1 **Strategies for the elimination of matrix effects in the LC-MS/MS analysis of the**

2 **lipophilic toxins okadaic acid and azaspiracid-1 in molluscan shellfish**

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9

10 **Abstract**

11 Considerable efforts are being made worldwide to replace in vivo assays with

12 instrumental methods of analysis for the monitoring of marine biotoxins in shellfish.

13 Analysis of these compounds by the preferred technique of LC-MS/MS is challenged

14 by matrix effects associated with shellfish tissue components. In methods validation,

15 assessment of matrix interferences is imperative to ensure the accuracy of analytical

16 results. We evaluated matrix interferences in the analysis of okadaic acid (OA) and

17 azaspiracid 1 (AZA1) in mollucscan shellfish by using a conventional acidic method

18 on electrospray triple stage quadrapole (TSQ) and hybrid quadrupole time of flight

19 (QToF) instruments, with matrix matched standards for several species. Using the

20 acidic method, we found no matrix interferences for OA, and matrix suppression for

21 AZA1, with the TSQ instrument; in contrast, we found matrix enhancement for OA,

22 and no matrix interference for AZA1, with QToF. The suppression of AZA1 signal

23 on the TSQ instrument was due to interfering compounds carried over from previous

24 injections. The degree of suppression was dependent on the tissue type, ranging from

25 20 to 70%.   Several strategies were evaluated to eliminate these interferences,

26 including the partitioning of the extract with hexane, optimization of the

27 chromatographic method, and the use of on-line SPE. The use of an alkaline method

28 and a modified acidic method eliminated matrix suppression for AZA1 on the TSQ

29 instrument, while an on-line SPE method proved effective in eliminating matrix

30 enhancement of OA on the QToF.

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Keywords:

Lipophilic marine biotoxins, Azaspiracid, Okadaic acid, LCMS, on line SPE, matrix
effects.

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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 32 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, 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/kg of toxins from the OA group (sum of OA, DTX1, DTX2 and their esters) and 160 μg/kg of toxins from the AZA group (sum of AZA1, -2 and -3). Currently the mouse bioassay (MBA) is the EU reference method for the detection of marine biotoxins in shellfish. However, there are a number of issues associated with this method in terms of sensitivity, accuracy, and ethics, and considerable efforts are underway to replace it with instrumental methods.

1   The mouse bioassay (MBA) will likely be replaced by liquid chromatography-

2   mass spectrometry (LC-MS/MS) as the reference method for the detection of marine

3   biotoxins in shellfish by the year 2011 (Community Reference Laboratories for

4   Marine Toxins meeting, Oct 2009). LC-MS/MS is considered the technique of choice,

5   offering improved sensitivity, selectivity, and accuracy as well as being faster and

6   automated. However, quantification using LC-MS/MS in biological matrices is often

7   challenging because of matrix effects which alter the accuracy and the precision of the

8   method. Matrix effects are believed to be caused by endogenous compounds co-

9   eluting with the analyte of interest, and competing for ionisation in the electrospray

10   (ESI) source [14, 15].

11   A number of different approaches have been taken to eliminate or to correct

12   for matrix effects in LC-MS/MS analyses including sample clean-up, standard

13   addition, matrix-matched standards, internal standards, and changes in

14   chromatographic conditions (e.g., pH of the mobile phase, nature of stationary phase).

15   Sample clean-up can be performed using liquid-liquid extraction (LLE) or solid phase

16   extraction (SPE), the latter of which is available with a variety of stationary phases

17   (normal and reverse phase, ion exchange, and immunoaffinity material with

18   antibodies specific to the analyte). Other clean-up methods specific to a given

19   application also exist (e.g., protein precipitation for analysis of blood). A successful

20   clean-up step for LC-MS/MS analysis implies that the sample is free of the

21   compound(s) responsible for matrix effects and that the recovery of the analyte of

22   interest is high. The aim of this approach is therefore to eliminate or minimise matrix

23   effects while ensuring acceptable accuracy. SPE has the added benefit of pre-

24   concentrating samples which can be useful when dealing with low level toxins. A

25   recent study showed that marine lipophilic toxins could be enriched by a factor of 10

26   using SPE, while eliminating sample impurities [16]. 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.

