ISSUE 15 | 2023

Analytix Reporter

Sample Preparation of Serum by BioSPME Prior to Determination of Free Testosterone

Supelco®
Analytical Products

ISSUE 15 | 2023

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Dear Reader,

One of the greatest adages that was ever told to me in my analytical chemistry upbringing is, “Garbage in, garbage out.” Though simple in messaging, this saying truly governs how the quality of data generated in any experiment is only as good as the sample and method that are employed. From a chromatographic standpoint, one can have the greatest column in the world, in mint condition, with a fully validated method ready to employ, but if the sample is “garbage quality,” meaning full of matrix compounds, interfering analytes, and other undesirable components, your data will likely be “garbage quality.”

This situation is especially true when considering clinical samples submitted for liquid chromatography analysis. Be it a serum, urine, plasma, or any other biological sample, there is a myriad of compounds that can interfere and lead to inaccurate results (or damage to your instrumentation). The proper selection of sample preparation aids can enable the clinical researcher to prepare a sufficiently clean sample that can facilitate accurate results with no damage to the analytical instrumentation or consumables like the HPLC column.

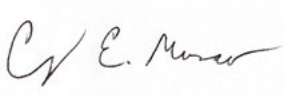
One example of such sample preparation aid is based on the HybridSPE® technology. Plasma/serum samples contain an abundance of proteins and phospholipids that can cause e.g., severe ion suppression. The HybridSPE® technology combines the simplicity of protein precipitation and selective removal of phospholipids all in one, allowing for high quality clean-up of plasma/serum samples. This technology comes in an assortment of formats: traditional SPE cartridges, 96-well plates, and Dispersive Pipet Extraction (DPX) tips, thus enabling high-throughput. Another technology that aids clinical researchers in sample clean-up, especially for urine samples, is based on a Hydrophilic-Lipophilic Balanced (HLB) sorbent. The Supel™ Swift HLB utilizes a modern version of such a sorbent which is fast interacting and can extract analytes with a wide range of polarities (logP values) while interfering compounds are stripped away. This product is also available in SPE cartridges, 96-well plates, and DPX tips.

Finally, I would like to mention one last technology, which is the focus of this issue’s cover story, is Biocompatible Solid Phase MicroExtraction (BioSPME) utilizing the Supel™ BioSPME pin device to extract small molecules out of a biological sample. With a proprietary polymer layer that prevents protein binding, the Supel™ BioSPME device was used to examine the amount of free testosterone in serum samples. Read on to learn more about this novel technology, that can be automated by a liquid handler, and how it was applied in a clinical setting.

All these unique sample preparation technologies will help you “take the garbage out” of your samples leading to reliable and accurate results.

Happy Resolving!

Sincerely yours,



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CLINICAL & FORENSIC

Optimizing Serum Sample Preparation for Free Testosterone Determination: A Comparative Analysis Using BioSPME

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Introduction

The industry's gold standard for sample preparation of free hormones from serum has been associated with equilibrium dialysis.[1] It is the free portion of hormones, including testosterone, that is responsible for the biological activity.[2] Free testosterone accounts for approximately 1-2% of total testosterone.[3] In males, this generally falls in the range of 20–230 pg/mL and for females in the range of 0.6–10 pg/mL (0.8-1.4% of total).[4] Solid phase microextraction (SPME) is a relatively new method to employ for the measurement of free concentration and one that has recently been incorporated into a 96-pin device for use with conventional well plates. Biocompatible SPME, or BioSPME, has been shown to be a fast sample preparation technique.[5]

Methods

The BioSPME sample preparation method for the 200 µL samples utilized a Supel™ BioSPME C18 (**Figure 2**) 96-pin device with a Hamilton® STARlet system. Although the method includes multiple steps, it was developed to have only a total processing time of 1 hour (**Figure 1**). A volume of 500 µL acetonitrile was used for the conditioning, and the same volume of water for the wash solution. The desorption and derivatization steps were performed off-line. The acetonitrile desorption solution, 50 µL, contained 25 pg/mL D_3-testosterone prior to derivatization. Derivatization was performed with an addition of 200 mM hydroxylamine hydrochloride, 100 µL, at 60 °C for 20 min with agitation at 600 rpm.[6] The free concentration of testosterone in serum samples was determined using simultaneously extracted calibrators (10 – 200 pg/mL) prepared in phosphate-buffered saline and analyzed by the method described in **Table 1**. Quantifier and qualifier transitions were utilized for the natural and isotopically labeled testosterone and derivatized testosterone (**Table 2**).

Injection volumes of 60 µL of the final, prepared samples of the total 150 µL were used to accommodate reinjections if required.

Table 1. LC-MS conditions

LC Conditions	
Instrument:	Agilent 1290
Column:	Ascentis® Express C18, 5 cm x 2.1 mm I.D., 2.7 µm (**53822-U**)
Mobile phase:	[A] Water with 0.1% formic acid; [B] Acetonitrile with 0.1% formic acid
Gradient:	**Time (min) / A% / B%** 0 / 95 / 5 2 / 95 / 5 3 / 5 / 95 6 / 5 / 95
Flow rate:	0.6 mL/min
Column temp.:	40 °C
Detector:	MS/MS (see below and **Table 2**)
Injection:	60 µL
MS Conditions	
Instrument:	AB Sciex 6500+ TripleQuad™
Curtain gas:	26
Collison gas:	4
Ion spray voltage:	3000 V
Temperature:	650 °C
Ion source gas 1:	50
Ion source gas 2:	55

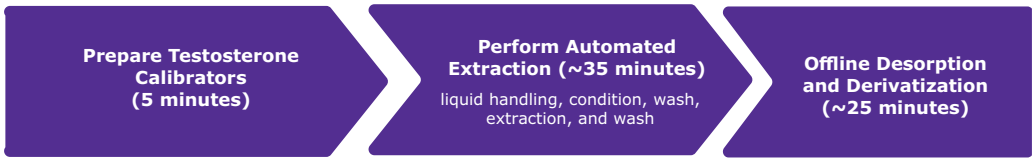

Figure 1. Overview of sample preparation prior to analysis by LC-MS/MS. A detailed outline of the automation is shown in **Figure 2**.

Table 2. MS Transitions monitored

Analyte		Q1	Q3	Dwell (ms)	DP (V)	EP (V)	CE (V)	CXP (V)
Testosterone	Quantifier	289.2	97.2	40	85	10	51	10
	Qualifier	289.2	109.2	40	85	10	33	10
D_3-Testosterone	IS	292.2	97.2	40	85	10	51	10
	IS	292.2	109.2	40	85	10	33	10
Te – NHOH	Quantifier	304.2	112.0	40	85	10	33	10
	Qualifier	304.2	124.0	40	85	10	30	10
D_3–Te-NHOH	IS	307.2	112.0	40	85	10	33	10
	IS	307.2	124.0	40	85	10	30	10

*Te-NHOH represents the derivatized testosterone with hydroxylamine

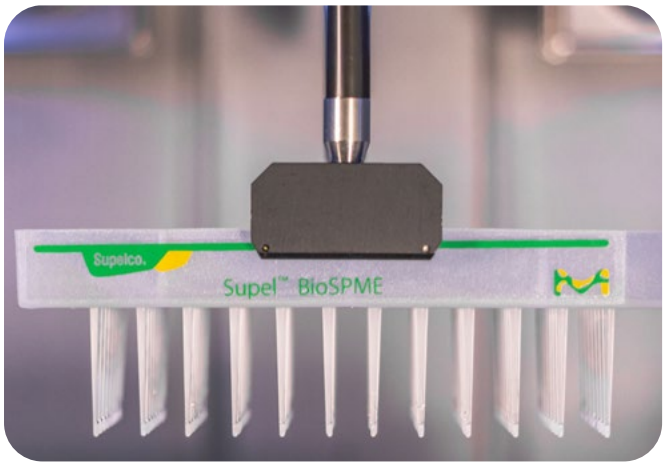

Figure 2. (Right) Overview of the steps the Hamilton® Starlet Robot performs in the automated version. (Above) Grippers from Hamilton® Starlet moving the Supel™ BioSPME Device.

Steps of the Liquid Handler

Liquid Handling	Optional	• Conditioning plate is filled with 500 µL of acetonitrile in corresponding consecutive wells • Wash plate is filled with 500 µL of water in corresponding consecutive wells
Conditioning	20 min, static	• Robot grips the BioSPME Pin device from the parked position and transfers it to the condition plate and submerges the pins in acetonitrile for 20 mins under static conditions.
Wash	10 s, static	• Robot transfers the BioSPME pin device from the conditioning plate to the wash plate and submerges the pins for 10 s under static conditions.
Extraction	10 min, 37 °C at 1200 rpm	• Robot transfers the pin device to the extraction plate, allowing extraction of the free testosterone to occur. The heated shaker is at 37 ºC and is mixing 1200 rpm for 10 minutes. • Glass-lined well-plate to prevent non-specific binding.
Wash	1 min, static	• Robot transfers the BioSPME pin device from the extraction back to the initial wash solution for 1 min under static condition.
Park		• Upon the second wash, the robot will transfer the BioSPME pin device back to the Home position. • Desorption and Derivatization will occur off-line

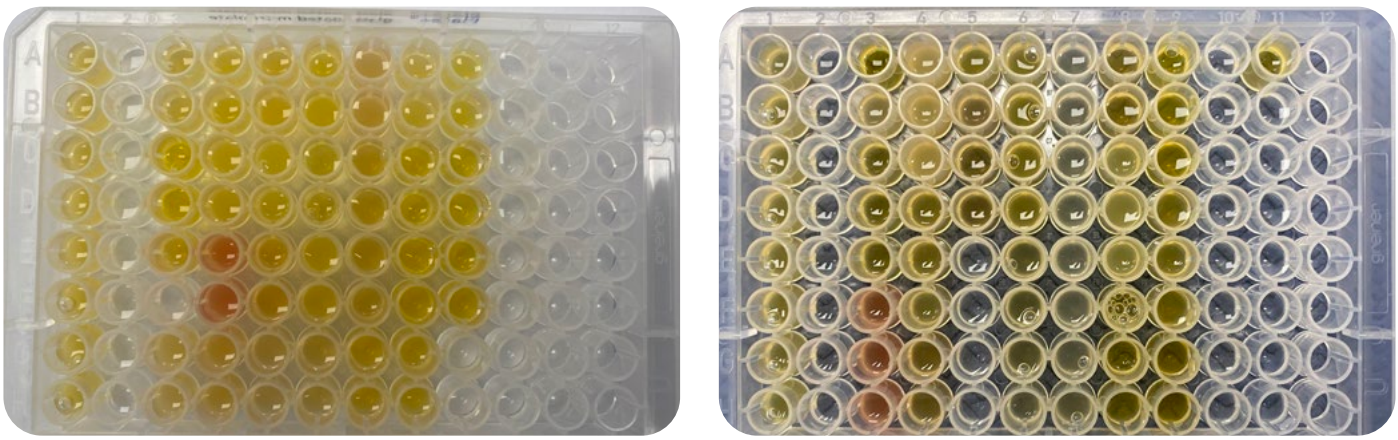

Figure 3. Actual samples for extraction 1 (left) and extraction 2 (right). Each loaded well contained 200 µL of either serum sample (yellow/red) or a calibrator prepared in phosphate buffered saline. Serum samples were not excluded if they appeared lipemic (cloudiness from lipids/fats), icteric (yellowing from bilirubin), or hemolytic (presence of ruptured red cells).

Free testosterone in male serum samples was previously determined by externally validated equilibrium dialysis-based methods at respective laboratories (Lab A, Lab L, and Lab Q). Samples were purchased from Lab A and were previously tested. The serum samples sent to Labs L and Q were collected in collaboration with the Clinical & Translational Science Institute at Pennsylvania State University. Aliquots of these samples were submitted for testing (extraction 1), with the remainder of the samples kept at -80 °C. A second extraction was performed after one month in the -80 ºC freezer (extraction 2). A total of 30 unique serum samples were tested using BioSPME in a randomized analysis. In general, samples were tested in duplicate or triplicate depending on availability of sample quantities. Images of the actual samples are shown in **Figure 3**.

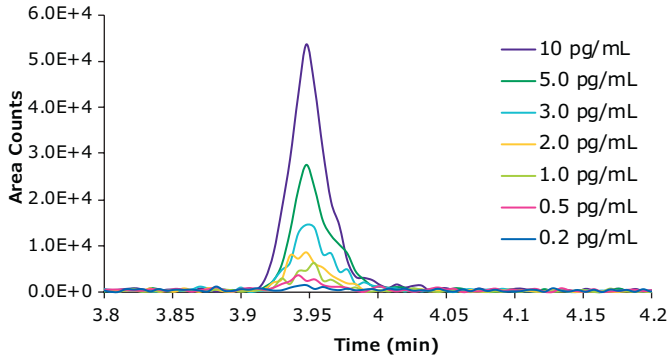

Figure 4. Chromatogram of Te-NHOH-1 at various concentrations ranging from 10 pg/mL down to 0.2 pg/mL in (1:2 acetonitrile:water).

Table 3. Accuracy and precision for injection of various concentrations of derivatized testosterone in 1:2 acetonitrile:water and the peak ratios for transitions monitored (n=12).

	Te-NHOH-1 Quantifier Transition			Te-NHOH-2 Qualifier Transition			Ratio Peak Area Counts Te-NHOH-1/Te-NHOH-2	
pg/mL in solution	pg/mL determined	RSD	S/N	pg/mL determined	RSD	S/N	Avg	RSD
0.2	0.13	68.0	3.8	< 0.00	n/a	13.1	1.31	66.1
0.5	0.43	20.6	4.6	0.22	47.4	32.1	1.00	20.7
1.0	1.06	8.0	30.5	0.80	9.5	62.4	1.09	15.1
2.0	2.05	5.3	55.3	1.76	4.6	112.5	1.17	9.0
3.0	3.17	5.1	77.2	2.87	6.7	160.9	1.17	8.5
5.0	5.15	3.2	161.7	4.99	2.9	294.5	1.16	4.1

Results:

The instrumental limit of detection, LOD, and lower limit of quantification, LLOQ, of the derivatized testosterone, Te-NHOH, were determined by serial dilution and n=12 injections (**Table 3**). The LOD was 0.2 pg/mL and LLOQ of the quantifier transition, Te-NHOH-1, was 1 pg/mL, with an RSD of 8.0%. The LLOQ of the qualifier transition, Te-NHOH-2, was 1 pg/mL, with an RSD of 9.5%. The peak integration ratio for the quantifier/qualifier was 1.10, with an RSD 15.1%. The sensitivity was achieved by replacing the 20 µL standard injection

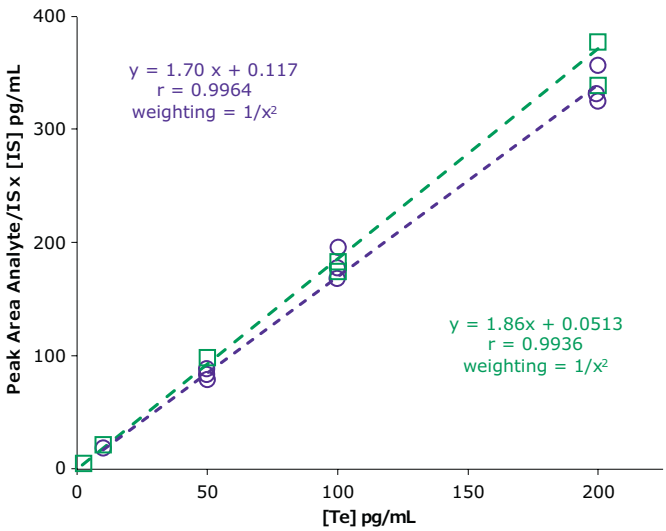

Figure 5. Extracted calibration curves from phosphate buffered saline for determination of free testosterone. Purple circles – extraction 1 and Green squares – extraction 2.

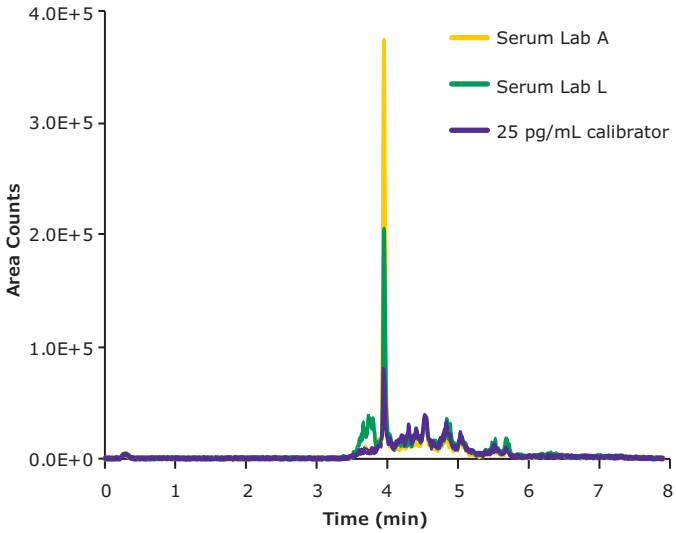

Figure 6. Total ion chromatograms for three different samples: 25 pg/mL extracted calibrator (purple), Lab L serum sample (green), and Lab A serum (yellow), each after the derivatization step.

loop on Agilent 1290 LC instrument with a 100 µL loop to allow for larger injection volumes. Representative chromatograms of the diluted samples down to 0.2 pg/mL are available in **Figure 4**.

