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Liquid chromatography—multiple tandem mass spectrometry for the determination of ten azaspiracids, including hydroxyl analogues in shellfish

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Abstract

Azaspiracids (AZAs) are a group of polyether toxins that cause food poisoning in humans. These toxins, produced by marine dinoflagellates, accumulate in filter-feeding shellfish, especially mussels. Sensitive liquid chromatography-electrospray ionisation mass spectrometry (LC-ESI-MS^n) methods have been developed for the determination of the major AZAs and their hydroxyl analogues. These methods, utilising both chromatographic and mass resolution, were applied for the determination of 10 AZAs in mussels (*Mytilus edulis*). An optimised isocratic reversed phase method (3 μm Luna-2 C_{18} column) separated 10 azaspiracids using acetonitrile/water (46:54, v/v) containing 0.05% trifluoroacetic acid (TFA) and 0.004% ammonium acetate in 55 min. Analyte determination using MS^3 involved trapping and fragmentation of the $[M + H]^+$ and $[M + H - H_2O]^+$ ions with detection of the $[M + H - 2H_2O]^+$ ion for each AZA. Linear calibrations were obtained for AZA1, using spiked shellfish extracts, in the range 0.05–1.00 μg/ml ($r^2 = 0.997$) with a detection limit of 5 pg (signal : noise = 3). The major fragmentation pathways in hydroxylated azaspiracids were elucidated using hydrogen/deuterium (H/D) exchange experiments. An LC-MS^3 method was developed using unique parent ions and product ions, $[M + H - H_2O - C_9H_{10}O_2R^1R^3]^+$, that involved fragmentation of the A-ring. This facilitated the discrimination between 10 azapiracids, AZA1–10. Thus, this rapid LC-MS^3 method did not require complete chromatographic resolution and the run-time of 7 min had detection limits better than 20 pg for each toxin.
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Keywords: Shellfish Poisoning; Toxins; Azaspiracids

1. Introduction

Azaspiracid Poisoning (AZP) is a recently identified human toxic syndrome that is caused by the consumption of shellfish that are contaminated by natural toxins. Azaspiracids (AZAs) were first identified in 1995 [1] following intoxications in The Netherlands, and the human symptoms, included nausea, vomiting, severe diarrhoea and stomach cramps [2,3]. Acute and chronic toxicological studies using mice have shown that AZAs caused widespread organ damage and induced tumours [4,5] but the mechanisms of toxicity remain to be elucidated [6]. Although AZAs were first identified in mussels that were cultivated in Ireland, a widespread European distribution of these toxins has recently been confirmed [7]. The EU regulatory limit for AZAs has recently been set at 0.16 μg/g total shellfish tissue.

Several major classes of polyether marine toxins, such as dinophysistoxins, pectenotoxins, yessotoxins and brevetoxins, are produced by marine dinoflagellates [8]. A common phytoplankton, belonging to the genus, *Protoperidinium*, has recently been discovered as the progenitor of AZAs [9]. These toxins accumulate in filter-feeding bivalve molluscs, including mussels (*Mytilus edulis*) [7] and scallops (*Pecten maximus*) [10] which can lead to the poisoning of human consumers.

Structurally, azaspiracids are polyether amino acids that have a 6,5,6-trispiroacetal moiety, rings A, B and C, together with a 2,9-dioxabicyclo[3.3.1]nonane ring that is fused with an azaspiro ring system, rings F, G, H, and I (Fig. 1A, Table 1) [1]. Three azaspiracids, AZA1–3, have been iden-

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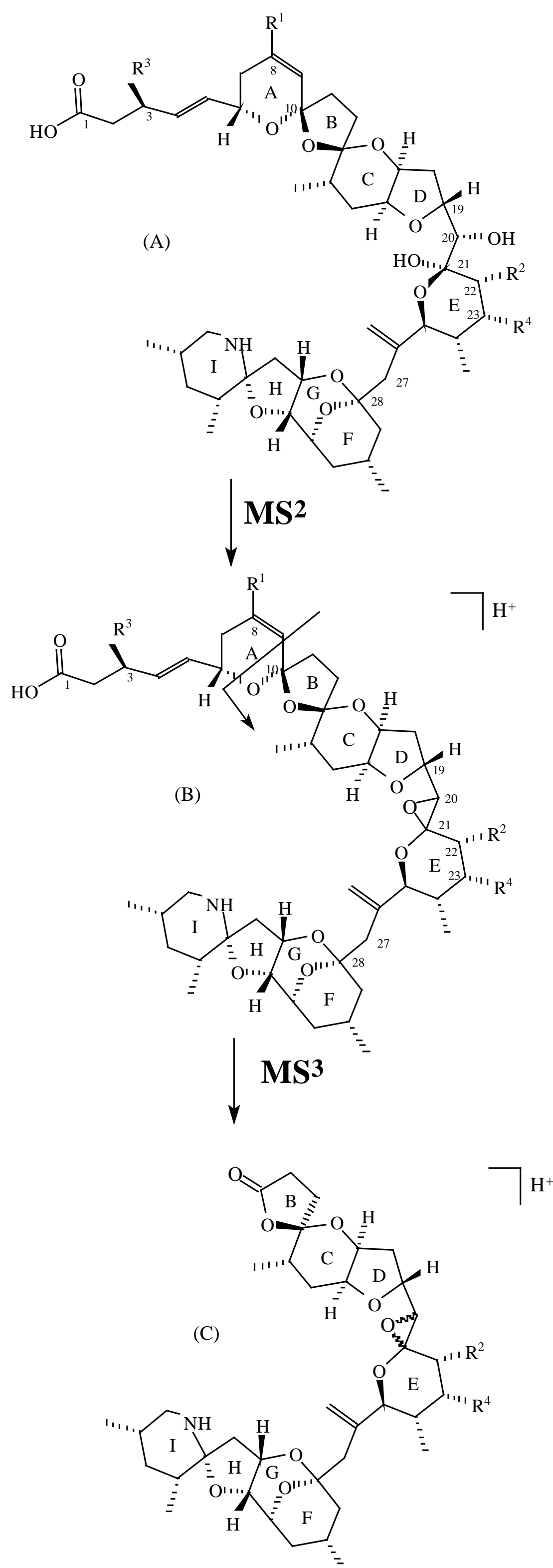

