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Journal of Chromatography A, 963 (2002) 353–361

JOURNAL OF CHROMATOGRAPHY A

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# Comparison of solid-phase extraction methods for the determination of azaspiracids in shellfish by liquid chromatography–electrospray mass spectrometry

Cian Moroney, Mary Lehane, Ana Braña-Magdalena, Ambrose Furey, Kevin J. James*

*Ecotoxicology Research Unit, Department of Chemistry, Cork Institute of Technology, Bishopstown, Cork, Ireland*

**Abstract**

Azaspiracids have been identified as the cause of a new toxic syndrome called azaspiracid poisoning (AZP) that has led to incidents of human intoxications throughout Europe following the consumption of mussels. Although five AZP toxins have been structurally elucidated to-date, azaspiracid (AZA1), 8-methylazaspiracid (AZA2) and 22-demethylazaspiracid (AZA3) are the predominant toxins. Separation of the three main AZP toxins was achieved using reversed-phase liquid chromatography (LC) and coupled to an electrospray ionisation source of an ion-trap mass spectrometer. Five reversed-phase ($C_{18}$) and three diol solid-phase extraction (SPE) cartridges were compared for their efficacy in the cleanup of shellfish matrix. The comparison was based on the optimum recoveries of AZA1, AZA2 and AZA3 from extracts of mussel tissues. LC–electrospray $MS^3$ analysis was used to quantify the AZP toxins in wash and eluate fractions in the SPE studies. Good recovery and reproducibility data were obtained for one diol SPE cartridge and two $C_{18}$ SPE cartridge types. 

*Keywords:* Solid-phase extraction; Shellfish poisoning; Azaspiracids; Toxins

## 1. Introduction

Acute human intoxications, following the consumption of shellfish, can occur due to contamination by natural toxins that originate in marine microalgae [1]. The toxins responsible for the most common types of shellfish toxic syndromes are regularly detected in many European countries but incidents of human intoxication are limited due to the implementation of shellfish toxin monitoring programmes. The most common shellfish toxic syndromes are diarrhetic shellfish poisoning (DSP) [2], paralytic shellfish poisoning (PSP) [3] and amnesic shellfish poisoning (ASP) [4]. However, a new toxic syndrome has recently been identified following human intoxications in several European countries following the consumption of mussels (*Mytilus edulis*) that were cultivated along the west coast of Ireland [5]. The human symptoms, nausea, vomiting, severe diarrhea and stomach cramps, were similar to DSP but typical DSP toxins were not present in sufficient quantities. Subsequently, a new group of toxins were identified and named azaspiracids as they contained an azaspiro ring fused with a 2,9-dioxabicyclo[3.3.1] nonane ring assembly [6] (Fig. 1). Azaspiracid (AZA1) and its 8-methyl and 22-demethyl analogues, AZA2 and AZA3, respectively are consistently the predominant toxins that have been found in shellfish from Ireland, the UK and Norway [7].

*Corresponding author. Tel.: +353-21-4326-317; fax: +353-21-4343-415.

*E-mail address:* kjames@cit.ie (K.J. James).

0021-9673/02/$ – see front matter 
PII: S0021-9673(02)00545-9

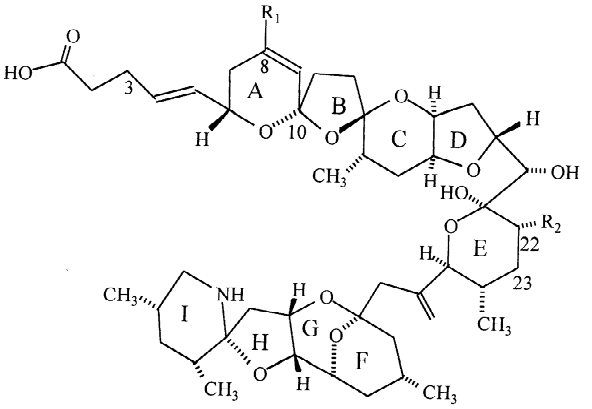

Fig. 1. Structures of AZP toxins, AZA1 ($R_1=H$; $R_2=CH_3$); AZA2 ($R_1=R_2=CH_3$); and AZA3 ($R_1=R_2=H$).

