

Non-Target PFAS Analysis in Dried Blood Spots Using the Agilent 6546 LC/Q-TOF with Profinder and FluoroMatch

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Abstract

This application note demonstrates the use of FluoroMatch Suite for the analysis and identification of per- and polyfluorinated substances (PFAS) using an Agilent 1290 Infinity II LC and Agilent 6546 LC/Q-TOF mass spectrometer with iterative exclusion (IE). FluoroMatch is applied to dried blood spots spiked with PFAS standards, and two workflows are described: using FluoroMatch Flow as an all-in-one solution where users simply drag .d files onto the interface, and using Agilent MassHunter software for peak picking and FluoroMatch Modular for downstream steps. FluoroMatch Flow covers file conversion, peak picking, blank filtering, annotation, homologous series detection, and confidence reporting, and it generates interactive visualizations for users to verify findings and discover new PFAS. Results show a low false positive rate and false negative rate in dried blood spots (4% and 0%, respectively, using Profinder and FluoroMatch Modular). In addition, rarely screened PFAS were discovered in blood which have seldom been reported. The workflow using Profinder produced a smaller feature table, reducing artifact peaks and in-source fragments as compared to the FluoroMatch Suite peak picking, while maintaining similar coverage.

Introduction

The number of unknown PFAS in various environment and human samples is estimated to exceed several thousand homologues. Total adsorbable organic fluorine has suggested the presence of significantly more PFAS not identified through targeted screening.¹⁻³ Because the source, exposure, and toxicity are unknown for PFAS, and there is strong potential for PFAS to pose significant environmental and human health hazards, it is essential to characterize and identify the unknown portion of PFAS.² To address this challenge, high-resolution mass spectrometry provides the sensitivity and universality to cover a significant portion of PFAS measurements. The 6546 LC/Q-TOF provides the necessary sensitivity, dynamic range, resolution, and mass accuracy stability required to increase the coverage of PFAS when acquiring data. Furthermore, by using the Agilent iterative exclusion algorithm when acquiring data-dependent MS/MS, greater MS/MS coverage can be obtained. Because fragmentation is needed to confirm or approximate PFAS structure and determine whether a feature may or may not be a PFAS, this increased MS/MS coverage leads to increased PFAS coverage.

A current bottleneck is processing data, and researchers at Yale University have partnered with Innovative Omics and Agilent Technologies to release an open-source solution: FluoroMatch Suite⁴⁻⁶, which can streamline the process. This software covers file conversion, peak picking and alignment, blank filtering, annotation, data organization by homologous series and confidence, and data visualization for confirmation and discovery of unknowns. This application note demonstrates the application of FluoroMatch Flow using an entire open-source workflow, and separately, using results from Agilent MassHunter Profinder software in combination with FluoroMatch Modular, on dried blood spots acquired using a 6546 LC/Q-TOF. The use of dried blood spots is unique because dried blood spots are easy to obtain, transport, and store. Because nearly every newborn baby in the United States has blood spot cards collected, the resulting data can both be used to understand the wide range of PFAS humans are exposed to, and to monitor that exposure over time. In this application note, over 90 potential PFAS in dried blood spots were detected and annotated. Twenty one unlabeled PFAS were spiked onto cards to determine both the false positive and false negative rates of different software workflows. Using MassHunter Profinder, FluoroMatch Modular, and FluoroMatch Visualizer, 0% false negative and false positive rates were obtained.

Experimental

Reagents and chemicals

All reagents and solvents were HPLC or LC/MS grade.

Sample preparation

Sample preparation has been described in-depth in a previous publication.⁷ Briefly, blank whole blood (UTAK, product number 44600-WB(F)) was spotted onto blood spot cards (QIAGEN QIAcard FTA classic) and air dried in a fume hood for one hour at room temperature. An area of 491 mm² was sectioned from the dried blood spot (DBS) card and collected using stainless steel scissors that were precleaned with methanol (Fisher Scientific, HPLC grade). A similar-sized section of the blank portion of the card was also cut adjacent to the dried blood spot for filtering out any PFAS introduced from the card, sample handling, transport, storage, or acquisition. DBS cards were placed in individual 15 mL polypropylene Falcon tubes (Corning, product number 21008-918) and spiked with a mixture of 21 native PFAS standards (AccuStandard), and ¹³C-labeled internal standards (Wellington Laboratories) (Table 1). DBS were dried in capped tubes for 30 minutes at room temperature and then stored at -20 °C until extraction.

DBS sample extraction consisted of adding sodium hydroxide (Fisher Scientific, 20 mM) prepared in methanol (1 mL) to sample tubes. Samples were shaken for 30 minutes with four stainless steel beads (Spex SamplePrep, 2.8 mm), sonicated for 10 minutes, and centrifuged for 20 minutes (4,000 rpm) at room temperature. Resulting supernatants were transferred into 2 mL polypropylene vials (Agilent, part number 5191-8150) and were extracted two additional times (20 mM sodium hydroxide in 500 µL methanol). Pooled supernatant for each sample (approximately 1,500 µL per sample collected) was filtered three times using a precleaned polypropylene filter cartridge with 1,000 µL methanol (**Agilent Bond Elut Lipid Extraction, part number 5610-2041**) and evaporated to dryness using a positive pressure manifold (Agilent, part number **5191-4101**). Dried samples were reconstituted with 50 µL of methanol and 50 µL ultrapure water (Milli-Q Integral 5). The reconstituted sample was centrifuged at 14,000 rpm for 10 minutes at 10 °C. This supernatant (~ 90 µL) was transferred to an Agilent (part number **5190-2242**) polypropylene vial with insert positioned in a LC/MS vial and sealed with an Agilent (part number **5191-8151**) cap and stored at -20 °C. Extracted card blanks and dried blood spot samples were analyzed together with blank and standard-spiked solvent samples. To prepare a pooled extract for analysis, 50 µL of nine dried blood spot samples were evaporated to low volume and reconstituted with 200 µL of 1:1 methanol to water.

