Current situation on analysis of marine toxins

Abstract: Marine toxins are a food safety concern worldwide. This review discusses current analytical methods for those toxins that are legally regulated in Europe, namely domoic acid, saxitoxin, okadaic acid, yessotoxin, pectenotoxin and azaspiracids, and all their analogues, and those that are not regularly monitored but are a threat to humans, such as tetrodotoxin, ciguatoxins and cyclic imines. Because of legislative changes that were implemented in 2011, most legally required methods are chromatographic. Saxitoxin and domoic acid are monitored by high-performance liquid chromatography with fluorescence and ultraviolet detection, respectively. All other toxins are monitored nowadays by means of chromatographic separation and mass spectrometry detection.

Keywords: analytical standards; chromatography; marine toxins; mass spectrometry.

*Corresponding author: Luis M. Botana, Department of Pharmacology, Veterinary School, Faculty Veterinaria, Universidad de Santiago de Compostela, Campus de Lugo, Lugo 27002, Spain, e-mail: Luis.Botana@usc.es

Ana M. Botana: Department of Analytical Chemistry, Universidad de Santiago de Compostela, Campus de Lugo, Lugo 27002, Spain

Paz Otero: Department of Pharmacology, Veterinary School, Faculty Veterinaria, Universidad de Santiago de Compostela, Campus de Lugo, Lugo 27002, Spain

Paula Rodríguez: Department of Pharmacology, Veterinary School, Faculty Veterinaria, Universidad de Santiago de Compostela, Campus de Lugo, Lugo 27002, Spain

Amparo Alfonso: Department of Pharmacology, Veterinary School, Faculty Veterinaria, Universidad de Santiago de Compostela, Campus de Lugo, Lugo 27002, Spain

Introduction

Phycotoxins are an acute worldwide seafood safety concern; hence, their detection and quantification in food with reliable methods is important. The methods must be validated according to international guidelines to ensure adequate results and to protect public health and reduce their economic impact.

Harmful algal blooms is the term used to describe a proliferation of marine microalgae (dinoflagellates, diatoms) that carries a toxic effect and, in many cases, economic losses. The microalgae are usually present in the plankton in low concentrations, but under some environmental conditions they can rapidly multiply in dense blooms. These microalgae produce phycotoxins, which accumulate in the digestive organs of filter-feeding shellfish, zooplankton and herbivorous fishes and through the trophic chain can produce human intoxications. The toxins are generally considered to be secondary metabolites and therefore not essential to the basic metabolism and growth of the microorganism (Van Dolah 2000). These episodes previously regarded as sporadic and localised phenomena and restricted to certain geographic areas have started to show up as recurrent and ubiquitous in any coast of the world and sometimes cause great damage to the local fishing industry and also to the natural communities of marine organisms (Vieites and Cabado 2008). Different countries have established standards about the total amount of phycotoxins that can be present in seafood products and also about the testing methods for detecting these molecules. Legal requirements for paralytic shellfish toxins [usually named paralytic shellfish poisoning (PSP)], amnesic shellfish toxins [amnesic shellfish poisoning (ASP)], lipophilic toxins, ciguatoxins (CTXs), brevetoxins or tetrodotoxins (TTXs) have been set around the world (Rodríguez-Velasco 2008). The European legislation establishes a maximum level of toxins in bivalve molluscs, echinoderms, tunicates and marine gastropods (the whole body or any part edible separately) of 800 μg of PSP equivalent/kg, 20 mg of domoic acid (DA)/kg, 160 μg of okadaic acid (OA) equivalents/kg, 160 μg of azaspiracids (AZAs) equivalents/kg and 1 mg of yessotoxin equivalent/kg, and the total absence of TTX and CTX (European Commission 2004a) (see structures in Figure 1). To check compliance with the limits laid down, several methods including mouse bioassay (MBA), high-performance liquid chromatography (HPLC) with fluorescence (FL), ultraviolet (UV) or mass spectrometry (MS) detection, immune assays or functional assays have been proposed. However, not all these methods are officially accepted as a validation process is necessary (European Commission 2007). MBA has been for many years considered as the reference monitoring method for phycotoxins, although the European Commission established that to be in accordance with animal health protection, all possible replacement, refinement and reduction of animals must be taken into account when laboratory animal methods are...
Figure 1 Structures of representative toxins.
used (Hess et al. 2006), and therefore alternative methods should be applied. Any method proposed as an alternative to the biological assay should not be less effective than the biological method, and the implementation should provide an equivalent level of public health protection. Following these premises, several methods were validated as alternatives to the MBA (Botana et al. 2009, 2010, Campbell et al. 2011). In the case of PSP toxins, the total content of toxins in the edible parts of molluscs must be officially detected by MBA (European Commission 2005) and also by the HPLC Lawrence method with pre-column oxidation as published in the Association of Official Analytical Chemists Official Method2205.06 (European Commission 2006). If the results are challenged, the reference method shall be the biological method. To detect PSP toxins, other analytical non-validated methods are available, such as an HPLC method with post-column oxidation (Oshima et al. 1976, Rodriguez et al. 2010), plasmon resonance biosensors (Foñfria et al. 2007) or enzyme immunoassays [enzyme-linked immunoassay (ELISA)] (Huang et al. 1996, Gare et al. 2010), based on both the use of antibodies and several functional assays based on the mechanism of action of the toxins (Vieytes et al. 1993, Louzzao et al. 2001). In the case of ASP, the total content of toxins in edible parts of molluscs may be detected by using either an HPLC method with UV detection (HPLC-UV) or an ELISA method (European Commission 2007). In this case, if results are challenged, the reference method shall be the HPLC method. For the detection of lipophilic toxins, a series of MBA procedures, differing in the test portion of flesh and the solvents used for extraction, were the official method for many years (European Commission 2005); however, after July 2011 a liquid chromatography (LC) tandem MS (LC-MS/MS) method was approved to be applied as the reference method for the detection of these toxins (Commission Regulation 2011). In addition to this, several methods based on the mechanism of action of each lipophilic toxin group have been developed (Vieytes et al. 1997, Alfonso et al. 2004, Vilarino et al. 2009, Otero et al. 2011b). There are no official methods to detect the presence of TTX or CTX even though several LC-MS/MS methods can be used to detect these toxins (Otero et al. 2010, Rodriguez et al. 2012).

As was mentioned earlier, the presence of phycotoxins is a dynamic and ubiquitous process in any coast of the world and therefore the presence of new analogues, new toxins or toxins from different locations are issues that must be taken into account when any analytical method is used. However, the legislation also established that in addition to the amount of toxin, the total toxicity, which is calculated by using conversion factors based on the available toxicity data of each toxin, should be calculated (Commission Regulation 2011). In this sense, the toxicity equivalent factor (TEF) of each compound compared with the potency of the reference compound must be applied to calculate the total toxicity of a sample (Botana et al. 2010). The Working Group on marine biotoxins, as part of the Contaminants Panel at the European Food Safety Agency (EFSA), has defined, on the basis of current knowledge, the TEF that should be used for the conversion of analytical results into toxic concentrations in each toxin group (European Commission Panel 2009b). Another essential item to implement and to validate any chemical detection method is the availability of standards as the identification of one toxin by using other analogues can lead to gross errors (Otero et al. 2011a). In addition, pure toxins in high amount are necessary to calculate the toxicity and TEF of each molecule to achieve results to protect human health. It can be concluded that analytical methods are useful tools to quantify the amount of known marine toxins in seafood samples, but several important details such as toxicity, calibrants and new toxins should be considered.

