Optimization of a rapid QuEChERS sample treatment method for HILIC-MS2 analysis of paralytic shellfish poisoning (PSP) toxins in mussels

Elsevier Editorial System(tm) for Food Control
Manuscript Draft

Manuscript Number:

Title: Optimization of a rapid QuEChERS sample treatment method for HILIC-MS2 analysis of paralytic shellfish poisoning (PSP) toxins in mussels

Article Type: Research Article

Keywords: Paralytic shellfish poisoning (PSP) toxins; hydrophilic interaction liquid chromatography-tandem mass spectrometry (HILIC-MS2); mussels; QuEChERS; experimental design optimization.

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Abstract: A rapid and simple QuEChERS sample treatment was proposed for the development of a selective hydrophilic interaction liquid chromatography-ESI-MS2-based method for the determination of saxitoxins (STXs) in mussel samples. Among different sorbents, ABS Elut-NEXUS phase, composed of polystyrene cross-linked with 50% divinyl benzene and poly(methyl methacrylate), provided the best results. The effects of experimental parameters, including sorbent amount, vortexing time and centrifugation time were investigated and optimized by experimental design. In particular, regression models and desirability functions were applied to find the experimental conditions providing the highest global extraction response. The method was validated under the optimized conditions; detection and quantification limits in the 3-159 μg/kg and 7-436 μg/kg ranges were respectively obtained, except for C2 for which highest values were calculated due to its low ESI ionization efficiency. Finally, the analysis of twenty-eight mussel samples permitted to detect and quantify some of the investigated STXs, proving the applicability of the devised method.

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DIPARTIMENTO DI CHIMICA

Editorial Office

Food Control

Parma, 16th March, 2015

Dear Editor,

enclosed I am sending an electronic copy of the paper "Optimization of a rapid QuEChERS sample treatment method for HILIC-MS^2 analysis of paralytic shellfish poisoning (PSP) toxins in mussels" by Monica Mattarozzi, Marco Milioli, Federica Bianchi, Antonella Cavazza, Silvia Pigozzi, Anna Milandri, Maria Careri.

The novelty of the present work relies on the development and optimization of a rapid sample treatment procedure, based on a fast protein precipitation step (within 30 min) and QuEChERS (quick, easy, cheap, effective, rugged and safe) clean-up, for the determination of paralytic shellfish poisoning toxins (saxitoxins) in mussels. The effects of main QuEChERS experimental factors as well as those of their interactions on saxitoxin response were investigated by 2^3 full factorial design; then, the global optimal conditions were found by multicriteria method of desiderability functions. The analysis of extracts was performed by hydrophilic interaction liquid chromatography coupled with tandem mass spectrometry detection (HILIC-MS^2), using pseudo selective reaction monitoring (pseudo-SRM) as acquisition mode. Differently from EU official recognized method, the extraction of different MS^2 transitions permitted the detection and quantitation of each toxin, individually; in addition, the possibility to record and visualize full MS^2 product ion spectra gives great advantages on identification reliability. After validation for the assessment of analytical quality parameters, the applicability of the method was proved through the analysis of mussel samples: six out of twenty-eight samples resulted naturally contaminated with some of the saxitoxins under investigation.

The manuscript has not been published elsewhere and it is not currently under a submission procedure to another Journal.

We hope that the manuscript could be of interest for *Food Control*.

Best regards

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Optimization of a rapid QuEChERS sample treatment method for HILIC-MS^2 analysis of paralytic shellfish poisoning (PSP) toxins in mussels

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Abstract

A rapid and simple QuEChERS sample treatment was proposed for the development of a selective hydrophilic interaction liquid chromatography-ESI-MS^2-based method for the determination of saxitoxins (STXs) in mussel samples. Among different sorbents, ABS Elut-NEXUS phase, composed of polystyrene cross-linked with 50% divinyl benzene and poly(methyl methacrylate), provided the best results. The effects of experimental parameters, including sorbent amount, vortexing time and centrifugation time were investigated and optimized by experimental design. In particular, regression models and desirability functions were applied to find the experimental conditions providing the highest global extraction response. The method was validated under the optimized conditions; detection and quantification limits in the 3-159 µg/kg and 7-436 µg/kg ranges were respectively obtained, except for C2 for which highest values were calculated due to its low ESI ionization efficiency. Finally, the analysis of twenty-eight mussel samples permitted to detect and quantify some of the investigated STXs, proving the applicability of the devised method.

