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Application Note

A Fast and Sensitive UPLC-MS/MS Method for the Detection of Lipophilic Marine Biotoxins in Shellfish

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Abstract

This application note demonstrates a method designed to cover all of the regulated compounds below their reporting level.

Introduction

Marine biotoxins include several families of hydrophilic and lipophilic compounds with varying degrees of toxicity. They can be found in saltwater environments around the world as a result of naturally occuring harmful algal blooms (HABs) which are very difficult, if not impossible, to predict or control. Filter feeding bivalve molluscs farmed for human consumption are unaffected by the toxins, but accumulate their metabolites in the hepatopancreas and flesh. If eaten, the concentrated toxins can cause a variety of illnesses in humans, and indeed are named for their effects – e.g. Diarrhetic, Amnesic and Paralytic Shellfish Poisoning (DSP, ASP, and PSP) toxins.

Due to the high potential risk to human health, shellfish harvests are subject to stringent testing for such compounds. If found above certain levels, harvesting must be suspended which can have severe economic consequences. Hence it is important for any analytical method used to be fast and accurate. However, the only currently 'official' prescribed method for the detection of these toxins in the EU is the mouse bioassay (MBA) where the effects of exposure to shellfish extracts on live mice are observed. Not only is this politically sensitive in today's society, the technique is not specific to any one particular toxin, and is not quantitative. In order to investigate alternative methods for the DSP toxins, BIOTOX, an EU funded project run under the Food Quality and Safety Priority (FP6-2003-Food-2A) was commissioned in 2005. The principal aims of the twelve European partner laboratories involved are to develop a reference multi-toxin method based on LC-MS and to use this to validate alternative, cost-effective monitoring methods such as immunoassays. The compounds of particular interest are those whose regulatory limits are outlined in EU Commission Decision 2002/225/EC (amendment to EU directive 91/942/EEC).

Aim of the Work

This note describes a method designed to cover all of the regulated compounds below their reporting level. For the large number of samples typically submitted for analysis and the potential for changes to the dispersity of toxins due to environment and climate, the method is applicable to many of the other lipophillic

toxins.

Shellfish extracts are a notoriously complex matrix, and ion suppression or enhancement in the mass spectrometer can be problematic, so part of this study was devoted to the investigation and reduction of matrix effects. To achieve this, advanced chromatographic techniques, together with state-of-the-art MS/MS instrumentation as outlined below were employed.

Waters ACQUITY UPLC is an advanced separation system which utilizes a 1.7 µm stationary phase particle size to improve resolution and peak shape in a shorter run-time. This offers enhanced selectivity and greater sensitivity of the detector, but means that the mass spectrometer is required to run much faster in order to maintain the integrity of the data.

Waters Quattro Premier XE Tandem Quadrupole Mass Spectrometer is equipped with traveling-wave ion guides, which enables very rapid clearing of the collision cell between MRM transitions. Not only does this drastically reduce cross-talk, but allows for the extremely short dwell and inter-scan delay times necessary for UPLC analyses. These features, as well as the proven robustness and high sensitivity of the ZSpray source and the ability to rapidly switch between ionization modes, make this the instrument of choice for such multi-analyte methods for all types of sample.

Experimental

Methods

Shellfish Extraction

Triple methanolic extraction was performed by weighing 2 g of sample in a 50 mL plastic centrifuge tube, to which 6 mL of methanol were subsequently added. The extracts were vortex mixed for 1 minute at 2,500 rpm and centrifuged for 15 minutes at 6,000 rpm. The supernatant was transferred into a 20 mL volumetric flask and the pellet was re-extracted in the same manner. The third extraction was carried out by adding an additional 6 mL of methanol and blending the extract at high speed (ultraturrax) at 11,000 rpm for 1 minute.

After the final centrifugation, the supernatant was transferred to the volumetric flask with the two previous extracts. For standard addition experiments, stock solutions of AZA 1 and OA ranging from 1 mg/mL to 30 ng/mL were prepared and added to the volumetric flasks. The volume was then completed up to the mark. The solutions were filtered using 0.2 mm filters prior to dispensing into capped LC vials ready for analysis.

