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# Analytix Reporter

## Drugs of Abuse Analysis in Urine with New HLB Solid Phase Extraction 96-well Plates for Clean-up





Supelco®
Analytical Products

ISSUE 13 | 2022

# Analytix Reporter




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Dear Reader,

Continuing advancements in the field of bioanalytical testing are markedly important in the areas of pharmaceutical drug discovery and development, forensics and toxicology, as well as clinical testing. The technologies we have today enable us to easily accomplish feats such as identifying a specific drug or toxin present in a forensic sample, selecting suitable pharmaceutical drug candidates along the drug discovery and development process, and responding rapidly with analytical solutions to new designer drugs of abuse as they enter the market.

The increasing sensitivity of MS detection and enhanced speed and separation efficiencies of LC columns and instrumentation allows us to analyze drugs, contaminants, and toxins at unprecedentedly low levels and rapid speeds. Technological advances continue to open doors to new ways of solving perpetual problems, meanwhile, the advancements in automation enable increased efficiencies as laboratories can realize high throughput analysis of thousands of samples per day.

Sample preparation often plays a pivotal role in allowing the achievement of fast and reliable results. Supelco® Analytical products offer comprehensive, reliable sample preparation and workflow solutions for the most pressing issues faced by bioanalytical laboratories. For example, the presence of phospholipids in biological fluids is one of the major causes of ion suppression in LC-MS workflows. To tackle this problem, we developed the HybridSPE® technology, a patented innovation using zirconia-coated silica to remove phospholipids rapidly and effectively. More recently, we introduced the Supel™ Swift HLB phase chemistry, a patent pending copolymer with dual polarity, enabling an efficient extraction of a broad range of compounds from various matrices, including urine, serum, and plasma. We offer these innovative phase chemistries, not only in a classical cartridge format, but also to aid automation in 96-well plates and dispersive extraction pipette tips (DPX) to accommodate a breadth of equipments and capabilities. This new DPX tip format offers a rapid, automated, dispersive solid phase extraction technique in both HybridSPE® and Supel™ Swift HLB chemistries; a perfect fit for bioanalytical applications using liquid handling robotics. Another installment of advancing automated sample preparation is our BioSPME technology, allowing a more efficient analyte extraction for protein binding studies and free analyte analyses.

While we continue to advance our phase chemistries and sample preparation configurations, our primary goal remains to enable our customers to efficiently achieve reliable and accurate results. That is why we are continuously working on a comprehensive and innovative analytical product portfolio to cater to your challenging workflow demands.

Sincerely yours,

**Candace Price**
Global Product Manager,
Sample Preparation

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CLINICAL & FORENSIC

# Analysis of Drugs of Abuse in Urine After Cleanup with New Supel™ Swift HLB Solid Phase Extraction 96-well Plates

M. James Ross, Olga Shimelis, Hugh Cramer, Teresa Marsala; Analytix@milliporesigma.com

Supel™ Swift HLB SPE is a new, proprietary, and patent pending copolymer having both hydrophilic and lipophilic functional groups. It is intended for use as a sorbent material in solid phase extractions (SPE) prior to instrumental analysis, such as LC-MS/MS. The dual polarity of Supel™ Swift HLB makes it ideal for extracting a broad range of compounds from aqueous matrices and is appropriate for samples in food & environmental applications as well as biological samples such as urine, serum, and plasma. The hydrophilic and lipophilic balanced (HLB) property of the polymer material enables the retention of a broad spectrum of compounds having a wide range of polarities and log P values (-0.9 - to 4.7).


**Figure 1.** The Supel™ Swift HLB 96-well plate, 30 mg of HLB sorbent/well

In this study, we demonstrate the cleanup of urine samples by HLB solid phase extraction for the analysis of opioids via LC-MS/MS. The 96-well SPE format **(Figure 1)** utilized is optimal for clinical and other laboratories working in a high-throughput environment.

During the analysis of drugs of abuse in urine, the drug and their metabolites (e.g. morphine) can be present glucuronide form **(Figure 2)**. In these cases, hydrolysis using a β-glucuronidase enzyme is performed prior to LC-MS analysis of the samples to ensure that the free form of the drug can be analyzed in the samples under investigation. Subsequently, the samples require a cleanup prior to injection into the LC-MS instrument. Solid phase extraction remains the most convenient method for use in such sample cleanups.

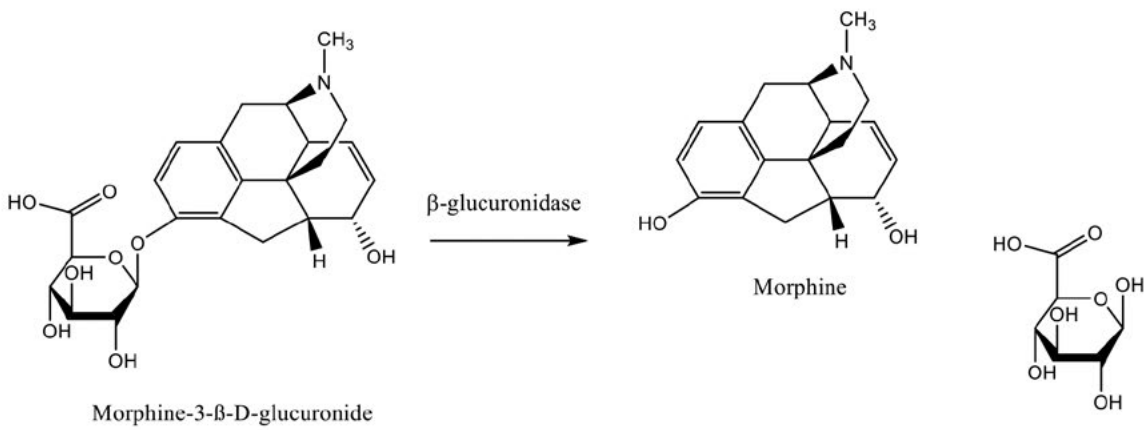


**Figure 2.** β-Glucuronidase hydrolysis of morphine-3-β-D-glucuronide to the free analyte, morphine.

# Methods

## Recovery of Analytes

Synthetic urine, SigMatrix Urine Diluent, was spiked with "Pain Management Multi-Component Opiate Mixture-13 solution" diluted to 100 ng/mL for 12 of the 13 compounds and at 10 ng/mL for fentanyl. A list of the components and the transitions monitored is available in **Table 1.** The following internal standards were added at 10 ng/mL: oxycodone-$D_3$, (±)-methadone-$D_9$, oxymorphone-$D_3$, hydrocodone-$D_3$, cis-tramadol-$^{13}C$, $D_3$, meperidine-$D_4$. The MS transitions monitored with these internal standards are shown in **Table 2.**

A β-glucuronidase solution at a concentration of 10 kU/g was prepared in 0.1 M phosphate buffer (pH 6). The bulk sample solution comprised of 3:1:1 SigMatrix urine diluent:β-glucuronidase (10 kU, pH 6.0):phosphate buffer (pH 6.0). The samples underwent digestion for 2 hours at 60 °C with mixing at 200 rpm. The hydrolysis conditions used were previously found to be optimum for using β-glucuronidase enzyme from limpets. The samples were cooled, and the sample solutions adjusted to pH 9 with an ammonium hydroxide solution. The samples were then processed on a Supel™ Swift HLB 96-well plate containing 30 mg/well of HLB sorbent as outlined in **Figure 3.** After sample processing, 75 µL of cleaned sample was diluted with 175 µL of LC/MS grade water to decrease the final organic component to 30%. Samples were analyzed on a Sciex 3200 QTrap MS instrument with an Agilent 1290 LC (separation parameters are shown in **Table 3**). Analytes were quantified by a 5-point external calibration curve using standards prepared daily from methanol stock solutions stored in glass vials. Injected calibrator solutions contained 10 ng/mL of the previously outlined internal standards in 70:30 methanol:water.

**Table 1.** Analytes in the "Pain Management Multi-Component Opiate Mixture-13 solution" with their MS-MS detection parameters

| Compound | log P | $pK_a$ | Retention Time (min) | Q1 | Q3 | DP (V) | CE (V) | EP (V) | CXP (V) | Internal Standard |
|---|---|---|---|---|---|---|---|---|---|---|
| Morphine | 0.9 | 8.2 | 1.59 | 286.1 | 128.1 | 63 | 71 | 8 | 4 | Oxymorphone-$D_3$ |
| Oxymorphone | 0.8 | 8.2 | 1.73 | 302.1 | 284.2 | 46 | 23 | 5.5 | 4 | Oxymorphone-$D_3$ |
| Hydromorphone | 1.1 | 8.2 | 1.89 | 286.1 | 185.3 | 61 | 37 | 5.5 | 6 | Oxymorphone-$D_3$ |
| Naloxone | 1.9 | 7.9 | 2.38 | 328.2 | 310.2 | 41 | 23 | 9 | 6 | Oxycodone-$D_3$ |
| Codeine | 1.4 | 8.2 | 2.70 | 300.1 | 114.9 | 61 | 61 | 8 | 8 | Oxycodone-$D_3$ |
| Naltrexone | 1.9 | 8.4, 9.9 | 2.75 | 328.2 | 310.2 | 41 | 23 | 9 | 6 | Oxycodone-$D_3$ |
| Oxycodone | 0.7 | 8.5 | 2.83 | 316.3 | 241.1 | 61 | 38 | 8 | 3 | Oxycodone-$D_3$ |
| Hydrocodone | 1.2 | 8.2 | 2.84 | 300.2 | 199.2 | 56 | 35 | 6.5 | 6 | Hydrocodone-$D_3$ |
| Tramadol | 1.3 | 9.4 | 3.66 | 264.2 | 57.9 | 31 | 33 | 6.5 | 6 | cis-Tramadol-$^{13}C$, $D_3$ |
| Meperidine | 2.7 | 8.6 | 3.98 | 248.2 | 220.3 | 51 | 29 | 9 | 4 | Meperidine-$D_4$ |
| Fentanyl | 4.1 | 9.0 | 4.60 | 337.2 | 188.3 | 46 | 29 | 9 | 4 | Meperidine-$D_4$ |
| Buprenorphine | 5.0 | 8.3 | 4.67 | 468.3 | 55.1 | 86 | 85 | 8 | 4 | Meperidine-$D_4$ |
| Methadone | 3.9 | 9.2 | 4.95 | 310.2 | 265.2 | 31 | 19 | 4 | 4 | Methadone-$D_9$ |

**Table 2.** Internal standards used with the 13 pain management compounds and their MS-MS detection parameters

| Internal Standard | Retention Time (min) | Q1 | Q3 | DP (V) | CE (V) | EP (V) | CXP (V) |
|---|---|---|---|---|---|---|---|
| Hydrocodone-$D_3$ | 2.84 | 303.2 | 199.2 | 56 | 35 | 6.5 | 6 |
| Meperidine-$D_4$ | 3.98 | 525.2 | 224.3 | 51 | 29 | 9 | 4 |
| (±)-Methadone-$D_9$ | 4.95 | 319.2 | 268.2 | 31 | 19 | 4 | 4 |
| Oxycodone-$D_3$ | 2.83 | 319.3 | 244.1 | 61 | 38 | 8 | 3 |
| Oxymorphone-$D_3$ | 1.73 | 305.1 | 287.2 | 46 | 23 | 5.5 | 4 |
| cis-Tramadol-$^{13}C$, $D_3$ | 3.66 | 268.2 | 57.9 | 31 | 33 | 6.5 | 6 |

| | |
|---|---|
| **Pre-extraction** | 5 kU of β-glucuronidase & mixture of analytes incubated for 2 hours at 60 °C, cooled and adjusted to pH 9 |
| **Conditioning** | 1000 µL 5% methanol in water |
| **Loading** | 500 µL of the hydrolyzed urine sample, pH 9 |
| **Wash** | 1000 µL of 5% methanol in water |
| **Elution** | 500 µL of 100% methanol with 5% formic acid |
| **Post-extraction** | Dilute 75 µL of the eluate with 175 µL of water |

**Figure 3.** Sample Preparation and SPE Method

**Table 3.** Analytical conditions for Sciex 3200 QTrap and Agilent 1290 LC instruments

| | |
|---|---|
| **Column:** | Ascentis® Express Phenyl-Hexyl 10 cm x 2.1 mm I.D., 2.7 µm (**53336-U**) |
| **Mobile Phase:** | [A] Water with 0.1% formic acid<br>[B] Acetonitrile with 0.1% formic acid |
| **Gradient:** | 10% to 45% [B] in 3 min, 45% to 100% [B] in 2 min and hold 2.4 min |
| **Flow Rate:** | 0.300 mL/min |
| **Detector:** | MS, ESI(+), Scheduled MRM |

## Matrix Effects on Ionization

Samples were prepared and processed as described earlier except for the spiking of analytes and internal standards. The cleaned matrix was spiked after processing with both analytes and internal standards. These samples were quantified by a 5-point external calibration curve as described earlier.

# Results and Discussion

## Percent Recovery

A representative chromatogram of an SPE cleaned-up sample spiked at 100 ng/mL (except for fentanyl at 10 ng/mL) is shown in **Figure 4**. Overall, 12 of the 13 analytes showed relative recoveries of 73 to

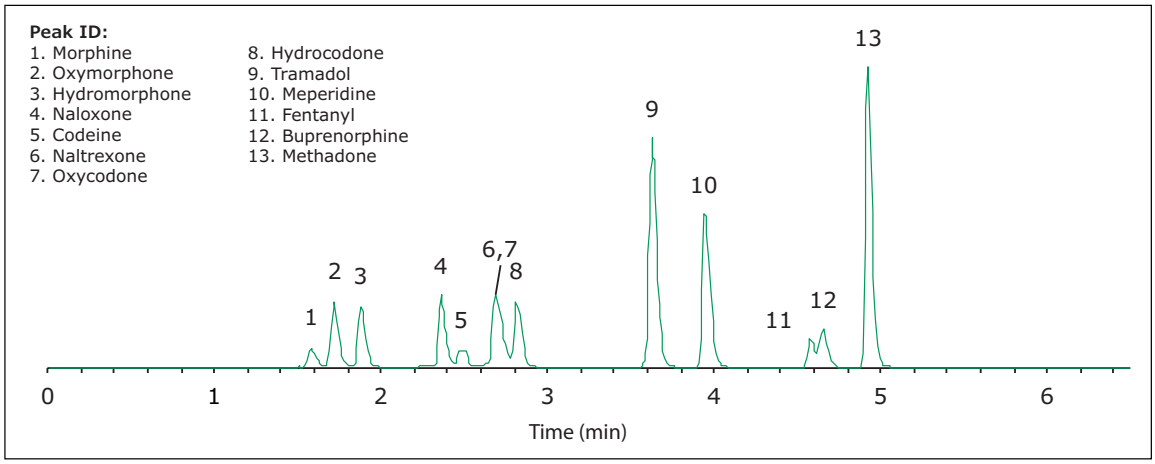


**Figure 4.** Representative chromatogram of the spiked urine-mimic samples after cleanup with SPE.

105% (n=96) with an overall recovery of 88% as shown in **Table 4** and **Figure 5**. The lower recovery for buprenorphine is attributed to a log P ~5, which would have led to non-specific binding.

For the thirteen analytes, the RSDs associated with the recoveries were <7.2% (n=96) showing consistency across the plate. Absolute recoveries are shown in **Figure 6**.

Without using the assigned internal standard, the absolute recovery across the plate for 12 of the 13 analytes was 70.5% (omitting buprenorphine). Nine of the 13 analytes show recovery at ≥70% as shown in **Figure 6** across the 96 wells.

**Table 4.** Percent recovery across the Supel™ Swift HLB 96 well plate. Analytes were spiked at 100 ng/mL (except for fentanyl 10 ng/mL)

| Compound | 1. Morphine | 2. Oxymorphone | 3. Hydromorphone | 4. Naloxone | 5. Codeine | 6. Naltrexone | 7. Oxycodone |
|---|---|---|---|---|---|---|---|
| Recovery (%) | 88% | 94% | 94% | 74% | 105% | 75% | 92% |
| RSD (%) | 5.4% | 4.1% | 5.5% | 6.9% | 7.2% | 6.2% | 6.7% |
| **Compound** | **8. Hydrocodone** | **9. Tramadol** | **10. Meperidine** | **11. Fentanyl** | **12. Buprenorphine** | **13. Methadone** | **Overall*** |
| Recovery (%) | 90% | 89% | 93% | 73% | 44% | 90% | 88% |
| RSD (%) | 4.6% | 2.0% | 3.3% | 6.1% | 5.8% | 2.7% | 10.8 |

*Omits buprenorphine

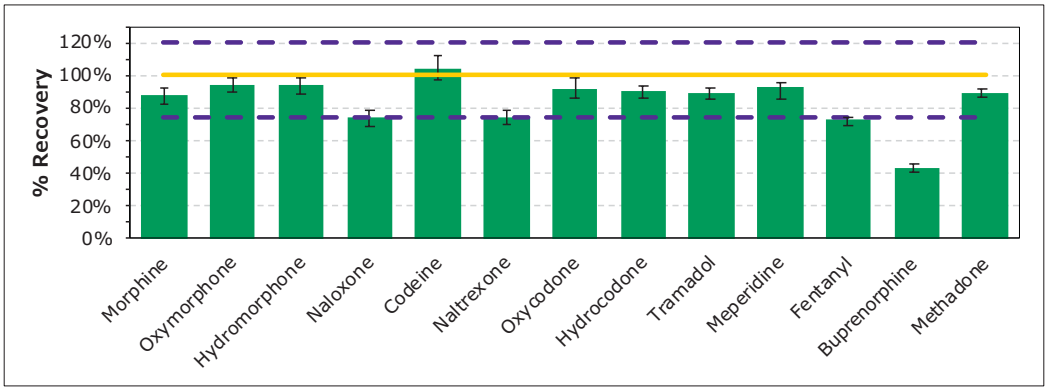


**Figure 5.** Relative percent recovery. Analytes were spiked at 100 ng/mL with exception of fentanyl at 10 ng/mL. Purple dash lines represent 75 and 120% recovery, with the gold solid line representing 100% recovery. Analytes are listed in elution order.

