ISSUE 10 | 2021

Analytix
Reporter

Applications Employing New 2.7 µm Porous Graphitic Carbon Particles for U/HPLC

Supelco®
Analytical Products

For questions &
comments contact us at
Analytix@merckgroup.com

Dear Reader,

Though high-performance liquid chromatography (HPLC) celebrated its 50th anniversary as a technique in analytical chemistry back in 2016, the challenges that analytical scientists face, on a daily basis, continue to push the industry towards new and innovative solutions. Being a provider of chromatography solutions for nearly all those 50 years, our team of HPLC R&D scientists have led the charge to meet the demands of the industry. Three such innovations I would like to showcase here in more detail:

The Chromolith® line of HPLC columns, with its groundbreaking monolithic technology, enables analytical scientists to achieve rapid and robust separations of analytes without elevated backpressures and with minimal sample preparation needed. This column technology has simplified LC workflows of many laboratories and provided higher sample throughput. Not satisfied with stopping there, further work has led to the creation of a wide pore format, making this platform applicable for large molecules and therefore catering to the needs of today's biopharmaceutical industry. The next development in the Chromolith® line, offering a 2.0 mm inner diameter (I.D.) geometry for improved sensitivity and performance, will be launched later this year.

With the Ascentis® Express line of columns, we were the first that adopted the superficially porous particle technology (SPP) in order for analytical scientists to achieve ultra-high performance liquid chromatography (UHPLC) efficiency even with standard HPLC instrumentation. By combining a range of particle sizes and chemistries, this industry-recognized portfolio of columns has enabled scientists to efficiently resolve a myriad of compounds. New additions to the portfolio, Ascentis® Express PAH and Ascentis® Express PFAS, will help accurately solve challenges in monitoring those contaminants.

The latest addition to the Supelco® HPLC portfolio, the Supel™ Carbon LC, is based on a patented, synthetic process for creating porous graphitic carbon (PGC) particles. This column technology enables scientists to simplify methods for analyzing polar compounds using the familiar reversed phase approach and without headaches caused by a lack of reproducibility due to method conditions that are more challenging to control. Several applications have been developed using this new column technology; turn to page 3 to learn more about this exciting new addition to the portfolio.

The Supelco® HPLC portfolio was built on a foundation of innovative technologies driven by innovative scientists. We helped to solve challenges accurately in HPLC's past 50 years, and we will continue to do so for HPLC's next 50 years and beyond!

Happy Resolving!

Yours sincerely,

Cory E. Muraco
Global Product Manager, Liquid Chromatography

SigmaAldrich.com/HPLC

For your personal copy, sign up at
SigmaAldrich.com/analytix

Applications of Newly Developed 2.7 µm Porous Graphitic Carbon Particles in U/HPLC

C. Corman, HPLC R&D Sr. Scientist; C. Muraco, Global Product Manager Liquid Chromatography Technologies; M. Ye, HPLC R&D Manager; C. Frantz HPLC R&D Sr. Scientist; and W. Maule, HPLC R&D Sr. Scientist, Analytix@merckgroup.com

Introduction:
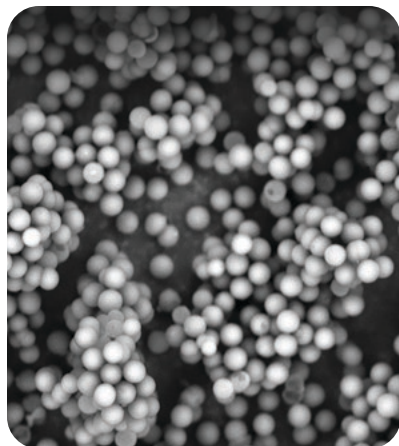
Since the 1960s, the scientific discipline of high-performance liquid chromatography (HPLC) has been dominated by silica particle-based column technologies. From irregular silica gels of the 1960s and 1970s to present-day superficially porous particles (SPPs), sub-2 µm fully porous particles (FPPs), and silica-based monoliths, the scientific literature is teeming with information on the applications of silica particle packed columns for a wide range of analyte polarities. Although there are advantages and disadvantages to each of these different particle modalities, in general, all silica-based packings have three common flaws: (1) a limited pH range for bonded phase stability (2) a limited temperature range for bonded phase stability, and (3) secondary interactions from active, silanol species (though, this can be a benefit when needed to elicit resolution by ionic interactions).

Back in the early days of chromatography, liquid chromatography (LC) utilized carbon particles. However, due to the nonlinear adsorption isotherms produced by then available, active carbon particles, they were not regarded as suitable for HPLC applications.[1] Through the late 1970s and 1980s, researchers optimized porous graphitic carbon (PGC) particles for less active surfaces, leading to the commercialization of carbon particle packed HPLC. The following years saw optimization of synthetic procedures to bring down the particle size from 10 µm to 3 µm, yielding higher efficiencies in the resolution of analytes. Finally, a breakthrough, novel, synthetic process was developed over the last two years to create a smaller (2.7 µm) carbon particle with a narrower particle size distribution (PSD), higher mechanical stability, and reproducibility—resulting in improved efficiencies in the separation of challenging analytes.

Summary of Fundamentals: Polar Retention on PGC

Porous graphitic carbon offers unique retention mechanisms that are beyond the scope of standard reversed phase supports. The presumption that a support made entirely of carbon atoms would behave like a perfect, non-polar phase without reactive silanol species formed the basis for PGC's development. However, further research revealed that this was not the case. The retention properties of PGC turned out to be different from traditional non-polar phases.[2] Studies by both Möckel et al. and Tanaka et al. showed mixed properties when comparing PGC to ODS (C18) phases.[3,4] While comparing PGC to ODS, the researchers discovered that, in some cases, a change in substituent, regardless of its polarity, could induce an increase in retention while on the ODS column, retention decreased. The researchers believed that polarizability of the analytes and graphitic surface were at play for the results they observed. Ross and others also observed similar behavior and came up with the term PREG (Polar Retention Effect on Graphite) to explain this distinctive characteristic of PGC.[1] While the exact mechanism for this phenomenon is still not clear, their explanation is still generally accepted. Those researchers surmised that the effect might be because of the uneven charge distribution in analytes due to delocalized electrons or polar functional groups and the high polarizability of the delocalized electrons of graphite. That is, when the polar analyte approaches the graphite surface, it produces an induced dipole. This interaction is heavily dependent on the polarizability of the surface, charge distribution of the analyte, and the orientation of the functional groups as they approach the graphite surface (**Figure 1**).

Even if the explanation for PREG is still unclear, it is obvious that PGC has special retentive properties, making it more suitable for polar compounds than standard reversed-phase approaches. Some key takeaways from PGC's ability to retain analytes are:

- PGC will behave like the conventional ODS phases in many ways for many analytes.
   - Typically, higher organic percentages must be used in the mobile phase as compared to ODS.
- Not every polar analyte will retain strongly on PGC, but PGC tends to retain polar compounds stronger than ODS phases.

- Analyte size and shape are important considerations. Planar compounds, especially aromatics, are good target analytes as the orientation can force an interaction (see **Figure 2** for analyte alignment example).

- Since PGC can have varying interaction strengths with analytes based on their electron distribution (size, shape, and orientation factors), it can discriminate between closely related compounds such as isomers or similar compounds with minor differences in functional groups.
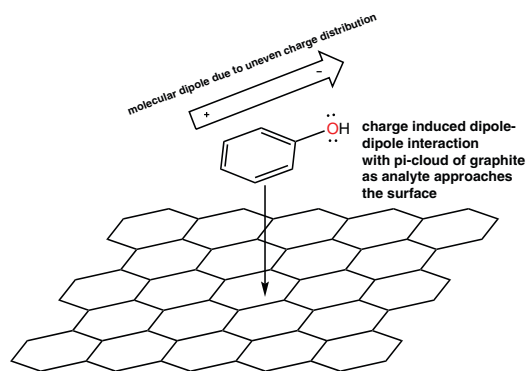

Figure 1. Illustration of Induced Dipole-Dipole Interaction as Analyte Advances Towards the Graphitic Plane.
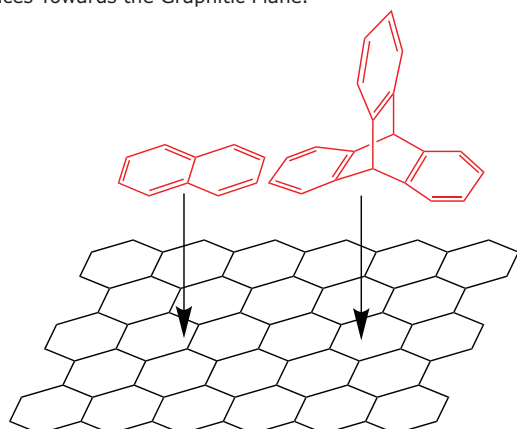

Figure 2. Representation of Analyte (red) Alignment with the Graphitic Surface (black). Analytes that can align their surface area flat against the graphitic plane have a stronger Interaction and tend to retain for longer as a result. More surface area contact attributes itself to longer retention than less surface area contact. An example illustrated here is naphthalene (left, stronger interaction) vs triptycene (right, relatively weaker interaction although one additional aromatic ring).

Applications

Analysis of Highly Polar Pesticides

One application where PGC may be of benefit is the coelution of polar analytes of interest within the void volume of an RP column. One example of this is the analysis of challenging polar pesticides such as glyphosate and related analogues. As seen in **Figure 3**, the use of a conventional C18 column, in this case an Ascentis® Express C18, 5.0 cm x 3.0 mm I.D.; 2.7 µm column, does not result in the retention of such compounds and coelution is observed. In this
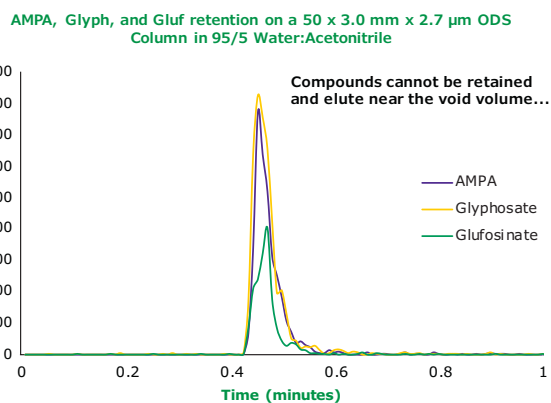
Figure 3. Three Polar Pesticides on an ODS Stationary Phase. No separation occurs and the compounds elute at or near the void volume of the column.
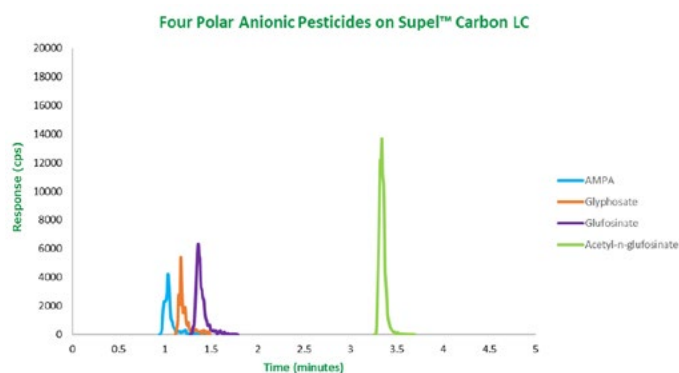
Order	Compound	Q1/Q3	Ret. (min)
1	Aminomethylphosphonic acid (AMPA) 4 µg/mL	110/79	1.03
2	Glyphosate 4 µg/mL	168.1/124	1.17
3	Glufosinate 4 µg/mL	180.1/63	1.36
4	Acetyl-n-glufosinate 2 µg/mL	222.2/136	3.34

Figure 4. Four Polar Pesticides Separated Using a PGC Stationary Phase (see Chromatographic Conditions in **Table 1**). Unlike the ODS column seen in **Figure 3**, PGC retains these polar compounds.

Table 1 Chromatographic Conditions for **Figure 4**

instrument:	Agilent™ 1290 HPLC, SCIEX™ 3200 Q Trap LC/MS/MS		
column:	Supel™ Carbon LC, 5.0 cm x 3.0 mm I.D., 2.7 µm (**59991-U**)		
mobile phase:	[A] 20 mM ammonium hydrogen carbonate in water; pH 9 adjusted with ammonium hydroxide; [B] Acetonitrile		
gradient:	**Time (min)**	**%A**	**%B**
	0	100	0
	1.0	100	0
	4.0	60	40
	7.5	0	100
	10.0	0	100
	10.1	100	0
	18.1	100	0
flow rate:	0.3 mL/min		
column temperature:	40 °C		
detector:	MS ESI(-), multiple reaction monitoring		
injection:	6 µL		
sample:	AMPA 4 µg/mL, glyphosate 4 µg/mL, glufosinate 4 µg/mL, acetyl-n-glufosinate 2 µg/mL; in mobile phase [A]		

case, 95:5 water:acetonitrile was used to protect the ODS phase from potential de-wetting. However, the Supel™ Carbon LC column was able to retain four polar pesticides (aminomethylphosphonic acid, glyphosate, glufosinsate, and acetyl-n-glufosinsate) using a simple gradient with a Mass Spectrometry (MS) friendly buffer (**Figure 4**).

Analysis of Paraquat and Diquat - Converting an Existing HILIC Method to a PGC Method with a Simpler Gradient

PGC may also aid in improving a pre-existing method that might be using a more complicated buffer setup. An example of this method conversion is the improvement of a hydrophilic interaction chromatography (HILIC) method for the separation of two polar herbicides, paraquat and diquat (**Figure 5**). Typically, some form of HILIC (**Figure 6**) or reversed-phase is used to retain both these compounds. The two herbicides are organic salts, highly soluble in water, and insoluble in most organic solvents. Also, both are weakly retained in reversed-phase chromatography

(RPC). In addition, RPC methodology requires heavy ion-pairing to improve the interaction strength, and peak shape of the analytes. While HILIC makes sense as it is better at retaining hydrophilic species than conventional reversed-phase, it still requires aggressive buffering and the peak shapes are not ideal (**Figure 6**).

Considering PGC's ability to retain polar analytes, as well as the structure of the analytes, paraquat and diquat are easily retained without the need for an aggressive 200 mM buffer concentration. **Figure 7** shows paraquat and diquat being retained using a simple gradient and 0.1% difluoroacetic acid (DFA) as a modifier to improve peak shape. The resolution is noticeably better, and the gradient can be adjusted for a faster analysis, when desired.

Figure 5. Analyte Structures – (Left) Paraquat, (Right) Diquat.

column:	Ascentis® Express HILIC, 10 cm x 2.1 mm I.D., 2.7 µm (**53939-U**)
mobile phase:	[A] 200 mM ammonium TFA; [B] acetonitrile; (20:80, A:B) isocratic
flow rate:	0.4 mL/min
column temp.:	60 °C
detector:	UV/Vis, 257 nm (paraquat) and 308 nm (diquat)
injection:	1 µL
sample:	50 mg/L in mobile phase

1. Diquat
2. Paraquat
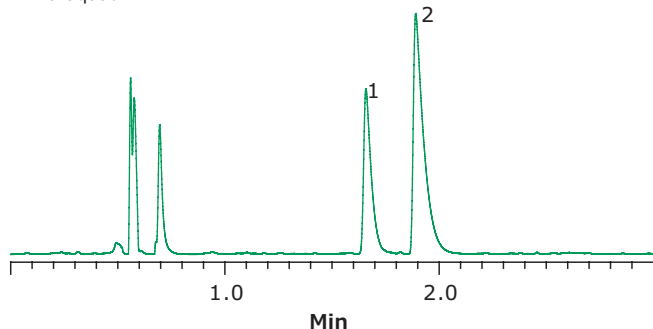
Figure 6. Separation of Paraquat and Diquat Using HILIC Conditions.

column:	Supel™ Carbon LC, 10 cm x 2.1 mm I.D., 2.7 µm (**59986-U**)
mobile phase:	(A) water (0.1% (v/v) DFA; (B) acetonitrile (0.1% (v/v) DFA
gradient:	

Time (min)	%A	%B
0	100	0
1	100	0
11	85	15
11.1	100	0
20.1	100	0

flow rate:	0.500 mL/min
column temp.:	55 °C
detection:	UV, 290 nm
injection:	2 µL
sample:	diquat – 10 µg/mL & paraquat – 100 µg/mL in mobile phase (A), water with 0.1% DFA

Figure 7. Paraquat/Diquat Separation by PGC. Better peak shape is achieved without the need for an overly concentrated buffer. In this case, DFA acts as an ion pair reagent. Elution order: 1) Paraquat - 4.362 min 2) Diquat - 7.295 min.

