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This is the author's manuscript

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/1740724 since 2020-06-09T10:18:07Z

Published version:

DOI:10.1016/j.chroma.2020.461038

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03 May 2024

Optimization and validation of a method based on QuEChERS extraction and liquid chromatographic-tandem mass spectrometric analysis for the determination of perfluoroalkyl acids in strawberry and olive fruits, as model crops with different matrix characteristics

Cristina Vanessa Agata Scordo[a], Leonardo Checchini[a], Lapo Renai[a], Serena Orlandini[a], Maria Concetta Bruzzoniti[b], Donatella Fibbi[c], Laila Mandi[d], Naaila Ouazzani[d], Massimo Del Bubba[a]

[a] Department of Chemistry "Ugo Schiff", University of Florence, Via della Lastruccia 3 – 50019 Sesto Fiorentino, Florence, Italy

[b] Department of Chemistry, University of Turin, Via Pietro Giuria 5 – 10125 Turin, Italy

[c] GIDA S.p.A., Via di Baciacavallo 36, 59100 Prato, Italy

[d] National Center for Research and Studies on Water and Energy (CNEREE), Cadi Ayyad University, Marrakech, Morocco

Abstract

A QuEChERS method was optimized and validated for the LC-MS/MS analysis of perfluoro-n-pentanoic acid (PFPeA), perfluoro-1-butanesulfonic acid (PFBuS), perfluoro-n-hexanoic acid (PFHxA), perfluoro-n-heptanoic acid (PFHpA), perfluoro-1-hexanesulfonic acid (PFHxS), perfluoro-n-octanoic acid (PFOA), perfluoro-n-nonanoic acid (PFNA), perfluoro-1-octanesulfonic acid (PFOS) and perfluoro-n-decanoic acid (PFDA) in freeze-dried strawberry and olive, as model fruits characterized by very different chemical compositions. The method was evaluated for apparent recovery, intra-day and inter-day precision, matrix effect and recovery. The method optimized for strawberry provided for most compounds absolute values of matrix effect (|ME%|) ≤11%, except for PFHxA, which showed a signal suppression of 22%. The extraction efficiency was tested at the spike levels 500-5000 pg/g d.w. for PFPeA, PFBuS, and PFHxA, and 100-1000 pg/g d.w. for the other target analytes, evidencing as a whole recoveries in the range of 65-89%. For olive fruits, due to their high fat content, an ultrasound-assisted extraction was necessary to obtain an efficient sample disgregation so as to increase the extraction yield and its precision. Moreover, a d-SPE clean-up with GCB allowed to achieve |ME%| ≤8% (except for PFBuS, which showed a signal enhancement of 19%) and recoveries calculated at the aforementioned spike levels were in the range 75-97%. The two methods provided very good linearity (R^2≥0.9984) from 10000 pg/g down to compound specific quantification limits, which were included in the ranges of 2.9-393 pg/g and 2.6-127 pg/g for strawberry and olive fruit, respectively. The methods were applied to the analysis of PFAAs in strawberry and olive fruits commercially available in two Italian supermarkets, as well as obtained under irrigation with various treated wastewaters (TWWs), evidencing in both cases a higher PFAAs occurrence in olives than in strawberry. However, PFAAs concentrations determined in the investigated fruit matrixes were quite low, being their sum 1.9 ng/g d.w. in the worst case (i.e. olive fruits grown under irrigation with TWWs).

Keywords: LC-MS/MS; d-SPE; perfluorinated compounds; fruit safety; organic micropollutants; irrigation by wastewater.

1 Introduction

Food safety is a very important and topical issue, since foodstuffs can be contaminated through different ways, alongside production and distribution stages [1]. This contamination does not only concern chemical substances intentionally used within the food production chain, such as pesticides in agriculture. In fact, foodstuffs can come into contact with various environmental micropollutants before human consumption [2].

Among organic micropollutants, those most recently identified in environmental matrices and/or recognized as environmentally hazardous, are obviously of major concern in the scientific community and have been included in the so-called group of "emerging contaminants" (ECs). ECs comprise a large group of chemicals, such as pharmaceuticals, personal care products, and also perfluoroalkyl acids (PFAAs) [3, 4], the latter widely used since 1960 for the industrial production of various consumer products [5-8]. The large industrial use of PFAAS derives from their peculiar physicochemical properties. In fact, PFAAs are synthetic chemicals in which carbon-hydrogen bonds are replaced by carbon-fluorine bonds, which is known as one of the strongest linkage in organic chemistry and entails extremely stable at high temperatures (typically up to 150°C) [9]. Moreover, they are neither flammable nor degradable under strong acidic or basic conditions, resistant to oxidizing agents and photolysis and not subjected to biodegradation processes [10, 11]. As a consequence of their large use, as well as their chemical and biological recalcitrance, PFAAs are characterized by an incomplete removal in wastewater treatment plants (WWTPs) and a wide occurrence in different water ecosystems have been reported [12-14].

Results of toxicological studies on animals have indicated that two of the most common PFCs, perfluorooctanesulfonate (PFOS) and perfluorooctanoic acid (PFOA), may affect fatty acid metabolism and the reproductive system as well as induce adverse effects in liver and other tissues [15, 16]. Furthermore, human biomonitoring data evidenced that PFAAs bind to proteins and, for PFOA and PFOS, has been estimated a bio-elimination time from the human body of 3.8 and 5.4 years, respectively [17-21]. These characteristics have led the European Food Safety Authority (EFSA) to establish maximum tolerable weekly intakes (TWIs) of 13 ng/kg body weight for PFOS and 6 ng/kg body weight for PFOA [22]. Moreover, PFOS has been added to the list of persistent organic pollutants (POPs) under the Stockholm Convention on POPs [23] .

It should also be remarked that physicochemical properties (e.g. lipophilicity and acidity) of these compounds greatly vary depending on the chain length and the acidic group present in the molecule. Based on these considerations, PFAAs represent an interesting group of model micropollutants to be investigated for their possible transfer to crops. Moreover, PFAAs have been found at low

concentrations in crops [24, 25], and therefore very sensitive methods are needed for their determination, thus highlighting the challenging character of this issue in analytical chemistry.

Few studies regarding crop contamination by PFAAs are reported in literature [24-30], some of them using the QuEChERS approach for the extraction of target analytes [25, 29]. In these works, however, the entire analytical procedure (i.e. extraction and clean-up) is applied without evaluating the influence of the two individual steps on matrix effect (ME). In this way, it is not clear whether the clean-up procedure leads to an effective advantage in terms of reduction of the matrix effect (ME), the investigation of which, on the other hand, is overlooked in most cases [24, 25, 28-30]. It should also be noted that the only methodological work concerning PFAAs determination in food, focuses on the analysis of a wide range of products (e.g. fruit, vegetables and cheese), proposing for all the matrices investigated the same complex clean-up procedures [27]. However, such a procedure, necessary for fatty foods, might be superfluous in the case of simple matrices with low or null fat content.

Among crops that could be adopted as models for investigating the contamination of fruits, strawberry (*Fragaria x ananassa*) and olive (*Olea europaea* L.) are surely very interesting, due to their remarkably different matrix characteristics. In fact, strawberry is characterized by a very high water content, which accounts for 85-90% irrespective of the cultivar considered [31]. Conversely, olive is one of the most lipophilic fruit, being it rich in fats, with a water content approximately included between 50% and 70%, depending on the cultivar considered [32]. Hence, olive fruit is very interesting to be investigated for the transfer of hydrophobic compounds, such as longer chain perfluorocarboxylic acids. In this regard, according to literature, the cultivars showing among the highest fruit fat content and corresponding low water percentage were “Frantoio” (<50%), “Picual” and “Koroneiki” (about 50%) [32].

Based on the considerations reported above, the aim of this study was to develop a rapid, sensitive and green method for the determination of nine PFAAs in strawberry and olive fruits using the QuEChERS extraction and the dispersive solid-phase extraction (d-SPE) as clean-up approach, followed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The optimized method was applied to the evaluation of PFAA occurrence in: (i) strawberry and olive fruits commercially available in Italian supermarkets, and (ii) in strawberry (cultivar “Camarosa”) and olive fruits (cultivar “Frantoio”) obtained under irrigation with various treated wastewaters (TWWs). In fact, even though the wastewater reuse for irrigation is an efficient tool of reducing water shortage and it is widely adopted in various Mediterranean countries [33], the presence of PFAAs in TWWs [12, 34, 35] could negatively affect human health, owing to their possible transfer from recycled water to crops.

2 Materials and methods

2.1 Chemicals and materials

Perfluoro-n-pentanoic acid (PFPeA, CAS 2706-90-3), perfluoro-1-butanesulfonic acid (PFBuS, CAS 375-73-5), perfluoro-n-hexanoic acid (PFHxA, CAS 307-24-4), perfluoro-n-heptanoic acid (PFHpA, CAS 375-85-9), perfluoro-1-hexanesulfonic acid (PFHxS, CAS 355-46-4), perfluoro-n-octanoic acid (PFOA, CAS 335-67-1), perfluoro-n-nonanoic acid (PFNA, CAS 375-95-1), perfluoro-1-octanesulfonic acid (PFOS, CAS 1763-23-1) and perfluoro-n-decanoic acid (PFDA, CAS 335-76-2) were purchased from Wellington Laboratories Inc. (Guelph, ON, Canada) as methanolic solutions, each one at the concentration of 50 µg/mL. The ^{13}C mass-labelled PFAA mixture containing the aforementioned compounds, each one at 2 µg/mL in methanol, was purchased from Wellington Laboratories Inc.

Sodium chloride (purity ≥ 99.0%) and anhydrous magnesium sulphate (purity ≥ 99.5%) used for QuEChERS extraction were obtained from Sigma-Aldrich (St. Louis, MO, USA). Water, methanol and acetonitrile (LC-MS grade) used for LC analyses and/or QuEChERS extraction, were obtained from Carlo Erba (Milan, Italy). Ammonium acetate and formic acid (MS grade, purity ≥ 99.0%) respectively used for the preparation of the LC eluents and the acidification of the extracts, were purchased from Sigma-Aldrich.

Endcapped octadecylsilica (C18), primary secondary amine (PSA) and graphitized carbon black (GCB) used for d-SPE clean-up of the QuEChERS extracts were purchased from Agilent Technologies (Santa Clara, CA, USA). Minisart® regenerated cellulose syringe filters, used for the filtration at 0.2 µm of the QuEChERS extracts, were supplied by Sartorius (Gottinga, Germany).

2.2 Samples

The strawberry (cultivar "Camarosa") and olive (cultivar "Frantoio") fruit samples used for the optimization and validation of the analytical method were collected within an experimentation conducted at the scientific campus of the University of Florence (Italy) and aimed at testing the possible transfer of chemical contamination in fruits irrigated with four different types of TWWs and tap water (TW) as control. More in detail, in this study, the effluents from the following WWTPs located in the area of Prato (Italy), were used: (i) "Baciacavallo" activated sludge plant (TWW_1); (ii) "Macrolotto 1" refining system of the Baciacavallo effluent (TWW_2); (iii) "Macrolotto 2" refining system of the Baciacavallo effluent (TWW_3) and (iv) "Calice" activated sludge plant (TWW_4). Full details of the treatment stages implemented in the four aforementioned WWTPs are reported in the paragraph S.1 of the *Supplementary material* section. All the TWWs contained PFAAs at concentrations ranging from a few to tens of ng/L.

Strawberry and olive fruits were harvested in July and November 2018, respectively. After collection, the fruits were transported to the laboratory, immersed in liquid nitrogen, freeze-dried and finally stored at -20 °C until they were analysed. Moreover, to test the applicability of the developed method, commercially available strawberry and olive fruits were purchased in two supermarkets.

2.3 LC-MS/MS analysis

Separation of target analytes was performed on a Shimadzu (Kyoto, Japan) chromatographic system consisting of two LC-20AD pumps (maximum allowed backpressure 600 bar), a SIL-30AC autoinjector (maximum allowed backpressure 1000 bar) equipped with a 20 µL loop, a CTO/20AC thermostatted column compartment and a CBM-20A module controller. A delay column ZORBAX SB-C18 (Agilent Technologies, 4.6 x 75 mm, particle size 3.5 µm) was installed between the mixer and the sample injector, in order to minimize the PFAA blank contributions due to the LC system (i.e. solvents and/or tubing).

