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Determination of lipophilic marine biotoxins in aquatic products by liquid chromatography coupled with triple quadrupole mass spectrometry

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Abstract

Lipophilic marine biotoxins include Azaspiracid-1 (AZA-1), Azaspiracid-2 (AZA-2), Azaspiracid-3 (AZA-3), Pectenotoxin-2 (PTX 2), Okadaic acid (OA), Dinophysistoxin-2 (DTX-2), Dinophysistoxin-1 (DTX-1), Yessotoxin (YTX), and 1-Homoyessotoxin (Homo-YTX) were extracted with methanol, followed by cleaning up with solid phase extraction technique (SPE). Lipophilic toxins were confirmed and quantified by liquid chromatography coupled with triple quadrupole mass spectrometry (LC/MS/MS) using calibration curves on the solvent. The quantification limits of this method satisfied the requirements of the European Maximum Residue Limit (MRLs) with 25 µg/kg for the YTX group and 10 µg/kg for AZA, OA, and PTX groups. To validate the effectiveness of this method, matrices of clams, fish, and mixed seafood were collected and analyzed (recovery ranged from 92.4 - 101.5%, and relative standard deviation was less than 20%). The method was used successfully to participate in a proficiency testing program organized by Quasimeme (z-score in the range of ±2)

Keywords*: Lipophilic marine biotoxins, LC-MS/MS, SPE, Shellfish.*

1. INTRODUCTION

Among over 5000 known species of marine phytoplankton, about 300 species can sometimes proliferate in such numbers that result in changing the color of water on the surface of the sea, algal bloom. Algal bloom is a natural phenomenon and it might be beneficial to aquaculture [1]. However, some research indicated that about 40 species of Dinoflagellates and Diatoms family have the capacity to produce potent toxins (Phycotoxins) [2]. Phycotoxins can accumulate in edible species such as fish, crabs, and shellfish [3-4] and will cause poisoning for consumers [2]. Based on their physicochemical properties, marine toxins are divided into two groups: hydrophilic and hydrophobic. The toxins AZA 1, AZA

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2, AZA 3, PTX 2, OA, DTX 2, DTX 1, YTX, and Homo-YTX are all strongly hydrophobic. Therefore, these groups of substances are called Lipophilic marine biotoxins.

Bivalve mollusks make a major contribution to Vietnam's total seafood export turnover (US$141.6 million, 2021 according to VASEP), especially to the EU, US, Japan, and Canadian markets. Therefore, the quality requirements of these products are always strictly controlled for markets such as the EU and US. For bivalve mollusks, the parameters of marine biological toxins: memory-causing toxin ASP (Amnesic Shellfish Poisoning), toxin-causing diarrhea DSP (Diarrhetic Shellfish Poisoning), and toxin-causing paralysis PSP (Paralytic Shellfish poisoning) belong to the national monitoring program and always be the leading criterion to evaluate product quality. Currently, methods are being used such as mouse bioassay [5-6], high-performance liquid chromatography method using 9-anthryl-diazomethane (ADAM) [7] reagent to generate fluorescent derivatives possesses several drawbacks as cannot detect specifically and selectively all toxins, consume solvents and standard materials, have a high limit of quantification, and consume time for analysis [5-7].

Faced with the above situation, we have developed a method to simultaneously determine the Lipophilic marine biotoxin content in Vietnam that meets the maximum residue limit (MRL) of Europe [8] (Table 1).

Table 1. *Maximum residue levels of Lipophilic toxins according to EU*

Toxin group	*MRL (µg/kg)*
OA	160
AZA	160
YTX	1000

2. SUBJECTS AND METHODS

2.1 Subjects

Samples of aquatic products including fish, clams, and mixed aquatic products (seafood mix: clams, flour, vegetables) were sampling from the national Agro, Forestry, Fisheries quality assurance department branch 4. The sample was determined to be free of Lipophilic marine biotoxins, suitable for method development and validation.

