Simultaneous Determination of 20 Polyfluoroalkane Substances in Dietary Milk by QuEChERS Combined with On-Line Interference Trapping LC-MS/MS Technique

Application Note

Food

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Abstract

This application note describes a sensitive and reliable method for determination of 20 polyfluoroalkane substances (PFASs) in milk based on the work published by Yu, et al. previously [1]. The milk sample was initially extracted and cleaned up using an optimized QuEChERS protocol, followed by an online interference trapping LC-MS/MS analysis. Using stable isotope labeled internal standards for calibration, the linear dynamic ranges for 20 PFASs were determined within 3–4 orders of magnitude, with correlation coefficients ≥0.997. The LOQs for the two types of PFASs, perfluorinated carboxylic acids (PFCAs) and perfluorinated sulfonic acids (PFSAs), were 0.010 µg/L and 0.050 µg/L, respectively. The average recoveries at three spiking levels for all compounds ranged from 72.8% to 111% with the RSD (n = 6) within 1.20–14.9%. The developed method is sensitive and reliable, thus can be applied to real sample survey.

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Introduction

Polyfluoroalkane substances (PFASs) are one class of emerging persistent organic contaminants. They are highly stable and transportable over long distance, and are extremely difficult to biodegrade over time in the environment [1]. PFASs have been reported worldwide from the natural environment, food, and human serum. The ubiquitous presence of PFASs in the natural environment has raised increasing concern for their long-term impacts to human health and wide life. Studies using animal models have demonstrated that PFOS and PFOA, the two major PFASs, can induce a range of toxicological responses [2-3]. The daily tolerance levels for humans were issued by EFSA in 2012 with the maximum daily intake of PFOA and PFOS at 1,500 and 150 ng/kg bw/d, respectively [4]. Hence, reliable methodologies are required for routine monitoring the levels of PFASs in variety of food products.

Dietary exposure is the primary route for PFASs enrichment in vivo. Milk, one of the main diets, is often detected with elevated levels of PFASs from many regions of the world, which is consistent with the persistence and bio-accumulative features of PFASs. In the past decades, a number of studies were reported focusing on development of methods for determination of PFASs in milk [5-8]. However, due to the interference from the matrix, from the extraction solvent and apparatus, and from the HPLC system, these methods often face the challenge of reproducibility and reliability. To further improve the PFASs measurement accuracy and reliability in milk, the QuEChERS approach combined with an on-line trapping LC-MS/MS method and the least-biased quantitation using stable isotope labeled internal standards was recently developed [1] and was described in this application note, and it was demonstrated that the method can potentially be used in screening of the common PFASs in real milk products.

Materials and Methods

Reagents and materials

The 20 PFASs and 9 isotopically labeled internal standards were purchased from Wellington Laboratories (Guelph, Canada), with purity greater than 98%. Methanol and acetonitrile were of HPLC grade and obtained from Merck (Darmstadt, Germany); ammonium acetate (HPLC grade) was from TEDIA (Fairfield, OH); sorbents such as primary and secondary amine (PSA), C18, and graphitized carbon black and other conventional analytical reagents were obtained from local vendors; water was purified through Milli-Q system with a resistivity of 18.2 MΩ·cm.

Instrumental conditions

LC configuration

- Agilent Infinity UHPLC 1290 binary pump (G4220A)
- High performance AutoSampler (G4226A)
- AutoSampler ThermoStat (G1330B)
- Thermostatted Column Compartment SL (G1316B)

LC conditions

Trapping column	Agilent ZORBAX Eclipse Plus C18, 4.6 × 50 mm, 5 µm
Analytical Column	Agilent Poroshell 120 Eclipse Plus C18, 2.1 × 100 mm, 2.7 µm
Column temperature	30 °C
Injection volume	5 µL
Needle wash	Flushport (100% methanol), 5 seconds
Mobile phase	A) water containing 5 mM ammonium acetate B) methanol containing 5 mM ammonium acetate
Gradient flow rate	0.2 mL/min

Gradient elution profile was shown in Table 1

Table 1. LC Gradient Elution Profile

Time	Sol. A (%)	Sol. B (%)
0	90	10
3	30	70
13	0	100
14	90	10
20	90	10

MS configuration

Agilent Triple Quadrupole 6460 mass spectrometer with Jet Stream ionization source

