Journal of Chromatographic Science 2015;53:373–379
doi:10.1093/chromsci/bmu054 Advance Access publication June 16, 2014

Article

Solid-Phase Extraction-Based Ultra-Sensitive Detection of Four Lipophilic Marine Biotoxins in Bivalves by High-Performance Liquid Chromatography–Tandem Mass Spectrometry

Lanyun Fang*, Xunping Yao, Li Wang and Jige Li

Ningbo Key Laboratory of Poison Research and Control, Ningbo Municipal Center for Disease Control and Prevention, Ningbo 315010, China

*Author to whom correspondence should be addressed. Email: flyxiangzi@yahoo.com; flyxiangzi@gmail.com

Received 13 June 2013; revised 11 April 2014

A solid-phase extraction (SPE) method for ultra-sensitive determination of four lipophilic marine biotoxins in bivalve samples by coupling high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS-MS) was developed. Azaspiracid-2 (AZA2), pectenotoxins-2, spirolide (SPX) and gymnodimine were simultaneously determined by HPLC–MS-MS in a positive multiple reaction monitoring mode. Separation was achieved on a reversed-phase C18 column with an acetonitrile–water gradient containing formic acid. During the analysis, solvent effects on the analytes were eliminated by using 1 : 1 water–methanol as dissolving solvent instead of pure methanol. Matrix effects in post-SPE extract and crude extract were seriously evaluated. Increased matrix effects in post-SPE extract countervailed the concentration purpose to some extent. The limits of detection of the SPE–HPLC–MS-MS method were determined to be in the range of 0.013–0.085 μg kg^{-1}, and the linear range of the method was in the range of 0.128–55.2 ng mL^{-1} for the detected toxins. The proposed method was validated in terms of linearity (matrix-matched standard curves), precision, recovery, repeatability and limits of quantification. The recoveries of fortified samples at three different concentration levels were satisfactory, and the intra- and interday precisions were <7 and 10%, respectively.Several bivalve samples were analyzed to demonstrate the applicability of the proposed method. Different target toxins were detected in different kind of bivalves. Among them, AZA2 and SPX1 were first detected in Chinese shellfish. The levels of detected toxins were below the current European Union regulatory limits.

Introduction

Marine biotoxins are produced by marine phytoplankton and can bioaccumulate in filter feeding bivalves. When consumed by humans, bivalves contaminated with these biotoxins can lead to intoxications in humans in symptoms such as gastrointestinal distress, nausea, vomiting and abdominal pain, a typical diarrhea symptom (1). Lipophilic marine toxins are produced by various microalgals such as *Dinophysis*, *Azadinum spinosum*, *Alexandrium ostenfeldii*, *Karenia selliformis* and *Protoceratium reticulatum* (1, 2). The large number of lipophilic marine toxins can be grouped according to the symptoms they may cause or according to their chemical structure. They can be classified to several groups including okadaic acid (OA) and its analogs such as dynophysistoxins (DTXs), yessotoxins (YTXs), azaspiracids (AZAs), pectenotoxins (PTXs) and cyclic imine (CI) toxins. OA, DTXs and AZAs cause diarrhetic effects while PTXs and YTXs do not exert diarrhetic effects but coexist with OA-group toxins and DTXs (2). They were all included within diarrhetic shellfish poisoning (DSP) toxins. Gymnodimines (GYMs) and spirolides (SPXs) are CI toxins that show neurotoxicity when administrated orally or injected intraperitoneally in mice (3). The European Union defined the maximum levels of 13 lipophilic marine toxins permitted in bivalves to be placed on the market in the Regulation (EC) No. 853/2004 with the exception of CI toxins (4). Oral and intraperitoneal toxicities of GYM and SPXs have been established in mice (2), but the human health effects of the CI toxins have not yet been determined and currently no regulatory limits for this group have been promulgated.

The official reference method for the surveillance of lipophilic marine toxins, mainly DSP toxins, in the European Union is the mouse bioassay (MBA). However, the lipophilic MBA is very slow and does not allow quantification or identification of a toxin group (5). This method frequently produces false-positive result especially when GYM and SPXs are present in the sample (3). There are growing demands of the replacement of MBA by some confirmatory methods. Recently, high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS-MS) emerged as a powerful instrumental analytical method for the determination of several lipophilic marine toxins and is considered to be a feasible alternative to the MBA, offering excellent sensitivity, specificity, reliability and rapidity (6). This realizes the specific detection of individual toxins in a single chromatographic run. Various HPLC–MS-MS methods were described for the determination of lipophilic marine toxins including OA and DTXs (7–9), YTXs (10, 11), AZAs (12), PTXs (13) as well as their combinations (3, 4). These methods mostly used a C8 or C18 column in combination with an isocratic or gradient elution with mobile phase of water–acetonitrile containing ammonium formate and formic acid to separate the toxins. In the sample pretreatment, they usually used organic solvents, mainly methanol, to extract lipophilic marine toxins from the homogenized shellfish samples. The extracted solutions were combined, centrifuged, filtered and injected to the LC–MS-MS. This extraction procedure may be called solid–liquid extraction. A solid-phase extraction (SPE) method was developed for the enrichment and cleanup of four lipophilic marine toxins (OA, PTX, AZA and YTX) in different bivalves and processed shellfish products. The method reached a concentration factor of 10, and the limits of quantification (LOQs) for the four toxins were determined to be 1 μg kg^{-1} (14). Regueiro's team coupled a security guard column to LC–MS-MS and accomplished an automated online SPE-LC–MS-MS method using column-switching techniques for the determination of lipophilic marine toxins. Using this coupling method, the loading volume of the sample was restricted to microliter level and no concentration effect can be obtained (15).