2.2 Reagents and standards

The reagents and standards were used in this study are LC-MS grade:

- Okadaic acid (CRM-OA-c), CAS No.: 78111-17-8, NRC
- Pectenotoxin-2 (CRM PTX2), CAS No.: 97564-91-5, NRC
- Azaspiracid-1 (CRM AZA1) CAS No.: 214899-21-5, NRC
- Azaspiracid-2 (CRM AZA2), CAS No.: 265996-92-7, NRC
- Azaspiracid-3 (CRM AZA3), CAS No.: 265996-93-8, NRC
- Dinophysistoxin-1 (CRM DTX1), CAS No.: 81720-10-7, NRC
- Dinophysistoxin-2 (CRM DTX2), CAS No.: 139933-46-3, NRC

- Yessotoxin (CRM YTX), CAS No.: 112514-54-2, NRC
- Homo - Yessotoxin (CRM YTX), CAS No.: 196309-94-1, NRC

The mixed working standard solution YTX, h-YTX 500 µg/L, and 200 µg/L for the remaining substances were prepared in methanol and used within 6 months under storage conditions at ± 30°C.

Other solvents and chemicals including acetonitrile (ACN), methanol, (MeOH), formic acid 98 - 100%, ammonium formate ≥ 99%, ammonium solution 25%, sodium hydroxide 99%, hydrochloric acid 37% are provided by Merck (Germany).

- Oasis PRIME HLB solid phase extraction column 3cc/60mg, Waters or equivalent.

2.3. Equipment

Liquid chromatography system (Acquity UPLC H-Class plus) coupled with a mass spectrometer (Xevo TQs Micro) and data analysis software Masslyn 4.2, Waters (USA).

- Analytical column: CSH C18 1.7 µm × 2.1 mm × 150 mm, Waters (USA).
- 5,000 rpm Centrifuge, Sigma or equivalent.
- 500 rpm shaker, IKA or equivalent.
- Vortex machine, Hwashin or equivalent.
- Homogeneous crusher, KCH or equivalent.
- Digital balance with resolution 0.1 g, Satorius or equivalent
- Analytical balance with resolution 0.00001 g, Satorius or equivalent
- Micropipette 20, 100, 1,000, and 5 mL, eppendorf or equivalent
- Other sample processing tools.

2.4. Methods

2.4.1. Sampling and sample preparation [13]

Remove inedible parts such as scales, bones, fins, tails, and shells (for shrimp, crabs, clams), collect only the edible part. The sample is drained and then grind with a blender. Transfer the sample to the plastic bags and encode the identification number. Unused portion are stored at 2 - 8°C for 24 hours.

2.4.2. Sample extraction and clean-up

2.4.2.1. Extraction (refer to the European method [9])

The sample was weighed accurately 1.00 g ± 0.05 g of the homogenized samples was weighed into a 15 mL polypropylene tube and 4 mL methanol added. The mixture was vortexed for 3 min and centrifuged at 4,000 rpm for 10 min. Extraction was performed twice. The supernatants were combined, transfer to a 10 mL volumetric flask and bring to volume with methanol.

2.4.2.2. Clean-up [11]

1 mL of extract was diluted with 9 mL of deionized water. The Oasis Prime HLB cartridge was conditioned with 3 mL of methanol, followed by 3 mL of water under gravity. The entire extract was passed through the HLB column. The column was rinsed twice, each time with 3 mL of deionized water, elute with 3 mL of methanol. Final extracts were

evaporated to dryness under a stream of nitrogen and re-dissolved in 1 mL of methanol. The supernatants were pass through a pore size 0.25 mm polytetrafluoroethylene (PTFE) filter and analyzed by UPLC-MS/MS.

2.4.3. Method

Lipophilic toxins were separated on a CSH column and detected on a triple quadrupole mass spectrometer using MRM (Multiple reaction monitoring) mode, details are presented in Tables 2.

Table 2. *Settings for HPLC-MS/MS system [12]*

HPLC	***MS/MS***
- Column: CSH C18 1.7 µm × 2.1 mm × 150 mm. - Flow rate 0.4 mL/min - Injection volume: 5 µL - Column temperature: 40ºC - Sample injection chamber temperature: 15ºC - Run time: 10 minutes - Mobile phase A: 0,05% ammonium in water - Mobile phase B: 0,05% ammonium in 90% ACN - LC gradient (refer to Table 4)	- Ionization mode: ESI +, ESI - - Source temperature: 150°C - Desolvation temperature: 500°C - Desolvation gas : 800 L/h - Cone gas: 30 L/h - Collision gas: argon at 2.93×10^{-3} mbar

2.4.3.1. Evaluate the influence of the sample matrix

To determine the matrix effect, the slope of a calibration curve built on the matrices was compared with the calibration curve on the solvent. If the difference between these slopes is not more than 10%, the influence of the matrix is considered to be insignificant [10].

