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**Research Article**

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# Rapid DA determination of ASP in shellfishes by SPE-HPLC-MS/MS

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## ABSTRACT

*A method of the high performance liquid chromatography-tandem mass spectrometry was established to detect acids shellfish poisoning (domoic acid) residues in aquatic products. Toxin was extracted with 50% methanol by ultrasonic. The supernatant was purified by solid phase extraction column to purification after centrifugation, then was subjected to HPLC-MS/MS analysis. Under the optimized conditions of LC and MS, DA showed a good linear relationship when its concentrations were 20.0 to 1000.0 μg/ L ($R^2$>0.999). Under the added concentrations between 20.0 and 100.0 μg/ kg level, the average recovery rates were 71.3% to 84.1%, and the coefficients of variation were 4.4% to 4.6%. The detection limit of DA was 5.0 μg/ kg. The method above was sensitive and accurate to determine DA of ASP in shellfishes.*

**Key words:** Shellfishes; acids shellfish poisoning (ASP); domoic acid; high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS); solid-phase extraction (SPE).

***Abbreviations**: ASP, acids shellfish poisoning; DA, domoic acid; HPLC-MS/MS, high performance liquid chromatography-tandem mass spectrometry; SPE, solid-phase extraction; ELISA, enzyme-linked immunosorbent assay.*

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## INTRODUCTION

Aquaculture in coastal areas has developed rapidly in recent years around the world. As a result of the long-term development, marine environment has seriously deteriorated and red tides outbreak frequently. Shellfish toxin, a class of organic compounds, is caused by ingesting toxic algae of shellfish. This process is a long-term accumulation in vivo with universality and sudden [1]. There is no suitable antidote in the world so far. If people eat shellfishes contained accumulation of toxin or its products, it may cause different levels of poisoning or even death [2-4]. It is thus clear that the safety problem of aquatic products quality is closely related to human health and life safety. At present, methods of analyzing the shellfish poison include the biological method (mice bioanalysis), enzyme-linked immunosorbent assay (ELISA), high performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS) and so on [5]. Compared with the technology of other countries, the detection and analysis ability of shellfish poison is weak in China. LC-MS/ MS and some new applications of testing technology have been seldom applied in domestic. The LC-MS/ MS method, which has the advantages of high sensitivity and good selectivity, is an ideal detection and analysis method of shellfish poison with a great application potential. Automatic solid phase extraction (SPE), which can stabilize and increase the reproducibility and recovery by SPE column purification, is a widely used method of sample pretreatment. Therefore, a SPE-HPLC/ MS/ MS method combined the technique of SPE with LC-MS was established to detect and analyse the ASP (DA).

## EXPERIMENTAL SECTION

1.1 Instrument, equipments and materials.

Instrument and equipments. Triple tandem quadrupole mass spectrometer (Agilent 6410B, Agilent company, Santa Clara, USA) equipped with electrospray ion source (ESI) and liquid chromatograph was supplied by Agilent company (Agilent 1200SL, Agilent company, Santa Clara, USA). Automatic solid-phase extraction apparatus (Rapid Trace II type, Caliper company, Tampa Bay, USA). Ultrasonic cleaning machine (KQ5200DA type, Shanghai Precision Instrument Co., Ltd., Shanghai, China). Preparation system of ultrapure water (Synergy ultrapure water system Synergy type, Millipore, Massachusetts, USA).Supelclean LC-SAX solid phase extraction column (SUPELCO company, Bellefonte, USA).

Materials. Materials were shellfish samples in our lab (frozen boiled vacuum Short Necked Clams, - 20$^{o}$C cryopreservation).ASP standard was Domoic Acid purchased from International research council of Canada with the molecular formula of $C_{15}H_{21}NO_6$, the molecular weight of 311.3 g/ mol, and the concentration of 327.1 ± 6.8 μmoles/ L (20 $^{o}$C).

Formic acid, acetone, methanol were purchased from Fisher company (chromatographically pure, Fisher company, Roanoke, USA). Acetonitrile was purchased from Waters company (chromatographically pure, Waters company, Milford Massachusetts, USA). Water of this experiment was ultrapure water (Synergy ultrapure water system Synergy type, Millipore, Massachusetts, USA).

