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Liquid chromatography with electrospray ion trap mass spectrometry for the determination of five azaspiracids in shellfish

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Abstract

Azaspiracid poisoning (AZP) is a new human toxic syndrome that is caused by the consumption of shellfish that have been feeding on harmful marine microalgae. A liquid chromatography–mass spectrometry (LC–MS) method has been developed for the determination of the three most prevalent toxins, azaspiracid (AZA1), 8-methylazaspiracid (AZA2) and 22-demethylazaspiracid (AZA3) as well as the isomeric hydroxylated analogues, AZA4 and AZA5. Separation of five azaspiracids was achieved on a C_{18} column (Luna-2, 150×2 mm, 5 μm) with isocratic elution using acetonitrile–water containing trifluoroacetic acid and ammonium acetate as eluent modifiers. Using an electrospray ionisation (ESI) source with an ion-trap mass spectrometer, the spectra showed the protonated molecules, $[M+H]^+$, with most major product ions due to the sequential loss of two water molecules. A characteristic fragmentation pathway that was observed in each azaspiracid was due to the cleavage of the A-ring at C_9–C_{10} for each toxin. It was possible to select unique ion combinations to distinguish between the isomeric azaspiracids, AZA4 and AZA5. Highly sensitive LC–MS^3 analytical methods were compared and the detection limits were 5–40 pg on-column. Linear calibrations were obtained for AZA1 in shellfish in the range 0.05–1.00 μg/ml ($r^2 = 0.9974$) and good reproducibility was observed with a relative standard deviation (%RSD) of 1.8 for 0.9 μg AZA1/ml ($n = 5$). The %RSD values for the minor toxins, AZA4 and AZA5, using LC–MS^3 (A-ring fragmentation) were 12.3 and 8.1 (0.02 μg/ml; $n = 7$), respectively. The selectivity of toxin determination was enhanced using LC–MS–MS with high energy WideBand activation.

Keywords: Azaspiracid poisoning; Toxins; Azaspiracids

1. Introduction

Human poisonings in The Netherlands in 1995 were caused by the consumption of contaminated mussels (*Mytilus edulis*) from Killary Harbour, Ireland. The associated human symptoms consisted of nausea, vomiting, severe diarrhoea and stomach cramps. This severe gastrointestinal disturbance was not due to the presence of known diarrhetic shellfish poisoning (DSP) toxins, okadaic acid and dinophysistoxin-2, which were present only at low concentrations. Azaspiracid was isolated from mussels and structurally elucidated [1,2]. Azaspiracid contains fused polyether rings similar to other classes of marine toxins [3] but there are also rare structural features including a trispiro ring and an azaspiro ring assembly, together with a terminal carboxylic acid group (Fig. 1). A second intoxication incident in

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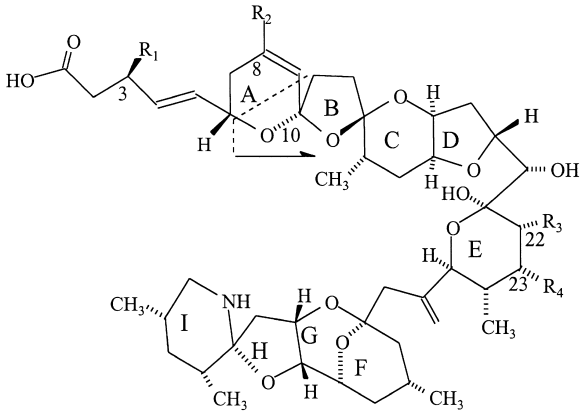

Fig. 1. Structures of the five known azaspiracids, AZA1–AZA5 (the hashed line indicates a characteristic MS fragmentation pathway).