2.4.3.2. Method validation

The analytical method is validated according to the guidelines of EC 657/2002, SANTE/2020/12830, NMKL Protocol No.6 standards, including parameters of linearity ($R^2 \geq 0.99$), accuracy (recovery in the range of 70 - 120%, reproducibility and repeatability with RSD ≤ 20%), limit of quantitation (LOQ). The samples used for validation included clams, basa, and mixed. Lipophilic toxin-free samples were used as blanks, and mixed standards were added at concentrations appropriate to the standard (here we spiked at the expected LOQ and 2 × LOQ) and analyzed by two analysts on 3 different days). The LOQ validated at a concentration of 25 µg/kg for the YTX group and 10 µg/kg for the remaining groups. The recovery and reproducibility must meet quantitative criteria (recovery within 70 - 120%, RSDR less than 20%).

3. RESULTS AND DISCUSSION

3.1 Optimization of chromatographic conditions dectector

A standard solution of 200 µg/L of each substance was injected directly into the probe (without going through the column) to optimize the parameters of parent ion, daughter ion, fragmentation potential, and collision energy. The results are presented in Table 3.

Table 3. *Fragmentation conditions used for quantification of lipophilic toxins*

Analyte	*ESI*	*Precursor (m/z)*	*Daughter ion (m/z)*	*Well time (s)*	*Collision potential (V)*	*CE (eV)*
OA, DTX2	NEG	803.5	255.2 **113**	0.02	65	57 47
DTX1	NEG	817.5	255.2 **113**	0.02	65	60 55
YTX	NEG	570.4	396.4 **467.4**	0.02	40	55 55
Homo-YTX	NEG	577.4	403.4 **474.4**	0.04	55	38 32
AZA1	POS	842.4	654.5 **362**	0.02	50	55 55
AZA2	POS	856.4	654.5 **672.5**	0.02	30	45 55
AZA3	POS	828.5	640.5 **362**	0.02	50	55 55
PTX2-b	POS	876.5	823.5 **212.5**	0.02	40	50 40

OA and DTX2 have the same fragmentation conditions, therefore need to be separated by liquid chromatography with a resolution of $R_s > 1$ and calculated using the formula [6]:

$$R_s = \frac{2x(RT_2 - RT_1)}{(W_1 + W_2)}$$

In which:

- RT_1 and RT_2 are the retention times of OA and DTX2, with RT_2 being larger than RT_1.

- W_1 and W_2 are the peak widths of OA and DTX2 at base.

3.2 Gradient program and Separation optimization of OA, DTX2

OA and DTX2 have the same fragmentation conditions, so the separation of these two substances on the chromatographic column by the retention time is a must. In the gradient program optimized (see Table 4) based on the EU standard method (see Table 5), two compounds OA and DTX2 were separated and identified (refer to Figure 1) with $R_s > 1$.

Table 4. *Elution gradient program*

Time (min)	*A%*	*B%*
0.0	90	10
0.5	90	10
1.5	75	25
3.5	55	45
5.0	5	95
6.5	5	95
6.6	90	10

Table 5. *Reference elution gradient program [9]*

Time (min)	*A%*	*B%*
0.0	90	10
1.0	90	10
10.0	10	90
13.0	10	90
15.0	90	10
19.0	90	10