Chromatographic separation was obtained using a KINETEX XB-C18 column (100 x 3 mm, particle size 2.6 µm) equipped with a guard column of the same phase (Phenomenex, Torrance, CA, USA), thermostatted at 50°C. Eluent A: aqueous solution of 2 mM ammonium acetate. Eluent B: methanolic solution of 2 mM ammonium acetate. A flow rate of 0.35 mL/min was adopted. The chromatographic gradient was the following: 10% B for 2 min, from 10% to 90% in 9.20 min, from 90% to 100% in 0.01 min, 100% for 6.79 min, from 100% to 10% in 2.50 min and final isocratic 10% for 5.5 min to allow system re-equilibration. Total analysis time (including system re-equilibration) was 26 min and maximum backpressure 180 bar. The injection volume was set to 10 µL. In order to minimize MS source contamination, from 0 min to 8 min and after the elution of the last peak until the start the system re-equilibration, the LC eluate was diverted to waste by means of a Vici (Schenkon, Switzerland) two-position six-port valve model HT (maximum allowed backpressure 500 bar), installed upstream the mass spectrometer.

The Shimadzu LC system was coupled to a 5500 QTrap mass spectrometer (Sciex, Framinham, MA, USA), by a Turbo V® interface equipped with an electrospray ionization (ESI) probe. MS/MS analysis was carried out using a time-scheduled Multiple Reaction Monitoring (MRM) mode in negative ionization. For each investigated compound, the most intense transition was used for quantification and the second most intense, when present, for confirming the identification. All the optimized MRM parameters are reported in **Table S1** of the *Supplementary material*.

Source dependent parameters were optimized in flow injection analysis at optimal LC flow and mobile phase composition, and were as follows: Curtain Gas (CUR) 50, Collision Gas (CAD) medium (8), Temperature (TEM) 650°C, Gas 1 (GS1) 50, Gas 2 (GS 2) 50, and IonSpray Voltage (IS) -4500 V. Chromatograms were acquired by the 1.6.2 version of software Analyst (Sciex). Criteria

proposed by the Commission Decision 2002/657/CE were adopted for identity confirmation [36]. Peak attribution and quantitative determination were performed using MultiQuant software version 3.0.2 (ABSciex).

2.4 QuEChERS extraction and clean-up procedure

Polypropylene (PP) and polyethylene (PE) plastic ware was used for all the analytical steps. Under the optimized experimental conditions, the following extraction and clean-up procedures were adopted for strawberry and olive fruits, respectively.

2.4.1 Strawberry

One gram of freeze-dried fruit, which appears as a homogeneous powder, was weighed into a 50 mL centrifuge tube and 5 mL of LC-MS water is added. The mixture was hand shaken for 15 s and vortex-mixed for 1 min. Then, 10 mL of acetonitrile were added and the mixture was further hand shaken for 15 s and vortex-mixed for 1 min. Afterwards, 2 g of NaCl and 2 g of $MgSO_4$ were added, and the obtained mixture underwent to additional hand shaking (15 s) and vortex mixing (1 min). The tube was centrifuged at 9000×g and T=4°C for 5 min. The acetonitrile supernatant is collected and finally filtered through a 0.2 µm filter in a 15 mL graduated tube. Hence, the extraction procedure lasted about 10 min. The filtered extract was acidified with formic acid (0.1% *v/v*) before LC-MS/MS analysis.

2.4.2 Olive

One gram of freeze-dried fruit, which appears as an inhomogeneous oily solid, was weighed into a 50 mL centrifuge tube and 5 mL of LC-MS water is added. The mixture was hand shaken for 15 s and vortex-mixed for 1 min. Then, 10 mL of acetonitrile were added and the mixture was further hand shaken for 15 s, and sonicated for 90 sec (pulsed mode, 10 sec on and 5 sec off, power 750 W), using a VC750 sonication probe (Sonics & Materials, Newtown, CT, USA). Afterwards, 2 g of NaCl and 2 g of $MgSO_4$ were added, and the obtained mixture underwent to additional hand shaking (15 s) and vortex mixing (1 min). The tube was centrifuged at 9000×g and T=4°C for 5 min.

The acetonitrile supernatant was treated with 400 mg of GCB and 150 mg of $MgSO_4$ per mL of extract. The resulting mixture was hand-shaken for 15 s, vortex mixed for 1 min, centrifuged at 9000×g and T=4°C for 5 min. and the recovered solvent filtered at 0.2 µm. The extract is finally made up to the original volume underwent to the clean-up, using the acetonitrile of the procedural blank, which is in our case the solvent that does not contain the matrix, brought through the entire analytical procedure in the same manner as a test sample [37]. As a whole, the overall procedure lasted about 20 min. Before LC-MS/MS analysis, the filtered extract, was acidified to pH=2.50±0.05 with formic acid (0.1% *v/v*)

2.5 Method performance evaluation and validation

In this work, the different method optimization steps were evaluated and validated by calculating the performance parameters following reported. In this regard, taking into account that the QuEChERS extracts, both as such and cleaned-up, contain salts which may affect the ionization of analytes, performance evaluations of the various analytical optimization steps were performed, when appropriate, in comparison with standard solutions prepared in the corresponding matrix-free procedural blank.

2.5.1 Linearity of the methods

The analysis of strawberry and olive fruits is performed using two methods, which differ for the presence of the d-SPE clean-up in the case of olives. For both matrixes, the method linearity was assessed by spiking in triplicate 1 g d.w. aliquots with ^{13}C labelled PFAA standards at the following concentrations: 10, 50, 100, 500, 1000, 5000, and 10000 pg/g d.w. The spiked samples underwent to the overall analytical protocol and finally analysed by LC-MS/MS after addition of formic acid 0.1% (*v/v*).

2.5.2 Apparent recovery

The apparent recovery percentage (AR%) [38] of the methods were assessed, following the IUPAC indications [39], by spiking in pentaplicate 1 g d.w. aliquots with ^{13}C labelled PFAA standards at 500 (level 1) and 5000 (level 2) pg/g d.w. for PFPeA, PFBuS, and PFHxA, and at 100 (level 1) and 1000 (level 2) pg/g d.w. for the other PFAAs (C_{spiked}). PFAAs concentrations in the spiked samples were then quantified (C_{found}) using a standard calibration curve prepared in the matrix-free procedural blank and AR% calculated according to the following equation.

$$AR\% = \frac{C_{found}}{C_{spiked}} \cdot 100$$

Based on the procedure described above, apparent recovery takes into account the combined effect of both matrix effect and reduced recovery during partition stages (e.g. between water and acetonitrile or acetonitrile and d-SPE sorbent) due to the presence of matrix components [40].

2.5.3 Instrumental precision

Intra-day and inter-day instrumental precision were assessed by analysing from run-to-run over 1 and 5 days the final extracts deriving from the study of the apparent recovery in strawberry and olive fruits, at level 1 concentrations.

2.5.4 Matrix effect

Matrix effect percentage (ME%) was defined as:

$$ME\% = \left(\frac{S_{matrix}}{S_{solvent}} \cdot 100\right) - 100$$

where S_{matrix} and $S_{solvent}$ are the slopes of calibration lines in matrix and in procedural blank, respectively [12]. Accordingly, ME% values higher or lower than zero indicate the presence of signal enhancement or suppression in comparison with the instrumental response observed in procedural blank. In fact, matrix components co-eluting with target analytes may alter their ionization, thus affecting the sensitivity and accuracy of the method when real samples are analysed [40].

2.5.5 Recovery

The recovery percentage (R%) was defined as [41]:

$$R\%=AR\%-ME\%$$

Standard deviation of R% ($\sigma_{R\%}$) was calculated according to error propagation rules

$$\sigma_{R\%}=\sqrt{\sigma^2_{AR\%}+\sigma^2_{ME\%}+2\sigma_{AR\%ME\%}}$$

where: $\sigma_{AR\%}$, $\sigma_{ME\%}$ are the experimental standard deviations of AR% and ME% and $\sigma_{AR\%ME\%}$ is the covariance of the two variables.

2.5.6 Method detection and quantification limits

For each target PFAA, instrumental detection limit (IDL) and quantification limit (IQL) were evaluated by replicated (n=5) analysis of procedural blanks, according to the following equation [42]:

$$IQL\ (IDL) = \frac{k\cdot\sigma_b}{S}$$

where: k is the critical value given by $2t_{(1-\alpha)}$ with n-1=4 degree of freedom and α= 0.05 (k= 4.264 and k=10, for IDLs and IQLs, respectively); σ_b is the standard deviation of the procedural blank and S is the slope of the calibration curve in procedural blank. The method detection (MDL) and quantification (MQL) limits in fruit samples were obtained by multiplying IDL and IQL by the value of AR% and dividing by 100.

2.5.7 Method accuracy and uncertainty

To the best of our knowledge, no reference material is available for the analysis of PFAAs in strawberries and olives. Consequently, in accordance with the specifications of Eurachem [43], the evaluation of the accuracy and uncertainty of the two methods was performed adopting for both matrices the spiking procedure. TW samples, which showed PFAAs concentrations below MDLs or MQLs, were chosen for performing the evaluation of method accuracy. Fruit aliquots of 1g d.w. were spiked in pentaplicate with ^{13}C labelled and unlabelled PFAA standards at 500 pg/g d.w. for PFPeA, PFBuS, and PFHxA, and at 100 pg/g d.w. for the other PFAAs. The spiked samples were then extracted following the procedures described in paragraph 2.4 and analysed for labelled and unlabelled spiked PFAAs using standard calibration curves prepared in the matrix-free procedural blank. The concentrations determined in each spiked sample for labelled compounds allowed

297 calculating AR%, as described in paragraph 2.5.2. The concentrations of unlabelled PFAAs
298 determined through the standard calibration curve ($C_{unlabelled}$) were corrected for AR% using the
299 equation reported below, thus obtaining the actual concentration in each spiked samples (C_{actual}).

300 $$C_{actual} = \frac{C_{unlabelled}}{AR\%} \cdot 100$$

301 Accuracy (Ac%) of the methods was finally calculated as the mean percentage ratio between C_{actual}
302 and C_{spiked}, determined in each spiked sample according to the following equation.

303 $$Ac\% = \frac{C_{actual}}{C_{spiked}} \cdot 100$$

304 *2.5.8 Analysis of real samples*

305 Quantification of target analytes in real samples was performed by the isotope dilution method by
306 spiking 500 pg/g of ^{13}C labelled standards of each target analyte in 1 g d.w. aliquots of fruit samples
307 and keeping them in the dark at 4°C overnight before extraction, in order to calculate the AR% of the
308 whole analytical procedure. Quantification was performed by means of external calibration lines,
309 prepared in the corresponding procedural blank, taking into account the AR% of each analyte.

310 2.6 Statistical analysis

311 The plot of data, as well as of calibration lines and the evaluation of linearity parameters were
312 performed using Microsoft® Excel 2016 (Redmond, WA, USA). Analysis of variance and contrast
313 tests for comparisons of means were performed by the Games-Howell non-parametric test, using the
314 Minitab software packages version 17.0.1 (Minitab Inc., State College, PA, USA).

315 **3 Results and discussion**

316 PFAAs herein investigated are reported in **Table 1**, which provides compound abbreviation,
317 molecular structures, molecular masses, pKa, and values of the logarithm of the octanol-water
318 partition constants (log K_{OW}). Target analytes included both sulfonic and carboxylic derivatives,
319 covering a wide range of polarity (log K_{OW} at pH=7 included from -1.81 to 4.15).

320 3.1 Optimization of the chromatographic conditions

321 Chromatographic analysis of PFAAs is usually affected by contaminations derived from eluents and
322 instrument parts made of fluorinated polymers (e.g. eluent lines, degassing apparatus, low pressure
323 mixing chamber). This problem is commonly faced by using the so-called “delay columns”,
324 containing a stationary phase able to strongly interact with PFAAs and installed between the eluent
325 mixer and the sample injector [12], thus allowing PFAAs released by the instrument and/or present
326 in the eluent to be delayed compared to those in the sample. As a drawback of this option, the
327 backpressure of the chromatographic system is more or less incremented, depending both on the
328 characteristics of the “delay” and of the analytical columns.

In this study, to reduce problems of contamination, LC pumps without any internal component made of fluorinated polymers were used, all the eluent pipelines were in stainless steel and no on-line degasser was employed. Moreover, an analytical pellicular column was used allowing to obtain chromatographic performances comparable to those previously achieved using fully porous columns with sub-2 µm particle size, but with much lower backpressure values, even though with an analysis time about 6 min. longer [12]. Hence, it is possible to use high retentive trap columns of various diameters and lengths packed with fully porous particles of relatively small size (e.g. 3.5 µm), maintaining backpressures values below 200 bar, which translates in a lesser needing of maintenance of LC system.

The LC elution gradient adopted in this study was properly optimized based on the conditions elsewhere reported for the analysis of PFAAs in water samples [12]. Furthermore, the chromatographic shape of the early-eluted PFAAs (i.e. PFBus and especially PFPeA) was strongly influenced by the acidity of the injected samples. Accordingly, formic acid was added to the extracts at 0.1% (*v/v*) to obtain narrower and quite symmetric peaks of PFPeA and PFBus (see **Figure 1**). **Figure 2** illustrates a reconstructed LC-MS/MS MRM chromatogram of the quantifier and qualifier transitions of target PFAAs obtained by injecting 10 µL of a standard solution (500 ng/L each) in acetonitrile acidified with 0.1% (*v/v*) formic acid.

