DEVELOPMENT AND VALIDATION OF A NOVEL APPROACH FOR THE ANALYSIS OF MARINE BIOTOXINS

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ABSTRACT

Harmful algal blooms (HABs) which can produce a variety of marine biotoxins are a prevalent and growing risk to public safety. The aim of this research was to investigate, evaluate, develop and validate an analytical method for the detection and quantitation of five important groups of marine biotoxins in shellfish tissue. These groups included paralytic shellfish toxins (PST), amnesic shellfish toxins (AST), diarrheic shellfish toxins (DST), azaspiracids (AZA) and neurotoxic shellfish toxins (NST).

A novel tandem liquid chromatographic (LC) approach using hydrophilic interaction chromatography (HILIC), aqueous normal phase (ANP), reversed phase (RP) chromatography, tandem mass spectrometry (MSMS) and fluorescence spectroscopic detection (FLD) was designed and tested. During method development of the tandem LC setup, it was found that HILIC and ANP columns were unsuitable for the PSTs because of the lack of chromatographic separation power, precluding them from being used with MSMS detection. In addition, sensitivity for the PSTs at regulatory limits could not be achieved with MSMS detection, which led to a RP-FLD combination. The technique of RP-MSMS was found to be suitable for the remaining four groups of biotoxins. The final method was a combination of two RP columns coupled with FLD and MSMS detectors, with a valve switching program and injection program.

A novel sample preparation method was also developed for the extraction and clean-up of biotoxins from mussels. It was determined that Strata-X was a suitable sorbent for use in the clean-up of mussel extracts. A validation study was carried out on the developed method *via* analysis of certified reference materials for AZAs, DSTs and ASP, and naturally contaminated mussel material for PSTs. A major limitation to this research was the scarcity and the restrictions in obtaining and receiving biotoxin reference materials. In addition, no reference materials were available for brevetoxins. Therefore, spiking trials were conducted for brevetoxins and it was found that no recoveries could be observed, possibly due to irreversible binding to matrix components.

Determination of measurement uncertainty was performed based on the validation data. The method was shown to be capable of meeting current regulatory needs with respect to specificity, analytical range, and limits of detection for PSTs and DSTs. Low recoveries were observed for AZAs and ASTs, which may be accounted for by the application of correction factors determined on a per batch basis.

DECLARATION OF ORIGINALITY

I, Bing Cheng Chai, declare that the Ph.D. thesis entitled "Development and Validation of a novel approach for the analysis of Marine Biotoxins" is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Signed:_____

Bing Cheng Chai

Date:_____

Dedicated to my parents and to Eleanor

Whatever your hand finds to do, do it with your might, for there is no work or thought or knowledge or wisdom in Sheol, to which you are going.

— Ecclesiastes 9:10 ESV

And whatever you do, in word or deed, do everything in the name of the Lord Jesus, giving thanks to God the Father through him.

— Colossians 3:17 ESV

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PRESENTATIONS ARISING FROM RESEARCH

Chai B.C. (2012) "Developing a more sensitive and rapid method for the detection of Paralytic Shellfish Toxins", Australian Institute of Food Science and Technology (AIFST) Young Members' Presentation Evening, Deakin University, Burwood, VIC, Australia.

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LIST OF ABBREVIATIONS

AOAC	Association of Analytical Communities
AST	Amnesic Shellfish Toxins
AZA	Azaspiracid
CCFFP	Codex Committee on Fish and Fishery Products
CITAC	Cooperation on International Traceability in Analytical Chemistry
CRM	Certified Reference Material
DA	Domoic Acid
DTX	Dinophysitoxin
EFSA	European Food Safety Authority
ESI	Electrospray ionisation
FLD	Fluorescence Detection
GCB	Graphitised carbon black
GUM	The ISO Guide to the Expression of Uncertainty in Measurement
HAB	Harmful algal bloom
HPLC	High Performance Liquid Chromatography
ISO	International Standards Organisation
LC-FLD	Liquid Chromatography — Fluorescence detection
LC-MSMS	Liquid Chromatography — Tandem Mass Spectrometry
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantitation
LOR	Limit of reporting
LRM	Laboratory Reference Material
m/z	Mass to charge ratio
MeOH	Methanol

MRM	Multiple Reaction Monitoring
MSMS	Tandem Mass Spectrometry
NMI	National Measurement Institute
NRCC	National Research Council Canada
NST	Neurotoxic Shellfish Toxins
OA	Okadaic Acid
PbTx	Brevetoxin
PP	Polypropylene
PST	Paralytic Shellfish Toxins
PVDF	Polyvinylidene Difluoride
QC	Quality Control
RM	Reference Material
RSD	Relative standard deviation
SARDI	South Australian Research and Development Institute
SD	Standard deviation
SPE	Solid Phase Extraction
SSR	Sample to Solvent Ratio
STX	Saxitoxin
TEF	Toxicity Equivalence Factor

LIST OF APPENDICES

1 GENERAL INTRODUCTION

1.1 Harmful Algal Blooms

Cyanobacteria, dinoflagellates, algae and diatoms are small aquatic organisms which are found globally, from freshwater lakes to temperate oceans. Collectively, these organisms can be grouped as phytoplankton. Of the phytoplanktons, 300 marine species are able to multiply rapidly to form dense biomasses known as Harmful Algal Blooms (HABs)(Daneshian *et al.*, 2013; Gerssen *et al.*, 2010a). HABs can cause mass fish deaths either through oxygen depletion or by physically clogging gills, but the greatest risk to fauna is presented by toxic secondary metabolites produced by phytoplankton. These biotoxins may affect the liver (hepatotoxins), nervous system (neurotoxins), and skin (dermatoxins)(Zanchett and Oliveira-Filho, 2013). In the temperate latitudes of Europe, South Africa, Asia, Australia, North America and South America, toxin-producing species of HABs can cause amnesic, azaspiracid, diarrhetic, neurotoxic and paralytic shellfish poisonings (Berdalet *et al.*, 2015). Thus, HABs pose a significant risk to public health, recreation, tourism, aquaculture and marine ecosystems due to their adverse effects on the environment, which threatens water quality, health of living resources and economies of nearby populations (McLean and Sinclair, 2012).

Marine biotoxins are divided into hydrophilic and lipophilic classes, based on their chemistry and aqueous solubility. Within these two classes, biotoxins are grouped based on their associated syndrome (Table 1-1). Syndromes associated with biotoxin exposure are usually named after the effects that they cause or the name of producer species. For

Table 1-1 Marine biotoxin intoxication syndromes and associated organisms

Toxic syndrome	Toxins	Producer species	References
Paralytic Shellfish Poisoning	Saxitoxins and analogs	*Alexandrium, Gymnodinium, Pyrodinium bahamense* var. *compressum,* Cyanobacteria species (*Lyngbya, Anabaena, Cylindrospermopsis, Aphanizomenon, Phlanktothrix*)	(Wiese, 2010) (Amade, 2014)
Amnesic Shellfish Poisoning	Domoic Acid and analogs	*Pseudo-nitzschia* spp.,Red algae such as *Chondria armata*, *Digenea simplex*, and *Alsidium corallinum*	(Lefebvre, 2010)
Diarrhetic Shellfish Poisoning	Okadaic Acid, Dinophysistoxins	*Dinophysis* spp., *Prorocentrum*	(Valdiglesias, 2013)
Azaspiracid Poisoning	Azaspiracids and analogs	*Azadinium* spp., *Protoperidinium* spp.	(Kilcoyne, 2014) (Kalaitzis ,2010)
Neurotoxic Shellfish Poisoning	Brevetoxins	*Karenia brevis* spp.	(Turner, 2015)

example, Paralytic Shellfish Toxins (PSTs) are neurotoxins which can cause paralysis, while brevetoxins are termed because they are produced by the diatom *Karenia brevis*. Some species of the diatom *Pseudo-nitzschia* are capable of producing the biotoxin responsible for Amnesic Shellfish Poisoning (ASP). In freshwater, several species of cyanobacteria are also known to produce PSTs (Wörmer *et al.*, 2011). Crustaceans, shellfish and fish that feed on these phytoplankton accumulate biotoxins and transmitting them to humans and animals further up the food chain (Farre *et al.*, 2013). They can also be transmitted through the ingestion of contaminated water (Batoréu *et al.*, 2005).

In recent decades, an increase in toxic bloom events has been observed. Analysis of HAB trends in the North American region has revealed that PST-producing dinoflagellates and AST-producing diatoms have been observed in varying intensities and frequencies along the west coast of Canada, USA, and Mexico (Lewitus *et al.*, 2012). In Turkey, more frequent observances of AST contamination have been reported (Dursun *et al.*, 2015), while in Ireland, there have been increasing closures of shellfish harvesting sites due to AZAs and DSPs found over the permitted levels (James *et al.*, 2002; Kilcoyne, 2015). The occurrence of HAB-forming organisms usually suited to milder waters have also been observed in waters off Norway (Edwards *et al.*, 2006). As a result, phytoplankton and their associated biotoxins are also increasingly being found in places with no record of HABs: In Iceland, the north coast of Eyjafjordur and west coast of Breidafjordur experienced a bloom of *Alexandrium* spp. in June 2009, contaminating blue mussels with paralytic shellfish toxins (PSTs), which led to extensive closures of harvesting sites, the first biotoxin related closure reported in Iceland (Burrell *et al.*, 2013). It was recently reported that oyster harvests were closed for the first time in the Gulf of Mexico due to confirmed presence of okadaic acid (DSTs) (Deeds *et al.*, 2010). Similarly, first reports have been published of AST detected in abalone species off the southern coastline of Australia (Malhi *et al.*, 2014).

Causes for this increase have been attributed to natural and anthropogenic factors such as include dispersion of biotoxin producing species *via* ocean currents, artificial dispersion *via* ballast water discharge, aquaculture, and increased monitoring of water bodies with more sensitive techniques (Anderson *et al.*, 2012; Bolch and de Salas, 2007;

Smayda, 2007). Climate change has also been cited as one of the major drivers of the increase in HABs (Silva *et al.*, 2015). Increasing sea surface temperatures have also been linked to the increase in dinoflagellate populations in the north-east Atlantic, where they outcompete native diatom populations (Edwards and Richardson, 2004). Warmer sea surface temperatures and water stratification increases the growth rate of dinoflagellates, and nutrient depletion at the surface favours the survival of dinoflagellates (Bopp *et al.*, 2005). Climate models predict more frequent blooms of biotoxin producing dinoflagellates (van der Fels-Klerx *et al.*, 2012), leading to an increase in the window of opportunity for blooms, with earlier and more persistent blooms (Moore *et al.*, 2011).

1.1.1 Factors Affecting Biotoxin Production

Biotoxin production by phytoplankton is affected by environmental and genetic factors (Pistocchi, 2014). Nutrient availability factors such as iron content in water, environmental parameters such as irradiance, temperature, salinity or inorganic nutrients have been shown to affect biotoxin content and composition for several different *Alexandrium* strains (Etheridge and Roesler, 2005; He *et al.*, 2010). Relationships between photosynthesis or growth rate and total toxicity were not found, suggesting that environmental factors directly influence toxicity.

Dinoflagellates such as *Alexandrium* are more competitive in nutrient-limited conditions due to higher nutrient affinities and capability to utilise organic nutrients compared to competitive species (Laabir *et al.*, 2013). Dramatic changes in biotoxin composition in one *A. fundyense* isolate were observed when grown in nitrogen- and phosphorous-limited semi-continuous cultures (Etheridge and Roesler, 2005). In South America, seasonal blooms of several toxic species of *Alexandrium* (*A. tamarense*, *A. catenella*, *A. minutum* and *A. tamiyavanichii*) have been well documented, and Montoya *et al.* (2010) showed that the proportion of the PST gonyautoxin (GTX) 1 & 4 content increased exponentially with the increase of *in situ* nitrate concentration. In contrast, the proportion of GTX2 & GTX3 decrease exponentially as nitrate concentration increased. Total biotoxin content increased as nitrogen concentration increased. The authors proposed that changes observed in toxin composition are induced by changes in nitrogen in their surrounding environment.

Biotoxin production can also be a survival response to presence of predator signalling compounds: In response to zooplankton lipids, Selander *et al.* (2015) have found that some *Alexandrium* species become 20 times more toxic. Holland and Kinnear (2013) have proposed that biotoxins acts as a defense mechanism against predators and also as a physiological aid, participating in nutrient absorption pathways.

Apart from extreme nutrient deprivation conditions that may cause a shift in the biotoxin profile, isolates in exponential growth phase tend to maintain their molecular fingerprint of biotoxins in culture. This makes it possible to compare biotoxin profiles from *Alexandrium* species and strains in long-term cultures, even if the isolates were not collected at the same time. These biotoxin profiles have been proposed to be used to trace the source organisms in biotoxin outbreaks (Wong *et al.*, 2011). However, significant changes in biotoxin composition have been reported in cells exposed to different stresses (Etheridge and Roesler, 2005; Poulton *et al.*, 2005). Nevertheless, diverse biotoxin profiles have been observed in different phytoplankton populations globally.

In Australia, HAB species that present a risk to shellfish include *Pseudo-nistzchia* (Ajani *et al.*, 2012), *Alexandrium tamarense* (Campbell *et al.*, 2013), *A. minutum*, *A. catenella*, and *Gymnodimium catenatum* (Parker, 2002).Table 1-2 shows the different PST profiles produced by the aforementioned species.

Table 1-2 Biotoxin profiles of different dinoflagellates

Organism	Dominant Toxins	References
G. catenatum	GTX6, C3, C4	Costa *et al.*, 2014
Alexandrium spp.	GTX1, GTX4	Lefebvre *et al.*, 2008
Alexandrium spp.	STX, NEO, GTX2, GTX3	Etheridge *et al.*, 2005
A. minutum	GTX2, GTX3, GTX1, GTX4, STX	Abouabdellah *et al.*, 2008
A catenella	C2, GTX2, GTX3, dcGTX2	Alvarez *et al.*, 2009, Krock *et al.*, 2007
A tamarense, A ostenfeldii	GTX2, GTX3, STX	Burrell *et al.*, 2013
Alexandrium tamarense/catenella	C12, GTX1, GTX4, GTX2, GTX3, NEO	Montoya *et al.*, 2010
Alexandrium tamarense	C2, GTX4	Kim and Shin, 2015

There is a lag time between peak of the bloom and toxicity maximum. For example, the maximum toxicity of mussels was measured 13 days after peak cell counts of a bloom of *Gymnodinium catenatum* (Costa *et al.*, 2014). Quantification becomes particularly important during this period since the highest risk of human poisoning occurs at this stage. After the peak of the bloom, a gradual decrease of C3 & C4 and GTX6 concentration, suggesting a depuration process in mussels.

1.1.2 Effects on Humans

Although the primary means of marine biotoxin exposure in humans is through consumption of toxic seafood (James *et al.*, 2010), it is not the only route of exposure. Humans can be exposed to marine biotoxins through physical contact with water during an active bloom (Weirich and Miller, 2014), and inhalation of aerosolised brevetoxins

when the wave action near beaches break up cells of brevetoxin-producing diatoms (Backer *et al.*, 2003; Fleming *et al.*, 2009; Pierce *et al.*, 2003). Exposure to aerosols containing brevetoxins have been linked with aggravated asthma symptoms (Bean *et al.*, 2011; Kirkpatrick *et al.*, 2011).

Table 1-3 Symptoms of shellfish poisoning in humans (Munday and Reeve, 2013)

Toxin	Reported effects in humans
Paralytic Shellfish Toxins	Nausea, paresthesia, tachycardia, muscular paralysis, respiratory failure, death
Amnesic Shellfish Toxins	Vomiting, diarrhoea, abdominal pain, confusion, memory loss, seizure, coma, death
Diarrhetic Shellfish Toxins	Nausea, vomiting, diarrhoea, abdominal pain
Azaspiracids	Nausea, vomiting, diarrhoea, abdominal pain
Brevetoxins	Nausea, vomiting, diarrhoea, chill, sweating, dysaesthesia, hypotension, paresthesia of lips, face and extremities, cramps, paralysis, seizures and coma after ingestion. Rhinorrhoea, cough, bronchoconstriction after inhalation

1.1.3 Impact on Animals

Accumulation of biotoxins by filter-feeding shellfish, crustaceans, and molluscs can affect multiple trophic levels in marine environments and food chains, impacting wild and aquaculture marine animals such as mussels, lobsters and salmon (Sephton *et al.*, 2007). Mortality events for larger animals such as dolphins (Fire *et al.*, 2011), seals (Jensen *et al.*, 2015), sea lions (Brodie *et al.*, 2006) and marine birds (Shumway *et al.*, 2003) have been recorded. Non-traditional vectors such as gastropods and plankton-eating fish have also been found to pass biotoxins along marine food webs (Jen *et al.*, 2014). In filter-feeding shellfish, dinoflagellates are drawn in from surrounding water and digested in the gut, releasing biotoxins which are absorbed and transported to other parts of the shellfish (Li *et al.*, 2005) without causing mortality.

Substantial mortalities of aquacultured Atlantic salmon at two sites in the Bay of Fundy (New Brunswick, Canada) in September 2003 were associated with a bloom of *Alexandrium fundyense*, a PST producing dinoflagellate. The zooplankton sampled contained PSTs matching the profile of blooming *A. fundyense* cells (Sephton *et al.*, 2007).

In the Portuguese coastal region, sardines are the most abundant planktivorous fish and a major component of the marine food web. Sardines consume phytoplankton, which include *G. catenatum*. The PST profile characterised in sardine samples in a study conducted by Costa *et al.* (2010) showed same sulfocarbamoyl and decarbamoyl toxins found in the consumed algae with minor differences in relative abundance of each biotoxin.

Intracellular biotoxins can be released into the environment *via* excretion or lysis of phytoplankton cells, posing another route of exposure *via* direct absorption from the water by aquatic animals. Studies conducted by Lefebvre (2008) have confirmed that the ingestion of PSTs *via* algal or zooplankton vectors is a route of PST exposure causing acute toxicity in adult and larval fish during toxic blooms. Extracellular STX exposure has been shown to impair the physiology and behaviour of developing fish larvae, causing a complete loss of sensorimotor function, caused delayed hatching and malformations and mortalities in zebrafish larvae (Lefebvre, 2008). However, the stability of extracellular PSTs in water may also be affected by upwelling events or river plumes and may not persist long at seawater pH unless stabilisation is achieved by complexation with other substances (Rue and Bruland, 2001).

1.1.4 Economic Impacts

The threat of marine biotoxins is not only a public health issue, but is also a threat to parts of the global economy, which will be discussed in the following paragraphs. HAB outbreaks often result in the death of marine life and closure of contaminated fisheries, while the continual expenditure required for the maintenance and running of monitoring programs present a major cost to global economies. They can form international trade barriers: In Thailand, the risk of biotoxin contamination from traditional shellfish

aquaculture practices caused a voluntary export ban to be enforced until internationally recognised shellfish sanitation programs were established (Chalermwat *et al.*, 2003).

The growing HAB threat will affect countries with large aquaculture industries such as Iceland, where marine products accounted for 42% of total export value in 2009 (Burrell *et al.*, 2013). New Zealand also has a large aquaculture industry valued at NZ$1.5 billion in 2010 (Rhodes *et al.*, 2013). Losses to local businesses such as shellfish farms, beach businesses, and other seafood related industries from one local PSP outbreak in Maine, USA were estimated at US$6 million per year (Boesch *et al.*, 1997). In Malaysia, fish die-offs due to HAB was estimated to cause RM$20 million loss in one incident (Lim *et al.*, 2012). In Australia, a PST contamination of Tasmanian shellfish during an unexpected HAB event was estimated to have a total financial impact of AU$23 million across the commercial fisheries and marine farming sector (Campbell *et al.*, 2013).

In addition, the cost of operating a monitoring program is also significant: In America, most states operate their own monitoring program, which cost up to US$200,000 annually. In Australia, shellfish safety monitoring is operated by each state, guided by a federal framework. States which have a large shellfish and aquaculture industry such as New South Wales, South Australia, Tasmania, and Victoria have larger operating costs (Ajani *et al.*, 2012; FRDC, 2011). The total costs of managing cyanobacterial risks were estimated to cost AUD$180-240 million annually, although this figure includes costs associated with joint management, urban and rural extractive users and non extractive users of Australian water resources (Steffensen, 2008).

The main concern with respect to shellfish associated with marine biotoxins is that most shellfish species can accumulate and tolerate high concentrations of biotoxins. Shellfish such as mussels and oysters are usually harvested live and appear no different to uncontaminated shellfish.

1.2 Control and Monitoring of Harmful Algal Blooms

Many of the biotoxins which have been identified in the past half century are now monitored with various chemical, biochemical or molecular-based methods (Moreira *et al.*, 2014). Due to monitoring programs being implemented by governments around the world, a decrease in the number of cases of poisoning has been observed (Bean *et al.*, 2005). A report by Hallegreaeff (2014) places the fatality rate of poisoning cases at 15% (300 out of 2000 cases reported annually worldwide).

New tools have been developed to aid in monitoring the conditions of water and likelihood of toxic HABs. One of them is Solid Phase Adsorbent and Toxin Tracking (SPATTs) which are passive bags of adsorbent material which are left in open water for a time to adsorb toxins and can be analysed by chemical methods (Fux, 2008; McCarthy *et al.*, 2014; Zendong *et al.*, 2014).

Additionally, measures can be implemented to minimise human exposure to these chemicals. One technique involves removal of HAB or toxins from water bodies when they occur. This can be done by application of modified clay to blooms to flocculate algae in water (Lu *et al.*, 2015), which was found to reduce one group of PST (gonyautoxins) by 82% and simultaneously reduce phosphate and nitrate concentration, which are macronutrients needed for phytoplankton growth. This method has been reported to have some success in China (Pan *et al.*, 2011) and Malaysia (Lim *et al.*, 2012). More recently, removal of PSTs *via* probiotic lactic acid bacteria (*Lactobacillus rhamnosus* GG and LC-705) have been reported by Vasama *et al.* (2014). The mechanism of this removal is thought to be a binding of biotoxins to components of the bacteria, as no differences were found between viable and non-viable forms of the bacteria. This finding is a positive initial result for management of biotoxin levels in water bodies.

The driving force for progress in the area of marine biotoxins monitoring and control is the fact that there are no current known antidotes to marine biotoxin intoxication (Silva *et al.*, 2015). Therefore, monitoring is a critical part of safety assurance. Underpinning

all monitoring programs are measurement techniques to determine risk and toxicity. Monitoring algal cell counts in water is an indirect measurement of toxicity risk as a high algal cell count may not necessarily mean that shellfish are toxic. Direct measurement of shellfish tissue remains a critical tool in ensuring edible shellfish safety.

1.2.1 Establishing regulatory limits

In Australia, the Imported Foods Inspection Scheme (IFIS) is a food inspection program overseen by the Department of Agriculture. Its purpose is to monitor imported food for compliance with Australian food reference standards. Bivalve molluscs such as mussels and oysters are categorised as risk foods. This means that it has been assessed as having medium to high risk to consumer health (Imported Food, 2015). Limits of biotoxin content have been established to protect human health. Australia follows international guidance in setting regulatory limits for marine biotoxins (Table 1-4). However, there are some differences in the levels established by Australia and Europe.

The maximum allowed limits of phycotoxins are established based on data derived from past poisoning incidents (FAO, 2004a). Toxicology data from animal trials are also used to determine toxicity and are used to revise as more information is gathered. Risk assessments take into account epidemiological data such as consumption frequencies, portion size variations between populations, and toxicological information. Using this data, limits are also set designed to provide covers up to the 97.5th percentile of global population (Toyofuku, 2006).

Table 1-4 Biotoxin limits set for bivalve molluscs in Australia (Australian Government, 2015) and Europe (EFSA, 2009a)

Toxin	Legislated toxins	Australian Limits	European Limits
Domoic Acid	DA and analogs	20 mg DA/kg	20 mg DA/kg
STX	STX and analogs	0.8 mg STX equivalents/kg	0.8 mg STX equivalents/kg

Toxin	Legislated toxins	Australian Limits	European Limits
DST	OA, DTX1/2/3	0.2 mg OA equivalents/kg	0.16 mg OA equivalents/kg
NST	Brevetoxin 1/2 and derivatives thereof	0.8 mg BTX2 equivalents/kg	Not Regulated
AZAs	Azaspiracid 1/2/3	Not Regulated	0.16 mg AZA1 equivalents/kg

The current regulatory limit for PSTs was established in the 1930s based on experiments on mice. The initial results of PST testing were expressed in terms of Mouse Units (MU), one unit being the amount of total biotoxin that killed a 20 g mouse within 15 min (EFSA, 2009b). As chemical methods became viable and reference standards of PSTs became available, the relationship between MU and toxin amount was determined: 1 MU was found to be equivalent to 0.2 µg of STX (Wekell *et al.*, 2004). This relationship was used to convert the mouse units from mouse bioassay into microgram equivalents. The mouse bioassay has a detection limit of 200 MU, which is 40 µg STX eq/100 g shellfish. The 80 µg STX eq/100 g shellfish is thought to have originated from Californian authorities who instituted quarantine measures when 2 mg of shellfish extract contained 2 MU (Wekell *et al.*, 2004).

Amnesic shellfish poisoning (ASP) caused by domoic acid and its isomers is a relatively newer syndrome, first being observed in 1987 in Prince Edward Island, Canada. After this episode, two workshops held in Ottawa, Canada and California, USA established the current regulatory limit of 20 mg/kg based on analysis of uneaten mussels recovered from the outbreaks and symptoms exhibited by victims (Wekell *et al.*, 2004).

1.2.2 Emerging Toxins

Despite the success of the current monitoring system, emerging toxins and new analogs of known toxins remain a concern. There is a large gap in knowledge about epidemiology and toxicological effects of sublethal chronic exposure to these toxins

(FAO, 2004a; Paredes *et al.*, 2011; Picot *et al.*, 2011; van Egmond, 2004). Risk assessments have yet to be done on many groups of toxins, which include hazard monitoring of HAB species, risk assessment and management of biotoxins in foods and non-foods, and registration of analytically-verified intoxications in humans (Daneshian *et al.*, 2013). In addition to the major groups of monitored marine biotoxins discussed above, there are also compounds that have recently been identified but have not yet reached the stage of having quantitative limits placed on them, due to a lack of toxicological data or no established link between illness and exposure to these emerging toxins e.g. cyclic amines, the yessotoxins and pectenotoxins, palytoxins and tetrodotoxin.

1.2.2.1 Cyclic Imines

Cyclic imines (CI) are a newly-described family of structurally related marine toxins (Silva *et al.*, 2015). Within the cyclic imine group are spirolides, gymnodimines, pinnatoxins, and pteriatoxins. The main common feature of members in this group is the presence of an imine moiety as part of a bicyclic ring system. Some organisms that produce cyclic imines also produce other marine biotoxins, such as *Karenia* (NSTs), *Alexandrium* and *Gymnodinium* (PSTs). Cyclic imine intoxication leads to neurological symptoms in mice (Paredes *et al.*, 2011). To date, human intoxications have not been reported (McNabb *et al.*, 2012).

1.2.2.2 Yessotoxins and Pectenotoxins

Pectenotoxins (PTXs) are a group of lipophilic toxins originating from Dinophysis species throughout the world. PTXs were originally grouped together with Yessotoxins (YTX) as the Diarrhetic Shellfish Toxins. However, in recent years, due to increased knowledge of its toxicology, PTXs have been reclassified into its own group. Current known forms of PTXs are PTX-1 to −14, PTX-2sa and 7-epi-PTX-2-sa (Li *et al.*, 2010). Intraperitoneal injection of PTXs in mice appear to be highly toxic. However, PTXs appear to be of low toxicity when administered orally (Higman *et al.*, 2014; Liu *et al.*, 2011).

1.2.2.3 Palytoxin

Palytoxins (PlTx) are a class of potent non-protein marine toxins, whose main biological target is the biological mechanism which maintains cellular ionic concentrations critical to normal cell functions (Ciminiello *et al.*, 2010). Originally isolated from the marine zoanthid *Palythoa*, it was subsequently found in dinoflagellates of the genus *Ostreopsis* (Lenoir *et al.*, 2004). Analogs of palytoxin have also been identified: ovatoxins, ostreocins, and mascarenotoxin. Mouse studies have provided evidence of lower oral toxicity compared to intravenous or intraperitoneal injection (Ciminiello *et al.*, 2010). Electrospray ionization-tandem mass spectrometry (ESI-MS/MS) shows great potential for rapid and sensitive identification of marine biotoxins in contaminated material (Ciminiello *et al.*, 2010).

1.2.2.4 Tetrodotoxin

Tetrodotoxin (TTX) is a well-known toxin occurring in pufferfish (Jang and Yotsu-Yamashita, 2006). They have a similar mode of action to PSTs, binding to the sodium channel in nerves and blocking normal mode of signal transfer. The source of TTX in pufferfish has been found to be endosymbiotic bacteria which naturally inhabit the gut of pufferfish (Bane *et al.*, 2014). Increasingly, TTX has been found outside of Asia in gastropods and fish (McNabb *et al.*, 2014). Although saxitoxin has been found in pufferfish flesh (Landsberg *et al.*, 2006), TTX has not been reported in shellfish. Consequently, TTX is only a concern in fugu or pufferfish and there are no limits established for shellfish.

1.2.2.5 Ciguatera Fish Poisoning

Ciguatera fish poisoning (CFP) is associated with ciguatoxin, maitotoxin, and gambierols. Ciguatoxins have distinctions between regions, leading to giving CTXs a prefix such as Indian Ocean ciguatoxin (I-CTX), Pacific Ocean ciguatoxin (P-CTX), and Caribbean Ocean ciguatoxin (C-CTX). The chemical structure of these compounds have only begun to be described in the last two decades (Caillaud *et al.*, 2010). Due to structural similarities with DSTs, toxins associated with CFP are suited to be analysed using mass spectrometry (Yogi *et al.*, 2014). Currently, no simple and reliable tests are available for ciguatera in finfish (Stewart and McLeod, 2014). Ciguatera toxins in

finfish are currently determined by mouse bioassay. Although it is a validated method, the tests have to be performed on a per-fish basis. Unlike shellfish, fish are mobile and ciguatera precursors undergo a complex biotransformation before final ciguatoxin formation and accumulation in predator finfish.

1.3 Significance and Aim of Research

There is a limited expertise in marine biotoxin analysis due to difficulties in obtaining reference materials and the costs associated with operating test methods. Currently there is only one major supplier of marine biotoxin reference materials and reference standards for global testing bodies. These limitations prevent progress in developing efficient and effective techniques to support food security and comply with regulations for public safety. Global trade will also benefit by having more efficient closing and opening of fisheries and harvesting sites, reducing the economic impact of HABs globally. Although there are methods that are validated for lipophilic and hydrophilic toxins, none are able to analyse both classes of toxins simultaneously. Therefore, there is a need for an improved and rapid analytical methods to respond to increasing threats of HABs.

The novel tandem LC setup used by Pyke *et al.* (2015) for metabolomics studies has been demonstrated to be able to separate both hydrophilic and lipophilic compounds for analysis by MSMS in a short amount of time. This tandem LC setup shows great potential for application to the analysis of marine biotoxins as they may also suitable for MS detection.

Based on the gaps highlighted above, the aim of this research is to develop a tandem LC method for the simultaneous detection and quantification of PSTs, ASTs, DSTs, NSTs and AZAs, with a focus on the ability to detect and quantify these five major groups at levels relevant to current regulatory limits.

To achieve this aim, a three phase approach was taken:

1. Development of a novel instrument setup incorporating liquid chromatography and a suitable detector. Hydrophilic Interaction Chromatography (HILIC) and Aqueous Normal Phase (ANP) chromatography, which are emerging analytical separation techniques for hydrophilic compounds were evaluated. Four columns belonging to these classes were compared for compatibility with tandem LC

instrument configuration and integration with traditional Reversed Phase (RP) chromatography.

2. Development and optimisation of a new extraction method for the extraction of both lipophilic and hydrophilic toxins from shellfish tissue.
3. Validation of the novel tandem LC method and assessment of suitability for routine monitoring of marine biotoxins.

1.4 Thesis Organisation

This thesis has been arranged in seven chapters. The next chapter discusses the literature surrounding marine biotoxins and their detection methods. Chapter 3 will list the materials, instruments and methods used. Chapter 4 deals with the process of method development leading up to the design and testing of the tandem LC capable of multi-toxin analysis. Chapter 5 will describe the process taken to develop a sample preparation technique suitable for both hydrophilic and lipophilic toxins, with optimisation of the cleanup procedure. In Chapter 6, method validation was performed to determine method performance criteria such as linearity, limit of detection, limit of quantitation, accuracy, etc. Finally, Chapter 7 will summarise the conclusions drawn from the research and discusses potential areas for further investigation and development.

2 LITERATURE REVIEW

2.1 Introduction

The aim of this chapter is to give more detailed information on the chemistries of the five groups of marine biotoxins studied in this research project. Following that, methods for the analysis of these biotoxins will be summarised and the complexities in analysis of these compounds will be discussed, along with progress made in recent years.

2.2 Hydrophilic Biotoxins

2.2.1 Amnesic Shellfish Toxins (AST)

The first reported case of AST intoxication was in 1987, where contaminated mussels from Prince Edward Island, Canada caused three deaths and more than 100 people to be admitted to hospital for gastrointestinal and neurological illness (La Barre *et al.*, 2014; Perl *et al.*, 1990). The mouse bioassay was a critical tool in the identification of this toxin, linking the illnesses to a large bloom of the diatom *Nitzschia pungens*. Domoic acid (DA) was subsequently linked to the event as the causative compound. Although its chemical structure was determined in 1982, domoic acid was not linked to seafood poisoning until the outbreak in Prince Edward Island.

2.2.1.1 Chemistry and Sources

Domoic acid is a water-soluble cyclic amino acid (Figure 2-1). It belongs to the kainoid class of compounds, and has been isolated from several red algae species and a number of diatom species (FAO, 2004b). More than 10 species of diatoms are known to produce this toxin, mostly from the genus *Pseudonitzschia* (Lefebvre and Robertson, 2010). To date, several isomers of DA have also been identified (Tasker, 2014). Isodomoic acid A, B, and C were identified as minor constituents in the red alga *Chondria armata*. Other known isomers of domoic acid are isodomoic acid A–H and 5'-epi-Domoic acid (Figure 2-1). Romero *et al.* (2011) have shown that AST composition is not dependent on geographical latitude, but is a characteristic of diatom strain and sub-strain. For example, strains of the diatom *Nitzschia navis-varingica* collected in the Philippines were found to contain mostly isodomoic acid B while those collected from Indonesia and Japan contained mostly domoic acid. It was previously thought that isodomic acid B would be in higher proportion at lower latitudes.

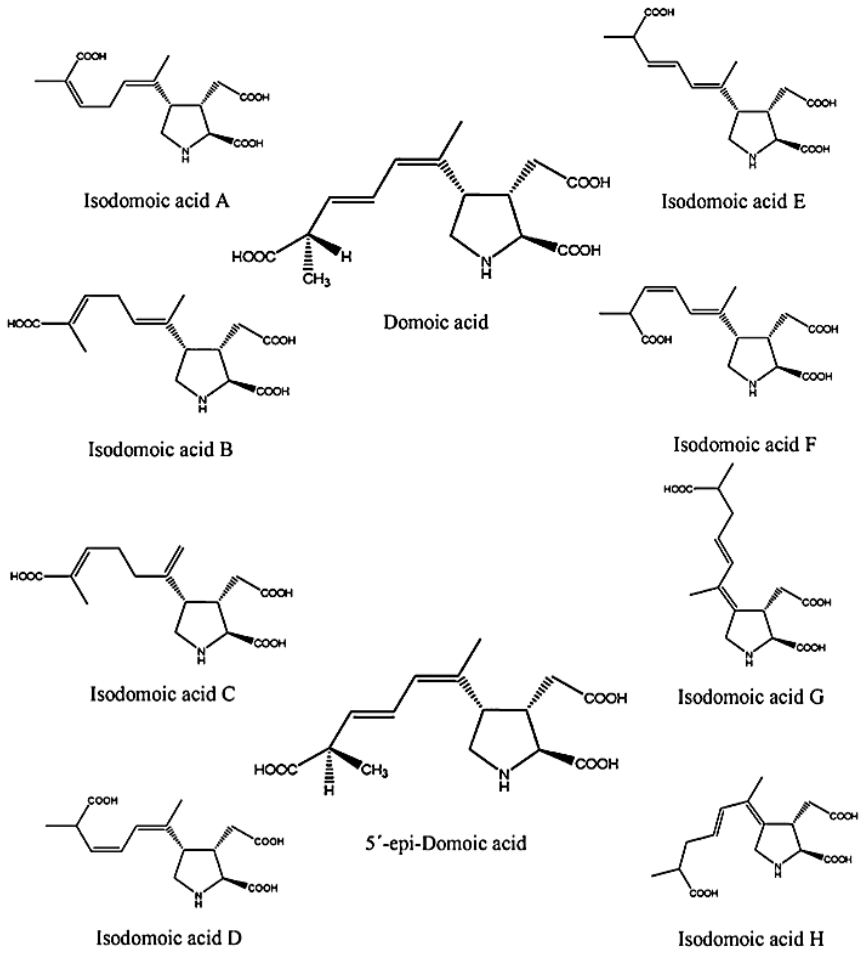


Figure 2-1 Chemical structures of Amnesic Shellfish Toxins (FAO, 2004b)

2.2.1.2 Toxicity and Mechanism of Action

Amnesic Shellfish Poisoning manifests itself *via* gastrointestinal (vomiting, diarrhoea, and abdominal cramps) and neurological effects (headaches and short-term memory loss). Domoic acid is a glutamate receptor activator, specifically targeting ionotropic glutamate receptors (Pulido, 2014). These receptors form cation-specific ion channels and regulate fast excitatory transmission in the central nervous system. In the hippocampus (the region associated with memory), domoic acid exposure can lead to amnesia and memory loss issues (Lefebvre and Robertson, 2010).

The toxicity of isodomoic acid A (IA), Isodomoic acid B (IB), and Isodomoic acid C (IC) is reported to be significantly lower than DA alone. In mouse studies conducted by Munday *et al.*, (2008), intraperitoneal administration of the toxins found LD_{50} of DA at 6.0 mg/kg while no deaths occurred for IA, IB and IC at the same dosage. IC was also dosed at 20 mg/kg with no deaths observed. These isomers are not included in sample toxicity tests due to their low presence compared to domoic acid. Nevertheless, because of structural similarities of these isomers, there is a possibility that marine animals such as shellfish, fish and mammals may act as vectors of the toxin isomers, which could potentially be converted from IB to IA and finally to DA in the animal tissue *via* enzymes or other chemical mechanisms, although there is no report showing these bioconversions in the animal tissue.

Recently, experiments performed by Funk *et al.* (2014) have shown that kidney damage occurred in a strain of Black Swiss mice fed with DA-contaminated shellfish at 5 µg/kg, which is significantly lower than current regulated limits (20 mg/kg). DA showed preferential accumulation in the kidney. As a result, there may be a need to revise the regulated amount to account for risks of long term subchronic exposure to this toxin. Detection methods may need to be able to detect up to 0.5-500 µg/kg of domoic acid, which is 40-40000 times lower than the currently adopted limit (EFSA, 2009a). Currently, no levels have been set for DA isomers.

2.2.2 Paralytic Shellfish Toxins (PST)

PSTs cause paralytic shellfish poisoning (PSP). They are a class of biotoxins that bind to sodium channels within the cell, disrupting normal cell signaling pathways. The disruption of cell signaling pathways may lead to nausea, tingling sensations around the lips and fingers, paralysis, and potentially death. These toxins are produced by both prokaryotes and eukaryotes (Orr *et al.*, 2013).

PSTs were originally detected on the Pacific Coast of the United States in 1937 and described by Schantz and Magnussen (1961). Since then, PSTs have been found in many other locations. This is partially due to the progressive implementation of monitoring programs, which have grown in parallel with the development of shellfish aquaculture, but also perhaps due to a true increase in the frequency of toxic outbreaks (Álvarez *et al.*, 2009).

2.2.2.1 Chemistry and Sources

Dinoflagellates from the genus *Alexandrium*, *Gymnodinium*, and *Pyrodinium* have been identified as PST producers (Shumway *et al.*, 2003). Several genera of cyanobacteria are also known to produce this toxin (*Lyngbya*, *Anabaena*, *Cylindrospermopsis*, *Aphanizomenon*, and *Phlanktothrix*)(Amade *et al.*, 2014).

Fifty-seven analogs of PST have been identified to date (Wiese *et al.*, 2010). These biotoxins have a common 3,4,6-trialkyltetrahydropurine structure and the following subgroups are formed by modifications of the four R-group side chains (Figure 2-2): These analogs fall under the following subgroups: N-sulfocarbamoyl (C1-4, GTX5 and GTX6), carbamate (GTX1-4, STX, NEO), decarbamoyl (dcGTX1-4, dcSTX, dcNEO), Deoxydecarbamoyl (doGTX2,3, doSTX), benzoate (GC1-6; GC1a-6a; GC1b-6b), C11-hydroxy (M1-5), Angola (A-D), and Lyngbya (LWT1-6)(Humpage *et al.*, 2010). The recently identified benzoate analogs have been found to have a slight lipophilic property (Baker *et al.*, 2003).

Depending on the pH of its environment, the R groups of PST analogs can have different net charge states, ranging from neutral (0) to net positive (+1 or

+2)(Dell'Aversano *et al.*, 2005). This zwitterionic property of the PSTs has been used for separation and isolation of PSTs toxins from a sample based on cationic exchange mechanisms (Turner *et al.*, 2009).

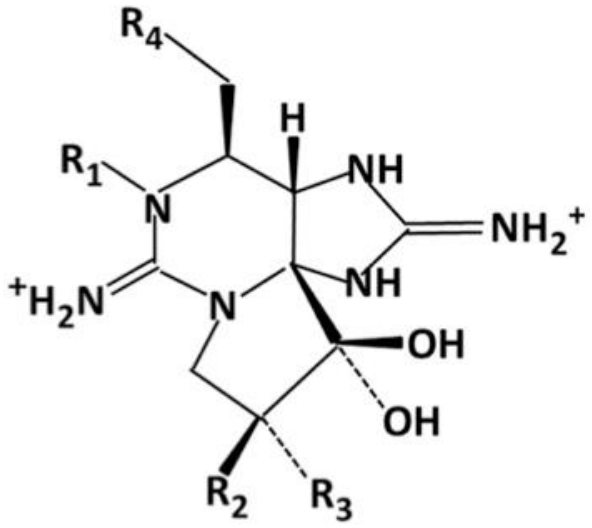


Figure 2-2 General structure of PSTs (Suarez-Isla, 2015)

2.2.2.2 Toxicity and Mechanism of Action

PSTs are neurotoxins, binding irreversibly with Site IV of the sodium channel in cells, which prevent action potentials from propagating along nerves and leads to paralysis (Mattei and Legros, 2014). They are also known to interact with calcium and potassium channel (Cusick and Sayler, 2013). Saxitoxin is one of the most potent naturally-occurring toxins known, having a fatal dose of 1 mg for a standard human model weight of 70 kg (Wiese *et al.*, 2010). However, other analogs of saxitoxin have different toxicities due to structural differences which directly influence its binding ability to sodium channel receptors. PST exposure also triggers the oxidative stress response in animals, exacerbating its toxic effect (Ramos *et al.*, 2014). PST exposure studies in cats have suggested that these toxins move freely between the extracellular and intercellular space (Andrinolo *et al.*, 2002) and also the blood-brain barrier (Cianca *et al.* 2007), which may explain why PST intoxication is so rapid.

The regulation of saxitoxin production in PST-producing organisms along with their metabolic role is poorly understood. The broad production of PSTs by prokaryotes and eukaryotes suggests lateral acquisition of PST biosynthetic genes, which may have aided cyanobacterial homeostasis under high pH or sodium conditions (Murray *et al.*,

2011). However, other studies have shown no effects of pH on toxin content (Holland and Kinnear, 2013): STX is a known sodium channel blocker, which slows down Na^+ uptake in highly saline environments. Ion channels are not only found in nerve cells, but are also used for motility and nutrient uptake in simple organisms.

