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Strategies for the elimination of matrix effects in the liquid chromatography tandem mass spectrometry analysis of the lipophilic toxins okadaic acid and azaspiracid-1 in molluscan shellfish

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ABSTRACT

Considerable efforts are being made worldwide to replace in vivo assays with instrumental methods of analysis for the monitoring of marine biotoxins in shellfish. Analysis of these compounds by the preferred technique of liquid chromatography tandem mass spectrometry (LC–MS/MS) is challenged by matrix effects associated with the shellfish tissues. In methods validation, assessment of matrix interferences is imperative to ensure the validity and accuracy of results being produced. Matrix interferences for the analysis of okadaic acid (OA) and azaspiracid 1 (AZA1) were assessed using acidic methods on electrospray triple stage quadrupole (TSQ) and hybrid quadrupole time of flight (QToF) instruments by the use of matrix matched standards for different tissue types. Using an acidic method no matrix interference and suppression was observed on the TSQ for OA and AZA1 respectively, whilst the opposite was observed on the QToF; matrix enhancement for OA and no matrix interference for AZA1. The suppression of AZAs on the TSQ was found to be due to interfering compounds being carried over from previous injections. The degree of suppression is very much dependant on the tissue type ranging from 15 to 70%. Several strategies were evaluated to eliminate these interferences, including the partitioning of the extract with hexane, optimisation of the chromatographic method and the use of on-line SPE. Hexane clean up did not have any impact on matrix effects. The use of an alkaline method and a modified acidic method eliminated matrix suppression for AZA1 on the TSQ instrument while an on-line SPE method proved to be effective for matrix enhancement of OA on the QToF.

1. Introduction

Diarrhetic shellfish poisoning (DSP) is a human illness caused by the consumption of shellfish contaminated with the lipophilic marine biotoxins okadaic acid (OA) and dinophysistoxins (DTX). DSP toxins are produced by marine dinoflagellate species of the genus *Dinophysis* and *Prorocentrum*, and are accumulated in filter-feeding molluscan shellfish. The DSP syndrome was first reported in Japan in 1978, and the occurrence of DSP toxins is now a worldwide issue with frequent *Dinophysis* outbreaks documented in Europe, Asia, South and North America over the past 20 years [1–4]. DSP symptoms include nausea, vomiting, gastrointestinal disturbances, and stomach pain [5].

In 1995, the presence in shellfish of another lipophilic marine toxin, azaspiracid (AZA), was responsible for diarrhetic illnesses in several individuals who consumed shellfish harvested in Ireland [6]. The AZA group now includes more than 24 analogs that are either produced by phytoplankton, products of biotransformation in shellfish or by-products of toxin storage [7]. However, only AZA1, -2 and -3 are regulated by the European Union [8]. AZAs have been found in shellfish from several European countries, Morocco, Eastern Canada, Japan and more recently in shellfish from Chile [9–13]. The symptoms of azaspiracid shellfish poisoning (AZP) are similar to that of DSP, and include nausea, vomiting, diarrhea, and stomach cramps.

The EU has set maximum levels of AZP and DSP toxins in shellfish destined for human consumption. These are 160 μg OA equiv./kg from the OA group (sum of OA and DTX) and including pectenotoxin (PTX) and 160 μg AZA equiv./kg from the AZA group (sum of AZA1, -2 and -3) [14]. Currently the mouse (or rat) bioassay (MBA) is the EU reference method for the detection of OA group and AZA toxins in shellfish. A recent study has shown that the detection limit of the MBA is adequate for the current regulatory limit of AZAs [15], however, sensitivity is an issue at the lower levels [16,17]. Furthermore, additional concerns relating to accuracy and ethics are prompting substantial efforts to replace it with instrumental methods.

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It is anticipated that the MBA will be replaced by LC–MS/MS as the reference method for the detection of marine biotoxins in shellfish by the year 2011 [18]. LC–MS/MS is considered the technique of choice as it offers improved sensitivity, selectivity and accuracy as well as being faster and automated. However, quantification using LC–MS/MS in biological matrices is often challenging because of matrix effects which alter the accuracy and the precision of the method. Matrix effects are believed to be caused by endogeneous compounds co-eluting with the analyte and competing for ionisation in the electrospray (ESI) source [19,20].

A number of different approaches have been taken to eliminate or to correct for matrix effects in LC–MS/MS analyses including sample clean up, standard addition, matrix matched standards, internal standards or changes in chromatographic conditions such as the pH of the mobile phase or the nature of stationary phase.

Sample clean-up can be performed using liquid–liquid extraction (LLE) or solid phase extraction (SPE) which is available with a variety of stationary phases (normal and reverse phase, ion exchange and immunoaffinity material with antibodies specific to the analyte). SPE also has the benefit of pre-concentrating samples which can be useful when dealing with low levels of toxins. Two recent reports have shown this technique to be effective in raising sensitivity as well as eliminating sample impurities [21,22], however, its effectiveness in overcoming matrix effects was not clearly demonstrated in these studies. Dilution of extracts has also been reported to reduce matrix interferences [15,23], yet such an approach compromises the sensitivity of the method.

In addition to sample clean up, various approaches have been used to correct for matrix effects. Quantification using matrix matched standards entails the production of a calibration curve in solutions with the exact same composition as the samples by extracting blank material or by reconstructing the matrix artificially and spiking the analyte at different concentrations. Although this approach is perfectly acceptable when the sample matrix is identical in all samples being analysed its application for the monitoring of marine toxins in shellfish is limited. Indeed, the production of matrix matched standards in all shellfish species (up to 10 different varieties) that are typically encountered in monitoring laboratories is impractical. Furthermore, the production of a calibration curve in extracts of a given species, does not imply that the matrix composition of another extract of the same species but from a different location and/or harvested at a different time of the year will be identical since environmental factors and food source will influence the composition of the shellfish tissues e.g. lipid content.

