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

This is a pre print version of the following article:

*Original*

Optimization of a rapid QuEChERS sample treatment method for HILIC-MS2 analysis of paralytic shellfish poisoning (PSP) toxins in mussels / Mattarozzi, Monica; Milioli, Marco; Bianchi, Federica; Cavazza, Antonella; Pigozzi, Silvia; Milandri, Anna; Careri, Maria. - In: FOOD CONTROL. - ISSN 0956-7135. - 60(2016), pp. 138-145. [10.1016/j.foodcont.2015.07.027]

*Availability:*

This version is available at: 11381/2802622 since: 2021-10-20T09:42:14Z

*Publisher:*

Elsevier Ltd

*Published*

DOI:10.1016/j.foodcont.2015.07.027

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(Article begins on next page)

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  $\mu\text{g}/\text{kg}$  and 7-436  $\mu\text{g}/\text{kg}$  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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# UNIVERSITÀ DEGLI STUDI DI PARMA

## DIPARTIMENTO DI CHIMICA

*Editorial Office*

*Food Control*

Parma, 16<sup>th</sup> 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<sup>2</sup> 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<sup>3</sup> full factorial design; then, the global optimal conditions were found by multicriteria method of desirability functions. The analysis of extracts was performed by hydrophilic interaction liquid chromatography coupled with tandem mass spectrometry detection (HILIC-MS<sup>2</sup>), using pseudo selective reaction monitoring (pseudo-SRM) as acquisition mode. Differently from EU official recognized method, the extraction of different MS<sup>2</sup> transitions permitted the detection and quantitation of each toxin, individually; in addition, the possibility to record and visualize full MS<sup>2</sup> 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

Dr. Monica Mattarozzi

1 **Optimization of a rapid QuEChERS sample treatment method for HILIC-MS<sup>2</sup> analysis of**  
2 **paralytic shellfish poisoning (PSP) toxins in mussels**

3  
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35 **Abstract**

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

48

49 **Keywords:** Paralytic shellfish poisoning (PSP) toxins; hydrophilic interaction liquid  
50 chromatography-tandem mass spectrometry (HILIC-MS<sup>2</sup>); mussels; QuEChERS; experimental  
51 design optimization