Keywords: Paralytic shellfish poisoning (PSP) toxins; hydrophilic interaction liquid chromatography-tandem mass spectrometry (HILIC-MS^2); mussels; QuEChERS; experimental design optimization

1. Introduction

Since bivalve mollusks are filter feeders, they can accumulate neurotoxins, such as saxitoxins (STXs) produced by marine algae and cyanobacteria. STXs belong to the group of paralytic shellfish poisoning (PSP) toxins, causing severe food poisoning in case of contaminated seafood consumption. STXs are closely related tetrahydropurines, that can be classified into carbamate, N-sulfocarbamoyl, decarbamoyl and deoxydecarbamoyl toxins on the basis of their side chain nature. To protect public health, European Regulation (EC) No. 853/2004 established that live bivalve mollusks placed on the market for human consumption must not contain PSP toxins in total quantities (measured in the whole body or any part edible separately) that exceed 800 µg/kg limit. When STXs determination is performed by liquid chromatography (LC), the content of the STX-group toxins is expressed as the sum of STX di-hydrochloride (di-HCl) equivalents: results for individual toxins are converted into STX equivalents by application of the toxicity equivalency factors (TEFs). Monitoring programs and food quality controls for marine biotoxins have been established in many countries. According to Commission Regulation (EC) No. 1664/2006 (Commission Regulation, 2006) the mouse bioassay (MBA) and the Association of Official Analytical Chemists (AOAC) liquid chromatography with pre-column derivatization and

fluorescent detection (LC-FLD)-based method are the officially prescribed analytical approaches in the European Union for the detection of STX-group toxins (Association of Official Analytical Chemists, 2005a, 2005b). The advantages of MBA are rapidity and the possibility to assess total profile toxicity, whereas LC-FLD can be automated and permits sensitive and selective toxin determination. However, both of these approaches have some limitations; in particular, besides undesirability for ethical reasons, MBA cannot be automated and is characterized by high variability of the results due to specific animal characteristics (Campbell, Vilariño, Botana, & Elliott, 2011). LC-FLD is time consuming and laborious because STXs have to be post- or pre-column derivatized to allow their fluorescence detection (Association of Official Analytical Chemists, 2005b, 2011); moreover, STXs determination is quite complex due to the overlapping of oxidation products of different STX analogues. More recently, hydrophilic interaction liquid chromatography (HILIC) coupled to tandem mass spectrometry (MS/MS or MS^2) has been proposed for STXs identification and quantification in algal (Halme, Rapinoja, Karjalainen, & Vanninen, 2012; Lajeunesse et al., 2012; Watanabe et al., 2013) and shellfish (Dell'Aversano, Hess, & Quilliam, 2005; Sayfriz, Aasen, & Aune, 2008; Turrell, Stobo, Lacaze, Piletsky, & Piletska, 2008; Zhuo et al., 2013) samples. In fact, LC-MS/MS approach represents a valid alternative because it can determine STXs individually, without the need of any derivatization step. However, the LC-MS/MS determination of STXs in mollusks still represents a challenging analytical issue due to high complexity of the matrix, which causes strong signal suppression interferences (Zhuo et al., 2013). As a consequence, in order to permit the implementation of LC-MS/MS-based method in official control monitoring, toxin extraction and sample purification have to be properly developed and optimized in order to reach the requested sensitivity and accuracy for STXs determination in very complex matrices. In addition, taking into account that the developed method should meet the criteria for routine analysis, sample treatment procedure should be rapid, easy and reproducible. The quick, easy, cheap, effective, rugged, and safe (QuEChERS) method has been initially developed as sample treatment procedure for the analysis of pesticide residues in food (Cervera, Portolés, López, Beltrán, & Hernández, 2014; González-Curbelo, Lehotay, Hernández-Borges, & Rodríguez-Delgado, 2014; He et al., 2015) then it has been applied also for the determination of different classes of compounds in various matrices (Anzillotti, Odoardi, & Strano-Rossi, 2014; Bragança, Plácido, Paíga, Domingues, & Delerue-Matos, 2012; Cerqueira et al., 2014; Kung, Tsai, Ku, & Wang, 2015; Lucatello et al., 2015; Regueiro, Álvarez, Mauriz, & Blanco, 2011; Rúbies et al., 2015; Zhuo et al., 2013). QuEChERS method involves micro-scale extraction and extract purification using dispersive solid-phase extraction (d-SPE), giving advantages especially in terms of fast and simple analysis, low costs and minimal solvent consumption. Up to now, the only

