Supplementary materials of the Manuscript: "Development of a comprehensive two-dimensional liquid chromatographic mass spectrometric method for the non-targeted identification of poly- and perfluoroalkyl substances in aqueous film-forming foams." - By Renai et al.

S1. Chemicals and reagents

Table S1 − Composition details of the Perfluorinated Native Compound Standard mixture (PFC-24-10X) purchased by AccuStandard. Numbering for results discussion (#), InChiKey identification code, molecular weight (MW), molecular formula, and exact mass of the pseudo-molecular ion of each compound are also reported. Perfluoro-n-hexanoic acid (PFHxA), perfluoro-n-heptanoic acid (PFHpA),per-fluoro-n-octanoic acid (PFOA), Perfluoro-n-nonanoic acid (PFNA), Perfluoro-n-decanoic acid (PFDA), perfluoro-n-undecanoic acid (PFUnDA), perfluoro-n-dodecanoic acid (PFDoDA), perfluoro-n-tridecanoic acid (PFTrDA), perfluoro-n-tetradecanoic acid (PFTeDA), N-methylperfluorooctanesulfonamidoacetic acid (Me-FSA), N-ethylperfluorooctanesulfonamidoacetic acid (Et-FSA), Perfluoro-n-butanoic acid (PFBuA), perfluoro-n-pentanoic acid (PFPeA), perfluoro-1-butanesulfonic acid (PFBuS), perfluoro-1-pentanesulfonic acid (PFPeS), perfluoro-1-hexanesulfonic acid (PFHxA), perfluoro-1-heptanesulfonic acid, (PFHpS), perfluoro-1-octanesulfonic acid (PFOS), perfluoro-1-nonanesulfonic acid (PFNS), perfluoro-1-decanesulfonic acid (PFDS), 1H,1H,2H,2H-perfluoro-1-hexanesulfonic acid (4:2-FTS), 1H,1H,2H,2H-perfluoro-1-octanesulfonic acid (6:2-FTS), 1H,1H,2H,2H-perfluoro1-decanesulfonic acid (8:2-FTS), and perfluorooctane sulfonamide (PFOSA).

Compound	#	InChIKey	MW	Formula	$[M-H]^-$ mass
PFHxA	1	PXUULQAPEKKVAH-UHFFFAOYSA-N	314.05	C6HF11O2	268.9825*
PFHpA	2	ZWBAMYVPMDSJGQ-UHFFFAOYSA-N	364.06	C7HF13O2	318.9793*
PFOA	3	SNGREZUHAYWORS-UHFFFAOYSA-N	414.07	C8HF15O2	368.9761*
PFNA	4	UZUFPBIDKMEQEQ-UHFFFAOYSA-N	464.08	C9HF17O2	418.9729*
PFDA	5	PCIUEQPBYFRTEM-UHFFFAOYSA-N	514.08	C10HF19O2	468.9697*
PFUnDA	6	SIDINRCMMRKXGQ-UHFFFAOYSA-N	564.09	C11HF21O2	518.9665*
PFDoDA	7	CXGONMQFMIYUJR-UHFFFAOYSA-N	614.1	C12HF23O2	568.9633*
PFTrDA	8	LVDGGZAZAYHXEY-UHFFFAOYSA-N	664.1	C13HF25O2	618.9601*
PFTeDA	9	RUDINRUXCKIXAJ-UHFFFAOYSA-N	714.11	C14HF27O2	668.9569*
Me-FSAA	10	QNDHIRFIMVNHBN-UHFFFAOYSA-N	571.21	C11H6F17NO4S	569.9668
Et-PFOSAA	11	QBBNOVJQZHTCNZ-UHFFFAOYSA-N	621.22	C12H6F19NO4S	619.9636
PFBuA	12	YPJUNDFVDDCYIH-UHFFFAOYSA-N	214.04	C4HF7O2	168.9889*
PFPeA	13	CXZGQIAOTKWCDB-UHFFFAOYSA-N	264.05	C5HF9O2	218.9857*
PFBuS	14	JGTNAGYHADQMCM-UHFFFAOYSA-N	300.1	C4HF9O3S	298.9425
PFPeS	15	ACEKLXZRZOWKRY-UHFFFAOYSA-N	350.1	C5HF11O3S	348.9393
PFHxS	16	QZHDEAJFRJCDMF-UHFFFAOYSA-N	400.11	C6HF13O3S	398.9360
PFHpS	17	OYGQVDSRYXATEL-UHFFFAOYSA-N	450.12	C7HF15O3S	448.9329
PFOS	18	YFSUTJLHUFNCNZ-UHFFFAOYSA-N	500.13	C8HF17O3S	498.9297
PFNS	19	MNEXVZFQQPKDHC-UHFFFAOYSA-N	550.13	C9HF19O3S	548.9265
PFDS	20	HYWZIAVPBSTISZ-UHFFFAOYSA-N	600.14	C10HF21O3S	598.9233
Hx-FTS	21	TXGIGTRUEITPSC-UHFFFAOYSA-N	328.15	C6H4F9O3S	326.9737
Oc-FTS	22	VIONGDJUYAYOPU-UHFFFAOYSA-N	428.17	C8H4F13O3S	426.9674
D-FTS	23	ALVYVCQIFHTIRD-UHFFFAOYSA-N	528.18	C10H4F17O3S	526.9610
PFOSA	24	RRRXPPIDPYTNJG-UHFFFAOYSA-N	499.15	C8H2F17NO2S	497.9457

