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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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1 **An innovative method based on QuEChERS extraction coupled to desorption electrospray**  
2 **ionization-high resolution mass spectrometry for screening the presence of paralytic shellfish**  
3 **toxins in clams**

4

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25 **Abstract**

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

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

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## 49 **Introduction**

50 Marine toxins are produced as secondary metabolites by bacteria and microalgae like  
51 dinoflagellates, diatoms, or cyanophyceae [1]. Changes in environmental conditions are the major  
52 factor responsible for the appearance of harmful algae blooms associated with the production of  
53 these toxic compounds. Being able to be accumulated through the marine food-chain, marine toxins  
54 can contaminate seafood causing food poisoning with different neurological and gastrointestinal  
55 illnesses [2]. Paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP), amnesic  
56 shellfish poisoning (ASP), neurotoxic shellfish poisoning (NSP) are some of the most important  
57 syndromes associated with marine toxin contamination. Among them, PSP is related to the presence  
58 of paralytic shellfish toxins (PST), called also Saxitoxins –STXs-, a complex group of more than  
59 20 water-soluble molecules characterized by a tetrahydropurine structure (Fig. 1). These  
60 compounds are able to inhibit neuronal transmission through blockage of voltage dependent sodium  
61 channels [3,4] causing signs of paralysis [5]. Due to their high hazard, a low maximum residue limit  
62 has been laid down in bivalve mollusks by the European Union (EU) legislation [6]: in fact,  
63 mollusks can be placed on the market for human consumption only if the PST content does not  
64 exceed the limit laid down in the regulation, i.e. 800 STX di-HCl eq/kg. The reference methods  
65 accepted in the EU for the analysis of these toxins are both the mouse bioassays [7] and the  
66 Lawrence method based on liquid chromatography with pre-column derivatisation and fluorescence  
67 detection (LC-FLD) [8]. Since a great number of shortcomings are related to the use of the mouse  
68 bioassay, i.e. high detection and quantitation limits, poor repeatability and presence of ethical issues  
69 related to the use of live animals, great efforts have been produced by the scientific community to  
70 develop reliable analytical methods able to detect and quantify each single toxin. In addition, taking  
71 into account that the Lawrence method does not allow the correct quantitation of coeluting  
72 analogues, and that the overlapping of oxidation products of different STX analogues is observed  
73 when pre-column derivatization is applied, a number of methods based on liquid chromatography

74 coupled to tandem mass spectrometry (LC-MS/MS) have been developed to obtain a reliable  
75 determination of these marine toxins [9-12].

76 Recently, ambient ionization approaches like desorption electrospray ionization–mass spectrometry  
77 (DESI-MS) have been successfully applied with advantages in terms of reduced analysis time and  
78 reduced sample preparation [13-15]. Taking into account the absence of chromatographic  
79 separation, high resolution mass spectrometry (HRMS) [16,17] is of pivotal importance for the  
80 univocal identification of the analytes. The use of high resolution full scan analysis as an alternative  
81 to selected reaction monitoring-based methods is really appealing: in fact, only the specificity of  
82 accurate mass and the full spectrum acquisition in HRMS is able to guarantee the correct  
83 identification of the investigated compounds. Despite these advantages, the determination of  
84 epimeric pairs still represents a limiting factor of this technique, since analogues cannot be  
85 individually resolved. Anyway, this drawback can be easily overcome taking into account that when  
86 positive samples are detected, a complete separation of PSP toxins can be further achieved by using  
87 alternative techniques like hydrophilic interaction liquid chromatography (HILIC) [10,18]. To the  
88 best of our knowledge, there has been no report of the analysis of marine toxins by DESI-HRMS  
89 technique.