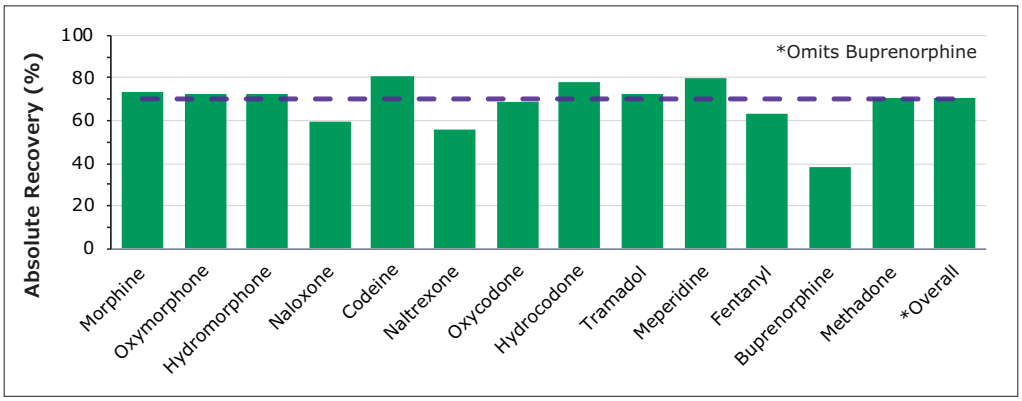


**Figure 6.** Absolute percent recovery Analytes were spiked at 100 ng/mL with exception of fentanyl at 10 ng/mL. Purple dash lines represent 70% absolute recovery. Analytes are listed in elution order.

## Matrix Effects

The impact of matrix components was calculated by comparing the signal response of the analyte in 70:30 methanol:water (representing 100%) to a sample that was cleaned using the SPE procedure outlined and was post-spiked (final extracted samples had 30% methanol present). Across the 13 analytes, minimal to no matrix effects (suppression or enhancement) ±10% was observed for most of the analytes as shown in **Figure 7**. Two analytes that were suppressed the most were naloxone (-30%) and naltrexone (-20%). These suppression values would have lead to the lower absolute recovery reported in **Figure 6** but were corrected for in relative recovery by use of an internal standard **(Figure 5)**.

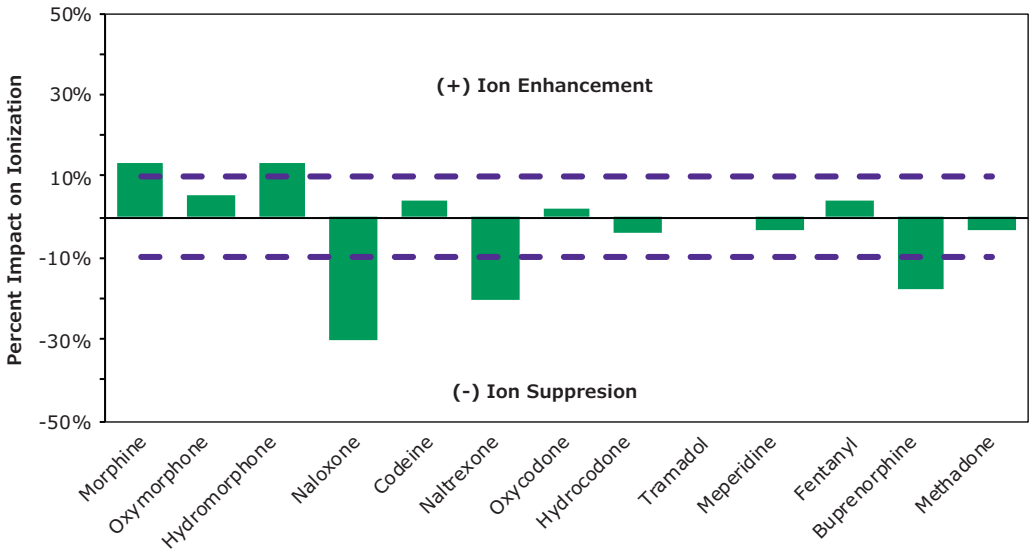


**Figure 7.** Matrix effects (ion suppression and ion enhancement) across the 13 analytes. Purple dash lines represent ±10% impact on ionization.

## Summary

Supel™ Swift HLB SPE is a hydrophilic and lipophilic polymer SPE phase designed for the extraction of a broad range of compounds from complex aqueous sample matrices. In this study, we demonstrated the utility of this SPE phase to prepare urine samples for the analysis of a series of pain management drugs readily available as a premade mixture. No post-extraction concentration or dry down of samples was required. The relative recoveries of the analytes were in the range of 73-105% with one exception of buprenorphine. The reproducibility across the entire plate was excellent with ≤7.2% RSD. Minimum matrix effects (±10%) were observed after Supel™ Swift HLB SPE cleanup. The developed SPE method can be applied to analyze a wider range of analytes in urine.

## Materials

| Description | Cat. No. |
|---|---|
| **SPE & HPLC** | |
| Supel™ Swift HLB 96-well plate, 30 mg/well | **57494-U** |
| Ascentis® Express Phenyl-Hexyl, 100 × 2.1 mm ID, 2.7 µm | **53336-U** |
| **Solvents & Reagents** | |
| Acetonitrile, hypergrade for LC-MS LiChrosolv® | **1.00029** |
| Methanol, hypergrade for LC-MS LiChrosolv® | **1.06035** |
| Water for chromatography (LC-MS Grade) LiChrosolv® | **1.15333** |
| Ammonium hydroxide solution puriss. p.a., reag.ISO, reag. Ph. Eur., ~25% $NH_3$ basis | **30501-M** |
| Formic acid for LC-MS LiChropur™, 97.5-98.5% (T) | **00940** |
| Sodium dihydrogen phosphate monohydrate for analysis EMSURE® ACS, Reag. Ph Eur | **1.06346** |
| di-Sodium hydrogen phosphate heptahydrate for analysis EMSURE® ACS | **1.06575** |
| β-Glucuronidase from limpets (Patella vulgata) Type L-II, lyophilized powder, 1,000,000-3,000,000 units/g solid | **G8132** |
| SigMatrix Urine Diluent | **SAE0074** |
| **Accessories** | |
| PlatePrep 96-well Vacuum Manifold, starter kit | **575650-U** |
| Seal Plate Film, Pk.100 | **Z369659** |
| Eppendorf® Deep Well Plate 96/1000 µL PCR Clean, volume 1000 µL, white border with clear wells, Pk.20 | **DWP961000W2-EP** |
| Vials, screw top, clear glass (vial only) | **27379** |
| **Certified Reference Materials (Cerilliant®)** | |
| Pain Management Multi-Component Opiate Mixture-13 solution,100 µg/mL each component (10 µg/mL Fentanyl), 1 mL | **P-071** |
| Hydrocodone-$D_3$ solution, 100 µg/mL in methanol, 1 mL | **H-005** |
| Meperidine-$D_4$ solution, 100 µg/mL in methanol, 1 mL | **M-036** |
| (±)-Methadone-$D_9$ solution, 100 µg/mL in methanol, 1 mL | **M-088** |
| Oxycodone-$D_3$ solution, 100 µg/mL in methanol, 1 mL | **O-005** |
| Oxymorphone-$D_3$ solution, 100 µg/mL in methanol, 1 mL | **O-019** |
| cis-Tramadol-$^{13}C$, $D_3$ hydrochloride solution, 100 µg/mL in methanol, 1 mL | **T-029** |

Learn more about Supel Swift HLB at **SigmaAldrich.com/supelswifthlb**

See our comprehensive offer of CRMs for drugs of abuse testing at **SigmaAldrich.com/cerilliant**

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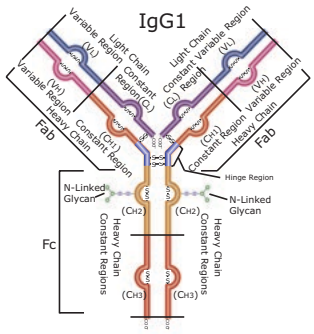
PHARMA & BIOPHARMA

# Analysis of Therapeutic Monoclonal Antibody Trastuzumab using BIOshell™ A400 Protein C4 Column

*Fast and high-resolution analysis of intact monoclonal antibodies (mAbs)*

Sundaram Palaniswamy, Segment Lead, Pharma QC; Aditya Pratihast, Application Scientist; Analytix@milliporesigma.com




## Abstract

Although the majority of small molecules analysed by reversed phase have a mass below 1500 Da, there is a growing need to improve the performance of HPLC columns for the separation of therapeutic proteins and protein drug conjugates. This application note demonstrates a fast and reproducible reversed phase method with high-resolution for the analysis of intact therapeutic monoclonal antibody, trastuzumab. Separation and quantification were achieved using a BIOshell™ A400 Protein C4 column in less than 5 minutes, and more importantly, the optimised method was able to monitor degradation compounds created by heat stress studies.

## Introduction

Over the past few years, monoclonal antibodies (mAbs) have become the best-selling drugs in the pharmaceutical market, and in 2018, eight of the top 10 best-selling drugs worldwide were biologics. The global therapeutic monoclonal antibody market was valued at approximately $115 billion in 2018 growing up to $300 billion by 2025. And although as of December 2019, 79 therapeutic mAbs have been approved by the US FDA for sales worldwide, there is a significant potential for the number to increase.[1] HPLC is a well-established method for the analysis of intact mAbs by Size Exclusion and Ion Exchange chromatography. However, technological advancements in the field of Reversed Phase (RP) have made them promising tools for the analysis on intact proteins.[2] Intact mAbs are yet analyzed with limited success using wide pore, fully porous particles due to their large molecular size leading to slow mass transfer and long analysis times. Superficially porous particles have overcome these challenges, but have lower loadability and still a rather limited offering of different stationary phases. Moreover, highly resolving core-shell columns easily separate intact mAbs quickly and with high efficiency.

Here, we have demonstrated the suitability of the BIOshell™ A400 Protein C4 column for a fast and high-resolution separation of intact trastuzumab using RP-HPLC. Retention time and area precision of the method were excellent, demonstrating the suitability of the column. Further we also showcase quantification and robustness that is highly suitable for biopharmaceutical QC applications.

## Experimental

### Equipment and Sample

The study was performed on a Shimadzu LC-2010CHT HPLC System. The therapeutic trastuzumab was purchased from a local pharmacy.

### Methods

Chromatographic parameters for intact trastuzumab using a BIOshell™ A400 Protein C4 column are shown in **Table 1**.

**Table 1.** Chromatographic parameters used for RP HPLC analysis of trastuzumab

| LC Parameters | | | |
|---|---|---|---|
| **Column:** | BIOshell™ A400 Protein C4, 100 x 2.1 mm I.D., 3.4 µm (**66825-U**) | | |
| **Mobile Phase:** | [A] Water + 0.1% TFA;<br>[B] Acetonitrile + 0.09% TFA | | |
| **Gradient Program:** | **Time** | **%A** | **%B** |
| | 0 | 95 | 5 |
| | 1 | 95 | 5 |
| | 2 | 80 | 20 |
| | 6 | 50 | 50 |
| | 8 | 5 | 95 |
| | 8.1 | 95 | 5 |
| **Post Time:** | 2 minutes | | |
| **Flow rate:** | 1 mL/min | | |
| **Autosampler Temp.:** | 5 °C | | |
| **Column Temp.:** | 80 °C | | |
| **Detector:** | UV 280 nm, 20 Hz | | |
| **Injection volume:** | 10 µL | | |
| **Sample:** | 1 mg/mL trastuzumab (1:10 dilution of formulation in mobile phase A) | | |

## Linearity, Limit of Quantitation (LOQ) and Limit of Detection (LOD)

The calibration curve was constructed with nine standard concentrations of trastuzumab from 1 to 25 µg/mL. The mAb concentration that provided a signal-to-noise ratio (S/N) > 3 was considered as LOD and S/N > 10 was considered as LOQ.

## Forced Degradation Studies

We compared the chromatographic profiles of native and heat-stressed trastuzumab for monitoring degraded products. For the forced degradation studies, 1 mg/mL of trastuzumab was exposed to 10 ppm hydrogen peroxide ($H_2O_2$) followed by heating at 80 °C for 60 min. An aliquot of 10 µL was used for RP HPLC analysis.

# Results and Discussion

## Intact Trastuzumab Analysis

For the HPLC analysis, a BIOshell™ A400 Protein C4, 3.4 µm HPLC Column with core-shell particles and 400 Å pore size delivered reproducible, fast and high-resolution separation of intact trastuzumab, making it suitable for biopharma development and QC applications. **Figure 1** demonstrates excellent peak shape and overlays of six replicates in less than 5 minutes under the chromatographic conditions.

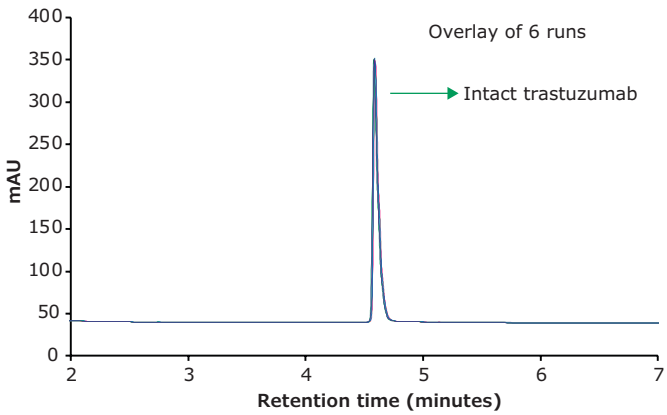



**Figure 1**. RP-HPLC analysis of trastuzumab on a BIOshell™ A400 Protein C4, 100 x 2.1 mm, 3.4 µm HPLC column.

## Precision of Retention Time and Area

**Table 2** shows the average Retention Time (RT) and Area RSDs from six replicates of trastuzumab injections. The Retention Time and Peak Area RSDs were less than 0.1% and 0.29 %, respectively, which demonstrates excellent reproducibility of the method and, thus, the precision of the method.

**Table 2.** Retention time and peak area precision (n = 6) for trastuzumab (1 mg/mL)

| | Mean | RSD (%) |
|---|---|---|
| Retention Time (min) | 4.58 | 0.1 |
| Peak Area | 987268 | 0.29 |

## Limit of Detection and Limit of Quantitation

The LOD and LOQ were 0.125 µg/mL and 0.25 µg/mL, respectively, for trastuzumab, indicating that the method was sensitive. Observed LOD and LOQ values of trastuzumab are reported in **Table 3**. Representative chromatograms on same scale for 2 calibration runs & blank are shown overlayed in **Figure 2**.

**Table 3**. LOD, LOQ, and mean area and retention time (n = 3)

| | Concentration (µg/mL) | Mean Area (n=3) | Retention Time (min) |
|---|---|---|---|
| LOD | 0.125 | 9562 | 4.58 |
| LOQ | 0.25 | 21977 | 4.58 |

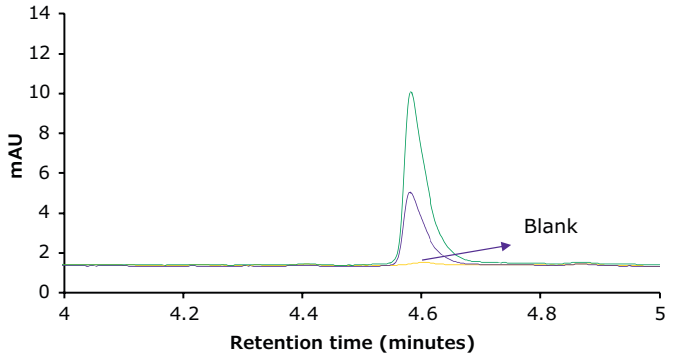



**Figure 2**. Overlay of representative chromatograms on same scale for 2 calibration runs & blank.

## Linearity

Linearity curves for trastuzumab were constructed from 1 µg/mL up to 25 µg/mL in this study using area response and concentration of trastuzumab. The average peak areas are listed in **Table 4**. The linearity curve for trastuzumab is shown in **Figure 3**.