*most intense ion corresponding to $[M-H-CO2]^-$ in-source fragmentation.

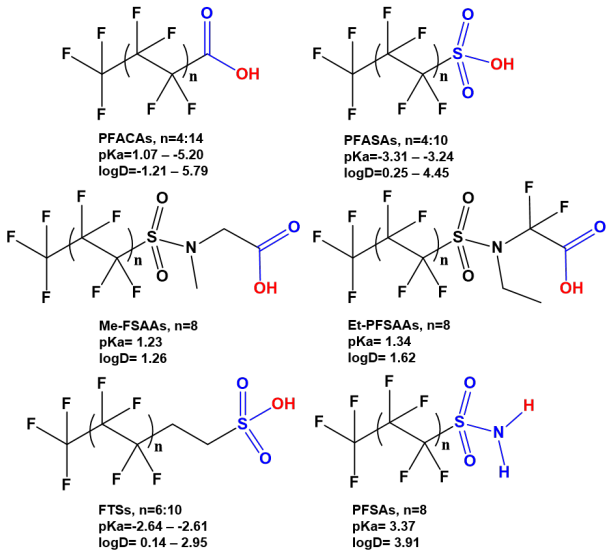

Figure S1 − Molecular structures and physicochemical properties of the perfluoroalkyl carboxylic acids (PFACAs), perfluoroalkyl sulfonic acids (PFASAs), N-methylperfluorosulfonamidoacetic acids (Me-FSAAs), N-ethylperfluorosulfonamidoacetic acids (Et-FSAAs), fluorotelomer sulfonic acids (FTSs), and perfluoroalkyl sulfonamides (PFSAs), occurring inside the Perfluorinated Native Compound Standard mixture (PFC-24-10X) purchased by AccuStandard, listed in Table S1. Ionic moieties are highlighted in blue, whereas the H-donor functions are in red. Physicochemical data and structures were calculated by the Chemicalize software (https://chemicalize.com).