Conversion of N-sulfocarbamoyl toxins such as C1/C2 to the corresponding carbamoyl analogues (GTX2/GTX3) can occur in shellfish tissues due the activity of glutathione-S-transferase enzymes (Nogueira *et al.*, 2004). These mechanisms are yet to be fully elucidated. These conversions have been found to occur spontaneously in response to physicochemical factors such as pH (Krock *et al.*, 2007). PSTs are highly water soluble and degrades rapidly in alkaline solutions (Stewart and McLeod, 2014).

The current regulatory limit for PSTs in shellfish is 800 µg STX.diHCl equivalents/kg. (EFSA, 2009a). "DiHCl" refers to the dichloride salt form of saxitoxin, which includes two chlorine ions.

2.3 Lipophilic Biotoxins

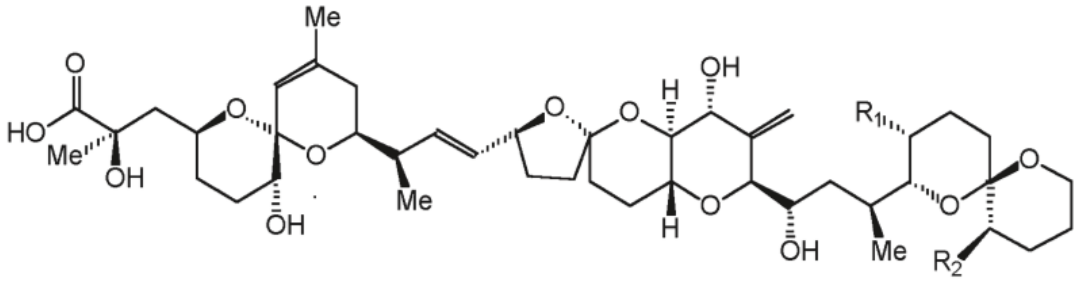
For this research, three lipophilic marine biotoxin groups were studied: Diarrheic Shellfish Toxins (DSTs), Azaspiracids (AZAs), and Neurotoxic Shellfish Toxins (NSTs).

2.3.1 Diarrheic Shellfish Toxins (DST)

The DST group consists of okadaic acid and dinophysistoxins (DTXs). OA was originally identified in black sponges, but later traced to *Prorocentrum* and *Dinophysis* species of dinoflagellates (Valdiglesias *et al.*, 2013). Diarrheic Shellfish Poisoning (DSP) was first described in detail in 1978 following outbreaks in Japan involving mussels and scallops (Stewart and McLeod, 2014). Japan and Europe are the regions most affected by this toxin (FAO, 2004c). Japanese researchers traced the toxin to the dinoflagellate *Dinophysis fortii* and subsequently named the toxin dinophysistoxin (Gerssen *et al.*, 2010a).

2.3.1.1 Chemistry and Sources

They are a group of lipophilic heat soluble polyethers (Sosa and Tubaro, 2015). DTX1 is the methylated derivative of OA while DTX2 is an isomer of OA (Louzao *et al.*, 2015). DTX3 describes a group of toxins with OA modified at the R1 group with long chain fatty acids (Figure 2-3).



R_1	R_2	
CH_3	H	Okadaic acid
CH_3	CH_3	DTX-1
H	CH_3	DTX-2

Figure 2-3 Chemical structures of Okadaic Acid and Dinophysistoxins (Holmes and Teo, 2002)

2.3.1.2 Toxicity and Mechanism of Action

Okadaic acid and its analogs are specific inhibitors of serine/threonine protein phosphatase 1 and 2A, binding to a hydrophobic groove near the active sites of these enzymes (Sosa and Tubaro, 2015). Severe effects on the gastrointestinal system are characteristic of DST poisoning: Diarrhoea, nausea, vomiting, abdominal pain, and chills can appear between 30 mins to 4 hours after ingestion of DSTs (Valdiglesias *et al.*, 2013). However, full recovery usually occurs within three days (FAO, 2004c). This may lead to underreporting of poisoning incidents as the symptoms may be confused with indigestion or food spoilage.

The exact mechanism of action of DSP is still not fully understood. However, it is thought that inhibition of protein phosphatases cause diarrhea upon ingestion (Munday and Reeve, 2013; Valdiglesias *et al.*, 2013). More recent studies have linked the diarrhetic effects of okadaic acid to modulation of neuropeptide Y secretion by

neuroblastoma cells (Louzao *et al.*, 2015). Neuropeptide Y inhibits gastrointestinal motility and water and electrolyte secretion throughout the intestine, and interactions with OA may trigger metabolic pathways which cause diarrhoea *in vivo*.

New toxicological data published by Louzao *et al.* (2015) have shown that DTX1 is more likely to disrupt and pass through the intestinal monolayer, which increases its toxicity compared to OA. In light of this information, there may be a need to revise the Toxicity Equivalence Factor (TEF) for DTX1. TEFs will be further discussed in Section **Error! Reference source not found.**. The current regulatory limit for DST in shellfish is 160 µg OA equivalents/kg (EFSA, 2009a).

2.3.2 Azaspiracids (AZA)

Azaspiracids (AZAs) are polyether marine toxins that have been associated with severe gastrointestinal intoxications. It was first associated with human intoxications in 1995 after several people in the Netherlands consumed Irish mussels from Killary Harbour (James, Moroney, *et al.*, 2003). Azaspiracids were originally grouped together with the DSTs due to similar symptoms, but it was subsequently recognised as unique from DSTs (Twiner, 2008).

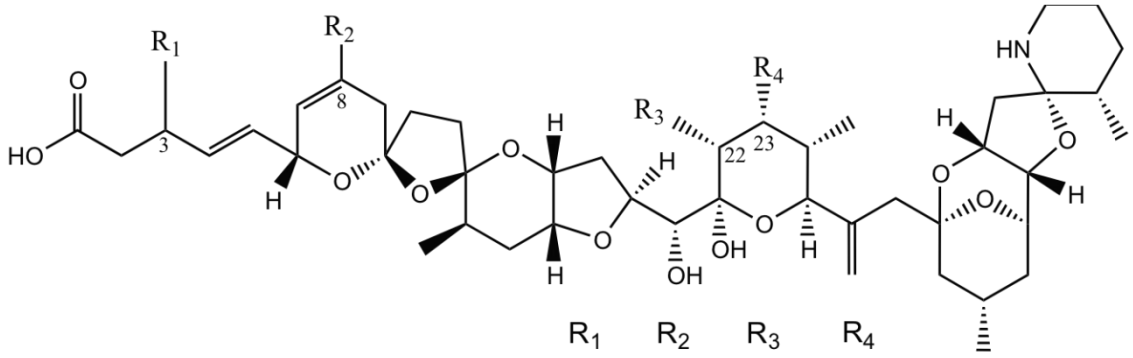
2.3.2.1 Chemistry and Sources

AZAs are known to be produced by marine dinoflagellates from the genera *Azadinium* and *Amphidoma* (Hess *et al.*, 2015) and are so called due to their chemical structure: a cyclic amine (Aza group), a unique tri-spiro-assembly and a carboxylic acid group (Twiner, 2008). Shortly after determination of the first azaspiracid toxin (AZA1), four additional analogs were discovered, AZA2 and AZA3 differing only in the methyl group positions (Figure 2-4), while AZA4 and AZA5 are hydroxyl analogs of AZA3 (Hess *et al.*, 2015). The carboxylic acid group and cyclic imine structure within AZAs cause the toxin to be ionised over the whole pH range, which gives it a slight water-soluble property.

In recent years, numerous analogs of AZAs have been identified, bringing the number up to AZA41 (Hess *et al.*, 2015, Gu *et al.*, 2013; Kilcoyne *et al.*, 2014; Kilcoyne,

Twiner, *et al.*, 2015; Krock *et al.*, 2012; Rehmann *et al.*, 2008). However, the toxicities of these now analogs are unknown and therefore not considered when testing for toxicity of samples. Analytical reference standards are currently available only for AZA 1, 2 and 3.

AZAs have been shown to depurate only slowly in shellfish, which could be due to toxin-binding proteins (Twiner *et al.*, 2012). Shellfish are also known to transform AZAs *via* hydroxylation and carboxylation reactions, possibly facilitated by enzymes (Rehmann *et al.*, 2008).



	R_1	R_2	R_3	R_4
AZA1	H	H	CH_3	H
AZA2	H	CH_3	CH_3	H
AZA3	H	H	H	H
AZA4	OH	H	H	CH_3
AZA5	H	H	H	OH

Figure 2-4 Chemical structure of Azaspiracids 1-5

2.3.2.2 Toxicity and Mechanism of Action

The mechanism of toxicity of the azaspiracids are currently unknown. However, symptoms of AZA intoxication have been reported to be similar to DSP toxins: nausea, vomitting, diarrhea, and abdominal cramps (Twiner, 2008). Toxicological studies on mice *via* intraperitoneal injection of AZA toxins revealed neurotoxin-like symptoms such as sluggishness, respiratory difficulties, spasms and paralysis (Hess *et al.*, 2015). At physiological pH, AZA1 exists as a zwitterion (i.e., contains both a positive and negative charge but is electrically neutral), which would give detergent-like properties to this molecule (James, Moroney, *et al.*, 2003). In addition, they could have many different charge states depending on pH, which varies throughout the body e.g. mouth, stomach, intestines. This overall neutral but potentially ionic character may result in

greater possibilities for interaction of AZA with its biological target, and slow down elimination from the body. AZAs have also been found to bind to proteins, which gives it protection against extreme pH conditions. However, when exposed to simulated human conditions, the amount of AZAs observed increased. This implies that after ingestion of contaminated shellfish, AZAs can become more toxic in the gut after ingestion of shellfish (Alfonso *et al.*, 2008)

The current regulatory limit for AZAs in shellfish is 160 µg AZA1 equivalents/kg (EFSA, 2009a).

2.3.3 Neurotoxic Shellfish Toxins (NST)

Exposure to brevetoxins causes Neurotoxic Shellfish Poisoning (NSP). These toxins have been detected in cockles, mussels and oysters (Ishida, Nozawa, Nukaya, *et al.*, 2004), and persist in sediments and the community of small organisms around seagrass (Hitchcock *et al.*, 2012).

2.3.3.1 Chemistry and Sources

Brevetoxins are a group of lipophilic polyether toxins produced by the dinoflagellate species *Karenia brevis*. Two structural backbones (Type A and B) are the basis of all known analogs of brevetoxin. Brevetoxins 1, 7 and 10 have the Type A backbone while Brevetoxins 2, 3, 5, 6, 8, and 9 have the Type B backbone. Figure 2-5 shows one example of Type A and Type B brevetoxins. Upon ingestion by shellfish, brevetoxins can undergo transformation, such as transformation of Brevetoxin 5 to Brevetoxin B5 (Ishida, Nozawa, Hamano, *et al.*, 2004). Brevisulcatic toxins and *Karenia brevisculata* toxins were novel forms of toxins identified recently from dinoflagellates harvested from New Zealand (Holland *et al.*, 2012). These toxins were found to have binding activity to sodium channels as well as haemolytic and cytotoxic effects when tested on cultured cell lines.

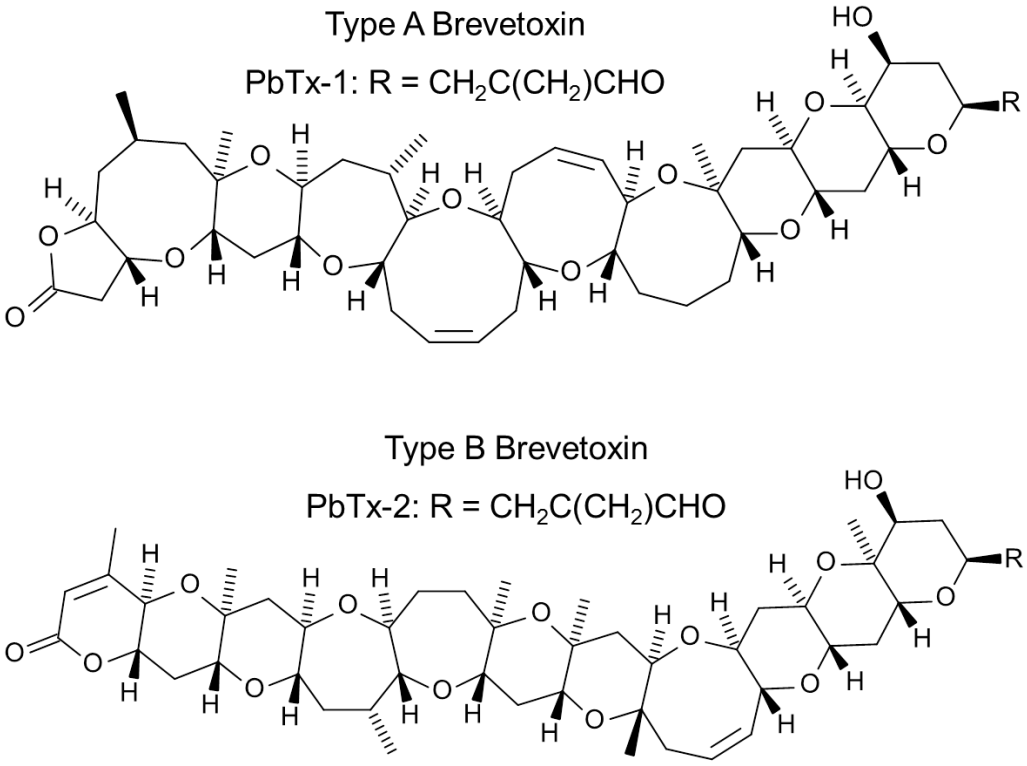


Figure 2-5 Chemical structures of Brevetoxins (Turner, Higgins, *et al.*, 2015)

2.3.3.1 Toxicity and Mechanism of Action

Brevetoxins target Site V of the α-subunit of voltage-gated sodium channels, but unlike other neurotoxins like saxitoxin and tetrodotoxin, brevetoxins are channel activators. Although it is called Neurotoxic Shellfish Poisoning, intoxication also produces gastrointestinal symptoms along with neurological (abdominal pain, vomitting, nausea, ataxia, paresthesia, reversal of temperature sensation)(Plakas and Dickey, 2010). In toxicity studies on mice, intraperitoneal injection of brevetoxins, abdominal breathing, along with elevated respiration rates were observed.

The current regulatory limit set for NSTs in shellfish is 800 mg PbTX-2 equivalents/kg (Brovedani *et al.*, 2015). However, to date, this level has only been adopted in the USA, New Zealand and Australia (Turner, Higgins, *et al.*, 2015).

2.4 Methods of Analysis

Seafood safety testing for marine biotoxins started after initial reports of illnesses were eventually linked to seafood and seafood products consumed in particular seasons and

locations. Early test methods relied on introduction of suspected toxic samples to various animal models (Campbell *et al.*, 2011). Useful bioassays were then refined and modified to serve as quantitative tools, and have since become ubiquitous for detecting harmful concentrations of marine algal toxins (Rourke and Murphy, 2014; Stewart and McLeod, 2014).

Methods that are currently employed for the detection of marine toxins are divided into analytical and biological groups (Vilariño *et al.*, 2010). Analytical methods enable identification of toxins measured based on their physiochemical properties, giving qualitative as well as quantitative information on the shellfish toxin profiles. Biological methods do not reveal toxin profiles within a sample but give an overall estimate of the toxin content. Under the biological methods group are immuno-based techniques, receptor-based techniques, and cell or tissue-based techniques.

The Codex Alimentarius is an international food standards established by the Food and Agriculture Organisation of the United Nations which was later joined by the World Health Organisation. The Codex is maintained by the Codex Alimentarius Commission, which is divided into many general committees, commodity committees and ad hoc task forces. One of these committees, the Codex Committee on Fish and Fishery Products (CCFFP) is responsible for maintaining and updating international food standards related to fresh and frozen fish and marine products that are traded internationally. In recent years, the CCFFP has recognised the importance of standardising testing methods for marine biotoxins and have begun discussions on method performance criteria necessary for international bodies to agree on standards related to testing and reporting of marine biotoxin analyses (CCFFP, 2012). At the current stage, draft method performance criterias have been discussed at an international level, but have yet to be finalised. However, these criterias serve a useful starting point for method development.

2.4.1 Reference Materials

Reference materials play a critical role in validating any newly developed method, both biological and analytical. For marine biotoxin analysis, certified mussel tissue and purified toxin reference standards enable the development and further refinement of methods, increasing confidence in the results obtained from these methods. Commercial availability of certified reference materials are critical to the maintenance and continuous validation of analytical methods (Otero and Alfonso, 2014). Reference materials are used to routinely calibrate measurement systems to support the enforcement and inspection of biotoxin risk sites.

Hydrophilic and lipophilic toxins can be chemically synthesized, produced by laboratory biosynthesis, or isolated and purified from harvest of toxic phytoplankton from the environment. Synthesis pathways for several PSTs (Bhonde and Looper, 2011; Fleming and Bois, 2006; Iwamoto and Nagasawa, 2010; Tsuchiya *et al.*, 2015) and AZAs have been published (Nicolaou, Frederick, *et al.*, 2006; Nicolaou, Koftis, *et al.*, 2006), but the processes are costly and carries a high risk due to the product being a highly potent toxin. Raw material for purification and standard production can be sourced from natural toxic blooms, but is more unpredictable and variable in toxin content and profile (Vilariño *et al.*, 2010). Therefore, standard production is still primarily through culture of toxic dinoflagellates and large scale chromatography and fractionation (Kilcoyne *et al.*, 2014).

A significant barrier for the production of marine biotoxin chemical standards comes from their listing under the Chemical Weapons Convention, which severely limits the work allowed to be done with this group of compounds (Anderson, 2012). Despite these challenges, much effort has been invested into production of chemical standards such as isolation and purification of toxins from algal cultures and contaminated shellfish (Watanabe *et al.*, 2011). These efforts have led to material such as irradiated freeze-dried mussel and oyster tissue, and certified contaminated samples (Alfonso *et al.*, 1993). Data from stability studies of these chemical standards are also important to evaluate the longevity of such materials so production costs can be kept low (Indrasena and Gill, 2000; McCarron *et al.*, 2006; McCarron, Emteborg, *et al.*, 2011).

2.4.2 Biological Methods

2.4.2.1 Bioassay-based Methods

The Mouse Bioassay (MBA) was first created by Sommer and Myer in the 1930s (Humpage *et al.*, 2010) and is a prime example of a biological method for marine toxin detection. In this assay, shellfish extracts are injected into the intraperitoneal cavity of mice, and time to death is recorded in the case of toxic samples. This result is compared against a dose-death time curve. However, it is now known that there is no correlation between the specific activity of saxitoxin analogs and lethal doses in mouse bioassays (Munday and Reeve, 2013), implying that dose-death time curves cannot be applied to all toxins and have to be determined individually for each toxin. Previously, it was assumed that the relative toxicities of the saxitoxin analogs could be predicted by their behavior in the mouse bioassay.

In addition, the MBA does not comply with modern standards of animal welfare. Despite the progress away from the MBA, it still remains an important method for developing countries without the resources to use advanced chemical methods, as well as in situations where there are unidentified toxins (Vilariño *et al.*, 2010). The MBA is able to detect biotoxins at regulatory levels and has a long history of consumer protection. It is also an established and validated method. In some countries where cost, regulatory inertia and the lack of analytical chemistry skills and infrastructure continue to be issues, the use of MBA for shellfish safety testing appears likely to continue. Considering that the MBA is likely to be used for shellfish testing for some years to come, regulated hypothermia and other nonlethal endpoints has been suggested in place of time of death measurements (Stewart and McLeod, 2014).

In places where a move away from the MBA has been encouraged, other methods have been developed for the testing of marine biotoxins. Bioassays based on other animal models such as locusts and cockroaches have been trialled (Cook *et al.*, 2006; Ruebhart *et al.*, 2011). However, the inherent variability of biological systems leads to the same weaknesses in these methods as the MBA. In addition, these methods depend on conservation of sodium channel structure between species, requires skilled technicians, maintenance of colonies, which are similar issues with mouse bioassay.

In recent years, the MBA has come under criticism from the scientific community, animal welfare groups as well as risk assessment authorities, mostly in Europe and America (Campbell *et al.*, 2011). Multiple weaknesses of the MBA have also been highlighted: non-specificity, false positives, interference by certain metals, effects of sex, strain and weight of the animals, the effects of pH of the injected solution and poor inter-laboratory agreement (Guy and Griffin, 2009). Up until 2011, the MBA was the reference method for lipophilic toxins, but work on validation of alternative assays have led to the adoption of LC-MSMS by the European Union as the reference method for such toxins (Otero and Alfonso, 2014). This decision took effect on 1 July 2011, and the mouse bioassay was phased out after 31 December 2014. The number of mice needed for bioassays is expected to decrease substantially over the coming years as alternative testing methods are adopted (Turrell *et al.*, 2007). New Zealand's response following its first shellfish biotoxin event in 1993 used an estimated 80,000 mice annually; however, this declined considerably over ensuing years with the introduction of phytoplankton monitoring and chemical methods for toxin detection.

2.4.2.2 Immuno-based Methods

Immuno-based methods depend on biotoxins binding to specific antibodies which are then measured to give a "Yes or No" tests for the presence of biotoxins. In some cases, an ELISA test can give semi-quantitative responses by measuring the intensity of the band formed on the strip. These tests have been applied for use as a shipboard screening method on board harvesting ships (Jellett *et al.*, 2002; Turner, Tarnovius, *et al.*, 2015). Examples of immuno-based methods are ELISA kits (Campbell *et al.*, 2009; Sato *et al.*, 2014; Tsumuraya *et al.*, 2014), magnetic microspheres (Devlin *et al.*, 2011), receptor binding assay (AOAC, 2011a), membrane biosensors (Campàs *et al.*, 2007), tissue culture assays (Kogure *et al.*, 1987), fluorescence planar waveguide biosensors (Meneely *et al.*, 2013), and most recently, the optoelectronic mouse (Campbell *et al.*, 2014).

ELISAs have been converted into test strip formats for rapid shipboard or harvest site assays. However, these kits have been reported to have a high false positive rate of up to 14% (Inami *et al.*, 2004, Gerssen *et al.*, 2010a), and the antibodies embedded are

specific to particular forms of PSTs often have a limited sensitivity to other analogs (Guy and Griffin, 2009). In a situation where there is poor cross reactivity of antibodies with other analogs of biotoxin, the total toxin levels may be underestimated. For instance, Lefebvre *et al.* (2008) concluded that ELISA may underestimate toxin levels by as much as 61 times in samples with STX. In addition, although many toxins are haptens (small molecular weight compound which elicits antibody production), some analogs fail to stimulate antibody response in many inoculated animal models. Furthermore, ethical issues arise in the production of these antibodies, as animals have to be inoculated with small doses of the toxins in order for them to produce the antibodies. Even in cases where the ethics are approved and overseen, the limited availability of purified toxins to stimulate antibody response in animals presents a challenge in sustainable antibody production (Kavanagh *et al.*, 2015).

Other biological methods which are similar to the MBA have applied for marine biotoxin analysis. Functional or receptor based assays give an overall toxicity of a sample based on affinity or binding onto receptors. These new techniques include saxiphilin binding (Robillot *et al.*, 2009), radioligand binding, sodium channel binding (Campbell *et al.*, 2007), the use of recombinant sodium channels (Vélez *et al.*, 2001). cell-based assays using neuroblastoma (Humpage *et al.*, 2010) and rat brain preparations (Manger *et al.*, 2014; Vilariño *et al.*, 2009).

Multiplexed immunosensors have been shown to be able to detect several classes of biotoxins: paralytic, diarrheic and amnesic shellfish toxins (Fraga *et al.*, 2013). However, the preparation of reagents for this analysis is complex and the sourcing of antigens remains an issue to be solved. Biosensors are another type of sensor which have been developed for marine biotoxin analysis. Biosensors rely on the binding of toxins to receptors grafted onto a substrate which generates a current to a detector. This has been developed for ASTs (Vilariño *et al.*, 2010). The advantages are sensitivity and ease of use, low cost of operation, and no specialised instrumentation required. However, standardisation between laboratories of cell-based methods are difficult and requires the maintenance of cell colony (Vilariño *et al.*, 2013).

2.4.3 Analytical Methods of Detection

There has been a significant shift in support of alternative methods, stemming from ethical objections to animal assays in the light of available chemical methods (Hess *et al.*, 2006). Development of analytical methods for the analysis of marine biotoxins have advanced steadily since regulations discouraging the use of the MBA have been introduced. Analytical methods involve the use of instruments for detection and in most cases also involve a separation phase to facilitate the identification of individual toxins. Because these methods do not use biological tissues or substrates as part of the testing process, there are no ethical issues compared to bioassays or immuno-based methods. These methods are also able to provide toxin profile information: analyses of toxin profiles, particularly involving confirmatory methods such as LC–MS, can provide new insights into patterns and sources of toxin accumulation in marine food webs (Krock *et al.*, 2007). Separation by chromatography coupled with the use of UV and FLD detection (Quilliam, 2003), and capillary electrophoresis methods have been reported (Keyon *et al.*, 2014). More recently, a quantitative NMR (qNMR) method was published for PSTs (Watanabe *et al.*, 2010).

2.4.3.1 Hydrophilic Toxins

A fluorescence method for PST analysis was developed in 1975 using alkaline hydrogen peroxide oxidation of STX to form a fluorescent pyrimidine purine (Ben-Gigirey *et al.*, 2015). Further development of this fluorescence-based method led to a HPLC-FLD method using periodic acid as an oxidation agent, culminating in a standardised method with prechromatographic oxidation published as the AOAC 2005.06 (AOAC, 2006). Refinement and extension of the AOAC 2005.06 method to include additional toxins such as decarbamoylneosaxitoxin (dcNEO) and decarbamoylgonyautoxin 2 and 3 (dcGTX2 & 3) was performed by Turner *et al.* (2009) and Ben-Gigirey *et al.* (2012). For other less commonly occuring toxins, the lack of calibrants for GTX6 and C3&4 can be mitigated by indirect quantification after acid conversion into NEO, described by Costa *et al.* (2014).

Fluorescence is a powerful detection method, with sensitivities quoted at fentogram (Morgan and Smith, 2010) and picogram levels (Snyder *et al.*, 2010), dependent on the analyte. Fluorescence detection is selective and has the potential to reduce noise from

non-fluorescing compounds in sample extracts. It is robust, economical, and an alternative for compounds that do not respond well to MS. Some compounds which are not-fluorescent can be made to be compatible through chemical derivatisation. Newer analogs such as M1-M4 and the benzoate analogs are not fluorescent.

There are two methods for the fluorimetric analysis of PSTs, the precolumn oxidation method (AOAC 2005.06) and the postcolumn oxidation method (AOAC, 2011b; Rourke *et al.*, 2008). Derivatisation is the chemical modification of compounds to change their nature to be more amenable to a particular detection mode (Qi *et al.*, 2014; Santa, 2010). Derivatisation may be performed to stabilise compounds in samples, improve extraction efficiency and selectivity, adjust retention time, and aid in detection (Xu *et al.*, 2011). For the postcolumn oxidation method, several drawbacks have been described, namely, complex instrumentation due to the addition of a reaction after the column with a long tubing for derivatisation procedure. The longer flow path leads to wider chromatographic peaks. In addition, multiple analysis conditions are required for quantitation of the full range of hydrophilic toxins (Vale *et al.*, 2008), which results in unpredictable column lifetime (Rourke and Murphy, 2014). For both precolumn and postcolumn oxidation methods, identification of biotoxins is based on retention time of reference standards compared with sample peaks. The identification process may have interferences from coelution of naturally fluorescent sample components. Even though there are known drawbacks of the oxidation step, such as toxins producing the same products that introduces uncertainty to toxicity calculations, the sensitivity of the FLD method has been demonstrated to be suitable for regulatory monitoring purposes (Turner *et al.*, 2009) and both fluorescence methods have been implemented by regulatory bodies around the world.

2.4.4 Mass Spectrometry

In recent years, mass spectrometric (MS) detectors have become more ubiquitous in food testing laboratories as these detectors become more sensitive, selective, robust, easy to use, and compact (Hird *et al.*, 2014). The increased adoption of this detector in analytical environments have also been due to the falling costs of operation and the recognition of its strengths in identification of compounds using MRM transitions and

ion ratios. In addition, the mass filtering of MS detectors also reduce chemical noise, leading to greater sensitivity.

Mass spectrometric detectors can be paired with liquid chromatography (LC) systems. The eluent from the column has to be ionised before it can enter the vacuum chamber as charged ions. This happens at the source of the detector, where heated gas is passed over the incoming flow from the LC. The solvent is evaporated and charged ions are formed (Figure 2-6). This form of ionisation is known as Electrospray Ionisation (ESI). It is considered a 'soft' form of ionisation because molecular ions are formed without significant fragmentation. The flow from the LC passes through a charged capillary, which produces a charge separation at the surface of the liquid as it exits the tip, forming a Taylor cone. As the droplets reduce in size through evaporation, the excess charge on the surface causes the compounds within to form ions which are drawn into the mass spectrometer's vacuum chamber through a counter electrode (Cech and Enke, 2002).

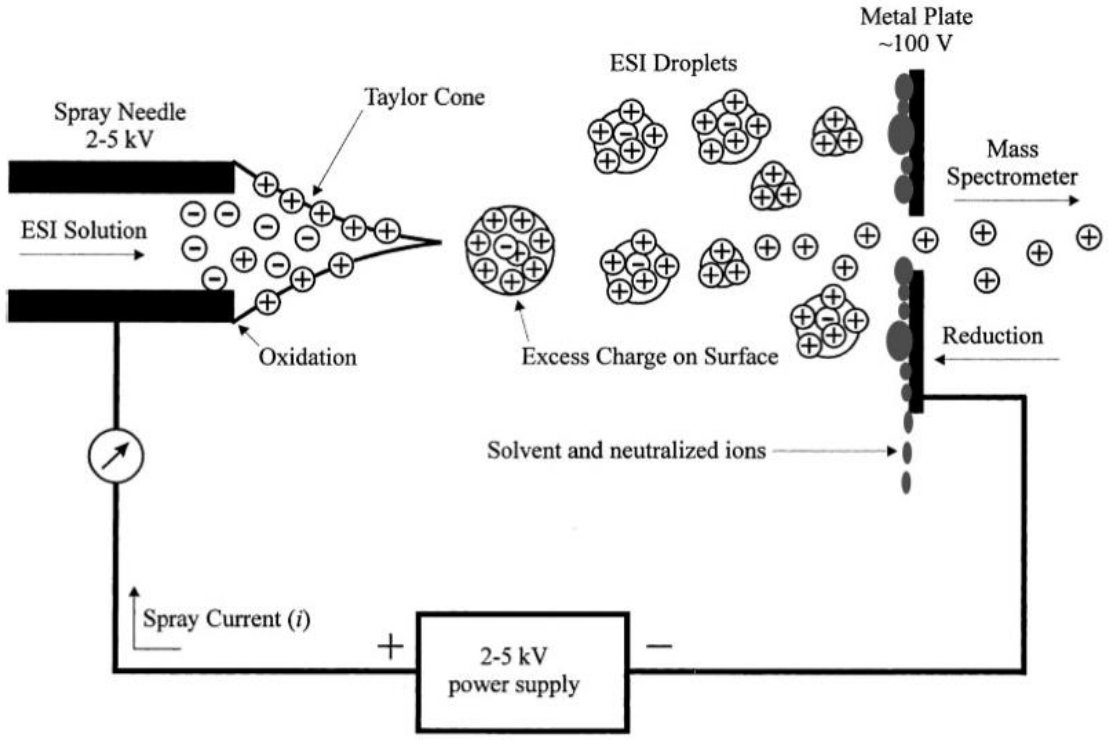


Figure 2-6 Diagram of ion formation in ESI source (Cech and Enke, 2002)

Further confirmation of toxin identity can be carried out using tandem MS detectors, which fragments the molecular ion in a collision cell placed between two mass filters

(Figure 2-7). The parent-to-product ion transitions that are monitored in tandem MS are called multiple reaction monitoring (MRM) transitions. In modern detectors, several parameters can be adjusted to maximise the sensitivity, such as the energy applied in the collision cell, the voltage applied across the inlet between the source and the focussing lens (fragmentor voltage), and cell accelerator voltage. The fragmentation patterns of the parent ion as well as precursor/product ion ratios can be used to identify compounds with high accuracy. The ability to analyse fragments from a parent ion is also useful for structure elucidation of toxins determination of novel analogs (Kilcoyne *et al.*, 2014; Quilliam *et al.*, 1993).

Recent advances in liquid chromatography–mass spectrometry (LC–MS) methods for PSP toxins use hydrophilic interaction liquid chromatography (HILIC), which allows separation of polar analytes without the use of ion pair reagents and highly aqueous buffered mobile phases. In addition, the use of LC–MS is advantageous as it offers sensitive and selective detection that does not require derivatisation of the toxins. LC–MS can provide additional confirmatory information, *via* fragmentation of the compound, making it less dependent on the retention time of individual PSP toxins (Turrell *et al.*, 2007). Several methods have been published on the analysis of PSTs using LC-MS and LC-MSMS (Blay *et al.*, 2011; Dell'Aversano *et al.*, 2004; Diener *et al.*, 2007), and also lipophilic toxins (García-Altares *et al.*, 2013; Gerssen *et al.*, 2009b; Haiyan *et al.*, 2014; James, Sierra, *et al.*, 2003; Nozawa *et al.*, 2003; Turner, Higgins, *et al.*, 2015; Zhuo *et al.*, 2014) and ASTs (Beach *et al.*, 2014; Ciminiello *et al.*, 2005; Hess *et al.*, 2005; Picot *et al.*, 2012; Romero *et al.*, 2011; Wang *et al.*, 2007; 2012).

LC-MSMS techniques are capable of identifying a specified toxin in a shellfish extract, but for quantitation, the response of the instrument to this toxin must be established, and this requires calibration with chemical standards. One issue affecting sensitivity arise from matrix interference which can affect instrument response (Kilcoyne and Fux, 2010; Kruve *et al.*, 2010; Turner *et al.*, 2012). For some toxins, the matrix interference can be mitigated by analysis with different polarities. Mass spectrometry of domoic acid is possible in positive and negative mode possible due to presence of both carboxylic acid (-COOH) and amine (-NH) functionalities (Dell'Aversano *et al.*, 2011). Signal suppression of 53% was observed in positive mode, while only 3% suppression was observed for negative mode. In addition, the dilution of the sample before analysis can

be done to reduce the amount of matrix that enters the instrument. For domoic acid, the concentration of matrix that would induce minimum suppression was determined to be 0.0625 g/mL for positive mode and 0.125 g/mL for negative mode, showing a higher resistance to matrix effects for negative ionisation.

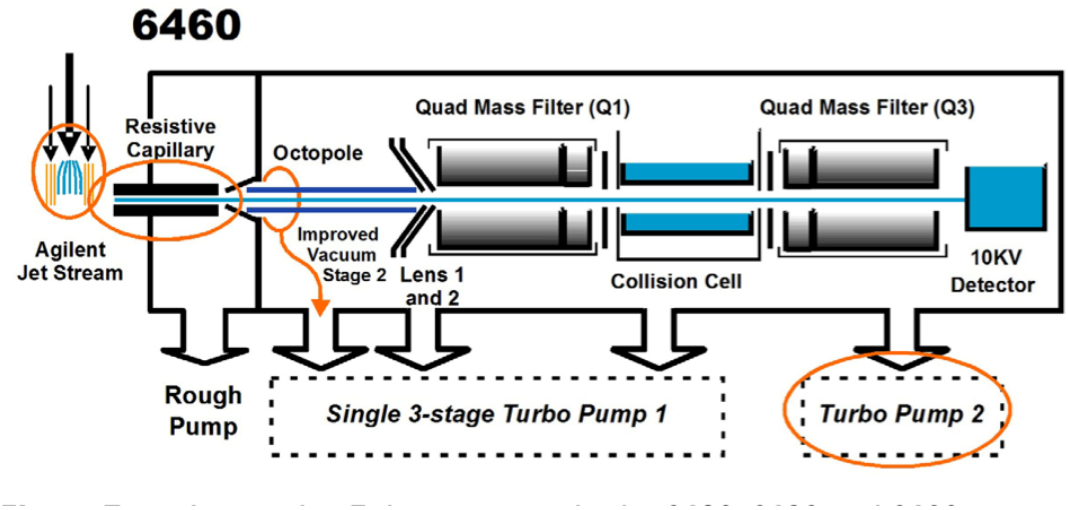


Figure 2-7 Scheme of tandem mass spectrometer (Agilent Technologies, 2015)

Source geometry design differences between different manufacturers has been found to affect mobile phase desolvation efficiency which directly influences the amount of sample reaching the mass filter and detector (Periat *et al.*, 2014). Therefore, MSMS source design have an effect on matrix effect and sensitivity (Stahnke *et al.*, 2012), which reduces the transferability of methods between laboratories. For the purposes of method development, the analytes of interest must be validated or verified on the instrument that is being used.

2.5 Tandem LC

The simultaneous analysis of non-polar and polar analytes in a sample is a formidable challenge. In the field of metabolomics, where snapshots of cellular processes are required, an accurate picture of both hydrophilic and lipophilic components can be obtained using either 2D LC or by running individual lipophilic and hydrophilic extracts on instruments. In 2D LC, eluent from one column (first dimension) is collected in a loop and sent *via* a switching valve to a second column (second dimension) with a different stationary phase so that coeluting compounds from the first column can be

further separated. The different selectivity of the second column allows for orthogonal separation of coeluting compounds and better identification of compounds over a larger range of polarity, which uses columns of different separation mechanisms to enable better identification of compounds over a larger range of polarity. However, because fractions have to be collected before further separation on the second column, 2D LC methods tend to have long analysis times per sample. Sample backlogs were one of the key factors cited for the release of PST contaminated shellfish to the market in 2012 (Campbell *et al.*, 2013). The other approach is tandem LC, where the chromatographic columns can be placed either in series or in parallel (Chalcraft and McCarry, 2013). Both approaches have been applied to polar and nonpolar molecules in metabolomics. In the first case, Chen *et al.* (2016) have reported a method which couples a RP and HILIC column in series. However, they have only demonstrated the method on algal samples and it has a long runtime of 65 minutes per sample.

New methods have been published which enable a much more rapid analysis of both hydrophilic and lipophilic extracts of complex biological samples such as plasma, blood, and tissue extracts. These methods are different from 2D LC systems and tandem LC in series in that the separations are performed in parallel, enabled by having an extra pump in the system. In addition, the flowpath in tandem LC setups incorporate a small trap column after the autosampler which retains compounds of one polarity while unbound compounds are separated downstream *via* an analytical column with a second pump. A valve diverts the flow to the second column and compounds on the trap column are washed off by a change in solvent composition to be separated by the second column. An example of a tandem LC setup is given in Figure 2-8. Tandem LCs have been described for metabolite profiling (Klavins *et al.*, 2014; Ortmayr *et al.*, 2015). Because the separation can be performed in parallel, tandem LC setups have a much shorter run time compared to 2D LC setups.

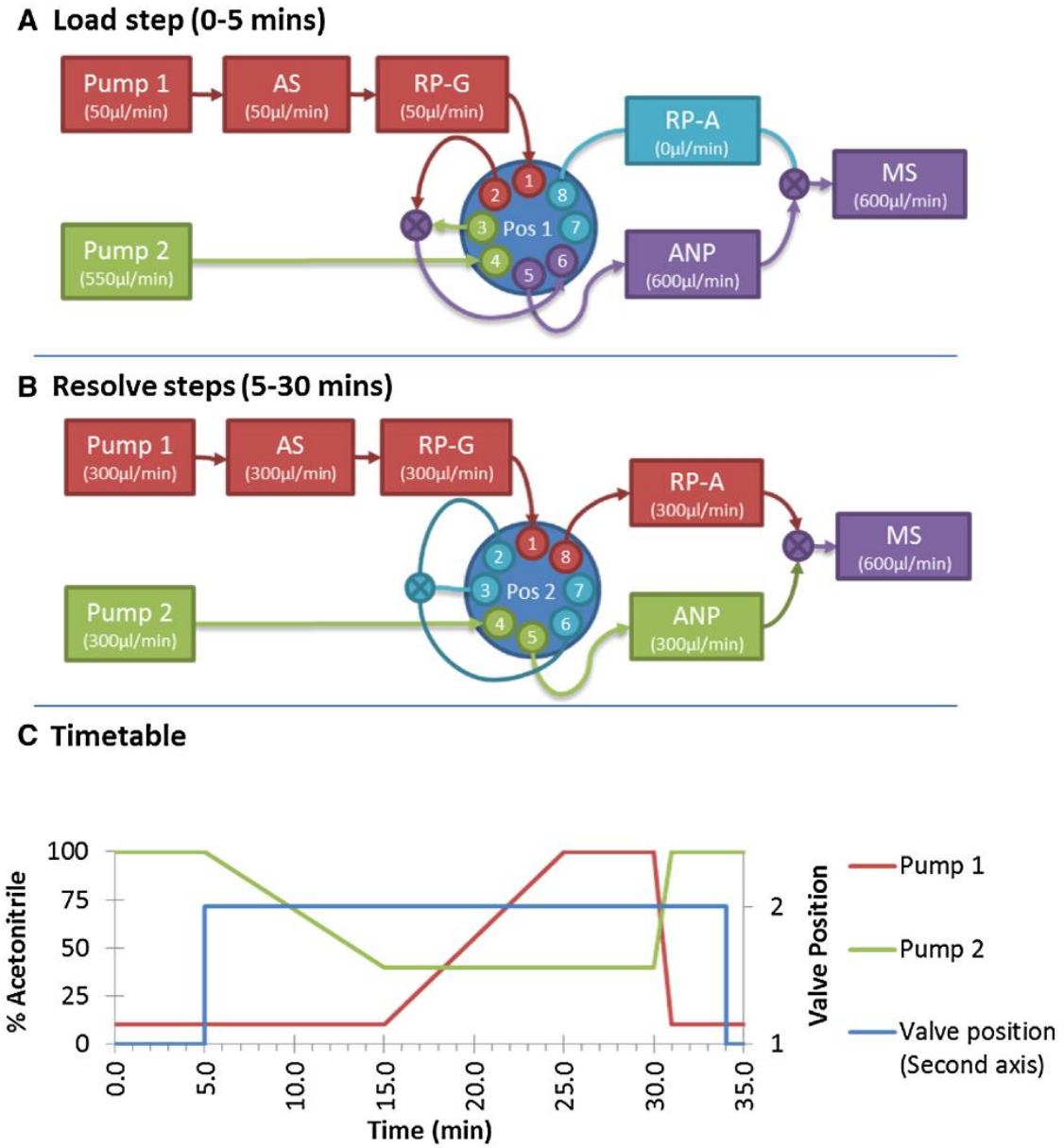


Figure 2-8 Schematic of a tandem LC setup (Pyke *et al.*, 2015)

The benefits of tandem LC setups include faster analytical runtimes due to parallel chromatography, flexibility in choosing between tandem and single column analysis, ease of changing detector combination, and utilisation of orthogonal separation modes (Pyke *et al.*, 2015). Using columns with different selectivities, orthogonal column combinations such as HILIC and RP can be used to cover a wide range of polarities. HILIC separates polar hydrophilic compounds while RP separates non polar compounds. Porous graphitised carbon and reversed phase columns have been used in orthogonal separation for metabolomics analysis of biological extracts (Ortmayr *et al.*, 2015).

For implementation of tandem LC for a particular analysis, several aspects have to be considered, such as compatibility between the different chromatographic columns, the solvent composition of the sample to be injected, flow rates of the two pumps, and detector suitability for the analytes.

From a review of the literature, Hydrophilic Interaction Chromatography (HILIC) and Reversed phase (RP) chromatography were found to be used for marine biotoxin analysis. Aqueous Normal Phase (ANP) was also chosen to be investigated as part of this research due to the dual separation capability.

