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## Approaches to evaluate matrix effects in the LC-MS analysis of three regulated lipophilic toxin groups in mussel matrix (*Mytilus edulis*)<sup>\*</sup>

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### Abstract

Liquid chromatography coupled to mass spectrometry (LC-MS) is seen as an integral part of methods of choice for the replacement of animal tests in the determination of lipophilic shellfish toxins. However, these techniques are prone to matrix effects that need to be considered when developing and validating methods. The analysis of shellfish is a challenging task due to the complexity of the shellfish matrix and the number of shellfish species encountered in monitoring laboratories. Therefore, it is crucial that the cause and the extent of matrix effects is fully understood in order to apply corrective measures to the analytical method and to develop efficient sample clean-up steps. This paper presents different approaches to evaluate matrix effects associated with the analysis of okadaic acid (OA), azaspiracid-1 (AZA1) and pectenotoxin-2 (PTX2) in cooked and raw mussel flesh. Post extraction addition and standard addition experiments were carried out, and analysed using various LC-MS methods. Gradient and isocratic elution were compared and ultra performance liquid chromatography, using C8 and C18 Acquity BEH columns, was evaluated for the extent of matrix effects. When matrix effects were observed, OA and PTX2 were always prone to signal enhancement and AZA1 to signal suppression. For all the toxins studied, matrix effects were dependant on chromatographic conditions. UPLC separation using a C8 column significantly reduced matrix effects compared to the other conditions assessed. Furthermore, sample dilution has proven to be an efficient way of reducing matrix effects associated with OA analysis.

**Keywords:** LC-MS; UPLC-MS; matrix effects; OA; PTX2; AZA1

### Introduction

Marine toxins are compounds naturally produced by harmful algal blooms (HABs) and accumulate in shellfish due to their filter-feeding nature. The consumption of contaminated shellfish can result in severe illness; among them diarrhetic shellfish poisoning (DSP) is a frequently observed symptom around the world. Lipophilic toxins belonging to the okadaic acid (OA) group are associated with DSP outbreaks and toxins belonging to the pectenotoxin (PTX) group usually co-occur with the OA group as these groups of toxins can be produced by the same organisms e.g. *Dinophysis* species. Azaspiracid poisoning (AZP) symptoms are

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3 similar to those induced by DSP and are caused by toxins belonging to the azaspiracid group.  
4 In order to protect shellfish consumers, these toxins are regulated by the European Union  
5 (EU), which set maximum levels of toxins that should not be exceeded when placing shellfish  
6 products on the market (Anonymous, 2004). National reference laboratories (NRL) must  
7 conduct shellfish testing following the EU reference method as described in the legislation  
8 (Anonymous, 2005). The EU reference method for the determination of lipophilic marine  
9 toxins is the mouse bioassay (MBA) and in addition to ethical issues concerning the use of  
10 animals in laboratories, the MBA suffers from major drawbacks such as little information on  
11 toxin composition, risks of false positives and false negatives as well as non suitability for  
12 rapid sample turnaround in monitoring laboratories. Over the last decade, research groups  
13 have evaluated LC-MS equipped with electrospray (ESI) sources as an alternative method for  
14 the determination of lipophilic toxins (James et al., 1999; Draisci et al., 1999; Goto et al.,  
15 2001; Vale and Sampayo, 2002; MacKenzie et al., 2002; Fernandez Amandi et al., 2002;  
16 Ciminiello et al., 2003; Stobo et al., 2005; Miles et al., 2005; Aasen et al., 2005; McNabb et  
17 al., 2005). The use of LC-MS for the monitoring of biotoxins has a great potential for the  
18 replacement of MBA as long as appropriate quality control criteria are implemented to ensure  
19 precision, sensitivity, selectivity, reproducibility and robustness of the analytical method. The  
20 US food and drug administration (FDA) guideline for the validation of bioanalytical methods  
21 outlines the need for the evaluation of matrix effects to ensure that those parameters will not  
22 be compromised (Anonymous, 2001). Furthermore, a recent review on the validation and  
23 qualification of spectroscopy based analytical methods also addresses the issues of matrix  
24 effects within the method validation process (Careri and Mangia, 2006). The consequences of  
25 matrix effects are the over- or under- estimation of the true concentration of analyte present in  
26 the sample affecting both accuracy and precision of the analytical method. LC-MS using ESI  
27 ionisation is a technique prone to various matrix effects that are not fully understood.  
28 Furthermore, matrix effects are analyte dependant and highly variable which make them  
29 difficult to predict. The first observation of matrix effects was reported in 1993 (Tang and  
30 Kebarle, 1993) and attributed to the competition between analytes and co-eluting matrix  
31 components. Later, King et al. (King et al., 2000) described matrix effects through several  
32 experiments to assess the influence of gas phase and solution phase processes in signal  
33 suppression mechanisms. The authors suggested that the release of neutral molecules into the  
34 gas phase does not seem to be the cause of matrix effects and that the main cause of ionisation  
35 suppression in biological extracts are likely to be induced by changes in the properties of the  
36 droplets due to the presence of non volatile solutes. While signal suppression has been  
37 reported in numerous papers and appears to be frequently encountered when analyses of  
38 biological samples are performed, observations of signal enhancement are much scarcer.

