New Analytical Frontiers

The number of PFAS compounds found on current analyte lists represents less than 1 % of the potential environmental contaminants that could be contributed by this large class of compounds. However, researchers continue to identify additional PFAS compounds with potential human health and environmental impacts, thereby increasing the scope of the problem. Consequently, it is inevitable that PFAS analyte lists will continue to grow, and, future analytical challenges - sample preparation, chromatograpy and mass spectrometry - will become more complex and difficult to overcome. Therefore, we close this PFAS Guide with two visionary technical notes which propose new analytical approaches that will help meet the evolving PFAS challenge.

1. pH-Variable LC Mobile Phase Gradient

PFAS Analysis Based Upon a pH-Variable LC Mobile Phase Gradient

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Introduction

Polyfluoroalkyl substances (PFAS) have been an environmental concern ever since the 1970s when initial reports of potential adverse health effects first came to light. While the analysis of PFAS compounds has been ongoing for some time in academia, they are a fairly recent addition to the suite of analyses commonly performed by commercial environmental laboratories. The only official methods for the analysis of PFAS in drinking water are EPA 537/537.1 and EPA 533 and there are currently no official methods for the analysis of PFAS in complex environmental matrices such as Wastewater, Sediment, and Soil. Although ASTM has released methods for the analysis of PFAS in complex matrices (ASTM D7979 and D7968), they have not gained widespread use within the environmental testing community. As PFAS analyte lists continue to expand and matrices become ever more complex, we anticipate the need for a scalable analytical framework that will enable the development of analytical methods for a wider range of PFAS compounds and matrices. In this Technical Note we present such a framework, based upon the use of a variable pH mobile phase gradient, which could facilitate the expansion of PFAS analyte lists beyond those in common use today.

Method Limitations

Most PFAS methods in use today employ an ammonium acetate (NH_4OAc) mobile phase at a pH of 7 and with a concentration between 2 and 20 mM. Although EPA methods 537.1 and 533 both specify 20 mM NH_4OAc, EPA's method flexibility criteria allow for the use of alternative mobile phases (1, 2). This allowance is useful in pursuing potentially better eluent systems and allowing the analyst to run various PFAS methods on the same instrument using the same column and similar mobile phase. The benefit of changing the eluent system is the ability to change analyte selectivity and potentially analyte resolution. Selectivity differences can also be useful when trying to discriminate analytes from matrix interferences. However, the drawback to changing eluent systems is that it takes time and can create other issues associated with differing mobile phase composition.

Recently introduced regulations in California (3) have significantly expanded the PFAS target analyte list to include compounds such as PFBA, PFMBA, PFHxDA and PFOcDA, which have very large differences in hydrophobicity. This presents a significant analytical challenge because PFHxDA (C16) and PFOcDA (C18) are very hydrophobic with limited solubility in water. The predicted solubility of PFOA (C8) and PFOcDA (C18) are 480,000 and 0.00047 ng/L respectively, using the WS-KOWIN from the USEPA EPISuite Software (4).

In addition, chromatographic analysis of PFBA in an extract that is > 90 % organic results in poor peak shape for this early eluting compound. Most methods that can successfully analyze for PFBA are either direct injection (100 % water), a 1:1 water-methanol dilution or have at least 20 % water in the extract (EPA 533). Some methods (ASTM D7979, D7968 and EPA 8327) add acetic acid to the extract to help improve the peak shape of PFBA. However, this results in poorer chromatographic performance for the longer chain PFHxDA (C16) and PFOcDA (C18).

A New Strategy

In recognition of these limitations, we have pursued a new chromatographic strategy using a 100 % organic system (for long chain PFAS solubility) and variable mobile phase pH to provide good chromatography for PFBA and other early eluting PFAS compounds. By staying within the confines of the NH_4OAc mobile phase composition but employing pH as a variable, one can realize the potential advantages mobile phase variation allowed by EPA while avoiding the primary disadvantages. This approach could be useful in overcoming the difficulty of expanding the analyte lists of the existing PFAS methods to incorporate both the hydrophilic shorter chain compounds and the extremely hydrophobic longer chain compounds.

Technical Approach

This work specifically focused on a secondary chemical characteristic of most PFAS compounds: the hydrophilic or polar functional head of the molecule which are either carboxylic or sulfonic acids which can be charged or neutral, depending on the pH of the eluent. Chromatographers can take advantage of secondary interactions by employing a mobile phase in which a pH gradient is performed, i.e. changing the pH of the mobile phase over time. Mobile phase pH becomes important when analytes contain acidic, basic or both functional groups. The mobile phase pH determines the charge state (protonation state) of the analyte and thereby influences its interactions with the mobile and stationary phase. This technique allows for more control of the ionic interactions between the PFAS analytes within a column's stationary phase and the mobile phase. This is analogous to the WAX SPE technique used in EPA method 533, wherein the ion exchange mechanism allows for stronger interaction with the shorter-chain PFAS compounds than does the styrenedivinylbenzene (SVDB) SPE sorbent used in method 537.1 which operates primarily in a reversed phase mode. Shorter chain PFAS compounds have a lower degree of binding ability due to their shorter chain length and thus often pass through, owing to binding mechanisms that rely exclusively or primarily on a reversed phase interaction.

1. pH-Variable LC Mobile Phase Gradient (continued)

In this new technique, the mobile phase at the beginning of the run has a low pH (~ pH 3.9) and changes over time to a higher pH (~ pH 9.3). This protonates or deprotonates the functional heads of the various PFAS compounds over time, depending upon the pKa of the functional group. This correspondingly changes the elution profile for the separation, in terms of both relative and absolute retention times. In principle, the protonation of short-chain, anionic PFAS will lead to greater retention, while the deprotonation of the later-eluting, long-chain PFAS may lead to less retention, thereby compressing the chromatogram. This will lead to less suppression from non-retained interferences, and shorter run times, allowing greater sample throughput. Separating interferences from early eluting analytes is particularly important when there is only one sensitive MRM transition available, as in the case of PFBA and PFPeA. It is reasonable to think that these orthogonal retention mechanisms (hydrophobicity vs. ionizability or pKa) could offer greater opportunity to resolve complex PFAS mixtures. This Technical Note provides an illustration of the potential power of this approach.

Experimental Conditions

Instrumentation and Consumables. All PFAS analyses were performed on an Agilent® 1100 HPLC with a Thermo Scientific® TSQ Vantage triple quadrupole mass spectrometer. All samples were prepared using a Phenomenex Strata®-X-AW 200 mg 33 µm in a 6cc format (pn: 8B-S038-FCH). The LC column employed was a Phenomenex Kinetex® C18 EVO 5 µm 100 x 2.1 mm (pn: 00B-4633-AN).

Reagent Preparation. Eluents: (1A) Ammonium Acetate (NH_4OAc) was prepared at 20 mM by dissolving 1.54 g NH_4OAc into 1.0 L of water. LC-MS methanol (MeOH) was used for (1B). Acetic acid (HOAc) was prepared at 20 mM by diluting 1.22 mL of glacial acetic acid into 1.0 L of water (2A). Basic methanol was prepared by diluting 1.46 mL of concentrated. Ammonium Hydroxide (NH_4OH) into 1.0 L of LC-MS methanol. Reference materials were purchased from Wellington Labs (Guelph, Canada) and diluted into LC-MS methanol for analysis.

Mass Spectrometer Operating Conditions: The capillary and vaporizer temperature were 250 ˚C and 300 ˚C respectively. The sheath and aux gas were held at 40 arb and 50 arb respectively. The ESI voltages for positive and negative mode were +3.0/-2.5add spacekV. See Appendix 1 for MS/MS Parameters.

LC Operating Conditions: A moderate organic gradient profile was used in both analyses being compared. The only difference between the two LC systems was the pH modifiers that were used in the aqueous and organic eluents. To illustrate the effect of improved peak shape and selectivity differences solely due to the pH modifiers, the times used to change from aqueous to high organic were identical.

Results and Discussion

Although it is difficult to determine the actual pH in any eluent system especially in the presence of methanol and a particular stationary phase, this was estimated in an offline experiment. In order to ascertain the pH change as 20 mM HOAc mixes with the 25 mM NH_4OH, the pH was measured offline for different mixture ratios of this binary system. The measured pH values are shown in **Table 3**. Based on this data, it is estimated that the gradient pH elution profile has a pH no wider than 3.9 and 9.3 from start to finish respectively.

