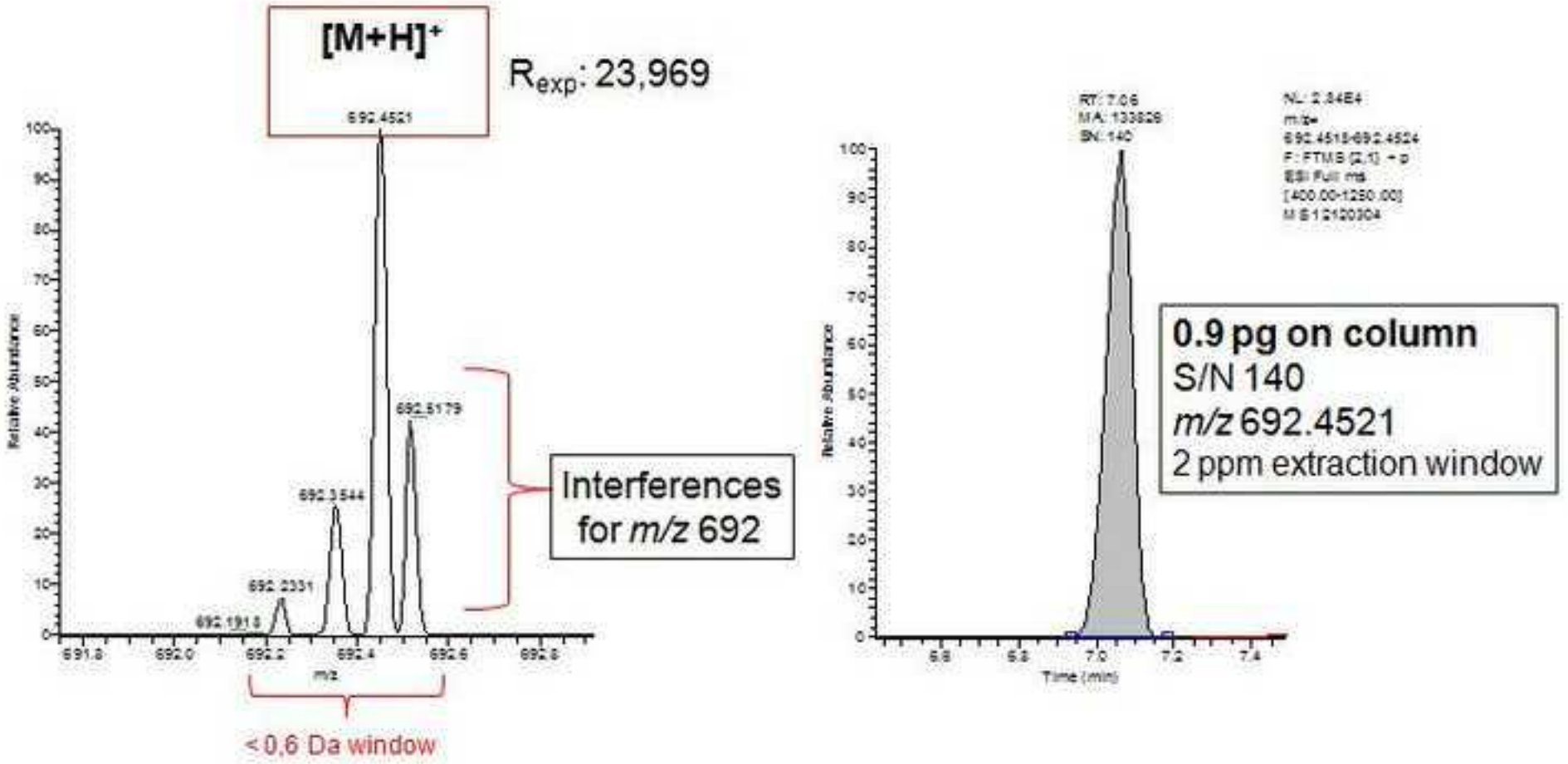
***Graphical Abstract**



Highlights

- We propose that liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) can be a very useful technique for multi-toxin detection and quantification, due to the capacity to acquire in full scan with good sensitivity and better selectivity.
- A LC-HRMS method for determination of major groups of lipophilic toxins has been developed and validated.
- The use of HRMS confirmation criteria can help to avoid false positives.
- The identification and confirmation criteria validated in the present study can contribute to define new parameters to implement HRMS in complex analysis, such as it is the case for lipophilic marine toxins in mussels.

Determination of lipophilic marine toxins in mussels by liquid chromatography coupled to high resolution mass spectrometry. Validation study.

Albert Domènech[a,b], Nuria Cortés-Francisco[b], Oscar Palacios[b], José M. Franco[c], Pilar Riobó[c], José J. Llerena[d], Stefania Vichi[a], Josep Caixach[b*]

[a] *Food Science and Nutrition Department, XaRTA (Catalonian Reference Network on Food Technology), Campus de l'Alimentació de Torribera, University of Barcelona, Av/ Prat de la Riba, 171 08921 Sta. Coloma de Gramenet, Spain.*

[b] *Mass Spectrometry Laboratory / Organic Pollutants, IDAEA-CSIC, c/ Jordi Girona, 18-26, 08034, Barcelona, Spain.*

[c] *Instituto de Investigaciones Marinas [U. A. Fitoplancton Tóxico (CSIC-IEO)], Av/ E. Cabello,6; 36208 Vigo, Pontevedra, Spain.*

[d] *Quality Assurance Unit, CID-CSIC, c/ Jordi Girona,18-26, 08034, Barcelona, Spain.*

* corresponding author: Dr. Josep Caixach, Mass Spectrometry Laboratory / Organic Pollutants, IDAEA-CSIC, c/ Jordi Girona, 18-26, 08034, Barcelona, Spain. Tel: (+34) 93 400 61 00. Fax: (+34) 93 204 59 04. E-mail address: josep.caixach@idaea.csic.es

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Abstract

A multitoxin method has been developed for quantification and confirmation of lipophilic marine biotoxins in mussels by liquid chromatography coupled to high resolution mass spectrometry (HRMS), using an Orbitrap-Exactive HCD mass spectrometer. Okadaic acid (OA), Yessotoxin, Azaspiracide 1, Gymnodimine, 13-desmethyl spirolide C, Pectenotoxin 2 and Brevetoxin B were analyzed as representative compounds of each lipophilic toxin group. HRMS identification and confirmation criteria were established. Fragment and isotope ions and ion ratios were studied and evaluated for confirmation purpose. In depth

characterization of full scan and fragmentation spectrum of the main toxins was carried out. Accuracy (trueness and precision), linearity, calibration curve check, limit of quantification (LOQ) and specificity were the parameters established for the method validation. The validation was performed at 0.5 times the current European Union permitted levels. The method performed very well for the parameters investigated. The trueness, expressed as recovery, range from 80 to 94 %, the precision, expressed as intralaboratory reproducibility, range from 5 % to 22 % and the LOQs range from 0.9 to 4.8 pg on column. Uncertainty of the method was also estimated for OA, using a certified reference material. A top–down approach considering two main contributions: those arising from the veracity studies and those coming from the precision's determination, was used. An overall expanded uncertainty of 38 % was obtained.

1. Introduction

Lipophilic marine biotoxins accumulate in filter–feeding shellfish and can develop into a food safety risk [1–3]. These toxins are produced by diverse microorganisms as deeply detailed in Paz et al., [3]. Lipophilic marine toxins can be classified in several groups such as okadaic acid (OA) and dinyphysistoxins (DTXs), yessotoxins (YTXs), azaspiracides (AZAs), pectenotoxins (PTXs), cyclic immines and brevetoxins [3]. A toxin of each group has been selected as representative tracer. OA, yessotoxin (YTX), azaspiracide 1 (AZA1), gymnodimine (GYM), 13–desmethyl spirolide C (SPX1), pectenotoxin 2 (PTX2) and brevetoxin B (PbTx–2) are selected as are the most common as it emerges from the EFSA opinions [4–9] and certified reference materials are available [10]. The current permitted levels by the legislation in shellfish are: for the sum of OA, DTXs and PTXs 160 μg kg^{-1} of OA equivalents, for the sum of YTXs 1000 μg kg^{-1} YTX equivalents and for the sum of AZAs 160 μg kg^{-1} AZA1 equivalents [11]. For the cyclic immines group (spirolides and gymnodimines) and for the brevetoxins group there are no legal limits yet. However, the European Food Safety Authority (EFSA) is issuing several opinions for each toxin group, which recommends a revision of these legal limits (lowering it, except for YTXs) [4–9].

