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Matrix effects, Development of Clean-up and LC-techniques Contributing Towards a Reference LCMS Method for the Analysis of Lipophilic Marine Toxins

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Matrix effects, development of clean-up and LC- techniques contributing towards a reference LCMS method for the analysis of lipophilic marine toxins

A Thesis submitted to Dublin Institute of Technology, Kevin Street for the Degree of M.Phil.
by

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Declaration

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This thesis was prepared according to the regulations for postgraduate study by research of the Dublin Institute of Technology and has not been submitted in whole or in part for an award in any other Institute or University.

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Abbreviations

ASP	amnesic shellfish poisoning
AZA	azaspiracid
AZP	azaspiracid poisoning
CRL	community reference laboratory
CRM	certified reference material
CV	coefficient of variation
DA	domoic acid
DAD	diode array detection
DI	de-ionised
DTX	dinophysistoxin
DSP	diarrhetic shellfish poisoning
ELISA	enzyme linked immuno sorbent assay
EU	European Union
FAO	Food and Agricultural Organisation
HABs	harmful algal blooms
HP	hepatopancreas
HPLC	high performance liquid chromatography
IMARES	institute for Marine Resources and Ecosystem Studies
LCMS	liquid chromatography - mass spectrometry
LLE	liquid-liquid partitioning
LOD	limit of detection
LOQ	limit of quantification
LRM	laboratory reference material
MBA	mouse bioassay
MI	Marine Institute
MIN	minute
MeOH	methanol
MSS	matrix strength standards
MW	Molecular weight
NRL	National Reference Laboratory
NSVS	Norwegian School of Veterinary Science
NSP	neurotoxin shellfish poisoning
NVI	National Veterinary Institute of Norway
OA	okadaic acid
PDA	photo diode array
PSP	paralytic shellfish poisoning
PTX	pectenotoxin
QC	quality control
QUASIMEME	Quality Assurance of Information for Marine Environmental Monitoring in Europe
Q-ToF	quadrupole time-of-flight
RSD	relative standard deviation
SD	standard deviation

SIM	single ion monitoring
SOP	standard operating procedure
SPE	solid phase extraction
SRM	selective reaction monitoring
SSR	solvent to sample ratio
STX	saxitoxin
TIC	total ion count
TSQ	triple stage quadrupole
UV	ultra violet
WP	work package
WS	working standard
YTX	yessotoxin

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Abstract

The most widely used reference method in Europe for the detection and monitoring of lipophilic marine toxins is the Mouse Bioassay (MBA) as first described by Yasumoto *et al.*, (1978). The MBA offers a good level of protection to human health and is capable of detecting the overall toxicity of previously known toxins. However, there are drawbacks associated with the use of the MBA as it is both expensive and time consuming and can give false positives due to interferences (Suzuki *et al,* 1996). The problems associated with the original mouse bioassay have led to several modifications (Yasumoto *et al*, 1984; Lee *et al*, 1987).

Recent EU Regulations 853/2004, 854/2004, 2074/2005 and 1664/2006 (Anon 2004a; Anon 2004b; Anon 2005a and Anon 2006a) set out details of which toxins should be monitored and the corresponding regulatory limits and methods to be used. They also permit the use of alternative methods, provided they are fully validated and can offer at least an equivalent level of protection for human health.

LCMS is emerging as one of the most promising analytical methods available for the analysis of marine toxins. However, none of the available LCMS based methods are fully validated for all of the regulated toxin groups (OA, DTXs, YTX, PTX and AZAs) and therefore the replacement of the MBA as a reference method is not yet feasible (Hess *et al,* 2006). This study focuses on particular aspects in the development of LCMS methodology and was carried out as part of an EU funded project called BIOTOX which was brought about to develop and validate alternative methods to the MBA.

Several aspects of the LC method were examined including, the column type and gradient elution conditions. The columns put forward were examined using: resolution between components of the mixture and the theoretical plate model of chromatography (plate number (N) and plate height (H)). The BDS Hypersil C8 emerged as the column to be advised for the majority of the lipophilic toxins included in the regulations (OA, DTX1, DTX2, PTX2,

AZA1, AZA2 and AZA3). An additional LC method was developed using a basic mobile phase to include the detection of YTX.

A study examining the MS conditions (ionisation mode, acquisition mode and number of transitions) showed that the choice of MS conditions plays a significant role in the results obtained. It was found that the analysis of the OA toxin group in negative ionisation mode gave more accurate results. The choice of acquisition mode for OA was not found to cause a significant variability in results. For AZAs and PTX2 (analysed in positive ionisation mode only) the choice of acquisition mode was important; parent ion monitoring was shown to give the most variable results compared to single and double transition monitoring.

Oyster and Scallop tissue were used to examine trends of matrix effects in shellfish extracts. Similar trends were found between two different MS detectors (Quadrupole Time of Flight and Triple Stage Quadruple) of the same manufacturer and equipped with identical ionisation sources. Ion suppression effects were observed for AZA1 (up to 15%) and ion enhancement effects were observed for OA (ranging from 0 to 40%) and PTX2 (ranging from 45 to 100%).

Two sample clean-up schemes, Liquid-liquid extraction (LLE) and Solid Phase Extraction (SPE) were investigated, with a view to removing or at least minimising matrix effects. A prerequisite for each of the sample clean-up schemes was good recovery of all toxins using one procedure.

The LLE procedure used a hexane extraction to remove any fats from the extract, and a dichloromethane (DCM) partitioning step to isolate the toxins. Recovery losses were incurred with additional partitioning steps; further losses were attributed to the evaporation/reconstitution step. Using the optimised conditions LLE recoveries of approximately 80% were obtained for OA and AZA1.

An array of different SPE sorbent phases were evaluated: Oasis HLB^{TM} (Waters), Strata SDB-L and Strata X (Phenomenex), Isolute Env + (Biotage/IST) and Bond Elut LRC

Certify (Varian). The load, wash and elution steps were optimised. The Strata-X and the Oasis HLB ™ cartridges (co-polymer sorbents) gave the best recoveries for OA, AZA1, PTX2 and YTX respectively.

Both clean-up schemes were evaluated for their effectiveness in the removal of matrix effects using oyster, mussel and scallop extracts. In the LLE study matrix effects were observed for OA (ion enhancement, 14%) and AZA1 (ion suppression, 36%) in the crude extract. LLE demonstrated a clean up effect for AZA1 only.

A small-scale study between three laboratories highlighted the difficulties for evaluating the clean-up effect for SPE. At the Marine Institute (MI) the matrix effects arising from the extracts were variable. No matrix effects were observed for OA or AZA1 whereas ion enhancement of 70% was observed for PTX2. Substantially different degrees of matrix effects were reported between laboratories (using the same tissues and spiking standards) and only one laboratory reported a significant clean-up effect using SPE. From the results obtained during the course of this thesis LLE and SPE have potential in removing matrix effects under certain conditions.

1. Introduction

1.1. Overview

This introductory chapter provides a brief overview of the work that was undertaken during the study period. The economic importance of the Aquaculture industry in Ireland is outlined. Marine toxins are classified according to the symptoms they produce; general descriptions regarding the individual syndromes are given, their related toxin groups and the effects that they have on human health are considered. More specific information is given on the OA and AZA toxin groups that were the focus of this study.

Descriptions of various methods that have been developed to monitor for these toxins are outlined. Within these descriptions the technique of liquid chromatography mass spectrometry (LCMS) is introduced. LCMS is now fast emerging as a possible tool to fulfil current European legislation for the monitoring of marine toxins. This legislation permits the introduction of alternative methods to the customary biological testing method (mouse bioassay) to be implemented into EU regulatory monitoring programs.

A general overview of LCMS systems is given, with information on the combination of the two techniques. The ionisation process is considered to introduce and explain the theory of matrix effects that can occur in LCMS analysis. A description of matrix effects is outlined along with descriptions of various sample purification techniques, which could be employed to remove them.

Finally, the advantages for introducing LCMS into a monitoring system as a replacement for the current testing method are considered.

1.2. Economic importance of the shellfish industry in Ireland

Shellfish are recognised worldwide as a nutritionally valuable food source. Increasing demand in Europe has led to significant growth in the Irish shellfish industry in recent decades. Ireland's aquaculture industry plays an important role in economic activity generating substantial revenue, in particular to the coastal communities. The main bivalve species being produced in Ireland at present are: mussel (*Mytilus edulis*) (in both rope and bottom culture), pacific oyster (*Crasostrea gigas*), native oyster (*Ostrea edulis*) clam (*Tapes philippinarium*) and scallop (*Pecten maximus*). The mussel industry in Ireland is the largest aquaculture sector in terms of tonnage and second only to salmon in terms of value (Anon 2007).

A report published annually by the Marine Institute (MI) and Bord Iascaigh Mhara (BIM) indicated that in 2006 the shellfish industry was valued at approximately €63 million, employing a total of 1,722 people. The figure below represents the percentage contribution of each shellfish species to the overall value (Anon, 2007).

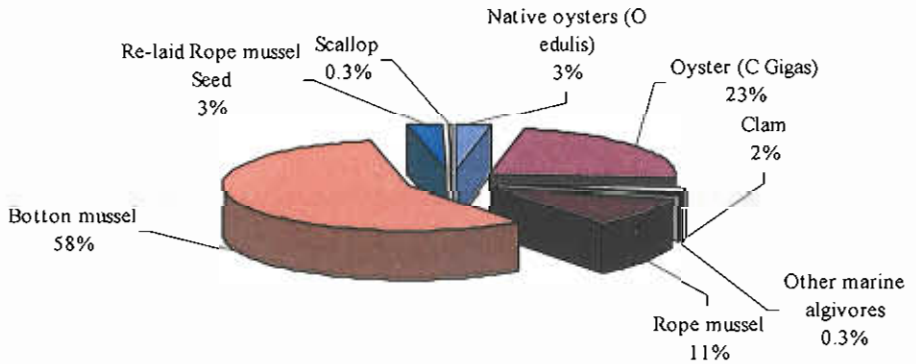


Figure 1-1: Aquaculture production value, percentage contributed from each species harvested in 2006 (Information obtained from Status of Irish Aquaculture report, 2006 (Anon, 2007)).

1.3. Production and distribution of algal toxins

Microscopic planktonic algae (phytoplankton) of the world's oceans are critical food for filter-feeding bivalve shellfish such as oyster, mussel, scallop, and clam. Amongst the estimated 5000 existing marine algal species, approximately 300 can sometimes occur in such high numbers (blooming) that they discolour the surface of the sea resulting in the so-called "red tides" (Hallegraeff *et al*, 1995; Lindahl *et al*, 1998). These events are referred to as Harmful Algal Blooms (HABs). Only about 40 of the marine algal species have the capacity to produce secondary metabolites "phycotoxins", which, when consumed by filter feeding bivalves can find their way into the human food chain (Fernandez *et al*, 2001). It is unclear as to why some marine algal produce toxins. These toxins have no apparent explicit role in the internal systems of the organism and produce very specific activities in mammals. Botana (1996) suggests that the producers use the toxins as a means to compete for space, fight predation or as a defence against the overgrowth of other organisms. Phycotoxins can pose a serious threat to human health and shellfish industries worldwide (Trevino, 1998).

The reported incidents of poisoning from consumption of contaminated shellfish have increased over previous decades prompting awareness into this subject. Hallegraeff (1995) suggested factors that may be responsible for this increase, they include:

- Increased scientific awareness of toxic species.
- Increased utilisation of coastal waters for aquaculture.
- Stimulation of plankton blooms by cultural eutrophication and/or unusual climate conditions
- Transport of dinoflagellates as resting cysts either in ships' ballast water or associated with translocation of shellfish stocks from one area to another.

1.4. Classification of marine toxins

Various classes of marine toxins have been identified, which can find their way into the food chain and cause a variety of gastrointestinal and neurological illnesses, in some cases leading to death.

Shellfish toxins have traditionally been classified according to the symptoms that arise from human consumption and to date five groups of syndromes have been distinguished: Diarrhetic shellfish poisoning (DSP); Azaspiracid shellfish poisoning (AZP); Paralytic shellfish poisoning (PSP); Amnesic shellfish poisoning (ASP); and Neurotoxic shellfish poisoning (NSP). However, a recent FAO/IOC/WHO working group suggested a division based on the chemical characteristic of each toxin group (Anon, 2005c).

The various toxin groups, causative organisms, human symptoms and mechanism of action are outlined in Table 1-1. The toxin groups of interest during this thesis (OA, PTX, YTX and AZA) are described in more detail in sections 1.4.1, 1.4.2, 1.4.3, and 1.4.4 respectively.

