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THE IMPLEMENTATION OF LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY FOR THE OFFICIAL CONTROL OF LIPOPHILIC TOXINS IN SEAFOOD: SINGLE-LABORATORY VALIDATION UNDER FOUR CHROMATOGRAPHIC CONDITIONS

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Abstract

We performed a comprehensive study to assess the fit for purpose of four chromatographic conditions for the determination of six groups of marine lipophilic toxins (okadaic acid and dinophysistoxins, pectenotoxins, azaspiracids, yessotoxins, gymnodimine and spirolides) by LC-MS/MS to select the most suitable conditions as stated by the European Union Reference Laboratory for Marine Biotoxins (EURLMB). For every case, the elution gradient has been optimized to achieve a total run-time cycle of 12 min. We performed a single-laboratory validation for the analysis of three relevant matrices for the seafood aquaculture industry (mussels, pacific oysters and clams), and for sea urchins for which no data about lipophilic toxins have been reported before. Moreover, we have compared the method performance under alkaline conditions using two quantification strategies: the external standard calibration (EXS) and the matrix-matched standard calibration (MMS). Alkaline conditions were the only scenario that allowed detection windows with polarity switching in a 3200 QTrap mass spectrometer, thus the analysis of all toxins can be accomplished in a single run, increasing sample throughput. The limits of quantification under alkaline conditions met the validation requirements established by the EURLMB for all toxins and matrices, while the remaining conditions failed in some cases. The accuracy of the method and the matrix effects where generally dependent on the mobile phases and the seafood species. The MMS had a moderate positive impact on method accuracy for crude extracts, but it showed poor trueness for seafood species other than mussels when analyzing hydrolyzed extracts. Alkaline conditions with EXS and recovery correction for OA were selected as the most proper conditions in the context of our laboratory. This comparative study can help other laboratories to choose the best conditions for the implementation of LC-MS/MS according to their own necessities.

Keywords

Lipophilic marine toxins; liquid chromatography-mass spectrometry; seafood; method validation; matrix effects

1 Introduction

Lipophilic marine toxins accumulate in seafood, causing remarkable economic losses in the aquaculture sector [1] and posing a risk to human health. To protect consumers, the European Union demands the monitoring of some lipophilic marine toxins [2] and limits their maximum permitted levels (MPLs) in edible shellfish tissues [3]: 160 μg/kg in okadaic acid (OA) equivalents for OA, dinophysistoxins (DTXs) and pectenotoxins (PTXs) together; 1 mg/kg for yessotoxins (YTXs) and 160 μg/kg for azaspiracids (AZAs). Other lipophilic marine toxins are not yet regulated in the European Union, like cyclic imines mainly comprising spirolides (SPXs) and gymnodimines (GYMs). The reference method to control lipophilic toxins in the European Union was the bioassay with mice or rats until January of 2011 [2]. The European Food Safety Authority (EFSA) indicated in 2009 the disadvantages of these bioassays [4]: ethical concerns, limited specificity, high variability in results, and insufficient detection capability for some toxins. According to the European Commission and the EFSA, the analytical methods based on liquid chromatography tandem mass spectrometry (LC-MS/MS) were a good alternative to replace the bioassays once the methods were validated and proved to be effective to protect consumers.

The LC-MS/MS multi-toxin methods to analyze lipophilic toxins in seafood can work under different chromatographic conditions. Separation of lipophilic toxins under acidic chromatographic conditions was first proposed by Quilliam *et al.* in 2001[5], studied in depth by McNabb *et al.* in 2005 [6] and widely used since then [7-10]. The European Union Reference Laboratory for Marine Biotoxins (EURLMB) also applies acidic conditions and validated its method in-house in 2011 [11]. Gerssen *et al.* proposed in 2009 [12] a multi-toxin method with alkaline conditions and in-house validated it in 2010 [13]; these conditions gained popularity in the last years [14-16]. Less extreme pH conditions were proposed by Stobo *et al.* [17] using ammonium acetate as buffer (pH 6.8) and by These *et al.* [18] using ammonium bicarbonate (pH 7.9).

Two institutions organized interlaboratory collaborative exercises to validate their LC-MS/MS methods in 2010: the EURLMB and the Dutch Institute of Food Safety (RIKILT). The EURLMB validated its Standardized Operating Procedure (SOP) [19] for OA, PTXs and AZAs (the participants could voluntarily include YTXs). The SOP stipulated the extraction protocol and the alkaline hydrolysis step, recommended a list of MS/MS transitions to monitor and suggested several chromatographic conditions to quantify lipophilic toxins by external standard calibration (EXS). The RIKILT validated its method for all regulated lipophilic toxins under alkaline chromatographic conditions and the quantification strategy of matrix-matched standard calibration (MMS) [20]. The success of both inter-laboratory studies demonstrated the effectiveness of the methods based on LC-MS/MS to replace the animal bioassays and promoted the approval of the Regulation (EC) no 15/2011 [2], which settled the method validated under the coordination of the EURLMB as the reference technique for the detection of lipophilic marine toxins in bivalve molluscs in Europe. This regulation applies from July 1st, 2011

and allows the use of mice and rats bioassays for lipophilic toxin determination until December 31st, 2014.

The EURLMB SOP referenced in the Regulation (EC) no 15/2011 [2] fixes neither the LC-MS/MS conditions nor the recovery correction approach [11], and this may trouble laboratories implementing the methods for control purposes. The EURLMB SOP provides several elution gradients and three possible chromatographic conditions as examples, allowing the analysts to choose the most convenient one: acidic conditions buffered with ammonium formate/formic acid; and basic conditions with ammonia or ammonia and ammonium bicarbonate as buffer. However, the selection of the chromatographic conditions requires a wide-scope study of the alternatives, since the pH and the buffer system of the mobile phases affect many parameters of the method: the selectivity of chromatographic separations, the ionization yields at the electrospray ionization source, the sensitivity of the MS response, the elution order and the matrix effects. Matrix effects can be corrected or compensated by, among other strategies, standard addition, SPE clean-up, sample dilution, and matched standard calibration (MMS) — the strategy used by RIKILT [17,21-25].

According to the literature and the conclusions from interlaboratory trials, several chromatographic conditions seem feasible for the analysis of marine toxins. However, neither study compared different elution conditions nor assessed their impact on the methods performance. This paper is a comprehensive comparative study on the suitability of different experimental approaches suggested in the EURLMB SOP. We optimized and in-house validated four chromatographic conditions [6,13,17,18] under the same experimental settings: same instrumentation, chromatographic column, sample preparation protocol, reagents, standards and analyst. We studied the separation and quantification of six groups of lipophilic toxins (all regulated in the EU plus GYMs and SPXs) at three concentration levels (0.5, 1 and 1.5 times the MPLs) with four relevant matrices for the seafood industry (mussels, pacific oysters, clams and sea urchin). We also assessed two quantification strategies (EXS and MMS) under alkaline conditions and studied matrix effects in detail. The aim of the work was to guide other labs in the decision-making process to select the most appropriate conditions for their LC-MS/MS method to analyze lipophilic toxins in seafood.

2 Materials and Methods

2.1 Standards and chemicals.

Certified reference standard solutions were purchased from the Institute for Marine Bioscience of the National Research Council (NRC) from Halifax (Canada): okadaic acid (OA, 14.3 ± 1.5 µg/mL), yessotoxin (YTX, 5.3 ± 0.3 µg/mL), pectenotoxin-2 (PTX2, 8.6 ± 0.3 µg/mL), azaspiracid-1 (AZA1, 1.24 ± 0.07 µg/mL), 13-desmethyl spirolide-C (SPX1, 7.0 ± 0.4 µg/mL, and gymnodimine-A (GYMA, 5.0 ± 0.2 µg/mL).

Certified reference standard solutions for dinophysistoxin-1 (DTX1) and dinophysistoxin-2 (DTX2) were not available, thus a sample of mussel (*Mytilus galloprovincialis*) naturally contaminated with OA, DTX1 and DTX2 from the inter-laboratory proficiency test for lipophilic toxins organized by the EURLMB in 2010 was used to calculate the retention time (t_R) of DTX1 and the chromatographic resolution between OA and DTX2. The samples from the proficiency test for lipophilic toxins organized by the EURLMB in 2011 were used to calculate the relative t_R of AZA2 and AZA3 compared to AZA1; and homo-yessotoxin (homoYTX), 45-hydroxy-yessotoxin (45-OHYTX) and 45-hydroxy-homo-yessotoxin (45-OHhomoYTX) compared to YTX. Unfortunately, none of the samples had PTX1 to be included in the study.

