



CHAPTER 1

Analysis of marine toxins: gaps on food safety control of marine toxins

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Analysis of marine toxins and gaps on food safety control

The field of marine toxins has been deeply studied in recent decades, but there are a lot of variables that need to be understood. These relate to the occurrence of harmful algal blooms, the production of different analogues of the same group of toxins, their accumulation and biotransformation in shellfish or in fish, and the associated hazards for human consumers, both from acute and long-term exposure.

Different factors are related to the proliferation of a determined alga in a zone: physical considerations, such as temperature or light, chemical parameters like nutrients, oxygen or pollutants, and biological relationships between kinds of algae and shellfish. These proliferations can develop suddenly, due to the germination of cysts from ocean sediments under appropriate environmental conditions, (Camacho *et al.*, 2007) and are referred to as harmful algal blooms (HABs) if marine toxins are detected during them.

Human poisoning during HABs has occurred in the past, modifying habits of populations in coastal and tropical areas. For example, Native Americans from the west coast of North America did not eat shellfish when bioluminescence was observed in the sea, because this phenomenon was related to a toxic algal bloom (Hallegraeff, 2004). Nowadays, it has been described an increase in the frequency, the intensity and the duration of HABs, and also an occurrence in areas where they had not been described in the past (Hallegraeff, 2004). For example, ciguatoxins and palytoxins have appeared in temperate waters some years ago (Perez-Arellano *et al.*, 2005; Ciminiello, 2006; Aligizaki *et al.*, 2008; Boada *et al.*, 2010; Amzil *et al.*, 2012), while tetrodotoxin was described as the source of a human intoxication in Europe (Rodriguez *et al.*, 2008) and azaspiracids were detected in Morocco or Portugal (Vale *et al.*, 2008; Elgarch *et al.*, 2008).

The factors involved in these changes are not completely known. Human activity and climate change have influenced, through the eutrophication of coastal waters (due to fertilizers, pollutants, organic matter), global warming and the transport of toxic algae from endemic to non-endemic areas in ship ballast waters or by new marine



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currents. Also, contaminated bivalves can harbour viable cells or cysts when they are transported to non-contaminated areas (Camacho *et al.*, 2007; Hallegraeff, 2004; Van Dolah, 2000; Moore *et al.*, 2008).

Prediction of HABs is not possible at the moment, this task being one important objective for many investigation groups around the world (Hallegraeff, 2010). Another problem for controlling HABs is the continuous detection of new analogues and toxins, followed by the description of new toxigenic algal species. This happened with *Azadinium spinosum* some years ago (Krock *et al.*, 2009; Tillmann *et al.*, 2009).

Marine toxins are natural metabolites produced during HABs. Their functions in the producer organisms have not yet been determined, but they develop very specific activities in mammals, such as binding to different cation or anion channels. Much hypotheses have been postulated relating these molecules to the interaction with prey or predators, or the gain of territories (Botana *et al.*, 1996). Different classifications of marine toxins have been established, based on their chemical structures, their physical properties or their mechanisms of action. However, the continuous isolation and description of new analogues and new groups of toxins makes the establishment of a definitive classification difficult.

Initially, marine toxins were classified in different groups depending on their toxic symptoms in humans, defining five groups of toxins: paralytic, neurotoxic, diarrheic, ciguateric and amnesic. Since then, new toxins with other symptomatology have been described, like azaspiracids, cyclic imines, yessotoxins, pectenotoxins, tetrodotoxins or palytoxin, so other classifications (see Table 1.1) have been established.

Contamination of shellfish and fish due to HABs generates problems for human health and economic losses, due to ecological damage and closures of harvesting sites. To reduce these impacts, monitoring programs have been implemented in many countries, with sampling of shellfish and phytoplankton, and specific legislation has been established, with regulatory limits for each toxin based on the available scientific data (EU Reg 853/2004; EU Commission Reg 786/2013). Although the consumption of contaminated seafood is the most important human exposure route, inhalation and skin contact during recreational or occupational activities must be considered in certain cases (Backer *et al.*, 2005; Fleming *et al.*, 2005), mainly for brevetoxins and palytoxins. Such effects usually appear when algal scum is observed.

Risk assessment of shellfish toxins is required to establish regulatory limits in shellfish for human consumption. In this sense, some considerations can be listed, such as

Table 1.1 Example of classification of marine toxins.

Group	Toxins
Paralytic toxins	Saxitoxins, Gonyautoxins, N-sulfocarbamoyl-11 hidroxysulfatotoxins
Diarrheic toxins	Okadaic acid, dinophysistoxins
Lipophilic toxins	Pectenotoxins, yessotoxins
Amnesic toxins	Domoic acid and analogues
Neurotoxic toxins	Ciguatoxins, maitotoxins, brevetoxins, cyclic imines, policavernosides, gambierol
Palytoxins	Palytoxins, ostreocins, ovatatoxins
Azaspiracids	Azaspiracids
Other toxins	Tetrodotoxins



the presence of many analogues of the same toxin group in the contaminated samples, the metabolism of these toxins by predators generating other toxic molecules (Bricelj & Shumway, 1998; Suzuki *et al.*, 2005; Jauffrais *et al.*, 2012; Abraham *et al.*, 2012), the effect of the presence of one group of toxins in the toxicity of another one, or the fact that some compounds toxic to rodents have never been recorded as toxic to humans (Botana, 2012). The contribution of each component of a toxic mixture to the total toxicity must be studied, establishing toxicity equivalence factors (TEFs) (Aune *et al.*, 2007), together with the study of the absorption and distribution of toxins after an oral exposition. Moreover, the effects of toxin consumption at low levels (sub-regulatory) in long-term exposure must be determined.

