DEVELOPMENT AND VALIDATION OF A METHOD FOR THE ANALYSIS OF THIRTEEN ORGANOPHOSPHORUS FLAME RETARDANTS IN MILK USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED WITH TRIPLE QUADRUPOLE TANDEM MASS SPECTROMETRY

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Introduction

Organophosphorus flame retardants (OPFRs) are a class of widely used plasticizers and flame retardants and they have been defined as high production volume chemical (HPVC).[1] As additive flame retardants, OPFRs can be easily released into the surrounding environments and they have been found to be ubiquitous in various environments.[2] Previous studies have indicated that exposure to OPFRs may cause various adverse effects on animals and humans.[3] Therefore, the increasing production and widespread existence of OPFRs have caused concerns regarding the potential toxicity and risks to human health.

The objective of the present study was to develop a simple and robust analytical methodology for the measurement of 13 currently used OPFRs in human/cow milk. Modified quick, easy, cheap, effective, rugged, and safe (QuEChERS) and solid-phase extraction (SPE) techniques were used for sample preparation, stable isotope dilution coupled with HPLC-MS/MS were used for quantification and qualification. Multiple parameters affecting the pretreatment were optimized to obtain a good compromise between analyte recoveries and extract clean-up. A series of parameters were validated, such as linearity, limits of detection (LODs), limits of quantification (LOQs), recovery and precision. Finally, the validated method was applied to analyze OPFRs in cow milk samples collected in Shouguang (a well-known FR producing region in China) and human milk samples collected in Beijing.

Materials and methods

Chemicals and materials

HPLC-grade methanol (MeOH), acetonitrile (ACN) and formic acid (FA) were provided by Fisher Scientific (Massachusetts, America). Two QuEChERS absorbents, primary secondary amine (PSA) and octadecyl-modified silica (C_{18}), were provided by Agilent Technologies (Palo Alto, CA, USA). ProElut PLS extraction cartridges (150 mg/6 mL, 30 μm) was provided by Dikma (Beijing, China).

Standard solutions of 13 OPFRs, including tri-*n*-butyl phosphate (TnBP), tris(1,3-dichloro-2-propyl) phosphate (TDCIPP), tris(2-chloroethyl) phosphate (TCEP), tri-*n*-propyl phosphate (TPrP), 2-ethylhexyl diphenyl phosphate (EHDPP), tris(2-ethylhexyl) phosphate (TEHP), bis(2,3-dibromopropyl) phosphate (DDBPP), tri(3-chloropropyl) phosphate (TCIPP), triphenyl phosphate (TPhP), trimethyl phosphate (TMP), tetrakis(2-chloroethyl)dichloroisopentyl diphosphate (V6), tris(2,3-dibromopropyl) phosphate (TDBPP) and tris(2-butoxyethyl) phosphate (TBOEP) (>98% purity), were all purchased from AccuStandard Inc. (New Haven, CT, U.S.A.). Six deuterated or ^{13}C-labeled internal standards, including d_{12}-TCEP, d_{21}-TPrP, $^{13}C_6$-TBOEP, d_{15}-TDCIPP, $^{13}C_{18}$-TPhP and d_{27}-TnBP, were obtained from Wellington Laboratories Inc. (Guelph, ON, Canada).

Samples

Human breast milk samples (n=9) were obtained in 2018 from 9 healthy volunteers living in Beijing (China). Mothers were not documented occupational exposure to OPFRs and their ages ranged between 30 and 39 years.

Cow milk samples were purchased at local markets in Shouguang town (Shandong Province) in 2016, Shouguang is a well-known organic FR production area in China.

Sample preparation

After freeze-drying, a 0.5 g±0.01 g dried milk (human breast milk or cow milk) and 5 ng each of the internal standards were added to a 15-mL polypropylene (PP) centrifuge tube and mixed with 5 mL of 0.5% formic acid in ACN. After vortexing for 1 min, the tube was then shaken by an orbital shaker for 2 h, and then ultrasonicated for 10 min. After centrifugating at 5000 rpm for 10 min, the supernatant was transferred into another precleaned PP tube. Then, d-SPE sorbents, comprising 150 mg of PSA, 150 mg of C_{18} and 250 mg of $MgSO_4$, were added. The tube was vortexed for 1 min and then centrifuged at 5000 rpm for 5 min. The supernatant was collected and then purified by passage through a ProElut PLS extraction cartridge (150 mg/6 mL). The cartridges were preconditioned with 5 mL of ACN, after the loading of the sample extract (Fraction 1), the cartridge was eluted with 3.5 mL of ACN (Fraction 2). The purified extracts (both Fractions 1 and 2) were mixed and evaporated to dryness under N2. The residues were dissolved with 200 μL of methanol and filtered through a 0.22-μm filter membrane for HPLC-MS/MS analysis.

