Agilent 6460 LC-QQQ – Highly Sensitive and Robust Analysis for Lipophilic Marine Toxins in Shellfish

Application Note

Environmental, Food Safety

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Abstract

Marine biotoxins are increasingly threatening the human health in many parts of the world. While the toxins are formed by microscopic planktonic algae of several genera usually at very low concentrations, they can be accumulated in bivalve molluscs to reach toxic doses. The consumption of contaminated shellfish or fish can lead to human poisoning or even death. In animals and humans there are four recognized symptom types of shellfish poisoning: Diarrhetic shellfish poisoning (DSP), paralytic shellfish poisoning (PSP), neurotoxic shellfish poisoning (NSP) and amnesic shellfish poisoning (ASP).

The official standard reference method in the EU (Commission Regulation EC No. 2074/2005) for monitoring of lipophilic biotoxins is the mouse bioassay (MBA). Recently the MBA has been considered to be inadequate (The EFSA Journal, 2009, 1306, 1-1) by the European Food Safety Authority (EFSA) because of high variability, insufficient detection capability and limited specificity. A specific, alternative method for the determination of marine biotoxins with low limits of detection (LOD) has been requested.

This document describes a highly sensitive LC-MS/MS method for the determination of lipophilic marine toxins, including ocadaic acid (OA), dinophysistoxins (DTX toxins) and polyether toxins like azaspiracids, pectenotoxins and yessotoxins. Preliminary tests have shown that this method is also applicable to further lipophilic toxins like domoic acid, gymnodimine or spirolides, if reference compounds are available.

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Introduction

Marine biotoxins are formed as secondary metabolites by marine planctonic algae typically at very low concentrations. During an algal bloom the concentrations of the toxins can reach toxic levels in particular due to the accumulation of the toxins in bivalve mollusks. In the last two decades the number and intensity of harmful algae blooms has increased and a bigger number of toxic compounds have been found in the marine food chain (Marine biotoxins. FAO Food and Nutrition Papers (80) 2004). A more sensitive and more reliable method for the determination of lipophilic marine toxins has been requested by the European Food Safety Authority (EFSA) since the current official standard reference method in the EU, the mouse bioassay has been considered to be inadequate because of high variability, insufficient detection capability and limited specificity.

Your Challenges

The challenge is to have a sensitive and robust analytical method available for the determination of lipophilic marine toxins, including ocadaic acid, dinophysistoxins (DSP toxins) and polyether toxins like azaspiracids, pectenotoxins and yessotoxins in seafood. The method should have the potential to be extended to further lipophilic toxins like domoic acid, gymnodimine or spirolides. The limits of detection (LODs) need to be below the maximum residue limits (MRLs) which are specified for azaspiracids, the sum of okadaic acid, dinophysistoxins and pectenotoxins, and yessotoxins under EU legislation. Quantitation and confirmation of compounds at a trace level can be complicated by the matrix. As reference material for many compounds is not available tools are required to quantify several compounds based on the response of others.

Our integrated approach

Modern methods for the analysis of marine biotoxins are based on physicochemical techniques like LC-MS/MS. Triple quadrupole mass spectrometry allows for drastic reduction or elimination of matrix interferences. The Multiple Reaction Monitoring (MRM) is based on the detection of a secondary "product ion" produced by the collisional dissociation of an analyte "precursor ion". Whereas the analyte precursor ion (isolated in MS1 by a SIM mechanism) has the same selectivity as SIM, the resultant product ions (isolated in MS2 by a SIM mechanism) are more likely to be unique to the target compounds leading to an increased selectivity of the MRM. The combination of unique product ions (more selectivity) and the elimination of background noise results in consistently low limits of detection even for complex matrices. The method described here is a highly sensitive and specific method for the analysis of shellfish samples for lipophilic marine toxins using the Agilent 6460 Triple Quadrupole LC-MS system in MRM mode in combination with the Agilent 1200 SL Rapid Resolution HPLC and the Agilent MassHunter Workstation software. The described sample clean-up procedure is extremely simple, and thus the method is highly applicable to routine analysis. It allows for the analysis of OA, DTX-1, DTX-2 including their esters after hydrolysis, YTX, OH-YTX, PTX-1, PTX-2, AZA-1, AZA-2, and AZA-3. Due to the lack of commercially available standards some of the toxins have to be quantified using the calibration response of other compounds using the *CopyCalibrationLevel.quant.script.*

Methods and Operation

Modern methods for analysis of marine biotoxins are based on LC-MS/MS with Triple Quadrupole systems. The sample preparation is simply done by liquid extraction and subsequent filtration. Separation is based on HPLC and quantification is done by LC-ESI-MS/MS (MRM –positive and negative mode).