Table 1. Overview of the 21 PFAS native standards included in the dried blood spot analysis.

Chemical Class	Chain	Analyte	CAS	Abbreviation	Parent Ion Formula	Molecular Ion Exact Mass	Retention Time (min)
Fluorotelomers	4:2	Sodium 1H,1H,2H,2H-perfluoro-1-hexanesulfonate	27619-93-8	4:2 FTS	$C_6F_9H_4SO_2^-$	326.973	5.3
	6:2	Sodium 1H,1H,2H,2H-perfluoro-1-octanesulfonate	27619-94-9	6:2 FTS	$C_8F_{13}H_4SO_2^-$	426.969	7.4
	8:2	Sodium 1H,1H,2H,2H-perfluoro-1-decanesulfonate	27619-96-1	8:2 FTS	$C_{10}F_{17}H_4SO_2^-$	526.961	8.6
Perfluoroalkane Sulfonic Acids	4	Potassium perfluoro-1-butanesulfonate	29420-49-3	PFBS	$CF_3(CF_2)_3SO_3^-$	298.942	6.0
	5	Sodium perfluoro-1-pentanesulfonate	630402-22-1	PFPeS	$C_5F_{11}SO_3^-$	348.939	7.3
	6	Potassium perfluoro-1-hexanesulfonate (linear and branched)	3871-99-6	PFHxS	$C_6F_{13}SO_3^-$	398.937	7.8
	7	Sodium perfluoro-1-heptanesulfonate	21934-50-9	PFHpS	$C_7F_{15}SO_3^-$	448.933	8.4
	8	Potassium perfluoro-1-octanesulfonate (linear and branched)	2795-39-3	PFOS	$C_8F_{17}SO_3^-$	498.931	9.3
	9	Sodium perfluoro-1-nonanesulfonate	98789-57-2	PFNS	$C_9F_{19}SO_3^-$	548.926	9.8
	10	Sodium perfluoro-1-decanesulfonate	2806-15-7	PFDS	$C_{10}F_{21}SO_3^-$	598.925	10.0
Perfluoroalkyl Carboxylic Acids	4	Perfluoro-n-butanoic acid	375-22-4	PFBA	$CF_3(CF_2)_3COO^-$	212.979	2.7
	5	Perfluoro-n-pentanoic acid	2706-90-3	PFPeA	$CF_3(CF_2)_4COO^-$	262.975	4.7
	6	Perfluoro-n-hexanoic acid	307-24-4	PFHxA	$C_6F_{11}O_2^-$	312.973	5.9
	7	Perfluoro-n-heptanoic acid	375-85-9	PFHpA	$C_7F_{13}O_2^-$	362.970	7.2
	8	Perfluoro-n-octanoic acid	335-67-1	PFOA	$CF_3(CF_2)_5COO^-$	412.966	7.7
	9	Perfluoro-n-nonanoic acid	375-95-1	PFNA	$CF_3(CF_2)_6COO^-$	462.963	8.2
	10	Perfluoro-n-decanoic acid	335-76-2	PFDA	$C_{10}F_{19}O_2^-$	512.961	9.3
	11	Perfluoro-n-undecanoic acid	2058-94-8	PFUnA	$C_{11}F_{21}O_2^-$	562.957	9.9
	12	Perfluoro-n-dodecanoic acid	307-55-1	PFDoA	$C_{12}F_{23}O_2^-$	612.955	10.4
	13	Perfluoro-n-tridecanoic acid	72629-94-8	PFTTrDA	$C_{13}F_{25}O_2^-$	662.950	10.8
14	Perfluoro-n-tetradecanoic acid	376-06-7	PFTeDA	$C_{14}F_{27}O_2^-$	712.948	11.2	

Instrumentation

LC system

Agilent 1290 Infinity II LC, including:

- Agilent 1290 Infinity II high speed pump (G7120A)
- Agilent 1290 Infinity II multisampler with thermostat (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent InfinityLab PFC-free HPLC Conversion Kit (part number 5004-0006)

MS system

Agilent 6546 LC/Q-TOF with an Agilent Jet Stream technology source.

Methods

Liquid chromatography was used to separate PFAS on a 1290 Infinity II LC. Full LC method parameters are described in Table 2. Briefly, an analytical separation was carried out on an Agilent InfinityLab Poroshell 120 EC-C18 column with background delayed with an Agilent InfinityLab PFC delay column. Mobile phases consisted of 2 mM ammonium acetate in water and 95:5 acetonitrile:water. The gradient separation took place across 12 minutes with a ramp to 100% organic (Table 2). Dried blood spot card extracts (10 μ L) were injected onto the column, with a 0.4 μ L/min flow rate across the column, maintained at 40 °C.

Table 2. Chromatographic conditions.