HPLC-MS/MS Analysis

A Waters Acquity ultra high performance liquid chromatographic system coupled with an Xevo TQ-S triple quadrupole mass detector (Waters, MA, USA) was used for instrumental analysis. The analytes were separated in an Acquity UPLC BEH C_{18} column (100×2.1 mm i.d., 1.7 μm) connected to a Waters Van Guard precolumn (5×2.1 mm i.d., 1.7 μm), and the column temperature was maintained at 40°C. The inject ion volume was 5 μL. Pure water (comprising 0.01% formic acid) (A) and methanol (comprising 10 mmol/L ammonium acetate) (B) were used as mobile phase at a constant flow rate of 0.2 mL/ min. The gradient elution started with 30% B, changed linearly to 80% B in 4 min, maintained for 4 min, increased to 100% B in 0.1 min, held for 3.5 min, and finally returned to the initial conditions (30% B), held for 3 min for column back-conditioning.

The MS detector was equipped with an electrospray ionization (ESI) probe and operated in positive mode. Sample quantification was performed in multiple reaction monitoring (MRM) mode, and the transitions selected for monitoring and quantitation. Because corresponding deuterated or ^{13}C-labeled ISs for some analytes are unavailable at present, they shared IS with other analytes.

Results and discussion

Optimization of sample pretreatment

Parameters that influence the sample pretreatment, including extraction solvents, the amount of clean-up sorbents and the volume of eluting solvent were investigated and optimized. 0.5±0.01 g of freeze-dried cow milk spiked with 5 ng of each analyte and internal standard was applied for the tests, and all the tests were carried out in triplicate.

ACN is the most frequently used extraction solvent in original QuEChERS technique, because it can effectively extract analytes with a wide range of polarities, and the co-extract has less fat content, which can effectively reduce the pressure of food sample purification and impurity removal.[4] However, ACN is less effective in extracting high fat-solubility compounds, and its extraction ability is usually enhanced by adding additives.[4] Whether the target analytes in the matrix can be completely extracted is a key factor affecting the entire method, and considering that OPFRs will be hydrolyzed under strong acid/alkaline condition, the extraction efficiencies of ACN, 0.1% FA-ACN and 0.5%FA-ACN were compared. As shown in Figure 1a, the recoveries of OPFRs increases rapidly as the percentage of FA increased from 0 to 0.1%. Subsequently, along with the percentage of FA increased to 0.5%, the recoveries of TMP, TCEP, V6 and TDBPP slightly enhanced, and the recoveries of the rest OPFRs showed no significant change, furthermore, the recoveries of most OPFRs have been approached 100%, which indicated that the target analyte has been almost completely extracted from the milk sample. Finally, 0.5%FA-ACN was employed in subsequent tests.

Next, two commonly used sorbents, C_{18} and PSA, were used to remove coextracted impurities from the extraction. C_{18} is essential for improving clean-up efficiency in high lipid content matrices, and PSA is normally used to enhance the purification of polar impurities such as sugars and fatty acids. In our study, 150 mg of C_{18} was used and the combination of PSA (100, 150 and 200 mg) were optimized. Figure 1b indicates the change of the recoveries of OPFRs along with the variation of PSA, and the best recoveries for most analytes were obtained when a combination of 150 mg of C_{18} and 150 mg of PSA were used. Moreover, the increase of PSA only led to a slight change of the recoveries of DDBPP, TBOEP, TnBP, TEHP and EHDPP, whereas at the same time significant decreases of the recovery of TMP (from 106.3% to 69.8%) were observed. Considering the costliness of PSA, and to obtain satisfactory and stable recoveries, the combination of 150 mg of C_{18} and 150 mg of PSA was chosen for further tests.

ProElut PLS SPE cartridge is a reversed-phase adsorbent, and it has both a hydrophilic group (pyrrolidone group) and a hydrophobic group (divinylbenzene). Due to its good adsorption effect on different polarities/acid-base and neutral compounds, ProElut PLS has been extensively applied in sample preparation.[5] And this motivated the use of ProElut PLS in this research. Proper eluting procedure is a crucial step for SPE to sufficiently elute the analytes from the cartridge. In this study, ACN was found to be the best eluting solvent, and the impact of eluting volume (2.5, 3.5 and 4.5 mL of ACN) on recoveries was investigated. As illustrated in Figure 1c, the recoveries increased as the ACN increased from 2.5 to 3.5 mL, and reached the highest value at the volume of 3.5 mL except for TDCIPP and EHDPP. Larger volume of ACN would result in subsequent prolonged evaporation time, and which would dramatically increase the loss of some volatile analytes including TMP, TCEP, TCIPP and TPhP. Finally, 3.5 mL of ACN was selected as the optimum volume. In summary, in our developed pretreatment methodology, 0.5% FA-ACN was used for sample extraction, and a combination of 100 mg of C18 and 150 mg of PSA was applied for QuEChERS clean-up, and 3.5 mL of ACN was the best for ProElut PLS SPE elution.