Table 1.
LC Conditions (neutral, pH=7)

	20 mM NH_4OAc	MeOH
Time	% A	% B
0.00	95	5
1.20	55	45
3.60	35	65
11.00	10	90
13.00	10	90
13.01	95	5
17.00	95	5

Table 2.
LC Conditions (gradient pH)

	20 mM HOAc	25 mM NH_4OH in MeOH
Time	% A	% B
0.00	95	5
1.20	55	
3.60	35	65
11.00	10	
13.00	10	90
13.01	95	5
17.00	95	5

Table 3.
Measured pH of a Binary Mixture of Eluents

20 mM HOAc	25 mM NH_4OH in MeOH	
% A	% B	Actual pH
100	0	3.62
95	5	3.86
90	10	4.17
80	20	4.55
70	30	5.14
60	40	5.77
50	50	6.45
40	60	7.13
35	65	8.15
30	70	8.52
20	80	8.98
10	90	9.33
0	99.5	10.25

1. pH-Variable LC Mobile Phase Gradient (continued)

One of the first notable improvements using the new gradient pH upon injecting an extract containing PFAS in 100 % methanol is that the peak shape for PFBA is drastically improved due to shifting the equilibrium of unprotonated PFBA to a protonated form. Protonated PFBA will interact with the nonpolar stationary phase much more than the mobile phase causing increased retention and a better focused peak. This is illustrated in **Figures 1** and **2**; PFBA (light blue). Under the commonly used eluent system of 20 mM NH_4OAc, PFBA and PFMPA exhibit severe fronting in 100 % methanol (required for PFODA solubility). However, using the gradient pH profile, these peaks are focused much better on the column.

Additionally, the latest eluters (PFTrA, PFTeDA, PFHxDA and PFODA) not only elute early, but the peak height is noticeably higher. The increase in height would improve detection limit with a greater s/n. This indicates that NH_4OH, which increases in concentration as the organic (methanol) gradient progresses, is affecting analyte retention by shifting their equilibrium to a deprotonated anion since the anions favor interactions with the mobile phase and the neutral analyte favors interaction with the stationary phase. In fact, the NH_4OH must be present in slightly higher molar concentration than the HOAc in order to move the pH into the slightly basic range.

Figure 1.
Chromatogram of 48 PFAS using 20 mM NH_4OAc (pH=7)

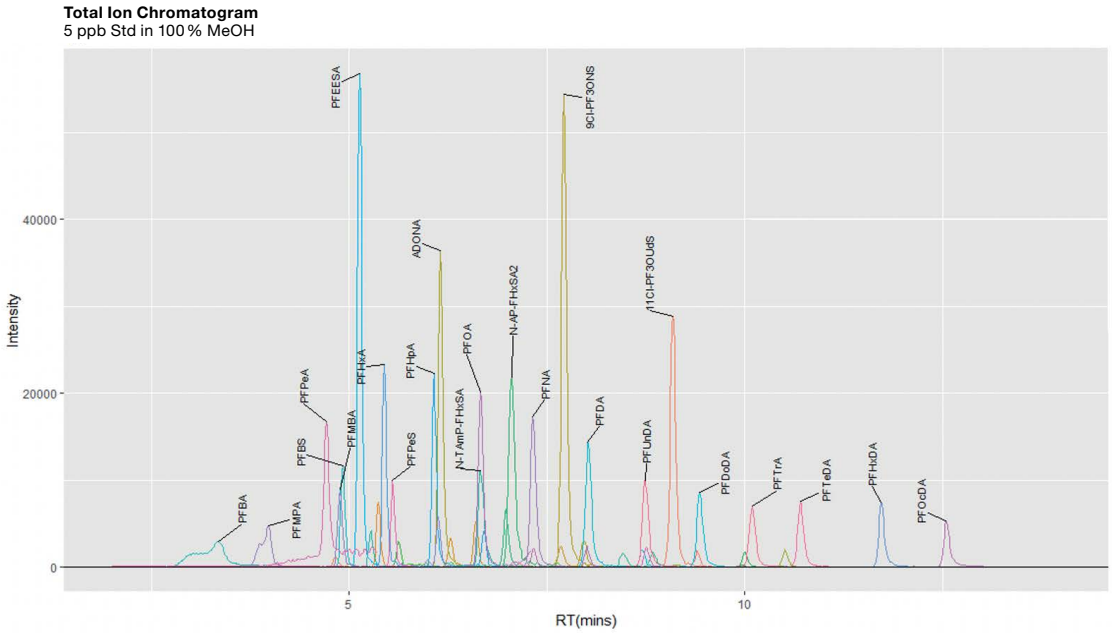
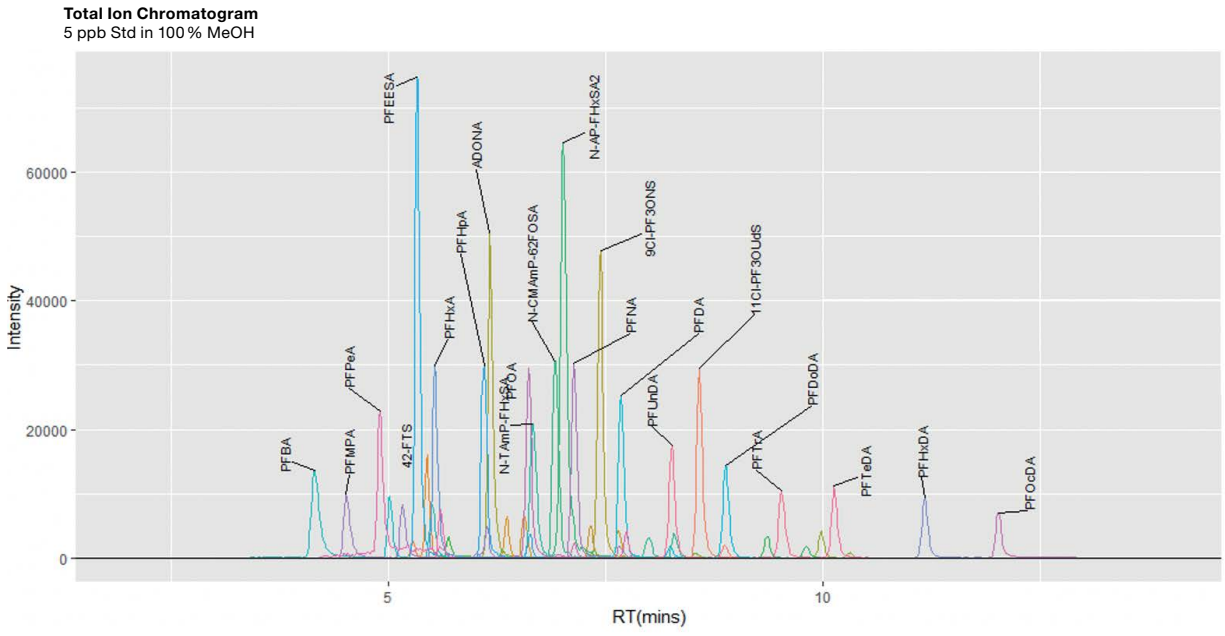


Figure 2.
Chromatogram of 48 PFAS using 20 mM HOAc and 25 mM NH_4OH (varied pH from 3.9 to 9.3)



1. pH-Variable LC Mobile Phase Gradient (continued)

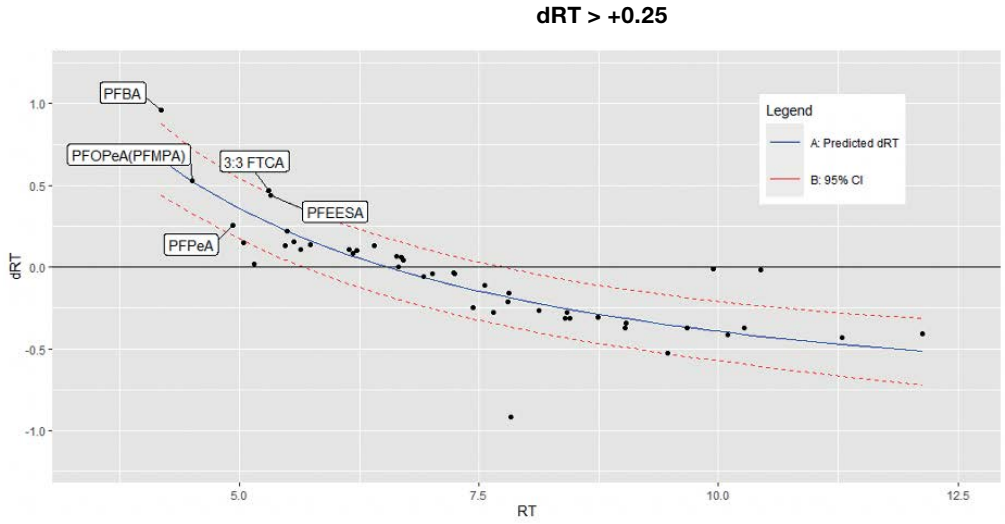
The selectivity of these two mobile phase systems was further investigated to see how they affect different PFAS compounds varying in chain length.