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46 The present study focussed on the assessment of matrix effects associated with the  
47 analysis of OA, AZA1 and PTX2 in mussel matrices and on how chromatographic parameters  
48 affected the degree of matrix effects observed. Matrix effects were assessed using post  
49 column infusion, post extraction addition to check the influence of analyte/matrix ratio and  
50 standard addition. Because post column infusion requires acquisition with a stable ESI spray a  
51 step gradient method was developed and compared with conventional gradient methods. In  
52 addition, the influence of solvent to sample ratio (SSR) employed for the extraction procedure  
53 as well as the introduction of a gentle heat treatment of the shellfish flesh in place in some  
54 NRLs (e.g. Denmark and Germany) were evaluated for the degree of matrix effects.  
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## 59 **Materials and methods**

### 60 *Solvents and reagents*

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Acetonitrile and methanol were purchased as Pestiscan-grade solvents from Labscan (Dublin, Ireland). Formic acid and ammonium formate were obtained from Sigma-Aldrich (Steinheim, Germany). Water was obtained from a reversed-osmosis purification system (Barnstead, Dublin, Ireland). OA and PTX2 certified reference materials (CRM) were purchased from the National Research Council (Halifax, Canada). The AZA1 standard solution was purified at the Marine Institute from naturally contaminated mussels.

#### *HPLC and UPLC conditions*

HPLC and UPLC analyses were performed using a Waters Acquity system. A binary mobile phase was used, with phase A (100 % aqueous) and phase B (95 % aqueous acetonitrile), both containing 2 mM ammonium formate and 50 mM formic acid.

The HPLC separation was achieved using a BDS Hypersil C<sub>8</sub> column (50 x 2.1 mm, 3 µm particle size; guard column, 10 x 2.1 mm, 3 µm particle size). The flow rate was set at 0.25 mL/min and a volume of 5 µL was injected into the LC system. The column temperature was 25 °C and the sample temperature was 5 °C. A gradient elution was employed, starting with 30 % B, rising to 90 % B over 8 min, held for 0.5 min, then decreased to 30 % B in 0.5 min and held for 3 min to equilibrate at initial conditions before the next run started. Alternatively, a step gradient elution using acidic mobile phases (as above) was carried out starting with 57% B until 4.5 min, rising to 75 % B over 0.5 min, held for 10 min, decreased to 57 % in 0.5 min and held for 3 min before the next injection.

The UPLC separation was achieved on an Acquity UPLC BEH C<sub>18</sub> column (100 x 2.1 mm, 1.7 µm particle size, in-line filter 0.2 µm) and on an UPLC BEH C<sub>8</sub> column (100 x 2.1 mm, 1.7 µm particle size, in-line filter 0.2 µm) (Waters Co, Hartford, MA). The flow rate was set at 0.4 mL/min and a volume of 5 µL was injected into the LC system. The column temperature was 30 °C and the sample temperature was 5 °C. The gradient started at 30 % B, raised to 90 % B in 3 min, and was held there for 1.5 min. After decreasing to 30 % B in 0.1 min the system was equilibrated for 2 min before the next run started. A total run time of 6.6 min was achieved.