**Table 4.** Summary of linearity range (n = 3) for trastuzumab

| Trastuzumab Conc. (µg/mL) | Average Area |
|---|---|
| 1 | 95,961 |
| 2 | 194,821 |
| 4 | 394,886 |
| 6 | 593,986 |
| 8 | 791,940 |
| 10 | 984,370 |
| 15 | 1,480,051 |
| 20 | 1,940,216 |
| 25 | 2,447,554 |

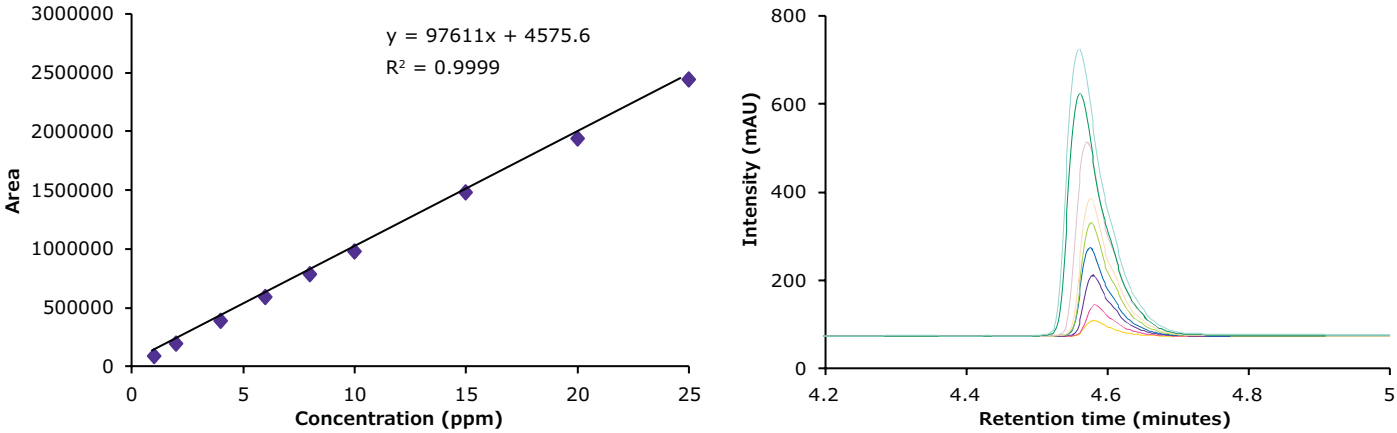



**Figure 3**. Linearity curve with nine standard concentrations of trastuzumab ranging from 1 to 25 µg/mL showing excellent coefficient values. Also shown are chromatogram overlays for the linearity ranges.

## Trastuzumab Degradation Studies

We compared the intact and stressed trastuzumab using RP-HPLC to evaluate if this method is stability indicating. Any deviations in peak RT or Area as a result of stress were considered degradation products.

**Figure 4** compares the RP-HPLC profile of unstressed and heat stressed trastuzumab. The profiles indicate that the BIOshell™ A400 Protein C4, HPLC column was able to distinguish between unstressed and stressed trastuzumab based on the peak shape and area.

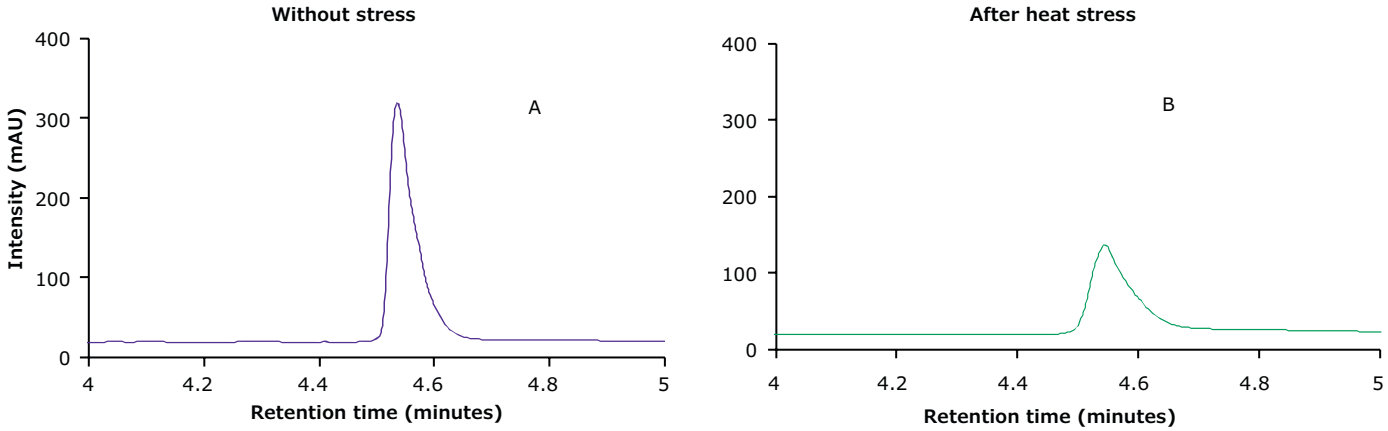



**Figure 4**. BIOshell™ A400 Protein C4, 100 x 2.1 mm, 3.4 µm RP-HPLC profiles of unstressed (A) and heat stressed trastuzumab sample (B)

## Conclusion

Analysis of intact mAbs provides a first level of interrogation of size, post translational modification and heterogeneity. RP-HPLC analysis of mAbs requires large pore sizes, a hydrophobic stationary phase and appropriate chromatographic methods. In this application note a simple LC-UV method for the analysis of intact trastuzumab was showcased. Using a BIOshell™ A400 Protein C4 column, a high resolution and rapid separation of intact trastuzumab was developed. Area and RT precision of the method were excellent and showed the reliability of the method. The calibration curves with nine standard concentrations of trastuzumab had excellent coefficient of linearity values displaying that the method was quantitative and accurate. The LOD and LOQ for trastuzumab were found to be 0.125 µg/mL and 0.25 µg/mL, respectively, indicating the method was sensitive. In addition, heat stressed studies demonstrated that the BIOshell™ A400 Protein C4 column was able to monitor degraded mAbs and the method could be used for stability studies.

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## Featured Products

| Description | Cat. No. |
|---|---|
| BIOshell™ A400 Protein C4, 100 x 2.1 mm I.D., 3.4 µm | **66825-U** |
| LiChrosolv® Acetonitrile Isocratic Grade | **1.14291** |
| Trifluoroacetic acid HPLC grade | **302031** |
| Water for chromatography LiChrosolv® | **1.15333** |

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PHARMA & BIOPHARMA

# Analysis of Oligonucleotides by SEC-MALS

*Presented application data courtesy from Tosoh Bioscience*

Anders Fridström, Analytical Scientific Liaison Manager; Analytix@milliporesigma.com

## Abstract

The importance of oligonucleotides in the generation of new pharmaceuctical therapies has been increasing in recent years with 10 FDA approved therapies in 2020.[1] This emerging field of therapies often requires improved or alternative analytical methods to accelerate development and assure the safety of the drug. This article describes the ability of ultra-high performance size exclusion chromatography to distinguish N and N-1 oligonucleotide species.

## Introduction

In recent years, several oligonucleotide drugs for gene silencing, such as short interfering RNA (siRNA) and antisense oligonucleotides (ASOs) have been approved and microRNA (miRNA) and aptamers are being developed as therapeutic platforms. The promising CRISPR-Cas system also requires a specific RNA moiety - guiding RNA - to recruit and direct the Cas nuclease activity.

Therapeutic oligonucleotides are produced through a synthetic, solid-phase chemical synthesis. Despite improvements in oligonucleotide synthesis, and despite the most ardent post-synthesis clean-up, there is always some heterogeneity with regards to oligonucleotide distribution. Monitoring of impurities in this distribution is a fundamental aspect of process and quality control. This fundamental assessment is typically done by capillary gel electrophoresis (CGE) or anion exchange chromatography. Here, we present the ability of size exclusion chromatography (SEC) to discriminate oligonucleotides differing by one base in length. The 2 µm silica-based stationary phase, TSKgel® UP-SW2000, with a pore size of 125 Å, was used in combination with UHPLC and UHPLC-MALS systems.[2]

## Analysis of Oligonucleotides by SEC[2]

TSKgel® UP-SW2000 is a recently developed silica-based 2 µm, 125 Å pore size SEC column designed for the separation of small proteins, peptides, and oligonucleotides. The column can be used both in HPLC and UHPLC systems and is ideally suited for method transfer from conventional silica-based size exclusion columns to UHPLC technology. Two, 30 cm TSKgel® UP-SW2000 columns in series were used to analyze a mixture of two oligonucleotides differing by only one base.

## Materials and Method

| | |
|---|---|
| **Columns:** | TSKgel® UP-SW2000, 2 x 300 x 4.6 mm I.D., 2 µm (**823514**) |
| **Mobile phase:** | [A] 50 mM phosphate buffer, pH 6.7, [B] 300 mM sodium chloride, 0.03% W/V sodium azide |
| **Flow rate:** | 0.2 mL/min |
| **Detection:** | UV, 260 nm |
| **Injection:** | 10 µL |
| **Sample:** | 19-mer (5'-AATTCATCGGTTCAGAGAC-3') & 20-mer(5'-GAATTCATCGGTTCAGAGAC-3') |

## Results

**Figure 1** demonstrates that UP-SW2000 can be used to separate a 20-mer and its N-1 19-mer.

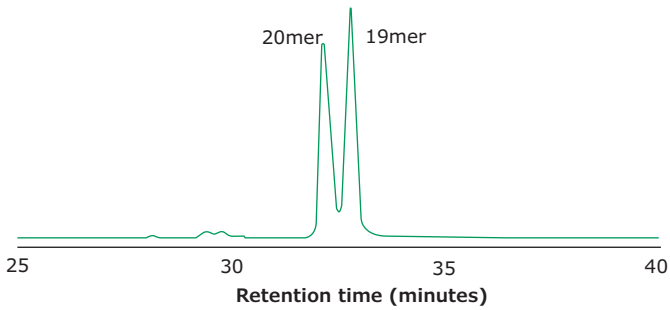


**Figure 1**. Separation of N and N-1 Oligonucleotides.

## SEC-MALS Analysis of Oligonucleotides[2]

Crude and purified oligonucleotide samples were analyzed by SEC-MALS using LenS3® multi-angle light scattering detector.

## Materials and Method

| | |
|---|---|
| **UHPLC:** | Thermo Fisher Dionex Ultimate 3000 UHPLC system with $LenS_3$® MALS |
| **Column:** | TSKgel® UP-SW2000 300 x 4.6 mm I.D., 2 µm (**823514**) |
| **Mobile phase:** | 0.5 M Sodium chloride, 0.1 M EDTA, pH 7.5; 0.1 M sodium sulfate; 0.03% w/v sodium azide in 0.1 M phosphate buffer |
| **Flow rate:** | 0.3 mL/min |
| **Column temp:** | Room temperature |
| **Detection:** | UV, 260 nm |
| **Injection vol.:** | 10 µL |
| **Sample:** | 20 Bases custom oligonucleotide with MW= 6141 Da (purified sample 0.3 mg/mL; crude sample 1 mg/mL) |

## Results

**Figure 2** shows the comparison of chromatograms of the crude and purified oligonucleotide samples and **Figure 3** shows the molecular weight distribution of the unpurified 20-mer. The molecular weight trace clearly indicates the presence of higher and lower molecular weight impurities.

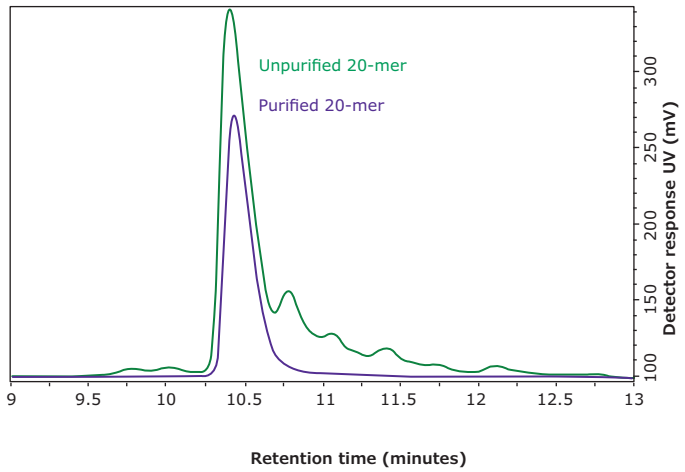


**Figure 2**. Overlay of unpurified and purified 20-mer UV chromatograms.

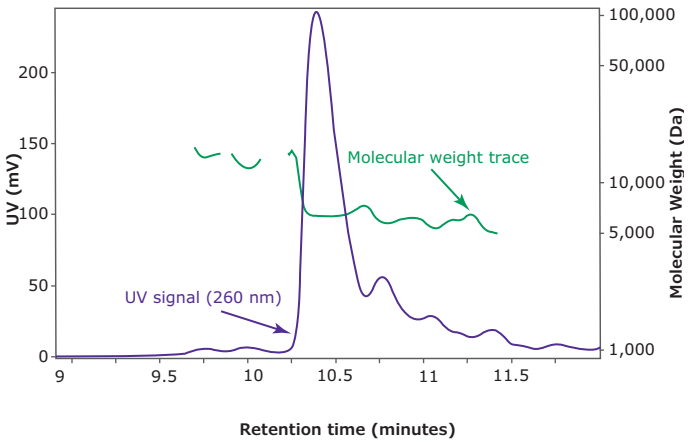


**Figure 3**. Molecular weight distribution (green) of the unpurified 20-mer.

The peak analysis (**Figure 4** and **Table 1**) allows a molecular weight profiling of the product and the impurities. The MALS analysis of the purified sample (**Figure 5**) proves the high purity of the 20-mer oligonucleotide. The good reproducibility of retention time and calculated molecular weight of the purified 20-mer is shown in **Table 2** (triplicate injection).

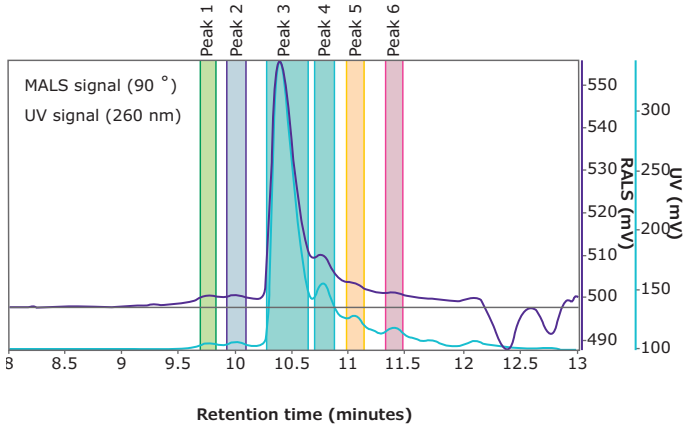


**Figure 4**. Peak analysis of the unpurified 20-mer.

**Table 1.** Molecular weight profiling

| Peak | Retention time | % RSD | MW (Da) | % RSD |
|---|---|---|---|---|
| 1 | 9.774 | 0.1% | 13,599 | 2.1% |
| 2 | 10.012 | 0.0% | 11,550 | 1.9% |
| 3 | 10.398 | 0.1% | 6,398 | 0.7% |
| 4 | 10.776 | 0.1% | 5,751 | 1.5% |
| 5 | 11.053 | 0.1% | 5,177 | 2.3% |
| 6 | 11.422 | 0.2% | 4,446 | 5.5% |

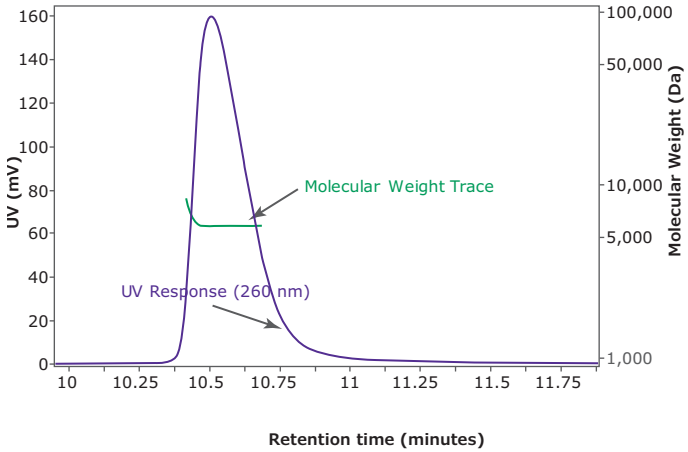


**Figure 5**. Molecular Weight distribution (green) of the purified 20-mer.

**Table 2.** Reproducibility of retention time

| Injection | Retention time (Min) | MW (Da) |
|---|---|---|
| 1 | 10.431 | 6.066 |
| 2 | 10.443 | 6.023 |
| 3 | 10.445 | 6.038 |
| Average | 10.440 | 6.042 |
| % RSD | 0.1% | 0.3% |

## Conclusion

TSKgel® UP-SW2000 is a size exclusion column designed for UHPLC analysis of biomolecules having molecular weight of 1 to 150 kDa. The separation range is ideally suited to analyze small proteins or peptides and their aggregates.

This study shows that this column can also be used to analyze oligonucleotides by (U)HPLC. Multi-angle light scattering detection delivers additional information on the molecular weight of the oligonucleotide and any impurities present in the sample.

