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Lipophilic Toxins Analyzed by Liquid Chromatography−Mass Spectrometry and Comparison with Mouse Bioassay in Fresh, Frozen, and Processed Molluscs

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The search for alternative methods to the mouse bioassay (MBA) has intensified over recent years. The present work analyzes seven different species of shellfish (clams, small scallops, small clams, mussels, oysters, cockles, and edible whelks) in fresh, frozen boiled, and canned presentations using liquid chromatography−mass spectrometry (LC-MS/MS), and the results are compared with the same samples analyzed through MBA. The toxins studied were OA, DTX1, DTX2, YTX, PTX2, and AZA1, which are legislated in the EU, and SPX1, which is not regulated yet. Consistent results between LC-MS/MS and MBA were found in 69% of the samples, whereas 26% of MBA showed "false-positive" results with respect to the toxins analyzed. No "false negatives" were observed. The possibility of LC-MS/MS as an alternative or complementary technique to MBA is discussed.

KEYWORDS: LC-MS/MS; lipophilic toxins; DSP; MBA

INTRODUCTION

Marine phytoplankton, especially several species of dinoflagellates and diatoms, produce different types of phycotoxins that can enter into the food chain. Shellfish act as one of the main vectors transmitting them to humans. The frequency and intensity of harmful algal blooms (HABs), the number of new toxins detected, the geographical areas affected, and intoxication episodes are increasing (*1*). This is a great concern for public authorities and for the fishery industry because these toxic episodes have an important impact on the economy producers and on the public health (*1*−*6*).

Marine toxins are classified according to their effects on humans and also according to their water solubility. Several research papers have been published concerning the epidemiological data of different groups of toxins (*3, 6*). Eleven types of toxins have been identified, each being responsible for different symptoms and toxicological effects (*2, 7*−*14*): diarrheic shellfish poisoning (DSP), yessotoxins (YTX), pectenotoxins (PTX), azaspiracid shellfish poisoning (AZP), paralytic shellfish poisoning (PSP), amnesic shellfish poisoning (ASP), neurotoxic shellfish poisoning (NSP), cyclic imines, tetrodotoxin, ciguatera fish poisoning (CFP), and palytoxin. Our study includes lipophilic toxins belonging to the DSP, YTX, PTX, and AZP groups:

DSP is caused by okadaic acid (OA) and dinophysistoxins (DTXs). This group of toxins is produced by dinoflagellates that belong to the genera *Dinophysis* and *Prorocentrum*. In addition to diarrhetic symptoms, they are tumor promoters in animal test systems and induce apoptosis in several human cell lines.

YTX is produced mainly by the algae *Protoceratium reticulatum*, but it was also detected during a bloom of *Lingulodinium polyedrum* in the Adriatic Sea. There are no reports of human intoxication, and its regulation is based on results from animal experiments.

PTX arises from *Dinophysis* spp., and it appears always with toxins from the OA group, although they do not share the same mechanism of action as OA-group toxins.

For AZP it has been recently discovered that the genus *Protoperidinium* acts as a vector for azaspiracids (personal communication). It produces symptoms similar to those of DSP and also neurotoxic effects or breathing difficulties.

Industry demands the previous analysis of the raw material, which has to match the legal requirements. In the EU the maximum value of DSP toxins allowed is 160 μg of OA equiv/kg of shellfish for OA, DTX, and PTX present at the same time in edible tissues (equivalent to 40 MU/kg), YTX maximum level is 1 mg of YTX equiv/kg of shellfish, and AZP toxins maximum permitted level is 160 μg of AZA equiv/kg (see **Table 1**) (*14*−*16*). The reference method in the EU for detection of lipophilic phycotoxins in molluscs and other invertebrates, the mouse bioassay (MBA) based on Yasumoto's method, basically involves an intraperitoneal injection of shellfish extracts in mice followed by observation of mouse survival time (*15*−*18*). MBA has been used to protect consumers for the past decades, although it has some disadvantages because little or no information about the exact toxin composition of the sample is