S2. Chromatographic conditions for the individual optimization of 1D and 2D

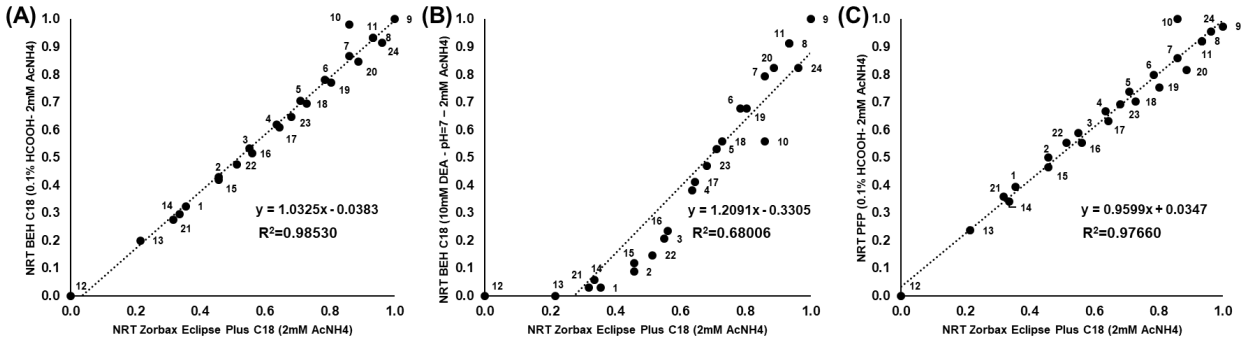

Figure S2 − Normalized retention time (NRT) scatter plots and regression trend lines of the investigated RPLC×RPLC conditions using the PFAS training set. The equation of the regression trend line and the relative correlation coefficient (R2) are also displayed in each plot. The A and B eluents and elution program are the same reported for setup 1 in Table S2. Acquity BEH C18 (100×2.1 mm, 1.7 µm) and Kinetex PFP (100×2.1 mm, 1.7 µm) were purchased from Waters (Millford, MA, USA) and Phenomenex (Torrance, California, USA), respectively. 1=PFHxA, 2=PFHpA, 3=PFOA, 4=PFNA, 5=PFDA, 6=PFUnDA, 7=PFDoDA, 8=PFTrDA, 9=PFTeDA, 10=Me-FSA, 11=Et-FSA, 12=PFBuA, 13=PFPeA, 14=PFBuS, 15=PFPeS, 16=PFHxS, 17=PFHpS, 18=PFOS, 19=PFNS, 20=PFDS, 21=Hx-FTS, 22=Oc-FTS, 23=D-FTS, 24=PFOSA.

Table S2 − 1D-LC setups investigated to evaluate the orthogonality of the selected stationary phases in respect of the training set of PFASs. %B = percentage of the organic modifier during gradient elution. %C = percentage of the 1M AcNH4 aqueous solution during the gradient elution.

Setups	Analytical Column	Mobile Phases	Flow rate (mL/min)	Time (min)	%B	%C
1	Zorbax Eclipse Plus C18	A: 2mM AcNH4 B: ACN 2mM AcNH4	0.4	0.00-1.00	20	0
				1.00-17.00	100	0
				17.00-19.00	100	0
				19.00-20.00	20	0
				20.00-25.00	20	0
2	Acclaim WAX-1	A: Water 20mM AcNH4 (pH 5.5) B: ACN:H2O 9:1 20mM AcNH4	0.4	0.00-1.00	45	0
				1.0-21.00	100	0
				21.00-22.0	100	0
				22.0-22.5	45	0
				22.5-28	45	0
3	Atlantis C18 AX	A: Water 20mM AcNH4 (pH 5.5) B: ACN:H2O (9:1 20mM AcNH4	0.4	0.00-1.00	45	0
				1.0-21.00	100	0
				21.00-22.0	100	0
				22.0-22.5	45	0
				22.5-28	45	0
4	Acclaim WAX-1	A: Water 20mM AcNH4 (pH 5.5) B: ACN:H2O 9:1 20mM AcNH4	0.4	0.00-1.00	65	0
				1.0-21.00	100	0
				21.00-22.0	100	0
				22.0-22.5	65	0
				22.5-28	65	0
5	Acclaim WAX-1	A: Water (pH 5.5) B: ACN C: 1M AcNH4 solution	0.4	0.00-1.00	45	1
				1.0-21.00	95	5
				21.00-22.0	95	5
				22.0-22.5	45	1
				22.5-28	45	1

