Determination of Lipophilic Marine Biotoxins in Molluscs by LC-MS/MS using Offline Extraction

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Introduction

In recent years many countries have had to deal with the consequences of toxic microalgal blooms in both marine and fresh water, such as the deaths of wild animals and domestic livestock.

Several cases of poisoning in humans have been associated with the direct consumption of shellfish, fish, or water contaminated by algal toxins. People may also come into contact with toxins during recreational activities along sea coasts affected by episodes of algal blooms. Depending on the type of toxin involved, there are forms of mild and usually self-limiting symptoms, characterized by gastrointestinal disorders or allergic-like episodes. Much more severe symptoms of the neurological type can lead to death.

The foods most frequently involved in episodes of human poisoning are represented by bivalve molluscs. These shellfish can accumulate and concentrate any biotoxins present in the plankton they ingest through filtering large quantities of water for trophical reasons. It is not possible to evaluate shellfish edibility by an organoleptic examination alone. While human ingestion of contaminated food with biotoxins can lead to the onset of different clinical symptoms, in shellfish it usually has only marginal effects. An important risk factor lies in the thermostability of such molecules which are not completely inactivated by common physical treatments for fish products (cooking, smoking, salting, freezing, housing), but remain virtually unchanged in the finished product.

There are a series of regulations issued by the European Union (EU) that relate to marine biotoxins. One is Regulation (EC) No 853/2004 which concerns the control of lipophilic toxins, establishing maximum levels for lipophilic toxins in bivalve molluscs destined for human consumption:[1]

- For okadaic acid, dinophysistoxins, and pectenotoxins together – 160 micrograms of okadaic acid equivalents per kilogram
- For yessotoxin – 1 milligram of yessotoxin equivalent per kilogram
- For azaspiracids – 160 micrograms of azaspiracid equivalents per kilogram

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In the past, aside from bioassays on mice, most analytical techniques developed for the determination of marine biotoxins in bivalve molluscs have been based on offline methodologies. These include methods involving solid phase extraction (SPE) or liquid-liquid extraction (LLE) followed by high pressure liquid chromatography (HPLC) with fluorimetric or UV-diode array detection, as well as detection by liquid chromatography coupled with mass spectrometry (LC-MS).

The EU Commission Regulation (EC) No 15/2011, amending Regulation (EC) No 2074/2005 about the testing methods for detecting marine biotoxins in bivalve molluscs, describes an LC-MS/MS procedure as the reference method for the quantification of lipophilic marine biotoxins – namely okadaic acid, pectenotoxin 2, azaspiracid 1, and yessotoxin.[2,3] Moreover, dinophysistoxin 1 (DTX-1) and dinophysistoxin 2 (DTX-2) can be quantified by the calibration curve of okadaic acid, pectenotoxin 1 by calibration of pectenotoxin 2, azaspiracid 2 and 3 by calibration of azaspiracid 1 and 45-OH-, and 45-homo-OH-yessotoxin by the calibration of yessotoxin.

In accordance with current European regulations, we propose a quick, selective, sensitive, and accurate analytical method for the determination of lipophilic marine biotoxins in bivalve molluscs using an LC-MS/MS method.

Goal

Our goal is to validate analytical procedures proposed in "*EU-Harmonised Standard Operating Procedure for determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS – Version 3*" by LC-MS/MS using offline extraction.[4]

Experimental

Sample Preparation

About 1 kg of bivalve molluscs (*Mytilus Galloprovincialis*) were cleaned with water and put in a solution of NaCl (3.5 g/L). After opening, the molluscs were washed with fresh water, their flesh was removed and placed on a stainless steel net, and they were washed again with deionized water. The whole collected raw tissue, not less than 150 g, was chopped and blended by a mixer.

Extraction procedure

9 mL of 100% methanol (gradient quality) were added to 2.00 ± 0.05 g of blended tissue, put into a centrifuge tube, and mixed by vortex for 3 minutes at maximum speed. After centrifugation at 4000 rpm for 10 minutes, the supernatant solution was transferred into a vial.

A second aliquot of 9 mL of 100% methanol was further added to the residual tissue pellet and homogenized for 1 minute by Ultra-turrax® (*IKA®, USA*) at 12,000 rpm and the mixture was centrifuged at 4000 rpm for 10 minutes. Then the supernatant solution was transferred and combined with the first extract and made up to 20 mL with 100% methanol. When not immediately analyzed, the solution was stored at -20 °C.

Spikes of toxin standard solutions can be added to the blended tissue before the extraction procedure.

Purification Procedure

The organic extract was purified by being passed through a C18 SPE cartridge preliminarily conditioned with 1 mL of 100% methanol. A 0.45 µm syringe filter was placed at the end of the cartridge to improve purification.