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Table 1. Legislated Lipophilic Biotoxins and Legal Limits Allowed for Commercial Products in Some Countries (*48*)

biotoxins		maximum levels			
		EU/Chile/New Zealand	Australia	Japan/South Korea	USA
DSP toxins	OA + DTX + PTX	160 μg of OA equiv/kg	0.2 mg of OA equiv/kg	5 MU/100 g	0.2 ppm of OA + DTX 1
	AZA	160 μg of AZA equiv/kg			
	YTX	1 mg of YTX equiv/kg			

given. Also, it has been studied that for some toxins, intraperitoneal toxicity has little relation to oral toxicity and in addition to that, the awareness of employing animals for ethical reasons is increasing. Results of the mouse bioassay are affected by strain, gender, age, and weight of mice and nonbiotoxin components in shellfish extracts, such as free fatty acids, which can lead to either positive or negative results (*19*). False positives increase economical losses for the shellfish industry, and also it must be considered that this method is qualitative and does not give any information about the evolution of the toxic event (if it is increasing or if natural depuration processes are taking place). Nowadays, within the EU, it is a requirement to take all steps to refine, reduce, and replace (3R) the use of animals employed in bioassays and scientific experiments (*20, 21*). In this sense the EU allows the possibility of using alternative methods to MBA to be implemented in regulatory monitoring programs if they provide an equivalent level of protection to consumers and if they are validated according to an international protocol (*18*). Altogether, research has focused on the development of alternative techniques to MBA for DSP monitoring as screening methods (*22*−*26*) or chemical methods (*27*−*32*).

Nowadays, LC-MS/MS is considered to be a well-accepted technique for the quantitative determination of pesticides, drugs, and environmental contaminants, and it has been successfully used for phycotoxin detection and quantification due to its efficient toxin separation, high selectivity, high sensitivity (lower limits of detection than MBA), and accurate and precise quantification. Several monitoring laboratories employ LC-MS/MS as an alternative technique to MBA following an in-house validation of the method. However, different species of shellfish and also different preparations of the same species could interfere with quantification by LC-MS/MS methods (*33*−*36*). The use of internal standards enables the assessment of which matrix effects are influencing the quantification, but internal standards are not currently available for lipophilic marine toxins. Then, to investigate matrix effects in shellfish, each case must be studied individually.

This work establishes a comparison between results obtained by the reference method MBA and an alternative technique employing LC-MS/MS in several preparations of molluscs (bivalves and gastropods). To gain this objective we developed a LC-MS/MS method for the detection and quantification of OA, DTX1, DTX2, YTX, PTX2, AZA1, and SPX1, although SPX1 is not legislated in the EU (**Figure 1**). Different shellfish products were studied, including clams, cockles, edible whelks, mussels, small clams, oysters, and small scallops under different preparations (fresh, frozen, boiled, and canned with different sauces). Toxin profiles found in each case were described and compared with qualitative MBA results.

MATERIALS AND METHODS

Reagents and Standards. Chemicals used for the LC-MS/MS method were high-purity chromatography grade acetonitrile and methanol (Scharlau Chemie), analytical grade ammonia and formic acid (Scharlau Chemie), and purified water (Milli-Q System, Waters). For MBA, acetone (Analema) and Tween 60 (Roig-Farma) were employed.

Marine toxins YTX, PTX2, SPX1, and AZA1 were obtained from the National Research Council (NRC) Halifax, Canada. OA was obtained from Sigma, and DTX1 was obtained from Wako Chemicals (Osaka, Japan).

For LC-MS/MS calibration and quantification, stock solutions were prepared as mixtures in methanol.

Samples. Samples of shellfish species included in this work (mussels, oysters, cockles, clams, small clams, edible whelks, and scallops) were analyzed in different preparations, fresh, frozen, boiled, and canned. Most of the samples came from self-controls that industries carry out before selling their products. Their origins were mainly from Europe, and most of them came from Spain.

