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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 (NH₄OAc) 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 NH₄OAc, 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 California⁵ 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 EPI Suite Software³. 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 NH₄OAc 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.

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 lesser 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 200mg 33µm in a 6cc format (pn: 8B-S038-FCH). The LC column employed was a Phenomenex Kinetex® C18 EVO 5µm 100 x 2.1 mm (pn: 00D-4633-AN).

Reagent Preparation. Eluents: (1A) Ammonium Acetate (NH₄OAc) was prepared at 20 mM by dissolving 1.54 g NH₄OAc 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 conc. Ammonium Hydroxide (NH₄OH) 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 15 arb respectively. The ESI voltages for positive and negative mode were +3.0/-2.5 kV. 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.

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

	20 mM NH ₄ OAc	MeOH
Time	% A	% B
0.00	5	95
1.20	45	55
3.60	65	35
11.00	90	10
13.00	90	10
13.01	5	95
17.00	5	95

Table 2.
LC Conditions (gradient pH)

	20 mM HOAc	25 mM NH ₄ OH in MeOH
Time	% A	% B
0.00	5	95
1.20	45	55
3.60	65	35
11.00	90	10
13.00	90	10
13.01	5	95
17.00	5	95

Results and Discussion

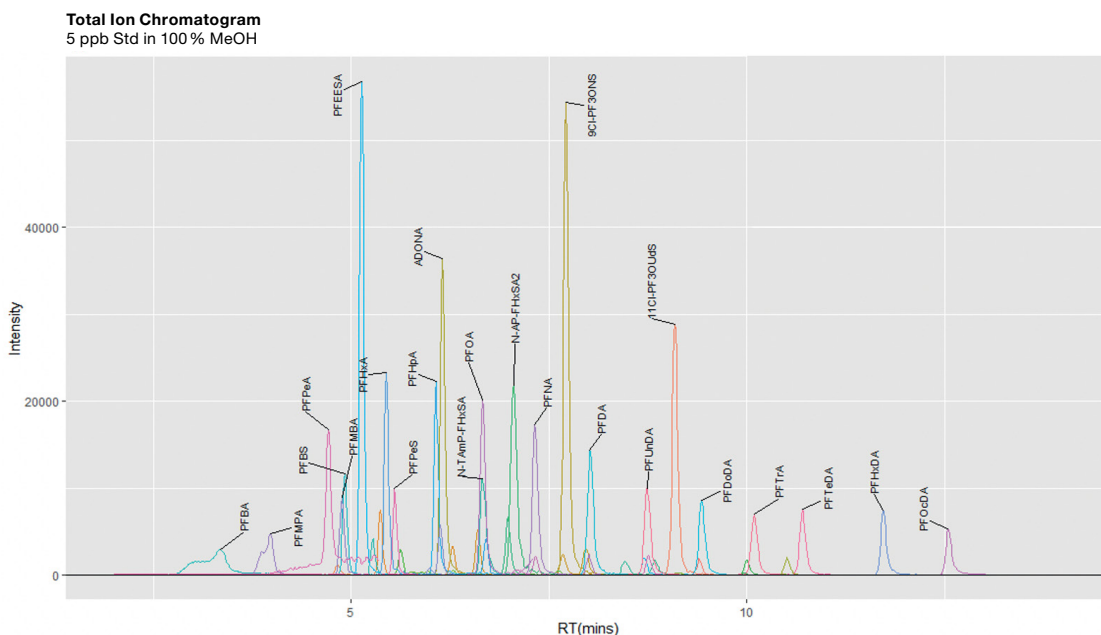
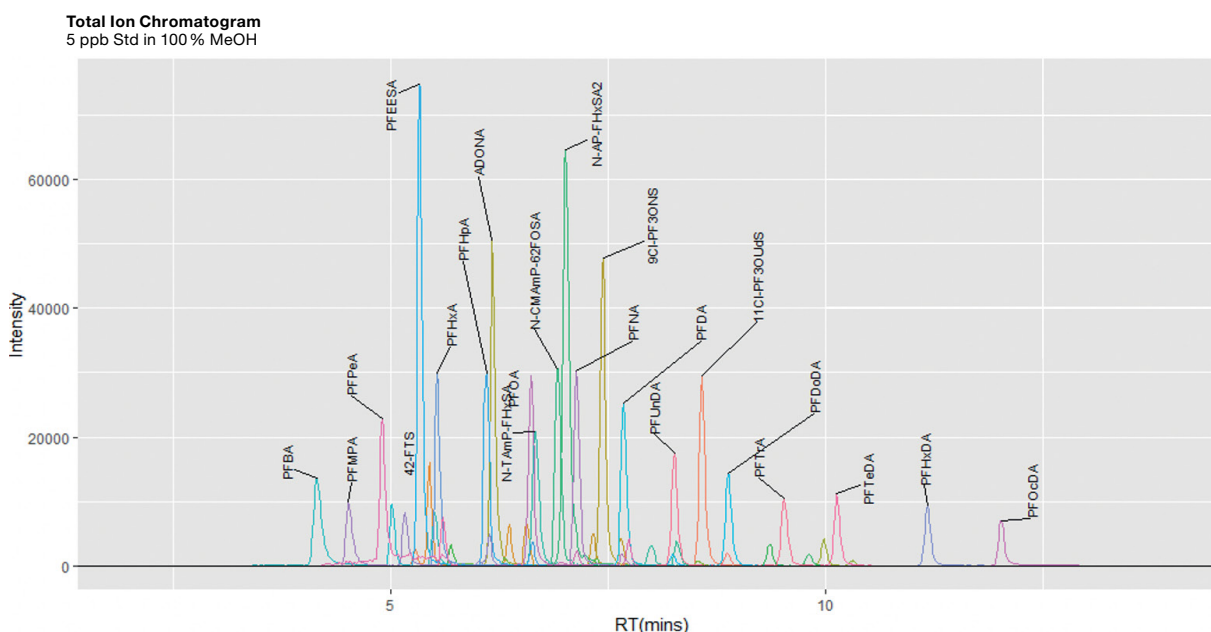
Although it is difficult to determine the actual pH in any eluent system especially in the presence of Methanol and a particular stationary phase, this was estimated in an offline experiment. In order to ascertain the pH change as 20 mM HOAc mixes with the 25 mM NH₄OH, the pH was measured offline for 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 3.
Measured pH of a Binary Mixture of Eluents