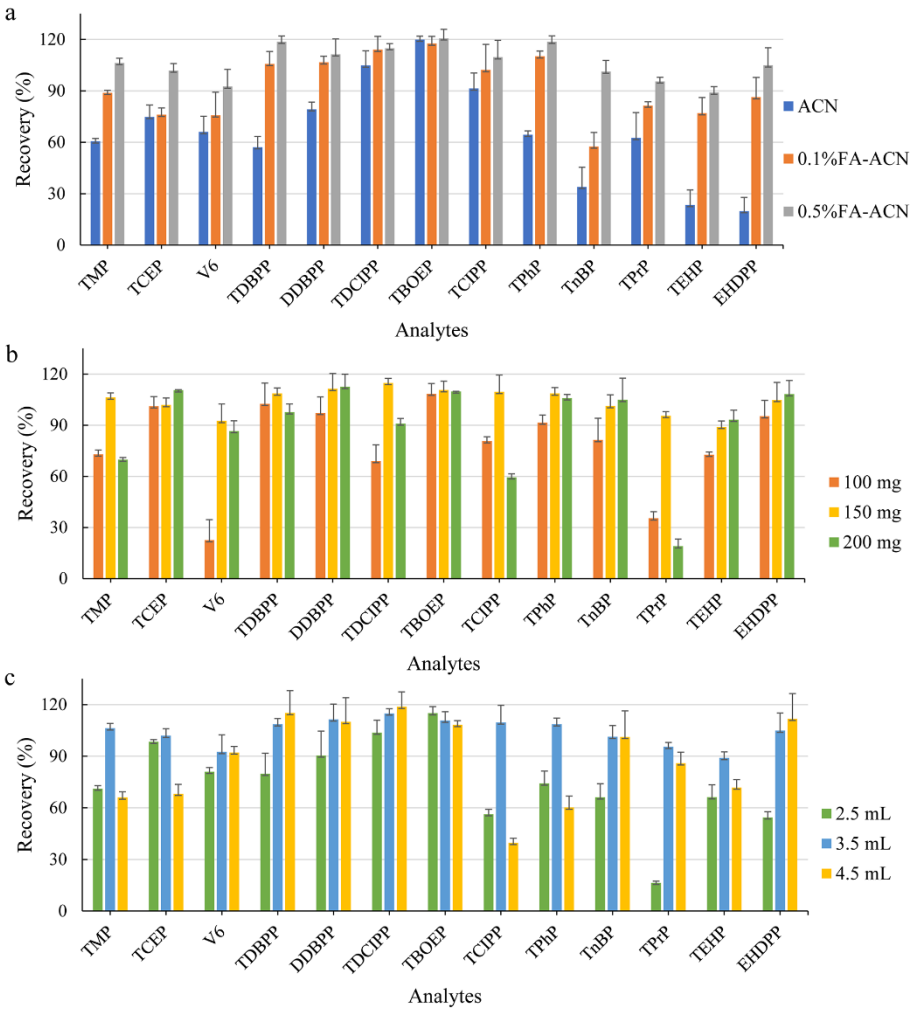

Figure 1 Effect of extraction solvents (a), the amount of PSA (b) and the volume of eluting solvent (c) on the recovery of 13 OPFRs (n=3)

Analytical performance characteristics

The calibration curves were constructed by least-squares linear regression analysis of the peak area ratios (the analytes and corresponding ISs) against standard solutions at five concentration levels ranging from 1 to 100 ng/mL with ISs at 5 ng/mL. For each concentration level, three replicates were analyzed. The results listed in Table 1 show that our method had a good linearity from 1 to 100 ng/mL with correlation coefficients (r) higher than 0.9990 (except for TCEP, r=0.9988). The LOD and LOQ were estimated by analyzing the cow milk samples at signal/noise ratios of 3 and 10, respectively. The LOD and LOQ values achieved by the proposed method were 0.001-0.3 ng/mL and 0.004-1.0 ng/mL, respectively, which showed that this method was sufficiently sensitive to monitor the 13 OPFRs.

The precision and accuracy of our methodology were estimated by analyzing the spiked cow milk samples at two levels (2.5 and 25 ng/mL), and five replicates were performed at each level. As shown in Table 1, satisfactory results were achieved for most OPFRs. The mean recoveries of the 13 OPFRs were in the range of 75.0%-115.8% (spiked at 2.5 ng/mL) and 76.7%-124.8% (spiked at 25 ng/mL, except for TCIPP), respectively, with the RSDs lower than 13.09% for all analytes (except for TMP, RSDs of 27.85% and 20.46%). At the higher spiked level, the low recovery of TCIPP (47.0%) might be the result of lacking commercially available labeled IS, and the high RSD values of TMP may also be the same reason.

