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Simultaneous detection of three biotoxins causing diarrhetic shellfish poisoning (okadaic acid, dinophysistoxin-1, dinophysistoxin-2) in oyster by LC-MS/MS

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ABSTRACT

A LC-MS/MS method was developed for simultaneous determination of okadaic acid, dinophysistoxin-1 and dinophysistoxin-2 in shellfish. The development of such a method was necessary for specific determination of these toxins, which was not assured by available officical methods in Vietnam using mouse bioassay and HPLC with fluorescent detection. Chromatographic separation of toxins was done on a ultra performance C18 column (50 mm x 2.1 mm; 1.8 µm) and isocratic elution was carried out with a acetonitrile – water mixture (35 : 65, v/v) containing 0.1% formic acid and 0.01 M of ammonium formate as mobile phase. The detection of toxins was done with electrospray ionization in negative mode, with the ESI ion source set at 5500 V, using one precursor ion (m/z = 803 for okadaic acid and dinophysistoxin-2, m/z = 817 for dinophysistoxin-2) and two product ions (m/z = 563 and 255) for identification of each toxin and the intensity of product ion m/z = 255 for quantitative determination of each toxin. The method was fully validated and proved to be reliable for intended purpose. The method was applied to analyze 39 oyster samples collected in Thanh Hoa province (Vietnam), in which 6 samples contained trace amounts of okadaic acid (3.1 – 9.5 ppb), 2 other samples contained trace amounts of dinophysistoxin-2 (4.4 and 6.4 ppb). The toxin detection in oyster showed a seasonal variation with higher frequency in dry season. No oyster sample was detected with toxin level higher than accepted limit (160 ppb).

1. INTRODUCTION

Food safety, including the safety of seafood, in recent years has became a hot topic in Vietnam, especially after serious marine environmental incident in coastal area of Mid-Vietnam provinces in 2016. So far, many poisoning symptoms in human due to consuming toxic seafood have been recorded in the world, including diarrheic shellfish poisoning (DSP)[1]. This disease was caused by a group of natural toxins consisting of okadaic acid (OA)[2], dinophysistoxin-1 (DTX-1), dinophysistoxin-2 (DTX-2)[3] and derivatives, mostly esterified ones, of OA, DTX-1, DTX-2, which were generally known as dinophysistoxin-3 (DTX-3). These toxins were produced in the nature by some dinoflagellate species belonging to the genus *Dinophysis* such as *D.fortii*[1] , *D.caudata*[1] and by some species of the genus *Prorocentrum*

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such as *P. lima* and *P. concavum*[1]. The first DSP outbreak was recorded in Japan in 1978[4], following by other outbreaks in Europe[5], North America[6] and South America[7]. In South East Asia, although officially no DSP outbreak has been detected yet, but algae bloom of Dinophysis species accompanied by high levels of DSP toxins in green mussels has been recorded in Philippines[8], while persistent low levels of DSP toxins were found in green mussels in Singapore[9]. Among the dinoflagellate species producing OA and DTXs, *D. caudata* has been detected in Vietnamese sea[10], therefore, the bivalves shellfish in Vietnamese coastal area can be exposed to OA and DTXs and accumulate these toxins in their bodies. Currently, DSP toxins (principally OA and DTXs) were among natural toxins to be controlled in shellfish according to requirements of authority in many countries. In general, the accepted limit for DSP toxins in shellfish was 0.16 mg of okadaic acid equivalent per kg of shellfish meat[11,12]. In Vietnam, the same limit is applied, more from the need to conform with requirements of stringent import markets than from the knowledge of actual presence of OA and DTXs in shellfish at Vietnamese sea coast because the risk of their accumulation in shellfish has not yet been thoroughly studied. The content of OA and DTXs in shellfish can be estimated indirectly through the toxicity on testing animal (mouse bioassay method-MBA)[13], or can be determined directly by physicochemical analytical technique, such as liquid chromatography with fluorescent detector[14,15] or mass spectrometry detection[16-18]. In Europe, LC-MS/MS was selected as preference technique to determine OA and DTXs in shellfish[17] due to its high specificity and sensitivity, enabling direct analyze of toxin without the need of derivatization as with fluorescent detection. However, in Vietnam, no official LC-MS/MS was yet developed for the determination of these toxins in shellfish. Moreover, no study using LC-MS/MS for accurate assessment of OA and DTXs in shellfish was carried out in Vietnam, leaving an important gap on comprehensive knowledge about the actual presence of these toxins in seafood along Vietnamese seacoast. In order to contribute to a better understanding of DSP risk in Vietnam, in this paper, an UPLC-MS/MS was developed and applied to determine OA, DTX-1 and DTX-2 in oyster, a shellfish widely used for human consume in Vietnam. This method was rapid (running time was 7 minutes), simple (using isocratic elution instead of gradient elution for chromatographic separation of toxin, and a simple methanol extraction for sample preparation), and will be used as analytical tool for a more long-term and widespread study on the actual contamination rate and level of OA and DTXs in shellfish along the seacoast of Vietnam.