3.2 Optimization of the QuEChERS procedure

The QuEChERS approach generally involves two steps: (i) a water/acetonitrile salting-out liquid/liquid partition of target compounds extracted from the solid matrix and (ii) a d-SPE for the clean-up of the acetonitrile extract. Sonication can be also applied to enhance extraction efficiency [44]. Within the general QuEChERS approach, these analytical steps must be properly optimized considering the characteristics of both target analytes and matrix [45].

The QuEChERS method has been originally developed for the analysis of polar pesticides in fresh fruit and vegetables [46, 47], which have a high water content. When dried samples are analysed, their rehydration before QuEChERS extraction is therefore recommended for increasing the recovery of target analytes [44]. The anhydrification of the samples and its subsequent rehydration with known volumes of water should be preferred to the analysis of fresh samples. In fact, this approach allows performing the analysis under standardized conditions from the point of view of the amount of water present in the extraction mixture. It should be also noted that the quantity of water vary considerably from fruit to fruit and within the same fruit, even from variety to variety [32]. In this latter regard, it should be emphasized that for commodities with less than 80% of water content, the addition of an extra-amount of water is important in order to weaken interactions of analytes with matrix and to ensure their adequate partitioning [48]. The amount of rehydration water herein added to the freeze-

363 dried samples – i.e. 5 mL of water to 1 g d.w. of fruit – was chosen in order to restore the original
364 water percentage of strawberry (about 90%), obtaining at the same time a water excess in olive fruits
365 compared to its original content (about 50%).
366 *3.2.1 Matrix-free partition tests*
367 The development of the QuEChERS method involved preliminarily the evaluation of the water-to-
368 acetonitrile partition of target analytes. In particular, using 5 mL-aliquots of a standard solution of
369 PFAAs (500 ng/L each) in LC-MS water and 2 g of NaCl and $MgSO_4$, the extent of partition was
370 evaluated using 2.5, 5 and 10 mL of acetonitrile. The tests were carried out as fully described in the
371 *Supplementary material*. The results of these experiments highlighted that the variation of acetonitrile
372 volume did not significantly affect the recovery values (**Figure S1** of the *Supplementary material*).
373 Moreover, in order to evaluate the effect of water phase pH on the partition, 5 mL-aliquots of a PFAA
374 standard solution in LC-MS water as such (pH=6.57±0.05), as well as acidified with 0.1% formic
375 acid (*v/v*) (pH=2.50±0.05) were extracted with 10 mL of acetonitrile, following the aforementioned
376 procedure. The acidification gave rise to a slight recovery increase for the more polar
377 perfluorocarboxylates (e.g. PFPeA and PFHxA), which was however not statistically significant (data
378 not shown).
379 *3.2.2 Strawberry fruits*
380 The sample/acetonitrile ratio is a crucial parameter affecting the recovery of target analytes and an
381 excess of solvent compared to the amount of dried sample underwent to analysis is generally
382 suggested for improving the extraction efficiency [44]. Hence, the QuEChERS extraction procedure
383 described in the paragraph 2.4.1 was evaluated for AR% and ME%, using the aforementioned
384 different volumes of acetonitrile (i.e. sample/water/acetonitrile 1/5/2.5, 1/5/5 and 1/5/10 *w/v/v*),
385 keeping constant to 2 g each the amount of NaCl and $MgSO_4$ as salting-out agents. These tests were
386 performed by spiking 1 g-aliquots of fruits with 100 µL of a ^{13}C-labelled PFAA working solution in
387 methanol containing each target analyte at 50 ng/mL and keeping them in the dark at 4°C overnight
388 before analysis.
389 **Figure 3** illustrates the results obtained for AR% and ME% in strawberry. The mean values of AR%
390 (**Fig. 3A**) showed an increasing trend with the increase of the acetonitrile volume. In particular, only
391 the use of 10 mL provided in almost all cases statistically higher recoveries compared to the lower
392 solvent volumes. These recoveries were in the ranges of 48-77% (2.5 mL), 57-78% (5 mL) and 67-
393 93% (10 mL) that are in most cases lower than those observed in the matrix-free partition tests. This
394 finding evidenced that the matrix exerted a significant influence on (i) the water-to-acetonitrile
395 partition of target analytes and/or (ii) their electrospray mass ionization, the latter being identified as
396 ME%. Accordingly, ME% was evaluated obtaining the results reported in **Fig. 3B**. In almost all cases,

a signal suppression due to the matrix was highlighted, being the only significant exception PFDA. However, the extent of matrix suppression was compound-dependent and for PFBuS, PFOA, PFNA PFOS and PFDA, |ME|<20% were observed irrespective of the volume of acetonitrile used. In this regard, it should be noted that signal enhancement/suppressions of 20% are commonly considered to have a negligible influence on the performances of analytical methods [49-51]. PFPeA and PFHpA showed ME ≤20% with the use of 5-10 mL of acetonitrile. Conversely, for PFHxA and PFHxS, only the use of 10 mL of acetonitrile allowed to decrease the signal suppression to values ≤20%, whereas solvent volumes of 5 mL, and above all 2.5 mL, entailed suppressive ME% as high as 30-45%. Accordingly, for strawberry, the sample/water/acetonitrile ratio of 1/5/10 *w/v/v* permits the direct PFAA LC-MS/MS analysis of the QuEChERS extract without any clean-up process, thus increasing the analytical throughput of the procedure (total analysis time of about 36 min.).

3.2.3 Olive fruits

As regards the analysis of olive fruits, the freeze-dried matrix appears as an inhomogeneous oily solid that persists as such also after the rehydration process and the vortex extraction with acetonitrile. Hence, this classic QuEChERS extraction was compared with an ultrasound-assisted procedure (USAE-QuEChERS) consisting of the sonication of the sample for 90 sec (pulsed mode, 10 sec on and 5 sec off, power 750 W) by the use of a sonication probe. Both QuEChERS and USAE-QuEChERS gave rise to an oily and strongly pigmented extract (see **Figure S2** in the *Supplementary material* section), even using 10 mL of acetonitrile, thus evidencing the need of a clean-up step before LC-MS/MS analysis. This finding is in agreement with elsewhere reported recommendation for the analysis of fruits characterized by high contents of fats, waxes and pigments, such as olives [48].

Preliminary matrix-free tests were performed in order to evaluate how PSA, C18 and GCB interact with target analytes in the absence of matrix. These phases were selected as the ones commonly employed in the clean-up step of QuEChERS protocols for the analysis of various food matrices [44], including olive fruits [48]. Moreover, the use of PSA/C18 and PSA/GCB 1:1 (*w/w*) mixtures is reported in official methods for pesticides residues analysis in foods [52]. In these procedures, variable ratios between the volume of extract and the mass of sorbent have been used, depending on the matrix and target analytes considered. In this regard, olive fruits can be considered a very complex matrix, since the clean-up of olive oil extracts for PFAA analysis needed amounts of d-SPE sorbents as high as 200 mg per mL of extract, even after a gel permeation pre-purification step [53].

The matrix-free tests were herein conducted using 10 mL-aliquots of a standard solution of PFAAs (500 ng/L each) in acetonitrile (derived from the USAE-QuEChERS procedural blank), employing 150 mg of $MgSO_4$ and 300 mg of sorbent per mL of solvent. **Figure S3** of the *Supplementary material* section illustrates the results of these tests. For most analytes, the use of PSA gave rise to recoveries

well below 50% (range 14-47%, median 37%). This finding is in accordance with the nature of anion exchanger of PSA, and the specific indication of its use in d-SPE clean-up of QuEChERS extracts for the removal of organic acids [52]. Conversely, C18 and GCB showed a very similar behaviour with recoveries included in the ranges of 89-106% and 88-102%, respectively. Hence, both phases were tested for the evaluation of ME%.

The optimization of the d-SPE clean-up was carried out by evaluating the extent of ME% in both QuEChERS and USAE-QuEChERS approaches after the clean-up and then assessing the AR% of the overall procedure. More in detail, the evaluation of ME% was performed by extracting 1 g of freeze-dried matrix with 10 mL of acetonitrile and 2 g each of NaCl and $MgSO_4$, and treating the resulting extract with 150 mg of $MgSO_4$, together with 200, 300 and 400 mg of C18 or GCB per mL of extract. The cleaned-up extract was spiked with ^{13}C-labelled PFAAs at 0, 50, 100, 500 and 1000 ng/L, comparing the slopes of these calibration lines in matrix with those in the procedural blank prepared by vortex mixing and centrifuging LC-MS acetonitrile together with the d-SPE sorbent.

Figure S4 and **Figure S5** of the *Supplementary material* illustrate the results of the d-SPE clean-up using C18 (**Figs. S4A and S5A**) and GCB (**Figs. S4B and S5B**), on QuEChERS and USAE-QuEChERS extracts, respectively. For both extraction approaches, the use of C18 showed |ME%| in most cases higher than 20%, irrespective of the amount of sorbent used. Conversely, for GCB, a decreasing trend with increasing the amount of the added sorbent was observed. This finding is consistent with the fact that GCB is known to adsorb compounds which exhibit strong hydrophobic interactions, such as pigments, which cannot be efficiently removed by C18 [53] and are probably responsible for the residual matrix effect observed in **Fig. S4A and Fig. S5A**. In particular, the use of 400 mg GCB allowed for achieving a strong reduction of the matrix effect, with |ME%| well below 20% for most analytes and in the worst cases of about +20% (i.e. for PFBuS with both procedures), being them as a whole considered negligible. However, generally higher standard deviations were observed for the QuEChERS method, compared to the USAE approach.

Based on the results of ME, the AR% was then evaluated for both QuEChERS and USAE-QuEChERS procedures, using 400 mg of GCB per mL of extract for the d-SPE clean-up (see **Figure 4**). The USAE-QuEChERS procedure provided extraction efficiencies slightly higher than the QuEChERS one and characterized by a much higher precision. These results were probably due to the sample disgregation achieved by using the ultrasound probe, which allow for obtaining a homogenous fine suspension of the sample in the extraction mixture.

Therefore, LC-MS/MS determination of PFAAs in olive fruits was performed by using the USAE-QuEChERS procedure, with a total analysis time of about 50 min.

3.3 Method validation

After method optimization, a validation study was performed for both investigated matrixes to assess: (i) linearity of the methods optimized for strawberry and olive fruits over proper spike ranges, depending on the compound investigated; (ii) AR% at two spike levels; (iii) instrumental intra-day and inter-day precision at two spike levels, expressed as relative standard deviation of peak area (RSD%); (iv) ME% over proper linear calibration ranges, depending on the compound investigated; (v) R% at two spike levels; (vi) MDLs and MQLs; (vii) Ac% at spike level 1. Note that different spike levels were chosen depending on matrix and compound considered (**Table 2**). More in detail, in strawberry, level 1 and level 2, were respectively chosen as 500 and 5000 pg/g d.w. for PFPeA, PFBuS, and PFHxA, whereas for the other analytes they were 100 pg/g d.w. and 1000 pg/g d.w. In olive, AR% of PFHxS, PFNA and PFDA was evaluated at 100 pg/g d.w. and 1000 pg/g d.w., whilst for the other analytes the levels were 500 and 5000 pg/g d.w.

The method showed very high linearity, with determination coefficients in all cases ≥ 0.9984. Intra-day precision at level 1, as measured by RSD%, was in the ranges of 2.9-7.1 and 1.8-7.2 for strawberry and olive fruits, respectively. At the level 2 the precision was slightly higher (RSD% of 2.1-6.1 for strawberry and 1.3-5.0 for olive), in agreement with its higher concentration (one order of magnitude). The inter-day RSD% were higher than the corresponding ones determined in the same day, remaining however in most cases below 9%. The evaluation of ME% confirmed the absence of significant signal suppression/enhancement in strawberry extracts, even without applying any clean-up process. It should also be noted that the d-SPE treatment adopted for olive extract was able to reduce for almost all analytes the |ME%| to values below 10%, with the only exception of PFBuS which showed a mean amplification effect of 19%. Hence, for both fruits, the matrix matched calibration is not necessary. The extraction procedures developed for the two matrices allowed not only to obtain small alterations of the ESI ionization capacity due to the matrix (|ME% |<20%), but also to efficiently recover PFAAs from both fruits. The R% values, in fact, were generally high, being in any cases >70%, with the only exceptions of PFHpA (65%) in the strawberry at the lowest fortification level (100 pg/g d.w.). The higher recovery observed for olive fruit (75-97%) compared to strawberry (65-89%) is probably due to the adoption of the USAE-QuEChERS. This procedure, in fact, led to the sample disgregation, thus allowing to obtain a better recovery efficiency, which on average was however limited to 10%. The proposed methods were also characterized by a very high sensitivity, being MQLs in most cases included in between ppt and tens of ppt, with very few exceptions that however showed values well below ppb levels. The evaluation of the overall Ac% of the methods highlighted values included in the ranges of 99-109 and 98-114 for strawberry and olive, respectively. The values higher than 100% found for most analytes in both matrices correspond to native concentrations in TW samples in between ppt and tens of ppt, that are lower than MDLs or MQLs.