S3. SAFD input parameters and 2D feature detection algorithm

The on-purpose SAFD-based algorithm for 2D feature detection was developed for processing the acquired 2D-LC-HRMS data for both method development and sample analyses. First, the raw 2D-LC-HRMS files were converted into .mzXML open format via MSConvert provided by the ProteoWizard package. Briefly, the number of scans to be converted was set to remove the system dead volume and re-equilibration time according to the setups used (see Table S2 and Table 1), setting a peak intensity threshold (absolute intensity) of 1.0×10^6. Subsequently, the files were processed using the SAFD algorithm applying the input parameters reported in **Table S3**. As an outcome of this first step, a feature list of all the identified *m/z* at a given t_R of the first dimension is obtained, in which each row represents a potential 2D feature occurring in one or more modulations. In the latter case, the same *m/z* is repeated in the feature list with a different first dimension t_R according to the set modulation time (e.g., 1 min), creating redundancies that hamper the correct feature detection. Thus, the 2D features that are eluted in two or more modulation were grouped to extract a "unique" feature implementing the following steps in the script: (i) identification of the features according to the defined mass error on the measured exact mass (e.g., 0.02 Da for 100-1000 mass range with a 35k resolution), (ii) removal of features occurring in too far away or (iii) in too many modulations throughout the entire first dimension analysis time. (iv) The grouped 2D features are then filtered to keep only the row with the maximum intensity, from which the first and second dimension adjusted retention times (t_{R1D} and t_{R2D}, respectively) are calculated using the following equations.

$$t_{R1D} = t_{Ri} - t_{Dwell2D}$$
$$t_{R2D} = t_{R1D} - (t^*_{R1D})$$

Where t_{Ri} is the retention time of the first dimension extracted using SAFD algorithm, $t_{Dwell2D}$ is the system delay on the second dimension, and t^*_{R1D} is the defect rounded t_{R1D}. A new list is then built describing the identified 2D features by their exact mass, t_{R1D}, t_{R2D}, maximum intensity, and the modulation times in which they occur. The obtained 2D feature list was used to build the scatter plots (t_{R1D} vs t_{R2D}) representing the two-dimensional separation space.

Table S3 – Input parameters used for processing spectra files with SAFD algorithm.

Input	Description	Values
mz_thresh	The *m/z* range to be imported	80-1000
int_thersh	Total ion current minimum absolute intensity for the datapoint to be imported	1.0E6
max_numb_iter	The maximum number of iterations performed by the algorithm	1.0E3
res	The mass resolution of the instrument	35000
max_t_peak_w	The maximum number of scans within a chromatographic feature	500
min_ms_w	The minimum peak width in the mass domain	0.01
r_thresh	The R2 threshold for the Gaussian fit	0.75
min_int	The minimum intensity for the features to be detected	1.0E6
sig_inc_thresh	The parameter for the detection of the overlapping features in the time domain	5
S2N	The signal to noise ratio for the feature list filtering set as an integer	3
min_peak_w_s	Remove the features that have less seconds than this threshold	3

S4. 2D-LC-MS/MS method development

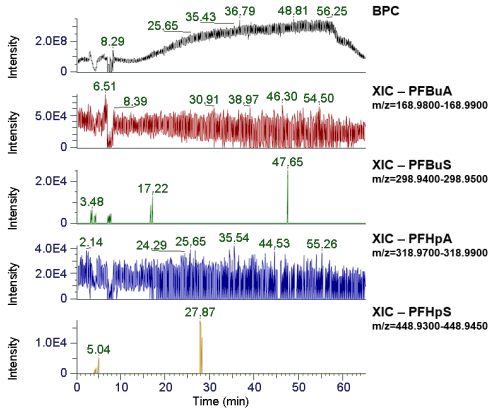

Figure S3 – Evalaution of system contamination by a blank injection (water/methanol 80/20, *v/v%*). BPC is displayed together with the XICs of PFBuA, PFBuS, PFHpA, and PFHpS as representative PFASs for contamination assessment.

