

Determination of okadaic acid related toxins from shellfish (*sinonovacula constricta*) by high performance liquid chromatography tandem mass spectrometry

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ABSTRACT

Consumption of shellfish contaminated with algal toxins produced by marine dinoflagellates can lead to diarrhetic shellfish poisoning (DSP). It was therefore essential that there are analytical techniques to identify and quantify DSP toxins in shellfish. This new methodology could facilitate DSP monitoring and create a means of rapidly responding to incidents threatening public health. In the last years there were different analytical methods for DSP, such as mouse bioassay and LC-FLD. With the development of instrument, Liquid chromatography-mass spectrometry was substituted for other analytical methods with its good sensitivity and selectivity and without derivatization for the determination of DSP. In this report, a high performance liquid chromatography-tandem mass spectrometric (HPLC-MS/MS) method was developed for the simultaneous determination of okadaic acid (OA) and dinophysistoxins(DTX-1) in *Sinonovacula constricta*. Optimization of pretreatment experiment was carried out to maximize recoveries and the effectiveness. The analytes were determined under multi-reactions monitoring (MRM) scan type with tandem mass analyzer using negative ion electrospray ionization (-ESI) mode. Finally, the detection and identification of OA and DTX-1 were based upon their retention times (RT) and the fragmentation patterns of their mass spectra. The method of LOQ for the two poisons was $0.02 \text{ mg} \cdot \text{kg}^{-1}$. The real sample test showed that this method could be used for sensitive, fast, and accurate determination of the two diarrhetic shellfish poisons in shellfish.

Keywords: *Sinonovacula Constricta*; High Performance Liquid Chromatography-Tandem Mass Spectrometry; Okadaic Acid; Dinophysistoxins-1

1. INTRODUCTION

Among the phycotoxin-related toxic phenomena, Diarrhetic Shellfish poisoning (DSP) is a severe gastrointestinal illness caused by consumption of shellfish contaminated with toxigenic dinoflagellates. Toxins responsible for DSP intoxication belong to the group of the lipophilic marine biotoxins. The main cause of worldwide DSP syndrome (Yasomotor *et al.*, 1993) are okadaic acid (OA) and its derivatives named dinophysistoxins (DTXs). These toxins have been shown to be potent phosphatase inhibitors, a property which can cause inflammation of the intestinal tract and diarrhea. OA and its 35-methyl derivative named dinophysistoxin-1 (DTX-1) (Figure 1) have also been shown to have tumors-promoting activity (Sylvaine *et al.*, 2002). In order to prevent human intoxication, many monitoring program of shellfish toxicity have been established in many developed countries. According to the current regulation with respect to this issue, the maximum permitted level for marketable shellfish is $0.16 \mu\text{g}$ OA equivalent/g shellfish meat (Emilia *et al.*, 2008).

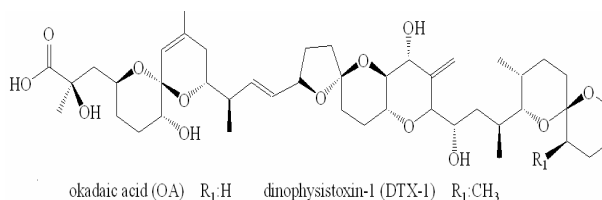


Figure 1. Structure of Okadaic acid (OA) and Dinophysistoxins-1 (DTX-1).

Although, routine monitoring of shellfish for DSP toxins is generally carried out using mouse bioassay (Pamela *et al.*, 1997; Nuria *et al.*, 2007), this approach suffers from poor reproducibility, low sensitivity, and interferences from certain endogenous compounds. Thus, instrumental methods offer the possibility of precise, sensitive and automated determination of the individual DSP toxins. The most of the previous studies on determination of DSP toxin profiles employed methods that targeted acidic polyether toxins. In fact, such toxins can be derivatized by using fluorometric derivatization reagents, which namely dramatization 9-anthryldiazomethane (ADAM) (Kevin *et al.*, 1997), 4-bromo-methyl-7-methoxycoumarin (Br-Mmc) (Shen *et al.*, 1997), 1-bromoacetylpyrene (BAP) (Jose C *et al.*, 2000), 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone (BrDMEQ) and 9-chloromethylantracene (CA) (Nogueiras *et al.*, 2003), then followed by quantification using liquid chromatography with fluorimetric detection(LC-FLD).The major disadvantage of this method is that toxins lacking the carboxylic acid functionality cannot be revealed. Although these were obvious improvements of the LC methods based on fluorometric derivatization reagents, they were unstable and not always available.

