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Graphene based pipette tip solid phase extraction of marine toxins in shellfish muscle followed by UPLC–MS/MS analysis ☆

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ABSTRACT

Graphene is a novel carbonic material with great potentials for the use as sorbent due to its ultrahigh surface area. Herein, we report the use of graphene as sorbent in solid-phase extraction (SPE) using pipette tip as cartridge namely GPT-SPE, together with ultra-performance liquid chromatography tandem mass spectrometry (UPLC – MS/MS), for the analysis of lipophilic marine toxins (LMTs), including yessotoxins (YTX), okadaic acid (OA), dinophysistoxin-1 (DTX1), gymnodimine (GYM), spirolides-1 (SPX1), pectenotoxin-2 (PTX2) and azaspiracid-1 (AZA1) in shellfish. The GPT-SPE procedure was optimized and the performance of graphene was fully validated. Results with high-sensitivity and good reproducibility was obtained and compared with that of other sorbents like C18 silica, multi-walled carbon nanotubes (MWCNTs), commercial Oasis HLB, and Strata-X for the extraction of LMTs, which showed superiority and advantages of graphene, such as good recoveries, stability and compatibility with various solvents. In order to exhibit the potentials of graphene as an excellent sorbent material, 67 mussel samples from six coastal cities of China were analyzed. OA was found to be the dominant contaminant, while YTX was also detected with low level.

1. Introduction

Lipophilic marine toxins (LMTs) are produced by several microalgae species and frequently bioaccumulated in filter-feeding molluscan shellfish, like mussels, oysters and clams [1]. Consumption of phycotoxins contaminated marine products may cause severe intoxications in humans such as diarrhetic shellfish poisoning (DSP), which is one of the most common syndromes all around world and results in adverse effects like gastrointestinal disorder, diarrhoea, abdominal cramps, nausea and vomiting [2]. On the basis of their chemical structures, several classes of LMTs can be discerned. Okadaic acid (OA) and its derivatives dinophysistoxin-1 (DTX-1), dinophysistoxin-2 (DTX-2) and dinophysistoxin-3 (DTX-3) are most responsible for diarrhea, showing activities of phosphatase inhibiting and tumor-promoting [3,4]. Yessotoxins (YTX) have an adverse effect on cardiac mussel cells and on defattening liver cells [5]. Gymnodimine (GYM) and spirolides (SPX) produce neurotoxic symptoms when administrated orally or injected intraperitoneally in mice [6,7]. Pectenotoxins (PTX) are mildly diarrhetic and hepatotoxic, while azaspiracids (AZA) can induce pathological changes to the liver, pancreas, thymus and spleen of mice [8,9].

Due to the server adverse effect of LMTs, major concern for public health authorities as well as for the shellfish industries has risen. In European Union (EU) legislation, maximum levels (ML) have been established for various LMTs in marine products when placing them on market for human consumption [11]. The permitted levels for the sum of OA, DTX, and PTX is set at 160 μg OA-equivalents kg^{-1}, the sum of relevant YTX is set at a total of 1 mg YTX-equivalents kg^{-1} and the sum of relevant AZA at 160 μg AZA-1 equivalents kg^{-1} -edible shellfish [12]. Regulatory limits for GYM and SPX have not yet been established. In order to meet the requirement of EU, much effort has been put in the development of novel methods recently for effective preparing samples and detecting possible different LMTs [13,14]. Liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC–ESI–MS/MS) is arising as the most promising strategy for quantification of LMTs, which combine efficient separation power with generic detection for different toxin groups. Meanwhile, solid-phase extraction with different sorbents, such as Cyano, C8, C18 and resins, were usually utilized for reducing matrix effects. Fux et al. described an UPLC–MS/MS method to analyze 21 LMTs in 6.6 min following triplicate methanolic extraction, but signal suppression and enhancement was observed for OA and AZA1

☆ *Notes*: The authors declare no competing financial interest.

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[15]. Gerssen et al. compared various columns and the pH of mobile phase for the separation and detection of LMTs [16], meanwhile, they also developed an optimized SPE procedure by testing ion-exchange, silica-based, and mixed-function sorbents [17].

Graphene is a new class of carbon nanomaterial, which has recently sparked much interest for both fundamental science and applied research [18]. Notably, its unique two-dimensional double-sided polyaromatic scaffold with high specific surface area holds great promise for sorption material with high loading capacity [19]. Moreover, the special π–π electrostatic stacking property endows it a strong affinity for carbon-based ring structures [20,21]. By far, graphene has already successfully applied in the field of MALDI matrix [22], graphene-assisted matrix solid-phase dispersion [23], graphene–polymer composite based stir rod sorptive extraction [24], solid-phase microextraction [25], and so on.

