The analysis of lipophilic marine toxins

Development of an alternative method

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ARJEN GERSSEN

Thesis

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ABSTRACT

Lipophilic marine toxins are produced by certain algae species and can accumulate in filter feeding shellfish such as mussels, scallops and oysters. Consumption of contaminated shellfish can lead to severe intoxications such as diarrhea, abdominal cramps and vomiting. Methods described in European Union (EU) legislation to test for the presence of these toxins are based on a mouse or rat bioassay. These assays are unethical and have a poor sensitivity and selectivity. The aim of this thesis is to develop an alternative method based on liquid chromatography - tandem mass spectrometry (LC-MS/MS) for the quantitative analysis of lipophilic marine toxins.

LC-MS/MS methods described in literature for the determination of lipophilic marine toxins used an acidic chromatographic system. Under acidic conditions peak shape and separation of a number of toxins preferably analyzed in electrospray ionization negative (ESI^-) and positive (ESI^+) were poor. A LC-MS/MS method with alkaline chromatographic conditions in which we were able to analyze 28 different toxins in a single analysis in separated retention time windows operating in either ESI^- or ESI^+ was developed. Furthermore, a clean up procedure based on solid phase extraction (SPE) was developed to reduce the amount of matrix effects (ion suppression and enhancement). A combination of SPE clean up and alkaline chromatographic conditions resulted in reduced matrix effects for all matrices tested (mussel, scallop and oyster).

The developed SPE & LC-MS/MS method was in-house validated at regulatory limits based on EU Commission Decision 2002/657/EC. With respect to accuracy, repeatability, reproducibility, decision limit, specificity and ruggedness the method performed well. The method also performed excellently in view of possible new limits that are four- to five-fold lower than current limits for some toxins.

Finally a screening method based on LC orbitrap MS was developed for 85 marine toxins of which most are not stated in EU legislation. The screening used in-house developed software which made it possible to reduce the complex data files and screen for a large number of toxins within seconds.

This thesis will contribute to the replacement of the animal assays that are still prescribed in EU legislation for the determination of lipophilic marine toxins in shellfish.

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General Introduction

Arjen Gerssen
Irene E. Pol-Hofstad
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Hester J. van den Top
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Marine Toxins: Chemistry, Toxicity, Occurrence and Detection, with Special Reference to the Dutch Situation, Toxins 2010; 2: 878.

GENERAL INTRODUCTION

Of the 5 000 phytoplankton species known to date under specific circumstances about 300 of them have a high proliferation rate, resulting in high density algae clouds: blooms. The circumstances for bloom development are not fully understood yet, but specific climatic and hydrographic conditions play a role in the formation of blooms [1-3]. Blooms are sometimes beneficial for aquaculture and marine biology [4]. However, of the 300 phytoplankton species mentioned above more than 40 species belonging to the classes of dinoflagellates and diatoms are known to produce phycotoxins (marine toxins) [5]. The abundance of these toxic phytoplankton species can vary from thousand until a few million cells per liter. The high abundance blooms of these toxic phytoplankton species are named harmful algae blooms (HABs). It has been suggested that certain phytoplankton species produce toxins to compete for space with other phytoplankton species [6].
Phycotoxins can accumulate in various marine species such as fish, crabs or filter feeding bivalves (shellfish) such as mussels, oysters, scallops and clams. In shellfish, toxins mainly accumulate in the digestive glands without causing adverse effects on the shellfish itself. However, when substantial amounts of contaminated

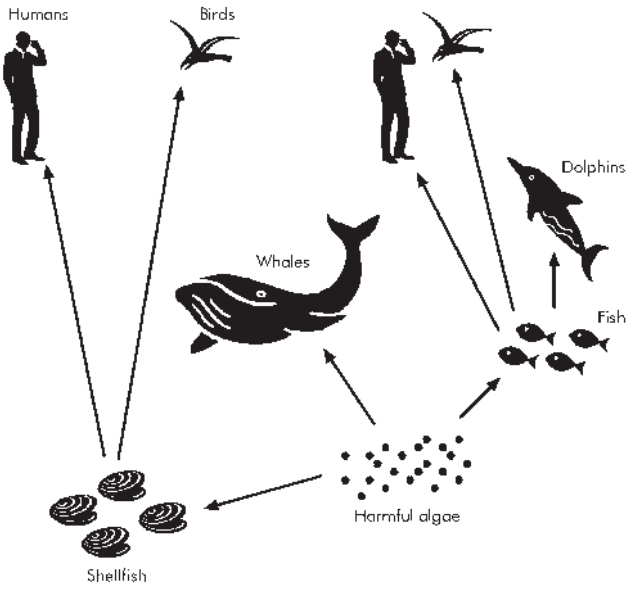

Figure 1.1 Harmful algae blooms in the food chain and their routes of exposure.

shellfish are consumed this may cause severe intoxication of the consumer (Fig. 1.1). Throughout the world, toxins produced by algae (including freshwater cyano toxins) are held responsible for approximately 60 000 human intoxications yearly [7]. Shellfish toxins also cause damage to wildlife [8, 9] and have a negative economic impact on recreation, tourism and shellfish industry. In Europe an estimated annual loss of 720 M€ for the recreation and tourism industry and 166 M€ for the shellfish industry is due to the occurrence of algae blooms [10, 11]. In order to prevent intoxication of the consumer by shellfish toxins, legislation has been developed and monitoring programs have been established worldwide [12, 13]. In this introduction an overview is given of the various types of poisoning syndromes, their corresponding algae and toxins. Furthermore, alternative methods are reviewed that have been developed to replace the animal bioassays that are currently used for the detection of lipophilic marine toxins.

Poisoning Syndromes and Corresponding Toxins

Based on their chemical properties marine shellfish toxins can be divided in two different classes: hydrophilic and lipophilic toxins. Toxins associated with the syndromes Amnesic Shellfish Poisoning (ASP) and Paralytic Shellfish Poisoning (PSP) are hydrophilic and have a molecular weight (MW) below 500 Da. Toxins responsible for Neurologic Shellfish Poisoning (NSP), Diarrhetic Shellfish Poisoning (DSP), Azaspiracid Shellfish Poisoning (AZP) and other toxins such as pectenotoxins, yessotoxins and cyclic imines all have as common denominator a MW above 600 Da (up to 2 000 Da). These toxins have strong lipophilic properties. Therefore, these toxins are generally called lipophilic marine toxins.

Hydrophilic toxins

Amnesic Shellfish Poisoning (ASP)

The diatom *Pseudo-nitzschia pungens* is one of the most important species of the more than 10 known producers of domoic acid (DA) (Fig. 1.2), the toxin responsible for ASP (Table 1.1). In addition, a number of toxic DA isomers have been described in the literature [14]. The primary action of DA is on the hippocampus, which is involved in processing memory and visceral functions [15]. DA is a neurotoxin that binds with a high affinity to glutamate receptors. This

binding leads to opening of the membrane channels (permeable to sodium). This, in turn, leads to an increased sodium influx and membrane depolarization. The adverse effects reported are gastrointestinal disorders, nausea, vomiting, abdominal cramps and diarrhea. Furthermore, also headache, dizziness and loss of the short-term memory can occur [16, 17].

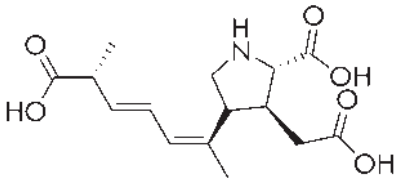

Figure 1.2 Chemical structure of domoic acid (DA).

ASP intoxication in humans was first reported in 1987 at Prince Edward Island, Canada [18]. During this toxic episode three people died and more than 100 were admitted to the hospital after consuming blue mussels (*Mytilus edulis*) with high levels of DA [17]. DA occurrence in shellfish is a global issue. In recent years shellfish containing DA have been reported in the USA, Canada, France, United Kingdom (UK), Spain, Ireland and Portugal [18-23]. The European Union (EU) has established a permitted level of 20 mg DA/kg shellfish. In 2009, the European Food Safety Authority (EFSA) published an opinion on DA [24]. In this opinion the panel recommended that it is safe to consume shellfish which contain less than 4.5 mg DA/kg shellfish in order to not exceed the acute reference dose (ARfD). DG SANCO (responsible for health and consumer protection in the EU) will discuss the EFSA opinion with the different EU member states and this may result in new legislation.

Paralytic Shellfish Poisoning (PSP)

Dinoflagellates of the *Alexandrium* genus are the producers of saxitoxins [saxitoxin, (Fig. 1.3)], the group of toxins responsible for PSP (Table 1.1). Within the saxitoxin group around 30 different analogues have been detected [45]. Not every analogue exhibits the same toxicity and nowadays for the most prominent analogues, toxic equivalent factors (TEF) have been established [46]. Saxitoxin

Table 1.1 Various marine toxin groups and their responsible algae.

	Toxin group	Syndrome	Genus	Species	Reference
Hydrophilic toxins	Domoic acid	ASP	*Pseudo-nitzschia*	*australis, calliantha, cuspidata, delicatissima, fraudulenta, galaxiae, multiseries, multistriata, pseudodelicatissima, pungens, seriata, turgidula*	[25]
	Saxitoxins	PSP	*Alexandrium*	*angustitabulatum, catenella, fundyense, lusitanicum, minutum, tamarense, tamiyavanichii*	[26-28]
			Gymnodinium	*catenatum*	[26]
			Pyrodinium	*bahamense*	[26]
Lipophilic toxins	Brevetoxins	NSP	*Karenia*	*brevis, brevisulcata, mikimotoi, selliformis, papilionacea*	[8, 29] [30]
			Chantonella	*cf. verruculosa*	[30]
	Okadaic acid, dinophysistoxins and pectenotoxins[1]	DSP	*Phalacroma*	*rotundatum*	[31]
			Prorocentrum	*arenarium, belizeanum, concavem, lima*	[32]
			Dinophysis	*acuminata, acuta, arenarium, caudate, fortii, mitra, norvegica, ovum, rotundata, sacculus, tripos*	[33-38]
	Yessotoxins	DSP	*Protoceratium*	*reticulatum*	[29, 39]
			Lingulodinium	*polyedrum*	[29]
			Gonyaulax	*polyhedra*	[29]
	Azaspiracids	AZP	*Azadinium*	*spinosum*	[40]
	Spirolides		*Alexandrium*	*ostenfeldii, peruvianum*	[41,42]
	Gymnodimines		*Karenia*	*selliforme*	[43]
			Gymnodium	*mikimotoi*	[44]

[1)] Pectenotoxins do not induce diarrhea but are produced by the same algae as the DSP toxins OA and DTXs.

causes inhibition of the voltage-gated sodium channel resulting in a reduced action potential [47]. Adverse effects of intoxication with saxitoxins start with tingling or numbness around the lips. These effects spread to the neck and face. In a progressed state, prickly sensation of fingertips, headache, dizziness, nausea, vomiting and diarrhea can occur. Even temporary blindness has been reported [46, 48]. When high levels of saxitoxins are consumed also the motor nerves are affected, resulting in respiratory difficulties and other muscular paralytic effects [49]. Eventually, this may lead to death [50].

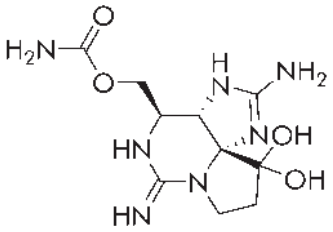

Figure 1.3 Chemical structure of saxitoxin (STX).

First reports of PSP intoxication are from 1920 in California, USA when at least six people died [51]. Until the 1970's PSP toxins were only detected in European, North American and Japanese waters. Nowadays, saxitoxins have been reported in Chile, South-Africa, Australia and other countries as well [52-54]. In most countries monitoring programs have been established to protect the consumer. The EU has established a permitted level of 800 μg saxitoxin 2HCl equivalents/kg shellfish. Recently (2009) the EFSA published an opinion on the saxitoxin group [46]. In this opinion it is recommended a safe level is as low as 75 μg saxitoxin 2HCl equivalents/kg in order to avoid exceeding the ARfD [46].

Lipophilic toxins

Neurologic Shellfish Poisoning (NSP)

NSP is caused by brevetoxins [brevetoxin-2, (Fig. 1.4)]. These are produced by the algae species of the *Karenia* genus (Table 1.1) [8, 30]. Brevetoxins cause opening of the voltage-gated sodium channels, leading to an influx of sodium in the cells and to a complete blockade of the neuronal excitability [55]. Adverse effects

observed are diarrhea, vomiting, cramps, rapid reduction of the respiratory rate and cardiac conduction disturbances which can lead to a coma and eventually to death [30]. In addition to consumption of brevetoxin-contaminated shellfish, intoxication can occur due to inhalation of aerosols produced by breaking waves at the shoreline [56, 57]. Inhalation of brevetoxin aerosols may result in respiratory problems and eye and nasal membrane irritation. Until now NSP intoxications have been limited to the USA (Gulf of Mexico and Florida) and New Zealand [58, 59]. As these toxins have not been found in Europe no legislation has been set for these toxins and no monitoring programs have been established. In the USA, legislation has been set by the Food and Drug Administration (FDA); the current regulatory limit is 800 μg brevetoxin-2 (PbTx-2) equivalents/kg shellfish [60]. At the time of writing, the EFSA had not published a scientific opinion on NSP-type toxins.

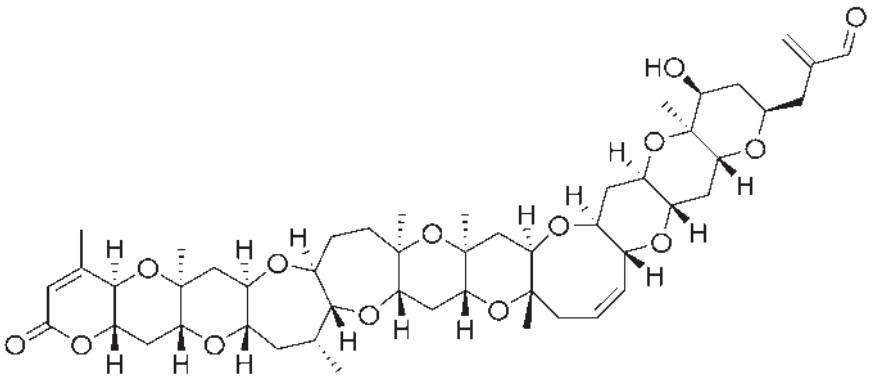

Figure 1.4 Chemical structure of brevetoxin (PbTx-2).

Diarrhetic Shellfish Poisoning (DSP)

Okadaic acid [OA, (Fig. 1.5)], dinophysistoxin-1 (DTX1) and -2 (DTX2) as well as the esterified forms of OA, DTX1 and DTX2 are produced by the *Dinophysis* genus (Table 1.1) [35]. Toxins of the OA group inhibit the serine and threonine phosphatases PP1 and PP2A [61]. This inhibition leads to hyperphosphorylation of proteins involved in the cytoskeletal junctions that regulate the permeability of the cell, resulting in a loss of cellular fluids [62]. Consumption of shellfish contaminated with high levels of OA-type toxins will result in adverse effects such as gastrointestinal disorder, diarrhea, abdominal cramps, nausea and vomiting

[63]. Furthermore, OA and DTX1 have been shown to be tumor promoting substances in animal tests [64].

The first documented human intoxication caused by DSP toxins was in The Netherlands in 1961 [65]. Nowadays, high levels of OA group toxins are repeatedly reported in shellfish or algae along the coasts of Europe (UK, Ireland, Denmark, Sweden, Norway, France, Spain, Italy, Portugal, The Netherlands and Belgium), Canada, South America (Chile), Japan, Australia and Africa (Morocco) [63, 66, 67]. TEF values for OA, DTX1 and DTX2 have been established (Table 1.2) [68, 69]. Within Europe the permitted level for the total amount of OA, DTXs and PTXs in shellfish has been set at 160 μg OA-equivalents/kg shellfish. In 2008, the EFSA panel concluded in their opinion on OA and analogues that OA and DTXs should not exceed 45 μg OA-equivalents/kg shellfish in order not to exceed the ARfD. For PTXs, a separate EFSA opinion has been prepared [70].

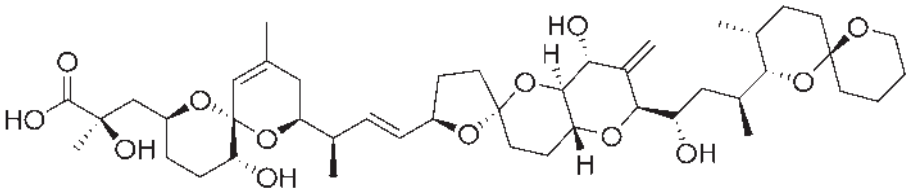

Figure 1.5 Chemical structure of okadaic acid (OA).

Pectenotoxins (PTXs) [pectenotoxin-2, (Fig. 1.6)] are produced by the same phytoplankton species as toxins of the OA group, the *Dinophysis* genus [33]. Approximately 15 different PTXs have been described to date [71, 72]. Pectenotoxin-2 (PTX2), pectenotoxin-2 seco acid (PTX2sa) and 7-epi pectenotoxin-2 seco acid (7-epi PTX2sa) are the predominant analogues in European shellfish [73]. The toxicity after i.p. or oral administration in mice of PTXs is considered to be comparable. After i.p. injection of PTX2, liver damage such as the generation of vacuoles and deformation of hepatocytes has been observed [74]. Oral administration of PTX2 resulted in histopathological changes in the liver and stomach of mice but no diarrhea has been observed [75]. No human intoxications by PTXs have been reported yet. As discussed earlier, PTXs are currently included in the European legislation in the OA group but EFSA has recently suggested that the

PTXs should be classified individually. The EFSA panel proposed a permitted level of 120 μg/kg PTX2 equivalents [70].

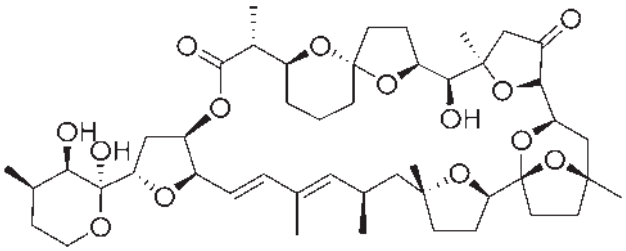

Figure 1.6 Chemical structure of pectenotoxin-2 (PTX2).

Yessotoxins (YTXs) [yessotoxin, (Fig. 1.7)] are produced by the dinoflagellates *Proceratium reticulatum* and *Lingulodinium polyedrum* [39, 76]. Until now up to 90 YTX analogues have been identified [77]. The most abundant toxins found in shellfish are YTX and the metabolites 45OH-YTX, 44COOH-YTX and their corresponding 1a-homologues [78]. Some analogues of YTX have only been found in certain regions such as adriatoxin in the Adriatic sea [79]. When injected i.p. the toxicity of YTX is relatively high, with a LD_{50} for mice of 750 μg/kg. In contrast, oral administration of high levels of YTX (7.5 and 10 mg/kg) did only result in some swelling of the heart muscle cells of mice [80]. Until now, no human intoxications caused by consumption of YTX contaminated shellfish have been reported. YTXs levels exceeding the current EU regulatory level (1 mg/kg) have

Table 1.2 Toxic equivalent factors of lipophilic marine toxins.

Toxin	TEF	Reference
Okadaic acid	1	[68]
Dinophysistoxin-1	1	
Dinophysistoxin-2	0.6	
Yessotoxin	1	[83]
1a-homo yessotoxin	1	
45OH yessotoxin	1	
45OH-1a-homo yessotoxin	0.5	
Azaspiracid-1	1	[84]
Azaspiracid-2	1.8	
Azaspiracid-3	1.4	

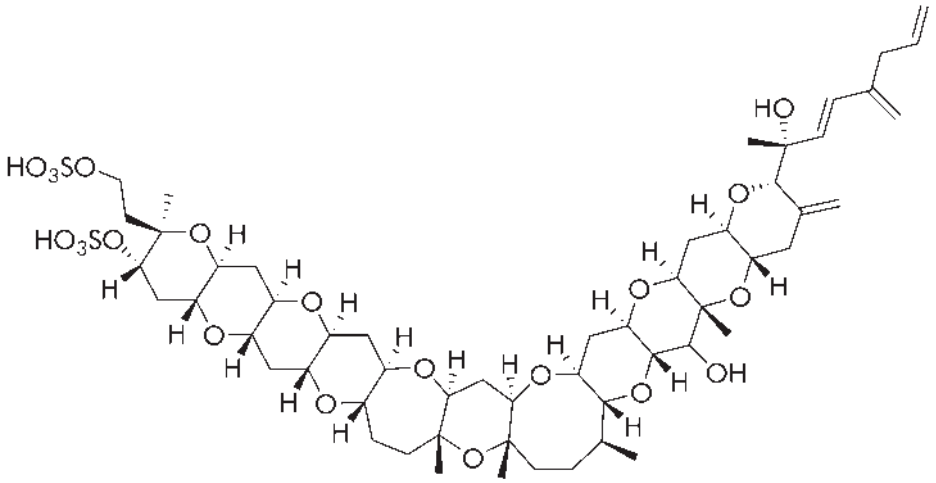

Figure 1.7 Chemical structure of yessotoxin (YTX).

occasionally been found in Italy, Norway and Portugal [78, 81, 82]. EFSA has suggested that a consumer is protected when shellfish do not exceed a concentration of 3.75 mg YTX-equivalents/kg shellfish [83]. EFSA identified YTX, 1a-homo-YTX, 45OH-YTX and 45OH-1a-homo-YTX as the most important YTXs present in shellfish. For these toxins TEFs have been established (Table 1.2) [83].

Diarrhetic Shellfish Poisoning is caused by OA and its DTX analogues. YTXs and PTXs are often included in the group of DSP toxins as they often co-occur with OA and DTX analogues although they do not cause diarrhea. Therefore, removal of these toxins from the DSP group should be considered. To our opinion lipophilic marine toxins is a better term to classify the toxins belonging to these groups.

Azaspiracid Shellfish Poisoning (AZP)

For years azaspiracids [azaspiracid-1 (Fig. 1.8)] were thought to be produced by *Protoperidinium crassipes* [85], although a clear correlation between high algae counts and toxin levels was lacking [86]. Recently, it was discovered that the AZAs are produced by a minute dinoflagellate [40, 86]. This dinoflagellate, *Azadinium spinosum*, is smaller (12–16 μm) than any of the other toxin-producing

dinoflagellates known so far. Until now, 24 different AZAs have been described, with azaspiracid-1 (AZA1), -2 (AZA2), -3 (AZA3) as the predominant ones [87]. The mechanism of action is not yet fully understood, but in-vitro experiments in mammalian cell lines showed alterations in the cytoskeletal structure, and an effect on the E-cadherin system, which is responsible for the cell-cell interactions [88-90]. This could explain the toxic effects such as gastrointestinal disorder, diarrhea and abdominal cramps that are observed during AZP intoxication [85, 91]. In 1995, the first intoxication due to AZP was reported, when in The Netherlands at least eight people got ill after consumption of mussels imported from Ireland. The rat bioassay, normally applied to detect OA type toxins, revealed the presence of diarrhetic toxic activity, where the mouse bioassay lacked detection of these toxins. Since then several AZP outbreaks have occurred in Ireland and by now AZAs have been detected in Ireland, UK, Norway, France, Portugal, Northern Africa (Morocco), South America (Chile) and the USA [67, 85, 92-97]. According to current EU legislation the total amount of AZAs should not exceed 160 μg/kg AZA1 -equivalents [98]. Recently, EFSA reviewed all available toxicity data and suggested that a safe level of AZA toxins in shellfish is below the ARfD of 30 μg AZA-1 equivalents/kg shellfish [84]. Furthermore, EFSA suggested TEFs for three most important AZAs (Table 1.2) [84].

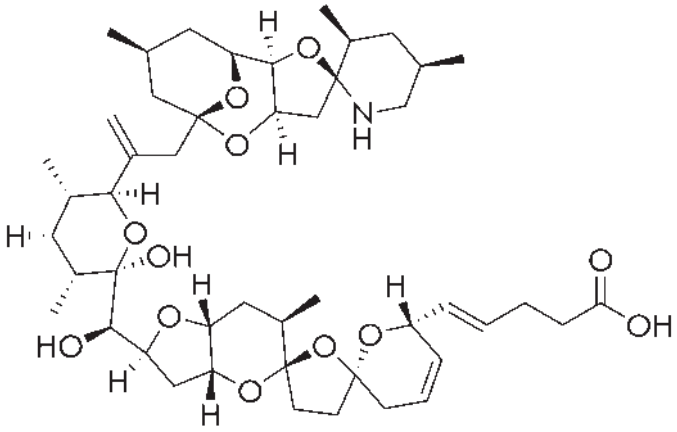

Figure 1.8 Chemical structure of azaspiracid-1 (AZA1).

Spirolides and gymnodimines (cyclic imines)

Spirolides (SPXs) [13-desmethyl spirolide C (Fig. 1.9)] and gymnodimines are toxins belonging to the cyclic imine group. SPXs are produced by *Alexandrium ostenfeldii* (Table 1.1) [41, 99]. Approximately 10–15 different SPXs (including esters) have been found in either algae or shellfish [100-102].

The mechanism of action is not yet completely understood, but i.p. injection of shellfish extracts containing SPXs or gymnodimines is causing death of the test animal within minutes [103]. For this reason these toxins have been classified as fast-acting toxins. Intoxications of humans with cyclic imines have not been reported yet. SPXs have been found, however, in algae and shellfish from Norway, Canada, Denmark, Spain and Chile [95, 100, 104], while gymnodimines thus far has only been detected in algae and shellfish from New-Zealand [44]. Currently, there is no EU-legislation for the cyclic imines. This toxin group was recently reviewed by the EFSA [164].

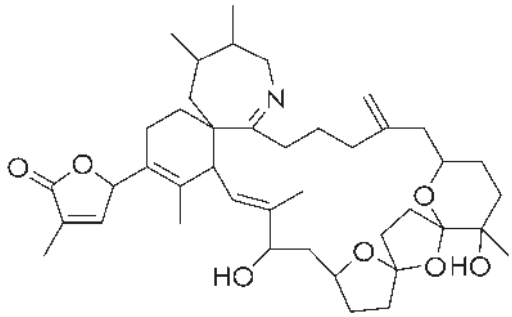

Figure 1.9 chemical structure of 13-desmethyl spirolide C (SPX1).

METHODS OF ANALYSIS

For the determination of marine toxins various biological (in-vivo and in-vitro), biochemical and chemical methods have been described in the literature. However, for lipophilic marine toxins chemical methods for long were not available. In this paragraph, an outline will be given on the official methods stated in European legislation and alternative methods developed in recent years.

The last decade has seen a strong increase in peer-reviewed papers on lipophilic marine toxins (Fig. 1.10). In general, method development and method validation

for lipophilic marine toxins was hampered for many years by the lack of (certified) standards and (certified) reference materials. As shown in figures 1.4 – 1.9 the chemical structures of the toxins are complex and, consequently, it is too difficult and expensive to synthesize them [105]. Therefore, standards need to be isolated from either contaminated shellfish or algae [106, 107]. In recent years considerable efforts have been made to expand the number of available toxins. In 2005, only small amounts of reliable reference standards were available for OA and PTX2. In 2007 YTX, AZA1 and SPX1 became available. Since then, of all important lipophilic marine toxin groups at least one certified standard is available (OA, PTX2, YTX, AZA1 and SPX1). It is expected that other important reference standards such as DTX1, DTX2, AZA2 and AZA3 will become available in the course of 2010.

Current official methods described in legislation and their limitations

EU legislation prescribes a biological test for the determination of OA, DTXs, YTXs, PTXs and AZAs in shellfish. This biological test can be a mouse (MBA) or a rat bioassay (RBA). The MBA was developed in Japan and the RBA in The Netherlands in the 1970s [65, 108]. Various laboratories have adjusted the MBA which has resulted in different protocols [109, 110]. In Europe a detailed procedure has been described by the Community Reference Laboratory on marine toxins (CRL-MB, Vigo, Spain) in order to standardize the protocol for the MBA [111]. Shellfish extracts are prepared by acetone extraction followed by liquid-liquid partitioning with dichloromethane or diethylether. After evaporation the extract is reconstituted in 1% polysorbate 20 solution. These extracts are injected i.p. into three male mice with a body weight of 20 g. Preferably the hepatopancreas of the shellfish should be used, as most toxins tend to concentrate in that part, only about AZAs there can be a discussion if these toxins diffuse into the shellfish flesh [91, 112]. If at least two out of the three mice die within 24 hours after injection, the sample is considered positive for lipophilic marine toxins [13]. Unfortunately, low levels of SPXs can also cause mouse death, even within minutes [103]. This indicates that the MBA lacks specificity. A strong point of the assay is that it can signal possible new emerging marine toxins. The RBA, an official EU method that is only applied in The Netherlands, is based on consumption of shellfish (see also section

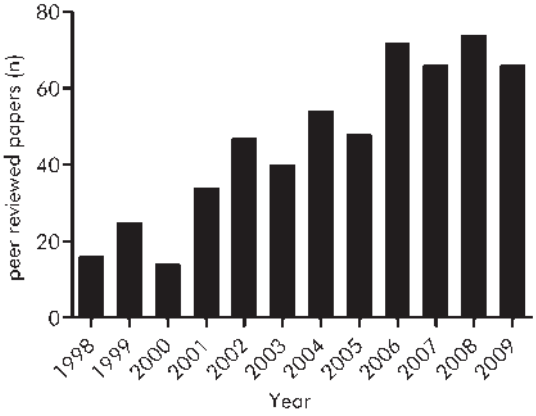

Figure 1.10 Number of peer reviewed publications for lipophilic marine toxins in the last decade.

'Occurrence of toxic events in The Netherlands'). Starved (24h) female rats are fed with 10 g of hepatopancreas of the shellfish. After 16h the consistency (softening) of the faeces is investigated. Severe diarrhea corresponds with toxin levels around the current EU legislation (160 μg/kg OA-equivalents or 160 μg/kg AZA1-equivalents) [68]. A major drawback of the RBA is that YTXs and PTXs are not detected at the regulatory limit because they do not induce diarrhea. Furthermore, the analyst needs to build up experience for a precise interpretation of the test results (texture of faeces). More in general, the limitations of the MBA and RBA are lack of specificity and sensitivity, no elucidation of the toxin profile is possible, and the frequent generation of false positive results. For these reasons, within Europe many countries now use a combination of an animal test and a chemical test (Table 1.3). Furthermore, the MBA in particular is becoming increasingly unacceptable for ethical reasons and this provides a strong impetus to out phase and replace the MBA.

From a worldwide perspective, the regulation of the lipophilic marine toxins differs widely. These differences are related to the presence or absence of the toxins in specific regions and on the methodology applied. In the USA the FDA has only installed OA and DTX1 legislation, while no routine monitoring programs for these toxins have been established yet (Table 1.4) [60, 114]. Canadian guidelines only mention maximum levels for OA and DTX1 in digestive glands [115]. In Japan, the

level has been expressed in mouse units (MU) which is a common way to express the regulatory limit when the MBA is applied [114]. In Australia and New Zealand a regulatory limit has been established for OA and DTX1, DTX2 and DTX3 [116]. In Europe most types of lipophilic marine toxins can be found in shellfish and as a result EU legislation covers OA, DTXs, PTXs, YTXs and AZAs.

Development of alternative methods

In-vitro assays

Functional assays are currently being developed as alternatives to the bioassays. Functional assays are based on the toxicological mode of action of a group of toxins in a biological process. Advantages of functional assays are their potential for high-throughput screening, detection of new toxins, while there is no need for applying TEF values. Still, false positives or negatives can occur due to matrix substances present in the extract or due to metabolic activation. It is extremely difficult to develop a functional assay that will comprise all lipophilic marine toxins in a single assay. Until now, functional assays have been developed for the OA group toxins, YTXs, PTXs and SPXs. Toxins of the OA group can be determined by protein phosphatase 2A (PP2A) inhibitor assays using fluorometric detection. Several of these assays have been published in recent years [117-119]. A good correlation between the MBA and the PP2A fluorometric assay has been obtained in several laboratories [117, 120]. Furthermore, for the OA group toxins and PTXs a cytotoxicity assay based on actin fillament depolymerization in a BE(2)-M17 neuroblastoma cell line has been developed [121]. For the OA group toxins and YTXs an assay was developed based on the reduction of cell-cell adhesion in MCF-7 and Caco-2 cells leading to an accumulation of E-cadherin [122, 123]. Also AZA1 showed an effect on the cell-cell adhesion and E-cadherin influx, but these results have not resulted in a functional assay format yet [88]. Unfortunately, with respect to OA and YTX the reproducibility of the assay was rather poor. Therefore the assay should be made more robust prior to routine application. Recently, a fluorescence polarization inhibition assay has been developed for SPXs. The assay uses nicotinic acetylcholine receptor-enriched membranes of the marbled electric ray (*Torpedo marmorata*) and is capable to analyze contaminated mussels with SPX concentrations in the range of 70–700 μg/kg [124].

Table 1.3 Methods used for the official control of lipophilic marine toxins.

Country	OA and DTXs	AZAs	PTXs	YTXs	Reference
Norway	MBA Chemical	Chemical	Chemical	MBA Chemical	[113]
Sweden[1]	MBA Chemical	MBA Chemical	MBA Chemical	MBA Chemical	[113]
Finland[2]					[113]
Denmark	MBA Chemical	Chemical	Chemical	Chemical	[113]
Ireland	MBA Chemical	MBA Chemical	MBA Chemical	MBA Chemical	[113]
United Kingdom	MBA	MBA	MBA	MBA	[113]
Germany	Chemical	Chemical	Chemical	Chemical	[113]
The Netherlands	RBA Chemical	RBA			[113]
Belgium	MBA	MBA	MBA	MBA	[113]
France	MBA	MBA	MBA	MBA	[113]
Austria	MBA Chemical	MBA Chemical	MBA Chemical	MBA Chemical	[113]
Portugal	MBA Chemical Biochemical	Chemical	Chemical	MBA	[113]
Spain	MBA	MBA	MBA	MBA	[113]
Italy	MBA Chemical	MBA	MBA	MBA Chemical	[113]
Greece	MBA Chemical	MBA			[113]
Turkey	MBA	MBA	MBA	MBA	[114]
Canada	MBA	MBA	MBA	MBA	[114]
United States[3]					[114]
Venezuela	MBA	MBA	MBA	MBA	[114]
Brazil[3]					[114]

Table 1.3 continued.

Country	OA and DTXs	AZAs	PTXs	YTXs	Reference
Chili	MBA	MBA	MBA	MBA	[114]
Uruguay	MBA	MBA	MBA	MBA	[114]
Republic of Korea	MBA Chemical	MBA Chemical	MBA Chemical	MBA Chemical	[114]
Japan	MBA	MBA	MBA	MBA	[114]
Thailand	MBA	MBA	MBA	MBA	[114]
New Zealand	Chemical	Chemical	Chemical	Chemical	[113]

MBA = mouse bioassay, RBA = rat bioassay.
Chemical = high performance liquid chromatography (HPLC), LC fluorometric detection (LC-FLD), LC mass spectrometry (LC-MS), LC tandem MS (LC-MS/MS), Biochemical = enzyme linked immunosorbent assay (ELISA).
[1)] Samples for the MBA are conducted in Norway.
[2)] MBA test for DSP is prohibited.
[3)] No monitoring established.

Of the functional assays developed thus far, most promising results have been obtained with the PP2A assay for the OA group toxins and the nicotinic acetylcholine receptor assay for SPXs. However, successful validation (single- and inter-lab) of these methods is still lacking.

Biochemical methods

In immunochemical methods antibodies are used that show affinity with specific structural parts of a toxin. Analogues of these toxins can often be detected by cross -reactivity, but no information is gained about differences in toxicity. Therefore, methods such as enzyme-linked immunosorbent assay (ELISA) can only be used for screening of shellfish samples. For some of the lipophilic marine toxin groups immunochemical methods have been developed. For the OA group an ELISA has been converted to a lateral flow immunochromatographic (LFI) format. The test strips allow the analysis of toxins on site without the use of lab facilities [125]. In principle, this would enable shellfish industry to carry out these tests themselves. A recent study on these test trips showed that a relative high number of samples (45%) were misidentified as positive [126]. Further research is needed to make this

Table 1.4 Permitted levels for lipophilic marine toxins.

Country or Continent	OA, DTXs (μg/kg)	PTXs (μg/kg)	AZAs (μg/kg)	YTXs (μg/kg)	MBA (MU/kg)	Reference
Europe	160 WF	Included in OA	160	1 000		[12]
United States	200	NR	NR	NR		[60]
Canada	1 000 DG	NR	NR	NR		[115]
Japan					50 (~200μg/ kg OA-eq)	[114]
Australia and New Zealand	200 WF	NR	NR	NR		[116]

WF = Whole shellfish flesh, DG = digestive glands and NR = not regulated, MU = mouse unit.

LFI suitable for routine monitoring purposes. Other biochemical methods that are currently under development for the OA group make use of amperometric immunosensors and immunobiosensors using surface plasmon resonance (SPR) [127, 128]. A sensitive ELISA for YTX has been developed with good correlation to a chemical method based on liquid chromatography / mass spectrometric detection. Its working range would make this ELISA suitable for routine monitoring [129, 130]. The advantage of this YTX ELISA is the cross-reactivity towards many YTX analogues [129], although it is unclear whether these analogues are toxic. Other promising biochemical methods for YTXs are SPR based biosensors, a resonance mirror bioassay and fluorescence polarization [131-133]. For the PTXs, AZAs and SPXs no biochemical methods are available yet. Most promising results have been obtained with the OA and the YTX group ELISA. Provided proper validation is carried out, these rapid screening biochemical methods can be used for high sample throughput analysis of shellfish toxins.

Chemical methods

In the 1980's, the first chemical detection methods developed for the OA group toxins were based on liquid chromatography (LC) coupled to fluorometric detection (LC-FLD). As most lipophilic marine toxins lack chromophores, a derivatisation step

was required. For toxins of the OA group 9-anthryldiazomethane (ADAM) [134] and for PTXs and YTXs 4-[2-(6,7–dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalinyl)ethyl]-1,2,4-triazoline-3,5-dione (DMEQ-TAD) have been used as derivatisation reagents [135, 136]. A major drawback of LC-FLD is its limited selectivity for the OA group toxins as well as for the PTXs and YTXs. The derivatisation step is rather laborious and can be critical. For AZAs and SPXs no LC-FLD methods have been developed. This is probably due to the fact that these toxins were only discovered in the mid 1990s when LC (tandem) mass spectrometry [LC-(MS)/MS] became increasingly popular.

In recent years much effort has been put in the development of LC-MS/MS methods that are dedicated to either detecting the specific classes of lipophilic marine toxins or detecting as many as possible different lipophilic marine toxins in a multi-toxin method. Many of the methods developed for specific classes of lipophilic marine toxins focused on either structure elucidation or on discovery of new lipophilic marine toxins. For example for the OA group toxins LC-MS/MS techniques have been used to identify new DTXs [137-140]. By now up to 40 different toxins belonging to the OA toxin group have been identified using LC-MS/MS [140, 141]. Several LC-MS/MS methods have been developed to detect new toxins (YTXs and PTXs) in either algae or shellfish [71, 77, 142-145]. Furthermore, LC-MS/MS has been used to investigate the transformation of toxins into metabolites. The conversion of YTX to 45OH-YTX and 44COOH-YTX and the conversion of PTX2 to PTX2sa have been studied by LC-MS/MS [75, 78, 146]. Another LC-MS/MS method was developed to determine up to 24 different AZAs in a single analysis [87]. Some dedicated methods were used to study the metabolic processes taking place when AZA contaminated mussels are heat-treated [147]. Also, with the help of LC-MS/MS new SPX analogues have been identified that are either produced in algae or in shellfish [101, 102].

Most of the methods described above were used for research purposes and were not intended for the monitoring programs. Nowadays, several LC-MS/MS methods are available to determine most or all toxin classes belonging to the lipophilic marine toxins. The first two multi-toxin LC-MS/MS methods for lipophilic marine toxins were developed in 2001 [148, 149]. Unfortunately, one method did not include the YTXs [148] while the other one used a laborious sample clean up

procedure based on liquid-liquid extraction and various solid phase extraction procedures [149]. Therefore, these methods were not suitable for routine monitoring programs. In 2005 two new multi-toxin methods were developed that included toxins from all regulated lipophilic marine toxin classes in the EU [150, 151]. These methods were in-house validated and good performance characteristics were obtained. Drawbacks were the exclusion of spirolides in one method [151] and poor chromatography for some compounds in the other one [150]. In 2007 a very high pressure liquid chromatography (VHPLC)-MS/MS method was developed. With this method it was possible to analyze 21 marine lipophilic toxins in only 6.6 minutes [152]. It should be mentioned that the separation and detection could only be accomplished by the newest generation LC and MS equipment. This VHPLC-MS/MS method has not been validated yet. The latest developed multi-toxin method was published in 2009 [153]. By a different choice of chromatographic conditions, all chromatography problems have been solved and the method has been in-house validated [98]. All prominent lipophilic marine toxins were included in this method (**Chapter 3** of this thesis). Currently, for this method a full collaborative validation study according to international guidelines is in preparation.

Occurrence of Toxic Events in the Netherlands

In The Netherlands until now only DSP has occurred, the other toxic syndromes (ASP and PSP) have not been reported. Only in 2002 one shellfish sample has been tested positive for domoic acid (unpublished data provided by M Poelman). Therefore, this historic overview only deals with the DSP syndrome. The first incidences outside The Netherlands were reported in Japan (1976 and 1977) [109]. Japanese researchers identified *Dinophysis fortii* as the algae producing this toxin. Therefore, the toxin was named Dinophysistoxin and the poisoning syndrome was named Diarrhetic Shellfish Poisoning (DSP) [154]. In 1982 the structure of the causative toxin, dinophysistoxin-1, was finally elucidated [155].

In The Netherlands the first incidences of poisoning associated with consumption of mussels were reported in July and August 1961 [65]. People that had consumed mussels experienced abdominal cramps, vomiting and severe diarrhea. At the same time, in the Eastern Scheldt and the Wadden Sea high concentrations of the

dinoflagellates *Prorocentrum micans*, *P. triestinum*, *P. minimum* and *Dinophysis acuminata* were reported. In the following years, these algae were isolated from the gastrointestinal tract (hepatopancreas) of the mussels. Following this episode, human intoxications re-occurred in The Netherlands in 1971 (mussels from the Eastern Scheldt), 1976, 1979 (mussels from the Wadden Sea) and 1981 (mussels from the Eastern Scheldt and Wadden Sea) [156-158]. In 1979 a rat bioassay was developed for the detection of these toxins and to prevent human intoxication [65] and this RBA was adopted as the official method of control for the detection of diarrhea causing toxins in The Netherlands. The monitoring program for DSP toxins in the Netherlands includes an early warning system and the pre-market analysis of shellfish on the presence of ASP, PSP and DSP toxins. The early warning system monitors the various potential toxic algae in sea water. The RBA was used to test if *P. micans* and *P. minimum* were responsible for the adverse effects observed in 1961. However, mussels contaminated with cultivated algae were fed to rats, but no adverse effects were observed [65]. Therefore, it remained doubtful if these algae were responsible for the toxin production. In 1981 it was demonstrated that in the Netherlands the responsible algae for the toxin production in the Eastern Scheldt and Wadden Sea was *D. acuminata* [159]. In 1986 and 1987 DSP toxins were again detected in the Wadden Sea, but due to the established monitoring program shellfish areas were closed and no human intoxications were reported [160, 161]. In October 1989, a minor episode of DSP toxicity occurred in the Wadden Sea; no incidences of human illness were reported. The production area was closed during the presence of DSP toxins. In 2002 *D. acuminata* caused the presence of DSP toxins in mussels from the Wadden Sea. This was followed by a closure of the production area for several weeks (unpublished data provided by M Poelman). By means of an LC-MS method low levels of toxins could be detected in mussels several weeks before the RBA picked up levels above the EU regulatory limit. In this case intoxication of local fishermen was observed, while the RBA detected levels of DSP toxins after closure of the fishing area (unpublished data provided by M Poelman). In 2005 and 2007 the presence of *D. acuminata* in the Wadden Sea triggered the application of a (delayed) monitoring program using LC-MS/MS. Analysis showed the presence of OA in mussels at levels well below the current regulatory limit, ranging from 18 till

68 μg OA equivalents/kg shellfish. The presence of high numbers of *D. acuminata* triggered analysis of shellfish by LC-MS/MS again in 2009. No detectable amounts of any DSP toxins were found. These results and also those obtained on earlier occasions indicate that there is no clear correlation between the counts of potential toxic algae and toxic events (Fig. 1.11). With respect to the EFSA opinion there are some concentrations found in 2005 and 2007 that are above the ARfD of 45 μg OA equivalents/kg shellfish. Therefore, in case legislation is changed towards the EFSA opinion more positive samples will be found in The Netherlands.
Overall, in the last decade in shellfish of Dutch waters only low levels of OA equivalents have been found.

THESIS OUTLINE

The MBA and RBA are still being used as the official methods in Europe for the detection of lipophilic marine toxins. Within the European Union there is a growing resistance against the use of animal tests for routine monitoring purposes. Based on the 3R's (Reduction, Refinement and Replacement) for animal experiments there is an urgent need for alternative methods [162]. Therefore, the EU has funded a research project (BIOTOX) within the Framework 6 Program on Food Quality and Safety (Food-CT-2004-514074), that focused on the development of cost-effective tools for risk management and traceability systems for marine biotoxins in seafood [163]. A major part of the research described in this thesis was conducted in the frame work of this project. It includes development and validation of alternative methods based on LC-MS/MS for the analysis of lipophilic marine toxins. In **Chapter 2** the performance of four different types of mass spectrometers for the detection of PTX2 and OA was investigated. The mass spectra obtained were used to propose fragmentation schemes for these toxins. In **Chapter 3** the development of a new LC-MS/MS multi-toxin method for the detection of marine lipophilic toxins is presented. The core of this method is an alkaline mobile phase system that has not been used before in the marine toxin field. The results were compared with existing multi-toxins methods. It is well known in LC-MS/MS analysis that matrix effects (signal suppression and signal enhancement) can lead to an under- or overestimation of the concentration. Therefore, improvement of the clean up by

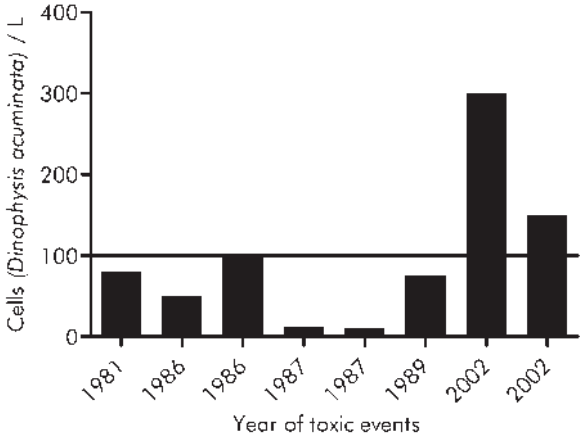

Figure 1.11 Number of *Dinophysis acuminata* cells per liter of sea water on the corresponding years of toxin detection. The line indicates an action limit, above 100 cells per liter corrective measures are taken.

means of solid phase extraction was studied (**Chapter 4**). The matrix effects for a number of different shellfish matrices were investigated before and after the clean up procedure. In **Chapter 5** the validation of the developed methods on the current regulatory limits is described. Method performance criteria based on the proposed EFSA permitted levels were additionally investigated for OA and AZA1. In **Chapter 6** a screening method based on LC with high resolution (100 000 at Full Width Half Maximum) MS is described which is in theory able to screen for several hundreds of marine lipophilic toxins by making use of special data reduction and library searching software.

REFERENCES

[1] Davis TW, Berry DL, Boyer GL, Gobler CJ. The effects of temperature and nutrients on the growth and dynamics of toxic and non-toxic strains of *Microcystis* during cyanobacteria blooms. *Harmful Algae* 2009; **8**: 715.

[2] Moore SK, Trainer VL, Mantua NJ, Parker MS, Laws EA, Backer LC, Fleming LE. Impacts of climate variability and future climate change on harmful algal blooms and human health. *Environ Health* 2008; **7 Suppl 2**: S4.

[3] Peperzak L. Future increase in harmful algal blooms in the North Sea due to climate change. *Water Sci Technol* 2005; **51**: 31.

[4] Benemann JR. Microalgae aquaculture feeds. *J Appl Phycol* 1992; **4**: 233.

[5] Daranas AH, Norte M, Fernandez JJ. Toxic marine microalgae. *Toxicon* 2001; **39**: 1101.

[6] Botana AM, Rodriguez Veijtes M, Alfonso A, Louzao M, Phycotoxins: paralytic shellfish poisoning and diarrhetic shellfish poisoning. In *Handbook of food analysis - Residues and other food component analysis*, Nollet LML, Ed. Dekker: New York, 1996; 1147.

[7] Van Dolah FM, Ramsdell JS. Review and assessment of in vitro detection methods for algal toxins. *J AOAC Int* 2001; **84**: 1617.

[8] Landsberg JH, Flewelling LJ, Naar J. *Karenia brevis* red tides, brevetoxins in the food web, and impacts on natural resources: Decadal advancements. *Harmful Algae* 2009; **8**: 598.

[9] Miller G. Wildlife biology - confused pelicans may have lingered too long up north. *Science* 2009; **323**: 449.

[10] Morgan KL, Larkin SL, Adams CM. Firm-level economic effects of HABS: A tool for business loss assessment. *Harmful Algae* 2009; **8**: 212.

[11] Hoagland P, Scatasta S, The economic effect of harmful algal blooms. In *Ecology on Harmful Algae*, Graneli E, Turner T, Eds. Springer: Berlin, 2006; **189**: 391.

[12] European Parliament, Council. Commission directive 2004/853/EC specific hygiene rules for food of animal origin. *Off J Eur Commun* 2004; **L226**: 22.

[13] European Parliament, Council. Laying down implementing measures for certain products under Regulation (EC) No 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No 854/2004 of the European Parliament and of the Council and Regulation (EC) No 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004. *Off J Eur Commun* 2005; **L338**: 40.

[14] Clayden J, Read B, Hebditch KR. Chemistry of domoic acid, isodomoic acids, and their analogues. *Tetrahedron* 2005; **61**: 5713.

[15] Adams AL, Doucette TA, James R, Ryan CL. Persistent changes in learning and memory in rats following neonatal treatment with domoic acid. *Physiol Behav* 2009; **96**: 505.

[16] Kumar KP, Kumar SP, Nair GA. Risk assessment of the amnesic shellfish poison, domoic acid, on animals and humans. *J Environ Biol* 2009; **30**: 319.

[17] Jeffery B, Barlow T, Moizer K, Paul S, Boyle C. Amnesic shellfish poison. *Food Chem Toxicol* 2004; **42**: 545.

[18] Perl TM, Bedard L, Kosatsky T, Hockin JC, Todd EC, Remis RS. An outbreak of toxic encephalopathy caused by eating mussels contaminated with domoic acid. *N Engl J Med* 1990; **322**: 1775.

[19] Bill BD, Cox FH, Horner RA, Borchert JA, Trainer VL. The first closure of shellfish harvesting due to domoic acid in Puget Sound, Washington, USA. *Afr J Mar Sci* 2006; **28**: 435.

[20] Campbell DA, Kelly MS, Busman M, Bolch CJ, Wiggins E, Moeller PDR, Morton SL, Hess P, Shumway SE. Amnesic shellfish poisoning in the king scallop, *Pecten maximus*, from the west coast of Scotland. *J Shellfish Res* 2001; **20**: 75.

[21] Blanco J, Cano J, Marino MDC, Campos MJ. Effect of phytoplankton containing paralytic shellfish and amnesic shellfish toxins on the culture of the king scallop *Pecten maximus* in Malaga (SE Spain). *Aquat Living Resour* 2006; **19**: 267.