Since it is known that the official reference methods, the rat bioassay and the mouse bioassay, have to be replaced for analytical and ethical questions, alternative methods are necessary. European Union proposed to replace it for liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) [12], before 31 December 2014.

To date, LC–MS/MS has been the most used technique, providing high sensitivity and selectivity [13]. However, with this technique it is mandatory to detect compounds that are pre–selected and it is sensible to matrix effects. Several methods of multitoxin analysis are described in the literature [13–16]. Some of them are intra– or inter–laboratory validated [15, 17, 18], but the analysis of lipophilic toxins by means of mass spectrometry still generates some controversies [19–22].

In the present study, liquid chromatography coupled to high–resolution mass spectrometry (LC–HRMS) is used. It has been shown that it is a very useful technique for toxins detection due to the capacity to resolve interferences from complex matrixes [23–27], such as mussels, acquiring in full scan with good sensitivity and better selectivity. Confirmation and quantification is essential to verify the results, avoiding false positives. For this reason, having as much identification and confirmation criteria as possible will be very useful.

To the best of our knowledge, none of the LC–HRMS methods for toxin analysis in literature combine both quantification and confirmation criteria. The aim of this study is to develop a method for the quantitative determination of lipophilic marine toxins in mussels based on HRMS. Identification criteria using high resolution (50,000 *m/z* 200 full width at half maximum – FWHM) and mass accuracy better than 5 ppm (in all the mass range of the study) were used. Fragment and isotope ions and ion ratios were studied and evaluated for confirmation purpose. In depth characterization of full scan and fragmentation spectrum of the main toxins was carried out. Moreover, the performance of the quantification method using HRMS was evaluated by a validation study. The following validation parameters, accuracy (trueness and precision), linearity, calibration curve check, limit of quantification (LOQ) and specificity were

established for all the toxins and last but not least, the uncertainty of the method was estimated for OA.

2. Experimental Section

2.1 Chemicals and materials

Methanol (SupraSolv) was acquired from Merck (Darmstad, Germany). Acetonitrile (LC–MS, Chromasolv, ≥ 99.9 %) and ammonium hydroxide solution (≥ 25 %) were purchased from Sigma–Aldrich (Stenheim, Germany). Water was deionized and passed through a Milli–Q water–purification system (Millipore, Billerica, MA, USA).

Some of the Certified Reference Materials (CRMs) comprising calibration solutions and mussel tissue were acquired from the NRC Certified Reference Materials Program (Halifax, NS, Canada): AZA1 (CRM–AZA1 1.24 ± 0.07 µg mL^{-1}), PTX2 (CRM–PTX2 8.6 ± 0.3 µg mL^{-1}), SPX1 (CRM–SPX1 7.06 ± 0.4 µg mL^{-1}), GYM (CRM–GYM 5 ± 0.2 µg mL^{-1}), blank mussel tissue with OA (CRM–DSP–MUS–b 10.1 ± 0.8 µg g^{-1}) and mussel tissue matrix (CRM–Zero–Mus). OA (10 µg mL^{-1}) and YTX (3 µg mL^{-1}) were purchased from N'Tox (Saint Jean d'Illac, France). PbTx–2 (100 µg, 95 % purity) was purchased from Latoxan (Valence, France).

2.2 Analytical procedure

Matrix–matched calibration curves were prepared with homogenate blank mussel extracts in the range between 0.2 and 150 ng mL^{-1}, which corresponded to 2 – 1500 µg kg^{-1} in sample, depending on each toxin. The ranges of the matrix–matched calibration curves for each toxin are shown in Table 1 Mussel extractions were made following the EU–Harmonised Standard Operating Procedure [10].

To carry out the method validation for the lipophilic toxins included in the study at the level of 0.5 times the legislation limit, a blank mussel (CRM–Zero–Mus) was spiked for each toxin: 80 µg kg^{-1} for OA, AZA1, PTX2 and 500 µg kg^{-1} for YTX. For SPX1 and GYM the lowest concentration, 80 µg kg^{-1}, was considered as no legal limit was set. Moreover, to estimate the uncertainty of the method for

OA the CRM-DSP-MUS was analyzed five times. The instrumental performance was evaluated with matrix-matched standards at the level of 1 and 25 ng mL^{-1}; as if they were real sample extracts.

2.3 Instrumentation

2.3.1 Liquid Chromatography

The LC system consisted of a Surveyor MS Plus pump and an Accela Open AS autosampler kept at 15 ºC (Thermo Fisher Scientific, San Jose, California). A 5 µL injection volume was used. A HypersilGold C18 (50 mm x 2.1 mm, 1.9 µm) (Thermo Fisher, Scientific, Bremen, Germany) was used for the separation of toxins at a flow rate of 300 µL min^{-1}. Mobile phase A was water and B was acetonitrile/water (90:10), both containing 6.7 mM ammonium hidroxyde [13]. The gradient started at 20 % of B and was kept this composition for 3.5 min. Then, it was increased to 90 % of B in 16 min and kept 3 min, then returns to initial conditions of 20 % of B maintaining it for 11 min for the column equilibration. The total duration of the method was 30 min.

2.3.2 High Resolution Mass Spectrometry

Mass spectrometry analyses were carried out with an Orbitrap-Exactive HCD (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray source (H-ESI II). Nitrogen (purity > 99.999 %) was used as sheath gas, auxiliary gas and collision gas. The instrument was daily calibrated in both positive and negative modes. Three time segments were set. First segment (0 min –3.5 min) working in negative mode without and with all ion fragmentation (AIF) (HCD 60 eV), second segment (3.5 min – 8.25 min) in positive mode without and with AIF (HCD 50 eV) and third segment (8.25 min – 30 min) in positive mode without and with AIF (HCD 20 eV). The mass range was *m/z* 400–1250 in full scan and *m/z* 70–1200 in AIF mode.

The resolution was 50,000 (*m/z* 200, FWHM) at a scan rate 2 Hz, the automatic gain control (AGC) was set as "high dynamic range" with a maximum injection time of 100 ms.

Optimized parameters were spray voltage, capillary voltage, skimmer voltage, tube lens voltage, capillary temperature, heater temperature, sheath gas flow rate and auxiliary gas flow rate. OA was used for optimization in negative mode and SPX1 and PbTx–2 for positive mode. In both modes the final parameters were: spray voltage of 3.25 kV, capillary temperature of 375 ºC, heater temperature of 250 ºC, sheath gas flow rate of 45 (arbitrary units) and auxiliary gas flow rate of 15 (arbitrary units). In negative ESI capillary voltage of –92.5 V, tube lens voltage of –190 V and skimmer voltage of –44 V were used. In positive ESI capillary voltage of 77.5 V, tube lens voltage of 175 V and skimmer voltage of 32 V were used.

The data was processed with Xcalibur 2.1 software (Thermo Fisher Scientific, Bremen, Germany). Automatic identification/quantification can be performed. The peaks are found in the chromatogram by the exact mass of diagnostic, fragment and isotope ion, the mass accuracy (± 5 ppm extraction window) and the retention time window. However, a manual verification is necessary to avoid false positives or false negatives and correct peak integration. To calculate the theoretical accurate mass (*m/z* calculated), the mass of the electron has been taken into account as 0.00055 Da [28]. Moreover, to apply the identification and confirmation criteria of the present study, our own excel files had to be built.

2.4 Validation

The quantification method for lipophilic toxins was proved to be fit for purpose carrying out a validation study to assure reliable results and prevent false positive and false negative results. The validation was based on diverse methodologies, such as EU Comission Decision 2002/657/EC [29] and the studies presented in Table 2, as no specific guidelines are set for analysis of marine biotoxins using HRMS. Statistical validation of the method developed was performed evaluating the parameters described in Table 2. Uncertainty of the method was also estimated for OA using a blank mussel tissue with a certified amount of OA.