Fig. 1. (A) Structures of azaspiracids (see Table 1). (B) Epoxy-azaspiracid ions formed due to water loss from $[M + H]^+$ ions. (C) Fragment ions formed from $[M + H - H_2O]^+$ ion due to A-ring fragmentation. See Table 2 for ion masses of protonated azaspiracids and the ions 1B and 1C.

Table 1
Azaspiracids (AZA1–10)

Toxin	R^1	R^2	R^3	R^4
AZA1	H	CH_3	H	H
AZA2	CH_3	CH_3	H	H
AZA3	H	H	H	H
AZA4	H	H	OH	H
AZA5	H	H	H	OH
AZA6	CH_3	H	H	H
AZA7	H	CH_3	OH	H
AZA8	H	CH_3	H	OH
AZA9	CH_3	H	OH	H
AZA10	CH_3	H	H	OH

tified in phytoplankton and they are the predominant toxins in shellfish. AZA2 and AZA3 are the 8-methyl and 22-demethyl analogues of AZA1, respectively [2]. Toxins that have been found in low abundance in shellfish include AZA4 and AZA5, which are the 3- and 23-hydroxy analogues, respectively, of AZA3 [11] and AZA6, which is an isomer of AZA1 [12,13]. AZA7–10 [14] are hydroxy analogues of AZA1 and AZA6 (Fig. 1, Table 1). The hydroxy analogues, AZA4, AZA5 and AZA7–10, are most likely the products of bioconversion in shellfish as they are not found in phytoplankton.

The determination of AZA1–3 in shellfish is possible using a single quadrupole mass spectrometer provided that a solid phase extraction (SPE) clean-up is used [11]. However, the development of a SPE protocol, that can be successfully applied to all 10 AZAs, may be problematic [14]. The high selectivity of multiple tandem MS is required for determining the minor azaspiracid contaminants in shellfish. LC-MS/MS, using triple quadrupole instruments [15–17] and LC-MS^3, using an ion-trap instrument, have been developed for the determination of AZAs [12,18,19]. The aim of this study was to develop multiple tandem MS methods for the simultaneous analysis of the predominant azaspiracids, as well as their minor analogues, in biological tissues. These methods employed both chromatographic resolution and mass selection of fragments ions in multiple tandem MS to permit the resolution of isomers.

2. Materials and methods

2.1. Chemicals and toxin standards

HPLC-grade acetonitrile and water were purchased from Labscan (Dublin, Ireland) and trifluoroacetic acid (TFA), deuterated methanol (CD_3OD) and water (D_2O) were obtained from Sigma-Aldrich (Dorset, UK). Azaspiracid standards, AZA1–3, were isolated from toxic mussels (*Mytilus edulis*) as described previously [2]. Contaminated mussels were collected from various locations on the west coast of Ireland and extraction for analysis of azaspiracids was carried out as described previously [19].

2.2. Liquid chromatography conditions

The LC system for the LCQ ion-trap mass spectrometer was a Waters 2690 Alliance (Waters Corporation, Milford, MA, USA) and the API 3000 triple quadrupole MS was linked to an Agilent 1100 series instrument (Agilent, Palo Alto, CA, USA). Both systems included a thermostated autosampler, operated at 4 °C. Isocratic chromatography was performed using two eluent compositions, designated I and II, at a flow rate of 200 μl/min, with a reversed phase column (Luna-2, 3 μm, 150 mm × 2.0 mm, Phenomenex, Macclesfield, UK) at 40 °C. The injection volume was 5 μl and the eluent flow was diverted to waste for 1 min after sample injection. Eluent I) was acetonitrile/water (46:54, v/v) and eluent II) was acetonitrile/water (65:35, v/v) and both contained 0.05% trifluoroacetic acid (TFA) and 0.004% ammonium acetate.

