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Short communication

Determination of non-halogenated, chlorinated and brominated organophosphate flame retardants in herring gull eggs based on liquid chromatography–tandem quadrupole mass spectrometry

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ABSTRACT

Numerous triester organophosphate flame retardants (OPFRs) have been used for several decades and continue to be used in a variety of commercial products. We developed a sensitive quantitative method for the analysis of, seven non-halogenated, three chlorinated and two brominated OPFRs of known or possible environmental relevance in herring gull eggs. This method is based on a simple two-step sample extraction followed by liquid chromatography–electrospray ionization(+)-tandem mass spectrometry. Instrumental detection limits and method limits of quantification (MLOQs) among the 12 OPFRs ranged from 0.01 to 0.12 ng/mL and 0.06 to 0.20 ng/g, respectively. The mean OPFR recovery efficiencies of replicate analyses ($n = 6$) were very quantitative and ranged from 89% to 104%, with the two brominated OPFRs being somewhat lower but reproducible, i.e., 67% and 72%, respectively. Essentially negligible matrix effects were indicated by a standard addition approach that revealed mean percent signal recoveries ($n = 5$ replicates) of 89–106% for most OPFRs. In the analysis of $n = 13$ herring gull eggs from the Channel-Shelter Island colony (Lake Huron), tris(2-chloroisopropyl) phosphate (<MLOQ – 4.1 ng/g wet weight, ww), tris(2-chloroethyl) phosphate (<MLOQ – 0.6 ng/g ww) and tris(2-butoxyethyl) phosphate (<MLOQ – 2.2 ng/g ww) were detected and/or quantified.

1. Introduction

Organophosphate esters, particularly triesters, are widely used as flame retardants and plasticizers in a variety of commercial products such as plastics, foam, textiles, furniture and many others. The usage of triester organophosphate flame retardants (referred to collectively as OPFRs) dates back to the 1960s [1]. Contemporary production of OPFRs has been continuous and in high volume. For example, the estimated annual consumption of OPFRs was almost twice as that of all brominated flame retardants (BFRs) combined in Western Europe, and increased from approximately 83,000 tons/year in 2001 to 91,000 tons/year in 2006 [1]. In the U.S. each of tris(1,3-dichloro-2-propyl) phosphate (TDCPP), triphenyl phosphate (TPP) and tris(2-chloroisopropyl) phosphate (TCPP) had production volumes estimated between 1 and 10 million pounds in 1986 and 1990 and increased to between 10 and 50 million pounds in 1994, 1998, 2002 and 2006 [2].

Despite the high production volumes and wide applications of OPFRs, knowledge is limited worldwide on their environmental contamination. Currently available OPFR environmental studies have mainly focused on abiotic compartments (e.g., air, dust, water, sediments) [3–7], whereas little is known regarding wildlife and human exposure [8–11]. Bioaccumulation potentials of OPFRs are not adequately understood, likely resulting from the scarcity of investigations in environmental biota. In two rare studies, several OPFRs exhibited total concentrations of up to 1900 ng/g lipid weight (lw) in fish from Swedish coasts and up to 525 ng/g lw in fish from Malina Bay, Philippines [10,11].

The majority of available OPFR studies used gas chromatography (GC)–mass spectrometry (MS) or -nitrogen–phosphorus detection (NPD) as the primary instrumental analysis tools [12]. Although several recent studies reported analytical methods based on liquid chromatogram (LC)–tandem MS, these methods were mostly developed for abiotic samples, such as drinking and surface waters and sediments [5,13–15]. The objective of the present study was to develop a highly efficient and sensitive LC–MS-based analytical method, as well as an efficient extraction procedure, for a broad

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suite of high production OPFRs of known or expected environmental relevance in lipid-rich biological samples. The developed method was then applied to investigate OPFR contamination in herring gull (*Larus argentatus*) eggs from the Laurentian Great Lakes of North America.

2. Materials and methods

2.1. Chemical standards and samples

Twelve triester OPFRs (Table 1) were purchased from Sigma–Aldrich (Oakville, ON, Canada) except for tris(2,4-dibromopropyl) phosphate (TDBPP) (AccuStandard, New Haven, CT, USA) and tris(2-bromo-4-methylphenyl) phosphate (TBMPP) (custom synthesized by GL Chemtec International, Oakville, ON, Canada). The deuterated tributyl phosphate (d_{27}-TBP), purchased from Cambridge Isotope Laboratories (Andover, MA, USA), was used as the internal standard (I.S.) for quantification of OPFRs.