52

53 **1. Introduction**

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

69 fluorescent detection (LC-FLD)-based method are the officially prescribed analytical approaches in  
70 the European Union for the detection of STX-group toxins (Association of Official Analytical  
71 Chemists, 2005a, 2005b). The advantages of MBA are rapidity and the possibility to assess total  
72 profile toxicity, whereas LC-FLD can be automated and permits sensitive and selective toxin  
73 determination. However, both of these approaches have some limitations; in particular, besides  
74 undesirability for ethical reasons, MBA cannot be automated and is characterized by high  
75 variability of the results due to specific animal characteristics (Campbell, Vilariño, Botana, &  
76 Elliott, 2011). LC-FLD is time consuming and laborious because STXs have to be post- or pre-  
77 column derivatized to allow their fluorescence detection (Association of Official Analytical  
78 Chemists, 2005b, 2011); moreover, STXs determination is quite complex due to the overlapping of  
79 oxidation products of different STX analogues. More recently, hydrophilic interaction liquid  
80 chromatography (HILIC) coupled to tandem mass spectrometry (MS/MS or MS<sup>2</sup>) has been  
81 proposed for STXs identification and quantification in algal (Halme, Rapinoja, Karjalainen, &  
82 Vanninen, 2012; Lajeunesse et al., 2012; Watanabe et al., 2013) and shellfish (Dell'Aversano, Hess,  
83 & Quilliam, 2005; Sayfriz, Aasen, & Aune, 2008; Turrell, Stobo, Lacaze, Piletsky, & Piletska,  
84 2008; Zhuo et al., 2013) samples. In fact, LC-MS/MS approach represents a valid alternative  
85 because it can determine STXs individually, without the need of any derivatization step. However,  
86 the LC-MS/MS determination of STXs in mollusks still represents a challenging analytical issue  
87 due to high complexity of the matrix, which causes strong signal suppression interferences (Zhuo et  
88 al., 2013). As a consequence, in order to permit the implementation of LC-MS/MS-based method in  
89 official control monitoring, toxin extraction and sample purification have to be properly developed  
90 and optimized in order to reach the requested sensitivity and accuracy for STXs determination in  
91 very complex matrices. In addition, taking into account that the developed method should meet the  
92 criteria for routine analysis, sample treatment procedure should be rapid, easy and reproducible.  
93 The quick, easy, cheap, effective, rugged, and safe (QuEChERS) method has been initially  
94 developed as sample treatment procedure for the analysis of pesticide residues in food (Cervera,  
95 Portolés, López, Beltrán, & Hernández, 2014; González-Curbelo, Lehotay, Hernández-Borges, &  
96 Rodríguez-Delgado, 2014; He et al., 2015) then it has been applied also for the determination of  
97 different classes of compounds in various matrices (Anzillotti, Odoardi, & Strano-Rossi, 2014;  
98 Bragança, Plácido, Paíga, Domingues, & Delerue-Matos, 2012; Cerqueira et al., 2014; Kung, Tsai,  
99 Ku, & Wang, 2015; Lucatello et al., 2015; Regueiro, Álvarez, Mauriz, & Blanco, 2011; Rúbies et  
100 al., 2015; Zhuo et al., 2013). QuEChERS method involves micro-scale extraction and extract  
101 purification using dispersive solid-phase extraction (d-SPE), giving advantages especially in terms  
102 of fast and simple analysis, low costs and minimal solvent consumption. Up to now, the only

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

112

## 113 **2. Materials and methods**

### 114 *2.1. Chemicals*

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

128

### 129 *2.2. Samples*

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

135

### 136 *2.3. Sample treatment*



137 An aliquot of  $1.0\pm 0.02$  g of homogenate was weighted into a centrifuge tube and extracted twice  
138 with 1 ml of 0.1% formic acid by vortexing for 10 min. After each extraction, the resulting slurry  
139 was centrifuged at 8000 rpm for 10 min; both supernatants were combined and centrifuged again at  
140 8000 rpm for 10 min. For protein precipitation, 1 ml of cold methanol was added to the extract and  
141 the tube was placed in freezer ( $-20\text{ }^{\circ}\text{C}$ ) for 30 min. After centrifugation at 8000 rpm for 10 min, the  
142 supernatant was reduced to a volume of 1 ml under nitrogen flow at  $40\text{ }^{\circ}\text{C}$ . Sample cleanup was  
143 performed by dispersive solid-phase extraction with ABS Elut-NEXUS phase. Under final  
144 optimized conditions, 10 mg of solid phase were directly weighed into a 1.5 ml eppendorf tube,  
145 then the extract was added and vortexed for 5 min. After centrifugation at 8000 rpm for 1 min,  
146 extracts were filtered on  $0.2\text{ }\mu\text{m}$  PTFE syringe filter (Pall Corporation, Port Washington, New York,  
147 USA) and stored at  $-20\text{ }^{\circ}\text{C}$  until analysis.