For deuterated studies, acetonitrile (CD_3CN)/D_2O (65:35, v/v) was used with the same eluent modifiers. The LC system was flushed (2 h) with deuterated mobile phase before injection of azaspiracids dissolved in 50% deuterated methanol (CD_3OD)/D_2O. The mass spectra showed complete H/D exchange as the protonated form of the ions were not observed in the full-scan spectrum.

2.3. Ion-trap mass spectrometry

Mass spectral analyses were performed using a LCQ ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA), with electrospray ionisation (ESI) in positive

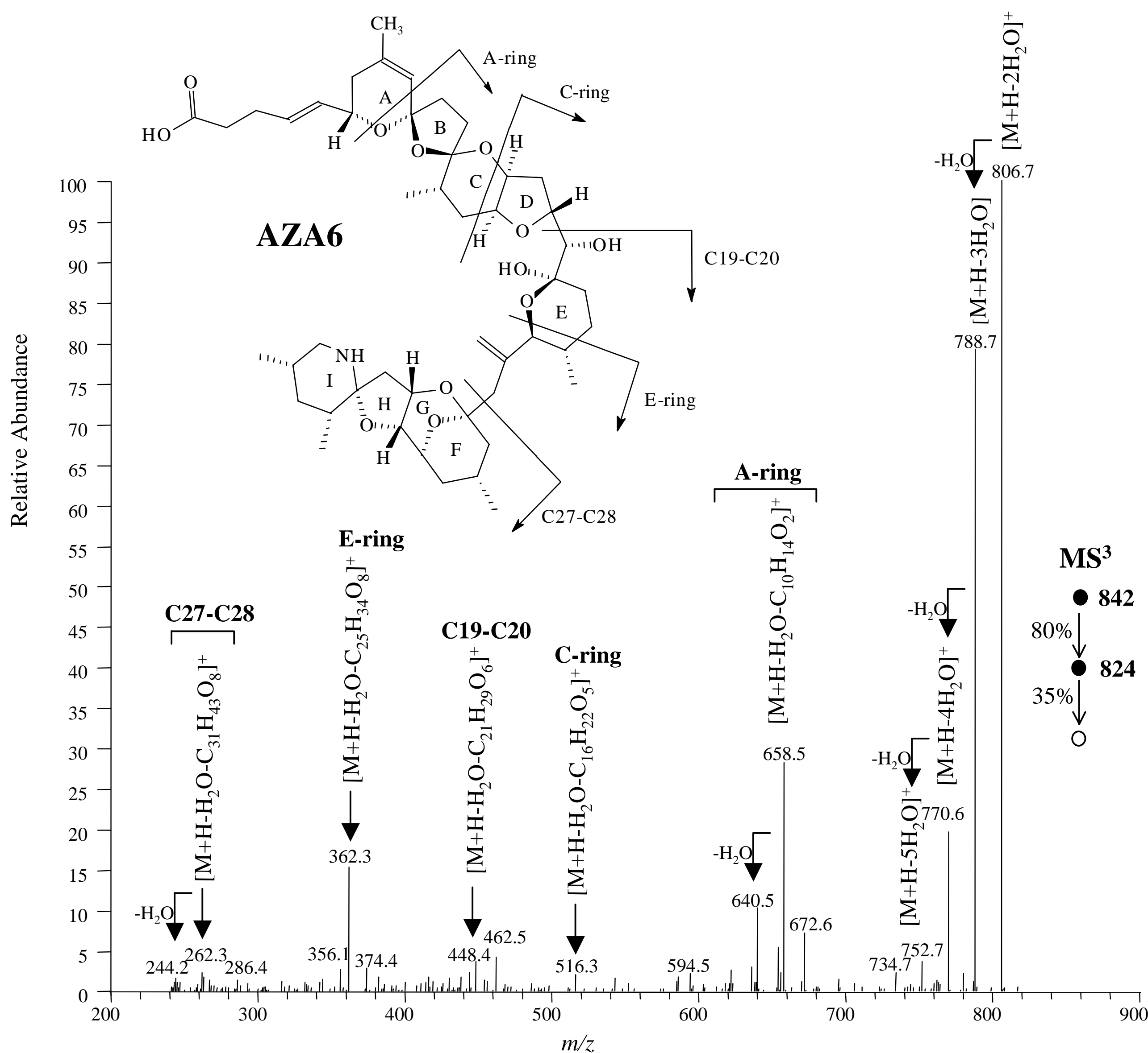

Fig. 2. Spectrum of AZA6 produced by positive ESI using an ion-trap in MS^3 mode. The major ions are assigned and the five backbone fragmentation processes are shown (inset).

mode, as detailed previously [12]. The ESI needle voltage was 4 kV, producing a spray current of approximately 80 μA. The capillary voltage was 10 V and the heated capillary temperature was 200 °C. The sheath gas flow rate used was 80 (arbitrary units) and the auxiliary gas was set to 20 (arbitrary units). The LCQ was tuned for azaspiracids (AZA1) and the voltages on the lenses were optimised in TunePlus (Excalibur software) whilst infusing a standard solution (1 μg AZA1/ml methanol) at the rate of 3 μl/min. The ion optics voltages were optimised: tube lens offset (15 V); octapole 1 offset (−3 V); octapole 2 offset (−7 V); inter octapole lens voltage (−16 V); trap dc offset voltage (−10 V). The microscan value was set at 7 ms and the maximum inject time was 250 ms.