#### *MS systems and conditions*

Two MS detection systems were used for quantitative LC-MS analysis. A triple quadrupole (QqQ) Quattro Ultima and a hybrid quadrupole-time of flight Q-ToF Ultima (Micromass Ltd., Manchester, UK) both equipped with a z-spray ESI source. Multiple-reaction-monitoring (MRM) acquisition mode was used for quantification using the triple quadrupole, analysing two transitions for OA and AZA1 and one fragment ion for PTX2. Monitored transitions were as follows (precursor > fragment): OA 803.5>255.5 and 803.5>803.5 in negative ionisation mode; AZA1 842.5>654.4 and 842.5>672.4 and PTX2 876.5>823.5 in positive ionisation mode. The cone voltages were 40 V and 60 V in negative and positive ionization mode respectively. The collision energy was set at 50 eV for the three analytes. The acquisition mode used for Q-TOF quantification was fragment ion scan, where the precursor ion was isolated by the first quadrupole, fragmented in the collision cell, and the final fragmentation spectrum obtained by the TOF. The same precursor ions as those reported for the triple quadrupole were chosen. The cone voltages were 50 V and 90 V and the collision energies were 40 eV and 50 eV in negative and positive ionisation mode, respectively.

#### *Shellfish homogenate preparation and heat treatment conditions*

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3 Toxin free mussels (*Mytilus edulis*) were collected from various locations around Ireland. The  
4 mussels were shucked and their flesh homogenised in a kitchen blender. All experiments  
5 described hereafter were carried out using the pooled homogenate. Heat treated flesh was  
6 obtained by immersing a closed centrifuge tube containing the homogenate (2 and 10 g for  
7 standard addition and post extraction addition respectively) in a 80 °C water bath for 10 min.  
8 This approach was taken in order to avoid water loss and the subsequent increase in matrix  
9 content concentration.  
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### 12 *Post column infusion*

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15 Post column infusion was carried out using the step gradient conditions with Q-TOF  
16 detection. A standard solution of AZA1 (700 ng/ml) was infused using a T piece while 5 µl of  
17 a blank mussel flesh extract (SSR 10, i.e. 2 g extracted into 20 ml) was injected.  
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### 20 *Post-extraction addition (PEA) – Influence of analyte/matrix ratio*

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22 The PEA-extraction was carried out by weighing aliquots (10 g) of mussel flesh into a 50 ml  
23 centrifuge tube and 10 ml of methanol (100%) were added. The samples were homogenised  
24 for 2 min using a multitube vortex mixer (Alpha Laboratories, V400) and were blended at  
25 high speed (11,000 rpm) with an Ultraturrax™ (IKA) for 1 min. Then, the tubes were  
26 centrifuged for 15 min at 4500 rpm before the supernatant was decanted. The SSR for these  
27 extracts was 1 ml/g. For further analysis 10 extracts were combined and used as pooled  
28 extract. Extracts of raw and heat-treated shellfish tissue were used to prepare the samples for  
29 the post extraction addition. In order to reach different matrix strengths various amounts of  
30 pooled extract were transferred into a 10 mL volumetric flask. Aliquots of 0.4 mL of standard  
31 stock solution were spiked into the extracts and the volume was completed up to the mark  
32 with methanol. Aliquots were filtered into HPLC vials using 0.2 µm filters. The concentration  
33 in the spiked samples ranged from 16.6 to 18.3 ng/mL for OA, PTX2 and AZA1. The overall  
34 variability (including procedural and instrumental variability) was assessed by adding 0.4 ml  
35 of stock solution to 9.6 ml of methanol (n=5). These solutions were quantified using  
36 calibration standards and used to calculate the matrix effects observed in extracts. Two  
37 aliquots (10 ml) of extracts of raw and heat-treated mussels (SSR 1) were inserted into glass  
38 vials and dried using an oven at 105°C. After 15 hours the dry residues were determined  
39 gravimetrically. The amount of dry residue in extracts (SSR 1) of raw and heat-treated  
40 mussels were 38.6 ± 3.1 mg/ml and 41.9 ± 0.1 mg/ml respectively.  
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### 45 *Standard addition*

