

Pushing the limits of standard-free PFAS screening

4D workflow including trapped ion mobility, Kendrick mass analysis, and *in-silico* fragment and CSS value predictions

Abstract

Accurate and comprehensive screening for the ever-growing number of per- and polyfluoroalkyl substances (PFAS) is challenged by many factors, including a lack of reference standards. A broad, standard-free approach to directly screen for PFAS in water samples was tested using the timsTOF Pro 2 system, applying Kendrick mass analysis to filter the data set. Paired with powerful MetaboScape®

data analysis tools, the 4D nature of the data collected, with high mass accuracy, near 95% MS/MS fragmentation coverage, and reproducible CCS ion mobility values, enabled confident identification of targeted PFAS, along with putative identification of untargeted PFAS. The workflow offers a promising direction for standard-free PFAS screening to support environmental protection.

Keywords:
PFAS, persistent organic pollutants (POPs), environmental screening, standard-free, timsTOF Pro 2, trapped ion mobility, MetaboScape, Kendrick mass analysis, CCS prediction

Introduction

Per- and polyfluoroalkyl substances (PFAS) have been a health and environmental concern for more than 70 years, shortly after their development for use in many household and industrial products [1-3]. At present, nearly 5000 compounds are included within the commercial PFAS family [4], all with at least one perfluorinated methyl group (-CF₃) or at least one perfluorinated methylene group (-CF₂-). As indicated by their classification as persistent organic pollutants (POPs) and so-called "Forever Chemicals," many of these compounds readily migrate and accumulate within the environment, often within ground and surface waters, and principally by this route have been found in humans, animals, and plants. Safe and effective means of removal and remediation have been challenged by their unique stability – a feature which initially led to their

widespread use based on their water and fire-retardant properties. Greater knowledge and increasing awareness of the harmful effects of the bioaccumulative exposure to many of these compounds have led to more stringent legislation for their use and disposal and have expanded monitoring requirements for an ever-growing list of PFAS, among other exposomic targets, within the EU, the US, and other parts of the world [5-9].

Successful PFAS screening may be challenged by many factors (Table 1). Both instrumental (hardware) and computational (software) approaches can contribute to addressing these challenges. The inclusion of 3D structure as a distinguishing feature has led to the development of instrumentation incorporating ion mobility spectrometry (IMS), which has proven to be a powerful tool

Table 1
Challenges to PFAS screening

The assessment of “total PFAS” requires the analysis of thousands of compounds that are highly diverse in mass and chemical properties, such as functional groups.
Degradation products are not uncommon, especially in longer PFAS.
Reference standards are not available for all compounds.
PFAS molecular databases and tandem mass spectral libraries are limited.
In addition to linear structures, PFAS isomers occur that cannot always be separated by HPLC.

in the detection of PFAS and other POPs in biofluids and environmental samples [10-15]. More recently, the convergence of (structure-based) theoretical and experimentally-derived identification characteristics has further developed modern detection capabilities for this growing class of hazardous pollutants.

A standard-free, 4D PFAS suspect screening workflow using the timsTOF Pro 2 instrument and MetaboScape software was tested for the direct analysis of water samples. The timsTOF Pro 2 system enabled multi-dimensional accurate mass compound separation and ultra-fast data-dependent MS/MS acquisition powered by PASEF. The use of trapped ion mobility separation improved resolution quality within the MS and MS/MS data pools, increasing the number of features detected while providing characteristic collision cross-section (CCS) values for each. PFAS signals were filtered from the complex data sets by applying Kendrick mass analysis. Based on the molecular structures of an assembled list of well-known and characterized PFAS contaminants, *in-silico* fragmentation patterns and CCS values were predicted and matched against the observed data, and corresponded to those which could be matched based on analytical standards. The uniquely rich nature of the data collected also enabled rapid

Experimental

Control and spiked (5 ng/mL) municipal water samples were kindly provided by the University of Amsterdam. Water samples (10 μ l) were analyzed in triplicate on the timsTOF Pro 2 instrument, using a VIP-HESI ion source (both Bruker Daltonics). Instrument