The main pathological changes caused by azaspiracid include necroses in the lamina propria of the small intestine, thymus and spleen together with lymphocyte injury and fatty changes in the liver [8]. This new shellfish toxic syndrome has been named azaspiracid poisoning (AZP) and several liquid chromatography–mass spectrometry (LC–MS) methods have been developed for the determination of AZP toxins in shellfish. Although intraperatoneal mouse bioassays, which were based on assays that were developed for DSP toxins [2], have been employed for the regulatory control of AZP toxins in shellfish in Europe, these have repeatedly failed to prevent acute human intoxications [9]. AZP toxins are not confined to the digestive glands of shellfish but are distributed through all tissues [10]. The Food Safety Authority in Ireland has imposed a limit of 0.16 μg total azaspiracids/g shellfish tissue, to be determined using LC–MS methods.

LC–MS–MS, using atmospheric pressure chemical ionisation (APCI) and selected reaction monitoring (SRM), has been developed for the determination of AZA1 [11]. LC–$MS^3$, using electrospray ionisation (ESI) and an ion-trap mass spectrometer, has recently been used for the determination of the predominant AZP toxins in shellfish [12]. These analytical methods utilised the precursor–product ions combinations, ($[M+H-nH_2O]^+$; $n=1$–3). However, pre-concentration and cleanup of shellfish extracts using a diol solid-phase extraction (SPE) cartridge was required for a less selective LC–ESI-MS analysis [13].

This study was undertaken to optimise a number of SPE methods for the efficient cleanup of shellfish extracts so as to facilitate the development of analytical methods for the determination of AZP toxins.

## 2. Experimental

### 2.1. Reagents and toxin standards

Azaspiracids, AZA1, AZA2 and AZA3, were isolated from toxic mussels (*Mytilus edulis*) using procedures similar to those described previously [6,7]. Quantitative data on AZP toxins in this study are related to a standard AZA1 toxin sample that was kindly provided by Professors T. Yasumoto and M. Satake, Tohoku University, Sendai, Japan. HPLC-grade acetonitrile, water, chloroform (stabilised with amylene) and chloroform (stabilised with 1% ethanol) were purchased from Labscan (Dublin, Ireland). Trifluoroacetic acid (TFA) was obtained from Sigma–Aldrich (Dorset, UK).

### 2.2. Extraction of AZP toxins from shellfish

The extraction procedure has been detailed elsewhere [14] and in brief, homogenised mussel tissues (ca. 20 g, accurately weighed) were extracted with acetone (2×8 ml). The combined extracts were made up to 25 ml with acetone and an aliquot (2.5 ml) was evaporated using nitrogen (TurboVap, Zymark, MA, USA). Analytes were extracted with ethyl acetate (2×2 ml), which was evaporated under nitrogen, and the residue was taken up in the appropriate solvent prior to SPE.

### 2.3. Liquid chromatography–mass spectrometry (LC–MS)

The LC–MS system consisted of a Finnegan MAT LCQ *Classic* ion-trap mass spectrometer with Xcaliber software (Thermo-Finnigan, San Jose, CA, USA) and a Waters (Milford, MA, USA) 2690 Alliance HPLC system, which 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) that was maintained at 35 °C. Isocratic chromatography was carried out using acetonitrile–water (70:30) containing 0.05% TFA 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–multiple MS ($MS^n$) experiments were carried out using an LCQ mass spectrometer equipped with an ESI source and operated in positive polarity. The MS system was tuned using AZA1 standard solution (1 μg/ml) which was infused at 3 μl/min with monitoring of the $[M+H]^+$ ion at 842.5. 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). 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.

Multiple tandem MS produced collision-induced dissociation (CID) spectra and were obtained using the protonated molecule for each toxin which fragmented similarly giving major ions due to the sequential loss of water molecules. The optimised relative collision energies (RCEs) were 25% for $MS^2$, 33% for $MS^3$ experiments. AZA2 and AZA3 standards were not available in sufficient amounts to produce full calibration data but were used in spectral studies and to confirm toxin identity in shellfish.