© The Author 2014. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oup.com

Downloaded from https://academic.oup.com/chromsci/article/53/2/373/307470 by guest on 24 April 2024

The European Food Safety Authority (EFSA) has published reports to suggest a lowering of the regulatory limits from 160 μg OA equiv./kg (shellfish meat) to 45 μg OA Eq./kg for OA group and from 160 μg AZA Eq./kg to 30 μg AZA Eq./kg for the AZA group (14). It indicates that the decrease of regulatory limits of lipophilic marine toxins is a global trend because of their potential toxicity to the seafood. Along the Chinese coast, more than eight shellfish poisoning events were recorded, especially, the region of East China Sea has been highlighted as high risk of harmful algal bloom in recent years (16). The first official report due to DSP event occurred in the cities of Ningbo and Ningde, the exact regions of East China Sea coast, which resulted in >200 people feeling ill after eating mussels. It is an urgent task to conduct routine marine toxins monitoring programs as well as risk assessment in these regions. The background data of the pollution level of seafood by these marine toxins are fundamentally needed.

The work presented here describes the development of a highly sensitive HPLC–MS-MS method for the simultaneous analysis of four lipophilic marine toxins including GYM, SPX1, azaspiracids-2 (AZA2) and pectenotoxin-2 (PTX2). The aim of this work is to provide a robust method having enough sensitivity to find out the background pollution level of the seafood by these marine toxins. In favor of applying an SPE pretreatment, the enrichment factor is up to 7.5. Combined with HPLC–MS-MS detection, the method lowers the LOQs to 0.037–0.28 μg kg^{-1} for the toxins. Furthermore, the simultaneous detection of GYM, SPX1, AZA2 and PTX2 using the SPE–HPLC–MS-MS method was seldom reported.

Experimental

Chemicals and standards

Water was deionized and passed through a Milli-Q water purification system (Millipore, Bedford, MA, USA). Acetonitrile (HPLC supra gradient), methanol (absolute, HPLC grade), ammonia (analytical grade) and formic acid (98–100%) were purchased from Merck kGaA (Darmstadt, Germany). Ammonium acetate of HPLC grade was from Tedia Company Inc. (Fairfield, OH, USA). GYM (NRC-CRM-GYM 5.0 ± 0.2 μg mL^{-1}), 13-desmethyl spirolide C (DesMeC) (NRC-CRM-SPX1 7.0 ± 0.4 μg mL^{-1}), AZA2 (NRC-CRM-AZA2 1.28 ± 0.05 μg mL^{-1}) and PTX2(NRC-CRM-PTX2 8.6 ± 0.3 μg mL^{-1}) certified calibration solutions were purchased from the National Research Council, Institute for Marine Biosciences, Halifax, Canada.

Stock solutions and calibration curve standards

A mixed stock solution containing GYM (100 ng mL^{-1}), SPX1 (140 ng mL^{-1}), PTX2 (172 ng mL^{-1}) and AZA2 (25.6 ng mL^{-1}) was prepared in 1 : 1 methanol–water. Calibration curve standards were prepared by dilution of this stock solution in blank scallop extract. Blank scallop extract was obtained after the extraction and SPE procedure the same as all the other samples. The concentration of each toxin of the calibration standard was 200, 100, 50, 25, 12.5, 6.25 and 3.125 times dilution of the stock solution. Quantification was carried out by an external calibration method using the acquired matrix-matched standard curves.

Extraction of samples

All bivalve samples were acquired at the retail market. Blue mussels (*Mytulis edulis*), oysters (*Crassostrea gigas*), clams and scallops were harvested in the East China Sea. Triple methanolic extraction was performed to extract the toxins from the samples: weighing ~2 g amount of shellfish (whole flesh) homogenate into a 15 mL plastic centrifuge tube to which 5 mL CH_3OH was added. The extracts were vortex mixed for 1 min, standing for 10 min and centrifuged for 5 min at 10,000 rpm. The supernatant was transferred to a 25-mL volumetric flask. The extraction was repeated for two more times. The supernatants were combined, transferred to the volumetric flask, and the total volume was made up to the mark with water.

Optimized SPE procedure

Oasis HLB 3cc (60 mg) extraction cartridges were finally chosen for the SPE procedure. After conditioning of SPE cartridges using 3 mL CH_3OH and 3 mL water, the as-prepared extracts in water : CH_3OH (2 : 3) solution were loaded on the cartridge. Washing the cartridges with 5 mL of water : CH_3OH (3 : 1) solution, the analytes were eluted by 2.5 mL CH_3OH and 2.5 mL CH_3OH containing 5% ammonia. After evaporation to dryness under an N_2 stream, the residue was reconstituted with 2 mL of water : CH_3OH (1 : 1) solution and filtered through a 0.20-μm membrane into clean sample vial prior to LC–MS-MS analysis.

Liquid chromatography–mass spectrometry

All standards and samples were analyzed using a Shimadzu UFLC XR liquid chromatography system (Shimadzu Corporation, Kyoto, Japan) coupled with an AB SCIEX Triple Quad™ 5500 mass spectrometer (Applied Biosystems Instrument Corporation, California, USA; made in Toronto, Canada). A reversed-phase Shim-pack XR-ODS HPLC packed column (150 × 2.0 mm i.d.; particle size, 2.2 μm) was used for the separation. Mobile phase A was water containing 2 mM ammonium acetate and 0.1% formic acid, and mobile phase B was acetonitrile containing 0.1% formic acid. A gradient was run at a flow rate of 0.4 mL/min starting at 20% B, which was increased linearly to 70% B in 5 min then to 85% B in 1 min. It was kept at 85% B for 2 min and returned to 20% B in 1 min. An equilibration time of 1 min was allowed prior to the next injection. The injection volume was 5 μL, and the temperature of the column was set at 40°C.