2.MATERIALS AND METHODS

2.1.Objects of study

Oyster samples: 39 oyster samples were collected in Thanh Hoa province in the period from September 2016 to May 2017. All samples belonging to the species *Crassostrea rivularis*.

2.2. Standards, chemicals, reagents

- Toxin standards: OA (Batch No: 20141119, 8.4 ppm solution in methanol), DTX-1 (Batch No: 20151219, 8.5 ppm solution in methanol), DTX-2 (Batch No:20150819, 3.8 ppm solution in methanol): All standards obtained from National Research Council Canada (NRCC) (Ottawa, Ontario, Canada).

- HPLC grade solvents: Methanol, acetonitrile purchased from Merck (Darmstad, Germany).

- PA grade chemicals: ammonium format, formic acid, isopropyl acetate purchased from Merck (Darmstad, Germany).

2.3. Instrumentation

- UPLC column: Zorbax Eclipse Plus C18 (2.1 x 50 mm; 1.8mm) purchased from Agilent (Santa Clara, CA, USA).

- UPLC-MS/MS system: UPLC apparatus LC20AD-XR of Shimadzu (Kyoto, Kyoto, Japan) connected to ABSciex Triple Quad 5500 of Sciex (USA).

2.4.Method

2.4.1. Sample preparation procedure

Oyster samples were stored at 4-8°C with dry ice and transported within 24 hours from sampling sites to laboratory. At laboratory, samples were stored at -75°C. Before analysis, samples were defrosted; the shellfish meat was taken out from shells and homogenized before extraction procedure.

To extract toxins from shellfish matrix, many studies have used methanol[17,19,20] or methanol-water mixture at different composition[21] and proved that these solvents were efficient for extracting OA and DTXs from shellfish tissues. However, the volume of solvent used for extraction was quite important, up to 10 ml per g of shellfish tissue[17,19], leading to a significant dilution of toxin concentrations comparing to original levels in shellfish. In our preliminary analysis, with this degree of dilution, it was virtually impossible to detect toxins at very low levels in shellfish, even with the use of UPLC-MS/MS. Our preliminary experiments also found that the use of methanol-water mixture for toxin extraction resulted in co-extraction of more other matter from shellfish matrix in comparison to the use of methanol. This problem exaggerated when water content in solvent mixture increased from 10% to 20%, and these co-extracting matters can cause serious problem for the UPLC-MS/MS system. To eliminate completely these co-extracting matters, another cleaning step by liquid-liquid extraction or solid phase extraction was necessary (results not showed). Therefore, methanol was chosen as extracting solvent and the volume of methanol used for extraction was investigated to find the smallest volume permitting a complete extraction of toxins. Artificially contaminated samples were prepared by spiking toxin standard to shellfish meat and homogenized to obtained the level of each toxin (OA, DTX-1 and DTX-2) about 50 ppb (ng/g of shellfish meat). These samples was extracted first with 3 ml of methanol, then stepwise with 2 ml of methanol until the extraction no longer contain toxin. On 6 artificially contaminated samples, experimental results showed that a 2-steps extraction was sufficient to extract all spiked toxins from sample matrix, and validation results also showed good precision and accuracy. Therefore, a 2-steps extraction with 3 ml and 2 ml of methanol consecutively was enough. To minimize the problem of co-extracting and simplify the sample preparation process, after methanol extraction, the extracting liquid was treated by centrifuging at room temperature and at 4°C. The supernatant liquid obtained after centrifuging at room temperature showed turbidity when mixed with equal volume of acetonitrile or after 24 hours keeping in refrigerator at 4°C, whereas no turbidity was found with supernatant liquid obtained after centrifuging at 4°C. This showed that the centrifuge step at low temperature helped eliminate effectively co-extracting matters and obtain cleaner extraction liquid. To concentrate the toxins and permit lower detection limit of toxin in shellfish, after centrifuging at 4°C, the obtained supernatant was evaporated under nitrogen flow and the residue was reconstituted in 500 µl of methanol to inject into LC-MS/MS system. This sample preparation procedure was simple, with good extraction efficiency for all toxins (see Table 3) and permitting a 2-folds concentration of toxin levels when injecting to UPLC-MS/MS system. From the results obtained from examining suitable extracting conditions, the following sample preparation was used in this study:

About 1 g of homogenized shellfish meat was precisely weighed into a 15-ml centrifuge tube, mix with 3 ml of methanol to extract the toxin by vortex shaking in 2 minutes. The tube was centrifuged at 14,000 rpm at 4°C in 5 minutes, then the liquid was transferred immediately to another 15-ml centrifuge tube. The shellfish meat portion in the first tube was extracted with another 2 ml of methanol by vortex shaking in 2 minutes, then centrifuged at 14,000 rpm at 4°C in 5 minutes, and the liquid layer was collected immediately into the second

tube. The liquid in second tube was evaporated under nitrogen flow and the residue was dissolved with 500 µl of methanol. To determine the free content of OA, DTX-1 and DTX-2 in shellfish samples, the obtained solution was injected directly into the UPLC-MS/MS system.

