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An innovative method based on quick, easy, cheap, effective, rugged, and safe extraction coupled to desorption electrospray ionization-high resolution mass spectrometry for screening the presence of paralytic shellfish toxins in clams

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ABSTRACT

The capabilities of desorption electrospray ionization-high resolution mass spectrometry (DESI-HRMS) were tested for screening the presence of some paralytic shellfish toxins in clams. A quick, easy, cheap, effective, rugged, and safe (QuEChERS) approach is proposed for sample clean-up. QuEChERS extraction was optimized by using a full factorial design followed by the multicriteria method of the desirability functions. Quantitation limits in the microgram per kilogram range proved reliability of the method for the detection of the investigated toxins in accordance to the rules laid down by European legislation. The optimized QuEChERS-DESI-HRMS based-method allowed for a rapid reliable screening of the investigated compounds at levels of interest, thus being useful for high-throughput analyses.

1. Introduction

Marine toxins are produced as secondary metabolites by bacteria and microalgae like dinoflagellates, diatoms, or cyanophyceae [1]. Changes in environmental conditions are the major factor responsible for the appearance of harmful algae blooms associated with the production of these toxic compounds. Being able to be accumulated through the marine food-chain, marine toxins can contaminate seafood causing food poisoning with different neurological and gastrointestinal illnesses [2]. Paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP), amnesic shellfish poisoning (ASP), neurotoxic shellfish poisoning (NSP) are some of the most important syndromes associated with marine toxin contamination. Among them, PSP is related to the presence of paralytic shellfish toxins (PST), called also Saxitoxins – STXs-, a complex group of more than 20 water-soluble molecules characterized by a tetrahydropurine structure (Fig. S1). These compounds are able to inhibit neuronal transmission through blockage of voltage dependent sodium channels [3,4] causing signs of paralysis [5]. Due to their high hazard, a low maximum residue limit has been laid down in bivalve mollusks by the European Union (EU) legislation [6]: in fact, mollusks can be placed on the market for human consumption only if the PST content does not exceed the limit laid down in the regulation, i.e. 800 STX di-HCl eq/kg. The reference methods accepted in the EU for the analysis of these toxins are both the mouse bioassays [7] and the Lawrence method based on liquid chromatography with pre-column derivatisation and fluorescence detection (LC–FLD) [8]. Since a great number of shortcomings are related to the use of the mouse bioassay, i.e. high detection and quantitation limits, poor repeatability and presence of ethical issues related to the use of live animals, great efforts have been produced by the scientific community to develop reliable analytical methods able to detect and quantify each single toxin. In addition, taking into account that the Lawrence method does not allow the correct quantitation of coeluting analogues, and that the overlapping of oxidation products of different STX analogues is observed when pre-column derivatization is applied, a number of methods based on liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) have been developed to obtain a reliable determination of these marine toxins [9–13].

Recently, ambient ionization approaches like desorption electrospray ionization–mass spectrometry (DESI–MS) have been successfully applied with advantages in terms of reduced analysis time and reduced sample preparation [14–16]. Taking into account the absence of chromatographic separation, high resolution mass spectrometry (HRMS) [17,18] is of pivotal importance for the univocal identification of the analytes. The use of high resolution full scan analysis as an alternative to selected reaction monitoring-

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based methods is really appealing: in fact, only the specificity of accurate mass and the full spectrum acquisition in HRMS is able to guarantee the correct identification of the investigated compounds. Despite these advantages, the determination of epimeric pairs still represents a limiting factor of this technique, since analogues cannot be individually resolved. However, it has to be considered that when a pair of isomers are characterized by similar toxicity equivalency factors [19], analogues could not be resolved since similar values are obtained when these toxins are expressed as STX equivalents. Taking into account the maximum residue limit of 800 STX di-HCl eq/kg, it is obvious that when analogues have similar TEF values, their individual contribution to this regulatory value is not significantly different. However, when positive samples are detected, a complete separation of PSP toxins can be further achieved by using alternative techniques like hydrophilic interaction liquid chromatography (HILIC) [10,20]. To the best of our knowledge, there has been no report of the analysis of marine toxins by DESI-HRMS technique.

However, when complex matrices like mollusks have to be analyzed, sample pre-treatment can be required to obtain analyte enrichment. Although solvent extraction followed by pre-concentration before instrumental analysis is one of the most common approaches for analyte enrichment, valid alternatives to time-consuming and laborious liquid–-liquid extraction can be utilized. Recently, the quick, easy, cheap, effective, rugged, and safe (QuEChERS) technique has been proposed as rapid sample treatment procedure for analysis of samples of food [21–23], environmental [24] and toxicological concern [25].

The aim of this study, that was performed in the framework of a research project devoted to the assessment of safety and quality of Mediterranean seafood products, was the development of a rapid QuEChERS-DESI-HRMS method for the detection of some saxitoxins in clams. The optimization of the instrumental parameters and the proper choice of both the DESI support and the extraction conditions allowed to prove the capability of the method to screen and quantitate the investigated compounds at trace levels in clams.