MS conditions

Ionization mode	Negative ionization
Scanning mode	Multiple reaction monitoring (MRM)
Capillary voltage	3,500 V
Nozzle voltage	500V
Nebulizer pressure	45 psi
Dry gas temperature	300 °C
Dry gas flow rate	6 L/min
Curtain gas	45 psi
Sheath gas temperature	260 °C
Sheath gas flow rate	11 L/min

Sample collection

The cow milk samples of major brands were collected nationwide in 50 mL low-density polyethylene (LDPE) bottles directly and shipped frozen into the laboratory, or left liquid and transported on ice into the laboratory followed by transferring into 50 mL LDPE bottles upon receipt. The samples were then frozen at –20 °C until analyzed.

Sample extraction and cleanup

QuEChERS protocol was optimized for preparation of PFASs from milk matrix. Briefly, milk sample (5.0 mL), the internal standard compounds (1.0 ng each), and 5.0 mL of pure water were transferred into a 50-mL LDPE centrifuge tube. The mixture was then homogenized for one minute, combined with 10.0 mL of acetonitrile and 30 µL of concentrated hydrochloric acid, and vortexed for one minute sequentially. The resulting mixture was further combined with 2 g of sodium chloride, followed by one minute of vortexing and 10 minutes of centrifugation at 5,000 rpm. The obtained acetonitrile layer was transferred into a clean glass testing tube and subjected to nitrogen drying with a final volume of approximately 4 mL.

The above milk extract was transferred to a 15-mL LDPE centrifuge tube pre-filled with 60 mg of PSA, 40 mg of C18, and 10 mg of graphitized carbon black. The tube was then vortexed for 5 min and followed by centrifugation at 5,000 rpm for 10 minutes. The resulting supernatant solution was transferred to a clean 15-mL LDPE centrifuge tube and dried under nitrogen at 45 °C. The obtained residue was dissolved in 1 mL methanol. The solution was filtered through 0.22-µm Whatman nylon membrane, and then transferred to 2-mL glass vial for LC-MS/MS analysis.

Quality assurance and quantitation

There are many factors contributing to quantitation bias of PFASs. To minimize such bias, possible sources of contamination have to be considered. The fluoropolymer materials such as poly-(tetrafluoroethylene) (PTFE) tubing in the HPLC system was replaced with the stainless steel tubing, and the teflon centrifuge tube generally used during sample preparation was changed to LDPE tube. The instrument drift was consistently monitored using quality control standards. When the quantity for the quality control standard was beyond the range of ±20% of its theoretical value, a new set of calibration curve was then established for the quantitation of the subsequent samples. The limit of detection (LOD) and limit of quantitation (LOQ) for the method were estimated at the lowest spiking concentration at which an S/N could reach 3 and 10, respectively. Solvent blank, solvent standards, and matrix-spiked standards were all monitored during each set of samples. When necessary, background subtraction was applied to further avoid the quantitation bias.

Results and Discussion

Optimization of HPLC-MS/MS conditions

To monitor the PFASs with sufficient selectivity and sensitivity, standard compounds of PFASs were initially infused into the MS spectrometer to optimize the acquisition parameters. MS full scan was first acquired to obtain the fragment voltage for precursor ions at which their highest intensity can be observed. Product ion scanning was further applied to optimize the collision energy for specific fragment ions at which high intensity can be reached. Most fragments from perfluorinated carboxylic acids (PFCAs) are species releasing the carboxyl group ($[M-H-44]^-$) and those from perfluorinated sulfonic acids (PFSAs) are species releasing the sulfonate group ([M-H-80]) and the fluorinated sulfonate group ($[M-H-99]^-$). These fragments with high intensity and selectivity were then selected for MRM detection of each compound, which are listed in Table 2.

