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UPLC/MS/MS Method for the Routine Quantification of Regulated and Non-Regulated Lipophilic Marine Biotoxins in Shellfish

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APPLICATION BENEFITS

A rapid, robust method for the analysis of both regulated and non-regulated lipophilic marine biotoxins in shellfish. Compared to the conventional HPLC/MS/MS method, the speed of analysis increased 4-fold: (from 20 min to 5 min per analysis). This method is also able to meet the regulated levels of detection that can be used instead of the mouse bioassay.

WATERS SOLUTIONS

ACQUITY UPLC® System

ACQUITY UPLC BEH C_{18} column

Xevo® TQ-S Mass Spectrometer

TargetLynx™ Application Manager

QUANPEDIA™ Database

KEY WORDS

Shellfish, biotoxins, lipophilic, phycotoxins, mussels, clams, oyster, diarrhetic shellfish poisoning, DSP

INTRODUCTION

Consumption of shellfish (mussels, oysters, clams, etc.) contaminated with biotoxins can cause severe intoxications in humans, such as diarrhetic shellfish poisoning (DSP). Due to their lipophilic properties, DSP toxins are often classified as lipophilic marine biotoxins. Marine biotoxins are naturally produced by different types of phytoplankton and are, therefore, also named phycotoxins. The complexity of the lipophilic marine biotoxins lies in the variety of physiochemical properties, such as carboxylic acids, sulfonic acids, and amino and imino functionalities.

In the European Union (EU) legislation, various toxin groups are regulated and describe how these toxins should be monitored in official control programs. The lipophilic biotoxins that should be monitored, shown in Figure 1A, are okadaic acid (OA), dinophysistoxin-1, -2, -3 (DTX1, -2, -3), where DTX3 are the ester forms of OA, DTX1 and -2, respectively, pectenotoxin-1, -2 (PTX1, -2), yessotoxin (YTX), 45OH yessotoxin (45OH YTX), homo yessotoxin (homoYTX), 45OH homo yessotoxin (45OH homoYTX), azaspiracid-1, -2 and -3 (AZA1, -2, -3).[1]

Before July 2011, the official method was a bioassay based on the oral administration of shellfish meat to a rat or the intraperitoneal injection of a shellfish extract in mice. There were two main issues with this method: first, it was perceived as unethical; and second, the method was not scientifically robust

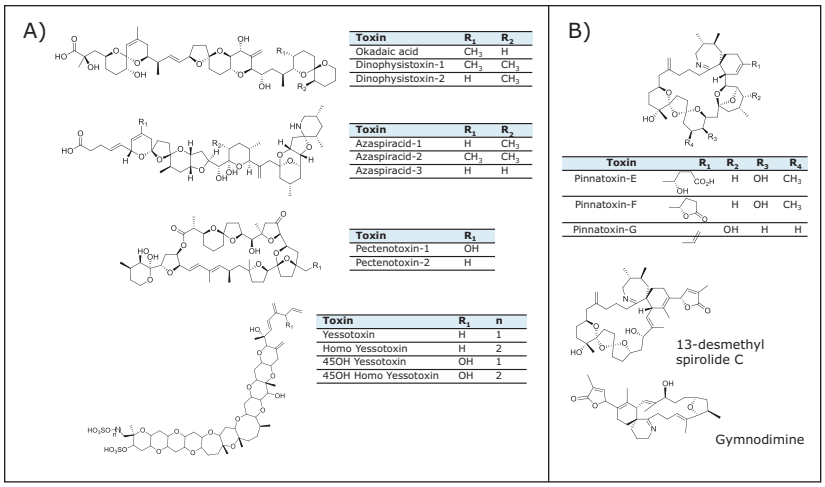

Figure 1. Chemical structure of a) regulated toxins and b) non-regulated cyclic imines.

enough to determine trace amounts of specific toxins. The presence of cyclic imines would cause a physical reaction using the bioassay – the result often fatal for the animal. Cyclic imines, shown in Figure 1B, are also classified as lipophilic marine toxins based on their physiochemical properties. Although these toxins are not currently regulated, the European Food Safety Authority (EFSA) has indicated that more data on toxicity, as well as their occurrence in shellfish, should be collected.

Since July 2011, the official method for control of shellfish on the presence of lipophilic marine biotoxins has been LC/MS/MS.[2] The EU reference LC/MS/MS method is based on a fixed extraction procedure followed by separation using conventional LC separation, with either an acidic mobile phase or alkaline mobile phase and detection by tandem quadrupole MS.