Commercially available chicken eggs, purchased from a local Ottawa (ON, Canada) supermarket, were pooled and used for method development and validation. The finalized quantitative method was then applied to screen for and determine OPFR concentrations in homogenates of individual herring gull eggs ($n = 13$) collected from the Channel-Shelter Island (Lake Huron) in 2010. This annual egg collection was part of Environment Canada's Great Lakes Herring Gull Monitoring Program [16].

2.2. Sample preparation

All analyses were carried out in the Organic Contaminants Research Laboratory (OCRL), NWRC (Ottawa, Canada). Approximately 1 g of chicken egg homogenate was ground with diatomaceous earth (DE; J.T. Baker, NJ, USA). After spiking with 10 ng of d_{27}-TBP (I.S.), the sample was subjected to accelerated solvent extraction (Dionex ASE 200, Sunnyvale, CA, USA) with 50:50 dichloromethane:hexane (DCM:HEX) at 100 °C and 1500 psi. After moisture removal through sodium sulfate and then gravimetric determination of lipid content using 10% of the lipid extract, the remaining extract was cleaned and separated on a 1 g ISOLUTE aminopropyl silica gel SPE column (Biotage, Charlotte, NC, USA) packed into a 6 mL Supelclean™ glass cartridge (Sigma–Aldrich). The SPE column was pre-washed with 15 mL 50:50 DCM:methanol, 15 mL DCM and 20 mL HEX to clean and condition the silica gel absorbent. After the sample was loaded, the first fraction was eluted with 2 mL 20:80 DCM:HEX and was discarded. The second fraction that contained target OPFRs were eluted with 4 mL 20:80 DCM:HEX, followed by 8 mL DCM. After evaporation to dryness under constant nitrogen flow, the residue was re-dissolved with 200 μL methanol, and filtered through a centrifugal filter (0.2 μm Nylon membrane, 500 μL; VWR, Mississauga, ON, Canada). The resulting filtrate was transferred to a vial for instrumental analysis.

2.3. Liquid chromatography–electrospray-tandem quadrupole mass spectrometry analysis

The separation and quantification of the target OPFRs was performed on a Waters 2695 high performance liquid chromatography (LC) system coupled to a Waters QuattroUltima tandem quadrupole mass spectrometer (MS/MS) (Waters, Milford, MA, USA). The LC system was equipped with a Waters Xterra® phenyl column (2.1 mm × 100 mm, 3.5 μm particle size) and the column temperature was kept as 40 °C. The mobile phases consisted of water (A) and methanol (B), both spiked with 0.1% formic acid (v/v). The mobile phase flow rate was 0.2 mL/min and the following gradient was employed: 5% B ramped to 70% B in 3 min (linear) and then ramped to 80% B in 12 min (linear), followed by a linear increase to 95% B in 3 min (held for 12 min) and then a change to 5% B in 1 min (held for 15 min). A 10 μL of aliquot of the sample was injected into the LC system.

The MS system was equipped with an electrospray ionization (ESI) probe operated in positive mode. High purity nitrogen and argon were used as nebulizing and collision gas, respectively. The detection and quantification of OPFR analytes was performed in the selected reaction monitoring (SRM) mode using the most abundant parent and daughter ions for individual OPFRs. The other operation parameters for MS were optimized as follows: capillary voltage: 4.0 kV; source temperature: 100 °C; probe temperature: 300 °C; cone gas flow: 150 L/h; desolvation gas flow: 700 L/h. The compound-dependent operation parameters and SRM transitions are listed in Table 1.

