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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

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ABSTRACT

A new method for the analysis of lipophilic marine biotoxins (okadaic acid, dinophysistoxins, azaspiracids, pectenotoxins, yessotoxins, spirolids) in fresh and canned bivalves has been developed. A QuEChERS methodology is applied; *i.e.* the analytes are extracted with acetonitrile and clean-up of the extracts is performed by dispersive solid phase extraction with C_{18}. The extracts are analyzed by ultra-high performance liquid chromatography coupled to a hybrid quadrupole-Orbitrap mass spectrometer, operating in tandem mass spectrometry mode, with resolution set at 70,000 (m/z 200, FWHM). Separation of the analytes, which takes about 10 min, is carried out in gradient elution mode with a BEH C_{18} column and mobile phases based on 6.7 mM ammonia aqueous solution and acetonitrile mixtures. For each analyte the molecular ion and 1 or 2 product ions are acquired, with a mass accuracy better than 5 ppm. The quantification is performed using surrogate matrix matched standards, with eprinomectin as internal standard. The high-throughput method, which has been successfully validated, fulfills the requirements of European Union legislation, and has been implemented as a routine method in a public health laboratory.

1. Introduction

Marine biotoxins (MBTXs) are produced by microalgae. Depending on climatic conditions algae blooms can occur and consequently, in these scenarios, the concentration of these toxins in sea water increases considerably. During these events, filter feeding bivalve molluscs can accumulate relatively high concentration levels of toxins. Although MBTXs are not toxic to these species, they may produce severe effects on humans if seafood contaminated with MBTXs is consumed.

MBTXs can be classified according to polarity into two major groups, *i.e.* hydrophilic and lipophilic toxins. Hydrophilic toxins, such as domoic acid and saxitoxins can produce amnesic shellfish poisoning or paralytic shellfish poisoning respectively [1]. Lipophilic toxins comprise several families of compounds, such as azaspiracids (AZAs), pectenotoxins (PTXs), spirolids (SPXs), yessotoxins (YTXs), brevetoxins (BTXs), ciguatoxins (CTXs) or okadaic acid group (OA), which comprises dinophysistoxin derivatives (DTXs). Their toxic effects are diverse [1], including gastrointestinal disorders or neurological symptoms.

To protect public health, maximum concentration limits for some MBTXs in bivalves, as well as official control programs, have been set up in many countries. In the European Union, Regulations (EC) No 853/2004 [2] and (EU) 786/2013 [3] establish the permitted limits of lipophilic MBTXs in live bivalve molluscs (Table 1).

Mouse bioassay has been the reference method for the official control of lipophilic MBTXs [4]. This assay provides information

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Table 1
Limits for lipophilic MBTXs in bivalve mollusks according to European regulations [2,3] and toxicity equivalence factors (TEF), as defined by EFSA [5].

Marine biotoxins	Permitted limit	Toxicity equivalence factor		
		Analogue	TEF	Results expression
Okadaic acid, Dinophysistoxins Pectenotoxins	160 μg kg^{-1} of OA equivalents	OA	1	μg OA equivalents kg^{-1}
		DTX1	1	μg OA equivalents kg^{-1}
		DTX2	0.6	μg OA equivalents kg^{-1}
		PTX1	1	μg PTX equivalents kg^{-1} *
		PTX2	1	μg PTX equivalents kg^{-1} *
Yessotoxins	3.75 mg kg^{-1} of YTX equivalents	YTX	1	mg YTX equivalents kg^{-1}
		hYTX	1	mg YTX equivalents kg^{-1}
		45-OH-YTX	1	mg YTX equivalents kg^{-1}
		45-OH-hYTX	0.5	mg YTX equivalents kg^{-1}
Azaspiracids	160 μg kg^{-1} of AZA equivalents	AZA1	1	μg AZA equivalents kg^{-1}
		AZA2	1.8	μg AZA equivalents kg^{-1}
		AZA3	1.4	μg AZA equivalents kg^{-1}

* To express the results according to EU legislation, under the guidance of the EU Reference Laboratory on MBTXs, PTXs are considered equivalent to OA, and thus the TEF for PTX1 and for PTX2 is taken as 1, expressed as μg OA equivalents kg^{-1}.

about total toxicity, but has several drawbacks; in addition to bioethical issues, the method lacks selectivity, the sensitivity for some compounds is rather limited (*e.g.* okadaic acid group), and the reproducibility of results is poor [5].

Due to these reasons, in 2011 the European Regulation (EU) 15/2011 [6] established that official controls should be performed with analytical methods based on liquid chromatography (LC) with tandem mass spectrometry (MS/MS) detection, which is now considered the reference technique, and from 2015 LC–MS/MS is mandatory for official controls.