2.5.1 Reversed Phase Chromatography

Reversed phase chromatography is the most common type of LC separation and is performed using a non-polar stationary phase with a polar mobile phase for elution of compounds (Moldoveanu and David, 2013). It is usually a first choice for the separation of both neutral and ionic samples, e.g. by using a column with C18 (octyldecyl modified silica) stationary phase and a mobile phase mix of water and a solvent such as methanol or acetonitrile (Snyder *et al.*, 2010). Modern columns are packed with a high purity silica with a low metal content and a high proportion of silanol groups on the particle surfaces (Type B silica)(Borges, 2015)

The retention mechanisms in RP chromatography is based on polar interactions of molecules with the stationary phase and mobile phase. The elution order in RP chromatography is approximately in order of decreasing polarity, i.e. leven oiess polar compounds are retained more strongly on the stationary phase and elute last (Snyder *et al.*, 2010). When the composition of mobile phase contains more acetonitrile or methanol, it becomes less polar (“stronger”) and increases the strength of interactions between analyte and mobile phase, leading to elution of the compound from the stationary phase.

Because of the ubiquity of RP chromatography, it has been used in marine biotoxins analysis. RP chromatography has been employed for the analysis of lipophilic toxins

(EURLMB, 2015; García-Altares *et al.*, 2013; Gerssen *et al.*, 2010b; McCarron *et al.*, 2014; Rúbies *et al.*, 2015; These *et al.*, 2011).

PSTs require derivatisation before separation in RP chromatography systems. However, even in cases where the derivatization is used, the mechanism employed for the separation utilises ionic interactions rather than polarity: high buffer mobile phases (up to 100 mM) and very small fraction of acetonitrile are required for derivatised PSTs.

2.5.2 Hydrophilic Interaction Chromatography

Hydrophilic Interaction Chromatography has emerged in recent years as a powerful separation technique that has a different selectivity to traditional RP chromatography. Hydrophilic compounds have weak retention in RP chromatography systems and often elute close to the hold-up volume, so that separation is difficult (Jandera, 2011). HILIC is able to retain polar compounds, and is very amenable to MS methods, due to a high percentage of volatile eluent entering the source, which aids in the desolvation process. HILIC has been applied in situations where polar analytes cannot be separated by conventional reverse phased chromatography. These separations include drug-like compounds from snake venom, *Panax* plants, apple juice and mulberries (Nguyen and Schug, 2008).

The retention mechanisms responsible for HILIC have been widely investigated in recent years. Hydrophilic, hydrophobic, electrostatic, hydrogen bonding, dipole–dipole, pi-pi interaction, and shape-selectivity have been examined to determine which of these forces exert the most effect on retention and selectivity of hydrophilic and polar compounds (Dinh *et al.*, 2011). Polar silanols on the surface of stationary phase particles hold a thin layer of water in which hydrophilic analytes partition into, retaining them (Figure 2-9)(Dinh *et al.*, 2013). Due to the hydrated layer having a critical effect on separation mechanism, HILIC is particularly sensitive to buffers in the mobile phase. Mobile phase was also a critical factor in HILIC chromatography such as proportion of water and ionic strength related to the concentration of buffer salts and pH (Heaton *et al.*, 2014). Ionic strength affects the hydrated silica layer, which in turn affects peak shape and resolution.

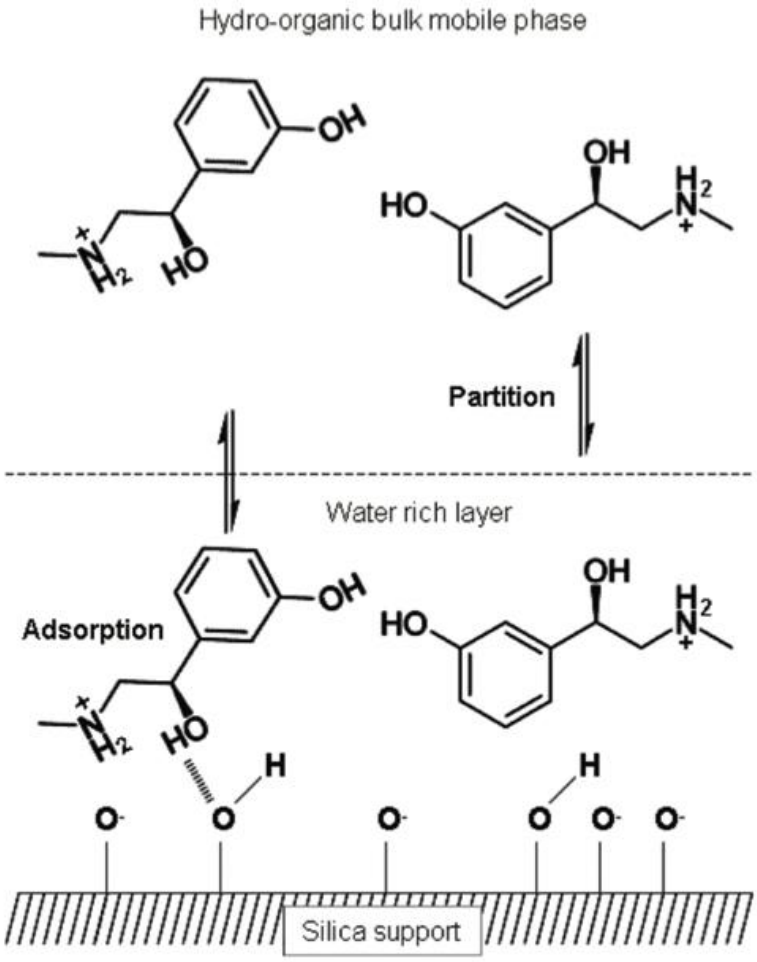


Figure 2-9 Partition and adsorption mechanism in HILIC retention (Heaton and Smith, 2012)

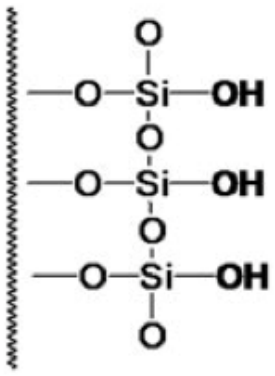


Figure 2-10 Chemical surface composition of underivatised or bare silica (McCalley, 2007).

Stationary phase modification of the TSKgel Amide80 comes under neutral group (Guo and Gaiki, 2011) and are less polar than bare silica. Retention mechanisms for neutral HILIC stationary phases are predominantly polar interactions or hydrogen bonding between functional groups on the stationary phase surface and polar groups of the analytes. ZIC HILIC stationary phase contains sulfoalkylbetaine moiety (Ikegami *et al.*, 2008) and comes under zwitterionic group, which contain both anionic (SO_3^-) and catioinic (N^+) groups (Figure 2-11). For the separation of hydrophilic PSTs, use of the TSKgel Amide80 column has been reported by Dell'Aversano *et al*. (2005), Sayfritz *et al.* (2008), and Mattarozzi *et al*. (2016). ZIC HILIC A method using bare silica columns was reported by Johnson et al (2009).

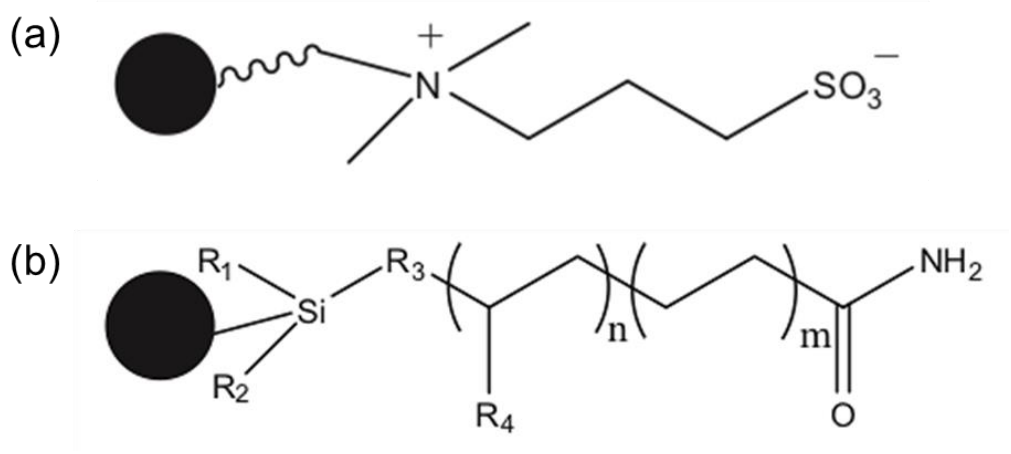


Figure 2-11 Functional groups of ZIC HILIC (a) and TSKgel Amide80 (b) stationary phases (Guo and Gaiki, 2011)

2.5.3 Aqueous Normal Phase (ANP) Chromatography

Aqueous Normal Phase (ANP) chromatography is another emerging chromatographic technology for the analysis of polar molecules. The basis of this separation is due to the use of Type C silica, in which silicon hydride groups replacing most of silanol (Si-OH) groups on the surface of the stationary phase particles (Figure 2-12)(Pesek and Matyska, 2005). Silanol groups are able to interact with analytes which can contribute to poor peak shape and irreversible binding (Pesek and Matyska, 2009). Type C silica is a relatively new material compared to established C18 reversed phase columns, only available commercially from 2006. This difference leads to selectivity differences

between the ubiquitous Type B silica columns described in Section 2.5.1 used for most chromatographic separations.

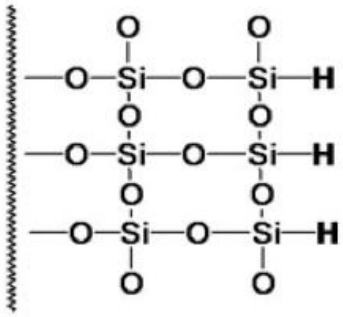


Figure 2-12 Chemical surface composition of silica hydride (Pesek and Matyska, 2009)

Currently, the retention mechanism behind silica hydride is not fully understood, but this material has shown ability to retain both polar and non polar analytes as discussed by Kulsing *et al.* (2014). In contrast to HILIC stationary phases, the silica hydride groups are less likely to retain water, which leads to a thinner water layer on the surface of the stationary phase particles (Pesek *et al.*, 2013). The proposed methods of retention are based on a double layer model (Figure 2-13)(Kulsing *et al.*, 2014). In Figure 2-13A, the inner layer contains strongly adsorbed ions while the diffuse layer contains loosely bound ions. The collective dipole model illustrated in Figure 2-13B shows mechanism more similar to HILIC, where a layer of enriched water forms on the surface of the silica bead. The absence of a water layer means a rapid reequilibration period after sample injection. The silica in a Diamond Hydride chromatographic column is classified as Type C silica. Diamond Hydride columns are based on silica hydride material but also contain a small amount of carbon (about 2%) which is chemically bonded to the stationary phase surface (Parmar, 2013), which enables a greater selectivity for polar compounds.

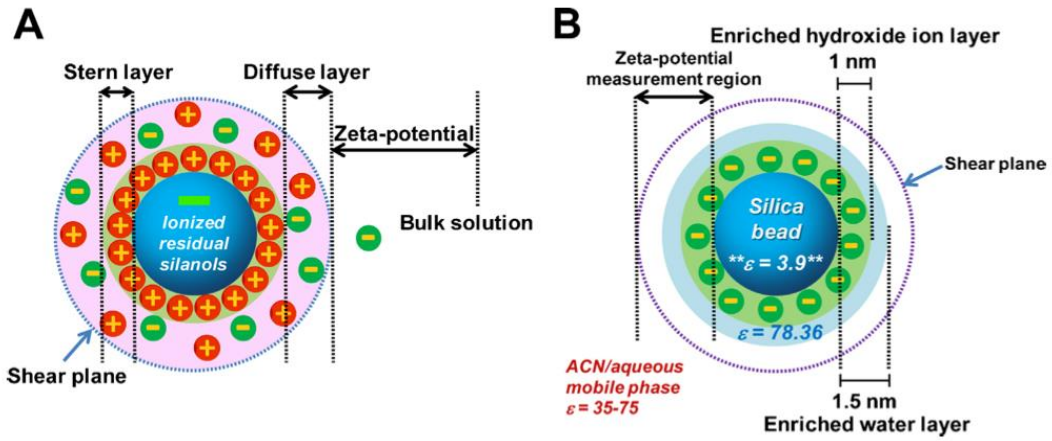


Figure 2-13 Mechanism of ANP retention (Kulsing *et al.*, 2014)

As ANP chromatography shares many features of HILIC stationary phases in terms of separation mechanisms, it is worth investigating its potential in application to the analysis of hydrophilic compounds such as paralytic shellfish toxins.

2.6 Toxicity Equivalence Factors

The necessity of Toxicity Equivalence Factors (TEF) arises from the recognition that functional group modifications affect the binding ability of toxins to the receptors, thus varying the severity of toxin analogs within each toxin group. For simplification of reporting and to express overall toxicity, the amount of toxins are usually converted in relation to a reference compound within each group of toxins (**Error! Reference source not found.**). For example, within the DSPs, results for DTX1 and DTX2 are converted to Okadaic Acid equivalents (OA Eq.) using the Toxicity Equivalence factor of 1.0 and 0.6, respectively. The result reported for a sample containing 3 different DSPs would be reported as "mg OA equivalents/kg".

The accuracy of calculated toxicity results depend on the accuracy of the TEFs applied, especially when a mix of different toxins is present in the shellfish. The TEF value for each toxin is determined through toxicology experiments such as administration of purified toxins to animals *via* several routes (Munday *et al.*, 2013) or instrumental measurement using receptor based binding assays (Alonso *et al.*, 2015). In recent years, as more toxicological data has been generated, there have been proposals to revise the

TEFs in light of new information gathered from tissue culture experiments (Perez *et al.*, 2011), along with mice and feline exposure trials (Andrinolo *et al.*, 2002; Munday *et al.*, 2013). Recently, work done by Munday *et al.* (2013) indicate that neosaxitoxin and decarbamoyl saxitoxin have higher toxicities than saxitoxin, which may lead to revisions of the existing TEFs for these toxins. Work done by Louzao *et al.*, (2015) also suggest a revision of TEF for DTX1.

Table 2-1 Currently adopted TEF values (EFSA, 2009a)

Toxin group	Toxin	TEF value
PST	C1	N/A
	C2	0.1
	GTX1	1
	GTX2	0.4
	GTX3	0.6
	GTX4	0.7
	GTX5	0.1
	dcGTX2	0.2
	dcGTX3	0.4
	NEO	1
	dcNEO	0.4
	STX	1
	dcSTX	1
AZA	AZA1	1
	AZA2	1.8
	AZA3	1.4
DST	DTX1	1
	DTX2	0.6
	OA	1
AST	DA	N/A
NST	Brevetoxins	N/A

2.7 Conclusion

In the course of the literature review, the diverse chemistries of the five groups of marine biotoxins were presented. Since the discovery and characterisation of these biotoxins, many methods have been developed for the analysis of these compounds in shellfish. Some of these methods include bioassays which require the use and sacrifice of animals. In recent years, there has been a shift away from bioassays and a move towards analytical methods of analysis, which are more sensitive and can provide toxin profile data. Currently, the Codex Committee for Fish and Fishery Products (CCFFP) are in the process of drafting a method performance criteria which can be applied to methods for biotoxin analysis, providing a common standard for the diverse biological and chemical methods employed around the world. Nevertheless, analytical methods have been recognised on an international level as a potential common platform for future marine biotoxin analysis, and developments in analytical methods have led to the application of mass spectrometry for the analysis of marine biotoxins. Therefore, mass spectrometry has also been utilised for the analysis of the marine biotoxins studied in this research. It is important to develop a method which is able to analyse the groups of regulated marine biotoxins. However, a fundamental issue to be addressed relating to the diverse chemical natures of the biotoxins, which are lipophilicity and hydrophilicity. Tandem LC has been described as a novel method that is able to bring these two polarities together in a single analysis. Therefore, the application of this method to the area of marine biotoxins is worth pursuing.

3 GENERAL REAGENTS AND MATERIALS

3.1 Introduction

In this chapter, an overview of the materials and methods used throughout this research will be presented. The contents include sample preparation procedures, procedures associated with method development and validation as well as the tandem LC setup. Subsequent chapters will further discuss specific materials and methods relevant to the procedures within the chapter.

3.1.1 Chemicals and Reagents

For all experiments, deionised water was produced onsite with a Milli-Q™ water purification system (Resistivity = 18.2 MΩ•cm at 25 °C) (Merck Millipore, VIC, Australia). For LC mobile phase, acetonitrile (LCMS grade) was purchased from Burdick and Jackson (Honeywell™, SA, Australia). Ammonium formate (≥99.0%, HPLC grade), ammonium hydroxide (28.0%-30.0% NH_3 basis), formic acid (LCMS eluent additive) and acetic acid (LCMS eluent additive) were obtained from Sigma Aldrich™ (NSW, Australia).

Sodium hydroxide, sodium chloride and disodium hydrogen phosphate (Ultra Grade) salts were obtained from Sigma Aldrich™ (Castle Hill, NSW, Australia). For the

oxidation of PSTs for fluorescence detection, hydrogen peroxide solution (H_2O_2 30% w/w) and periodic acid were obtained from Sigma Aldrich™ (Castle Hill, NSW, Australia). Glacial acetic acid was obtained from Merck Millipore™ (Bayswater, VIC, Australia)

Biotoxin reference standards were obtained from the National Research Council Canada (Nova Scotia, Canada). These included PST reference standards for C1&2, GTX1&4, GTX2&3, GTX5, dcGTX2&3, dcSTX, STX, dcNEO, NEO, AST reference standard for domoic acid, DST reference standards for OA, DTX-1, DTX-2 and AZA reference standards for AZA-1, AZA-2, AZA-3. NST reference standards for PbTx-1 and PbTx-2 were obtained from the University of North Carolina (Wilmington, US). Eprinomectin (Purity by assay = 92.4%) was obtained from Sigma Aldrich (NSW, Australia). All reference standards were stored at -20 °C or 4 °C according to supplier's instructions. Chemical Abstract Services (CAS) Registry numbers, as well as lipophilicity found in literature as partition coefficient (Log *P*), and supplied concentrations are tabulated in Table 3-1.

Table 3-1 Chemical properties, log*P* values and supplied concentration for chemicals used in this research

Toxin	CAS Registry Number	log *P*	Concentration supplied (µg/mL)
C1	80173-30-4	-4.37	53.9
C2	80226-62-6	-4.37	16.1
GTX1	60748-39-2	-3.78	24.8
GTX4	64296-26-0	-3.86	8.1
GTX2	60508-89-6	-3.86	45.2
GTX3	60537-65-7	-3.86	17.2
GTX5	64296-25-9	-4.48	24.7
dcGTX2	86996-87-4	-2.41	40.9
dcGTX3	87038-53-7	-3.96	9.2
NEO	64296-20-4	-3.86	20.7
dcNEO	68683-58-9	-2.7	10.1

Toxin	CAS Registry Number	log *P*	Concentration supplied (µg/mL)
STX diHCl	35554-08-6	-2.51	24.7
dcSTX	58911-04-9	-2.71	21.4
AZA1	214899-21-5	4.95	1.30
AZA2	265996-92-7	4.97	1.22
AZA3	265996-93-8	4.24	1.04
DTX1	81720-10-7	5.61	15.2
DTX2	139933-46-3	5.25	3.8
OA	78111-17-8	5.13	8.4
DA	14277-97-5	-1.79	101.8
Eprinomectin	123997-26-2	5.12	Neat powder

3.1.2 Equipment

Three different SPE cartridges were used in the course of this research: Strata-X™ (500 mg, 3 mL) and Strata-X-CW™ (250 mg, 3 mL) which were obtained from Phenomenex (Lane Cove, NSW, Australia); and Supelco™ EnviCarb (250 mg, 3 mL) which was obtained from Sigma Aldrich™ (Castle Hill, NSW, Australia).

The chromatography equipment used during this project was a 1260 Infinity series LC from Agilent Technologies™ (Mulgrave, VIC, Australia) which consisted of a solvent degasser (G4225A), binary pump (G1312B), autosampler (G1367D), thermostat (G1330B) and a thermostatted column compartment (G1316A) with 10 port-2 position valve (G1160A). In addition, a quaternary pump (G1311A), coupled to a solvent degasser (G1379B) was used for the tandem LC setup. This system was connected to either a triple quadrupole mass spectrometry detector (G6460A) or a fluorescence detector (G1321A).

HILIC columns used in this study were TSKgel Amide80™, 150 x 2.1mm, 3 µm obtained from Tosoh Biosciences™ (Tokyo, Japan), ZIC HILIC™, 150 x 2.1, 3.5 µm,

obtained from Merck™ KgaA (Darmstadt, Germany), Kinetex HILIC, 100 x 2.1 mm, 1.7 µm obtained from Phenomenex™ (Lane Cove, NSW, Australia) and Diamond Hydride™, 150 x 2.1 mm, 2.2 µm from MicroSolv Technologies™ (New Jersey, US). Reversed Phase columns used were: Poroshell 120 EC-C18, 75 x 2.1 mm, 2.7 µm and Poroshell 120 SB-C18, 100 x 4.6 mm, 2.7 µm from Agilent Technologies™ (Mulgrave, VIC, Australia). Guard columns used were Poroshell 120 SB-C18, 5 x 4.6 mm, 2.7 µm from Agilent Technologies™ (Mulgrave, Australia) and SecurityGuard Ultra Cartridge C18 2.1 mm and KrudKatcher Ultra 0.5 µm, sourced from Phenomenex (Lane Cove, NSW, Australia).

3.1.3 Shellfish Samples

Mussels grown and harvested in Mount Martha, Victoria, Australia were purchased from a local fish market. These mussels were tested for presence of PST with the routine AOAC 2005.06 method implemented in the lab (AOAC, 2006). Frozen whole mussels sourced from the Tasmanian PST outbreak of 2012 (Campbell *et al.*, 2013) were obtained from the South Australian Research and Development Institute (SARDI). They were known to contain STX, GTX2&GTX3, GTX5 and C1&C2 by external laboratory tests (Madigan T., Personal Communication, 10 October, 2014). Contaminated mussels were received frozen in retail packaging of 1.2 kg whole mussels and stored at -20 °C until required.

Prior to analysis, mussels were defrosted, shucked and debearded under running tap water, and placed on a woven wire sieve with 2 mm aperture size (Endecotts, London, UK). The tissue was rinsed with deionised water thrice and drained. Tissues were then homogenised using a Ultraturrax homogeniser (IKA, Germany) for 5 min. The homogenate was divided into 5 g aliquots in 50 mL PP tubes and stored at -20 °C until extraction. This process was carried out on both PST contaminated and toxin-free mussels.

Mussel Certified Reference Materials (CRM) were obtained from the National Research Council Canada (Nova Scotia, Canada). These included CRM-Zero-Mus, CRM-AZA-Mus and CRM-DSP-Mus. CRM‑Zero‑Mus is a mussel tissue (*Mytilus edulis*) matrix CRM intended for use as a negative control for the analysis of paralytic shellfish toxins

(PST), domoic acid (DA) and its isomers, okadaic acid (OA) and diniphysistoxins (DTX), azaspiracids (AZA), yessotoxins (YTX), pentenotoxins (PTX) and gymnodimine (GYM). Lot: 200604, Unit: 1813.

CRM‑AZA‑Mus is a certified reference material (CRM) prepared from naturally contaminated mussel tissues (*Mytilus edulis*). Each bottle contained about 8 g of homogenised mussel with AZAs. The certified values for which are: AZA1 at 1.16 µg/g, AZA2 at 0.273 µg/g and AZA3 at 0.211 µg/g. Lot: 200603, Unit: 3412 and 3448.

CRM‑DSP‑Mus‑c is a thermally sterilized homogenate of *Mytilus edulis* and a minor amount of the dinoflagellate *Prorocentrum lima*. Each bottle of CRM contained about 4 g of mussel homogenate. The toxin levels certified for this material are: okadaic acid at 1.07 µg/g, dinophysistoxin-1 at 1.07 µg/g, dinophysistoxin-2 at 0.86 µg/g, and domoic acid at 11.8 µg/g. Lot: 201314, Unit: 1691 and 1791.

4 DESIGNING THE TANDEM LC-FLD-MSMS

4.1 Introduction

The literature reviewed in Chapter 2 highlighted the potential for improved efficiencies in biotoxin analysis through the use of a tandem LC setup that can quantify both hydrophilic and lipophilic components through the same chromatographic injection. Previous metabolomics research by Pyke *et al.* (2015) proposed an adsorption column with a valve-enabled flow switching system that allowed a single injection to be separated and separated through two orthogonal chromatographic columns.

The aim of this part of my study was to develop a chromatographic system that is fit for purpose in the analysis of target biotoxins. This aim will be addressed through five steps:

1. Determination and confirmation of MRM transitions for biotoxins
2. Investigate suitable columns for tandem LC instrumentation, with a focus on emerging separation techniques for PSTs such as HILIC and ANP.
3. Trial, design and implementation of the tandem LC instrument for marine biotoxin analysis.
4. Optimisation of instrument conditions such as mobile phase composition, injection volume, detector settings
5. Evaluation of designed tandem LC-FLD-MSMS instrument

4.2 Determination of Biotoxin Multiple Reaction Monitoring Transitions

MRM transitions allow the detector to filter out background ions and be selective for the toxins of interest based on mass to charge (*m/z*) ratio of both the precursor and fragment ions. For this research, the precursor and product ion for the hydrophilic and lipophilic biotoxins were sourced from the literature. Dell'Aversano *et al.* (2005), Boundy *et al.* (2015) and Rossignoli *et al.* (2015) have published transitions for hydrophilic toxins, while Brana Magdalena *et al.* (2014) and Gerssen *et al.* (2009a) have reported MS transitions for lipophilic toxins. However, direct implementation without verification should not be assumed due to differences between the source geometry of mass spectrometers, which is known to affect performance between different MSMS detectors (Periat *et al.*, 2014). Therefore, MRMs and their associated parameters have to be verified on the MSMS detector used for a particular piece of research. Optimisation of MS parameters such as fragmentor voltage, collision energy, polarity, source temperature, MS drying gas flow, MS sheath gas flow, MS capillary voltage and MS nozzle voltage were performed to obtain the settings for the highest sensitivity.

Eprinomectin was used as a substitute for the targeted lipophilic toxins. As previously mentioned, biotoxin reference materials are expensive and a limited amount was available for this research. Eprinomectin was used throughout this research in two ways, firstly as a system monitoring compound to check that the flow switching for the tandem LC was operating correctly. The second use for eprinomectin was as a surrogate for lipophilic toxins. Eprinomectin has been used as an internal standard for lipophilic toxin analysis (Rúbies *et al.*, 2015) and has some similarities in chemical structure to the DSTs, AZAs and Brevetoxins, with similar logP and Polar Surface Area values (Figure 4-1).

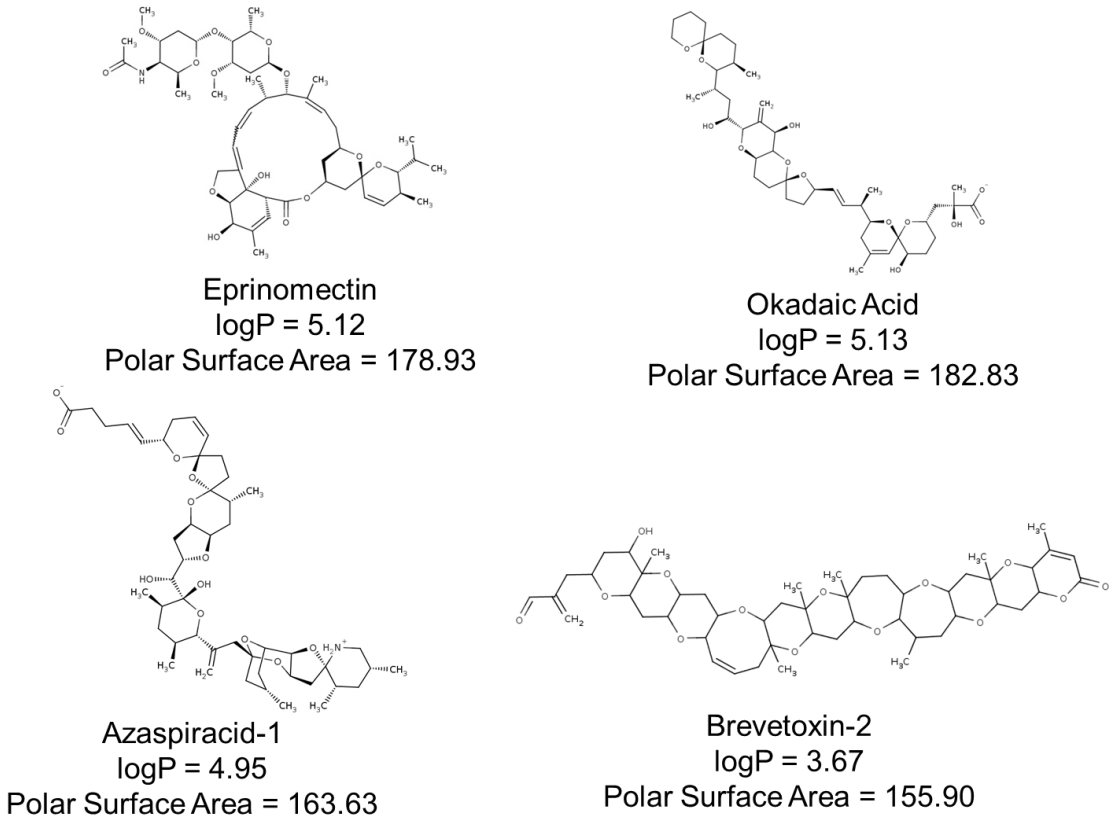


Figure 4-1 Structure and logP values of Eprinomectin compared to some lipophilic toxins

4.2.1 Materials and Methods

Marine biotoxin reference standards for PST, AST, AZAs and DSTs were obtained from the National Research Council Canada (Nova Scotia, Canada): C1&2, GTX1&4, GTX2&3, GTX5, dcGTX2&3, dcSTX, STX, dcNEO, NEO, DA, AZA-1, AZA-2, AZA-3, OA, DTX-1, and DTX-2. Brevetoxins PbTx-1 and PbTx−2 were purchased from the University of North Carolina (Wilmington, US). Eprinomectin, which was used as a lipophilic toxin substitute.

The liquid chromatography system used was a Agilent Technolgies 1260 LC system with a degasser, binary pump, cooled autosampler (set at 5 °C), and column compartment. The LC was coupled to a 6460A MSMS detector (Agilent Technologies, Mulgrave, VIC, Australia). MassHunter Optimizer tool (Agilent MassHunter Optimizer B.06.00, Agilent Technologies, Mulgrave, VIC, Australia) was used to verify MRM transitions and determine optimal detector parameters such as fragmentor voltage, collision energy, dwell time, and cell accelerator voltage for PST, AST, AZA, DST,

PbTx, and eprinomectin. Biotoxin reference standards and eprinomectin used for MS optimisation were diluted with water to a concentration of 800 µL/mL and directly introduced into the MS with a mobile phase composition of 0.1 vol% formic acid in H_2O:Acetonitrile (1:1 v/v) set to a flow rate of 0.2 mL/min.

4.2.2 Results and Discussion

The precursor and product ions for PSTs observed during the MRM optimisation process correspond to those reported in literature (Dell'Aversano *et al.*, 2005; Sleno *et al.*, 2004). For eprinomectin, the method reported by Rúbies *et al.* (2015) for lipophilic toxin analysis did not monitor the molecular ion $[M+H]^+$ but only the sodium adduct $[M+Na]^+$. However, the molecular ion MRMs determined by the optimisation process (Table 4-1) were in agreement to those in other reports (Kinsella *et al.*, 2009).

For the AST and DST group of toxins, both positive and negative MRMs were found. These MRM transitions can serve as confirmation ions as they should have the same retention time for both positive and negative MRMs. These transitions may also respond differently and one ionisation may be preferable to another depending on matrix. Therefore, both positive and negative MRMs were kept.

For this research, MRM verification was performed using an infusion technique without a column. The results were able to confirm MRM transitions and fragmentation patterns reported in literature (Table 4-1). No retention times were reported for PSTs because trials were conducted on several different analytical columns with different separation conditions. However, lipophilic toxins were analysed using a C18 analytical column and this column was used throughout the project. The retention times were determine to be the following: Domoic acid (RT = 4.00 min), AZA1 (RT = 8.17 min), AZA2 (RT = 8.35 min), AZA3 (RT = 7.85 min), OA (RT = 7.46 min), DTX1 (RT = 8.31 min), DTX2 (RT = 7.71 min), PbTX-1 (RT = 8.7 min), PbTX-2 (RT = 8.82 min) and Eprinomectin (RT = 8.88 min).

Table 4-1 MRM transitions determined by LC-MSMS

Compound	Precursor Ion (m/z)		Product Ion (m/z)	Fragmentor Voltage (V)	Collision Energy	Polarity
C1	396.1	→	316.1	111	4	+
	316.1	→	220.1	149	20	
	273.1	→	148.1	144	16	
C2	396.1	→	378.1	100	10	+
	396.1	→	298.1	111	16	
GTX1	332.1	→	314.1	149	16	+
	332.1	→	254.1	149	16	
GTX2	316.1	→	298.1	135	16	+
	316.1	→	220.1	135	20	
GTX3	396.1	→	378.1	200	6	+
	396.1	→	316.1	111	4	
	396.1	→	298.1	111	16	
GTX4	412.1	→	332.1	116	8	+
	412.1	→	314.1	116	4	
dcGTX2	273.1	→	255.1	144	16	+
	273.1	→	148.1	160	16	
	273.1	→	126.1	150	18	
dcGTX3	353.1	→	255.1	111	12	+
STX	300.1	→	204.1	134	24	+
	300.1	→	179.1	134	20	
	300.1	→	138.1	134	24	
dcSTX	257.1	→	222.1	131	16	+
	257.1	→	126.1	131	16	
NEO	316.1	→	138.1	135	28	+
	316.1	→	298.1	135	16	
	316.1	→	220.1	135	20	
dcNEO	273.1	→	126.1	150	18	+
	273.1	→	195.1	150	21	
DA (+)	312.1	→	266.1	15	10	+
	312.1	→	248.2	130	14	
	312.1	→	161.1	15	15	
DA (-)	310	→	266	86	4	-
	310	→	222.1	86	13	
AZA1	842.6	→	824.6	220	29	+
	842.6	→	806.6	200	50	
	842.6	→	672.4	220	53	
AZA2	856.5	→	838.5	220	29	+
	856.5	→	820.4	200	50	

Compound	Precursor Ion (m/z)		Product Ion (m/z)	Fragmentor Voltage (V)	Collision Energy	Polarity
	856.5	→	672.4	220	53	
AZA3	828.5	→	810.5	220	29	+
	828.5	→	658.4	220	53	
OA (+)	827.4	→	809.4	340	52	+
	827.4	→	723.4	340	52	
OA (-)	803.5	→	255.2	295	54	-
	803.5	→	113.1	295	74	
DTX1 (+)	841.5	→	823.5	260	46	+
	841.5	→	737.5	260	54	
DTX1 (-)	817.5	→	255.2	295	54	-
	817.5	→	113.1	295	74	
DTX2 (+)	827.5	→	809.5	260	46	+
	827.5	→	723.5	260	54	
DTX2 (-)	803.5	→	255.2	295	54	-
	803.5	→	113.1	295	74	
PbTX-1	867.5	→	849.4	156	5	+
	867.5	→	831.5	156	17	
PbTX-2	895.5	→	877.4	180	8	+
	895.5	→	319.2	180	8	
Eprinomectin	914.5	→	186.1	150	15	+
	914.5	→	154.1	150	15	

4.3 Column Selection for Lipophilic and Hydrophilic Biotoxins

Having determined the appropriate detector settings for the instrument, the next step in the process was to investigate a selection of columns to obtain suitable chromatographic separation for the toxins. In order to ensure optimal results from a tandem LC setup, it was important to carry out a selection process to identify the combination of columns that will yield the best performance.

As lipophilic toxins have been well studied in the past, the focus here is on separation performance for hydrophilic toxins. Three HILIC columns were compared: TSKgel Amide80 and ZIC HILIC (Dell'Aversano *et al.*, 2005; Diener *et al.*, 2007; Halme *et al.*, 2012; Rossignoli *et al.*, 2015) and Kinetex HILIC. The underivatised Kinetex HILIC column was chosen in order to investigate the potential for improved chromatographic resolution based on core shell stationary phase technology (Heaton and McCalley,

2014). For ANP chromatography, Diamond Hydride was selected to investigate its applicability for marine biotoxin analysis (Pesek *et al.*, 2013).

4.3.1 Materials and Methods

4.3.1.1 Hydrophilic Toxins

A mix of PSTs (C1&2, GTX1&4, GTX2&3, dcGTX2&3, NEO, dcNEO, STX and dcSTX) were diluted in water to a concentration of 800 µg/mL for each compound in the mix. A 5 µL injection of the standard mixture was selected. Chromatography was performed with a Agilent Technologies 1260 Infinity LC system with a quadratic pump connected to a 6460A MSMS detector (Mulgrave, VIC, Australia). Mobile phases used was as follows: Channel A; 10% formic acid solution in water, Channel B: 100 mM ammonium formate, Channel C: MilliQ water, Channel D; Acetonitrile. Formic acid was maintained at 0.1 vol% throughout the analytical run by setting Channel A to supply 1% at a constant rate. The gradient started at 90% D for 5 min, then ramping to 50% D over the next 10 min and held at 50% D for 13 min before returning to 90% D over 5 min and holding for a further 5 min. The flow rate was set at 0.8 mL/min for the ZIC HILIC, TSKgel Amide80, and Diamond Hydride columns, but for the Kinetex HILIC column, the flow rate was reduced to 0.2 mL/min due to smaller particle size and higher back pressure. The Kinetex column is packed with smaller particle size and thus gives higher back pressure for a given flow rate. MS source settings were as follows: Drying Gas Temperature 300 °C, Drying Gas Flow 5 L/min, Nebulizer 60 psi, Sheath Gas Temp 250 °C, Sheath Gas Flow 10 L/min, Capillary 3500 V (+/-), Nozzle voltage 500 V (+/-).

4.3.1.2 Lipophilic Toxin Chromatography

From literature, the chemistry of lipophilic toxins are better suited to be separated by reversed phase (RP) chromatography. Therefore, a standard C18 column was identified to be an appropriate starting point for method development. Following Braña Magdalena *et al.* (2014), a C18 column was used for the separation of lipophilic toxins. MRMs for the lipophilic toxins were applied from the previous optimisation step. Lipophilic toxins were diluted in methanol and a 5 µL injection was used for the study.

The mobile phase was 2 mM ammonium formate 0.1% v/v formic acid in water, B was Acetonitrile 0.1 vol% formic acid. The flowrate used was 0.4 mL/min, and the gradient program used started at 100% A, held for 1 min, changing to 20% A over the next 6 min and held at 20% A for 0.5 min. The solvent composition then returned to 100% A over 1 min and maintained for 4 min. The total gradient time was 12.5 min.

4.3.2 Results and Discussion

4.3.2.1 Hydrophilic Biotoxins

The column selection process with reference standards experimentally determined that PST separation was unsatisfactory with HILIC chromatography. Poor peak shapes were observed in Kinetex HILIC, with no retention of C toxins and peak tailing for STX. ZIC HILIC showed less tailing and taller peaks, but isomers were not resolved and STX toxins had a very long elution time.

Replication of the chromatographic separations reported using TSKgel Amide80 (Dell'Aversano *et al.* 2005) and ZIC HILIC (Diener *et al.* 2007) was unsuccessful. Both TSKgel Amide80 and ZIC HILIC were only able to separate the toxins replicate the chromatographic separation as stated in literature even with further in-house chromatographic optimisation in attempts to gain similar chromatography profile: Changes to a shallower/steeper mobile phase gradient, staring mobile phase composition, changes in mobile phase flow rate, increased column temperature (40 °C) and injection volume did not produce a desirable outcome. Shown in Figure 4-2 is an example of the separation attained using TSKgel Amide80 and ZIC HILIC with the optimised condition. A toxin mixture (containing 12 compounds) was used for this

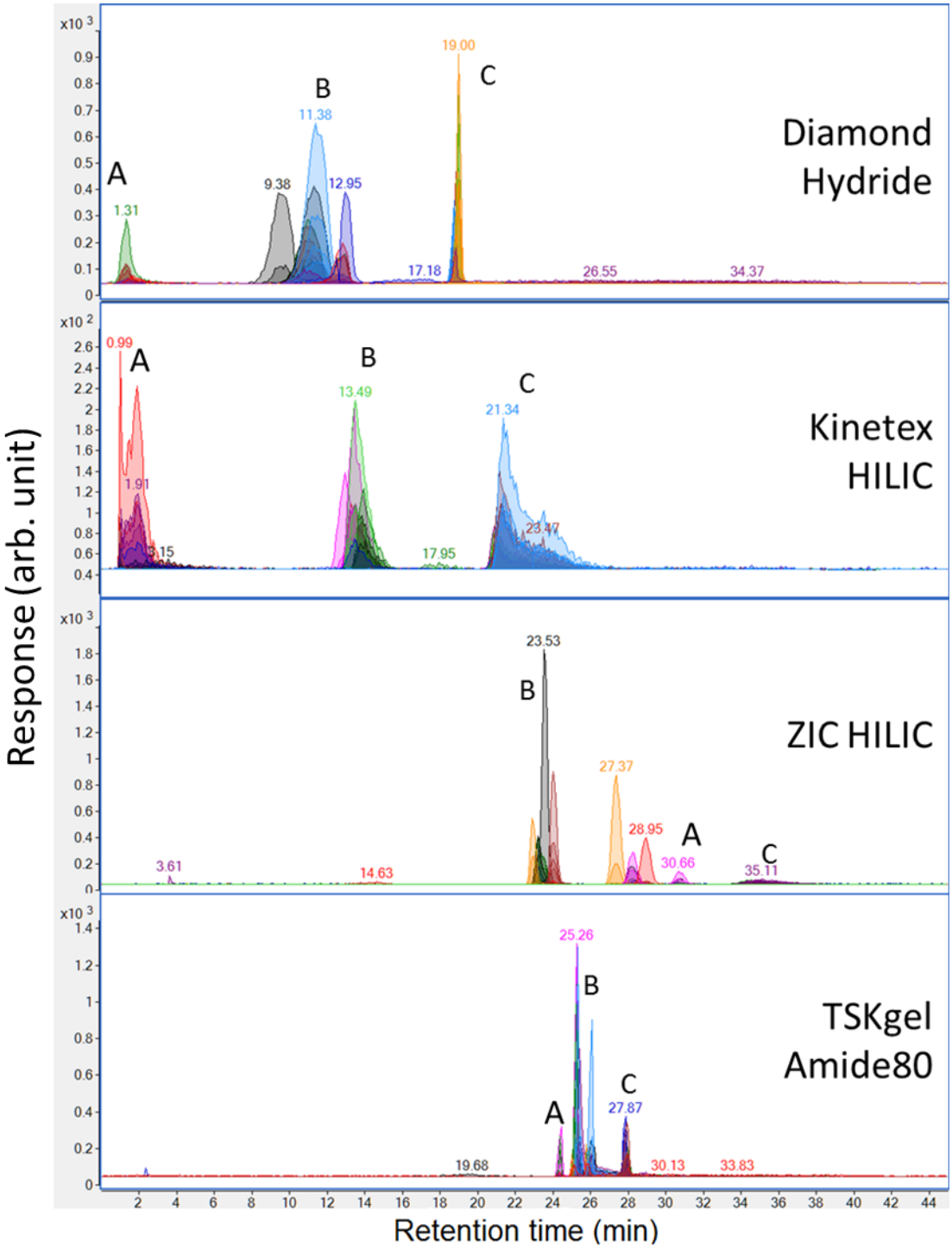


Figure 4-2 Chromatograms of PST reference standards at 800 µg/mL on trialed columns. Elution regions are shown for C toxins (A), GTX toxins (B) and STX, dcSTX, NEO and dcNEO (C)

based on their groups and not individually. However, LC conditions reported by Dell'Aversano *et al.* (2005) could not be replicated using the current LC set up as UHPLC instrument reported is able to run at higher backpressure, thereby providing

increased resolution *via* increase theoretical plates. TSKgel Amide80 showed poor separation of isomeric analogs such as GTX2 and GTX3.