For method validation, a clean shellfish sample was spiked with standard substances of OA, DTX-1 and DTX-2 at 4 levels for each toxin: 2.5 ng/g, 5 ng/g, 20 ng/g and 40 ng/g.

2.4.2. Standard preparation procedure

The standard solution of OA, DTX-1, DTX-2 obtained from NRCC was precisely diluted with methanol to desired concentration. Single standard solutions at 100 ppb were prepared for OA, DTX-1 and DTX-2 separately for peak identification. Mix standard solution containing all the 3 toxins at concentration from 5 ppb to 100 ppb were prepared for method validation and determination of toxins in shellfish samples.

2.4.3. Analysis procedure with UPLC-MS/MS system

For analyzing OA, DTX-1 and DTX-2 by mass spectrometry, the most common ionization technique was electrospray ionization (ESI) in both positive mode (ESI^+)[22] and, negative mode (ESI^-)[17,20,23-25], between them ESI^- was more commonly used. In ESI^- mode, MRM detection was applied with $[M-H]^-$ precursor ion of toxin (m/z 803 with OA and DTX-2 and m/z 817 with DTX-1) and product ions with m/z 113, 255 and 563 being used for identification of toxins, and quantitative determination was done with product ion m/z 255[17,20,23-25]. The use of ESI^- mode was preferred because studies have showed that at negative ion mode, OA and DTX-1, DTX-2 all gave precursor ion [M-H] with high intensity, and the $[M-H]^-$ ion of OA (m/z 803) and DTXs (m/z 817 for DTX-1 and m/z 803 for DTX-2) all produced product ions having m/z 563, 255, and 113, a characteristics highly specific for DSP toxins, in which product ion m/z 255 had highest intensity, suitable for quantitative application[26, 27]. In contrast, the fragmentation pathways of OA and DTXs in positive ion mode was more diversified and more complicated for quantitative application[27], therefore ESI^+ mode was less common in analysis of OA and DTXs than ESI^- mode. In this study, ESI^- mode was used for MS/MS detection of toxins with the $[M-H]^-$ precursor ion and 2 product ion m/z 255 and 563 for identification of toxins, and product ion m/z 255 for quantitative analysis.

In ESI^- mode, because $[M-H]^-$ ion and its product ions of OA and DTX-2 having the same value of m/z, these two toxins can not be distinguished by MS/MS detection and must be separate by UPLC process. The separation of OA and DTXs were done mainly by reverse phase liquid chromatography on C18 column[17,22,25], C8 column[23,24], with mobile phase using mixture of acetonitrile and water containing ammoniac at basic pH [17,23] or a mixture of acetonitrile and water at acidic pH containing formic acid [20,22] or formic acid and ammonium formate [17,24,28]. In this study, a conventional HPLC column (packed with 5 µm particles) and a UPLC column (packed with 1.8 µm particles), both contained C18 stationary phase, were tried in the preliminary study with mobile phase consisted of acetonitrile – water containing formic acid and ammonium formate. With the conventional column, the use of gradient elution was needed to obtain complete separation between OA and DTX-2 and an acceptable separation time (10 minutes) at flow rate of 1.0 mL per minute (results not showed). With the UPLC column, at flow rate 0.3 ml per minute with isocratic elution, OA and DTX-2 were completely separated and all toxins were eluded within 6 minutes in optimized conditions. Therefore, the final method was established with UPLC column Zorbax Eclipse Plus. In terms of chromatographic resolution and separation time, the UPLC conditions used in this study were comparable to those published in other papers [19,20,29]. From studying published works by other authors and experimental results to optimize analysis conditions, the following LC-MS/MS method was used for analyzing OA, DTX-1 and DTX-2 in this study:

The chromatographic separation of OA, DTX-1 and DTX-2 was done in UPLC column Zorbax Eclipse Plus C18 (2.1 x 50 mm; 1. 8mm) maintained at 25°C. The isocratic elution of toxins was done with a mixture of acetonitrile – water (35 : 65, v/v) containing 0.1% of formic acid and 0.01 mM of ammonium formate as mobile phase. The flow rate of mobile phase was maintained at 0.3 ml per minute. Injection volume was 5 µl. The detection of OA, DTX-1 and DTX-2 was realized by using MS/MS detector equipped with an ESI source and set at ESI^- mode with working conditions as per Table 1. The detection of OA, DTX-1 and DTX-2 was done in MRM mode by monitoring 1 precursor ion and 2 product ions for each toxin (Table 2), in which product ion with m/z = 255 was used for quantitative determination of the toxins and product ion with m/z = 563 was used for identification purpose.