1.2 Analysis conditions

1.2.1 Chromatographic analysis conditions. Agilent XDB-C18 column (1.8 μm, 4.6 mm×50 mm). Sample room temperature was 4 $^{o}$C. Column temperature was 25 $^{o}$C. Injection volume was 20 μL. Mobile phase A was ultrapure water containing 0.1% formic acid, B was acetonitrile. Elution method was isocratic elution, A/B (60/40, V/V). Flow rate was 0.2 mL/ min.

1.2.2 Mass spectrometry conditions. Electrospray ion source (ESI), positive ion ($ESI^+$) ionization and Multiple reaction monitoring (MRM) mode were used. Ion source temperature was 350 $^{o}$C. Atomization gas pressure was 30 psi (Table 1).

**Table 1 The parameters of MRM**

| Analyte | Parent ion/ amu | Daughter ion/ amu | Dwell time/ msec | Collision Gas energy/ eV | Fragmentor/ V |
|---|---|---|---|---|---|
| DA | 312.3 | 160.8 | 200 | 15 | 120 |
| | | 265.7* | 200 | 10 | 120 |

*Note: * quantify daughter ion*

1.3. Extraction and purification of samples

1.3.1 Extraction conditions of samples. Shellfish products were decorticated. 100.0 g of the edible shellfish organization was taken to be centrifuged at 1000 r/ min for 5 min. Then 2.0 g of the samples was taken in 50 mL centrifuge tube. After 2 min of vortex shock with 5 mL 50% methanol-water solution, the samples was subjected to ultrasonic extraction for 10 min, and was centrifuged at 4000 r/ min for 10 min subsequently [6]. Repeat the above operation with 5 mL 50% methanol-water solution. The supernatant was prepared to be enriched and purified with solid-phase extraction apparatus.

1.3.2 Purification conditions of samples. Rapid Trace II Automatic solid phase extraction apparatus parameters (Table 2).

**Table 2 Parameters of SPE**

| Step | Solvent | Volume /mL | Flow rate / (mL/ min) |
|---|---|---|---|
| Activation | MeOH | 6.0 | 1.5 |
| | $H_2O$ | 3.0 | 1.5 |
| | 50% MeOH-$H_2O$ | 3.0 | 1.5 |
| Loading | supernatant | 5.5 | 1.0 |
| | | 5.5 | 1.0 |
| Elution | 50% Aceton-$H_2O$ | 5.0 | 1.0 |
| Collection | 0.1 M formic acid aqueous solution | 1.5 | 0.8 |
| | | 1.5 | 0.8 |
| Wash program | $H_2O$ | 6.0 | 6.0 |

First, methyl alcohol, water and 50% methanol-water solution were successively used to preprocess the Supelclean LC-SAX solid phase extraction column[7,8]. Then the supernatant was added and eluted with 10% acetone aqueous solution. At last it was eluted with 0.1% formic acid aqueous solution. Eluent was directly passed through 0.25 μm millipore filter, then the filtrate was analyzed by HPLC-MS/MS.

## RESULTS AND DISCUSSION

2.1 Selection and optimization of extraction solvent. Something such as the lipid in shellfishes was also extracted while extracting DA, so that it could affect the detection limit and recovery of this method[9]. Therefore, it has significant meaningful to select and optimize extraction solvent. 50% methanol-water solution, acetone and 50% acetonitrile-water solution were selected as DA extraction solvent. Comparing the extraction effect, repeated experiments showed that the average recovery rate of 50% methanol-water was 78.6%, the average recovery rate of acetone was 65.9%, the average recovery rate of 50% acetone-water was 68.1%. So 50% methanol-water as extraction solvent had the highest recovery rate of DA. Considered the solvent of DA standard is methanol, therefore, 50% methanol-water solution was used as extraction solvent (Fig. 1).