Kinetex HILIC and Diamond Hydride columns were trialed in an attempt to attain suitable chromatographic separation for the mixed toxin reference standards. Kinetex HILIC was chosen as it is a core shell stationary phase which can improve resolution without increasing backpressure, while Diamond Hydride as it has a separation mechanism similar to HILIC except with a lower water retention thereby requiring less time to reequilibrate between analyses. As shown in Figure 4-2, both columns under optimised condition was only able to separate the toxin groups not individuals within each group. The Diamond Hydride column showed no retention for the neutral C toxin group, and other PST groups showed fronting peaks.

One of the critical requirements for MS analysis of PSTs is the adequate separation of analogs. Due to the in-source fragmentation and desulfonation of the C-toxins, their mass spectra are almost identical to those of their respective desulfonated gonyautoxins. In the mass spectra of C-toxins, pseudo-molecular ions are never observed, but their respective [M+H-SO_3] fragments are detectable (Krock *et al.*, 2007). Figure 4-4 shows that C1 and GTX2 both share the same transition (396 to 316) and C2 and GTX3 share the 396 to 298 transition. It is worth noting that there is a minor response after the GTX2 peak which may be GTX3.

4.3.2.2 Lipophilic Biotoxins

Inhouse chromatography verification on C18 Reversed Phase columns was found to be suitable for the lipophilic toxins, with good peak symmetry and resolution (Figure 4-3). Due to similar MRM transitions, the critical peaks OA and DTX2 must be resolved for unambiguous identification of these two toxins. Unique transitions for DTX1 and AZA2 overcame chromatographic coelution observed due to chromatograms being able to be separated from each other using analysis software.

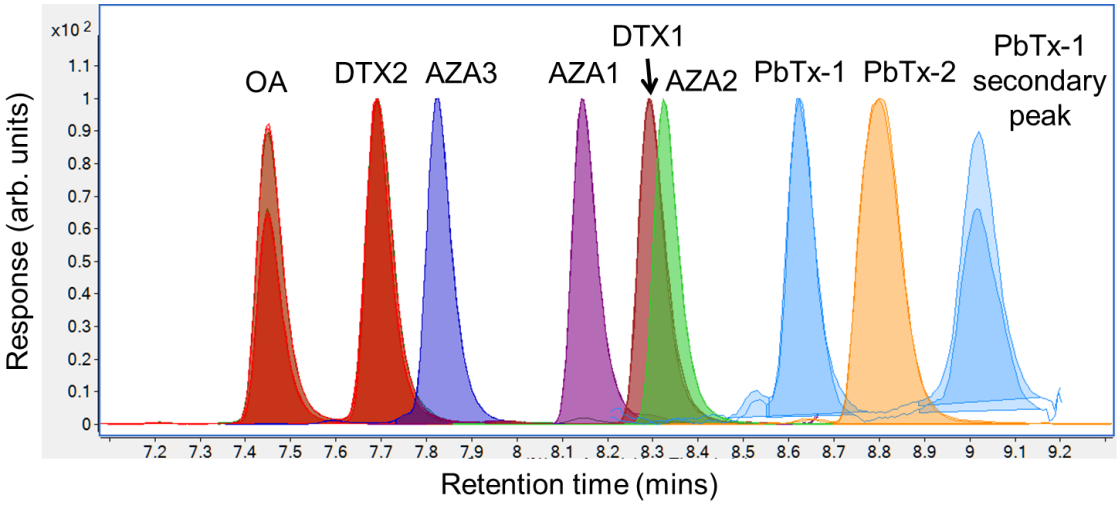


Figure 4-3 Chromatogram of lipophilic toxin mix (scaled to largest chromatogram)

4.3.2.3 Discussion

Shifting of retention times haven been observed in LC analysis of relatively simple algal extracts and have been suspected to be caused by matrix components (Foss *et al.*, 2012). Wang *et al.* (2010) has shown that fragmentation patterns may be affected by mobile phase pH, challenging the assumptions that ESI spectra generated are independent of ionisation environment. This effect is more pronounced on compounds containing more than one ionisable group. Saxitoxins have two ionisable guanidium (HNC_2) groups (Figure 2-2). Dell'Aversano *et al.* (2005) identified different sites being ionised within the toxin molecule to be the cause of different fragmentation pathways. The chemical instability of the N-sulfocarbamoyl C-toxins is reflected in their mass spectra. The N–S bond is the weakest in the molecule, and desulfonation of the C-toxins readily occurs in the ion-source of the mass spectrometer. In addition, C toxins have a low ESI efficiency, which affects sensitivity on MS detectors (Halme *et al.*, 2012). ESI is also unable to cope with high flow rates because it causes source saturation, negatively affecting ionisation efficiency.

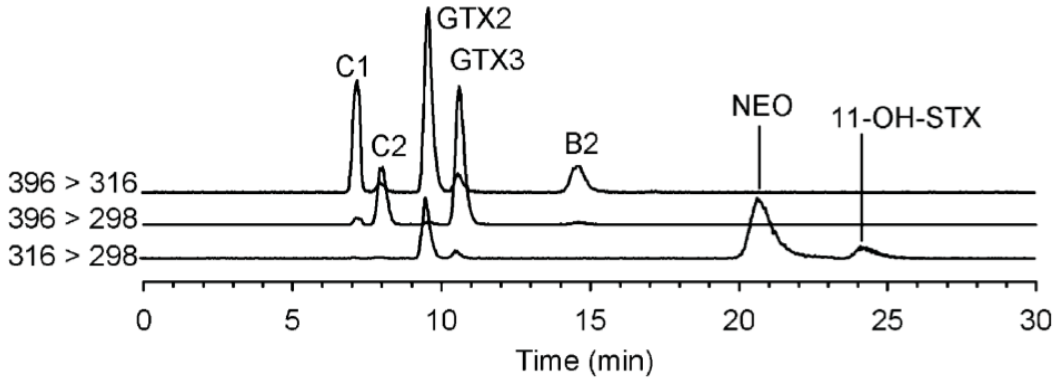


Figure 4-4 Multiple toxin peaks in MRM transitions for PSTs (Dell'Aversano *et al.*, 2005)

Overall, it was determined that the chromatographic responses seen using HILIC and ANP chromatography would be unsuitable for integration into a tandem LC setup and ultimately a different route would need to be explored. Additionally, due to shared ion transitions between several PSTs, chromatographic resolution between analogs must be achieved in order to correctly identify the toxins. This critical resolution was not achieved through HILIC or ANP chromatography. It was therefore decided that the AOAC method for PSTs should be trialed for incorporation into a tandem LC setup and modified to work in conjunction with the RP chromatography shown to be suitable for lipophilic toxins.

A review of the AOAC 2005.06 precolumn oxidation method was made to determine if it could be adapted to the tandem LC setup with MS detection. Careful review showed that it could not as it is not suitable for MS analysis due to three main parameters, which are: low MS sensitivities of derivatised biotoxins, poor chromatography of underivatised forms of PSTs on C18 RP chromatography used in the precolumn oxidation method, and the high concentration of buffer salts in the mobile phase which is unsuitable for the MS detector desolvation process. A high concentration of buffer in the mobile phase was required to achieve chromatographic separation of the derivatised forms of the PSTs, and therefore cannot be changed to a simpler MS-compatible mobile phase such as formic acid or acetic acid solutions. Considering these factors, fluorescence detection was chosen to replace MS detection of PSTs.

4.4 Fluorescence Chromatography for Paralytic Shellfish Toxins

Due to the limitations encountered in the trial of HILIC columns, RP chromatography was selected for the analysis of the PSTs. Chemical modification *via* derivatisation of the toxins is required for retention on RP columns and also for fluorescence detection. For example, exposure of STX to hydrogen peroxide under alkaline conditions forms 8-amino-6-hydroxymethyl-2-iminopurine-3(2H)-propionic acid, which is highly fluorescent (Figure 4-5a).

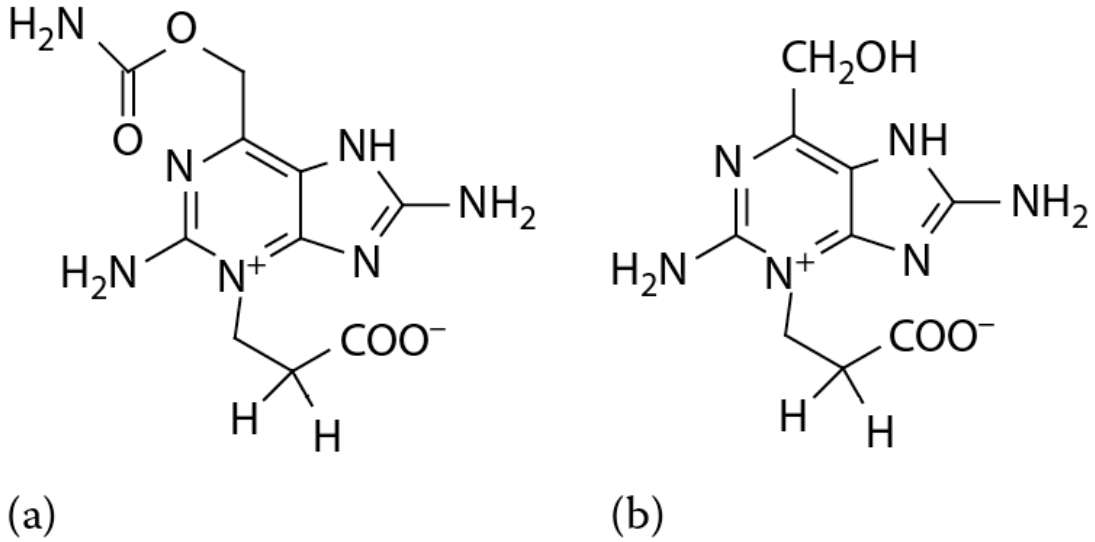


Figure 4-5 Chemical structures of peroxide oxidised forms of STX (a) and dcSTX (b)(Quilliam *et al.*, 1993)

Work done by Quilliam *et al.* (1993) in identifying the chemical structures of PST derivatives also demonstrated low sensitivity of these oxidised biotoxins by ESI-MSMS. It is noted that fluorescence detection can be susceptible to interferences by background from sample matrix or mobile phase components (Morgan and Smith, 2010). This can be overcome by running blank samples as well as underivatised samples alongside the derivatised samples. Subtraction of any peaks from these control samples can correct for these interferences for a more accurate result.

4.4.1 Materials and Methods

PST contaminated mussel tissues were extracted according to AOAC 2005.06 method and oxidised *via* peroxide and periodate oxidation. The oxidised samples were analysed

on a LC system with a fluorescence detector (Settings were Excitation = 340 nm and Emission = 395 nm, with PMT gain = 11). The accredited method at the National Measurement Institute used a Polaris C18-A column. The new column trialed was a superficially porous C18 column, which was chosen to test for potential improvements in peak shape, resolution and overall runtime.

The mobile phases used were 0.1 M ammonium formate in water (A) and 0.1 M ammonium formate in 5 % acetonitrile (B), both adjusted to pH 6 with 0.1 M acetic acid. The flow rate was set to 2 mL/min for the Polaris column, while for the Poroshell column it was set to 1 mL/min due to higher backpressure from the Poroshell column. The gradient started at 100 % A and decreased to 95% A over the first 5 minutes, further decreasing to 30% A over the next 4 minutes and returning to 100% A over the next 2 minutes and holding at 100% A for 3 minutes.

4.4.2 Results and Discussion

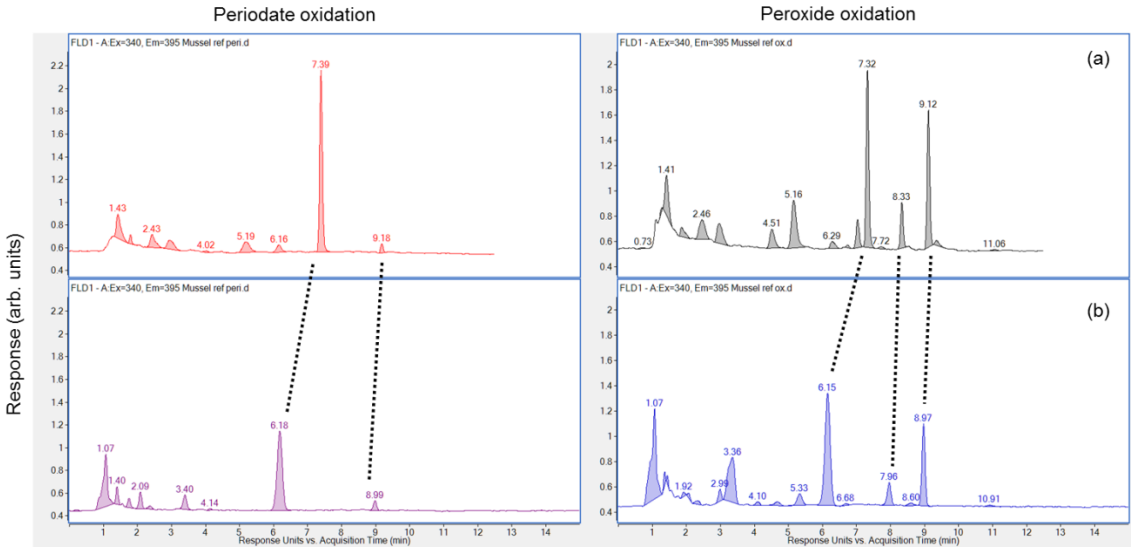


Figure 4-6 Fluorescence chromatograms of PST contaminated mussels with Poroshell column (a) and Polaris C18 column (b). Dashed lines indicate corresponding toxin peaks between the two columns.

The Poroshell column showed superior resolution and peak heights compared to the fully porous Polaris column. Toxin profiles were similar for both periodate and peroxide oxidation (Figure 4-6). In general, the toxin peaks eluted later in the Poroshell column compared to the Polaris column due to a lower flow rate, but the peak shape was improved and peak area was greater when using the superficially porous column. An

added advantage to the Poroshell column with the lower flow rate is a lower solvent consumption for each analysis.

4.5 Setting up tandem LC system with Selected Columns and Detectors

Fluorescence chromatography was found to be suitable for the analysis of PSTs. Therefore, the postcolumn derivatisation method was chosen to be incorporated with the lipophilic method in a tandem LC setup.

The final analysis cycle consists of three phases: extract injection, separation, and re-equilibration (Figure 4-7). In the load phase (A), the figure shows the valve positions and flow directions during the extract injection. Mobile phases from both pumps are mixed at the T-junction (ii) and the combined flow goes through the C18 analytical column for PST analysis through the fluorescence detector (FLD). The hydrophilic toxins are not retained on the C18 trap (i) while the lipophilic toxins are retained. In the second phase (B), the 10 port-2 position valve switches and the flow from Pump 1 is directed to the analytical column for lipophilic toxins (iii). Pump 2 performs the solvent gradient for chromatography of fluorescent PST derivatives. Pump 1 also performs the lipophilic toxin separation gradient, which washes the lipophilic toxins from the trap for separation on the analytical column connected to the MSMS source. The instrument reverts to the (A) position for re-equilibration at 11.51 min. Figure 4-7C is a graph showing the change in solvent composition and valve position over the duration of the chromatographic run. Table 4-2 shows the valve position, flow rates and proportion of solvent B for both Pump 1 and Pump 2.

The advantage of this method lies in the time savings of in analyzing multiple classes of marine biotoxins from one sample instead of having to perform two or three methods to obtain a result. This gives a faster turnaround time and also a fuller picture of the toxin content of a particular shellfish sample.

Table 4-2 Flow rates, solvent ratios and valve positions for the tandem LC-FLD-MSMS system

Time (min)	Pump1 (%B)	Pump1 Flow rate (mL/min)	Pump2 (%B)	Pump2 Flow rate (mL/min)	Valve position	MS valve position
0	0	0.4	0	0.6	1	Divert
1	0	0.4	0	0.6	1	Divert
1.01		0.4	0	1	2	Divert
1.2		0.4	0	1	2	Divert
3		0.4	5	1	2	To MS
7	80	0.4	70	1	2	To MS
7.5	80	0.4	70	1	2	To MS
8.5	0	0.4	0	1	2	To MS
9.5	0	0.4	0	1	2	To MS
10.5	0	0.4	0	0.6	2	Divert
11.5	0	0.4	0	0.6	2	Divert
11.51	0	0.4	0	0.6	1	Divert
12.5	0	0.4	0	0.6	1	Divert

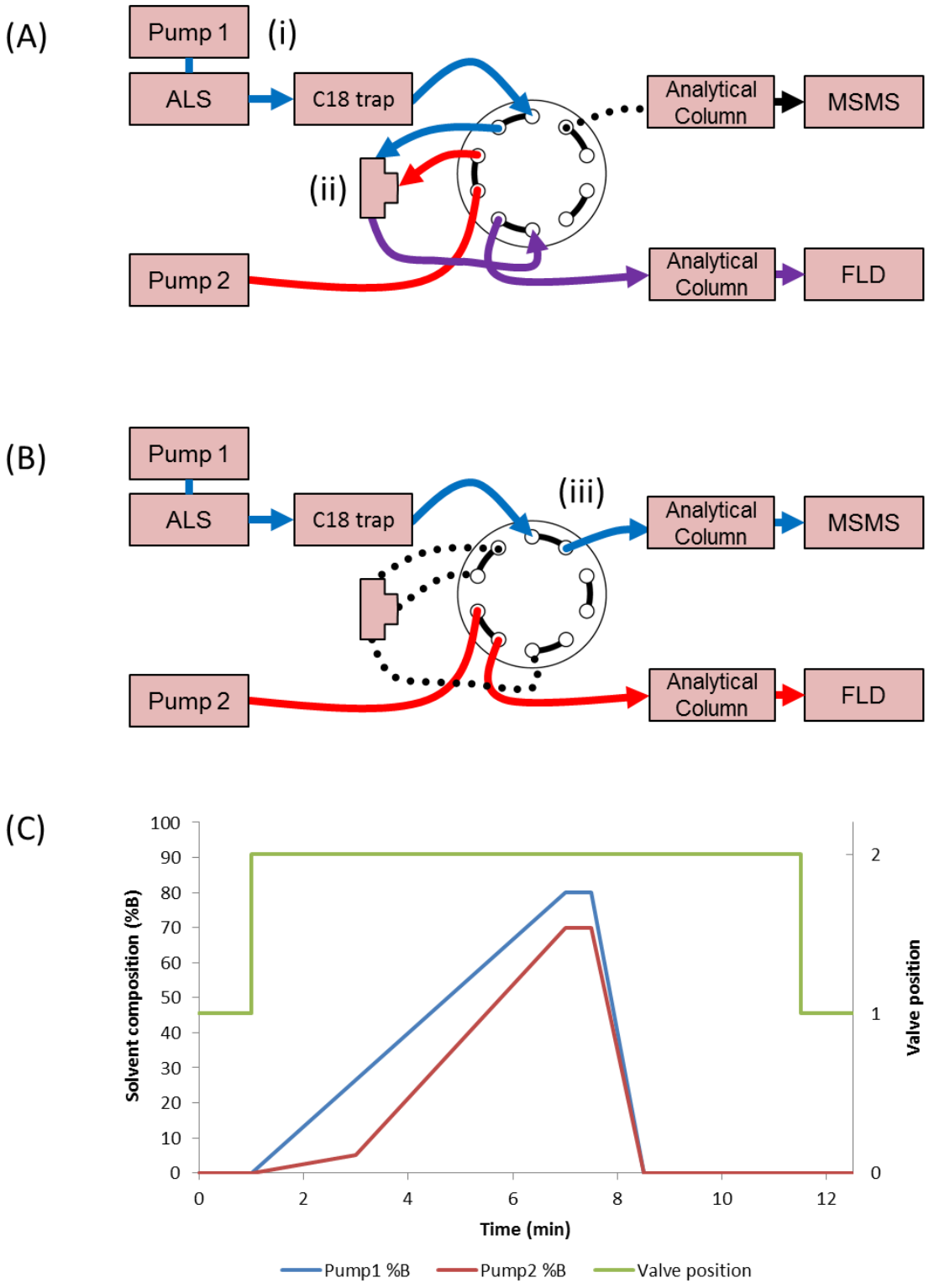


Figure 4-7 Schematic of tandem LC –FLD-MS setup with fluorescence detector and C18 trap

4.6 Combined Lipophilic and Hydrophilic Extracts on Tandem LC-FLD-MSMS

Optimisation of the tandem LC method was undertaken while a sample preparation was being developed. As described in Chapter 5, the sample preparation method developed yielded two extracts. The first extract contains PSTs and the second cantains lipophilic toxins and domoic acid. Experiments were carried out to evaluate whether both extracts could be combined into one vial and injected onto the tandem LC system for trapping with a C18 guard column. In this study, the hydrophilic extract containing PSTs was oxidised and combined with the lipophilic extract before introduction into the tandem LC-FLD-MSMS.

4.6.1 Material and Methods

Periodate oxidation of Strata-X SPE cleaned extracts (Section 5.7) was standardised relative to peroxide oxidation by doubling the concentration of periodic acid from 0.01 M to 0.02 M. 292 µL of periodate oxidant was combined with 100 µL of hydrophilic extract and allowed to react for 2 min, followed by addition of 3 µL acetic acid. Then, 45 µL of lipophilic extract was added to the vial and 30 µL of the combined extract was injected into the tandem LC system. This method is a modification of the AOAC 2005.06 method by doubling concentration of periodic acid in oxidation solution and approximately halving total oxidant volume added (0.06 M periodic acid, 292 µL oxidant). This is supported by work by Turner *et al.* (2012) on King scallops where periodate ratios up to 200% of standard concentrations was found to not change the toxin peak areas significantly. Peroxide oxidation was carried out according to the AOAC 2005.06 method: 250 µL of 1.0 M NaOH is added to a vial, followed by 25 µL of 10 % hydrogen peroxide solution, followed by 100 µL of hydrophilic extract, allowing to react for 2 minutes. Then 20 µL of acetic acid is added to the vial, followed by 45 µL of lipophilic extract. 30 µL of the combined extract was injected into the tandem LC system.

4.6.2 Results and Discussion

Analysis of the combined extract showed that lipophilic toxins are broken down by oxidation agents periodate and peroxide (Figure 4-9). Chromatograms show a complete loss of DA (Figure 4-8) and eprinomectin after exposure to peroxide and periodate oxidation solutions. Figure 4-9 shows a graph comparing the peak areas of lipophilic toxins after exposure to oxidation agents. Azaspiracids showed a complete loss after being exposed to 0.06 M periodate oxidant, while a <50% loss was observed with exposure to peroxide oxidant for AZA1 and AZA2. In the case of AZA3, an increase in around 30% relative to a control, which may indicate that the peroxide oxidant has a conversion process of for AZA1 and AZA2 into AZA3. For Brevetoxins, there was no detection of both PbTx-1 and PbTx-2 after exposure to peroxide oxidant while smaller losses were observed for periodate oxidants. For DSTs, a decrease of about 40% was observed for OA, DTX1 and DTX2 after exposure to either periodate and peroxide oxidant.

Overall, the derivatisation agents were found to be severely detrimental to the detection of lipophilic toxins, leading to large signal losses when analysed by MSMS. This indicates that either a degradation or conversion process happens when lipophilic toxins are exposed to the oxidation agents. From the results of this experiment, PST derivatised extracts were separated from underivatised lipophilic toxins and domoic acid into two vials to prevent degradation of MSMS toxins from exposure to PST derivatising agents.

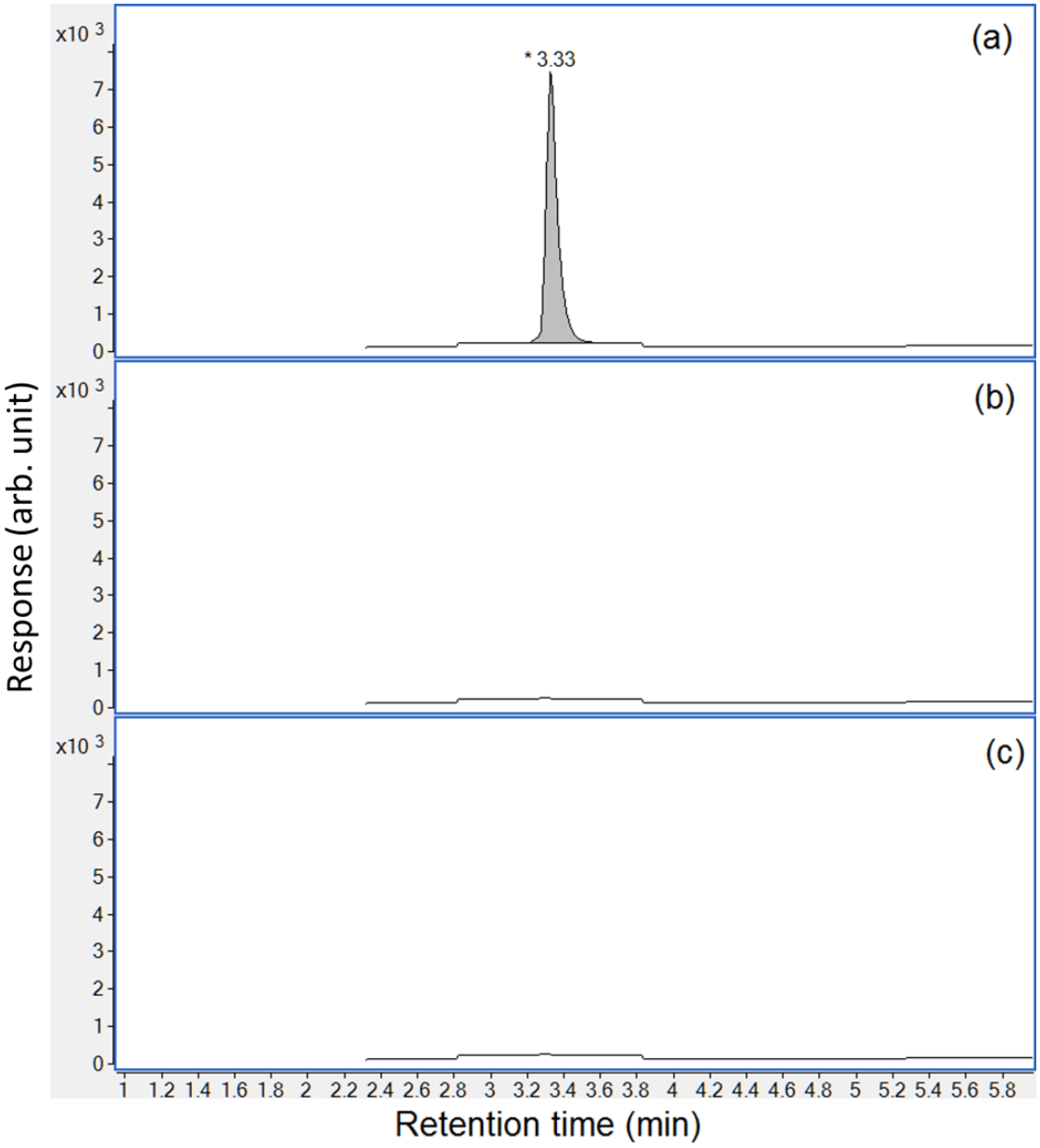


Figure 4-8 Loss of Domoic Acid when exposed to water (a), periodate oxidant (b) and peroxide oxidant (c)

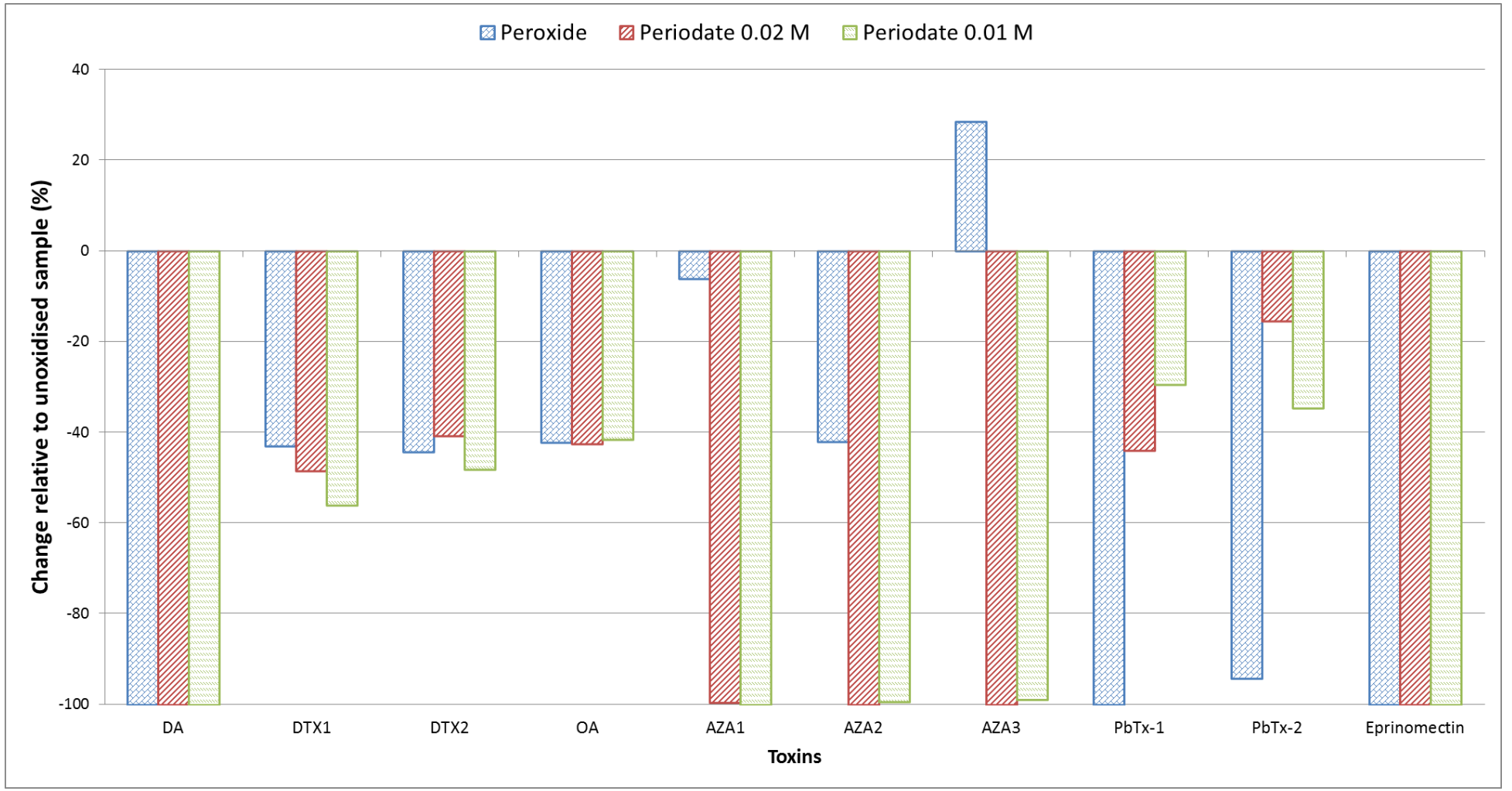


Figure 4-9 Decrease in lipophilic toxins peak areas when exposed to periodate and peroxide oxidation agents

4.7 Final Tandem LC-FLD-MSMS Design

This required a change in the instrument setup (Figure 4-7). Instead of combining the flows from both columns into the MSMS *via* a T-piece as described by Pyke *et al.* (2015), the PSTs would be analysed using FLD. The trap column was replaced with an inline filter prior to the column for column protection. For separation of PSTs, a C18 column with FLD detection was employed, while C18 column connected to MSMS was used for lipophilic toxins and AST analysis.

The analysis cycle consists of three phases: hydrophilic extract injection, lipophilic extract injection, and re-equilibration (Figure 4-7). In the load phase (A), the figure shows the valve positions and flow directions during the extract injection (i). Mobile phases from both pumps are mixed at the T-junction (ii) and the combined flow goes through the C18 analytical column for PST analysis through the fluorescence detector (FLD). In the second phase (B), the 10 port-2 position valve switches and the flow from Pump 1 is directed to the analytical column for lipophilic toxins (iii). The ALS injects the lipophilic extract while Pump 2 performs the solvent gradient for chromatography of fluorescent PST derivatives. Pump 1 also performs the lipophilic toxin separation gradient which flows into the MSMS source. The instrument reverts to the (A) position for re-equilibration at 11.51 min. Figure 4-7C is a graph showing the change in solvent composition and valve position over the duration of the chromatographic run. Table 4-2 shows the valve position, flow rates and proportion of solvent B for both Pump 1 and Pump 2. The sample injector program that was implemented for the two injections is described in Appendix A.

Both separations employ C18 superficially porous columns. For PST analysis, a high concentration of buffer (100 mM) is used. It also has a highly aqueous mobile phase gradient (5% acetonitrile in B), while for lipophilic toxins and AST, low buffer (2 mM) and high proportion of solvent in mobile phase (up to 80% acetonitrile) is required to elute the toxins from the analytical column. A high buffer concentration is not recommended for MS analysis as the salts can deposit on the source after mobile phase vaporisation. Early eluting components of the sample that do not contain analytes of

interest can also deposit on the source and decrease sensitivity over time. To reduce exposure of the source to sample components which contribute to source contamination, the flow of mobile phase from the analytical column is diverted away to waste until the analytes elute from the column.

The final tandem LC-FLD-MSMS setup has a 12.5 minute analysis time, which is an improvement to the AOAC 2005.06 official PST method runtime of 15 minutes. Although the overall time gained may be marginal, lipophilic toxin analysis is also performed simultaneously. The tandem LC-FLD-MSMS method also incorporates improvements such as the inclusion of an inline particulate filter prior to the column for prevention of backpressure buildup due to column clogging from sample microparticles over time and guard columns for both analytical columns to further prevent column clogging. The porous-shell stationary phase technology in the analytical columns improves chromatographic resolution and reduces solvent consumption.

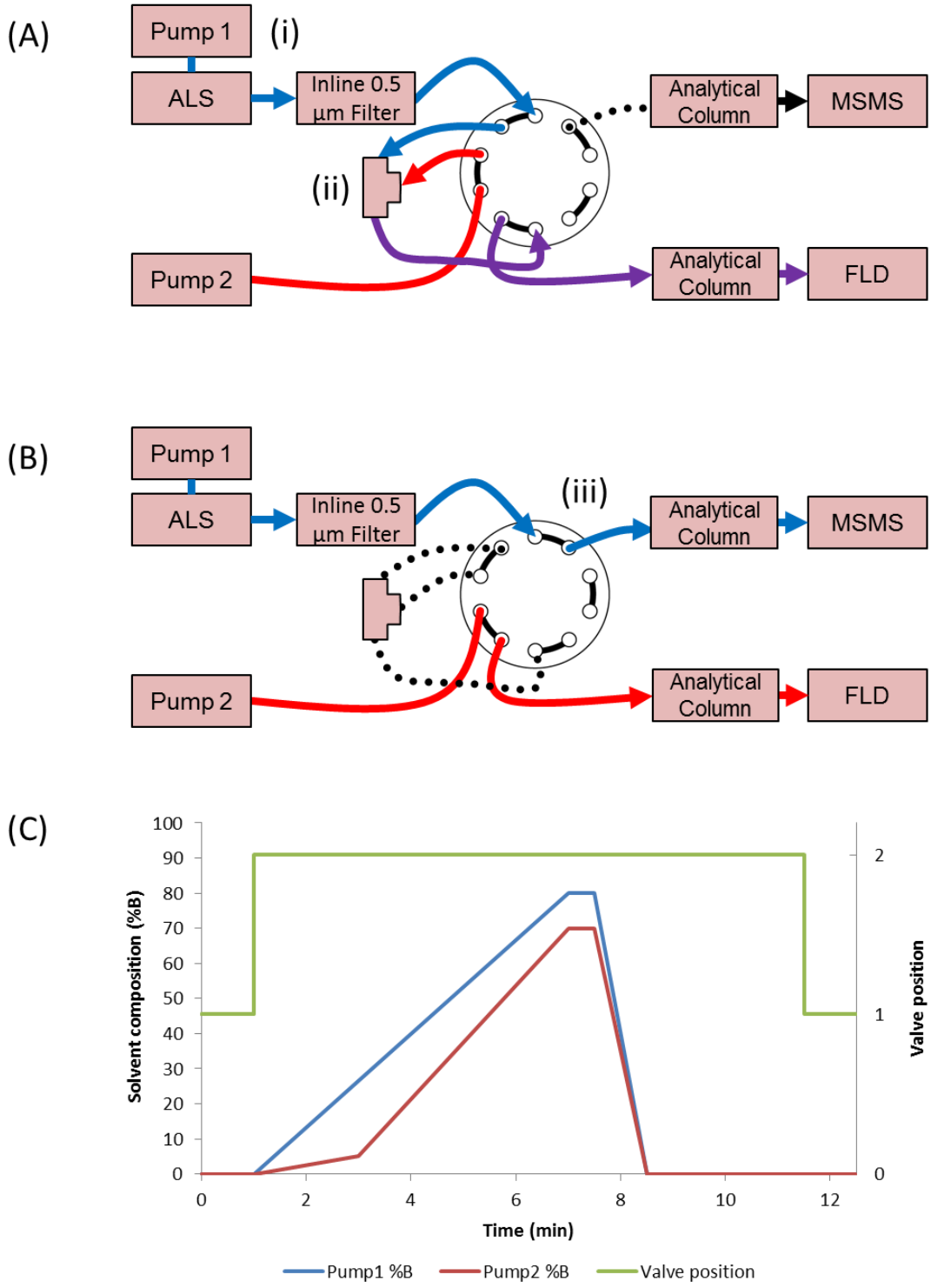


Figure 4-10 Scheme of final tandem LC-FLD-MS setup with inline filter replacing C18 trap

4.8 Conclusion

A tandem LC-MSMS setup was designed for the simultaneous analysis of hydrophilic and lipophilic marine biotoxins in shellfish. Reversed phase chromatography and MSMS was found to be suitable for lipophilic toxins AZA, DST, Brevetoxins and AST. Emerging chromatographic separation techniques were investigated for suitability for tandem LC setups. However, HILIC and ANP were found to be unsuitable for PSTs in terms of peak shape, resolution and sensitivity. Therefore, reverse phased chromatography with fluorescence detection was used for PST analysis. This required derivatisation of the samples before analysis using either periodate or peroxide oxidation agents. Subsequently, the tandem LC-FLD-MSMS system was set up combining the FLD and MSMS chromatographic systems. Because of the need for derivatisation of PSTs in the fluorescence method, an experiment was conducted to determine the stability of other marine toxins in the presence of the PST derivatising agents. It was found that the oxidising agents used for derivatisation of PSTs also significantly degraded other toxins, reducing the response of the lipophilic toxins in the MSMS detector. As a result, hydrophilic extracts containing PSTs were oxidised separately in one vial and lipophilic extracts in another vials, one injected after the other. This dual injection method was able to overcome the challenge of degradation of other marine biotoxins.

Having tested the instrumental setup with reference standards, the finalised method was used in optimising sample preparation and validation documented in Chapters 5 and 6.

5 COMBINED SAMPLE PREPARATION FOR HYDROPHILIC AND LIPOPHILIC MARINE BIOTOXINS

5.1 Introduction

Chapter 4 presented a novel measurement approach for the detection of marine biotoxins through a single analytical step. To ensure the success of any measurement, however, there is a need to ensure that sample extraction from the shellfish tissue can be performed quickly and completely. Furthermore, for a tandem analysis it is important to ensure that any cleanup and/or derivatisation does not negatively impact the quantification of other components. This will be one of the more challenging aspects for the array of compounds targeted in this research.

Generally, the goal of sample preparation is to isolate components of interest from a matrix in order to facilitate analysis (Chen *et al.*, 2008). The steps in sample preparation have the purpose of changing the state of the sample from its original form into

something that is suitable for the analytical system used. In the process, matrix components that can adversely affect the results of the analysis are removed or reduced, improving the accuracy and sensitivity of results. The reduction of matrix components entering the ESI source also maintains cleanliness for the MSMS detector. In addition, cleaned up samples also reduces column fouling and contributes to reduced downtime of the instrument.

Solid-liquid extraction (SLE) is the most common sample preparation methods in pesticide residue analysis in food, where solid samples are extracted with an appropriate solvent. Often, samples are homognised in order to maximise the surface area in contact with the extraction solvent. The solvent can be polar (e.g. water) or non-polar (e.g. dichloromethane), depending on the nature of the analyte and the matrix being tested. The pH of the extraction solvent also affects the efficiency of the extraction as solubilities of analyte molecule change depending on their dissociation state (Moldoveanu and David, 2015). Thorough mixing causes the analyte to partition from the solid sample into the liquid solvent, which are then separated. Physical processes such as decanting, centrifugation or filtration can be used to separate the solid sample from the extraction solvent.

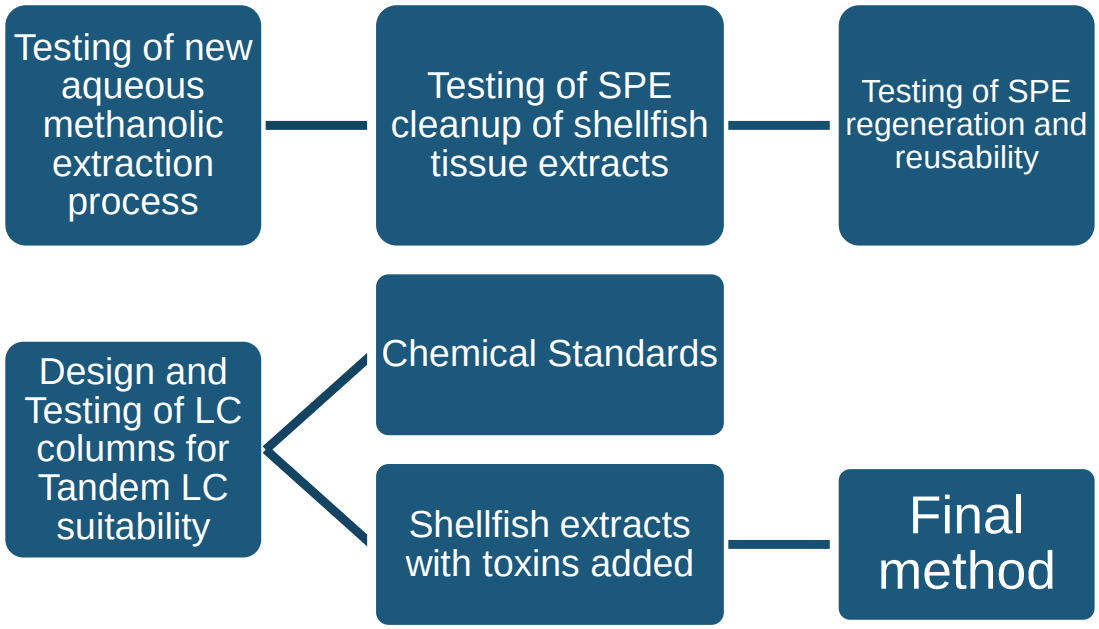


Figure 5-1 Timeline of method development process

5.1.1 Biotoxin extraction methods

For the extraction of marine biotoxins from shellfish samples, aqueous solvents are used for hydrophilic biotoxins, while lipophilic biotoxin extraction is carried out by methanolic solvents. The highly water soluble PSTs have been found to degrade in alkaline to neutral solutions (Stewart and McLeod, 2014). Therefore, acidic solvents are used in the extraction of this class of marine biotoxins (Wekell *et al.*, 2004). The AOAC 2005.06 precolumn oxidation HPLC-FLD method has become widely adopted after undergoing interlaboratory validation studies (Turner *et al.*, 2010). It is one of the internationally recognised methods for the detection of PSTs in shellfish. with prechromatographic derivatisation of PST with either periodate or peroxide solutions. Periodate oxidation derivatises all N-hydroxylated toxins while peroxide oxidation acts only on non-N-hydroxylated toxins, but produces a derivative with a stronger fluorescence response. The extraction and cleanup procedure for this method can be found in Appendix B. Heat is applied to shellfish tissues to denature proteins, stabilise the matrix and increase extraction efficiency (McCarron and Hess, 2005).

