Validated Method for the Analysis of Lipophilic Marine Biotoxins in Bivalves by Liquid Chromatography—High-Resolution Mass Spectrometry

Complete method: Rúbies, A. et al, New method for the analysis of lipophilic marine biotoxins in fresh and canned bivalves by liquid chromatography coupled to high resolution mass spectrometry: A Quick, Easy, Cheap, Efficient, Rugged, Safe approach, J. Chrom. A, 2015 Mar 20;1386:62-73. doi: 10.1016/j.chroma.2015.01.088

Highlights

- The successfully validated method for the confirmatory, quantitative analysis of lipophilic marine biotoxins meets the requirements of European Union legislation.
- The QuEChERS strategy provides effective and reliable sample preparation.
- UHPLC with HRMS/MS delivers excellent results in the quantitative analysis of MBTXs.
- Calibration with surrogate matrix-matched standards (SMMS) provides reliable quantitative results.
- The lower limit quantitation of the method is 25 µg/kg.

Introduction

A harmful algal bloom is a serious environmental problem that results when the population of microscopic algae in a water system rapidly increases. The algae have the ability to produce marine biotoxins (MBTXs), which are dangerous to humans and other aquatic life. These biotoxins can accumulate in filter-feeding bivalve mollusks and, in turn, pose significant food safety risks to humans who ingest the shellfish, including gastrointestinal illnesses or neurological disorders.

Adequate testing for biotoxins is necessary to ensure public safety and long-term viability of commercial shellfish markets. Maximum concentration levels for MBTXs in bivalves have been established by many countries. European Union Regulations (EC) No 853/2004[1] and (EU) 786/2013[2] define permitted limits of lipophilic MBTXs in live bivalve mollusks (Table 1). As of 2015, LC-MS/MS is mandatory for official controls.

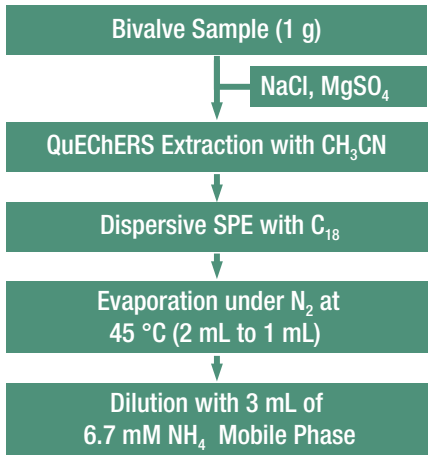
Table 1. Permitted limits for marine biotoxins according to EU regulations.[1,2]

Marine Biotoxins	Permitted Limit
Okadaic acid Dinophysistoxins Pectenotoxins	160 µg/kg of okadaic acid equivalents
Yessotoxins	3.75 mg/kg of yessotoxins equivalents
Azaspiracids	160 µg/kg of azaspiracids equivalents

Experimental

Sample Preparation

A sample to acetonitrile to C_{18} proportion of 1:5:0.025 produced the best recovery of analytes. To detect and quantify the total content of the okadaic acid group toxins, an additional hydrolysis step was required to transform the esters of the toxins.



LC-MS Conditions

UHPLC analysis was performed using the Thermo Scientific™ Accela™ UHPLC system with a C18 column (100 × 2.1 mm, 1.7 µm). Mobile phases were (A) 6.7 mM ammonia aqueous solution and (B) acetonitrile. The injection volume was 5 µL, and the flow rate was 0.25 mL/min. A Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap mass spectrometer, operating in tandem mass spectrometry mode, was used for analysis.

Spray voltage:	3.5 kV (positive ionization), 1.5 kV (negative mode)
Sheath gas (N_2):	30 arb. units
Capillary temperature:	300 °C
S-lens:	50 arb. units
Heater temperature:	200 °C
Normalized collision energy:	15% to 34% for the various compounds
Resolution:	70,000 (*m/z* 200, FWHM)
Scan rate:	2 Hz
Automatic gain control:	5 × 104
Maximum injection time:	35–100 ms

Thermo Scientific™ TraceFinder™ software version 3.1 was used for data processing.