3. Results and Discussion

3.1 Optimization of conditions

In addition to optimizing the experimental parameters for efficient toxin ionization, in the development of the method, there were some critical aspects that had to be addressed.

Acidic, neutral and basic mobile phase composed by water with acetonitrile or methanol at different proportions, were tested under two chromatographic columns, Hypersil Gold C18 (50 mm x 2.1 mm, 1.9 µm) and Mediterranea Sea 18 (100 mm x 2.1 mm, 3.0 µm) (Teknokroma, Barcelona, Spain). Hypersil Gold was chosen because better peak shape and separation were obtained. The basic conditions were selected because the negatively charged toxins eluted early and separately from the positive ones, so it allows to set different time segments. As the mass analyzer cannot switch polarity fast enough working in both scan modes (full scan and AIF), having independent time segments for each polarity mode permits to rapidly switch full scan and AIF, providing in the same injection information from molecular ions and fragments ions.

Firstly, the aim was to work with only 2 time segments. Nevertheless, changes in the conditions were needed to obtain better signal for PbTx–2. After testing several gradients, PbTx–2, was delayed to the end of the chromatogram. As a consequence, 3 time segments were set: the first in ESI– for OA and YTX; the second in ESI+ for AZA1, GYM, SPX1 and PTX2; and the third for PbTx–2 in ESI+, with a lower HCD voltage. For each time segment only one HCD voltage was possible, in order to obtain enough data points per peak, so the HCD voltage was optimized in each case to had at least one intense fragment for every toxin.

3.2 Mass spectral characterization

Mass spectral characterization is indicated in Figure 1 showing also the fragment ions obtained by AIF experiment. At the optimum working conditions specified in the previous section, each compound was identified and several fragment ions were obtained as described below. Some of these fragment ions have been already described in literature [25–27] but in the present manuscript all the fragment ions have been evaluated with confirmation purpose.

The diagnostic ion was selected by taking the most selective and intense peak either the deprotonated / protonated molecule or an adduct. Non–desirable adducts consequence of basic mobile phase conditions were avoided by meticulous ion source parameters optimization. Major diagnostic ion and few signal distribution was achieved for almost all the toxins.

OA can be analyzed either in positive or negative ESI mode. Better sensitivity was obtained when the deprotonated molecule at *m/z* 803.4587 $[M–H]^-$ was extracted from the full scan experiment. At 50 HCD voltage the fragment ions generated were at *m/z* 785.4482 $[C_{44}H_{65}O_{12}]^-$, at *m/z* 255.1238 $[C_{13}H_{19}O_5]^-$ and at *m/z* 113.0608 $[C_6H_9O_2]^-$. The *m/z* 255.1238 was chosen for being the most intense.

The Orbitrap–MS mass spectrum of YTX showed an intense diagnostic ion at *m/z* 1163.4587 $[M–2H+Na]^-$. In full scan mode there were some other characteristic ions of YTX at lower intensities, such as at *m/z* 1141.4717 $[M–H]^-$, at *m/z* 570.2322 $[M–2H]^{2-}$ and also in full scan there were fragment ions at *m/z* 1061.5149 $[M–SO_3]^-$ and at *m/z* 467.1669 $[C_{42}H_{62}O_{19}S_2]^{2-}$. Although it is not desirable to have ion source fragmentation, during the optimization of ion source conditions, it has been observed that with higher voltages and temperatures the sensitivity increased. HCD fragment ions were at *m/z* 855.3842 $[C_{42}H_{63}O_{16}S]^-$ and at *m/z* 96.9601 $[HSO4]^-$. The *m/z* 96.9601 was the most intense, but it was considered not suitable, due to the fact that this *m/z* region presented many interferences from solvent and mussel matrix (data not shown). The fragment ion at *m/z* 467.1669 was chosen, although it was a source fragment, because it presented good stability in all the concentration range.

AZA1 produced the protonated molecule at *m/z* 842.5049 $[M+H]^+$. The fragment ions generated in the HCD cell were a water loss of the protonated molecule at *m/z* 824.4943 $[C_{47}H_{70}NO_{11}]^+$, two water losses of the protonated molecule at *m/z* 806.4838 $[C_{47}H_{68}NO_{10}]^+$ and at *m/z* 672.4106 $[C_{38}H_{58}NO_9]^+$. The *m/z* 824.4943 was used as fragment ion due to its high intensity.

The GYM mass spectrum revealed that the water loss of the protonated molecule at *m/z* 490.3316 $[C_{32}H_{44}NO_3]^+$ was more intense than the protonated molecule at *m/z* 508.3421 $[M+H]^+$. That occurred as a consequence of the high voltage and temperature of the method that were necessary for the other toxins. HCD fragment ions were at *m/z* 392.2948 $[C_{27}H_{38}NO]^+$, at *m/z* 162.1277 $[C_{11}H_{16}N]^+$, at *m/z* 136.1121 $[C_9H_{13}N]^+$ and at *m/z* 121.0886 $[C_8H_{11}N]^+$. The fragment ion at *m/z* 121.0886 was chosen because it was the most intense.

SPX1 produced the protonated molecule at *m/z* 692.4521 $[M+H]^+$ and a water loss at *m/z* 674.4415 $[C_{42}H_{60}NO_6]^+$. The HCD mass spectrum showed several fragment ions: a water loss at *m/z* 674.4415 $[C_{42}H_{60}NO_6]^+$, at *m/z* 444.3108 $[C_{27}H_{42}NO_4]^+$ and at *m/z* 164.1430 $[C_{11}H_{18}N]^+$. The fragment ion at *m/z* 164.1430 was chosen because it was the most intense and characteristic.

PTX2 formed some adducts at full scan mode, with ammonium at *m/z* 876.5104 $[M+NH_4]^+$, with sodium at *m/z* 881.4658 $[M+Na]^+$ and with potassium at *m/z* 897.4397 $[M+K]^+$. In full scan there was also a double water loss of the protonated molecule at *m/z* 823.4647 $[C_{47}H_{67}O_{12}]^+$. In the HCD mass spectrum there were several water losses from the protonated molecule (for instance, at *m/z* 823.4647 and at *m/z* 805.4512) and some fragment ions generated were at *m/z* 213.1121 $[C_{11}H_{17}O_4]^+$ and at *m/z* 195.1016 $[C_{11}H_{15}O_3]^+$ (data not shown). The fragment ion at *m/z* 823.4647 was chosen, because although it was a source fragment, it presented good stability in all the concentration range.

PbTx–2 produced the protonated molecule at *m/z* 895.4838 $[M+H]^+$ and the sodium and potassium adducts at *m/z* 917.4658 $[M+Na]^+$ and at *m/z* 933.4397 $[M+K]^+$. HCD fragment ions were several water losses from the protonated molecule at *m/z* 877.4733 $[C_{50}H_{69}O_{13}]^+$ and at *m/z* 859.4627 $[C_{50}H_{67}O_{12}]^+$. The fragment ion at *m/z* 877.4733 was chosen for being the most intense.

3.3 Determination of lipophilic marine toxins

Lipophilic toxins were separated by reverse phase chromatography coupled to an Orbitrap–Exactive HCD mass spectrometer. As shown in Figure 2 seven toxins were separated in 10 min. After an in depth characterization of full scan and fragmentation spectra, a diagnostic ion, a fragment ion and an isotope ion were chosen for each toxin and were included in Table 3. The choice of the diagnostic ions changed depending on background interferences [30]. The fragment ion chosen was the one giving higher signal and the most stable ion fragment ratio. The M+1 isotope ion was chosen in all the cases for the calculation of the isotope ion ratio.

In the present study, the chromatographic separation of PbTx–2 was achieved in alkaline conditions. However, it should be highlighted that PbTx–2 was poorly ionized by the conditions of the method, although meticulous optimization of parameters was carried out. For these reasons, the identification and confirmation was not possible at the concentrations of interest. Further validation was not performed.

3.3.1 Identification and confirmation criteria

The identification and confirmation criteria adopted in the present study are detailed in Table 2. The use of confirmation by a second ion is very helpful to prevent false positives [23].