Although ASTs also come under the hydrophilic group of marine biotoxins, they exhibit retention on reversed phased columns similar to lipophilic toxins. Braña Magdalena *et al.* (2014) have reported an extraction method where AST is extracted together with lipophilic toxins. For the extraction of lipophilic marine biotoxins, there is a strong agreement in literature for the use of methanol as the extraction solvent. For lipophilic toxins such as AZAs and DSTs, methanol is used for extraction of AZAs and DSTs (MacKenzie *et al.*, 2002; Gerssen *et al.*, 2009b). The EU Harmonised Standard Operating Procedure for lipophilic toxins (EURLMB, 2015) describes a methanolic extraction of shellfish tissue. This method does not require a cleanup step. However, Gerssen *et al.* (2009b) has validated a lipophilic marine biotoxin sample preparation method which utilised polystyrene-divinylbenzene SPE cleanup (Strata-X), which reduced the matrix effects and improved accuracy. The details of the extraction and cleanup method reported by Gerssen which was used as a strating point in this research can be found in Appendix C.

while reported methods for brevetoxin extraction use a 80% methanol solution (Ishida, Nozawa, Hamano, *et al.*, 2004; Turner, Higgins, *et al.*, 2015)

The methods that form the basis of a combined hydrophilic and lipophilic toxin extraction method are summarised in Table 5-1. As shown in the table, there are several commonalities between the three methods, namely, the use of LC chromatography and the use of Strata-X SPE for the cleanup of the extracts.

Table 5-1 Summarised marine biotoxin extraction methods

	PSTs	AZA, DSTs, AST	Brevetoxins
Reference	AOAC, 2006 Harwood *et al.*, (2013)	Braña Magdalena *et al.* (2014)	McNabb *et al.*, (2012)
Extraction mass (g)	5	2	2
Extraction solvent	1% acetic acid	Methanol	80% Methanol
Heating step	100 ˚C for 5 min	N/A	60 ˚C for 20 min
Final Solvent-to-Sample ratio	2:1	10:1	10:1
Extract Cleanup	Strata-X SPE	Strata-X SPE	Strata-X SPE
Analytical method	HPLC-FLD	HPLC-MSMS	HPLC-MSMS
Notes			Hexane partitioning before SPE cleanup

5.1.2 Solid Phase Extraction

In the last several decades, Solid Phase Extraction (SPE) has grown to become an important tool for sample cleanup. SPE is based on partitioning between a solid phase (sorbent) and a liquid phase (sample)(Buszewski and Szultka, 2012). It is also called "digital chromatography" since it employs the same principles as LC, albeit with much larger solvent polarity differences when loading and eluting components (Majors, 2010). One advantage of SPE as a sample cleanup method is that the sorbent can be modified with different functional groups to have a high affinity to a particular group of analytes. There are many choices of sorbent backbones such as silica, graphitic carbon, or polymers (Wen *et al.*, 2014). For silica-based sorbents, common bound ligands are Octyl (C8), Octyldecyl (C18), amino (NH_2), carboxylic acid (COOH) while for polymeric resins, polystyrene-divinylbenzene (PS-DVB) and poly(divinylbenzene-co-

N-vinylpyrrolidone) are the most widely used forms (Buszewski and Szultka, 2012). These different sorbents and functional groups exert a wide range of interaction forces with analytes: hydrogen bonds, ionic, dipole-dipole, π-π, van der Waals forces, etc.

SPE has been applied to many phases and has been made available in formats such as single cartridges, disks, 96-well plates, pipette tips, as well as small columns which can be placed in the LC flowpath for on-line cleanup (Żwir-Ferenc and Biziuk, 2006). A general outline of the SPE process is given in Figure 5-2. There are two modes of SPE: analyte retention or matrix adsorption. In analyte retention, the analyte is bound to the sorbent and is released when a strong solvent is passed through the cartridge. In matrix adsorption, the matrix components are retained on the sorbent and the analytes are eluted during the wash step.

The key considerations when developing a SPE method are the choice of sorbent and the the sorbent capacity, as well as composition and volume of elution solvents (Hennion, 1999; Pichon, 2000). In an ideal situation, all the analyte binds to the sorbent under loading conditions but would elute completely when exposed to the elution solvent (Bielicka-Daszkiewicz, 2015).

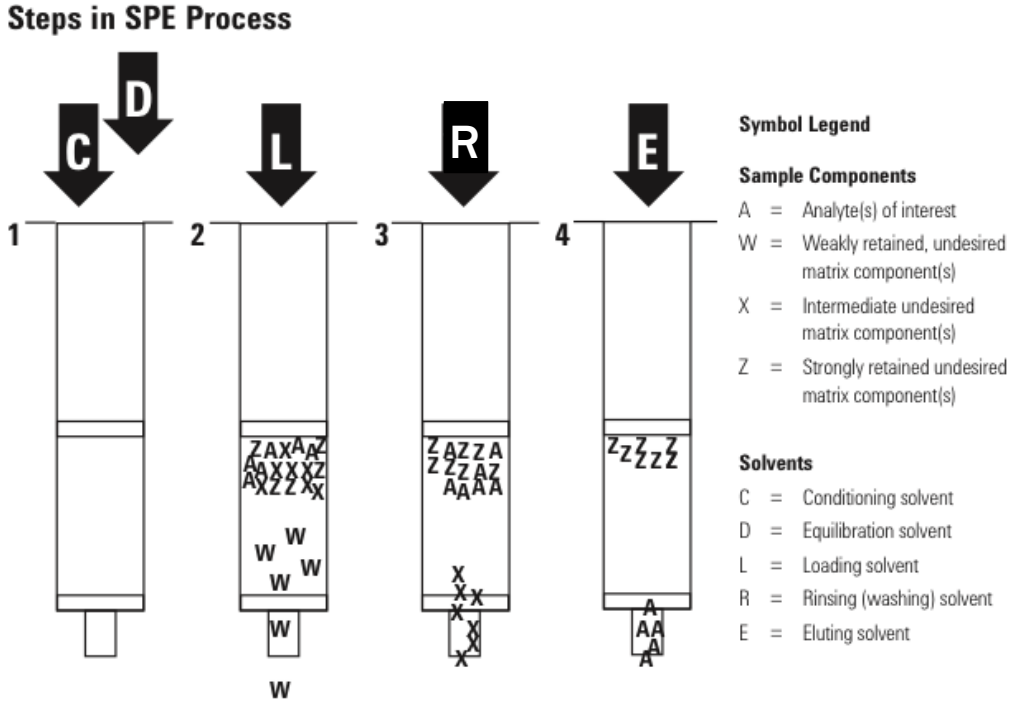


Figure 5-2 General scheme of SPE cleanup, showing the sequence of conditioning, load, wash and elution steps with position of analytes and matrix components at each stage (Majors, 2013)

Strata-X is styrene-divinylbenzene (SDVB) polymer that has been surface-modified with a modified N-Vinylpyrrolidone functional group that contains three mechanisms of retention: π-π bonding, dipole-dipole interactions and hydrophobic interactions. This enables it to exhibit both hydrophilic and lipophilic retention (Bielicka-Daszkiewicz and Voelkel, 2009). Improved results by replacing octadecyl-modified silica C18 SPE cartridges with polymeric Strata-X SPE cartridges has been described in literature (CEFAS, 2010; Harwood *et al.*, 2013). The Strata-X polymeric sorbent has also been applied as filled passive adsorption bags for monitoring marine biotoxins in open seas (Zendong *et al.*, 2014).

SPE has been applied to the analysis of marine biotoxins, both hydrophilic and lipophilic. For PSTs in shellfish, Sayfritz *et al.* (2007) has reported method using acetonitrile extraction solvent and a freeze step to separate acetonitrile from water. The water fraction is then cleaned up with poly(divinylbenzene-co-N-vinylpyrrolidone) SPE followed by activated carbon SPE. However, this was found to be irreproducible when repeated by Harju *et al.* (2015), who suggested the cause to lie in the partitioning step of

Acetonitrile/Water after freezing and retention of toxins in the discarded acetonitrile fraction.

Graphitized carbon black (GCB) has been shown to be suitable for very polar (water soluble) analytes due to the interactions between the surface of the carbon and the analytes (Hennion, 2000). These properties of GCB were employed by Boundy (2015) and Turner (2015) to develop a sample preparation method for PSTs. The SPE sorbent retains planar interferents from shellfish extracts and does not bind to the toxins. The GCB cleanup has been suggested to make shellfish extracts suitable for LC-MS by a desalting mechanism. Salts can disrupt the chromatographic mechanism and introduce high variability of separation, in addition to fouling the detector source when non-volatile salts deposits form due to the evaporative process of ESI. Salts can also form adducts with the target compounds, which may reduce the abundances of the molecular ion and thus the signal of the compound detected by the MSMS.

5.1.3 Combined Toxin Extraction and Cleanup Method

Developing a method for analysis of hydrophilic and lipophilic marine biotoxins requires a sample preparation and cleanup procedure that is compatible with both classes of toxins. For this study, after considering similar applications for the literature, an aqueous methanolic solution with an acidic pH was determined to be the most appropriate choice. This was because this solvent would give a pK_a close to that of the analyte and should maximise its efficiency. Common extraction procedures have been proposed for AST and PSTs (Vale and Sampayo, 2002), with good results. Building on these reports, a multi-toxin extraction method was developed, with consideration of different toxin solubilities for lipophilic and hydrophilic marine biotoxins. Typical extraction procedures for lipophilic and hydrophilic toxins are summarised in Table 5-1, based on the AOAC 2005.06 (AOAC, 2006) for PSTs and Gerssen *et al.* (2009) for lipophilic toxins. The proposed combined method for both classes of toxins is shown in the middle column, with indication of steps from the original extraction in their respective colours. In addition to the combined extraction, a cleanup step must also be considered. The lipophilic and hydrophilic cleanup methods reported by Gerssen *et al.*

and the AOAC has been summarised in Figure 5-33, with the proposed combined cleanup method shown in purple.

The proposed extraction and cleanup methods would need to be tested in combination with the tandem LC-FLD-MSMS described in Chapter 4. This chapter was focused on the nature of the extraction and cleanup of real and simulated samples. The suitability of the proposed extraction was tested. In the first experiment, the comparison of carbon and polymeric SPE sorbents was conducted to evaluate the compatibility of lipophilic toxins with carbon SPE. Following that, the more suitable sorbent would be further optimised by measuring the elution volumes required to fully elute the compounds of interest from the SPE cartridge. In the interest of reducing tissue mass extracted, a smaller sample mass was extracted and compared to the original mass stated in the AOAC 2005.06 method. This was due to the fact that limited certified reference materials were available for the validation process and a smaller extracted mass would yield more replicates per unit of certified reference material. The fractionation of PSTs through cationic exchange (Strata-X-CW) SPE was also verified, in order to assess any changes that may have been brought about by the new extraction and cleanup method. Finally, regeneration of the polymeric Strata-X SPE cartridges was investigated. Polymeric sorbents are more stable to high pH solvents and may be able to be cleaned and reused with solvents such as methanol with ammonia.

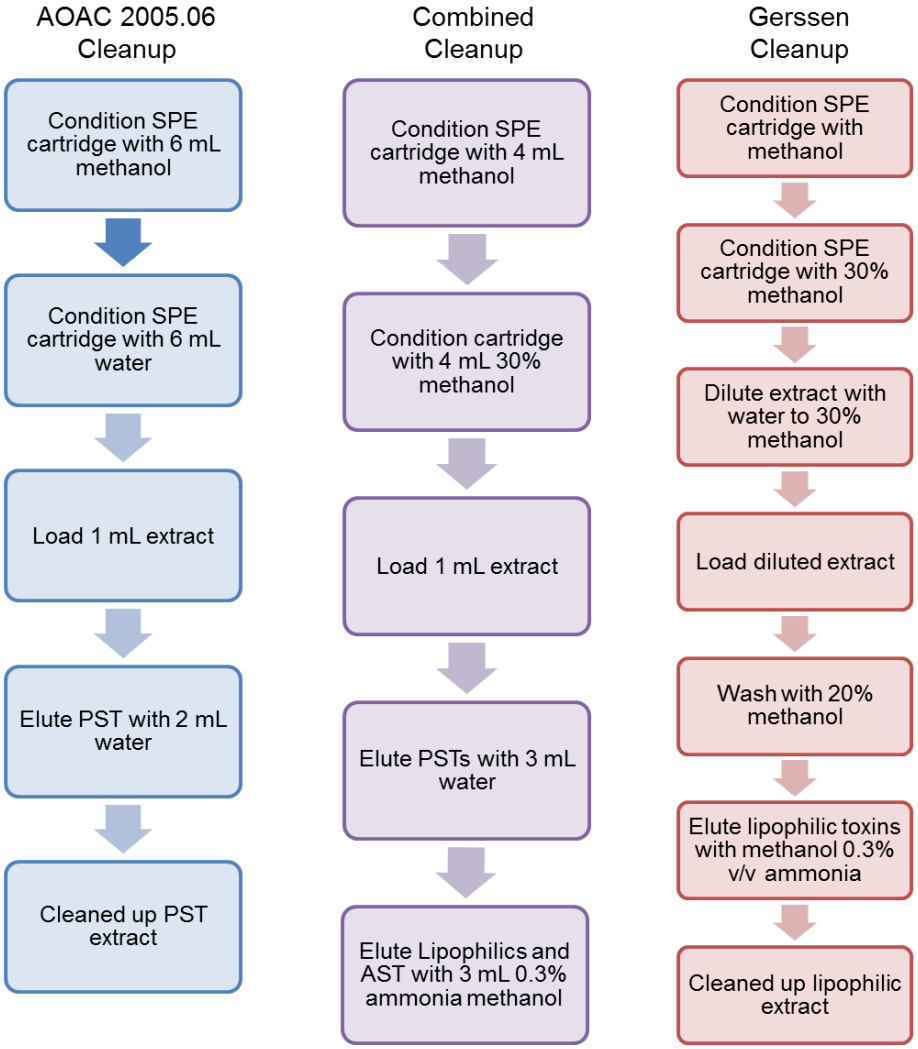


Figure 5-3 Flowchart of shellfish tissue cleanup methods from the AOAC 2005.06 method for hydrophilic PSTs (in blue) and from Gerssen *et al.* (2009a) (in red) for lipophilic toxins. The proposed combined method is shown in purple.

5.2 Comparison of Carbon and Polymeric SPE

For the cleanup investigation, Strata-X Polymeric SPE was compared against EnviCarb Graphitic Carbon SPE. Polymeric sorbents were chosen due to their resistance to high pH solvents used for elution. 0.3 vol% ammonium hydroxide in methanol has a high pH value which would dissolve silica based material.

5.2.1 Materials and Methods

Mussels naturally contaminated with PSTs were used for this experiment for hydrophilic toxin recovery. Mussels were extracted using method described in Section 5.1. Eprinomectin was used in place of lipophilic toxin to evaluate toxin recoveries from Strata-X and EnviCarb SPE cartridges. A 5 µL portion of eprinomectin standard in methanol (100 µg/mL) was added to 1 mL of 30% methanol solution. All analyses were performed in triplicate.

For Strata-X SPE cleanup, Strata-X SPE cartridges (500 mg, 3 mL) were conditioned with 4 mL of methanol followed by 4 mL of 30% methanol solution. A 1 mL portion of eprinomectin standard or PST mussel extract in 30% methanol was loaded onto the cartridge. Then, 3 mL of deionised water was used to elute hydrophilic toxins, and the fraction was collected. A 3 mL volume of 0.3% v/v ammonium hydroxide in methanol was passed through the column to elute lipophilic toxins and the fraction was collected in a separate 15 mL polypropylene (PP) tube.

EnviCarb SPE cleanup was performed according to the method reported by Turner *et al.* (2015): EnviCarb SPE cartridges (250 mg, 3 mL) were conditioned with 3 mL of 20% Acetonitrile + 0.25% acetic acid followed by 3 mL of 0.025 vol% ammonia solution. 400 µL of Eprinomectin standard or PST mussel extract was loaded followed by a wash with 700 µL of MilliQ water which was discarded. Then, 2 mL of 20% Acetonitrile + 0.25% acetic acid was passed through the cartridge to elute the hydrophilic toxins. This fraction was collected in a 15 mL PP tube. 2 mL of 0.3% v/v ammonium hydroxide in methanol was passed through the column to lipophilic toxins and the fraction was collected in a separate 15 mL PP tube. Peroxide oxidation was performed on the hydrophilic fractions and both were analysed with the tandem LC-FLD-MSMS system.

5.2.2 Results and Discussion

The chromatograms of the extracts cleaned up *via* Strata-X and EnviCarb SPE are shown in Figure 5-4 and Figure 5-5. Eprinomectin was not eluted from the EnviCarb cartridge even when a strong solvent was applied (Methanol with 0.3 vol% ammonium hydroxide). Planar compounds like eprinomectin are known to bind strongly to

graphitised carbon material, with GCB containing various functional groups at the sorbent surface (Hennion, 2000). These groups are positively charged, which produces an anion exchange mechanism. Eprinomectin was used as a surrogate based on its similar chemical and steric properties to lipophilic toxins such as AZAs, DSTs and Brevetoxins (Shown previously in Figure 4-1). Low recovery of eprinomectin from the EnviCarb cartridge indicated that this type of sorbent was unsuitable for use with lipophilic toxins.

In contrast, Strata-X SPE cartridges exhibited retention of lipophilic components which can be eluted from the sorbent with methanol 0.3 vol% ammonia, which has previously been reported by Gerssen *et al.* (2010).

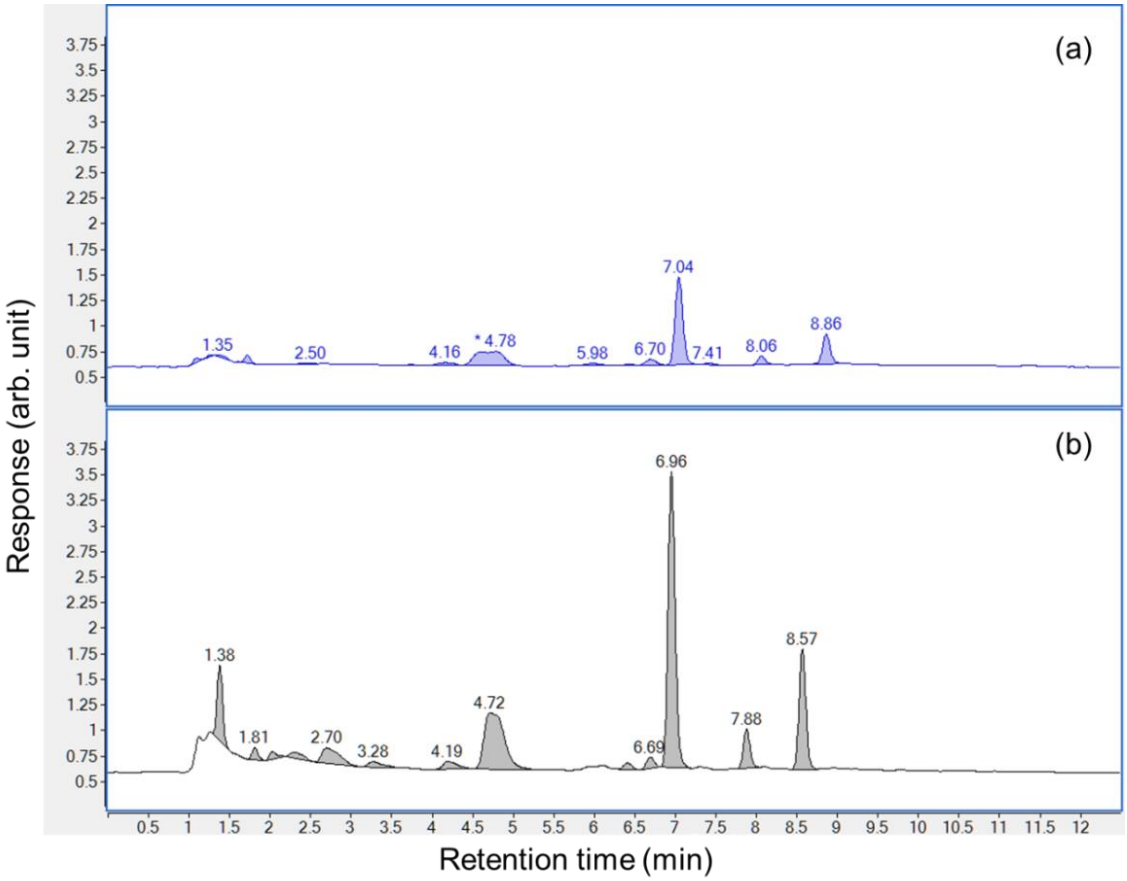


Figure 5-4 FLD chromatograms of peroxide oxidised PST LRMs after EnviCarb SPE (a) and Strata-X SPE (b) cleanup

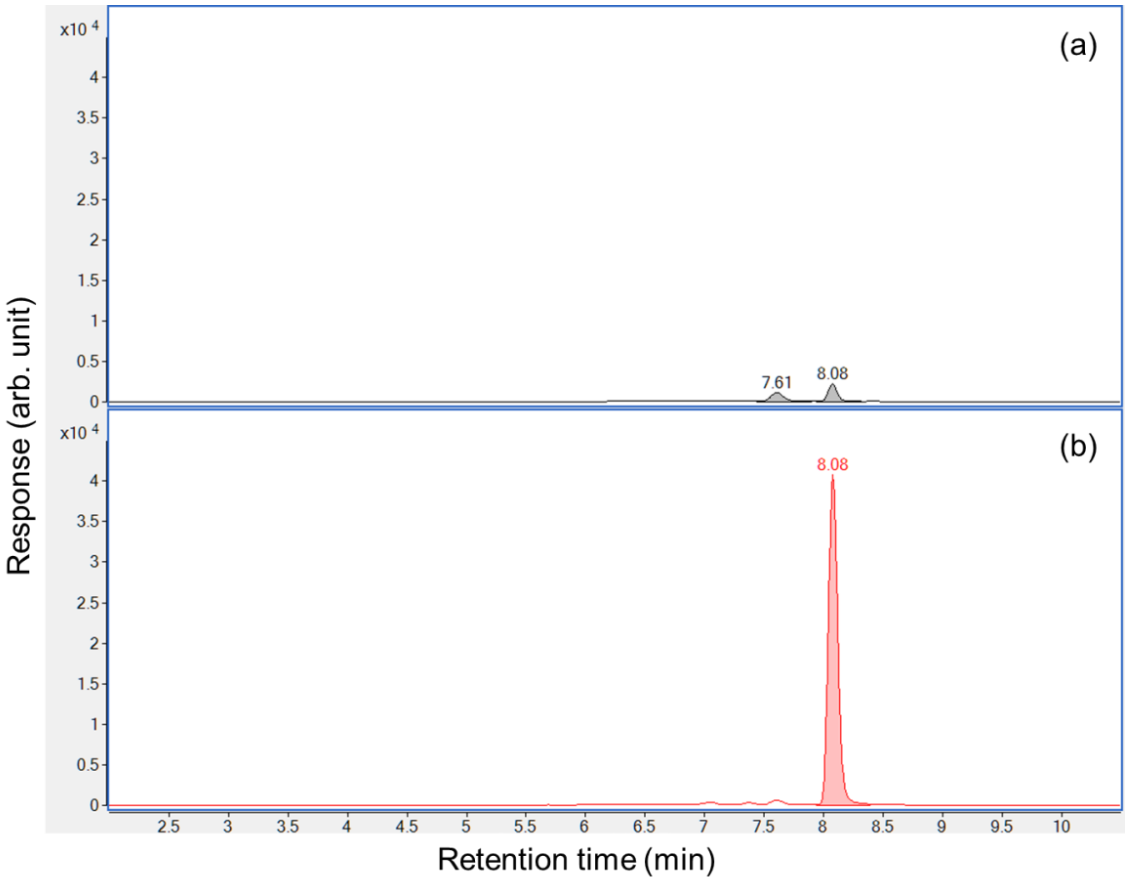


Figure 5-5 Chromatogram of eprinomectin recovery from EnviCarb SPE (a) and Strata-X SPE (b)

5.2.3 Conclusion

A comparison of Strata-X and Envicarb SPE was performed, using mussel tissue naturally contaminated with PSTs to represent hydrophilic toxins. Eprinomectin was used to represent lipophilic toxins. Low recoveries of eprinomectin from Envicarb SPE was observed relative to Strata-X SPE. The low recoveries indicated that low recoveries may also extend to chemically similar compounds such as the lipophilic toxins. Therefore, Strata-X SPE was chosen for the optimisation of the SPE elution volumes.

5.3 Optimisation of SPE elution volumes

After determining that Strata-X is suitable for both hydrophilic and lipophilic toxins, the cleanup procedure was optimised to ensure the correct analyte fractions were collected. Therefore, it was necessary to assess the volume of eluent necessary for complete elution of hydrophilic toxins from the Strata-X SPE cartridge.

5.3.1 Materials and Methods

PST LRM extracts were loaded onto conditioned Strata-X cartridges and washed sequentially with 1 mL aliquots of deionised water. The fractions were collected individually from the SPE cartridge, up to 10 mL. The ten fractions were oxidised by peroxide oxidation prior to analysis by tandem LC-FLD-MSMS. This experiment was performed in triplicate.

5.3.2 Results and Discussion

Figure 5-6 shows the chromatograms of only the first 6 fractions from Strata-X cleanup. Among the ten fractions analysed, PSTs were observed only in the 2nd, 3rd and 4th fraction, corresponding to the first 3 mL of wash. No peaks were detected in fractions 5-10, showing that 3 mL is sufficient to elute all PSTs from the cartridge. There were also no peaks detected in the first fraction after loading. This shows that immediately after the load step, the first fraction of eluent from the cartridge is the conditioning solvent as the sample moves down into the sorbent bed. From this result, it was decided that the first fraction to elute after sample loading should be discarded with the conditioning solvents and that 3 mL of water would be used to elute hydrophilic toxins from the Strata-X SPE cartridge. This maximises sensitivity of the PSTs by collecting only the fractions that contain the toxins, while maintaining the final extract volume at 4 mL.

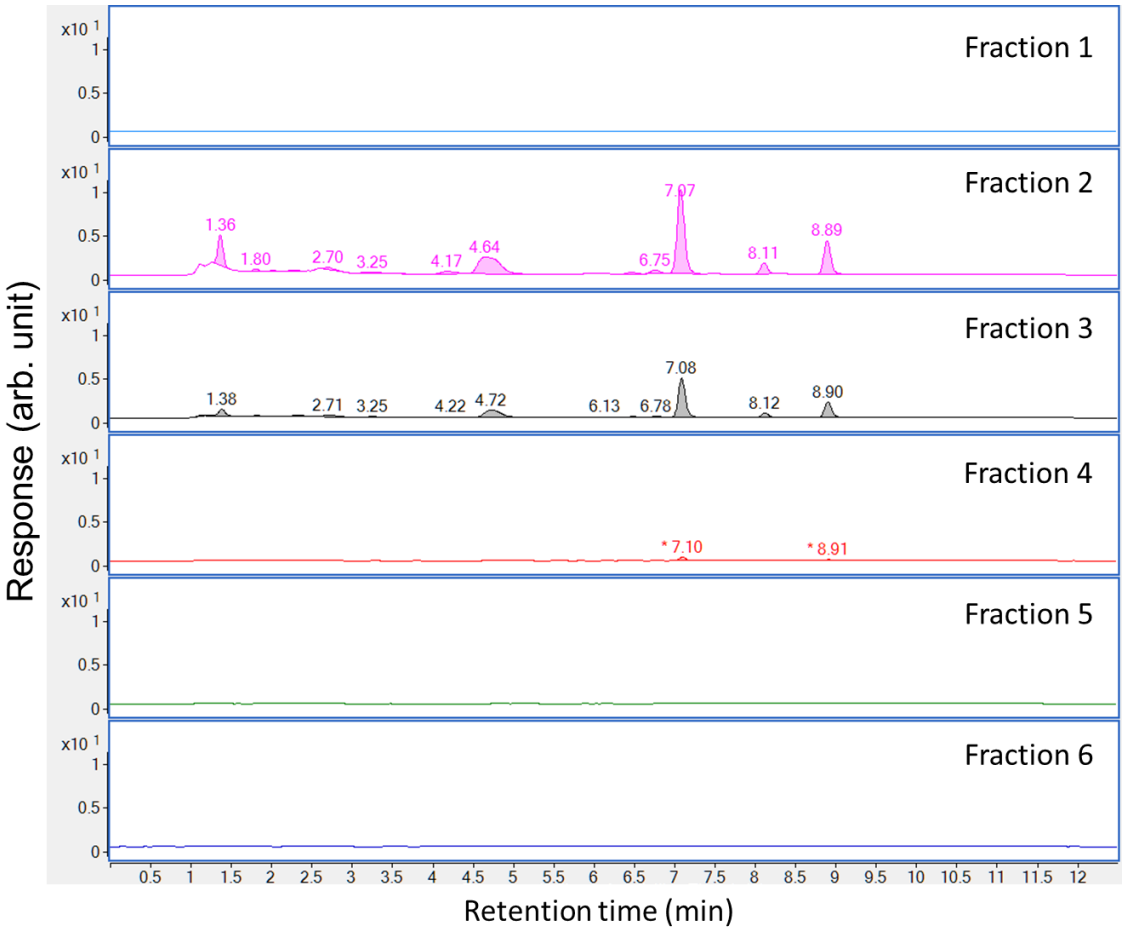


Figure 5-6 FLD chromatograms of six consecutive 1 mL elutions collected from Strata-X SPE cartridge

5.3.3 Conclusion

The optimisation of elution volumes for biotoxins from Strata-X SPE showed that 3 mL was sufficient for the complete elution of hydrophilic biotoxins from 500 mg of sorbent. This was 1 mL more than what was stated in the AOAC 2005.06 method for PSTs. However, the final volume of extract was still maintained at 4 mL due to the discarding of the first 1 mL after sample loading.

5.4 Optimisation of Extraction Sample Mass

Reported marine biotoxin extractions use a range of sample masses, from 2 g for lipophilic toxins (EURLMB, 2015) to 5 g for PSTs (AOAC, 2006). The EURLMB lipophilic toxin method specifies 20 mL of extract from 2 g of sample while the PST extraction method extract specifies 10 mL of extract from 5 g of sample. However, only

1 mL of extract was needed for the Strata-X SPE cleanup procedure. A SSR of 2:1 was chosen to be investigated with the aim of maximising the number of replicates of CRM and LRM that can be analysed. A secondary aim was to reduce solvent consumption while maintaining method performance. In addition, less waste was generated throughout the procedure, reducing the cost per sample.

5.4.1 Materials and Methods

To compare 5 g and 1 g masses for sample extractions, naturally PST-contaminated mussel tissue was used. For the extraction of 5 g, 3 mL of methanol was added in a 50 mL PP tube, which was capped and vortexed for 90 s. This was then centrifuged for 10 min at 3000 rpm and the supernatant is decanted into a 15 mL PP tube. Another 3 mL of 1% acetic acid solution was added to the remaining pellet and is resuspended by vortexing for 90 s. The tube was placed in boiling water for 5 min, then in a cold water bath. The sample was centrifuged for 10 min at 3000 rpm and the supernatants were combined. The extract was then topped up to 10 mL with deionised water.

For the extraction of 1 g of sample, the procedure was the same as for 5 g sample, except that extraction was carried out in 2 mL PP spin tubes. The volume of methanol was reduced to 600 µL for the first extraction and 600 µL of 1% acetic acid was used for the second extraction. The spin tubes were heated in a 100 °C heating block for 5 min. The supernatants were combined in a separate 2 mL PP spin tube and topped up to 2 mL with deionised water. As with 5 g samples, the solvent sample ratio was maintained at 2:1. Sample extracts were cleaned up *via* Strata-X SPE and analysed on the tandem LC-FLD-MSMS setup.

5.4.2 Results and Discussion

Toxin profiles are shown in the chromatograms shown in Figure 5-7, and toxin peak areas are presented in Table 5-2.

Table 5-2 Comparison of toxin peak areas between 5 grams and 1 gram extraction mass (± 1 Std Dev), *n*= 3

Toxin	Periodate oxidation		Peroxide oxidation	
	1 gram	5 grams	1 gram	5 grams
C1&2	2.39 ± 0.06	2.37 ± 0.13	8.33 ± 1.30	8.16 ± 1.20
GTX2&3	16.95 ± 0.83	15.52 ± 0.77	16.46 ± 2.85	15.71 ± 1.61
GTX5	N/A	N/A	2.15 ± 0.05	2.28 ± 0.18
STX	1.33 ± 0.12	1.39 ± 0.10	6.74 ± 0.36	7.07 ± 0.44

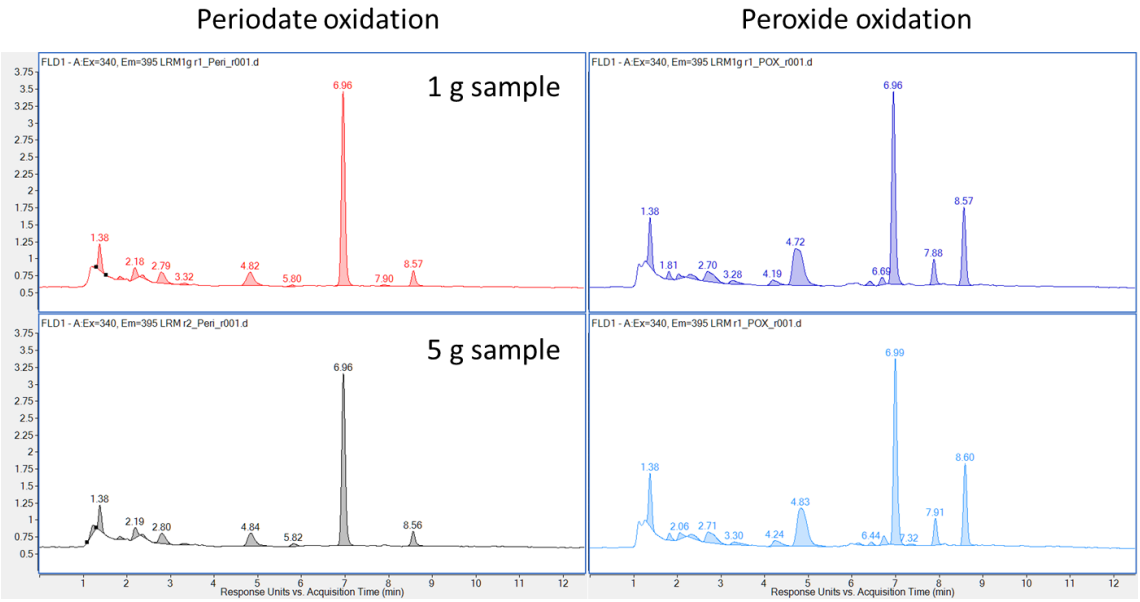


Figure 5-7 FLD chromatograms of 5 g and 1 g PST LRM after periodate and peroxide oxidation

No significant differences (<10%) were observed between 5 g and 1 g sample extractions when sample to solvent ratios were maintained. Therefore, it was confirmed to be suitable to proceed using 1 g sample mass for homogenous samples such as CRMs.

5.4.3 Conclusions

Overall, it was demonstrated in highly homogenous samples that the extraction efficiency of 1 g is the same as for 5 g of sample when the solvent to sample ratio was maintained. Using a smaller mass will be advantageous in terms of additional replicates from a given mass. For example, Certified Reference Materials are expensive and are shipped in small amounts such as 4 g and 8 g portions (Section 3.1.3). However, it should be noted that routine samples may not be very homogeneous. Therefore, it is advisable to use a larger sample mass during routine analysis when possible to ensure that a representative sample is analysed.

5.5 Verification of Strata-X-CW Fractionation of PSTs

To verify that the fractionation process for hydrophilic toxins is not affected by the new extraction and cleanup procedure, the hydrophilic fraction from the Strata-X cleaned up samples were further fractionated with weak cationic exchange SPE Strata-X-CW, according to the CEFAS method validation report (CEFAS, 2008). This fractionation was necessary for a full quantification of a positive extract as this allowed for subgroups of PSTs to be separated according to overall charge (Turner and Hatfield, 2012).

5.5.1 Materials and Methods

Strata-X-CW cartridges were conditioned with 4 mL 0.01 M ammonium acetate, followed by loading 2 mL of PST extract (previously purified with Strata-X SPE), followed by 3 mL of water. The first fraction is expected to contain neutral C toxins. 3 mL of 0.3 M NaCl was then passed through the cartridge and collected in another 15 mL PP tube. The second fraction was expected to contain the GTX toxins (with +1 overall charge). Finally, 3 mL of 2.0 M NaCl was passed through the SPE cartridge and is collected in a new 15 mL PP tube. The third fraction was expected to contain STX, dcSTX, NEO, and dcNEO (with +2 overall charge).

5.5.2 Results and Discussion

Figure 5-7 shows the fluorescence chromatograms of PST LRMs after fractionation with Strata-X-CW SPE. Toxin profiles were shown as C1&2, GTX2&3, GTX5 and STX. The toxins were found in the expected fractions according to overall compound

charges (CEFAS, 2008). C1&2 were found in Fraction 1 while GTX2&3 and GTX5 were found in Fraction 2. STX was found in Fraction 3.

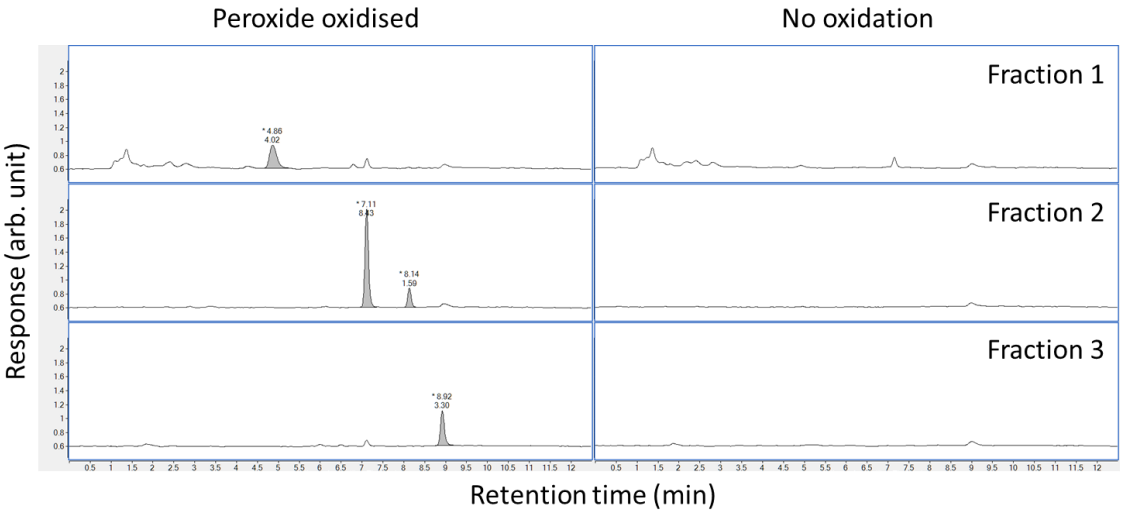


Figure 5-8 Peroxide oxidation and controls of PST LRM after fractionation using Strata-X-CW SPE

5.5.3 Conclusions

Shellfish extracts purified with the proposed method was fractionated using cationic exchange SPE. This was done to ensure that the new extraction and cleanup method would not affect the fractionation process. Toxins were found to elute in the expected fractions without any carryover. Therefore, extracts from the developed procedure was suitable for fractionation using Strata-X-CW.

5.6 Regeneration and Reuse of Strata-X Cartridges

EnviCarb SPE cartridges were trialed in this research due to reusability reported by Turner *et al.* (2015). They were able to regenerate EnviCarb SPE cartridges by flushing the sorbent with methanol followed by water. Polymeric SPE sorbents such as Strata-X may also have the advantage of being able to be recycled as they are robust when exposed to high pH solvents. Therefore, for this research, it was hypothesised that Strata-X can be regenerated and reused for multiple loadings of marine biotoxin extracts. Although this may be unacceptable practice for regulators, research bodies with limited resources may find it an interesting possibility to reuse cartridges.

5.6.1 Materials and Methods

Strata-X SPE cartridges were conditioned, loaded with PST contaminated mussel extracts, washed, eluted and reconditioned a total of six times. After the elution step, cartridges were reconditioned with 4 mL of methanol and 4 mL of 30% methanol before reloading with another aliquot of mussel extract. Six loadings were done in order to demonstrate stability after multiple uses and to evaluate any physical deterioration of sorbent. This experiment was performed in triplicate and samples were analysed after peroxide oxidation. The peroxide oxidation procedure is outlined in Appendix B.

5.6.2 Results and Discussion

The results of the experiment are shown in Figure 5-9. The figure shows a stacked chromatogram of 6 elutions from 1 SPE cartridge. The profile for all 6 elutions were shown to be similar. Figure 5-10 shows a bar graph comparing the peak areas for each toxin in the 6 loading and regeneration cycle. Higher variability of peak areas were observed with the earlier eluting C1, C2, GTX2 and GTX3, whereas a low variability of GTX5 and STX was observed. The regenerated Strata-X cartridges was shown in this study to be more suitable for later eluting toxins such as GTX5 and STX. However, early eluting toxins such as C1&2 and GTX2&3 was not suitable for cleanup with regenerated SPE cartridges.

