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Molecularly imprinted polymer for selective extraction of domoic acid from seafood coupled with high-performance liquid chromatographic determination

Wen-Hui Zhou [a,c,*], Xiu-Chun Guo [b,c], Heng-Qiang Zhao [c], Si-Xin Wu [a], Huang-Hao Yang [c,**], Xiao-Ru Wang [c]

[a] The Key Laboratory for Special Functional Materials of MOE, Henan University, Kaifeng, PR China
[b] Pharmaceutical College of Henan University, Kaifeng, PR China
[c] The First Institute of Oceanography of SOA, Qingdao, PR China

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ABSTRACT

In this work, a highly selective sample cleanup procedure that combining molecular imprinting technique (MIT) and solid phase extraction (SPE) was developed for the isolation of domoic acid (a fascinating marine toxin) from seafood samples. The molecular imprinting polymer (MIP) for domoic acid was prepared using 1,3,5-pentanetricarboxylic acid as the template molecule instead of domoic acid. 4-Vinyl pyridine was used as the functional monomer and ethylene glycol dimethacrylate was used as the cross-linking monomer. The obtained imprinted polymer showed high affinity to domoic acid and was used as selective sorbent for the SPE of domoic acid from seafood samples. An off-line molecularly imprinted solid phase extraction (MISPE) method followed by high-performance liquid chromatography (HPLC) with diode-array detection for the detection of domoic acid was also established. Good linearity was obtained from 0.5 mg L^{-1} to 25 mg L^{-1} ($R^2 > 0.99$) with a quantitation limit of 0.1 mg L^{-1}, which was sufficient to determine domoic acid at the maximum level permitted by several authorities. The mean recoveries of domoic acid from mussel extracts were 93.4–96.7%. It was demonstrated that the proposed MISPE–HPLC method could be applied to direct determination of domoic acid from seafood samples.

1. Introduction

Domoic acid, the chemical responsible for amnesic shellfish poisoning (ASP), was originally isolated from the macroscopic red alga called *Chondria armata* in Japan [1]. Domoic acid is mainly produced by diatoms of the genus Pseudo-nitzschia [2], and is accumulated in shellfish edible tissue during algal blooms. On ingestion by humans, contaminated shellfish may cause different symptoms, including vomiting, diarrhea, dizziness, seizures and permanent loss of short-term memory [3]. The first reported outbreak of ASP occurred in a localized area of eastern Prince Edward Island, Canada in 1987 [4]. In that outbreak, hundreds of people become ill after eating contaminated mussels (*Mytilus edulis*), including four fatalities. Since this incident, the Canadian authorities as well as the European Union and the USA imposed an action limit of 20 μg g^{-1} domoic acid in wet tissues of shellfish.

Following the 1987 outbreak of ASP, several analytical methods have been developed for the quantitative determination of domoic acid, including liquid chromatography–mass spectrometry (LC–MS) [5,6], capillary electrophoresis (CE) [7,8], gas chromatography–mass spectrometry (GC–MS) [9], radioimmunoassay (RIA) [10], enzyme-linked immunosorbent assay (ELISA) [11,12], thin-layer chromatography (TLC) [13], cytotoxicity assay (CA) [14,15], and surface plasmon resonance (SPR) [16]. And domoic acid has been detected using different methods in several regions of the worldwide [17–22]. Although these methods are becoming more common in some laboratories, HPLC is often the only analytical tool available in many research institutes and regulatory agencies responsible for monitoring the occurrence of toxins (domoic acid, in this case). So, new method base on HPLC is more suited for routine domoic acid analyses and monitoring. Therefore, a reliable, simple and low cost method with high sensitivity and selectivity for determination of domoic acid residues in seafood is still highly demanded.

In view of the seafood-matrix, pretreatment in order to concentrate and purify domoic acid for analysis should be executed. SPE is a common method that is usually used to clean up a sample before using a chromatographic or other analytical method to quantitate the amount of analyte(s). However, SPE still has

* Corresponding author at: The Key Laboratory for Special Functional Materials of MOE, Henan University, Jinming Campus, Kaifeng, PR China.
** Corresponding author.
E-mail address: zhouwh@henu.edu.cn (W.-H. Zhou).