Processing of shellfish samples must be studied, because it can produce changes in the levels or the distribution of toxins (CONTAM, 2009). In some cases, these changes can be associated to water losses, and they can be different for various analogues of the same group of toxins (McCarron *et al.*, 2008). In risk assessment, another important parameter is the standard portion, which had been fixed at 100 g. In recent years it has been determined that a portion size of 400 g would be more appropriate, to include high consumers (CONTAM, 2010), resulting in more restrictive regulatory limits (Paredes *et al.*, 2011).

Legislation must be revised when new toxins are detected in a specific region and they are not regulated (Perez-Arellano *et al.*, 2005; Aligizaki *et al.*, 2008; Rodriguez *et al.*, 2008), and also if the scientific data suggests that regulated toxins do not pose a risk for human health (EU Commission Reg 786/2013).

There are many methods available for detection and/or quantification of marine toxins, with different advantages and drawbacks, depending on the kind of samples to be analyzed (microalgae, seafood, sea water, etc.), the scope of the analysis (identification or quantification) or the number of samples to be analyzed.

Mouse bioassays (MBAs) have been used for many years for the detection and quantification of marine toxins in samples (AOAC, 1980). These assays vary depending on the kind of toxin to be analyzed, but they consist of the intraperitoneal injection of white mice with extracts obtained from the samples (Hess *et al.*, 2006; Botana, 2008). The death of two of the three mice tested in a determined time period means a positive result. This method has a number of disadvantages: its detection limits are near the regulatory limits; it is not specific (which means that each toxin from the sample cannot be individually identified and quantified); and non-toxic interferences can appear (Holland, 2008). From a practical point of view, these methods are expensive, because animals must achieve determined characteristics, such as a given weight, which means that they require housing. In addition, there are ethical issues related to animal welfare, because animals used are sacrificed at the end of the assay and suffer different cruel symptoms.

To guarantee a higher level of safety in commercial products, and to implement early warning systems, these bioassays must be substituted by alternative methods, which allow the identification and quantification of each one of the analogues of each group of toxins. Nevertheless, it must be considered that the mouse bioassay determines the overall toxicity of the compounds in the sample, whereas the alternative methods quantify each toxin group or even each analogue individually (Otero *et al.*, 2011). In the future, the mouse bioassays will be done for detecting new or unknown marine toxins, not for routine monitoring (EU Commission Reg 15/2011, 2011).

Development of these alternatives has been, and continues to be slow, mainly due to the scarcity of reference materials and standards, and the need for determining the



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toxicity of each analogue and/or its mechanism of action. However, there are a lot of methods designed for the detection and/or quantification of each toxin group, each having advantages and disadvantages (CONTAM, 2008a, b, c; 2009a, b, c, d, e; 2010a, b, c). Using these alternative methods can produce gaps in the analysis of emerging toxins, since they only detect their targets, not the unexpected toxins that can appear in the samples. This was the case of the first tetrodotoxin episode in Spain, with samples tested negative using Lawrence *et al.* (1995) and Oshima (1995) HPLC methods for PSP toxins (Rodríguez *et al.*, 2008). Nowadays, another important objective is the development of methods to screen many samples in the same assay, with the simultaneous detection of various toxin groups (Gago-Martínez *et al.*, 1996).

The initial need of substituting the bioassays (Hess *et al.*, 2006) was solved with the design of methods using liquid chromatography separation of the toxins, and fluorescence, UV or absorbance detection, which provide an analytical quantification of the toxins in a sample. If the target compound did not show this kind of signal, it was derivatized or followed various purification procedures. Some years ago, this initial option was completed and improved using mass spectrometry detection, which allows the quantification of each individual analogue (Quilliam, 2003) if reference materials and standards are available. This is not the current situation in many cases, so the concentration of one analogue is estimated with a reference material or another analogue of the same group of toxins (EU-RL-MB, 2011). This temporary solution can lead to false quantifications in some cases, since the signal obtained in mass spectrometry depends on the chemical structure of the compounds and can differ between analogues (Otero *et al.*, 2011). After this quantification, the total toxicity of the sample is calculated using the TEF of each analogue (Botana *et al.*, 2010), which is not available for all the toxic compounds described, due to the lack of standards.

In spite of the limitations of liquid chromatography-mass spectrometry, it has been selected as the official monitoring method for marine lipophilic toxins in shellfish samples in Europe (EU Commission Reg 15/2011, 2011). An example of this method for basic and acid mobile phases using HPLC appears in Table 1.2.

The MS/MS detection is performed in multiple reaction monitoring (MRM) mode using two transitions per toxin: OA and DTX-2 (m/z 803.5/255.0, m/z 803.5/113.0), DTX-1 (m/z 817.5/255.0, m/z 817.5/113.0), YTX (m/z 1141.5/1061.7, m/z 1141.5/855.0), 45-OH-YTX (m/z 1157.5/1077.7, m/z 1157.5/871.5), homoYTX (m/z 1155.5/1075.5, m/z 1155.5/869.5), 45-OH-homo-YTX (m/z 1171.5/1091.5, m/z 1171.5/869.5), PTX-1 (m/z 892.5/821.0, m/z 892.5/213.2), PTX-2 (m/z 876.5/823.4, m/z 876.5/213.2), AZA-1 (m/z 842.5/824.5, m/z 842.5/806.5), AZA-2 (m/z 856.5/838.5, m/z 856.5/820.5), AZA-3 (m/z 828.5/810.5, m/z 828.5/792.5).

With the exception of OA and YTX toxins groups, which are ionized in negative mode, the remaining lipophilic toxins are preferably ionized in positive mode. The transition with the highest intensity is used for quantification, while the transition with the lowest intensity is used for confirmatory purposes. According to the legislation (EU Reg 853/2004; EU Commission Reg 786/2013), the toxin amounts found in shellfish for human consumption must be lower than 3.75 mg eq YTX/kg, 0.16 mg eq AZA-1 and 0.16 mg eq OA/kg (for OA and PTX toxin group).