application of QuEChERS-based sample clean-up for the HILIC-MS/MS analysis of ten PSP toxins in sea food, in particular in *Scomberomorus niphonius*, oysters and blood clams, has been reported by Zhou et al. (2013). In a research program, dealing with the development of new analytical strategies for the assessment of safety and quality of Mediterranean seafood products, in the present work an experimental design was used to study the effects of parameters associated to d-SPE clean-up for the development of a rapid sample treatment for the HILIC-MS^2 determination of saxitoxins in mussels. The reduction of the time required for sample treatment, not only in terms of final extract purification by d-SPE but also in terms of protein precipitation, paves the way to high throughput analysis of PSP toxins in very complex food matrices like shellfishes.

2. Materials and methods

2.1. Chemicals

Certified reference chemicals of PSP toxins (STX, saxitoxin; NEO, neosaxitoxin; GTX1-5, gonyautoxins; C1 and C2, N-sulfocarbamoyl toxins; dcSTX, dacarbamoyl saxitoxin; dcNEO, decarbamoyl neosaxitoxin; dcGTX2 and dcGTX3, dacarbamoyl gonyautoxins) were purchased from the NRC Certified Reference Materials Program (Institute for Marine Bioscience, Halifax, Canada): some certified solutions are a mix of two STXs, i.e. GTX1 and GTX4, GTX2 and GTX3, dcGTX2 and dcGTX3, C1 and C2. Acetonitrile (HPLC grade), methanol (HPLC grade), formic acid (≥ 98 %) were purchased from Sigma Aldrich (Milan-Italy). Ammonium formate (97 %) was purchased from Janssen Chimica (Beerse, Belgium). ABS Elut-NEXUS 30 mg cartridges, Q-Sep QuEChERS tubes containing 150 mg magnesium sulfate, 50 mg PSA (primary and secondary amine) and 50 mg C18, Q-Sep QuEChERS tubes containing 150 mg magnesium sulfate, 50 mg PSA, 50 mg C18, 7.5 mg GCB (graphitized carbon black) were from Restek (Milan, Italy), whereas Supel QuE Z-Sep/C18 tubes (Z-Sep 120 mg, C18 300 mg) were from Supelco (Milan, Italy). Water was obtained with a MilliQ element A10 System (San Francisco, CA, USA).

2.2. Samples

Mussels used as “blank” test matrix for method development and validation were purchased from local supermarkets. A total of twenty-eight mussel samples from different Italian sea areas were analyzed: 10 were supplied by National Reference Laboratory for Marine Biotoxins (Cesenatico, FC, Italy), whereas the other samples were purchased from local supermarkets. Mussels were shucked and the soft edible part was homogenized and stored in the freezer (-20°C) until analysis.

2.3. Sample treatment

An aliquot of 1.0±0.02 g of homogenate was weighted into a centrifuge tube and extracted twice with 1 ml of 0.1% formic acid by vortexing for 10 min. After each extraction, the resulting slurry was centrifuged at 8000 rpm for 10 min; both supernatants were combined and centrifuged again at 8000 rpm for 10 min. For protein precipitation, 1 ml of cold methanol was added to the extract and the tube was placed in freezer (-20 °C) for 30 min. After centrifugation at 8000 rpm for 10 min, the supernatant was reduced to a volume of 1 ml under nitrogen flow at 40°C. Sample cleanup was performed by dispersive solid-phase extraction with ABS Elut-NEXUS phase. Under final optimized conditions, 10 mg of solid phase were directly weighed into a 1.5 ml eppendorf tube, then the extract was added and vortexed for 5 min. After centrifugation at 8000 rpm for 1 min, extracts were filtered on 0.2µm PTFE syringe filter (Pall Corporation, Port Washington, New York, USA) and stored at -20°C until analysis.