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 same composition as the sample extracts, by extracting control material or by reconstructing the matrix artificially, and spiking the analyte at different concentrations. This implies that the sample matrix is available in the laboratory and that it is free of analyte, and that standards are available in sufficient quantities. 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 species) 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 components in 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 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 DSP toxins [17]. Although the method is very powerful and widely accepted, its use in monitoring laboratories remains limited for a number of reasons. Accurate determination of the response factor in a given extract should be performed over a significant range of concentrations (e.g., 50, 100, and 200 % of the

1   initial concentrations), while ensuring that the upper limit of quantification of the

2   method will not be exceeded. This implies that the analyte concentration in the sample

3   should first be determined using an external calibration in order to determine the

4   amounts of standard that should be added. Obviously, multi-analyte analyses of

5   compounds in the sample at different concentrations would complicate the matter

6   further. Also, the preparation and addition of spiking solutions can lead to analyst

7   errors and increase the duration of sample preparation and overall analysis time, as

8   four or five injections are required instead of a single injection. The use of an internal

9   standard can be a very efficient approach to ensure that satisfactory accuracy is

10 obtained through the different steps of the analytical method. Unfortunately, the total

11 or partial synthesis of the isotopically labelled compound is required and currently no

12 such compounds are available for DSP and AZP toxins, to our knowledge.

13   We examined matrix effects associated with analysis of lipophilic marine

14 toxins in shellfish tissues on two LC-MS/MS instruments, QToF and TSQ, using ESI

15 source and identical LC conditions. Matrix interferences were assessed using matrix-

16 matched standards for six different shellfish species/tissues; *Mytilis edulis*,

17 *Crassostrea gigas, Ostrea edulis, Ensis siliqua, Pecten maximus* adductor muscle

18 (meat), and *Pecten maximus* gonad. Where interferences were observed, we describe

19 efforts made to overcome them. The performances of the methods employed were

20 also evaluated in terms of sensitivity, accuracy, and precision.

21 **2. Materials and methods**

22 **2.1. Solvents and reagents**

23   Acetonitrile, methanol, and hexane were purchased as pestican grade solvents

24 from Labscan (Dublin, Ireland). Formic acid, ammonium formate, and ammonium

25 hydroxide were obtained from Sigma Aldrich (Steinheim, Germany). Water was

26 obtained from a reverse-osmosis purification system (Barnstead, Dublin, Ireland). OA

1 and AZA1 certified reference materials (CRM) were obtained from the NRC (Halifax,

2 Canada).

3 **2.2. LC-MS/MS**

4 Two LC-MS/MS systems were used; a Micromass triple stage quadrupole

5 (TSQ) Ultima coupled to a Waters 2695 LC, and a Micromass time-of-flight (QTof)

6 Ultima coupled to a Waters 2795 LC. Both systems were equipped with a z-spray ESI

7 source.

8 The TSQ was operated in selected reaction monitoring (SRM) mode and the

9 following transitions were monitored: OA, m/z 803.5>255.1 and 803.5>803.5 in

10 negative ionisation mode; AZA1 m/z 842.5>654.4 and 842.5>672.4, AZA2

11 856.5>654.4 and 856.5>672.4, AZA3 828.5>640.4 and 828.5>658.4 in positive

12 ionisation mode. The cone voltages were set at 70 V and 60 V in negative and positive

13 ion modes, respectively, and the collision voltage was set at 40 V. Cone and

14 desolvation gas flows were set at 100 and 800 L/h, respectively, while the source

15 temperature was set at 150°C.

16 The QTof was operated in fragment ion scan (FIS) mode monitoring for the

17 same precursor ions as those reported for the TSQ. The cone voltages were set at 80 V

18 and 40 V, and collision energy voltages at 30 V and 50 V, for negative and positive

19 modes, respectively. Cone and desolvation gas flows were set at 100 and 750 L/h,

20 respectively, while the source temperature was set at 140°C. Quantification was

21 performed by summing the ions of m/z 824.5, 672.5, 654.5 and 362.5 for AZA1 (and

22 the equivalent fragment ions for AZA2 and -3) and the ions of m/z 803.5 and 255.1

23 for OA.

24 **2.2.1. Acidic gradient method**

25 A gradient elution method was set with an acidic binary mobile phase, with

26 phase A (100% aqueous) and phase B (95% acetonitrile), each containing 2 mM

1   ammonium formate and 50 mM formic acid, following the method of Quilliam et al.,

2   2001 [18]. The gradient elution started with 30% B, increased to 90% B over 8 min,

3   held for 2.5 min, decreased to 30% B in 0.5 min and held for 4 min to equilibrate the

4   system before the next injection. The chromatographic separation was achieved using

5   a Hypersil BDS C8 column; 50 x 2.1 mm, 3 µm with a guard column of the same

6   stationary phase 10 x 2.1 mm, 3 µm (Thermo Scientific, Runcorn, UK). The flow rate

7   was set at 0.25 ml/min and the injection volume at 5 µl. The column and sample

8   temperatures were set at 25°C and 6 °C, respectively.