Upon close examination of the ΔRT data there were certain analytes (e.g PFOSA) that indicated possible differences in selectivity. In order to evaluate significant selectivity differences between the two eluent systems that were not obvious, a statistical approach was used. This is necessary because not every slight change in RT or resolution may be significant. First, a least squares regression was performed on the ΔRT as a function of RT of the new method. The equation that was used to model the change in the two systems is listed in equation (1) where a, b, c are the coefficients for the intercept, linear term, and inverse term respectively:

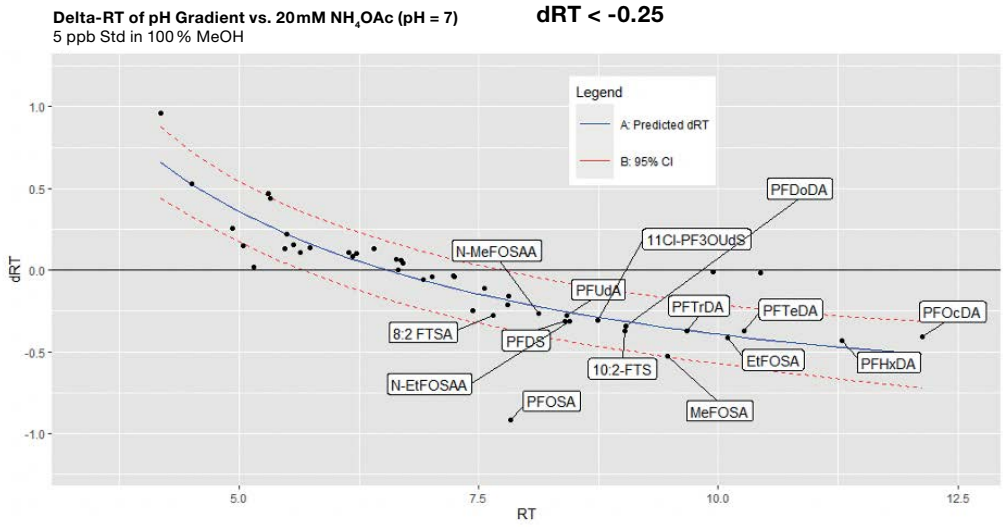
$$\Delta RT = a + b \cdot RT + c \cdot \frac{1}{RT} \qquad (1)$$

To validate the regression model and the prediction interval of significance at 95 %, a Global Validation of Linear Models Assumptions (GVLMA) was used (5) The plots in **Figure 3** highlight the most important aspects of the advantages of this new system. These are increased retention for early eluters (3a), decreased elution for late eluters (3b), and significant selectivity differences (3c). To evaluate significant differences, the x-axis shows the retention time (RT) for the new mobile phase and the y-axis shows the ΔRT relative to the neutral ammonium acetate mobile phase.

Figure 3.
Notable Mobile Phase Elution Changes
a) PFAS Analytes with Increased Retention



b) PFAS Analytes with Decreased Retention

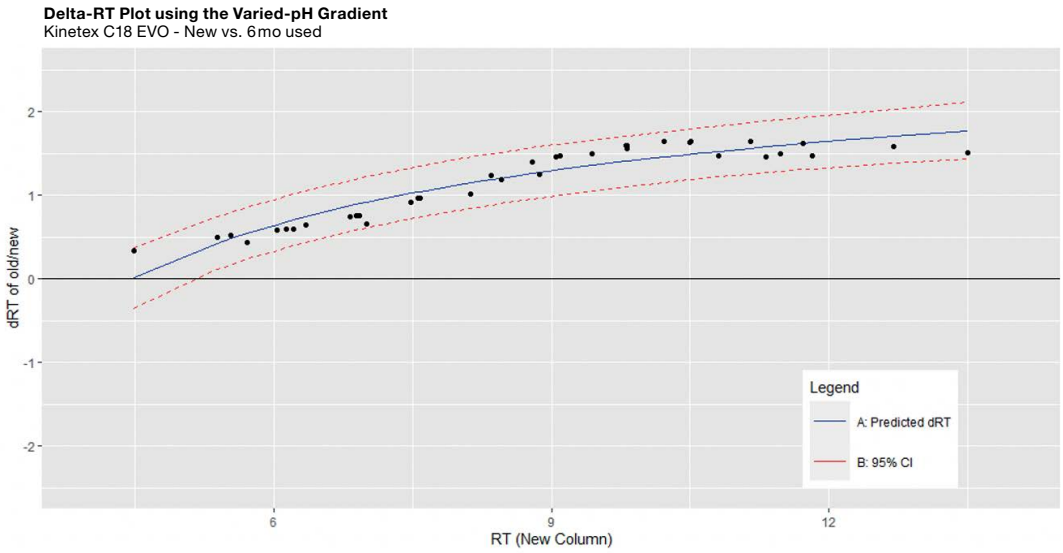


1. pH-Variable LC Mobile Phase Gradient (continued)

It is also worth noting that this new eluent system also has an effect on sensitivity for certain compounds. Specifically, N-TAmP-FHxSA, N-CMAmP-62FOSA, and N-AP-FHxSA2 (which are detected in ESI+) had an increase in response more than 2x in the new pH gradient eluent system (**Figure 1-2**).

Lastly, the robustness of the stationary phase was examined by evaluating a "well used" LC column versus a brand new column. The "well used" column had been used to analyze thousands of samples over approximately six months. This included drinking water extracts as well as non-potable aqueous and soil extracts. The Kinetex® EVO C18 showed reasonable robustness and, although some retention is lost over time, there was no significant ($P<0.05$) selectivity difference observed. Again, the GVLMA cross-validation was used (**Figure 4**) to detect significant elution order changes (ie: all analytes had statistically the same elution order) although "absolute" elution order was different in some cases.

Figure 4.
Retention Difference of New vs Used column Under Varied pH conditions



Conclusion

The objective of using a pH gradient mobile phase for PFAS analysis is that it allows the analyst to widen the scope of analyte chemistry to properly chromatograph short-chain and long-chain PFAS in 100 % organic extracts as well as change the selectivity of the method. This holds true for any analyte panel outside the scope of method EPA 537.1 and EPA 533, in that the absolute and relative retention of some analytes are different than when using a standard organic gradient with ammonium acetate (NH_4OAc).

Additionally, this solution may provide the ability to move certain peaks away from interferences and high ion suppression zones at the beginning of the chromatographic run. It may also allow for the inclusion of other PFAS analytes with a minimal redevelopment and optimization. The pH gradient method shows excellent robustness and reproducibility, with stable PFAS analyte retention times, even when using different columns, systems, and analysts. The changes in retention times (both absolute and relative) offer another tool for more complex PFAS mixtures - either those with more PFAS analytes or from working with dirtier matrices.

Moving forward, this promising mobile phase gradient approach could be combined with work investigating alternative HPLC stationary phases to determine optimal conditions for PFAS panels that are much broader in scope and chemistry. In principle, this approach should allow the separation of an even wider class of PFAS including non-volatile short-chain PFAS. Preliminary data suggest that the use of Formic acid (ie: 25 mM HOFo) instead of 25 mM HOAc can drop the pH slightly lower; closer to pH = 3. This has the benefit of increased retention for TFA, TFMS, and PFPrA in extracts that are 100 % methanol.