2.4. Triple stage quadrupole mass spectrometry

An API 3000 (Applied Biosystems, Warrington, UK) mass spectrometer (triple stage quadrupole) was used with a Turbo Ionspray interface. The ion source dependant parameters were optimised as follows; nebuliser gas 8 (arb), curtain gas 12 (arb), Ionspray voltage (IS) 5500 V, temperature (TEM) 400 °C. Multiple reaction monitoring (MRM) experiments were developed using voltages optimised for pseudo-molecular ion production; declustering potential (DP) 60 V, focusing potential (FP) 400 V, entrance potential (EP) 10 V, and product ion generation; collision cell exit potential (CXP) 10 V, collision gas (CAD) 3 (arb).

3. Results and discussion

LC-tandem MS is undoubtedly the method of choice for the determination of trace quantities of analytes in complex biological matrices [20]. A number of LC-MS approaches were examined with the aim of developing a method for the simultaneous determination of the predominant azaspiracids and their bioconversion analogues. There are two main challenges in the development of a rapid LC-MS method for the simultaneous analysis of azaspiracids and their hydroxyl analogues in biological tissues. Firstly, AZAs differ widely in relative abundance and secondly, these toxins include two pairs of isomers and a group of four isomers (Fig. 1, Table 1). The following LC-MS methods that were developed for the determination of individual AZAs utilised both chromatographic resolution and mass selectivity.

3.1. Fragmentation pathways for azaspiracids using electrospray ion-trap MS

A major fragmentation pathway in azaspiracids involves multiple water losses producing the ions, $[M+H-nH_2O]^+$ ($n = 1$–5). There are several sites in azaspiracids at which water losses can occur and the first water loss is the facile dehydration at the C20–21 diol. This was readily demonstrated by hydrogen/deuterium (H/D) exchange experiments. To illustrate, the deuterated molecule ions in the full-scan spectra of AZA6 and AZA4 were $m/z = 847$; $[M(d_4) + D]^+$ and $m/z = 850$ $[M(d_5) + D]^+$, respectively. The corresponding protonated ions, $[M + H]^+$, were at $m/z = 842$ (AZA6) and $m/z = 844$ (AZA4). The MS^2 spectra showed that the first water loss in the non-deuterated experiments became a 20 Da loss ($-D_2O$) in the deuterated experiments. This represents compelling evidence that the proposed epoxide formation was the first step in the fragmentation process