The aim of this study was to investigate the potential of graphene as SPE sorbent for the purification of LMTs. After that, graphene was used as sorbent for developing a microscale SPE using pipette tip as cartridge, namely GPT-SPE. The eluates from GPT-SPE were analyzed by UPLC–MS/MS detection. The performance of graphene was compared with several other sorbents or commercial SPE cartridge including C18, MWCNTs, HLB and Strata-X. Finally, the proposed method was applied to the analysis of the tissue from commercial available shellfish.

2. Materials and methods

2.1. Chemicals and reagents

The chemical standards of YTX, OA, DTX1, GYM, SPX1, PTX2 and AZA1 were all purchased from Lix Biotech Co., Ltd (Beijing, China). For linearity studies, working solutions were freshly prepared by appropriate dilution of the stock solution with LC mobile phase (concentrations ranging from 0.1 to 200 ng mL^{-1}). Acetonitrile, methanol and formic acid were chromatographic grade and obtained from Merck (Darmstadt, Germany). High purity water with a resistivity of 18.2 MΩ cm^{-1} was obtained from a Milli-Q water system (Millipore, Bedford, MA, USA).

2.2. GPT preparation

Graphite oxide (GO) was synthesized using modified improved method proposed by Marcano [26]. Briefly, a 9:1 mixture of concentrated H_2SO_4/H_3PO_4 (360:40 mL) was added to a mixture of graphite flakes (3.0 g, 1 wt equiv) and $KMnO_4$ (18.0 g, 6 wt equiv) and the reaction was heated to 50 °C and stirred for 12 h. Then, the reaction was cooled to room temperature and poured onto ice (400 mL) with 30% H_2O_2 (20 mL) causing the color turning to yellow along with bubbling. For workup, the mixture was filtered and the filtrate was centrifuged (10,000 rpm for 2 h). The residue was washed with 1.0 L of HCl (10%) and 1.0 L of water. The obtained solid was dialyzed against water for one week, and then dried by lyophilization. The tapping mode AFM image of isolated GO was shown in Fig. 1.

Graphene was synthesized by hydrazine reduction of GO. About 1.0 g of GO was weighted and exfoliated in 1.0 mL of water by ultrasonication for 1 h. Then, hydrazine hydrate was added to the dispersion with the weight ratio of hydrazine to GO being 7:10. This dispersion was heated at 95 °C overnight, and the reduced GO gradually precipitated as black solid. The final product washed thoroughly with water and methanol, and then dried by lyophilization.

A mass of 2 mg graphene were packed into a 200 μL pipette tip. Polypropylene materials were set as frits at both sides to hold the sorbent (a bed of 1.2 cm height of graphene was obtained). Before use, the GPT was washed with 1.0 mL ethyl acetate, methanol and ultra-pure water step by step.

