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An innovative method based on QuEChERS 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 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.

Keywords: Saxitoxins; Desorption electrospray ionization; High resolution mass spectrometry; QuEChERS; Mollusks

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. 1). 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 [9-12].

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 [13-15]. Taking into account the absence of chromatographic separation, high resolution mass spectrometry (HRMS) [16,17] 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-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. Anyway, this drawback can be easily overcome taking into account that 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) [10,18]. 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 [19-21], environmental [22] and toxicological concern [23].

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.

Materials and methods

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 (≥ 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).

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 [24]. 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 x 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 minutes. After centrifugation at 8000 x 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 x g for 1 min, the extract was filtered on 0.2µm PTFE syringe filter (Pall Corporation, Port Washington, NY, USA) and submitted to DESI-HRMS analysis (more than 40 samples can be analysed in less than 100 min)

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^{TM} 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: 50V; capillary voltage: 15V; 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).

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 [25,26]. 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 [27].

Method validation

Method validation was performed according to EURACHEM guidelines [28] following the same procedure reported in previous studies [29,30]. 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 concentration values i.e. detection limit (LOD) and quantitation limit (LOQ) by projection of the corresponding signals y_D and y_Q through a calibration plot y=f(x) onto the concentration axis.

Calibration curves (six concentration levels, three replicated measurements for each level) were evaluated by analyzing blank clam samples spiked with the proper amount of the investigated analytes. Homoschedasticity was verified by applying the Bartlett test. Mandel’s fitting test was also performed to check the goodness of fit and linearity. The significance of the intercept (significance level 5%) was established by running a Student’s *t*-test. Intra-day repeatability and intermediate precision were calculated in terms of RSD % on two concentration levels, performing three replicates at each level. Intermediate precision was estimated over three days verifying homoscedasticity of the data and performing the analysis of variance (ANOVA) at the confidence level of 95%.

Real samples analysis

Ten clam samples were purchased in local markets. Two samples were supplied by National Reference Laboratory for Marine Biotoxins (Cesenatico, FC, Italy). After extraction, 1 µl of dcNEO (used as internal standard) followed by 1 µl of the toxin extract were spotted onto HTC printed slides and submitted to DESI-HRMS analysis.

Results and discussion

Desorption electrospray ionization-high resolution mass spectrometry was evaluated as a new tool for screening the presence of PST in mollusks. Preliminary experiments were carried out in order to evaluate the best instrumental conditions for toxin detection: our findings proved that DESI-HRMS signals of the investigated toxins could be obtained only by using HTC-printed slides. The only exceptions were C1&C2: in fact, their detection was not feasible also when high concentration levels were used. As already stated in a previous study [31], this behavior could be explained taking into account the low ionization efficiency of these compounds. Additional experiments performed by changing both the geometrical configuration of the DESI source and by adding NaCl onto each HTC spot prior to analysis were not useful to increase sensitivity and selectivity. Instead, the choice of the proper spray composition was of paramount importance for the detection of the investigated compounds. In fact, when the MeOH:H_2O (1:1) with 0.1% FA spray composition was utilized, most of the investigated toxins could not be detected or very low signal intensities were obtained. These drawbacks were solved by using AcN:H_2O (1:1) with 0.1% FA spray composition: by operating under these conditions a noticeable increase in sensitivity (Fig. 2) was obtained for all the PSP toxins, thus allowing the detection of all the investigated compounds.

HRMS was of primary importance for the correct identification of the analytes. As shown in Fig. 3, very complex mass spectra were obtained, thus requiring exact mass measurements to assess the presence of PSP toxins. Despite the advantages of HR, a limiting factor still persists when DESI-MS experiments are performed: the absence of a chromatographic separation prior to analysis does not allow the correct identification of isomers. As a consequence, the separation of dcGTX2,3, GTX2,3 and GTX1,4 was not feasible. However, it has to be noticed that the same drawback is related to the use of the official LC-FLD method: consequently, when each toxin has to be detected, alternative methods based on hydrophilic interaction liquid chromatography (HILIC) have to be used [10, 18]. Table 1 lists the chemical structures, the *m/z* values and the mass accuracy of the investigated analytes. As shown in the table, the most significant ions correspond both to the $[M+H]^+$ ion and to the loss of a SO_3 group.