3.3.1.1 Mass accuracy and precision

In the present study, mass accuracy and precision expressed as parts per million (ppm) were used. Precision has received limited coverage in the literature, but it is important, and both accuracy and precision should be considered, when dealing with accurate mass measurements [31]. In Table 3 are listed mass accuracy and precision of each toxin obtained from matrix–matched calibration curves. Observed *m/z*, mass accuracy and mass precision are averages from all the points of the calibration curve, except for PbTx–2. Mass accuracy and precision were calculated using root–mean–square to avoid positive and negative values

cancelling each other. Mass accuracy in most of cases was ranging between 1 and 2.5 ppm (see Table 3) for diagnostic, fragment ions and isotope ions. So a maximum of 5 ppm of error was permitted to the software for peak identification. Precision was ranging from 0.23 to 2.12 ppm, which indicates a good stability in the mass measurement. For PTX2 a high variability in the mass measurement was observed between high and low points of the calibration curve.

3.3.1.2 High resolution

The experimental resolution was better than 20,000 (FWHM) in all the mass range of interest, in order to achieve the criteria listed in Table 2. As it is shown in Figure 3a) high resolution is necessary to resolve the interferences coming from the matrix.

3.3.1.3 Retention time

Retention time in standards and samples must agree, so a restrictive tolerance has been set. Only peaks with values lower than three times the standard deviation (SD) from the mean of the matrix–matched calibration curve retention times had been considered.

3.3.1.4 Ion ratio

As it has been previously said, the incorporation of additional parameters and criteria for confirmation of positive results is recommended. In the present study, the ion ratio is defined as the ratio between the diagnostic ion and the confirmation ion. The confirmation ion can be a fragment ion or an isotope ion, so two different ion ratios were evaluated. The tolerance of the ion ratios must not exceed those from Decision 2002/657/EC [29]: if the ion ratio is under 2, a ± 20 % of maximum ratio tolerance is accepted, if it is between 2 and 5, ± 25 % is accepted, if it is between 5 and 10, a ± 30 % is accepted and if it is more than 10, a ± 50 % is accepted.

Fragment ion ratio

The fragment ion ratio, defined as the ratio between the area of the diagnostic ion and the area of the fragment ion, has been used to confirm peak identity in the samples. The average ion ratio for each toxin has been established. This was done

after studying the ion ratios of the diagnostic ions with all the fragments obtained, evaluating its stability in all the concentration range. HCD fragments were preferably used, but for some toxins it was mandatory to use source fragments due to the hard ionization conditions. The definitive ion ratios are listed in Table 3. For OA the fragment ion ratio was 6.39 with a relative standard deviation (RSD) of 7 %. In the case of YTX a value of 0.72 was obtained with a RSD of 17 %. For AZA1 the ion ratio was 4.47 with a RSD of 17 %. GYM obtained a value of 14.44 with a RSD of 13 %. For SPX1 the fragment ion ratio was 5.13 with an RSD of 15 %. PTX2 obtained a value of 5.28 with an RSD of 40 %.

To confirm a finding as an actual positive the ion ratio of the sample should be in agreement with the ion ratio of the matrix–matched calibration curve.

Isotope ion ratio

The isotope ion ratio, defined as the ratio between the monoisotope ion (diagnostic ion) and the isotope ion (M+1, corresponding to the natural isotope ^{13}C), has been calculated as a confirmation criteria. It was possible to perform it because in every case the diagnostic ion has relatively high *m/z* (490.3316 to 1163.4537), M+1 was always around 50% of it and the isotope ion ratio was stable in all the concentration range. Moreover, with the software used it is possible to determine the theoretical isotope ion ratio of each compound and it has been observed that all isotope ion ratios were very similar to the theoretical ratio. The isotope ion ratios are listed in Table 3. For OA it was 2.17 with a RSD of 15 %. In the case of YTX a value of 1.62 was obtained with a RSD of 7 %. For AZA1 the isotope ratio was 2.08 with a RSD of 10 %. GYM obtained a value of 2.96 with a RSD of 9 %. For SPX1 the isotope ion ratio was not possible to calculate in all the levels of the calibration curve, due to interferences in the M+1 and in M+2. PTX2 obtained a value of 1.84 with a RSD of 18 %.

After analyzing all the data, an optimum confirmation ion for each toxin was selected [23]. For OA, although fragment ion ratio had acceptable values, at low concentration levels of the matrix–matched calibration curve was not possible to use it, so the isotope ion ratio was used as confirmation criteria for this toxin. In the case of YTX, AZA1 and GYM both ratios can be used as they had the same

sensibility and acceptable values of the ion ratios in the studied concentration range of the calibration curves. For SPX1 the fragment ion ratio should be used as there were interferences due to the complex matrix in the M+1 and in M+2 isotope ions. The interference was detected by analyzing the isotope ion ratio of a calibration curve without matrix. For PTX2 the isotope ion ratio was used because better sensibility can be achieved, instead of using the fragment ion ratio, as this had an unacceptable RSD.

3.4 Validation study

The suitability of the quantification method for lipophilic toxins was evaluated by a validation study. Firstly, a freeze-dried blank mussel spiked at 0.5 times the legislation limit was used as no reference material containing all the toxins at low level (80 µg kg^{-1}, except for YTX 500 µg kg^{-1}) was available. Validation was performed at this level as we are near de MRL as it's recommended by the Comission Decision 2002/657/EC [29]. Afterwards, a mussel tissue reference material containing a certified amount of OA was analyzed to estimate the uncertainty of the method.

3.4.1 Validation Parameters

3.4.1.1 Accuracy

The accuracy of a method can be defined taking in consideration its trueness (closeness of agreement between the average of a number of tests results and an accepted reference value) and its precision (closeness of agreement between test results) [32].

Trueness

In the present study, trueness is expressed as the recovery of fortified mussel samples (n=3), spiked at concentration levels of 0.5 times the legislation limit. Table 4 shows that recoveries were in the range of 80 – 94 %. These values are acceptable according to Comission Decision 2002/657/EC [29], which states that

the accuracy (as recovery) of a method with analyte levels above 10 µg kg^{-1} must be ranging between 80 – 110 %.

Precision

The precision, expressed as intralaboratory reproducibility of the method, was determined in terms of relative standard deviation (RSD_R) from n=3 recovery experiments at 0.5 times the legislation limit.

The precision of the method was ranging between 5 % and 22 % as listed in Table 4. This precision is totally acceptable according to the Horwitz equation [29]. It should be highlighted that this equation gives unacceptable high values for concentrations below 100 µg kg^{-1}. As set in the Comission Decision 2002/657/EC [29], the highest variation acceptable is 23 % at 100 µg kg^{-1}, and this method presents a maximum variation of 22 % for OA at lowest concentration (80 µg kg^{-1}), so the values obtained were acceptable.

3.4.1.2 Linearity

Matrix–matched calibration curves were run every day. A minimum of 5 points for each calibration curve were required. Linearity was considered acceptable when the regression coefficient was ≥ 0.98 [10] with residuals lower than 30 % [33]. For all the matrix–matched calibration curves injected the correlation was acceptable, obtaining values between 0.9806 and 0.9993 (Table 1). Due to the high linear range chosen for the curves (the range of concentration studied ranged from 0.2 to 150 ng mL^{-1}) to fulfill with residuals values lower than 30 %, a weighted curve in concentration was adopted (1/x) and it was not forced to go through the origin.

3.4.1.3 Calibration Curve Check (intra–batch response drift)

The response drift of the method was checked by comparing a level of the matrix–matched calibration curve at the beginning of the analysis with the same level analyzed after the samples. The difference could not exceed a 30%. Fresh calibration curves were needed. It is especially important in this case as no internal standards are available and evaporation of matrix–matched calibration curves may occur.

3.4.1.4 Limit of Quantification (LOQ)

LOQ was determined by the lowest point of the calibration curve which was possible to confirm fulfilling the criteria established in section 3.3.1 (see Identification and Confirmation Criteria). Table 1 shows the instrumental LOQ, expressed as picograms on column, obtained for each toxin. In Figure 3 the spectrum and the extracted ion chromatogram from SPX1 (*m/z* 692.4521) at 0.18 pg μl^{-1} are shown. These low values from LOQ are quite interesting in a middle future because the EFSA has proposed new legislation limits and, for the majority of them, these are much lower than the actual ones.

