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Determination of domoic acid in shellfish by liquid chromatography with electrospray ionization and multiple tandem mass spectrometry

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Abstract

Amnesic shellfish poisoning is a potentially lethal human toxic syndrome which is caused by domoic acid (DA) that originates in marine phytoplankton belonging to the *Pseudonitzschia* genus. A new sensitive liquid chromatographic–mass spectrometry (LC–MS) method has been developed for the determination of DA in various marine biological samples. The characteristic fragmentation pathways for DA were established using multiple stage MS on selected daughter ions, which were sequentially trapped and fragmented. Chromatography was performed using a gradient of acetonitrile–water (5:95 to 40:60), containing trifluoroacetic acid (0.05%), over 25 min at 0.2 ml/min with a C_{18} column (Luna-2, 150×2.0 mm, 5 μm). Using electrospray ionisation, multiple tandem MS experiments were performed with an ion-trap mass spectrometer (Finnigan MAT LCQ). The protonated DA molecule was the precursor ion, m/z 312, and the relative collision energies were optimised for multiple MS (MS^n, n=2–4) studies. LC–MS^3 using the ions, m/z 266 and 220, from the loss of two HCOOH molecules, produced the best sensitivity data. Calibration data for various MS modes were: MS (0.05–10 μg DA/ml, r^2=0.9973); MS^2 (0.025–10 μg DA/ml, r^2=0.9997); MS^3 (0.025–10 μg DA/ml, 0.9994). The detection limits (3:1 signal:noise) were better than 0.02 μg DA/ml for LC–MS, 0.014 μg DA/ml for LC–MS^2 and 0.008 μg DA/ml for LC–MS^3. This method was applied to determine DA in scallop (*Pecten maximus*) tissues, which subsequently led to the closure of several shellfish harvesting sites on the west coast of Ireland. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Shellfish poisoning; *Pecten maximus*; Food analysis; Domoic acid; Toxins

1. Introduction

During the winter of 1987, 153 people suffered from acute intoxication after consuming cultured blue mussels (*Mytilus edulis*) originating from Prince Edward Island. The cause of the intoxication was ascertained to be domoic acid (DA), a potent neurotoxic amino acid and a natural product of some marine phytoplankton [1] (Fig. 1). This was the first recorded human intoxication, the symptoms produced included nausea, disorientation, temporary amnesia and in more serious cases, especially, elderly people and/or those with gastric lesions, persistent short term memory loss and/or coma resulted [2,3]. The syndrome thus produced was

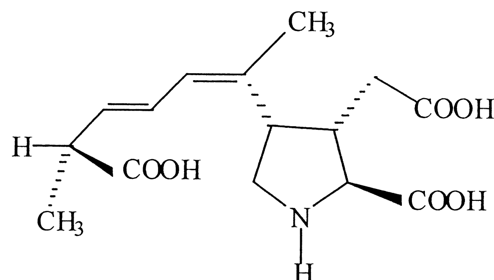

Fig. 1. Structure of domoic acid.

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called amnesic shellfish poisoning (ASP). The phytoplankton responsible for this incident of intoxication was identified as the pennate diatom, *Nitzchia pungens f. multiseries,* and the scallops grazing on this phytoplankton concentrated the toxin in their mid-gut gland (hepatopancreas) [4]. Since this initial occurrence several other incidents have occurred at various sites throughout Canada, the USA and Europe. In Monterey Bay, CA, USA, domoic acid was identified in anchovies and pelicans, the source of domoic acid on this occasion was found to be *Pseudonitzschia australis* [5]. In Washington and Oregon states, USA, domoic acid was found in razor clams and crabs harvested in November 1999 [6]. In Canada, control mechanisms have been put in place to prevent harvesting if domoic acid reaches a concentration of greater than or equal to 20 μg DA/g in edible tissues and this limit has now been adopted in Europe [7].