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48 Mussel homogenate aliquots (2 g) were weighed into 50 mL centrifuge tubes and 6 mL of  
49 methanol (100%) were subsequently added. Samples were homogenised using a multitube  
50 vortex mixer for 1 min and centrifuged (Analytica Jouan, CR 422) at 4500 rpm for 5 min. The  
51 supernatant was decanted into a 20 mL volumetric flask. Then, 6 mL of methanol were added  
52 to the remaining pellet and the homogenization and centrifugation steps were repeated. The  
53 supernatant was added to the volumetric flask. An additional 6 mL of methanol were added to  
54 the pellet, blended using an Ultraturrax at 11,000 rpm for 1 min and subsequently centrifuged.  
55 The supernatant was transferred to the volumetric flask and the volume was completed to the  
56 mark with methanol. A SSR of 10 mL/g was obtained.  
57

58 Extracts of raw and heat-treated shellfish tissues were used to prepare the samples for the  
59 standard addition. The solutions were prepared in 10 mL volumetric flasks containing 9 mL  
60 of shellfish extract. Increasing volumes of standard stock solution were then added to the

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3 flasks and the volume was completed up to the mark with methanol. In addition, another set  
4 of solution was prepared by halving (4.5 mL of extract in 10 mL flask) the matrix strength.  
5 The solutions were filtered using 0.2 µm filters (Schleicher & Schuell) into HPLC vials for  
6 the analysis. The concentrations of the standard addition solutions ranged from 4 to 33 ng/mL  
7 for OA and PTX2, and from 11 to 91 ng/mL for AZA1. Reference solutions were prepared by  
8 spiking varying volumes of standard stock solution (100, 200 and 400 µl) in methanol and  
9 quantified using calibration standards. Two Aliquots (10 ml) of extracts of raw and heat-  
10 treated mussels were inserted into glass vials and dried using an oven at 105°C. After 15  
11 hours the dry residues were determined gravimetrically. The amount of dry residue in extracts  
12 (SSR 10) of raw mussel and heat-treated mussels were  $7.25 \pm 0.25$  mg/ml and  $7.38 \pm 0.48$   
13 mg/ml respectively.  
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## Results

### *Matrix effects associated with AZA1*

Figure 1 shows an example of chromatograms obtained for an AZA1 standard solution at 75.3 ng/mL and for the post column infusion of AZA1 when a blank mussel extract (SSR 10) was injected. The chromatograms were acquired on the Q-Tof following a HPLC step gradient elution. In these conditions, AZA1 eluted at 11.06 min (Figure 1A) and no signal disturbances seemed to take place in this region (Figure 1B). The post column infusion of AZA1 showed that, when the step gradient was raised to high organic mobile phase composition several components that lead to strong signal enhancement are eluting between 6.36 to 9.86 min. As the compounds all elute before AZA1, the results suggest that AZA1 analysis in these conditions is not affected by matrix effects. This was subsequently checked by standard addition of AZA1 in mussel matrix with a SSR of 10. The slopes, standard deviations (SD) and matrix effects are shown in Table 1.

The response observed for AZA1 in extract of raw mussel was 22.3 % lower than the response observed in methanol (Table 1). The post-extraction addition of AZA1 demonstrated that significant matrix effects are taking place at low matrix strength (Figure 2B). This is in agreement with the standard addition experiment as ca. 20 % signal suppression took place in solution with a dry residue of 7 mg/mL, equivalent to the dry residue of the SSR 10 mussel extract. This suggests that dilution of the matrix will have little effect on the degree of matrix effects observed. Table 2 shows that indeed, dilutions of the extract did not provide a significant reduction of signal suppression in HPLC and UPLC-C<sub>18</sub> analyses on the QqQ. However, analysis of AZA1 on the QqQ using UPLC equipped with a C<sub>8</sub> column demonstrated that matrix effects were eliminated in all extracts.