2.4. LC-MS/MS analysis

Chromatographic separation was performed on a HPLC system (Thermo Electron Corporation, San Josè, CA, USA) coupled with a LTQ XL linear ion trap mass spectrometer (Thermo Electron Corporation) equipped with a pneumatically assisted electrospray (ESI) interface. The system was controlled by the Xcalibur software (Thermo Electron Corporation). The mobile phase was delivered by the Surveyor chromatographic system (Thermo Electron Corporation) equipped with a 200-vial capacity sample tray. A volume of 10 ml of each extract was injected into TSKgel Amide-80 150 mm x 2.00 mm, 3 µm, (Tosoh Bioscience, San Francisco, CA, USA) column, thermostated at 35 °C, at a flow rate of 200 ml/min. A binary solvent gradient was used for the analysis: solvent A consisted of 95 % acetonitrile and 5 % aqueous solution containing 20mM ammonium formate and 26 mM formic acid (pH=3.2), solvent B consisted of 95 % water and 5 % aqueous solution containing 20 mM ammonium formate and 26 mM formic acid (pH=3.2). STXs were separated with the following gradient: solvent B was set at 20 % for 15 min, delivered by a linear gradient from 20 to 40 % in 15 min and to 50 % in 5 min; then, it was maintained at 50 % for 3 min, delivered from 50 to 70 % in 2 min, and finally maintained at 70 % for 5 min before column re-equilibration.

The sheath gas (nitrogen, 99.99% purity), the auxiliary gas (nitrogen, 99.99% purity) and the sweep gas (nitrogen, 99.99% purity) were delivered at flow rates of 50, 20 and 5 arbitrary units, respectively. Optimized conditions of the source were set as follows: ESI voltage, 4.5 kV; capillary voltage, 15 V; capillary temperature, 275 °C; tube lens, 50 V. Pseudo-selected reaction monitoring (pseudo-SRM) was used as MS^2 acquisition mode; extracted ion chromatograms were obtained by extraction of individual fragment ion currents using Xcalibur software. Precursor ions, normalized

collision energies (CE) and the MS^2 transitions used for validation and quantitation purposes are given in Table 1.

2.5. Experimental design and optimization procedure

The experiments were carried out on blank mussels samples spiked with PSP toxins at: 100 µg/kg for STX, dcSTX, NEO, GTX5, 2145 µg/kg for GTX1, 700 µg/kg for GTX4, 1315 µg/kg for GTX2, 500 µg/kg for GTX 3, 6680 µg/kg for C1, 2000µg/kg for C2, 260 µg/kg for dcNEO and dcGTX3, 1154 µg/kg for dcGTX2. A 2^3 two-levels full factorial design (FFD) followed by the multicriteria method of desirability functions was carried out (Box, Hunter, & Hunter, 1978). The effects of amount of ABS Elut-NEXUS phase (ABS), vortexing time (V) and centrifugation time (C) were evaluated. Low and high levels were: 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 (Carlson, 1992). All statistical analyses were carried out by using the statistical package SPSS 10.0 for Windows (SPSS, Bologna, Italy).

2.6. Method validation

Method validation was carried out according to Eurachem guidelines (Eurachem Guide, 1998) using not contaminated mussels as blank matrix.

Detection (y_D) and quantitation (y_Q) limits were expressed as signals based on the mean blank ($\bar{x}_b$) and the standard deviation of blank responses (s_b) as follows: $y_D = \bar{x}_b + 2t\, s_b$ and $y_Q = \bar{x}_b + 10\, s_b$, where t is the constant of the t-Student distribution (one-tailed) at 95% confidence level. The value of $\bar{x}_b$ and s_b were calculated performing ten blank measurements. The concentration values of the detection limit (LOD) and quantitation limit (LOQ) were obtained by projection of the corresponding signals y_D and y_Q through a calibration plot y=f(x) onto the concentration axis. Matrix-matched calibration curves were built up and linearity was established over the calibration range for all the analytes. Five concentration levels were analyzed performing three measurements at each concentration level. Homoscedasticity was verified by applying the Bartlett test; lack-of-fit and Mandel's fitting tests were also performed to check the goodness of fit and linearity. The significance of the intercept (significance level 5%) was established running a 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%.

Trueness was evaluated in terms of recovery rate (RR%) by spiking mussel homogenate at two concentration levels (LOQ level and intermediate calibration level for each STXs) and calculated as percent ratio found to added amount: all the measurements were replicated three times.

3. Results and discussion

3.1. HILIC-ESI-MS/MS separation and detection

In the first step of the work, flow injection analysis (FIA) of certified standard solutions was performed in order to optimize ESI-MS^2 parameters, and to record the STXs mass spectra. Attention was paid to the choice of the optimum normalized CE energy to be used for each toxin fragmentation, by exploring the range between 20 and 45, with the aim of identifying the best value able to provide an ideal fragmentation pattern for quantitative purposes. The MS^2 transitions selected for validation and quantitative analysis were the ones giving the best signal-to-noise ratio in matrix extract (see Table 1).