Table 1-1: Shellfish toxin groups, causative organisms, symptoms and modes of action

Toxin group (main toxins)	Causative organism(s)	Symptoms	Mode of action
Azaspiracid (azaspiracids)	Unknown*	Chills, headaches, diarrhoea, nausea and vomiting, stomach cramps	Unknown
Brevetoxin (brevetoxins)	*Karenia brevis*	Reduction in respiratory rate, cardiac condition disturbances and a reduction in body tempcrature	Na+ Channel activator
Domoic acid (domoic acid)	*Pseudo- snitzchia spp.*, Benthic diatoms	Diarrhoea, nausea and vomiting, stomach cramps, headache and memory loss and even death in severe cases	Glutamate receptor agonist
Okadaic acid (okadaic acid, dinophysistoxins	*Dinophysis spp.*, *Prorocentrum spp.*	Diarrhoea, nausea and vomiting, stomach cramps	Protcin phosphatase inhibitor
Pectenotoxin (Pectenotoxins)	*Dinophysis spp.*,	Unkown	Unknown
Saxitoxin (saxitoxin, neosaxitoxin,gonya utoxins)	*Alexandrium spp.*, *Gymnodinium catenatum*, *Pyrodinium bahamese*, cyanobacteria	Tingling sensation, progressive paralysis and death	Na^+ Channel blocker
Yessotoxin (Yessotoxins)	*Protoceratium reticulatum* *Linggulodinium polyedrun*	Unknown	Unknown
Cyclic Iimines (spirolides, gymnodime, pinnatoxins, pteriatoxins)	*Alexandrium ostenfaldi*, *Karenia selliforme*	Unknown	Unknown

* Suspected Protoperidinium

1.4.1. Okadaic acid group (OA)

OA and its derivatives named dinophysistoxins (DTX1, DTX2 and DTX3) are lipophilic toxins that accumulate in the fatty tissue of shellfish and are responsible for DSP (Figure 1-2 and Table 1-2).

DSP was first reported in Japan in the 1970's when more than 150 people were reported to be suffering from vomiting and diarrhoea (Yasumoto *et al*, 1978). DTX1 was the DSP toxin present and was isolated from mussels by Murata (1982).

Similar poisoning incidences were reported from the Netherlands and the toxin responsible was identified as OA (Kumagi *et al*, 1983), which was first isolated from sponge (Tachibana *et al*, 1981). DTX2 was first isolated from Irish mussels (Hu *et al*, 1992a) and can be the dominant toxin of the OA group in Irish shellfish (Carmondy *et al*, 1995). DTX3 was first isolated from toxic scallops originating in Japan (Yasumoto *et al*, 1989) and was found to be a mixture of 7 O-acyl derivatives of DTX1. To date DTX3 has not been detected in phytoplankton samples suggesting that the acylation of DTX1 to DTX3 takes place in the HP of shellfish (Yasumoto *et al*, 1989). DTX3 has been used to collectively name the toxins in which the 7-hydoxy functions of OA, DTX1 and DTX2 have been acylated with fatty acids (FA) (Fernandez *et al*, 1996).

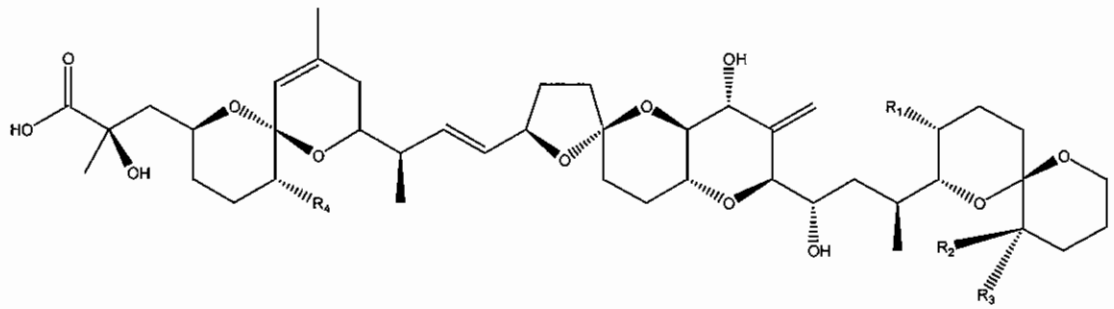


Figure 1-2: Chemical structure of OA

Table 1-2: Molecular weight (MW) and substitution locations of OA and DTX aualogues

Abbreviation	Chemical name	MW	R1	R2	R3	R4
OA	Okadaic acid	804.5	CH_3	H	H	OH
DTX1	35-methyl-okadaic acid	818.5	CH_3	CH_3	H	OH
DTX2	31-desmethyl-35-methyl-okadaic acid	804.5	H	H	CH_3	OH
DTX3	Fatty acid esters of DTXs	Variable	H/CH_3	H/CH_3	H/CH_3	FA

Human symptoms of DSP include diarrhoea, nausea, vomiting and abdominal pain which can start from 30 minutes after ingestion. Complete recovery typically occurs within three days after consumption. (Van Egmond *et al*, 1993).

The OA group has the most significant impact on the Irish aquaculture industry of any of the other toxin groups; OA and DTX2 are regularly detected in mussels in excess of the regulatory limits (Hess *et al*, 2003) resulting in closures of production areas.

The OA group of toxins is produced by dinoflagellates such as certain *Dinophysis* and *Prorcentrum* species (Quilliam, 1995). The toxins are all heat stable polyether lipophilic compounds. These compounds are potent inhibitors of the protein phosphatases PP1 and PP2 and the adverse effects of the toxins are considered to be as a direct result of this activity (Hu *et al*, 1992a). This property is linked to inflammation of the intestinal tract and diarrhoea in humans (Van Apeldoorn, 1998; Hallegraeff *et al*, 1995). OA and DTX1 are also tumour promoters *in vitro*; this property is linked to the inhibition properties displayed by this group of toxins (Draisci *et al*, 1996).

1.4.1.1. Methods of analysis

The current EU reference method for the OA toxin group is the customary biological testing method (including the rat and mouse bioassay (MBA)). In the MBA the toxins are extracted from shellfish, this extract is injected intraperitoneally into male mice with a body weight of about 20g and their survival is monitored for up to 24 hours.

The first chemical analyses of the OA toxin group were based on liquid chromatography-fluorometric detection (LC-FLD). For fluorometric detection to be possible a derivitisation step is required. Lee *et al*, (1987) reported a method involving the sequential extraction of shellfish tissue with MeOH, ether and chloroform; derivitisation with 9-anthryldiazomethane (ADAM); silica Sep-Pak clean up followed by determination by HPLC with fluorescence detection

A number of rapid methods have been developed for seafood toxins including in vitro cell toxicity assays, receptor protein assays and immunoassays such as enzyme-linked (ELISA) (Quilliam, 2001). The development of antibodies has initiated the development of immunoassays for OA however these assays cannot be used for accurate quantification due

to differences in cross reactivity (Quilliam, 1995). Despite the speed, high sensitivity and relative low cost associated with the use of immunoassays they cannot be used for the precise quantitative analysis of toxin groups that possess a variable range of toxins. In addition it is generally recognised that confirmation of positives is still required (Quilliam, 2001).

The recent developments in LCMS methodology for the analysis of marine toxins are promising; several methods have been reported for OA, DTX1 as well as DTX2 toxins (Quilliam, 1995; James *et al*, 1997; Draisci *et al*, 1998c; Holmes *et al*, 1999; Suzuki *et al*, 2000).

1.4.1.2. Regulations and monitoring

The MBA is the most widely used method for the monitoring of OA and enforcement of the regulatory limits in Europe. Regulation (EC) No. 853/2004 states that:

- " The maximum level of OA and DTXs together, in edible tissues (whole body or any part edible separately) of molluscs, echinoderms, tunicates and marine gastropods is 160 µg OA eq/kg " (Anon, 2004a)

Regulation 2074/2005 requires that:

- Alternative analytical methods such as LCMS can be used in combination with the MBA however when discrepancies are experienced the MBA should be considered as the reference method. (Anon, 2005a; Anon, 2006a)

1.4.2. Pectenotoxins (PTX)

Pectentoxins are neutral toxins consisting of polyether-lactones. Pectenotoxins were first isolated in Japan by Yasumoto *et al*, (1985) from contaminated scallops (*Patinopeceten yessonesis*). PTXs differ from the OA toxins in that they have a larger carbon backbone and a lactone ring rather than an open structure (Figure 1-3) (Yasumoto *et al*, 1984) but are associated to the OA toxins as they have been shown to be produced by certain *Dinophysis* species that also produce some of the OA toxins (Lee *et al*, 1989; Draisci *et al*, 1996). PTXs to date have not been associated with any human poisonings suggesting that the toxins may not pose a serious threat to human heath (Miles *et al*, 2004). Structural alteration amongst the PTXs originates at the C-43 position where oxidation occurs. PTX1, PTX2 and PTX6 appear to be the most important PTXs (Table 1-3). Since PTX2 is only found in phytoplankton it has been suggested that an oxidation occurs in the HP of shellfish producing other PTXs (Draisci *et al*, 1996).

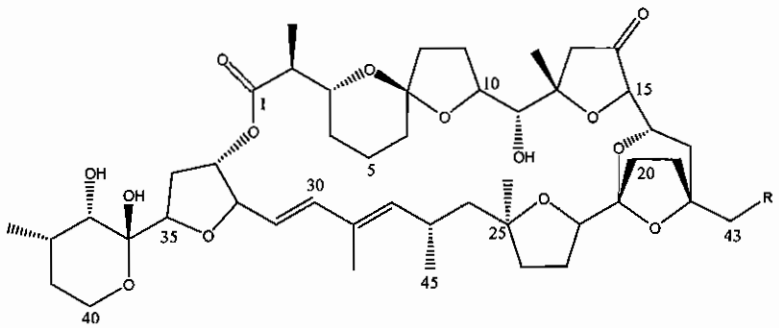


Figure 1-3: Chemical structure of Pectenotoxins

Table 1-3: MW and substitute locations of PTX1, PTX2 and PTX6

Abbreviation	Chemical name	MW	R
PTX1	Pectenotoxin-1	874.5	CH_2OH
PTX2	Pectenotoxin-2	858.5	CH_3
PTX6	Pectenotoxin-2	888.5	COOH

1.4.2.1. Methods of analysis

The MBA is the currently the most commonly used method for the regulation of PTX2 toxins in Europe.

The application of LC-FLD has been reported for the detection of PTX2 following derivitisation with 4-[2-(6, 7–dimethoxy-4-methyl-3-oxo-3, 4-dihydroquinoxalinyl) ethyl]-1, 2, 4- triazoline-3, 5-dione (DMEQ-TAD) (Sasaki *et al*, 1999).

Specific LCMS methods for the analysis of PTX have been reported (Suzuki *et al*, 1998 Suzuki and Yasumoto, 2000; Suzuki *et al*, 2001).

Multi-toxin methods, which have incorporated the OA group, PTX and YTX toxins, have also been reported (Suzuki *et al*, 2005; Goto *et al*, 2001; Stobo *et al*, 2005; Quilliam *et al*, 2001; Mc Nabb *et al*, 2005).

1.4.2.2. Regulations and monitoring

The MBA is currently the most widely used method for the monitoring of PTX in Europe. Regulation (EC) No 853/2004 requires that:

- "The maximum level of PTX and OA, DTXs together, in edible tissues (whole body or any part edible separately) of molluscs, echinoderms, tunicates and marine gastropods is 160 µg OA eq/kg" (Anon, 2004a)

Regulation No 2074/2005a requires that:

- Alternative analytical methods such as LCMS can be used in combination with the MBA however when discrepancies are experienced the MBA should be considered as the reference method (Anon, 2005a; Anon, 2006a)

1.4.3. Yessotoxin (YTX)

Yessotoxin (YTX) is a disulphated compound along with its derivative 45-hydroxyyessotoxin (45-OH-YTX) (Murata *et al*, 1987) (
Figure 1-4). This ladder shaped polycyclic ether compound is produced by the dinoflagellate *Protoceratium reticulatum* (Satake *et al*, 1997).

Yessotoxin was first isolated from the digestive organs (hepatopancreas) of scallops (*Patinopecten yessoenis*) collected from Mitsu Bay, Japan in 1986 (Murata *et al*, 1987). Numerous analogues and derivatives of YTX have been reported to date (Hess and Aasen, 2007a), only a selection is shown.

YTX has shown to give positive results when using the MBA (Murata *et al*, 1987) and has previously been included in the OA toxin family however the chemistry and toxicology of YTXs differ significantly. YTX do not yield diarrhoearoea (Terao *et al*, 1990) they attack the cardiac muscle in mice after injection while the desulphated YTX attacks the liver (Murata *et al*, 1987; Satake *et al*, 1996; Ciminiello *et al*, 2000b). However, a molecular mechanism of action has not been conclusively elucidated.