148

#### 149 *2.4. LC-MS/MS analysis*

150 Chromatographic separation was performed on a HPLC system (Thermo Electron Corporation, San  
151 Josè, CA, USA) coupled with a LTQ XL linear ion trap mass spectrometer (Thermo Electron  
152 Corporation) equipped with a pneumatically assisted electrospray (ESI) interface. The system was  
153 controlled by the Xcalibur software (Thermo Electron Corporation). The mobile phase was  
154 delivered by the Surveyor chromatographic system (Thermo Electron Corporation) equipped with a  
155 200-vial capacity sample tray. A volume of 10 ml of each extract was injected into TSKgel Amide-  
156 80 150 mm x 2.00 mm,  $3\text{ }\mu\text{m}$ , (Tosoh Bioscience, San Francisco, CA, USA) column, thermostated  
157 at  $35\text{ }^{\circ}\text{C}$ , at a flow rate of 200 ml/min. A binary solvent gradient was used for the analysis: solvent  
158 A consisted of 95 % acetonitrile and 5 % aqueous solution containing 20mM ammonium formate  
159 and 26 mM formic acid ( $\text{pH}=3.2$ ), solvent B consisted of 95 % water and 5 % aqueous solution  
160 containing 20 mM ammonium formate and 26 mM formic acid ( $\text{pH}=3.2$ ). STXs were separated  
161 with the following gradient: solvent B was set at 20 % for 15 min, delivered by a linear gradient  
162 from 20 to 40 % in 15 min and to 50 % in 5 min; then, it was maintained at 50 % for 3 min,  
163 delivered from 50 to 70 % in 2 min, and finally maintained at 70 % for 5 min before column re-  
164 equilibration.

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

171 collision energies (CE) and the MS<sup>2</sup> transitions used for validation and quantitation purposes are  
172 given in Table 1.

173

#### 174 2.5. *Experimental design and optimization procedure*

175 The experiments were carried out on blank mussels samples spiked with PSP toxins at: 100 µg/kg  
176 for STX, dcSTX, NEO, GTX5, 2145 µg/kg for GTX1, 700 µg/kg for GTX4, 1315 µg/kg for GTX2,  
177 500 µg/kg for GTX 3, 6680 µg/kg for C1, 2000 µg/kg for C2, 260 µg/kg for dcNEO and dcGTX3,  
178 1154 µg/kg for dcGTX2. A 2<sup>3</sup> two-levels full factorial design (FFD) followed by the multicriteria  
179 method of desirability functions was carried out (Box, Hunter, & Hunter, 1978). The effects of  
180 amount of ABS Elut-NEXUS phase (ABS), vortexing time (V) and centrifugation time (C) were  
181 evaluated. Low and high levels were: ABS: 10-100 mg, V: 30-300 s, C: 60-1200 s. The best  
182 regression models were obtained by a forward search stepwise variable selection algorithm and the  
183 optimal conditions were evaluated by the global desirability D (Carlson, 1992). All statistical  
184 analyses were carried out by using the statistical package SPSS 10.0 for Windows (SPSS, Bologna,  
185 Italy).

186

#### 187 2.6. *Method validation*

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

190 Detection ( $y_D$ ) and quantitation ( $y_Q$ ) limits were expressed as signals based on the mean blank ( $\bar{x}_b$ )  
191 and the standard deviation of blank responses ( $s_b$ ) as follows:  $y_D = \bar{x}_b + 2t s_b$  and  $y_Q = \bar{x}_b + 10 s_b$ ,  
192 where  $t$  is the constant of the t-Student distribution (one-tailed) at 95% confidence level. The value  
193 of  $\bar{x}_b$  and  $s_b$  were calculated performing ten blank measurements. The concentration values of the  
194 detection limit (LOD) and quantitation limit (LOQ) were obtained by projection of the  
195 corresponding signals  $y_D$  and  $y_Q$  through a calibration plot  $y=f(x)$  onto the concentration axis.

196 Matrix-matched calibration curves were built up and linearity was established over the calibration  
197 range for all the analytes. Five concentration levels were analyzed performing three measurements  
198 at each concentration level. Homoscedasticity was verified by applying the Bartlett test; lack-of-fit  
199 and Mandel's fitting tests were also performed to check the goodness of fit and linearity. The  
200 significance of the intercept (significance level 5%) was established running a t-test.

201 Intra-day repeatability and intermediate precision were calculated in terms of RSD % on two  
202 concentration levels, performing three replicates at each level. Intermediate precision was estimated  
203 over three days verifying homoscedasticity of the data and performing the analysis of variance  
204 (ANOVA) at the confidence level of 95%.