LC-MS/MS Preparation. *Extraction Procedure.* Extraction of the toxins for LC-MS/MS analysis was carried out employing the whole body of the different specimens previously shucked and homogenized with a homogenizer. Two grams of the homogenized sample was weighed, and 18 mL of 100% MeOH was added and mixed using a vortex mixer for 3 min at full speed and centrifuged at 2500*g* for 10 min at 4 °C. Then, 2.5 mL of the supernatant was evaporated to dryness with nitrogen at 40 °C, and the residue was resuspended in 1 mL of 100% MeOH, filtered through a 0.2 μm syringe filter (Waters), and injected into the LC-MS/MS.

Hydrolysis of Esters. For determination of the ester forms (DTX3) of OA, DTX1, and DTX2, 2.5 mL of the supernatant obtained after the centrifugation step described above was subjected to an alkaline hydrolysis procedure, based on that of Mountfort (*37*). Then, 313 μL of 2.5 M NaOH was added to 2.5 mL of the extract, and the vial was sealed and heated at 76 °C for 40 min, followed by neutralization with 250 μL of HCl (2.5 M). Final pH was checked and adjusted between 4 and 6. Then samples were evaporated to dryness with nitrogen, resuspended in MeOH, and injected into the LC-MS/MS as described above.

LC-MS/MS Analysis. The analysis was performed using a Thermo Finnigan LC-MS/MS (model LCQ Advantage), which consisted of an ion trap mass spectrometer detector, a syringe pump, and a data system.

Separations were performed by using a Phenomenex Luna C18(2) column (5 μm × 150 × 2 mm) regulated at 25 °C. The mobile phase consisted of acetonitrile/water (90:10) in channel A, 100% acetonitrile in channel B, and 100% water containing 20 mM ammonium and 500 mM formic acid in channel C.

Mobile phase composition was constant and consisted of 20% phase A, 70% B, and 10% phase C for all of the toxins included in the study except SPX1. In this case constant buffer composition consisting of 30% phase A and 70% phase B was used. The mobile phase rate was set at 0.2 mL/min, and the injection volume was 10 μL.

For each toxin, a selected reaction monitoring (SRM) technique was established for monitoring of product ions from selected parent ions of each toxin, and collision energies were optimized in each case (see **Table 2**).

The LC-MS/MS method applied in this work was based on that of McNabb et al. (*29*). These authors reported chromatographic conditions for separation of a wide range of DSP toxins using an acetonitrile gradient with acidic buffer.

In the present work LC-MS/MS detection was optimized for the toxins OA, DTX1, DTX2, YTX, SPX1, PTX2, and AZA1. Electrospray ionization (ESI) with positive and negative mode was employed depending on the toxin analyzed. MS/MS of selected daughter ions from the parent ions of each toxin was performed as observed in **Table 2**. The multiple reaction monitoring (MRM) technique achieves a high degree of sensitivity, although to provide the most sensitive detection for toxins included, the instrument was