[22] James KJ, Gillman M, Amandi MF, Lopez-Rivera A, Puente PF, Lehane M, Mitrovic S, Furey A. Amnesic shellfish poisoning toxins in bivalve molluscs in Ireland. *Toxicon* 2005; **46**: 852.
[23] Vale P, Sampayo MA. Domoic acid in Portuguese shellfish and fish. *Toxicon* 2001; **39**: 893.
[24] Alexander J, Benford D, Boobis A, Ceccatelli S, Cravedi J-P, Domenico AD, Doerge D, Dogliotti E, Edler L, Farmer P, Filipic M, Fink-Gremmels J, Furst P, Guerin T, Knutsen HK, Livesey C, Machala M, Mutti A, Schlatter J, van Leeuwen R, Verger P. Marine biotoxins in shellfish - Domoic acid. *The EFSA Journal* 2009; **1181**: 1.
[25] Bates S, Trainer VL, The ecology of harmful diatoms. In *Ecology on Harmful Algae*, Graneli E, Turner T, Eds. Springer: Berlin, 2006; **189**: 81.
[26] Beppu R, Nojima K, Tsuruda S, Gomez-Delan G, Barte-Quilantang M, Taniyama S, Sagara T, Nishio S, Takayama H, Miyazawa K, Asakawa M. Occurrence of PSP-producing dinoflagellate *Alexandrium tamiyavanichii* in Bingo-Nada, the central coastal water of the Seto Inland Sea, Hiroshima Prefecture, Japan. *Mar Pollut Bull* 2008; **56**: 758.
[27] Martin JL, Hanke AR, LeGresley MM. Long term phytoplankton monitoring, including harmful algal blooms, in the Bay of Fundy, eastern Canada. *J Sea Res* 2009; **61**: 76.
[28] MacKenzie L, de Salas M, Adamson J, Beuzenberg V. The dinoflagellate genus *Alexandrium (Halim)* in New Zealand coastal waters: comparative morphology, toxicity and molecular genetics. *Harmful Algae* 2004; **3**: 71.
[29] Wang DZ. Neurotoxins from marine dinoflagellates: A brief review. *Mar Drugs* 2008; **6**: 349.
[30] Watkins SM, Reich A, Fleming LE, Hammond R. Neurotoxic shellfish poisoning. *Mar Drugs* 2008; **6**: 431.
[31] Caroppo C, Congestri R, Bruno M. On the presence of *Phalacroma rotundatum* in the southern Adriatic Sea (Italy). *Aquat Microb Ecol* 1999; **17**: 301.
[32] Hu TM, Curtis JM, Walter JA, Wright JLC. Identification of DTX-4, a new water-soluble phosphatase inhibitor from the toxic dinoflagellate *Prorocentrum-Lima*. *J Chem Soc Chem Comm* 1995; 597.
[33] Draisci R, Lucentini L, Giannetti L, Boria P, Poletti R. First report of pectenotoxin-2 (PTX-2) in algae (*Dinophysis fortii*) related to seafood poisoning in Europe. *Toxicon* 1996; **34**: 923.
[34] Jorgensen K, Andersen P. Relation between the concentration of *Dinophysis acuminata* and diarrheic shellfish poisoning toxins in blue mussels (*Mytilus edulis*) during a toxic episode in the Limfjord (Denmark), 2006. *J Shellfish Res* 2007; **26**: 1081.
[35] MacKenzie AL, Beuzenberg V, Holland PT, McNabb P, Suzuki T, Selwood A. Pectenotoxin and okadaic acid-based toxin profiles in *Dinophysis acuta* and *Dinophysis acuminata* from New Zealand. *Harmful Algae* 2005; **4**: 75.
[36] Ten-Hage L, Delaunay N, Pichon V, Coute A, Puiseux-Dao S, Turquet J. Okadaic acid production from the marine benthic dinoflagellate *Prorocentrum arenarium Faust* (*Dinophyceae*) isolated from Europa Island coral reef ecosystem (SW Indian Ocean). *Toxicon* 2000; **38**: 1043.
[37] Raho N, Pizarro G, Escalera L, Reguera B, Marin I. Morphology, toxin composition and molecular analysis of *Dinophysis ovum* Schutt, a dinoflagellate of the "*Dinophysis acuminata* complex". *Harmful Algae* 2008; **7**: 839.
[38] Spatharis S, Dolapsakis NP, Economou-Amilli A, Tsirtsis G, Danielidis DB. Dynamics of potentially harmful microalgae in a confined Mediterranean Gulf-Assessing the risk of bloom formation. *Harmful Algae* 2009; **8**: 736.
[39] Loader JI, Hawkes AD, Beuzenberg V, Jensen DJ, Cooney JM, Wilkins AL, Fitzgerald JM, Briggs LR, Miles CO. Convenient large-scale purification of yessotoxin from *Protoceratium reticulatum* culture and isolation of a novel furanoyessotoxin. *J Agric Food Chem* 2007; **55**: 11093.
[40] Tillmann U, Elbrachter M, Krock B, John U, Cembella A. *Azadinium spinosum* gen. et sp. nov. (*Dinophyceae*) identified as a primary producer of azaspiracid toxins. *Eur J Phycol* 2009; **44**: 63.
[41] Cembella AD, Lewis NI, Quilliam MA. The marine dinoflagellate *Alexandrium ostenfeldii* (*Dinophyceae*) as the causative organism of spirolide shellfish toxins. *Phycologia* 2000; **39**: 67.
[42] Touzet N, Franco JM, Raine R. Morphogenetic diversity and biotoxin composition of *Alexandrium* (*Dinophyceae*) in Irish coastal waters. *Harmful Algae* 2008; **7**: 782.

[43] Miles CO, Wilkins AL, Stirling DJ, MacKenzie AL. Gymnodimine C, an isomer of gymnodimine B, from *Karenia selliformis*. *J Agric Food Chem* 2003; **51**: 4838.
[44] Seki T, Satake M, Mackenzie AL, Kaspar HF, Yasumoto T. Gymnodimine, a new marine toxin of unprecedented structure isolated from New-Zealand oysters and the dinoflagellate, *Gymnodinium Sp*. *Tetrahedron Lett* 1995; **36**: 7093.
[45] Dell'Aversano C, Walter JA, Burton IW, Stirling DJ, Fattorusso E, Quilliam MA. Isolation and structure elucidation of new and unusual saxitoxin analogues from mussels. *J Nat Prod* 2008; **71**: 1518.
[46] Alexander J, Benford D, Cockburn A, Cradevi J-P, Dogliotti E, Domenico AD, Fernandez-Cruz ML, Fink-Gremmels J, Furst P, Galli C, Grandjean P, Gzyl J, Heinemeyer G, Johansson N, Mutti A, Schlatter J, van Leeuwen R, van Peteghem C, Verger P. Marine biotoxins in shellfish - saxitoxin group. *The EFSA Journal* 2009; **1019**: 1.
[47] Lagos N, Andrinolo D, Paralytic shellfish poisoning (PSP): toxicology and kinetics. In *Seafood and Freshwater Toxins: Pharmacology, Physiology and Detection*, Botana LM, Ed. Marcel Dekker: New York, 2000; 203.
[48] Report of the joint FAO/IOC/WHO ad hoc expert consulation on biotoxins in bivalve mollucs. *FAO/IOC/WHO report* 2004.
[49] de Carvalho M, Jacinto J, Ramos N, de Oliveira V, Pinho e Melo T, de Sa J. Paralytic shellfish poisoning: clinical and electrophysiological observations. *J Neurol* 1998; **245**: 551.
[50] Azanza MPV. Philippine foodborne-disease outbreaks (1995-2004). *J Food Safety* 2006; **26**: 92.
[51] Meyer KF, Sommer H, Schoenholz P. Mussel poisoining. *Am J Prev Med* 1928; **2**: 365.
[52] Pitcher GC, Cembella AD, Joyce LB, Larsen J, Probyn TA, Sebastian CR. The dinoflagellate *Alexandrium minutum* in Cape Town harbour (South Africa): Bloom characteristics, phylogenetic analysis and toxin composition. *Harmful Algae* 2007; **6**: 823.
[53] Krock B, Seguel CG, Cembella AD. Toxin profile of *Alexandrium catenella* from the Chilean coast as determined by liquid chromatography with fluorescence detection and liquid chromatography coupled with tandem mass spectrometry. *Harmful Algae* 2007; **6**: 734.
[54] Robertson A, Stirling D, Robillot C, Llewellyn L, Negri A. First report of saxitoxin in octopi. *Toxicon* 2004; **44**: 765.
[55] Al-Sabi A, McArthur J, Ostroumov V, French RJ. Marine toxins that target voltage-gated sodium channels. *Mar Drugs* 2006; **4**: 157.
[56] Pierce RH, Henry MS, Blum PC, Hamel SL, Kirkpatrick B, Cheng YS, Zhou Y, Irvin CM, Naar J, Weidner A, Fleming LE, Backer LC, Baden DG. Brevetoxin composition in water and marine aerosol along a Florida beach: Assessing potential human exposure to marine biotoxins. *Harmful Algae* 2005; **4**: 965.
[57] Kirkpatrick B, Fleming LE, Backer LC, Bean JA, Tamer R, Kirkpatrick G, Kane T, Wanner A, Dalpra D, Reich A, Baden DG. Environmental exposures to Florida red tides: Effects on emergency room respiratory diagnoses admissions. *Harmful Algae* 2006; **5**: 526.
[58] Heil DC. *Karenia brevis* monitoring, management, and mitigation for Florida molluscan shellfish harvesting areas. *Harmful Algae* 2009; **8**: 608.
[59] Ishida H, Nozawa A, Nukaya H, Tsuji K. Comparative concentrations of brevetoxins PbTx-2, PbTx-3, BTX-B1 and BTX-B5 in cockle, *Austrovenus stutchburyi*, greenshell mussel, *Perna canaliculus*, and Pacific oyster, *Crassostrea gigas*, involved neurotoxic shellfish poisoning in New Zealand. *Toxicon* 2004; **43**: 779.
[60] Fish and fisheries products hazards and control guidance. *Food and Drug Administration* 2001; Chapter 6: Natural Toxins (A chemical Hazard).
[61] Honkanen RE, Codispoti BA, Tse K, Boynton AL, Honkanan RE. Characterization of natural toxins with inhibitory activity against serine/threonine protein phosphatases. *Toxicon* 1994; **32**: 339.
[62] Vale C, Botana LM. Marine toxins and the cytoskeleton: okadaic acid and dinophysistoxins. *FEBS J* 2008; **275**: 6060.

[63] Garcia C, Truan D, Lagos M, Santelices JP, Diaz JC, Lagos N. Metabolic transformation of dinophysistoxin-3 into dinophysistoxin-1 causes human intoxication by consumption of O-acyl-derivatives dinophysistoxins contaminated shellfish. *J Toxicol Sci* 2005; **30**: 287.

[64] Manerio E, Rodas VL, Costas E, Hernandez JM. Shellfish consumption: a major risk factor for colorectal cancer. *Med Hypotheses* 2008; **70**: 409.

[65] Kat M, The occurrence of *prorocentrum* species and coincidental gastrointestinal illness of mussel consumers. In *Toxic dinoflagellate blooms*, Taylor DL, Seliger HH, Eds. Elsevier: 1979; 215.

[66] Scoging A, Bahl M. Diarrhetic shellfish poisoning in the UK. *Lancet* 1998; **352**: 117.

[67] Elgarch A, Vale P, Rifai S, Fassouane A. Detection of diarrheic shellfish poisoning and azaspiracid toxins in Moroccan mussels: comparison of the LC-MS method with the commercial immunoassay kit. *Mar Drugs* 2008; **6**: 587.

[68] Alexander J, Audunsson GA, Benford D, Cockburn A, Cradevi J-P, Dogliotti E, Domenico AD, Fernandez-Cruz ML, Fink-Gremmels J, Furst P, Galli C, Grandjean P, Gzyl J, Heinemeyer G, Johansson N, Mutti A, Schlatter J, van Leeuwen R, van Peteghem C, Verger P. Marine biotoxins in shellfish - okadaic acid and analogues. *The EFSA Journal* 2008; **589**: 1.

[69] Aune T, Larsen S, Aasen JAB, Rehmann N, Satake M, Hess P. Relative toxicity of dinophysistoxin -2 (DTX-2) compared with okadaic acid, based on acute intraperitoneal toxicity in mice. *Toxicon* 2007; **49**: 1.

[70] Alexander J, Benford D, Cockburn A, Cradevi J-P, Dogliotti E, Domenico AD, Fernandez-Cruz ML, Fink-Gremmels J, Furst P, Galli C, Grandjean P, Gzyl J, Heinemeyer G, Johansson N, Mutti A, Schlatter J, van Leeuwen R, van Peteghem C, Verger P. Marine biotoxins in shellfish - pectenotoxin group. *The EFSA Journal* 2009; **1109**: 1.

[71] Miles CO, Wilkins AL, Hawkes AD, Jensen DJ, Selwood AI, Beuzenberg V, Mackenzie AL, Cooney JM, Holland PT. Isolation and identification of pectenotoxins-13 and -14 from *Dinophysis acuta* in New Zealand. *Toxicon* 2006; **48**: 152.

[72] Miles CO, Wilkins AL, Samdal IA, Sandvik M, Petersen D, Quilliam MA, Naustvoll LJ, Rundberget T, Torgersen T, Hovgaard P, Jensen DJ, Cooney JM. A novel pectenotoxin, PTX-12, in *Dinophysis spp*. and shellfish from Norway. *Chem Res Toxicol* 2004; **17**: 1423.

[73] Vale P, Sampayo MAD. Pectenotoxin-2 seco acid, 7-epi-pectenotoxin-2 seco acid and pectenotoxin-2 in shellfish and plankton from Portugal. *Toxicon* 2002; **40**: 979.

[74] Espina B, Rubiolo JA. Marine toxins and the cytoskeleton: pectenotoxins, unusual macrolides that disrupt actin. *FEBS J* 2008; **275**: 6082.

[75] Miles CO, Wilkins AL, Munday R, Dines MH, Hawkes AD, Briggs LR, Sandvik M, Jensen DJ, Cooney JM, Holland PT, Quilliam MA, MacKenzie AL, Beuzenberg V, Towers NR. Isolation of pectenotoxin-2 from *Dinophysis acuta* and its conversion to pectenotoxin-2 seco acid, and preliminary assessment of their acute toxicities. *Toxicon* 2004; **43**: 1.

[76] Bowden BF. Yessotoxins-polycyclic ethers from dinoflagellates: Relationships to diarrhetic shellfish toxins. *Toxin Rev* 2006; **25**: 137.

[77] Miles CO, Samdal IA, Aasen JAB, Jensen DJ, Quilliam MA, Petersen D, Briggs LM, Wilkins AL, Rise F, Cooney JM, MacKenzie AL. Evidence for numerous analogs of yessotoxin in *Protoceratium reticulatum*. *Harmful Algae* 2005; **4**: 1075.

[78] Aasen JAB, Samdal IA, Miles CO, Dahl E, Briggs LR, Aune T. Yessotoxins in Norwegian blue mussels (*Mytilus edulis*): uptake from *Protoceratium reticulatum*, metabolism and depuration. *Toxicon* 2005; **45**: 265.

[79] Ciminiello P, Fattorusso E, Forino M, Magno S, Poletti R, Viviani R. Isolation of adriatoxin, a new analogue of yessotoxin from mussels of the Adriatic sea. *Tetrahedron Lett* 1998; **39**: 8897.

[80] Aune T, Sorby R, Yasumoto T, Ramstad H, Landsverk T. Comparison of oral and intraperitoneal toxicity of yessotoxin towards mice. *Toxicon* 2002; **40**: 77.

[81] Draisci R, Ferretti E, Palleschi L, Marchiafava C, Poletti R, Milandri A, Ceredi A, Pompei M. High levels of yessotoxin in mussels and presence of yessotoxin and homoyessotoxin in dinoflagellates of the Adriatic Sea. *Toxicon* 1999; **37**: 1187.

[82] Vale P, Botelho MJ, Rodrigues SM, Gomes SS, Sampayo MAD. Two decades of marine biotoxin monitoring in bivalves from Portugal (1986-2006): A review of exposure assessment. *Harmful Algae* 2008; **7**: 11.

[83] Alexander J, Benford D, Cockburn A, Cradevi J-P, Dogliotti E, Domenico AD, Fernandez-Cruz ML, Fink-Gremmels J, Furst P, Galli C, Grandjean P, Gzyl J, Heinemeyer G, Johansson N, Mutti A, Schlatter J, van Leeuwen R, van Peteghem C, Verger P. Marine biotoxins in shellfish - yessotoxin group. *The EFSA Journal* 2008; **907**: 1.

[84] Alexander J, Benford D, Cockburn A, Cradevi J-P, Dogliotti E, Domenico AD, Fernandez-Cruz ML, Fink-Gremmels J, Furst P, Galli C, Grandjean P, Gzyl J, Heinemeyer G, Johansson N, Mutti A, Schlatter J, van Leeuwen R, van Peteghem C, Verger P. Marine biotoxins in shellfish - azaspiracid group. *The EFSA Journal* 2008; **723**: 1.

[85] James KJ, Saez MJF, Furey A, Lehane M. Azaspiracid poisoning, the food-borne illness associated with shellfish consumption. *Food Addit Contam A* 2004; **21**: 879.

[86] Krock B, Tillmann U, John U, Cembella AD. Characterization of azaspiracids in plankton size-fractions and isolation of an azaspiracid-producing dinoflagellate from the North Sea. *Harmful Algae* 2009; **8**: 254.

[87] Rehmann N, Hess P, Quilliam MA. Discovery of new analogs of the marine biotoxin azaspiracid in blue mussels (*Mytilus edulis*) by ultra-performance liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom* 2008; **22**: 549.

[88] Ronzitti G, Hess P, Rehmann N, Rossini GP. Azaspiracid-1 alters the E-cadherin pool in epithelial cells. *Toxicol Sci* 2007; **95**: 427.

[89] Twiner MJ, Hess P, Dechraoui MYB, McMahon T, Samons MS, Satake M, Yasumoto T, Ramsdell JS, Doucette GJ. Cytotoxic and cytoskeletal effects of azaspiracid-1 on mammalian cell lines. *Toxicon* 2005; **45**: 891.

[90] Ito E, Satake M, Ofuji K, Kurita N, McMahon T, James KJ, Yasumoto T. Multiple organ damage caused by a new toxin azaspiracid, isolated from mussels produced in Ireland. *Toxicon* 2000; **38**: 917.

[91] James KJ, Lehane M, Moroney C, Fernandez-Puente P, Satake M, Yasumoto T, Furey A. Azaspiracid shellfish poisoning: unusual toxin dynamics in shellfish and the increased risk of acute human intoxications. *Food Addit Contam A* 2002; **19**: 555.

[92] Torgersen T, Bremnes NB, Rundberget T, Aune T. Structural confirmation and occurrence of azaspiracids in Scandinavian brown crabs (*Cancer pagurus*). *Toxicon* 2008; **51**: 93.

[93] Amzil Z, Sibat M, Royer F, Savar V. First report on azaspiracid and yessotoxin groups detection in French shellfish. *Toxicon* 2008; **52**: 39.

[94] Vale P, Bire R, Hess P. Confirmation by LC-MS/MS of azaspiracids in shellfish from the Portuguese north-western coast. *Toxicon* 2008; **51**: 1449.

[95] Alvarez G, Uribe E, Avalos P, Marino C, Blanco J. First identification of azaspiracid and spirolides in *Mesodesma donacium* and *Mulinia edulis* from Northern Chile. *Toxicon* 2010; **55**: 638.

[96] Lopez-Rivera A, O'Callaghan K, Moriarty M, O'Driscoll D, Hamilton B, Lehane M, James KJ, Furey A. First evidence of azaspiracids (AZAs): A family of lipophilic polyether marine toxins in scallops (*Argopecten purpuratus*) and mussels (*Mytilus chilensis*) collected in two regions of Chile. *Toxicon* 2009; **55**: 692.

[97] Klontz KC, Abraham A, Plakas SM, Dickey RW. Mussel-associated azaspiracid intoxication in the United States. *Ann Intern Med* 2009; **150**: 361.

[98] Gerssen A, van Olst EHW, Mulder PPJ, de Boer J. In-house validation of a liquid chromatography tandem mass spectrometry method for the analysis of lipophilic marine toxins in shellfish using matrix matched calibration. *Anal Bioanal Chem* 2010; **397**: 3079.

[99] MacKinnon SL, Walter JA, Quilliam MA, Cembella AD, LeBlanc P, Burton IW, Hardstaff WR, Lewis NI. Spirolides isolated from Danish strains of the toxigenic dinoflagellate *Alexandrium ostenfeldii*. *J Nat Prod* 2006; **69**: 983.

[100] Aasen JAB, MacKinnon SL, LeBlanc P, Walter JA, Hovgaard P, Aune T, Quilliam MA. Detection and identification of spirolides in Norwegian shellfish and plankton. *Chem Res Toxicol* 2005; **18**: 509.

[101] Aasen JAB, Hardstaff W, Aune T, Quilliam MA. Discovery of fatty acid ester metabolites of spirolide toxins in mussels from Norway using liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom* 2006; **20**: 1531.
[102] Ciminiello P, Dell'Aversano C, Fattorusso E, Forino M, Grauso L, Tartaglione L, Guerrini F, Pistocchi R. Spirolide toxin profile of Adriatic *Alexandrium ostenfeldii* cultures and structure elucidation of 27-hydroxy-13,19-didesmethyl spirolide C. *J Nat Prod* 2007; **70**: 1878.
[103] Richard DJA, Arsenault E, Cembella AD, Quilliam MA, Investigations into the toxicology and pharmacology of spirolides, a novel group of shellfish toxins. In *Proceedings of the 9th conference on Harmfull algal blooms*, Hallegraeff GM, Blackburn SI, Bolch CJ, Lewis RJ, Eds. Hobart, Australia, 2000; 383.
[104] Villar Gonzalez A, Rodriguez-Velasco ML, Ben-Gigirey B, Botana LM. First evidence of spirolides in Spanish shellfish. *Toxicon* 2006; **48**: 1068.
[105] Nicolaou KC, Chen JS. Total synthesis of complex heterocyclic natural products. *Pure Appl Chem* 2008; **80**: 727.
[106] Alfonso C, Alfonso A, Otero P, Rodriguez P, Vieytes MR, Elliot C, Higgins C, Botana LM. Purification of five azaspiracids from mussel samples contaminated with DSP toxins and azaspiracids. *J Chromatogr B* 2008; **865**: 133.
[107] Rundberget T, Sandvik M, Larsen K, Pizarro GM, Reguera B, Castberg T, Gustad E, Loader JI, Rise F, Wilkins AL, Miles CO. Extraction of microalgal toxins by large-scale pumping of seawater in Spain and Norway, and isolation of okadaic acid and dinophysistoxin-2. *Toxicon* 2007; **50**: 960.
[108] Yasumoto T, Hashimoto.Y, Bagnis R, Randall JE, Banner AH. Toxicity of surgeonfishes. *B Jpn Soc Sci Fish* 1971; **37**: 724.
[109] Yasumoto T, Oshima Y, Yamaguchi K, Occurrence of new type of toxic shellfish in Japan and chemical properties of the toxin. In *Toxic dinoflagellate blooms*, Taylor DL, Seliger HH, Eds. Elsevier: 1979; 395.
[110] Yasumoto T, Murata M, Oshima Y, Matsumoto GK, Clardy J, Diarrhetic shellfish poisoning. In *Seafood toxins : based on a symposium at the 186th meeting of the American Chemical Society, Washington, D.C., August 28 - September 2, 1983*, Ragelis EP, Ed. ACS: Washington, 1984; 207.
[111] Community Reference laboratory for marine biotoxins, *EU harmonised standard operating procedure for detection of lipophilic toxins by mouse bioassay, version 5 June 2009*. http:// www.aesan.msps.es/en/CRLMB/web/home.shtml (15-07-2009)
[112] Hess P, Nguyen L, Aasen JAB, Keogh M, Kilcoyne J, McCarron P, Aune T. Tissue distribution, effects of cooking and parameters affecting the extraction of azaspiracids from mussels, *Mytilus edulis*, prior to analysis by liquid chromatography coupled to mass spectrometry. *Toxicon* 2005; **46**: 62.
[113] Anon. Approaches to validation and regulatory acceptance of alternative methods to the mouse bioassay as method of reference. In *Analysis of marine biotoxins*, Anon (eds). Bunderinstitut fur risikobewertung: Berlin, 2005.
[114] Van Egmond HP, van Apeldoorn ME, Speijers GJA. Marine Biotoxins. In *FAO food and nutrition paper*, anon (eds). Food and Agriculture Organization of the united nations: Rome, 2004.
[115] Fish products standards and methods manual - Canadian guidelines for chemical contaminants and toxins in fish and fish products. *Canadian food inspection agency* 2007; Appendix 3.
[116] Contaminants and natural toxicants. *Food Standards Australia New Zealand* 2009; Standard 1.4.1.
[117] Gonzalez JC, Leira F, Fontal OI, Vieytes MR, Arevalo FF, Vieites JM, Bermudez-Puente M, Muniz S, Salgado C, Yasumoto T, Botana LM. Inter-laboratory validation of the fluorescent protein phosphatase inhibition assay to determine diarrhetic shellfish-toxins: intercomparison with liquid chromatography and mouse bioassay. *Anal Chim Acta* 2002; **466**: 233.
[118] Vieytes MR, Fontal OI, Leira F, deSousa JMVB, Botana LM. A fluorescent microplate assay for diarrheic shellfish toxins. *Anal Biochem* 1997; **248**: 258.

[119] Honkanen RE, Stapleton JD, Bryan DE, Abercrombie J. Development of a protein phosphatase-based assay for the detection of phosphatase inhibitors in crude whole cell and animal extracts. *Toxicon* 1996; **34**: 1385.
[120] Hummert C, Reichelt M, Luckas B. New strategy for the determination of microcystins and diarrhetic shellfish poisoning (DSP) toxins, two potent phosphatases 1 and 2A inhibitors and tumor promoters. *Fresen J Anal Chem* 2000; **366**: 508.
[121] Leira F, Alvarez C, Cabado AG, Vieites JM, Vieytes MR, Botana LM. Development of a F actin-based live-cell fluorimetric microplate assay for diarrhetic shellfish toxins. *Anal Biochem* 2003; **317**: 129.
[122] Rossini GP. Functional assays in marine biotoxin detection. *Toxicology* 2005; **207**: 451.
[123] Pierotti S, Albano C, Milandri A, Callegari F, Poletti R, Rossini GP. A slot blot procedure for the measurement of yessotoxins by a functional assay. *Toxicon* 2007; **49**: 36.
[124] Vilarino N, Fonfria ES, Molgo J, Araoz R, Botana LM. Detection of gymnodimine-A and 13-desmethyl C spirolide phycotoxins by fluorescence polarization. *Anal Chem* 2009; **81**: 2708.
[125] Laycock MV, Jellett JF, Easy DJ, Donovan MA. First report of a new rapid assay for diarrhetic shellfish poisoning toxins. *Harmful Algae* 2006; **5**: 74.
[126] Vale P, Gomes SS, Lameiras J, Rodrigues SM, Botelho MJ, Laycock MV. Assessment of a new lateral flow immunochromatographic (LFIC) assay for the okadaic acid group of toxins using naturally contaminated bivalve shellfish from the Portuguese coast. *Food Addit Contam A* 2009; **26**: 214.
[127] Campas M, de la Iglesia P, Le Berre M, Kane M, Diogene J, Marty JL. Enzymatic recycling-based amperometric immunosensor for the ultrasensitive detection of okadaic acid in shellfish. *Biosens Bioelectron* 2008; **24**: 716.
[128] Llamas NM, Stewart L, Fodey T, Higgins HC, Velasco MLR, Botana LM, Elliott CT. Development of a novel immunobiosensor method for the rapid detection of okadaic acid contamination in shellfish extracts. *Anal Bioanal Chem* 2007; **389**: 581.
[129] Briggs LR, Miles CO, Fitzgerald JM, Ross KM, Garthwaite I, Towers NR. Enzyme-linked immunosorbent assay for the detection of yessotoxin and its analogues. *J Agric Food Chem* 2004; **52**: 5836.
[130] Samdal IA, Aasen JAB, Briggs LR, Dahl E, Miles CO. Comparison of ELISA and LC-MS analyses for yessotoxins in blue mussels (*Mytilus edulis*). *Toxicon* 2005; **46**: 7.
[131] Alfonso C, Alfonso A, Vieytes MR, Yasumoto T, Botana LM. Quantification of yessotoxin using the fluorescence polarization technique and study of the adequate extraction procedure. *Anal Biochem* 2005; **344**: 266.
[132] Fonfria ES, Vilarino N, Vieytes MR, Yasumoto T, Botana LM. Feasibility of using a surface plasmon resonance-based biosensor to detect and quantify yessotoxin. *Anal Chim Acta* 2008; **617**: 167.
[133] Pazos MJ, Alfonso A, Vieytes MR, Yasumoto T, Vieites JM, Botana LM. Resonant mirror biosensor detection method based on yessotoxin-phosphodiesterase interactions. *Anal Biochem* 2004; **335**: 112.
[134] Lee JS, Yanagi T, Kenma R, Yasumoto T. Fluorometric-determination of diarrhetic shellfish toxins by high-performance liquid-chromatography. *Agr Biol Chem* 1987; **51**: 877.
[135] Sasaki K, Takizawa A, Tubaro A, Sidari L, Loggia RD, Yasumoto T. Fluorometric analysis of pectenotoxin-2 in microalgal samples by high performance liquid chromatography. *Nat Toxins* 1999; **7**: 241.
[136] Yasumoto T, Takizawa A. Fluorometric measurement of yessotoxins in shellfish by high-pressure liquid chromatography. *Biosci Biotechnol Biochem* 1997; **61**: 1775.
[137] Miles CO, Wilkins AL, Hawkes AD, Jensen DJ, Cooney JM, Larsen K, Petersen D, Rise F, Beuzenberg V, Mackenzie AL. Isolation and identification of a cis-C(8)-diol-ester of okadaic acid from *Dinophysis acuta* in New Zealand. *Toxicon* 2006; **48**: 195.
[138] Paz B, Daranas AH, Cruz PG, Franco JM, Pizarro G, Souto ML, Norte M, Fernandez JJ. Characterisation of okadaic acid related toxins by liquid chromatography coupled with mass spectrometry. *Toxicon* 2007; **50**: 225.

[139] Paz B, Daranas AH, Cruz PG, Franco JM, Napolitano JG, Norte M, Fernandez JJ. Identification and characterization of DTX-5c and 7-hydroxymethyl-2-methylene-octa-4,7-dienyl okadaate from *Prorocentrum belizeanum* cultures by LC-MS. *Toxicon* 2007; **50**: 470.
[140] Torgersen T, Wilkins AL, Rundberget T, Miles CO. Characterization of fatty acid esters of okadaic acid and related toxins in blue mussels (*Mytilus edulis*) from Norway. *Rapid Commun Mass Spectrom* 2008; **22**: 1127.
[141] Torgersen T, Miles CO, Rundberget T, Wilkins AL. New esters of okadaic acid in seawater and blue mussels (*Mytilus edulis*). *J Agric Food Chem* 2008; **56**: 9628.
[142] Suzuki T, Beuzenberg V, Mackenzie AL, Quilliam MA. Liquid chromatography - mass spectrometry of spiroketal stereoisomers of pectenotoxins and the analysis of novel pectenotoxin isomers in the toxic dinoflagellate *Dinophysis acuta* from New Zealand. *J Chromatogr A* 2003; **992**: 141.
[143] Suzuki T, Horie Y, Koike K, Satake M, Oshima Y, Iwataki M, Yoshimatsu S. Yessotoxin analogues in several strains of *Protoceratium reticulatum* in Japan determined by liquid chromatography-hybrid triple quadrupole/linear ion trap mass spectrometry. *J Chromatogr A* 2007; **1142**: 172.
[144] Blanco J, Alvarez G, Uribe E. Identification of pectenotoxins in plankton, filter feeders, and isolated cells of a *Dinophysis acuminata* with an atypical toxin profile, from Chile. *Toxicon* 2007; **49**: 710.
[145] Miles CO, Wilkins AL, Selwood AI, Hawkes AD, Jensen DJ, Cooney JM, Beuzenberg V, MacKenzie AL. Isolation of yessotoxin 32-O-[beta-L-arabinofuranosyl-(5'->1")-beta-L-arabinofuranoside] from *Protoceratium reticulatum*. *Toxicon* 2006; **47**: 510.
[146] Suzuki T, Mackenzie AL, Stirling D, Adamson J. Pectenotoxin-2 seco acid: a toxin converted from pectenotoxin-2 by the New Zealand Greenshell mussel, *Perna canaliculus*. *Toxicon* 2001; **39**: 507.
[147] McCarron P, Kilcoyne J, Miles CO, Hess P. Formation of azaspiracids-3, -4, -6, and -9 via decarboxylation of carboxyazaspiracid metabolites from shellfish. *J Agric Food Chem* 2009; **57**: 160.
[148] Quilliam MA, Hess P, Dell' Aversano C In *Recent developments in the analysis of phycotoxins by liquid chromatography-mass spectrometry*; de Koe WJ, Samson RA, van Egmond HP, Gilbert J, Sabino M, Eds. Mycotoxins and Phycotoxins in Perspective at the turn of the century: Wageningen, The Netherlands, 2001; 383.
[149] Goto H, Igarashi T, Yamamoto M, Yasuda M, Sekiguchi R, Watai M, Tanno K, Yasumoto T. Quantitative determination of marine toxins associated with diarrhetic shellfish poisoning by liquid chromatography coupled with mass spectrometry. *J Chromatogr A* 2001; **907**: 181.
[150] McNabb P, Selwood AI, Holland PT. Multiresidue method for determination of algal toxins in shellfish: Single-laboratory validation and interlaboratory study. *J AOAC Int* 2005; **88**: 761.
[151] Stobo LA, Lacaze JPCL, Scott AC, Gallacher S, Smith EA, Quilliam MA. Liquid chromatography with mass spectrometry - detection of lipophilic shellfish toxins. *J AOAC Int* 2005; **88**: 1371.
[152] Fux E, McMillan D, Bire R, Hess P. Development of an ultra-performance liquid chromatography-mass spectrometry method for the detection of lipophilic marine toxins. *J Chromatogr A* 2007; **1157**: 273.
[153] Gerssen A, Mulder PPJ, McElhinney MA, De Boer J. Liquid chromatography - tandem mass spectrometry method for the detection of marine lipophilic toxins under alkaline conditions. *J Chromatogr A* 2009; **1216**: 1421.
[154] Aune T, Yndestad M, Diarrhetic shellfish poisoning. In *Algal toxins in seafood and drinking water*, Falconer IR, Ed. Academic Press: London, 1993; 87.
[155] Murata M, Shimatani M, Sugitani H, Oshima Y, Yasumoto T. Isolation and structural elucidation of the causative toxin of the diarrhetic shellfish poisoning. *B Jpn Soc Sci Fish* 1982; **48**: 549.
[156] Ministry of Agriculture Nature Management and Fisheries. *Jaarverslag Visserij* 1971; 394.
[157] Ministry of Agriculture Nature Management and Fisheries. *Jaarverslag Visserij* 1976; 292.
[158] Ministry of Agriculture Nature Management and Fisheries. *Jaarverslag Visserij* 1981; 170.
[159] Kat M. Diarrhetic mussel poisoning in The Netherlands related to the dinoflagellate *Dinophysis acuminata*. *Antonie Van Leeuwenhoek* 1983; **49**: 417.

[160] Ministry of Agriculture Nature Management and Fisheries. *Jaarverslag Visserij* 1986; 64.
[161] Ministry of Agriculture Nature Management and Fisheries. *Jaarverslag Visserij* 1987; 163.
[162] European Parliament, Council. Commission Decision 1986/609/EC. *Off. J. Eur. Commun.* 1986; **L358**: 1.
[163] *Framework 6 Program on Food Quality and Safety - BIOTOX, development of cost-effective tools for riskmanagment and traceability systems for marine biotoxins in seafood [(FP6-2003-Food-2A), contract number 514074].* www.biotox.org
[164] Alexander J, Benford D, Cockburn A, Cradevi J-P, Dogliotti E, Domenico AD, Fernandez-Cruz ML, Fink-Gremmels J, Furst P, Galli C, Grandjean P, Gzyl J, Heinemeyer G, Johansson N, Mutti A, Schlatter J, van Leeuwen R, van Peteghem C, Verger P. Marine biotoxins in shellfish - cyclic imines. *The EFSA Journal* 2010; **1628**: 1.

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Mass spectrometric analysis of the marine lipophilic biotoxins pectenotoxin-2 and okadaic acid by four different types of mass spectrometers

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ABSTRACT

The performances of four different mass spectrometers [triple-quadrupole (TQ), time-of-flight (ToF), quadrupole time-of-flight (QToF) and ion trap (IT)] for the detection of the lipophilic marine toxins pectenotoxin-2 (PTX2) and okadaic acid (OA) were investigated. The spectral data obtained with the different mass spectrometric analyzers were used to propose fragmentation schemes for PTX2 in positive electrospray mode and OA in negative electrospray mode. TQ data were used to obtain product ions, while ToF and QToF-MS produced accurate mass data of the precursor ion and product ions, respectively. IT data provided a better understanding of the fragmentation pathways using MS^n experiments. With respect to analytical performance, all four mass analyzers showed a good linearity ($R^2>0.97$) and repeatability (CV<20%). Detection limits (S/N=3) were the lowest on triple-quad MS; 12.2 pg and 2.9 pg on-column for PTX2 and OA, respectively.

INTRODUCTION

Shellfish species that accumulate phycotoxins through filtration of algae from the surrounding water can cause several syndromes when consumed by humans. The most common syndromes are Paralytic Shellfish Poisoning (PSP), Amnesic Shellfish Poisoning (ASP) and Diarrhetic Shellfish Poisoning (DSP). The important sources of DSP toxins are dinoflagellates of the *Dinophysis* and *Prorocentrum* genera. DSP toxins can accumulate in filter-feeding shellfish such as scallops, clams, oysters and mussels. Human consumption of DSP contaminated shellfish can lead to several gastrointestinal disorders such as diarrhea, nausea, vomiting and abdominal cramps. DSP is therefore a serious problem for public health and the shellfish industry [1, 2].

The most prominent members of the group of DSP toxins are the lipophilic toxins okadaic acid (OA) and derivatives of this toxin, dinophysistoxin-1 (DTX1), DTX2 and their ester derivatives (DTX3). These DSP toxins can coexist with the lipophilic pectenotoxins (PTXs). Azaspiracids (AZAs) and yessotoxins (YTXs) are other lipophilic marine toxins that can be present in shellfish samples. PTXs have also shown to be hepatotoxic [3], while YTXs have an adverse effect on the cardiac muscle cells [4] and on defattening of liver cells. On top of its diarrheagenic properties, OA is also a tumor promoter [5].

At present, mouse or rat bioassays are being used for the determination of DSP toxins in shellfish. Currently this is the reference method prescribed in EU legislation [6]. The first analytical-chemical analysis of lipophilic marine toxins was based on liquid chromatography-fluorometric detection (LC-FLD); this method was only applicable for OA. For FLD of the toxins, derivatisation with a fluorescent substituent is required [7, 8]. However, FLD is not applicable to all lipophilic marine toxins owning to the lack of a carboxylic acid functionality in some of the toxins. Therefore, nowadays MS is the method of choice for quantification and identification of lipophilic marine toxins [9, 10]. MS is not limited to the presence of specific functional groups. Another advantage of MS compared to FLD is that no laborious and critical derivatisation techniques are required.

The MS techniques used in this study are based on either tandem MS or ToF-MS or a combination of both. Tandem MS uses two stages of mass analysis; these two steps in mass analysis can be either in space or in time. In tandem-in-space the

selection of the ion of interest, the collision induced dissociation (CID) and the analysis of the induced fragments are occurring simultaneously at different places in the instrument. Tandem-in-space can be achieved on a triple-quadrupole (TQ) or a hybrid quadrupole ToF (QToF)-MS. TQ-MS experiments are widely used for the determination of lipophilic marine toxins such as OA, DTXs, PTXs, AZAs and YTXs [11-14]. With tandem-in-time the events take place in the same space but are separated in time [i.e. ion trap (IT)]. IT mass spectrometers are often used for MS^n experiments of lipophilic marine toxins, where the predominant fragments are similar to those in tandem-in-space MS [15, 16].

New techniques like high resolution ToF-MS also offer the potential for the identification of unknown compounds. The ToF technique is interesting because of its capability to perform accurate mass measurements using high-resolution spectral data [resolution > 10 000 at full width half maximum (FWHM)]. The aim of this work is: (1) to elucidate the fragmentation pathways of PTX2 and OA using four different mass spectrometric techniques (TQ-MS, ToF-MS, QToF and IT) and (2) to compare the information about linearity, repeatability and sensitivity obtained by these mass spectrometers in order to judge their suitability for application in routine shellfish monitoring. We have chosen three mass spectrometers, TQ-MS, QToF and IT, which are already used in routine biotoxin

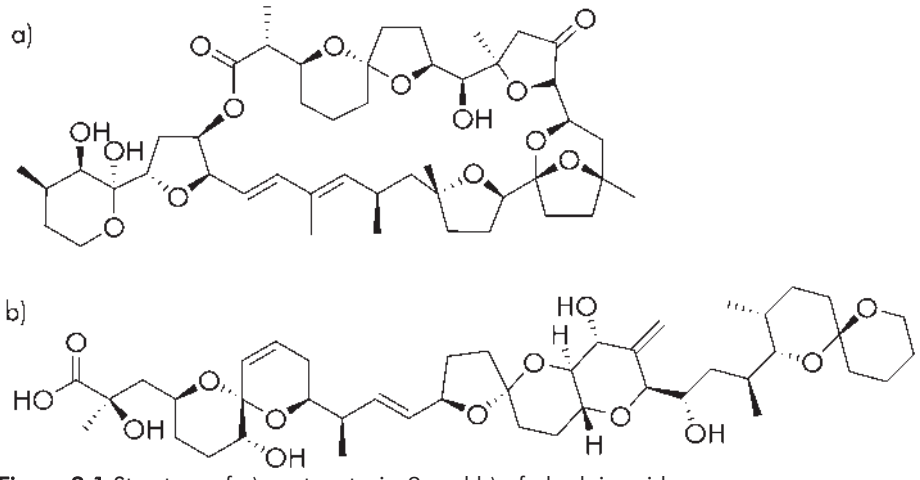

Figure 2.1 Structure of a) pectenotoxin-2 and b) of okadaic acid.

analysis. Furthermore, we chose a more innovative technique like ToF-MS. At the time of this study, other lipophilic marine toxins, e.g. azaspiracids and yessotoxins, were not yet commercially available. Therefore we had to limit this study to OA and PTX2 (Fig. 2.1).

EXPERIMENTAL

Reagents and standards

Water was deionized and passed through a Milli-Q water purification system (Millipore, Billerica, MA, USA). Formic acid (98-100%) and ortho-phosphoric acid (85%) were purchased from Merck, Darmstadt, Germany. Ammonium formate (>97%) and Leucine-enkephalin were purchased from Sigma Aldrich, Steinheim, Germany. Acetonitrile [High performance LC (HPLC) supra gradient] and methanol (absolute) were purchased from Biosolve, Valkenswaard, The Netherlands. PTX2 (CRM-PTX2 8.6±0.3 μg/ml) and OA (CRM-OA-b 24.1±0.8 μg/ml) were purchased from the National Research Council, Institute for Marine Biosciences (NRC-CNRC), Halifax, Canada. The direct infusion experiments were carried out using a 200 ng/ml PTX2 or OA solution in methanol. The linearity was determined using solutions of PTX2 or OA with the concentrations of 5, 10, 20, 40 and 60 ng/ml in methanol. Matrix-matched standards were prepared by carrying out a triplicate extraction of 2 g mussel homogenate (*Mytilus edulis*) with 6 ml methanol. After adding 6 ml of methanol the sample was homogenized by Vortex mixing. The extract was centrifuged (3 000 × *g*, 5 min) and the supernatant transferred to a volumetric flask (20 ml). After the third extraction the volumetric flask was made up with methanol. Extracts were spiked with PTX2 and OA at the concentrations 5, 10, 20, 40 and 60 ng/ml. The repeatability was determined using a 40 ng/ml PTX2 and OA standard solution (n=10).

Triple-quadrupole (TQ-MS, LC-MS/MS)

Mass spectrometry was performed using a Micromass Quattro Micro II LC-MS/MS (Waters-Micromass, Manchester, UK) instrument equipped with a Z-spray interface. Detailed settings are given in Table 2.1. The multiple reaction monitoring (MRM) channels that were monitored for product ions of PTX2 ($[M+NH_4]^+$ m/z 876.5) were m/z 195.1, 213.1, 275.2, 439.3, 457.3, 551.3, 805.5 and 823.5. Product

ions of OA ($[M-H]^-$ *m/z* 803.5) monitored were *m/z* 113.0, 151.1, 209.1, 255.1, 321.2, 563.3 and 785.5. Positive and negative ionization mode were applied in separate runs.

An Agilent HP 1100 series (Palo Alto, CA, USA) HPLC system was used equipped with a Thermo Electron BDS Hypersil C_8 50 x 2.1 mm column with 3 μm particles. The LC conditions used were adapted from Hess *et al.* [17]. Eluent A was H_2O and B was acetonitrile/H_2O (95:5 v/v), both containing a fixed buffer concentration of 2 mM ammonium formate and 50 mM formic acid. The column temperature was set at 30°C and the flow rate was set at 0.2 ml/min. The gradient started at 0 min, 30% B and increased linearly to 90% B in 8 min. The 90% B was kept for 2.5 min and returned in 0.5 min to 30% B. An equilibration time of 4 min was allowed before the next injection. The vial compartment of the autosampler was kept at 10°C and a 10 μl injection volume was used. Direct infusion experiments were carried out with a Hamilton 500 μl injection needle infusing at 10 μl/min. During direct infusion a flow of 0.05 ml/min 30% B was applied to simulate a more realistic environment.

Time of Flight (LC-ToF-MS)

Mass spectrometry was performed on a LCT Premier (Waters-Micromass, Manchester, UK) instrument equipped with a Z-spray interface. For the negative

Table 2.1 Summary of the general MS settings.

	PTX2 ESI^+			OA ESI^-		
	TQ-MS	ToF-MS	QToF-MS	TQ-MS	ToF-MS	QToF-MS
Capillary (kV)	3.2	2.5	3.0	3.2	2.5	3.0
Cone (V)	25	50	50	60	50	50
Source temp (°C)	120	120	120	120	120	120
Desolvation temp (°C)	350	350	350	350	350	350
Cone gas flow (l/hr)	50	40	40	50	40	40
Desolvation gas flow (l/hr)	500	700	700	500	700	700
Collision Energy (eV)	27		27	50		50
Gas Cell Pirani pressure ($\times 10^{-3}$ mbar)	3.0		3.0	3.0		3.0

and positive ion mode only the polarity changed but all other settings were kept the same (Table 2.1). To induce in-source CID a voltage of 60V for PTX2 and 85V for OA was applied to an aperture which is localized between the ion transfer optics. This type of fragmentation can be compared with applying a high cone voltage on single- or triple-quad analyzers. The lock mass was used to continuously recalibrate the mass axis. The lock mass in the positive mode was set at *m/z* 556.2771 and in the negative mode at *m/z* 554.2615 using 500 pg/ml leucine enkephalin in acetonitrile/H_2O (1:1) containing 0.1% formic acid infused at a flow rate of 0.02 ml/min and sampled during 1 s every 5 s.
An ACQUITY Ultra performance LC (UPLC) system (Waters, Manchester, UK) was used as a conventional HPLC system. The analytical column and the LC settings and conditions were identical to those of the TQ-MS experiments.

Quadrupole Time of Flight (LC-QToF-MS)
Mass spectrometry was performed on a QToF Micro (Waters-Micromass, Manchester, UK) instrument equipped with a Z-spray interface. Positive and negative ion modes were applied in separated runs. Except for the polarity and collision energy all the other settings were kept the same (Table 2.1). The lock mass in the negative mode was set at *m/z* 782.8074 and in the positive mode at *m/z* 784.8230 using one of the cluster ions of 0.1% phosphoric acid in acetonitrile/H_2O (1:1). An ACQUITY UPLC system was used as a conventional HPLC. The analytical column and LC settings and conditions were identical to the TQ-MS experiments.

Ion-trap (LC-IT-MS)
Mass spectral analysis was performed on a IT Advantage (Thermo Finnigan, San Jose, CA, USA) instrument. The electrospray ionization (ESI) probe with a positive polarity had a needle voltage of 5 kV and a capillary voltage of 3 V. The sheath gas flow rate used was 25 (arbitrary units) and the auxiliary gas was set at 15 (arbitrary units). For a negative polarity, the needle voltage was set at -5 kV, the capillary voltage was set at -5 V. For both polarities the capillary temperature was set at 150°C. The IT was tuned for both PTX2 (ESI^+) and OA (ESI^-) and the voltages on the lenses were optimized in TunePlus (Xcalibur software) while infusing a

standard solution in methanol from either 800 ng/ml PTX2 or 800 ng/ml OA at a flow rate of 5 µl/min. The optimized ion optic settings were as follows: lens voltage (ESI^+ -19.4 V, ESI^- 16.3 V), multipole 1 offset (ESI^+ -3.7 V, ESI^- 4.1 V), multipole 2 offset (ESI^+ -6.4 V, ESI^- 6.6 V), Multipole r.f. amplitude (ESI^+ 400, ESI^- 560), coarse trap d.c. offset (ESI^+ -10.1, ESI^- 9.8) and fine trap d.c. offset (ESI^+ -10.2 V, ESI^- 9.75 V). A Thermo Finnigan surveyor HPLC system (Thermo Finnigan, San Jose, CA, USA) was used. Column, LC settings and conditions were identical to the TQ-MS experiments.

RESULTS AND DISCUSSION

Fragmentation pathways

To investigate fragmentation pathways, experiments were carried out by direct infusion of PTX2 and OA standard solutions. These experiments were also done in order to optimize the instrument ionization conditions for each of the analytes.

Pectenotoxin-2 (PTX2)

For a PTX2 standard solution, a higher sensitivity was obtained in ESI^+ than in ESI^-. In ESI^-, PTX2 formed a formic acid adduct ($[M-H+CHOOH]^-$ *m/z* 903.5). CID produced the $[M-H]^-$ precursor ion (*m/z* 857.5) at high collision energies but no detectable fragments were formed (20-80 eV). In ESI^+, PTX2 was observed as an ammoniated precursor ion of *m/z* 876.5 $[M+NH_4]^+$. Figure 2.2a shows the CID mass spectrum of ammoniated PTX2 measured on the TQ-MS. These data are in accordance with those described in literature [18, 19]. The most abundant fragment ions observed for PTX2 result from successive water losses $[M+H-nH_2O]^+$ (n=1-5). Loss of water, however, is not a very specific fragmentation. In our view, the more specific (but less abundant) fragment ions result from skeletal fragmentation of the molecule, yielding the fragments *m/z* 195.1, 213.1, 275.2, 439.2, 457.2 and 551.2 (Table 2.2, Fig. 2.2).

Using the ToF-MS, an accurate mass of the ammoniated precursor ion could be retrieved within a mass error of 2-3 mDa (5 ppm). An optional feature of the ToF Premier is the production of in-source CID spectra (Fig. 2.2b) by aperture voltage fragmentation and single MS data with high resolution in a single run. The in-source CID fragments are a result of the collision of the precursor ion with the

nitrogen gas molecules present in the aperture region. This in-source CID offers the opportunity to obtain accurate masses of fragment ions with a resolution of 10 000 (FWHM). A disadvantage of performing in-source CID on a ToF instrument is the intrinsic loss of detectability at higher aperture voltages. At an aperture voltage of 60 V, which is required to induce sufficient in-source fragmentation of PTX2, the loss of detectability is estimated to be close to 80%. As a consequence, only solutions containing high PTX2 concentrations can be analyzed in this manner, e.g. by direct infusion experiments (Table 2.2). Furthermore, it can be seen from Table 2.2 that using in-source CID on a ToF does not always provide accurate masses of product ions; the accurate masses determined tend to be higher than the theoretical masses calculated. The loss of detectability is most likely related to the high aperture voltages applied; when the precursor ion is fragmented in the aperture region, scattering of the formed fragments may occur in the ion tunnel resulting in loss of sensitivity.

Although operating at a resolution of only 5 000 (FWHM), the QToF provided accurate masses for most fragments with a mass error below 5 mDa (Fig. 2.2c). Compared to the ToF-MS results the accurate mass of the product ions are better in the QToF-MS.