2.4. Evaluation of matrix effects

Six replicates of 1 g chicken egg homogenate and an additional six replicates of 2 g chicken egg homogenate were extracted and cleaned-up using the method described previously, and with no standards spiked before extraction. The final extract containing the OPFRs was reconstituted in 100 μL of methanol and then divided into two sub-samples, A and B (50 μL each). Sub-sample A was spiked with 50 μL of a standard solution containing 12 OPFRs

Table 1
Optimized instrumental parameters, selected reaction monitoring (SRM; mass-to-charge (m/z)) transitions and performance evaluation results (instrumental linearity, inter- and intra-day precision and instrumental detection limits (IDLs)) for organophosphate flame retardants (OPFRs) analyzed by liquid chromatography–electrospray ionization(+)-tandem mass spectrometry.

Compound	Acronym	SRM transition (m/z)	Cone voltage (V)	Collision energy (eV)	Linearity, R^2 (0.5–100 ng/mL)	Precision (RSD%) (50 ng/mL)		IDL (ng/mL)
						Intra-day	Inter-day	
Tris(2-chloroethyl) phosphate	TCEP	284.9 > 63	35	25	0.993	3	5	0.03
Tripropyl phosphate	TPrP	225.3 > 99	40	5	0.999	4	5	0.06
Tris(2-chloroisopropyl) phosphate	TCPP	329.1 > 99	35	20	0.999	5	5	0.05
Tris(1,3-dichloro-2-propyl) phosphate	TDCPP	430.9 > 99	60	25	0.999	5	7	0.01
Triphenyl phosphate	TPP	327.1 > 77.1	100	40	0.999	3	4	0.06
Tris(2,3-dibromopropyl) phosphate	TDBPP	698.6 > 99	55	30	0.999	6	5	0.12
Tributyl phosphate	TBP	267.1 > 99	35	20	0.999	4	5	0.01
Tricresyl phosphate	TCrP	369.1 > 91	90	40	0.997	5	8	0.1
2-Ethylhexyl-diphenyl phosphate	EHDPP	363.2 > 250.8	35	10	0.999	2	4	0.05
Tris(2-butoxyethyl) phosphate	TBEP	399 > 199	35	15	0.994	5	6	0.03
Tris(2-bromo-4-methylphenyl) phosphate	TBMPP	604.9 > 90	110	70	0.994	5	7	0.1
Tris(2-ethylhexyl) phosphate	TEHP	435.3 > 99	50	20	0.999	4	3	0.05
Deuterated tributyl phosphate (I.S.)	d_{27}-TBP	294.3 > 102	35	20	n/a	3	3	0.01

and d_{27}-TBP (I.S.) at a concentration of 50 ng/mL per compound. Sub-sample B was a duplicate control and spiked only with 50 μL of methanol. An external standard solution (S) was prepared by combining 50 μL of the 50 ng/mL standard solution with 50 μL methanol. By comparing the response differences of the analytes in the sub-samples A and B to the responses of the analytes in the external standard, a matrix effect (ME) value was calculated as:

$$\text{ME}\ (\%) = 100 \times \frac{(A_i - B_i)}{(S_i)} \quad (1)$$

where A_i, B_i and S_i are the chromatographic peak areas of the analyte (i) in sub-samples A and B and external standard solution (S), respectively. The analyte signals may be suppressed or enhanced by the co-eluted contents in the samples if ME (%) is lower or higher than 100%, respectively. Sub-sample A and B were 1:1 dilutions from the original 100 μL chicken egg extract, which mimicked the matrix of an actual sample extract with a volume of 200 μL.

2.5. Overall recoveries and method limit of quantification

The recovery efficiencies of target OPFRs throughout the analytical method were evaluated using chicken egg homogenates. Each of six replicates of 1 g chicken egg homogenate was spiked with 10 ng each of target OPFRs and d_{27}-TBP (I.S.), and was subjected to the analytical method described previously. Recoveries were determined using internal calibration as the quantification technique.