QuEChERS clean-up

Taking into account that mollusks represent a very complex matrix, a sample treatment procedure was required prior to DESI-HRMS analysis. In fact, the direct deposition of the homogenate onto the HTC slide was not feasible owing to the possible contamination of the ion source due to the blowing up of matrix particles. The feasibility of a QuEChERS approach was evaluated: as reported in a previous study [9], prior to QuEChERS clean-up, a protein precipitation step was required. Our findings [24] showed that by using cold methanol for 30 min at -20 °C, the complete precipitation of proteins could be achieved. This result proved to be useful in speeding-up the entire analytical process, in fact, this is a very rapid time if compared with that (4 hours) reported by Zhou and coworkers [9] in a study on blood clam and oyster shellfish matrices. Different types of QuEChERS and the ABS Elut-NEXUS phase were also evaluated. By contrast to the results achieved by Zhuo et al. [9], the use of QuEChERS containing GCB did not improve clean-up efficiency, in fact, discoloration was observed only after one day. The best results were achieved by using the ABS Elut-NEXUS phase, a polymeric sorbent made of polystyrene cross-linked with 50% divinyl benzene and poly(methyl methacrylate) characterized by a high surface area that offers a non-polar retention mechanism. It can be stated that the mixed polarity of this phase can be more suitable for the removal of interfering compounds with respect to the C18 adsorbent. On the basis of these findings, extraction conditions were optimized by performing an experimental design. By contrast to a One Variable at A Time approach, in which variables are changed independently from the others, the experimental design allows to simultaneously evaluate the effects of all the variables involved in the study as well as their interaction. Regression models for each toxin were obtained, thus providing useful information about their behaviour. In addition, by applying the multicriteria method of the desirability functions, regression models can be further used for optimization purposes. By using a 2^3 full factorial design and the multicriteria method of desirability functions, the optimal extraction conditions were found in correspondence to an ABS phase amount of 10 mg, a vortexing time of 5 min and a centrifugation time of 1 min obtaining the global desirability of D=0.78. The regression models used to search for the highest global QuEChERS-DESI-HRMS recovery within the explored domain and the values of the single desirability d_i are reported in Table 2. The good values of both single desirabilities and global desirability proved that the clean up conditions were satisfying for all the investigated toxins.

As a general comment, it can be stated that without the QuEChERS clean up, no DESI-HRMS signal could be obtained, thus suggesting the strong importance of this purification step before instrumental analysis. Similar results were also obtained in an our previous study in which HILIC-MS analyses were performed [24]: again, without sample cleanup, a strong pressure increase due to the clogging of column was observed, thus enforcing the impact of sample cleanup on this kind of matrices.

Finally, method validation was carried out under the optimized extraction conditions.

Method validation

The QuEChER-DESI-HRMS method was validated and applied for the quantitation of the investigated toxins in clams. Taking into account that signal instability is a common phenomenon when DESI-MS analyses are carried out, an internal standard was used to achieve repeatable responses. As already proposed by Halme et al. [31], one of the PSP toxin was used as internal standard: more precisely, in our study NEO was chosen as internal standard: this choice was due to the fact that this toxin is usually detected less frequently and possesses a lower toxicity in comparison to the other toxins. In addition, taking into account the rapidity of DESI-MS analysis, the presence of this toxin in real samples can be assessed prior to its addition as internal standard.

Interesting results were obtained with LOD and LOQ values in the microgram per kilogram range, thus proving the potential of the method for the determination of these compounds in accordance with the rules laid down by the European Union legislation. Good linearity was also proved over one order of magnitude for all the analytes by applying Mandel's fitting test (Table 3).

Method precision was assessed for each toxin by testing two concentration levels, i.e. the LOQ and the highest concentration level used for the calibration curve. Good results were obtained both in terms of intra-day repeatability and intermediate precision: RSD values lower than 4.1% at the highest concentration and lower than 13% at the lowest one were calculated for intra-day repeatability. As for intermediate precision, ANOVA showed that mean values were not significantly different among the 3 days obtaining p values > 0.05. RSD lower than 23% at both concentration levels were calculated. Extraction recoveries ranging from 62±7% to 99±4% (n=3) were calculated at the same concentration levels used for the evaluation of precision (Table 4), thus showing the good efficiency of the developed method in terms of extraction recovery as well as of precision.

Finally, the developed method was applied for the analysis of 20 different samples taken from local supermarkets and 2 samples supplied by National Reference Laboratory for Marine Biotoxins (Cesenatico, FC, Italy). All the samples were from different regions of Italy: 2 samples from Emilia Romagna, 5 from Veneto, 3 from Liguria, 4 from Toscana, 3 from Marche and 3 from Sardegna. No paralytic shellfish toxins were detected in the analyzed samples taken from local markets; by contrast, 410 and 860 µg STX di-HCl eq/kg total toxicity, respectively were observed for the two samples supplied by the National Reference Laboratory for Marine Biotoxins (Cesenatico, FC, Italy). The achieved results were furtherly verified by applying both the HILIC-MS^2 method developed in our laboratory [24] obtaining a good agreement among the achieved results: 460 and 980 µg STX di-HCl eq/kg total toxicity were obtained by using the HILIC-MS^2 method whereas 440 and 840 µg STX di-HCl eq/kg total toxicity the mouse bioassay method, respectively, thus assessing the reliability of the DESI-HRMS method for screening and quantitate paralytic shellfish toxins.

Conclusions

In the present work, a QuEChERS-DESI-HRMS method was developed and validated for screening the presence of paralytic shellfish toxins in clams taken from Italian sea. Although isomers could not be detected individually, high resolution proved to be a powerful tool for the analysis of the investigated compounds, allowing the identification of PSP toxins in complex matrices like mollusks. The suitability for high-throughput quantitative purposes, makes the method a valid alternative to more laborious approaches for quality control applications.

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