Agilent 1290 Infinity II LC	
Parameter	Value
Analytical Column	Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 100 mm, 2.7 μm
Delay Column	Agilent InfinityLab PFC delay column, 4.6 × 30 mm
Column Temperature	40 °C
Injection Volume	10 μL
Autosampler Temperature	6 °C
Needle Wash	IPA:acetonitrile
Mobile Phase	A) 2 mM ammonium acetate in water B) 95:5 acetonitrile:water
Flow Rate	0.4 mL/min
Gradient Program	Time (min) B (%)
	0.0 15
	1.0 15
	1.5 25
	7.0 60
	10.0 100
	12.0 100
12.1 15	
Stop Time	12.5 min
Post Time	3 min

High-resolution mass spectrometry data were acquired on an Agilent 6546 LC/Q-TOF in negative ion mode across an *m/z* range of 40 to 1,000. Full MS method parameters are described in Table 3. Five repeated injections of samples were acquired using Agilent data-dependent iterative exclusion analysis for increased MS/MS coverage (iterative MS/MS). MS/MS spectra were acquired at collision energies of 10 and 40 V (Table 3).

Software

The Innovative Omics FluoroMatch Suite contains multiple software and workflow options, which can be implemented for PFAS data processing, annotation, and visualization.

In this study, two workflows were implemented and compared:

1. Using Agilent MassHunter Profinder for recursive peak picking, adduct, dimer, and other artifact reduction, with Microsoft Excel for blank filtering, and FluoroMatch Modular for annotation, scoring, and homologous series grouping
2. Using FluoroMatch Flow for peak picking, blank filtering, annotation, scoring, and homologous series grouping

In both cases, FluoroMatch Visualizer was used to visualize the resulting features, annotations, and fragmentation data.

Table 3. Agilent 6546 Q-TOF iterative auto MS/MS parameters.

Agilent 6546 LC/Q-TOF	
Parameter	Value
Gas Temperature	120 °C
Gas Flow	10 L/min
Nebulizer (psig)	25
Sheath Gas Temperature	390 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	2,000 V
Nozzle Voltage	0 V
Fragmentor	135 V
Skimmer	65 V
OctopoleRF Vpp	750
Reference Mass	<i>m/z</i> 119.03632, <i>m/z</i> 980.016375 (-)
MS and MS/MS Range	<i>m/z</i> 40 to 1,000 (150 to 1,200), (67 to 1,000)
Minimum MS and MS/MS Acquisition Rate	2 spectra/s (8 spectra/s, 3 spectra/s)
Isolation Width	Medium
Collision Energy	10, 40 eV
Maximum Precursors Per Cycle	3
Precursor Abundance-Based Scan Speed	Yes
Use MS/MS Accumulation Time Limit	Yes
Reject Precursors That Cannot Reach Target TIC	No
Threshold for MS/MS	1,000 or 0.001%
Active Exclusion Enabled	Yes
Purity	100%, 50%
Isotope Model	Common organic
Sort Precursors	By abundance only
Static Exclusion Ranges	Not used, but <i>m/z</i> 112.9856, 980.01638, and 119.0363 were excluded in the preferred list
Iterative MS/MS: Mass Error Tolerance	± 20 ppm
Iterative MS/MS: RT Exclusion Tolerance	± 0.1 min

In both workflows, the iterative exclusion data (five iterative injections of dried blood spot samples) were used for both peak picking and annotation. For both FluoroMatch Modular and FluoroMatch Flow, instructions on setting up and running the software are available as YouTube tutorials at:

<https://innovativeomics.com/software/>

(navigate to FluoroMatch or other software of interest and scroll down to the tutorials section).

1. For FluoroMatch Flow, download and install the software from innovativeomics.com/software.
2. Double-click **FluoroMatch_Flow.bat** to open the interface (Figure 1). Warning messages may indicate the wrong version of Java. 64-bit Java should be installed via the instructions in the manual and video tutorials.
3. Before importing files by dragging the vendor .d files onto the interface, files that do not follow a specific naming convention should be renamed. All files should end in `_Neg.d`, and files used for MS/MS annotation should have `ddMS2` in the name (regardless of whether they are targeted MS/MS data, iterative exclusion data, or traditional data-dependent data).

For full-scan data for peak picking, vendor files should be renamed to include target in the file name if they are being used in the untargeted peak picking step. The FluoroMatch Flow MZmine peak picking strategy has two steps. First, it uses file names with target in the name to determine all peaks in your samples in an untargeted fashion. Then, it targets these peaks across all samples (including the ones with

target in the name). In this instance, all files were used for the untargeted peak picking step and were labeled with target. Generally, 5 to 15 target files are recommended, which often will be pools or other reference samples which are expected to have the majority of PFAS. Therefore, the majority of peaks will be found to target across remaining samples. The more samples included, the more peaks will be determined, but the longer the software will take to run.

Note that in this instance, because the iterative exclusion data were also used for peak picking, the files were copied. One was named with `ddMS2` and `_Neg.d` in the name for annotation, while the other one was named with `target` and `_Neg.d` in the name for peak picking. As long as a minimum of 10 to 15 full scans are acquired across most peaks, MS/MS experiments can also be used for peak picking. Blank filtering parameters were set to 80th percentile of samples having to be greater than the average of the extraction blanks (blank area of dried blood spot card) plus three times the standard deviation of the extraction blanks.