3.4.1.5 Blank Quality Control (QC) / Specificity

Extracted blank mussel (n=15) was analyzed as a real sample to study signals obtained from the matrix and to evaluate if interferences that lead to false positive results were obtained. The good specificity of the technique (working in high resolution 50,000 – *m/z* 200, FWHM and with extracted ion window of 5 ppm) makes possible to have no signal at all in the blank mussel for any of the toxins, except for SPX1. Nevertheless it must be noted that the blank mussel is not certified for the absence of SPX1, so the obtained signals could be attributed to its presence in the sample. However, in all the positive results for SPX1, the concentration was below the LOQ or it can not be confirmed.

3.4.2 Uncertainty estimation for OA

Uncertainty is a quantitative indicator of the confidence in the analytical data and describes the range around a reported or experimental result within which the true value can be expected to lie within a defined probability (confidence level) [30].

The uncertainty of the whole method at the interest level was estimated following a top–down approach [34] considering two main contributions: those arising from the veracity studies and those coming from the precision's determination.

Those values have been derived from the analysis of a reference material (with a certified value of 10.1 ± 0.8 μg g^{-1}). To achieve the interest level, a dilution of 1/50 was done following the EU-Harmonised Standard Operating Procedure [10].

Before the final uncertainty's estimation, the compatibility index between the results from our laboratory and the CRM was checked. The two values were compared following the methodology proposed by the Institute for Reference Materials and Measurements [35]. This procedure takes into account the difference between the certified value and the measurement result, as well as their respective uncertainties. No significant difference between the measurement result and the certified value was detected.

A value for expanded uncertainty (k = 2) of 38 % was obtained, which is in agreement with the expected value arising from the specialized literature and meets the criteria of SANCO 12495/2011 [30].

3.5 Instrumental Quality Parameters

In addition to the validation of the overall method (extraction and instrumental analysis), instrumental quality parameters were assessed. As shown in Table 5 instrumental trueness, repeatability ($iRSD_r$) and reproducibility were evaluated at two levels (1 and 25 ng mL^{-1} matrix-matched standards). Instrumental trueness (n = 6) were ranging from 91 to 116 % in 1 ng mL^{-1} and from 94 to 111 % in 25 ng mL^{-1}. Repeatability ($iRSD_r$) (n = 5) values were ranging from 4 to 10 % in 1 ng mL^{-1} and from 1 to 7 % in 25 ng mL^{-1}. Reproducibility ($iRSD_R$) was tested in 6 different days obtaining values from 7 to 16 % in 1 ng mL^{-1} and from 9 to 14% in 25 ng mL^{-1}.

Conclusions

A sensitive LC–HRMS method for quantification of major groups of marine lipophilic toxins has been developed and validated. The method performed very well for the parameters investigated. Ion ratios as confirmation criteria were deeply studied. It was observed, that both fragment ion ratio and isotope ion ratio can be used to confirm a positive result, but for each compound one or the other can be more suitable. The use of the HRMS criteria can help to prevent false results. Interferences coming from the matrix can be identified because data is acquired in full scan mode so matrix effects are minimized. It has been shown that HRMS provides incomparable confirmatory performances with excellent quantitative capabilities. Further studies are necessary to include more toxins of each group studied and more toxin groups. Moreover, this study can contribute to define new parameters based on HRMS, for complex matrix analysis, as it is the case for lipophilic marine toxins in mussels.

References

1. Food and Agriculture Organization of the United Nations. Marine Biotoxins. FAO Food and Nutrition Paper 80. (2004). Rome

2. P. Hess, Anal. Bioanal. Chem., 397 (2010) 1683–1694.

3. B. Paz, P. Riobó, J.M. Franco, Rapid. Commun. Mass Spectrom., 25 (2011) 3627–3639.

4. EFSA Panel on Contaminants in the Food Chain (CONTAM), EFSA Journal 589 (2008) 1–62

5. EFSA Panel on Contaminants in the Food Chain (CONTAM) EFSA Journal 907 (2008) 1–62

6. EFSA Panel on Contaminants in the Food Chain (CONTAM), EFSA Journal 723 (2008) 1–52

7. EFSA Panel on Contaminants in the Food Chain (CONTAM), EFSA Journal 1109 (2009) 1–47

8. EFSA Panel on Contaminants in the Food Chain (CONTAM), EFSA Journal 8(6) (2010) 1628

9. EFSA Panel on Contaminants in the Food Chain (CONTAM), EFSA Journal 8(7) (2010) 1677

10. European Union Reference Laboratory for Marine Biotoxins, EU-RL-MB SOP, (2011) Version 4

11. European parliament, Regulation 2004/853/ EC, Off. J. Eur. Commun. 139 (2004) 55–205.

12. European Comission, Commission Regulation 2011/15/EC, Off. J. Eur. Commun. 279 (2011) 5–7

13. A. Gerssen, P.P. Mulder, M.A. McElhinney, J. de Boer, J. Chromatogr. A, 1216 (2009) 1421–1430.

14. E. Fux, D. McMillan, R. Bire, P. Hess, J. Chromatogr. A, 1157 (2007) 273–280.

15. P. McNabb, A.I. Selwood, P.T. Holland, AOAC, 88 (2005) 761–772.

16. L.A. Stobo, J.C.L.1. Lacaze, A.C Scott, S. Gallacher, E.A Smith, M.A. Quilliam, AOAC, 88 (2005) 1371–1382.

580 17. A. Gerssen, E.H. van Olst, P.P. Mulder, J. de Boer, Anal. Bioanal Chem., 397
581 (2010) 3079–3088.

582 18. H.J. van den Top, A. Gerssen, P. McCarron, H.P. van Egmond, Food Addit
583 Contam. Part A. Chem. Anal. Control. Expo. Risk Assess., 28 (2011) 1745–1757.

584 19. P. Otero, A. Alfonso, C. Alfonso, P. Rodriguez, M.R. Vieytes, L.M. Botana,
585 Anal. Chem., 83 (2011) 5903–5911.

586 20. A. Gago-Martinez, J.M. Hungerford, Anal. Chem., 84 (2012) 475–475.

587 21. A. Gerssen, d.T. van, H.P. van Egmond, Anal. Chem., 84 (2012) 476–477.

588 22. P.T. Holland, P. McNabb, M.A. Quilliam, Anal. Chem., 84 (2012) 478–480.

589 23. H.J. Mol, P. Zomer, M. Koning, Anal. Bioanal. Chem., 403 (2012) 2891–
590 2908.

591 24. A. Kaufmann, Anal. Bioanal. Chem., 403 (2012) 1233–1249.

592 25. A. Gerssen, P.P. Mulder, J. de Boer, Anal. Chim. Acta, 685 (2011) 176–185.

593 26. P. Blay, J.P. Hui, J. Chang, J.E. Melanson, Anal. Bioanal. Chem., 400 (2011)
594 577–585.

595 27. Z. Škrabáková Z J. O'Halloran, F.N.A.M. van Pelt, K.J. James, Rapid
596 Commun. Mass. Spectrom., 24 (2010) 2966–2974.

597 28. Webb K, Bristow AWT, Sargent M, Stein Methodology for Accurate Mass
598 Measurement of Small Molecules. Best Practice Guide. LGC Ltd, London BK
599 2004.

600 29. European Comission, 2002/657/EC. , Off. J. Eur. Commun. 221 (2002) 8–36

601 30. European Comission, Directorate General Health and Consumer Protection,
602 SANCO/12495/2011. (2012).

603 31. "Guide for authors", J. Am. Soc. Mass. Spectrom.

604 32. International Organization for Standardization (ISO), ISO Guide 5725 (1994)
605 1–1994.

606 33. E. Pitarch, C. Medina, T. Portolés, F.J. López, F. Hernández, Anal. Chim.
607 Acta, 583 (2007) 246–258.

