Comparison and Validation of the QuEChERSER Mega-Method for Determination of Per- and Polyfluoroalkyl Substances in Foods by Liquid Chromatography with High-Resolution and Triple Quadrupole Mass Spectrometry

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Abstract:

Instances of food contamination with per- and polyfluoroalkyl substances (PFAS) continue to occur globally, but sample preparation and analytical methods are quite limited and often monitor for a small percentage of known PFAS. This study aimed to evaluate, validate, and compare performance of two instruments with the recently developed "quick, easy, cheap, effective, rugged, safe, efficient, and robust" (QuEChERSER) sample preparation mega-method – a method developed to monitor chemicals over a broad range of physicochemical properties. Initial evaluation of the QuEChERSER mega-method for determination of PFAS in food demonstrated recoveries, matrix interferences, and lipid removal comparable to (or better than) US Food and Drug Administration (FDA) and USDA Food Safety and Inspection Service (FSIS) methods. Subsequent validation of QuEChERSER in beef, catfish, chicken, pork, liquid eggs, and powdered eggs on a high-resolution mass spectrometer achieved acceptable recoveries (70–120%) and precision (RSDs ≤ 20%) for all 33 target analytes at the 1 and 5 ng g^{-1} levels and 67–88% of analytes at the 0.1 ng g^{-1} level, depending on the matrix. Additional validation was performed by tandem mass spectrometry on a triple quadrupole instrument. This approach provided no non-detects and better recoveries at the 0.1 ng g^{-1} level than the HRMS method but exhibited more variability at 1 and 5 ng g^{-1} spiking levels. Analysis of NIST SRMs 1946 and 1947 gave accuracies of 70-117%. These results demonstrate the capability of combining PFAS analysis with a mega-method previously validated for 350 analytes, while collecting non-target data for future retrospective analysis of emerging alternatives with a high-resolution mass spectrometry method.

Keywords:

PFAS; LC-HRMS; LC-MS/MS; meat; fish; eggs; sample preparation

Abbreviations[1]

[1] Per and polyfluoroalkyl substances (PFAS); EFSA (European Food Safety Authority); Food and Drug Administration (FDA); US Department of Agriculture (USDA); Food Safety Inspection Service (FSIS); Triple quadrupole (QqQ);primary secondary amine (PSA); graphitized carbon black (GCB); methanol (MeOH); acetonitrile (MeCN); instrument top sample preparation (ITSP); dispersive-SPE (d-SPE); polypropylene (PP); high-resolution mass spectrometry (HRMS); electron ionization (EI); matrix-matched (MM); reagent-only (RO); National Institute of Standards and Technology (NIST); standard reference material (SRM); multiple reaction monitoring (MRM); total ion chromatogram (TIC); polytetrafluoroethylene (PTFE); perfluorocarboxylic acids (PFCAs); perfluorosulfonic acids (PFSAs); perfluorobutanoic acid (PFBA); perfluoropentanoic acid (PFPeA); perfluorohexanoic acid (PFHxA);

1. Introduction

Per- and polyfluoroalkyl substances (PFAS), a class of compounds known for their persistence, ability to bioaccumulate, and potential adverse health outcomes pose a significant threat to environmental and human health [1-3]. Used in a variety of consumer and industrial products [4, 5], both legacy and emerging PFAS have led to global occurrences of water, soil, and foodstuff contamination [6-8] through industrial and wastewater discharge or the leaching of food packaging and textiles [9, 10]. Despite recent regulations limiting production of some PFAS, the stable contaminants are still frequently detected alongside a growing number of emerging PFAS – compounds designed to replace legacy chemicals while imparting the same properties, often with minimal understanding of their potential health effects and long-term fate [11, 12]. The complex and evolving suite of PFAS contaminants requires thorough screening approaches to best understand exposure [13]. Diet has been considered a major source of PFAS exposure [14], with the extent of exposure depending largely on location and type of diet. PFAS has been found capable of accumulating in plants [15, 16] and livestock when contaminated water, bio sludge, or feed is employed [17-20] or when foods are exposed to PFAS throughout processing, packaging, and preparation steps [21].

Studies examining PFAS in food have focused on seafood [22-24] or target only a handful of analytes, and of the total diet studies conducted, most are from European countries [14, 25-35]. A recent review [36] focusing on the European market found exceedance of tolerable weekly intake values outlined by the European Food Safety Authority (EFSA) from all major food sources (fish, meat, eggs, fruits, and vegetables). The report also highlights the need for additional dietary exposure surveys and methods

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perfluoroheptanoic acid (PFHpA); perfluorooctanoic acid (PFOA); perfluorononanoic acid (PFNA); perfluorodecanoic acid (PFDA); perfluoroundecanoic acid (PFUdA); perfluorododecanoic acid (PFDoA); perfluorotridecanoic acid (PFTrDA); perfluorotetradecanoic acid (PFTeA); perfluorobutanesulfonic acid (PFBS); perfluoropentanesulfonic acid (PFPeS); perfluorohexanesulfonic acid (PFHxS); perfluoroheptanesulfonic acid (PFHpS); perfluorooctanesulfonic acid (PFOS); perfluorononanesulfonic acid (PFNS); perfluorodecanesulfonic acid (PFDS); sodium dodecafluoro-3H-4, 8-dioxanonanoate (NaDONA); perfluoro-2-methyl-3-oxahexanoic acid (HFPO-DA); 9-chlorohexadecafluoro-3-oxanonane-1-sulfonate (9Cl-PF3ONS); 11-chloroeicosafluoro-3-oxaundecane-1-sulfonic acid (11Cl-PF3OUdS); perfluoro-3-methoxypropanoic acid (PFMPA); perfluoro-4-methoxybutanoic acid (PFMBA); perfluoro(2-ethoxyethane)sulfonic acid (PFEESA); nonafluoro-3,6-dioxaheptanoic acid (NFDHA); 1H,1H, 2H, 2H-perfluorohexane sulfonic acid (4:2FTS); 1H,1H, 2H, 2H-perfluorooctane sulfonic acid (6:2FTS); 1H,1H, 2H, 2H-Perfluorodecane sulfonic acid (8:2FTS); N-ethyl perfluorooctanesulfonamidoacetic acid (NEtFOSAA); N-methyl perfluorooctanesulfonamidoacetic acid (NMeFOSAA); perfluoro-1-butanesulfonamide (FBSA); perfluoro-1-hexanesulfonamide (FHxSA); perfluoro-1-octanesulfonamide (FOSA)

adapted to emerging PFAS with low detection limits. Typically, a QuEChERS (or similar) approach followed by SPE cleanup is used to extract PFAS from food and has been demonstrated effective on a variety of matrices, including meats [37], seafood [38, 39], fruits and vegetables [40-43], dairy [38, 44], and processed foods [45, 46]. However, SPE cleanup can inevitably result in loss of some PFAS classes along with unwanted co-extractives. Due to the ubiquity of PFAS, these manifolds and disposables can also lead to sample contamination. Therefore, an ideal PFAS method will provide excellent cleanup efficiency (remove unwanted matrix interferences) over minimal steps (avoid contamination and loss) and capture the large, expanding list of PFAS.