The extracted calibration curve for free testosterone quantification, range of 10–200 pg/mL, had a R=0.9964 and 0.9936 using a $1/(x^2)$ regression (**Figure 5**). Representative total ion chromatograms of three different samples are presented in **Figure 6**. A representative chromatogram of the monitored transitions is shown in **Figure 7**. Correlation plots between the free testosterone determined by validated equilibrium dialysis (Lab A, range 20.3–194.4 pg/mL) and the BioSPME method prior to LC-MS/MS yields a linear correlation of y = 0.917x – 6.23, R^2 = 0.954 (extraction 1) and y = 0.956x – 1.57, R^2 = 0.976 (extraction 2). Extraction 2 was performed over a month later after undergoing a freeze/thaw cycle and storage at -80 °C in the interim (**Figure 8**). Including the additional samples from Labs L and Q, linear correlations of y = 0.923x – 6.37, R^2=0.921 (extraction 1), and y = 0.941x + 0.15, R^2=0.960 (extraction 2) (**Figure 9**). In either correlation graph, the R^2 is above 0.92 and indicates an almost ideal correlation between the two methods. When considering the existence of a y-intercept, it may result from a couple of different reasons. These include different sample preparation methods, instrumentations, and analysts. Another variable that is unaccounted for is the state of the serum samples, as some of the samples were pretested (and underwent freeze-thaw cycles) while others were shipped across the country.

The derivatized internal standard peak area counts, D_3-Te-NHOH, on a per-well basis were monitored and used as a quality check. An average internal peak area count across all tested wells was 5.84 x 10^4 ± 0.61 x 10^4 (RSD 10.6%) and 6.40 x 10^4 ± 0.58 x 10^4 (RSD 9.1%) for extraction 1 and extraction 2 respectively. The percent difference from the average for extraction 1 and extraction 2 is

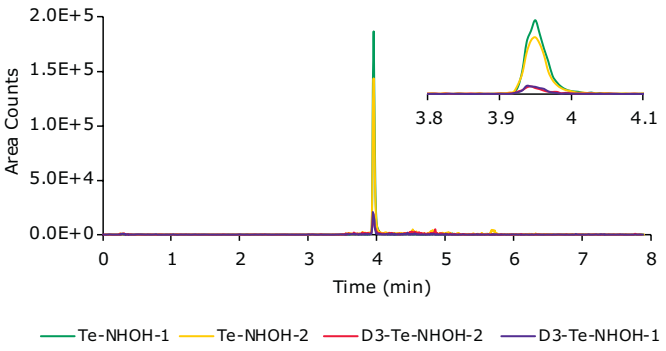

Figure 7. Representative chromatogram (Lab A #1) showing the transitions monitored. Zoomed-in window to highlight the peak.

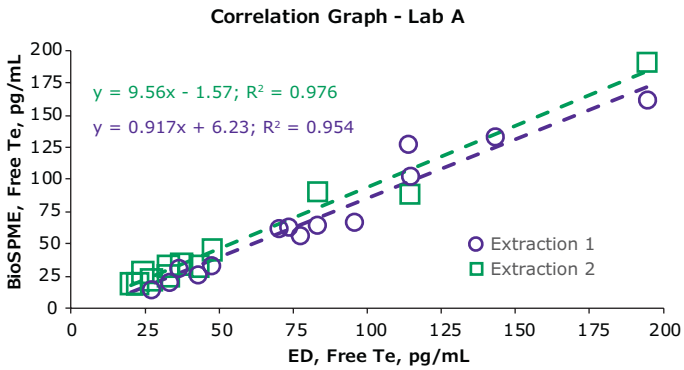

Figure 8. Correlation of free testosterone for Lab A samples between two different sample preparation methods; BioSPME, determined internally by R&D, and equilibrium dialysis (ED), pre-determined externally. Purple circles for extraction 1, and Green squares for extraction 2.

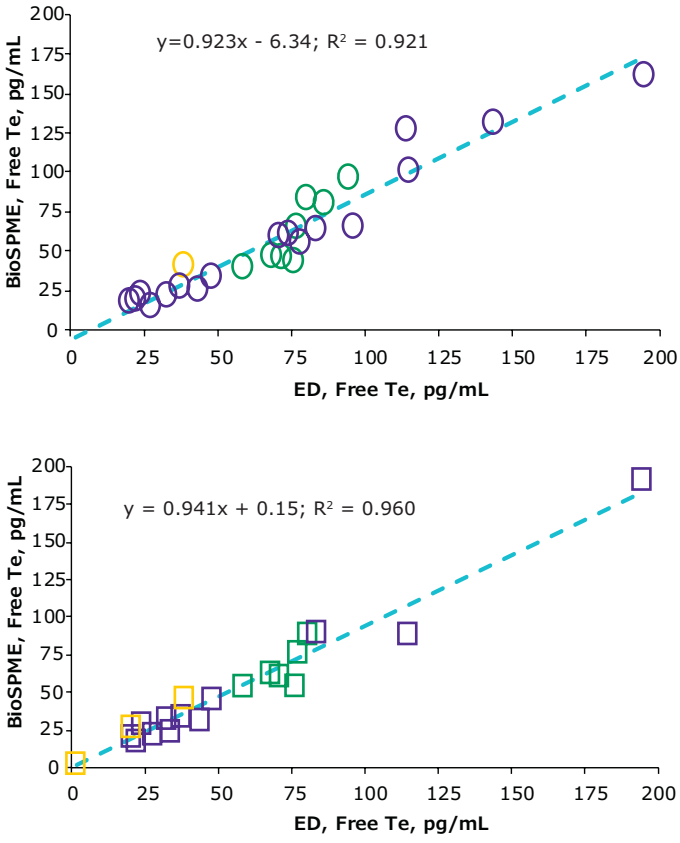

Figure 9. Correlation of free testosterone for all samples; Lab A (purple), Lab L (yellow), and Lab Q (green) between two different sample preparation methods; BioSPME, determined internally by R&D, and equilibrium dialysis (ED), determined externally using a CLIA validated equilibrium dialysis LC-MS/MS method. Circles for extraction 1 (top); squares for extraction 2 (bottom).

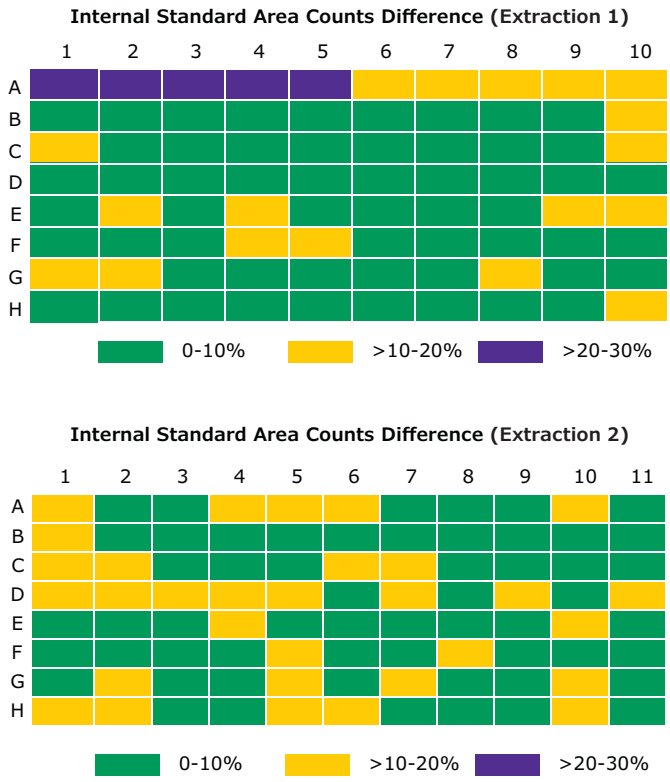

Figure 10. The percent difference from the average internal standard area counts, D3-Te-NHOH-1, Avg = 5.82 x 10^4 for extraction 1 (top) and Avg = 6.40 x 10^4 for extraction 2 (bottom), on a per well basis across the plate. Columns 2 and 10 were testosterone calibrators, remaining columns were samples.

shown in **Figure 10**. This uncertainty is contributed by the pipetting for desorption and derivatization plus from LC-MS/MS measurement. In all samples, the amount of underivatized testosterone was below the limit of detection indicating that derivatization process was complete.

Conclusion:

A BioSPME extraction method prior to analysis by LC-MS/MS was developed, and the evaluated results showed a strong correlation (R^2 = 0.92–0.96) for serum samples analyzed by externally validated equilibrium dialysis LC-MS/MS for free testosterone. The BioSPME method was automated by using a Hamilton® Starlet Robotic system and can be adapted to other robotic liquid handlers that have gripper functionality. The time to process one 96-well plate was approximately an hour. The developed LC-MS/MS detection method used derivatization of the final extract by hydroxylamine hydrochloride to increase the sensitivity for the detection of free testosterone.

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Description	Cat. No.
BioSPME & HPLC	
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Supel™ BioSPME C18 96-Pin Devices, 10 pack	**59683-U**
Positioning Adapter (for Automation of Supel™ BioSPME), 1 pack	**59686-U**
Ascentis® Express C18 5 cm x 2.1 mm I.D., 2.7 µm	**53822-U**
Acetonitrile, LC-MS LiChrosolv®	**1.00029**
Water, LC-MS LiChrosolv®	**1.15333**
Hydroxylamine hydrochloride ReagentPlus®, 99%	**159417**
Phosphate Buffer Solution	**P5358**
Certified reference materials - Cerilliant®	
Testosterone solution 1.0 mg/mL in acetonitrile, 1 mL	**T-037**
Testosterone-d3 (16, 16, 17-d3) solution 100 µg/mL in acetonitrile, 1 mL	**T-046**
Accessories	
Nunc® 96 DeepWell™ plate, non-treated, U-bottom natural polypropylene wells, maximum volume 1.3 mL, non-sterile	**P8241**
Corning® Thermowell PCR 96 well plates, polypropylene, conical bottom, clear, Pk.25	**CLS6551**
Plate Glass Coated Microplates 96-Well Microplate, glass coated, round well; U-Shape, 7.2mm dia	**Available from NS3**
MultiTherm™ shaker with heating	**Z755753**
Texan™ reagent reservoir for multichannel pipettes	**R9259**
Zone-Free™ Sealing Films	**Z721646**
SealPlate Film	**Z369659**
Corning® Stripwell™ accessories "egg crate" strip holder	**CLS2572**

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Supel™ BioSPME devices are to be used for sample preparation of serum and plasma for the subsequent analysis and concentration determination of free analytes via LC-MS and LC-MS/MS. The Supel™ BioSPME devices are to be used with compatible automation instruments via gripper paddle maneuver, or manually via hand maneuver through the sample preparation workflow steps. Supel™ BioSPME devices are for R&D use only. Not for drug, household, or other uses.

A Comparison of Superficially Porous Particle Column Chemistries for Peptide Mapping

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Introduction

Superficially porous particles (SPP) have proven themselves as an efficient alternative to fully porous particles in HPLC separations. Higher efficiency per backpressure unit is achieved with these particles, in comparison with fully porous particles, and the advantages of this particle technology have been reported in the literature.[1] These higher efficiencies are due to shorter diffusion paths within, and narrower particle size distributions of the SPP.

In this article, we compare three different superficially porous particle chemistries, from the BIOshell™ line of U/HPLC columns, in terms of their performance in the separation of peptides and peptide mapping. The quality parameters evaluated include peak width at half maximum (FWHM, full width half maximum), peak capacity, resolution between selected peak pairs, and theoretical plates (N).

For system suitability testing, a mixture of synthetic peptides in the MSRT Calibration Mix (cat.no. **MSRT1**) was first used to compare the cyano, phenyl-hexyl, and C18 bonded phases, prior to performing the same comparisons with a tryptic digest of the monoclonal antibody reference material NISTmAb, a humanized $IgG1_k$ monoclonal antibody. Each column was identical in terms of physical dimensions, pore size, particle size and mobile phases, and gradient conditions were kept constant for all tests performed (**Table 1**).

Experimental Methods

The system suitability mix was prepared according to the instructions on the data sheet of the **MSRT1** but with a final acetonitrile concentration of 1.6%. The injection volume was 10 µL.

Digestion of the NISTmAb reference material (**NIST8671**) was performed with a low artifact digestion buffer (**EMS0011**) using instructions provided in the product information sheet. To look for oxidized and deamidated forms of peptides, trypsin was added and digestion performed overnight in ammonium carbonate. In this way, higher amounts of oxidized methionine and deamidated asparagine were generated on some peptides to evaluate chromatographic separations.

The analysis was performed under the instrument and gradient conditions shown in **Tables 2-3**.

Table 2. LC Instrumental conditions used for peptide analysis

LC Conditions	
LC Instrument:	Waters Acquity UPLC®
Column:	see **Table 1**
Mobile phase:	[A] 0.1% Formic acid in water; [B] 0.1% Formic acid in acetonitrile (ACN)
Gradient:	**Time (min) / A% / B%** 0 / 99.5 / 0.5 60 / 65 / 35 61 / 3 / 97 68 / 3 / 97 69 / 99.5 / 0.5 80 / 99.5 / 0.5
Flow rate:	0.2 mL/min
Pressure:	See **Table 5**
Column temp.:	60 °C
Detector:	MS (see **Table 3** for conditions)
Injection:	10 µL
Sample(s):	1. MRST1: MS RT Calibration Mix with final acetonitrile concentration of 1.6% 2. Tryptic digest of NISTmAb

Table 1. Superficially porous silica particle chemistries evaluated

Column	Pore Size (Å)	Particle Size (µm)	I.D. x L	Matrix active group	Max. pressure (bar)	Max. temp. (°C)
BIOshell™ A160 Peptide C18	160	2.7	2.1 mm X 150 mm	C18 (octadecyl) bonding phase, diisobutyloctadecyl	600	90
BIOshell™ A160 Peptide Phenyl-Hexyl	160	2.7	2.1 mm X 150 mm	Phenyl-Hexyl (dimethylphenyl-hexylsilane) bonding phase	600	90
BIOshell™ A160 Peptide CN	160	2.7	2.1 mm X 150 mm	Cyano bonding phase	600	90

Table 3. Mass spectrometry parameters

MS Conditions	
Instrument:	Thermo QE Plus
Polarity:	Positive ion
Spray voltage:	4.0 kV
Capillary temperature:	320 °C
Sheath gas:	10
Aux gas:	5
S-Lens:	50 V
m/z Range:	200-2000
ddMS2:	Top10

Results & Discussion

A system suitability test mix is a recommended way to monitor the performance of a chromatographic system prior to submitting valuable samples for analysis. **MSRT1** is a mix of 14 isotopically labeled peptides whose sequences are shown in **Table 4**. Each peptide is labeled with an isotopically labeled form of either ($^{13}C_6$, $^{15}N_1$) leucine [L], ($^{13}C_6$, $^{15}N_2$) lysine [K], or ($^{13}C_6$, $^{15}N_4$) arginine [R].

Table 4. MSRT1 peptide sequences. Labelled amino acids in brackets.

Peptide #	Peptide Sequence
1	RGDSPASSP[K]
2	GLV[K]
3	LGGNETQV[R]
4	AEFAEVS[K]
5	SGFSSVSVS[R]
6	ADEGISF[R]
7	DISLSDY[K]
8	LVNEVTEFA[K]
9	DQGGELLSL[R]
10	GLFIIDD[K]
11	LGEYGFQNA[L]
12	YWGVASFLQ[K]
13	TDELFQIEGLKEELAYL[R]
14	AVQQPDGLAVLGIFL[K]

Injection of this mix on each of the three columns (**Figure 1**) indicates increasing retention of peptides in the order of cyano, phenyl-hexyl, and C18 under identical chromatographic conditions. The reason for this result is most likely derived from the retention mechanisms in the chromatographic system. Under the mobile phase conditions utilized, the analytes would behave mostly by a partitioning mechanism, with an increasing retention with the degree of hydrophobicity of the ligands on the silica surface. For the system suitability mix, the peptide sample solution consisted of 1.6% acetonitrile after following the instructions in the package insert. Even with this low organic solvent composition, some polar peptides were not retained on the cyano column even with the low 0.5% starting organic composition of the mobile phase. This result serves as a reminder to keep organic content as low as possible when introducing samples on columns, both for the mobile phase and the sample solution.

Peak widths are sharpest (full-width half max, FWHM) on the C18 column, ranging from 0.07 to 0.11 min, intermediate on the phenyl-hexyl at 0.09 to 0.18 min, and widest on the cyano phase at 0.12 to 0.16 min.

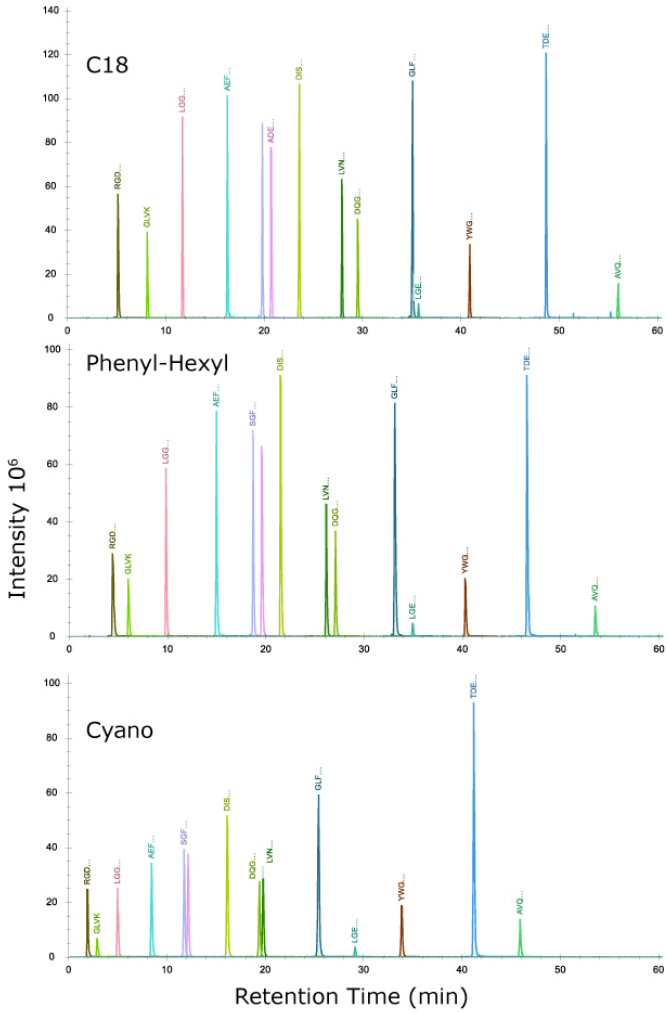

Figure 1. Injection of system suitability mix, **MSRT1**, containing 14 isotopically labelled peptides across molecular weights of 423.3 to 2176.1, onto the three column chemistries indicated. The sequences of the peptides are provided in **Table 4**.