Acetonitrile (ACN) hypergrade for LC-MS, methanol (MeOH) gradient grade for HPLC and formic acid puriss, 98.0% were purchased from Merck (Darmstadt, Germany). Ammonium bicarbonate and ammonium acetate (both elution additive for LC-MS), ammonium hydroxide (28% in water; ≥99.99% trace metals basis), ammonium formate for HPLC ≥99.0% and sodium hydroxide puriss. p.a were purchased from Sigma-Aldrich (Steinheim, Germany). Hydrochloric acid 37% was purchased from Panreac Quimica (Barcelona, Spain). Ultrapure water was obtained though a Milli-Q purification system (resistivity >18 MW·cm) from Millipore (Bedford, MA).

2.2 Preparation of extracts

Blue mussels (*Mytilus galloprovincialis*), pacific oysters (*Crassostrea gigas*), clams (*Ruditapes philippinarum*) and sea urchins (*Paracentrotus lividus*) were collected from the seafood harvesting areas of Catalonia, Spain (NW Mediterranean Sea) in 2010 and 2011. A triple-step extraction with MeOH was performed on whole tissues according to the procedure proposed by Gerssen *et al.* [13], but samples were homogenized with a hand blender instead of with an Ultra Turrax homogenizer. We chose this extraction procedure to ensure the recovery of the more lipophilic OA and DTX esters [13]. The protocol used 1 g of tissue (keeping the tissue:extractant volume ratio at 1:10, v/v) saving expensive certificate standards required for spikings. We used an analytical balance Sartorius 1702 (Goettingen, Germany), a vortex-mixer MS2 Minishaker (IKA Labortechnik, Staufen, Germany), and a centrifuge Jouan MR 23i (Thermo Fisher Scientific Inc., Waltham, MA, USA). Crude extracts were filtered through polytetrafluoroethylene (PTFE) 0.2 μm membrane syringe filters.

2.3 Alkaline hydrolysis

The alkaline hydrolysis of the samples was performed according to the EURLMB SOP [11] based on the protocol initially developed by Mountfort *et al.* [26].

2.4 Chromatographic separation

Toxins were separated on a Waters X-Bridge™ C8 (guard column 2.1 x 10 mm, 3.5 µm particle size, column 2.1 x 50 mm, 3.5 µm particle size; Waters, Milford, MA, USA) in an Agilent 1200 LC system (Agilent Technologies, Santa Clara, CA) consisting of a binary pump (G1312B), four channel degasser (G1379B), thermostated low carry-over autosampler (G1367C + G1330B), and column oven (G1316B). Four elution systems were tested:

• *Mobile phases in acidic conditions (pH 2)* according to McNabb *et al.* [6]: Mobile phase A consisted of 2 mM of ammonium formate and 50 mM of formic acid in ultrapure Milli-Q water. Mobile phase B consisted of 2 mM of ammonium formate and 50 mM of formic acid in 95/5 v/v ACN/Milli-Q water.

• *Mobile phases in close to neutrality conditions (pH 6.8)* according to Stobo *et al.*[17]. Mobile phase A consisted of 5 mM of ammonium acetate in ultrapure Milli-Q water. Mobile phase B consisted of 5 mM of ammonium acetate in 95/5 v/v ACN/Milli-Q water.

• *Mobile phases in slightly alkaline conditions (pH 7.9)* according to These *et al.*[18]: Mobile phase A consisted of 5 mM of ammonium bicarbonate in ultrapure Milli-Q water. Mobile phase B consisted of 5 mM of ammonium bicarbonate in 95/5 v/v ACN/Milli-Q water. Mobile phase B was kept in the ultrasonic bath for 10 min to dissolve the buffer.

• *Mobile phases in alkaline conditions (pH 11)* according to Gerssen *et al.*[12,13]: Mobile phase A consisted of 6.7 mM of ammonia in ultrapure Milli-Q water. Mobile phase B consisted of 6.7 mM of ammonia in 90/10 v/v ACN/Milli-Q water.

The mobile phases were filtrated through 0.2 µm nylon-membrane filters and the pH of aqueous mobile phases was measured with a CyberScan pH1100 (EUTECH Instruments, Thermo Fisher Scientific Inc., Waltham, MA, USA).

The column oven temperature was set at 30 °C and the flow rate was 0.5 mL/min. Gradient programs are shown in Table 1. We optimized a total run time of 12 min for all gradients, including column conditioning (Table 1) and included a step of 100% mobile phase B for 1 min to flush late eluting compounds [24], thus extending the lifespan of the column. The diverter valve was programmed to deliver the eluent from column to waste for the first 1.5 min in all gradients.

Injection volume was optimized at 10 µL under alkaline conditions and 5 µL for the other conditions after testing the loading capacity of the column. The sample compartment was set at 4 ºC. The outer surface of needle was flushed with MeOH in the autosampler before every injection.

The column used for the whole study was ethylene-bridged hybrid (BEH). This column is designed to work at variable pH from 2 to 11. Before switching mobile phases, the system was purged and the column was washed with mixtures of ACN/Milli-Q water (95% to 0% water) at 0.2 mL/min for two hours and conditioned with 20% mobile phase B at 0.5 mL/min for 20 min before running gradient five times. Column equilibration was done at the beginning of each batch with the mobile phases used for analysis running the same gradient of analysis five times. At the end of each batch, the

column was washed with mixtures of ACN/Milli-Q water for 25 min to remove lipophilic interferences and buffers.

2.5. Mass spectrometry

We used a triple quadrupole 3200 QTRAP® mass spectrometer (MS) equipped with a TurboV electrospray ion source (Applied Biosystems, Foster City, CA). The MS was operated in the multiple reaction monitoring (MRM) mode, selecting two product ions per toxin to allow quantification (the most intense transition) and confirmation (two confirmation ions for GYMA). Table 2 shows a summary of the MS/MS settings for lipophilic toxins analysis. The MS/MS conditions were based on the recommended values in the EURLMB SOP [11] for a 3200 QTRAP® MS. The selection of the precursor ions was based on the literature [6,13,17,18]. We chose the double charge precursor ion ($[M-2H]^{2-}$) to monitor YTXs under pH 11 [12] but we decided to maintain the ammonium adduct ($[M+NH_4]^+$) instead of the sodium adduct ($[M+Na]^+$) to monitor PTXs under pH 6.8 since the reference paper [17] only applied single-quadrupole MS analysis, thus it does not provide information about fragmentation or MS/MS parameters from the precursor $[M+Na]^+$ for PTXs.

Mass spectrometric detection was performed in both negative (-ESI) and positive polarity (+ESI). Under pH 2, pH 6.8 and pH 7.9, two different injections were needed per sample: the toxins OA, DTX1, DTX2 and YTXs were detected in the -ESI, while the +ESI was used to detect SPX1, GYMA, AZAs and PTXs. The alkaline mobile phase allows polarity switching from negative to positive mode to analyze all toxins in two detection windows during the same run: the first retention time window was programmed during the first 4.5 min in negative ESI mode to detect OA, DTX1, DTX2, and YTXs; the second retention time window lasted 7.5 min in positive ESI mode to analyze SPX1, GYMA, AZAs, PTXs.Resolution of the quadrupoles was set at unit.

2.6 Quality requirements posed by the EURLMB

We checked in every batch the quality control criteria stated by the EURLMB SOP [11] regarding resolution, limits of quantification (LOQs) and linearity.

Resolution (*Rs*) between the isomers OA and DTX2 was calculated according to Equation 1:

$$R_s = 2\,(t_{R(DTX-2)} - t_{R(OA)})/(W_{(OA)} + W_{(DTX2)}) \quad \text{Equation 1}$$

Where t_R means retention time and W means peak width (both in minutes). The resolution for each chromatographic condition was assessed as the average resolution of six replicates in a reference sample naturally contaminated with OA and DTX2. The EURLMB requests resolution between OA and DTX2 to be greater than one [11].

LOQs were evaluated with three replicate blank samples of each matrix spiked at the theoretical LOQs (calculated with blank homogenized tissues spiked with OA, PTX2, SPX1, GYMA and AZA1 at 80 µg/kg and with YTX at 250 µg/kg), analyzed by triple injection, as the concentration that met a S/N of ten for the most abundant fragment and a S/N greater than three for the transition used for confirmation. Noise was calculated with a blank sample of each matrix at the expected retention times of the toxins. Methods validated under the specification of the EURLMB SOP [11] shall reach LOQs as low as 40 µg/kg for AZA1 and OA, 50 µg/kg for PTX2 and 60 µg/kg for YTX.