In the latest decade, liquid chromatography coupled with mass spectrometry (LC-MS) using atmospheric-pressure ionization (API) has proven to be the most valuable instrumental tool for direct determination of toxins without derivatization (Rosa *et al.*, 1995; Toshiyuki and Takeshi, 2000; Shinya and Katsuo, 2001; Patrizia *et al.*, 2006). It has both high sensitivity and selectivity, which makes it possible to determine DSP by direct injection. Meanwhile, Liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Lincoln Mackenzie *et al.*, 2002; Patricia *et al.*, 2004; Suzuki *et al.*, 2004; Beatriz *et al.*, 2007) has been shown to be a valuable analytical tool for identifying and quantifying the shellfish poisons and their metabolites. Moreover, it is a particularly useful method for handling very small samples with low analytical concentrations.

The primary aim of this work was to develop a rapid and sensitive method for the simultaneous determination and confirmation of OA, DTX-1 in shellfish at low levels by means of high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS).

2. MATERIALS AND METHODS

2.1. Materials and Reagents

Shellfish used as the negative control and for spiking and for test were collected from the southeastern coast, Zhejiang, China in June 2009. The adductor muscle and digestive glands were separated from other tissues, homogenized and kept frozen at -20°C until used.72 samples collected from 8 different areas were tested (Figure 2).



Figure 2. Origins of *Sinovacuula constricta*.

HPLC grade solvents (acetonitrile, methanol) and analytical grade solvents (n-hexane, chloroform, acetone, acetic acid) were purchased from Tedia (Ohio, USA). Distilled water was passed through a Milli-Q water purification system (Millipore, France). Sep-Pak silica plus cartridge columns (500 mg, 3 mL) were purchased from Supelco (Milford, MA, USA). Purified OA standard ($\geq 95\%$) was purchased from Sigma-Aldridge (Dublin, Ireland), DTX-1(90%) was purchased from Wako(Osaka, Japan).

2.2. Sample Extraction and Purification

The Homogenized shellfish hepatopancreas (2 g) was mixed with 10 mL 80% methanol for 1min in a 50 mL polypropylene tube, after ultrasonic extraction during 5 min, then centrifuged for 10 min at 4000 rmin⁻¹. An aliquot (5 mL) of the supernatant was transferred to another 15 mL tube, washed with 5 mL hexane. The hexane layer was aspirated to waste and 1 mL water was added to the residual solution, and then was extracted with 6 mL chloroform. The water layer was transferred to another 15 mL tube and extracted with chloroform (2 mL \times 3 mL) again. The chloroform extracts were combined and evaporated to dryness under nitrogen at 60°C and reconstituted in 1ml 20% hexane-acetone.

A Sep-Pak silica cartridge was conditioned sequentially with 10mL acetone, 10 mL methanol and 10 mL 20% hexane/acetone. Then the column was load with the 1mL extract sample and washed with 1ml 20% hexane/acetone followed by 10 mL 3% methanol/acetone. After drying, the remaining toxins were eluted with 10 mL 40% methanol/acetone and evaporated to dryness under nitrogen at 45°C .Then the residue was dissolved in 1mL 80% methanol. Finally the extract was filtrated through 0.22 μm organic filter and analyzed by HPLC-MS/MS.

2.3. HPLC-MS/MS Analysis

HPLC-MS/MS was performed on an HP 1100 series liquid chromatograph (Agilent, Palo Alto, CA, USA), coupled to an API 3000 triple quadrupole mass spectrometer (Applied Biosystem) with an atmospheric pressure ionization source and an electrospray ionization (ESI) interface. The instrumentation was controlled using Analyst v.1.2 software.

Chromatographic separations of OA and DTX-1 were carried out under the following combinations of column and mobile phases: Zorbax XDB C18 (2.1 mm × 150 mm, 5 μm, Agilent) with the mobile phase, acetonitrile - 0.1% acetic acid (70:30, v/v). The column temperature and flow-rate were kept at 30°C and 0.25 mL·min⁻¹, respectively. 10 μL of sample were injected onto the column at the room temperature.