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several limitations. One of the main disadvantages of the classical SPE sorbents (C_8, C_{18}, etc.) is low selectivity. The molecular imprinting technique (MIT) is one of the most attractive methods for obtaining selective recognition abilities and the corresponding MIP represents robust and stable artificial receptors for analyte of interest. So combining MIT with SPE technique is possible to combine the advantages of both molecular recognition and traditional separation methods. Thus, molecularly imprinted solid phase extraction (MISPE) presents the high specificity, selectivity and sensitivity of the molecular recognition mechanism and the high resolving power of separation methods [23].

In view of the high toxicity and cost of the target molecules, dummy imprinting is a viable strategy for the synthesis of MIP for domoic acid. In the present work, the MIP toward domoic acid was prepared through bulk polymerization method using 1,3,5-pentanetricarboxylic acid (PTA) as the dummy template molecule, 4-vinyl pyridine (4-VP) as the functional monomer and ethylene glycol dimethacrylate (EGDMA) as the cross linking agent. The obtained imprinted polymer showed high affinity to domoic acid and was successfully applied as special SPE sorbent for selective extraction of domoic acid from seafood samples. The method had been validated and applied to determine domoic acid from mussel extract with satisfactory results. To our knowledge, this work represents the first attempt of using MIP as selective SPE sorbents for the determination of domoic acid from mussel extract.

2. Experimental

2.1. Materials and chemicals

4-Vinyl pyridine (4-VP), ethylene glycol dimethacrylate (EGDMA) were purchased from Alfa and distilled to remove the polymerization inhibitor before use. 1,3,5-Pentanetricarboxylic acid (PTA) was procured from TCI (Shanghai, China) and used without any further purification. Domoic acid was purchased from Sigma–Aldrich and used without any further purification. Azobis(isobutyronitrile) (AIBN) was the product of Sinopharm Chemical Reagent Beijing Co., Ltd. (Beijing, China) and was recrystallized prior to use. Acetonitrile and methanol were all of HPLC grade and procured from Merck. All water used was obtained from a Millipore Milli-Q purification system. Blue mussels were obtained from a local fish market in Qingdao.

A 100 mg L^{-1} domoic acid stock solution was prepared in acetonitrile and placed in the dark. More dilute standards were prepared by appropriate dilution.

2.2. Instrumentation and conditions

HPLC analysis was performed on the Agilent 1100 series HPLC system containing a G1322A Vacuum Degasser, a G1312A Binary Pump, a G1313A ALS Autosampler, a G1316A Column Compartment and a G1315B diode array detector. Separation was carried out on an Agela Venusil XBP-C_{18} column (250 mm × 4.6-mm-i.d., particle size 5 μm). The column thermostat was set at room temperature. The mobile phase was water–acetonitrile–trifluoroacetic acid (750:250:1 (v:v)) and its flow rate was set at 0.6 mL min^{-1}. The detection wavelength was set to 242 nm.

ESI–TOF–MS analysis was performed on a 6210 time-of-flight mass spectrometer (Agilent, USA) equipped with an electrospray ionization (ESI) interface. The mass spectrometer conditions were optimized for domoic acid detection as follows. The collision induced dissociation (CID) voltage was set at 100 V. A spray voltage of 4.0 kV was employed and the velocity of drying gas was 11.0 L min^{-1}. The temperature of the heated transfer capillary was 350. The mass spectrometer was scanned from m/z 150 to 650 in full scan mode.

2.3. Preparation of MIP

Generally, MIP was prepared using bulk polymerization method by dissolving 0.3 mmol of template PTA, 6 mmol of functional monomer 4-VP and 30 mmol of cross-linker EGDMA in 5 mL of toluene in a 20 mL borosilicate glass bottle. This mixture was stirred over night at room temperature for the formation of a complex of template and monomers. After adding 0.25 mmol of initiator AIBN, the solution was saturated with dry nitrogen for 10 min, then the bottle was equipped with a rubber cap. At last, the bottle was placed in a thermostated oil bath and polymerized at 60 °C for 24 h. After polymerization, the polymer was ground with a mortar and pestle, and sieved between 200 mesh and 400 mesh screens to give particles with size dimensions between 37 and 74 μm. After that, the particles were repeatedly suspended in acetone to remove the small particles. Then the product was extracted with ethanol containing 20% acetic acid using a Soxhlet apparatus for 48 h to make sure that the template could not be detected in the filtrate. At last, the product was washed with ethanol for three times and dried under vacuum at room temperature.