90 However, when complex matrices like mollusks have to be analyzed, sample pre-treatment can be  
91 required to obtain analyte enrichment. Although solvent extraction followed by pre-concentration  
92 before instrumental analysis is one of the most common approaches for analyte enrichment, valid  
93 alternatives to time-consuming and laborious liquid-liquid extraction can be utilized. Recently, the  
94 quick, easy, cheap, effective, rugged, and safe (QuEChERS) technique has been proposed as rapid  
95 sample treatment procedure for analysis of samples of food [19-21], environmental [22] and  
96 toxicological concern [23].

97 The aim of this study, that was performed in the framework of a research project devoted to the  
98 assessment of safety and quality of Mediterranean seafood products, was the development of a rapid

99 QUEChERS-DESI-HRMS method for the detection of some saxitoxins in clams. The optimization  
100 of the instrumental parameters and the proper choice of both the DESI support and the extraction  
101 conditions allowed to prove the capability of the method to screen and quantitate the investigated  
102 compounds at trace levels in clams.

103

## 104 **Materials and methods**

### 105 *Chemicals*

106 PSP toxins: saxitoxin (STX), decarbamoylsaxitoxin (dcSTX), neosaxitoxin (NEO),  
107 decarbamoylneosaxitoxin (dcNEO-used as internal standard), gonyautoxin 1&4 (GTX1,4),  
108 gonyautoxin 2&3 (GTX2,3), decarbamoylgonyautoxin 2&3 (dcGTX2,3), gonyautoxin 5 (GTX5)  
109 and N-sulfocarbamoyl-gonyautoxin-2 and-3 (C1&2) were purchased from the NRC Certified  
110 Reference Materials Program (Institute for Marine Bioscience, Halifax, Canada). Formic acid ( $\geq$   
111 98% purity, FA), acetonitrile and methanol (both HPLC grade) were from Sigma Aldrich (Milano,  
112 Italy). Ammonium formate salt (97%, purity) was from Janssen Chimica (Beerse, Belgium). Water  
113 was obtained with a Millipore Milli-Q Element A10 System (Merck KGaA, Darmstadt, Germany).  
114 ABS Elut-NEXUS 30 mg cartridges, Q-Sep QuEChERS tubes containing 150 mg magnesium  
115 sulfate, 50 mg PSA and 50 mg C18, Q-Sep QuEChERS tubes containing 150 mg magnesium  
116 sulfate, 50 mg PSA, 50 mg C18, 7.5 mg GCB were from Restek (Milano, Italy), whereas Supel  
117 QuE Z-Sep/C18 tubes (Z-Sep 120 mg, C18 300 mg) were from Supelco (Milano, Italy). Glass,  
118 poly(methyl methacrylate) (PMMA) and hydrophobic (HTC)-printed slides were purchased from  
119 Prosolia™, Inc. (Indianapolis, IN, US).

120

### 121 *Sample treatment*

122 Clams were purchased from a local supermarket and used as blank matrix. The same procedure  
123 used in a previous study was used for the extraction of the investigated PSP toxins [24]. Briefly, 1 g

124 of clam homogenate was weighted into a centrifuge tube and extracted twice with 1 ml of 0.1%  
125 (v/v) formic acid by vortexing for 10 min. The sample was then centrifuged at 8000 x g for 10 min  
126 and the supernatants combined together. One ml of cold methanol was added and the tube was  
127 placed in an ice-bath for 30 minutes. After centrifugation at 8000 x g for 10 min at 15°C, proteins  
128 were removed and the remaining supernatant was reduced to a volume of 1 ml under a nitrogen  
129 flow at 40°C. Sample clean-up was performed by QuEChERS technique by adding 10 mg of ABS  
130 Elut-NEXUS phase to the extract and vortexing for 5 min. Finally, after the last centrifugation at  
131 8000 x g for 1 min, the extract was filtered on 0.2µm PTFE syringe filter (Pall Corporation, Port  
132 Washington, NY, USA) and submitted to DESI-HRMS analysis (more than 40 samples can be  
133 analysed in less than 100 min)