Alternatives to this analytical methodology have been designed, based on the biological receptors of the toxins (i.e. functional methods), or on the binding with antibodies. These kinds of methods do not identify analogues; they only indicate the presence of a group of toxins. However, these methods are easy to use and are cheaper than



Table 1.2 Chromatographic conditions of the multi-toxin LC-MS/MS method to detect the lipophilic marine toxins using acid and basic mobile phases.

	Chromatography with acid mobile phase			Chromatography with basic mobile phase		
Column	BDS-Hypersil C8, 50 mm × 2 mm, 3 μm particle size			X-Bridge C18, 150 mm × 3 mm, 5 μm particle size		
Flow	0.2 mL/min			0.25–0.4 mL/min		
Injection volume	5 μL			5–10 μL		
Column T	25 °C			40 °C		
Mobile phase	A: Water (both containing 2 mM ammonium formate and 50 mM formic acid)			A: Water (both containing 6.7 mM ammonium hydroxide (pH 11))		
	B: ACN (95%)			B: ACN (90%)		
	Time (min)	Mobile phase A (%)	Mobile phase B (%)	Time (min)	Mobile phase A (%)	Mobile phase B (%)
	0	70	30	0-1	90	10
	8	10	90	10	10	90
Gradient	11	10	90	13	10	90
	11.5	70	30	15	90	10
	17	70	30	19	90	10

liquid chromatography with mass detection, and functional assays also supply information about the toxicity of samples.

Functional assays use the mechanism of action of toxins for their detection and quantification. To design these assays, it is necessary to identify the receptor targets for all the toxins (Botana *et al.*, 2009), and to design the best detection method for the interaction between the receptor and the toxin, both in solution (Vieytes *et al.*, 1997; Alfonso *et al.*, 2005; Fonfría *et al.*, 2010; Otero *et al.*, 2011) or on some kind of surface (Pazos *et al.*, 2004; Fonfría *et al.*, 2007). One drawback of this methodology is the availability of the receptor, and the need to assure its stability during the assays. In this sense, the synthesis of cloned receptors would be useful (Campbell *et al.*, 2011).

Assays based on toxin recognition by an antibody can be used for the routine detection of marine toxins. The most important disadvantage of these assays is the need for large quantities of pure toxins to obtain anti-toxin antibodies (Hirama, 2005). These molecules show high specificity and sensitivity and, depending on its specificity, an antibody may be able to detect only one toxin or various members of a group of toxins. Nowadays, other methodology is being studied, such as the use of aptamers (oligonucleotides or peptides that bind to specific targets), which has many advantages compared to antibodies (Jayasena, 1999), and the same applications (Handy *et al.*, 2013).

In the field of detection methods, the main tasks that need to be solved are the validation of the alternative methods described, the multi-detection of several groups of toxins in a single assay (Fraga *et al.*, 2013; McNamee *et al.*, 2013), and the miniaturization and the portability for the on-site testing.



Gaps on food safety control for marine toxins by chemical methods

Marine toxins pose a significant food safety risk when they accumulate in shellfish, and adequate testing for biotoxins is required to ensure public safety and the long-term viability of commercial shellfish markets. In developed countries, there is a legislated specific requirement for regular and controlled analysis of seafood for the presence of phycotoxins. In Canada, South America and Japan, MBA is the method used for the official control of marine toxins, while in New Zealand, chromatographic methods are used for their monitoring programmes (Gerssen *et al.*, 2011).

From July 2014, in Europe, it is no longer possible officially to analyze for the presence of lipophilic marine toxins in shellfish by MBA (EU Commission Reg 15/2011, 2011). New regulations replaced the MBA for lipophilic marine toxin detection, which had been used up to now as the reference method, by the LC-MS/MS approach. This change has resulted in supporter groups and other groups against this law. There is also controversy and debate about whether the chemical method is an effective method to protect human health. Since this technique has been evaluated, and it is considered to be successful by many laboratories (Gerssen *et al.*, 2009; Blay *et al.*, 2011), this section highlights some gaps in the food safety control of marine toxins when they are monitored by chemical methods.

The legislated group of lipophilic marine toxins consists of four different chemical groups: okadaic acid (OA) and its derivatives, yessotoxins (YTXs) azaspiracids (AZAs) and pectenotoxins (PTXs). These are the most frequently toxins that have appeared along European coasts, together with the spirolides (SPXs), for which no legislation has yet been established.

Only few analogues of each group are legislated, and their quantity has to be referred to a predominant compound of the group, called the reference compound (RC). For instance, in the case of the AZA group, results should be expressed as milligrams of AZA-1 equivalents per kg of whole flesh, using TEFs for AZA-2 and AZA-3, and the other AZA analogues are considered of low relevance. To be able to determine the toxicity of a seafood sample by LC-MS/MS, the use of TEFs is necessary.

Similarly, the use of TEFs requires the knowledge of the toxicity of each analogue present in a sample in order to translate analytical data into total toxicity. However, the toxicity for some toxins varies considerably, depending on the administration route, oral or *i.p.* administration (Tubaro *et al.*, 2003; Munday *et al.*, 2008). Correct TEF values are necessary since, if the TEFs are not applied properly, this can greatly affect the final results. Oral toxicity studies provide information that compare better to human food poisoning, because they take into account the ratio of the absorption of the toxic molecule in the digestive system of mammals.

However, given the lack of sufficient information available in regard to the oral route, the European Food Safety Authority (EFSA) panel proposed TEFs based on acute effects after *i.p.* administration (CONTAM, 2009b). The limited toxicological information does not allow the setting of TEFs for the oral route for any of the toxin groups, and EFSA mentions that the TEF values should be revised when studies on acute oral toxicity data for the relevant analogues of each toxin group become available.