20 mM HOAc	25 mM NH ₄ OH 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

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 NH₄OAc, 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 (PFT₄A, PFT₄DA, PFH₄DA 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 NH₄OH, 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 NH₄OH 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 NH₄OAc (pH = 7)**Figure 2.**Chromatogram of 48 PFAS using 20 mM HOAc and 25 mM NH₄OH (varied pH from 3.9 to 9.3)

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:

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

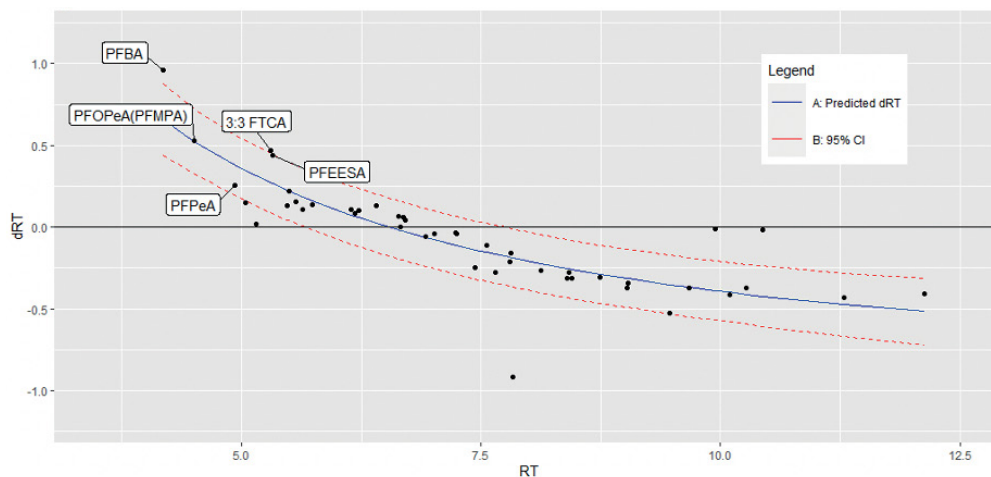
To validate the regression model and the prediction interval of significance at 95 %, a Global Validation of Linear Models Assumptions (GVLMA) was used⁴. The plots in **Figure 3** highlight the most important aspects of the advantages of this new system. These are increased retention for early eluters (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