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## Featured Product

| Description | Cat. No. |
|---|---|
| TSKgel® UP-SW2000, phase diol, 300 × 4.6 mm, 2 µm | **823514** |

## Related Products

| Description | Cat. No. |
|---|---|
| EDTA disodium salt suitable for HPLC, LiChropur™, 99.0-101.0% (KT) | **79884** |
| Potassium dihydrogen phosphate anhydrous for HPLC LiChropur™ | **5.43841** |
| Sodium azide, purum p.a., ≥99.0% (T) | **71290** |
| Sodium chloride for HPLC LiChropur™ | **5.43832** |
| di-Sodium hydrogen phosphate anhydrous for HPLC LiChropur™ | **5.43838** |
| Sodium sulfate suitable for HPLC LiChropur™, 99.0-101.0% (T) | **80948** |
| Sodium azide, purum p.a., ≥99.0% (T) | **71290** |

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# Synthetic Carbon Adsorbents for Host Cell Protein Removal in Monoclonal Antibody Purification

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## Introduction

Monoclonal antibodies (mAbs) are a class of critical biotherapeutic drugs. The demand for mAbs is steadily increasing. But the depletion of host cell proteins (HCP) in the manufacturing of mAbs remains a challenge. HCPs can represent an immune response risk after the administration of mAbs if not reduced to appropriate levels. Removal of HCPs is typically done via a Protein A (PA) chromatography capture step, carried out in bind-and-elute mode. Following the Protein A purification step, the feed is passed through two polishing steps in the form of ion exchangers (IEX), namely cation exchange chromatography (CEX) and anion exchange chromatography (AEX). The remaining impurities are mostly low molecular weight, hydrophobic HCPs, that are difficult to remove either because of their physiochemical properties[1], or their non-specific association with the antibody[2]. These impurities represent the group of critical HCPs which need to be addressed in order to achieve the highest possible purification.

Efforts to further reduce HCP content beyond that achieved with the standard PA-IEX downstream processes are being considered. One such solution is to add additional polishing steps like hydrophobic interaction chromatography (HIC) between the PA and IEX chromatographic steps. In this work, a post-Protein A (PPA) pool was applied to three different carbon-based adsorbents in flow-through mode to test their HCP depletion behavior under dynamic conditions.

## Methods and Materials

### Post Protein A Pool

The PPA pool was produced by treating a clarified cell culture harvest of CHO-K1 containing mAb02 with Protein A chromatography. For this purpose, a 205 x 26 mm (108 mL) column packed with ProSep® Ultra Plus Protein A resin was used on an Äkta™ Pure 25 system. A high salt wash is typically performed to further reduce the HCP content; however, the salt wash was omitted here to keep the HCP content high for the purpose of challenging the adsorbents. The HCP content of the PPA used in this study was 6,000 ppm. After thirty minutes of virus inactivation, the PPA pool was titrated to pH 5 with 2 M tris(hydroxymethyl)aminomethane.

### Adsorbent Materials

Two Carboxen® synthetic carbon adsorbents and a commercially available depth filter were investigated for their HCP removal capability and mAb recovery. Carboxen® 1032 and Carboxen® 569 are commercially available (Merck KGaA, Darmstadt, Germany) synthetic carbon adsorbents with particle sizes in the range of 20-40 mesh. For this study, customized 50 µm (approx. 270 mesh) particles of these two synthetic carbons were used. The depth filter was selected for comparative purposes. The depth filter adsorbent bed comprises of traditional naturally sourced activated carbon and cellulose.

A schematic drawing of Carboxen® synthetic carbon particles is given in **Figure 1**. The synthetic carbons are highly engineered derivatized resins which have been designed to withstand high pressures of up to 110 MPa. Resins used in bio chromatography undergo plastic deformation and traditional activated carbons are known to fracture and generate fines at far lower pressures.

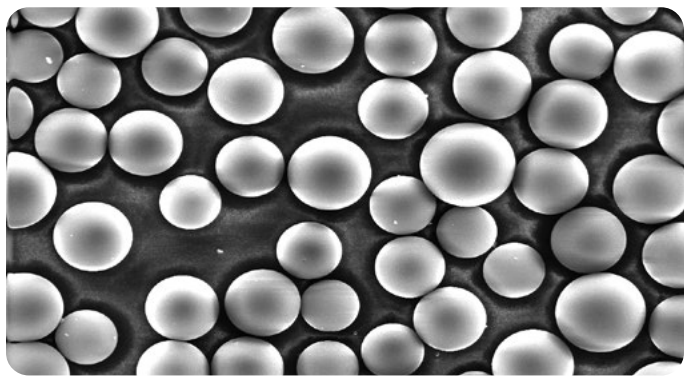

**Figure 1**. Carboxen® synthetic carbon particle.

**Table 1.** Carboxen® synthetic carbon material properties

| | Surface Area ($m^2/g$) | Surface pH (ASTM D6851) | 1 = most hydrophilic 10 = most hydrophobic | Pore Structure (%) | | | Ball Pan Hardness (ASTM D3802) |
|---|---|---|---|---|---|---|---|
| | | | | Micro (< 20 Å) | Meso (20 - 500 Å) | Macro (>500 Å) | |
| **Carboxen® 563** | 500 | 6.8 | 7 | 38 | 24 | 38 | 99.8 |
| **Carboxen® 564** | 400 | 8.7 | 8 | 47 | 26 | 27 | 99.8 |
| **Carboxen® 569** | 500 | 8.6 | 9 | 45 | 32 | 23 | 99.4 |
| **Carboxen® 572** | 1000 | 9.5 | 4 | 48 | 23 | 29 | 99.1 |
| **Carboxen® 1005** | 1000 | 8.0 | 10 | 47 | 26 | 27 | 99.6 |
| **Carboxen® 1032** | 800 | 3.0 | 1 | 49 | 0 | 51 | 98.6 |
| **Carboxen® 1033** | 400 | 7.0 | 3 | 38 | 0 | 62 | 99.7 |
| **Carboxen® 1034** | 1200 | 10.5 | 2 | 52 | 3 | 45 | 98.9 |

Functionalization of carbon surfaces with oxygen functional groups alters the materials' pH and hydrophobic/hydrophilic properties. The pH of Carboxen® synthetic carbons can be tailored from 2.5 to 10.5. The pH can be selected to meet the application needs. The surface chemistry dictates the hydrophobic/hydrophilic properties and with proper oxygen functionalization, the synthetic carbon adsorbent can be made anywhere from hydrophilic to extremely hydrophobic. The Carboxen® synthetic carbons are activated through physical means. Therefore, there is no chemical residual left on the carbon that could create a chemical leaching concern in use. The carbon particles are of high purity and pass all the extractable and leachable tests as outlined in both USP 43 - NF 38 and Ph. Eur 10. As they are produced from a synthetic source, Carboxen® synthetic carbons are inherently free of heavy metals and other inorganic content found in naturally derived adsorbents.

While eight Carboxen® synthetic carbons are commercially available, 1032 and 569 were particularly selected since their properties are markedly different and thus a performance difference was anticipated between the two. Carboxen® 569 is highly hydrophobic and does not contain any ligand or attached surface functionality, and it can be thought of as a super phenyl phase. Carboxen® 569 has a point of zero charge at pH 8.8. Carboxen® 1032 is functionalized with oxygenated surface functional groups that give the material more of a hydrophilic character and a point of zero charge at pH 4.1. Material properties of the eight commercially available materials are given in **Table 1.**

## Experimental Procedure

The Carboxen® materials were packed into Super Compact Columns (Götec Labortechnik GmbH, Germany) with a column volume of 300 µL. The columns' inner diameter was 5 mm and the bed depth, 1.5 cm. The depth filter was used directly. Prior to the purification process, the depth filter was flushed with Milli-Q® water for 100 L/m² and conditioned with 50 mM acetate buffer for 300 L/m² at a flux of 300 L/h/m², corresponding to 2.5 mL/min. Packed columns containing Carboxen® synthetic carbons were flushed and conditioned with 50 mM acetate buffer for 50 column volumes at a flow rate of 0.5 mL/min.

For the HCP removal analysis, a pre-defined volume of 200 mL of the PPA pool, pH 5, was applied to each adsorbent. To maintain a residence time of 90 s within the devices, flow rates of 0.2 mL/min and 2 mL/min (corresponding to a flux of 240 L/h/m²) for the columns and the filter were applied, respectively. The PPA pool and each flow-through fraction were characterized by size exclusion chromatography (SEC) to determine the mAb load (onto each adsorbent) and mAb recovery. A Tosoh TSKgel® Super SW3000 SEC column was used on an Ultimate™ 3000 UHPLC system with diode-array detector. HCP breakthrough was measured with ELISA. A CHO Host Cell Proteins 3rd generation ELISA-Assay Kit F550-1 from Cygnus Technologies was utilized. The procedure was carried out according to the manufacturer's instructions.

## Results and Discussion

For comparison of the different adsorbents regarding their suitability for HCP depletion, the filter area was converted to volume based on its given dimensions. The HCP breakthrough was then plotted against the load of HCP (**Figure 2A**) and the load of mAb (**Figure 2B**) per adsorbent volume. Since the comparison was made at an HCP breakthrough of 10%, only this range is displayed.

Carboxen® 1032 had the best performance among the three materials and showed a very good depletion of HCPs. At an HCP breakthrough of 10%, 2300 g of mAb per liter of adsorbent could be applied on Carboxen® 1032 and 14 g of HCP per liter of adsorbent could be depleted. The depth filter performed the worst among the three materials tested for HCP depletion, with a depletion of about 3 g of HCP per liter of adsorbent after an application of 500 g of mAb per liter of adsorbent. Carboxen® 569 showed a performance in-between that of the other two materials tested. On Carboxen® 569, 900 g of mAb per liter of adsorbent could be applied, and 5 g of HCP per liter of adsorbent could be depleted. All three materials tested had a high mAb recovery, upward of 95%.

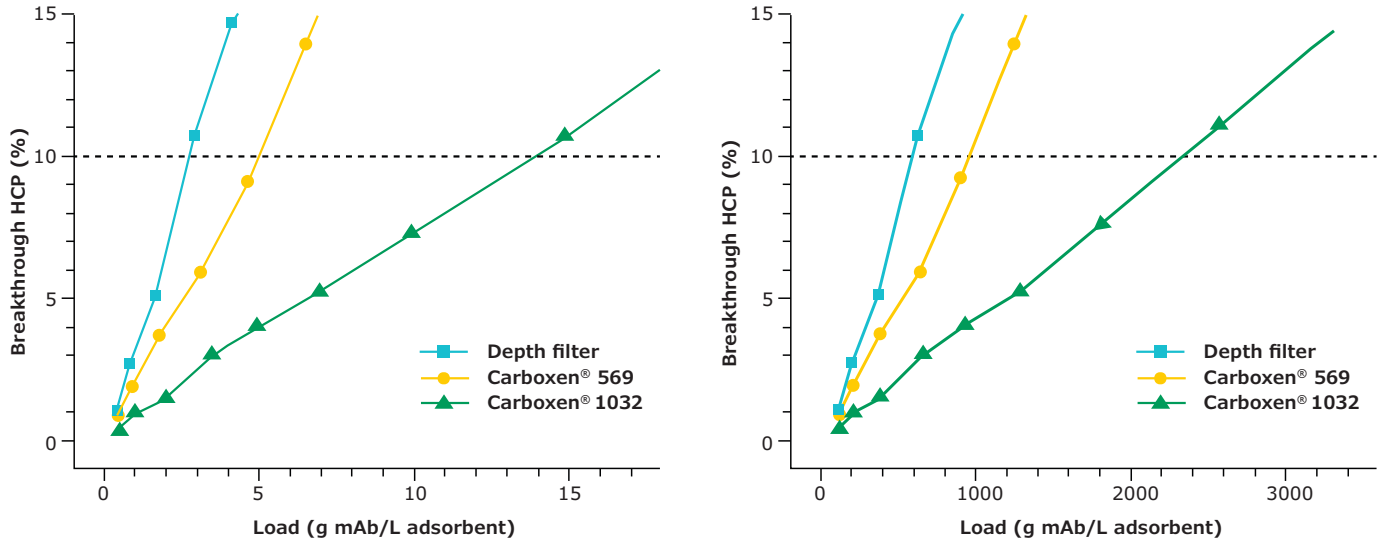


**Figure 2.** HCP breakthrough plotted against A) load of HCP and B) load of mAb per unit volume of adsorbent.

Carboxen® 1032 is assumed to be slightly negatively charged at pH 5, as its point of zero charge is lower than pH 5. Considering the excellent HCP retention obtained by this material, it is likely that most of the proteins are positively charged or uncharged under the given conditions. This observation leads to the assumption that most proteins have an isoelectric point of 5 or higher. In contrast, Carboxen® 569 is assumed to be strongly positively charged at pH 5 as it has a point of zero charge greater than pH 5. The depth filter contains activated carbon as an adsorbent and is also likely to be positively charged at pH 5. Therefore, the positively charged proteins are repelled by these adsorbents.

Carboxen® 1032 worked best, out of all the materials investigated in this study, and under these specific conditions. However, PPA pools are highly variable as are the purification conditions (e.g. pH and conductivity) and the polishing media needs to fit accordingly. There are eight commercially available Carboxen® synthetic carbons that cover a wide range of material properties with dispersion pHs ranging from 2.5 to 11, and from extremely hydrophobic to hydrophilic materials. Likely there is a Carboxen® synthetic carbon or a mixture that can optimize HCP depletion while maintaining high mAb yield in most mAb purification processes. This feat could be achieved by finding the best suited pH and conductivity of the PPA. For example, Carboxen® 569 is a strongly hydrophobic material. An increase in conductivity is expected to improve the HCP removal, as protein charges can be shielded by the added salt. Furthermore, pH variations could lead to different HCP depletion due to their different isoelectric points.

## Conclusion

The Carboxen® synthetic carbons demonstrated excellent host cell protein reduction and high mAb yield. Carboxen® 1032 had the best performance among the three materials tested and showed a very good depletion of host cell proteins. At a HCP breakthrough of 10%, 2300 grams of monoclonal antibody per liter of adsorbent could be applied on Carboxen® 1032 and 14 grams of HCP per liter of adsorbent could be depleted. With the addition of a Carboxen® synthetic carbon polishing step to the standard PA-IEX downstream processes, difficult-to-remove lower molecular weight and hydrophobic HCPs can be depleted. With an added synthetic carbon purification step, downstream ion exchangers would bear a lower burden because of the reduction in impurities, so that their durability can be increased. Ideally, the implemented step can replace one of the downstream ion exchangers. These points would lead to a reduction in production costs. In an alternative configuration, the Carboxen® synthetic carbon can act as a final polishing step to target the critical HCPs that are not removed in the standard PA-IEX process. Carboxen® synthetic carbons can ultimately lead to the production of a safer mAb product for the patient.

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ENVIRONMENTAL

# Analysis of PFAS Extractables in Polyethersulfone (PES) Syringe Filters

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## Abstract

Guidelines and methods for measuring perfluoroalkyl substances (PFAS) in various matrices have been established by regulatory agencies in recent years. A key consideration for any PFAS method is to avoid contamination that can impact the accuracy of data, including those coming from sample preparation techniques such as filtration. In this article, EPA Method 537.1 was used to demonstrate that the Millex® syringe filters with PES (polyethersulfone) Millipore Express® membranes did not give any detectable levels of PFAS contamination.

## Introduction

PFAS are poly- and perfluorylalkyl substances known as "forever chemicals" and comprise a group of over 4,000 varieties of long- and short-chain perfluorinated compounds.[1] The excellent properties and broad use of PFAS, such as in firefighting foams and water-repellant clothing, has led to persistent accumulation of these man-made chemicals in environmental and biological matrices. These compounds have potential negative impact on humans health and aquatic life including liver damage, cancer, weakened immune system and high cholesterol.[1-3]

In response, agencies in the US and Europe have taken regulatory action. The Stockholm Convention proposed regulations for two of the commonest PFAS compounds—perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS)—with certain exemptions, effective in 2020. The US Environmental Protection Agency (EPA) published an Action Plan in 2019 followed by recommendations for testing water matrices for PFAS compounds under the Safe Drinking Water Act in early 2020, with a drinking water advisory concentration of 70 parts per trillion (ppt). The European Union (EU) proposed a PFOA limit of 25 parts per billion (ppb) in mid-2020. In response to regulatory proposals and actions, academic and industrial testing labs have developed analytical methods for testing and monitoring PFAS in a variety of matrices. These regulations are important to understanding the extent of human exposure and environmental contamination to inform future remediation efforts.

Currently, most of the analytical methods for water matrices involve solid phase extraction (SPE) for sample preparation after the addition of internal standards and fortification with surrogate standards to the sample, prior to Liquid Chromatography and tandem Mass Spectrometry (LC-MS/MS) detection of selected PFAS analytes (usually 20-30 compounds). **Table 1** lists some examples of current methods for PFAS testing.