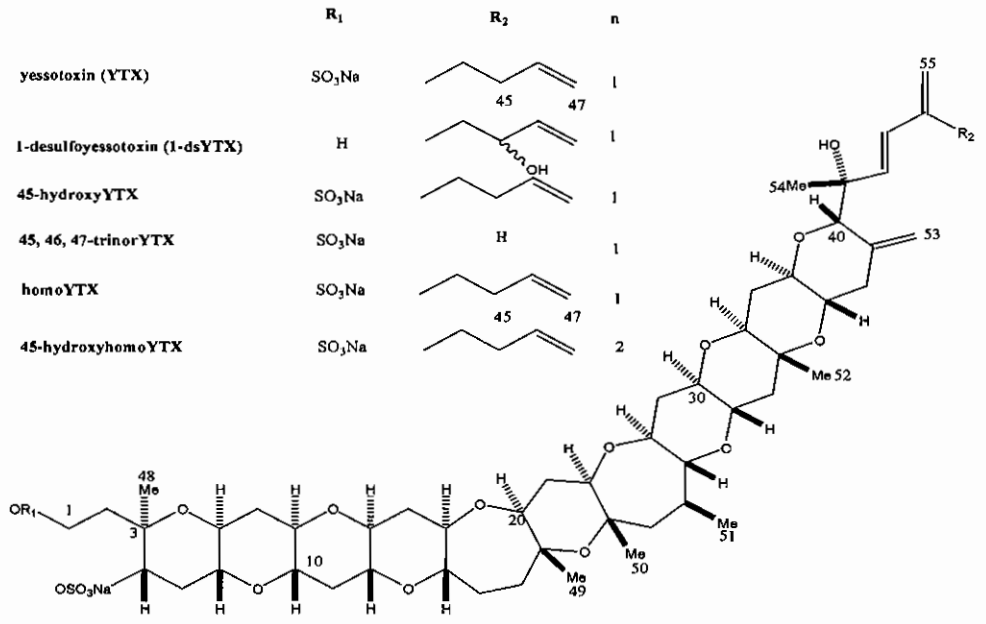


Figure 1-4: Chemical structure of Yessotoxin and associated analogues

1.4.3.1. Methods of analysis

The MBA is the most widely used method for the regulatory control of YTX in Europe.

The application of LC-FLD has been reported for the detection of YTX following derivitisation with 4-[2-(6, 7 –dimethoxy-4-methyl-3-oxo-3, 4-dihydroquinoxalinyl) ethyl]-1, 2, 4- triazoline-3, 5-dione (DMEQ-TAD) (Yasumoto and Takizawa, 1997). The presence of a conjugated diene functionality in the side chain of YTX like compounds is a prerequisite for this method, the lack of conjugated double bonds in some of the YTX derivatives (not shown) makes this method unreliable for the detection of the full suite of YTX compounds. However, the four regulated toxins (YTX, 45-OH-YTX, homo-YTX and 45-OH-homo-YTX) may be detected with this method.

Specific LCMS methods for the analysis of YTX have also been reported (Ciminiello *et al*, 2003; Aasen *et al*, 2005).

Multi-toxin methods, which have included the YTX toxins, along with the OA group and PTX, have also been reported (Goto *et al*, 2001; Quilliam *et al*, 2001; Mc Nabb *et al*, 2005; Stobo *et al*, 2005 Suzuki *et al*, 2005).

1.4.3.2. Regulations and monitoring

The MBA is the most widely used method for the regulation of YTX in Europe. Regulation (EC) No 853/2004 states that:

- " The maximum levels of YTXs in edible tissues (whole body or any part edible separately) should not exceed 1mg YTX eq/kg" (Anon, 2004a)

Regulation (EC) No 2074/2005 requires that:

- Alternative analytical methods such as LCMS can be used in combination with the MBA however when discrepancies are experienced the MBA should be considered as the reference method (Anon, 2005a; Anon, 2006a)

1.4.4. Azaspiracid shellfish toxins

AZA was discovered in 1995 when at least 8 people became ill in the Netherlands after eating mussels cultivated in Ireland (Killary harbour). The symptoms resembled those of DSP including nausea, vomiting, stomach cramp, and diarrhoearheoa. However, very low concentrations of the OA toxins were found upon testing of the samples for the presence of OA and DTXs. In addition to the symptoms associated with the OA toxins, a slowly progressive paralysis was observed in the MBA from these same extracts. It was then that azaspiracid was identified and the new syndrome azaspiracid poisoning (AZP) was named (Satake *et al*, 1998b). Since then, methyl (AZA2) and desmethyl (AZA3) analogues and a further eight hydroxyl analogues have also been reported (Ofuji *et al*, 1999a; Ofuji *et al*, 2001; Brombacher *et al*, 2002; James *et al*, 2003a)

The symptoms of AZP resemble those of DSP, sickness occurs between three and eighteen hours after consumption of contaminated shellfish and full recovery can typically occur after two to five days (McMahon and Silke, 1996; James *et al*, 2004).

Several other European countries including the UK, Norway, the Netherlands, France, Italy, Spain and Portugal have reported the occurrence of either cases of poisoning and/or presence of contaminated shellfish.

Recent research suggests that *Protoceratum crassipes* may be the source organism (James *et al*, 2003b), however, Aasen *et al*, (2006) reported that there is little evidence for *Protoperidium crassipes* being the causative organism of AZAs in Norweigan shellfish. Recent reports from Ireland have shown no correlation between high levels of AZAs found in Irish shellfish in 2005 and the presence of *Protoperidium crassipes* in phytoplanktons samples (Moran *et al*, 2007).

Azaspiracids differ from any of the previously known nitrogen containing toxins found in shellfish or dinoflagellates. They have unique spiro-ring assemblies, a cyclic amine instead of a cyclic imine group. The carboxyl and amine functions in the azaspiracids "appear to form an intramolecular ion-pair", resulting in an overall lower polarity for the molecule coupled with a lower reactivity of both functions (Quilliam, 2003). Such an ion pair would be formed

between the carboxylic acid function and the cyclic amine function of AZA. The generic structure for AZA1 is shown in Figure 1-5, the associated analogues are shown in Table 1-4.

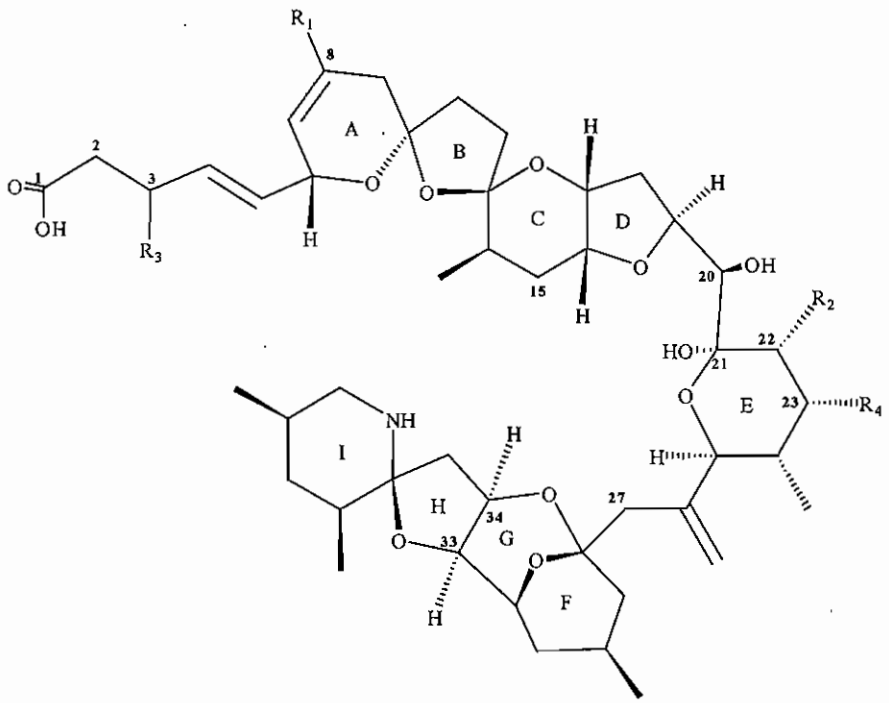


Figure 1-5: Chemical structure of AZA1

Table 1-4: (MW) and substitution locations of AZA and analogues

Abbreviation	Chemical name	MW	R1 (C8)	R2 (C22)	R3 (C3)	R4 (C23)
AZA1	Azaspiracid	841.5	H	CH_3	H	H
AZA2	8-methyl-azaspiraicd	855.5	CH_3	CH_3	H	H
AZA3	22-desmethyl-azaspiracid	827.5	H	H	H	H
AZA4	22-desmethyl-3-hydroxy-azaspiracid	843.5	H	H	OH	H
AZA5	22-desmethyl-23-hydroxy-azaspiracid	843.5	H	H	H	OH

1.4.4.1. Methods of analysis

The MBA is the official reference method in the EU for the detection of AZAs; extracts are injected intraperitoneally in mice, following the same MBA procedure as in the detection of the OA group toxins. The AZA response is characterised by hopping, scratching and progressive paralysis and even death, which is atypical for OA toxins (Satake *et al*, 1998a).

As in the OA group AZAs lack a chromophore, which prevents the use of analytical methods based on HPLC-UV. There have been no reports on the derivitisation of AZA1 for the development of a method based on HPLC with fluorescence detection. The toxicological mode of action of the AZA group is still unclear and this has prohibited the development of antibodies for the production of functional assays.

Several LC-MS methods have been reported for azaspiracid group (Oufiji *et al*, 1999b; Draisci *et al*, 2000; Lehane *et al*, 2002). Multi-toxin methods incorporating AZAs have also been reported (Quilliam *et al*, 2001; Aasen *et al*, 2003; Mc Nabb *et al*, 2005; Stobo *et al*, 2005; Fux *et al*, 2007a).

As with the OA toxin group further validation and implementation of an LCMS method for the analysis of AZAs is restricted by the lack of commercially available standards and reference materials.

1.4.4.2. Regulations and monitoring

Regulation (EC) No 853/2004 states that:

- " Maximum levels of AZA toxins in bivalve molluscs, echinoderms, tunicates and marine gastropods (whole body or any part edible separately) shall be 160µg/kg " (Anon, 2004a)

Regulation (EC) No 2074/2005 requires that:

- The mouse bioassay is the preferred method of analysis. Analytical methods such as LCMS and immunoassays can be used in combination or alone, provided that, they can detect the correct analogues (AZA1, -2 and -3) and are not less effective than the biological methods and offer at least and equivalent level of protection to human health. When discrepancies occur between the biological and alternative methods the mouse bioassay is considered the reference method (Anon, 2005a; Anon, 2006a)

1.5. Liquid Chromatography coupled to mass spectrometry (LCMS)

Liquid Chromatography/Mass Spectrometry (LCMS) refers to the combination of High performance liquid chromatographic (HPLC) separation with mass spectrometric (MS) detection. The combination of these two techniques allows the analysis of virtually any molecular species, including thermally labile, non-volatile, and high molecular weight species. Over 80% of known organic species are amenable to separation using liquid chromatography. Mass spectrometry is capable of providing structural information, molecular weight, empirical formula, and quantitative information about a specific analyte (Ardrey, 2004).

HPLC was first developed in the early twentieth century by the Russian botanist Michael Tswett who successfully fractionated petroleum ether extracts of chlorophyll and other plant pigments on narrow glass columns packed with dry calcium carbonate (Tswett, 1906).

Nowadays separation by HPLC is carried out by passing a mixture in a liquid mobile phase through an analytical column packed with a stationary phase. The components of the sample are separated within the column. The time taken to pass through the analytical column is called the retention time (Ardrey, 2004). The emerging fractions can be measured by using a variety of detectors such as ultraviolet (UV) or MS. The detected components are recorded as peaks of which the corresponding area correlates with the amount of the compound. Preparative scale HPLC is performed to achieve purification however during the course of this thesis HPLC was carried out at a smaller analytical scale.

The advantage of MS over other detection methods is the sensitivity provided. A full scan spectrum, and positive identification, can be obtained from picrogram (pg) amounts of analyte with high accuracy and precision (Herbert and Johnstone, 2003). The principle of MS detection is the production of ions that are subsequently filtered according to their mass to charge (m/z) ratio.

The combination of HPLC with MS started in the early 1970s and is enabled by the use of appropriate interfacing, the primary purpose being the removal of the mobile phase. The interface can have dual functions acting both as an inlet and an ionisation source. The earliest interface was a continuous moving belt (loop), which operates by placing the liquid emerging from the LC column onto a belt as a succession of drops. They are heated at a low

temperature to evaporate any solvent and leave mixture components behind (Herbert and Johnstone, 2003). This method has been replaced by more recent developments such as particle beam and electrospray, and because these newer techniques have no moving parts they are more robust.