Toxin	R_1	R_2	R_3	R_4
AZA1	H	H	CH_3	H
AZA2	H	CH_3	CH_3	H
AZA3	H	H	H	H
AZA4	OH	H	H	H
AZA5	H	H	H	OH

Arranmore Island, Ireland, was caused by mussels containing azaspiracid along with several new toxins, 8-methylazaspiracid (AZA2), 22-demethylazaspiracid (AZA3) [4] and two minor azaspiracids, AZA4 and AZA5, which are the 3-hydroxy and 23-hydroxy analogues of AZA3 [5]. Azaspiracid differs significantly from the DSP toxins in that it can induce pathological changes to the liver, pancreas, thymus and spleen of mice [6]. Since these new compounds represent a new class of toxins from both structural and pathological considerations, a new toxic syndrome, azaspiracid poisoning (AZP), was declared [4]. Azaspiracids, in common with other major classes of polyether marine toxins such as dinophysistoxins, pectenotoxins, yessotoxins and brevetoxins, have been identified in marine dinoflagellates [7] and shellfish accumulate these toxins through normal grazing. The discovery of azaspiracids in shellfish is therefore another example of the impact of toxins from harmful algae on human health [8]. Azaspiracids have also recently been identified in shellfish from Norway and England and this suggests that AZP is potentially widespread in Europe and responsible for some unexplained human toxic events [9].

Various intraperitoneal mouse bioassays, that were developed for DSP toxins [10], have been employed for the regulatory control of azaspiracids in shellfish in Europe but these have failed to prevent acute human intoxications [7]. Azaspiracids are not always confined to the digestive glands of shellfish but can be distributed through all tissues [11] and shellfish intoxication periods can be prolonged [1,7].

One of the early applications of ion-spray liquid chromatography–mass spectrometry (LC–MS) was the determination of marine neurotoxins in shellfish [12]. LC–MS also proved to be a powerful technique in the investigation of the structurally more complex polyether toxins in shellfish and marine phytoplankton [13–15]. Micro-liquid chromatography, coupled with mass spectrometry and tandem mass spectrometry (LC–MS–MS), has been proposed as a universal method for marine toxins [16,17], and the simultaneous determination of various groups of DSP toxins has recently been reported [18]. An LC–MS–MS method, using atmospheric pressure chemical ionisation (APCI) with a triple quadrupole MS instrument, has been developed for the determination of azaspiracid in shellfish [19]. For maximum sensitivity and specificity, selected reaction monitoring (SRM) LC–MS–MS analysis was implemented using the precursor–product ion combinations ($[M+H-nH_2O]^+$; $n=1$–3). Recently, a method for the simultaneous determination of the three main azaspiracids, AZA1, AZA2 and AZA3, in shellfish extracts was developed using electrospray ionisation (ESI) LC–MS analysis. In this protocol, pre-concentration and clean-up of shellfish extracts was required using a diol solid-phase extraction (SPE) cartridge [20].

The primary aim of this research was to develop a robust and sensitive analytical method for the determination of the five known azaspiracids, AZA1–AZA5, in shellfish. We now report the development of an ion-trap multiple tandem MS method which is highly selective and requires minimal sample preparation steps.

2. Experimental

2.1. Reagents and toxin standards

Azaspiracid toxins, AZA1, AZA2 and AZA3, were isolated from toxic mussels (*Mytilus edulis*) using procedures similar to those described previously. Contaminated shellfish were collected from Killary Harbour and Arranmore Island, Ireland. Due to the small amounts of available standard toxins, quantitative data on all azaspiracids in this study are related to a standard AZA1 that was isolated from mussels [2]. HPLC-grade methanol, acetonitrile and water were purchased from Labscan (Dublin, Ireland) and trifluoroacetic acid (TFA) from Sigma–Aldrich (Dorset, UK).

2.2. Sample treatment

Mussel (*M. edulis*) tissue (up to 20 g) was homogenised (Ultra Turrax, IKA, Germany) for 1 min. Homogenised tissue (ca. 5 g) was accurately weighed into a 50 ml centrifuge tube, acetone (8 ml) was added and the mixture was again homogenised for 1 min, followed by centrifugation at 3000 rpm for 3 min. The supernatant was transferred to a volumetric flask (25 ml) and this extraction procedure was repeated, combined with the first extract and made up to volume with acetone. An aliquot (2.5 ml) was evaporated using nitrogen at 40 °C to remove acetone (TurboVap, Zymark, MA, USA). To the remaining aqueous residue, water (0.5 ml) and ethyl acetate (2 ml) were added with vortex mixing for 1 min. The solution was centrifuged at 3000 rpm for 3–4 min and the ethyl acetate layer was transferred to a glass tube (10 ml). The extraction with ethyl acetate (2 ml) was repeated and the combined extracts were evaporated using nitrogen. The residue was reconstituted with acetonitrile (200 μl) using sonication and this solution (3–5 μl) was used for LC–MS analysis.