Chromatographic separation was followed by positive ion electrospray ionization (ESI) tandem mass spectrometry in the multiple reaction monitoring mode. Per analyte at least two transitions were monitored using the settings in brackets: GYM: 508.2 > 490.4 (declustering potential, DP: 200 V, collision energy, CE: 34 eV), 508.2 > 392.2 (DP: 200 V, CE: 47 eV) and 508.2 > 162.0 (DP: 200 V, CE: 55 eV); SPX1: 692.4 > 674.4 (DP: 200 V, CE: 44 eV) and 692.4 > 656.5 (DP: 200 V, CE: 50 eV); PTX2: 876.5 > 823.5 (DP: 200 V, CE: 30 eV) and 876.5 > 805.5 (DP: 200 V, CE: 35 eV) and AZA2: 856.5 > 838.5 (DP: 150 V, CE: 42 eV) and 856.5 > 820.4 (DP: 150 V, CE: 58 eV). The conditions of mass spectrometer were as follows: ion spray voltage = 5.5 kV; curtain gas = 40 psi; collision gas = 6 psi; source temperature = 500°C, ion source gas 1 = 50 psi; ion source gas 2 = 50 psi; entrance potential = 10 V and collision cell exit potential = 11 V.

Downloaded from https://academic.oup.com/chromsci/article/53/2/373/307470 by guest on 24 April 2024

Results

SPE recovery of standards

Standard solutions of analytes prepared in CH_3OH [GYM (2.0 ng mL^{-1}), SPX1 (2.8 ng mL^{-1}), PTX2 (3.44 ng mL^{-1}) and AZA2 (0.51 ng mL^{-1})] were applied to the optimized SPE procedure. Recovery rates of four toxins were investigated and determined by comparison of the peak intensity of the analytes after and before the SPE procedure. Results showed that the recovery rates of four toxins were in the range of 96–105%.

Method performance characterization

In order to assess the performance of the proposed method, the main analytical performance parameters were thoroughly evaluated.

Matrix-matched standard curves (linear range)

Matrix effects are known to be a general problem regarding the quantification by LC–MS in biological samples. In this study, four target toxins experience ion suppression after the SPE procedure. Reliable quantification can be obtained using matrix-matched standard curves because matrix-matched standard curve is a calibration curve in solutions with the exact same composition as the samples. Matrix-matched standards were prepared by spiking the analytes at a series of different concentrations in post-SPE free toxin extract (the matrix). Table I clearly demonstrates the linear range, the regression equation, the correlation coefficient and the number of calibration points of the target marine toxins. The linearities of four toxins in matrix were excellent with correlation coefficients ~0.999.

Limits of detection and limits of quantification

The limit of detection (LOD) was defined as the injected concentration that produces signal of 3-fold of noise (S/N). A dilution series of standard toxins were used to fortify a series of processed blank extract that created a series of matrix-matched samples of varying concentration. Analyze the series of samples until the S/N value of each analytes approaching 3 (17). The LODs in the matrix expressed as concentration were determined to be in the range of 0.013–0.085 ng mL^{-1}. Taking into account the weighing amount and the final fixing volume, the method sensitivity was calculated to be 0.013–0.085 μg kg^{-1} for the target marine toxins. The detection limits of the present method are much below the EFSA suggestion, and the method was more sensitive than other published articles (14, 15). In LC–MS-MS analyses, the limit of identification (concentration at which the method can reliably produce the correct ion ratios used for identification) can be defined as LOQ, which produces signal of 10-fold of noise. Likewise, a series of gradually diluted standard mixtures in the processed blank extract until the S/N value of each analytes approaching 10 was analyzed. It was turned out that the LOQs of the toxins in matrix were 0.037–0.28 μg kg^{-1} ($N = 4$). At this analytical range, the analytes can be reliably identified. The diagnosis ion transition, the secondary ion transition and the retention time of the analytes guarantee the identification of the analytes in matrix.

Recovery of the method

According to the Commission Decision 2002/657/EC, if there is no certified reference material (CRM) available, the recovery has to be determined by experiments using fortified blank matrix. In the present method, fortified scallop samples at three different concentration levels (low, middle and high) were prepared by adding appropriate standard mixtures to the scallop homogenate and then experience the SPE procedure. The amount of each analyte thus calculated by the matrix-matched standard curve was compared with former fortified amount, and the recovery rate of the method was obtained. Table II shows the detailed recovery results. It can be seen that the average recovery rates of four targets at three different concentration levels were in the range of 71–101%. In order to clearly demonstrate the recovery results, extract ion chromatograms of four target toxins of their most intense ion transition in fortified blank scallop (A) at lower concentration level and corresponding standard solution in processed blank matrix (B) were provided as Figure 1. It can be seen from Figure 1 that the extract ion chromatograms of four target toxins except GYM were very clean. The targets can be easily identified by characteristic fragmentations and retention time. Toward GYM, some interfering peaks were observed in the extract ion chromatogram of the most intense ion transition of 508.2 > 490.4 and the peaks were overlapped to some extent. It is difficult to identify the exact peak of GYM. So, other two

Table II
Recoveries of Four Toxins at Three Different Fortified Concentration Levels