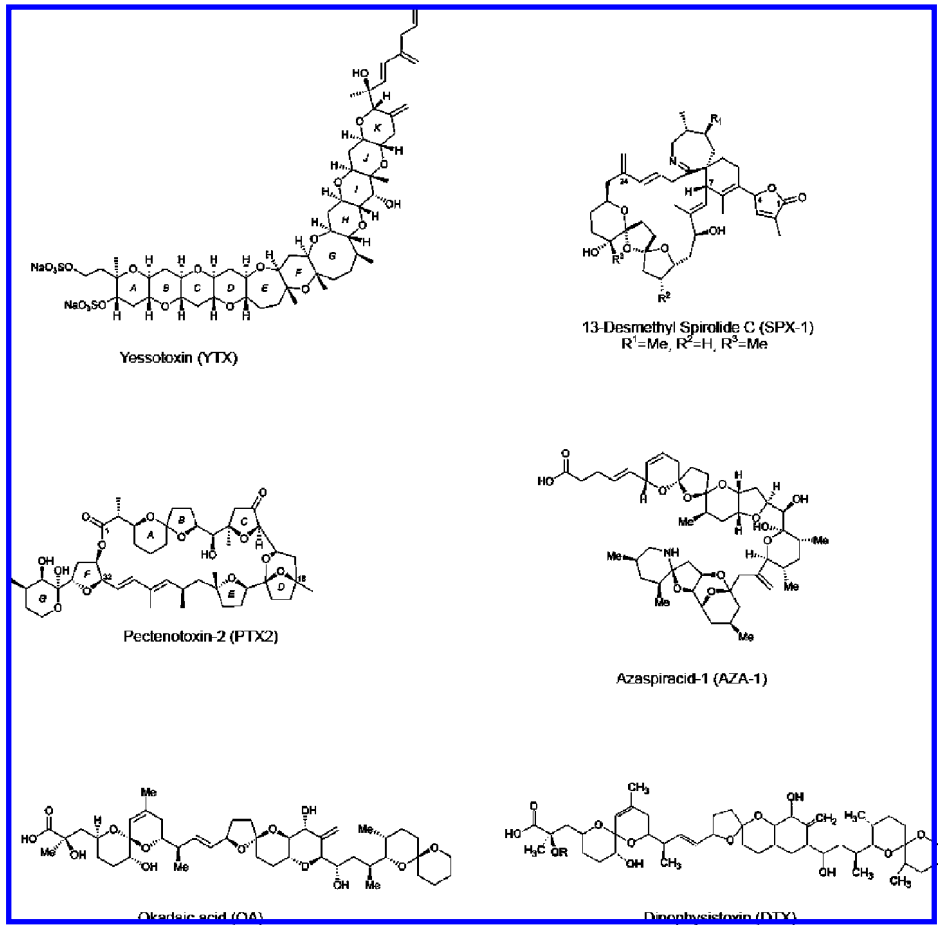

Figure 1. Structures of lipophilic marine toxins monitored using LC-MS/MS method: yessotoxin (YTX), 13-desmethylspirolide C (SPX1), pectenotoxin-2 (PTX2), azaspiracid-1 (AZA1), okadaic acid (OA), and dinophysistoxin (DTX).

Table 2. LC-MS/MS Parameters for Determination of DSP Toxins

phycotoxin analyzed	ion polarity mode[a]	MRM transition	RT[b]	collision energy (eV)
OA	ESI−	803.5 → 255.2 803.5 → 563.4 803.5 → 785.5	5.06 ± 0.03	38
DTX1	ESI−	817.5 → 255.2 817.5 → 563.4 817.5 → 785.5	8.56 ± 0.07	38
DTX2	ESI−	803.5 → 255.2 803.5 → 563.4 803.5 → 785.5	5.87 ± 0.05[c]	38
SPX1	ESI+	692.5 → 444.4 692.5 → 674.3	2.38 ± 0.01	92
PTX2	ESI+	876.5 → 823.5	6.73 ± 0.04	92
AZA1	ESI+	842.6 → 806.5 842.6 → 824.5	5.89 ± 0.07	43
YTX	ESI−	1141.5 → 924.5 1141.5 → 1061.5	6.93 ± 0.19	38

[a] ESI, electrospray ionization. [b] RT, retention time (minutes). Average ± standard deviation ($n = 12$ injections). [c] $n = 3$ injections.