**Analysis of PSP toxins**

PSP is a fatal human syndrome produced by a naturally occurring group of neurotoxic alkaloids present in several species of dinoflagellates. This group of toxins constitutes the most spread worldwide affecting the whole of Europe, South Africa, India, Morocco, eastern Asian coast and North and South America as well.

Saxitoxin (STX) is the first described and most studied compound of the group (it is the representative compound), and 57 other analogues also have been researched (Wiese et al. 2010). Traditionally, STX analogues have been composed only of hydrophilic compounds and divided into subgroups on the basis of substituent side chains: carbamate [STX, neo-STX and gonyautoxins (GTX1–4)], decarbamoyl (dc-STX, dc-neo-STX and dc-GTX1–4) and N-sulfocarbamoyl (GTX5–6 and C1–4). These groups present different toxicities, with the carbamoyl analogues being the most toxic, followed by decarbamoyl analogues with intermediate toxicity, and N-sulfocarbamoyl analogues the least toxic (Vale et al. 2008a,b). Figure 1 shows the chemical structure of the more often found compounds in toxic dinoflagellates and poisoned seafood.

More recently, analogues from a novel family of PSP toxins containing a hydrophobic side chain were isolated and structurally characterised from Australian strains of *Gymnodinium catenatum* and designated GC1-GC3 (Negri
Other hydrophilic analogues of STX with an acetate R₄ side chain were reported in the freshwater filamentous cyanobacterium *Lyngbya wollei* but not in the marine environment so far (Onodera et al. 1997).

PSP toxins specifically block the excitation current in nerve and muscle cells by means of site one of the sodium channel (Messner and Catterall 1986); therefore, the accumulation of PSP toxins in shellfish creates a serious public health problem and affects the fisheries industry. Sommer and Meyer (1937) were the first to develop a method to determine PSP toxins: their MBA method established the basis for that. This is the reference method internationally accepted to quantify PSP toxicity, and it is used worldwide in monitoring programmes. The methods used to determine PSP toxins are fluorimetric assays, HPLC with fluorimetric detection (either pre-column or post-column oxidation), LC/MS and capillary electrophoresis (CE) methods.

The HPLC methods are widely used to quantify PSP toxins present in seafood samples, and they are also useful to provide the PSP profile because chromatographic methods are identification methods as well. These toxins have only a weak chromophore group, and it must be modified before detection: when they are oxidised in alkaline solution, a purine is formed that becomes fluorescent at acidic pH. This reaction can be either a pre-column one or a post-column one, and obtained purines are monitored with an FL detector.

Bates and Rapoport (1975) first studied the oxidative alkaline conditions of PSP toxins to get fluorescent compounds; then, Buckley et al. (1978) added them to their method and established the basis of the HPLC method for these compounds with the post-column reaction.

In 1984, Oshima et al. (1984) proposed a method based on alkaline oxidation to produce highly fluorescent derivatives, but with problems in separating toxins such as GTX1, GTX4 and GTX3, GTX5. Afterwards, they described a method that was able to separate almost all the PSP toxins with three different eluents according to the basicity of the three groups of toxins (group I: C1–C4; group II: GTX1,4, GTX5 (B1) and GTX6 (B2), dc-GTX1,4; group III: NEO, dc-STX and STX) (Oshima et al. 1987). In a later work, Oshima (1995) improved the system and included an extract cleaning procedure. The first disadvantage is that it is a time-consuming method for the analysis of PSP toxins.

In the group of pre-column methods, the method developed by Lawrence and Menard (1991) needs to be highlighted. One of the biggest disadvantages was the inability to easily distinguish between different toxin groups with similar toxicity.

Following this, the method was modified by changing chromatographic conditions to reduce analysis time and improve performance (Lawrence et al. 1995). In 2005, the Lawrence method was adopted as the official method to detect PSP toxins and then approved by the European Union (EU) for monitoring these toxins (Association of Official Analytical Chemists 2005, European Commission 2006). It is based on the pre-column oxidation of PSP toxins with hydrogen peroxide and sodium periodate followed by fluorimetric detection. It was validated for the determination of STX, NEO, GTX2,3, GTX1,4, dc-STX, GTX5 (B1), C1,2 and C3,4 in molluscs (mussels, clams, oysters and scallops). In 2009 a validation study was undertaken for the extension of this method to two more toxins: dc-NEO and dc-GTX2,3 (Turner et al. 2009).

Pre- and post-column HPLC methods, despite the many benefits of each, which includes an increased sensitivity to low concentrations of toxins and less variability in results, also present some drawbacks that should be resolved. In the case of hydrophobic analogues, they are retained by C18 resins (Negri et al. 2003), because of which the use of HPLC methods in monitoring programmes will miss the presence of hydrophobic analogues (Vale 2008, Vale et al. 2009). LC-MS methods are also being developed to obtain a good characterisation of these compounds: Electrospray ionisation MS (ESI-MS) is effective for the detection of polar PSP toxins (Quilliam 2003, Dell’Aversano et al. 2005); however, variable retention times for PSP toxins in different seafood matrixes were observed. At present, it is not possible to achieve a complete separation of all PSP toxins, and the sensitivity of MS detection is not good enough to control seafood toxin at the regulatory limit established (Diener et al. 2007).

Although the analysis of PSP toxins provides a profile of the composition of a given toxic episode, the use of surface plasmon resonance biosensors has reached a considerable level of development (Campbell et al. 2007). The high sensitivity of this approach allows fast throughput, as sample extraction and detection is rather simple, plus the chips can be used many times (Fonfria et al. 2007).

For this technology, an antibody-based method has been described and validated both at the single laboratory level (Campbell et al. 2010) and in an interlaboratory study (van den Top et al. 2011b).

## Analysis of DA

DA, belonging to the kainoid class of compounds, is the chemical responsible for ASP. It was originally isolated as
a product from the red algae *Chondria armata* and subsequently isolated from other red algae and several species of diatoms, mainly of the genus *Pseudo-nitzschia* (Bates et al. 1989, Lundholm et al. 1994). DA has also been detected in several species of molluscs worldwide including the United States, New Zealand, Mexico, several European countries such as France, Portugal, Ireland and Spain and the Croatian coast (Horner et al. 1993, Mígez et al. 1996, Vale, 2001, Ujevic et al. 2010). Although the DA is the main toxin found in a variety of shellfish species, other minor analogues, about 10 isomers of DA (isodomoic acids A–H and DA 5’ diasteriomer), have been identified in marine samples (Wright et al. 1990, Zaman et al. 1997).