3.4 Comparison with previously published methods

The main characteristics of the method herein proposed can be compared with those reported in the elsewhere published papers regarding method development for assessing fruit contamination by PFAAs. However, as far as we know, no data have been published on PFAA determination in olive or other "fatty fruits", whilst very few papers are present in literature concerning PFAAs analysis in fruits with high water content [25, 27-29] (**Table 3**). One of these researches focused on PFAAs determination in strawberry [28], employing a method previously developed for vegetables [54]. In this study a laborious extraction procedure was proposed, using a high percentage of dichloromethane in the extraction mixture, thus providing a non-green analytical approach and making necessary dryness evaporation in order to achieve compatibility with the complex clean-up process adopted and LC-MS/MS instrumental analysis. As a whole, the analytical protocol lasted for about 8 hours and provided quite low apparent recoveries (on average 45%), which could be ascribed to the complexity of the method, while it is not possible to estimate the contribution of the matrix effect, since it has not been investigated. Moreover, the sensitivity resulted fairly low, being MQLs in the range of 27-2910 pg/g w.w.

Much lower MQLs (1-4 pg/g w.w.) were reported by D'Hollander and co-workers [24] in a study regarding strawberry and other water-rich fruits (e.g. orange, apple and melons). However, the method envisaged a very high extraction time (about 16 h) and no information was reported regarding method performances in terms of recovery and matrix effect.

Genualdi et al. [29] proposed a classical QuEChERS protocol for the analysis of PFAAs in cranberry (*Vaccinium macrocarpon* Ait.), based on acidic water-to-acetonitrile partition of target analytes followed by dispersive solid-phase extraction (d-SPE) with a sorbent mixture of PSA and GCB and LC-MS/MS determination. The entire analytical procedure was quite rapid, since it lasted about 40 min. However, the method showed very high MDLs (240-2300 pg/g w.w. for the same PFAAs herein analysed, corresponding to extrapolated MQLs in the range 792-7590 pg/g w.w., see **Table 3**), despite the very high intrinsic sensitivity of the mass detector used and the high AR% obtained. In this latter regard, it should be noted that the high recovery values reported have been obtained notwithstanding the use of PSA, which was herein found not suitable for the d-SPE step due to its strong sorption properties towards target analytes.

A more complex QuEChERS procedure was optimized by Sznajder-Katarzynska and co-workers [25] for the determination of PFAAs in apple, on the basis of a method previously developed for the analysis of PFOA and PFOS in honey [55]. Major differences compared to the classical QuEChERS approach were the use of an ultrasound assisted partition of target compounds and a d-SPE clean-up

step with styrene-divinylbenzene. The optimized method achieved a sensitivity in the range 6-27 pg/g w.w. with a total analysis time of about 70 min.

An analytical method aimed at determining PFAAs in very different kinds of food, including some fruits, was developed by Ballesteros et al. [27], whose method was characterized by high apparent recoveries, moderate matrix effect and MQLs included in the range 3.5-60 pg/g w.w. In order to make the procedure suitable for a wide range of matrices, including fatty food, a complex clean-up strategy was required, with a consequent increase of the total analysis time (about 2 hours). However, results herein obtained showed that such a complex clean-up procedure is unnecessary in the case of relatively simple matrices with low or null fat content such as strawberry, and also for fatty fruits such as olives it seems oversized.

Based on the discussion reported above, the method herein proposed represented a general improvement in terms of simplicity and total analysis time (36 min), as well as sensitivity in comparison with previously published methods focusing on the determination of PFAAs in fruits.

3.5 Method application to real samples

The optimized methods have been applied to the analysis of strawberry and olive fruits purchased in two Italian supermarkets, and obtained under controlled irrigation conditions with TW and different TWWs (see paragraph 2.2). PFAAs concentrations found in these samples are shown in **Table 4**, as mean and standard deviation of three independent determinations of each fruit sample. As an example, **Figure 5** shows the overlapped quantifier and qualifier transitions of PFOA and PFOS in marketed strawberry and olive fruits, compared to spiked procedural blanks.

As regards strawberry, in both commercial samples PFPeA and PFBuS were not detected. Moreover, sample labelled as “Market 2” showed a higher number of detected analytes and generally higher concentrations than “Market 1”. PFHxS was by far the most abundant compound determined in commercially available strawberry, being it present at concentrations of 148 and 790 pg/g d.w., in “Market 1” and “Market 2”, respectively. Regarding PFOA and PFOS, for which TWIs have been provided by EFSA (6 and 13 ng/kg body weight), they were detected only in “Market 2” sample, at 27 and 90 pg/g d.w. These concentrations are low from the food intake point of view even assuming a very high daily dose of strawberry, such as 500 g of fresh fruit, since they correspond to daily ingestions approximately 30-40 times lower than the TWI for PFOS and PFOA, respectively. Only one paper reported data regarding PFAAs occurrence in marketed strawberries, collected in Norway, Belgium and Czech Republic [24]. In that study, PFAAs were found to be present in only one sample, which evidenced the occurrence of PFBuS, PFHxA, PFHxS, PFOA and PFOS (58-192 pg/g w.w.) and a total PFAAs concentration of 611 pg/g w.w., corresponding to about 6110 pg/g d.w. that is a value higher than the ones herein found in fruits from Italian markets. Interestingly, strawberries

cultivated using TWWs did not show PFAAs concentrations above the quantification limits irrespective of the kind of wastewater used for irrigation, suggesting that the contamination observed in marketed strawberries could derive from contamination sources other than irrigation water. In this regard, it must be considered that the absence of contamination or its very low extent have been observed in the presence of irrigation with TWWs containing PFAAs at very different concentration levels, ranging from few to hundreds of ng/L.

A different scenario was observed for PFAAs concentrations in olives (see **Table 4**), compared to strawberries, since higher values were found in olive samples, both in the case of marketed products and the ones obtained under irrigation with TWWs. In particular, both commercial samples showed higher concentrations of PFPeA and PFBuS, compared to the other PFAAs. Moreover, "Market 2" sample contained all the investigated PFAAs at concentrations well above the quantification limits, with PFOA and PFOS at 124-137 and 39-95 pg/g d.w. These values, corresponding to about 60-70 and 20-50 pg/g w.w., even though higher than those previously reported for strawberry, can be considered low in relation to TWIs established by EFSA for PFOA and PFOS, considering that olives are a food much less consumed, compared to strawberry. Olive samples irrigated with the same TWWs applied to strawberries showed a higher occurrence of PFAAs. More in detail, PFPeA and PFHxA were the analytes determined at the highest concentrations, whereas PFOA and PFOS were never quantified. The higher accumulation of PFAAs in olive fruits than in strawberry could be related to their very different composition in terms of water and fat content.

4 Conclusions

The method optimized and validated in this research represents an improvement in terms of simplicity and/or analysis time and/or sensitivity, compared to previously published analytical procedures focusing on the determination of PFAAs in fruits. Moreover, the method herein proposed for PFAAs analysis in olives represents the first validated analytical approach for this fruit matrix.

Since the analytical approach proposed has been validated on two types of fruit, which are very different for their content of water and fatty substances, it is reasonable to assume that it can be successfully applied to a wide range of fruit products.

The application of the proposed method to the analysis of real samples evidenced that fruits commercially available at supermarkets contained concentrations generally higher than those grown under controlled watering conditions, using tap water but also TWWs for their irrigation, thus suggesting that commercial crops can be exposed to a wide range of PFAAs contamination sources, in addition to irrigation water. In any case, the quantities of PFOA and PFOS that can be ingested through the analysed fruits, even taking into account much higher daily doses than those calculated

on the basis of surveys carried out by major international organizations, are lower than the TWIs established by EFSA. However, considering that the intake of these compounds derive from the ingestions of various foodstuffs, all of them possibly contaminated by PFOA and PFOS [22], the concentrations herein determined could be considered noteworthy.

Acknowledgements

This research was supported by the grant 13-069 (IRRIGATIO project) under the ERANET MED 2014 call, which is gratefully acknowledged. Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR) is also acknowledged. Authors also wish to thank Susan Mary Cadby for her linguistic revision of the paper.

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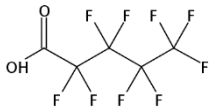
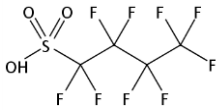
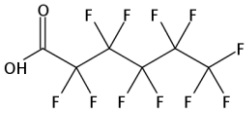
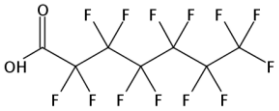
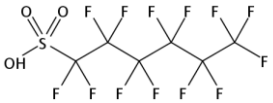
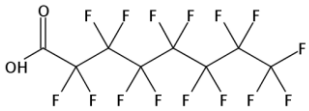
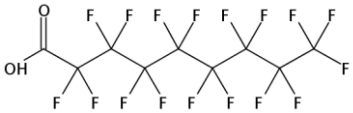
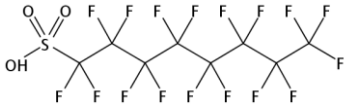
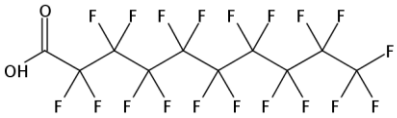
Table 1 – Abbreviations, molecular structures, molecular masses, pKa* and log K_{OW}* values (calculated at pH=7) of perfluoroalkyl acids (PFAAs) investigated in this study.

PFAAs	Abbreviation	Molecular Structure	Molecular Mass	pK_a	log K_{OW}
Perfluoro-n-pentanoic	PFPeA		264.05	0.40	0.64
Perfluoro-1-butanesulfonic	PFBuS		300.10	-3.57	-1.81
Perfluoro-n-hexanoic	PFHxA		314.05	0.42	1.24
Perfluoro-n-heptanoic	PFHpA		364.06	0.47	1.97
Perfluoro-1-hexanesulfonic	PFHxS		400.11	-3.34	-0.45
Perfluoro-n-octanoic	PFOA		414.07	0.50	2.69
Perfluoro-n-nonanoic	PFNA		464.08	0.52	3.42
Perfluoro-1-octanesulfonic	PFOS		500.13	-3.27	1.01
Perfluoro-n-decanoic	PFDA		514.08	0.52	4.15

* Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994-2019 ACD/Labs)

Table 2 – Linearity of the method expressed as determination coefficient (R^2) calculated over proper spike ranges[(a)], apparent recovery (AR%), instrumental intra-day and inter-day precisions (expressed as relative standard deviation of peak area, RSD %), matrix effect (ME%), recovery (R%), method detection (MDLs) and quantification (MQLs) limits (pg/g d.w.), accuracy (Ac%). For AR%, ME%, R%, and Ac%, values in bracket represent the standard deviation. AR%, RSD% and R% were calculated at two spike levels, whereas Ac% at the spike level 1[(b)]. MDLs and MQLs were calculated based on AR% of the lowest spike level (level 1).