### 2.4. SPE

SPE studies were carried out using extracts of two mussels tissue homogenates. One containing a high level of AZP toxins; AZA1 (2.6 μg/g); AZA2 (0.6 μg/g); AZA3 (0.5 μg/g) and the other contained a lower level of toxins; AZA1 (0.10 μg/g); AZA2 (0.02 μg/g); AZA3 (0.02 μg/g). The distribution studies of the AZP toxins in the load, wash and elution fractions were carried out using the 'high' level AZP shellfish extract. Detailed recovery studies were performed on two shellfish samples containing AZP toxins, designated 'high' and 'low' reflecting their relative concentrations.

Five $C_{18}$ SPE materials (all 3 ml) were used in this study as follows: (A) Discovery $DSC_{18}$ (Supelco, Bellefonte, PA, USA); (B) Supelclean ENVI-18 (Supelco); (C) IST Isolute $C_{18}$(EC) (International Sorbent Technology, Hengoed, UK); (D) Bakerbond $C_{18}$ Polarplus (J.T. Baker, Deventer, Netherlands); (E) Supelclean $LC_{18}$ (Supelco).

SPE conditioning: methanol–water (80:20, v/v; 6 ml); sample load: methanol–water (80:20, v/v; 2 ml); SPE wash methanol–water (80:20, v/v; 6 ml); SPE elution methanol (8 ml). Sixteen fractions (1 ml each) from the load, wash and elution SPE steps were collected for AZP toxin analysis. Recovery studies ($n=9$) were carried out separately using the same SPE conditions and fractions 9–16, eluted with methanol, were combined and the total AZP toxins determined.

Three Diol-SPE materials (all 3 ml) were studied as follows: (A) Supelclean LC-Diol (Supelco); (B) IST Isolute SPE Diol (International Sorbent Technology); (C) Sep-Pak Vac Diol (Waters). In addition to comparing the performance of these diol phases, two analytical grades of chloroform were compared. One grade of chloroform had 1.0% ethanol as stabiliser and the other was stabilised with amylene (50 ppm).

SPE conditioning: methanol (5 ml), chloroform (5 ml); sample load: chloroform (1 ml); SPE wash: chloroform (5 ml); SPE elution chloroform–methanol (50:50, v/v; 7 ml). Thirteen fractions (1 ml each) from the load, wash and elution SPE steps were collected for AZP toxin analysis. Recovery studies ($n=9$) were carried out using the same SPE conditions and fractions 7–13, were combined and the total toxins were determined.

## 3. Results and discussion

Sample preparation for the determination of phycotoxins in shellfish can be problematic due, in part, to an extensive variation in the toxin content. As with most marine intoxications, the concentration of AZP toxins in individual mussels from the same batch can vary greatly, up to 10-fold (unpublished data), and it is therefore important to use a representative sample [15,16]. Most analytical protocols