1. pH-Variable LC Mobile Phase Gradient (continued)

Appendix 1.
Instrumental Conditions for MS/MS Analysis and RT Data

						Retention Time Data	
Analyte	Precursor	Product	CE	S-Lens	Polarity	Gradient pH	Constant pH=7
PFBA	213	169	9	35	-	4.18	3.22
PFMPA	229	85	12	35	-	4.51	3.98
PFPeA	263	219	9	38	-	4.93	4.68
3:3-FTCA	241	177	8	37	-	5.30	4.83
PFEESA	315	135	23	90	-	5.32	4.88
PFBS	299	80	36	100	-	5.04	4.89
PFMBA	279	85	12	40	-	5.15	5.13
NFHDA	295	201	10	33	-	5.50	5.28
4:2-FTS	327	307	20	110	-	5.48	5.35
PFHxA	313	269	9	47	-	5.57	5.42
PFPeS	349	80	41	100	-	5.64	5.53
HFPO-DA	285	169	8	37	-	5.74	5.60
PFHpA	363	319	9	56	-	6.15	6.04
PFHxS	399	80	44	120	-	6.18	6.10
ADONA	377	251	11	60	-	6.22	6.12
5:3-FTCA	341	237	13	57	-	6.41	6.28
6:2-FTS	427	407	22	130	-	6.64	6.57
PFOA	413	369	9	62	-	6.69	6.63
N-TAmP-FHxSA	499.1	60	37	140	+	6.66	6.66
PFHpS	449	80	46	110	-	6.71	6.66
N-CMamP-6:2FOSA	571.1	440	31	140	+	6.92	6.98
N-AP-FHxSA	485.1	85	34	130	+	7.01	7.05
PFNA	463	419	10	65	-	7.24	7.27
PFOS	499	80	46	105	-	7.25	7.29
9Cl-PF3ONS	530.9	351	28	120	-	7.56	7.67
7:3FTCA	441	337	11	70	-	7.44	7.68
8:2-FTS	527	507	27	130	-	7.65	7.93
PFDA	513	469	10	75	-	7.81	7.97
PFNS	549	80	48	130	-	7.80	8.01
N-MeFOSAA	570	419	20	120	-	8.13	8.40
PFUnDA	563	519	10	85	-	8.42	8.69
PFDS	599	80	49	110	-	8.40	8.71
PFOSA	498	78	34	110	-	7.84	8.75
N-EtFOSAA	584	419	20	120	-	8.45	8.76
11Cl-PF3OUdS	630.9	451	30	120	-	8.74	9.05
PFDoDA	613	569	12	92	-	9.04	9.38
10:2-FTS	627	607	31	150	-	9.03	9.40
MeFOSE	616	59	15	90	-	9.95	9.96
MeFOSA	512	169	30	110	-	9.48	10.00
PFTrA	663	619	12	101	-	9.67	10.05
EtFOSE	630	59	15	91	-	10.45	10.46
EtFOSA	526	169	30	120	-	10.10	10.51
PFTeDA	713	669	12	108	-	10.27	10.64
PFHxDA	813	769	12	120	-	11.29	11.72
PFOcDA	913	869	13	140	-	12.13	12.54

References

1) U.S. Environmental Protection Agency, Method 537.1—Determination of Selected Per- And Polyfluorinated Alkyl Substances In Drinking Water By Solid Phase Extraction And Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS). November 2018.

2) U.S. Environmental Protection Agency, Method 533— Determination of Per- And Polyfluoroalkyl Substances In Drinking Water By Isotope Dilution Anion Exchange Solid Phase Extraction And Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS). November 2019.

3) Analytical Reporting Limits for PFAS compliance with DoD Table B-15 of QSM, Version 5.1 or later (updated 07/22/20) https://www.waterboards.ca.gov/pfas/docs/reporting_limits_dod_qsm_v5_1_or_later_july_22_2020.pdf

4) U.S. Environmental Protection Agency, Estimation Program Interface Suite (EPISuite) Software, v4.11, 2000-2017. https://www.epa.gov/tsca-screening-tools/download-epi-suitetm-estimation-program-interface-v411

5) Peña, Edsel A., and Elizabeth H. Slate. "Global Validation of Linear Model Assumptions." Journal of the American Statistical Association, vol. 101, no. 473, 2006, pp. 341–54, doi:10.1198/016214505000000637.

2. Column Chemistry Considerations

Column Chemistry Considerations for Full Coverage of PFAS Analyte Ranges

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Introduction

Per- and polyfluorinated alkyl substances (PFAS) are man-made chemicals, that have been widely used since the 1940s. They have been employed in a large variety of consumer products, such as nonstick cookware, food containers, stain and water repellent fabrics, polishes, waxes, paints, and cleaning products and are now widely distributed in the global environment. A significant source of PFAS environmental contamination has been the widespread use of PFAS-containing aqueous firefighting foams (AFFF), which are known to migrate into groundwaters at airports and military bases. Further environmental exposure to PFAS comes from industrial production facilities (e.g. chrome plating, electronics, manufacturing, or oil recovery). Living organisms, including plants, animals, and humans, can accumulate PFAS compounds in their tissue, which can build up over time and impact their health.[1-3] A total of 9,252 PFAS are listed in EPA's most recent list of PFAS substances. [4] However, only a handful of these, such as perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS), have been widely monitored in the environment or have been thoroughly studied for their toxicological effects.

Common Chromatographic Approaches

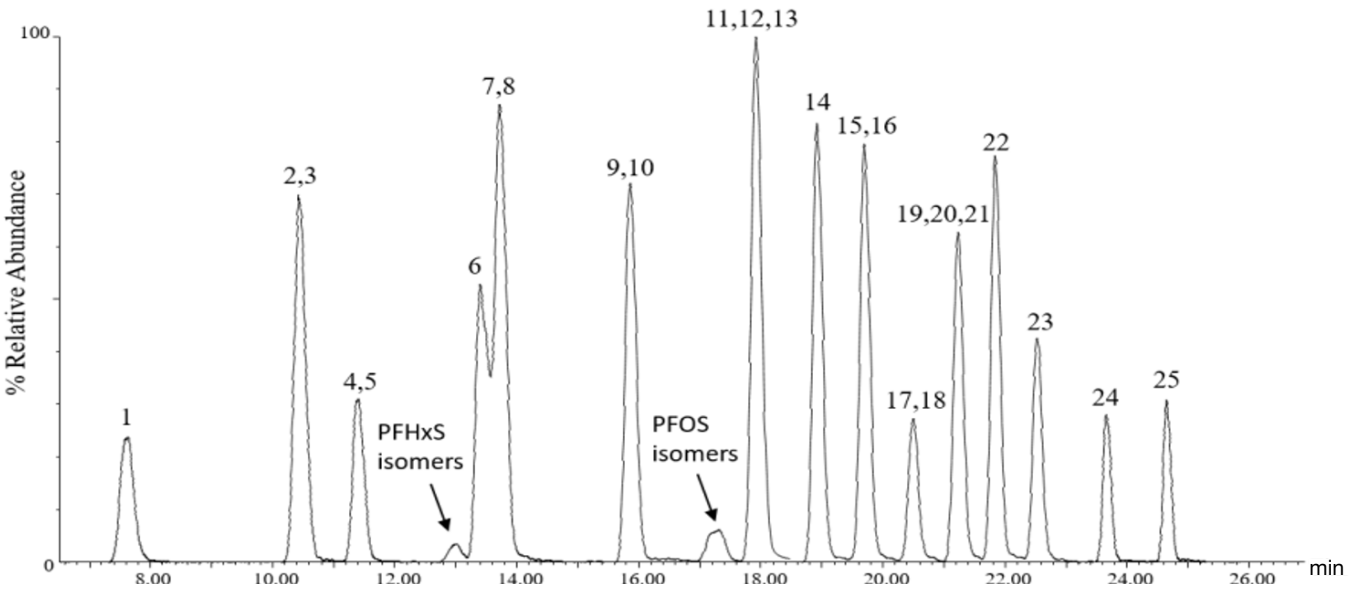
PFAS compounds are typically determined by LC-MS/MS and LC-HRMS instrumentation. The use of mass spectrometry detection has played a significant role in the quantitation of specific compounds where standards are available. Where standards are not available, the use of time of flight (TOF) and Orbitrap™ MS detectors are used to semi-quantify unknown PFAS compounds. The chromatographic separation of PFAS compounds in currently validated methods typically involves a reversed phase mechanism using a C18 or Phenyl column in an acidic-methanol eluent. For example, EPA method 537.1 uses a C18 column (5 µm, 2.1 x 150 mm C18) and EPA Method 533 was validated using a C18 Phenomenex Gemini® column (3 µm, 2 x 50 mm). Conversely, ASTM D7979 and EPA 8327 were validated using a Phenyl-Hexyl column (1.7 µm, 2.1 × 100 mm), ISO 21675 used a C18 column (5 µm, 2 × 50 mm) and the Department of Agriculture CLG-PFAS 2.01 method used a C8 column, Phenomenex Luna® C8(2) (3 µm, 2 x 50 mm).

