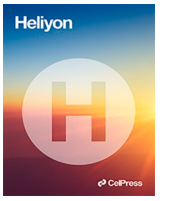
Heliyon 9 (2023) e21610

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Determination of neurotoxic shellfish poisoning toxins in shellfish by liquid chromatography-tandem mass spectrometry coupled with dispersive solid phase extraction

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A R T I C L E I N F O

Keywords:
Neurotoxic shellfish poisoning
Dispersive solid phase extraction
Brevetoxins
Shellfish
Liquid chromatography-tandem mass spectrometry

A B S T R A C T

An innovative method based on dispersive solid phase extraction (d-SPE) in conjunction with LC-MS/MS had been developed for the simultaneous quantitative determination of three brevetoxins (BTXs), which can result in neurotoxic shellfish poisoning (NSP), in shellfish. The toxins were extracted with a 50 % acetonitrile (v/v) and cleaned by alumina-neutral sorbent. After chromatographic separation on a C_{18} column, the analytes were qualitatively and quantitatively detected using multiple reaction monitoring (MRM) in positive ionization mode. The created approach was validated by SANTE 11312/2021. The LOQs were 5 μg/kg for each toxin, below the advised regulatory limit of 800 μg BTX-2/kg. The mean recoveries of brevetoxins were in the range of 75.9 %–114.1 %, and the ranges of their intra- and inter-day precisions were 0.9–9.7 % and 0.6–7.2 %, respectively. The matrix effects for three BTXs in four shellfish matrices were in the range of 85.6 %–114.8 %. The method demonstrated great consistency and high sensitivity, and it can meet the requirements of daily monitoring.

1. Introduction

Brevetoxins (BTXs or PbTxs), a group of marine biotoxins that can cause neurotoxic shellfish poisoning (NSP), is naturally produced by the marine dinoflagellate of the *Karenia brevis* [1]. BTXs can be divided into A-type (mainly including Brevetoxin-1 (BTX-1), etc.) and B-type (mainly including Brevetoxin-2 (BTX-2), Brevetoxin-3 (BTX-3), etc.), based on the different skeleton of the structure, with the A-type consisting of 10 fused ether rings and the B-type consisting of 11 fused ether rings (Fig. S1). BTXs will build up in shellfish because they filter-feed on a lot of toxin-containing algae [2]. Although BTXs are not poisonous to these shellfish, they can have serious and harmful consequences on humans and higher animals that consume seafood contaminated with them. BTXs are neurotoxins with a strong binding affinity for the receptors on voltage gate sodium channels, which can cause symptoms such as vomiting, spasms, muscle pain, reversal of hot and cold sensations, dizziness, ataxia, bradycardia, vomiting, and coma [1,3]. Similar to other marine biotoxins, BTXs are heat stable, acid resistant, and cannot be damaged by traditional cooking methods. Outbreaks of NSP caused by the consumption of BTX toxin-containing shellfish were discovered early along the coast of the Florida area [4,5], the Gulf of Mexico [6], and the Hauraki coast of New Zealand [7–9]. However, several factors (such as climate change, ballast water transfer, etc) could conduce to

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https://doi.org/10.1016/j.heliyon.2023.e21610
Received 10 May 2023; Received in revised form 23 October 2023; Accepted 24 October 2023
Available online 27 October 2023

the growth or proliferation of BTX-producing phytoplankton worldwide [3]. In recent years, the algae of *Karenia brevis* or BTX toxins were also found in other countries. In China, the outbreak of the algae of *Karenia brevis* was found in the coastal regions of Zhejiang Province, and this marine alga had also been identified in the Yangtze Estuary and the coastal regions of Guangdong Province [10]. BTXs were discovered in mussels from a lagoon on the island of Corsica, marking the first detection in France (Mediterranean Sea) [11]. Given its threat to human health, the FAO, IOC, and WHO have jointly reported the safe limit of BTXs concentration in shellfish tissue samples as 800 μg BTX-2/kg (equivalent to 20 mouse units MUs/100g), and the recommended safety limit for BTX-toxins in France is 180 μg BTX-3/kg [11].