The non-imprinted polymer (NIP) particles were prepared and washed using the same recipe but without the addition of the template PTA.

2.4. Steady-state binding studies

Solutions containing known amounts of domoic acid were prepared in acetonitrile. An amount of 5 mg of the polymer particles was weighed and put into a 5 mL of domoic acid standard solution, and then slightly shaken on a horizontal shaker for 7 h at room temperature in the dark. Final domoic acid concentrations were determined by HPLC using UV detection at 242 nm. The amount of domoic acid bound to the polymer particles was calculated by subtracting the amount of free domoic acid from the initial amount added to the mixture.

2.5. Preparation of MISPE column

MISPE and NISPE columns were prepared by packing 150 mg of the respective polymers particles in a 1.0 mL glass syringe (2 cm × 0.9 cm i.d.). The syringe tube was thoroughly cleaned and dried, and attached with two sieve plates at the bottom end and the top end, respectively. Prior to loading the sample, the MISPE column was washed with water containing 5% ammonium hydroxide to remove the residual template from the synthetic procedure until the template could not be detected in the filtrate.

2.6. Preparation of mussel extract

Mussel extract was prepared from edible tissues of blue mussels and 50% aqueous methanol (v/v) according to the method proposed by López-Rivera et al. [24]. Then, the mussel extract was treated in a centrifuge to remove the insoluble compounds, and a known concentration of domoic acid was added into the extracts. At last, the real concentration of domoic acid after MISPE procedure was determined by HPLC.

2.7. SPE for domoic acid standard solutions

Before loading samples, the MISPE column was previously conditioned with 2 mL of deionized water and acetonitrile, successively. After 4 mL of domoic acid standard solutions at different concentrations were passed through the columns at