In France, Scotland and Spain, domoic acid has been detected in various shellfish [8–11]. Because the incidences of this toxin are so widespread, this emphasizes the need for rapid identification and screening of this toxin. To this end, several analytical methods have been developed for the quantitative determination of domoic acid in shellfish and marine phytoplankton. Domoic acid, and related amino acids, possess a conjugated diene moiety, regulatory agencies have employed a liquid chromatographic method with photodiode-array ultraviolet detection (LC–UV) which has a detection limit of 0.5 μg DA/g tissue, more than 100 times better than mouse bioassays [12]. Capillary electrophoresis has been applied to the determination of DA in shellfish but two solid-phase extraction (SPE) steps using strong cation-exchange (SCX) and strong anion-exchange (SAX) phases were required [13]. The derivatisation reagent, 9-fluorenylmethylchloroformate (FMOC), has been used for toxin analysis in phytoplankton and in seawater samples using fluorimetric detection, LC–FL [14]. Although improved detection limits were reported in comparison to the UV-diode array detection (DAD) method, this method was not successful for the analysis of shellfish tissue associated with chromatographic interferences due to reagent products and other compounds in the matrix. Another derivatising reagent, 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) has been successfully applied to the analysis of DA in marine phytoplankton and shellfish tissue samples. This fluorescent derivatisation method is very sensitive and does not suffer from any chromatographic interferences. This isocratic fluorimetric LC method has a detection limit of 6 ng DA/g tissue and is readily applicable to automation [15]. LC–MS techniques have also been employed to analyse domoic acid in samples. Fast atom bombardment (FAB) MS and FAB-MS–MS spectrometry studies have been carried out on domoic acid and have provided characteristic fragment ions to allow for the rapid screening of marine samples [16–20].

This study describes the qualitative and quantitative analysis of domoic acid by LC–multiple MS (MS^n) using a Finnegan MAT LCQ ion-trap mass spectrometer. The method involves a minimum amount of sample preparation and is capable of detecting domoic avid at very low levels as well as providing characteristic fragmentation patterns for this toxin and its isomers. MS to MS^{2-5} experiments were carried out on certified domoic acid standard spiked into blank shellfish extracts. The method also allows for the rapid screening of the toxin and its isomers in shellfish samples. Quantitation studies were carried out using MS^n and these studies proved to be both highly sensitive and selective. Various fragmentation pathways were followed by selecting different daughter ions for subsequent multiple tandem MS experiments.

2. Experimental

2.1. Chemicals

MUS-1B, certified reference shellfish material (38.3+0.8 μg DA/g) and DACS-1C, certified calibration solution (100 μg DA/ml) were obtained from the National Research Council, (Halifax, Canada). Acetonitrile and water were LC grade and were purchased from Labscan, (Dublin, Ireland). Trifluoroacetic acid (TFA) was purchased from Sigma–Aldrich (Dublin, Ireland).

2.2. Shellfish sample preparation

Samples were extracted according to a modifica-

tion of a procedure described by Quilliam et al. [21]. Briefly, 4 g of tissue homogenate was accurately weighed into a graduated centrifuge tube. Methanol–water (1:1, 16 ml) were added and homogenised (3 min at 10 000 rpm). The resulting slurry was then centrifuged at 3000 rpm for 10 min. The supernatant was filtered through a 0.45 μm filter into a screw-capped vial. For the direct analysis, extracts (1 ml) were diluted to 10 ml with water and a 5 μl aliquot was injected for LC–MS^n analyses. For clean-up and concentration, the extracts (5 ml) were subjected to SPE using a SAX cartridge (3 ml, J.T. Baker, Deventer, Netherland), conditioned with methanol (6 ml), water (3 ml) and finally with methanol–water (1:1, 3 ml). Filtered supernatant (5 ml) was loaded onto the cartridge and washed with acetonitrile–water (10:90). A 0.1 *M* formic acid (pH 2.92) solution was added to the cartridge, the first fraction (0.5 ml) was discarded and the following fractions (3.0 ml) were collected for analysis.

2.3. Liquid chromatography–mass spectrometry

LC–MS analysis was carried out using a Hewlett-Packard HPLC system (HP 1100 series) linked to a Finnigan MAT LCQ mass spectrometer (ThermoFinnegan, San Jose, CA, USA). The MS was equipped with an electrospray ion-spray (ESI) interface. The LC system (Hewlett-Packard, HP1100, UK) consisted of a pump (BinPump, G1312A), degasser (G1322A), autosampler (ALS, G1329A), thermostat (ALSTherm, G1330A), column oven (ColComp, G1316A) and diode-array detector (DAD, G1315A). Gradient chromatography was performed using acetonitrile–water (5:95–40:60) over 25 min containing 0.05% TFA, solvent flow (0.2 ml/min) with a Luna C_{18}(2) column (150×2.0 mm, 5 μm, Phenomenex, Macclesfield, UK) at 40°C.