As previously demonstrated by Dell'Aversano et al. (2005) the separation of STXs compounds can be performed by HILIC chromatography, which is based on a complex retention mechanism, consisting of partitioning, adsorption, ionic interactions and sometimes even hydrophobic interactions (Guo & Gaiki, 2011). Since ionic strength was found to represent an important parameter strongly affecting the retention of polar compounds in HILIC (Ihunegbo, Tesfalidet, & Jiang, 2010; Núñez, Gallart-Ayala, Martins, & Lucci, 2012), in this work, with the aim of optimizing the mobile phase composition in terms of ionic strength, three concentrations of a buffer solution were tested. The buffer, chosen on the basis of previous studies (Dell'Aversano et al., 2005; Sayfritz et al., 2008), was constituted by ammonium formate and formic acid at pH 3.2. The concentration of the buffer components were varied from 2 to 20 mM for ammonium formate, and from 3.6 to 26 mM for formic acid, while keeping constant the pH value at 3.2, taking care of remaining in the ratio of buffer activity. The effect on retention times was evaluated on a mixture of STXs standards. Results showed that the increase of the ionic strength determined a decrease in the retention times of all analytes. In detail, while increasing ionic strength by a factor of about 10, the observed decrease of retention times was found to be between 18 and 32%, depending on the toxin. Since analytes separation and peaks efficiency was not strongly affected, the buffer characterized by a higher ionic strength, i.e. 20 mM ammonium formate and 26 mM formic acid, was selected as best mobile phase in order to reduce analysis time. These results support the hypothesis of the occurrence of electrostatic interactions between the stationary phase and the analytes, resulting from a competition between the analyte and the buffer ions, as previously suggested (Núñez et al., 2012).

In Fig. 1 a representative HILIC-MS^2 chromatogram relative to the analysis of an extract from a mussel blank matrix fortified with a mixture of the investigated STXs is reported. Regardless of some co-eluted peaks, the developed HILIC-MS^2 method permitted to separate individual contribution of each toxin to the recorded signal, by extracting different and characteristic MS^2 transitions. This acquisition mode represents a great advantages over LC-FLD-based method, since overcomes co-elution problems. In addition, the LTQ mass analyzer offers the possibility to perform pseudo-SRM acquisition to record and visualize full MS^2 product ion spectra for each toxin of interest. This technique, rather than acquiring only a few selected parent-to-product ion transitions, as triple quadrupole does, gives great advantages on identification reliability and on reduction of false positive rate.

3.2. QuEChERS clean-up and Experimental design

Mussels represent a quite complex matrix, rich in proteins and fats. Therefore, the analysis of toxins requires a complex sample pre-treatment aimed at extracting and purifying the analytes of interest and remove interfering compounds. Different purification steps have been set up after STXs extraction, usually performed by acidic media (Dell'Aversano et al., 2005; Zhuo et al, 2013; Sayfritz et al. 2008).

A first step regards protein precipitation. To this aim, different precipitation agents such as acetonitrile, trichloroacetic acid and methanol were investigated. The effect of precipitation time (between 30 and 4 hours) and temperature (room temperature and -20 °C) was also evaluated. Among them, cold methanol provided the most abundant precipitation within 30 min at -20°C.

As already reported in a previous study on blood clam and oyster shellfish matrices (Zhuo et al., 2013), a QuEChERS protocol was developed and optimized in order to obtain a rapid clean-up of the mussel extract. Preliminary experiments were carried out to select the best sorbents for the QuEChERS protocol. As described in the experimental section, the performances of different commercially available QuEChERS tubes were tested. Our results proved that all of the three C18-based sorbent phases (i.e. Q-Sep QuEChERS and Supel QuE Z-Sep/C18) were not suitable for the clean-up of the PSP toxins, as a strong reduction of toxin chromatographic responses was observed. In addition, the use of GCB did not improve clean-up efficiency since only a partial discoloration of the solution could be obtained only after one day. For this reason, a QuEChERS protocol using the ABS Elut-NEXUS phase, consisting of polystyrene cross-linked with 50% divinyl benzene and poly(methyl methacrylate) was evaluated. It proved to be the best choice, probably since the occurrence in this phase of two different components acting with a double mechanism results to

promote a better removal of interfering compounds respect to the C18 sorbent, without entrapping the analytes of interest.