Despite the fact that the chemical method for lipophilic marine toxin detection has been developed as a multi-toxin detection method (Gerssen *et al.*, 2009; Blay *et al.*, 2011), it is only used for limited number of toxins, four analogues of YTX toxin group,



three analogues of each OA and AZA toxin group and two of PTX toxin group (11 or 12 toxins at most). This is one of the main drawbacks of the LC-MS/MS method in order to protect human health.

The number of compounds that can be analyzed in a single run is limited. LC-MS/MS technology is a targeted method, and the target toxins should be selected before the run. The toxins usually selected for LC-MS/MS analysis comprise the 12 regulated toxins, but outside of the monitoring there may be many additional analogues which may also be of toxicological relevance. For example, the OA toxin group exists outside of OA, DTX-1 and DTX-2; these toxins can also be present in the form of fatty acid-containing esters (Rossignoli *et al.*, 2011). The AZA group and PTX group both consist out of over 20 possible analogues (Gerssen *et al.*, 2010). For YTX group toxins, over 36 natural derivative analogues have been identified, and more than 90 analogues have been described (Miles *et al.*, 2005). However, for routine monitoring control, experts on biotoxins considered that is not practical to fully determine the presence of all toxin levels in shellfish. If no toxic adequate information about a analogue is available, it is proposed that new compounds present in shellfish at less than five percent of the RC should not be regulated (FAO/IOC/WHO, 2011).

The identification and quantification of marine toxins in order to protect human health has become the main part of the work based in HPLC, by coupling detection systems which measure the different physical and chemical properties of the molecule (Ciminiello *et al.*, 2011). The most common detection systems for lipophilic marine toxins are quadrupole (Q) and triple quadrupole (TQ) mass spectrometers (Otero *et al.*, 2011; These, *et al.*, 2011), ion trap (IT) (Rodriguez *et al.*, 2008), time-of-flight (ToF) (Meisen *et al.*, 2009) or hybrid instrument (IT-ToF; Q-ToF; IT-LC/ESI-MS/MS) (Ferranti *et al.*, 2009). Toxins are ionized in negative or positive mode, then separated by the m/z ratio and, finally, detected and registered in the chromatograms by converting the ion flow in an electric signal.

However, this signal is not always the same between different analyzers and MS methods. This fact can give rise to different toxin concentrations when a calibration curve is used (Otero *et al.*, 2011). As shown in a comparative study, the quantification of three lipophilic toxins – OA, DTX-1 and DTX-2 – was affected by several parameters, such as MS detection method, mobile-phase-solvent brands and equipment (Otero *et al.*, 2011). The study demonstrated that OA, DTX-1 and DTX-2 toxin amount was increased or decreased, depending on the MS detection method used to quantify. Two MS methods were used; one with the specific transitions for OA and DTXs (four transitions), while the other method included the transitions for six lipophilic toxins (a total of 10). Quantities analyzed by LC-MS/MS using the MS method with 10 transitions were considerably lower than those obtained by the method with four transitions. This meant that the number of transitions included in the MS methods, not fixed in the reference method (EU-RL-MB, 2011), affected the toxin quantification.

Figure 1.1 shows the OA quantification by a TQ instrument using three mobile phases composed by three acetonitrile brands and two MS methods. As can be observed, the toxin amount can be increased up to 42% or decreased up to 40%, depending on the ACN and mass method used. These results show that this method may result in large errors in the concentration of a toxic sample.

For example, a sample containing 200 $\mu\text{g}/\text{kg}$ would be detected as 120 $\mu\text{g}/\text{kg}$, hence negative. By contrast, a sample containing 120 $\mu\text{g}/\text{kg}$ of OA, considered as negative by EU regulations, can be quantified as 174.4 $\mu\text{g}/\text{kg}$. From the perspective of public health protection, the worst case is in which a positive sample for EU legislation, and

