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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

Jane Kilcoyne [a,*], Elie Fux [b]

[a] Marine Institute, Renville, Oranmore, Co. Galway, Ireland
[b] U.S. Food and Drug Administration, Gulf Coast Seafood Laboratory, Dauphin Island, AL 36528, USA

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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.

* Corresponding author. Tel.: +353 91 387376.
E-mail address: jane.kilcoyne@marine.ie (J. Kilcoyne).

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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