The first method developed to detect the compound was LC-UV (Lawrence et al. 1989), and a protocol involving aqueous methanol extraction and strong anion-exchange (SAX) clean-up has been applied extensively to the analysis of DA and its known isomers in shellfish and fish tissues on a regulatory basis. Nowadays, the LC-UV remains the most widely used method, and it has been validated and standardised as the reference method for DA quantification (International 2000, European Committee for Standardization 2003). Furthermore, ELISA has also been validated (Kleivdal et al. 2007) and is used officially in the EU for screening purposes. These methods have limits of detection (LODs) low enough to detect DA at a concentration of 4.5 mg/kg of shellfish meat. This dose was established as the maximum amount that a portion of 400 g of meat should contain for not exceeding the acute reference dose of 30 mg DA/kg body weight (European Commission Panel 2009a).

Several other biological methods have been developed to detect and quantify DA in seawater and tissue samples, such as electrochemical ELISA (Micheli et al. 2004), radioimmunoassay (Lawrence et al. 1994), receptor binding assay (Lefebvre et al. 2010), cytotoxicity assay (Beani et al. 2000, Leira et al. 2003) and surface plasmon resonance (Le Berre and Kane 2006, Traynor et al. 2006, Stevens et al. 2007). Some of these techniques, such as enzyme immunoassay and ELISA, sometimes may require little clean-up of shellfish samples and have the advantages of being easy and fast (Yu et al. 2004). However, the cross-reactivity with similar toxins will result in false positives and limit the demonstration of toxicity (He et al. 2010).

DA contains a characteristic conjugated diene chromophore with strong absorbance at 242 nm, which permits its detection by LC-UV at concentrations as low as 4–80 ng/ml, depending on the sensitivity of the detector (Mafra et al. 2009). This is suitable for regulatory purposes; however, false positives are frequently encountered because of interferences from crude extracts (Hess et al. 2001). Mainly, tryptophan and its derivatives, normally contained in shellfish and finfish tissues, may be eluted close to DA with some columns and chromatographic conditions. Thus, there are other alternatives, such as FL (James et al. 2000, Chan et al. 2007) and chemiluminescence detection (Kodamatani et al. 2004). As DA concentrations in *Pseudo-nitzschia* cultures and phytoplankton field samples are often much lower, a more sensitive method of detection is required. The most common technique for the analysis of DA in seawater samples uses a derivatising agent, which results in low LODs (Pocklington et al. 1990), but this procedure involves some problems. Among them, a poor selectivity because many compounds in the sample, such as amino acids, can be derivatised and interfere with DA detection. Also, the handling during the derivatisation step is time consuming for the use of LC-FL on a large scale. In addition to the LC-UV and LC-FL, various extraction and screening techniques, such as thin-layer chromatography (Quilliam et al. 1998), CE (Gago-Martinez et al. 2003), gas chromatography coupled to MS (GC-MS) (Pleasance et al. 1990), LC-MS/MS (Wang et al. 2007, Mafra et al. 2009) and ultraperformance liquid chromatography with tandem MS detection (UPLC-MS/MS) (de la Iglesia et al. 2008), have allowed the detection of novel compounds inside of the DA group in new coastal areas of the world.

As the seafood matrix can interfere in the detection of trace levels, pre-treatment steps to concentrate and purify DA are needed. Solid-phase extraction (SPE) is the most common method used for the clean-up of shellfish samples before using a chromatography or other analytical technique to quantify the toxin amount (Zhao et al. 1997, Quilliam et al. 1998, James et al. 2000, Pardo et al. 2007, He et al. 2010). So, several methods have been based on SAX and strong cation exchange (Pleasance et al. 1990, Furey et al. 2001, Gago-Martinez et al. 2003, Ciminiello et al. 2005). The purification with SPE cartridges results in a valid approach for the routine monitoring of DA in shellfish to prevent the matrix effect and signal suppression. However, this is a manual time-consuming procedure; even some authors concluded that an additional step with an SPE cartridge did not significantly improve the recovery of DA from shellfish samples (Powell et al. 2002).

Therefore, there are many analytical methods that do use a sample clean-up and could be avoided when the pH of the mobile phase in the LC methods is properly adjusted (He et al. 2010). Table 1 summarises several chemical methods, specifying the sample clean-up procedure in different matrices and the LOD obtained in each method.

MS techniques are being increasingly used in some laboratories, and today, LC-MS/MS is perhaps the most
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<table>
<thead>
<tr>
<th>Method</th>
<th>Matrices</th>
<th>Treatment sample (clean-up)</th>
<th>LODs</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td>LC-UV</td>
<td>Mussels</td>
<td>Molecularly imprinted polymer-solid-phase extraction (MISPE) SPE</td>
<td>0.1 mg/ml</td>
<td>(Ciminiello et al. 2005)</td>
</tr>
<tr>
<td>LC-UV</td>
<td>Fur seals (fluidics)</td>
<td>vertisement and derivatisation</td>
<td>8 ng/ml</td>
<td>(European Commission Panel 2009a)</td>
</tr>
<tr>
<td>UPLC-MS/MS</td>
<td>Phytoplankton, seawater samples</td>
<td>Without sample pre-concentration and derivatisation</td>
<td>&lt;1 ng/ml</td>
<td>(Traynor et al. 2006)</td>
</tr>
<tr>
<td>LC-UV</td>
<td>Phytoplankton, seawater samples</td>
<td>SPE and post-column derivatisation</td>
<td>42 pg/ml (on column)</td>
<td>(Powell et al. 2002)</td>
</tr>
<tr>
<td>UPLC-MS/MS</td>
<td>Shellfish and phytoplankton</td>
<td>SPE and pre-column derivatisation (shellfish) Without SPE (phytoplankton)</td>
<td>≤1 ng/ml</td>
<td>(He et al. 2010)</td>
</tr>
<tr>
<td>LC-ESI-MS</td>
<td>Seawater</td>
<td>SPE-C18 (with Empore disks)</td>
<td>0.02 ng/ml</td>
<td>(Gago-Martinez et al. 2003)</td>
</tr>
<tr>
<td>LC-FL</td>
<td>Seawater</td>
<td>Without clean-up</td>
<td>0.05–0.09 mg/kg</td>
<td>(McNabb et al. 2005)</td>
</tr>
<tr>
<td>LC-FL</td>
<td>Mussels</td>
<td>SPE-C18 cartridges</td>
<td>30 pg/ml</td>
<td>(Quilliam et al. 1998)</td>
</tr>
<tr>
<td>LC-FL</td>
<td>Shellfish and phytoplankton</td>
<td>Pressurised liquid extraction (PLC) with purification inside the extraction cell</td>
<td>0.2 μg/ml</td>
<td>(Wang et al. 2007)</td>
</tr>
<tr>
<td>LC-UV</td>
<td>Mussels and scallops</td>
<td>Without SPE clean-up</td>
<td>25 ng/ml</td>
<td>(Doucette et al. 2009)</td>
</tr>
<tr>
<td>LC-UV-CLD</td>
<td>Mussels</td>
<td>Without any pre-concentration or derivatisation steps</td>
<td>0.008 μg/ml</td>
<td>(Hess et al. 2001)</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Seawater and phytoplankton</td>
<td>SPE-C18 cartridges</td>
<td>1–4 pg/ml (on column)</td>
<td>(Mosher et al. 1965)</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Mussels, clams, cockles</td>
<td>Pressurised liquid extraction (PLC) with purification inside the extraction cell</td>
<td>10 μg/ml</td>
<td>(Miyazawa and Noguchi 2001)</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Mussels</td>
<td>SPE clean-up: reversed phase and SCX cartridges</td>
<td>1 μg/ml</td>
<td>(Pocklington et al. 1990)</td>
</tr>
<tr>
<td>LC-UV</td>
<td>Clams and scallops</td>
<td>SAX cartridges</td>
<td>0.47 μg/ml</td>
<td>(Kodamatani et al. 2004)</td>
</tr>
<tr>
<td>CE-EIA</td>
<td>Mussels, oysters, clams and scallops</td>
<td>–</td>
<td>0.02 ng/ml</td>
<td>(Noguchi and Arakawa 2008)</td>
</tr>
<tr>
<td>CE</td>
<td>Mussels, razor clams and anchovy</td>
<td>SAX clean-up</td>
<td>0.15 μg/ml</td>
<td>(Pleasance et al. 1990)</td>
</tr>
</tbody>
</table>