Using flow injection analysis (FIA) at 3 μl/min, the mass spectrometer was tuned using certified domoic acid standard solution (1 μg DA/ml) and the optimised *Tune Plus* parameters were as follows: capillary temperature of 195°C, source voltage (kV) of 4.20, source current (μA) of 100, sheath gas flow 80 (arb), auxilliary gas flow 20 (arb), capillary voltage of 3 V. Optimised lens values were: tube lens offset of 25 V, octapole Rf Amp (Vp–p) of 400, octapole 1 offset (V) of −3, octapole 2 offset (V) of −5.50, interoctapole lens (V) of −16, trap d.c. offset (V) of −10, maximum ion time of 400 and an ion time (MS) of 5.00. The optimum collision energies for LC–MS^{2-5} experiments were in the range 16–18%. The main advantage of the ion-trap MS detector is that during a chromatographic run several scan events can be performed simultaneously. For conclusive identification of domoic acid in shellfish, five scan events were carried out and these included MS, MS^2, MS^3, MS^4 and MS^5. A divert time of 2 min was set at the start of each chromatographic run to prevent early eluting matrix material from blocking the heated capillary region of the MS system and contaminating the ion optic region.

3. Results and discussion

LC–MS techniques have been employed in the past for the analysis of the neurotoxin domoic acid (Fig. 1) [17–20]. Quilliam et al. [17] has used both FAB-MS and FAB-MS–MS spectrometry to analyse this toxin and its isomers. Full mass spectra from these experiments shows the molecular ion for the toxin at m/z 312 $[M+H]^+$, a peak at m/z 623 is indicative of the dimer ion $[2\ M+H]^+$. The fragmentation pattern produced in the MS–MS spectra consists mainly of water and CO or formic acid losses. A peak appearing at m/z 74 was attributed to the fragment ion $[CO_2H\text{–}CH{=}NH_2]^+$, these results would seem to suggest the complete disruption of the proline ring during collision induced dissociation in the positive ion mode. Negative FAB-MS analysis was also carried out and yielded the following fragments; m/z 310 $[M-H]^-$, m/z 266 and 222 resulting from successive losses of CO_2 from $[M-H]^-$ acid anion.

For this study, a Finnigan MAT LCQ ion-trap mass spectrometer was used to determine DA in shellfish tissue. The MS was equipped with an ESI interface and operated in positive ion mode. Initially, MS^n ($n=1$–5) experiments were performed on DA to establish a characteristic fragmentation pattern and thus allow unambiguous identification. This was achieved by trapping daughter ions at each successive stage of multiple tandem MS experiments and fragmenting them to produce new daughter ions. The MS conditions were optimised in order to discrimi-

nate in favour of trapping the ions of interest at each successive stage and the optimised method was used to confirm the presence of domoic acid in shellfish samples.

3.1. Optimisation of MS fragmentations for domoic acid

Full-scan MS in the positive ion mode gave the molecular ion $[M+H]^+$ for domoic acid as the base peak at *m/z* 312. Using an optimised relative collision energy (RCE) of 16% for MS^2, two prominent peaks were observed in the mass spectrum, at *m/z* 266 and 294 $[M+H-H_2O]^+$ with relative abundances of 100 and 27%, respectively. The *m/z* 266 peak resulted from the loss of a formic acid molecule $[M+H-HCOOH]^+$ or water and carbon dioxide $[M+H-H_2O-CO_2]^+$. MS^3 experiments were conducted separately on the ions at *m/z* 266 and 294. Firstly, on the *m/z* 294 ion, an optimised RCE of 18% yielded a base peak of *m/z* 248 as a result of a loss of a formic acid molecule and a peak at *m/z* 276 corresponding to the loss of a second water molecule was also apparent in the spectrum (Fig. 3C). MS^3 experiments were conducted also on the *m/z* 266 fragment ion and yielded the spectrum (Fig. 3D): Using 16% RCE, the base peak observed was at *m/z* 248 due to the loss of a water molecule. Also observed were two prominent peaks at *m/z* 220 and 193 with a relative abundance of 77 and 86% due to a loss of formic acid and $[C_2H_4O_2N]$, respectively. MS^4 experiments were performed on the daughter ions of *m/z* 266 namely *m/z* 248, 220, and 193 and the following results were obtained: MS^4 on the ion at *m/z* 248 yielded a base peak of *m/z* 202, due to the loss of a formic acid molecule. MS^4 on the ion at *m/z* 220 ion yielded three prominent fragment ions one at *m/z* 202 due to the loss of water, one ion at *m/z* 174 due to the loss of formic acid and another ion at *m/z* 119. MS^4 on the ion at *m/z* 193 yielded a fragment ion at *m/z* 175 due to the loss of a water molecule (Fig. 2). Although MS^5 experiments produced fragmentation data, reproducibility was poor.