Analyte	Background value (μg kg^{-1})	Fortified value (μg kg^{-1})	Determined value (μg kg^{-1})	Recovery rate (%)	Average recovery rate (%)
GYM	0	1.0	0.709	70.9	71
		4.0	2.88	72.0	
		15.0	10.5	70.0	
SPX1	0	1.4	1.02	73.0	77
		5.6	4.64	82.9	
		21.0	15.75	75.0	
AZA2	0	0.256	0.254	99.2	101
		1.024	1.020	99.6	
		3.84	3.96	103.0	
PTX2	0	1.72	1.74	101.0	99
		6.88	6.69	97.2	
		25.8	25.5	98.7	

Table I
The Parameters of Matrix-Matched Standard Curves, LODs and LOQs in Matrix[a]

Analyte	Linear regression equation	Linear range (ng mL^{-1})	Number of calibration points	Correlation coefficient	LOD (μg kg^{-1})	LOQ (μg kg^{-1})
PTX2	$y = 4.92 \times 10^3x + 499$	0.86–55.2	7	0.9996	0.085	0.28
AZA2	$y = 6.05 \times 10^4x - 368$	0.128–8.2	6	0.9988	0.013	0.037
SPX1	$y = 1.1 \times 10^4x + 743$	0.7–44.8	7	0.9996	0.070	0.22
GYM	$y = 4.01 \times 10^4x + 979$	0.5–16.0	6	0.9990	0.052	0.16

[a]x, the concentration of analyte; y, the peak area of analyte.

Downloaded from https://academic.oup.com/chromsci/article/53/2/373/307470 by guest on 24 April 2024

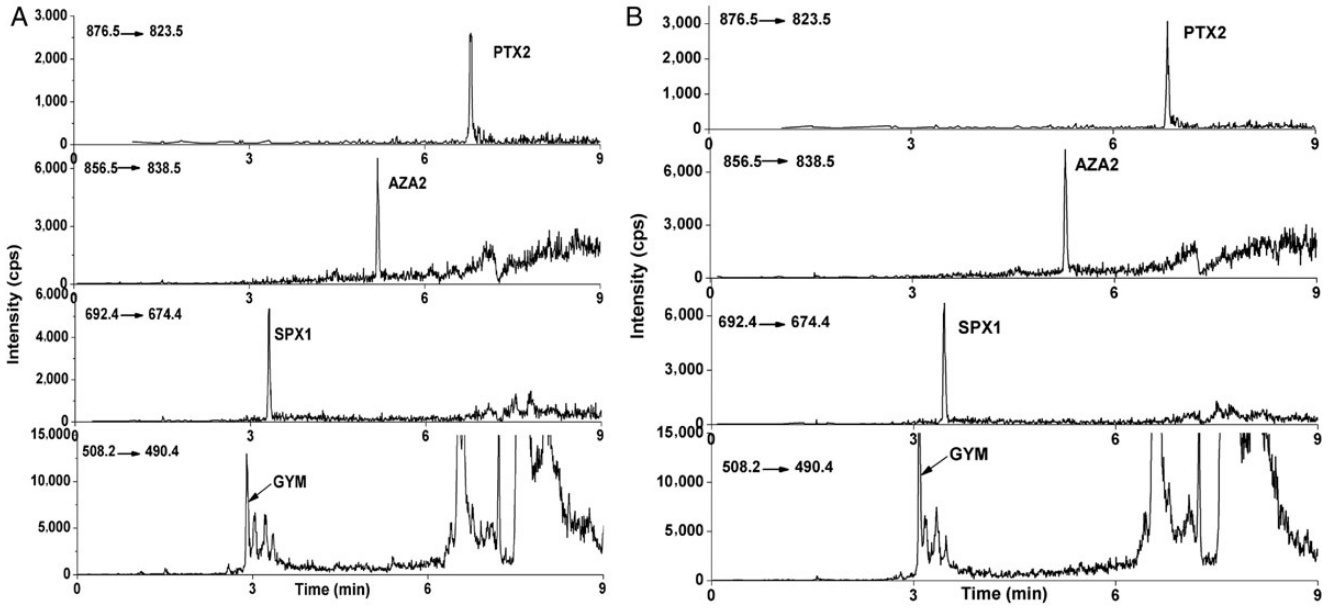

Figure 1. Extract ion chromatograms of four target toxins in fortified blank scallop (A) and corresponding standard solution in processed blank matrix (B) under optimal conditions. Fortified concentrations of four toxins: PTX2 (1.72 μg kg^{-1}), AZA2 (0.256 μg kg^{-1}), SPX1 (1.4 μg kg^{-1}) and GYM (1.0 μg kg^{-1}). Separation and MS conditions were described in Experimental.

characteristic transitions, 508.2 > 392.2 and 508.2 > 162.0, were used together to identify GYM.

Repeatability and precision

The precision of the method was assessed using a processed blank scallop spiked of multitoxins at certain concentrations [PTX2 (3.44 μg kg^{-1}), AZA2 (0.512 μg kg^{-1}), SPX1 (2.8 μg kg^{-1}) and GYM (2.0 μg kg^{-1})]. The intraday precision was determined with six replicates analysis on the same day, whereas the interday precision was evaluated using three replicates in each of five consecutive day. Both intra- and interday precision studies presented satisfactory results for all targets, with RSDs <7 and 10%, respectively.

The repeatability of the method was represented as the precision of examining five parallel fortified blank scallop. PTX2 and AZA2 have excellent repeatability (RSDs < 4%), whereas GYM and SPX1 show relative poor repeatability with RSDs ~12%.

