Development and validation of an LC–MS/MS method for the quantitation of 30 legacy and emerging per- and polyfluoroalkyl substances (PFASs) in human plasma, including HFPO-DA, DONA, and cC6O4

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Abstract

Per- and polyfluoroalkyl substances (PFASs) include persistent organic pollutants whose spread is still ubiquitous. Efforts to substitute substances of high concern with fluorinated alternatives, such as HFPO-DA (GenX), DONA (ADONA), and cC6O4, have been made. The aim of this work was to develop and validate an isotopic dilution liquid chromatography-tandem mass spectrometry (LC–MS/MS) method suitable to quantify 30 PFASs in human plasma. Analytes included legacy PFASs (PFOA, PFOS, and PFHxS), fluorinated alternatives (PFBA, PFBS, 6:2 FTSA, HFPO-DA, DONA, and cC6O4), and newly identified compounds (F-53B and PFECHS). The sample preparation was rapid and consisted of simple protein precipitation and centrifugation. Calibration standards and quality control solutions were prepared with a human pooled plasma containing relatively low background levels of the considered analytes. A complete validation was carried out: the lower limits of quantitation (LLOQs) ranged from 0.009 to 0.245 µg/L; suitable linearity (determination coefficients, $R^2$ 0.989–0.999), precision (2.0–19.5%, relative standard deviation), and accuracy (87.9–113.1% of theoretical) were obtained for considered concentration ranges. No significant variations of analyte responses were recorded under investigated storage conditions and during matrix effect tests. The external verification confirmed the accuracy of the method, although limited to 12 analytes. The method was also applied to 38 human plasma samples to confirm its applicability. The developed assay is suitable for large-scale analyses of a wide range of legacy and emerging PFASs in human plasma. To our knowledge, this is the first published method including cC6O4 for human biomonitoring.

Keywords Per-/polyfluoroalkyl substances · PFAS · LC-MS/MS · Fluorinated alternatives · Emerging PFAS · Per/polyfluoroalkyl acids

Abbreviations

11Cl-PF3OuDS Commercial solution containing the 8:2 Cl-PFESA standard
4:2 FTSA 1H,1H,2H,2H-Perfluorohexanesulfonic acid
6:2 Cl-PFESA 2-(6-Chloro-1,1,2,2,3,3,4,4,5,5,6,6-dodecafluorohexoxy)-1,1,2,2-tetrafluoroethanesulfonic acid
6:2 FTSA 1H,1H,2H-Perfluorooctanesulfonic acid
8:2 Cl-PFESA 2-(8-Chloro-1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8-hexadecafluorooctoxy)-1,1,2,2-tetrafluoroethanesulfonic acid
8:2 FTSA 1H,1H,2H-Perfluorodecanesulfonic acid

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| Abbreviation | Description |
|--------------|-------------|
| 9Cl-PF3ONS   | Commercial solution containing the 6:2 Cl-PFESA standard |
| ARPAV        | Regional Agency for Environmental Protection and Prevention of Veneto |
| ARPA Lombardia | Regional Agency for Environmental Protection of Lombardy |
| CAS          | Chemical Abstracts Service |
| cC6O4        | (cis/trans)-Perfluoro([5-methoxy-1,3-dioxolan-4-yl]oxy)acetic acid |
| CE           | Collision energies |
| d3-N-MeFOSAA | N-Methyl-d3-perfluoro-1-octanesulfonamidoacetic acid |
| d5-N-EtFOSAA | N-Ethyl-d5-perfluoro-1-octanesulfonamidoacetic acid |
| DF           | Dilution factor |
| DONA         | 3-H-Perfluoro-4,8-dioxanonanoic acid |
| EDTA         | Ethylenediaminetetraacetic acid |
| EMA          | European Medicines Agency |
| FDA          | Food and Drug Administration |
| G-EQUAS      | German External Quality Assessment Scheme |
| HFPO-DA      | 2,3,3,3-Tetrafluoro-2-(heptafluoropropoxy)propanoic acid |
| ICH          | International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use |
| ICI-EQUAS    | Interlaboratory Comparison Investigations and External Quality Assurance Schemes |
| IS           | Internal standards |
| LC–ESI–MS/MS | Liquid chromatography-electrospray ionisation tandem mass spectrometry |
| LLOQ         | Lower limit of quantitation |
| M2-4:2 FTS   | Sodium 1H,1H,2H,2H-perfluoro-1-[1,2-13C2]hexanesulfonate |
| M2-6:2 FTS   | Sodium 1H,1H,2H,2H-perfluoro-1-[1,2-13C2]octanesulfonate |
| M2-8:2 FTS   | Sodium 1H,1H,2H,2H-perfluoro-1-[1,2-13C2]decanesulfonate |
| M2PFDoA      | Perfluoro-n-[1,2-13C2]dodecanoic acid |
| M2PFTeDA     | Perfluoro-n-[1,2-13C2]tetradecanoic acid |
| M3HFPO-DA    | 2,3,3,3-Tetrafluoro-2-(1,1,2,2,3,3,3-heptafluoropropoxy)-13C3-propanoic acid |
| M3PFBS       | Sodium perfluoro-1-[2,3,4-13C3]butanesulfonate |
| M3PFHxS      | Sodium perfluoro-1-[1,2,3-13C3]hexanesulfonate |
| M4PFBA       | Perfluoro-n-[13C4]butanoic acid |
| M4PFHxA      | Perfluoro-n-[1,2,3,4,6-13C5]hexanoic acid |
| M5PFPeA      | Perfluoro-n-[13C5]pentanoic acid |
| M6PFDA       | Perfluoro-n-[1,2,3,4,5,6-13C6]decanoic acid |
| M7PFUdA      | Perfluoro-n-[1,2,3,4,5,6,7-13C7]undecanoic acid |
| M8FOSA       | Perfluoro-1-[13C8]octanesulfonamide |
| M8PFOA       | Perfluoro-n-[13C8]octanoic acid |
| M8PFOS       | Sodium perfluoro-1-[13C8]octanesulfonate |
| M9PFNA       | Perfluoro-n-[13C9]nonanoic acid |
| NaDONA       | Commercial solution containing the DONA standard |
| N-EtFOSAA    | N-Ethylperfluoroctane sulfonamidoacetic acid |
| N-MeFOSAA    | N-Methylperfluoroctane sulfonamidoacetic acid |
| P5Me ODOMXOA | Commercial solution containing the cC6O4 standard |
| PFAAs        | Perfluoroalkyl acids |
| PFAC-24PAR   | Commercial solution containing the 24 native standards |
| PFASs        | Per-/polyfluoroalkyl substances |
| PFBA         | Perfluorobutanoic acid |
| PFBS         | Perfluorobutanesulfonic acid |
| PFCAs        | Perfluoroalcohol carboxylic acids |
| PFDA         | Perfluorodecanoid acid |
| PFDoDA       | Perfluorododecanoic acid |
| PFDS         | Perfluorodecanic sulfonic acid |
| PFEAs        | Per-/polyfluoroalkyl ether acids |
| PFECAs       | Per-/polyfluoroalkyl ether carboxylic acids |
| PFESAs       | Per-/polyfluoroalkyl ether sulfonic acids |
| PFHxS        | Perfluoroheptanoic acid |
| PFHpS        | Perfluoroheptanesulfonic acid |
| PFHpA        | Perfluoroheptanoid acid |
| PFHzA        | Perfluoroheptanesulfonic acid |
| PFHzS        | Perfluoroheptanesulfonic acid |
| PFNA         | Perfluorononanoic acid |
| PFNS         | Perfluorononanesulfonic acid |
| PFOA         | Perfluoroctanoic acid |
| PFOS         | Perfluoroctanesulfonic acid |
| PFOSA        | Perfluoroctanesulfonic acid |
| PFPeA        | Perfluoropentanoic acid |
| PFPeS        | Perfluoropentanesulfonic acid |
| PFSAs        | Perfluoroalkylsulfonic acids |
Development and validation of an LC–MS/MS method for the quantitation of 30 legacy and emerging…

Perfluorinated Compounds (PFCs) are man-made compounds containing an aliphatic portion characterised by at least one perfluorocarbon moiety. The excellent strength of the carbon–fluorine bond makes perfluorocarbon moieties chemically inert and thermally stable [1]. Despite being an attractive industrial property, perfluorocarbon resistance to degradation raises concerns about environmental fate and human health impact. Indeed, among perfluorooalkyl acid (PFAA) subclasses, perfluoroalkyl-carboxylic and sulfonic acids (PFCA and PFSA) count respectively perfluorooctanoic acid (PFBA) and perfluorooctanesulfonic acid (PFBS), partially fluorinated substances (fluorotelomers, e.g. 6:2 fluorotelomersulfonic acid, 6:2 FTSA), and PFASs containing fluorinated carbon chain interspersed with heteroatoms (e.g. per-/polyfluoroalkyl ether acid family, PFEAs) have been conceived, with the aim of replacing traditional compounds with safer alternatives [17].

