METHOD DEVELOPMENT FOR THE DETERMINATION OF ORGANOPHOSPHATE ESTER FLAME RETARDANTS IN FISH SAMPLES BY MODIFIED QuEChERS FOLLOWED BY GC-MS/MS

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SUMMARY

PHÁT TRIỂN PHƯƠNG PHÁP XÁC ĐỊNH CHẤT CHỐNG CHÁY CƠ PHỐT PHO Ở MẪU CÁ BẰNG PHƯƠNG PHÁP QuEChERS CẢI TIẾN KẾT HỢP GC-MS/MS

Chất chống cháy cơ phốt pho được sử dụng rộng rãi như hợp chất ngăn chặn sự cháy trong nhiều lĩnh vực, đặc biệt là ngành điện – điện tử. Do đó, các hợp chất này dễ dàng phát tán ra môi trường, gây ô nhiễm đất, nước, không khí và sinh vật. Tuy nhiên, các nghiên cứu phân tích hàm lượng các hợp chất chống cháy cơ phốt pho ở cá biển còn chưa được quan tâm ở Việt Nam. Bằng việc cải tiến phương pháp chiết QuEChERS, cụ thể là tối ưu điều kiện hóa hơi dung môi, kết hợp sắc kí khí ghép nối khối phổ hai lần, phương pháp phân tích trong nghiên cứu này phù hợp để phân tích hàm lượng vết hợp chất chống cháy trong cá biển Việt Nam. Dung môi hóa hơi dưới dòng khí N_2 ở 1°C tối ưu được hiệu suất thu hồi của các chất nằm trong khoảng 82 – 102%. Với phương pháp được phát triển, hiệu suất thu hồi của các hợp chất ở nồng độ 1, 50 và 100 ng/g lần lượt dao động trong các khoảng 91,3 – 108,2%, 92,8 – 103,7% và 92,4 – 103,7%, giới hạn phát hiện và giới hạn định lượng của phương pháp lần lượt từ 0,05 đến 0,10 ng/g và từ 0,15 đến 0,30 ng/g, và các giá trị lệch chuẩn tương đối của độ tái lặp phương pháp đều nhỏ hơn 20%. Phương pháp được ứng dụng để phân tích trên 15 mẫu cá (thuộc 5 loài, Psettodes erumei, Sillago sihama, Rastrelliger kanagurta, Decapterus macarellus, Selaroides leptolepis), với hàm lượng ΣOPFR dao động trong khoảng 39,0 – 181,5 ng/g khối lượng khô. Kết quả phân tích các mẫu cá cho thấy các hợp chất có chứa clo trong cấu trúc có hàm lượng lớn hơn các hợp chất chỉ chứa các chuỗi hidrocacbon.

Từ khóa. Chất chống cháy cơ phốt pho, QuEChERS, GC-MS/MS, cá biển, Việt Nam

1. INTRODUCTION

Organophosphorus flame retardants (OPFRs) have found widespread utilization as flame retardants in various commercial applications, such as electronic devices, in addition to their role as plasticizers or additives in lubricants [1,2]. As a result of the lack of intermolecular forces in polymeric materials, the liberation of OPFRs into the surrounding environment can readily transpire through mechanisms such as volatilization, migration, or solubilization [1]. Numerous investigations have meticulously documented the presence of these compounds in environment as well as marine fish [3]. It is noteworthy that these organisms may potentially serve as a source of dietary intake for human consumption. Elevated

levels of OPFRs have been linked to adverse health effects such as neurotoxicity, reproductive toxicity, carcinogenic, mutagenic, and endocrine-disrupting effects [4].

Accelerated solvent extraction and Soxhlet extraction are two frequently used sample preparation techniques utilized for the analysis of OPFRs in biological matrices. The Soxhlet extraction method typically spans a duration of 18 to 24 hours, while rapid extraction necessitates the use of solvent systems that are challenging to retrieve and incur high operational costs [5]. Hence, it is essential to use a sample extraction technique that fulfills certain criteria, including the utilization of eco-friendly solvents in minimal quantities, a streamlined and expeditious procedure, and the minimal presence of contaminants within the sample matrix. Therefore, it can be concluded that the QuEChERS approach is capable of satisfying the aforementioned conditions [6].