Separation of Vitamin D2 and D3 Metabolites and Their Epimers

Finally, an application highlighting the necessity of PGC materials is the separation of vitamins: more specifically, Vitamin D and its metabolites. Vitamin D metabolites have been used as biomarkers for various possible disease states and vitamin deficiencies. The tests are based on the level of two metabolites in blood: 25-hydroxyvitamin D2 and D3. It was found that these biomarkers could be further metabolized through a C3-epimerization pathway, resulting in two additional forms of metabolites, C3-epi-25 hydroxyvitamin D2 and D3 (**Figure 8**). Also, a recent topic has centered around the epimer having the same biological function as the non-epimers. It is necessary to detect the level of the epimers among the metabolites using LC-MS. Since the epimer and its non-epimer metabolite have the same m/z ratio, it is necessary to separate the epimers from their corresponding non-epimer metabolites prior to MS detection.[5-7]

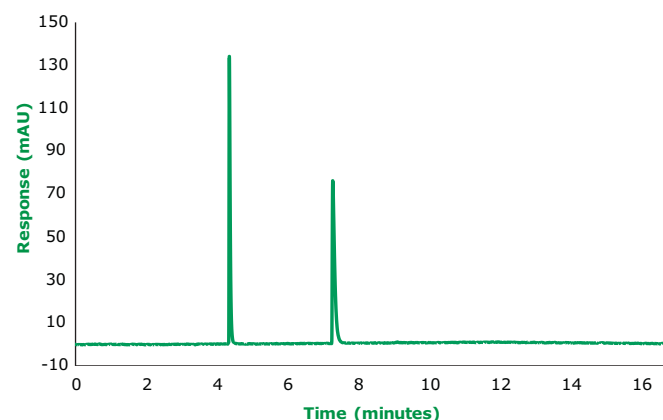| instrument:	Dionex Ultimate™ 3000		
column:	Supel™ Carbon LC, 10 cm x 2.1 mm I.D., 2.7 µm (**59986-U**)		
mobile phase:	(A) 2-propanol (B) tetrahydrofuran		
gradient:	**Time (min)**	**%A**	**%B**
	0	100	0
	15	30	70
	20	30	70
	20.1	100	0
	30.1	100	0
flow rate:	0.3 mL/min		
column temp.:	25 °C		
detection:	UV, 275 nm		
injection:	2 µL		
sample:	vitamin D2 & D3 test mix, in 100% ethanol (D2 – 100 µg/mL, epi-D2 – 50 µg/mL, D3 – 25 µg/mL, epi-D3 – 50 µg/mL)		
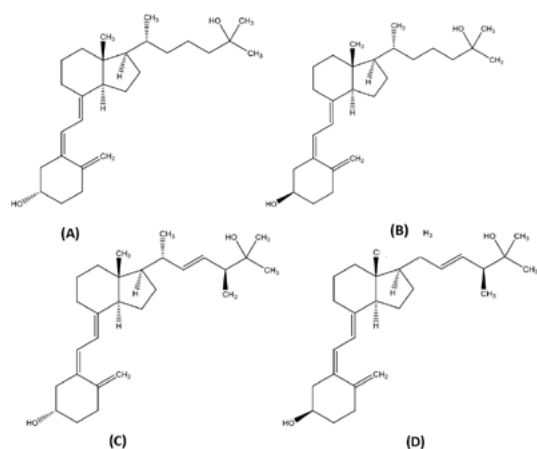
Figure 8. Structures of 25-Hydroxyvitamin D2 and D3 and Their Respective Epimers. (A) 25-(OH)-D3 (B) 3-epi-25-(OH)-D3 (C) 25-(OH)-D2 (D) 3-epi-25-(OH)-D2
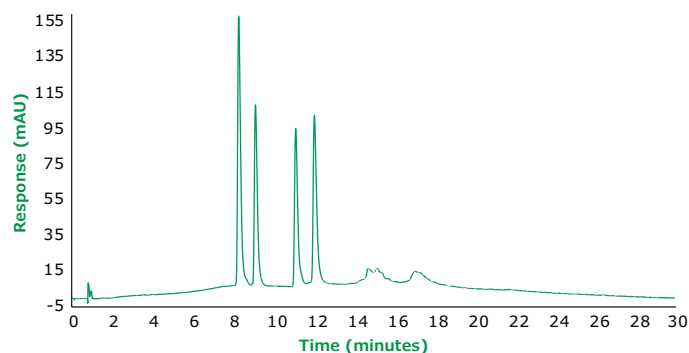
Figure 9. Separation of 25-Hydroxyvitamin D2 and D3 and Their Epimers on Supel™ Carbon LC. Elution order:
1) 3-epi-25-(OH)-D3 – 8.294 min 2) 25-(OH)-D3 – 9.125 min
3) 3-epi-25-(OH)-D2 – 11.126 min 4) 25-(OH)-D2 – 12.062 min

For this reason, an application is required to resolve all four of the compounds. PGC has shown to be effective at resolving both vitamin D2 and D3 as well as the epimer analogues. By utilizing a simple gradient with strong organic mobile phases, all four compounds can be fully resolved in a reasonable analysis time (**Figure 9**).

Summary

Porous graphitic carbon is a novel stationary phase and gives the chromatographer an additional chemistry option in the separation of challenging compounds, beyond the realm of conventional silica-based reversed-phase chromatography. While in many respects, PGC may behave like a reversed-phase column, it also offers the advantages of enhanced temperature, solvent, and pH stability. Moreover, because of the unique properties of graphite, highly polar compounds (that may need HILIC or ion-exchange conditions) can be retained on a PGC column. Although its retention mechanisms are yet to be elucidated, it is clear that PGC has unique retentive properties towards polar compounds – especially planar molecules or analytes with double bond conjugation that can interact with the electron cloud of graphite. PGC is a unique stationary phase amongst more conventional HPLC stationary phases and further advancements in PGC particle design may result in even better resolving power for a wider range of compounds.

References:

1. Knox, J. and Ross, P. *Advances in Chromatography*, 1997, 37, 73-119

2. C. West, C. Elfakir, M. Lafosse. *Journal of Chromatography*, A, 2010, 1217 3201–3216.

3. H. J. Möckel, A. Braedikow, H. Melzer & Gaby Aced A Comparison of the Retention of Homologous Series and Other Test Solutes on an Ods Column and a Hypercarb Carbon Column, *Journal of Liquid Chromatography*, 1991, 14:13, 2477-2498

4. Nobou Tanaka, Tetsuya Tanigawa, Kazuhiro Kimata, Ken Hosoya, Takeo Arai, Selectivity of carbon packing materials in comparison with octadecylsilyl- and pyrenylethylsilylsilica gels in reversed-phase liquid chromatography, *Journal of Chromatography*, A, 1991, Volume 549, 29-41

5. Lutsey PL, Eckfeldt JH, Ogagarue ER, Folsom AR, Michos ED, Gross M. The 25-hydroxyvitamin D3 C-3 epimer: distribution, correlates, and reclassification of 25-hydroxyvitamin D status in the population-based Atherosclerosis Risk in Communities Study (ARIC). *Clin Chim Acta.,* 2015 Mar 10, 442, 75-81. doi: 10.1016/j.cca.2014.12.036. Epub 2015 Jan 9. PMID: 25578393; PMCID: PMC4339618.

6. Karras, S. N., Kotsa, K., Angeloudi, E., Zebekakis, P., & Naughton, D. P. The Road Not So Travelled: Should Measurement of Vitamin D Epimers during Pregnancy Affect Our Clinical Decisions?. *Nutrients,* 2017, 9 (2), 90. https://doi.org/10.3390/nu9020090

7. Shah, I., James, R., Barker, J. *et al*. Misleading measures in Vitamin D analysis: A novel LC-MS/MS assay to account for epimers and isobars. *Nutr J*, 2011, 10, 46 https://doi.org/10.1186/1475-2891-10-46

Featured Products

Description:	Cat. No.
HPLC Columns	
Supel™ Carbon LC, 10 cm x 2.1 mm, 2.7 µm	**59986-U**
Supel™ Carbon LC, 5 cm x 3.0 mm, 2.7 µm	**59991-U**
Ascentis® Express C18, 5 cm x 3.0 mm, 2.7 µm	**53811-U**
Ascentis® Express HILIC, 10 cm x 2.1 mm, 2.7 µm	**53939-U**
Solvents, Reagent & Standards	
Water, from an Milli-Q® IQ water system	**ZIQ7003T0**
Water for UHPLC-MS LiChrosolv®	**1.03728**
Acetonitrile, gradient grade for HPLC, Sigma-Aldrich™	**34851-4L**

Description:	Cat. No.
Acetonitrile for UHPLC-MS LiChrosolv®	**1.03725**
2-Propanol for HPLC, 99.5% , Sigma-Aldrich™	**439207-4L**
Tetrahydrofuran for HPLC, ≥99.9%, inhibitor-free, Sigma-Aldrich™ *	**439215-4L**
Ethyl alcohol, Pure - 200 proof, HPLC/spectrophotometric grade*	**459828-4L**
Ammonium Hydrogen Carbonate for LC-MS LiChropur™	**5.33005**
Ammonium Hydroxide, 28% NH₃ in water, ≥ 99.99% Trace Metals Basis	**338818**
Triethylammonium acetate buffer for HPLC, 0.98-1.02 M	**69372**
Difluoroacetic Acid, 98%, Sigma-Aldrich™	**142859-5G**
Diquat dibromide monohydrate, PESTANAL®, analytical standard, 250 mg	**45422**
Paraquat dichloride hydrate, PESTANAL®, analytical standard, 100 mg	**36541**
25-Hydroxyvitamin D2 solution 50 µg/mL in ethanol, ampule of 1 mL, certified reference material, Cerilliant®	**H-073**
3-epi-25-Hydroxyvitamin D2 solution, 100 µg/mL in ethanol, 98% (CP)	**753556**
25-Hydroxyvitamin D3 solution 100 µg/mL in ethanol, ampule of 1 mL, certified reference material, Cerilliant®	**H-083**
3-epi-25-Hydroxyvitamin D3 solution, 50 µg/mL in ethanol, certified reference material, Cerilliant®	**E-086**

*(not in all countries available, see SigmaAldrich.com for alternatives)

Related Products

Description:	Cat.No.
Reagents	
2-Propanol hypergrade for LC-MS LiChrosolv®	**1.02781**
Difluoroacetic acid for LC-MS LiChropur™, ≥97.5% (GC)	**00922**
25-Hydroxyvitamin D calibration solutions, NIST® SRM® 2972a	**NIST2972A**

Supel™ Carbon LC Column

Unique Retention and Method Development Options

Based on a porous graphitic carbon (PGC) material, this new HPLC Column provides distinct application advantages over classical silica:

- Retention of very polar compounds e.g. pesticides and amino acids (without need for HILIC conditions)
- Temperature stability up to 250 °C
- pH stability (1-14)
- Unique retention mechanism
- Compatibility with any solvent
- Unique shape selectivity

SigmaAldrich.com/carbonLC

Supelco®
Analytical Products

New Reference Materials for Extractables & Leachables Testing

Expanding our E&L portfolio including a reference material for Irganox 1010 degradation product 7,9-Di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione

Matthias Nold, Product Manager Reference Materials, Analytix@merckgroup.com
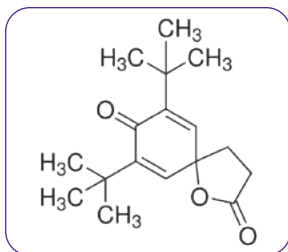

Figure 1. 7,9-Di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione (Cat. No. **78478**)

In previous issues of Analytix Reporter we introduced new neat reference materials (issue 6) and two calibration mixes for extractables and leachables testing (issues 7 & 8). Now we present a further expansion of our portfolio of reference materials for extractables and leachables.

Extractables and leachables (E&L) are chemical substances that can potentially migrate from polymers of packaging materials, tubings or medical devices into a pharmaceutical product and affect safety and quality of a product. That's why extensive extractables and leachables studies are required from manufacturers to show that their products cannot expose patients to harmful amounts of such chemicals. Extractables and Leachables are also relevant in Food Safety testing, where they are covered by Food Contact Materials regulations.

To facilitate identification and quantification of extractables and leachables, we offer a comprehensive portfolio of reference materials. A list of more than 100 certified reference materials and analytical standards for commonly found extractables and leachables can be found on our website at
SigmaAldrich.com/extractablesandleachables

This portfolio is now complemented by 18 new reference materials including several alkanes that are frequently detected in extractables and leachables studies by GC. Also among the new products is the 7,9-Di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione, CAS: 82304-66-3 (**Figure 1**) which is a probably genotoxic degradation product of polymer additive Irganox 1010, and which has been reported to be found in drug products[1] as well as in mineral water.[2]

All these products are certified by quantitative NMR (qNMR) in accordance to ISO 17025. The quantitative values are traceable to NIST SRM and the stated uncertainty is taking into account stability and homogeneity of the material.

Newly Added Reference Materials for Extractables and Leachables Testing

| Description	CAS	Qty.	Cat. No.
Benzophenone	119-61-9	100mg	**78274**
Bis(2,4-di-tert-butylphenyl) phosphate	69284-93-1	100mg	**90048**
1-Decene	872-05-9	100mg	**78270**
Didodecyl 3,3'-thiodipropionate	123-28-4	100mg	**78132**
7,9-Di-tert-butyl-1-oxaspiro[4.5] deca-6,9-diene-2,8-dione	82304-66-3	50mg	**78478**
Docosane	629-97-0	100mg	**78290**
Eicosane	112-95-8	100mg	**78292**
Ethyl 4-ethoxybenzoate	23676-09-7	100mg	**78699**
Ethylene glycol butyl ether	111-76-2	100mg	**78263**
Hexadecane	544-76-3	100mg	**78293**
Isophorone	78-59-1	100mg	**78345**
Isovaleric acid	503-74-2	100mg	**78266**
Octadecane	593-45-3	100mg	**78294**
1-Octadecene	112-88-9	100mg	**78295**
1-Octene	111-66-0	100mg	**78340**

References

1. Singh et al. Identification of leachable impurities in an phthalmic drug product originating from a polymer additive Irganox 1010 using mass spectroscopy", Journal of Pharmaceutical and Biomedical Analysis, Vol. 152, 2018, 197 - 203.

2. BfR Opinion Nr. 007/2011, 2 February 2011; BfR assesses analyses of substances with hormone-like activity in natural mineral waters.

Protein Binding Determination - Comparison Study of Techniques & Devices

Supel™ BioSPME vs Rapid Equilibrium Dialysis

M. James Ross, Senior R&D Scientist; Olga Shimelis, R&D Manager Sample Preparation; Candace Price, Product Manager Sample Prep - SPE and BioSPME, Analytix@merckgroup.com

Introduction
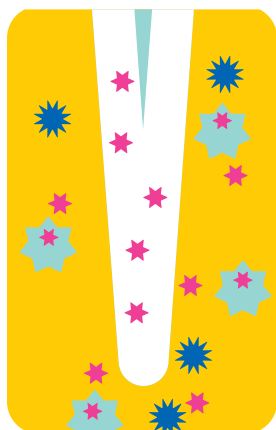
An important aspect of drug discovery is understanding the interaction of the drug candidate with plasma proteins and lipids. The binding of drugs to the proteins and lipids is referred to as the plasma protein binding (PPB or F_b). The molecular attributes of a drug can provide useful insights into its number of interactions with the protein. In general, organic acids have a single binding site with albumin, whereas, organic bases have multiple bindings sites associated with glycoproteins.[1] In addition to albumin, other proteins commonly associated with drug binding are alpha-1-acid glycoprotein (AAG) and lipoproteins, such as very high-density lipoprotein (VHDL) and low-density lipoprotein (LDL).[2] When measuring the pharmacologic potency of a drug, it is the free fraction or unbound fraction (F_u) of a drug that is generally considered responsible for its activity as described by the free drug hypothesis.[3,4,5]

Determining the protein binding properties of a drug is important to understand the amount of free drug available in the blood. So far, equilibrium membrane dialysis has been the traditional technique used to measure drug protein binding. The technique involves equilibration of a drug rich plasma sample with a drug-free buffer across a membrane. This allows the free drug to migrate across the membrane and prevents the protein bound drug from moving into the buffer. This equilibrium takes more than 24 hours to establish. Other techniques such as rapid equilibrium dialysis further reduce the workflow time from >24 hours to 6 hours by using specifically designed devices.

In this study, the Supel™ BioSPME 96-Pin device is used to measure drug protein binding. Supel™ BioSPME 96-Pin devices have been developed using solid phase microextraction, SPME, technology to extract free unbound analytes from biological fluids. These devices consist of a 96-pin plate, with the tips of pins coated with a thin layer of adsorbent particles. The patented binder within the coating allows the small analytes of interest to bind, while preventing macromolecules from binding. This enables a robust, selective, and non-exhaustive extraction of free analytes, having both qualitative and quantitative applications. The 96-pin configuration allows direct sampling from 96 well plates and is compatible with robotic liquid handling systems, providing a fully automated high-throughput methodology.

In this study, the Supel™ BioSPME 96-Pin device workflow is compared with a rapid equilibrium dialysis technique to measure drug protein binding. A series of compounds with molecular weights in the range of 230-750 Da and a hydrophobicity (LogP) range of 1.5-5 were utilized for comparison of protein binding values, and determined using each technique. In addition to measured values and accuracy, the study compared overall sample cleanliness and workflow time of each technique.

Experimental

Determination of Protein Binding using Supel™ BioSPME 96-Pin Device

Human plasma and phosphate buffered saline (PBS) were spiked at a therapeutically relevant concentration and incubated for one hour at 37 °C while shaking at 300 rpm. After the incubation, 200 µL of plasma and PBS were loaded into wells in separate columns of an extraction well plate (n = 8). The protein binding determination workflow using the Supel™ BioSPME 96-Pin device was conducted with an automated robotic liquid handling system. Briefly described in **Figure 1**, the pin device is statically (without shaking) conditioned for twenty minutes in isopropanol, then transferred into a new well plate containing water for 10 seconds (wash step). This is followed by the extraction step, where the pin device is transferred into the preloaded extraction well plate and the analyte extraction takes place while shaking at 1200-1250 rpm at 37 °C for 15 minutes. The pin device is returned to the water solution for a 60 second wash and then transferred into a desorption well plate for the final step. The desorption solution is a mixture of
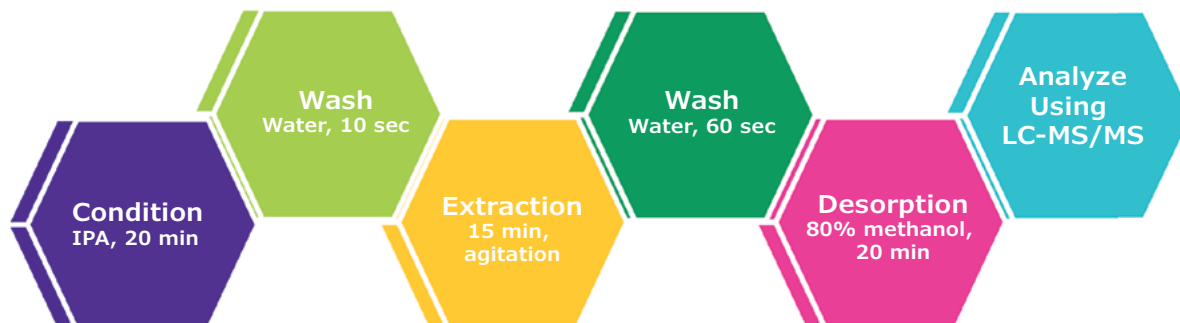
Figure 1. Overview of the Steps in Determining the Free Fraction of Drug in Human Plasma by Supel™ BioSPME 96-Pin Device.

80:20 methanol:water, and the pin device undergoes desorption for 20 minutes under static conditions. **Figure 2** shows the Supel™ BioSPME 96-Pin device being maneuvered by the automated liquid handing system gripper. **Figure 3** represents the extraction of free unbound analyte onto the Supel™ BioSPME 96-Pin device. The amount extracted does not greatly impact the concentration of free analyte, and is termed non-depletive. As the buffer solution is considered 100% free, the Supel™ BioSPME 96-Pin device extracts more from buffer than from plasma.

Figure 2. Supel™ BioSPME 96-Pin Device Maneuvered by Automated Liquid Handing System Gripper.
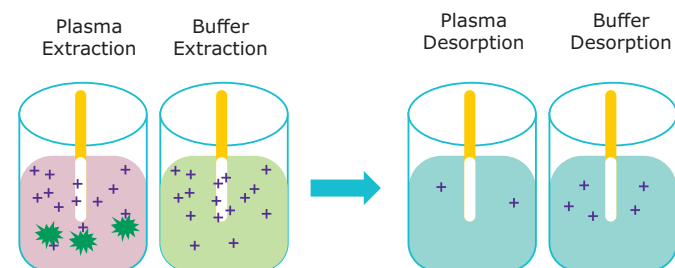
Figure 3. Representation of the extraction step (left) removing free analytes from plasma (pink) and buffer (green) and the analytes releasing into the desorption solution (blue).