9   **2.2.2. Acidic gradient method with a 100% B flush**

10   A modified gradient method with acidic mobile phase was also evaluated. The

11   gradient started with 30% B, increased to 90% B over 8 min with a flow rate of 0.25

12   ml/min, held for 5 min, increased to 100% B at 0.4 ml/min, held for 5 min, and

13   returned to 30% B at 0.25 ml/min which was held for 4 minutes to equilibrate the

14   system.

15   **2.2.3. Alkaline method**

16   The alkaline method followed that of Gerssen *et al*, 2009 [19]; a binary mobile

17   phase was used, with phase A (100% aqueous) and phase B (90% acetonitrile) each

18   containing 6.7 mM ammonium hydroxide. Separation was achieved using a Waters X

19   bridge, C18 column (150 x 3 mm, 5 µm). The flow rate was set at 0.25 ml/min and the

20   injection volume at 5 µl. The column and sample temperatures were set at 25°C and

21   6°C, respectively. A gradient elution was employed, starting with 10% B which was

22   held for 1 min and increased linearly to 90% over 9 min. The mobile phase was held

23   at 90% B for 3 min and returned to 10% B in 2 min. The system was then allowed to

24   equilibrate for 4 min.

2.2.4 Column switching method

For the on-line SPE method a binary mobile phase was used with phase A (100% aqueous) and phase B (95% acetonitrile) each containing 2 mM ammonium formate and 50 mM formic acid. The loading column was an Oasis HLB, 5 µ, 2.1 x 20 mm column and LC separation was achieved using a Hypersil BDS C8 column; 50 x 2.1, 3 µm; guard column, 10 x 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 at which time the switch valve directed the flow onto the analytical column and the flow was reduced to 0.02 ml/min. After 3 seconds the flow was changed to 0.075 ml/min and the % B was increased from 20% to 30% over 27 sec. The % B was then increased 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) contaminated with both OA group and AZA toxins was extracted using the procedure described below (preparation of matrix-matched standards). An aliquot (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 collected in a centrifuge tube and an aliquot transferred into a LC vial for analysis.

An aliquot (1 ml) of the hexane layer was pipetted into LC vials and dried down under nitrogen. Dried residues were re-solubilised with 200 µl of methanol with vortex mixing for 30 sec. The sample was transferred into an insert vial for analysis. Three methanol standards were run directly after three injections of the LRM extract

1   in addition to the 'clean' LRM extract, followed by a four point calibration curve (all

2   performed in triplicate).

3   **2.4. Preparation of matrix-matched standards**

4   For each tissue type, uncontaminated raw samples tested as part of the routine

5   monitoring programme in Ireland were selected from different harvesting dates and

6   sites (around the coasts of Ireland). The species chosen for investigation were *M.*

7   *edulis*, *C. gigas, O. edulis, E. siliqua, P. maximus* meat, *P. maximus* gonad, with the

8   *M. edulis* and *C. gigas* being the most commonly analysed routinely. The shellfish

9   were shucked, homogenised, and aliquoted for extraction. Two g of tissue was

10   extracted by vortex mixing for 1 min with 9 ml of methanol, centrifuged at 5,000 rpm

11   for 5 min, and the supernatant decanted into a 20 ml volumetric flask. The remaining

12   pellet was further extracted using an ultra turax for 1 min with an additional 9 ml of

13   methanol, centrifuged at 5,000 rpm for 5 min, and the supernatant decanted into the

14   same 20 ml volumetric flask which was brought to volume with methanol. The

15   standards were prepared in 25 ml volumetric flasks containing 20 ml of filtered

16   (Whatmann, 0.2 µm, cellulose acetate filter) tissue extract. For the *M. edulis* matrix-

17   matched standards, aliquots of standard stock solution were added to the flasks and

18   the volume was brought to the mark with methanol, yielding toxin concentrations

19   ranging from 2.5 – 280 ng/ml for OA and 0.8 – 92 ng/ml for AZA1.

20   Spiked tissue samples were prepared for the following tissues: *C. gigas, O.*

21   *edulis, E. siliqua, P. maximus* meat and *P. maximus* gonad. For the spiked tissue

22   samples 1 ml of stock standard solution was added to the flasks and the volume

23   brought to the mark with methanol, such that the final concentration was 10 ng/ml and

24   6 ng/ml for OA and AZA1 (equivalent to 125 µg/kg and 75 µg/kg in tissue),

25   respectively.

1 For all the matrix-matched standards, a sample to solvent ratio (SSR) of 12.5 was

2 obtained which reflects the routine monitoring extraction method.