2.3. Liquid chromatography–mass spectrometry

The LC–MS system consisted of a Finnigan MAT LCQ Classic ion-trap mass spectrometer (Thermo-Finnigan, San Jose, CA, USA) and a Waters 2690 Alliance (Waters, Milford, MA, USA) HPLC system, that included a binary pump and a thermostated autosampler to maintain the sample vials at 4 °C. The analytical column was a Luna-2 C_{18} (5 μm, 150×2.0 mm, Phenomenex, Macclesfield, UK) which was maintained at 35 °C. Except where otherwise stated, isocratic chromatography was carried out using acetonitrile–water (60:40 or 46:54) containing 0.05% trifluoroacetic acid TFA and 0.5 m*M* ammonium acetate at a flow-rate of 200 μl/min. For the first and last minute of the chromatographic run, the LC eluent was diverted to waste.

LC–MS and LC–MS^n experiments were carried out using an LCQ mass spectrometer equipped with an electrospray ionisation (ESI) source and operated in positive polarity. The voltage on the ESI needle was set at 4 kV, producing a spray current of approximately 80 μA. The capillary voltage was set at 10 V and the temperature of the heated capillary was 200 °C. The sheath gas flow-rate used was 60 (arbitrary units) and the auxillary gas was set to zero (arbitrary units). To tune the LCQ for azaspiracids, the voltages on the lenses were optimised in the TunePlus function of the Excalibur software whilst infusing a standard solution (1 μg AZA1/ml methanol) at the rate of 3 μl/min. The optimised lens voltages were as follows: tube lens offset (0.0 V); octapole 1 offset (−3.0 V); octapole 2 offset (−7.0 V); inter-octapole lens voltage (−16.0 V); trap DC offset voltage (−10.0 V). The microscan value was set at 3 ms and the maximum inject time was 50 ms.

2.4. Multiple tandem MS experiments

Four scan events were prescribed to run simultaneously in the LCQ mass spectrometer. The first event was a full-scan MS to acquire data on ions in the range of 50–1500 m/z. The scan events 2–4 were MS–MS experiments that were carried out on protonated molecule ions of AZA1–AZA5 (see Table 1). For the MS^3 analysis of AZA1–AZA5, the same values were applied to the TunePlus parameters as for MS–MS. The optimised relative collision energies (RCE) were 30% for MS^2 and 35% for MS^3. WideBand activation was applied over a mass range that extended to 20 m/z below that of the parent ion. This by-passed one water loss stage and a

Table 1
Mass spectral data for five azaspiracids, AZA1–AZA5

Toxin	$[M+H]^+$	$[M+H-H_2O]^+$	$[M+H-2H_2O]^+$	$[M+H-H_2O-C_9H_{10}O_2R_1R_2]^+$ (% abundance)[a]
AZA1	842.5	824.5	806.5	672.4 (12%)
AZA2	856.5	838.5	820.5	672.4 (30%)
AZA3	828.5	810.5	792.5	658.4 (22%)
AZA4	844.5	826.5	808.5	658.4 (35%)
AZA5	844.5	826.5	808.5	674.4 (38%)

[a] Relative abundance to the base peak, $[M+H-H_2O]^+$, using WideBand activation.

high RCE of 93% was used in order to achieve extensive fragmentation of the selected ions. The activation time was also optimised and this value was 250 ms. Azaspiracids were determined using two LC–MS^3 methods with the target parent and fragment ion combinations presented in Table 1:

MS^3 method 1

$$[M+H]^+ \rightarrow [M+H-H_2O]^+ \rightarrow [M+H-2H_2O]^+$$

MS^3 method 2

$$[M+H]^+ \rightarrow [M+H-H_2O]^+ \rightarrow [M+H-H_2O-C_9H_{10}O_2R_1R_2]^+$$

3. Results and discussion

As with most marine intoxications, the concentration of azaspiracids in individual mussels from the same batch can vary by up to 500% and it is therefore important to ensure representative sampling. Also, although the azaspiracids were usually concentrated in the digestive glands of mussels during the initial stages of intoxication, they can be distributed throughout the total tissue [11]. Therefore, the entire shellfish tissues should be used for azaspiracid determinations. Most analytical methods for other polyether toxins, such as the DSP toxins belonging to the okadaic acid and pectenotoxin classes, require only the use of the digestive glands as these toxins are found almost entirely in these organs.

3.1. Mass spectral studies on azaspiracids

Positive electrospray ionisation (ESI) of the standards, AZA1, AZA2 and AZA3, gave protonated molecule ions, $[M+H]^+$, at m/z 842, 856 and 828, respectively. Full-scan collision-induced dissociation (CID) experiments were carried out on azaspiracids using an RCE of 40% for each toxin. These source-CID experiments produced signals due to $[M+H]^+$, $[M+H-H_2O]^+$ and $[M+H-2H_2O]^+$ for each of the five azaspiracids, AZA1–AZA5. For example, AZA3 showed signals at m/z 828.5, 810.5 and 792.5 (Fig. 2A). Spectra from the source-CID experiments performed on AZA4 and AZA5 are shown in Fig. 2B and C. Thus, virtually identical spectra were obtained for these isomeric compounds and this showed that fragmentation gave signals that were from a sequential loss of water molecules from the ions at m/z 844 (Fig. 2B and C). This fragmentation, with multiple losses of water molecules, is typical of polyether shellfish toxins, including OA analogues [13].

The selected precursor ions can also undergo CID in the ion trap by collision with helium to produce ions that can be selectively and repeatedly trapped and fragmented to give MS^n spectral data. To achieve good selectivity in analysis, MS^2 and MS^3 experiments on azaspiracids were performed using optimised RCE values of 25 and 35% on the $[M+H]^+$ and the $[M+H-H_2O]^+$ ions, respectively. The optimised RCE in the context of this work refers to the selection of an energy at which the relative abundance of the parent ion in the fragmentation spectra was $<5\%$, and the main fragment ion was $[M+H-H_2O]^+$. However, important characteristic ions that were observed for azaspiracids were due to fragmentation of the A-ring (Fig. 1) which produced $[M+H-H_2O-C_9H_{10}O_2R_1R_2]^+$ ions at m/z 672.4 (AZA1, AZA2) or m/z 658.4 (AZA3). Table 1 lists the ions that were formed by the A-ring fragmentation of the $[M+H-H_2O]^+$ ions and the hydroxylated analogues of AZA3 showed characteristic ions at m/z 658.4 (AZA4) and m/z 674.4 (AZA5). It was therefore possible to select different precursor and product ion combinations for AZA4 and AZA5 that would allow the selective determination of these toxins using LC–MS^3.

3.2. LC–MS and LC–MS^3 analysis of azaspiracids

The three most abundant azaspiracid toxins were well separated from each other using isocratic re-