Linearity was estimated from the calibration curves analyzed before and after the analysis of a set of samples (six to eight samples). The correlation coefficients of the quantification curves had to be greater than 0.98 to ensure linearity; the deviation of the slopes between consecutive calibration curves has to be lower than 25% to be considered as acceptable, as requested in the EURLMB SOP [11].

Sensitivity of the method was evaluated as the slope of the external standard calibration curves for each toxin.

2.7 Validation parameters

The in-house validation study relied on the concepts described in Taverniers *et al.* [27], the guidelines proposed by the Regulation (EC) 657/2002 on performance criteria for analytical methods [28], and the methodology applied by de la Iglesia *et al.* [29].

The accuracy of the methods was assessed by the intermediate precision and the trueness. The spikings were done on blank homogenized tissue instead of on extracts in MeOH to make the validation process as comprehensive as possible.

The intermediate precision was expressed as the relative standard deviation (RSD in %). It was calculated for each matrix (mussels, pacific oysters, clams and sea urchins) at three different concentration levels of OA, PTX2, SPX1, GYMA and AZA1 (80 µg/kg, 160 µg/kg and 240 µg/kg) and two concentration levels of YTX (250 µg/kg and 500 µg/kg) spiked in blank homogenized tissues and quantified using external standard calibration curves. Four replicates spread over four consecutive days were analyzed by single injection using daily fresh mobile phase. The RSD was transformed to HorRat value as the ratio between the experimental RSD and the predicted RSD according to the Horwitz equation [30] (Equation 2), which is dependent on the concentration (*C*) spiked for the intermediate precision assessment.

$$\text{HorRat} = \text{RSD}(\%)\ \text{experimental} / 2^{(1-0.5\log C)} \quad \text{Equation 2}$$

The Regulation (EC) 657/2002 [28] suggests that for in-house laboratory validation, the experimental RSD should not exceed the expected RSD (HorRat < 1). Intermediate

precision was only calculated when at least three out of the four replicates met the quality requirements regarding linearity.

Trueness in terms of recovery was calculated for each sample matrix at the three concentration levels (two concentration levels for YTX) using the four replicates analyzed by single injection in consecutive days and quantified using external standard calibration curves. Recovery in percentage was calculated by comparing the quantifications by external calibration with the theoretical spiked concentration. The Regulation (EC) 657/2002 [28] recommends correcting the quantification with the mean recovery only if trueness falls between 80% and 110%.

We used the same batch sequences for all chromatographic conditions. The matrices were injected always in the same order, grouped by its concentration level (from low to high concentration). Blanks of MeOH were analyzed before and after calibration curves and sets of samples to assess potential carry-over problems.

Chromatographic selectivity was based on t_R of the analytes that have commercial standard solutions (at least one representative for each group of lipophilic toxins). For analogues without standards available, we used the relative retention time (RRT) compared to the representative toxin.
The drift in t_R in the samples compared to those in the standard solutions was acceptable below 3%, as stated in the EURLMB SOP [11]. Mass spectrometric selectivity was assessed with the transitions monitored in the MS/MS system, proposed by the EURLMB SOP [11] and by Gerssen *et al.* [12] for the determination of YTX under alkaline conditions. The maximum permitted tolerances for relative ion intensities were taken from Regulation (EC) 657/2002 [28] and were checked in all matrices analyzed, spiked at the MPL (0.5 times the MPL for YTX) during three consecutive days. The presence of potential interferences was assessed by analyzing blank samples for all matrices.

2.8 Calibration strategies and matrix effects assessment

The external standard calibration curves were prepared in MeOH (LC-MS grade) from an initial multi-toxin stock solution of 400 ng/mL of OA, PTX2, SPX1, GYMA and AZA1, and 625 ng/mL of YTX. The calibration curves had six levels in the range of 5 to 60 ng/mL of OA, PTX2, SPX1, GYMA and AZA1 and 8 to 94 ng/mL of YTX.
The in-house validation of the four chromatographic conditions was done using the external standard calibration strategy (EXS) to quantify the spiked samples. This calibration strategy saves the expensive certified standard solutions, assuming the calibration curves prepared in MeOH lasts longer than those involving seafood matrices. Nevertheless, the matrix-matched standard (MMS) calibration strategy has been reported to compensate matrix effects caused by seafood tissues in the determination of lipophilic toxins [13]. The MMS calibration strategy consists on the

preparation of the calibration curve in a solvent with the same composition as the matrix of interest, usually in extracts of blank tissues of the same seafood species analyzed [24], thus the influence of the matrix interferences would affect equally to samples and standards.

We performed a comparative study between the External Standard calibration (EXS) and the matrix-matched standard calibration (MMS) prepared with blank mussel extracts. The study tested if matrix effects were species dependent and if MMS improved method accuracy compared to EXS.

We spiked homogenated seafood tissues by adding the standards on the tissues and vortex-mixing them for 1 min. One blank sample of each matrix was spiked at three different concentration levels of OA, PTX2, SPX1, GYMA and AZA1 (80 µg/kg, 160 µg/kg and 240 µg/kg) and two concentration levels of YTX (250 µg/kg and 500 µg/kg), injected in triplicate and quantified with a five level calibration curve (5 to 40 ng/mL) prepared in MeOH to assess the EXS strategy. The same spiked samples were injected in triplicate and quantified against a five level calibration curve (5 to 40 ng/mL) prepared in blank mussel extracts to assess the MMS strategy. The quantification of the hydrolyzed spiked samples was performed by triple injection against an hydrolyzed EXS calibration curve and against an hydrolyzed MMS calibration curve in mussels, both spiked with OA before the hydrolysis (five levels from 5 to 40 ng/mL).
We also studied species dependence in matrix effects for OA (free and total OA after hydrolysis), YTX, PTX2, AZA1, SPX1 and GYMA in mussels, oysters, clams and sea urchins using the four chromatographic conditions. Matrix effects (ME) were estimated as the ratio between the slopes of a five level calibration curve (5 to 40 ng/mL) prepared in extracts of the blank seafood matrices, and the same curve prepared in MeOH. Values of ME lower than one mean the matrix inhibits the signal; ME higher than one means signal enhancement. If the slope of both calibration curves are equal (ME = 1), the matrix would have no effect on the sensitivity of the method. Each calibration level was analyzed by single injection under pH 2, pH 6.8 and pH 7.9; three injection replicates were analyzed under alkaline conditions.

2.9 Statistical analysis.

Statistical calculations were performed using SPSS 17.0. The significance tests used to evaluate the influence of the species in the matrix effect was a One-Way ANOVA (one test per toxin), supported by a Levene Test of Homogeneity of Variances, and a Post Hoc Tukey HSD Test when the ANOVA test showed significant differences in the mean between groups (species). Alpha was set at 0.05 (95% confidence) for all tests and experiments.

3 Results and discussion

3.1 Implementation of LC-MS/MS methods according to the EURLB-SOP quality requirements

We expected t_R and elution order of the toxins to change under different chromatographic conditions [12] (Figure 1), since the charge state of the toxins is influenced by the pH of the mobile phase. Under pH 2, YTX coeluted with PTX2, and the "–ESI toxins" (OA and YTX) eluted in the same time window as the "+ESI toxins" (GYMA, SPX1, PTX2 and AZA1). The shift from acidic to almost neutral conditions reduced OA t_R and slightly alkaline conditions increased the t_R of the cyclic imines. When pH was modified from pH 7.9 to pH 11, t_R of OA, YTX and AZA1 became shorter, thus the "–ESI toxins" eluted at the beginning of the chromatogram and "+ESI toxins" eluted afterwards. This change in the elution order enabled detection windows to be set with different polarity in our 3200 QTRAP® and analyze all toxins in the same run.

Our results of t_R and elution orders (Figure 1 and Table 3) agreed with those explained in Gerssen *et al.* [12]. We also observed a narrower peak for YTX once the pH was set close to neutrality in relation to acidic conditions. AZA1 t_R was the most shortened by pH changes (3.2 min difference over 9 pH units, Table 3) and peaks widened when pH changed from acid to alkaline conditions.