### *Matrix effects associated with PTX2*

In HPLC step gradient conditions with Q-Tof detection, standard addition of PTX2 in extracts of raw mussels with a SSR of 10 resulted in 15.9 % signal enhancement when compared to the signal obtained in methanol (Table 1). The post-extraction addition experiment in the same conditions demonstrated that signal enhancement is taking place with solutions of low matrix strength, as shown in Figure 2A. Signal enhancement was reduced as the matrix strength increased and signal suppression was observed in extracts exceeding dry residues of ca. 25 mg/ml. PTX2 analysis using QqQ and UPLC equipped with a C<sub>8</sub> column in extracts with a SSR of 10 completely overcame matrix effects as no significant differences between the standard solution slope and the standard addition slope were observed, as shown in Table 2.

### *Matrix effects associated with OA*

The results obtained from the post-extraction addition of OA in extracts of raw and heat-treated mussel flesh analysed by gradient HPLC-QqQ are shown in Figure 3. The pattern observed for both matrices were similar and therefore, heat treatment of mussel flesh does not appear to reduce the signal enhancement observed for OA analysis. Furthermore, Figure 3 suggests that the highest degree of matrix effects was obtained for extracts with the same amount of dry residue as extract of SSR of 10, and that the matrix effects observed for an extract of SSR 20 would be reduced. This was also observed in standard addition experiments analysed by gradient HPLC as well as UPLC equipped with a C<sub>8</sub> column and with a C<sub>18</sub>



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3 column, as shown in Table 2. Furthermore, analyses in UPLC conditions always led to less  
4 matrix effects than the HPLC conditions. OA analyses were less prone to signal enhancement  
5 in diluted extracts (SSR 20) of raw mussel flesh when UPLC with a C<sub>8</sub> column or a C<sub>18</sub>  
6 column was used, as no significant differences were observed between the standard addition  
7 and the standard curve slopes.  
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## 10 Discussion

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12 In the present study we demonstrated that the choice of analytical conditions for the analysis  
13 of lipophilic toxins in mussels (*Mytilus edulis*) can have a major impact on the accuracy of the  
14 quantitative results. The evaluation of matrix effects was carried out using three approaches.  
15 The post column infusion is a dynamic way of assessing matrix effects since in given  
16 chromatographic conditions the retention times of compounds disturbing the analysis can be  
17 monitored. However, in our conditions, correlation between post column infusion and post  
18 extraction as well as standard addition experiments was difficult to establish. The post column  
19 infusion of AZA1 did not provide any evidence of matrix effects, while clear signal  
20 suppression was observed when the standard addition and the post extraction addition of  
21 AZA1 were performed.  
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24 Correlation between post extraction addition and standard addition experiments  
25 required the use of a “matrix strength figure”. It was chosen to express matrix strength as the  
26 concentration of dry residue in extracts since co-eluting non-volatile compounds are often  
27 associated with signal suppression in LC-MS (King et al., 2000). The ratios between the  
28 amount of dry residue obtained in standard addition and post extraction addition extracts were  
29 5.8 and 5.2 for raw and heat treated mussel flesh respectively, while the ratios of tissue  
30 homogenate used for the extractions were 5 for raw and heat treated flesh. This suggests that a  
31 single step extraction (used for the preparation of post extraction addition solutions) and a  
32 triple extraction (used for the preparation of standard addition solutions) are equally efficient  
33 towards non-volatile compounds recoveries and thus, post extraction addition and standard  
34 addition experiments could be directly compared assuming that non-volatile compounds are  
35 largely responsible for matrix effects.  
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38 The assessment of matrix effects associated with AZA1 analysis using post extraction  
39 addition and standard addition were in agreement. Standard addition in an extract with SSR of  
40 10 and post extraction addition in extract with similar concentration of non-volatile material  
41 to the standard addition extracts resulted in ca 22 % signal suppression.  
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44 When matrix effects associated with PTX2 analysis were assessed by post extraction  
45 addition a 7 % signal enhancement was observed in extracts with similar amount of dry  
46 residue than the standard addition solutions. The degree of matrix effects measured by the  
47 standard addition experiment exhibited a signal enhancement of 15.9 %. The difference  
48 observed (9 %) was not considered to be significant when taking into account the systematic  
49 variability of the analytical method.  
50