The new QuEChERS mega-method (QuEChERSER; more than QuEChERS) captures a broader polarity range than QuEChERS and has already been validated in a variety of matrices for environmental contaminants, veterinary drugs, and pesticides [47-49]. This approach could overcome the loss/time/contamination challenge for PFAS analysis while covering a new analyte class with an already existing high-throughput, efficient method.

In addition to capturing a wide array of PFAS in sample preparation, detection methods should allow simultaneous analysis of compounds with and without available standards, including non-targeted screening of emerging PFAS and their byproducts [50]. Traditionally, PFAS screening relied on LC coupled with MS/MS to achieve selectivity and sensitivity for the select number of standards available, which despite improvements, still lags behind the number of existing PFAS (>9000). High-resolution mass spectrometers help overcome this issue by combining targeted and non-targeted analysis [51-55]. Selectivity is achieved by high-resolution accurate mass while advancements in databases, data mining software, and fragmentation prediction algorithms [56, 57] have led to improved suspect and non-target screening capabilities. High-resolution instrumentation combined with a comprehensive and unspecific sample preparation method is necessary to keep up with the changing PFAS landscape.

This study aimed to (1) compare recoveries, matrix effects and cleanup efficiency of QuEChERSER against previously reported US Food and Drug Administration (FDA) and USDA Food Safety and Inspection Service (FSIS) PFAS extraction methods for food, (2) validate the method in beef, chicken, catfish, pork, liquid eggs, and powdered eggs at three spiking levels, and (3) compare quantitative performance of high-resolution (Q-Orbitrap) and low-resolution (triple quadrupole) mass

spectrometers based on instrumental limits of quantitation, matrix effects, and recoveries. Analysis of standard reference materials and incurred catfish allowed for further method performance evaluation.

2. Methods and Materials

2.1. Chemicals and Tissue Samples

Stock solutions were purchased from Wellington Laboratories (Guelph, Ontario, Canada) and included a 30-compound mixture containing carboxylates (C4-C14), sulfonates (C4-C10; linear and branched), 4:2FTS, 6:2FTS, 8:2FTS, NaDONA, 9Cl-PF3ONS, 11Cl-PF3OUdS, FBSA, FHxSA, FOSA, HFPO-DA, NMeFOSAA and NEtFOSAA, as well as a 4-compound mixture of the emerging perfluorochemicals PFEESA, NFDHA, PFMBA, and PFMPA (representative structures presented in Figure 1). The solutions were mixed to create a 500 ng mL^{-1} stock solution of 34 perfluorochemicals for method development. Twenty isotopically-labeled internal standards (M4PFBA, M5PFPeA, M5PFHxA, M4PFHpA, M8PFOA, M9PFNA, M6PFDA, M7PFUdA, M2PFDoA, M2PFTeDA, M8FOSA, d3-NMeFOSAA, d5-NEtFOSAA, M3PFBS, M3PFHxS, M8PFOS, M2-4:2FTS, M2-6:2FTS, M2-8:2FTS, and M3HFPO-DA from Wellington Laboratories) were prepared as a 250 ng mL^{-1} stock solution in methanol. Formic acid, ammonium acetate, and Optima LC-MS grade solvents (water, acetonitrile, and methanol) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Salts, $MgSO_4$/NaCl (4/1, w/w), and sorbents, primary secondary amine (PSA) and graphitized carbon black (GCB), were from UCT (Bristol, PA, USA).

Beef, catfish, pork, and chicken tissue were collected from supermarkets in Philadelphia, PA. Frozen tissue was roughly chopped using an acetone-rinsed knife and homogenized with dry ice in a Robot Coupe RSI 2Y1 (Ridgeland, MS, USA). Brown and white eggs were also collected from local supermarkets, combined and vortexed. Powdered eggs were mixed with water in a 1:1 ratio (w/w) and vortexed just prior to extraction. The egg powder and homogenized samples were kept at –20 °C until processing.

2.2. Sample Preparation for Method Evaluation

Newly developed QuEChERSER (more than QuEChERS) method has been validated for pesticides, environmental contaminants, and veterinary drugs. As a preliminary assessment, we compared this new mega-method to previously reported food safety PFAS methods in terms of recoveries, matrix effects, and cleanup efficiency for beef, catfish, and eggs. Recoveries and cleanup efficiency were assessed for each matrix in triplicate at a 16 or 40 ng g^{-1} spiking level such that 100% recovery would result in a final extract concentration of 8 ng mL^{-1}, regardless of extraction method, to assist in cleanup comparisons. Because the amount of tissue used for extraction varies among the three compared methods, 1 g was selected as a reasonable mid-point for evaluation. For matrix effect determination, an additional set of 1 g samples were extracted according to each method and spiked with standards (discussed in 2.2.5 Method Comparisons).

2.3. QuEChERSER Method

Following the QuEChERSER protocol (Figure 2), 5 mL of 4:1 (v/v) acetonitrile/water was added to 1 g tissue samples spiked with standard and internal standard mixtures. Samples were vortexed, shaken for 10 min on a platform mixer, centrifuged at 3711 rcf for 3 min, and then 0.2 mL of supernatant was transferred to a polypropylene (PP) microcentrifuge tube in duplicate. Duplicate volumes were transferred to determine the effect of final solvent composition on PFAS recoveries. Once evaporated to near dryness with N_2 gas, 0.8 mL of methanol or initial mobile phase (95:5 H_2O:MeOH) was added. Samples were vortexed, centrifuged at 12,500 rcf for 5 min, and then the supernatant was transferred to a PP autosampler vial for analysis (LC portion). The remaining initial extract was added to 1 g of salt (4:1 (w/w) $MgSO_4$/NaCl), shaken for 1 min, and centrifuged for 3 min at 3711 rcf. The acetonitrile layer was transferred to an autosampler vial for Instrument Top Sample Preparation (ITSP) cleanup (GC portion). The GC portion was analyzed by GC-MS for cleanup efficiency comparisons (discussed in Section 2.7) and by LC-MS to determine the recovery of PFAS in the GC portion.

2.4. US Food and Drug Administration Method

The US FDA method for determining 16 PFAS in food is based on a QuEChERS approach followed by SPE cleanup of samples with positive detections. Briefly, 1 g of tissue was weighed into a PP tube, spiked with standard and internal standard mixtures at 16 and 2 ng g^{-1}, respectively, and then 1 mL

of water, 2 mL of acetonitrile, and 30 µL of formic acid were added. Samples were shaken for 1 min and then 1.5 g of salts (4:1 $MgSO_4$/NaCl) were added before shaking again for 5 min. After centrifugation at 10,000 rcf for 5 min, 1 mL of supernatant was added to dispersive-SPE (d-SPE) sorbent (180 mg of $MgSO_4$, 60 mg of PSA, and 30 mg of GCB) and the remainder was transferred to a PP vial for ITSP cleanup. Samples taken through d-SPE cleanup were vortexed, shaken for 2 min, and centrifuged for 5 min at 10,000 rcf. The supernatant was filtered through a 0.45 µm nylon filter vial and transferred to a PP autosampler vial.