3.2. LC–MS^n analysis of domoic acid in shellfish

Optimisation of the voltages on ion optics region of the MS detector and subsequent calibration studies

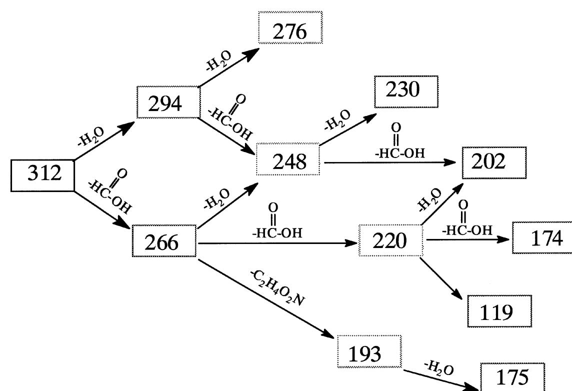

Fig. 2. MS fragmentation pathway for domoic acid (positive ion mode).

were carried out using certified domoic acid standard (DACS-1C). For shellfish studies using this method, a certified reference mussel material containing DA (MUS-1B) was used for method development. With this LC–MS method it is possible to analyse quantitatively crude methanol–water shellfish extracts. However to avoid blockage of the heated capillary due to repeated injection of a large number of crude samples, a solid-phase extraction (SPE) method with a strong anion-exchanger (SAX) was used [21]. The average recovery of domoic acid from MUS-1B using SPE clean-up was 92% ($n=5$). Calibration studies were carried out on certified domoic acid standard spiked into scallop tissue. Fig. 3 shows the total ion chromatogram (TIC) peaks and their associated mass spectra obtained from LC–MS^{2-3} experiments. The MS^3 spectra showed extensive fragmentation of domoic acid including the ion at *m/z* 193 corresponding to a loss of 74 amu $[-C_2H_4O_2N]$ as a result of the disruption of the proline ring (Fig. 2). Therefore the MS^3 spectrum provides a means for the identification of DA in marine samples.

For the calibration studies four scan events were carried out simultaneously (MS^{1-4}). Fragment ions were selected from each MS^n stage and calibration curves were constructed from the corresponding total ion chromatogram (TIC) peak areas. Linear calibration plots were obtained using the LC_{Quan} function in the LCQ Navigator software. Fragment ions selected for quantitation were as follows: from the MS spectra 312 *m/z*, from the MS^2 spectra 266 *m/z*,