2. Materials and methods

2.1. Chemicals

PSP toxins: saxitoxin (STX), decarbamoylsaxitoxin (dcSTX), neosaxitoxin (NEO), decarbamoylneosaxitoxin (dcNEO – used as internal standard), gonyautoxin 1&4 (GTX1,4), gonyautoxin 2&3 (GTX2,3), decarbamoylgonyautoxin 2&3 (dcGTX2,3), gonyautoxin 5 (GTX5) and N-sulfocarbamoyl-gonyautoxin-2 and-3 (C1&2) were purchased from the NRC Certified Reference Materials Program (Institute for Marine Bioscience, Halifax, Canada). Formic acid ($\geq$ 98% purity, FA), acetonitrile and methanol (both HPLC grade) were from Sigma Aldrich (Milano, Italy). Ammonium formate salt (97%, purity) was from Janssen Chimica (Beerse, Belgium). Water was obtained with a Millipore Milli-Q Element A10 System (Merck KGaA, Darmstadt, Germany). ABS Elut-NEXUS 30 mg cartridges, Q-Sep QuEChERS tubes containing 150 mg magnesium sulfate, 50 mg PSA and 50 mg C18, Q-Sep QuEChERS tubes containing 150 mg magnesium sulfate, 50 mg PSA, 50 mg C18, 7.5 mg GCB were from Restek (Milano, Italy), whereas Supel QuE Z-Sep/C18 tubes (Z-Sep 120 mg, C18 300 mg) were from Supelco (Milano, Italy). Glass, poly(methyl methacrylate) (PMMA) and hydrophobic (HTC)-printed slides were purchased from Prosolia™, Inc. (Indianapolis, IN, US).

2.2. Sample treatment

Clams were purchased from a local supermarket and used as blank matrix. The same procedure used in a previous study was used for the extraction of the investigated PSP toxins [13]. Briefly, 1 g of clam homogenate was weighted into a centrifuge tube and extracted twice with 1 ml of 0.1% (v/v) formic acid by vortexing for 10 min. The sample was then centrifuged at 8000*g* for 10 min and the supernatants combined together. One ml of cold methanol was added and the tube was placed in an ice-bath for 30 min. After centrifugation at 8000*g* for 10 min at 15 °C, proteins were removed and the remaining supernatant was reduced to a volume of 1 ml under a nitrogen flow at 40 °C. Sample clean-up was performed by QuEChERS technique by adding 10 mg of ABS Elut-NEXUS phase to the extract and vortexing for 5 min. Finally, after the last centrifugation at 8000*g* for 1 min, the extract was filtered on 0.2 μm PTFE syringe filter (Pall Corporation, Port Washington, NY, USA) and submitted to DESI-HRMS analysis (more than 40 samples can be analyzed in less than 100 min)

2.3. DESI-HRMS analysis

All the analyses were carried out using a LTQ Orbitrap XL hybrid FTMS instrument (ThermoFisher Scientific Inc., San Jose, CA, USA), equipped with a Omni Spray™ ion source (Prosolia Inc.) and by operating in the positive ion mode.

The experimental conditions were as follows: solvent flow: 1 μl/min; spray voltage: 3 kV; tube lens voltage: 50 V; capillary voltage: 15 V; capillary temperature: 250 °C; nitrogen pressure: 8.5 bar; incident angle: 55° tip-to surface distance 2 mm; surface-to-inlet distance 0.5 mm; tip-to-inlet distance 4 mm. Two different spraying solvents were tested: methanol:water (1:1, v/v) with 0.1% FA and acetonitrile:water (1:1, v/v) with 0.1% FA. The sample plate was positioned on a moveable 1-D stage. Preliminarily, full scan accurate mass spectra in the 100–500 amu range were acquired to determine appropriate masses for each analyte. Identification and quantification of target compounds was performed using the accurate mass of the analytes within a mass window of 5 ppm. Quantitation was performed by using the extracted ion chromatograms by selecting the appropriate m/z value for each analyte. Signal acquisition and data processing were performed using the Xcalibur 2.0 software (Thermo Fisher).

2.4. Optimization procedure

The experiments were carried out on blank clam samples spiked with paralytic shellfish toxins at 300 μg/kg for STX, dcSTX, NEO, GTX5, 1200 μg/kg for GTX1,4, 1080 μg/kg for GTX2,3 and 1620 μg/kg for dcGTX2,3. A 2^3 two-levels full factorial design (FFD) followed by the multicriteria method of desirability functions was carried out [26,27]. The effects of amount of ABS Elut-NEXUS phase (ABS), vortexing time (*V*) and centrifugation time (*C*) were evaluated. Low and high levels were the following: ABS: 10–100 mg, *V*: 30–300 s, *C*: 60–1200 s. The best regression models were obtained by a forward search stepwise variable selection algorithm and the optimal conditions were evaluated by the global desirability D [28].

2.5. Method validation

Method validation was performed according to EURACHEM guidelines [29] following the same procedure reported in previous studies [30,31]. Not contaminated clam samples were used as blank matrix. Briefly, detection (y_D) and quantitation (y_Q) limits, preliminary expressed as signals based on the mean and the standard deviation of blank responses, were calculated in terms of