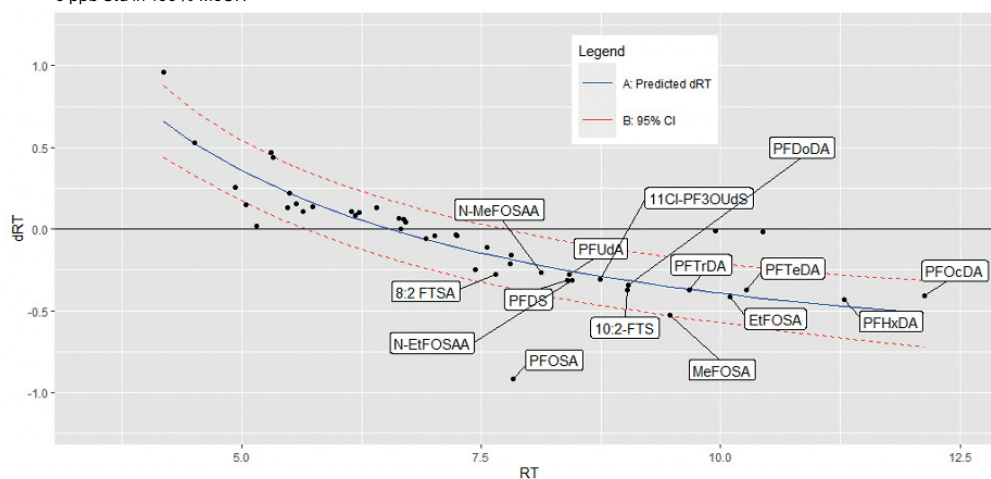
dRT > +0.25



b) PFAS Analytes with Decreased Retention

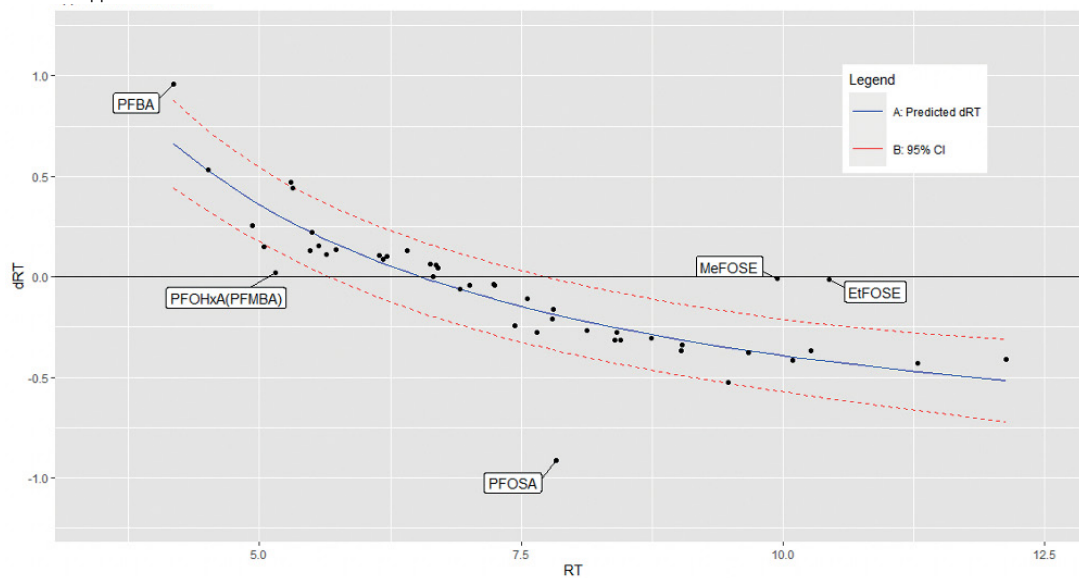
Delta-RT of pH Gradient vs. 20mM NH_4OAc (pH = 7)
5 ppb Std in 100 % MeOH