PFEAs include per-/polyfluoroalkyl ether carboxylic and sulfonic acids (PFECAs and PFESAs) subclasses. Among PFECAs, HFPO-DA (CAS n° 13,252–13-6, also known as GenX), DONA (CAS n° 919,005–14-4, also known as its ammonium salt ADONA), and cC6O4 (CAS n° 1,190,931–41-9) are PFECA substitutes of traditional polymerisation surfactants (as PFOA) [18, 19]. HFPO-DA has been widely encountered in environmental samples [18, 20, 21] and rarely in human biological samples derived from the general population [14], while DONA has only been detected in surface water [22] and in human plasma samples derived from areas impacted by fluorochemical plants [23]. cC6O4 has recently been found in Italy’s largest river basin (Po River, Veneto region, Italy) [24], and it is constantly monitored by regional environmental protection agencies ARPAL [25] and ARPAV [26].

Even though intended as a better option than PFOA, HFPO-DA and PFBS have been recently classified as “substances of very high concern”, according to REACH regulation [27, 28].

The production of perfluoroethylcyclohexane sulfonate (PFECHS) and chlorinated polyfluoroalkyl ether sulfonic acids (e.g. F-53B-related components 6:2 Cl-PFESA and 8:2 Cl-PFESA) is long-standing; nevertheless, their presence has only recently been ascertained in the environment [18, 20, 29–35] and in human biological matrices [36–40]. For these reasons, it is common for authors to refer to these substances as “emerging/alternative”.

Biological monitoring of both legacy and emerging PFASs is a useful approach to carry out integrated and representative assessment of human exposure to these substances. Several methods have been developed for the quantification of different chemical classes of PFASs in human serum and plasma, by implementing liquid chromatography coupled with tandem mass spectrometry as a suitable analytical technique for high sensitivity and selectivity. Before injection, plasma and serum samples are usually treated to remove proteins [41–45] and/or purified with solid-phase extraction (SPE) [16, 46–58]. However, only a few methods considered the emerging compounds HFPO-DA [16, 43, 44], F-53B...
[16, 53, 56], DONA [16, 43], or PFECHS [43, 45]. Also, we did not find in the scientific literature methods determining cC6O4 in human blood matrices.

The aim of this work was to develop and validate a high-throughput LC–MS/MS method for the determination of 30 PFASs in human plasma, including both legacy and emerging compounds.

Materials and methods

Chemicals

The analytes are reported in Table 1. All analytical standard solutions were purchased from Wellington Laboratories (Guelph, Canada): a mixture containing 24 native standards (PFAC-24PAR), a mixture containing 19 mass-labelled standards (MPFAC-24ES), six separate solutions each containing one emerging PFAS (P5MeODIOXOAc, NaDONA, 9Cl-PF3ONS, 11Cl-PF3OUdS, HFPO-DA, PFECHS), and a solution containing another mass labelled standard (M3HFPO-DA). Names, concentrations, and other specifications of each compound present in these commercial standard solutions are reported in the supplementary material (Table S1 and S2). HPLC-grade acetonitrile, HPLC-grade methanol, and analytical-grade glacial acetic acid were purchased from Sigma-Aldrich (Milan, Italy). Analytical-grade isopropyl alcohol was purchased from Carlo Erba Reagents (Val-de-Reuil, France), analytical-grade 25% ammonia solution was purchased from Merck (Darmstadt, Germany), while purified water was obtained through a Milli-Q Plus ultra-pure system (Millipore, Milford, MA, USA).

Human plasma samples

Human plasma samples were leftovers obtained from patients of the hospital where the laboratory is located, which had blood collected for other routine clinical measurements. The regulation of the hospital allows the use of routine leftover samples for method development, optimization, and validation as long as they are anonymised. The blood was drawn in EDTA anticoagulant polypropylene tubes and centrifuged at 1127 × g for 15 min to separate plasma, which was transferred to another polypropylene tube. The obtained sample was stored frozen (− 20 °C) until use.

Blank matrix

Since certain PFASs are widespread in the general population, we analysed several unknown plasma samples and selected the ones characterised by reduced content of the analytes. In particular, the samples with PFOA, PFOS, and PFHxS concentrations up to their first 5th percentile were chosen and mixed to obtain the blank matrix (human pooled plasma). It was stored frozen (− 20 °C) in a polypropylene tube until use.

Standard solution preparation

Commercial solutions were transferred from their amber glass ampoules to glass vials previously rinsed with methanol (screw cap with PTFE liner), and were finally stored at − 20 °C.

The six standard solutions containing the emerging PFASs were diluted (dilution factor, DF: 25) and mixed in methanol. This solution and the PFAC-24PAR stock solution were diluted with the blank matrix (DF: 50) to obtain the highest calibration standard, which was further diluted with the blank matrix to prepare both the remaining calibration standards and the quality control solutions (QCs). For each analyte, the concentrations ranged from the lower (LLOQ) to the upper limit of quantitation (ULOQ) (Table 2 and Table S3). The method validation was conducted on two QCs per analyte, except for the sum of branched isomers of PFOS, for which high background levels were detected in blank matrix and allowed only one QC level to be included (Table 2 and Table S3).

An internal standard working solution, containing the 20 isotopic labelled standards, was prepared in methanol by diluting and mixing M3HFPO-DA (DF: 25,000) and MPFAC-24ES (DF: 2500).

All prepared standard solutions were stored (− 20 °C) in 2.0-mL glass vials (screw cap with PTFE liner) until use.

Sample preparation

An aliquot of 20 µL of plasma sample was dispensed into polypropylene conical tubes, and 80 µL of the internal standard working solution was added. Since the internal standard working solution was prepared in methanol, this step was also aimed to crash plasma proteins. The obtained solution was thoroughly vortexed and centrifuged at 10,500 × g for 15 min. The supernatant was then collected and transferred in an autosampler vial (screw cap with self-sealing PTFE septa) containing a 250-µL polypropylene insert. The vial was finally placed in the thermostated autosampler until analysis. The same procedure was followed for unknown samples, sample blanks (unspiked blank matrix), procedural blanks (methanol treated as an unknown sample), calibration standard solutions, and QCs.