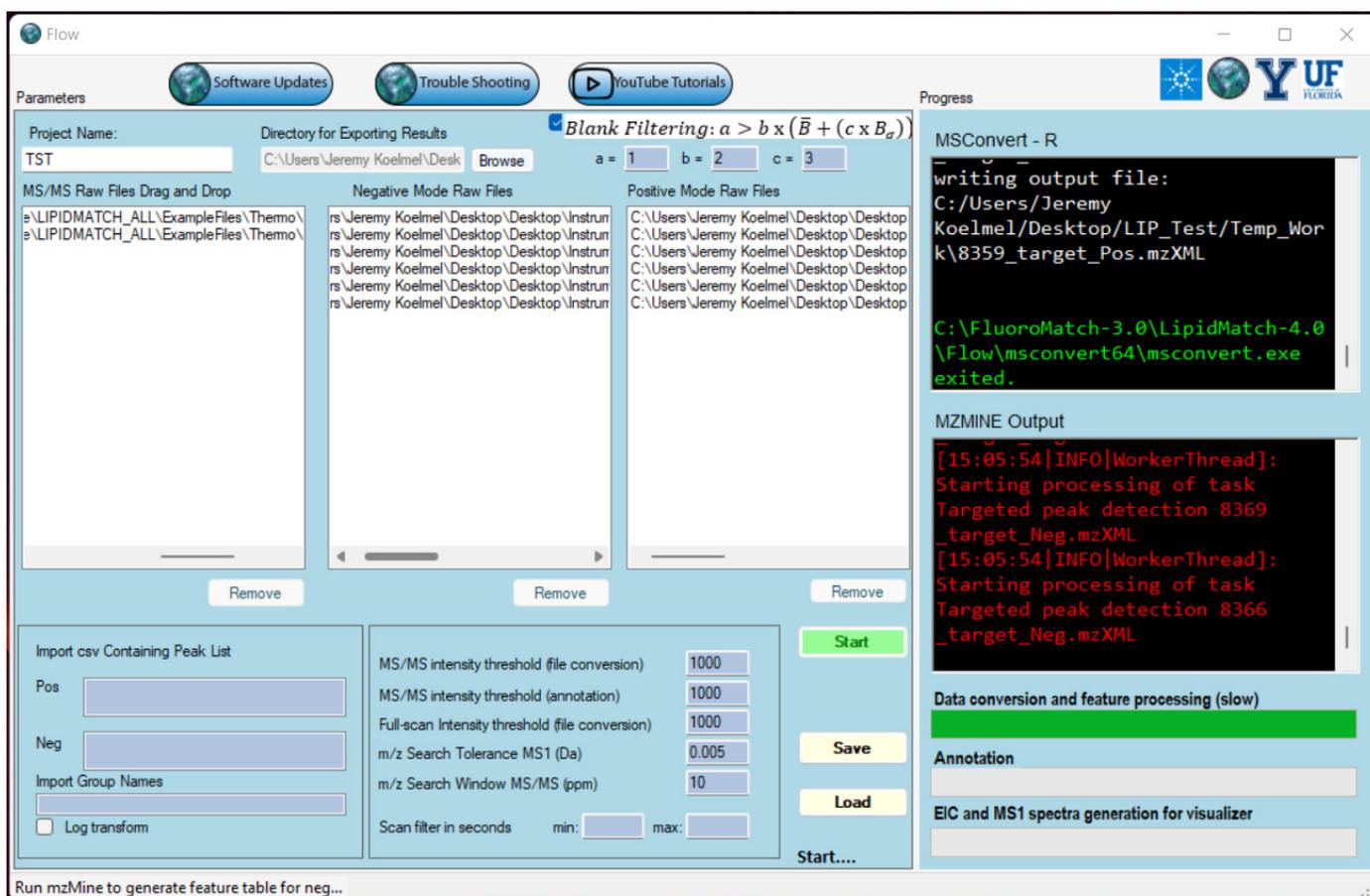


Figure 1. In the Innovative Omics FluoroMatch Flow interface, raw vendor files (.d in this case) are dragged onto the interface with the correct naming conventions.

Features not used in this application note that may also be useful are: statistical analysis, targeted peak detection for known compounds, and visualizations of EICs, MS1 spectra, isotope labeling, and statistics.

For MassHunter Profinder software, the parameters listed in Table 4 were used after selecting Batch Recursive Feature Extraction.

Table 4. Agilent MassHunter Profinder software parameters.

Profiler Parameter	Value
Use Peaks with Height \geq	600
Ion Species	-H, +Cl, +CH ₃ COO
Neutral Losses	H ₂ O, CO ₂ , SO ₃ , CO ₂ F, SO ₃ F
Limit Charge States To	1 to 2
Alignment RT Tolerance	0.1 min
Alignment Mass Tolerance	5 mDa / 20 ppm
MFE Score \geq	70
MFE Filter Matches (Depends on Experimental Design)	50% of files in at least one sample group
Expected Data Variation MS Mass	2.0 mDa, 5.6 ppm
Expected Data Variation MS Isotope Abundance	7.5%
MS/MS Mass	5.0 mDa, 7.5 ppm
Retention Time	0.115 min
Peak Filters, Absolute Height \geq	600 points
Score (Tgt) \geq	50
A Compound Must Satisfy the Checked Find by Ion Filter Criteria	50% of files in at least one sample group

After the files underwent recursive peak picking in Profinder, the results were exported as a CSV feature table. The m/z values (neutral masses) in a new column were converted to the $[M-H]^-$ m/z value as a required column for FluoroMatch Modular (as FluoroMatch Modular requires adduct m/z values, not neutral m/z values). A Microsoft Excel formula was used to subtract 1.00783 (H mass) and add 0.00055 (electron mass). A column title row ID was also added, and feature numbers were assigned in simple numerical order (1, 2, 3) from the first to last row of the Excel sheet, using the Excel fill-down operation. The resulting table was saved using "_Neg.csv" at the end of the filename. The five iterative injections were used to run msconvert (ProteoWizard tools) and converted to .ms2 format using the parameters in the manual for Agilent .d files. The only exception was that the threshold peak filter (absolute) was set to 30 counts. All .ms2 files and the feature table (.csv) were put into an input folder on the desktop.