Table 1 Some representative analytical chemical methods for the detection of DA in a range of matrices.
CE, capillary electrophoresis; CE-EIA, capillary electrophoresis-enzyme immunoassay; DA, domoic acid; EIA, enzyme immunoassay; GC-MS, gas chromatography-mass spectrometry; LC-ESI-MS, liquid chromatography-enzyme immunoassay-tandem mass spectrometry; LC-FL, liquid chromatography with fluorescence detection; LC-MS/MS, liquid chromatography with tandem mass spectrometry; LC-UV, liquid chromatography with ultraviolet detection; LC-UV-CLD, liquid chromatography-ultraviolet-chemiluminescence detection; LODs, limits of detection; SAX, strong anion exchange; SCX, strong cation exchange; UPLC-MS/MS, ultraperformance liquid chromatography with tandem mass spectrometry; TLC, thin layer chromatography.

important and legally accepted confirmatory tool, providing a high sensitivity, accuracy and selectivity for DA and its isomers in crude extracts (McNabb et al. 2005). Nevertheless, LC-UV is often the only analytical instrument available in many research institutes and regulatory agencies responsible for monitoring the occurrence of marine toxins. Although the LOD of DA can be improved by using different injection volumes in the LC-UV, the matrix complexity may result in some degree of uncertainty about the peak’s identity at low DA levels (<3 ng/ml), when a sensible UV spectrum cannot be obtained (Mafra et al. 2009). In this case, confirmation by LC-MS is sometimes necessary and should also be required whenever DA is reported in a new geographical area. Some studies on algal production of DA to track the toxin in seawater, as an early alert for toxin accumulation in marine organisms, are available for remote, subsurface detection (Doucette et al. 2009).
Overall, an improvement in techniques based on LC and/or MS to achieve reliable, simple and low-cost methods is essential for DA detection in any sample matrix.

**Analysis of TTX**

TTX, a well-known potent neurotoxin, was first isolated from puffer fish (Mosher et al. 1965) and has also been found in a wide variety of animals including arthropods, echinoderms, mollusks, worms, newts or frogs (Miyazawa and Noguchi 2001, Noguchi and Arakawa 2008). Neither its biochemical path nor its true origin is fully clarified, as three hypotheses point to its origin: endogenous (Lehman et al. 2004), through food chain (Lin and Hwang 2001, Kono et al. 2008) or through symbionts (Narita et al. 1987, Wang et al. 2008).

Although TTX exists mainly in tropical waters worldwide, this toxin has appeared on European coasts (Katikou et al. 2009). It is possibly due to the migration of toxic species from the Red Sea to the Mediterranean Sea through the Suez Canal, a phenomenon known as Lessesian migration (Golani 1998, Galil and Zenetos 2002). This migration may happen because of the opening of new corridors allied to the increase in water temperature as a result of climate change. So, marine species typical of tropical and subtropical waters are adapted to waters less warm, and changes in environmental conditions lead to poisoning incidents in new geographical areas. In fact, recent intoxication cases caused by the consumption of TTX bearers have been described in the Israeli coast (Noguchi and Ebesu 2001), through food chain (Kawabata 1978, Yasumoto 1991). However, the MBA is not completely satisfactory, due to its low sensibility. MBA is not suitable for measuring TTX in biological samples as it cannot distinguish between TTX and STX or TTX analogues. Other biological methods have been developed for TTX detection, such as ELISA, methods that use antibodies specific to the toxin (Raybould et al. 1992, Neagu et al. 2006, Zhou et al. 2007), tests with cultured neuroblastoma, hemolytic assays (Hamasaki et al. 1996) and also techniques using biosensors (Kreuzer et al. 2002, Neagu et al. 2006, Yakes et al. 2011). Nevertheless, to obtain specific information of a sample, as the toxic profile or the amount of an individual toxin, chemical methods have been developed, including immunoaffinity chromatography (Kawatsu et al. 1999), GC-MS (Man et al. 2010), HPLC with FL detection (HPLC-FL) (O’Leary et al. 2004, Jen et al. 2007) and HPLC-UV (Yu et al. 2010). Although these techniques ensure low LODs and high sensitivity to detect TTX, they have some limitations. In the case of immunoaffinity or ELISA, both methods require a costly monoclonal antibody or reagent (Leung et al. 2011), and the GC-MS requires a complex extraction procedure (Jen et al. 2008). However, HPLC-FL shows differences in the FL intensities of some TTX analogues compared with TTX itself, which causes problems in quantifying (Shoji et al. 2001). For these reasons, analytical methods based on LC-MS and LC-MS/MS have been developed (Jang et al. 2010, Chen et al. 2011, Rodriguez et al. 2012). These LC methods, together with appropriate extraction procedures, have been able to determine the presence of TTX, not only in the remains of fish and other animals but also in the blood and urine of poisoned patients (Tsai et al. 2006, Rodriguez et al. 2008). Moreover, these methods provide better LODs when compared with HPLC-FL or UV (Jen et al. 2008). Most of the LC-MS instruments are equipped with an atmospheric pressured ionisation (API) fitted with an ESI source based on collision-induced dissociation (CID) of a triple quadrupole mass analyser. For the detection of TTXs, the ESI interface operates in the positive ionisation mode. So, TTX and its are regulations for TTX in countries such as Japan and Korea, where official control does not regulate the amount of toxin in fish that can be placed on the market, but restaurants that want to serve species containing TTX require special licenses. EFSA has not published any documents related to the risks involving TTX, but some authors claim that the minimum lethal dose in humans is 2 mg, though it may vary with factors such as age, health and sensitivity to the toxin (Noguchi and Ebesu 2001, Cohen et al. 2009).