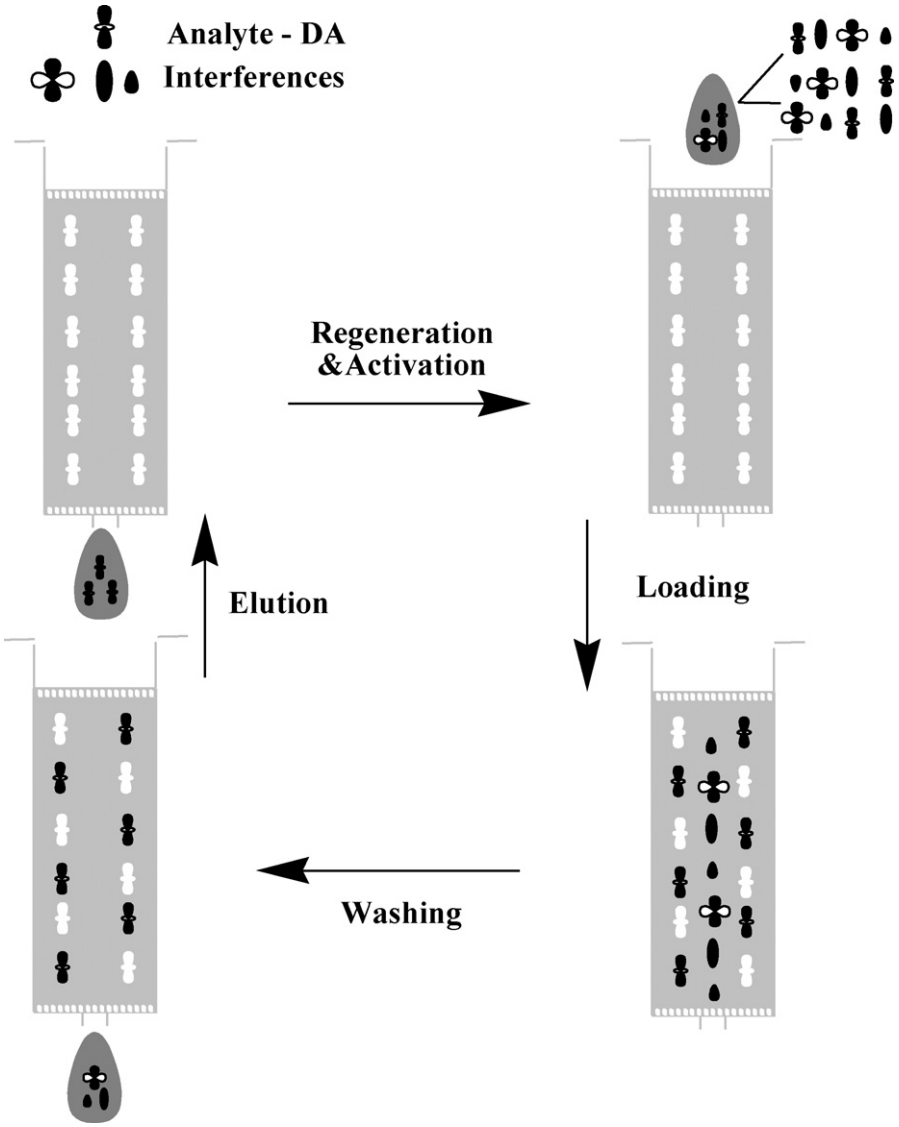

Fig. 1. SPE procedure using MIP as the sorbent for selective extraction of domoic acid.

a flow rate of 0.25 mL min^{-1}, the columns were washed with 2 mL of water and 2 mL of acetonitrile at the same flow rate. The analyte retained on the column was eluted with 3 mL methanol containing 5% acetic acid. The eluent was evaporated to dryness under vacuum at 25 °C, and the residue was reconstituted into 1 mL of mobile phase for further HPLC analysis.

2.8. SPE for spiked mussel extract

To validate the performance of MISPE for the biological matrix, the MIP particles and corresponding NIP particles were packed individually into cartridges to compare their efficiency of extracting domoic acid from mussel extract. 4 mL of the mussel extract spiked at 0.5 mg L^{-1} of domoic acid was passed through the MISPE (or NISPE) column as described above. Finally, the elution fractions were dried under a gentle nitrogen stream, and the residues were reconstituted with acetonitrile to final volume of 1 mL for subsequent HPLC analysis and extraction recoveries were calculated using the constructed calibration curve, respectively.

3. Results and discussion

3.1. Preparation of MIPs

In the previous study, researchers have reported that domoic acid could be assembled with a kainate-type subunit of the glutamate receptor [25]. Furthermore, the studies by Kubo et al. also suggested that the acidity and spatial location of the COOH groups in the template molecules were important for the selective recognition of domoic acid [26,27]. As a structurally related analog of domoic acid, PTA has similar molecular weight, shape and functional groups with domoic acid and could be used as the dummy template molecule. In order to avoid or reduce the interrupt from the solvent which compete off the template molecules from their binding sites, toluene was selected as the solvent in our synthesis procedure. For a successful imprinting, adequate template: functional monomer molar ratios have to be used [28]. In most of case, the molar ratio between template and functional monomers could be approximately set from 1:3 to 1:5 (for the simplest case of one group in the template and functional monomer). Taking into account of the structure of PTA and possible application in polar solvents, the molar ratio between template and functional monomers

was selected as 1:20. The molar ratio of monomer to cross-linker was selected at 1:5 to ensure the formation of defined recognition sites with polymers [29].

The MIP with high affinity for domoic acid was prepared by bulk polymerization method in this work. And the obtained imprinted polymer was used as selective sorbent for the solid phase extraction of domoic acid from seafood samples (Fig. 1). In the non-covalent imprinting approach (in this case), hydrogen bond play an important role in the recognition process. We therefore assume that hydrogen bond interaction between the functional monomer (pyridine group, in this case) and the target (carboxy group, in this case) is responsible for the selective binding of domoic acid. And the capable of binding domoic acid from water may due to the formation of multiple hydrogen bonds.

3.2. Evaluation of the recognition efficiency of MIP

After MIP and NIP synthesis, equilibrium binding experiments were performed at room temperature with MIP and NIP. Fig. 2 shows experimental binding isotherms of the MIP (and NIP) amount versus domoic acid concentrations. In the 0.5–25 mg L^{-1} domoic acid concentration range, the MIP exhibits a higher capacity for domoic acid than the NIP. The weak adsorption of domoic acid on NIP is due to non-specific interaction with the polymer matrix.

