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Analytix Reporter

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by Direct Injection of Samples Using a Monolithic Silica Column

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Analytix Reporter

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Our analytical products find their way into many different labs, industries and applications around the world. The more we correspond with you who use our products, the more we learn about what you need to provide reliable analytical results under sometimes challenging circumstances.

In this issue of the Analytix Reporter newsletter we cover topics where methods could be optimized, difficult analytical problems be solved or new solutions to an analytical challenge could be offered; all through collaboration with the scientific analytical community. Of course, we always respect confidentiality and we never share any sensitive information from our collaborators. Nevertheless, we often garner new information that we can share with you. Applications in this issue cover simplified analysis of an anti-parasite agent in seawater in an aquarium, sensitive iron detection by photometric tests, determination of PAHs in paprika powder, ginsenosides finger printing by HPTLC, on-line removal of phospholipids from plasma and serum samples prior to LC-MS, and a new superficially porous particle (SPP) column for improved biomolecule separation for mAbs QC. All these applications are complemented by a growing number of reference materials and high quality analytical solvents and reagents.

The topics in this newsletter came about through working on tricky problems in our analytical community. We hope you find them of interest and inspiration. If you have a tough analytical challenge then please let us know how we can help you.

Happy Analysis!

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ENVIRONMENTAL

Praziquantel Determination in Aquarium Water by Direct Injection of Samples Using a Monolithic Silica Column

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Introduction

Praziquantel (PZQ) is used in the aquarium industry to treat monogenea and other parasites. This is often done through a bath immersion treatment, where medications are dissolved in aquarium water rather than dosed directly to a target organism.

Depending on the details of the treatment, it can be done in a separate holding tank or directly in the system being treated. In order to determine whether the therapeutic level of the medication is reached, and for how long that level persists, it is important to test medication concentrations in the treatment water. The current methodology for the determination of PZQ in aquarium water utilizes solid phase extraction (SPE) filtration to separate the analyte from the aquarium water sample. The extract is then analyzed using high performance liquid chromatography (HPLC).

Treatments may not be monitored to the extent desirable due to the time and cost of materials involved in the analysis. The goal of this study was to simplify the analysis and to decrease overall analysis time primarily by eliminating the SPE extraction steps.

Experimental

The current method was developed by Walt Disney World Resort Epcot, The Living Seas,[1] which referenced a Journal of Chromatography article[2] for the determination of Praziquantel in serum. While the HPLC determination of this analyte in serum would require extraction because of the sample matrix, seawater in small injection volumes should not present a problem so long as the organic concentration of the mobile phase is kept low enough that salts will not precipitate from solution. At 40% acetonitrile in the mobile phase, the salts in the sample do not precipitate out of solution. By using a monolithic silica column, SPE sample prep can be eliminated because of this support's ability to tolerate dirty samples. Changes to acetonitrile concentration of the moblie phase, injection size, and a simplified sample prep increase the method's sensitivity and efficiency and add up to significant time savings. These improvements are further discussed below. Additionally, these changes reduce the acetonitrile consumption for sample processing and completely eliminate the use of methanol. This will reduce the amount of solvent waste generated by the method.

The following experiments were performed on a Waters ACQUITY Arc with PDA detection at 220 nm.

Results/Discussion

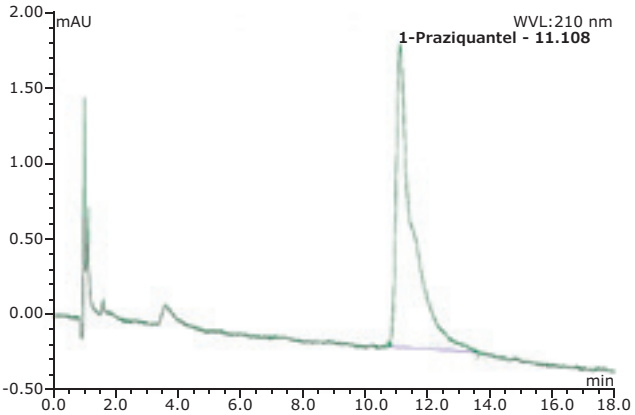
Separation Improvements

The chromatogram in **Figure 1** is from the Georgia Aquarium. It is a 50 µL injection, and mobile phase is 40% acetonitrile (ACN). Because of the fairly large injection volume, the Praziquantel peak is overloading the column as evidenced by poor peak shape.

A Chromolith® HighResolution RP-18 endcapped 100-4.6 HPLC column was used for the remainder of the chromatograms presented below.

The analysis in **Figure 1**, that utilized a Partisil ODS3 column, was duplicated on a Chromolith HR. **Figure 2** (purple line) is a 50 µL injection of a 2 mg/L PZQ standard in ACN. The analysis time was greatly reduced from 14 minutes in **Figure 1** to about 2 minutes on the Chromolith column. Peak symmetry was greatly improved with the Chromolith column. To simulate direct injection of a seawater sample, a standard was prepared in water containing 3.5% NaCl (**Figure 2**, green line). In this chromatogram you can see the baseline disturbance of the injection volume as the injection solvent elutes in the dead volume.

Figure 1. 1 mg/L PZQ in salt water, 40% ACN, 50 µL injection



HPLC:	Dionex UltiMate 3000
Column:	Partisil 5µm ODS3 250x4.6mm
Mobile phase:	40% acetonitrile (ACN)
Gradient:	Isocratic
Flow Rate:	1.6 mL/min
Column Temp:	30°C
Detector:	UV/Vis at 210 nm
Injection:	50 µL
Sample:	1mg/L PZQ in artificial salt water

(continued on next page)

Figure 3 is again a 2 mg/L PZQ sample injected on a Chromolith HR, but the injecton volume was reduced to 10µL. Peak resolution and shape are significantly improved while sensitivity is maintained even with the lower injection volume.

In **Figure 4**, a reduction in acetonitrile concentration in the mobile phase is studied. The mobile phase flow rate was also reduced to 1 mL/min. The acetonitrile concentrations are from 40% acetonitrile (purple trace) to 60% (yellow trace).

For the remainder of these experiments, a 50% acetonitrile mobile phase was used (represented by the green trace chromatogram in **Figure 4**) as it presents a good compromise of analysis time and solvent use.

At this point we have optimized the SPE separation from the original Disney technique to decrease organic solvent usage, and improve separation efficiency. In the next section, we demonstrate sample preparation without SPE.

Sample Preparation Improvements

Figure 5 is a 10 µL direct injection (no SPE) of a treatment water sample from Georgia Aquarium containing 5.2 mg/L PZQ. This demonstrated that the elimination of sample prep did not adversely affect the analysis.

Treatment samples that were received from Georgia Aquarium were spiked with 10mg/L PZQ. In **Figure 6** the original sample (green trace) containing 5.2mg/L PZQ and the spiked sample (purple trace) are overlaid.

Figure 2. PZQ in salt water (green) and in acetonitrile (purple), 2 mg/L PZQ, 40% ACN, 50 µL injection.

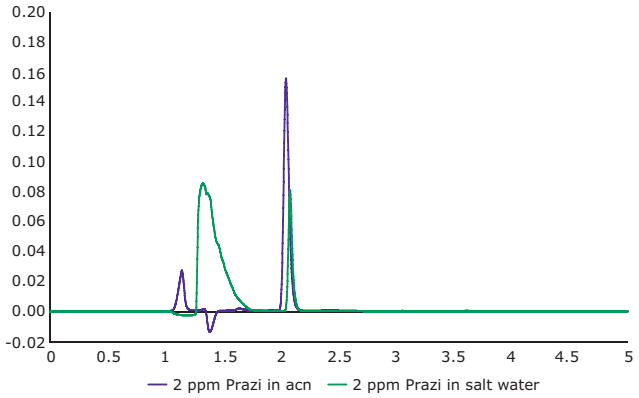


Figure 3. PZQ in salt water (green) and in acetonitrile (purple), 2 mg/L, 40% ACN, 10 µL injection.

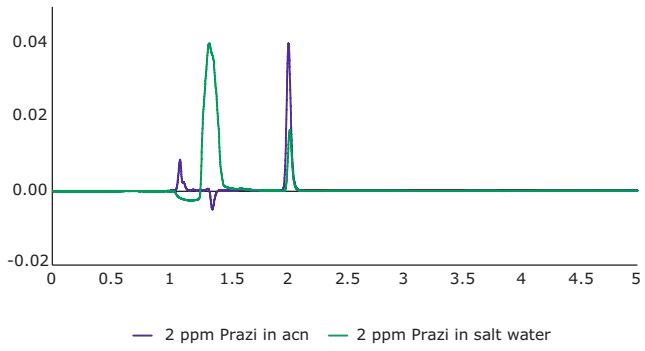


Figure 4. Acetonitrile concentration options, 2mg/L PZQ, 1 mL/min flow rate

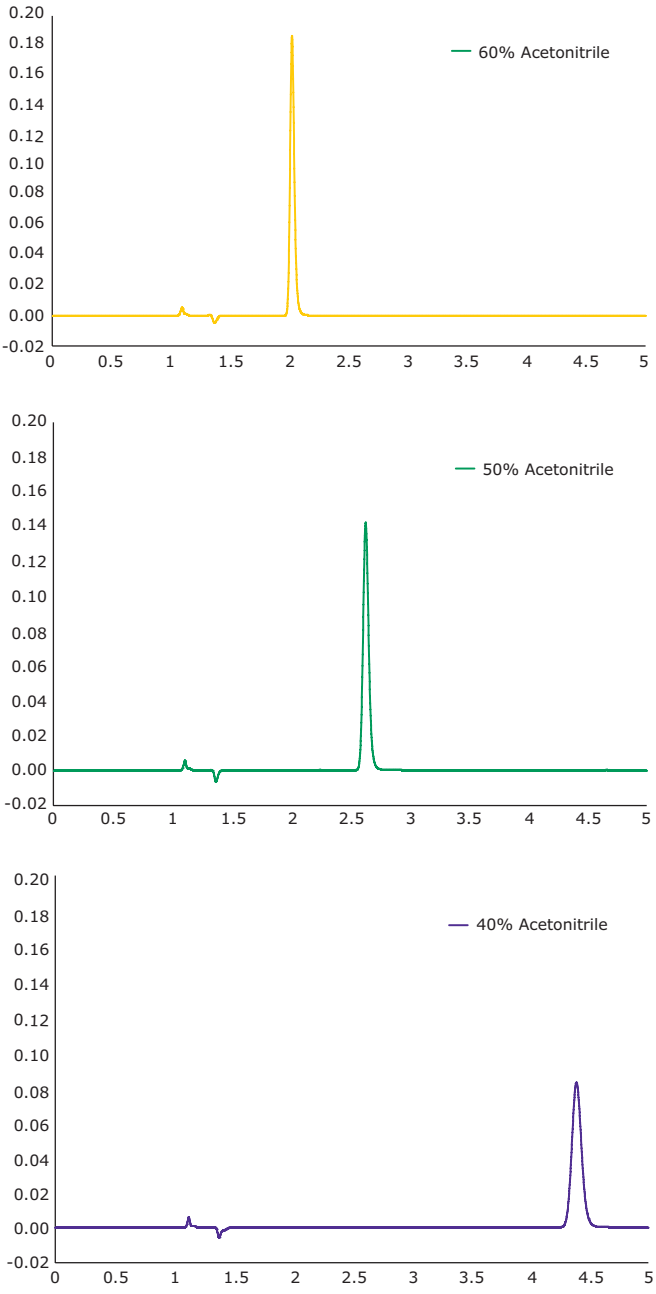


Figure 5. Sample NH03-3 from Georgia Aquarium analyzed without prior SPE extraction

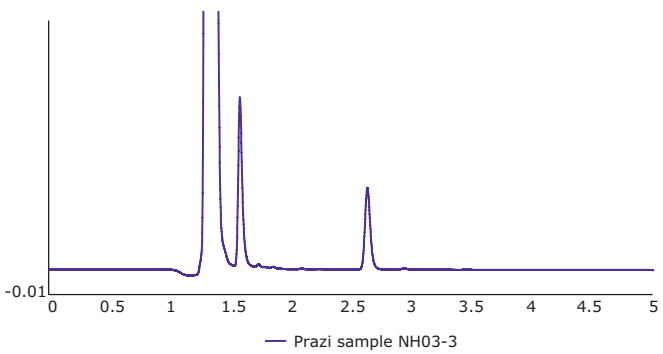


Figure 6. Sample NH03-3 from Georgia Aquarium, direct injection (green) and spiked with 10mg/L PZQ (purple)

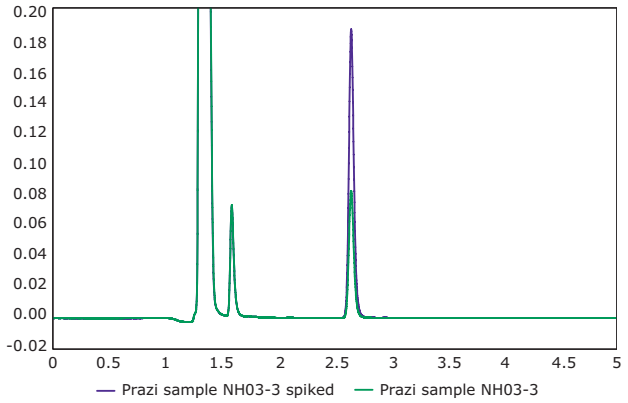


Figure 7. Calibration curve of 5 standards from 1.25mg/L to 20mg/L PZQ in ACN

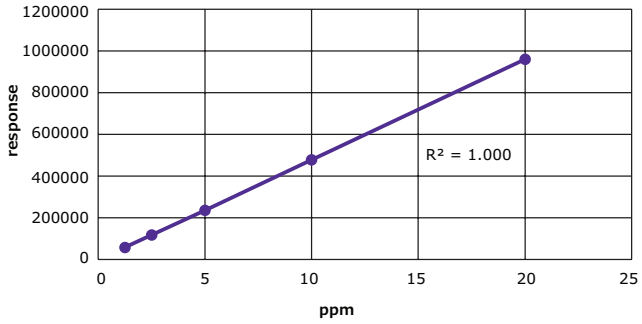


Figure 8. Chromatogram overlay of 6 PZQ standards, 0.625mg/L

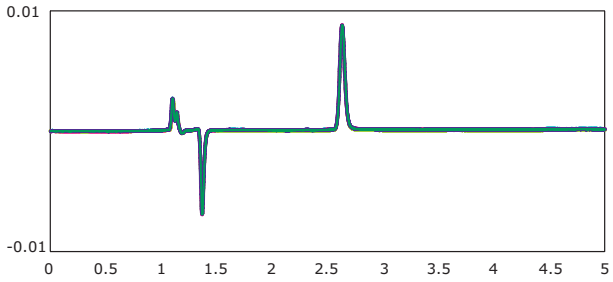
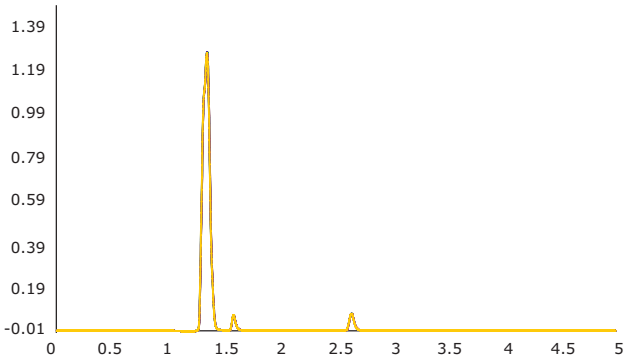


Figure 9. 10 injections from a series of 500 repeat injections of a composite sample from PZQ treatments at Georgia Aquarium



Spike recovery was calculated and is displayed in **Table 1** for several treatment samples.

Table 1. Spiked recoveries of treatment samples from Georgia Aquarium

Sample	Determined Amount	Spike Amount	Spike Recovery
NH03-6 spiked	10.02	10.00	96.92%
NH03-6	0.33		
NH03-4 spiked	14.96	10.00	96.50%
NH03-4	5.31		
Water blank spiked	9.71	10.00	97.11%
Water blank	0.00		
3.5% Salt Water blank spiked	9.75	10.00	97.49%
3.5% Salt Water blank	0.00		

The HPLC system was calibrated using 5 standards prepared in ACN between 1.25 and 20 mg/L Praziquantel. The calibration curve and data are displayed in **Figure 7** and the correlation is 1.000.

The method detection limit (MDL) was calculated from 6 replicate injections of a 0.625mg/L standard. MDL was calculated to be 0.015mg/L using the students' t-test method based on the data represented in **Table 2** and **Figure 8**.