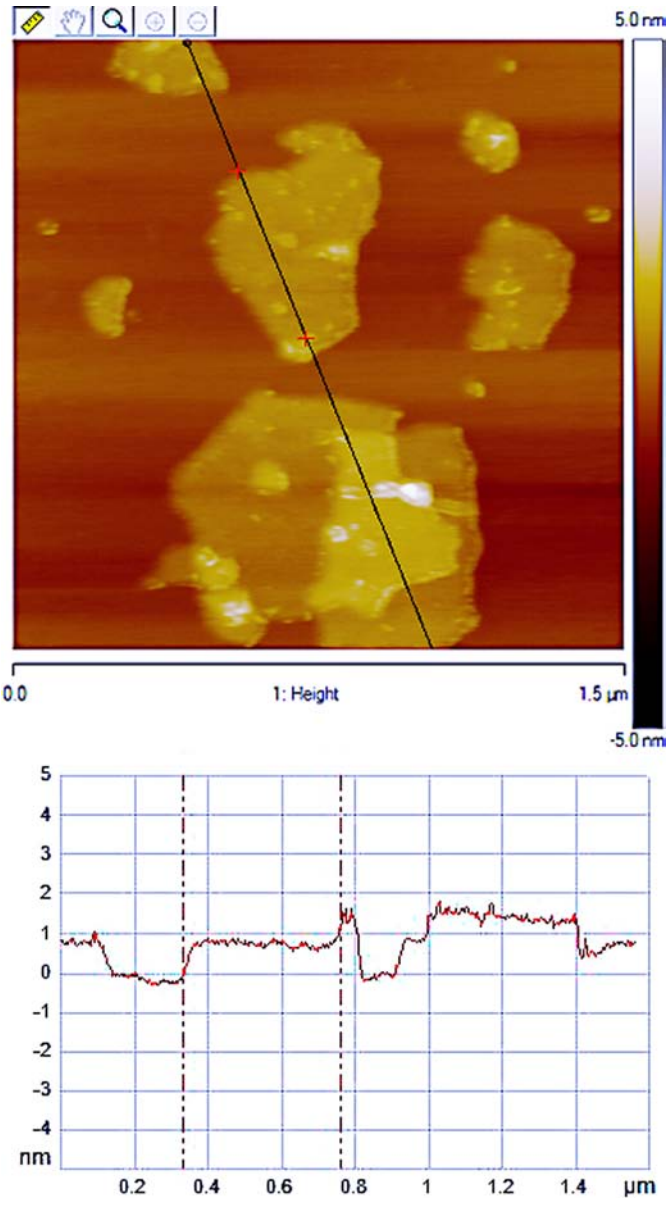

Fig. 1. Tapping mode image of isolated graphene oxide sheets on freshly cleaved mica, the height is about 1 nm, indicating a single graphene oxide sheet.

2.3. Sample preparation

The shellfish tissues were homogenized using an electric blender. 0.2 g of shellfish homogenate was extracted in triplicate with 3 mL methanol by ultrasonic assisted extraction (UAE) with a Model VCX-130 ultrasonic probe of 20 kHz and 130 W (Sonics & Materials Inc., Newton, USA). A 12 mm diameter probe tip was inserted into the sample and the power level was fixed at 50% amplitude for all experiments. The extraction was performed for 15 min with temperature maintained at 4 °C, and then the sample tube was centrifuged to separate the liquid extract from the solid residue. A 100 μL aliquot of the supernatant was mixed with diluted stock solutions, dried with nitrogen flow and re-suspended in 200 μL water for GPT-SPE.

The liquid sample was aspirated into the conditioned GPT cartridge, and dispensed back into the same sample tube. These two steps are referred to as one aspirating/dispensing cycle.

In this work, extraction of seven LMTs was performed by at least 20 repeated aspirating/dispensing cycles, and the eluate was dispensed as waste. After washing with 5% acetonitrile, the GPT was placed on a vacuum manifold and dried under vacuum for 3 min to remove any traces of water. Finally, analytes were eluted from the tip with 200 μL 5% ammonium hydroxide (25%) in acetonitrile as basic condition or 0.5% formic acid in acetonitrile as acidic condition by 10 repeated aspirating/dispensing cycles. The obtained eluate was ready for LC−MS/MS analysis.

2.4. LC−MS/MS

Chromatographic analysis was performed on a Waters UPLC system (Waters, Milford, MA) which was equipped with a quaternary pump, an autosampler, a vacuum degasser and Empower workstation (Waters, Milford, MA). The separation was achieved on a Gemini-NX column (50 mm × 2 mm; i.d., 3 μm, Phenomenex, USA). A gradient UPLC method was employed for separation with the mobile phase A: 10 mM ammonium hydroxide aqueous solution and mobile phase B: acetonitrile. The gradient profile was carried out as follows: 20% B (initial), 20–50% B (0–2.5 min), 50–95% B (2.5–4.0 min), 95–100% B (4.0–6.0 min). The flow rate was 0.6 mL min^{-1}. The column temperature was set at 30 °C, and injection volume of 5 μL was selected. All of the LMTs were eluted within 5 min. During the rest time the column was cleaned, readjusted to the initial conditions and equilibrated.

Two triple-quadrupole linear ion trap mass spectrometers (both 3200Q-Trap and 4000Q-Trap, Applied Biosystems, Foster City, CA) equipped with electrospray ionization (ESI) were tested in both positive and negative ionization mode. Instrument control, data acquisition and the processing were performed using the associate Analyst 1.5.1 software. MS/MS data acquisition was performed in the multiple-reaction monitoring (MRM) mode. According to European Union Decision 2002/657/EC, for the confirmation of the presence of an illegal veterinary drug at least 4 identification points are necessary (1 point was earned with the precursor ion and 1.5 points were earned with each product ions). Therefore, two transitions between precursor ion and the two most abundant product ions were monitored: one for quantitative determination and the other for qualitative analysis. In order to obtain maximum sensitivity for identification and detection of LMTs, the ion source temperature (TEM) was set at 500 °C, and ion spray voltages (IS) were set at 5.5 kV and –4.5 kV in positive and negative ion modes, respectively. Ion source gas1 (GS1) and ion source gas2 (GS2) were used as the drying and nebulizer gases at a back pressure of 35 psi and 30 psi, respectively. Curtain gas (CUR) was 35 psi. The analyte specific parameters (declustering potential (DP), entrance potential (EP) for precursor ions, collision energy (CE) and collision cell exit potential (CXP) for product ions) are shown in Table 1.