Table 2. MRM Parameters for MS/MS Detection of 20 PFASs and Their Corresponding Internal Standards

No.	Compound	Precursor ion	Product ion	Fragment (V)	CE (ev)	Internal standard
1	PFBA	213	169*	70	1	MPFBA
2	PFPA	263	219*	70	1	MPFBA
3	PFHxA	313	269*/119	70	1/9	MPFHxA
4	PFHpA	363	319*/169	90	1/8	MPFHxA
5	PFOA	413	369*/169	90	2/9	MPFOA
6	PFNA	463	419*/219	110	3/9	MPFNA
7	PFDA	513	469*/269	120	2/10	MPFDA
8	PFUnDA	563	519*/319	120	2/11	MPFUnDA
9	PFDoDA	613	569*/169	140	3/15	MPFDoDA
10	PFTrDA	663	619*/169	140	3/14	MPFDoDA
11	PFTeDA	713	669*/269	140	4/18	MPFDoDA
12	PFHxDA	813	769*/169	150	4/19	MPFDoDA
13	PFOcDA	913	869*/219	150	6/23	MPFDoDA
14	PFBS	299	99*/80	190	31/32	MPFHxS
15	PFPS	349	99*/80	200	34/40	MPFHxS
16	L-PFHxS	399	99*/80	230	33/38	MPFHxS
17	L-PFHpS	449	99*/80	250	36/42	MPFHxS
18	L-PFOS	499	99*/80	260	55/59	MPFOS
19	L-PFNS	549	99*/80	280	49/65	MPFOS
20	L-PFDS	599	99*/80	300	50/65	MPFOS
21	MPFBA	217	172*	70	1	—
22	MPFHxA	315	270*	70	2	—
23	MPFOA	417	372*	70	2	—
24	MPFNA	468	423*	100	1	—
25	MPFDA	515	270*	110	10	—
26	MPFUnDA	565	520*	110	3	—
27	MPFDoDA	615	570*	120	2	—
28	MPFHxS	403	103*	220	33	—
29	MPFOS	503	99*	270	54	—

* Quantitative MRM

L- before a compound name (16-20) indicates a levorotary isomer

M before a compound name (21-29) indicates monoisotope-labeled compounds used as internal standards

Based on the weak polarity of PFASs, the C18 column was selected and a gradient elution profile (Table 1) was established for separation. It should be noted that interference from LC system can decrease the accuracy of PFASs measurement, particularly when the levels of the analytes are low. Even though PTFE tubing has been avoided in the LC system, the PFASs interference can also result from the mobile phases which may elevate the background level and lower the detection sensitivity (Figure 1A). To further remove the interference from the LC system, a short C18 trapping column was inserted between solvent mixer and the autosampler to trap the interference. With such setting, the effect of interference from the LC system on the detection accuracy can be efficiently minimized as the interference was delayed to elute out of column due to the large void volume of the trapping column (Figure 1B). Using such a trapping/analytical column combination, the typical MRM chromatograms for 20 PFASs can be achieved as shown in Figure 2.

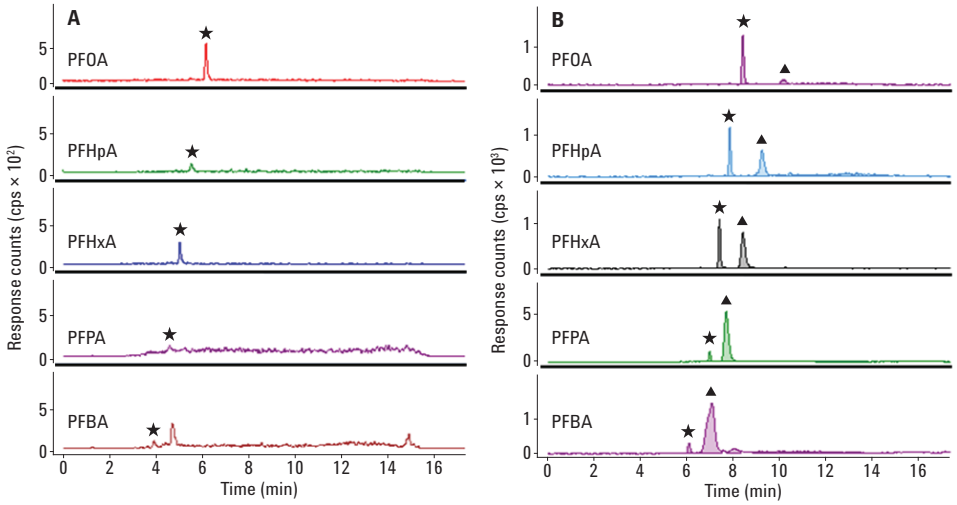

Figure 1. Pre-injection trapping allows separation of target compounds from the background interference. A) Without trapping column; B) with trapping column. A star indicates the target compound, and a ▲ is an interference compound. Note: the retention time for each compound in B is delayed 2.3 minutes due to the increase of void volume introduced by the trapping column which delayed the gradient profile.