LC–MS/MS analysis

The LC–MS/MS system consisted of an Agilent 1260 liquid chromatograph (Agilent Technologies, Cernusco sul Naviglio, Italy) coupled with a Q-Trap 5500 mass spectrometer.
Table 1  List of the acronyms for analytes, grouped by compound class. For each compound, the molecular structure, monoisotopic mass, Chemical Abstracts Service (CAS) registry number, quantifier (quant.), qualifier (qual.), and internal standard (IS) multiple reaction monitoring (MRM) transitions, chromatographic retention time (RT), and collision energy (CE) are reported. Abbreviations are reported in the homonymous section.

| Compound class | Analytes | Molecular structure | Monoisotopic mass (g/mol) | CAS | MRM transition | Precursor ion (m/z) | Product ion (m/z) | RT (min) | CE (V) |
|----------------|----------|---------------------|---------------------------|-----|----------------|--------------------|------------------|-----------|--------|
|                | PFBA     | ![PFBA structure](image1) | 213.996 | 375-22-4 | Quant. | 213 | 169 | 3.6 | -12 |
|                | PPFpA    | ![PPPfpA structure](image2) | 263.98 | 2706-90-3 | Quant. | 263 | 219 | 8.7 | -12 |
|                | PFHxA    | ![PFHxA structure](image3) | 313.98 | 307-24-4 | Quant. | 313 | 269 | 10.7 | -13 |
|                | PFHpA    | ![PFHpA structure](image4) | 363.98 | 375-85-9 | Quant. | 363 | 319 | 11.9 | -13 |
|                | PFBA     | ![PFBA structure](image5) | 217.05 | 307-24-4 | Quant. | 217 | 122 | 12.9 | -13 |
|                | PPFpA    | ![PPPfpA structure](image6) | 268.99 | 2706-90-3 | Quant. | 268 | 223 | 8.7 | -12 |
|                | PFHxA    | ![PFHxA structure](image7) | 318.98 | 307-24-4 | Quant. | 318 | 273 | 10.7 | -13 |
|                | PFHpA    | ![PFHpA structure](image8) | 367.98 | 375-85-9 | Quant. | 367 | 322 | 11.9 | -13 |
| PFCAs          | PFOA     | ![PFOA structure](image9) | 413.97 | 335-67-1 | Quant. | 413 | 369 | 12.9 | -15 |
|                | PFNA     | ![PFNA structure](image10) | 463.97 | 375-95-1 | Quant. | 463 | 419 | 13.7 | -15 |
|                | PFDA     | ![PFDA structure](image11) | 513.97 | 335-76-2 | Quant. | 513 | 469 | 14.5 | -15 |
|                | PFUnDA   | ![PFUnDA structure](image12) | 563.96 | 2058-94-8 | Quant. | 563 | 519 | 15.2 | -15 |
|                | PFNA     | ![PFNA structure](image13) | 463.97 | 375-95-1 | Qual. | 463 | 169 | 12.9 | -24 |
|                | PFDA     | ![PFDA structure](image14) | 513.97 | 335-76-2 | Qual. | 513 | 169 | 14.5 | -24 |
|                | PFUnDA   | ![PFUnDA structure](image15) | 563.96 | 2058-94-8 | Qual. | 563 | 269 | 15.2 | -26 |
|                | NFOSA    | ![NFOSA structure](image16) | 498.95 | 754-91-6 | Quant. | 498 | 78 | 17.0 | -90 |
|                | N-MeFOSAA| ![N-MeFOSAA structure](image17) | 570.97 | 2355-31-9 | Quant. | 570 | 419 | 15.4 | -27 |
|                | N-EtFOSAA| ![N-EtFOSAA structure](image18) | 584.99 | 2991-50-6 | Quant. | 584 | 419 | 16.0 | -28 |
|                | PPFs     | ![PFs structure](image19) | 299.95 | 375-73-5 | Quant. | 299 | 80 | 10.9 | -47 |
|                | PFPeA    | ![PFPeA structure](image20) | 349.95 | 2706-91-4 | Quant. | 349 | 80 | 12.3 | -79 |
|                | a-PHAs: linear isomer | ![a-PHAs structure](image21) | 399.94 | 355-46-4 | Quant. | 399 | 80 | 13.3 | -85 |

1. Abbreviations are reported in the homonymous section.
Table 1 (continued)

| Compound class | Analytes | Molecular structure | Monoisotopic mass (g/mol) | CAS | MRM transition | Precursor ion (m/z) | Product ion (m/z) | RT (min) | CE (V) |
|----------------|----------|---------------------|---------------------------|-----|----------------|---------------------|------------------|----------|------|
| PFHxS branched isomers | | | 399.94 | IS: M3PFHxS | 402 | 80 | -90 |
| | | | | | Quant. | 399 | 80 | -85 |
| | | | | | Qual. | 399 | 99 | -40 |
| | | | | | IS: M3PFHxS | 402 | 80 | -90 |
| PFHpS | | | 449.94 | | 375-92-8 | Quant. | 449 | 80 | -100 |
| | | | | | Qual. | 449 | 99 | -85 |
| | | | | | IS: M3PFHxS | 402 | 80 | -90 |
| n-PFOS linear isomer | | | 499.94 | | 1763-23-1 | Qual. | 499 | 80 | -110 |
| | | | | | IS: M3PFOS | 507 | 80 | -115 |
| PFOS branched isomers | | | 499.94 | IS: M3PFOS | 507 | 80 | -115 |
| | | | | Quant. | 499 | 80 | -110 |
| | | | | Qual. | 499 | 99 | -95 |
| PFNS | | | 549.93 | 68259-12-1 | Quant. | 549 | 80 | -125 |
| | | | | Qual. | 549 | 99 | -105 |
| | | | | IS: M3PFOS | 507 | 80 | -115 |
| PFDS | | | 599.93 | 335-77-3 | Quant. | 599 | 80 | -130 |
| | | | | Qual. | 599 | 99 | -115 |
| | | | | IS: M3PFOS | 507 | 80 | -115 |
| PFECHS | | | 461.94 | 646-83-3 | Quant. | 461 | 381 | -9 |
| | | | | Qual. | 461 | 99 | -70 |
| | | | | IS: M3PFOS | 507 | 80 | -115 |
| 4:2-FTSA | | | 327.98 | 757124-72-4 | Quant. | 327 | 307 | -26 |
| | | | | IS: M2-4:2-FTSA | 329 | 309 | -27 |
| 6:2-FTSA | | | 427.98 | 27619-97-2 | Quant. | 427 | 407 | -32 |
| 8:2-FTSA | | | 527.97 | 39108-34-4 | Qual. | 427 | 81 | -74 |
| | | | | IS: M2-4:2-FTSA | 429 | 409 | -33 |
| | | | | Quant. | 527 | 507 | -37 |
| | | | | Qual. | 527 | 81 | -90 |
| | | | | IS: M2-8:2-FTSA | 529 | 509 | -37 |
| HPO-DA | | | 329.98 | 13252-13-6 | Quant. | 285 | 169 | -10 |
| | | | | Qual. | 329 | 169 | -18 |
| | | | | IS: M3HPO-DA | 332 | 287 | -8 |
| DONA | | | 377.98 | 919005-14-4 | Quant. | 377 | 251 | -16 |
| | | | | Qual. | 377 | 85 | -40 |
| | | | | IS: M4PFHpA | 367 | 322 | -13 |
| cC6O4 | | | 339.96 | 1199931-41-9 | Quant. | 339 | 113 | -15 |
| | | | | Qual. | 339 | 85 | -35 |
| | | | | IS: M5PFHxA | 318 | 273 | -13 |
| 6:2-PFESA | | | 531.90 | 756426-56-1 | Quant. | 531 | 351 | -38 |
| | | | | Qual. | 531 | 83 | -75 |
| | | | | IS: M5PFOS | 507 | 80 | -115 |
| 8:2-PFESA | | | 631.90 | 763051-92-9 | Quant. | 631 | 451 | -40 |
| | | | | Qual. | 631 | 83 | -85 |
| | | | | IS: M5PFOS | 507 | 80 | -115 |
Table 2 Results of method validation. For each analyte, lower and upper limits of quantitation (LLOQ and ULOQ), mean $R^2$ for investigated linearity range, and within-run precision and accuracy at LLOQ are reported. Within- and between-run precision and accuracy, stability (short term and long term), and matrix effect were calculated at QC levels.