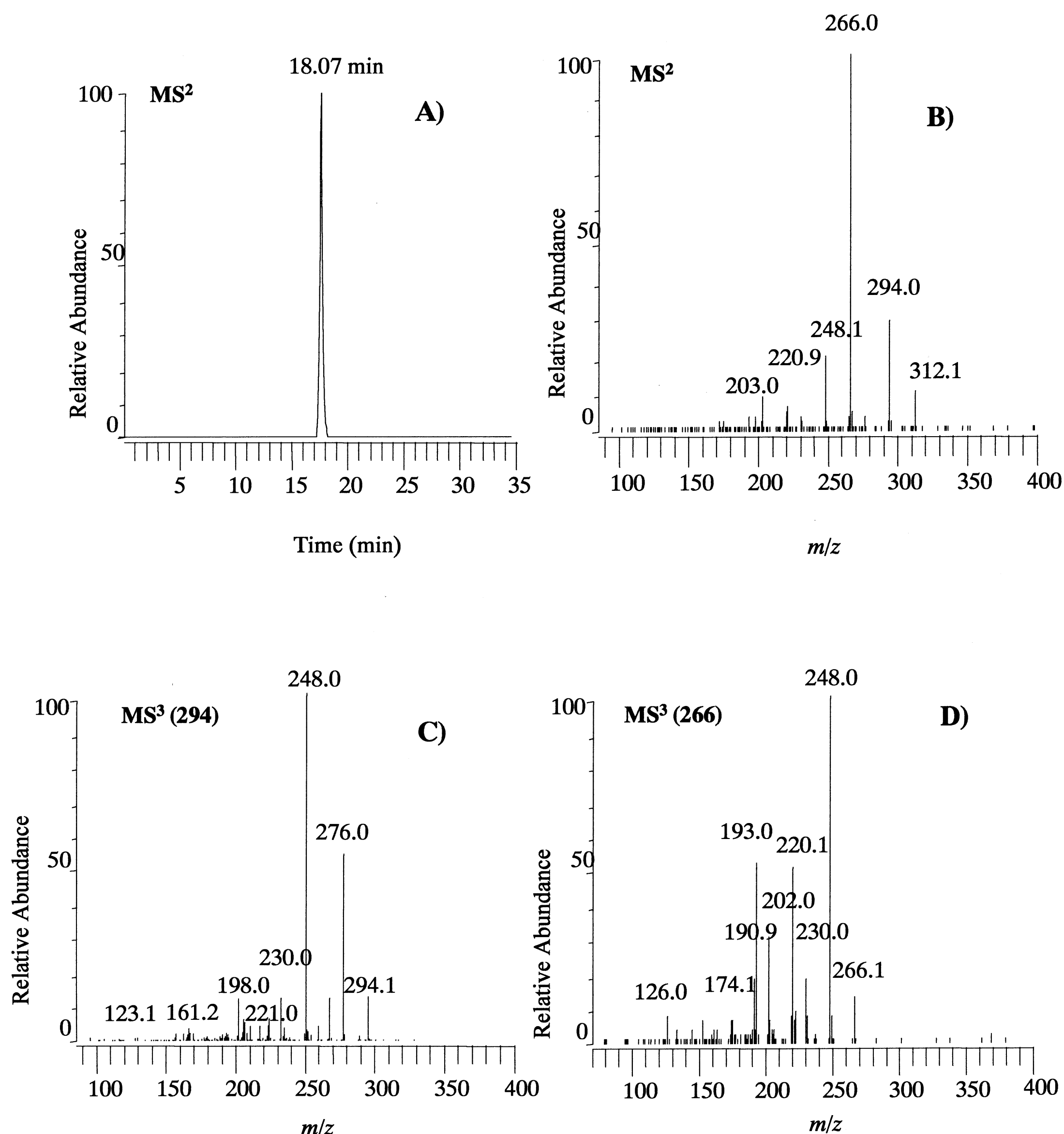

Fig. 3. Total ion chromatograms (A) and associated mass spectrum (B) from the LC–MS^2 analyses of the DA certified standard, DACS-lC with SPE clean-up (0.05 μg/ml). The amount of domoic acid is equivalent to 0.25 ng on-column. Conditions: Luna C_{18} column (150×2.0 mm, 5 μm); gradient HPLC analysis over 25 min using a mobile phase: acetonitrile–water containing 0.05% TFA, (5:95–15:85) for the first 5 min; (15:85–25:75) for the next 5 min; (25:75–40:60) for the following 5 min and from (40:60 to 5:95) for the final 5 min; temperature 40°C; flow-rate 0.2 ml/min; injection volume 5 μl. (C) The MS^3 spectrum that was obtained by trapping and fragmenting the ion, m/z=294. (D) The MS^3 spectrum that was obtained by trapping and fragmenting the ion, m/z=266.

from the MS^3 spectra 194 m/z and from the MS^4 spectra 175 m/z (Fig. 2). These fragments were selected as they showed the greatest reproducibility in terms of peak area. Calibration plots using extracts from scallop tissue containing 0.05–10 μg DA/ml for MS (n=3); containing 0.025–10 μg DA/ml for MS^2 (n=3) and containing 0.025–10 μg DA/ml MS^3 (n=3) were obtained. All plots were linear and