The data of the static absorption experiment were further processed with the Scatchard equation to estimate the binding parameters of the MIPs. A dissociation constant (K_d = 9.47 μmol L^{-1}) and a maximum number of binding sites (B_{max} = 32.21 μmol g^{-1}) for MIP were calculated from the Scatchard equation. Meanwhile the dissociation constant (K_d = 31.25 μmol L^{-1}) and the maximum number of binding sites (B_{max} = 16.76 μmol g^{-1}) for NIP were calculated from the NIP binding data. A large K_d means low affinity to the target, while a large B_{max} means high binding capacity to the target. When compared to NIP, the MIP has a higher binding capacity and higher affinity to domoic acid. The difference in domoic acid binding affinity to the MIP and NIP clearly indicates the role of the imprinting process in the formation of specific binding sites.

3.3. SPE for domoic acid standard solutions

The recoveries of standard solutions containing domoic acid (0.5–50 mg L^{-1}) are calculated and summarized in Table 1. It is clear that MISPE columns have good recoveries for domoic acid at different concentrations except for high concentration. The reduction of domoic acid recovery in high concentration is probably due to the low loading capacity of the MISPE column. A standard calibration curve (Fig. 3) for domoic acid analysis was constructed, which was linear (R^2 > 0.99) over the concentration range from 0.5 mg L^{-1} to 25 mg L^{-1} with a detection limit of 0.1 mg L^{-1}. Thus, this method could be used to detect some seafood samples polluted by domoic acid.

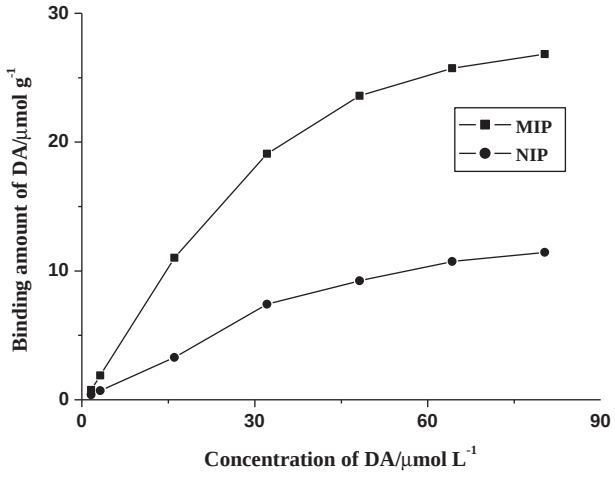

Fig. 2. Binding isotherm for the molecularly imprinted polymer (MIP, ■) and non-molecularly imprinted polymer (NIP, ●) particles. Experiment was conducted by the addition of 5 mg of particles in 5 mL of DA in methanol at room temperature.

Table 1
The recoveries of domoic acid in standard solutions.

Conc. (mg L^{-1})	Recovery (%)	R.S.D.[a] (%)
0.5	96.2	4.53
1.0	96.9	2.67
2.0	97.5	1.82
5.0	96.8	2.09
10.0	95.3	3.23
25.0	89.3	2.3
50.0	67.4	2.41

[a] All the results represent mean values of three measurements.

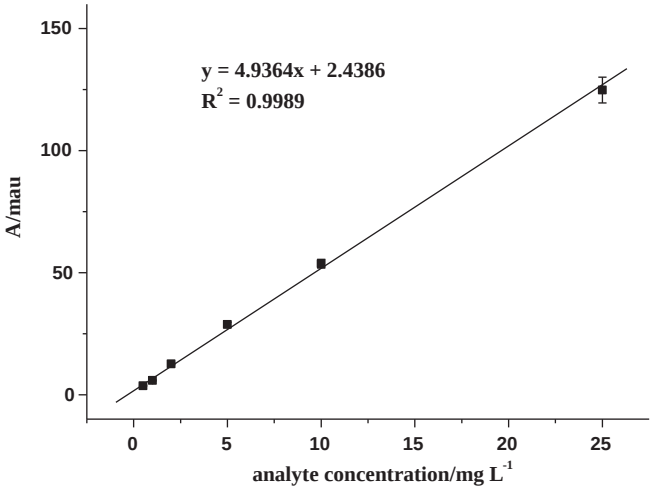

Fig. 3. Calibration curve for quantitative analysis of domoic acid.