51 The results obtained from OA post extraction addition suggested that matrix effects  
52 can be reduced when the matrix strength of the extract decreases. This was confirmed in  
53 HPLC and UPLC analyses, where matrix effects were reduced significantly in diluted extracts  
54 of raw and heat treated mussel. This approach was not successful for AZA1 and PTX2 (Table  
55 2, Figure 2). The use of UPLC always reduced matrix effects associated with OA analysis. In  
56 addition, it was observed that the UPLC stationary phase seems to play a major role in the  
57 matrix effects associated with AZA1 and PTX2. Thus, the use of UPLC with a C<sub>18</sub> column for  
58 their analyses led to a higher degree of matrix effects than those observed in HPLC. This may  
59 be due to the higher capacity of retention of disturbing lipophilic compounds by the C<sub>18</sub>  
60 columns. The use of UPLC with a C<sub>8</sub> column proved to be the most suitable method for the

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3 quantification of OA, AZA1 and PTX2 in mussels. In addition to the gain in accuracy, UPLC  
4 also offers a more rapid sample turnaround as the time of analysis was halved compared with  
5 the HPLC method.  
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7 Matrix effects encountered in lipophilic toxins analysis from shellfish by LC-MS have  
8 not been extensively published. Stobo et al. (Stobo et al., 2005) reported the results from  
9 standard addition experiments where extracts of four shellfish species were spiked with OA,  
10 PTX2 and AZA1 at concentrations ranging from 1.5 to 50 ng/ml using 6 calibration solutions.  
11 The results obtained from extracts of raw mussel (*Mytilus edulis*) flesh led to 130 % signal  
12 enhancement for OA, 44 % signal suppression for AZA1 and 25 % signal suppression for  
13 PTX2 compared to the response in standard solutions. (Ito and Tsukada, 2002) investigated  
14 matrix effects associated with analyses of OA, DTX1, YTX and PTX6 in hepatopancreas of  
15 scallops by standard addition using three spiked solutions ranging from 50 to 500 ng/ml. Ion  
16 suppression was observed for all compounds ranging from 15 % (PTX6) to 33 % (OA)  
17 despite a liquid/liquid partitioning procedure with chloroform. Matrix effects associated with  
18 OA and AZA1 analysis were also evaluated in the course of the development of a UPLC  
19 method for the detection of 21 lipophilic toxins (Fux et al., 2007). The UPLC was equipped  
20 with the BEH C<sub>18</sub> column coupled to a last generation triple quadrupole (Waters Premier XE).  
21 The standard addition in extracts of raw mussels (*Mytilus edulis*) demonstrated signal  
22 suppression of 17 and 23 % for OA and AZA1 respectively. The differences observed may be  
23 due to the differences in source geometries or to differences in chemical composition of the  
24 shellfish flesh. Similarly to this study, heat treatment of the flesh reduced matrix effects  
25 associated with analysis of AZA1 using UPLC with a C<sub>18</sub> column.  
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## 30 Conclusions

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32 This study demonstrated the challenges associated with the validation of LC-MS methods for  
33 the quantification of lipophilic toxins. Matrix effects were strongly dependent on the  
34 chromatographic conditions. Both signal enhancement and signal suppression were observed  
35 depending on the toxin considered. Dilution of the matrix strength did provide a way to  
36 reduce matrix effects associated with OA analyses and was not successful with PTX2 and  
37 AZA1. In addition to the gain in analysis time, the UPLC method with a C<sub>8</sub> column showed  
38 the least matrix effects towards the lipophilic toxins studied.  
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43  
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22 Figure 1: Examples of (A) AZA1 standard and (B) AZA1 post column infusion  
23 chromatograms obtained by step gradient HPLC with Q-ToF detection.

