A FAST AND SENSITIVE MULTI-ANALYTE UPLC-MS/MS METHOD FOR THE DETECTION OF DSP AND OTHER LIPOPHILIC MARINE BIOTOXINS IN SHELLFISH

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OVERVIEW

The high sensitivity and selectivity of LC-MS/MS have proven to be reliable and effective in many areas of analysis where validated quantitation and confirmation are required. Recent advances in the technologies involved show great potential for the technique in the field of biotoxin research, where the detection of shellfish toxins is becoming increasingly reliant on instrumental methods.

The diversity of structures and chemistries exhibited by these compounds, the extreme complexity of the matrices and the requirement for a fast and efficient analysis have given rise to considerable analytical challenges. The high resolution offered by Ultra-Performance LC helps to minimise co-elution of analytes and matrix and in turn reduces suppression effects in the mass spectrometer, increasing overall sensitivity. The latest generation of tandem-quadrupole mass spectrometers use novel ion optics and high performance electronics, enabling fast switching between MRM transitions and polarity switching, to allow for more analytes in a single run.

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 difficult to predict or control. Filter-feeding bivalve molluscs farmed for human consumption are unaffected by the toxins, but the parents and their metabolites accumulate 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 & 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 be very costly. Hence it is important for any analytical method used to be fast and accurate. However, the only currently 'official' prescribed method 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)

Described here is a method designed to cover all of the regulated compounds below their reporting level. Due to 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 should be fast, and applicable to as many of the other lipophillic toxins as possible. Shellfish extracts are a notoriously complex matrix, and ion suppression 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 are employed.

ACQUITY UPLC is an advanced separation system which utilises a 1.7 µm stationary phase particle size to improve resolution and peak shape in a shorter run-time[(1)]. This offers enhanced selectivity and greater sensitivity at the detector, but means that the mass spectrometer is required to run much faster in order to maintain the integrity of the data.

The Quattro Premier XE tandem quadrupole mass spectrometer is equipped with travelling-wave ion guides, which enable very rapid clearing of the collision cell between MRM transitions[(2)]. 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 Z-Spray source and the ability to rapidly switch between ionisation modes make this the instrument of choice for such multi-analyte methods for all types of sample.

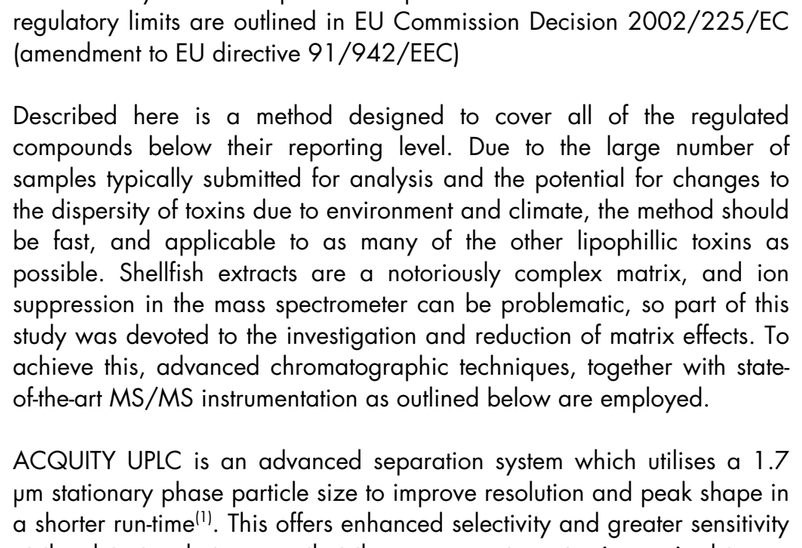

Figure 1. Waters Micromass Quattro Premier XE with Waters ACQUITY UPLC system

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 2500 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 µm filters prior to dispensing into capped LC vials ready for analysis.

This is the method suggested as the BIOTOX reference method for shellfish extraction by the Marine Institute.