Table 1. Working condition of ESI source

Parameter	Optimal value	Unit
Ion source voltage	-4500	V
Ion source temperature	450	°C
Ion source Gas 1	30	Psi
Ion source Gas 2	30	Psi
Curtail Gas	25	Psi
Collision Gas Pressure	7	Psi

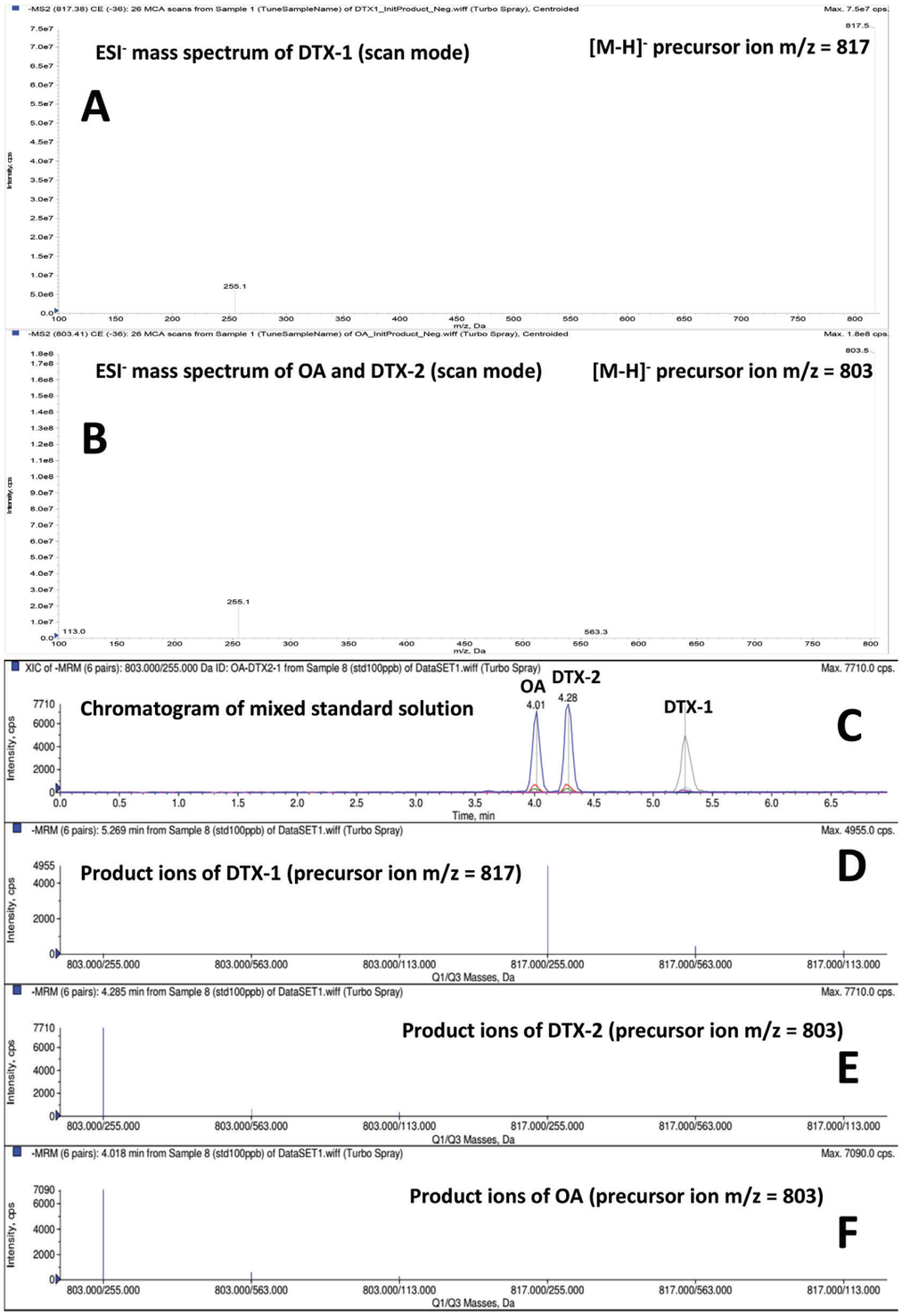
Table 2. Conditions for forming product ions

Toxin	Precursor ion (m/z)	Product ion (m/z)	Declustering Potential (V)	Capillary Energy (eV)	Collision cell exit Potential (V)
OA	803	255	-85	-60	-17
		563		-55	-17
DTX1	817	255	-85	-60	-17
		563		-55	-17
DTX2	803	255	-85	-60	-17
		563		-55	-17

3.RESULTS

3.1.Method validation

The method as described at 2.4 was able to isolate OA, DTX-1 and DTX-2 from oyster matrix and identify them by the chromatographic separation and the presence of specific ions of each toxins in MS/MS detection. Typical mass spectra and chromatograms of OA, DTX-1, DTX-2 were presented in Figure 1. To assure the reliability of analytical results, the sample preparation procedure and the UPLC-MS/MS analysis procedure were validated in term of specificity, linearity, precision, accuracy, limit of quantitation (LOQ) and limit of detection (LOD) according to the requirements for analytical methods of AOAC International and European Commission.