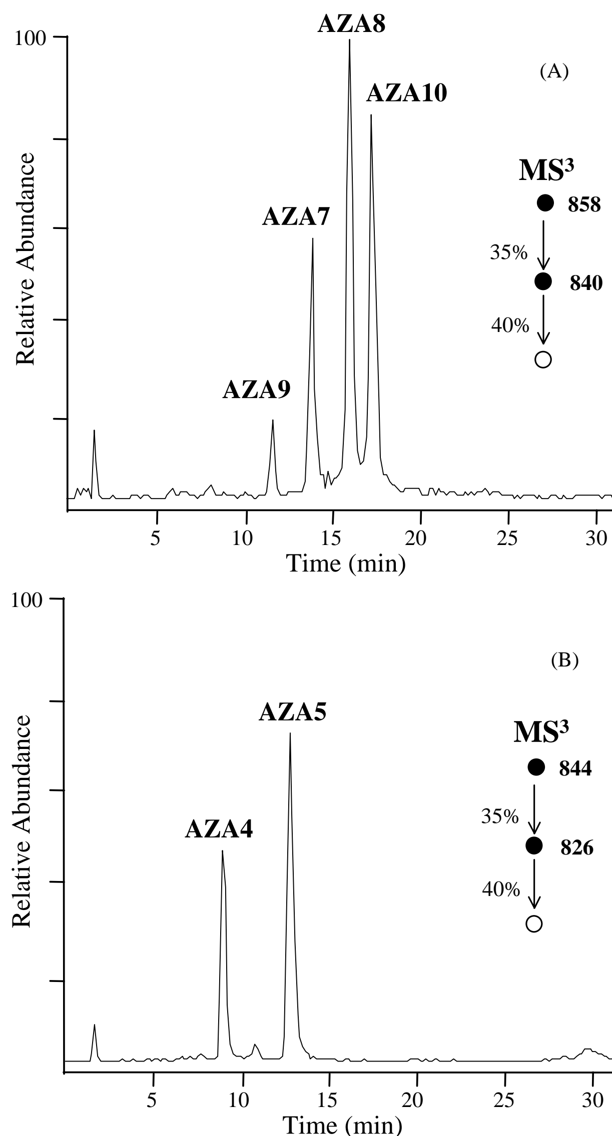

Fig. 3. Chromatograms from the LC-MS^3 (method I) analysis of azaspiracids in an extract from mussels (*M. edulis*) using the following targeted ions: $[M+H]^+$, $[M+H-H_2O]^+$ and $[M+H-2H_2O]^+$ (see Table 1), corresponding to 10 azaspiracids. (A) 11.2 min (AZA9), 13.4 min (AZA7), 15.4 min AZA8, 16.5 min AZA10; (B) 8.6 min AZA4, 13.0 min AZA5. The predominant toxins (not shown) were at 28 min (AZA3); 36.5 min (AZA6); 42 min (AZA1); 56 min (AZA2). Chromatographic conditions: Luna-2 C_{18} column (3 μm, 150 mm × 2.0 mm) at 40 °C; mobile phase was acetonitrile/water (46:54, v/v) containing 0.05% trifluoroacetic acid (TFA) and 0.004% ammonium acetate; flow rate was 200 μl/min.

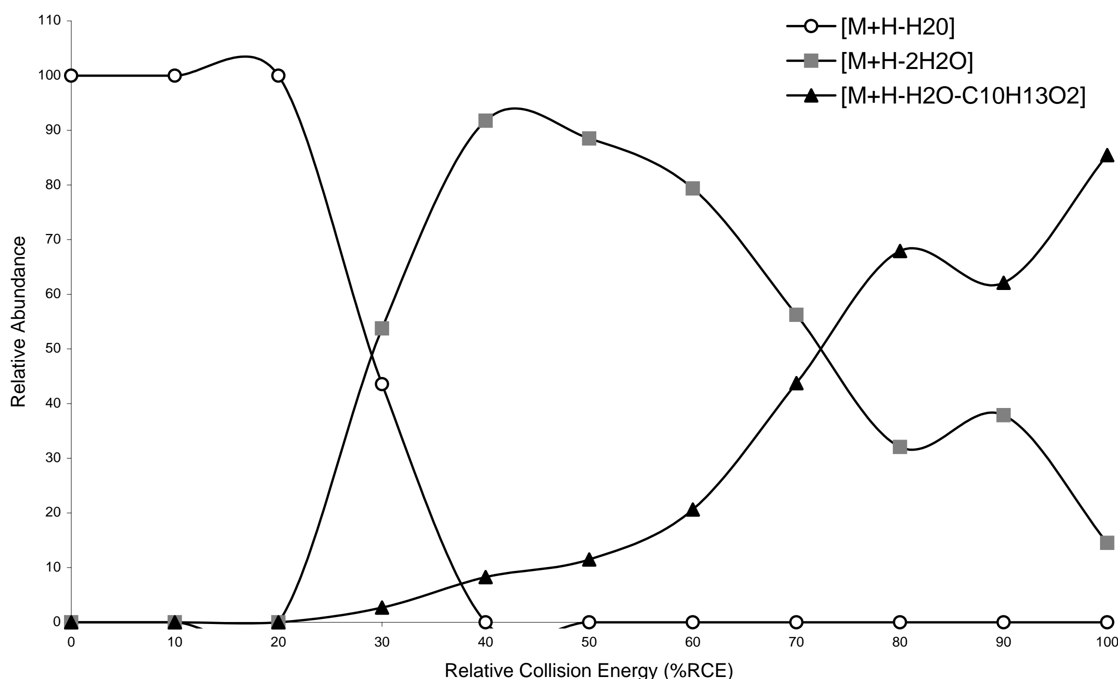

Fig. 4. Plot of the relative collision energy (RCE) vs. the abundance of the $[M + H - H_2O]^+$ ion and selected product ions in the MS^3 stage, generated from AZA6 in the quadrupole ion-trap MS. The selected ions were, $m/z = 824$ $[M + H - H_2O]^+$; $m/z = 806$ $[M + H - 2H_2O]^+$; $m/z = 658$ $[M + H - H_2O - C_{10}H_{13}O_2]^+$ (from the A-ring fragmentation of 824).

(Fig. 1B). To acquire further evidence of the fragmentation pathways, MS^n ($n = 2 - 4$) experiments were performed on deuterated and non-deuterated azaspiracids and spectra were compared.

An illustration of the spectral interpretation that is possible using a prescribed MS^n experiment is shown in Fig. 2. This spectrum was obtained from the MS^3 of AZA6 and the assignments are proposed for the major ions, which are due to multiple water losses from the protonated molecule ion and fragmentation of the azaspiracid backbone. These major fragmentation processes (Fig. 2 inset) included the A-ring, C-ring, C19–20, E-ring and C27–28. An intrinsic part of MS method development is the optimisation of the relative collision energies (RCE). In this example, these values were 40% (MS^2) and 80% (MS^3), and were selected to produce optimum abundance of ions (Fig. 1C) from the fragmentation of the A-ring, $[M + H - H_2O - C_9H_{10}O_2R^1R^3]^+$. The typical process for optimisation of the RCE values is discussed in Section 3.3.