Table 2. Replicate analysis of a 0.625mg/L standard used to calculate MDL

Replicate	1	2	3	4	5	6	Avg	Sd	MDL*
ppm	0.674	0.675	0.676	0.686	0.678	0.675	0.677	0.004	0.015

*Students' t -test

To evaluate if column life will be affected by multiple injections of the seawater sample matrix, a series of 500 injections of a composite of the samples provided by Georgia Aquarium was performed. **Figure 9** is an overlay of 10 injections from the 500 injection sequence. These injections were equally spaced (every 50 injections) throughout the sequence. Both retention time and response remained stable over 500 injections. The column pressure did increase throughout the run from about 950 psi to 1800 psi. The lower column pressure at the beginning of the 500 injection series was easily restored by flushing the system with 90% water and 10% ACN. This showed that the column and HPLC system can be flushed clean. It is recommended to include a similar flush step at the end of each sequence of samples to properly maintain system pressure.

Table 3 is a comparison of the method described in this article with the original SPE method for detecting PZQ in seawater. The samples are from treatments carried out at Georgia Aquarium. The results of the two methods are comparable.

(continued on next page)

Table 3. A comparison of the SPE method run at Georgia Aquarium and the Chromolith method described in this article.

Sample Name	Retention Time	Chromolith Method Result	Result from GA Aquarium SPE Method
Prazi sample NH03-1	2.635	nd	nd
Prazi sample NH03-2	2.645	5.17	5.1692
Prazi sample NH03-3	2.644	5.21	5.3906
Prazi sample NH03-4	2.644	5.32	5.6451
Prazi sample NH03-5	2.645	4.34	5.4443
Prazi sample NH03-6	2.646	0.20	1.3211
Prazi sample NH03-7	2.635	nd	nd
Prazi sample NQ01-1	2.635	nd	0.0627

Conclusions

With the new method, HPLC analysis time was reduced from 18 minutes per sample to 5 minutes, which provided time and cost savings for the lab. Because the monolithic column will tolerate "dirty" samples, seawater samples can be directly injected into the instrument, eliminating SPE sample prep. This reduces analysis time by an additional 20 minutes per sample. Total time saved is about 30 minutes per sample, as outlined in **Table 4**.

Table 4. A comparison of the time required for the SPE method and the method described in this article.

	Time Per Sample (min)	
Method Step	SPE Method	New Method
SPE Extraction	20	0
Sample Prep for HPLC	3	3
HPLC Analysis	18	5
Total Time Spent:	41	8

In addition, 84% less acetonitrile is consumed due to elimination of extraction solvents and shorter analysis time. This makes it a greener method by reducing solvent waste produced in the lab.

The new method provides reliable and sensitive results, with recoveries >95% and a method detection limit (MDL) of 0.015 mg/L. The development of the new method makes Praziquantel testing more efficient and cost effective, which will allow an increase in the measurement frequency for monitoring treatments.

Instrument and Conditions

Final Method:	
instrument:	Waters Acquity Arc with PDA
column:	Chromolith® High Resolution RP-18 Endcapped 100 x 4.6 mm (1.52022)
mobile Phase:	50% Acetonitrile (AX0142) / 50% Water (Milli-Q)
flow Rate:	1 mL/min.
column Temp:	30 °C
detector:	PDA @220 nm
injection Volume:	10 µL
sample:	Seawater (Filtered)

References

1. Crowder, J. and T. Charanda. 2004. Determination of Praziquantel in seawater. 1st AQUALITY symposium. Oceanario De Lisboa, Portugal.
2. Xiao, S., B.A. Catto, and L.T. Webster, Jr. 1983. Quantitative determination of Praziquantel in serum by high-performance liquid chromatography. Journal of Chromatography, 275: 127 – 132.

Featured Products

Description	Cat.No.
HPLC	
Chromolith® High Resolution RP-18 Endcapped 100-4.6	**1.52022**
Solvents	
Acetonitrile: Omnisolv®*	**AX0142**
Water was from a Milli-Q® Advantage lab water system	
Standard	
Praziquantel VETRANAL™, 250 mg	**46648**

*only available in North America

Related Products

Chromolith® High Resolution RP-18 Endcapped 150-4.6	**1.52023**
Chromolith® HighResolution RP-18 Endcapped 5-4.6 guard cartridges (3 pieces)	**1.52025**
Chromolith® 5-4.6 guard cartridge holder	**1.52032**
Acetonitrile gradient grade for liquid chromatography LiChrosolv®	**1.00030**

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ENVIRONMENTAL

Sensitive Determination of Iron in Drinking Water, Mineral Water, Groundwater, and Spring Water Using Rapid Photometric Tests

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The quality of drinking water is regulated by a variety of guidelines, such as the EU Council Directive 98/83[1,2] and WHO guideline.[3] The key principles used to define these limits consider both health hazards and sensory and technical reasons. Iron, for example, does not exhibit a risk for health in concentrations usually found in drinking water.[2,3] However, increased concentrations of iron result in the formation of iron hydroxide products, which can form deposits in water pipe systems and a brown discoloration of the water.[4]

To ensure the supply of clear and colorless water, country-specific limits have been set for drinking water. The limit for iron set by the EU directive is 0.2 mg/L Fe,[2] while the U.S. EPA specifies 0.3 mg/l Fe.[5] To prevent the formation of iron deposits in water pipe systems, a limit of 0.02 mg/L should not be exceeded.[6] To ensure that the specified limits are met, drinking water is, in many cases, subjected to a treatment step in which the iron is precipitated. This method virtually eliminates any iron content, reducing the iron concentration to the lower ppb range.[6]

Analytical methods

Highly sensitive analytical methods for trace level quantification include flame atomic absorption spectroscopy (flame AAS, F-AAS) and optical emission spectrometry with inductively coupled plasma (ICP-OES). Depending on the dosage volume, the measuring range of the F-AAS method according to DIN EN ISO 38406-32 is 0.002–0.020 mg/L Fe. The limit of quantification (LOQ) for the ICP-OES method according to DIN EN ISO 11885 is 0.002 mg/L Fe.[7,8] In our lab an LOQ of 0.0007 mg/L Fe is achieved by ICP-MS according to the ICH Q2 standard.

Analysis of iron using analytical test kits (rapid photometric methods)

A practical alternative for swift, sensitive results without investment in expensive instruments are rapid photometric methods. Test kits are generally characterized by their ease of use and speed of the procedure. The choice of the method depends on the application, the measuring range, and the required accuracy. In the case of iron, two sensitive photometric methods can be chosen.

The determination of iron using the 1,10-phenanthroline method according to APHA 3500-Fe B and DIN 38406-1 enables photometric measurement down to a level of 0.01 mg/L, which is entirely sufficient for many samples.[9]

If lower LOQs are required, the triazine method can be chosen. In this method, all iron ions are reduced to iron (II) ions. These react in a thioglycolate-buffered medium containing a triazine derivative to form a red-violet complex, which is subsequently determined photometrically.[10] Using a 100 mm cell and the Prove 600 UV-VIS spectrometer, LOQs for iron as low as 0.0025 mg/L can be achieved. Due to iron removal treatment and the naturally low iron content of most drinking water, preference should be given to the more sensitive triazine method. The Spectroquant® Iron Test (Cat. No. 114761) has an overall measuring range of 0.0025-5.00 mg/L Fe. In the Spectroquant® photometers, the methods are pre-programmed, so no time-consuming calibration curve must be created.

Sample preparation and performance of the measurement with Spectroquant® Iron Test

Samples must first be acidified with nitric acid to stabilize the iron, while carbonic acid-containing samples must also be degassed in an ultrasonic bath. A detailed description of the measurement procedure is given in the application “Sensitive Measurement of Iron in Water”.[11]

Method comparison of ICP-MS vs. Spectroquant® Iron Test

The iron content of five different mineral waters was determined by Spectroquant® test kit and ICP-MS. All samples were below the LOQ of the respective method (0.0007 mg/L for ICP-MS, 0.0025 mg/L for Spectroquant® test kit.

The five samples were spiked with iron at three different concentration levels by standard addition, and the respective recovery rates were determined by the photometric method. The results are shown in **Table 1** and **Figure 1**.

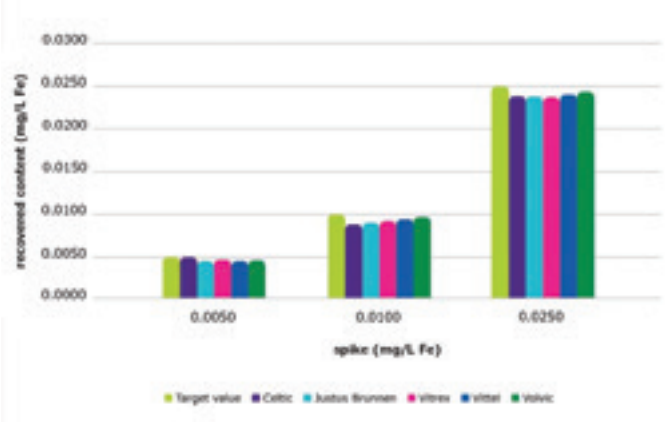
The added concentrations of iron were accurately recovered. The recovery rates in the spiked samples ranged between 89% and 99% over all experiments, with an average recovery rate of 95%.

(continued on next page)

Table 1. Iron content recovered after standard addition

Mineral water	Addition [mg/L Fe]	Recovered concentration [mg/L Fe]	Recovery rate
Celtic natural	0.0050	0.0050	99%
	0.0100	0.0089	89%
	0.0250	0.0239	96%
Justus Brunnen medium	0.0050	0.0046	91%
	0.0100	0.0091	91%
	0.0250	0.0239	96%
Vitrex natural	0.0050	0.0048	95%
	0.0100	0.0093	93%
	0.0250	0.0238	95%
Vittel natural	0.0050	0.0046	91%
	0.0100	0.0095	95%
	0.0250	0.0241	97%
Volvic natural	0.0050	0.0047	93%
	0.0100	0.0098	98%
	0.0250	0.0244	98%

Figure 1: Results of the standard addition



An even higher accuracy can be achieved by a custom calibration curve. **Table 2** shows the performance characteristics of the pre-programmed method for Cat. No. 114761 determined according to DIN 38402 A51 and ISO 8466-1 compared with a manually made calibration curve for the measurement range 0.0005 – 0.0100 mg/l Fe using the photometric test kit. The calibration curve is shown in **Figure 2**.

At 4.35%, the coefficient of variation of the custom calibration curve is 3.3 times higher than that of the pre-programmed method. This is due to the fact that at these lower concentrations, the deviations have a stronger relative effect in the custom calibration. Seen in absolute terms, the custom calibration procedure provides considerably lower method errors, as shown by the values of the method standard deviation and the method confidence interval for P=95%, which are 13 to 14 times lower than those of the pre-programmed method.

In the case of the standard additions, the use of such a custom calibration resulted in a further enhancement of the recovery rate, which now achieved a mean value of 101%. The individual values are between 95% and 106% (see **Table 3**).

Figure 2: Calibration curve for the measuring range 0.0005–0.0100 mg/L Fe

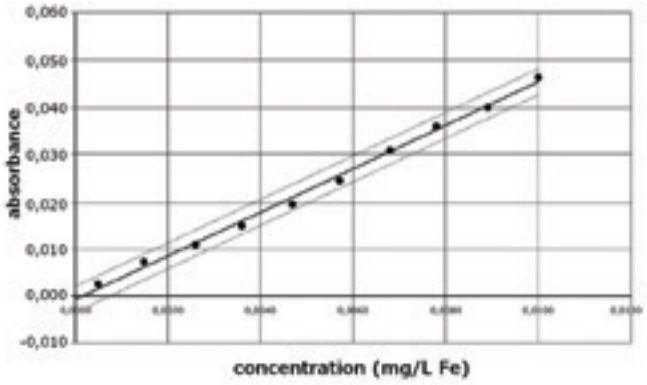


Table 2: Comparison of performance characteristics

	Pre-programmed method 0.0025 – 0.5000 mg/L Fe	Custom calibration 0.0005 – 0.0100 mg/L Fe
Method standard deviation [mg/L]	± 0.00328	± 0.00023
Method coefficient variation [%]	± 1.31	± 4.35
Confidence interval (P=95 %) [mg/L]	± 0.0079	± 0.0006

Table 3: Iron content recovered after standard addition with custom calibration

Mineral water	Addition [mg/L Fe]	Recovered concentration [mg/L Fe]	Recovery rate
Celtic natural	0.0050	0.0053	106%
	0.0100	0.0095	95%
	0.0250	0.0255	102%
Justus Brunnen medium	0.0050	0.0049	97%
	0.0100	0.0097	97%
	0.0250	0.0255	102%
Vitrex natural	0.0050	0.0051	102%
	0.0100	0.0099	99%
	0.0250	0.0254	102%
Vittel natural	0.0050	0.0049	97%
	0.0100	0.0102	102%
	0.0250	0.0257	103%
Volvic natural	0.0050	0.0050	99%
	0.0100	0.0105	105%
	0.0250	0.0261	104%

Since mineral waters have only low iron content, the experiments were also carried out using samples of groundwater and spring water, whose iron concentrations are naturally higher due to the lack of any water treatment. The measurement was carried out using the pre-programmed method. Here again the measurement results were verified by reference analysis using the ICP-MS method. **Table 4** shows a comparison of the results obtained with the two methods.

Table 4: Iron content of groundwater and spring water – comparison of ICP-MS and Spectroquant® Iron Test 114761

	Concentration [mg/L Fe]	
Groundwater and spring water	ICP-MS	Spectroquant® Iron Test 114761
Spring water Bad König	0.0047	0.0041
Spring water Höchst Himmelsleiter	0.0043	0.0051
Spring water Breitenbrunn	0.0022	< 0.0025
Spring water Vielbrunn	0.0017	< 0.0025
Spring water Rai-Breitenbach	0.0059	0.0051
Groundwater Bensheim	2.70	2.71

The results yielded by the Spectroquant® Iron Test are in agreement with those obtained using the ICP-MS method. Due to the very high iron content of the Bensheim groundwater sample of 2.7 mg/L Fe, in deviation from the defined procedure, a 10 mm cell was used. The recovery rate here was 100%. These results show that even very high concentrations of iron can be precisely determined by means of the iron test.

In the case of the low iron concentrated spring water samples, the measurement results differed only by a maximum value of 0.0008 mg/L. Even those iron concentrations that are below the LOQ of the photometric method were confirmed by the ICP-MS measurements.

Summary

The Spectroquant® Iron Test offers a good alternative to ICP or AAS when it comes to determining the iron content in drinking water, mineral water, groundwater, and spring water. The method yields results comparable to those obtained by the ICP-MS method and is easy to perform. For all laboratories for which the purchase of an ICP-OES or ICP-MS system is inexpedient for economic reasons, the Spectroquant® Iron Test Cat. No. 114761 offers a swift, sensitive, and precise alternative for the determination of the iron content of drinking water, mineral water, groundwater, and spring water.

Chemicals, samples, and instruments used:

All measurements were conducted using a Prove 600 photospectrometer. The reference system was a Thermo Fisher Scientific HR-ICP mass spectrometer (method on the Element 2 device).