The standard addition method eliminates the need for the availability of a blank matrix and only requires the analyte to be available as a calibration solution of sufficient concentration. This method has been used to deal with matrix suppression in the analysis of scallops for diarrhetic shellfish toxins [24]. Although the method is very powerful and widely accepted, its use in monitoring laboratories remains limited for a number of reasons, primarily due to increased sample preparation and analysis time.

The use of internal standards is a very efficient approach to ensure that satisfactory accuracy is obtained through the different steps of the analytical method. Unfortunately, the total or partial synthesis of the isotopically labelled compound is required and currently no such compounds are available for the DSP and AZA toxins to our knowledge.

Elimination or reduction of matrix effects to an acceptable level can also be achieved through modifications of the chromatographic conditions to change the selectivity towards the interfering compounds and/or the analyte.

We examined matrix effects associated with shellfish tissues on two LC–MS/MS instruments; a QToF and a TSQ, using ESI sources and identical LC conditions. Matrix interferences were assessed using matrix matched standards for six different tissue types; *M. edulis*, *C. gigas*, *O. edulis*, *E. siliqua*, *P. maximus* meat, *P. maximus* gonad and where interferences are observed we describe efforts made to overcome them. The performances of the methods employed were also evaluated in terms of sensitivity, accuracy and precision.

2. Materials and methods

2.1. Solvents and reagents

Acetonitrile, methanol and hexane were purchased as pestican grade solvents from Labscan (Dublin, Ireland). Formic acid, ammonium formate and ammonium hydroxide were obtained from Sigma–Aldrich (Steinheim, Germany). Water was obtained from a reverse-osmosis purification system (Barnstead, Dublin, Ireland). OA and AZA1 certified reference materials (CRM) were obtained from the NRC (Halifax, Canada).

2.2. LC–MS/MS

Two LC–MS/MS systems were used; a Micromass triple stage quadrupole (TSQ) Ultima coupled to a Waters 2695 HPLC and a Micromass time-of-flight (QToF) Ultima coupled to a Waters 2795 HPLC. Both systems were equipped with a z spray ESI source. The TSQ was operated in multiple reaction monitoring (MRM) mode and the following transitions were monitored: OA, m/z 803.5 > 255.5 and 803.5 > 803.5 in negative ionisation mode; AZA1, m/z 842.5 > 654.4 and 842.5 > 672.4, AZA2 856.5 > 654.4 and 856.5 > 672.4, AZA3 828.5 > 640.4 and 828.5 > 658.4 in positive ionisation mode. The cone voltages were set at 70 V and 60 V in negative and positive modes respectively and the collision voltage was set at 40 V in both modes. Cone and desolvation gas flows were set at 100 and 800 l/h respectively while the source and desolvation temperatures were set at 150 °C and 350 °C respectively.

The QToF was operated in fragment ion scan (FIS) mode monitoring for the same precursor ions as those reported for the TSQ. The cone voltages were set at 80 V and 40 V in negative and positive modes respectively. The collision energy was set at 30 V in negative mode and 50 V in positive mode. Cone and desolvation gas flows were set at 100 and 750 l/h respectively while the source and desolvation temperatures were set at 140 °C and 350 °C respectively. Quantification was performed by summing the ions of m/z 824.5, 672.5, 654.5 and 362.5 for AZA1 (and the equivalent fragment ions for AZA2 and -3) and the ions of m/z 803.5 and 255.1 for OA.

2.2.1. Acidic gradient method

A gradient elution method was set with an acidic binary mobile phase, with phase A (100% aqueous) and phase B (95% aqueous acetonitrile), each containing 2 mM ammonium formate and 50 mM formic acid following the method of Quilliam et al. [25]. The gradient elution started with 30% B, increased to 90% B over 8 min, held for 2.5 min, decreased to 30% B in 0.5 min and held for 4 min to equilibrate the system before the next injection. The chromatographic separation was achieved using a Hypersil BDS C8 column; 50 mm × 2.1 mm, 3 μm with a guard column of the same stationary phase 10 mm × 2.1 mm, 3 μm (Thermo Scientific, Runcorn, UK). The flow rate was set at 0.25 ml/min and the injection volume at 5 μl. The column and sample temperatures were set at 25 °C and 6 °C respectively.

We assessed matrix effects for several shellfish tissues over a number of months. The spike samples and *M. edulis* matrix matched standards were ran in triplicate against methanol standards (seven levels) using in-house validated and accredited methods of analysis for the monitoring of lipophilic toxins.

A matrix-matched standard curve was prepared with *M. edulis* in order to compare response factors over the range of concentrations

representative of naturally contaminated shellfish. The accuracy was calculated as a percentage of difference between the slopes obtained in methanol and in the *M. edulis* extracts. The accuracies reported for all other shellfish species were calculated from spiked samples at a single concentration. Within each batch all samples were analyzed by triplicate injection.

2.2.2. Acidic gradient method with a 100% B flush

A modified gradient method with acidic mobile phase was also evaluated. The gradient started with 30% B at 0.25 ml/min, increased to 90% B over 8 min, held for 5 min, increased to 100% B at 0.4 ml/min, held for 5 min and set back to 30% B at 0.25 ml/min which was held for 4 min to equilibrate the system.