It is essential to devise a methodology for the analysis of organophosphorus flame retardant chemicals in fish specimens using the enhanced QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) technique, which can effectively mitigate the impact of the sample matrix.

The primary research aim of this project is to provide a methodology for the measurement of OPFR chemicals in fish samples. This will be achieved by enhancing the QuEChERS technique, with a particular focus on improving the conditions for solvent vaporization and validating the analytical method. Subsequently, the aforementioned methodology was used to examine and conduct an initial assessment of the occurrence of OPFRs in marine fish specimens procured from local markets situated in Hanoi. The obtained results serve as empirical data that reflects the present condition of OPFR contamination in marine fish.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Six OPFR standards, including tri-butyl phosphate (TBP), *tris*(2-chloroethyl) phosphate (TCEP), *tris*(2-chloroisopropyl) phosphate (TCPP, 3 isomer compounds), *tris*(2,3-dichloropropyl) phosphate (TDCPP), tri-phenyl phosphate (TPhP) and *tris*(2-butoxyethyl) phosphate (TBEP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The internal standard utilized in this study was deuterated TPhP (TPhP-d_{15}), whereas the surrogate was deuterated TCEP (TCEP-d_{12}). These compounds were obtained from Cambridge Isotope Laboratories, Inc. (CIL, Andover, MA, USA). The individual stock standard solutions were 1000 mg/L in methanol. A stock mixture standard solution with a concentration of 50 mg/L was prepared from individual stock standard solutions in methanol. The working standard was made by dissolving it in a solution of methanol. The internal standard and surrogate standard were produced in methanol at a concentration of 1 mg/L. The standard solutions were held in vials made of dark amber glass at a temperature of -20 °C.

The chemicals used in the investigation, namely acetonitrile (MeCN), toluene, and *n*-hexane, were of analytical grade purity above 99% and were procured from Merck (Darmstadt, Germany). Sodium chloride (NaCl), anhydrous magnesium sulfate ($MgSO_4$), and glacial acetic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). The Captiva EMR-Lipid (EMR, 100 mg) and the Bond Elut primary secondary amine cartridge (PSA, 500 mg) were obtained from Agilent Technologies (Santa Clara, California, United States).

2.2. Sample collection

A selection of fish species, including flounder (*Psettodes erumei*, n=3), goby (*Sillago sihama*, n=3), mackerel (*Rastrelliger kanagurta*, n=3), pompano (*Decapterus macarellus*, n=3), and yellowstripe scad (*Selaroides leptolepis*, n=3), were purchased at the local market in Hanoi**.** These species are classified as near-shore species and frequently utilized in Vietnam. The fish samples were securely wrapped in aluminum foil and afterwards sent to the laboratory. Subsequently, the fish specimen received an external washing process using ultrapure water,

followed by drying using dust-free paper. The first step included recording the measurements of body length and weight. Then, muscle samples were obtained, subjected to lyophilization, and ground in a Universal Cutting Mill (PULVERISETTE 19, Idar-Oberstein, Germany). Subsequently, the powders were stored at -20 °C until analysis.

2.3. Sample preparation

The approach for sample preparation was conducted by following a previously established protocol with a modification [7], specifically, the solvent vaporization conditions. The recovery performance of the analytical process is directly impacted by the conditions under which solvent vaporization occurs. A total of 25 mL of MeCN:toluene (5/1, v/v) was spiked at the OPFRs level of 100 ng/mL (n = 3) and subjected to vaporization under three different conditions: (i) using a rotovap; (ii) with a nitrogen gas flow at room temperature; and (iii) with a nitrogen gas flow at 1°C. As a result, the final sample preparation process as follows: a mass of 1 g of homogenized sample was measured and transferred into a 50-mL centrifuge tube. A 10 µL of the surrogate standard of 1 mg/L was introduced prior to the commencement of sample processing. A volume of 10 mL of ultrapure water and 10 mL of MeCN containing 1% acetic acid were introduced into the tube, followed by vortexing for a duration of one minute. A mixture of 1 g of NaCl and 4 g of $MgSO_4$ was introduced into the tube and vigorously agitated manually. Subsequently, the mixture was subjected to vortexing for a duration of 1 minute, followed by centrifugation at a speed of 7000 rpm for a period of 30 min (Hettich Universal 320, Germany). Then, a 5 mL volume of extract was introduced into the SPE column which was composed of two kinds of sorbents, namely PSA and EMR and had been preactivated using 5 mL of MeCN containing 1% acetic acid. The loading of the extract onto the column was carried out at a flow rate of 2 mL/min. Subsequently, the analyte was then eluted from the column using 20 mL of a mixture including MeCN and toluene at a volumetric ratio of 5:1. The elution and extraction solvents were concentrated to a final amount of 5 mL using a centrifugal evaporator (Genevac Ltd., Ipswich, Suffolk, UK). Following that, the volume was then reduced to complete dryness by using a nitrogen stream at a temperature of 1 °C. The duration of the solvent vaporization step amounts to a total of 40 min. Subsequently, the solid residue was dissolved again using a 1 mL *n*-hexane solvent solution containing 10 ng/mL of TPhP-d_{15}.