Featured products

Description	Cat. No.
Spectroquant® Prove 600 UV/VIS spectrophotometer 1,8 nm spectral bandwidth	173018
Spectroquant® Iron Test 0.0025-5.00 mg/L Fe	114761
Iron Standard Solution CertiPUR® 1000 mg/l in 0.5 mol/l HNO_3	119781
Nitric acid 65% for analysis EMSURE® ISO	100456
Water Ultrapur	101262

To read more about the Spectroquant® line for Spectrophotometric Analysis visit us at **SigmaAldrich.com/spectroquant**

References

1. Ordinance of the DVGW (Deutscher Verein des Gas- und Wasserfach, German Technical and Scientific Association for Gas and Water), Verordnung über die Qualität von Wasser für den menschlichen Gebrauch, TrinkwV 2001, edition of 10 March 2016.
2. Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption, 1998.
3. World Health Organization, Guidelines for Drinking Water Quality, 2011.
4. H. D. Belitz, W. Grosch, P. Schieberle, Lehrbuch der Lebensmittelchemie, Springer Berlin Heidelberg, 2001.
5. United States Environmental Protection Agency, 2012 Edition of the Drinking Water Standards and Health Advisories, EPA 822-S-12-001, 2012.
6. DVGW (Deutscher Verein des Gas- und Wasserfaches, German Technical and Scientific Association for Gas and Water), Enteisenung und Entmanganung; Teil 1: Grundsätze und Verfahren, Arbeitsblatt W 223-1-2005-02, 2005.
7. DIN 38406-32:2000-05, Deutsche Einheitsverfahren zur Wasser, Abwasser- und Schlammuntersuchung – Kationen (Gruppe (E), Bestimmung von Eisen mittels Atomabsorptionsspektrometrie, 2000.
8. DIN EN ISO 11885:2009-09, Wasserbeschaffenheit – Bestimmung von ausgewählten Elementen durch induktiv gekoppelte Plasma-Atom-Emissionsspektrometrie (ICP-OES), 2009.
9. Package leaflet Spectroquant® Iron Test, Cat. No. 100796, June 2016.
10. Package leaflet Spectroquant® Iron Test, Cat. No. 114761, June 2016.
11. https://www.SigmaAldrich.com/catalog/product/mm/114761

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FOOD & BEVERAGE

Determination of PAHs in Paprika Powder Using a Novel SPE Cartridge

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Introduction

Paprika is a spice made from dried sweet peppers, and is used for flavor and color in many types of cuisine. Contamination with polynuclear aromatic hydrocarbons (PAHs) can occur when pepper plants are exposed to these pollutants in the environment and/or during the drying process. The use of herbs in cooking and food production has become increasingly popular, thus exposure to PAHs, specifically those with carcinogenic properties, is of concern. The European Union (EU) has gone so far as to regulate the maximum allowable levels of several PAHs in dried herbs and spices. Specifically for paprika, the maximum allowable levels have been set to 10 ng/g for benzo[a]pyrene and 50 ng/g total for the sum of benzo[a]pyrene, chrysene, benzo[a]anthracene and benzo[b]fluoranthene.[1]

The sample preparation methods used in the testing of PAHs in herbs and spices require solvent extraction, followed by a cleanup step. The cleanup methods that have been used include gel permeation chromatography (GPC), and solid phase extraction (SPE) with silica gel. [2,3] In the last several years, a new approach for cleanup of high background samples to be analyzed for PAHs has utilized a dual layer SPE cartridge containing a Florisil® top layer and Z-Sep/C18 bottom layer. This cartridge, the Supelclean™ EZ-POP NP, has been used for direct extraction of PAHs from edible oils and for cleanup of extracts of biota samples.[4,5] In this work, PAHs were analyzed from paprika samples using an optimized QuEChERS extraction followed by cleanup with the EZ-POP NP SPE cartridge. Compared to QuEChERS cleanup with loose sorbents, the EZ-POP NP yielded a much cleaner extract. Optimization of the extraction and EZ-POP NP cleanup procedures resulted in recoveries of >70% at a spiking level of 10 ng/g; calculated without internal standard correction.

Experiment

Paprika samples were obtained at a local grocery store. Samples for evaluation of method accuracy and reproducibility were spiked at 10 ng/g with a mixture containing the PAHs listed in **Table 1**. After spiking, samples were allowed to equilibrate for 1 hour prior to extraction. Sample extracts were prepared using the procedure described in **Figure 1**. Extracts were then subjected to SPE cleanup using the EZ-POP NP cartridge, as detailed in **Figure 2**. Analysis was done by GC/MS/MS using the conditions shown in **Table 2** and the transitions in **Table 1**. An internal standard mixture was added to the extracts just prior to the GC analysis, and was used only for monitoring instrument performance. Samples were quantitated against a matrix-matched calibration curve prepared in unspiked paprika extract.

Table 1. PAHs Analyzed and Transitions Used for GC/MS/MS Analysis

Peak #	Compound	RT	MRM 1	CE	MRM 2	CE
1	Naphthalene-d_8 (IS)	5.21	136/108	20	136/84	25
2	Naphthalene	5.25	128/102	20	128/78	20
3	Acenaphthylene	7.62	152/126	30	152/102	30
4	Acenaphthene	7.88	153/127	30	152/126	30
5	Fluorene	9.2	166/165	15	165/164	20
6	Phenanthrene	13.5	178/152	25	176/150	25
7	Anthracene	13.71	178/151	30	176/150	25
8	Fluoranthene-d_{10} (IS)	20.76	212/208	35	210/208	15
9	Fluoranthene	20.91	202/152	30	201/200	15
10	Pyrene	22.95	201/200	15	200/174	25
11	Benz[a]anthracene	29.42	228/226	30	114/101	10
12	Cyclopenta[cd]pyrene	29.72	226/225	30	226/224	40
13	Chrysene	29.81	228/226	30	113/112	10
14	5-Methyl chrysene (IS)	31.7	242/241	20	241/239	30
15	Benzo[b]fluoranthene	33.60	252/250	30	126/113	10
16	Benzo[k]fluoranthene	33.67	252/250	30	126/113	10
17	Benzo[j]fluoranthene	33.71	252/250	30	126/113	10
18	Benzo[a]pyrene	34.64	252/250	35	125/124	10
19	Perylene-d_{12}	34.84	264/260	35	130/116	15
20	Dibenz[a,h]anthracene	37.13	278/276	35	125/124	10
21	Indeno[1,2,3-cd] pyrene	37.2	138/137	10	137/136	15
22	Benzo[g,h,i]perylene	38.13	138/137	15	137/136	15
23	Dibenzo[a,l]pyrene	41.77	302/301	20	302/300	40
24	Dibenzo[a,e]pyrene	43.27	302/301	40	302/300	40
25	Coronene (IS)	43.76	300/299	35	300/298	50
26	Dibenzo[a,i]pyrene	44.29	302/301	40	302/300	40
27	Dibenzo[a,h]pyrene	44.68	302/301	20	302/300	40

Results & Discussion

GC/MS/MS Conditions. The analysis conditions were optimized for PAHs. Higher injection port and MS temperatures, along with a pressure pulsed injection and a 2 mm ID inlet liner, were used to improve response and peak shape. The oven temperature program was based on that used by Anderson et al., and was necessary to provide chromatographic resolution of isomers such as benzo[b], [k] and [j] fluoranthene, that cannot be distinguished by their MRM transitions[6]. This temperature program, in combination

(continued on next page)

Figure 1. Sample Extraction Procedure Used for PAHs from Paprika

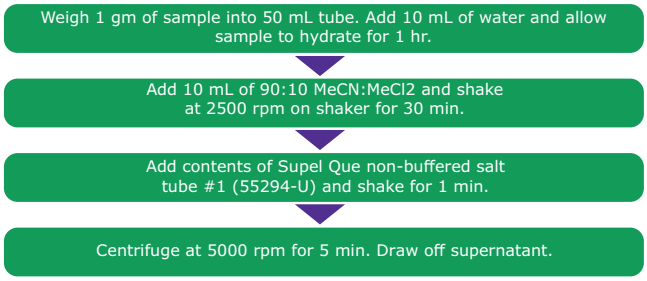


Figure 2. Cleanup Procedure Using EZ-POP NP SPE for Paprika Extract

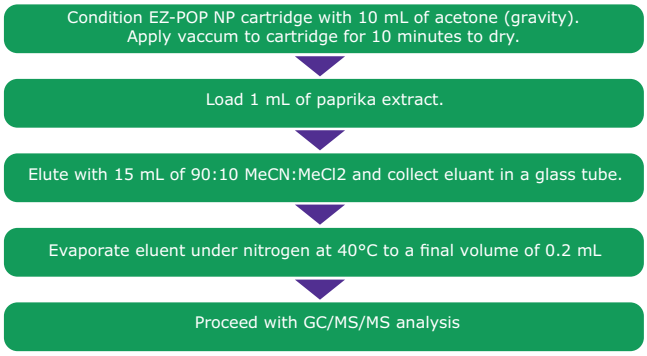


Figure 3. GC/MS/MS Analysis of Benzo [b], [k], and [j] Fluoranthenes on the SLB®-PAH MS Columns; 10 ppb in Paprika Extract

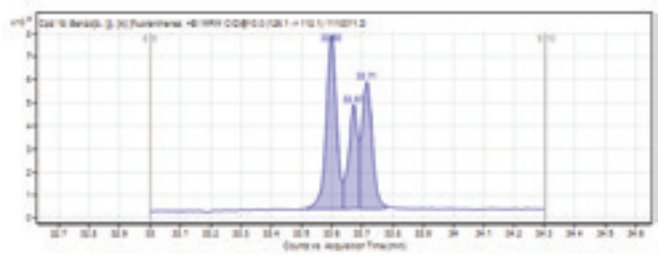


Figure 4. Effect of Extraction Solvent on Recovery of 5 & 6 Ring PAHs from Spiked Paprika (10 ng/g)

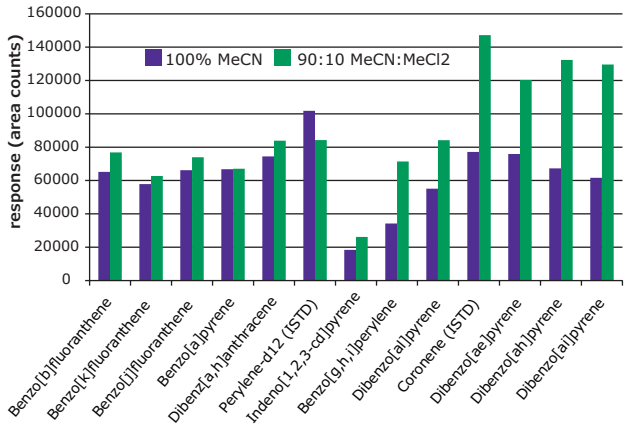


Table 2. GC Analysis Conditions

column	SLB®-PAHms, 30 m x 0.25 mm I.D., 0.25 µm (28340-U)
oven	60 °C (1 min), 40 °C/min to 180 °C, 3 °C/min to 230 °C, 1.5 °C/min to 235 °C (10 min), 15 °C/min to 280 °C (3 min), 15 °C/min to 350 °C (15 min)
inj. temp	300 °C
carrier gas	helium, 0.8 mL/min, constant
detector	MRM, per Table 1
MS source temp.	250 °C
Quadrupole temps	200 °C (Q1 & Q2)
injection	1 µL, pulsed splitless (60 psi until 0.75 min, splitter on at 0.75 min)
liner	2 mm I.D. FocusLiner™ with taper

with the GC capillary column (SLB-PAHms), provided adequate resolution of all isomeric sets. An example of this is shown in **Figure 3** for the isomers benzo [b], [k] and [j] fluoranthene. These isomers cannot be separated on a 5% phenylmethylsilicone or "X-5" type phase.

Optimization of Sample Preparation Procedure. Sample extraction was done based on a "QuEChERS-like" approach. The composition of the extraction solvent was optimized from the 100% acetonitrile normally used for QuEChERS to increase recovery of the heavier PAHs. **Figure 4** presents a comparison of absolute response for the heavier 5 & 6 ring PAHs obtained from paprika spiked at 10 ng/g, and extracted with 100% acetonitrile and a mixture of 90:10 acetonitrile:methylene chloride. The resulting extracts were analyzed before SPE cleanup to evaluate the effect of the extraction solvent only. The 5&6 ring PAHs showed better recovery using the 90:10 mixture, as indicated by the higher responses. As expected, the extract produced using the 90:10 mixture showed more background, visible as oily residue. For this reason, higher percentages of methylene chloride in the extraction solvent were not evaluated.

For cleanup, the EZ-POP NP SPE cartridge provided better cleanup than QuEChERS. The EZ-POP NP cartridge was originally developed for analysis of PAHs from edible oils, specifically olive oil. Acetonitrile is the elution solvent recommended for use with edible oil samples, as it provides a balance between analyte recovery and background retention. Previous work showed that the addition of methylene chloride increased PAH recovery; but it had the unwanted side effect of increasing the amount of background in the final extract.[4] However, since the solvent extracts of the paprika contained substantially less oil, it was possible to modify the cleanup procedure to increase recoveries of the 5 and 6 ring PAHs without increasing background to an unacceptable level. Specifically, the composition of the elution solvent was changed to include methylene chloride. **Figure 5** shows a comparison of absolute response obtained for 4-6 ring PAHs using different elution solvents during the EZ-POP NP cleanup. The samples processed were paprika (10 ng/g spiking level) extracted using 90:10 acetonitrile:methylene chloride. The responses of all PAHs increased with the addition of methylene chloride to the elution solvent. The difference in responses between 10 and 20% addition was minimal for some compounds; however, the background in the final extract did increase. Thus, 90:10 acetonitrile:methylene chloride was chosen as the elution solvent to be used in the EZ-POP NP cleanup.

Background Reduction. In the case of dry samples such as paprika, QuEChERS cleanup using loose sorbents does not provide enough capacity for reducing background

Figure 5. Effect of Elution Solvent Composition Used During EZ-POP NP Cleanup on Recovery of 4-6 Ring PAHs from Spiked Paprika (10 ng/g)

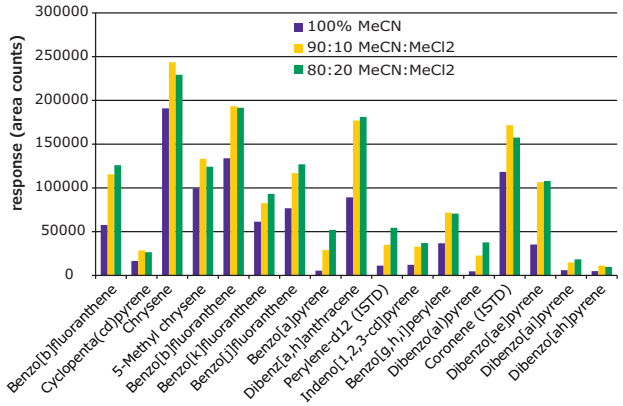


Figure 6. Paprika Extracts Cleaned Using EZ-POP NP SPE and QuEChERS with Similar Sorbents. Z-Sep, Z-Sep/C18 and Z-Sep+ are QuEChERS cleanups

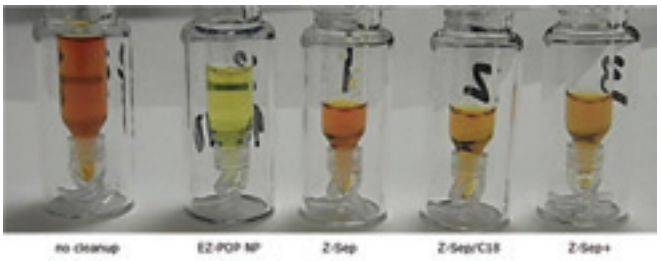


Figure 7. GC/MS Scan Comparison of Paprika Extracts; EZ-POP NP, QuEChERS with Z-Sep+, and No Cleanup.

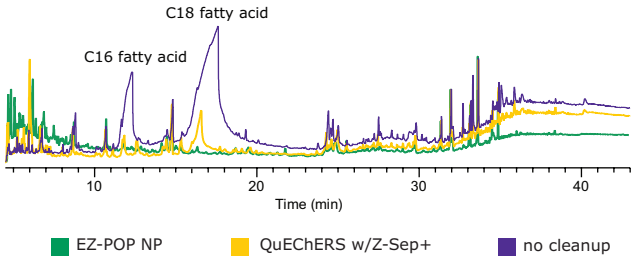
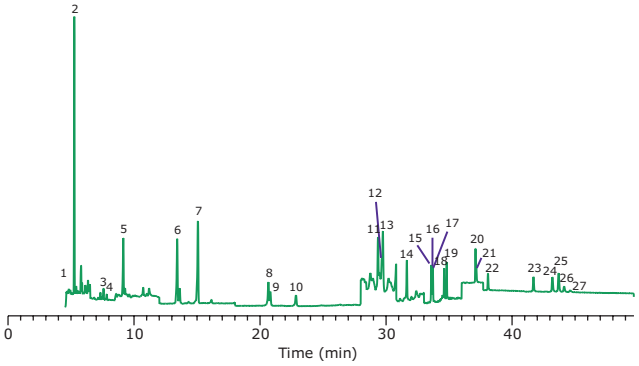


Figure 8. GC/MS/MS Analysis of PAHs in Paprika, Spiked at 10 ng/g, After Cleanup Using EZ-POP NP. Peak IDs are as indicated in Table 1.



to an acceptable level. If analysis is for pesticides, dual layer SPE cartridges containing carbon and primary secondary amine (PSA) or aminopropyl functionalized silica can be used for cleanup. However, for PAHs, carbon cannot be used as it will strongly retain these compounds, resulting in poor recovery. EZ-POP NP does not contain carbon, and thus is suitable for PAHs. Its cleanup capacity was compared directly to QuEChERS using blends containing similar sorbents to those in the EZ-POP NP cartridge. Extracts of paprika generated using 90:10 acetonitrile:methylene chloride and cleaned using EZ-POP NP and QuEChERS are shown in **Figure 6.** Compared to no cleanup, both approaches reduced color, with the EZ-POP NP having the lightest color. The sample cleaned with Z-Sep showed some oily residue, as seen on the inside of the vial. Using GC/MS-scan for comparison (**Figure 7**), the EZ-POP NP cleanup produced the lowest background, especially with regard to removal of C16 and C18 fatty acids. Only Z-Sep+ data is shown here, as it had the lowest background of the three QuEChERS sorbents included in the comparison. Using the final, optimized cleanup method with EZ-POP NP, all PAHs were detected free of background as shown in **Figure 8**.