2.2.3. Alkaline method

The alkaline method followed that of Gerssen et al. [26]; a binary mobile phase was used, with phase A (100% aqueous) and phase B (90% aqueous acetonitrile), each containing 6.7 mM ammonium hydroxide. Separation was achieved using a Waters X bridge, C18 column (150 mm × 3 mm, 5 μm). The flow rate was set at 0.25 ml/min and the injection volume was set at 5 μl. The column and sample temperatures were set at 25 °C and 6 °C respectively. A gradient elution was employed, starting with 10% B which was held for 1 min and increased linearly to 90% over 9 min. The mobile phase was held at 90% B for 3 min and returned to 10% B in 2 min. The system was then allowed to equilibrate for 4 min.

2.2.4. On-line SPE method

For the on-line SPE method a binary mobile phase was used, with phase A (100% aqueous) and phase B (95% aqueous acetonitrile), each containing 2 mM ammonium formate and 50 mM formic acid. The loading column was an Oasis HLB, 5 μm, 2.1 mm × 20 mm column and HPLC separation was achieved using a Hypersil BDS C8 column; 50 mm × 2.1 mm, 3 μm; guard column, 10 mm × 2.1 mm, 3 μm (Thermo Scientific, Runcorn, UK). The flow rate was set at 0.2 ml/min and the injection volume was 10 μl. The column and sample temperatures were set at 25 °C and 6 °C respectively. The sample was initially injected onto the loading column with 20% B for 2 min after which time the switch valve directed the flow onto the analytical column and the flow was reduced to 0.02 ml/min. After 3 s the flow was increased to 0.075 ml/min and the % B was increased from 20% to 30% over 27 s. The % B was then increased further to 100% over 10 min, held for 18 min, then decreased to 30% B over 0.5 min and held for 9 min. The system was then equilibrated for 3 min at 20% B and a flow rate of 0.2 ml/min. The switching valve was set to direct the flow to waste after 23 min.

2.3. Partitioning of shellfish extract with hexane

A laboratory reference material (LRM) prepared with *M. edulis* tissue and contaminated with both OA group and AZA toxins was extracted using the extraction described below (preparation of matrix matched standards). A set volume (5 ml) of the filtered extract was partitioned with 15 ml of hexane. The sample was shaken vigorously for 1 min and the layers were allowed to settle. The LRM extract (bottom layer) was then collected in a centrifuge tube and an aliquot transferred into a HPLC vial for analysis.

A set volume (1 ml) of the hexane layer was then pipetted into HPLC vials and dried down under nitrogen. Dried residues were re-solubilised with 200 μl of methanol with vortex mixing for 30 s. The sample was transferred into an insert vial for analysis. Three methanol standards were run directly after three injections of the non-partitioned LRM extract in addition to the partitioned LRM extract, followed by a four point calibration curve (all performed in triplicate).

2.4. Preparation of matrix matched standards

For each tissue type, uncontaminated raw samples tested as part of the routine monitoring programme in Ireland were selected from different harvesting dates and sites (around the coasts of Ireland). The extraction procedure described in this study has been used for several years in the shellfish toxins monitoring program in Ireland [27]. The shellfish were shucked, homogenised and aliquoted for extraction where 2 g of tissue was extracted by vortexing for 1 min with 9 ml of methanol, centrifuged at 5000 rpm for 5 min and the supernatant decanted into a 20 ml volumetric flask. The remaining pellet was further extracted using an Ultra Turrax for 1 min with an additional 9 ml of methanol, centrifuged at 5000 rpm for 5 min and the supernatant decanted into the same 20 ml volumetric flask which was then brought to volume with methanol. The standards were prepared in 25 ml volumetric flasks containing 20 ml of filtered (Whatmann, 0.2 μm, cellulose acetate filter) tissue extract. For the *M. edulis* matrix matched standards increasing volumes of standard stock solution were added to the flasks and the volume was brought to the mark with methanol with toxin concentrations ranging from 2.5 to 280 ng/ml for OA and 0.8 to 92 ng/ml for AZA1.

Spiked tissue samples were prepared for the following tissues: *C. gigas*, *O. edulis*, *E. siliqua*, *P. maximus* meat and *P. maximus* gonad. For the spiked tissue samples 1 ml of stock standard solution was added to the flasks and the volume brought to the mark with methanol such that the final concentration was 10 ng/ml and 6 ng/ml for OA and AZA1 (equivalent to 125 μg/kg and 75 μg/kg in tissue) respectively.

For all the matrix matched standards a sample to solvent ratio (SSR) of 12.5 was obtained which reflects the routine monitoring extraction method.

2.5. Statistical analysis

Statistical calculations were carried out using Sigmastat 3.0. The significance test used to compare species and methods was the two-way analysis of variance Holm–Sidak test. Alpha was set at 0.05 (95% confidence) for all experiments.

3. Results and discussion

3.1. Assessment of matrix effects using the acidic gradient method

The average concentrations and standard deviations shown in Table 1 were calculated from five batches acquired over several months. The accuracy of AZA1 measurements on the TSQ in the different species of shellfish ranged from 64.2 to 83.1%. Signal suppression was consistently observed and was significantly different between the shellfish species ($p = 0.009$). When the same method was performed on the QToF the accuracy ranged from 97.1 to 104.6% without significant differences between species ($p = 0.467$).

The accuracy observed for OA using the acidic method also greatly varied between the two instruments (Table 1). Acceptable accuracies were achieved on the TSQ which ranged from 94.3 to 110.9%. The two-way ANOVA test revealed that the accuracy was statistically different between the shellfish species ($p < 0.001$). The pairwise multiple comparison procedure results demonstrated that the accuracy obtained for OA in *O. edulis* (110.9%) and for *M. edulis* (108.0%) were not significantly different ($p = 0.343$) but were significantly different when compared to the other shellfish species (p values ranging from <0.001 to 0.041). The accuracy obtained for OA analysis on the QToF with the acidic method was affected by signal enhancement and ranged from 114.6 to 130.9% with a significant difference between the shellfish species ($p = 0.008$).