LC Method

LC System:	Waters ACQUITY UPLC
Mobile Ph. A:	H_2O + 2mM CHO_2NH_4 + 50mM CHOOH
Mobile Ph. B:	95% aqueous MeCN + 2mM CHO_2NH_4 + 50mM CHOOH
Column:	ACQUITY UPLC BEH C_{18} 1.7 µm 2.1 x 100 mm with 2 µm in-line filter
Flow Rate:	0.4 mL/min
Injection Vol.:	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 Method

Instrument:	Micromass Quattro Premier XE tandem quadrupole mass spectrometer
Ionisation Mode:	ES+/ES-
Capillary V:	+/- 2.5kV
Desolvation Gas:	850L/h N_2 at 350°C
Cone Gas:	50L/h N_2
Source T:	120°C
Acquisition:	MRM mode
Cone V:	See table 1, below
Collision E:	See table 1, below
Collision Gas:	Ar at 4.5e-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. of confirmed analytes with MRM transitions and specific instrument parameters

RESULTS & DISCUSSION

Due to the diversity in the chemistries of the various toxins as illustrated in figure 2, 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. Figure 3 shows example chromatograms of all the compounds analysed, compiled from four samples.

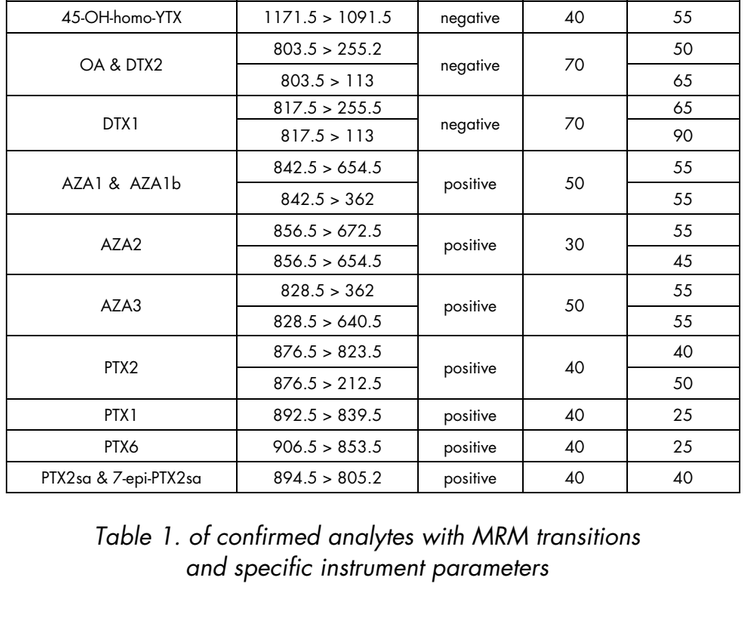

Figure 2. Example toxin structures - Okadaic Acid, Pectenotoxin 2 and Yessotoxin

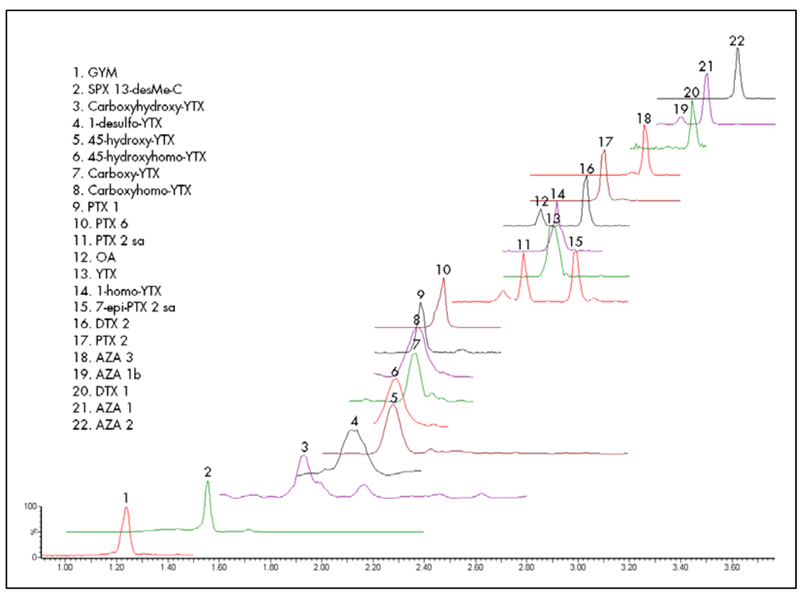

Figure 3. Example chromatograms compiled from 4 sample sets containing all confirmed toxins

One trace for each compound is shown here, although the acquisition method actually contains 31 transitions in 16 overlapping functions to include confirmatory ions for the most common toxins. This clearly shows the demands imposed on the mass spectrometer are considerable. In order to acquire sufficient data-points for quantitation across each peak, switching between polarities and the individual MRM transitions must be very rapid. This is effectively achieved using the T-Wave device in the collision cell[(2)] as demonstrated by the quantitation results shown in figure 4 below. The TargetLynx quantitation software used here includes additional features designed specifically for use in the food safety arena where results must be confirmed before reporting. In the example below, the measured concentration of OA in one sample is shown in red, indicating that the ratio of the primary (quantitation) and secondary (confirmatory) ions fall outside the specified tolerance of 10%, compared to standards.