Compound	R^2	AR%		$RSD\%_{intra\text{-}day}$		$RSD\%_{inter\text{-}day}$		ME%	R%		MDLs	MQLs	Ac%
		level 1	level 2	level 1	level 2	level 1	level 2		level 1	level 2			
Strawberry													
PFPeA	0.9992	73 (8)	74 (6)	7.1	5.4	10.9	7.6	-5 (4)	78 (11)	79 (11)	109	393	109 (9)
PFBuS	0.9987	85 (7)	76 (6)	5.9	5.3	9.8	6.9	-4 (5)	89 (12)	80 (8)	29	104	107 (7)
PFHxA	0.9984	59 (5)	63 (7)	4.3	4.1	7.3	5.5	-22 (3)	81 (9)	85 (9)	40	146	106 (5)
PFHpA	0.9993	74 (4)	89 (8)	3.7	2.9	9.4	7.1	9 (5)	65 (9)	80 (13)	7.2	26	103 (7)
PFHxS	0.9988	66 (6)	71 (2)	5.4	2.1	9.7	8.2	-7 (2)	73 (8)	78 (4)	10	37	103 (7)
PFOA	0.9989	64 (3)	70 (3)	2.9	3.1	7.9	6.5	-11 (3)	75 (5)	81 (5)	8.6	31	107 (5)
PFNA	0.9993	64 (4)	72 (5)	4.1	3.6	8.6	7.0	-8 (4)	72 (7)	80 (7)	1.3	4.9	106 (6)
PFOS	0.9985	76 (8)	81 (8)	6.4	6.1	9.2	8.1	2 (6)	74 (11)	79 (14)	5.7	20	108 (9)
PFDA	0.9988	66 (4)	67 (4)	3.8	3.3	6.1	4.5	-4 (3)	71 (6)	72 (6)	0.8	2.9	99 (5)
Olive													
PFPeA	0.9997	93 (4)	88 (2)	3.0	2.2	6.8	6.1	-4 (2)	97 (6)	92 (4)	35	127	110 (6)
PFBuS	0.9991	105 (9)	102 (3)	1.8	1.3	5.5	4.3	19 (3)	86 (11)	83 (4)	24	85	107 (7)
PFHxA	0.9994	82 (3)	84 (3)	5.4	2.2	8.6	4.8	-4 (4)	86 (7)	88 (7)	26	94	114 (7)
PFHpA	0.9992	82 (5)	93 (4)	7.2	5.0	8.2	7.1	4 (3)	78 (5)	89 (6)	21	74	98 (9)
PFHxS	0.9997	83 (5)	91 (2)	5.1	4.2	8.9	7.3	-6 (4)	89 (7)	97 (5)	2.4	8.6	102 (8)
PFOA	0.9997	67 (7)	84 (3)	3.4	3.0	6.7	5.4	-8 (3)	75 (10)	92 (7)	22	78	106 (5)
PFNA	0.9997	86 (6)	89 (6)	3.1	1.8	4.8	3.6	-3(2)	89 (7)	92 (8)	0.7	2.6	102 (6)
PFOS	0.9994	87 (8)	92 (4)	5.3	1.3	9.6	3.5	3 (3)	84 (9)	89 (5)	5.8	21	99 (7)
PFDA	0.9997	87 (4)	87 (4)	4.1	3.7	7.3	6.4	4 (3)	83 (6)	83 (6)	2.7	9.8	105 (7)

[(a)] R^2 values were calculated in the following ranges. Strawberry – (i) PFNA and PFDA 10-10000 pg/g d.w.; (ii) PFHpA, PFHxS, PFOA and PFOS 50-10000 pg/g d.w.; (iii) PFBuS, PFPeA and PFHxA 500-10000 pg/g d.w. Olive – (i) PFHxS, PFNA and PFDA 10-10000 pg/g d.w.; (ii) PFBuS, PFHxA, PFHpA, PFOA and PFOS 50-10000 pg/g d.w.; (iii) PFPeA 500-10000 pg/g d.w.

[(b)] Spike levels for PFPeA, PFBuS, and PFHxA were 500 pg/g d.w. (level 1) and 5000 pg/g d.w. (level 2), whereas for the other analytes were 100 pg/g d.w. (level 1) and 1000 pg/g d.w. (level 2).

Table 3 – Main characteristics of the analytical method herein proposed for the analysis of target PFAAs in strawberry, in comparison with those provided by elsewhere published studies in various fruit matrixes, using different extraction/clean-up methods followed by LC-MS/MS instrumental determination. AR%=apparent recovery percentage. ME% = matrix effect percentage; MQL = method quantification limit (pg/g wet weight); n.i. = not investigated; n.r. = not reported.

Matrix	Method details	PFAAs	AR%	ME%	MQL	Reference
Strawberry	QuEChERS without clean-up. Total analysis time 36 min.	PFPeA	73	-5	39	This study
		PFBuS	85	-4	10	
		PFHxA	59	-22	15	
		PFHpA	74	9	2.6	
		PFHxS	66	-7	3.7	
		PFOA	64	-11	3.1	
		PFNA	64	-8	0.5	
		PFOS	76	2	2.0	
		PFDA	66	-4	0.3	
Strawberry	Ultrasonic assisted extraction with CH_3OH+1% NH_4OH/CH_2Cl_2 1/1 (v/v). Multiple clean-up processes. Total analysis time ≈ 8 h.	PFPeA	45[a]	n.i.	143[b]	[28]
		PFBuS			71[b]	
		PFHxA			1450[b]	
		PFHpA			2910[b]	
		PFHxS			27[b]	
		PFOA			737[b]	
		PFNA			79[b]	
		PFOS			29[b]	
Strawberry	Ultrasonic treatment and shaking extraction with CH_3OH+10 mM KOH. Poly(styrene-co-pyrrolidone-co-divinylbenzene) functionalized with piperazine SPE clean-up. Total analysis time ≈ ~~17~~ 16 h.	PFBuS	n.i.	n.i.	2	[24]
		PFHxA			2	
		PFHpA			4	
		PFHxS			4	
		PFOA			1	
		PFNA			1	
		PFOS			2	
		PFDA			4	
Cranberry Bog	QuEChERS with PSA/GCB 2/1 (w/w) d-SPE clean-up. Total analysis time ≈ 40 min.	PFPeA	106	n.i.	2112[c]	[29]
		PFBuS	104		7590[c]	
		PFHxA	101		792[c]	
		PFHpA	101		3102[c]	
		PFHxS	91		2607[c]	
		PFOA	94		1386[c]	
		PFOS	109		1782[c]	
Apple	QuEChERS with poly(styrene-co-divinylbenzene) SPE clean-up. Total analysis time ≈ 70 min.	PFPeA	89	n.r.	14	[25]
		PFBuS	89		27	
		PFHxA	91		24	
		PFHpA	97		20	
		PFHxS	85		17	
		PFOA	89		12	
		PFNA	95		14	
		PFOS	91		6	
		PFDA	92		15	
Apple and Orange	Ultrasonic assisted extraction with THF/water 75/25 (v/v). Multiple clean-up processes. Total analysis time ≈ 2 h.	PFPeA	n.r.	≤10[d]	60	[27]
		PFBuS	n.r.		50	
		PFHxA	93-103		15	
		PFHpA	n.r.		15	
		PFHxS	81-120		25	
		PFOA	82-98		10	
		PFNA	82-103		5	
		PFOS	81-120		3.5	
		PFDA	84-96		5	

[a] Average recovery across all analytes. [b] Calculated from method ~~detection~~ quantification limits reported in the original manuscript on a dry weight basis, dividing by 10 (i.e. the wet weight-to-dry weight ratio, considering a humidity percentage of 90%). [c] Calculated from method detection limits reported in the original manuscript, multiplying by 3.3. [d] Absolute value of matrix effect reported for all target analytes without specifications.

Table 4 – Mean concentrations (pg/g d.w.) and standard deviation (n=3, in bracket) of PFAAs in strawberry and olive fruits commercially available at supermarkets and grown under irrigation with tap water (TW) and treated wastewaters (TWWs).

Compound	Market 1	Market 2	TW	TWW_1	TWW_2	TWW_3	TWW_4
Strawberry							
PFPeA	<109[a]	<109[a]	<109[a]	<109[a]	<109[a]	<109[a]	<109[a]
PFBuS	<29[a]	<29[a]	<29[a]	<29[a]	<29[a]	<29[a]	<29[a]
PFHxA	<40[a]	40[a]-146[b]	<40[a]	40[a]-146[b]	<40[a]	<40[a]	40[a]-146[b]
PFHpA	<7.2[a]	7.2[a]-26[b]	<7.2[a]	<7.2[a]	<7.2[a]	<7.2[a]	<7.2[a]
PFHxS	148	790	<10[a]	<10[a]	<10[a]	<10[a]	<10[a]
PFOA	<8.6[a]	27	<8.6[a]	8.6[a]-31[b]	8.6[a]-31[b]	8.6[a]-31[b]	8.6[a]-31[b]
PFNA	<1.3[a]	47	<1.3[a]	<1.3[a]	<1.3[a]	<1.3[a]	<1.3[a]
PFOS	<5.7[a]	90	<5.7[a]	<5.7[a]	<5.7[a]	<5.7[a]	<5.7[a]
PFDA	54	35	<0.8[a]	<0.8[a]	<0.8[a]	<0.8[a]	<0.8[a]
Total PFAAs	202	989	n.d.	n.d.	n.d.	n.d.	n.d.
Olive							
PFPeA	227	682	35[a]-127[b]	1408	740	576	1019
PFBuS	403	185	24[a]-85[b]	<24[a]	24[a]-85[b]	<24[a]	114
PFHxA	<26[a]	33	26[a]-94[b]	481	209	103	256
PFHpA	<21[a]	107	<21[a]	21[a]-74[b]	21[a]-74[b]	<21[a]	21[a]-74[b]
PFHxS	<2.4[a]	22	<2.4[a]	2.4[a]-8.6[b]	2.4[a]-8.6[b]	<2.4[a]	<2.4[a]
PFOA	124	137	22[a]-78[b]	22[a]-78[b]	22[a]-78[b]	<22[a]	22[a]-78[b]
PFNA	42	35	<0.7[a]	<0.7[a]	<0.7[a]	<0.7[a]	<0.7[a]
PFOS	39	95	<5.8[a]	5.8[a]-21[b]	5.8[a]-21[b]	<5.8[a]	5.8[a]-21[b]
PFDA	2.7[a]-9.8[b]	15	<2.7[a]	20	2.7[a]-9.8[b]	<2.7[a]	12
Total PFAAs	835	1311	n.d.	1909	949	679	1401

[(a)] MDL in real sample. [(b)] MQL in real sample. n.d. = not determined.

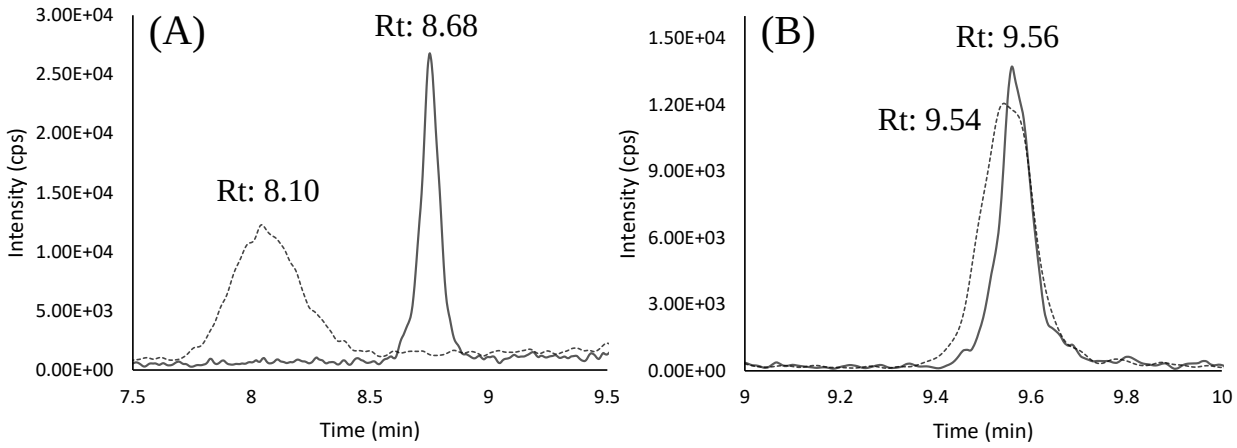


Figure 1 – MRM chromatograms of (A) PFPeA and (B) PFBuS reference standard solutions (concentration 500 ng/L) prepared in the QuEChERS procedural blank as such (dashed line) and acidified with 0.1% (*v/v*) formic acid (solid line).