608 34. Eurolab (2007). Measurement uncertainty revisited: Alternative approaches to
609 uncertainty evaluation, Technical report No. 1/2007

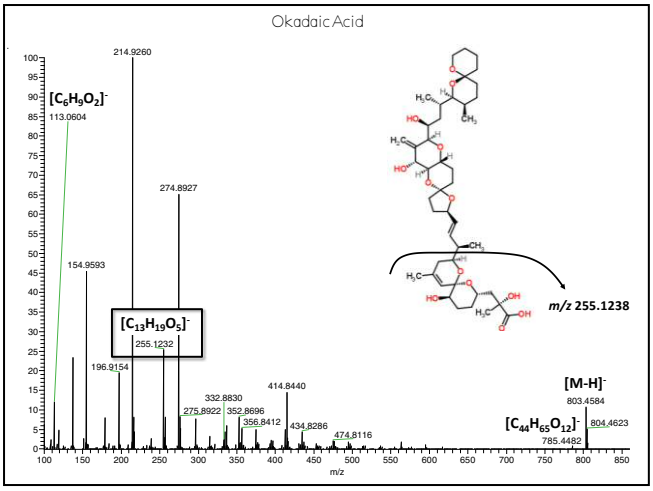
610 35. T. Linsinger, Application Note 1: Comparison of a measurement result with
611 the certified value. European Commission – Joint Research Centre Institute for
612 Reference Materials and Measurements (IRMM), 2010

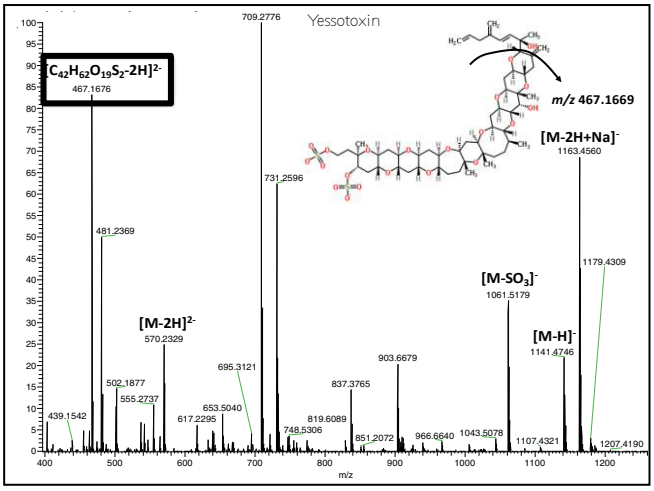
Figure captions

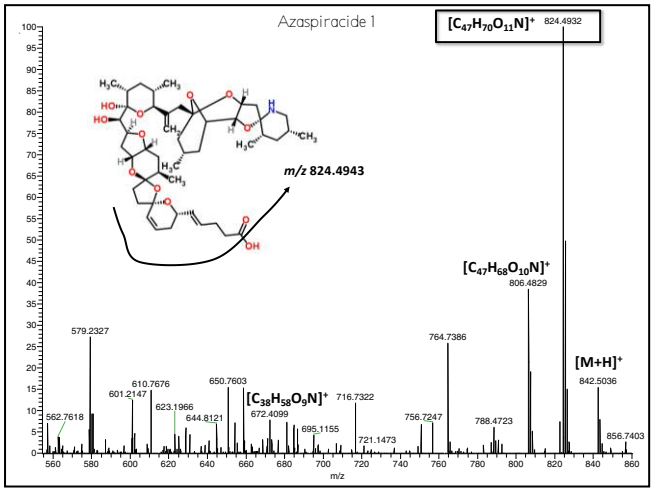
Figure 1. Mass spectral characterization of all the toxins. For okadaic acid the fragmentation spectrum at 60 HCD voltage is shown. For azaspiracide 1, gymnodimine, 13-desmethyl spirolide C the fragmentation spectra at 50 HCD voltage are shown. For yessotoxin and pectenotoxin 2 the full scan spectra are shown. For brevetoxin B the fragmentation spectrum at 20 HCD voltage is shown. All the spectra correspond to matrix-matched standard at 50 ng mL^{-1}, except for brevetoxin B that correspond to 1µg mL^{-1}.

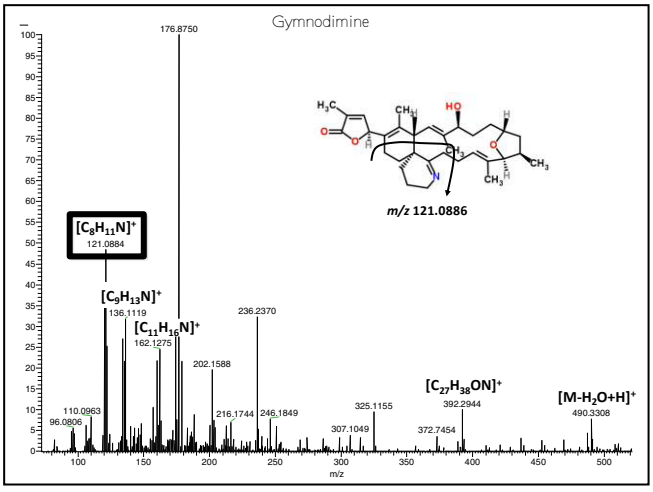
Figure 2. Extracted ion chromatogram of the lipophilic marine toxins, showing a) diagnostic ions and b) fragment ions, with an extraction window of 5 ppm.

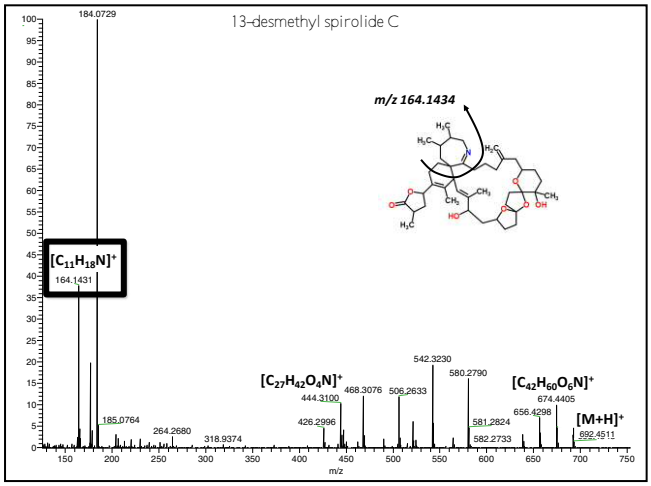
Figure 3. a) Spectrum and b) Extracted ion chromatogram from SPX1 (*m/z* 692.4521) at 0.18 pg μL^{-1}.

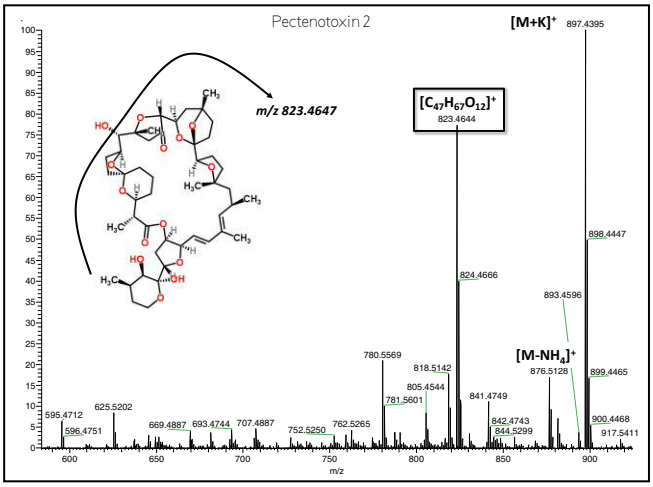












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660 Figure 1.
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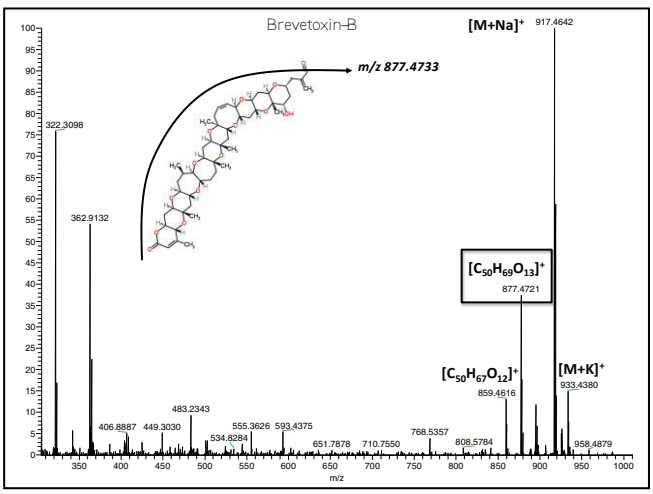