| Analyte | Linearity and sensitivity | Spiked concentration (µg/L) | Precision | Accuracy | Stability | Matrix effect |
|---------|---------------------------|-----------------------------|-----------|----------|-----------|--------------|
|         | LLOQ (µg/L) | ULOQ (µg/L) | Mean $R^2$ (n = 6) | Precision at LLOQ (%RDS) (n = 5) | Mean accuracy at LLOQ (%theoretical) (n = 5) | Within-run precision (%RSD) (min–max) (n = 5) | Between-run precision (%RSD) (n = 25) | Within-run accuracy (%theoretical) (n = 5) | Between-run accuracy (%theoretical) (n = 25) | Short-term stability (% variation) | Long-term stability (% variation) | Matrix effect (%RSD) (n = 7 plasma samples) | Matrix effect (%theoretical) (n = 7 plasma samples) |
|---------|-------------|-------------|------------------|-------------------|-----------------------------|----------------------------------|---------------------------------|----------------------------------|---------------------------------|-----------------------------|-----------------------------|---------------------------------|---------------------------------|
| PFBA    | 0.078       | 5.00        | 0.997            | 1.8               | 97.5                        | 0.625                            | 3.9 (1.7–6.5)                  | 5.4                              | 97.7 (89.6–117.5)           | 98.3                        | 102.2                       | 103.8                       | 10.2 (104.5)                  | 104.9 (110.7)               |
|         |             |             |                  | 2.50               | 2.7 (1.2–3.7)               | 2.7                              | 2.7 (1.2–3.7)                  | 2.7                              | 2.7 (1.2–3.7)                  | 2.7 (1.2–3.7)               | 2.7 (1.2–3.7)               | 2.7 (1.2–3.7)               | 2.7 (1.2–3.7)               |
| PFPeA   | 0.020       | 1.25        | 0.998            | 10.7               | 116.7                       | 0.078                            | 3.8 (1.7–5.9)                  | 8.8                              | 91.8–106.4                     | 99.2                        | 99.4                       | 105.1                       | 6.6 (110.7)                  | 108.8 (111.7)               |
|         |             |             |                  | 0.078               | 7.3 (2.8–9.2)               | 7.3                              | 7.3 (2.8–9.2)                  | 7.3                              | 7.3 (2.8–9.2)                  | 7.3 (2.8–9.2)               | 7.3 (2.8–9.2)               | 7.3 (2.8–9.2)               | 7.3 (2.8–9.2)               |
| PFHxA   | 0.039       | 2.50        | 0.996            | 8.3                | 104.2                       | 0.078                            | 3.2 (1.6–4.1)                  | 4.3                              | 93.2–101.1                     | 98.9                        | 100.4                      | 96.8                       | 4.9 (111.7)                  | 105.6 (108.8)               |
|         |             |             |                  | 0.078               | 6.6 (4.1–11.5)              | 6.6                              | 6.6 (4.1–11.5)                 | 6.6                              | 6.6 (4.1–11.5)                 | 6.6 (4.1–11.5)             | 6.6 (4.1–11.5)             | 6.6 (4.1–11.5)             | 6.6 (4.1–11.5)             |
| PFHpA   | 0.039       | 2.50        | 0.997            | 16.2               | 101.4                       | 0.078                            | 12.6 (8.7–15.8)                | 12.3                             | 91.2–103.7                     | 94.7                        | 105.0                      | 18.7                       | 98.8 (108.8)                | 105.6 (108.8)               |
|         |             |             |                  | 0.078               | 4.2 (2.3–7.2)               | 4.2                              | 4.2 (2.3–7.2)                  | 4.2                              | 4.2 (2.3–7.2)                  | 4.2 (2.3–7.2)               | 4.2 (2.3–7.2)               | 4.2 (2.3–7.2)               | 4.2 (2.3–7.2)               |
| PFOA    | 0.156       | 40.0        | 0.997            | 17.9               | 101.7                       | 0.078                            | 11.4 (6.0–16.9)                | 11.2                             | 67.7–118.3                    | 95.9                        | 105.5                      | 78.4                       | 107.5 (108.5)               | 110.7 (110.7)               |
|         |             |             |                  | 0.078               | 4.2 (3.3–6.3)               | 4.2                              | 4.2 (3.3–6.3)                  | 4.2                              | 4.2 (3.3–6.3)                  | 4.2 (3.3–6.3)               | 4.2 (3.3–6.3)               | 4.2 (3.3–6.3)               | 4.2 (3.3–6.3)               |
| PFNA    | 0.078       | 5.00        | 0.996            | 12.2               | 119.5                       | 0.625                            | 7.3 (2.7–9.4)                  | 7.4                              | 96.6 (82.6–107.7)            | 95.9                        | 95.8                       | 100.1                       | 5.1 (103.4)                 | 103.4 (103.4)               |
|         |             |             |                  | 0.625               | 4.2 (3.2–5.2)               | 4.2                              | 4.2 (3.2–5.2)                  | 4.2                              | 4.2 (3.2–5.2)                  | 4.2 (3.2–5.2)               | 4.2 (3.2–5.2)               | 4.2 (3.2–5.2)               | 4.2 (3.2–5.2)               |
| PFDA    | 0.078       | 5.00        | 0.997            | 14.3               | 114.0                       | 0.625                            | 7.1 (3.7–11.9)                 | 7.5                              | 85.9–106.1                    | 99.0                        | 94.6                       | 98.7                       | 7.8 (104.3)                 | 108.7 (104.3)               |
|         |             |             |                  | 0.625               | 5.6 (5.1–6.4)               | 5.6                              | 5.6 (5.1–6.4)                  | 5.6                              | 5.6 (5.1–6.4)                  | 5.6 (5.1–6.4)               | 5.6 (5.1–6.4)               | 5.6 (5.1–6.4)               | 5.6 (5.1–6.4)               |
| PFUnDA  | 0.078       | 5.00        | 0.998            | 9.8                | 99.2                        | 0.625                            | 4.7 (1.8–7.4)                  | 5.5                              | 88.6–104.7                    | 97.6                        | 95.7                       | 8.9                        | 105.4 (105.4)               | 108.7 (105.4)               |
|         |             |             |                  | 0.625               | 5.7 (3.3–7.7)               | 5.7                              | 5.7 (3.3–7.7)                  | 5.7                              | 89.0–102.0                    | 97.7                        | 100.0                      | 94.2                       | 6.2 (113.0)                 | 113.0 (113.0)               |
| Analyte   | LLOQ (µg/L) | ULOQ (µg/L) | Mean R² | Precision at LLOQ (%RSD) (n = 6) | Mean accuracy at LLOQ (%theoretical) (n = 5) | Spiked concentration (µg/L) | Precision | Within-run accuracy (%RSD) (min–max) (n = 5) | Between-run precision (%RSD) (n = 25) | Accuracy | Within-run accuracy (%theoretical) (n = 5) | Between-run accuracy (%theoretical) (n = 25) | Stability | Short-term stability (% variation) | Long-term stability (% variation) | Matrix effect | Matrix effect (%theoretical) (n = 7 plasma samples) |
|----------|-------------|-------------|---------|----------------------------------|------------------------------------------|---------------------------|----------------|------------------------------------------|---------------------------------------|---------|----------------------------------------|------------------------------------------|-----------|----------------------------------|-----------------------------------|---------------|----------------------------------------|
| PFDcDA   | 0.039       | 2.50        | 0.997   | 9.0                              | 105.3                                    | 0.078                     | 6.4 (3.5–11.2) | 7.4                                      | 96.0 (76.9–110.0)                      | 97.1     | 99.9 (111.6)                          | 97.1 (102.9)                          | 99.7      | 110.3                            | 6.4 (110.7)               | 11.0 (113.3) |
| PFTdDA   | 0.020       | 1.25        | 0.997   | 15.8                             | 103.1                                    | 0.078                     | 6.4 (4.0–7.6) | 13.7                                     | 98.5 (80.6–116.1)                      | 99.5     | 105.8 (87.4)                          | 99.5 (103.5)                          | 105.8     | 87.4                             | 11.1 (133.5)              | 99.7 (112.1) |
| PFTeDA   | 0.020       | 1.25        | 0.999   | 8.1                              | 97.6                                    | 0.078                     | 5.0 (3.3–7.3) | 5.2                                      | 96.8 (85.5–110.2)                      | 98.7     | 97.4 (100.0)                          | 97.4 (100.0)                          | 102.0     | 103.2                            | 5.4 (112.1)               | 100.0 (104.0) |
| PFOSA    | 0.010       | 1.25        | 0.998   | 9.3                              | 117.0                                    | 0.078                     | 3.8 (3.1–4.8) | 3.9                                      | 94.8 (84.7–105.4)                      | 97.3     | 96.5 (105.2)                          | 96.5 (100.0)                          | 101.3     | 102.0                            | 8.3 (100.0)                | 99.8 (104.1) |
| N-MeFOSAA| 0.039       | 2.50        | 0.997   | 7.7                              | 107.8                                    | 0.078                     | 12.9 (7.8–20.0) | 15.1                                     | 98.8 (82.2–123.9)                      | 99.7     | 89.1 (93.8)                           | 99.1 (97.1)                           | 102.7     | 107.8                            | 19.2 (97.1)                | 104.0 (104.1) |
| N-EtFOSAA| 0.039       | 2.50        | 0.997   | 18.6                             | 107.1                                    | 0.078                     | 14.3 (6.4–20.6) | 14.8                                     | 103.7 (77.8–157.3)                     | 101.2    | 108.8 (106.6)                         | 108.8 (91.6)                          | 103.0     | 107.8                            | 10.3 (106.9)               | 92.0 (112.7) |
| PFBS     | 0.035       | 2.22        | 0.998   | 13.9                             | 90.8                                    | 0.069                     | 5.6 (4.5–6.3) | 6.5                                      | 101.7 (80.5–136.3)                     | 95.3     | 113.5 (96.3)                          | 113.5 (105.4)                         | 102.7     | 92.1                             | 6.6 (112.6)                | 90.5 (110.7) |
| PFPeS    | 0.018       | 1.18        | 0.998   | 12.6                             | 114.3                                    | 0.074                     | 8.6 (4.1–11.8) | 9.2                                      | 102.0 (82.0–128.3)                     | 103.0    | 105.9 (113.0)                         | 105.9 (90.5)                          | 102.5     | 97.6                             | 19.2 (106.9)               | 99.8 (110.9) |
| Analyte          | Linearity and sensitivity |                  | Spiked concentration (µg/L) |                  |                  |                  | Stability | Matrix effect |                  |                  |             |
|------------------|---------------------------|------------------|-----------------------------|-------------------|------------------|------------------|-----------|----------------|----------------|------------------|-------------|
|                  | LLOQ (µg/L) | ULOQ (µg/L) | Mean $R^2$ ($n = 6$) | Precision at LLOQ (%RSD) ($n = 5$) | Mean accuracy at LLOQ (%theoretical) ($n = 5$) | Precision Within-run precision (%RSD) (min–max) ($n = 5$) | Between-run precision (%RSD) ($n = 25$) | Accuracy Within-run accuracy (% theoretical) ($n = 5$) | Between-run accuracy (% theoretical) ($n = 25$) | Stability Short-term stability (% variation) | Matrix effect Matrix effect (µSDD) | Matrix effect (µSDD) (n=7 plasma samples) |
| n-PFHxS          | 0.116        | 7.42          | 0.995                       | 7.0               | 101.0            | 0.464                                           | 4.6 (1.8–7.0) | 5.6             | 101.5 (74.5–114.7) | 97.7             | 104.6   | 111.0 | 10.2 | 98.6 |
|                  |             |               |                             | 1.85              |                  |                                                                                   | 98.3 (87.1–115.7) | 98.5             | 102.4 | 100.4 | 7.8  | 103.2 |
| PFHxS: Σ branched isomers | 0.013       | 0.862        | 0.996                       | 14.5              | 93.0             | 0.108                                           | 9.5 (4.9–16.0) | 11.9            | 100.0 (82.7–124.1) | 97.6             | 104.1   | 92.2  | 8.7  | 104.0 |
|                  |             |               |                             | 0.431             |                  |                                                                                   | 96.5 (83.2–108.7) | 96.8             | 105.6 | 98.5  | 9.5  | 103.1 |
| PFHpS            | 0.074       | 4.76          | 0.997                       | 8.3               | 113.1            | 0.595                                           | 6.8 (4.8–9.9) | 9.1             | 98.1 (87.8–111.4) | 98.6             | 99.1    | 87.8  | 18.8 | 108.0 |
|                  |             |               |                             | 2.38              |                  |                                                                                   | 98.5 (87.8–109.3) | 99.3             | 108.0 | 85.5  | 18.9 | 105.6 |
| n-PFOS           | 0.229       | 29.3          | 0.997                       | 14.2              | 101.6            | 0.457                                           | 15.4 (10.0–20.3) | 16.3            | 113.1 (76.8–152.5) | 107.2            | 108.2   | 89.9  | 7.7  | 91.6  |
|                  |             |               |                             | 1.829             |                  |                                                                                   | 95.8 (81.5–109.8) | 96.5             | 104.1 | 104.8 | 8.1  | 102.2 |
| PFOS: Σ branched isomers | 0.245     | 7.84          | 0.997                       | 10.8              | 99.9             | 0.490                                           | 8.9 (6.1–14.4) | 9.0             | 92.6 (73.5–112.7) | 97.1             | 100.4   | 104.8 | 13.5 | 98.3  |
| PFNS             | 0.038       | 2.40          | 0.997                       | 5.0               | 107.8            | 0.075                                           | 6.7 (4.3–7.5) | 6.8             | 95.5 (82.6–111.5) | 99.9             | 96.2    | 114.5 | 14.1 | 104.9 |
|                  |             |               |                             | 0.601             |                  |                                                                                   | 98.2 (88.1–109.1) | 100.2            | 99.4  | 105.7 | 11.1 | 105.3 |
| PFDS             | 0.019       | 1.21          | 0.997                       | 7.2               | 98.5             | 0.076                                           | 12.1 (7.9–16.7) | 17.6            | 96.1 (80.6–118.1) | 98.2             | 97.2    | 110.9 | 8.6  | 110.1 |
|                  |             |               |                             | 0.604             |                  |                                                                                   | 101.7 (92.6–113.1) | 103.4            | 97.8  | 102.8 | 7.6  | 111.3 |
| PFECHS           | 0.009       | 1.16          | 0.993                       | 16.0              | 95.8             | 0.072                                           | 15.7 (7.8–21.2) | 17.5            | 103.4 (82.8–133.0) | 101.8            | 95.8    | 87.9  | 9.7  | 94.7  |
|                  |             |               |                             | 0.578             |                  |                                                                                   | 97.9 (88.5–109.8) | 98.2             | 109.5 | 90.3  | 9.9  | 101.9 |
Table 2 (continued)