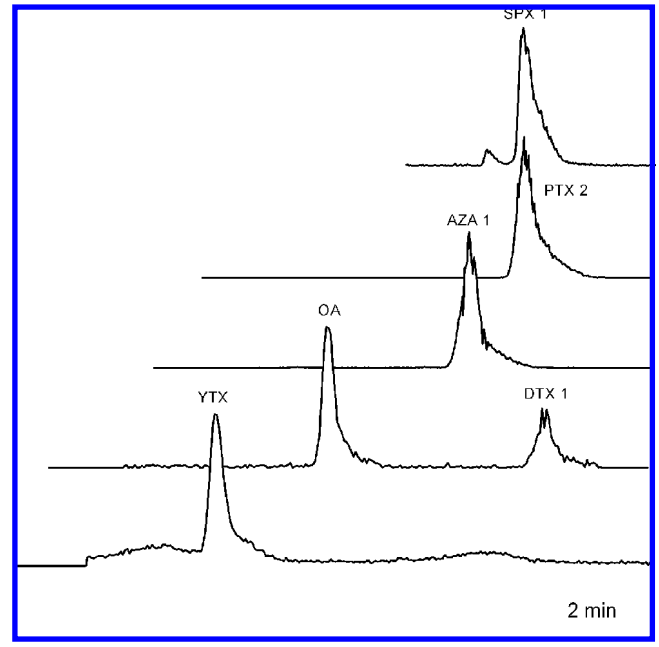

Figure 2. LC-MS/MS chromatograms for YTX, OA, DTX1, AZA1, PTX2, and SPX1. Detection was achieved using selected reaction monitoring (SRM) with mass transitions $m/z > m/z$ detailed in **Table 1**.

optimized. The optimization was done automatically by infusion of each standard into the ESI source using a syringe pump. This process was done by monitoring the precursor ion, and the final methods were built using the most intense product ions obtained in each case (**Figure 2**).

To quantify the toxins, standards of different concentrations were prepared from stocks solutions of OA, DTX1, and YTX, and different calibration curves were built in each case. Standards of OA and DTX1 were prepared at levels of 10, 20, 100, 150, and 200 ng/mL and for YTX at levels of 100, 500, 1000, 1250, and 1500 ng/mL. DTX2 was

Table 3. Recovery of Phycotoxins Added to Fresh Mussel Samples[a]

phycotoxin	recovery (%)
OA	92
DTX1	113
YTX	92

[a] Recovery (%) is representive of three extracts (by triplicate).

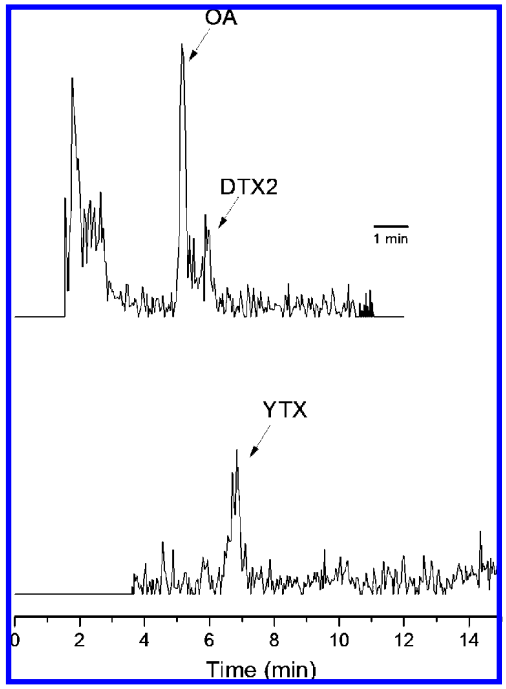

Figure 3. Chromatograms representing two positive samples containing OA, DTX2, and YTX.

quantified using the calibration curve for OA and assuming a relative response factor (RRF) of 1 (*14, 29*). Due to the low probability of finding PTX2, AZA1, and SPX1 these toxins were analyzed only for detection. Retention times are shown in **Table 2**.

The instrument control, data processing, analysis, and quantification were conducted by using Xcalibur software.

Mouse Bioassay. MBA was performed according the European Harmonized Protocol based on that of Yasumoto (*38, 39*). Acetone extraction of the whole flesh or the hepatopancreas of molluscs (according to the standard operating procedure for detection of okadaic acid, dinophysistoxins and pectenotoxins by mouse bioassay) was followed by evaporation and resuspension of the residue in a 1% solution of Tween 60 surfactant. One milliliter aliquots of the extract were ip injected into three male mice and observed for 24 h. The death of two of the three mice within 24 h was interpreted as a positive result. On the contrary, if none or only one of the mice died within this time, the test was considered to be negative (*15*−*18*).