The predicted peak capacity of the setups investigated for the development of the method was obtained as reported below. First, peak capacity values on the first and second dimensions (${}^{1D}n_c$ and ${}^{2D}n_c$, respectively) were calculated using the following equations.

$$ {}^{1D}n_c = 1 + \frac{{}^{1D}t_g}{w_1}; \quad {}^{2D}n_c = 1 + \frac{{}^{2D}t_g}{w_2} $$

$1t_g$ and $2t_g$ are the gradient times on the two dimensions (see Table 1), and w_1 and w_2 are the averaged baseline peak widths in first and second dimension, respectively. In detail, baseline peak widths were extracted as the difference between the maximum and minimum t_R inside the SAFD report of 1D and 2D-LC-HRMS data acquired on the standard mixture of the training set of PFASs.
These values were then set into the equation for the calculation of the predicted peak capacity ($n'_{c,2D}$) of the system, as reported by Li and co-workers.

$$ n'_{c,2D} = \frac{{}^{1D}n_c \cdot {}^{2D}n_c}{\sqrt{1 + 3.35\left(\frac{{}^{2}t_c \cdot {}^{1}n_c}{{}^{1}t_g}\right)^2}} $$

Where $2t_c$ is the modulation time adopted as reported in Table 1.

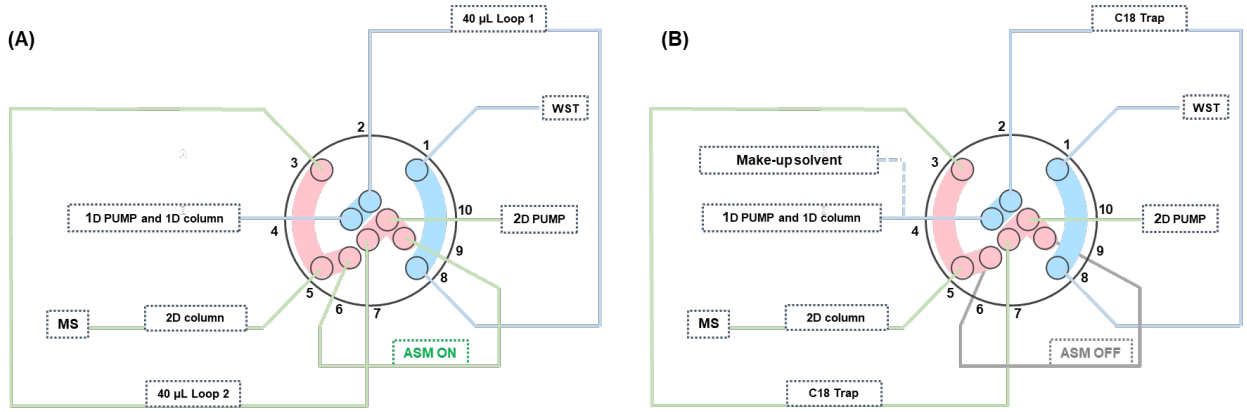

Figure S4 − 2D-LC valve system adopted for the modulation strategies here investigated. (A) Active solvent modulation (ASM) and (B) stationary phase assisted modulation (SPAM).