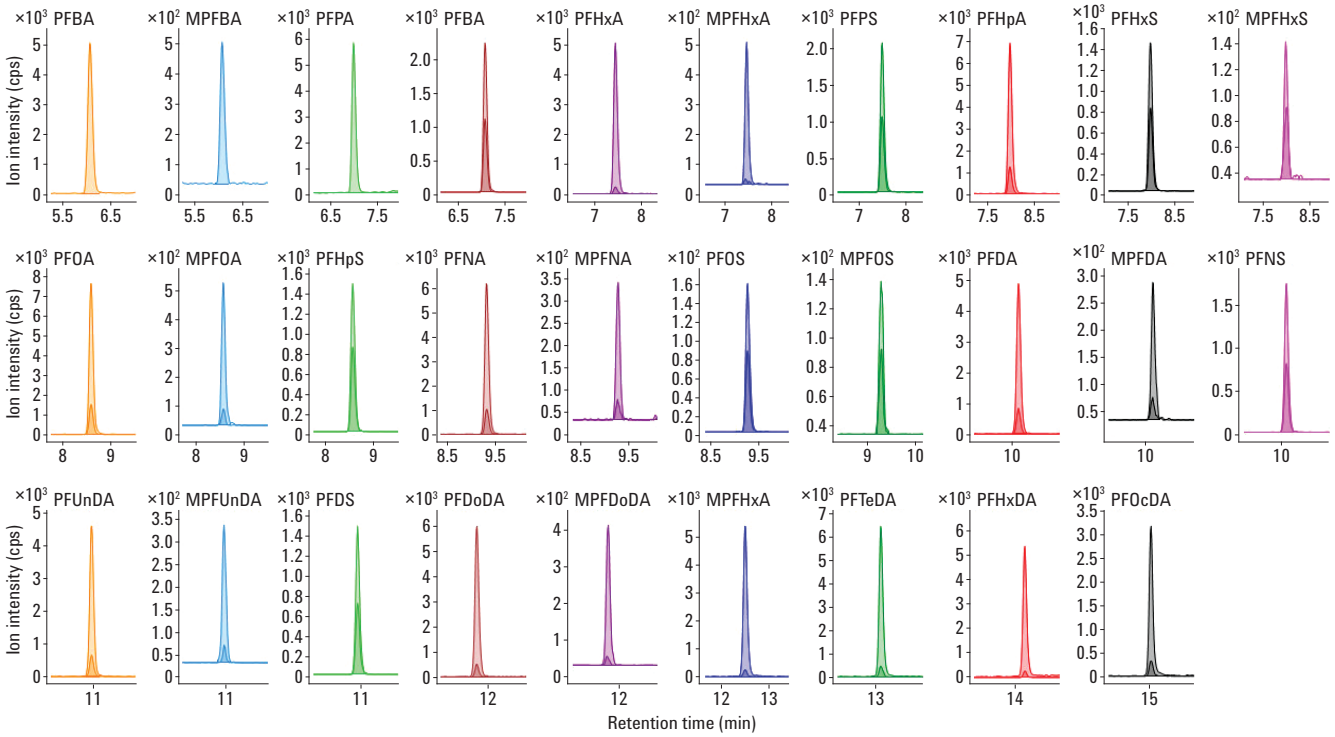

Figure 2. The typical MRM chromatograms for 20 PFASs and the nine internal standards under the gradient elution. The concentration of each analyte was 10.0 µg/L, and the internal standard concentration was 1.0 µg/L. Compounds starting with 'M' are mono-isotopic-labeled internal standards.