Table 1
Accuracy and precision data (expressed as percentages) obtained on QToF and TSQ with the acidic method (average ± SD; *n*, no. of injections, *p*, no. of concentration points).

Analyte	Species	Acidic			
		TSQ		QToF	
AZA1	*M. edulis* (*p* = 7)	82.6 (*n* = 18)	±7.8	102.7 (*n* = 15)	±11.3
	C. gigas (*p* = 1)	83.1 (*n* = 13)	±4.5	104.6 (*n* = 21)	±7.8
	O. edulis (*p* = 1)	69.8 (*n* = 13)	±6.8	101.2 (*n* = 18)	±3.6
	E. siliqua (*p* = 1)	73.5 (*n* = 12)	±7.3	101.1 (*n* = 21)	±5.4
	P. max meat (*p* = 1)	79.3 (*n* = 13)	±13.6	103.3 (*n* = 21)	±5.5
	P. max gonad (*p* = 1)	64.2 (*n* = 13)	±3.6	97.1 (*n* = 21)	±3.1
OA	*M. edulis* (*p* = 7)	108.0 (*n* = 18)	±8.4	130.9 (*n* = 18)	±7.7
	C. gigas (*p* = 1)	102.4 (*n* = 13)	±3.2	114.6 (*n* = 18)	±16.4
	O. edulis (*p* = 1)	110.9 (*n* = 13)	±8.3	130.5 (*n* = 18)	±18.1
	E. siliqua (*p* = 1)	94.3 (*n* = 12)	±6.7	119.3 (*n* = 18)	±12.7
	P. max meat (*p* = 1)	98.3 (*n* = 13)	±3.5	119.7 (*n* = 15)	±23.3
	P. max gonad (*p* = 1)	101.3 (*n* = 13)	±5.1	125.9 (*n* = 18)	±11.0

Comparison of the results between instruments show that the apparent recoveries observed on the QToF were always higher than on the TSQ regardless of the species and the method used.

During analysis of AZA1 on the TSQ it was noted that the injection of a standard after the injection of a number of tissue extracts led to a lower response than when injected after a calibration curve. The degree of suppression was dependant on the type of tissue extract. This phenomenon is illustrated in Fig. 1 which shows the response of three consecutive injections of an AZA1 standard (104 ng/ml) after three injections of three shellfish extracts prepared from five different species. A six point calibration curve was systematically run after the three injections of the AZA1 standard and used to calculate the concentrations reported in Fig. 1. Depending on the tissue type the degree of suppression ranged from 15 to 70%. In this instance *P. maximus* gonad tissue appeared to be the worst offender while the clams (*T. philippinarium*) had the least effect. Injections of the AZA1 standard after the oyster, mussel and scallop extracts have shown that the first injections are equally affected by signal suppression while the third injection led to a significantly higher response. These results suggest that either later eluting compounds, or compounds lingering in the source are responsible for the signal suppression observed. This phenomenon is not observed for the analysis of OA on the QToF.

It was also noted that the results for the suppression obtained for the shellfish extracts in Fig. 1 were dissimilar to those obtained in Table 1. This may be due to the fact that although some of the extracts used in the two separate experiments were from the same species, they were harvested at different locations and times. This would suggest that the use of matrix matched standards from extracts other than the sample, can lead to erroneous results.

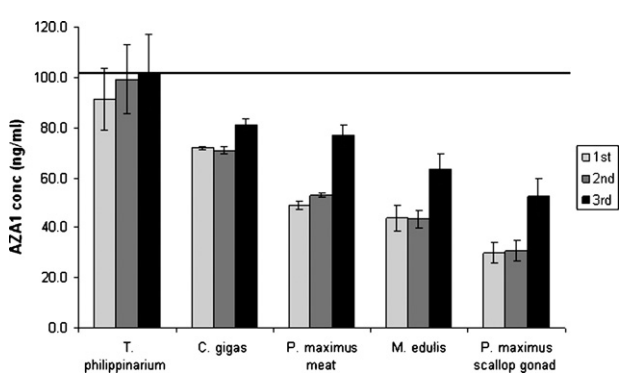

Fig. 1. Concentration obtained for three consecutive injections of a standard of AZA1 (104 ng/ml shown as the bold line) on the TSQ using gradient elution following three injections of various shellfish tissue extracts. The error bars show the standard deviations obtained from the mean (*n* = 3).

The within-day precision obtained with the acidic method for OA ranged from 1 to 10% on both instruments while the between-day precision over at least 5 days was 8% on both the QToF and the TSQ (Table 2). The analysis of AZA1 using the acidic method on the QToF demonstrated excellent precision as the within-day precision ranged from 2 to 5% and a between-day precision of 11% (Table 2). The results obtained for AZA1 with the acidic method on the TSQ were not as good with within-day precision ranging from 3 to 16%. The high variation on day five was due to a lower response of the first set of solutions that was injected compared to the second and the third replicate set (Table 2). A between-day precision of 8% was observed over 5 days.

3.2. Methods to address matrix effects

3.2.1. Partitioning of extract with hexane

The LRM was extracted following the same procedure used for the other shellfish as described in Section 2.

As part of our experiment we investigated the recoveries of OA and AZA1 (analysis of OA on TSQ and AZA1 on QToF) in the methanolic (and hexane) fraction after the hexane partitioning (data not shown). The recoveries were satisfactory for both compounds (>95%).