IT data provided no additional information about the fragmentation pathways of PTX2. PTX2 gave predominantly a series of water losses; skeletal fragments were less abundant (Fig. 2.2d). In order to increase the sensitivity, wide band activation was used in MS^3 mode. By selecting a larger mass window of 38 Da instead of 3 Da three fragments (the ammoniated precursor ion together with two fragments formed from subsequent water losses) were selected for further fragmentation. However, when applying a relative collision energy (RCE) of 48% or higher the only additional fragment obtained was the *m/z* 733.3 fragment, resulting from water loss of *m/z* 751.2.

By combining the mass data obtained by the various mass analyzers a fragmentation pathway for PTX2 can be proposed (Fig. 2.3).

Table 2.2 Fragments determined for PTX2 (ESI^+).

Elemental composition	Theoretical mass (*m/z*)	Observed experimental mass (*m/z*)					
		ToF-MS	Deviation (mDa)	QToF-MS	Deviation (mDa)	TQ-MS	IT-MS
$[C_{47}H_{70}O_{14} + NH_4]^+$	876.5109	876.5081	-2.8	876.5041	-6.8	876.4	876.5
$[C_{47}H_{68}O_{13} + H]^+$	841.4738	841.4752	+1.4	841.4730	-0.8	841.4	841.4
$[C_{47}H_{66}O_{12} + H]^+$	823.4633	823.4604	-2.9	823.4614	-1.9	823.4	823.4
$[C_{47}H_{64}O_{11} + H]^+$	805.4527	805.4554	+2.7	805.4498	-2.9	805.3	805.3
$[C_{47}H_{62}O_{10} + H]^+$	787.4421	787.4504	+8.3	787.4390	-3.1	787.3	787.3
$[C_{29}H_{42}O_{10} + H]^+$	551.2856	551.2973	+11.7	551.2858	+0.2	551.2	551.2
$[C_{29}H_{40}O_9 + H]^+$	533.2751	533.2739	-1.2	533.2739	-1.2	533.2	533.2
$[C_{28}H_{41}O_5 + H]^+$	457.2954	457.2985	+3.1	457.2960	+0.6	457.2	457.2
$[C_{28}H_{38}O_4 + H]^+$	439.2848	439.2871	+2.3	439.2842	-0.6	439.2	439.2
$[C_{17}H_{22}O_3 + H]^+$	275.1647	275.1733	+8.6	275.1643	-0.4	275.2	No[1]
$[C_{11}H_{16}O_4 + H]^+$	213.1127	213.1196	+6.9	213.1163	+3.6	213.1	No[1]
$[C_{11}H_{14}O_3 + H]^+$	195.1021	195.1140	+11.9	195.0996	-2.5	195.1	No[1]

[1] Not possible to observe this fragment.

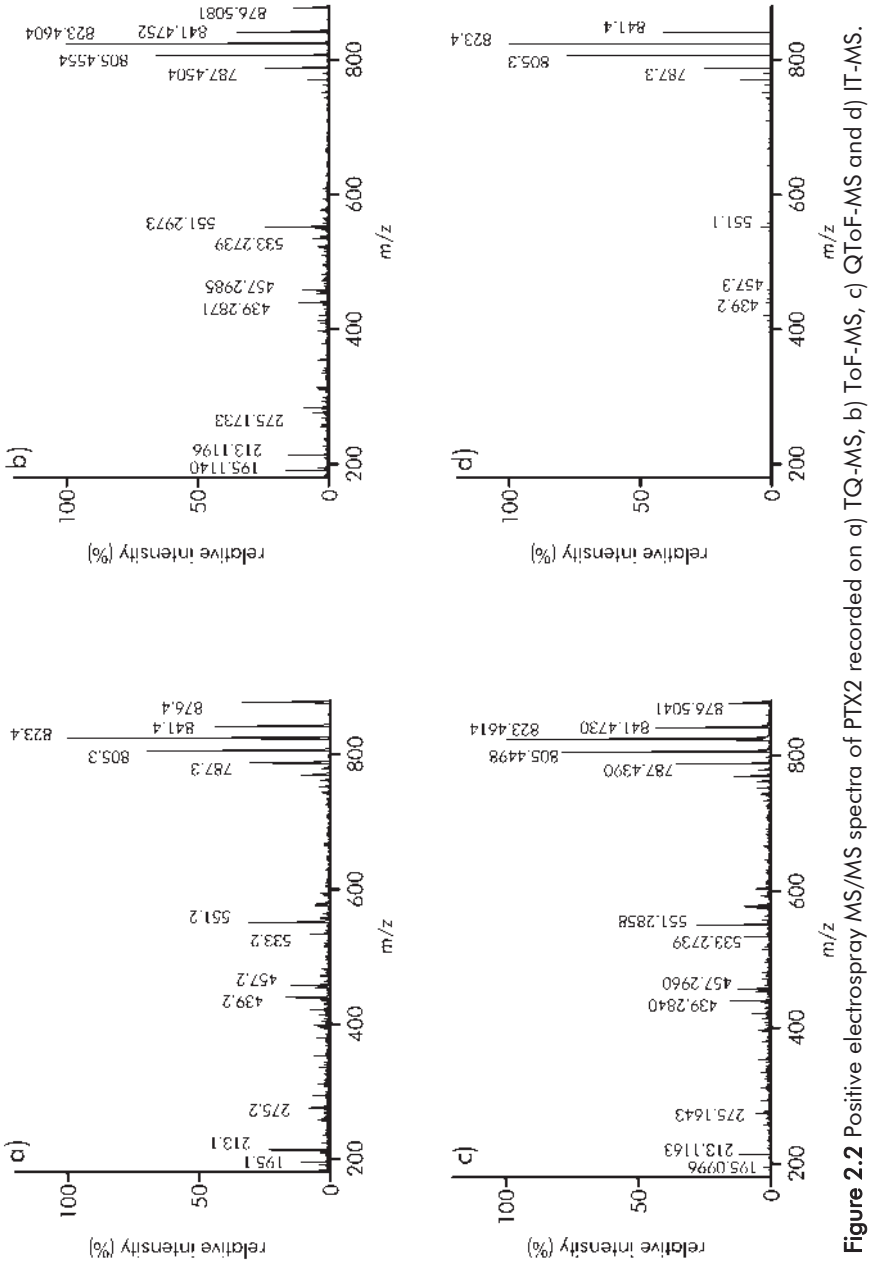

Figure 2.2 Positive electrospray MS/MS spectra of PTX2 recorded on a) TQ-MS, b) ToF-MS, c) QToF-MS and d) IT-MS.

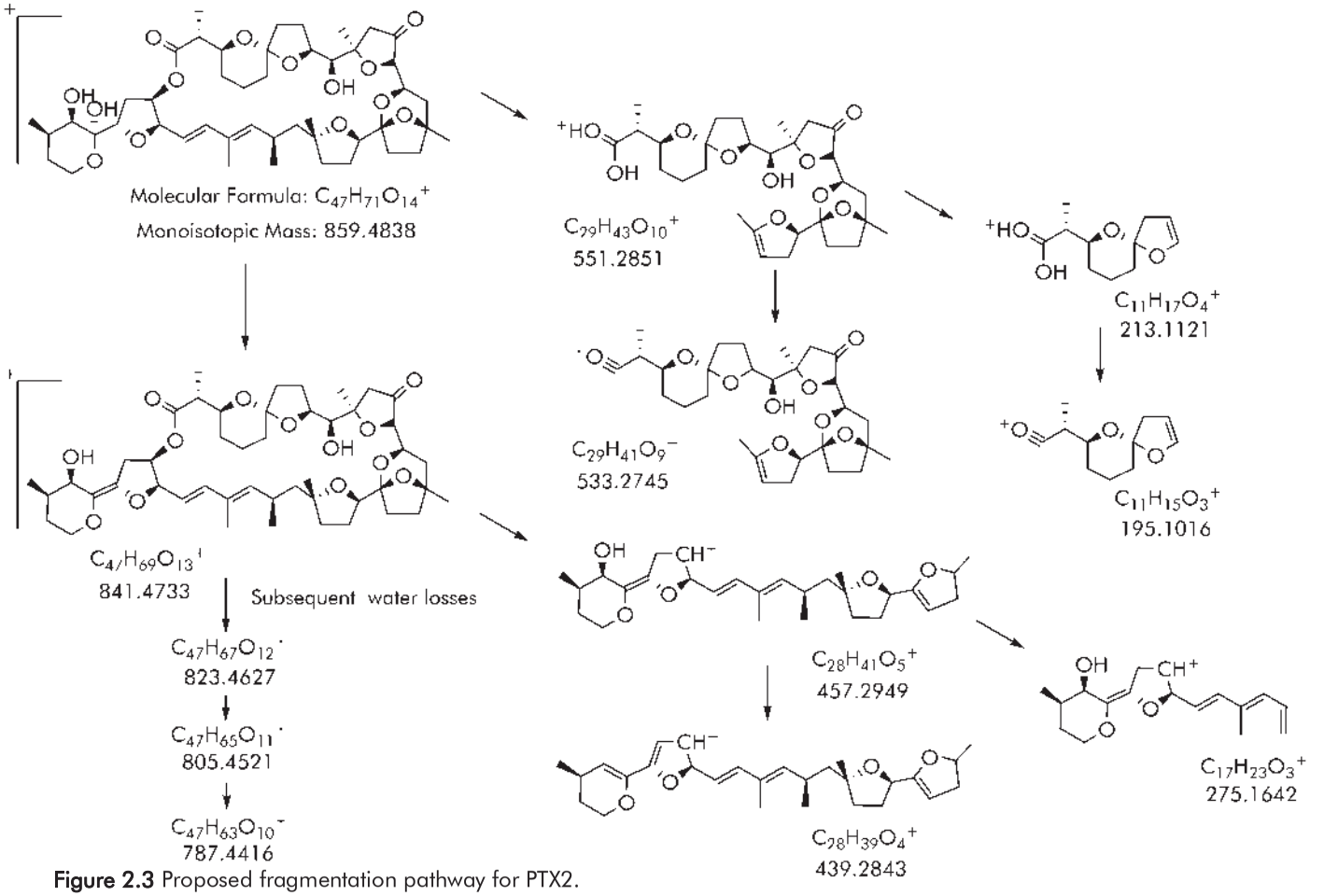

Figure 2.3 Proposed fragmentation pathway for PTX2.

Okadaic Acid (OA)

For OA the sensitivity in ESI^- was better than in ESI^+ [20]. In the ESI^+ mode, OA was observed as an ammoniated precursor ion ($[M+NH_4]^+$ *m/z* 822.5). The fragments produced were mainly from successive losses of water. In the ESI^- mode OA produced a deprotonated precursor ion of *m/z* 803.5 $[M-H]^-$. TQ-MS fragmentation of OA in the ESI^- mode resulted in the production of the skeletal fragments with *m/z* 563.3, 545.3, 321.2, 255.1, 209.1, 150.9 and 112.9 (Table 2.3, Fig. 2.4a).

The ToF-MS yielded an accurate mass for OA $[M-H]^-$ with a mass error below 2 mDa. As discussed above for PTX2, in-source induced fragmentation resulted in the production of fragment ions, but with reduced mass accuracy. At lower aperture voltages an improved mass accuracy was obtained at the expense of a lower detectability for the resulting skeletal fragments.

The QToF experiments provided some additional information on the fragmentation (Fig. 2.4c). Again, the obtained mass accuracy for the fragments was better using the QToF-MS than by in-source CID on the ToF-MS.

Additional information on the fragmentation pathways of OA could be retrieved using the IT mass spectrometer (Fig 2.4d). For OA in MS^2, the two most intense fragments observed were *m/z* 563.3 and 255.0. When performing MS^3 on the *m/z* 563.3 fragment, two new fragments were formed at *m/z* 545.1 and *m/z* 255.0. This shows that the *m/z* 255.0 fragment is formed from the precursor ion as well as the *m/z* 563.3 fragment. When isolating the *m/z* 255.0 fragment and applying a RCE of 32%, the *m/z* 209.1 and 112.8 fragments were the most abundant. At a slightly higher RCE of 33% the most abundant fragment observed was *m/z* 151.0. The proposed fragmentation pathway for OA is shown in figure 2.5.

Table 2.3 Fragments determined for OA (ESI^-).

Elemental composition	Theoretical mass (*m/z*)	Observed experimental mass (*m/z*)					
		ToF-MS	Deviation (mDa)	QToF-MS	Deviation (mDa)	TQ-MS	IT-MS
$[C_{44}H_{68}O_{13} - H]^-$	803.4582	803.4578	-0.4	803.4567	-1.5	803.5	803.3
$[C_{44}H_{66}O_{12} - H]^-$	785.4476	785.4531	+5.5	785.4454	-2.2	785.5	785.3
$[C_{30}H_{44}O_{10} - H]^-$	563.2856	563.2849	-0.7	563.2874	+1.8	563.3	563.3
$[C_{30}H_{42}O_9 - H]^-$	545.2751	No[1]		545.2769	+1.8	545.3	545.2
$[C_{19}H_{30}O_4 - H]^-$	321.2071	321.2098	+2.7	321.2077	+0.6	321.2	321.2
$[C_{13}H_{20}O_5 - H]^-$	255.1232	255.1266	+3.4	255.1301	+6.9	255.1	255.0
$[C_{12}H_{18}O_3 - H]^-$	209.1178	209.1279	+10.1	209.1219	+4.1	209.1	209.1[2]
$[C_9H_{12}O_2 - H]^-$	151.0759	151.0896	+13.7	151.0804	+4.5	150.9	151.0[2]
$[C_6H_{10}O_2 - H]^-$	113.0603	113.0568	-3.5	113.0576	-2.7	112.9	112.8[2]

[1)] Not possible to observe this fragment.
[2)] Fragment observed in MS^3.

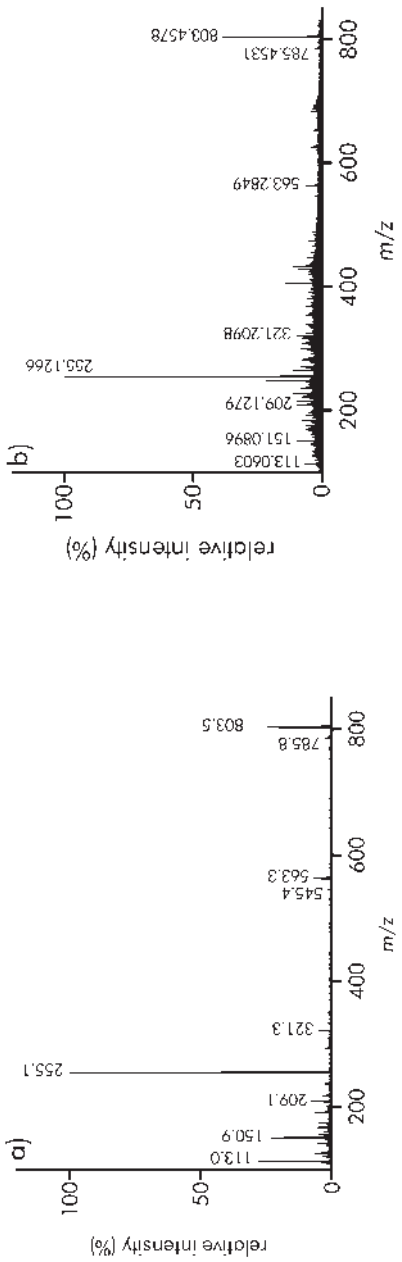

Figure 2.4 Negative electrospray MS/MS spectra of OA recorded on a) TQ-MS, b) ToF-MS, c) QToF-MS and d) IT-MS.

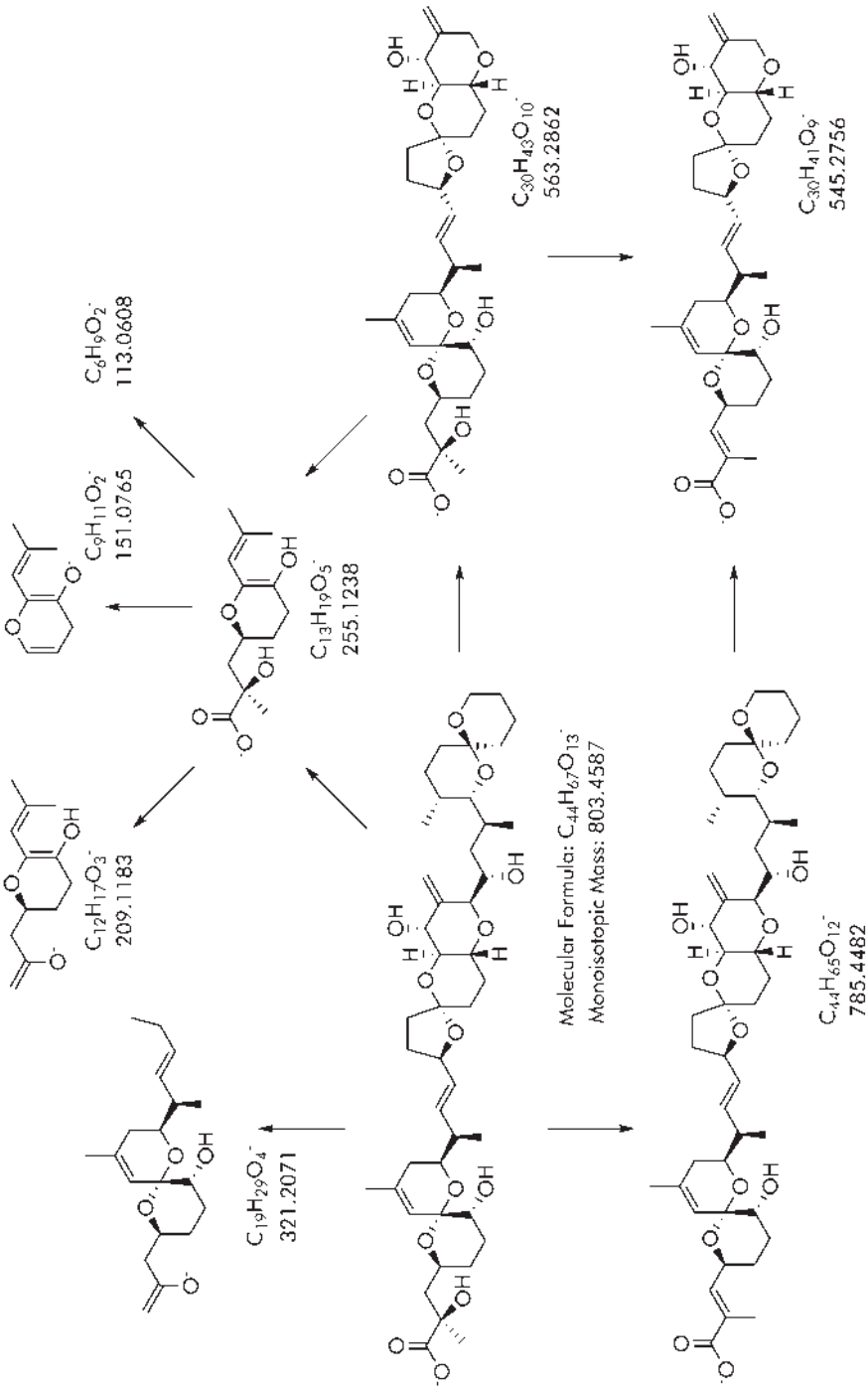

Figure 2.5 Proposed fragmentation pathway for OA.

Linearity, repeatability and detection limits

The TQ-MS showed good linearity and repeatability for both PTX2 and OA (Table 2.4). The detection limit (LOD) was calculated using a signal-to-noise value of three (S/N=3), for two MRM channels. For PTX2 the abundant *m/z* 876.5 > 823.5 transition and the less abundant but more specific *m/z* 876.5 > 213.1 transition were chosen. For OA the MRM channels *m/z* 803.5 > 255.1 and 803.5 > 151.1 were used (Table 2.5). Matrix-matched standard and standard solution concentrations were used at such levels that the S/N values were close to 3. For PTX2, higher LODs were obtained for spiked extracts compared to methanolic standard solutions, indicating ion suppression for spiked extracts. For OA, lower LODs were obtained for spiked extracts compared to methanolic standard solutions, indicating ion enhancement for spiked extracts. Therefore, for routine analysis either extensive sample clean up or matrix-matched standards should be used to correct for these ion suppression and enhancement effects.

In our study linearity, repeatability and LOD on the ToF-MS were good (Tables 2.4 and 2.5). With the QToF, we used phosphoric acid as mass reference compound, which worked well, without contamination of the cone and baffle. However, when using phosphoric acid on the ToF-MS the cone and baffle become contaminated rather quickly. Therefore, leucine-enkaphalin was used instead on the ToF-MS. Linearity on the QToF was good, but repeatability and LOD were less promising compared to ToF-MS and TQ-MS (Tables 2.4 and 2.5). A major disadvantage of the QToF Micro is that it is not possible to switch polarity from ESI^+ to ESI^- and vice versa during or between two runs without manually putting the system on standby. This is necessary in order to recondition the voltage of the micro channel plate (MCP). Newer types of QToF mass spectrometers have an improved resolution and also an option to do polarity switching in a single run.

As expected, linearity and repeatability of the IT-MS were somewhat lower compared to the other mass spectrometric instruments. Furthermore, on the IT-MS, fragments of OA could be observed only in extracts with OA concentrations >40 ng/ml. However, when the precursor ion was selected with single ion monitoring a signal could be obtained from the standard with the lowest concentration (5 ng/ml). Therefore, the single ion monitoring data were used for the determination of the linearity and repeatability. With PTX2 no sensitivity

problems were observed. For PTX2 the *m/z* 823.5 and 805.5 fragments were used, due to the fact that the range of the ion-trap was not sufficient to obtain the fragment with *m/z* 213.1 (Table 2.4). This low-mass cut-off for IT-MS is around 25-30% of the *m/z* of the selected precursor ion.

Table 2.4 Linearity and repeatability determined in MeOH for the different mass analyzers.

	Linearity (5-60 ng/ml) (R^2)		Repeatability (n=10) at 40 ng/ml (%)	
	PTX2	OA	PTX2	OA
TQ-MS	>0.990	>0.990	3.6	9.0
ToF-MS	>0.990	>0.990	3.4	2.8
QToF-MS	>0.990	>0.990	8.0	12.8
IT-MS	>0.980	>0.970	6.8	17.8

Table 2.5 LOD determined for the different mass analyzers (calculated at S/N=3 in picograms on-column).

	Standard in methanol		Spiked mussel extract	
	PTX2 (pg)	OA (pg)	PTX2 (pg)	OA (pg)
TQ-MS[1]	1.9	7.6	12.2	2.9
TQ-MS[2]	3.7	13.7	22.3	7.5
ToF-MS	1.7	8.1	14.6	11.0
QToF-MS	40.6	108.0	12.5	224.2
IT-MS[3]	1.7	73.5	3.6	7.3[4]

[1)] PTX2: *m/z* 823.5 fragment; OA: *m/z* 255.1 fragment.
[2)] PTX2: *m/z* 213.1 fragment; OA: *m/z* 151.1 fragment.
[3)] PTX2: sum of *m/z* 823.5, 805.5, 551.3 fragments; OA: *m/z* 255.1 fragment.
[4)] OA: SIM at *m/z* 803.5.

CONCLUSIONS

In this study, the spectra obtained by the different mass analyzers successively contributed to the elucidation of the fragmentation pathways of PTX2 and OA. In general, to elucidate fragmentation pathways a combination of fragmentation data observed by e.g. an IT-MS and high resolution data obtained by, e.g. QToF-MS, is needed.

All mass analyzers had good linearity and repeatability for PTX2 and OA. The LOD for PTX2 on all mass analyzers was sufficiently low to use them for routine analysis. On the QToF-MS the LOD for OA was too high to use it for routine analysis, but on the other mass analyzers the LOD was good. The most sensitive mass analyzer is the TQ-MS. This mass analyzer is most frequently used for routine quantitative analysis of shellfish samples. For example, OA on TQ-MS system is ten-fold more sensitive than the 9-anthryldiazomethane (ADAM) fluorescence method. On the TQ-MS the LOD for OA was around 3 μg/kg, while for ADAM derivatisation an LOD of 30 μg/kg was reported [21]. The application of LC-MS/MS for lipophilic shellfish toxin analysis is reliable and sensitive enough to be a good candidate to replace the controversial mouse assays which are still prescribed in European legislation.

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REFERENCES

[1] Yasumoto T, Murata M. Marine toxins. *Chem Rev* 1993; **93**: 1897.

[2] Toyofuku H. Joint FAO/WHO/IOC activities to provide scientific advice on marine biotoxins (research report). *Mar Pollut Bull* 2006; **52**: 1735.

[3] Terao K, Ito E, Yanagi T, Yasumoto T. Histopathological studies on experimental marine toxin poisoning .1. Ultrastructural-changes in the small-intestine and liver of suckling mice induced by dinophysistoxin-1 and pectenotoxin-1. *Toxicon* 1986; **24**: 1141.

[4] Terao K, Ito E, Oarada M, Murata M, Yasumoto T. Histopathological studies on experimental marine toxin poisoning .5. The effects in mice of yessotoxin isolated from *Patinopecten-yessoensis* and of a desulfated derivative. *Toxicon* 1990; **28**: 1095.

[5] Fujiki H, Suganuma M, Yoshizawa S, Nishiwaki S, Winyar B, Sugimura T. Mechanisms of action of okadaic acid class tumor promoters on mouse skin. *Environ Health Persp* 1991; **93**: 211.

[6] European Parliament, Council. Commission directive 2004/853/EC specific hygiene rules for food of animal origin. *Off J Eur Commun* 2004; **L226**: 22.

[7] Lee JS, Yanagi T, Kenma R, Yasumoto T. Fluorometric-determination of diarrhetic shellfish toxins by high-performance liquid-chromatography. *Agr Biol Chem Tokyo* 1987; **51**: 877.

[8] Yasumoto T, Fukui M, Sasaki K, Sugiyama K. Determinations of marine toxins in foods. *J AOAC Int* 1995; **78**: 574.

[9] McNabb P, Selwood AI, Holland PT. Multiresidue method for determination of algal toxins in shellfish: Single-laboratory validation and interlaboratory study. *J AOAC Int* 2005; **88**: 761.

[10] Stobo LA, Lacaze JPCL, Scott AC, Gallacher S, Smith EA, Quilliam MA. Liquid chromatography with mass spectrometry - detection of lipophilic shellfish toxins. *J AOAC Int* 2005; **88**: 1371.

[11] Jorgensen K, Jensen LB. Distribution of diarrhetic shellfish poisoning toxins in consignments of blue mussel. *Food Addit Contam A* 2004; **21**: 341.

[12] Puente PF, Saez MJF, Hamilton B, Lehane M, Ramstad H, Furey A, James KJ. Rapid determination of polyether marine toxins using liquid chromatography-multiple tandem mass spectrometry. *J Chromatogr A* 2004; **1056**: 77.

[13] Draisci R, Giannetti L, Lucentini L, Ferretti E, Palleschi L, Marchiafava C. Direct identification of yessotoxin in shellfish by liquid chromatography coupled with mass spectrometry and tandem mass spectrometry. *Rapid Commun Mass Spectrom* 1998; **12**: 1291.

[14] Fux E, McMillan D, Bire R, Hess P. Development of an ultra-performance liquid chromatography-mass spectrometry method for the detection of lipophilic marine toxins. *J Chromatogr A* 2007; **1157**: 273.

[15] Lehana M, Saez MJF, Brana-Magdalena A, Ruppen Canas I, Sierra MD, Hamilton B, Furey A, James KJ. Liquid chromatography - multiple tandem mass spectrometry for the determination of ten azaspiracids, including hydroxyl analogues in shellfish. *J Chromatogr A* 2004; **1024**: 63.

[16] James KJ, Sierra MD, Lehane M, Brana-Magdalena A, Furey A. Detection of five new hydroxyl analoques of azaspiracids in shellfish using multiple tandem mass spectrometry. *Toxicon* 2003; **41**: 227.

[17] Hess P, McMahon T, Slattery D, Swords D, Dowling G, McCarron M, Clarke D, Gibbons W, Silke J, O'Cinneide M. Use of LC-MS testing to identify lipophilic toxins, to establish local trends and interspecies differences and to test the comparability of LC-MS testing with the mouse bioassay: an example from the Irish Biotoxin monitoring programme 2001. *Emerg Mar Phyc Anal Meth* 2005; 43.

[18] James KJ, Bishop AG, Draisci R, Palleschi L, Marchiafava C, Ferretti E, Satake M, Yasumoto T. Liquid chromatographic methods for the isolation and identification of new pectenotoxin-2 analogues from marine phytoplankton and shellfish. *J Chromatogr A* 1999; **844**: 53.

[19] Miles CO, Wilkins AL, Samdal IA, Sandvik M, Petersen D, Quilliam MA, Naustvoll LJ, Rundberget T, Torgersen T, Hovgaard P, Jensen DJ, Cooney JM. A novel pectenotoxin, PTX-12, in *Dinophysis spp*. and shellfish from Norway. *Chem Res Toxicol* 2004; **17**: 1423.

[20] Suzuki T, Yasumoto T. Liquid chromatography-electrospray ionization mass spectrometry af diarrhetic shellfish-poisoning toxins okadaic acid, dinophysistoxin-1 and pectenotoxin-6 in bivalves. *J Chromatogr A* 2000; **874**: 199.

[21] Quilliam MA. Analysis of diarrhetic shellfish poisoning toxins in shellfish tissue by liquid-chromatography with fluorometric and mass-spectrometric detection. *J AOAC Int* 1995; **78**: 555.

3

Liquid chromatography tandem mass spectrometric method for the detection of marine lipophilic toxins under alkaline conditions

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ABSTRACT

A new LC-MS/MS method for the separation and detection of the most prominent marine lipophilic toxin groups comprising okadaic acid, dinophysistoxins, yessotoxins, azaspiracids, pectenotoxins, spirolides and some okadaic acid fatty acid esters has been developed. With this method 28 different lipophilic marine toxins can be analyzed in a single run. Separation was achieved with an acetonitrile/water gradient containing ammonium hydroxide (pH 11). All toxins were stable under these alkaline conditions. Compared to chromatography using an acidic gradient, the limit of detection (LODs) for okadaic acid, yessotoxin, gymnodimine and 13-desmethyl spirolide C was improved two- to three-fold, mainly due to better peak shapes. The azaspiracids and pectenotoxin-2 showed comparable LODs under acidic and alkaline conditions. A major advantage of the developed method is that toxins can be clustered in retention time windows separated for positively and negatively ionized molecular ions. Therefore, there is no need for rapid polarity switching or two separate runs for one sample. The new method showed good repeatability and reproducibility and is an important step in the development of alternatives to the animal tests currently in use for shellfish toxin analysis.

INTRODUCTION

Consumption of shellfish contaminated with phycotoxins can cause severe intoxications in humans such as Diarrhetic Shellfish Poisoning (DSP). Due to their lipophilic properties DSP toxins are often classified as lipophilic marine toxins. Members of the DSP toxin group are okadaic acid (OA) and its derivatives dinophysistoxin-1 (DTX1), dinophysistoxin-2 (DTX2) and dinophysistoxin-3 (DTX3) (Fig. 3.1a) [1]. The latter comprises the fatty acid acyl esters of OA, DTX1 and DTX2. Other lipophilic marine toxin groups are yessotoxins (YTXs) (Fig. 3.1b), azaspiracids (AZAs) (Fig. 3.1c), pectenotoxins (PTXs) (Fig. 3.1d), gymnodimine (GYM) (Fig. 3.1e) and spirolides (SPXs) (Fig. 3.1e). YTXs have an adverse effect on cardiac mussel cells and on defattening liver cells [2]. AZAs cause diarrhetic effects [3] while PTXs are mildly diarrhetic and hepatotoxic [4]. GYM and SPXs produce neurotoxic symptoms when administrated orally or injected intraperitoneally in mice [5,6]. The European Union has established legislation for 13 lipophilic marine toxins [7]; while GYM and the SPXs are not yet under legislation. For the detection of these toxins EU legislation prescribes a mouse bioassay. However, this method regularly produces false positives especially when other toxins such as GYM and SPXs are present in the samples. These toxins can cause death of the test animal in the bioassay even at low concentrations [8]. Intoxications in humans caused by GYM and SPXs have not been reported, however.

In recent years much effort has been put in the development of LC-MS/MS methods that are dedicated to either detecting the specific classes of lipophilic marine toxins or detecting as much as possible different lipophilic marine toxins in a multi-toxin method. In the literature several methods for the separation of OA, DTX1, DTX2 and the esters (DTX3) have been described. Most of these methods use a 50 mm C_8 or a 150 mm C_{18} column in combination with a water/acetonitrile gradient containing ammonium formate and formic acid or a water/methanol gradient containing ammonium acetate [9-12]. For YTXs several methods have been described where ammonium acetate or ammonium formate is used [13,14]. Miles *et al.* were able to analyze over 90 analogues of YTX present in algae extracts using a 50 mm C_{18} column in combination with a water/acetonitrile gradient containing ammonium formate and formic acid [14]. Amandi *et al.* indicated that using a C_{18} column in combination with an isocratic mobile phase

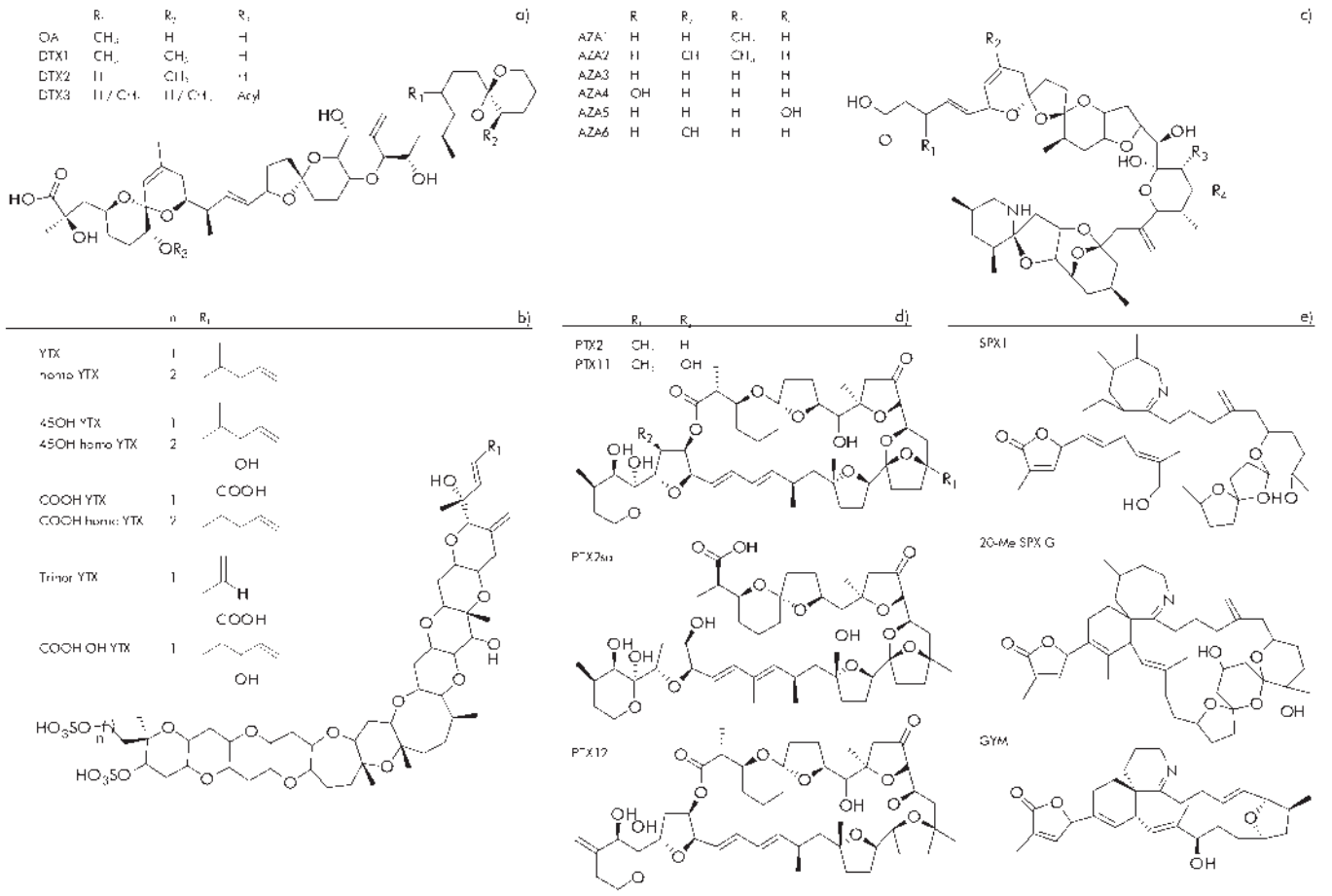

Figure 3.1 Structures of the toxins included in the final method. a) Okadaic acid and dinophysistoxins, b) yessotoxins, c) azaspiracids, d) pectenotoxins, e) spiroimine toxins.

(water/acetonitrile 40:60 v/v) containing ammonium acetate resulted in poor peak shapes for YTXs [13]. In general, YTXs tend to elute as broad peaks under acidic conditions with a full-width-half-maximum (FWHM) up to minutes [15]. Rehmann *et al.* were able to separate over 21 different AZA analogues using very-high-pressure liquid chromatography (VHPLC) in combination with an VHPLC C_{18} column with a water/acetonitrile gradient containing ammonium formate and formic acid [16]. Miles *et al.* used a C_8 as well as a C_{18} column to separate different PTXs using isocratic and gradient elution with water/acetonitrile containing ammonium formate and formic acid [17,18]. Aasen *et al.* described a method for the separation of SPXs using a 50 mm C_8 column and a water/acetonitrile gradient containing ammonium formate and formic acid [8].

Recently, some methods for the simultaneous analysis of several lipophilic marine toxin groups have been developed. Fux *et al.* described an VHPLC method to analyze 21 lipophilic marine toxins in only 6.6 min using a water/acetonitrile gradient containing ammonium formate and formic acid. The MS method used rapid polarity switching and therefore complete separation of toxins preferably analyzed in negative or positive ionization mode was not necessary [19]. Stobo *et al.* developed a method that comprises all toxins mentioned in the European Union (EU) legislation. By using a water/acetonitrile gradient containing ammonium acetate at neutral pH (6.8) a favourable separation and good peak shapes were obtained [20]. The DTX3 toxins could only be analyzed after alkaline hydrolysis to free OA, DTX1 and DTX2. Mc Nabb *et al.* developed a multi-toxin method that used an acidic water/acetonitrile gradient. Good peak shapes were obtained but the toxins preferably analyzed in different ionization modes were overlapping [21]. Quilliam *et al.* described a multi-toxin method for the various lipophilic toxins based on an acidic water/acetonitrile gradient. Good peak shapes were obtained but it should be pointed out that the YTX group was not included in this method [22].

In all except one of the multi-toxin methods mentioned above a water/acetonitrile gradient at low pH (2-4) has been used for the separation of the toxins. With these mobile phase systems the chromatographic separation of some of the 13 lipophilic marine toxins under legislation can be problematic. In most of the methods some toxins which are analyzed most preferably in negative or positive ionization mode

are co-eluting [19,21-23]. When the mass spectrometer is not capable of fast polarity switching during analysis, the samples need to be analyzed in two separate runs.

Many lipophilic marine toxins contain functionalities (SO_3H, COOH, NH_2, =NH) that can be protonated or deprotonated depending on the pH of the solvent. Therefore, the pH of the mobile phase will have an impact on their chromatographic behaviour. This can be seen from the method of Stobo *et al.*, which utilises a gradient at neutral pH resulting in an improved separation of toxins analyzed preferably in positive and negative ionization. It was anticipated that a change of pH to alkaline conditions could result in an even better chromatographic separation by using the newer type of cross-linked silica based C_{18} column materials which are stable up to pH 12. In this paper a newly developed LC-MS/MS method is presented in which 28 lipophilic marine toxins are separated with a mobile phase at alkaline pH. The new method was compared to an established method for the separation of lipophilic marine toxins [22,23]. Special attention was paid to the stability of the toxins under the alkaline conditions. In addition, the new LC method was used to separate some brevetoxin metabolites (BTXs) which can cause Neurotoxic Shellfish Poisoning (NSP).

EXPERIMENTAL

Reagents and standards

Water was deionized and passed through a Milli-Q water purification system (Millipore, Billerica, MA, USA). Formic acid (98-100%) was purchased from Merck, Darmstadt, Germany. Ammonium formate (>97%) was purchased from Sigma Aldrich, Steinheim, Germany. Acetonitrile (HPLC supra gradient) and methanol (absolute, HPLC grade) were purchased from Biosolve, Valkenswaard, The Netherlands. Ammonium hydroxide (25%) was purchased from VWR international, Amsterdam, The Netherlands. Okadaic acid (OA) (CRM-OA-b 24.1±0.8 μg/ml), yessotoxin (YTX) (CRM-YTX 5.3±0.3 μg/ml), azaspiracid-1 (AZA1) (CRM-AZA1 1.24±0.07 μg/ml), pectenotoxin-2 (PTX2) (CRM-PTX2 8.6±0.3 μg/ml), gymnodimine (GYM) (CRM-GYM 5.0±0.2 μg/ml) and 13-desmethyl spirolide C (SPX1) (CRM-SPX1 7.0±0.4 μg/ml) were purchased from the National Research Council, Institute for Marine Biosciences (NRC-CNRC), Halifax, Canada.

Laboratory Reference Material (LRM) containing OA, DTX1-2, YTX, AZA1-3 and PTX2; material containing OA-esters and contaminated material with AZAs were kindly donated by Dr. P. Hess from the Marine Institute, Oranmore, Ireland. Shellfish material contaminated with YTXs, *Dinophysis acuta* extracts and mussel extract containing PTXs were kindly donated by Dr. M. Sandvik, National Veterinary Institute, Oslo, Norway. SPX contaminated material was kindly donated by Dr. J. Aassen from The Norwegian School of Veterinary Science, Oslo, Norway. Shellfish material containing brevetoxin metabolites was kindly donated by Dr. G.J. Doucette from the Center for Coastal Environmental Health and Biomolecular Research, Charleston, SC, USA.

Preparation of standards and extracts

A stock solution of 200 ng/ml containing OA, YTX, AZA1, PTX2, GYM and SPX1 was prepared in methanol. From this stock dilutions were made in the range of 1-50 ng/ml ($n=7$) in blank mussel extract (*Mytilus edulis*). Blank mussel extract was prepared by homogenizing 100 g of whole flesh mussel tissue (Eastern Scheldt, The Netherlands) with a T25 Ultra Turrax mixer at 24 000 rpm (IKA® Works Inc., Wilmington, NC, USA). One gram of this shellfish homogenate was extracted in triplicate with 3 ml methanol. After each addition of methanol the extract was Vortex-mixed during 1 minute. After Vortex-mixing the extract was centrifuged 5 min at 2 000 $\times$ *g*. The supernatant was transferred to a volumetric flask of 10 ml and after the third extraction the volume was made up to 10 ml with methanol. The crude shellfish extract was filtered through a HT tuffryn 0.2 μm membrane filter (Pall Corp., East Hills, NY, USA) prior to spiking or analysis.

Chromatographic separation

A Waters Alliance 2690 (Milford, MA, USA) HPLC system was equipped with two different LC columns. The column heater was kept at 40°C. The vial compartment of the autosampler was kept at 10°C and a 10 μl injection volume was used. It should be mentioned that the Alliance system with its low pressure mixing chamber is quite sensitive to gas formation from ammonia containing mobile phases. This system should therefore be carefully degassed and kept under helium during analysis.

HPLC method using a Hypersil column

The method using an acidic mobile phase was adapted from Hess *et al.* [23]. A Thermo Finnegan BDS Hypersil C_8 (50 × 2.1 mm, 3 μm) column was used. Mobile phase A was water and B was acetonitrile/water (95:5 v/v), both containing 2 mM ammonium formate and 50 mM formic acid (pH = 2.6). A gradient was run at a flow rate of 0.2 ml/min starting at 30% B, which was increased linearly to 90% B in 8 min. It was kept at 90% B for 2.5 min and returned in 0.5 min to 30% B. An equilibration time of 4 min was allowed prior to the next injection. Additionally the column was tested for a neutral mobile phase system which was adapted from the method of Stobo *et al.* [20]. Water was used as mobile phase A and acetonitrile/water (95:5 v/v) as mobile phase B, both containing 5 mM ammonium acetate (pH 7). The gradient and runtime were kept the same as for the acidic gradient.

HPLC method using a XBridge column

A Waters XBridge C_{18} (150 × 3 mm, 5 μm) column was used. Mobile phase A was water and B was acetonitrile/water (90:10 v/v), both containing 6.7 mM ammonium hydroxide (pH = 11). A flow rate of 0.4 ml/min was used. A gradient started at 10% B, was kept at 10% for 1 minute and was then increased linearly to 90% B in 9 min. The mobile phase composition was kept at 90% B for 3 min and returned to 10% B in 2 min. An equilibration time of 4 min was allowed before the next injection. Additionally, an acidic and a neutral mobile phase system in combination with the XBridge column were also investigated. The composition of acidic and neutral mobile phase system was as described in the '*HPLC method using a Hypersil column*' section. The gradient and the runtime were kept the same as for the alkaline gradient.

Mass spectrometry

Mass spectrometric detection was performed with a Micromass Quattro Ultima tandem mass spectrometer (Waters-Micromass, Manchester, UK) equipped with an electrospray ionization interface (ESI). The mass spectrometer was operated in both negative and positive ESI. In both modes a capillary voltage of 2.8 kV, a desolvation gas temperature of 350°C at a N_2 flow of 600 l/h, a source temperature of 120°C and a nebulizer gas (N_2) flow of 100 l/h was used. Argon

was used as collision-induced dissociation (CID) gas at a pressure of 2.5×10^{-3} mbar. The cone voltage and collision energy were optimized by direct infusion experiments under acidic and alkaline conditions (Table 3.1). For those toxins for which insufficient material was available the cone voltage and collision energy were estimated based on structure comparison with the available toxin standards. Two product ions were selected for each toxin, to allow quantification as well as identification of the specific toxin. With the acidic HPLC method (Hypersil column) two separated injections were done with the MS operating separately in ESI^- and in ESI^+. For the detection of the toxins retention time windows were set. With the alkaline HPLC method three different retention time windows were applied. In the first window the MS was operated in ESI^- and in the second and third window in ESI^+. The first window contained 22 transitions (12 different toxins), the second 12 (6 toxins) and the third window 20 transitions (10 toxins). The dwell-time was set at 60 msec per transition.

Investigated parameters of the HPLC methods

Methanolic mussel extracts spiked with the toxin standards and extracts from various contaminated shellfish materials were analyzed on the Hypersil column with the acidic HPLC method and on the XBridge column with the alkaline HPLC method. From the chromatograms obtained the retention time, retention time stability during a series of injections and the peak width at FWHM ($n=3$) of the various toxins were determined. For the determination of the limit of detection (LOD) of each toxin, a signal-to-noise ratio of 3 was extrapolated from the lowest abundant product ion of the toxin present in the lowest spiked methanolic mussel extract.

Stability toxins under alkaline conditions

The LRM extract containing OA, DTX1-2, YTX, AZA1-3 and PTX2 was used to investigate the stability of the toxins under alkaline conditions. One ml of methanolic extract was mixed with 125 μl of increasing ammonium hydroxide concentrations: 0, 0.133, 0.667, 1.33, 4 and 13.3 M corresponding to 0, 15, 74, 148, 591 and 1478 mM in the extract. The solutions were mixed and kept at room temperature or at 60°C for 1 hour. After 1 hour the solutions were neutralized by

addition of a solution of 125 µl formic acid solution, in a concentration which matched with the added ammonium hydroxide. All experiments were done in duplicate.

Table 3.1 MS/MS conditions used for the Multiple Reaction Monitoring (MRM) acquisition windows for the detection of marine lipophilic toxins.

Toxin	ESI mode	Precursor ion (*m/z*)	Product ion (*m/z*)		Cone voltage (V)	Collision energy (eV)
			1	2		
OA	ESI^-	803.5	255.2		60	45
				113.1	60	50
DTX1	ESI^-	817.5	255.2		60	45
				113.1	60	50
DTX2	ESI^-	803.5	255.2		60	45
				113.1	60	50
16:0 OA-ester	ESI^+	1060.5 [1]	769.5	305.2	60	20
22:6 OA-ester	ESI^+	1132.5 [1]	769.5	305.2	60	20
YTX	ESI^-	1141.5 [2]	1061.5	855.4	45	40
		570.4 [3]	467.4	396.4	75	30
1a-homo-YTX	ESI^-	1155.5 [2]	1075.5	869.4	45	40
		577.4 [3]	474.4	403.4	75	30
45OH-YTX	ESI^-	1157.5 [2]	1077.5	855.4	45	40
		578.4 [3]	467.4	396.4	75	30
45OH-1a-homo-YTX	ESI^-	1171.5 [2]	1091.5	869.4	45	40
		585.4 [3]	474.4	403.4	75	30
44COOH-YTX	ESI^-	1173.5 [2]	1093.5	855.4	45	40
		586.4 [3]	467.4	396.4	75	30
44COOH-1a-homo-YTX	ESI^-	1187.5 [2]	1107.5	869.4	45	40
		593.4 [3]	474.4	403.4	75	30
Trinor-YTX	ESI^-	1101.5 [2]	1021.5	855.4	45	40
		550.4 [3]	467.4	396.4	75	30
44COOH-45OH-YTX	ESI^-	1189.5 [2]	1109.5	855.4	45	40
		593.4 [3]	467.4	396.4	75	30

[1] Present as $[M+NH_4]^+$.
[2] Present as $[M-H]^-$ under acidic conditions.
[3] Present as $[M-2H]^{2-}$ under alkaline conditions.

Table 3.1 continued.

Toxin	ESI mode	Precursor ion (*m/z*)	Product ion (*m/z*)		Cone voltage (V)	Collision energy (eV)
			1	2		
AZA1	ESI^+	842.5	824.5		35	30
				672.4	35	40
AZA2	ESI^+	856.5	838.5		35	30
				672.4	35	40
AZA3	ESI^+	828.5	810.5		35	30
				658.4	35	40
AZA4	ESI^+	844.5	826.5		35	30
				658.4	35	40
AZA5	ESI^+	844.5	826.5		35	30
				674.4	35	40
AZA6	ESI^+	842.5	824.5		35	30
				658.4	35	40
PTX2	ESI^+	876.5 [1]	823.5	213.1	40	30
PTX11	ESI^+	892.5 [1]	839.5	213.1	40	30
PTX12	ESI^+	874.5 [1]	821.5	213.1	40	30
PTX2sa	ESI^-	875.5	179.2	137.2	70	50
SPX1	ESI^+	692.5	444.2		40	40
				164.3	40	50
20-Me SPX G	ESI^+	706.5	346.2		40	40
				164.3	40	50
SPX unknown 1	ESI^+	708.5	346.2		40	40
				164.3	40	50
SPX unknown 2	ESI^+	694.5	458.3		40	40
				164.3	40	50
GYM	ESI^+	508.2	490.2		50	25
				162.2	50	45
Cys BTX-A	ESI^+	990.5	901.5	869.5	40	35
Cys BTX-A S-oxide	ESI^+	1006.5	919.5	869.5	40	35
Cys BTX-A glycine	ESI^+	1047.5	901.5	869.5	40	35
Cys BTX-B	ESI^+	1018.5	929.5	879.5	40	35
Cys BTX-B S-oxide	ESI^+	1034.5	947.5	879.5	40	35

[1)] Present as $[M+NH_4]^+$.

