Application Note

Environmental

Analysis of Per- and Polyfluoroalkyl Substances (PFASs) in Biological Fluid Using a Novel Lipid Removing Sorbent and LC-MS/MS

Authors

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Abstract

Efficient sample preparation prior to LC-MS/MS analysis of per- and polyfluoroalkyl substances (PFASs) is an important consideration for environmental contamination research laboratories performing multiresidue analysis. Phospholipids (PPLs) have been identified as a major cause of matrix effects in the LC-MS/MS analysis of plasma samples. This Application Note describes plasma sample preparation and LC-MS/MS analysis of PFASs using in-well PPT followed by PPL removal using the Agilent Captiva EMR—Lipid cartridge. Captiva EMR—Lipid cartridges produced cleaner eluents, with removal of over 99 % of unwanted PPLs from the plasma matrix, and over 75 % recovery of target analytes, with RSDs <14 %. Analysis of PFASs at 5 ng/mL yielded ideal peak shapes with good signal-to-noise (S/N). Calibration curves for all PFASs from 0.1–50 ng/mL were linear, with an R^2 >0.992.

Introduction

Per- and polyfluoroalkyl substances (PFASs) are man-made compounds widely used as surfactants, fire-retardants, waterproofing, and nonstick and nonstain agents. Their unique properties also make them persistent and ubiquitous in the environment and in animals. Research suggests that PFASs can cause reproductive and developmental problems such as liver, kidney, and immune effects, tumors, and changes in cholesterol. When PFASs are ingested by drinking or eating, they are readily absorbed, but slowly cleared, and can accumulate in animal tissue. Studies have shown that PFASs with carbon chains longer than seven carry the most risk for bioaccumulation[1].

Efficient sample preparation prior to LC-MS/MS analysis of PFASs is an important consideration for multiresidue analysis. Sample preparation is used to reduce system contamination, improve data integrity and method selectivity, and to enhance analytical sensitivity. Two of the major interferences found in plasma are proteins and phospholipids (PPLs). PPLs have been identified as a major cause of matrix effects in LC-MS/MS bioanalyses due to competition for space on the surface of droplets formed during electrospray ionization (ESI)[2].

Common sample preparation techniques for plasma, serum, and whole blood in research laboratories include protein precipitation (PPT), solid phase extraction (SPE), liquid-liquid extraction (LLE), and supported liquid extraction (SLE). Each technique has advantages and disadvantages in terms of speed, cost, and quality of data generated. For example, PPT, LLE, and SLE do not remove PPLs, and SPE is more time-consuming and complicated to perform. However, of these techniques, PPT is most widely used. Using PPT, proteins are easily removed by adding an organic crash solvent such as ACN or MeOH into bio-fluid samples in a prescribed ratio. As the proteins are denatured, they form a precipitate that can be removed by filtration or centrifugation. PPLs are not removed by PPT because they are soluble in the organic crash solvent.

A sample preparation method that eliminates certain sample preparation steps, including off-line PPT, centrifugation, and dilution, while allowing streamlined in-well PPT and PPL removal, is highly desirable. This Application Note describes an approach that relies on Agilent Captiva EMR—Lipid to remove interferences, particularly PPLs, without analyte loss, in a simple pass-through format. The resulting extract is cleaner, reducing potential ion suppression, and column and mass spectrometer contamination.

Extraction of PFASs from plasma was performed using in-well PPT followed by PPL removal using a Captiva EMR—Lipid cartridge. Subsequent quantitative analysis was performed using an Agilent 6495 Triple Quadrupole LC/MS system. Efficiency of PPLs removal was evaluated. Method reproducibility and recovery for the PFASs evaluated were also determined.

Experimental

Reagents and Chemicals

Table 1 lists the PFASs analyzed. All standards and internal standards were purchased from Wellington Laboratories (Guelph, ON, Canada). LC-MS grade ammonium acetate was purchased from Sigma-Aldrich. All solvents were LC-MS grade or higher, and were obtained from Burdick and Jackson (Muskegon, MI, USA).

Table 1. PFASs and IS analyzed, with corresponding triple quadrupole MRM acquisition parameters.