The ratio between sample and sorbent amounts, and the time of contact are known to be important parameters affecting interactions during QuEChERS purification. Therefore, in order to optimise conditions for 1 ml of mussel extract purification, an experimental design was performed in terms of amount of sorbent to be used, time of contact and centrifugation time. By using a 2^3 full factorial design and the multicriteria method of desirability functions, the optimal experimental 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 a global desirability of D=0.84. Table 2 lists the regression models used to search for the highest global QuEChERS-LC-MS^2 recovery within the explored domain and the values of the single desirability (di). As a result, very good single desirability values were obtained for all the PSP toxins, thus proving the suitability of the clean-up process for all the investigated toxins.

3.3. Method validation and application

The method was validated by using the experimental settings providing the optimised conditions. Results are reported in Table 3.

LOD and LOQ values in the µg/kg range were obtained, thus demonstrating the potentiality of the method to verify compliance of mussel samples with the law. The high LOD and LOQ values recorded for C1 and C2 are due to their low ionization efficiency, observed also in standard solutions, as has been pointed out previously by Halme et al. (2012) using an ESI-linear ion trap system for mass spectrometry detection. However, these high values do not represent a major drawback in the use of this analytical approach as the TEF for C2 is 0.1 (European Food Safety Authority, 2009), i.e. the toxicity of C2 is very low compared to STX. As for other performance method parameters, good linearity was proved in the calibration range for all the analytes by applying Mandel's fitting test. Method precision was evaluated testing two concentration levels, LOQ and upper calibration limit for each toxin. Good results were obtained both in terms of intra-day repeatability and intermediate precision with RSD values lower than 16%. As for intermediate precision, ANOVA performed on the data acquired over three days showed that the mean values were not significantly different ($p > 0.05$). The comparison between response factor obtained in standard solution and in matrix extract showed a significant ionization suppression, in the 43-84% range, due to a strong matrix effect. For this reason, matrix-matched calibration curves were used for quantitation. Good trueness in terms of method recoveries were obtained, with values ranging from 79 (±3) to 113 (±4)%.

Finally, the method was applied for the analysis of 28 real mussel samples. No traces of STXs were detected (<LOD) in samples purchased from local supermarket, demonstrating safety of commercially available mussels. By contrast, traces of some STXs were detected and quantified in six out of ten samples supplied by National Reference Laboratory for Marine Biotoxins (Table 4). The possibility to individually determine the investigated STXs in naturally contaminated mussel samples proves method applicability and reliability.

4. Conclusions

The optimization of QuEChERS based sample treatment, associated with fast protein precipitation step, permitted the development of a rapid method for HILIC-ESI-MS2 analysis of STXs in a very complex matrix such as mussels. Full factorial design and multicriteria method of desirability functions permitted not only to study single factor and their interaction effects on single toxin but also to identify the best experimental conditions for all the STXs investigated. Finally, the method resulted able to individually detect and quantify STXs in naturally contaminated samples.

Acknowledgments

The Project was funded by the "Italian Ministry for the University and Research (MIUR)" within the Projects of National Interest (PRIN) "Assessment of quality and safety of Mediterranean seafoods by "omics" sciences". Project ID: 2012TLC44W.

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Figure captions

Fig. 1. HILIC-ESI-MS^2 extracted chromatograms of the STXs under investigation from the analysis of a mussel extract (STXs were spiked before sample treatment at the following concentrations: C1, 6680 µg/kg; C2, 2000 µg/kg; GTX2, 789 µg/kg; GTX1, 613 µg/kg; dcGTX2, 444 µg/kg; GTX3, 300 µg/kg; GTX4, 200 µg/kg; dcGTX3, 100 µg/kg; GTX5, 100 µg/kg; STX, 100 µg/kg; dcSTX, 100 µg/kg; NEO, 100 µg/kg; dcNEO, 1000 µg/kg).

Highlights

- Rapid QuEChERS-based sample treatment for determination of saxitoxins in mussels.
- Experimental design and desiderability functions for QuEChERS protocol optimization.
- HILIC-ESI-MS^2 with pseudo-SRM acquisition mode for saxitoxin analysis.
- Method validation and application to mussel samples.