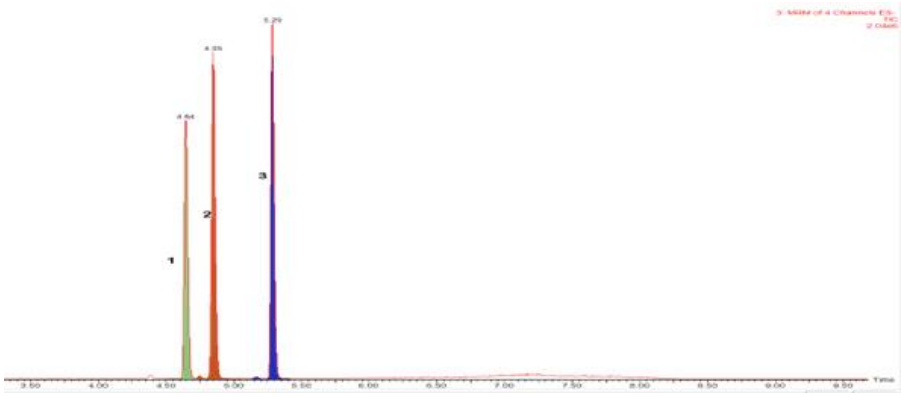

Figure 1. *Chromatography of the eluate using the optimized gradient program. OA (1), DTX2 (2), DTX1 (3)*

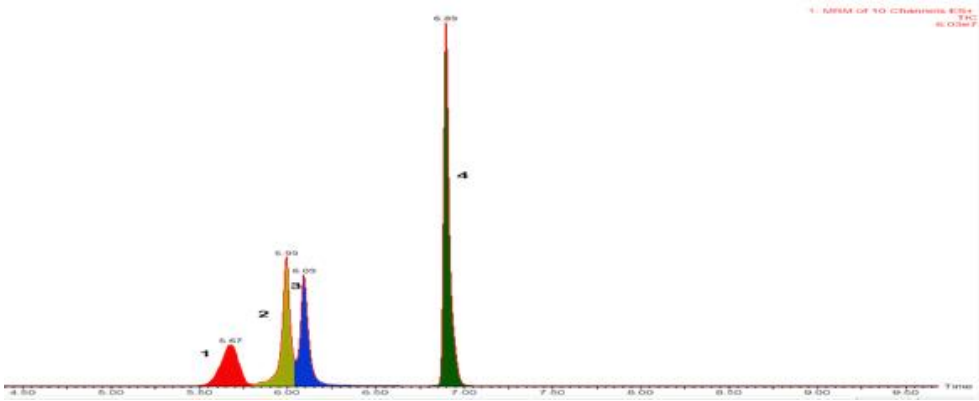

Figure 2. *Chromatography of the eluate using the optimized gradient program. AZA3 (1), AZA2 (2), ZAZ1 (3), PTX2 (4)*

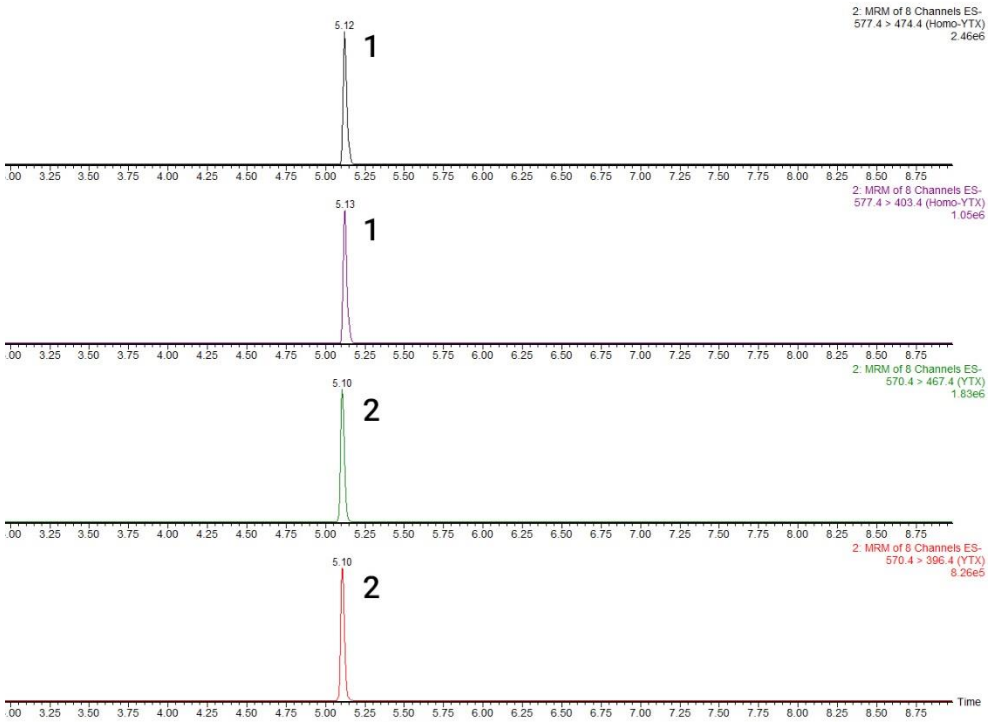

Figure 3. *Chromatography of the eluate using the optimized gradient program. t Homo-YTX (1), YTX (2)*