| Analyte   | Linearity and sensitivity | Spiked concentration (µg/L) | Precision | Accuracy | Stability | Matrix effect |
|-----------|---------------------------|----------------------------|-----------|----------|-----------|---------------|
|           | LLOQ (µg/L) | ULOQ (µg/L) | Mean $R^2$ | Precision at LLOQ (%RDS) | Between-run precision (%RSD) | Mean accuracy at LLOQ (%theoretical) | Between-run accuracy (%theoretical) | Short-term stability (% variation) | Long-term stability (% variation) | Matrix effect (%RSD) | Matrix effect (%theoretical) |
| 4:2 FTSA  | 0.018 | 1.17 | 0.997 | 13.3 | 95.4 | 0.073 | 9.4 (7.2–12.3) | 9.3 | 97.7 (82.1–115.2) | 98.8 | 99.6 | 95.5 | 8.4 | 109.0 |
| 6:2 FTSA  | 0.019 | 1.19 | 0.995 | 16.1 | 112.4 | 0.074 | 5.1 (3.3–7.4) | 5.2 | 99.2 (83.1–127.8) | 102.5 | 99.1 | 94.1 | 9.1 | 111.8 |
| 8:2 FTSA  | 0.038 | 1.20 | 0.994 | 18.9 | 96.6 | 0.075 | 9.0 (6.5–11.0) | 9.0 | 94.0 (82.7–109.1) | 97.8 (72.7–131.9) | 99.8 | 88.8 | 112.5 | 16.8 | 112.0 |
| HFPO-DA   | 0.020 | 1.25 | 0.989 | 15.2 | 95.3 | 0.078 | 6.7 (2.0–9.7) | 7.4 | 100.2 (80.0–131.7) | 100.2 | 109.6 | 90.3 | 9.4 | 102.2 |
| DONA      | 0.018 | 1.18 | 0.996 | 15.3 | 101.5 | 0.074 | 6.6 (5.4–11.5) | 8.5 | 91.8 (78.0–104.5) | 92.6 | 90.3 | 9.4 | 97.4 |
| cC6O4     | 0.020 | 1.25 | 0.994 | 15.4 | 90.9 | 0.078 | 4.1 (2.5–7.1) | 5.3 | 94.6 (84.8–107.6) | 94.2 | 89.1 | 9.7 | 104.6 |
| 6:2 Cl-PFESA | 0.018 | 1.17 | 0.997 | 18.9 | 103.9 | 0.073 | 6.6 (4.7–9.0) | 9.2 | 99.6 (85.9–119.5) | 106.9 | 97.5 | 9.3 | 107.5 |
| 8:2 Cl-PFESA | 0.009 | 1.18 | 0.997 | 10.6 | 96.8 | 0.074 | 4.7 (1.8–8.7) | 6.4 | 103.9 (95.2–113.9) | 110.3 | 104.9 | 10.9 | 102.9 |
Mass spectrometer parameters were manually optimised for M3HFPO-DA. Since not all analogue internal standards were available, the remaining analytes were paired with structurally similar mass labelled standards (Table 1). Other general mass spectrometer parameters were manually optimised for data elaboration. The analyte response (area ratio) was recorded as the ratio between the peak area of the native standard and the peak area of the assigned internal standard.