RESULTS AND DISCUSSION

The retention that compounds experience on reversed phase HPLC columns is governed by their lipophilic properties and by the presence of polar or ionic groups, which can interact with the stationary phase. The pH of the mobile phase is an important parameter which can significantly influence the amount of interaction between the toxins and the stationary phase. Changes in the elution order of the various groups of lipophilic marine toxins can be expected when the pH of the mobile phase is changed (Fig. 3.2). The elution order will depend on the charge state of the toxins (Table 3.2). OA and DTXs will be in a neutral state under acidic conditions but negatively charged under alkaline conditions. As YTX is a strong acid containing two sulphonic acid groups the charge state will be negative under both acidic and alkaline conditions. AZAs are amphoteric compounds as they contain both a carboxylic group and a substituted amino function. At a low pH AZAs should be positively charged while at high pH they will be negatively charged. PTXs do not contain ionisable groups while GYM and SPXs contain an imino group that will be in a positive charge state at low pH and neutral at high pH. In general, compounds that are in their ionic state will elute at a lower percentage organic solvent in the mobile phase than when they are in their neutral state. Based on the charge state we can expect reduced retention for OA and DTXs under alkaline compared to acidic conditions. For GYM and SPXs retention should be increased. Retention for YTXs and PTXs should be largely unaffected by the change of pH. With respect to the AZAs the net effect of pH changes is unclear as the compound will remain in an ionized form. Changing the pH of the mobile phase may also have an effect on the peak shape because a change in charge state of the toxin will affect its interaction with the stationary phase.

In initial experiments, the Hypersil and XBridge columns were compared using six reference standards (OA, YTX, AZA1, PTX2, SPX1, GYM) under acidic, neutral and alkaline mobile phase conditions. The Hypersil column material consists of end-capped silica which has a working range between pH 2-9; therefore only the acidic and neutral mobile phase were applied to this column. The XBridge column, which contains a cross-linked type of silica, is stable over a broader pH range (pH 2-12); therefore all three mobile phase systems were applied for this column.

Under acidic conditions a slightly improved peak shape was obtained for YTX with the XBridge compared to the Hypersil column (Fig. 3.2a-c). Also the peak shape of SPX1 and GYM was significantly better on the XBridge column compared to the Hypersil column using the acidic conditions. The other toxins were not affected by the difference in column material at low pH. Under neutral conditions the peak shape of OA was poor on the Hypersil column, but good on the XBridge column (Fig. 3.2d-e). Stobo *et al.* reported no particular problems for OA on the Hypersil column under neutral conditions [20]. For the other toxins the peak shape was good under neutral conditions, although overlapping of toxins preferably analyzed in ESI^+ and ESI^- occurred on both columns. With the exception of AZA1, for all tested toxins the peak width at FWHM was at least three-fold better on the XBridge column with the alkaline mobile phase than on the Hypersil column with the acidic mobile phase (Fig. 3.2a-e, Table 3.3). It can be concluded that both the change in pH of the mobile phase and the change in column material from traditional silica (Hypersil) to cross-linked silica (XBridge) contributed to a better peak shape. As the use of a Hypersil column in combination with an acidic mobile phase is most often cited in literature, this HPLC method was compared with the new developed alkaline HPLC method. A large suit of toxin standards and toxins present in extracts of naturally contaminated materials were used to compare the two HPLC methods. For each group of lipophilic marine toxins a detailed discussion on the results obtained with both methods will be given below.

Table 3.2 Functional groups and charge state of the marine lipophilic toxin groups.

		Net charge state	
Toxin	Functional group	pH 3	pH 11
OA / DTXs	Carboxylic acid	Neutral	Negative
YTXs	Sulfonic acid (2×)	Negative	Negative
AZAs	Carboxylic acid and amino	Positive	Negative
PTXs	None	Neutral	Neutral
GYM / SPXs	Imino	Positive	Neutral
BTXs	Amino acid	Positive	Negative

OA and DTXs

OA, DTX1 and DTX2 were baseline separated under both acidic and alkaline conditions. Separation between OA and DTX2 is important as they have similar precursor and product ions but a different toxicity [24]. The linearity of a set of OA matrix matched standards was good with both methods (R >0.999). OA and DTXs are preferably analyzed in ESI^-, although the use of the ESI^+ is also possible. In ESI^- less matrix effects were observed [25]. The LOD of OA was based on the weaker transition in negative mode, *m/z* 803.5 > 113.1. The LOD of OA with the acidic

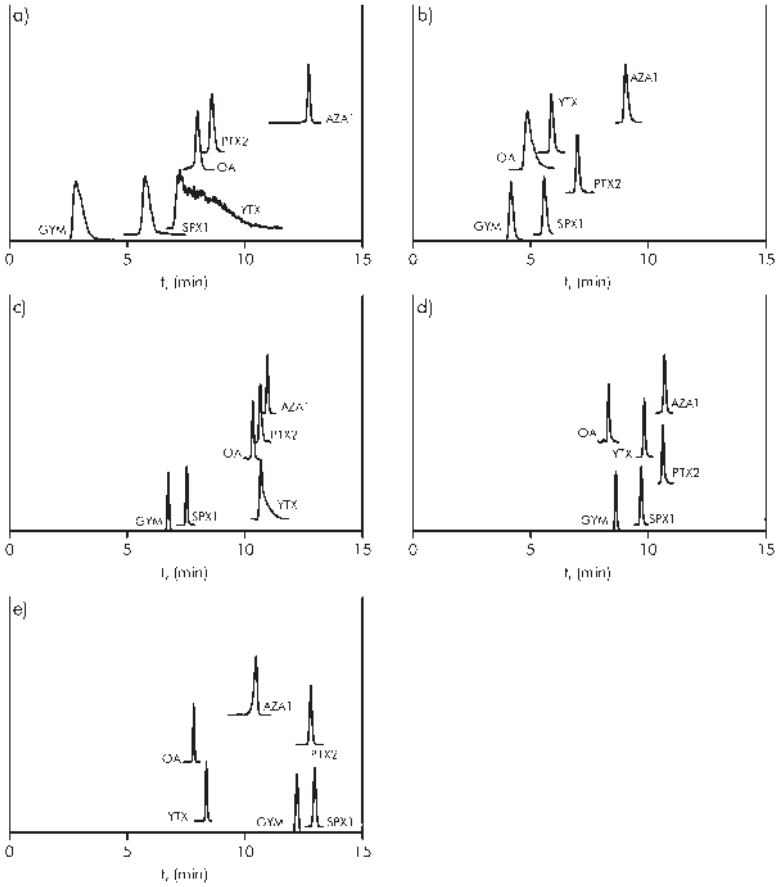

Figure 3.2 Separation of lipophilic marine toxin standards using a) the Hypersil column under acidic LC conditions, b) the Hypersil column under neutral conditions, c) the XBridge column under acidic conditions, d) the XBridge column under neutral conditions and e) the XBridge column under alkaline conditions.

method was 22 pg on-column and with the alkaline method 9 pg on-column (Table 3.3). The improvement is partly due to the fact that with the latter method OA elutes as a more narrow peak and partly because with the alkaline mobile phase the ionization efficiency is improved. Furthermore, a few DTX3 (acyl esters of OA) toxins were analyzed by means of both LC methods. Due to their long lipophilic acyl chains (C_{14}-C_{22}) these esters will typically elute at the end of the gradient at a high percentage acetonitrile. Two esters of OA (16:0 and 22:6) were selected as representatives of the DTX3 group. Both esters did not elute from the column with the acidic gradient. With the alkaline gradient the esters eluted in the last part of the chromatogram together with the PTXs, GYM and SPXs. As the latter toxins are preferably analyzed in ESI^+, the OA esters were also recorded in ESI^+.

YTXs

Chromatography of YTX was rather problematic under acidic conditions as a broad peak and severe tailing were observed, resulting in an estimated baseline peak width of 2-3 min (Fig. 3.2a-c). Under alkaline conditions the peak shape of all YTXs dramatically improved, resulting in baseline peak widths of only 10-15 seconds (Fig. 3.2e, Table 3.3). Although the charge state (-2) of YTX is the same at pH 3 and pH 11, there may be some secondary interactions (ion exchange) taking place at low pH, due to protonation of the silanol groups on the stationary phase, resulting in 'smearing' of the chromatographic peak. Interestingly, the observed ionization state in the MS of YTX and its analogues depends on the pH of the mobile phase. Under acidic conditions YTX is mainly observed in a single negatively charged state, while under alkaline conditions the double negatively charged precursor ion, *m/z* 570.4 ($[M\text{-}2H]^{2-}$) is predominant. The ionization efficiency is affected by the change in pH resulting in the selection of different precursor to product ion transitions under acidic and alkaline conditions. Transition *m/z* 1141.5 > 855.5 was used for the determination of the YTX LOD with the acidic method and *m/z* 570.4 > 396.4 for the alkaline method. Linearity of the YTX standard solution range was good under both conditions ($R > 0.999$) but a three-fold improvement in LOD was obtained under alkaline conditions, mainly due to a much narrower peak. Facilitated by the increased sensitivity and better peak shapes, it is possible to analyze the lower abundant YTX analogues that can

Table 3.3 LC characteristics of the marine lipophilic toxin groups (avg±SD, n=3).

Acidic Hypersil method			
Toxin	t_r (min)	FWHM (s)	LOD in *Mytilus edulis* extract (pg on-column)
OA	8.02±0.01	13.5±1.2	22.1
DTX1	9.36±0.01	10.8±1.4	
DTX2	8.44±0.01	10.6±0.9	
16:0 OA ester	Not eluted		
22:6 OA ester	Not eluted		
YTX	7.04±0.05	12.9±0.4	6.1
1a-homo-YTX	7.07±0.03	19.6±4.6	
45OH-YTX	4.92±0.17	29.0±4.2	
45OH-1a-homo-YTX	5.07±0.61	33.3±24.8	
44COOH-YTX	5.71±0.06	17.5±1.9	
44COOH-1a-homo-YTX	5.73±0.11	15.5±1.4	
Trinor-YTX	5.94±0.01	15.0±1.2	
44COOH-45OH-YTX	Not detected		
AZA1	12.70±0.00	7.8±0.1	1.1
AZA2	13.02±0.02	7.8±0.1	
AZA3	12.12±0.02	8.1±0.4	
AZA4	10.58±0.02	8.9±0.2	
AZA5	11.16±0.00	9.9±1.2	
AZA6	12.46±0.00	8.3±0.5	
PTX2	8.53±0.02	12.3±1.2	6.9
PTX11	8.09±0.02	4.8±0.1	
PTX12	8.99±0.00	9.9±2.5	
PTX2sa	8.14±0.04	10.0±0.7	
SPX1	5.78±0.10	16.1±4.6	1.9
20-Me SPX G	6.41±0.00	14.8±2.0	
SPX unknown 1	6.60±0.03	22.9±4.3	
SPX unknown 2	7.46±0.04	6.5±2.2	
GYM	2.86±0.13	18.1±6.4	14.1

Table 3.3 continued.

	Basic XBridge method		
Toxin	t_r (min)	FWHM (s)	LOD in *Mytilus edulis* extract (pg on-column)
OA	7.81±0.00	4.3±0.2	9.1
DTX1	8.59±0.00	3.2±0.3	
DTX2	8.03±0.00	4.3±0.2	
16:0 OA ester	12.32±0.02	3.6±0.5	
22:6 OA ester	12.20±0.00	4.6±1.2	
YTX	8.32±0.02	4.9±0.2	2.2
1a-homo-YTX	8.34±0.01	4.9±0.1	
45OH-YTX	7.67±0.00	4.3±0.1	
45OH-1a-homo-YTX	7.67±0.01	4.7±0.2	
44COOH-YTX	7.36±0.00	5.1±0.2	
44COOH-1a-homo-YTX	7.34±0.03	9.2±0.3	
Trinor-YTX	7.85±0.00	5.4±0.8	
44COOH-45OH-YTX	7.29±0.01	6.2±0.7	
AZA1	10.50±0.01	10.1±0.2	1.1
AZA2	10.75±0.02	8.2±0.1	
AZA3	9.60±0.01	16.6±1.7	
AZA4	9.25±0.03	13.0±0.5	
AZA5	9.01±0.01	8.3±1.3	
AZA6	9.80±0.03	13.9±0.6	
PTX2	12.80±0.01	7.2±0.4	7.4
PTX11	12.61±0.00	4.8±0.1	
PTX12	12.96±0.03	9.9±2.5	
PTX2sa	7.79±0.01	3.2±0.3	
SPX1	12.95±0.01	7.3±0.4	0.8
20-Me SPX G	12.96±0.00	4.3±0.1	
SPX unknown 1	13.07±0.01	4.4±0.4	
SPX unknown 2	14.65±0.01	5.6±1.5	
GYM	12.19±0.01	7.0±0.7	3.7

be present in contaminated shellfish samples such as 1a-homo-YTX, 45OH-YTX, 45OH-1a-homo-YTX), 44COOH-YTX, 44COOH-1a-homo-YTX, trinor-YTX and 44COOH-45OH-YTX) (Fig. 3.3).

AZAs

AZAs were sufficiently separated under both acidic and alkaline conditions, although under acidic conditions the separation between AZA1 and AZA2 as well as the peak shapes were somewhat better. For some, but not all AZAs, an increase in peak width was observed with the alkaline gradient. Perhaps at pH 11 the AZAs are not yet fully in their deprotonated state giving room to secondary interactions with the stationary phase. Using the Hypersil gradient the AZAs were actually eluting outside the gradient. However, retention times were reproducible, therefore the run time was not extended. Linearity for AZA1 under acidic conditions and alkaline conditions was good ($R > 0.998$) while the LOD for AZA1 for both

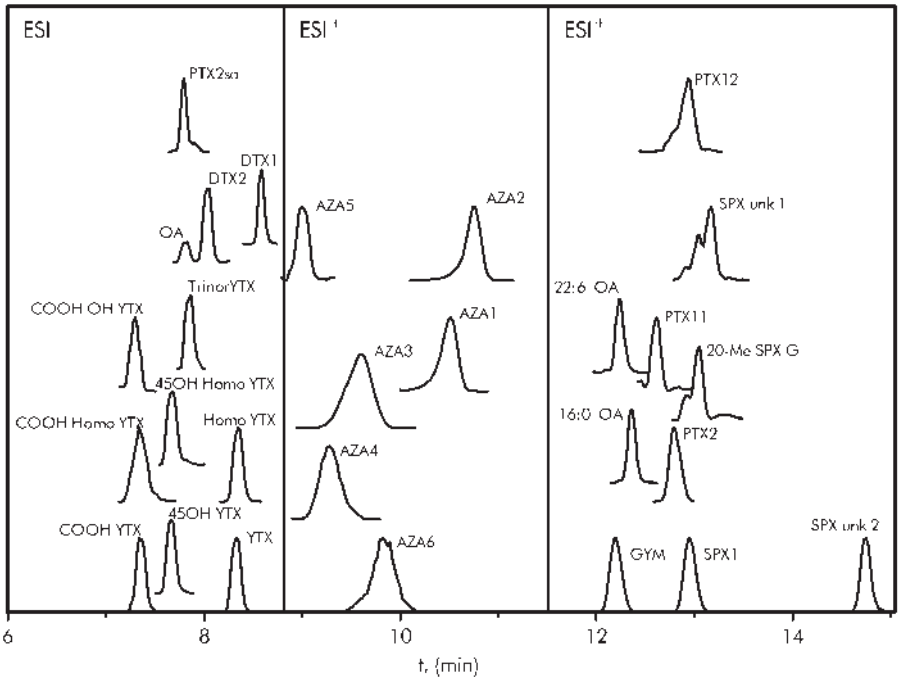

Figure 3.3 Separation of 28 toxins under alkaline conditions. The toxins were clustered in three MRM time windows allowing MS/MS detection without the need of continuous ESI polarity switching.

methods were comparable. Compared to the elution order under acidic conditions the elution order of AZA4 and AZA5 was reversed: AZA5 eluted before AZA4 with the alkaline method. AZA4 and 5 are the most 'hydrophilic' members of the AZA group that were included in this method, containing an additional hydroxy group and no extra methyl groups.

PTXs

PTX2 [retention time (t_r) 8.53 min] was practically co-eluting with DTX2 (t_r 8.44 min) when the acidic Hypersil method was used. Because DTX2 is recorded preferably in ESI^- and PTX2 with ESI^+ this poses a complication for their analysis. To solve this problem either fast positive negative switching or a double injection with separate positive and negative recording is required. It is also possible to analyze PTX2 in negative ionization mode, but at the expense of sensitivity (data not shown). Under alkaline conditions PTX2 eluted much later (t_r 12.80 min) than DTX2 (t_r 8.03 min) (Fig. 3.3). Peak characteristics and the LOD for PTX2 were comparable for both methods. In the new method also some PTX analogues (PTX11 and PTX12) that can be present in shellfish have been included. Unfortunately, PTX1, which is under EU legislation, was not available as standard nor present in contaminated shellfish. However, it can be anticipated that PTX1, being like PTX11 a hydroxylated PTX2 analogue, will just elute before PTX2 in the third window.

GYM and SPXs

Due to their positive charge state the spiroimine toxins GYM and SPX1 are early eluting toxins under acidic conditions at 2.86 and 5.78 min, respectively. Under alkaline conditions these toxins are in a neutral state and as a result they eluted much later at 12.19 min for GYM and 12.95 min for SPX1. Furthermore, with the acidic mobile phase peaks were quite broad and somewhat tailing, a situation that was greatly improved with the alkaline mobile phase on the XBridge column (Fig. 3.2a-e). Due to the improved peak shape lower LODs for GYM and SPX1 were obtained (Table 3.3). In the SPX contaminated shellfish extracts two spirolides were identified for which a structure has been proposed: SPX1 and 20-methyl spirolide G [8]. Two more spirolides were present in the extract with mass $[M+H]^+$ *m/z*

708.5 and 694.5. These toxins have been reported as well but their structures have not been elucidated [8]. The unidentified spirolides contain a specific fragment of *m/z* 164.1 that is typical for C/D and G spirolide types (Table 3.3 and Fig. 3.3).

BTXs

In addition to the classes of lipophilic marine toxins discussed above, some brevetoxin metabolites were studied for their chromatographic behaviour under alkaline conditions (Fig. 3.4). BTXs are lipophilic toxins produced by the dinoflagellate *Karenia brevis*. *Karenia brevis* blooms are frequently reported in the US Gulf of Mexico and New Zealand [26]. BTXs produced by *Karenia brevis* are rapidly metabolized to cysteine conjugates when accumulated in shellfish [27,28]. In the methods described thus far often a mobile phase containing acetic acid is used for the separation of BTXs [26,29]. With the alkaline LC method the cysteine, cysteine S-oxide and cysteine glycine metabolites of brevetoxin with the type A

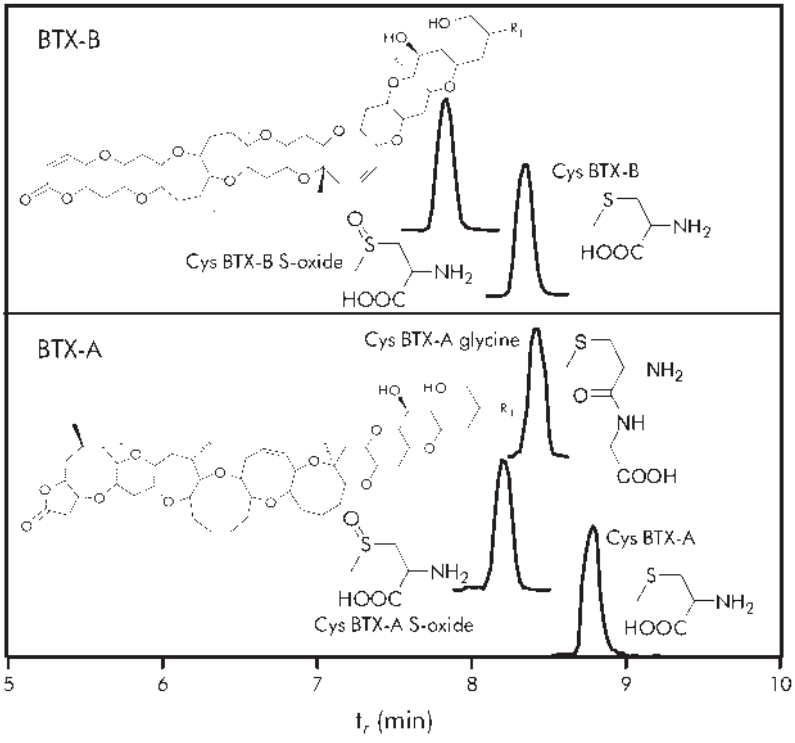

Figure 3.4 Separation of brevetoxin cysteine metabolites using the alkaline LC method.

(BTX-A) and type B (BTX-B) backbone could be separated and analyzed (Fig. 3.4). Due to the presence of the ionic amino acid group the brevetoxins elute in the same region of the chromatogram as OA and YTX. Unfortunately they are best analyzed in ESI^+. For this reason, it is not possible to analyze them simultaneously with the other lipophilic marine toxins without polarity switching. Linearity and LODs could not determined as no purified BTX standards were available.

Stability of the toxins under alkaline conditions

A specific concern of the new method was the stability of OA, DTXs, YTX, AZAs and PTX2 in the presence of ammonium hydroxide solution. It is known that heating in 2.5 M NaOH is detrimental for most lipophilic marine toxins except for OA, DTX1 and DTX2 [12]. However, NH_4OH is a much weaker base than NaOH and the concentration in the mobile phase is only around 7 mM. To test the stability of the toxins, a mussel extract (*Mytilus edulis*) containing a mix of the relevant toxins was mixed with ammonium hydroxide solutions with increasing molarity. The extracts were kept for 1 hour at room temperature and at 60°C (to mimic more stringent conditions) and peak area's were compared before and after the addition of ammonia. Figure 5 clearly shows that even at 60°C and in combination with high concentrations ammonium hydroxide solution all the investigated toxins were stable. The recovery of each individual toxin was more than 90%.

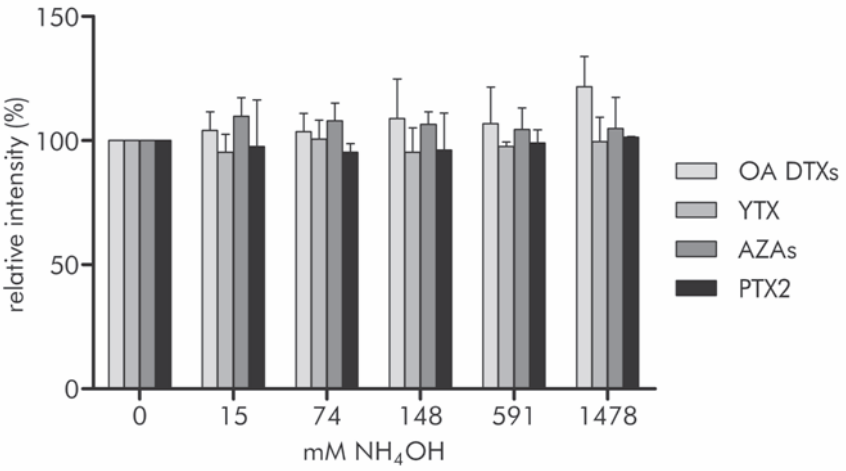

Figure 3.5 Stability of the lipophilic marine toxin groups in the presence of NH_4OH at 60°C for a 1 hour period.

Performance characteristics of the alkaline LC method

Retention time stability is an important parameter because drifting of the toxins in the chromatogram can lead to compounds eluting outside their retention windows. To determine the retention time stability a long term experiment (24 hr) with different matrices (mussels, oysters and scallops) containing OA, YTX, AZA1 and PTX2 was performed using the alkaline LC method. The retention time stability for all toxins was very good with an RSD below 0.5% (n=76).

The use of a multi-toxin reference material to determine performance characteristics is preferable. Unfortunately, certified multi-toxin materials are not yet available for the lipophilic marine toxin group. However, within the EU project BIOTOX a reference material has been produced containing a number of relevant marine toxins (OA, DTX1-2, YTX, 45OH-YTX, AZA1-3, PTX2). This material has been used for a feasibility (inter-laboratory) study, in which 13 laboratories analyzed the sample with their own in-house developed methods (mostly methods in which an acidic gradient was used). Full details of this study will be published elsewhere. From the material three samples were analyzed daily for two days. From the data obtained the repeatability, reproducibility and accuracy was determined. The repeatability of the alkaline LC method was good ranging from 0.4% for OA to 12.9% for PTX2 (Table 3.4). The reproducibility ranged from 5.6% for AZA2 to 18.3% for PTX2. The data on accuracy obtained for OA, DTX1, DTX2, 45OH-YTX, and AZA2 were within the 95% confidence interval (C.I.). Data obtained with the alkaline method for YTX, AZA1, AZA3 and PTX2 were just outside the 95% C.I., but were not significantly different from the average values found, based on the outlier tests applied (Grubbs, Dixons and Nalimov). It can be concluded that the alkaline method performed quite well for the detection of lipophilic marine toxins in shellfish.

CONCLUSION

The use of a mobile phase containing ammonium hydroxide instead of formic acid substantially improved the separation of the lipophilic marine toxins. Furthermore the LODs for a number of toxins improved two- to three-fold, partly due to better peak shapes and partly due to an improved ionization efficiency; e.g. the LOD of OA improved significantly from 22 pg under acidic conditions to 9 pg on-column

Table 3.4 Preliminary validation results obtained in a feasibility (inter-laboratory) study.

	Toxin								
	OA	DTX1	DTX2	YTX	45OH-YTX	AZA1	AZA2	AZA3	PTX2
Day 1									
Concentration (μg/kg) (n=3)	253.6	267.4	947.2	925.5	135.8	392.1	107.4	115.0	117.5
Repeatability (%)	0.4	4.2	1.1	2.8	2.9	1.5	0.9	4.3	9.4
Day 2									
Concentration (μg/kg) (n=3)	206.9	224.0	773.4	797.9	134.2	382.4	98.7	106.5	88.3
Repeatability (%)	11.6	0.9	3.4	8.1	9.1	9.4	5.1	10.7	12.9
Average (μg/kg)	230.2	245.7	860.3	861.7	135.0	387.2	103.0	110.8	102.9
Reproducibility (%)	12.9	10.1	11.3	9.6	6.0	6.1	5.6	8.2	18.3
Feasibility									
Average (μg/kg)	235.3	267.7	739.2	667.6	142.7	340.2	116.2	128.0	139.6
95% C.I. (μg/kg)	27.4	48.6	174.0	181.7	76.6	29.2	18.8	14.6	24.1
No of labs after removal outliers	9	10	9	9	7	11	11	9	8

under alkaline conditions. The peak shape for YTX and analogues substantially improved when run under alkaline conditions. Only some of the AZAs were slightly negatively affected by the alkaline gradient but the increased peak widths did not result in dramatically increased LODs. A significant advantage of the 'new' alkaline method compared to the 'old' acidic and neutral methods is the clustering of compounds in retention time windows. All negatively charged toxins elute early in the chromatogram; and these compounds are best monitored in the ESI^- mode. The AZAs elute in the middle part of the chromatogram, while PTXs, GYM and SPXs elute in the last part. These toxin groups are best analyzed in the ESI^+ mode. A negative to positive mode switching moment can be incorporated in the method to enable the analysis of all relevant toxins including some relevant DTX3 in a single run. In our method the difference in t_r between DTX1 (the last eluting toxin recorded in ESI^-) and AZA5 (the first eluting toxin recorded in ESI^+) is 0.4 min. Under reproducible chromatographic conditions this is a sufficient time span to switch from one MRM window to the next. At this moment 28 toxins are included in the new method but there is potential to add even more toxins. The alkaline mobile phase does not lead to degradation of any of the lipophilic marine toxins present in a tested mussel extract. Furthermore, the XBridge column used contains the same column material as the UPLC BEH column, therefore in order to reduce analysis time the method can easily be transferred to the newer ultra high performance LC systems. The developed alkaline LC method can also be used for other marine toxin groups such as the brevetoxins. With respect to repeatability and reproducibility the alkaline method showed very good results.

Matrix effects are an important issue in the analysis of biological samples analyzed with ESI MS. We have carried out a study on these effects observed for lipophilic marine toxins under acidic and alkaline HPLC conditions. The results obtained with this study and the development of a solid phase extraction procedure to reduce matrix effects will be presented in **Chapter 4**. This method, after an in-house validation study that is presented in **Chapter 5**, will hopefully contribute to the replacement of the animal test, which is still the official method prescribed by the EU.

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REFERENCES

[1] Fujiki H, Suganuma M, Yoshizawa S, Nishiwaki S, Winyar B, Sugimura T. Mechanisms of Action of Okadaic Acid Class Tumor Promoters on Mouse Skin. *Environ Health Persp* 1991; **93**: 211.

[2] Terao K, Ito E, Oarada M, Murata M, Yasumoto T. Histopathological studies on experimental marine toxin poisoning .5. The effects in mice of yessotoxin isolated from *Patinopecten-yessoensis* and of a desulfated derivative. *Toxicon* 1990; **28**: 1095.

[3] Ito E, Satake M, Ofuji K, Kurita N, McMahon T, James K, Yasumoto T. Multiple organ damage caused by a new toxin azaspiracid, isolated from mussels produced in Ireland. *Toxicon* 2000; **38**: 917.

[4] Terao K, Ito E, Yanagi T, Yasumoto T. Histopathological studies on experimental marine toxin poisoning .1. Ultrastructural-changes in the small-intestine and liver of suckling mice induced by dinophysistoxin-1 and pectenotoxin-1. *Toxicon* 1986; **24**: 1141.

[5] Richard DJA, Arsenault E, Cembella AD, Quilliam MA In *Investigations into the toxicology and pharmacology of spirolides, a novel group of shellfish toxins*, Proceedings of the 9th conference on Harmfull algal blooms, Hobart, Australia, 7-11 February, 2000; Hallegraeff GM, Blackburn SI, Bolch CJ, Lewis RJ, Eds. Hobart, Australia, 2000; 383.

[6] Gill SS, Murphy M, Clausen J, Richard DJA, Quilliam MA, MacKinnon SL, LaBlanc P, Mueller RW, Pulido OM. Neural injury biomarkers of novel shellfish toxins, spirolides: A pilot study using immunochemical and transcriptional analysis. *Neurotoxicology* 2003; **24**: 593.

[7] European Parliament, Council. Commission directive 2004/853/EC specific hygiene rules for food of animal origin. *Off J Eur Commun* 2004; **L226**: 22.

[8] Aasen JAB, MacKinnon SL, LeBlanc P, Walter JA, Hovgaard P, Aune T, Quilliam MA. Detection and identification of spirolides in Norwegian shellfish and plankton. *Chem Res Toxicol* 2005; **18**: 509.

[9] Paz B, Daranas AH, Cruz PG, Franco JM, Pizarro G, Souto ML, Norte M, Fernandez JJ. Characterisation of okadaic acid related toxins by liquid chromatography coupled with mass spectrometry. *Toxicon* 2007; **50**: 225.

[10] Paz B, Daranas AH, Cruz PG, Franco JM, Napolitano JG, Norte M, Fernandez JJ. Identification and characterization of DTX-5c and 7-hydroxymethyl-2-methylene-octa-4,7-dienyl okadaate from *Prorocentrum belizeanum* cultures by LC-MS. *Toxicon* 2007; **50**: 470.

[11] Suzuki T, Beuzenberg V, Mackenzie AL, Quilliam MA. Discovery of okadaic acid esters in the toxic dinoflagellate *Dinophysis acuta* from New Zealand using liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom* 2004; **18**: 1131.

[12] Doucet E, Ross NN, Quilliam MA. Enzymatic hydrolysis of esterified diarrhetic shellfish poisoning toxins and pectenotoxins. *Anal Bioanal Chem* 2007; **389**: 335.

[13] Amandi MF, Furey A, Lehane M, Ramstad H, James KJ. Liquid chromatography with electrospray ion-trap mass spectrometry for the determination of yessotoxins in shellfish. *J Chromatogr A* 2002; **976**: 329.

[14] Miles CO, Samdal IA, Aasen JAB, Jensen DJ, Quilliam MA, Petersen D, Briggs LM, Wilkins AL, Rise F, Cooney JM, MacKenzie AL. Evidence for numerous analogs of yessotoxin in *Protoceratium reticulatum*. *Harmful Algae* 2005; **4**: 1075.

[15] Ciminiello P, Dell'Aversano C, Fattorusso E, Forino M, Magno S, Poletti R. Direct detection of yessotoxin and its analogues by liquid chromatography coupled with electrospray ion trap mass spectrometry. *J Chromatogr A* 2002; **968**: 61.

[16] Rehmann N, Hess P, Quilliam MA. Discovery of new analogs of the marine biotoxin azaspiracid in blue mussels (*Mytilus edulis*) by ultra-performance liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom* 2008; **22**: 549.

[17] Miles CO, Wilkins AL, Hawkes AD, Jensen DJ, Selwood AI, Beuzenberg V, Mackenzie AL, Cooney JM, Holland PT. Isolation and identification of pectenotoxins-13 and -14 from *Dinophysis acuta* in New Zealand. *Toxicon* 2006; **48**: 152.

[18] Miles CO, Wilkins AL, Samdal IA, Sandvik M, Petersen D, Quilliam MA, Naustvoll LJ, Rundberget T, Torgersen T, Hovgaard P, Jensen DJ, Cooney JM. A novel pectenotoxin, PTX-12, in *Dinophysis spp*. and shellfish from Norway. *Chem Res Toxicol* 2004; **17**: 1423.

[19] Fux E, McMillan D, Bire R, Hess P. Development of an ultra-performance liquid chromatography-mass spectrometry method for the detection of lipophilic marine toxins. *J Chromatogr A* 2007; **1157**: 273.
[20] Stobo LA, Lacaze JPCL, Scott AC, Gallacher S, Smith EA, Quilliam MA. Liquid chromatography with mass spectrometry - detection of lipophilic shellfish toxins. *J AOAC Int* 2005; **88**: 1371.
[21] McNabb P, Selwood AI, Holland PT. Multiresidue method for determination of algal toxins in shellfish: Single-laboratory validation and interlaboratory study. *J AOAC Int* 2005; **88**: 761.
[22] Quilliam MA, Hess P, Dell' Aversano C In *Recent Developments in the analysis of phycotoxins by liquid chromatography-mass spectromety;* de Koe WJ, Samson RA, van Egmond HP, Gilbert J, Sabino M, Eds. W.J. de Koe: Wageningen, The Netherlands, 2001; 383.
[23] Hess P, McMahon T, Slattery D, Swords D, Dowling G, McCarron M, Clarke D, Gibbons W, Silke J, O'Cinneide M In *Use of LC-MS testing to identify lipophilic toxins, to establish local trends and interspecies differences and to test the comparability of LC-MS testing with the mouse bioassay: an example from the Irish Biotoxin monitoring programme 2001*, Molluscan Shellfish Safety, Santiago de Compostela, Spain, 2003 Villalba A, Reguera A, Romalde JL, Beiras R, Eds. Santiago de Compostela, Spain, 2003; 57.
[24] Aune T, Larsen S, Aasen JAB, Rehmann N, Satake M, Hess P. Relative toxicity of dinophysistoxin -2 (DTX-2) compared with okadaic acid, based on acute intraperitoneal toxicity in mice. *Toxicon* 2007; **49**: 1.
[25] Mc Elhinney MA. Matrix effects, development of clean-up and LC techniques contributing towards a reference LCMS method for the analysis of lipophilic marine toxins. Dublin Institute of Technology, Dublin, 2007.
[26] Twiner MJ, Dechraoui MYB, Wang Z, Mikulski CM, Henry MS, Pierce RH, Doucette GJ. Extraction and analysis of lipophilic brevetoxins from the red tide dinoflagellate *Karenia brevis*. *Anal Biochem* 2007; **369**: 128.
[27] Wang Z, Plakas SM, El Said KR, Jester ELE, Granade HR, Dickey RW. LC/MS analysis of brevetoxin metabolites in the Eastern oyster (*Crassostrea virginica*). *Toxicon* 2004; **43**: 455.
[28] Plakas SM, Wang Z, El Said KR, Jester ELE, Granade HR, Flewelling L, Scott P, Dickey RW. Brevetoxin metabolism and elimination in the Eastern oyster (*Crassostrea virginica*) after controlled exposures to *Karenia brevis*. *Toxicon* 2004; **44**: 677.
[29] Roth PB, Twiner MJ, Wang Z, Dechraoui MYB, Doucette GJ. Fate and distribution of brevetoxin (PbTx) following lysis of *Karenia brevis* by algicidal bacteria, including analysis of open A-ring derivatives. *Toxicon* 2007; **50**: 1175.

4

Solid phase extraction for the removal of matrix effects in lipophilic marine toxin analysis by liquid chromatography - tandem mass spectrometry

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ABSTRACT

The potential of solid phase extraction (SPE) clean up has been assessed to reduce matrix effects (signal suppression or enhancement) in the liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis of lipophilic marine toxins. A large array of ion exchange, silica based and mixed function SPE sorbents was tested. Polymeric sorbents were found to retain most of the toxins. Optimization experiments were carried out to maximize recoveries and the effectiveness of the clean up. In LC-MS/MS analysis the observed matrix effects can depend on the chromatographic conditions used, therefore two different HPLC methods were tested, using either an acidic or an alkaline mobile phase. The recovery of the optimized SPE protocol was around 90% for all toxins studied and no break-through was observed. Matrix effects are determined by comparing spikes in crude and cleaned extracts with spikes in methanol. In crude extracts, all toxins suffered from matrix effects, although in varying amounts. Most serious effects were observed for okadaic acid (OA) and pectenotoxin-2 (PTX2) in the positive electrospray ionization mode (ESI^+). SPE clean up on polymeric sorbents in combination with the alkaline LC method resulted in a substantial reduction of matrix effects to less than 15% (apparent recovery between 85-115%) for OA, yessotoxin (YTX) in ESI^- and azaspiracid-1 (AZA1), PTX2, 13-desmethyl spirolide C (SPX1) and gymnodimine (GYM) in ESI^+. In combination with the acidic LC method the matrix effects after SPE were also reduced but nevertheless approximately 30% of the matrix effects remained for PTX2, SPX1 and GYM in ESI^+. It was concluded that SPE of methanolic shellfish extracts can be very useful for reduction of matrix effects. However, the type of LC and MS methods used is also of great importance. SPE on polymeric sorbents in combination with LC under alkaline conditions was found the most effective method.

INTRODUCTION

Bivalves such as mussels, scallops and oysters are filter feeding organisms that can accumulate marine biotoxins produced by algae. The most common intoxications in Europe caused by the consumption of shellfish contaminated with marine toxins are Diarrhetic Shellfish Poisoning (DSP), and to a lesser degree Paralytic Shellfish Poisoning (PSP). Toxins responsible for DSP intoxication belong to the group of the lipophilic marine biotoxins. In this paper five different groups of lipophilic marine biotoxins are considered: The first group are the already mentioned DSP toxins of which the predominant toxins are okadaic acid (OA), dinophysistoxin-1 (DTX1), dinophysistoxin-2 (DTX2) and dinophysistoxin-3 (DTX3) (Fig. 4.1a). DSP toxins cause diarrhea, nausea, vomiting and abdominal cramps [1, 2]. The second group are the yessotoxins (YTXs) (Fig. 4.1b). YTXs have an adverse effect on the cardiac muscle cells in mice, but intoxications in humans so far have not been reported [3, 4]. The third group are the azaspiracids (AZAs) (Fig. 4.1c), in which AZA1, -2 and -3 are the predominant toxins. Azaspiracids show adverse effects comparable to OA and DTXs; nausea, diarrhea, stomach cramps, etc. Although the adverse effects are comparable to those of DSP, the mode of action of AZAs is different and only partially elucidated [5, 6]. The fourth group are the pectenotoxins (PTXs) (Fig. 4.1d). The diarrhetic effects of PTXs are not clear and toxicity is only observed after intraperitoneal injection in mice [7, 8]. The fifth group are the spiroimine toxins; spirolides (SPXs) and gymnodimine (GYM) (Fig. 4.1e). These toxins show adverse neurological effects on the respiratory tract, comparable with saxitoxin (PSP toxin). The spiroimine toxins can cause death of mice within minutes after intraperitoneal injection [9].

EU regulation 2004/853/EC prescribes which lipophilic toxins should be monitored and determined in the edible parts of shellfish [10]. The permitted levels for the sum of OA, DTXs and PTXs is set at 160 μg/kg, the sum of relevant YTXs is set at a total of 1 mg/kg and the sum of relevant AZAs at 160 μg/kg [10]. The spiroimines are currently not under EU legislation, but may become regulated in the future. The EU prescribes a mouse or rat bioassay as the reference test for lipophilic marine toxins in shellfish. On the national level, alternative methods may be used if it can be guaranteed that the obtained results are coinciding with the results of the official reference test. The animal assays have some serious

Figure 4.1. Structure of a) okadaic acid and dinophysistoxins 1-3, b) yessotoxin, c) azaspiracids 1-3, d) pectenotoxin-2, e) 13-desmethyl spirolide C (SPX1) and gymnodimine.

drawbacks with respect to sensitivity, detectability of the individual toxins and with respect to ethics. In recent years, analytical methods based on liquid chromatography (LC) coupled to mass spectrometry (MS) have been developed as an alternative for the detection of lipophilic marine toxins in crude methanolic shellfish extracts [11-14]. However, it is well known that in LC-MS/MS analysis matrix effects (ion suppression or ion enhancement) can lead to an under- or overestimation of the concentration. In order to overcome matrix effects, different approaches can be used, including internal standards, standard addition or the removal of matrix effects by various clean up techniques. Unfortunately, in the field of marine toxin analysis internal standards are not available yet. Ito *et al.* demonstrated that standard addition can be an effective tool to eliminate matrix effects [15]. Due to the scarcity of standards the standard addition approach is expensive and not generally feasible in routine analysis. Different techniques have been tested for the clean up of methanolic shellfish extracts. Liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are the clean up techniques most often used in the analysis of lipophilic marine biotoxins. Various organic solvents have been used for the LLE of the crude extracts [16-19]. After LLE the organic phase is evaporated to dryness and reconstituted in methanol, acetonitrile or acetone. The evaporation step may have a negative effect on the solubility and stability of the toxins. From a (pre-)validation round conducted within the EU BIOTOX project it became clear that LLE did not give satisfactory results with respect to accuracy, reproducibility and repeatability [20].

Most SPE methods are directed to the clean up or isolation of an individual toxin or toxin group. SPE clean up has been combined with LC-fluorescence detection (FLD) for the determination of OA and DTXs in shellfish. Puech *et al.* described the use of immuno-affinity cartridges with satisfactory recoveries (>55%) [21] and Quilliam *et al.* used aminopropylsilica cartridges with excellent recoveries (>95%) [22]. For the determination of OA, DTX, YTX and PTX toxins with LC-MS, Goto *et al.* published a rather laborious method using two parallel SPE procedures with recoveries in the range of 69-134% [23]. A C_{18} cartridge was used to isolate YTX and 45OH-YTX from the crude methanolic extract, while for the isolation of OA and DTX1 LLE was applied, followed by SPE on a silica cartridge. Stobo *et al.* used a polymeric type SPE cartridge to isolate AZAs from crude extracts [12] and Moroney *et al.* used a

diol type SPE sorbent for the clean up of AZA1-3 from shellfish extracts [24], however, the effectiveness of the clean up has not been described in detail.
To our knowledge there has not yet been a multi-toxin SPE method developed that comprises the predominant lipophilic marine toxins from each of the five toxin groups. In this paper an SPE method is presented for the isolation and clean up of lipophilic marine toxins from all toxin groups from crude methanolic extracts. The efficiency of the SPE method in the removal of interfering matrix components resulting in a reduction or removal of ion suppression / enhancement effects was tested for three different shellfish species: mussel, scallop and oyster. Recently, Fux *et al*. have shown that matrix effects can be heavily dependent on the chromatographic system used [25]. To study this in more detail two separate LC-MS/MS methods were applied, using very different mobile phase systems [11, 26]. For OA and PTX2 the experiments were also conducted with two electrospray ionization modes.

EXPERIMENTAL

Reagents and standards

Water was deionized and passed through a Milli-Q water purification system (Millipore, Billerica, MA, USA). Formic acid (98-100%) was purchased from Merck, Darmstadt, Germany. Ammonium formate (>97%) was purchased from Sigma-Aldrich, Zwijndrecht, The Netherlands. Acetonitrile (HPLC supra gradient) and methanol (absolute) were purchased from Biosolve, Valkenswaard, The Netherlands. Ammonium hydroxide (25%) was purchased from VWR international, Amsterdam, The Netherlands. OA [certified reference material (CRM)-OA-b 24.1±0.8 μg/ml], YTX (CRM-YTX 5.3±0.3 μg/ml), AZA1 (CRM-AZA1 1.24±0.07 μg/ml), PTX2 (CRM-PTX2 8.6±0.3 μg/ml), 13-desmethyl spirolide C (SPX1) (CRM-SPX1 7.0±0.4 μg/ml) and gymnodimine (GYM) (CRM-GYM 5.0±0.2 μg/ml) were purchased from the National Research Council, Institute for Marine Biosciences (NRC CNRC), Halifax, Canada. A laboratory reference material (LRM) was prepared from mussel (*Mytilus edulis*) containing OA, DTX1, -2, YTX, AZA1, -2, -3 and PTX2 at the Marine Institute, Ireland. An extract in methanol with a solvent-to-sample ratio of 10 was prepared from this LRM homogenate.

Preparation of standard solutions and extracts

A stock solution containing 320 ng/ml OA, 400 ng/ml YTX, 200 ng/ml AZA1 and 320 ng/ml PTX2 and a stock solution of 250 ng/ml SPX1 and GYM was prepared in methanol. Blank mussel (*Mytilus edulis*), scallop (*Pecten maximus*) and oyster (*Crassostrea gigas*) extracts were prepared by homogenizing 100 g of whole flesh with a T25 ultra turrax mixer at 24 000 rpm (IKA® Works Inc., Wilmington, NC, USA). 2 g of this shellfish homogenate was extracted in triplicate with 6 ml methanol. After each addition of methanol the extract was vortex mixed during 1 minute, after which the extract was centrifuged 5 min at 2 000 × *g*. The methanolic extracts were combined in a volumetric flask of 20 ml, and the volume was made up to 20 ml with methanol. The crude shellfish extract was filtered through a 0.2 μm high temperature (HT) resistance Tuffryn membrane filter (Pall Corp., East Hills, NY, USA) prior to spiking. The crude methanolic extract was spiked at a concentration of 16 ng/ml OA, 20 ng/ml YTX, 10 ng/ml AZA1 and 16 ng/ml PTX2. In an additional experiment the same crude blank mussel extract was spiked with 12.5 ng/ml SPX1 and GYM.

Liquid chromatography

An Alliance 2690 (Waters, Milford, MA, USA) HPLC system was used in combination with a Thermo Electron BDS Hypersil C_8 (50 × 2.1 mm, 3 μm) column and with a Waters XBridge C_{18} (150 × 3 mm, 5 μm) column. For both columns the temperature was set at 40°C.

The Hypersil column was used under acidic conditions (pH = 2.6). Mobile phase A was water and B was acetonitrile/water (95:5 v/v), both containing a fixed concentration of 2 mM ammonium formate and 50 mM formic acid. A gradient was run at a flow rate of 0.2 ml/min; it started with 30% B, which was increased linearly to 90% B in 8 min. The composition was kept for 2.5 min at 90% B and was in 0.5 min returned to 30% B. An equilibration time of 4 min was allowed before the next injection.

The XBridge column was used under alkaline conditions (pH = 11) with water as mobile phase A and acetonitrile/water (90:10 v/v) as mobile phase B. Both mobile phases contained 6.7 mM ammonium hydroxide. A gradient was run at a flow rate of 0.4 ml/min; the gradient started with 10% B, which was after one min increased

Table 4.1 Multiple reaction monitoring (MRM) acquisition parameters for the selected lipophilic marine toxins.

Toxin	ESI mode	Precursor (*m/z*)		Product (*m/z*)	Cone (V)	Collision energy (eV)
OA	ESI^-	$[M\text{-}H]^-$	803.5	113.1	60	50
				151.1	60	50
				255.2	60	45
	ESI^+	$[M+Na]^+$	827.5	723.3	70	45
				791.4	70	45
				809.3	70	45
YTX	ESI^-	$[M\text{-}H]^-$	1141.5	1061.5	45	40
	ESI^-	$[M\text{-}2H]^{2-}$	570.4	396.4	75	40
				467.4	75	40
AZA1	ESI^+	$[M+H]^+$	842.5	654.4	35	40
				672.4	35	40
				824.5	35	30
PTX2	ESI^-	$[M\text{-}H]^-$	857.4	137.2	90	45
				155.2	90	45
				179.2	90	45
PTX2	ESI^+	$[M+NH_4]^+$	876.5	213.1	40	30
				805.5	40	30
				823.5	40	30
SPX1	ESI^+	$[M+H]^+$	692.5	164.3	40	50
				444.2	40	40
				674.4	40	30
GYM	ESI^+	$[M+H]^+$	508.2	121.4	50	45
				162.2	50	45
				490.2	50	25

linearly to 90% B in 9 min. The composition was kept at 90% B for 3 min and was in 2 min returned to 10% B. An equilibration time of 4 min was allowed before the next injection. For both the acidic and alkaline LC method the vial compartment of the autosampler was kept at 10°C and a 10 μl injection volume was used.

Mass spectrometry

Mass spectrometry was performed using a Micromass Quattro Ultima tandem mass spectrometer (Waters-Micromass, Manchester, UK) equipped with an electrospray ionization interface (ESI). The mass spectrometer was operated in the negative ESI (ESI^-) and the positive ESI (ESI^+) mode, with a capillary voltage of 2.8 kV, a desolvation temperature of 350°C at a nitrogen gas flow rate of 600 l/h, a source temperature of 120°C, and a cone gas flow rate of 100 l/h. Argon was used as collision induced dissociation (CID) gas at a pressure of 2.3×10^{-3} mbar. The cone voltage and collision energy were optimized by direct infusion experiments under acidic and alkaline conditions (Table 4.1). For both LC gradients negative and positive ionization were applied in separate runs. OA and PTX2 were analyzed in both the ESI^- and ESI^+ mode while YTX was analyzed only in the ESI^- mode and AZA1, SPX1 and GYM only in the ESI^+ mode, respectively. For the acidic and alkaline LC method a solvent delay of respectively 2 and 3 min was used to reduce contamination of the MS system.

Solid phase extraction

Automated SPE system (Symbiosis Pharma)

Development of the SPE procedure was performed using an array of cartridges and sorbents from different brands. Due to a very limited availability of standards, optimization of the SPE method was carried out using the LRM extract. SPE sorbent screening was performed with an automated SPE system, the Symbiosis Pharma (Spark Holland, Emmen, The Netherlands). The Symbiosis Pharma consists of a temperature controlled stacker, a temperature controlled autosampler (Reliance), a high pressure dispenser (HPD single), a high pressure mix with a solvent selection manifold, a gradient pump set and an automatic cartridge exchanger (ACE). With the Symbiosis Pharma the following sorbents were tested: Cyano, C_2, C_8, C_8 end-capped (C_8 ec), C_{18}, C_{18} high definition (C_{18} HD), general purpose resin (GP) and strongly hydrophobic resin (SH), all cartridges containing 12.5 mg sorbent from Spark Holland, Emmen, The Netherlands, and weak cation-exchange (WCX), strong cation-exchange (MCX), weak anion-exchange (WAX) and strong anion-exchange (MAX) cartridges containing 2.5 mg sorbent from Waters, Etten-leur, The Netherlands (Table 4.2).

All cartridges were conditioned with 1 ml methanol and equilibrated with 1 ml methanol/water (30:70 v/v). The crude methanolic shellfish extract was diluted with water to an organic strength of methanol/water (30:70 v/v) and 25 µl of the diluted extract was automatically transferred to the top of the cartridge. The Cyano, C_2, C_8, C_8 ec, C_{18}, C_{18} HD, GP and SH cartridge were washed with 1 ml methanol/water (20:80 v/v) and subsequently eluted with 500 µl methanol containing 1% v/v ammonium hydroxide (25%). The ion exchange cartridges (WCX, MCX, WAX and MAX) were washed with 1 ml 5% v/v ammonium hydroxide (25%) in water, methanol/water (30:70 v/v) or 2% v/v formic acid in water. Elution was carried out with 500 µl 5% v/v ammonium hydroxide (25%) in methanol, methanol or 2% v/v formic acid in methanol. The Symbiosis SPE extracts were analyzed by LC-MS/MS using the alkaline LC gradient.