3.2. Isocratic LC chromatographic separation of 10 azaspiracids

In many LC-MS methods for the analysis of trace analytes, the high selectivity of MS obviates the requirement for good chromatographic resolution [20]. However, when confronted with the determination of multiple isomers, especially at low natural abundance, the LC-MS method must either deliver chromatographic resolution or distinguish between isomers by mass selectivity using characteristic fragment ions. In a previous study, we demonstrated that AZA1–5 could be determined using LC-MS^3 methods that involved C_{18}-bonded columns and acetonitrile/water with trifluoroacetic acid (TFA) and ammonium acetate eluent

Table 2
Masses of the parent and major fragment ions of azaspiracids

Toxin	$[M + H]^+$	$[M + H - H_2O]^+$	$[M + H - 2H_2O]^+$	$[M + H - H_2O - C_9H_{10}O_2R^1R^3]^+$
AZA1	842.5	824.5	806.5	672.4
AZA2	856.5	838.5	820.5	672.4
AZA3	828.5	810.5	792.5	658.4
AZA4	844.5	826.5	808.5	658.4
AZA5	844.5	826.5	808.5	674.4
AZA6	842.5	824.5	806.5	658.4
AZA7	858.5	840.5	822.5	672.4
AZA8	858.5	840.5	822.5	688.4
AZA9	858.5	840.5	822.5	658.4
AZA10	858.5	840.5	822.5	674.4

modifiers [19]. For azaspiracids, the most sensitive LC-MS[3] method involved trapping and fragmentation of the parent and the product ions; $[M + H]^+ \rightarrow [M + H - H_2O]^+ \rightarrow [M + H - 2H_2O]^+$. A feature of ion-trap MS is that there can be an improvement in detection sensitivity in multiple MS modes. This is attributed to the reduction in background noise (N) being more dramatic than the decline in analyte signal (S) giving an improved S/N in MS^2 and MS^3 stages [21]. In multiple MS modes, the detection sensitivity obviously depends on the production and trapping of abundant fragment ions. Through the implementation of this LC-MS^3 method, the separation of the 10 azaspiracids, AZA1–10, was achieved in 60 min (Fig. 3). The selected chromatographic conditions were developed primarily to separate the four isomers, AZA7–10, as the selected masses, *m/z* 858 $[M + H]^+$, *m/z* 840 $[M + H - H_2O]^+$ and *m/z* 822 $[M + H - 2H_2O]^+$, do not distinguish between these compounds (Fig. 2A). The elution times for the other azaspiracids were; 28 min (AZA3); 36.5 min (AZA6); 42 min (AZA1); 56 min (AZA2). The detection limits ($S/N = 3$) of this method were $(1-2) \times 10^{-14}$ M, which is equivalent to 5 pg on-column. Although this chromatography may be acceptable for preparative LC, the long chromatographic analysis is unacceptable in routine LC-MS where the instrument time required per sample is a major cost factor.

Attempts to develop a gradient method were unsuccessful for the chromatographic resolution of the hydroxyl analogues. The separation of AZA7–10 was achieved an initial isocratic stage (acetonitrile/water 46:54 for 19 min) prior to a rapid terminal gradient (increasing to acetonitrile/water 80:20 over 3 min). Although the total run time was reduced to 24 min, an additional 25 min was required for equilibration in order to generate reproducible chromatographic data.

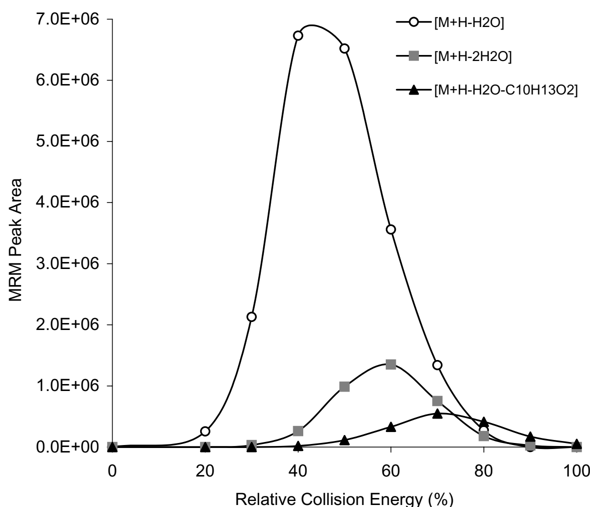

Fig. 5. Plot of the relative collision energy (RCE) vs. the MRM peak area of the three product ions, $m/z = 824$ $[M+H-H_2O]^+$; $m/z = 806$ $[M+H-2H_2O]^+$; $m/z = 658$ $[M+H-H_2O-C_{10}H_{13}O_2]^+$ generated by MS/MS of the AZA6 parent ion using a triple stage quadrupole MS.

3.3. *Effect of collision energies on the relative abundance of fragment ions*

Fragmentation studies were conducted on AZA6 by multiple tandem MS using a quadrupole ion trap MS (ThermoFinnigan LCQ) and a triple quadrupole MS (Applied Biosystems API 3000). In the ion-trap experiments, the RCE was ramped, in 10% increments, to 100% in MS^2 mode, and the relative abundance of the fragmentation ions $[M + H - H_2O]^+$, $[M + H - 2H_2O]^+$ and $[M + H - H_2O - C_9H_{10}O_2R^1R^3]^+$ were measured. The MS^2 collision energy ramping experiments (not shown) indicated that although the product ion from A-ring fragmentation increased proportionally with applied RCE, its relative abundance was less than 15%. The development of a robust LC-MS^2 method was problematic in the absence of a plateau region for target ion abundance as a function of RCE. The most abundant ion in the MS^2 spectra is due to the loss of a single water molecule, $[M + H - H_2O]^+$, and there was a broad abundance maximum at RCE = 30–45%.