**Table 1.** Examples of methods for PFAS testing

| Country | Method | Matrix | Limit/Range | Sample Prep | Analytical Method |
|---|---|---|---|---|---|
| USA | EPA 533 | Drinking water | 1.6-16 ng/L | SPE | LC-MS/MS |
| | EPA 537.1 | Drinking water | 0.53-6.3 ng/L | SPE | LC-MS/MS |
| | EPA Draft 1633 | Aqueous, soil, biosolids, sediment, tissue | 1.6-40 ng/L (typical, aqueous) | SPE; Filtration | LC-MS/MS |
| | SW-846 Method 8327 | Non-potable groundwater, surface water, wastewater | 10-400 ng/L | SPE; Filtration | LC-MS/MS |
| | ASTM D7979-20 | Water matrix (no drinking water) | 0.7-107 ng/L (MDL) | Solvent extraction; Filtration | LC-MS/MS |
| | EPA Draft 1633 | Aqueous, soil, biosolids, sediment, tissue | 1.6-40 ng/L (typical, aqueous) | SPE; Filtration | LC-MS/MS |
| | ASTM D7968-17a | Environmental solids: soil, sediment, sludge | 25-20,000 ng/kg | Solvent extraction; Filtration | LC-MS/MS |
| | FDA Method C-010.01 | Food (bread, lettuce, milk, fish) | 0.05-5 ng/g | QuEChERS; Filtration; SPE | LC-MS/MS |
| EU | ISO 25101 | Drinking water, groundwater, surface water (fresh and sea water) | 2-10,000 ng/L for PFOS; 10-10,000 ng/L for PFOA | SPE | LC-MS/MS |
| | ISO 21675 | Drinking water, naturalwater, waste water (< 2g/L solid particulate material) | ≥0.2 ng/L (LOQ) | Filtration as needed; SPE | LC-MS/MS |

Abbreviations: MDL = minimum detection limit; LOQ = limit of quantitation; SPE = solid phase extraction; PFAS = perfloroalkyl substances; PFOA = perflurooctanoic acid; PFOS = perfluorooctanesulfonic acid; LC-MS/MS = liquid chromatography tandem mass spectrometry

## The Need for Sample Filtration in PFAS Testing

Drinking water is considered a "clean" matrix and often does not require filtration as part of sample preparation. However, methods such as SW-846 Method 8327, ASTM D7968, ASTM D7979 and ISO 21675 involve matrices that could have a higher degree of particulate matter such as wastewater. Particulates in solution must be removed prior to LC-MS/MS, as they can be detrimental to sample analysis, column longevity and overall instrument function. These methods identify the need for filtration using membranes in a syringe filter format.

In recent studies, there has been concern over contamination of PFAS samples from a variety of sources, including collection bottles, storage vials, tubing components, and any other plastic that comes into contact with the sample.[4] This includes membrane filters and housing. In this study, syringe filters with polyethersulfone (PES) membranes and polypropylene (PP) housing were tested for PFAS using EPA method 537.1. While this method does not require filtration as part of the sample preparation step, it was used to provide a clean sample to test extractable contamination levels, if any, in the syringe filters.

## Experimental

To determine if the sample filtration media contributes to PFAS contamination, samples were analyzed using EPA 537.1. Briefly, a 250 mL PFAS-free DI water sample was spiked with surrogates. The internal standard spike of 0.08 ppb was used for QC blanks. The sample bottles and tubes were rinsed with basic methanol. The samples were first filtered through non-sterile Millex® syringe filters with PES Millipore Express® membranes, followed by extraction using styrene divinylbenzene (SDVB) SPE cartridge. Three lots of non-sterile Millex® syringe filters with PES Millipore Express® membranes were tested. The SPE eluate was concentrated to 1 mL in 96:4% (v/v) methanol:water prior to LC-MS/MS analysis using a C18 column.

**Figure 1** is the schematic of the experimental procedure. **Table 2** shows the LC-MS/MS conditions.

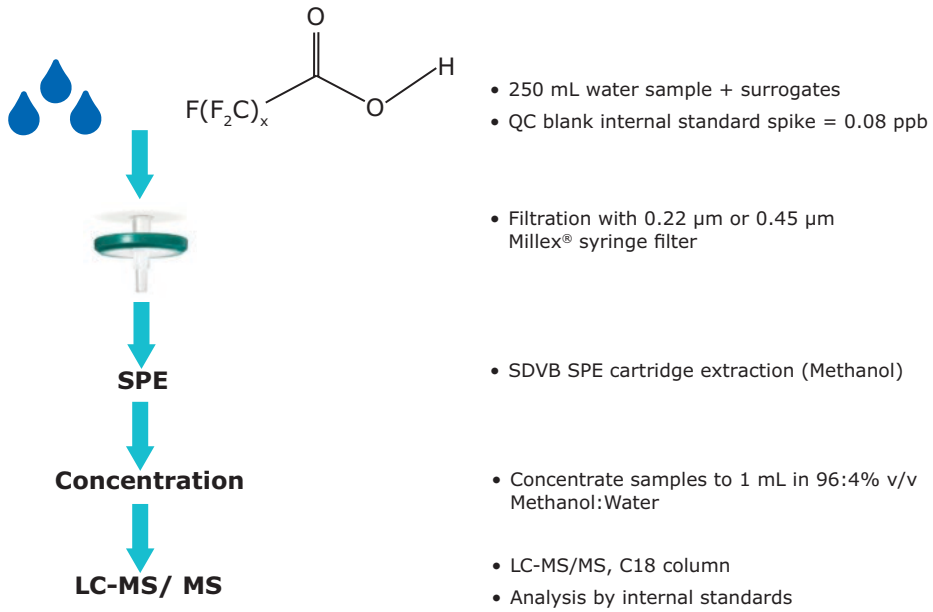



**Figure 1**. Schematic outline for testing Millex® syringe filters for PFAS contamination.

**Table 2.** LC-MS/MS conditions used for measuring PFAS

| | |
|---|---|
| **Column:** | C18, 100 x 2.1 mm I.D., 2.7 µm superficially porous particles |
| **Mobile phase:** | [A] Water, 0.1% (v/v) acetic acid;<br>[B] Methanol (MeOH), 0.1% (v/v) acetic acid |
| **Gradient:** | |

| Time (min) | A % | B % | Flow (mL/min) |
|---|---|---|---|
| 0-0.0 | 65% | 35% | 0.4 |
| 0-7.0 | 0% | 100% | 0.4 |
| 7.0-10.0 | 0% | 100% | 0.7 |
| 10.0-11.0 | 0% | 100% | 0.7 |
| 11.0-15.0 | 65% | 35% | 0.4 |

| | |
|---|---|
| **Flow rate:** | See gradient table |
| **Detection:** | MS/MS, ESI(-), details of MS/MS conditions can be requested from the author |
| **Column temp:** | 50.0 °C |
| **Injection volume:** | 3-5 µL autosampler injection |
| **Sample :** | SPE eluate concentrated to 1 mL methanol: water, 96:4% (v/v) |

## Results & Discussion

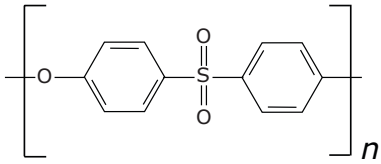
PFAS extractables in nonsterile Millex® filters with PES membranes were measured according to EPA Method 537.1, with some additional PFAS compounds not required by the method including next-generation PFAS compounds (GenX). In all three lots tested for 0.22 µm and 0.45 µm syringe filters with PES membranes, no PFAS contaminants were detected even with the very low minimum detection limits (MDL) of the method (**Table 3**). These results suggest that nonsterile Millex® syringe filters with PES membranes are reliable and appropriate for use in filtration of samples in the analysis of PFAS compounds in environmental matrices that require filtration prior to further clean-up, by solid phase extraction for example, and/or LC-MS/MS analysis.

**Table 3.** Detection of PFAS after filtration with nonsterile Millex® filters with PES membranes using LC-MS/MS according to EPA 537.1

| | | | | Millex® PES | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 0.22 µm | | | 0.45 µm | | |
| Compound | Abbreviation | RL (ppb) | MDL (ppb) | Lot1 | Lot2 | Lot3 | Lot1 | Lot2 | Lot3 |
| **Perfluoroalkylcarboxylic Acids** | | | | | | | | | |
| Perfluorobutanoic acid | PFBA | 0.0040 | 0.0020 | | | | | | |
| Perfluoropentanoic acid | PFPeA | 0.0020 | 0.0010 | | | | | | |
| Perfluorohexanoic acid | PFHxA | 0.0020 | 0.0010 | | | | | | |
| Perfluoroheptanoic acid | PFHpA | 0.0020 | 0.0010 | | | | | | |
| Perfluorooctanoic acid | PFOA | 0.0020 | 0.0010 | | | | | | |
| Perfluorononanoic acid | PFNA | 0.0020 | 0.0010 | | | ND – not detected in filtrate | | | |
| Perfluorodecanoic acid | PFDA | 0.0020 | 0.0010 | | | | | | |
| Perfluoroundecanoic acid | PFUnDA | 0.0020 | 0.0010 | | | | | | |
| Perfluorododecanoic acid | PFDoDA | 0.0020 | 0.0010 | | | | | | |
| Perfluorotridecanoic acid | PFTrDA | 0.0020 | 0.0010 | | | | | | |
| Perfluorotetradecanoic acid | PFTeDA | 0.0020 | 0.0010 | | | | | | |
| **Perfluoroalkylsulfonic Acids, Perfluorooctanesulfonamides, and Perfluorooctanesulfonamidoacetic Acids** | | | | | | | | | |
| Perfluorobutanesulfonic acid | PFBS | 0.0020 | 0.0010 | | | | | | |
| Perfluoropentanesulfonic acid | PFPeS | 0.0020 | 0.0010 | | | | | | |
| Perfluorohexanesulfonic acid | PFHxS | 0.0020 | 0.0010 | | | | | | |
| Perfluoroheptanesulfonic acid | PFHpS | 0.0020 | 0.0010 | | | | | | |
| Perfluorooctanesulfonic acid | PFOS | 0.0020 | 0.0010 | | | ND – not detected in filtrate | | | |
| Perfluorononanesulfonic acid | PFNS | 0.0020 | 0.0010 | | | | | | |
| Perfluorodecanesulfonic acid | PFDS | 0.0020 | 0.0010 | | | | | | |
| PFOSA | PFOSA | 0.0040 | 0.0020 | | | | | | |
| N-MeFOSAA | MeFOSAA | 0.0040 | 0.0020 | | | | | | |
| N-EtFOSAA | EtFOSAA | 0.0040 | 0.0020 | | | | | | |
| **Fluorotelomer Sulfonates and Next Generation PFAS Analytes** | | | | | | | | | |
| 4:2 Fluorotelomer sulfonate | 8:2 FTS | 0.0080 | 0.0020 | | | | | | |
| 6:2 Fluorotelomer sulfonate | 6:2 FTS | 0.0080 | 0.0020 | | | | | | |
| 8:2 Fluorotelomer sulfonate | 8:2 FTS | 0.0080 | 0.0020 | | | | | | |
| HFPO-DA | GenX | 0.0040 | 0.0020 | | | ND – not detected in filtrate | | | |
| ADONA | ADONA | 0.0080 | 0.0020 | | | | | | |
| 9Cl-PF3ONS (F-53B Major) | -- | 0.0080 | 0.0020 | | | | | | |
| 11Cl-PF3OUdS (F-53B Minor) | -- | 0.0080 | 0.0020 | | | | | | |

Abbreviations: RL = reporting limit (ppb); MDL = minimum detection limit (ppb); ND = not detected above both RL and MDL.

A




B

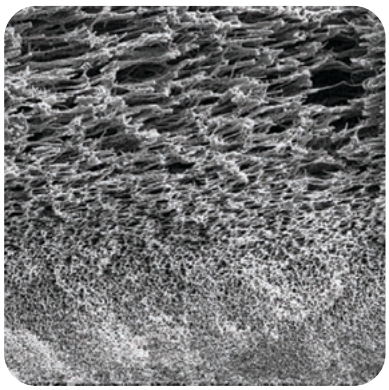


**Figure 2**. (A) Polyethersulfone membrane repeating unit (B) SEM image of PES membrane showing its asymmetric pore structure.

The microporous membrane in the nonsterile Millex® syringe filters used in this study is the Millipore Express® PES membrane. **Figure 2** shows the repeating unit of the polymer and an SEM image of the membrane. PES is commonly used as an alternative to cellulose membranes and is known for its thermal stability, durability and resistance to acidic and alkaline solutions. This membrane is excellent for aqueous filtration applications, offer fast flow, high filter capacity and low protein binding. The unique asymmetric structure of Millipore Express® PES membranes extends filtration capacity, allowing them to tolerate higher particle loads and protein concentrations that may be found in water samples tested using SW-846 Method 8327, ASTM D7968, ASTM D797 and ISO 21675 PFAS methods. Nonsterile, 33mm Millex® syringe filters with PES membranes have polypropylene housing and the membranes are not manufactured using fluorinated compounds.

Millex® syringe filters with both nylon membrane and nylon membrane with a glass fiber prefilter were also tested. Similar results were obtained - no PFAS contaminants were detected.

## Conclusion

Increasing concern about the threat of PFAS to human health has sparked a cascade of regulatory action in the US and Europe. While the primary focus has been on drinking water, other matrices containing higher particulates are also under investigation. Samples with higher particulates will require filtration prior to LC-MS/MS analysis or further SPE cleanup. Using EPA Method 537.1, no PFAS extractables were detected in nonsterile Millex® syringe filters with PES membranes. Therefore, when filtration of higher particulate samples is needed in a PFAS workflow, nonsterile Millex® syringe filters with PES membranes or nylon membranes (with or without glass fiber prefilter) are suitable options.

**References**

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3. Pérez F, Nadal M, Navarro-Ortega A, Fàbrega F, Domingo JL, Barceló D, Farré M. 2013. Accumulation of perfluoroalkyl substances in human tissues. Environment International. 59354-362. http://dx.doi.org/10.1016/j.envint.2013.06.004
4. USEPA, 2020a. Method 537.1, version 2.0, Determination of Selected Per- and Polyfluorinated Alkyl Substances in Drinking Water by Solid Phase Extraction and Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS). U.S. Environmental Protection Agency, Washington, DC. https://cfpub.epa.gov/si/si_public_record_Report.cfm?dirEntryId=343042&Lab=NERL

## Featured Products

| Description | Cat. No. |
|---|---|
| **Syringe Filters** | |
| Millex-GP Syringe Filter, PES 0.22µm | **SLGP033N** |
| Millex-HP Syringe Filter, PES 0.45µm | **SLHP033N** |
| Millex Syringe Filter, Nylon, 0.20µm | **SLGN033** |
| Millex Syringe Filter, Nylon, HPF, 0.20µm | **SLGNM25** |

## Related Products

| Description | Cat. No. |
|---|---|
| **SPE - Supelclean™** | |
| ENVI-Chrom P 6mL/500 mg SPE Tube | **57226** |
| ENVI-WAX™ SPE Tube, 200 mg/6 mL, Pk.30 | **54056-U** |
| ENVI-WAX™ SPE Tube, 500 mg/6 mL, Pk.30 | **54057-U** |
| **HPLC column** | |
| Ascentis® Express PFAS 10 cm x 2.1mm ID, 2.7 µm | **53559-U** |
| Ascentis® Express PFAS Delay column 5 cm × 3.0 mm | **53572-U** |
| **Solvents & Reagents** | |
| Methanol for chromatography (LC-MS grade) LiChrosolv® | **1.06035** |
| Water for chromatography (LC-MS grade) LiChrosolv® or ultrapure water from a Milli-Q® IQ 7 series water purification system | **1.15333** |
| Acetic Acid for HPLC LiChropur™ | **5.43808** |

See more information, also on the results with the nylon filters, at **SigmaAldrich.com/pfassamplefiltration**

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

ENVIRONMENTAL

# LC-MS Analysis of PFAS Compounds in EPA Methods 537.1, 533 and 8327

## *Ascentis® Express PFAS HPLC Columns*

Petra Lewits, Product Manager Analytical Chromatography, HPLC Column; Cory Muraco, Product Manager, Liquid Chromatography Technology; Analytix@milliporesigma.com

PFAS (Per- and poly-fluoroalkyl substances) are persistent, man-made organic compounds, widely found in the environment. Recent awareness has brought attention to the toxicity of these substances.

The EPA has developed, validated, and published three methods to support the analysis of 29 PFAS in drinking water, Method 533, 537 and 537.1. EPA 8327 covers the analysis of selected per- and polyfluoroalkyl substances (PFAS) in prepared extracts of various matrices (e.g., waters and solids) by liquid chromatography/tandem mass spectrometry (LC-MS/MS) analysis.

The Ascentis® Express PFAS HPLC column is designed for the separation of novel and legacy short chain and long chain PFAS compounds containing branched and linear isomers, whilst adhering to EPA methodology requirements.

Furthermore, a specific PFAS delay column prevents background PFAS contamination from interfering with the sample results in quantitative LC-MS methods.

The selectivity data for the different EPA method compounds under the conditions outlined in **Table 1** is displayed in **Figure 1** and **Table 2**.