3.3 The sample cleaning efficiency of the HLB column

A sample was spiked at 10 µg/kg was analyzed using the SPE procedure as outlined in 2.4.2. The results are shown in Figurer 4.

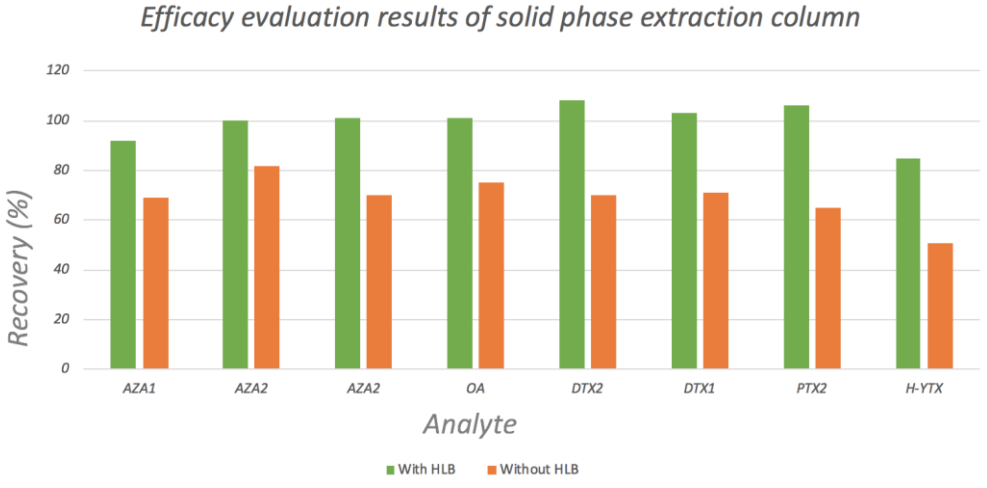

Figure 4. *Efficacy evaluation results of solid phase extraction column*

The study results showed that the cleaning efficiency of the solid-phase extraction column (85 - 108%) is better than that of not using the SPE column (51 - 82% due to the failure to remove interfering components such as Lipids, proteins, colors, etc.). The use of solid phase extraction columns is appropriate for the analytical procedure.

3.4 The matrix effects

Matrix effects were investigated by preparing the calibration curve on the clam, catfish, mix sample and the solvent [10]. The results are presented in Table 6.

Table 6. *Results of evaluating the influence of the matrices*

Analytes	***Matrix***		
	Clams	***Catfish***	***Mix sample***
	Ratio (%)		
AZA 1	2.2	9.0	7.6
AZA 2	2.7	2.9	3.2
AZA 3	0.1	8.0	10.0
PTX 2	0.5	10.0	7.2
OA	5.1	7.8	3.7
DTX 2	7.2	5.0	7.6
DTX 1	2.7	6.6	8.7
Homo-YTX	2.7	10.1	5.3

The data showed that the ratio between the slope a of the calibration curve on the solvent and the one on matrices ranges from 0.1 - 10.1% for analytes and matrices. Thus, the influence of the matrices on the analytical results is acceptable [10], and the curve built on solvent can be used for analysis.

3.5. Method validation

The method was validated according to EC 657/2002 guidelines, SANTE/2020/1283.

3.5.1. Calibration curve

The calibration curve of the Lipophilic toxin analysis method was developed based on the correlation between the concentration and the peak area of the analytes. The calibration curve was investigated at the following concentrations: 0, 25, 50, 75, 100, and 125 µg/kg for the YTX group and 0, 10, 20, 30, 40, and 50 µg/kg for the remaining groups on solvent and sample matrices. The results are shown in Table 7.