Phthalic acids have a chemical structure similar to that of domoic acid [26], so phthalic acids could be selected as interfering substances for examining the interfering effects from similar species and the recognition selectivity of the MIP. In our previous study, we have reported that MIP prepared using 1,3,5-pentanetricarboxylic acid as the template molecule could discriminate domoic acid from phthalic acids easily [30]. So the proposed method should have good selectivity for the determination of domoic acid.

3.4. SPE for spiked mussel extract

To validate the performance of MISPE for the seafood-matrix, the MIP and corresponding NIP were packed individually into cartridges to compare their efficiency of extracting domoic acid from mussel extracts. Fig. 4a is the HPLC chromatogram of mussel extract added with 2 mg L^{-1} domoic acid, in which the complexity of the mussel extract matrix background is evident. The extraction on MISPE column can successfully clean up the mussel extract matrix, thus allowing the extraction of domoic acid with high selectivity (Fig. 4b). The result showed that the recovery of 0.5 mg L^{-1} domoic acid in mussel extract was 93.4 ± 4.9% (n = 3). Contrary to this, NISPE column showed no such selectivity (Fig. 4c). Hence MIP offers itself as a simple and straightforward technique for the direct analysis of domoic acid in mussel extract without lengthy sample cleanup.

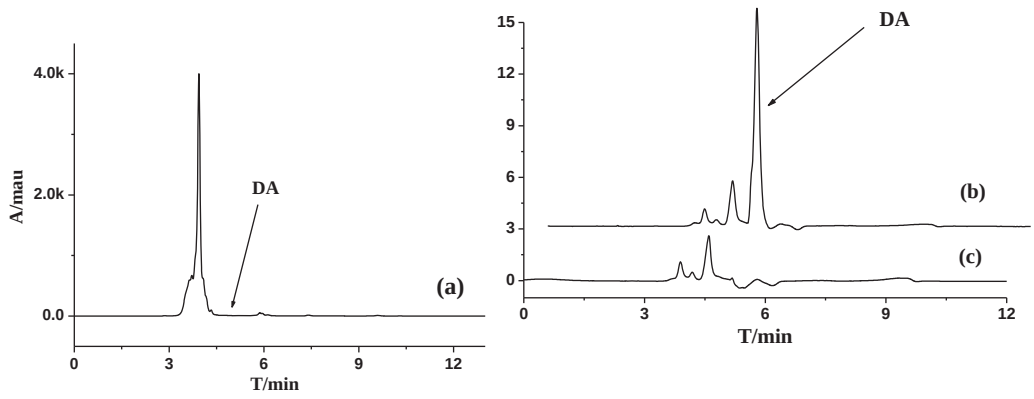

Fig. 4. HPLC chromatograms of (a) mussel extract spiked with 2 mg L^{-1} domoic acid, and after extraction of the mussel extract containing 0.5 mg L^{-1} domoic acid on MISPE column (b) and NISPE column (c).