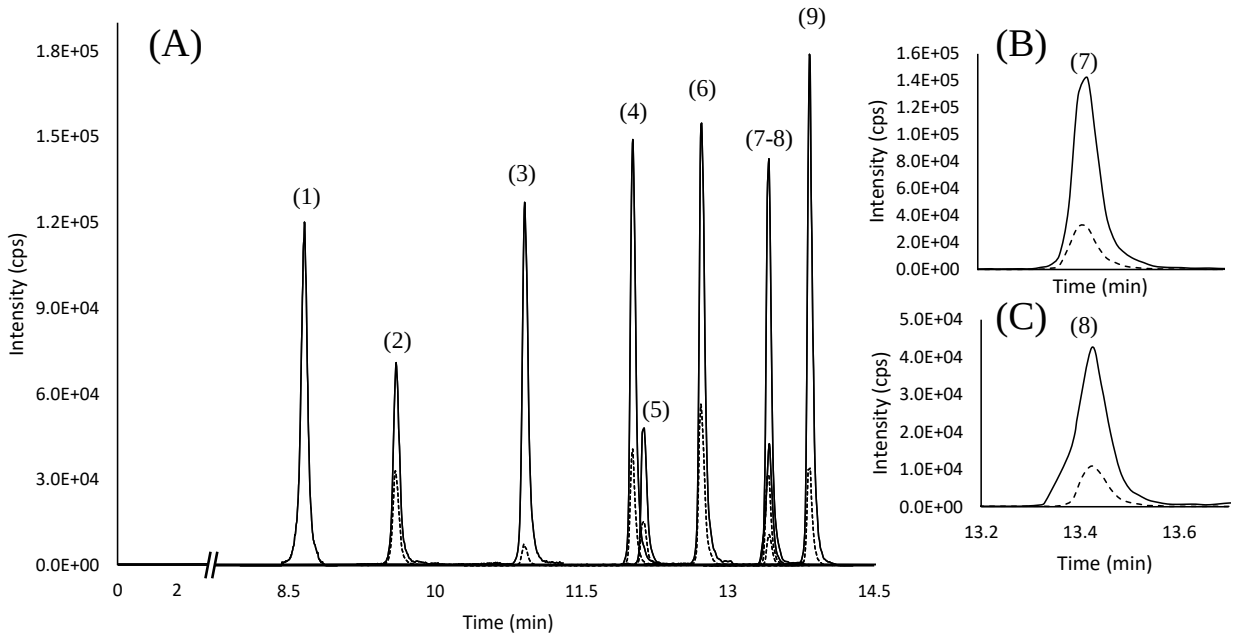


Figure 2 – Reconstructed MRM chromatogram of the quantifier (solid line) and qualifier (dashed line) transitions of (A) target PFAAs obtained by injecting 10 µL of a standard mixture in the procedural blank acidified with 0.1% formic acid, at a concentration level of 500 ng L^{-1} each. (1) PFPeA; (2) PFBuS; (3) PFHxA; (4) PFHpA; (5) PFHxS; (6) PFOA; (7) PFNA; (8) PFOS; (9) PFDA. Magnifications of peaks (7) and (8) are shown in boxes (B) and (C), respectively.

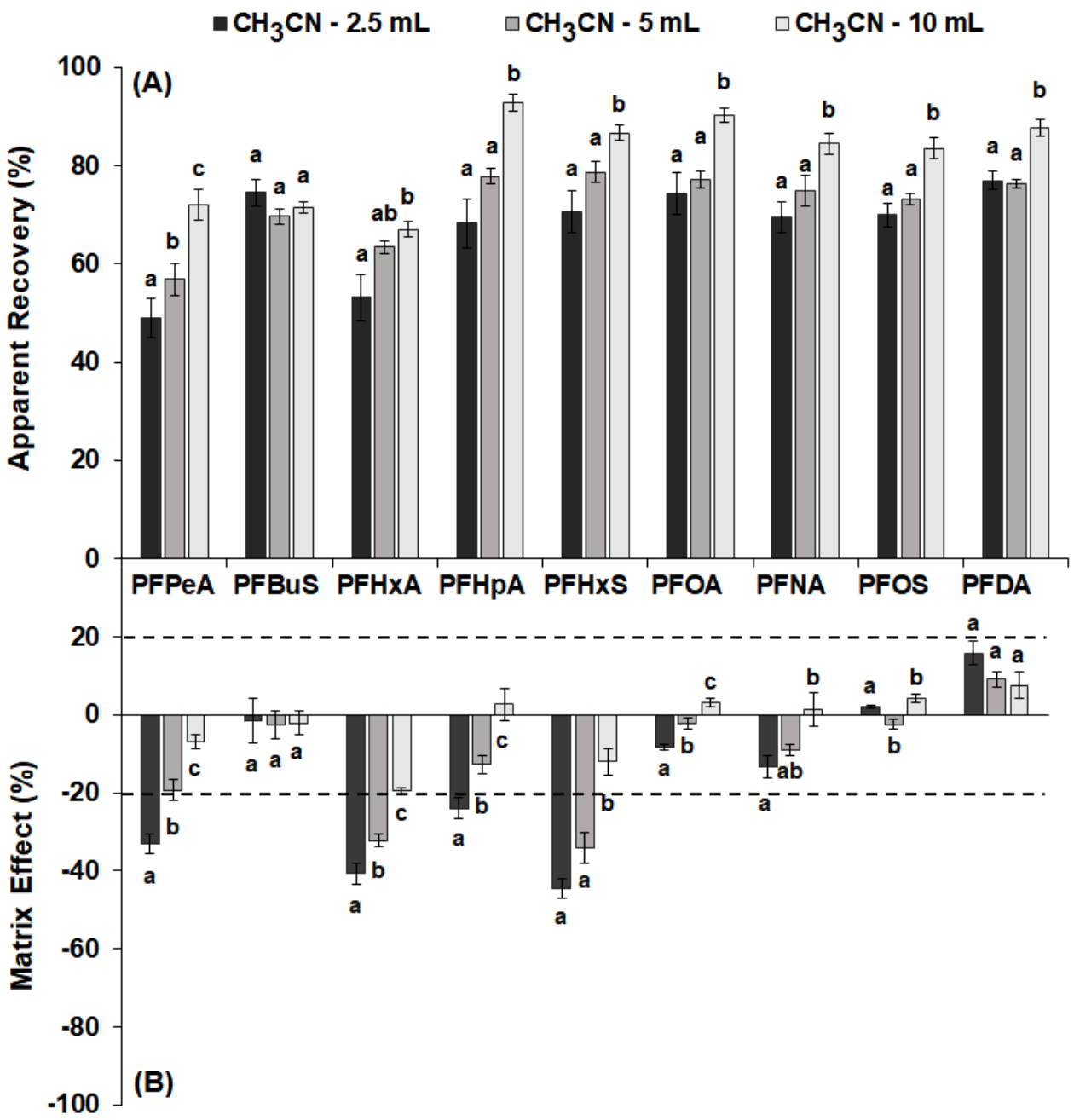


Figure 3 – Mean values (n=3) of apparent recoveries (A) and matrix effects (B) of the QuEChERS extraction performed on 1 g dry weight-aliquots of strawberry fruits spiked with ^{13}C-labelled PFAAs at 5 ng/g each, rehydrated with 5 mL of LC-MS water and extracted using 2.5, 5 and 10 mL of acetonitrile, and 2 g each of NaCl and $MgSO_4$. Error bars represent standard deviation. Within a same compound, different letters indicate statistically significant differences according to the Games-Howell non-parametric test (P<0.05).

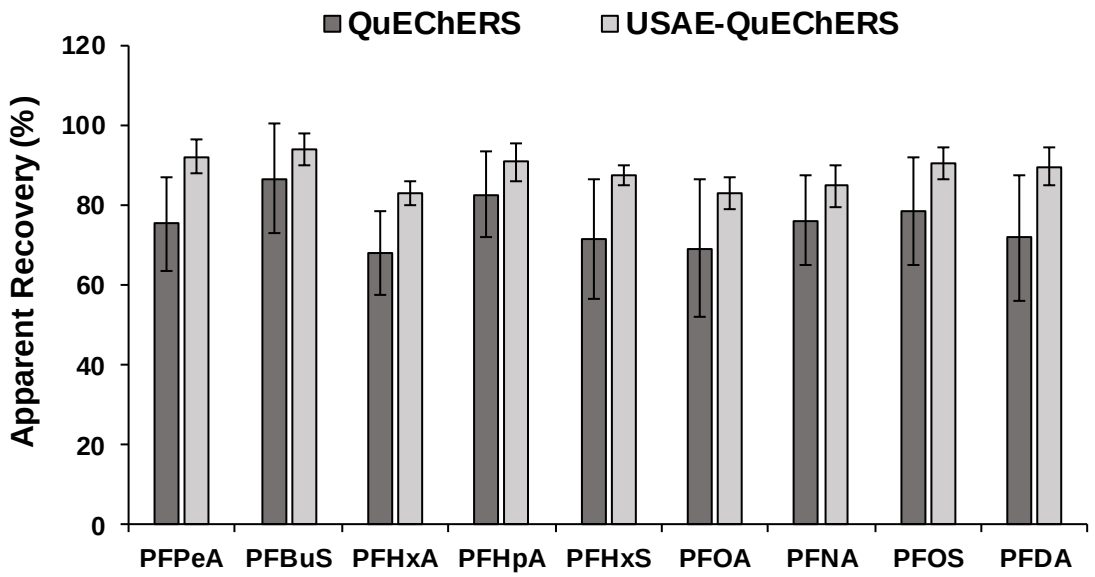


Figure 4 – Mean values (n=3) of apparent recoveries determined for the QuEChERS and USAE-QuEChERS extractions, coupled with d-SPE clean-up performed with 400 mg of GCB sorbent per mL of olive fruit extract. The extraction was performed on 1 g dry weight-aliquots of olive fruits fruits spiked with ^{13}C-labelled PFAAs at 5 ng/g, rehydrated with 5 mL of LC-MS water and treated with 10 mL of acetonitrile and 2 g each of NaCl and $MgSO_4$. Error bars represent standard deviation.

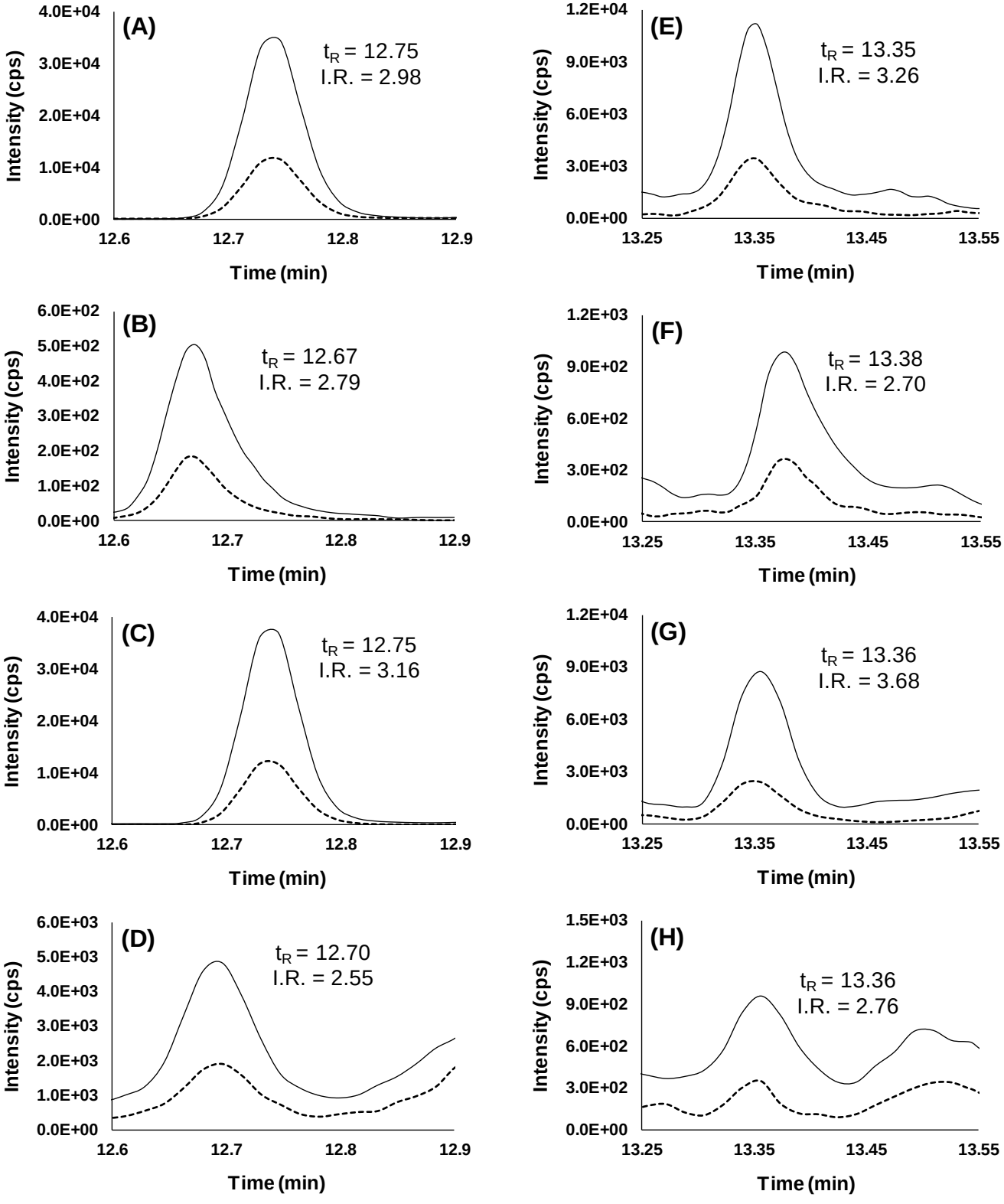


Figure 5 – Overlapped MRM quantifier (solid line) and qualifier (dashed line) transitions, retention time (t_R, min) and ion ratio of: (A) PFOA, 100 ng/L solution in strawberry procedural blank; (B) PFOA in strawberry "Market 2" sample; (C) PFOA, 100 ng/L solution in olive procedural blank; (D) PFOA in olive "Market 2" sample; (E) PFOS, 100 ng/L solution in strawberry procedural blank; (F) PFOS in strawberry "Market 2" sample; (G) PFOS, 100 ng/L solution in olive procedural blank; (H) PFOS in olive "Market 2" sample.