The aims of this study were to produce a much faster routine analysis than the conventional LC method under alkaline conditions, and include additional non-regulated compounds that are of interest to EFSA. In this application note, various classes of lipophilic marine biotoxins including some of the non-regulated cyclic imines were analyzed in a five-minute analysis using a Waters® ACQUITY UPLC System coupled with a Xevo TQ-S. UPLC® technology allows for analytical run times to be reduced without compromising peak resolution and quality, while the Xevo TQ-S (tandem quadrupole mass spectrometer) will provide ultra-high sensitivity for the detection, and allows the simultaneous acquisition of multiple reaction monitoring (MRM) transitions in alternating electrospray positive (ESI^+) and negative ionization (ESI^-) modes, which is required for the analysis of these lipophilic marine biotoxins.

Standard preparation

Certified standards OA, DTX1, -2, PTX2, YTX, AZA1, -2, -3, gymnodimine (GYM), and 13-desmethyl spirolide C (SPX1) were purchased from the National Research Council, Institute for Marine Biosciences (NRC-CNRC), Halifax, Canada. Semi-purified standards for pinnatoxin-E (PnTX-E), -F (PnTX-F) and -G (PnTX-G) were obtained from Cawthron Institute, Nelson, New Zealand. For each toxin, a standard stock solution was prepared in methanol. From the stock solutions, the Matrix Matched Standards (MMS) calibration curves were prepared in blank mussel extract. Blank extract was prepared according to the sample extraction procedure. The levels of the matrix matched standard were 0, 0.125, 0.25, 0.5, 1.0, and 1.5 times the validation level (which for the regulated toxins is similar to the permitted levels, as shown in Table 1).

		(MMS) (µg/kg)					
Lipophilic marine biotoxin	**EU permitted level**	**0**	**0.125**	**0.25**	**0.5**	**1**	**1.5**
OA, DTX1, DTX2, PTX2	160 µg OA-equivalents/kg*	0	20	40	80	160	240
AZA1, -2, -3	160 µg AZA1 equivalents/kg*	0	20	40	80	160	240
Yessotoxins	1000 µg YTX equivalents/kg*	0	125	250	500	1000	1500
GYM, PnTX-E, PnTX-F	200 µg/kg*	0	25	50	100	200	300
SPX1	100 µg/kg*	0	12.5	25	50	100	150
PnTX-G	50 µg/kg*	0	6.25	12.5	25	50	75
OA, DTX1, DTX2, PTX2	160 µg OA-equivalents/kg*	0	20	40	80	160	240
AZA1, -2, -3	160 µg AZA1 equivalents/kg*	0	20	40	80	160	240
Yessotoxins	1,000 µg YTX equivalents/kg*	0	125	250	500	1000	1500
GYM, PnTX-E, PnTX-F	200 µg/kg*	0	25	50	100	200	300
SPX1	100 µg/kg*	0	12.5	25	50	100	150
PnTX-G	50 µg/kg*	0	6.25	12.5	25	50	75

*Table 1. The EU permitted levels and recommended reporting levels for the lipophilic marine biotoxins. * Edible shellfish*

EXPERIMENTAL

UPLC conditions

System:	ACQUITY UPLC
Run time:	5.0 min
Column:	ACQUITY UPLC BEH C_{18} 2.1 X 100 mm, 1.7 µm
Column temp.:	40 °C
Sample temp.:	10 °C
Mobile phase A:	Water containing 6.7 mM ammonium hydroxide
Mobile phase B:	9:1 acetonitrile/water containing 6.7 mM ammonium hydroxide
Weak wash:	9:1 water/acetonitrile
Strong wash:	9:1 acetonitrile/water
Flow rate:	0.6 mL/min
Injection volume:	5 µL

Time (min)	Flow rate (mL/min)	%A	%B	Curve
Initial	0.6	70	30	N/A
0.5	0.6	70	30	6
3.5	0.6	10	90	6
4.0	0.6	10	90	6
4.1	0.6	70	30	6
5.0	0.6	70	30	6

MS conditions

Mass spectrometer:	Xevo TQ-S
Ionization mode:	ESI - / +
Capillary voltage:	3.0 kV
Source temp.:	150 °C
Desolvation temp.:	500 °C
Desolvation gas:	800 L/h
MRM method:	See Table 2.

Sample extraction

Homogenized whole flesh shellfish tissue (1 g) was extracted in triplicate with 3-mL methanol. After each addition of methanol, the extract was vortex-mixed. After vortex-mixing, the extract was centrifuged for 5 min at 2000 g, and the supernatant was transferred to a 10-mL volumetric flask. After the third extraction (9 mL in total), the volume was made up to 10 mL with methanol. The crude shellfish extract was then filtered through a 0.2-µm membrane filter, prior to spiking or analysis.