The mass spectrometer was operated by electrospray in negative ion mode (ESI-) with multiple reaction monitoring (MRM) for the detection of OA, DTX-1. The monitored ions were the [M-H]⁻ precursor ions at m/z of 803.6 (OA), 817.4 (DTX-1), respectively and the most abundant product ion observed for each toxin. The MS parameters were optimized for the ionization of standard toxins using flow injection analysis. Two different productions were used to verify the selectivity for determination of OA and DTX-1 as shown in Table 1. The optimized MRM experiment was established for the concurrent determination of the aforementioned toxins using the following conditions: Ionspray Voltage -4500 V, Auxiliary Gas Speed 7 L·min⁻¹, Turbo Ionspray Source Temperature 500°C, Nebulizer Gas 9 psi, Curtain Gas 8 psi, Collision Gas 8 psi, Focusing Potential -260 V, Entrance Potential -9 V, and Cell Exit Potential -13 V. All gases in the MRM experiment were high-purity nitrogen gas. Other optimization of MS conditions as shown in **Table 1**.

2.4. HPLC-MS/MS Assay

Stock solutions (10 mg·L⁻¹) of individual shellfish toxin standards (OA, DTX-1) were prepared by dissolving in methanol. A mixed stock solution (1 mg·L⁻¹) containing two standards was prepared from stock solutions of individual standards by mixing and diluting with methanol. Different calibration standards (20, 50, 100, 200, 500, 800 μg·L⁻¹) were prepared by appropriate dilution of the

mixed stock solution with methanol. The standards were injected directly into the HPLC-MS/MS system. The calibration curve was obtained by the peak area (y-axis) plotted against the concentration of toxins standard (x-axis). The qualitative analysis of OA and DTX-1 of the experimental samples were performed based on the retention time and the ion ration of standard solution.

2.5. The Experiment of Recovery, Precision and Accuracy

Homogenized negative shellfish hepatopancreas (2 g), which spiked with 0.02, 0.1, 0.2 and 0.4 mg·kg⁻¹ mixed standard solution of OA and DTX-1 respectively, were pretreated as section 2.2 and then analyzed by HPLC-MS/MS. Three replicate samples at each concentration were analyzed on the same day. The percentage of recovery was calculated by comparing the concentration obtained according to the calibration curve with the actual spiked concentration of standard solution (OA, DTX-1). The precision was evaluated by coefficients of variation (CV %) and the accuracy was estimated based on the average percentage of recovery.

3. RESULTS AND DISCUSSION

3.1. HPLC-MS/MS Condition Analysis

The mass spectra of each compound were measured in the positive and the negative ion modes for the precursor ion full-scan of toxins standard (1 mg·L⁻¹). It was found that the detection sensitivity for the toxins studied was better in negative rather than in positive mode with the precursor ion [M-H]⁻ at m/z 803.6 for OA, m/z 817.4 for DTX-1, respectively. The fragmentation of the target toxins was optimized to efficiently generate several product ions from each precursor ion by collision-induced dissociation (CID) and shown in **Figure 3**. Selecting two precursor/product ion combinations (Q1/Q3 pairs) as monitor ions to verify the selectivity and determination of toxins, which were m/z 803.6/255.0, 803.6/563.4 for OA, m/z 817.4/255.0 and 817.4/113.1 for DTX-1 and shown in **table 1**. To achieve optimum sensitivity and selectivity, MRM was implemented and the optimization of MS conditions as shown in the aforementioned experiment.

Table 1. Optimization of the partial MS condition.

Analyte	Precursor ion (m/z)	Product ions (m/z)	Declustering Potential (V)	Collision Energy (V)	Retention time (min)
Okadaic Acid (OA)	803.6	255.1* 563.1	-70	-60 -68	3.38
Dinophysistoxins-1 (DTX-1)	817.4	255.1* 113.1	-110	-68 -94	5.70

*Quantificational ion.

The polar solvent (methanol, acetonitrile) usually is used as mobile phase for reverse phase column C18. It was found that the efficiency of ionization with 70% methanol was inferior to that obtained in 70% acetonitrile; meanwhile ionization efficiency would be intensified with 0.1% acetic acid (Toshiyuki and Takeshi, 2000). So 70% acetonitrile containing 0.1% acetic acid was selected as the mobile phase of LC-MS/MS.

3.2. Sample Extraction and Purification

Table 2 listed the recovery of the preliminary extraction and elution with different solutions at the spiked level of $0.1 \text{ mg} \cdot \text{kg}^{-1}$ from standard toxins. The recovery of toxins from 80% methanol extracts were 94.2% for OA and 90.6% for DTX-1, slightly lower than 80% acetonitrile extracts. Besides the 90% methanol (Hirofumi *et al.*, 2001) gave least residue than other organic solvent (methanol, acetone) as extractant. Considering that the toxicity of acetonitrile was more harmful than methanol and wasted more time during evaporation under nitrogen. And there is no obvious difference for the recovery between 80% and 90% for methanol-water. Thus, experiment chose 80% methanol as extractant insuring against good recovery.