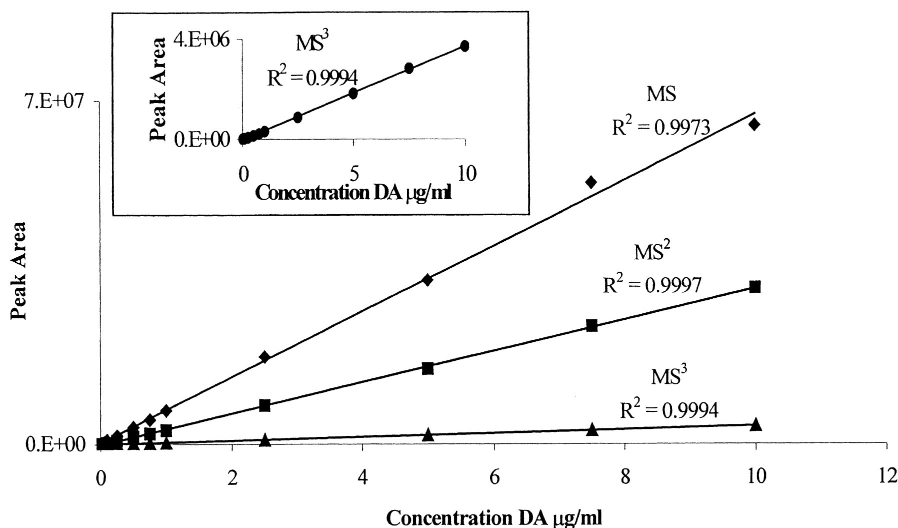

Fig. 4. Average calibration plots using extracts from scallop tissue containing 0.05–10 µg DA/ml for MS (n=3); containing 0.025–10 µg DA/ml for MS^2 (n=3) and containing 0.025–10 µg DA/ml MS^3 (n=3) were obtained. All plots were linear and yielded r^2 values of 0.9973, 0.9997 and 0.9994, respectively. Insert shows the plot obtained from MS^3 analysis of shellfish tissue spiked with DACS-1C.

yielded r^2 values of 0.9973, 0.9997 and 0.9994, respectively. Fig. 4 shows the calibration plots for LC–MS^{1-3}. The correlation co-efficients and signal-to-noise ratios unproved for each successive stage of MS. The detection limits (3:1 signal:noise) were better than 0.025 µg DA/ml (125 pg DA/5 µl injection) for LC–MS^1, 0.014 µg DA/ml (70 pg DA/5 µl injection) for LC–MS^2 and 0.008 µg DA/ml (40 pg DA/5 µl injection) for LC–MS^3. The improvement in the signal-to-noise ratio can be expected because in the MS^2 and MS^3 stages, interference ions are removed from the trap. RSD values range from 2.5% for 5 µg DA/ml (n=5) to 4.0% for 0.5 µg DA/ml (n=5) to 5.5% for 0.05 µg DA/ml (n=5). The calibration curve obtained for the 175 m/z ion in the MS^4 spectra showed poor linearity (r^2=0.8442), and the ions obtained in the MS^5 spectra were not reproducible. This analytical method was applied to scallop hepatopancreas from samples collected from the west coast of Ireland. Fig. 5A and B shows total ion chromatograms (TIC) and the associated mass spectra obtained from LC–MS^{1-4} experiments on these samples. Also apparent in chromatograms (Fig. 5A) are a number of isomers of DA. Research is on-going to isolate these compounds for MS^n studies.

4. Conclusion

An LC–MS^n method has been developed which allows for the rapid, unambiguous identification and quantitation of domoic acid in shellfish samples. The method is straight-forward and requires a minimum of sample clean-up with or without a pre-concentration step and is readily automated. This method is very sensitive and the ability to carry out four MS scan events simultaneously (MS^{1-4}), allows the unequivocal identification of domoic acid in all shellfish tissue. This method is applicable for use in clinical studies of domoic acid intoxication and for environmental toxicological monitoring.