PAH Spike Recoveries and Analysis of Unknown Samples. Recovery and reproducibility data for analyses of paprika samples spiked at 10 ng/g is presented in **Table 3**. All recovery values were determined without internal standard correction and thus are absolute. Overall, recoveries were very good (>70%) with RSD values of ≤15%. The exceptions were fluorene and dibenzo[a,h] pyrene. There was some matrix interference eluting with the fluorene peak (present in both MRMs monitored), and for dibenzo[a,h]pyrene, response was erratic from the spikes, which resulted in poor reproducibility.

Table 3. Recovery and Reproducibility for PAHs from Paprika, Spiking Level of 10 ng/g (n = 3)

n=3	avg. % Recovery	% RSD
Naphthalene	124%	11%
Acenaphthene	78%	13%
Acenaphthylene	76%	15%
Fluorene	52%	27%
Phenanthrene	102%	4%
Anthracene	78%	12%
Fluoranthene	95%	7%
Pyrene	100%	3%
Benz[a]anthracene	99%	9%
Cyclopenta[c,d]pyrene	70%	12%
Chrysene	99%	6%
5-Methyl chrysene	91%	10%
Benzo[b]fluoranthene	89%	6%
Benzo[k]fluoranthene	82%	6%
Benzo[j]fluoranthene	97%	8%
Benzo[a]pyrene	91%	12%
Dibenz[a,h]anthracene	84%	6%
Indeno[1,2,3-cd]pyrene	86%	12%
Benzo[g,h,i]perylene	80%	5%
Dibenzo[a,l]pyrene	108%	12%
Dibenzo[a,e]pyrene	82%	5%
Dibenzo[a,i]pyrene	108%	15%
Dibenzo[a,h]pyrene	147%	36%

In addition to the paprika used for spike recovery, two more brands were analyzed unspiked. One was labeled as “smoked paprika” and thus was expected to contain substantially higher PAH levels. Both brands were analyzed in duplicate, and the results are summarized in **Table 4**. Several PAHs were detected in

(continued on next page)

Table 4. Analysis of Paprika in Unspiked Samples
(% RPD = % reproducibility)

	Brand A		Brand B*	
n=2	Conc. (ng/g)	%RPD	Conc. (ng/g)	%RPD
Naphthalene	69.8	5%	302	3%
Acenaphthene	3.0	10%	211.6	1%
Acenaphthylene	ND		27.7	4%
Fluorene	ND		272.2	6%
Phenanthrene	25.1	2%	2833	56%
Anthracene	ND		677	4%
Fluoranthene	ND		995	8%
Pyrene	ND		884	7%
Benz[a]anthracene	ND		177.6	7%
Cyclopenta[c,d]pyrene	7.1	1%	99	7%
Chrysene	ND		103.1	8%
5-Methyl chrysene	ND		0.6	25%
Benzo[b]fluoranthene	ND		10.8	5%
Benzo[k]fluoranthene	ND		ND	
Benzo[j]fluoranthene	ND		6.4	5%
Benzo[a]pyrene	ND		8.3	6%
Dibenz[a,h]anthracene	ND		ND	
Indeno[1,2,3-cd]pyrene	ND		2.3	2%
Benzo[g,h,i]perylene	ND		2.6	5%
Dibenzo[a,l]pyrene	ND		ND	
Dibenzo[a,e]pyrene	ND		ND	
Dibenzo[a,i]pyrene	ND		ND	
Dibenzo[a,h]pyrene	ND		ND	

*smoked paprika

brand A; but none were at levels above the EU limits for herbs and spices. The smoked sample, on the other hand, contained detectable levels of all four of the PAHs listed in the EU regulation. Benzo[a]pyrene was present below the maximum allowable level of 10 ng/g; however, the combined levels of this PAH plus benzo[a] anthracene, chrysene and benzo[b]fluoranthene were well above the 50 ng/g maximum. This is to be expected, since the smoking process produces PAHs which subsequently end up in the paprika. However, the EU regulation specifically states that since smoked herbs are generally consumed in such small quantities, they are exempt from these regulatory limits.

Heavier PAHs can be difficult to analyze at low levels by GC/MS due to issues with response and peak shape. Instrument performance can rapidly decline with the injection of dirty samples. To determine if this was occurring with analysis of the paprika extracts, internal standards of non-target PAHs from 4-6 rings were added to the extracts prior to analysis. Response of these compounds was then monitored throughout the run. No decline in response was observed for any of the internal standards, and the variation in area counts throughout the run was ≤15% for each. This indicates that the cleanup was adequate to produce a rugged GC method.

Conclusions

A sample preparation method was developed for the low level analysis of PAHs in paprika powder. The extraction protocol utilized a "modified QuEChERS" approach in which methylene chloride was added to the extraction solvent to increase recoveries. Cleanup was achieved using SPE with the EZ-POP NP cartridge with the addition of methylene chloride to the acetonitrile elution solvent. This modification resulted in absolute recoveries of the 4-6 ring PAHs of >80% with RSD values < 15% for most. The SPE cleanup reduced background in the extract, especially for C16 and C18 fatty acids, significantly compared to QuEChERS cleanup using loose sorbents. Analysis of the PAHs on an application specific GC column (the SLB®-PAHms) was necessary to chromatographically resolve isomeric sets which cannot be discerned based on MRMs.

References

1. Commission Regulation (EU) 2015/1933 of 27 October 2015 amending Regulation EC/1881/2006 as regards maximum levels for polycyclic aromatic hydrocarbons in cocoa fibre, banana chips, food supplements, dried herbs and dried spices. *Off. J. Eur. Union* **2015**, *L282/11*.
2. Rozentale, I.; Lun, A. Y.; Zacs, D.; Bartkevics, V., The occurrence of polycyclic aromatic hydrocarbons in dried herbs and spices. *Food Control.* **2018**, *83*, 45-53.
3. Monado-Maraña, O.; Pérez, R.L.; Escandar, G.M.; Muñoz de Peña, A.; Galeano-Díaz, T., Combination of liquid chromatography with multivariate curve resolution-alternating least- squares (MCR-ALS) in the quantitation of polynuclear aromatic hydrocarbons present in paprika samples. *J. Agric. Food Chem.* **2016**, *64,* 8254-8262.
4. Stenerson, K.K.; Shimelis, O.; Halpenny, M.; Espenschied, K.; Ye, M.M, Analysis of polynuclear aromatic hydrocarbons in olive oil after solid-phase extraction using a dual-layer sorbent cartridge followed by high-performance liquid chromatography with fluorescence detection. *J. Agric. Food Chem.* **2015,** *63*, 4933-4939.
5. Cabillic, J.; Fallot, C.,Final Report on the Feasibility Study of QuEChERS Extraction and / or Purification for Analysis of PAHs in Biota, AQUAREF Report 2015 - 22 p.
6. Anderson, K.A.; Szelewski, M.J.; Wilson, G; Quimby, B.D.; Hoffman, P.D., Modified ion source triple quadrupole mass spectrometer gas chromatograph for polycyclic aromatic hydrocarbon analyses. *J. Chrom. A.* **2015,** *1419*, 89-98.

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Capillary Column Nut for Agilent® MS	**28034-U**
Vials	
Certified Vial Kit, Low Adsorption (LA), 2 mL, amber w PTFE-silicone septa, pk of 100	**29652-U**

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High-Performance Thin-Layer Chromatography: A Fast and Efficient Fingerprint Analysis Method for Medicinal Plants

HPTLC Fingerprint of Ginsenosides

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Ginsenosides are triterpene saponins. Most ginsenosides are composed of a dammarane skeleton (17 carbons in a four-ring structure) with various sugar moieties (*e.g.* glucose, rhamnose, xylose and arabinose) attached to the C-3 and C-20 positions.

Over 30 ginsenosides have been identified and classified into two categories:

- 20(S)- protopanaxadiol (PPD) (Rb1, Rb2, Rb3, Rc, Rd, Rg3, Rh2, Rs1)
- 20(S)-protopanaxatriol (PPT) (Re, Rf, Rg1, Rg2, Rh1). The difference between PPTs and PPDs is the presence of a carboxyl group at the C-6 position in PPDs.

Moreover, several rare ginsenosides, such as the ocotillol saponin F11 (24-R-pseudoginsenoside) and the pentacyclic oleanane saponin Ro (3,28-o-bisdesmoside) have also been identified. Asian ginseng (*Panax ginseng*) is commonly known as the true ginseng.

In this article, HPTLC methods suitable for the analysis of ginsenosides are presented, using CAMAG equipment, Merck TLC plates, analytical standards and extract reference materials. The extract reference materials are manufactured by HWI Analytik and exclusively distributed by Merck Sigma-Aldrich.

Detection of ginsenosides in the HPTLC fingerprint of different Panax species (roots and root extracts) is obtained by following the HPTLC methods of the Ph. Eur. monograph,[1] USP DSC 2015 monograph[2] and the method of the HPTLC Association (International Association for the Advancement of HPTLC)[3] by comparison of the R_F values and colors of reference substances and matching zones in the root extract. Depending on the regulation followed, one of the three methods of identification can be used.

Recommended CAMAG Devices:

Automatic TLC Sampler (ATS 4), Automatic Developing Chamber (ADC 2), TLC Visualizer 2, Chromatogram Immersion Device 3, TLC Plate Heater 3, and visionCATS

Derivatization Reagent:

Anisaldehyde[1] or sulfuric acid reagent[2,3]

Sample:

0.015 g/mL extract (HWI extract) in 70% methanol[1]

Note: Deviation from methods 2 and 3 for the sample preparation

Standards:

Standard solutions of ginsenosides were prepared in a concentration of 0.5 mg/mL in methanol.

Note: Deviation from method 3 for the application volumes

Chromatography Following USP <203> [4]:

Stationary phase: HPTLC Si 60 F_{254} 20 x 10 cm (Merck)

Sample application: 4 µL each of test solution and 2 µL of standards are applied as 8 mm bands, 8 mm from lower edge, 20 mm from the left edge[1, 3]

Note: Deviation from method 3 for the sample preparation and the application volume of reference and test solutions

4 µL each of test solution and 4 µL of standards are applied as 8 mm bands, 8 mm from lower edge, 20 mm from the left edge[2]

Note: Deviation from method 2 for the sample preparation

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Developing solvent: Ethyl acetate – water – butanol 25:50:100 (*v/v/v*) - upper layer[1]

Dichloromethane – ethanol – water 70:45:6.5 (*v/v/v*)[2]

Chloroform – ethyl acetate – methanol – water 15:40:22:9 (*v/v/v/v*)[3]

Development: In the ADC 2 with an unsaturated chamber and after conditioning at 33% relative humidity for 10 min using a saturated solution of magnesium chloride[1]

Development is performed with ADC 2, saturated for 20 minutes with the developing solvent (filter paper). Prior to the development the plate is conditioned for 10 min to a relative humidity of 33% (with a saturated solution of $MgCl_2$).[2, 3]

Developing distance: 70 mm (from lower edge)[1, 2]
80 mm (from lower edge)[3]

Plate drying: 5 min in a stream of cold air

Derivatization: The plate is immersed (immersion speed: 3 cm/s, immersion time: 0 s) into anisaldehyde reagent (mixture of 0.5 mL of *p*-anisaldehyde, 10 mL of glacial acetic acid, 85 mL of methanol, and 5 mL of sulfuric acid) with the Chromatogram Immersion Device 3 and heated for 5 min at 105°C.[1]

The plate is immersed (immersion speed: 3 cm/s, immersion time: 0 s) into sulfuric acid reagent (10% in methanol) with the Chromatogram Immersion Device **3** and heated for 5 min at 100 °C.[2, 3]

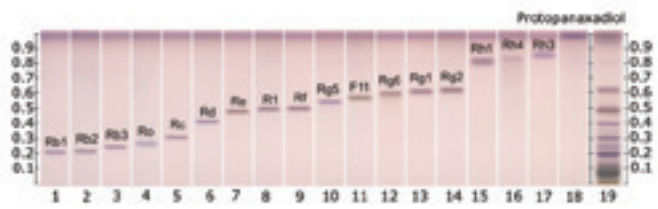
Evaluation: Documentation under method 3 is with 100 °C white light[1, 2, 3] and UV 366 nm[2, 3] after derivatization with the TLC Visualizer 2

Results and Discussion:

HPTLC chromatograms of ginsenoside standards and a *Panax ginseng* root extract

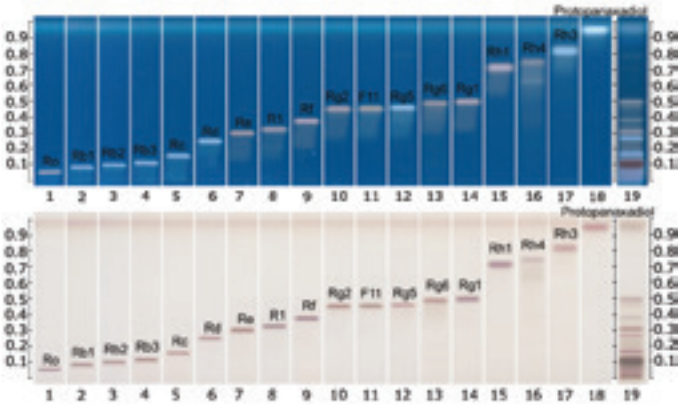
HPTLC chromatograms after derivatization. Tracks 1-17: ginsenosides, track 18: protopanaxadiol, track 19: *Panax ginseng* root extract (article no.: 05115001 batch: HWI01294)

Method 1: According to the Ph. Eur.[1]



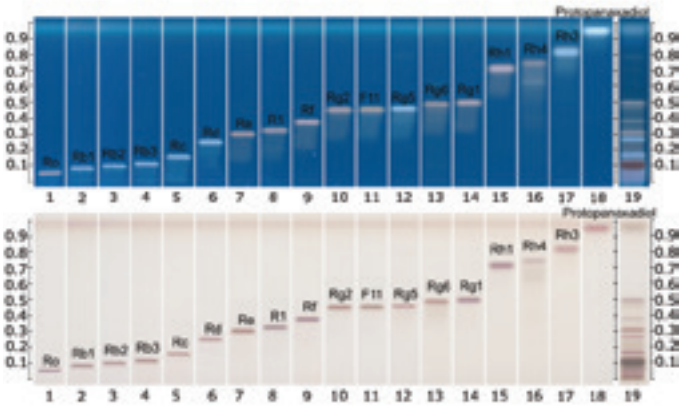
HPTLC chromatograms under white light after derivatization

Method 2: According to the Tienshi ginseng method from[2]



HPTLC chromatograms under UV 366 nm and under white light after derivatization

Method 3: According to the HPTLC Association[3]

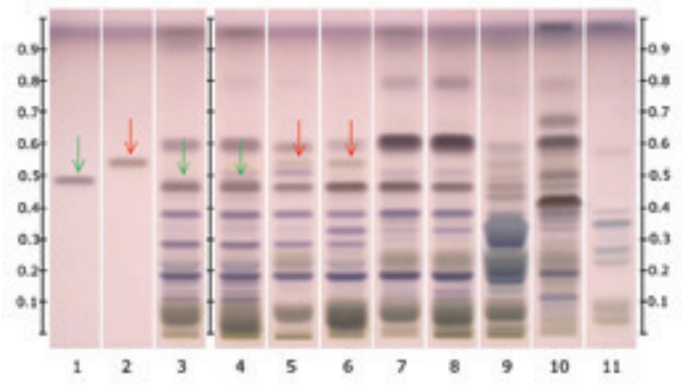


HPTLC chromatograms under UV 366 nm and under white light after derivatization.

HPTLC chromatograms of plants containing ginsenosides (different Panax species)

P. ginseng, P. quinquefolium, P. notoginseng, P. japonicus, P. vietnamensis roots and root extracts were collected and analyzed. The *P. ginseng* root extract (article no.: 0511-50-01 batch: HWI01294) was used as botanical reference material to identify Asian ginseng (the ginsenoside Rf should be present and F11 absent).