Table 2
Instrument parameters

MS		timsTOF Pro 2	
Source		VIP-HESI	
Ionization		ESI negative mode	
Acquisition mode		PASEF MS/MS, 100 ms ramp time, 2 PASEF MS/MS ramps per cycle	
Calibration		Automatic data recalibration using sodium formate for mass calibration and Agilent Tuning Mix for mobility calibration	
LC		Elute UHPLC with column oven (heat/cool), using Elute PFAS Kit*	
Column		Bruker Intensity Solo 1.8 C18-2 (2.1 x 100 mm)	
Column oven temp.		40°C	
Flow rate		0.4 mL/min	
Injection volume		10 μ l	
Mobile phase		A: H ₂ O/MeOH (99:1) + 5 mM ammonium acetate B: MeOH + 5 mM ammonium acetate	
Gradient	Time	Flow rate	Composition
	0.00 min	0.200 mL/min	4.0% B
	0.10 min	0.200 mL/min	4.0% B
	1.00 min	0.200 mL/min	18.3% B
	2.50 min	0.223 mL/min	50.0% B
	14.00 min	0.400 mL/min	99.9% B
	16.00 min	0.480 mL/min	99.9% B
	16.10 min	0.480 mL/min	4.0% B
	19.00 min	0.480 mL/min	4.0% B
	19.10 min	0.200 mL/min	4.0% B
20.00 min	0.200 mL/min	4.0% B	

* The Elute PFAS Kit (Bruker Daltonics part no. 1894795) includes a Restek PFAS delay column and PEEK tubing to improve the integrity of aqueous sample analysis, avoiding system-related PFAS contamination

segregation and putative identification of untargeted PFAS in spiked water samples, using MetaboScape tools to mine public databases for candidate compounds for comparisons of elemental compositions, fragmentation patterns, and CCS values. This high-resolution approach combines both speed and analytical separatory power with MetaboScape data handling and processing tools with great potential for broad screening of PFAS pollutants.

parameters are detailed in Table 2. All data analyses were made within MetaboScape (Bruker Daltonics).

The complete data curation workflow is shown in Figure 1.

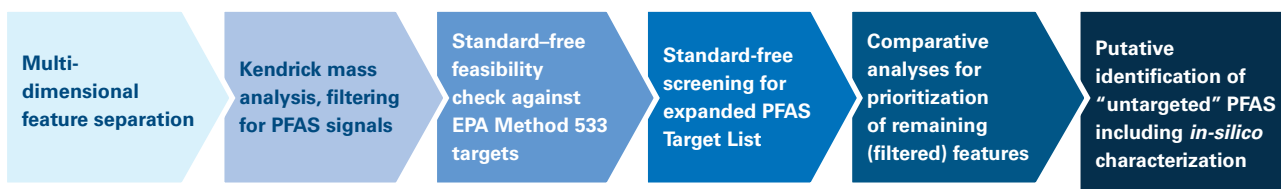


Figure 1
Overview of timsTOF Pro 2 and MetaboScape workflow for standard-free PFAS screening.

TIMS and the power of PASEF for high-quality MS and MS/MS

Trapped ion mobility spectrometry (TIMS) has changed the landscape for proteomic, metabolomic, and lipidomic screening and discovery workflows [10,11], and its potential utility for environmental analysis is clear within this exploratory study. The extra dimension of orthogonal compound separation based on ion mobility enables short LC run times with no loss in peak capacity, and its Parallel

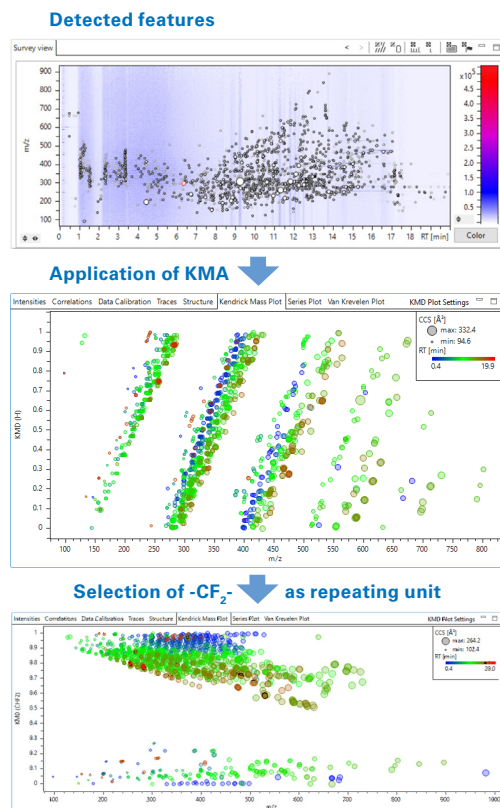
Accumulation Serial Fragmentation (PASEF) acquisition method [12] enables ultra-fast (>100 Hz) data-dependent MS/MS collection, with unsurpassed coverage from single injections. More than 1500 MS features were detected in the tested water samples, along with (automatically collected) MS/MS fragmentation data for nearly 95% of these MS signals.