Hexane did not appear to have any effect on matrix suppression for the AZAs on the TSQ with no significant differences being observed between the partitioned (hexane) LRM and the crude LRM (Fig. 2). The suppression is still observed for the subsequent LRM and standard injections for both partitioned and non-partitioned samples and reflects what was observed for the different tissue types (see Fig. 1).

Table 2
Within and between days precision obtained with the acidic method calculated on the percentage of difference in response factor between a set of spiked solutions of *M. edulis* extracts and methanol. A set of seven solutions equivalent to 0.063–3.5 mg/kg for OA and 0.010–1.150 mg/kg for AZA1 was injected in triplicate on each day.

Days	*n* = 3	OA QToF	OA TSQ	AZA QToF	AZA1 TSQ
1	Average	135.6	100.8	105.7	81.5
	Stdev	5.3	8.9	3.2	5.5
2	Average	132.0	108.1	86.0	82.0
	Stdev	7.2	4.1	2.8	7.8
3	Average	137.8	113.1	96.8	85.8
	Stdev	4.5	4.4	4.1	3.3
4	Average	129.5	100.6	108.5	86.3
	Stdev	9.9	7.4	4.9	4.4
5	Average	120.2	117.3	116.6	77.6
	Stdev	3.2	0.8	3.9	15.1
6	Average	130.5	–	–	–
	Stdev	3.9	–	–	–
Average		130.9	108.0	102.7	82.6
Stdev		7.7	8.4	11.3	7.8

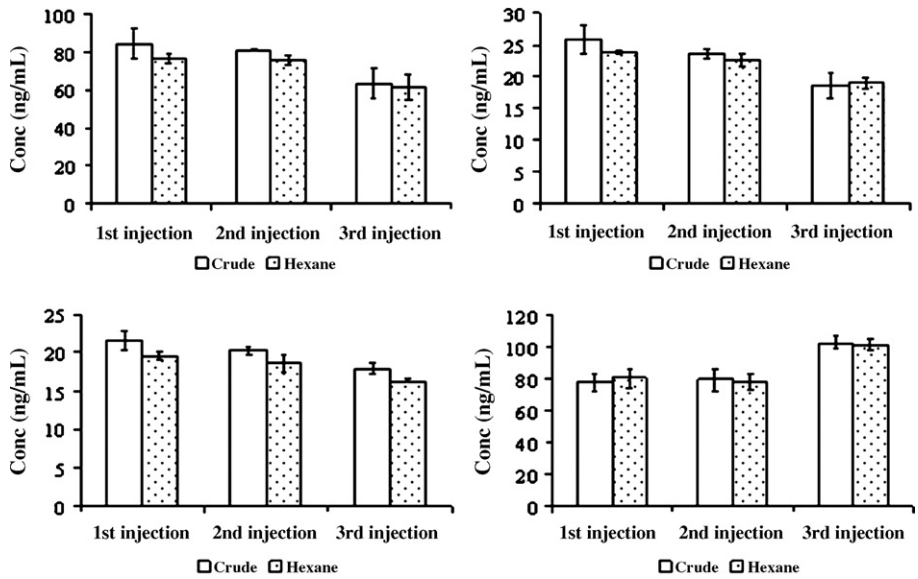

Fig. 2. Average concentrations of AZAs ($n = 3$) obtained by injection of three successive LRM extracts and three successive LRM extracts after hexane partitioning on the TSQ. Each series of three injections were separated by the injection of three successive standard solutions. (A) Concentration of AZA1 in partitioned and non-partitioned LRM. (B) Concentration of AZA2 in partitioned and non-partitioned LRM. (C) Concentration of AZA3 in partitioned and non-partitioned LRM. (D) Concentrations of AZA1 standards (104 ng/mL) after the injection of three LRM and three partitioned LRM.

Furthermore, the signal suppression effect observed in AZA1 standards after the injection of shellfish extracts presented in Fig. 1 was also examined. The results from Fig. 2D show that the two injections of a methanolic standard of AZA1 (104 ng/ml) that followed three injections of the LRM were affected by signal suppression as the average concentrations were 78.0 ± 5.6 and 79.4 ± 7.1 ng/ml for the first and second injections respectively. It is only on the third injection of the standard that the concentration measured (102.7 ± 4.1 ng/ml) returned within the expected theoretical concentration.

The effect of hexane partitioning on the signal enhancement effect observed for OA on the QToF instrument was also evaluated. Similarly to the above results, the hexane partitioning did not eliminate the matrix effects observed (data not shown).

These findings are in agreement with the results reported by Ito and Tsukada [24]. In this study the partitioning of scallop extracts with hexane and chloroform was evaluated for the reduction of signal suppression observed by LC–MS when the analysis of OA, DTX1, yessotoxin and pectenotoxin-6 was attempted. This clean-up procedure had no effect on the matrix effects observed. The LC–MS method from McNabb et al. also included a hexane partitioning step prior to injection but there is no information regarding the potential benefits of this clean-up step on matrix effects [28]. Although the partitioning step does not eliminate matrix effects, its application enables a higher degree of cleanliness in the source and in the system without detrimental effect on the accuracy.

3.2.2. Alkaline method

Changing the selectivity of the method may help to overcome matrix interferences. The use of an alkaline method for the separation of lipophilic toxins was reported to increase the sensitivity for the OA group of toxins and enable better separation of the DSP (including PTX2) and AZA group of toxins. This separation allows analysis of both groups of toxins in the one run without having to alternate the mass spectrometer polarity [26]. An additional study found that SPE on polymeric sorbents combined with an alkaline method can significantly reduce matrix interferences for both OA and AZA1 [22].