Analysis steps
Liquid Phase Extraction
HPLC Analysis with linear gradient
Detection by LC-ESI-MS/MS (MRM).

Sample preparation steps
An amount of 2 g of cooked, grinded shellfish tissue is weighed. Addition of 9 mL of methanol (80 %). This extraction procedure is done twice. The two extracts are combined and filled up to 50 mL in a volumetric flask. An aliquot is filtered to remove all remaining particles (filter RC 0.45 µm) and 10 µL of this sample extract is injected for the LC-MS/MS analysis.

Structures of analytes (example)

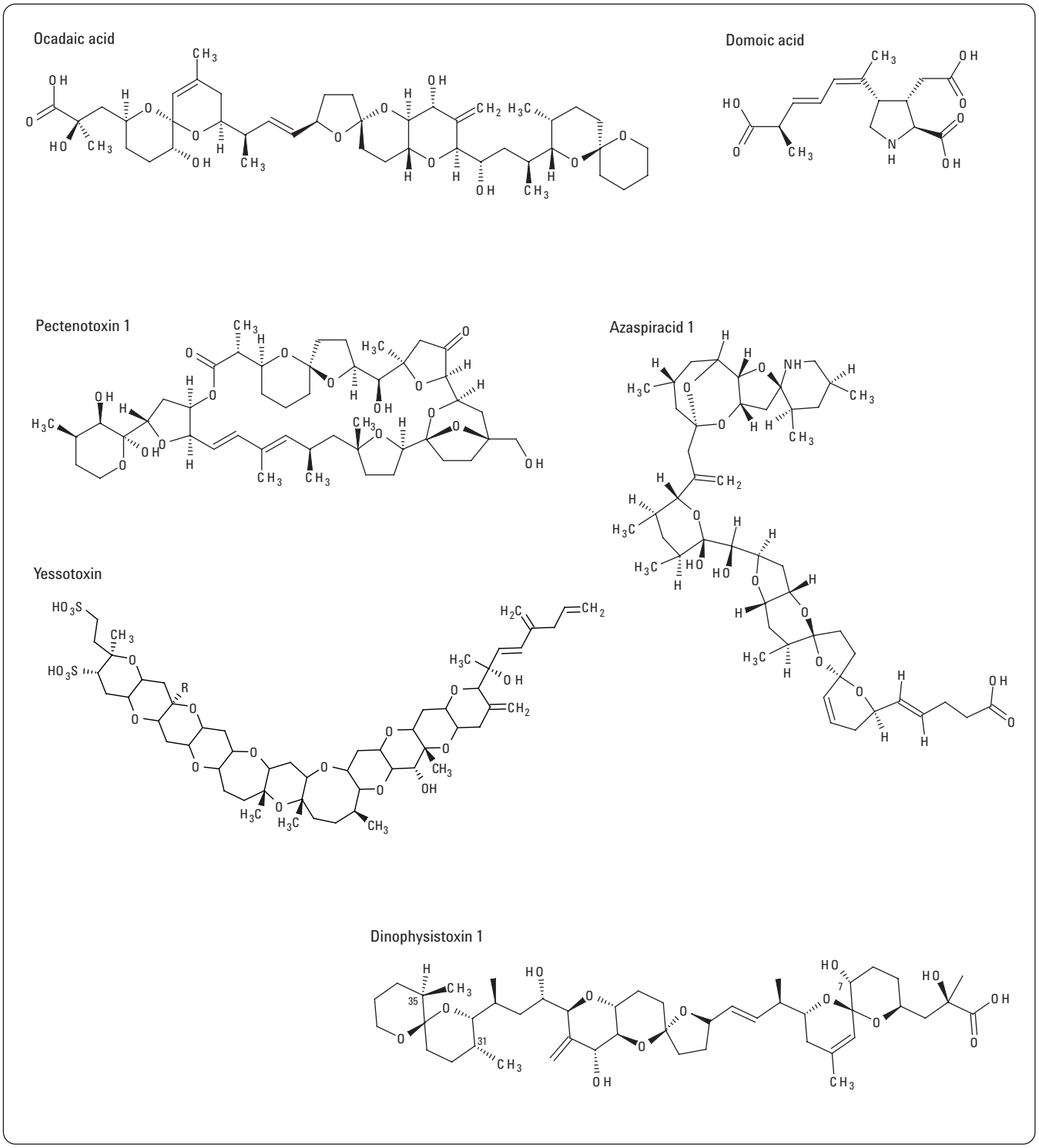

Figure 1
Chemical formulas of some marine toxins.

LC-MS/MS method

The total run time required to determine lipophilic marine toxins is less than 30 min. – The method runs in positive ionization mode for OA, DTX-1, DTX-2, PTX-1, PTX-2, AZA-1, AZA-2 and AZA-3. YTX is done in a separate run which requires a different column and mobile phase. The extract (10 µL) is injected directly to the LC-MS/MS system. For the LC method a solvent mixture of 0.1 % formic acid in Water (A) and MeOH (B) for positive ionization and 2 mM ammonium acetate in water (A) and MeOH (B) for negative ionization is used as mobile phase on a linear gradient. The column (Phenomenex Luna 5 µm C18(2) 100 Å 150 x 2.0 mm [pos. mode], ZORBAX Eclipse Plus C 8 4.6 x 75 mm 3.5 µm [neg. mode]) is held in an oven at 30 °C with a flow-rate of 0.2 mL/min.

Gradient* Time [min]	Solvent ratio B [%]
0	5
10	85
22	85
23	5
30	5

Table 1
Gradient settings.
*Applicable for positive and negative ionization mode.