Taking the average FWHM values for retained peptides yielded values of 0.093 for the C18 phase, 0.115 for the phenyl-hexyl phase, and 0.141 for the cyano phase (**Table 5**).

Using a peak capacity calculation of PC = 1+ t_g/W_h, where t_g is the length of the linear gradient, and W_h is the average FWHM, PC values were 643 for the C18, 524 for phenyl-hexyl, and 426 for the cyano column. The average number of plates was also calculated using the equation N = 5.545 $(t_r/FWHM)^2$ for each peak (t_r = retention time of each peak) and then taking the average to yield the number of plates on the C18 column of 593,000, the phenyl-hexyl 389,000, and the cyano 159,000. These values reflect both the greater retention and sharper peaks obtained, overall, on the C18 column.

Table 5. Column performance comparison

Column	Average FWHM (min)	Peak Capacity	Theoretical plates	Average Peak Resolution	Column Backpressure (psi)*
BIOshell™ A160 Peptide C18	0.093	643	593,000	4.9	1,633
BIOshell™ A160 Peptide Phenyl-Hexyl	0.115	524	389,000	3.3	1,859
BIOshell™ A160 Peptide CN	0.141	426	159,000	3.0	1,443

* Water/acetonitrile = 50%:50%, 0.2 mL/min at 60 °C

Table 6. Select critical pairs from **MSRT1**

Pair/Peptide Number	Peptides
1	
1	**RGDSPASSP[K]**
2	**GLV[K]**
2	
5	**SGFSSVSVS[R]**
6	**ADEGISF[R]**
3	
10	**GLFIIDD[K]**
11	**LGEYGFQNA[L]**

Figure 2 shows the separation achieved with three critical pairs of peptides (**Table 6**) on the three column chemistries while the average resolution of the three pairs is shown in **Table 5**.

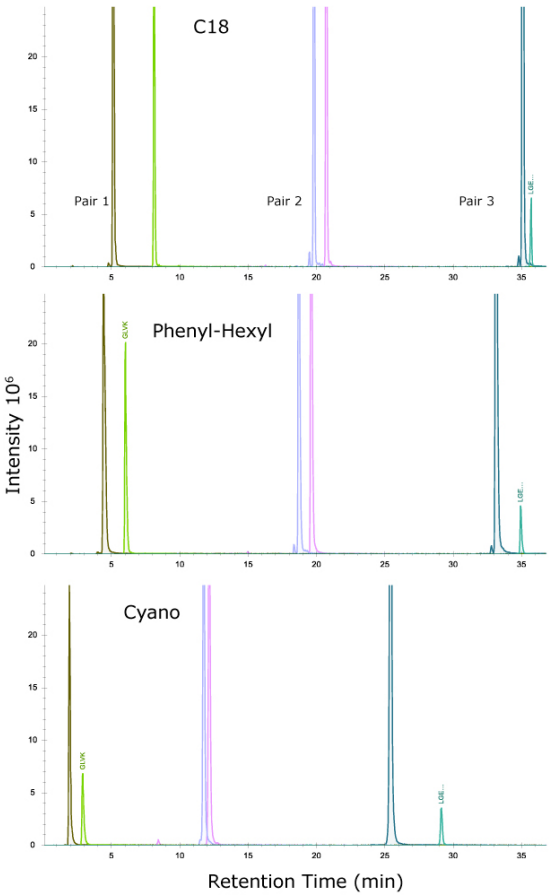

Figure 2. Separation of three peptide pairs from **MSRT1** on the three column chemistries C18, phenyl-hexyl, and cyano.

As another measure of column performance, several pairs of adjacent peaks were selected to calculate resolution (R), where $R = 2\,\Delta Z/(W_A+W_B)$, Z is the difference in retention time between the two peaks, and W_A and W_B are the widths at the baseline of the two peaks.

As seen in **Figure 2**, and perhaps most interesting, the three columns have slightly different selectivities, particularly for the last eluting pair, where the cyano column outperforms the C18 and phenyl-hexyl columns. This result illustrates the importance of phase chemistry when trying to achieve alternative selectivity for peptides. Due to the pi electrons in the triple bond of the cyano ligand, aromatic amino acids in the peptide may interact more through pi-pi stacking interactions than just through London dispersion forces, as observed on the C18 column. The cyano column does not perform as well with pairs 1 and 2, with pair 2 not being fully resolved. The C18 and phenyl-hexyl columns both show good retention of pair 1, the early eluting peptides.

The use of a system suitability mix can be recommended for regular evaluation of columns over time, to check system performance before submitting precious samples, and to make comparisons when evaluating new column chemistries.

Column Comparison with A Digested Monoclonal Antibody - NISTmAb

We next compared the columns using a tryptic digest of NISTmAb to create the separations for heavy chain (HC) and light chain (LC) peptides shown in **Figure 3**. Again, the same trend is observed in overall retention, with C18 being the most retentive and cyano the least. All three columns performed equally well in terms of sequence coverage with values of 87% or greater for all three chemistries on the heavy chain and 97% coverage of the light chain. Peak widths at FWHM across all peptides in the digest gave equivalent results as the **MSRT1** system suitability mix with C18 performing the best, followed by the phenyl-hexyl and then the cyano columns.

The box and whisker plot in **Figure 4** illustrates the distribution of peaks widths obtained across the three columns for all HC and LC peptides. Interestingly, the broadest peak on each of the columns was a peptide containing three prolines. It has been reported[2] that peptides containing several proline molecules can suffer from broad peaks and peak splitting due to cis-trans isomerization of proline-proline bonds as well as other proline-amino acid bonds.

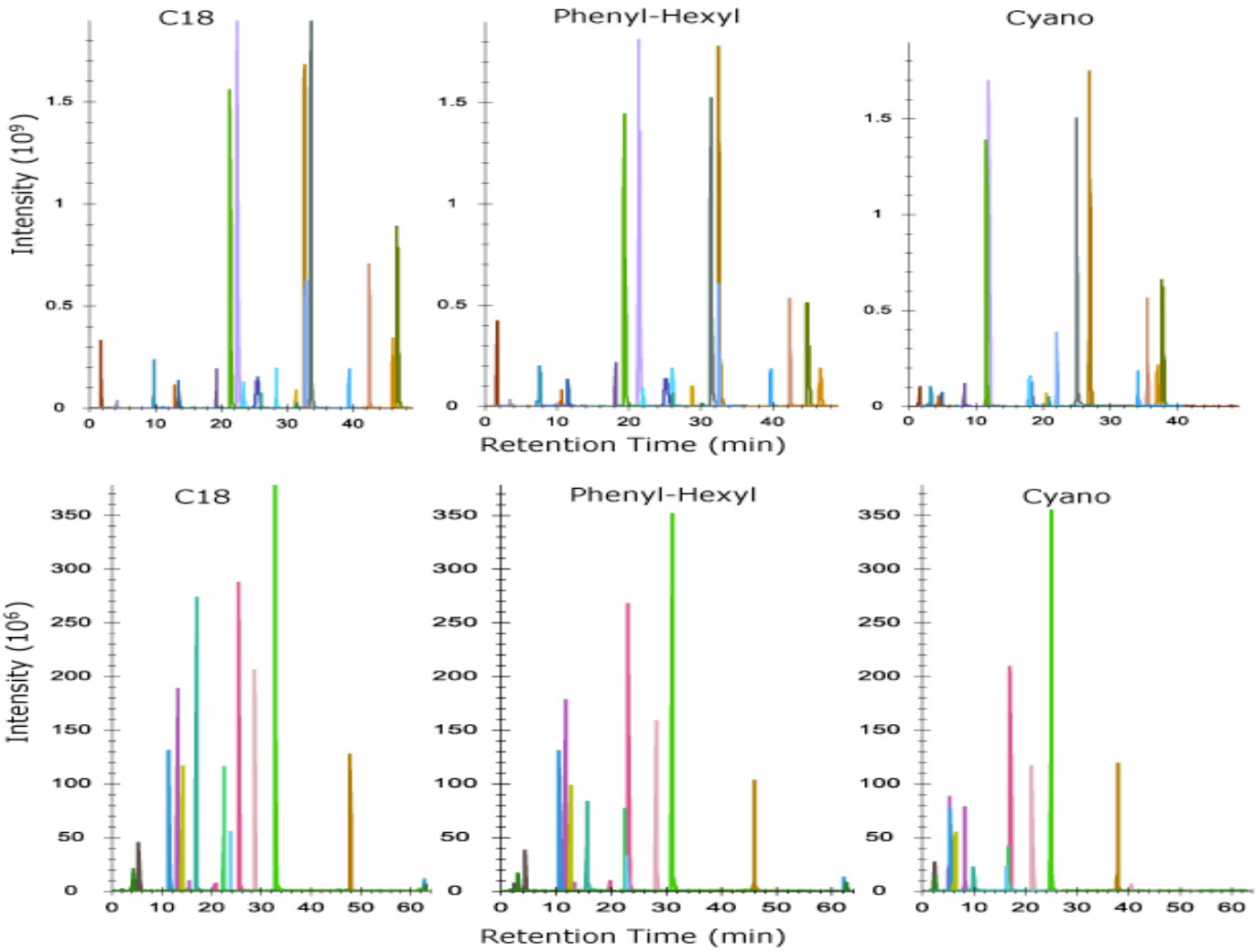

Figure 3. Elution profile of peptides from heavy chain (top) and light chain (bottom) using tryptic digest of NISTmAb on three different column chemistries shown.

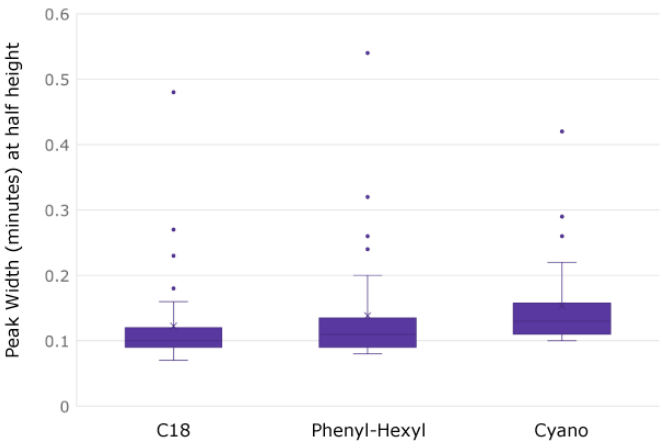

Figure 4. Peaks widths for NISTmAb peptides separated on three different column chemistries. The broadest peak observed on all columns is EPQVYTLPPSR due to three proline molecules and cis-trans isomerization (see text).

The number of theoretical plates was calculated based on the retention time and FWHM for all the heavy and light chain peptides as done previously with **MSRT1** (**Figure 5**). The results again show the C18 column to be the best-performing phase chemistry followed by the phenyl hexyl and then cyano chemistries.

Selectivity comparison

The digestion of NISTmAb was performed under conditions expected to yield greater amounts of deamidated asparagine as described. All three columns separated deamidated from unmodified peptide very well (**Figure 6**), allowing for the determination of this modification in the quality control of therapeutic proteins. Separation of oxidized methionine on peptides resulted in those peptides eluting earlier than the native form by two minutes or more (data not shown).

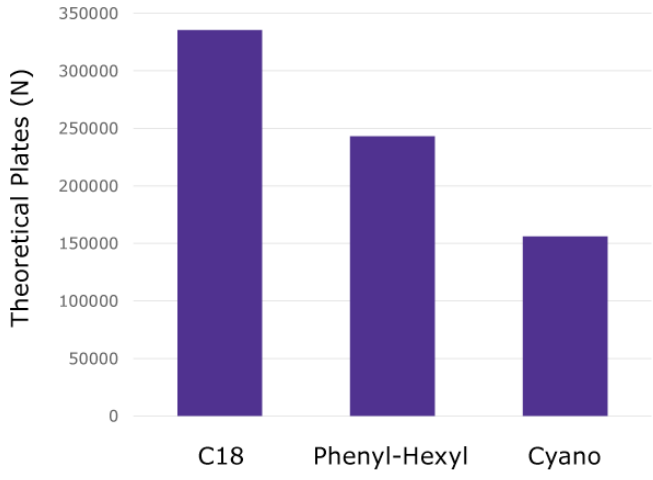

Figure 5. Average number of plates calculated from all NISTmAb heavy chain and light chain peptides on each column chemistry.

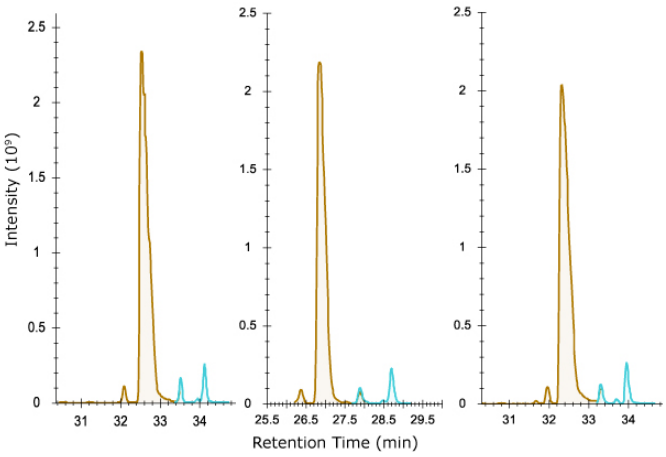

Figure 6. Separation of deamidated (blue) from unmodified NISTMab peptide FNWYVDGVEVHNAK (brown). The three deamidated forms are presumed to be a result of aspartic and isoaspartic acid isomers formed. Use of electron activated dissociation may aid in elucidating these identities.

Conclusion

Three superficially porous particles (SPP) with different bonded chemistry were evaluated for their ability to perform peptide mapping type experiments. Each column was of the same dimensions and operated under the same set of mobile phase and gradient conditions. A system suitability mix of 14 isotopically-labeled peptides was first used to evaluate peak widths, peak capacity, theoretical plates, and resolution of three peptide pairs. As expected, the C18 chemistry provided the best retention of peptides with phenyl-hexyl phase next, followed by cyano. Peak widths generally followed the same sequence with C18 again performing the best and, therefore, providing the highest peak capacity, and plates. Regarding the resolution of three selected peak pairs, it is apparent that the different phases do offer slight differences in selectivity so that, in some cases, a cyano or phenyl-hexyl chemistry may outperform a C18 phase. Use of the MSRT Calibration Mix for LC-MS has then proven to be useful in the performance evaluation of these columns.

The same trends were generally observed during the analysis of a monoclonal antibody digest, yet all performed equally well in terms of the sequence coverage provided. In this comparison, a high concentration of a relatively pure mAb was used so that differences in sequence coverage achieved from column to column were not revealed as they might have been with a complex digest containing a range of protein concentrations. In a more complex sample, one might expect the C18 column to show better performance as a result of the resolution, peak capacity, and plate number. The ability to separate deamidated forms of asparagine containing peptides from the unmodified form is shown nicely on all three columns. Overall, the C18 chemistry provides the narrowest peaks, greatest retention, and highest peak capacity of the three columns. In some cases, evaluation of cyano and phenyl-hexyl chemistries may be desirable for the separation of critical peak pairs due to differences in selectivity.

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Description	Cat. No.
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BIOshell™ A160 Peptide C18, 15 cm x 2.1 mm, 2.7 µm	**66905-U**
BIOshell™ A160 Peptide Phenyl-Hexyl, 15 cm x 2.1 mm, 2.7 µm	**577528-U**
BIOshell™ A160 Peptide CN, 15 cm x 2.1 mm, 2.7 µm	**66969-U**
Acetonitrile, LiChrosolv® hypergrade for LC-MS	**1.00029**
Water, LiChrosolv® for LC-MS	**1.15333**
Formic acid, LiChropur™ for LC-MS	**5.33002**
Samples and System Suitability Reagents	
NISTmAb Humanized IgG1$_k$ Monoclonal Antibody (NIST® RM 8671)	**NIST8671**
MS RT Calibration Mix Proteomics Retention Time Standard for LC-MS	**MSRT1**
Sample Preparation	
Low Artifact Digestion Buffer	**EMS0011**
Iodoacetamide (IAM)	**A3221**
Tris(2-carboxyethyl)phosphine hydrochloride solution (TCEP), 0.5 M, pH 7.0 (aqueous solution; pH was adjusted with ammonium hydroxide)	**646547**
SOLu-Trypsin	**EMS0004**

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Analysis of Oligonucleotide Standard 6 Mix by Liquid Chromatography-UV

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Introduction

With the COVID-19 pandemic, oligonucleotides (oligos) have proven their importance in diagnostic and therapeutic applications. Currently, 11 oligonucleotide drugs crossing many disease areas have been approved by the FDA.[1, 2] Obstacles preventing quicker development of oligonucleotide therapeutics include the challenges of unfavorable absorption, distribution, metabolism, excretion, and toxicity (ADMET) studies for many clinical trials.[2] Some strategies have been developed to tackle the challenges, such as chemical modification to improve drug delivery.

Synthetic oligonucleotides are typically small, single- or double-stranded modified nucleic acids.[2] There are many established techniques for oligonucleotide analysis and characterization, including capillary gel electrophoresis (CGE), ion exchange chromatography (IEX), and ion pair reversed-phase liquid chromatography (IP-RPLC). Generally, HPLC purification of oligonucleotides is very challenging due to the similarity of oligonucleotide structures, very polar characteristics, presence of truncated and/or modified oligos, ease of self-association into a variety of conformations, and affinity for metal surfaces.[1,2] This application describes the separation of an internally produced oligonucleotide standard (Oligo Standard 6) mix, which includes six oligonucleotides, on Supelco® Chromolith® RP-18e columns.