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

208

### 209 **3. Results and discussion**

#### 210 *3.1. HILIC-ESI-MS/MS separation and detection*

211 In the first step of the work, flow injection analysis (FIA) of certified standard solutions was  
212 performed in order to optimize ESI-MS<sup>2</sup> parameters, and to record the STXs mass spectra.

213 Attention was paid to the choice of the optimum normalized CE energy to be used for each toxin  
214 fragmentation, by exploring the range between 20 and 45, with the aim of identifying the best value  
215 able to provide an ideal fragmentation pattern for quantitative purposes. The MS<sup>2</sup> transitions  
216 selected for validation and quantitative analysis were the ones giving the best signal-to-noise ratio  
217 in matrix extract (see Table 1).

218 As previously demonstrated by Dell'Aversano et al. (2005) the separation of STXs compounds can  
219 be performed by HILIC chromatography, which is based on a complex retention mechanism,  
220 consisting of partitioning, adsorption, ionic interactions and sometimes even hydrophobic  
221 interactions (Guo & Gaiki, 2011). Since ionic strength was found to represent an important  
222 parameter strongly affecting the retention of polar compounds in HILIC (Ihunegbo, Tesfalidet, &  
223 Jiang, 2010; Núñez, Gallart-Ayala, Martins, & Lucci, 2012), in this work, with the aim of  
224 optimizing the mobile phase composition in terms of ionic strength, three concentrations of a buffer  
225 solution were tested. The buffer, chosen on the basis of previous studies (Dell'Aversano et al.,  
226 2005; Sayfritz et al., 2008), was constituted by ammonium formate and formic acid at pH 3.2. The  
227 concentration of the buffer components were varied from 2 to 20 mM for ammonium formate, and  
228 from 3.6 to 26 mM for formic acid, while keeping constant the pH value at 3.2, taking care of  
229 remaining in the ratio of buffer activity. The effect on retention times was evaluated on a mixture of  
230 STXs standards. Results showed that the increase of the ionic strength determined a decrease in the  
231 retention times of all analytes. In detail, while increasing ionic strength by a factor of about 10, the  
232 observed decrease of retention times was found to be between 18 and 32%, depending on the toxin.  
233 Since analytes separation and peaks efficiency was not strongly affected, the buffer characterized by  
234 a higher ionic strength, i.e. 20 mM ammonium formate and 26 mM formic acid, was selected as  
235 best mobile phase in order to reduce analysis time. These results support the hypothesis of the  
236 occurrence of electrostatic interactions between the stationary phase and the analytes, resulting from  
237 a competition between the analyte and the buffer ions, as previously suggested (Núñez et al., 2012).

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

248

### 249 *3.2. QuEChERS clean-up and Experimental design*

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

255 A first step regards protein precipitation. To this aim, different precipitation agents such as  
256 acetonitrile, trichloroacetic acid and methanol were investigated. The effect of precipitation time  
257 (between 30 and 4 hours) and temperature (room temperature and -20 °C) was also evaluated.  
258 Among them, cold methanol provided the most abundant precipitation within 30 min at -20°C.  
259 As already reported in a previous study on blood clam and oyster shellfish matrices (Zhuo et al.,  
260 2013), a QuEChERS protocol was developed and optimized in order to obtain a rapid clean-up of  
261 the mussel extract. Preliminary experiments were carried out to select the best sorbents for the  
262 QuEChERS protocol. As described in the experimental section, the performances of different  
263 commercially available QuEChERS tubes were tested. Our results proved that all of the three C18-  
264 based sorbent phases (i.e. Q-Sep QuEChERS and Supel QuE Z-Sep/C18) were not suitable for the  
265 clean-up of the PSP toxins, as a strong reduction of toxin chromatographic responses was observed.  
266 In addition, the use of GCB did not improve clean-up efficiency since only a partial discoloration of  
267 the solution could be obtained only after one day. For this reason, a QuEChERS protocol using the  
268 ABS Elut-NEXUS phase, consisting of polystyrene cross-linked with 50% divinyl benzene and  
269 poly(methyl methacrylate) was evaluated. It proved to be the best choice, probably since the  
270 occurrence in this phase of two different components acting with a double mechanism results to