2.5. USDA Food Safety and Inspection Service (FSIS) Method

FSIS method CLG-PFAS 2.03 is based on solvent extraction followed by lipid and protein freeze-out for 16 PFAS target analytes. One gram of tissue was spiked with standard and internal standard mixtures and then 5 mL of methanol was added. Samples were vortexed, left to sit for 30 min at room temperature and then kept at –20 °C for one hour. Frozen samples were centrifuged at 3500 rpm for 22 min at 4 °C. An aliquot of supernatant was transferred to a PP autosampler vial for LC-HRMS analysis and another aliquot transferred for ITSP cleanup.

2.6. Instrument Top Sample Preparation (ITSP) Automated Cleanup

ITSP automated cleanup was examined as an additional cleanup step for the FSIS method and LC portion of the QuEChERSER method (ITSP is already used as a cleanup step for GC analysis) and in lieu of d-SPE cleanup in FDA's method. A mini-SPE blend containing $MgSO_4$/PSA/C18/CarbonX from ITSP Solutions (Hartwell, GA, USA) was evaluated. As previously described, ITSP is performed using a robotic PAL system by CTC Analytics where 300 µL of extract is added to mini-SPE cartridges at 2 µL s^{-1} and approximately 220 µL of cleaned extract is collected.

2.7. Method Comparisons

Trueness of each method was evaluated by spike recoveries and matrix effects. Recoveries were calculated as the ratio of extracted concentration to spiked concentration. To evaluate matrix effects, beef, catfish, and eggs were processed with each method and cleanup combination, spiked with target analytes at a final concentration of 1 ng mL^{-1} (n=3), and then analyzed. An additional set of solvent-only

spikes was generated by spiking methanol with target analytes at the same concentration (1 ng mL^{-1}). Absolute matrix effects were calculated as the percent difference in peak area between matrix and solvent-spiked samples, while relative matrix effects were calculated using an internal standard calibration curve. All matrix effects were calculated such that a positive value represents ion enhancement and negative value represents ion suppression. Cleanup efficiency, as percent co-extractive removal, was calculated by comparing the total peak area of GC-Electron Ionization (EI)-MS total ion chromatograms of the pre-ITSP matrix blanks to d-SPE, ITSP, and QuEChERSER GC-portion extracts. GC-EI-MS conditions were previously described [47-49].

2.8. Method Validation

After initial evaluation of method performance, QuEChERSER was validated for six matrices (beef, catfish, chicken, pork, liquid eggs, and powdered eggs) at three spiking levels (0.1, 1, and 5 ng g^{-1}; equivalent to 0.05, 0.5, and 2.5 ng mL^{-1} in final extracts). A matrix blank, reagent blank, and five replicates of each spiking level were processed according to the QuEChERSER protocol outlined above for 0.5 g of tissue. To boost detectability, 1 mL of supernatant was transferred for drying and then reconstituted to 0.4 mL by adding 0.2 mL of methanol (2.5-fold concentration compared to 4-fold dilution in original method). Matrix-matched (MM) and reagent-only (RO) calibration curves were generated from matrix blanks and solvent, respectively, spiked with standards and internal standards after processing. A 5-point calibration curve prepared from 0.05 to 5 ng mL^{-1} was used for quantification of most analytes. Linearity was assessed for each matrix and final linear ranges were determined by visual inspection and calculating the RSD of relative response factors ($R^2 > 0.99$ and RSD of RRF < 20%). LODs and LOQs were determined by multiplying the standard deviation of samples spiked at 0.04 ng g^{-1} by 3.3 and 10, respectively. Spiked samples were necessary for signal quantification, as blanks resulted in a peak area of zero for most analytes in the high-resolution mass spectrometry (HRMS) method. Matrix effects were calculated by subtracting the slope of the RO calibration curve from the MM calibration curve and dividing by the RO slope for each matrix (($MM_{slope} - RO_{slope}$) / RO_{slope} × 100%), such that a negative value corresponds to ion suppression and positive value ion enhancement. Finally, analysis of incurred catfish

(domestic, wild-caught) samples from the USDA FSIS and SRMs 1947 and 1946 from NIST (Gaithersburg, MD USA) allowed for determination of method accuracy.

2.9. Liquid Chromatography Mass Spectrometry

2.9.1. Chromatographic Separation

A Waters Acquity LC System was fitted for PFAS analysis using the Waters PFAS solutions kit (Milford, MA, USA) to minimize background contamination. All solvent lines were replaced with peek tubing and a delay column was installed to prevent coelution of background PFAS and PFAS from samples. Chromatographic separation was achieved with a 1.7 µm, 2.1 x 100 mm ACQUITY BEH C18 column equipped with a 1.7 µm, 2.1 x 5 mm Acquity BEH C18 guard column (Waters Corp., Milford, MA, USA) maintained at 50 °C. The following gradient elution program with 95:5 H_2O:MeOH (A) and MeOH (B) both containing 2 mM ammonium formate was used: initial 5% B (held for 0.5 min), increased to 50% B over 0.5 min, increased to 100% B over 9 min (held for 2 min) and then returned to the initial 5% B over 0.5 min and before 2.5 min of equilibration (total run time = 15 min). Injection volume was 5 µL and solvents were diverted to waste for the first minute to avoid contamination of the source with highly polar material. The Acquity system was coupled to both HRMS and MS/MS systems by a contact closure connection.

2.9.2. UHPLC-HRMS Analysis and Data Processing

A full-scan HRMS method was developed on a Q-Exactive Plus Hybrid Quadrupole-Orbitrap™ system (Thermo Fisher Scientific, Bremen, Germany). Instrumental settings were as follows: spray voltage –2500 V, capillary temperature 300 °C, sheath gas pressure 40, auxiliary gas pressure 10, auxiliary gas heater temperature 250 °C, and the S-lens radio frequency set to 50. The spectrometer was operated in full-scan negative ionization mode scanning 150–1000 *m/z* with full width at half maximum resolution set at 70,000 and automatic gain control at 3×10^6. Mass calibration was performed before every batch.

Quantitation and data processing were completed in Tracefinder™ (Version 4.1, Thermo Fisher Scientific) using a 5 ppm mass extraction window for parent mass ions. Peak areas were generated using

the summation peak integration function and quantified by 1/X weighted internal standard calibration curves. Analyte details are provided in Table S1.