LC Conditions

System:	Waters ACQUITY UPLC
Mobile phase A:	H_2O + 2 mM $CHOONH_4$ + 50 mM CHOOH
Mobile phase B:	95% aqueous MeCN + 2 mM $CHOONH_4$ + 50 mM CHOOH
Column:	ACQUITY UPLC BEH C_{18} 1.7 µm, 2.1 x 100 mm + 2 µm in-line filter
Flow rate:	0.4 mL/min
Injection volume:	10 µL
Column temp.:	30 °C
Gradient:	t = 0 min 30% B t = 3 min 90% B t = 4.5 min 90% B t = 4.6 min 30% B
Cycle time:	6.6 min

MS Conditions

System:	Waters Quattro Premier XE
Ionization mode:	ES+/ES capillary
Volt:	+/- 2.5kV
Desolvation gas:	850 L/h N_2 at 350 °C

Cone gas:	50 L/h N_2
Source temp.:	120 °C
Acquisition:	MRM mode
Cone V:	See Table 1
Collision energy:	See Table 1
Collision gas:	Ar at 4.5 e^{-3} mbar

Compound	MRM Transition	Mode	Cone Voltage/V	Collision Energy/Ev
GYM	508.3 > 392.4	positive	50	35
	508.3 > 490.4			24
Spx-13-desMeC	692.5 > 164.2	positive	50	60
	692.5 > 444.4			55
Carboxyhydroxy-YTX	1189.5 > 1109.5	negative	45	40
YTX	1141.5 > 1061.5	negative	40	55
	1141.5 > 925			55
1-Desulfo-YTX	1061.5 > 981.5	negative	45	40
45-OH-YTX	1157.5 > 1077.5	negative	40	55
Carboxy-YTX	1173.5 > 1094.5	negative	40	55
Homo-YTX	1155.5 > 1075.5	negative	40	55
Carboxyhomo-YTX	1187.5 > 1107.5	negative	40	55
45-OH-Homo-YTX	1171.5 > 1091.5	negative	40	55
OA & DTX2	803.5 > 255.2	negative	70	50
	803.5 > 113			65
DTX1	817.5 > 255.5	negative	70	65
	817.5 > 113			90
AZA1 & AZA1b	842.5 > 654.5	positive	50	55
	842.5 > 362			55
AZA2	856.5 > 672.5	positive	30	55
	856.5 > 654.5			45
AZA3	828.5 > 362	positive	50	55
	828.5 > 640.5			55
PTX2	876.5 > 823.5	positive	40	40
	876.5 > 212.5			50
PTX1	892.5 > 839.5	positive	40	25
PTX6	906.5 > 853.5	positive	40	25
PTX2sa & 7-Epi-PTX2sa	894.5 > 805.2	positive	40	40

Table 1. Confirmed analytes with MRM transitions and specific instrument parameters.

Software

The data were acquired using Waters MassLynx Software and processed using the TargetLynx Application Manager.

Results and Discussion

Due to the diversity in the chemistries of the various toxins as illustrated in Figure 1, the chromatographic method was designed not to be optimal for any particular class, but applicable to all the compounds of interest. Hence using the final, generic method, good separation was achieved for most compounds, although some co-elute or have broader peak widths than may ideally be desirable. The high selectivity of the MS/MS method enables easy differentiation of such peaks.