(dark green = proteins, purple = analyte)

The extraction plates used in this study included both plastic and glass-coated. The choice of the plate depended on the properties of the compound and how well the compound behaved in buffer solution. More hydrophobic compounds, such as ketoconazole and imipramine were found to exhibit non-specific binding to plastic and thus had better extraction efficiency from glass-coated 96-well plates. Extractions of erythromycin and propranolol were also performed using glass-coated plates because of their higher extraction efficiency values, in comparison to extraction from plastic plates.

Protein Binding Determination by Rapid Equilibrium Dialysis

Rapid equilibrium dialysis was performed as directed by the product instruction sheet. Two hundred microliters of human plasma "spiked" at a therapeutically relevant concentration and 400 µL of phosphate buffered saline (PBS) were loaded into corresponding chambers of the rapid equilibrium dialysis device, in at least triplicate sets. Dialysis proceeded for at least 4 hours while covered and shaking at 300 rpm and 37 ºC on an Eppendorf® shaker. At the end of dialysis, 50 µL of the spiked plasma was mixed with 50 µL of clean (unspiked) PBS, and 50 µL of the dialysate (buffer compartment) was mixed with 50 µL of clean plasma. This was done to ensure matrix consistency. Next, protein precipitation was initiated with the addition of 300 µL of ice-cold acetonitrile to each sample before centrifugation at 5,000 rpm for 10 minutes at 4 ºC. Finally, the supernatant was transferred into glass vials for analysis by LC-MS/MS.

Samples prepared using both the Supel™ BioSPME 96-Pin device and the rapid equilibrium dialysis device were analyzed by LC-MS/MS. The chromatographic and mass spectrometric analyses were performed on an Agilent 1290 / AB Sciex 6500 LC-MS/MS system following the conditions described in **Table 1**. Quantitation was performed using an external calibration in the desorption solution.

Table 1. LC-MS/MS Conditions for Monitoring Analytes in Protein Binding Determination

column:	Ascentis® Express Biphenyl 10 cm x 2.1 mm, 2.7 µm (**64065-U**)
mobile Phase:	[A] 5 mM ammonium acetate, 0.1% acetic acid in 95% water and 5 % acetonitrile
	[B] 5 mM ammonium acetate, 0.1% acetic acid in 95% acetonitrile and 5% water

gradient:	Time (min)	A (%)	B (%)
	0.0	90	10
	0.5	90	10
	3.0	10	90
	5.0	10	90
	5.1	90	10
	7.1	90	10

flow rate:	0.4 mL/min
column temp:	40 °C
detector:	MS, ESI(+) Scheduled MRM (MRM details can be requested from the author)
injection:	dependent upon analyte; 5 – 20 µL

Results & Discussion

The Supel™ BioSPME method determines the free concentration of analyte in plasma by comparing it with the extraction of the same analyte from buffer samples; where 100% of the analyte is considered to be free of protein binding. Supel™ BioSPME 96-pin devices were directly compared with a rapid equilibrium dialysis technique, as it is often considered the standard approach and workflow of choice for plasma protein binding determination. Supel™ BioSPME 96-pin devices show numerous advantages over the rapid equilibrium dialysis technique, in terms of time savings, sample cleanliness, and a simplified workflow; while
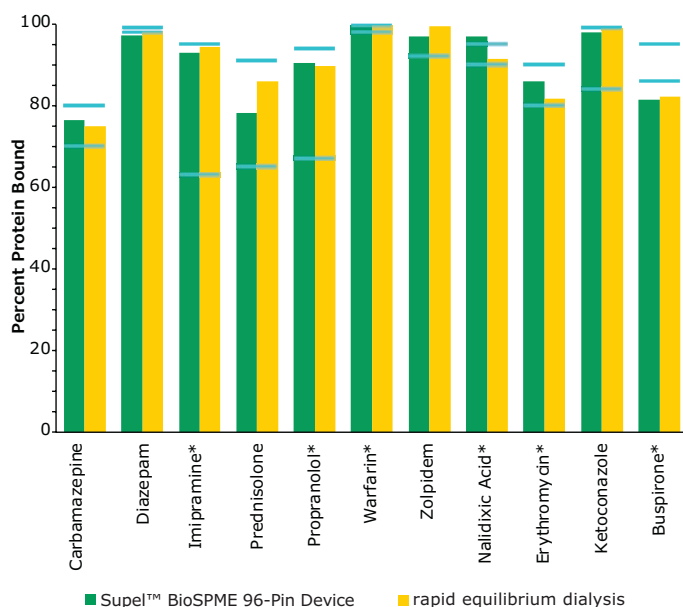
Figure 4. Comparison of Protein Binding Values Between Supel™ BioSPME 96-Pin Device and Rapid Equilibrium Dialysis Methods. The blue lines indicate the published protein binding literature value intervals. Compounds with asterisks are charged at physiological pH.

still maintaining the same high standards of accuracy and reproducibility needed by the bioanalytical laboratories performing this analysis. A comparison of the protein binding values obtained using the rapid equilibrium dialysis method and Supel™ BioSPME method is shown below in **Figure 4**.

The data from **Figure 4** is shown in tabular form in **Table 2**. The values from the BioSPME method are in good agreement with values determined using rapid equilibrium dialysis devices and the reported literature values.

Table 2. Protein Binding Values (F_B) for Nine Compounds from Plasma Using Supel™ BioSPME at a Sample Volume of 200 µL (n=8).

Analyte	Concentration Spiked (ng/mL)	Supel™ BioSPME F_B(%)	rapid equilibrium dialysis F_B (%)	Literature Values* F_B (%)
Carbamazepine	100	76.4	75.0	70-80%
Diazepam	100	97.3	98.2	98-99%
Imipramine	100	92.6	94.4	63-95%
Prednisolone	100	78.2	86.1	65-91%
Propranolol	100	90.5	89.7	67-94%
Warfarin	2500	99.8	99.7	98.1-99.6%
Zolpidem	100	96.9	99.5	92%
Nalidixic Acid	2000	97.0	91.4	90-95%
Erythromycin	500	81.8	81.7	90%
Ketoconazole	500	96.8	99.0	84-99%
Buspirone	100	81.6	82.3	86-95%

* Values obtained from peer reviewed journals and/or listed on the FDA drug label. Sources available from the author upon request.

Comparison of Workflow Time: Supel™ BioSPME Device vs Rapid Equilibrium Dialysis

As high throughput laboratories are always interested in optimizing efficiencies as much as possible, the time to perform each one of the workflows was evaluated for comparison purposes. The Supel™ BioSPME 96-Pin device workflow (<2 hours) takes one third of the amount of time the rapid equilibrium dialysis workflow (6 hours) takes, as shown in **Table 3**. This provides the opportunity to increase throughput three-fold, allowing the liquid handling instrument to be used for other assays, and giving the scientist time for other projects.

Table 3. Comparison of Time Requirement by Method

Supel™ BioSPME Method		Rapid Equilibrium Dialysis Method	
Step	Time (min)	Step	Time (min)
Sample Prep	60	Sample Prep	60
Condition	20	Dialysis	240
Wash	0.2	Post sample preparation	40
Extraction	15	Centrifugation	10
Wash	1	Transfer into vials for analysis	10
Desorption	15		
Total	<2 Hours	Total	6 Hours

Conclusion

The Supel™ BioSPME 96-Pin device technique has shown significant time savings for protein binding determination when compared with the rapid equilibrium dialysis method. Since the workflow for the Supel™ BioSPME 96-Pin device is less than two hours compared to the rapid equilibrium dialysis method, it triples the throughput. In addition, the format of the Supel™ BioSPME 96-Pin device allows for a fully automated robotic method, without the need for additional hardware, i.e. a centrifuge, as required when performing the rapid equilibrium dialysis method. This translates to increased productivity and reduced manual steps for the laboratory. The accuracy of the protein binding values obtained using the Supel™ BioSPME 96-Pin device are in agreement with those obtained using the rapid equilibrium dialysis method; as demonstrated with 10 compounds with varying LogP values. The patented binder and coating of the Supel™ BioSPME 96-Pin device allows for selective extraction of target analytes, while excluding larger macromolecules, to provide a fast and accurate drug protein binding measurement.

References

1. Cervelli, M., & Russ, G. - Principles of Drug Therapy, Dosing, and Prescribing in Chronic Kidney Disease in Renal Replacement Therapy. Comprehensive Clincial Nephrology (Fourth Edition), Chapter 73, 871-893(2010), https://doi.org/10.1016/B978-0-323-05876-6.00073-3.

2. Scheife, R. (1989; 23). Protein binding: what does it mean? DICP, https://doi.org/10.1177/106002808902300706.

3. Eichel, E. Protein binding changes and drug interactions: What do we know? Current Psychiatry. 2018 October;17(10):38-41

4. Smith, D., Di L, & Kerns, E. The effect of plasma protein binding of in vivo efficacy: misconceptions in drug discovery. Nature Reviews Drug Discovery volume 9, 929–939 (2010), https://doi.org/10.1038/nrd3287.

5. Wanat, K. Biological barriers, and the influence of protein binding on the passage of drugs across them Molecular Biology Reports, 47, pages 3221–3231 (2020) https://doi.org/10.1007/s11033-020-05361-2.

6. Hug, M., Tascon, M., Nazdrajic, E., Rozkowska, A., & Pawliszyn, J. Measurement of free Drug Concentration from Biological Tissue by Solid-Phase Microextraction: In Silico and Experimental Study. Analytical Chemistry, 7719-7728 (2019).

See the full application notes at
SigmaAldrich.com/BioSPME

Featured Products

Description	Cat. No.
BioSPME & HPLC	
Supel™ BioSPME 96-Pin device	**59680-U**
Ascentis® Express Biphenyl column 10 cm x 2.1 mm, 2.7 µm	**64065-U**
2-Propanol hypergrade for LC-MS LiChrosolv®	**1.02781**
Water, for chromatography (LC-MS Grade) LiChrosolv®	**1.15333**
Methanol, hypergrade for LC-MS LiChrosolv®	**1.06035**
Phosphate buffered saline BioPerformance Certified, pH 7.4	**P5368**
Reference Materials (CRMs all Cerilliant®, except PHR1039)	
Carbamazepine solution, 1.0 mg/mL in methanol, CRM, 1 mL	**C-053**
Carbamazepine-d10, 100 µg/mL in methanol, CRM, 1 mL	**C-094**
Diazepam solution, 1.0 mg/mL in methanol, CRM, 1 mL	**D-907**
Diazepam-d5,100 µg/mL in methanol, CRM, 1 mL	**D-910**
Imipramine hydrochloride solution, 1 mg/mL (as free base) in methanol, CRM, 1 mL	**I-902**
Imipramine-d3 maleate, 100 µg/mL (as free base) in methanol, CRM, 1 mL	**I-903**
Prednisolone solution, 1.0 mg/mL in acetonitrile, CRM, 1 mL	**P-121**
Propranolol solution, 1.0 mg/mL in methanol (as free base), CRM, 1 mL	**P-055**
Warfarin solution, 1.0 mg/mL in acetonitrile, CRM, 1 mL	**W-003**
Zolpidem solution, 1.0 mg/mL in methanol, CRM, 1 mL	**Z-017**
Nalidixic acid analytical standard, 100 mg	**97023**
Erythromycin, Pharmaceutical Secondary Standard, CRM, 1 g	**PHR1039**
Accessories	
Holder for Supel™ BioSPME 96-Pin device	**59686-U**
Corning® 96 Well Storage Microplate V-bottom clear, polypropylene, bag of 25 ×, sterile, lid: no, Pk.100	**CLS3357**
Nunc® 96 DeepWell™ plate, non-treated, U-bottom natural polypropylene wells, maximum volume 1.3 mL, non-sterile, Pk.50	**P8241**
Seal Plate Film, non-sterile, Pk.100	**Z369659-100EA**

Supel™ BioSPME 96-Pin Devices

For a 3x **Faster Plasma Protein Binding Study** Workflow compared to Rapid Equilibrium Dialysis Methods.

SigmaAldrich.com/BioSPME

Analysis of Fingolimod Hydrochloride According to USP Monograph Guidelines

Unites States Pharmacopeia (USP43-NF38) Monograph

In this article, a rapid, accurate, and simple method is presented for the total chromatographic purity analysis of fingolimod hydrochloride using High Performance Liquid Chromatography equipped with a Diode Array Detector (HPLC-DAD). The experimental conditions follow guidelines, with minor, but allowed modifications from the USP43-NF38 monograph methods for the assay and organic impurity profiling of fingolimod hydrochloride. Using the 33-minute gradient method from USP and a Purospher® STAR RP-18 HPLC column (150 x 3.0 mm, 3 µm), baseline separation of fingolimod and its impurities was achieved. A 0.1% solution of phosphoric acid in water and acetonitrile were used as the mobile phase for the gradient elution.

Chemical Structures of Fingolimod and its Impurities
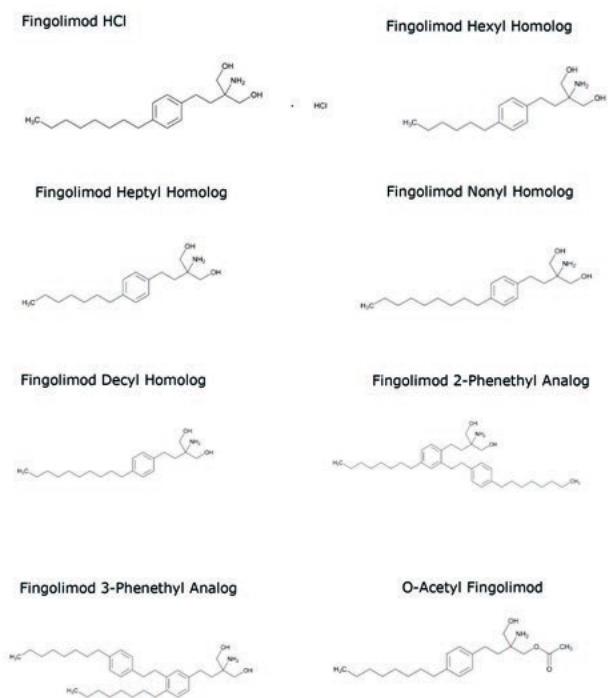
Experimental

Conditions	
column:	Purospher® STAR RP-18 Hibar RT (3µm) 150x3.0 mm
detection:	UV @ 215 nm (DAD)
mobile phase:	[A]: 0.1% phosphoric acid in water [B]: acetonitrile
gradient:	

Time (min)	A (%)	B (%)
0	80	20
20	5	95
23	5	95
23.1	80	20
33	80	20

flow rate:	0.8 mL/min
pressure:	135-300 bar
temperatures:	column: 40 °C; autosampler: 10 °C
injection volume:	5 µL

Sample	
diluent:	mobile phase A:B (50:50)
test solution:	dissolve 15 mg of USP Fingolimod Hydrochloride RS in 25 mL diluent (0.6 mg/mL).
system suitability solution:	dissolve 15.0 mg of USP Fingolimod for System Suitability using 25 mL diluent (0.6 mg/mL).
standard solution:	dilute 1.0 mL of the test solution to 100.0 mL using mobile phase further dilute 1.0 mL of this solution to 10.0 mL using the mobile phase (0.003 mg/mL)
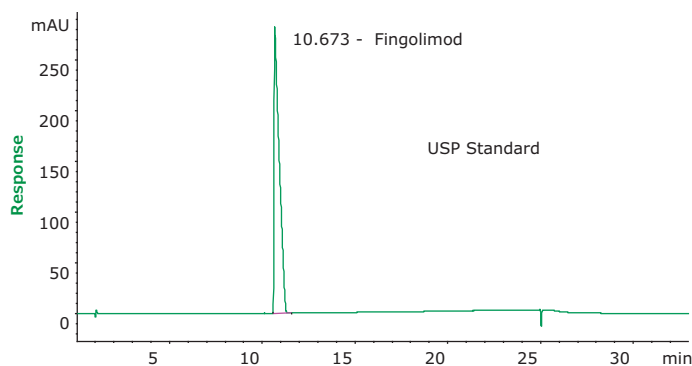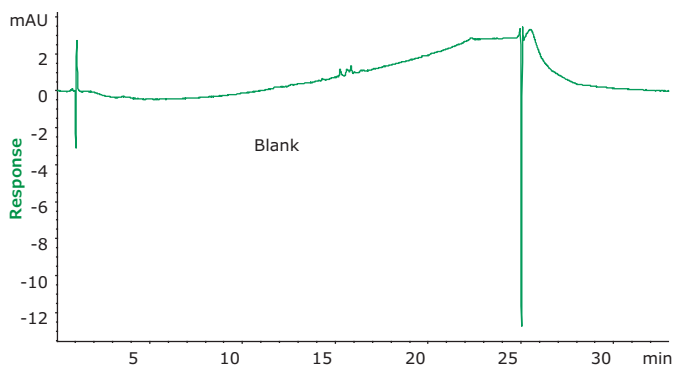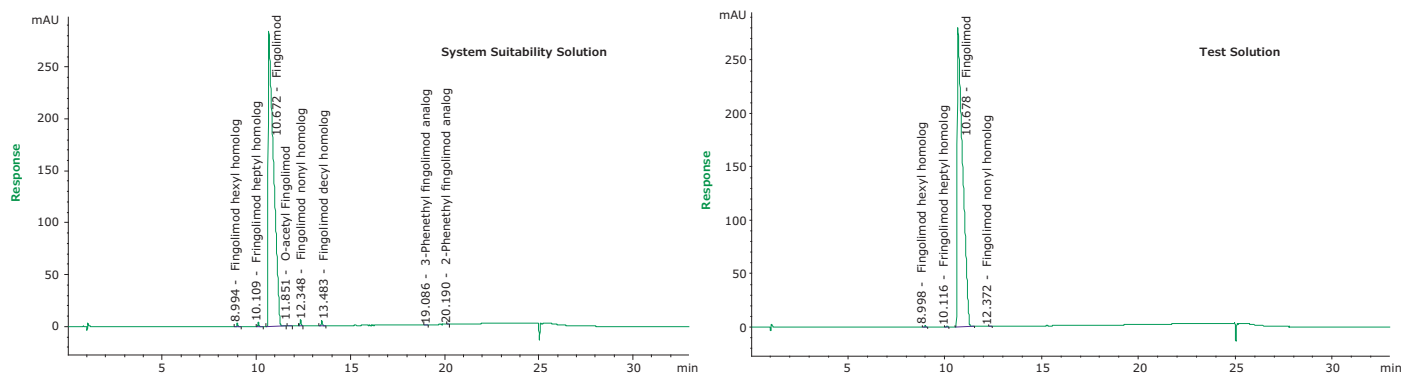
Specificity (System Suitability Solution)

Peaks	Compound	Retention Time (min)	RRT	Requirement (USP43-NF38)	Resolution	Requirement (USP43-NF38)	Tailing	Requirement (USP43-NF38)
1	Fingolimod Hexyl Homolog	9.0	0.84	0.82	-	-	1.0	-
2	Fingolimod Heptyl Homolog	10.1	0.94	0.93	11.6	-	1.1	-
3	Fingolimod	10.7	1.00	1.00	2.1	-	4.4	Not more than (NMT) 5
4	O-Acetyl Fingolimod	11.8	1.10	-	3.0	-	0.7	-
5	Fingolimod Nonyl Homolog	12.3	1.15	1.13	4.2	1.2 (between peak 4 and 5)	1.2	-
6	Fingolimod Decyl Homolog	13.5	1.26	1.23	9.8	-	1.4	-
7	3-Phenethyl Fingolimod Analog	19.8	1.85	1.97	42.1	-	0.8	-
8	2-Phenethyl Fingolimod Analog	20.2	1.89	2.00	2.8	0.8 (between peak 7 and 8)	1.2	-

Repeatability (System Suitability Solution)

Peaks	Compound	Area Response (N=3)	Standard Deviation	RSD (%)	RSD (%) (USP43-NF38)
3	Fingolimod	5496.1	2.3	0.04	NMT 0.73%

Conclusion

A rapid, accurate, and simple method for the total chromatographic purity analysis of Fingolimod hydrochloride by HPLC-DAD was developed, well within the boundaries of the USP43-NF38 monograph methods. The applied conditions met the system suitability criteria, and the method demonstrated good resolution/selectivity, reproducibility, and sensitivity.