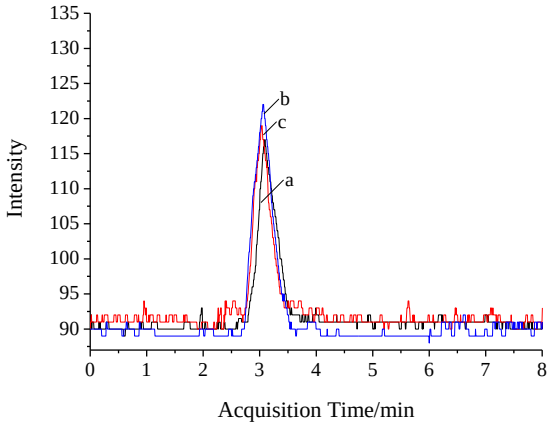


**Fig 1. Chromatograms for the extration solvent of (a)acetone, (b)50% methanol-water, (c)50% acetone-water**

2.2 Qualification of mass spectrum and optimization of mass spectrum conditions. DA standard liquid (1 μg/ mL) was directly injected. Mass spectrometry and MS/MS scanning in positive ion mode was used in order to obtain DA molecular ion peak m/z 312.3 and information about fragment ion. Furthermore, the result showed that 312.3/265.7 and 312.3/160.8 which could be confirmed were qualitative ion pair, and 312.3/265.7 was quantitative daughter ion (Fig. 2). Meanwhile, collision gas energy and fragmentor were optimized repeatedly to obtain the optimum parameters of the mass spectrum.

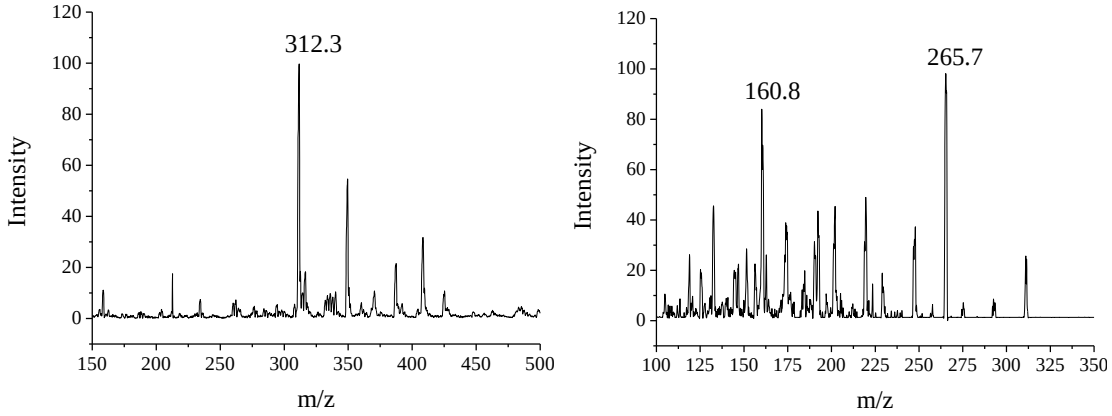


**Fig 2. MS and MS/MS chromatograms of DA**

Methanol or acetone was used as mobile phase for chromatographic separation [10]. Compared with methanol, acetone was more likely to gasify, and it had lower viscosity of reagent and column pressure, so here acetone was chosed as mobile phase. DA was analyzed with positive ion scanning using acetone solution which contained formic acid or ammonium formate as mobile phase to improve ionization efficiency. 0.1% formic acid was added in mobile

phase water in order to improve the peak shape to make the result more accurate. The appearance times of DA standard and blank sample were 3.088 min and 3.061 min consistently. The good peak shape could express good separation with impurities (Fig. 3 and Fig. 4).

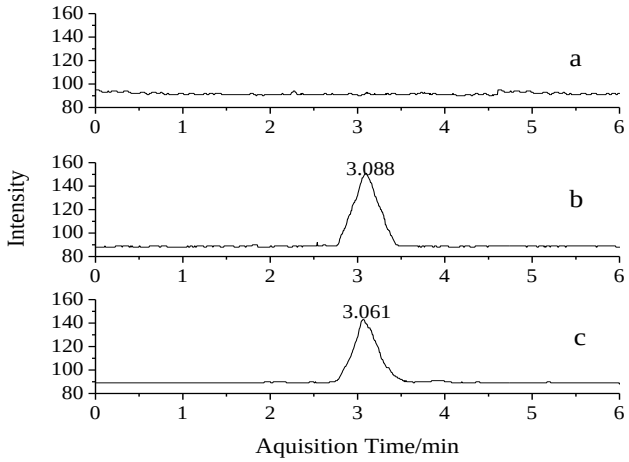


**Fig 3. chromatograms for the separation of the (a) blank sample, (b) standard solution(100 ng/ mL), (c) blank sample with DA**