PFAS Chromatographic Challenges

While these methods are generally adequate for a limited list of analytes, the large number of potential PFAS analytes that could potentially be present in a sample will inevitably challenge simple chromatographic separation approaches. This phenomenon was seen early in the development of the EPA drinking water methods. EPA 537.1 when validated, identified several overlapping peaks which can be seen in **Figure 1** as demonstrated by peaks, 2,3; 4,5; 7,8; 9,10; 11,12,13; 15,16; 17, 18; 19, 20, 21.

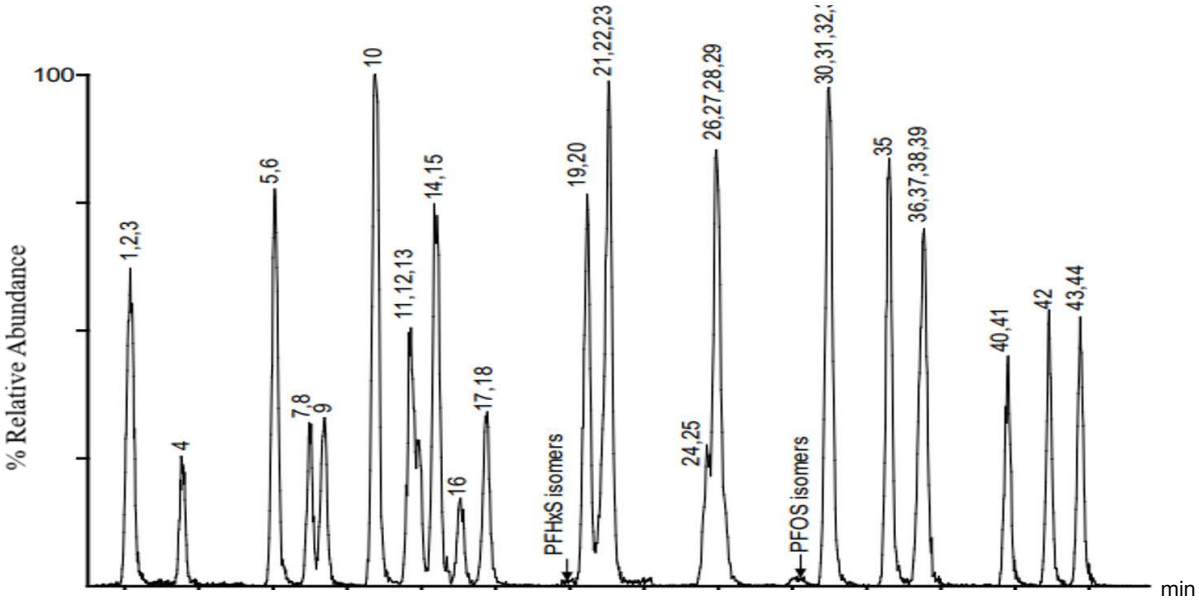
2. Column Chemistry Considerations (continued)

Figure 1.
Example chromatogram for reagent water fortified with method 537.1 analytes at 80 ng/L



Likewise, when EPA 533 was developed and validated with an expanded list of PFAS compounds, it also shows several overlapping peaks, as seen in **Figure 2**.

Figure 2.
Example chromatogram for reagent water fortified with method 533 analytes at 80 ng/L



2. Column Chemistry Considerations (continued)

Whereas many of these overlapping peaks can be successfully resolved by the mass analyzer, the potential presence of isobaric homologues and unresolved matrix interferences point to the continuing need for good chromatographic separation to assure reliable identification and quantitation. Although the problem may be manageable for today's small analyte lists, the challenge will inevitably grow as new PFAS compounds are added for investigational or regulatory purposes.

Looking to the Future

Current PFAS methods primarily rely upon C18 solid phase chemistry and simple methanol-ammonium acetate mobile phase gradients. These methods do not make full use of all the tools in the chromatographer's toolbox, nor need they, given today's limited analyte lists. However, this simple situation will inevitably change and there will be a need to develop more sophisticated chromatographic methods to tease out the subtle chemical and structural differences between closely related PFAS compounds. Chief among these will be the application of novel stationary phases and mobile phases to exploit the different interactions between closely related PFAS molecules. This Tech Note was designed to provide a vision of the potential power of such new chromatographic approaches.

Scope

In this Tech Note we will present data for a select list of PFAS compounds (**Table 1**) that were selected to reflect some of the chemical diversity of the PFAS universe. This color-coded grouping will be used to illustrate the differences in chromatographic retention time and elution order between various stationary phases including C8, C18, Phenyl-Hexyl, Biphenyl and F5 which can have significantly different sorptive properties. We will also examine how differing mobile phase polarity (e.g., methanol vs. acetonitrile) influences chromatographic performance for these various phases. Ideally, this information can be used to enhance chromatographic resolution as the list of PFAS compounds continues to increase. The goal is to provide insights that will allow method developers to identify useful separation strategies.

Table 1.

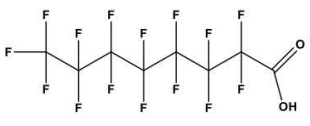
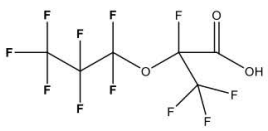
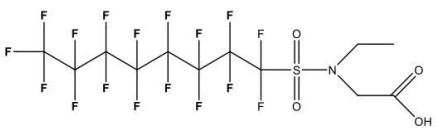
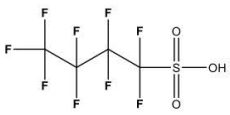
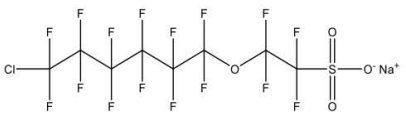
Chemical Name	Abbreviation
Perfluoroalkyl carboxylic acids (PFCAs)	
Perfluorohexanoic acid	PFHxA
Perfluoroheptanoic acid	PFHpA
perfluoro-n-octanoic acid	PFOA
perfluoro-n-nonanoic acid	PFNA
perfluoro-n-decanoic acid	PFDA
perfluoro-n-undecanoic acid	PFUdA
perfluoro-n-dodecanoic acid	PFDoA
perfluoro-n-tridecanoic acid	PFTrDA
perfluoro-n-tetradecanoic acid	PFTeDA
Perfluoroalkyl ether carboxylic acids (PFECA)	
hexafluoropropylene oxide-dimer acid	HFPO-DA
dodecafluoro-3H-4,8-diosanonanoate	NaDONA
Perfluorooctane sulfonamides and derivatives	
N-methylperfluoro-1-octanesulfonamidoacetic acid	N-MeFOSAA
N-ethylperfluoro-1-octanesulfonamidoacetic acid	N-EtFOSAA
Perfluorinated sulfonic acids (PFSAs)	
Perfluorobutanesulfonic acid	L-PFBS
perfluoro-1-hexanesulfonate	L-PFHxS
perfluoro-1-octanesulfonate	L-PFOS
Chlorinated polyfluoroalkyl ether sulfonic acids (Cl-PFESAs)	
9-chlorohexadecafluoro-3-oxanonane-1-sulfonate	9Cl-PF3ONS
11-chloroeicosalfluoro-3-oxaundecane-1-sulfonate	11Cl-PF3OUdS

Method Variables

PFAS Chemistries

There are established, validated methods set forth by the EPA and ISO for chromatographic separation of PFAS compounds using specific types of columns and packing materials. Unfortunately, not all PFAS compounds can be separated with sufficient accuracy using these methods because of the different types of functional groups that are on different PFAS compounds. In the select list that was used, there are 5 categories of PFAS compounds as shown in **Figure 3**, with an example of each. Owing to the variety of functional groups that can potentially be found on PFAS compounds, there are a variety of HPLC column chemistries that could aid enhanced separation.

Figure 3.