RESULTS

LC-MS/MS Quantification. The recovery percentage was obtained by adding a standard solution to the mussel extracts, and each aliquot of each extract was measured three times (in triplicate). Then, analyses of spiked tissues with toxins have been repeated at least three times to check our method. For OA and DTX1, 200 μL of a standard solution of 1000 ng/mL was added on a mussel homogenate, and each aliquot was quantified in triplicate injections. For YTX 200 μL of a standard solution of 1250 ng/mL was added, and one aliquot was quantified in triplicate injections. Results were 92% recovery for OA and YTX and 113% for DTX1 as observed in **Table 3**.

Sample Analysis. The LC-MS/MS technique and MBA method were applied to samples selected for this study. Seven different shellfish species were analyzed: mussels (*Mytilus* sp.), cockles (*Cerastoderma* sp.), clams (*Tapes* sp., *Ruditapes* sp.), small clams (*Donax* sp.), oysters (*Crassostrea gigas*), small scallops (*Clamys varia*), and edible whelks (*Buccinum undatum*), including not only fresh and frozen products but also different types of processed products; 32% of the samples analyzed were canned (see **Table 4**).

LC-MS/MS. LC-MS/MS was employed to detect the presence of toxins in the extracts obtained following the extraction method described. The presence of any of the toxins in a sample was considered when a peak was detected with the expected mass to charge ratio at the expected retention time. This was confirmed by performing MS/MS mode and comparing spectra obtained with standards under the same conditions. In the samples studied we detected OA, DTX2, DTX1, YTX, PTX2, and AZA1. Although recent studies have reported the presence of SPX1 in Spanish waters (*40*), it was not found above the LOD for any of the samples analyzed. OA was the most prevalently found toxin, appearing in mussels, cockles, clams, and scallops, followed by DTX2 (in mussels, cockles, and small scallops), YTX (in mussels), and finally AZA1, which appeared only in small scallops. Any of the studied toxins was detected in small clams, oysters, and edible whelks.

OA, DTX1, and YTX quantitation was carried out by comparing the area of the peak at the considered *m/z* (803.5 for OA and DTX2, 817.5 for DTX1, and 1141.5 for YTX) as explained above. These positive samples were fresh, boiled, and canned mussels and also canned cockles. No sample was positive for YTX above the legal limit (YTXs > 1000 μg/kg), although this toxin was found in fresh mussels. A total of 12 samples (mussels, cockles, clams, and small scallops) had some of the quantified toxins (OA, DTXs, and YTX), although at levels under the legal limits allowed in the EU. AZA1 appeared in four samples of small scallops; in three of these samples was also found OA. Finally, in 40 of 78 samples none of the analyzed toxins were detected. **Figure 3** shows a chromatogram of positive samples for OA, DTX2, and YTX.

MBA. Results employing MBA showed 31 positive samples; at least two mice died within 24 h after ip injection of the samples. These positive samples were 20 samples of mussels, 6 of cockles, 3 of small scallops, and 2 of clams. The other 43 samples analyzed were negative as shown in **Table 5**. There are four samples for which we cannot quantify the amount of toxins present because we did not quantify AZAs (the bottom section of **Table 5**). Then, from 78 samples analyzed we found 69% matches, 26% "false positives", and 5% of samples that cannot be assigned to any group.

DISCUSSION

This work deals with marine biotoxins detection by LC-MS/MS and MBA in fresh, frozen, and processed samples of molluscs and compares results obtained with both methods. There are some previous works that study marine biotoxins detection and quantification by LC-MS and LC-MS/MS (*27*−*32*); these studies analyzed a limited number of fresh species. On the contrary, in this paper seven different species of molluscs and several presentations, fresh, frozen, boiled, and canned with different sauces, were analyzed.