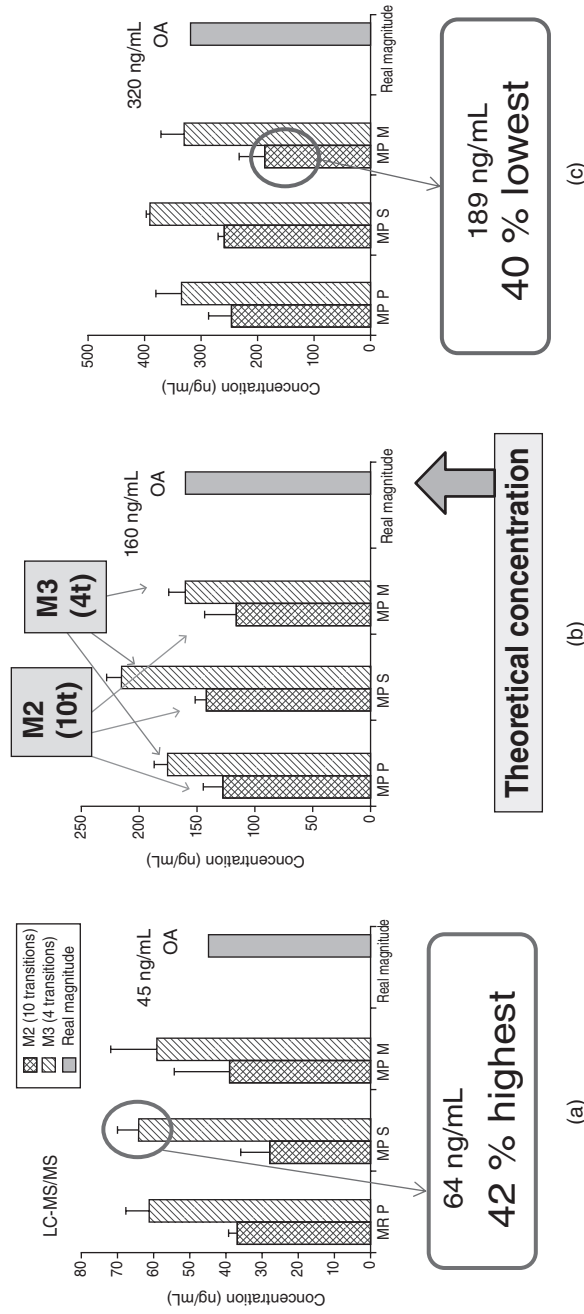


Figure 1.1 Effect of acetonitrile (ACN) from the mobile phase on the quantification of OA by LC-MS/MS. MP P: mobile phase containing ACN from Panreac. MP S: mobile phase containing ACN from Sigma. MP M: mobile phase containing ACN from Merk. Each toxin was quantified using two MS methods: an MS method that includes 10 toxin transitions; and an MS method that includes four toxin transitions. Mean \pm SEM of $n = 3$ experiments. Each graphic represent the concentrations in methanol for OA at 45 ng/mL; (a) at 160 ng/mL; (b); and 320 ng/mL (c). Black column: theoretical concentration of toxin. Open columns: toxin concentration obtained by each MS method.



thus toxic, reaches the market and intoxicates the consumers. This happened recently in Ireland, where several illnesses associated with DSP were reported following the consumption of frozen mussel meats. The implicated batches were removed from sale (https://www.fsai.ie/news_centre/food_alerts/SDLM_mussels_recall.html). The use of LC-MS/MS method to monitor the lipophilic marine toxin has been frequent in Ireland since 2001.

An important issue to have into account in the toxin monitoring is the LOD of the detection systems. Studies using four analyzers showed variability in the LODs for PTX-2 and OA, TQ-MS being the most sensitive mass analyzers (Gerssen *et al.*, 2008). In fact, the determination of some lipophilic toxins using IT-ToF detectors can be a problematic. Figure 1.2 shows the analysis of OA at different concentrations, using an ultra-high liquid chromatography (UPLC) method coupled to IT-TOF system. The separation was performed in a Waters Acquity UPLC[®] BEH C₁₈ (100 mm × 2.1, 1.7 μm) with an acid mobile phase, composed of water and acetonitrile 95% (both containing 2 mM ammonium formate and 50 nM formic acid) at a flow of 0.4 mL/min. This is a column, an acid mobile phase and a gradient usually employed for these toxins.

As it can be observed in the chromatogram of Figure 1.2a, OA standard at a concentration of 250 ng/mL eluted in 3.9 minutes. However, when 10 ng/mL OA is injected (Figure 1.2b), it is not possible to determine the peak, since it is confused with the noise. Also, despite the fact that the chromatographic conditions and method parameters were optimized, 1 ng/mL OA (Figure 1.2c) is not observed.

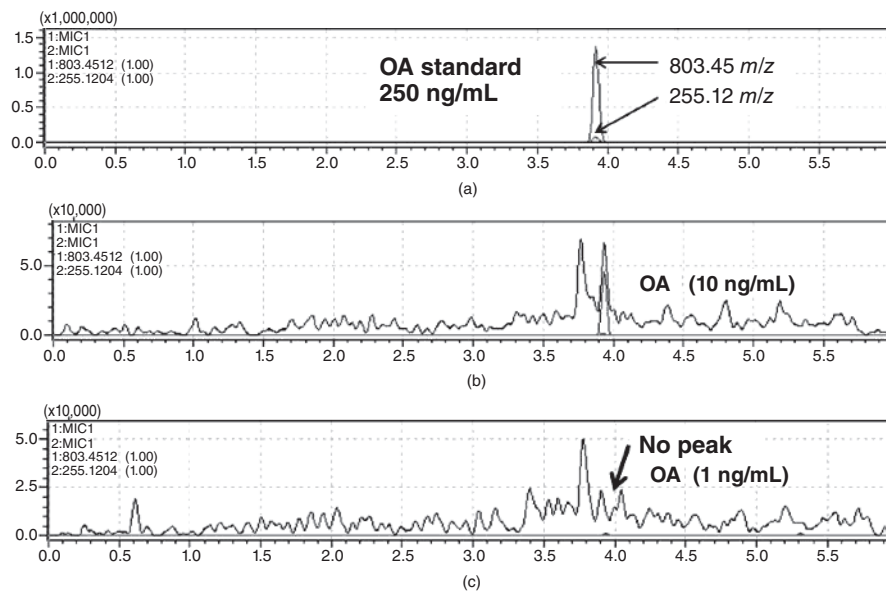


Figure 1.2 Chromatogram of OA standard at different concentrations: 250 ng/mL (a), 10 ng/mL (b) and 1 ng/mL (c), obtained in the LCMS-IT-TOF system from Shimadzu (Kyoto, Japan). The column used for the identifications was a Waters Acquity UPLC[®] BEH C₁₈ (100 mm × 2.1, 1.7 μm) with a mobile phase, composed of water (a) and acetonitrile 95% (b), both containing 2 mM ammonium formate and 50 nM formic acid. The mobile phase flow rate was 0.4 mL/min and the injection volume was 5 μL. Intensity units in counts per second (cps).



LODs and LOQs of the mass analyzers are important considerations, since the lipophilic marine toxin detection protocol is based in weighting $2.00 \text{ g} \pm 0.05 \text{ g}$ of tissue homogenate and, after an extraction procedure, the resulted supernatant has to be combined and made up to 20 mL of 100% methanol. With this protocol, the original sample is highly diluted and, therefore, sensitive methods are necessary in order to protect human health.

Use of standards

An important problem in the field of marine toxins is the lack of standards, needed in toxicological studies and also in routine quantification of shellfish and phytoplankton samples. Due to this, different kinds of materials are used in research and quantification, without a stated traceability, and sometimes obtaining unreliable data.