The FluoroMatch Modular script in the FluoroMatch download was then opened in a text editor (FluoroMatch-2.6\FluoroMatch_Modular\FluoroMatch_Modular_2o6.r) and copied and pasted into R, version 3.3.3, included in the FluoroMatch distribution (FluoroMatch-2.6\Background_Files_FluoroMatch_Flow\FluoroMatch_Flow\R-3.3.3\bin\x64\Rgui.exe). Table 5 shows the parameters that were used with FluoroMatch Modular.

Table 5. Innovative Omics FluoroMatch Modular parameters.

FluoroMatch Modular Parameters	Value
m/z ppm Window (MS/MS Confirmation Criteria)	30 (\pm 15 ppm)
RT Window (for Aligning MS/MS Scans to Features)	0.1 min
Isolation Window (for Aligning MS/MS Scans to Features)	0.4 Da
Minimum Scans (for MS/MS Annotation)	1
Minimum Intensity (for MS/MS Annotation)	30

After running the two workflows (FluoroMatch Modular with Profinder, and FluoroMatch Flow), the resulting files were imported into FluoroMatch Visualizer: NegIDed_FIN.csv (final annotated feature table organized by homologous series and annotation confidence) and Neg_rawMSMS.csv (annotated MS/MS spectrum). Note that the Power BI FluoroMatch Visualizer file is found in the FluoroMatch Suite distribution (FluoroMatch-2.6\FluoroMatch_Visualizer\2022_05_01_Visualizer_v9_FIN.pbix). Microsoft Power BI Desktop can be downloaded and installed for free, and must be installed separately before use. In the Power BI FluoroMatch Visualizer file, the data were imported in the home tab of the Power Query editor, under Data Source Settings.

Note that the user analyzing the data (JPK) was blind to the fact that the nonlabeled standards were spiked into samples. Therefore, true false negative and false positive rates could be calculated for the software and manual review using FluoroMatch Visualizer.

Results and discussion

Discovery of new or rarely screened PFAS in serum using iterative MS/MS, state-of-the-art Q-TOF LC/MS technology, and the FluoroMatch Suite

After running the software both with and without Profinder (see Methods), and manual verification with FluoroMatch Visualizer, 29 PFAS across five homologous series were assigned with high confidence (Table 4). Of the 29 high-confidence annotations, 21 were spiked in standards, four were contaminants from standard solutions, and four were likely endogenous. FluoroMatch Visualizer results after using Profinder are shown in Figure 1. These series were identified as fluorotelomer perfluoroalkyl sulfonic acids (FTS), perfluoroalkyl carboxylic acids (PFCA), perfluoroalkyl ether carboxylic acids (PFECA), perfluoroalkyl sulfonic acids (PFSA), and perfluoroalkyl ether sulfonic acids. Of these, one C4 isomer of PFAS and all three PFECA were endogenous (Table 4). Some of these novel chemicals were discovered for the first time in human serum using the 6546 LC/Q-TOF and FluoroMatch Suite, including potential limitations and advantages of dried blood spots that have been previously described. Of the three PFECA, either the species were rarely observed, or were previously unobserved in serum. Using iterative MS/MS, 14% more features had MS/MS as compared to using a single data-dependent analysis alone (73 of 523 features with MS/MS). Thirty-nine of these 73 features had some PFAS-related MS/MS evidence (potential fluorine containing fragment).

Comparison of Profinder and FluoroMatch Flow (MZmine) results

After blank filtering, 1,921 features were detected in Profinder and 17,301 were detected using FluoroMatch Flow (which deploys MZmine and recursive peak picking in the background). The difference in the number of features is large, but could be attributed to how each software groups features. Profinder uses compound groups whereas MZmine reports individual features. Profinder's feature groups were used with FluoroMatch Modular and yielded a lower false negative rate (missed standard annotations) compared to FluoroMatch Flow. When combining FluoroMatch and Profinder, 100% of the spiked-in standards were annotated, suggesting a 0% false negative rate for PFAS with known structures. For FluoroMatch Flow, the C14 PFCA was not peak picked, and hence a 5% false negative rate (20/21) was calculated. Coverage of nonspiked-in standards was also similar for both software workflows. Out of the 29 manually verified PFAS discovered using FluoroMatch Flow, only two were not peak picked using Profinder, both of which were trace (not well-formed) peaks. These were a C6 PFSA isomer (contaminant from standards) and C4 PFECA isomer (likely endogenous, see Table 6).