Figure 1

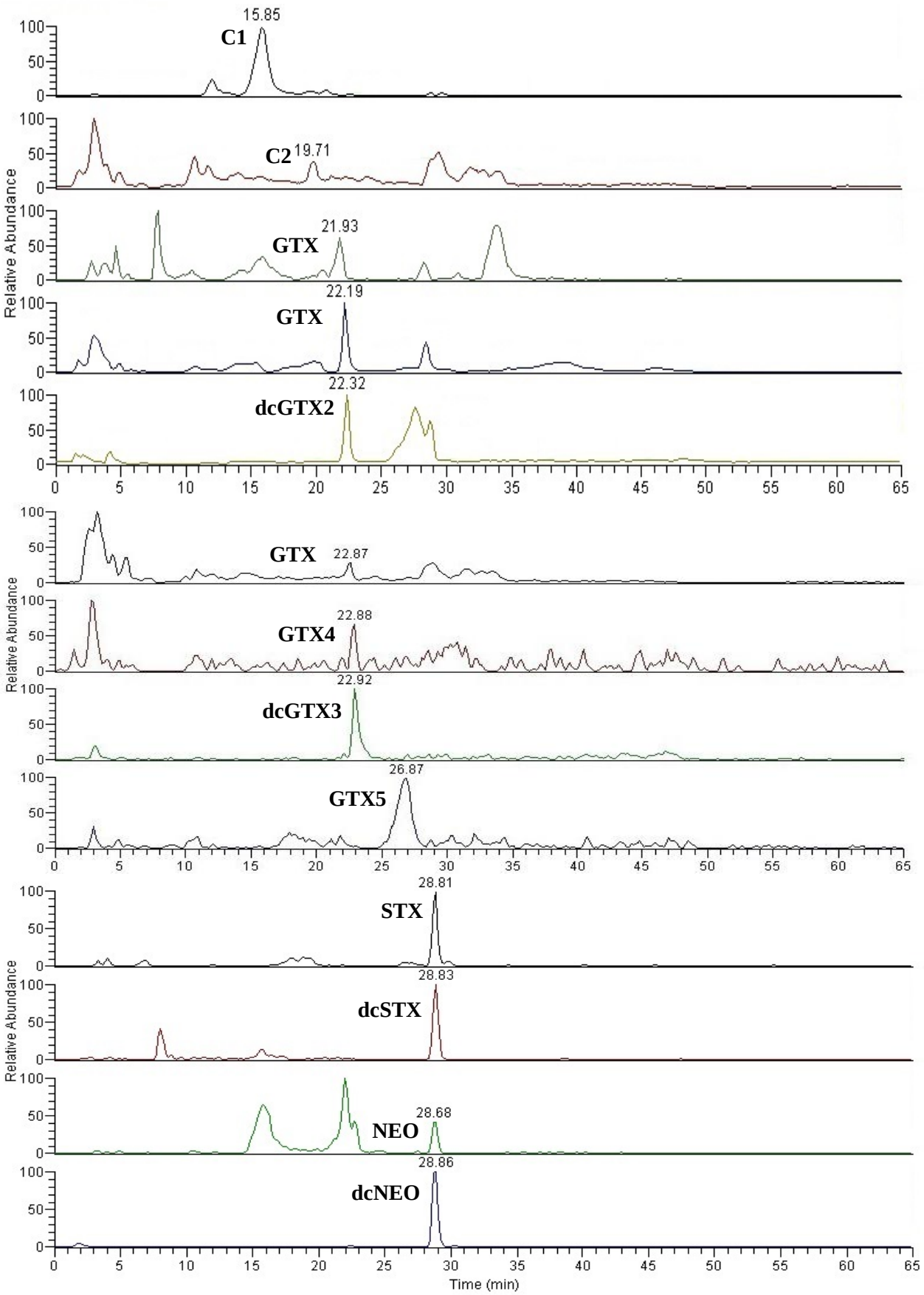

Fig. 1.

Table 1

MS^2 transitions used for method validation and STXs quantitation.

PSP toxin	*m/z* precursor ion > *m/z* product ion transition	CE (normalized units)
STX	300 > 221	40
NEO	316 > 237	25
GTX1	332 > 314	40
GTX2	316 > 257	25
GTX3	396 > 378	40
GTX4	412 > 332	30
GTX5	380 > 300	25
C1	396 > 316	40
C2	396 > 378	40
dcSTX	257 > 126	40
dcNEO	273 > 179	40
dcGTX2	273 > 255	40
dcGTX3	353 > 273	30

Table 2

Regression models[a] and single desirabilities (d) calculated for the PSP toxins.