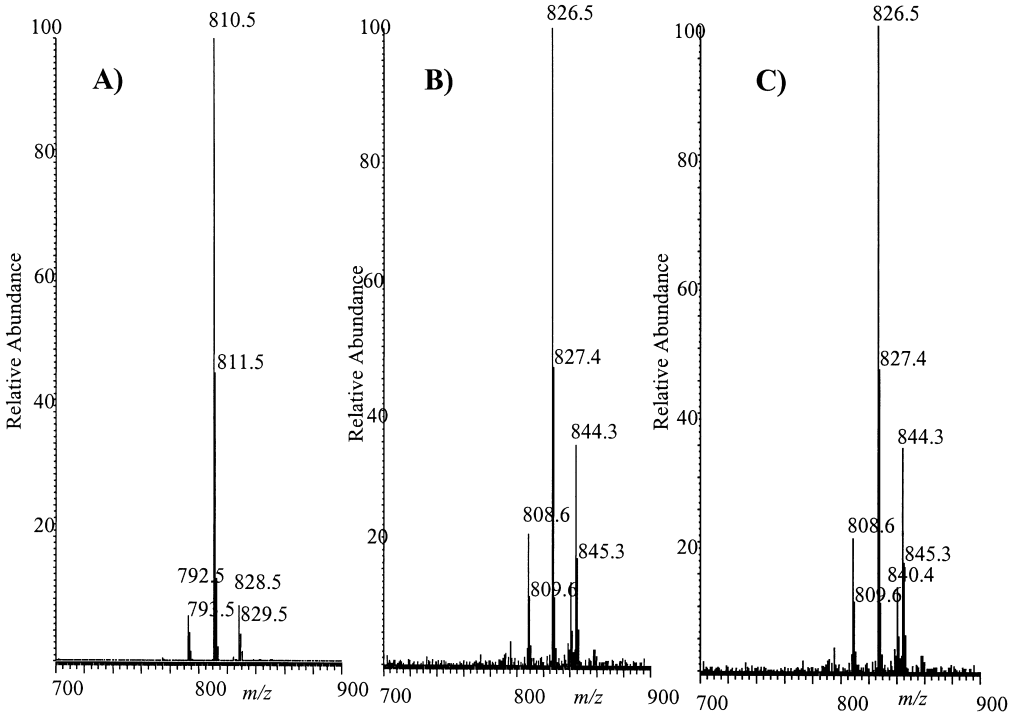

Fig. 2. Source-CID mass spectra of three azaspiracids, (A) AZA3, (B) AZA4 and (C) AZA5.

versed-phase LC, eluting with acetonitrile–water (70:30) containing trifluoroacetic acid (0.05% TFA). The use of TFA as an eluent modifier was important in order to achieve good peak shapes but when either formic acid or acetic acid were used, it was difficult to obtain reproducible data. It is generally advisable to limit the amount of polar matrix constituents entering the MS source and this was conveniently achieved by diverting the eluent from the column to waste for the first minute of the chromatographic run prior to MS detection. Using LC–MS^3 method 1, by selecting the parent ion and the product ions from the sequential loss of two water molecules ($[M+H]^+$, $[M+H-2H_2O]^+$, Table 1), good signal intensities and optimum detection limits were achieved. Obviously, selectivity is enhanced using multiple tandem MS but, unlike a triple quadrupole MS, multiple tandem MS using an ion-trap instrument also proved to be more sensitive than LC–MS. This suggests that the reduction in background noise in multiple tandem MS is more dramatic than the decline in analyte signal. Fig. 3 shows the typical chromatograms that were obtained. The three predominant azaspiracid toxins, AZA3 (3.29 min), AZA1 (4.28 min) and AZA2 (5.03 min), have different molecular masses and are readily determined using LC–MS. Unfortunately, the AZA4 and AZA5 isomers (2.42 min) were not resolved using these chromatographic conditions.

Detailed calibration studies were performed for AZA1 in LC–MS and two LC–MS^3 modes (Fig. 4). It was possible to select unique parent ion–product ion combinations for each of the major azaspiracid toxins, AZA1, AZA2 and AZA3, that are found in shellfish, using the LC–MS^3 method 1 that targeted the $[M+H-2H_2O]^+$ ions. Calibration data for AZA1, using spiked shellfish extracts, showed good linearity in the range 0.05–1.00 μg/ml ($r^2 = 0.9974$) and the detection limit (signal-to-noise ratio 3) was 5 pg. When using the shellfish extraction protocol outlined in Section 2.2, this is equivalent to 0.4 ng AZA1/g shellfish tissue. The LC–MS^3 method 2, that targeted the A-ring fragmentation ion of AZA1, $[M+H-H_2O-C_9H_{12}O_2]^+$, at m/z 672.4, produced