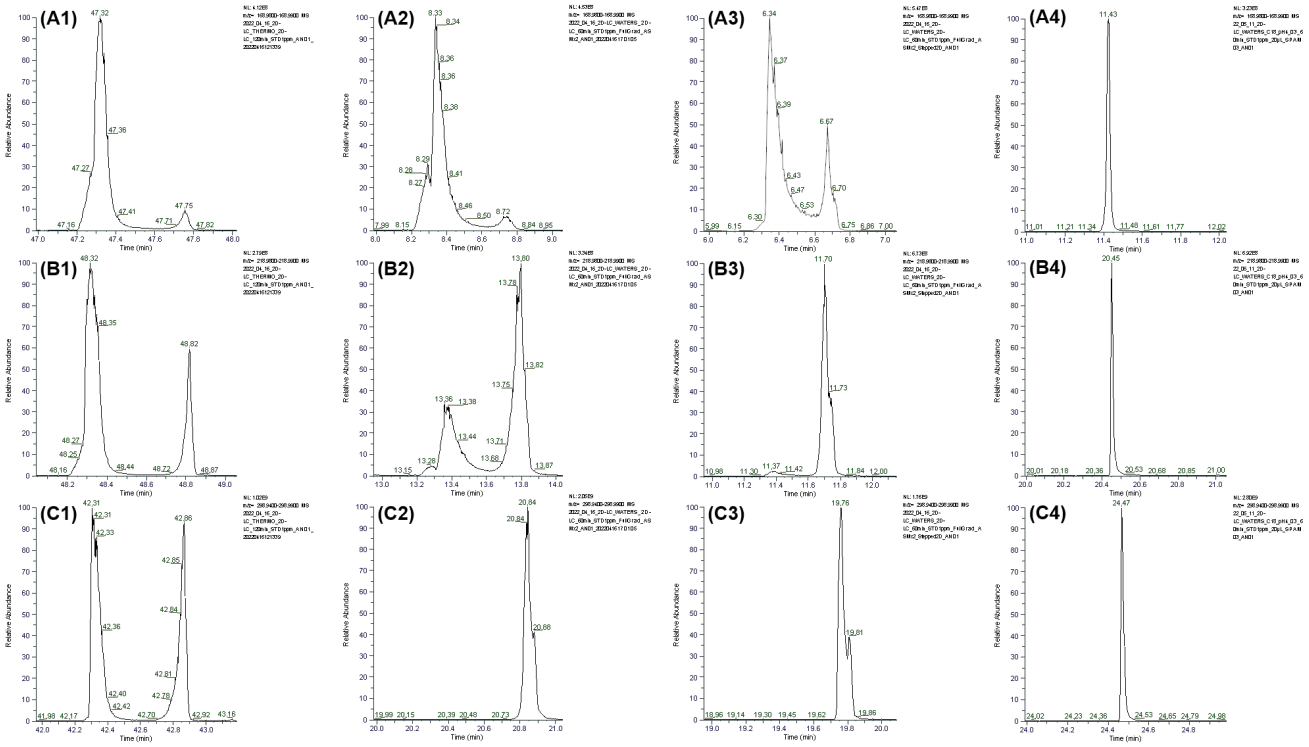

Figure S5 − XICs showing the peak splitting effect from setups 1, 2 and 3 (as numbered in columns; using ASM modulation), whereas (column 4, SPAM) the sharpness increase obtained using setup 4 conditions for (A rows) PFBuA (analyte 12 of Table S1), (B rows) PFpeA (analyte 13 of Table S1), and (C rows) PFBuS (analyte 14 of Table S1).

Table S4 – Values of predicted peak capacity ($n'_{c,2D}$) and undersampling correction factor (1/β) of the investigated setups reported in Table 1.

Setup	${}^{1D}n_c$	${}^{2D}n_c$	$n'_{c,2D}$	1/β
1	4.3	11	37	0.8
2	19.5	5.7	80	0.7
3	19.5	7.4	104	0.7
4	27	14.4	181	0.5
5	51	6.4	228	0.7
6	51	9.6	342	0.7