2.9.3. UHPLC-MS/MS Analysis and Data Processing

To compare quantitative performance of a HRMS method with the more common targeted MS/MS approach, method validation samples were also analyzed on a SCIEX 6500 QTRAP™ MS/MS system (Foster City, CA, USA) operating in multiple reaction monitoring (MRM) mode. Transitions were optimized for a subset of analytes and compared with the US FDA method's transitions reported on SCIEX QTRAP 6500+ instrument. Parameters were similar to previously reported values, so those were employed for common analytes. For additional analytes, a standard mixture was infused by syringe to optimize declustering potential, entrance potential, collision energy, and exit potential in Analyst® (Version 1.6.2, SCIEX). A scheduled MRM method was generated using a 30 s MRM window and target scan time of 0.5 s. Source parameters were as follows: curtain gas 40 au, ion spray voltage –4500 V, source temperature 350 °C, ion source gas 1 and 2 at 50 au, and collisionally activated dissociation gas set to medium.

Quantitation and data processing was performed in MultiQuant™ (Version 3.0, SCIEX). Peak areas of the quantification ion were generated using the summation peak integration function and concentrations calculated by 1/X weighted internal standard calibration curves. Method details are provided in Table S2.

3. Results and Discussion

3.1. Method Comparison

To determine the suitability of QuEChERSER for PFAS analysis in food, a method comparison study was conducted to compare previously reported US FDA and USDA FSIS methods with the new mega-method in three representative USDA FSIS-regulated foods (beef, catfish, and eggs). Generally, the FDA and QuEChERSER methods performed better than the FSIS method, especially in beef and catfish matrices (Figure 3). Recoveries were similar among QuEChERSER (89 ± 9%), FDA (103 ± 14%), and FSIS (89 ± 6%) methods in eggs with most falling within the accepted range of 70–120%. Both FDA

and QuEChERSER methods had acceptable recoveries in beef (106 ± 14% *vs.* 88 ± 10%) and catfish (101 ± 14% *vs.* 84 ± 11%) with FDA method's values slightly better when considering the average across all target analytes. GenX (HFPO-DA) was not included in the FSIS method comparison summary due to insufficient recoveries across all matrices. Similarly, the sulfonamido acetic acids (NEtFOSAA and NMeFOSAA) and corresponding internal standards were not recovered well by the FDA method in eggs. Therefore, despite slightly lower internal standard corrected recoveries in the QuEChERSER method, it performed the best across all compound classes, likely due to simple cleanup steps where losses (and contamination) are less likely to occur than with d-SPE or filtration. Individual recoveries are reported in Table S3. Due to decreased detectability of HFPO-DA compared to other analytes, the spiking concentration of M3-HFPO-DA was increased 5-fold for subsequent method validation (i.e., 2 ng g^{-1} spike of 19 internal standards and 10 ng g^{-1} spike of M3-HFPO-DA).

Absolute matrix effects were quantified by comparing peak areas in extracted matrix and solvent, while relative matrix effects (internal standard corrected) were calculated as the difference between spiked concentrations and those determined with internal standard calibration curves. Matrix effects were averaged across all analytes for each of the three methods to provide a visual overview of the methods' performance (Figure 4). Error bars represent standard deviation and show matrix effect variability among analytes for a given method. For all three methods, average absolute and relative matrix effects were within ±20%, indicating limited matrix interferences for most compounds, regardless of approach (Tables S4-6). Catfish had the greatest amount of ion suppression and enhancement, particularly with FDA's d-SPE cleanup. The FSIS method had consistent ion suppression near −20% for all matrices that was not corrected with an internal standard calibration curve. QuEChERSER performed well overall, with the average relative matrix effect between 0 and −10% for all analytes (n=3), except for FBSA in catfish (−12%).

Co-extractive removal was assessed by integrating the GC-MS full-scan total ion chromatograms (TICs) of pre-cleanup matrix blank extracts and comparing the peak area to that of post-cleanup extracts. Due to the simple extraction technique of the FSIS method, only a post-freezing extract was analyzed to avoid clogging the GC. Similarly, to avoid introducing water to the GC, a salted-out GC portion of QuEChERSER extracts were used to compare cleanup efficiency instead of the LC portion (Figure 2).

Overall, the pre-cleanup extracts collected for comparison were post-freezing for FSIS, pre-d-SPE for FDA, and post-salt out for QuEChERSER. In terms of peak area, catfish had the largest amount of matrix in pre-cleanup FDA and QuEChERSER extracts, while egg had the most in FSIS extracts (Figure 5A). It was expected that pre-cleanup FDA and QuEChERSER GC-portion extracts would be similar due to the QuEChERS approach of FDA and thus similar liquid extraction and salt-out protocol, whereas methanol in the FSIS method would lead to a different profile with more extractants present. While not identical, the sample equivalents were similar between methods (0.16, 0.2 and 0.22 g of tissue mL^{-1} for QuEChERSER, FSIS and FDA methods, respectively). Pre-cleanup extracts were then processed according to their respective methods and analyzed again by GC-MS TICs to calculate percent peak area remaining (Figure S1). The percent peak area remaining for FDA-d-SPE was 82% for beef and 64% for catfish. Due to precipitation of egg in the FDA pre-cleanup sample, no percent removal comparison could be made, but the d-SPE egg sample had a peak area that was 20% and 34% of pre-cleanup beef and catfish. Cleanup of the QuEChERSER GC-portion was performed by automated ITSP, according the original QuEChERSER protocol, and is further discussed below.

Method cleanup efficiency was also compared by LC-ESI(–)-HRMS TICs of post-cleanup matrix blanks. This approach allowed for a comparison among methods using the LC portion of QuEChERSER. In agreement with GC-MS results, LC TICs suggest QuEChERSER extracts are the cleanest and contain the least amount of co-extractives/lipids of the three methods and matrices (Figure 5B), in part due to smaller tissue equivalents in the final extracts. Peak intensities were at least two-fold higher in the FSIS and FDA extract TICs. Relatively small amounts of extracted lipids, combined with competitive recoveries and minimal matrix effects, suggests a QuEChERSER approach for PFAS extraction offers an improvement over the food extraction methods investigated herein for beef, catfish, and eggs.