**Method validation**

The method was thoroughly validated following the guidelines reported by the Food and Drug Administration (FDA) [59], the Italian Society of Clinical Biochemistry and Clinical Molecular Biology (SiBioC) [60], and the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) [61].

**Linearity**

The standard solutions used for calibration curves were prepared as reported in the “Standard solution preparation” section. Fourteen calibration standard solutions, covering a wide range of concentrations, were injected twice within each analytical sequence, along with a repetition of six replicates of blank matrix. For each analyte, the calibration curve consisted of at least six non-zero calibrators and was calculated by plotting the blank subtracted area ratios as y-values and the known concentrations (µg/L) as x-values; a 1/x weighted least-squares linear regression was computed. Linearity was assayed by calculating the mean coefficient of determination, $R^2$: three independent calibration curves were prepared and analysed separately in three analytical batches over the course of 6 months. The acceptance criteria followed to ensure the quality and reproducibility of each calibration curve are those described in the “calibration curve” section of the FDA guidelines. The use of blank subtracted calibration curve was in agreement with ICH guideline M10 on bioanalytical method validation [61].

**Selectivity and carryover effect**

In order to evaluate the selectivity of the method, procedural blanks (unspiked methanol prepared as an unknown sample) and blank samples (unspiked blank matrix) were analysed both with and without adding the internal standard solution; solvent blanks (pure methanol) were also analysed. The presence of interfering peaks with the same retention time of quant. transitions or internal standard transitions was verified. Moreover, the interference from internal standards was
evaluated by comparing the quant. chromatograms obtained by analysing replicates of pooled plasma samples with those obtained by analysing replicates of pooled plasma samples prepared without internal standards.

To evaluate the carryover effect, two analyses of the solvent blanks were carried out right after every analysis of the highest calibration standard level.

**Sensitivity**

For each analyte, the lower limit of quantification (LLOQ) was calculated using the following formula: \( \text{LLOQ} = \frac{(10 \text{SE}_q + q)}{m} \), where \( q \) is the intercept of the calibration curve (calculated only if positive), \( m \) is the slope, and \( \text{SE}_q \) is the standard error of \( q \) \[62\]; if the intercept was negative, the formula was reduced to \( 10 \text{SE}_q/m \). LLOQ was obtained as a mean from three independent calibration curves prepared and analysed separately in three analytical batches over the course of 1 month. Within-run precision and accuracy (“Precision and accuracy” section) at LLOQ concentrations were further experimentally determined by analysing five independent replicates of spiked blank matrix: for each analyte, the LLOQ was confirmed if the mean accuracy was within ±20% of the theoretical value, and the RSD % was ≤ 20%.

**Precision and accuracy**

To test the method precision, the preparation and analysis of each QC sample were repeated five times per run (within-run), and for five different runs over the course of 2 weeks (between-run). Relative standard deviations (\%RSDs) of the blank subtracted area ratios were calculated among the analyses carried out within each run; the within-run precision was calculated as the mean of these \%RSDs. The between-run precision was calculated as the \%RSD among all the analyses.

To test the accuracy of the method, the preparation and analysis of each QC sample were repeated five times per run (within-run), and for five different runs over the course of 8 months (between-run). Accuracy was calculated by dividing the calculated concentrations in the spiked samples by the theoretical spiked concentration and multiplying by 100 (\% theoretical). For each analyte, the within-run accuracy was calculated as the average of the mean accuracies obtained within each analytical batch, while the between-run accuracy was calculated as the mean accuracy obtained from all analyses.

**Stability**

Short-term stability was tested to verify the stability of the prepared samples while stored at 10 °C in the autosampler: two replicates of QC samples were analysed right after preparation and following 1 week of storage. The short-term stability was calculated as the % ratio between the area ratios obtained from the analyses of the stored QCs and those obtained by analysing freshly prepared QCs.

Long-term stability was tested to verify the stability of analytes in the matrix, from the sample collection to the analytical measurement while kept at −20 °C. Blank matrix was spiked with the native standard solutions at the concentration of QCs; then, for each level, an aliquot was immediately prepared and analysed, along with the calibration curve, while another aliquot was frozen at −20 °C. After 1 month, the second aliquot was defrosted at room temperature, prepared, and analysed along with a freshly prepared calibration curve. Long-term stability was calculated as the % ratio between calculated concentrations of the stored QC samples and those obtained with freshly prepared QCs.

**Matrix effect**

Seven plasma samples (previously screened for relatively low background levels of analytes), each derived from different individuals, were spiked with the native standards at QC concentrations, in duplicate. For each analyte, the area ratio was subtracted by the area ratio obtained in the corresponding non-spiked sample, and the results were compared among the seven different samples. Between-sample precision and accuracy were determined at each QC level.

**External verification**

The accuracy of the method was further verified for 12 analytes (PFPeA, PFHxA, PFHpA, PFNA, PFDA, PFUnDA, PFDoDA, PFBS, PFHxS, PFHpS, and PFOS), through the analyses of serum samples which had been stored in the frame of the interlaboratory comparison investigations and external quality assurance schemes (ICI-EQUAS) carried out during the HBM4EU project \[63–65\]. We did not participate in this exercise, but we used these samples as reference standard material. The sera were stored frozen (−20 °C) until use. For each ICI-EQUAS round, two levels (low and high) of considered PFASs were available; the samples from three different rounds (2, 3, and 4) were analysed in two independent analytical sequences 6 months apart from each other. Accuracy and Z-score were calculated to compare our results to reference values reported in the HBM4EU final reports. Mean Z-scores were calculated using the following expression: \( Z = \frac{(x - C)}{\sigma T} \), where \( x \) is our calculated concentration, \( C \) is the reference concentration, and \( \sigma T \) is a fit-for-purpose targeted standard deviation calculated as 0.25*\( \sigma \). According to the ICI-Equas guidelines, Z-scores ≤ 2 are considered satisfactory \[66\].

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Furthermore, we participated in the round 67 of the German External Quality Assessment Scheme (G-EQUAS) for the external verification of PFOA and PFOS. In this case, two serum samples were analysed in blind; the results were submitted and then compared to those obtained from reference laboratories [67, 68].

**Analytical sequence**

For routine measurements, a typical sequence consisted of a few injections of pure methanol, followed by blank samples, all the fourteen calibration standard solutions, QCs, a set of unknown samples interspersed by repetitions of QCs, and finally a second injection of the calibration standard solutions. Each sequence was considered acceptable if at least 75% and a minimum of six non-zero calibrator levels were within ±15% of their theoretical concentrations, except at LLOQ, for which an inaccuracy up to ±20% was accepted, and if at least 67% of all QC samples were within ±15% of their theoretical values, with at least 50% of QC samples per level were within ±15% of their theoretical values [59].