Optimization of the sample extraction and cleanup procedure

QuEChERS procedure has been widely used in the analysis of pesticide residues and other residues in various food matrices. It involves two major steps: sample extraction and cleanup. Acetonitrile is the common solvent for extraction. Here we compared pure acetonitrile, and acidified acetonitrile using formic acid and hydrochloric acid (HCl). As shown in Figure 3, non-acidified solvent shows the lowest recovery for most PFASs, with 15 out of 20 compounds falling in the 70–120% of recovery threshold; using formic acid/acetonitrile, 16 compounds display recovery within 70–120%; while using HCl/acetonitrile, 19 out of 20 compounds have recoveries in the range of 70–120%. Hence, HCl is the better acidified reagent. The amount of HCl was further optimized, and 30 µL of HCl per 10 mL acetonitrile (0.3% HCl) was eventually selected as the extraction solvent.

The conventional sorbents such as PSA, C18 and graphitized carbon black are commonly used during QuEChERS cleanup step to remove organic/fatty acids, lipids, and pigments, respectively. To achieve better recovery, the amount of these sorbents was optimized. As shown in Figure 4A, the highest recoveries were found for PFASs at 60 mg PSA, and they could reach highest when 40 mg C18 was used (Figure 4B). Graphitized carbon black did not show clear effects on the recoveries of PFASs, which might be due to the low levels of pigment in milk. Eventually, a sorbent mixture containing 60 mg PSA, 40 mg C18, and 10 mg graphitized carbon black was finally selected for cleanup.

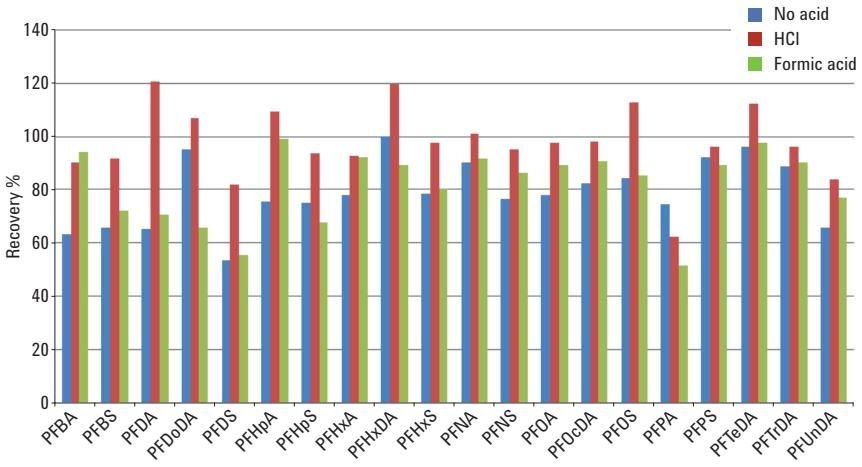

Figure 3. Comparison of the extraction efficiency using three different solvents.