See our complete offering for HPLC at
SigmaAldrich.com/HPLC

Featured Products

Description	Cat. No
Purospher® STAR RP-18 Hibar RT 15 cm x 3.0 mm, 3 µm	**1.50414**
Acetonitrile gradient grade for liquid chromatography LiChrosolv® Reag. Ph Eur	**1.00030**
Water for chromatography (LC-MS grade) LiChrosolv® or tap fresh from an appropriate Milli-Q® ultrapure water system	**1.15333**
Phosphoric Acid for HPLC, LiChropur™, 85%	**49685**
Fingolimod Hydrochloride United States Pharmacopeia (USP) Reference Standard	**1270480**
Fingolimod System Suitability Mixture United States Pharmacopeia (USP) Reference Standard	**1270526**
Syringe Filter Millex-HV Durapore® (PVDF) membrane, hydrophilic, Non-sterile, 0.45 µm pore size, 13 mm diameter,	**SLHVX13NK**

Purospher® STAR HPLC and UHPLC Columns

Excellent Peak Symmetry, Reproducibility, and a pH Stability from pH 1.5 to 10.5 – Column-to-Column & Lot-to-Lot.

SigmaAldrich.com/HPLC

LC-MS Analysis of Serum for a Wide Analyte Range Using a Novel HLB SPE Phase

M. James Ross, Senior Scientist, Analytix@merckgroup.com

Abstract

In this study, a mixture of twenty compounds, the majority pharmaceuticals, with LogP values ranging from -0.89 to 4.65 were analyzed in calf serum, highlighting the broad utility of the new hydrophilic lipophilic balanced (HLB) SPE phase, Supel™ Swift HLB.

Introduction

The preparation of biological samples can have a large impact on the reproducibility and accuracy of their analytical results.[1] But, solid phase extraction (SPE) provides an opportunity to reduce matrix effects such as ion suppression and aid in the reliability of consistent results. Hydrophilic-Lipophilic Balanced (HLB) cartridges contain a sorbent offering good wettability for hydrophilic compounds, in addition to providing reverse phase retention.[2] These properties allow HLB cartridges to effectively handle a broad range of compounds with varying properties. In this study, a mixture of twenty example compounds with LogP values ranging from -0.89 to 4.65 were analyzed from spiked calf serum using a Supel™ Swift HLB SPE cartridge for cleanup and LC-MS determination.

Experimental

A series of twenty analytes and sixteen internal standards as listed in **Table 1** were spiked into calf-serum and allowed to equilibrate for an hour. The calf-serum sample was diluted with an equal volume of 0.4% aqueous formic acid and mixed before the sample was loaded onto the Supel™ Swift HLB SPE cartridge (1 mL/30 mg), and another commercially available HLB cartridge (1 mL/30 mg) for comparison.

Table 1. The Twenty Analytes Listed in Order of Elution and 16 Internal Standards (if applicable) Analyzed in Calf-Serum by LC-MS/MS

Analyte	Usage	LogP	RT (min)	MRM Quant.	MRM Qual.	Internal Standard Analyte	RT (min)	Monitored Transition
Nizatidine	antacid	0.77	1.36	332.1/155.1	332.1/131.1	na	-	-
Amiloride	diuretic	-0.89	2.80	230.0/171.0	230.0/115.9	5-(N,N-dimethyl) amiloride	5.24	258.0/199.0
Benzoylecgonine	cocaine metabolite	-0.59	3.28	290.1/168.0	290.1/105.0	Benzoylecgonine-D3	3.28	293.1/171.0
Imidacloprid	insecticide	0.87	4.16	256.0/208.9	256.0/175.0	Imiacloprid-D4	4.16	260.0/213.0
Mirtazapine	antidepressant	3.21	4.60	266.2/195.1	266.2/166.9	N-desmethylmirtazapine	4.6	252.1/195.1
Nevirapine	HIV antiviral	2.49	4.98	267.1/226.1	267.1/107.1	Abacavir	3.56	287.2/191.1
Methapyrilene	antihistamine	3.11	4.99	262.2/217.0	262.2/107.1	Methapyrilene-dimethyl-D6	4.99	268.2/217.0
Imiquimod	anti-tumor	2.65	5.16	241.1/185.1	241.1/167.9	na	-	-
Buspirone	anxiolytic	1.78	5.36	386.2/122.0	386.2/95.1	Buspirone-D8	5.36	384.2/122.0
Hydroquinidine	antiarrhythmic	2.82	5.40	327.3/172.2	327.3/160.2	Quinine	5.12	325.2/172
Mesoridazine	neuroleptic drug	3.57	5.72	387.1/98.2	387.1/126.2	Chlorpromazine	7.6	319.1/246.0
Mianserin	antihistamine	3.83	6.23	265.2/208.2	265.2/193.1	Mianserin-D3	6.23	268.2/208.2
Haloperidol	antipsychotic	3.66	6.80	367.1/123.2	367.1/165.2	Haloperidol-D4	6.9	380.1/127.2
Imipramine	antidepressant	4.28	6.93	281.1/86.0	281.1/165.1	Imipramine-D3	6.93	284.1/89.0
Atrazine	herbicide	2.2	7.13	216.1/174.0	216.1/68.0	Atrazine-D5	7.14	221.1/179.0
Amitriptyline	antidepressant	4.81	7.17	278.1/191.0	278.1/233.2	Amitriptyline-D3	7.17	281.1/191.0
Clarithromycin	antibiotic	3.24	7.50	748.5/590.1	748.5/158.0	na	-	-
Losartan	antihypertensive	4.06	7.50	423.2/207.2	423.2/235.2	na	-	-
Nefazodone	antidepressant	4.65	7.91	470.2/274.2	470.2/246.2	MCPP-D8*	4.21	205.2/157.8
Loratadine	antihistamine	4.55	10.51	383.3/337.2	383.1/267.2	Loratadine-D5	10.51	388.1/337.2

*MCPP-D8 stands for 1-(3-Chlorophenyl)piperazine-D8

Recovery and Ion Suppression/Enhancement

The analytes were monitored at two different transitions of quantifier and qualifier (for confirmation), by a scheduled MRM method. Samples were analyzed against matrix-matched calibration curves. The external standard calibration used six concentrations of the analytes between 20 to 150 ng/mL and a fixed concentration of 50 ng/mL for the internal standards when applicable.

Analytes were spiked in triplicates into calf-serum at concentrations of 100 ng/mL, with a fixed internal

standard of concentration 50 ng/mL, before their processing by either the 3-step or 5-step method as shown in **Figure 1**. The samples were dried and resuspended in starting mobile phase before duplicate analysis by LC-MS/MS on an Agilent 1290 Infinity LC attached to a Sciex 3200 QTRAP® system. The LC conditions including the starting mobile phase are listed in **Table 2**. A representative trace from recovery is shown in **Figure 2**.
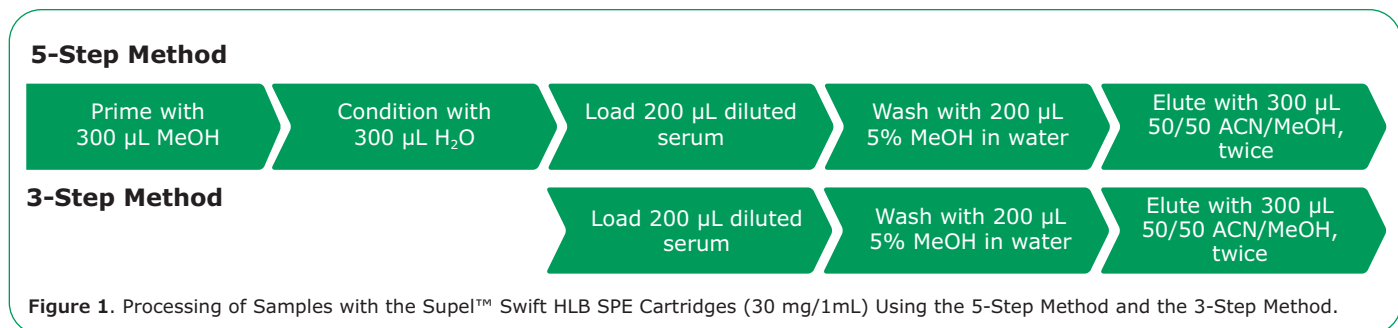
5-Step Method

Prime with 300 μL MeOH → Condition with 300 μL H$_2$O → Load 200 μL diluted serum → Wash with 200 μL 5% MeOH in water → Elute with 300 μL 50/50 ACN/MeOH, twice

3-Step Method

Load 200 μL diluted serum → Wash with 200 μL 5% MeOH in water → Elute with 300 μL 50/50 ACN/MeOH, twice

Figure 1. Processing of Samples with the Supel™ Swift HLB SPE Cartridges (30 mg/1mL) Using the 5-Step Method and the 3-Step Method.

Table 2. LC-MS/MS Analysis Conditions for Recovery and Ionization Effects Study

column:	Ascentis® Express RP-Amide, 10 cm x 2.1 cm I.D., 2.7 μm (**53913-U**)
mobile phase:	[A] 5 mM ammonium acetate, 0.1% acetic acid in water; [B] 5 mM ammonium acetate, 0.1% acetic acid in 95% acetonitrile and 5% water
gradient:	95% A, 5% B held for 1 min; to 43% B in 6 min; held at 43% B for 1 min; to 61.5% B in 2 min, to 95% A and 5% B in 1 min, held for 3 mins.
flow rate:	0.4 mL/min
column temp:	40 °C
detector:	MS, ESI(+) scheduled MRM (see **Table 1**)*
injection:	2 μL

*more details can be found in Application Note "LC-MS analysis of plasma samples using HLB SPE cartridges"

— Nizatidine	— Buspirone	— Clasrithromycin	— Nevirapine	— Imipramine
— Benzoylecgonine	— Mesoridazine	— Nefazodone	— Imiquimod	— Amitriptyline
— Mirtazapine	— Haloperidol	— Amiloride	— Hydroquinidine	— Losartan
— Methapyrilene	— Atrazine	— Imidacloprid	— Mianserin	— Loratadine
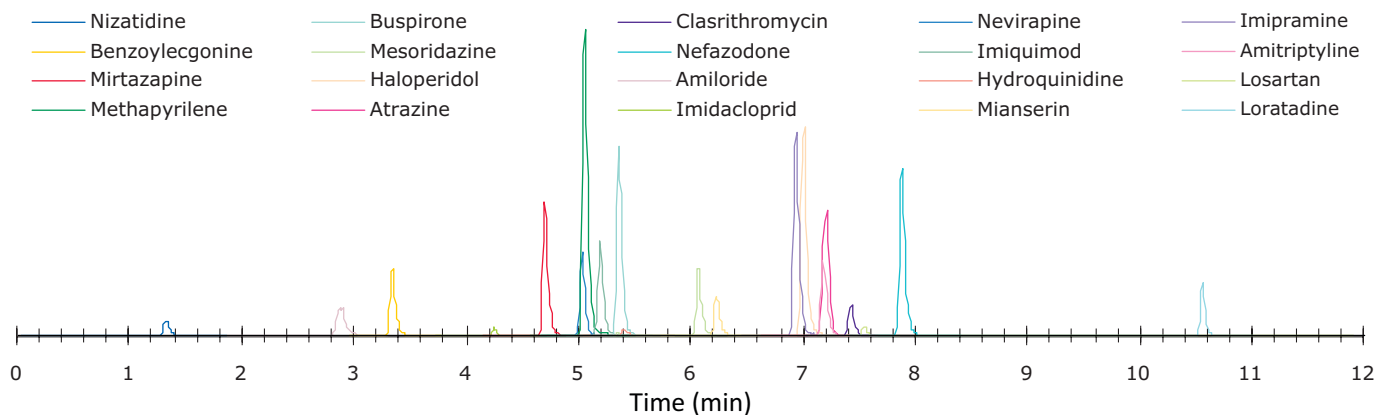
Figure 2. Representative Chromatogram of Analytes and Internal Standards for 5-Step Method Showing Individual Analytes. For Retention Times see **Table 1**.

Results & Discussion

The recoveries of the 20 analytes following their SPE cleanup from both the 3-Step and 5-Step methods of the Supel™ Swift HLB SPE cartridges are presented in **Table 3** and **Figure 3**. Overall, the 5-Step method showed better recovery (100.7 ±6.8%) compared to the 3-Step process (85.1 ±4.2%), partly because

of the two earliest eluting compounds (amiloride and nizatidine, both below 70% recovery in 3-Step method). Under the 5-Step process, all twenty analytes had recoveries between 80% and 120%. For 16 of the 20 analytes, the 3-Step process achieved recoveries in the 80% to 120% range.

Table 3. Summary of % Recovery Using the Supel™ Swift HLB SPE Cartridge and Commercially Available HLB Cartridge

	Supel™ Swift HLB SPE cartridge				Commercially Available HLB SPE cartridge	
	3-Step		5-Step		5-Step	
	% Recovery	RSD (%)	% Recovery	RSD (%)	% Recovery	RSD (%)
Amiloride	53.6	2.9	98.7	3.0	98.2	1.0
Benzoylecgonine	89.3	1.0	102.7	1.7	104.7	1.6
* Nizatidine	47.2	3.0	92.9	3.5	18.7	1.3
Imidacloprid	91.6	0.7	105.9	4.2	91.1	4.5
* Imiquimod	96.9	4.1	102.9	2.5	104.2	1.0
Buspirone	88.7	2.3	97.9	3.2	84.4	4.4
Atrazine	88.5	1.3	109.3	1.5	107.0	3.8
Nevirapine	98.0	3.4	112.2	3.0	104.4	3.7
Hydroquinidine	88.1	1.2	110.7	4.0	100.3	5.9
Methapyrilene	89.0	6.5	105.1	3.4	93.9	3.3
Mirtazapine	98.1	1.0	112.7	6.6	127.4	9.9
* Clarithromycin	76.0	6.4	93.4	11.7	110.9	7.8
Mesoridazine	91.6	0.7	112.9	24.7	86.2	9.7
Haloperidol	82.5	1.4	99.0	4.3	90.9	5.8
Mianserin	81.2	0.7	95.6	0.2	96.7	1.7
Imipramine	82.7	0.7	98.0	0.4	102.7	0.8
Loratadine	73.2	1.0	82.6	1.2	131.7	2.4
*Losartan	91.9	18.8	84.8	23.2	80.0	6.3
Nefazodone	107.8	6.7	100.4	4.4	199.4	16.8
Amitriptyline	82.5	2.5	97.0	2.6	96.1	2.3

*No internal standards used, represent absolute recoveries
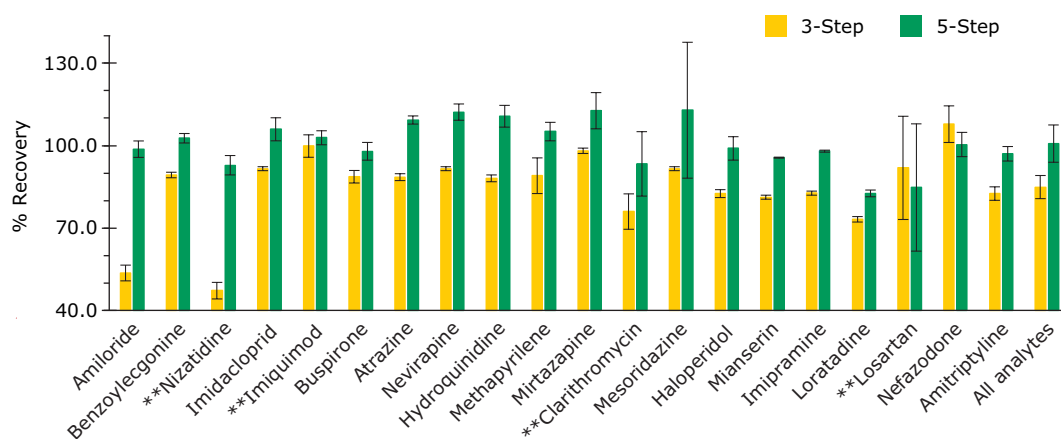
** Analytes did not have an internal standard.

Figure 3: Summary of Recovery for the 3-Step and 5-Step Process Using Supel™ Swift HLB SPE cartridges. Analytes are Ordered by Increasing LogP Values.
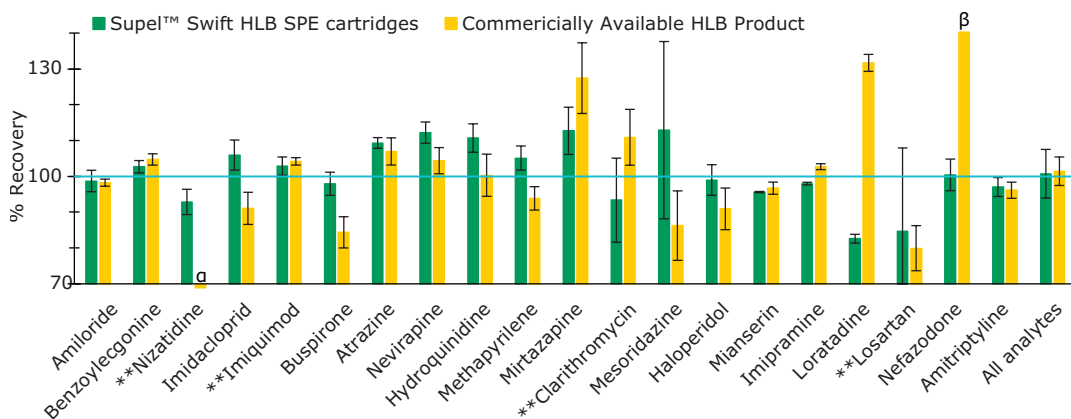
** Analytes did not have an internal standard.