24 Figure 2: Post extraction addition of PTX2 (A) and AZA1 (B) analysed using step gradient  
25 HPLC conditions and Q-TOF detection. Bold lines represent the precision obtained by  
26 duplicate injection of spiked matrix free solutions. The error bars were calculated from  
27 duplicate injection.  
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29 Figure 3: Post extraction addition of OA in HPLC gradient conditions with QqQ detection in  
30 extracts of raw and heat treated mussel flesh. Bold lines represent the precision obtained by  
31 duplicate injection of spiked matrix free solutions (n=5). The error bars were calculated on  
32 triplicate injections.  
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Table 1. Slopes, intercepts (Int.) and correlation coefficients ( $R^2$ ) obtained for a set of standard solution and standard addition solutions of AZA1 and PTX2 using the Hypersil C8 column with step gradient conditions and QTOF detection. Matrix effects (ME) are reported as the percentage of deviation of the slope obtained in raw mussel (RM) matrix from the slope obtained in standards. Standard deviations (SD) were calculated from four injections of standards and duplicate injections of standard addition solutions.

Toxin	Matrix	Slope $\pm SD$	Int. $\pm SD$	$R^2$ $\pm SD$	Concentration range	Number of solutions	ME %
AZA1	RM SSR	8.032	-36.70	0.9950	12.9 – 103.9	5 + blank	- 22.3
	10	$\pm 0.032$	$\pm 0.84$	$\pm 0.0008$			
	Stds	10.344 $\pm 0.617$	-30.45 $\pm 6.00$	0.9985 $\pm 0.0003$	1.5 – 149.9	7	
PTX2	RM SSR	2.184	-2.40	0.9938	10.7 – 89.3	5 + blank	15.9
	10	$\pm 0.098$	$\pm 0.22$	$\pm 0.0011$			
	Stds	1.884 $\pm 23.1$	-3.77 $\pm 0.53$	0.9971 $\pm 0.0018$	0.9 – 91.3	7	

Table 1. Slopes, intercept (Int), correlation coefficients ( $R^2$ ) and percentage of matrix effects (ME) observed for OA, AZA1 and PTX2 added to extracts of heat-treated mussels (HM) and raw mussel (RM) flesh and standards using separations by HPLC with a  $C_8$  column and UPLC with a  $C_8$  or  $C_{18}$  column. Detection was performed on the Quattro Ultima (QqQ).