Data filtering by Kendrick mass analysis – a legacy approach reapplied

Kendrick mass analysis [16], originally used for the analysis of petroleum hydrocarbons, capitalizes on the CF_2 moiety shared within PFAS. Kendrick mass analysis “redefines” the mass of the CF_2 repeating unit (exact mass 49.9968 amu) as an integer value (50 amu), resulting in a mass defect relative

to the true (IUPAC) mass. Homologous compounds differing only by the number of repeating CF_2 units have an identical Kendrick mass defect (KMD), and those within the same greater fluorohydrocarbon family have similar KMD values. Applying this recalibration to the collected data pool, the PFAS fluorohydrocarbons have similar Kendrick mass defects (between -0.25 and +0.1) and align horizontally when KMD is plotted against m/z (Figure 2). Of the ~1500 features detected in the tested water samples, ~200 candidate PFAS signals were indicated following this data filtering. All other features (from other compound classes) were deselected and excluded from further analysis.

Figure 2
Overview of complete feature detection and data filtering for PFAS via Kendrick mass analysis (KMA), plotting m/z vs. the Kendrick mass defect (KMD). Sphere colors in the Kendrick Mass Plot view indicate relative retention times and sphere sizes indicate relative CCS (collision cross section) values for each compound.



Testing the standard-free screening workflow

One well-known list of PFAS contaminants of concern within drinking water was defined in 2019 by US EPA Method 533 [17]. Reference data exists for these 25 compounds using many common analytical methods, typically including retention times (RTs), MS/MS data, and, in some cases, ion mobility-based CCS values.

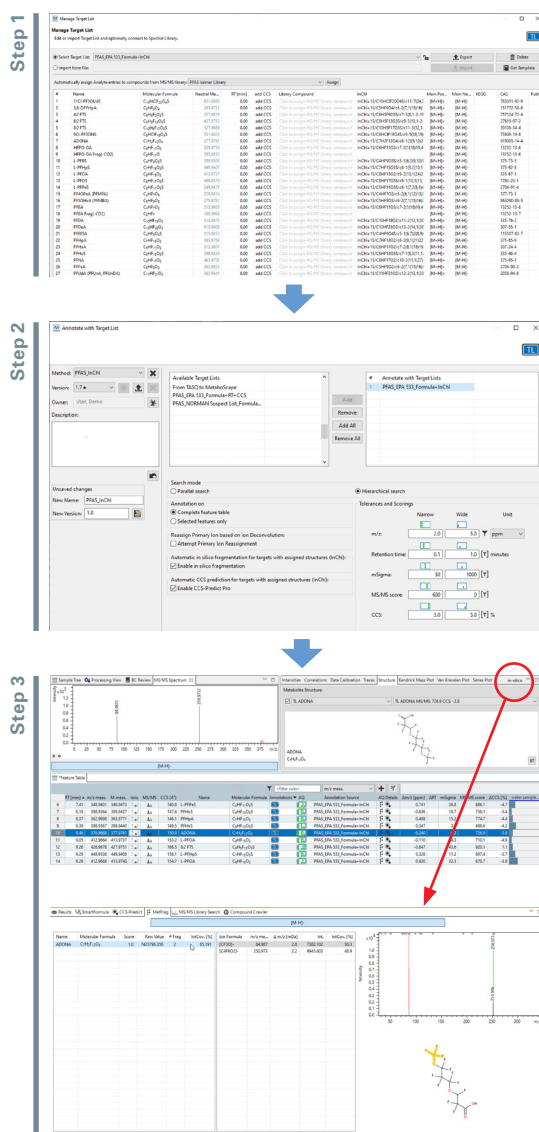
As discussed, separation of compounds by ion mobility capitalizes on differences in the 3D size and shape of molecular species of

interest. This physical property is an identifying characteristic that can also be predicted based on the spatial arrangements of the elements within. The recognition of the unique utility of ion mobility and the development of systems such as the timsTOF Pro 2, capable of reliable and reproducible assessment of this characteristic, has recently led to dramatic growth in reference databases and the inclusion of CSS values in public databases (e.g., PubChem) [13,18-20].