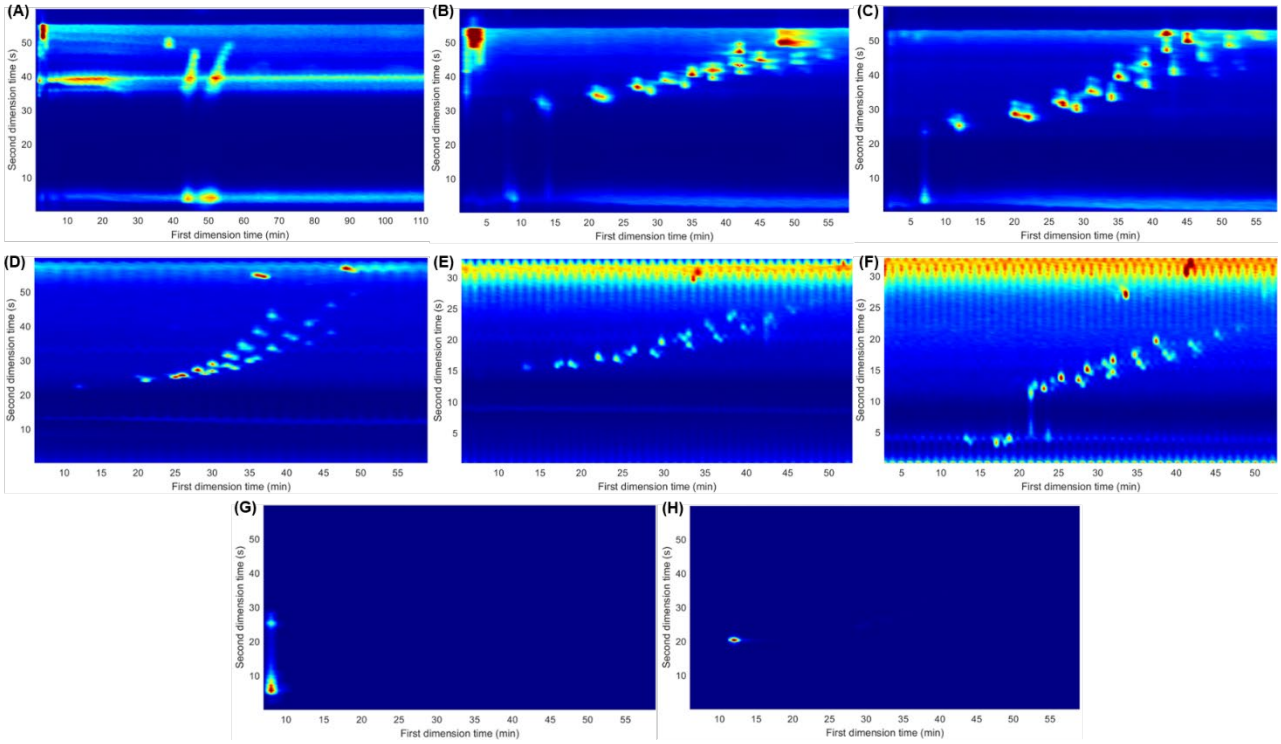

Figure S6 – LC×LC contour plots of (A-F) TICs of the setups investigated for method development, reported in Table 1 and related to Figure 3 of the main text. EICs of the pseudo-molecular ion at *m/z* 168.99 related to PFBuA evidencing (G) the peak splittin using ASM and (H) the correct peak focusing adopting SPAM. Contour plots were generated using MOREPEAKS software available at 10.5281/zenodo.6375413.

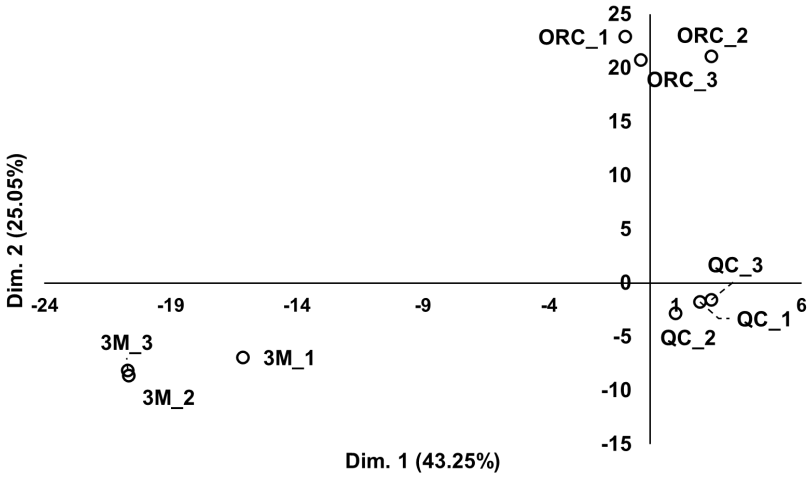

Figure S7 − Score plot of first (Dim. 1) and second (Dim. 2) components of the PCA performed on the intensities of aligned features detected in 3M, ORC, and pooled samples (QCs).