Table 7. *Parameters of the calibration curve on solvent and on matrices*

No	***Analyte***	***Matrix***	***a-slope***	***b-intercept***	***R^2***
1	AZA 1	Solvent	2947	-22588	0.9946
2	AZA 2		1609	-12230	0.9981
3	AZA 3		2576	-19944	0.9952
4	PTX 2		7973	-54513	0.9986
5	OA		64	-437	0.9976
6	DTX 2		70	-484	0.9969
7	DTX 1		90	-668	0.9972
8	YTX		9	-183	0.9964

No	*Analyte*	*Matrix*	*a-slope*	*b-intercept*	R^2
9	Homo-YTX		10	-129	0.9950
1	AZA 1	Catfish	2581	-15354	0.9985
2	AZA 2		1362	-8560	0.9994
3	AZA 3		2071	-8729	0.9962
4	PTX 2		6975	-44864	0.9989
5	OA		55	-307	0.9964
6	DTX 2		67	-329	0.9943
7	DTX 1		84	-583	0.9942
8	YTX		11	-217	0.9976
9	Homo-YTX		13	-238	0.9986
1	AZA 1	Seafood mix	2723	-15884	0.9991
2	AZA 2		1558	-11043	0.9999
3	AZA 3		2319	-15966	0.9978
4	PTX 2		7400	-57435	0.9924
5	OA		61	-556	0.9931
6	DTX 2		92	-875	0.9951
7	DTX 1		98	-851	0.9975
8	YTX		14	-357	0.9970
9	Homo-YTX		18	-449	0.9963
1	AZA 1	Clams	448	779	0.9960
2	AZA 2		225	-65	0.9971
3	AZA 3		525	1199	0.9933
4	PTX 2		267	297	0.9967
5	OA		73	97	0.9982
6	DTX 2		244	57	0.9993
7	DTX 1		260	527	0.9949
8	Homo-YTX		25	47	0.9973

3.5.2. Limit of quantification

The limit of quantification (LOQ) is defined as the minimum concentration of an analyte that can be quantified by the method with repeatability, reproducibility less than 20%, and recovery efficiency from 80 - 120%. In addition, the method only makes sense when the LOQ is equal to or less than the maximum residue limit set by international

organizations. According to the results presented in section 3.5.3, at concentrations of 25 µg/kg (h-YTX) and 10 µg/kg (remaining substances) all meet this regulation. So LOQ completely meets the requirements with the maximum residue limit (MRL) of the EU markets.

3.5.3. Recovery, reproducibility and repeatability

Repeat analysis of standard addition samples in 3 days, 5 samples per day by 2 analysts at 25 µg/kg for YTX and 10 µg/kg for the rest of the analytes, 5 samples at 50 µg/kg for YTX and 20 µg/kg for the remaining analytes on clam, catfish and Seafood mix. The recovery, repeatability, and internal reproducibility results are shown in Table 8.

Table 8. *Recovery, reproducibility and repeatability of the method on matrices*

Matrix	***Analytes***	***Recovery H (%)***	***Repeatability RSD_r (%)***	***Internal reproducibility RSD_p (%)***
Clams	AZA 1	101,2	3.7	4.3
	AZA 2	98,7	4.7	5.2
	AZA 3	97,3	2.9	3.5
	PTX 2	99,5	1.9	2,5
	OA	99,2	7.4	7.6
	DTX 2	99,2	6.6	6,8
	DTX 1	100,7	8.9	9,2
	Homo-YTX	98,7	3.9	4,2
Catfish	AZA 1	100,9	1.5	1,5
	AZA 2	101,8	1.5	1,6
	AZA 3	99,3	1.3	1,4
	PTX 2	101,1	2.6	3,4
	OA	101,3	4.5	4,7
	DTX 2	99,5	3.1	3,4
	DTX 1	101,0	3.5	3,8
	Homo-YTX	98,3	2.6	2,7
Seafood mix	AZA 1	98,8	2.1	2,3
	AZA 2	99,5	2.5	2,8
	AZA 3	101,5	2.7	2,8
	PTX 2	99,0	1.5	1,8
	OA	98,4	4.9	5,2
	DTX 2	100,4	3.9	4,3
	DTX 1	98,0	4.3	4,6
	Homo-YTX	92,4	4.7	5,7

3.6. Proficiency testing

This method was used to analyze mollusk samples from the proficiency testing program (BT11) "Round 2021-2" organized by Quasimeme International organization with lab code Q3216B4. The published results (refer to Table 9) showed that the method satisfied accuracy and precision (all the z-score values were less than 2, and the z-score values populated at both z-negative and z-positive indicate that the results from this procedure are not subject to systematic error.) and was suitable for sample analysis requirements for the national surveillance program on bivalve mollusks.