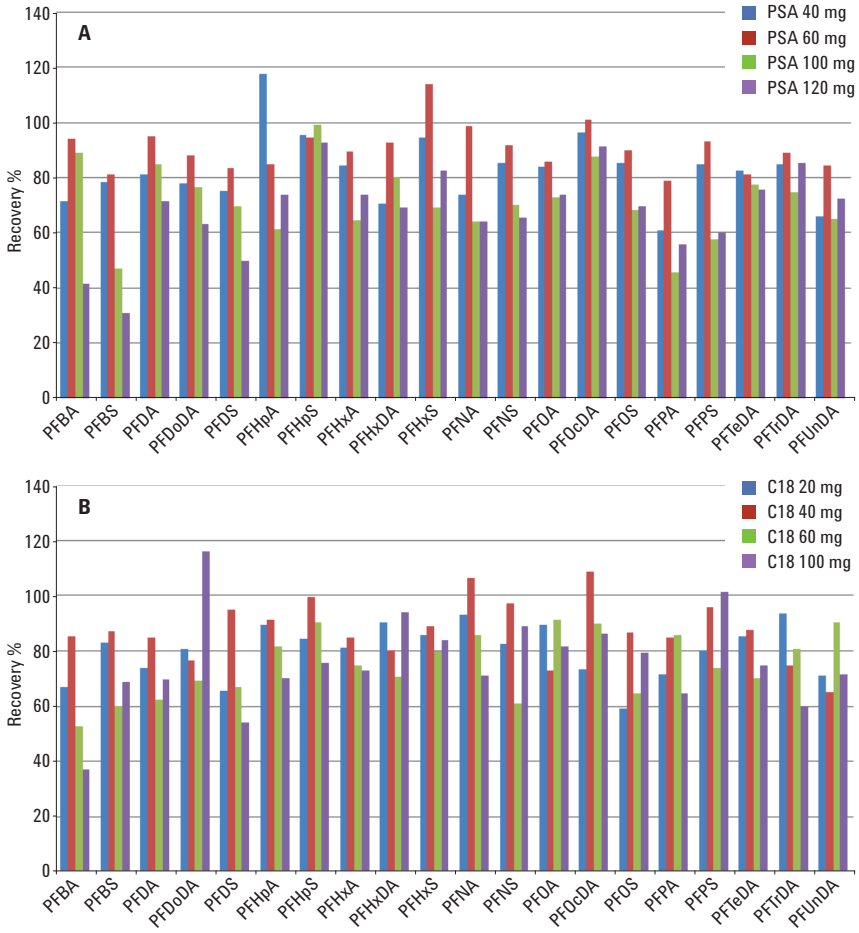

Figure 4. Cleanup efficiency using various amounts of PSA (A) and C18 (B).

Calibration linearity and detection sensitivity

To efficiently reduce the matrix effect and improve measurement accuracy, stable isotope-labeled internal standard was applied for quantitation. For those compounds without isotopic analogs, they were assigned an isotope labeled internal standard with similar chemical structure. The internal standard for each compound is shown in Table 2. The calibration solutions were then prepared containing various amount of analytes and fixed amount of internal standards. A calibration curve was further established by plotting the peak area ratios of analytes to the corresponding internal standards versus the amount of added analytes. As shown in Table 3, all PFASs showed very good linearity in the range of 0.010–20.0 µg/L, with the correlation coefficients (γ^2) all higher than 0.997.

To obtain the LOD and LOQ of the method, seven concentrations of each PFAS (0.0010, 0.0030, 0.0050, 0.010, 0.030, 0.050, and 0.10 µg/L) were spiked into the blank milk matrix and subjected to analysis. The levels which generated chromatographic peaks with an S/N of 3 and 10 for the quantifier ions were set as LOD and LOQ of the corresponding compound, respectively. As shown in Table 3, the LODs and LOQs for PFCAs were 0.0030 µg/L and 0.010 µg/L, respectively, and those for PFSAs were 0.010 µg/L and 0.050 µg/L, respectively.

Table 3. Dynamic Linear Ranges and Limits of Detection and Quantitation

Compounds	Linear range (µg/L)	Linear equation	γ^2	LOD/(µg/L)	LOQ/(µg/L)
PFBA	0.010–2.0×10	y = 1.0832x+0.0840	0.9992	0.0030	0.010
PFPA	0.010–2.0×10	y = 1.1292x+0.1905	0.9995	0.0030	0.010
PFHxA	0.010–2.0×10	y = 1.3487x+0.1783	0.9996	0.0030	0.010
PFHpA	0.010–2.0×10	y = 1.4805x+0.1522	0.9991	0.0030	0.010
PFOA	0.010–2.0×10	y = 1.7161x+0.0583	0.9997	0.0030	0.010
PFNA	0.010—2.0×10	y = 1.7989x+0.1209	0.9989	0.0030	0.010
PFDA	0.010–2.0×10	y = 9.8660x+1.8285	0.9994	0.0030	0.010
PFUnDA	0.010–2.0×10	y = 1.6208x+0.0636	0.9999	0.0030	0.010
PFDoDA	0.010–2.0×10	y = 1.5565x+0.0906	0.9989	0.0030	0.010
PFTrDA	0.010–2.0×10	y = 1.5234x+0.0611	0.9996	0.0030	0.010
PFTeDA	0.010–2.0×10	y = 2.2169x-0.0877	0.9984	0.0030	0.010
PFHxDA	0.010–2.0×10	y = 1.3530x+0.0682	0.9991	0.0030	0.010
PFOcDA	0.010–2.0×10	y = 0.7745x+0.0331	0.9983	0.0030	0.010
PFBS	0.050–2.0×10	y = 1.3518x-0.0647	0.9975	0.0030	0.010
PFPS	0.050–2.0×10	y = 1.3764x+0.0438	0.9994	0.010	0.050
L-PFHxS	0.050–2.0×10	y = 1.0978x+0.2054	0.9980	0.010	0.050
L-PFHpS	0.050–2.0×10	y = 1.1167x-0.0016	0.9999	0.010	0.050
L-PFOS	0.050–2.0×10	y = 1.5211x+0.0652	0.9996	0.010	0.050
L-PFNS	0.050–2.0×10	y = 1.2969x+1.3288	0.9994	0.010	0.050
L-PFDS	0.050–2.0×10	y = 1.2589x+0.0561	0.9997	0.010	0.050