3 **2.5. Statistical analysis**

4 Statistical calculations were performed using Sigmastat 3.0. The significance

5 test used to compare species and methods was the two-way analysis of variance

6 Holm-Sidak test. Alpha was set at 0.05 for all experiments.

7

8 **3. Results**

9 **3.1. Assessment of matrix effects**

10 We assessed matrix effects on two different LC-MS/MS instruments, a QToF

11 and a TSQ, using an acidic gradient method for several shellfish tissues over a number

12 of months. The spike samples and *M. edulis* matrix-matched standards were run in

13 triplicate against methanol standards (seven levels) using validated and accredited

14 methods of analysis for the monitoring of lipophilic toxins.

15 A matrix-matched standard curve was prepared with *M. edulis* in order to

16 compare response factors over the range of concentrations representative of naturally

17 contaminated shellfish. The accuracy was calculated as a percentage of difference

18 between the slopes obtained in methanol and in the *M. edulis* extracts. The accuracies

19 reported for all other shellfish species were calculated from spiked samples at a single

20 concentration. Data were compiled from five batches acquired over several months.

21 Within each batch all samples were analyzed by triplicate injection. A two-way

22 ANOVA analysis was performed using the different species and days of analyses as

23 variables and the concentrations obtained intra and inter batches (n = 12-18) as the

24 response.

25 The accuracy of AZA1 measurements on the TSQ instrument in the different

26 species of shellfish ranged from 64.2 to 83.1% (Table 1). Signal suppression was

1 consistently observed and was significantly different between the shellfish species (p

2 = 0.009). When the same method was performed on the QToF the accuracy ranged

3 from 97.1 to 104.6% without significant differences between species (p = 0.467).

4   The accuracy observed for OA using the acidic method also greatly varied

5 between the two instruments (Table 1). Acceptable accuracies were achieved on the

6 TSQ, ranging from 94.3 to 110.9%. The two-way ANOVA test revealed that the

7 accuracy was statistically different between shellfish species (p<0.001). The pairwise

8 multiple comparison procedure results demonstrated that the accuracy obtained for

9 OA in *O. edulis* (110.9 %) and for *M. edulis* (108.0 %) were not significantly different

10 (p = 0.343) but were significantly different when compared to the other shellfish

11 species (p values ranging from <0.001 to 0.041). The accuracy obtained for OA

12 analysis on the QToF with the acidic method was affected by signal enhancement and

13 ranged from 114.6 to 130.9% with a significant difference between the shellfish

14 species (p = 0.008).

15   Comparison of the results between instruments show that the apparent

16 recoveries observed on the QToF were always higher for both AZA1 and OA than on

17 the TSQ regardless of the species. During analysis of AZA1 on the TSQ it was noted

18 that the injection of a standard after the injection of a number of tissue extracts led to

19 a lower response than when injected after a calibration curve. The degree of

20 suppression was dependent on the type of tissue extract. This phenomenon is

21 illustrated in Figure 1 which shows the response of three consecutive injections of an

22 AZA1 standard (104 ng/ml) after three injections of shellfish extracts prepared from

23 five different species. A six point calibration curve was systematically run after the

24 three injections of the AZA1 standard and used to calculate the concentrations

25 reported in Figure 1. Depending on the tissue type the degree of suppression ranged

26 from 15 to 70%. *P. maximus* gonad tissue had the greatest effect while the clams (*T.*

1   *philippinarium*) had the least. Injections of the AZA1 standard after the oyster,

2   mussel, and scallop extracts demonstrated that the first and second injections are

3   equally affected by signal suppression while the third injection led to a significantly

4   higher response. These results suggest that either later eluting compounds, or

5   compounds lingering in the source, are responsible for the signal suppression

6   observed. This phenomenon was not observed for the analysis of OA on the QToF.

7   The within-day precision obtained with the acidic method for OA ranged from

8   1 to 10 % on both instruments while the between-day precision over at least five days

9   was 8% on both the QToF and the TSQ (Table 2). The analysis of AZA1 using the

10   acidic method on the QToF demonstrated excellent precision as the within-day

11   precision ranged from 2 to 5 % and a between-day precision of 11% (Table 2). The

12   within-day precision for AZA1 using the acidic method on the TSQ ranged from 3 to

13   16%. The high variation on day five was due to a lower response of the first set of

14   solutions that was injected compared to the second and the third replicate set (Table

15   2). A between-day precision of 8 % was observed over five days.