Currently, there is no official method for TTX detection, but the MBA has been used in many cases to determine TTX toxicity in food matrices (Katikou et al. 2009). However, the MBA is not completely satisfactory, due to its low sensibility. MBA is not suitable for measuring TTX in biological samples as it cannot distinguish between TTX and STX or TTX analogues. Other biological methods have been developed for TTX detection, such as ELISA, methods that use antibodies specific to the toxin (Raybould et al. 1992, Neagu et al. 2006, Zhou et al. 2007), tests with cultured neuroblastoma, hemolytic assays (Hamasaki et al. 1996) and also techniques using biosensors (Kreuzer et al. 2002, Neagu et al. 2006, Yakes et al. 2011). Nevertheless, to obtain specific information of a sample, as the toxic profile or the amount of an individual toxin, chemical methods have been developed, including immunoaffinity chromatography (Kawatsu et al. 1999), GC-MS (Man et al. 2010), HPLC with FL detection (HPLC-FL) (O’Leary et al. 2004, Jen et al. 2007) and HPLC-UV (Yu et al. 2010). Although these techniques ensure low LODs and high sensitivity to detect TTX, they have some limitations. In the case of immunoaffinity or ELISA, both methods require a costly monoclonal antibody or reagent (Leung et al. 2011), and the GC-MS requires a complex extraction procedure (Jen et al. 2008). However, HPLC-FL shows differences in the FL intensities of some TTX analogues compared with TTX itself, which causes problems in quantifying (Shoji et al. 2001). For these reasons, analytical methods based on LC-MS and LC-MS/MS have been developed (Jang et al. 2010, Chen et al. 2011, Rodriguez et al. 2012). These LC methods, together with appropriate extraction procedures, have been able to determine the presence of TTX, not only in the remains of fish and other animals but also in the blood and urine of poisoned patients (Tsai et al. 2006, Rodriguez et al. 2008). Moreover, these methods provide better LODs when compared with HPLC-FL or UV (Jen et al. 2008). Most of the LC-MS instruments are equipped with an atmospheric pressured ionisation (API) fitted with an ESI source based on collision-induced dissociation (CID) of a triple quadrupole mass analyser. For the detection of TTXs, the ESI interface operates in the positive ionisation mode. So, TTX and its are regulations for TTX in countries such as Japan and Korea, where official control does not regulate the amount of toxin in fish that can be placed on the market, but restaurants that want to serve species containing TTX require special licenses. EFSA has not published any documents related to the risks involving TTX, but some authors claim that the minimum lethal dose in humans is 2 mg, though it may vary with factors such as age, health and sensitivity to the toxin (Noguchi and Ebesu 2001, Cohen et al. 2009).
analogues are analysed by MS, in which positive ionisation produces a typical molecular ion of [M+H]+ for each one. Because of the continuous emergence of new TTX analogues, the most recent 8-epi-type TTX analogues found in newts (Kudo et al. 2012), some authors prefer to operate with the MS in the multiple reaction monitoring (MRM) mode to quantify the individual toxins. However, others use the MS operating in the single ion monitoring mode or the single ion recording mode, such that only a selected m/z value is detected in the analysis. On the contrary, the MRM mode not only allows detecting the precursor molecule but also tracks multiple product ions from the fragmentation pattern in the same run. In this case, only one product ion is used in the quantification and the others to confirm the toxin.

Today, the investigation is focused in developing LC-MS methods that lead to a good separation and identification of all TTX analogues in any sample with low LODs. So, different analytical columns and LC conditions are tested by researchers to improve the detection of TTX. Table 2 summarises several LC-MS/MS methods to detect TTX and its analogues. The toxin separation is typically achieved in reverse-phase columns and with solvents containing an ion pair reagent (i.e., ammonium heptafluorobutrate). But this ion pair sometimes tends to remain in the MS, causing spectral noise (Shoji et al. 2001, Rodríguez et al. 2012). Also, because of the polarity of TTXs, some of them are not well retained in these columns. To solve all these problems, the hydrophilic interaction liquid chromatography (HILIC) is becoming more useful in the analysis of TTXs (Chen et al. 2011, Chulaneltra et al. 2011, Cho et al. 2012, Kudo et al. 2012, Rodríguez et al. 2012, Silva et al. 2012). The HILIC technique employs low aqueous/high polar organic solvents without ion pair reagents, resulting in increased sensitivity compared with the reverse phase. So, TTX, which is the more polar compound between its analogues, elutes with the higher proportion of water in a gradient elution. Also, problems with noise peaks are reduced in HILIC columns. Almost all TTX analogues show a common major product ion, which is usually 162 (m/z) formed from the molecular ions in MS/MS, suggesting that this product ion can be used in the quantification. However, there are analogues such as TTX and 4-epi-TTX that have the same molecular weight and the same fragmentation pattern; therefore, a complete separation is needed to quantify them. As a result, the HILIC separation system is very useful for the simultaneous quantification of TTXs by LC-MS/MS in the MRM mode. Figure 2 shows an example of a chromatogram obtained by LC-ESI-CID-MS/MS from puffer fish sample. The TTX analogues eluted in a HILIC column and detected under the MRM mode were the following: 5,6,11-trideoxy-TTX (m/z 272>254/162), 5-deoxy-TTX, and 11-deoxy-TTX (m/z 304>286/176), 4,9-anhydro-TTX (m/z 302>256/162), 11-nor-TTX-6(R)-ol and 11-nor-TTX-6(S)-ol (m/z 290>272/162), 4-epi-TTX and TTX (m/z 320>302/162).

In summary, the LC-MS/MS technology is the most appropriate and chosen by some laboratories worldwide for detecting TTXs. One problem is the difficulty in obtaining purified TTX analogues for calibration. TTX is frequently used as an internal standard for their quantification, and this could lead to errors in the toxin amount. Nevertheless, because of the timely appearance of TTX in new geographical areas, monitoring programmes coupled to a suitable and standardised analytical method should be developed and for this, certified standards of TTXs would be necessary.

Detection of lipophilic marine toxins

Marine lipophilic toxins are a heterogeneous group of toxins with different molecular weights and specific chemical characteristics (Table 3). This group includes yessotoxins (YTXs), AZAs, pectenotoxins, gymnodimines (GYMs), spirolides (SPXs), CTXs and diarrhetic shellfish poisoning (DSP). Members of the DSP toxin group are OA and its derivatives dinophysistoxin-1 (DTX-1), dinophysistoxin-2 (DTX-2) and dinophysistoxin-3. To detect them, a number of analytical methods have been developed in the last decades, comprising MBAs, in vitro assays and chemical assays (Botana et al. 2012). Each method has its merits and drawbacks, and ultimately it is the responsibility of regulators and analysts to decide upon the most appropriate technology for their particular situation. The regulation of lipophilic marine toxins worldwide differs widely. In Japan, Canada and South America, MBA is the method used for the official control of these toxins, while in New Zealand, chromatographic methods have been used for their monitoring programmes (McNabb 2008, Gerssen et al. 2011). In Europe, chromatographic techniques coupled to MS were also the method of choice to replace MBAs to detect lipophilic marine toxins (Commission Regulation 2011). MBA, which has been used for many years as an exclusive reference method for lipophilic marine toxin detection, was replaced by the LC-MS/MS approach with the new regulation.

