Analysis on 10 cm x 2.1 mm I.D. Column



C. Use Column Dimensions and Flow Rate to Enhance Sensitivity without Loss of Resolution



High Purity LC-MS Mobile Phase Additives and Water

Shyam Verma

shyam.verma@sial.com

With improved LC-MS technology and speed of analysis there is greater demand for high purity of chemicals used for sample preparation, mobile phases and post-column additives. Alkali ions, plasticizers and surfactants are particularly problematic, as they are widespread and interfere strongly with LC-MS causing higher background noise and the formation of adducts. Sigma-Aldrich offers solvents, additives, and reagents that are specifically designed to meet the requirements of high-purity and consistency.

CHROMASOLV® Mobile Phase additives are selective chemicals commonly added to the mobile phase or introduced post-column prior to the interface to influence analyte ionization. Most often, the objective is to improve the signal quality. On the other hand, some additives are used to suppress unwanted signals or selectively enhance the signal of specific compounds in a mixture, for example, glycosidic species in a mixture of peptides.

Organic acids like formic and acetic acid are among the most commonly used additives. Two fundamental reasons for use of these additives are:

1. Many chromatographic separations benefit in terms of retention and/or peak shape under acidic conditions since any silanol activity is suppressed.
2. Most MS measurements are done in positive ion mode, which is accomplished by the addition of a proton to form the molecular ion $[M+H]^+$. The low molecular weight organic acids mentioned above exhibit necessary acidity and volatility to provide an excess of cations for this purpose.

Volatile and low molecular weight organic acids improve ionization and resolution of a wide range of molecules.

CHROMASOLV Water is a high purity product with quality suitable for both gradient HPLC and MS applications, offers tremendous advantages over other non-gradient grade water available in the market. It can be used in both UV and MS detection methods without any compromise.

+ Featured Products

Description	Qty.	Cat. No.
LC-MS CHROMASOLV Mobile Phase Additives and Water		
Trifluoroacetic acid	50 mL	40967
Formic acid	10 x 1 mL, 50 mL	56302
Acetic acid	50 mL	49199
Propionic acid	50 mL	49916
Ammonium formate	50 g	55674
Ammonium acetate	25 g	49638
Ammonium bicarbonate	50 g	40867
Ammonium hydroxide solution 25%	100 mL	44273
Triethylamine	50 mL	65897
Water	1 L	39253

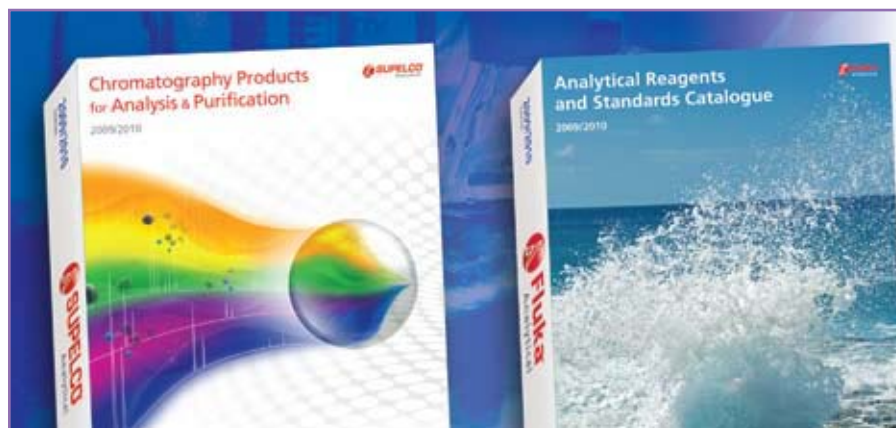
All chemicals are "puriss p.a."

A complete product listing can be found online at:
sigma-aldrich.com/lc-ms-solvents

20% Off Mobile Phase Additives and Water!

Receive 20% off the list price.

Please quote promotion code 992 when placing your order. Offer valid until June 1, 2009.



NEW! Analytical Catalogs Now Available

To request these valuable resources, complete the information on the attached postcard and return it to us by mail or fax, or request online at sigma-aldrich.com/supelco-catalog

Sigma-Aldrich Introduces New Standards Kit for ASTM D5501: Determination of Ethanol Content of Denatured Fuel Ethanol

Vicki Yearick, Steve Cecil
techservice@sial.com

Current public focus on reduction of the use of fossil fuels and replacement of these fuels with cleaner-burning renewable fuels has played a key role in the growth of the fuel ethanol industry. Large demand for blending ethanol into gasoline began with its use as an oxygenated gasoline additive. Currently the US EPA has set the denatured ethanol-to-gasoline blend rate at 10 percent, but discussions are being held to increase this level to 15 percent or higher. Automotive companies are also producing flex-fuel vehicles; able to operate on ethanol-to-gasoline blends of up to 85 percent denatured ethanol, called E85.

With all this increased attention to fuel ethanol, producers and blenders have increased awareness of mandated fuel ethanol specifications, outlined in ASTM D4806 - *Specification for Denatured Fuel Ethanol for Blending with Gasoline for Use as Automotive Spark-Ignition Engine Fuel*.

Ethanol producers are required by law to render the fuel ethanol unfit for human consumption by adding a denaturant, typically natural gasoline. ASTM D4806 requires the fuel ethanol to contain a minimum of 92.1% ethanol by volume with the denaturant volume ranging from 1.96% to 4.76%.

Producers and blenders must monitor and report the content of ethanol and the denaturant to show they are in compliance with state and federal laws. Monitoring is accomplished by following the analytical method ASTM D5501 - *Determination of Ethanol Content of Denatured Fuel Ethanol by Gas Chromatography*.

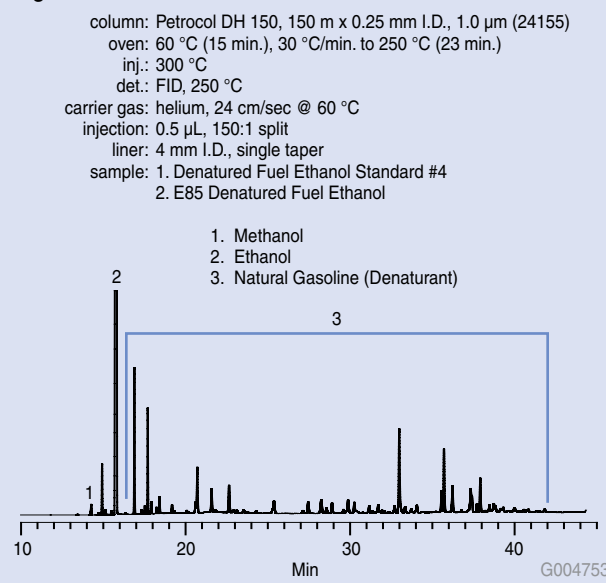
The GC method ASTM D5501 specifies the use of temperature program and a flame ionization detector to analyze the sample on a long polydimethylsiloxane capillary column, such as the Supelco Petrocol™ DH 150 (Cat. No. 24155). The method cites establishing peak identification, followed by the quantitation of ethanol.

The identification of ethanol and methanol is performed by injecting into the GC column a mixture containing known amounts of each alcohol in proportion to what is expected in the final blend, using n-heptane as a solvent. Retention times of the fuel ethanol sample are then compared to the analytical standard to verify identity.

Quantitation requires preparation of six multi-component calibration solutions, each containing ethanol, methanol and n-heptane in varying concentrations, to establish a linearity curve for the GC system. Because D4806 specifies a minimum ethanol content of 92.1% for denatured fuel ethanol, the ethanol content found in the six solutions range from 92 to 97%. N-heptane is included in the solutions in-place of the denaturant.

The D5501 calibration solutions and the denatured fuel ethanol samples are individually analyzed using the column conditions and temperature program shown in Figure 1. The mass relative response factors for the fuel ethanol sample are calculated to the nearest 0.01 mass percent, and then compared to the values obtained for each of the six calibration solutions to determine the ethanol content in the denatured fuel ethanol sample for reporting purposes.

Figure 1. E85 Denatured Fuel Ethanol



Preparation of these calibration standards is time consuming and requires maintaining an inventory of high purity raw materials. Calibration standards preparation is made easier by using Sigma-Aldrich's ASTM D5501 Denatured Fuel Ethanol Standards Kit. This kit contains pre-made Supelco brand multi-component, quantitative solutions covering the required range for accurate calibration, per the ASTM D5501. A certificate of analyses is provided for each calibration solution. To learn more about this kit and suggested analytical columns, please visit sigma-aldrich.com/biofuel

+ Featured Products

Description	Cat. No.
ASTM D5501 Denatured Fuel Ethanol Standard Kit	40361-U
Kit contains seven ampuls of varying concentrations. Prepared wt/wt.	
Solution 1 Ethanol:Heptane:Methanol (92%:7.40%:0.60%)	
Solution 2 Ethanol:Heptane:Methanol (93%:6.50%:0.50%)	
Solution 3 Ethanol:Heptane:Methanol (94%:5.60%:0.40%)	
Solution 4 Ethanol:Heptane:Methanol (95%:4.70%:0.30%)	
Solution 5 Ethanol:Heptane:Methanol (96%:3.80%:0.20%)	
Solution 6 Ethanol:Heptane:Methanol (97%:2.90%:0.10%)	
Solution 7 Ethanol:Heptane:Methanol (98%:1.95%:0.05%)	
Petrocol DH 150 capillary column 150 m x 0.25 mm I.D., 1.0 µm	24155

Monitor Food Antioxidants with Sigma-Aldrich Antioxidant Standards Kit

Antioxidants are added to food and other products to prevent rancidity. Although the mechanisms are not clearly understood, the antioxidants react with free radicals and peroxides slowing the rancidity. Certain other additives greatly enhance the effectiveness of antioxidants. Metal scavenger and chelating agents, such as citric acid and citrates, tie up the trace metals and greatly reduce their catalytic activity. Synergism between antioxidants has also been noted, and many commercial antioxidant mixes are formulated to contain mixtures of the antioxidants. The most common of these mixtures contain both butyl-hydroxyanisole (BHA) and butyl-hydroxytoluene (BHT).