General Procedures

Oligo Standard 6 is an internal (in-house) system suitability mix for HPLC-UV evaluation of oligonucleotide separations. The standard contains six components with molecular weights of 3588.3 Da (Oligo 1), 4157.93 Da (Oligo 2), 7580.83 Da (Oligo 3), 10014.35 Da (Oligo 4), 6116.97 Da (Oligo 5), and 4395.8 Da (Oligo 6) following their elution order on Chromolith® RP-18e columns tested here.

Reagent Preparation

50 mM Triethylammonium acetate (TEAA)

To prepare 1 L of 50 mM TEAA, 50 mL of TEAA (commercial 1 M solution) was added into 950 mL of HPLC grade water and mixed well.

20 mM Triethylammonium acetate (TEAA)

To prepare 1 L of 20 mM TEAA, 20 mL of TEAA (commercial 1 M solution) was added into 980 mL of HPLC grade water and mixed well.

5 mM Triethylammonium acetate (TEAA)

To prepare 1 L of 5 mM TEAA, 5 mL of TEAA (commercial 1 M solution) was added into 995 mL of HPLC grade water and mixed well.

Sample Preparation

5 µM Oligo Standard 6 sample
1 mL of HPLC grade water was added into the sample vial which contains 5 nmol each of the six Oligo components and mixed well.

HPLC-UV System Setup and Data Analysis

Essential settings of the HPLC-UV chromatography system for analysis of Oligo Standard 6 are listed in **Table 1**.

Table 1. HPLC-UV general system settings.

Instrument Setup	
Instrument:	Agilent 1260 Infinity II
Software:	Agilent ChemStation
Columns:	Chromolith® Performance RP-18e, 100 x 4.6 mm; Chromolith® HighResolution RP-18e, 100 x 2.0 mm / 50 x 2.0 mm
Mobile phase:	[A] 5–50 mM TEAA; [B] Acetonitrile
Gradients:	See **Figure 1**, **3,** and **4**
Flow rate:	0.4–3 mL/min (see text)
Autosampler temp.:	5 °C
Column temp.:	25 °C; 40 °C (2 mm I.D. columns)
Detector:	UV; 260 nm
Injection:	3-5 µL (see text)
Run time:	12 min
Sample:	5 µM Oligo Standard 6 in water

Results and Discussion

With the linkage of phosphate groups, oligonucleotides tend to stick to metal surfaces present in stainless steel column hardware and the LC system, resulting in reduced sensitivity and inaccurate quantitation. Researchers have made a variety of efforts to mitigate this adsorption inside instrumentation, such as treating the system with EDTA, 2 mL/min high pH mobile phase, or utilizing bio-inert HPLC system components.[3] Conventional HPLC columns are typically packed in metal columns, exposing metal surfaces with positive charge, that can adsorb acidic molecules, such as oligonucleotides containing phosphate groups. Chromolith® HPLC columns are made of highly porous monolithic rods of silica, with an innovative bimodal pore structure and packed in metal-free PEEK (polyetheretherketone) columns, which make it a good candidate for oligonucleotide analysis.

Chromolith® Performance RP-18e, 4.6 mm I.D. column

High Flow Rate Test

To improve separation efficiencies, the particle size of packing material is usually reduced. Conventional HPLC columns typically contain 5, 3, 2, and even sub 2 µm silica particles. However, the smaller particle size causes higher back pressure, affecting the assay throughput, robustness, and column lifetime. The optimal solution would be a column that offers faster throughput without too high back pressure. Chromolith® columns are not packed with silica particles, but rather are a single rod of high-purity, silica gel. Their unique construction enables highly efficient separations at accelerated speeds, ideal for high throughput analysis.[4]

Figure 1 shows the separation of Oligo Standard 6 on a Chromolith® Performance RP-18e column under a flow rate of 2 mL/min with 25 pmol on column for each oligonucleotide.

50 mM of TEAA was used as mobile phase A and acetonitrile as mobile phase B, with a gradient of 5% B, ramping to 15% B in 10 minutes. Typical back pressure at 3 mL/min is 50 bar, which is beneficial for high throughput assays.

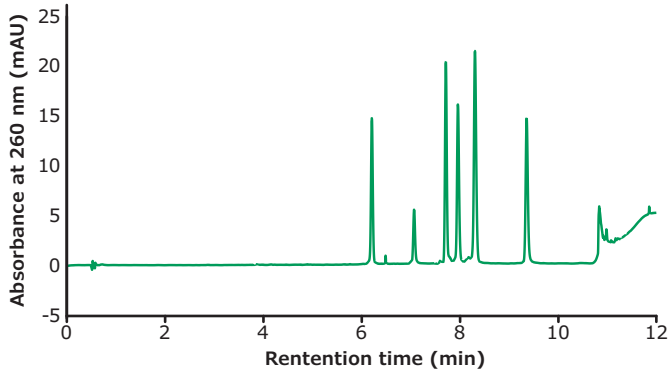
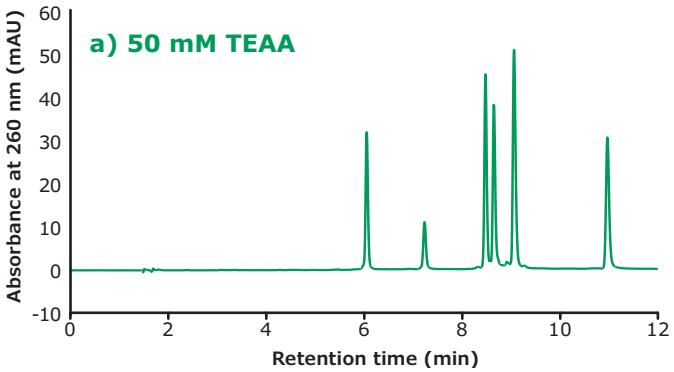


Figure 1. Oligo Standard 6 separation on Chromolith® Performance RP-18e, 100 x 4.6 mm column at flow rate of 3 mL/min with a gradient of 5% B to 15 % B in 10 minutes. Mobile phase A: 50 mM TEAA in water; Mobile phase B: acetonitrile. Injection volume: 5 µL (25 pmol on column).

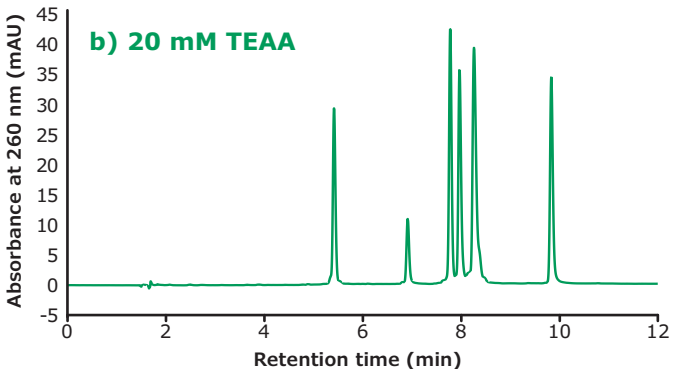
Ion-Pairing Additive Concentration Test

In the qualitative and quantitative analysis of oligonucleotide impurities, ion-pair reversed phase liquid chromatography has been the dominant technique. The ion-pairing reagents added in mobile phase are typically several alkylammonium salts, which are adsorbed on the column sorbent with the positive charges exposed to interact with the negatively charged oligonucleotides. Triethylammonium acetate (TEAA) is one of the commonly used ion-paring reagents in LC-UV analysis of oligonucleotides. Optimizing ion-pairing additive concentration is important to achieve efficient separation while minimizing cost from additive consumption. In this work, an optimization of TEAA concentration was conducted.



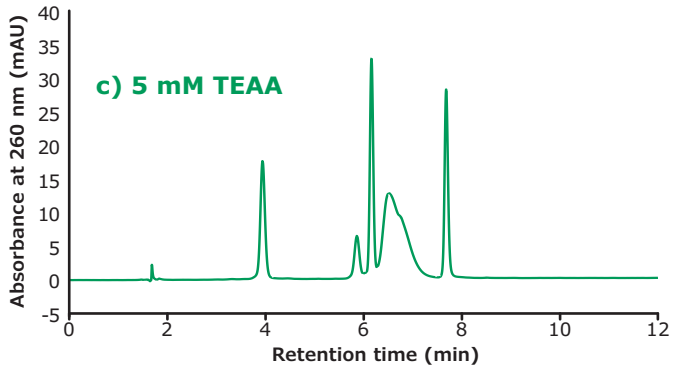
50 mM TEAA

Peak ID	RT (min)	Peak Area	Peak Height	Resolution (USP)
Oligo 1	6.051	113.191	32.336	
Oligo 2	7.232	45.290	11.023	12.549
Oligo 3	8.476	153.993	45.496	13.167
Oligo 4	8.647	136.388	38.111	1.938
Oligo 5	9.058	205.826	50.765	4.293
Oligo 6	10.964	142.741	30.822	17.499



20 mM TEAA

Peak ID	RT (min)	Peak Area	Peak Height	Resolution (USP)
Oligo 1	5.418	110.992	29.261	
Oligo 2	6.916	44.280	10.811	15.225
Oligo 3	7.780	158.234	42.434	8.820
Oligo 4	7.969	145.570	35.631	1.917
Oligo 5	8.263	215.256	39.189	2.522
Oligo 6	9.835	144.133	34.367	13.334



5 mM TEAA

Peak ID	RT (min)	Peak Area	Peak Height	Resolution (USP)
Oligo 1	3.942	121.392	17.759	
Oligo 2	5.865	40.805	5.964	10.811
Oligo 3	6.163	157.698	32.526	1.925
Oligo 4/5	6.517	368.327	12.332	0.749
Oligo 6	7.685	143.739	28.318	2.462

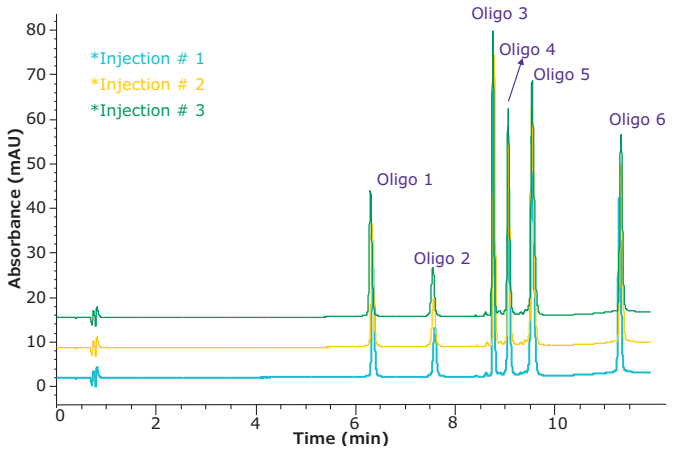
Figure 2. Oligo Standard 6 separation on Chromolith® Performance RP-18e, 100 x 4.6 mm column with different TEAA concentration in mobile phase A: a) 50 mM TEAA; b) 20 mM TEAA; and c) 5 mM TEAA. Resolution is calculated between each two adjacent peaks.

Figure 2 shows the different concentrations of TEAA tested in mobile phase A with acetonitrile as mobile phase B in the separation. In this case, five microliters (25 pmol) of Oligo Standard 6 sample were injected on column at a flow rate of 1 mL/min with a gradient of 8% B to 15% B in 10 minutes for each test. With 50 mM of TEAA in mobile phase A, the oligonucleotides were well separated with the retention time as indicated in **Figure 2**. When the TEAA concentration was lowered to 20 mM, Oligo 1 to 6 eluted in the same order but with less retention on the column. With the exception of Oligos 1 and 2, the resolution between each peak pair is seen to be lower as well. When TEAA concentration was further lowered to 5 mM, Oligos 4 and 5 were not separated, which indicates the ion-pairing strength not to be high enough to separate these two oligonucleotides. Comparing the peak heights of the six Oligos under the three different TEAA concentrations, 50 mM TEAA produced the highest peak height as shown in the table in **Figure 2**. Therefore, the ion-pairing additive concentration needs to be optimized based on the characteristics of the oligonucleotides.

Chromolith® HighResolution RP-18e, 2 mm I.D. Columns

The Chromolith® HighResolution (HR) column possesses 1.15 µm macropores compared with 2 µm on the Chromolith® Performance column. This modification results in higher separation efficiency and better peak shape. Although this creates higher back pressure, the back pressure of this column is still less than half of that of any particulate column of similar efficiency.[4]

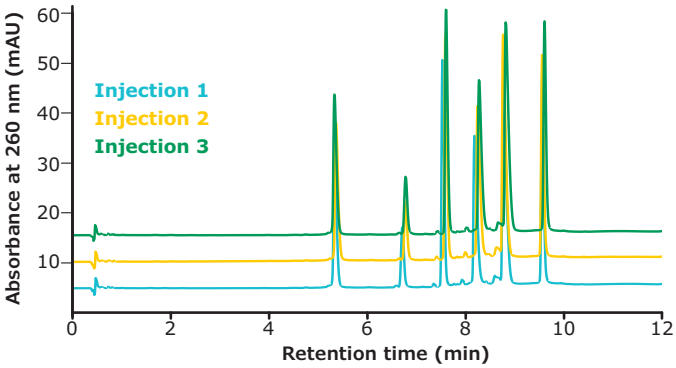
Here, 3 µL of Oligo Standard 6 sample was injected onto a Chromolith® HighResolution RP-18e, 100 x 2.0 mm column at 0.4 mL/min with a gradient of 8% B to



Peak ID	RT (min)	Peak Area	Peak Height	Resolution (USP)
Oligo 1	6.337	102.148	27.621	
Oligo 2	7.568	50.707	10.807	11.691
Oligo 3	8.781	167.619	65.201	13.279
Oligo 4	9.072	150.772	47.522	3.972
Oligo 5	9.539	228.983	53.013	4.936
Oligo 6	11.327	159.908	39.979	17.292

Figure 3. Oligo Standard 6 separation on Chromolith® HighResolution RP-18e, 100 x 2.0 mm column. Mobile phase A: 50 mM TEAA in water, Mobile phase B: acetonitrile; gradient: 8% B to 15% B in 10 minutes at flow rate of 0.4 mL/min, column temp.: 40 °C, Injection: 3 µL (15 pmol on column).

15% B in 10 minutes. **Figure 3** is an overlay of three injections showing consistent retention and response. 50 mM TEAA concentration was used as mobile phase A and acetonitrile as mobile phase B. The resolution between Oligo 4 and 5 is 4.936. A shorter Chromolith® HighResolution RP-18 column, 50 x 2 mm, was compared using the same conditions as in **Figure 3** but with slightly more injected on column (5 µL or 25 pmol). As shown in **Figure 4**, on a 50 x 2 mm column, all six oligonucleotides were eluted within 10 minutes, with the resolution between Oligo 4 and 5 of 3.921. Thus, Chromolith® HR RP-18e column is capable of oligonucleotide analysis using LC-MS compatible flow rates.



Peak ID	RT (min)	Peak Area (mAU*min)	Peak Height (mAU)	Resolution (USP)
Oligo 1	5.343	261.641	56.765	
Oligo 2	6.794	106.792	23.310	12.458
Oligo 3	7.618	357.882	90.331	7.641
Oligo 4	8.292	316.582	61.032	5.746
Oligo 5	8.836	479.91	84.329	3.921
Oligo 6	9.625	307.511	84.625	6.631

Figure 4. Oligo Standard 6 separation on Chromolith® HighResolution RP-18e, 50 x 2.0 mm column. Mobile phase A: 50 mM TEAA in water, Mobile phase B: acetonitrile; gradient: 8% B to 15% B in 10 minutes at flow rate of 0.4 mL/min, column temp.: 40 °C, Injection: 5 µL (25 pmol on column).

Conclusion

In this application note, the separation of Oligo Standard 6, an internally created HPLC-UV system suitability mix, was demonstrated on Chromolith® Performance and Chromolith® HighResolution RP-18e columns. Flow rates up to 3 mL/min were evaluated on Chromolith® Performance column with excellent separation of the six oligos, indicating that it is ideal for high throughput assays. The results of the ion-pairing reagent optimization experiments indicate that 50 mM TEAA provides the best separation and sensitivity for Oligo Standard 6. Separation of Oligo Standard 6 on the Chromolith® HighResolution column with a flow rate of 0.4 mL/min produced better resolution of Oligo 4 and 5 compared to the 3 mL/min method on Chromolith® column, with resolution (USP) of 3.9 vs 1.9 for the oligos 4/5. This result demonstrates that the Chromolith® HighResolution (HR) column is suitable for oligonucleotide analysis by LC-MS with mass spectrometer favorable flow rates tested here. In addition, the polymeric column housing can be used as part of a metal-free, or bio-inert HPLC system.

Acknowledgement

The authors would like to thank Pierre Potier for providing the Oligo Standard 6 mix and for technical support.

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4. Chromolith® HPLC columns brochure Race through your Separations on Any System (BR8065EN, 10/2021).

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Description	Cat. No.
HPLC Columns	
Chromolith® Performance RP-18 endcapped L × I.D. 100 mm × 4.6 mm	**1.02129**
Chromolith® HighResolution RP-18 endcapped L × I.D. 100 mm × 2.0 mm	**1.52322**
Chromolith® HighResolution RP-18 endcapped, L × I.D. 50 mm × 2.0 mm	**1.52321**
Chemicals & Reagents	
Triethylammonium Acetate, 1 M Solution	**90358**
Water, HPLC Grade	**270733**
Acetonitrile, for UHPLC, suitable for mass spectrometry (MS)	**900667**
Instruments & Consumables	
Eppendorf ThermoMixer® F1.5	**EP5384000012**
Vials, amber glass, volume 2 mL	**27344**
Pipette 0.5–10 µL	**EP4924000223**
Pipette 10–100 µL	**EP4924000258**
Pipette 100–1000 µL	**EP4924000282**
Pipette tips 0.1–20 µL box	**Z640204**
Pipette tips 2–200 µL box	**Z640220**
Pipette tips 50–1000 µL box	**Z640247**

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PHARMA & BIOPHARMA

UHPLC-MS Bottom-Up Analysis of Trastuzumab on a BIOshell™A160 Peptide C18 Column

Saving solvent by using a smaller I.D column

Cory Muraco, Product Manager Liquid Separations, Analytix@milliporesigma.com

Introduction

Bottom-up analysis (also called peptide mapping) is a routine assay performed by analysts in the biopharmaceutical industry as determining the primary structure of a biotherapeutic is a critical quality attribute (CQA). Narrow inner diameter (I.D.) columns with 15 cm lengths are typically employed for this analysis in order to achieve high resolution and sensitivity. However, peptide mapping methods require a long run time and, therefore, utilize larger volumes of solvent than shorter methods. This requirement leads to higher costs of the method in terms of higher volumes of solvent used as well as an additional expense in disposing the used solvent. This article demonstrates the use of a new, 1.5 mm I.D. column in reducing solvent consumption for peptide mapping techniques without compromise in method performance (**Figure 1**) as can be derived from 98% sequence coverage on both columns.