Regarding false positives (when no manual validation was performed), Profinder (in combination with FluoroMatch Modular) outperformed FluoroMatch Flow. A single false positive was obtained using the Profinder workflow, determined as an annotated C4 PFCA isomer which did not follow the correct retention time and m/z pattern (Figure 2; Table 4). Hence, a false positive rate of approximately 4% was obtained (1/28). In contrast, without manual validation, FluoroMatch Flow annotated three compounds which did not follow the correct retention time and m/z trends, and annotated nine compounds (PFCs) which were in-source fragments. Hence, FluoroMatch Flow had a 30% false positive rate. It is important to note that when using FluoroMatch Visualizer to manually validate software annotations, there was a 0% false positive rate for both workflows. It is also important to note that both workflows had a 0% false positive rate for compounds automatically assigned with a level-2 Schymanski score (score A in FluoroMatch); false positives only occurred when considering members of a series with lower annotation confidence.

Table 6. Comparison of features with manually verified annotations from Agilent MassHunter Profinder software listed with Innovative Omics FluoroMatch Modular (FM) versus FluoroMatch Flow.

Chemical Class	Profinder + FM			FluoroMatch Flow		
	Formula	m/z	RT	Formula	m/z	RT
Perfluoroalkyl Carboxylic Acids (PFCA)	C ₄ HF ₇ O ₂ *	212.9792	2.0	C ₄ HF ₇ O ₂ *	212.9791	2.0
	C₄HF₇O₂	212.9792	4.6	C₄HF₇O₂	212.9792	4.6
	C ₅ HF ₉ O ₂ *	262.9758	4.3	C ₅ HF ₉ O ₂ *	262.9759	4.3
	C ₆ HF ₁₁ O ₂ *	312.9727	5.3	C ₆ HF ₁₁ O ₂ *	312.9727	5.3
	C ₇ HF ₁₃ O ₂ *	362.9696	6.1	C ₇ HF ₁₃ O ₂ *	362.9696	6.1
	C ₈ HF ₁₅ O ₂ *	412.9663	6.8	C ₈ HF ₁₅ O ₂ *	412.9662	6.8
	C ₉ HF ₁₇ O ₂ *	462.9630	7.5	C ₉ HF ₁₇ O ₂ *	462.9630	7.5
	C ₁₀ HF ₁₉ O ₂ *	512.9601	8.1	C ₁₀ HF ₁₉ O ₂ *	512.9599	8.1
	C ₁₁ HF ₂₁ O ₂ *	562.9565	8.8	C ₁₁ HF ₂₁ O ₂ *	562.9567	8.8
	C ₁₂ HF ₂₃ O ₂ *	612.9533	9.3	C ₁₂ HF ₂₃ O ₂ *	612.9532	9.3
	C ₁₃ HF ₂₅ O ₂ *	662.9503	9.7	C ₁₃ HF ₂₅ O ₂ *	662.9502	9.7
	C₁₄HF₂₇O₂*	712.9470	10.0			
Perfluoroalkyl Sulfonic Acids (PFSA)	C ₄ HF ₉ O ₃ S*	298.9429	5.1	SO₃HF	98.9556	5.8
	C ₄ HF ₉ O ₃ S	298.9429	5.4	C ₄ HF ₉ O ₃ S*	298.9429	5.1
	C ₅ HF ₁₁ O ₃ S*	348.9396	6.3	C ₄ HF ₉ O ₃ S	298.9429	5.4
	C ₆ HF ₁₃ O ₃ S*	398.9365	7.1	C ₅ HF ₁₁ O ₃ S*	348.9397	6.3
	C ₇ HF ₁₅ O ₃ S*	448.9332	7.8	C ₆ HF ₁₃ O ₃ S*	398.9364	7.1
	C ₈ HF ₁₇ O ₃ S [§]	498.9301	8.2	C₆HF₁₃O₃S[§]	398.9364	6.9
	C ₈ HF ₁₇ O ₃ S*	498.9302	8.5	C ₇ HF ₁₅ O ₃ S*	448.9333	7.8
	C ₉ HF ₁₉ O ₃ S*	548.9270	9.1	C ₈ HF ₁₇ O ₃ S [§]	498.9300	8.2
	C ₁₀ HF ₂₁ O ₃ S*	598.9237	9.6	C ₈ HF ₁₇ O ₃ S*	498.9301	8.5
				C ₉ HF ₁₉ O ₃ S*	548.9268	9.1
			C ₁₀ HF ₂₁ O ₃ S*	598.9236	9.5	
Perfluoropolyether Carboxylic Acids (PFCEA)	C ₄ HF ₇ O ₃	228.9743	3.1	C₄HF₇O₃	228.9739	3.5
	C ₅ HF ₉ O ₃	278.9710	4.7	C ₄ HF ₇ O ₃	228.9741	3.1
				C ₅ HF ₉ O ₃	278.9708	4.7
Perfluoropolyether Sulfonic Acids (PFESA)	C ₃ HF ₇ O ₄ S [§]	264.9409	4.6	CHF₃O₄S	164.9507	4.3
	C ₄ HF ₉ O ₄ S [§]	314.9381	5.8	C ₃ HF ₇ O ₄ S [§]	264.9411	4.6
				C ₄ HF ₉ O ₄ S [§]	314.9383	5.8
Fluorotelomer Perfluoroalkyl Sulfonic Acids (FTS)	C ₆ H ₅ F ₉ O ₃ S*	326.9741	5.0	C ₆ H ₅ F ₉ O ₃ S*	326.9740	5.0
	C ₈ H ₅ F ₁₃ O ₃ S*	426.9677	6.6	C ₈ H ₅ F ₁₃ O ₃ S*	426.9677	6.6
	C ₁₀ H ₅ F ₁₇ O ₃ S*	526.9614	7.9	C ₁₀ H ₅ F ₁₇ O ₃ S*	526.9613	7.9
Perfluorocarbons (PFC)				C₂F₉H	118.9926	4.6
				C₃F₉H	168.9894	2.0
				C₄F₉H	218.9861	4.3
				C₅F₉H	268.9830	5.3
				C₆F₉H	318.9797	6.1
				C₇F₉H	368.9765	6.8
				C₈F₉H	418.9732	7.5
				C₉F₉H	468.9701	8.1
			C₁₀F₉H	518.9667	8.7	