2.5. Method validation

Validation of methods was performed with respect to various parameters including linearity, limit of detection (LOD), limit of quantification (LOQ), and intra- and inter-day precision and accuracy, recovery, and stability. Method linearity (peak area vs. concentration) was evaluated using linear regression analysis at six concentrations of each standard solution. LOD and LOQ were calculated on the basis of signal to noise ratio ($S/N=3$ for LOD and $S/N=10$ for LOQ) on the mass spectrograms of the tested shellfish tissue samples. Precision was determined in terms of repeatability, by running six extracts of shellfish tissue samples spiked at three levels (low, mediate and high) on a single day, and reproducibility was quantified using six replicate samples spiked at the same levels on five different days. Recovery studies were carried out on commercial blank shellfish tissue samples and the values were determined at three different levels (low, mediate and high). Three replicates were performed at each spiking level to determine the relative standard deviation (RSD).

Table 1
Optimized MS/MS parameters for the tested lipophilic marine toxins.

Compounds	Retention time[a]	Precursor ion	Transitions[b]	DP[c]	EP[c]	CE[c]	CXP[c]
Negative-ion mode							
OA	1.21	$[M-H]^-$	**803.5 > 255.3** [d]	60	10	65	8
			803.5 > 563.5	60	10	65	8
YTX	1.61	$[M-H]^-$	**1141.6 > 1061.6**	45	10	60	12
			1141.6 > 925.5	45	10	60	12
DTX1	1.97	$[M-H]^-$	**817.5 > 255.3**	65	10	60	10
			817.5 > 563.5	65	10	60	10
Positive-ion mode							
AZA1	2.60	$[M+H]^+$	**842.5 > 824.5**	50	10	60	10
			842.5 > 654.5	50	10	60	10
GYM	3.40	$[M+H]^+$	**508.3 > 490.3**	45	12	35	10
			508.3 > 392.3	45	12	35	10
SPX1	3.86	$[M+H]^+$	**692.5 > 164.5**	45	10	55	10
			692.5 > 444.3	45	10	55	10
PTX2	3.95	$[M+NH_4]^+$	**876.5 > 823.5**	40	11	40	10
			876.5 > 213.0	40	11	40	10

Ion Spray Voltages (IS): 5.5 kV (positive ion mode) and −4.5 kV (negative ion mode); Curtain Gas (CUR): 35 psi; Ion Source Gas1 (GS1): 35 psi; Ion Source Gas2 (GS2): 30 psi; Temperature (TEM): 500 °C; Interface Heater: on; Collision Activated Dissociation (CAD): Medium.

[a] Unit: min.
[b] Unit: *m/z*.
[c] Unit: eV.
[d] Bold transitions are used for quantitative analysis.

3. Results and discussion

A new sample preparative method was developed for a reliable quantification of individual LMTs present in biological samples with regard to national regulations. With the optimized LC method, YTX, OA, DTX1, GYM, SPX1, PTX2 and AZA1 can be separated well. However, YTX, OA and DTX1 were better analyzed in positive-ion mode, while the rests were performed better in negative-ion mode. For this reason, it is impossible to detect them simultaneously without polarity switching. Optimization of MRM conditions was carried out by direct infusion of standard solutions without separation. ESI provided single charged precursor ions for all LMTs except for YTX, which also yielded a double negatively charged precursor ion ($[M-2H^{2-}]$) at *m/z* 570.4. The retention time of LMTs, MS/MS ion transitions, and electronic conditions were optimized and summarized in Table 1.

3.1. Optimization of GPT-SPE conditions

Generally, the GPT-SPE procedure contains three critical steps: sample loading, matrix washing, and analytes recovering. The operating parameters were optimized involving in these three steps to investigate the analytical potential of graphene used as PT-SPE sorbent for extracting LMTs. It must be kept in mind that these conditions were a compromise resulting from the chemically diverse set of LMTs and may be far from optimal for some compounds.