Analytes stability in matrix

Because of no incurred material available, a blank scallop spiked of multitoxins at lower concentrations [PTX2 (3.44 μg kg^{-1}), AZA2 (0.512 μg kg^{-1}), SPX1 (2.8 μg kg^{-1}) and GYM (2.0 μg kg^{-1})] was prepared and homogenized by an IKA-Werke Ultra-Turrax T25 Basic homogenizer (IKA Works GmbH & Co., Staufen, Germany; made in Guangzhou, China). Divide the material into five aliquots to plastic vials and seal them. Analyze one aliquot immediately. Store the remaining aliquots at −20°C and analyze them after 5, 10 and 15 days. Table III shows the determined values of four toxins in the spiked scallop after varying storage time, and the bias (deviation toward the first day determined value) was calculated. Results showed that no degradation of the toxins in the scallop was found after 15 days storage at −20°C. Besides, the certificates of the toxins show that the toxins are valid for 1 year from the time of original packaging if stored unopened at the recommended storage conditions of −12°C.

Table III
Analytes Stability in Scallop

Analyte	Storage time (days)	Determined value (μg kg^{-1}, $N = 3$)	Bias (%)
GYM	0	1.42 ± 0.11	0
	5	1.35 ± 0.10	4.9
	10	1.24 ± 0.08	12.7
	15	1.28 ± 0.12	9.9
SPX1	0	2.21 ± 0.17	0
	5	2.11 ± 0.13	4.5
	10	2.20 ± 0.15	4.1
	15	2.03 ± 0.11	8.1
PTX2	0	3.39 ± 0.18	0
	5	3.31 ± 0.20	2.4
	10	3.49 ± 0.17	2.9
	15	3.21 ± 0.13	5.3
AZA2	0	0.520 ± 0.028	0
	5	0.510 ± 0.023	1.9
	10	0.503 ± 0.036	3.3
	15	0.518 ± 0.024	0.4

Discussion

Solvent effect on analyte peak shape

The selectivity and sensitivity of LC–MS-MS analysis not only depends on the ionization technique and mass spectrometer but also on the conditions of LC technique. The composition of the solvent to dissolve the sample generally affects the peak shape and peak intensity of analytes in LC–MS-MS. In previous published papers, the researchers prepared the stock solution of marine toxins in absolute methanol (3, 4, 14). But in our experiment, we observed that GYM exhibited two peaks in the ion extracted chromatogram when prepared in absolute methanol as shown in Figure 2, a. The front peak was broad and distorted,

Downloaded from https://academic.oup.com/chromsci/article/53/2/373/307470 by guest on 24 April 2024

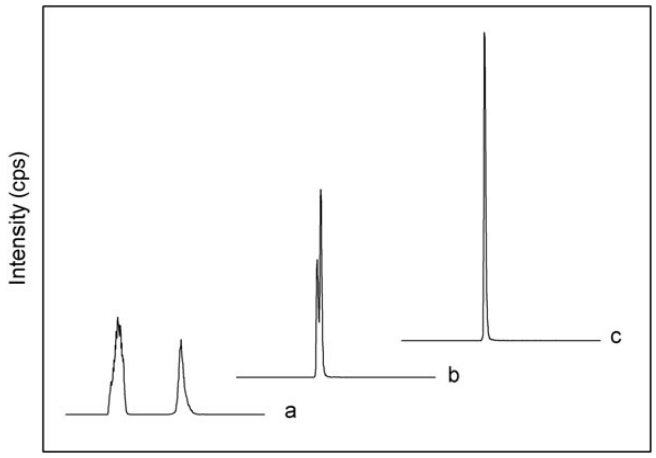

Figure 2. Extract ion chromatograms of GYM (508.2 > 490.4) when prepared in different solvents: (a) in absolute methanol, (b) in 1 : 1 of mobile phase A : mobile phase B and (c) in 1 :1 of water : methanol. Separation and MS conditions were the same as Figure 1.

which was not ascribed to an isomer. It was caused by the solvent effect. The solubility of absolute methanol is much stronger than the eluent composed of acetonitrile and water, which makes the analyte peak abnormal. When the standard solution of multitoxins was prepared in 1 : 1 mobile phase A : mobile phase B, a split peak was observed for GYM (Figure 2, b). Compared with Figure 2, a, the peak shape improved and the front peak disappeared. It may be due to the composition consistency between solvent and mobile phase. But the organic additives in the solvent and the real-time changing of the mobile phase probably resulted in the peak splitting. The above phenomenon seriously affects the identification and quantification of the analytes. It is indispensable to find a suitable solvent to eliminate the solvent effect so that good peak shape can be obtained. In light of the arguments presented above, an attempt to dissolve the standard multitoxins in 1 : 1 water : methanol (v/v) was made. An excellent sharp and symmetrical peak were displayed (Figure 2, c). The moderate solubility and neutral environment provided by 1 : 1 water : methanol guarantee the good performance of the peak.

A similar result was also observed for SPX1 analyzed under certain conditions. But the shape of PTX2 and AZA2 peaks is different from GYM and SPX1, which was sharp and symmetrical in all the three situations (data not shown). Presumably, under our chromatographic condition, the order of eluted analyte was GYM, SPX1, AZA2 and PTX2. More polar compounds may have low retention on reversed-phase column and are eluted with low organic solvent content, and their ionization efficiency in LC–ESI–MS can be low (18). Normally, the ionization efficiency is better for compounds that are eluted at higher organic solvent content. So GYM and SPX1 may show a higher tendency to be affected by the solvent and eluent so that GYM and SPX1 exhibit different peak shapes. As a result, we used 1 : 1 water : methanol as dissolving solvent instead of pure methanol to eliminate the solvent effects.