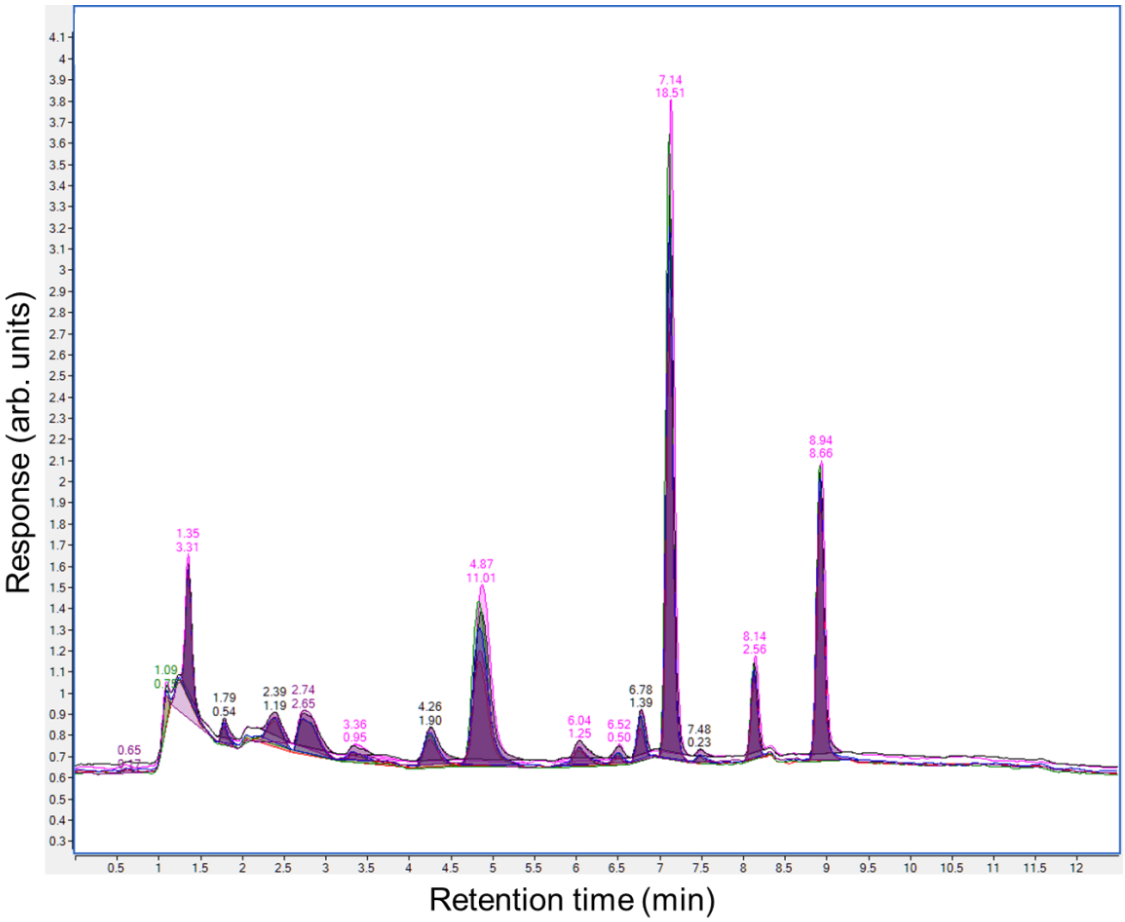


Figure 5-9 Overlaid chromatograms of 6 replicates of PST LRM extracts using regenerated Strata-X SPE cartridges

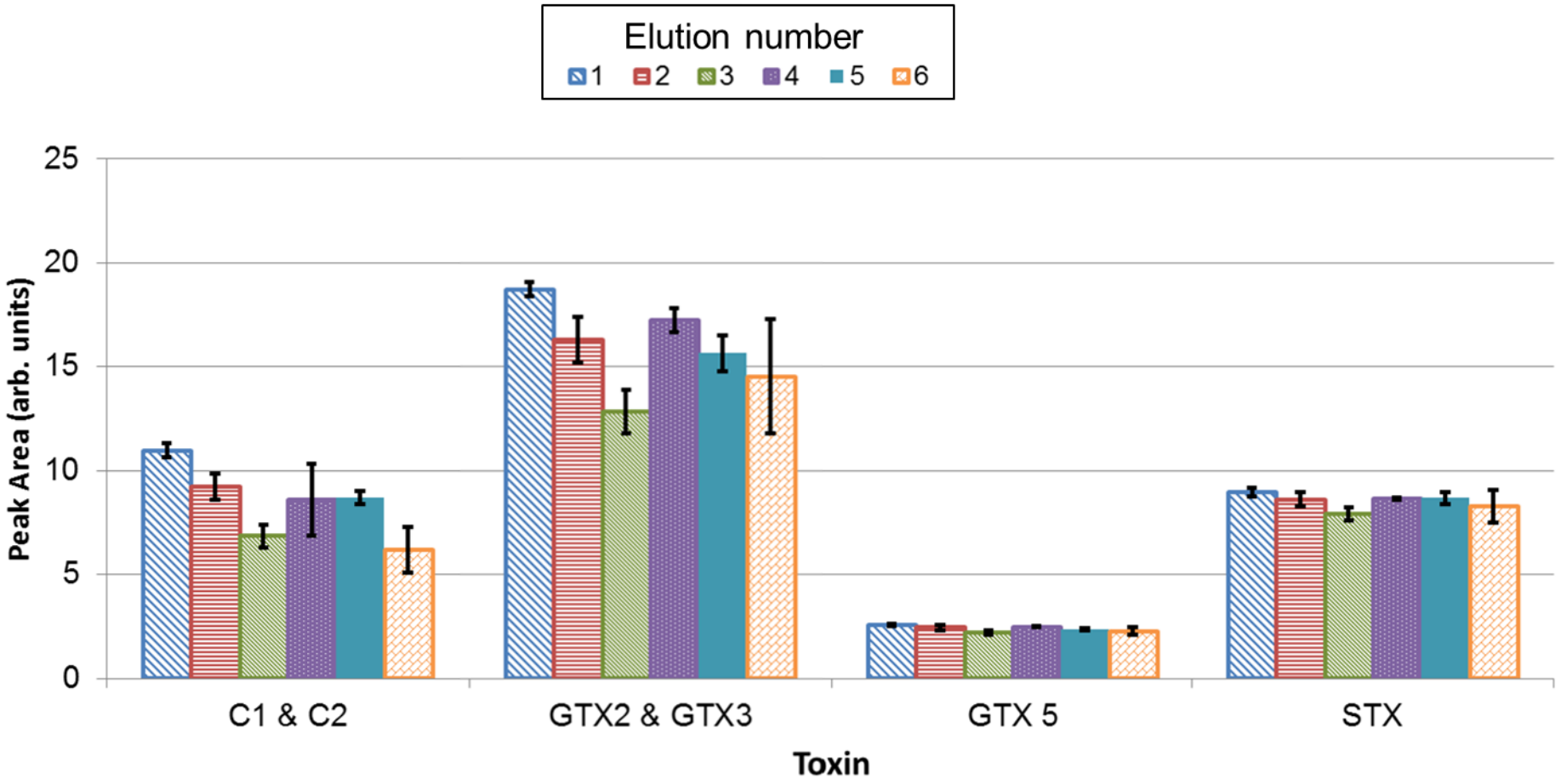


Figure 5-10 Peak areas of PSTs from 6 load, elute and regeneration cycles of Strata-X SPE cartridges

5.7 Final Sample Preparation Method

Based on the prior sections, the finalisation of the sample preparation protocol demonstrated that the experimentally determined sample preparation procedure was suitable for biotoxin analysis. A hybrid extraction method was developed where the sample was (1) extracted by methanol in order to extract lipophilic toxins, followed by (2) a second extraction with 1% acetic acid solution and heating to denature and cook the shellfish tissue and extract hydrophilic toxins (Figure 5-11). The combined methanol and water extract was then cleaned up using a polymeric SPE sorbent as mentioned.

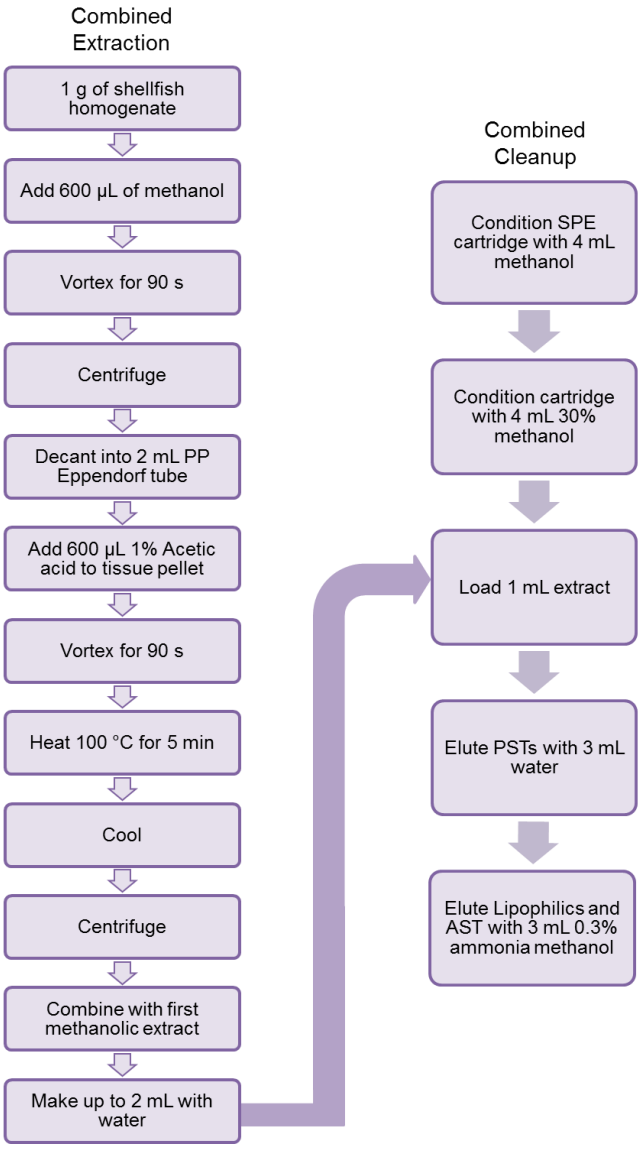


Figure 5-11 Final extraction and clean-up scheme

5.8 Preliminary Validation of Sample Preparation Method

Following the finalisation of the sample preparation and clean up described above, a preliminary method validation was performed using certified reference materials and mussels artificially spiked with toxins. The preliminary validation was performed to determine the suitability of the experimental conditions established for the analysis of mussel tissue. These mussel materials are described in Section 3.3.1. A limited amount of biotoxin reference material necessitated the use of eprinomectin as a substitute for lipophilic toxins in evaluation of extraction and cleanup conditions. The preliminary validation was used to assess the method with actual lipophilic toxin reference standards and certified reference materials, highlighting any weaknesses which may require further investigation.

5.8.1 Preliminary Recovery Data for PSTs

For this experiment, PST-free mussels were spiked with PSTs. C1&2, GTX2&3 and NEO was spiked at 400 µg/kg. These toxins were chosen to represent the groups with 0, +1, and +2 overall charge, respectively. Analyses were performed in triplicate. Recoveries observed for these toxins in a mussel matrix are shown in Table 5-3. The preliminary validation was performed in triplicate.

Table 5-3 Recoveries of several PSTs from spiking experiment (* denotes the recommendation for Recovery Correction)

Toxin	**Spiked amount (µg/kg)**	**Mean recovery (%), *n*=3**
C1&2	400	81 ± 6.0
GTX2&3	400	46 ± 3.3*
NEO	400	76 ± 0.91

The recoveries ranged between 46% to 81% for the three toxins analysed. For the PST validation, toxin-free mussel tissue were spiked with 0.4 µg/g of C1&2, NEO and GTX2&3. Results with recoveries falling outside 80-120% but with a low relative standard deviation can be reported after correcting for recoveries (Thompson *et al.*,

1999). In this case, if a real samples was analysed and GTX2&3 was detected the result could be reported with a recovery correction based on the recovery measured for GTX2&3 for the same batch.

5.8.2 Preliminary Recovery Data for DSTs

A sample of the certified reference material (CRM-DST-Mus) was analysed using the above developed method. The recoveries are shown in Table 5-4. The recovery values obtained show that the developed method is suitable for the analysis of DSTs at regulatory limits. The positive ionisation MRMs were found to be more sensitive and therefore were used for recovery calculations.

Table 5-4 Recoveries for CRM-DSP-Mus

Toxin	**Certified amount (µg/g)**	**Mean Recovery (%), *n*=3**
DTX1 (+)	1.07 ± 0.08	98 ± 6.4
DTX2 (+)	0.86 ± 0.11	111 ± 8.8
OA (+)	1.07 ± 0.08	113 ± 6.2

5.8.3 Preliminary Recovery Data for ASTs

The certified mussel reference material used for DSTs (CRM-DST-Mus) also contained domoic acid and was used for determination of AST recovery. The concentration of domoic acid in this material was certified to be 11.8 ± 0.6 µg/g. The average recovery for DA (+) in the reference material was 20 ± 3.45 % (*n*=3). Although the recovery was low, the RSD for the recoveries was low. In this case if a real samples was analysed and DA was detected the result should be reported with a recovery correction based on the DA recovery achieved for the same batch. The possible major source for this low recovery come from matrix interference. Confirmation of the effects of matrix interference on the DA was demonstrated though a comparison study by decreasing the amount of matrix while adding a constant amount of DA. Toxin-free mussel extracts which had undergone Strata-X cleanup was used as the matrix. The purified extracts were diluted with methanol at various levels before addition of domoic acid reference

standards at 3.33 µg/mL (equivalent to 20 mg/g of mussel tissue, the regulatory limit for DA).

Figure 5-12 shows the peak areas of domoic acid (positive and negative ionisation) at various dilutions of the matrix. For negative ionisation, there is a clear trend of increase in peak area as the amount of matrix is diluted with methanol. For positive ionisation, there seems to be an enhancement effect at 10% matrix as the peak area is higher than when the reference standard is measured in pure methanol. At 25% matrix in solution, the peak area is similar to the control in pure methanol. However, from 50% matrix to undiluted matrix, the same trend of decreasing peak areas was observed.

The results of the matrix dilution experiment show that the detector is affected by matrix components that are in the extract even after Strata-X cleanup.

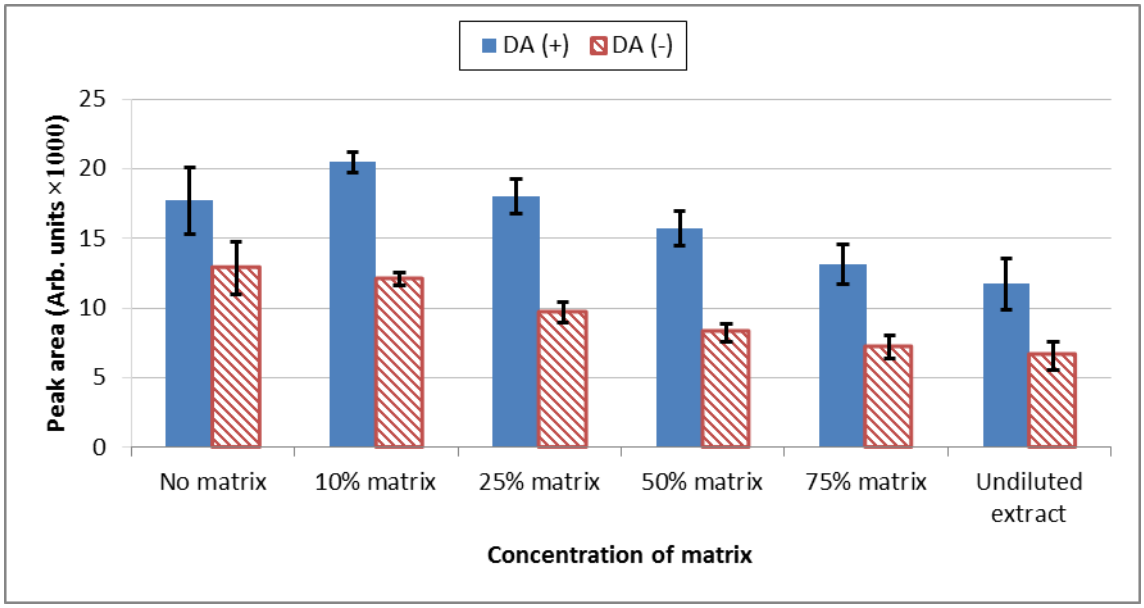


Figure 5-12 Change in peak area f domoic acid in different matrix concentrations

5.8.4 Matrix Effects

Matrix effects are a concern in detection techniques which rely on ionisation of analytes, such as MS. Coeluting components of the sample can cause either suppression or enhancement of the signal, which affects accuracy of results (Trufelli *et al.*, 2011)

Overcoming suppression in LCMS analysis for the hydrophilic PSTs has been explored by Dell'Aversano *et al.* (2005) and Harju *et al.* (2015). One of the methods that has been proposed is matrix dilution, where the extract is diluted with MS compatible solvents (acetonitrile). This serves to reduce the amount of coextracting material which enters the source with the analyte, thus reducing suppression of the analyte signal.

However, a critical problem arose related to the sensitivity of the instrument, particularly for the detection of PSPs. This problem stems from matrix effects from the sample. Matrix effects are a well documented phenomenon in ESI MS (Kruve and Leito, 2013), especially in shellfish samples (Zhuo *et al.*, 2013). To overcome matrix effects, extrapolative dilution of the sample extracts has been suggested (Dell'Aversano *et al.*, 2005; Kruve and Leito, 2013). However, dilution of samples will reduce the overall sensitivity of the instrument. One way to overcome matrix effects is to do matrix matching. However, the extent of matrix effect is variable from sample to sample, and matrix matched calibrations may not account for all the variability in each analysis (Mattarozzi *et al.*, 2015). Furthermore, the limited availability and access to reference materials needs to be seriously considered if matrix matching to be included in biotoxin analyses. Resulting benefit compared to the cost incurred will not be justifiable for matrix matching for routine analyses.

5.8.5 Preliminary Recovery Data for PbTx-1 and PbTx-2

No CRMs are available for brevetoxins. Recovery data was obtained through spiking trials using chemical standards. Toxin-free mussel tissue was spiked with standards of brevetoxins: 1 g of mussel homogenate was spiked at 800 ug/g of PbTx-1 and PbTx-2. No recoveries were able to be obtained from this sample.

The sources of low recoveries were identified from literature, which were sample ionisation disruption (matrix suppression) and metabolic activity. These transformations alter the recoverability of brevetoxins from a sample of shellfish tissue. Additionally, the unstable nature of brevetoxins also complicates the process of creating suitable reference materials with predictable properties for method development. To investigate the source of no recovery observed for brevetoxins, a matrix dilution experiment was

perfomed. In this experiment, the matrix concentration was varied by dilution with methanol while adding a constant concentration of brevetoxin reference standard. The matrix dilution trial showed no significant differences or discernible trends for detector response (Figure 5-13 and Figure 5-14). As the matrix solution that was used had already undergone the sample preparation procedure, the results support the hypothesis that irreversible binding of brevetoxins to matrix components may cause the low recovery of brevetoxins from shellfish samples (Plakas and Dickey, 2010). Previously, McNabb *et al.* (2012) reported recoveries of 61% for brevetoxin-2 from shellfish tissue spiking trials. In their method, a hexane wash step was included, which may improve the recovery of brevetoxins. Ishida *et al.* (2004) had reveiwed several extraction methods for brevetoxins, comparing methanol and dichloromethane extraction of shellfish. They found that methanolic extraction was suitable for PbTx-1 while dichloromethane was suitable for PbTx-2. However, the best recovery obtained was relatively low (44%). The literature revealed that there are limited brevetoxin methodologies that can analyse for brevetoxins in shellfish effectively.

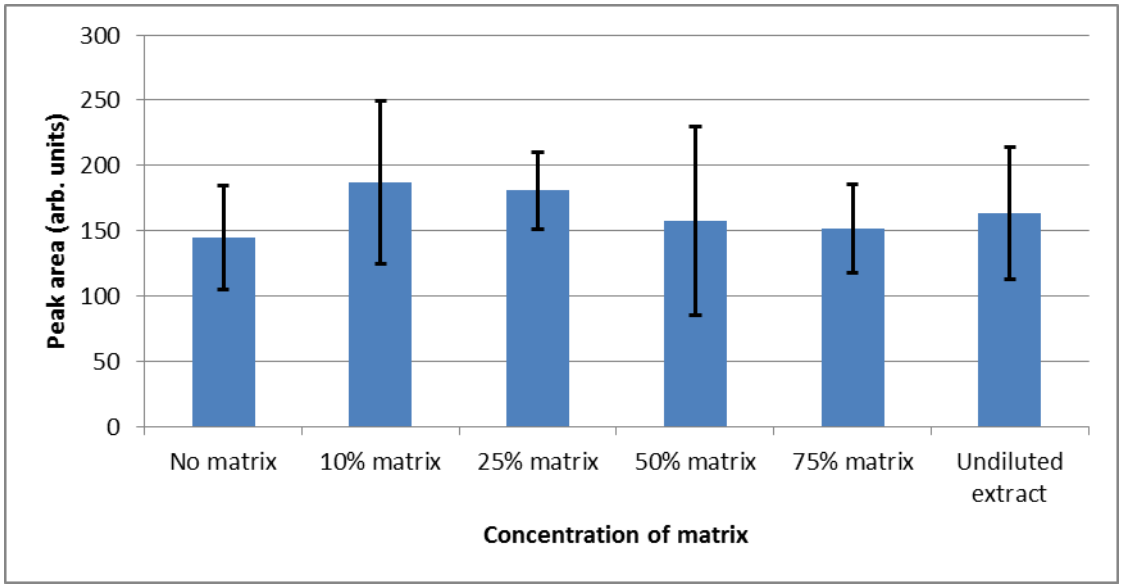


Figure 5-13 Peak area of brevetoxin 1 (PbTx-1) at different concentrations of matrix extract in solution

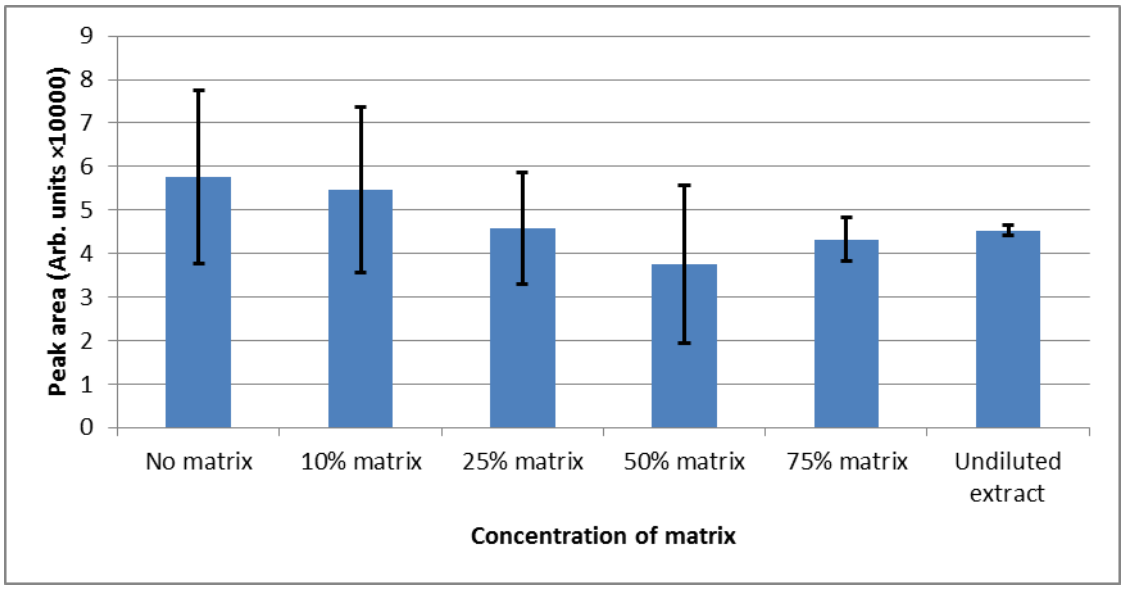


Figure 5-14 Peak area of brevetoxin 2 (PbTx-2) at different concentrations of matrix extract in solution

Overall, the developed sample extraction and cleanup method was found to be unsuitable for brevetoxins as no recoveries of either brevetoxin-1 and -2 were observed. As a result of this preliminary study for brevetoxins, this group was not included in the methodology for the validation procedure carried out in Chapter 6.

5.8.6 Preliminary Recovery Data for AZA-CRM-Mus

CRM-AZA-Mus material was extracted according to the developed method. This material was certified to contain AZA toxins. The amount of toxins measured is shown in Table 5-5.

Table 5-5 Recoveries for CRM-AZA-Mus

Toxin	Certified amount (µg/g)	Mean Recovery (%) *n*=3
AZA1	1.16 ± 0.10	16 ± 2.2*
AZA2	0.273 ± 0.024	35 ± 2.6*
AZA3	0.211 ± 0.023	20 ± 2.1*

To further investigate the low recoveries of the AZAs, a dilution of the extract was performed. Extracts were diluted with methanol and analysed. This is in contrast to the

matrix dilution performed for brevetoxins. The lipophilic extracts from the SPE cleanup procedure were diluted by a factor of 2, 5, 10 and 20. Matrix suppression effects were reduced when the samples were diluted prior to analysis. An increased detector response of about 33% was found for AZA2 and AZA3 and 89% increase in AZA1 response was found with a 10-fold dilution (Figure 5-15).

Modifications of AZA profiles due to extraction or sample processing are (1) epimerization, (2) 22-decarboxylation, and (3) formation of methyl derivatives (Rehmann *et al.*, 2008; Hess *et al.*, 2015). However, these esters were only observed in methanol extracts stored at room temperature or higher for prolonged periods (i.e., several months). In addition, high temperatures (around 90 °C) have been found to significantly alter the AZA toxin profile in mussel tissue (Kilcoyne, McCarron, *et al.*, 2015). Azaspiracids have been found to degrade rapidly under acidic conditions (Alfonso *et al.*, 2008), and it has been demonstrated that matrix suppression may be reduced by using alkaline conditions for analysis. However, columns packed with a special stationary phase which can withstand high pH mobile phases are needed for this analysis (silica dissolves at pH 8)(Kilcoyne and Fux, 2010).

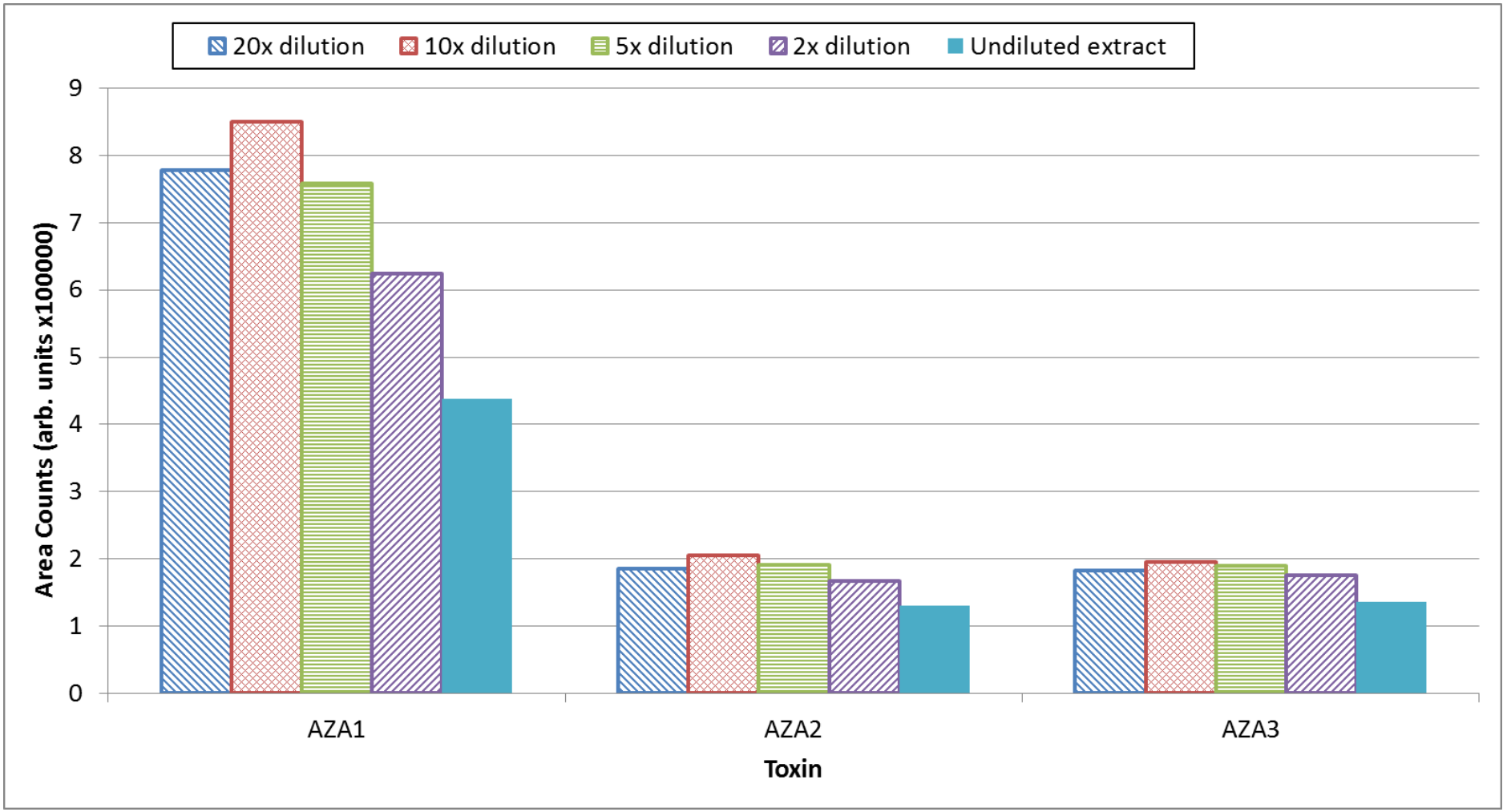


Figure 5-15 Peak areas of CRM-AZA-Mus at various dilution levels (average of two replicates)

5.9 Conclusion

A combined extraction method with the ability to extract both lipophilic and hydrophilic toxins from shellfish tissue was developed and optimised. Strata-X polymeric SPE was found to be suitable for both hydrophilic and lipophilic toxins. Elution volumes for the cleanup protocol were optimised to ensure correct toxins fractions were collecting while maintaining final extract volume at 4 mL. Regenerated Strata-X cartridges were also found to be suitable only for late eluting toxins such as STX and GTX5. Other conditions such as solvent types and amount, range of pH , centrifugation time, vortex time were not investigated due to the fact that there is a large consensus in literature for these conditions e.g. methanol and acetic acid solutions were superior for lipophilic and hydrophilic toxins, respectively.

A preliminary validation study was carried out prior to the full method validation in order to ensure that the method performed suitably. The small scale validation also allowed for adjustments to be made during development since there was a limited amount of chemical standards and certified reference material available. It was found that PST and DST recoveries were within the proposed guidelines. Improved recoveries were gained for AZAs through a 10-fold dilution of the extract. However, this dilution decreases the absolute amount of toxin entering the detector, and therefore should be applied when a positive sample is encountered.

No recoveries of brevetoxins was observed, indicating that the sample extraction and cleanup method developed was not suitable for this group of biotoxins. Furthermore, during the time of this research, no mussel reference materials for brevetoxins were available to conduct further investigations. As a result, this group was excluded from the method validation study detailed in Chapter 6. Currently brevetoxin samples are recommended to be analysed using the method reported by McNabb *et al.* (2012), which contains a hexane cleanup step.

6 VALIDATION OF TANDEM LC-FLD-MSMS METHOD

6.1 Introduction

In chapters 4 and 5, the development of a novel tandem LC-FLD-MSMS method in order to extract and measure biotoxins in marine shellfish in a single injection was described. In order to fully understand the potential for this technique a validation needed to be performed. The purpose of method validation is to establish fitness for purpose of the analytical procedure. Method validation is a critical part of evaluating method reliability and consistency. Validation also provides confidence in the results from the method and determines its limitations, establishing boundaries for its application.

The parameters which are assessed in the validation process include accuracy, selectivity, detection limit, quantitation limit, linearity, recoverability and robustness (Feinberg, 2007; Ruiz-Angel *et al.*, 2014). In the validation process, aspects of the method are evaluated, such as accuracy/recovery, precision (repeatability, intermediate precision and reproducibility), linearity and application range, limit of detection (LOD)/limit of quantitation (LOQ), selectivity/specificity, robustness, ruggedness, uncertainty, trueness, stability and system suitability studies.

6.1.1 Linearity

The linearity evaluates the relationship between concentration and detector response, in order to ensure that the measurement has a predictable correlation across a calibration range. Typically, in a linearity study the linearity should be evaluated across 0-150% of the expected measurement range (NATA, 2013). Typically, a calibration curve will consist of 5 data points, in which a line of best fit (using least squares regression) is implemented to establish a relationship between concentration and instrument response. The linear model commonly used is $y = a + bx$, with y as the intercept (in this case instrumentation response), b is the slope of the regression equation and x is the x intercept (or concentration).

6.1.2 Selectivity

The aspect of selectivity includes measurement of the target analytes which can be distinguished reliably from other compounds within the sample (non-targeted compounds). An example of this is the utilisation of specific detectors to measure specific compounds, such as fluorescence detection for compounds with a fluorophore. Using unique MRM transitions for compounds analysed by MSMS is a selective process.

6.1.3 Limit of Detection/Quantitation

The limit of detection for an analysis is required when methods are designed for analytes that are close to zero. This is is determined by determining the concentration of analyte which produces a signal-to-noise ratio of 3:1. This can be achieved experimentally by dilution of standards until the S/N is reached. Limit of Quantitation is determined by multiplying the LOD by 3. The LOD and LOQ are reported based on standard concentration in solvent. The reported value is an expression of the amount in reference to the original sample mass or volume. For example, 10 µg/kg in a sample is defined as 10 µg of the analyte per a kg of actual sample.

6.1.4 Repeatability

Repeatability is the estimate of standard deviation/variance between repeated measurements. It measures the short-term variation between batches of analyses, and can be used to estimate the likely difference between replicate measurements within a batch. This is usually tested through repeating the analysis at different times with the

same conditions. For this research, instrument repeatability was assessed by repeated analysis of reference standards due to limited amounts of reference materials.

6.1.5 Measurement Uncertainty

Measurement uncertainty is the quantitative analysis using validation data to determine error associated with analytical results. The validation data will be used to evaluate the spread results from different replicate conditions and bias in the procedure. In the last two decades MU has been implemented within laboratories to assist with estimating error in measurement and uncertainty associated with measurements. Most measurement uncertainty approaches stem from the ISO guide to calculating uncertainty in measurement (GUM). This guide was initially applied to physical measurements, but has since been extended to chemical and biological measurements. Measurement uncertainty is essence the standard deviation of the associated parameters that contribute to the results. Overall, measurement uncertainty encompasses two uncertainty sources, those associated with bias corrections, and those associated with random effects. Measurement uncertainty is the summation of these two sources of uncertainty. Uncertainty quantifies the doubt inherent in any analytical measurement and helps analysts to make reliable decisions with quantifies risk of false compliance and non-compliance (Rozet *et al.*, 2011).

There are two common approaches for measurement uncertainty determination this include : “bottom up” and “top down”. In the top down approach, measurement uncertainty is determined using the standard deviation of the overall results. The measurement uncertainty is calculated by incorporating validation data over a period of time and operators variables as this would maximise the potential error probabilities. For example, the standard deviation between replicates over a period of time. On the other hand, in the bottom up approach, the sum of the errors for each associated parameter with the analytical procedure is calculated. These include things such as error in the glassware used, standard preparation, recovery value, and errors with duplicate analysis.

The bottom up is a more comprehensive approach, which identifies and quantifies as many potential sources of error. Commonly, an experienced chemist would be able to identify the critical stages of the analytical procedure where the source of error is significant. The main barrier for using this approach is the complexity which increases with the complexity of the method. For this research the MU was determined using the bottom up approach as this enable the research to identify possible sources of error to assist in improving the method (Konieczka and Namieśnik, 2010).

6.2 Validation Results

6.2.1 Linearity

A series of dilutions over the range of from zero to twice the regulated limit were performed for each individual toxin standard (0.2, 0.5, 1.0, 1.5, 2.0 times regulated limits). Through this experiment, the following linearity ranges were calculated (Table 6-1), with the calibration range reported as µg/g of each toxin. The PSTs are reported as STX equivalents (STX.eq) using factors described in . This demonstrated the linear relationship between the toxin concentrations and the detector response over the measured range. The linearity graphs for each compound can be found in Appendix E.

Table 6-1 Linearity of standards

Toxin group	Toxin	Calibration range (ug/g)	Linearity (R^2)
PST	C1&2	0-1600 STX eq	0.997
	GTX1&4	0-1600 STX eq	0.994
	GTX2&3	0-1600 STX eq	0.995
	GTX5	0-1600 STX eq	0.999
	dcGTX2&3	0-1600 STX eq	0.997
	NEO	0-1600 STX eq	0.999
	dcNEO	0-1600 STX eq	0.999
	STX	0-1600 STX eq	0.999
	dcSTX	0-1600 STX eq	0.999
AZA	AZA1	0-320	0.993
	AZA2	0-320	0.992
	AZA3	0-320	0.993

Toxin group	Toxin	Calibration range (ug/g)	Linearity (R^2)
DST	OA(+)	0-1280	0.993
	OA(-)	0-1280	0.995
	DTX1(+)	0-1280	0.989
	DTX1(-)	0-1280	0.992
	DTX2(+)	0-1280	0.988
	DTX2(-)	0-1280	0.985
AST	DA(+)	0-20000	0.992
	DA(-)	0-20000	0.995

6.2.2 Selectivity

Selectivity assessment for hydrophilic and lipophilic biotoxins was performed by analysis of blank solvents compared to blanks spiked with biotoxin reference standards. It was shown using the tandem LC-FLD-MSMS setup for this research, that the samples without the biotoxin can be clearly distinguished from samples with biotoxins. This was achieved using unique MRM transitions for AZAs, DSTs, and ASTs. For the PSTs, fluorescence detection was used, which was based on a PST-specific derivatisation procedure using periodic acid or hydrogen peroxide to produce unique fluorescent derivatives at specific retention times which enabled confirmation.

Anlaysis of CRM-ZERO-Mus was used as a baseline comparison for toxin detection. This sample showed no chromatographic peaks except for one corresponding to the added eprinomectin.

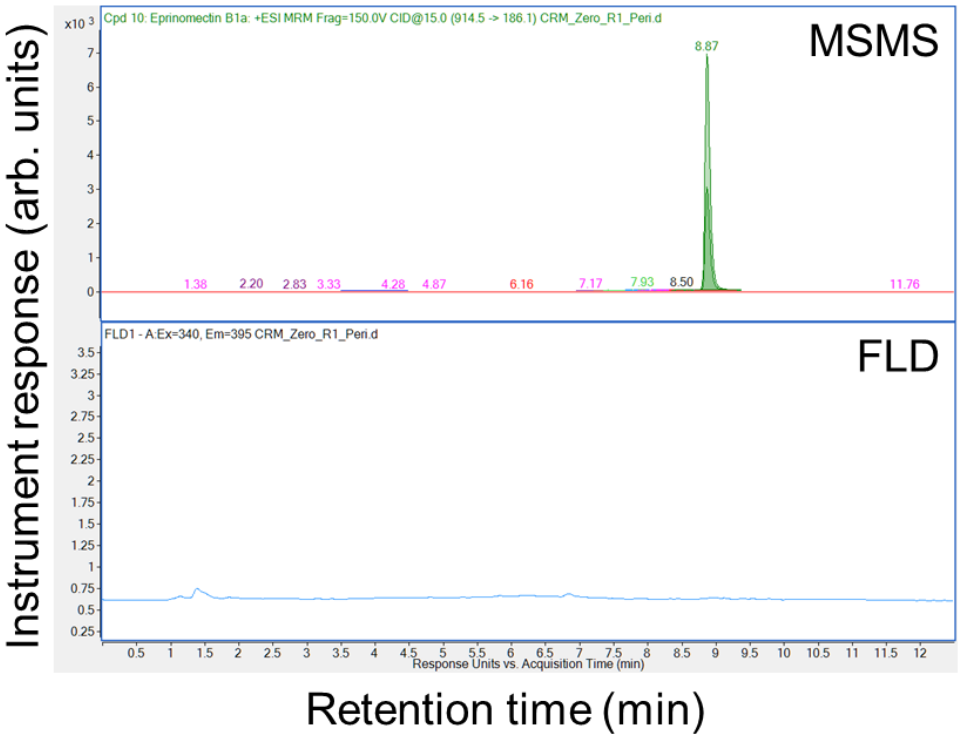


Figure 6-1 MSMS and FLD chromatograms of CRM-Zero-Mus

CRM-DSP-Mus showed peaks for domoic acid, OA, DTX2, DTX1 and eprinomectin for the MSMS detector and showed no peaks from the fluorescence detector (Figure 6-2).

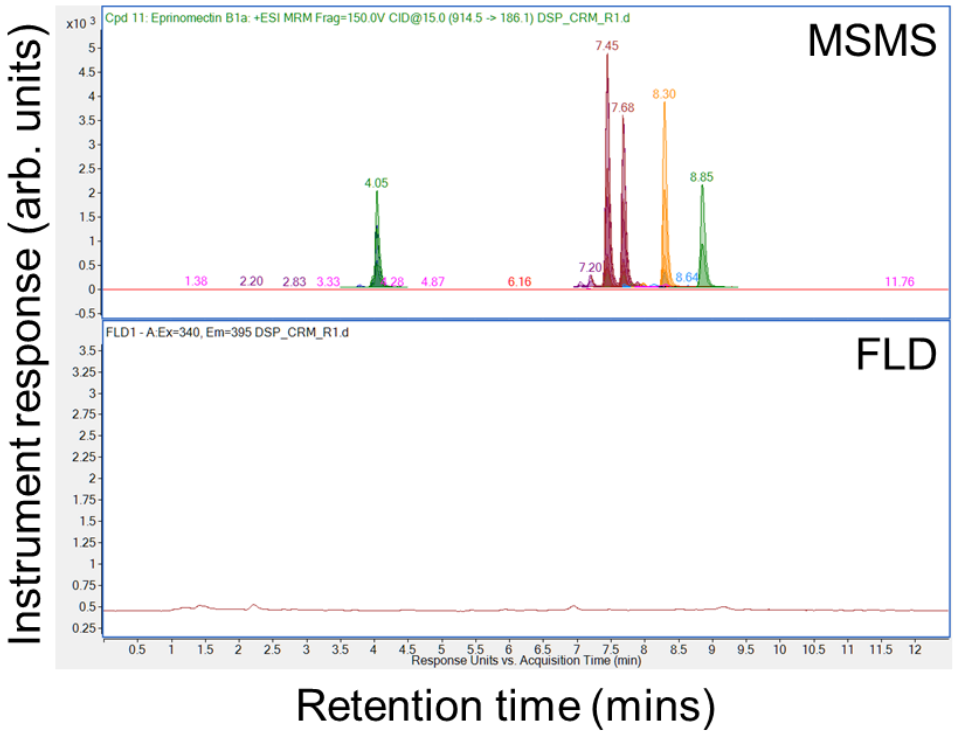


Figure 6-2 MSMS and FLD chromatograms of CRM-DSP-Mus

Analysis of CRM-AZA-Mus showed chromatograms for AZA3, AZA1, and AZA1, along with eprinomectin. Several peaks were observed in the FLD signal, but the retention times did not correspond to any known peaks for PSTs (Figure 6-3).

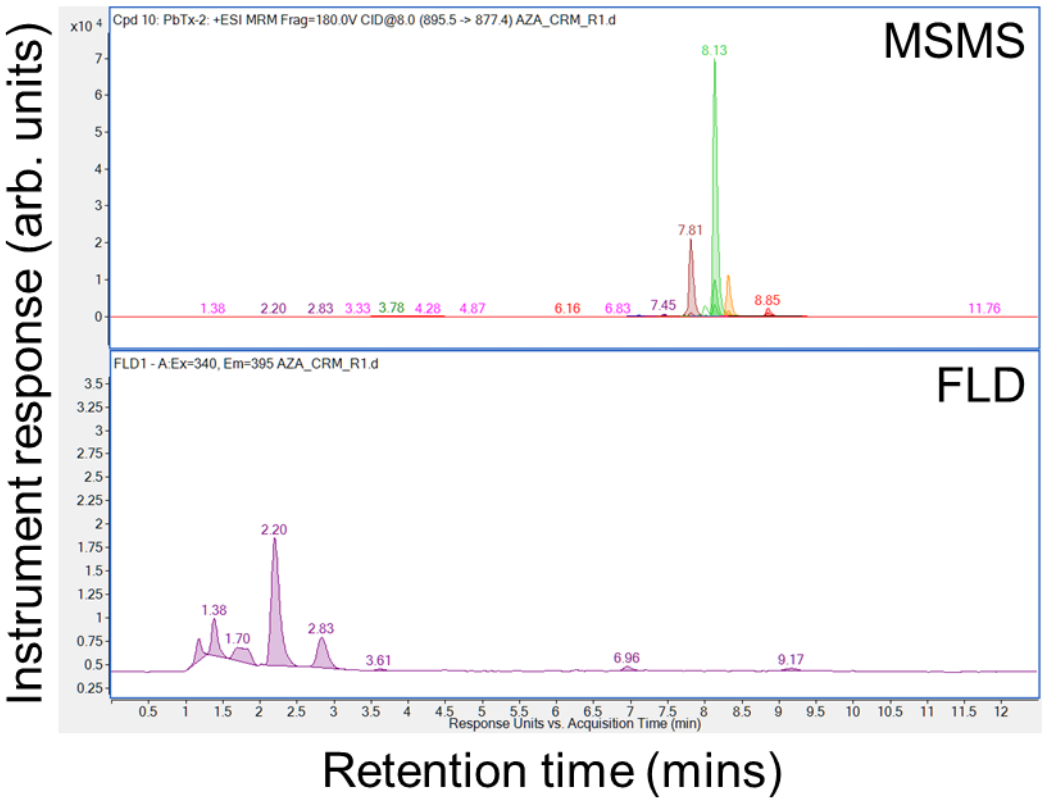


Figure 6-3 MSMS and FLD chromatograms of CRM-AZA-Mus

Figure 6-4 shows the MSMS and FLD chromatograms from the analysis of PST LRM. There were no peaks in the MSMS signal except for eprinomectin. For the FLD signal, periodate oxidised extracts showed a strong toxin peak corresponding to GTX2&3 at 7.39 minutes.