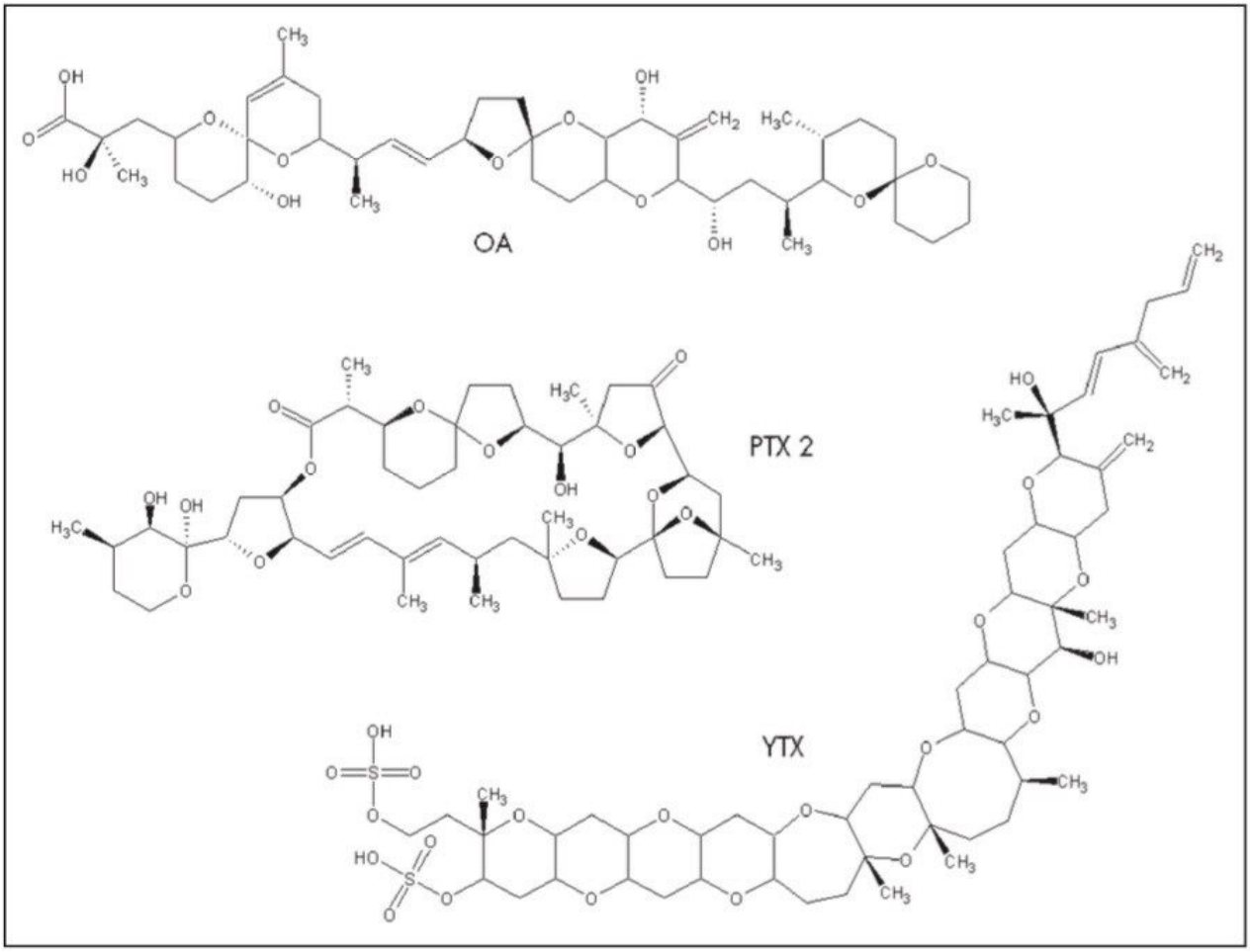

Figure 1. Example toxin structures: okadaic acid, pectenotoxin 2, and yessotoxin.

Figure 2 below shows example chromatograms of all the compounds analyzed, compiled from four samples. All compounds elute within 3.5 minutes, so with column re-equilibration time taken into account, a 6.6 minute cycle time between injections is enabled.