In order to determine the amount of DTX3 (ester forms of OA, DTX1, and -2), the extracts were also subjected to alkaline hydrolysis using 2.5-M sodium hydroxide. After heating the alkaline mixture for 40 min at 76 °C, the mixture was cooled to room temperature, and subsequently neutralized using 2.5 M hydrochloric acid.

Compound name	Parent (*m/z*)	Daughter (*m/z*)	Ionisation	Dwell (s)	Cone (V)	Collision (eV)	Standard available
trinor YTX	550.4	396.4	Negative	0.003	75	30	No
	550.4	467.4	Negative	0.003	75	30	
YTX	570.4	396.4	Negative	0.003	75	30	Yes
	570.4	467.4	Negative	0.003	75	30	
homoYTX	577.4	403.4	Negative	0.003	75	30	No
	577.4	474.4	Negative	0.003	75	30	
45OH YTX	578.4	396.4	Negative	0.003	75	30	No
	578.4	467.4	Negative	0.003	75	30	
45OH Homo YTX	585.4	403.4	Negative	0.003	75	30	No
	585.4	474.4	Negative	0.003	75	30	
COOH YTX	586.4	396.4	Negative	0.003	75	30	No
	586.4	467.4	Negative	0.003	75	30	
COOH OH YTX	593.4	396.4	Negative	0.003	75	30	No
	593.4	403.4	Negative	0.003	75	30	
COOH Homo YTX	593.4	467.4	Negative	0.003	75	30	No
	593.4	474.4	Negative	0.003	75	30	
OA/DTX2	803.5	113.1	Negative	0.003	80	60	Yes
	803.5	255.2	Negative	0.003	80	45	
DTX1	817.5	113.1	Negative	0.003	80	60	Yes
	817.5	255.2	Negative	0.003	80	45	
GYM	508.2	162.2	Positive	0.003	60	55	Yes
	508.2	490.2	Positive	0.003	60	40	
SPX1	692.5	164.3	Positive	0.003	60	55	Yes
	692.5	444.2	Positive	0.003	60	40	
PnTX-G	694.5	164.3	Positive	0.003	60	55	Yes
	694.5	676.5	Positive	0.003	60	40	
20-Me SPX G	706.5	164.3	Positive	0.003	60	55	No
	706.5	346.2	Positive	0.003	60	40	
PnTX-F	766.5	164.3	Positive	0.003	60	55	Yes
	766.5	748.5	Positive	0.003	60	40	
PnTX-E	784.5	164.3	Positive	0.003	60	55	Yes
	784.5	766.5	Positive	0.003	60	40	
AZA3	828.5	658.4	Positive	0.003	35	40	Yes
	828.5	792.5	Positive	0.003	35	30	
AZA6	842.5	658.4	Positive	0.003	35	40	Yes
AZA1	842.5	672.4	Positive	0.003	35	40	Yes
AZA1/6	842.5	824.5	Positive	0.003	35	30	Yes/No
AZA4	844.5	658.4	Positive	0.003	35	40	No
AZA5	844.5	674.4	Positive	0.003	35	40	No
AZA4/5	844.5	826.5	Positive	0.003	35	30	No
AZA2	856.5	672.4	Positive	0.003	35	40	Yes
	856.5	838.5	Positive	0.003	35	30	
PTX12	874.5	213.1	Positive	0.003	40	30	No
	874.5	821.5	Positive	0.003	40	30	
PTX2	876.5	213.1	Positive	0.003	40	30	Yes
	876.5	823.5	Positive	0.003	40	30	
PTX11	892.5	213.1	Positive	0.003	40	30	No
	892.5	839.5	Positive	0.003	40	30	
PTX2sa	894.5	213.1	Positive	0.003	40	30	No
	894.5	805.2	Positive	0.003	40	30	

Table 2. MRM transitions of the various lipophilic marine biotoxins.

Single-day validation

A single lab single-day validation study was performed in order to assess the performance of the developed UPLC/MS/MS method. The following characteristics criteria were assessed: linearity, recovery, repeatability, within-laboratory reproducibility (empirically calculated from the repeatability), selectivity, and decision criteria (CCα). Blank shellfish from the Dutch national monitoring program were used for validation. The shellfish species that were included in the validation were mussels *(Mytilus edulis)*, oysters *(Crassosrea gigas)*, cockles *(Cerastoderma edule)*, and ensis *(Ensis directus)*. In order to ensure that the shellfish species used for the validation were blank, they were subject to analysis for the toxins shown in Table 2 prior to the beginning of the study. Seven different shellfish samples (four mussels, one oyster, one cockle, and one ensis) were extracted and spiked at 0.0, 0.5, 1.0, and 1.5 times the validation level.