In order to ensure consistent product quality, these additives must be monitored. In the past, food analysts had to go to multiple vendors to obtain good quality standards of all the antioxidants they may be required to monitor. Sigma-Aldrich offers an antioxidant standards kit containing several of the antioxidants listed in Association of Official Analytical Chemists Method 983.15: Phenolic Antioxidants, Fats, and Butter Oil plus ethoxyquin, an additional antioxidant commonly used in spices and other food products, and in cosmetics. Each antioxidant has been evaluated for purity, then packaged

neat under nitrogen. A certificate of analysis for each antioxidant is included with the kit.

Reference

1. Official Methods of Analysis (17th Ed.), Method 983.15. Association of Official Analytical Chemists, Arlington, VA USA (1996)

+ Featured Products

Description	Cat. No.
Phenolic Antioxidant Kit 2	40048-U
500 mg each, individually packaged	
<i>tert</i> -Butylhydroquinone (PG)	
Ethoxyquin	
2,3- <i>tert</i> -Butyl-4-hydroxyanisole (BHA)	
2,6-Di- <i>tert</i> -butyl-4-hydroxymethylphenol (IONOX 100)	
3,5-Di- <i>tert</i> -butyl-4-hydroxytoluene (BHT)	
Lauryl gallate	
Nordihydroguaiaretic acid (NDG)	
Octyl gallate	
Propyl gallate	

! Related Information

For assistance in selecting the proper chromatographic column for analysis of antioxidants, please contact the Sigma-Aldrich Technical Service Department at techservice@sial.com or, visit us at sigma-aldrich.com

TRADEMARKS: Agilent – Agilent Technologies; Ascentis, CHIROBIOTIC, CHROMASOLV, CYCLOBOND, Discovery, Equity, HybridSPE, Petrocol, SLB, SP, SPB, Supel, Supelco, Sup-
Herb, VOCOL – Sigma-Aldrich Biotechnology LP; Carbowax – Union Carbide Chemicals & Plastics Technology Corp.; Fused-Core – Advanced Materials Technologies, Inc.

How to Optimize GC Separations: Start With the Proper Column

Leonard M. Sidisky, Katherine K. Stenerson,
and Michael D. Buchanan
mike.buchanan@sial.com

Introduction

An optimized chromatographic separation begins with the column. The selection of the proper capillary column for any application should be based on four significant factors: stationary phase, column I.D., film thickness, and column length. The practical effects of these factors on the performance of the column are discussed briefly in this article, in order of importance. Note that this information is general. Specific situations may warrant exceptions to these guidelines.

Factor 1 – The Stationary Phase

Choosing a stationary phase is the most important step in selecting a column. A stationary phase is the film coated on the inner wall of a capillary column, and should be selected based on the application to be performed. The differences in the chemical and physical properties of injected organic compounds and their interactions with the stationary phase are the basis of the separation process. When the strength of the analyte-phase interactions differs significantly for two compounds, one is retained longer than the other. How long they are retained in the column (retention time) is a measure of these analyte-phase interactions.

Changing the chemical features of the stationary phase alters its physical properties. Two compounds that co-elute (do not separate) on a particular stationary phase might separate on another phase of a different chemistry, if the difference in the analyte-phase interactions is significant. This is the reason for providing a wide variety of capillary column phases. Each phase provides a specific combination of interactions for each chemical class of analytes.

Established Applications

Gas chromatography, first established in the 1950's, is a mature analytical technique with many established applications. Therefore, it is probable that literature, such as written methodology or journals, exists stating which stationary phases have successfully been used for a given application. Additionally, column manufacturers routinely publish phase selection charts, such as the example shown in Table 1, that is specific to the environmental testing industry. Charts like this are conveniently arranged by

industry to simplify the process of selecting the proper phase. Simply locate the application within that chart to identify a recommended column phase.

Table 1. Phase Selection Chart of Supelco GC Columns for Environmental Applications

	GC-MS/ GC Volatiles	GC-MS Semi-volatiles	GC Semi-volatiles (Pest/PCBs)	GC-MS Dioxins	GC-MS PCB Congeners	GC-MS PBDE Congeners
SPB™-Octyl					✘	
SLB-5ms		✘	✘	✘	✘	✘
SPB-624	✘					
VOCOL™	✘					
SPB-608			✘			
Sup-Herb™			✘			
Equity®-1701			✘			
SPB-50			✘			
SPB-225				✘		
SP™-2331				✘		
SLB™-IL100					✘	

New Applications

For new applications, there is often no existing reference to provide guidance. In these 'method development' instances, one must have some knowledge of the chemistry of the compounds to be analyzed. Phase selection is based on the general chemical principle that "likes dissolves like." A non-polar column is the recommended starting point for the analyses of non-polar compounds. Likewise, polar columns are usually recommended for the separation of polar compounds. Table 2 lists several recommended phases for each group of compound polarities.

Non-polar compounds are generally composed only of carbon and hydrogen atoms and contain carbon-carbon single bonds. Normal hydrocarbons (n-alkanes) are the most common non-polar compounds analyzed by capillary gas chromatography. Non-polar capillary columns separate these compounds very well. Interaction between non-polar compounds and a non-polar phase are dispersive, meaning that they are governed by Van der Waals forces. These are intermolecular attractions that increase with the size of the compound. Thus, larger compounds with higher boiling points have longer retention. Elution order generally follows the boiling points of the compounds.

Table 2. Phase Polarity Based on Compound Polarity

Compound Polarity	Compound Examples	Recommended Phases
Non-Polar		
C and H atoms only C-C bonds	alkanes	Petrocol, SPB-Octyl, Equity-1, SPB-1, SLB-5ms, Equity-5, SPB-5
Polar		
Primarily C and H atoms; Also contain Br, Cl, F, N, O, P, S	alcohols, amines, carboxylic acids, diols, esters, ethers, ketones, thiols	SPB-624, OVI-G43, VOCOL, SPB-20, Equity-1701, SPB-35, SPB-50, SPB-225, PAG, Omegawax, SPB-1000, Nukol, SUPELCOWAX 10
Polarizable		
C and H atoms only C=C or C≡C bonds	alkenes, alkynes, aromatic hydrocarbons	SP-2330, SP-2331, SP-2380, SP-2560, SP-2340, TCEP

Polar compounds are composed primarily of carbon and hydrogen atoms, but also contain one or more atoms of bromine, chlorine, fluorine, nitrogen, oxygen, phosphorus, or sulfur. Alcohols, amines, carboxylic acids, diols, esters, ethers, ketones, and thiols are typical polar compounds analyzed by capillary GC. Intermediate polar or polar capillary columns separate these compounds well. In addition to dispersive interactions, interactions between polar compounds and the phase include dipole, π - π , and/or acid-base interactions. Separations are determined by differences in the overall effects of these interactions.

Polarizable compounds are compounds composed of carbon and hydrogen, but contain one or more double or triple carbon-carbon bond. These compounds include alkenes, alkynes and aromatic (benzene-ring containing) hydrocarbons. Highly polar capillary columns are generally used to separate these compounds.

Bonded vs. Non-Bonded

Bonded phases are immobilized/chemically bonded (crosslinked) within the tubing, while non-bonded phases are simply coated on the wall. Generally, a bonded phase is preferred, because it has less bleed during use, can be used to higher temperatures, and, when necessary, can be rinsed with solvents to remove accumulated non-volatile materials. When a bonded phase is not available, such as for the highly polar phases, look for a stabilized phase. These phases are not as permanent as bonded phases (cannot be rinsed), but have greater thermal stability than non-bonded phases. For some applications, the only choice is a non-bonded phase. In these instances, extra care must be taken so the maximum temperature limit is not exceeded.

Factor 2 – Column I.D.

The current range of commercially available capillary column internal diameters enables the balancing of two factors: efficiency (number of theoretical plates) and

Table 3. Effects of Column I.D.

Internal Diameter (mm)	Efficiency: Plates/Meter (N/m)	Efficiency: Total Plates (N)	Capacity: Each Analyte (ng)
0.53	1,300	39,000	1000-2000
0.32	2,300	69,000	400-500
0.25	2,925	87,750	50-100
0.20	3,650	109,500	<50
0.18	4,050	121,500	<50
0.10	7,300	219,000	<10

Theoretical values for 30 m long columns, calculated @ a k' = 6.00 and 85% coating efficiency

sample capacity (amount of any one sample component that can be applied to the column without causing the desired sharp peak to overload). Optimizing one of these factors requires a sacrifice from the other. The ideal I.D. for a given application is dependent on the analytical needs.

High efficiency is observed chromatographically as narrow and well-resolved peaks. The efficiency of a capillary column, measured in plates (N) or plates per meter (N/m), increases as the I.D. of the column decreases. This is one of the basic principles behind Fast GC (see Supelco publication T407096 JTW for further details). If the sample to be analyzed contains many analytes, or has analytes that elute closely together, the most-narrow I.D. capillary column that is practical should be selected. Note that very narrow bore columns, such as 0.10 or 0.18 mm I.D., may require specialized equipment, such as a GC with a pressure regulator that allows a higher column head pressure.