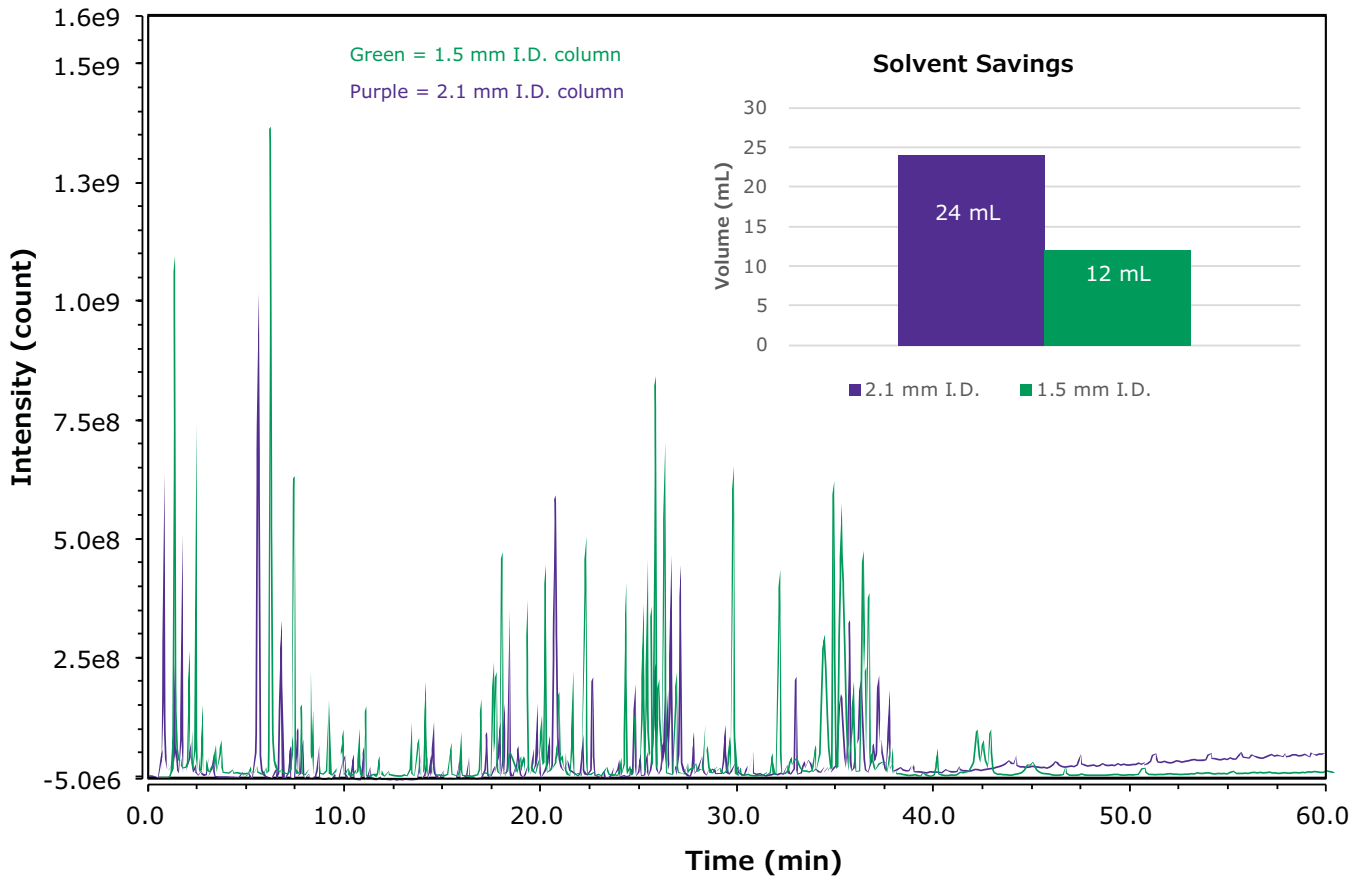

Figure 1. Bottom-up analysis of trastuzumab on BIOshell™ A160 Peptide C18 columns with 2.1 (purple) and 1.5 mm (green) inner diameter (conditions see **Table 1**).

Table 1. LC-MS Conditions for bottom-up analysis of trastuzumab.

Conditions:	
Column:	BIOshell™ A160 Peptide C18, 15 cm x 2.1 or 1.5 mm I.D., 2.7 µm
Mobile phase:	[A] Water (0.1% (v/v) DFA); [B] Acetonitrile (0.1% (v/v) DFA*)
Gradient:	2 – 50% B in 60 min
Flow rate:	0.2 mL/min (1.5 mm I.D.) or 0.4 mL/min (2.1 mm I.D.)
Column temp.:	60 °C
Detector:	MSD, ESI-(+)
MS Conditions:	
Spray voltage:	3.8 kV
Capillary temp:	320 °C
Sheath gas:	35
Aux gas:	10
RF lens:	50
Injection:	2.0 µL
Sample:	Trastuzumab tryptic digest, 1.25 mg/mL, 1.5 M guanidine hydrochloride, 0.5% (v/v) formic acid

*To read more about the use if DFA as mobile phase modifier for LC-MS, see the article

Difluoroacetic Acid as an Efficient Mobile Phase Modifier for the LC-UV/MS Analysis of Proteins

in Analytix Reporter - Issue 12, **SigmaAldrich.com/analytix**

Conclusion

This application note described the use of a new 1.5 mm I.D. column to reduce solvent consumption in peptide mapping workflows without compromising the efficiency. As noted, 50% less solvent was consumed, as compared to a 2.1 mm I.D. column, using the 1.5 mm I.D. column as the optimum flow rate for this column is 0.2 mL/min. This observation translates to only 12 mL of solvent being used in this assay versus 24 mL using a 2.1 mm I.D. column. By using less solvent, the cost per sample is reduced as well as the cost of waste disposal, making this a greener method. Finally, sensitivity, in general, was improved using the 1.5 mm I.D. column vs. the 2.1 mm I.D. column, enabling more accurate quantitation of signature peptides as well as the detection of post-translational modifications.

Featured Products

Description	Cat. No.
BIOshell™ A160 Peptide C18, 15 cm × 1.5 mm I.D., 2.7 µm	**66922-U**
BIOshell™ A160 Peptide C18, 15 cm × 2.1 mm I.D., 2.7 µm	**66905-U**
Water, for UHPLC, suitable for MS	**900682**
Acetonitrile, for UHPLC, suitable for MS	**900667**
Guanidine hydrochloride, ≥99% (titration), organic base and chaeotropic agent	**G4505**
Difluoroacetic acid, for LC-MS, LiChropur™	**00922**
Formic acid, for LC-MS LiChropur™, 97.5-98.5% (T)	**00940**

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ENVIRONMENTAL

LC-MS Analysis of PFAS Compounds in EPA Method 533 using Supelclean™ ENVI-WAX™ SPE

Lara Rosenberger, Yannick Hövelmann, Olga Shimelis, Analytix@milliporesigma.com

Introduction

Per- and polyfluoroalkyl substances (PFAS) have been in use since the 1940s. Consisting of over 4700 different compounds, PFAS substances are used in almost every facet of modern life. The utility of these compounds resulted in their rapid adoption in consumer goods manufacturing. PFAS compounds can now be found in food packaging, cookware, cosmetics, stain and water repellants, firefighting foams, and are commonly used in many manufacturing processes. While incredibly useful, these compounds also carry a risk to health that we have only recently started to understand clearly.

PFAS compounds are also commonly known as “forever chemicals” which means they do not break down in the environment like other chemicals. This persistence can result in the concentration of these compounds growing to levels that are unsafe for human exposure and that can cause negative health effects such as low infant birth weights, effects on the immune system, cancer, and thyroid hormone disruption.

PFAS detection plays therfore a crucial role in safeguarding public health and the environment. PFAS detection in water is essential for assessing water quality and to identifying potential health risks. To achieve accurate measurements and quantification of these contaminants in water samples, various PFAS analysis methods are employed.

Multiple regulatory methods, such as EPA 537 and 533, detail the extraction of PFAS analytes from drinking water using SPE cartridges followed by LC-MS/MS analysis. For EPA method 533, weak anion exchange (WAX) cartridges are specified and should contain 500 mg of the mixed-mode polymeric adsorbent. Supelclean™ ENVI-WAX™ SPE cartridges are direct equivalent to the specified SPE in EPA method 533. This application note demonstrates the extraction of 25 analytes from water using Supelclean™ ENVI-WAX™ SPE.

Experimental

The procedure from EPA method 533 was followed for sample collection and sample preparation. Supelclean™ ENVI-WAX™ SPE 500 mg/6 mL cartridges (**54057-U**) were used with a Visiprep™ vacuum manifold (**57030-U**) for processing the samples. The large volume sampling kit (57275) was also used but the Teflon tubing was replaced with silicone tubing (1/8" diameter). The Teflon guides in the original manifold were replaced with stainless-steel solvent guides (**57027**). Analysis of the samples was done using an Agilent 6495C LC-MS/MS instrument. Ascentis® Express PFAS HPLC Column, 2.7 µm, 15 cm x 2.1 mm (**53560-U**) was used as an analytical column. In addition, an Ascentis® Express PFAS Delay Column, 2.7 µm, 5 cm x 3.0 mm (**53572-U**) was used (**Table 1**). The chromatogram of 25 compounds in a calibration standard is shown in **Figure 1**.

UHPLC-MS grade water samples were tested for PFAS contamination and found to be free of 25 analytes as per the EPA method 533. The water was spiked at 10 or 40 ng/L with 25 analytes to demonstrate the performance of Supelclean™ ENVI-WAX™ SPE cartridges for this method. 250 mL of water samples were loaded onto 500 mg/6 mL SPE cartridges, and eluted using methanol with 2% (v/v) ammonium hydroxide; the resulting eluate was evaporated to dryness and reconstituted into 1.0 mL of 4% (v/v) methanol in water for LC-MS/MS detection.

Following the performance assessment of the method using Supelclean™ ENVI-WAX™ SPE, a tap water sample was analyzed using the same methodology for the presence of 25 PFAS compounds.

Filters Suitable for PFAS Analysis

Read more about recommended Millex® syringe filter and cut disc membrane filters at **SigmaAldrich.com/pfassamplefiltration**

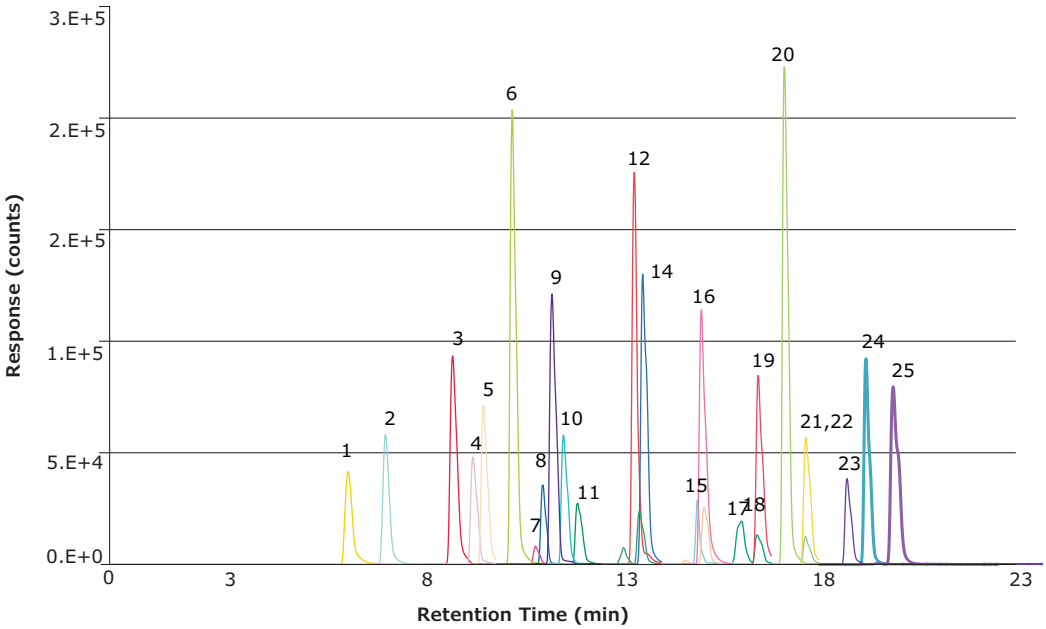

Figure 1. 25 PFAS compounds at 50 ppb in 96:4 water:methanol (v/v) solvent.

Table 1. LC-Conditions for analysis of 25 PFAS compounds

Chromatography Conditions	
Column:	Ascentis® Express PFAS, 2.7 µm, 15 cm x 2.1 mm (**53560-U**)
Delay column:	Ascentis® Express PFAS Delay Column, 2.7 µm, 5 cm x 3.0 mm (**53572-U**)
Mobile Phase:	[A] 20 mM Ammonium acetate; [B] Methanol
Gradient:	

Time (min)	%A	%B
Inital	95.0	5.0
0.5	95.0	5.0
3.0	60.0	40.0
16.0	20.0	80.0
18.0	20.0	80.0
20.0	5.0	95.0
22.0	5.0	95.0
25.0	95.0	5.0
35.0	95.0	5.0

Flow Rate:	0.25 mL/min
Injection:	10 µL
Detector:	MS, MRM (see **Table 2**)
Samples:	Water samples (spiked and unspiked) extracted by SPE

Table 2. MRM trasition used for 25 PFAS compounds in EPA method 533

Peak	Compound		MRM
1	PFBA	Perfluorobutanoic acid	213.0->169.0
2	PFMPA	Perfluoro-3-methoxypropanoic acid	229.0->85.0
3	PFPeA	Perfluoropentanoic acid	263.0->219.0
4	PFBS	Perfluorobutanesulfonic acid	298.9->80.0
5	PFMBA	Perfluoro-4-methoxybutanoic acid	279.0->85.1
6	PFEESA	Perfluoro(2-ethoxyethane)sulfonic acid	314.5->135.0
7	NFDHA	Nonafluoro-3,6-dioxaheptanoic acid	295.0->201.0
8	4:2FTS	1H,1H,2H,2H-Perfluorohexane sulfonic acid	327.0->307.0
9	PFHxA	Perfluorohexanoic acid	313.0->269.0
10	PFPeS	Perfluoropentanesulfonic acid	348.9->80.0
11	HFPO-DA	Hexafluoropropylene oxide dimer acid	285.0->169.0
12	PFHpA	Perfluoroheptanoic acid	363.0->319.0
13	PFHxS	Perfluorohexanesulfonic acid	389.9->80.0
14	ADONA	4,8-Dioxa-3H-perfluorononanoic acid	377.0->251.0
15	6:2 FTS	1H,1H,2H,2H-Perfluorooctane sulfonic acid	427.0->406.9
16	PFOA	Perfluorootanoic acid	413.0->369.0
17	PFHpS	Perfluoroheptanesulfonic acid	448.9->80.0
18	PFOS	Perfluorooctanesulfonic acid	498.9->80.0
19	PFNA	Perfluorononanoic acid	463.0->419.0
20	9Cl-PF3ONS	9-Chlorohexadecafluoro-3-oxanonane-1- sulfonic acid	530.9->350.9
21	8:2FTS	1H,1H,2H,2H-Perfluorodecane sulfonic acid	527.0->507.0
22	PFDA	Perfluorodecanoic acid	513.0->469.0
23	PFUnA	Perfluoroundecanoic acid	563.0->519.1
24	11Cl-PF3OUdS	11-Chloroeicosafluoro-3-oxaundecane-1-sulfonic acid	631.0->451.0
25	PFDoA	Perfluorododecanoic acid	613.0->569.0

Results and Discussion

The background evaluation of the method using all SPE consumables and accessories resulted in excellent low background values (shown in **Table 3**). The result for screening all compounds in the UHPLC-MS solvent was at or below the lower limit of detection (LLOD) of the LC-MS/MS instrument.

Table 3. Results of background testing for the evaluation

Compound	Background in UHPLC-MS water (ng/L)*
PFBA	Below LLOD*
PFMPA	2[1]
PFPeA	Below LLOD
PFBS	Below LLOD
PFMBA	Below LLOD
PFEESA	Below LLOD
NFDHA	Below LLOD
4:2FTS	Below LLOD
PFHxA	3[2]
PFPeS	Below LLOD
HFPO-DA	Below LLOD
PFHpA	Below LLOD
PFHxS	Below LLOD
ADONA	Below LLOD
6:2 FTS	Below LLOD
PFOA	Below LLOD
PFHpS	Below LLOD
PFOS	Below LLOD
PFNA	Below LLOD
9Cl-PF3ONS	Below LLOD
8:2FTS	Below LLOD
PFDA	Below LLOD
PFUnA	Below LLOD
11Cl-PF3OUdS	Below LLOD
PFDoA	Below LLOD

[1] LCMRL (Lowest Concentration Minimum Reporting Level) is 5.3 ng/L per EPA method 533

[2] LCMRL is 3.8 ng/L per EPA method 533

*LLOD were 2-6 ppt for all compounds

Per EPA method 533 the recovery of the laboratory spiked blank water samples should fall in the range 70-130% with reproducibility of better than 20%. **Figure 2** demonstrates the recoveries from laboratory spiked UHPLC-MS water blanks, where the recoveries for 25 compounds met the EPA method requirements. **Figure 3** presents the %RSD for each of the 25 compounds indicating that the less than 20% RSD requirement was met.