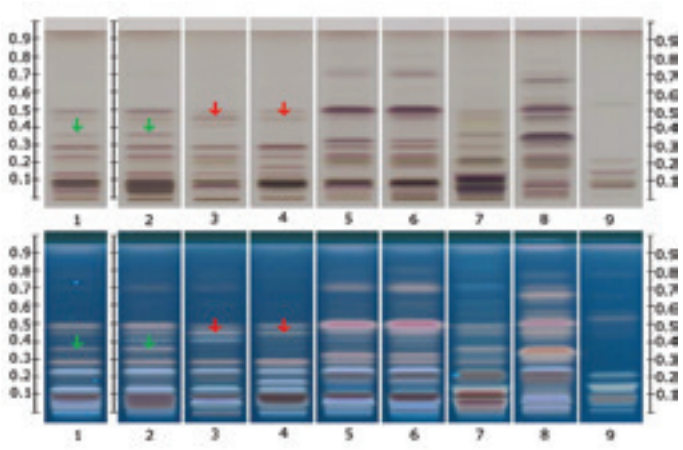
Method 1: According to the Ph. Eur.[1]



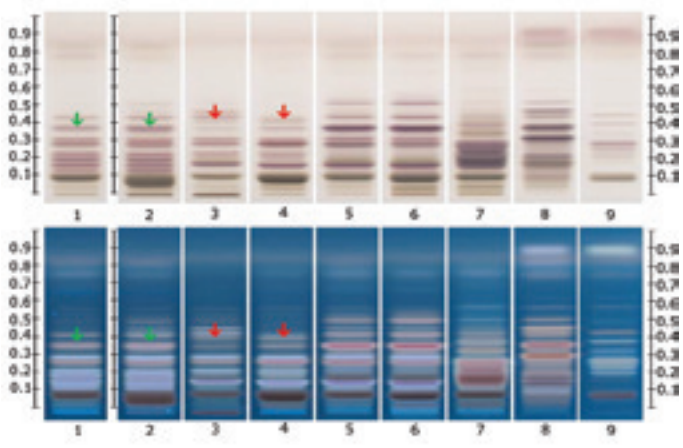
HPTLC chromatogram under white light after derivatization.

Track 1: ginsenoside Rf (green arrow); **2:** ginsenoside F11 (red arrow); **3:** *P. ginseng* root extract (article no.: 0511-50-01 batch: HWI01294, ginsenoside Rf highlighted with the green arrow); **4:** P. ginseng root (ginsenoside Rf highlighted with the green arrow); **5:** *P. quinquefolium* root extract (American ginseng, ginsenoside F11 highlighted with the red arrow); **6:** *P quinquefolium* root (American ginseng, ginsenoside F11 highlighted with the red arrow); **7:** *P. notoginseng* root extract; **8:** *P. notoginseng* root; **9:** *P. japonicas* root; **10:** *P. vietnamensis* root; **11:** wild Vietnamese ginseng root.

Method 2: According to the Tienshi ginseng method from[2]



Method 3: According to the HPTLC Association[3]



Method 2 & 3 Parameter:

HPTLC chromatograms under white light and UV 366 nm after derivatization with ginsenoside Rf (green arrows) and ginsenoside F11 (red arrows).

Track 1: *P. ginseng* root extract (article no.: 05115001 batch: HWI01294, ginsenoside Rf highlighted with the green arrows); **2:** *P. ginseng* root (ginsenoside Rf highlighted with the green arrows); **3:** *P. quinquefolium* root extract (American ginseng, ginsenoside F11 highlighted with the red arrows); **4:** *P quinquefolium* root (American ginseng, ginsenoside F11 highlighted with the red arrows); **5:** *P. notoginseng* root extract; **6:** *P. notoginseng* root; **7:** *P. japonicas* root; **8:** *P. vietnamensis* root; **9:** wild Vietnamese ginseng root.

All shown methodologies are suitable for detection of ginsenosides in different Panax species. Ginsenoside Rf is unique to Asian ginseng while F11 is found exclusively in American ginseng. Thus the Rf/F11 ratio is used as a phytochemical marker to distinguish American ginseng from Asian ginseng. In the botanical reference material used (*Panax ginseng* root extract, article no.: 0511-50-01) the presence of Rf and absence of F11 could be confirmed with all methods and it is therefore suited for identification of Asian ginseng.

Methods 1 and 2 have the advantage in that they are in accordance with official monographs in the pharmacopoeias (Ph. Eur. resp. USP). Method 3 is an alternative method provided by the HPTLC Association. The method is improved for the separation of Rf and F11 to better distinguish American and Asian ginseng. The derivatization with sulfuric acid reagent (methods 2 and 3) leads to different colored zones under UV 366 nm, useful for identification.

Featured Products

Description	Package Size	Cat. No.
Analytical Standards		
Ginsenoside Rb1*	10mg	**170580**
Ginsenoside Rb2	10mg	**41868**
Ginsenoside Rb3	10mg	**42635**
Ginsenoside Rc	5mg	**44987**
Ginsenoside Rd	10mg	**01518**
Ginsenoside Re*	10mg	**03000590**
Ginsenoside Re	10mg	**77960**
Ginsenoside Rf*	10mg	**01580590**
Ginsenoside Rg1*	10mg	**00370580**
Ginsenoside Rg2	10mg	**08171**
Ginsenoside Rg3	10mg	**64139**
Ginsenoside Rg5	5mg	**43016**
Ginsenoside Rh1	10mg	**56805**
Ginsenoside Rh3	5mg	**43084**
Ginsenoside Rh4	5mg	**42776**
Ginsenoside Ro	10mg	**94381**
Notoginsenoside R1	10mg	**77089**
Protopanaxadiol	10mg	**62685**
Protopanaxatriol	10mg	**42476**
Pseudoginsenoside F11	10mg	**67530**

*HWI reference standard

(continued on next page)

Featured Products (cont.)

Description	Package Size	Cat. No.
Extract Reference Material		
Panax ginseng extract Quantitative Markers: Ginsenoside Rb1, Rb2, Rc, Rd, Re, Rf, Rg1 and Rg2 Qualitative Markers**: Ginsenoside Rb1 and Rg1	500mg	**05115001**
TLC Plates		
HPTLC glass plate Silica gel 60 F254 20×10 cm	50 Plates	**1.05642**

(**: traceable to HWI primary pharmaceutical standards)

References

1. Ginseng root: Monograph in Ph. Eur. 8.0, 01/2008:2383. European Directorate for the Quality of Medicines and HealthCare, Strasbourg, France.
2. Tienchi Ginseng Root and Rhizome Dry Extract: Monograph in USP 40-NF35 (2017). United States Pharmacopeial Convention, Rockville, MD, USA.
3. HPTLC identification method for *Panax ginseng*, HPTLC Association (www.hptlc-association.org) (accessed May 22, 2016)
4. <203> High-Performance Thin-Layer Chromatography Procedure for Identification of Articles of Botanical Origin in USP 39-NF34. (2016), United States Pharmacopeial Convention, Rockville, MD, USA

Find all available analytical standards for phytochemicals listed on **SigmaAldrich.com/medicinalplants**

For all ginsenoside standards see **SigmaAldrich.com/panax**

An overview of all plant extract reference materials can be found at **SigmaAldrich.com/plantextracts**

NUTRITIONAL SUPPLEMENTS

New Phytochemical Standards from HWI Pharma Solutions

Matthias Nold, Product Manager, Reference Materials, **matthias.nold@sial.com**

We are proud to be an exclusive distributor of the reference standards manufactured by HWI-Pharma Solutions in Rülzheim (Germany).[1] Since the launch of the first series of products in January 2011, the product range has been continuously expanded and currently consists of more than 120 products.

The quantitative value is determined by **quantitative NMR** (qNMR).[2] This is a direct relative method, which is increasingly used for the quantification of organic compounds, as an alternative to the much more laborious mass balance approach. The certificate delivered with these products also contains a chromatographic purity value.

Recently, several new products have been added to the portfolio (see **Table 1**). A complete listing can be found online at **SigmaAldrich.com/phytopharma**.

Table 1.Newly added HWI reference standards of phytopharmaceuticals

Description	Package Size	Cat. No.
Benzyl acetate	100mg	**05880595**
Berberine chloride	50mg	**00900585**
Carminic acid	25mg	**03320585**
(±)-β-Citronellol	100mg	**05630590**
Ectoine	100mg	**02380595**
Hydroxyectoine	100mg	**02390595**
Isoxanthohumol	25mg	**05890580**
DL-Kavain	25mg	**05790585**
Alpha-onocerin	10mg	**05800590**
Patchouli alcohol	10mg	**05690595**

References:

1. G. Förster, F. Michel, M. Nold; Analytix 1/2010 page 11.
2. M. Veith; Analytix 1/2010 page 14.

PHARMA & BIOPHARMA

BIOshell™ IgG 1000 Å C4 UHPLC Column for Improved Biomacromolecule Separations

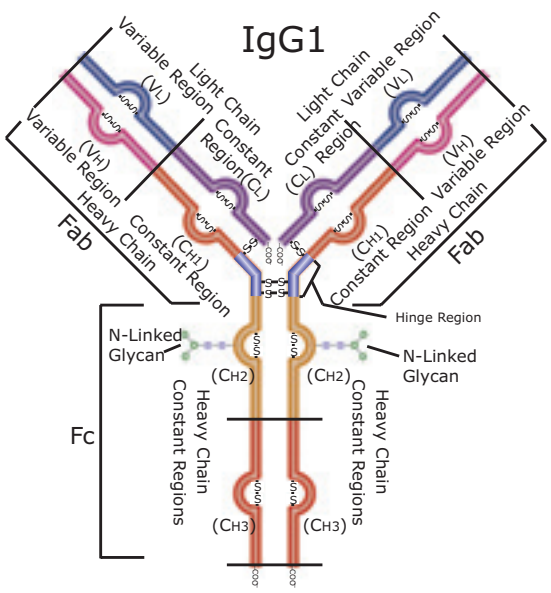
Cory E. Muraco, Senior R&D Scientist, Technology and Workflow R&D, **cory.muraco@sial.com**

Introduction

Monoclonal antibodies (mAbs) are widely manufactured by many biopharmaceutical companies to treat a myriad of diseases ranging from Alzheimer's disease, Parkinson's disease, ulcerative colitis, and many types of cancers. Most recombinant therapeutic mAbs belong to the human immunoglobulin G (IgG) category among the immunoglobulin superfamily. A schematic of an IgG antibody is depicted in **Figure 1**.

A general IgG antibody is composed of two light chains (LC) that are tethered to two heavy chains (HC) through disulfide bonds. In addition, due to the fact that the LC and HC are composed of amino acids with reactive side chains, IgG's can be post-translationally modified through phosphorylation, methylation, oxidation, and nitrosylation, among other modifications. These modifications may change the binding affinity of the mAb so that it binds either the wrong antigen, does not bind any antigen, or associates with the wrong cell surface receptor. Biopharmaceutical companies need to develop rigorous methods to assess lot-to-lot reproducibility of their candidate biologic drug, and the above mentioned modifications are known as Critical Quality Attributes (CQAs) that both the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) monitor. Due to these stringent requirements from regulatory bodies, much research has been pursued in the past 20 years to develop accurate, robust, and high-throughput methods to assess biopharmaceutical purity and structure.

Figure 1. Graphical depiction of a human IgG1 antibody. Note the structural complexity of the different domains of the antibody.[1]



Ultra-high performance liquid chromatography (UHPLC) has emerged as a promising technique to characterize these biomacromolecules. Most biopharmaceutical R&D laboratories, as well as quality control (QC) laboratories, have ready access to this type of instrumentation. Due to the lower system dispersion, lower dead volume, and higher upper pressure limit of these instruments, biopharmaceutical companies have been able to develop methods that not only probe the finest structural details of a candidate drug, but have enabled QC labs to assay hundreds of sample in a single day.

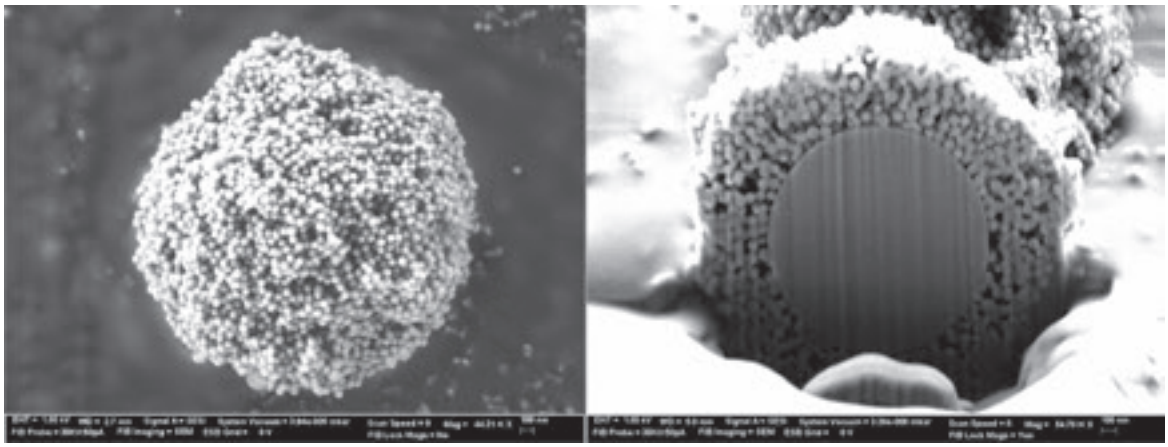
Besides advances in UHPLC instrumentation, there have been many advances in the field of HPLC column and stationary phase development. Two main types of particle morphology are prevalent in the industry today: fully porous particles (FPPs) and superficially porous particles (SPPs, also called core-shell or Fused-Core™ particles). To take advantage of the low dispersion of UHPLC instrumentation, columns with sub-2 µm FPPs with pore sizes of 300 Å have been used to accommodate for the larger hydrodynamic radii of biomacromolecules. These columns have been the industry standard since the mid 2000's. However, these columns suffer limitations when analyzing larger or more complex proteins like mAbs and antibody-drug conjugates (ADCs). The relatively small pore size, in addition to a totally porous architecture, restricts the free diffusion of large molecules through the particle. This architecture concomitantly results in an increase in the mass transfer term of the van Deemter equation, leading to peak tailing, loss of resolution, and low recovery.

In recent years, the use of columns packed with SPPs has been in vogue, especially in the area of biologic characterization. Historically, Horvath and Kirkland pioneered the concept and initial synthetic techniques for producing SPPs in the late 1960's to early 1970's.[2,3] The past 40 years have seen a resurrection and renaissance of these particles' use in UHPLC, and now, advanced versions of these particles are available for several different application areas. These applications include small molecule pharmaceutical separations, pesticide analysis, glycan analysis, chiral separations, and large molecule separations. **Figure 2** shows a scanning electron microscopy (SEM) image of an SPP. Note the presence of the solid silica core in the SPP.

The current BIOshell™ line of columns utilizes SPP technology to provide an alternative to sub-2 µm FPPs for biomolecule separations. Recently, a new addition to the BIOshell™ line of columns has been introduced: the BIOshell™ IgG 1000 Å C4 column. This column is

(continued on next page)

Figure 2. SEM image of the BIOshell™ IgG 1000 Å C4 SPP particle. Left panel shows the porous outer layer (shell) of the particle. Right panel is a cutaway of the particle, revealing the solid, non-porous silica core. Data courtesy of Advanced Materials Technology (AMT).



packed with 2.7 µm SPPs that are composed of a 0.5 µm shell thickness and a 1.7 µm solid silica core. The particle is also composed of 1000 Å pores, permitting the unrestricted analysis of mAbs, ADCs, and other, much larger, biomacromolecules. Advantages over columns packed with FPPs are numerous: the SPP shows significant advantage in mass transfer, leading to less band spreading; columns packed with SPPs are more uniformly packed than columns composed of FPPs, leading to a lower Eddy dispersion (A term) in the van Deemter equation; and larger particle sized SPPs have efficiencies similar to or better than sub-2 µm FPPs, leading to the ability of the analyst to run at higher flow rates with less risk of on-column frictional heating due to elevated column backpressure. The remainder of this article will highlight, in more detail, advantages of the BIOshell™ IgG 1000 Å C4 column as compared to commercial FPP columns for large molecule separations.