The LC–ESI(+)-MS/MS instrumental detection limit (IDL) was defined as the concentration of each analyte giving a signal five times the standard deviation of the background noise in the chromatogram. Any analyte that has instrumental responses below IDL was considered as non-detectable (n.d.). The method limit of quantification (MLOQ) was evaluated by replicate analyses (n = 8) of chicken egg homogenate (1 g) spiked with 30 μL of a standard solution containing target OPFRs (10 ng/mL per compound) and 20 μL of the d_{27}-TBP (I.S.) solution (200 ng/mL). The MLOQs for individual

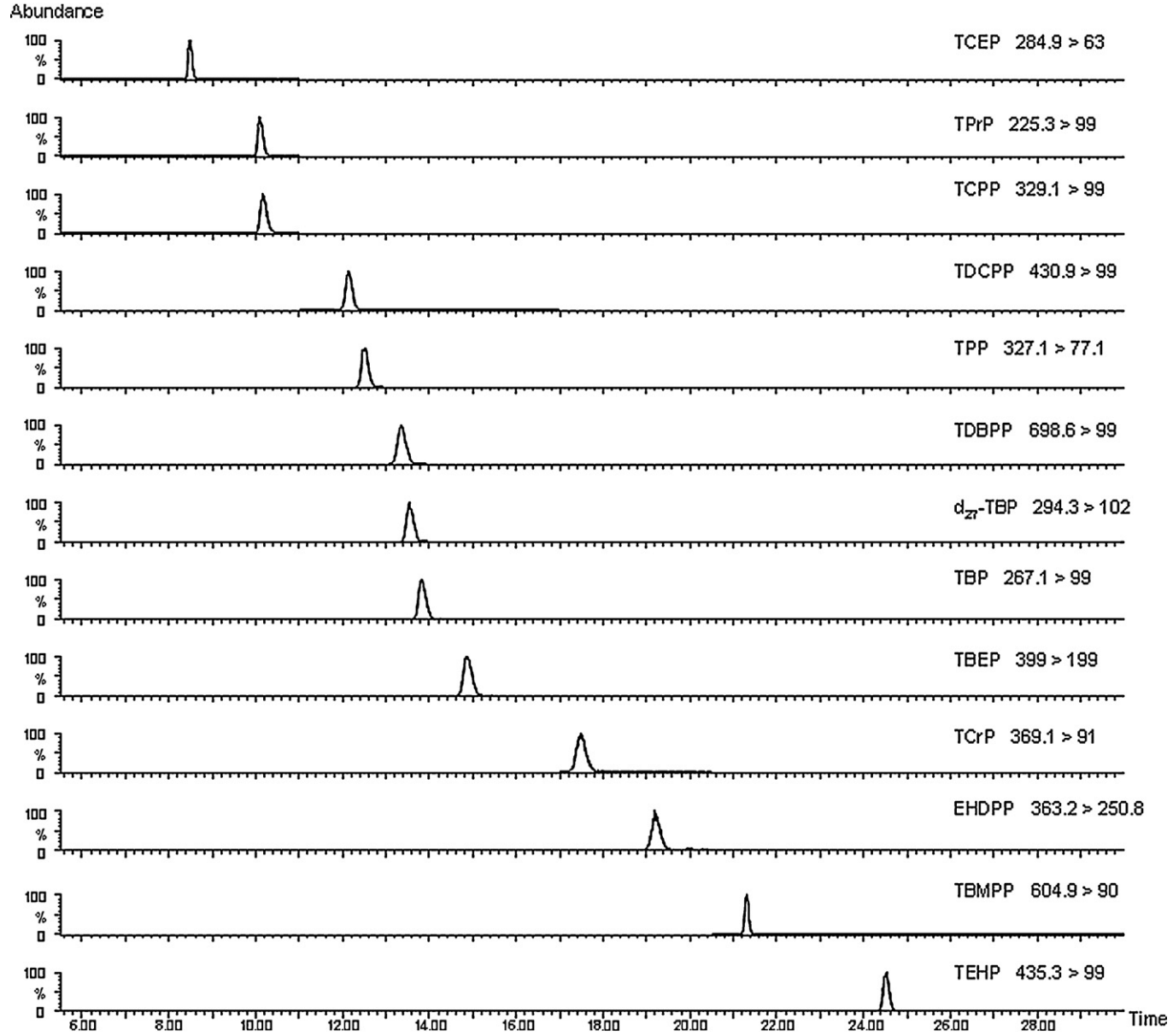

Fig. 1. Selected reaction monitoring (SRM) chromatograms for a standard mixture solution containing 12 organophosphate flame retardants (OPFRs) and an internal standard, under optimized LC–ESI(+)-MS/MS conditions. Four acquisition windows are used, i.e., 0–11 min; 11–17 min; 17–20.5 min; and 20.5–30 min. Refer to Table 1 for the full chemical names of the OPFR abbreviations.