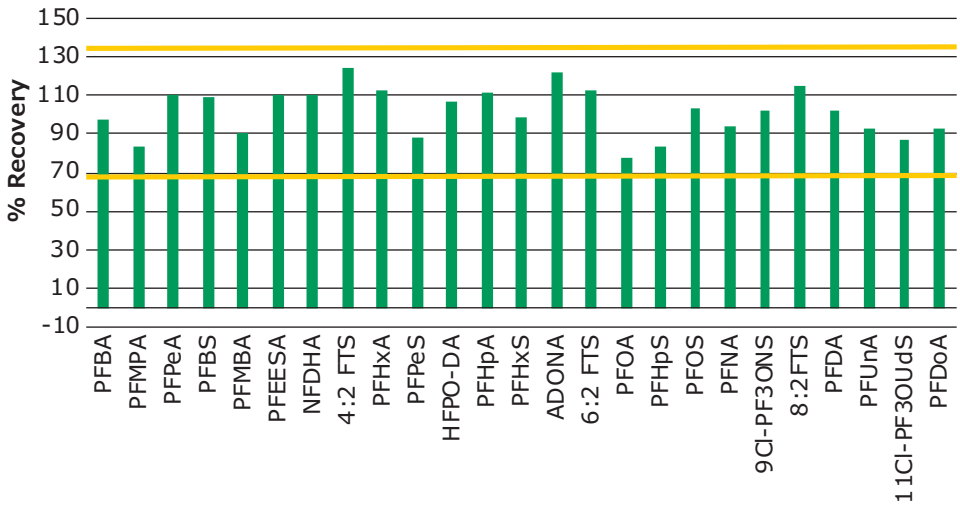

Figure 2. Recoveries of 25 analytes spiked into UHPLC-MS grade water samples. Most analytes were spiked at 10 ng/L, perfluorosulfonic acids were spiked at 40 ng/L. 3 replicate measurements were performed.

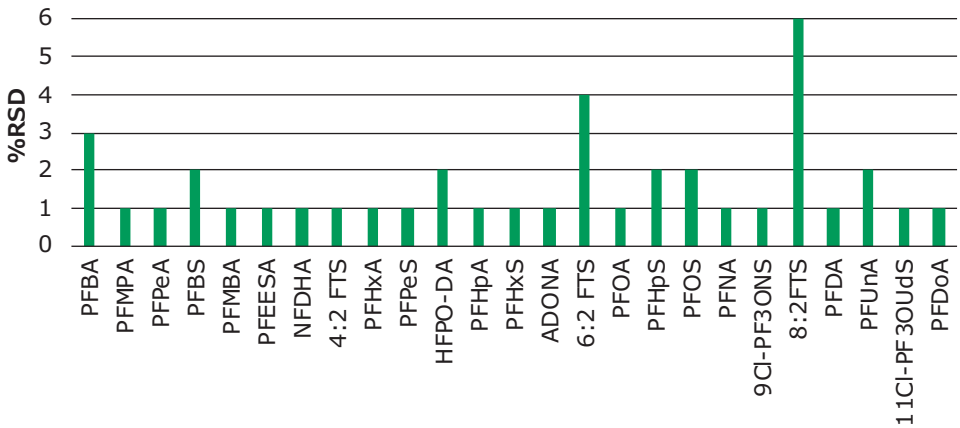

Figure 3. %RSD for recoveries of the 25 analytes spiked into UHPLC-MS water samples. 3 replicate measurements were performed.

A drinking water sample was also analyzed using EPA 533 method. No analytes were detected in the sample above 0.5 ng/L concentrations, and most were below LLOD.

Conclusions

The workflow for EPA method 533 is presented in this article. All 25 compounds were recovered with acceptable accuracy and precision using Supelclean™ ENVI-WAX™ SPE cartridges, Visiprep™ vacuum manifold, Ascentis® Express PFAS columns and UHPLC-MS grade solvents. The background from all consumables and LC system was low and acceptable for detecting low levels of PFAS analytes.

Featured & Related Products

Description	Cat. No
Supelclean™ ENVI-WAX™ SPE 500 mg/6 mL cartridges, pk of 30	**54057-U**
Supelclean™ ENVI-WAX™ SPE 200 mg/6 mL cartridges, pk of 30	**54056-U**
Visiprep™ vacuum manifold	**57030-U**
Stainless steel solvent guides for vacuum manifold, pk of 12	**57027**
Ascentis® Express PFAS HPLC Column, 2.7 µm, 15 cm x 2.1 mm	**53560-U**
Ascentis® Express PFAS Delay Column, 2.7 µm, 5 cm x 3.0 mm	**53572-U**
Water UHPLC suitable for mass spectrometry	**900682**
Methanol UHPLC suitable for mass spectrometry	**900688**
Standards	
Perfluoro-3,6-dioxaheptanoic acid, analytical standard, 100 mg	**94712**
Perfluorobutanoic acid, analytical standard, 25 mg	**68808**
Perfluorodecanoic acid, analytical standard, 25 mg	**43929**
Perfluorododecanoic acid, analytical standard, 50 mg	**92291**
1,1,2,2-Tetrafluoro-2-(1,1,2,2,2-pentafluoroethoxy) ethanesulfonic acid, analytical standard, 100 mg	**93896**
Perfluoroheptanoic acid, analytical standard, 25 mg	**43996**
Perfluorohexanoic acid, analytical standard, 25 mg	**43809**
Heptadecafluorooctanesulfonic acid potassium salt, analytical standard, 100 mg	**89374**
Heptadecafluorooctanesulfonic acid, 100 µg/mL in methanol, analytical standard, 1 mL	**33607**
Perfluorooctanoic acid, analytical standard, 100 mg	**33824**
Pentadecafluorooctanoic acid, 100 µg/mL in methanol, analytical standard, 1 mL	**33603**
Perfluoropentanoic acid, analytical standard, 25 mg	**68542**
Perfluoroundecanoic acid, analytical standard, 50 mg	**80444**
Perfluorotetradecanoic acid, analytical standard, 50 mg	**80312**

Find more information on PFAS testing at **SigmaAldrich.com/PFAS**

ENVIRONMENTAL

Fast and Efficient Separation of 18 PAHs [EPA 610 and EPA 8310 + 2 compounds] using an Ascentis® Express PAH HPLC Column

Petra Lewits, Global Product Manager for HPLC Columns, Analytix@milliporesigma.com

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are toxic compounds commonly found in the environment because of incomplete combustion of fuels, such as coal, tar, and crude oil, among others. These compounds' carcinogenic, mutagenic, and teratogenic nature makes them compounds of concern to environmental organizations around the world. Consequently, there are many regulatory methods in place for PAH testing in environmental samples like air, soil, water, as well as food samples to protect human health.

PAH analysis in water at trace levels demands a highly sensitive method. In this application, we demonstrate an analysis for the separation of 16 + 2 standard PAH compounds (**Table 2**) mentioned in EPA method 610 and 8310 on an Ascentis® Express PAH column.

The Ascentis® Express PAH is a non-endcapped, trifunctional C18 phase with a proprietary manufacturing process, designed on superficially porous particle (SPP) technology, to provide a fast and efficient separation of PAH compounds. The analysis was completed with a resolution value of at least 1.5 in under 5 minutes for EPA method 610. The column gave better detection sensitivity with fluorescence detection in comparison to UV and a fully porous particle (FPP) sub-2 µm column.

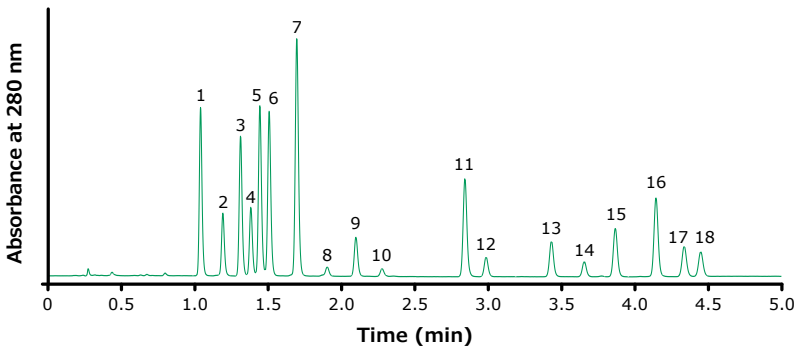

Figure 1. HPLC Separation of 18 PAHs with UV detection (peak ID see **Table 2**).

Experimental

Separation of 18 PAHs: EPA 610 with UV detection

Here a separation under 5 min for the 16 PAH in EPA 610 plus 1-methylnapthalene and 2-methylnaphthalene is shown using a 5 cm x 4.6 mm column (**Table 1**) is shown (**Figure 1**).

Table 1. Chromatographic conditions for determination of 18 PAHs by HPLC-UV

Column:	Ascentis® Express 90 Å PAH, 2.7 µm, 5 cm x 4.6 mm (**53539-U**)
Mobile phase:	[A] Water; [B] acetonitrile
Gradient:	**Time** / **%B**: 0.0 / 50; 4.0 / 100; 6.0 / 100; 6.1 / 50
Flow rate:	1.8 mL/min
Pressure:	256 bar
Column temp:	30 °C
Detector:	UV @ 280 nm; Data Rate: 100 Hz; Response Time: 0.025 s; Flow Cell: 1 µL
Injection:	2 µL
Diluent:	Methanol

Table 2. Peak identification for the 18 PAH compounds measured

Peak Number	Compound
1	Naphthalene
2	Acenaphthylene
3	1-Methylnaphthalene
4	2-Methylnaphthalene
5	Acenaphthene
6	Fluorene
7	Phenanthrene
8	Anthracene
9	Fluoranthene
10	Pyrene
11	Benzo[a]anthracene
12	Chrysene
13	Benzo[b]fluoranthene
14	Benzo[k]fluoranthene
15	Benzo[a]pyrene
16	Dibenzo[a,h]anthracene
17	Benzo[g,h,i]perylene
18	Indeno[1,2,3-c,d]pyrene

Separation of 18 PAHs: UV and Fluorescence Detection (FLD)

For this comparison of an FLD to a UV method, the chromatographic conditions were the same as for the above shown UV detection (**Table 1**), except for the changed injection volume of 0.3 µL. The chosen FLD settings were Ex: 260/ Em: 350/440/500.

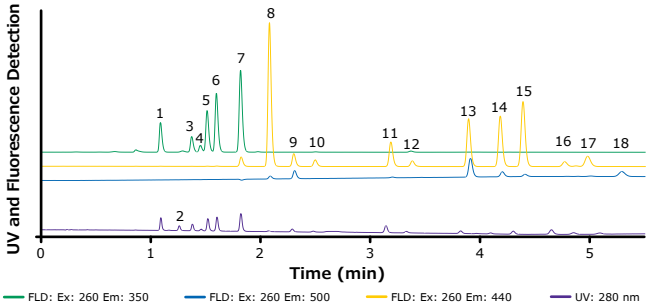

Figure 2. Separation of 18 PAHs with fluorescence and UV detection (peak ID see **Table 2**).

FPP vs. SPP: Comparison for PAH Analysis using EPA 8310 + 2 compounds

Comparison of the superficially porous particle (SPP) Ascentis® Express PAH column to a fully porous particle column (FFP) in the market, both 5 cm x 4.6 mm I.D. under the conditions outlined in **Table 3** is shown in **Figure 3**.

Table 3. Chromatographic conditions for SPP vs. FFP column comparison

Column:	Ascentis® Express 90 Å PAH, 2.7 µm, 5 cm x 4.6 mm I.D. (**53539-U**)
Competitor column:	FPP 95 Å PAH, 1.8 µm, 5 cm x 4.6 mm
Mobile phase:	[A] Water; [B] acetonitrile
Gradient:	Time / %B: 0.0 / 50; 4.0 / 100; 5.0 / 100; 5.1 / 50
Flow Rate:	1.8 mL/min
Pressure Drop:	Ascentis® Express PAH: 256 bar
	Competitor Column FPP: 344 bar
Column temp.:	30 °C
Detector:	UV @ 280 nm; Data Rate: 100 Hz; Response Time: 0.025 S; Flow Cell: 1 µL
Injection:	2 µL
Diluent:	Methanol

The Ascentis® Express PAH outperforms a fully porous particle (FPP) sub-2 µm column for a fast, 5 min separation of EPA method 8310 + 2 compounds demonstrating improved speed and resolution at lower backpressure (**Figure 3**).

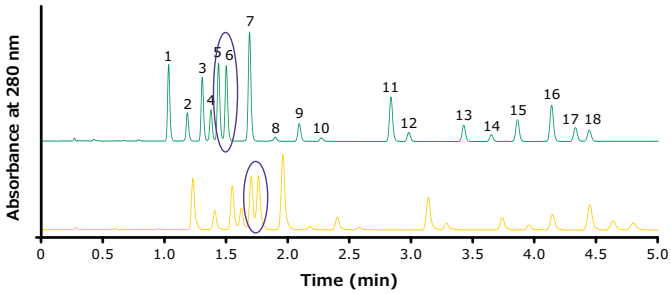

Figure 3. Comparison of Ascentis® Express PAH (green) and fully porous particle (FPP) sub-2 µm column (yellow) for a fast, 5 min separation of method EPA 8310 + 2 PAHs (Peak IDs see **Table 2**).

Highly Efficient Separation of 18 PAHs on 3 mm I.D. Column

A column with 3 mm I.D. was used for the separation shown in **Figure 4** with the conditions described in **Table 4**. This column dimension provided more LC-MS suitability and higher sensitivity of the method.

Table 4. Chromatographic conditions for separation of 18 PAHs on a 10 cm x 3 mm I.D. column

Column:	Ascentis® Express 90 Å PAH, 2.7 µm, 10 cm x 3 mm (**53535-U**)
Mobile phase:	[A] Water; [B] acetonitrile
Gradient:	Time / %B: 0.0 / 50; 8.0 / 100; 10.0 / 100; 10.1 / 50
Flow rate:	0.77 mL/min
Pressure drop:	Initial Back Pressure: 263 bar
Column temp.	30 °C
Detector:	UV @ 280 nm; Data Rate: 100 Hz; Response Time: 0.025 s; Flow Cell: 1 µL
Injection:	2 µL
Diluent:	Methanol

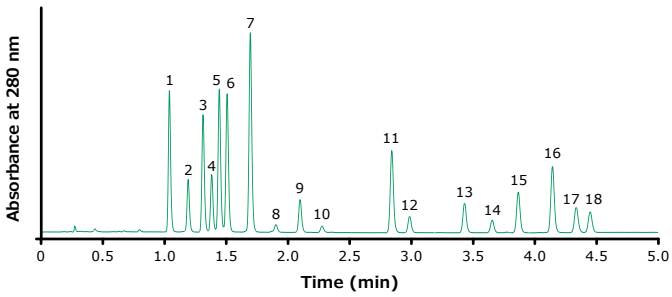

Figure 4. Fast and sensitive separation of 18 PAHs on Ascentis® Express PAH, 2.7 µm, 10 cm x 3.0 mm I.D (peak IDs see **Table 2**).

Conclusion

The Ascentis® Express PAH column delivers a method-specific, robust, and high-efficiency separation of 16 + 2 standard PAH compounds with a resolution value of at least 1.5 in under 5 minutes for EPA 8310. Using a fluorescence detector, the method's sensitivity increases substantially in comparison to UV detection.

The Ascentis® Express PAH outperforms a fully porous particle (FPP) sub-2 µm column for a fast, 5 min separation of method EPA 8310+2 compounds demonstrating improved speed and resolution. A 3 mm I.D. HPLC column enables more sensitive results, suitability for LC-MS use and solvent savings.

Featured Products

Description	Cat. No.
Ascentis® Express PAH, 2.7 µm, 5 cm x 4.6 mm, 90 Å	**53539-U**
Ascentis® Express PAH, 2.7 µm, 10 cm x 3 mm, 90 Å	**53535-U**
Acetonitrile gradient grade for liquid chromatography LiChrosolv® Reag. Ph Eur	**1.00030**
Water for chromatography (LC-MS Grade) LiChrosolv® (or tap fresh water from a suitable Milli-Q® system)	**1.15333**
Reference Materials and Standards	
EPA 610 Polynuclear Aromatic Hydrocarbons Mix, certified reference material, in methanol: methylene chloride (1:1)	**CRM48743**
Naphthalene, certified reference material, TraceCERT®, 100 mg	**91489**
Acenaphthylene, certified reference material, TraceCERT®, 100 mg	**92549**
1-methylnaphthalene, certified reference material, TraceCERT®	**38383**
2-methylnaphthalene, certified reference material, 1000 µg/mL in methanol	**44637-U**
Acenaphthene, certified reference material, TraceCERT®, 100 mg	**05426**
Fluorene, certified reference material, TraceCERT®, 100 mg	**56849**
Phenanthrene, certified reference material, TraceCERT®, 100 mg	**73338**
Anthracene, certified reference material, TraceCERT®, 100 mg	**07671**
Fluoranthene, analytical standard	**45504**
Pyrene, certified reference material, TraceCERT®, 100 mg	**18868**
Benzo[a]anthracene, certified reference material, TraceCERT®, 50 mg	**75451**
Chrysene, certified reference material, TraceCERT®, 50 mg	**94035**
Benzo[b]fluoranthene, certified reference material, TraceCERT®, 50 mg	**30958**
Benzo[k]fluoranthene, certified reference material, TraceCERT®, 10 mg	**03323**
Benzo[a]pyrene, certified reference material, TraceCERT®, 50 mg	**51968**
Dibenzo[a,h]anthracene, certified reference material, TraceCERT®, 10 mg	**91861**
Benzo[g,h,i]perylene, certified reference material, TraceCERT®, 10 mg	**55488**
Indeno[1,2,3-c,d]pyrene, certified reference material, TraceCERT®, 10 mg	**94377**

Read more on the Ascentis® Express and other HPLC columns at **SigmaAldrich.com/HPLC**

ENVIRONMENTAL

Testing Drinking Water - An Overview of National & International Regulations

Gunter Decker, Johanna Tornatzky, Global Product Manager for Point of Use, Analytix@milliporesigma.com

Introduction

Contaminated water is a threat to human health and the environment. As a result, national and international regulatory agencies like the U.S. Environmental Protection Agency (USEPA), World Health Organization (WHO), EU, and other regulators have set official drinking water testing methods and wastewater testing methods. For the analysis of drinking water, these methods must be used to assure a certain quality of the results and to ensure a benchmark of health and safety.