dRT < -0.25



c) PFAS with Minimal and Significant Selectivity Changes

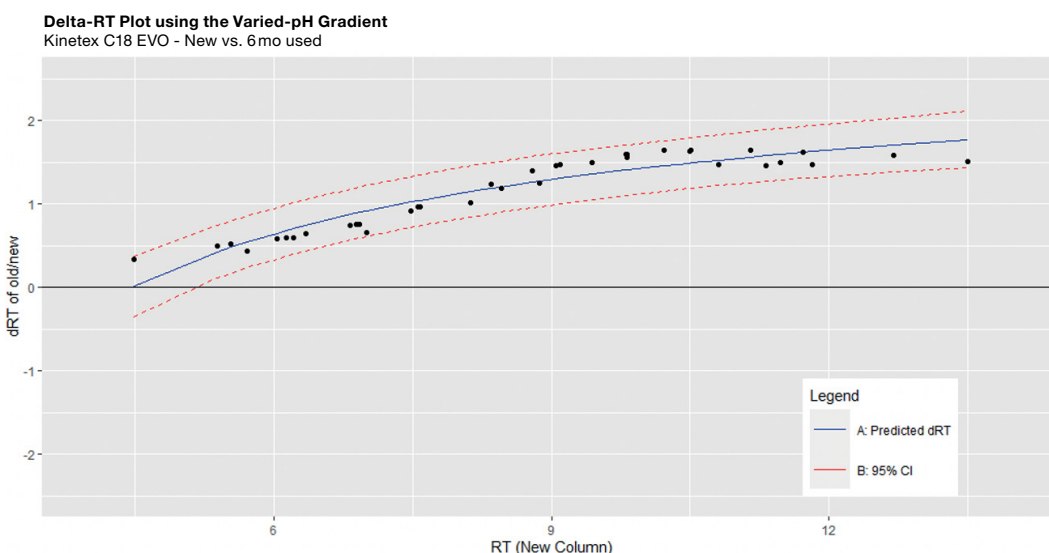
Delta-RT of pH Gradient vs. 20mM NH_4OAc (pH = 7)
5 ppb Std in 100 % MeOH



It's also worth noting that this new eluent system also has an effect on sensitivity for certain compounds. Specifically, N-TAmP-FHx-SA, N-CMAmP-62FOSA, and N-AP-FHxSA2 (which are detected in ESI+) had an increase in response more than 2 x 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.

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

Analyte	Precursor	Product	CE	S-Lens	Polarity	Retention Time Data	
						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
PFTriA	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

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- 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.
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- 5) 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




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Strata SDB-L SPE 500 mg/6 mL tubes, 30/pk	8B-S014-HCH
Verex™ Vial, 9 mm Screw, PP, 1.7 mL, 1000/pk	AR0-39P0-13
Verex Vial, 9 mm Screw, PP, 300 µL, 1000/pk	AR0-39P2-13
Verex Vial, 9 mm Screw, PP, 700 µL, 1000/pk	AR0-39P1-13
Vial Cap Verex™ Cert+ Cap (one-piece), 9 mm, PE w/ Starburst pre-Slit, 2 mL, 1000/pk	AR0-89P6-13-C



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