-MS2 (817.38) CE (-36): 26 MCA scans from Sample 1 (TuneSampleName) of DTX1_InitProduct_Neg.wiff (Turbo Spray), Centroided
Max. 7.5e7 cps.
ESI- mass spectrum of DTX-1 (scan mode)
[M-H]- precursor ion m/z = 817
A
255.1
m/z, Da
-MS2 (803.41) CE (-36): 26 MCA scans from Sample 1 (TuneSampleName) of OA_InitProduct_Neg.wiff (Turbo Spray), Centroided
Max. 1.8e8 cps.
ESI- mass spectrum of OA and DTX-2 (scan mode)
[M-H]- precursor ion m/z = 803
B
255.1
113.0
563.3
m/z, Da
XIC of -MRM (6 pairs): 803.000/255.000 Da ID: OA-DTX2-1 from Sample 8 (std100ppb) of DataSET1.wiff (Turbo Spray)
Max. 7710.0 cps.
Chromatogram of mixed standard solution
OA
4.01
DTX-2
4.28
DTX-1
C
Intensity, cps
Time, min
-MRM (6 pairs): 5.269 min from Sample 8 (std100ppb) of DataSET1.wiff (Turbo Spray)
Max. 4955.0 cps.
Product ions of DTX-1 (precursor ion m/z = 817)
D
803.000/255.000
803.000/563.000
803.000/113.000
817.000/255.000
817.000/563.000
817.000/113.000
Q1/Q3 Masses, Da
-MRM (6 pairs): 4.285 min from Sample 8 (std100ppb) of DataSET1.wiff (Turbo Spray)
Max. 7710.0 cps.
Product ions of DTX-2 (precursor ion m/z = 803)
E
-MRM (6 pairs): 4.018 min from Sample 8 (std100ppb) of DataSET1.wiff (Turbo Spray)
Max. 7090.0 cps.
Product ions of OA (precursor ion m/z = 803)
F