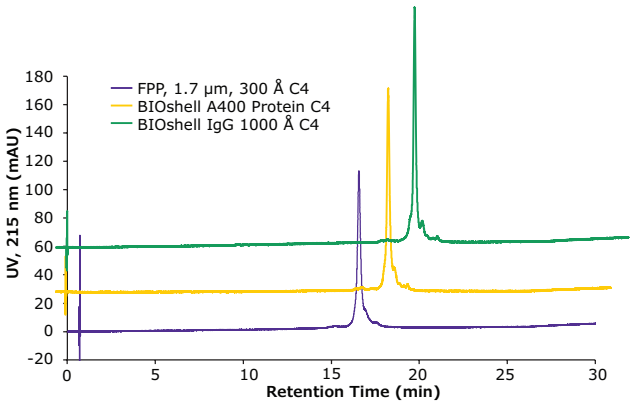
Efficiency Advantage of the BIOshell™ IgG 1000 Å C4 Column

As noted in the previous paragraph, the efficiency gain observed when comparing SPP and FPP particle architecture is due mostly to the short diffusion path within the SPP architecture, thus enhancing mass transfer. In addition, the BIOshell™ IgG column has 1000 Å pores, therefore minimizing any secondary size-exclusion effects that could lead to band broadening and loss of resolution. These concepts are illustrated in **Figure 3** in which the SigmaMAb monoclonal antibody standard is assayed on three columns: BIOshell™ IgG 1000 Å C4, BIOshell™ A400 Protein C4, and a 1.7 µm, 300 Å FPP packed column. Note the lower peak width, at half height, of the main antibody peak as well as improved resolution of minor variants surrounding the main antibody peak, observed on the BIOshell™ IgG column.

In order to ensure that this trend observed in **Figure 3** was translatable to any mAb, a series of mAbs was assayed on the same columns, using the same chromatographic method, and the peak width at half height, a measure of efficiency, was compared. **Figure 4** summarizes those results. None of the mAbs assayed generated peak widths, at half height, greater than 0.23 min on the BIOshell™ IgG 1000 Å C4 column whereas this value was consistently higher on the other two columns.

Figure 3. Comparisons of chromatographic results of the analysis of SigmaMAb, a recombinant IgG1 antibody standard.

Conditions	
Column:	As indicated
Mobile Phase:	[A] 70:30 water (0.1% TFA): acetonitrile (0.1% TFA)
	[B] 50:50 water (0.1% TFA): acetonitrile (0.1% TFA)
Gradient:	0% B to 50% B in 25 min
	50% B to 100% B in 5 min
Flow Rate:	0.4 mL/min
Column Temp.:	75 °C
Detector:	UV, 215 nm
Injection:	1.0 µL
Sample:	SigmaMAb (MSQC4), 1 g/L, water (0.05% TFA)



Reduced Mass Transfer Effects from the BIOshell™ IgG Column

One method to gauge the efficiency of a column operating at high flow rates is to examine the peak volume of an analyte at varying flow rates. For SPPs, the mass transfer term of the van Deemter equation is relatively unaffected by flow rate. Thus, theoretically, peak volume should show relatively little change with increasing flow rate but should change (i.e., increase) for analytes assayed with FPP-packed columns. Using SigmaMAb as the analytical probe, this investigation was

Figure 4. Comparison of peak widths, at half height, for a series of five monoclonal antibodies.

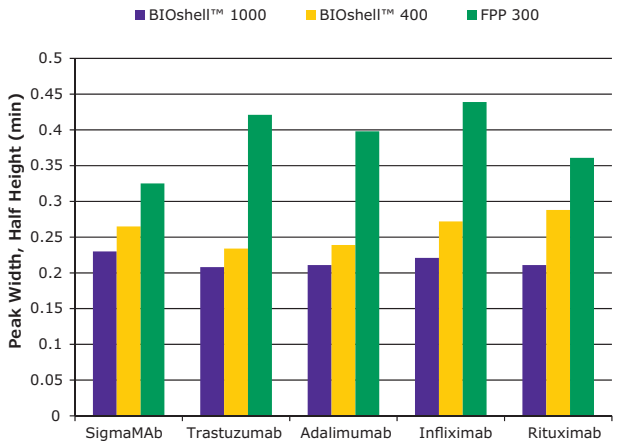


Figure 5. SigmaMAb peak volume versus flow rate. Note how the two columns packed with SPPs have shallow slopes compared to the FPP column. Conditions were the same as outlined in Figure 3, except in each case, gradient volume was held at 10 mL. Peak volume was calculated as peak width at half height multiplied by flow rate.

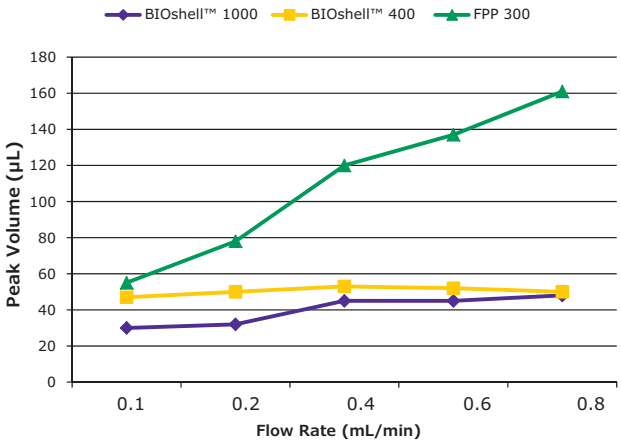
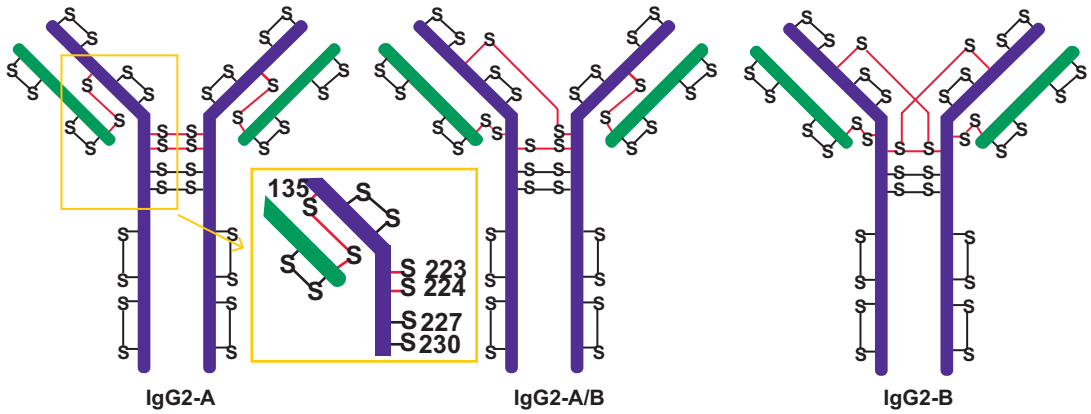


Figure 6. Major disulfide isoforms of IgG2 antibodies. The inset in the yellow square shows the amino acid numbers of the cysteines in the hinge region. The "S" with the red line connecting it denotes cysteines active in disulfide bond scrambling. Adapted from Reference 4.



conducted using the BIOshell™ IgG 1000 Å C4 column, BIOshell™ A400 Protein C4, and the FPP, C4 column used previously. **Figure 5** summarizes the results. The two BIOshell™ columns, as expected, show little change in peak volume with increasing flow rate while the FPP 300 column shows a steep increase due to the effect on the C term in the van Deemter equation.

Resolving Cysteine Variants of IgG2 Antibodies with the BIOshell™ IgG Column

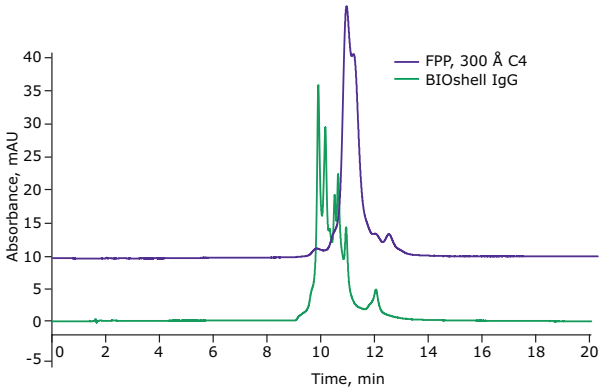
Because of their reduced effector function activity, IgG2 antibodies are becoming the favored format for some protein therapeutics. All IgG2 biologics, however, are composed of different ratios of IgG2 isoforms that only differ by the pattern of disulfide bonding in the hinge region. **Figure 6** displays some of the possible isoforms of an IgG2 antibody.[4]

Because some of these isoforms may have immunogenic effects on a patient, and to ensure lot-to-lot reproducibility of a biologic, a method is required to resolve these different variants. Recently, an analytical reversed-phase chromatography (RPC) method was developed to resolve these different variants on the BIOshell™ IgG 1000 Å C4 column. **Figure 7** compares the chromatographic results of using a column packed with FPP versus using the BIOshell™ IgG column in resolving different disulfide bond isoforms of denosumab. Notice the drastically improved resolution of disulfide bond isoforms using the BIOshell™ IgG column over the column packed with FPPs. Using this RPC method with the BIOshell™ IgG column, in combination with techniques such as redox amplification, thiol tagging, and mass spectrometry, it would be possible to identify and confirm the peaks in **Figure 7**.

(continued on next page)

Figure 7. Analysis of denosumab, an IgG2 antibody, by RPC.

Conditions:	
Column:	As indicated
Mobile Phase:	[A] 88:10:2 water (0.1% DFA): acetonitrile (0.1% DFA): n-propanol (0.1% DFA)
	[B] 10: 20:70 water (0.1% DFA): acetonitrile (0.1% DFA): n-propanol (0.1% DFA)
Gradient:	14% B to 24% B in 20 min
Flow Rate:	0.2 mL/min
Column Temp.:	80 °C
Detector:	UV, 280 nm
Injection:	2.0 µL
Sample:	Denosumab, 2.0 mg/mL, water (0.1% DFA)



Conclusion:

As the market for new drugs is slowly overtaken by biologics, the challenges in determining the purity of a new drug will be daunting. New column technology, like the BIOshell™ IgG 1000 Å C4 column, will help scientists develop new methodologies to resolve these challenges. The BIOshell™ IgG column, incorporating SPPs, allows for high speed, high efficiency separations without a drastic increase in backpressure. As regulatory agencies require biopharmaceutical companies to add more CQAs in the monitoring of new drugs, the BIOshell™ IgG column can provide a valuable addition to the analyst's tool box.

For more information about our BIOshell™ HPLC Columns for Biopolymer Separations please visit **SigmaAldrich.com/BIOshell**

References

1. C. E. Muraco, *LC/GC North America*, **35**, 734-745 (2017)
2. C. G. Horvath, B. A. Preiss, S. R. Lipsky, *Anal. Chem.*, **39**, 1422-1428 (1967)
3. J. J. Kirland, *Anal. Chem.* **41**, 218-220 (1969)
4. L. M. Jones, W. Cui, H. Zhang, M. L. Gross, *J. Am. Soc. Mass Spectrom.* **24**, 835-842 (2013)

Featured Products

Description	Cat.No.
BIOshell™ IgG 1000 Å C4, 10 cm x 2.1 mm I.D., 2.7 µm	**63288-U**
BIOshell™ A400 Protein C4, 10 cm x 2.1 mm I.D., 3.4 µm	**66825-U**
EXP® Pre-Column Filter	**51163-U**
EXP® Pre-Column Filter Cartridges	**51164-U**
SILu™Lite SigmaMAb Universal Antibody Standard human	**MSQC4**
Water for chromatography (LC-MS Grade) LiChrosolv®	**115333**
Acetonitrile hypergrade for LC-MS LiChrosolv®	**100029**
Trifluoroacetic acid, ≥99%, purified by redistillation, for protein sequencing*	**299537**

*Product available in North America

Related Products

BIOshell™ IgG, C4 2.7 µm Guard Cartridge 5mm x 2.1mm, Pk.3	**63291-U**
Guard Cartridge Holder, for use with Ascentis® Express & BIOshell™ Guard Columns, pk of 1	**53500-U**
Trifluoroacetic acid for protein sequence analysis	**1.08178**

CLINICAL & FORENSIC

LC/MS/MS Analysis Using On-line Cartridges for the Removal of Phospholipids from Protein Precipitated Biological Fluid Samples

Hillel Brandes, Separations R&D, **hillel.brandes@sial.com**

Introduction

Phospholipids (PLs) are abundantly (at the mg/mL level) present in biological fluids such as: blood, plasma, serum, and cerebrospinal fluids, among others. PLs are often co-extracted with a broad range of analytes of interest during sample preparation. The phospholipids present in a sample are notorious for producing various issues in LC/MS-based bioanalysis. PLs may cause ion suppression or, in rarer cases, ion enhancement, during MS detection. They also tend to build up on a reversed-phase (e.g., C18 and C8) column, fouling the chromatographic separation and ultimately shortening the column lifetime. Consequently, the accuracy, reproducibility, and sensitivity of LC/MS bioanalyses may be greatly compromised if the PLs are not removed.

HybridSPE® Phospholipid technology has been developed for selective and rapid depletion of phospholipids from biological samples prior to LC/MS analysis of small molecules. The technology utilizes the affinity of zirconia particles for selective binding and removal of phospholipids. The technology was introduced a few years ago in two product formats: 96-well filter plates for high throughput sample preparation and individual cartridges for low throughput applications. Here a new product format, an on-line cartridge, is described as an alternative option of phospholipid removal and sample preparation. The setup of the on-line cartridges with a LC/MS column is devised and the efficiency for phospholipid removal from protein precipitated plasma samples has been evaluated. Applicability of the system was demonstrated with three sets of compounds with different physiochemical properties.

Experimental

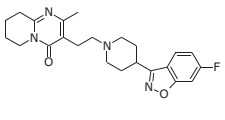
Materials

Supel™ Genie HybridSPE® on-line cartridge (2 cm length x 4.0 mm i.d), Rat Plasma K2-EDTA (Lampire Cat. # 7306407); Protein precipitation solvent: Acetonitrile with 1% formic acid or methanol with 1% (w/v) ammonium formate.

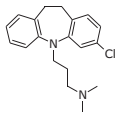
Sample Processing Procedure: The rat plasma or rat plasma spiked with analytes was protein precipitated by vortex mixing the plasma samples with the precipitation solvent at a 1:3 ratio. The mixture was then centrifuged at 10,000 rpm x 3 min and the resulting supernatant was collected for LC/MS analysis.

Three sets of analytes

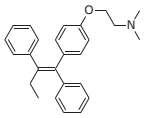
Set-1: Basic analytes



Risperidone

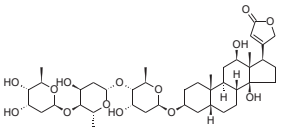


Clomipramine

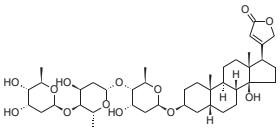


Tamoxifen

Set-2: Polar neutral analytes

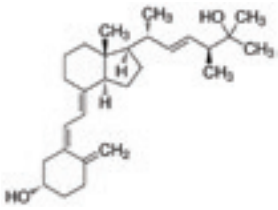


Digoxin

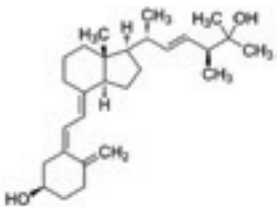


Digitoxin

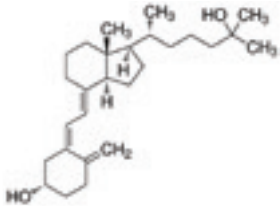
Set-3: Non-polar neutral analytes



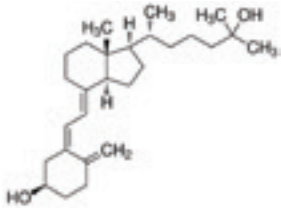
25-Hydroxyvitamin D_2



3-*epi*-25-Hydroxyvitamin D_2



25-Hydroxyvitamin D_3



3-*epi*-25-Hydroxyvitamin D_3

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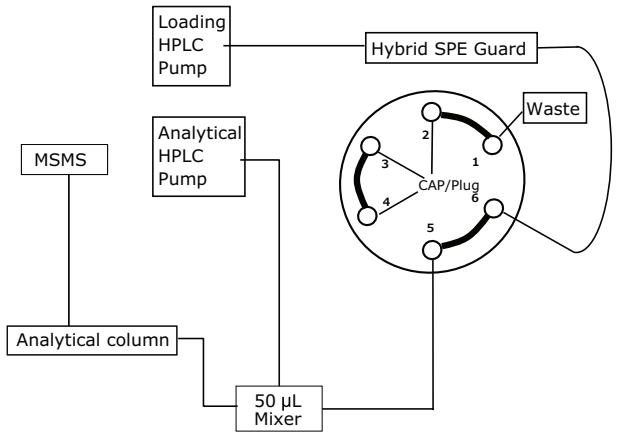
HybridSPE®-LC/MS setup

As shown in **Figure 2**, the setup consists of two pumps, one for HPLC separation, and the other for loading protein-crashed samples and washing the HybridSPE® on-line cartridges. A 50 µL tee is employed for mixing of sample loading mobile phase and the HPLC separation mobile phases. The 2-position switching valve allows for washing the cartridges once the samples are loaded onto the HPLC column.