LC Conditions for the Thermo Scientific Hypersil GOLD Column

System	Thermo Scientific Accela UHPLC
Solvent A	100% water with 2 mM ammonium formate and 50 mM formic acid
Solvent B	95% acetonitrile + 5% water with 2 mM ammonium formate and 50 mM formic acid
Flow Rate	200 µL/min
Gradient	The mixture started at 30% solvent B (8.0 min) followed by a linear gradient up to 90% solvent B in 3.0 min. It went up to 30% of solvent B in 0.5 min. This composition was maintained for 5.5 min.
Analytical Column	Hypersil GOLD™; 50 × 2.1 mm, particle size 1.9 µm, part number 25002-052130

H-ESI II Source Conditions

Ion Source Polarity	Positive Ion Mode	Negative Ion Mode
Spray Voltage	3000 V	2700 V
Capillary Temperature	270 °C	270 °C
Vaporizer Temperature	240 °C	240 °C
Sheath Gas Pressure (N_2)	15 units	15 units
Auxiliary Gas Pressure (N_2)	5 units	5 units

MS/MS Setup

MS analysis was carried out on a Thermo Scientific TSQ Quantum Ultra triple quadrupole mass spectrometer equipped with a heated electrospray ionization probe (H-ESI II).

Collision Gas (Ar)	1.5 mTorr
Q1/Q3 Peak Resolution	0.7 u (unit mass resolution)
Scan Time	0.100 s
Scan Width	0.500 *m/z*
Data Acquisition Mode	SRM

Analyte	ESI Mode	Parent Mass	Product Mass	Collision Energy	Tube Lens
AZA-1	ESI+	842.3	806.1	51	207
			824.2	42	207
AZA-2	ESI+	856.3	838.1	42	214
			820.2	49	214
AZA-3	ESI+	828.3	792.2	48	192
			810.0	40	192
PTX-2	ESI+	876.3	841.3	35	205
			823.0	40	205
			805.3	41	205
DTX-1	ESI-	817.0	255.0	69	197
			113.1	67	197
DTX-2	ESI-	803.15	255.3	61	207
			113.1	50	207
YTX	ESI-	1141.5	1061.7	50	240
		570.2	467.3	40	240
OA	ESI-	803.3	254.9	68	216
			113.1	50	216

Table 1: Selected ion transitions (*m/z*) of the studied compounds and optimized collision energy and tube lens value for the TSQ Quantum Ultra triple quadrupole mass spectrometer

The optimization of selective reaction monitoring (SRM) parameters was performed by direct infusion of standards. Collision-induced dissociation (CID) data were recorded for each analyte including optimum collision energies for the selected ion transitions.

Table 1 summarizes all the mass transitions found for each analyte and its relative collision energy (CE) and tube lens values.

Results and Discussion

To ensure thorough validation of the method, neat standard solutions, standard addition on purified extracts, and spiked blank tissue extracts were prepared and compared.

Table 2 lists the correlation coefficients (r^2) indicating the linearity of the calibration curves for the three types of samples analyzed; five concentrations of the sample solution are considered (2, 5, 10, 20, and 50 µg/kg or similar).

To assess the inter-day repeatability of the method, ten replicates of spiked samples were analyzed between days. Solutions were prepared containing all the toxins in the five different concentrations used to perform the calibration curves (2, 5, 10, 20, and 50 µg/kg or similar).

The repeatability of the method expressed as the coefficient of variation percentage (CV %) has been rated less than 20% as shown in Table 3.

Analyte	Neat Solution	Spiked Purified Extract	Spiked Extract
AZA-1	0.9932	0.9965	0.9970
AZA-2	0.9973	0.9964	0.9901
AZA-3	0.9972	0.9958	0.9993
DTX-1	0.9964	0.9995	0.9953
DTX-2	0.9973	0.9966	0.9965
YTX	0.9999	0.9923	0.9988
PTX-2	1.0000	0.9977	0.9927
OA	0.9955	0.9924	0.9927

Table 2: Correlation Coefficient (r^2) of the calibration curves for the three types of samples analyzed in the concentration range of 2–50 µg/kg)

Analyte	Standard Deviation	Repeatability	CV%
AZA-1	0.25	0.79	20
AZA-2	0.29	0.90	18
AZA-3	0.43	1.37	17
DTX-1	0.18	0.55	2
DTX-2	0.22	0.68	9
YTX	0.12	0.40	12
PTX-2	0.39	1.36	20
OA	0.16	0.48	4

Table 3: Values of CV% obtained for the repeatability of the lower concentrated curve point (2 µg/kg)

The calculations, of limit of detection (LOD) and limit of quantification (LOQ) were made in accordance with the *UNICHIM Manual N. 179/0* where the calculation of the limit of detection is made through the calibration curve of the instrument used for analysis.[5]

To estimate the LOD and LOQ of the method (Table 4), ten samples were prepared by adding standard solution to 500 mg of homogenized mussel flesh and repeating the extraction procedure according to the method in "*EU-Harmonised Standard Operating Procedure for determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS – Version 3*". LOD and LOQ are expressed in µg/Kg. Recoveries are shown in Table 5.