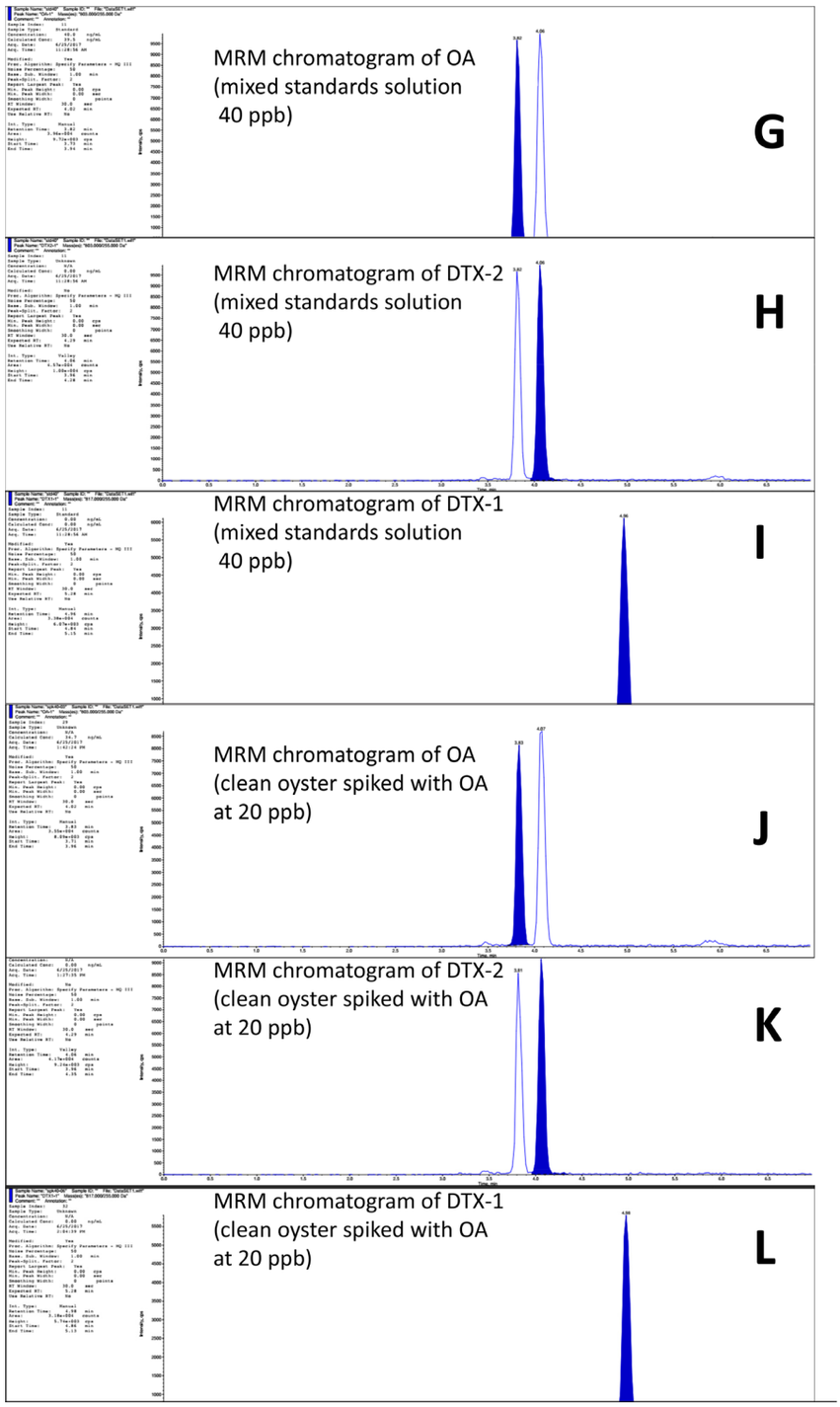


Figure 1. Typical mass spectra and chromatograms of OA, DTX-1 and DTX-2. A: Mass spectra of DTX-1 at ESI^- scan mode; B: Mass spectra of OA and DTX-2 at ESI^- scan mode; C:Chromatogram of mixed standard solution; D:Product ions of DTX-1; E:Product ions of DTX-2; F:Product ions of OA; G:Chromatogram of OA in MRM mode of mixed standard solution 40 ppb; H: Chromatogram of DTX-2 in MRM mode of mixed standard solution 40 ppb; I:Chromatogram of DTX-1 in MRM mode of mixed standard solution 40 ppb; J:Chromatogram of OA in MRM mode of spiked shellfish sample; K: Chromatogram of DTX-2 in MRM mode of spiked shellfish sample; L: Chromatogram of DTX-1 in MRM mode of spiked shellfish sample (sample was spiked with toxin standards at 20 ppb (the toxin concentration in solution injected to LC-MS/MS apparatus was concentrated 2 times).

In terms of specificity, the identification of OA, DTX-1 and DTX-2 by MS/MS detector with 1 precursor ion and 2 product ions for each toxin assured IP = 4, meeting the requirements of EC[30]. In chromatogram, the peaks of OA, DTX-1, DTX-2 were all fully resolved on chromatograms (see Figure 1C), which was important because OA and DTX-2 must be distinguished by their respective retention times because they gave precursor ion and product ions with identical value of m/z in ESI mode (Figure 1B, 1E, 1F). Furthermore, the chromatograms obtained with clean shellfish sample spiked with standard toxins also showed that this method was able to determine OA, DTX-1 and DTX-2 in shellfish without interference from the sample matrix (Figure 1J, 1K, 1L). Therefore, the method with conditions and procedures as being described at 2.4 is specific for the determination of OA, DTX-1 and DTX-2 in shellfish.

In terms of linearity, accuracy, precision, LOD and LOQ, the validation results were summarized in Table 3. The obtained results in Table 3 proved that the method met requirements of AOAC in term of accuracy and precision. The LOD, LOQ of the method for each toxin were 0.75 ng and 2.5 ng per g of oyster meat, respectively. These limits were far below the general accepted limit of 160 ppb for total amount of toxins causing DSP, expressed in OA or equivalent of OA, in shellfish for human consume. It showed that the method was sensitive enough for control of OA, DTX-1 and DTX-2 in oysters, and this method was also more sensitive than some other methods already published[15,20].