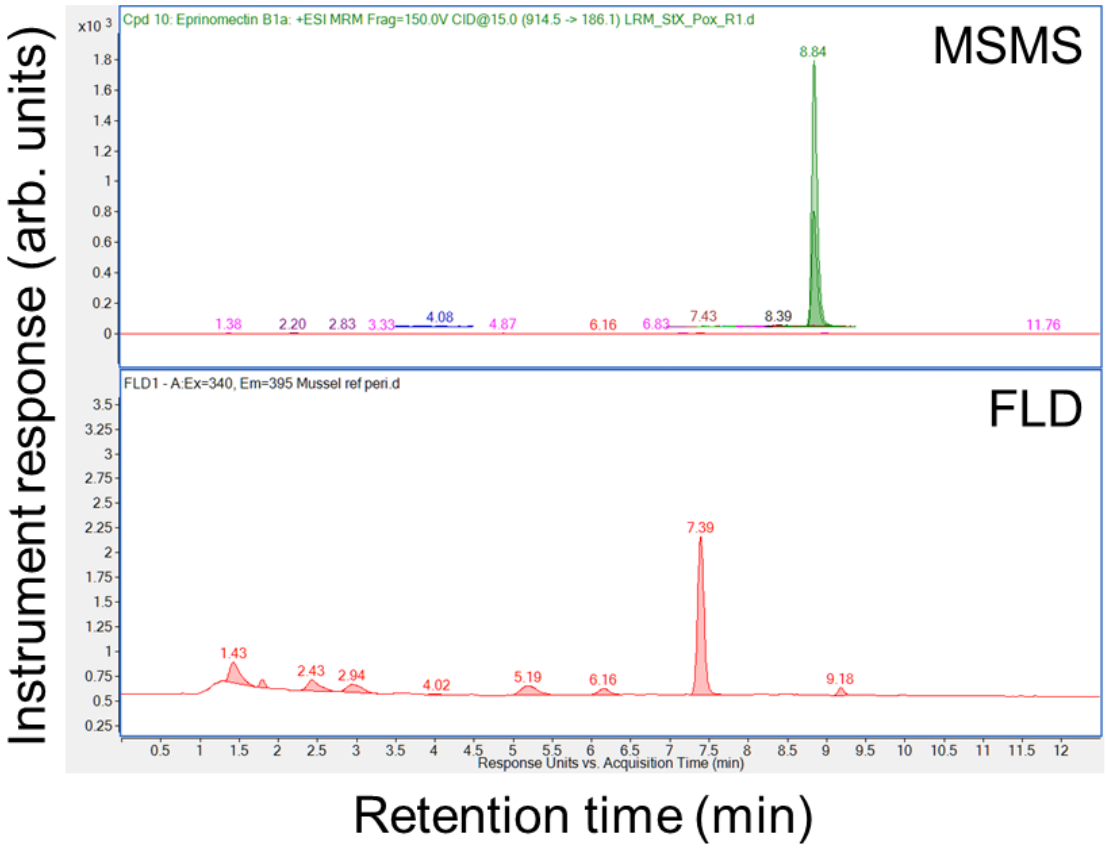


Figure 6-4 MSMS and FLD chromatograms of PST LRM

6.2.3 Limit of Detection and Quantitation

A series of dilutions of biotoxin reference standards were analysed to determine the limit of detection (LOD) of the instrument. PSTs were oxidised by both periodate and peroxide, in ranges between 1 to 100 ng/mL. Other biotoxins were diluted serially and analysed. The (LOD) was determined by assessing signal to noise ratios (Determined by RMS algorithm in analysis software) and calculated incorporating sample preparation dilution factors (8 for hydrophilic toxins and 6 for lipophilic toxins) to give a value corresponding to sample mass. For PSTs, the values were converted to STX equivalents using their respective TEFs.

The LOQ was calculated by multiplying LODs by a safety factor of 3. and recoveries. Table 6-2 shows the LOD and LOQ values for the toxins.

Table 6-2 Limit of Detection and Quantitation determined for marine biotoxins

Toxin group	Toxin	LOD (µg/kg)	LOQ (µg/kg)	Current regulatory limits (ug/kg)
PST	C1&2	20	67	800 (STX eq)
	GTX1&4	200	666	
	GTX2&3	5	17	
	GTX5	20	67	
	dcGTX2&3	16	53	
	NEO	40	133	
	dcNEO	16	53	
	STX	80	266	
	dcSTX	80	266	
AZA	AZA1	0.8	3	160
	AZA2	0.8	3	
	AZA3	0.8	3	
DST	DTX1 (+)	8	27	160
	DTX2 (+)	8	27	
	OA (+)	8	27	
AST	DA (+)	30	100	20000

6.2.4 Repeatability/Reproducibility

For this research method repeatability was assessed through multiple analyses of CRMs and PST-contaminated mussel samples over two separate days. This could only be

performed for AZA CRM as the material arrived in 8 g bottles which allowed for two analyses with three repliactes in each batch.

Table 6-3 Repeatability and reproducibility data for AZA and PSTs (*n*=3)

Toxin	Recovery of first batch (%)	Recovery of second batch (%)	RSD (%)
AZA1	18.5 ± 2.0	19.6 ± 1.8	2.9
AZA2	7.8 ± 2.3	7.3 ± 2.9	3.3
AZA3	24.3 ± 1.1	27.4 ± 1.1	6.0

The data from the two batches showed good agreement between values determined on two separate days.

6.2.5 Accuracy

Determination of accuracy was based on recovery data as shown in Table 6-4. The minimum number of replicates recommended by NATA is 7 (NATA, 2013), but limited amounts of material restricted the number of data points to 3.

Table 6-4 Toxin recoveries from CRMs and spiked mussel tissues

Toxin	Certified/spiked value (ug/g)	Recovery (%)	SD	RSD (%)
AZA1	1.16	16	2.2	11
AZA2	0.273	35	2.6	34
AZA3	0.211	20	2.1	8
DTX1 (+)	1.07	98	6.4	10
DTX2 (+)	0.86	111	8.8	12
OA (+)	1.07	113	6.2	6
DA (+)	11.8	20	3.45	16

Toxin	Certified/spiked value (ug/g)	Recovery (%)	SD	RSD (%)
C1&2	400	81	6	7
GTX2&3	400	46	3.3	4
NEO	400	76	0.91	6

The data showed acceptable recoveries for DSTs (DTX1, DTX2 and OA) and two PSTs (C1&2, NEO). For AZA1, AZA2 and AZA3, low recoveries were observed (<40%) and DA (20%), and for GTX2&3. However, for the low recovery biotoxins, a low RSD was found (RSD <16%) with the exception of AZA2, indicating good reproducibility.

6.2.6 Measurement Uncertainty

Measurement uncertainty was determined using the validation data generated for accuracy, linearity, and repeatability. A calculation tool developed at NMI was used, which incorporated four major sources of uncertainty in the estimate: recovery (RSD_{rec}) and duplicate data (RSD_{dup}), standard preparation error (RSD_{std}) and the error associated with calibration (RSD_{calib}). RSD_{rec} was estimated using validation data from Section 6.2.5. The calculations also take into account duplicates in the RSD_{dup} component of the estimate. For RSD_{std}, uncertainties related to volume, temeprature fluctuations and stock concentration was considered. The biotoxin reference standards from the NRCC were supplied with a certificate containing uncertainties associated with each material. For biotoxin pairs such as C1&2 and GTX2&3, the highest uncertainty of each pair provided was used for the measurement uncertainty determination. This was done in order to maintain a conservative estimation. Finally, RSD_{calib} was calcuated using the data of response against concentration which generates a regression curve and assesses the linearity and residuals.

Table 6-5 shows a summary of the measurement uncertainty for each biotoxin. The calculated measurement uncertainty of each individual toxin has a confidence interval of 95% and a coverage factor of 2. Figures 6-5 to 6-8 show the relative contributions of each component to the overall uncertainty for each toxin.

Table 6-5 Uncertainties associated with toxin measurements

Toxin group	Toxin	Uncertainty at midpoint of calibration (%)
PSTs	C1&2	25
	GTX2&3	39
	NEO	23
AZAs	AZA1	33
	AZA2	96
	AZA3	26
DSTs	DTX1 (+)	41
	DTX1 (-)	39
	DTX2 (+)	43
	DTX2 (-)	33
	OA (+)	47
	OA (-)	47
ASTs	DA (+)	40
	DA (-)	32

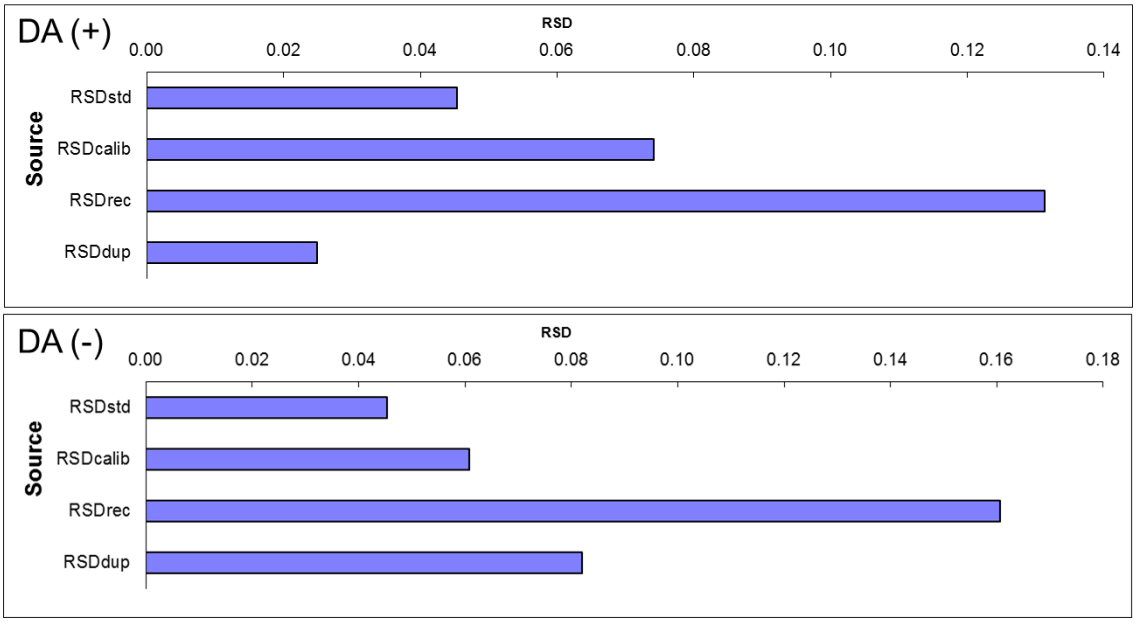


Figure 6-5 Measurement Uncertainty charts for AST

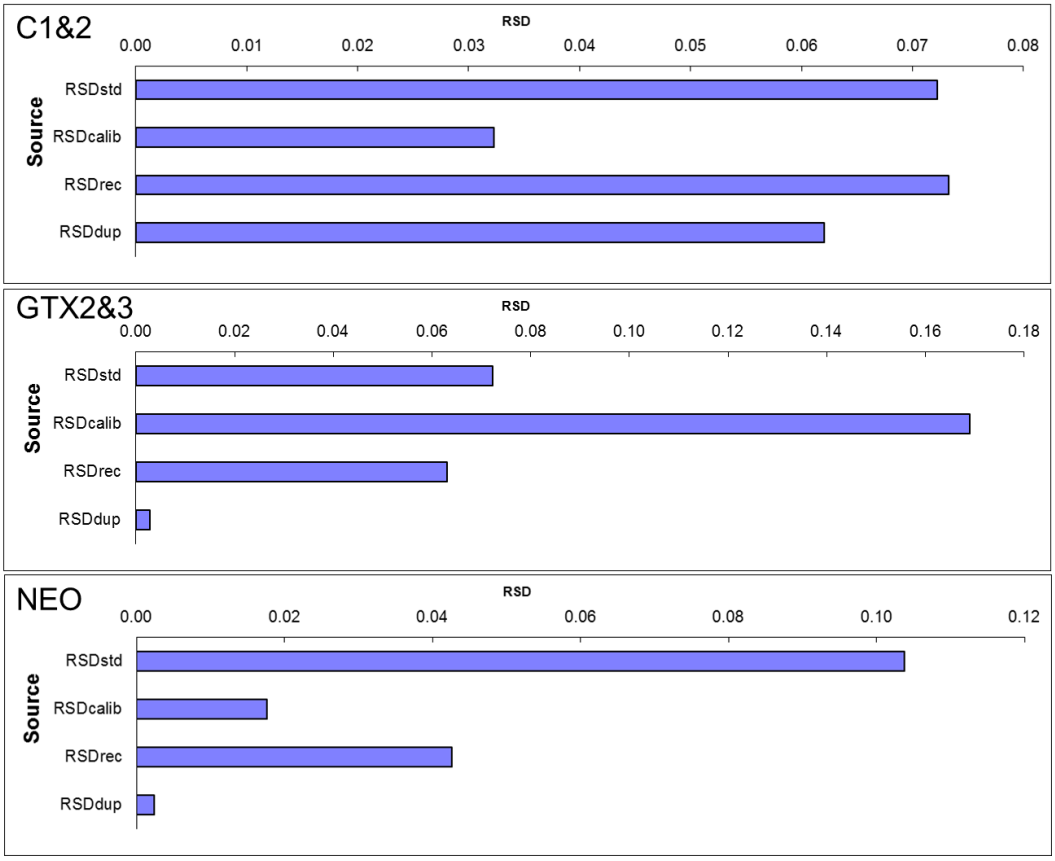


Figure 6-6 Measurement Uncertainty charts for PSTs

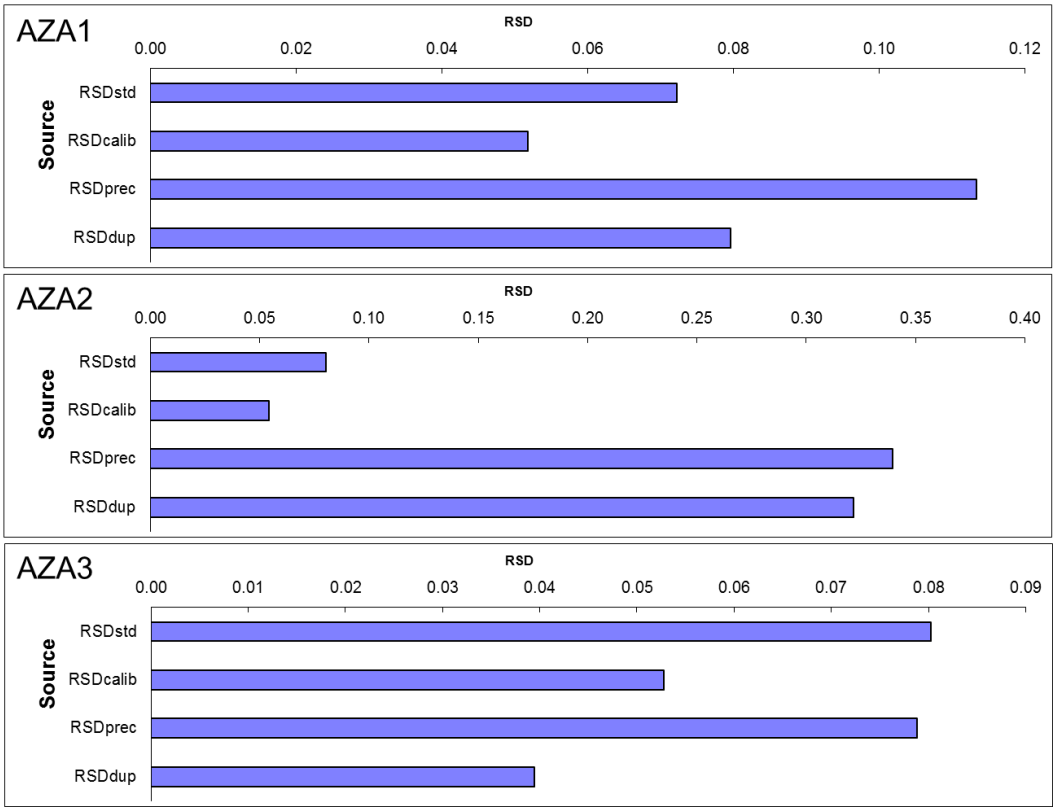


Figure 6-7 Measurement Uncertainty charts for AZAs

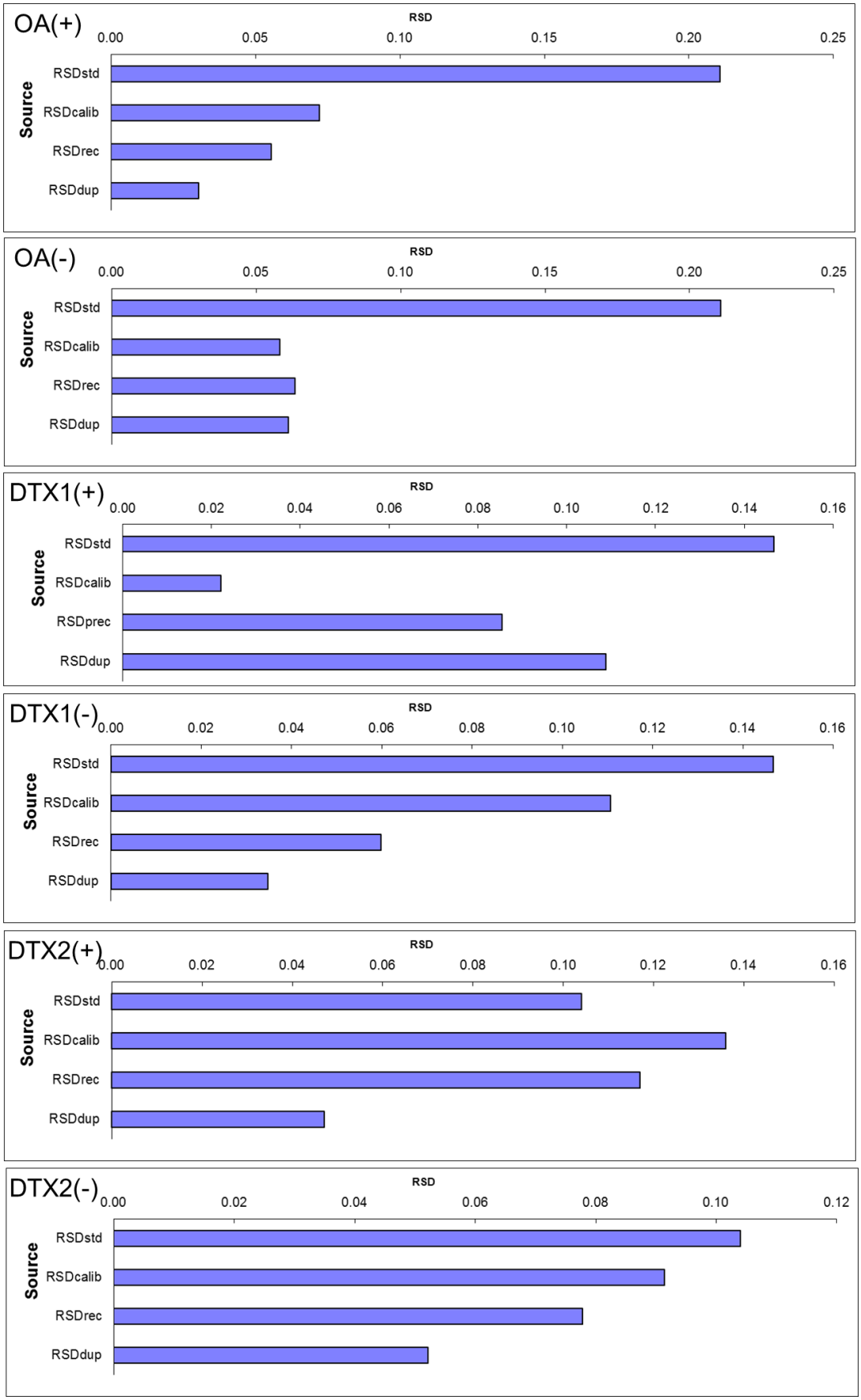


Figure 6-8 Measurement Uncertainty charts for DSTs

Overall, the Measurement Uncertainty for most toxins was nominally given 35% except for OA with 47%. The measurement uncertainties were considered satisfactory for this trace level work (Anstis P, Personal Communication, 9 March 2016). However, a 96% uncertainty was found for AZA2 (Figure 6-7), indicating that this method is not suitable for AZA2 analysis but could be used as a qualitative screen at a higher level. Closer examination of the data was able to show that the greatest source of error was associated with the purity of the reference standard (with an error of 10.5%) and the poor recovery data from the certified reference material. The high uncertainty associated with the standard may be explained by the fact that the biotoxin reference standards are produced through a complex procedure *via* purification of a mass culture of algae. At present, there is a limited source of reference materials and suppliers. This presents a large obstacle towards analytical methods and procedures for marine biotoxin determination. At this stage, the measurement uncertainties presented here demonstrates that the method is fit for monitoring purposes at the current regulatory limits.

6.3 Conclusion

A validation study was conducted to assess the performance characteristics of the developed method. Certified reference materials and mussel tissue spiked with chemical standards were used to assess characteristics such as linearity, LOD, LOQ, LOR, selectivity, accuracy, and repeatability (only for AZAs). It was found that linearity for the toxins were acceptable between 0.2 to 2 times the regulatory limits for PSTs, AST, DST, and AZA. Selectivity was shown by comparing results from the analysis of certified blank mussel tissue (CRM-Mus-Zero) and certified mussel tissues for the AST, DST, and AZA groups (CRM-DST-Mus and CRM-AZA-Mus). For PSTs, a naturally contaminated mussel tissue was analysed. The selectivity assessment showed that the FLD and MSMS detector was able to distinguish between samples without any biotoxins and samples containing biotoxins.

Measurement uncertainty was also estimated from four sources and was used to evaluate the uncertainties associated with the toxin measurement using the developed method. Analysis of the measurement uncertainty revealed that the largest sources were

from reference standard dilutions and also recoveries. The validation data show that the developed method is suitable for PSTs, DSTs, AST and AZA1 and AZA3. AZA2 was found to have a large associated measurement uncertainty (96%). Although the recoveries were low for AZA1, AZA3, DA, GTX2&3 and STX, these toxins had a standard deviation of <16%, indicating that the results were consistent.

7 CONCLUSIONS AND FUTURE WORK

7.1 Conclusions and Recommendations

A novel tandem LC-FLD-MSMS method capable of simultaneous analysis of lipophilic and hydrophilic marine biotoxins was developed and validated. The validation process has shown the technique is well suited for analysis of PSTs, DST, AST, and AZAs but would be subjected to high errors for AZA2 with a measurement uncertainty of 96% This high measurement uncertainty was due to uncertainty related to precision and duplicate analysis. Regardless, the analysis is able to combine several analyses which were performed separately into a single platform. The total time of analysis is 12.5 min for each sample. In addition, the burden on sample preparation is reduced by having a single method for the extraction and cleanup of the shellfish tissue.

A number of emerging columns were explored for their potential to improve separation performance and detection using mass spectrometry. The results of this comparison were not promising, with the conclusion that HILIC and ANP columns were found to be largely unsuitable for separation of the PSTs. Therefore, the new method would need to employ existing separation and detection technologies to achieve the aim of multiple toxin detection of PSTs together with other biotoxins. Derivatisation of the PSTs using periodate or peroxide oxidation agents produced fluorescent derivatives which can be

separated and detected *via* reversed phase chromatography and fluorescence detection. However, in the case of the other biotoxins such as AST, DSTs, AZAs and NSTs, RP chromatography coupled to MSMS detection was found to be suitable.

The simultaneous analysis of lipophilic and hydrophilic components was based on a similar concept used in the analysis of metabolites in cell cultures and was implemented and modified to suit five class of marine biotoxins, PSTs, AST, DSTs, AZAs and NSTs, with the possibility of incorporating new classes as they are discovered and regulated. The tandem LC-FLD-MSMS that was set up in this project incorporates a two phase analysis. In the first phase, the hydrophilic extract from the sample preparation procedure is injected into the system. After a short delay, the flow path of the system is changed via a switching valve and the second injection containing lipophilic toxins and AST is injected into the system. Fluorescence detection is used for the derivatised PSTs while the other toxins are detected using MSMS.

The developed method also incorporated an extraction protocol followed by a SPE cleanup for five marine biotoxin groups (PSTs, ASTs, DSTs, AZAs and NSTs). A method combining hydrophilic and lipophilic extraction with acetic acid solution and methanol was developed and tested. Polymeric SPE was chosen for the cleanup step, which also separated the biotoxins into two fractions, one containing PSTs and the other containing ASTs and lipophilic biotoxins. Preliminary validation using certified reference materials revealed that AZAs experienced significant matrix effects, which reduced the sensitivity. As a result, an additional sample dilution step was included to minimise matrix effects. This dilution step was shown to improve recoveries of AZAs by 33-89%. Preliminary validation also showed that the developed method was not suitable for brevetoxins as no recoveries for brevetoxins at regulatory limits were observed. Further investigation found that this was due to non instrumental factors, such as irreversible binding of toxins to matrix components or metabolic processes. Non-instrumental matrix factors cannot be overcome by dilution, unlike AZAs. Therefore, brevetoxins were excluded from the validation procedure.

Finally, the method was evaluated with several CRMs and LRMs. Certified Reference Material with a known amount of toxins were also tested using this method and recoveries were in the range of certified values. The method uncertainties determined for this method. The suitability of this method was evaluated using reference standards and toxin spiked recoveries. The LODs and LORs were determined. Selectivity was determined by comparing mussel samples known to be free of toxins and certified reference materials for biotoxins.

The research recommend that each batch of samples should be analysed with a combination of quality control steps including solvent blanks, matrix blanks, internal standards, toxin-free mussel tissue spiked with known concentrations of selected biotoxins in replicate, CRMs when available and calibration standards made out using appropriate solvents. Batches of samples with recoveries falling outside 80-120% of expected values but with a relative standard deviations of <15% are recommended to be reported after correcting for recoveries. This study has shown that compounds including GTX2&3, DA, AZA1, AZA2 and AZA3 should be reported with recovery corrections based on the recovery each analytical batch.

Overall, the new approach developed here has opened a new field of exploration for the analysis of marine biotoxins and presents a roadmap that may be suitable to other systems that would benefit from the simultaneous analysis of compounds with a large range of polarities such as clinical, environmental or industrial analytical scenarios. Furthermore, the reduced time required for the analysis of samples by this new approach will be of significance in Australia, and internationally, to facilitate a rapid response to serious health issues as they are occurring.

7.2 Future Work

While this work has opened a new field of analysis, there is further work that should be performed to ensure the ongoing relevance of the technique. The method uncertainty has highlighted that the main sources of uncertainty in the current technique comes from the reference standards used. In order to reduce uncertainty from this source, the purity

of the standards should be considered and also aspects such as uncertainty related to recovery.

While the analysis has proven successful for a range of compounds, brevetoxins have proven to be difficult to analyse as a result of matrix effects. This has been highlighted by other studies and researchers are yet to find a solution. For brevetoxins, further sample preparation steps may be required, such as contact with organic solvents such as hexane to remove fat soluble components. However, the removal of fat soluble components from a sample may also remove lipophilic toxins, so care must be taken to ensure that any additional steps in sample preparation will not affect the other lipophilic or hydrophilic toxins within a sample.

There is a great need to pursue this area of research due to the increasing pressures of aquaculture and changing climates, which affect the duration and intensity of algal blooms. There is a need to have a sensitive and selective analytical tool with a broad spectrum of toxins analysed. Currently available methods only screen for single classes of toxins and therefore for a complete picture require multiple analyses.

Further optimisation of this method can be explored in terms of investigating different column selectivities, different separation conditions, and changes in sample preparation approaches to improve recoveries. Further developments in HPLC technology will enable broader application of this approach of marine biotoxin analysis. Technological advances such as higher pressure systems such as UHPLC will enable a faster runtime, as well as increased sensitivity due to higher peak heights or lower flow into MS source, which lowers desolvation load for the source. Other advances which may open up new avenues of exploration, such as the proliferation of sensitive detectors such as OrbiTraps and ion mobility MS (Poyer *et al.*, 2013). High resolution QTOF data can be used to create a spectral library for confirmation of compounds which are detected by MS. In some circles, direct injection of samples into detectors has been thought to hold promise for this field of analysis, but the challenges associated with sample cleanliness and the similarity of toxin analogs remain.

New stationary phase chemistries with novel separation mechanisms may provide promising results in the context of marine biotoxin analysis. Some of the ones described below are not yet commercially available, but it will be interesting to see what improvements they offer to marine biotoxin analysis. Emerging stationary phase chemistries which have been described in literature are monolithic columns, hybrid core shell silica embedded with carbon nanoparticles, dendritic polymer-modified silica, and carboxylate-modified porous graphitic carbon. Monolithic columns are polymeric rods which are used as stationary phases. They are tolerant of high pH conditions, which may improve the chromatography of lipophilic and hydrophilic toxins (Gama *et al.*, 2012; Jandera *et al.*, 2012). Hybrid core shell silica embedded with carbon nanoparticles have recently been described by Ibrahim *et al.* (2014), which has been shown to have selectivity for positional isomeric pairs. Application of this stationary phase to marine biotoxin analysis may improve separation of the isomeric analogs such as GTX toxins, C toxins, and domoic acid isomers. Dendritic polymer-modified silica (Li *et al.*, 2014), which has shown to have a mixed-mode exchange mechanism may potentially improve separation of a broad range of analytes, especially relevant to analytes with a range of net charges such as PSTs. Finally, carboxylate-modified porous graphitic carbon has been reported to have a high electrostatic and hydrophilic character, and a separation mechanism which can be adjusted through modifying the pH of mobile phase (Wahab *et al.*, 2013).

As stated earlier, the tandem LC approach used here can act as a road map to the development of other techniques that require the analysis of compounds with a large range of polarities. The flexibility with regards to columns and detectors within the technique described in this work would make it eminently suitable to adaptation to other fields. One particular area of growing concern would be pesticide residues analysis in food, where a large number of analytes are required to be extracted and analysed. In recent years, the number of pesticides which are of concern are increasing. The tandem LC method may be applied to this area to increase the number of compounds analysed and also expand the types of samples that can be analysed in one method. By utilizing the approach taken in the development of this technique, it would be possible to simplify techniques and improve analysis times across a range of pesticide residues analysis and other analytical contexts.

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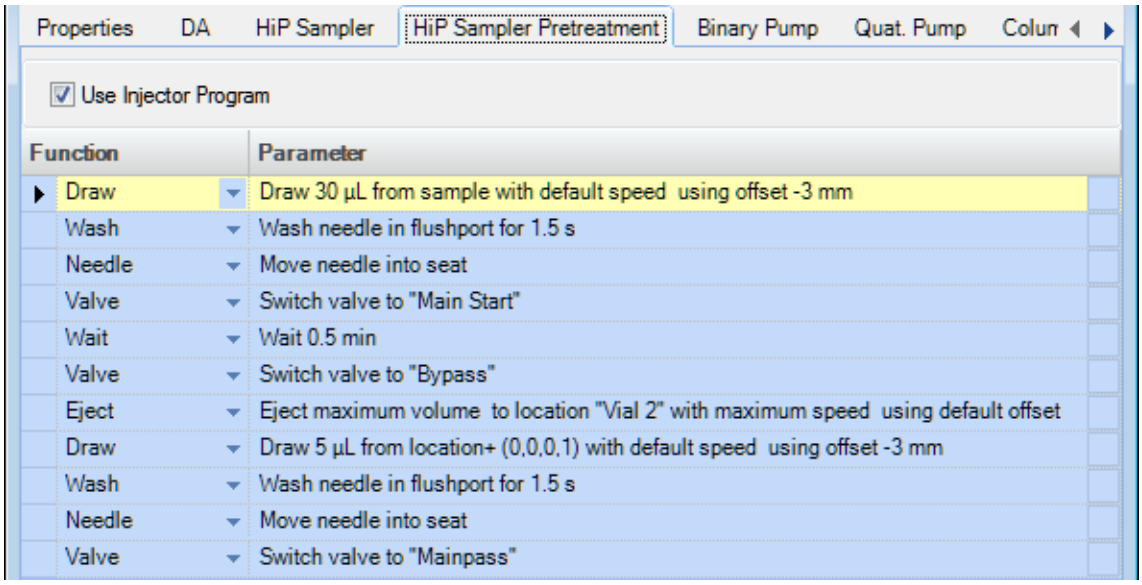
APPENDICES

A. SAMPLE INJECTION PROGRAM FOR TANDEM LC-FLD-MSMS



A 1 Injector program implemented for tandem LC-FLD-MSMS

Figure A 1 shows the injector program that was used for the final tandem LC-FLD-MSMS. In the first phase, 30 µL of sample was drawn from the vial containing the oxidised hydrophilic extract which is then introduced to the chromatographic column. Then, the system waits for 30 sec before resetting the sample draw piston, ejecting the loop contents in an empty vial on the sample tray (Vial 2). Following that, 5 µL of sample is drawn from the adjacent vial, containing the lipophilic extracts. This is then introduced into the chromatograpic column for the lipophilic toxins.

B. SUMMARISED EXTRACTION METHOD FROM AOAC (2006) AND CLEANUP METHOD FROM HARWOOD *ET AL.* (2013)

For the extraction step, 2.0 g of homogenised shellfish tissue was transferred to a 50 mL polypropylene (PP) certrifuge tube. Then 3 mL of 1% acetic acid solution was added to the homogenate before mixing with a vortex. Then, the sample was heated to 100 °C in a water bath for 5 min and centrifuged for 10 min at 3600 × *g*. The supernatant was decanted into a new PP tube and the extraction was repeated without the heating step. The supernatants were combined and was then made up to 10 mL with deionised water.

For the cleanup step, Strata-X SPE cartridge (500 mg, 3 ml) was loaded onto a vacuum manifold. This was first conditioned with 6 mL of methanol followed by 6 mL of water. 1 mL of the shellfish extract was passed through the cartridge before washing with 2 mL of water. The effluent is collected in a 15 mL PP tube and is adjusted to pH 6.5 with 0.2 M NaOH, confirmed using pH indicator paper. The extract was then made up to 4 mL with water.

The SPE cleaned extract can then be oxidised by either periodate or peroxide oxidation before injection into the HPLC system. For periodate oxidation, 500 µL of periodate oxidant was dispensed into a 2 mL HPLC vial followed by 100 µL of the SPE cleaned shellfish extract. The vial was mixed well and left to react for 1 min before addition of 5 µL acetic acid. The AOAC method calls for addition of a matrix modifier solution made from Pacific oysters, but omission of the matrix modifier for periodate oxidation has been justified by experiments showing no adverse effects when it was omitted (Harwood *et al.*, 2013; Turner A.D., Personal communication, 24 September, 2015).

For peroxide oxidation, 250 µL of 1 M NaOH was dispensed into a 2 mL HPLC vial together with 25 µL of 10% hydrogen peroxide solution, followed by 100 µL of SPE cleaned shellfish extract. The vial was mixed well and left to react for 2 min before addition of 20 µL acetic acid.

C. SUMMARISED EXTRACTION AND CLEANUP METHOD FOR LIPOPHILIC BIOTOXINS (GERSSEN *ET AL.*, 2009A)

2 g of homogenised shellfish tissue was extracted in triplicate with 6 mL of methanol. After each addition of methanol, the extract was vortex mixed for 1 min, after which the extract was centrifuged for 5 min at 2000 × g. The supernatants were combined in a 20 mL volumetric flask and made up to 20 mL with methanol. The crude extract was filtered through a 0.2 um membrane filter. The crude extract is diluted in a new tube by adding 2.8 mL of deionised water to 1.2 mL of crude extract.

For extract cleanup, a Strata-X SPE cartridge (30 mg, 1 mL) was mounted on a vacuum manifold. The first conditioning solvent was 1 mL of methanol. Then, the cartridge was conditioned with 1 mL of 30% methanol. The diluted crude extract was applied to the cartridge. The cartridge was then washed with 1 mL 20% methanol. Finally, the lipophilic toxins were eluted from the cartridge using 1.2 mL of methanol containing 0.3 vol% of ammonium hydroxide. The purified extract is then transferred to a HPLC vial for analysis.

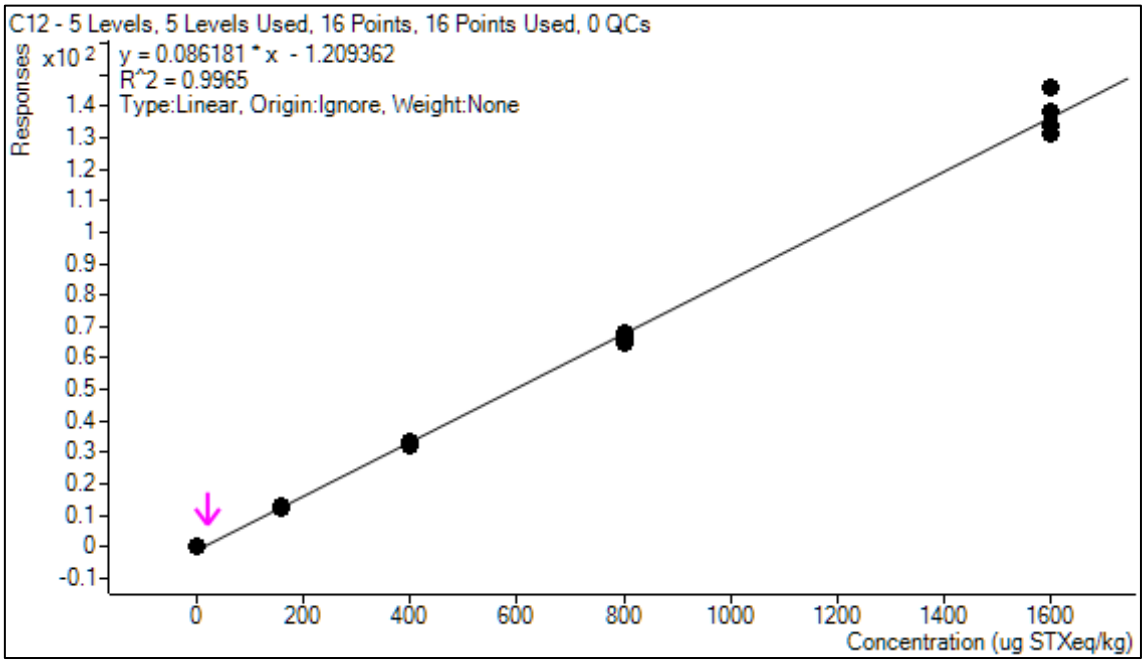
D. SUMMARISED EXTRACTION AND CLEANUP METHOD FOR BREVETOXINS (MCNABB *ET AL.,* 2012)

Shellfish to be analysed were first cleaned with a stiff brush. They were then shucked and drained on a sieve for 5 min. Then the tissues were homogenised with a blender.

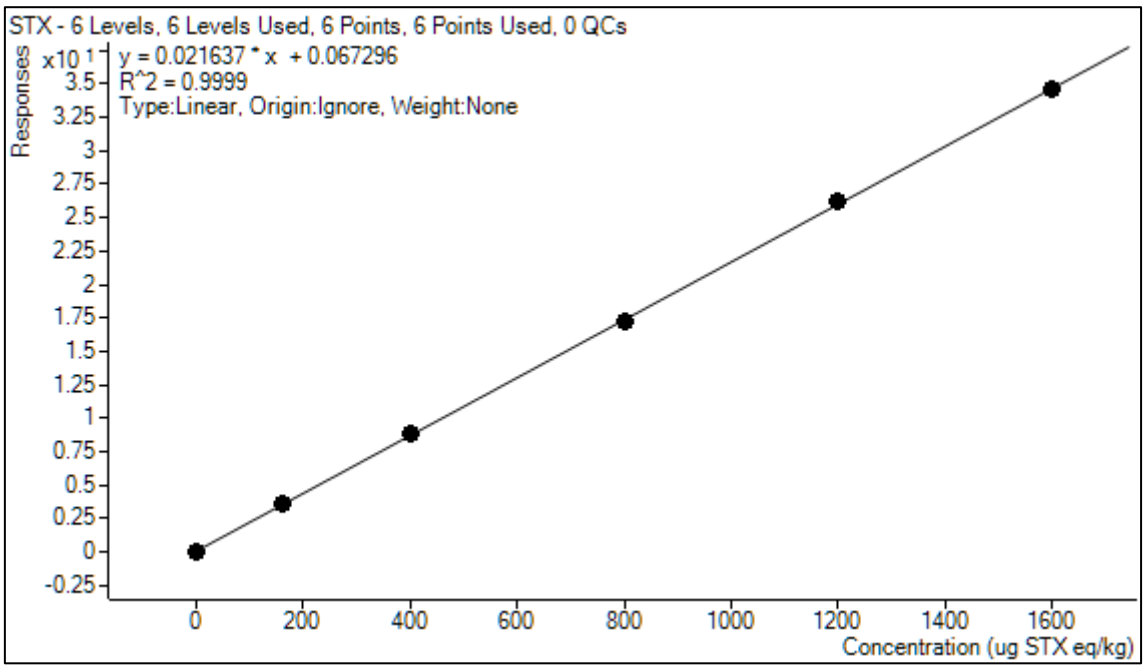
For extraction, 2.0 g of homogenised shellfish tissue were transferred into a 50 mL graduated polyethylene tube. Then, 9 mL of 80% methanol solution was added to the tube and the contents were mixed with a vortex mixer. Samples were then heated in a water bath at 60 °C for 20 min. Following that, samples were cooled in an ice bath and certrifuged until a firm pellet formed (3000 × *g* for 10 min). The supernatant was decanted into a clean 50 mL tube, and the extraction process was repeated. The extracts were combined and made up to the 20 mL marke with deionised water. Then, 8 mL of this extract was transferred into a clean 50 mL tube and 10 mL of *n*-hexane was added. After gentle mixing, the sample was centrifuged until the phases separated (3000 × *g* for 5-10 min). The hexane layer was removed by aspiration, and 5 mL of the bottom methanol layer was transferred into a clean 50 mL tube. The methanol fraction is diluted by adding 15 mL of deionised water before loading onto the SPE cartridge.

The Strata-X SPE cartridge (60 mg, 3 mL) was mounted on a vacuum manifold and conditioned with 3 mL of 100% methanol followed by 3 mL of 25% methanol. The diluted methanol extract is loaded onto the cartridge. The SPE cartridge is then rinsed with 4.5 mL of 25% methanol which was eluted to waste. Brevetoxins were eluted with 4.5 mL of 100% methanol. The cleaned up extract was made up to 5 mL with water and 1 mL of this was transferred to an autosampler vial for analysis.