All of the LCMS analysis that is described in this thesis made use of an electrospray ionisation source (ESI), which functions as both an inlet and an ionisation source. The ESI interface operates by nebulising a solution using a strong electric field. This produces a spray of small-charged droplets from which the solvent is removed by evaporation, leaving sample ions to pass straight into the anlayser region of a mass spectrometer (Herbert and Johnstone, 2003).

Various mass analysers are available such as Quadrupole, Time of Flight, Sector and Ion trap. The two main LCMS systems used during this thesis were: Triple Quadrupole (QqQ) and Quadrupole Time of Flight (QToF).

1.6. Matrix effects

When LCMS was first developed, one of its most attractive capabilities was the possibility of limited sample preparation and high throughput analysis, considering the LC column solely as a loading system. Even with the dramatic improvements that can be achieved by the application of LCMS, there are still some problems associated with the technique that can invalidate quantitative results. One such drawback is the occurrence of matrix effects. Matrix effects were originally discussed by Kerbale and Tang (1992), and can lead to a significant increase or decrease in the response of an analyte in a sample compared to a pure standard solution.

Matrix effects are attributed to interferences, which co-elute along with the analyte of interest. The mechanism by which matrix effects occur is still unclear but is thought to be as a result of competition between the interfering compound and the analyte during the ionisation process, resulting in either ion suppression or enhancement. Bonfiglio *et al*, (1999) reported that the chemical nature of the compound has a significant effect on the degree of matrix effects; it was reported that the most polar of four compounds displayed the highest degree of matrix effects.

Matrix components may also qualitatively interfere with the mass spectrometric detection of analytes. For instance, metal complexation may lead to adduct ions (pseudo-molecular ions), thereby reducing the abundance of the molecular ion. Such adducts may also be more stable and the resulting spectrum may have a lower abundance of specific fragments.

Matrix effects can be a cause of significant errors in the accuracy and precision of a method (Annesley, 2003), therefore, the evaluation of matrix effects is required as part of quantitative method development. The FDA guidelines (FDA, 2007) for method validation advise, "In the case of LC-MS-MS based procedures, appropriate steps should be taken to ensure the lack of matrix effects throughout the application of the method". However, it is not stated how to evaluate the presence of matrix effects or how to eliminate them.

Various methods exist for evaluating matrix effects; the use of matrix matched standards is an approach which compares the signal obtained from a neat set of standard solutions (usually a calibration line) which represents the reference peak areas (relative 100% response value) to the same set of standards prepared in pre-extracted sample. Ito and Tsukada, (2001)

applied this approach to correct for matrix effects experienced in LCMS analysis of OA toxins in scallops and reported that the values of OA toxins were approx 19-42% lower than the values obtained from the standard solutions in MeOH. The rates of signal suppression were constant and independent of the concentration of OA toxins in the solution.

Stobo *et al*, (2005) also used matrix-matched standards to investigate the impact of matrix effects on detector response using cockle, mussel, oyster and scallop material and reported that matrix effects varied considerably depending on toxin and shellfish type. The signal for OA in matrix was enhanced ranging from 180 to 230% when compared to standards in solvent; AZA1 was suppressed to values ranging from 52 and 55%. The responses of YTX and PTX2 were least affected (75-100% of standard response).

McNabb *et al*, (2005) evaluated the matrix effect by diluting a standard mixture with solvent or blank shellfish tissue extract. There was a 10-12% enhancement of the PTX2 response, 21-23% suppression for the YTX response and no significant effects on the response of OA.

Whilst these approaches can compensate for matrix effects and can produce results, which are more quantitatively accurate, they do not account for the loss of sensitivity that is accompanied by signal suppression/enhancement effects and the variability between series of samples. The only way to avoid such difficulties is to take steps to eliminate or significantly reduce matrix effects. The process of sample extraction and purification is the most direct means of obtaining maximum sensitivity and signal reproducibility.

Various clean up procedures have been applied to shellfish extracts to remove or at least minimise matrix effects. Hummert *et al*, (2000) applied gel permeation chromatography (GPC) to raw mussel extracts for the detection of the OA toxins (OA, DTX1, -2) demonstrating a recovery of approx 70% for the entire method protocol (consisting of extraction, cleanup and LCMS determination). Goto *et al*, (2001) conducted a study, which focused on purification schemes for the individual groups (OA, PTX, YTX). By applying a liquid liquid extraction (LLE) procedure and two solid phase extraction (SPE) methods, it was reported that SPE effectively removed components that were responsible for signal suppression of 50%.

1.7. Sample extraction and purification

1.7.1. Sample clean up

1.7.1.1. Liquid-liquid extraction (LLE)

LLE is a classical clean up method that is based on the selective partitioning of the toxins into one of two immiscible phases one of which contains the analyte of interest. The analyte then migrates into the other phase until equilibrium is reached. LLE using hexane can be useful in the removal of lipids and also waxes which can cause interferences in the MS. LLE is a relatively inexpensive clean up technique that does not require highly specialised staff or expensive equipment.

LLE has been reported as part of shellfish sample pre-treatment procedures by a number of authors (Quilliam, 1995; Ito and Tsukada K, 2001; Fernandez *et al*, 1996; Mc Nabb *et al*, 2005). All of these methods use either a hexane-washing step alone or include a subsequent washing step using chloroform or dichloromethane (DCM) to isolate and purify the analyte further. DCM has been suggested to replace chloroform for toxicological reasons.

Although LLE has been used in laboratories successfully for a number of years it is now being used less frequently. LLE is a time consuming technique and often requires an evaporation step, which may lead to recovery losses and is now being replaced by more modern clean up techniques such as SPE and GPC.

1.7.1.2. Solid phase extraction (SPE)

SPE is a sample preparation technique that uses both a solid sorbent and a liquid phase to isolate an analyte in solution. The analyte of interest may either preferentially adsorb to the sorbent, or may remain in the liquid phase. If the analyte is adsorbed onto the sorbent it can be desorbed by washing with an appropriate solvent. If SPE is performed in this way, it is similar in practice to LLE, where now the solid sorbent has replaced the immiscible liquids. A wide variety of sorbents are available commercially, each offering different selectivity. By passing a liquid through the sorbent bed under vacuum the technique is now a form of column chromatography. The attributes of SPE offer more separation power, specificity, and selectivity compared to the previously described LLE. Higher recoveries can be achieved with better reproducibility.

Methods that have incorporated an SPE step into the sample pre-treatment after an initial LLE step have been reported: Quilliam (1995) reported an SPE method using an aminopropylsilica column for the clean up of OA toxins in mussels after an initial LLE step (Hexane and chloroform). It was found that after the LLE step the extracts were still visibly dirty (ranging in colour from golden brown to black), a further cleanup step would serve to protect the column, reduce interferences and allow further concentration of the extract. A reference material was used to test the effectiveness of the aminopropylsilica column; a recovery of approximately 95% was reported. Evaporation of the cleaned extract followed by reconstitution in MeOH resulted in pale-yellow solutions indicating a clean up effect. While removal of coloured substance shows a potential clean-up, it does not constitute proof of the removal of the substances intefering with the analyte. Unfortunately, the paper did only report recovery but did not elaborate on the efficiency of the clean-up in removing matrix effects for the subsequent LC-MS determination of OA.

Goto *et al*, (2001) reported a clean up procedure for the OA toxins, YTX and YTXOH using solid phase extraction on a silica cartridge (SPE Sep-Pak silica), samples containing YTXOH were purified separately using a reversed phase cartridge column (SPE Sep-Pak C18). The Silica SPE effectively removed contaminants, which caused ion suppression however low recoveries were achieved for YTXOH. Recoveries for the remaining toxins ranged from approx. 70-134%. The reversed phase cartridge column achieved recoveries of approx. 70%.

For the AZA toxins Moroney *et al*, (2002) compared two SPE extraction methods, five reversed phase (C18) and three diol solid phase extraction cartridges were compared for their efficacy in the cleanup. Good recovery and reproducibility were reported for one diol (84-97%) and two C18 cartridge types (71-98%).

1.8. Lipid content of Shellfish

The consumption of shellfish offers generous amounts of proteins, vitamins and minerals, essential fatty acids accompanied by a low fat and calorie content, this is represented in Table 1-4 (Pigott *et al*, 1990).

Table 1-5: Proximate composition of shellfish species (raw edible portion) (Data extracted from Pigott *et al*, 1990)

	Water (%)	Protein (%)	Lipid (%)	Cal/100g
Oyster	82	8.6	2.4	66-91
Scallop	77	15.1	1	81
Clam	81.7	9.7	1.2	63

Fish are generally classified in terms of their lipid content; lipids are the group of food components commonly known as fats, sterols, waxes etc. (compounds not soluble in water). Lipids play a vital role in marine ecosystems as both a source of energy and as a structural aid in the cell membrane (Copeman *et al*, 2003).

Dominant components of lipids in shellfish are triglycerides, free fatty acids, sterols and phospholipids. The lipophilic toxins are considered as polar lipids themselves, they are extracted from shellfish tissue using organic solvents and an extraction step using hexane can remove the co-occurring non-polar lipids. When considering the use of a hexane- step the possible losses of OA toxins of low polarity (DTX3), which may be solubilised in the hexane layer, must be considered. This step must be avoided when analysing samples of unknown origin and with unknown OA toxin profiles (Fernández *et al*, 1996).

The toxins can be isolated using a number of solvents such as acetonitrile or MeOH. Other classes of lipid are therefore contained within the organic extract depending on the solvent used in the extraction. MeOH is the preferred extraction solvent for the lipophilic toxins and this solvent is also used to isolate phospholipids.

Lipids can be selectively extracted from shellfish tissue using a solvent mixture of chloroform-MeOH-water as described by Bligh and Dyer (1959). Further isolation of the various lipid classes can be achieved using techniques such as thin layer chromatography

(TLC), column chromatography and more recently solid phase extraction (Lacaze *et al*, 2007). During this thesis, total lipid content only was considered due to time constraints.

Phospholipids are present in high concentration in biological matrices and have been shown to cause matrix effects in LCMS analysis. This is thought to be due to the effect that they have on desolvation of the mobile phase in the ESI source or as a result of competition during the ionisation process for excess charges on the droplet surface (Enke, 1997).

Hydrolysis of lipids yields free fatty acids and this hydrolysis can occur naturally within shellfish tissue during frozen storage. It has also been reported that the mouse bioassay can suffer interferences from free fatty acids (FFA) in that sufficient amounts of FFA can kill mice after intraperitoneal injection (Takagi *et al*, 1984). The oxidation and hydrolysis of lipids in shellfish during frozen storage can cause serious deterioration in quality (Jeong *et al*, 1990).

1.9. Rationale for the replacement of the mouse bioassay

The mouse bioassay is the most widely used method for the analysis of marine toxins in Europe. The method involves the controversial issue of the use of mammals in testing and also suffers the drawbacks associated with the use of mammalian bioassays such as: poor reproducibility, low sensitivity and interferences from other components of the extract. In addition, the MBA measures total toxicity only and does not give quantitative information about the individual toxins (Hess *et al*, 2006).

As the information obtained from the MBA is so limited, the build up to toxic events cannot be monitored effectively. Recent EU legislation has been introduced to clarify the monitoring requirements. The legislation outlines the monitoring requirements, the corresponding concentration limits to be imposed and the methods to be used in fulfilment of the legislation. It also allows the use of alternative methods provided they offer at least an equivalent level of protection for human health.

LCMS is emerging as one of the most promising analytical methods available for marine toxins as it allows the simultaneous analysis of toxins in a single procedure and also provides quantitative information on the individual toxins. Quilliam, (2003) suggested factors that demonstrate how LCMS meets all of the necessary requirements of a laboratory involved in monitoring and research of marine toxins such as:

- Universal detection capability
- High sensitivity, selectivity and specificity
- Minimal sample preparation
- Ability to deal with structural diversity, identification of new toxins
- Separation of complex mixtures
- Precise and accurate quantification
- Wide linear range
- Automation

Numerous multitoxin methods using LCMS have been reported such as: (Thompson *et al*, 2002; Aasen *et al*, 2003; McNabb *et al*, 2005; Stobo *et al*, 2005; Fux *et al*, 2007). The EU

regulations state that the reference method should be " Internationally accepted" however to date, a full validation of practical and quantitative methods is not currently available.

There are challenges to be overcome before it is possible to implement LCMS into a routine monitoring programme. Toxin classes must be well characterised in terms of their structure, as with each new year the situation becomes more complex with the emergence of new toxin analogues. The lack of commercially available standard solutions is a prohibiting factor, which needs to be addressed.