OPFRs were calculated by multiplying the standard deviations generated from replicate analyses with a Student's *t*-value appropriate for a 99% confidence level.

Procedural blank (DE only; spiked with d_{27}-TBP) assays ($n = 6$) were conducted to evaluate contamination from proposed methodology. For the OPFRs consistently present in the blanks, their mean contamination levels in blanks were compared to previously proposed MLOQs, and the higher values were used as final MLOQs. In the analysis of environmental samples, the OPFR concentrations were reported after blank subtraction.

3. Results and discussion

3.1. SRM transitions and instrumental performance

Table 1 lists *m*/*z* values for the parent and daughter ions of the most intense SRM transitions for OPFR quantification under optimized ESI(+)-MS/MS conditions. For each of the examined OPFRs the parent ion used was that of the molecular ion $[M+H]^+$. Among the screened compounds, the two brominated OPFRs (TDBPP and TBMPP) had SRM transitions of *m*/*z* 698.6 > 99 amu and 604.9 > 90 amu, respectively. To our knowledge, for TDBPP and TBMPP, there have been no reports of any LC–MS/MS-based analytical methods or their application to a sample matrix.

Using the most abundant SRM transitions for each OPFR and the optimal mass spectrometer operation parameters (Table 1), we acquired the LC–ESI(+)-MS/MS mass chromatograms with sufficient chromatographic and mass resolved separation (Fig. 1). The mean IDLs ranged from 0.01 to 0.12 ng/mL (Table 1), which are at the same magnitudes as those reported in a recent OPFR study in fish tissues based on a LC–MS/MS system [11]. To evaluate the precision of instrumental performance, intra- and inter-day injections of a standard solution (50 ng/mL per compound) were conducted under reproducible conditions. The relative standard deviations (RSDs) were less than 6% and 8% for intra- and inter-day analyses, respectively (Table 1).

3.2. Matrix effects

Using the standard addition approach described previously, we determined the mean ME% values from five replicate analyses of 1 g chicken egg homogenate, which ranged from 89% to 106% for most examined OPFRs (Table 2). Ionization suppression was only observed for TBMPP and tris(2-ethylhexyl) phosphate (TEHP), where the matrix effect was still rather minimal as indicated by ME% values 77% and 82%, respectively. As our monitoring species are primarily lipid-rich avian eggs, the sample matrix was anticipated to be a good challenge as to the effectiveness of sample clean-up and OPFR isolation, as well as the quality of the subsequent LC–ESI(+)-MS/MS analysis. Therefore, we increased the extraction weight of chick egg homogenate to 2 g and re-evaluated the matrix effects. For most OPFRs, the mean ME% values were only slightly reduced relative to previous measurements for 1 g egg homogenate. However, the extent of ionization suppression for TBMPP and TEHP was improved, i.e., the ME% values increased to 90% and 96%, respectively (Table 2). Regardless, our analytical methodology demonstrated minimal matrix effects in the analysis of target OPFRs in lipid-rich samples such as avian eggs.

3.3. Overall recoveries and method limits of quantification

Except for TDBPP and TBMPP that had somewhat lower mean % recoveries (i.e., 67% and 72%, respectively), the mean recoveries for the rest of OPFRs ranged from 89% to 104%, with standard deviations lower than 16% (Table 2). Our analytical method used a specialized aminopropyl SPE for OPFR isolation and sample clean-up, and eliminated a gel permeation chromatography (GPC) step that was used as a universal procedure to remove bulk lipid interferences from the biological extracts. This greatly reduced solvent and time consumption, and resulted in precise recovery efficiencies for the 12 OPFRs under study. The somewhat lower recoveries of the two brominated OPFRs may be due to matrix effects that suppress ionization signals, and/or stronger absorption (than the other OPFRs) with SPE bed materials or glass tubes.