16   **3.2. Methods to address matrix effects**

17   **3.2.1. Partitioning of extract with hexane**

18   A mussel tissue laboratory reference material (LRM) was used in this

19   experiment. The LRM was extracted following the same procedure used for the other

20   shellfish as described in the materials and methods section. We investigated the

21   recoveries of OA and AZA1 in the methanolic fraction after the hexane partitioning

22   (data not shown). The recoveries were satisfactory for both compounds (> 95%).

23   Hexane did not appear to have any effect on matrix suppression for the AZAs on the

24   TSQ, with no significant differences being observed between the partitioned LRM

25   and the crude LRM (Figure 2). The suppression is still observed for the subsequent

1 LRM and standard injections for both partitioned and non partitioned samples and

2 reflects what was observed for the different tissue types (see Figure 1).

3 The successive injections of the crude extracts of the LRMs demonstrated a

4 declining signal response to AZA1, AZA2 and AZA3 with respect to the number of

5 injections. Partitioning the extracts with hexane did not modify the trend as there were

6 no differences in the concentrations of AZA1, AZA2 and AZA3 observed in crude

7 extracts and extracts that were partitioned with hexane.

8 Furthermore, the signal suppression effect observed in AZA1 standards after

9 the injection of shellfish extracts presented in Figure 1 was also examined. The results

10 show that the two injections of a methanolic standard of AZA1 (104 ng/ml) that

11 followed three injections of the LRM were affected by signal suppression as the

12 average concentrations were 78.0 ± 5.6 and 79.4 ± 7.1 ng/ml for the first and second

13 injections respectively. It is only on the third injection of the standard that the

14 concentration measured (102.7 ± 4.1 ng/ml) returned within the expected theoretical

15 concentration.

16 The effect of hexane partitioning on the signal enhancement effect observed

17 for OA on the QToF instrument was also evaluated. Similarly to the above results, the

18 hexane partitioning did not eliminate the matrix effects observed (data not shown).

19 **3.2.2. Alkaline method**
20 Changing the selectivity of the method may help to overcome matrix

21 interferences. The use of an alkaline method for the separation of lipophilic toxins

22 was reported to increase the sensitivity for the OA group of toxins and enable better

23 separation of the DSP (including PTX2) and AZA group of toxins. This separation

24 allows analysis of both groups of toxins in the one run without having to alternate the

25 mass spec polarity [19]. An additional study found that SPE on polymeric sorbents

1 combined with an alkaline method can significantly reduce matrix interferences for

2 both OA and AZA1 [20].

3

4   The alkaline method was run on both the QToF and TSQ instruments without

5 any sample pre-treatment to determine any impact on matrix interferences. To assess

6 the matrix effects, methanol standards were run with matrix matched standards in

7 triplicate and the slopes compared. Excellent results were obtained when the analyses

8 were performed on the TSQ with accuracies of 90.9 to 108.1 % for AZA1 and 97.2 to

9 104.4 % for OA (Table 3). There was no statistically significant difference between

10 the species (p = 0.083 and 0.278 for AZA1 and OA, respectively). Signal

11 enhancement was systematically observed for both OA and AZA1 when the QToF

12 was used with the alkaline method. For AZA1 the accuracy ranged from 107.7 to

13 135.5% with a significant difference observed between species (p<0.01) while the

14 accuracy for OA ranged from 122.8 to 127.4 % without significant difference between

15 species (p = 0.928). By using the alkaline method, the AZA1 suppression effect on

16 the TSQ was overcome. Analysis of three injections of a *P. maximus* gonad extract

17 followed by three standard injections yielded 98% ± 1.1 recovery for the AZA1 (and

18 OA) in the standard compared with 38% ± 12 recovery for AZA1 using the acidic

19 method.

20   The precision of OA measurements by the alkaline method ranged from 0.4 to

21 11 % within-day on both instruments (Table 4). Between-day precision was 9.5 and

22 8.3% on the QToF and the TSQ, respectively. The precision obtained for AZA1 using

23 the alkaline method was also acceptable. Within-day precision ranged from 2 to 14 %

24 on both instruments, and between-day precision of 9.2 and 16.6% on the QToF and

25 TSQ, respectively.

26

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 one-month period. The average and standard deviations (n=12) for the six shellfish species are shown in Table 5.

The introduction of the 100% ACN flush eliminated the suppression effects observed in the analysis of AZA1 on the TSQ. Accuracies ranged from 89.3 to 103.7 %. The highest bias was observed for *P. maximus gonad* as was the case without flush. The two-way ANOVA indicated that the differences in the mean values between species of shellfish 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 by the original acidic gradient on the TSQ demonstrated acceptable accuracies, the method with the 100% ACN 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 accuracies obtained for OA on the QToF ranged between 117.3 to 171.4%. A significant statistical difference was observed between species (p<0.001). 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). A previous study showed an enhancement effect of 50% for OA on this instrument using the acidic method [21].