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)	Retention time (min)
PFTrDA	663	618.7	8	3.16
PFDoA	613	268.7	20	2.92
PFDoA-$^{13}C_2$	614.9	570	5	2.92
N-EtFOSAA	584	526	20	3.40
10:2 FTA	577	463	6	2.51
N-MeFOSAA	570	511.8	24	3.23
N-MeFOSAA d_3	573	515	24	3.23
PFUdA	563	519	8	2.70
PFDA	513	468.6	8	2.48
PFDA	513	218.7	16	2.48
PFDA-$^{13}C_2$	514.9	469.9	5	2.48
PFOS	498.9	99	50	2.61
PFOS	498.9	80	50	2.61
PFOS-$^{13}C_4$	502.9	80	50	2.61
8:2 FTA	477	393	14	2.12
PFNA	462.9	418.9	5	2.25
PFNA	462.9	169	17	2.25
PFOA	412.9	368.9	5	2.00
PFOA-$^{13}C_4$	416.0	371.9	5	2.00
PFHxS	398.9	99	45	2.10
PFHxS-$^{13}C_3$	401.9	79.8	52	2.10
6:2 FTA	377	293	18	1.51
PFHpA	362.9	319	5	1.67
PFHxA	313	268.6	4	1.07
PFHxA-$^{13}C_2$	314.9	269.9	5	1.07
PFBS	298.9	98.9	29	1.15
PFPeA	263	218.7	0	0.63
PFBA	213	168.7	4	0.47
PFTeDA	712.9	668.5	8	3.33

Solutions

A combined standard working solution of PFASs was made at 10 ug/mL in methanol. The isotopically-labeled PFASs were combined in a working solution at 10 ug/mL in methanol, and used as internal standard (IS). All working solutions were stored in polypropylene vials with snap caps and polypropylene-lined septa to prevent the PFASs from sticking to the glass and to avoid contamination.

Calibration Standards and Quality Control Samples

Prespiked quality control (QC) samples were fortified with standard working solution to the appropriate concentrations in replicates of seven. The QC samples were low QC (LQC), middle QC (MQC), and high QC (HQC), corresponding to 1, 5, and 20 ng/mL in plasma, respectively. The IS was spiked at 10 ng/mL at each QC level.

Blank matrix after cleanup by Captiva EMR—Lipid was post-spiked with a corresponding working solution to yield 1, 10, and 20 ng/mL concentrations of PFASs. The IS was spiked to a final concentration of 10 ng/mL, in replicates of five.

Matrix-matched calibration curves were prepared with the standard working solution. Blank matrix after Captiva EMR—Lipid was post-spiked to correspond to 0.1, 1, 5, 10, 25, and 50 ng/mL in plasma. The IS was spiked at 10 ng/mL at each calibration level.

Equipment and Instrumentation

Table 2 provides the list of the equipment and instrumentation used to perform the analysis.

LC-MS/MS Analysis

An Agilent 1290 Infinity II LC System coupled to an Agilent 6495 Triple Quadrupole LC/MS system was used for the LC-MS/MS analysis. Tables 3 and 4 provide the LC and MS conditions. The sample extracts (4 µL) were directly injected into the LC system.

Table 1 provides the triple quadrupole dynamic multiple reaction monitoring (DMRM) acquisition parameters for each PFAS compound monitored. To evaluate PPL removal by Captiva EMR—Lipid, 11 major PPL compound precursor ions, and the product ion fragment at *m/z* 184 were monitored, as shown in Table 5.

Table 2. Equipment and instrumentation used for sample preparation and analysis.

Component	Part number
Sample Preparation	
Agilent Captiva EMR—Lipid, 1 mL cartridge	5190-1002
Agilent Vac Elut SPS 20 Manifold with collection rack for 13 × 100 mm test tubes	12234101
VWR 13 × 100 mm culture tubes 8 mL polypropylene	
Eppendorf pipettes and repeater pipettor (VWR, NJ, USA)	
Liquid Chromatography System	
Agilent 1290 Infinity II LC System	
Agilent ZORBAX Eclipse Plus 95Å C18, 4.6 × 50 mm, 3.5 µm (delay column)	959943-902
Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 50 mm, 2.7 µm	699775-902T
Agilent 1290 Infinity inline filter 0.3 µm	5067-6189
Crimp/snap-top polypropylene vials, 1.0 mL, 100/pk	5182-0567
Crimp/snap caps with polypropylene septa, 100/pk	5182-0542
Mass Spectrometry System	
Agilent 6495 Triple Quadrupole LC/MS system with iFunnel Technology	
Agilent MassHunter Software (Ver. 08.00)	

Table 3. LC conditions.