Several LC–MS methods for lipophilic MBTXs analysis in shellfish samples have been reported (Table 2). The extraction of analytes from tissue is performed with methanol [7–18]. Some methods avoid the clean-up step [7–12], but others consider solid phase extraction (SPE) with C_{18} [13,14] or polymeric [14–18] cartridges. Obviously, the inclusion of a clean-up step increases the analysis time per sample, but in the case of complex extracts it can be a good strategy to decrease the ion suppression effects on MS analysis, as well as to ensure the good instrument maintenance and to extend the lifetime of the chromatographic column. The QuEChERS (Quick, Easy, Cheap, Efficient, Rugged, Safe) approach, *i.e.* the combination of an extraction using acetonitrile with a high salt content and clean-up using d-SPE, was initially developed for the analysis of pesticides [19], and it has also been applied to the analysis of antibiotics, mycotoxins, polycyclic aromatic hydrocarbons, etc. in different types of food samples with highly positive results [20–25]. The QuEChERS strategy was adopted in this study due to its simplicity and high throughput. To our knowledge there is no previous publication in the literature reporting the application of QuEChERS to the analysis of marine biotoxins.

Chromatographic separation is a key issue in the analysis of MBTXs by LC–MS, and several approaches at acidic, neutral or basic pH using different columns, based on C8 [7,8,18] or C_{18} [7,9–17] phases, have been reported.

The triple quadrupole (QqQ) analyzer in the multiple reaction monitoring (MRM) acquisition mode is, currently, the most common approach for MS analysis [7,8,13,15–17]. It provides excellent sensitivity and, to ensure selectivity, two MRM transitions are usually monitored for each analyte. However the scenario is changing with the introduction of high resolution (HR) MS instruments in routine laboratories. Time of flight (TOF) and, specially, Orbitrap based instruments offer very high resolution, and excellent accuracy and stability of mass measurements, which makes the new generation of HRMS instruments very attractive and valuable tools in MBTXs analysis. The studies on screening analysis [10,26], as well as on quantitative and confirmatory methods for lipophilic MBTXs [11,12] using LC- Orbitrap–MS have shown the suitability of HRMS for both untargeted and targeted analysis of MBTXs in complex samples. Moreover, hybrid TOF and Orbitrap MS instruments, usually presented as Q-TOF and Q-Orbitrap configurations, appear as indispensable tools for confirmatory analysis to fulfil the established legislation based on LC–MS/MS [6]. The capabilities of high resolution and accurate mass measurements of the second mass analyzer with pre-selection of the molecular ion in the first mass analyzer (Q), provides extra sensitivity and selectivity [27,28]. In comparison to QqQ, product ions can be acquired in high resolution mode so that isobaric interferences are resolved. Moreover, the accurate masses of the molecular and product ions are measured, providing additional confirmatory information and helping to eliminate false results [29].

Here we present a high-throughput confirmatory quantitative method for the analysis of regulated MBTXs (AZAs, PTXs, SPXs, YTXs, OA and DSPs) in shellfish samples, based on QuEChERS and UHPLC-Q-Orbitrap-HRMS. Thus, after the extraction of the analytes with acetonitrile, a simple clean-up by dispersive solid phase extraction (d-SPE) with C_{18} is performed, and the extracts are injected into the chromatographic system. The calibration is carried out using surrogate matrix matched standards (SMMS), by spiking blank samples with standards and internal standard before extraction. The method, which has been validated, has been included in the accreditation scope of the laboratory of the Agència de Salut Pública de Barcelona (LASPB) according to ISO 17025 [30], and it is currently applied in routine analysis.

2. Experimental

2.1. Chemical and reagents

Standards of pectenotoxin-2 (PTX2) (7.27 ± 0.33 μg mL^{-1}, ≥99%), 13-desmethyl spirolide C (SPX1) (6.89 ± 0.45 μg mL^{-1}, ≥97%), azaspiracid-1 (AZA1) (11.91 ± 0.87 μg mL^{-1}, ≥99%), azaspiracid-2 (AZA2) (11.73 ± 0.78 μg mL^{-1}, ≥99%), azaspiracid-3 (AZA3) (10.40 ± 0.51 μg mL^{-1}, ≥99%), yessotoxin (YTX) (7.91 ± 0.64 μg g^{-1}, ≥97.9%), homoyessotoxin (hYTX) (7.78 ± 0.67 μg g^{-1}, ≥98.4%), okadaic acid (OA) (24.92 ± 1.82 μg g^{-1}, ≥98.9%), dinophysistoxin-1 (DTX1) (2.55 ± 0.20 μg g^{-1}, ≥98%),