All conditions met the quality requirements for OA-DTX2 resolution. The best resolutions between OA-DTX2 calculated according to Equation 1 were 1.67 and 1.55 under pH 6.8 and pH 7.9, respectively. Resolutions achieved with elution at pH 2 and pH 11 were lower (1.09 and 1.01, respectively) though still fulfilled the quality criteria [11].

The external calibration curves of the NRC standards confirmed that the elution system does have an effect on sensitivity (Table 4 and Figure 2). Alkaline conditions showed the highest sensitivity for all toxins but AZA1; the improvements in sensitivity for YTX and PTX2 were remarkable: after normalizing sensitivity data with injection volumes, YTX sensitivity was five times better under alkaline conditions than under acidic conditions, while PTX2 sensitivity increased almost three-fold. Chromatographic conditions under pH 6.8 and 7.9 generally showed lower sensitivities than acidic conditions, especially for PTX2 under pH 6.8 and for YTX in both cases.

In our case and following the EURLMB SOP [11] requirements, only the alkaline conditions could be implemented as a multi-toxin method, since it was the only one proving acceptable LOQs for all regulated toxins (Table 5), including YTX (less than 60 μg/kg), with our middle-class 3200 QTRAP® MS. The analysis of YTX under acidic conditions gave high LOQs (Table 5), from 272.6 μg/kg (in sea urchin) to 377.1 μg/kg (in mussel), influenced by the poor chromatographic peak shape of YTX under pH 2 (Figure 1). Although more alkaline pH improved YTX peak shape, the detection capability for YTX under pH 6.8 and pH 7.9 was still too low (Table 4) and the

458 theoretical LOQs for YTX under these pH were found over 300 μg/kg, therefore
459 experimental LOQs were not evaluated to save valuable standards. Conditions under pH
460 6.8 also failed to provide LOQs for PTX2 lower than 50 μg/kg in most of the matrices.
461 The lowest LOQs for AZA1 and GYMA were achieved under pH 7.9 (7.1 μg/kg and 2.3
462 μg/kg respectively, average for the four matrices), while the lowest LOQs values for
463 OA, PTX2 and SPX1 were found under alkaline conditions (6.5 μg/kg, 11.9 μg/kg and
464 8.6 μg/kg respectively, average for the four matrices).

465 This study confirms that the selection of the proper chromatographic condition can
466 contribute to better LOQs. Alkaline conditions provided better LOQs for YTX because
467 of three reasons: first, they allowed 10 μL of sample injection (instead of 5 μL as in the
468 rest of the conditions) without peak broadening caused by column overloading; second,
469 the double charged species monitored as the precursor ion of YTX were highly selective
470 and sensitive [13]; and finally, alkaline pH seems to reduce secondary interactions
471 between the sulfonic acids of YTX and the stationary phase of the column [12],
472 resulting in narrower peaks with better S/N ratios (Figure 1). The ionization yield of
473 YTX at pH 6.8 and 7.9 has not been studied in detail (nor in this study neither in the
474 literature), thus the selection of a different precursor ion might increase YTX sensitivity
475 under these elution systems. Nevertheless, the maximum permitted level for the YTXs
476 is 1 mg/kg, thus other conditions could be also applied and still be efficient to monitor
477 the YTXs according to the Regulation (EC) 853/2004 [3].

478 Low sensitive instruments may require the reconsideration of the extraction procedure
479 to achieve better LOQs, by reducing the extraction volume or applying pre-
480 concentration steps, but matrix effects and recoveries should be carefully taken into
481 account when applying these strategies.

482 The correlation of the calibration curves calculated by least-squares adjustment was not
483 always satisfactory. Although all chromatographic conditions had correlation
484 coefficients less than 0.98 in some specific occasions, we realized that some toxins
485 (especially YTX) and chromatographic conditions (particularly pH 7.9) are more prone
486 to have linearity problems.
487
488 A major change in the slope (response drift over 25%) of two consecutive calibration
489 curves means the sensitivity of the method for a certain toxin is not stable during the
490 batch, which occurred in 12.5% of the calibration curves of SPX1 and PTX2 analyzed
491 under pH 6.8, and in 25% of the curves of YTX with pH 7.9. Acidic and alkaline
492 conditions kept the sensitivity constant for all toxins in all batches (none of the batches
493 had a slope drift larger than 25%). Changes in sensitivity were unlikely due to carry-
494 over problems, since we did not find any toxin signal in control blank samples analyzed
495 after positive control samples or high concentration standards. However, response drifts
496 were more frequent for those toxins with poor sensitivities under certain
497 chromatographic conditions.
498

499 3.2 Methods performance
500
501 The alkaline conditions had the best overall performance in terms of precision (Table
502 6). For AZA1, alkaline conditions provided HorRat values below one in all matrices and
503 concentrations, but other of conditions were also precise enough in most cases at
504 medium and high concentrations. The precision in the analysis of GYMA spiked in
505 mussels was only satisfactory under alkaline conditions, but acidic conditions had better
506 precision in sea urchins. The HorRat values for OA (both crude and hydrolyzed) were in
507 general very high (up to 3.4 in mussels spiked at 0.5 times the MPL analyzed under
508 acidic conditions after hydrolysis). The precision for crude OA in mussels under
509 alkaline conditions was good, but in sea urchins the acidic conditions would provide
510 better HorRat values at medium and high concentrations. For PTX2 and SPX1, alkaline
511 conditions generally gave better results in terms of precision.
512 The intermediate precision for YTX was generally insufficient under all
513 chromatographic conditions but slightly better under pH 11. Since alkaline conditions
514 were the only one providing LOQs lower than 60 μg/kg for YTX, they were the best
515 choice for the analysis of YTX.
516
517 Trueness was expressed as recovery (Table 6). The recovery of the lipophilic toxins
518 resulted to be dependent on the chromatographic conditions, since the pH and the buffer
519 in the mobile phase can affect the ionization yield of the toxins and the elution of
520 potential interferences present in the matrix.
521 The recoveries for AZA1 were mostly lower than 70% for all matrices under pH 6.8 and
522 7.9 and slightly better under pH 2, but the toxin concentration was overestimated under
523 alkaline conditions. The recoveries for GYMA were generally low under all
524 chromatographic conditions (slightly better under pH 6.8), but especially under pH 2,
525 with recoveries below 85%. The recoveries of OA strongly depended on the pH: the
526 overestimation of crude OA under alkaline conditions was remarkable, while the
527 recoveries generally fell in the range of 80% to 120% under pH 2 and were slightly
528 lower under pH 7.9. The hydrolyzed OA also resulted in overestimation under pH 11,
529 but the recoveries were generally lower than those for the crude OA in all cases. The
530 recoveries for PTX2 were generally low under acidic conditions and under pH 6.8 and
531 7.9, but they fell between 80% and 110% in most cases under pH 11, thus it would be
532 possible to correct the concentration using the mean recovery [28] Recovery correction
533 can also be applied for SPX1 quantification under alkaline conditions, while SPX1 were
534 under-quantified with pH 2 and pH 7.9 and over-quantified with pH 6.8. The YTX
535 recovery under pH 2, pH 6.8 and pH 7.9 were not reliable since most measurements
536 were imprecise and the LOQs were too high. Under alkaline conditions, recoveries for
537 YTX were always below 80%.
538
539 Improvements in precision and trueness enhance accuracy. Precision benefits from
540 replicate injections of the sample and more data points per peak. The EURLMB
541 validated its method using double injection [11], but the SOP allows single injection

whenever possible to increase sample throughput and save standards, as aimed in this study, but this approach showed to be sometimes insufficient and double or triple injection is encouraged. The number of acquired points per peak of transitions used for quantification may also be increased by reducing the dwell time of confirmatory transitions (assuming proper S/N and relative ion intensities ratios). Besides, clustering of "–ESI toxins" and "+ESI toxins" is very useful to increase the sample throughput of instruments with slow polarity switching, but it still provide a benefit even in modern instruments since the less time invested in polarity switches, the more data points acquired per peak. Trueness is improved by correction in recovery with certified reference materials or in-house internal reference materials when the firsts are not available.

The deviation in t_R for all toxins in the spiked samples compared to those in the standards never exceeded 3%. The stability of the pH in the mobile phase ensures the retention times remain constant along the analysis. Alkaline mobile phase was prone to changes in pH (likely due to the evaporation of the ammonium hydroxide) and we observed AZAs t_R were very sensitive to those slight changes. Thus, alkaline mobile phases should be freshly prepared daily. When there is no available standard to obtain the t_R of a toxin, the relative t_R can provide additional identification points complementary to the MRM transitions. Moreover, it may be interesting to get relative t_R under different elution conditions for toxin analogues for which standards are not commercially available, especially when derivatives are present in samples at very low concentration and acquisition of a full product ion spectrum is not possible. Retention times behaviour under different chromatographic conditions can provide additional identification points.