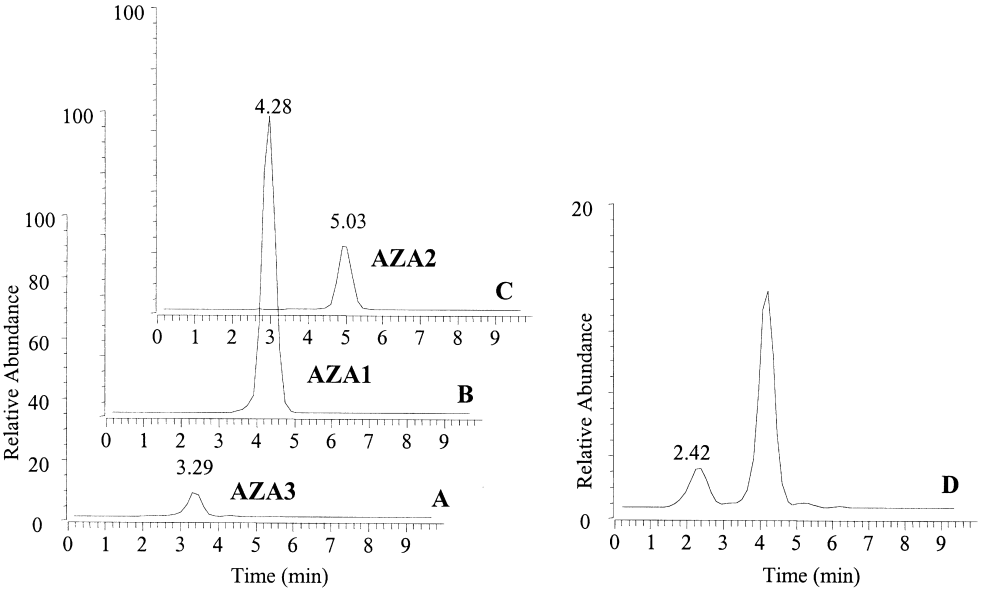

Fig. 3. Chromatograms (A)–(D) from the LC–MS^3 (method 1) analysis of azaspiracids in an extract from mussels (*Mytilus edulis*) harvested in Arranmore Island, Ireland. The targeted ions, $[M+H]^+$, $[M+H-H_2O]^+$, $[M+H-2H_2O]^+$ (see Table 1), corresponding to five azaspiracids: (A) 3.29 min (AZA3), (B) 4.28 min (AZA1), (C) AZA2, (D) AZA4/AZA5. Scan range was 235–900 and the total concentration of AZA1–AZA5 in shellfish was 3.2 μg/g. Chromatographic conditions: Luna-2 C_{18} column (5 μm, 150×2.0 mm) at 40 °C; mobile phase, acetonitrile–water (70:30) containing 0.05% TFA; flow-rate, 200 μl/min.

good calibration data (Fig. 4) ($R^2 = 0.9991$) but the detection limit (40 pg AZA1 on-column) was somewhat higher than in method 1. The Food Safety Authority in Ireland has imposed a provisional regulatory limit of 0.16 μg/g for total AZA1, AZA2 and AZA3 in shellfish tissues and these LC–MS^3 methods are therefore applicable for regulatory control of azaspiracids.

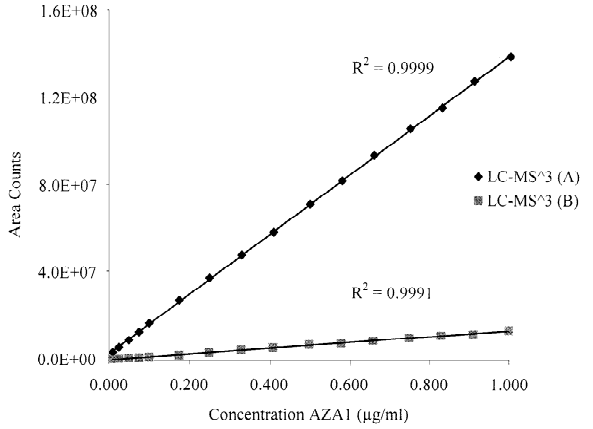

Fig. 4. Calibration data for the determination of AZA1 in mussel tissue (0.05–0.10 μg AZA1/ml) using LC–MS^3 (method 1) and LC–MS^3 (method 2).