An example of this need appears in the European legislation (EU Commission Reg 15/2011, 2011), which establishes the use of a validated technique of liquid chromatography coupled to mass spectrometry for the routine detection of certain lipophilic toxins, 'both for the purposes of official controls at any stage of the food chain and own-checks by food business operators'. In this document, it is mentioned that other methods could be applied for this purpose if they fulfil the criteria stipulated by the European Union Reference Laboratory on marine biotoxins, but they should be intra-laboratory validated and tested under recognized proficiency test schemes. These activities could only be done using reference standards which guarantee the traceability of the final results.

Also, contradictory results in toxicology studies of marine toxins can be related to the use of different standards from various sources, or to the instability of these compounds in the solutions used during this kind of studies. For example, production of pectenotoxins by the same dinoflagellate as okadaic acid and dinophysistoxins led to the study of the toxicology of pectenotoxins using contaminated solutions (Miles *et al.*, 2004; Munday, 2008). Due to this, pectenotoxins were considered diarrheic compounds for many years. Other examples are different lethal doses obtained for the same compounds by different authors, using toxins obtained from various suppliers (Otero *et al.*, 2012; Munday *et al.*, 2012).

The terminology associated with reference materials and standards is large and confusing. Different names are used for different kinds of materials with various applications (see Table 1.3), but all of them can be used to guarantee the quality of the results obtained in a laboratory. This characteristic is influenced by several parameters, one of these being the traceability of the results, which is needed to establish their reliability and to compare measurements from different methods and places. These materials are also used for quality control, calibration of equipments and method validation.

Certified reference materials (CRMs) must be used for the complete development and validation of analytical methods (ILAC-G9, 2005) for the quantification of marine toxins. These kinds of materials guarantee the traceability of the measurements (ISO Guide 35, 2006), and their accuracy and must be produced and certified according to strict procedures that fulfil the requirements of ISO Guide 34 (2009) and 35 (2006), or similar. Characteristics such as stability, homogeneity or uncertainty, associated to the certified value, must be clearly described in the certificate of the material (ISO Guide 31, 2000) supplied by the manufacturer. Other parameters from the certificate must

**Table 1.3** Definitions associated to reference materials and standards.

Reference material (RM): <i>'material, sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process'</i> (ISO Guide 30 (2008)).
Certified reference materials (CRMs): <i>'reference materials characterized by a metrologically valid procedure for one or more specified properties, accompanied by a certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability'</i> (ISO Guide 30 (2008)).
Primary standard: <i>'standard that is designated or widely acknowledged as having the highest metrological qualities and whose value is accepted without reference to other standards of the same quantity, within a specified context'</i> (ISO Guide 30 (1992)).
Secondary standard: <i>'standard whose value is assigned by comparison with a primary standard of the same quantity'</i> (ISO Guide 30 (1992)).
Measurement standard (calibrant): <i>'realization of the definition of a given quantity, with stated quantity value and associated measurement uncertainty, used as reference'</i> (VIM, 2008).
Working standards: standards <i>'used routinely to calibrate or verify measuring instruments or measuring systems'</i> (VIM, 2008).

be considered for obtaining reliable results, like period of validity, conditions of storage or intended use.

There are two types of certified reference materials of marine toxins: solutions of one/more toxins in appropriate solvents with a determined concentration; and tissues contaminated with one/more toxins (matrix reference materials).

Matrix reference materials are used to perform recovery studies for complete method validations, to study sample pre-treatments (extractions, partitions, derivations, evaporations, clean-ups, etc.) and for quality control purposes. Their composition and their characteristics are similar to routine tested shellfish samples, so that the behaviour of the target analyte would be the same in both materials. Due to this, matrix reference materials must be used to calibrate methods sensitive to different types of matrix (ISO Guide 32, 1997), and their use provides an estimation of the percentage of recovery of a target analyte at the end of a series of procedures.

These kinds of materials are scarce, since their production is difficult. They can be obtained from contaminated natural tissues, or from non-contaminated natural tissues spiked with the target toxins. Once the initial material has been selected, homogeneity and stability of the toxin in the matrix must be assured, which is a difficult task due to the characteristics of the tissues. Another problem is the amount of materials needed, since all possible shellfish tissues (mussels, clams, oysters, cockles, etc.) can contain different groups and analogues of marine toxins. Also, the comparability of naturally contaminated and spiked tissues must be assured, since the availability of the native analyte during different procedures such as extractions can be different to the availability of the spiked analyte, which is free on the surface of the tissue.

If CRMs for a group of toxins are not available, purified materials can be used for certain applications. These materials have been characterized by different methods (fluorescence, absorbance, mass spectrometry, NMR, etc.), which guarantee their purity and quantity but, usually, other parameters, such as their traceability, homogeneity or the stability, have not been completely studied due to their limited availability.



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Also, control materials must be used to guarantee the quality of the measurements performed by each laboratory. These materials must be measured in every batch of routine samples and fulfil some requirements: an assigned value for the target toxin, a similar physical form than the problem samples, a demonstrated stability during large time periods and a high availability (Thompson & Wood, 1995). These materials can be prepared by each laboratory, matching the characteristics needed for a determined sample pre-treatment or quantification procedure, but they do not guarantee the traceability of the measurements performed, so their uses can be in quality assurance, recovery studies or checking of the stability of a system or an instrument (EURACHEM, 1998).

Commercial availability of marine toxins reference materials and standards is limited, due to the laborious procedures needed to obtain them. In a first step, toxins can be purified from contaminated shellfish or fish samples or from cultures of the producer microorganism, through a large chain of steps controlled by appropriate quantification procedures. Once the target compound is obtained, it must be characterized using complex analytical and statistical procedures that guarantee the traceability of the results, defining its quantity, purity, homogeneity, stability, the uncertainty associated to the measured values, and so on. Later, materials must be handled and stored using adequate methods and equipments, to maintain their characteristics. All of these activities need high technical knowledge and are time-consuming, so that suppliers of marine toxins are limited. Some years ago, only the National Research Council of Canada and the Japan Food Research Laboratory could provide these materials, but at present there is also a European supplier company, Laboratorio CIFGA, from Spain. These suppliers develop the activities previously listed and maintain adequate post-distribution services to guarantee that all of the information related to the production, characterization and use of the materials is available for their clients.