The alkaline method was run on both the QToF and TSQ instruments without any sample pre-treatment to determine any impact on matrix interferences.

To assess the matrix effects methanol standards were run with matrix matched standards in triplicate and the slopes compared (Table 3).

Table 3
Accuracy and precision data (expressed as percentages) obtained on QToF and TSQ with alkaline method (average ± SD; *n*, no. of injections, *p*, no. of concentration points).

Analyte	Species	Alkaline			
		TSQ		QToF	
AZA1	*M. edulis* ($p = 7$)	103.2 ($n = 12$)	±16.6	135.5 ($n = 12$)	±9.2
	C. gigas ($p = 1$)	108.1 ($n = 9$)	±9.5	118.7 ($n = 12$)	±13.2
	O. edulis ($p = 1$)	101.1 ($n = 9$)	±3.2	131.3 ($n = 12$)	±13.0
	E. siliqua ($p = 1$)	90.9 ($n = 9$)	±4.5	107.7 ($n = 12$)	±11.2
	P. max meat ($p = 1$)	102.1 ($n = 9$)	±4.3	107.9 ($n = 12$)	±7.3
	P. max gonad ($p = 1$)	97.9 ($n = 9$)	±2.9	125.7 ($n = 12$)	±20.6
OA	*M. edulis* ($p = 7$)	103.9 ($n = 12$)	±8.3	122.8 ($n = 15$)	±9.5
	C. gigas ($p = 1$)	106.2 ($n = 9$)	±3.6	123.4 ($n = 12$)	±13.2
	O. edulis ($p = 1$)	97.2 ($n = 9$)	±4.8	127.4 ($n = 12$)	±7.2
	E. siliqua ($p = 1$)	99.5 ($n = 9$)	±3.2	126.0 ($n = 12$)	±15.9
	P. max meat ($p = 1$)	101.6 ($n = 9$)	±8.0	124.3 ($n = 12$)	±17.8
	P. max gonad ($p = 1$)	99.2 ($n = 9$)	±6.4	126.7 ($n = 12$)	±13.5

Table 4
Within and between days precision obtained with the alkaline method calculated on the percentage of difference in response factor between a set of spiked solutions of *M. edulis* extracts and methanol. A set of seven solutions equivalent to 0.063–3.5 mg/kg for OA and 0.010–1.150 mg/kg for AZA1 was injected in triplicate on each day.

Days	Replicates	OA QToF	OA TSQ	AZA QToF	AZA1 TSQ
1	Average	130.6	109.8	141.1	120.7
	Stdev	9.3	4.9	9.0	5.8
2	Average	114.5	111.8	134.9	114.2
	Stdev	4.9	3.2	13.7	9.1
3	Average	127.8	93.1	134.6	80.5
	Stdev	11.1	2.9	2.8	8.3
4	Average	115.2	107.6	131.6	95.5
	Stdev	7.5	0.4	10.7	9.8
5	Average	125.7	97.1	–	105.2
	Stdev	4.2	6.6	–	3.6
Average		122.8	103.9	135.5	103.2
Stdev		9.5	8.3	9.2	16.6

Excellent results were obtained when the analyses were performed on the TSQ using the alkaline method with accuracies of 90.9–108.1% for AZA1 and 97.2–104.4% for OA (Table 3). There was no statistically significant difference between the species ($p = 0.083$ and 0.278 for AZA1 and OA respectively). Signal enhancement was systematically observed for both OA and AZA1 when the QToF was used with the alkaline method. For AZA1 the accuracy ranged from 107.7 to 135.5% with a significant difference observed between species ($p < 0.01$) while the accuracy for OA ranged from 122.8 to 127.4% without significant difference between species ($p = 0.928$).

By using the alkaline method the AZA1 suppression effect on the TSQ was overcome without any sample pre-treatment; analysis of three injections of a *P. maximus* gonad extract followed by three standard injections yielded 98 ± 1.1% recovery for the AZA1 (and OA) in the standard compared with 38 ± 12% recovery for AZA1 using the acidic method.

The precision of OA measurements using the alkaline method ranged from 0.4 to 11% on both instruments (Table 4). Between-day precision was 9.5 and 8.3% on the QToF and the TSQ respectively. The precision obtained for AZA1 using the alkaline method was also acceptable with within-day precisions ranging from 2 to 14% on both instruments and between-day precisions of 9.2 and 16.6% on the QToF and TSQ respectively.

The accuracies for OA and AZA1 using the acidic and the alkaline methods were reported in extracts of mussels (*M. edulis*), scallops (*P. maximus*) and oysters (*C. gigas*) [22]. The crude extracts spiked with OA (equivalent to 160 μg/kg) using a SSR of 10 showed that, with the acidic method and analysis of OA in the negative ESI mode, signal enhancement was observed in scallops and oysters (128.8 and 123.6% respectively) while an acceptable accuracy was obtained in mussels (104.7%). The use of alkaline method led to excellent accuracies in crude extracts of mussels and in scallops (99.3 and 98.9%) while signal suppression was observed in oysters (79.6%). Therefore, a systematic decrease in the response (>20%) was observed when the alkaline method was used.

This trend was not observed in our study. In the past, signal enhancement (50%) was observed when the analysis of OA in crude extracts of mussels was performed on the same instrument and using the same acidic method [23]. Although the same species of mussels were used (*M. edulis*), the flesh composition may have been different enough than in the present study to induce differences in the degree of matrix effects observed.