To test the feasibility of a standard-free screening approach, two so-called “Target Lists” were created within MetaboScape based on the PFAS listed with EPA Method 533. While both lists included compound names and molecular formulae, only one included experimentally-derived reference values for RT and CCS for each PFAS target. The other included the IUPAC International Chemical Identifier (InChI, <https://www.inchi-trust.org/>) for each, providing the 3-dimensional structural information necessary to generate *in-silico* fragments and to predict CCS values within MetaboScape using MetFrag and CCSPredict Pro, respectively (Figure 3). Using automated interrogation of the collected data pool, the same 19 PFAS were detected within the water samples using the experimentally derived or the “theoretical” Target List. The multi-dimensional nature of the data collected enabled confident identification with either approach, supporting further standard-free analyses within the data set (Figure 4).

Extending standard-free suspect screening for PFAS

Beginning with the PFAS defined within US EPA Method 533, an expanded Target List of 52 commonly monitored PFAS was created within MetaboScape. As in the feasibility test, InChI identifiers were included along with the target compound names and molecular formulae to enable *in-silico* derivation of identification parameters. A total of 46 monitored and regulated PFAS were detected within the water samples based on the analytical parameters of parent



Creation of Target List(s), including compound names, elemental composition and InChI structures

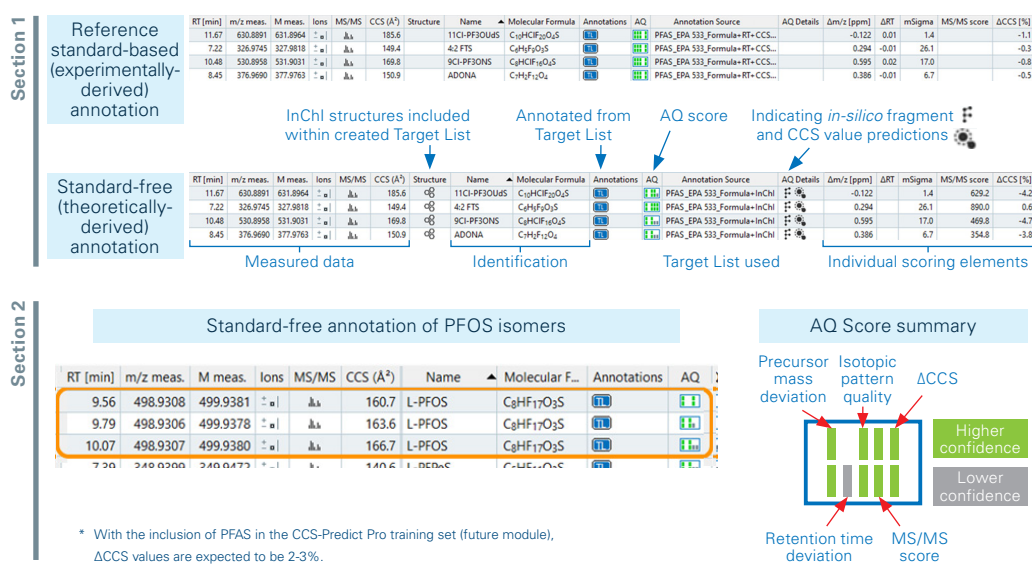
Compound annotation / interrogation of filtered data pool using one or more Target Lists

Feature annotation, with observed / *in-silico* fragment matching and CCS value comparison

Figure 3 Assignment of data features for suspect PFAS identification from a target list.

Figure 4
Comparison of experimentally and theoretically derived feature annotation.

Both annotations use chemical formulas for accurate mass determination (deviation noted as $\Delta m/z$) and isotopic pattern prediction (isotopic pattern quality quantitated as mSigma). The standard workflow includes reference data for ΔRT and ΔCCS calculations, while *in-silico* MS/MS fragmentation patterns (MetFrag) and CCS values (CCS-Predict Pro) are used for the standard-free workflow. AQ (Annotation Quality) score thresholds may be customized by the user. ΔCCS values are slightly higher* for the standard-free workflow.



Untargeted PFAS identification via MetaboScope

The putative identification of additional PFAS within the remaining (unannotated, filtered) signals focused on mass features of statistical significance between the two groups of water samples (spiked and control). Within MetaboScope, automated t-test based comparisons enabled feature prioritization in support of a PFAS suspect discovery workflow. Elemental compositions of features of interest were calculated using SmartFormula, considering mass accuracy and the isotopic pattern observed. CompoundCrawler was then used to search public databases (including PubChem, ChemSpider, and ChEBI) for structures matching the calculated elemental compositions. As with the known Target List,

in-silico fragmentation data and theoretical CCS values for candidate structures were generated using MetFrag and CCSPredict Pro, respectively. By following this 4D workflow, additional PFAS that were not included in the suspect Target List can be putatively identified (Figure 5). A secondary screening using characteristic PFAS fragments (e.g., for branched or linear forms) can support further target discernment.