Figure 2. The setup of on-line HybridSPE® cartridge with LC/MS



HybridSPE®-LC/MS conditions for Set-1 and -2 analytes

Instrument:	Shimadzu™ LCMS-8030 with 2DLC setup
HPLC column:	Ascentis® Express F5 10 cm x 2.1 mm (53569-U)
Mobile phase:	(A) Water/10 mM ammonium formate; (B) methanol
Gradient:	0% B% for 4 min, to 75% B in 0.5 min, held for 8 min
Flow:	0.3 mL/min
Column temperature:	45 °C
Sample loading flow:	0.1 mL/min
Sample loading solvent:	methanol with 10 mM ammonium formate
Injection Vol:	1 µL
Detection:	MS, ESI(+), MRM mode

HybridSPE®-LC/MS conditions for Set-3 analytes

Instrument:	Shimadzu™ LCMS-8030 with 2DLC setup
HPLC column:	Ascentis® Express C18 5 cm x 2.1 mm (53822-U)
Mobile phase:	(A) Water; (B) 90% Acetonitrile, each with 10 mM ammonium formate
Gradient:	0% B% for 4 min, to 80% B in 2 min, held for 1.5 min
Flow:	0.3 mL/min
Column temperature:	35 °C
Sample loading flow:	0.1 mL/min
Sample loading solvent:	80% acetonitrile with 50 mM ammonium formate
Injection Vol:	1 µL
Detection:	MS, ESI(+), MRM mode

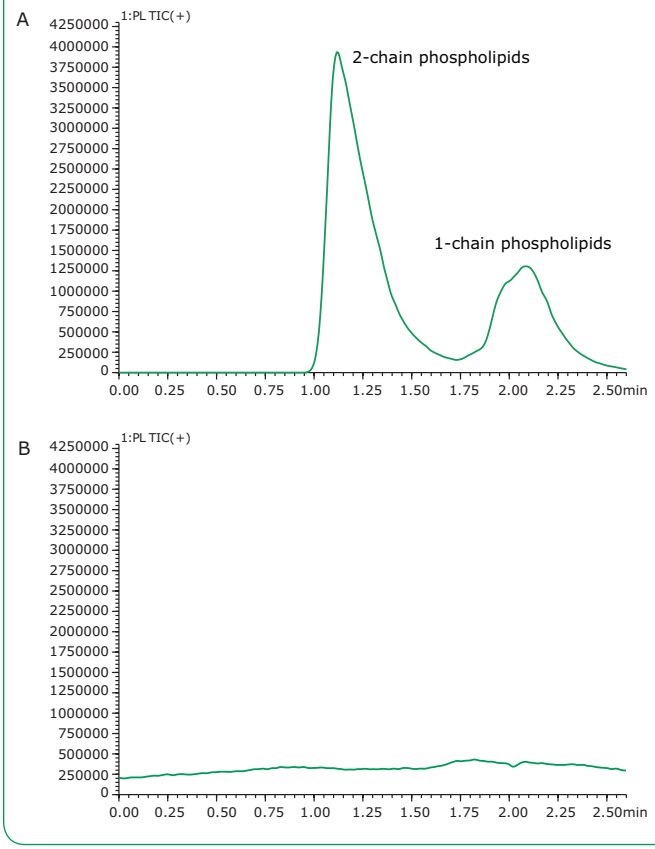
HybridSPE®-LC/MS conditions for phospholipid detection:

Instrument:	Shimadzu™ LCMS-8030 with 2DLC setup
HPLC column:	Ascentis® Express OH5 5 cm x 2.1 mm (53749-U)
Mobile phase:	(A) Water; (B) 90% Acetonitrile, each with 10 mM ammonium formate
Isocratic:	85% B
Flow:	0.3 mL/min
Column temperature:	35 °C
Sample loading flow:	0.2 mL/min
Sample loading solvent:	90% acetonitrile with 10 mM ammonium formate
Injection:	1 µL
Detection:	MS, ESI(+), MRM mode
Phospholipid ions:	precursor ions 496, 520, 522, 524, 758, 782, 786, and 810, product ions are all 184

Results and Discussion

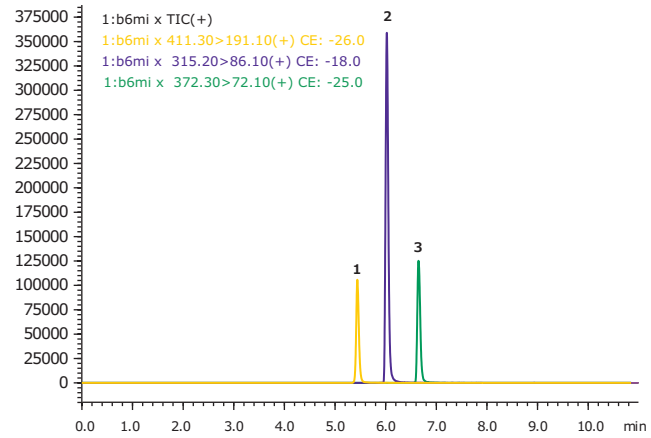
The Supel™ Genie HybridSPE® on-line cartridges are designed for removal of phospholipids from more than 100 injections of 1 µL of protein precipitated plasma samples. **Figure 3**(A) shows the phospholipids in the rat plasma, if not removed, give rise to two broad peaks of high intensity. The two peaks correspond to the phospholipids containing one and two fatty acyl chain(s), respectively. When the HybridSPE® on-line cartridge is set up with the LC/MS (**Figure 2**), no phospholipid peaks were detected, even at the 120th injection of the same rat plasma sample (see **Figure 3**(B)). The results demonstrate the HybridSPE® cartridges are capable of elimination of the phospholipids from 120 consecutive injections of 1 µL of protein precipitated plasma samples.

Figure 3. (A) Phospholipids in plasma sample without a HybridSPE® cartridge; (B) #120th injection of the same plasma sample with a HybridSPE® on-line cartridge set up with LC/MS (see Figure 2 for the setup).



The applicability of the HybridSPE® on-line cartridges has been demonstrated with three sets of analytes including basic and neutral classes. As can be seen from **Figure 4-6**, narrow and symmetric peaks were observed for all of the tested analytes with a peak width at half height <6 s and tailing factors 0.9-1.3, respectively. **Table 1** summarizes the recoveries and reproducibilities of the target compounds using the on-line SPE method. For all of the tested analytes, recoveries of 94-102% were obtained and reproducibilities of 1-5% were achieved.

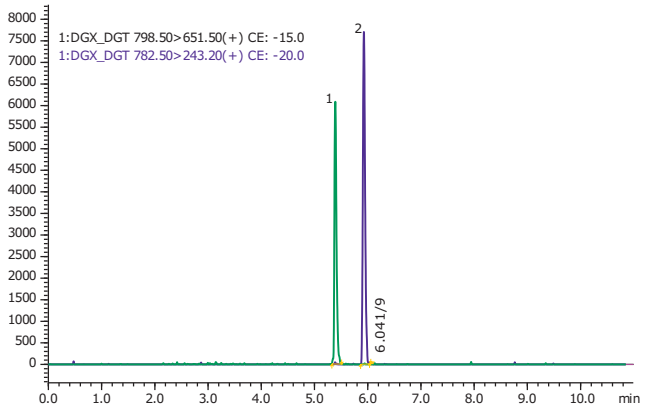
Figure 4. Representative LC/MS chromatogram of basic (Set-1) analytes



Peak	Analyte	Peak width at 50% height (s)	Taliling factor
1	Risperidone	3.54	1.2
2	clomipramine	3.78	1.3
3	Tamoxifen	3.36	1.2

- All peaks are narrow: <4s peak width at half height
- Both peaks are symmetric, with tailing factors of 1.2-1.3
- Baseline is low and clean: no interference peaks

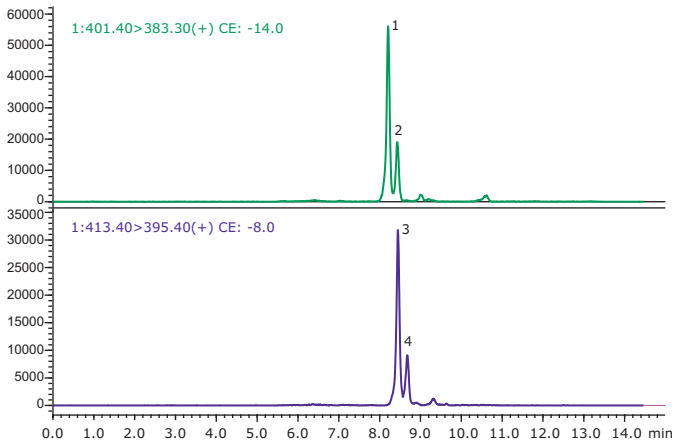
Figure 5. Representative LC/MS chromatogram of polar neutral (Set-2) analytes



Peak	Analyte	Peak width at 50% height (s)	Taliling factor
1	Digoxin	2.52	1.3
2	Digitoxin	2.70	1.2

- Both peaks are narrow: <3s peak width at half height
- Both peaks are symmetric, with tailing factors of 1.2-1.3
- Baseline is low and clean: no interference peaks

Figure 6. Representative LC/MS chromatogram of non-polar neutral (Set-3) analytes



Peak	Analyte	Peak width at 50% height (s)	Taliling factor
1	25-OH D3	4.74	0.9
2	3-*epi*-25-OH D3	4.80	0.9
3	25-OH D2	4.62	1.0
4	3-*epi*-25-OH D2	5.10	0.9

- All peaks are narrow: <6s peak width at half height
- Both peaks are symmetric, with tailing factors of 0.9-1.0
- Baseline is low and clean: no interference peaks

(continued on next page)

Table 1. Analyte's Recovery and Reproducibility

Analyte	Retention time (mm	MRM Quantifier	Avg. Recovery*	%RSD, n=20
Digoxin	5.4	798.5/651.5	96%	4.9
Digitoxin	5.9	782.5/243.2	97%	2.2
Risperidone	5.4	411.3/191.3	102%	1.5
clomipramine	6.0	315.2/86.1	94%	1.1
Tamoxifen	6.6	372.3/72.2	98%	1.4
25-OH D3	8.2	401.4/383.3	102%	1.2
3-*epi*-25-OH D3	8.4	401.4/383.3	102%	2.5
25-OH D2	8.4	413.4/395.4	100%	2.0
3-*epi*-25-OH D2	8.6	413.4/395.4	99%	4.2

* The recovery was calculated by comparison of the peak area of the spiked analytes in plasma to those of the neat analytes at the same concentration.

Summary

An on-line cartridge for phospholipid removal with LC/MS analysis has been successfully developed. The performance testing demonstrates the on-line cartridges are capable of removing >95% of phospholipids from 1 µL of plasma samples even after 120 consecutive injections. Three applications have been established using on-line HybridSPE® with LC/MS detection. Recovery of the analytes is 94%-102%, with a reproducibility of 1%-5%. For all of the tested analytes, narrow and symmetric peaks were observed, peak width at half height <6 s and tailing factors 0.9-1.3, respectively.

Featured Products

Description	Cat. No.
Sample Preparation	
Supel™ Genie HybridSPE® On-line Starter Kit	**55324-U**
Supel™ Genie HybridSPE® On-line SPE Cartridge, pk. of 2	**55326-U**
Supel™ Genie HybridSPE® On-line SPE Cartridge, pk. of 6	**55327-U**
LC	
Ascentis® Express C18 5 cm x 2.1 mm, 2.7 µm	**53822-U**
Ascentis® Express F5 10 cm x 2.1 mm, 2.7 µm	**53569-U**
Ascentis® Express OH5 5 cm x 2.1 mm, 2.7 µm	**53749-U**

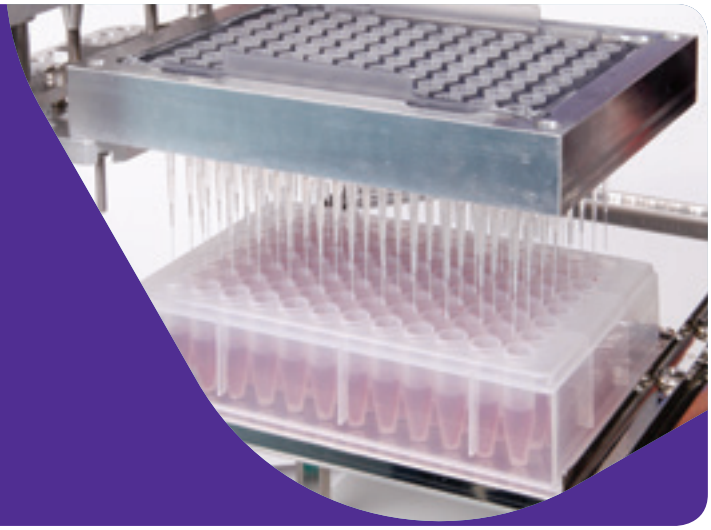
Related Products

Description	Cat. No
Sample Preparation	
Supel™ Genie RP-Amide On-line Starter Kit	**55516-U**
Supel™ Genie RP-Amide On-line SPE Cartridge, pk. of 2	**55519-U**
Supel™ Genie RP-Amide On-line SPE Cartridge, pk. of 6	**55522-U**
Supel™ Genie C8 On-line Starter Kit	**55274-U**
Supel™ Genie C8 On-line SPE Cartridge, pk. of 2	**55512-U**
Supel™ Genie C8 On-line SPE Cartridge, pk. of 6	**55515-U**
Solvents	
Water for chromatography (LC-MS Grade) LiChrosolv®	**1.15333**
Acetonitrile hypergrade for LC-MS LiChrosolv®	**1.00029**

Did you know...

... that the HybridSPE® material is also available in 96-well plates for protein & phospholipid removal in one off-line step?

Read more under
SigmaAldrich.com/hybridspe



Tips & Tricks: Sensitivity Gains in LC-MS

Stephan Altmaier, Head of Instrumental Analytics R&D, **stephan.altmaier@merckgroup.com**

Introduction

The prerequisite of any highly sensitive analysis via HPLC-MS (high performance liquid chromatography coupled to mass spectrometry) is the use of ultrapure solvents and reagents and careful handling of all associated materials, consumables, and systems. This prevents any contamination throughout the entire sample handling process from preparation to MS detection, and improves sensitivity.

In the following sections, various measures and options for maximized LC-MS sensitivity and low limit of detection (LOD) are shown. Each and every tip avoids contaminations causing signal suppression, adduct formation, elevated background noise and increased spectrum complexity.

Solvents & Additives - General

Typical solvents utilized in LC-MS include water, acetonitrile, methanol, isopropanol and *n*-propanol. Additives such as acids (e.g., formic acid), bases (e.g., ammonia) or buffers (e.g., ammonium acetate) are used to enable the protonation or deprotonation of the analytes.

The quality of the above-mentioned solvents and additives strongly influences the sensitivity of MS detection; therefore, utilization of MS grade solvents and ultrapure additives is mandatory. Make sure that these reagents are labeled as LC-MS grade by the manufacturer.

Generally, organic solvents for HPLC, such as acetonitrile and methanol, are available in three qualities: Isocratic grade, gradient grade and hypergrade for LC-MS LiChrosolv®. For MS analysis, hypergrade quality solvents should be used to ensure best performance and reliable results.

With regard to water, bottled or Milli-Q® ultrapure water from water purification systems are suitable for use with MS instrumentation. In case of low water consumption, bottled water is preferable, whereas Milli-Q® water is suggested in an environment with higher consumption. Milli-Q® systems deliver type I water and are a perfect match with LC-MS analysis. They should be used/flushed regularly in order to maintain or even further improve water quality.

Buffers are utilized to set and control the pH of a specific chromatographic separation and to protonate or deprotonate analytes in solution, which can support the electrospray ionization process. For LC-MS, only volatile buffers and additives such as ammonium formate or acetate or triethylamine should be utilized. The use of nonvolatile buffers (e.g., sulfates, phosphates, borates) will cause precipitation in the MS source and ultimately result in tedious cleaning procedures. High buffer concentrations might lead to signal suppression.