The number of aspirating/dispensing cycles is a critical parameter for extraction recovery by the PT-SPE [27]. The extraction profiles of LMTs were examined by comparing the analyte abundance from the spiked shellfish samples against the number of aspirating/dispensing cycles. Extraction of the compounds reached equilibrium after 20 aspirating/dispensing cycles at both high

(100 ng mL^{-1}) and low (1 ng mL^{-1}) concentrations, and this number of aspirating/dispensing cycles was used in subsequent experiments giving an extraction time of approximately 1 min.

The ionization efficiency of LMTs in ESI source may be affected by matrix interference. Given that matrix of crude extract from spiked shellfish sample is quite complex, the percentage of organic modifier for clean-up of the sample matrix was also studied. Washing solvents with 0, 5, 10, and 20% acetonitrile were tested and the results indicate that no desorption was detected when the acetonitrile concentration was equal to or lower than 10%. However, more co-extracted matter retained on the GPT-SPE column when high purity water was used as solvent. When acetonitrile concentration was 20%, the recoveries of all the three tested compounds were decreased (data were not shown). Consequently, 5% acetonitrile was adopted as the optimum washing solvent.

In the elution process, the GPT was transfer to a 1.5 mL vial which contained 200 μL 5% ammonium hydroxide (25%) in acetonitrile. The analytes were eluted by aspirating/dispensing the solvent through the tip several times. The number of aspirating/dispensing cycles was at least 5, and more cycles used for desorption did not substantially alter the results for the compound tested, which was agree well with studies elsewhere [23]. In order to achieve sufficient recovery, 10 sequential aspirating/dispensing cycles (approximately 10 s) with 200 μL of basic acetonitrile were used in the elution step.

The retention behavior of LMTs on graphene is governed by their lipophilic properties and polar or ionic groups. Therefore, the pH value of eluting solvents is quite important for the quantitative recoveries of LMTs during the GPT-SPE procedure by influence the interaction between the LMTs and the graphene. Generally, chemicals that are in their ionic state will elute at a lower percentage organic solvent in the mobile phase than when they are in their neutral state. Gerssen et al. have already described the charged state of LMTs, under both acidic and basic conditions [16]. In brief, YTX is a strong acid and will be always negatively charged, OA and DTX1 will be neutral under acidic condition but be negative under basic condition, GYM and SPX1 will be positively charged at low pH and neutral at high pH due to the imino group, PTX2 will stay neutral since no ionizable groups contained, and AZA1 will be in a positively charge at a low pH while be in a negative charge at high pH. The GPT were eluted with alkaline, neutral, and acidic solutions to study the retention of the LMTs on the graphene. The results were summarized and shown in Fig. 2. The best results were got by using basic eluting solvent, where the recoveries of GYM, SPX1, PTX2 and AZA1 were nearly to 90%, obviously higher than that under other conditions. However, the recoveries of YTX, OA and DTX1 were comparatively lower, especially that of OA, which is only about 78%, lower than that under acidic and neutral conditions with recoveries 83% and 80%, respectively. Acidic condition was not utilized due to its low YTX recovery, only about 58%. In generally, basic condition was used given to its comparatively better performance.

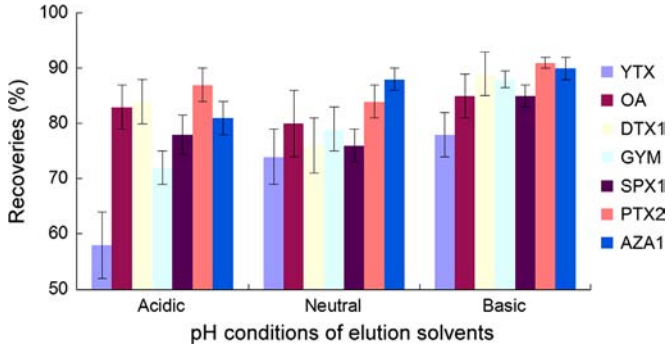

Fig. 2. Recoveries of the tested seven lipophilic marine toxins using elution solvents under basic condition using 5% ammonium hydroxide (25%) in acetonitrile, neutral condition, and acidic condition using 0.5% formic acid in acetonitrile (standard deviation, $n=3$).