Supplementary material of the Manuscript:

"Optimization and validation of a method based on QuEChERS extraction and liquid chromatographic-tandem mass spectrometric analysis for the determination of perfluoroalkyl acids in strawberry and olive fruits, as model crops with different matrix characteristics"

by Cristina Vanessa Agata Scordo et al.

S.1 Treatment stages adopted for the production of treated wastewaters

The Baciacavallo wastewater treatment plant (WWTP) is the core of the centralized treatment system of the textile industrial district and domestic sewage of the Prato area (Italy). It is essentially constituted by equalization, primary sedimentation, biological oxidation, sedimentation, flocculation and a final refinement with ozone to remove colour and residual organic micropollutants (TWW_1). The TWW_1 is further treated by clariflocculation, sand filtration and activated carbon obtaining the TWW_2 (Macrolotto 1 refining system). In parallel, TWW_1 is treated by clariflocculation, sand filtration and finally mixed with the Bisenzio river water, obtaining the TWW_3 (Macrolotto 2 refining system). Calice WWTP is the second largest facility in the aforementioned area, devoted to the treatment of both domestic and industrial wastewater, together with leachate from landfills and sewages from septic tanks after a pre-treatment with a membrane biological reactor. The Calice WWTP essentially consists of equalization, primary sedimentation, denitrification, biological oxidation, sedimentation, clariflocculation, sand filtering and ozonation (TWW_4).

S.2 Optimization of mass parameters

The precursor and product ions, as well as compound-dependent parameters used for tandem mass spectrometric analysis of PFAAs, are reported in **Table S1**.

Table S1 – Optimized MRM parameters for the investigated analytes. Letters A and B in bracket after each compound abbreviation refer to the quantifier and qualifier transitions, respectively. Q1: precursor ion; Q3: product ion; DP: declustering potential; EP: entrance potential; CE: collision energy; CXP: collision exit potential.

PFAAs	Q1 (Da)	Q3 (Da)	DP (V)	EP (V)	CE (eV)	CXP (V)
PFBuS (A)	299	80	-120	-10	-66	-12
PFBuS (B)	299	99	-120	-10	-37	-13
PFPeA (A)	263	219	-53	-10	-12	-20
PFHxA (A)	313	269	-50	-10	-12	-23
PFHxA (B)	313	119	-50	-10	-25	-13
PFHxS (A)	399	80	-30	-10	-85	-10
PFHxS (B)	399	99	-30	-10	-42	-12
PFHpA (A)	363	319	-50	-10	-13	-28
PFHpA (B)	363	169	-50	-10	-25	-15
PFOA (A)	413	369	-55	-10	-13	-32
PFOA (B)	413	169	-55	-10	-24	-14
PFOS (A)	499	80	-120	-10	-105	-7
PFOS (B)	499	99	-120	-10	-95	-9
PFNA (A)	463	419	-60	-10	-15	-38
PFNA (B)	463	219	-60	-10	-25	-17
PFDA (A)	513	469	-67	-10	-15	-40
PFDA (B)	513	219	-67	-10	-25	-19
^{13}C3-PFBuS (A)	302	80	-110	-10	-70	-12
^{13}C3-PFBuS (B)	302	99	-110	-10	-35	-13
^{13}C5- PFPeA (A)	268	223	-40	-10	-15	-20
^{13}C5-PFHxA (A)	318	273	-45	-10	-13	-23
^{13}C5-PFHxA (B)	318	120	-45	-10	-30	-13
^{13}C3-PFHxS (A)	402	80	-110	-10	-85	-10
^{13}C3-PFHxS (B)	402	99	-110	-10	-45	-12
^{13}C4-PFHpA (A)	367	322	-60	-10	-15	-28
^{13}C4-PFHpA (B)	367	169	-60	-10	-20	-15
^{13}C8-PFOA (A)	421	376	-70	-10	-14	-32
^{13}C8-PFOA (B)	421	172	-70	-10	-28	-14
^{13}C8-PFOS (A)	507	80	-110	-10	-113	-7
^{13}C8-PFOS (B)	507	99	-110	-10	-85	-9
^{13}C9-PFNA (A)	472	427	-60	-10	-17	-38
^{13}C9-PFNA (B)	472	223	-60	-10	-28	-17
^{13}C6-PFDA (A)	519	474	-80	-10	-17	-40
^{13}C6-PFDA (B)	519	219	-80	-10	-28	-19

S.3 Optimization of the QuEChERS procedure

The development of the QuEChERS method involved preliminarily the evaluation of the water-to-acetonitrile partition of target analytes in the presence of NaCl and $MgSO_4$. In particular, using 5 mL-aliquots of a standard solution of PFAAs (500 ng/L each) in LC-MS water and 2 g of each salt, the extent of partition was evaluated using 2.5, 5 and 10 mL of acetonitrile. The tests were carried out as following described. Acetonitrile was added to the PFAA standard solution and the QuEChERS procedure mentioned above (paragraph 2.4 of the main text) was performed. The acetonitrile supernatant derived from the centrifugation is collected and made up to 10 mL with acetonitrile of the procedural blank in order to compensate the different suppressive ME due to the diverse relative proportions among water, salts and acetonitrile evaluated in the partition experiments. The extracts are then filtered at 0.2 µm and analysed by LC-MS/MS, comparing the chromatographic areas of the partition experiments with calibration lines based on PFAA standard solutions prepared with the corresponding procedural blanks. **Figure S1** illustrates the results of these experiments, which highlighted recovery percentages in the ranges of 88-104%, 83-99% and 84-108% for the acetonitrile volumes of 2.5 mL, 5 mL and 10 mL, respectively. In particular, it should be noted that high recovery values were achieved also for PFBuS and PFHxS, which showed negative values of the log K_{OW} and that, for almost all PFAAs, the variation of acetonitrile volume did not significantly affect the recovery values.

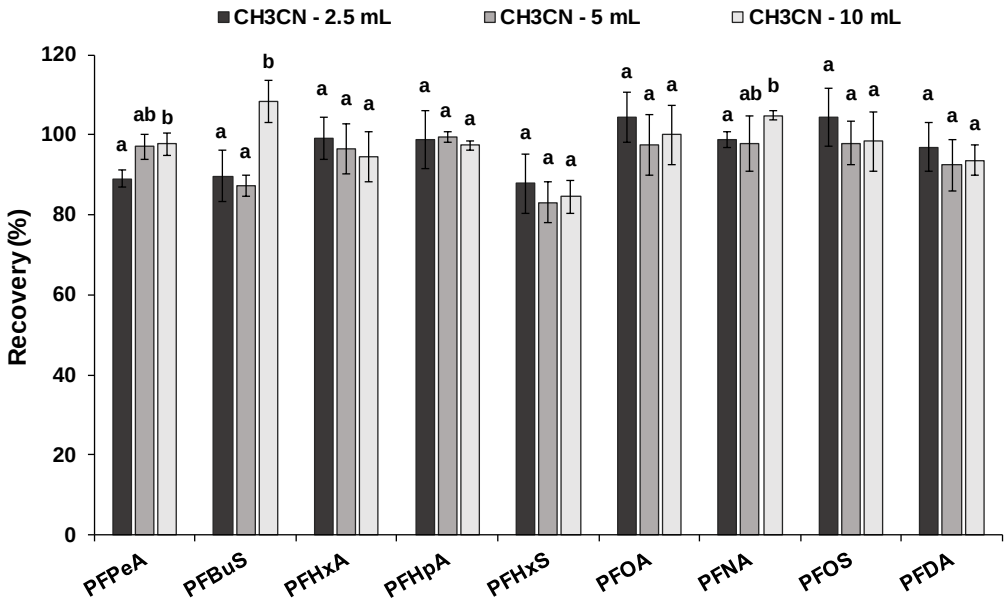


Figure S1 – Mean recovery values (n=3) of the water-to-acetonitrile partition experiments performed on PFAA standard solutions (500 ng/L) prepared in LC-MS water (pH=6.57±0.05), using 2.5, 5.0 and 10 mL of acetonitrile. Error bars represent standard deviation. Within a same compound, different letters indicate statistically significant differences according to the Games-Howell non-parametric test (P<0.05).

Figure S2 highlights the detail of the oily and strongly pigmented acetonitrile extract obtained treating one gram of freeze-dried olive fruit (cultivar "Frantoio") as specified in the paragraph 2.4.2 of the main text. Briefly, the freeze-dried fruit is rehydrated with 5 mL of LC-MS water and the mixture hand shaken for 15 s and vortex-mixed for 1 min. Then, 10 mL of acetonitrile are added and the mixture is further hand shaken for 15 s, and sonicated for 90 sec. Afterwards, 2 g of NaCl and 2 g of $MgSO_4$ are added, and the obtained mixture additionally hand shaken (15 s) and vortex mixed (1 min). The tube was centrifuged at 9000×g and T=4°C for 5 min, obtaining the aforementioned oily and pigmented extract.

Figure S2 – Detail of the oily and strongly pigmented QuEChERS extract obtained in this study for the extraction of the "Frantoio" cultivar olives.

The results of the matrix-free tests clean-up procedure employing PSA, C18 and GCB sorbents were illustrated in **Figure S3**.

Figure S4 and **Figure S5** illustrate the trends of the matrix effect (ME%), expressed as percentage of suppression (negative values) or enhancement (positive values) of the signal, determined for the QuEChERS and USAE- QuEChERS extracts of olive fruits.

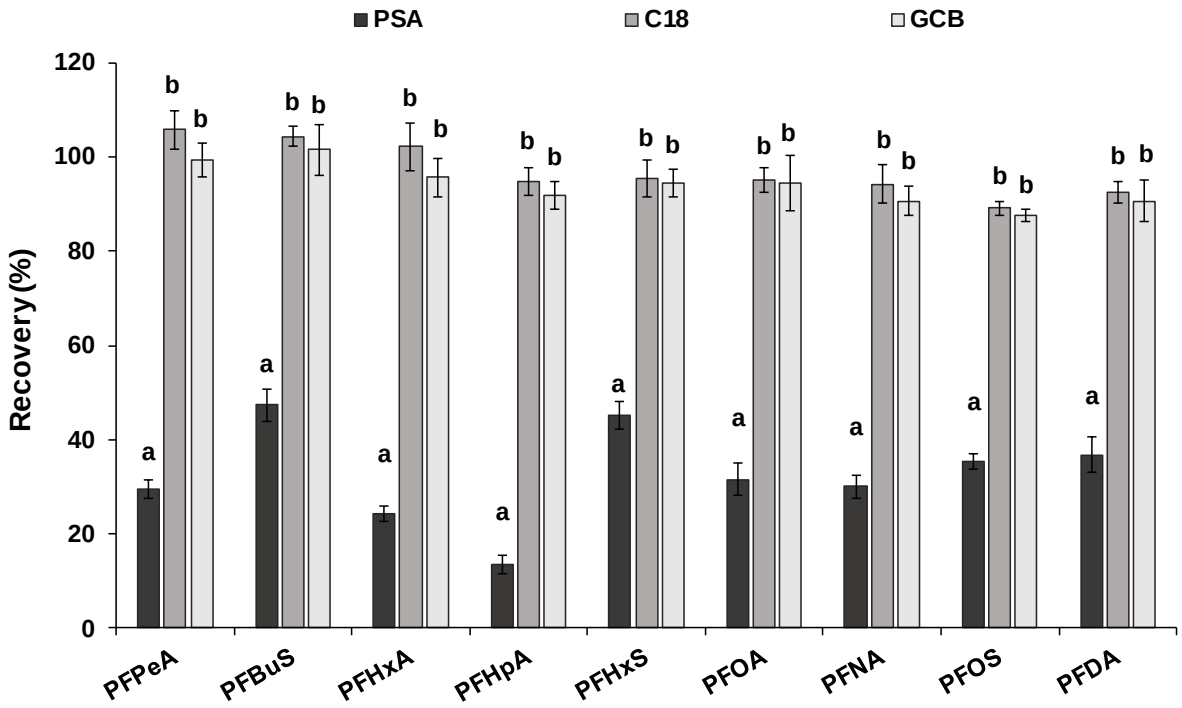


Figure S3 – Mean recovery values (n=3) of the matrix-free d-SPE partition experiments performed on PFAA standard solutions (500 ng/L in LC-MS acetonitrile deriving from the QuEChERS extraction procedural blank), containing 150 mg/mL of $MgSO_4$, using 300 mg/mL of PSA, C18 and GCB. Error bars represent standard deviation. Within a same compound, different letters indicate statistically significant differences according to the Games-Howell non-parametric test ($P<0.05$).