	Hypersil GOLD		
Analyte	**LOD (µg/Kg)**	**LOQ (µg/Kg)**	**Outliers (Huber Test)**
AZA-1	0.56 ± 0.18	1.11	NO
AZA-2	0.93 ± 0.31	1.86	NO
AZA-3	1.28 ± 0.43	2.57	NO
DTX-1	5.66 ± 1.02	10.45	NO
DTX-2	0.71 ± 0.23	1.42	NO
YTX	1.67 ± 0.56	3.33	NO
PTX-2	1.40 ± 0.46	2.79	NO
OA	3.95 ± 1.32	7.91	NO

Table 4: LOD and LOQ of the method

Analyte	**Spiked Purified Extract**	**Spiked Extract**
AZA-1	96 ± 11	97 ± 11
AZA-2	101 ± 9	94 ± 14
AZA-3	104 ± 10	99 ± 6
DTX-1	101 ± 6	101 ± 7
DTX-2	101 ± 6	108 ± 42
YTX	99 ± 15	102 ± 17
PTX-2	103 ± 13	102 ± 20
OA	95 ± 7	93 ± 18

Table 5: Recovery values, where
$R\% = \{[(\mu g/Kg)_{CALCULATED}/(\mu g/Kg)_{THEORETICAL}]*100\}$

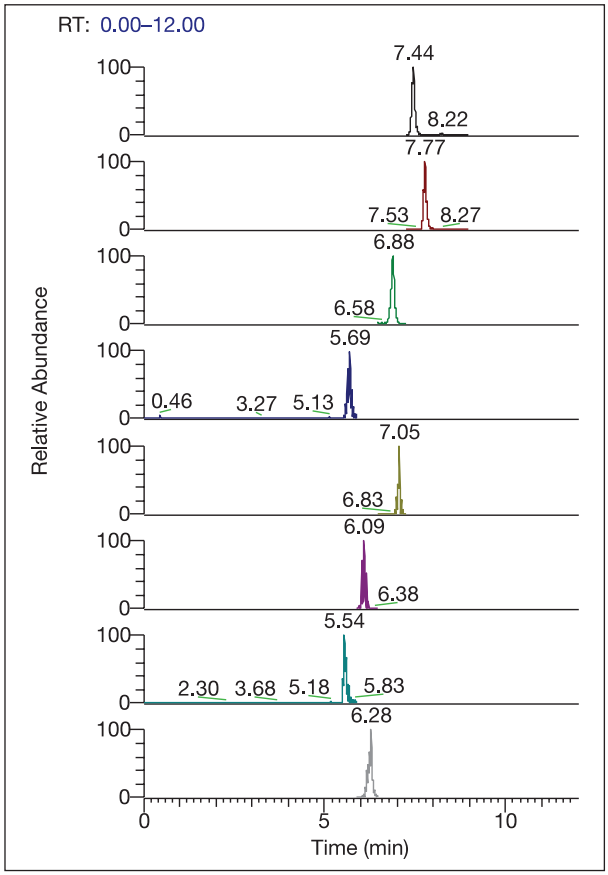

Figure 1: Chromatogram of sample containing 40 ppb of toxins (Retention Time: 7.44 min – AZA-1; 7.77 min – AZA-2; 6.88 min – AZA-3; 5.69 min – OA; 7.05 min – DTX-1; 6.06 min – DTX-2; 5.54 min – YTX; 6.28 min – PTX-2

Conclusion

This method proved to be selective, sensitive, accurate, and reproducible. It can be successfully applied for the quantitative determination of several classes of lipophilic marine biotoxins in bivalve mollusc samples.

References

1. Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April **2004**. *Official Journal of the European Communities* L139, 55-255.
2. Commission Regulation (EU) No 15/2011 of 10 January **2011**. *Official Journal of the European Union*. L6, 3-9.
3. Commission Regulation (EC) No 2074/2005 of 5 December **2005**. *Official Journal of the European Communities*, L388, 27-59.
4. European Union Reference Laboratory for Marine Biotoxins (EU-RL-MB), Agencia Española de Seguridad Alimentaria y Nutrición (AESAN). *EU-Harmonised Standard Operating Procedure for determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS – Version 3*. **2011**.
5. UNICHIM, Manual N. 179/0. *Linee guida per la convalida di metodi analitici nei laboratori chimici - criteri generali*. Milano. **1999**.

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