3.2. ITSP Evaluation

Prior to method validation, automated ITSP was evaluated for clean-up efficiency of QuEChERSER (GC-portion) extracts, as well as an alternative to the current d-SPE approach used by the FDA method. Since the GC-portion of QuEChERSER utilizes ITSP for cleanup, investigating this approach on spike-recovery samples allowed for determination of PFAS recoveries in the GC fraction of

311 the method. Applying the same TIC approach described above, 68%, 34%, and 63% of peak area
312 remained after ITSP cleanup of QuEChERSER beef, catfish, and egg extracts. This was better than the
313 percent removal of lipids provided by d-SPE in the FDA method and similar to cleanup of FDA extracts by
314 ITSP (Figure S1). Therefore, ITSP provides a reasonable, automated alternative to traditional d-SPE
315 cleanup of beef, catfish, and egg extracts. This was also supported by evaluation of spike recoveries and
316 matrix effects. Notably, at the time of analysis, cartridges contained PTFE septa, but PFAS-free kits are
317 now available. This inherently led to contamination with long-chain PFCAs appearing as ion enhancement
318 and elevated recoveries, but still led to median matrix effects and recoveries within ±20% and 70–120%
319 (Figure S2), respectively. As previously noted, HFPO-DA, NEtFOSAA and NMeFOSAA were less
320 sensitive than other PFAS, so limited recovery of their internal standards led to issues with recovery
321 calculations but informed future spiking levels for method validation. PFMPA was the only analyte to show
322 major differences in recoveries between the LC portion of QuEChERSER and GC portion with ITSP. This
323 analyte uses labeled-PFBA as a surrogate which could be inflated from ITSP contamination, though a
324 similar trend was not present for FDA-ITSP cleanup. Another possibility is spiking level differences. All
325 samples were spiked such that the amount of sample and PFAS was identical between methods for lipid
326 removal (i.e., the GC fraction of QuEChERSER contained a different amount of PFAS than the LC
327 portion). Therefore, if analysis of PFAS in the GC fraction is preferred (or necessary for additional cleanup
328 of complex matrices), method validation at various spiking levels should be performed. Nevertheless,
329 results are promising given the acceptable recoveries and cleanup found by our preliminary GC portion
330 results. Moving forward, method validation of the LC portion of QuEChERSER was conducted due to its
331 simplicity combined with sufficient evidence from the evaluation step suggesting its suitability for PFAS.

332 **3.3. Method Validation**

333 Initial method comparison results suggested the QuEChERSER method is an acceptable
334 approach for PFAS quantitation, thus method validation was performed for FSIS-regulated foods (beef,
335 catfish, chicken, pork, liquid eggs, and powdered eggs) at three spiking levels (0.1, 1, and 5 ng g^{-1}) in
336 quintuplicate. All validation samples were analyzed using the same Orbitrap HRMS method developed for
337 sample preparation comparisons and then reanalyzed with targeted MS/MS analysis on a SCIEX

QTRAP. Chromatographic separation was performed on the same UHPLC system for both MS methods, so retention times were essentially the same and ranged from 2.7 min (PFBA; omitted from validation) to 8.8 min (PFTeA; C14 carboxylic acid). PFBA was not resolved from a background peak and thus exhibited poor linearity due to integration issues, so it was dropped from the validation study. The method provided separation of branched and linear isomers of sulfonamidoacetic acids and sulfonates (Figure S3), but all values reported herein are the sum of both. Linearity and linear ranges were similar among matrices for a given instrumental method (R^2 > 0.99 for 96% and 98% of analytes in HRMS and QqQ; Tables S7-8). The QqQ method provided broader linear ranges, with more analytes maintaining linearity at the 0.05 ng mL^{-1} level, particularly for the longer-chain carboxylic acids, sulfonamides, and next-generation PFAS (FOSAAs, HFPO-DA, and NFDHA). With the exception of PFPeA, this ultimately led to improved recoveries for the low-level spike in the QqQ method when compared to the HRMS method (Figure 6 and Tables S9-10). Acceptable recoveries of PFPeA were achieved for all levels and matrices in the HRMS method due to limited background signal, whereas an elevated background for the PFPeA transition (263 > 219) led to linearity challenges, increased limits of detection, and less than ideal recoveries with the QqQ method. For mid and high-level spikes, the HRMS method performed best with all analytes providing acceptable recoveries (70–120%) and RSDs (< 20%).

Matrix effects were evaluated for each analysis type by comparing the slopes of calibration curves prepared in matrix and solvent. For HRMS-analysis, 98% of calculated matrix effects were within ±20% (total = 198) while 96% of calculated matrix effects were within ±20% for QqQ-analysis. Individual matrix effects are presented for both methods in Table S11. While both methods experienced similar amounts of ion suppression or enhancement with medians around 0%, the HRMS method performed better with fewer analytes experiencing ionization effects, as shown by the tighter boxplots in Figure 6. Regardless of analysis type, matrix effects should have a limited impact on the method's quantitative ability.

LOD and LOQ values were calculated by spiking seven replicates of matrix blanks (for each matrix) at 0.04 ng g^{-1}. The standard deviation of the analyte signal was multiplied by 3.3 and 10 for LODs and LOQs, respectively. Due to lack of signal in the pork matrix, spikes were increased to 0.08 ng g^{-1} for this group. Targeted methods are commonly performed on triple quadrupoles due to the selectivity and

sensitivity achieved with product ion selection, while HRMS is typically viewed as a more qualitative instrument for rapid screening and generation of large complex data sets. However, excellent selectivity can also be achieved with high-resolution accurate mass when interferences are not present [58]. Limited noise was observed in the extracted mass window of the PFAS parent ions (5 ppm), which provided lower LODs and LOQs in the HRMS method than the QqQ method (Figure 6) despite better detectability, lower linear range, and thus improved recoveries at the low spiking level in the QqQ method. The difference in LOQs was most pronounced for pork, but all matrices showed lower medians and less variance in HRMS-derived LOQs. Individual LODs and LOQs are reported in Tables S7-8, with most below 50 ng kg^{-1}. HFPO-DA (in most matrices) and PFTeA in catfish had the highest LOQs for HRMS, while QqQ had more analyte variability between matrices. It is important to note that differences in ion sources, as well as mass analyzers, contribute to observed differences in the methods' quantitative abilities.

Another benefit of HRMS is the ability to differentiate between PFOS and bile acids. Cholic acids are known interferents of PFOS, particularly in egg or liver samples, that coelute when chromatographic separation is not optimized. Because the parent ions are not resolved by unit-resolution LC-MS, false positives and inaccurate quantification can occur. For method validation on the MS/MS system, the quantitation ion for PFOS was switched from *m/z* 499 > 80 to *m/z* 499 > 99 in eggs. However, high-resolution accurate mass was able to differentiate between these compounds, which ultimately decreases the chances of a false positive. Quantitative analysis in full-scan mode of a high-resolution mass spectrometer also allows for concurrent non-target and suspect screening of contaminants. With continual emergence of PFAS alternatives and limited standard availability, non-target screening combined with targeted analysis is essential to more completely characterizing the magnitude of PFAS contamination.

Due to reports of PFAS losses in glass vials, especially under aqueous conditions, the stability of our target analytes was investigated. Solutions were prepared in 25% or 100% methanol and stored in glass or PP vials with PP snap caps at –20 and 4 °C. The largest contributor to changes in signal/concentration was evaporation from vials with PP snap caps. The effect was significant at 4 °C with polypropylene vials, where total solvent evaporation occurred after 14 days. Overall, our results suggest storage is unaffected by solvent type or storage temperature when using glass vials for the 33 analytes investigated (Figure S5). If internal standards are present (e.g., sample extracts) in glass vials, recoveries

remain near 100% for 30 days. Note that polypropylene silicone screw caps for polypropylene vials were used instead of snap caps throughout the validation study and no evaporation was observed.