Perfluoroalkyl carboxylic acids (PFCAs)	Perfluoroalkyl ether carboxylic acids (PFECAs)	Perfluoro sulfomamide carborylic acids (PFSACAs)
 Perfluoro-n-octanoic acid	 Hexafluoropropylene oxide-dimcer acid	 N-ethylperfluoro-1-octanesulfonamidoacetic acid
Perfluorinated sulfonic acids	**Chlorinated polyfluoroalkyl ether sulfoninic acids**	
 Perfluorobutanesulfonic acid	 9-chlorohexadecafluoro-3-oxanonane-1-sulfonate	

2. Column Chemistry Considerations (continued)

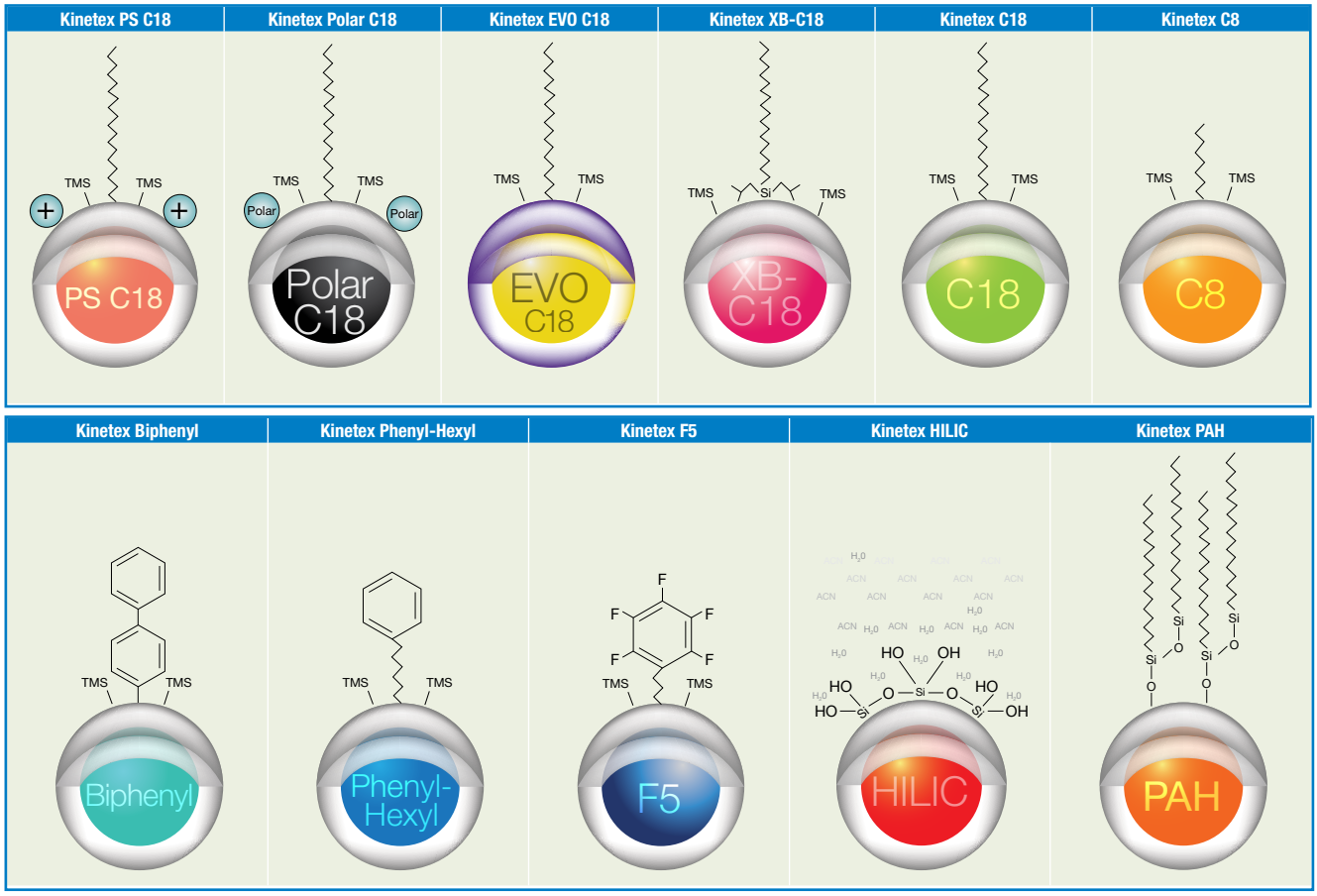
Solid Phase Chemistries

A representation of the different solid phase chemistries that are available in Phenomenex HPLC columns that could be used in PFAS separations is presented in **Figure 4**. This wide variety of ligand chemistries – combined with differences in porosity and other morphological variations – was developed to offer a wide range of variables for method development.

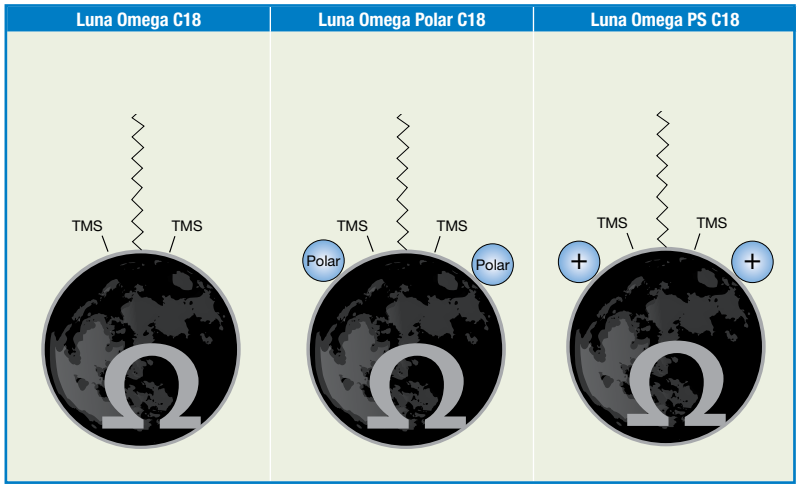
Different combinations of these variables serve to enhance the separation of polar compounds, increase surface areas, add pH stability, decrease system backpressures, etc. These, and additional column properties, provide chromatographers with a high degree of flexibility with which to tackle challenging separations.

Figure 4.
Available column chemistries appropriate for PFAS compound separation

Kinetex® Core-Shell 1.3, 1.7, 2.6, and 5 µm



Luna® Omega Fully Porous 1.6, 3, and 5 µm



2. Column Chemistry Considerations (continued)

Mobile Phase Chemistries

However, in addition to column selection, chromatographers can also make changes in mobile phase polarity to further enhance selectivity. For example, EPA method 533 was altered in several ways to enhance separation of the selected PFAS compounds used in the present study. In the first elution regime, the percentage of methanol was increased at run initiation and then further increased to a higher percentage than had been previously used in the published method. This decreased the overall run time but kept the percentage increase of methanol roughly the same. This elution regime will be referred to later in this Tech Note as "533 Similar" (**Table 2**).

In the second elution regime, acetonitrile was added to the mobile phase at a ratio of 80:20 methanol:acetonitrile to increase mobile phase polarity (but with all other factors remaining the same as in the "533 Similar" elution regime). This second elution regime will be referred to as "533 Acetonitrile Altered" (**Table 3**). The results from these two elution regimes will be addressed separately. Clearly, there are many other potential mobile phase variations that could be investigated. However, the two variations presented here will suffice to demonstrate the power of mobile phase polarity combined with solid phase chemistry variation to effect PFAS chromatographic behavior.

Table 2.

EPA 533 - As Published		
Time (min)	% 20 mM Ammonium Acetate	% Methanol
Initial	95	5
0.5	95	5
3	60	40
16	20	80
18	20	80
20	5	95
22	5	95
25	95	5
35	95	5

40 → 80 in 13 min 3.08 % per min

533 Similar		
Time (min)	% 20 mM Ammonium Acetate	% Methanol
Initial	55	45
15	10	90
21	10	90
21.5	55	45

40 → 90 in 15 min 3.0 % per min

Table 3.

533 Similar		
Time (min)	% 20 mM Ammonium Acetate	% Methanol
Initial	55	45
15	10	90
21	10	90
21.5	55	45

533 Acetonitrile Altered		
Time (min)	% 20 mM Ammonium Acetate	% 80-20 Methanol Acetonitrile
Initial	55	45
15	10	90
21	10	90
21.5	55	45

Results and Discussion

For ease of comparison, all chromatographic data will be presented in tabular format with the chromatography columns on the left, the PFAS compounds across the top, and the specific analyte retention times under the PFAS compounds. The highlighted boxes identify two compounds that have overlapping retention times ($\Delta RT \leq 5$ seconds) and the arrows at the bottom indicate when two compounds have changed elution order. The different PFAS compound classes are represented by the colors referenced in **Table 1**. This representation is a more insightful way to present the data because overlaying or stacking individual chromatograms makes it very difficult to compare results across columns. The two mobile phase chemistry regimes identified above will now be discussed separately.