Optimization of SPE procedure

SPE has been developed as an alternative to liquid–liquid extraction owing to its efficiency, simplicity, less organic reagent consumption and easy to isolate and concentrate sample. An SPE procedure generally consists of four distinct steps: column conditioning, sample loading, washing and elution. The loading and eluting efficiency are very crucial to ensure the analyte recovery. So the loading and elution conditions were systematically investigated.

Table IV
Concentration Distribution of Analytes (%) in Different Collected Fraction Depending on the Percentage of Water in Loading Solution Applied to SPE

Analyte	Collected fraction	Percentage of water in loading solutions applied to SPE (%)					
		0	25	35	40	50	75
GYM	Filtrate	58	0	0	0	0	0
	Washing effluent	25	1.4	0	0	0	0
	Eluate	6	91	89	93	98	103
SPX1	Filtrate	62	0	0	0	0	0
	Washing effluent	25	0	0	0	0	0
	Eluate	4	86	89	92	91	91
AZA2	Filtrate	60	7	0	0	0	0
	Washing effluent	22	0	0	0	0	0
	Eluate	10	88	86	91	92	94
PTX2	Filtrate	56	0	0	0	0	0
	Washing effluent	31	0	0	0	0	0
	Eluate	4	96	99	103	87	102

Loading

Oasis HLB sorbent is a kind of porous copolymer composed of hydrophobic divinylbenzene and hydrophilic *N*-vinylpyrrolidone monomer. They are universally applied in the adsorption of acidic, neutral and basic compounds. It is expected that four lipophilic marine toxins (GYM, SPX1, AZA2 and PTX2) will be adsorbed on HLB columns because of their hydrophobic characteristic. After triple methanolic extraction from shellfish, the lipophilic toxins were dissolved in CH_3OH. The most important thing is to decide the percentage of water had to be added to the extracts for optimizing analyte adsorption on HLB columns. Details were as followed: 1.5 mL multitoxin standard solutions of GYM (2.0 ng mL^{-1}), SPX1 (2.8 ng mL^{-1}), PTX2 (3.44 ng mL^{-1}) and AZA2 (0.51 ng mL^{-1}) in 0, 20, 35, 40, 50 and 75% water in solution were applied to the preconditioned HLB columns, washed with 1 mL of water, dried and eluted by 3 mL of absolute CH_3OH. To facilitate calculation, the volumes of filtrates, washing effluents and eluates were accurately collected and analyzed separately. Concentration distribution expressed as percentage of toxin amounts was determined by comparison of the peak intensity of different fraction with that of multitoxins standard solution applied to SPE. The results were demonstrated in Table IV.

It can be seen from Table IV that applying absolute CH_3OH solutions to the SPE, all four toxins could be detected in filtrates as well as in washing effluents. They were almost not detected in the eluates. This indicated that 100% CH_3OH solution was so strong that the analytes cannot completely adsorbed onto the cartridge. Increasing the percentage of water in the loading solution to 25%, no analytes were detected except AZA2 could be found a little bit (7%) in the filtrate and GYM could still be detected in the washing effluent (1.4%). When water percentage was up to 35%, no analytes could be detected at all in the filtrates and washing effluents. The recoveries of the analytes in the eluates were satisfactory. Continuous increasing of water percentage to higher level did not bring larger recovery rates. Hence, a dilution of the methanolic extracts by 40% water is employed for exhaustive loading of the analytes onto the SPE cartridges.

Downloaded from https://academic.oup.com/chromsci/article/53/2/373/307470 by guest on 24 April 2024

Washing and elution

Washing steps can remove salts and very polar substances whose retention qualities are lower than the target analytes. Generally, a washing procedure before elution only a solvent with a decreased amount of organic component can be applied. We can draw a conclusion from the results of loading conditions that a solution with >40% water can avoid analytes losing. To be conservative, water : CH_3OH (3 : 1) solution (water percentage, 75%) was chosen as the washing solution. Our experiments also proved that the washing conditions did not bring any loss of the target analytes.

After washing, part of interfering substances flow out of the column and the target analytes were still retained. Strong eluent should be adopted for the analyte elution. With respect to HLB column, absolute CH_3OH was usually used as the eluent. But it seems that for the desorption of amine containing analytes an eluent with a basic pH is recommended (14). Thus, for elution of the analytes, three different eluents: 5 mL CH_3OH (A), 2.5 mL CH_3OH plus 2.5 mL 1% ammonia in CH_3OH (B) and 5 mL 1% ammonia in CH_3OH (C) were examined. It turned out that B and C gave satisfactory recovery result for each individual analyte (recovery rate, >90%), but A showed lower recovery rate for SPX1. To facilitate evaporation, eluent B was applied. During the SPE procedure, the flow rate of loading and elution was carefully set at 1 mL/min. Too fast flow rate may lead to retaining and elution deficiency.

Matrix effects

Matrix effects are a general problem regarding the quantification by LC–MS, and they are believed to be caused by nonvolatile endogenous compounds co-eluting with the analyte which deteriorate or support the ionization process. Ion suppression or ion enhancement may lead to under- or overestimation of the true concentration of analyte present in the sample, which affects both accuracy and precision of the analytical method. Matrix effects can be eliminated or corrected by means of sample cleanup, internal standards, standard addition, matrix-matched standards or changes in chromatographic conditions (19).