Sample Preparation Steps

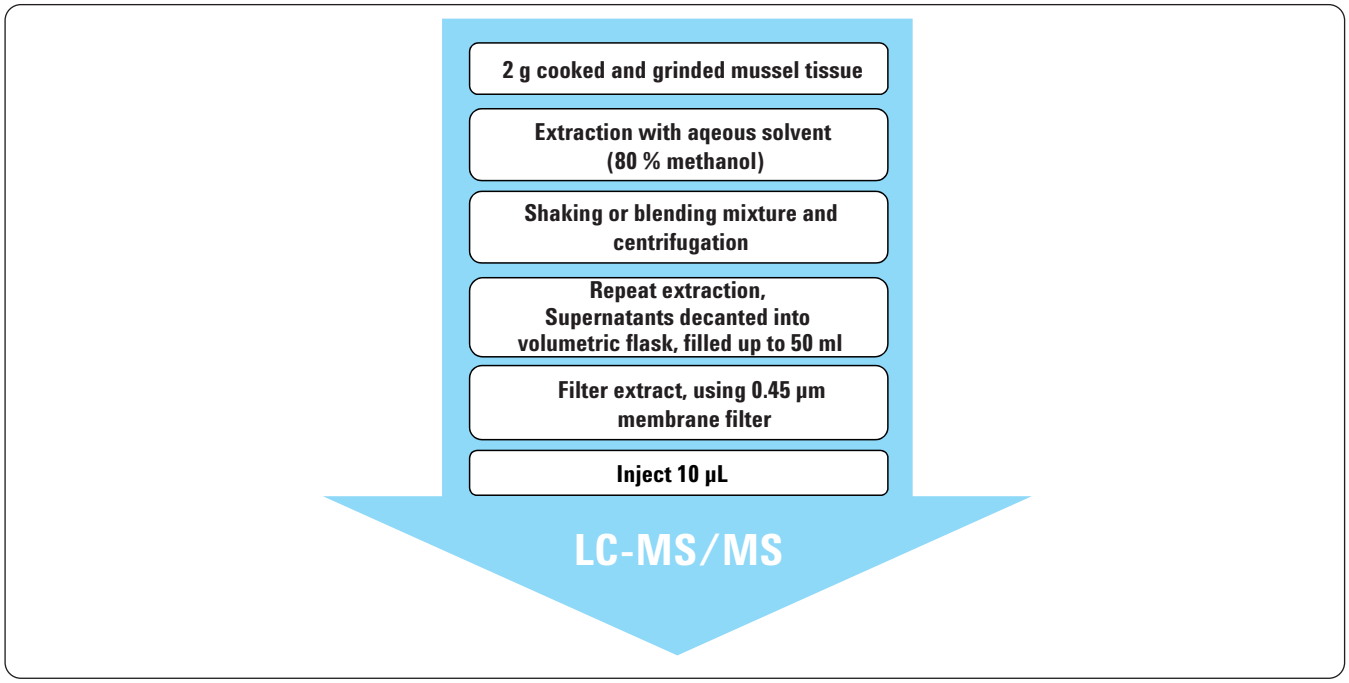

Figure 2
Sample preparation procedure for the determination of marine toxins in shellfish.
According to McNabb, P., A.I. Selwood, and P.T. Holland, Multiresidue method for determination of algal toxins in shellfish. J AOAC Int, 2005. 88: p. 761-772
Chapela, M.J., et al., Lipophilic toxins analyzed by liquid chromatography-mass spectrometry and comparison with mouse bioassay in fresh, frozen, and processed molluscs. J Agric Food Chem, 2008. 56(19): p. 8979-86.
Moutfort, D.O., T. Suzuki, and P. Trueman, Protein phosphatase inhibition assay adapted for determination of total DSP in contaminated mussels. Toxicon, 2001. 39: p. 383-390