134

#### 135 *DESI-HRMS analysis*

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

139 The experimental conditions were as follows: solvent flow: 1 µL/min; spray voltage: 3 kV; tube  
140 lens voltage: 50V; capillary voltage: 15V; capillary temperature: 250°C; nitrogen pressure: 8.5 bar;  
141 incident angle: 55°; tip-to surface distance 2 mm; surface-to-inlet distance 0.5 mm; tip-to-inlet  
142 distance 4 mm. Two different spraying solvents were tested: methanol:water (1:1, v/v) with 0.1%  
143 FA and acetonitrile:water (1:1, v/v) with 0.1% FA. The sample plate was positioned on a moveable  
144 1-D stage. Preliminarily, full scan accurate mass spectra in the 100-500 amu range were acquired to  
145 determine appropriate masses for each analyte. Identification and quantification of target  
146 compounds was performed using the accurate mass of the analytes within a mass window of 5 ppm.  
147 Quantitation was performed by using the extracted ion chromatograms by selecting the appropriate

148  $m/z$  value for each analyte. Signal acquisition and data processing were performed using the  
149 Xcalibur 2.0 software (Thermo Fisher).

150

#### 151 *Optimization procedure*

152 The experiments were carried out on blank clam samples spiked with paralytic shellfish toxins at  
153 300  $\mu\text{g}/\text{kg}$  for STX, dcSTX, NEO, GTX5, 1200  $\mu\text{g}/\text{kg}$  for GTX1,4, 1080  $\mu\text{g}/\text{kg}$  for GTX2,3 and  
154 1620  $\mu\text{g}/\text{kg}$  for dcGTX2,3. A  $2^3$  two-levels full factorial design (FFD) followed by the multicriteria  
155 method of desirability functions was carried out [25,26]. The effects of amount of ABS Elut-  
156 NEXUS phase (ABS), vortexing time (V) and centrifugation time (C) were evaluated. Low and  
157 high levels were the following: ABS: 10–100 mg, V: 30–300 s, C: 60–1200 s. The best regression  
158 models were obtained by a forward search stepwise variable selection algorithm and the optimal  
159 conditions were evaluated by the global desirability D [27].

160

#### 161 *Method validation*

162 Method validation was performed according to EURACHEM guidelines [28] following the same  
163 procedure reported in previous studies [29,30]. Not contaminated clam samples were used as blank  
164 matrix. Briefly, detection ( $y_D$ ) and quantitation ( $y_Q$ ) limits, preliminary expressed as signals based  
165 on the mean and the standard deviation of blank responses, were calculated in terms of  
166 concentration values i.e. detection limit (LOD) and quantitation limit (LOQ) by projection of the  
167 corresponding signals  $y_D$  and  $y_Q$  through a calibration plot  $y=f(x)$  onto the concentration axis.

168 Calibration curves (six concentration levels, three replicated measurements for each level) were  
169 evaluated by analyzing blank clam samples spiked with the proper amount of the investigated  
170 analytes. Homoscedasticity was verified by applying the Bartlett test. Mandel's fitting test was  
171 also performed to check the goodness of fit and linearity. The significance of the intercept  
172 (significance level 5%) was established by running a Student's  $t$ -test. Intra-day repeatability and



173 intermediate precision were calculated in terms of RSD % on two concentration levels, performing  
174 three replicates at each level. Intermediate precision was estimated over three days verifying  
175 homoscedasticity of the data and performing the analysis of variance (ANOVA) at the confidence  
176 level of 95%.

177

#### 178 *Real samples analysis*

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

183

#### 184 **Results and discussion**

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

198 under these conditions a noticeable increase in sensitivity (Fig. 2) was obtained for all the PSP  
199 toxins, thus allowing the detection of all the investigated compounds.