The present study employed SPE as a cleanup and concentration procedure and focused on the assessment of matrix effects using standard addition method. Standard addition was carried out by spiking the marine toxins into the post-SPE toxin-free scallop extract (the gray column) and comparing the results with that of the standard solution in 1 : 1 water : methanol (v/v) (the black column). The results were clearly shown in Figure 3. All four toxins experience ion suppression by inherent co-eluting substances, and the degrees of signal suppression were ~15–45%. This is the case of a concentration factor of 7.5 (15 mL concentrated to 2 mL) after the SPE procedure. There were differences between the results obtained in this work and those previously reported. For example, the AZA ion suppression reported by Stobo *et al.* (4) (AZA1, 44 and 48% for mussels and oysters, respectively) was higher than the suppression observed in this study (AZA2, 22% for scallop). This may be due to the differences in instruments, chromatographic conditions, sample source, the pretreatment method and the solvent to sample ratio, etc. To evaluate whether an SPE procedure was valuable for concentration purpose, the matrix effects of the respective analytes in a spiked crude extract without a concentration step were also carried out. The data showed no significant matrix effects were observed for four target marine toxins in crude extract (15 mL) compared with standard solution. Taking into account the matrix effects in the post-SPE toxin-free scallop extract (2 mL), the contrast results indicated that a concentration factor higher than 2 obtained by SPE procedure is helpful to improve method sensitivity.

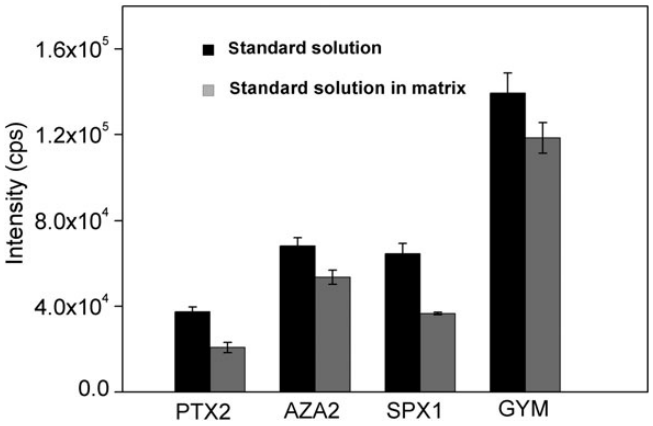

Figure 3. Matrix effects evaluated by standard addition method. Concentrations of toxins in standard solution and spiked toxin-free scallop extract after SPE were: PTX2 (1.72 ng mL^{-1}), AZA2 (0.256 ng mL^{-1}), SPX1 (1.4 ng mL^{-1}) and GYM (1.0 ng mL^{-1}). The error bars represent RSDs of four replicate experiments.

Application to real samples

Several kinds of bivalves including blue mussels, oysters and scallops were analyzed by the proposed method. Considering the potential different matrix effects caused by different kinds of samples, the concentration of detected target toxins was calculated based on the ratio of response generated from the detected sample to a fortified scallop sample of known concentration.

Among 40 bivalves, AZA2 was found in two blue mussels at the level of ~0.035 μg kg^{-1} which is near the quantification limit. PTX2 and SPX1 were found at the levels of 0.40 and 0.56 μg kg^{-1}, respectively, in one oyster. This oyster was selected as a blind sample distributed to three analysts. They followed the procedure of the proposed method to find out the type and concentration of the toxin in the sample. Their results were consistent with ours and the mean and standard deviation of their data were: PTX2, 0.32 ± 0.03 μg kg^{-1}, and SPX1, 0.51 ± 0.04 μg kg^{-1}, respectively. The levels of detected toxins were below the current European Union (EU) regulatory limits. No traces of four toxins were detected in scallops. With comparison to two published papers dealing with Chinese shellfish (6, 16), AZA2 and SPX1 were first detected in Chinese shellfish.

Conclusion

A highly sensitive HPLC–MS-MS method with an SPE pretreatment for the simultaneous determination of four lipophilic marine toxins including GYM, SPX1, AZA2 and PTX2 was presented. During the experiment, solvent effect was observed. By dissolving the standard multitoxins in 1 : 1 water : methanol (v/v), solvent effect was avoided and sharp, symmetrical peaks of GYM and SPX1 were obtained. Applying a concentration factor

Downloaded from https://academic.oup.com/chromsci/article/53/2/373/307470 by guest on 24 April 2024

of 7.5 with the SPE procedure, the LOQs for the analyzed toxins were determined to be 0.037–0.28 μg kg^{-1}. The LOQs were much lower than other published articles (14, 15), which are an important aspect in view of a possible lowering of the regulatory limits. Since matrix effects in post-SPE extract were more significant than crude extract without a concentration step, matrix effects in post-SPE extract and crude extract were seriously investigated. It is shown that matrix effects do not expand proportionately as the concentration factor. A concentration factor >2 is helpful to improve method sensitivity.

The present method was successfully applied to analyze the target toxins in Chinese shellfish harvested from the East China Sea. For a reliable quantitative analysis, a spiked blank extract was processed in the same way as the unknown samples and used for correction of matrix effects. Our study reports on the first occurrence of AZA2 and SPX1 in Chinese shellfish in favor of the high method sensitivity. The maximum levels of toxins found in Chinese shellfish were lower than the recommended safety limit of AZAs and PTXs (GYM and SPXs not included). Hence, the risk of acute effects resulting from human exposure to these toxins due to the consumption of shellfish from Chinese coast can be regarded as low. Although GYM and SPX1 were not currently included in the EU regulation, the data obtained can provide a primary basis for establishing regulatory control in China.