Mass Spectrometer Settings and Jet Stream Parameter

Agilent 6460 QQQ ESI JetStream Source parameter

Gas Temperature:	300 °C
Gas Flow:	5 L/min
Nebulizer:	45 psi
Sheath Gas Temp:	250 °C
Sheath Gas Flow:	11 L/min
Capillary:	+ 3500 V
	- 3500 V
Nozzle Voltage:	+/- 500 V
Delta EMV	400

Agilent 6410 QQQ ESI-Source parameter

Ionization:	ESI
Gas Temperature:	300 °C
Gas Flow:	10 L/min
Nebulizer:	43 psi
Capillary:	+ 4500 V
	- 5200 V
Delta EMV:	400

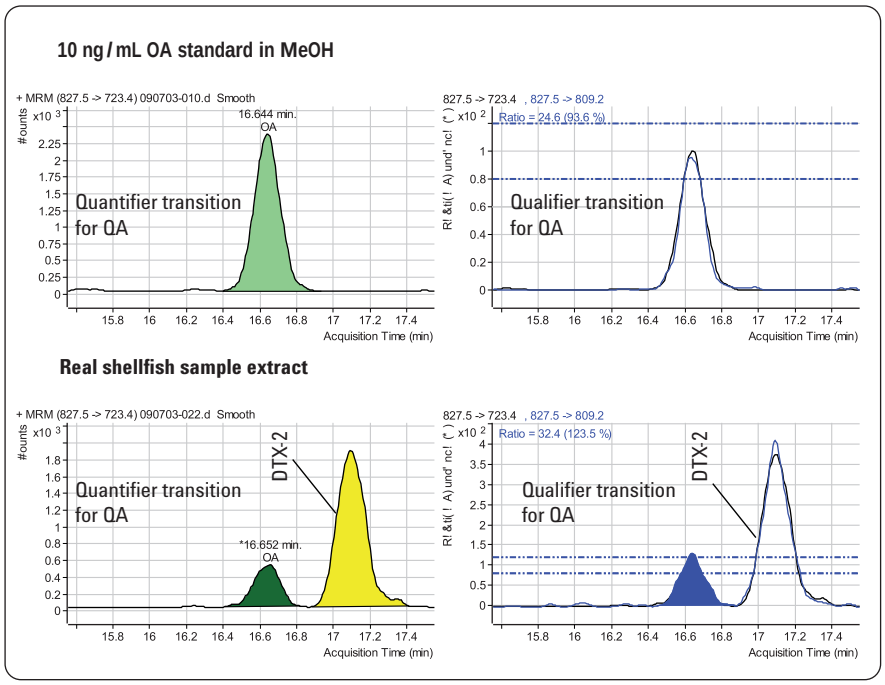

Figure 3
OA standard and real shellfish sample, containing OA and DTX-2 (same sample as shown in figure 4).
OA 23 µg/kg and DTX-2 130 µg/kg.