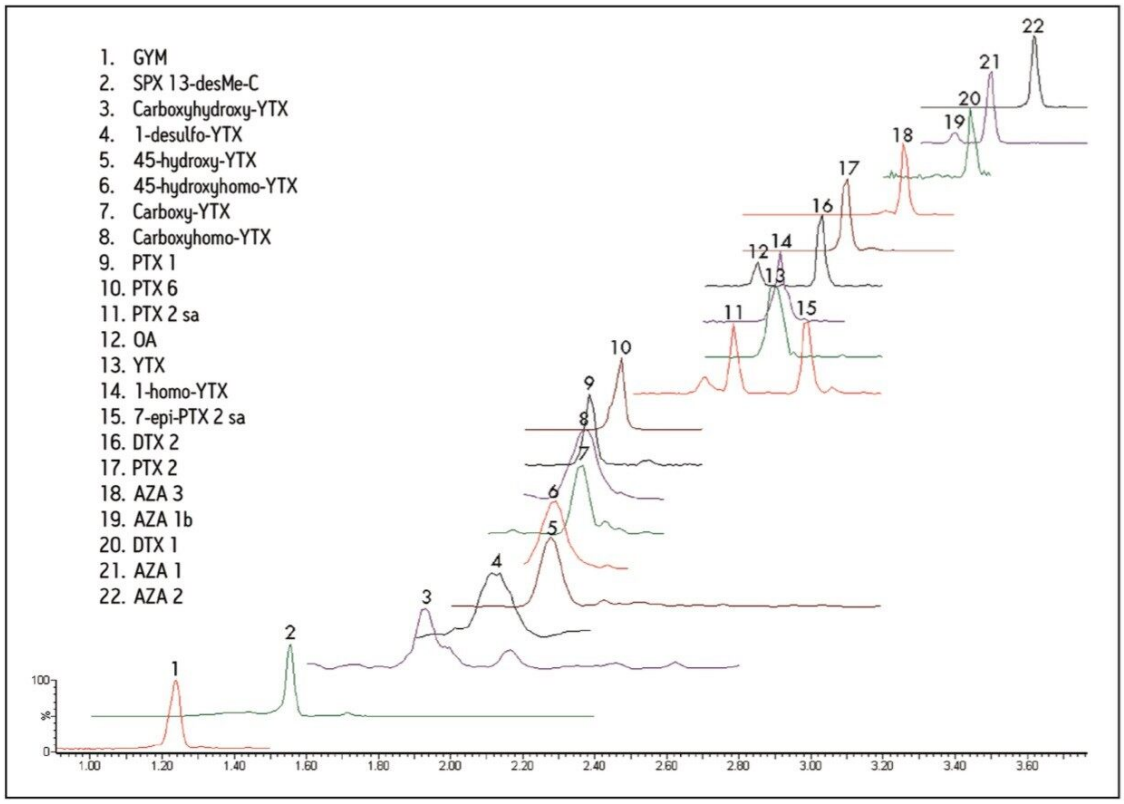

Figure 2. Example chromatograms compiled from four sample sets containing all confirmed toxins.

Quantification and Confirmation

The ability of the mass spectrometer to rapidly switch between transitions in both ionization modes enables sufficient data-points to be acquired for quantification across each peak. For the most important compounds, two transitions were selected to allow confirmation of quantified components by the relative abundance of the two product ions. TargetLynx Application Manager, an advanced software package containing standard quantitative as well as confirmatory tools, was employed to process the data.

Figure 3 of the TargetLynx results browser shows the calibration curve and results for okadaic acid in a sample set. The central overview bar highlights in red two samples containing trace amounts of yessotoxin, whose concentration cannot be confirmed by the product ion ratio, due to a very low result below the limit of quantification.