2.4. Gas chromatography-tandem mass spectrometry

The approach for instrument operation was conducted by following a previously established protocol [8]. The studies were conducted using a Trace 1310 gas chromatography system connected to a TSQ9000 triple quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The chromatographic separation of the target chemicals was performed using an HP-5MS capillary column (30 m x 0.25 mm inside diameter and 0.25 µm film thickness) manufactured by Agilent (Santa Clara, CA, USA). The oven-temperature programme used for this purpose was as follows [8]: The starting temperature was set at 80 °C (held for 2 min), raised to 230 °C (10 °C/min, held for 5 min), raised 270 °C (°C/min, held for 1 min), and raised to 300 °C (30 °C/min, held for 5 min). The whole duration of the experiment was 37 minutes, which included a solvent delay of 4 minutes. The carrier gas used in this experiment was helium, which was maintained at a consistent flow rate of 1.2 mL/min. In the splitless mode, the injection volume used was 1 µL. The triple quadrupole mass spectrometer was used in electron ionization mode with an energy of 70 eV. The temperatures assigned to the inlet, transferline, and ion source were 250 °C, 290 °C, and 230 °C, respectively. The quantification process used the selected reaction monitoring (SRM) mode, which included the use of one quantifier transition (Q) and one qualifier transitions (C) to accurately identify each chemical. Table 1 displays the specifications of the triple quadrupole mass spectrometer.

Table 1: Retention times (RT) and electron ionization tandem mass spectrometry (EI-MS/MS) parameters

Compounds	RT	SRM transition (m/z, collision energy (V))	
		Q	C
TCEP	14.69	249>125 (8)	249>63 (14)
TCEP-d_{12}	14.73	261>131 (10)	213>148 (12)
TCPP_1 TCPP_2 TCPP_3	15.04, 15.20, 15.34	125>117 (6)	125>99 (10)
TBP	16.15	127>81 (22)	127>99 (6)
TDCPP	21.69	191>75 (8)	191>155 (6)
TPhP-d_{15}	23.08	341>339 (8)	341>223 (22)
TPhP	23.27	326>233 (8)	326>169 (28)
TBEP	23.45	125>99 (8)	199>57 (12)

3. RESULTS AND DISCUSSION

3.1. Optimization of solvent evaporation

To optimize the analytical sensitivity, it is recommended to employ the method of analyte concentration via solvent evaporation. The aim of this investigation was to evaluate three distinct methodologies, specifically the rotary evaporator (rotovap), nitrogen gas at ambient temperature, and the combined approach of centrifugal vacuum and nitrogen gas at 1 °C. The results are depicted in Figure 1. The rotovap, operates through the rotation of the evaporator, thereby enhancing the surface area conducive to mass transfer. Moreover, the utilization of nitrogen gas necessitates the procedure of extracting the gaseous element to the interface of the solvent. As per Dąbrowski's research [9], these techniques enhance the vaporization rate by reducing the solvent's partial pressure in the gaseous phase above the liquid. The utilization of a nitrogen stream elicits more advantageous results in terms of recovery in comparison to the rotovap technique. Vacuum centrifuges employ the utilization of centrifugal force to induce surface boiling solely on the surface of the liquid. This methodology functions to impede the formation of gas bubbles that possess the capability to displace the liquid, thereby reducing the duration required for the evaporation of the specimen. Therefore, the optimization of low temperature performance serves the dual purpose of simultaneously reducing evaporation and preventing analyte degradation.