Sample capacity increases with column I.D., and the greatest capacity is provided from wide bore columns (0.53 mm I.D.). Wide bore columns can accommodate a larger mass of each analyte in a sample than narrow bore capillary columns. Exceeding the sample capacity of a column will result in skewed peaks and decreased resolution. Therefore, if the samples to be analyzed contain compounds at high concentrations, or represent a wide range of concentrations, then a wide bore column should be considered. If the proper I.D. is chosen, the column should allow the system to provide sufficient sensitivity for the minor components without being overloaded with the major components. The analyst must decide if the loss in efficiency resulting from using a wide bore column is problematic for their application. Note that the nature of the sample components and the polarity of the phase will affect sample capacity. Non-polar phases have higher capacities for non-polar analytes, and polar phases have higher capacities for polar analytes.

The effects of column I.D. on efficiency and sample capacity are represented in Table 3. As shown, 0.25 mm I.D. columns provide adequate plates/meter for most

(continued on page 14)

Table 4. Effects of Film Thickness

	0.10 to 0.25 μm film	1 to 5 μm film
Benefits	Sharper peak shape May increase resolution Decreased column bleed Increased signal-to-noise Increased max. temp.	Reduced interaction with tubing Increased analyte capacity
Drawbacks	Increased interaction with tubing Decreased analyte capacity	Increased peak width May decrease resolution Increased column bleed Decreased max. temp.
Other	Decreased retention Decreased elution temperature	Increased retention May increase resolution Increased elution temperature
Uses	High boiling point analytes Semivolatiles Trace analyses	Low boiling point analytes Volatiles, gases High analyte concentrations

(continued from page 13)

applications while allowing acceptable sample capacity. Because of this compromise between efficiency and sample capacity, 0.25 mm is the most popular I.D. for capillary GC columns. Columns with a smaller or larger I.D. allow the user to optimize either efficiency or capacity, based on the requirements of their application.

Factor 3 – Film Thickness

As listed in Table 4, the benefits of decreasing film thickness are sharper peaks (which may increase resolution) and reduced column bleed; both resulting in increased signal-to-noise. Additionally, the column's maximum operating temperature will be increased. The drawbacks are increased analyte interaction with the tubing wall, and decreased analyte capacity. Decreasing film thickness also allows analytes to elute with shorter retention times and at lower temperatures, which may be desirable or undesirable, depending on the application.

Thinner film columns, i.e. 0.10 to 0.25 μm , should be used for analytes with high (>300 °C) boiling points (such as pesticides, PCBs, FAMES, phthalate esters, and other semivolatile compounds), or for trace analyses.

The benefits of increasing the film thickness are reduced analyte-tubing interaction and increased sample capacity. The drawbacks of increasing the film thickness are increased peak widths (which may reduce resolution), increased column bleed, and a reduced maximum operating temperature for the column. Increasing film thickness also leads to increased analyte retention (may also increase resolution, specifically for compounds with low k') and increased elution temperature. Depending on the application, these last effects may be either desirable or undesirable.

Thick film columns, i.e. 1 to 5 μm , are best suited for analytes with low boiling points (such as volatile organic

compounds and gases). These types of analytes are retained longer on the thicker film, which may eliminate the need for subambient oven conditions. A thicker film will also increase capacity, thus making the column more compatible for higher concentration samples than a thinner film column.

Phase Ratio (β)

Effects of phase film thickness are interdependent with column I.D. The phase ratio, beta (β), expresses the ratio of the gas volume and the stationary phase volume in a column:

$$\beta = \frac{\text{column radius } (\mu\text{m})}{2 \times \text{film thickness } (\mu\text{m})}$$

In contrast to relative terms ("thick film" and "thin film"), β values establish a distinct ranking for columns. As a general rule, select columns by β values as follows:

β Value	Uses
<100	Highly volatile, low molecular weight compounds
100-400	General purpose analyses Wide range of compounds
>400	High molecular weight compounds Trace analyses

β values are also useful when changing column I.D. and film thickness combinations for a particular analysis, because columns with the same phase ratio will provide very similar retention times and elution order under the same analytical conditions. Figure 1 depicts this phenomenon.

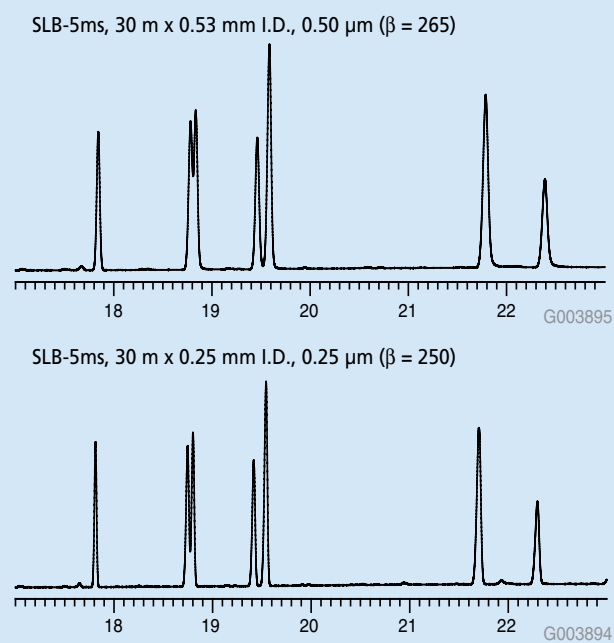
Figure 1. Columns with Similar β Values

Table 5. Effects of Column Length

Column Length (m)	Inlet Pressure (psi)	Peak 1 Retention (min)	Peak 1/2 Resolution (R)	Efficiency: Total Plates (N)
15	5.9	8.33	0.8	43,875
30	12.0	16.68	1.2	87,750
60	24.9	33.37	1.7	175,500

Theoretical values for 0.25 mm I.D. columns with 85% coating efficiency, 145 °C isothermal analyses, helium at 21 cm/sec, k'(peak 1) = 6.00

Factor 4 – Column Length

The last of the four significant factors to consider when selecting a column is length. A longer column will provide greater resolution than a shorter column. However, there are practical limits to increasing column length. With an isothermal analysis, a 60 m column does in fact increase resolution by almost 40%, relative to a 30 m column, but will increase the analysis time and also the head pressure required to move analytes through the column. Selecting a column length is a compromise between speed and head pressure on one side, and resolution on the other. Table 5 summarizes the effects of column length on various performance and operating parameters of 0.25 mm I.D. columns.

It should be stressed that doubling column length will NOT double resolution (resolution only increases according to the square root of the column length). If resolution between a critical pair is less than 1, doubling column length will not bring it to baseline (resolution value of at least 1.5). Increasing column length to increase resolution should be considered as a last resort. A more effective approach to increasing resolution is to reduce column I.D.

Shorter columns, such as those <15 m, are generally used when great resolution is not required, such as for screening purposes or for simple samples whose components are dissimilar in chemical nature. However, if column I.D. is decreased along with length, resolution can be maintained, or in some cases, actually increased.

Generally a 30 m column provides the best balance of resolution, analysis time, and required column head pressure. In some cases, a 30 m column with a thicker film may be as useful as a 60 m column for achieving a separation.

Use a 60 m column when higher resolution is required. Samples that are highly complex or contain volatile analytes are commonly analyzed on 60 m columns.

Very long, >100 m columns are also available for use when there is a need for extremely high resolution, such as in the detailed analysis of very complex samples (such as gasoline). Due to the extreme length of these columns, high head pressures are required to maintain column flow.

Conclusion

In the complete chromatographic system, the column has the greatest influence on the quality of the separation and resulting success of a any given application. When selecting the column, the first and most important attribute to consider is the chemistry of the stationary phase, as it will have a direct influence on column selectivity, or ability to separate sample components. Next, the choice of column I.D. and film thickness must be balanced to meet the needs of efficiency, sample capacity, retention, elution temperature, and bleed required for the analysis. Lastly, column length should be chosen with consideration of the resolution and analysis time requirements of the application. By considering the four factors of column choice discussed here, analysts should be able to make informed and logical choices with regards to column choice, thus increasing the chance that their application will be successful.

References

1. Harold McNair and James Miller, "Basic Gas Chromatography" (1997), Wiley, ISBN 0-471-17261-8.
2. David Grant, "Capillary Gas Chromatography" (1996), Wiley, ISBN 0-471-95377-6.

! Related Information

For column selection, request *GC Column Selection Guide: Achieve Optimal Method Performance*, T407133 (KCX). This brochure contains twelve easy to read column phase selection charts. These charts detail common applications performed in ten distinct industries, plus two applications that are independent of any industry.

For valuable information concerning the technique of Fast GC, request *Fast GC: A Practical Guide for Increasing Sample Throughput without Sacrificing Quality*, T407096 (JTW). Included in this brochure are both practical considerations and theoretical discussions.



GC Analysis of Glycols and Diols

Katherine K. Stenerson and Michael D. Buchanan
mike.buchanan@sial.com

Introduction

Glycol and diol compounds are used to solubilize active ingredients for use in many consumer products, including cosmetics, ballpoint pen inks, wood stains, and lacquers. They are also used as co-monomers in polymerization reactions to form polyesters and polyurethanes. (1) For safety and quality purposes, proper identification and purity checks of these raw materials are necessary.