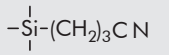
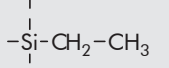
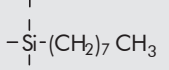
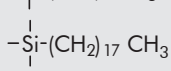
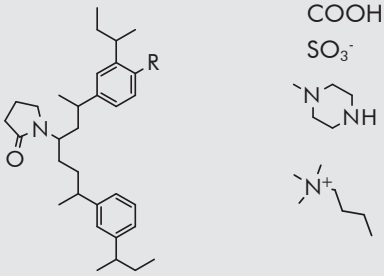
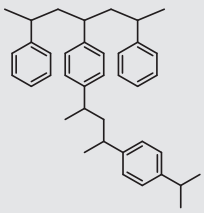
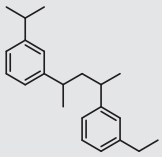
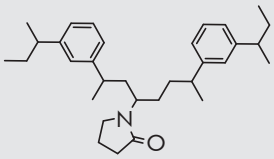
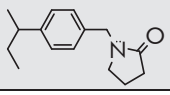
Initially selected SPE protocol

In order to optimize the off-line SPE two different cartridges containing polymeric sorbents were used: 60 mg 3 cc Oasis® HLB (Waters, Etten-leur, The Netherlands) and 60 mg 3 cc Strata™-X (Phenomenex, Torrance, CA, USA). The retention capacity of both cartridges was investigated by application of LRM extract diluted before application with 60% water. The cartridges were subsequently washed with methanol/water (50:50 v/v) and eluted with methanol. The wash and elution solvents and volumes were optimized during the method development stage for the cartridge with the best retention capacity. Furthermore, break-through, wash losses and recoveries, based on the mass spectrometric analysis of the toxins present in the LRM material, before and after SPE clean up were determined. Based on the results obtained the final SPE protocol was defined.

Final SPE protocol

After further optimization (see 'optimization of the SPE clean up protocol') a 30 mg 1 cc Strata™-X cartridge was selected. It was activated with 1 ml of methanol. Prior to application of the sample the cartridge was equilibrated using 1 ml methanol/water (30:70 v/v). Crude methanolic shellfish extract (1.2 ml) was diluted with 2.8 ml water and applied on the cartridge. The cartridge was washed with 1 ml methanol/water (20:80 v/v) in order to remove polar compounds. Finally the

Table 4.2 Sorbents tested for clean up of marine lipophilic toxins.

Type of cartridge		Structure
Silica	Cyano	
	C_2	
	C_8 and C_8 ec	
	C_{18} and C_{18} HD	
Ion exchange	WCX	
	MCX	
	WAX	
	MAX	
Polymer	SH	
	GP	
	Oasis-HLB	
	Strata™-X	

toxins were eluted from the cartridge using 1.2 ml methanol containing 0.3% v/v ammonium hydroxide. The purified extracts were transferred to an HPLC vial and analyzed by LC-MS/MS. Various parameters were investigated (Fig. 4.2). The recovery of the SPE clean up step was determined by comparing the MS response of individual toxins spiked to the crude extract pre SPE with the same toxins spiked to the purified extract post SPE. Secondly, the matrix effect observed for each toxin in the crude extract was determined for both LC gradients by comparing the toxins spiked to crude extract with the toxins spiked to methanol. In the same way, the matrix effects observed in the purified extracts, obtained after SPE, were determined by comparing the purified extract with the toxins spiked to methanol. Finally, the apparent recovery, defined as the combination of the (reduced) matrix effect and the recovery of the SPE was established by comparing the pre SPE toxin spiked extracts with the toxins spiked to methanol. All experiments were repeated 5 times for each matrix, the results were based on the total area of three transitions, except for YTX where under acidic conditions one transition was used (Table 4.1). At low pH the predominant precursor ion was the single charged molecule at *m/z* 1141.5 $[M\text{-}H]^-$, while at high pH the predominant precursor was the doubly charged molecule $[M\text{-}2H]^{2-}$, at *m/z* 570.4. Therefore the $[M\text{-}H]^-$ ion was used with the acidic LC conditions and the with the $[M\text{-}2H]^{2-}$ with the alkaline LC conditions.

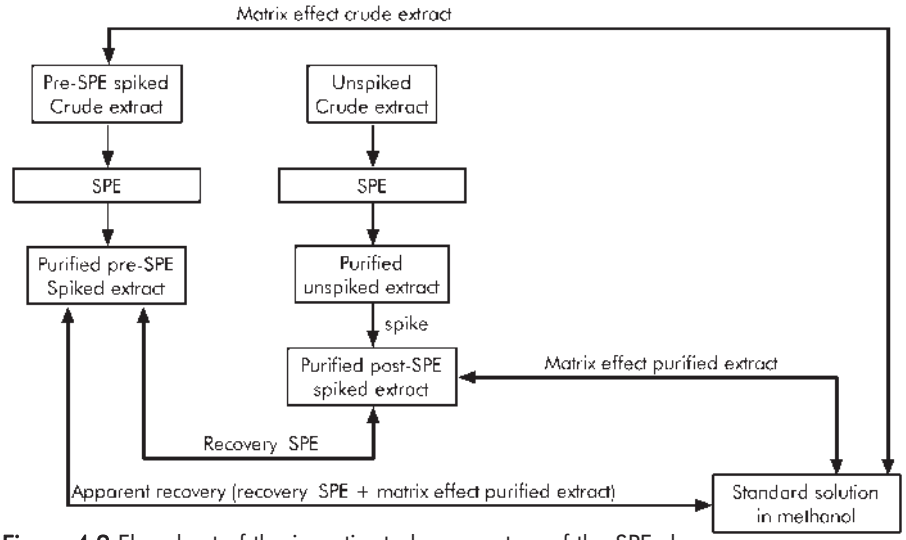


Figure 4.2 Flowchart of the investigated parameters of the SPE clean up.

RESULTS AND DISCUSSION

Initial method development

The first step of the SPE method development focused on finding an appropriate sorbent which retained the various toxin groups. With the Symbiosis Pharma system a rapid sorbent screening of 12 different sorbents ranging from very polar to strongly hydrophobic was performed (see Table 4.2 for the chemistry of the sorbents). On most cartridges, retention may be expected based on the lipophilic character of the toxins tested. Additional retention due to the presence of ionisable functional groups may play an important role when ion exchange cartridges are used.

Based on the chemistry of the ion exchange sorbents, a prediction could be made for the interaction of the various toxins with these sorbents, indicating the difficulties to be expected in finding an appropriate sorbent which will retain all lipophilic marine toxins. To study the retention of the toxins on the various ion exchange sorbents, the cartridges were washed and eluted with alkaline, neutral and acidic solutions. All wash and elution combinations were tested per ion exchange cartridge. In this way the cartridges were also used in their non ideal or even opposite polarity of wash and elution conditions. For example, the recommended conditions for a MAX cartridge are alkaline wash followed by acidic elution to obtain optimal retention and recovery for compounds containing a carboxylic acid group. OA, which contains a carboxylic acid group, was very well retained on the MAX cartridge as expected; but also the WAX cartridge (specific for strong acids) yielded a high recovery under most of the applied wash and elution conditions, with the exception of the alkaline wash and acidic elution (<25%) (Fig. 4.3). YTX contains two sulfonic acid groups and should be retained on a WAX cartridge. Good recoveries on the WAX cartridge were obtained for YTX, when a neutral or acidic wash was applied in combination with alkaline elution (Fig. 4.3). However, the recovery for YTX was poor on the MAX, WCX and MCX cartridge regardless the conditions applied. AZA1 is an amphoteric compound containing a carboxylic acid as well as an amino group, and could be retained on the MAX as well as on the MCX cartridge. The MAX cartridge gave good recoveries for AZA1 with an alkaline wash followed by acidic elution and with a neutral wash and alkaline elution. Furthermore, reasonable recoveries were obtained for AZA1 on

the MCX and WCX cartridge with an acidic or neutral wash and neutral or alkaline elution (Fig. 4.3). SPX1 and GYM contain an imine group which should be retained best on an MCX cartridge. However, SPX1 was also well retained on the WCX cartridge under most conditions (Fig. 4.3). PTX2, a neutral compound, does not contain any specific functional groups. For this reason it is difficult to predict its behavior on the different cartridges. In practice PTX2 had some retention on all cartridge types. The best recoveries were obtained on the MAX and WCX cartridges under acidic wash conditions. Reasonable recoveries were obtained on the MAX and WAX cartridges using an alkaline wash and acidic elution.

Figure 4.3 shows that only in two instances (MAX and WAX with acidic wash and alkaline elution) two out of five toxins were retained with high efficiency (>90%). Only in one occasion (WAX cartridge with a neutral wash and neutral elution step), four out of five toxin groups could be recovered with more than 50% yield. It is evident that it will be very difficult to find an ion exchange cartridge and the appropriate wash and elution conditions at which for all five toxin groups good recoveries are obtained.

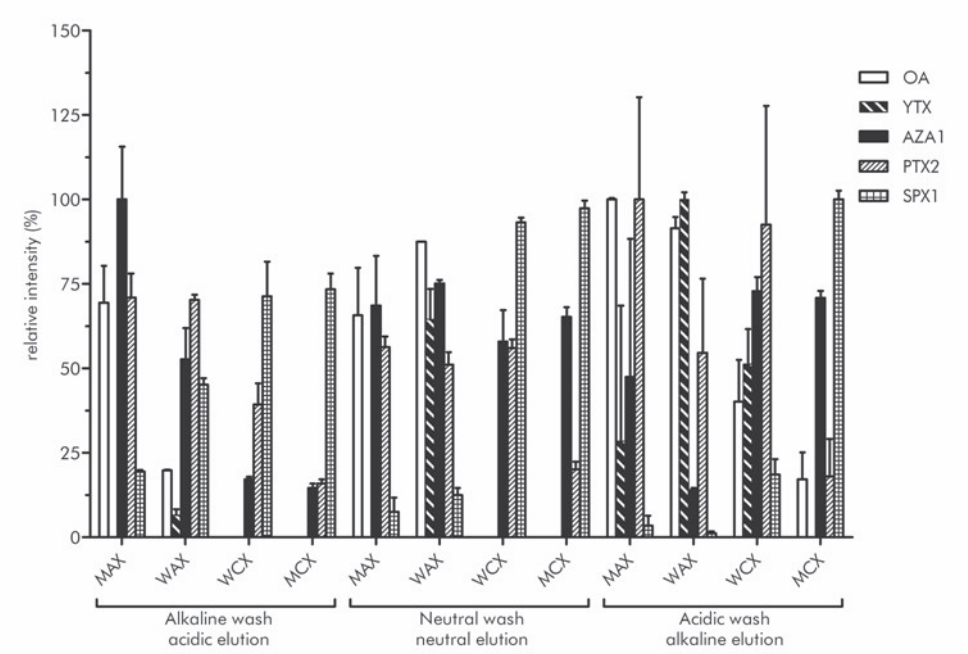


Figure 4.3 Sorbent screening of different ion exchange cartridges using the Symbiosis Pharma automated SPE system. Highest signal intensity per individual toxin set at 100%. Wash with methanol/water (50:50 v/v) and elution with methanol (n=2).

With respect to the silica based sorbents that were tested with the Symbiosis Pharma system, OA gave good recoveries on all the cartridges that were investigated, with the exception of the CN cartridge (Fig. 4.4). For YTX good recoveries were obtained with the C_{18} HD and GP cartridge, while AZA1 showed good recoveries on all the cartridges except on the SH cartridge. The recovery of PTX2 was good on all cartridges with the exception of the CN cartridge. From the data shown in figure 4.4 it is clear that the C_{18} HD and GP cartridges showed overall the best recoveries of the five toxin groups.

In conclusion, for the ion exchange cartridges and silica based cartridges for each toxin different optimum conditions and different optimal sorbents were obtained. Especially for YTX it was difficult to find suitable conditions that would match with the other toxins. As none of the cartridges dealt with above is capable to retain all five toxin groups the focus changed towards general purpose polymeric cartridges. These polymeric cartridges can be applied for a broad range of compounds. Two different brands of polymeric sorbents were selected for further investigation, Oasis® hydrophilic lipophilic balance (HLB) and Strata™-X. Because the Strata™-X cartridge was not available for the Symbiosis Pharma system, further optimization experiments were performed off-line. With polymeric cartridges such as Oasis® HLB and Strata™-X retention of compounds is based on their polar and lipophilic interactions.

Optimization of the SPE clean up protocol

In comparison with the previous tested sorbents (ion exchange and silica) the polymeric cartridges are capable to retain all the lipophilic marine toxin groups. From figure 4.5 it can be seen that OA, DTX1, DTX2 and YTX were better retained on the Strata™-X than on the Oasis® HLB cartridge during the application and wash step. AZA1, -2, -3 and PTX2 were slightly better retained on Oasis® HLB. Overall Strata™-X performed somewhat better. Therefore, the Strata™-X cartridge was used for further optimization experiments. Volume and organic solvent strength of the applied crude extract, wash step and elution step were optimized.

Solvent strength crude extract

The volume and organic solvent strength of the crude shellfish LRM extract tolerated during application on the cartridge was investigated. No break-through was observed when the crude methanol extracts (2 ml) were diluted to methanol/ water (30:70 v/v) in water prior to application to the SPE cartridge. At a higher percentage methanol break-through of OA, DTX1, DTX2 and YTX was observed. Increasing volumes of crude sample extract (up to 6 ml diluted to methanol/water (30:70 v/v) by mixing with 14 ml water) were applied to the 60 mg cartridge. For none of the extracts break-through of any of the toxins was observed. Thus, if necessary, a concentration step can be incorporated into the extraction protocol.

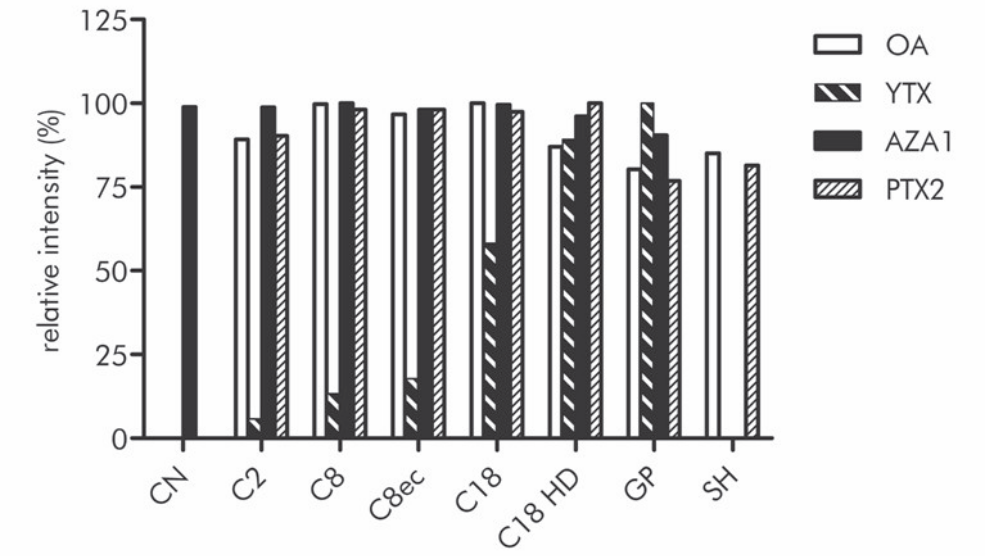


Figure 4.4 Sorbent screening using the Symbiosis Pharma automated SPE system. Highest signal intensity per individual toxin set at 100%. Wash with methanol/water (20:80 v/v) elution with 1% v/v NH_4OH in methanol.

Wash step

The organic solvent strength of the wash step (3 ml) was optimized by using 0-70% v/v methanol/water mixtures with increments of 10%. OA, DTX1, DTX2 and YTX started to elute when wash solutions were used with more than 30% methanol. AZAs were retained on the cartridge with up to 50% methanol and PTX2 did not elute even with 70% methanol. A wash step of methanol/water (20:80 v/v) was

incorporated to avoid losses during washing.

Next, the effect of using acidic, neutral or alkaline methanol/water (20:80 v/v) as wash solvent was investigated. The acidic wash solvent contained 1% v/v formic acid and the alkaline wash solvent 1% v/v ammonium solution. As no significant differences in recovery of the toxins were obtained between the various wash solvents, a neutral wash step of methanol/water (20:80 v/v) was incorporated in the final method.

Elution step

Elution was performed with 2 ml methanol. To estimate the amount of remaining toxins on the cartridge a second elution step with 2 ml methanol, collected in a second vial, was incorporated. The first elution resulted in an almost complete recovery (>90%) of OA, DTX1-2, AZA1-3 and PTX2 but for YTX the recovery was somewhat lower (60-70%). Around 10-20% of YTX was eluted in the second step. The recovery of YTX correlated with the pH of the wash step used during clean up. With an alkaline wash step the recovery of YTX was higher (80-90%), while with an

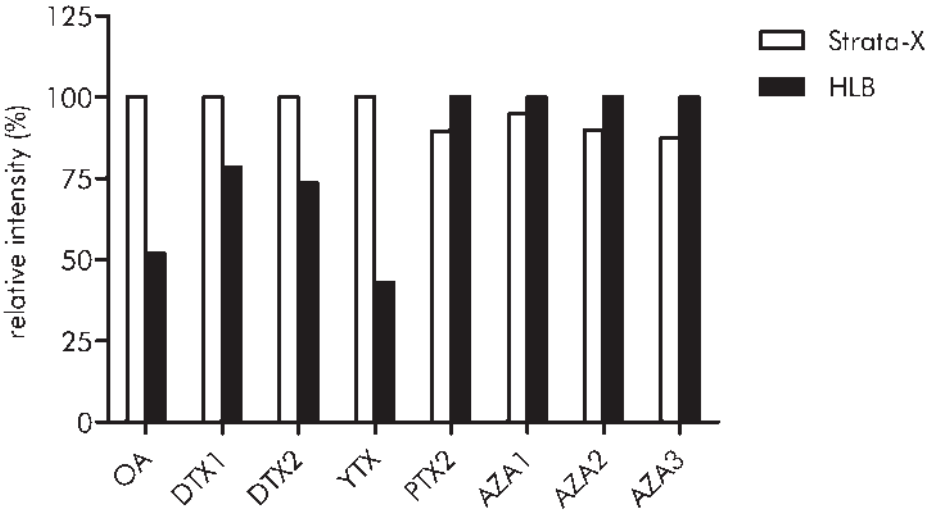


Figure 4.5 Relative intensity of selected toxins on two different sorbents with the preliminary SPE protocol. Highest intensity per individual toxin is set at 100%.

acidic wash step it was lower (50-60%). By adding 0.3% v/v ammonia solution to the elution solvent the recovery could be increased to around 90% for YTX without affecting the recoveries of the other toxins. Using higher concentrations of ammonia did not further improve recoveries.
The stability of the toxins under alkaline conditions in the presence of ammonia has been investigated previously [11]. No degradation of any of the toxins was observed even at concentrations of 12.5% v/v ammonia in water.

Performance of the optimized protocol
All optimization experiments were conducted with 60 mg Strata™-X cartridges. Although this may be a very practical size to be used in routine monitoring of shellfish samples it was decided to downscale the procedure to 30 mg cartridges in order to save valuable toxin standards.
To study the efficacy of the optimized SPE protocol with respect to recovery and matrix effects, methanolic extracts with a solvent-to-sample ratio of 10, from blank mussels, scallops and oysters were spiked with OA, YTX, AZA1, PTX2. In a second experiment, SPX1 and GYM were spiked to a blank mussel extract to check if the developed method could also be applied for these toxins as well. Toxin concentrations were chosen such that they were at (16 ng/ml OA and PTX2) or somewhat below (20 ng/ml YTX and 10 ng/ml AZA1) the current permitted level in EU legislation [10]. For SPX1 and GYM there is currently no legislation established. These toxins were added to the mussel extract at a concentration of 12.5 ng/ml.
It was anticipated that the chromatographic conditions used prior to MS detection can be of importance to the observed matrix effects [25]. Changing the pH of the mobile phase may lead to an altered separation of toxins and matrix components. To investigate the effect of chromatographic separation, two different established HPLC methods were used [11, 27], one using acidic conditions and the other one alkaline conditions. As was shown before, the elution profile of the toxins is quite different under these conditions [11].
The ion ratios of the various transitions were reproducible with RSDs lower than 15% (n=70, data not shown). The only exception was PTX2 analyzed in the ESI^- mode in combination with the acidic LC method. Due to an eight-fold lower sensitivity, compared to ESI^+, an RSD of around 25% was obtained.

Recovery of the SPE method

The SPE clean up procedure worked well for OA, YTX, AZA1, PTX2, SPX1 and GYM yielding good to excellent recoveries (Tables 4.3-4.5). The average recovery of all toxins and matrices combined was very good, 90.0±6.0% as determined with the alkaline LC gradient, and 92.6±6.9% with the acidic LC gradient. For individual toxins the recovery varied between 74.6±2.9% for YTX in oyster extract (obtained with the alkaline method) and 102.9±10.7% for OA in oyster extract (measured in ESI^- with the acidic gradient). In general the relative standard deviations (RSD) obtained with the alkaline method (max. 12.5% for GYM) were better compared to those of the acidic method (max. 22.3% for AZA1). Ideally, the recoveries calculated with both methods should not be significantly different, because the recovery of the SPE should not be influenced by the chromatographic system used. Indeed, most of the recoveries obtained were not significantly different ($p=0.05$) with both methods, with the exception of OA (ESI^-) in mussel and in oyster and YTX in oyster. For OA no explanation can be given for the differences in the recovery obtained. For YTX the differences in recovery can be explained by the peak shape of YTX that was rather poor under acidic conditions, which hampered an accurate integration of ion signals.

Matrix effects in the crude and SPE purified extracts

In the crude scallop and oyster extracts substantial ion enhancement was found for OA when run with the acidic method. The ion enhancement for OA in the scallop extract was as high as 103% in ESI^+ and 29% in ESI^-. When the samples were run with the alkaline method for OA severe signal suppression of up to 70-80% was observed in ESI^+, but only minor suppression (<20%) was observed in ESI^- (Tables 4.3-4.5). The SPE clean up reduced the signal enhancement for OA in the samples analyzed under acidic conditions to less than 20% in ESI^+ with a large relative standard deviation of 22.8% and in ESI^- the matrix effect was reduced to <15% with RSD's below 15%. With the alkaline method only minor ion suppression or enhancement (<10%) was observed, except for mussel and oyster extracts for which severe suppression (40-50%) was found in ESI^+. In ESI^+ enhancement and suppression effects for OA are stronger than in ESI^-. This is true for both applied methods, but especially for the alkaline method for which significant suppression is

observed after SPE clean up. OA, DTX1 and DTX2 are, therefore, preferably analyzed in the ESI^- mode.

For YTX only moderate matrix effects were observed in the crude extracts (Tables 4.3-4.5). The largest effect was observed for the mussel extract run under acidic conditions resulting in 25% ion enhancement. Under alkaline conditions the oyster extract gave 20% ion suppression for YTX. SPE clean up contributed to a reduction of the suppression and enhancement effects for YTX in the various extracts to 15% for both LC gradients. The only exception was the mussel extract analyzed with the acidic gradient, for which an enhancement of around 20% was found. For YTX significantly smaller RSD's were obtained with the alkaline method (5.0-6.2%) than with the acidic method (12.2-19.4%). This can be attributed to the fact that the peak shape of YTX is much better under alkaline conditions [11].

Suppression effects for AZA1 in the crude extracts were in the order of 20-40% with the acidic, and 10-20% with the alkaline method. After SPE clean up still some suppression (20% in scallop extracts) was found with the acidic gradient while with the alkaline gradient the suppression was reduced to <5%. For both methods the relative standard deviations obtained were good (<15%) except for the crude scallop extract analyzed under acidic conditions (RSD 35.9%).

For PTX2 the differences in observed matrix effects in the crude extracts between ESI^+ and ESI^- were not as large as for OA. PTX2 analyzed in ESI^+ showed under acidic conditions signal enhancement of around 40%, while in ESI^- minor enhancement was observed (<15%). When applying the alkaline method, significant ion suppression was observed for PTX2 with both ESI modes, ranging from 15% for oyster in ESI^+ to 43% for the scallop extract in ESI^-. In the purified extract PTX2 revealed ion enhancement of 20-30% in ESI^+ under acidic conditions, while in ESI^- minor enhancement was observed (<15%). Relatively high relative standard deviations of more than 15% were obtained in ESI^-, which can be explained by the low sensitivity of PTX2 in ESI^-. The alkaline gradient largely removed the matrix effects resulting in ion suppression or enhancement effects of less than 15% for all matrices in ESI^+ and ESI^-.

In the crude mussel extract only minor suppression occurred for SPX1 and GYM with both gradients (<15%). Surprisingly, for the extract analyzed after SPE the suppression increased to 35% with the acidic conditions, while for the alkaline

Table 4.3 Performance parameters of the optimized SPE protocol for mussel extracts.

					Percentage Matrix effects					
			Recovery SPE (%)		Crude (%)		After SPE (%)		Apparent Recovery (%)	
Toxin	Ionization	LC conditions	Avg (n=5)	RSD	Avg (n=5)	RSD	Avg (n=5)	RSD	Avg (n=5)	RSD
OA	ESI⁻	Alkaline	90.0	6.4	99.3	3.8	104.9	4.4	94.5	6.0
		Acidic	102.5	4.7	104.7	8.1	106.9	7.5	109.5	8.4
OA	ESI⁺	Alkaline	93.6	4.8	**23.8**	8.4	**61.3**[1]	8.2	**57.3**[1]	7.5
		Acidic	92.5	10.1	**61.1**	12.8	**119.2**[1]	14.1	110.3[1]	**16.0**
YTX	ESI⁻	Alkaline	90.0	4.7	95.4	4.6	109.9	5.0	99.0	5.3
		Acidic	93.2	8.3	**124.9**	14.5	**122.4**	**19.4**	114.0	**20.7**
AZA1	ESI⁺	Alkaline	88.6	3.5	88.1	5.7	98.2[1]	6.5	86.9	5.8
		Acidic	85.5	**22.3**	**84.3**	10.3	105.6[1]	9.6	90.2	**23.3**
PTX2	ESI⁻	Alkaline	89.3	5.3	**64.7**	8.0	101.1[1]	5.9	90.3[1]	5.6
		Acidic	95.5	**17.4**	105.9	**16.0**	106.8	**19.4**	102.0	**20.2**
PTX2	ESI⁺	Alkaline	90.0	3.9	**56.5**	5.3	86.7[1]	5.3	**78.0**[1]	5.5
		Acidic	89.0	8.4	**139.5**	7.7	**128.1**	11.4	114.0[1]	13.0
SPX1	ESI⁺	Alkaline	100.7	11.6	89.4	5.0	95.6	10.3	96.2	7.8
		Acidic	96.3	13.4	95.7	6.0	**64.9**[2]	11.6	**62.6**[2]	8.8
GYM	ESI⁺	Alkaline	96.9	12.5	**81.1**	6.4	95.8[1]	11.3	92.9[1]	9.0
		Acidic	89.0	9.8	92.5	4.5	**70.3**[2]	9.4	**62.5**[2]	5.4

Numbers given in **Bold**: when RSD above 15% or when recovery, crude, after SPE or apparent recovery are below 85% or above 115%. [1)] significantly improved compared to crude extract ($p \leq 0.05$), [2)] significant deterioration compared to the crude extract ($p \leq 0.05$).

Table 4.4 Performance parameters of the optimized SPE protocol for scallop extracts.

					Percentage matrix effects					
			Recovery (%)		Crude (%)		After SPE (%)		Apparent Recovery (%)	
Toxin	Ioniza-tion	LC conditions	Avg (n=5)	RSD	Avg (n=5)	RSD	Avg (n=5)	RSD	Avg (n=5)	RSD
OA	ESI^-	Alkaline	93.6	3.2	98.9	4.7	104.4	5.1	97.8	4.3
		Acidic	102.3	9.7	**128.8**	10.6	85.5[1]	12.5	87.5[1]	11.2
OA	ESI^+	Alkaline	85.8	4.2	**17.6**	**19.3**	101.2[1]	14.3	86.9[1]	14.6
		Acidic	91.9	11.3	**203.1**	**19.9**	**115.4**[1]	**19.9**	106.1[1]	**19.9**
YTX	ESI^-	Alkaline	88.5	3.6	98.2	6.0	113.5	6.2	100.4[1]	5.5
		Acidic	94.8	10.2	105.9	11.8	99.0	13.0	93.9	13.0
AZA1	ESI^+	Alkaline	86.4	3.0	89.0	4.9	102.8[1]	4.4	88.8	5.0
		Acidic	86.4	7.2	**59.1**	**35.9**	**80.0**	14.5	**69.1**	13.9
PTX2	ESI^-	Alkaline	89.9	4.7	**56.7**	7.9	88.7[1]	6.7	**79.7**[1]	7.0
		Acidic	94.2	17.2	**115.6**	**17.6**	90.5	**18.0**	85.3	12.2
PTX2	ESI^+	Alkaline	90.1	2.8	**71.1**	5.1	94.1[1]	4.9	**84.8**[1]	5.0
		Acidic	88.5	8.7	**128.4**	9.9	111.4	10.2	98.6[1]	9.3

Numbers given in **Bold**: when RSD above 15% or when recovery, crude, after SPE or apparent recovery are below 85% or above 115%. [1)] significantly improved compared to crude extract ($p \leq 0.05$), [2)] significant deterioration compared to the crude extract ($p \leq 0.05$).

Table 4.5 Performance parameters of the optimized SPE protocol for oyster extracts.

			Recovery (%)		Percentage matrix effects: Crude (%)		After SPE (%)		Apparent Recovery (%)	
Toxin	Ioniza-tion	LC conditions	Avg (n=5)	RSD	Avg (n=5)	RSD	Avg (n=5)	RSD	Avg (n=5)	RSD
OA	ESI^-	Alkaline	85.8	5.0	**79.6**	7.0	93.4[1]	7.1	**80.1**	7.0
		Acidic	102.9	10.7	**123.6**	9.5	90.9[1]	13.6	93.6[1]	**15.3**
OA	ESI^+	Alkaline	94.4	5.2	**19.0**	9.5	**44.9**[1]	10.0	**42.4**[1]	9.7
		Acidic	94.1	6.7	**140.5**	**22.9**	**117.7**	**22.8**	110.7	**22.3**
YTX	ESI^-	Alkaline	**74.6**	2.9	**80.3**	4.9	103.9[1]	5.2	**77.6**	5.2
		Acidic	89.7	10.8	109.7	11.8	97.0	12.2	86.9	12.0
AZA1	ESI^+	Alkaline	**84.1**	3.8	**83.5**	3.8	100.6[1]	3.6	**84.6**	4.5
		Acidic	**78.2**	7.2	**73.6**	10.7	**83.7**	7.5	**65.5**	7.6
PTX2	ESI^-	Alkaline	93.1	6.6	**67.7**	13.4	114.2[1]	11.9	106.2[1]	11.9
		Acidic	101.6	11.5	106.2	12.9	85.2	14.1	86.6	**15.7**
PTX2	ESI^+	Alkaline	94.3	5.4	85.4	5.4	102.9[1]	5.1	97.0[1]	4.9
		Acidic	89.1	7.2	**140.2**	10.8	**131.8**	11.7	**117.4**	12.4

Numbers given in **Bold**: when RSD above 15% or when recovery, crude, after SPE or apparent recovery are below 85% or above 115%. [1)] significantly improved compared to crude extract ($p \leq 0.05$), [2)] significant deterioration compared to the crude extract ($p \leq 0.05$).

method the suppression remained minimal at less than 5%.
It can be concluded that SPE clean up resulted in the reduction of matrix effects for most toxins studied. However, the amount of reduction was variable and depended on the chromatographic conditions used. Overall, the clean up was more efficient and more reproducible in combination with the alkaline method. As shown in tables 4.3, 4.4 and 4.5 most toxins analyzed with the alkaline gradient gave a significant ($p \leq 0.05$) reduction in matrix effects after SPE clean up compared to the crude extracts.

Apparent recovery of the SPE method
The apparent recovery, defined as the combined effect of the SPE recovery and the remaining matrix effects is shown in tables 4.3, 4.4 and 4.5. In general, an improvement was obtained when the apparent recoveries were compared with the corresponding recoveries obtained for the crude extracts. For the acidic gradient the apparent recovery varied between 62.5% for SPX1 and GYM in mussel and 117.4% for PTX2 in the oyster extract. For the alkaline method the apparent recovery varied between 77.6% for YTX in oyster and 100.4% for YTX in the scallop extract. Furthermore, the SD's obtained with the alkaline method were significantly better than with the acidic method. In general, the overall recovery of the SPE clean up combined with the alkaline method was more reproducible than with the acidic method.

CONCLUSION

The SPE clean up on the Strata™-X cartridge resulted in reduced matrix effects with both LC methods. However, when SPE was used in combination with the alkaline method the matrix effects after SPE were reduced to less than 15% for all toxins analyzed in their preferred mode regardless of the matrix used. For the acidic method, differences in matrix effects were less substantial between the crude extract and extract after SPE. The matrix effects obtained in crude mussel extracts were in accordance with the results obtained by Fux *et al*. [25]. Only for two toxins (OA and AZA1) in purified mussel extract the matrix effects were less than 15%. The observed RSDs of the results were much smaller with the alkaline method as well (<15%).

When the apparent recovery is compared to the crude extract, a significant improvement ($p \leq 0.05$) was only obtained for a few toxins (Tables 4.3-4.5). At first sight, one could consider not to implement the SPE procedure. The loss of toxins encountered during SPE (average recoveries around 90%) attributed to this modest improvement in apparent recovery. However, when the crude extract and the extract after SPE are compared a significant reduction ($p \leq 0.05$) in matrix effects was obtained for most toxins analyzed by the alkaline method and for some analyzed with the acidic method. Therefore, the apparent recovery should be corrected for the loss of toxins encountered during SPE. There are several methods to correct for these losses. First, a correction factor matching the loss of recovery could be used, which should be determined during validation of the method. The second option is to use matrix-matched standards. When these matrix-matched standards would be applied to the SPE procedure, they would give the correction for the recovery of the SPE step. Differences observed between the acidic and alkaline chromatographic conditions after SPE clean up using Strata™-X indicate that the clean up could possibly still be further improved. This will, however, not be easy because the large range of lipophilicities involved. Single-toxin group SPE clean up could be considered as an alternative, but obliviously only at the expense of much more time. The use of the Symbiosis Pharma system for the automated screening of various SPE sorbents was advantageous in this study. With this system, the testing of sorbents including the testing of various wash and elution solvents could be done more effectively compared to the time consuming offline optimization experiments. In summary, this study showed that a multi-toxin SPE method with good recoveries is now available.

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REFERENCES

[1] Yasumoto T, Murata M. Marine toxins. *Chem Rev* 1993; **93**: 1897.

[2] Toyofuku H. Joint FAO/WHO/IOC activities to provide scientific advice on marine biotoxins (research report). *Mar Pollut Bull* 2006; **52**: 1735.

[3] Terao K, Ito E, Oarada M, Murata M, Yasumoto T. Histopathological studies on experimental marine toxin poisoning .5. The effects in mice of yessotoxin isolated from *Patinopecten-yessoensis* and of a desulfated derivative. *Toxicon* 1990; **28**: 1095.

[4] Aune T, Sorby R, Yasumoto T, Ramstad H, Landsverk T. Comparison of oral and intraperitoneal toxicity of yessotoxin towards mice. *Toxicon* 2002; **40**: 77.

[5] Twiner MJ, Hess P, Dechraoui MYB, McMahon T, Samons MS, Satake M, Yasumoto T, Ramsdell JS, Doucette GJ. Cytotoxic and cytoskeletal effects of azaspiracid-1 on mammalian cell lines. *Toxicon* 2005; **45**: 891.

[6] Ito E, Satake M, Ofuji K, Kurita N, McMahon T, James KJ, Yasumoto T. Multiple organ damage caused by a new toxin azaspiracid, isolated from mussels produced in Ireland. *Toxicon* 2000; **38**: 917.

[7] Miles CO, Wilkins AL, Munday R, Dines MH, Hawkes AD, Briggs LR, Sandvik M, Jensen DJ, Cooney JM, Holland PT, Quilliam MA, MacKenzie AL, Beuzenberg V, Towers NR. Isolation of pectenotoxin-2 from *Dinophysis acuta* and its conversion to pectenotoxin-2 seco acid, and preliminary assessment of their acute toxicities. *Toxicon* 2004; **43**: 1.

[8] Miles CO, Wilkins AL, Munday JS, Munday R, Hawkes AD, Jensen DJ, Cooney JM, Beuzenberg V. Production of 7-epi-pectenotoxin-2 seco acid and assessment of its acute toxicity to mice. *J Agric Food Chem* 2006; **54**: 1530.

[9] Richard DJA, Arsenault E, Cembella AD, Quilliam MA, Investigations into the toxicology and pharmacology of spirolides, a novel group of shellfish toxins. In *Proceedings of the 9th conference on Harmfull algal blooms*, Hallegraeff GM, Blackburn SI, Bolch CJ, Lewis RJ, Eds. Hobart, Australia, 2000; 383.

[10] European Parliament, Council. Commission Directive 2004/853/EC Specific hygiene rules for food of animal origin. *Off J Eur Commun* 2004; **L226**: 22.

[11] Gerssen A, Mulder PPJ, McElhinney MA, De Boer J. Liquid chromatography - tandem mass spectrometry method for the detection of marine lipophilic toxins under alkaline conditions. *J Chrom A* 2009; **1216**: 1421.

[12] Stobo LA, Lacaze JPCL, Scott AC, Gallacher S, Smith EA, Quilliam MA. Liquid chromatography with mass spectrometry - detection of lipophilic shellfish toxins. *J AOAC Int* 2005; **88**: 1371.

[13] McNabb P, Selwood AI, Holland PT. Multiresidue method for determination of algal toxins in shellfish: Single-laboratory validation and interlaboratory study. *J AOAC Int* 2005; **88**: 761.

[14] Fux E, McMillan D, Bire R, Hess P. Development of an ultra-performance liquid chromatography-mass spectrometry method for the detection of lipophilic marine toxins. *J Chromatogr A* 2007; **1157**: 273.

[15] Ito S, Tsukada K. Matrix effect and correction by standard addition in quantitative liquid chromatographic-mass spectrometric analysis of diarrhetic shellfish poisoning toxins. *J Chromatogr A* 2002; **943**: 39.

[16] Jorgensen K, Jensen LB. Distribution of diarrhetic shellfish poisoning toxins in consignments of blue mussel. *Food Addit Contam A* 2004; **21**: 341.

[17] Furey A, Brana-Magdalena A, Lehna M, Moroney C, James KJ, Satake M, Yasumoto T. Determination of azaspiracids in shellfish using liquid chromatography/tandem electrospray mass spectrometry. *Rapid Commun Mass Spectrom* 2002; **16**: 238.

[18] Puente PF, Saez MJF, Hamilton B, Lehane M, Ramstad H, Furey A, James KJ. Rapid determination of polyether marine toxins using liquid chromatography-multiple tandem mass spectrometry. *J Chromatogr A* 2004; **1056**: 77.

[19] Vale P, Sampayo MAD. Esterification of DSP toxins by Portuguese bivalves from the Northwest coast determined by LC-MS - a widespread phenomenon. *Toxicon* 2002; **40**: 33.

[20] Van Loco J, Kotterman MJJ, Hess P *Report on results of the validation study (D29), within the 6th Framework project 'BIOTOX: Development of cost-effective tools for risk management and traceability systems for marine biotoxins in seafood' Contract no: 514074* 2008.

[21] Puech L, Dragacci S, Gleizes E, Fremy JM. Use of immunoaffinity columns for clean-up of diarrhetic toxins (okadaic acid and dinophysistoxins) extracts from shellfish prior to their analysis by HPLC fluorimetry. *Food Addit Contam A* 1999; **16**: 239.

[22] Quilliam MA. Analysis of diarrhetic shellfish poisoning toxins in shellfish tissue by liquid-chromatography with fluorometric and mass-spectrometric detection. *J AOAC Int* 1995; **78**: 555.

[23] Goto H, Igarashi T, Yamamoto M, Yasuda M, Sekiguchi R, Watai M, Tanno K, Yasumoto T. Quantitative determination of marine toxins associated with diarrhetic shellfish poisoning by liquid chromatography coupled with mass spectrometry. *J Chromatogr A* 2001; **907**: 181.

[24] Moroney C, Lehane M, Brana-Magdalena A, Furey A, James KJ. Comparison of solid-phase extraction methods for the determination of azaspiracids in shellfish by liquid chromatography-electrospray mass spectrometry. *J Chromatogr A* 2002; **963**: 353.

[25] Fux E, Rode D, Bire R, Hess P. Approaches to the evaluation of matrix effects in the liquid chromatography-mass spectrometry (LC-MS) analysis of three regulated lipophilic toxin groups in mussel matrix (*Mytilus edulis*). *Food Addit Contam A* 2008; **25**: 1024.

[26] Hess P, McMahon T, Slattery D, Swords D, Dowling G, McCarron M, Clarke D, Gibbons W, Silke J, O'Cinneide M In *Use of LC-MS testing to identify lipophilic toxins, to establish local trends and interspecies differences and to test the comparability of LC-MS testing with the mouse bioassay: an example from the Irish Biotoxin monitoring programme 2001*, Molluscan Shellfish Safety, Santiago de Compostela, Spain, 2003 Villalba A, Reguera A, Romalde JL, Beiras R, Eds. Santiago de Compostela, Spain, 2003; 57.

[27] Quilliam MA, Hess P, Dell' Aversano C In *Recent developments in the analysis of phycotoxins by liquid chromatography-mass spectrometry*; de Koe WJ, Samson RA, van Egmond HP, Gilbert J, Sabino M, Eds. Mycotoxins and Phycotoxins in Perspective at the turn of the century: Wageningen, The Netherlands, 2001; 383.

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In-house validation of a liquid chromatography tandem mass spectrometry method for the analysis of lipophilic marine toxins in shellfish using matrix matched calibration

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ABSTRACT

A liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the quantitative analysis of lipophilic marine toxins in shellfish extracts (mussel, oyster, cockle and ensis) was in-house validated using the European Union (EU) Commission Decision 2002/657/EC as guideline. The validation included the toxins okadaic acid (OA), yessotoxin (YTX), azaspiracid-1 (AZA1), pectenotoxin-2 (PTX2) and 13-desmethyl spirolide C (SPX1). Validation was performed at 0.5, 1 and 1.5 times the current EU permitted levels, which are 160 μg/kg for OA, AZA1 and PTX2 and 1 000 μg/kg for YTX. For SPX1 400 μg/kg was chosen as target level as no legislation has been established yet for this compound. The method was validated for determination in crude methanolic shellfish extracts and for extracts purified with solid phase extraction (SPE). Extracts were also subjected to hydrolysis conditions in order to determine the performance of the method for OA and dinophysistoxin (DTX) esters. The toxins were quantified against a set of matrix matched standards instead of standard solutions in methanol. In order to save valuable standard the toxin standard was spiked to methanolic extract instead of to the homogenate. This was justified by the fact that the extraction efficiency is high for all relevant toxins (>90%). The method performed very well with respect to accuracy, intra-day precision (repeatability), inter-day precision (within-lab reproducibility), linearity, decision limit (CC_α), specificity and ruggedness. At the permitted level the accuracy ranged from 102 to 111%, the repeatability 2.6-6.7% and the reproducibility 4.7-14.2% in crude methanolic extracts. The crude extracts performed less satisfactory with respect to the linearity (<0.990) and the change in LC-MS/MS sensitivity during the series (>25%). SPE purification resulted in a greatly improved linearity and signal stability during the series. Recently the European Food Safety Authority (EFSA) has suggested that in order to not exceed the acute reference dose the levels should be below 45 μg/kg OA-equivalents and 30 μg/kg AZA1-equivalents. A single day validation was successfully conducted at these levels. In case regulatory levels are lowered towards the EFSA suggested values, the official methods prescribed in legislation (mouse and rat bioassay) will no longer be sensitive enough. The presented validated LC-MS/MS method has the potential to replace these animal tests.

INTRODUCTION

Filter-feeding shellfish species such as mussels, oysters and ensis can be contaminated with various types of lipophilic marine toxins. These lipophilic marine toxins are produced by specific phytoplankton species such as *Dinophysis acuta*, *Protoceratium reticulatum* and *Alexandrium ostenfeldii* [1-4]. Consumption of shellfish contaminated with lipophilic marine toxins can cause severe intoxications [5, 6]. The lipophilic marine toxin group comprises okadaic acid (OA), dinophysistoxins (DTXs), yessotoxins (YTXs), azaspiracids (AZAs), pectenotoxins (PTXs) and spirolides (SPXs). From these toxins OA, DTXs and AZAs are known to cause gastrointestinal disorders in humans [7, 8]. For the other toxins (YTXs, PTXs, SPXs) no cases of intoxication in humans have been reported yet, but these toxins have been found lethal or at least highly toxic to mice when intraperitoneally injected [9-11]. Legislation and routine monitoring programs have been established in order to protect the consumer [12]. The permitted levels in whole flesh shellfish have been set for the sum of all relevant OA, DTXs and PTXs at 160 μg/kg, for the sum of relevant YTXs at 1 000 μg/kg and for the sum of the relevant AZAs at 160 μg/kg [12]. For SPXs no legislation has been established yet. At the time that the current legislation was established toxicity data on most toxins was scarce or even lacking. More data has become available since, and recently the European Food Safety Authority (EFSA) has published several opinions in which this new toxicological information is evaluated. From the EFSA documents it can be seen that the current permitted levels for OA, DTXs and for the PTXs and AZAs may not be sufficient to exclude the risk of intoxication [13-15], while for the YTXs the permitted level overestimates the toxicity [16]. The EFSA has proposed the following protection levels: the sum of OA and DTXs at 45 μg/kg, for AZAs 30 μg/kg, for PTXs 120 μg/kg and for YTXs 3750 μg/kg [13-16]. It may be expected that the EFSA opinions will initiate a discussion among the different member states of the European Union (EU) on whether or not the current permitted levels in the EU legislation should be changed. Lowering of permitted levels may have a serious impact on the shellfish industry. It will also have an impact on the methods that can be applied to monitor legislation compliance. It is highly unlikely that the official reference methods currently prescribed in legislation, the rat bioassay (RBA) and the mouse bioassay (MBA) can be adapted to the proposed levels [17]. These

assays are under discussion anyway because of ethical reasons. Many years, EU legislation prescribes the need for a reduction, refinement and replacement of animal experiments [18]. Therefore, there is an urgent need for alternative methods that are sensitive enough for all relevant toxins. Alternative methods such as biochemical or chemical methods are promising to replace the current animal tests. Alternative biochemical methods, such as a protein phosphatase inhibition assay (PP2A) and an enzyme-linked immunosorbent assay (ELISA) have been developed for OA (including DTXs) and for the YTXs, respectively [19, 20]. These methods all focus on a specific lipophilic marine toxin group. Alternative chemical methods that comprise all lipophilic marine toxin classes are based on liquid chromatography (LC) coupled to (tandem) mass spectrometry [MS(/MS)]. Some of these LC-MS based multi-toxin methods can be used for routine monitoring purposes [21-24]. One of the drawbacks of LC-MS/MS methods is their sensitivity to matrix effects. Matrix effects can lead to an under- or overestimation of the concentration present in shellfish. To remove or reduce these matrix effects several clean up methods have been developed [25, 26]. In this paper we present the results of a in-house validation study of our recently published LC-MS/MS method [24]. The effect of solid phase extraction (SPE) for sample clean up was also studied and included in the validation.

Table 5.1 Current permitted levels in EU legislation and levels proposed by EFSA.

Toxin	Legislation (μg/kg)	EFSA opinion (μg/kg)
OA and DTXs	160[1]	45
PTXs		120
YTXs	1000	3750
AZAs	160	32

[1)] Including pectenotoxins.

EXPERIMENTAL

Reagents and standards

Water was deionized and passed through a Milli-Q water purification system (Millipore, Billerica, MA, USA). Acetonitrile (HPLC supra gradient) and methanol (absolute, HPLC grade) were purchased from Biosolve, Valkenswaard, The Netherlands. Ammonium hydroxide (25%) and hydrochloric acid (37%) were purchased from VWR international, Amsterdam, The Netherlands. Sodium hydroxide was purchased from Merck, Darmstadt, Germany. The certified reference materials (CRM) okadaic acid (OA) (CRM-OA-b 24.1±0.8 μg/ml), yessotoxin (YTX) (CRM-YTX 5.3±0.3 μg/ml), azaspiracid-1 (AZA1) (CRM-AZA1 1.24±0.07 μg/ml), pectenotoxin-2 (PTX2) (CRM-PTX2 8.6±0.3 μg/ml) and 13-desmethyl spirolide C (SPX1) (CRM-SPX1 7.0±0.4 μg/ml) and MusB (CRM-MusB 10.1 μg/g OA) were purchased from the National Research Council, Institute for Marine Biosciences (NRC-CNRC), Halifax, Canada.

Preparation of extracts

Homogenates of blank mussels (*Mytilus edulis*), oysters (*Crassosrea gigas*), cockles (*Cerastoderma edule*) and ensis (*Ensis directus)* were prepared by homogenizing 100 g of whole flesh tissue with a T25 Ultra Turrax mixer at 24 000 rpm (IKA® Works Inc., Wilmington, NC, USA). One gram of shellfish homogenate was extracted in triplicate with 3 ml methanol. After each addition of methanol the extract was Vortex-mixed during 1 minute. After Vortex-mixing the extract was centrifuged 5 min at 2 000 × *g*. The supernatant was transferred to a volumetric flask of 10 ml and after the third extraction the volume was made up to 10 ml with methanol. The crude shellfish extract was filtered through a HT tuffryn 0.2 μm membrane filter (Pall Corp., East Hills, NY, USA) prior to spiking.

Determination of extraction efficiency

To determine the extraction efficiency samples naturally contaminated with OA, DTX2 and DTX3 and a sample with OA, DTX2 and AZA1, -2 and -3 were extracted in duplicate. The homogenate (1 g) was extracted four times with 3 ml methanol. After each extraction the supernatant was transferred to a volumetric flask of 10 ml and volume was made up to 10 ml with methanol. After the four methanol

extractions a fifth extraction was performed with 3 ml acetone. The acetone extract was evaporated to dryness and reconstituted in methanol. From each extraction step the relative amount of toxin transferred was calculated. Furthermore, for the extraction efficiency six CRM MusB samples containing 10.1 μg/g OA and six blank shellfish samples spiked at 0.5 times the permitted level (PL) with YTX, AZA1, PTX2 and SPX1 were extracted is the same way as described in the paragraph about the preparation of extracts.

Preparation of matrix matched standards

A mixed standard stock solution containing 320 ng/ml OA, AZA1 and PTX2 and 2 000 ng/ml YTX and 800 ng/ml SPX1 was prepared in methanol. Matrix matched standards (MMS) were used to construct a calibration curve. Blank extracts (1.8 ml) were spiked with respectively 0, 25, 50, 100 and 150 μl mixed stock solution, representing 0, 0.25, 0.5, 1 and 1.5 × PL (Table 5.1). For SPX1 no permitted level has been established yet. Therefore, in this study a concentration of 400 μg/kg was chosen as the target level. The total volume of each extract was adjusted to 2 ml with methanol.

Solid phase extraction (SPE) clean up

The SPE procedure was carried out as described by Gerssen et. al [25]. Strata-X cartridges, 30 mg 1 ml (Phenomenex, Torrance, CA, USA) were conditioned and equilibrated using 1 ml of methanol and methanol/water (30:70 v/v), respectively. The methanolic shellfish extracts (1.2 ml) were diluted with 2.8 ml water. After loading the 4 ml of diluted extract on the cartridge, the cartridge was washed with 1 ml methanol/water (20:80 v/v). Finally, the toxins were eluted from the cartridge with 1.2 ml methanol containing 0.3% v/v of a 25% ammonium hydroxide solution in water.

Preparation of extracts for determination of the performance characteristics

Blank mussel and oyster extracts, different than the ones used for the MMS, were spiked. The extract (1.8 ml) was spiked with 50, 100 and 150 μl (0.5, 1, 1.5 × PL, respectively) of the mixed standard stock solution. The total volume was made up to 2 ml by adding 150, 100 and 50 μl methanol, respectively. After spiking, an

aliquot (1.2 ml) of the extract was purified with solid phase extraction (SPE) before analysis. The remainder of the extract was analyzed without further clean up. On a separate occasion eight different shellfish extracts (two mussels, two oysters, two cockles, two ensis) were prepared and spiked at 0.5 × PL to determine the inter-species repeatability.