RESULTS AND DISCUSSION

The individual stock solutions of the cyclic imines were diluted in methanol to obtain tuning parameters with IntelliStart™ Technology. IntelliStart greatly simplifies the use of LC/MS systems by automating instrument setup, compound tuning, and performing system suitability checks. The *m/z* of the various toxins, as well as cone voltages and collision energies, are shown in Table 2. In order to save valuable toxin standards, extracts were spiked instead of raw shellfish homogenate, which saves a factor 10 in standards. Spiking to extracts is justified by the fact that the extraction efficiency is very high (>90%) for all relevant toxins. Therefore, the spiking of extracts is very unlikely to lead to false negative results. The developed UPLC/MS/MS method is based on the chromatography earlier described by Gerssen *et al.*[3] The UPLC separation provided good results for all different toxins classes; only peak shapes for azaspiracids were somewhat negatively affected by the alkaline conditions. Due to the high selectivity and sensitivity of the Xevo TQ-S, this is not problematic for routine applications, as the peaks can still be easily detected at the sensitivity levels required by regulation. The resulting MRM chromatograms from the five-minute UPLC separation of various toxins are shown in Figure 2.

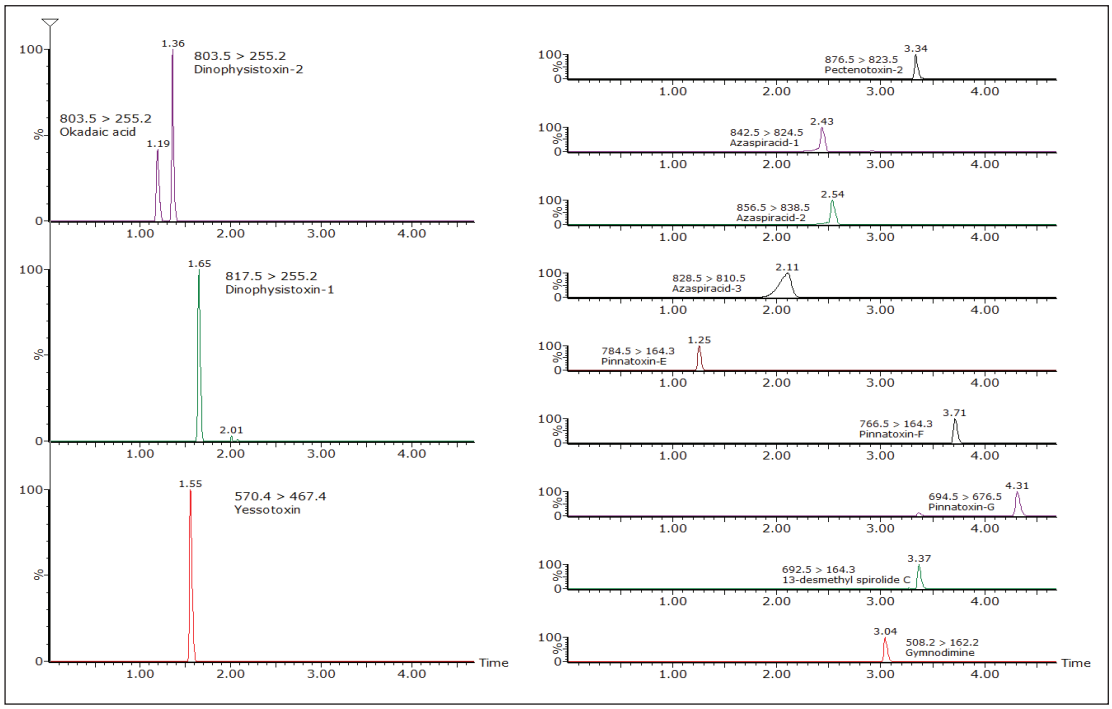

Figure 2. MRM chromatograms of matrix matched standard in mussel matrix at 1.0x validation level.

For each compound, two optimized MRMs are shown: one for confirmation and the other for quantification. The ion ratio between the results of two MRMs is one of the analytical criteria that will determine whether or not a biotoxin has been positively identified. Another regulatory criterion is the maximum residue limit (MRL) of the compound. Within the processing software, TargetLynx, it is possible to automatically flag parameters that are set within the regulatory framework so that the analyst can very easily see when these criteria have not met the permitted levels.