Accuracy and precision

To test the accuracy and precision of the method, spiking test was conducted. The spiking levels for PFSAs were set at 0.050 (LOQ level), 0.50, and 2.0 µg/L, and those for PFCAs were set at 0.010 (LOQ level), 0.50, and 1.0 µg/L. Six replicates were performed at each spiking level with blank matrix as control. As shown in Figure 5, the recoveries for all PFASs ranged from 72.8–111%, with RSD (n = 6) within 1.20–14.8% (n = 6). The results indicate that the method is highly accurate and repeatable.

Real sample analysis

Here, 46 milk samples randomly collected from various manufacturers across China were analyzed by the developed method. Totally, 16 compounds were detected positively in these samples. Among them, PFPA, PFOA, and PFOS were found in approximately 50% of the total tested samples with levels ranging from LOD to 0.12 µg/L. PFBA, PFNA, PFHxDA, and PFTrDA were found more frequently, occurred in 41 out of 46 (89%) milk samples, with levels ranging from LOD to 0.37 µg/L. The other nine PFASs including PFHxA, PFHpA, PFDA, PFTeDA, PFOcDA, PFPS, PFHxS, PFHpS, and PFDoDA were occasionally found in some samples, and the levels were relatively low, ranging from LOD to 0.092 µg/L, except for PFOcDA which was detected at a level as high as 0.33 µg/L in one sample. In summary, total amount of PFASs in the 46 milk samples ranged from 0.19-0.66 µg/L. It should be noted that the levels for two PFASs with longer carbon chains, PFTrDA and PFHxDA, are particularly high in two sampling sites, ranging from 0.048 to 0.11 µg/L and from <0.010 to 0.23 µg/L, respectively. Such high levels have not been reported previously. It may be related to the local industrial contamination and requires further investigation.

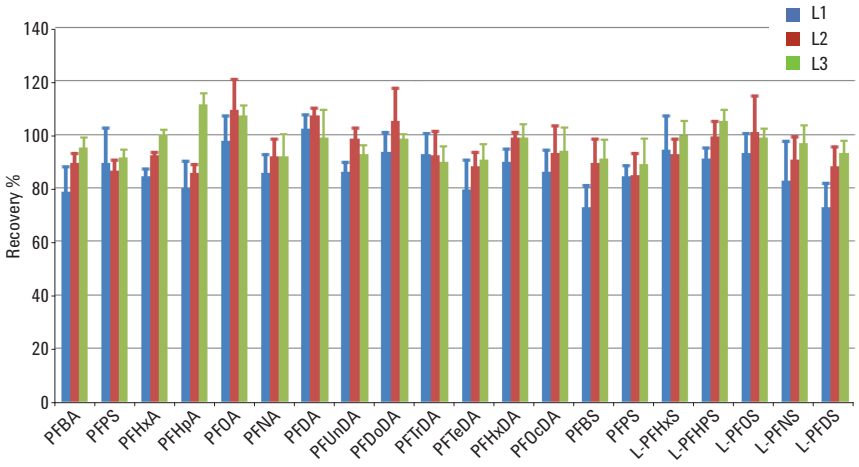

Figure 5. Recovery and precision for the 20 PFASs at the specified spiking levels. Note: n = 6 for each level. L1, L2, and L3 for PFCAs are 0.010, 0.50, 1.0 µg/L, respectively; those for PFSAs are 0.050, 0.50, and 2.0 µg/L, respectively. The upper error bar is the standard deviation of six replicative measurements.

Conclusion

A QuEChERS protocol combined with an online interference trapping LC-MS/MS method was developed and described in this application note. The method has the advantages of low background, high sensitivity, high accuracy and precision, as well as wide dynamic linear range, and thus can be applied for real milk sample analysis.

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