In the study by Gerssen et al. [22], the crude extracts spiked with AZA1 (equivalent to 100 μg/kg) using a SSR of 10 showed that, with the acidic method, signal suppression was observed in mussel, scallops and oysters (accuracies of 84.3, 59.1 and 73.6% respectively). The use of alkaline method systematically led to better accuracies (88.1, 89.0 and 83.5% in the crude extracts of mussels, scallops and oysters respectively). The results we obtained on the TSQ (same instrument as in Gerssen et al.) are in agreement with these observations and the suppression effect observed for AZA1 using the acidic method was eliminated when the alkaline method was used. The suppression effect in the analysis of AZA1 has been reported for numerous shellfish species on different instruments with various chromatographic methods [26,23,29,30]. The results we obtained for AZA1 on the QToF with the acidic method are consistent with a previous study performed on this instrument [31] and within acceptable accuracies. However, signal enhancement was observed when the alkaline method was used.

3.2.3. *Modified acidic gradient method with 100% organic solvent flush*

Standards and matrix matched standards were run in triplicate in each batch to assess the impact on matrix enhancement for OA on the QToF and matrix suppression for AZA on the TSQ. Four batches were run over a 1-month period. The average and standard deviations ($n = 12$) for the six shellfish species are shown in Table 5.

The introduction of the 100% acetonitrile flush for the analysis of AZA1 on the TSQ resulted in improved accuracies when compared to the results shown in Table 1. The suppression effect observed previously was eliminated and the accuracies ranged from 89.3 to 103.7%. Interestingly the highest bias was observed for *P. maximus* gonad which was also the case with the short acidic gradient method. The two-way ANOVA indicated that the differences in the mean values between shellfish species were significant ($p < 0.001$). The analysis of OA in the different shellfish species on the TSQ led to excellent accuracies, ranging from 98.2 to 105.8%. Although the analysis of OA using the short acidic gradient on the TSQ demonstrated acceptable accuracies, the method with the 100% acetonitrile flush provided more consistent results between species. After allowing for the effect of the days of analysis, the two-way ANOVA indicated that the difference between the mean values obtained for the different shellfish species was not significant ($p = 0.496$).

The signal enhancement observed in the analysis of OA with the QToF remained critical with the 'flushing' method. The accuracies ranged between 117.3 and 171.4%. A significant statistical difference was observed between species ($p < 0.001$). Investigations

Table 5
Accuracy and precision data (expressed as percentages) obtained on QToF and TSQ with the modified acidic gradient method with 100% organic solvent flush (average ± SD; *n*, no. of injections, *p*, no. of concentration points).

Species	AZA1		OA			
	TSQ		TSQ		QToF	
M. edulis ($p = 7$)	103.7 ($n = 12$)	±7.7	100.7 ($n = 12$)	±10.3	162.4 ($n = 12$)	±11.6
C. gigas ($p = 1$)	103.4 ($n = 12$)	±7.1	102.8 ($n = 12$)	±13.6	150.6 ($n = 12$)	±21.5
O. edulis ($p = 1$)	94.8 ($n = 12$)	±8.8	105.8 ($n = 12$)	±12.1	164.4 ($n = 12$)	±13.1
E. siliqua ($p = 1$)	94.9 ($n = 12$)	±6.8	100.4 ($n = 12$)	±9.3	134.9 ($n = 12$)	±11.8
P. max meat ($p = 1$)	97.4 ($n = 12$)	±5.4	98.2 ($n = 12$)	±8.0	117.3 ($n = 12$)	±10.2
P. max gonad ($p = 1$)	89.3 ($n = 12$)	±10.8	100.3 ($n = 12$)	±11.2	171.4 ($n = 12$)	±15.2

showed that the pronounced enhancement effect was not related to the flushing step as the same results were obtained when using the shorter acidic method and with a new analytical column (data not shown).

Our results indicated that the suppression of AZA1 on the TSQ was caused either by late eluting compounds or due to compounds lingering in the source from previous injections. In order to determine which was the case, an experiment was performed using the acidic method which consisted of two injections of an *O. edulis* extract followed by the injection of an AZA1 standard in triplicate. The above procedure was then repeated with modifications. The flow going through the column was stopped after the injections of the *O. edulis* extract, the column was replaced with a union and the mobile phase B set at a flow rate of 0.4 ml/min for 5 min (as is the case with the acidic flush method). After 5 min the column was installed on the system and allowed to equilibrate for 3 min before the next injection of AZA1 standard. The experiment was repeated in triplicate.

As observed previously the AZA1 standard was suppressed by 17 ± 3% after two injections of the *O. edulis* extract using the acidic method. The suppression was still observed even after the source was flushed (18 ± 5%) indicating that the interfering compounds were strongly retained on the column.

3.2.4. On-line SPE

The use of two columns for the separation of compounds from complex mixtures such as shellfish provides another dimension to conventional liquid chromatography. This approach has been successfully used for both single laboratory and collaborative study validations for the determination of low level agricultural residues in soft drinks by LC–MS/MS [32,33].

The performance of a combination of two columns was evaluated for OA analyses on the QToF using the acidic method. An Oasis HLB column was used as the initial column to trap OA from the matrix. The column was then back flushed onto the analytical column, the BDS Hypersil C8 for further separation. The approach was adapted from a method used for the analysis of phycotoxins in plankton cells [34]. The accuracy of the method was evaluated using the same approach as that for OA and AZA1 using the acidic and the alkaline methods. All solutions were injected in triplicate on 5 separate days over a 5-month period. Acceptable accuracies were obtained in all shellfish species which ranged from 86.5 to 102.6% (Table 6). Comparison of these results with those obtained using the acidic method on the QToF (Table 1) demonstrates that the use of a second column significantly reduced the matrix effects that were associated with OA analysis in shellfish species.

The between-day precision obtained using the column switching method was acceptable for all shellfish species with relative standard deviations ranging from 5.7 to 11.4% (Table 6).