3.4. Incurred samples and SRM Analysis

NIST standard reference materials (SRMs) 1946 and 1947 were extracted in triplicate and analyzed using both HRMS and QqQ methods. PFNA, PFDA, PFDoA, PFUdA, PFTrDA, PFOS, FBSA, and FOSA were detected in all replicates of at least one SRM (Table 1), with accuracy ranging between 70 and 117%. PFOS was the only compound with a reported uncertainty, and these accuracies ranged between 98 and 103% for the HRMS method and 116 and 117% for the QqQ method. Compounds not listed with reference values in the SRM certificate were also detected. These measurements were within an order of magnitude of the concentrations reported by the inter-laboratory study evaluating the standard reference material [59].

Six samples of incurred domestic, wild-caught catfish tissue were obtained from the USDA FSIS to confirm the PFAS extraction capability of QuEChERSER. PFOS was the most frequently detected compound and at the highest levels. PFDA, PFUdA, PFDoA, PFTrDA, PFDS, and FOSA were also present above limits of detection in at least one of the samples (Table 2). Also, comparison of measurements by QqQ and HRMS instrumental methods (Table 2) demonstrate a close agreement between generated values, as well as those determined by FSIS a year prior.

4. Conclusions

The QuEChERSER mega-method was compared against other US federal agency methods for PFAS analysis in foodstuff and demonstrated an improvement in matrix effects, cleanup efficiency, and recoveries for most of the 33 target analytes, which is double the number of PFAS previously investigated by these methods. An evaluation of ITSP cleanup also demonstrated a fast and automated cleanup approach for laboratories where GC analysis with high-throughput and parallel cleanup is preferred, since GC fractions can be later analyzed on an LC-MS method. Following preliminary method evaluation, a validation experiment in six FSIS-regulated foodstuffs provided excellent results for all analytes at 1 and 5 ng g^{-1} levels and for 67–88% of analytes at the 0.1 ng g^{-1} level using LC-HRMS. Additional analysis on a triple quadrupole instrument obtained similar results, with all analytes validated at 5 ng g^{-1}, 91–100% at 1

421 ng g^{-1}, and 70–91% at 0.1 ng g^{-1} spiking levels. With minor adjustments, this method can easily be
422 implemented in laboratories already analyzing environmental contaminants, mycotoxins, veterinary drugs,
423 and pesticides with a QuEChERS approach. Additionally, the ability of this extraction technique to capture
424 a broad range of chemicals may support monitoring studies where targeted and non-targeted screening
425 are combined to better support characterization of dietary exposure to PFAS. Future studies might
426 perform validation in additional food products and evaluate recovery of other PFAS classes.
427

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Disclaimer: Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. This research was supported by the U.S. Department of Agriculture, Agricultural Research Service.

Figures and Tables:

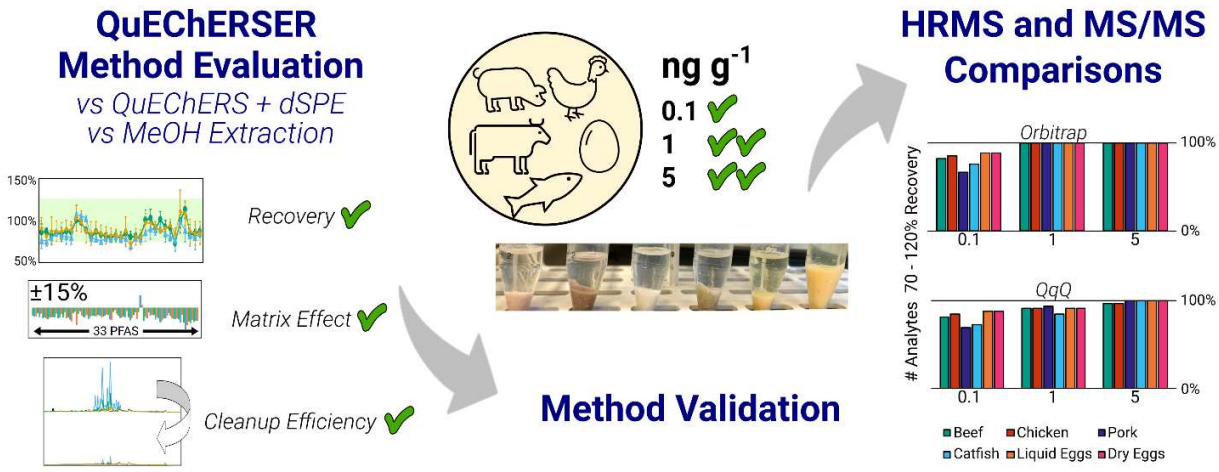


Table of Content Figure

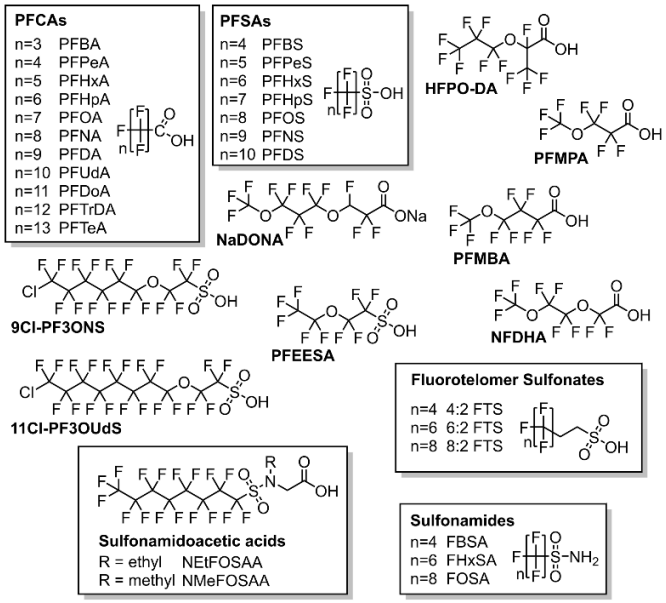


Figure 1. Structures of PFAS included in this study.