α/β Commercially Available HLB (not shown, off scale): Nizatidine: 18.7 ± 1.3% and Nefazodone: 199.4 ± 16.8%

Figure 4. Percent Recovery of 5-Step Method for Supel™ Swift HLB SPE Cartridges and a Commercially Available HLB Product. Analytes are in order of Increasing LogP values.

In addition to evaluating the performance of the Supel™ Swift HLB SPE cartridges, its performance was also compared with another commercially available HLB cartridge. Both cartridges contained 30 mg of resin and had a max sample volume of 1 mL. To compare the effectiveness of these cartridges, the 5-Step method was employed for the same set of analytes and under similar conditions. **Figure 4** and **Table 3** represent the recovery using the two cartridges. As previously mentioned, all analytes had a recovery in the range of 80-120% when using the Supel™ Swift HLB SPE cartridges. This is in contrast to the other commercially available cartridge where only 16 of the 20 analytes (80%) were recovered in the 80-120% range.

Beyond recovery rates, another important factor to consider when performing SPE is the impact of residual background on ion suppression and/or ion enhancement. The ion suppression/enhancement is an indication of how well the SPE step removes matrix components (in this case from calf-serum). As displayed in **Figure 5**, for 16 of the 20 analytes (80%) processed with Supel™ Swift HLB SPE cartridges, there was minimal impact on ionization (≤ +/- 10%). For the remaining 4 analytes, ion enhancement but no ion suppression was observed. This is in stark contrast to the other commercially available cartridge, where 14 of the 20 analytes (70%) showed an ionization suppression (13 of the 14) or enhancement (1 of 14) of
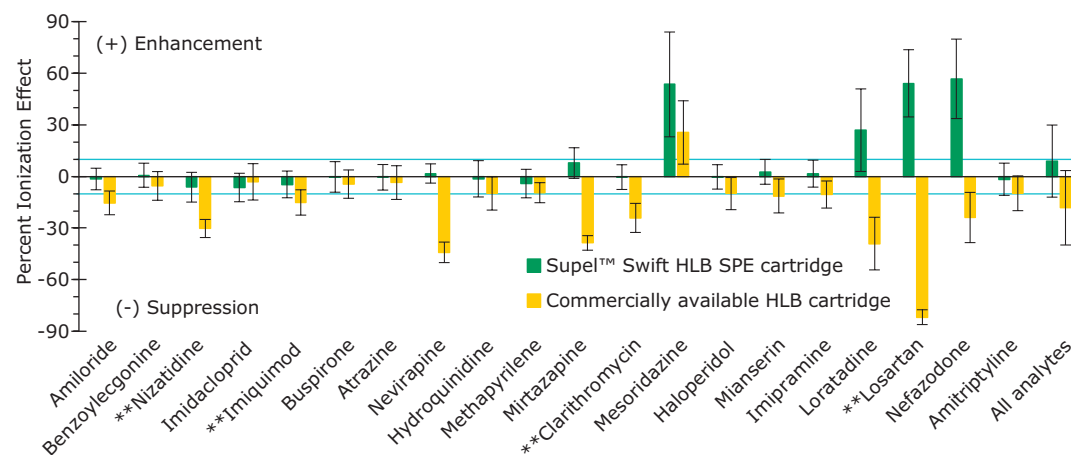

Figure 5. Signal Suppression or Enhancement Effects for Supel™ Swift HLB SPE Cartridges and Another Commercially Available HLB Product Using the 5-Step Method. Analytes are Arranged in Order of their Increasing LogP Values.

more than 10%. To conclude, samples prepared using the Supel™ Swift HLB SPE cartridges showed less ion suppression/enhancement when compared to another commercially available cartridge.

Conclusion

Through the use of twenty analytes with various LogP values, the Supel™ Swift HLB SPE cartridges demonstrated excellent recoveries (100% of analytes in the range of 80-120% with the 5-step method) and minimal effects on analyte ionization for 80% of the analytes, indicating a good matrix removal.

The advantages of the Supel™ Swift HLB SPE cartridges were further demonstrated on their comparison to a commercially available HLB cartridge under the same set of conditions.

To view the full data set of this application, download the Application Note "LC-MS analysis of plasma samples using HLB SPE cartridges" on **SigmaAldrich.com/SupelSwiftHLB.**

References

1. Andrade-Eiora, A. *et al.* Solid-phase extraction of organic compounds: A critical review (Part I). *TrAC Trends in Analytical Chemistry*, Volume 80, **2016**, Pages 641-654, doi: https://doi.org/10.1016/j.trac.2015.08.015

2. Michopoulos, F.L. et al. UPLC-MS-Based Analysis of Human Plasma for Metabolomics Using Solvent Precipitation or Solid Phase Extraction. *J Proteome Res* **2009**, 2114-2121. doi:https://doi.org/10.1021/pr801045q

Featured Products

Description	Cat. No.
SPE & HPLC	
Visiprep™ SPE Vacuum Manifold DL, 12-port model	**57044**
Supel™ Swift HLB SPE, 30 mg/1 mL, Pk.108	**57493-U**
Ascentis® Express Phase RP-Amide 10 cm x 2.1 cm, 2.7 µm particle size	**53913-U**
Solvents, Reagents, and Accessories	
Acetic acid for LC-MS LiChropur™	**5.33001**
Acetonitrile hypergrade for LC-MS LiChrosolv®	**1.00029**
Ammonium acetate for mass spectrometry, LiChropur™, eluent additive for LC-MS	**73594**
Ammonium formate eluent additive for LC-MS, LiChropur™, ≥99.0%	**70221**

Description	Cat. No.
Methanol HPLC Plus, ≥99.9%	**646377**
Disposable Liners for Visprep™ DL Manifolds, pk of 100	**57059**
MRQ30 Clear CD Vial™, Blue cap, Pre-slit TEF/Silicone septa, volume 1.2 mL, pk. 100	**23187-U**
Certified Reference Materials (CRMs) & Analytical Standards	
Abacavir sulfate, Pharm. Secondary Standard; CRM, 500 mg	**PHR1256**
Amiloride hydrochloride hydrate, ≥98% (HPLC), powder, research chemical, 1 g	**A7410**
Amitriptyline hydrochloride, Pharm. Secondary Standard; CRM, 1 g	**PHR1384**
Amitriptyline-D$_3$ hydrochloride, 1.0 mg/mL in methanol (as free base), CRM, Cerilliant®, 1 mL	**A-121**
Atrazine, CRM, TraceCERT®, 50 mg	**90935**
Atrazine-D$_5$, PESTANAL®, analytical standard, 10 mg	**34053-R**
Benzoylecgonine solution, 1.0 mg/mL n methanol, CRM, Cerilliant®, 1 mL	**B-004**
Benzoylecgonine-D$_3$, 1.0 mg/mL in methanol, CRM, Cerilliant®, 1 mL	**B-008**
Buspirone-D$_8$ hydrochloride, 100 µg/mL in methanol (as free base), CRM, Cerilliant®, 1 mL	**B-055**
1-(3-Chlorophenyl)piperazine-D$_8$ hydrochloride 100 µg/mL in methanol (as free base), CRM, Cerilliant®, 1 mL	**C-112**
Clarithromycin, Pharm. Secondary Standard; CRM, 500 mg	**PHR1038**
N-Desmethylmirtazapine, 1.0 mg/mL in acetonitrile, CRM, Cerilliant®, 1 mL	**D-086**
Dexamethasone, United States Pharmacopeia (USP) Reference Standard	**1176007**
5-(N,N-dimethyl) amiloride hydrochloride, research chemical, 25 mg or 100 mg	**A4562**
Haloperidol, Pharm. Secondary Standard; CRM, 400 mg	**PHR1724**
Haloperidol-D$_4$, 100 µg/mL in methanol, CRM, Cerilliant®, 1 mL	**H-002**
Hydroquinidine, 95%, 5 g	**359343**
Imidacloprid-D$_4$, PESTANAL®, analytical standard, 10 mg	**34170**
Imipramine hydrochloride, Pharm. Secondary Standard; CRM, 1 g	**PHR1797**
Imipramine-D$_3$ maleate, 100 µg/mL in methanol (as free base), CRM, Cerilliant®, 1 mL	**I-903**
Loratadine, Pharm. Secondary Standard; CRM, 1 g	**PHR1376**
Methapyrilene hydrochloride, analytical standard, 1 g	**442641**
Mianserin Hydrochloride, 1.0 mg/mL in methanol (as free base), CRM, Cerilliant®, 1 mL	**M-919**
Mianserin-D$_3$, 100 µg/mL in methanol, CRM, Cerilliant®, 1 mL	**M-901**
Mirtazapine, 1.0 mg/mL in methanol, CRM, Cerilliant®, 1 mL	**M-128**
Nefazodone Hydrochloride, 1 mg/mL in methanol (as free base), CRM, Cerilliant®, 1 mL	**N-119**

See our complete offering of Supel™ Swift HLB SPE products at **SigmaAldrich.com/SupelSwiftHLB**

Methods for Analysis of the California List of Pesticides in Cannabis

Geoffrey Rule, Principal Scientist, Analytix@merckgroup.com

Abstract

Methods to quantify the California list of pesticides from dried cannabis (hemp), using both GC-MS/MS and LC-MS/MS are described with a special focus on evaluation of matrix effects and use of internal standards.

Introduction

Growers and processors of cannabis must show their products to be safe as per individual state requirements. Generally, requirements include testing to ensure that the cannabis flower is free of pesticides.[1] In this application note we demonstrate how Supelco analytical standards, instrument consumables, and reagents can be used to analyze low levels of pesticides in cannabis and, in particular, the 66 pesticides required by the State of California.

QuEChERS extraction has been widely adopted for preparation of samples in the analysis of pesticides from a variety of agricultural matrices. But it shows some limitations due to the broad range of physical and chemical properties of the pesticides. As a consequence, there is a trend of minimal sample clean-up by instrument vendors, and a simple solvent extraction proposed by some. In other cases, a simple flow-through or chemical filtration type clean-up is proposed where the solvent extract is allowed to pass through an SPE cartridge of some type to remove unwanted matrix material. While these goals are admirable, they may or may not always be successful based on the number of analytes required, reporting limits, the instrumentation available as well as the matrix being extracted. This makes proper understanding of matrix effects, extraction recovery, and use of isotope labelled internal standards critical in many cases.

In this article we describe the determination of 66 pesticides from the California list of pesticides, in a locally obtained hemp sample using both LC-MS/MS and GC-MS/MS. A flow-through, interference removal clean-up procedure is utilized for the analysis. Methods are also outlined to evaluate the matrix effects and extraction recovery — two essential aspects of developing rugged methods. In addition, we describe the use of analyte protectants for compounds best analyzed by gas chromatography. The use of stable isotope labelled (SIL) internal standards is also discussed with a focus on the advantages provided by them.

Methods

One gram of coarse ground hemp was weighed into a 50 mL centrifuge tube. Two ceramic homogenization pellets were added along with 15 mL of acetonitrile. The sample was extracted manually, with vigorous shaking for 5 min, and then centrifuged for 10 min at 2800 rpm. The entire supernatant was then removed and passed through a Discovery® DSC-18 solid phase extraction cartridge (6 mL, 500 mg) by gravity flow. This was followed by two additional extractions, each with 5 mL acetonitrile. The eluents from the three extractions were combined and the final volume was brought up to 25 mL with acetonitrile. Aliquots of this solution were then placed in separate autosampler vials for both LC-MS/MS and GC-MS/MS analysis.

The conditions developed for both the LC-MS/MS and GC-MS/MS analysis methods are shown in **Tables 1** and **2**. Calibration curves were prepared using both the blank matrix extract, that had gone through the extraction procedure, and pure solvent or mobile phase. To cover the range for California requirements, a total of nine calibration standards were utilized at 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 1.5, 3 and 5 µg/g hemp equivalent. To evaluate analyte recovery, samples are prepared by "spiking" hemp with a pesticide solution pre-extraction, to best represent actual plant samples. In our case, we spiked hemp samples at concentrations of 0.1 and 3 µg/g for extraction recovery experiments. Experiments can be performed in the same fashion for both LC-MS and GC-MS evaluation of suppression or enhancement effects, and determination of extraction recovery (**Figure 1**). A comparison of the solvent based curve with the one prepared in the blank matrix extract (post-extract spike) reveals the extent to which matrix components are suppressing or enhancing chromatographic peak intensities. Comparison of the post-extract spiked curve with the samples prepared by spiking prior to extraction (pre-extract spike), provides a means of evaluating analyte losses from the extraction, or sample clean up procedure.
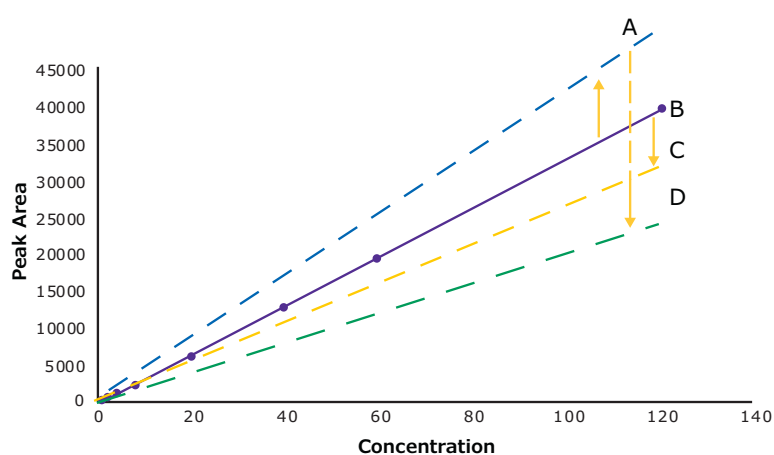
Table 1. LC-MS/MS Analysis Conditions

LC-MS/MS Conditions	
instrumentation:	Agilent 1290 series HPLC and autosampler with 6460 QQQ
column:	Ascentis® RP-Amide, 10 cm x 2.1 mm, 3 µm particles (**565301-U**) with RP-Amide guard column, 2 cm x 2.1 mm I.D., 5 µm (**565372-U**)
mobile phase:	[A] 2 mm ammonium formate, 0.1% formic acid, 2% methanol in Milli-Q water
	[B] 2 mm ammonium formate, 0.1% formic acid, 5% Milli-Q® water in acetonitrile

gradient	Time (min)	A (%)	B (%)
	0.0	100	0
	1.0	100	0
	14.0	0	100
	17.0	0	100
	17.5	100	0
	20.0	100	0

flow rate:	0.4 mL/min
column temp:	40 ° C
detector:	MS/MS, ESI (+) dMRM Acquisition Mode*
injection:	12 µL

* For a list of transitions used please contact the author

Table 2. GC-MS/MS Analysis Conditions

GC-MS/MS Conditions	
column:	SLB®-5ms 30 m × 0.25 mm, 0.25 µm (**28471-U**)
oven:	60°C (1 min), 40 °C/min to 170 °C, 10 °C/min to 310 °C (3 min)
injector:	solvent vent mode: 60 °C (0.35 min), 600 °C/min to 300 °C; 5 psi until 0.3 min, split vent flow 50 mL/min at 1.5 min
carrier gas:	helium, 1.2 mL/min, constant flow
detector:	MS/MS
injection:	2 µl, solvent vent splitless injection with 0.2 µL sandwich of analyte protectant solution
liner:	4 mm ID dual tapered liner
sample:	hemp extract in acetonitrile

A Post-extract curve showing enhancement

B Curve in pure solvent

C Post-extract curve showing suppression

D Pre-extract curve showing extraction recovery

B to A Enhancement

B to C Suppression

A-C to D Extraction Recovery

(Peak area A/B -1)x(100) = % Enhancement

(1- Peak area C/B)x(100) = % Suppression

(D/C)x(100) or (D/A)x(100) = % Extraction Recovery

Figure 1. Matrix Enhancement, Suppression and Recovery Evaluation

An additional means of evaluating suppression effects in LC-MS is through use of a "tee-infusion" experiment.[2] In this experiment a syringe pump is used to infuse a solvent solution of the analytes of interest into a tee fitting placed between the column and the mass spectrometer (**Figure 2**). The infusion flow rate and concentration are typically quite low, in the order of 10 µL/min or so, and 100-200 pg/µL. A blank matrix extract is prepared and injected to the LC system while the analytes are monitored over the course of the LC gradient. Comparing injections of the blank matrix extract with a similar injection of mobile phase indicates where matrix components elute during the run and their impact on analyte signal intensity.
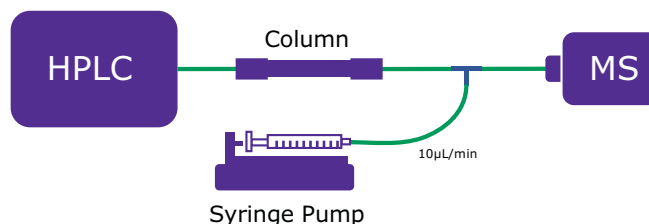
Figure 2. Illustration of Tee-Infusion Experimental Set-Up

Use of analyte protectants in gas chromatography

Suppression or enhancement effects may be observed in GC-MS also. The causes are different than electrospray ionization (ESI) LC-MS but the effects can be studied in a similar fashion. In GC, the suppression or enhancement effects generally result from the loss or degradation of analytes in the hot injection port, liner, and column inlet when injected in relatively clean extracts or pure solvent. In case of more complex sample extracts, the matrix components can protect analytes from this degradation by blocking the active sites present in these regions of the GC. The matrix therefore causes an enhancement effect, and presents as an improved analyte peak shape and intensity. To ameliorate the situation, several compounds have been identified that will reduce analyte degradation if injected simultaneously with the analyte.[3, 4] Compounds such as sorbitol and gulonic acid lactone, gluconic acid lactone, shikimic acid, and 3-ethoxy-1,2-propanediol are examples of compounds found to reduce analyte degradation. Some protectants are also shown to be most effective during specific periods of a chromatographic run, for example during early, middle, or late stages of the run, and for particular analytes. In

our work, a solution of two compounds was prepared for use (**Table 3**). The solution was placed on the autosampler and an injection method was created to "sandwich" the sample extract within the protectant solution.

With a study of extraction recoveries and matrix effects on peak intensities, additional efforts can be directed at either sample clean-up or in adjusting chromatographic conditions where necessary. The choice of appropriate internal standards can also be made to generate reliable methods for any given matrix.