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*

16

1   extract followed by the injection of an AZA1 standard in triplicate. The above

2   procedure was then repeated (in triplicate), however this time the flow going through

3   the column was stopped after the injections of the *O. edulis* extract, the column was

4   replaced with a union and the mobile phase B set at a flow rate of 0.4 ml/min for 5

5   min (as is the case with the acidic flush method). After 5 min the column was installed

6   on the system and allowed to equilibrate for 3 min before the next injection of AZA1

7   standard. As observed previously the AZA1 standard was suppressed by $17 \pm 3\%$

8   after 2 injections of the *O. edulis* extract using the acidic method. The suppression

9   was still observed even after the source was flushed $(18 \pm 5\%)$ indicating that the

10   interfering compounds were strongly retained on the column.

11   **3.2.4. Column switching**

12   The performance of a combination of two columns was evaluated for OA

13   analyses on the QToF using the acidic method. An Oasis HLB column was used as

14   the initial column to trap OA from the matrix. The column was then back flushed onto

15   the analytical column, the BDS Hypersil C8, for further separation. The approach was

16   adapted from a method used for the analysis of phycotoxins in plankton cells [22].

17   The accuracy of the method was evaluated using the same approach as that for OA

18   and AZA1 using the acidic and the alkaline methods. All solutions were injected in

19   triplicate on five separate days over a five month period. Acceptable accuracies were

20   obtained in all shellfish species, ranging from 86.5 to 102.6 % (Table 6). Comparison

21   of these results with those obtained using the acidic method on the QToF

22   demonstrates that the use of a second column significantly reduced the matrix effects

23   that were associated with OA analysis in shellfish species. The between-day precision

24   obtained using the column switching method was acceptable for all shellfish species

25   with relative standard deviations ranging from 5.7 to 11.4%. The sensitivity of the

26   column switching method was comparable to the acidic method on the same

1   instrument with a LOD equivalent of 16 µg/kg tissue (Table 7). Attempts to shorten

2   the run time (from 43 min) by adjusting the gradient conditions and/or flow rates were

3   unsuccessful.

4

5   **4. Discussion**

6   **4.1. Assessment of matrix effects**

7   We assessed matrix interferences on two LC-MS/MS instruments for the

8   quantification of OA and AZA1 in a variety of shellfish species using an acidic

9   mobile phase adapted from a method developed by Quilliam et al. [18]. When matrix

10   effects were observed, several strategies were evaluated to reduce them. Most of the

11   results presented in this study were obtained by the analysis of spiked samples in

12   order to provide a comparison of instrumental methods which could then be used in a

13   formal single laboratory validation. The extraction procedure described in this study

14   has been used for several years in the shellfish toxins monitoring program in Ireland

15   [23]. Matrix suppression was observed in the analysis of AZA1 on the TSQ while

16   matrix enhancement was observed for OA on the QToF.

17   **4.2. Assessment of strategies to overcome matrix interferences**

18   **4.2.1. Partitioning with hexane**

19   The signal suppression effect observed in the analysis of AZA1 on the TSQ

20   can be due to late eluting compounds or compounds lingering in the source from

21   previous injections which compete for ionization. In reversed phase chromatography,

22   lipophilic compounds can be retained on the stationary phase for extended periods and

23   their removal prior to subsequent injections was therefore investigated. Partitioning

24   the shellfish extracts with hexane was investigated, however no impact on the

25   suppression effect was observed. This finding is in agreement with the results reported

26   by Ito and Tsukada [17]. In this study the partitioning of scallop extracts with hexane

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and chloroform was evaluated for the reduction of signal suppression observed in LC-MS analysis of OA, DTX1, yessotoxin and pectenotoxin-6. This clean-up procedure had no effect on the matrix effects observed. The LC-MS method from McNabb et al. (2005) 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 [24]. 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.

4.2.2. Gradient with organic solvent flush

The introduction of a flush with 100% organic solvent to the acidic method eliminated the matrix suppression observed for AZA1 on the TSQ and the results suggested that the suppression was caused either by late eluting compounds or by compounds lingering in the source. The results of an additional experiment suggested that matrix effects were observed as a result of the elution of interfering compounds that were strongly retained on the stationary phase. The introduction of a flush step did not have any detrimental effects on the results obtained for OA on the TSQ as the accuracies observed were satisfactory, similarly to the results obtained with the short acidic gradient discussed above. However, the signal enhancement observed in the analysis of OA with the QToF remained critical.