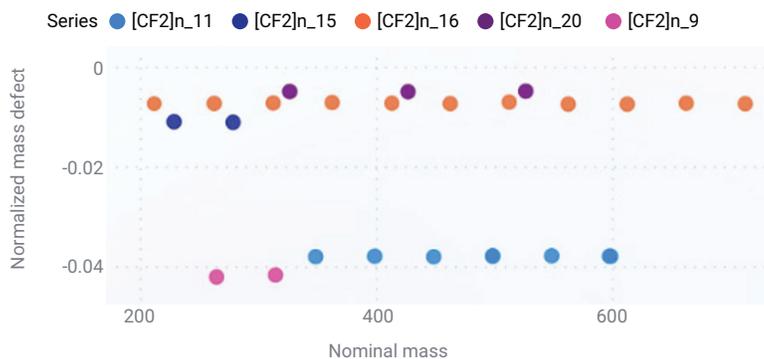
Bolded black text indicates those values that were unique to a workflow. **Blue** indicates features that were false positives (did not belong to a series or were in-source fragments (PFCs)).

* Standards spiked into the samples to denote false negative and false positive rates.

§ Annotated compounds that were contaminants from standards.

Note: If there is no symbol, compounds were likely endogenous in the DBS.

A Kendrick mass defect plots (normalized to CF₂)



B Retention time versus *m/z* plot

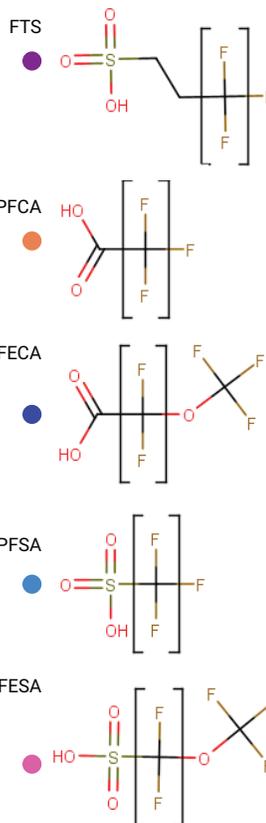
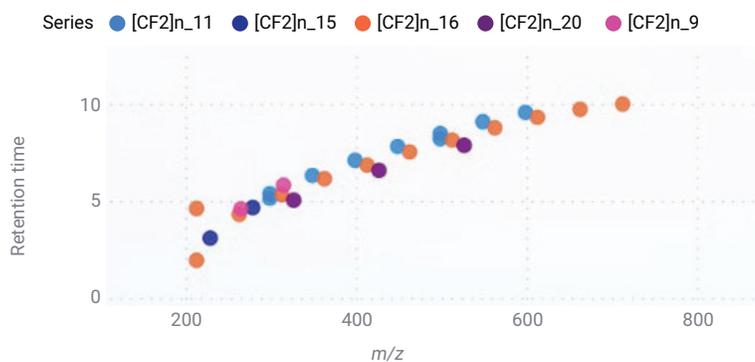


Figure 2. Mass defect normalized to CF₂ versus nominal mass plots (Figure 2A) and retention time versus *m/z* plots (Figure 2B) from the Innovative Omics FluoroMatch Visualizer for series with the highest confidence annotations. Data are from Agilent MassHunter Profinder software peak picking with FluoroMatch Modular annotation/homologous series grouping.

The main reduction in the false positive rate using Profinder was due to the fact the Profinder combines dimers, adduct, and other ESI-related artifacts to ensure good peak and isotopic behavior and eliminate duplicates. Profinder algorithms significantly simplify the resulting dataset without cost to comprehensive coverage. For example, a series was assigned as perfluorocarbons after peak picking using FluoroMatch Flow. It was determined that this series resulted

from in-source fragmentation of PFCAs (Figure 3), and Profinder had eliminated these artifacts. Figure 4 shows the simplification in the visualization of PFAS series after filtering by confident assignments and chemical series. While nearly the same number of confident series were detected in both workflows, FluoroMatch Flow shows a much higher number of standalone features which do not form series and other nonannotated features, suggesting higher false positives.