STX (d=0.87)

y = 18490 (±590) - 1570 (±470) x_1 – 930 (±270) x_2 – 1600 (±270) x_3 – 1000 (±530) x_1x_2 + 3000 (±900) x_1^2 + 2860 (±900) x_2^2

dcSTX (d=0.87)

y= 12300 (±550) - 1500 (±490) x_1 - 870 (±490) x_3 - 1400 (±550) x_1x_2 + 2950 (±740) x_1^2

NEO (d=0.82)

y = 26160 (±760) - 1160 (±630) x_2 - 1340 (±630) x_3 - 1940 (±700) x_1x_2 + 4300 (±1100) x_1^2 + 3500 (±1100) x_2^2

dcNEO (d=0.88)

y = 5030 (±320) - 460 (±120) x_1x_2 + 770 (±380) x_2^2 - 690 (±480) x_3^2

C1 (d=0.95)

y = 10930 (±470) + 830 (±120) x_1 + 740 (±120) x_2 - 1380 (±470) x_1x_2 + 1520 (±640) x_1^2

C2 (d=0.90)

y = 6680 (±500) + 710 (±120) x_1 + 890(±120) x_2 - 1240 (±420) x_3 - 890 (±470) x_1x_2 + 1470 (±750) x_1^2 + 1810(±750) x_2^2

GTX1 (d=087)

y = 22200 (±830) + 1632 (±690) x_2 - 2200 (±690) x_3 - 2440 (±770) x_1x_2 + 3400 (±1200) x_1^2 + 3750 (±1240) x_2^2

GTX2 (d=0.87)

y = 19230 (±620) + 1390 (±230) x_2 - 1010 (±230) x_3 - 1710 (±440) x_1x_2 - 1290 (±440) x_2x_3 + 2640 (±880) x_1^2

GTX3 (d=0.87)

y = 790 (±140) - 380 (±40) x_3 - 280 (±30) x_2x_3 + 800 (±120) x_1^2

GTX4 (d=0.87)

y = 930 (±150) + 180 (±40) x_2 + 250 (±80) $x_1x_2x_3$ + 450 (±220) x_3^2

GTX5 (d=0.75)

y = 2900 (±320) - 1170 (±260) x_3 - 430 (±290) x_1x_2 + 440(±290) x_2x_3 + 1120 (±470) x_1^2 + 660 (±470) x_2^2

dcGTX2 (d=0.65)

y = 23400 (±660) - 1290 (±890) x_1 + 2040 (±890) x_2 + 1230 (±890) x_3 - 2640 (±990) x_1x_3

dcGTX3 (d=0.75)

y = 3050 (±300) - 600 (±280) x_1x_2 - 410 (±280) x_1x_3 + 990 (±440) x_1^2 + 840 (±440) x_2^2

[a] x_1 = ABS phase amount (ABS); x_2 = vortexing time (V); x_3 = centrifugation time (C)

Table 3

Validation data for the analysis of saxitoxins in mussels.

PSP toxin	LOD (µg/kg)	LOQ (µg/kg)	Calibration range (µg/kg)	Calibration curve $y=a(\pm s_a)x$
STX	3	7	10-1000	19.1(±0.6)x
NEO	11	27	30-1000	5.2(±0.3)x
GTX1	12	35	100-4500	34.7(±1.9)x
GTX2	20	65	70-4000	1.5(±0.2)x
GTX3	95	271	500-1500	3.2(±0.3)x
GTX4	159	436	500-1500	0.34(±0.02)x
GTX5	14	33	100-1000	16.5(±0.3)x
C1	140	289	500-5000	3.4(±0.1)x
C2	708	1452	1500-5000	2.2(±0.2)x
dcSTX	3	7	100-1000	13.7(±0.4)x
dcNEO	14	50	100-1000	4.6(±0.3)x
dcGTX2	37	80	200-4500	10.9(±0.1)x
dcGTX3	6	20	50-1000	1.9(±0.1)x

Table 4

Results of the analysis of positive naturally contaminated mussel samples (all supplied by National Reference Laboratory for Marine Biotoxins).

	Toxin concentration (µg/kg)[a]												
Sample	STX	NEO	GTX1	GTX2	GTX3	GTX4	GTX5	C1	C2	dcSTX	dcNEO	dcGTX2	dcGTX3
1	47±1	49±2	n.d.	212±23	n.d.	n.d.	2325±24	611±80	n.d.	n.d.	n.d.	n.d.	n.d.
2	46±1	93±7	1134±106	177±20	n.d.	n.d.	2107±227	1590±40	n.d.	n.d.	n.d.	n.d.	n.d.
3	29±1	78±11	n.d.	91±11	n.d.	n.d.	1056±52	530±73	n.d.	n.d.	n.d.	n.d.	n.d.
4	22±4	50±3	964±45	83±10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5	n.d.	n.d.	1128±130	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6	n.d.	n.d.	511±20	127±12	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

[a] n.d.: Not detected (<LOD)