The described MetaboScope-driven comparative analyses could be used to survey PFAS profiles at different collection points or over time, assess incident-driven contaminations, or to evaluate the efficacy of PFAS removal and remediation schemes.

Discussion

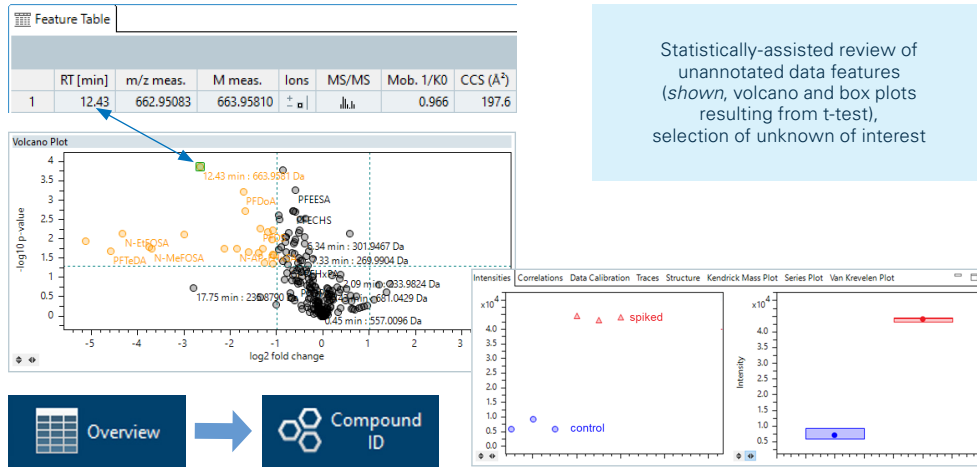
A four-dimensional approach to find "forever" PFAS - today and tomorrow

Accurate and comprehensive screening of the diverse and growing classes of PFAS present within environmental samples is challenged by many factors, including a lack of reference standards. The physical (hardware-driven) separatory dimensions of the timsTOF Pro 2 system combine to deliver highly resolved and accurate mass MS signals along with excellent MS/MS quality and coverage, while reliably and reproducibly providing characteristic CCS values for the PFAS pollutants. Coupled with the data curation capabilities within the MetaboScope software - including the use of Kendrick mass

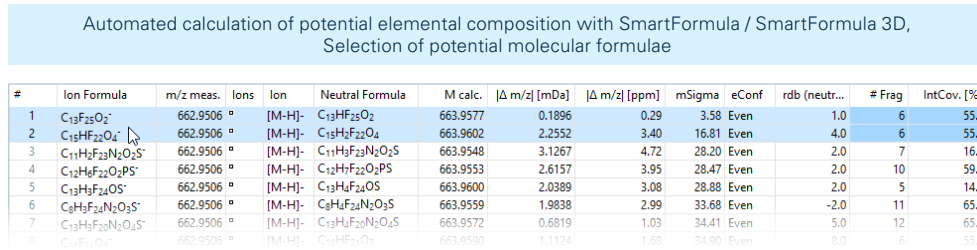
analysis and tools for *in-silico* prediction - both suspected and unexpected PFAS could be detected within the tested water samples.

This standard-free workflow offers a promising approach for rapid PFAS screening in various environmental samples, with the added advantage of supporting high-throughput sample analyses with short gradient times and ultra-rapid MS/MS data collection. This study was designed for a qualitative evaluation rather than a quantitative approach; however, preliminary sensitivity assessments indicate detection sensitivity for most PFAS between