Funding

The work was financially supported by Ningbo Agricultural and Social Development Foundation (No. 2011C50057), Ningbo Natural Science Foundation (No. 2012A610184) and Ningbo Innovative Research Team Program for Key Science and Technology (No. 2012B82018).

References

1. Gerssen, A., Mulder, P.P.J., de Boer, J.; Screening of lipophilic marine toxins in shellfish and algae: development of a library using liquid chromatography coupled to orbitrap mass spectrometry; *Analytica Chimica Acta*, (2011); 685: 176–185.
2. Paredes, I., Rietjens, I.M.C.M., Vieites, J.M., Cabado, A.G.; Update of risk assessments of main marine biotoxins in the European Union; *Toxicon*, (2011); 58: 336–354.
3. Gerssen, A., Mulder, P.P.J., McElhinney, M.A., de Boer, J.; Liquid chromatography tandem mass spectrometry method for the detection of marine lipophilic toxins under alkaline conditions; *Journal of Chromatography A*, (2009); 1216: 1421–1430.
4. Fux, E., McMillan, D., Bire, R., Hess, P.; Development of an ultra-performance liquid chromatography–mass spectrometry method for the detection of lipophilic marine toxins; *Journal of Chromatography A*, (2007); 1157: 273–280.
5. Botana, L.M., Alfonso, A., Botana, A., Vieytes, M.R., Vale, C., Vilarino, N., *et al.*; Functional assays for marine toxins as an alternative, high-throughput screening solution to animal tests; *Trends in Analytical Chemistry*, (2009); 28: 603–611.
6. Liu, R., Liang, Y., Wu, X., Xu, D., Liu, Y., Liu, L.; First report on the detection of pectenotoxin groups in Chinese shellfish by LC–MS/MS; *Toxicon*, (2011); 57: 1000–1007.
7. Paz, B., Daranas, A.H., Cruz, P.G., Franco, J.M., Pizarro, G., Souto, M.L., *et al.*; Characterisation of okadaic acid related toxins by liquid chromatography coupled with mass spectrometry; *Toxicon*, (2007); 50: 225–235.
8. Paz, B., Daranas, A.H., Cruz, P.G., Franco, J.M., Napolitano, J.G., Norte, M., *et al.*; Identification and characterization of DTX-5c and 7-hydroxymethyl-2-methylene-octa-4,7-dienyl okadaate from Prorocentrum belizeanum cultures by LC-MS; *Toxicon*, (2007); 50: 470–478.
9. Doucet, E., Ross, N.N., Quilliam, M.A.; Enzymatic hydrolysis of esterified diarrhetic shellfish poisoning toxins and pectenotoxins; *Analytical and Bioanalytical Chemistry*, (2007); 389: 335–342.
10. Amandi, M.F., Furey, A., Lehane, M., Ramstad, H., James, K.J.; Liquid chromatography with electrospray ion-trap mass spectrometry for the determination of yessotoxins in shellfish; *Journal of Chromatography A*, (2002); 976: 329–334.
11. Miles, C.O., Samdal, I.A., Aasen, J.A.G., Jensen, D.J., Quilliam, M.A., Petersen, D., *et al.*; Evidence for numerous analogs of essotoxin in Protoceratium reticulatum; *Harmful Algae*, (2005); 4: 1075–1091.
12. Rehmann, N., Hess, P., Quilliam, M.A.; Discovery of new analogs of the marine biotoxin azaspiracid in blue mussels (Mytilus edulis) by ultra-performance liquid chromatography/tandem mass spectrometry; *Rapid Communication Mass Spectrometry*, (2008); 22: 549–558.
13. Miles, C.O., Wilkins, A.L., Hawkes, A.D., Jensen, D.J., Selwood, A.I., Beuzenberg, V., *et al.*; Isolation and identification of pectenotoxins-13 and -14 from Dinophysis acuta in New Zealand; *Toxicon*, (2006); 48: 152–159.
14. These, A., Scholz, J., Preiss-Weigert, A.; Sensitive method for the determination of lipophilic marine biotoxins in extracts of mussels and processed shellfish by high-performance liquid chromatography tandem mass spectrometry based on enrichment by solid-phase extraction; *Journal of Chromatography A*, (2009); 1216: 4529–4538.
15. Regueiro, J., Rossignoli, A.E., Álvarez, G., Blanco, J.; Automated on-line solid-phase extraction coupled to liquid chromatography tandem mass spectrometry for determination of lipophilic marine toxins in shellfish; *Food Chemistry*, (2011); 129: 533–540.
16. Li, A., Ma, J., Cao, J., McCarron, P.; Toxins in mussels (Mytilus galloprovincialis) associated with diarrhetic shellfish poisoning episodes in China; *Toxicon*, (2012); 60: 420–425.
17. Gu, Q., Shi, X., Yin, P., Gao, P., Lu, X., Xu, G.; Analysis of catecholamines and their metabolites in adrenal gland by liquid chromatography tandem mass spectrometry; *Analytica Chimica Acta*, (2008); 609(2): 192–200.
18. Kostiainen, R., Kauppila, T.J.; Effect of eluent on the ionization process in liquid chromatography mass spectrometry; *Journal of Chromatography A*, (2009); 1216: 685–699.
19. Kilcoyne, J., Fux, E.; Strategies for the elimination of matrix effects in the liquid chromatography tandem mass spectrometry analysis of the lipophilic toxins okadaic acid and azaspiracid-1 in molluscan shellfish; *Journal of Chromatography A*, (2010); 1217: 7123–7130.

Downloaded from https://academic.oup.com/chromsci/article/53/2/373/307470 by guest on 24 April 2024