Of the few known OPFR methodological studies that reported MLOQs, such quantitative sensitivity was based primarily on an estimation of the signal-to-noise ratios (e.g., S/N = 10) of analyte peaks in the respective chromatograms [8,13,17]. In a few other studies only method limits of detection (MLODs) or instrumental LODs were reported [6]. Using a standard addition approach, where MEs were negligible, we determined the MLOQs for individual OPFRs in a matrix (chicken egg) very similar to our environmental samples (herring gull eggs). Considering a sample size of 1 g egg homogenate (containing approximately 10% lipids), the determined MLOQs ranged from 0.06 to 0.20 ng/g wet weight (ww) (or 0.6–2 ng/g lw) for the examined OPFRs (Table 2). Of the exceedingly limited biota monitoring studies, Marklund-Sundkvist et al. [10] reported the MLODs (defined as three times the noise level)

Table 2
Mean percent matrix effects, overall recoveries and method limits of quantification (MLOQs) of target organophosphate flame retardants (OPFRs) (standard deviations in parentheses).

OPFR[a]	Mean matrix effects (%)[b]		Mean overall recoveries (%)[c] ($n = 6$)	MLOQs[d] (ng/g wet weight)
	1 g egg homogenate ($n = 5$)	2 g egg homogenate ($n = 5$)		
TCEP	98 (2)	94 (4)	101 (10)	0.10
TPrP	95 (15)	99 (5)	96 (15)	0.10
TCPP	101 (6)	95 (5)	89 (10)	0.20
TDCPP	94 (8)	90 (6)	90 (8)	0.06
TPP	98 (8)	94 (7)	93 (8)	0.10
TDBPP	89 (6)	79 (9)	72 (14)	0.15
TBP	99 (5)	89 (5)	100 (12)	0.10
TCrP	103 (5)	94 (7)	96 (11)	0.12
EHDPP	106 (5)	92 (7)	94 (16)	0.09
TBEP	90 (8)	86 (5)	92 (7)	0.15
TBMPP	77 (30)	90 (23)	67 (19)	0.1
TEHP	82 (19)	96 (22)	104 (13)	0.07
d_{27}-TBP (I.S.)	95 (6)	89 (7)	81 (3)	n/a

[a] See Table 1 for chemical name of OPFR abbreviation.
[b] Matrix effects were evaluated based on spiked extracts of 1 g and 2 g samples of chicken egg homogenates.
[c] Overall recoveries of OPFR analytes and internal standard were evaluated based on internal and external calibration, respectively.
[d] MLOQs were evaluated based on spiked 1 g chicken egg homogenate samples.

Table 3
Concentrations[a] (ng/g wet weight) of the organophosphate flame retardants (OPFRs) TCEP, TCPP, TBEP, TPP and TDCPP in the herring gull eggs ($n = 13$) collected (2010) from the colony site at the Channel-Shelter Island (Lake Huron) in the Great Lakes of North America.

Individual egg homogenates	Lipid%	TCEP[b]	TCPP[b]	TBEP[b]	TPP[b]	TDCPP[b]
1	6.0	0.23	0.37	0.23	<MLOQ	n.d.
2	7.5	0.16	0.41	0.24	<MLOQ	<MLOQ
3	7.4	<MLOQ	0.21	0.65	<MLOQ	<MLOQ
4	8.0	0.16	0.32	0.45	n.d.	<MLOQ
5	7.7	0.12	0.20	0.57	<MLOQ	<MLOQ
6	8.1	0.23	0.40	2.2	0.13	<MLOQ
7	7.5	0.21	0.42	0.44	0.13	0.17
8	6.9	<MLOQ	0.22	0.16	<MLOQ	n.d.
9	6.8	0.28	0.60	0.41	<MLOQ	n.d.
10	8.0	0.17	0.21	0.62	<MLOQ	<MLOQ
11	10	0.55	1.4	0.49	0.11	<MLOQ
12	8.7	<MLOQ	4.1	0.70	<MLOQ	0.11
13	7.6	0.20	<MLOQ	0.40	<MLOQ	<MLOQ

[a] Reported values were already subjected to blank subtraction.
[b] See Table 1 for the complete chemical names of the OPFR abbreviations.

of 11 ng/g lw for TDCPP, 23 ng/g lw for tris(2-butoxyethyl) phosphate (TBEP), and 0.05–11 ng/g lw for other OPFRs using a GC–MS based analytical methodology. However, such MLODs were determined based on a large sample size (e.g., approximately 30 g of fish muscle tissue). In contrast, our present methodology analyzed OPFRs in bird eggs in the 1 g size range, and resulted in sub-ppb MLOQs (Table 2). Another recent fish study also reported sub-ppb MLOQs for several OPFRs using a LC–MS/MS based analytical technique, although such MLOQs were calculated as the amount giving 10 times the standard deviation of the peak area for blank replicates [11].

