A Simple and Rapid Method for Quantitative HPLC MS/MS Determination of Selected Perfluorocarboxylic Acids and Perfluorosulfonates in Human Serum

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1. Introduction

Perfluoroalkyl and polyfluoroalkyl substances (PFASs) are a class of synthetic organic chemicals containing fully (per) or partly (poly) fluorinated carbon chains with different functional groups used since 1950s in various industrial and commercial products (fire foams, clothes, protective coatings for carpets, pots, paints, and insecticides) [1]. For many years, they have spread in the environment (water, soil, and biota) and have also entered the food chain [2–6]. Their chemical-physical properties are thermal and chemical resistance, impermeability to oil and water, and surfactant properties. These chemical-physical properties combined with high industrial production and frequent and widespread use have made PFAS persistent contaminants capable of spreading easily in the environment without degrading but bioaccumulating and bioconcentrating in the food chain [7, 8]. Human exposure to these substances can pose a risk to public health [9–13]; for this reason, the scientific community in the last few years is implementing a series of monitoring measures for these substances in the environment and in humans. In particular, human biomonitoring is the most effective way to characterize human exposure to PFAS. We therefore developed a very simple method for the analysis of PFAS in human serum by liquid chromatography tandem mass spectrometry. The sample preparation is based...
on the denaturation of proteins with acetonitrile, volume
reduction, and injection: no extraction or purification in the
solid phase is necessary.

2. Materials and Methods

The Laboratory of the Italian National Institute of Health
(ISS), Human Exposure to Environmental Contaminants
Unit, has developed the method described in this paper.

We ordinarily use the method hereafter described for the
analysis of serum samples within the framework of various
national and international projects and in interlaboratory
comparison exercises [10, 14–18], Ring Exercise Test for
Persistent Organic Pollutants in Human Serum https://
www.inspq.qc.ca/en/cqt/eqas/amap/description [19]. Re-
results obtained by our laboratory in the interlaboratory
comparison exercises are reported in detail in supplemen-
tary data 2. Reports of the interlaboratory comparison ex-
cercises are in supplementary data 3.

2.1. Sample Preparation. Sampling is usually performed by
Italian local sanitary units in charge for blood withdrawal,
which we provide with specific instructions to avoid PFAS
contamination, particularly contaminations deriving from
the presence of teflon-coated materials or similar (these are
among the major causes of analytical interferences found in
PFAS analysis). We recommend using polycarbonate or
high-density polyethylene materials to collect or store the
samples. Blood withdrawal (about 5 mL) must be performed
with a serum tube containing no anticoagulant (may contain
coaulation activator and separator gel) that must be placed in
a suitable rack in a vertical position (avoiding as far as
possible any solicitation of the contents) and left at room
temperature until the clot is completely formed (about
30 min) and then centrifuged (centrifuge within 2 hours
after collection) at about 3500 g for 15 minutes at 20°C.

After centrifugation, the tube must be removed from the
centrifuge and placed in a rack, and the serum was removed from
the tube with a pipette, transferred into a 15 mL
polypropylene tube with a properly labelled Falcon-type
screw cap, and immediately frozen keeping the tube in a
vertical position inside a suitable test tube rack. 15mL
screw cap, and immediately frozen keeping the tube in a
polypropylene test tubes used for storing and shipping the
samples. Blood withdrawal (about 5 mL) must be performed
with a serum tube containing no anticoagulant (may contain
coaulation activator and separator gel) that must be placed in
a suitable rack in a vertical position (avoiding as far as
possible any solicitation of the contents) and left at room
temperature until the clot is completely formed (about
30 min) and then centrifuged (centrifuge within 2 hours
after collection) at about 3500 g for 15 minutes at 20°C.

2.2. Chemicals. High-purity chemical 13C-labelled internal
standards of perfluorobutanolic acid (PFBA), perfluoroheaxonic
acid (PFHxA), perfluorooctanoic acid (PFOA), perfluorohexane
sulfonate (PFHS), perfluorooctane sulfonate (PFOS), per-
fluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA),
perfluoroundecanoic acid (PFUDA), and perfluorododecanoic
acid (PFDOA) are purchased from Wellington Laboratories
(Wellington Laboratories Inc., Ontario, Canada, N1G 3M5). The
13C-labelled internal standard solution is prepared by with-
drawing, with a 500 μL syringe, 2.5 mL of a commercial mixture
of PFAS 13C or 18O-labelled identified as “MPFAC-MX,” at a
concentration of 2000 ng/mL diluted with acetonitrile up to the
volume of 25 mL in a class A glass flask and stored in the re-
frigerator at 4 ± 3°C until use. The serum samples are analyzed for
nine perfluorocarboxylic acids: PFBA, PFPeA, PFHxA,
PFHpA, PFOA, PFNA, PFDA, PFUdA, and PFDOA and four
perfluorosulfonates: PFBS, PFHxS, PFHpS, and PFOS; unla-
belled standards of these PFASs are purchased from Wellington
Laboratories (Wellington Laboratories Inc., Ontario, Canada,
N1G 3M5). The unlabelled PFAS standard solution identified as
“PFAS,” at a concentration of 200 ng/mL, is prepared by
withdrawing 1 mL of a commercial mixture of natural PFAS
identified as “PFAC-MXB,” at a concentration of 2000 ng/mL
diluted with acetonitrile up to the volume of 10 mL in a glass
class A flask. This solution is stored at 4 ± 3°C until use. The
injection standard solution (13C14 PFHpA 50 ng/mL in aceto-
nitrile) identified as “13C14PFHpA” is prepared by withdrawing
1 mL of a commercial mixture of 13C14 PFHpA 50 μg/mL and
diluted with acetonitrile at a volume of 1000 mL in a glass
class A flask. It is stored at 4 ± 3°C until use. HPLC-grade acetonitrile
(≥99.9% purity) is purchased from VWR Chemicals (Radar,
Pennsylvania, United States), and HPLC-grade water is pur-
chased from J.T. Becker (Thermo Fisher Scientific, Hampton,
New Hampshire, United States).