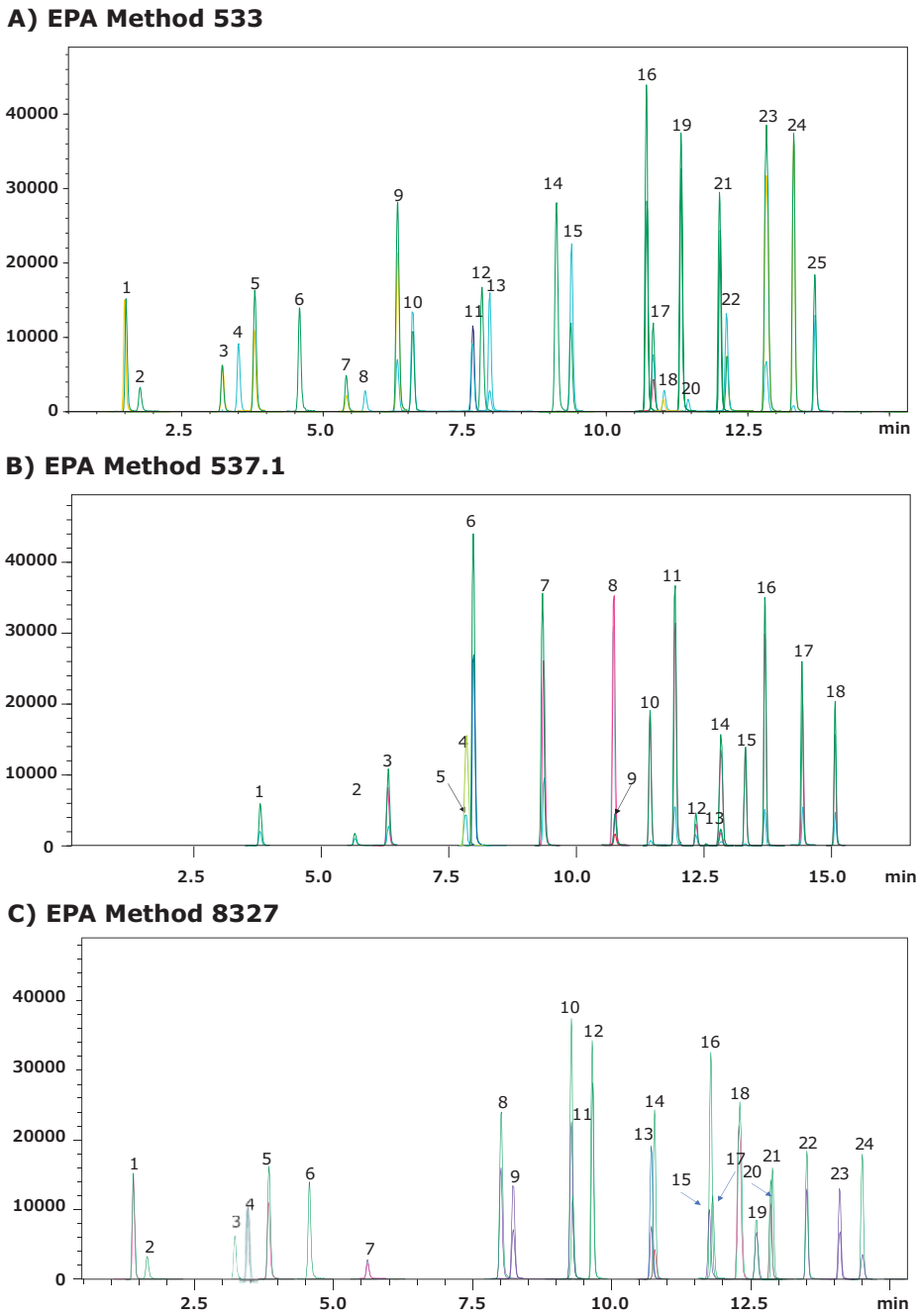


**Figure 1**. Analysis of PFAS compounds with Ascentis Express PFAS column and PFAS Delay column under A) EPA Method 533 conditions; B) EPA Method 537.1 conditions and C) EPA Method 8327 conditions (Peak IDs in **Table 2**).

**Table 1**. LC-MS Conditions

| LC Conditions | |
|---|---|
| **Analytical Column:** | Ascentis® Express PFAS, 100 x 2.1 mm I.D., 2.7 µm (**53559-U**) |
| **Delay Column:** | Ascentis® Express PFAS Delay, 50 x 3 mm I.D., 2.7 µm (**53572-U**) |
| **Mobile Phase A:** | [A] 10 mM Ammonium acetate; [B] methanol |
| **Gradient:** | |

| Time | %B |
|---|---|
| 0.0 | 33.0 |
| 18.0 | 98.0 |
| 18.1 | 100.0 |
| 21.0 | 100.0 |
| 21.1 | 33.0 |
| 26.0 | End |

| | |
|---|---|
| **Flow Rate:** | 0.4 mL/min |
| **Pressure:** | 485 bar |
| **Temperature:** | 35 °C |
| **Injection Volume:** | 2.0 µL |
| **Sample Solvent:** | Methanol (96%), water (4%) |
| **MS Conditions:** | |
| **Detection:** | ESI(-) MS/MS |
| **LC System:** | Shimadzu Nexera X2 |
| **ESI LCMS system:** | Shimadzu LCMS-8040 |
| **Spray Voltage:** | -2.0 kV |
| **Nebulizing gas:** | 2 L/min |
| **Drying gas:** | 15 L/min |
| **DL temp:** | 250 °C |

**Table 2.** MS transition and retention data

| | | EPA 533 | | EPA 537.1 | | EPA 8327 | |
|---|---|---|---|---|---|---|---|
| **Compound** | **Transition** | **Peak #** | **$t_R$ (min)** | **Peak #** | **$t_R$ (min)** | **Peak #** | **$t_R$ (min)** |
| PFBA | 213.0000>169.0000 | 1 | 1.36 | | | 1 | 1.36 |
| 4:2FTS | 229.0000>85.0000 | 2 | 1.89 | | | 2 | 1.89 |
| PFPeA | 263.0000>219.0000 | 3 | 3.22 | | | 3 | 3.22 |
| PFBS | 299.0000>80.0000 | 4 | 3.81 | 1 | 3.789 | 4 | 3.81 |
| PFHpS | 279.0000>85.0000 | 5 | 3.97 | | | 5 | 3.97 |
| PFPeS | 315.0000>135.0000 | 6 | 4.79 | | | 6 | 4.79 |
| PFMPA | 327.0000>307.0000 | 7 | 5.43 | | | | |
| PFHxA | 313.0000>269.0000 | 8 | 5.68 | 2 | 5.639 | 7 | 5.68 |
| PFEESA | 349.0000>80.0000 | 9 | 6.10 | | | | |
| HFPO-DA | 285.0000>169.0000 | 10 | 6.34 | 3 | 6.307 | | |
| PFHpA | 363.0000>319.0000 | 11 | 7.76 | 4 | 7.723 | 8 | 7.76 |
| PFHxS | 399.0000>80.0000 | 12 | 7.99 | 5 | 7.936 | 9 | 7.99 |
| ADONA | 377.0000>250.9000 | 13 | 8.01 | 6 | 7.978 | | |
| FOSA | 427.0000>407.0000 | | | | | 10 | 9.30 |
| PFOA | 413.0000>369.0000 | 14 | 9.40 | 7 | 9.368 | 11 | 9.40 |
| PFMBA | 449.0000>80.0000 | 15 | 9.51 | | | | |
| PFDS | 295.0000>201.0000 | | | | | 12 | 9.70 |
| PFNA | 463.0000>419.0000 | 16 | 10.75 | 8 | 10.715 | 13 | 10.75 |
| PFOS | 499.0000>80.0000 | 17 | 10.79 | 9 | 10.762 | 14 | 10.79 |
| 9Cl-PF3ONS | 530.9000>351.0000 | 18 | 11.46 | 10 | 11.439 | | |
| PFNS | 527.0000>507.0000 | | | | | 15 | 11.84 |
| PFDA | 513.0000>469.0000 | 19 | 11.89 | 11 | 11.857 | 16 | 11.89 |
| 8:2FTS | 549.0000>80.0000 | 20 | 11.90 | | | 17 | 11.90 |
| N-MeFOSAA | 570.0000>419.0000 | | | 12 | 12.336 | 18 | 12.37 |
| 6:2FTS | 498.0000>78.0000 | 21 | 12.68 | | | 19 | 12.68 |
| NFDHA | 599.0000>80.0000 | 22 | 12.85 | | | | |
| PFUnA | 563.0000>519.0000 | 23 | 12.86 | 13 | 12.822 | 20 | 12.86 |
| N-EtFOSAA | 584.0000>419.0000 | | | 14 | 12.827 | 21 | 12.87 |
| 11Cl-PF3OUdS | 630.7000>451.0000 | 24 | 13.33 | 15 | 13.311 | | |
| PFDoA | 613.0000>569.0000 | 25 | 13.71 | 16 | 13.690 | 22 | 13.71 |
| PFTrDA | 663.0000>619.0000 | | | 17 | 14.435 | 23 | 14.45 |
| PFTeDA | 713.0000>669.0000 | | | 18 | 15.083 | 24 | 15.10 |

## Featured Products

| Product list | Cat. No. |
|---|---|
| Ascentis® Express PFAS, 100 x 2.1 mm I.D., 2.7 µm | **53559-U** |
| Ascentis® Express PFAS Delay, 50 x 3 mm I.D., 2.7 µm | **53572-U** |
| Methanol for chromatography (LC-MS grade) LiChrosolv® | **1.06035** |
| Water for chromatography (LC-MS grade) LiChrosolv® or ultrapure water from a Milli-Q® IQ 7 series water purification system | **1.15333** |
| Ammonium acetate suitable for mass spectrometry (MS), LiChropur™, eluent additive for LC-MS | **73594** |

Learn more about PFAS analysis at **SigmaAldrich.com/PFAS**

See also our reference materials under organic pollutants at **SigmaAldrich.com/standards**

FOOD & BEVERAGE

# Navigating Disinfection Control in Food and Beverage Manufacturing

Saskia Neubacher, Product Manager, Point of Use; Analytix@milliporesigma.com

Cleaning and disinfection is important in the production environment in beverage processing

## An Overview of Disinfection Control

Disinfection is the process of cleaning using compounds that either eliminate bacteria and other disease-causing organisms or reduce them to levels not harmful to health. Disinfection control is a key process in food manufacturing and comprises of several steps: ensuring the correct concentrations of disinfecting reagents are used, checking that disinfection has been completed sufficiently, and testing that it has been accomplished without leaving any contaminating residues. Disinfection control makes use of the chemical reactions caused by disinfectants to determine their absence or presence, or more precisely their concentration.

It is important to note that, in contrast, hygiene monitoring is a related and sometimes overlapping process that it is generally preventative. Hygiene monitoring also involves ensuring that cleaning procedures have been implemented adequately and that no contaminating residues remain, but additionally can involve allergen monitoring and prevention of microbial growth.

Both of these processes are critical in food and beverage industry where preventing food-borne illnesses is a key objective in maintaining safe manufacturing. Here, we focus on disinfection control and how to navigate the numerous options in its methods and instrumentation.

## Considerations in Choosing Disinfectants

One of the primary concerns in selecting a disinfectant is to maintain the quality of the final manufactured product. As such, a disinfectant in food and beverage industry must not be toxic or leave toxic residues. It should also be fast-acting in order to minimize disruption to the manufacturing process. Additionally, it should not leave any odor or taste residue that could affect the end product. In order to facilitate the removal of any residues, it is for instance helpful if a disinfectant has good solubility so that it may be rinsed away after disinfection is complete.

The compatibility of the disinfectant with the equipment and methods used in manufacturing is also important. For example, it should work well on the type/material of the surface being disinfected without damaging the equipment. It should also be compatible in terms of other physical factors such as temperature and pH, remaining effective under the conditions it is used in.

Finally, the method of disinfection control required of a given disinfectant can also affect which disinfectant is chosen.

## Disinfection Control with Photometry

A number of photometric methods can be used to assess commonly-used disinfectants such as peroxide, peracetic acid, chlorine, quaternary ammonium compounds, ozone, and more. There are two options for instrumentation in these types of measurements: high-tech benchtop instruments like the Spectroquant® Prove spectrophotometer, or convenient mobile instruments like the Spectroquant® Move family of colorimeters.

### Benchtop Spectrophotometers

For disinfection control conducted in a laboratory setting, benchtop spectrophotometers like the Spectroquant® Prove can be used. One key advantage of these instruments is that they have a broad measuring range. The Spectroquant® Prove 600, for

example, can detect ultra low concentrations if that is required by a disinfection control method. They also use Live ID codes which automatically detect the test method, lot number, expiry date, and calibration updates to streamline analysis and documentation.

## On-the-Spot Analysis with Colorimeters

Often the convenience of portable instruments is important, especially when it would be most appropriate to conduct disinfection control in the field. Spectroquant® Move colorimeters are portable instruments that allow analysis directly at the sampling site.


Spectroquant® Move Colorimeter for disinfection control

These instruments are robust: they are waterproof according to IP68 standard and are therefore suitable for use in wet or dusty environments. They also give fast results and are easy to use. The Spectroquant® Move 100 is designed for use with over 100 pre-programmed parameters for versatility. The Spectroquant® Move DC, on the other hand, is designed specifically for disinfection control, and can measure five essential parameters: chlorine, ozone, chlorine dioxide, cyanuric acid, and pH.

## Test Strip Methods for Disinfection Control

An alternative to wet chemistry is using test strips. Both of the methods discussed here can be conducted on-site, and share this advantage with Spectroquant® Move Colorimeters. Additionally, test strip analysis circumvents the need for handling liquid chemicals or using glass vials in the field to enhance safety, which can be an important factor in choosing a disinfection control procedure.

## Reflectometry with Test Strips

Disinfection control using Reflectoquant® test strips and the RQflex® 20 reflectometer for read-out can be an attractive option because it can be done in the field, is easy to conduct, produces little waste, and yields quantitative results. An additional benefit is that tests are barcoded with batch-specific calibration information for accuracy and precision, and results are traceable. Test strips are available for 23 different parameters, and the portfolio includes compounds commonly investigated in the food and beverage industry.

RQflex® 20 reflectometer for disinfection control

## Did you know...

… that the FREE MQuant® StripScan smart phone app for reliable and consistent test strip readout and documentation got expanded to 10+ parameters, including Peracetic Acid and Peroxide?

**SigmaAldrich.com/mquant-stripscan**

## Test Strips as a Standalone Method

Visual tests using a color chart for comparison with test strips are also an option for semi-quantitative disinfection control. The MQuant® line of test strips can be used for this type of analysis and has the benefit of being the most economical of the methods discussed here. Test strips offer an additional advantage of not requiring any instrumentation, and consequently no electricity, to carry out analyses. MQuant® test strips are robust, easy to use, and safe.


MQuant® test strips for disinfection control

A smartphone app, MQuant® StripScan, is available to perform color comparison if desired in order to improve precision, as well as provide documentation of results and traceability.

## A Closer Look at Common Disinfectants

### Peracetic Acid

Peracetic acid is often used to disinfect drinking water bottles and surfaces in production. Its determination by titration is complex, with many steps requiring use of glassware. It is also time-consuming, taking about 15 minutes for each measurement.

MQuant® test strips, in contrast, can give a semi-quantitative result within a minute, thereby cutting down the analysis time significantly. For a quantitative and documented result, Reflectoquant® test strips can be used to get a result in just a few simple steps and comparably a short period of time.

### Hydrogen Peroxide

Another disinfectant frequently used in food and beverage manufacturing is peroxide. Tests for its levels are available in the Spectroquant® line as well as in for test strips both for use with the RQflex® 20 reflectometer and for the standalone MQuant® method. Several different concentration ranges are available for all of the methods.

### Ozone

Ozone is a powerful antioxidant and is also used as a disinfectant. Spectroquant® test kits can be used for a quantitative determination of ozone. Alternatively, MQuant® rapid liquid test kits with color cards or color disk comparators can also be used to determine ozone concentrations.

### Chlorine

Chlorine is used in the food and beverage industry for its ability to quickly eliminate bacteria and other microbes in water. It is used in potable water disinfection and washing vegetables, among other things. Spectroquant® kits can be used for quantitative chlorine testing, whereas MQuant® test strips and rapid liquid tests for semi-quantitative and fast results.