Table 1 Method validation of the QuEChERS-SPE-UPLC-MS/MS method

Analyte	Linear range (ng/mL)	Regression equation	r	LOD (ng/mL)	LOQ (ng/mL)	Spiked (ng/mL)	Recovery (%)	RSD (%, n = 5)
TMP	1-100	$y = 0.4693x - 0.4111$	0.9998	0.02	0.07	2.5 25	97.7 79.0	27.85 20.46
TCEP	1-100	$y = 0.1815x + 1.7352$	0.9988	0.04	0.15	2.5 25	84.2 81.3	8.45 2.60

TCIPP	1-100	$y = 0.0967x + 0.2368$	0.9999	0.005	0.02	2.5 25	75.0 47.0	9.15 8.09
TPrP	1-100	$y = 0.0849x - 0.0435$	0.9999	0.003	0.01	2.5 25	107.9 110.7	1.43 3.36
TDBPP	1-100	$y = 0.0563x - 0.0228$	0.9998	0.09	0.3	2.5 25	115.8 116.4	5.21 2.91
DDBPP	1-100	$y = 0.1116x - 0.0307$	0.9997	0.3	1.0	2.5 25	107.7 124.8	10.49 7.04
TDCIPP	1-100	$y = 0.9014x + 9.6462$	0.9999	0.007	0.02	2.5 25	102.1 113.7	10.93 9.72
TPhP	1-100	$y = 0.6641x + 0.0692$	0.9999	0.001	0.004	2.5 25	92.8 99.5	6.65 2.18
TBOEP	1-100	$y = 0.0867x - 0.0553$	0.9999	0.004	0.01	2.5 25	106.7 107.9	2.04 2.71
TnBP	1-100	$y = 0.1077x + 0.0137$	0.9999	0.004	0.01	2.5 25	109.1 112.5	8.22 11.06
EHDPP	1-100	$y = 0.009x + 0.0029$	0.9999	0.02	0.06	2.5 25	81.9 76.7	2.31 5.10
V6	1-100	$y = 0.026x - 0.0456$	0.9996	0.2	0.6	2.5 25	112.2 120.0	13.09 10.93
TEHP	1-100	$y = 0.0346x - 0.0177$	0.9999	0.008	0.03	2.5 25	98.8 107.5	7.03 5.85

y: OPFRs peak area/internal standard peak area; *x*: mass concentration, ng/mL

Normally, after sample pretreatment, there are still a few residual impurities in the extract, which will cause matrix effects. In order to evaluate the influence of sample matrices on quantification, the matrix effect (ME) was also investigated. The cow milk sample were performed by modified QuEChERS and SPE to obtain the eluents. The matrix-matching solutions and reagent standard solutions were prepared by adding working solutions into the eluents and MeOH respectively to achieve identical spiked levels (25 ng/mL), and then these two solutions were directly determined by HPLC-MS/MS. The ME was defined as the ratio of the peak area of the analyte in the matrix-matching solutions and reagent standard solutions, expressed as % relative response. In this way, the value of ME higher than 100 suggests a signal enhancement, and lower than 100 means a signal suppression.[6] Significant signal suppression was found for several analytes, especially for TEHP (ME=24%), TCEP (ME=40%) and TDCIPP (ME=50%). However, for V6 (ME=115%), TBOEP (ME=112%) and EHDPP (ME=143%), signal enhancement was observed. For TDBPP, DDBPP and TPhP, slight matrix effects were observed, with MEs varied between 92% to 108%. Therefore, isotopically labeled ISs are essential for compensating the matrix effects.

Analysis of real samples

The proposed method was further applied for the monitoring of OPFRs in cow milk (9 cases) and human milk (9 cases) samples. During the analysis process, one procedural blank was performed together with each sample batch and then blank concentrations were subtracted from the sample concentrations.

In the 9 human milk samples, 11 OPFRs were found, and the DFs of 7 OPFRs (TCEP, TDCIPP, TCIPP, TnBP, TPrP, TEHP and EHDPP) reached 100%. Median levels of the 11 detected OPFRs in human milk ranged from <LOD to 1.47 ng/mL, and TEHP showed the highest median level, followed by TCIPP (0.73 ng/mL) and TPhP (0.49 ng/mL). For the cow milk samples, only 9 OPFRs can be detected, and only TnBP reached a 100% DF, which indicated that OPFRs are more ubiquitous in human milk. Median levels of the 9 detected OPFRs in cow milk ranged from <LOD to 0.32 ng/mL, and TCEP showed the highest median level. The application of the developed method in real samples showed that OPFRs are ubiquitous in cow/human milk.

Acknowledgements

This study was supported by the National Key R&D Program of China (2017YFC1600500), and the Shandong Natural Science Foundation of China (ZR2018LB034).

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