The sensitivity of the column switching method was comparable to the acidic method on the same instrument with a limit of detection (LOD) equivalent of 16 μg/kg tissue (Table 7). Attempts to shorten the run time (from 43 min) by adjusting the gradient conditions and/or flow rates were unsuccessful.

Table 6
Accuracy (expressed as a percentage) of the column switching method on the QToF (acidic mobile phase) for OA in different shellfish species (average ± SD; *n*, no. of injections, *p*, no. of concentration points).

Shellfish species	Average OA recovery ± SD ($n = 15$)
M. edulis ($p = 7$)	95.1 ± 11.4
C. gigas ($p = 1$)	101.4 ± 10.2
O. edulis ($p = 1$)	90.4 ± 5.7
E. siliqua ($p = 1$)	86.5 ± 8.6
P. max meat ($p = 1$)	93.5 ± 6.7
P. max gonad ($p = 1$)	102.6 ± 10.9

Table 7
LODs (μg/kg) for AZA1 and OA on the TSQ and the QToF with the acidic and alkaline method determined in mussel extracts.

	Acidic (μg/kg)		Alkaline (μg/kg)	
	TSQ	QToF	TSQ	QToF
AZA1	0.3	3	0.5	5
OA	10	20	5	10

3.3. Method performances

A fit for the purpose analytical method should meet the minimum performances for specific parameters set by international organizations [35–39]. The validation parameters include selectivity, accuracy, precision, range, sensitivity and ruggedness (the FDA and ICH guidelines also include the assessment of the stability of the analytes). When LC–MS/MS methods are used the selectivity of the method is generally excellent and the absence of response in several blank samples is usually sufficient to demonstrate the specificity of a given method.

3.3.1. Sensitivity

The LODs observed for OA and AZA1 on both instruments and using the acidic and alkaline methods are shown in Table 7. The alkaline method allowed for a two fold improvement in sensitivity for OA compared to the acidic method. The LOD achieved for AZA1 was better with the acidic method than with the alkaline method by a factor of 1.7 on both instruments. The TSQ was 10 times more sensitive than the QToF for AZA1.

3.3.2. Accuracy

In the AOAC guideline, acceptable accuracy is a function of the concentration and the purpose of the analysis. An accuracy of 75–125% is considered acceptable for methods of quantification at ppb levels, as in this study. The FDA guideline [37] defines an acceptable accuracy as being 15% of the actual value except at the lower limit of quantification (LOQ) at which 20% is acceptable. Therefore, the accuracy that we obtained for OA on the TSQ and for AZA on the QToF with the acidic method, as well as for both OA and AZA1 on the TSQ with the alkaline method, meet the requirements of the AOAC and the FDA guidelines.

3.3.3. Precision

According to the AOAC guidelines, repeatability is defined as the degree of agreement of results when conditions are maintained as constant as possible with the same analyst, reagents, equipment, and instruments performed within a short period of time. The repeatability varies with concentration and a theoretical calculated value can be obtained from the Horwitz equation (1) where *C* is the concentration of the analyte expressed a mass fraction.

$$\mathrm{RSD}_r = C^{-0.15} \quad (1)$$

The HORRAT formula (Eq. (2)) allows for the calculation of a ratio that should fall between 0.5 and 2 in order to consider the repeatability as satisfactory.

$$\mathrm{HORRAT}_r = \frac{\mathrm{RSD}_r(\mathrm{found})}{\mathrm{RSD}_r(\mathrm{calculated})} \quad (2)$$

Therefore, acceptable precisions for the extracts spiked with OA should have relative standard deviations ranging from 2.8 and 11.2 while acceptable precisions for AZA1 should range from 3.0 to 12.1. Almost all the standard deviations of the analyses carried out with both instruments were within the acceptable range. The FDA guidelines define acceptable precision as a RSD obtained from five measurements being less than 15% and less than 20% at the lower

LOQ. Therefore, according to the FDA guidelines, acceptable precisions were obtained for OA and AZA1 using both acidic and alkaline methods for all shellfish species on the TSQ, except for *M. edulis*, using the alkaline method for which 16.6% RSD was observed.

We demonstrated that the within-day precision is greatly affected by a suppression effect for the AZAs. The injection of several shellfish extracts strongly suppressed the response in the samples analyzed after the shellfish extracts. When the alkaline and modified acidic methods were evaluated this phenomenon was not observed.

4. Conclusions

We demonstrated the impact of matrix interference in the LC–MS/MS analysis of low-level toxins in molluscan shellfish, and strategies to overcome this. Contrasting results were obtained on two different LC–MS/MS instruments, using an acidic method, even with the same source type (ESI), using the same LC conditions (and samples) and the analyses performed by a single analyst. Significant differences were observed between shellfish species.

Partitioning the sample with hexane proved unsuccessful in overcoming the interferences observed for OA on the QToF and AZAs on the TSQ.

Matrix suppression for AZA1 was overcome using an acidic method with an organic solvent flush and alternatively by an alkaline method.

Matrix enhancement observed for OA on the QToF was eliminated only by an on-line SPE method.

In the author's lab the alkaline method is the method of choice for the TSQ while the acidic method (using on-line SPE for OA analysis) is the preferred procedure for the QToF.

Introduction of LC–M/MS as the primary method for the regulatory monitoring of biotoxins in shellfish will be quite challenging, considering the variety of instrumentation and techniques available.

This study clearly demonstrates that different LC–MS/MS instruments can produce very dissimilar results due to matrix interferences and that it is necessary to initially evaluate matrix effects and where present implement procedures to eliminate and/or correct for them.

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