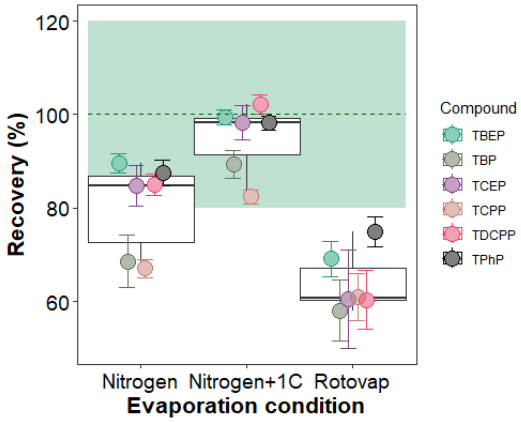

Figure 1: The recovery of analytes was shown in detail for solvent evaporation conditions

3.2. Method validation

The analytical procedures were subjected to validation in accordance with the guidelines stipulated by the European Commission (SANTE/11312/2021). The outcomes of the method validation procedure are exhibited in Table 2. The linearity was assessed over a concentration range spanning from 1 to 100 ng/mL, with all regression coefficient values surpassing 0.99. The assessment of repeatability (RSD_R) and reproducibility (RSD_{wr}) was conducted by analyzing the relative standard deviation (%RSD) at three discrete concentration levels in blank fish sample. The experiment consisted of replicating six samples at each

concentration level on a daily basis, with this process being done over the span of two consecutive days. The assessment of precision was performed by quantifying the recuperation of analyses at three discrete concentrations (1, 50, and 100 ng/g) in blank fish matrices, with six replicates executed for each concentration level. Precisely, a mass of 1 gram of the aforementioned fish sample was intentionally enriched with the meticulously determined substance and subsequently subjected to analysis in accordance with the developed procedure. As a result, with respect to the notion of repeatability, the observed RSD_r values exhibited a range of variability from 2.1% to 9.4%. Similarly, in the context of reproducibility, the observed range of RSD_{wr} values exhibited variations ranging from 6.7% to 11.4%. Both of the aforementioned values exhibited a percentage that is below 20%. The method detection limit (MDL) was ascertained through the quantification of the target analyte's quantity in fish extracts. The aforementioned extracts were obtained from freeze-dried samples weighing 1 g and were generated using a modified methodology for sample preparation. The MDL was determined by evaluating the ion signal-to-noise ratio with a minimum requirement of three. The method quantitation limit (MQL) was determined by multiplying the MDL by a factor of 3.3. Consequently, the MDLs and the MQLs for the analytes were determined to be 0.10 ng/g and 0.30 ng/g, respectively. The evaluation of the matrix effect involved the comparison of the slope of the calibration curves prepared using the matrix to that of the calibration curves prepared using the solvent. As a result, the matrix effect seen in our investigation exhibited a range of -11.2% to +10.2%, aligning with the recommendations provided in SANTE/11312/2021. Based on the aforementioned findings, it is conclusively demonstrated that the optimized methodology employed in this investigation is well-suited for the quantification of minute concentrations of OPFRs within fish matrices.

Table 2: Method quality parameters

Compounds	Range linearity (ng/mL)	R^2	% Recovery			% Repeatability (% Reproducibility)			MDL (ng/g)	MQL (ng/g)	ME (%)
			1 (ng/g)	50 (ng/g)	100 (ng/g)	1 (ng/g)	50 (ng/g)	100 (ng/g)			
TCEP	1-100	0.9999	95.2	95.4	97.9	8.5 (10.6)	7.4 (8.5)	3.1 (6.7)	0.05	0.15	10.2
TCPP	1-100	0.9998	107.9	96.7	103.4	7.7 (11.4)	2.5 (9.7)	6.2 (6.1)	0.05	0.15	9.6
TBP	1-100	0.9981	101.6	103.7	92.4	7.1 (9.1)	7 (10.9)	4.8 (10.7)	0.05	0.15	-6.5
TDCPP	1-100	0.9992	100.6	92.8	100.6	9.4 (6.8)	7.1 (7.7)	4.2 (9.0)	0.05	0.15	-11.2
TPhP	1-100	0.9997	91.3	94.8	103.7	6.3 (9.1)	4.3 (9.6)	2.1 (11.3)	0.10	0.30	-6.8
TBEP	1-100	0.9996	108.2	95.2	99.6	5.9 (8.5)	6.6 (8.2)	6.9 (9.9)	0.10	0.30	-3.4