To date, more than 200 lipophilic marine toxins have been described in the literature (Gerssen et al. 2011, Rodríguez et al. 2012). The LC-MS/MS approach is the most appropriate and used by some laboratories worldwide for detecting these toxins.
Separation Eluents Toxins \([M+H]^+\) (m/z) Matrices LODs References

Reverse phase

30 m mm ammonium heptafluorobutyrate in 1 m mm ammonium acetate buffer (pH 5.0) 320, 336, 302, 290, 304 and unknown analogues (348, 330) Amphibian – (Stobo et al. 2005)

ZIC-HILIC

(A) 10 m mm ammonium formate and 10 m mm formic acid in water (B) acetonitrile:water (80:20) in 5 m mm ammonium formate and 2 m mm formic acid (3:7 v/v) 320, 302, 304, 272 Puffer fish 0.09–0.2 ng/ml (Villar-Gonzalez et al. 2008)

HILIC

16 m mm ammonium formate/acetonitrile (3:7 v/v) 256, 272, 288, 302, 304, 320, 336 Puffer fish 0.9 pmol/injection (Jang et al. 2010)

HILIC

(A) 1% formic acid/water and (B) 100% methanol 320 Blood serum 0.1 ng/ml (Wang et al. 2008)

Reverse phase

1% acetonitrile, 10 m mm trimethylamine and 10 m mm ammonium formate in water 320, 272 Gastropods and human fluids (blood and urine) <10 ng/ml (Gerssen and McLaughlin 2008)

HILIC

16 m mm ammonium formate/acetonitrile (3:7 v/v) 320, 302, 304, 288, 272 Puffer fish <0.5 nmol/g (Cho et al. 2012)

ZIC-HILIC

(A) 10 m mm ammonium formate and 10 m mm formic acid in water (B) acetonitrile:water (80:20) in 5 m mm ammonium formate and 2 m mm formic acid 320, 302, 304 Puffer fish – (McNabb 2008)

HILIC

10 m mm ammonium formate/acetonitrile (22:78 v/v) 272, 288, 290, 302, 304, 320, 336 Puffer fish 0.10 ng/ml (Kudo et al. 2012)

HILIC

1% acetic acid in acetonitrile/1% acetic acid in water (88:12 v/v, pH 4.1) 320 Blood 0.32 ng/ml (Gerssen et al. 2011)

Reverse phase

(A) 1% acetonitrile, 20 m mm heptafluorobutyric acid, 20 m mm ammonium hydroxide and 10 m mm ammonium formate in water (B) and same mixture (A) with 5% acetonitrile 320, 290, 304, 302 Puffer fish – (Rodríguez et al. 2012)

HILIC

(A) 10 m mm ammonium formate and 10 m mm formic acid in water (B) acetonitrile:water (95:5) in 5 m mm ammonium formate and 2 m mm formic acid 320, 290, 304, 272 Puffer fish 16 ng/ml (Rodríguez et al. 2012)

HILIC

Not clear 254, 270, 272, 286, 288, 290, 302, 304, 320 Netws – (Botana et al. 2012)

HILIC

(A) 10 m mm ammonium formate and 10 m mm formic acid in water (B) acetonitrile:water (95:5) in 5 m mm ammonium formate and 2 m mm formic acid 320, 272 Gastropods 1.7 ng/ml (Cohen et al. 2009)

<table>
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<th>Separation</th>
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<td>Puffer fish</td>
<td>–</td>
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<td>Puffer fish</td>
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<td>Puffer fish</td>
<td>16 ng/ml</td>
<td>(Rodríguez et al. 2012)</td>
</tr>
<tr>
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<td>320, 272</td>
<td>Gastropods</td>
<td>1.7 ng/ml</td>
<td>(Cohen et al. 2009)</td>
</tr>
</tbody>
</table>

Table 2: Summary of several LC-MS/MS methods to detect TTX and its analogues.

HILIC, hydrophilic interaction liquid chromatography; LC-MS/MS, liquid chromatography with tandem mass spectrometry; LODs, limits of detection; TTX, tetrodotoxin; ZIC-HILIC, Highly polar Zwitterionic hydrophilic interaction liquid chromatography.

However, most LC-MS/MS methods are focused on the analysis of the 13 toxins that are legislated in the EU (Table 3). GYMs and SPXs are usually included in the multi-toxin detection methods despite the fact that they are not legislated in the EU. Hence, LC-MS/MS methods detect different marine toxin groups in a short period of time (Stobo et al. 2005, McNabb 2008, Gerssen et al. 2011), and they are classified according to the chromatographic solvents used. Basic or acid mobile phases can be used with an elution gradient. Table 4 summarises the LC characteristics of two types of chromatography developed for the monitoring of lipophilic marine toxin. If an acid chromatography is employed, the mobile phase is composed of water and ACN/water (95:5), both containing 50 m m formic acid and 2 m mm ammonium formate (Alfonso et al. 2008, Villar-Gonzalez et al. 2008, Otero et al. 2010, Blay et al. 2011). Mobile phases for basic chromatography are composed of water and ACN/water (90:10), both containing 0.05% ammonia, 2 m mm bicarbonate or 6.7 m mm ammonium hydroxide (pH 11) (Gerssen et al. 2009, 2011). MRM data are detailed in Table 4. The transition with the highest intensity is used for quantification, while the...
transition with the lowest intensity is used for confirmatory purposes. With the exception of OA and YTX toxins groups, which are ionised in the negative mode, the remaining lipophilic toxins are preferably ionised in the positive mode. If the instrument is able to simultaneously operate in positive and negative ionisation modes, the MS methods include transitions for the toxins that are ionised in both ionisation modes. If it is not capable of detecting all toxins in a single injection, compounds are detected in two separated runs, one with the equipment operating in the positive mode and the other with the equipment operating in the negative mode.

This technique has been evaluated, and it is considered to be successful by many laboratories (Gerssen et al. 2009, Blay et al. 2011). The short, narrow-bore column packed with 3 μm Hypersil-BDS-C8 phase and the X-Bridge C18 are two of the most widely used columns, which are capable of separating a wide range of toxins by using rapid gradient (Villar-Gonzalez et al. 2007, Ciminiello et al. 2010, Gerssen et al. 2011). The method with both basic and acid mobile phases was also adjusted to the new technologies, UPLC-MS/MS (Fux et al. 2007, Rundberget et al. 2011), and more than 20 analogues were separated in only 6.6 min (Fux et al. 2007). One advantage of the acid against basic chromatography is that the first one facilitates good separation of acidic OA analogues by suppressing the ionisation of the carboxyl groups and preventing deleterious ion exchange interactions with residual silanol groups in the stationary phase (Suzuki and Quilliam 2011). Nevertheless, the chromatography of compounds included in the YTX group can be problematic under acidic conditions (Gerssen et al. 2009). LODs for lipophilic toxins achieved by the LC-MS/MS approach are low (Table 4), and toxins can be detected at levels below the current regulatory limit. For both acid and basic chromatography conditions, these LODs are lower for toxins ionised in the positive mode than in the negative one, specially for SPXs (Blay et al. 2011, van den Top et al. 2011a).