Analyte	Polarity	Prec Ion m/z	Prod Ion m/z	Frag [V]	CE [eV]	Quantifier
OA and DTX-2	pos	827.5	723.4	220	55	X
	pos	827.5	809.2	220	45	
DTX-1	pos	841.5	737.2	220	55	X
	pos	841.5	823.2	220	45	
PTX-1	pos	897.5	555.3	230	70	X
	pos	897.5	853.5	230	60	
PTX-2	pos	881.5	539.3	230	70	X
	pos	881.5	837.5	230	60	
PTX-2sa*	pos	899.5	855.5	230	60	X
	pos	899.5	557.3	230	70	
YTX	neg	1141.5	1061.3	135	35	X
	neg	1141.5	925.5	135	60	
Homo-YTX*	neg	1155.4	1075.5	135	35	X
OH-YTX	neg	1157.4	1077.5	135	35	X
OH-Homo-YTX*	neg	1171.4	1091.5	135	35	X
AZA-1	pos	842.5	824.5	200	40	X
	pos	842.5	806.5	200	55	
AZA-2	pos	856.5	838.5	200	40	X
	pos	856.5	820.5	200	55	
AZA-3	pos	828.5	810.5	200	40	X
	pos	828.5	792.5	200	55	

*Transitions based on literature information

Table 2
MRM transitions and MS settings.

Results

The method has been validated within an international collaborative study. The collaborative study was conducted in the framework of the working group §64 LFGB "Phycotoxins", which is hosted by the federal Office of Consumer Protection and Food Safety (BVL).

Compound	LOD	LOQ
[1]OA	6 µg/kg	20 µg/kg
[1]DTX-1 & 2	6 µg/kg	20 µg/kg
[2]AZA-1 to 3	6 µg/kg	20 µg/kg
[1]PTX-1 & 2	6 µg/kg	20 µg/kg
[3]YTX	10 µg/kg	35 µg/kg

[1] MRL in raw mussel material for sum of OA, DTX-1 & 2, PTX-1 & 2: 160 µg/kg OA equivalents

[2] MRL in raw mussel material for sum of azaspiracids: 160 µg/kg AZA-1 equivalents

[3] MRL in raw mussel material for sum of yessotoxins: 1000 µg/kg YTX equivalents

Table 3
LODs and LOQs of method for lipophilic marine toxins in cooked, grinded mussels.

Reproducibility S.D. is in the range from approx. 10 % to 35 % (depends on matrix, concentration, analyte). Extraction recovery is in the range from 75 % to 102 % (depends on analyte and matrix).

Benefits

- Highly sensitive and selective determination of marine toxins in shellfish with Agilent 6460 Triple Quadrupole LC-MS system and Agilent 1200 SL Rapid Resolution HPLC
- Simple and cost-effective sample preparation and easy workflow for routine sample analysis with high reliability
- Compliance with recent EFSA guidelines
- Flexibility to add other lipophilic toxins

Chromatogram of real shellfish samples

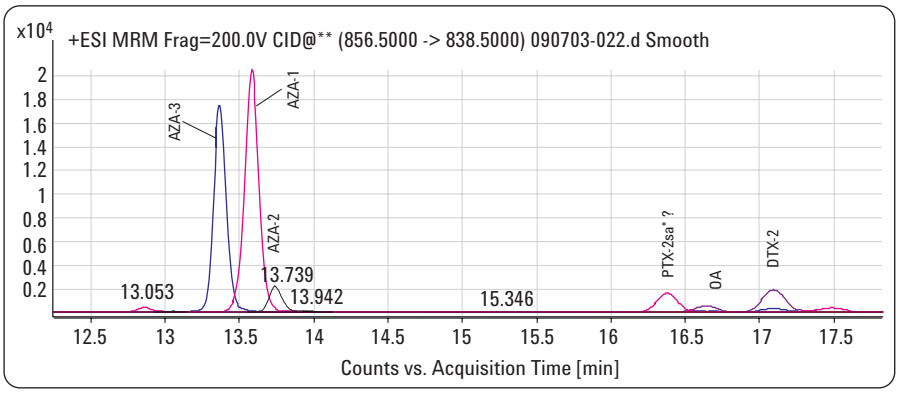

Figure 4
Real blue mussel sample extract.
Concentration: 96 µg/kg AZA-1, 22 µg/kg AZA-2, 50 µg/kg AZA-3, 23 µg/kg OA and 130 µg/kg DTX-2.
*PTX-2sa, tentatively assigned on the basis of transitions from literature, no standard available for PTX-2sa.