	Matrix	HPLC $C_8$ <sup>1</sup>				UPLC $C_8$ <sup>2</sup>				UPLC $C_{18}$ <sup>3</sup>			
		Slope	Int.	$R^2$	ME %	Slope	Int.	$R^2$	ME %	Slope	Int.	$R^2$	ME %
OA	RM SSR	116.0±	-135.0	0.9982	<b>50.4</b>	78.0	8.3	0.9969	<b>21.2</b>	92.1	10.9	0.9971	<b>19.3</b>
	10	6.7	±3.7	±0.0019		±8.1	±13.6	±0.0023		±5.8	±18.1	±0.0032	
	RM SSR	104.2±	33.3	0.9926	<b>35.0</b>	70.5	55.3	0.9975	<b>9.5</b>	81.6	99.1	0.9976	<b>5.7</b>
	20	3.6	±11.4	±0.0066		±6.6	±86.7	±0.0020		±8.5	±59.6	±0.0028	
	HM SSR	115.0±	-43.2	0.9961	<b>49.0</b>	85.3	-97.8	0.9956	<b>32.5</b>	98.3	-111.9	0.9942	<b>27.2</b>
	10	17.9	±111.0	±0.0006		±5.5	±24.5	±0.0042		±5.8	±2.7	±0.0050	
	HM SSR	99.0 ±	88.5	0.9973	<b>28.3</b>	75.7	70.1	0.9950	<b>17.6</b>	90.6	99.7	0.9932	<b>17.4</b>
20	1.9	±33.7	±0.0022	±9.8		±65.1	±0.0041	±6.4		±56.7	±0.0040		
Std	77.2 ±	-13.7	0.9987		64.4	14.3	0.9994		77.2	57.6	0.9946		
		9.7	±152.1	±0.0016		±5.5	±121.4	±0.0002		±3.9	±140.4	±0.0071	
AZA1	RM SSR	1321.1	149.2	0.9997	<b>-9.3</b>	694.8	29.5	0.9994	<b>-2.4</b>	667.4	393.2	0.9997	<b>-34.4</b>
	10	±126.9	±3.7	±0.0003		±49.7	±437.1	±0.0003		±62.1	±818.2	±0.0004	
	RM SSR	1189.5	2838.8	0.9987±0	<b>-18.4</b>	692.5	850.2	0.9996	<b>-2.7</b>	744.1	938.3	0.9990	<b>-26.8</b>
	20	±13.1	±112.9	.0002		±45.4	±251.7	±0.0003		±101.1	±31.6	±0.0010	
	HM SSR	1196.2	-49.1	0.9995±0	<b>-17.9</b>	712.1	115.2	0.9994	<b>0.1</b>	825.1	-377.5	0.9779	<b>-18.9</b>
	10	±17.9	±859.1	.0005		±62.0	±116.7	±0.0005		±26.8	±1017	±0.0094	
	HM SSR	1232.0	1576	0.9989±	<b>-15.4</b>	715.6	157.5	0.9997	<b>0.1</b>	885.6	-469.4	0.9981	<b>-12.9</b>
20	±107	±172	0.0011	±74.3		±563.8	±0.0003	±28.8		±726.0	±0.0000		
Std	1456.8	-1947	0.9995±		711.7	-497.9	0.9995		1016.9	-1095	0.9967		
	±132	±530	0.0001		±46.4	±384.3	±0.0004		±76.6	±1109	±0.0052		
PTX2	RM SSR	1119.9	-580.8	0.9989±0	<b>45.3</b>	364.1	169.6	0.9994	<b>-1.5</b>	432.6	-221.9	0.9962	<b>92.5</b>
	10	±30.0	±165.4	.0012		±16.6	±52.5	±0.0005		±40.3	±124.7	±0.0024	
	RM SSR	1083.6	423.6	0.9981±0	<b>40.6</b>	433.3	-5.6	0.9995	<b>17.2</b>	380.6	113.1	0.9970	<b>69.4</b>
	20	±22.2	±89.8	.0008		±31.4	±117.9	±0.0002		±33.4	±86.0	±0.0034	
	HM SSR	1188.2	-576.3	0.9993±0	<b>54.2</b>	393.1	71.0	0.9978	<b>6.4</b>	397.5	-696.6	0.9942	<b>76.8</b>
	10	±196	±440.0	.0001		±33.5	±58.6	±0.0028		±39.2	±86.3	±0.0033	
	HM SSR	1042.2	347.8	0.9996±	<b>35.2</b>	439.4	21.5	0.9990±0	<b>18.9</b>	399.8	-62.9	0.9944	<b>77.9</b>
20	±60.3	±33.7	0.0002	±32.7		±40.1	.0012	±102.4		±311.5	±0.0012		
Std	770.7 ±	119.4	0.9994±		369.7	126.1	0.9996±0		224.7	167.7	0.9923		
	80.8	±404.8	0.0007		±24.1	±130.7	.0002		±29.9	±291.7	±0.0106		

<sup>1</sup> Standard slope was obtained from triplicate injections of a set of 7 solutions and slopes in mussel matrices were obtained from triplicate injections of a set of five solutions

<sup>2</sup> Standard slope was obtained from four replicate injections of a set of 7 solutions and slopes in mussel matrices were obtained from triplicate injections of a set of five solutions

<sup>3</sup> Standard slope was obtained from six replicate injections of a set of 7 solutions and slopes in mussel matrices were obtained from duplicate injections of a set of five solutions