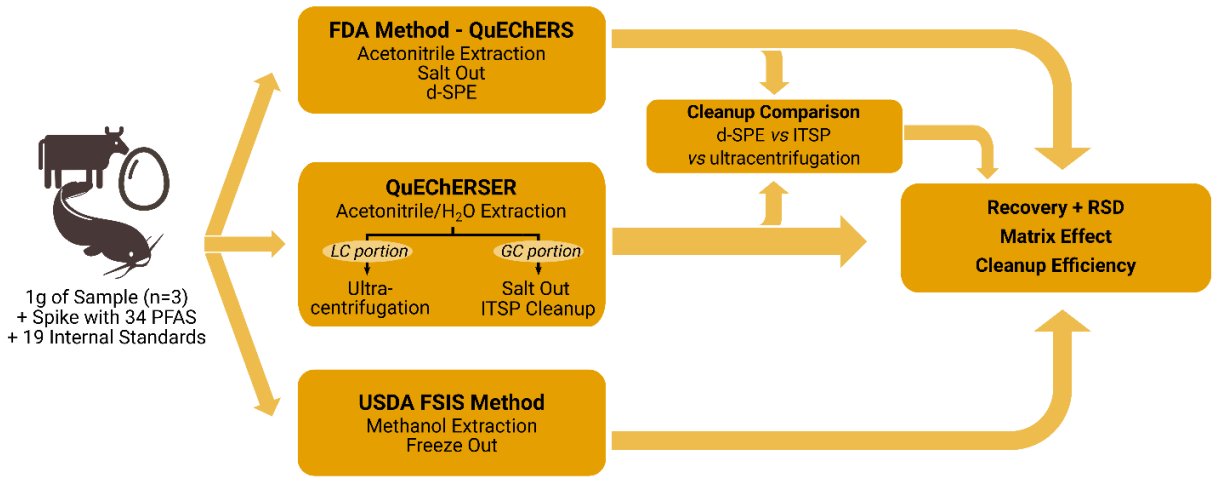


Figure 2. Workflow used to compare three PFAS extraction methods (USDA FSIS, US FDA, and new QuEChERSER mega-method) in beef, catfish, and eggs. ITSP was also compared with traditional d-SPE (FDA) and ultracentrifuge (QuEChERSER) cleanup.

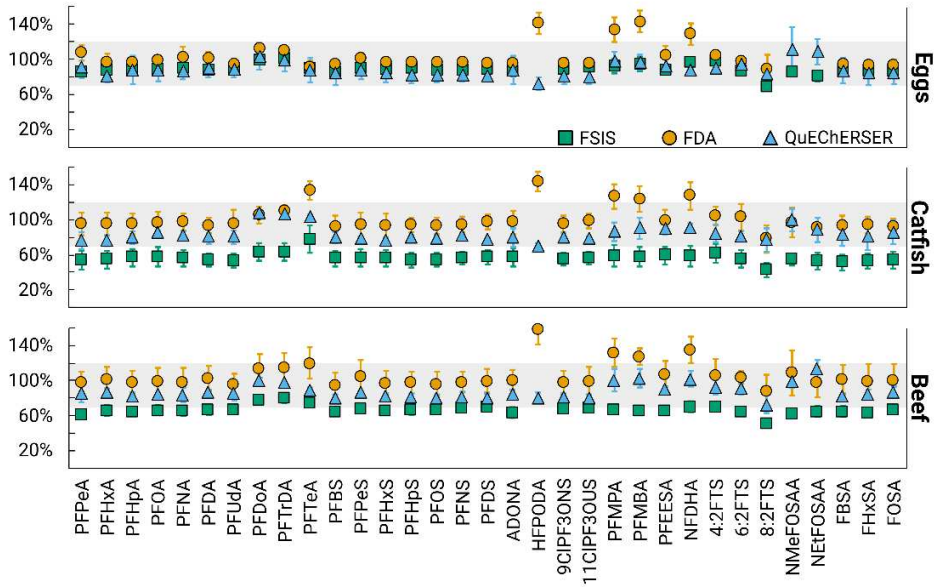


Figure 3. Spike recoveries of 33 PFAS analytes are shown for beef, catfish, and eggs using three extraction techniques. Data points and error bars represent the average and relative standard deviation (n=3). An acceptable range of 70-120% is highlighted in each panel.

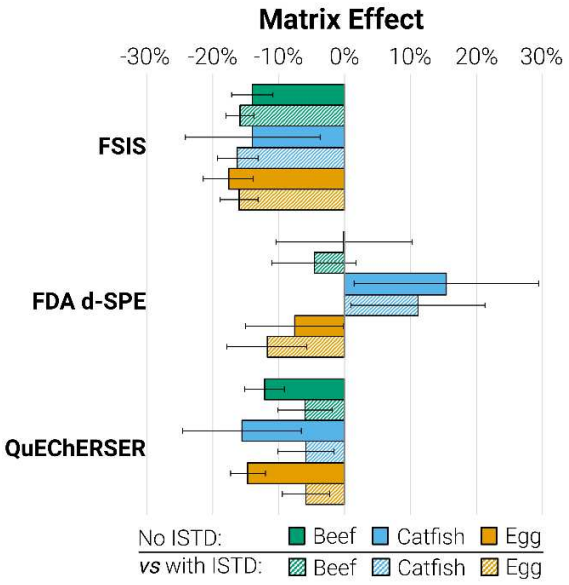


Figure 4. Average matrix effect (for all PFAS target analytes) shows how well and uniformly (small error bars; small standard deviation) each method performs.