3.2. Comparison of sorbents

Experiments were carried out to assess the effect of several sorbents on the yield and selectivity of the GPT-SPE technique. The performance of different SPE materials was tested, including both polar and non polar, such as C18, hydrophilic-lipophilic balance (HLB), commercial Strata-X, multi-walled carbon nanotubes (MWCNTs), and novel graphene. The extraction efficiencies of different solid phase sorbents are depicted in Fig. 3. The best results were obtained when using graphene as PT-SPE sorbent, followed by commercial Strata-X. Graphene was an ideal sorbent for recovering LMTs from biological sample because the recoveries of DTX1, PTX2 and AZA1 were nearly to 90%. The recoveries of YTX, OA, GYM and SPX1 were comparatively lower but still satisfactory, ranging from 78% to 87%. Strata-X is popular in LMTs analysis, and in this experiment, the performance of Strata-X is generally similar with graphene, except YTX and SPX1, with recoveries 74% and 76%, respectively. MWCNT is a popular and versatile material in SPE procedure. In fact, its recovering ability of LMTs is somewhat weaker than that of graphene. The reason could be ascribed to the double-sided polyaromatic scaffold structure of graphene, which made it an ultra-high specific surface area material with high loading capacity, while the MWCNT only has one-side available to the target chemicals. HLB provided generally most terrible recovery results because the recovery of YTX species was only about 54%. This could be well explained by the strong acidic property of the YTX, which made it difficult to recover. C18 was a most commonly used SPE sorbent in the studies of residues in food, bioactive compounds in herbal medicines and metabolites in "omics" [28]. In this experiment, although the recoveries of OA, PTX2 and AZA1 were satisfactory, the recoveries of the rest LMTs were only ranging from 68.0% to 77.5% when C18 was used as PT-SPE sorbent. Therefore, graphene performed well and was chosen as PT-SPE sorbent to recover LMTs from shellfish tissues.

3.3. Method validation

Method validation was implemented according to the study reported before [29]. The following performance studies carried out: specificity, linearity, limit of detection (LOD) and limit of quantification (LOQ), accuracy and precision, recovery and reproducibility.

The method specificity was evaluated for any interfering peaks in the retention time window of the respective target analyte by testing blank control samples without adding chemical standards. The latent interference from co-eluting matrix constituents on the ionization of

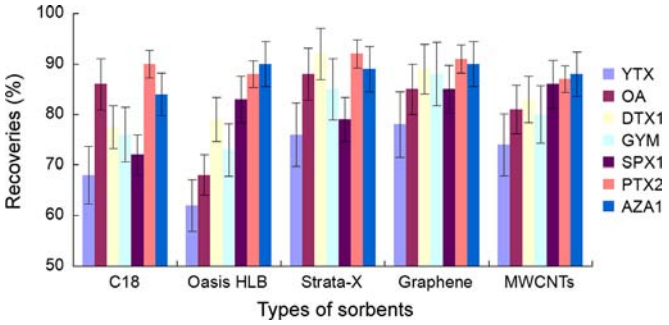

Fig. 3. Performance comparison of five types of sorbents or commercial SPE cartridges, including C18, HLB, MWCNTs, commercial Strata-X cartridge and novel graphene (standard deviation, $n=3$).

analyte, i.e. the potential ion suppression or enhancement, was expressed as matrix effect (ME). LMT standard solutions with three concentrations were tested following the method proposed previously with moderate modifications, and the ME was then calculated as: ME (%)=(1—Area of spiked standards/Area of standards in pure solvent) × 100%. ME=0 indicate the absence of matrix effect, otherwise denoting ionization suppression (ME > 0) or enhancement (ME < 0) [30,31]. The higher the absolute value of ME, the stronger the matrix interference. Experiments were carried out using several shellfish tissue matrices spiked at both high (100 ng mL^{-1}) and low (1 ng mL^{-1}) concentrations. Matrix effects below 6% with RSD≤6% were obtained for all shellfish tissue matrices, which indicated that the ionization suppression or signal enhancement did exist but not very significant.