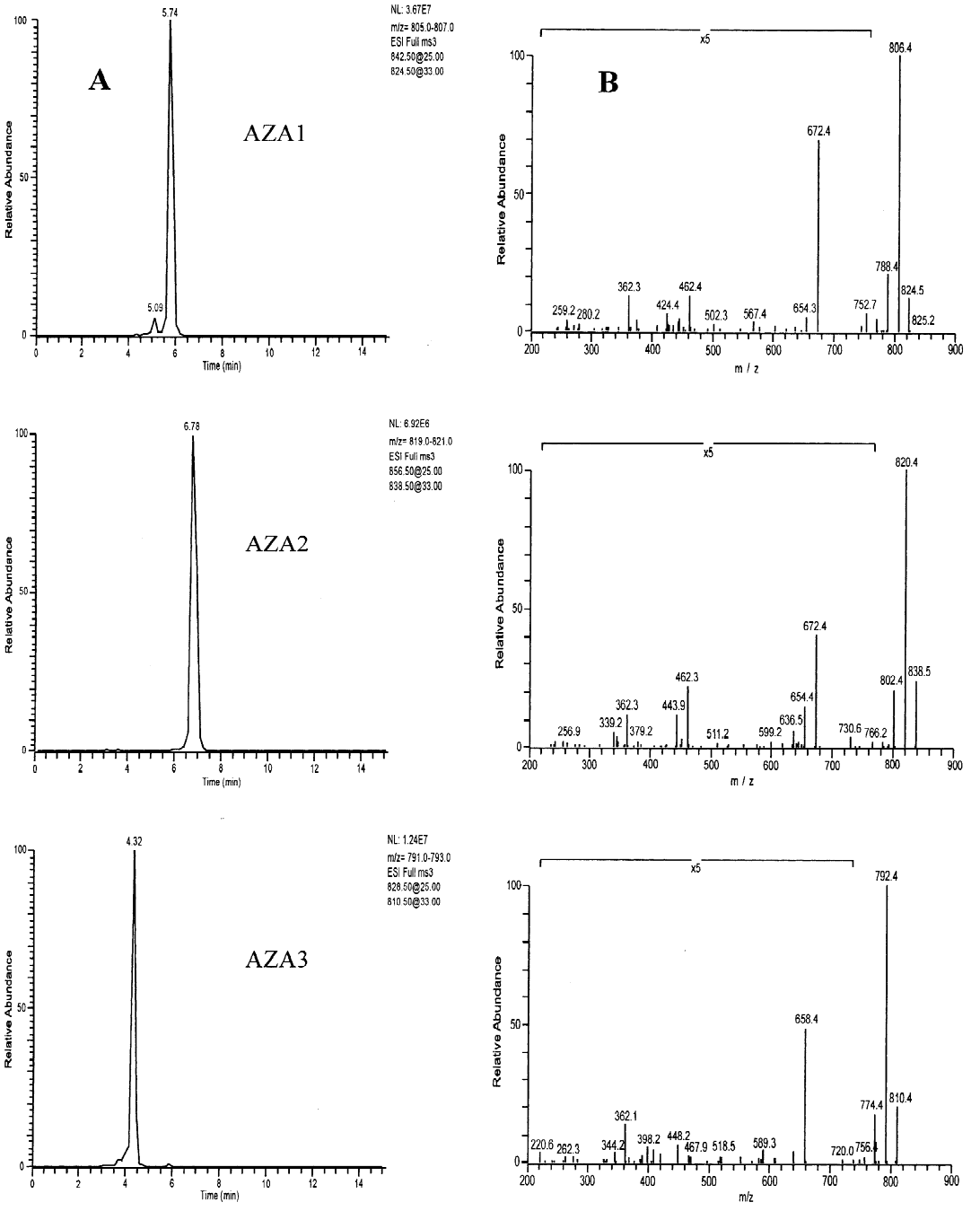


Fig. 2. (A) Chromatograms from the LC–$MS^3$ analysis of AZP toxins in mussel tissues. The targeted ions, $[M+H]^+$, $[M+H-H_2O]^+$, $[M+H-2H_2O]^+$ corresponding to the three predominant AZP toxins in shellfish: (a) 4.32 min (AZA3); (b) 5.47 min (AZA1); and (c) 6.78 min (AZA2). Scan range was 235–900 and the total concentration of AZA1–AZA3 in shellfish was 3.7 μg/g. Chromatographic conditions: Luna-2 $C_{18}$ column (5 μm, 150×2.0 mm) at 40 °C; mobile phase was acetonitrile–water (70:30) containing 0.05% TFA; flow-rate was 200 μl/min. (B) Mass spectra, corresponding to the chromatogram opposite Fig. 2A, AZA1 (5.47 min), AZA2 (6.78 min), AZA3 (4.32 min), respectively. The base peak in the $MS^3$ spectra results from the successive loss of two water molecules $[M+H-2H_2O]^+$. Fragmentation of the A-ring produces ions at *m*/*z* 672.4 (AZA1), *m*/*z* 672.4 (AZA2) and *m*/*z* 658.4 (AZA3).