Comparison between MBA and LC-MS/MS has been studied before by other authors, but these previous studies used only samples from Japan and Scotland. Moreover, none of these reports analyzed canned samples, and the method used for MBA was not based on the European harmonized protocol (*41, 42*). This paper studies molluscs from different European places and

Table 4. DSP Toxin Profiles Found in Bivalves Studied

commercial sample	product presentation	samples	phycotoxin detected	range[a] (μg/kg)
mussels (*Mytilus* sp.)	fresh	27	OA	45−567
			DTX2	141−276
			YTX	230−734
			PTX2	detected*
	frozen	2	OA	55−85
	boiled	7	OA	48−58
			DTX2	<LOQ−204
	canned in hot pickled sauce	2	none	<LOD
	canned in pickled sauce	5	OA	<LOQ−643
			DTX2	<LOQ−281
	canned in brined sauce	2	OA	<LOQ−199
			DTX2	<LOQ
cockles (*Cerastoderma* sp.)	fresh	1	OA	<LOQ−108
	boiled	2	none	<LOD
	canned in brined sauce	13	OA	104−1929
			DTX2	37−625
clams (*Tapes* sp., *Ruditapes* sp.)	frozen	5	OA	<LOQ
	canned in brined sauce	1	none	<LOD
small clams (*Donax* sp.)	fresh	1	none	<LOD
oysters (*Crassostrea gigas*)	fresh	4	none	<LOD
small scallops (*Chlamys varia*)	frozen	5	OA	<LOQ
			DTX1	<LOQ
			DTX2	<LOQ
			AZA1	detected*
	canned in scallops sauce	2	none	<LOD
edible whelk (*Buccinum undatum*)	frozen	1	none	<LOD
total		78		

[a] LOD = 2 μg/kg. LOQ = 36 μg/kg. *, detected but not quantified.

Table 5. Comparison between Toxicity Obtained by MBA and LC-MS/MS of the Samples

Matches between MBA and LC-MS/MS	
positive	11
negative	43
total	54

MBA, False Positives	
LC-MS/MS, no toxins detected	8
LC-MS/MS, toxins detected, OA + DTXs < 160 μg/kg; YTXs < 1000 μg/kg	12
total	20

LC-MS/MS, AZA-1 Detected	
MBA, positive (LC-MS/MS, OA detected)	3
MBA, negative (LC-MS/MS, OA not detected)	1
total	4

compares MBA (following the European harmonized protocol) with LC-MS/MS to determine the possibilities of employing it as a complementary or alternative technique to the EU reference method. These studies are required to make progress in the implementation of analytical methods in marine biotoxins monitoring.

In our work a high degree of correspondence between MBA and LC-MS/MS was found. As we can see in **Table 5** we found 54 of 78 consistent results, 11 positive samples and 43 negative samples, with both methods. This represents a 69% agreement between LC-MS/MS and MBA. On the other hand, we detected 26% of what is called in other studies "MBA false positives" with respect to OA, DTX, YTX, PTX2, and AZA1. This group of false positives is divided into two groups depending on the toxins detected. One group consists of eight samples in which none of the toxins analyzed by LC-MS/MS were detected; these samples were three fresh mussels, two frozen clams, one canned clam, and two canned mussels in pickled sauce. The other group consisted of 12 samples in which lipophilic toxins detected were under the legal limit. In both cases the positive results obtained with MBA might be due to the presence of other toxins not analyzed by LC-MS/MS or, alternatively, the presence of more than one toxin under the legal limit, although they could be detected by MBA. In this context other analogues of PTX (PTX1), YTX (45 OH YTX, homo YTX, and 45 OH homo YTX), and AZAs (AZA2 and AZA3) are legislated, but no reference materials for detecting these toxins are avaliable yet. Also, it is known that palitoxin, brevetoxin, gymnodimine, spirolides, pinnatoxin, and ciguatoxin give a positive response in MBA, although they are not currently regulated by the EU (*14*).

In addition, in canned mussels the substances used for the manufacture of some sauces might interfere with results, causing false positives (unpublished data). The European Food Safety Authority (EFSA) has recently published a compilation of results

concerning only OA-group toxins, and 29% of MBA false-positive results were detected. On the contrary, also 13% of tested samples were negative in MBA, although they exceed the regulatory limit for OA-group toxins (*14*). However, in our work no MBA false negatives were detected.