Hydrolysis

To determine the amount of esters of OA, DTX1 and DTX2 present in the shellfish sample alkaline hydrolysis can be performed [27]. However, as no esterfied standards of OA and DTX were available, the performance of the method was tested by subjecting OA to alkaline hydrolysis conditions. For the validation of hydrolysis an MMS as well as spikes containing OA were prepared at 0, 0.25, 0.5, 1 and 1.5 times the current PL (Table 5.1). In a test tube, 250 µl of 2.5 M sodium hydroxide solution was added to 2 ml spiked extract. The closed tube was mixed and placed in a water bath at 76°C. After 45 min the hydrolyzed extract was cooled to room temperature and neutralized with 250 µl 2.5 M hydrochloric acid. To check for evaporation of methanol during heating of the test tubes, these were weighed before and after hydrolysis (n=20). An aliquot of 1.2 ml was purified with SPE before analysis the remainder was analyzed without further purification.

LC-MS/MS analysis

Chromatographic separation was achieved using a Shimadzu HPLC system (Shimadzu, 's-Hertogenbosch, The Netherlands) consisting of a degasser (DGU-20A^3), a binary pump system (LC20-AD), an autosampler (SIL-HTc) and a column oven (CTO-20A). Separation was achieved on a Waters XBridge C_{18} (150 × 3 mm, 5 µm) column. Mobile phase A was water and B was acetonitrile/water (90:10 v/v), both containing 6.7 mM ammonium hydroxide (pH = 11). A flow rate of 0.4 ml/min was used. The gradient started at 10% B. This composition was kept for 1 min and was the changed linearly in 9 min to 90% B. The mobile phase composition was kept at 90% B for 3 min and returned to 10% B in 2 min. An equilibration time of 4 min was allowed before the next injection. An injection volume of 10 µl was used and the column temperature was kept at 40°C. Mass spectrometric detection was performed using a Mircromass Quattro Ultima tandem

mass spectrometer (Waters-Micromass, Manchester, UK) equipped with an electrospray ionization interface (ESI). The mass spectrometer was operated in both negative and positive ESI. In both modes a capillary voltage of 2.8 kV, a desolvation gas temperature of 350°C at a N_2 flow of 600 l/h, a source temperature of 120°C and a nebulizer gas (N_2) flow of 100 l/h were used. Argon was used as collision-induced dissociation (CID) gas at a pressure of 2.5×10^{-3} mbar. The cone and collision energy were optimized for each toxin. Two product ions were selected for each toxin, to allow quantification as well as identification of the specific toxin: OA *m/z* 803.5 > 255.2 [cone voltage (CV): 60 V, collision energy (CE): 45 eV], OA *m/z* 803.5 > 113.1 (CV: 60 V, CE: 50 eV), YTX *m/z* 570.4 > 467.4 (CV: 75 V, CE: 30 eV), YTX *m/z* 570.4 > 396.4 (CV: 75 V, CE: 30 eV), AZA1 *m/z* 842.5 > 824.5 (CV: 35 V, CE: 30 eV), AZA1 *m/z* 842.5 > 672.4 (CV: 35 V, CE: 40 eV), PTX2 *m/z* 876.5 > 823.5 (CV: 40 V, CE: 30 eV), PTX2 *m/z* 876.5 > 213.1 (CV: 40 V, CE: 30 eV), SPX1 *m/z* 692.5 > 444.2 (CV: 40 V, CE: 40 eV) and SPX1 *m/z* 692.5 > 164.3 (CV: 40 V, CE: 50 eV).

Validation parameters investigated

The method was validated using the EU Commission Decision 2002/657/EC as guideline. Seven replicates, at each of the three spiked levels (0.5, 1 and 1.5 × PL), were analyzed. Analysis was carried out on three separate occasions using two different types of blank shellfish extract (day 1 oyster, day 2 mussel and day 3 mussel). In this study the accuracy, intra-day precision (repeatability), inter-day precision (within-lab reproducibility), linearity, decision limit (CC_α), specificity and ruggedness were determined.

RESULTS AND DISCUSSION

EU legislation demands that the validation of an alternative method for marine toxins should be carried out according to an internationally recognized protocol [28]. Commission Decision 2002/657/EC describes the performance characteristics of analytical methods for so called group A and B substances in products of animal origin [29]. As mentioned in Council Directive 1996/23/EC group B substances comprises compounds such as veterinary drugs, environmental contaminants and mycotoxins [30]. Therefore, we decided to use Commission

Decision 2002/657/EC as the basis for the validation of the lipophilic marine toxins analytical method.

Extraction efficiency

In former validation studies an important aspect was the consequent use of methanolic solutions of toxins for the construction of calibration curves [22, 23]. We have recently shown that significant matrix effects can be observed in shellfish extracts and that one of the ways to compensate for these effects is to use matrix matched standards [25]. In order to save valuable toxin standards, it is preferred to add toxin standards to methanolic extracts rather than to the shellfish homogenate itself. Spiking to extracts is justified when the extraction efficiency is very high (>90%) for all relevant toxins. To determine the extraction efficiency two naturally contaminated shellfish samples were extracted with methanol (4 ×) and acetone (1 ×). As can be seen in figure 5.1, after 3 methanol extractions more than 90% of the toxin content was extracted. Even the more lipophilic OA and DTX esters were extracted with over 90% efficiency. Furthermore, when the CRM MusB material was subjected to the normal procedure of 3 extractions with 3 ml methanol the recovery of OA was 97.2±5.1% (n=6). Extraction of six different matrices [mussel, cockles, ensis and oyster (n=6)] spiked with YTX, AZA1, PTX2 and SPX1 at 0.5 × PL resulted in average recoveries of 93.5%, 97.0%, 93.2% and 96.9%, respectively.

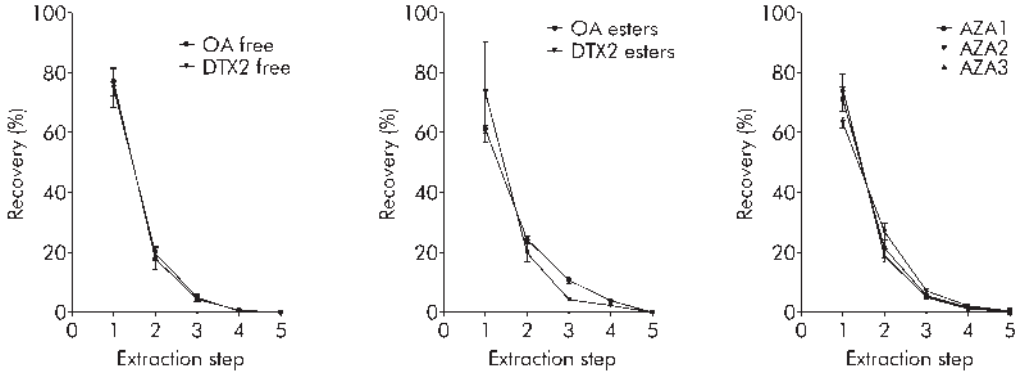

Figure 5.1 Repeated extraction from shellfish to investigate the toxin extraction efficiency. Methanol was used for the first four extractions, acetone for the final extraction.

Therefore, it is very unlikely that spiking of extracts will lead to false negatives. In the validation study extracts were analyzed with and without SPE purification in order to determine the effect of an additional clean up step on the performance parameters of the method.

Accuracy

The accuracy of the method has been determined instead of the trueness, because no certified incurred materials at the regulatory limit are available. The accuracy was determined by comparing the amount of toxin spiked to the extract with the amount of toxin found. Decision 2002/657/EC points out that the accuracy of a method with analyte levels above 10 μg/kg should be between 80-110%. Overall, good accuracies were obtained (Table 5.3); only in a few instances slightly elevated accuracies were obtained. The lowest accuracy obtained was 94% for YTX spiked at 1.5 × PL and analyzed after SPE clean up. The highest accuracy found was 119% for PTX2 spiked at 1.5 × PL in the crude extract. Consequently, with respect to accuracy it was concluded that SPE clean up resulted only in a modest improvement.

Intra-day repeatability (RSD_r)

The intra-day repeatability (RSD_r) of the crude extracts as well as that of the cleaned extracts was good (Table 5.3). The repeatability for the samples analyzed without SPE clean up varied between 2.5% for AZA1 spiked at 1 × PL and YTX spiked at 1.5 × PL and 12.0% for PTX2 spiked at 0.5 × PL. For samples analyzed after SPE the RSD_r varied between 3.3% for YTX spiked at 1.5 × PL and 10.7% for SPX1 spiked at 1 × PL. Overall, the repeatability for most of the toxins in the crude extracts was somewhat better than that of the SPE cleaned extracts. For the hydrolyzed extracts containing OA the repeatability was somewhat better when SPE clean up was applied. To check if the higher RSD_r of the crude hydrolyzed extracts was caused by evaporation of the methanol during heating, the weight of the test tubes before and after hydrolysis was recorded. The loss in weight was 0.14±0.08% (n=20), which is negligible. It was anticipated that purification of the extracts would result in an improved repeatability. However, this was not shown. One possible explanation is that SPE clean up introduces an extra error (variation

in recovery of the SPE) in the results. This recovery error would more or less counterbalance the positive effect of the SPE on the system performance which will be discussed in the paragraph about linearity.

To investigate whether inter-species differences between the various shellfish matrices play a role, eight different shellfish extracts (two mussels, two oysters, two cockles and two ensis), originating from different regions and sampled on two different occasions (March and June 2009) were analyzed after spiking at 0.5 × PL (Table 5.2). These shellfish extracts were quantified against an MMS calibration curve prepared from an unrelated blank mussel extract. The repeatability obtained was good with an average RSD_r of 5.4% for the crude extracts and 5.1% for the SPE cleaned extracts. The poorest repeatability between the species was found for OA in the crude extract (RSD_r of 6.9%) and for OA in cleaned extract (RSD_r of 6.5%). This experiment shows that effects of inter-species and inter-season differences are relatively small. This also means that a set of MMS prepared in a particular shellfish extract can be used without problems for other shellfish matrices.

Table 5.2 Accuracy and repeatability of crude extracts of various shellfish species spiked at 0.5 PL (n=1).

		Average concentration found (µg/kg)				
Sampled	Sample	OA	YTX	AZA1	PTX2	SPX1
March	Mussel	84.1	523.5	80.8	78.0	218.5
	Oysters	79.7	512.9	77.1	76.2	204.7
	Ensis	83.8	498.6	73.6	82.3	208.1
	Cockle	91.2	527.1	82.8	84.6	203.9
June	Mussel	92.9	517.9	78.3	85.5	237.9
	Oysters	95.4	501.8	82.0	82.4	196.0
	Ensis	84.2	518.6	83.2	75.5	204.4
	Cockle	79.9	521.9	72.8	72.7	193.3
	RSD_r (%)	6.9	2.0	5.2	5.9	6.8
	Accuracy (%)	108.0	103.1	98.5	99.6	104.2

Table 5.3 Multiple day validation results for the analysis of lipophilic marine biotoxins in shellfish (mussel and oyster) (n=21).

		Fortification level µg/kg	Accuracy %	RSD_r %	RSD_R %	CC_α[1] µg/kg	Linearity[2]
OA	Without SPE	80	103	7.2	10.1		
		160	103	4.6	12.4	194	0.997-1.000
		240	101	3.7	15.6		
	With SPE	80	111	7.4	8.5		
		160	98	7.2	9.1	184	0.995-0.999
		240	96	6.3	7.5		
	Hydrolysis, without SPE	80	99	8.4	9.0		
		160	106	6.7	14.2	200	0.993-0.998
		240	106	4.8	11.0		
	Hydrolysis, with SPE	80	103	4.6	4.9		
		160	100	5.9	6.1	176	0.992-1.000
		240	98	3.7	6.6		
YTX	Without SPE	500	100	3.7	7.2		
		1000	102	2.8	10.4	1175	0.998-1.000
		1500	102	2.5	12.0		
	With SPE	500	106	4.5	4.6		
		1000	98	4.8	6.6	1106	0.999-1.000
		1500	94	3.3	3.8		

AZA1	Without SPE	80	102	5.4	7.0		
		160	110	2.5	4.7	173	0.995-1.000
		240	113	2.9	7.3		
	With SPE	80	102	3.5	3.6		
		160	100	7.6	8.5	182	0.999-1.000
		240	99	4.0	4.6		
PTX2	Without SPE	80	95	12.0	17.5		
		160	111	5.4	5.6	176	0.996-1.000
		240	119	3.6	3.7		
	With SPE	80	103	6.8	7.4		
		160	104	7.6	8.3	182	0.999-1.000
		240	104	6.0	7.0		
SPX1	Without SPE	200	106	4.3	6.0		
		400	108	2.7	8.5	460	0.999-1.000
		600	109	2.5	11.8		
	With SPE	200	106	7.1	7.3		
		400	97	10.7	10.8	469	0.996-1.000
		600	96	6.7	7.8		

[1)] At 95% confidence interval.
[2)] Minimum-maximum correlation obtained from the MMS series analyzed before and after the sample extracts.

Within-laboratory reproducibility (RSD_R)

The within-laboratory reproducibility (RSD_R) was good for all toxins analyzed (Table 5.3). The highest RSD_R was obtained for PTX2 (17.6%) analyzed at 0.5 × PL in crude extract. After SPE purification this improved to a RSD_R of 9.9% (Table 5.3). The lowest RSD_R was obtained for AZA1 (4.6%) at 1.5 × PL analyzed after SPE clean up. In general, the RSD_R were better in the SPE cleaned extracts, especially for OA and YTX, which are recorded in ESI negative mode.

The within-laboratory reproducibility can also be expressed as HorRat values [31], which can be calculated using the following equation:

$$HorRat_R = RSD_R \text{ (obtained)} / RSD_R \text{ (calculated)}$$

in which the RSD_R (obtained) is the relative standard deviation of the measured reproducibility and RSD_R (calculated) is the relative standard deviation of the precision calculated by the Horwitz equation [31]:

$$RSD_R = 2^{(1-0.5 \log C)}$$

in which C is the concentration of the toxin expressed in g/g sample. The within-laboratory reproducibility is considered as acceptable when the HorRat value is <2. Below 1.5 it is considered as good and below 1 as excellent. From figure 5.2 it can be concluded that the within-laboratory reproducibility was excellent for all extracts analyzed. The highest HorRat value was obtained for PTX2 (17.6%, HorRat = 0.8) analyzed at 0.5 × PL in the crude extract. As already mentioned above, the RSD_R is on average is slightly better for the extracts cleaned by SPE (average HorRat of 0.4±0.1) than for the crude extracts (average HorRat of 0.5±0.2).

Linearity

MMS calibration was run at the beginning and at the end of each series and the linearity of both curves was calculated by the least-squares method. Linearity was considered acceptable when the correlation was at least 0.990. For all MMS series analyzed in the crude and purified extracts the correlation was good [0.992 or higher (Table 5.3)]. No internal standards are available that can be used to correct for changes in sensitivity during analysis. Therefore, a more or less constant

sensitivity during the analytical series is very important. This was checked by combining the MMS series before and after the sample extracts into one calibration curve. The correlation coefficient of this calibration curve should be 0.990 or better. This was the case for the samples which were purified with SPE. For these series combined calibration curves could be constructed with correlations ≥ 0.990 for all toxins analyzed, indicating that a loss or change in sensitivity during the series is not a serious problem (max 22% for YTX). In contrast, in the crude extracts all toxins except YTX (21%) suffered from a drift in sensitivity by more than 25% (max 137.2% for PTX2), resulting in combined calibration curves with a correlation of less than 0.990 (0.854 for PTX2). It is clear that the stability of the LC-MS/MS system remained more constant over a longer period of time when cleaned extracts were injected. This is especially important when long series have to be run. Alternatively, the series can be kept short, or a control sample should be included that is analyzed at regular intervals during the series.

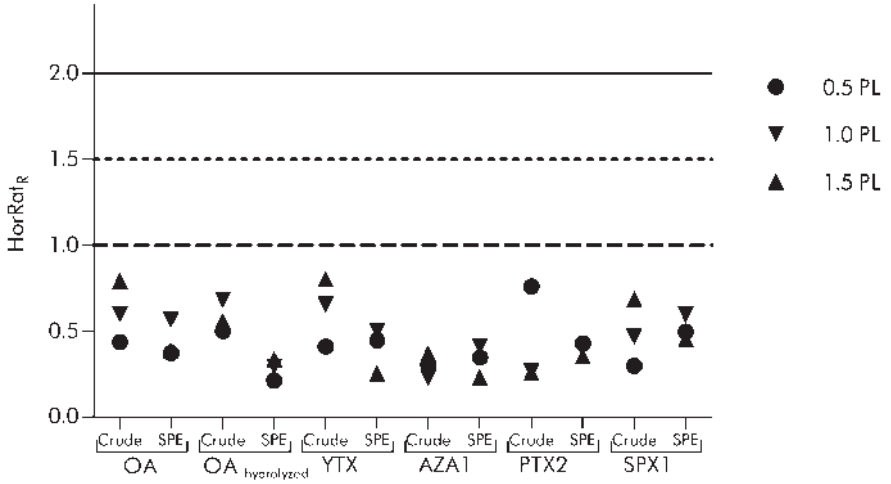

Figure 5.2 HorRat values obtained for the analysis of the various lipophilic marine toxins in crude shellfish extract and in SPE-purified shellfish extract.

Decision limit CC_α

Twenty samples were fortified with the various toxins at the permitted levels and analyzed. For these samples the within-laboratory reproducibility standard deviation (SD_R) was calculated. CC_α can be determined using the equation:

$$CC_\alpha = PL + t \times SD_R$$

in which PL is the permitted level for the toxins in μg/kg, *t* is 1.64 from a *one*-tailed *t*-distribution with *P*=0.05 (with an infinite number degrees of freedom). If the concentration of a toxin in a sample is found at or above the CC_α it can be concluded with a probability of 1-α or 95% (α = 5%) that the sample is above the PL and thus non-compliant. No significant differences were obtained for the CC_α between the crude extracts and the SPE purified extracts, except for YTX and hydrolyzed OA. For these toxins SPE clean up resulted in a lower CC_α (Table 5.3).

Specificity

Twenty one different blank samples (seven mussels, four oysters, eight cockles, two ensis) were analyzed to determine if interfering peaks were present in the selected mass traces representing the different toxins. In none of the analyzed samples interfering responses were detected (Fig. 5.3).

Ruggedness

The ruggedness of the method for small variations which can accidently happen within a laboratory was tested. No major changes (different MS settings etc) were investigated. The Vortex mixing time was extended from one min to two min; the speed of the centrifuge was increased from 2 000 to 2 500 × *g* and with SPE the cartridges were eluted by an extra minute. For the ruggedness, the results obtained should be within the standard deviation of the within-laboratory reproducibility. For all toxins tested under the mentioned conditions the method performed within this limit.

Single-day validation of OA and AZA1 at EFSA proposed levels

EFSA has recently proposed new PLs for most of the lipophilic marine toxins. For OA and AZA1 the proposed safety levels are much lower (45 μg/kg for OA and

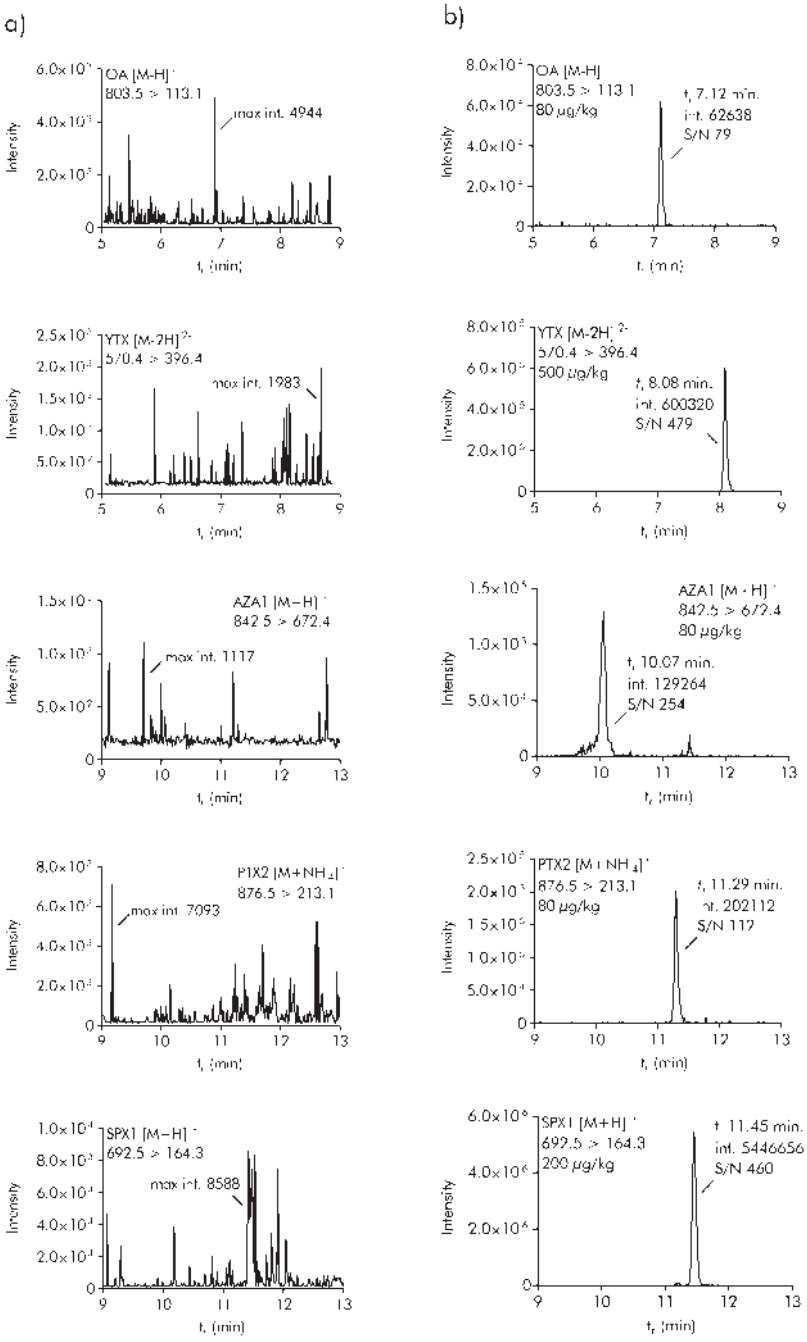

Figure 5.3 LC-MS/MS chromatograms of selected transitions (weakest transition shown) of a) a blank mussel extract and b) a blank mussel extract spiked with OA, YTX, AZA1, PTX2 and SPX1 at 0.5 PL.

30 μg/kg for AZA1) than the current PLs. With regard to the PTXs, the proposed PL of 120 μg/kg falls within the range of the validation performed (80, 160, 240 μg/kg). As EFSA has suggested to increase the YTX PL from 1 000 μg/kg to 3 750 μg/kg, it was considered less important to determine the performance of the method at this higher concentration. In order to determine if the method for OA and AZA1 also performs well at the levels proposed by EFSA an additional single-day validation was carried out (Table 5.4). The performance characteristics obtained for OA and AZA1 at the low levels were good with regard to accuracy, repeatability and sensitivity (Table 5.4). Therefore, if in the future the EU would decide to lower the PLs for OA and AZA1, this method is capable of analyzing these toxins with a high degree of confidence.

Application of the method to the routine monitoring programme in The Netherlands
During 2007 and 2008 a total of 623 shellfish samples that were collected in the Dutch monitoring program were analyzed by the rat bioassay as well as by the LC-MS/MS method. The monitoring included 491 mussel (*M. edulis*), 43 oyster (*C. Gigas*), 41 cockle (*C. edule)* and 48 ensis (*E. directus)* samples. All samples gave

Table 5.4 Single-day validation results obtained for the analysis of lipophilic marine biotoxins in shellfish (mussel) at the levels proposed by the European Food Safety Authority (n=7) [1].

		Fortification level μg/kg	Accuracy %	RSD_r %	CC_α μg/kg	Linearity[2]
OA	Without SPE	22.5	96	10.1		
		45	113	6.1	53.3	0.998-1.000
		67.5	108	5.4		
AZA1	With SPE	16	107	2.1		
		32	106	2.7	34.5	0.999-1.000
		48	104	1.2		

[1)] The validation was conducted before the document for the AZAs was published. The proposed permitted level for AZA (30 μg/kg) slightly differs from the level chosen in this study (32 μg/kg).
[2)] Minimum-maximum correlation calculated from the MMS series analyzed.

negative results with the rat bioassay. With LC-MS/MS low levels of OA were detected in 37 mussel samples from the Wadden Sea during the 2007 season. These levels were above the limit of detection (LOD) of 1.9 μg/kg [signal-to-noise (S/N) of 3 for the strongest transition]. In figure 5.4 the maximum concentrations found in the specific areas are given. OA concentrations found above the limit of quantitation (LOQ) [S/N=6 (16.4 μg/kg) for the weakest transition] ranged from 18.2 μg/kg till 67.5 μg/kg okadaic acid. These concentrations were well below the current PL but some exceed the safety levels proposed by EFSA. These results indicate that in case the regulatory limits are lowered, animal tests such as the rat bioassay will lack sensitivity to meet these limits. Spirolides (SPX1) were the only other toxins that were found in the Dutch shellfish harvesting areas. In 2007 and 2008 SPX1 was detected in 15 mussel samples above the LOQ (1.6 μg/kg), ranging in concentrations from 2.3 to 9.6 μg/kg.

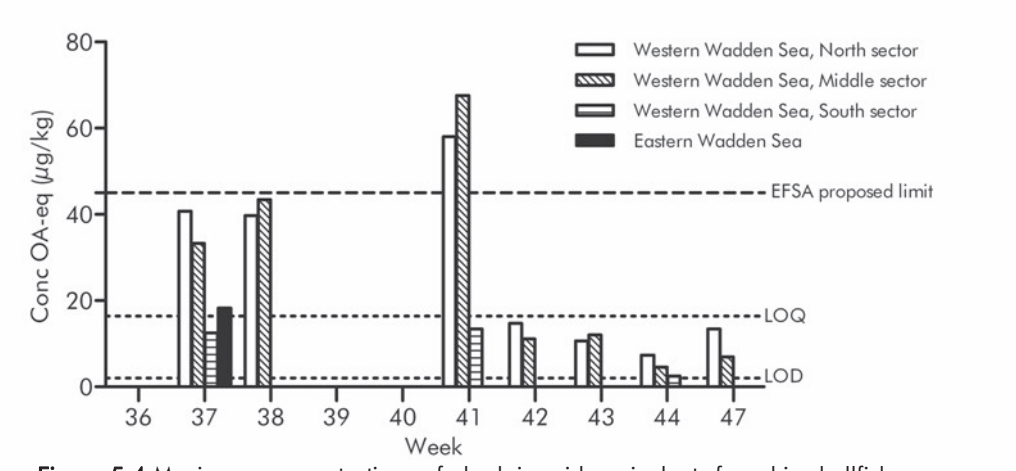

Figure 5.4 Maximum concentrations of okadaic acid equivalents found in shellfish (mussels) taken from production area's in the Dutch Wadden Sea in 2007.

CONCLUSION

A recently developed LC-MS/MS method was validated, both in combination with and without SPE purification using the European Commission Decision 2002/657/EC as guideline. MMS was used instead of spiking standards in methanol to construct calibration curves. The use of MMS largely eliminates matrix effects (ion suppression/enhancement).

The method performed very well for the parameters investigated. Only minor differences were observed between the crude extract and SPE purified extract. The largest difference observed was the change in sensitivity that occurred during analysis of the crude extracts. For longer series (>20 samples) it is advised to incorporate an SPE clean up step, although this will lead to a more time consuming method. Furthermore, it was shown that matrix matched standards in blank mussel extracts can be used to quantify other matrices such as oyster, cockle and ensis. The species differences did not have a significant effect on the method. The validated method also performed well at low concentrations for OA and AZA1. Therefore, we recommend the use of this method for the analysis of lipophilic marine toxins instead of the currently used, less sensitive and animal unfriendly mouse and rat bioassays.

ACKNOWLEDGEMENTS

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REFERENCES

[1] Gerssen A, Pol-Hofstad IE, Poelman M, Mulder PPJ, van den Top HJ, de Boer J. Marine toxins: Chemistry, toxicity, occurence and detection, with special reference to the Dutch situation. *Toxins* 2010; **2**: 878.

[2] Cembella AD, Lewis NI, Quilliam MA. The marine dinoflagellate *Alexandrium ostenfeldii* (*Dinophyceae*) as the causative organism of spirolide shellfish toxins. *Phycologia* 2000; **39**: 67.

[3] Rundberget T, Sandvik M, Larsen K, Pizarro GM, Reguera B, Castberg T, Gustad E, Loader JI, Rise F, Wilkins AL, Miles CO. Extraction of microalgal toxins by large-scale pumping of seawater in Spain and Norway, and isolation of okadaic acid and dinophysistoxin-2. *Toxicon* 2007; **50**: 960.

[4] Ciminiello P, Dell'Aversano C, Fattorusso E, Forini M, Magno S, Guerrini F, Pistocchi R, Boni L. Complex yessotoxins profile in *Protoceratium reticulatum* from north-western Adriatic sea revealed by LC-MS analysis. *Toxicon* 2003; **42**: 7.

[5] James KJ, Saez MJF, Furey A, Lehane M. Azaspiracid poisoning, the food-borne illness associated with shellfish consumption. *Food Addit Contam A* 2004; **21**: 879.

[6] Klontz KC, Abraham A, Plakas SM, Dickey RW. Mussel-associated azaspiracid intoxication in the United States. *Ann Intern Med* 2009; **150**: 361.

[7] Yasumoto T, Murata M, Oshima Y, Matsumoto GK, Clardy J, Diarrhetic shellfish poisoning. In *Seafood toxins : based on a symposium at the 186th meeting of the American Chemical Society, Washington, D.C., August 28 - September 2, 1983*, Ragelis EP, Ed. ACS: Washington, 1984; 207.

[8] James KJ, Lehane M, Moroney C, Fernandez-Puente P, Satake M, Yasumoto T, Furey A. Azaspiracid shellfish poisoning: unusual toxin dynamics in shellfish and the increased risk of acute human intoxications. *Food Addit Contam A* 2002; **19**: 555.

[9] Paz B, Daranas AH, Norte M, Riobo P, Franco JM, Fernandez JJ. Yessotoxins, a group of marine polyether toxins: an overview. *Mar Drugs* 2008; **6**: 73.

[10] Richard DJA, Arsenault E, Cembella AD, Quilliam MA, Investigations into the toxicology and pharmacology of spirolides, a novel group of shellfish toxins. In *Proceedings of the 9th conference on Harmfull algal blooms*, Hallegraeff GM, Blackburn SI, Bolch CJ, Lewis RJ, Eds. Hobart, Australia, 2000; 383.

[11] Miles CO, Wilkins AL, Munday R, Dines MH, Hawkes AD, Briggs LR, Sandvik M, Jensen DJ, Cooney JM, Holland PT, Quilliam MA, MacKenzie AL, Beuzenberg V, Towers NR. Isolation of pectenotoxin-2 from *Dinophysis acuta* and its conversion to pectenotoxin-2 seco acid, and preliminary assessment of their acute toxicities. *Toxicon* 2004; **43**: 1.

[12] European Parliament, Council. Commission directive 2004/853/EC specific hygiene rules for food of animal origin. *Off J Eur Commun* 2004; **L226**: 22.

[13] Alexander J, Audunsson GA, Benford D, Cockburn A, Cradevi J-P, Dogliotti E, Domenico AD, Fernandez-Cruz ML, Fink-Gremmels J, Furst P, Galli C, Grandjean P, Gzyl J, Heinemeyer G, Johansson N, Mutti A, Schlatter J, van Leeuwen R, van Peteghem C, Verger P. Marine biotoxins in shellfish - okadaic acid and analogues. *The EFSA Journal* 2008; **589**: 1.

[14] Alexander J, Benford D, Cockburn A, Cradevi J-P, Dogliotti E, Domenico AD, Fernandez-Cruz ML, Fink-Gremmels J, Furst P, Galli C, Grandjean P, Gzyl J, Heinemeyer G, Johansson N, Mutti A, Schlatter J, van Leeuwen R, van Peteghem C, Verger P. Marine biotoxins in shellfish - azaspiracid group. *The EFSA Journal* 2008; **723**: 1.

[15] Alexander J, Benford D, Cockburn A, Cradevi J-P, Dogliotti E, Domenico AD, Fernandez-Cruz ML, Fink-Gremmels J, Furst P, Galli C, Grandjean P, Gzyl J, Heinemeyer G, Johansson N, Mutti A, Schlatter J, van Leeuwen R, van Peteghem C, Verger P. Marine biotoxins in shellfish - pectenotoxin group. *The EFSA Journal* 2009; **1109**: 1.

[16] Alexander J, Benford D, Cockburn A, Cradevi J-P, Dogliotti E, Domenico AD, Fernandez-Cruz ML, Fink-Gremmels J, Furst P, Galli C, Grandjean P, Gzyl J, Heinemeyer G, Johansson N, Mutti A, Schlatter J, van Leeuwen R, van Peteghem C, Verger P. Marine biotoxins in shellfish - yessotoxin group. *The EFSA Journal* 2008; **907**: 1.

[17] Chapela MJ, Reboreda A, Vieites JM, Cabado AG. Lipophilic toxins analyzed by liquid chromatography-mass spectrometry and comparison with mouse bioassay in fresh, frozen, and processed molluscs. J Agric Food Chem 2008; **56**: 8979.

[18] European Parliament, Council. Regulation (EC) no. 609/1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. *Off J Eur Commun* 1986; **L358**: 1.

[19] Vieytes MR, Fontal OI, Leira F, deSousa JMVB, Botana LM. A fluorescent microplate assay for diarrheic shellfish toxins. *Anal Biochem* 1997; **248**: 258.

[20] Briggs LR, Miles CO, Fitzgerald JM, Ross KM, Garthwaite I, Towers NR. Enzyme-linked immunosorbent assay for the detection of yessotoxin and its analogues. *J Agric Food Chem* 2004; **52**: 5836.

[21] Fux E, McMillan D, Bire R, Hess P. Development of an ultra-performance liquid chromatography-mass spectrometry method for the detection of lipophilic marine toxins. *J Chromatogr A* 2007; **1157**: 273.

[22] McNabb P, Selwood AI, Holland PT. Multiresidue method for determination of algal toxins in shellfish: Single-laboratory validation and interlaboratory study. *J AOAC Int* 2005; **88**: 761.

[23] Stobo LA, Lacaze JPCL, Scott AC, Gallacher S, Smith EA, Quilliam MA. Liquid chromatography with mass spectrometry - detection of lipophilic shellfish toxins. *J AOAC Int* 2005; **88**: 1371.

[24] Gerssen A, Mulder PPJ, McElhinney MA, De Boer J. Liquid chromatography - tandem mass spectrometry method for the detection of marine lipophilic toxins under alkaline conditions. *J Chromatogr A* 2009; **1216**: 1421.

[25] Gerssen A, McElhinney MA, Mulder PPJ, Bire R, Hess P, De Boer J. Solid Phase Extraction for the removal of matrix effects in lipophilic marine toxin analysis by liquid chromatography – tandem mass spectrometry. *Anal Bioanal Chem* 2009; **394**: 1213.

[26] Goto H, Igarashi T, Yamamoto M, Yasuda M, Sekiguchi R, Watai M, Tanno K, Yasumoto T. Quantitative determination of marine toxins associated with diarrhetic shellfish poisoning by liquid chromatography coupled with mass spectrometry. *J Chromatogr A* 2001; **907**: 181.

[27] Mountfort DO, Suzuki T, Truman P. Protein phosphatase inhibition assay adapted for determination of total DSP in contaminated mussels. *Toxicon* 2001; **39**: 383.

[28] European Parliament, Council. Laying down implementing measures for certain products under Regulation (EC) No 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No 854/2004 of the European Parliament and of the Council and Regulation (EC) No 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004. *Off J Eur Commun* 2005; **L338**: 40.

[29] European Parliament, Council. Regulation (EC) no. 657/2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Off J Eur Commun* 2002; **L221**: 8.

[30] European Parliament, Council. Regulation (EC) no. 23/1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664/EEC. *Off J Eur Commun* 1996; **L125**: 10.

[31] Horwitz W, Albert R. The Horwitz ratio (HorRat): A useful index of method performance with respect to precision. *J AOAC Int* 2006; **89**: 1095.

6

Screening of lipophilic marine toxins in shellfish and algea using liquid chromatography coupled to orbitrap mass spectrometry

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Submitted for publication

ABSTRACT

Most liquid chromatography (LC) mass spectrometry (MS) methods used for routine monitoring of lipophilic marine toxins focus on the analysis of the 13 toxins that are stated in European Union legislation. However, to date over 200 lipophilic marine toxins have been described in the literature. To fill this gap, a screening method using LC coupled to high resolution (HR) orbitrap MS (resolution 100 000) for marine lipophilic toxins has been developed. The method can detect a wide variety of okadaic acid (OA), yessotoxin (YTX), azaspiracid (AZA) and pectenotoxin (PTX) group toxins. To build a library of toxins, shellfish and algae samples with various toxin profiles were obtained from Norway, Ireland, United Kingdom, Portugal and Italy. Each sample extract was analyzed with and without collision induced dissociation fragmentation. Based on their mass and specific fragmentation pattern, 85 different toxins were identified comprising 33 OA, 26 YTX, 18 AZA and 8 PTX group toxins. A major complication of full scan HRMS is the huge amount of data generated (file size), which restricts the possibility of a fast search. A software program called *metAlign* was used to reduce the orbitrap MS data files. The 200-fold reduced data files were screened using an additional software tool for *metAlign*: 'Search_LCMS'. A search library was constructed for the 85 identified toxins. The library contains information about compound name, accurate mass, mass deviation (<5 ppm), retention time (min) and retention time deviation (<0.2 min). An important feature is that the library can easily be exchanged with other instruments as the generated *metAlign* files are not brand-specific. The developed screening procedure was tested by analysing a set of known positive and blank samples, processing them with *metAlign* and searching with Search_LCMS. A toxin profile was determined for each of the contaminated samples. No toxins were found in the blank sample, which is in line with the results obtained for this sample in the routine monitoring program (rat bioassay and LC-MS/MS).

INTRODUCTION

Consumption of filter feeding shellfish contaminated with lipophilic marine toxins can lead to intoxications in humans with signs such as abdominal cramps, vomiting and gastro-intestinal disorders. Lipophilic marine toxins are produced by various algae species such as *Dinophysis acuta*, *Azadinum spinosum* and *Protoceratium reticulatum* [1-3]. The group of lipophilic marine toxins consists of five different chemical groups: okadaic acid (OA) and its derivatives (Fig. 6.1a), yessotoxins (YTXs) (Fig. 6.1b), azaspiracids (AZAs) (Fig. 6.1c), pectenotoxins (PTXs) (Fig. 6.1d) and spirolides (SPXs) (Fig. 6.1e). In order to protect shellfish consumers, monitoring programs and legislation have been established in Europe for most of these toxin groups except for the spirolides for which no legislation has been established yet. Monitoring of these toxins in shellfish should officially be done with a mouse or rat bioassay [4]. Apart from the fact that these tests are nowadays considered unethical, the results are often of a poor precision as both the methods lack sensitivity and selectivity. Recently, several chemical methods based on liquid chromatography tandem mass spectrometry (LC-MS/MS) have been developed [5-8]. For quantitative analysis these LC-MS/MS methods have proven to provide excellent sensitivity and selectivity [5]. The main drawback of LC-MS/MS methods is the limited number of compounds that can be analyzed in a single run. LC-MS/MS is a target method and the target toxins should be selected before the run. In addition to its excellent sensitivity, selective fragmentation allows confirmation of a compound. The suit of toxins usually selected for LC-MS/MS analysis comprises the most abundant lipophilic marine toxins found in shellfish, which are stated in legislation (Table 6.1) [4]. Besides this select group of toxins to date a large number (>200) of lipophilic marine toxins have been described in the literature [9-13]. Many of these additional marine toxins may also be of toxicological relevance. In order to monitor all these toxins other, better suited LC-MS techniques are required, most notably, full scan MS with accurate mass measurement. It is possible to search in the recorded data multi-targeted for a theoretically unlimited number of compounds. Another important aspect of full scan MS techniques is the possibility to perform a retrospective search for newly described toxins. Full scan mass spectrometers that are currently available for LC-MS applications are either Time-of-Flight (ToF) or orbitrap-MS instruments. Compared to triple quadrupole

Table 6.1 Toxins that should be analyzed in routine monitoring programs according to EU legislation.

Toxin group	OA and DTXs	YTXs	AZAs	PTXs
	Okadaic acid	Yessotoxin	Azaspiracid-1	Pectenotoxin-1
	Dinophysistoxin-1	1a-homo yessotoxin	Azaspiracid-2	Pectenotoxin-2
	Dinophysistoxin-2	45OH yessotoxin	Azaspiracid-3	
	Dinophysistoxin-3 (7-O acyl esters)	45OH 1a-homo yessotoxin		

mass analyzers (MS/MS), ToF- and orbitrap-MS provide a much higher mass resolution and mass precision. A major drawback of the full scan mass spectrometers, apart from a somewhat lower sensitivity, is the amount and complexity of the data generated. Furthermore, software tools for fast library searching of marine toxins in shellfish have not yet been well developed. More in general, tools for data handling are often brand-specific and this seriously complicates/limits the exchangeability of libraries and data generated by different instruments. Therefore, a lipophilic marine toxin search library based on high resolution (HR) MS data was developed. Furthermore, the use of a generic library for the screening of lipophilic marine toxins in shellfish and algae is described and discussed.

EXPERIMENTAL

Chemicals and standards

Water was deionised and passed through a Milli-Q water purification system (Millipore, Billerica, MA, USA). Acetonitrile (HPLC supra gradient) and methanol (absolute, HPLC grade) were purchased from Biosolve, Valkenswaard, The Netherlands. Ammonium hydroxide (25%) was purchased from VWR International, Amsterdam, The Netherlands. OA (CRM-OA-c 14.3±1.5 μg/ml), yessotoxin (YTX) (CRM-YTX 5.3±0.3 μg/ml), azaspiracid-1 (AZA1) (CRM-AZA1 1.24±0.07 μg/ml) and pectenotoxin-2 (PTX2) (CRM-PTX2 8.6±0.3 μg/ml) were purchased from the National Research Council, Institute for Marine Biosciences (NRC-CNRC), Halifax, Canada. Laboratory Reference Material (LRM) extract containing OA, dinophysistoxin-1, -2 (DTX1, -2), YTX, AZA1-3 and PTX2; shellfish material

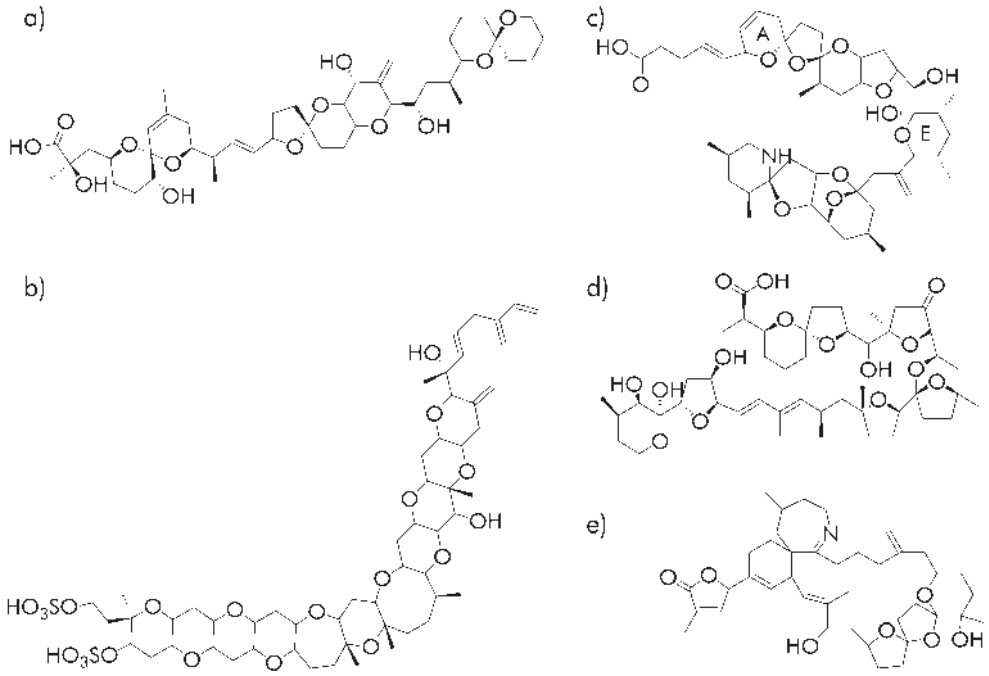

Figure 6.1 Chemical structure of a) okadaic acid, b) yessotoxin, c) azaspiracid-1, d) pectenotoxin-2sa, e) 13-desmethyl spirolide C.

containing OA-esters and shellfish material contaminated with AZAs were kindly donated by Dr. P. Hess from the Marine Institute, Oranmore, Ireland. Shellfish material contaminated with OA and DTXs was kindly donated by Dr. S. Morris from CEFAS, Weymouth, United Kingdom. Dried extracts of shellfish contaminated with OA and DTXs were kindly donated by Dr. P. Vale from Instituto de Investigação das Pescas e do Mar, Lisabon, Portugal. Algae pellets containing YTXs were kindly donated by Dr. C. Dell'Aversano from Dipartimento di Chimica delle Sostanze Naturali, Universitá degli Studi di Napoli "Federico II", Napoli, Italy. Shellfish and algae material contaminated with YTXs, *Dinophysis acuta* extracts and mussel extract containing PTXs were kindly donated by Dr. M. Sandvik, National Veterinary Institute, Oslo, Norway.

Preparation of extracts

Sample pre-treatment was adapted to the constitution in which the samples were presented. Intact or steam cooked shellfish material was homogenized with a T25 Ultra Turrax mixer at 24 000 rpm (IKA® Works Inc., Wilmington, NC, USA). One gram of shellfish homogenate was extracted in triplicate with 3 ml methanol. After each addition of methanol the extract was Vortex-mixed during 1 min. After Vortex-mixing the extract was centrifuged 5 min at 2 000 × *g*. The supernatant was transferred to a volumetric flask of 10 ml and after the third extraction the volume was adjusted to 10 ml with methanol. For algae pellets, 2 ml methanol was added and the toxins were extracted in an ultrasonic bath during 20 min (Branson, Danbury, CT, USA). Dried extracts were reconstituted in 2 ml methanol. All methanolic extracts were filtered through a 0.2 μm PTFE membrane filter (Minisart SRP4, Satorius Stedim Biotech GmbH, Goettingen, Germany) prior to analysis.

Liquid chromatography – mass spectrometry (LC-orbitrap-MS)

The LC system consisted of an Accela quartenary pump and an Acella autosampler including a column oven (Thermo Scientific, Bremen, Germany). For the separation of the toxins a Waters XBridge C_{18} (150 × 3 mm, 5 μm) column was used (Waters, Etten-leur, The Netherlands). The column was kept at a temperature of 40°C. Mobile phase A was water and B acetonitrile, both containing 6.7 mM ammonium hydroxide (pH = 11). A flow rate of 0.4 ml/min was used. A gradient was started at 30% B, was kept at this composition for 1 min and was then increased linearly to 100% B in 19 min. The mobile phase composition was kept at 100% B for 4 min and returned to 30% B in 1 min. An equilibration time of 5 min was allowed before the next injection. Of the methanolic extract 10 μl was injected.

MS detection was performed with an Exactive orbitrap (Thermo Scientific, Bremen, Germany) at an ultra high resolution of 100 000 [full width half maximum (FWHM)], resulting in a scan time of 1 sec. The orbitrap was equipped with a heated electrospray ionization interface (HESI). The mass spectrometer was operated in both negative and positive HESI (ESI^-, ESI^+) in separate runs. In both modes a spray voltage of 4 kV, a capillary temperature of 250°C and a heater temperature of 300°C was used. In ESI^- a capillary voltage of -95 V, tube lens voltage of -190 V and a skimmer voltage of -46 V was used. In ESI^+ a capillary

voltage of 47.5 V, a tube lens voltage of 90 V and a skimmer voltage of 22 V was used. Fragmentation took place in a high collision dissociation (HCD) cell. The HCD cell settings were optimized using OA, YTX, PTX2 and AZA1 standard solutions. In ESI^- an optimal collision energy of 65 eV was determined, in ESI^+ 50 eV. Prior to each sequence the instrument was calibrated, in ESI^- by infusion of a mixture of sodium dodecyl sulphate, taurocholic acid, Ultramark 1621 and acetic acid in acetonitrile/methanol/water (2:1:1 v/v/v) (Sigma Aldrich, Darmstadt, Germany). In ESI^+ the instrument was calibrated using a mixture of caffeine, MFRA (Met-Arg-Phe-Ala), Ultramark 1621 and acetic acid in acetonitrile/methanol/water (2:1:1 v/v/v) (Sigma Aldrich, Darmstadt, Germany). Each sample was analyzed in duplicate in ESI^-, ESI^+ and with and without HCD fragmentation.

Data processing

Data reduction of the orbitrap-MS data was done with the software package *metAlign* [14], which is freely available at http://www.metalign.nl/uk/. *MetAlign* reduced the file size by noise elimination and peak picking. Optimization of noise elimination, threshold and peak picking settings have been described earlier by Lommen *et al*. [14]. The following settings were used for noise elimination; mass resolution of 100 000, amplitude range for accurate mass determination from 20 000 to 999 999 999 counts, 'echo suppression' interval around the mass peak of 0.04 Da with a 5 percent amplitude of the mass peak, 'forest suppression'

Table 6.2a Part of the (comma separated values) Search_LCMS file for a targeted search in ESI^- on OA/DTX toxins.

Compound	*m/z*	Mass window (Da)	Retention time (min)	Retention time window (min)
Okadaic acid	803.4587	0.0040	8.4	0.2
Dinophysistoxin-1	817.4744	0.0041	9.9	0.2
Dinophysistoxin-2	803.4587	0.0040	8.9	0.2
16:0 OA	1041.6884	0.0052	18.8	0.2
16:0 DTX2	1041.6884	0.0052	19.0	0.2

Table 6.2b Part of the (comma separated values) Search_LCMS file for a untargeted search in ESI^- on OA/DTX toxins. Common fragments are included in the search.

Compound	*m/z*	Mass window (Da)
all	803.4587	0.0040
all	255.1238	0.0013
all	785.4482	0.0039

interval around the mass peak of 1 Da with a 3 percent amplitude of the mass peak and a 0 Da offset. A maximum peak amplitude of 10e8 was allowed, the threshold level was set at 10 000 counts and the average peak width at 10 scans. Sets of reduced files can be quickly scanned for large numbers of compounds using the newly developed add-on tool for *metAlign;* Search_LCMS. The search list [comma separated values list (CSV file)] (Table 6.2) used in this study contains all lipophilic marine toxins that were found in Scifinder Scholar. The CSV search list consists of the component name, component mass, mass tolerance (in Da), retention time and retention time tolerance (in min). It is also possible to perform an untargeted search by scanning for a specific mass throughout the whole chromatogram. In that case the component name should be 'all' and no retention time and retention time tolerance are defined (Table 6.2b). The allowed mass deviation in all cases was set at 5 ppm.