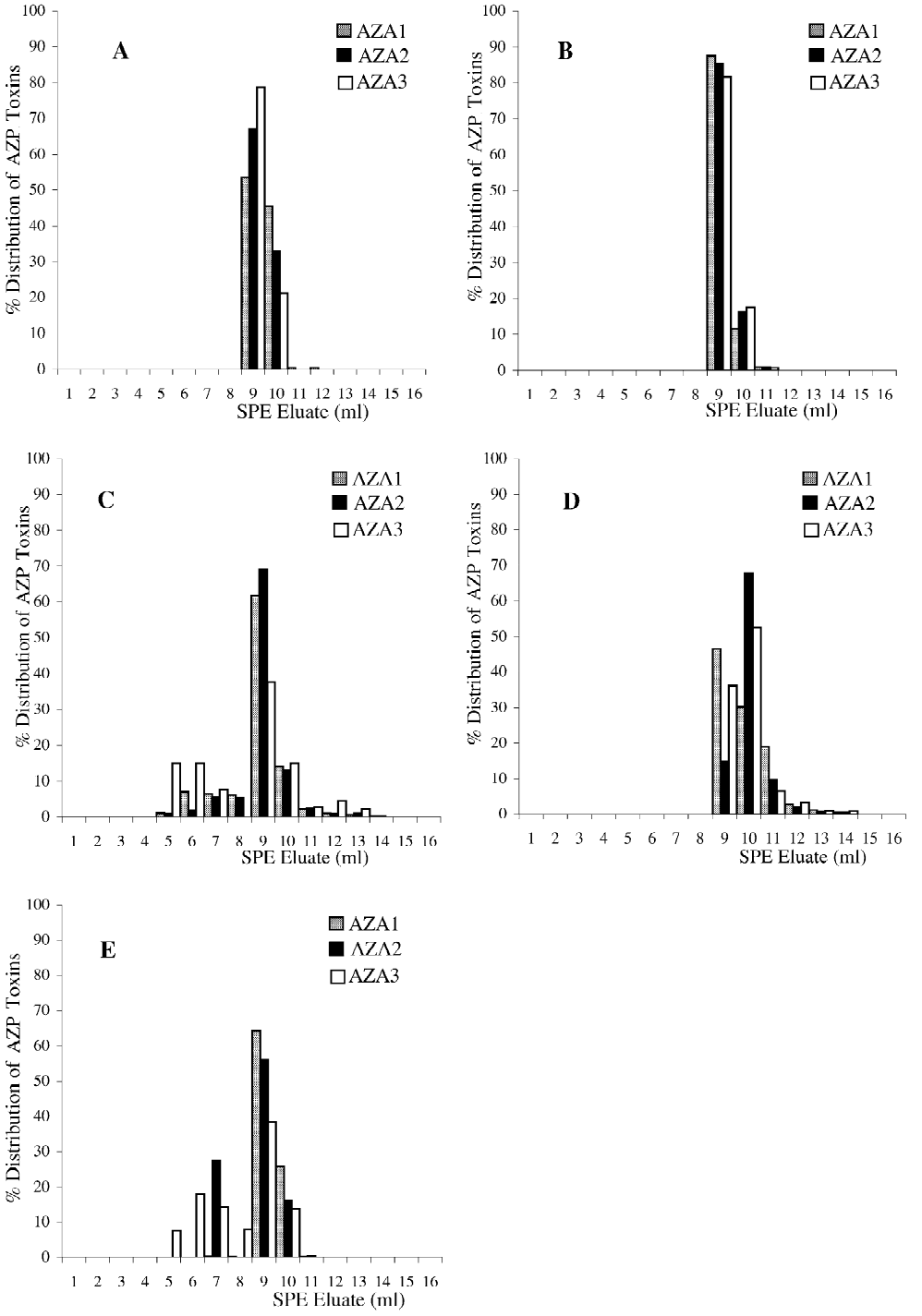


Fig. 3. The distribution of AZP toxins in the collected fractions (1 ml each), obtained using five SPE $C_{18}$ cartridge types. Fractions 1 and 2 (load), fractions 3–8 (wash) and fractions 9–16 (elution). The data show the relative % distribution of each toxin, AZA1–AZA3. A $C_{18}$ = Discovery DSC-18; B $C_{18}$ = Supelclean ENVI-18; C $C_{18}$ = IST Isolute $C_{18}$(EC); D $C_{18}$ = Bakerbond $C_{18}$ Polarplus; and E $C_{18}$ = Supelclean LC-18.

for the determination of polyether toxins in shellfish use only the digestive glands where these toxins are usually concentrated. However, AZP toxins can be distributed throughout the total tissues and therefore the entire shellfish tissues was used for toxin determination [10].