Method linearity (peak area vs. concentration) was evaluated based on the analysis of the data with six levels of concentration, by linear regression with intercepts and $1/x$ weighting factor. All of the analytes were linear in concentrations up to the level of 200 ng mL^{-1}, and the correlation coefficients (R^2) of the calibration curves were in the range of 0.9963–0.9996 ($n=6$), which indicated good linearity between the peak area (y) and investigated compound concentration (x, ng mL^{-1}). LOD and LOQ were calculated on the basis of signal to noise ratio ($S/N=3$ for LOD and $S/N=10$ for LOQ) on the mass spectrograms of the tested shellfish tissue samples. The LOD and LOQ were less than 1.5 μg kg^{-1} and 3.9 μg kg^{-1} (Table 2), which showed enough sensitivity for conducting the determination process. Recovery studies were carried out on spiked blank shellfish tissue samples and the values were determined based on the ratio of the measured concentration/the fortified level × 100% at three different levels. The recovery of assay was easily influenced at extremely high concentration of target analytes, partially due to MS/MS in-source fragmentation [31]. This problem was solved by diluting the injecting concentration and decreasing the sampling volume. It suggested that there was no relevant difference in extraction recovery at acceptable range of concentrations. Accuracy and precision was determined in terms of repeatability, by running seven extracts of shellfish tissue samples spiked at three different levels (listed in Table 3) on a single day, and reproducibility was quantified using five replicate samples spiked at the same levels on five different days. Three replicates were performed at each spiking level to determine the relative standard deviation (RSD). The RSD of intra- and inter-day accuracy for LMTs in shellfish tissue extracts were in the ranges of 1.01–3.56% and 1.54–4.64%, respectively. These data indicate that the repeatability, intermediate precision, and bias values of the assay are regarded as quite satisfactory. The stability was expressed as a percentage of the theoretical value. For short-term stability, the RSD of all the analytes were lower than 5.4 at three spiking levels, which indicated that the rural samples could be prepared and handled during the period of a minimum of 12 h without any indication of degradation. For long-term stability, the recoveries became unstable after one month storage, maybe due to enzymatic hydrolysis or biotransformation of LMTs. These studies suggest that shellfish tissue samples containing LMTs can be handled under normal laboratory conditions without significant loss of compound.

Table 2
Parameters of linearity, limit of detection (LOD) and limit of quantification (LOQ).

Analyte	R^2[a]	Linearity[b]	LOD[c]	LOQ[c]
OA	0.9991	1.5–150	1.5	3.9
YTX	0.9963	2.0–200	1.4	3.7
DTX1	0.9996	2.0–200	1.0	3.1
AZA1	0.9993	0.1–100	0.2	0.6
GYM	0.9987	0.5–100	0.1	0.2
SPX1	0.9989	0.5–100	0.1	0.2
PTX2	0.9992	1.0–100	0.2	0.5

[a] Regression coefficient.
[b] ng mL^{-1}.
[c] μg kg^{-1} l.

Table 3
The method precision and recovery at three different concentrations for GPT-SPE coupled with UPLC–MS/MS analysis of lipophilic marine toxins from shellfish muscle samples.

Compounds	Spiked levels (μg kg^{-1})	Intra-day precision ($n=7$)		Inter-day precision ($n=5$)	Recovery	
		Mean value (μg kg^{-1}) ± SD	RSD %	RSD %	Mean value (μg kg^{-1}) ± SD	RSD %
OA	2.0	1.87 ± 0.06	2.11	2.15	1.77 ± 0.16	5.57
	10.0	9.93 ± 0.44	1.47	1.54	8.53 ± 0.84	3.54
	100.0	97.03 ± 3.25	3.38	4.47	86.03 ± 2.15	7.34
YTX	2.0	1.89 ± 0.04	1.28	3.31	1.61 ± 0.54	4.84
	10.0	10.05 ± 0.15	1.01	2.05	7.25 ± 0.96	5.88
	100.0	103.68 ± 0.48	2.12	3.20	75.38 ± 3.28	6.65
DTX1	2.0	2.02 ± 0.03	2.57	2.60	1.82 ± 0.09	4.78
	10.0	9.38 ± 0.84	2.48	3.58	9.26 ± 0.64	5.20
	100.0	91.60 ± 5.69	3.56	4.63	92.60 ± 4.69	7.01
AZA1	1.0	1.09 ± 0.04	2.06	2.15	0.92 ± 0.04	5.61
	10.0	10.21 ± 0.25	1.02	1.61	9.24 ± 0.44	3.51
	100.0	97.68 ± 0.48	3.06	3.51	92.34 ± 3.11	4.29
GYM	0.5	0.47 ± 0.03	1.21	3.26	0.43 ± 0.03	4.92
	10.0	10.05 ± 0.26	2.13	3.03	8.81 ± 0.43	5.83
	50.0	48.89 ± 2.04	3.25	3.27	48.96 ± 1.24	6.63
SPX1	0.5	0.48 ± 0.03	2.1	2.21	0.40 ± 0.06	5.47
	10.0	9.35 ± 0.56	1.58	1.60	8.56 ± 0.64	3.54
	50.0	51.89 ± 1.94	3.39	2.48	44.66 ± 2.11	5.33
PTX2	1.0	1.07 ± 0.03	1.56	2.58	0.88 ± 0.02	4.75
	10.0	9.83 ± 0.34	2.52	3.58	9.67 ± 0.53	5.27
	100.0	99.03 ± 2.25	3.56	4.64	96.32 ± 4.46	7.00