**Method application and statistical analyses**

The developed method was applied to 38 plasma samples collected from the local general adult population (see “Human plasma samples” section for details). Non-quantifiable values were replaced with half of the LLOQ, then descriptive statistics was applied (median, 5th and 95th percentiles). Statistical analyses were performed using the R software (version 4.0.5) [69], with the Rstudio interface (Version 1.4.1106 RStudio, PBC, Boston, MA, USA), and the tidyverse package [70].

**Results**

**Method validation**

Figure 1 shows the extracted ion chromatograms of quant. transitions obtained from an analysis of blank sample spiked with the analytical standards. The analytes are separated and eluted in 18 min. For both PFHxS and PFOS, the linear isomer was separated from the branched isomers, which were independently quantified as sum of all the possible branched isomers.

**Linearity**

The mean $R^2$ of each analyte ranged from 0.989 to 0.999 (Table 2), thus showing a good linearity for the considered concentration ranges.

**Selectivity and carryover effect**

As expected, all human plasma samples analysed contained many PFASs, some of which were detected at trace levels and others at significant concentrations (PFOA, PFOS, and PFHxS). The background concentration of each analyte found in the blank matrix used for method validation is reported in Table S4. Nonetheless, no interfering peaks having the same retention time of internal standard and quantifier transitions were detected, except for a co-eluting peak from the matrix at a close retention time of the quant. transition of PFBS that affected its sensitivity. Zero calibrators were free of interference at the retention time of the internal standards, according to FDA guidelines; the contribution of internal standards to the peak area of quant. transitions was not significant.

Over the course of 1 year, no significant carryover effect was observed as no considerable peaks in solvent blanks were observed, according to FDA guidelines.

**Sensitivity**

We found a good match between theoretically calculated LLOQ and the experimental verification. Indeed, the precision and the accuracy at LLOQ ranged respectively from 1.8 to 18.9% (%RSD), and from 90.8 to 119.5% (%theoretical) (Table 2). LLOQ values ranged from 0.009 to 0.078 µg/L for most compounds, with the exception of PFOA (0.156 µg/L), n-PFHxS (0.116 µg/L), n-PFOS (0.229 µg/L), and PFOS $\sum$ branched isomers (0.245 µg/L) for which we observed the highest background levels in blank matrixes. However, these levels were still suitable for an adequate quantitation of these compounds in samples from the general population (see “Method application” section).

**Precision and accuracy**

The results of the within- and between-run accuracy and precision tests are reported in Table 2. The within-run mean %RSD of blank subtracted area ratios ranged from 2.7 to 15.7%, while overall between-run %RSD ranged from 3.0 to 17.6%. Within-run mean accuracy ranged from 87.9 to 113.1%, while between-run accuracy ranged from 93.6 to 107.2%. The analyses were performed over the course of 8 months, thus showing the robustness of the method.

**Stability**

The results of the short-term stability (prepared samples stored for 1 week at 10 °C in the autosampler) ranged from 87.4 to 113.5%, while the results of the long-term stability (QC samples stored at –20 °C for 1 month) ranged from...
84.9 to 114.5% (Table 2), thus showing no significant alterations of analyte responses over time.

Matrix effect

The matrix effect, calculated as %RSD of the blank subtracted area ratios among seven different plasma samples, ranged from 4.7 to 19.7%, while the calculated concentrations ranged from 90.5 to 113.3% of the theoretical values (Table 2).

External verification

The results of the analyses of the ICI-Equas samples are presented in Fig. 2 and reported in the supplementary material (Table S5). If compared with reference values established by expert laboratories, mean accuracy ranged from 82.1 to 119.2% and the mean Z-score ranged from 0.1 to 0.8. The participation in the G-Equas round 67 for PFOA and PFOS was evaluated as satisfactory (Table S6 and Fig. S1).

Method application

The results of the method application to 38 plasma samples are reported in Table 3. PFOA, PFNA, PFDA, n-PFHxS, n-PFOS, PFOS Σ branched isomers, and PFECHS were always detected (≥ LLOQ). We obtained high detection frequencies (≥ 70% and < 100%) for other long-chain PFCAs (PFUnDA and PFTrDA) and some PFSA homologues (PFPeS, PFHxS Σ branched isomers, and PFHpS), while the short-chain perfluoroalkyl acids PFBA and PFBS were found in few samples. PFOA, n-PFOS, and PFOS Σ branched isomers showed the highest median levels (1.497, 1.909, and 1.267 µg/L, respectively), followed by n-PFHxS (0.580 µg/L) and C9–11 PFCAs homologues. The emerging analytes, HFPO-DA, DONA, and cC6O4, were mostly not quantifiable in the considered samples.

Discussion

In this work, a method for the determination of 30 PFASs in human plasma has been set up and fully validated. The target analytes were carefully chosen in order to include both legacy PFASs belonging to different chemical classes and emerging fluorinated compounds whose environmental diffusion could be on the rise.

The development of this analytical method presented some challenges. In order to delay the possible PFAS contaminations derived from the HPLC system, a trap column was installed before the autosampler compartment, as suggested by previous applications [42, 49–51, 71, 72]. It has been reported that PFASs, in particular those with a long perfluoroalkyl chain, if diluted in water, can be adsorbed by laboratory material such as polypropylene [73] or glass [74], while this effect is not expected in pure undiluted biological samples characterised by abundant matrix components, or in samples dissolved mainly in an organic solvent [75–77]. For these reasons, dilution of standard solutions in water were avoided and, in general, the numbers of subsampling steps were kept as low as possible to reduce possible losses and/or contaminations. Further verifications were conducted.
analysing procedural blanks, in order to assess any contribution to overall contamination from every step of the entire measurement procedure, from the blood sampling to the collection in autosampler vials with PTFE-septum caps. PFASs leaching from PTFE-containing labware are recognised as sources of interference during PFAS analyses [77]; indeed, during the method development, we also tested polypropylene (PP) caps. While eliminating the risk of PFAS contaminations, the use of PP caps determined a significant evaporation of methanol as the PP cap does not re-seal after needle puncture. To demonstrate the suitability of PTFE-containing caps, replicates of procedural blanks in contact with both materials (PP and PTFE caps) were analysed. No signal differences were recorded among the two preparations, as confirmed consistently in different analytical sequences. Therefore, we considered the use of PFTE-containing caps as an adequate analytical practice, as long as the monitoring of interferences is routinely conducted through the analysis of procedural blanks.

Unlike other PFASs, HFPO-DA presented a peculiar fragmentation behaviour as an in-source fragmentation was observed, as previously described [78]. In order to increase sensitivity, the most intense fragment generated was chosen for both the native compound and its related isotopic labelled internal standard, despite the latter not matching the quant. transition; however, we verified its suitability through the good results obtained within method validation.

The sample preparation involved the protein precipitation with an organic solvent, the centrifugation, and the injection of the supernatant onto the HPLC system. During method development, we took into consideration a further purification step with a solid-phase extraction (SPE) using weak anionic exchange (WAX) cartridges (Waters, Sesto San Giovanni, Italy) (data not shown). Indeed, WAX cartridge can be very useful for the analysis of strong acidic compounds as PFASs [79]. Nevertheless, after performing some experiments, we decided to avoid its use for some reasons: (1) a matrix effect test suggested that the usage of SPE did not improve the burden of the matrix effect for the analytes and that the additional manual steps required affected reproducibility; (2) considerable contamination with PFBA derived from WAX cartridges was observed, as declared by the producer [80]; and (3) one of the considered analytes is not a strong acidic compound (PFOSA), thus requiring the collection and analysis of the eluate derived from the cartridge washing step, in turn reducing the throughput of the assay. Only a few other methods analysed PFASs with a sample preparation consisting only of a protein precipitation without further cleaning [42–44, 81]. The main advantages are the few steps and short time required for sample preparation, the low amount of solvent used, and the lower cost for consumables.

The main strength of the present method is the quantification of several emerging PFASs, with great sensitivity: LLOQs of the present work were lower for HFPO-DA, F-53B-related analytes, and DONA [16, 43, 44, 56] and comparable for PFECHS [43] by comparing them with those reported by published methods. To the best of our knowledge, this is the first method able to quantify cC6O4 in human blood matrices.