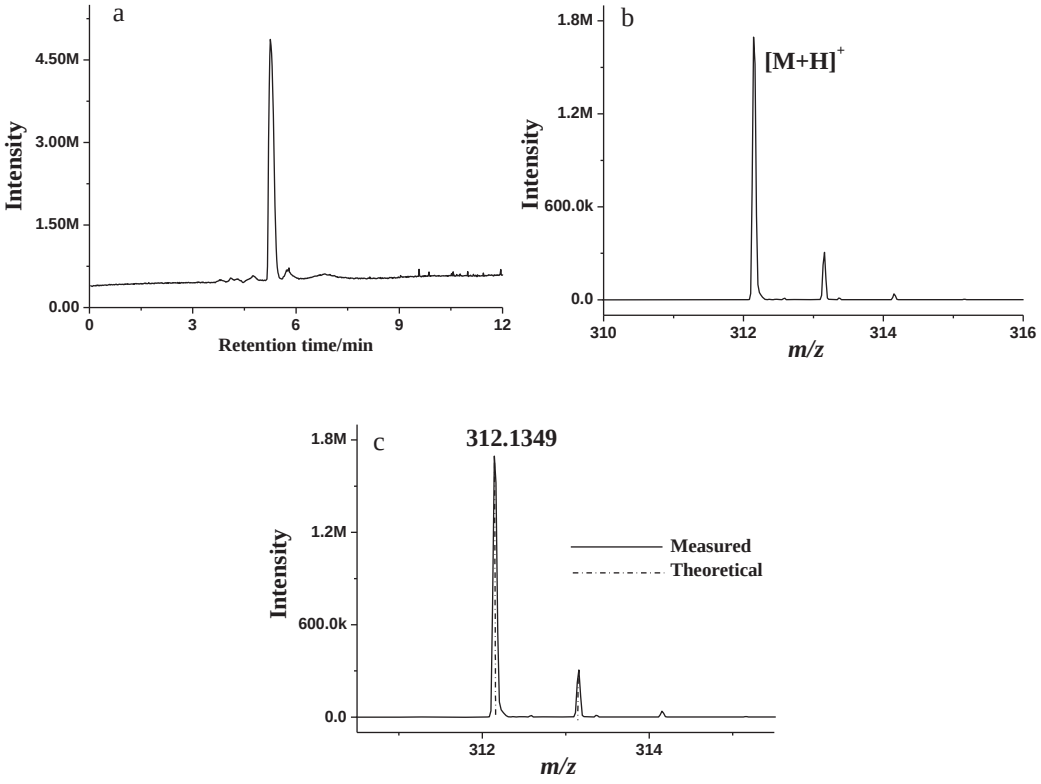

Fig. 5. The total ion chromatogram (EIC, a) of the elution fraction after MISPE procedure from mussel extracts; MS spectra (b) of the peak at 5.26 min in the positive mode; observed mass ($[M+H]^+$) of domoic acid and its isotopics compared with calculated isotopics (c).

3.5. Identification of domoic acid by HPLC–ESI–TOF–MS

In order to confirm the extraction of domoic acid on MISPE column, ESI–TOF–MS analysis was performed on a 6210 time-of-flight mass spectrometer equipped with an ESI interface. The total ion chromatogram (TIC) and MS spectra of peak at 5.26 min of the elution fraction from mussel extract are shown in Fig. 5. The peak at 5.26 min observed on TIC was attributed to *m*/*z* 312, meanwhile was not found from blank (Fig. 5a). Positive mass spectra of the peak at 5.26 min showed its molecular ion peaks, $[M+H]^+$: *m*/*z* 312, which is in agreement with the molecular weight of domoic acid being 311 (Fig. 5b). The typical isotopic pattern of the domoic acid was calculated by the isotope calculator and was found to be consistent (including position and intensity) with the pattern obtained from the main distribution in the spectrum (Fig. 5c). This provides a direct evidence for the extraction of domoic acid on MISPE column.

Table 2
Regeneration property experiment of the MISPE column. The domoic acid concentration used for binding was 0.500 mg L^{-1}.

Added Conc. (mg L^{-1})	Detected Conc.[a] (mg L^{-1})	Recovery (%)	R.S.D. (%)
0.500	1.928	96.4	3.97
	1.884	94.2	
	1.952	97.6	
	1.970	98.5	
	2.096	104.8	

[a] The extractions were conducted with an enrichment factor of 4.

3.6. Reproducibility of the MISPE column

The MIP can be used again to adsorb the interested analyte after regeneration, that is one of the important advantages of

MIPs. In order to reuse the polymers after each extraction, a short regeneration step was performed by washing with 3 mL of water:ammonium hydroxide (95:5 (v:v)). The results (Table 2) showed that the MISPE column could be used 5 times and maintained their adsorption capacity at an almost constant value. This confirmed the reliability and efficacy of the proposed method for the analysis of domoic acid residues in real samples.

4. Conclusions

The MIP toward domoic acid was prepared using a dummy imprinting strategy by bulk polymerization method. PTA, 4-VP and EGDMA were used as the dummy template molecule, the functional monomer and cross-linker, respectively. The obtained MIP showed good selectivity and affinity for domoic acid, and was used as selective sorbent for the solid phase extraction of domoic acid from seafood samples. Accordingly, a method was successfully developed for analysis of domoic acid in seafood samples by using the obtained MISPE column coupled with HPLC. The limit of quantitation of the proposed method was 0.1 mg L^{-1}, which is below the maximum residue limits established by different authorities. The high recoveries and satisfied precisions for analyte proved that the proposed method was valid for the analysis of domoic acid in complex matrixes (mussel extract).

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.02.004.

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