For the measurement of BTXs, several techniques had been developed, including the mouse bioassay (MBA) [12], enzyme-linked immunosorbent assay (ELISA) [13,14], receptor binding assays (RBAs) [15], cytotoxicity assays [16], capillary electrophoresis (CE) based immunoassay [17], spectroscopic ellipsometry [18], high-performance liquid chromatography (HPLC) [19] and liquid chromatography-tandem mass spectrometry (LC-MS/MS) [10,20–23]. The MBA method gives information about overall toxicity but has several limitations, including limited selectivity, poor reproducibility, and bioethical concerns [24]. The receptor binding assay does not have a high level of specificity because it reacts to all toxins that bind to sodium channel receptors [24]. The cytotoxicity assay is centered on the effects of BTXs on voltage-gated sodium channels of grown neuronal cells, with the disadvantages of low specificity, low throughput, and interference of co-extracted substances [24]; ELISA is frequently used for routine screening but is susceptible to false positives or false negatives. Spectroscopic ellipsometry was a label-free biosensing method, but with the interference of other marine biotoxins such as okadaic acid [18]. The HPLC method has a low detection sensitivity, and is susceptible to co-outflow interference. LC-MS/MS, the currently preferred method, has the advantages of high selectivity and sensitivity.

The main cleanup procedures reported for the determination of BTXs are the solid phase extraction (SPE) method, with the SPE columns of Strata-X [20,21,23], HLB [10], and C18 [22], etc. Although SPE cleanup procedures have good purification effects, also have the disadvantages of large solvent consumption, costly, long preparation time, and high operational requirements. Recently, dispersive solid phase extraction (d-SPE), which mainly relies on adsorbents to selectively adsorb impurities (non-adsorbing targets) to achieve the purpose of purifying samples, has been widely used in the pretreatment of shellfish matrix for the determination of marine biotoxins [25–27]. However, the d-SPE cleanup method used for the concurrent detection of BTXs is lacking.

In this study, we present a confirmatory quantitative method for the measurement of BTXs in shellfish samples, based on the d-SPE cleanup approach combined with LC-MS/MS. The parameters that affect the recovery of BTXs in the extraction and cleanup procedure, were carefully investigated and optimized.

2. Materials and methods

2.1. Sampling

Samples of several species of bivalve mollusks (scallop, mussel, oyster, and blood clam) were purchased from the local markets of Zhoushan (Eastern China, 121°30′-123°25′ N, 29°32′-31°04′ E) during 2016–2022. The samples of shellfish were rinsed with pure water. The closed-shell muscle of shellfish was opened with a knife. The whole tissues of shellfish were homogenated by a knife grinder of Retsch GM 200. The shellfish samples were stored at −20 °C until the analyses were conducted. The use of bivalve mollusks was inspected by the animal care welfare committee of Zhoushan municipal district center for disease control and prevention (No. zsjk-2020-003). To prepare the fortified samples and the matrix-matched calibration standards, blank shellfish samples were measured to confirm that they were free of BTXs.

2.2. Equipment

The Acquity H-Class system is connected with a TQ-XS Triple Quadrupole Mass spectrometer (Waters, Milford, MA, USA) that has a heated electrospray ionization (HESI) source. The LC equipment includes a binary pump, degasser, solvent reservoir, temperature-controlled column oven, and refrigerated autosampler. For data collection and analysis, the software of MassLynx 4.2 was employed.