RESULTS AND DISCUSSION

Since the introduction of HR bench-top instruments, the definition of HRMS is under debate. For ToF-MS instruments high resolution is generally defined as 10 000 (FWHM) or higher, while for orbitrap-MS HR starts at 50 000 (FWHM). HR results in a better separation between two closely related masses. A second benefit of the

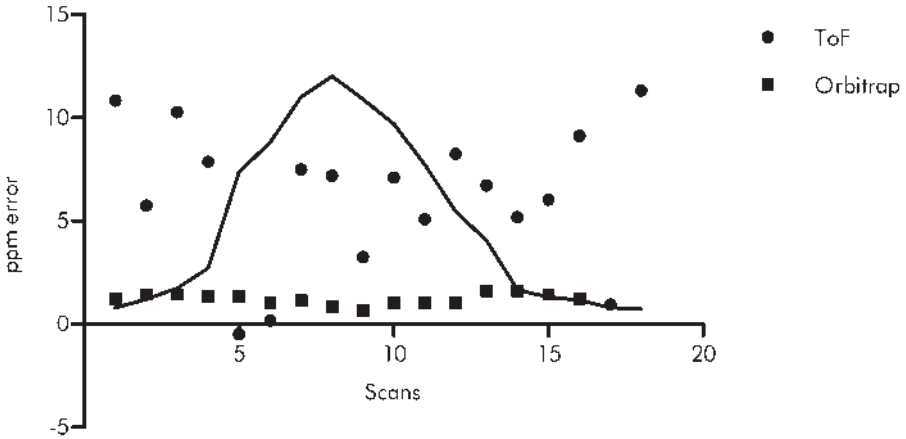

Figure 6.2 Mass error observed within individual scans for a chromatographic peak of AZA1 recorded by Time-of-Flight (ToF) and orbitrap MS.

orbitrap is the HCD cell which produces fragments with relatively small mass errors (<5 ppm). In the older generation ToF and QToF instruments in-source fragmentation can be used, but generally the mass errors for the produced fragments are quite high (up to 30 ppm) [15]. Drawback of the orbitrap is the relation between scan time and resolution. At a resolution of 100 000 the scan time is one sec. The scan rate for ToF-MS instruments is often 200 ms or less. This makes the orbitrap less suitable to detect the sharp peaks (<5 sec) that are generated under very high pressure chromatography (VHPLC) conditions. Furthermore, mass accuracy varies between instruments and this may complicate the identification of unknowns. A typical example is given in figure 6.2, in which the mass accuracy throughout the chromatographic peak has been recorded on a

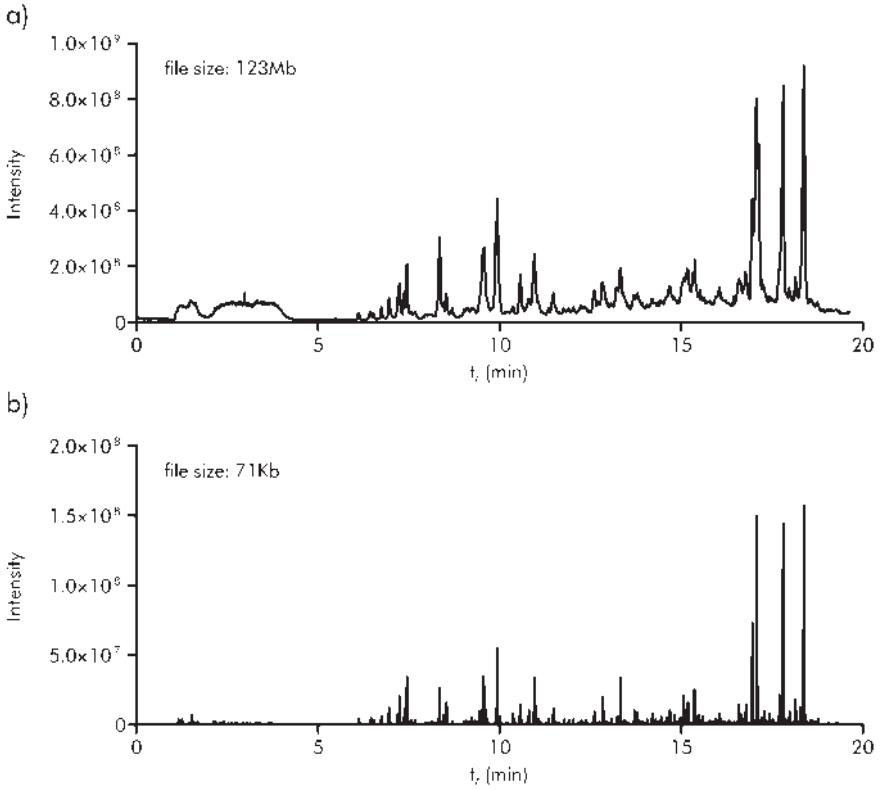

Figure 6.3 Full scan chromatogram of a) raw data file and b) *metAlign* reduced data file of a shellfish extract.

ToF and an orbitrap instrument. Mass accuracy is almost constant for an orbitrap (1 to 2 ppm) while for a ToF-MS instrument operating at a resolution of 10 000 the mass accuracy was shifting by more than 10 ppm. In the present study the orbitrap-MS was used to enable correct identification of the toxins and to build the database.

Data processing

A major complication of full scan MS is the amount of data generated. Per analysis this can be as much as 200 Mb, depending on runtime, scan speed and resolution. Storage and data handling becomes problematic for longer series. In most cases the software provided by the manufacturer is not equipped for adequate data reduction and does not offer fast search possibilities for screening. To overcome this limitation data files generated with the orbitrap have been data reduced by using *metAlign*. After applying *metAlign*, the data file size was reduced ca. 200-fold without the loss of any specific mass spectrometric information (Fig. 6.3). Search_LCMS was then used to perform a quick search (within seconds) on the relevant precursor masses and fragments.

Development of a lipophilic marine toxins library

For the creation of a search library it is important to identify the toxins correctly. Information on the elemental composition and molecular weight of the precursor alone is not sufficient to identify a compound unequivocally. Additional confirmation by means of characteristic fragments may assist in the identification of compounds. Therefore, the samples were analyzed with and without collision induced fragmentation. Toxins were identified by using the 'all' function in Search_LCMS for the specific fragments and precursor ions in, respectively, the data collected with and without HCD fragmentation. A toxin was added to the library when the mass accuracy of the parent ion was below 5 ppm and the fragmentation pattern included one or more fragments that are specific for OA, YTX, AZA or PTX. In this way in total 33 OA, 26 YTX, 18 AZA and 8 PTX group toxins were tentatively identified (Tables 6.3-6.6).

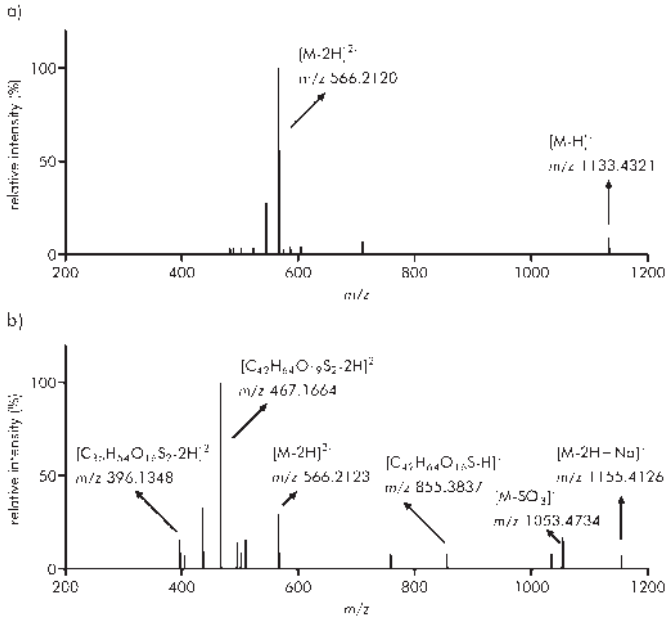

Figure 6.4 Mass spectra of an (unknown) yessotoxin ($C_{52}H_{78}O_{23}S_2$) eluting at 8.9 min recorded a) without fragmentation and b) with fragmentation at 65 eV.

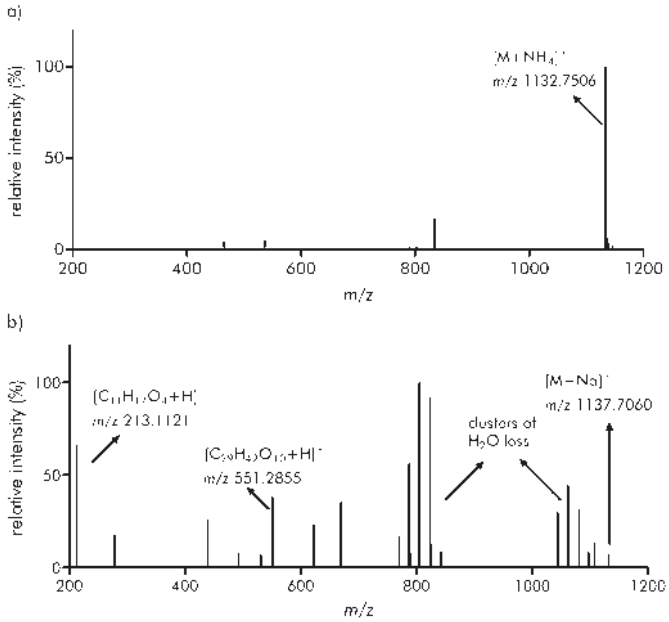

Figure 6.5 Mass spectra of 16:0 pectenotoxin-2sa ester recorded a) without fragmentation and b) with fragmentation at 50 eV.

Table 6.3 Overview of OA group toxins found in shellfish extracts and added to the search library.

Compound Name	t_r (min)	Elemental composition	$[M-H]^-$ *m/z* obs.	*m/z* calc.	Mass error (ppm)	Fragment[1] *m/z* obs.	Mass error (ppm)	Fragment[2] *m/z* obs.	Mass error (ppm)
OA	8.4	$C_{44}H_{68}O_{13}$	803.4606	803.4587	2.3	785.4498	2.0	255.1241	1.0
DTX2	8.9	$C_{44}H_{68}O_{13}$	803.4601	803.4587	1.6	785.4493	1.4	255.1240	0.6
DTX1	9.9	$C_{45}H_{70}O_{13}$	817.4757	817.4744	1.6			255.1239	0.3
14:0 OA	13.5	$C_{58}H_{94}O_{14}$	1013.6586	1013.6571	1.4	785.4495	1.7	255.1242	1.4
14:0 DTX2	13.9	$C_{58}H_{94}O_{14}$	1013.6586	1013.6571	1.5	785.4492	1.3	255.1236	-0.9
14:1 OA	15.6	$C_{58}H_{92}O_{14}$	1011.6428	1011.6415	1.4	785.4495	1.6		
14:1 DTX2	16.0	$C_{58}H_{92}O_{14}$	1011.6427	1011.6415	1.2	785.4487	0.7		
14:3 OA	17.0	$C_{58}H_{88}O_{14}$	1007.6112	1007.6102	1.0				
14:3 DTX2	17.4	$C_{58}H_{88}O_{14}$	1007.6119	1007.6102	1.8				
15:0 OA	17.9	$C_{59}H_{96}O_{14}$	1027.6740	1027.6728	1.2	785.4491	1.2	255.1234	-1.7
15:0 DTX2	18.2	$C_{59}H_{96}O_{14}$	1027.6736	1027.6728	0.8	785.4484	0.3	255.1235	-1.3
16:0 OA	16.1	$C_{60}H_{98}O_{14}$	1041.6890	1041.6884	0.5	785.4490	1.0	255.1235	-1.4
16:0 DTX2	16.4	$C_{60}H_{98}O_{14}$	1041.6892	1041.6884	0.8	785.4489	0.9	255.1236	-0.9
16:1 OA	17.3	$C_{60}H_{96}O_{14}$	1039.6739	1039.6728	1.1	785.4492	1.3	255.1237	-0.3
16:1 DTX2	17.6	$C_{60}H_{96}O_{14}$	1039.6725	1039.6728	-0.2	785.4495	1.7	255.1239	0.3
16:2 OA	18.8	$C_{60}H_{94}O_{14}$	1037.6589	1037.6571	1.7	785.4490	1.1	255.1221	-6.7
16:2 DTX2	19.0	$C_{60}H_{94}O_{14}$	1037.6588	1037.6571	1.6	785.4493	1.4	255.1237	-0.4

Table 6.3 continued.

Compound Name	t_r (min)	Elemental composition	$[M-H]^-$ *m/z* obs.	*m/z* calc.	Mass error (ppm)	Fragment[1] *m/z* obs.	Mass error (ppm)	Fragment[2] *m/z* obs.	Mass error (ppm)
17:1 OA	18.2	$C_{61}H_{98}O_{14}$	1053.6898	1053.6884	1.3	785.4495	1.7	255.1238	-0.3
17:1 DTX2	18.5	$C_{61}H_{98}O_{14}$	1053.6894	1053.6884	1.0	785.4494	1.5		
18:0 OA	15.7	$C_{62}H_{102}O_{14}$	1069.7205	1069.7197	0.7	785.4496	1.8		
18:0 DTX2	16.0	$C_{62}H_{102}O_{14}$	1069.7207	1069.7197	0.9	785.4497	2.0		
18:1 OA	16.6	$C_{62}H_{100}O_{14}$	1067.7046	1067.7041	0.5	785.4490	1.0	255.1237	-0.5
18:1 DTX2	16.9	$C_{62}H_{100}O_{14}$	1067.7047	1067.7041	0.6	785.4491	1.2	255.1239	0.4
18:2 OA	17.7	$C_{62}H_{98}O_{14}$	1065.6893	1065.6884	0.9	785.4492	1.3	255.1238	0.0
18:2 DTX2	18.0	$C_{62}H_{98}O_{14}$	1065.6906	1065.6884	2.1	785.4495	1.7		
18:3 OA	18.9	$C_{62}H_{96}O_{14}$	1063.6741	1063.6728	1.2	785.4491	1.2	255.1237	-0.4
18:3 DTX2	19.2	$C_{62}H_{96}O_{14}$	1063.6738	1063.6728	1.0	785.4482	0.1	255.1239	0.2
18:4 OA	20.2	$C_{62}H_{94}O_{14}$	1061.6587	1061.6571	1.5	785.4494	1.5	255.1238	-0.1
18:4 DTX2	20.5	$C_{62}H_{94}O_{14}$	1061.6588	1061.6571	1.6	785.4488	0.7	255.1237	-0.6
20:5 OA	16.4	$C_{64}H_{96}O_{14}$	1087.6740	1087.6728	1.1	785.4495	1.7	255.1236	-0.8
20:5 DTX2	16.7	$C_{64}H_{96}O_{14}$	1087.6740	1087.6728	1.1	785.4492	1.3	255.1240	0.5
22:6 OA	17.2	$C_{66}H_{98}O_{14}$	1113.6901	1113.6884	1.5	785.4492	1.3	255.1236	-0.9
22:6 DTX2	17.5	$C_{66}H_{98}O_{14}$	1113.6903	1113.6884	1.7	785.4490	1.1	255.1237	-0.5

[1] Fragment $[C_{44}H_{65}O_{12}\text{-}H]^-$ *m/z* 785.4482.
[2] Fragment $[C_{13}H_{20}O_5\text{-}H]^-$ *m/z* 255.1238.
Blank means not detected.

Table 6.4 Overview of YTX group toxins found in algae and shellfish extracts and added to the library.

Compound Name	t_r (min)	Elemental composition	$[M-H]^-$ *m/z* obs.	*m/z* calc.	Mass error (ppm)	$[M-2H]^{2-}$ *m/z* obs.	Mass error (ppm)	$[M-2H+Na]^-$ *m/z* obs.	Mass error (ppm)
Unknown[6]	6.8	$C_{48}H_{72}O_{21}S_2$	1047.3966	1047.3935	3.0	523.1936	1.9	1069.3765	1.5
Unknown[6]	7.8	$C_{48}H_{72}O_{21}S_2$	1047.3966	1047.3935	3.0	523.1930	0.8	1069.3760	1.0
Unknown	7.8	$C_{54}H_{82}O_{24}S_2$	1177.4579	1177.4565	1.2	588.2257	2.7	1199.4379	0.0
44-oxotrinor-YTX	8.1	$C_{52}H_{78}O_{22}S_2$	1117.4363	1117.4354	0.8	558.2140	1.0	1139.4194	2.3
Unknown	8.3	$C_{55}H_{84}O_{24}S_2$	1191.4722	1191.4721	0.0	595.2334	2.5	1213.4541	0.5
Unknown	8.3	$C_{60}H_{74}O_{21}S_2$	1195.4250	1195.4248	0.2	597.2086	0.7	1217.4066	0.3
41-formyl-39-oxo-YTX	8.3	$C_{48}H_{72}O_{23}S_2$	1079.3851	1079.3833	1.7	539.1886	2.1	1101.3660	1.2
44,45-diOH-YTX	8.4	$C_{55}H_{84}O_{23}S_2$	1175.4788	1175.4772	1.3	587.2356	2.0	1197.4599	1.1
45OH-YTX[7]	8.5	$C_{55}H_{82}O_{22}S_2$	1157.4673	1157.4667	0.5	578.2302	1.8	1179.4499	1.6
45OH-44COOH-YTX	8.5	$C_{55}H_{82}O_{24}S_2$	1189.4941	1189.4929	1.1	594.2434	2.0	1211.4772	2.4
Unknown[6]	8.6	$C_{48}H_{72}O_{21}S_2$	1047.3956	1047.3935	2.0	523.1935	1.8	1069.3761	1.2
44-oxotrinor-YTX	8.6	$C_{52}H_{78}O_{22}S_2$	1117.4370	1117.4354	1.5	558.2146	2.0	1139.4184	1.4
Unknown	8.7	$C_{54}H_{80}O_{22}S_2$	1143.4544	1143.4510	2.9	571.2225	2.1	1165.4348	2.1
Unknown	8.8	$C_{54}H_{80}O_{22}S_2$	1143.4520	1143.4510	0.9	571.2225	2.1	1165.4348	2.1
Unknown	8.9	$C_{52}H_{78}O_{23}S_2$	1133.4321	1133.4303	1.6	566.2120	1.9	1155.4126	0.8
45OH-YTX[7]	8.9	$C_{55}H_{82}O_{22}S_2$	1157.4690	1157.4667	2.0	578.2305	2.3	1179.4494	1.2
45OH-YTX[7]	8.9	$C_{55}H_{82}O_{22}S_2$	1157.4688	1157.4667	1.8	578.2310	3.3	1179.4519	3.3
Unknown	8.9	$C_{52}H_{78}O_{23}S_2$	1133.4320	1133.4303	1.5	566.2125	2.8	1155.4110	-0.6
32-O-diglycoside-YTX	8.9	$C_{65}H_{98}O_{29}S_2$	1405.5571	1405.5563	0.6	702.2756	2.4		
45OH-YTX[7]	9.0	$C_{55}H_{82}O_{22}S_2$	1157.4678	1157.4667	1.0	578.2307	2.6	1179.4504	2.0
45,46,47-trinor-YTX	9.0	$C_{52}H_{78}O_{21}S_2$	1101.4428	1101.4404	2.1	550.2172	2.1	1123.4224	0.5
44COOH-YTX	9.0	$C_{55}H_{82}O_{23}S_2$	1173.4624	1173.4616	0.7	586.2277	1.9	1195.4457	2.3
32-O-monoglycoside-YTX	9.0	$C_{60}H_{90}O_{25}S_2$	1273.5162	1273.5140	1.7	636.2531	0.5		
YTX	9.1	$C_{55}H_{82}O_{21}S_2$	1141.4734	1141.4717	1.4	570.2327	1.7	1163.4547	1.4
1a-homo-YTX	9.7	$C_{56}H_{84}O_{21}S_2$	1155.4892	1155.4874	1.5	577.2411	2.7	1177.4689	0.1
45OH-1a-homo-YTX	8.7	$C_{56}H_{84}O_{22}S_2$	1171.4802	1171.4823	-1.8	585.2384	2.5		

Table 6.4 continued.

Compound Name	$[M\text{-}SO_3]^-$ *m/z* obs.	Mass error (ppm)	Fragment[1] *m/z* obs.	Mass error (ppm)	Fragment[2] *m/z* obs.	Mass error (ppm)	Fragment[3] *m/z* obs.	Mass error (ppm)
Unknown[6]	967.4384	1.7	855.3839	-0.4	467.1674	2.2	396.1357	2.1
Unknown[6]	967.4384	1.7			467.1667	0.8	396.1353	1.2
Unknown	1097.5001	0.4	855.3839	-0.4	467.1664	0.2	396.1346	-0.6
44-oxotrinor-YTX	1037.4803	1.7	855.3851	1.0	467.1671	1.6	396.1356	1.8
Unknown	1111.5151	-0.2	855.3840	-0.3	467.1664	0.2	396.1350	0.3
Unknown	1115.4681	0.1	855.3866	2.7	467.1676	2.6	396.1359	2.7
41-formyl-39-oxo-YTX	999.4260	-0.6	855.3847	0.5	467.1662	-0.2	396.1355	1.5
44,45-diOH-YTX	1095.5198	-0.5	855.3839	-0.4	467.1663	-0.1	396.1349	0.1
45OH-YTX[7]	1077.5118	1.8			467.1671	1.7	396.1354	1.5
COOH OH-YTX	1109.5378	1.5	855.3857	1.7	467.1674	2.3	396.1359	2.6
Unknown[6]	967.4376	1.0	855.3853	1.2	467.1671	1.7	396.1354	1.5
44-oxotrinor-YTX	1037.4782	-0.3	855.3842	-0.1	467.1664	0.2	396.1348	0.0
Unknown	1063.4959	1.6	855.3857	1.7	467.1673	2.0	396.1357	2.2
Unknown	1063.4959	1.6	855.3857	1.7	467.1675	2.5	396.1361	3.1
Unknown	1053.4734	-0.1	855.3837	-0.6	467.1664	0.0	396.1348	-0.1
45OH-YTX[7]	1077.5112	1.3	855.3857	1.7	467.1672	1.8	396.1357	2.0
45OH-YTX[7]	1077.5118	1.8	855.3867	2.8	467.1675	2.6	396.1358	2.4
Unknown	1053.4740	0.5	855.3841	-0.2	467.1667	0.8	396.1346	-0.5
32O-diglycoside-YTX	1325.5982	-0.9						
45OH-YTX[7]	1077.5121	2.1	855.3857	1.7	467.1673	2.0	396.1356	1.9
45,46,47-trinor-YTX	1021.4833	-0.3			467.1663	-0.2	396.1348	0.0
COOH-YTX	1093.5068	1.9	855.3857	1.7	467.1673	2.0	396.1356	1.8
32O-monoglycoside-YTX	1193.5581	0.8			467.1674	2.3	396.1350	0.4
YTX	1061.5175	2.4	855.3858	1.8	467.1671	1.6	396.1362	3.5
					Fragment[4] *m/z* obs.	mass error (ppm)	Fragment[5] *m/z* obs.	mass error (ppm)
1a-homo-YTX	1075.5325	1.8			474.1752	2.2	403.1436	2.3
45OH-1a-homo-YTX	1091.5252	-0.3			474.1742	0.1	403.1430	0.9

[1)] Fragment $[C_{42}H_{64}O_{16}S\text{-}H]^-$ *m/z* 855.3843, [2)] fragment $[C_{42}H_{64}O_{19}S_2\text{-}2H]^{2-}$ *m/z* 467.1663, [3)] fragment $[C_{35}H_{54}O_{16}S_2\text{-}2H]^{2-}$ *m/z* 396.1348, [4)] fragment $[C_{43}H_{66}O_{19}S_2\text{-}2H]^{2-}$ *m/z* 474.1742, [5)] fragment $[C_{36}H_{56}O_{16}S_2\text{-}2H]^{2-}$ *m/z* 403.1427.

[6)] This elemental composition corresponds to enone-YTX, keto-YTX or nor oxo-YTX.

[7)] fragments and precursor correspond to (an isomer) of 45OH-YTX.

Table 6.5 Overview of AZA group toxins found in the sample extracts and added to the search library.

Compound Name	t_r (min)	Elemental composition	$[M+H]^+$ *m/z* obs.	*m/z* calc.	Mass error (ppm)	Elemental composition[1]	*m/z* obs.	Mass error (ppm)
AZA1	14.1	$C_{47}H_{71}NO_{12}$	842.5049	842.5049	0.0	$C_{38}H_{57}NO_9$	672.4107	0.2
AZA2	14.7	$C_{48}H_{73}NO_{12}$	856.5204	856.5205	-0.1	$C_{38}H_{57}NO_9$	672.4107	0.2
AZA3	12.0	$C_{46}H_{69}NO_{12}$	828.4890	828.4892	-0.3	$C_{37}H_{55}NO_9$	658.3948	-0.2
AZA4	11.4	$C_{46}H_{69}NO_{13}$	844.4839	844.4841	-0.3	$C_{37}H_{55}NO_9$	658.3950	0.1
AZA5	10.8	$C_{46}H_{69}NO_{13}$	844.4840	844.4841	-0.2	$C_{37}H_{55}NO_{10}$	674.3894	-0.7
AZA6	12.5	$C_{47}H_{71}NO_{12}$	842.5044	842.5049	-0.6	$C_{37}H_{55}NO_9$	658.3953	0.5
AZA8	11.9	$C_{47}H_{71}NO_{13}$	858.4995	858.4998	-0.3	$C_{38}H_{57}NO_{10}$	688.4060	0.8
AZA9	12.1	$C_{47}H_{71}NO_{13}$	858.4995	858.4998	-0.4	$C_{37}H_{55}NO_9$	658.3949	-0.1
AZA10	11.3	$C_{47}H_{71}NO_{13}$	858.4998	858.4998	0.0	$C_{37}H_{55}NO_{10}$	674.3884	-2.1
AZA11/12	12.4	$C_{48}H_{73}NO_{13}$	872.5166	872.5154	1.3			
AZA11/12	13.8	$C_{48}H_{73}NO_{13}$	872.5158	872.5154	0.4			
AZA13	10.3	$C_{46}H_{69}NO_{14}$	860.4788	860.4791	-0.3	$C_{37}H_{55}NO_{10}$	674.3909	1.5
AZA14	11.3	$C_{47}H_{71}NO_{14}$	874.4951	874.4947	0.5	$C_{38}H_{57}NO_{10}$	688.4046	-1.4
AZA15	10.7	$C_{47}H_{71}NO_{14}$	874.4942	874.4947	-0.6	$C_{37}H_{55}NO_{10}$	674.3898	-0.1
AZA16/17	8.8	$C_{47}H_{69}NO_{14}$	872.4799	872.4791	0.9			
AZA18/19	8.9	$C_{48}H_{71}NO_{14}$	886.4954	886.4947	0.8			
AZA20/21	8.4	$C_{47}H_{69}NO_{15}$	888.4734	888.4740	-0.7			
AZA22/23	8.8	$C_{48}H_{71}NO_{15}$	902.4905	902.4896	1.0			

[1)] Fragment of A-ring.

Table 6.5 continued.

Compound Name	Fragment[2] *m/z* obs.	Mass error (ppm)	Mass error (ppm) $-H_2O$	$-2\ H_2O$	$-3\ H_2O$
AZA1	362.2685	-1.3	0.0	0.3	0.0
AZA2	362.2684	-1.5	-0.2	0.1	-0.1
AZA3	362.2684	-1.6	-0.2	-0.3	-0.2
AZA4	362.2684	-1.5	-0.1	-0.1	-0.1
AZA5	362.2684	-1.5	0.3	-0.2	0.0
AZA6	362.2683	-1.7	-0.4	-0.9	0.3
AZA8	362.2683	-1.9	-0.3	-0.2	0.2
AZA9	362.2684	-1.6	-0.5	-0.4	2.9
AZA10	362.2684	-1.5	-0.1	0.2	0.8
AZA11/12			-0.2	-0.6	
AZA11/12			-0.6	-1.1	
AZA13	362.2681	-2.3	-0.5	-0.6	-0.3
AZA14	362.2683	-1.7	-0.3	0.0	-0.4
AZA15	362.2685	-1.4	-0.3	-0.2	-0.1
			$-H_2O$	$-H_2O\ -CO_2$	$-2\ H_2O\ -CO_2$
AZA16/17	362.2684	-1.6	-0.4	-0.3	-0.7
AZA18/19	362.2682	-2.2	-0.4	-0.6	-0.4
AZA20/21	362.2678	-3.1	-0.2	-0.5	-1.2
AZA22/23	362.2684	-1.6		0.8	

[2)] Fragment of E-ring $[C_{22}H_{35}NO_3+H]^+$ *m/z* 362.2689.

Table 6.6 PTX group toxins found in various sample extracts and added to the search library.

Compound Name	t_r (min)	Elemental composition	$[M+NH_4]^+$ *m/z* obs.	*m/z* calc.	Mass error (ppm)	$[M+Na]^+$ *m/z* obs.	Mass error (ppm)
PTX2sa[1]	8.8	$C_{47}H_{72}O_{15}$	894.5225	894.5210	1.7	899.4763	-0.1
PTX2sa[1]	8.9	$C_{47}H_{72}O_{15}$	894.5212	894.5210	0.3	899.4764	0.0
PTX11	16.2	$C_{47}H_{70}O_{15}$	892.5053	892.5053	-0.1	897.4607	0.0
PTX2	17.6	$C_{47}H_{70}O_{14}$	876.5103	876.5104	-0.1	881.4656	-0.3
14:0 PTX2sa	17.0	$C_{61}H_{98}O_{16}$	1104.7173	1104.7193	-1.8	1109.6744	-0.3
16:0 PTX2sa[1]	17.4	$C_{63}H_{102}O_{16}$	1132.7517	1132.7506	0.9	1137.7053	-0.6
16:0 PTX2sa[1]	18.9	$C_{63}H_{102}O_{16}$	1132.7511	1132.7506	0.4	1137.7059	-0.1

Compound Name	Fragment[2] *m/z* obs.	Mass error (ppm)	Fragment[3] *m/z* obs.	Mass error (ppm)	Mass error (ppm) -2 H_2O	-3 H_2O	-4 H_2O	-5 H_2O
PTX2sa[1]	551.2855	0.8	213.1120	-0.4	0.2	0.2	0.5	0.1
PTX2sa[1]	551.2842	-1.5	213.1120	-0.3	0.0	0.2	0.0	2.9
PTX11	551.2846	-0.8	213.1121	-0.1	0.4	0.0	-0.6	1.6
PTX2			213.1118	-1.3	0.0	0.8	0.1	
14:0 PTX2sa	551.2851	0.1	213.1120	-0.6	0.6	0.7	1.0	0.6
16:0 PTX2sa[1]	551.2839	-2.0	213.1117	-1.7	-0.7	-1.5	-2.0	-1.3
16:0 PTX2sa[1]	551.2855	0.9	213.1121	-0.2	-0.1	1.2	0.7	1.6

[1)] Observed due to epimerization at C-7 position.
[2)] Fragment $[C_{29}H_{42}O_{10}+H]^+$ *m/z* 551.2850.
[3)] Fragment $[C_{11}H_{17}O_4+H]^+$ *m/z* 213.1121.

For the OA group toxins, including the various esters, two specific fragments (*m/z* 255.1237 and *m/z* 785.4482) are often observed in ESI^- (Table 6.3) in the extracts. Using this approach a wide variety of 7-O-acylated OA and DTX2 esters were identified in the various shellfish samples. The ester profile corresponds to what has been reported by others [16, 17]. With respect to the elution order of OA and DTX2 esters it is reasonable to assume that OA esters elute first, like the parent compounds [5]. Unfortunately, no samples (shellfish or algae) were available containing DTX1 esters or OA-diol esters.

YTX derivatives containing two sulfonic acid groups produce the following ions in ESI^-: $[M-H]^-$, $[M-2H+Na]^-$ and $[M-2H]^{2-}$. Furthermore, the specific YTX fragments that are often observed are *m/z* 855.3843, *m/z* 467.1663 and *m/z* 396.1348 [9, 18]. 1a-homo-YTX derivatives produce *m/z* 474.1742 and *m/z* 403.1427 as common fragments (Table 6.4). Correct identification of YTX group toxins was complicated by the fact that most toxins elute in a short time span and fragmentation patterns are quite similar. This illustrates the limitation of HCD fragmentation in an orbitrap-MS. All ions are transferred to the HCD cell without the possibility to carry out a selected precursor ion fragmentation. Based on the presence of the specific ions $[M-H]^-$, $[M-2H+Na]^-$ and $[M-2H]^{2-}$, in combination with the presence of the specific fragments, YTX group toxins were tentatively identified. Several known YTX toxins were found in the Norwegian algae and shellfish samples but also at least five YTXs were observed in the algae sample with to us unknown structures (Table 6.4). Miles *et al* already described that numerous unknown YTX derivatives can be present in algae [9]. In figure 6.4 the precursor mass spectrum and the fragmentation spectrum of an unknown YTX derivative with elemental composition of $C_{52}H_{78}O_{23}S_2$ is shown as an example.

Characteristic for the fragmentation observed for AZA toxins, are the subsequent losses of up to 5 water molecules and the fragmentation of the A and E ring. Fragmentation of the A ring results in fragments with *m/z* 688.4055, 674.3898, 672.4106 or 658.3949 depending on the substituents. E ring fragmentation produces one fragment with *m/z* 362.2689 for all AZAs (Table 6.5). The AZA group of toxins is relatively small. According to the literature there are 32 possible AZAs, of which to date 20 have been found [12]. In the shellfish samples contaminated with AZAs, AZA toxins 1-15 could be identified based on the

Table 6.7 Detection of lipophilic marine toxins in shellfish samples, results obtained with Search_LCMS. Samples include a mix of highly contaminated mussel extracts; sample 1, 2 and 3 are contaminated mussel extracts; sample 4 is a blank mussel extract. Threshold intensity has been set at 1 000.

		Sought	Mix		Sample 1		Sample 2		Sample 3		Sample 4	
Compound name	Sought mass *m/z*	t_r (min)	Intensity	Mass error (ppm)	Intensity	Mass error (ppm)	Intensity	Mass error (ppm)	Intensity	Mass error (ppm)	Intensity	Mass error (ppm)
OA	803.4587	8.4	207823	1.6	24329	2.4	118123	3.2	25044	2.3	0	
DTX2	803.4587	8.9	104543	2.8	108497	2.5	411109	2.8	0		0	
DTX1	817.4744	9.9	119958	1.9	0		109375	2.1	252134	2.2	0	
14:0 DTX2	1013.6571	17.4	10009	1.7	3867	1.6	1301	2.8	0		0	
15:0 OA	1027.6728	17.9	2880[3]	0.5	0		0		0		0	
15:0 DTX2	1027.6728	18.2	2880[3]	0.5	0		0		0		0	
16:1 OA	1039.6728	17.3	2565	2.2	0		0		0		0	
16:1 DTX2	1039.6728	17.6	19680	1.0	4876	1.7	2721	1.5	0		0	
16:0 OA	1041.6884	18.8	72610	1.4	27612	1.4	7869[3]	1.9	0		0	
16:0 DTX2	1041.6884	19.0	33109	1.7	27079	1.2	7869[3]	1.9	0		0	
17:1 OA	1053.6884	18.2	2150[3]	2.5	0		0		0		0	
17:1 DTX2	1053.6884	18.5	2150[3]	2.5	0		0		0		0	
18:3 DTX2	1063.6728	16.9	0		1009	1.8	0		0		0	
18:2 OA	1065.6884	17.7	4111[3]	2.7	0		0		0		0	
18:2 DTX2	1065.6884	18.0	4111[3]	2.7	1008	1.2	0		0		0	
18:1 OA	1067.7041	18.9	15207	1.9	3089	0.8	1136	4.1	0		0	
18:1 DTX2	1067.7041	19.2	5704	0.9	1102	0.1	1068	1.8	0		0	
18:0 OA	1069.7197	20.2	1815	1.9	1655	3.8	0		0		0	
20:5 DTX2	1087.6728	16.7	5385	0.8	0		1061	-0.9	0		0	
22:6 OA	1113.6884	17.2	0		0		0		0		0	
22:6 DTX2	1113.6884	17.5	6668	1.6	1279	0.5	1316	-1.8	0		0	

Table 6.7 continued.

Compound name	Sought mass *m/z*	Sought t_r (min)	Mix Intensity	Mass error (ppm)	Sample 1 Intensity	Mass error (ppm)	Sample 2 Intensity	Mass error (ppm)	Sample 3 Intensity	Mass error (ppm)	Sample 4 Intensity	Mass error (ppm)
Unknown[1]	523.1926	6.8	2022	4.3	0		0		0		0	
Unknown[1]	523.1926	7.8	259797	2.1	0		0		0		0	
YTX	570.2317	9.1	1380385	1.8	0		17243	3.5	28696	4.1	0	
45OH-YTX[2]	578.2291	8.5	5251	-0.2	0		0		1334	3.5	0	
45OH-YTX[2]	578.2291	8.9	1650	1.5	0		0		0		0	
45OH-YTX[2]	578.2291	9.0	7615	1.8	0		0		0		0	
AZA1	842.5049	14.1	1361843	-0.3	1024	2.7	152136	0.0	0		0	
AZA2	856.5205	14.7	378000	-0.1	0		23348	0.1	0		0	
AZA3	828.4892	12.0	70282	-0.1	0		15024	2.1	0		0	
AZA4	844.4841	11.4	149959	0.0	0		14305	-0.5	0		0	
AZA5	844.4841	10.8	1720	-0.5	0		0		0		0	
AZA6	842.5049	12.5	25096	-0.7	0		3873	-0.7	0		0	
AZA8	858.4998	11.9	50141[3]	-0.1	0		5010[3]	0.6	0		0	
AZA9	858.4998	12.1	50141[3]	-0.1	0		5010[3]	0.6	0		0	
AZA10	858.4998	11.3	1930	-0.9	0		0		0		0	
AZA14	874.4947	11.3	4584	-0.8	0		0		0		0	
AZA16/17	872.4791	8.8	5455	-0.2	0		1217	2.7	0		0	
AZA20/21	888.4740	8.4	2181	-0.1	0		0		0		0	
PTX2	876.5104	17.6	15724	0.5	0		106503	0.5	0		0	
PTX11	892.5053	16.2	0		0		2085	-1.5	0		0	
PTX2sa	894.5210	8.9	11803	1.2	4024	1.6	12195	0.9	5400	0.7	0	
14:0 PTX2sa	1104.7193	17.0	40951	0.7	12962	-0.2	14727	-0.4	20340	0.2	0	
16:0 PTX2sa[4]	1132.7506	17.4	14475	-0.1	0		0		4233	1.1	0	
16:0 PTX2sa[4]	1132.7506	18.9	79572	0.0	55628	0	25992	-2.3	33396	-0.2	0	

[1)] This elemental composition corresponds to: enone YTX, keto YTX or nor oxo YTX

[2)] Found mass corresponds to 45-OH YTX isomers

[3)] The retention window was set at 0.2 min. Closely eluting toxins (<= 0.2 min) with the same exact mass result in a duplicate identification due to the fact that Search_LCMS only gives the most intense peak for a mass in a retention time window.

[4)] 7-C epimers of PTX2sa esters.

fragmentation with the exception of AZA7, AZA11 and AZA12 for which no A and E ring fragments were observed, although for the latter two the precursor and subsequent water losses were detected. In total 18 AZAs were found in the Irish shellfish sample, all of which have been described earlier in literature (Table 6.5) [12].

The PTXs all fragment by a series (up to 5) of water losses and produce common fragments of *m/z* 213.1121 and *m/z* 551.2850 [15]. The latter fragment is specific for PTX2, PTX2sa, PTX11 and PTX12. Unfortunately, in this study only a few shellfish and algae samples containing PTX toxins were available, which limited the number of PTXs identified. During the initial analysis of the data precursor ions of PTX2sa esters were not included in the search list. During the review of the output file produced by Search_LCMS, pairs of the specific PTX fragments (*m/z* 213.1121 and *m/z* 551.2850) were found. Review of the spectra showed the water loss patterns and fragments that are characteristic for PTX2sa esters (Fig. 6.5). These spectra could be assigned to 14:0 and 16:0 PTX2sa esters which were recently described by Wilkins *et al* [13]. Literature on PTX esters is very scarce. This case nicely shows the potential of LC-orbitrap-MS as a screening method to detect new lipophilic marine toxins.

A CSV file has been constructed in which the 85 identified toxins have been incorporated. This library can be used for the efficient (fast) screening of shellfish samples. In this library information on the precursor ions of the 85 toxins are included. The selected (most abundant) precursor ions are: for the OA group $[M\text{-}H]^-$, YTX group $[M\text{-}2H]^{2-}$, AZA group $[M+H]^+$ and PTX group $[M+NH_4]^+$. In the future the data file can be easily expanded, in case more materials are analyzed that contain new toxins.

Linearity and sensitivity of the orbitrap

Linearity of the orbitrap-MS was assessed by injecting standard solutions in methanol containing OA, YTX, AZA1, and PTX2. Concentrations ranged from 2 till 16 ng/ml for OA, AZA1 and PTX2 and from 12.5 till 100 ng/ml for YTX. Linearity was acceptable for all toxins analyzed (correlation coefficient >0.98). The detection limit was based on the signal-to-noise ratio of the precursor ion. The noise levels were estimated at the beginning of the chromatogram and were

approximately 400 counts in ESI^- and 500 counts in ESI^+. The calculated detection limits (signal-to-noise = 3) were 1.4 pg, 21.3 pg, 5.4 and 1.1 pg on-column for OA, YTX, AZA1 and PTX2, respectively.

Screening of samples for toxins

The constructed library was used to screen shellfish samples that had not been used for the development of the toxin database. Six different samples were analyzed: a standard mix with OA, YTX, AZA1 and PTX2 (used as positive control sample), a mix of highly contaminated extracts, three samples with an unknown toxin profile and one sample that was reported as negative in the Dutch routine monitoring program by both the rat bioassay and by LC-MS/MS analysis [5]. With the low limits of detection it is possible to distinguish between blank samples and samples that contain toxins. The toxin profiles of the various samples are shown in Table 6.7. Most important is that the toxins spiked to the sample could be retrieved and that in the blank mussel no toxins were detected. The screening method is thus fit to discriminate contaminated samples from blank samples. When positive samples are found, a target method with proper calibration solutions should be used for correct quantitation.

Future extension of the library

The library that has been constructed can easily be transferred to other laboratories as it is a simple CSV data file. *MetAlign* settings for the data reduction are instrument dependent and should be optimized within each laboratory and for each instrument. Settings for noise elimination, threshold and peak picking are also instrument dependent. Ideally, the LC method, column material, mobile phase composition and gradient should be kept identical in order to keep the same elution order and approximate retention times of the toxins. However, even when these parameters are kept the same, retention times may shift due to differences in dead volumes, aging of the column, etc. To address this variability the Search_LCMS software comes with a Make_Retentions program, which makes it possible to correct retention times of each series by injecting a standard mix of known toxins. The obtained retention times of this standard mix can be used as a retention time index (reference) to fit the other retention times of the toxins.

CONCLUSION

This study described the potential of HR full scan accurate MS for marine toxin research. A lipophilic marine toxin library has been constructed that can be used for screening of shellfish samples or for the determination of the toxin composition in complex samples. The library is exchangeable between various instruments and different laboratories. Currently, efforts are undertaken to make this search library available online. When the library comes available online, it ensures that the most updated version will be available. Laboratories can share their information by adding new toxins to the library. By sharing this information search libraries will become more complete and toxin profiles can be determined more accurately. This developed library can be of interest for laboratories that are involved in monitoring of shellfish toxins. The approach described in this paper is also under development or already used in other fields of research such as pesticide or veterinary drug analysis in various biological matrices.

REFERENCES

[1] Rundberget T, Sandvik M, Larsen K, Pizarro GM, Reguera B, Castberg T, Gustad E, Loader JI, Rise F, Wilkins AL, Miles CO. Extraction of microalgal toxins by large-scale pumping of seawater in Spain and Norway, and isolation of okadaic acid and dinophysistoxin-2. *Toxicon* 2007; **50**: 960.

[2] Tillmann U, Elbrachter M, Krock B, John U, Cembella A. *Azadinium spinosum* gen. et sp. nov. (*Dinophyceae*) identified as a primary producer of azaspiracid toxins. *Eur J Phycol* 2009; **44**: 63.

[3] Paz B, Riobo P, Fernandez AL, Fraga S, Franco JM. Production and release of yessotoxins by the dinoflagellates *Protoceratium reticulatum* and *Lingulodinium polyedrum* in culture. *Toxicon* 2004; **44**: 251.

[4] European Parliament, Council. Laying down implementing measures for certain products under Regulation (EC) No 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No 854/2004 of the European Parliament and of the Council and Regulation (EC) No 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004. *Off J Eur Commun* 2005; **L338**: 40.

[5] Gerssen A, Mulder PPJ, McElhinney MA, De Boer J. Liquid chromatography - tandem mass spectrometry method for the detection of marine lipophilic toxins under alkaline conditions. *J Chromatogr A* 2009; **1216**: 1421.

[6] Fux E, McMillan D, Bire R, Hess P. Development of an ultra-performance liquid chromatography-mass spectrometry method for the detection of lipophilic marine toxins. *J Chromatogr A* 2007; **1157**: 273.

[7] Stobo LA, Lacaze JPCL, Scott AC, Gallacher S, Smith EA, Quilliam MA. Liquid chromatography with mass spectrometry - detection of lipophilic shellfish toxins. *J AOAC Int* 2005; **88**: 1371.

[8] McNabb P, Selwood AI, Holland PT. Multiresidue method for determination of algal toxins in shellfish: Single-laboratory validation and interlaboratory study. *J AOAC Int* 2005; **88**: 761.

[9] Miles CO, Samdal IA, Aasen JAB, Jensen DJ, Quilliam MA, Petersen D, Briggs LM, Wilkins AL, Rise F, Cooney JM, MacKenzie AL. Evidence for numerous analogs of yessotoxin in *Protoceratium reticulatum*. *Harmful Algae* 2005; **4**: 1075.

[10] Doucet E, Ross NN, Quilliam MA. Enzymatic hydrolysis of esterified diarrhetic shellfish poisoning toxins and pectenotoxins. *Anal Bioanal Chem* 2007; **389**: 335.

[11] Aasen JAB, Hardstaff W, Aune T, Quilliam MA. Discovery of fatty acid ester metabolites of spirolide toxins in mussels from Norway using liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom* 2006; **20**: 1531.

[12] Rehmann N, Hess P, Quilliam MA. Discovery of new analogs of the marine biotoxin azaspiracid in blue mussels (*Mytilus edulis*) by ultra-performance liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom* 2008; **22**: 549.

[13] Wilkins AL, Rehmann N, Torgersen T, Rundberget T, Keogh M, Petersen D, Hess P, Rise F, Miles CO. Identification of fatty acid esters of pectenotoxin-2 seco acid in blue mussels (*Mytilus edulis*) from Ireland. *J Agric Food Chem* 2006; **54**: 5672.

[14] Lommen A. MetAlign: interface-driven, versatile metabolomics tool for hyphenated full-scan mass spectrometry data preprocessing. *Anal Chem* 2009; **81**: 3079.

[15] Gerssen A, Mulder PPJ, van Rhijn JA, de Boer J. Mass spectrometric analysis of the marine lipophilic biotoxins pectenotoxin-2 and okadaic acid by four different types of mass spectrometers. *J Mass Spectrom* 2008; **43**: 1140.

[16] Torgersen T, Wilkins AL, Rundberget T, Miles CO. Characterization of fatty acid esters of okadaic acid and related toxins in blue mussels (*Mytilus edulis*) from Norway. *Rapid Commun Mass Spectrom* 2008; **22**: 1127.

[17] Vale P. Detailed profiles of 7-O-acyl esters in plankton and shellfish from the Portuguese coast. *J Chromatogr A* 2006; **1128**: 181.

[18] Canas IR, Hamilton B, Amandi MF, Furey A, James KJ. Nano liquid chromatography with hybrid quadrupole time-of-flight mass spectrometry for the determination of yessotoxin in marine phytoplankton. *J Chromatogr A* 2004; **1056**: 253.

7

Conclusions and outlook

Research described in this thesis has focused on the group of lipophilic marine toxins, which is the general term for a number of marine toxins with different structures such as diarrhetic shellfish poisons (DSP), yessotoxins (YTX), azaspiracids (AZAs), pectenotoxins (PTXs), gymnodimine (GYM) and spirolides (SPXs). Members of the DSP group are okadaic acid (OA), dinophysistoxin-1 (DTX1), dinophysistoxin -2 (DTX2) and dinophysistoxin-3 (DTX3) [1]. The latter comprises the fatty acid acyl esters of OA, DTX1 and DTX2. The European Union has established legislation for thirteen lipophilic marine toxins [2], while GYM and the SPXs are not yet under legislation. An animal bioassay is prescribed as the official method of control. Within the European Union (EU) more than 10 000 test animals (mice) are used annually for routine monitoring of lipophilic marine toxins in shellfish. This animal assay is under discussion for ethical reasons but also for lack of specificity and sensitivity. To improve the protection of the consumer and to replace the animal test, there is an urgent need for alternative methods. In the past 6 years the aim of several EU funded studies has been the development of alternative methods for the detection of lipophilic marine toxins. The DETECTOX project (Food-CT-2004-514055) focused on the development of a multi-channel biosensor chip based on Surface Plasmon Resonance (SPR) for the detection of the various classes of the lipophilic marine toxins. The project has resulted in a validated SPR biosensor assay kit for the detection of OA and DTXs [3]. Biosensor assays were developed for YTXs and AZAs as well, but these were not yet validated [4]. Biotoxmarin (Food-CT-2004-513967) focused on the isolation and chemical characterization of marine biotoxins in general and on the development of dipstick methods in particular. The project succeeded in the isolation of some new toxins such as the cytotoxins oxazinin-1 and -2 [5]. Unfortunately, no dipstick methods were successfully developed. The EU project BIOTOX (Food-CT-2004-514074) focused on the development of alternative methods for the detection of lipophilic marine toxins, for example based on liquid chromatography coupled to mass spectrometric detection (LC-MS). Research described in this thesis was partly financed by the BIOTOX project. In this last chapter the findings of our research are evaluated and discussed, and recommendations for future research are given.

Standards

The first challenge for this thesis research was the development of methods without the availability of reference standards, as at the start of this project in January 2005 for most of the toxins, except OA and pectenotoxin-2 (PTX2), no standards were commercially available. For this reason, in the study described in **chapter 2** only OA and PTX2 were included. By the end of 2006, very small amounts of semi-purified standards of yessotoxin (YTX) and azaspiracid-1 (AZA1) were obtained from partners within the BIOTOX project. These standards were isolated, respectively, from algae and from contaminated shellfish [6]. In 2007, YTX and AZA1 became commercially available and this contributed substantially to the development and the in-house validation of the analytical method as described in **chapters 3-5**.

Mass spectrometric detection

In the **chapters 2 and 6** the merits of MS are shown. In **chapter 2** four different MS techniques were used and fragmentation pathways for OA and PTX2 were proposed. The four techniques used were triple quadrupole MS (TQ-MS), time-of flight MS (ToF-MS), quadrupole ToF-MS (QToF-MS) and ion trap MS (IT-MS). Each technique has its advantages and disadvantages. TQ-MS is the most sensitive MS when used in multiple-reaction-monitoring (MRM) mode. These systems are frequently used in the routine analysis of many different compounds due to their excellent sensitivity and selectivity. In MRM mode the transitions to be monitored should be predefined (targeted approach). The maximum number of toxins that can be analyzed in a single run with a TQ-MS depends on the scan speed, as every MRM transition occurs in a certain time frame. With the (TQ-MS) LC-MS/MS method developed in **chapter 3** 28 different toxins could be analyzed in a single run; which could even be extended to a maximum of about 40. In ToF-MS the number of compounds screened in a single analysis is theoretically unlimited. Drawback of ToF-MS compared to TQ-MS is the lower sensitivity. In our case OA was 3-fold more sensitive on a TQ-MS. An advantage of the ToF-MS is that all ions are recorded with a high resolution [10 000 at full-width-half-maximum (FWHM)] and high accuracy (<10 ppm). Therefore, this technique can be used for multi-target screening of a large number of compounds [7]. As no compounds or

transitions have to be predefined it is also possible to search retrospectively the data for new, emerging or newly described toxins (**chapter 6**). With a QToF-MS fragmentation data can be obtained in addition to the ToF-MS data, which is useful for identification purposes. Unfortunately, the QToF-MS used in our experiments had a poor sensitivity and there was no possibility of switching polarities during a series. Therefore, OA and PTX2 experiments had to be done on separate occasions. Modern QToF-MS instruments are more sensitive and can switch polarities during a run. The last MS studied was an IT-MS. With this instrument it was possible to obtain MS^n fragments, which can be helpful in elucidating fragmentation pathways. A drawback of IT-MS is the mass cutoff range. For example, for OA the selected ion is *m/z* 803.5 and the cutoff limit is around *m/z* 240 (30% of the selected ion). In practice, fragmentation pathways could be elucidated by performing MS^n experiments on an IT-MS and a high resolution QToF-MS for accurately identifying the fragments produced.