Table 3. Summary of validation results

Criteria	*OA*	*DTX2*	*DTX1*
Linearity	5 – 100 ppb: y = 905.6x + 568.1 (R^2 = 0.997)	5 – 100 ppb: y = 786.4x + 674.9 (R^2 = 0.997)	5 – 100 ppb: y = 1082x – 184.0 (R^2 = 0.998)
Precision (n = 6 at each spiked level)	2.5 ppb: RSD = 5.6 % 5 ppb: RSD = 5.0 % 20 ppb: RSD = 2.9 % 40 ppb: RSD = 2.3 %	2.5 ppb: RSD = 5.4 % 5 ppb: RSD = 2.4 % 20 ppb: RSD = 1.9 % 40 ppb: RSD = 1.1 %	2.5 ppb: RSD = 8.0 % 5 ppb: RSD = 3.8 % 20 ppb: RSD = 3.5 % 40 ppb: RSD = 2.1 %
	Requirement of AOAC: RSD (%) ≤ 21 % with concentration from ≥ 10 ppb to 100 ppb [31].		
Accuracy (the recovery rate (%) with n = 6 at each spiked level)	2.5 ppb: 98 - 113 % 5 ppb: 92 - 105 % 20 ppb: 94 - 101 % 40 ppb: 87 - 93%	2.5 ppb: 99 - 107 % 5 ppb: 103 - 110 % 20 ppb: 93 - 98 % 40 ppb: 89 - 92 %	2.5 ppb: 73 - 91 % 5 ppb: 93 - 103% 20 ppb: 93 - 98 % 40 ppb: 89 - 94 %
	Requirement of AOAC: recovery from 60 – 115 % with concentrations from 10 ppb to <100 ppb and recovery from 80 – 110 % with concentrations from 100 ppb to < 1ppm [31].		
LOQ (ng/g of shellfish meat)	2.5	2.5	2.5
LOD (ng/g of shellfish meat)	0.75	0.75	0.75

3.2. Determination of OA, DTX-1 and DTX-2 levels in oyster

The amount of OA, DTX-1 and DTX-2 were determined for 39 samples of oyster (*C. rivularis*) collected in Thanh Hoa province. DTX-1 was not detected in any sample. In contrast, 6 oyster samples were detected as containing OA, in which 5 samples having OA level above the LOQ of analysis method, varying from 3.1 to 9.5 ppb. Another sample contained OA but at level lower than the LOQ of analysis method (see Table 4). Besides, 2 other samples were detected as containing DTX-2 at levels of 4.4 ppb and 6.4 ppb, respectively. And no sample was found containing more than one type of toxin. Typical chromatograms of oyster sample which did not contain toxin and those samples containing OA and DTX-2 were showed in Figure 2.