In procedural blanks we consistently encountered background levels for several OPFRs. Replicate procedural blank assays ($n = 6$) determined the mean (±standard deviation) contamination levels of TCPP, tris(2-chloroethyl) phosphate (TCEP) and TBP to be quantifiable at 0.15(±0.07), 0.10(±0.03) and 0.08(±0.01) ng/g ww, respectively, essentially at their MLOQs. Previous studies also observed unavoidable contamination of several OPFRs (e.g., TBP, TBEP, TPP and TCPP) in procedural blanks during the analysis of water and sediment samples [4–6]. Our initial sample clean-up trials tested different silica gel and Florisil sorbents from several manufacturers, and without exception all contained considerable OPFR background contamination. Commercially available plastic SPE cartridges were also found to contain TCPP and TBP. As a result, we strongly suggest that plastic equipment including plastic SPE cartridges should be avoided for ultratrace OPFR determination. To minimize the background OPFR contamination, we used solvent rinsed glass cartridges for SPE and pre-rinsed aminopropyl silica gel with solvents. Such procedures controlled the background OPFR levels in the sub-ppb range (Table 2).

3.4. Analysis of the Great Lakes herring gull eggs

TCPP, TCEP and TBEP were consistently detected in the gull eggs ($n = 13$) collected from the colony site at Channel-Shelter Island (Lake Huron), with levels ranging from <MLOQ – 4.1 ng/g ww, <MLOQ – 0.6 ng/g ww and <MLOQ – 2.2 ng/g ww, respectively (Table 3). TPP and TDCPP were quantifiable in a small fraction of the samples, with levels barely higher than their MLOQs. The rest of target OPFRs were generally non-detectable in any of the herring gull egg homogenates. None of TCPP, TCEP or TBEP had wet weight-based concentrations significantly correlated with lipid contents in the examined 13 individual eggs ($p > 0.05$).

Although TCEP, TCPP and TBEP have relatively low Log K_{ow} values (i.e., 1.44, 2.59 and 3.75, respectively) [1], their consistent detection in the present herring gull eggs indicated bioaccumulation potential. This raises the needs for the full evaluation of their bioavailability in the food web and ecosystem of these gulls and subsequently in their eggs at colony sites across the Great Lakes basin. Furthermore, the OPFR residue levels or lack thereof in the present gull eggs may be underestimated due to metabolic dealkylation of triester OPFRs to diester and monoester metabolites in the maternal gulls and/or selective triester OPFR transfer from mother to egg. Triester OPFRs can be metabolized to phosphoric acid diesters and monoesters, e.g., TEHP metabolism to di(2-ethylhexyl) phosphate (DEHP) and mono(2-ethylhexyl) phosphate (MEHP) [18], and TDCPP metabolism to di(1,3-dichloro-2-propyl) phosphate [19].

4. Conclusions

Overall, our OPFR analytical methodology demonstrated its advantages in several aspects: (1) easy and fast. With no need for bulk lipid removal by GPC there is a large reduction of time and solvent consumption; (2) sensitive and accurate. Both the LC–ESI(+)-MS/MS instrumentation and the whole methodology exhibited exceptional sensitivities in the detection of the target OPFRs. In addition to the demonstrated precision in instrumental analysis, good overall recoveries and minimum matrix effects, as well as negligible background contamination, collectively assure the data quality; (3) applicable to biotic sample monitoring and particularly wildlife research. Only a small sample size (i.e., 1 g egg homogenate) is required for the analysis using the established methodology. Therefore, the sample consumption is greatly reduced, which is extremely necessary for investigations using valuable and archived wildlife samples. In applying this methodology to real field samples, we successfully detected TCPP, TCEP and TBEP in herring gull eggs from the Great Lakes. In light of limited OPFR studies in environmental biota, highly sensitive and multiple OPFR residue methods are needed to fill these knowledge gaps.

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