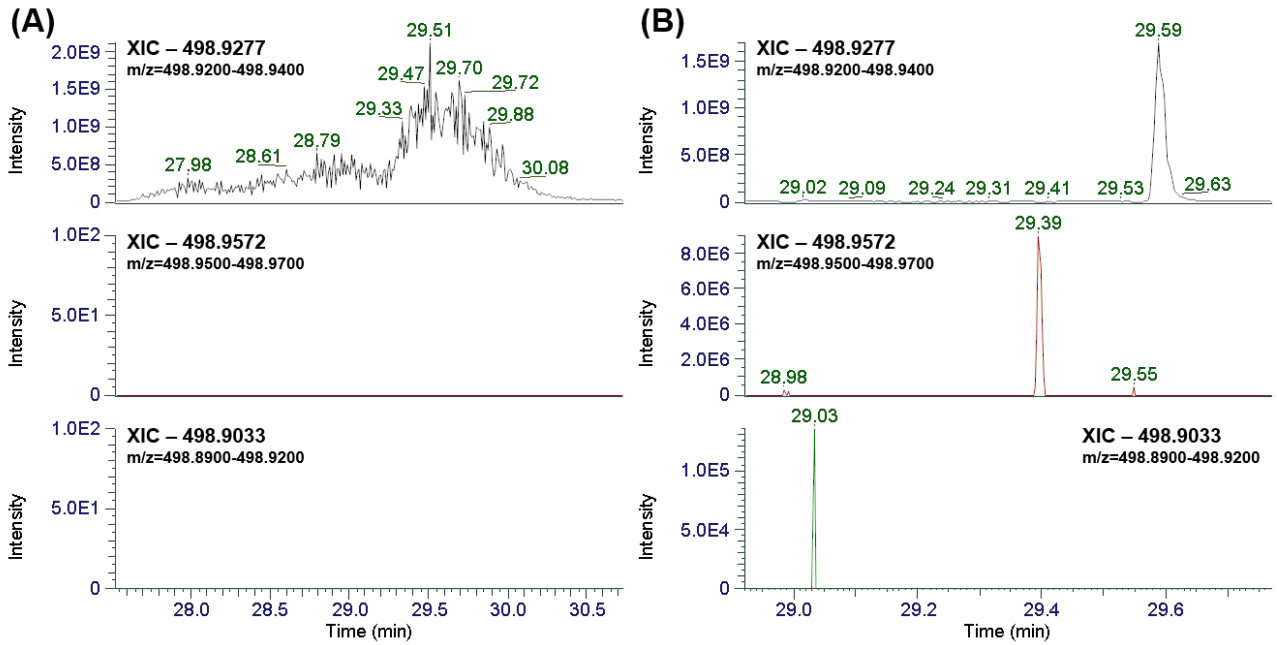

Figure S8 – Representative set of *m/z* features in 3M sample as XICs in (A) 1D-LC vs (B) one modulation of LC×LC configurations.

Table S5 – Annotated PFASs in the investigated samples by the match with the training set, described by compound name, experimental *m/z* (Da), mass accuracy in ppm (Δ), and retention time on first (t_{R1D}) and second (t_{R2D}) dimensions.

Compound	*m/z*	Δ	t_{R1D}	t_{R2D}
3M				
PFACAs				
PFHxA	268.9819	-2.2	18.20	0.05
PFHpA	318.9793	0.0	23.83	0.18
PFASAs				
PFBuS	298.9418	-2.3	16.01	0.06
PFPeS	348.9389	-1.1	21.64	0.19
PFHxS	398.9368	2.0	26.06	0.21
PFHpS	448.9329	0.0	29.38	0.23
PFOS	498.9277	-4.0	31.61	0.26
PFNS	548.9257	-1.5	34.36	0.26
PFDS	598.9235	0.3	36.58	0.28
ORC				
FTSs				
Oc-FTSs	426.9688	3.3	20.55	0.20
D-FTSs	526.9612	0.4	28.29	0.24