Column Phase Selection

The chemical properties of these compounds, particularly the multiple active hydroxyl (-OH) functional groups, must be considered when selecting an appropriate phase for GC analysis. A polar (polyethylene glycol/Carbowax® 20M) phase is the traditional choice for the separation of polar compounds, however, glycol and diol peaks will exhibit tailing on unmodified phases of this type due to the strong interaction between -OH functional groups. A non-polar column such as a poly(dimethylsiloxane) can be used to minimize these interactions, however it may not provide the retention and/or selectivity necessary for the separation.

Polar Column Choice: The SPB-1000

For a polar column, the SPB-1000 is a good choice. This column is made with a modified polyethylene glycol phase that incorporates acidic functional groups. These groups lend an acidic character to this column, acting as a tailing inhibitor for active, acidic analytes. Figure 1 illustrates the separation of a variety of glycols and diols on the SPB-1000.

Non-Polar Column Choice: The Equity-1

Poly(dimethylsiloxane) is a non-polar GC phase, and will not interact as strongly with polar compounds as a polar polyethylene glycol phase. As a result, retention will not be as strong, and tailing may be decreased. Figure 2 illustrates the separation on an Equity-1 column of the same mix shown in Figure 1. Notice the significant reduction in retention, and change in elution order as compared to the SPB-1000.

Conclusion

Phase selection is the most important factor when selecting a GC column. (2) There may be several phases of different chemistries that are able to perform an application, and the specific needs of the application should be considered when making a choice. For example, if it is necessary to analyze 2-ethoxyethanol for the presence of other glycols, the SPB-1000 may be a better choice than

Figure 1. Glycols and Diols on the SPB-1000

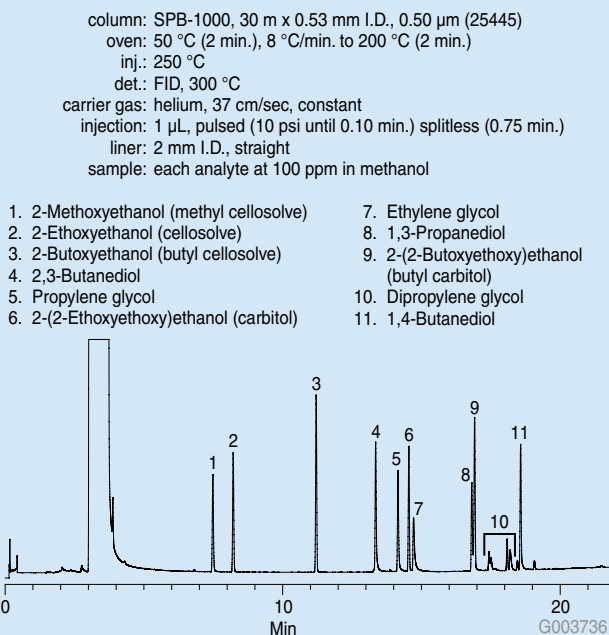
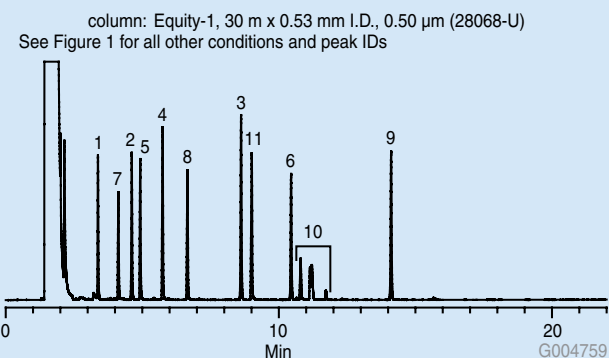


Figure 2. Glycols and Diols on the Equity-1



the Equity-1, as it will provide greater retention and better separation. Check available application information to help determine the best column choice. Also, consider consulting with the column manufacturer about your specific application. Supelco's Technical Service chemists can provide you with expert technical advice to aid in your column selection and method development.

References

- Richard J. Lewis Sr. editor, *Hawley's Condensed Chemical Dictionary*, Fourteenth Edition (2001), Wiley, ISBN 0-471-38735-5.
- M. D. Buchanan, *How to Optimize GC Separations: Use the Proper Column*, *Supelco Reporter* (April 2009); Vol. 27.2: 12.

Featured Products

Description	Cat. No.
SPB-1000, 30 m x 0.53 mm I.D., 0.50 μ m	25445
Equity-1, 30 m x 0.53 mm I.D., 0.50 μ m	28068-U

Troubleshooting Analyte Recovery when Using HybridSPE-Precipitation Technology

Craig Aurand, Charles Mi, Xioaning Lu, An Trinh, and Michael Ye

an.trinh@sial.com

Introduction

HybridSPE™-Precipitation (HybridSPE-PPT) is a new sample prep platform for pharmaceutical bioanalysis. The technology merges the simplicity of protein precipitation with the selectivity of SPE for the selective removal of proteins and phospholipids from biological plasma. Removal of these two key interferences greatly reduces the risk of ion-suppression during LC-MS-MS analysis for improved assay sensitivity and reproducibility. In Reporter issues 26.3 and 26.5, we provided an overview of HybridSPE-PPT and offered application examples that illustrate the benefits of the technology. In this article, we discuss non-phospholipid specific interactions between the HybridSPE phase and the sample that could potentially lead to low recovery of certain basic and acidic chelator compounds. We conclude our discussions with strategies for improving the recovery of such problematic compounds.

How does HybridSPE-PPT work?

When using 96-well HybridSPE-PPT, 100 μ L of plasma is added to the individual wells followed by 300 μ L precipitation agent (1% formic acid in acetonitrile). After a brief mixing step to adequately precipitate endogenous proteins, vacuum is applied to the plate. As the sample flows through the packed-bed/filter-frit assembly, both proteins

and phospholipids are concurrently removed. Proteins are physically removed by low porosity filters whereas phospholipids are chromatographically removed by the stationary phase. The resulting eluent is free of both phospholipids and proteins, and can be directly analyzed via LC-MS-MS.

The HybridSPE stationary phase is a patent pending zirconia coated silica that is highly selective towards phospholipids. Retention is based on a Lewis acid-base interaction between the empty zirconia d-orbitals (Lewis acid) and the electron pair of the phosphate moiety (Lewis base) inherent of all phospholipids. Phosphate is a very strong Lewis base and will preferentially interact with zirconia over other Lewis bases (Figure 1).

The Importance of Formic Acid

Most acidic pharmaceutical compounds contain carboxyl (-COOH) groups. When processing acidic compounds using HybridSPE-PPT, the HybridSPE Zr-Si stationary phase will likely co-retain acidic compounds along with phospholipids, resulting in low absolute recovery. To rectify the situation, formic acid is added to the precipitation agent and becomes part of the sample during HybridSPE processing. Formic acid is a stronger Lewis base than most -COOH groups found in acidic pharmaceutical compounds. As a result, formate ions will tie up the phase's zirconia ions, minimizing retention of acidic analytes of interest. Because formate is not a strong enough Lewis base to displace the phosphates, phospholipids preferentially retain on the HybridSPE-PPT phase.

In this application, we process three acidic compounds (ketoprofen, naproxen, and flunixin) and two neutral compounds using HybridSPE-Precipitation. The analytes were spiked into plasma at the level of 20 ng/mL. 100 μ L of plasma was precipitated with 300 μ L of one of two reagents prior to HybridSPE-PPT: 1) 1% formic acid in acetonitrile or 2) neat acetonitrile. The resulting HybridSPE-PPT eluent was analyzed by LC-MS-MS (MRM) using an Ascentis

(continued on page 18)

Figure 1. HybridSPE-PPT 96-well Schematic and Phospholipid Retention Mechanism

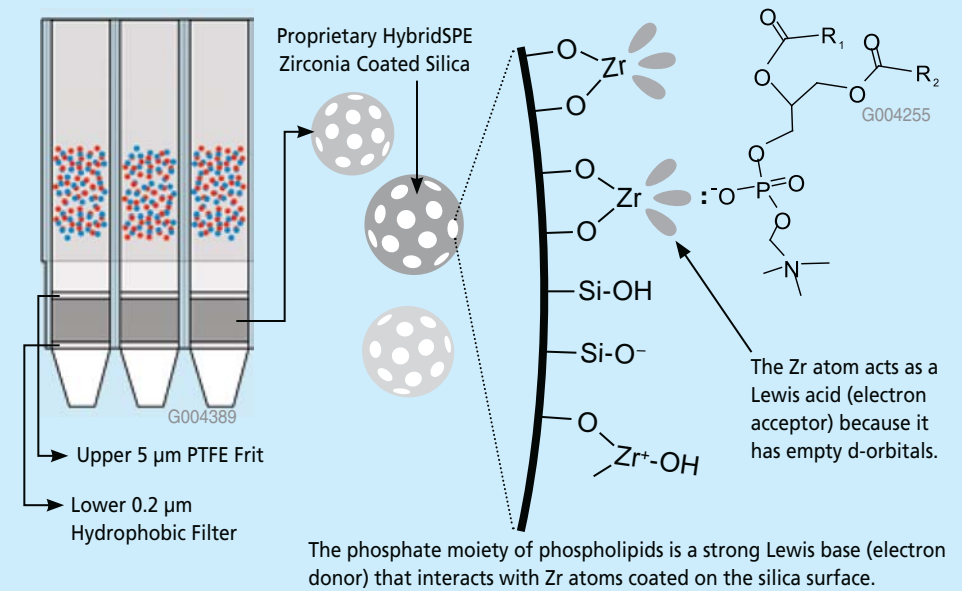
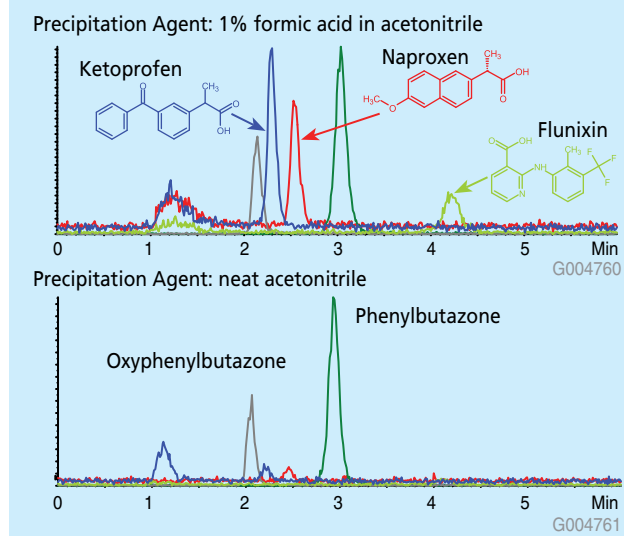