Following the recent confirmation of the structures of AZA4 and AZA5, as the 3- and 23-hydroxy analogues of AZA3, respectively, modifications to the chromatographic conditions were carried out to resolve these isomers. Fig. 5 shows the chromatogram that was obtained using LC–MS^3 (method 1) with acetonitrile–water (46:54) containing 0.5 m*M* ammonium acetate and 0.05% TFA. Remarkably, relatively small additions of ammonium acetate dramatically improved the separation of the isomers, with AZA4 eluting at 3.73 min and AZA5 eluting at 4.63 min. Using these conditions, the elution time for AZA1 (not shown) was 34.05 min. The chromatographic separation of these toxins was important to permit useful MS fragmentation experiments on each compound. The spectra shown in Fig. 5B and C clearly show a number of distinguishing features between these isomers, especially the ions at m/z 658.5 and m/z 674.4, which result from the frag-

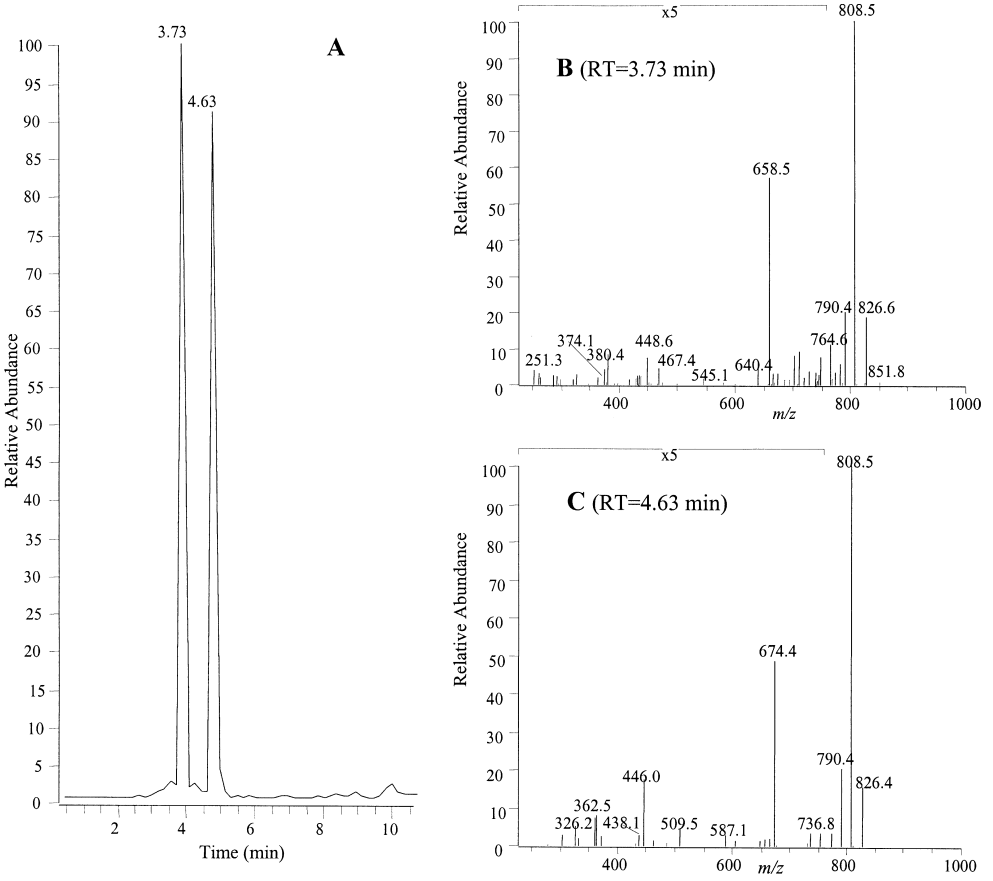

Fig. 5. (A) Chromatogram showing the separation of the isomers, AZA4 and AZA5, which was obtained using LC–MS^3 (method 1); AZA4 (3.73 min) and AZA5 (4.63 min). (B) and (C) are the mass spectra corresponding to AZA4 and AZA5, respectively. Chromatographic conditions were as in Fig. 3 except the mobile phase was acetonitrile–water (46:54) containing 0.5 m*M* ammonium acetate and 0.05% TFA.

mentation of the A-ring in the $[M+H-H_2O]^+$ ions from AZA4 and AZA5, respectively.