New risks in the EU

Marine algal toxins and the associated phytoplankton species are responsible for >60,000 toxicity incidents worldwide per year, with a mortality rate of 1.5% (Bourne, *et al.*, 2010). In Europe, episodes of human intoxication due to the consumption of marine toxins have been common in recent years, due to toxins which were not regularly monitored (Rodriguez *et al.*, 2008).

Climatic change is expected to affect food and feed safety, including seafood production. Climatologists and other experts in atmospheric and biological sciences point out that global temperatures are expected to increase between 1.8–5.8 °C by the late 21st century (Cáceres, 2012), and the highest temperature increases will be at high northern latitudes (Liu *et al.*, 2013). These predictions have been confirmed by long-term observations in European seas, where sea surface temperature rates have increased around 0.01 °C per year since the 1860s (Sarmiento *et al.*, 2010).

Moreover, the distribution of precipitation is expected to change, resulting in an increase in the number of extreme precipitation events, and even areas with decreasing precipitation (Liu *et al.*, 2013). It is likely that such changes will deeply affect different aspects of the structure and functioning of marine ecosystems. Blooms of dinoflagellates are estimated to occur more often (van der Fels-Klerx *et al.*, 2012). *Dinophysis* spp. dinoflagellate, which produces OA toxins, has been studied, and it was observed the occurrence of this dinoflagellate is increasing (van der Fels-Klerx *et al.*, 2012). If the



behaviour of *Dinophysis* spp. group is similarly to other dinoflagellates in the future, then the frequency of harmful algae blooms may also increase. This is also the case with *Alexandrium ostenfeldii* (*A. ostenfeldii*) dinoflagellate. This organism, which produces SPXs toxins, was originally considered a cold-water species (Gribble *et al.*, 2005), and was only described as a producer of neurotoxins associated with PSP (MacKinnon *et al.*, 2004). However, *A. ostenfeldii* has been present in recent years in temperate waters throughout the world. The presence of SPX in molluscs has increased considerably in Europe (Rundberget *et al.*, 2011).

Figure 1.3 shows the lipophilic analysis by LC-MS/MS of mussels purchased from markets originating in Galicia, Spain. Figure 1.3(a) and figure 1.3(b) show the chromatograms of the OA and SPX standards, respectively. As can be observed in Figure 1.3(c), the analysis of the mussel sample showed OA at 8.16 minute and SPX-1 (Figure 1.3.(d)) at 5.70 minutes at levels of 12.5 μg OA/kg and 41 μg SPX-1/kg. In the case of the OA, this amount is considerably lower than the maximum level

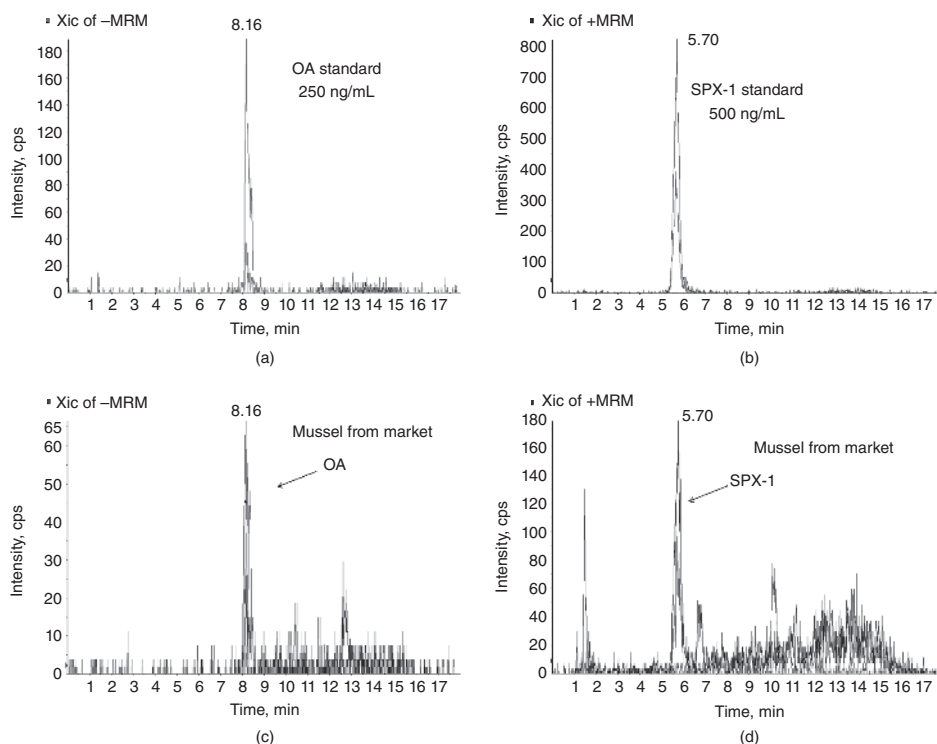


Figure 1.3 Chromatograms in negative mode of OA standard (a) and mussel from market (c). Chromatograms in positive mode of SPX-1 standard (b) and mussel from market (d). The equipment used was a HPLC system, from Shimadzu (Kyoto, Japan), coupled to a QTRAP LC/MS/MS system from Applied Biosystems (USA), which integrate a hybrid quadrupole-linear ion trap mass spectrometer equipped with an ESI source. The column used for the analysis was a BDS-Hypersil C8 (50 mm \times 2 mm, 3 μm) with a mobile phase, composed by water (a) and ACN 95% (b), both containing 50 mM formic acid and 2 mM ammonium formate. The mobile phase flow rate was 0.2 mL/min and the injection volume was 5 μL . Intensity units in counts per second (cps).



allowed (160 µg OA/kg) – about 13 times lower – and, as SPX are not legislated, there is therefore no maximum level. In this analysis, the homogenate of mussel was extracted following the official method and, in total, the lipophilic toxin amount in the mussel sample was 53.5 µg/kg. This sample is not a threat for consumers in the short term, since the toxin amount is amply below the official limit.