4.2.3. Alkaline method

The accuracies for OA and AZA1 using the acidic and the alkaline methods were recently reported in extracts of mussels (*M. edulis*), scallops (*P. maximus*), and oysters (C. *gigas*) [19]. 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

1 (104.7%). The use of alkaline method led to excellent accuracies in crude extracts of

2 mussels and in scallops (99.3 and 98.9%, respectively) while signal suppression was

3 observed in oysters (79.6%). Therefore, a systematic decrease in the response (> 20%)

4 was observed when the alkaline method was used in the study from Gerssen et al [20].

5 This trend was not observed in our study. In the past signal enhancement (50 %) was

6 observed when the analysis of OA in crude extracts of mussels was performed on the

7 same instrument using the same acidic method [21]. Although the same species of

8 mussels were used (*M. edulis*), the flesh composition may have been different enough

9 than in the present study to induce differences in the degree of matrix effects

10 observed.

11   In the study from Gerssen et al. [20], the crude extracts spiked with AZA1

12 (equivalent to 100 μg/kg) using a sample to solvent ratio of 10 showed that, with the

13 acidic method, signal suppression was observed in mussel, scallops and oysters

14 (accuracies of 84.3, 59.1 and 73.6%, respectively). The use of alkaline method

15 systematically led to better accuracies (88.1, 89.0 and 83.5 % in the crude extracts of

16 mussels, scallops and oysters, respectively). The results we obtained on the TSQ

17 (same instrument as in Gerssen et al.) are in agreement with these observations and

18 the suppression effect observed for AZA1 on the acidic method was eliminated when

19 the alkaline method was used. The suppression effect in the analysis of AZA1 has

20 been reported for numerous shellfish species on different instruments with various

21 chromatographic methods [19], [21], [25-26]. The results we obtained for AZA1 on

22 the QToF with the acidic method, which are consistent with a previous study

23 performed on this instrument [27] were within acceptable accuracies but signal

24 enhancement was observed when the alkaline method was used.

4.2.4. Column switching

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 [28-29]. The use of this method for the quantification of OA in shellfish using the QToF significantly improved the performances compared to the conventional acidic method. However, despite acceptable accuracy, precision, and sensitivity, the method is significantly longer than the acidic method.

4.3. Method performances

A fit for the purpose analytical method should meet the minimum performances for specific parameters set by international organizations [30-34]. 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.

4.3.1. Sensitivity

The limit of detections (LOD) observed for OA and AZA1 on both instruments using the acidic and alkaline methods are shown in Table 7. The alkaline method allowed for a two fold improvement in sensitivity 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 ten-fold more sensitive than the QToF for AZA1.

4.3.2. Accuracy

In the AOAC guideline [34], 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 [32] defines an acceptable accuracy as being 15% of the actual value except at the lower limit of quantification 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.

4.3.3. 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 as mass fraction.

(1) $RSDr = C^{-0.15}$

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

(2) $HORRATr = \dfrac{RSDr(found)}{RSDr(calculated)}$

Therefore, acceptable precisions for the extracts spiked with OA should have 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 defines 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. The injection of several shellfish extracts strongly suppressed the

response in the samples analyzed after the shellfish extracts. Therefore, when the

acidic method is used it is very important that samples are analysed randomly to

reduce bias in the quantification. When the alkaline and modified acidic methods were

evaluated this phenomenon was not observed.

5. Conclusions

We demonstrate the impact of matrix interference in LC-MS/MS analysis of low-level

toxins in molluscan shellfish, and strategies to overcome this. Contrasting results were

obtained on two different LCMS/MS instruments, even with the same source type

(ESI), using the same LC conditions (and samples), and with analyses performed by a

single analyst. Matrix suppression for AZA1 on the TSQ 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. Introduction of LC-MS/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.