For confirmation of the identity of marine toxins the same approach has been used as in the field of veterinary drugs in products of animal origin [8, 9]. Confirmation is based on the collection of identification points (IPs) (Table 7.1). For the confirmation of toxins three IPs are required. The number of IPs earned by a specific analysis depends on the technique used. Low resolution mass spectrometers such as TQ-MS and IT-MS, are able to gather 1 IP for the precursor and 1.5 IP for each product ion. With the use of at least two MRM transitions this will make 4 IPs, what is sufficient for confirmatory analysis. For high resolution MS (HRMS) (≥ 20 000 at FWHM) 2 IPs are earned for the precursor and 2.5 IPs for each product ion. Mass spectrometers with a resolution of 20 000 and higher can be used for confirmatory analysis when a precursor and product ion can be recorded. Unfortunately, the ToF and QToF-MS used **chapter 2** had a resolution of 10 000 and 5 000, respectively. Therefore, these instruments can only be used for screening purposes. Furthermore, when a ToF-MS is run in full scan mode with in-source fragmentation no precursor is selected. Therefore, it is impossible to be 100% sure that a product ion originates from a specific precursor. Consequently, such HRMS can 'officially' only be used for screening purposes [9]. An orbitrap MS is a type of ion-trap MS using fast Fourier transformation to obtain mass spectra with a much higher resolution (100 000) and higher mass accuracy (<5 ppm) than

Table 7.1 System of identification points according to EU legislation [9].

MS-technique	Identification points
Low resolution (LR) MS	1.0
$LRMS^n$ precursor ion	1.0
$LRMS^n$ product ion	1.5
High resolution (HR) MS	2.0
$HRMS^n$ precursor ion	2.0
$HRMS^n$ product ion	2.5

the ToF-MS used in **chapter 2**. In **chapter 6** the orbitrap MS has been used to create a method for screening of lipophilic marine toxins in shellfish samples. A major drawback of HRMS is the file size and limited ability to search these files rapidly for compounds of interest. Therefore, the software program *metAlign* was used to reduce the orbitrap MS files by a factor of up to 200 [10]. These reduced data files can be searched rapidly with an add-on software tool for *metAlign* called Search_LCMS. Search_LCMS uses a simple library which consists of a compound name, a compound mass, a mass tolerance (Da), a retention time (min) and a retention time tolerance (min). A library was constructed from toxins identified in various shellfish and algae samples that were donated by various European laboratories. In total 85 different lipophilic marine toxins were identified and stored in this library. An advantage compared to commercial software tools is that *metAlign* and Search_LCMS are instrument-independent and can handle data formats of most MS manufacturers. Furthermore, the library can easily be updated with newly identified toxins and re-analysis of recorded data can be done within seconds.

Chromatographic separation

The retention that compounds experience on reversed phase HPLC columns is governed by their lipophilic properties and by the presence of polar or ionic groups, which interact with the stationary phase. The pH of the mobile phase is an important parameter, which can significantly influence the amount of interaction between the toxins and the stationary phase. Most methods described for lipophilic marine toxins in the literature make use of an acidic mobile phase system [11-13]. These acidic conditions result in poor peak shapes for yessotoxins (**chapter 3**). It

has been suggested that under acidic conditions secondary interactions such as ion -exchange of the sulphate anions with the protonated silanol functions of the column material occur. A multi-toxin method in which neutral chromatographic conditions are applied has also been described [13]. This method showed better peak shapes for the various toxins. Unfortunately, in our laboratory we obtained a broad peak for OA under these conditions. Furthermore, toxins that are preferably analyzed in positive or negative electrospray ionization mode, ESI^+ and ESI^-, respectively, overlap in most of the applied methods. If the mass spectrometer is capable of rapidly switching between polarities, this overlap of toxins is not necessarily a problem [12]. However, as only the latest generation mass spectrometers are capable of performing rapid polarity switching, alternatives are desirable. In order to improve the separation of toxins, first the type of column material was investigated. A silica C_{18} (Hypersil) column, which is most often used for lipophilic marine toxin analysis, was replaced by a cross-linked silica C_{18} (XBridge) column. According to the improved peak shape of YTX the amount of secondary interaction was significantly reduced (**chapter 3**). Still, some overlap of the toxins preferably analyzed in ESI^+ and ESI^- occurred. A great advantage of the cross-linked silica is its pH stability, even under alkaline conditions. Changing the mobile phase pH from acidic to alkaline had a significant effect on the elution order. Due to the charged state of the toxins, OA and DTXs showed a reduced retention, while SPXs and GYMs showed an increased retention. The retention of YTXs and PTXs was not affected. Under alkaline conditions a very good peak shape was obtained for YTX as well as for the other toxins. The toxins were separated in different retention windows that operate in either ESI^+ or ESI^-. Therefore, there is no need for rapid polarity switching or performing two separate analyzes of the sample. Another important achievement was the baseline separation of OA and DTX2. As these toxins have similar transitions, they will appear in the same MRM window. However, the toxicity of OA and DTX2 is different: the toxic equivalency factor (TEF) for OA is 1 and for DTX2 0.6 (Table 7.2) [14]. Therefore, it is important that these two toxins are well separated in order to correctly estimate the toxicity level of the shellfish sample. More in general, the sensitivity of the various toxins in the new method is comparable or improved compared to the methods that use acidic chromatographic conditions. The possibility to change from two

Table 7.2 Toxic equivalent factors of the various lipophilic marine toxins.

Toxin	Toxic equivalency factors	Equivalent to
Okadaic acid	1	Okadaic acid
Dinophysistoxin-1	1	Okadaic acid
Dinophysistoxin-2	0.6	Okadaic acid
Yessotoxin	1	Yessotoxin
1a-homo yessotoxin	1	Yessotoxin
45OH-yessotoxin	1	Yessotoxin
45OH 1a-homo yessotoxin	0.5	Yessotoxin
Azaspiracid-1	1	Azaspiracid-1
Azaspiracid-2	1.8	Azaspiracid-1
Azaspiracid-3	1.4	Azaspiracid-1

injections per sample to a single one, saves valuable time per series. For the Dutch routine monitoring program this is important because the results should be reported within 30h after receipt of the samples. This includes sample preparation, sample extraction and clean up, LC-MS/MS analysis, data analysis and reporting.

Removal of matrix effects

LC-MS/MS methods are known to be sensitive to matrix effects (signal suppression or enhancement). These effects may lead to an under- or overestimation of the toxin concentration present in shellfish. In the literature various techniques have been described to address matrix effect problems in lipophilic marine toxin analysis. The use of standard addition to the shellfish sample is one of them [15]. Although standard addition will compensate for the matrix effects, this approach is not feasible for routine monitoring. Only for available standards, standard addition can be performed and not all relevant toxins standards are available yet. Furthermore, the standards available are expensive and for standard addition relatively large amounts are needed. In general, the standard addition approach is expensive and time-consuming because each sample has to be analyzed at least twice. Another approach is the use of internal standards. Unfortunately, suitable internal standards are not available (yet). Therefore, sample clean up is the most feasible option. Different techniques such as liquid-liquid extraction (LLE) and solid phase extraction (SPE) have been used in the past to clean up methanolic shellfish extracts [16-18]. Within the BIOTOX project the first approach was based on LLE

with hexane and dichloromethane. However, results of a pre-validation round showed a poor performance with regard to repeatability and reproducibility [19]. As an alternative, an SPE method was developed (**chapter 4**). In the literature a few SPE methods have been described for the clean up of some lipophilic marine toxins. Our goal was to develop a method that could be applied for the complete lipophilic marine toxin class. Obviously, methods for each individual toxin group based on, for example ion exchange cartridges, would give the strongest reduction of matrix effects. However, this is not feasible for routine monitoring. Therefore, the focus was directed to polymeric sorbents that are capable of retaining a wide variety of toxins. Wash and elution steps were optimized in order to get a maximum reduction in matrix effects while maintaining good recoveries. The method was developed for all available standards (OA, YTX, AZA1, PTX2, SPX1 and GYM) in three different shellfish matrices: mussels, scallops and oysters. Overall recovery of the applied SPE method was good with an average of 90 ± 6% for all toxins. Residual matrix effects were determined for both the alkaline and acidic chromatographic conditions. The SPE clean up on the polymeric cartridges resulted in reduced matrix effects with both LC methods. However, only when SPE was used in combination with the alkaline method, the matrix effects were reduced to less than 15% for all toxins analyzed in their preferred mode and regardless of the matrix used. The reduction in matrix effects between the crude extract and extract after SPE was less substantial in combination with the acidic chromatography. The use of matrix-matched standards applied to the SPE procedure, appeared to give the appropriate correction for the recovery of this step. This approach was used in the validation of the complete method.

Validation of the developed method

The method developed in **chapter 3 and 4** was in-house validated for the quantitative analysis of lipophilic marine toxins in shellfish extracts (mussel, oyster, cockle and ensis) using the EU Commission Decision 2002/657/EC as guideline [9]. From the thirteen toxins described in EU legislation for nine (DTX1-3, 45OH-YTX, 1a-homo-YTX, 45OH-1a-homo-YTX, AZA2-3 and PTX1) certified reference standards are still lacking. Therefore, the validation included only the toxins OA, YTX, AZA1, PTX2 and SPX1. As the chemical structures of the lacking toxin

standards are closely related to the available standards, it is assumed that the response in the LC-MS/MS system is comparable. To some extend the validity of this assumption can be questioned as the different toxins are eluting in different places in the chromatogram and it may be expected that matrix effects or ionization efficiencies will differ. In our case the response of OA, DTX1-2 and AZA1-3 showed comparable ion ratios indicating that fragmentation pathways are more or less comparable. Validation was performed at 0.5, 1 and 1.5 times the currently permitted levels in the EU, which are 160 μg/kg for OA, AZA1 and PTX2 and 1000 μg/kg for YTX [2]. For SPX1 400 μg/kg was chosen as target level as no legislation has been established yet for this compound. The method was validated for determination in crude methanolic shellfish extracts and for extracts purified with SPE. Extracts were also subjected to alkaline hydrolysis in order to determine the performance of the method for the dinophysistoxin esters (DTX3). The toxins were quantified against a set of matrix-matched standards instead of standard solutions in methanol. In order to save valuable standards, the toxin standard mixture was spiked to a part of the crude methanolic extract instead of to the shellfish homogenate. This was justified by the fact that the extraction efficiency was high for all toxins tested (>90%). The method performed very well with respect to accuracy, intra-day precision (repeatability), inter-day precision (within-lab reproducibility), linearity, decision limit (CC_α), specificity and ruggedness. Recently the European Food Safety Authority (EFSA) has suggested an acute reference dose of 45 μg/kg OA-equivalents and 30 μg/kg AZA1-equivalents [14, 20]. For YTXs and PTXs EFSA suggested an acute reference dose of 3750 μg/kg and 120 μg/kg, respectively [21, 22]. These new levels for OA and AZA1 suggested by EFSA are substantial lower than the current EU levels. To test the method for these new levels, a single day validation was successfully conducted. In case regulatory levels will be lowered towards the new EFSA values, the official methods prescribed in legislation (mouse and rat bioassay) will no longer be sensitive enough. The new LC-MS/MS method will be able to replace those animal tests. Still, in the EU results for toxin concentrations are only officially accepted for those toxins for which certified standards are available and which were included in the validation. When the remaining toxins become commercially available, they can be included in the method by an additional single day validation. When the in-house validation is

performed according to international guidelines and good results in proficiency testing programs are obtained, sufficient data will be collected to withdraw the animal test for routine analysis and replace it by the LC-MS/MS method. Of course, an internationally validated and harmonized protocol is preferable. Therefore, efforts are currently ongoing to carry out a full collaborative study on the developed method. In 2009, a small pre-trial was organized for the analysis of OA, DTXs and AZAs with four laboratories. Excellent results were obtained even at low levels of OA and AZAs (Table 7.3) resulting in HorRat values for the between-lab reproducibility below 1.5.

The LC-MS/MS method for the analysis of lipophilic shellfish toxins presented in this thesis is able to replace the current animal tests that are still prescribed in EU legislation as the official method for these toxins. When this method is adopted as official EU method, the unethical mouse bioassay, which has been used for almost half a century, can finally be phased out. Moreover, a more extensive suite of lipophilic toxins can reliably be determined in one run, and with a higher sensitivity.

Table 7.3 Average concentrations found in the test samples analyzed by four labs during a pre-trial exercise.

Toxin	Sample 1 (μg/kg±SD)	Sample 2 (μg/kg±SD)	Sample 3 (μg/kg±SD)	Sample 4 (μg/kg±SD)
OA	9.5±2.7	36.4±4.4	13±2.4	83.3±9.9
DTX1		120.7±17.2	44.5±6.4	
DTX2	2.5±1.2	36.5±6.0	12±2.6	173.9±15.3
Sum DTXs including TEFs	10.5±3.6	179.0±22.7	63.1±9.3	191.3±19.5
AZA1	34.2±3.4			
AZA2	8.2±1.2			
AZA3	14.8±6.2			
Sum AZAs including TEFs	69.7±10.6			

Outlook

A collaborative study on the developed LC-MS/MS method will be performed in the course of 2010. If successful, it is expected that the results will serve as a basis for the implementation of the method as official method for the analysis of lipophilic marine toxins in EU legislation. Certified standards are still needed for DTX-1, DTX-2, DTX-3, AZA-2 and AZA-3. It is expected that some of these toxins will become available in the course of 2010. Globally there is only one supplier of these toxins. It would be advantageous for the lipophilic marine toxin field if more research on the production of toxic algae and toxin isolation was conducted, in particular in Europe. This would also serve the preparation of one or more certified reference materials for these shellfish toxins in Europe.

Other interesting marine toxin groups that are considered as interesting for future research are toxins responsible for Paralytic Shellfish Poisoning (PSP), palytoxins, ciguatera toxins, cyclic imines and brevetoxins. For Paralytic Shellfish Poisoning (PSP) a laborious LC fluorometric method (LC-FLD) is the only official alternative to the mouse bioassay [23]. Robust and sensitive LC-MS methods for the detection of PSP toxins are still lacking. For these toxins more efforts should be undertaken to develop confirmatory methods based on LC-MS.

Due to global warming it may be expected that in the near future algae responsible for the production of palytoxins, ciguatera toxins and brevetoxins can survive in European waters. For example, ciguatera toxins were previously only found in fish in the Pacific, Caribbean and Indian Ocean, but recently they have emerged in the Eastern Mediterranean Sea [24]. EFSA has very recently prepared scientific opinions on palytoxins, cyclic imines and ciguatera toxins [25-27]. In order to measure these relatively new toxin groups standards should be made available and method development and validation will be needed. In addition to LC-MS/MS methods it is desirable to have fast tests such as ELISA tests or functional assays available for the screening of toxins. The functional assays also have the potential of identifying toxins with a comparable structure or mode of action, something which is not readily possible with LC-MS/MS. It is desirable to develop such tests and make them available for use in the shellfish industry. Dipstick tests would be a preferred format for that purpose.

REFERENCES

[1] Fujiki H, Suganuma M, Yoshizawa S, Nishiwaki S, Winyar B, Sugimura T. Mechanisms of Action of Okadaic Acid Class Tumor Promoters on Mouse Skin. *Environ Health Persp* 1991; **93**: 211.

[2] European Parliament, Council. Commission directive 2004/853/EC specific hygiene rules for food of animal origin. *Off J Eur Commun* 2004; **L226**: 22.

[3] Llamas NM, Stewart L, Fodey T, Higgins HC, Velasco MLR, Botana LM, Elliott CT. Development of a novel immunobiosensor method for the rapid detection of okadaic acid contamination in shellfish extracts. *Anal Bioanal Chem* 2007; **389**: 581.

[4] Alfonso C, Alfonso A, Pazos MJ, Vieytes MR, Yasumoto T, Milandri A, Poletti R, Botana LM. Extraction and cleaning methods to detect yessotoxins in contaminated mussels. *Anal Bioanal Chem* 2007; **363**: 228.

[5] Ciminiello P, Dell'Aversano C, Fattorusso E, Forino M, Magno S, Santelia FU, Moutsos VI, Pitsinos EN, Couladouros EA. Oxazinins from toxic mussels: isolation of a novel oxazinin and reassignment of the C-2 configuration of oxazinin-1 and -2 on the basis of synthetic models. *Tetrahedron* 2006; **62**: 7738.

[6] Rundberget T, Sandvik M, Larsen K, Pizarro GM, Reguera B, Castberg T, Gustad E, Loader JI, Rise F, Wilkins AL, Miles CO. Extraction of microalgal toxins by large-scale pumping of seawater in Spain and Norway, and isolation of okadaic acid and dinophysistoxin-2. *Toxicon* 2007; **50**: 960.

[7] Peters RJB, Bolck YJC, Rutgers P, Stolker AAM, Nielen MWF. Multi-residue screening of veterinary drugs in egg, fish and meat using high-resolution liquid chromatography accurate mass time-of-flight mass spectrometry. *J Chromatogr A* 2009; **1216**: 8206.

[8] European Parliament, Council. Regulation (EC) no. 23/1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664/EEC. *Off J Eur Commun* 1996; **L125**: 10.

[9] European Parliament, Council. Regulation (EC) no. 657/2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Off J Eur Commun* 2002; **L221**: 8.

[10] Lommen A. MetAlign: interface-driven, versatile metabolomics tool for hyphenated full-scan mass spectrometry data preprocessing. *Anal Chem* 2009; **81**: 3079.

[11] McNabb P, Selwood AI, Holland PT. Multiresidue method for determination of algal toxins in shellfish: Single-laboratory validation and interlaboratory study. *J AOAC Int* 2005; **88**: 761.

[12] Fux E, McMillan D, Bire R, Hess P. Development of an ultra-performance liquid chromatography-mass spectrometry method for the detection of lipophilic marine toxins. *J Chromatogr A* 2007; **1157**: 273.

[13] Stobo LA, Lacaze JPCL, Scott AC, Gallacher S, Smith EA, Quilliam MA. Liquid chromatography with mass spectrometry - detection of lipophilic shellfish toxins. *J AOAC Int* 2005; **88**: 1371.

[14] Alexander J, Audunsson GA, Benford D, Cockburn A, Cradevi J-P, Dogliotti E, Domenico AD, Fernandez-Cruz ML, Fink-Gremmels J, Furst P, Galli C, Grandjean P, Gzyl J, Heinemeyer G, Johansson N, Mutti A, Schlatter J, van Leeuwen R, van Peteghem C, Verger P. Marine biotoxins in shellfish - okadaic acid and analogues. *The EFSA Journal* 2008; **589**: 1.

[15] Ito S, Tsukada K. Matrix effect and correction by standard addition in quantitative liquid chromatographic-mass spectrometric analysis of diarrhetic shellfish poisoning toxins. *J Chromatogr A* 2002; **943**: 39.

[16] Jorgensen K, Jensen LB. Distribution of diarrhetic shellfish poisoning toxins in consignments of blue mussel. *Food Addit Contam A* 2004; **21**: 341.

[17] Puente PF, Saez MJF, Hamilton B, Lehane M, Ramstad H, Furey A, James KJ. Rapid determination of polyether marine toxins using liquid chromatography-multiple tandem mass spectrometry. *J Chromatogr A* 2004; **1056**: 77.

[18] Vale P, Sampayo MAD. Esterification of DSP toxins by Portuguese bivalves from the Northwest coast determined by LC-MS - a widespread phenomenon. *Toxicon* 2002; **40**: 33.

[19] Van Loco J, Kotterman MJJ, Hess P *Report on results of the validation study (D29), within the 6th Framework project 'BIOTOX: Development of cost-effective tools for risk management and traceability systems for marine biotoxins in seafood' Contract no: 514074* 2008.

[20] Alexander J, Benford D, Cockburn A, Cradevi J-P, Dogliotti E, Domenico AD, Fernandez-Cruz ML, Fink-Gremmels J, Furst P, Galli C, Grandjean P, Gzyl J, Heinemeyer G, Johansson N, Mutti A, Schlatter J, van Leeuwen R, van Peteghem C, Verger P. Marine biotoxins in shellfish - azaspiracid group. *The EFSA Journal* 2008; **723**: 1.

[21] Alexander J, Benford D, Cockburn A, Cradevi J-P, Dogliotti E, Domenico AD, Fernandez-Cruz ML, Fink-Gremmels J, Furst P, Galli C, Grandjean P, Gzyl J, Heinemeyer G, Johansson N, Mutti A, Schlatter J, van Leeuwen R, van Peteghem C, Verger P. Marine biotoxins in shellfish - yessotoxin group. *The EFSA Journal* 2008; **907**: 1.

[22] Alexander J, Benford D, Cockburn A, Cradevi J-P, Dogliotti E, Domenico AD, Fernandez-Cruz ML, Fink-Gremmels J, Furst P, Galli C, Grandjean P, Gzyl J, Heinemeyer G, Johansson N, Mutti A, Schlatter J, van Leeuwen R, van Peteghem C, Verger P. Marine biotoxins in shellfish - pectenotoxin group. *The EFSA Journal* 2009; **1109**: 1.

[23] Lawrence JF, Niedzwiadek B, Menard C. Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid chromatography with fluorescence detection: collaborative study. *J AOAC Int* 2005; **88**: 1714.

[24] Aligizaki K, Nikolaidis G. Morphological identification of two tropical dinoflagellates of the genera *Gambierdiscus* and *Sinophysis* in the Mediterranean Sea. *J Biol Res-Thessalon* 2008; **9**: 75.

[25] Alexander J, Benford D, Cockburn A, Cradevi J-P, Dogliotti E, Domenico AD, Fernandez-Cruz ML, Fink-Gremmels J, Furst P, Galli C, Grandjean P, Gzyl J, Heinemeyer G, Johansson N, Mutti A, Schlatter J, van Leeuwen R, van Peteghem C, Verger P. Marine biotoxins in shellfish - ciguatoxins. *The EFSA Journal* 2010; **1627**: 1.

[26] Alexander J, Benford D, Boobis A, Ceccatelli S, Cradevi J-P, Domenico AD, Doerge D, Dogliotti E, Edler L, Farmer P, Filipic M, Fink-Gremmels J, Furst P, Guerin T, Knutsen HK, Machala M, Mutti A, Schlatter J, van Leeuwen R, Verger P. Marine biotoxins in shellfish - palytoxin group. *The EFSA Journal* 2009; **1393**: 1.

[27] Alexander J, Benford D, Cockburn A, Cradevi J-P, Dogliotti E, Domenico AD, Fernandez-Cruz ML, Fink-Gremmels J, Furst P, Galli C, Grandjean P, Gzyl J, Heinemeyer G, Johansson N, Mutti A, Schlatter J, van Leeuwen R, van Peteghem C, Verger P. Marine biotoxins in shellfish - cyclic imines. *The EFSA Journal* 2010; **1628**: 1.

Summary

Marine toxins (phycotoxins) are natural toxins produced by at least 40 species of algae belonging to the classes of dinoflagellates and diatoms [1]. Blooms of these toxic phytoplankton species are named harmful algae blooms (HABs). Phycotoxins can accumulate in various marine species such as fish, crabs or filter feeding bivalves (shellfish) such as mussels, oysters, scallops and clams. In shellfish, toxins mainly accumulate in the digestive glands without causing adverse effects on the shellfish itself. However, when substantial amounts of contaminated shellfish are consumed by humans, this may cause severe intoxication of the consumer [2-4]. Based on their chemical properties marine toxins can be divided in two different classes: hydrophilic and lipophilic toxins. Toxins associated with the syndromes Amnesic Shellfish Poisoning (ASP) and Paralytic Shellfish Poisoning (PSP) are hydrophilic by nature and have a molecular weight (MW) below 500 Da. Toxins responsible for Neurologic Shellfish Poisoning (NSP), Diarrhetic Shellfish Poisoning (DSP), Azaspiracid Shellfish Poisoning (AZP) and other toxins such as pectenotoxins (PTXs), yessotoxins (YTXs) and cyclic imines [spirolides (SPX) and gymnodimine] all have as common denominator a MW above 600 Da (up to 2 000 Da). These toxins have strong lipophilic properties and are generally called lipophilic marine toxins. EU legislation prescribes animal tests (mouse or rat) as the official method for control of lipophilic marine toxins in shellfish [5]. More than 10 000 test animals (mostly mice) are used annually for routine monitoring of lipophilic marine toxins in shellfish within the European Union (EU). Besides the ethical aspects of this cruel animal test, it also contradicts with other EU legislation which states the reduction, refinement and replacement of animal tests [6]. Furthermore, these animal tests can produce false positive results and have a poor sensitivity and selectivity. In this thesis the development of an alternative method for the determination of lipophilic marine toxins is described, based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

In **chapter 2** the performance characteristics of four different mass spectrometers [triple-quadrupole (TQ), time-of-flight (ToF), quadrupole time-of-flight (QToF) and ion trap (IT)] for the detection of the lipophilic marine toxins okadaic acid (OA) and pectenotoxin-2 (PTX2) were investigated. The spectral data obtained with the different mass spectrometric analyzers were used to propose fragmentation

schemes for OA in negative electrospray (ESI^-) mode and PTX2 in positive electrospray (ESI^+) mode. TQ-MS was used to obtain product ions, while ToF and QToF-MS produced accurate mass data of the precursor ion and product ions, respectively. IT-MS data obtained from MS^n experiments provided a better understanding of the fragmentation pathways. With respect to analytical performance, TQ instruments produced the lowest detection limits, and were the most robust. Therefore, the TQ-MS was used for the development of a routine method.

In **chapter 3** a new LC-MS/MS method for the separation and detection of the most prominent marine lipophilic toxin groups comprising OA, dinophysistoxins (DTXs), yessotoxins (YTXs), azaspiracids (AZAs), pectenotoxins (PTXs), spirolides (SPXs) and okadaic acid fatty acid esters (DTX3) is described. With this method 28 different lipophilic marine toxins can be analyzed in a single run. Separation is achieved with an acetonitrile/water gradient containing ammonium hydroxide (pH 11). Traditionally, LC-MS/MS methods used acidic chromatographic conditions for the determination of lipophilic marine toxins. However, under acidic conditions peak shapes as well as separation of some toxins was poor. With the alkaline chromatographic conditions the limit of detection (LOD) for OA, yessotoxin (YTX), gymnodimine (GYM) and 13-desmethyl spirolide C (SPX1) was improved two- to three-fold. This improvement is mainly due to improved peak shapes. A major advantage of the developed method is that toxins can be clustered in retention time windows separated for positively and negatively ionized molecules. Therefore, there is no need for rapid polarity switching or for two separate runs to analyze a sample. For the new method a very good repeatability and reproducibility was obtained.

It is well known that LC-MS/MS analysis is sensitive for matrix effects (signal suppression or enhancement). This is also the case for lipophilic marine toxins. Therefore, in **chapter 4** the potential of solid phase extraction (SPE) clean up has been assessed to reduce matrix effects in the analysis of lipophilic marine toxins. A large array of ion-exchange, silica-based and mixed function SPE sorbents was tested. The toxins were best retained on polymeric sorbents. Optimization

experiments were carried out to maximize recoveries and the effectiveness of the clean up. This was done by optimization of the wash and elution conditions. Matrix effects were assessed using either an acidic or an alkaline chromatographic system as described in **chapter 3**. In combination with the alkaline LC method this resulted in a substantial reduction of matrix effects to less than 15%, while in combination with the acidic LC method approximately 30% of the matrix effects remained. The use of SPE resulted in a reduction of matrix effects with both LC methods but in combination with alkaline conditions the SPE method was the most effective.

Before a method can be officially used in the EU for routine analysis, the method needs to be validated. In **chapter 5** the in-house validation is described for the quantitative analysis of OA, YTX, AZA1, PTX2 and SPX1 in shellfish extracts (mussel, oyster, cockle and ensis). Dinophysistoxin-1, -2 and azaspiracid-2 and -3 were not included in the study because the certified standards were not available. The validation was performed using the EU Commission Decision 2002/657/EC as guideline. Validation was performed at 0.5, 1 and 1.5 times the current EU permitted levels, which are 160 μg/kg for OA, AZA1 and PTX2 and 1 000 μg/kg for YTX. For SPX1 400 μg/kg was chosen as target level as no legislation has been established yet for this compound. The method was validated for determination in crude methanolic shellfish extracts and for extracts purified with solid phase extraction (SPE). The toxins were quantified against a set of matrix matched standards instead of standard solutions in methanol. In order to save valuable standard the toxin standards were spiked to the methanolic extract instead of the shellfish homogenate. This was justified by the fact that the extraction efficiency is high for all relevant toxins (>90%). The method performed very well with respect to accuracy, intra-day precision (repeatability), inter-day precision (within-lab reproducibility), linearity, decision limit (CC_α), specificity and ruggedness. For crude extracts the method performed less satisfactory with respect to the linearity (<0.990) and the change in LC-MS/MS sensitivity during the series (>25%). This decrease in sensitivity could be attributed to contamination of the LC-MS/MS system. SPE purification resulted in a greatly improved linearity and signal stability during the series. Recently the European Food Safety Authority (EFSA) has published a number of opinions on the various toxin groups. The EFSA has

suggested that in order not to exceed the acute reference dose the levels should be below 45 μg/kg OA-equivalents and 30 μg/kg AZA1-equivalents. If these levels are adapted in legislation this means a 4-5 fold lower permitted limit than the current one. For these toxins q single day validation was successfully conducted at these levels.

In **chapter 6** a method is described which can be used to screen shellfish and algae samples for the presence of lipophilic marine toxins. The method described in the other chapters was mainly focussing on the 13 toxins stated in EU legislation. However, in the literature more than 200 different lipophilic marine toxins have been described. These 200 toxins cover of a wide variety of OA, YTX, AZA and PTX group toxins. To fill this gap, a LC coupled to high resolution (HR) orbitrap MS (resolution 100 000) screening method was developed. Shellfish and algae samples with various toxin profiles were obtained from Norway, Ireland, United Kingdom, Portugal and Italy. Based on their accurate mass and specific fragmentation pattern, 85 different toxins were detected, of which 33 OA, 26 YTX, 18 AZA and 8 PTX group toxins. A major drawback of full scan HRMS is the amount of data generated (file size), which makes it difficult to perform a fast search on the toxins. Therefore, the software program *metAlign* has been used to reduce the orbitrap MS files 200-fold by performing a baseline correction and noise elimination. These reduced data files were searched using an additional software tool for *metAlign*: 'Search_LCMS'. A search library was constructed for the 85 identified toxins. The library contains information about compound name, accurate mass, mass tolerance (Da), retention time (min) and retention time tolerance (min). In order to test the screening procedure a set of known positive and blank samples was analyzed and processed with *metAlign* and searched with Search_LCMS. For the positive samples the toxin profiles were determined. No toxins were found in the blank sample, in line with the routine monitoring program results (rat bioassay and LC-MS/MS).

Outlook

Now the developed method has been in-house validated, the next step will be a full collaborative study. If the outcome of that study is satisfactorily, the method

described in this thesis can be adopted in EU legislation and the mouse and rat bioassay can be finally abolished. Furthermore, research is needed for the production and isolation of lipophilic marine toxins and method development on functional assays and other new emerging toxins such as palytoxins, cyclic imines and ciguatera toxins.

REFERENCES

[1] Gerssen A, Pol-Hofstad IE, Poelman M, Mulder PPJ, van den Top HJ, de Boer J. Marine toxins: Chemistry, toxicity, occurence and detection, with special reference to the Dutch situation. *Toxins* 2010; **2**: 878.

[2] Aune T, Yndestad M, Diarrhetic shellfish poisoning. In *Algal toxins in seafood and drinking water*, Falconer IR, Ed. Academic Press: London, 1993; pp 87.

[3] Jeffery B, Barlow T, Moizer K, Paul S, Boyle C. Amnesic shellfish poison. *Food Chem Toxicol* 2004; **42**: 545.

[4] Botana AM, Rodriguez Veijtes M, Alfonso A, Louzao M, Phycotoxins: paralytic shellfish poisoning and diarrhetic shellfish poisoning. In *Handbook of food analysis - Residues and other food component analysis*, Nollet LML, Ed. Dekker: New York, 1996; pp 1147.

[5] European Parliament, Council. Laying down implementing measures for certain products under Regulation (EC) No 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No 854/2004 of the European Parliament and of the Council and Regulation (EC) No 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004. *Off J Eur Commun* 2005; **L338**: 40.

[6] European Parliament, Council. Regulation (EC) no. 609/1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. *Off J Eur Commun* 1986; **L358**: 1.

Samenvatting

Mariene toxines (fycotoxines) zijn natuurlijke toxines die worden geproduceerd door tenminste een 40-tal algensoorten die behoren tot de groep dinoflagellaten en diatomeeën [1]. Een bloei van deze toxische fytoplankton soorten wordt 'harmful algae bloom' (HAB) genoemd. Mariene toxines kunnen accumuleren in vis, krabben en schelpdieren zoals mosselen, oesters en andere tweekleppigen die zich voeden door het filtreren van zeewater. In schelpdieren accumuleren de toxines voornamelijk in het spijsverteringskanaal zonder dat dit nadelige effecten heeft voor het schelpdier zelf. Wanneer deze schelpdieren echter worden geconsumeerd door mens of dier kan dit tot een vergiftigingssyndroom leiden [2-4]. Gebaseerd op de chemische eigenschappen kunnen de mariene toxines in twee groepen worden ingedeeld: hydrofiele en lipofiele toxines. Toxines verantwoordelijk voor de vergiftigingsyndromen amnesische schelpdiervergiftiging (ASP) en paralytische schelpdiervergiftiging (PSP) zijn wateroplosbaar en hebben een molmassa onder de 500 Da. Toxines verantwoordelijk voor neurologische schelpdiervergiftiging (NSP), diaretische schelpdiervergiftiging (DSP) en azaspiracide schelpdiervergiftiging (AZP) en andere toxines zoals pectenotoxines (PTXs), yessotoxines (YTXs) en cyclische imines [spirolides (SPX) en gymnodinium] hebben als gemeenschappelijke eigenschap een molmassa boven de 600 Da (tot ongeveer 2 000 Da). Verder zijn deze toxines goed vet oplosbaar (lipofiel). Daarom worden deze toxines ook wel lipofiele mariene toxines genoemd. Wetgeving in de Europese Unie (EU) schrijft diertesten (muis of rat) voor als de officiële methode voor de controle van schelpdieren op de aanwezigheid van lipofiele mariene toxines [5]. Voor de routine analyze van mariene toxines in schelpdieren worden in de EU jaarlijks meer dan 10 000 proefdieren (voornamelijk muizen) gebruikt. Het gebruik van deze dieronvriendelijke test is in tegenspraak met andere EU wetgeving die de vermindering, verfijning en vervanging van dierproeven voorschrijft [6]. Verder kunnen de dierproeven vals positieve resultaten opleveren en heeft de test een lage gevoeligheid en selectiviteit. In dit proefschrift is de ontwikkeling van een alternatieve methode voor de bepaling van lipofiele schelpdiertoxinen beschreven, die gebaseerd is op vloeistof chromatografie gekoppeld aan tandem massaspectrometrie (LC-MS/MS).

In **hoofdstuk 2** zijn de prestatiekenmerken van vier verschillende massaspectrometers met elkaar vergeleken voor de detectie van okadaic acid (OA) en pectenotoxine-2 (PTX2). De massaspectrometers die gebruikt zijn, zijn een triple quadrupole (TQ), time-of-flight (ToF), quadrupole time-of-flight (QToF) en een ion-trap (IT). De spectrale data verkregen met de vier verschillende instrumenten zijn gebruikt om fragmentatieschema's op te stellen voor OA en PTX2 in respectievelijk negatieve electrospray ionisatie mode (ESI^-) en positieve ionisatie mode (ESI^+). De TQ-MS is gebruikt om product ionen te bepalen terwijl de ToF en QToF-MS zijn gebruikt om de accurate massa te bepalen van respectievelijk de precursor en product ionen. De IT-MS heeft geholpen om met behulp MS^n experimenten de fragmentatiepatronen beter te begrijpen. De TQ-MS had de beste prestatiekenmerken zoals de laagste detectie limiet en het systeem is erg robuust. Daarom is voor het vervolgonderzoek, de ontwikkeling van een routine methode, gebruik gemaakt van een TQ-MS.

In **hoofdstuk 3** is een nieuwe LC-MS/MS methode beschreven voor de scheiding en detectie van de belangrijkste groep van lipofiele mariene toxines, bestaande uit OA, dinophysistoxines (DTXs), yesstoxines (YTXs), azaspiraciden (AZAs), pectenotoxines (PTXs), SPXs en vetzuuresters van OA (DTX3). Met de nieuw ontwikkelde LC-MS/MS methode is het mogelijk om in een enkele analyse 28 verschillende lipofiele mariene toxines te analyseren. De nieuw ontwikkelde methode maakt gebruik van een gradiënt water/acetonitril/ammoniumhydroxide met een alkalische pH (pH 11). Traditioneel worden chromatografische scheidingen van lipofiele mariene toxines gedaan onder zure omstandigheden. Onder deze condities is de piekvorm en scheiding van enkele toxines echter matig. Onder alkalische condities is de detectielimiet voor OA, YTX, gymnodinium (GYM) en 13-desmethyl spirolide C (SPX1) een factor twee tot drie lager, als gevolg van een sterk verbeterde piekvorm. Het grootste voordeel van de ontwikkelde methode is dat de toxines die bij voorkeur in de negatieve mode ioniseren en de toxinen die in de positieve mode ioniseren in het chromatogram bij elkaar clusteren in retentietijdsegmenten. Hierdoor is het niet noodzakelijk om tijdens de analyse snel van polariteit te kunnen wisselen óf om twee aparte analyses uit te voeren op het monster. De nieuw ontwikkelde methode heeft een goede herhaalbaarheid en reproduceerbaarheid.

Het is bekend dat LC-MS/MS analyse gevoelig is voor matrixeffecten (signaal onderdrukking of versterking). Dit is ook het geval voor de analyse van lipofiele mariene toxinen. Daarom is in **hoofdstuk 4** een solid phase extractie (SPE) methode ontwikkeld. Een scala aan ionenwisselaars, op silica gebaseerde en mixed function SPE sorbentia is getest. Polymere sorbentia geven de meest optimale retentie voor de verschillende toxines. De SPE procedure is geoptimaliseerd voor de meest effectieve opschoning en de hoogste terugvinding. Dit is gedaan door de was- en elutie-omstandigheden te optimaliseren. Matrixeffecten zijn bepaald met behulp van de traditionele LC-MS/MS methode onder zure condities en met de nieuw ontwikkelde LC-MS/MS methode uit **hoofdstuk 3** onder basische condities. Combinatie van de SPE procedure en de chromatografie onder alkalische omstandigheden resulteerde in matrixeffecten van minder dan 15%, terwijl met de zure chromatografie nog steeds matrixeffecten ter grootte van 30% werden gevonden. SPE leverde voor beide chromatografische methoden een reductie van matrixeffecten op, maar in combinatie met de alkalische chromatografie werden de beste resultaten behaald.

Voordat een methode officieel binnen de EU kan worden toegepast zal deze gevalideerd dienen te worden. In **hoofdstuk 5** is de methode in-huis gevalideerd voor de kwantitatieve bepaling van OA, YTX, AZA1, PTX2 en SPX1 in schelpdierextracten (mossel, oester, kokkel en ensis). In verband met het ontbreken van gecertificeerde standaarden voor dinophysistoxine-1, -2 en azaspiracide-2 en -3 zijn deze toxines niet in de validatiestudie meegenomen. De validatie is uitgevoerd volgens de richtlijnen van EU document Commission Decision 2002/657/EC. De validatie is uitgevoerd op 0.5, 1 en 1.5 keer de toegestane limiet voor de verschillende toxines. Voor OA, AZA1 en PTX2 is de toegestane limiet 160 μg/kg en voor YTX 1000 μg/kg. Voor SPX1 is nog geen officiële limiet vastgesteld en is een streefwaarde van 400 μg/kg gekozen. De validatie is uitgevoerd voor zowel ruwe methanolextracten alsmede voor met SPE opgeschoonde methanolextracten. De verschillende toxines werden gekwantificeerd ten opzichte van standaardoplossingen in blanco schelpdierextract in plaats van tegen een reeks standaardoplossingen in methanol. Om de relatief dure standaarden te sparen is er tijdens de validatie gespiked aan het extract in

plaats van aan het schelpdierhomogenaat. Dit is gerechtvaardigd omdat de extractie-efficiëntie voor alle toxines hoog is (>90%). De methode presteerde uitstekend voor de opgeschoonde extracten op het gebied van accuraatheid, herhaalbaarheid, reproduceerbaarheid, lineariteit, beslissingslimiet, specificiteit en robuustheid. Voor de ruwe methanolextracten was met name de lineariteit minder goed (<0.990) en was er een groter verloop van gevoeligheid (>25%). Dit was te wijten aan de vervuiling van het LC-MS/MS systeem, iets wat bij de opgeschoonde extracten geen probleem was. Recentelijk zijn door de Europese autoriteit voor voedselveiligheid (EFSA) een aantal opinies uitgebracht over de verschillende groepen lipofiele mariene toxines. Om de acute referentie dosis niet te overschrijden mag men volgens de EFSA maximaal 45 μg/kg OA of 30 μg/kg AZA1 consumeren. Dit betekent dat, als de wetgeving hierop wordt aangepast, de toegestane limiet een factor 4-5 lager gaat worden. Er is voor deze toxines een succesvolle aanvullende validatie uitgevoerd op deze lagere niveaus.

In **hoofdstuk 6** is een methode beschreven die gebruikt kan worden om schelpdiermonsters en algen kwalitatief te screenen op de aanwezigheid van lipofiele mariene toxinen. De methode zoals beschreven in de voorgaande hoofdstukken richt zich voornamelijk op de 13 toxinen die in de EU wetgeving zijn opgenomen. Maar in de literatuur zijn inmiddels meer dan 200 lipofiele mariene toxinen beschreven. De lijst van 200 toxines omvat een grote variëteit aan OA, DTXs, YTXs, AZAs en PTXs. Om dit gat te dichten is er een LC- hoge resolutie orbitrap MS (resolutie 100 000) methode ontwikkeld. Schelpdier- en algenmonsters met verschillende toxinenprofielen werden verkregen uit Noorwegen, Ierland, Engeland, Portugal en Italië. Gebaseerd op de accurate massa en het specifieke fragmentatiepatroon zijn er 85 verschillende toxines gevonden waarvan 33 OA en DTXs, 26 YTXs, 18 AZAs en 8 PTXs. Een groot nadeel van hoge resolutie MS is de grootte van de databestanden, wat de mogelijkheden beperkt om snel data te kunnen doorzoeken op de verschillende toxines. Daarom is gebruik gemaakt van het softwareprogramma '*metAlign*' om de orbitrap data met een factor 200 te reduceren, door middel van basislijncorrectie en de verwijdering van ruis. Deze gereduceerde databestanden kunnen vervolgens snel worden doorzocht met behulp van Search_LCMS, een

additionele tool voor *metAlign*. De 85 geïdentificeerde toxines zijn in een zoekbibliotheek samen gebracht. Deze zoekbibliotheek bevat de componentnaam, de exacte massa, toegestane massa afwijking (Da), retentietijd (min) en toegestane afwijking van de retentietijd (min). Om de gecreëerde bibliotheek te testen is een set positieve en blanco schelpdiermonsters geanalyseerd. De data daarvan werden verwerkt met *metAlign* en vervolgens doorzocht met Search_LCMS. Voor de positieve monsters kon een toxineprofiel worden bepaald. In het blanco monster werd ook in deze screening geen toxinen aangetroffen dit is in overeenstemming met de resultaten van het routine monitoring programma (rattest en LC-MS/MS).

Vooruitzicht

Nu de methode is uitontwikkeld en in-huis is gevalideerd, is de volgende stap een gezamelijke inter-laboratorium validatiestudie. Als deze is uitgevoerd en de resultaten voldoen, wordt het mogelijk de methode, die in dit proefschrift is beschreven, in de EU wetgeving op te nemen en kunnen de muis- en rattest eindelijk worden afgeschaft. Daarnaast is er meer onderzoek nodig naar de productie en isolatie van lipofiele mariene toxines en methodeontwikkeling op het gebied van functionele assays en naar nieuwe toxines zoals palytoxines, cyclische imines en ciguatera toxines.

REFERENTIES

[1] Gerssen A, Pol-Hofstad IE, Poelman M, Mulder PPJ, van den Top HJ, de Boer J. Marine toxins: Chemistry, toxicity, occurence and detection, with special reference to the Dutch situation. *Toxins* 2010; **2**: 878.

[2] Aune T, Yndestad M, Diarrhetic shellfish poisoning. In *Algal toxins in seafood and drinking water*, Falconer IR, Ed. Academic Press: London, 1993; pp 87.

[3] Jeffery B, Barlow T, Moizer K, Paul S, Boyle C. Amnesic shellfish poison. *Food Chem Toxicol* 2004; **42**: 545.

[4] Botana AM, Rodriguez Veijtes M, Alfonso A, Louzao M, Phycotoxins: paralytic shellfish poisoning and diarrhetic shellfish poisoning. In *Handbook of food analysis - Residues and other food component analysis*, Nollet LML, Ed. Dekker: New York, 1996; pp 1147.

[5] European Parliament, Council. Laying down implementing measures for certain products under Regulation (EC) No 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No 854/2004 of the European Parliament and of the Council and Regulation (EC) No 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004. *Off J Eur Commun* 2005; **L338**: 40.

[6] European Parliament, Council. Regulation (EC) no. 609/1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. *Off J Eur Commun* 1986; **L358**: 1.

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Arjen

About the author

CURRICULUM VITAE

Arjen Gerssen was born on the 21st of March 1979 in Den Helder. In 1995 he finished his school for lower general secondary education (MAVO) at the OSG Nieuwediep in Den Helder. In the same year he started intermediate vocational education with specialization chemistry at the Bakhuis Roozeboom Institute in Beverwijk (MLO). During this study he performed two internships. At Mallinckrodt Medical in Petten he transferred LC methods to new equipment and studied the performance criteria. During the second internship performed at Corus in IJmuiden he worked on a routine quality control laboratory for the analysis of all types of waters (cooling, process, waste and drinking water). In 1999 he received his diploma and continued with a bachelor study Analytical Chemistry at Hogeschool Alkmaar in Alkmaar (HLO). In 2000 he decided to combine this study with courses at the VU University in Amsterdam. In 2001 he started his thesis research for his bachelor degree on the VU University at the department for Analytical Chemistry and Applied Spectroscopy on the at-line coupling of capillary electrophoresis and surface enhanced Raman spectroscopy. This research resulted in two peer reviewed papers. In 2002 he received his BSc degree in Alkmaar and continued with the master study Analytical Chemistry and Applied Spectroscopy at the VU University in Amsterdam. He did his master thesis research on fast excited-state intramolecular proton transfer resulting in a peer reviewed publication. In October 2004 he received his MSc degree. In January 2005 he was appointed at RIKILT Institute of Food Safety in Wageningen on the FP6 EU project BIOTOX. Research conducted in this EU project resulted in this thesis. At 1st of March 2009 Arjen started as scientific researcher at RIKILT.

LIST OF PUBLICATIONS

Gerssen A, Mulder PPJ, de Boer J. Screening of lipophilic marine toxins in shellfish and algae using liquid chromatography coupled to orbitrap mass spectrometry. 2010; submitted.

Gerssen A, van Olst EHW, Mulder PPJ, de Boer J. In-house validation of a liquid chromatography tandem mass spectrometry method for the analysis of lipophilic marine toxins in shellfish using matrix matched standards. *Anal Bioanal Chem* 2010; **397**: 3079.

Gerssen A, Pol-Hofstad IE, Poelman M, Mulder PPJ, van den Top HJ, de Boer J. Marine Toxins: Chemistry, Toxicity, Occurrence and Detection, with Special Reference to the Dutch Situation. *Toxins* 2010; **2**: 878

Gerssen A, McElhinney MA, Mulder PPJ, Bire R, Hess P, de Boer J. Solid phase extraction for removal of matrix effects in lipophilic marine toxin analysis by liquid chromatography -tandem mass spectrometry. *Anal Bioanal Chem* 2009; **394**: 1213.

Gerssen A, Mulder PPJ, McElhinney MA, de Boer J. Liquid chromatography-tandem mass spectrometry method for the detection of marine lipophilic toxins under alkaline conditions. *J Chromatogr A* 2009; **1216**: 1421.

Gerssen A, Mulder P, van Rhijn H, de Boer J. Mass spectrometric analysis of the marine lipophilic biotoxins pectenotoxin-2 and okadaic acid by four different types of mass spectrometers. *J Mass Spectrom* 2008; **43**: 1140.

Lommen A, Gerssen A, Oosterink JE, Kools HJ, Ruiz-Aracama A, Peters RJB, Mol HGJ. Ultra -fast searching assists in evaluating sub-ppm mass accuracy enhancement in U-HPLC/Orbitrap MS data. *Metabolomics* 2010; In press

Mol H, Lommen A, Zomer P, van der Kamp H, van der Lee M, Gerssen A. Data handling and validation in automated detection of food toxicants using full scan GC-MS and LC-MS. *LC-GC Europe* 2010; **23**

Smoluch M, Joshi H, Gerssen A, Gooijer C, van der Zwan G. Fast excited-state intramolecular proton transfer and subnanosecond dynamic stokes shift of time-resolved fluorescence spectra of the 5-methoxysalicylic acid/diethyl ether complex. *J Phys Chem A* 2005; **109**: 535.

Dijkstra RJ, Gerssen A, Efremov EV, Ariese F, Brinkman UAT, Gooijer C. Substrates for the at -line coupling of capillary electrophoresis and surface-enhanced Raman spectroscopy. *Anal Chim Acta* 2004; **508**: 127.

Seifar RM, Dijkstra RJ, Gerssen A, Ariese F, Brinkman UAT, Gooijer C. At-line coupling of capillary electrophoresis and surface-enhanced resonance Raman spectroscopy. *J Sep Sci* 2002; **25**: 814.

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Conferences and meetings

EFFI symposium, Developments in the field of food safety, Wageningen, The Nettherlands, 2005, oral presentation.

QUASIMEME workshop on shellfish toxin analysis, Nantes, France, 2006, oral presentation.

Dutch toxicology days, Wageningen, The Netherlands, 2006, poster presentation.

International workshop on liquid chromatography – tandem mass spectrometry for screening and trace level quatitation in environmental and food samples, Barcelona, Spain, 2006, oral presentation.

International Symposium on Recent Advances in Food Analysis: Prague, Czech Republic, 2007, poster presentation.

Safefood Biotoxin Research Network Meeting, Belfast, Ireland, 2008.

Contributed with oral presentations to the EU BIOTOX meetings

Progress meeting, Galway, Ireland, 2005

Progress meeting, Nantes, France, 2006

Midterm meeting, Brussels, Belgium, 2006

Progress meeting, Wageningen, The Netherlands, 2007

Progress meeting, Istanbul, Turkey, 2007

Final project meeting, Oslo, Norway, 2008

Courses

Postgraduate education in toxicology

Food toxicology, Wageningen, The Netherlands, 2005

Toxicological risk assessment, Wageningen, The Netherlands, 2005

Epidemiology, Utrecht, The Netherlands, 2006

Legal and regulatory toxicology, Utrecht, The Netherlands, 2006

Cell toxicology, Leiden, The Netherlands, 2006

Laboratory animal science, Utrecht, The Netherlands, 2007

Pathobiology, Utrecht, The Netherlands, 2007

Organ toxicology, Utrecht, The Netherlands, 2007

Mutagenesis and carcinogenesis, Leiden, The Netherlands, 2007

Toxicogenomics, Maastricht, The Netherlands, 2008

Other courses

Techniques for writing and presenting a scientific paper, Wageningen business school, Wageningen, The Netherlands, 2005

Acquity UPLC chemistry, instrumentation and software, Waters, Etten-Leur, The Netherlands, 2005

Project management, Wageningen UR, Wageningen, The Netherlands, 2010

Additional activities

Organisation of EU BIOTOX workshop on the feasibility study on certification of two biotoxin reference materials, Wageningen, The Netherlands, 2007

Internship at Center for Coastal Environmental Health and Biomolecular Research, National Ocean Service, National Oceanic and Atmospheric Administration, Charleston, South Carolina, USA, September 2006 – December 2006

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