2.3. Reagents and materials

The reference materials of BTX-1, BTX-2, and BTX-3 were purchased from Algalchem Inc (Taiwan). Ammonium formate, ammonium acetate, and formic acid (FA) were obtained from CNW (Germany). Methanol (MeOH) and acetonitrile (ACN) were of HPLC grade and purchased from Merck. Water was obtained from a Milli-Q water preparation system from Merck. The d-SPE sorbents of HC-C18 (particle size, 40–63 μm), Poly-Sery HLB (particle size, 60 μm), Carbon-GCB (particle size, 120–400 mesh), and PSA (particle, size 40–63 μm), were all obtained from CNW Technologies GmbH. The sorbent of Alumina-neutral (Brockmann I, particle size, 50–200 μm) was purchased from Acros Organic GmbH.

2.4. Sample treatment

A part of a homogeneous shellfish sample (0.1 ± 0.002 g) was weighed and put into a 2 mL polypropylene centrifuge tube, to which 0.9 mL of 50 % ACN was added. After vortex mixing for 10 min at a speed of 2300 rpm, the mixture was sonicated for 10 min, and then centrifuged at 15000 rpm for 10 min. The supernatant was transferred to a new 2 mL centrifuge tube, which was pre-filled with two hundred milligrams (±5 mg) alumina-neutral. The suspension was vortexed for 1 min and then centrifuged at 15000 rpm for 10 min.

The supernatant was passed through a 0.22 μm PTFE membrane filter prior to LC-MS/MS analysis.

2.5. LC-MS/MS conditions

LC separations were performed on an Acquity UPLC BEH C_{18} column (100 × 2.1 mm, 1.7-μm particle diameter, Waters) using an Acquity H-Class UPLC system. The mobile phases used in this method were 0.1 % FA (A) and ACN (B). The UPLC gradient was as follows: the initial mobile phase of 50 % B was held for 0.5 min, with a linear gradient to 65 % B at 1.5 min, held for 6.3 min, returned to 50 % B at 9.0 min, and held for 1.5 min before the subsequent injection. The mobile phase flow rate was 0.3 mL/min with a total run-time of 10.5 min.

The column effluent was directed into a triple quadrupole mass spectrometer equipped with a positive HESI interface. The optimal conditions for analysis included a spray voltage of 3 kV, a cone gas flow of 150 L/h, a desolvation gas flow of 1000 L/h, a desolvation temperature of 400 °C, and a collision gas flow of 0.15 mL/min. The optimal mass spectrum parameters of BTXs were summarized in Table S1. Two transitions were selected for each toxin for the MRM approach to meet the EU requirements for confirmatory analysis (Commission Decision 2002/657/EC). The relative intensities of the detected ions (ion ratio), expressed as a percentage of the intensity of the quantitative to qualitative ion transition, correspond to those of the calibration standard solutions, within the tolerances of ion ratio required by the EU.

2.6. Calibration

The original corresponding weight of 100 μg of BTX-1, BTX-2, and BTX-3 toxin standard were added to a volumetric flask to make 5.0 mL with MeOH. The stock standard solution was kept at −20 °C in the dark. Matrix-matched calibration standards were prepared by the addition of known amounts of the stock standard solution to appropriate volumes of the blank matrix extract. The concentrations of the calibration solutions were 0.5 μg/L, 1 μg/L, 2 μg/L, 5 μg/L, 10 μg/L, 20 μg/L, 50 μg/L, and 100 μg/L. Both solution- and matrix-matched calibration standards were freshly prepared before use.

3. Results and discussion

3.1. Optimization of chromatography conditions

For the chromatographic detection of BTXs, two columns of Acquity UPLC BEH C_{18} and Hypersil Gold C_{18} (100 × 2.1 mm, 1.9-μm particle diameter, Thermo Scientific) were contrasted in this study. Based on the target peak response and chromatographic peak shape, the column of Acquity UPLC BEH C_{18} was superior to the Hypersil Gold C_{18} column. For the constituents of the mobile phase, ACN and water with several modifiers (0.1 % FA, 0.1 % FA+2 mM ammonium formate, and 0.1 % FA+2 mM ammonium acetate) were investigated. The results showed that the chromatographic peak response values of BTXs decreased significantly when ammonium formate or ammonium acetate was added to the mobile phase system. The mobile phase consisting of ACN and 0.1 % FA aqueous solution yielded acceptable performance in terms of the peak intensity and peak shape of BTXs. As we know, different mobile phase gradient modes might also affect the chromatographic behavior of targets. The chromatographic peak intensities of three BTXs in the shellfish matrix of blood clam within two mobile phase gradient modes (including the mobile phase gradient mode A and the mobile phase gradient mode B, shown in Table S2) were contrasted in this study. As shown in Fig. S2, the peak intensities of all three BTXs by