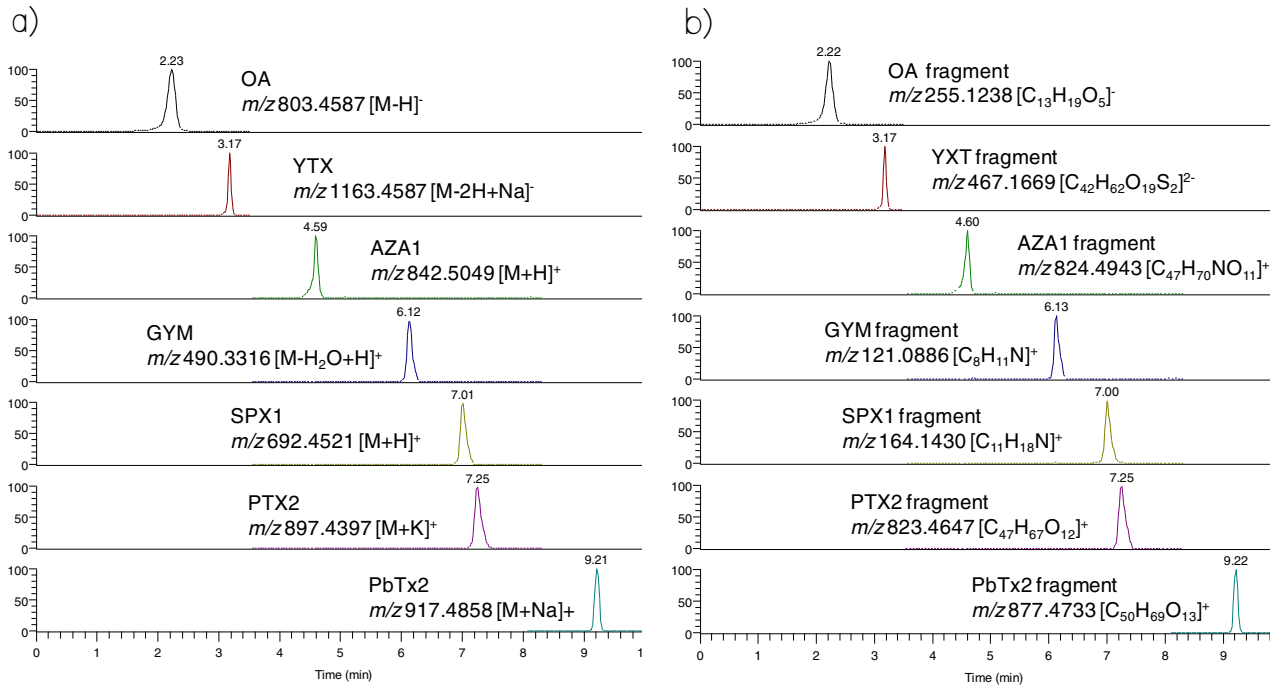
a)
2.23
OA
m/z 803.4587 [M-H]-
3.17
YTX
m/z 1163.4587 [M-2H+Na]-
4.59
AZA1
m/z 842.5049 [M+H]+
6.12
GYM
m/z 490.3316 [M-H2O+H]+
7.01
SPX1
m/z 692.4521 [M+H]+
7.25
PTX2
m/z 897.4397 [M+K]+
9.21
PbTx2
m/z 917.4858 [M+Na]+
Time (min)
b)
2.22
OA fragment
m/z 255.1238 [C13H19O5]-
3.17
YXT fragment
m/z 467.1669 [C42H62O19S2]2-
4.60
AZA1 fragment
m/z 824.4943 [C47H70NO11]+
6.13
GYM fragment
m/z 121.0886 [C8H11N]+
7.00
SPX1 fragment
m/z 164.1430 [C11H18N]+
7.25
PTX2 fragment
m/z 823.4647 [C47H67O12]+
9.22
PbTx2 fragment
m/z 877.4733 [C50H69O13]+
Time (min)

Figure 2.

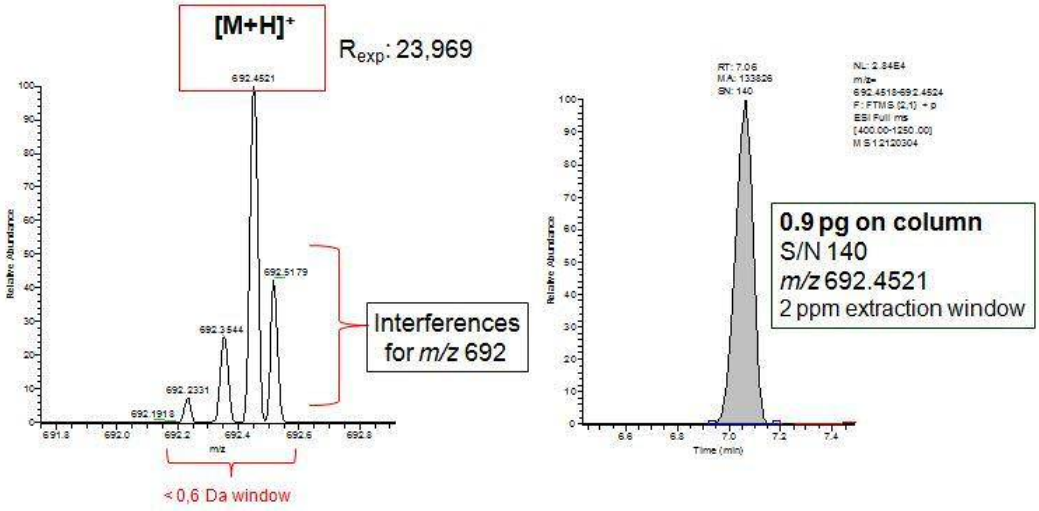
[M+H]+
Rexp: 23,969
692.4521
692.5179
692.3544
692.2331
692.1918
Interferences for m/z 692
< 0,6 Da window
Relative Abundance
m/z
RT: 7.06
MA: 133826
SN: 140
0.9 pg on column
S/N 140
m/z 692.4521
2 ppm extraction window
Time (min)

Figure 3.

687 Table 1. Identification and confirmation criteria. Validation parameters. Matrix–matched
688 calibration curves ranges. Values from n = 6 matrix–matched calibration curves. Linearity
689 expressed as regression coefficients and residuals. In italics data not used, as described in
690 section 3.3.1.4.

Toxin	RT (min) ± 3·SD	Fragment ion ratio ± tolerance	Isotope ion ratio ± tolerance	Matrix–matched calibration curves range	R^2	Residuals (minimum–maximum)	LOQ (pg on column)
Okadaic acid	2.27±0.12	*6.39 ± 30%*	2.17± 25%	0.5 – 150 ng mL^{-1}	0.9911 – 0.9988	0% – 29%	2.4
Yessotoxin	3.17±0.07	0.72 ± 20%	1.62 ± 25%	1 – 150 ng mL^{-1}	0.9825 – 0.9968	0% – 28%	4.8
Azaspiracide–1	4.58±0.08	4.47 ± 25%	2.08 ± 25%	0.5 – 50 ng mL^{-1}	0.9806 – 0.9955	2% – 26%	2.4
Gymnodimine	6.13±0.09	14.44 ± 50%	2.96 ± 25%	0.5 – 150 ng mL^{-1}	0.9832 – 0.9993	0%–23%	2.4
13–desmethyl spirolide C	7.02±0.09	5.13 ± 30%	–	0.2 – 150 ng mL^{-1}	0.9904 – 0.9992	0% – 25%	0.9
Pectenotoxin–2	7.25±0–08	*5.28 ± 30%*	1.84 ± 25%	0.5 – 50 ng mL^{-1}	0.9926 – 0.9961	1% – 30%	3.1

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Table 2. Identification and confirmation criteria, validation parameters. Comparison between studies.