1 **Acknowledgements**

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2   **Table 1: Accuracy and precision data (expressed as percentages) obtained on QToF and TSQ**
3   **instruments with the acidic method (values are the mean ± SD; n = 12-18).**
4
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6   **percentage of difference in response factor between a set of spiked solutions of *M. edulis* extracts**
7   **and methanol. A set of seven solutions equivalent to 0.063 to 3.5 mg/kg for OA and 0.010 to 1.150**
8   **mg/kg for AZA1 was injected in triplicate on each day.**
9
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12
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14   **percentage of difference in response factor between a set of spiked solutions of *M. edulis* extracts**
15   **and methanol. A set of seven solutions equivalent to 0.063 to 3.5 mg/kg for OA and 0.010 to 1.150**
16   **mg/kg for AZA1 was injected in triplicate on each day.**
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20   **SD; n =12).**
21
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24
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1   Table 1
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Analyte	Species	Acidic			
		TSQ		QToF	
AZA1	*M. edulis*	82.6	± 7.8	102.7	± 11.3
	C. gigas	83.1	± 4.5	104.6	± 7.8
	O. edulis	69.8	± 6.8	101.2	± 3.6
	E. siliqua	73.5	± 7.3	101.1	± 5.4
	P. maximus meat	79.3	± 13.6	103.3	± 5.5
	P. maximus gonad	64.2	± 3.6	97.1	± 3.1
OA	*M. edulis*	108.0	± 8.4	130.9	± 7.7
	C. gigas	102.4	± 3.2	114.6	± 16.4
	O. edulis	110.9	± 8.3	130.5	± 18.1
	E. siliqua	94.3	± 6.7	119.3	± 12.7
	P. maximus meat	98.3	± 3.5	119.7	± 23.3
	P. maximus gonad	101.3	± 5.1	125.9	± 11.0

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3 Table 2

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

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

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4 Table 4

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

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6 Table 5

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

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4 Table 6

Shellfish species	Mean OA recovery ± SD (n=3)
M. edulis	95.1 ± 11.4
C. gigas	101.4 ± 10.2
O. edulis	90.4 ± 5.7
E. siliqua	86.5 ± 8.6
P. maximus meat	93.5 ± 6.7
P. maximus gonad	102.6 ± 10.9

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13 Table 7

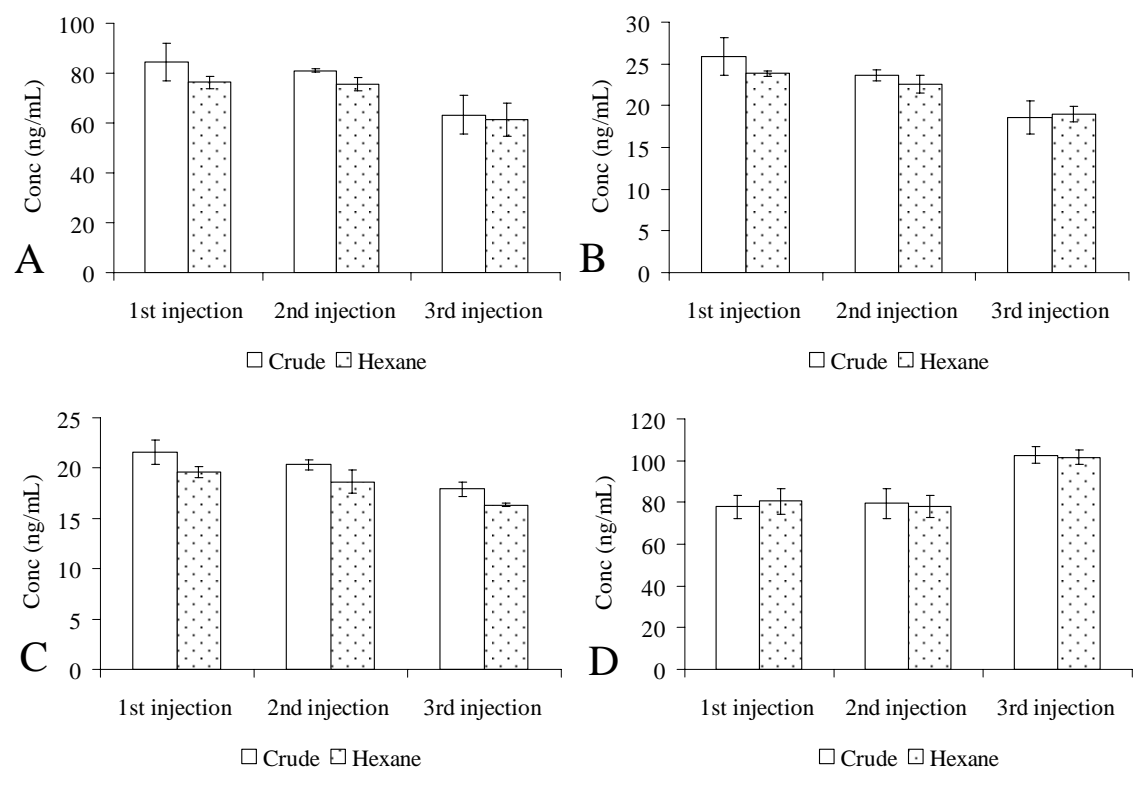|	Acidic		Alkaline	
	TSQ	QToF	TSQ	QToF
AZA1	0.3	3	0.5	5
OA	10	20	5	10

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