Parameter	Value
Flow rate	0.5 mL/min
Colum temperature	50 °C
Autosampler temperature	5 °C
Injection volume	4 µL
Mobile phase	A) 5 mM Ammonium acetate in water B) Acetonitrile
Needle wash: Multiwash	S1) H_2O S2) H_2O:ACN (50:50) S3) ACN 10 seconds each wash
Gradient	Time (min) %B 0.0 30 0.5 30 3.5 90 4.5 100
Stop time	5.0 minutes
Post time	1.5 minutes

Table 4. MS conditions.

Parameter	Value
Ionization mode	Negative ESI
Gas temperature	130 °C
Gas flow	15 L/min
Nebulizer	35 psi
Sheath gas heater	375 °C
Capillary voltage	2,000V
Vcharging	500
Delta electron multiplier voltage (EMV)	200
Polarity	Negative

Table 5. Triple quadrupole MRM acquisition parameters for PPLs.

Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)
808.4	184.4	30
806.4	184.4	30
786.4	184.4	30
784.4	184.4	30
760.4	184.4	30
758.4	184.4	30
704.4	184.4	30
524.4	184.4	30
522.4	184.4	30
520.4	184.4	30
496.4	184.4	30

Agilent MassHunter Software (Ver. 08.00) was used for instrument control, and for qualitative and quantitative data processing and analysis. Reproducibility and recovery of the method for PFASs were determined.

Sample Preparation Procedures

PFAS Extraction from Plasma

1. Add 400 µL of ACN (1 % FA) to the Captiva EMR—Lipid 1 mL cartridge.
2. Add 100 µL of spiked or blank human plasma, prespun.
3. Perform in-well mixing.
4. Pull a low vacuum of 2–4 psi for a controlled flow rate of 1 drop per 3–5 seconds.
5. Collect the extract in polypropylene test tubes.
6. Inject directly onto the LC-MS/MS system using polypropylene autosampler vials.

Because MeOH forms smaller precipitant particles than ACN, ACN is recommended to maximize PPT and avoid gelation prior to Captiva EMR—Lipid treatment. A ratio range of 1:3 to 1:5 (sample/solvent) is recommended. Plasma sample is added after the crash solvent. Acid (formic acid) helps break up proteins, and minimizes protein binding.

Preferably, active in-well mixing is done using wide-bore pipette tips. The vacuum initiates flow through the Captiva EMR—Lipid cartridge. A controlled flow rate of one drop per 3–5 seconds is recommended for optimal lipid removal. After sample elution off the cartridge, higher vacuum is applied to maximize sample recovery. Polypropylene collection tubes and autosampler vials are highly recommended to prevent PFAS loss due to sticking on glass surfaces.

PPL Removal Evaluation, PPT Only

1. Add 400 µL of ACN (1 % FA) to a test tube.
2. Add 100 µL of blank plasma, prespun.
3. Vortex on a Heidolph Multi Reax at 800–1,000 rpm for 5 minutes.
4. Centrifuge at 5,000 rpm for 5 minutes.
5. Pipette the supernatant into a polypropylene autosampler vial for LC-MS/MS analysis.

Results and Discussion

Unwanted Lipid Matrix Removal

The EMR—Lipid approach is simple and universally applicable to reducing matrix effects and improving analyte recoveries for the analysis of polar, midpolar, and nonpolar target analytes, in research laboratories. The EMR—Lipid sorbent selectively traps lipids by size exclusion and hydrophobic interaction (Figure 1). Unbranched hydrocarbon chains (lipids) enter the pores of the sorbent, but bulky analytes do not. Lipid chains that enter the sorbent are then trapped by hydrophobic interactions.