Figure 2. Comparison of Precipitation Agents (with and without formic acid) using HybridSPE-PPT



(continued from page 17)

RP-Amide column. Figure 2 compares the results between the two precipitation agents. From the results described in Figure 2, complete loss in recovery was observed for the three acidic compounds (ketoprofen, naproxen, and flunixin) when formic acid was not added to the precipitation agent. When formic acid is added to the precipitation agent, greater than 88% absolute recovery was observed for each of the acidic compounds (data not shown). In contrast, the two neutral compounds, phenylbutazone and oxyphenylbutazone were unaffected by the presence of formic acid resulting in high recovery under both conditions.

Troubleshooting Recovery of Chelator and Acidic Chelator Compounds

In our research thus far, we have found that certain chelator and acidic chelator compounds retain exceptionally strong on the Zr-Si stationary phase used in HybridSPE-PPT

Table 1. Relative Retention Strength of Lewis Bases to Zirconia

Lewis Base	Relative Retention Strength on Zirconia
Hydroxide	Strongest ↑ Weakest
Phosphate	
Fluoride	
Citrate	
Sulfate	
Acetate	
Formate	
Chloride	

resulting in low absolute recovery (< 40%) when using the recommended primary HybridSPE-PPT method (100 μ L plasma + 300 μ L formic acid in

acetonitrile). Such chelating compounds can be identified as having functional groups with oxygen atoms in the alpha and beta positions. To improve recovery, a Lewis base stronger than formate is required as a modifier in the

precipitation agent. Table 1 lists various Lewis bases and their relative retention strength on zirconia. From our experience, replacing formic acid with citric acid and adding a simple conditioning step can significantly increase the recovery of certain chelator and acidic chelator compounds. Figure 3 describes specific chelation functional groups and lists example compounds with such functional groups. Details for the secondary procedure we recommend are described in Table 3. By using the secondary procedure described in Table 3, recovery for chelator compounds can improve from <40% to 65-95%. Mechanistically, citric acid is a stronger Lewis base than formic acid, inhibiting retention of chelator compounds. However, citric acid is not a strong enough Lewis base to displace retention of phosphates (i.e., phospholipids).

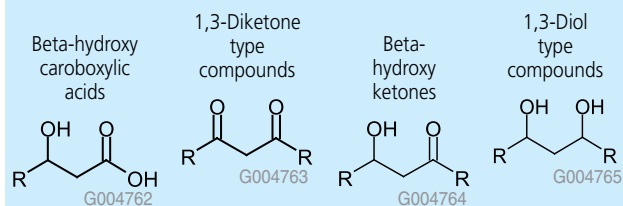
Troubleshooting Recovery of Basic and Neutral Compounds

Although the primary retention mechanism for HybridSPE is based on Lewis acid-base interactions between Zr ions on the stationary phase and negatively charged functional groups in the sample (e.g., phosphate moiety of phospholipids), secondary interactions derived from the silica surface can retain basic and neutral compounds resulting in poor recovery. These secondary interactions with silica surface include: 1) weak cation exchange and 2) HILIC interactions.

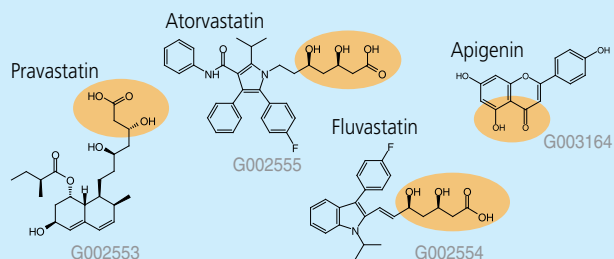
To disrupt any weak-cation exchange interactions between the silica silanol groups (Si-O⁻) and basic compounds (e.g., contains amine functional groups), formic acid should be substituted with ammonium formate. The primary procedure we recommend uses formic acid as part of the precipitation agent. As a result, H⁺ is the resulting counter-ion used to

Figure 3. Low Recovery Chelation Functional Groups with Example Compounds

Chelation functional groups that can lead to low HybridSPE-PPT recovery and may require citric acid secondary procedure:



Example compounds with chelation functional groups:



neutralize exposed silanol groups on the HybridSPE-PPT phase (Si-O⁻ => Si-OH). For some basic compounds, H⁺ is not a strong enough counter-ion to inhibit cation-exchange retention between silanol groups and basic compounds. This results in poor recovery. By replacing formic acid with ammonium formate, a stronger ammonium counter-ion (NH₄⁺) is employed. Ammonium ions are sufficient in counter-ion strength to inhibit most (if not all) basic compounds from interacting with silanol groups.

For more polar compounds, secondary HILIC interactions (e.g., hydrogen bonding) may occur between basic/neutral analytes of interest and the silica surface. These secondary HILIC interactions can be disrupted by substituting acetonitrile with methanol as the precipitation agent. To further minimize potential secondary HILIC interactions, the sample needs to be 25% aqueous prior to HybridSPE-PPT processing. Therefore, combining 100 µL plasma with 300 µL organic precipitation agent is recommended for HybridSPE-PPT. For smaller plasma volumes (e.g., 20-50 µL), the sample should be diluted with DI water to maintain a

Table 3. Summary of Recommended Primary and Secondary Procedures for 96-well HybridSPE-PPT

Primary Procedure (suitable for 80% of applications):

Recommended for most applications (basic, neutral, acidic analytes)

1. To each well, add 100 µL plasma followed by 300 µL 1% formic acid in acetonitrile. Mix the sample well (e.g., vortex).
2. Apply vacuum and collect the resulting eluent for LC-MS-MS analysis.
3. If low recovery is observed, proceed to Secondary Procedures.

Secondary Procedure (acidic & chelator compounds):

Recommended for low recovery chelator and acidic chelator compounds

1. Condition each well with 400 µL 0.5% citric acid in acetonitrile (until flow has ceased).
2. To each well, 100 µL plasma followed by 300 µL 0.5% citric acid in acetonitrile. Mix the sample well (e.g., vortex).
3. Apply vacuum and collect the resulting eluent for LC-MS-MS analysis.

Note:

Recovery of chelator compounds can improve from < 40% to 65-95%
Citric acid is a stronger Lewis base than formic acid inhibiting the retention of chelator compounds.

Citric acid is not a strong enough Lewis base to inhibit phosphates (phospholipids) from retaining on the HybridSPE phase.

Secondary Procedure (basic & neutral compounds):

Recommended for low recovery basic and neutral compounds

1. To each well, add 100 µL plasma followed by 300 µL 1% ammonium formate in methanol. Mix the sample well (e.g., vortex).
2. Apply vacuum and collect the resulting eluent for LC-MS-MS analysis.

Note:

Recovery of basic and neutral compounds can improve from < 40% to > 89%
NH₄⁺ (ammonium formate) is a stronger counter-ion than H⁺ (formic acid) inhibiting most basic compounds from interacting with HybridSPE silanol groups (Si-O⁻).
Methanol is a more polar solvent than acetonitrile further inhibiting any potential secondary HILIC interactions between the analyte and HybridSPE silica surface.

Table 2. Improvement of Absolute Recovery when Incorporating Ammonium Formate in the Precipitation Agent

Analyte (% Absolute Recovery)	Standard (no matrix) + 1% formic acid in MeCN	Standard (no matrix) + MeOH	Standard (no matrix) + 1% NH ₄ HCO ₂ in MeOH	Plasma + 1% NH ₄ HCO ₂ in MeOH
Mirtazapine (266/195)	0.0%	13.2%	96.0%	104.0%
Risperidone (411/191)	0.0%	10.4%	99.1%	123.3%
Olanzapine (313/256)	0.0%	13.6%	89.4%	56.4%

final sample volume of 100 µL prior to addition of 300 µL precipitation agent. If greater sensitivity is required after sample dilution, an evaporation and reconstitution step can be added prior to LC-MS-MS analysis.

In this study, 3 basic compounds experienced low recovery when using the primary method (100 µL plasma + 300 µL 1% formic acid in acetonitrile). 100 µL of spiked (20 ng/mL) plasma samples or standard samples (no matrix) were precipitated with 300 µL neat methanol, 1% formic acid in acetonitrile, or 1% ammonium formate in methanol prior to 96-well HybridSPE-PPT processing using the “In-well” precipitation method. Absolute recovery was assessed by reversed-phase LC-MS-MS (Table 2). From these results, significant improvements in HybridSPE-PPT recovery of the basic compounds observed when substituting 1% formic acid in acetonitrile with 1% ammonium formate in methanol as the precipitation agent.

Table 3 summarizes the primary and secondary procedures recommended when optimizing conditions for HybridSPE-PPT.