We did not detect interfering peaks in the blank samples for any toxin under any chromatographic conditions, but switching chromatographic conditions could serve as a strategy to get rid of matrix interfering compounds since the pH modifies the selectivity towards the compounds of the matrix, as proposed by Kilcoyne and Fux [24].

The relative ion intensities measured in the samples and in the calibration standards at comparable concentrations fell into the tolerance ranges proposed by the Regulation (EC) 657/2002 [28] in most cases. There were two small deviations out of the tolerance ranges: for PTX2 in sea urchin matrix analyzed under acidic conditions (1% out of the tolerance range) and for YTX in oysters analyzed under alkaline conditions (4% out of the tolerance range). The most important variation was found for YTX in oysters analyzed under acidic conditions (17% out of the tolerance range), probably related to the poor sensitivity and chromatographic peak shape of YTX under pH 2. Nevertheless, the matrix might alter the fragmentation ratios of an analyte [31], although this phenomenon has been barely studied.

3.3 Calibration strategies and matrix effects assessment

585 Matrix effects strongly varied depending on the toxin. Signal enhancement was
586 especially evident for OA in most matrices and chromatographic conditions. Overall
587 positive matrix effects were less important for PTX2, while AZA1 mostly tended to
588 signal suppression. Matrix effects for cyclic imines depended on chromatographic
589 conditions (Table 4), and generally suffered from signal suppression under acidic
590 conditions and moderate signal enhancement at more alkaline pH. The use of different
591 chromatographic conditions affects matrix effects by altering the elution order of
592 interferences, but this effect is difficult to assess, and it had not been systematically
593 studied before.
594
595 Matrix effects may explain deviations in recovery, a problem often reported in
596 lipophilic toxin determination by LC-MS/MS [17,21-25]. Signal suppression of AZA1,
597 SPX1 and GYMA under pH 2 could explain the low recovery of these toxins, while OA
598 signal enhancement correlated with the overestimation of OA in mussels and sea urchin.
599 Under pH 6.8 and pH 7.9, the strong signal suppression for AZA1 in all matrices may
600 explain the problems with trueness. Moreover, signal enhancement under pH 6.8 may
601 explain the recoveries over 110% for SPX1, while signal suppression for GYMA in sea
602 urchin and for SPX1 in clams correlated with insufficient recoveries.
603
604 The statistical analysis showed that matrix effects were species dependent for YTX and
605 GYMA (Figure 3): all seafood matrices enhanced YTX signal in the LC-MS/MS, but
606 the signal promotion was significantly lower ($p < 0.001$) in mussels than in the rest of
607 the matrices tested. GYMA signal suppression was significantly higher ($p = 0.032$) in
608 sea urchin matrix than in mussel matrix.
609
610 We assessed the accuracy of the method with EXS and MMS (Table 7). Precision was
611 evaluated as the HorRat value for intraday precision and trueness was assessed as
612 recovery. We found that the calibration strategy of MMS improved method accuracy for
613 the determination of GYMA and PTX2, since both toxins showed low recoveries
614 (below 80%) when the spiked samples were quantified against an EXS curve. Figure 3
615 shows that GYMA tended to suffer from signal inhibition by seafood matrices, thus
616 MMS would be a suitable approach to get satisfactory recovery values for this toxin.
617 The recoveries of AZA1 and SPX1 were slightly higher when spiked samples were
618 quantified against a MMS curve. However, the use of MMS did not have a great impact
619 in the correction of matrix effects in the determination of these toxins. Okadaic acid
620 tended to show a strong signal enhancement influenced by seafood matrices; this
621 observation agreed with the literature [11, 14, 21, 23]. The recoveries found for OA
622 when the spiked samples were quantified against an EXS curve ranged from 114%
623 (when 80 µg/kg were spiked in clams tissue), to 225% (when 240 µg/kg were spiked in
624 mussels tissue). The use of MMS drastically dropped the recovery values for OA,
625 ranging from 61% (240 µg/kg OA spiked in oyster) to 104% (160 µg/kg spiked in
626 mussel), and being over 70% in most of the cases. In the case of hydrolyzed samples,
627 the recovery of OA in hydrolyzed extracts decreased following the same trend.

However, only hydrolyzed mussel samples had good recoveries with MMS, the recoveries for other seafood species were below 80%, although MMS did not noticeably affect precision. Regarding YTX, recoveries drastically decreased with MMS compared to those found with EXS, which were extremely high during this experiment. Nevertheless, only the results for mussels were accurate, since MMS negatively affected precision for YTX and the variation among injections was too high to provide reliable results.

The species dependence of matrix effect may determine if MMS prepared in one species can compensate matrix effects for other species, but the previous studies on the topic did not reach a consensus. Gerssen *et al.* [13] proved that the MMS prepared in blank mussel extract can be used for matrix effect correction even in other seafood matrices, since the influence of the species in the method was negligible. On the other hand, several studies claimed that matrix effects seem to be species dependent. Stobo *et al.* [17] found that matrix effects varied depending on the type of seafood matrix, even for the same toxin. For example, signal suppression for AZA1 was more evident for king scallop than for mussels, cockles and oysters matrices. Kilcoyne and Fux [24] found that the differences in recovery of OA in spiked samples of several seafood tissues were statistically significant. Moreover, the degree of suppression of the AZA1 signal was also species dependent, and the article even warned about the possibility of differences in matrix effects between samples of the same species but collected in different locations due to differences in the diet and physiological state of the organisms. McCarron *et al.* [25] also highlighted the importance of finding a proper matrix to be used as a match in the MMS strategy.

Matrix effects in lipophilic toxins analysis have been extensively studied. Besides MMS, other groups have proposed several techniques to compensate matrix effects: solid phase extraction (SPE) clean-up [22,24], optimization of the chromatographic method [24], selection of the appropriate instrumentation [21,24], sample dilution [21], and standard addition [23,25]. All techniques their disadvantages, mostly related to the additional time and amount of standards needed, thus the selection of a proper strategy to deal with matrix effects is not trivial.

We demonstrated that matrix effects are species dependent for some lipophilic toxins in seafood, thus MMS may not be always suitable to compensate matrix effects under alkaline conditions. Besides, this strategy is more time and standards consuming than the EXS. In our laboratory of shellfish harvesting monitoring, we decided to use EXS as the quantification strategy, since we rarely analyze seafood samples with the toxins that benefit the most from the MMS (GYMA, PTX2 and AZA1). We correct OA recoveries (the most prevalent toxin in our study area) in mussels and oysters with the certified reference material of mussels naturally contaminated with OA, commercially available as CRM-DSP Mus b by the NRC (Canada), since the matrix effects for OA have been proved to be not species-dependent for crude extracts.

In the comparative study between quantification strategies, the low recoveries found for PTX2 using the EXS strategy (Table 7) were unexpected, since the in-house validation under pH 11 was performed with the same quantification strategy and the recoveries were satisfactory in that case (Table 6). The contradiction between both experiments, which were performed using the exact same method and spiked samples, might be explained by the number of replicates used: the in-house validation experiment assessed intermediate accuracy (four different spiked samples extracted in four days and analyzed by single injection), whereas the EXS strategy experiment evaluated intraday accuracy (one sample analyzed by triple injection in one day). PTX2 is rarely found in seafood matrices, since it is rapidly metabolized into PTX2-sa [32]. The analysis of YTX was very challenging, even under alkaline conditions. The poor precision of the method during the analysis with EXS strategy could explain the overestimation of YTX during this experiment (Table 7), which is contradictory with the recoveries found during the in-house validation process (Table 6). We expect that the routine application of the method and the definition of proper strategies for quality control, such as the participation in collaborative studies and the use of internal reference standards to correct recoveries, will help us to improve the quantitative determination of YTX in seafood samples. As a result of these experiments, we found indispensable to increase the number of replicates to achieve good accuracy in the analysis of YTX.