Table 9. *The results of the proficiency test program organized by Qusimeme*

Parameter	***Reported concentration (µg.Kg^{-1})***	***Z'-Score***
AZA1	63.43	-1.2
AZA2	22.58	-1.0
AZA3	17.72	-0.3
Total AZA group	128.87	-1.0
DTX1 free	14.44	1,5
DTX2 free	43.02	0.0
OA free	14.88	1.0
Total OA, DTX1, DTX2 free	53.13	0.8
DTX1total	16.94	0.1
DTX2 total	60.57	-1.4
OA total	41.26	-1.1
Total OA, DTX1, DTX2 hydrolysis	94.54	-1.1
PTX1	< LOQ	N/A
PTX2	< LOQ	N/A
Total OA and PTX group	94.54	-1,0
YTX	0.00877	&
Homo-YTX	<LOQ	N/A
45-OH-Homo-YTX	<LOQ	N/A
45-OH-YTX	0.00355	&
Total YTX group	0.0123	&

4. CONCLUSION

In this study, the lipophilic toxin group analysis method was developed and validated with a short run time (10 minutes, compared to the European standard method of 20 minutes

[9]). Compared with the European method [9], it can be saved a haft amount of solvent and time. A calibration curve on solvent has been used in this method, resulting in saving much of time and cost of sample analysis. Moreover, the accuracy and precision of this method were validated via the participation of proficiency test (all z-score values were in range less than ± 2), and the method has also been accredited that complied with ISO/IEC 17025:2017 by the Vietnam Bureau of Accreditation. The method has been applying for analysis of lipophilic toxins in routine samples for quality control, food safety and the national surveillance program. In the future, the method can be applied to studies on the existence of Lipophilic toxins in algae and marine animals in the waters of Vietnam.

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Xác định độc tố sinh học biển Lipophilic trong thủy sản bằng sắc ký lỏng khối phổ ba tứ cực

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Tóm tắt

Độc tố sinh học biển Lipophilic gồm Azaspiracid-1 (AZA-1), Azaspiracid-2 (AZA-2), Azaspiracid-3 (AZA-3), Pectenotoxin-2 (PTX 2), Okadaic acid (OA), Dinophysistoxin-2 (DTX-2), Dinophysistoxin-1(DTX-1), Yessotoxin (YTX) và 1-Homoyessotoxin (Homo-YTX) trong mẫu thủy sản được chiết bằng methanol. Sau đó, dịch chiết được làm sạch (loại màu và chất gây nhiễu) sử dụng kỹ thuật chiết pha rắn (SPE). Độc tố Lipophilic trong dịch chiết được khẳng định và định lượng bằng sắc ký lỏng khối phổ ba tứ cực (LC-MS/MS) sử dụng kỹ thuật ngoại chuẩn dựng trên dung môi. Giới hạn định lượng (25 µg/kg đối với nhóm YTX và 10 µg/kg đối với nhóm AZA, OA và PTX) hoàn toàn đáp ứng yêu cầu giới hạn dư lượng tối đa cho phép (MRL) của Châu Âu (EU) ở ngưỡng 160 µg/kg đối với nhóm OA, AZA và 1.000 µg/kg đối với nhóm YTX . Phương pháp đã được thẩm định trên nền mẫu thủy sản gồm nghêu, cá và thủy sản phối chế cho độ chính xác phù hợp (độ thu hồi từ 92,4%-101,5% và độ lệch chuẩn tương đối thấp hơn 20%). Phương pháp đã được sử dụng trong chương trình thử nghiệm thành thạo do tổ chức quốc tế Quasimeme tổ chức cho kết quả thỏa mãn (z-score trong khoảng ±2).

Từ khóa: *Độc tố sinh học biển Lipophilic, LC-MS/MS, SPE, nhuyễn thể 2 mảnh vỏ.*