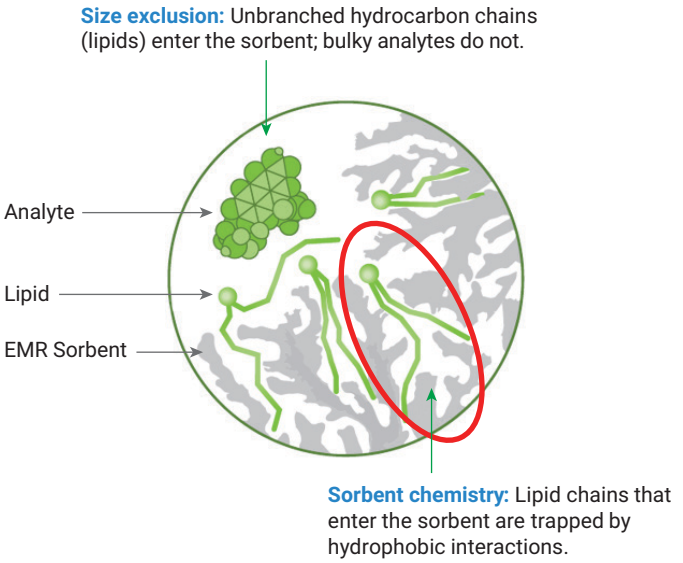

Figure 1. EMR—Lipid mechanism: size exclusion and hydrophobic interactions.

Though the PFAS structures shown in Figure 2 contain a long straight carbon chain, the carbon is attached to fluorine atoms, which are sterically larger than unbranched hydrocarbon chains. Therefore, they are sterically hindered from entering into the pores of EMR—Lipid sorbent.

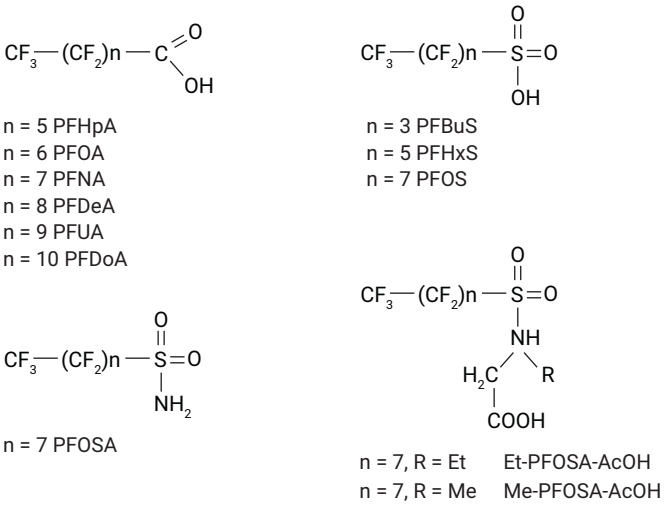

Figure 2. Molecular structures of PFASs.

Chromatographic Performance

The MRM chromatogram of plasma spiked at 5 ng/mL (Figure 3) shows the chromatographic performance that can be obtained using the EMR—Lipid protocol. Even at the 5 ng/mL level, ideal peak shapes due to reduced matrix effect and interferences resulted in good separation and signal-to-noise (S/N) for accurate integration. Using the 6495 Triple Quadrupole LC/MS system, accurate detection and quantification at levels of 0.1 ng/mL and lower can be achieved when performing analysis of PFASs in plasma.

PPL Removal

PPLs are the main constituents of cell membranes and the main class of compounds that cause significant matrix effect[3,4]. Glycerophophocholines and lysophosphatidylcholines represent 70 % and 10 % of the total plasma PPLs, respectively[5], and are the major source of matrix effects. To determine the efficiency of PPL removal from plasma using Captiva EMR—Lipid cartridge cleanup, 11 naturally occurring PPL compounds were monitored. Specifically, the ion fragment at *m/z* 184 was used to monitor the PPLs in plasma extract after PPT and Captiva EMR—Lipid removal.