Results

With the chromatographic and instrument conditions shown (**Tables 1** and **2**), we successfully met the California requirements for 57 of the 66 pesticides by LC-MS/MS. The HPLC conditions developed provided the separation of analytes as shown in **Figure 3** with daminozide being the earliest eluting compound and acequinocyl the latest.

Not surprisingly, results of the tee-infusion experiment indicate that acetonitrile extraction of hemp yields a large amount of cannabinoid material in the extract (**Figure 4**). The cannabinoids come off in the course of each chromatographic run and can cause variable degrees of suppression depending on the amount of each cannabinoid present. Samples expected to be high in THC and THCA might therefore benefit from slightly different chromatographic conditions than those for samples high in CBD and CBDA.

The GC-MS/MS conditions developed provided the separation of analytes as shown in **Figure 5** and allowed the successful determination of 40 compounds from the California list. The two instrumental methods (LC & GC) provide an overlap of 32 compounds, which may be considered advantageous for situations where one or more analyte-matrix combinations may be challenging on one instrument type but not the other. A tabulation of results for each pesticide, and by each method, is given in **Table 4**.

Table 3. Preparation of Analyte Protectant Solution

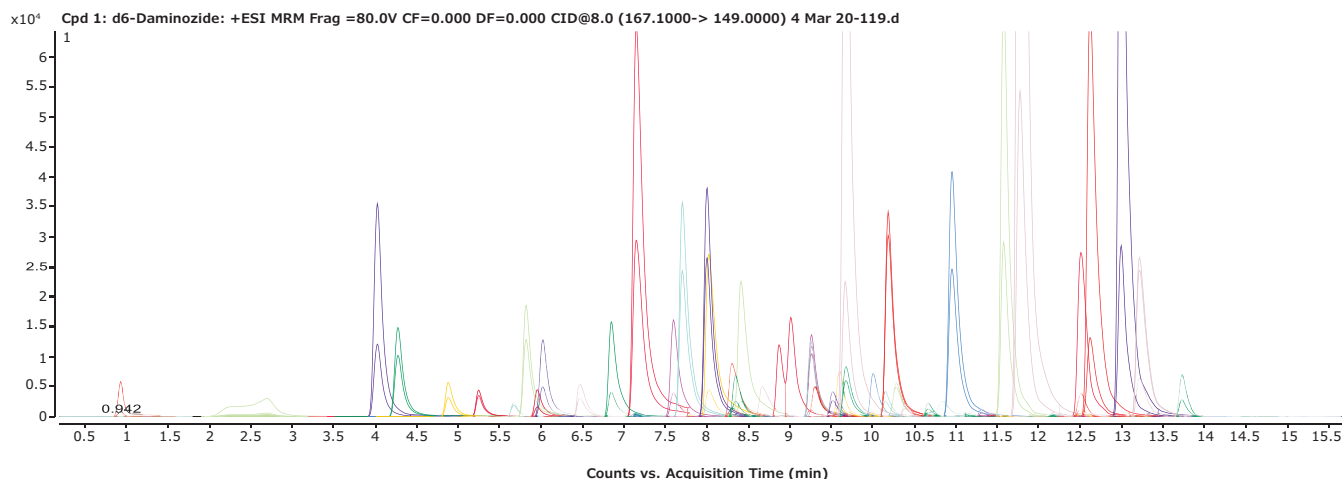| Step	Procedure
1	Weigh ~500 mg of D-Sorbitol into a 10 mL volumetric flask and add 6 mL of LC-MS grade acetonitrile. Bring to volume with Milli-Q® water (Solution A).
2	Weigh ~500 mg of L-Gulonic acid γ-lactone into a 10 mL volumetric flask and add 5mL of LC-MS grade acetonitrile. Bring up to volume with Milli-Q® water (Solution B).
3	Add 2 mL of Solution A with 4 mL of Solution B in a 10 mL volumetric flask and bring to volume with LC-MS grade acetonitrile
4	Place into appropriate autosampler vial for making sandwich injection with 0.2 µL of air gap above and 0.2 µL of the analyte protectant solution

Figure 3. Standard Injection of California Pesticides Prepared in Hemp and Analyzed by LC-MS/MS
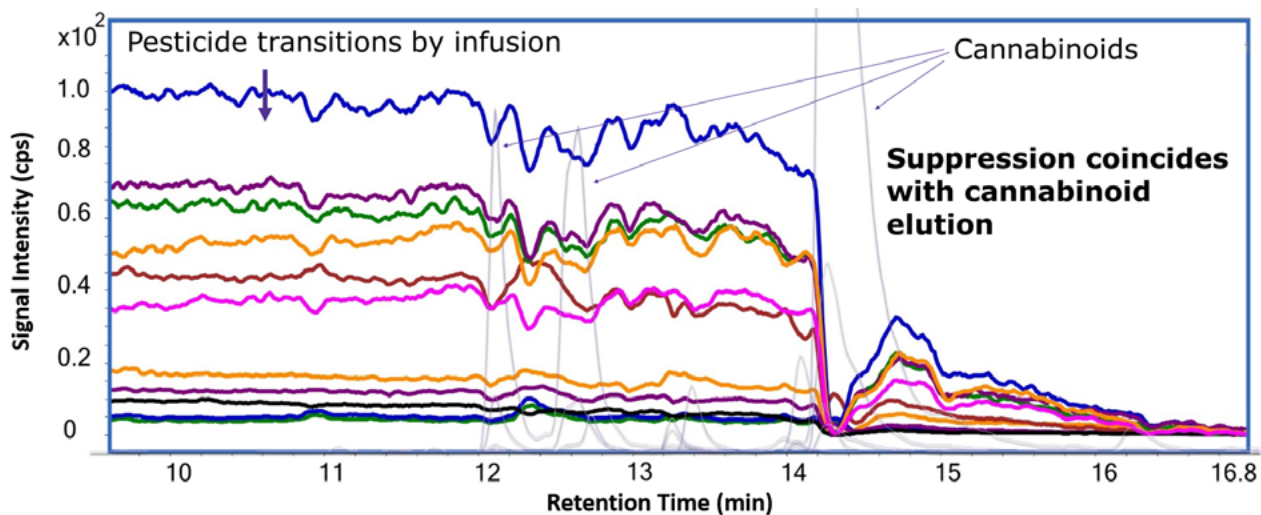
Figure 4. A portion of the Chromatographic Run in a Tee-Infusion Experiment - The colored traces indicate the signal intensity of several pesticides being infused over the course of a run. The cannabinoid peaks (grey) were collected as a separate chromatographic run and have been overlaid with the infusion data for illustration. The suppression occurring after 15 minutes is due to unidentified matrix components eluting from the column.
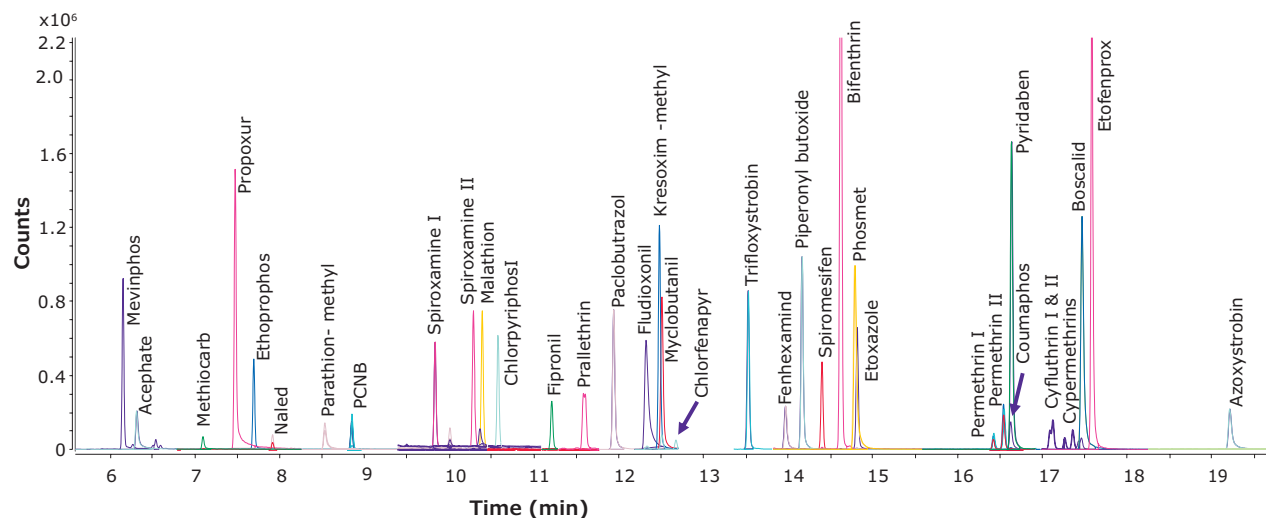
Figure 5. Standard Injection of California Pesticides Prepared in Hemp and Analyzed by GC-MS/MS

Table 4. Tabulated Results: California Pesticides at Minimum Action Level (MAL)[5]

Analyte	GC-MS/MS MAL met?	LC-MS/MS MAL met?	Analyte	GC-MS/MS MAL met?	LC-MS/MS MAL met?
Acephate	YES	YES	Chlorfenapyr	YES	YES
Acequinocyl	NO	NO	Chlorpyrifos	YES	YES
Acetamiprid	NO	YES	Clofentezine	NO	YES
Aldicarb	NO	YES	Coumaphos	YES	YES
Avermectin	NO	YES	Cyfluthrin I & II	YES	NO
Azoxystrobin	YES	YES	Cypermethrin I II III IV	YES	NO
Bifenazate	NO	YES	Daminozide	NO	YES
Bifenthrin	YES	YES	Diazinon	NO	YES
Boscalid	YES	YES	Dichlorvos	YES	YES
Captan	YES	YES	Dimethoate	NO	YES
Carbaryl	NO	YES	Dimethomorph	NO	YES
Carbofuran	NO	YES	Ethoprop	YES	YES
Chlorantraniliprole	NO	YES	Etofenprox	YES	YES
Chlordane I	YES	NO	Etoxazole	YES	YES
Chlordane II	YES	NO	Fenhexamid	YES	YES

Table 4. (cont.) Tabulated Results: California Pesticides at Minimum Action Level (MAL)[5]

Analyte	GC-MS/MS	LC-MS/MS	Analyte	GC-MS/MS	LC-MS/MS
	MAL met?	MAL met?		MAL met?	MAL met?
Fenoxycarb	NO	YES	Permethrins	YES	YES
Fenproximate	NO	YES	Phosmet	YES	YES
Fipronil	YES	YES	Piperonyl butoxide	YES	YES
Flonicamid	NO	YES	Prallethrin	YES	YES
Fludioxonil	YES	YES	Propiconazole	NO	YES
Hexythiazox	NO	YES	Propoxur	YES	YES
Imazalil	NO	YES	Pyrethrins	NO	YES
Imidacloprid	NO	YES	Pyridaben	YES	YES
Kresoxim-methyl	YES	YES	Spinetoram J	NO	YES
Malathion	YES	YES	Spinosyn A	NO	YES
Metalaxyl	NO	YES	Spinoteram L	NO	YES
Methiocarb	YES	YES	Spiromesifen	YES	YES
Methomyl	NO	YES	Spirotetramat	NO	YES
Mevinphos	YES	YES	Spiroxamine I	YES	YES
Myclobutanil	YES	YES	Spiroxamine II	YES	YES
Naled	YES	YES	Tebuconazole	NO	YES
Oxamyl	NO	YES	Thiacloprid	NO	YES
Paclobutrazol	YES	YES	Thiamethoxam	NO	YES
Parathion-methyl	YES	NO	Trifloxystrobin	YES	YES
PCNB	YES	NO			

Use of stable isotope labeled internal standards

The potential benefit of using stable isotope labeled (SIL) internal standards (IS) should always be considered when developing methods for challenging matrices or for particular analytes. While they do add to the cost of sample analysis, they make up for it in providing more accurate and rugged methods, even in the presence of matrix effects and recovery losses. Analog ISs, meaning compounds that are chemically only similar to the analyte, cannot guarantee the same advantages as SIL ISs due to differences in retention time and ionization efficiency (**Figure 6**). A SIL IS, on the other hand, is essentially identical to the analyte itself but differing only in mass. This means, once added to a sample the ratio of analyte to SIL IS will not vary through the sample preparation, chromatography, and analysis stages. Use of this ratio in quantitation therefore results in excellent accuracy. (See our isotope labeled pesticide standards at SigmaAldrich.com/ILSPesticides)
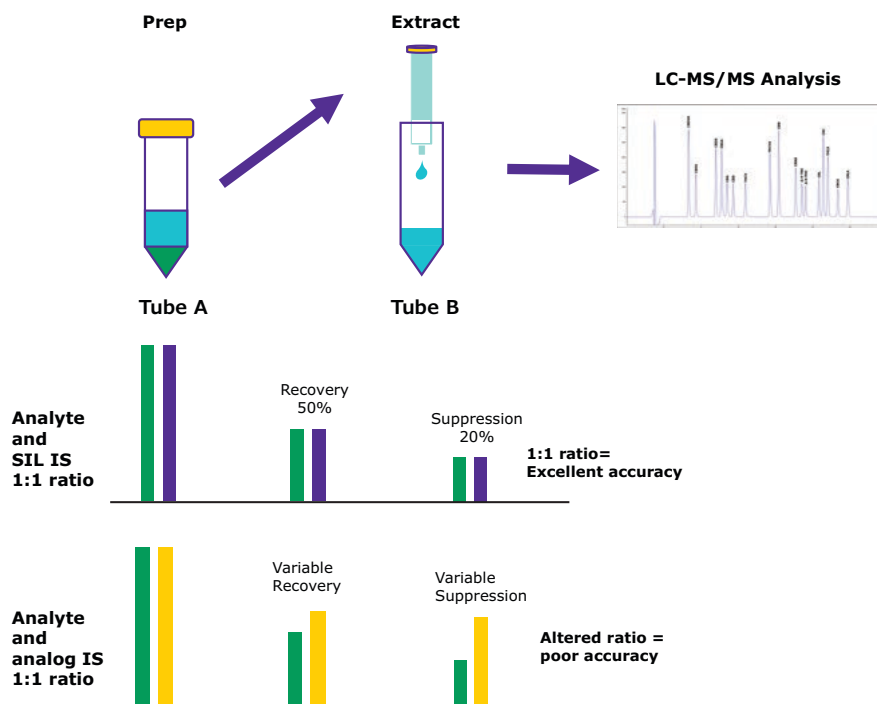

Figure 6. A Depiction of the Advantages Obtained by Use of SIL IS Over Analog IS in LC-MS/MS Analysis.

Conclusion

A method has been developed to quantify the California list of pesticides from dried cannabis (hemp), in accordance with the state requirements, utilizing a combination of both LC-MS/MS and GC-MS/MS. A single step, flow-through (interference removal) solid phase extraction cleanup is used to prepare sample extracts for both instrumental techniques. The linearity, recovery, and precision were satisfactorily achieved (not shown) and schemes for performing calibration, extraction recovery, and suppression/enhancement studies are provided.

A total of 57 pesticides were reported by LC-MS/MS and 40 using GC-MS/MS (**Table 4**). Due to high levels of interfering CBDA, one analyte, acequinocyl, was not detectable at minimum levels with the existing instrumentation. All other pesticides were reported with one or the other analytical technique to meet or exceed current California regulatory limits for each category.[5]

It is shown that a combination of GC-MS/MS and LC-MS/MS instrumentation provides an efficient way to analyze cannabis for pesticides.

Literature

1. Seltenrich, N. Into the Weeds: Regulating Pesticides in Cannabis. Enviro Health Persp. 2019; 127(4) doi.org/10.1289/EHP5265

2. Bonfiglio R, King RC, Olah TV, Merkle K. The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds. Rapid Commun Mass Spectrom. 1999;13(12):1175-1185. doi:10.1002/(SICI)1097-0231(19990630)13:12<1175::AID-RCM639>3.0.CO;2-0

3. Mastovská K, Lehotay SJ, Anastassiades M. Combination of analyte protectants to overcome matrix effects in routine GC analysis of pesticide residues in food matrixes. Anal Chem. 2005;77(24):8129-8137. doi:10.1021/ac0515576

4. Anastassiades M, Mastovská K, Lehotay SJ. Evaluation of analyte protectants to improve gas chromatographic analysis of pesticides. J Chromatogr A. 2003;1015(1-2):163-184. doi:10.1016/s0021-9673(03)01208-1

5. California Bureau of Cannabis Control, Text of Regulations; https://bcc.ca.gov/law_regs/cannabis_order_of_adoption.pdf

See our complete portfolio on pesticide and labeled pesticide reference materials at
SigmaAldrich.com/Pesticides

SigmaAldrich.com/ILSPesticides

For more such information on testing of cannabis visit us at **SigmaAldrich.com/Cannabis**

Featured Products

Description	Part Number
LC-MS/MS	
Discovery® C18 SPE Cartridges, 500 mg, 6 mL, Pk.30	**52604-U**
Ascentis® RP-Amide 100 x 2.1 mm, 3 µm	**565301-U**
Ascentis® RP-Amide Supelguard™ Guard Cartridge, 2 cm x 2.1 mm I.D., 5 µm, PK.2	**565372-U**
Ultrapure water from Milli-Q® system or bottled water	**Milli-Q® IQ 7005 or 1.15333**
Acetonitrile with 0.1% (v/v) Formic acid hypergrade for LC-MS LiChrosolv®	**1.59002**
Methanol hypergrade for LC-MS LiChrosolv®	**1.06035**
Water with 0.1% (v/v) Formic acid hypergrade for LC-MS LiChrosolv®	**1.59013**
Ammonium Formate, eluent additive for LC-MS, LiChropur™, ≥99.0%	**70221**
Formic acid 98% - 100%, for LC-MS LiChropur™	**5.33002**
50 mL Centrifuge Tubes, Pk.500	**T2318**
15 mL centrifuge tube, Pk.500	**T1818**
Certified Vial Kit – Amber (Autosampler vials), Pk.100	**29653-U**
Guard frit with holder	**803410**
Replacement frits	**803411**
GC-MS/MS	
SLB®-5ms, 30 m × 0.25 mm, df 0.25 µm	**28471-U**
Acetonitrile for gas chromatography ECD and FID SupraSolv®	**1.00017**
Molded Thermogreen® LB-2 Septa, solid discs, diam. 11 mm, Pk.50	**28676-U**
Inlet Liner Splitless Type, Dual-Taper Design (unpacked), Pk.5	**2048505**
Certified Vial Kit, Low Adsorption (LA), 2 mL, amber glass vial, natural PTFE/silicone septa (with slit), thread for 9 mm, Pk. 100	**29654-U**
Hamilton® syringe701N, volume 10 µL, needle size 26s ga (bevel tip), needle L 51 mm (2 in.)	**20734**
L-Gulonic acid γ-lactone, 95%	**310301**
D-Sorbitol, 99%	**240850**
Supelco® Helium Purifier, stainless steel fittings, 1/8 in	**27600-U**
OMI®-2 Purifier Tube, Pk.1	**23906**
OMI®-2 Purifier Holder, Pk.1	**23921**

Determination of Total Glucose and Xylose in Instant Coffee by Reverse Phase HPLC-UV

Eddy Tan, Application Scientist, Lee May May, Senior Application Scientist, Singapore Applications Laboratory, Analytix@merckgroup.com

Abstract

This is a method for the determination of total glucose and xylose in coffee samples by reverse phase HPLC-UV. The limit of detection for glucose and xylose is 53.2 and 33.8 ppm respectively for freeze dried coffee.