### 3.1. LC–$MS^3$ analysis of AZP toxins

AZA1, AZA2 and AZA3 were well resolved using isocratic reversed-phase LC, with acetonitrile–water (70:30) containing 0.05% TFA. However, since these toxins have different molecular masses, this separation is not essential for the successful implementation of an LC–$MS^3$ analytical protocol. Positive ESI of the standards, AZA1, AZA2 and AZA3, gave protonated molecular ions, $[M+H]^+$, at $m/z$ 842, 856 and 828, respectively. Fragmentation, with multiple losses of water molecules, is typical of polyether shellfish toxins, including OA analogues [17]. The selected precursor ions undergo CID in the ion-trap, following impact with helium gas, to produce ions that can be selectively trapped and fragmented to give multiple tandem MS spectral data. $MS^2$ and $MS^3$ experiments on AZP toxins were performed using optimised RCEs of 25 and 33% on the $[M+H]^+$ and the $[M+H-H_2O]^+$ ions, respectively. The RCE value is a percentage of the maximum collision energy achievable. In this context, the optimised RCE refers to the value required to produce a maximum intensity of targeted daughter ions whilst maintaining 2–5% of parent ions.

LC–$MS^3$ analysis, by selecting the parent ion and the product ions from the sequential loss of two water molecules, ($[M+H]^+$, $[M+H-2H_2O]^+$), gave good signal intensities and optimum detection limits. Although multiple tandem MS enhances selectivity, LC–$MS^3$ is more sensitive than LC–MS probably

Table 1
SPE recovery data for AZP toxins from extracts of mussel tissues

| | Toxin | 'High' | | | 'Low' | | |
|---|---|---|---|---|---|---|---|
| | | Range (%) | Mean (%) | RSD (%, $n=9$) | Range (%) | Mean (%) | RSD (%, $n=9$) |
| A $C_{18}$ | AZA1 | 94–99 | 98 | 1.6 | 88–96 | 92 | 3.0 |
| | AZA2 | 88–94 | 91 | 2.5 | 78–85 | 82 | 3.2 |
| | AZA3 | 90–97 | 95 | 2.9 | 68–73 | 71 | 2.6 |
| B $C_{18}$ | AZA1 | 94–99 | 97 | 1.9 | 78–85 | 82 | 3.1 |
| | AZA2 | 76–82 | 80 | 2.7 | 77–86 | 82 | 3.8 |
| | AZA3 | 92–97 | 96 | 2.2 | 78–84 | 81 | 2.3 |
| C $C_{18}$ | AZA1 | 88–94 | 93 | 3.1 | 68–75 | 72 | 4.2 |
| | AZA2 | 74–86 | 82 | 5.0 | 69–74 | 71 | 2.7 |
| | AZA3 | 75–83 | 81 | 3.9 | 67–73 | 70 | 3.5 |
| D $C_{18}$ | AZA1 | 22–64 | 43 | 37 | 14–43 | 28 | 40.0 |
| | AZA2 | 18–54 | 36 | 35 | 16–40 | 28 | 31.0 |
| | AZA3 | 15–49 | 26 | 51 | 11–44 | 27 | 43.0 |
| E $C_{18}$ | AZA1 | 76–83 | 80 | 3.3 | 82–91 | 87 | 3.5 |
| | AZA2 | 54–61 | 58 | 4.5 | 72–80 | 78 | 4.2 |
| | AZA3 | 68–83 | 78 | 7.2 | 79–85 | 82 | 2.7 |
| A Diol | AZA1 | 92–99 | 96 | 2.8 | 80–87 | 84 | 4.0 |
| | AZA2 | 89–97 | 94 | 3.3 | 83–90 | 87 | 3.3 |
| | AZA3 | 93–99 | 97 | 2.3 | 88–95 | 93 | 3.2 |
| B Diol | AZA1 | 76–85 | 82 | 4.7 | 75–81 | 78 | 2.9 |
| | AZA2 | 75–83 | 79 | 4.8 | 62–71 | 70 | 4.6 |
| | AZA3 | 79–84 | 82 | 2.1 | 77–83 | 80 | 2.5 |

A $C_{18}$ = Discovery DSC-18; B $C_{18}$ = Supelclean ENVI-18; C $C_{18}$ = IST Isolute $C_{18}$ (EC); D $C_{18}$ = Bakerbond $C_{18}$ Polarplus; E $C_{18}$ = Supelclean LC-18.
A Diol = Supelclean LC-Diol; B Diol = IST Isolute SPE Diol.
'High' tissue sample contained AZA1 (2.60 μg/g); AZA2 (0.60 μg/g); AZA3 (0.50 μg/g). 'Low' tissue sample contained (0.10 μg/g); AZA2 (0.02 μg/g); AZA3 (0.02 μg/g).