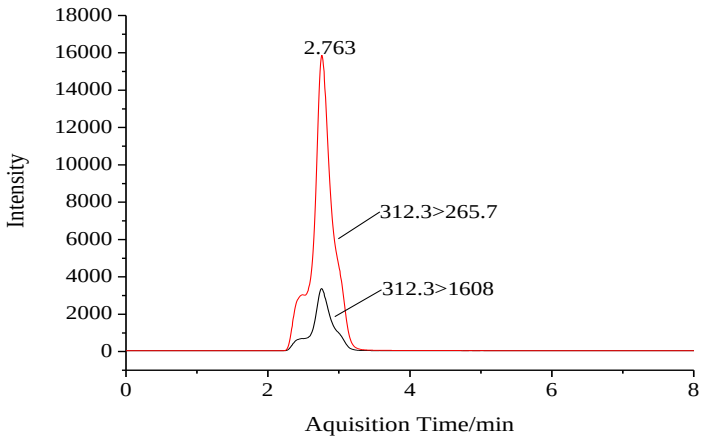


**Fig 4. MRM chromatogram of DA**

2.3 Methodology validation

2.3.1 Linear relation. Blank samples without DA was prepared into blank solution matrix according to 1.3, and the blank solution matrix was added with different concentrations of standard solution respectively as follow, six concentrations were 20.0, 50.0, 100.0, 200.0, 500.0 and 1000.0 μg/ L [11,12]. Then linear regression analysis was used to be determinated according to the three times of average peak area y and the corresponding concentrations of DA quantitative ion x, and calibration curve could be drawned(Fig. 5).

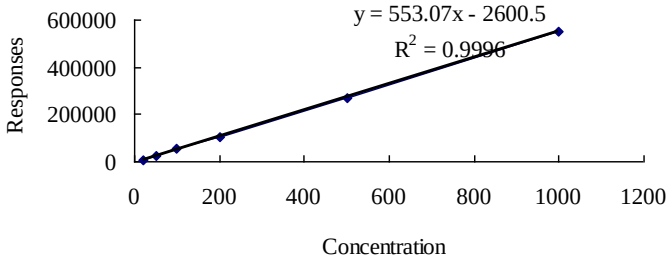


**Fig 5. Calibration curve of DA**

The calibration curve showed that there was a good linear relationship when the concentrations of DA were 20.0 to 1000.0 μg/ L, and the correlation coefficient $R^2$>0.999 satisfied the requirement of quantitative analysis.

2.3.2 Recovery, precision and detection limit. Blank samples without DA were selected to be added with DA standard solution at the concentrations of 20 μg/ kg, 50 μg/ kg, 100 μg/ kg to obtain the recovery and precision according to the established method. Each concentration was performed three parallel samples and determined. The result showed that the average recovery rates were 71.3% to 84.1% and relative standard deviation (RSD) were 4.4% to 4.6% (Table 3).

**Table 3 Recovery and degree prcision (n=3)**

| Name of sample | Adding concentration (μg/ kg) | Estimated value (μg/ kg) | | | Average recovery rate $\overline{X}$ (%) | Relative standard deviation RSD (%) |
|---|---|---|---|---|---|---|
| Short Necked Clam | 20.0 | 7.0 | 7.5 | 6.9 | 71.3 | 4.5 |
| | 50.0 | 39.1 | 36.9 | 40.4 | 77.6 | 4.6 |
| | 100.0 | 80.9 | 88.2 | 83.2 | 84.1 | 4.4 |

The detection limit of target object (DA) could be worked out by a method that the signal noise ratio ($R_{SN}$) of quantitative daughter ion (312.3/265.7) mass chromatographic peak was equal or greater than 3. By the formula of (1), the detection limit was 5.0 μg/ kg.

$$C_L = \frac{3 \times c}{R_{SN}} \times \frac{V}{m} \quad (1)$$

(c: adding concentration of blank sample; V: the ultimately constant volume; m: sample mass)

## CONCLUSION

This study established a method of extraction with 50% methanol by ultrasonic and determination of ASP (DA) in shellfishes by SPE-HPLC-MS/MS. This method can preferably eliminate the matrix interference of samples and has a good linearity, high sensitivity and degree of automation to determine DA in shellfishes simply, rapidly and effectively. And it can also completely satisfy the requirement of DA residual detection limit in shellfishes and its products domestic and overseas.

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