Chromatogram of real shellfish samples

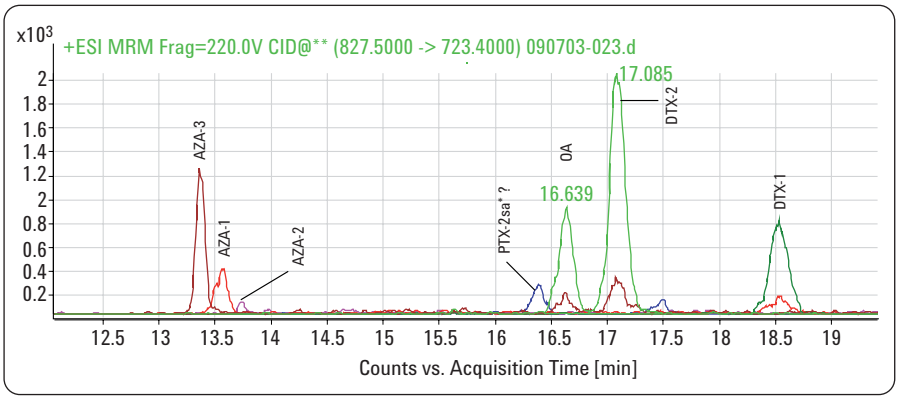

Figure 5
Real blue mussel extract, AZA-1 to -3 < LOQ 20 µg/kg.
Concentration: 37 µg/kg OA, 120 µg/kg DTX-2 and 69 µg/kg DTX-1.
*PTX-2sa, tentatively assigned on the basis of transitions from literature, no standard available for PTX-2sa.

Chromatogram of a QC sample

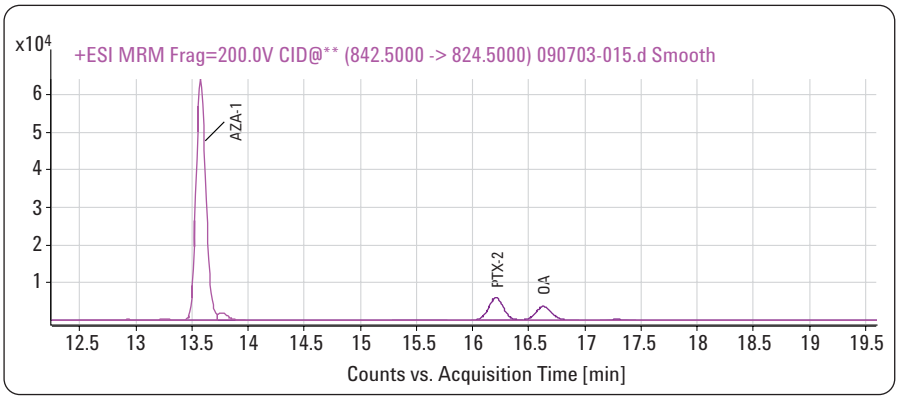

Figure 6
QC-Sample, 15 µg/kg for AZA-1, PTX-2 and OA, spiked in blue mussel extract.

Calibration curve (matrix matched)