271 promote a better removal of interfering compounds respect to the C18 sorbent, without entrapping  
272 the analytes of interest.  
273 The ratio between sample and sorbent amounts, and the time of contact are known to be important  
274 parameters affecting interactions during QuEChERS purification. Therefore, in order to optimise  
275 conditions for 1 ml of mussel extract purification, an experimental design was performed in terms  
276 of amount of sorbent to be used, time of contact and centrifugation time. By using a  $2^3$  full factorial  
277 design and the multicriteria method of desirability functions, the optimal experimental conditions  
278 were found in correspondence to an ABS phase amount of 10 mg, a vortexing time of 5 min and a  
279 centrifugation time of 1 min obtaining a global desirability of  $D=0.84$ . Table 2 lists the regression  
280 models used to search for the highest global QuEChERS-LC-MS<sup>2</sup> recovery within the explored  
281 domain and the values of the single desirability (di). As a result, very good single desirability values  
282 were obtained for all the PSP toxins, thus proving the suitability of the clean-up process for all the  
283 investigated toxins.

284

### 285 *3.3. Method validation and application*

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

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

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

311

#### 312 **4. Conclusions**

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

319

#### 320 **Acknowledgments**

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

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474 **Figure captions**

475 **Fig. 1.** HILIC-ESI-MS<sup>2</sup> extracted chromatograms of the STXs under investigation from the analysis  
476 of a mussel extract (STXs were spiked before sample treatment at the following concentrations: C1,  
477 6680 µg/kg; C2, 2000 µg/kg; GTX2, 789 µg/kg; GTX1, 613 µg/kg; dcGTX2, 444 µg/kg; GTX3,  
478 300 µg/kg; GTX4, 200 µg/kg; dcGTX3, 100 µg/kg; GTX5, 100 µg/kg; STX, 100 µg/kg; dcSTX,  
479 100 µg/kg; NEO, 100 µg/kg; dcNEO, 1000 µg/kg).