Although analytical methods are by far the target of most of the international efforts for the detection of this group of compounds, it should be highlighted that enzyme-based assays are available for high-throughput detection of these compounds (Vieytes et al. 1997, González et al. 2002, Rubiolo et al. 2012), and biosensor methods that use the surface plasmon resonance technology (Llamas et al.

**Figure 2** Mass chromatogram of the LC-ESI-CID-MS/MS obtained under MRM operation from the puffer fish sample. Toxins eluted in an XBridge Amide column (i.d. 2.1×150 mm; 3.5 μm) at 25°C, 0.2 ml/min. The LC was operated in gradient with eluent A consisting of 10 mm ammonium formate and 10 mm formic acid in water. Eluent B contained 95% acetonitrile and 5% water with a final concentration of 5 mm ammonium formate and 2 mm formic acid. Minutes 16 to 22 represent a broadening of the chromatogram where the toxins are eluted. CID, collision-induced dissociation; ESI, electrospray ionisation; i.d., inner diameter; LC, liquid chromatography; MRM, multiple reaction monitoring; MS, mass spectrometry; TIC, total ion chromatogram.

**Table 3** Chemical characteristics of lipophilic marine toxins.

<table>
<thead>
<tr>
<th>Toxin groups</th>
<th>Chemical class</th>
<th>Main compounds (M)</th>
<th>Formula</th>
<th>Toxin analogues covered by the EU legislation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Okadaic acid (OA)</td>
<td>Polyether, spiro-keto assembly</td>
<td>OA (804)</td>
<td>C_{44}H_{68}O_{13}</td>
<td>OA, DTX-1, DTX-2, DTX-3</td>
</tr>
<tr>
<td>Azaspiracid (AZA)</td>
<td>Polyether, second amine, 3-spiro ring</td>
<td>AZA-1 (841.5)</td>
<td>C_{47}H_{71}NO_{12}</td>
<td>AZA-1, AZA-2, AZA-3</td>
</tr>
<tr>
<td>Petecnotoxin (PTX)</td>
<td>Polyether, ester macrocycle</td>
<td>PTX-1 (874.5)</td>
<td>C_{47}H_{70}O_{14}</td>
<td>PTX-1, PTX-2</td>
</tr>
<tr>
<td>Yessotoxin (YTX)</td>
<td>Ladder-shaped polyether</td>
<td>YTX (1141)</td>
<td>C_{55}H_{82}O_{21}S</td>
<td>YTX, 45-OH-YTX, homo-YTX, 45-homo-YTX</td>
</tr>
<tr>
<td>Gymnodimines (GYMs)</td>
<td>Macrocycle; cyclic imine</td>
<td>GYM (507)</td>
<td>C_{52}H_{24}OS_{2}</td>
<td>None</td>
</tr>
<tr>
<td>Spirolides (SPXs)</td>
<td>Macrocycle; cyclic imine</td>
<td>SPX-1 (691.5)</td>
<td>C_{51}H_{18}NO_{3}</td>
<td>None</td>
</tr>
</tbody>
</table>

EU, European Union.
<table>
<thead>
<tr>
<th>Column</th>
<th>BDS-Hypersil C8, 50 mm×2 mm, 3 μm particle size</th>
<th>X-Bridge C18, 150 mm×3 mm, 5 μm particle size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow</td>
<td>0.2 ml/min</td>
<td>0.25–0.4 ml/min</td>
</tr>
<tr>
<td>Injection vol.</td>
<td>5 μl</td>
<td>5–10 μl</td>
</tr>
<tr>
<td>Column T</td>
<td>25°C</td>
<td>25°C–40°C</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>A: Water (both containing 2 mm ammonium formate) B: ACN (95%) and 50 mm formic acid)</td>
<td>A: Water (both containing 6.7 mm ammonium hydroxide (pH 11))</td>
</tr>
<tr>
<td>Gradient</td>
<td>Time (min) Mobile phase A (%) Mobile phase B (%)</td>
<td>Time (min) Mobile phase A (%) Mobile phase B (%)</td>
</tr>
<tr>
<td>0</td>
<td>70 30</td>
<td>0–1 90</td>
</tr>
<tr>
<td>8</td>
<td>10 90</td>
<td>10 90</td>
</tr>
<tr>
<td>11</td>
<td>10 90</td>
<td>13 10</td>
</tr>
<tr>
<td>11.5</td>
<td>70 30</td>
<td>15 90</td>
</tr>
<tr>
<td>17</td>
<td>30 30</td>
<td>19 90</td>
</tr>
<tr>
<td>LODs</td>
<td>OA: 22.1 pg, YTX: 6.1 pg, AZA-1: 1.1 pg; PTX-2: 6.9 pg, SPX-1: 1.9 pg, GYM: 14.1 pg</td>
<td>OA: 9.1 pg, YTX: 2.2 pg, AZA-1: 1.1 pg; PTX-2: 7.4 pg, SPX-1: 0.8 pg, GYM: 3.7 pg</td>
</tr>
<tr>
<td></td>
<td>OA: 10 μg/kg, AZA-1: 0.3 μg/kg, PTX-2: 10 μg/kg, SPX-1: 4 μg/kg</td>
<td>OA: 5 μg/kg, AZA-1: 0.5 μg/kg, AZA-2: 0 μg/kg, DTX-1: 5 μg/kg, DTX-2: 3 μg/kg, AZA-1: 2 μg/kg, AZA-2: 2 μg/kg, AZA-3: 3 μg/kg, PTX-2: 2 μg/kg, YTX: 16 μg/kg</td>
</tr>
</tbody>
</table>

**Table 4** Main characteristics of two types of liquid chromatography (LC) for the detection of lipophilic marine toxins: (1) chromatography under acidic conditions and (2) chromatography under basic conditions.

ACN, acetonitrile; AZA, azaspiracid; DTX, dinophysistoxin; GYM, gymnodimine; OA, okadaic acid; PTX, peptecnotoxin; SPX, spirolide; YTX, yessotoxin. *Limits of detection (LODs) of several lipophilic toxins are also indicated on it. MS/MS detection was performed in the multiple reaction monitoring (MRM) mode by 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), homo-YTX (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) and AZA-3 (m/z 828.5/810.5, m/z 828.5/792.5).
in fish of certain species, such as *G. javanicus*, *Lutjanus bohar*, *Plectropomus leopardus* and *Epinephelus fuscoguttatus* (Caillaud et al. 2010), not in bivalves. CTXs are potent polyether toxins issued from *Gambierdiscus* species of dinoflagellates. These toxins are usually found in areas between 35°N and 35°S latitude, mainly Indo-Pacific and Caribbean areas. However, in recent years, these toxins are increasingly appearing in countries not expected for their latitude such as waters close to the European and African continents (Perez-Arellano et al. 2005, Bentur and Spanier 2007, Otero et al. 2010). Its distribution is expected to shift towards other countries, and in fact this seems to have begun the trend. For this reason, some laboratories that did not normally include these compounds in the toxin analysis are now in need of improving the methods for detecting them.