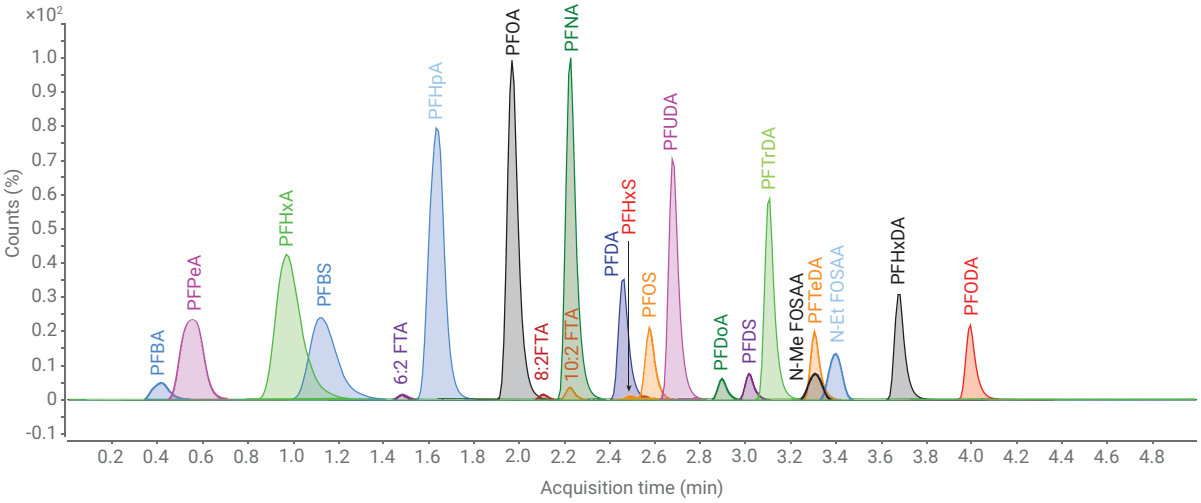

Figure 3. MRM chromatogram of plasma spiked at 5 ng/mL.

As shown in Figure 4, approximately 99 % (based on peak area comparison) of the PPLs monitored were eliminated from the extracted plasma samples compared to PPT alone, some of which would have coeluted with the target analytes. The high relative abundance of PPLs shown in Figure 4 (red trace) subjects the detector to potential saturation and could impact the quality of quantification. In addition, a high abundance of PPLs can contaminate an LC-MS system over time.

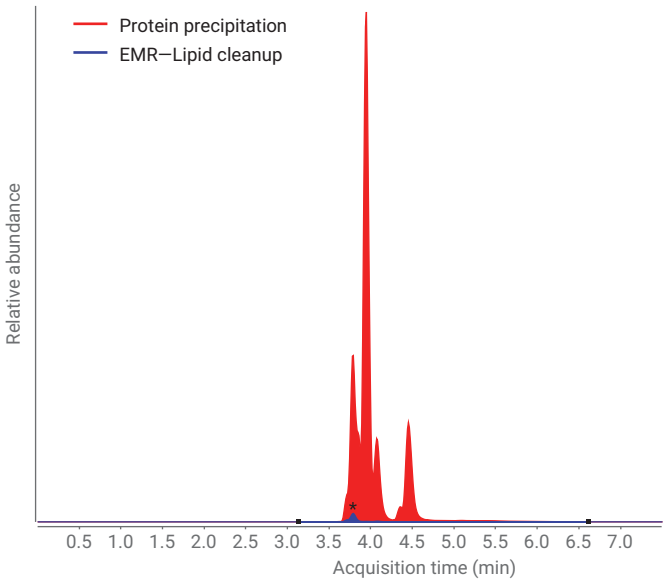

Figure 4. Overlay of MRM chromatograms of 11 PPLs monitored at *m/z* 184 after PPT only (red) and after Agilent Captiva EMR—Lipid cleanup (blue).

Quantitative Performance

Calibration curve linearity was evaluated. Figure 5 shows the calibration curves for PFOA and PFOS. Good linearity of response was observed at the six concentration levels tested (0.1 to 50 ng/mL) on the three separate occasions that they were generated. The average coefficient of determination (R^2) for all PFASs studied was greater than 0.992, with linearity from 0.1 to 50 ng/mL, linear fit, 1/x weighting.