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## Highlights

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

Figure 1

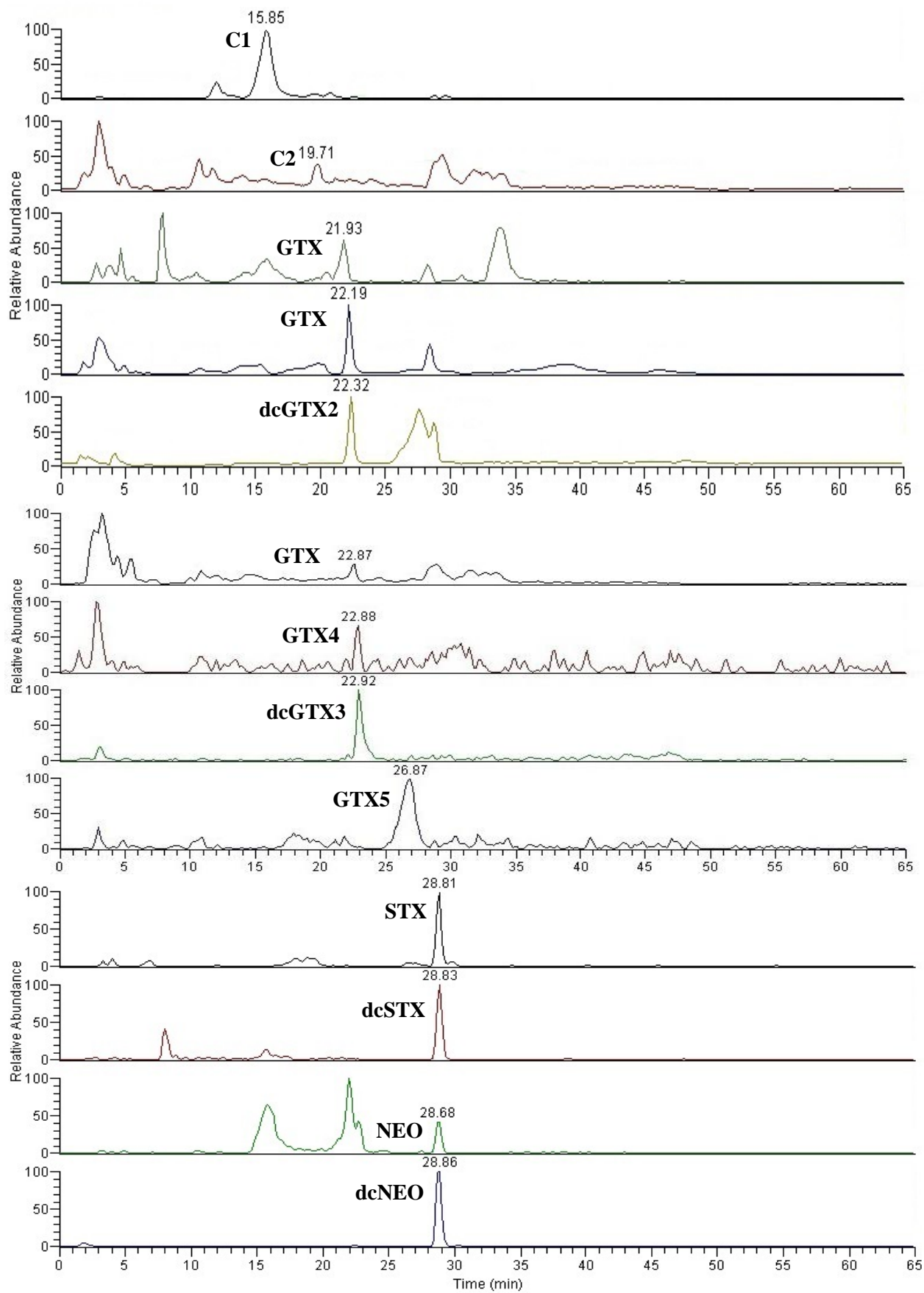


Fig. 1.

**Table 1**MS<sup>2</sup> transitions used for method validation and STXs quantitation.

PSP toxin	<i>m/z</i> precursor ion > <i>m/z</i> 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<sup>a</sup> and single desirabilities (d) calculated for the PSP toxins.**STX** (d=0.87)

$$y = 18490 (\pm 590) - 1570 (\pm 470) x_1 - 930 (\pm 270) x_2 - 1600 (\pm 270) x_3 - 1000 (\pm 530) x_1 x_2 + 3000 (\pm 900) x_1^2 + 2860 (\pm 900) x_2^2$$

**dcSTX** (d=0.87)

$$y = 12300 (\pm 550) - 1500 (\pm 490) x_1 - 870 (\pm 490) x_3 - 1400 (\pm 550) x_1 x_2 + 2950 (\pm 740) x_1^2$$

**NEO** (d=0.82)

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

**dcNEO** (d=0.88)

$$y = 5030 (\pm 320) - 460 (\pm 120) x_1 x_2 + 770 (\pm 380) x_2^2 - 690 (\pm 480) x_3^2$$

**C1** (d=0.95)

$$y = 10930 (\pm 470) + 830 (\pm 120) x_1 + 740 (\pm 120) x_2 - 1380 (\pm 470) x_1 x_2 + 1520 (\pm 640) x_1^2$$

**C2** (d=0.90)

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

**GTX1** (d=0.87)

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

**GTX2** (d=0.87)

$$y = 19230 (\pm 620) + 1390 (\pm 230) x_2 - 1010 (\pm 230) x_3 - 1710 (\pm 440) x_1 x_2 - 1290 (\pm 440) x_2 x_3 + 2640 (\pm 880) x_1^2$$