2. Column Chemistry Considerations (continued)

1. EPA 533 Similar

In order to determine how the selected PFAS compounds would elute and separate, seven different chromatography columns with different solid phase chemistries were examined. **Figure 5** displays columns that have C18-functionality or PAH-functionality. The PFAS elution order was generally consistent for most of the C18 phases, although specific elution times varied. The Kinetex® PAH column demonstrated two compound functional pairs with a reverse elution order: NaDONA (a perfluoroether carboxylic acid) vis-á-vis L-PFHxS (a perfluronated sulfonic acid) and PFUdA (a perfluoroalkyl carboxylic acid) vis-á-vis N-EtFOSSA (a perfluorooctane sulfonamide). In addition, there were slight differences in overlapping peaks amongst the various C18 phases, whereas the Kinetex PAH phase had only one overlapping pair. When compared to two C8 phases (**Figure 6**), the elution order was similar to the C18 phases, and the retention times were similar, but there were fewer overlapping peak pairs (one pair vs. 3 pairs).

However, the C8 phases also demonstrated two compound functionality pairs with a reverse order elution from the C18 phases: L-PFOS (a perfluoronated sulfonic acid) vis-á-vis PFNA (a perfluroalkyl carboxylic acid) and (again) PFUdA vis-á-vis N-EtFOSSA, presumably is response to the lower hydrophobicity of the C8 phase functionality. Interestingly, both C8 phases and the PAH phase had fewer overlapping peaks compared to the C18 phases, but in different parts of the elution order spectrum. This likely represents the greater contribution of pi-electron interaction with the PAH phase in contrast with more consistent hydrophobic interaction characteristic of the C18 phases. These variations are subtle rather than dramatic, but they offer insights into interactions between solid phase chemistry and PFAS compound class that could be useful for better separating adjacent compound pairs or shifting analytes away from mass spectral interferences.

Figure 5.
C18 and PAH summary

		L-PFBS	PFHxA	HFPO-DA	PFHpA	L-PFHxS	NaDONA	PFOA	PFNA	L-PFOS	9-Cl-PF3ONS	PFDA	N-MeFOSAA	PFUdA	N-EtFOSAA	11Cl-PF3OUdS	PFDoA	PFTrDA	PFTeDA
Gemini®	C18	5.28	7.37	8.23	9.53	10.02	10.09	11.41	13.12	13.12	13.58	14.28	15.03	15.35	15.36	16.05	16.34	17.23	18.11
Luna®Omega	Polar C18	4.15	6.18	7.10	8.31	8.38	8.47	10.16	11.43	11.43	12.34	12.58	13.30	14.04	14.05	14.38	14.59	15.47	16.29
Luna Omega	Ps C18	4.29	6.34	7.21	8.46	8.55	9.05	10.35	12.06	12.03	12.53	13.24	14.01	14.31	14.36	14.58	15.23	16.08	16.44
Kinetex	C18	3.36	6.38	7.38	9.44	10.04	10.07	12.03	13.48	13.52	14.4	15.1	15.41	16.17	16.15	16.5	17.15	18.06	18.48
Kinetex	XB-C18	3.27	5.30	6.18	7.56	8.10	8.13	9.54	11.31	11.34	12.24	12.51	13.28	13.58	14.02	14.31	14.56	15.47	16.31
Kinetex	Polar C18	3.05	4.59	5.49	7.18	7.33	7.37	9.16	10.54	10.57	11.52	12.17	12.51	13.25	13.27	14.03	14.25	15.18	16.05

		L-PFBS	PFHxA	HFPO-DA	PFHpA	NaDONA	L-PFHxS	PFOA	PFNA	L-PFOS	9-Cl-PF3ONS	PFDA	N-MeFOSAA	N-EtFOSAA	PFUdA	11Cl-PF3OUdS	PFDoA	PFTrDA	PFTeDA
Kinetex	PAH	1.24	2.07	2.60	3.99	4.27	4.31	5.81	7.59	7.76	8.61	9.16	9.73	10.32	10.52	11.14	11.69	12.75	13.71

Elution Order Shifts from C18

Elution Order Shifts from C18
Overlaps are Eliminated

Figure 6.
C8 summary

		L-PFBS	PFHxA	HFPO-DA	PFHpA	L-PFHxS	NaDONA	PFOA	L-PFOS	PFNA	9-Cl-PF3ONS	PFDA	N-MeFOSAA	N-EtFOSAA	PFUdA	11Cl-PF3OUdS	PFDoA	PFTrDA	PFTeDA
Luna	C8	5.41	7.51	8.38	9.51	10.04	10.15	11.33	12.47	12.49	13.28	13.53	14.12	14.40	14.46	15.13	15.33	16.14	16.48
Kinetex	C8	5.30	7.54	8.47	10.14	10.26	10.33	11.57	13.18	13.19	14.03	14.26	14.45	15.15	15.24	15.56	16.14	16.56	17.33

Elution Order Shifts from C18

2. Column Chemistry Considerations (continued)

Finally, additional differences are seen when comparing Kinetex® Biphenyl, Phenyl-Hexyl, and F5 columns. These phases were designed with different chemistries having varying polarities to provide better selectivity for aromatic compounds. However, these polarity differences and greater pi-electron interactability also come into play with the different PFAS chemistries, as evidenced by the various reverse order elution pairs from the C18 phases.

The elution order in the Kinetex Biphenyl and Phenyl-Hexyl columns are consistent, but markedly different from the Kinetex F5 column. The Biphenyl and F5 phases showed only one set of overlapping peaks, but the Phenyl-Hexyl column had 3 sets of overlapping peaks. Interestingly, the compound classes that overlapped were different between the Phenyl-Hexyl and Biphenyl columns (**Figure 7**).

Figure 7.
Phenyl Stationary Phase Summary

		L-PFBS	PFHxA	HFPO-DA	PFHpA	NaDONA	L-PFHxS	PFOA	PFNA	L-PFOS	PFDA	9-Cl-PF3ONS	N-MeFOSAA	PFUdA	N-EtFOSAA	PFDoA	11Cl-PF3OUdS	PFTrDA	PFTeDA
Kinetex	Biphenyl	1.47	2.06	2.19	3.67	4.00	4.43	5.53	7.02	7.49	8.64	8.21	9.23	9.24	9.88	10.16	10.56	10.96	11.67
Kinetex	Phenyl-Hexyl	2.53	3.89	4.57	5.97	6.39	6.42	7.69	9.10	9.35	10.30	10.37	11.13	11.33	11.69	12.21	12.31	13.01	13.67

Elution Order Shifts from C18

		L-PFBS	PFHxA	HFPO-DA	PFHpA	NaDONA	L-PFHxS	PFOA	PFNA	L-PFOS	9-Cl-PF3ONS	PFDA	N-MeFOSAA	PFUdA	N-EtFOSAA	11Cl-PF3OUdS	PFDoA	PFTrDA	PFTeDA
Kinetex	F5	3.37	5.12	5.84	7.49	7.85	7.98	9.45	11.10	11.35	11.88	12.47	13.37	13.63	13.84	14.09	14.61	15.47	16.18

Elution Order Shifts from C18

2. EPA 533 Acetonitrile Altered

Acetonitrile is a highly polar molecule and is often added to the mobile phase to alter how analytes interact with the solid phase. The previously discussed experimental sequence was repeated using a 80:20 methanol:acetonitrile mobile phase with the same PFAS compounds and HPLC columns. The C18 columns all still had a consistent elution order as compared to 533 Similar but displayed earlier retention times (**Figure 8**). However, compared to 533 Similar, the conditions of 533 Acetonitrile Altered resulted in a much larger number of retention time elution order shifts.

The addition of acetonitrile to the mobile phase increased the number of overlapping peaks for the Gemini® C18, Luna® Omega Polar C18, and Kinetex Polar C18 columns, but it conversely decreased the number of overlapping peaks for the Luna Omega PS-C18 and Kinetex C18 columns. In the Kinetex PAH column, the methanol:acetonitrile mobile phase also significantly changed the elution order as compared to methanol-only mobile phase, but with some differences in the effected compounds (**Figure 8**). However, with Kinetex PAH there were also more overlapping peaks, resulting in compromised separation for early eluters.