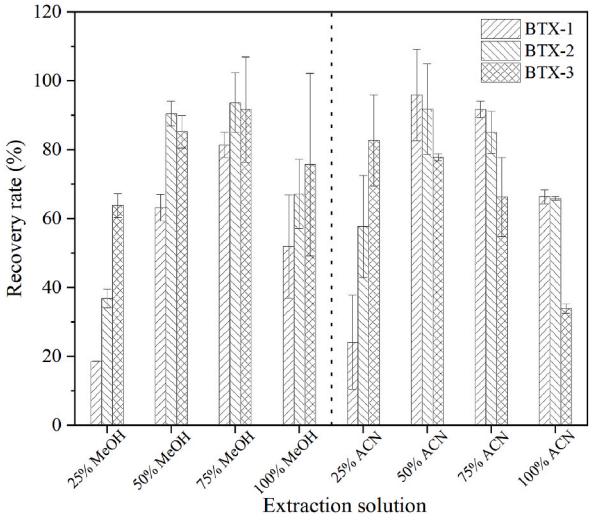

Fig. 1. The effects of extraction solution on recovery rates of three BTXs (n = 3).

using the mobile phase gradient mode A were greater than that by using the mobile phase gradient mode B. The chromatograms of BTXs in blood clam at the concentration of 5.0 μg/L on the two mobile phase gradient modes showed in Fig. S3. The optimal MRM chromatograms of BTXs were shown in Fig. S4.

3.2. Sample extraction

For the extraction of BTXs from shellfish tissues, a commonly selected extraction solvent was aqueous methanol (MeOH) or ACN. Experiments were conducted to study the extraction efficiency of a range of solvent concentrations (i.e., 25 % MeOH, 50 % MeOH, 75 % MeOH, 100 % MeOH, 25 % ACN, 50 % ACN, 75 % ACN, and 100 % ACN) from mussel samples spiked at 50 μg/kg. As shown in Fig. 1, when the extraction solvents were used as 75 % MeOH or 50 % ACN, the extraction recoveries of all three target toxins were relatively higher, and the recovery rate of each BTX toxin was higher than 78 %. The recoveries of BTXs with the extraction solution of 100 % MeOH were not good, and these results were similar to a previous report [28]. Subsequently, 75 % MeOH and 50 % ACN were selected as extraction solvents to investigate their effects on the extraction and recovery rate of BTX toxins in four shellfish matrices of mussel, oyster, scallop, and blood clam. As shown in Fig. 2A, when the extraction solution of 75 % MeOH was selected, good recoveries of there BTXs were found in the mussel matrix, but the poor extraction recoveries of BTX-1 and BTX-3 were found in the oyster matrix, and the low recovery rates of BTX-1 and BTX-2 were found in two shellfish matrices of scallop and blood clam. However, when the extraction solvent was 50 % ACN, the extraction recoveries of all three BTXs in four shellfish matrices ranged from 73.0 to 108.6 %, which complied with the requirements of [29] (see Fig. 2B). Additionally, the difference between 1-time extraction and 2 repeated extractions on the recovery rate of the target was also compared, and the results indicated that there was no appreciable difference between 1-time extraction and 2 repeated extractions. Therefore, a single extraction of 50 %ACN aqueous solution was used for sample extraction.