We highlight the selection of the mobile phase is a crucial step to implement the LC-MS/MS method: it affects chromatographic separation, sensitivity, LOQs, accuracy and matrix effects. We did not investigate the effect of LC conditions on the MS/MS behaviour, because we follow the recommendations stated in the EURLMB SOP and the amount of standards needed for that task is unaffordable by our laboratory. The impact of different elution conditions on tandem MS detection should be further investigated: mobile phase can affect the ionization yield and nature of precursor ions in the ESI source, but it may also alter the MS^2 spectra since ion fragmentation is not always independent of the ionization environment [33,34]. The next EURLMB SOP shall address this issue.

We consider unlikely that one single set of conditions could work perfectly for all toxin profiles and matrices, thus we would encourage the laboratories to include their priorities regarding toxin and samples types in the decision-making process to implement their methods. This concern has been faced before in marine toxin analysis: the suitability of HPLC methods for paralytic shellfish poisoning (PSP) depending on the toxin profile is already well known as one of the issues that are hindering the adoption of HPLC-FLD methods to replace the bioassays [35,36]. Nevertheless, the availability of different methods must be seen as a tool for the analyst to gain a better understanding of the marine toxins in environmental matrices.

4. Conclusion

The method based on LC-MS/MS for the determination of lipophilic toxins in seafood has been accepted by the European Union as a reliable technique to protect public health and reduce the use of animals for routine analysis. The EURLMB SOP [11] establishes a solid framework for the implementation of the LC-MS/MS method but it is not explicit enough concerning the chromatographic conditions and the matrix effect correction strategy that should be used. The current study is the first work aimed to compare the most common chromatographic alternatives for the determination of lipophilic toxins in seafood by LC-MS/MS in terms of functionality, quality criteria, validation parameters and quantification strategies under the same experimental settings (extraction and hydrolysis procedures, chromatographic column, MS instrument conditions, standards and reagents, and analyst). We chose the alkaline conditions, EXS calibration as quantification strategy, and recovery correction for OA with CRM-DSP Mus b to be implemented as the routine method. Alkaline conditions provide higher sample throughput, lower LOQs, and the best overall performance in terms of sensitivity and accuracy in the validation study. The EXS strategy combined with OA recovery correction by CRM-DSP Mus b demanded less time and standard investment and provided satisfactory results. The analysis of YTX was challenging and it is still being improved in our laboratory by increasing the number of injections, participating in collaborative studies and preparing internal reference standards to correct YTXs recoveries.

When selecting the best chromatographic conditions, factors such as the instrumentation available (regarding polarity switching, limits of detection, and sensitivity), the number of samples needed to analyze, the toxin profile and the sample matrices should be considered. The matrix effects should be examined carefully, especially when including a new toxin in the method or analyzing a new matrix. A proper selection process may be time and resources demanding, but we hope that this comparative study may serve as starting point to other laboratories implementing their own methods for lipophilic toxins determination in seafood by LC-MS/MS.

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Figure 1: Example of chromatograms of NRC CRM standard solution of AZA1, GYMA, OA, PTX2, and SPX1 at 50 ng/mL; YTX at 200 ng/mL, under four chromatographic conditions. The chromatogram for pH 11 was edited using samples from a collaborative study with homoYTX, 45-OH-YTX, 45-OH-homoYTX, AZA2, AZA3, and PTX2-sa; and using the CRM- Mus b with DTX1.

Figure 2: Comparison of external calibration curves of six lipophilic toxins under four elution system (different pH and buffer composition). Normalized for 5 µL of injection volume for pH 11 (*n*=2). Slope values are listed in Table 4.

Figure 3: Matrix effects (ME) under alkaline conditions, expressed as the ration between the slopes of a calibration curve prepared in methanolic seafood extracts against the slope of a calibration curve prepared in MeOH (*n*=3). ME > 1 means signal enhancement; ME < 1 means signal suppression; ME =1 means no matrix effect. * and # represent significant differences (Tukey Test, *p* value < 0.05)

Table 1: Optimized elution gradients for four chromatographic conditions for the analysis of six groups of lipophilic toxins.

pH 2		pH 6.8 and pH 7.9		pH 11	
Time (min)	% Mobile phase B	Time (min)	% Mobile phase B	Time (min)	% Mobile phase B
0	20	0	20	0	20
6	80	3	40	8	100
7.5	80	5	80	9	100
8	100	7.5	80	9.5	20
9	100	8	100	12	20
9.5	20	9	100		
12	20	9.5	20		
		12	20		

Table 2: Transitions monitored, dwell times, declustering potentials (DP), entrance potentials (EP), collision cell entrance potentials (CEP) and collisions energies (CE) for the detection of six groups of lipophilic toxins.

Toxin	Transitions (m/z)	Time (ms)	DP (V)	EP (V)	CEP (V)	CE (V)	Precursor ion
OA and DTX2	803.5 > 255.2	150	-115	-12	-46	-64	$[M-H]^-$
	803.5 > 113.1	150	-115	-10.5	-41	-68	
DTX1	817.5 > 255.2	150	-115	-12	-46	-64	$[M-H]^-$
	817.5 > 113.1	150	-115	-10.5	-41	-68	
YTX	1141.5 > 855.2	150	-60	-9	-54	-90	$[M-H]^-$
	1141.5 > 713.2	150	-60	-9	-54	-106	
YTX under pH 11	570.4 > 467.4	150	-75	-9	-54	-30	$[M-2H]^{2-}$
	570.4 > 396.4	150	-75	-9	-54	-30	
45-OHYTX	1157.5 > 855.2	150	-60	-9	-54	-90	$[M-H]^-$
	1157.5 > 713.2	150	-60	-9	-54	-106	
45-OHYTX under pH 11	578.4 > 467.4	150	-75	-9	-54	-30	$[M-2H]^{2-}$
	578.4 > 396.4	150	-75	-9	-54	-30	
homoYTX	1155.5 > 869.2	150	-60	-9	-54	-90	$[M-H]^-$
	1155.5 > 727.2	150	-60	-9	-54	-106	
homoYTX under pH 11	577.4 > 474.4	150	-75	-9	-54	-30	$[M-2H]^{2-}$
	577.4 > 403.4	150	-75	-9	-54	-30	
45-OHhomoYTX	1171.5 > 869.2	150	-60	-9	-54	-90	$[M-H]^-$
	1171.5 > 727.2	150	-60	-9	-54	-106	
45-OHhomoYTX under pH 11	585.4 > 474.4	150	-75	-9	-54	-30	$[M-2H]^{2-}$
	585.4 > 403.4	150	-75	-9	-54	-30	
SPX1	692.5 > 444.2	150	86	7	30	45	$[M+H]^+$
	692.5 > 426.3	150	86	7	30	45	
GYMA	508.4 > 202.4	150	60	8.5	25	55	$[M+H]^+$
	508.4 > 392.4	150	60	8.5	25	55	
	508.4 > 490.4	150	60	8.5	25	55	
PTX2 and 7-epi-PTX2	876.5 > 213.3	150	50	10	35	50	$[M+NH_4]^+$
	876.5 > 823.5	150	50	10	35	50	
PTX1	892.5 > 213.3	150	50	10	35	50	$[M+NH_4]^+$
	892.5 > 821.5	150	50	10	35	50	
PTX-2sa and 7-epi-PTX2sa	894.5 > 213.3	150	50	10	35	50	$[M+NH_4]^+$
	894.5 > 823.5	150	50	10	35	50	
AZA-1	842.5 > 362.3	150	75	12	40	70	$[M+H]^+$
	842.5 > 462.5	150	75	12	40	70	
AZA-2	856.5 > 362.3	150	75	12	40	70	$[M+H]^+$
	856.5 > 462.5	150	75	12	40	70	
AZA-3	828.5 > 362.3	150	75	12	40	70	$[M+H]^+$
	828.5 > 448.5	150	75	12	40	70	

Table 3: Average retention times in minutes (*n*=6) and average relative retention times (*n*=2; in brackets and *italic*) of six groups of lipophilic toxins.

	Retention times (min)			
	pH 2	pH 6.8	pH 7.9	pH 11
AZA1	7.9	6.6	6.3	4.7 *(1.0)*
AZA2				*(1.1)*
AZA3				*(0.9)*
OA-c	6.2	4.8	4.5	3.1
DTX1	7.0	5.9	5.7	3.7
DTX2	6.5	5.2	4.8	3.4
GYMA	4.3	4.4	6.0	5.8
PTX2	6.6	6.5	6.5	6.6 *(1.0)*
PTX2-sa				*(0.5)*
SPX1	4.9	5.5	6.4	6.3
YTX	6.4	6.2	5.9	3.8 *(1.0)*
HomoYTX				*(1.0)*
45-OH-YTX				*(0.84)*
45-OH-homoYTX				*(0.84)*

Table 4: Sensitivity of six groups of lipophilic toxins (slope of the calibration curve in methanol LCMS) and matrix effects (ME) under four chromatographic conditions, expressed as the ratio between the slopes of a calibration curve in methanolic seafood extracts against the slope of a calibration curve in methanol.