Nowadays, the most used chemical method used for detecting CTXs in shellfish samples is LC-MS/MS. Methods based on HPLC-UV and HPLC-FL have been also used for many years, but if they are compared with LC-MS/MS, it is obvious that both have disadvantages. As CTXs do not have characteristic chromophore groups in their structures (like DA), the use of HPLC-UV results in methods with poor sensitivity and selectivity (Caillaud et al. 2010). Despite this, several authors have used this technique as a strategy for the isolation and purification of CTXs prior to their characterisation in fish tissues and extracts of dinoflagellates (Lewis and Jones 1997; Lewis and Vernoux 1998, Satake et al. 1998, Hamilton et al. 2002a,b). However, the presence of primary hydroxy groups in some CTXs (P-CTX-1, P-CTX-2, P-CTX-3 and 2,3-dihydroxy-CTX-3C) suggested that these toxins could be derivatised into fluorescent esters and thus become suitable for HPLC-FL (Yasumoto et al. 1995). These analyses are performed in a Develosil ODS-5 column (4.6×250 mm) using an isocratic elution gradient with around 90% acetonitrile (Yasumoto et al. 1995). This method was successfully applied to samples of *Gambierdiscus toxicus* and to different carnivorous fish species, including *G. javanicus*, *Lutjanus bohar*, *Plectropomus leopardus* and *Epinephelus fuscoguttatus* (Caillaud et al. 2010). However, this approach lacks applicability as many CTX analogues such as P-CTX-3C do not exhibit derivatisation.

Later, the application of MS played a critical role in the structure elucidation of many CTX congeners (Lewis and Jones 1997, Hamilton et al. 2002a,b, Pottier et al. 2002a). Today, LC-MS/MS technology is widely used by many laboratories for their detection in contaminated samples. This technique is not the reference method, as happens in the case of other lipophilic toxins. Each laboratory uses LC conditions that are considered more properly and thus, the bibliography describes different methods of analysis. Prior to the chromatographic analysis of CTXs in fish samples, strict clean protocols are used (Hamilton et al. 2002a,b, Pottier et al. 2002a, Otero et al. 2010). Although these steps improve the sensitivity and reduce the matrix effects, it is certain that they do not remove metallic impurities, which result in the formation of adducts (Hamilton et al. 2002a, Otero et al. 2010).

LC systems comprise C18 columns, such as 5-μm Phenomenex Luna (Lewis et al. 2009) or 3.5-μm Zorbax 300SB (Hamilton et al. 2002a,b) and C8 columns such as 3-μm Phenomenex Hyperclone (Roeder et al. 2010) or 3-μm Phenomenex Luna (Dechraoui et al. 2005). Mobile phases are composed generally by acetonitrile and water, and the use of gradients of these organic
solvents buffered with 1 mM ammonium acetate improve the separation and detection of CTXs compared with an acetonitrile:water gradient modified with 0.1% trifluorocetic acid (Lewis and Jones 1997). MS detection is usually performed in the positive MRM mode (Perez-Arellano et al. 2005, Roeder et al. 2010) or in the Q1 mode selecting a range of mass (Hamilton et al. 2002a, Pottier et al. 2002a). In the last case, the MS spectrum will show the characteristic pattern of ion formation for CTX polyethers (multiple losses of water and the formation of sodium and ammonium adducts) (Pottier et al. 2002a). Each CTX gives rise to prominent ammonium, potassium and sodium adducts and losses of waters. An example of a typical chromatogram and spectrum of a CTX standard is shown in Figure 3. The compound is the synthetic 51-hydroxy-CTX-3C with $M_r$ 1038.5, and the identification was performed in a UPLC system coupled to an Xevo TQ MS mass spectrometer from Waters (Manchester, UK). The column used was a Waters Acquity UPLC BEH C18 (100×2.1 mm; 1.7 μm), and the mobile phase consisted of a gradient of acetonitrile/water. As can be observed, the ion 1039.5 m/z due to the [M+H]$^+$ is not the most prominent. However, the ions m/z 1077.5 [M+K]$^+$ and m/z 1061.5 [M+Na]$^+$ have a high intensity. In addition, the spectrum shows two water losses, m/z 1021.5, which corresponds to [M+H-H$_2$O], and m/z 1003.5, which corresponds to [M+H-H$_2$O].

Another consideration to be taken into account in the analysis of CTXs by LC-MS/MS is the big number of analogues belonging to this toxin group and the few standards available. To date, more than 50 different congeners have been identified (Litaker et al. 2010), and the presence of several CTXs with equal molecular weight in the same extract is very frequent. Up to six different analogues for the compound 1111.6 m/z (CTX-1B) eluting in different retention times have been described (Lewis and Jones 1997). This issue makes the identification of CTX difficult. Moreover, because of the lack of certified standards and reference materials and the limited amounts of contaminated material available for method development, the validation status of LC-MS/MS methods is very restricted and up to now no collaborative study has been undertaken.

Recently, a method with 14 reference toxins prepared by either synthesis or isolation from natural sources was used to identify and quantify 16 toxins from the CTXs group (Yogi et al. 2011). LC separation was performed in a Zorbax Eclipse Plus C18 column in <14 min by using a linear gradient of mobile phases: 5 mM ammonium formate and 0.1% formic acid in water and in methanol. The mass spectrometer operates in the positive mode for the monitoring of sodium adduct ions [M+Na]$^+$. This method has greatly reduced the time of analysis as up to now almost all methods described use run times of around 50 min (Hamilton et al. 2002a,b, 2009, Pottier et al. 2002b).

Figure 3 Chromatogram (A) and mass spectrum (B) of 51-OH-CTX-3C ($M_r$ 1038.5) standard at 1000 ng/ml, using selected ion recording (SIR) UPLC mode of 1039.5 m/z [M+H]$^+$. Analysis was performed in an ACQUITY UPLC system coupled with Xevo TQ MS mass spectrometer. Chromatographic identification was achieved in a Waters Acquity UPLC BEH C$_18$ column (100×2.1 mm; 1.7 μm). The mobile phase consisted of two components: acetonitrile/water (95:5) (A) and water (B), both containing 50 mM formic acid and 2 mM ammonium formate using a gradient elution. 51-OH-CTX-3C, 51-hydroxy-CTX-3C; UPLC, ultraperformance liquid chromatography.
References


Conclusions

Although there are many choices for the analysis and detection of marine toxins, the current legal requirement demands the use of LC-MS for most of the groups, with the exception of the DA and saxitoxin groups, which can be detected by HPLC. The chemical complexity of each toxin group, with new analogues being added every year, and their different profiles depending on the geographical area and the climate conditions highlight the importance of robust analytical methods and a good supply of analytical standards. Along with this demand, it is very important to continue with the development of high-throughput methods that may provide quick results about the presence of these very toxic compounds in food, as food safety will always be a concern for industries that deal with certain mollusks and certain types of fish.

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Ana M. Botana professor of Analytical Chemistry; 60 international papers.

Luis M. Botana full professor of Pharmacology at the University of Santiago de Compostela (USC), expert in marine toxin pharmacology; 200 international papers, editor of 8 books and author of 25 patents.

Paula Rodríguez and Paz Otero Postdoctoral researchers; 20 international papers.

Amparo Alfonso Professor of Pharmacology at the USC; 90 International papers, and 6 patents.