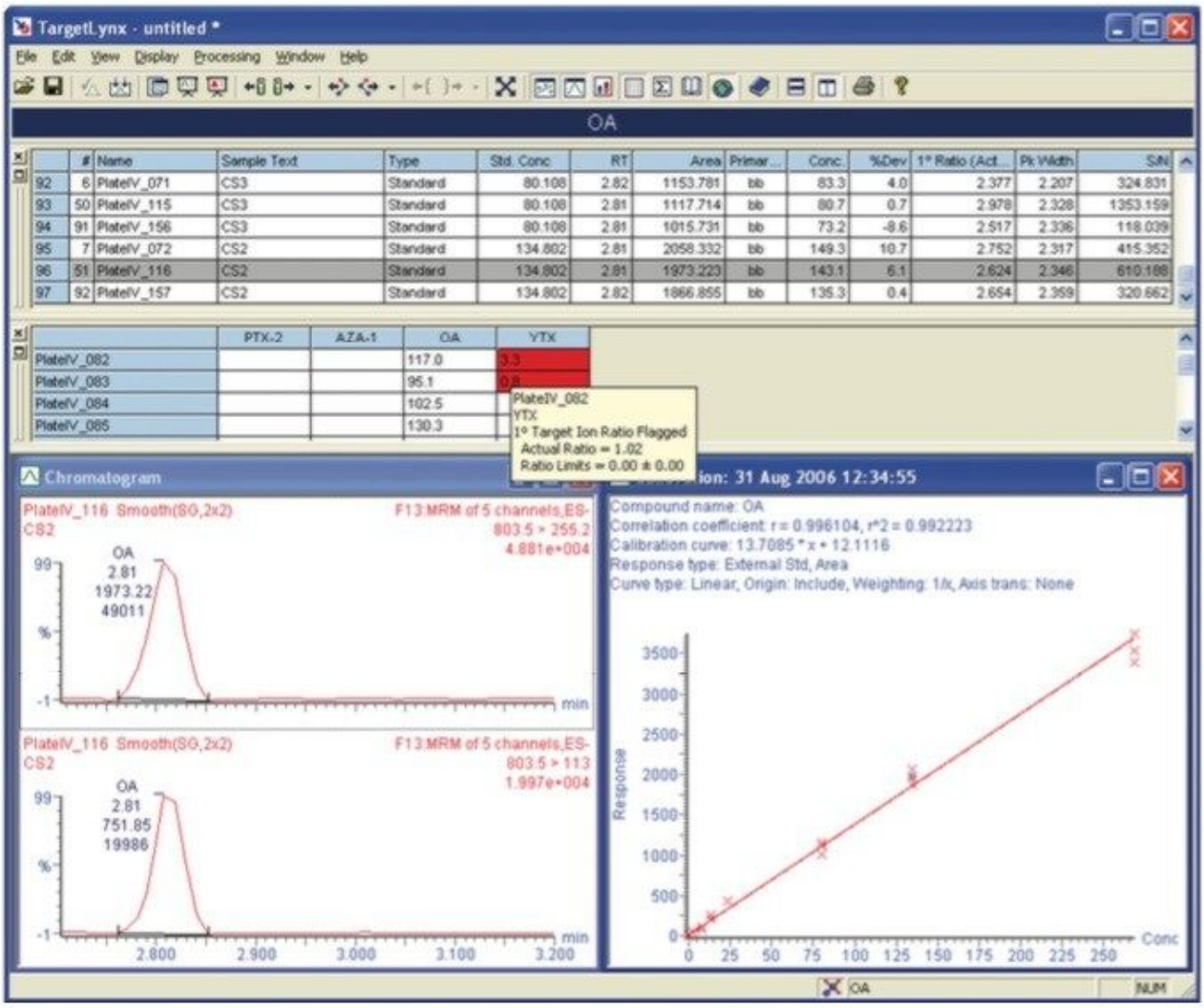

Figure 3. The TargetLynx report showing calibration curve for OA. Results highlighted in red are flagged when ion ratios are outside tolerance.

A certified reference material of mussel extract (CRMDSP-Mus-b), purchased from NRC, was used to determine the accuracy of the method. Duplicate analyses resulted in concentrations of 9.55 and 9.48 µg/g, indicating an average of 93.7% accuracy compared to the stated value of 10.1 ± 0.8 µg/g OA.

Table 2 overleaf summarizes the preliminary results, based on ~2,000 samples for the six known standard compounds.

Compound	Correlation coefficient	LOD (pg/mL)	LOQ (ng/mL)	Range (ng/mL)
OA	0.997 ± 0.0012	483.1	1.61	1.5-232
YTX	0.9969 ± 0.0009	336.3	1.12	2.8-56
AZA1	0.9996 ± 0.0004	32.8	0.11	0.4-77
PTX2	0.9993 ± 0.0007	47.7	0.16	0.5-96
GYM	0.9974 ± 0.0014	60	0.2	1.5-111
SPX 13-desMe-C	0.9966 ± 0.0037	22	0.07	1.0-80

Table 2. TargetLynx quantitation results for the five toxins of certified concentration and YTX, purified at Marine Institute.

Matrix Effects

When dealing with such complex matrices as shellfish extracts, it is imperative to consider any suppression or enhancement of analyte ionization due to co-eluting endogenous peaks. There are various methods of assessing this, and for this study, standard addition (matrix matched standards) and post-extraction addition of OA and AZA 1 were employed. Analyses were carried out on raw and cooked mussels and oysters in order to investigate how matrix effects can vary.

From the results of the standard addition experiments shown in Figure 4, it can clearly be seen for AZA 1, suppression due to the matrix as compared to solvent standards is very much dependent on the matrix, with raw mussels and oysters affected more than cooked.