Sensitivity and matrix effects

	pH 2 (*n*=1)	**pH 6.8** (*n*=1)	**pH 7.9** (*n*=1)	**pH 11** (*n*=3)
AZA1				
*MeOH**	*644.38*	*466.11*	*386.35*	*798.85*
Mussel	0.87	0.62	1.03	0.97
Oyster	0.74	0.72	1.00	0.91
Clam	0.96	0.78	0.77	1.18
Sea Urchin	0.94	0.75	0.98	1.00
GYMA				
*MeOH**	*333.81*	*216.09*	*319.44*	*1010.84*
Mussel	0.86	1.19	1.13	0.91
Oyster	0.72	0.87	1.03	0.80
Clam	0.89	0.88	1.12	1.02
Sea Urchin	0.73	1.19	0.96	0.58
OA-c				
*MeOH**	*105.93*	*88.78*	*96.01*	*200.89*
Mussel	1.65	1.92	1.55	2.09
Oyster	1.54	1.10	1.07	2.31
Clam	1.49	1.09	1.01	2.65
Sea Urchin	1.45	1.59	1.16	2.04
PTX2				
*MeOH**	*141.74*	*35.29*	*195.02*	*739.35*
Mussel	0.98	1.27	1.16	0.97
Oyster	0.95	1.10	1.09	1.05
Clam	1.00	1.46	1.27	1.07
Sea Urchin	0.88	1.28	1.08	1.09
SPX1				
*MeOH**	*1157.07*	*607.84*	*957.77*	*2885.69*
Mussel	0.69	1.03	1.22	1.05
Oyster	0.87	1.38	1.17	1.07
Clam	0.81	1.37	0.92	1.21
Sea Urchin	0.77	1.28	1.19	1.19
YTX				
*MeOH**	*32.11*	*13.21*	*9.58*	*335.16*
Mussel	0.94	1.34	1.16	1.80
Oyster	0.90	0.93	1.34	2.57
Clam	0.99	1.13	1.25	3.08
Sea Urchin	1.03	1.18	1.03	2.79

MeOH*: Slope of calibration curve in methanol

Table 5: LOQs (µg/kg) evaluated with blank homogenized samples of each matrix spiked at the theoretical LOQs (*n*=3) for six groups of lipophilic toxins.

	LOQs (µg/kg)			
	pH 2	pH 6.8	pH 7.9	pH 11
AZA1				
Mussel	8.7	12.4	9.2	6.0
Oyster	6.8	13.9	8.3	10.9
Clam	5.6	29.8	7.7	9.7
Sea Urchin	7.6	15.5	3.1	4.6
GYMA				
Mussel	6.1	4.7	1.5	13.2
Oyster	7.0	7.8	3.4	5.7
Clam	11.2	19.2	1.7	10.9
Sea Urchin	18.4	6.4	2.4	3.4
OA-c				
Mussel	14.9	26.9	22.0	3.6
Oyster	15.2	29.2	18.3	6.8
Clam	8.7	21.1	19.1	7.1
Sea Urchin	10.5	25.5	21.7	8.5
PTX2				
Mussel	25.6	71.6	5.3	13.4
Oyster	22.2	52.9	27.2	15.6
Clam	23.3	36.7	11.0	10.2
Sea Urchin	24.8	85.8	13.1	8.7
SPX1				
Mussel	33.7	8.2	55.7	14.7
Oyster	17.4	6.2	15.7	14.3
Clam	5.9	22.8	9.4	3.4
Sea Urchin	7.9	16.4	2.4	1.8
YTX				
Mussel	377.1	> 300.0	> 300.0	36.0
Oyster	340.3	> 300.0	> 300.0	15.8
Clam	312.9	> 300.0	> 300.0	12.4
Sea Urchin	272.6	> 300.0	> 300.0	16.3

Table 6: Trueness as recovery (R, in %) and precision as HorRat value (no units) for six groups of lipophilic toxins. Average values of four replicates spread over four consecutive days quantified using an external calibration curve with single injection. n.q.: Not quantified because the batch did not meet the linearity requirements.

	AZA1				GYMA				OA-c				OA Hydrolized				PTX2				SPX1				YTX			
	pH 2	pH 6.8	pH 7.9	pH 11	pH 2	pH 6.8	pH 7.9	pH 11	pH 2	pH 6.8	pH 7.9	pH 11	pH 2	pH 6.8	pH 7.9	pH 11	pH 2	pH 6.8	pH 7.9	pH 11	pH 2	pH 6.8	pH 7.9	pH 11	pH 2	pH 6.8	pH 7.9	pH 11
Mussel																												
80 µg/kg																									*250 µg/kg*			
R (%)	55.3	n.q.	27.4	120.8	41.2	70.8	70.8	73.2	93.3	93.2	73.7	150.5	162.0	96.8	94.7	155.4	67.4	56.0	32.5	93.8	35.8	61.1	50.6	99.0	94.7	113.8	214.9	76.1
HorRat	1.4	n.q.	1.2	0.3	1.7	1.0	1.1	0.8	0.5	0.9	1.5	0.7	3.4	2.2	1.9	1.5	2.5	0.6	1.9	0.5	2.3	1.2	0.8	0.6	1.3	0.5	0.7	1.1
160 µg/kg																									*500 µg/kg*			
R (%)	84.3	23.7	73.7	124.1	73.7	110.1	83.2	82.1	143.7	116.3	94.4	158.4	140.5	100.2	85.5	136.7	96.4	n.q.	n.q.	93.3	71.6	122.0	64.5	111.6	69.0	n.q.	n.q.	42.7
HorRat	1.6	1.3	1.9	0.7	1.5	1.5	1.2	1.4	1.3	1.6	1.2	0.9	1.1	1.6	0.6	1.4	2.4	n.q.	n.q.	1.1	2.2	1.0	0.5	0.4	1.2	n.q.	n.q.	0.9
240 µg/kg																												
R (%)	79.3	30.6	59.1	104.7	55.7	99.0	94.8	74.3	116.3	99.2	96.9	120.7	100.7	77.7	86.0	104.7	89.8	n.q.	n.q.	68.7	68.9	135.0	73.2	94.7				
HorRat	1.1	1.4	1.0	0.7	1.3	1.1	1.4	1.0	1.1	1.0	1.4	0.9	0.7	0.7	0.8	0.9	0.7	n.q.	n.q.	1.1	1.0	1.0	1.4	0.8				
Oyster																												
80 µg/kg																									*250 µg/kg*			
R (%)	58.5	n.q.	33.7	119.5	68.2	67.6	64.2	81.0	97.9	61.8	50.0	134.9	77.1	33.9	42.0	99.4	80.6	90.9	40.9	102.4	51.7	65.2	49.2	94.2	54.3	46.8	131.5	55.0
HorRat	1.6	n.q.	1.3	0.4	1.8	1.3	1.3	0.5	1.3	1.5	2.3	0.5	1.4	1.7	1.7	0.5	0.6	0.6	1.7	0.4	1.6	1.8	0.9	0.9	2.1	2.0	1.4	1.0
160 µg/kg																									*500 µg/kg*			
R (%)	76.7	34.3	60.7	105.9	62.3	90.5	74.2	74.8	104.6	71.5	59.2	119.7	72.1	41.4	45.2	77.1	82.2	96.9	56.2	88.4	73.5	89.3	63.7	102.2	40.8	n.q.	n.q.	37.4
HorRat	1.3	1.8	0.5	0.3	1.4	0.5	0.4	0.6	1.0	1.3	1.7	1.5	2.5	2.5	2.6	1.9	1.2	1.5	1.1	0.5	0.4	0.1	0.4	0.4	1.0	n.q.	n.q.	0.8
240 µg/kg																												
R (%)	78.6	41.2	67.5	100.0	71.6	101.1	87.0	83.9	109.1	78.7	77.0	124.8	82.8	46.5	57.6	89.8	94.1	n.q.	n.q.	77.4	84.1	135.6	78.1	95.5				
HorRat	0.9	0.5	0.4	0.5	1.3	0.9	0.5	0.5	0.9	0.8	0.6	1.0	2.2	2.1	2.9	0.8	0.2	n.q.	n.q.	0.6	1.4	1.7	1.1	0.8				
Clam																												
80 µg/kg																									250 µg/kg			
R (%)	63.4	n.q.	35.8	116.6	51.6	76.2	34.0	79.7	80.9	81.2	51.2	135.6	131.5	74.8	59.4	112.3	53.3	81.6	37.6	95.6	34.1	49.5	46.1	76.5	65.0	73.9	164.1	47.8
HorRat	0.7	n.q.	0.7	0.4	0.5	1.3	1.5	0.3	2.8	1.3	1.7	0.6	2.4	2.4	2.9	0.9	1.7	0.4	1.4	0.5	1.9	1.6	1.4	1.0	2.6	3.4	2.2	0.8
160 µg/kg																									500 µg/kg			
R (%)	101.3	48.7	64.2	104.6	78.4	105.5	65.1	81.7	103.7	107.6	87.0	116.1	106.9	86.1	89.8	92.5	84.8	131.1	76.8	85.8	62.8	131.7	70.7	82.4	61.4	n.q.	n.q.	31.3
HorRat	2.8	1.1	1.3	0.4	1.3	1.0	2.9	0.2	2.5	1.7	2.2	1.3	0.9	1.5	1.8	0.9	2.4	1.5	1.5	0.5	1.0	0.7	1.3	1.0	1.2	n.q.	n.q.	1.1
240 µg/kg																												
R (%)	73.1	45.5	59.4	99.3	67.5	99.1	48.6	91.4	105.9	88.6	75.6	123.3	103.4	60.4	50.8	93.1	75.9	n.q.	n.q.	74.2	66.8	138.1	72.5	82.1				
HorRat	0.9	0.9	1.5	0.3	1.7	1.5	1.3	0.3	1.7	0.9	1.1	1.0	1.0	0.8	0.6	0.8	1.0	n.q.	n.q.	0.4	1.2	0.8	0.8	0.6				
Sea Urchin																												
80 µg/kg																									*250 µg/kg*			
R (%)	63.0	n.q.	37.8	121.3	60.6	72.8	55.5	69.8	83.6	72.2	48.6	140.9	80.5	46.1	44.3	148.0	75.5	43.8	45.3	100.6	59.7	84.4	40.9	74.6	37.5	54.4	106.2	71.1
HorRat	1.3	n.q.	1.6	0.4	1.4	0.5	1.6	1.1	1.2	1.1	2.2	0.9	0.8	1.2	2.4	1.3	0.9	2.2	1.8	0.3	1.0	0.4	0.6	0.6	1.5	1.3	1.1	1.4
160 µg/kg																									*500 µg/kg*			
R (%)	81.7	27.5	58.6	122.9	81.3	84.5	73.7	85.8	112.2	81.5	66.7	150.5	85.2	58.0	56.5	126.6	87.3	n.q.	n.q.	94.6	72.6	109.2	61.9	100.6	43.6	n.q.	n.q.	35.5
HorRat	1.1	1.5	0.3	0.6	0.7	1.0	1.0	1.3	0.4	0.9	0.3	1.6	0.5	1.5	1.4	1.7	0.7	n.q.	n.q.	0.3	0.7	1.1	0.4	1.4	0.6	n.q.	n.q.	0.8
240 µg/kg																												
R (%)	85.9	37.0	62.4	98.8	76.4	83.3	77.8	73.2	118.3	83.6	68.3	107.5	84.4	55.1	58.3	85.2	76.7	n.q.	n.q.	78.0	76.0	133.7	66.5	73.0				
HorRat	0.8	1.4	0.3	0.6	0.9	0.3	0.7	1.4	0.4	0.8	0.4	1.0	1.2	1.3	1.5	0.9	1.0	n.q.	n.q.	0.8	1.0	0.2	0.9	1.2				