**GTX3** (d=0.87)

$$y = 790 (\pm 140) - 380 (\pm 40) x_3 - 280 (\pm 30) x_2 x_3 + 800 (\pm 120) x_1^2$$

**GTX4** (d=0.87)

$$y = 930 (\pm 150) + 180 (\pm 40) x_2 + 250 (\pm 80) x_1 x_2 x_3 + 450 (\pm 220) x_3^2$$

**GTX5** (d=0.75)

$$y = 2900 (\pm 320) - 1170 (\pm 260) x_3 - 430 (\pm 290) x_1 x_2 + 440 (\pm 290) x_2 x_3 + 1120 (\pm 470) x_1^2 + 660 (\pm 470) x_2^2$$

**dcGTX2** (d=0.65)

$$y = 23400 (\pm 660) - 1290 (\pm 890) x_1 + 2040 (\pm 890) x_2 + 1230 (\pm 890) x_3 - 2640 (\pm 990) x_1 x_3$$

**dcGTX3** (d=0.75)

$$y = 3050 (\pm 300) - 600 (\pm 280) x_1 x_2 - 410 (\pm 280) x_1 x_3 + 990 (\pm 440) x_1^2 + 840 (\pm 440) x_2^2$$

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<sup>a</sup>  $x_1$  = 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 ( $\mu\text{g}/\text{kg}$ )	LOQ ( $\mu\text{g}/\text{kg}$ )	Calibration range ( $\mu\text{g}/\text{kg}$ )	Calibration curve $y=a(\pm s_a)x$
STX	3	7	10-1000	$19.1(\pm 0.6)x$
NEO	11	27	30-1000	$5.2(\pm 0.3)x$
GTX1	12	35	100-4500	$34.7(\pm 1.9)x$
GTX2	20	65	70-4000	$1.5(\pm 0.2)x$
GTX3	95	271	500-1500	$3.2(\pm 0.3)x$
GTX4	159	436	500-1500	$0.34(\pm 0.02)x$
GTX5	14	33	100-1000	$16.5(\pm 0.3)x$
C1	140	289	500-5000	$3.4(\pm 0.1)x$
C2	708	1452	1500-5000	$2.2(\pm 0.2)x$
dcSTX	3	7	100-1000	$13.7(\pm 0.4)x$
dcNEO	14	50	100-1000	$4.6(\pm 0.3)x$
dcGTX2	37	80	200-4500	$10.9(\pm 0.1)x$
dcGTX3	6	20	50-1000	$1.9(\pm 0.1)x$

**Table 4**

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

Sample	Toxin concentration ( $\mu\text{g}/\text{kg}$ ) <sup>a</sup>												
	STX	NEO	GTX1	GTX2	GTX3	GTX4	GTX5	C1	C2	dcSTX	dcNEO	dcGTX2	dcGTX3
1	47 $\pm$ 1	49 $\pm$ 2	n.d.	212 $\pm$ 23	n.d.	n.d.	2325 $\pm$ 24	611 $\pm$ 80	n.d.	n.d.	n.d.	n.d.	n.d.
2	46 $\pm$ 1	93 $\pm$ 7	1134 $\pm$ 106	177 $\pm$ 20	n.d.	n.d.	2107 $\pm$ 227	1590 $\pm$ 40	n.d.	n.d.	n.d.	n.d.	n.d.
3	29 $\pm$ 1	78 $\pm$ 11	n.d.	91 $\pm$ 11	n.d.	n.d.	1056 $\pm$ 52	530 $\pm$ 73	n.d.	n.d.	n.d.	n.d.	n.d.
4	22 $\pm$ 4	50 $\pm$ 3	964 $\pm$ 45	83 $\pm$ 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 $\pm$ 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 $\pm$ 20	127 $\pm$ 12	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

<sup>a</sup> n.d.: Not detected (<LOD)