While a quality standard must always be maintained, regulations have increasingly allowed for more method flexibility in recent years. For example, the USEPA criteria for the recognition of slightly modified methods were pioneers in terms of simplifications. This paved the way to use test kits with the same chemistry like in official water testing methods. The revisions of the EU drinking water directives are also pursuing a similar approach. In the latest update of December 2020 [EU Directive 2020/2184], the measurement of uncertainty in conjunction with the limit of detection was defined as the criteria for method applicability. This means that any method can now be used in principle for the analysis, provided it meets the criteria. This also applies to the use of alternative methods like rapid tests.

To provide more convenient, while still reliable methods for users, rapid test methods following recognized standards (ISO, USEPA, and others) were developed. Respective certifications like ISO accordance or equivalency to EPA methods assure users that the ready-to-use test kits deliver comparable results so they can be used to comply with national regulations. These methods offer several additional benefits. One notable advantage is the reduced use of chemicals and sample volumes, resulting in decreased waste generation and enhanced user safety.

What WHO, USEPA and the EU say

WHO Drinking Water Limits and Methods[1]

The WHO declares access to safe drinking water to be a basic human right, being essential to human health and an element of an effective policy for health protection.

There are drinking water guidelines published for the maximum allowable levels of many parameters and the detailed information on individual parameters can be found in the subchapters of the guidelines.

In addition to imposing limits on parameters, the WHO is also clear on its guidelines for drinking water analysis methods. While it is not essential to use standard methods, it is crucial to ensure that the chosen methods are appropriately validated, and their precision and accuracy are determined before making any significant decisions based on the obtained results.

It is therefore necessary to ascertain that a given method has sufficient precision and accuracy, with an auditable quality control and quality assurance procedure to ensure credible results.

USEPA Drinking Water Limits and Methods

The USEPA states on its Groundwater and Drinking Water web page: “The National Primary Drinking Water Regulations (NPDWR) are legally enforceable primary standards and treatment techniques that apply to public water systems. Primary standards and treatment techniques protect public health by limiting the levels of contaminants in drinking water.”[2]

In 1974, the Safe Drinking Water Act (SDWA)[3] was enacted to protect and regulate public water supplies in the US. The SDWA authorized the USEPA to set enforceable standards for contaminants in drinking water in the interest of public health. Most approved analysis methods come from either the USEPA, American Public Health Association (APHA), or the American Society for Testing and Materials (ASTM).

Additionally, there are allowances for modified methods with strict guidelines in terms of how alternatives are implemented and categorized.[4] USEPA-equivalent methods may differ significantly from approved methods but must meet the criteria set out for procedural changes to be considered USEPA-compliant.

We received our first USEPA equivalency certificate in 1999 for a Spectroquant® photometric test kit and since then we continue to work closely with consultants to have more equivalent methods for a range of testing

parameters certified. To date, we provide equivalent methods to those in the Safe Drinking Water Act for the following parameters:

- Ammonium
- Chlorine
- COD
- ortho- and total Phosphate

For wastewater analysis, several equivalent methods to those in the Clean Water Act are available:

- Ammonium
- Chlorine
- Chromium (VI)
- COD
- Cyanide
- Nitrate
- Nitrite
- ortho- and total Phosphate
- Sulfate

For more detailed information, including approvals/equivalency documents, visit **SigmaAldrich.com/usepa**

EU Drinking Water Directive

The EU Drinking Water Directive (2020/2184 of Dec 16th, 2020)[5] established the legal framework to protect human health from the adverse effects of drinking water contamination, providing clear regulations for all member states. Like both WHO guidelines and USEPA regulations, limits on various water parameters were implemented in order to protect human health. In its most recent update in December 2020, an important change was included in terms of analytical quality assurance such that the performance characteristics required of a measurement were clearly defined. Any method that fulfills the requirements for Limit of Quantification (LOQ) and Uncertainty (k=2) of the measurement as a minimum can be used.

Annex III, Part B of the directive has the chemical and indicator parameters for which such performance characteristics are specified, including a table defining the minimum performance characteristic 'uncertainty of measurement'.

A comprehensive list of photometric ready to use test kits that meet these criteria can be found in our catalog that can be downloaded at **SigmaAldrich.com/wfa-catalog**

A general overview about drinking water testing with links to different focus topics can be found on **SigmaAldrich.com/water-testing**

For more information on photometric test kits visit us at **SigmaAldrich.com/photometry**

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FOOD & BEVERAGE

Headspace-SPME as a Versatile Monitoring Method for Early Detection of Insect Infestation in Rice

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Abstract

Isopentenols and polysulfides have been reported as potential early biomarkers for the presence of insects (moths and beetles) in rice. The aim of this study was to develop a headspace solid phase microextraction (HS-SPME-GC-MS) method for high-throughput analysis and detection of early volatile biomarkers (prenol, prenal, isopentenol, hexanal, dimethyl disulfide, dimethyl trisulfide, 2-methylfuran, and 2-pentylfuran) in rice as previously used experimentally as biomarkers.[1] After examination of 4 commercially available SPME coatings, Carboxen®-PDMS fiber coating was found to be most effective in the extraction and desorption of the volatile components compared to the other fibers. We demonstrated that HS-SPME can be used as a fast and versatile insect monitoring method in integrated pest management (IPM) programs.

Introduction

Stored grains can be infested by a variety of pests that can cause grain damage and affect their quality and nutritional standards. Pest infestation in stored rice is responsible for postharvest losses of 9% in developed countries and even larger worldwide. Typical insect pest control methods that implement chemical insecticides have been gradually replaced for modern stored-product integrated pest management (IPM) programs that represent an eco-friendly and environmentally safe approach for pest control.[2] IPM decision-making is based on knowledge of population dynamics and threshold insect density, where appropriate monitoring tools are of great importance.[1,2] A variety of monitoring methods are employed. For instance, pheromone traps are typically used as a monitoring method, in which adult insect are targeted. However, an adult female insect can produce hundreds of eggs before being detected which could delay pest control actions.[1,3] Thus, the use of new monitoring methods for early insect detection would be highly beneficial for fine-tuning and improving IPM programs.

All living organisms present in the environment produce wide range of volatile organic compounds (VOCs) in different stages of their life cycle. Nowadays, VOCs are used as biomarkers, particularly benzoquinones, hydrocarbons, alcohols, furans, and aldehydes are used as insect biomarkers which can be characteristic of a determinate insect species.[1-5] Monitoring methods that allow the detection of specific VOCs resulting from the activity of the larvae in early stages of insect infestations are needed in IPM programs. Thus, detecting the presence of insects at low densities and early stages of development allows to implement corrective actions and avoid total deterioration of stored grains.[1] In this regards, solid phase microextraction (SPME) is a viable alternative as a sample preparation method, as will be shown here. Compared to other preconcentration techniques, SPME is simple, inexpensive, and solvent-free. It is fully automatable, and no thermal desorption unit or modifications to the GC instrument are necessary. Compatible with all GC systems, SPME can be used by practically every laboratory. The objective of this study was to use SPME with GC-MS analysis as a method to detect insect biomarkers (**Figure 1**) as a tool for identification of early insect infestation in stored grains, such a rice.

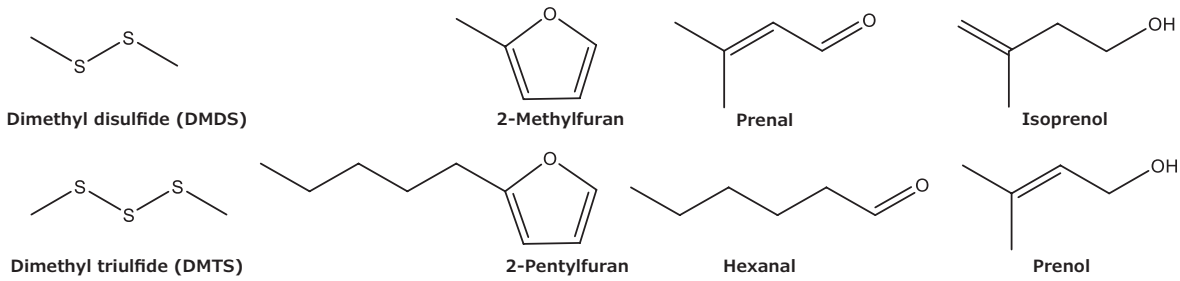

Figure 1. Characteristic VOCs compounds produced by insects at the larvae stage.

Experimental

The HS-SPME method optimization was achieved using spiked rice samples obtained from a local market with undetectable GC-MS level of studied analytes. During method development, fiber selectivity, extraction time (2, 5, 10, 15, 20 min), and temperature (30, 40, 50 and 60 °C) parameters were studied. For this purpose, 1 g of rice was spiked at 10 ng/g with 1 µL of a 10 µg/mL solution of analytes prepared in methanol. The HS-SPME-GC-MS method is summarized in **Table 1** and **2**.

Table 1. HS-SPME Sampling Conditions

Sample/ matrix:	1 g of rice spiked with 1 µL of standard mixture in 10 mL headspace vial
SPME fibers:	PDMS on fused silica core, 100 µm, 23 ga
	DVB-PDMS on nitinol core, 65 µm, 23 ga
	DVB-CAR-PDMS on Stableflex™, 50/30, 23 ga
	CAR-PDMS on nitinol core, 75 µm, 23 ga
Autosampler:	Gerstel MPS II with cooled tray holder
Incubation:	2 minutes at 40 °C
Agitation:	Rotation speed of sample at 250 rpm
Extraction:	Headspace at various extraction temperatures and times with stirring at 250 RPM
Desorption:	3 min at 300 °C for Carbon, 260°C for DVB based and PDMS coating
Fiber post-bake:	2 min at 300 °C for CAR-PDMS Fiber
	2 min at 270 °C for DVB-CAR-PDMS Fiber
	2 min at 250 °C for PDMS and DVB-PDMS Fibers

Table 2. GC-MS Conditions

Column:	SUPELCOWAX® 10, 60 m x 0.25 mm ID; 0.25 µm
Oven:	45 °C (hold 0.5 min) to 110 °C @ 3 °C/ min to 200 °C @ 20 °C/min (hold 0.1 min)
Inlet:	300 °C for CAR-PDMS Fiber
	260 °C for DVB-CAR-PDMS, PDMS and DVB-PDMS Fibers
Carrier gas:	Helium, 1 mL/min constant flow
Detector:	MS Quadrupole, full-scan and SIM Mode (m/z see below), Source at 230 °C, Quad at 150 °C, Electron energy at 70 eV
MSD interface:	250 °C
Injection:	Splitless for 180 s then vent at 20 mL/min
Liner:	0.75 mm ID SPME
MS Conditions SIM Mode	
m/z:	Dimethyl disulfide (DMDS, m/z: 94)
	Dimethyl trisulfide (DMTS, m/z: 126)
	Hexanal (m/z: 56)
	Isoprenol (m/z: 86)
	2-Methylfuran (m/z:82)
	Prenal (m/z: 84)
	Prenol (m/z: 86)
	2-Pentylfuran (m/z: 138)
	Benzene-$^{13}C_6$ (IS, m/z: 84)

Results and Discussion

HS-SPME Method Optimization Procedure

Coating selectivity: Fiber selectivity study was performed using PDMS, DVB/PDMS, CAR/PDMS and DVB/CAR/PDMS SPME fibers to evaluate the performance and effectiveness of each fiber coating chemistry on the headspace extraction of insect volatile biomarkers in a 10 ng/g spiked rice sample. The extraction conditions were as follows: equilibrium time of 2 min, extraction time of 10 min, and temperature of extraction of 40 °C, further sample preparation conditions are mentioned in the experimental section. Chromatographic biomarkers profiles using different SPME coating chemistries are shown in **Figure 2**. It can be observed that CAR-PDMS on nitinol and DVB-CAR-PDMS exhibit better analyte response for the sample tested.

The results of comparing different fibers are shown in **Figure 3**, which depict the average response (area counts) for the different tested fibers. Overall, CAR/PDMS fiber renders a good extraction performance for most of the analytes. Specially for small analytes, where the micropores present in the fiber retain and release these analytes efficiently. However, DVB-CAR-PDMS coating extracts more efficiently prenol in comparison to the rest of the analytes. This is due to the better interaction of this analyte with the DVB layer. Thus, CAR/PDMS on nitinol and DVB-CAR-PDMS on StableFlex (SF) were used for further HS-SPME method optimization.

The parameters for HS-SPME and GC/MS optimized methods are listed in **Table 1** and **2**. Peak identifications were assigned using MS spectral matching against reference spectra in the Wiley and NIST libraries. Additionally, confirmatory identification was done by comparing the MS spectra of the sample with analytical standards.

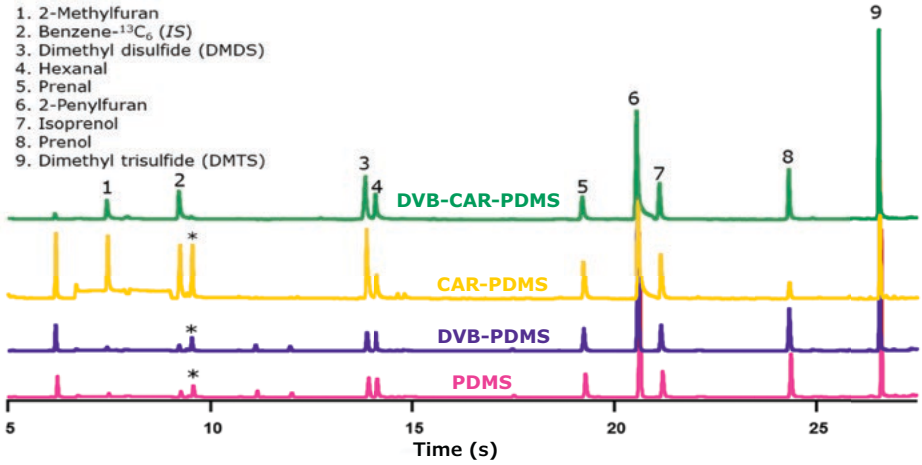

Figure 2. Chromatographic profile for selected biomarkers using four SPME coating chemistries. Sample: 1 g rice spiked at 10 ng/g of selected insect biomarkers. (* Fiber background)

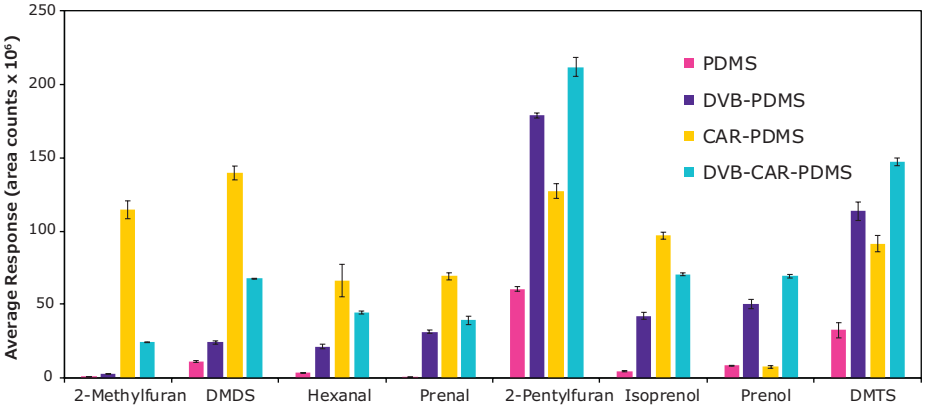

Figure 3. Evaluation of four SPME coating chemistries on the extraction of selected insect biomarkers.

Effect of extraction time:

The influence of the extraction time was investigated in the range from 2 to 20 min. **Figure 4** shows that the extraction efficiency for CAR-PDMS and DVB-CAR-PDMS increased as the extraction time increased up to 10 min, reaching the equilibrium for all the analytes. Thus, an extraction time of 10 min was selected for both coating chemistries.

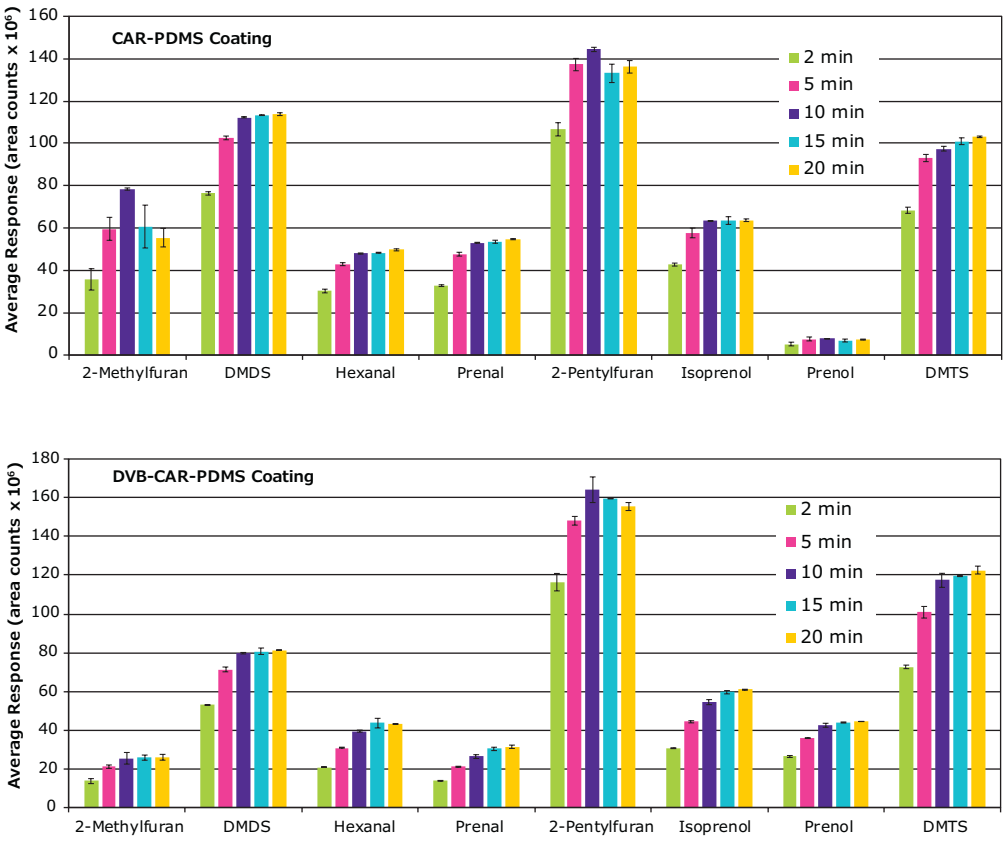

Figure 4. Extraction time for selected insect biomarkers via HS-SPME-GC-MS using CAR-PDMS and CAR-DVB-PDMS SPME fibers. Mean values and standard deviation of analyte peak area (n=3). Sample: 1 g rice spiked at 10 ng/g of selected insect biomarkers.