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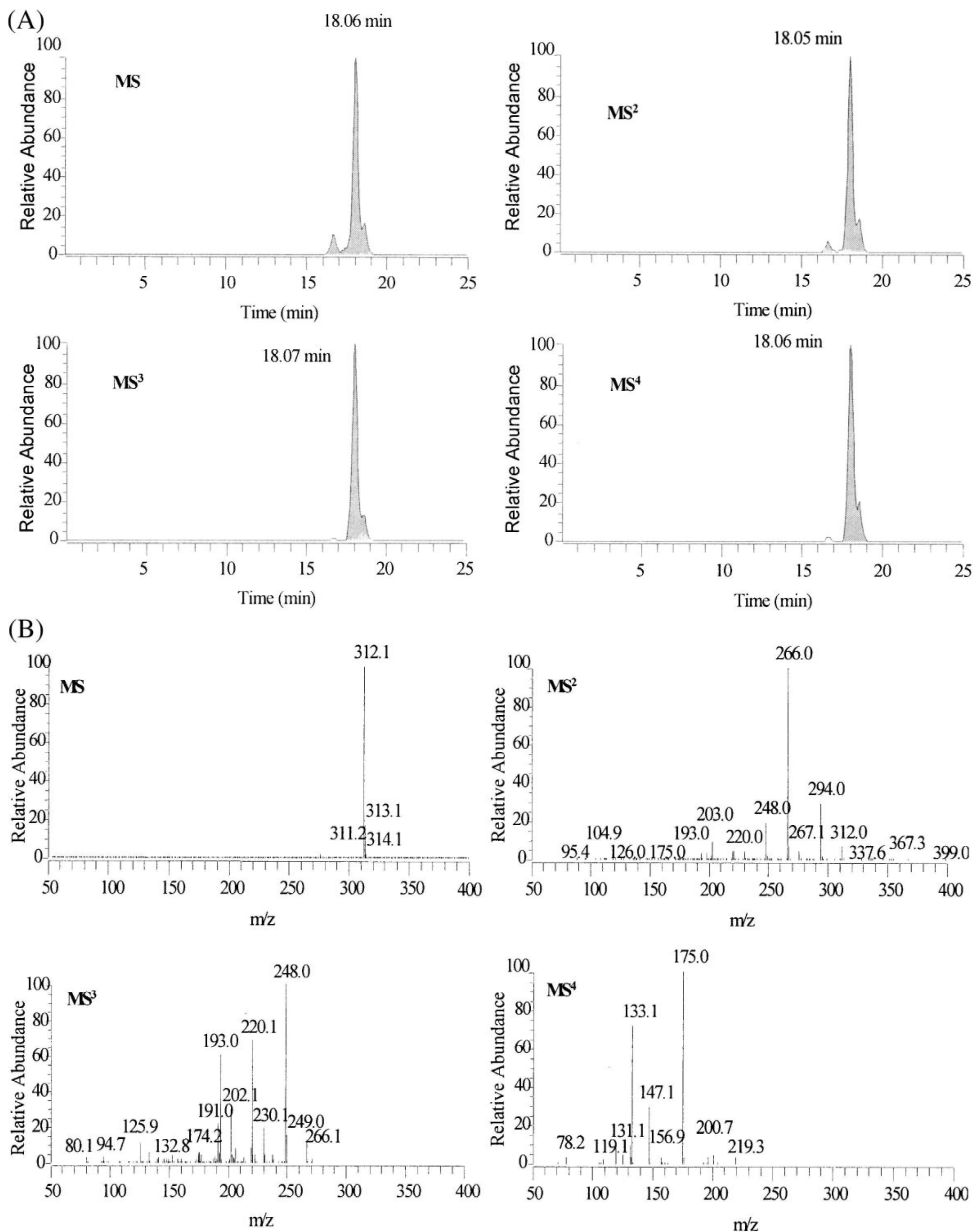

Fig. 5. (A) Total ion chromatograms (TIC) for the LC–MS^{1-4} analyses of domoic acid (DA) in scallop hepatopancreas tissue after SPE clean-up (1.2 μg DA/ml). The amount of domoic acid is equivalent to 6.0 ng on-column. The retention time for DA is 18.06 min. For other conditions see Fig. 3. (B) Associated MS^{1-4} mass spectra corresponding to the total ion chromatogram (TIC) in (A).

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