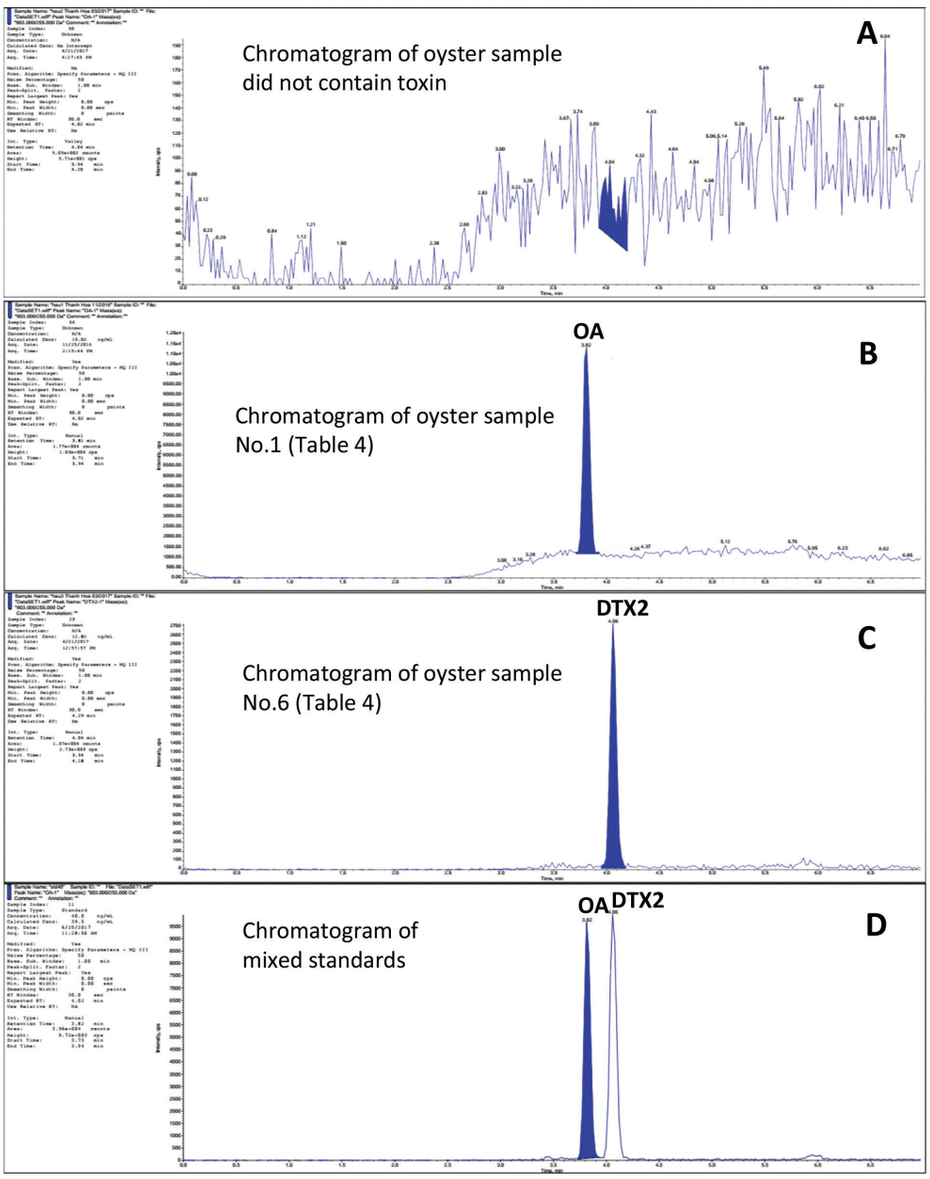


Figure 2. Typical chromatograms obtained with oyster sample. A: Chromatogram of oyster sample did not contain toxin; B: Chromatogram of oyster sample No.1 (Table 4); C: Chromatogram of oyster sample No.6 (Table 4); D:Chromatogram of mixed standards.

Table 4. Summary about shellfish samples containing toxins

No	Sampling date	Detected toxin	Level of toxin (ppb)
1	November 2016	OA	9.5
2	November 2016	OA	5.1
3	September 2016	OA	4.7
4	February 2017	OA	4.3
5	October 2016	OA	3.1
6	March 2017	DTX2	6.4
7	February 2017	DTX2	4.4
8	March 2017	OA	+

Note: n.d.: not detected; +: toxin detected at level lower than LOQ

4. DISCUSSION

Using LC-MS/MS for simultaneous qualitative and quantitative detection of DSP toxins was the current prevailing trend on selection of analytical technique. The conditions for chromatographic separation and for MS/MS detection of the method developed in this study were selected based on other published works (as mentioned in 2.4) and adjusted to obtain a simple, rapid and reliable analysis method (as confirmed by validation results in Table 3 and chromatographic results as in Figure 1 and Figure 2). However, the most remarkable contribution of this study is the sample preparation procedure by methanol extraction following by centrifuging at 4°C and a toxin concentrating step by evaporation and reconstitution. This sample preparation procedure did not require complex cleaning steps or the use of expensive solid phase extraction column, and enabling the detection of toxins in shellfish down to 0.75 ppb. The suitability of this sample preparation procedure as well as that of the whole analysis method for intended application was also confirmed from experimental results obtained from real oyster samples, in which toxins were extracted effectively from shellfish matrix and specifically analysed.

This study is the first work investigating the presence of OA and DTXs in the oyster species *C. revularis* in a coastal region of Vietnam. Within the scope of bibliographic research for this paper, the authors found only one long-term study on the presence of OA and DTXs in shellfish in South East Asia. In the work of Holmes MJ, et al[9], persistent low concentrations of DSP toxins (both OA and DTXs) were found in green mussel in the period between October 1995 and December 1997. The highest toxin level found in the work of Holmes MJ, et al was 23.7 ppb of OA, higher than the highest level of 9.5 ppb found in oyster sample in this study (see Table 4). Although there was yet no comparative study about the accumulation of DSP toxins in green mussel *Perna viridis* and *C. revularis*, a study in Korea has showed that the Pacific oyster *C. gigas* accumulated less DSP toxins than the mussel *Mytilus* spp. at the same sampling location[21]. Therefore, it was probable that the low levels of toxin found in oyster in this study come partly from the biological characteristics of *C. revularis*.

The sampling period in this study was from September 2016 to May 2017, or covering practically all the dry season in Vietnam (from November to April), and half of the wet season (from May to October). It was noteworthy to point out that 7 out of 8 samples containing toxin were detected during the period of dry season, and only 1 sample (in September 2016) was found containing toxin during the period of wet season (see Table 4). However, to reach a definite conclusion about possibility of a seasonal variation of toxin presence in oyster, the sampling must be prolonged to cover the whole period of wet season. So far, officially no

DSP outbreak was yet reported in South East Asia, but DSP toxins have been found at levels unsafe for human consume in green mussels[8], so the potential risk still existed. In this study, the finding of contaminated oyster almost each month from September 2016 to March 2017 showed that oysters at sampling location were in continual exposure with toxin-producing microalgae, but perhaps at low concentration of algae cells that could not produce important intake and accumulation of toxin in oysters.

Despite the presence of OA in 6/39 samples and that of DTX-2 in 2/39 samples, the detected levels of OA and DTX-2 in these samples were much lower than the accepted limit of 160 ppb imposed by authority in Vietnam as well as in many other countries. Therefore, these samples of oyster were still safe for human consume. However, long-term studies have also showed that the contamination of DSP toxins in shellfish at the same location may have significant year-to-year variation and even more significant between multiannual periods[21,32]. So the presence of OA and DTX-2 in oyster, even at very low levels, confirmed the potential risk of DSP outbreak in Vietnam and the necessity of monitoring toxins in shellfish for human consume in general.

5. ACKNOWLEDGEMENT

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