3.3. LC–MS–MS analysis of azaspiracids using WideBand activation

The identification of characteristic fragment ions for isomers has positive implications for the robustness of an LC–MS analytical method since each isomer can be determined using a selective MS mode even without complete chromatographic resolution. WideBand activation, using a high RCE, was examined to improve the abundance of characteristic ions due to ring fragmentations of azaspiracids. In this MS mode, resonance excitation energy is applied over a mass range that extends to 20 amu below the parent ion [21]. WideBand activation is useful for molecules, such as polyethers, that exhibit facile water losses in MS because the trapping and fragmentation of the $[M+H]^+$ and $[M+H-H_2O]^+$ ions can be achieved in a single scan. Increasing the RCE to 93% was found to produce the highest abundance of ions from A-ring fragmentations, $[M+H-H_2O-C_9H_{10}O_2R_1R_2]^+$, including AZA4 ($R_1=OH$; $R_2=H$, m/z 658.4) and AZA5 ($R_1=H$; $R_2=H$, m/z 674.4). The enhanced MS signal intensities that were produced permitted adequate detection for these azaspiracids that are typically present in low natural abundance (<5% of total azaspiracids) in shellfish. It is also possible to optimise a value for the

activation time, which is the time that the Rf frequency (Activation Q) is applied to fragment ions. A shorter activation time results in less fragmentation and values can range from 0.03 to 10 000 ms. The optimised value was determined to be 250 ms. To improve sample throughput, the mobile phase composition was changed to 60% acetonitrile and this gave elution times for the five azaspiracids in the range 3.0–8.6 min (Fig. 6). Despite the proximity of AZA4 and AZA5 in this chromatogram, the MS resolution that was achieved for these isomers was sufficient to permit the individual determination of each toxin. The %RSD values for the minor toxins, AZA4 and AZA5, using this method were 12.3 and 8.1 (0.02 μg/ml; $n=7$), respectively.

These LC–MS methods for the determination of multiple azaspiracids should assist the basic research into these recently discovered toxins in marine biological materials and also permit the efficient regulatory control of shellfish destined for human consumption.

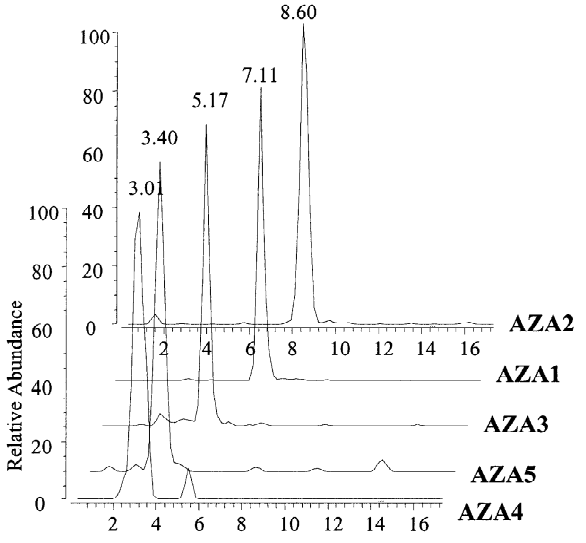

Fig. 6. Chromatograms of AZA1–AZA5 that were obtained using Wideband activation and targeting the product ions from A-ring fragmentations, $[M+H-H_2O-C_9H_{10}O_2R_1R_2]^+$ (see Table 1). The relative intensities of the chromatographic peaks for AZA1–AZA5 were as follows: AZA1 (43.6%), AZA2 (28.8%), AZA3 (21.9%), AZA4 (2.0%) and AZA5 (3.7%). Chromatographic conditions were as in Fig. 3 except the mobile phase was acetonitrile–water (60:40) containing 0.5 m*M* ammonium acetate and 0.05% TFA.

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