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

211

#### 212 *QuEChERS clean-up*

213 Taking into account that mollusks represent a very complex matrix, a sample treatment procedure  
214 was required prior to DESI-HRMS analysis. In fact, the direct deposition of the homogenate onto  
215 the HTC slide was not feasible owing to the possible contamination of the ion source due to the  
216 blowing up of matrix particles. The feasibility of a QuEChERS approach was evaluated: as reported  
217 in a previous study [9], prior to QuEChERS clean-up, a protein precipitation step was required. Our  
218 findings [24] showed that by using cold methanol for 30 min at  $-20\text{ }^\circ\text{C}$ , the complete precipitation  
219 of proteins could be achieved. This result proved to be useful in speeding-up the entire analytical  
220 process, in fact, this is a very rapid time if compared with that (4 hours) reported by Zhou and  
221 coworkers [9] in a study on blood clam and oyster shellfish matrices. Different types of QuEChERS  
222 and the ABS Elut-NEXUS phase were also evaluated. By contrast to the results achieved by Zhuo

223 et al. [9], the use of QuEChERS containing GCB did not improve clean-up efficiency, in fact,  
224 discoloration was observed only after one day. The best results were achieved by using the ABS  
225 Elut-NEXUS phase, a polymeric sorbent made of polystyrene cross-linked with 50% divinyl  
226 benzene and poly(methyl methacrylate) characterized by a high surface area that offers a non-polar  
227 retention mechanism. It can be stated that the mixed polarity of this phase can be more suitable for  
228 the removal of interfering compounds with respect to the C18 adsorbent. On the basis of these  
229 findings, extraction conditions were optimized by performing an experimental design. By contrast  
230 to a One Variable at A Time approach, in which variables are changed independently from the  
231 others, the experimental design allows to simultaneously evaluate the effects of all the variables  
232 involved in the study as well as their interaction. Regression models for each toxin were obtained,  
233 thus providing useful information about their behaviour. In addition, by applying the multicriteria  
234 method of the desirability functions, regression models can be further used for optimization  
235 purposes. By using a  $2^3$  full factorial design and the multicriteria method of desirability functions,  
236 the optimal extraction conditions were found in correspondence to an ABS phase amount of 10 mg,  
237 a vortexing time of 5 min and a centrifugation time of 1 min obtaining the global desirability of  
238  $D=0.78$ . The regression models used to search for the highest global QuEChERS-DESI-HRMS  
239 recovery within the explored domain and the values of the single desirability  $d_i$  are reported in  
240 Table 2. The good values of both single desirabilities and global desirability proved that the clean  
241 up conditions were satisfying for all the investigated toxins.

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

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

249

#### 250 *Method validation*

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

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

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

273 Finally, the developed method was applied for the analysis of 20 different samples taken from local  
274 supermarkets and 2 samples supplied by National Reference Laboratory for Marine Biotoxins  
275 (Cesenatico, FC, Italy). All the samples were from different regions of Italy: 2 samples from Emilia  
276 Romagna, 5 from Veneto, 3 from Liguria, 4 from Toscana, 3 from Marche and 3 from Sardegna.  
277 No paralytic shellfish toxins were detected in the analyzed samples taken from local markets; by  
278 contrast, 410 and 860  $\mu\text{g}$  STX di-HCl eq/kg total toxicity, respectively were observed for the two  
279 samples supplied by the National Reference Laboratory for Marine Biotoxins (Cesenatico, FC,  
280 Italy). The achieved results were furtherly verified by applying both the HILIC-MS<sup>2</sup> method  
281 developed in our laboratory [24] obtaining a good agreement among the achieved results: 460 and  
282 980  $\mu\text{g}$  STX di-HCl eq/kg total toxicity were obtained by using the HILIC-MS<sup>2</sup> method whereas  
283 440 and 840  $\mu\text{g}$  STX di-HCl eq/kg total toxicity the mouse bioassay method, respectively, thus  
284 assessing the reliability of the DESI-HRMS method for screening and quantitate paralytic shellfish  
285 toxins.

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287

## 288 **Conclusions**

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

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304 contaminated clam samples

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