2.3. Instrumental Analysis. Instrumental analysis is carried
out by HPLC (Waters Alliance 2695, Waters Corporation,
Milford, MA, USA). Chromatographic separation is
achieved using a Kinetex C18 Column (5 μm, 100 mm × 2.1 mm ID, 100 Å) supplied by Phenomenex
(Torrance, CA, USA) operated at a temperature of 45°C. A
cartridge column (YMC ODS-A 5 μm, 20 × 4.0 mm ID, 120 Å) supplied by YMC Europe (Dinslaken, Germany) is
attached in line before the column to trap residues from the
mobile phase or the system. An injection volume of 10 μL is
determined to be optimal considering the required sensitivity of the method and the chromatographic performance. Larger injection volumes have been shown to cause matrix effect. The mobile phases are an acetic acid/ammonium acetate solution in water (A) and acetonitrile (B). Solution A is prepared by adding 19 mg ammonium acetate and 4.5 mL acetic acid to 250 mL of water. We have investigated the effect of the pH of solution A on the chromatographic behavior of the analytes and found that an improvement of short-chained PFAS peak shapes is achieved at lower pH values; pH 2.24 resulted to be good enough to analyze short-chained PFASs and acceptable to limit system deterioration. The flow during the injection is 0.35 mL/min, and the HPLC pump gradient timetable is shown in Table 1. The HPLC is interfaced with a triple quadrupole mass spectrometer (Micromass Quattro micro TM API, Waters Corporation, Milford, MA, USA).

Analytes are detected by electrospray negative ionization in ion multiple reaction monitoring (MRM) mode, and argon is used as collision gas. A minimum of ten scans across the chromatographic peak is required to ensure adequate peak shape. Working conditions and acquisition parameters are reported in Tables 2 and 3.

### 3. Results

In human serum, perfluorosulfonates (particularly, PFHxS and PFOS) consist of significant quantities of branched isomers, whereas perfluorocarboxylic acids are predominantly linear. In our method, we quantify perfluorosulfonates as the sum of linear and branched isomers. For PFOS quantification, we selected the transition 499 > 80 as it is more sensitive than the m/z 499 > 99 transition and generally preferred when quantifying both linear and branched isomers. Data acquisition and processing are performed using MassLynx, ver. 4.1 and TargetLynx Application Manager. Analytes are quantified against a reference standard by applying the isotope dilution technique. The reference standard for PFAS quantification is prepared with each batch of test samples. The limits of quantification range are 0.01–0.5 ng/mL. Figures 2 and 3 show some examples of chromatograms, which illustrate the separation of perfluorocarboxylic acids and perfluorosulfonate compounds typically found in human serum.

As required by the internal control quality, each laboratory bottle and vial is tested before first use: washing acetonitrile is added, concentrated, and analyzed. If it is PFAS-free, then the glassware can be used. According to the quality system, for each batch of test samples (20 test samples in one batch), at least one procedural blank and one quality control sample (QC) are analyzed. The blank

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**Table 1: Water alliance 2695 HPLC pump gradient timetable.**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>A%</th>
<th>B%</th>
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<tr>
<td>0.00</td>
<td>90</td>
<td>10</td>
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<tr>
<td>0.10</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>4.00</td>
<td>0</td>
<td>100</td>
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<tr>
<td>7.00</td>
<td>90</td>
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**Table 2: MS tune conditions.**

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<table>
<thead>
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<th>Temperatures</th>
<th>Source temp (°C)</th>
<th>Desolvation temp (°C)</th>
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<tr>
<td></td>
<td>120</td>
<td>450</td>
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</table>