3.3. Sample clean-up

d-SPE clean-up method was a simple, rapid, and efficient pretreatment method, which mainly rely on adsorbents to selectively adsorb impurities (non-adsorbing targets) to achieve the purpose of purifying samples. The choice of adsorbent was one of the most important parameters of the d-SPE clean-up method. C_{18} was a kind of hydrophobicity silicone-based absorbent with a strong adsorption performance for non-polar compounds. HLB was an adsorbent with modified divinylbenzene polymers. The design of hydrophilic-lipophilic balancing (HLB) performance was suitable for the purification of acidic, alkaline, and neutral compounds. Graphite charcoal black (GCB) had a six-membered ring structure, so it had a strong affinity with plane molecules, especially suitable for being separated from various matrices to remove non-color substances and steroids. The main component of the PSA absorbent was polymerically bonded with an ethylenediamine-N-propyl phase that contained both primary and secondary amines (PSA), which could be combined with organic acids, pigment, metal ions, and phenolic compounds in the sample to remove the relevant interference in the substrate. Alumina-neutral was a strong polar adsorption agent, easy to retain the rich electronic compounds of the heterogeneous rings (containing nitrogen, phosphorus, sulfur, etc.), aromatic hydrocarbons, and organic amine compounds.

This study examined the adsorption of C_{18}, PSA, GCB, HLB, and alumina-neutral adsorbents. First of all, the adsorbents of 50 mg alumina-neutral, 50 mg PSA, 50 mg C_{18}, 50 mg HLB, and 5 mg GCB were selected for d-SPE cleanup pretreatment. As shown in Fig. 3, when the adsorbents of C_{18}, HLB, PSA, and GCB were used to purify the shellfish matrix, a part of the target was adsorbed, resulting in lower recoveries of BTXs. The adsorption effect of GCB was particularly obvious, the targets were completely adsorbed, resulting in the recovery rates of BTXs being almost zero. The good recoveries for all three BTXs were acquired, when the adsorbent of alumina-neutral was used for purification. The effects of the usage of alumina-neutral on the recoveries of BTXs were also discussed in this study. The recoveries of BTXs were improved slightly, with the increase of the alumina-neutral usage (see Fig. S5). The highest recovery was obtained when the usage of alumina-neutral was 200 mg, continued increase in the usage of neutral alumina with a slight decline in recoveries of BTXs. In contrast to the apparent color of the sample solution before or after d-SPE processing, the color of the sample

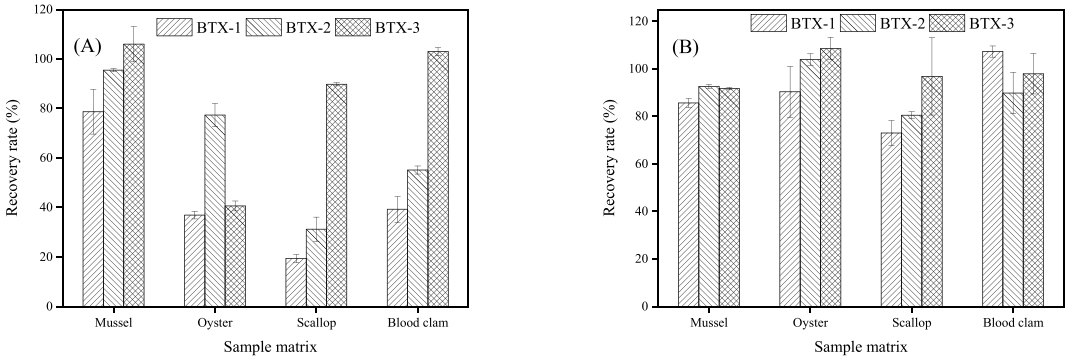

Fig. 2. (A): The recovery rates of BTXs in four kinds of shellfish with the extraction solvent of 75 % MeOH (n = 3); (B): The recovery rates of BTXs in four kinds of shellfish with the extraction solvent of 50 % ACN (n = 3).