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		Pitarch, 2007 [33]	EU-RL-MB SOP [10]	Gerssen, 2010[17]	SANCO / 12495 / 2011 [30]	Mol, 2012 [23]	Present study
Analytes applied to		Priority organic micropollutants	Lipophilic toxins	Lipophilic toxins	Pesticide residues	Pesticides	Lipophilic toxins
Matrix		Water	Molluscs	Shellfish	Food and feed	Vegetables and fruits	Mussel
Analytical technique		GC-MS/MS	LC-MS/MS	LC-MS/MS	HRMS	LC–HRMS/MS	LC–HRMS/MS
Purpose		Quantification	Quantification	Quantification	Quantification	Screening	Quantification
Identification and Confirmation Criteria							
Mass accuracy		—	—	—	< 5 ppm	< 5 ppm	< 5 ppm
High Resolution (at full width at half maximum – FWHM)		—	—	—	≥ 20,000 at the mass range of interest	≥ 20,000 at the mass range of interest	≥ 20,000 at the mass range of interest
Retention time (RT) drift		Agreement in RT between samples and standards	Not exceed 3 %	5 %	2.5 %	1 %	Mean ± 3 · SD (not relative to time)
Diagnostic ions		1 or 2 precursor ions	1 precursor ion	1 precursor ion	≥ 2 diagnostic ions	≥ 2 diagnostic ions	1 diagnostic ion
Fragment ions		At least two MS/MS transitions	At least two MS/MS transitions	Two product ions were selected for each toxin	At least 1 fragment ion	At least 1 fragment ion	1 fragment ion
Isotope ions		—	—	—	–	M+1, M+2	M+1
Ion ratio		Ratio between quantitative and confirmative transitions	Must be recorded	As described in Decision 2002/657/EC	Comparison of experimental ratio of samples and standards	Fragment ion ratio: ratio between diagnostic and fragment ion	Fragment ion ratio: ratio between diagnostic and fragment ion
						Isotope ion ratio: relation between diagnostic ion and M+1 or M+2	Isotope ion ratio: relation between diagnostic ion and M+1
Fragment-isotope ion ratio tolerance		Comparison of experimental ratio of samples and standards. As described in Decision 2002/657/EC	—	As described in Decision 2002/657/EC	As described in Decision 2002/657/EC	Independent of relative intensity between ions: ± 50%	As described in Decision 2002/657/EC
Validation parameters							
Accuracy	Trueness	Recovery 70–120 %	—	As described in Decision 2002/657/EC. Recovery 80 – 110 %	Recovery 70 –120 %	—	As described in Decision 2002/657/EC. Recovery 80 – 110 %
	Precision	RSDr < 20 %	—	Intraday repeatability and reproducibility. HorRat < 1.0	RSDr and RSD_R < 20 %	—	As described in Decision 2002/657/EC. RSD_R < 23 %

Sensitivity / LOD	the lowest fortification level tested	with the lowest intensity	strongest transition			
LOQ	Lowest level that can be validated with recovery (70 –120 %) and precision (RSD < 20 %)	—	S/N ≥ 6 for the weakest transition	Lowest level that can be validated with recovery (70 – 120 %) and precision (RSD < 20 %) and ≤ MRL	—	Lowest point of the calibration curve that fulfill all the identification and confirmation criteria
Blank Quality Control (QC) / Specificity	—	Methanol blank to be injected. No signal for lipophilic toxins (< LOD or < 10 % of the lowest calibration point)	21 different blank samples to determine interfering peaks	Blank reagent < 30 % LOQ	—	Mussel blank samples (n = 15) to determine interfering peaks. No signal for lipophilic toxins (< LOQ or do not fulfill the confirmation criteria)

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Table 3. Chemical formula, diagnostic ion, fragment ion, isotope ion, *m/z* calculated and observed for each ion. Mass error (RMS error) and in brackets the standard deviation (SD) of all the concentration levels of the calibration curve (r isotope ratio) and relative standard deviation (RSD) of the ion ratio calculated using all the concentration levels of th

Toxin	Chemical formula	Diagnostic ion	*m/z* calculated	*m/z* observed	Mass accuracy and precision	Fragment ion	*m/z* calculated	*m/z* observed	Mass accuracy and precision	Fragment ion ratio (% RSD)	Isotope ion
Okadaic acid	$C_{44}H_{68}O_{13}$	$[M-H]^-$	803.4587	803.4597	1.43 (0.53)	$[C_{13}H_{19}O_5]^-$	255.1238	255.1232	2.53 (0.39)	6.39 (7)	$[C_{43}{}^{13}CH_{67}O_{13}$
Yessotoxin	$C_{55}H_{82}O_{21}S_2$	$[M-2H+Na]^-$	1163.4537	1163.4558	2.04 (0.75)	$[C_{42}H_{62}O_{19}S_2]^{2-}$	467.1669	467.1676	1.59 (0.25)	0.72 (17)	$[C_{54}{}^{13}C\ H_{80}O_{21}S_2$
Azaspiracide–1	$C_{47}H_{71}NO_{12}$	$[M+H]^+$	842.5049	842.5048	0.83(0..92)	$[C_{47}H_{70}NO_{11}]^+$	824.4943	824.4933	1.28 (0.37)	4.47 (17)	$[C_{46}{}^{13}C\ H_{72}NO$
Gymnodimine	$C_{32}H_{45}NO_4$	$[M+H-H_2O]^+$	490.3316	490.3308	1.82 (0.86)	[C8H11N]+	121.0886	121.0885	1.41 (0.52)	14.44 (13)	$[C_{31}{}^{13}C\ H_{44}NO$
13-desmethyl spirolide C	$C_{42}H_{61}NO_7$	$[M+H]^+$	692.4521	692.4514	1.13 (0.81)	$[C_{11}H_{18}N]^+$	164.1434	164.1431	1.70 (0.23)	5.13 (15)	$[C_{41}{}^{13}C\ H_{62}NO$
Pectenotoxin–2	$C_{47}H_{70}O_{14}$	$[M+K]^+$	897.4397	897.4396	1.87 (2.09)	$[C_{47}H_{67}O_{12}]^+$	823.4647	823.4645	2.23 (2.05)	5.28 (40)	$[C_{46}{}^{13}C\ H_{70}O_{14}$
Brevetoxin B*	$C_{50}H_{70}O_{14}$	$[M+Na]^+$	917.4658	917.4692	–	$[C_{50}H_{69}O_{13}]^+$	877.4733	877.4720	–	–	$[C_{49}{}^{13}C\ H_{70}O_{14}$

*Brevetoxin B data from one single acquisition.

Table 4. Validation parameters for spiked mussel: trueness expressed as recovery experiments and precision as intralaboratory reproducibility (n = 3) at 0.5 times the legislation limit.

Toxin	0.5 times the legislation limit	Trueness (%)	RSD_R (%)
Okadaic acid	80 µg·kg^{-1}	94	22
Yessotoxin	500 µg·kg^{-1}	88	12
Azaspiracide–1	80 µg·kg^{-1}	80	8
Gymnodimine*	80 µg·kg^{-1}	90	5
13–desmethyl spirolide C*	80 µg·kg^{-1}	85	8
Pectenotoxin–2	80 µg·kg^{-1}	89	13

*For SPX1 and GYM as no legislation limit was set, the lowest concentration was taken.

727 Table 5. Instrumental quality parameters. Instrumental accuracy (trueness) (n=6),
728 instrumental repeatability ($iRSD_r$) (n=5) and intralaboratory reproducibility ($iRSD_R$)
729 (n=6).
730

Toxin	Accuracy: trueness	$iRSD_r$	$iRSD_R$	Accuracy: trueness	$iRSD_r$	$iRSD_R$
	1 ng mL^{-1}			25 ng mL^{-1}		
Okadaic acid	102%	9%	16%	102%	1%	13%
Yessotoxin	116%	6%	7%	111%	3%	12%
Azaspiracide–1	93%	7%	9%	94%	3%	9%
Gymnodimine	100%	4%	13%	102%	1%	16%
13-desmethyl spirolide C	94%	5%	14%	108%	2%	11%
Pectenotoxin–2	91%	10%	10%	101%	7%	14%

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