<table>
<thead>
<tr>
<th>Gas flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desolvation (L/hr)</td>
</tr>
<tr>
<td>Cone (L/hr)</td>
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**Table 3: Acquisition parameters.**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Trace</th>
<th>RT (min)</th>
<th>Dwell (s)</th>
<th>Cone (V)</th>
<th>Collision (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFBA</td>
<td>213 &gt; 169</td>
<td>3.60</td>
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<td>9</td>
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<td>PFPcA</td>
<td>263 &gt; 219</td>
<td>3.76</td>
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<tr>
<td>PFHxA</td>
<td>313 &gt; 269</td>
<td>3.88</td>
<td>0.02</td>
<td>15</td>
<td>10</td>
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<tr>
<td>PFHpA</td>
<td>363 &gt; 319</td>
<td>3.97</td>
<td>0.02</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>PFOA</td>
<td>413 &gt; 369</td>
<td>4.05</td>
<td>0.02</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>PFNA</td>
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<td>4.13</td>
<td>0.02</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>PFDA</td>
<td>513 &gt; 469</td>
<td>4.23</td>
<td>0.02</td>
<td>15</td>
<td>13</td>
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<tr>
<td>PFDoA</td>
<td>563 &gt; 519</td>
<td>4.31</td>
<td>0.02</td>
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<td>11</td>
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<tr>
<td>PFBS</td>
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<td>25</td>
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<td>0.02</td>
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<td>30</td>
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<tr>
<td>PFOS</td>
<td>499 &gt; 80</td>
<td>4.16</td>
<td>0.02</td>
<td>55</td>
<td>45</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Trace</th>
<th>RT (min)</th>
<th>Dwell (s)</th>
<th>Cone (V)</th>
<th>Collision (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFBA13C4</td>
<td>217 &gt; 172</td>
<td>3.60</td>
<td>0.02</td>
<td>12</td>
<td>9</td>
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<tr>
<td>PFHxA13C2</td>
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<td>3.88</td>
<td>0.02</td>
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<td>10</td>
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<tr>
<td>PFHpA13C4</td>
<td>367 &gt; 322</td>
<td>3.97</td>
<td>0.02</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>PFOA13C4</td>
<td>417 &gt; 372</td>
<td>4.05</td>
<td>0.02</td>
<td>15</td>
<td>11</td>
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<tr>
<td>PFNA13C5</td>
<td>468 &gt; 423</td>
<td>4.13</td>
<td>0.02</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>PFDA13C2</td>
<td>515 &gt; 470</td>
<td>4.23</td>
<td>0.02</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>PFDoA13C2</td>
<td>565 &gt; 520</td>
<td>4.31</td>
<td>0.02</td>
<td>15</td>
<td>11</td>
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<tr>
<td>PFBS</td>
<td>299 &gt; 99</td>
<td>3.84</td>
<td>0.02</td>
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<td>25</td>
</tr>
<tr>
<td>PFHxS18O2</td>
<td>399 &gt; 99</td>
<td>4.02</td>
<td>0.02</td>
<td>50</td>
<td>30</td>
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<tr>
<td>PFHpS</td>
<td>449 &gt; 99</td>
<td>4.08</td>
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<td>499 &gt; 80</td>
<td>4.16</td>
<td>0.02</td>
<td>55</td>
<td>45</td>
</tr>
</tbody>
</table>

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**Figure 1: Assessment of human exposure to PFAS through biomonitoring.**
and the control sample are spiked, extracted, and treated like the test samples. The concentration of the quality control sample is plotted on a control chart to verify that the procedure is under control. If the analytical batch results to be out of control, results are rejected and causes of the out of control are analyzed and corrected until the system quality control procedures give positive results. We have been using for years the described method for the analysis of PFAS in serum samples within the framework of various national and international projects and in interlaboratory comparison exercises and consider it suitable for routine analysis. Figure 4 shows an example of
the quality control charts we use for each batch of test samples (20 test samples in one batch). We have selected control charts of the most known and most determinable compounds in human serum, PFOS, and PFOA; control charts of all the determined analytes are reported in supplementary data 1.

Figure 3: Chromatogram (MRM) of a real serum sample (perfluorocarboxylic acids (left) and perfluorosulfonate compounds (right) are present in the sample at very low levels).
4. Conclusion

In the present study, a simple and fast analytical method was developed to simultaneously quantify 13 perfluoroalkyl compounds. This method has been developed for the quantification of 13 perfluorocarboxylic acids and perfluorosulfonate compounds (belonging to the class of alkyl per- and polyfluorinated substances (PFAS)) in human serum.

Data Availability

The reports of the AMAP and HBM4EU (with a good z-score) intercalibration exercises (https://www.hbm4eu.eu; AMAP Ring Exercise Test for Persistent Organic Pollutants in Human Serum https://www.inspq.qc.ca/en/ctq/ecas/amap/description [10, 15–18]) support the findings of this study.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The authors wish to thank all the participants involved in human biomonitoring national and international studies on PFAS.

Supplementary Materials

Supplementary data 1: quality control sample charts—quality control charts of PFAS concentrations in the quality control sample were analyzed and collected in our laboratory in the last 5 years. Supplementary data 2: control charts’ z-score of HBM4EU and AMAP—control charts of z-score values obtained in the last 3 years by our laboratory in the interlaboratory
comparison exercises organized within the HBM4EU project and the Arctic Monitoring and Assessment Programme (AMAP). Supplementary data 3: AMAP report 2016–2017—reports of the interlaboratory comparison exercises organized by the Arctic Monitoring and Assessment Programme (AMAP) in 2016–2017. (Supplementary Materials)

References