Conclusion

HybridSPE-PPT technology is a new sample prep platform designed for pharmaceutical bioanalysis. The technology combines the simplicity of protein precipitation and the selectivity SPE by specifically targeting the removal of precipitated proteins and phospholipids. The phospholipid removal mechanism is based on a Lewis acid-base interaction between the phosphate moiety inherent with all phospholipids and the Zr-Si stationary phase. Although a primary method that is suitable for most applications is available, low recovery can occur. In this report, we described the secondary interactions that can take place resulting in low recovery and strategies for how to troubleshoot these recovery issues.

For more information and to download the latest HybridSPE-PPT instruction sheet, please visit our website sigma-aldrich.com/hybridspe-ppt

Featured Products

Description	Cat. No.
HybridSPE-Precipitation	
96-well Plate, 50 mg/well, pk. 1	575656-U
Cartridge, 30 mg/1 mL, pk. 100	55261-U

ordering: 800-247-6628 (us only) / 814-359-3441 technical service: 800-359-3041 (US and Canada only) / 814-359-3041

Sample Handling

SUPELCO
Analytical

Using SPME in the Analysis of Drugs of Abuse

Katherine K. Stenerson and Bob Shirey

katherine.stenerson@sial.com

Forensic chemists are often called upon to perform determinations that include the analysis of unknown substances or biological fluids for the presence of illegal drugs. Many of these drugs can be analyzed by GC, either directly or in their derivatized forms, and when mass spectral detectors are used, positive identification and low levels of detection are possible. To enhance low-level detection, solid phase microextraction (SPME) can be used as part of the sample preparation process. In this article, we will discuss the use of SPME for the extraction and analysis of amphetamines and other drugs of abuse.

Analysis of Amphetamines

SPME has been used extensively for the analysis of amphetamines in urine. In this study, we examined its utility for the extraction of amphetamine compounds from plasma. This application could be useful in toxicology laboratories involved in postmortem drug testing. Also, the technique is suitable as a confirmation tool for clinical and forensic cases.

Experimental

Rat plasma was spiked at 50 µg/L with several species of amphetamine compounds, including the precursors ephedrine and pseudoephedrine. A summary of the sample preparation procedure, including SPME conditions, is presented in Table 1. The ethyl chloroformate derivatization reagent was added directly to the plasma sample/buffer mixture. The mixture was vortexed for 1 min, and extraction performed by heated-headspace SPME. Analysis was done by GC-MS/SIM on an SLB-5ms capillary column.

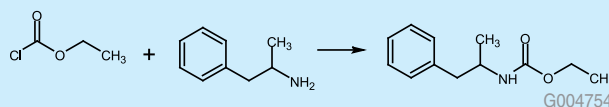
Table 1. Amphetamines; Derivatization and SPME Extraction Conditions

Sample:	In 4 mL vial: 0.5 mL rat plasma + 1.5 mL 25% NaCl in 5M K ₂ CO ₃ (saturated solution- undissolved salt crystals present)
Derivatization:	10 µL of 97% ethyl chloroformate, vortexed 1 min.
Fiber:	100 µm PDMS, 57300-U
Extraction:	Headspace, 75 °C, 15 min., with stirring at 300 rpm
Desorption:	275 °C, 6 min.

Derivatization

Due to the presence of an amine group in their structure, amphetamines are difficult to analyze by GC without derivatization. There are several different methods for derivatizing amphetamines. Ethyl chloroformate reacts with the amine group in amphetamine compounds to form carbamates, which are compatible with GC analysis. An example of this is illustrated in Figure 1 with amphetamine.

Figure 1. Derivatization of Amphetamine with Ethyl chloroformate

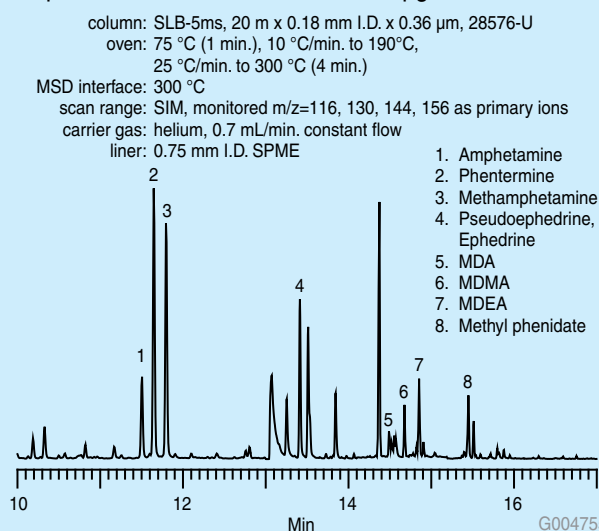


The derivatization reaction is unaffected by water, thus it can be done in an aqueous environment such as plasma. This allowed the derivatization reagent to be added directly to the buffer/plasma sample, and eliminated the need for the drying step that is often necessary prior to using other types of reagents.

Results

A chromatogram depicting the analysis of an extracted plasma sample is presented in Figure 2. Good response was obtained for all compounds, with matrix interference from the plasma minimized by the use of headspace SPME. Both pseudoephedrine and ephedrine yielded multiple derivatives with the ethyl chloroformate reagent, and the largest peak, resulting from the expected carbamate derivative, was chosen for the analysis.

Figure 2. SPME Headspace Extraction of Derivatized Amphetamines from Rat Plasma, 50 µg/L



Analysis of Other Drugs of Abuse

The speed and ease of SPME gives it utility as a screening tool in many field applications. In the case of drug analysis, there is interest in using it in combination with a portable GC-MS unit to extract and identify illegal drugs present in unknown powders found at crime scenes. In many instances, it may be necessary to test for several different types of drugs. In this study, we examined the utility of SPME for

extracting a varied array of drugs of abuse, and compared the results obtained with several different fiber chemistries.

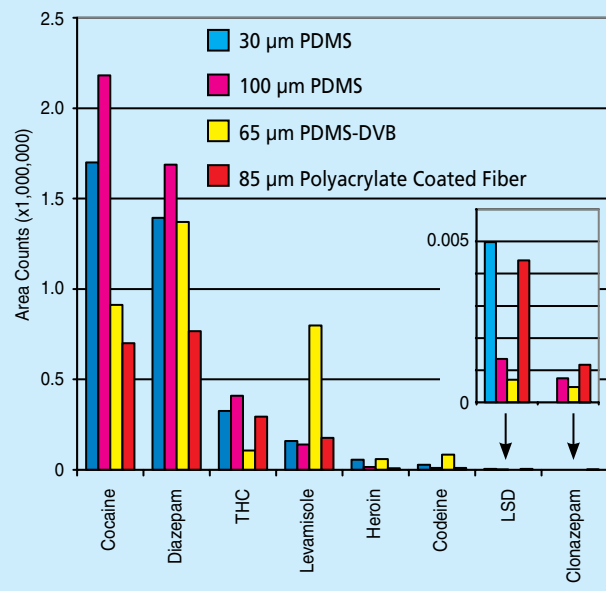
Experimental

The drugs chosen for this study include common psychotropic, stimulant, opiate, and hallucinogenic compounds. All can be analyzed by GC without prior derivatization, and were extracted in their native forms. Phosphate buffer (50 mM) containing 25% NaCl at pH=11 was spiked at 500 µg/L with each compound. (Higher recovery was observed for some of the drugs from basic solutions, compared to neutral and acidic solutions). The sample was then extracted by immersion SPME, with stirring, for 15 min. Analysis was done by GC-MS, in full scan mode, using an SLB-5ms capillary column.

Comparison of Fiber Chemistries

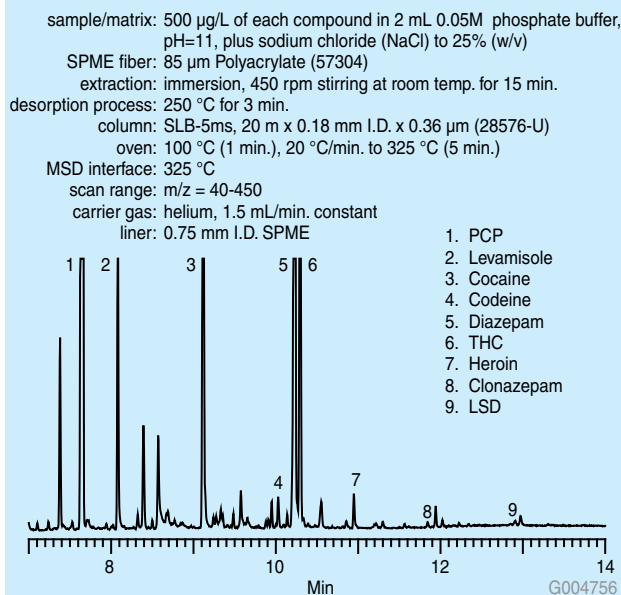
The extraction and analysis were performed using several different fiber chemistries, ranging from nonpolar to polar. A comparison of the absolute responses obtained for each compound between the two fibers is depicted in Figure 3. Phencyclidine responded well with all fiber types, and for reasons of scale, is not included in Figure 3. No one fiber was found to give optimal response for all compounds. As expected, the nonpolar PDMS fibers were best able to extract the less polar analytes and worked best for PCP, cocaine, diazepam and heroin. Clonazepam, followed by LSD, were the lowest responding compounds. The highest response for LSD was seen with the 30 µm PDMS fiber, indicating that desorption rather than extraction was the issue for this compound. The thinner coating on the 30 µm PDMS fiber may have allowed for more efficient desorption

Figure 3. Comparison of Absolute Response Between SPME Fibers



of this heavy compound than the 100 µm PDMS fiber. The analysis resulting from the extraction with the polar polyacrylate fiber is presented in Figure 4. Clonazepam response was the greatest with the polyacrylate fiber (PA), and LSD exhibited some response with this fiber as well. The other compounds showed reduced response on the polyacrylate compared with the less polar fibers, but enough that all were easily detectable at 500 µg/L.