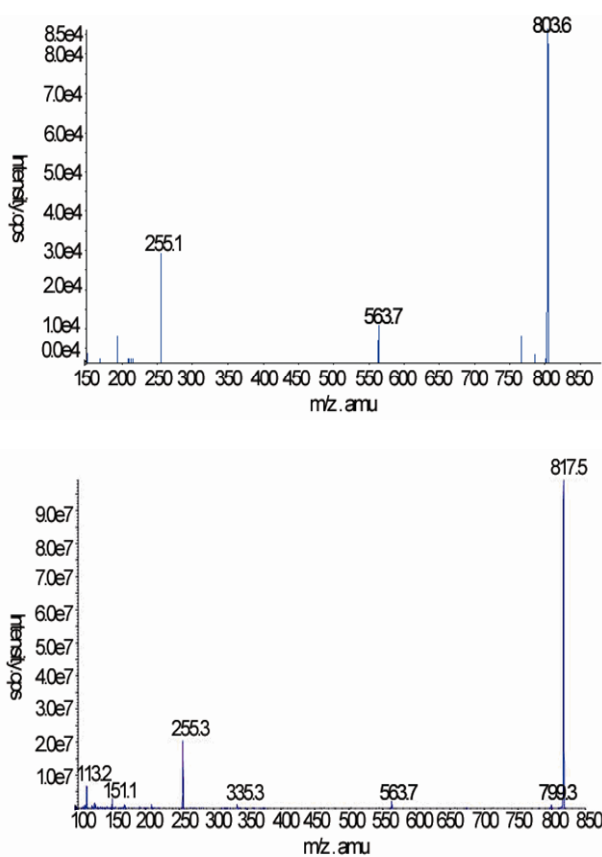


Figure 3. Product ions full-scan negative-ion ESI mass spectrums of OA (up) and DTX-1 (down).

Purification of extractant (OA and DTX-1) from the shellfish hepatopancreas was carried out by Sep-pak silica cartridge as previously described (Patrizia *et al.*, 2006) due to the significant suppression of ionization by contaminants. In test of elution for the toxins using three different rates of acetone–methanol, it shows that the recovery in 40% methanol/acetone was the highest (99% for OA, 97.8% for DTX-1) than others.

3.3. Method Evaluation (Recovery, Precision and Accuracy)

Figure 4 showed the chromatogram of the toxins (OA, DTX-1) standard with $200 \mu\text{g} \cdot \text{L}^{-1}$. From which it could

Table 2. Comparison of extract and elution from different solutions at the spiked level of $0.1 \text{ mg} \cdot \text{kg}^{-1}$.

Solution	Solution component	Recovery (% , n = 6)	
		OA	DTX-1
Extract solution	80% Methanol- water	94.2	90.6
	80% Acetonitrile- water	96.0	92.5
	40% Methanol-acetone	99.0	97.8
Elution solution	50% Methanol-acetone	93.2	95.2
	60% Methanol-acetone	91.5	84.4

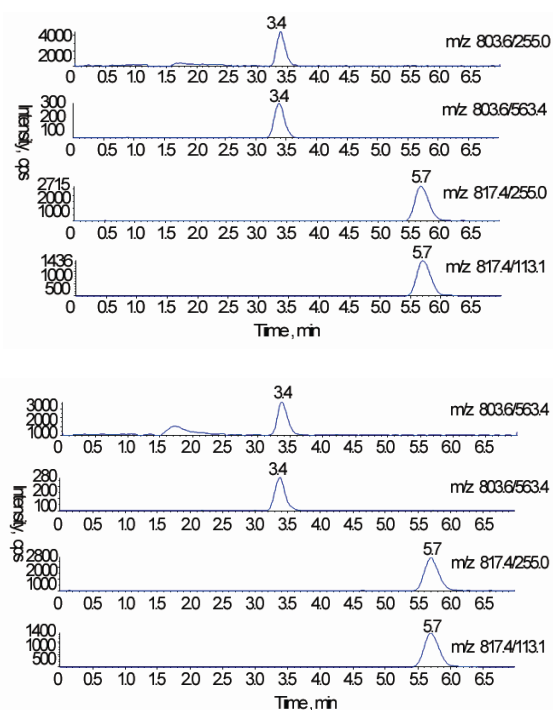


Figure 4. Chromatograms of the OA and DTX-1 standard solution ($200 \mu\text{g} \cdot \text{L}^{-1}$) (up) and the negative spiked sample ($0.1 \text{ mg} \cdot \text{kg}^{-1}$) (down).