Figure 8.
C18 and PAH Summary

		PFHxA	L-PFBS	HFPO-DA	PFHpA	NaDONA	PFOA	L-PFHxS	PFNA	PFDA	L-PFOS	N-MeFOSAA	N-EtFOSAA	9-Cl-PF3ONS	PFUdA	PFDoA	11Cl-PF3OUdS	PFTrDA	PFTeDA
Gemini	C18	2.42	2.55	2.97	3.88	4.45	5.55	5.47	7.25	8.89	9.08	9.10	9.82	10.24	10.43	11.85	12.96	13.15	14.40
Luna Omega	Polar C18	1.83	1.97	2.25	2.92	3.43	4.31	4.51	5.75	7.14	7.35	7.35	7.98	8.44	8.45	9.69	10.84	10.88	12.01
Luna omega	Ps C18	1.87	1.99	2.34	3.12	3.66	4.69	4.87	6.29	7.74	7.90	8.02	8.71	8.91	9.10	10.38	11.46	11.71	12.91
Kinetex	C18	1.42	1.47	1.69	2.30	2.79	4.12	4.31	6.40	8.28	8.45	8.54	9.28	9.62	9.90	11.36	12.51	12.72	14.00
Kinetex	Polar C18	1.32	1.39	1.58	1.99	2.38	3.13	3.29	4.45	5.90	6.09	6.16	6.80	7.21	7.29	8.53	9.62	9.65	10.76

Elution Order Shifts from "EPA 533 Similar"

		PFHxA	L-PFBS	HFPO-DA	PFHpA	NaDONA	PFOA	L-PFHxS	PFNA	PFDA	L-PFOS	N-MeFOSAA	PFUdA	9-Cl-PF3ONS	N-EtFOSAA	PFDoA	11Cl-PF3OUdS	PFTrDA	PFTeDA
Kinetex	XB-C18	1.37	1.42	1.63	2.23	2.69	3.68	3.76	5.27	6.80	6.87	7.26	7.94	7.96	8.20	9.52	10.57	10.82	12.08

Elution Order Shifts from "EPA 533 Similar"

2. Column Chemistry Considerations (continued)

		L-PFBS	PFHxA	HFPO-DA	PFHpA	NaDONA	L-PFHxS	PFNA	PFNA	L-PFOS	PFDA	N-MeFOSAA	N-EtFOSAA	9-Cl-PF3ONS	PFUdA	PFDoA	11Cl-PF3OUdS	PFTrDA	PFTeDA
Kinetex®	PAH	0.76	0.76	0.83	0.95	1.05	1.33	1.34	2.19	3.44	3.49	3.78	4.30	4.31	4.78	6.10	6.90	7.47	8.82

Elution Order Shifts from "EPA 533 Similar"

The methanol:acetonitrile mobile phase also resulted in more overlapping pairs and changes in elution order with the C8 columns (**Figure 11**). The elution order was consistent between the two C8 columns using this method, but there were many shifts in elution order compared to the methanol-only eluent. Finally, the methanol:acetonitrile method and the methanol-only method showed similar but not identical elution orders in the Kinetex Biphenyl and Phenyl-Hexyl Columns. The elution order with Kinetex F5 was less comparable with Kinetex Biphenyl and Phenyl-Hexyl columns with the acetonitrile altered eluent than previously seen with the methanol-only eluent. However, with the acetonitrile altered eluent, Kinetex F5 was more similar in elution order to the C18 columns than to the phenyl stationary phases.

The 533 Acetonitrile Altered method also showed increased overlapping peaks in all phenyl and F5 stationary phases (**Figure 12**), although the shorter run times may have contributed significantly to these increases. All things considered, the methanol:acetonitrile data demonstrate that mobile phase polarity (in conjunction with stationary phase chemistry) has a great deal of influence over the sorption behavior of the different classes of PFAS compounds and could be a powerful tool with which to influence chromatographic behavior.

Figure 9.
C8 Summary

		PFHxA	L-PFBS	HFPO-DA	PFHpA	NaDONA	PFOA	L-PFHxS	PFNA	PFDA	N-MeFOSAA	L-PFOS	N-EtFOSAA	9-Cl-PF3ONS	PFUdA	PFDoA	11Cl-PF3OUdS	PFTrDA	PFTeDA
Luna®	C8	2.05	2.15	2.34	3.22	3.55	4.51	5.07	6.22	7.48	7.53	8.04	8.28	9.05	9.09	10.25	11.33	11.36	12.42
Kinetex	C8	2.14	2.22	2.41	3.30	4.10	5.19	5.34	7.01	8.32	8.35	8.47	9.11	9.50	9.53	11.06	12.17	12.14	13.17

Elution Order Shifts from "EPA 533 Similar"

Figure 10.
Phenyl Stationary Phase Summary

		PFHxA	L-PFBS	HFPO-DA	PFHpA	NaDONA	PFOA	L-PFHxS	PFNA	PFDA	L-PFOS	N-MeFOSAA	N-EtFOSAA	9-Cl-PF3ONS	PFUdA	PFDoA	11Cl-PF3OUdS	PFTrDA	PFTeDA
Gemini®	C18	2.42	2.55	2.97	3.88	4.45	5.55	5.47	7.25	8.89	9.08	9.10	9.82	10.24	10.43	11.85	12.96	13.15	14.40

Benchmark: C 18 "EPA 533 Acetonitrile Altered"

		PFHxA	L-PFBS	HFPO-DA	PFHpA	NaDONA	PFOA	L-PFHxS	PFNA	PFDA	L-PFOS	N-MeFOSAA	9-Cl-PF3ONS	N-EtFOSAA	PFUdA	PFDoA	11Cl-PF3OUdS	PFTrDA	PFTeDA
Kinetex	F5	1.27	1.32	1.45	1.93	2.29	3.12	3.26	4.55	5.92	6.05	6.54	6.84	7.03	7.22	8.44	9.24	9.63	10.76

Elution Order Shifts from C18 Acetonitrile Altered

		PFHxA	L-PFBS	HFPO-DA	PFHpA	NaDONA	PFOA	L-PFHxS	PFNA	PFDA	N-MeFOSAA	L-PFOS	N-EtFOSAA	PFUdA	9-Cl-PF3ONS	PFDoA	PFTrDA	11Cl-PF3OUdS	PFTeDA
Kinetex	Phenyl-Hexyl	1.05	1.14	1.18	1.39	1.60	1.99	2.25	2.96	4.09	4.33	4.47	4.82	5.16	5.46	6.17	7.09	7.45	7.95
Kinetex	Biphenyl	0.85	0.90	0.90	0.99	1.07	1.20	1.32	1.55	2.11	2.32	2.37	2.68	2.81	3.23	3.59	4.31	4.83	5.05

Elution Order Shifts from C18 Acetonitrile Altered

2. Column Chemistry Considerations (continued)

Conclusions

The HPLC methodology in EPA methods 537, 537.1 and 533 are all based upon a C18 stationary phase and a methanol-water mobile phase. In this study we have shown that the use of alternative stationary phases of varying surface chemistry and eluents of varying polarity can significantly alter the sorption-elution characteristics of different classes of PFAS compounds. This orthogonal approach to PFAS HPLC chromatography should serve as a fruitful avenue for future method development. As analyte lists increase in size and complexity, a variety of HPLC column chemistries and eluent compositions will be needed to accommodate the wide range of PFAS related compounds that might be encountered such as polar acids, non-polar acids, esters, amides, sulfonamides, and telomere length, all of which can be complicated with branched vs. linear isomers.

The work presented here is merely illustrative and should be considered a starting point for column chemistry and mobile phase considerations for PFAS HPLC methodology. Even though the demonstration sample contained a nice mix of PFAS compounds with varied functional groups, there are certainly many more compounds in the 9000-strong (and growing) PFAS inventory that will challenge LC-MS methodology. National and state PFAS analyte panels are constantly being updated and expanded. There is increasing emphasis on identifying and quantifying PFAS related isomers, unique functional groups and degradation products across a wide range of sample matrices. With regulated detection and quantitation limits being driven lower and lower, sensitivity is a significant issue. The choice of HPLC column chemistry will play a significant role in successfully meeting all these future challenges.

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Perfluoroalkyl Substances **(PFAS)** Testing Guide

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