BIOTOX is a multi-disciplinary project primarily focused on the development, validation and standardisation of reference methods (LCMS) and cost effective assays for the identification and quantification of lipophilic marine toxins, which, have been included in the European legislation. These new methods will hopefully lead to reduction or replacement of the existing animal tests. Validation studies will ensure that the methods adhere to international validation standards.

1.10. Scope of work

This project was carried out as part of a EU project (BIOTOX) whose aim was to develop alternative methods to the MBA for the analysis of marine toxins. This work was focused on specific aspects of LCMS method development that are required in the development of a new reference LCMS method.

Although not the main focus of the thesis, some aspects of the LC and MS development and validation were undertaken, including collaboration with other institutes. The LC parameters were examined including: the effect that the analytical column and the gradient elution patterns play in the separation of the toxins. A study of the MS conditions was carried out to investigate the use of different ionisation and acquisition modes.

With the development of any LCMS method studies on matrix effects must to be conducted to gain understanding on the trends occurring. While matrix effects are known to be highly variable and difficult to control, it was still necessary to examine the behaviour of relevant toxins in various shellfish tissues, analysed using different MS analysers.

Two approaches to the study of matrix effects were undertaken; matrix matched standards were prepared using oyster and scallop material across a range of toxin concentrations which relate to the current regulatory limits. The lipid content of the shellfish material used during the study was determined to investigate any correlation between lipid content and the pattern of matrix effects.

The evaluation of two sample clean-up schemes was undertaken using LLE and SPE. The first task was to ensure sufficient recovery of toxins throughout the procedure and when the method development was complete the effectiveness of each technique in the removal of matrix effects was evaluated.

2. Materials and Methods

2.1. Introduction

The extraction and instrumental conditions described during the course of this chapter are generic methods, which have been used repeatedly throughout this thesis. Any development of methods undertaken is described in the relevant experimental section of that particular chapter.

2.2. Chemicals and standards

2.2.1. Chemicals

Methanol and acetonitrile were obtained as HPLC grade solvents; dichloromethane (DCM), n-hexane, isopropyl acetate (IPA), cyclohexane, isopropylacetate and chloroform were obtained as PESTISCAN grade. All solvents were purchased from Labscan (Stillorgan, Ireland). A reverse osmosis purification system (Barnsted Int., Dubuque, IA, USA) supplied deionised water. Trifluoroacetic acid (TFA), formic acid (FA), ammonium formate (AF) and ammonium hydroxide (NH_4OH) were purchased from Sigma-Aldrich (St. Louis, MO, USA)

2.2.2. Standards

OA, PTX2 and YTX calibration standards were prepared using certified calibration solutions produced by the National Research Council Canada (NRC CRM OA, NRC CRM PTX2, and NRC CRM YTX).

The AZA standards used were dilutions of an AZA1 solution, which was isolated in 2001 from mussels originating in Ireland (Killary Harbour 1996, Bantry Bay 2000) under supervision of Dr. M. Satake (Hess, 2001). At the time of this work there was no purified AZA calibrant available, either certified or non-certified.

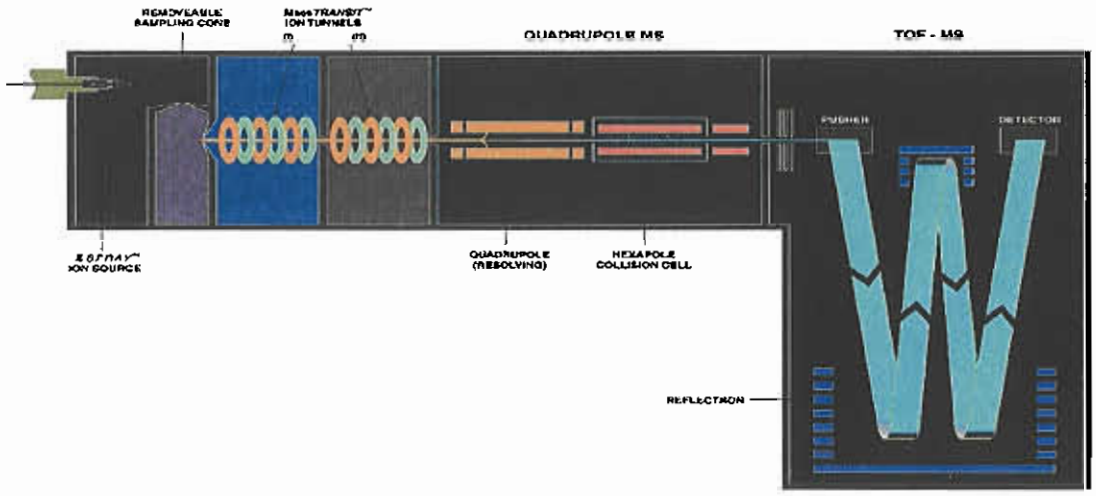
2.3. MS Systems

The two main MS systems (Quadrupole Time of Flight (QToF) and Triple Quadrupole (QqQ)) used during the course of this work are described below; where other systems were used there are appropriate descriptions given in that particular section.

2.3.1. Quadrupole Time of Flight (LC-QToF-MS)

Mass spectrometry was performed on a Q-ToF Ultima (Waters-Micromass Manchester, UK) equipped with a Z-spray interface coupled to an Alliance 2795 HPLC system. This system doesn't have the facility of automatic polarity switching and so the OA and AZA toxins were analysed separately (OA: ESI-, AZA: ESI+). The Q-ToF is represented in Figure 2-1. Ions are generated in the Z-spray source and are transferred to the quadrupole analyser (MS1). Upon leaving the quadrupole the ions flow into the orthogonal time of flight analyser (MS2). The ion beam is focused into the pusher from where a section of the ion beam is pulsed towards the reflectron, which then reflects ions back to the detector. As ions travel from the pusher to the detector they are separated in mass according to their flight times, with heavier ions (higher mass to charge (m/z) ratios) arriving later.

Figure 2-1: Instrument description for Quadrupole-Time of Flight



The source conditions for both ionisation modes are shown in Table 2-1.

Table 2-1: Source parameters used for Q-ToF analysis

Parameter	ESI +	ESI -
Capillary voltage (kV)	3.2	2.8
Source temperature (°C)	130	130
Desolvation temperature (°C)	350	350
Cone gas (L/hr)	50	50
Desolvation gas (L/hr)	500	550

2.3.2. Triple Stage Quadrupole (LC-TSQ-MS-MS)

Mass spectrometry was also performed using a Micromass Quattro Ultima triple stage quadrupole (TSQ) mass analyser (Waters Micromass Manchester,UK) also equipped with a Z-spray interface. The MS was coupled to an Alliance 2695 HPLC system. The TSQ (Figure 2-2) is capable of automatic polarity switching so both OA and AZA toxins could be monitored in a single analysis. Ions are generated in the Z-spray at atmospheric pressure. The ions are passed through a series of orifices into the first quadrupole where they are filtered according to their mass to charge (m/z) ratio. The mass separated ions then pass into the collision cell, where they either undergo decomposition or pass unhindered into the second quadrupole. The fragment ions are analysed by the second quadrupole. The transmitted ions are then detected and processed to the data system.

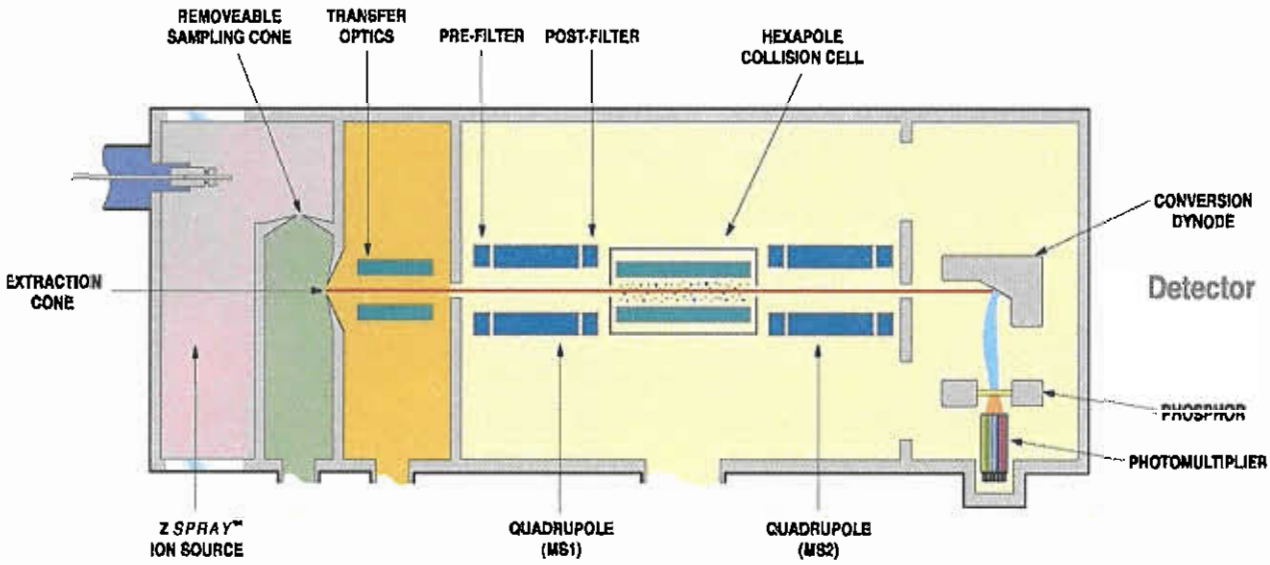


Figure 2-2: Instrument description for Triple Stage Quadrupo le

The source conditions are shown in Table 2-2.

Table 2-2: Source parameters used for TSQ analysis

Parameter	ESI +	ESI -
Capillary voltage (kV)	3.3	3.5
Source temperature (°C)	150	150
Desolvation temperature (°C)	350	350
Cone gas (L/hr)	121	121
Desolvation gas (L/hr)	770	770

2.4. Extraction procedure for lipophilic marine toxins

2.4.1. Routine Marine Institute extraction procedure

This duplicate extraction procedure was developed at the MI for use in the regulatory monitoring programmes (MI unpublished information). A sub-sample of tissue (2.0 ± 1.0 g) was weighed into 50ml polypropylene centrifuge tubes and extracted with MeOH (9ml). The samples were vortexed using a multi-tube vortex mixer (V400 Alpha Labs, UK), for 1 minute at full power. After a centrifugation step of 4000 rpm for 10 minutes (CR4-22 Jouan, Thermo Electron Corp., CA, USA), the supernatant was transferred to a 25ml volumetric flask. A second extraction, of the remaining pellet was carried out using an Ultra-turrax homogeniser ™ equipped with a T25 motor and a S25 probe (IKA-Werke, Staufen, Germany) at 11,000 rpm for 1 minute. After centrifugation the supernatant was transferred to the same 25ml volumetric flask, which contains the first addition of supernatant, the volume was completed to 25ml with MeOH. This gives a solvent to sample (SSR) ratio of 12.5. An aliquot of this solution was filtered through a 0.2-µm filter (Schleicher & Schuell, Whatman, UK) into HPLC vials (AGB Scientific Ltd., Dublin, Ireland) for analysis by LCMS.

2.4.2. BIOTOX extraction procedure

The following extraction procedure was developed as part of the BIOTOX project. An aliquot of shellfish extract (2g ± 0.1g) was weighed into a polypropylene tube and extracted a first time with 6mL of MeOH by vortexing for 1 minute on a multi-tube vortex mixer. After centrifugation at 3,000 rpm ± 2,000 rpm for 5 minutes the supernatant was decanted into a 20mL volumetric flask. The pellet was re-extracted with 6mL of MeOH and vortexed for 1 minute. Again the solution was centrifuged at 3,000 rpm ± 2,000 rpm for 5 minutes, the supernatant was decanted into the same volumetric flask. The pellet was extracted a third time with 6mL of MeOH using the Ultra-turrax ™ homogeniser for 1 minute at full power.

After another centrifugation step the supernatant was decanted in the same 20mL volumetric flask. The solution in the volumetric flask was completed to the mark with MeOH. This gave a SSR of 10. The crude extract was filtered through a 0.2-µm filter into HPLC vials for analysis by LCMS.

2.5. Analysis of lipophilic marine toxins by LCMS

For the analysis of the OA/AZA toxins three LCMS methods were adopted: one which is currently used as part of the regulatory monitoring programme (OA, DTX2 DTX1, AZA1, -2 and -3) at the Marine Institute and another two methods one of which have been developed as part of the BIOTOX project and for which various aspects of development will be described in this thesis. The majority of LCMS analysis was carried out using LC-TSQ-MS however the conditions used for LC-QToF-MS analysis are also outlined.