However, a matter of concern is that the legislation allows each toxin to be quantified regardless of the presence of other lipophilic toxins. This means that a sample up to 1 mg eq YTX/kg + up to 160 µg eq AZA-1+ up to 160 µg eq OA/kg (for OA and PTX toxin group) is allowed and, therefore, it could be available at the markets. This is another drawback of the new chemical method for lipophilic marine toxin detection with respect to the MBA. In the case of several toxin groups being present in a sample, the MBA detects all toxins together, and the chemical approach allows that a sample with higher levels of several lipophilic marine toxin groups is available at the markets, while the same sample detected by MBA is not available.

The most striking observation is the fact that toxic compounds such as Ciguatoxins (CTXs), Tetrodotoxins (TTXs) and Palitoxins (PITXs) are appearing in molluscs and gastropods in Europe, and these toxins do not have official methods for their detection. This is clearly a matter for concern, since this new phenomenon is probably to be attributed to a potential ecological change due to increased warm temperatures. TTX is one of the most potent neurotoxins, and is known to block the sodium ion channels responsible for nerve and muscle excitability (Kawatsu *et al.*, 1999). Unlike other biotoxins which are produced by dinoflagellates, the TTX is not produced by microalgae. Symbiotic bacteria have been involved in TTX genesis for marine animals (*Shewanella algae*, *S. putrefaciens*, *Vibrio* sp., *Pseudomonas* sp., and *Alteromonas tetraodonis*) (Crocchi *et al.*, 2006).

TTX food poisonings are typical in Asian tropical countries, and is caused by the consumption of blowfish or small gastropods contaminated with the toxin (Noguchi & Arakawa, 2008). However, a food poisoning incident resulting from the ingestion of a trumpet shell (species *Charonia lampas lampas*) contaminated with TTX involved a single person in the south of the Iberian Peninsula in October 2007 (Rodríguez *et al.*, 2008). The patient was a 49-year-old man, and he had bought the shell for personal consumption in a market in Malaga, Spain. The symptoms began minutes after ingestion of the mollusc, and included abdominal pain with nausea and vomiting, weakness, difficulty in articulating words and keeping the eyelids open, and difficulty breathing (Fernandez-Ortega *et al.*, 2010). Later on, TTX and analogues were again found in three species of *Monodonta liniata*, *Charonia lampas* and *Gibbula umbilicalis* collected in different locations in the coast of Portugal (Vila Nova de Milfontes, Angeiras and Memória) between 2009 and 2010 (Silva *et al.*, 2012). Despite these findings, detection methods for TTX are still not included in the European regulation.

PITXs are produced by *Ostreopsis* genera dinoflagellates (Ramos & Vasconcelos, 2010). This dinoflagellate is also typical from tropical waters, and it was first detected in Mediterranean warm water in the 1970s (Taylor, 1979). However, it was in more recent years when the big HABs were recorded in the European Mediterranean coast and North Africa, in the Atlantic coast of Portugal (Penna *et al.*, 2010; Parsons *et al.*, 2012) and also in Spain (Barroso Garcia *et al.*, 2008). Toxic outbreaks by *Ostreopsis* spp. represent a completely new problem, affecting mainly the Mediterranean (Penna *et al.*, 2005). It caused great concern after the 2005 episode occurring near Genoa (Liguria, Tyrrhenian Sea), when 209 people needed medical care (Brescianini



et al., 2006; Durando *et al.*, 2007). Therefore, the regulation of palitoxins in EU should be considered.

CTXs are potent polyether toxins produced by *Gambierdiscus* species of dinoflagellate (Satake *et al.*, 1993, 1997, 1998). CTXs open the sodium channels in excitable cells, and more than 50 analogues have been characterized to date. These compounds have high toxicity and may pose a high risk to consumers, since 1 ng/g are toxic to humans (Lehane & Lewis, 2000). CTXs have previously been only linked to Caribbean, Pacific and Indian areas, causing a human food-borne intoxication called ciguatera fish poisoning (CFP) (Dickey & Plakas, 2009; Dickey, 2008).

In the last decade, *Gambierdiscus* have been recorded in the East Mediterranean. The first record of the presence of *Gambierdiscus* in this area was in Crete in 2003 (Aligizaki *et al.*, 2008). Subsequently, in January 2004, a family of fishermen had symptoms after the ingestion of 26 Kg of a fish species, *Seriola rivoliana*, collected along the coast of the Canary Islands. A sample of 150 g of the fish was kept and was analyzed by an *in vitro* assay for specific toxins that act on sodium channels and LC-MS/MS methodology. The results were positive and the content of CTX in the sample was 1.0 mg/kg (Perez-Arellano *et al.*, 2005).

Later, the presence of organisms that produce CTXs was found in the Mediterranean, near Israel (Bentur & Spanier, 2007), and again in the Canary Islands (Boada *et al.*, 2010). In 2010, it was confirmed the presence of CTX from different origins, in two species of *Seriola dumerilli* and *Seriola fasciata* captured in waters belong to Madeira Archipelago, Portugal (Otero *et al.*, 2010). The detection of CTXs requires very sensitive analytical methods, since intoxications can occur at very low concentrations of the toxin, and a method for routine testing is not available worldwide as yet. All of these facts show that climate change should not be ignored in food safety management and research, particularly by Spanish and Portuguese regulatory agencies. The presence of new analogues and new toxins from other localizations are issues that must be taken into account when any analytical method is used.

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