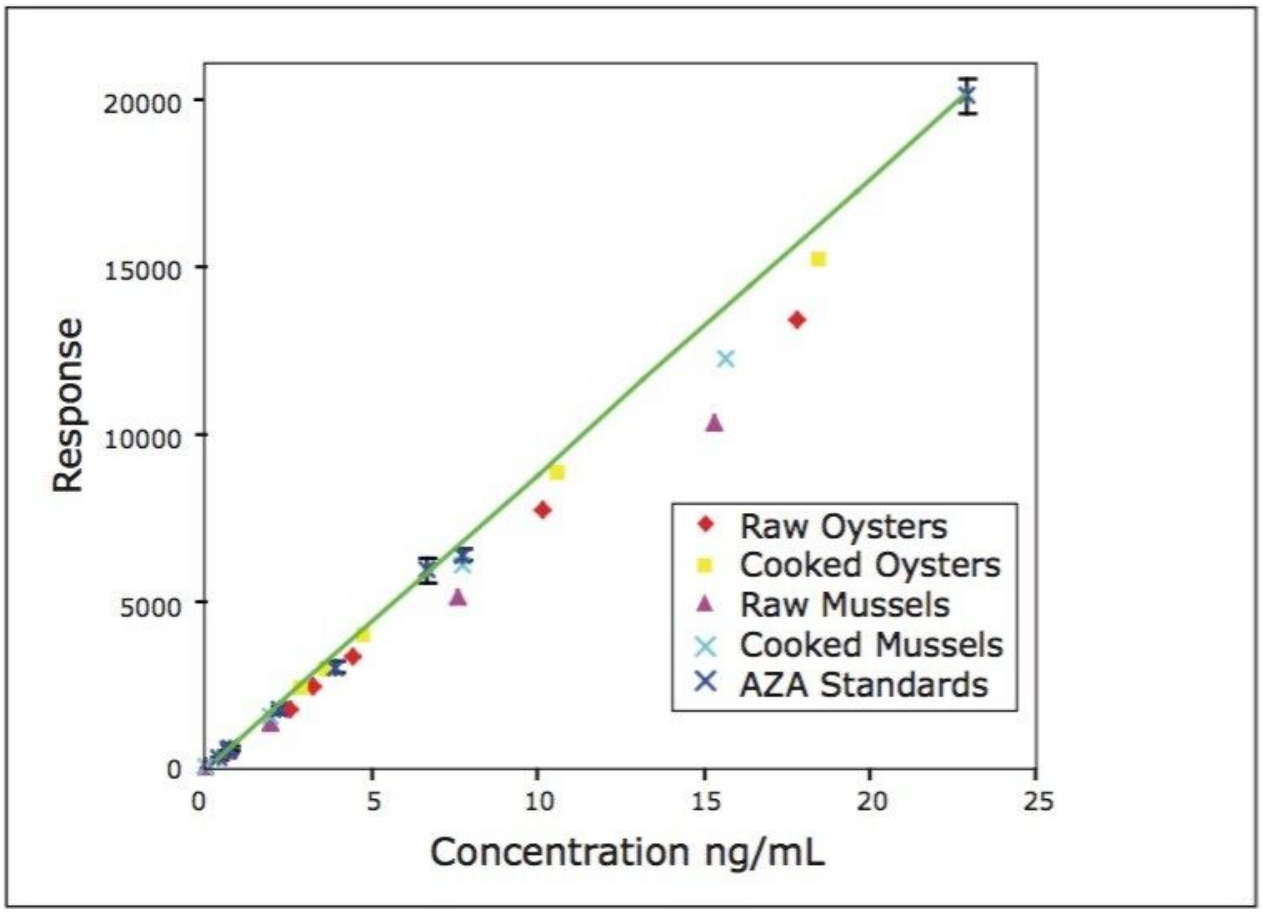

Figure 4. AZA 1 Standard addition results.

Figure 5 shows results for the post-extraction addition experiments. These demonstrate that suppression also becomes more problematic as the percentage of matrix increases (i.e. more sample or less extraction solvent).