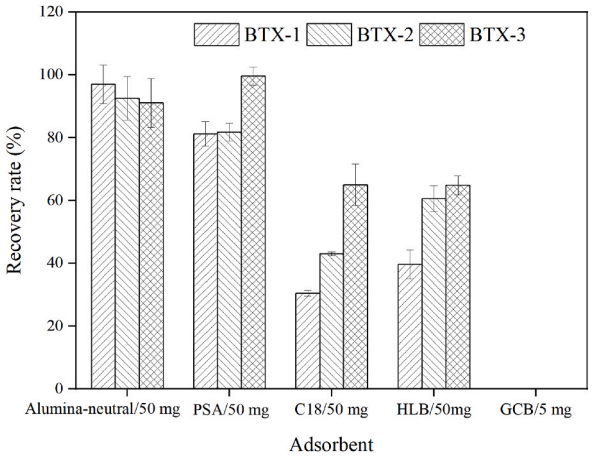

Fig. 3. The effects of d-SPE adsorbents on the recoveries of BTXs (n = 3).

solution which was not purified after extraction was pale yellow, and the apparent color of the sample solution with d-SPE cleanup treatment became lighter, which showed that d-SPE pretreatment can effectively remove impurities, and reduce the contamination of chromatography columns and mass spectrometers by complex sample matrices. In summary, 200 mg of alumina-neutral was used as an adsorbent for shellfish sample purification in this study.

3.4. Method validation

The validation process was done in accordance with Document SANTE 11312/2021 [29]. The matrix effect, specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), recovery, accuracy, and precision were calculated as validation parameters for the method in four shellfish matrices (mussel, oyster, scallop, and blood clam). The signal-to-noise ratios (the ratio between the signal intensity of each target obtained under multiple reaction monitoring (MRM) conditions and the intensity of noise in a spiked sample) of 3 and 10, respectively, were used to calculate the LOD and LOQ values of the target compound in the shellfish. The BTXs concentration of the spiked sample that was used for calculating the LOD and LOQ values was based on the concentration of the spiked sample that was closest to these limits.

3.4.1. Specificity

Specificity was measured by analyzing the reagent blank and the four representative shellfish matrices (mussel, oyster, scallop, and blood clam). The results were checked to see if any interfering peaks were presented in the analyte retention time window for each analyte. The specificity of this method was found to be satisfactory with no interfering peaks around target analytes in reagent blank and four shellfish matrix samples. These results implied that this approach could distinguish and measure the analyte even when there were other molecules present in the sample matrix.

Table 1
Calibration curves, matrix effects, LOD, and LOQ of the proposed method.

Matrix	Toxins	Ranges (ng/mL)	Curve	R^2	ME (%)[a]	LOD (μg/kg)	LOQ (μg/kg)
Solvent	BTX-1	0.5–100	y = 3546.3x - 1971.1	0.9998	/	/	/
	BTX-2	0.5–100	y = 2163.6x - 1904.3	0.9996	/	/	/
	BTX-3	0.5–100	y = 2468.0x - 1296.4	0.9998	/	/	/
Mussel	BTX-1	0.5–100	y = 3221.3x - 1490.1	0.9998	90.8	1.5	5
	BTX-2	0.5–100	y = 1876.2x - 1201.6	0.9994	86.7	1.5	5
	BTX-3	0.5–100	y = 2112.9x - 714.11	0.9998	85.6	1.5	5
Oyster	BTX-1	0.5–100	y = 3326.1x + 35.419	0.9998	93.8	1.5	5
	BTX-2	0.5–100	y = 1851.9x + 280.38	0.9999	85.6	1.5	5
	BTX-3	0.5–100	y = 2277.5x - 97.348	1.0000	92.3	1.5	5
Scallop	BTX-1	0.5–100	y = 3571.9x - 482.49	0.999	100.7	1.5	5
	BTX-2	0.5–100	y = 2284.9x - 613.34	0.9986	105.6	1.5	5
	BTX-3	0.5–100	y = 2554.9x + 455.18	0.9998	103.5	1.5	5
Blood clam	BTX-1	0.5–100	y = 3673.5x + 391.67	0.9999	103.6	1.5	5
	BTX-2	0.5–100	y = 2396.3x - 1198.1	0.9998	110.8	1.5	5
	BTX-3	0.5–100	y = 2832.2x - 1159.1	0.9998	114.8	1.5	5