Samples with AZA1 must be considered separately. Although some authors consider that azaspiracids accumulate mainly in the digestive gland (*43*), others have demonstrated that azaspiracids can migrate to other tissues (*44*). In our study four samples of frozen small scallops, all from the same geographical origin, presented AZA1. This toxin has been detected before in several European countries (*45*). Of the four samples, only one gave a negative result in MBA, maybe because the level of AZA1 present was not enough to kill the mice. In addition, three samples that gave a positive result in MBA had also OA, but at very low levels. This is probably due to the synergistic effects that toxins may have on the MBA results (*14*). In this sense there is a lack of information related to synergy among toxins. A study carried out by Dragunow et al. (*46*) showed that Neuro2a cells pretreated with 10 μM concentrations of gymnodimine showed significant vulnerability to okadaic acid. More studies about the additive, synergistic, or antagonistic effects of lipophilic toxin co-occurrence on MBA and on human health are required.

Several publications dealt with LC-MS/MS as the method of choice to detect and quantify marine biotoxins. Many authors considered this technique to be a suitable alternative to MBA. In some countries where testing of shellfish has been traditionally based on MBA, such as New Zealand, new methods based on LC-MS/MS are now in use (*29*). Also, some EU Member States are currently using LC-MS/MS data to supplement information generated by MBA by parallel testing (*14*).

One of the main advantages of the LC-MS/MS method is that its high sensitivity helps to interpret MBA results. This is clear in our study where some MBA negative samples contained at least one toxin under the legal limit of commercialization. On the other hand, some MBA positive samples had a mixture of two different toxins. The LC-MS/MS method does not present ambiguous results because it helps to resolve samples below and approaching the toxic thresholds. In these cases the accurate quantification of the toxins will serve to maintain in the market those samples close to the legal limit that gave positive results in MBA. Another advantage of LC-MS/MS is the possibility of differentiating some toxins such as YTXs and PTXs that give positive results in the traditional MBA used for their detection but may be of limited public health significance (*45, 47*). Also, it must be considered that OA is identified as a tumor promoter in rodents, and in this sense it is important to assess the health risk of shellfish consumption; however, MBA is not able to detect OA-group toxins below 160 μg of OA equiv/kg of shellfish meat. In addition, MBA is not able to detect ester forms (DTX3) of OA, DTX1, and DTX2 (*14*). These cases will be solved by employing a differentiating and quantitative technique such as LC-MS/MS, although its main disadvantage is the lack of some standards. Therefore, to improve this technique, continuing efforts are required to increase the range of certified reference standards available for marine biotoxins.

MBA's main advantage is that it provides a measure of total toxicity based on the biological response of the animal to the toxin. Then it protects human health, although this current European reference method can be validated for only some parameters (qualitative method) and not so extensively as the LC-MS/MS method because it is influenced by a large number of variables that should be controlled. MBA clearly gives an extra protection to the consumer because known toxins as well as unknown toxic compounds could be detected (*19*). However, this extra protection could also act as a shortcoming, causing economical loses to the producers as it could report false positives (*1*). Altogether, considering the advantages and shortcomings of MBA, this method needs to achieve a difficult balance between consumer protection and economical considerations. Taking into account recent EFSA conclusions, the development of LC-MS/MS methodology, among other methods, is very promising because it has a great potential to be an alternative or complementary technique to the mammalian assays (*14*).

ABBREVIATIONS USED

ASP, amnesic shellfish poisoning; AZA1, azaspiracid-1; AZP, azaspiracid shellfish poisoning; CFP, ciguatera fish poisoning; DTXs, dinophysistoxins; DSP, diarrhetic shellfish poisoning; equiv, equivalents; EU, European Union; HABs, harmful algal blooms; ip, intraperitoneal injection; LC-MS/MS, liquid chromatography−mass spectrometry/mass spectrometry; MBA, mouse bioassay; NSP, neurotoxic shellfish poisoning; OA, okadaic acid; PSP, paralytic shellfish poisoning; PTX2, pectenotoxin-2; SPX1, desmethyl spirolide-c; YTX, yessotoxin.

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