Table 7: Trueness as recovery (R, in %) and precision as HorRat value (no units) for the determination of lipophilic toxins under alkaline conditions by triple injection with two calibration strategies: External Standard calibration (EXS) and Matrix Matched Standard calibration (MMS).

		AZA1		GYMA		OA		OA Hydrolized		PTX2		SPX1			YTX	
		EXS	**MMS**	**EXS**	**MMS**	**EXS**	**MMS**	**EXS**	**MMS**	**EXS**	**MMS**	**EXS**	**MMS**		**EXS**	**MMS**
Mussel																
80	R (%)	99.6	110.0	63.8	92.8	175.7	83.0	211.3	81.1	61.8	98.0	98.5	117.0	*250*	232.8	109.1
µg/kg	HorRat	0.4	0.5	0.8	0.4	0.7	1.1	0.3	0.1	0.3	0.2	0.3	0.6	*µg/kg*	0.2	1.3
160	R (%)	104.7	102.8	68.1	83.6	196.9	103.8	222.3	81.5	59.6	88.9	90.8	90.5	*500*	173.3	71.4
µg/kg	HorRat	0.6	0.4	0.4	0.4	0.4	0.3	0.7	0.1	0.3	0.4	0.4	0.5	*µg/kg*	0.7	0.5
240	R (%)	114.3	110.2	69.4	89.4	225.3	84.4	289.7	106.6	66.1	85.0	109.1	98.4			
µg/kg	HorRat	0.5	0.3	0.0	0.5	0.5	0.9	0.8	0.8	0.2	0.5	0.5	1.0			
Oyster																
80	R (%)	108.5	120.4	79.6	108.1	135.7	80.7	159.0	55.4	66.4	103.5	87.4	119.0	*250*	147.2	57.7
µg/kg	HorRat	0.3	0.7	1.0	0.4	1.0	0.2	0.8	0.9	0.5	0.9	0.3	0.6	*µg/kg*	0.3	1.7
160	R (%)	96.1	111.6	75.3	95.2	137.5	66.2	186.2	49.0	56.1	91.4	78.0	105.1	*500*	115.5	40.1
µg/kg	HorRat	0.3	0.3	0.7	0.5	0.1	0.7	0.8	0.8	0.0	0.7	0.4	0.2	*µg/kg*	0.5	1.9
240	R (%)	101.2	100.9	79.3	92.1	154.2	61.5	158.3	49.5	68.4	81.8	81.1	98.6			
µg/kg	HorRat	0.3	0.6	0.2	0.4	0.0	0.5	1.6	0.4	0.1	0.2	0.1	0.2			
Clam																
80	R (%)	92.5	103.1	69.6	90.8	114.5	68.1	143.1	53.8	64.5	89.3	67.9	88.3	*250*	130.8	59.1
µg/kg	HorRat	0.3	0.6	0.8	0.6	0.9	0.3	0.8	0.7	0.1	0.7	0.5	0.2	*µg/kg*	0.6	1.3
160	R (%)	97.9	114.6	77.3	101.3	143.1	70.5	186.2	43.4	78.5	92.9	78.8	93.1	*500*	94.3	39.0
µg/kg	HorRat	0.3	0.4	0.9	0.1	0.3	0.8	0.8	0.6	0.2	0.4	0.8	0.5	*µg/kg*	0.3	1.1
240	R (%)	112.5	101.4	87.2	95.5	173.1	84.3	166.2	40.2	79.5	89.0	90.5	91.0			
µg/kg	HorRat	0.3	0.3	0.3	0.2	0.6	0.1	1.8	0.1	0.7	0.6	0.4	0.7			
Sea Urchin																
80	R (%)	99.7	115.0	55.0	95.9	140.0	69.7	201.8	45.5	74.5	91.9	77.7	82.3	*250*	156.2	64.9
µg/kg	HorRat	0.5	0.5	0.7	0.8	1.0	1.0	1.0	2.0	0.2	0.4	0.4	0.3	*µg/kg*	0.7	1.7
160	R (%)	109.1	108.7	63.1	98.4	138.7	84.8	194.1	39.2	80.6	91.1	81.4	91.6	*500*	136.1	48.1
µg/kg	HorRat	0.5	0.8	1.3	0.2	0.1	0.4	1.3	1.4	0.3	0.5	0.2	0.7	*µg/kg*	0.7	1.1
240	R (%)	117.3	110.0	62.3	93.5	181.6	77.8	160.4	52.6	77.8	96.3	85.9	82.8			
µg/kg	HorRat	0.1	0.6	0.4	0.3	1.0	0.2	1.6	1.2	0.0	0.0	0.6	0.9			