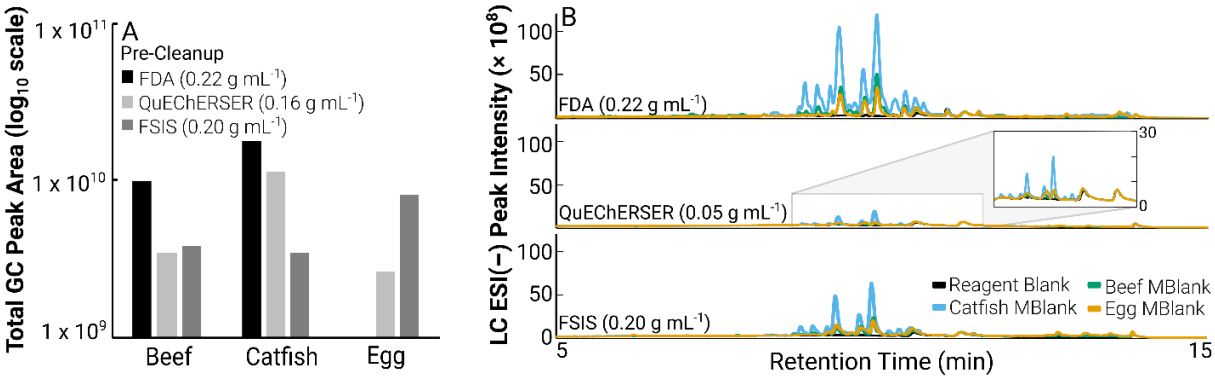
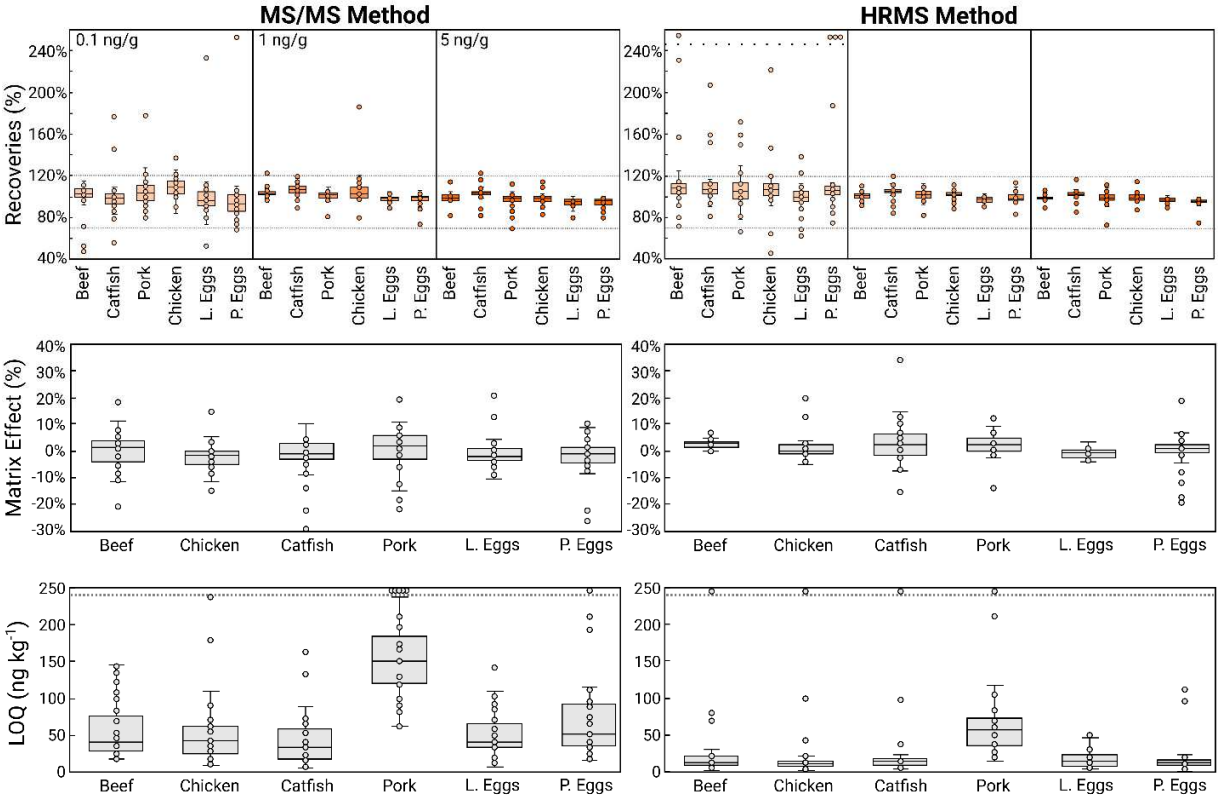


Figure 5. Cleanup efficiency for the three extraction methods. Total peak areas of pre-cleanup (initial extracts) are shown for total ion chromatograms (TICs) by GC-MS as an indicator of lipids extracted (A). TICs from LC-MS analysis of final matrix blank extracts (post-cleanup) show a decreased amount of lipids in QuEChERSER extracts compared to FDA and FSIS (B). Sample equivalents (g mL^{-1}) are shown in parenthesis for each sample preparation method.



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483

484 **Figure 6.** Boxplots comparing validation results for QuEChERSER with targeted MS/MS analysis (left
485 panels) and full-scan high-resolution analysis (right panels). Recoveries at three spiking levels, matrix
486 effects, and LOQs are presented for six matrices. Lines are representative of medians and all data points
487 are shown as circles. Data points above the dashed line are greater than the y-axis maximum.

488

Table 1. Accuracy of Results from SRM 1946 and 1947 Analysis with QuEChERSER

Analyte	SRM 1946	HRMS		QqQ	
	reference value (ng g^{-1} ± SD)	measured value (ng g^{-1} ± SD)	accuracy (% ± RSD)	measured value (ng g^{-1} ± SD)	accuracy (% ± RSD)
PFNA		0.17 ± 0.01		0.19 ± 0.02	
PFDA		0.14 ± 0.01		0.15 ± 0.01	
PFUdA		0.33 ± 0.01		0.35 ± 0.04	
PFTrDA		0.31 ± 0.02		0.32 ± 0.03	
PFOS	2.19 ± 0.08	2.15 ± 0.13	98 ± 6%	2.55 ± 0.22	116 ± 9%
Analyte	SRM 1947	HRMS		QqQ	
	reference value (ng g^{-1} ± SD)	measured value (ng g^{-1} ± SD)	accuracy (% ± RSD)	measured value (ng g^{-1} ± SD)	accuracy (% ± RSD)
PFNA	0.2	0.19 ± 0.01	93 ± 4%	0.19 ± 0.01	97 ± 8%
PFDA	0.26	0.18 ± 0.01	70 ± 7%	0.19 ± 0.01	72 ± 6%
PFUdA	0.28	0.284 ± 0.001	101 ± 1%	0.24 ± 0.02	86 ± 8%
PFDoA		0.11 ± 0.02		0.13 ± 0.01	
PFTrDA	0.2	0.15 ± 0.01	73 ± 9%	0.18 ± 0.02	91 ± 10%
PFOS	5.9 ± 0.39	6.06 ± 0.17	103 ± 3%	6.89 ± 0.13	117 ± 2%
FBSA		0.19 ± 0.01		0.21 ± 0.06	
FOSA		0.21 ± 0.01		0.24 ± 0.01	

Table 2. Measured concentrations (ng g^{-1}) in incurred catfish samples with QuEChERSER and comparison of QqQ and HRMS data with values previously measured by USDA FSIS using their method.

Sample	Method	Instrument	PFDA	PFUdA	PFDoA	PFTrDA	PFTeA	PFOS	PFDS	FOSA
Catfish 1	FSIS	QqQ						2.11		
	ARS	QqQ	0.28	0.28	0.33	0.13	0.12	2.02	0.13	0.17
		HRMS	0.27	0.26	0.38	<LOQ	<LOQ	1.69	0.14	0.13
Catfish 2	FSIS	QqQ						2.27		
	ARS	QqQ	0.16	0.10				2.91		
		HRMS	0.13	<LOQ				2.61		
Catfish 3	FSIS	QqQ						3.83		
	ARS	QqQ	0.40	0.61	0.21	0.31	0.20	4.98	0.20	
		HRMS	0.37	0.53	0.16	0.32		4.25	0.24	
Catfish 4	FSIS	QqQ						3.76		
	ARS	QqQ	0.42	0.22				6.67		
		HRMS	0.4	0.21				5.71		
Catfish 5	FSIS	QqQ						0.84		
	ARS	QqQ	0.22	0.50	0.31	0.10		1.39		
		HRMS	0.21	0.46	0.28	<LOQ		1.23		
Catfish 6	FSIS	QqQ		0.56						
	ARS	QqQ	0.10	0.68				0.22		
		HRMS	<LOQ	0.66				0.13		

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