[a] ME% = The slope of matrix-matched standard curve/The slope of solvent standard curve × 100 %.

3.4.2. Matrix effect

As a form of matrix effect, molecules from the sample matrix that coelute with the target compounds can interfere with the ionization process of the mass spectrometer. In this study, the matrix effect was quantified as a percentage ratio between the slopes of the calibration curve of matrix-matched and solvent standards. If the ratio of slopes ranged from 80 % to 120 %, no matrix effects were considered. The effects of signal suppression or enhancement were found if the ratios were below 80 % or above 120 %. In our experiment, the matrix effects for three BTXs in four shellfish matrices were in the range of 85.6 %–114.8 % (see Table 1). No significant signal enhancement or suppression was observed in any of the four matrices. The solution-matched external standard method could be selected for the quantitative determination of BTXs in shellfish samples, which can simplify the analytical process.

3.4.3. Linearity, LOD, LOQ

The results showed good linearity in the BTX concentration range studied (0.5–100 μg/L), with correlation coefficients (R^2) higher than 0.9986 in all cases (see Table 1). The LOD and LOQ values obtained for three BTXs in four shellfish matrices were 1.5 μg/kg and 5 μg/kg, respectively. The LOQ value of BTXs in shellfish was significantly lower than the safety limit of BTXs (800 μg/kg). Compared to the LODs and LOQs reported in the previous report, the LOD and LOQ values in this study were significantly lower than the previous methods (see Table 2).

3.4.4. Accuracy and precision

The accuracy and precision were measured using BTXs-spiked samples at low, medium, and high concentrations of 20 μg/kg, 100 μg/kg, and 250 μg/kg, respectively. The recoveries of the BTXs were in the range of 77.8–104.4 % in the mussel samples, 97.3–114.1 % in the oyster samples, 75.9–98.9 % in the scallop samples, and 77.2–107.5 % in the blood clam samples. The intra-day accuracy was established using an examination of six repetitions on the same day, whereas the inter-day precision was obtained using tests conducted over three consecutive days, with three duplicates per day. The intra- and inter-day precisions of the approach were expressed as relative standard deviations (RSD). Table 3 listed the intra- and inter-day results for BTXs recoveries and precisions, in which both the intra- and inter-day precision results are adequate for all compounds, with RSDs of less than 9.7 % and 7.2 %, respectively, which was approved by the SANTE standard of RSD 20 %.

3.5. Application to real samples

The developed d-SPE-LC-MS/MS method was then applied for the quantitative determination of BTX-1, BTX-2, and BTX-3 in 214 samples of shellfish, including mussel, oyster, scallop, and blood clam, obtained from the local fish markets of Zhoushan from 2016 to 2022. In any of the shellfish samples analyzed, none of the three toxins under examination were detected It was reported that the toxins of BTXs were also not found in the shellfish samples collected from South Korea, a neighbor of China, in 2017 [21]. This outcome demonstrated that, in terms of contamination with BTX toxins, the shellfish under investigation from Zhoushan was safe for human consumption.

4. Conclusions

In this work, a new analytical method was developed that involves simple extraction and cleanup followed by d-SPE-LC-MS/MS, which was then optimized for the quantitative determination of BTXs in shellfish. The optimized parameters used for method validation included specificity, linearity, LOD, LOQ, recovery, and precision with respect to the tissues of mussel, oyster, scallop, and blood clam. In conclusion, this method provides a simple, rapid, reliable, and cost-efficient approach for the detection of marine biotoxins, BTX-1, BTX-2, and BTX-3, in shellfish.