## Featured Products

| Description | Cat. No. |
|---|---|
| **Instruments** | |
| Spectroquant® Prove 100 VIS photometer | **173016** |
| Spectroquant® Prove 300 UV/VIS photometer 4 nm spectral bandwidth | **173017** |
| Spectroquant® Prove 600 UV/VIS photometer 1.8 nm spectral bandwidth | **173018** |
| Spectroquant® Move 100 colorimeter | **1.73632** |
| Spectroquant® Move DC colorimeter | **1.73635** |
| RQflex® 20 reflectometer | **1.17246** |
| **Spectroquant® tests** | |
| Chlorine Test (free chlorine) | **1.00598** |
| Chorine Test (total chlorine) | **1.00602** |
| Hydrogen Peroxide Test | **1.18789** |
| Hydrogen Peroxide Cell Test | **1.14731** |
| Ozone Test | **1.00607** |
| **Reflectoquant® Test Strips** | |
| Peracetic acid, 1-22.5 mg/L | **1.16975** |
| Peracetic acid, 20-100 mg/L | **1.17956** |
| Peracetic acid, 75-400 mg/L | **1.16976** |
| **MQuant® Test Strips** | |
| Chlorine Test (free chlorine) 0.5 - 10.0 mg/L | **1.16896** |
| Chlorine Test (free chlorine) 0.5 - 20 mg/L | **1.17925** |
| Chlorine Test (free chlorine) 25-500 mg/L | **1.17925** |
| Peroxide Test 0.2-20.0 mg/L | **1.16974** |
| Peroxide Test 20.0-100 mg/L | **1.17968** |
| Peracetic acid, 5-50 mg/L | **1.10084** |
| Peracetic acid, 20-160 mg/L | **1.17976** |
| Peracetic acid, 100-500 mg/L | **1.10001** |
| Peracetic acid, 500-2000 mg/L | **1.17922** |
| Peroxide, 0.5-25 mg/L | **1.10011** |
| Peroxide, 0.5-100 mg/L | **1.10081** |
| Peroxide, 100-1000 mg/L | **1.10337** |

View more resources on disinfection control at **SigmaAldrich.com/disinfectioncontrol**

# Titripac® Sustainable Packaging for Ready-to-Use Certipur® pH Buffers

Shailly Krishna Rajusth, Ingrid Hayenga, Product Managers Reference Materials; Analytix@milliporesigma.com

Ready-to-Use Certipur® pH Buffer solutions (4 L & 10 L) having pH values in the range of 1 to 12, are now also available in the sustainable Titripac® packaging format. This packaging ensures stability, reliability and high-quality buffer solutions - from the first to the last drop. This hermetically sealed packaging system effectively eliminates various contamination sources, such as air, carbon dioxide or even microorganisms during use. The outer carton can be compressed and simply be disposed together with other paper waste, and the internal liner bag can be easily folded together prior to its disposal, thereby reducing the waste volume (**Figure 1**).

Titripac® packaging is extremely easy-to-use. The integrated spout appears simply by pressing on the designated part of the pack. By opening the tap, the buffer solution can be withdrawn at any time – effortlessly and without the risk of contamination.

Titripac® packaging was awarded in 2016 with the Green Good Design Award due to its unique environmental benefits.


**Figure 1**. Compressed Titripac® components ready for efficient disposal.

## Titripac® Packaging Advantages

- Convenient to use – comes with integrated withdrawal tap (**Figure 2**)
- Minimized packaging waste (**Figure 3**)
- Reliable to the last drop: hermetically sealed packaging
- Stability assurance even after opening (**Figure 4**)
- For more detailed information on stability data for Titripac® packaging download the Technical Datasheet: Certified Reference Materials: Ready-to-use Certipur® buffer solutions in Titripac® packaging at **SigmaAldrich.com/physicalproperties**
- Technical datasheet now available on respective product description pages


**Figure 2**. Ready-to-Use Certipur® pH buffer solutions (4 L & 10 L).

Waste of 20 x 1 L solution in PE bottles = Waste of 2 x 10 L solution in Titripac® pack

**Figure 3**. Minimized packaging waste.

**Table 1**. Buffer solutions CRMs certified at 20 °C.

| pH | Packaging | Cat. No. |
|---|---|---|
| 2.00 | Titripac® 4 L | **1.09433.4000** |
| | Titripac® 10 L | **1.09433.9010** |
| 4.00 | Titripac® 4 L | **1.09435.4000** |
| | Titripac® 10 L | **1.09435.9010** |
| 4.00 (Red) | Titripac® 4 L | **1.09475.4000** |
| | Titripac® 10 L | **1.09475.9010** |
| 6.00 | Titripac® 4 L | **1.09437.4000** |
| 7.00 | Titripac® 4 L | **1.09439.4000** |
| | Titripac® 10 L | **1.09439.9010** |
| 7.00 (Green) | Titripac® 4 L | **1.09477.4000** |
| | Titripac® 10 L | **1.09477.9010** |
| 8.00 | Titripac® 4 L | **1.09460.4000** |
| 9.00 | Titripac® 4 L | **1.09461.4000** |
| | Titripac® 10 L | **1.09461.9010** |
| 9.00 (Blue) | Titripac® 4 L | **1.09476.4000** |
| | Titripac® 10 L | **1.09476.9010** |
| 10.00 | Titripac® 4 L | **1.09438.4000** |
| | Titripac® 10 L | **1.09438.9010** |
| 10.00 (yellow) | Titripac® 4 L | **1.09400.4000** |
| | Titripac® 10 L | **1.09400.9010** |

**Table 2**. Buffer solution CRMs certified at 25 °C.

| pH | Packaging | Cat. No. |
|---|---|---|
| 1.00 | Titripac® 4 L | **1.09441.4000** |
| 2.00 | Titripac® 4 L | **1.09442.4000** |
| 3.00 | Titripac® 4 L | **1.09444.4000** |
| 4.00 | Titripac® 4 L | **1.09445.4000** |
| 4.00 (Red) | Titripac® 4 L | **1.99054.4000** |
| 4.01 | Titripac® 4 L | **1.09406.4000** |
| 5.00 | Titripac® 4 L | **1.09446.4000** |
| 6.00 | Titripac® 4 L | **1.99036.4000** |
| 7.00 | Titripac® 4 L | **1.09407.4000** |
| 7.00 (Yellow) | Titripac® 4 L | **1.99057.4000** |
| 8.00 | Titripac® 4 L | **1.99038.4000** |
| 9.00 | Titripac® 4 L | **1.09408.4000** |
| 10.00 | Titripac® 4 L | **1.09409.4000** |
| 10.00 (Blue) | Titripac® 4 L | **1.99050.4000** |
| 11.00 | Titripac® 4 L | **1.99041.4000** |
| 12.00 | Titripac® 4 L | **1.99022.4000** |

## Stability of Buffer Solutions

The stability of Ready-to-Use Certipur® pH buffer solutions in Titripac® formats was tested under daily routine conditions. Every six months, the pH value was measured with a combined glass electrode after 5-point-calibration according to DIN 19268 with reference buffer solutions according to DIN 19266, IUPAC, NIST, Ph.Eur. and USP.

The following example stability graphs (**Figure 4**) display the measured pH values of the pH 4.00 buffer solutions in the available Titripac® 4 L & 10 L volumes over their shelf life. See the above mentioned Technical Datasheet for a comprehensive overview.

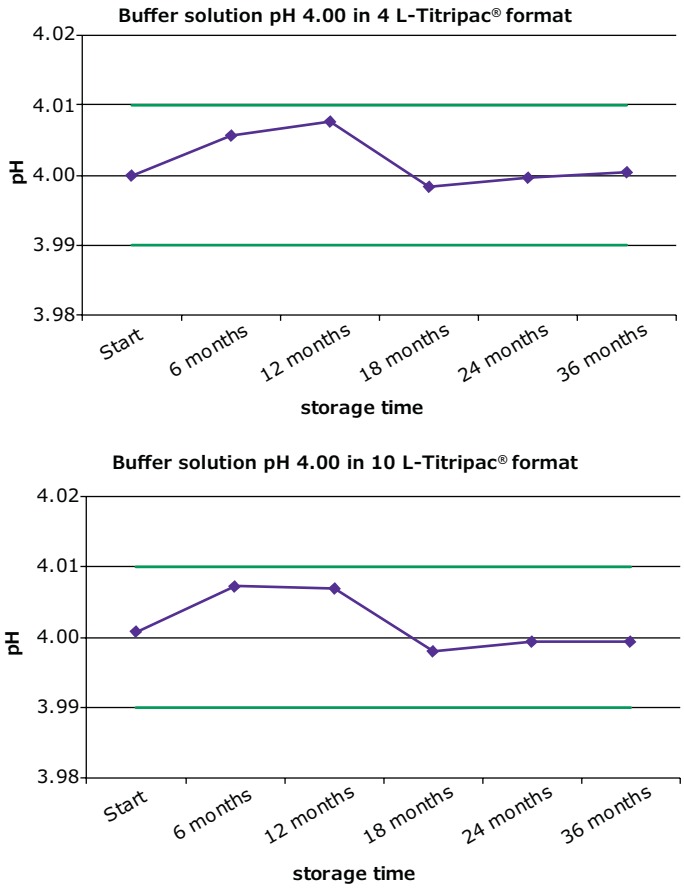


**Figure 4**. Stability graphs for storage up to 36 months for pH 4.00

For more information and to download the technical data sheet with the full stability data set, visit our website **SigmaAldrich.com/physicalproperties**

# The Carbon Enigma: Material Fundamentals and Retention Properties for Porous Graphitic Carbon (PGC) Stationary Phases

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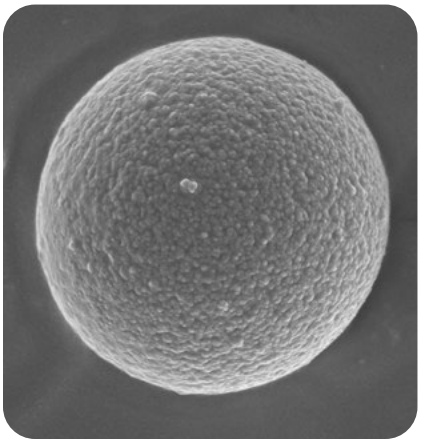
## Introduction



Despite its uniqueness compared to more conventional liquid chromatography (LC) stationary phases, Porous Graphitic Carbon (PGC) has been around since the 1980's. During that time, the goal was to generate a material that had the advantages of standard Reversed-Phase (RP) phases based on silica supports while eliminating some of the negative attributes, such as limited pH range, limited temperature stability, and secondary interactions from active surface silanols. What the researchers discovered was while there is some overlap with standard RP phases, some degree of graphitization gives the material other distinctive properties. Due to some of these characteristics specific to graphites, PGC has situated itself as more of a specialty phase for more challenging separations when other more traditional options do not work.

## Brief History Lesson

The first published papers on the topic of PGC date back to 1982 where Gilbert et al demonstrated the phase's usefulness in both Gas Chromatography (GC) and Liquid Chromatography (LC).[1] At the time, this material was referred to as Porous Glassy Carbon. Although the terminology is somewhat different, it is indeed very similar, and somewhat appropriate terminology, to describe the commercial PGC materials used in HPLC columns today. From here, the processing of this material was studied and optimized further by Professor John Knox and others at the University of Edinburgh,[2] leading to a material that was suitable for a packing in HPLC columns. Since then, another PGC stationary phase material is available, Supel™ Carbon LC, which, albeit, has many similarities to pre-existing PGC phases, but has some differences as well.

## Particle Morphology

In order to aid in understanding how retention is governed on a PGC stationary phase for liquid chromatography, some general prerequisite information about the material is needed. Graphite is one of the many allotropes of carbon. Graphite consists of stacked planes of six-membered carbon rings with each carbon atom arranged in a hexagonal or honeycomb-like lattice within each individual plane. The individual planes are referred to as graphene while graphite is essentially stacked layers of graphene planes (**Figure 1**).

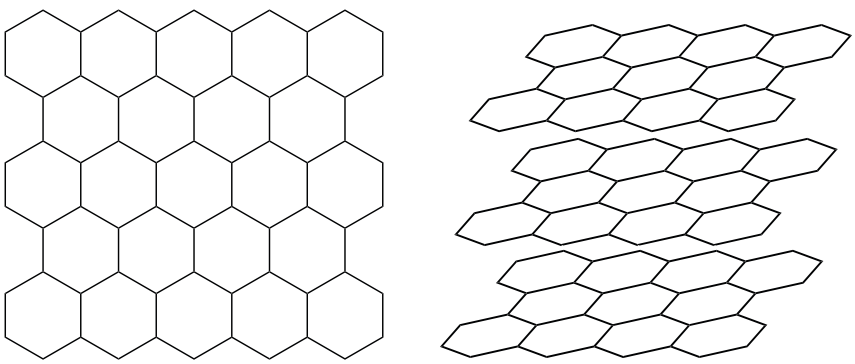


**Figure 1**. (Left) Hexagonal arrangement on a single plane of graphite. (Right) Stacked layers of graphene forming graphite

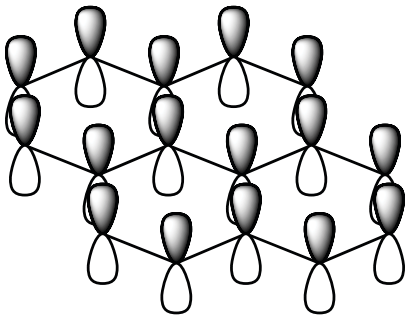


**Figure 2**. p-Orbitals on each carbon atom results in $sp^2$ hybridization.

Within a plane, each carbon atom is covalently bonded to three adjacent carbon atoms, but since carbon has four valence electrons available for bonding, the remaining valence electron is transposed perpendicular above or below the plane in p-orbitals (**Figure 2**). Since every carbon atom in the plane has a delocalized electron in its p-orbital, this overlap results in $sp^2$ orbital hybridization where all the delocalized electrons are free to move in the plane resulting in a continuous electron cloud called a pi-orbital. This phenomenon is sometimes referred to as the pi-cloud of graphite.

The layers are held together by weak van-der-Waals forces and, depending on the layer registration, the graphite can be considered two-dimensional or three-dimensional. Three-dimensional graphite has an ordered layer registration such as ABAB or ABCABC while two-dimensional graphite does not have specific ordering and the planes do not have perfect spacing and alignment with each other within the stacks.[2] This poor arrangement between layers is often described as turbostratic. While three-dimensional graphite exists, the PGC particles developed for chromatographic purposes have all been two-dimensional graphites as consequence for how the material is produced. The original term "Glassy" carbon which was later changed to "Graphitic" Carbon is worth noting because typically glassy carbons are produced from the pyrolysis of phenolic resins or similar resins under extreme heating, sometimes above 3000 °C which is not too dissimilar to current commercialized PGC phases. However, glassy carbons are non-graphitizable materials and no amount of heating will cause any degree of crystalline graphite formation. PGC, on the other hand, has some degree of crystalline graphite. When PGC is generated from an amorphous carbon template, extreme heating, temperatures above 2000 °C, result in carbon atom rearrangement to occur, forming graphitic layers in the form of ribbons which randomly become intertwined.[2] This weaving of graphitic ribbons is a primary distinction between a typical graphitic carbon black (GCB) and PGC, and lends itself to why PGC has the durability to be used in HPLC whereas GCB's do not. When magnified for perspective, as can be seen in the example illustration in **Figure 3**, the top surface of PGC is not completely flat but consists of sections of exposed graphitic ribbons that eventually end and meet adjacent graphitic planes that are part of the same or a different intertwined graphitic ribbon. To put it simply, the surface can be visualized as having flat sections and edges where flat sections converge against one another.

It has been hypothesized that the edge planes may play a role in PGC's chromatographic behavior since there is potential, unfilled valency on the edge carbons. Previous work downplays the importance of functional groups on the edge planes.[1,2,3] However, one study theorized that edge planes may have a more critical role than initially thought and played a part in oxidation of the stationary phase which resulted in altered retention characteristics.[4] Another theory is that the electron distribution on the graphitic plane favors localization near the extremities or edges resulting in a partial positive or neutral center and a progressively increasing negative charge towards the outermost edges resulting in analytes interacting in specific regions on the graphitic surface based on properties such as formal charge.[5,6] There are still unanswered questions in this respect, but further modeling and characterization is needed to enhance the current prevailing theories.


**Figure 3**. **A)** Zoomed in illustration of the different graphitic planes on the top surface of PGC. **B)** The intertwined graphitic ribbons that give PGC particles its robust strength. **C)** A cut section of a graphitic ribbon exposing multiple layers.

## Retention Properties

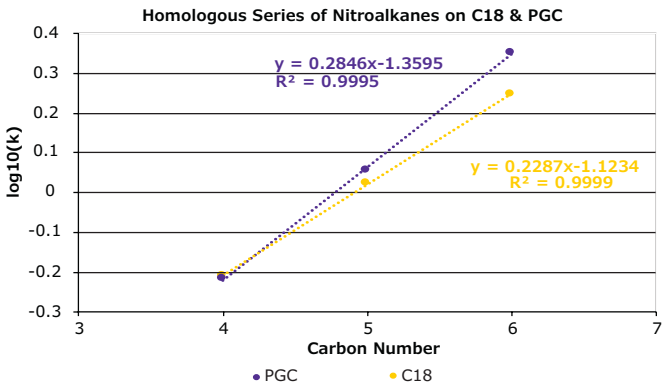
Many theoretical studies have been done to better understand the interactions that take place on a PGC surface and drives analyte retention in liquid chromatography. Unfortunately, most of the studies use different mobile phase setups with a wide variety of buffers and additives, thus further complicating the situation. This attribute becomes especially challenging because some additives that are commonly used with PGC adsorb strongly to the PGC surface, altering its chromatographic behavior. Nevertheless, these fundamental studies have helped reach some logical conclusions. To briefly summarize, as it is understood thus far, retention is governed by multiple competing factors:

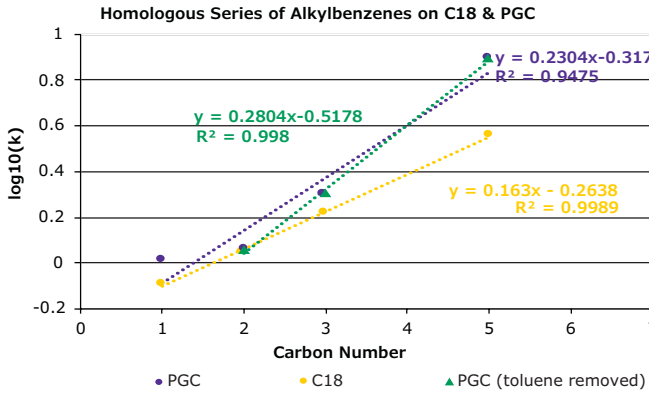
1. Dispersive Interactions – Similar in behavior to that seen in reversed phase chromatography and heavily driven by mobile phase strength (organic/aqueous ratio) and analyte properties.[7-10]
   a. Solvent/Solute interactions that form in a solution and can either be dipole-dipole or H-bonding or hydrophobic-hydrophilic repulsion interactions
   b. Solvent & solute dispersive forces (London) with graphite.
2. Electronic Interactions between polarizable groups of the analyte and the surface of graphite (often referred to as PREG – polar retention effect on graphite).[2,7,8,11] Not as well understood but is thought to stem from some form of charge induction or electron lone pair donating/accepting interactions with the pi cloud of graphite. This effect can be significant if there is pi-pi overlap due to unsaturation or aromaticity of the analyte. Likewise, the stereochemistry of the analyte plays an important role and the location of the polar functional groups with respect to the graphitic plane.
3. Retention is heavily influenced by the analyte size and shape whereby increased surface area contact of the analyte with the surface of graphite results in longer retention times than minimal surface area contact. This trait becomes especially noticeable with aromatic compounds (pi-pi overlap seems to be especially important with aromatics).