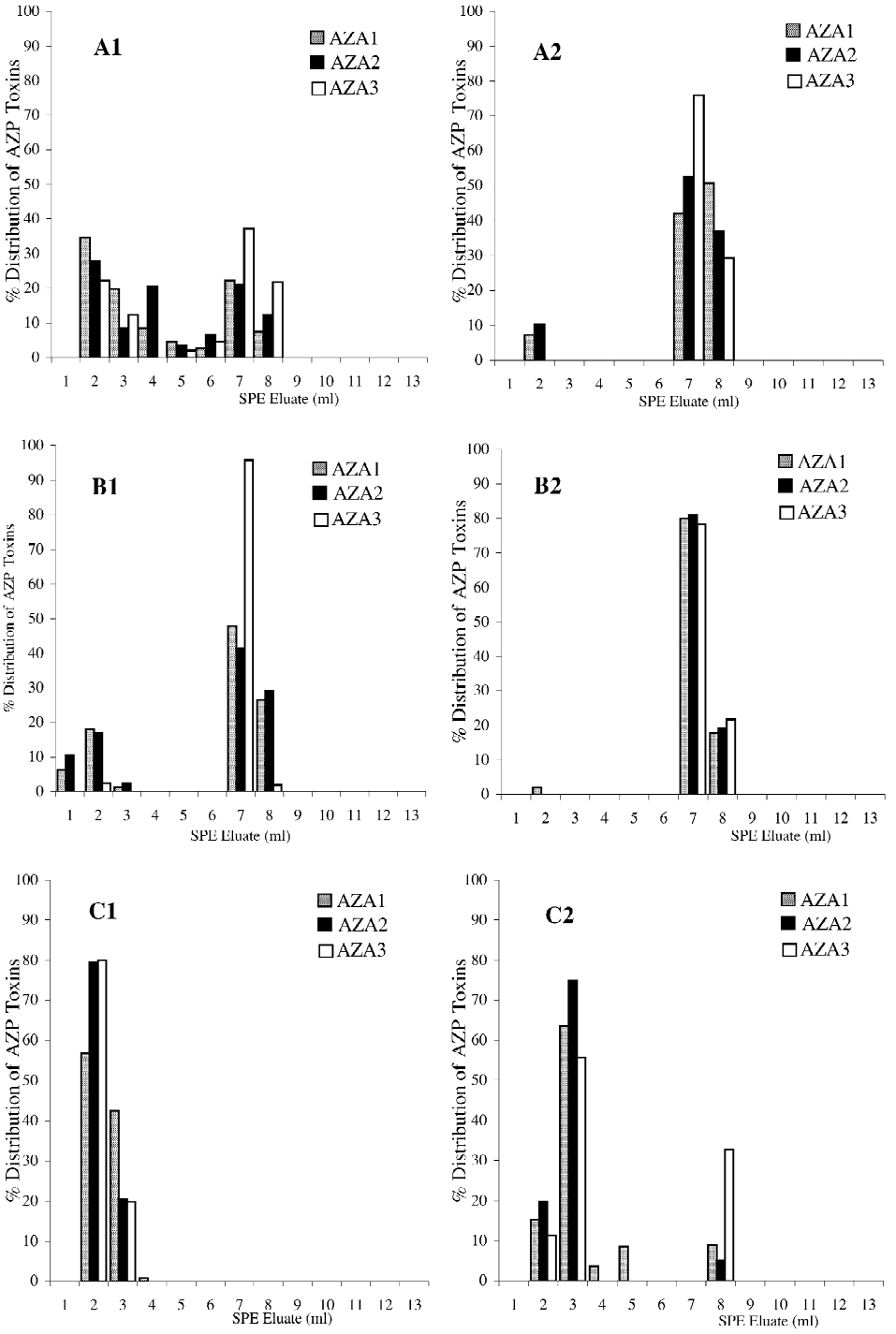


Fig. 4. The distribution of AZP toxins in the collected fractions (1 ml each), obtained using three SPE diol cartridge types. Fractions 1 (load), fractions 2–6 (wash) and fractions 7–13 (elution). The data show the relative % distribution of each toxin, AZA1–AZA3. A Diol = Supelclean LC-Diol; B Diol = IST Isolute SPE Diol; Sep-Pak Vac Diol. A1–C1 were obtained using chloroform containing 1% ethanol stabiliser and A2–C2 were obtained using chloroform without ethanol.