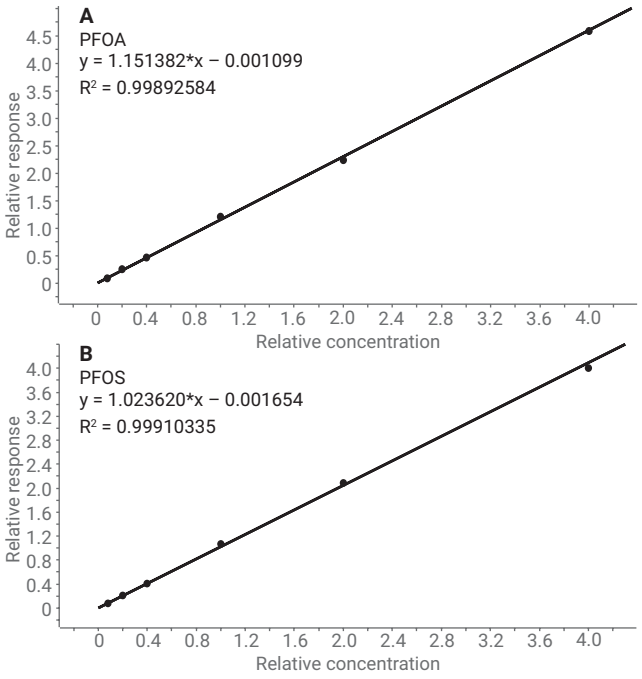

Figure 5. Calibration curves. A) PFOA, B) PFOS. Concentration range 0.1–50 ng/mL in plasma using protein precipitation followed with Agilent Captiva EMR—Lipid cleanup.

Method recovery and reproducibility (RSDs) for the 22 PFASs were determined by spiking the standard into plasma at 5 and 20 ng/mL in replicates of five. Overall recoveries were excellent and between 75 and 125 % (Figure 6). Most PFASs had recoveries of 90–110 %. The widely studied PFASs, PFOS and PFOA, had average recoveries of 92.7 ±6.6 % and 93.1 ±5.0 %, respectively, at both spiking levels in plasma. Relative standard deviations were acceptable, and ranged from 0.8 to 14 % at the 5 and 20 ng/mL levels.

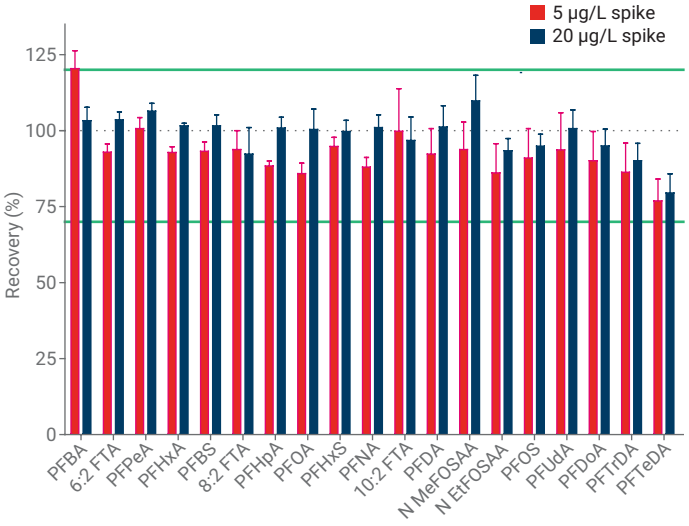

Figure 6. Recovery and RSD for the PFASs evaluated at 5 and 20 ng/mL.

Conclusion

This Application Note presents a simple and rapid workflow to prepare plasma samples for LC-MS/MS analysis of PFASs. Extraction of 22 PFASs from plasma was performed using in-well PPT followed by PPL removal using an Agilent Captiva EMR—Lipid cartridge in a pass-through format. Captiva EMR—Lipid efficiently removed 99 % of the unwanted PPLs from the plasma matrix, with excellent recovery of target analytes. The sample extract was cleaner than using PPT alone, thereby reducing the potential for ion suppression and LC-MS/MS system contamination and downtime. In-well PPT has the benefit of less sample handling and transfer.

Analysis of PFASs at 5 ng/mL yielded ideal chromatographic peak shapes and good S/N. Response for PFASs over six concentration levels (0.1–50 ng/mL) was linear, with an R^2 greater than 0.992. Recoveries were excellent at 75 % or higher, and RSDs less than 14 % for the PFASs tested. The results showed the method to be acceptable for multiresidue extraction and analysis of PFASs.

Captiva EMR—Lipid methodology can readily be incorporated into existing research laboratory workflows, and does not require additional sample preparation devices or glassware. In either 96-well plate or 1 mL cartridge formats, Captiva EMR—Lipid is compatible with automation, enabling high-throughput applications. The frit design provides easy and efficient elution of samples without clogging.

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