Figure 4. SPME Extraction of Drugs from Buffer Solution Using the Polyacrylate Fiber



Conclusion

We have shown SPME can be used in the analysis of amphetamine compounds by doing a pre-extraction derivatization. If ethyl chloroformate is used as the derivatization reagent, the reaction can be done directly in an aqueous environment, and by doing headspace; the method can be applied to challenging biological matrices such as plasma.

SPME can also be used in general determinations of other drugs of abuse. When extracting differing species of drug compounds, such as those often determined in a basic drug screening analysis, fiber chemistry should be considered. The polyacrylate fiber shows the most promise for use as a general “screening” fiber for this type of application. Other fiber types may be useful if it is necessary to optimize a method for specific drug compounds.

References

- Husek, Petr. Chloroformates in gas chromatography as general purpose derivatizing reagents. *J. Chrom. B* (1998), 717, 57-91.
- Wise, Jeff; Danielson, Terry; Mozayani, Ashraf; Li, Richard. Analysis of amphetamine, methamphetamine, methylenedioxyamphetamine and methylenedioxymethamphetamine in whole blood using in-matrix ethyl chloroformate derivatization and automated solid-phase microextraction followed by GC-MS. *Forensic Toxicology* (2008), 26, 66-70.

Pipette Tips for Micro-Purification – Priced to Meet Your Budget

Shyam Verma

shyam.verma@sial.com

Pipette tips are convenient and useful tools for extraction, concentration, and/or purification of complex bio-molecules through e.g. hydrophobic C18 interactions. Supel™-Tips are designed to purify and enrich femtomole to picomole quantities of desired analytes from small samples for subsequent analysis and identification with mass spectrometric and/or chromatographic techniques.

Supel-Tips contain a chromatography sorbent bed with particle of size 50-60 μm bonded at the working end of the 10 μL pipette tip with a proprietary high purity inert

adhesive. This sorbent bed acts as a solid phase extraction medium that adsorbs molecules of interest from the sample matrix. Subsequently the concentrated, desalted analytes are eluted for downstream analysis.

These micropipette tips are offered in 10 μL size in P10 polypropylene micropipettes in a 96 pack. They are compatible with most organic solvents, buffers and alkaline solutions, but are not compatible with concentrated inorganic acids, such as, hydrochloric and nitric acids.

The new Zirconia-silica and Titania-silica composite pipette tips are designed for rapid enrichment of phosphopeptides (see Figure 1).

In the current promotion below, the Supel-Tips are now offered at a markedly reduced price to meet your analytical budget.

Take advantage of the 20% price reduction on any of the products listed below until June 15, 2009.

+ Featured Products

Description	Qty.	Cat. No.
Supel-Tips Zr Pipette Tips, 10 μL	96	54266-U
Supel-Tips Ti Pipette Tips, 10 μL	96	54263-U
Supel-Tips C18 Pipette Tips, 10 μL	96	TPSC18-96EA
	960	TPSC18-960EA
Supel-Tips Carbon Pipette Tips, 10 μL	96	54227-U

For complete listing of our products or to request a free sample, please visit: sigma-aldrich.com/pipette-tips

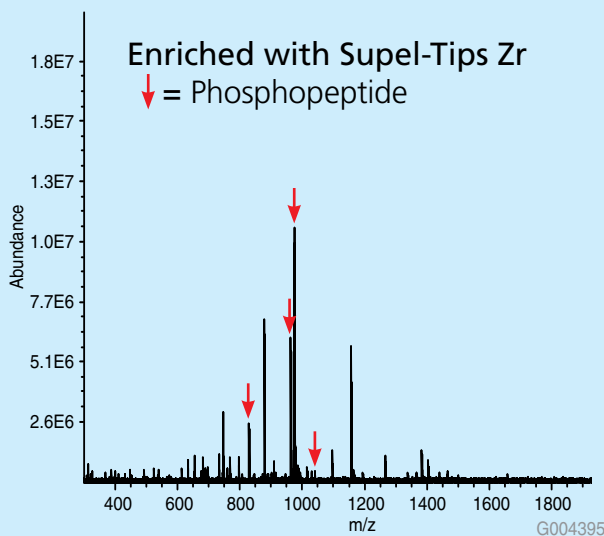
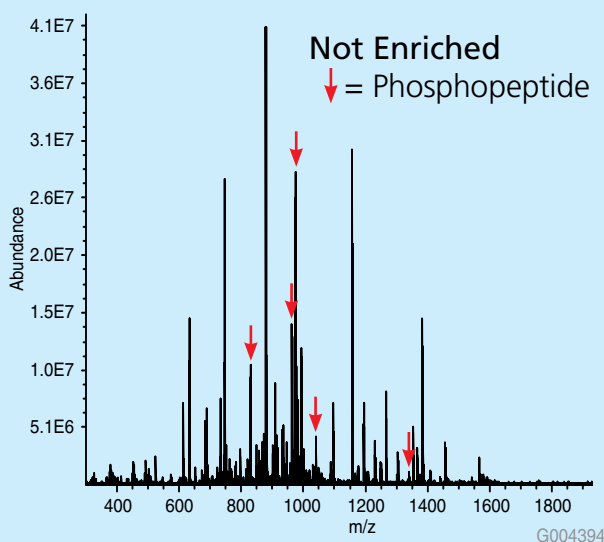
To request free literature or for assistance in selecting suitable products, contact Supelco Technical Service at 800-359-3041 (US and Canada) or 814-359-3041 or techservice@sial.com

20% Off Supel-Tips!

Receive 20% off the list price.

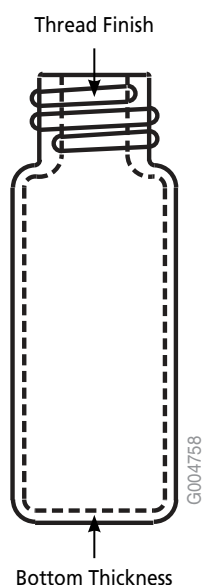
Please quote promotion code 987 when placing your order. Offer valid until June 15, 2009.

Figure 1. Identification of α -Casein Phosphopeptide



Certified Vials – Every Vial is Autosampler Compatible

There is a vast difference between each manufacturer's specifications for 12 x 32 mm autosampler vial bottom thickness and vial thread finish. For your autosampler vial to function correctly, both of these dimensions must be manufactured to very precise specifications.



The thickness of the glass on the bottom of the vial affects the distance that the autosampler needle can penetrate without making contact with the bottom of the vial. It is important for the needle to be set at the optimum depth to work properly. Ideally, the needle should withdraw the correct volume of sample without hitting the bottom of the vial.

The needle depth of the autosampler is determined by measuring an initial vial and deciding how deep the needle

will go. This value becomes difficult to determine correctly when the bottom thickness varies from vial to vial. Although the standard bottom thickness of 12 x 32 mm vials is 0.8 mm, it can vary in a package of vials by as much as +/- 0.4 mm (0.15").

Two things can happen if the bottom thickness specification varies. The autosampler needle may bend if it makes contact with the bottom of the vial. Secondly, if the technician sets the needle at a higher level to avoid hitting the bottom, much of the valuable sample will remain.

Neither outcome is acceptable in the laboratory. Consistency in analysis requires vials with consistent bottom thickness dimension in every vial. Our certified vials provide this feature. The thickness of the glass is carefully monitored to provide vials that have the same thickness every time.

Another key specification that causes problems for the lab technician is the thread finish of the vial. The interior of the cap thread must align perfectly with the exterior thread of the vial to provide a good seal. Any difference in these specifications will lead to sample evaporation and/or leakage when the parts are used together.

Purchasing products from different sources is often done to be economical, but in many instances the products ordered will not fit together properly. We recommend that you purchase these components from the same supplier to ensure that the parts are compatible. Our glass vials are checked carefully to make sure they work with the caps for which they are intended.

Our certified vials are manufactured to the strictest specifications possible. Each lot of vials is examined to ensure that there are no inconsistencies in bottom thickness and vial thread as well as height and diameter.