Another strength of the present method is that we used a pooled human plasma as a blank matrix: most of the previous method used similar animal blood matrices such
as calf serum and plasma, as a surrogate matrix containing lower amounts of ubiquitous PFASs [48, 54, 56, 82]. We bought commercial human pooled plasma from Biowest (Nuaillé, France), but it was not suitable for the method because of the high levels of PFOA and PFOS. Therefore, we screened several real human samples and created a pooled plasma mixing only those containing low levels of analytes (“Blank matrix” section). The main advantage of this approach is that, unlike other methods which used a surrogate bovine matrix, the calibration curves were prepared in real human plasma, thus allowing working with an ideal control matrix, matching the matrix of the unknown samples. A limitation is represented by the higher LLOQs obtained for some compounds, especially those of PFOA, n-PFHxS, n-PFOS, and PFOS ∑ branched isomers, which were still adequate for the quantitation of these compounds at the levels usually found in the general population (Table 3). LLOQs for all other compounds were

Table 3 Results of the application of the method to the plasma samples (n = 38). Data are reported as median and 5th and 95th percentiles, along with the number (and percentage) of samples with concentrations greater than or equal to the lower limit of quantitation.

| Analyte | 5th percentile (µg/L) | Median (µg/L) | 95th percentile (µg/L) | Samples ≥ LLOQ (percentage) |
|---------|-----------------------|---------------|------------------------|-----------------------------|
| PFBA    | <LLOQ                 | <LLOQ         | 0.104                  | 3 (8%)                      |
| PFPeA   | <LLOQ                 | <LLOQ         | <LLOQ                  | 0 (0%)                      |
| PFHxA   | <LLOQ                 | <LLOQ         | <LLOQ                  | 0 (0%)                      |
| PFHpA   | <LLOQ                 | <LLOQ         | 0.194                  | 18 (47%)                    |
| PFOA    | 0.952                 | 1.497         | 3.565                  | 38 (100%)                   |
| PFNA    | 0.274                 | 0.409         | 0.748                  | 38 (100%)                   |
| PFDA    | 0.106                 | 0.198         | 0.329                  | 38 (100%)                   |
| PFUnDA  | <LLOQ                 | <LLOQ         | 0.310                  | 29 (76%)                    |
| PFDoDA  | <LLOQ                 | <LLOQ         | 0.058                  | 5 (13%)                     |
| PFTrDA  | <LLOQ                 | 0.030         | 0.073                  | 27 (71%)                    |
| PFTeDA  | <LLOQ                 | <LLOQ         | <LLOQ                  | 1 (3%)                      |
| PFOSA   | <LLOQ                 | <LLOQ         | <LLOQ                  | 1 (3%)                      |
| N-MeFOSAA | <LLOQ              | <LLOQ         | 0.050                  | 3 (8%)                      |
| N-EtFOSAA | <LLOQ              | <LLOQ         | <LLOQ                  | 1 (3%)                      |
| PFBS    | <LLOQ                 | <LLOQ         | 0.086                  | 10 (26%)                    |
| PFPeS   | <LLOQ                 | 0.029         | 0.076                  | 28 (74%)                    |
| n-PFHS: linear isomer | 0.275               | 0.580         | 1.692                  | 38 (100%)                   |
| PFHxS: ∑ branched isomers | <LLOQ            | 0.035         | 0.079                  | 35 (92%)                    |
| PFHpS   | <LLOQ                 | 0.093         | 0.249                  | 29 (76%)                    |
| n-PFOS: linear isomer | 1.113               | 1.909         | 4.679                  | 38 (100%)                   |
| PFOS: ∑ branched isomers | 0.612              | 1.267         | 3.279                  | 38 (100%)                   |
| PFNS    | <LLOQ                 | <LLOQ         | <LLOQ                  | 0 (0%)                      |
| PFDS    | <LLOQ                 | <LLOQ         | <LLOQ                  | 0 (0%)                      |
| PFCHS   | 0.021                 | 0.041         | 0.135                  | 38 (100%)                   |
| 4:2-FTSA | <LLOQ               | <LLOQ         | <LLOQ                  | 0 (0%)                      |
| 6:2 FTSA | <LLOQ               | <LLOQ         | <LLOQ                  | 1 (3%)                      |
| 8:2 FTSA | <LLOQ               | <LLOQ         | <LLOQ                  | 1 (3%)                      |
| HFPO-DA | <LLOQ                 | <LLOQ         | <LLOQ                  | 0 (0%)                      |
| DONA    | <LLOQ                 | <LLOQ         | <LLOQ                  | 0 (0%)                      |
| cC6O4   | <LLOQ                 | <LLOQ         | <LLOQ                  | 1 (3%)                      |
| 6:2 Cl-PFESA | <LLOQ       | <LLOQ         | <LLOQ                  | 6 (16%)                     |
| 8:2 Cl-PFESA | <LLOQ       | <LLOQ         | <Lâ ŁO Q                | 0 (0%)                      |

For calculation of the median and 5th and 95th percentiles, the non-quantifiable values were replaced with half of the LLOQ. Replacement values were the following: PFBA: 0.039; PFPeA: 0.010; PFHxA: 0.020; PFHpA: 0.020; PFOA: 0.078; PFNA: 0.039; PFDA: 0.039; PFUnDA: 0.039; PFDoDA: 0.020; PFTrDA: 0.010; PFTeDA: 0.010; PFOSA: 0.005; N-MeFOSAA: 0.020; N-EtFOSAA: 0.020; PFBS: 0.018; PFPeS: 0.009; n-PFHxS: linear isomer: 0.025; n-PFHxS: ∑ branched isomers: 0.006; n-PFHxS: ∑ branched isomers: 0.122; PFNS: 0.019; PFDS: 0.010; PFECHS: 0.004; 4:2 FTSA: 0.009; 6:2 FTSA: 0.010; 8:2 FTSA: 0.019; HFPO-DA: 0.010; DONA: 0.009; cC6O4: 0.010; 6:2 Cl-PFESA: 0.009; and 8:2 Cl-PFESA: 0.004 µg/L.
considerably low and often lower than most of the others previously reported [83, 84].

An additional strength of this work is the extensive validation of the method, which was precise and accurate as shown by results collected over the course of 8 months (Table 2 and S5). The external verification of the method, even though performed only on a limited number of analytes, is a confirmation of its accuracy and robustness. The external verification also confirmed the stability of those PFASs in serum samples, as they were analysed at least 1 year after being prepared for the HBM4EU project, yielding accurate results. Furthermore, although our method was developed in plasma, the results obtained suggest the applicability of the method also on serum matrix for the analytes included in the ICI-Equas and the G-Equas. Therefore, the presence or absence of clotting factors in the matrix does not affect the capability of our method to properly quantify the considered analytes. Finally, another advantage of this method is the small amount of human plasma required to analyse a sample (20 µL).

Regarding PFHxS and PFOS, we were also able to separate the linear isomer from all the branched-chain isomers and quantified the latter as a sum of branched isomers by referring to its certified concentration. The importance of assessing human exposure to PFAS isomers has been reported [85].

The application of the method was intended to verify its performance and was applied only to a small subset of samples. As expected, PFOA, PFOS, PFHxS, and other long-chain PFAAs (PFNA, PFDA, PFUnDA, PFTrDA, PFHpS) were detected in most samples also showing the highest median concentrations. Among the emerging compounds, PFECsS were always found, while PFEAs as HFPO-DA, DONA, cC6O4, and F-53B-related analytes were mostly not quantifiable.

In conclusion, the present analytical method is a suitable tool for the biological monitoring of both traditional and emerging PFASs, for which human exposure may be on the rise; further studies are thus required to monitor their presence in larger populations and to assess their toxicokinetics and toxicological properties.

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Data availability All data and material are available upon request to the corresponding author.

Declarations

Ethics approval The use of routine leftover samples, which were used for the preparation of the human pooled plasma, is allowed by the hospital Fondazione IRCCS Ca’ Granda Ospedale Maggiore Policlinico, Milan, Italy, for method development, optimization, and validation, as long as samples are anonymised; therefore, it does not require a specific approval by the ethics committee.

Conflict of interest The authors declare no competing interests.

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