Table 2
Comparison of some LC-MS/MS methods for brevetoxins reported in the literature.

No	Year	Instrument method	Clean-up method	Matrix	Toxins	LOD (μg/kg)	LOQ (μg/kg)	Reference
1	2012	LC-MS/MS	SPE	shellfish	BTX-2	25	32	[20]
					BTX-3	39	50	
2	2014	LC-MS/MS	SPE	shellfish	BTX-1	8.25	NF	[21]
					BTX-2	13.6	NF	
					BTX-3	0.06	NF	
3	2018	LC-MS/MS	SPE	shellfish	BTX-1	7	25	[23]
					BTX-2	7	25	
					BTX-3	7	25	
4	2022	LC-MS/MS	d-SPE	shellfish	BTX-1	1.5	5	This study
					BTX-2	1.5	5	
					BTX-3	1.5	5	

NF: not found.

Table 3
The validation data of the proposed method.

Toxin	Spike level (μg/kg)	Mussel				Oyster				Scallop				Blood clam			
		Intra-day (n = 6)		Inter-day (n = 3)		Intra-day (n = 6)		Inter-day (n = 3)		Intra-day (n = 6)		Inter-day (n = 3)		Intra-day (n = 6)		Inter-day (n = 3)	
		Rec %	RSD %	Rec %	RSD %	Rec %	RSD %	Rec %	RSD %	Rec %	RSD %	Rec %	RSD %	Rec %	RSD %	Rec %	RSD %
BTX-1	20.0	83.0	6.3	87.9	4.5	110.6	2.1	112.3	1.5	82.3	8.5	89.2	6.8	77.2	4.2	77.9	1.3
	100.0	77.8	3.7	79.4	3.7	99.7	1.4	100.8	1.1	79.0	3.0	75.9	2.9	82.3	5.4	81.4	0.9
	250.0	85.3	2.9	82.1	4.2	97.3	1.0	97.8	0.6	77.9	4.9	78.3	4.5	82.7	6.9	84.5	1.6
BTX-2	20.0	87.3	5.9	90.7	3.0	110.4	6.8	114.1	4.3	90.7	8.7	98.9	7.2	81.8	7.7	81.9	1.4
	100.0	81.5	9.7	86.5	6.0	104.6	1.4	106.1	1.3	86.9	2.6	81.5	4.8	88.6	6.4	86.8	1.6
	250.0	92.4	2.6	87.7	5.8	100.5	0.9	100.5	0.7	81.1	3.5	82.7	4.1	86.4	5.0	87.7	1.2
BTX-3	20.0	102.6	3.5	104.4	1.7	102.9	5.4	104.4	2.6	76.7	6.4	78.1	4.1	107.5	2.4	104.9	2.3
	100.0	98.5	5.4	103.6	5.4	111.2	1.6	112.4	1.4	98.2	1.5	93.5	4.1	100.8	2.2	99.5	2.1
	250.0	100.1	2.1	97.8	3.1	107.4	0.8	107.6	0.7	95.8	2.1	95.6	1.3	93.4	2.0	94.4	1.1

Ethic statement

This study was approved by the animal care welfare committee of Zhoushan municipal district center for disease control and prevention (No. zsjk-2020-003).

Funding statement

This work was financially supported by the Science and Technology Program of Zhoushan (No.2021C31071); Medical and Health Science and Technology Program of Zhoushan (No.2020YA01).

Date availability statement

Data included in article/supplementary material/referenced in article.

CRediT authorship contribution statement

Li Fang: Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Fengmei Qiu:** Data curation, Funding acquisition, Methodology, Investigation, Validation.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Li Fang and Fengmei Qiu should be regarded as co-first authors because they both made equal contributions to this project. The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e21610.

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