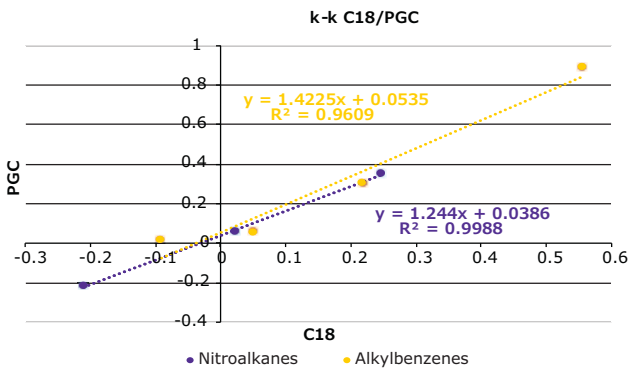
### Interaction with Hydrophobic Analytes

In theory, a surface comprising of all carbon atoms should be very hydrophobic and will behave like a long alkyl-chain, reversed-phase support, but this is not always the case for PGC. We examined a set of substituted alkyl benzenes and nitroalkanes on both a C18 column and PGC column (**Figure 4** & **Table 1**). In general, the compounds behave similarly but there are some differences as well.

The C18 column and the PGC column perform similar, and the k-k plot confirms that the relationship between



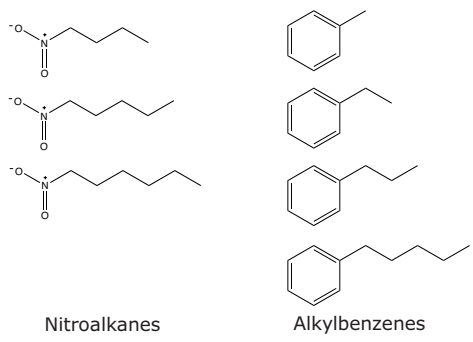




**Figure 4**. Hydrophobicity comparison between PGC and C18 with a set of nitroalkanes and alkylbenzenes. (Top) A series of nitroalkanes & alkyl benzenes log(k) data plotted against its substituent's carbon number on a PGC and C18 column. (Bottom Left) k-k Plot of log(k) data on both PGC & C18 which displays strong correlation ($R^2$ = 0.96). (Bottom Right) Analyte structures (see **Table 1** for conditions).

**Table 1.** Chromatographic Conditions.

| | |
|---|---|
| **Column:** | Ascentis® Express C18 50 x 3 mm I.D., 2.7 µm (**53811-U**); Supel™ Carbon LC 50 x 3 mm I.D., 2.7 µm (**59991-U**) |
| **Mobile phase:** | [A] Acetonitrile; [B] Water; (80/20 A/B for alkylbenzenes, 60/40 A/B for nitroalkanes) |
| **Flowrate:** | 0.5 mL/min |
| **Column Temp.:** | 25 °C |
| **Detector:** | UV, 190 nm & 210 nm |
| **Injection:** | 1.0 µL |
| **Sample:** | Alkylbenzenes – Toluene, Ethylbenzene, Propylbenzene, Pentylbenzene, 100 µg/mL ea. in mobile phase; Nitroalkanes – 1-Nitrobutane, 1-Nitropentane, 1-Nitrohexane 100 µg/mL ea. in mobile phase; Urea ($t_0$ marker) 1000 µg/mL in mobile phase |

these two phases across the two compound sets is quite linear. The homologous series plot shows that the PGC column has a slightly steeper slope indicating that it is more selective towards each methylene addition compared to the C18 column. Interestingly, for the alkylbenzenes the methylene addition from C1 (toluene) to C2 (ethyl benzene) does not follow the same slope pattern as the rest of data set. The reasoning for this outcome may be related to toluene's aromatic shape and the fact that ethyl benzene has free rotation and loses some molecular surface area that can contact the stationary phase surface.

### Interaction with Hydrophilic Analytes

PGC is known for its ability to retain more polar, hydrophilic analytes that typically are unretained on a standard, reversed-phase column such as C18. However, there is some confusion as to which type of compounds will and will not retain. We examined two sets of polar analytes.

As can be seen from the chromatographic results in **Figure 5** (conditions in **Table 2**), the C18 column is only able to retain acetone. However, on the PGC column, all the analytes in set two are retained and only acetone is slightly retained from set one. For the C18 column, it makes sense that it cannot retain these analytes. The compounds are very hydrophilic with negative log P values hydrophilic and the interaction of the octadecyl ligand is not strong enough to break the even stronger dipole-dipole interactions that these polar analytes are involved in with the bulk mobile phase (90% water / 10% acetonitrile). Thus, the analytes stay in solution and no retention is observed.

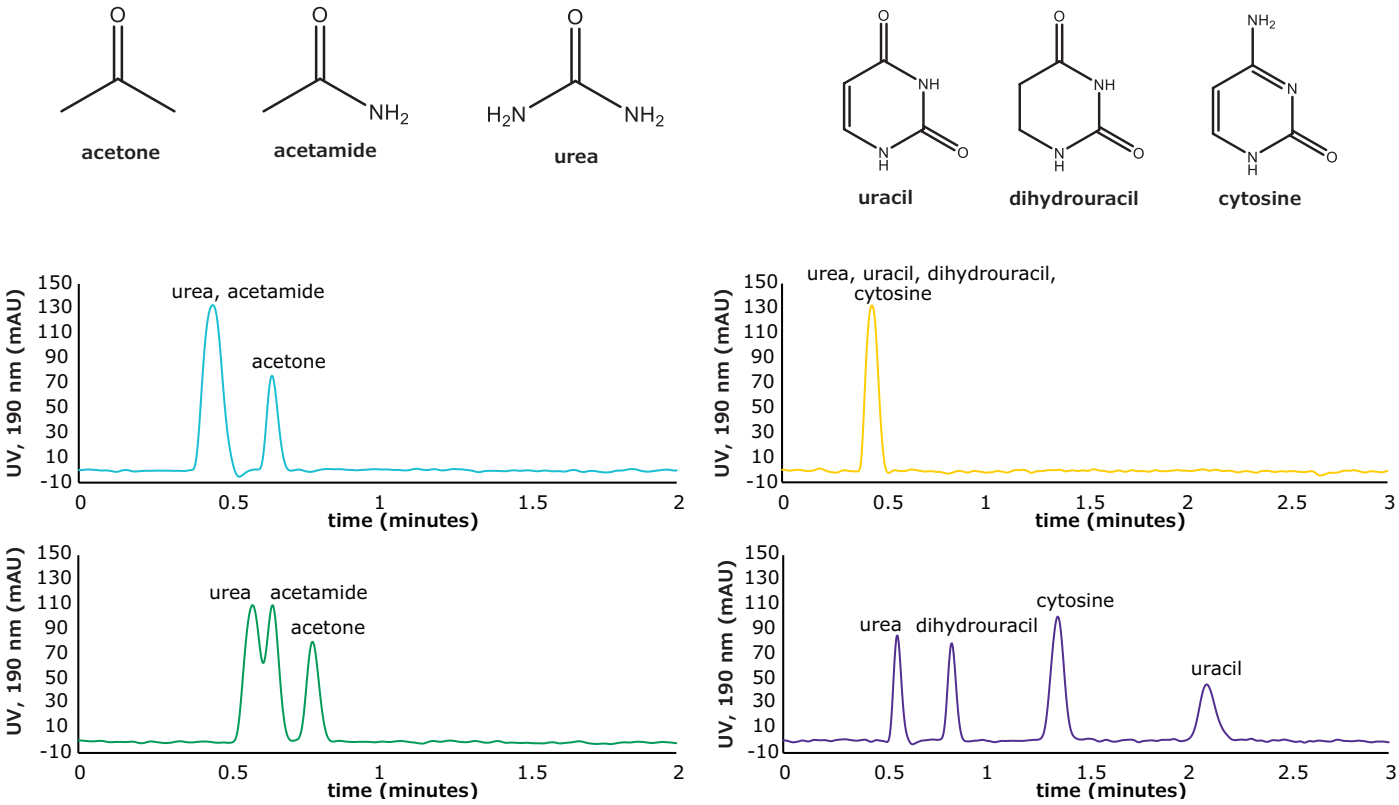



**Figure 5**. (Top) Set 1 & 2 analyte structures, (Middle) Set 1 & 2 chromatograms on C18, (Bottom) Set 1 & 2 chromatograms on PGC. In this case, the C18 column cannot retain any of the analytes except acetone. Conversely, the PGC column is able to retain cytosine and uracil from set 2, but no or poor retention of set 1 analytes is observed similar to the C18 performance (see **Table 2** for conditions).

**Table 2.** Chromatogarphic conditions

| | |
|---|---|
| **Column:** | Ascentis® Express C18 50 x 3 mm I.D., 2.7 µm (**53811-U**); Supel™ Carbon LC 50 x 3 mm I.D., 2.7 µm (**59991-U**) |
| **Mobile phase:** | [A] Acetonitrile; [B] Water; (10/90 A/B) |
| **Flowrate:** | 0.5 mL/min |
| **Column Temp.:** | 25 °C |
| **Detector:** | UV, 190 nm & 210 nm |
| **Injection:** | 1.0 µL |
| **Sample:** | Set 1 – Urea, Acetamide, Acetone, 100 µg/mL ea. in mobile phase; Set 2 – Urea, Dihydrouracil, Cytosine, Uracil, 100 µg/mL ea. in mobile phase |

The analyte interactions with the PGC column are completely different. For set one, it seems that for small, polar analytes, poor retention is observed similar to the C18 phase. All three analytes are planar, have pi electrons due to the carbonyl group, and an uneven charge distribution. These are good analyte criteria for retention on PGC. While this is all true, it seems that, for very small polar molecules, the uneven charge distribution is either not inducing the graphitic surface, or its contribution is not noticed because it is superseded by the mobile phases' proclivity to keep the solutes in solution. Additionally, with small, polar molecules, there is not enough molecular surface area to interact with the graphitic plane when compared to a much larger polar molecule.

Contrast that with the set two compounds, the polar, cyclic compounds, which can be retained. Uracil is a common $t_0$ marker in reversed-phase chromatography, but this cannot be used as such on PGC-based materials. Cytosine, which is even more hydrophilic than uracil is also retained. The most compelling detail about the elution order is the significantly earlier elution of dihydrouracil than uracil.

### The Importance of Analyte Stereochemistry

It has been well documented over the years that PGC exhibits unique shape selectivity for compounds based on their stereochemistry. Even some of the prior qualitative examples shown gleans of some aspects on this phenomenon. Why did the slope for the alkylbenzenes series improve in linearity when toluene was removed from the data set? Likewise, why does dihydrouracil show significantly less retention? Examining the latter option, uracil has a completely planar structure, but losing the double bond in the ring the molecule having to have some free rotation and dihydrouracil to be slightly bent. As a result, dihydrouracil is non-planar and cannot position as much surface area onto the graphitic plane compared to uracil (**Figure 6**). This aspect is an important factor that must be considered when using PGC as a stationary phase as analyte stereochemistry is a driving factor as to why PGC can retain, cannot retain, or discriminate similar compounds.

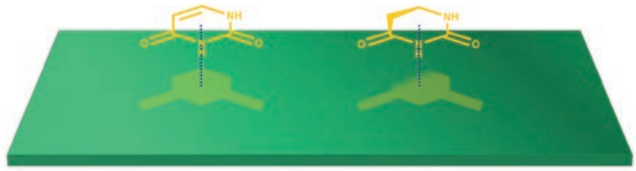


**Figure 6**. An illustration of how analyte shape affects how much maximal molecular surface area contact happens on the graphitic surface of PGC. Notice the larger shadow for the compound on the left versus the compound on the right, indicating more surface area interaction and stronger interaction with the stationary phase.

## Conclusions

Porous graphitic carbon is a unique stationary phase giving the chromatographer an additional chemistry option to separate challenging compounds beyond the realm of conventional reversed-phase chromatography. In many respects, the PGC column may behave like a reversed-phase column with enhanced temperature, and pH stability, but due to the special properties of graphite, polar compounds that may need HILIC or ion-exchange conditions can be retained as well. However, some aspects about the analyte's stereochemistry and potential surface area contact points need to be considered for there to be enough retention. Although the mechanisms are not fully understood, especially in regards to PREG, it is undeniable that PGC has unique retention properties towards polar compounds – especially planar molecules or analytes with double bond conjugation that can interact with the electron cloud of graphite. More research into these fundamental mechanisms will yield more accurate retention prediction models.

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|---|---|
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| Acetonitrile, suitable for HPLC, gradient grade, ≥99.9% | **34851** |
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| 1-Nitrohexane, 98% | **259497** |
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# HPLC Tips & Tricks: Optimizing Injection Volume

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The peak area has a linear relationship with injection volume/sample concentration, if the column and/or detector is not yet overloaded, and if the sample is dissolved in the mobile phase which flows through the column at the moment of injection (effects related to different sample diluents will be covered in one of the next HPLC tips & tricks). Commonly with sample load, we primarily associate the injection volume which, in HPLC, is usually determined by autosampler setting. In case of diluted samples, increasing sample volume can be considered as one option for improving detection limit/sensitivity. However, there are some effects related to increased injection volume and/or concentration in HPLC/UHPLC which take place: the column can be overloaded if too much sample is injected onto the column; the height of the peak will increase with a larger injection volume; the peak width will broaden with an increase in injection volume. The sum of previously mentioned effects would mean that increasing injection volume can result in a decrease of resolution between the separated compounds (in case of overloading). It is important to keep in mind that changing injection volume in general would affect the peak (height, volume and width) dependent on: the dimensions of the column (length and diameter), packing particle size and type (fully porous, superficially porous, or monolithic material as well as its surface area, functionalization density, chromatographic mode, and retention mechanism), and the peak retention (peak capacity value). Rule of thumb, for any chromatographic system change (shorter column, narrower column, smaller particle size, or shorter retention) that reduces the final volume of the peak eluting from the column, the volume of sample should be proportionally reduced to avoid loss of resolution. It is quite challenging to evaluate the sum of all these different parameters, therefore most often we turn to a more pragmatic approach and look only to column size and resolution minimum which is still acceptable. There is a generally accepted rule of thumb which recommends that the injection volume should be not more than 1%-2% of total column volume (considering a sample concentration of ~1 µg/µL, which is a standard value in analytical liquid chromatography); however, everyone could/should determine the actual maximal acceptable load by simply performing several different load injections and compare critical peak pair resolution or main peak chromatographic performance parameters. As an example, for the commonly used UHPLC column dimension of 50 x 2.1 mm which has 173 µL total volume, and a void volume of ~120 µL (considering a factor of 0.7 for a packing with fully porous particles) the injected volume should be in the range of 1.2 and 2.4 µL (when sample concentration is ~1 µg/µL), to limit band broadening and loss in resolution (few other examples: 4.6 mm I.D. and 50 to 250 mm length – ideal injection volume between 5.8 and 58 µL, 3 mm I.D. 50 to 150 mm length ideal injection volume between 2.5 and 14.8 µL. It is important to note that isocratic runs are much more prone to volume overloading effects than gradient methods. For isocratic runs, we can approximately calculate the recommended limit of injection volume by dividing peak retention volume (in µL, flow rate multiplied by peak elution time) by the square root of that peak efficiency (plates per column).

How might one see that the column is (volume) overloaded? When a very large volume of sample is injected into the HPLC column, the peaks begin to front more (peak symmetry factor < 1) and the retention time may decrease, resulting in a decline in column efficiency and separation resolution. The trick is to find the sweet spot, a balance between resolution and sensitivity (the sensitivity topic I already covered in issue 9 of this journal). Practically, it is easily done: start with the smallest volume that your injector can reproducibly inject and keep doubling it until max 3% of your column's volume is reached, then calculate limit of detection and resolution dependence on injection volume. Be prepared for the need to accept a compromise between detection limit and resolution.

In case you have any chromatography troubles, please let us know.

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