Effect of extraction temperature:

For the present study, the effect of the extraction temperature was examined in the range between 30 and 60 °C. The results shown in **Figure 5** indicate that there was no significant effect on the analyte response for most of the analytes between 30 and 40 °C for both fiber chemistries. However, the peak area exhibited a slight decrease when the temperature increased up to 60 °C, possibly due to the desorption of the volatile analytes from the coating. Therefore, the extractions were carried out at 30 °C.

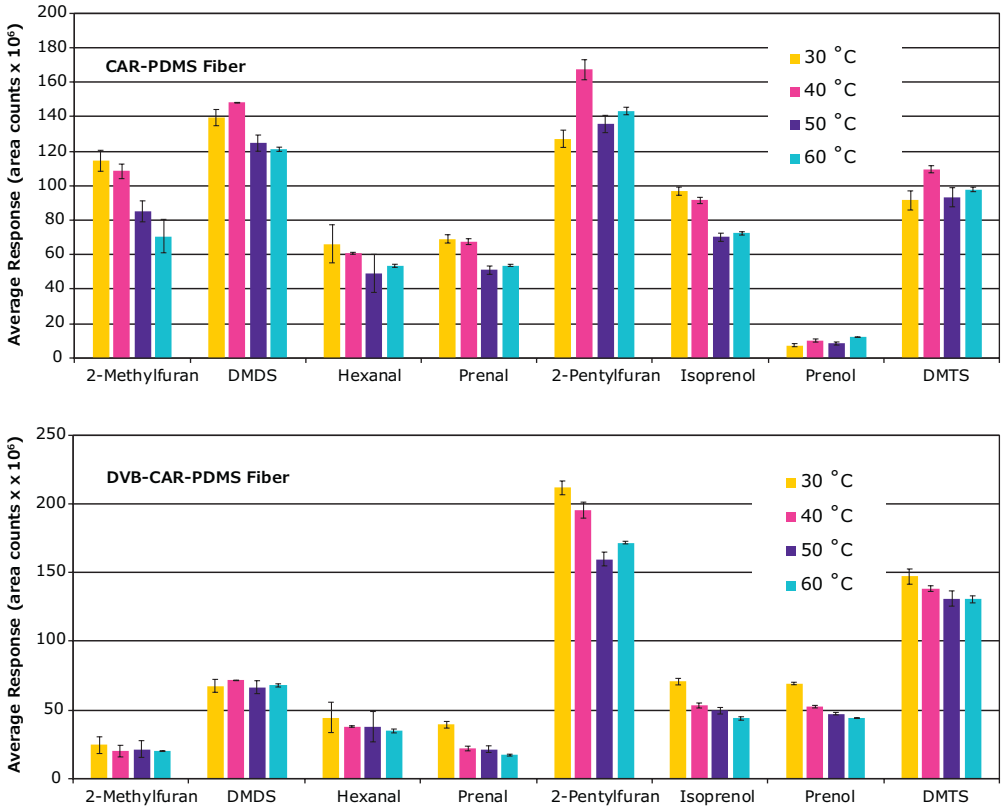

Figure 5. Extraction temperature for selected insect biomarkers via HS-SPME-GC-MS with CAR-PDMS and CAR-DVB-PDMS. Mean values and standard deviation of analyte peak area (n=3). Sample: 1 g rice spiked at 10 ng/g of selected insect biomarkers.

Recovery and Reproducibility

Table 3 depicts linearity, recovery and reproducibility values using CAR-PDMS and DVB-CAR-PDMS fibers. Linearity was obtained through the construction of a multipoint calibration curve, at seven different concentration levels from 2.5 ng/g - 200 ng/g and using benzene-$^{13}C_6$ (10 ng/g) as an internal standard. The calibration curve for each analyte was prepared by adding proper volumes of standard solution and IS into SPME vials containing 1.0 g of rice. Excellent linearity and accuracy for all the analytes were observed for both SPME fibers in the studied calibration range. CAR-PDMS and DVB-CAR-PDMS fibers exhibit accuracy values of 61-103% and 77-90%, respectively. Repeatability ≤ 10% RSD was observed for all the analytes for both fiber chemistries, and this was determined by analyzing 3 replicates of SPME extractions of rice samples spiked at 10 ng/g. As can be observed from **Table 3**, CAR-PDMS on nitinol exhibits higher extraction performance for all the analytes except prenol (recoveries: prenol 61%, other analytes 91-103%), which is likely due to a stronger retention of the analyte in the micropores present in the structure of the fiber. Thus, CAR-PDMS is an excellent fiber choice for detection of early volatile biomarkers in rice. However, DVB-CAR-PDMS can be used as a complementary fiber chemistry for the extraction of prenol.

Table 3. HS-SPME method accuracy and repeatability (n=3) in spiked rice samples using DVB-CAR-PDMS and CAR-PDMS SPME fibers

	DVB-CAR-PDMS on Stableflex™ (50/30 µm)			CAR-PDMS on nitinol core (75 µm)		
Analyte	R^2	% Recovery Spiked Sample*	%RSD*	R^2	% Recovery Spiked Sample*	%RSD*
Dimethyl disulfide	0.9975	90%	2	0.9975	99%	1
Dimethyl trisulfide	0.9960	77%	2	0.9980	95%	3
Hexanal	0.9988	84%	7	0.9984	94%	1
Isoprenol	0.9991	82%	5	0.9994	92%	2
2-Methylfuran	0.9976	77%	3	0.9998	90%	1
Prenal	0.9967	77%	10	0.9986	95%	1
Prenol	0.9962	82%	6	0.9893	61%	9
2-Pentylfuran	0.9992	88%	6	0.9997	103%	1

* 3 spiked samples - spike at 10 ng/g

Conclusions

A HS-SPME-GC-MS method has been developed for high-throughput analysis and detection of early volatile insect biomarkers in rice samples. Carboxen®-PDMS on nitinol fiber core was found to be most effective in the extraction and desorption of 2-methylfuran, DMDS, hexanal, prenal, 2-pentylfuran, isoprenol and dimethyl trisulfide compared to DVB-CAR-PDMS on Stableflex™ fiber core. However, it presented low extraction performance only for prenol. Thus, CAR-PDMS is an excellent fiber choice for detection of early volatiles indicating insect infestation in rice. However, DVB-CAR-PDMS can be used as a complementary fiber chemistry for the detection of prenol.

The HS-SPME-GC-MS method can be used in integrated pest management (IPM) programs as a fast and versatile monitoring approach/tool for identification of early insect infestation in store grains such a rice.

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Divinylbenzene-polydimethylsiloxane (DVB-PDMS), 65 µm, Nitinol, needle size 23 ga, for use with manual holder/autosampler	**57923-U**
Carboxen®-Polydimethylsiloxane (CAR-PDMS), 75 µm, Nitinol, needle size 23 ga, for use with manual holder/autosampler	**57907-U**
Divinylbenzene-Carboxen®-Polydimethylsiloxane (DVB-CAR-PDMS), 50/30 µm, Stableflex, needle size 23 ga, for use with manual holder/autosampler	**57298-U**
SPME fiber holder for Varian autosampler	**57331**
SPME fiber holder for CTC, Combipal, Gerstel MPS2	**57347-U**
SPME fiber holder for manual sampling	**57330-U**
0.75 mm Inlet Liner, Direct (SPME) Type, Straight Design	**2637505**
Molded Thermogreen® LB-2 Septa, with injection hole	**28338-U**
Headspace vial, screw top, 10 mL rounded bottom	**SU860099**
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Dimethyldisulfide, analytical standard	**68986**
Dimethyl trisulfide, analytical standard	**79592**
Hexanal, analytical standard	**18109**
Isoprenol (3-Methyl-3-buten-1-ol), 97%	**129402**
2-Methylfuran, analytical standard	**39587**
2-Pentylfuran, natural (US), ≥97%, FG	**W331715**
Prenal (3-Methylcrotonaldehyde), analytical standard	**08552**
Prenol (3-Methyl-2-buten-1-ol), 99%	**162353**

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Ensuring Safety at Every Bite

New Analytical Standards for Food Contact Materials Testing

Matthias Nold, Product Manager Reference Materials; Analytix@milliporesigma.com

Food contact materials (FCMs) are defined as packaging materials or other articles intended to come into contact with food during production, processing, storage, or consumption. They can be made out of a wide range of materials, including plastic, metal, glass, paper, cardboard, or ceramics.

Food contact materials and food packaging materials in particular play an important role in protecting food from microbial contamination, facilitating storage and prolonging shelf life of food, and thus contributing to reducing food waste. However, chemical components can migrate from FCMs into a food product and potentially cause harm to consumers which is why food contact materials underlie specific regulations in most regions of the world.[1]

For the European Union, plastic materials and articles intended to come into contact with food are regulated by EC regulation 10/2011.[2] It includes a comprehensive list of authorized substances that are allowed to be used in the manufacturing of plastic food contact materials and sets overall migration limits (OMLs) as well as for a considerable number of the listed substances also specific migration limits (SMLs).

As part of our extensive reference materials portfolio, we offer a big range of analytical standards and certified reference materials for regulated FCM substances **SigmaAldrich.com/fcm**. Specifically for compounds with SML listed in EC 10/2011, more than 100 products are available in our catalog, enabling for an efficient and reliable analysis of food contact materials. **Table 1** shows the most recent additions sorted by "FCM number" as listed in the positive list of regulation 10/2011. A comprehensive range of reference materials for EC 10/2011 can be viewed at **SigmaAldrich.com/EC10_2011**

Table 1. Recently launched analytical standards according to the positive list of EC 10/2011

FCM No.	Product Description	CAS	Pack Size	Quality Grade	Cat. No.
185	Ethylene glycol dimethacrylate	97-90-5	1 mL	Analytical Standard	**52465**
207	Bis(2-ethylhexyl) adipate	103-23-1	100 mg	Analytical Standard	**68995**
236	m-Phenylenediamine	108-45-2	100 mg	Analytical Standard	**52519**
261	Diethylenetriamine	111-40-0	1 mL	Analytical Standard	**51851**
292	Triisopropanolamine	122-20-3	100 mg	Analytical Standard	**90164**
293	Triethyl phosphite	122-52-1	1 mL	Analytical Standard	**50982**
305	Hexamethylenediamine	124-09-4	100 mg	Analytical Standard	**52003**
341	Bicyclo[2.2.1]hept-2-ene	498-66-8	100 mg	Analytical Standard	**51694**
377	(3-Aminopropyl)triethoxysilane	919-30-2	1 mL	Analytical Standard	**49863**
453	Vinyltrimethoxysilane	2768-02-7	1 mL	Analytical Standard	**52009**
463	Trimethylolpropane trimethacrylate	3290-92-4	1 mL	Analytical Standard	**52052**
471	Dimethyl 5-sulfoisophthalate sodium salt	3965-55-7	100 mg	Analytical Standard	**91338**
617	2-Acrylamido-2-methyl-1-propanesulfonic acid	15214-89-8	100 mg	Analytical Standard	**52473**
788	3-(Trimethoxysilyl)propyl methacrylate	2530-85-0	1 mL	Analytical Standard	**51822**

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HPLC Tips & Tricks: Getting Greener in HPLC

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Liquid chromatography is a widely used analytical technique in various fields such as pharmaceuticals, biotechnology, food and beverage, environmental monitoring, and more. The most popular type of liquid chromatography is reversed phase (~>75%). Up until now, the technique often employs acetonitrile and relatively large columns (the most used column dimension still is 250 x 4.6 mm). However, there are a few options for adjusting the mobile phase to improve the sustainability of chromatography without compromising its performance.

Use eco-friendly solvents: One of the biggest environmental impacts of liquid chromatography is the use of solvents. Replacing hazardous solvents with more environmentally friendly options such as water, ethanol, or other organic solvents that are bio-renewable, safe, non-toxic, and biodegradable can significantly reduce the environmental impact. Green solvents are an important component in making liquid chromatography more sustainable in general. However, higher viscosity/backpressure, UV cut-off, and temperature limits of the used solvent system might need to be considered. Here are some examples of green solvents potentially to be used in liquid chromatography:

Water: Water is the most commonly used solvent in liquid chromatography, especially in reversed-phase chromatography. This solvent can be considered as one of the greenest solvents. Hot water (superheated water from 75 to 180 °C) has been already proven to have the potential to reduce organic solvent percentage in the mobile phase.[1]

Ethanol: Ethanol is typically a bio-based solvent that can be produced from renewable sources such as fermentation of bio-waste. This solvent is non-toxic, biodegradable, and has a low environmental impact.

CO_2: Supercritical CO_2 is a green solvent that is used in supercritical fluid chromatography (SFC). This solvent is non-toxic, non-flammable, and can be easily recycled.

Other bio-based solvents: Bio-based solvents such as terpenes or lactic acid for sample extraction, and glycerol[2] or dimethyl carbonate[3] are gaining attention in liquid chromatography workflows. These solvents are derived from renewable sources and have low toxicity and a low environmental impact.

However, it is important to note that not all green solvents are suitable for every chromatographic application, so users should carefully consider the specific properties and requirements of their method before selecting an alternative green solvent. Also, for validated methods, it is not allowed to make any changes in mobile phase composition according to Pharmacopoeias without full re-validation.

Another set of improvements is related to the method setup and different instrumental solutions:

Optimized methods: HPLC method optimization can significantly reduce the consumption of solvents and the generation of waste. The environmental impact of an HPLC method can be reduced by reducing the column dimensions, in particular the column inner diameter, reducing the injection volume, using different gradient conditions, and/or reducing the run time. The most significant reduction in solvent consumption can be achieved by using shorter columns with smaller inner diameters. The loss in separation efficiency of a shorter column can be compensated by more efficient smaller particles or superficially porous particles to still obtain accurate and reliable results.

Use "greener" equipment: Modern liquid chromatography equipment is designed to be more energy-efficient. Using systems that recycle solvents and/or using a lower flow split ratio can help to reduce solvent consumption and waste generation.

Recycle waste: Instead of discarding the waste generated during the chromatographic process, it could be (partly) recycled or reused for other applications, thus reducing the overall environmental impact. This approach so far is only applicable for isocratic runs.

Choose sustainable suppliers: It is important to select suppliers who prioritize sustainability and offer environmentally friendly products. This fact includes suppliers who use recycled materials, source raw materials sustainably, and prioritize energy- and raw material-efficient production methods. Look out for e.g. high EcoVadis rating or Environmental, Social, Governance (ESG) rating from MSCI.

Consider alternative methods: In some cases, alternative analytical techniques such as capillary electrophoresis, supercritical fluid chromatography or sensorics-based methods may be more sustainable and have a lower environmental impact than liquid chromatography while still providing the needed analytical answer.

In conclusion, by considering and adopting the above mentioned strategies, in particular, the reduction of column dimensions, liquid chromatography can be made more sustainable, reducing its environmental impact and contributing to a more sustainable future.

To read more about our sustainability efforts, visit us at **SigmaAldrich.com/sustainable-chemistry**

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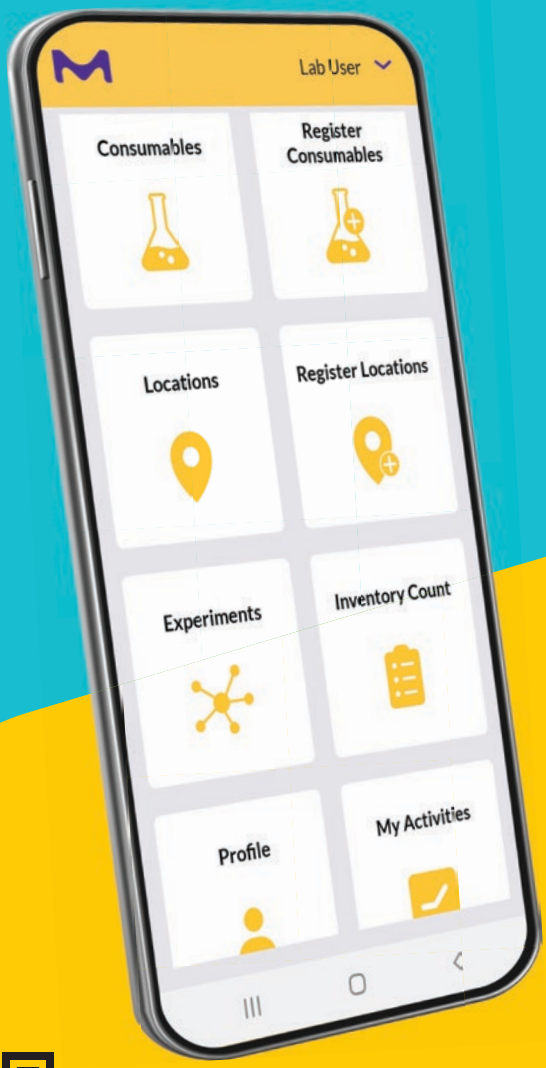

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Lit. No. MS_NL12294EN Ver. 1.0
48478
08/2023