Introduction

Coffee is an indispensable beverage for many people. The adulteration of coffee with coffee husks, cereal grains and soy beans to raise the profit margin is well known. Typical markers for such adulteration include glucose and xylose. Instant coffee is considered to be adulterated if it contains more than 2.46% total glucose and 0.45% total xylose.[1,2]

As sugars lack UV chromophores, their determination is typically accomplished by HPLC-RID (Refractive Index Detector)[3] or by anion exchange chromatography with a pulsed amperometric detector (HPAEC-PAD)[1,4]. The RID is less sensitive compared to the UV detector and often requires a longer time to stabilize. It is therefore not the detector of choice for many HPLC users. The HPAEC-PAD is a more expensive setup with a limited set of applications and separation columns. It is therefore not a common instrument.

Although there are established methods for sugar determination in coffee e.g. AOAC Method 995.13 and ISO Method 11292:1995, they all require the HPAEC-PAD instrument.

Here, we demonstrate the determination of total glucose and xylose using a procedure to release the sugars from the coffee followed by an SPE cleanup. The released sugars are next derivatized with a UV tag, 1-phenyl-3-methyl-5-pyrazolone (PMP).[5,6,7] A final clean up by liquid-liquid extraction with dichloromethane is done before HPLC injection. The standard addition technique was chosen as it corrects for varying levels of matrix interferences with different coffee samples.

Instruments & Samples

The analysis was performed on a Thermo Dionex UltiMate 3000 UHPLC. An ultrasonic bath was used to dissolve the coffee samples. For sample digestion and derivatization, a water bath and vortex mixer were used. The Visiprep™ vacuum manifold with a vacuum pump was employed for the SPE cleanup. A bench and a mini centrifuge were used to spin down the samples.

Freeze dried coffee and milk coffee mixture samples were purchased from a local grocery store. Freeze dried coffee refers to pure and instant soluble coffee granules or powder whereas milk coffee mixture would have sugar, milk, emulsifier, flavoring agents etc. compounded with instant coffee powder.

Method

Glucose and xylose standard solutions

Prepare 1 L of 1 M hydrochloric acid. Weigh 100 mg of glucose and 100 mg of xylose into a 10 mL volumetric flask. Add 6 mL of hot 1 M hydrochloric acid (~80 °C) and swirl gently. Sonicate for 10 minutes to dissolve completely before topping up to the mark with 1 M hydrochloric acid. Mix well before use.

Coffee sample solutions and reagent blank

Weigh 1.5 g of the freeze dried coffee sample into a 10 mL volumetric flask. For milk coffee mixture sample, use 0.5 g. Add 6 mL of hot 1 M hydrochloric acid (~80 °C) to both. Swirl gently and sonicate for 10 minutes. Ensure all solids are dissolved (milk solids will remain insoluble) before topping up to mark with 1 M HCl.

Spike in glucose and xylose at 400, 800 and 1600 ppm for freeze dried coffee samples as in **Table 1**. Do a

reagent blank using water in place of sample. For milk coffee samples, use 500 µl of sample solution instead.

Table 1. Glucose and Xylose Spiking for Freeze Dried Coffee Sample

Identity	Freeze dried coffee, µl	Glucose, µl	Xylose, µl	1M HCl, µl	Dilution factor	Glucose ppm	Xylose ppm
A	2000	0	0	3000	Nil	0	0
B	2000	200	200	2600	25.00	400	400
C	2000	400	400	2200	12.50	800	800
D	2000	800	800	1400	6.25	1600	1600

Acid digestion

Incubate all spiked solutions at 80 ±2 °C for 3 hours. Then cool to room temperature, spin down contents and filter through a Millex PTFE hydrophilic 0.45 µm filter into a new tube.

SPE cleanup

This SPE cleanup is necessary to remove oils, fats and other organics present in the coffee samples. Set up the SPE cartridges (LiChrolut® RP-18 200 mg/3 mL PP SPE tubes) on the Visiprep™ SPE vacuum manifold system. Connect this to the vacuum pump. Condition the SPE cartridges first with 2 x 3 mL methanol followed by 2 x 3 mL 1 M HCl. Next, place a 15 mL centrifuge tube as a receiver for each of the SPE cartridges. Transfer 1 ml of the filtrate from the acid digestion step into the SPE cartridge. Control flowrate for a dropwise elution.

Derivatization: Tagging the sugars with PMP (UV label)

Prepare 10 mL of 0.5 M PMP in methanol and 10 mL of 1.2 M sodium hydroxide. Pipette 200 µL from the SPE cleanup step into a 2 mL microcentrifuge tube. Add 200 µL of 1.2 M sodium hydroxide and vortex for 30 seconds. Pipette 100 µL into a 5 mL microcentrifuge tube. Add 100 µL of 0.5 M PMP and vortex for 1 minute. Spin down and incubate at 70 ±2 °C for 100 minutes in a water bath. Cool to room temperature for the next step.

Cleanup of sample for HPLC

Prepare 10 mL of 0.2 M hydrochloric acid. Add 100 µL of 0.2 M hydrochloric acid to the tagged sample. Vortex for 30 seconds and spin down contents. Add 1800 µL of water and 1500 µL of dichloromethane to it. Vortex for 1 minute and centrifuge for 2 minutes at 7000 RCF. Draw off the top aqueous layer into another 5 mL tube. Discard the dichloromethane. Repeat extraction of the aqueous layer with 1500 µL of dichloromethane twice more. Filter the aqueous layer through a 0.22 µm 13 mm Millex PTFE hydrophilic filter into a 2 mL HPLC vial. Seal vials and proceed to HPLC injection. Chromatographic conditions are in **Table 2**.

Table 2. Chromatographic Conditions

column:	Purospher® STAR RP-18e, 15 cm x 3 mm, 3 µm (**1.50750**) with guard cartridge, 4-4 mm (**1.50270**) and pre-column holder (**1.16217**)
mobile phase:	[A] 200 mM ammonium acetate (pH 6.8 ± 0.05); [B] acetonitrile; (78% A / 22% B; isocratic elution). All filtered through hydrophilic PTFE, 0.2 µm
flow rate:	0.4 mL/min
column temp:	35 °C
detector:	UV, 245 nm
pressure:	~200 bar
injection volume:	20 µL

Results and Discussion

Both glucose and xylose peaks were symmetrical and eluted at ~9.8 and ~11.5 minutes respectively (**Figure 1**). The freeze dried coffee has a more complex HPLC profile compared to the coffee mixture sample. See **Figure 2** for spiked freeze dried coffee sample.

The freeze dried coffee samples 1 and 2 had a total xylose content >0.42% w/w (**Table 3**). The coffee mixture samples 1 and 2 had a high glucose content
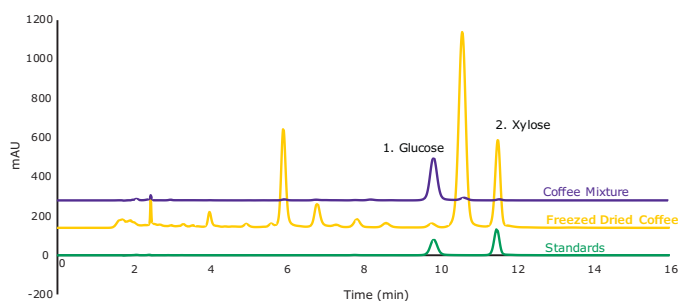
Figure 1. Coffee Mixture and Freeze Dried Coffee with Glucose and Xylose Standards

Sample	Peak	Compound	Retention Time (min)	Resolution	Peak Symmetry
Freeze dried coffee	1	Glucose	9.79	5.75	0.97
	2	Xylose	11.52	-	0.93
Coffee mixture	1	Glucose	9.83	5.72	1.03
	2	Xylose	11.55	-	0.93
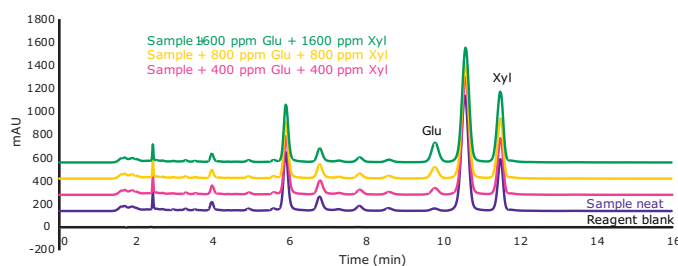
Figure 2. Freeze Dried Coffee Sample Spiked with Glucose and Xylose Standards

Glucose Standard Addition

$y = 0.0194x + 6.3314$
$R^2 = 0.9996$

Xylose Standard Addition

$y = 0.029x + 56.267$
$R^2 = 0.9999$

Linearity - Glucose	
Concentration (ppm)	**Mean Area**
0	6.1962
404.08	14.1098
808.16	22.4116
1616.32	37.5630
Slope	**0.0194**
STEYX	**0.3132**
LOD	**53.2 ppm**
LOQ	**161.2 ppm**

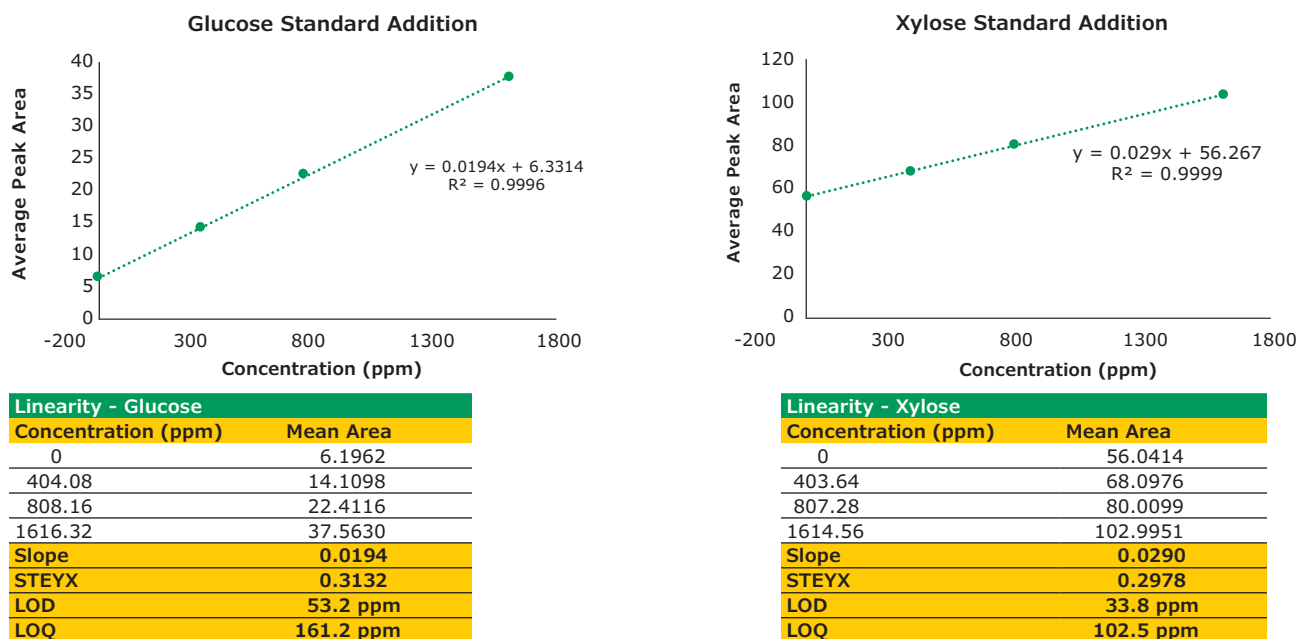| Linearity - Xylose	
Concentration (ppm)	**Mean Area**
0	56.0414
403.64	68.0976
807.28	80.0099
1614.56	102.9951
Slope	**0.0290**
STEYX	**0.2978**
LOD	**33.8 ppm**
LOQ	**102.5 ppm**

Figure 3. Standard Addition Calibration Plots and Data for Freeze Dried Coffee

>40% w/w as both have sugar and glucose syrup listed as ingredients.

Table 3. Results for Coffee Samples

Sample	Glucose (% w/w)	Xylose (% w/w)
Freeze dried coffee 1	0.35	4.10
Freeze dried coffee 2	0.55	3.23
Coffee mixture 1	43.31	0.68
Coffee mixture 2	46.59	0.13

CONCLUSION

We can determine total glucose and xylose in coffee by Reversed Phase HPLC-UV. This is a sensitive isocratic separation that can be completed by fifteen minutes with the Purospher® STAR RP-18e fully porous particle column. The method can be modified using Fused-Core® or Chromolith® columns for even faster separation while still applicable to conventional HPLC and to UHPLC instruments.

REFERENCES

1. ISO Method 11292:1995 - Instant coffee: Determination of free and total carbohydrate contents - Method using high-performance anion-exchange chromatography

2. https://www.beveragedaily.com/Article/2011/04/19/ISO-develops-new-standard-for-100-pure-soluble-coffee

3. Constantino, L. V., Zeffa, D. M., Koltun, A., Urbano, M. R., Sanzovo, A. W. S., and Nixdorf, S. L. Extraction of Soluble Sugars from Green Coffee Beans Using Hot Water and Quantification by a Chromatographic Method without an Organic Solvent. Acta Chromatographica, 2020, 32, 4, 242–246

4. AOAC Method 995.13 - Carbohydrates in Coffee

5. Honda S., Akao E., Suzuki S., Okuda M., Kakehi K., and Nakamura J. High-performance liquid chromatography of reducing carbohydrates as strongly ultraviolet-absorbing and electrochemically sensitive 1-phenyl-3-methyl-5-pyrazolone derivatives. Analytical Biochemistry, 1989, 180, 351–357

6. Wang W., Chen F., Wang Y., Wang L., Fu H., Zheng F. and Beecher L. Optimization of reactions between reducing sugars and 1-phenyl-3-methyl-5-pyrazolone (PMP) by response surface methodology. Food Chemistry, 2018, 254, 158-164

7. Zhang L., Xu Jin., Zhang L., Zhang W. and Zhang Y. Determination of 1-phenyl-3-methyl-5-pyrazolone-labeled carbohydrates by liquid chromatography and micellar electrokinetic chromatography. Journal of Chromatography, B, 2003, 793, 159-165

Featured Materials

Description	Cat. No.
HPLC Columns	
Purospher® STAR RP-18e, 3 µm, 150-3 mm, Hibar® RT	**1.50750**
Purospher® STAR RP-18e 4-4 mm Guard Cartridge	**1.50270**
Pre-column holder for LiChroCART® cartridges 4-4 for capillary connection	**1.16217**
Sample Prep, Reagents and Accessories	
LiChrolut® RP-18, 200 mg, 3 mL SPE PP Tube, Pk.50	**1.02014**
Visiprep™ SPE Vacuum Manifold	**57030-U**
Millipore® Chemical Duty Pump, 220 V/50 Hz	**WP6122050**
Acetonitrile isocratic grade for liquid chromatography LiChrosolv®	**1.14291**
Ammonium Acetate for analysis EMSURE® ACS, Reag. Ph Eur	**1.01116**
Dichloromethane for liquid chromatography LiChrosolv®	**1.06044**
Hydrochloric Acid Fuming 37%,for analysis EMSURE® ACS, ISO, Reag. Ph Eur	**1.00317**
Methanol for analysis EMSURE® ACS, ISO, Reag. Ph Eur	**1.06009**
Methanol for liquid chromatography LiChrosolv®	**1.06018**
3-Methyl-1-Phenyl-2-Pyrazoline-5-one (PMP), 99%	**M70800**
Sodium Hydroxide pellets for analysis EMSURE®	**1.06498**
Omnipore® 0.2 µm 47mm Membrane Filters	**JGWP04700**
Millex® - LCR 0.22 µm 13 mm filter unit, Hydrophilic PTFE	**SLCR013NL**
Millex® - LCR 0.45 µm 33 mm filter unit, Hydrophilic PTFE	**SLCR033NB**
HPF Millex® - LCR 0.45 µm 33 mm filter unit	**SLLGM25NS**
Reference Materials	
D-(+)-Glucose, Pharmaceutical Secondary Standard	**PHR1000**
D-(+)-Xylose, Pharmaceutical Secondary Standard	**PHR2102**

New Fapas® Food Matrix Reference Materials

Expanding our comprehensive offering of matrix reference materials for F&B testing

Matthias Nold, Product Manager Reference Materials, Analytix@merckgroup.com

Matrix reference materials are an important tool for an analytical laboratory in order to develop, validate, or verify the results of analytical methods. While neat (or solution) reference materials are usually used for calibration or identification purposes of specific analytes, matrix materials take into account matrix effects and can serve to account for bias during sample workup and preparation. Matrix Materials are characterised in their composition of specified major, minor, or trace chemical constituents. The material can be naturally contaminated, or the samples can be fortified by spiking the analytes of interest to a blank matrix.

The closer the nature of the chosen matrix reference material is to the tested samples, the better it can help to validate the results of a method.

Manufacturing of Food Matrix Reference Materials is a very laborious and time consuming process. Most of the Food Matrix Materials currently available on the market are manufactured either by metrological institutes (like the National Institute of Standards and Technology (NIST) or the European Joint Research Center (JRC)) or by Capitalise Proficiency Testing (CPT) providers with access to a robust set of analytical data from accredited labs.

Our offering of close to 200 food matrix materials including products from NIST and JRC is now complemented by 41 new reference materials (RMs) manufactured by Fapas®, a provider of proficiency testing schemes for food analysis. Fapas® is the proficiency testing branch of FERA, a center of excellence for interdisciplinary investigation and problem solving across plant and bee health, crop protection, sustainable agriculture, food and feed quality, and chemical safety in the environment, based in York (UK).

These Reference Materials (RM) are derived from materials used for proficiency testing schemes and undergo formal testing for both short-term and long-term stability. The products are delivered with an associated datasheet, which lists the reference values and their expanded uncertainty U. The value of U is not a performance limit but is the uncertainty relating to the reference value. RMs therefore have a greater degree of trust in their values than, for example, quality control materials and can be used for method calibration purposes. Fapas® RMs are manufactured in accordance to the principles of ISO 17034, but they are not certified reference materials (CRMs).

As shown in the product table below, the product range includes a variety of analytes and matrix types.