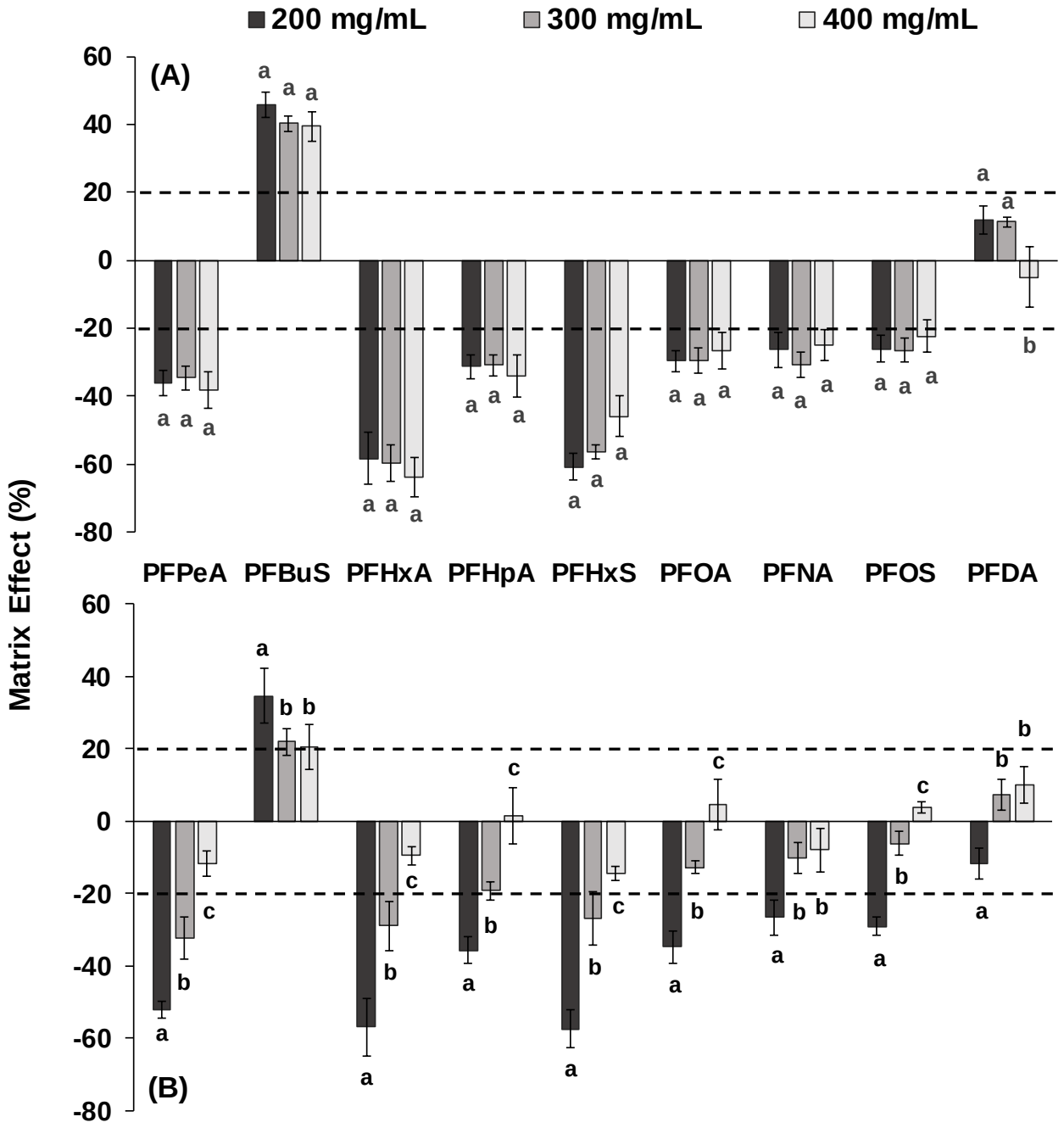


Figure S4 – Mean values (n=3) of matrix effects determined for the optimized QuEChERS extraction, coupled with d-SPE clean-up performed with 200, 300 and 400 mg of C18 (A) and GCB (B) sorbents per mL of olive fruit extract. Error bars represent standard deviation. Within a same compound, different letters indicate statistically significant differences according to the Games-Howell non-parametric test (P<0.05).

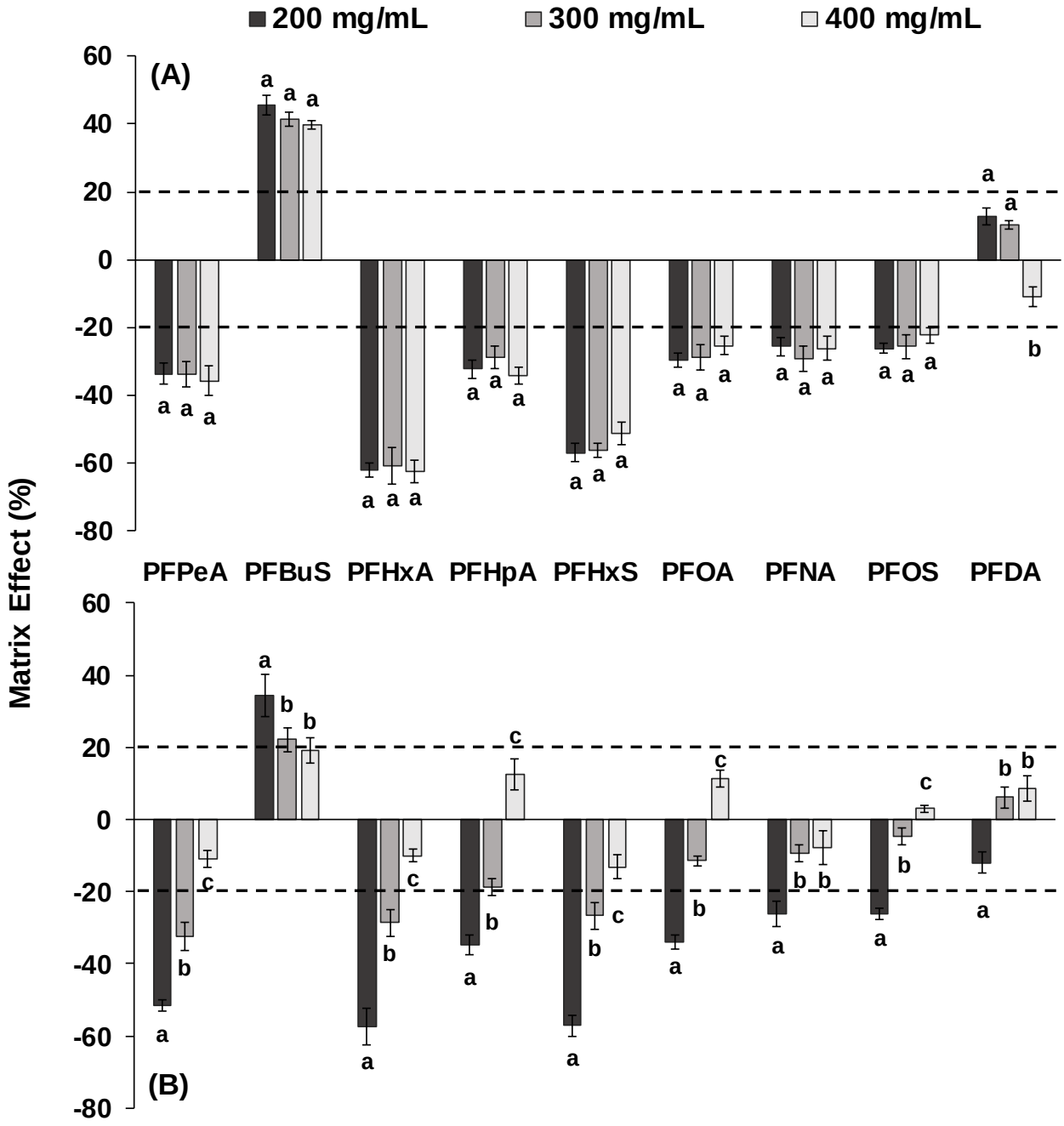


Figure S5 – Mean values (n=3) of matrix effects determined for the optimized USAE-QuEChERS extraction, coupled with d-SPE clean-up performed with 200, 300 and 400 mg of C18 (A) and GCB (B) sorbents per mL of olive fruit extract. Error bars represent standard deviation. Within a same compound, different letters indicate statistically significant differences according to the Games-Howell non-parametric test (P<0.05).

The QuEChERS protocols optimized and validated for the determination of PFAAs in freeze-dried strawberry and olive fruits are illustrated in **Figure S6**.

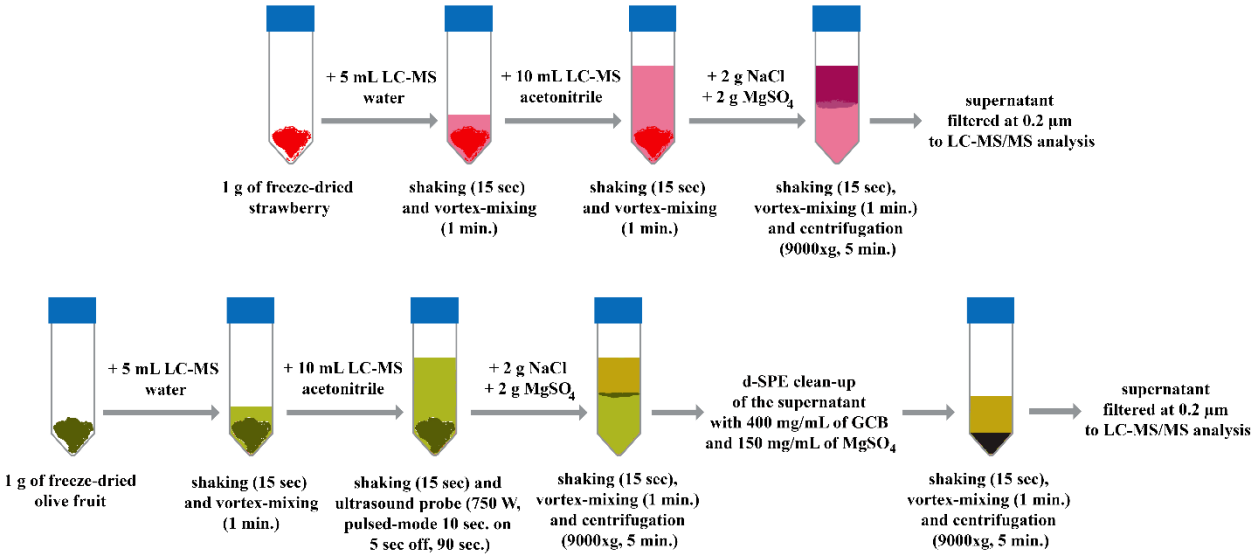


Figure S6 – Scheme of the QuEChERS protocols for the analysis of PFAAs in (A) strawberry and (B) olive fruits.

Table S2 – Characteristics of the calibration lines in the procedural blank.

Compound	Range (pg/g d.w.)	R^2	Slope	Slope error	Significance	Intercept	Intercept error	Significance
Strawberry								
PFPeA	500-10000	0.9992	43785	396	<0.05	-12892	2225	<0.05
PFBuS	500-10000	0.9987	42947	488	<0.05	3335	2742	0.25
PFHxA	500-10000	0.9984	81419	1043	<0.05	694	5858	0.90
PFHpA	50-10000	0.9993	103633	690	<0.05	2257	3169	0.48
PFHxS	50-10000	0.9988	61379	539	<0.05	1343	2474	0.60
PFOA	50-10000	0.9989	134297	1111	<0.05	911	5100	0.86
PFNA	10-10000	0.9993	104241	653	<0.05	917	2774	0.74
PFOS	50-10000	0.9985	54473	481	<0.05	-5833	2209	<0.05
PFDA	10-10000	0.9988	127352	1027	<0.05	581	4363	0.89
Olive								
PFPeA	500-10000	0.9997	33086	151	<0.05	1351	693	0.07
PFBuS	50-10000	0.9991	32628	250	<0.05	404	1151	0.73
PFHxA	50-10000	0.9994	84044	665	<0.05	-11339	3048	<0.05
PFHpA	50-10000	0.9992	101963	738	<0.05	-6669	3389	0.06
PFHxS	10-10000	0.9997	72345	298	<0.05	-5114	1267	<0.05
PFOA	50-10000	0.9997	102589	432	<0.05	-5354	1980	<0.05
PFNA	10-10000	0.9997	104497	442	<0.05	-2047	1879	0.28
PFOS	50-10000	0.9994	54980	462	<0.05	-3210	2119	0.15
PFDA	10-10000	0.9997	154130	660	<0.05	-11863	2804	<0.05