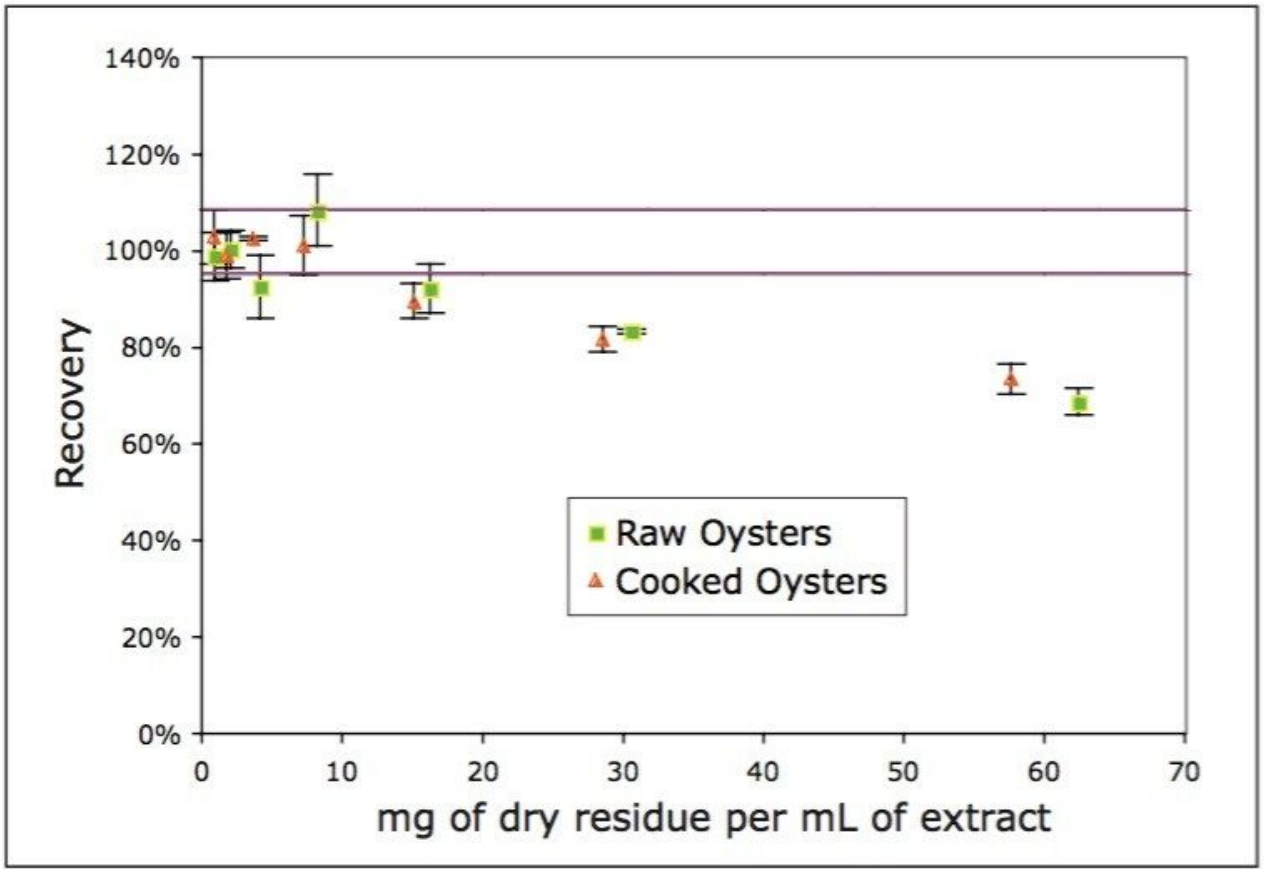

Figure 5. AZA 1 Post-extraction addition results.

From these two sets of analyses, it can be concluded that to minimize matrix effects, it is necessary to minimize the amount of matrix introduced. Using the methods described in this application note, this is easily achievable. With the high sensitivity shown, it is possible to perform the intensive extraction described, thus diluting the sample reducing the amount of matrix entering the source of the mass spectrometer. An extraction of 2 g flesh into 20 mL solvent is within the range shown not to suffer from matrix effects in this UPLC method.

Conclusion

- The UPLC-MS/MS method developed is fast and accurate.
- Quantitative and confirmatory transitions are included for the major analytes of interest.

- The ability of the Quattro Premier XE to perform multiple MRM transitions in both polarities allows analysis of many more toxins than are regulated in the EU.
- The high sensitivity of the method allows for more dilution and lower injection volume, hence matrix effects are minimized.

Acknowledgements

Certified reference materials of OA, PTX 2, YTX, GYM, and SPX 13-desMe-C were purchased from NRC, Canada. AZA1 was isolated from naturally contaminated mussels by Nils Rehmann at the Marine Institute.

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