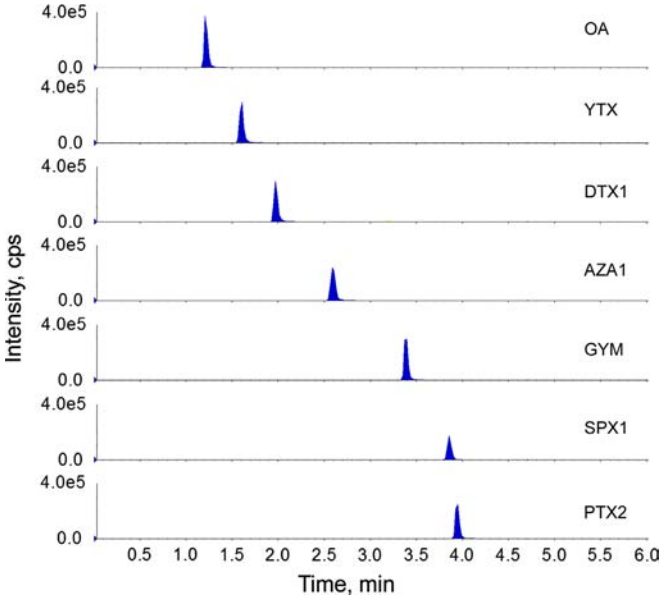

Fig. 4. UPLC–MS/MS chromatograms showing the separation of the tested seven lipophilic marine toxins (100 ng mL^{-1} standard solution) in both positive and negative ion modes.

3.4. Real sample application

The proposed methodology was applied to the determination of concentrations of LMTs in 67 shellfish samples from six different

Table 4
The quantities of lipophilic marine toxins in 67 shellfish samples from six geographical origins (μg kg^{-1}) (n=5).

Analytes	Jinzhou	Laizhou	Zhoushan	Shantou	Haikou	Hong Kong
Sinnovacula constricta	/[a]	/	–[b]	OA (2.5 μg kg^{-1})	–	–
Tegillarca granosa	/	/	–	–	–	–
Ruditapes philippinarum	–	OA (+[c])	–	/	/	/
Scapharca subcrenata	/	–	–	/	/	/
Barbati virescents	YTX (5.4 μg kg^{-1})	–	–	/	/	–

[a] "/": Shellfish samples from this area were not collected.
[b] "–": No LMT was detected in this sample.
[c] "+": Positive sample with content of LMT lower than LOQ.

geographic origins. Typical MRM chromatograms of LMTs in both positive and negative ion modes were showed in Fig. 4. Analytes can be easily discriminated and quantified, since no significant interference of the nearby components was observed in the quantitative analysis of each compounds. The contents of LMTs in the shellfish materials were summarized in Table 4. From the results of the tested samples, *Sinnovacula constricta* and *Ruditapes philippinarum* were detected as OA positive. *Barbati virescents* from Jinzhou was detected as YTX positive with concentration 5.4 μg kg^{-1} while free of the rest LMTs. Marine toxic pollution depends on many factors, including the physiological and genetic characteristics of shellfishes, the quantity and toxin content of algae, and the ability of shellfish on filtration, absorption, transformation and accumulation of toxin from algae. LMTs related algae, like *Dinophysis caudate*, *Dinophysis fortii*, *Dinophysis tripos*, *Prorocentrum lima* and so on, growth rapidly with the increase of temperature. Meanwhile, water-body eutrophication pollution of estuaries making red tide frequently took place in the East China Sea and the South China Sea, threatening the living conditions of shellfishes. Thus, qualitative and quantitative analysis of LMTs could be useful and even necessary to ensure the safety of shellfish and the successfully applied GPT-SPE combined UPLC–MS/MS method exhibited great advantage detecting the low concentrations of LMTs in shellfish.

4. Conclusion

The potential of graphene and the performance of GPT-SPE were evaluated by UPLC–MS/MS using seven LMTs as model analytes. The graphene-packed PT-SPE cartridges showed reliable and attractive analytical performance in the analysis of commercial shellfishes. When compared with other commercial sorbents, like C18, HLB, Strata-X, and MWCNTs, graphene was more effective than or as effective as these sorbents for SPE of trace YTX, OA, DTX1, GYM, SPX1, PTX2 and AZA1 under similar conditions. The overall method showed excellent specificity, linearity, reproducibility, high extraction efficiency and low detection limits. Therefore, we believe that graphene is a promising material for PT-SPE. The GPT-SPE is disposable and can't be used repeatedly since the bed of the sorbent may easily corrupted. Meanwhile, contamination may be induced if the GPT-SPE was repeatedly used for different samples. Further efforts should be made to extend the range of application.

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