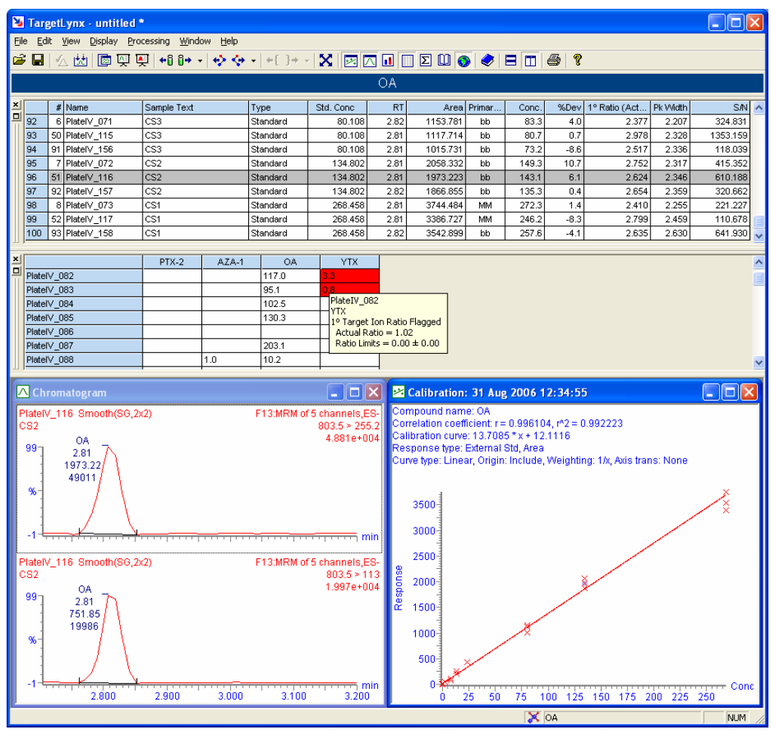

Figure 4. TargetLynx report showing calibration curve for OA. Results highlighted in red are flagged when ion ratios are outside tolerance.

In total, 22 toxins were analysed in the samples made available. Of these, five are available as certified standards, and AZA1 as a quality control standard. The quantitation results for these are shown in table 2. In order to investigate the accuracy of the method, a certified sample of mussel tissue (CRM-DSP-Mus-b) containing 10.1 ±0.8µg/g OA was purchased from NRC and analysed by the full method. Two separate extractions and duplicate analyses resulted in concentrations of 9.55 and 9.48µg/g, indicating an average of 93.7% accuracy.

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 six toxins of certified concentration

CONCLUSIONS

- **UPLC enables a fast analysis of multiple toxins**
- **The ability of the MS to switch rapidly between functions and polarities allows good, quantitative data to be acquired for all compounds, even when co-elution occurs**
- **All toxins are measurable considerably below their reporting level**
- **The method is efficient and accurate, and after validation could be suitable as a reference method**

References

1. Swartz M. E., UPLC™, An Introduction and Review, J. Liquid Chromatography and Related Technologies, 2005, 28(7-8), pp 1253-1263.
2. K. Giles, S. D. Pringle, K. R. Worthington, D. Little, J. L. Wildgoose and R. H. Bateman, *Rapid Communications in Mass Spectrometry*, 18 (2004) 2401.

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.

Samples containing the various YTX analogues were kindly shared by John Aasen of NSVS, Oslo and Anna Milandri of Centro Ricerche Marine, Cesenatico, Italy. PTX 1 & 6 contaminated scallop extracts were generously donated by Pr. Yasumoto of JFRL, Tokyo, Japan. The authors are very grateful for all these contributions, and also to NRC for the kind donation of a pre-certification sample of YTX standard.