In the MS^3 mode, the $[M+H-H_2O]^+$ ion was trapped and the RCE was ramped to 100% and the relative abundance of the $[M + H - 2H_2O]^+$ and $[M + H - H_2O - C_{10}H_{13}O_2]^+$ ions were measured. These data were plotted as the relative abundance of the selected fragment ions versus RCE (Fig. 4).

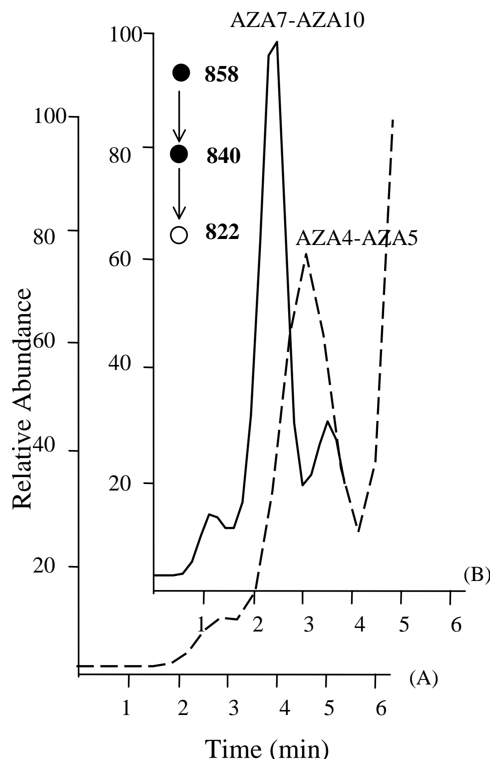

Fig. 6. Chromatograms from the LC-MS^3 (method II) (a) AZA4/AZA5 (hashed line) and (b) AZA7–10 using the following targeted ions: $[M+H]^+$, $[M+H-H_2O]^+$ and $[M+H-2H_2O]^+$ (see Table 1). Chromatographic conditions were as shown in Fig. 3 except that the eluent composition was changed to acetonitrile/water (65:35, v/v).

Fragmentation occurs at RCE > 20% and the main product ions were due to a second water loss, $[M+H-2H_2O]^+$, and A-ring fragmentation, $[M+H-H_2O-C_{10}H_{13}O_2]^+$. Table 2 lists the ions that were produced for protonated AZAs and their main product ions. It is apparent from this table, that only the product ions due to A-ring fragmentation have different masses and hence can be exploited to distinguish between AZAs. Therefore, the observation that the A-ring fragmentation process produced the highest abundance of ions at RCE = 80% in the ion-trap was important, since this allowed the selective determination of isomers whilst maintaining high sensitivity.

For comparison purposes, MRM experiments were conducted using a triple stage quadrupole MS (Applied Biosystems, API 3000). The $[M+H]^+$ precursor ions for AZA6 were selected and subjected to MS^2 analysis whilst ramping the collision energy. In the case of AZA6, the relative abundance of the selected product ions, $[M+H-H_2O]^+$, $[M+H-2H_2O]^+$ and $[M+H-H_2O-C_{10}H_{13}O_2]^+$, at various collision energies, showed that the abundance of product ions due to A-ring fragmentation was always low (ca. 8%) when compared to the single water-loss ion (Fig. 5). The major consequence of this observation was that the determination of azaspiracids, using an MRM experiment that selected A-ring fragment ions, was less sensitive than the MS^3 ion-trap method. The ion-trapping process discriminates in favour of retaining target ions and eliminating unwanted ions. This results in the reduction of the signal/noise ratio that is due mainly to the diminished background noise in MS^n [19,21].

3.4. Analyte selective LC-MS^3 determination of azaspiracids

The A-ring fragmentation of azaspiracids results in the loss of the C1–9 portion which contains the substituents, R^1 and R^3, leaving the residual ion containing R^2 and R^4 (Fig. 1C). Therefore, advantage can be taken of this process in MS^3 mode to select unique product ions for each isomeric azaspiracid. The isomers, AZA7–10, have the same combination of ions for the neutral loss of two consecutives water molecules, $m/z = 858 \rightarrow 840 \rightarrow 822$. However, they gave