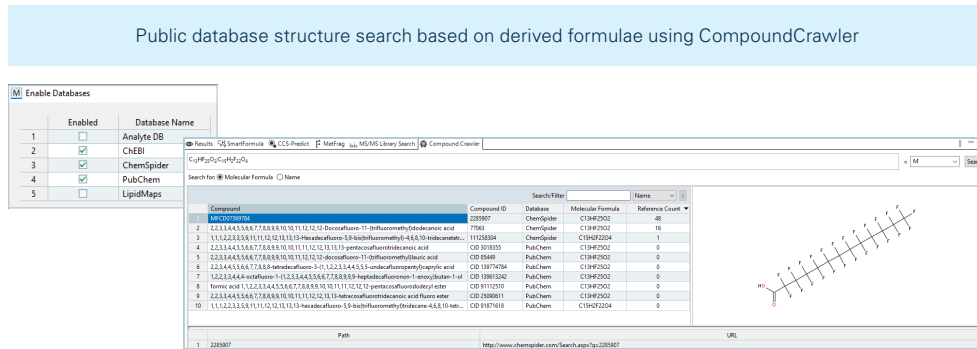
Step 1



Step 2



Step 3



Step 4

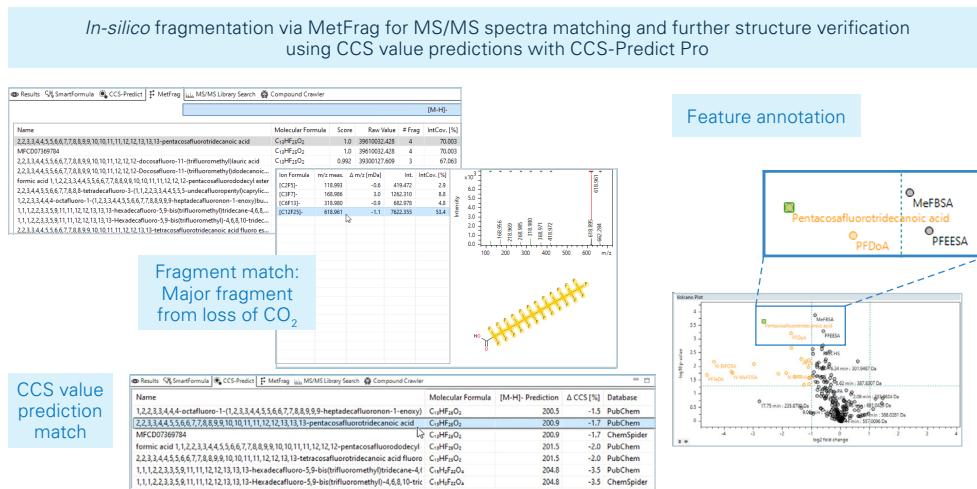


Figure 5 "Untargeted" PFAS identification workflow.

2 and 100 ng/L in direct analyses of higher sample volumes (200 μ L). Sample enrichment / concentration, for example, via on-line extraction (OLE), prior to analysis is common practice in many pollutant detection schemes. This enables even larger sample volumes (e.g., 1 mL) and thus higher sensitivity.

As could be expected from such a broad class of compounds, the detection of all PFAS using a single set of instrument conditions may be a challenging task, however, the collection speed and data-rich nature of every potential iteration of analysis, and the multiple data mining tools within MetaboScape, easily support exploratory screening schemes. As previously mentioned, isomeric forms are detected using this multi-dimensional workflow (e.g., Figure 4, Section 2, left). As isomers share multiple fragment ions, however, a high peak resolution is mandatory to obtain clean, compound-specific, MS/MS data required for confident fragment pattern matching against *in-silico* fragments or spectral libraries. Alternate instrument conditions with higher mobility resolution

have previously been demonstrated to be successful for the separation of co-eluting PFOS isomers [21].

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Conclusions

- A standard-free screening workflow using trapped ion mobility spectrometry enabled the detection of multiple PFAS pollutants in tested water samples.
- Separation by trapped ion mobility using PASEF provided a broad pool of high-quality MS and MS/MS data, with MS/MS coverage \approx 95%. Further, the CCS values determined provided a valuable compound characteristic for identification.
- The application of Kendrick mass analysis, filtering the data pool for mass defects due to the shared CF_2 moieties, reduced data complexity and has utility for unknown and exploratory PFAS screening.
- In targeted searches for the PFAS included within EPA Method 533, the same PFAS could be identified using Target Lists created with experimentally-derived (standard-based) and *in-silico* (standard-free) reference data, demonstrating the feasibility of the workflow.
- Forty-six governmentally monitored PFAS were rapidly identified in the tested water samples from an extended Target List created using structural information derived from InChI coding, with confident identification based on 4D feature differentiation.
- Statistical analyses using t-tests within MetaboScape permitted clear differentiation between the control and spiked water samples for the putative identification of additional PFAS using calculation of elemental compositions, structure search, and prediction of *in-silico* fragments and CCS values.
- This workflow shows excellent potential to push the analytical limits for standard-free PFAS screening to support improved environmental protection.

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