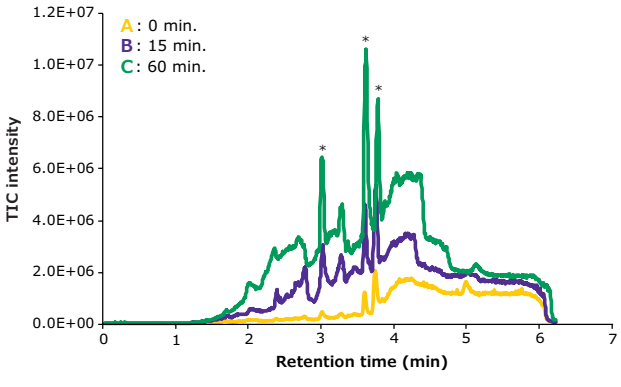
Buffers ionize an analyte molecule M, but the formation of adducts [M+buffer] with, e.g., ammonium, formate or acetate is possible. This causes additional signals with specific *m/z* values in a spectrum which may compromise quantitative analyses. Consequently, for samples with high salt load such as food, body fluids or tissue, a desalting step using solid phase extraction (SPE) (e.g., Supel™-Select HLB, or LiChrolut® and Supelclean® cartridges) is recommended.

Buffers should be prepared by titration of the respective acid and base, as their purity is normally higher than the related salts. If the use of salts is necessary, an MS analysis of those used should be performed prior to use application to determine if and what type of contaminant is present in the salts.

Impurities in or contaminants of solvents and additives can accumulate on the stationary phase and elute as ghost peaks in gradient runs (**Figure 1**). This scenario may occur when the column is equilibrated under highly aqueous conditions prior to a gradient run. Ghost peaks can even appear without equilibration if the concentration and/or retentivity of contaminants is high

Figure 1. Accumulation of contaminants on an HPLC column during equilibration and elution via a gradient profile; peaks attributed to plasticizers are marked with an asterisk (*).

Conditions:	
Instrument:	Bruker Esquire 6000plus
Mobile phase:	A: water/acetonitrile 95/5 (v/v) + 0.1% formic acid
	B: acetonitrile + 0.1% formic acid
Gradient:	0 min 100% A, 3 min 5% A, 5 min 5% A
Flow rate	0.4 mL/min
Temperature:	25 °C
Detector:	pos. ESI-MS (TICs)
Sample:	plasticizers added by the immersion of plastic tubing in aqueous solvent A



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and/or the starting conditions of a gradient are highly aqueous. To avoid ghost peaks in gradient runs, column equilibration time should be kept as short as possible and the flushing volume should not exceed ten column volumes.

Strong acids such as hydrochloric acid, sulfuric acid or nitric acid should be avoided because they tend to form strong ion pairs with analytes and therefore make the analyte unsuitable for any type of ionization. Additionally, some of these strong acids have unfavorable oxidizing properties.

Many laboratories use trifluoroacetic acid (TFA) in order to form ion pairs with peptides and proteins and to improve subsequent HPLC separation; however, TFA causes strong ion suppression of the analyte during MS detection and may as well contaminate the mass spectrometer. If the use of TFA is necessary, then a weak acid or isopropanol should be added to help decrease the signal suppression effect. Alternatively, difluoroacetic acid (DFA) is an option that decreases the signal suppression effect (as compared to using TFA).

Solvents & Additives – Storage & Handling

Solvents should be stored in the original manufacturer's bottle; this can be either surface treated amber or borosilicate glass. Adjustment of the bottle size to specific needs is recommended, because decanting/transferring to a different container, a source of contamination, should be avoided whenever possible. Avoid standard clear or soda-lime glass bottles. Leaching alkalines and silica can form adducts with analytes.

Bottles have to be sealed and connected to the HPLC system using professional caps, adapters, and tubing directly mounted to the solvent bottle. Any homemade solution will likely cause contamination of the solvent or eluent and could lead to the evaporation of organic solvents into the lab atmosphere.

Avoid plastic devices such as bottles, funnels, beakers, or gloves which can leach additives like plasticizers, anti-static agents, stabilizers or anti-slipping agents (**Figure 2**). The only exceptions are devices that have been tested for leachables and extractables by the manufacturer, e.g., pipette tips or syringes.

Laboratory Equipment

Cleaning of laboratory equipment and vessels can most simply be done by evaporation in a fume hood, as all reagents used in MS applications are volatile and of high purity. In cases where contamination is observed, flushing with MS grade solvents will be necessary in order to properly clean the equipment.

If a dishwasher needs to be used for any reason, it is critical that after washing, the vessels are flushed/rinsed with an MS grade solvent multiple times.

HPLC Column

The choice of an HPLC column dimension is guided not only by factors such as sample size, detection technique, and necessary loadability, but also by economic considerations such as reducing solvent consumption. A decrease in column internal diameter (i.d.), while geometrically scaling injection volume and flow rate accordingly, is a simple means of also improving sensitivity of a given separation.

Figure 2. Mass spectra of two Milli-Q® water samples stored in polypropylene (A) and clean amber glass bottles (B), respectively (bottom), and TICs of the same samples (top). The analyses were performed via direct injection of the solvents into the MS operated in positive ESI mode.

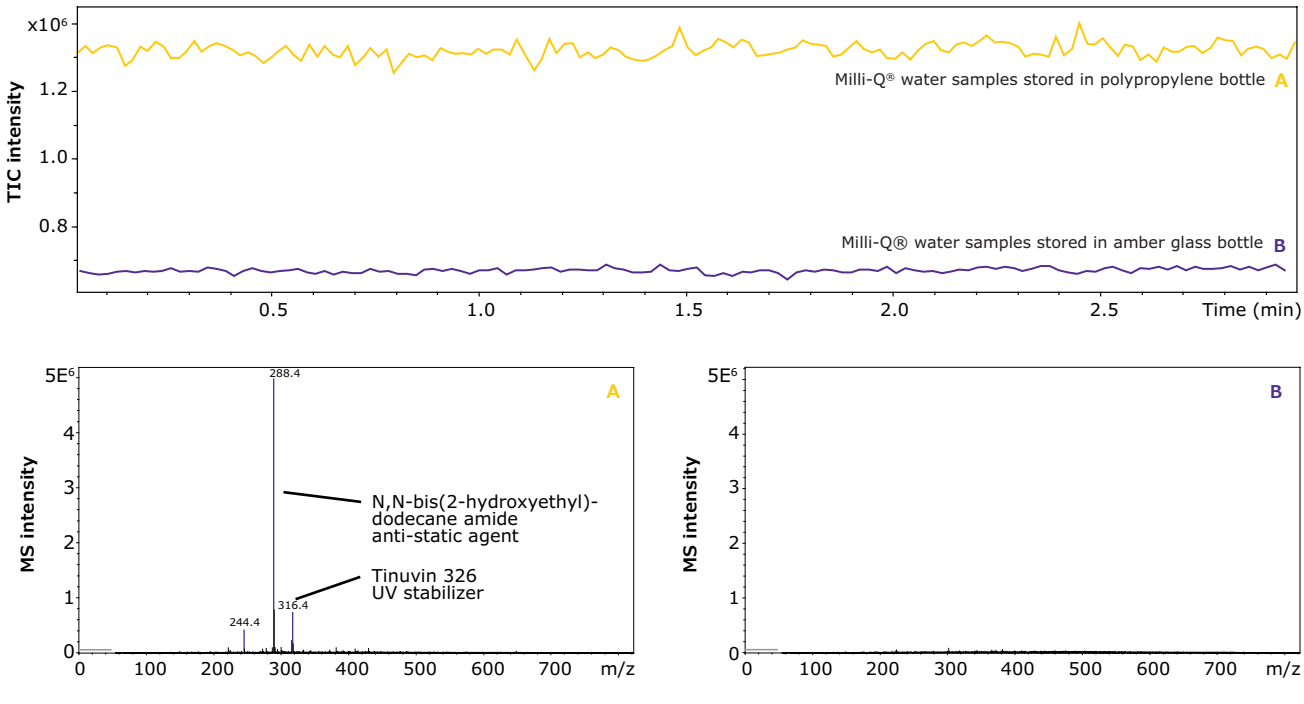
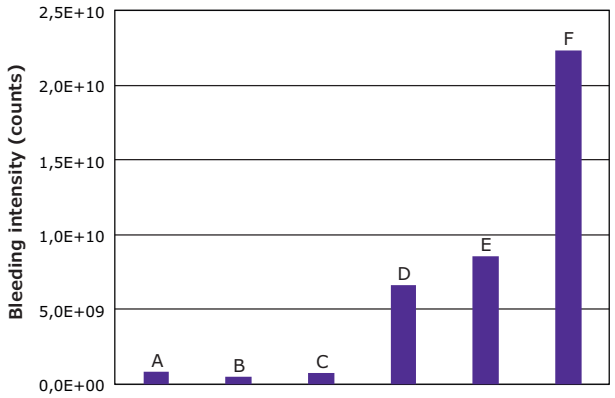


Figure 3. Quantification of HILIC column bleeding of various Merck columns in comparison with alternative products measured by mass spectrometry.
A: Merck Purospher® STAR Si 100-2.1
B: Merck Purospher® STAR ZIC®-HILIC 100-2.1
C: Brand A HILIC 100x2.1 (normal phase material)
D: Brand B HILIC 100x2 (ammonium sulfonic acid)
E: Brand C HILIC 100x2 (phosphorylcholine)
F: Brand D HILIC 100x2.1 (ammonium sulfonic acid)

Conditions:	
Column:	As indicated
Mobile phase:	acetonitrile/25 mM ammonium acetate pH 6.8 80/20 (v/v), 6 min.
Flow:	0.8 mL/min
Temperature:	50 °C
Detector:	Ion-trap MS (m/z 50-2000)



A possible and frequent but often overlooked source of contamination in an LC-MS run is the chromatographic column itself. Many of the silica-based bonded phases are inherently prone towards bond/phase cleavage by hydrolysis, mainly at acidic pH (e.g., below pH 2), a phenomenon referred to as column bleeding (**Figure 3**).

The use of a washing protocol can help to decrease the negative effect of column bleed. Alternatively, a column should undergo up to ten gradient runs from strongly aqueous to strongly organic before use with MS.

HPLC System

A proper setup of the HPLC system itself can contribute to increased sensitivity as well. An important parameter is the minimization of dead volume, i.e., the volume of all system parts from the injector to the detector cell, except for the HPLC column volume. Large dead volumes can cause peak broadening, tailing, or splitting and lead to poor resolution and decreased performance, and hence can decrease sensitivity and prevent detection of low abundant analytes. Consequently, all system parts (tubing, connectors, fittings) must contribute the smallest possible dead volumes.

Replace the pump inlet filter every 1 to 2 months or after changing from acetonitrile to methanol (or vice versa) as a solvent. This maintenance will lower the baseline noise and protect the system and column from pump debris.

Eluent filter frits (from solvent inlet filters) should be made out of stainless steel or PEEK rather than glass. Cleaning of the latter is tedious, as buffer residue is hard to remove, and silica and alkali might be leached out of the glass filter and form adducts.

General Recommendations

The specific requirements of different chromatographic problems might make the use of various mobile phase compositions necessary, ranging from aqueous to organic. As a general recommendation, the water content in an eluent used in LC-MS should be set to 5 to 80% in order to work trouble-free and with a stable spray.

If the water content is below 5%, buffers may precipitate in the eluent and the HPLC system. A countermeasure can be the use of a suitable organic solvent or a decrease of buffer concentration in the eluent. Buffer solubility in utilized solvents (and gradient range) should always be checked prior to analysis.

A water content of more than 80% might lead to a breakdown of the MS spray. Several options help to keep the MS spray working.

1. Decrease in the surface tension of the eluent by addition of a volatile organic solvent such as acetonitrile or methanol to the mobile phase after the LC system and in front of the MS source.
2. Reduction of the flow delivered to the MS by means of a split or column exchange.
3. Manipulation of the MS source conditions (increase in dry gas temperature or flow).

In order to avoid microbial contamination of both system and mobile phase, and phase collapse, water content of the mobile phase should not be set above 95%. If a highly aqueous mobile phase is necessary, 0.05% sodium azide can be added to the eluent. Alternatively, regular flushing of the HPLC system with organic solvent, preferably isopropanol or methanol, prior to standby is mandatory. Do not use acetonitrile, because acetonitrile can polymerize and block system valves.

Conclusion

Mass spectrometry is a powerful technique for identification and quantification of molecules within complex mixtures. The success of mass spectrometry strongly depends on reducing contamination throughout the entire LC-MS workflow: from sample preparation to equipment cleaning. An important first step in this process is the exclusive use of highest quality materials for LC-MS, including solvents, buffers, reagents and columns. The combination of ultra-pure solvents and reagents with contamination-free handling ensures maximized LC-MS sensitivity and low LODs.

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To find more information on our LC-MS solvents and reagents, please visit us at **SigmaAldrich.com/lc-ms**

IN ESSENCE

Water Determination in Liquefied Petroleum Gas using GC BID and Ionic Liquid Column Watercol™

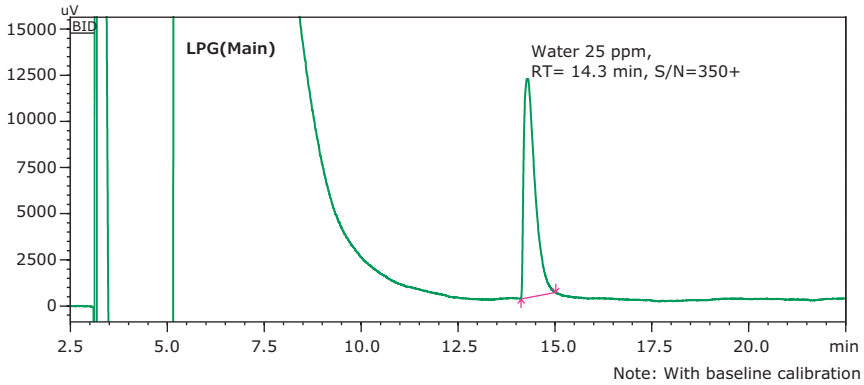
Shimadzu Application Data Sheet No.18

Water in petrochemical feedstocks can cause problems for processors. Freezing of pipe lines and valves and poisoning of expensive catalysts are just a few examples. Monitoring water in petroleum from an upstream source to the downstream processing plant is critical to insure uninterrupted operation.

A new determination option by GC can bypass undesirable chemical interference effects from the petroleum to the "Sulfur interaction" that can skew results obtained by traditional water determination techniques. Shimadzu's proprietary Barrier Ionization Discharge (BID) detector and Supelco's water analysis column "Watercol" are combined to separate and measure the water in a formulation of feedstock (**Figure 1**) and provide a sensitive and accurate result (**Table 1**). Measurements can be made down to sub-ppm level of water detection.

Instrument	
Gas chromatograph:	Tracera (GC-2010 Plus A + BID-2010 Plus)
Sample injection:	Valco Internal Liquid Sample Injector with Splitter Injection Unit
Gas purifier:	Supelco High Capacity Gas Purifier (Cat.#29541-U)
Analysis Conditions	
Column:	Watercol™ 1910, 60 m x 0.25 mm ID, 0.20 µm
Oven:	35 °C (2.0 min) – 5 °C/min – 150 °C (15 min) Total. 40 min
Carrier gas:	Helium 45 cm/sec (Column flow rate 3.78 mL/min)
Inj. volume:	2 µL
Split:	1:5
Transfer line temp.:	175 °C (After Internal Liquid Sample Injector to GC column Oven)
Detector temp.:	200 °C
Discharge gas vol.:	50 mL/min(He)

Figure 1. Chromatogram for water determination (25 ppm) in LPG. Quantification of Limit(S/N=10) and Detection of Limit(S/N=3.3) can be down to 0.66 ppm and 0.22 ppm respectively.



Note: With baseline calibration

Table 1: Repeatability of Water analysis(n=5).

Water	No.1	No.2	No.3	No.4	No.5	Average	Standard Deviation	%RSD
RT(min)	14.285	14.288	14.286	14.296	14.304	14.292	0.008	0.06
Area(mV•s)	244,037	249,854	246,884	242,950	238,428	244,430	4,296	1.76
Height(mV)	12,418	12,600	12,468	12,045	11,851	12,276	315	2.57
Conc.(ppm)	24.96	25.56	25.25	24.85	24.39	25.00	0.44	1.76

This application is also subject of the ASTM Work Item https://www.astm.org/DATABASE.CART/WORKITEMS/WK59649.htm

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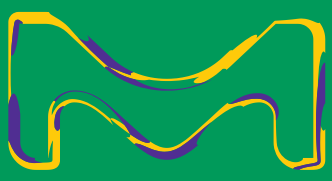

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