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Data

Evaluation of QuEChERS Method

The extraction method was tested on canned and fresh samples of various bivalve species. A comparison of the trueness of the spiked samples at 25 µg/kg in the different matrices showed no variations. In addition, the internal standard responses were the same in these samples.

Validation of Complete QuEChERS UHPLC-HRMS Method

For each analyte, the molecular ion and one or two product ions were acquired with a mass accuracy better than 5 ppm (Figure 1). Quantification was performed using SMMS, with eprinomectin as internal standard. A single SMMS calibration curve built from blank mussel samples was shown to be suitable for the analysis of different bivalve species, which is of importance to laboratories that analyze multiple types of shellfish.

The method was validated in mussel muscle. Specificity was evaluated by analyzing blank mussel samples, which were found to be free of endogenous interferences. In addition, blank clam, cockle, and razor clam samples were investigated and, again, no interfering peaks were observed at the retention time of the analytes.

The lower limit of the method was established as 25 µg/kg for the set of analytes. Linearity studies showed excellent results over the range 25–350 µg/kg, with coefficients of regression (r^2) greater than or equal to 0.9998. Residuals were below 25% at 25 µg/kg and below 15% for higher concentrations. Recovery ranged between 79% and 114%, while relative standard deviations ranged between 1.5% and 18.7%.

To further test the method, two mussel certified reference materials were analyzed and the data showed good agreement (Table 2).

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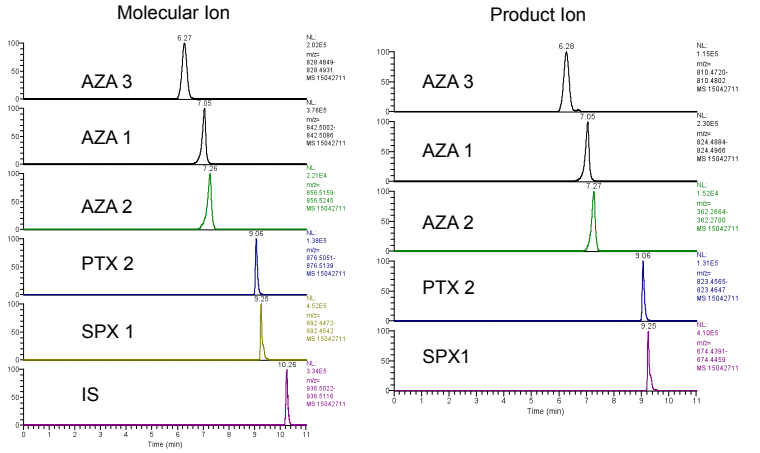

Figure 1. Extracted molecular ion chromatograms and extracted product ion chromatograms for a mixture of MBTXs.

Table 2. Analysis of certified reference materials.

MBTX	Certified Value		Determined Amount	
	Concentration (mg/kg)	Uncertainty (%)	Concentration (mg/kg)	Trueness (%)
Okadaic acid	0.361	0.034	0.336	93.1
Dinophysistoxin-1	0.206	0.019	0.189	91.8
Dinophysistoxin-3	0.283	0.054	0.287	101.4
Azaspiracid-1	1.16	0.100	1.06	91.4
Azaspiracid-2	0.273	0.024	0.256	93.8
Azaspiracid-3	0.211	0.023	0.222	105.2

References

1. Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 Laying Down Specific Hygiene Rules for Food of Animal Origin, OJ L 139, 30.4.2004, p. 55.
2. Commission Regulation (EU) No 786/2013 of 16 August 2013 Amending Annes III to Regulation (EC) No 853/2004 of the European Parliament and of the Council as Regards the Permitted Limits of Yessotoxins in Live Bivalve Molluscs, OJ L 220, 17.8.2013, p. 14.

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