2.5.1. MI method for the analysis of lipophilic toxins by LCMS

This multi toxin method was adapted from Quilliam *et al*, (2001) and was previously described by Hess *et al*, (2003). The analytical LC column was a Thermo electron BDS-Hypersil C8 column (50 x 2mm, 3µm) with a guard column (10 x 2mm, 3µm). The column was maintained at a temperature of 25°C in the column oven. An injection volume of 5uL was used

This method employs a binary mobile phase. For the preparation of the mobile phase a buffer solution was prepared firstly by dissolving ammonium formate (0.63g ± 0.02g) in distilled water, formic acid was added (11.6 ± 0.02g) and this was completed to a final volume of 250ml in a volumetric flask. The resulting concentration of buffer was 2mM ammonium formate and 50mM formic acid.

The aqueous (100%) mobile phase A was prepared using de-ionised water and 5 % of the buffer solution. Mobile phase B was prepared with acetonitrile (95%) and 5% of the stock buffer solution (pH of approx 3).

For the separation of the lipophilic toxins a gradient method was set up; with a flow rate of 0.25ml/min. The gradient started at 30% B at time zero and was raised to 90% B over 8 minutes. The gradient was held at 90% B for 0.5 minutes and then decreased to 30% B over 0.5 minutes. This %B was held for a further 3 minutes to allow equilibration before the next injection. Using this gradient, OA, DTX2 and DTX1 elute first (approx. 6-8 minutes), followed by AZA3, -1 and -2 (approx. 10-13 minutes).

The TSQ instrument is favoured in the routine monitoring programme as it offers enhanced sensitivity over the QTof, this method is currently accredited under Irish national accreditation board (INAB) to ISO 17025 standards. The TSQ is operated in selective reaction monitoring mode (MRM). No scanning takes place during MRM; the MS1 and MS2 quadrupoles only allow transmission of a specified parent ion, which gives a specified daughter ion to be monitored. This is the most selective and sensitive mode because only a specific ion, which fragments to produce the specific daughter ion, will be monitored for the whole of the scan time cycle. The MS conditions are outlined in Table 2-3.

When the QToF instrument was used with the above-described LC methods it was operated in ToF-MS-MS mode, where the molecular ion is isolated in the quadrupole and where after collision in the collision cell, the whole fragmentation spectrum is obtained in the detector (ToF). Collision energies of 50 and 35 eV (for AZA and OA group respectively) were applied in the collision cell for monitoring the following ions: AZA1 (m/z = 842.5), AZA2 (m/z = 856.5), AZA3 (m/z = 828.5), OA and DTX2 (m/z 803.5) and DTX1 (817.5). Quantification was carried out on the most abundant fragment selected from the TOF spectrum.

For both instruments linear calibrations were obtained using mixed AZA1 and OA standards with good regression data (average >0.998). AZA2 and-3 concentrations were quantified using the AZA1 standards, DTX1 and -2 concentrations are quantified using OA standards (assuming equal molar responses). AZA-1 to 3 are analysed in positive ionisation mode (ES+) whereas OA, DTX1 and 2 are analysed in negative ionisation mode (ES-).

Table 2-3: MS conditions for MI method using LC-TSQ-MS

Compound	Theoretical mass (m/z)	Transitions monitored	Cone voltage (V)	Collision energy (eV)	Ionisation mode
OA	803.5	803.5>803.5 803.5>255.1	50	40	-
DTX1	817.5	817.5>255.1 817.5>817.5	50	40	-
DTX2	803.5	803.5>803.5 803.5>255.1	50	40	-
AZA1	842.5	842.5>654.4 842.5>672.4	90	50	+
AZA2	856.5	856.5>654.4 856.5>672.4	90	50	+
AZA3	828.5	828.5>640.4 828.5>658.4	90	50	+
PTX2	876.5	876.5>823.5 876.5>876.5	30	30	+

2.5.2. BIOTOX method A (Hypersil BDS) for the analysis of lipophilic toxins by LCMS

The BIOTOX method is similar to the method described above; however, there were some minor differences. The mobile phase and the analytical column used were the same but the mobile phase was operated at a lower flow rate of 0.2ml/min and the column did not have a guard column attached.

The gradient also slightly differed, starting at 30% B and increased linearly to 90% B over 8 minutes. The gradient was held at 90% B for 2.5 minutes and then decreased to 30% B over 0.5 minutes. The gradient remained at 30 % B for 4 minutes to allow equilibration prior to the next injection. An injection volume of 10uL was used.

The MS conditions were as outlined above are the same when using the TSQ and QToF instruments.

2.5.3. BIOTOX method B (XBridge) for the analysis of lipophilic toxins by LCMS

The third method employed a basic mobile phase using ammonium hydroxide (pH of approximately 11). The aqueous mobile phase A was prepared using de-ionised water (100%) with ammonium hydroxide (0.05%). Mobile phase B was prepared using acetonitrile (95%), de-ionised water (5%) and with ammonium hydroxide (0.05%).

The XBridge column (C18, 5µm, 3.0 x 150mm) has a wider range of pH stability as the column is packed with a monomer, which contains a preformed ethylene bridge that imparts the high pH dissolution resistance of polymer material into a silica backbone. This allows the use of the basic mobile phase, which can improve the elution of acidic toxins from reversed phase columns. The mobile phase was operated at a flow rate of 0.4mL/minute.

A gradient elution was used starting at 10% B and increased linearly to 100% B over 10 minutes. The gradient was held at 100% B for 3 minutes and then decreased to 10% B over 1 minute. The gradient remained at 10 % B for 4 minutes to allow equilibration prior to the next injection. An injection volume of 5 uL was used. The same transitions as in both previous methods were monitored, however the cone voltage (V) and collision energies (eV) were different (as outlined in Table 2-4).

Table 2-4: MS conditions for BIOTOX method B for analysis using LC-TSQ-MS

Compound	Theoretical mass (m/z)	Transitions monitored	Cone voltage (V)	Collision energy (eV)	Ionisation mode
OA	803.5	803.5>255.2 803.5>113	60	50	-
DTX1	817.5	817.5>255.1 817.5>817.5	60	50	-
DTX2	803.5	803.5>255.2 803.5>113	60	50	-
YTX	1141.8	1141.8>1141.8 1141.8>1061.6	60	35	
AZA1	842.5	842.5>824.4 842.5>672.4	40	40	+
AZA2	856.5	856.5>654.4 856.5>672.4	40	40	+
AZA3	828.5	828.5>640.4 828.5>658.4	40	40	+
PTX2	876.5	876.5>823.5 876.5>551.3	40	30	+

3. LCMS method development and validation

3.1. Introduction

This chapter describes a number of collaborations that were undertaken with other laboratories in the study of LC separation (column type and gradient conditions). The early development of a further LCMS method for the main lipophilic toxins is also described.
The MS parameters were examined through an inter-laboratory study (Transition Study), which was coordinated by the Marine Institute involving a number of MS analysers.

3.2. Liquid chromatography (LC) separation

This section describes a collaborative study including three laboratories, to assess the LC separation of the lipophilic marine toxins. These trials were aimed towards deciding upon a set of LC conditions to be used in any further studies. The development of an additional LCMS method (BIOTOX method B) is also described; the need for an additional method arose due to certain difficulties that were being experienced in the preliminary method (BIOTOX method A). Both of the methods were outlined fully in the experimental chapter 2 and are referenced in the appropriate experimental section of the chapters where the method was used. This chapter outlines the development that was undertaken.
The column and gradient trials involved two partners from the BIOTOX project: RIKILT (Institute for Food Safety, Wageningen, The Netherlands) and NSVS (National School of Veterinary Science). Columns were selected from previously reported methods and from columns available within the collaborators laboratories.

3.2.1. BIOTOX LC method (A)

This method, described in section 2.5.2 was adapted from Quilliam *et al*, (2001) and later described by Hess *et al*, (2003) and uses a binary mobile phase and gradient elution which operates at a flow rate of 0.25ml/min. The analytical LC column was a Thermo electron BDS Hypersil C8 column (50 x 2mm, 3μm).

A series of columns were tested to investigate the role of the column choice in the separation of toxins. Following this a number of gradient elution patterns (as put forward by the participants in the study) were examined using the column of choice arising from the previous column experiments.

3.2.1.1. Preparation of materials

3.2.1.1.1. Toxins standards and reference materials

A laboratory reference material (LRM) prepared at the MI containing OA, DTX1 and-2, esters and AZA1, -2, -3 was used along with a CRM-OA-MUS-b (NRC Institute for Marine Biosciences, Halifax, Canada) containing OA and DTX1. PTX-2 standard (CRM-PTX2) was obtained from the NRC for spiking into the tissues. A sample of mussel HP contaminated with YTX, 45-OH-YTX and carboxy-YTX was sent from NSVS (Norway).

3.2.1.2. Extraction procedure

Toxins were extracted from the tissues using the BIOTOX extraction method as described in section 2.4.2.

3.2.1.3. LC method development

The LCMS analyses for this period of work were conducted using LC- QToF-MS (as described in section 2.3.1.).

3.2.1.3.1. Influence of column type on separation

The first series of experiments examined the influence of the LC column in the separation of the OA (OA, DTX1 and -2) and AZA (AZA1, -2 and -3) toxins groups. Four silica based reversed phase columns were included in this study. The columns and a description of their properties are shown below.

Table 3-1: Dimensions and physiochemical properties of the columns used in examining the influence of column type on separation

Column	Length (mm)	Internal diameter (mm)	Particle size (µm)	Pore size (Å)	Surface area (m^2/g)	Carbon load (%)
BDS Hypersil C8	50	2	3	130	170	7
Ace 3 C18	30	2	3	100	300	15.5
Luna C18	15	2	5	100	400	17.5
Supelco RP amide C16	150	2	5	180	200	11

The mobile phase as described in section 2.5 was tested in isocratic conditions in both negative and positive ionisation mode. Using isocratic elution the individual toxin groups are eluted using constant mobile phase composition and in a shorter time, this allowed for the examination of a number of columns in a shorter period. The mobile phase operated at a flow rate of 0.2ml/min when the MS was operating in negative ionisation mode and 0.25ml/min in positive ionisation mode.

The columns were evaluated using three parameters given below and which characterise the chromatographic process (Equation 3-1 to 3-3):

1. Resolution (R)
2. Plate number (N)
3. Plate height (H)

The separation of two components is of particular importance when one is being determined in the presence of the other; this is defined as the resolution (R), and is calculated as follows;

$$R = \frac{t_B - t_A}{0.5 \times (w_A + w_B)}$$

Equation 3-1: Calculation of resolution (R)

Where w_A and w_B *are the peak width of the detector responses from the two components measured in time units*

And t_B *and* t_A *are termed the retention time of the two components and correspond to the time taken for an analyte to elute from the analytical column.*

An approach for measuring how efficiently a column is performing is to apply the theoretical plate model of chromatography. The plate model presumes that in a chromatographic column a large number of equilibrations occur between the analyte, mobile phase and stationary phase. Each equilibrium is referred to as a plate (Niessen, 1999). The column efficiency is expressed, either by stating the number of theoretical plates in a column (N) (a larger value indicates more efficiency) or by stating the plate height (H) (a smaller value indicates a better efficiency).

$$N = 16\left(\frac{t_A}{w_A}\right)^2$$

Equation 3-2: Calculation of number of plates for the column to measure column efficiency

If the length of the column is L, then the plate height (H) is:

$$H = L / N$$

Equation 3-3: Calcnlation of plate height

3.2.1.3.2. Gradient elution patterns

The second series of experiments focused on designing an appropriate gradient elution pattern. Three gradient patterns were proposed by the participants and are outlined below.

1. Marine Institute gradient (previously outlined in section 2.4.2): starting with 30% B (95% acetonitrile) at time zero linearly rising to 90% B at 8 minutes. Then, 90% B was held for 2.5 minutes, decreased to 30% B over 0.5 minutes which was held again for 4 minutes until the next run;
2. RIKILT Gradient: starting with 30% B (95% acetonitrile) at time zero linearly rising to 90% B at 8 minutes. Then, 90% B was held for 0.5 minutes, decreased to 30% B over 1.5 minutes which was held again for 5 minutes until the next run;
3. NSVS Gradient: starting with 35% B (95% acetonitrile) at time zero linearly rising to 100% B at 6 minutes. Then, 100% B was held for 9 minutes. The system was run for 10 minutes at 35% B before the next injection.

3.2.1.4. Results

3.2.1.4.1. Influence of column type

The AZAs were well separated on all of the tested columns. The chromatographic parameters are described in Table 3-2 and the corresponding chromatograms are represented in figures 3-1 to 3-4. The elution order was the same regardless of the column, with AZA3 eluting first followed by AZA1 and finally AZA2. The columns were differentiated upon using chromatographic parameters such as: resolution between components of the mixture and the theoretical plate model of chromatography (plate number (N) and plate height (H)).