Certified vials are offered in a variety of styles that include 9 mm thread, crimp top, and snap top vials. For assistance in selecting the correct vial for your autosampler, visit sigma-aldrich.com/vials



+ Featured Products

Decription	Cat. No.
12 x 32 mm Certified Kits with 9 mm Thread, Blue Polypropylene Cap, Pack of 100	
2 mL clear glass, PTFE/silicone septa	29378-U
2 mL clear glass, PTFE/silicone septa with slit	29379-U
2 mL clear glass with graduated marking spot, PTFE/silicone	29381-U
2 mL clear glass with graduated marking spot, PTFE/silicone with slit	29384-U
2 mL amber glass, PTFE/silicone septa	29385-U
2 mL amber glass with graduated marking spot, PTFE/silicone	29386-U
2 mL amber glass with graduated marking spot, PTFE/silicone with slit	29387-U
12 x 32 mm Certified Kit with 10/425 Thread, Blue Polypropylene Cap, Pack of 100	
2 mL clear glass, PTFE/silicone septa	29432-U
12 x 32 mm Certified Kit, Snap Ring Design, Blue Polypropylene Cap, Pack of 100	
2 mL clear glass, PTFE/silicone septa	29421-U
12 x 32 mm Certified Vials with 11 mm Crimp Top, Pack of 100	
2 mL clear glass	29403-U
2 mL clear glass with graduated marking spot	29404-U
2 mL amber glass	29407-U
2 mL amber glass with graduated marking spot	29408-U

ordering: 800-247-6628 (us only) / 814-359-3441 technical service: 800-359-3041 (US and Canada only) / 814-359-3041

Accessories

SUPELCO
Analytical

Argentina

SIGMA-ALDRICH DE ARGENTINA S.A.
 Free Tel: 0810 888 7446
 Tel: (+54) 11 4556 1472
 Fax: (+54) 11 4552 1698

Australia

SIGMA-ALDRICH PTY LTD.
 Free Tel: 1800 800 097
 Free Fax: 1800 800 096
 Tel: (+61) 2 9841 0555
 Fax: (+61) 2 9841 0500

Austria

SIGMA-ALDRICH HANDELS GmbH
 Tel: (+43) 1 605 81 10
 Fax: (+43) 1 605 81 20

Belgium

SIGMA-ALDRICH NV/S.A.
 Free Tel: 0800 14747
 Free Fax: 0800 14745
 Tel: (+32) 3 899 13 01
 Fax: (+32) 3 899 13 11

Brazil

SIGMA-ALDRICH BRASIL LTDA.
 Free Tel: 0800 701 7425
 Tel: (+55) 11 3732 3100
 Fax: (+55) 11 5522 9895

Canada

SIGMA-ALDRICH CANADA LTD.
 Free Tel: 1800 565 1400
 Free Fax: 1800 265 3858
 Tel: (+1) 905 829 9500
 Fax: (+1) 905 829 9292

China

SIGMA-ALDRICH (SHANGHAI)
 TRADING CO. LTD.
 Free Tel: 800 819 3336
 Tel: (+86) 21 6141 5566
 Fax: (+86) 21 6141 5567

Czech Republic

SIGMA-ALDRICH spol. s r. o.
 Tel: (+420) 246 003 200
 Fax: (+420) 246 003 291

Denmark

SIGMA-ALDRICH DENMARK A/S
 Tel: (+45) 43 56 59 10
 Fax: (+45) 43 56 59 05

Finland

SIGMA-ALDRICH FINLAND OY
 Tel: (+358) 9 350 9250
 Fax: (+358) 9 350 92555

France

SIGMA-ALDRICH CHIMIE S.à.r.l.
 Free Tel: 0800 211 408
 Free Fax: 0800 031 052
 Tel: (+33) 474 82 28 00
 Fax: (+33) 474 95 68 08

Germany

SIGMA-ALDRICH CHEMIE GmbH
 Free Tel: 0800 51 55 000
 Free Fax: 0800 64 90 000
 Tel: (+49) 89 6513 0
 Fax: (+49) 89 6513 1160

Greece

SIGMA-ALDRICH (O.M.) LTD.
 Tel: (+30) 210 994 8010
 Fax: (+30) 210 994 3831

Hungary

SIGMA-ALDRICH Kft
 Ingyenes telefonszám: 06 80 355 355
 Ingyenes fax szám: 06 80 344 344
 Tel: (+36) 1 235 9055
 Fax: (+36) 1 235 9050

India

SIGMA-ALDRICH CHEMICALS
 PRIVATE LIMITED
 Telephone
 Bangalore: (+91) 80 6621 9600
 New Delhi: (+91) 11 4358 8000
 Mumbai: (+91) 22 2570 2364
 Hyderabad: (+91) 40 4015 5488
 Fax
 Bangalore: (+91) 80 6621 9650
 New Delhi: (+91) 11 4358 8001
 Mumbai: (+91) 22 2579 7589
 Hyderabad: (+91) 40 4015 5466

Ireland

SIGMA-ALDRICH IRELAND LTD.
 Free Tel: 1800 200 888
 Free Fax: 1800 600 222
 Tel: +353 (0) 402 20370
 Fax: + 353 (0) 402 20375

Israel

SIGMA-ALDRICH ISRAEL LTD.
 Free Tel: 1 800 70 2222
 Tel: (+972) 8 948 4100
 Fax: (+972) 8 948 4200

Italy

SIGMA-ALDRICH S.r.l.
 Numero Verde: 800 827018
 Tel: (+39) 02 3341 7310
 Fax: (+39) 02 3801 0737

Japan

SIGMA-ALDRICH JAPAN K.K.
 Tel: (+81) 3 5796 7300
 Fax: (+81) 3 5796 7315

Korea

SIGMA-ALDRICH KOREA
 Free Tel: (+82) 80 023 7111
 Free Fax: (+82) 80 023 8111
 Tel: (+82) 31 329 9000
 Fax: (+82) 31 329 9090

Malaysia

SIGMA-ALDRICH (M) SDN. BHD
 Tel: (+60) 3 5635 3321
 Fax: (+60) 3 5635 4116

Mexico

SIGMA-ALDRICH QUÍMICA, S.A. de C.V.
 Free Tel: 01 800 007 5300
 Free Fax: 01 800 712 9920
 Tel: 52 722 276 1600
 Fax: 52 722 276 1601

The Netherlands

SIGMA-ALDRICH CHEMIE BV
 Free Tel: 0800 022 9088
 Free Fax: 0800 022 9089
 Tel: (+31) 78 620 5411
 Fax: (+31) 78 620 5421

New Zealand

SIGMA-ALDRICH NEW ZEALAND LTD.
 Free Tel: 0800 936 666
 Free Fax: 0800 937 777
 Tel: (+61) 2 9841 0555
 Fax: (+61) 2 9841 0500

Norway

SIGMA-ALDRICH NORWAY AS
 Tel: (+47) 23 17 60 60
 Fax: (+47) 23 17 60 50

Poland

SIGMA-ALDRICH Sp. z o.o.
 Tel: (+48) 61 829 01 00
 Fax: (+48) 61 829 01 20

Portugal

SIGMA-ALDRICH QUÍMICA, S.A.
 Free Tel: 800 202 180
 Free Fax: 800 202 178
 Tel: (+351) 21 924 2555
 Fax: (+351) 21 924 2610

Russia

SIGMA-ALDRICH RUS, LLC
 Tel: +7 (495) 621 6037
 +7 (495) 621 5828
 Fax: +7 (495) 621 5923

Singapore

SIGMA-ALDRICH PTE. LTD.
 Tel: (+65) 6779 1200
 Fax: (+65) 6779 1822

Slovakia

SIGMA-ALDRICH spol. s r. o.
 Tel: (+421) 255 571 562
 Fax: (+421) 255 571 564

South Africa

SIGMA-ALDRICH
 SOUTH AFRICA (PTY) LTD.
 Free Tel: 0800 1100 75
 Free Fax: 0800 1100 79
 Tel: (+27) 11 979 1188
 Fax: (+27) 11 979 1119

Spain

SIGMA-ALDRICH QUÍMICA, S.A.
 Free Tel: 900 101 376
 Free Fax: 900 102 028
 Tel: (+34) 91 661 99 77
 Fax: (+34) 91 661 96 42

Sweden

SIGMA-ALDRICH SWEDEN AB
 Tel: (+46) 8 742 4200
 Fax: (+46) 8 742 4243

Switzerland

SIGMA-ALDRICH CHEMIE GmbH
 Free Tel: 0800 80 00 80
 Free Fax: 0800 80 00 81
 Tel: (+41) 81 755 2828
 Fax: (+41) 81 755 2815

United Kingdom

SIGMA-ALDRICH COMPANY LTD.
 Free Tel: 0800 717 181
 Free Fax: 0800 378 785
 Tel: (+44) 1747 833 000
 Fax: (+44) 1747 833 313
 SAFC (UK) Tel: 01202 712305

United States

SIGMA-ALDRICH
 P.O. Box 14508
 St. Louis, Missouri 63178
 Toll-Free: 800 325 3010
 Toll-Free Fax: 800 325 5052
 Call Collect: (+1) 314 771 5750
 Tel: (+1) 314 771 5765
 Fax: (+1) 314 771 5757

Vietnam

SIGMA-ALDRICH PTE LTD. VN R.O.
 Tel: (848) 3516 2810
 Fax: (848) 6258 4238

Internet

sigma-aldrich.com



Mixed Sources

Product group from well-managed
 forests, controlled sources and
 recycled fiber
www.fsc.org Cert no. SGS-COC-XXXXXX
 © 1996 Forest Stewardship Council

**World Headquarters**

3050 Spruce St., St. Louis, MO 63103
 (314) 771-5765
sigma-aldrich.com

Order/Customer Service (800) 325-3010 • Fax (800) 325-5052

Technical Service (800) 325-5832 • sigma-aldrich.com/techservice

Development/Bulk Manufacturing Inquiries SAFC® (800) 244-1173

©2009 Sigma-Aldrich Co. All rights reserved. SIGMA, SAFC, SAFC®, SIGMA-ALDRICH, ALDRICH, FLUKA, and SUPELCO are trademarks belonging to Sigma-Aldrich Co. and its affiliate Sigma-Aldrich Biotechnology, L.P. Sigma brand products are sold through Sigma-Aldrich, Inc. Sigma-Aldrich, Inc. warrants that its products conform to the information contained in this and other Sigma-Aldrich publications. Purchaser must determine the suitability of the product(s) for their particular use. Additional terms and conditions may apply. Please see reverse side of the invoice or packing slip.

LCY
 T209002

*Accelerating Customers'
 Success through Innovation and
 Leadership in Life Science,
 High Technology and Service*

SIGMA-ALDRICH®