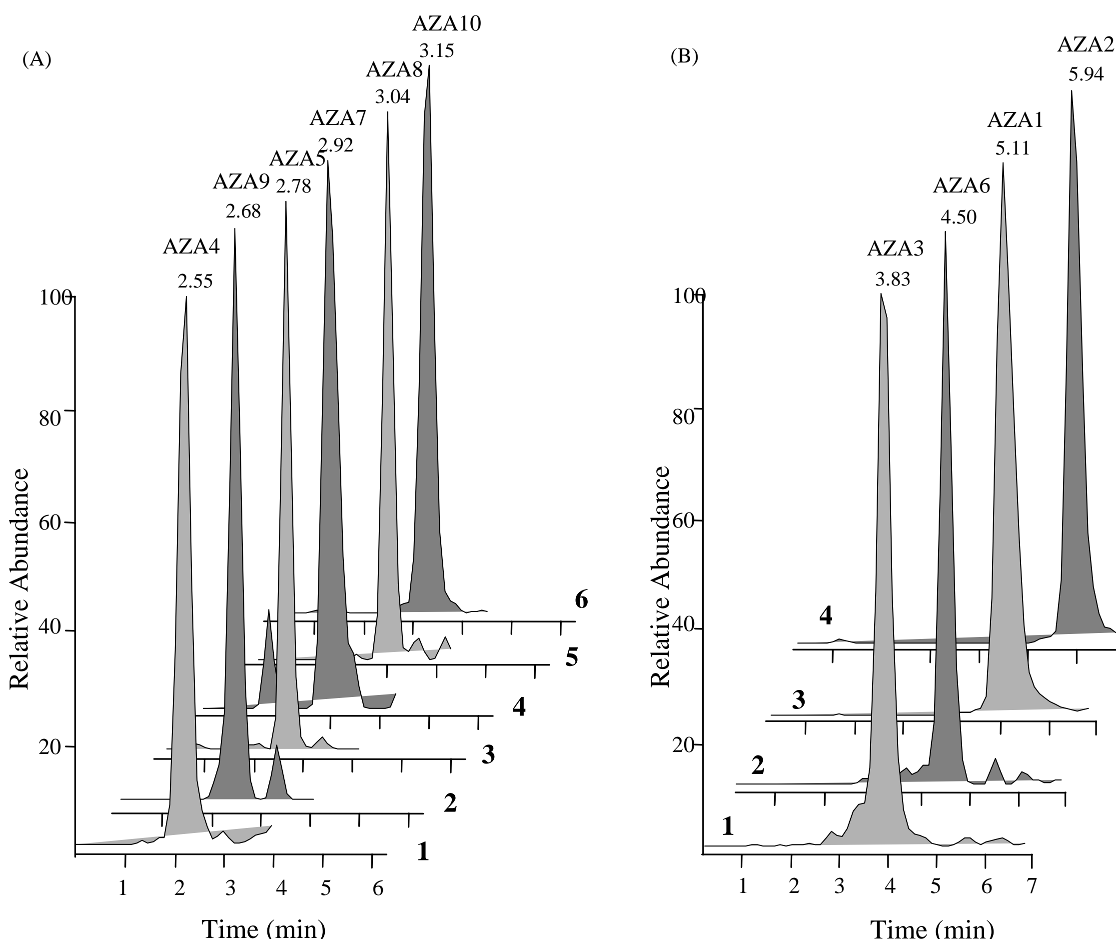

Fig. 7. Chromatograms from the LC-MS^3 (method II) analysis of azaspiracids in an extract from mussels (*M. edulis*) using the following targeted ions: $[M+H]^+$, $[M+H-H_2O]^+$ and $[M+H-H_2O-C_9H_{10}O_2R^1R^3]^+$ (see Table 1), corresponding to 10 azaspiracids. (A) Chromatograms of the 3- and 23-hydroxy azaspiracids, AZA4, AZA5 and AZA7–10. (B) Chromatograms of AZA1–3 and AZA6. Chromatographic conditions were as shown in Fig. 3 except that the eluent composition was acetonitrile/water (65:35, v/v).

different ions for the A-ring fragmentation, AZA7 ($m/z = 858 \rightarrow 840 \rightarrow 672$); AZA8 ($m/z = 858 \rightarrow 840 \rightarrow 688$); AZA9 ($m/z = 858 \rightarrow 840 \rightarrow 658$); AZA10 ($m/z = 858 \rightarrow 840 \rightarrow 674$). The chromatograms (Fig. 6A and B) were obtained from a shellfish extract containing AZA4, AZA5 and AZA7–10, but analysed using the non-selective consecutive water-loss ions for MS^3. Obviously, these AZAs cannot be individually determined using this method. The chromatograms from the same sample using the rapid isocratic LC-MS^3 method (II), developed to determine the 10 AZAs in a 7 min elution time, are shown in Fig. 7. AZAs were resolved due to the mass selectivity that was possible by using the A-ring fragment ions in MS^3 mode. The sensitivity of this method is 2–4 times less than the isocratic method in Section 3.2. This is a consequence of the requirement of 10 scan events in MS, one event for the detection of each toxin. By comparison, the isocratic method requires only five scan events. Typically, in extract from shellfish, the hydroxyl isomers, AZA4, AZA5, represented 5–8% of total AZAs and the isomers, AZA7–10, represented less than 2%. As an analytical method, this rapid elution LC-MS^3 procedure, specifying unique parent-product ion combinations is preferred, but for semi-preparative chromatography, gradient elution with column switching may be feasible.

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