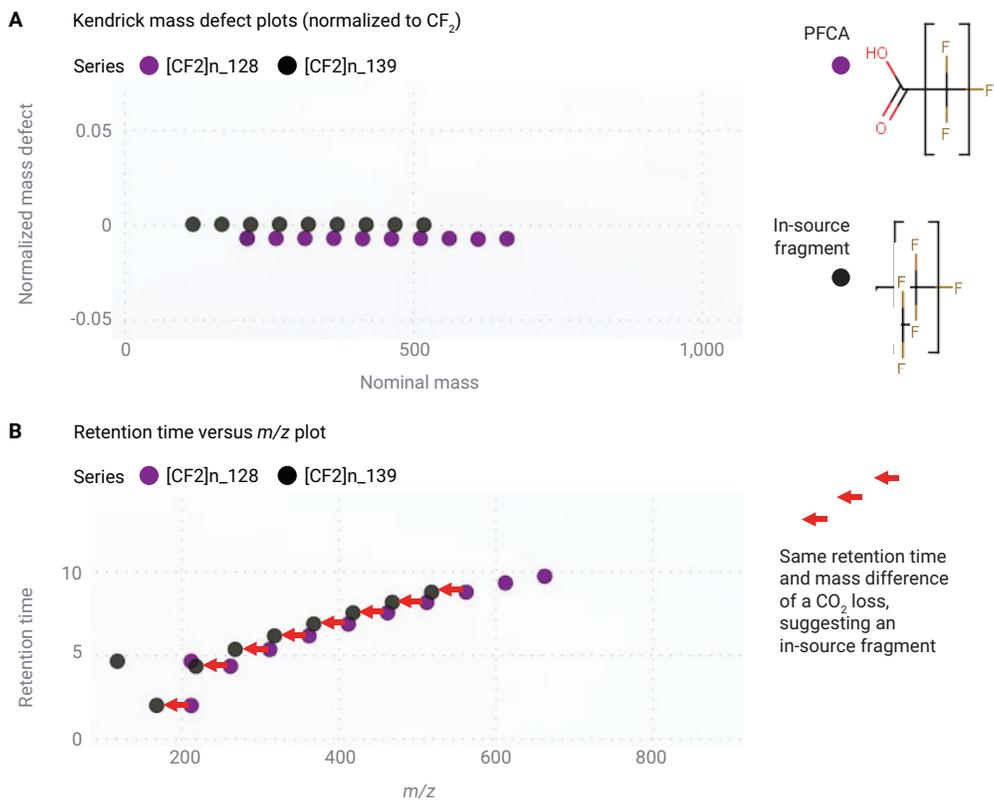


Figure 3. Agilent MassHunter Profinder software removes in-source artifacts (adduct, dimer, in-source fragment, etc.) to reduce the chance of false positives, including this misannotated PFC, which was an in-source fragment of PFCA. These plots are Innovative Omics FluoroMatch Visualizer outputs (red arrows manually added). In Profinder (Figure 2), no PFC features were retained after artifact removal.

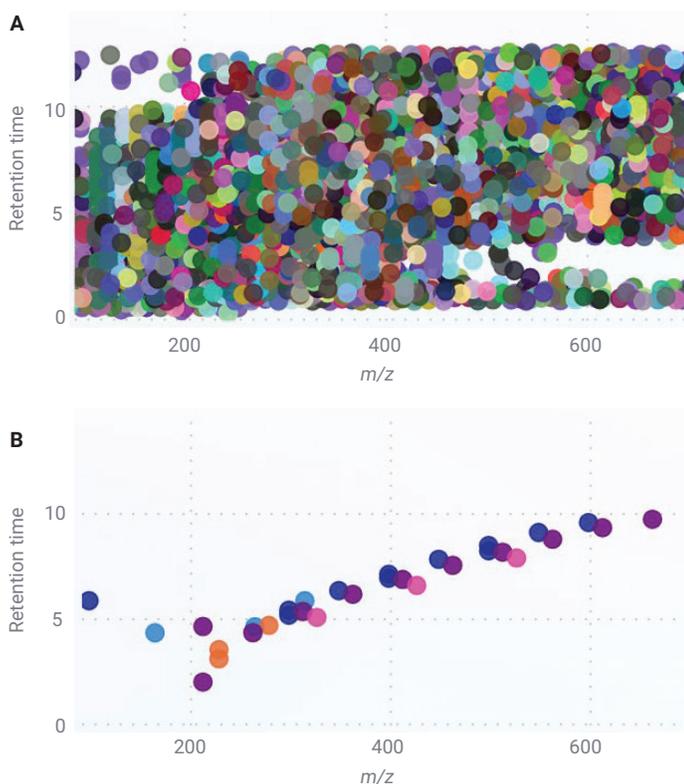


Figure 4. All features in retention time versus m/z space (A) versus only features in the PFAS-rich mass defect region, which formed confident series (B) assigned by FluoroMatch Modular using Agilent MassHunter Profinder software for peak picking.

Conclusion

Innovative Omics FluoroMatch Suite covers all steps of the PFAS data-processing workflow. When applied to the Agilent 6546 LC/Q-TOF for acquisition, a workflow was developed which was both comprehensive and accurate for discovering new and previously screened PFAS in dried blood spot cards. The user can take FluoroMatch Flow as an all-in-one solution by dragging their Agilent .d files onto the user interface and clicking **Start**. Alternatively, they can use Agilent MassHunter Profinder software with optimized parameters or the parameters described in this application note for peak picking, and use FluoroMatch Modular and FluoroMatch Visualizer for homologous series detection, annotation, and visualization of PFAS. Both workflows performed similarly, although Profinder algorithms significantly reduced the number of artifact peaks and false positives, improving the ease with which the data could be explored.

Dataset and software availability

FluoroMatch Suite can be downloaded at:
innovativeomics.com/software

The interactive dataset can be accessed at:
innovativeomics.com/datasets and
<https://medicine.yale.edu/lab/pollitt/projects/pfas/>

The raw 6546 (.d) spectral files can be accessed at:
<ftp://massive.ucsd.edu/MSV000091786/>
 (using FileZilla or other FTP client)

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RA45348.6518518518

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Printed in the USA, March 11, 2024
5994-7023EN