Table 3: Concentrations found in five different fish species

Compounds	Range *(Mean)* (ng/g dw)				
	Flounder	**Goby**	**Mackerel**	**Pampano**	**Yellowtripe scad**
TBP	1.3 - 9.4 *(5.7)*	4.3 - 4.8 *(4.5)*	2.4 - 9.0 *(5.5)*	5.6 - 23.4 *(14.5)*	1.4 - 3.1 *(2.1)*
TCEP	16.6 - 44.6 *(31.8)*	19.6 - 33.1 *(25.1)*	6.9 - 27.4 *(18.1)*	13.8 - 51.5 *(28.8)*	11.7 - 23.7 *(15.9)*
TBEP	6.5 - 22.2 *(16.2)*	7.2 - 27.4 *(14.3)*	3.2 - 13.1 *(8.2)*	6.7 - 36.9 *(18.1)*	3.4 - 12.3 *(9.0)*
TDCPP	1.5 - 16.7 *(9.7)*	3.6 - 27.2 *(13.2)*	0.6 - 66.6 *(24.0)*	6.7 - 36.7 *(17)*	2.9 - 14.7 *(7.6)*
TPhP	6.8 - 14.1 *(11.6)*	5.6 - 25.1 *(12.3)*	1.1 - 16.3 *(7.7)*	7.2 - 23.5 *(13.1)*	6.0 - 11.5 *(8.3)*
TCPP	38.1 - 41.6 *(39.8)*	28.3 - 43.2 *(36.8)*	12.9 - 18.2 *(16.3)*	27.3 - 43.9 *(35.0)*	17.2 - 29.6 *(23.7)*

3.3. Application to marine fish samples

The modified method was applied to samples of five fish species to determine organophosphate esters. The results are shown in Table 3.

All OPFR chemicals were shown to be present in all fish species, with TCPP being found at the highest concentrations. Additionally, it is seen that the average concentration of OPFR in each species exhibits a descending trend, with pompano having the highest mean value of 21.1 ng/g dw, followed by flounder with 19.1 ng/g dw, goby with 17.7 ng/g dw, mackerel with 13.3 ng/g dw, and yellowstripe scad with 11.1 ng/g dw. Chlorinated compounds, such as TCPP and TCEP, exhibit greater concentrations compared to alkyl/aryl esters due to their increased resistance to deterioration in aqueous environments. This resistance to degradation allows chlorinated compounds to persist in water, whereas other compounds are more prone to hydrolysis and subsequent removal in wastewater treatment facilities. Numerous studies have shown that the distribution of OPEs in fish exhibits notable variations across species and even within the same species, mostly attributable to disparities in eating behavior, body size, developmental stage, or metabolic capabilities [10, 11]. The investigation revealed that the average concentration of ΣOPFRs in all species was 4.11 ng/g wet-weight (ww), with an average moisture content of 75%.

4. CONCLUSION

A novel methodology has been effectively devised, presenting a more cost-effective and user-friendly approach for the quantification of six organophosphate esters in fish specimens. The efficacy of the modified QuEChERs extraction method, along with the use of innovative Captiva EMR-Lipid and Bond Elut PSA cartridges, in conjunction with GC-MS/MS, has been shown in the analysis of these chemicals in fish species with varying lipid content. Consequently, the technique that was developed exhibited a MDL of 0.10 ng/g and a MQL of 0.3 ng/g. Additionally, it is worth noting that the measured RSDs of the repeatability and reproducibility values were found to be below 20%. The methodology used in this research is well-suited for the quantification of trace levels of OPFRs in fish matrices. All of the variables mentioned in this research were seen in at least one species of fish. It was discovered that chlorinated compounds were present in greater amounts compared to alkyl/aryl esters. The average concentration of ΣOPFRs measured in this particular study was determined to be 4.11 ng/g ww.

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