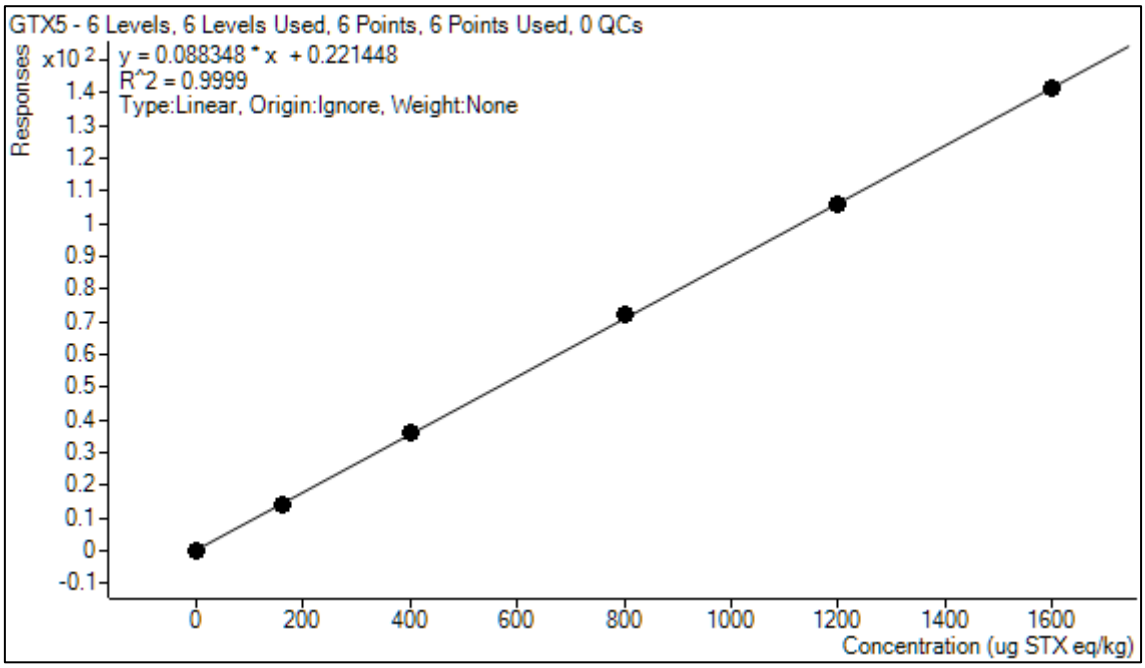
E. LINEARITY CHARTS FOR BIOTOXINS



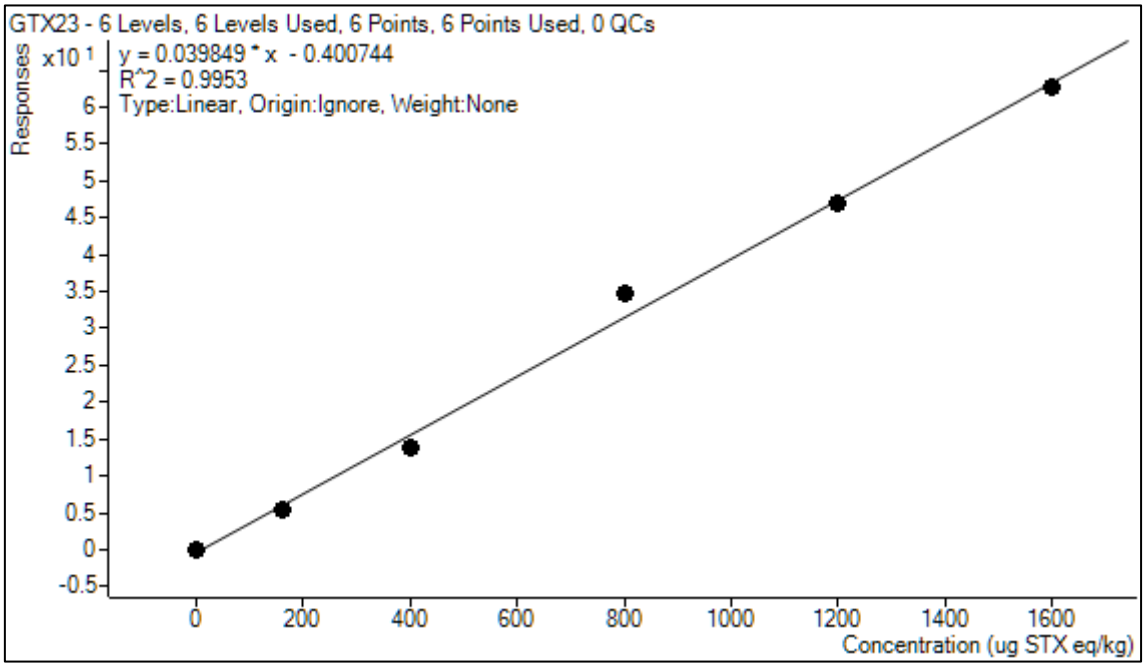
E 1 Linearity plot for C1&2



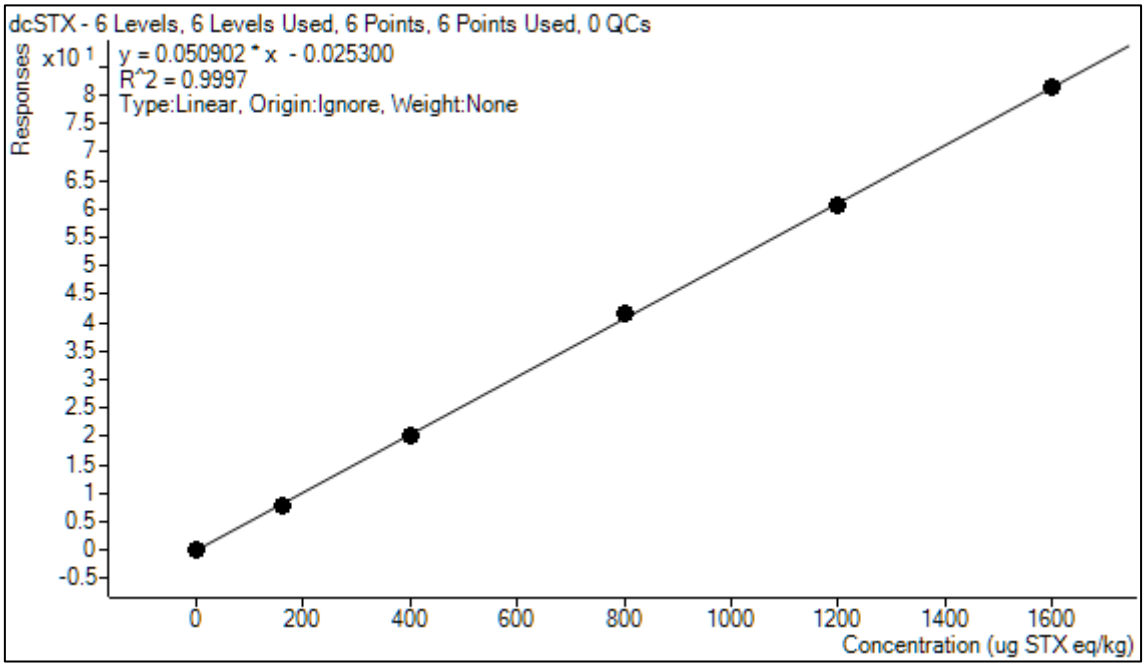
E 2 Linearity plot for STX



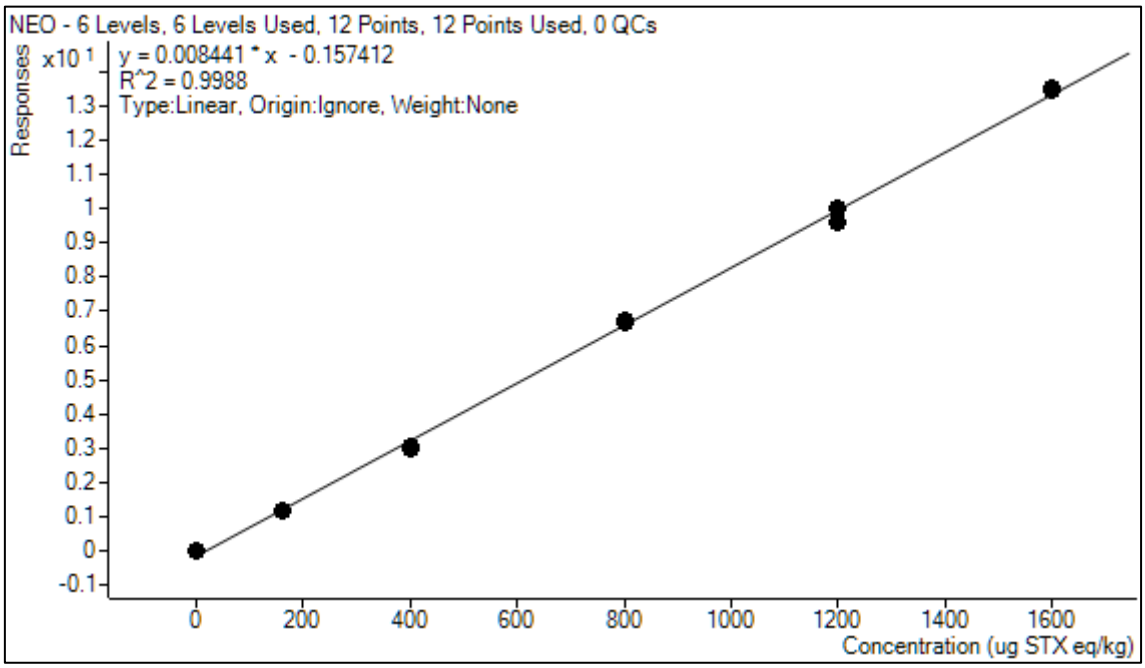
E 3 Linearity plot for GTX5



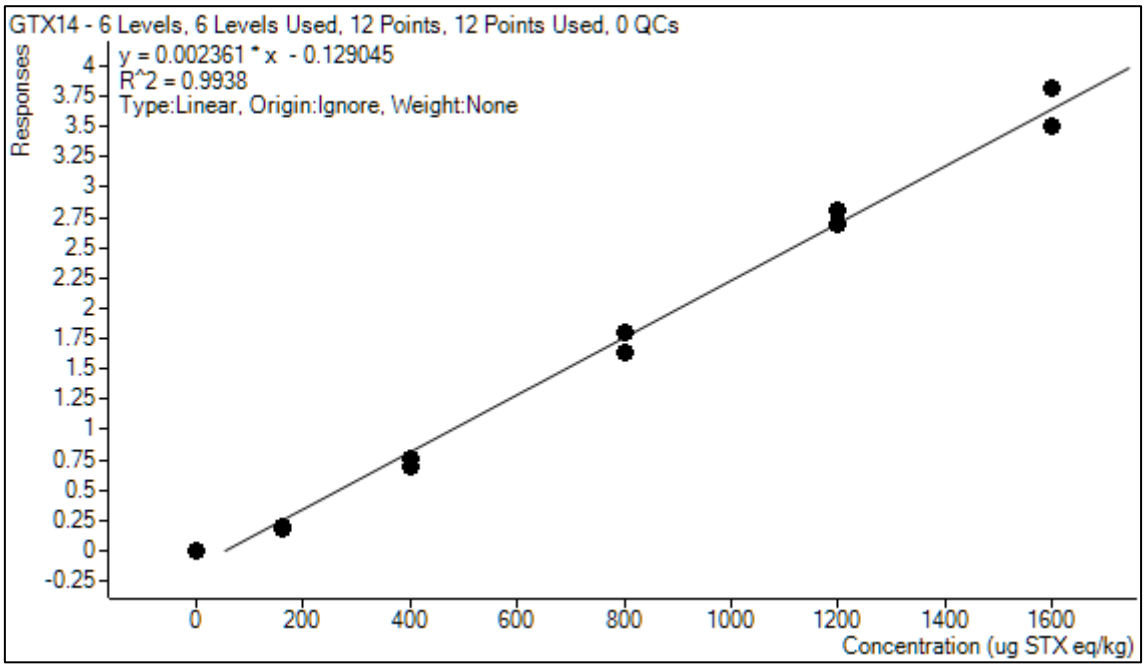
E 4 Linearity plot for GTX2&3



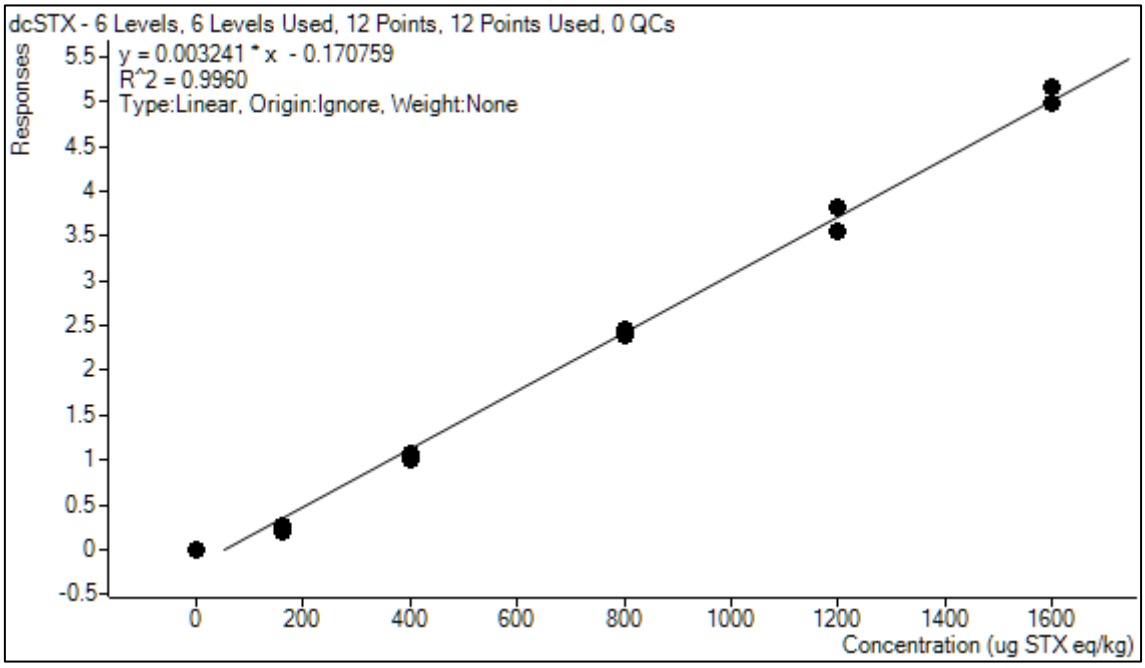
E 5 Linearity plot for dcSTX



E 6 Linearity plot for NEO



E 7 Linearity plot for GTX1&4



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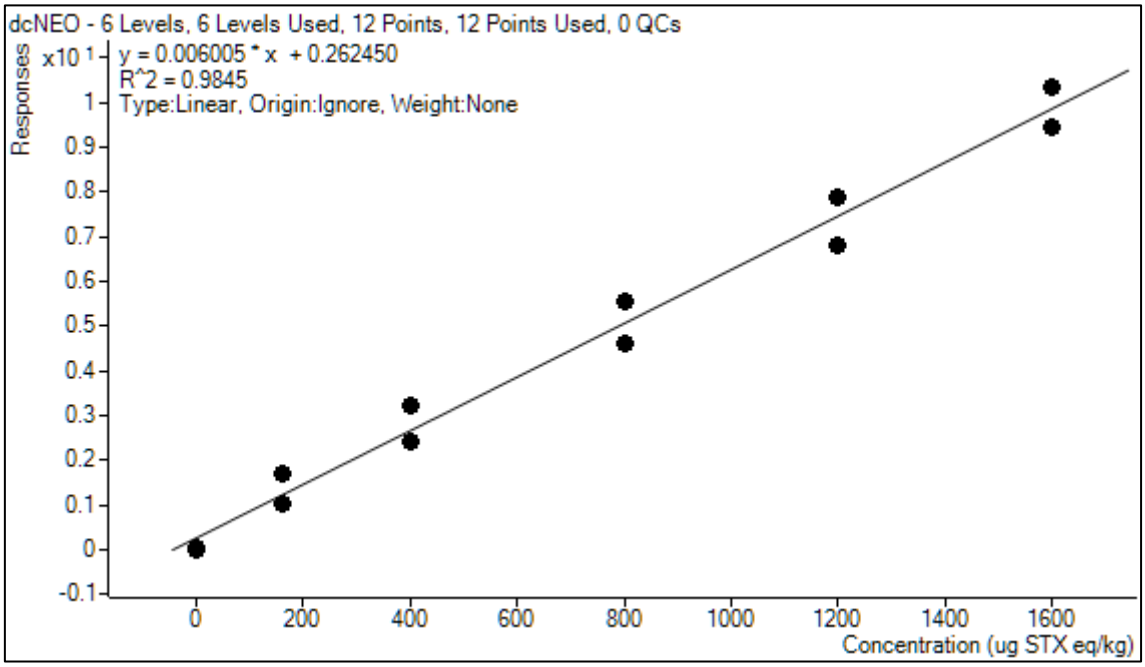
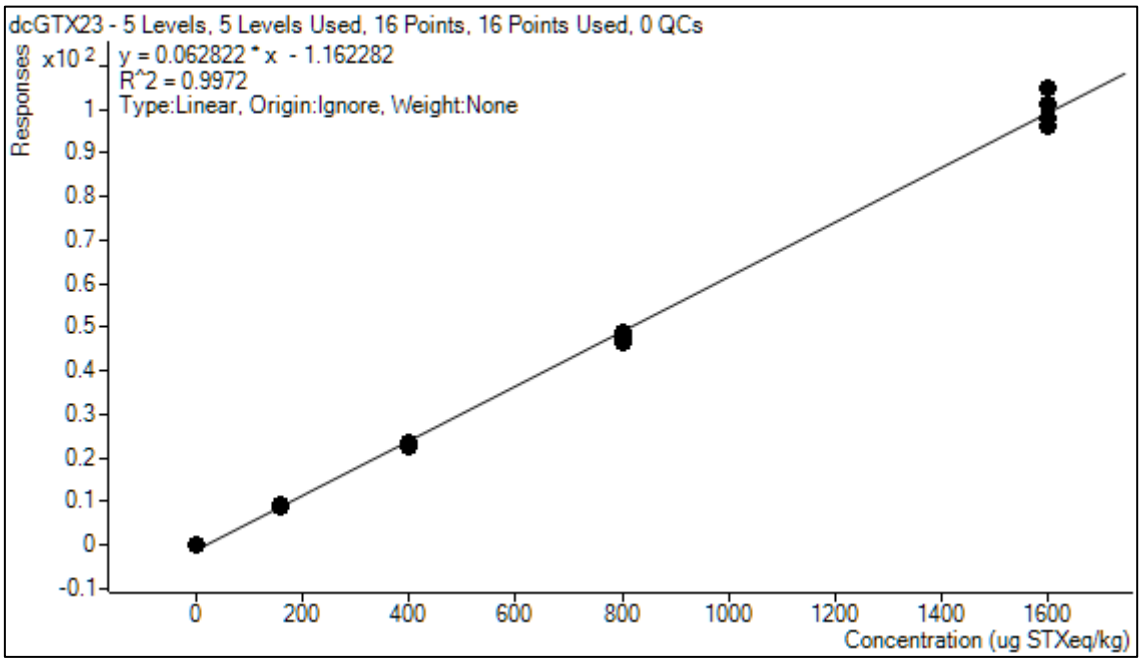
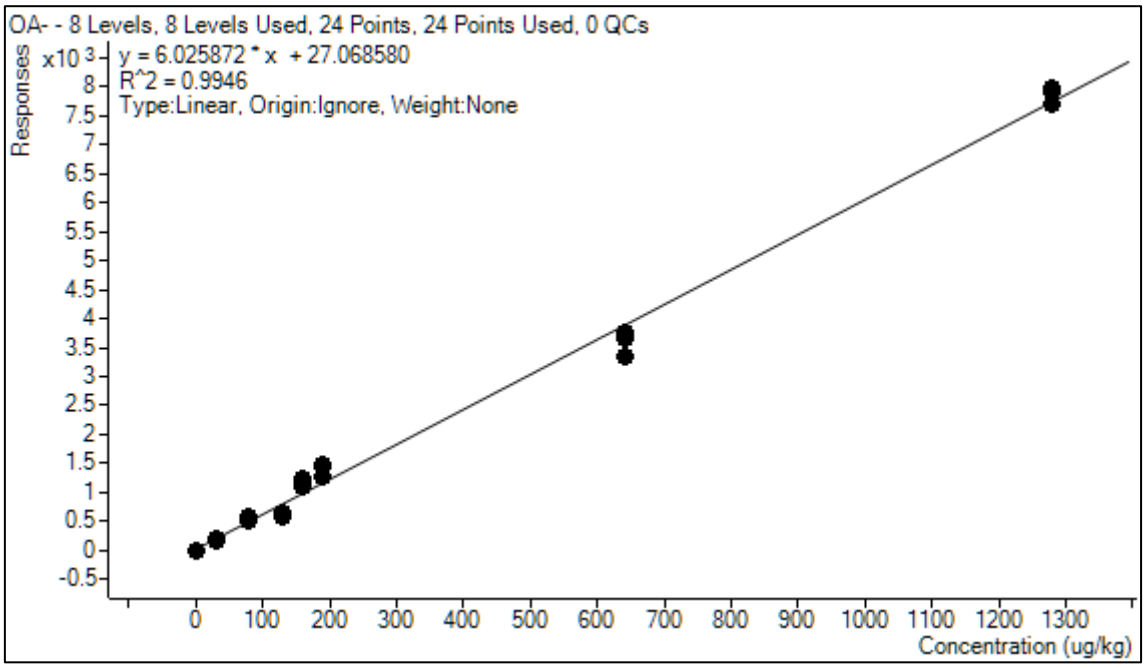


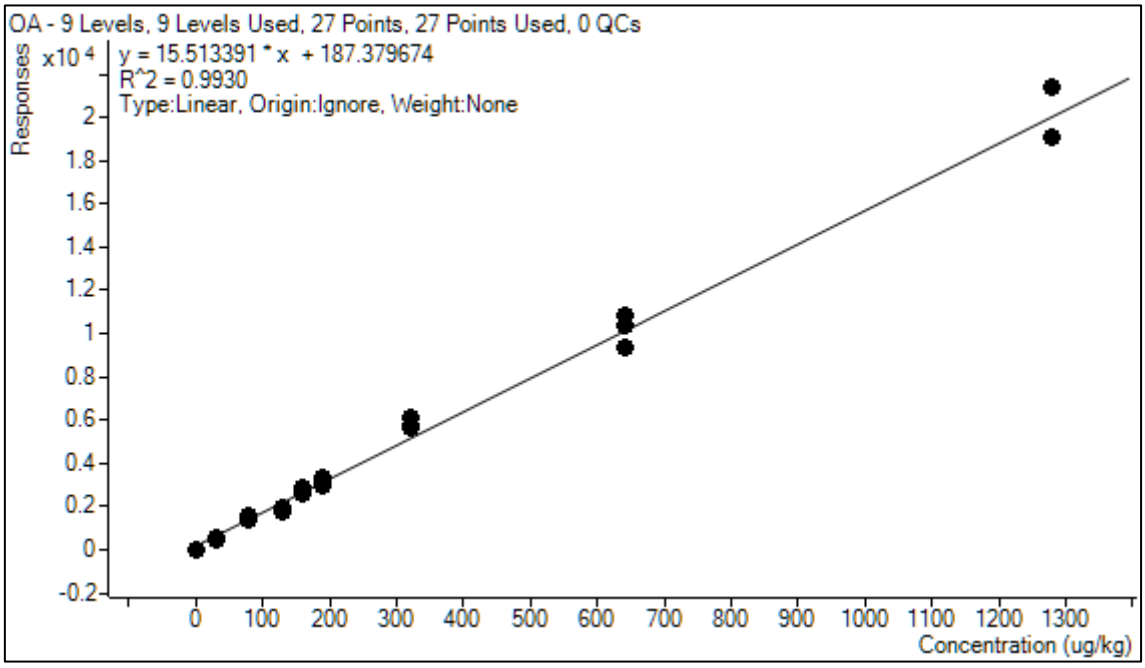
E 9 Linearity plot for dcNEO



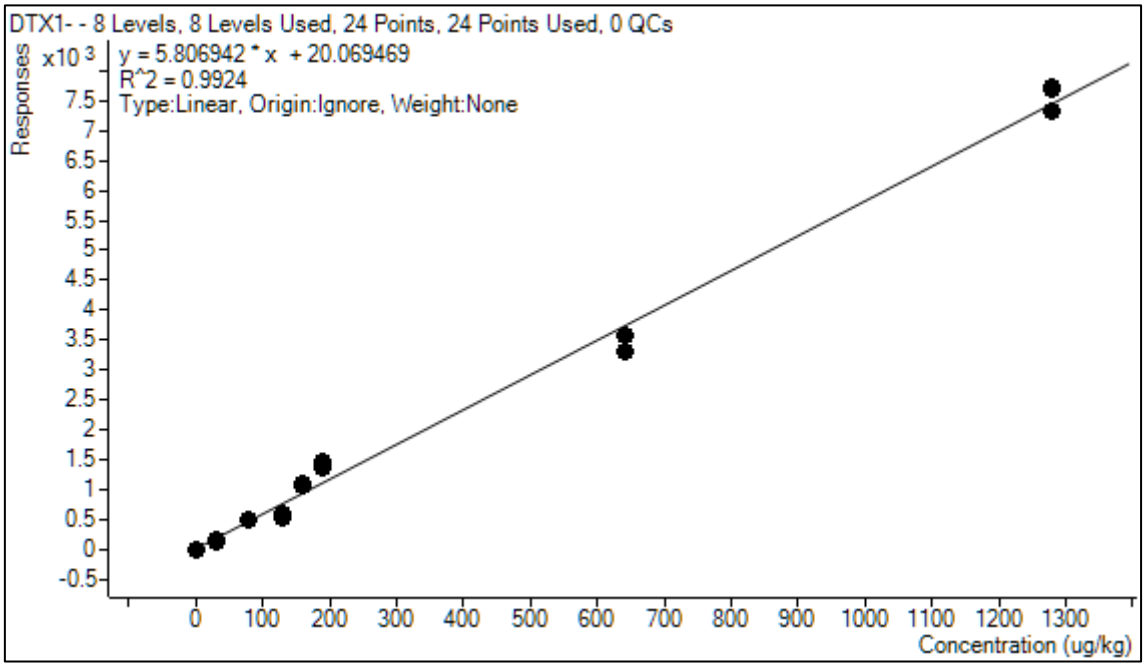
E 10 Linearity plot for dcGTX2&3



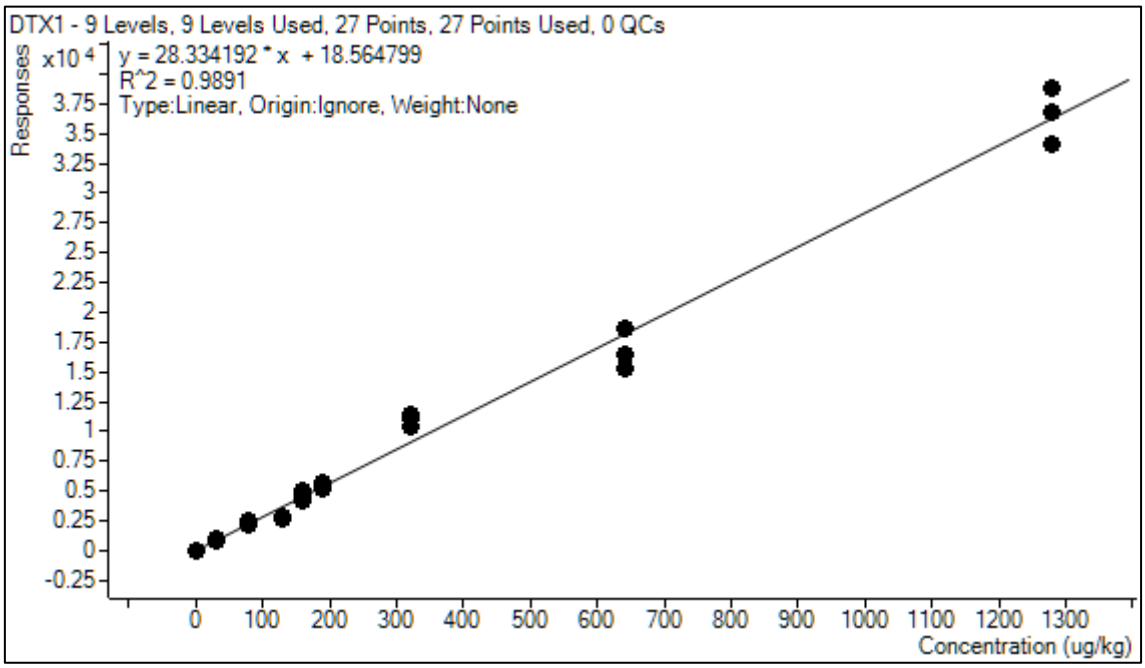
E 11 Linearity plot for OA (negative)



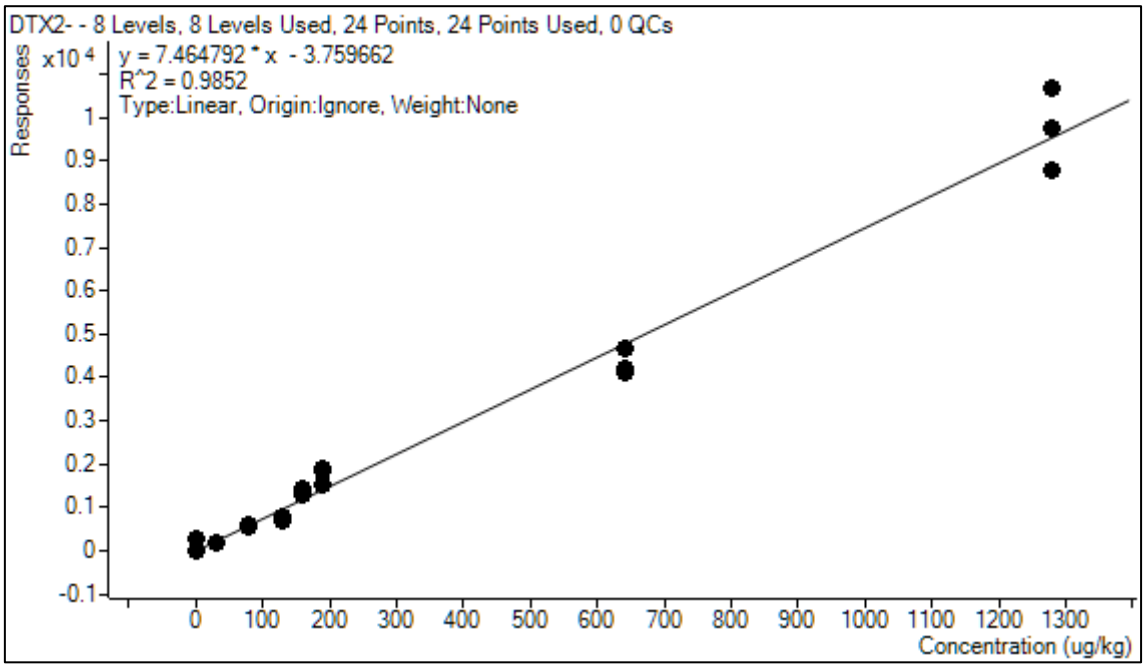
E 12 Linearity plot for OA (positive)



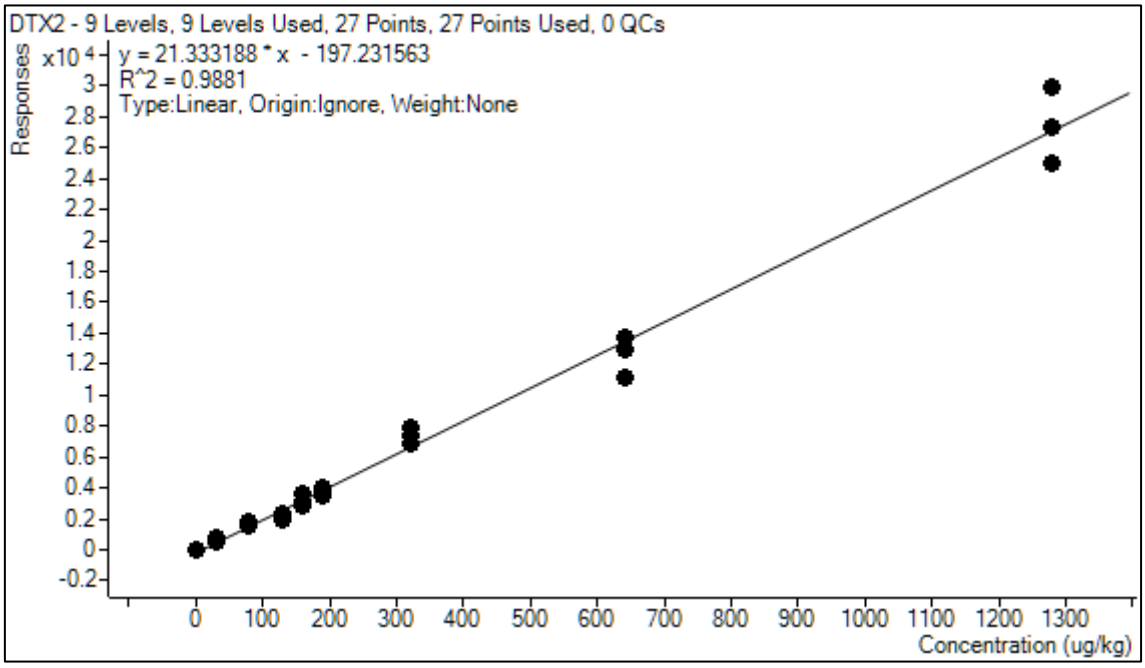
E 13 Linearity plot for DTX1 (negative)



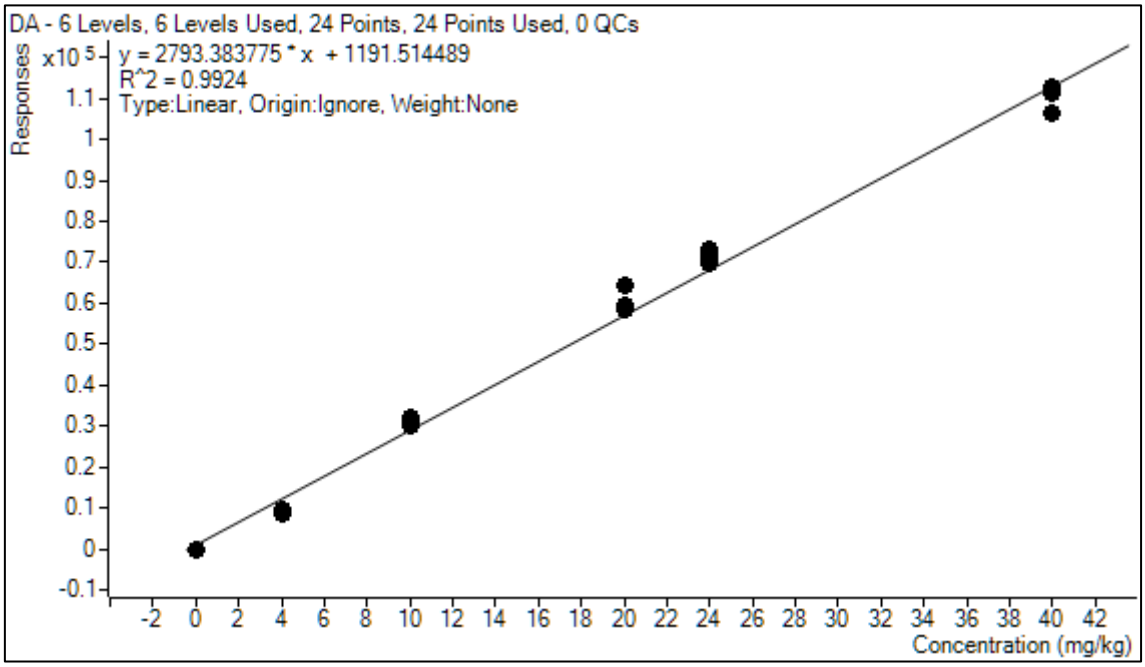
E 14 Linearity plot for DTX1 (positive)



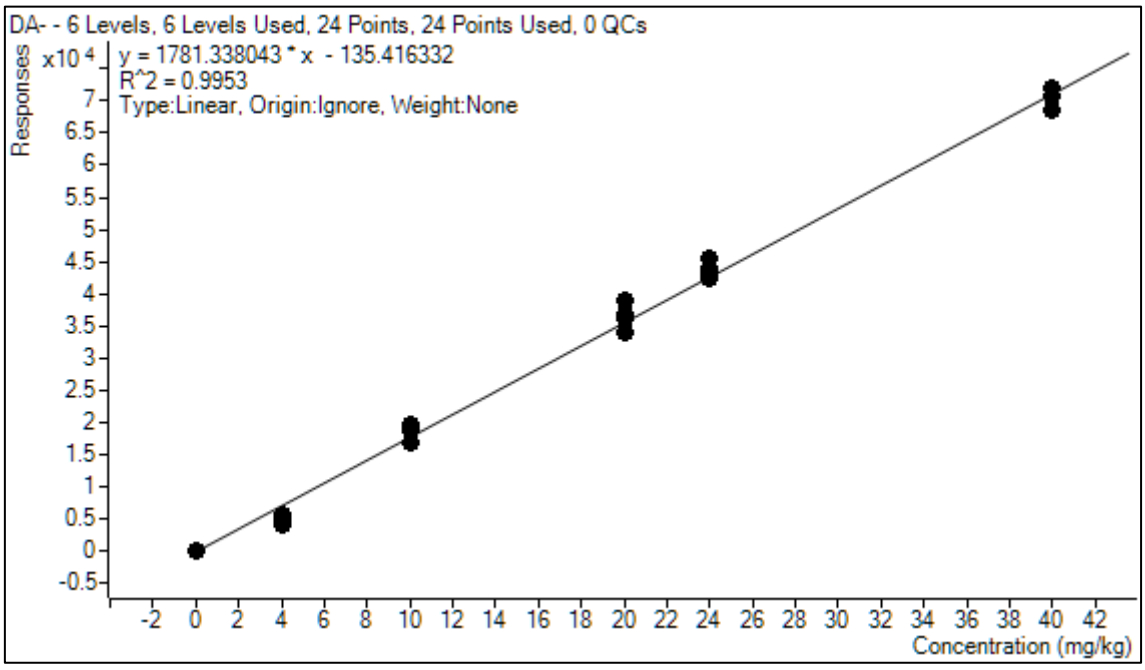
E 15 Linearity plot for DTX2 (negative)



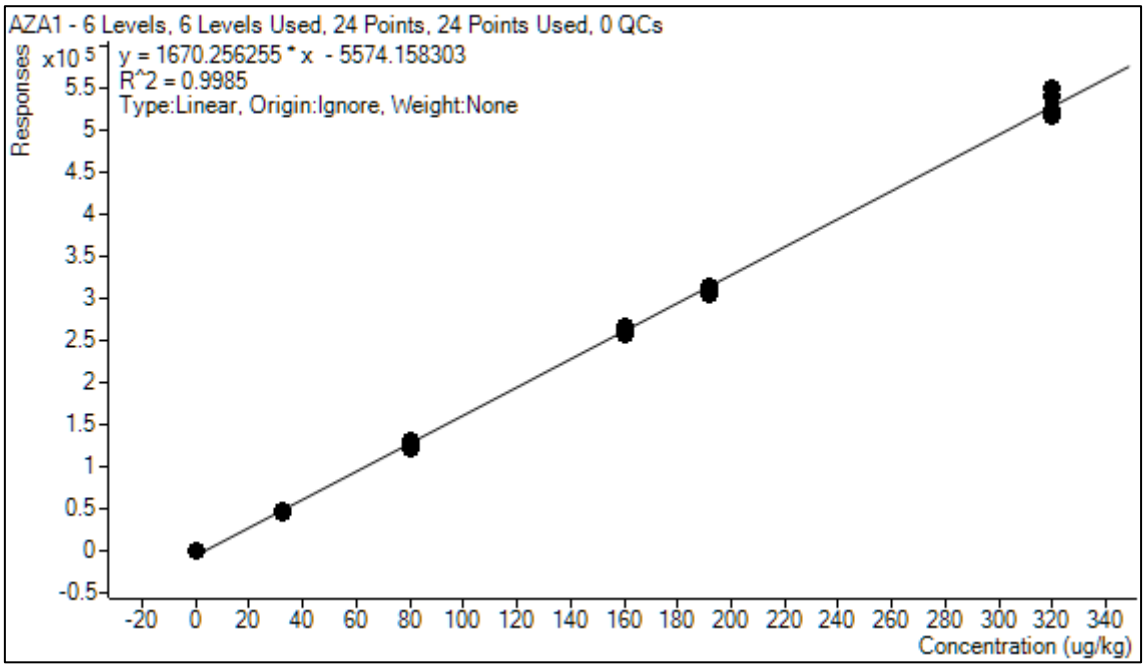
E 16 Linearity plot for DTX2 (positive)



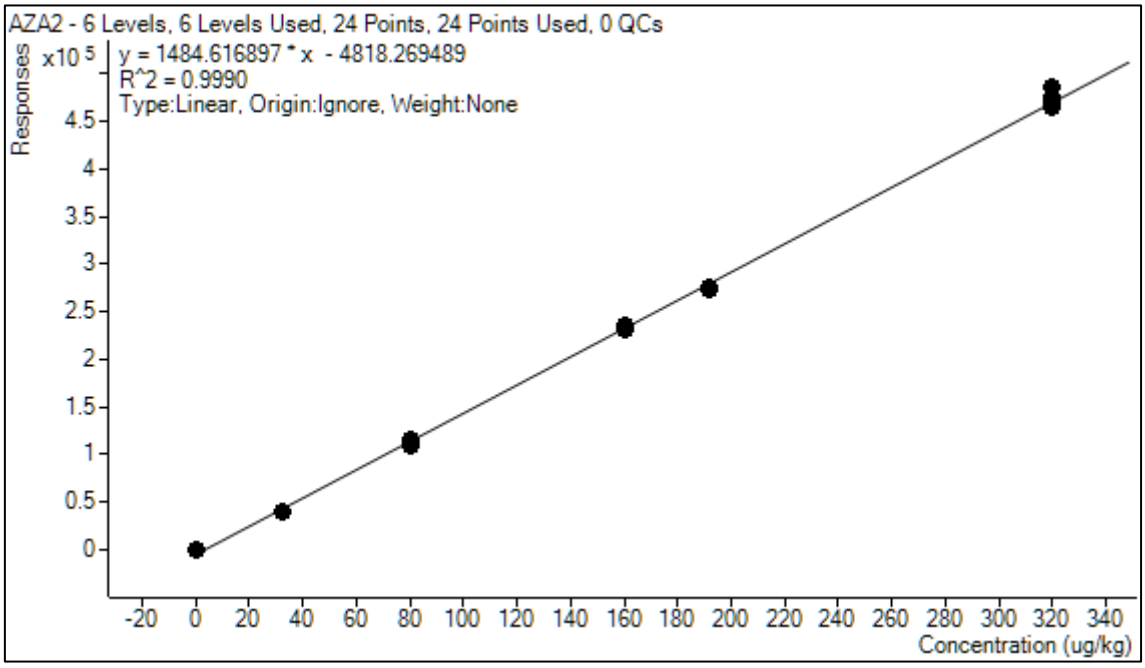
E 17 Linearity plot for DA (positive)



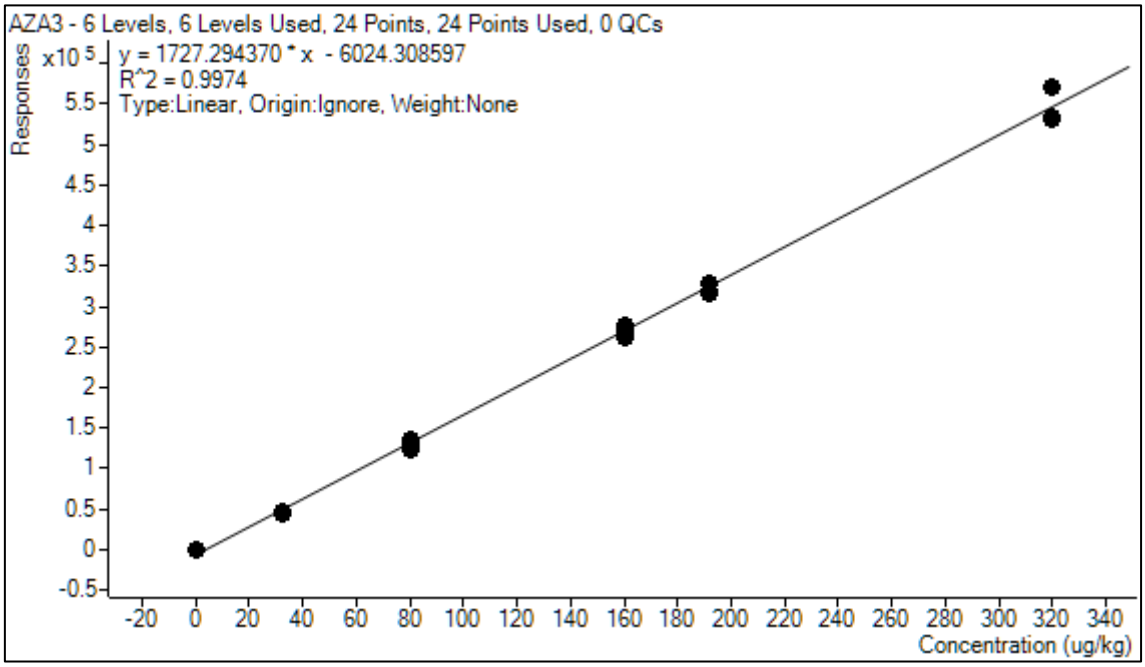
E 18 Linearity plot for DA (negative)



E 19 Linearity plot for AZA1



E 20 Linearity plot for AZA2



E 21 Linearity plot for AZA3