due to a reduced background signal [18]. Fig. 2A shows the typical chromatograms that are obtained. Since the three predominant azaspiracids, AZA3 (4.32 min), AZA1 (5.74 min) and AZA2 (6.78 min), have different molecular masses, and consequently different fragment ion masses, chromatographic resolution is not essential. Calibrations showed good linearity in the range 0.05–2.00 μg/g ($r^2=0.998$, $n=7$) with a detection limit (signal:noise=3) of 5 pg. Fig. 2B shows the mass spectra corresponding to the three toxins, AZA1–AZA3, that were obtained by sequential trapping and fragmentation of the parent ions, $[M+H]^+$, and the $[M+H-H_2O]^+$ ions.

### *3.2. SPE studies using reversed phases*

Based on the elution profiles, $C_{18}$ phases C and E performed poorly, with the loss of up to 30% of AZP toxins during the wash and load steps. AZA3 exhibited a lower sorption to $C_{18}$ phases than AZA1 and AZA2 and this is predictable since AZA3 elutes faster than AZA1 and AZA2 in LC (Fig. 2A). Based on the elution profiles (Fig. 3A and B), the A, B and D phases performed better as AZP toxins were not lost during the load or wash steps. However, the D $C_{18}$ phase required a larger volume to recover the AZP toxins (6 ml) whereas a lower volume was required for the other phases, A $C_{18}$ (2 ml) and B $C_{18}$ (3 ml). Although the D $C_{18}$ phase gave acceptable profile data (Fig. 3D), the reproducibility was poor as shown by the recovery study (Table 1). Two shellfish homogenates were used in these studies, one containing 3.7 μg/g total toxins ('high') and the other containing 0.14 μg/g total toxins ('low'). The A and B $C_{18}$ SPE cartridges gave acceptable reproducibility data at both 'high' and 'low' levels of AZP toxins. However, the B $C_{18}$ phase gave remarkably consistent recovery data for each of the individual toxins using the 'low' sample and is therefore preferable.

### *3.3. SPE studies using diol phases*

Initial studies using diol SPE phases were unsatisfactory despite a previous report of the efficacy of this phase for the cleanup of shellfish extracts prior to LC–MS analysis of AZP toxins [13]. Fig. 4 A1, B1 and C1, show that losses during the load and wash stages ranged from 46 to 100%. Since chloroform usually contains ca. 1% ethanol as a stabiliser, we repeated the study using chloroform containing 50 ppm amylene stabiliser, without ethanol. The remarkable changes in performance can be seen in Fig. 4, A2, B2 and C2, and these demonstrate that AZP toxins have a high affinity for ethanol. The dramatic effect of variations in the amount of ethanol stabiliser in chloroform on the reproducibility of silica SPE for derivatised shellfish toxins has previously been reported [19]. Also, B Diol SPE gave a more uniform elution of the three AZP toxins than A and 80% of AZP toxins were in fraction 7 which was the first 1 ml of the elution stage. Detailed recovery studies were carried out on the diol phases that showed good retention of AZP toxins during the load and wash steps. In the recovery studies using the A Diol phase, the mean recoveries for each of the AZP toxins were 94–97% with the 'high' tissue sample and 84–93% using the 'low' one.

Since LC–$MS^3$ is a highly specific analytical method for the determination of trace contaminants in complex matrices, the efficient SPE methods presented here for sample preparation should prove more useful in the development of alternative analytical methods for AZP toxins in shellfish.

## Acknowledgements

Funding from Enterprise Ireland (Strategic Research Programme) and the Higher Education Authority (Programme for Research in Third-Level Institutions-II) are gratefully acknowledged.

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