An acceptable value for the resolution would be a number greater than 1.5 (this would correspond to base-line resolved peaks) and all of the columns achieved this level of separation between toxins. The highest resolution values were achieved using the BDS Hypersil C8 column (followed by the Luna C18). The parameters, which relate to the column efficiency (plate height (N) and plate height (H)), indicated that the Luna column corresponded to the most efficient choice of column (highest plate number (6053) and lowest plate height (0.01).

Table 3-2: Chromatographic properties calculated from chromatograms obtained after LC-QToF-MS analysis in positive mode ionisation of LRM and CRM material

	BDS Hypersil C8	Luna C18	Ace C18	RP Amide C16
Resolution (R) AZA3 + AZA1	6.15	5.70	2.97	3.94
Resolution (R) AZA1 + AZA2	4.29	4.16	2.08	2.82
Plate number (N) AZA1	4386	6053	1310	4409
Plate height (H) AZA1	0.08	0.01	0.06	0.07

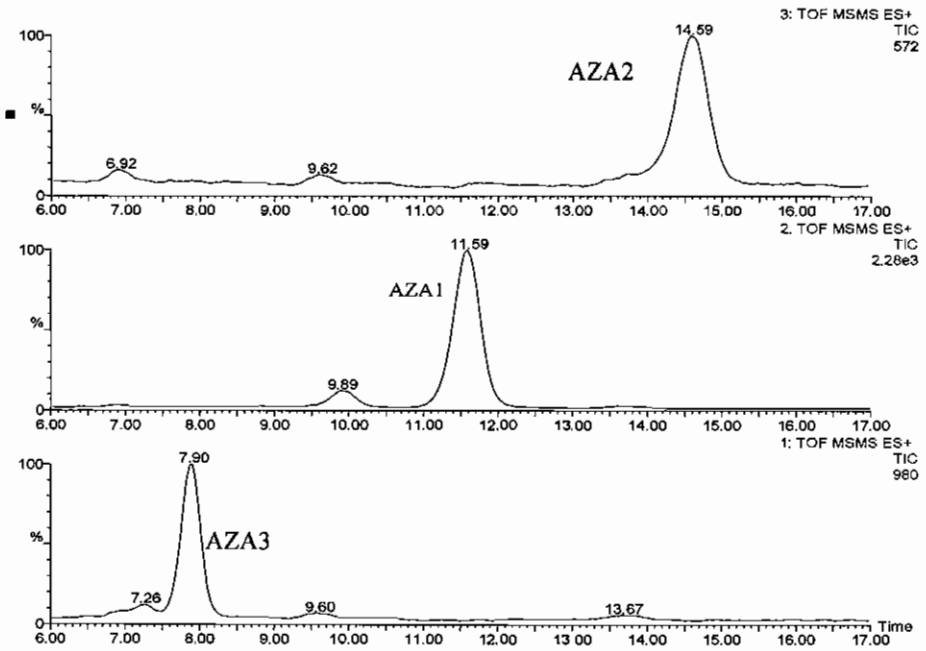


Figure 3-1: Separation of the AZAs on the BDS C8 column (50×2mm, 3µm) in isocratic conditions (60% B, 0.25ml/min; ES+)

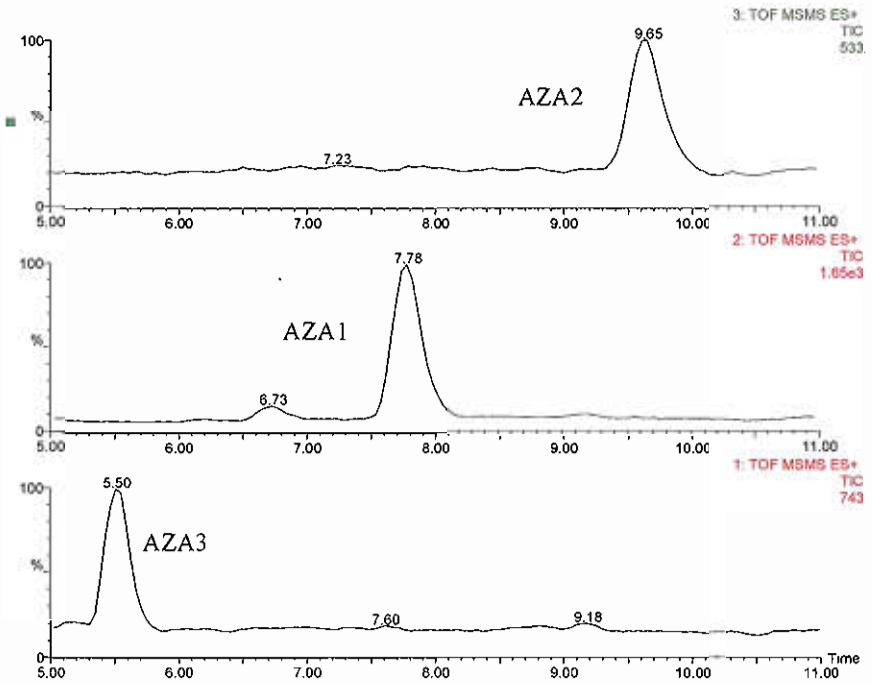


Figure 3-2: Separation of the AZAs on the Luna C18 column (150×2mm, 3µm) in isocratic conditions (60% B, 0.25ml/min; ES+)

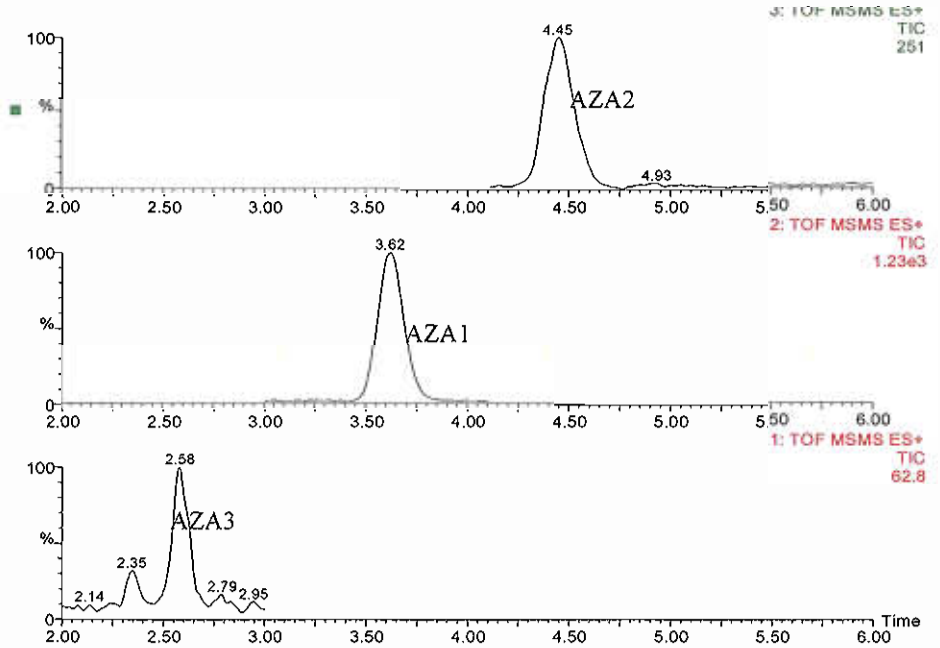


Figure 3-3: Separation of the AZAs on the Ace C18 column (30×2mm, 3µm) in isocratic conditions (60% B, 0.25ml/min; ES+)

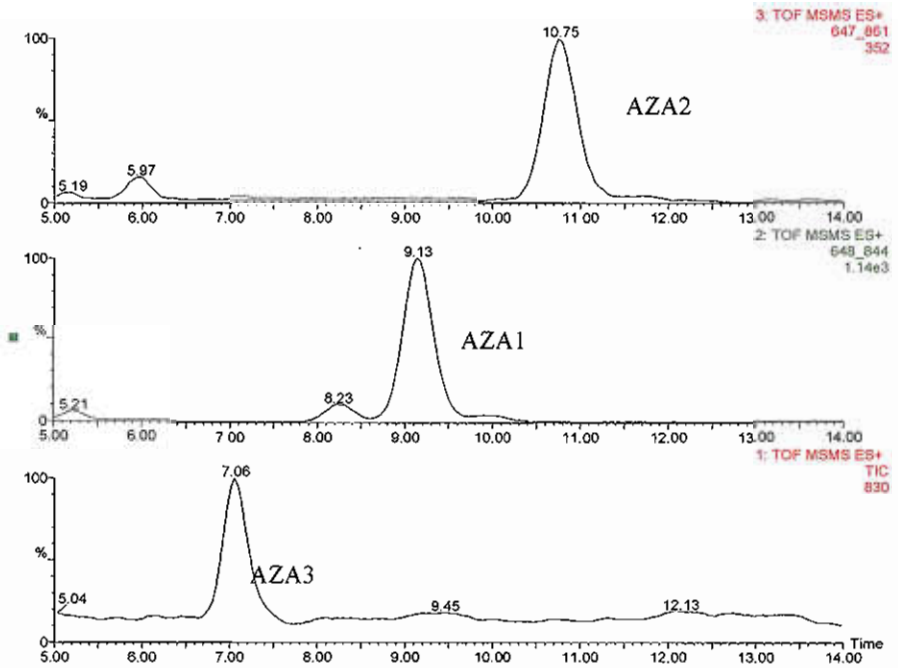


Figure 3-4: Separation of the AZAs on the RP Amide C16 column (150×2mm, 5µm) in isocratic conditions (60% B, 0.25ml/min; ES+)

The chromatograms obtained from the column trials for the OA group are presented below (Figure 3-5 to Figure 3-8). The elution order was the same regardless of the column type; OA eluted first, followed by DTX-2 and then DTX-1 eluted last. For the BDS Hypersil C8, ACE C18 and RP Amide columns the retention window was short (ranging between 2 and 3.2 min). The Luna C18 column (Figure 3-6) emerged as the most efficient column, offering the best resolution between OA and DTX2 (5.9) although there was a large amount of background noise associated with DTX1, which eluted almost 12.5 minutes after DTX2 (the compound identified as DTX1 is in fact something else because the MS acquisition file was set up incorrectly and did not monitor DTX1 during the appropriate retention window). This particular error was not recognised until after the results had been compiled and therefore it was not feasible to repeat the experiment.

Overall, the column which offered the best compromise for all of the toxins was the BDS Hypersil C8, and for this reason this column was used in the next series of experiments testing a number of gradient conditions.

Table 3-3: Chromatographic properties calculated from chromatograms obtained after LC-QToF-MS analysis in negative mode ionisation of LRM and CRM material

	Hypersil BDS C8	Luna C18	Ace C18	RP Amide C16
Plate number (N) OA	6300	10221	506	2240
Plate height (H) OA	0.08	0.01	0.06	0.07
Resolution (R) OA + DTX2	0.98	5.90	1.09	1.69
Resolution (R) DTX2 + DTX1	3.13	DTX1not identified	3.98	7.60

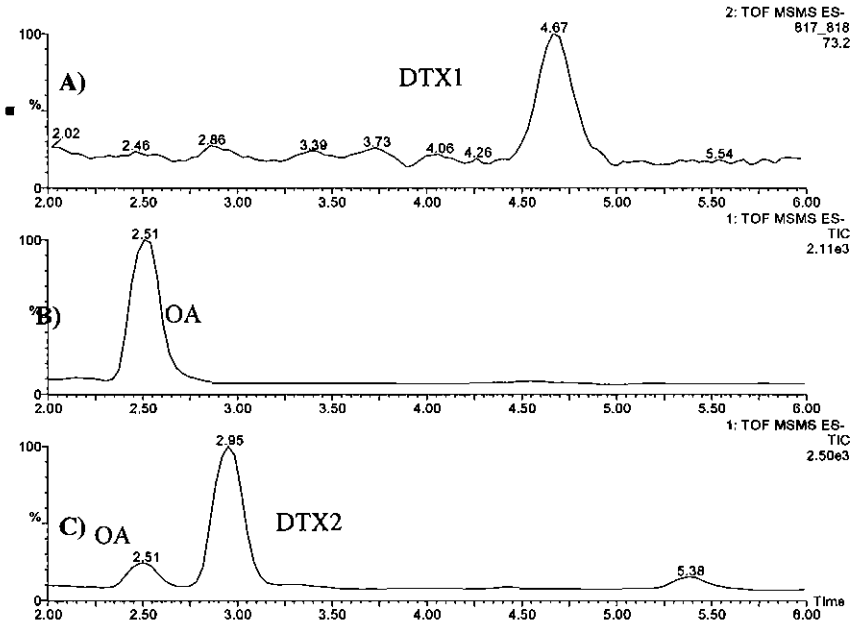


Figure 3-5: Separation of OA, DTX2 and DTX1 on the BDS Hypersil C8 column (50×2mm, 3µm) in isocratic conditions (55% B, 0.2ml/min, ES-). A, B) CRM; C) LRM