be seen there was a single and symmetric peak and the retention time is 3.4 min for OA with the monitor ion pairs m/z 803.6/255.0, 803.6/563.4 and 5.7 min for DTX-1 with m/z 817.4/255.0, 817.4/113.1. The down of **Figure 4** showed the chromatogram of negative sample was spiked with $0.1\text{mg}\cdot\text{kg}^{-1}$. And it could be seen there was no obvious interferential peaks when the sample was pretreated as described above to the section 2.2 experiment and detected with HPLC-MS/MS. The good linearity of the peak area plotted against concentrations for the toxins with the linear (OA: $y = 20-241$, $r = 0.9995$; DTX-1: $y = 141x-85$, $r = 0.9997$) over concentration ranging from $20\ \mu\text{g}\cdot\text{L}^{-1}$ to $800\ \mu\text{g}\cdot\text{L}^{-1}$.

Table 3 gives the recovery and coefficients of variation (CV %) data corresponding to negative samples that were spiked each with 0.02, 0.1, 0.2 and 0.4 mg OA and DTX-1 per 1kg of the hepatopancreas. The average recovery of OA and DTX-1 were decreased with the spiked level from 0.02 to $0.4\ \text{mg}\cdot\text{kg}^{-1}$ because of the matrix effect. This suggested that interferential compounds of the matrix reduced ionization efficiency of toxins. It is acceptant that the mean recoveries were within the range from 79.0% to 92.2%, and the CV% was lower than 11.6%. The signal to noise (S/N) was calculated from the ratio between analyte peak signal to base line and peak-to-peak noise signal. The S/N was above 10 for the mixed standard solution ($20\ \mu\text{g}\cdot\text{L}^{-1}$). The LOQ of the method at an S/N ratio of 10 were estimated to $0.02\ \text{mg}\cdot\text{kg}^{-1}$ for the both of OA and DTX-1.

3.4. Method Application

The developed method was applied to the analysis of 72 batches of that were collected from 8 different areas in Zhejiang province. **Table 4** showed the results of the 14 positive samples. 2 samples were found OA, with the concentration of $25.6\ \mu\text{g}\cdot\text{kg}^{-1}$ and $33.0\ \mu\text{g}\cdot\text{kg}^{-1}$, respectively. 12 samples were found DTX-1, with the concentration from $84.1\ \mu\text{g}\cdot\text{kg}^{-1}$ to $293.0\ \mu\text{g}\cdot\text{kg}^{-1}$.

Table 3. Recovery and CV% for the OA and DTX-1.

Analyte	Spiked level ($\text{mg}\cdot\text{kg}^{-1}$)	Recovery (% , $n = 6$) (mean \pm S.D)	CV (%)
OA	0.02	89.4 ± 7.9	8.87
	0.1	87.9 ± 10.2	11.6
	0.2	84.7 ± 7.3	8.57
	0.4	79.0 ± 8.5	10.8
DTX-1	0.02	92.2 ± 3.8	4.09
	0.1	90.8 ± 4.1	4.51
	0.2	89.9 ± 6.3	6.98
	0.4	84.2 ± 4.7	5.54

Table 4. Results of the positive samples.

No.	Sampling time	Sampling location	Concentration ($\mu\text{g}\cdot\text{kg}^{-1}$)
1	August,2007	Shensi	DTX-1: 293.0
2	Spetember,2007	Shensi	DTX-1: 208.4
3	December,2007	Shensi	OA: 25.6
4	March,2008	Putuo	DTX-1: 155.0
5	June,2008	Putuo	DTX-1: 192.4
6	Spetember,2007	Xiangshan	DTX-1: 246.0
7	October,2007	Xiangshan	DTX-1: 168.5
8	August,2007	Shanmen	OA: 33.0
9	Spetember,2007	Shanmen	DTX-1: 257.2
10	June,2008	Wenling	DTX-1: 84.1
11	Spetember,2007	Leqing	DTX-1: 176.2
12	Spetember,2008	Leqing	DTX-1: 105.0
13	July,2007	Dongtou	DTX-1: 144.3
14	Spetember,2008	Dongtou	DTX-1: 97.8

4. CONCLUSIONS

In conclusion, the proposed method, which offers a rather newly and rapid developed extraction, clean-up of the sample, was found to have acceptable reproducibility, high specificity and sensitivity and a detection capability which allows the detection of the diarrhetic shellfish poisons (OA and DTX-1) by means of HPLC-MS/MS under multi-reactions monitoring (MRM) scan with tandem mass analyzer using negative ion electrospray ionization (-ESI) mode and identification based upon their retention times and the fragmentation patterns of their mass spectra.

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