Matrix Type	Analyte Types	Description	Cat. No.
Animal feed	Biotoxins	Aflatoxins in animal feed	**FAP80738**
		Fusarium toxins in animal feed	**FAP83946**
		OA in animal feed	**FAP83947**
Beverages	Biotoxins	OTA in coffee (processed)	**FAP84207**
	Carbohydrates and Sweeteners	Soft drinks ingredients	**FAP80679**
	Organic Pollutants	Acrylamide in coffee	**FAP80591**
	Trace Elements	Metals in wine	**FAP80561**
		Metals in soft drink	**FAP80562**
Cereals	Biotoxins	Aflatoxins in maize	**FAP80868**
		Fumonisins in cereals	**FAP80926**
		Fusarium toxins in cereals	**FAP80916**
		Multi-Mycotoxins in cereals	**FAP82171**
		OA in cereals	**FAP80836**
	Organic Pollutants	Acrylamide in potato products	**FAP80659**
	Trace Elements	Metals in infant cereal	**FAP80551**
		Metals in rice	**FAP80469**
		Metals in wheat	**FAP80467**
		Nutritional elements in breakfast cereal	**FAP88984**
Dairy products	Ash, Carbohydrates, Moisture, Nitrogen, Total Fat	Proximates in condensed milk	**FAP85259**
	Biotoxins	Aflatoxin M1 in milk powder	**FAP80733**
	Organic Pollutants	Melamine in milk powder	**FAP80673**
		Pesticides and PCBs in infant formula	**FAP88987**
		Pesticides and PCBs in milk powder	**FAP89036**
	Trace Elements	Metals in milk powder	**FAP80527**
		Nutritional elements in infant formula	**FAP88659**
		Nutritional elements in milk powder	**FAP88819**
Fish and seafood	Allergens	Histamine in fish	**FAP79864**
	Nitrogen	Total Volatile Basic Nitrogen in fish	**FAP89089**
	Trace Elements	Metals in seafood	**FAP80466**
Fruits and vegetables	Biotoxins	Patulin in fruit	**FAP84209**
	Trace Elements	Metals in fruit products	**FAP80553**
		Metals in vegetable puree	**FAP80554**
Meat	Ash, Moisture, Nitrogen, Total Fat, Trace Elements	Nutritional in canned meat product	**FAP85276**
		Nutritional and Hydroxyproline in meat	**FAP84231**
Oils and fats	Organic Pollutants	PAH in oils and fats	**FAP79868**
		Pesticides and PCBs in fat	**FAP89005**
		Pesticides in oil	**FAP89004**
Processed food	Allergens	Nut allergen in biscuit	**FAP79867**
	Allergens, Protein	Nut allergen in choc, quant	**FAP79859**
Spices	Biotoxins	Mycotoxins in spices	**FAP80775**
	Trace Elements	Metals in spices	**FAP79875**

The complete range of food matrix materials can be browsed by matrix type and analyte type on
SigmaAldrich.com/foodmatrix.

Our full portfolio of reference materials can be found at **SigmaAldrich.com/Standards**

Certified Reference Materials of Brevetoxins

New product additions to our marine toxins certified reference materials range

Matthias Nold, Product Manager Reference Materials, Analytix@merckgroup.com

The pleasure of eating a good meal of fresh seafood can sometimes be abruptly dampened, if the food is contaminated by algal toxins accumulated through the food chain. Toxic algae can exponentially grow in unpredictably occurring algae blooms. Also, due to climate change such algae blooms can spread to new areas where they have been unknown before.

There is a big variety of naturally occurring marine toxins with very diverse chemical structures produced by various species of algae or phytoplankton. Some examples of marine toxin classes include amnesic shellfish toxins (domoic acid), diarrhetic shellfish toxins (okadaic acid and dinophysistoxins), and paralytic shellfish toxins (e.g. saxitoxin or neosaxitoxin).

Brevetoxins (BTX) are neurotoxins produced by the dinoflagellate Karenia brevis and are responsible for neurotoxic shellfish poisoning (NSP). Acute symptoms of NSP include nausea, vomiting, diarrhoea, parasthesia, cramps, bronchoconstriction, paralysis, seizures, and coma. Brevetoxins have complex cyclic polyether structures as shown in **Figure 1**.

Although neurotoxic shellfish poisoning (NSP) predominantly occurs in the Gulf of Mexico and the east coast of the US, it can also be found in other regions, such as New Zealand. Particularly notable was the NSP outbreak observed in the New Zealand Hauraki Gulf region in 1993. In these regions, the brevetoxins in shellfish are regulated. The US FDA and New Zealand sets the action level at 0.8 mg BTX-2 equivalents per kg shellfish (MPI BMS RCS 2018, ref US FDA). In Australia, the maximum level for BTX-group toxins is 20 MUs/100 g, but the BTX analogue is not specified (FSANZ, 2010). In the EU, brevetoxins are currently not regulated but the EFSA published a scientific opinion assessing the risks to human health related to the consumption of brevetoxin-(BTX) group toxins in shellfish and fish.[1]

While traditional methods such as the mouse bioassay or ELISA are still being used for detection of marine toxins, the use of LC-MS is gaining importance.[2] Therefore, the availability of well characterized, reliable reference materials is critical. One of the main challenges hereby is the limited availability of such materials. The toxins often need to be isolated from the producing algae, which is a very laborious process that typically yields only a few mg of purified material.
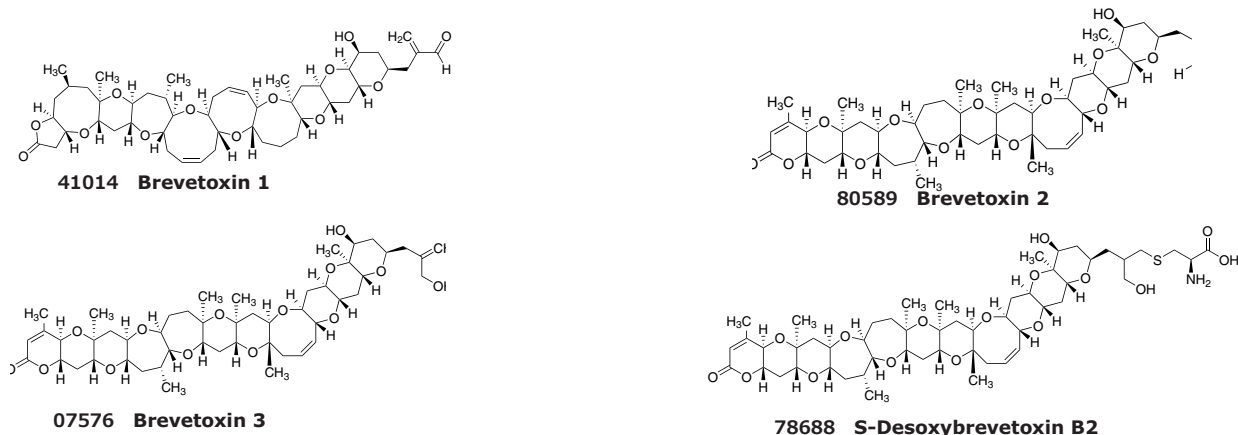

41014   **Brevetoxin 1**

80589   **Brevetoxin 2**

07576   **Brevetoxin 3**

78688   **S-Desoxybrevetoxin B2**

Figure 1. Chemical Structures of the Brevetoxins

In our ISO/IEC 17025 and ISO 17034 double accredited laboratory, we use a combination of quantitative NMR (qNMR) and Isotope Dilution MS (IDMS) enabling the manufacturing of Certified Reference Materials (CRMs) with very low quantities of starting materials.[3] A considerable number of marine toxin CRM solutions have been launched over the past years using this method. Recently, four new brevetoxins CRM solutions have been added to this range.

TraceCERT® Marine Toxin CRM Solutions for Brevetoxins

Description	Qty.	Cat No
Brevetoxin 1, 20 µg/g in acetonitrile	0.5 mL	**41014**
Brevetoxin 2, 20 µg/g in acetonitrile	0.5 mL	**80589**
Brevetoxin 3, 20 µg/g in acetonitrile	0.5 mL	**07576**
S-Desoxybrevetoxin B2, 20 µg/g in methanol	0.5 mL	**78688**

For more information and an up-to-date list of marine toxin CRMs please visit:
SigmaAldrich.com/marinetoxins

References:

1. EFSA Journal 2010; 8(7):1677

2. McNabb, Paul S.; Selwood, Andrew I.; et al. Journal of AOAC International, Volume: 95, Issue: 4, Pages: 1097-1105

3. Analytix Reporter, Issue 5, 2019

Determination of Water in Sunflower Oil by Karl Fischer Titration

Bettina Straub-Jubb, Product Manager Titration, Analytix@merckgroup.com

Sunflower oil is the most used cooking oil worldwide. It finds its use in food, as a frying oil, or in cosmetic products as a natural emollient. Additionally, it is used in the production of biodiesel and in some pharmaceutical and technical applications. Every year sunflower seeds produce more than 16 million tons of oil. Sunflower oil contains polyunsaturated fatty acids, such as linoleic acid, monounsaturated acids such as oleic acid, saturated fatty acids, and a high amount of vitamin E antioxidant.

The water content in sunflower oil influences the quality and shelf life of the oil and can chemically react with the oil's components, e.g. it can break up the ester bonds and form free fatty acids.

The amount of water in sunflower oil is typically 0.2 % or less, therefore the coulometric method is recommended and described in different norms e.g. DIN EN ISO 8534 - Animal and vegetable fats and oils -- Determination of water content -- Karl Fischer method (pyridine free).

Application Details

Special information concerning the sample

Due to the inadequate solubility of this sample in methanolic Karl Fischer reagent the addition of solubilizers (e.g. chloroform, decanol) is necessary. Because of the low water content of sunflower oil, coulometric Karl Fischer titration is the most appropriate method.

Titration method - Coulometry without diaphragm

Conditions	
Reagents	
Working Medium:	80 mL Anolyte Aquastar® (188079)
Solubilizer:	20 mL Decanol (**8.03463**)
Instrument parameters	**For end point indication (general recommendation)**
I(pol):	5 - 10 µA
U(EP):	50 - 100 mV
Stop Criterion:	drift < 10 µg/min
Sample size	
By weight:	~1 g (weight needs to be accurately determined)

Procedure

The Karl-Fischer reagent and solubilizer is placed in the titration cell without a diaphragm. The coulometer is started, and the solvent mixture is titrated dry. After pre-titration and stabilization of the drift, the sample (1 mL) is injected into the titration cell with a syringe (exact sample weight determination by weighing of syringe before and after injection) and the water content is determined. We recommend doing an instrument check before the sample titration and after a few sample titrations with a water standard 0.1 %.

Alternatively, we also have procedures available to determine water content in sunflower oils with the volumetric method and a low concentrated titrant. Find out more on our webpage at **SigmaAldrich.com/titration**

Featured Products

Description	Cat. No.
Anolyte for coulometric Karl Fischer Titration without diaphragm Aquastar®	**1.88079**
1-Decanol for synthesis	**8.03463**
Water standard 0.1% Standard for Karl Fischer Titration 1 g ≙ 1 mg H_2O Aquastar®	**1.88051**
Water Standard 0.01 % Standard for Karl Fischer Titration 1 g ≙ 0.1 mg H_2O Aquastar®	**1.88050**

Coulometric Titration— Faster and More Efficient

New Aquastar® coulometric reagent for Karl Fischer water determination

Bettina Straub-Jubb, Product Manager Titration, Analytix@merckgroup.com

Coulometric Karl Fischer titration is the most suitable method and especially evolved for determining very low levels of water in a wide variety of samples, providing accurate and reliable results and a fast titration.

The new coulometric Anolyte for cells without a diaphragm was formulated to improve coulometric titration, making the process even faster. Using this new reagent gives highly accurate and reproducible results.

Coulometry

The coulometric Karl Fischer Titration is an absolute method and therefore the method of choice for low water content samples. Coulometry generates iodine *in situ* through anodic oxidation at the generator electrode during the titration. Therefore, coulometric reagents do not contain iodine, but iodide. A very sensitive and accurate control of the iodine generation enables the precise determination of very low water concentration, down to 10 ppm.

The direct coulometric method is recommended only for liquid samples. For solid samples, an external water release is needed or the Karl Fischer oven technology can be used in combination with a coulometric titrator for samples who are thermally stable.

The coulometric Karl Fischer Titration does not require standardization, but a regular instrument check with an appropriate water standard is recommended.

New Karl Fischer Anolyte for cells without a diaphragm

Many samples dissolve easily in methanol - the alcohol used in coulometric reagents. However, some samples need the addition of co-solvent or buffer to dissolve or react properly. The new reagent can be used for a wide variety of samples, especially those that need a solubilizer or buffer to achieve accurate and precise titration results. Low water content in the range of 10 ppm to 10 000 ppm can be determined precisely and reproducibly. Oils, fats, ointments, strong acids, and bases can be determined with the addition of solubilizer or bases with a direct titration; and solid, insoluble samples, or samples causing side reactions with the Karl Fischer oven method or an external water release.

The new Anolyte can be used with all kind of samples and methods. It shows a very fast conditioning time and a very good drift stability. The sample titration is fast as well, and the results have an excellent precision.

This new coulometric Anolyte makes the water determination in samples with low water content much more efficient and accurate.

Benefits

- no crystallization
- extremely fast with efficient conditioning time
- very good drift stability
- rapid and reproducible results
- high accuracy and excellent precision

Description	Qty.	Cat. No.
Anolyte for coulometric Karl Fischer titration without diaphragm	500 mL	**1.88079**
Recommended Standards for Coulometry		
Water standard 0.1 % 1 mg/g water	10 x 8 mL	**1.88051**
Water standard 0.01 % 0.1 mg/g water	10 x 8 mL	**1.88050**
Water standard oil (15 -30 ppm)	10 x 8 mL	**1.88055**
Water standard oven 1%	5 g	**1.88054**

More about our Aquastar® Karl Fischer Reagents
SigmaAldrich.com/titration

For application support contact
aquastar@merckgroup.com

HPLC Tips & Tricks - Mobile Phase Preparation

Dr. Egidijus Machtejevas, Lead Expert, Analytical Science Liaison, Analytix@merckgroup.com

The most important part of a chromatographic set up is the column. But even though it provides retention, the final separation also depends strongly on the mobile phase (MP). The various effects offered by the mobile phase influence the retention and differential migration (selectivity) of the solutes through the column. Therefore, during method development and optimization, the separation might have to be tuned by changing mobile phase parameters, such as solvent type, additives (different buffers, ion pair reagents), or operating conditions (gradient time/steepness, temperature, flow rate). Often, problems with chromatographic separation are related to an incorrectly/inconsistently prepared mobile phase. Hence, an inclination to use simpler mobile phases can be observed for practical reasons of increased method robustness, easier method transfer, and ease of use (e.g. in clinical diagnostics or in process control).

Solvent/Mobile Phase Additive Purity

Most of you are familiar with the rule: "garbage in, garbage out." This adage is especially true when selecting the correct purity of mobile phase components. For example, it is an absolute must to use gradient grade solvents & reagents for gradient grade separations to get an accurate, reproducible and clean baseline (free of ghost peaks), and sensitive chromatographic separation (see also HPLC Tips and Tricks in Analytix Reporter 9 "Increase Your HPLC/UHPLC Method Sensitivity"). Using the correct and suitable grade of solvents based on the application (e.g. hypergrade for LC-MS for LC-MS methods, see more at SigmaAldrich.com/Solvents) also minimizes the chances of contaminations and extends the longevity of a chromatographic column. For cases requiring addition of any reagent like buffer, it is to be ensured that the reagent meets the required quality and has not passed its expiry date. The degradants from expired additives lead to ghost peaks in sample chromatograms. Certain additives degrade quicker, depending on their nature (for example 20 mM, pH 7 phosphate buffer). Improper/careless handling (for example left over solvent put back into bottle, bottle left on the lab bench without the cap closed, lost pipette tips floating inside the bottle and so on) of these reagents spoil chemicals quickly.

Mixing Mobile Phases

For isocratic separations with premixed mobile phases, solvent volumetric contractions in commonly used mixtures (water/acetonitrile, methanol or tetrahydrofuran) should be taken into account during their preparation. The only correct way to prepare such mobile phase mixtures is to separately take precisely measured volumes of the components and mix them. For example, to get a 70% organic mobile phase, 300 mL of water and 700 mL of organic solvent should be precisely measured separately and then combined together in a flask. But if only the water is measured precisely and the organic solvent is then added to make up the required final volume, due to the solvent mixture contraction, the resulting solvent strength will be a little higher (or weaker in case organic solvent was added first and water was added later). For premixing of MPs, attention should be taken to the toxic solvent fumes that might be emitted, under a fume hood.

Nowadays, gradients are generally correctly formulated using gradient pumps; however, some minor differences in retention behavior might be observed during comparison of the instruments with low-pressure and high-pressure gradient systems due to their mixing mechanisms.

Personally, in Reversed-Phase chromatography, I prefer HPLC methods with premixed mobile phases such as 5% acetonitrile in water and/or 5% water in acetonitrile. The rationale behind such preference is to increase degassing effectiveness, avoid mixture heating (e.g. methanol in water) or cooling (e.g. acetonitrile in water) upon mixing, and also to improve mixing efficiency by making the two mobile phases more similar in viscosity and surface tension. The limitations of such premixed solvents are that solvent strength of mobile phase B cannot be 100% and an extra step in mobile phase preparation – which is an additional, potential source of error. Typically, it is recommended to use mobile phase solvents directly out of their delivery containers to prevent additional chances of contamination.

The final step in the mobile phase preparation is filtration. There are many different types of filters that could be chosen based on the solvents being filtered. Recommended are membrane-type filters with pore sizes of at least 0.45 µm for HPLC systems, and 0.22 µm for UHPLC systems. Filtering removes particles from the prepared mobile phase and prevents clogging of the system and column.

To be continued in the next HPLC Tips & Tricks discussing mobile phase pH and buffer preparation issues.

In case you have any chromatography trouble, please contact us at **Analytix@merckgroup.com**

Unbeatable accuracy again and again

Because your work is time-critical and requires absolute precision, you need products you can rely on for all your demanding applications. That's why every product across the comprehensive Supelco® portfolio is meticulously tested against the tightest specifications to ensure the highest industry standards and compliance with applicable regulations. So for unbeatable accuracy every time, choose Supelco® analytical products.

For more information, please visit:

SigmaAldrich.com/SuccessReplicated
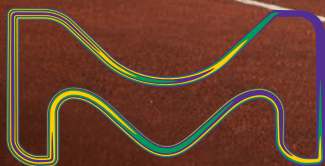
Supelco®
Analytical Products

To place an order or receive technical assistance

Order/Customer Service: **SigmaAldrich.com/order**
Technical Service: **SigmaAldrich.com/techservice**
Safety-related Information: **SigmaAldrich.com/safetycenter**

SigmaAldrich.com