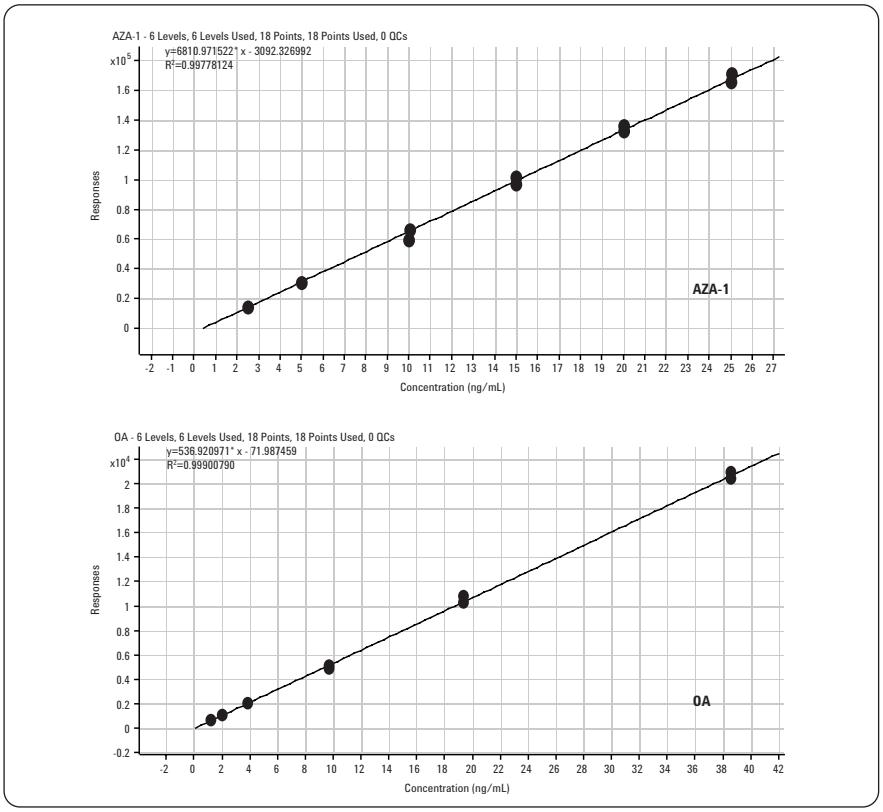

Figure 7
Calibration curve for AZA-1 and OA spiked in mussel extract, linear curve fit, origin included weighting none. Calibration range AZA-1 2.5 to 25 ng / mL and OA 1.1 to 38 ng / mL.

Calibration curve (in methanol)

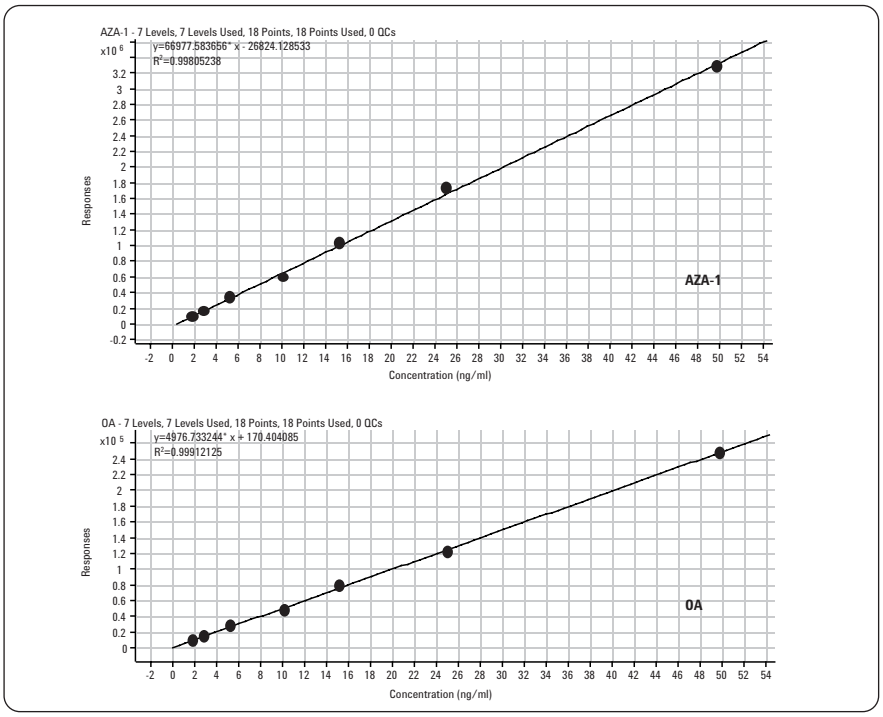

Figure 8
Calibration curve for AZA-1 and OA in MeOH, linear curve fit, origin included weighting none. Calibration Range 1.5 to 50 ng / mL for both.

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