Sensitivity for the various toxins was good, even at levels of 0.125 times the validation level (regulatory level for regulated toxins), a signal-to-noise above 3 could be obtained. Currently for some of the regulated toxins, standards are not yet available (PTX1, 45OH YTX, homoYTX, and 45OH homoYTX). In order to determine these toxins, MRM transitions are included in the method based on the structural relation with the toxin from which a standard is available, respectively PTX2 and YTX. Furthermore, MRM transitions of other non-regulated lipophilic toxins are included, which could be screened for using this confirmatory method, as described in Table 2. The method parameters that that have been determined in these experiments are now integrated into the Quanpedia Database, allowing any user of this system to have access to this method protocol.

Single-day validation

With respect to single-day validation, different method performance parameters were assessed, such as linearity, recovery, repeatability, within-laboratory reproducibility, selectivity and CCα, shown in Table 3.

Compound name	Validation level (µg/kg)	Recovery (%)	RSDr (%)	RSDrl (%)	CCα (µg/kg)
OA	160	99	2.7	4.1	171
DTX1	160	99	7.6	12.2	192
DTX2	160	102	2.6	4.1	171
YTX	1000	100	2.5	4.0	1070
AZA1	160	98	1.3	2.1	166
AZA2	160	98	1.9	3.0	168
AZA3	160	99	1.9	3.0	168
PTX2	160	103	8.7	13.9	197
GYM	200	99	3.9	6.3	221
SPX1[1]	100	108	14.6	23.4*	141
SPX1[2]	100	104	12.8	20.4	135
PinE	200	122	23.1	36.9*	347
PinF	200	91	5.1	8.1	224
PinG	50	102	3.9	4.8	54

*Table 3. Validation results of the single-day validation. [1]including the ensis matrix [2]without the ensis matrix, and *poor reproducibility.*

For all toxins, the linearity of the matrix matches standard calibration curve was excellent (R^2 > 0.990) for the concentration range used (0.125 - 1.5 x PL). The recoveries obtained for the various toxins were good and ranged from 91% to 104%. The only exception to this was PnTx-E, where a recovery of 122% was obtained. This early eluting (tr 1.2 min) compound tends to form methyl esters when extracts are prepared in methanol, and this behavior can be used to explain the higher than expected recovery, as well as the poor repeatability (RSDr 23.1%), when compared to the other analytes. For all regulated compounds, the repeatability observed was good. For the non-regulated toxins, only PnTx-E and SPX1 were somewhat higher than expected. For SPX1, this was caused by the ensis matrix by removing the results of the ensis and re-calculating the repeatability results were acceptable, respectively 14.6% and 12.8% with and without ensis matrix. In order to decide if a sample is non-compliant, the toxin level should be at or above the decision limit (CCα). The decision limit is a concentration at which it can be concluded with a probability of 1-α or 95% (α = 5%) that the sample is above the permitted level and thus non-compliant.

CONCLUSIONS

By using a UPLC system coupled with a tandem quadrupole MS (Xevo TQ-S), a robust, rapid method has been developed for the analysis of both regulated and non-regulated lipophilic marine biotoxins in shellfish. Compared to the conventional HPLC/MS/MS method, the speed of analysis increased four-fold, from 20 min to 5 min per analysis. The method is able to meet the regulated levels of detection that can be used instead of the mouse bioassay.

The use of IntelliStart allows the user to quickly and automatically determine the optimal settings and extend this method when more toxin standards become available. As this method (and all the instrument settings) has now been included into the Quanpedia Database, it is easy to implement this approach into other laboratories.

References

1. European Commission. Laying down implementing measures for certain products under Regulation (EC) No. 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No. 854/2004 of the European Parliament and of the Council and Regulation (EC) No 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004. Off J Eur Commun L 2005; 338:40-41.
2. European Commission. Commission Regulation (EU) No 15/2011 of 10 January 2011, amending Regulation (EC) No. 2074/2005 as regards recognized testing methods for detecting marine biotoxins in live bivalve molluscs. Off. J. Eur. Commun. 2011; L6:3-6.
3. Gerssen A, Mulder PPJ, McElhinney MA, De Boer J. Liquid chromatography - tandem mass spectrometry method for the detection of marine lipophilic toxins under alkaline conditions. *J Chromatogr A*. 2009; 1216:1421-1430.

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February 2013 720004601EN AG-PDF

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