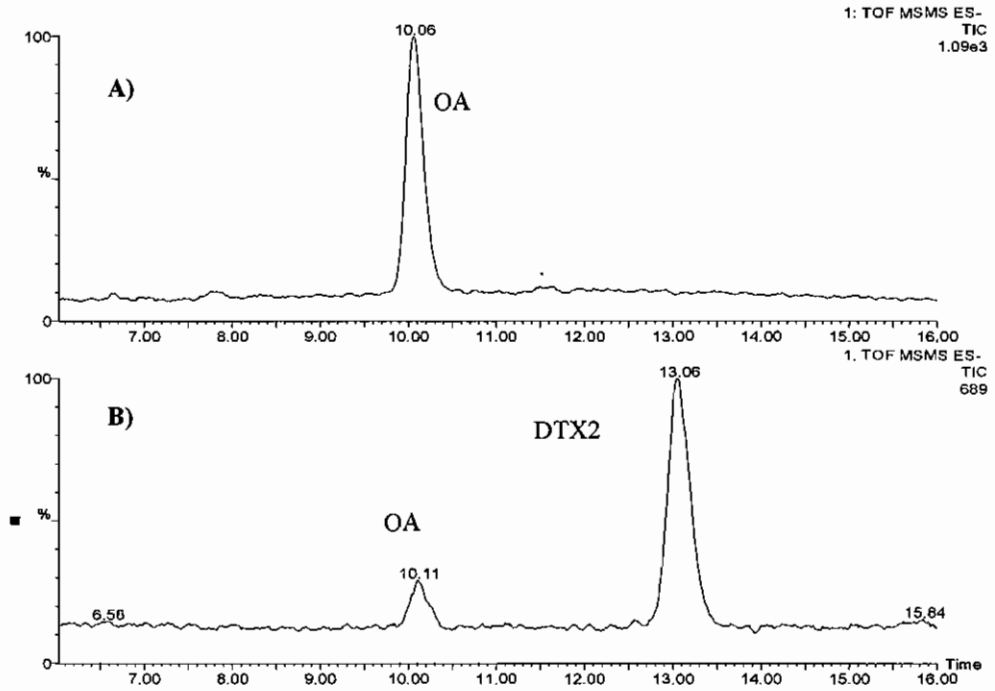


Figure 3-6: Separation of OA and DTX2 on the Luna C18 column (150×2mm, 3µm) in isocratic conditions (55% B, 0.2ml/miu, ES-). A) CRM; B) LRM

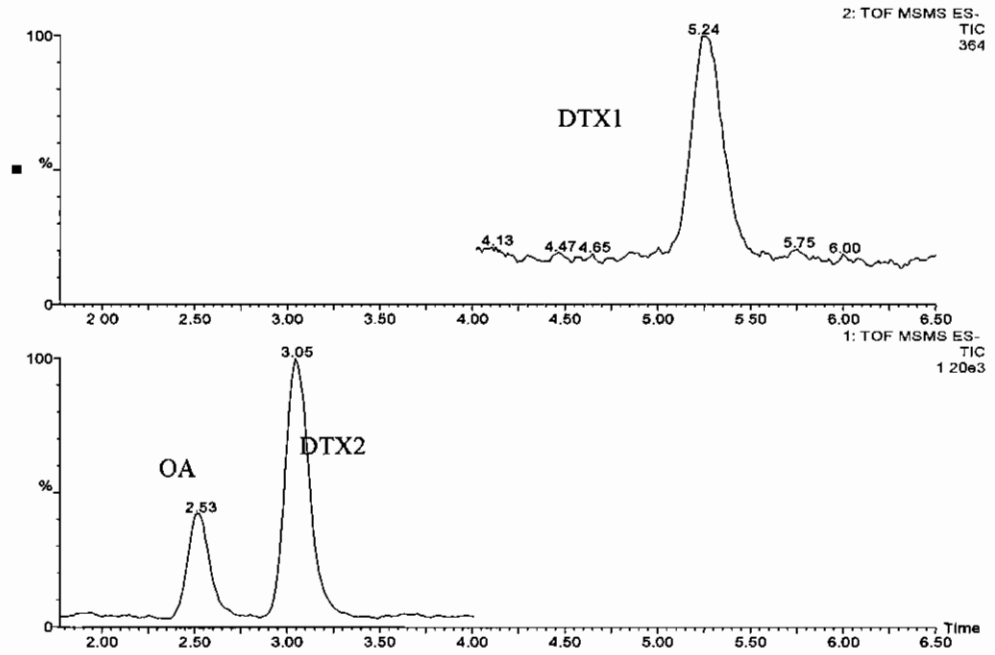


Figure 3-7: Separation of OA, DTX2 and DTX1 on the Ace C18 column (30×2mm, 3µm) in isocratic conditions (55% B, 0.2ml/min, ES-).

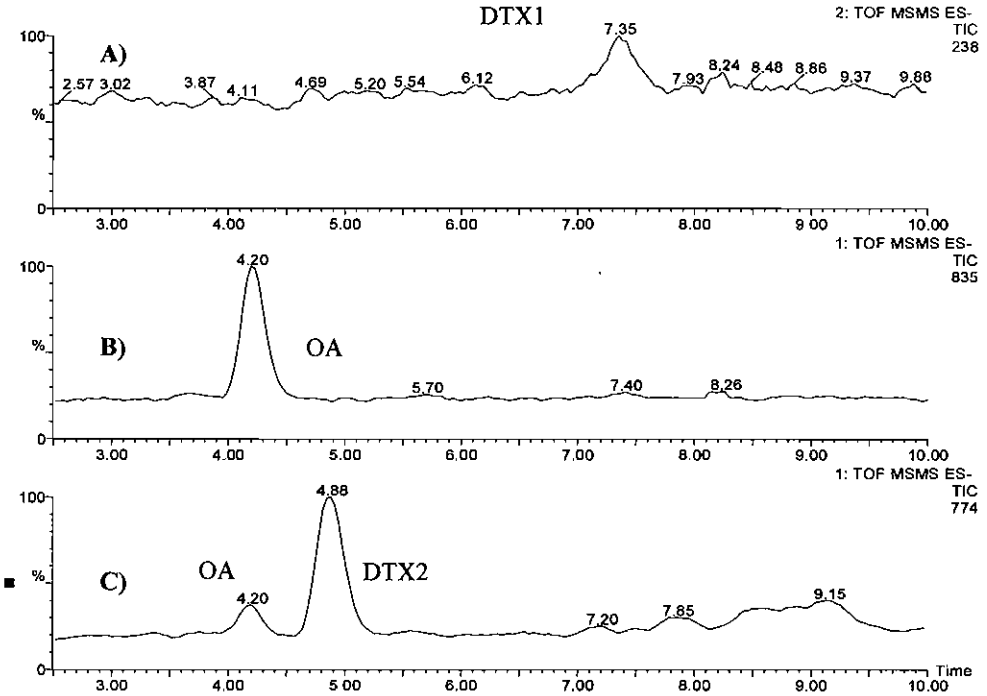


Figure 3-8: Separation of OA, DTX2 and DTX1 on the RP Amide C16 column (150×2mm, 5µm) in isocratic conditions (55% B, 0.2ml/min, ES-). a, b) CRM; c) LRM.

3.2.1.4.2. Gradient elution patterns

This analysis for this study was carried out using LC-QToF-MS and therefore separate runs were required for toxins monitored in positive (PTX2, AZA1, -2 and –3) and negative (OA, DTX1, -2, YTX, 45-OH-YTX and carboxy-YTX) mode ionisation.

An identical elution pattern was found in the MI and RIKILT gradient conditions, even though the gradient conditions differed slightly after 8.5 mins. Figure 3-9 represents the separation achieved at the MI. The AZA toxins eluted quite close together (approx 1.3 mins), which was much quicker than when using isocratic elution (Figure3-1). This resulted in sharp peaks and increased sensitivity. The toxins analysed in negative mode eluted in 2.82 mins and were well separated, although 45-OH-YTX and carboxy-YTX elute very close (0.3 mins).

Using the NSVS gradient conditions (Figure 3-10) the elution order was the same as in the MI/RIKILT conditions in both ionisation modes. The retention window for the AZAs was very narrow (approx 1 min), which was a limiting factor. In negative mode ionisation the toxins all eluted approx 1min later than when using the MI conditions and in 1.8 minutes, which was a very narrow elution window for good resolution of such a number of toxins. The same narrow elution window was experienced for 45-OH-YTX and carboxy-YTX (0.25 mins).

There was some evidence of co-elution of some toxins (PTX2/DTX-2 and DTX1/AZA3) under all gradient conditions. For DTX1/AZA3, this may have been due to the absence of a guard column, which otherwise would have seen the AZA toxins eluting between 10-14 minutes.

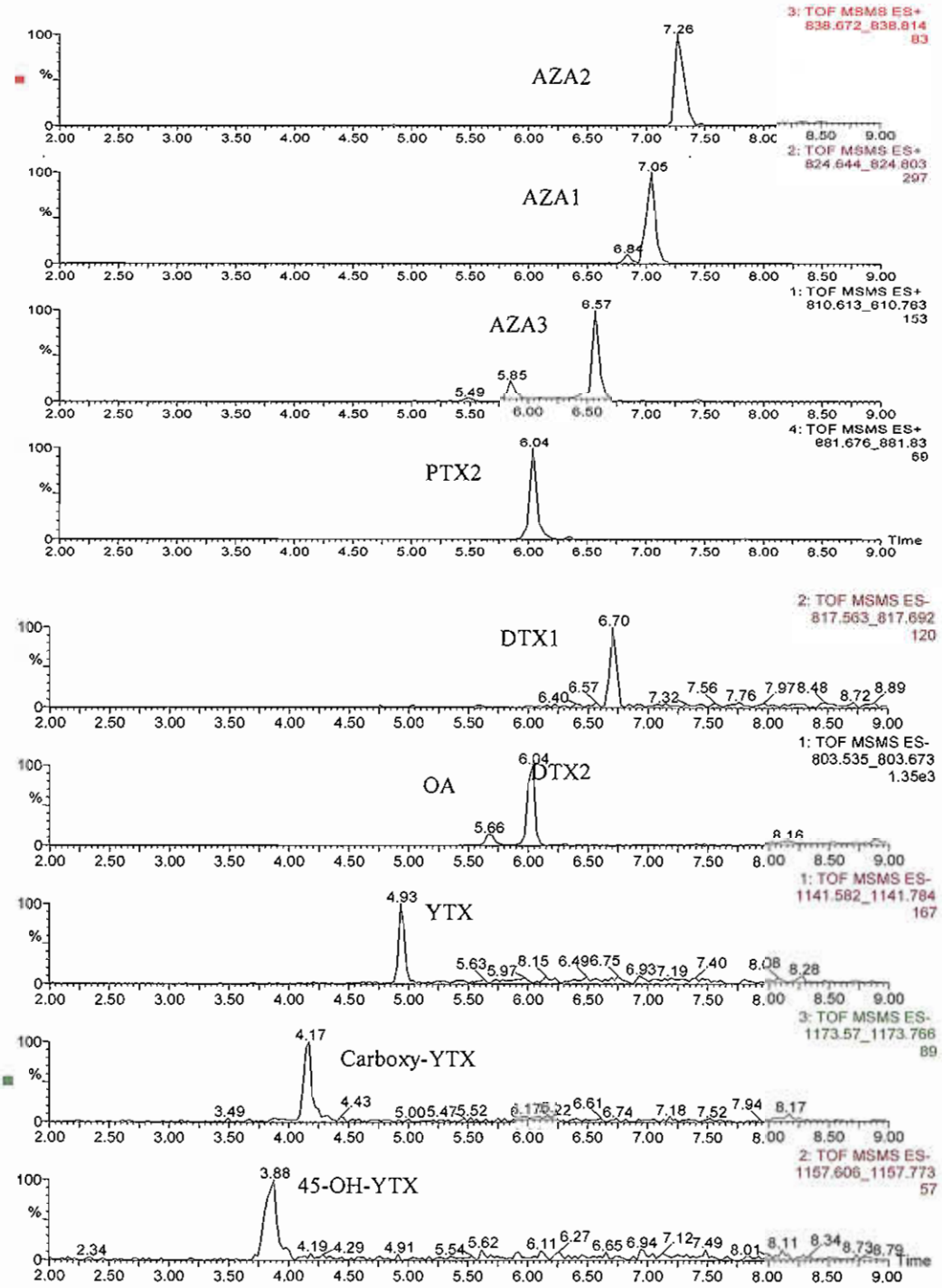


Figure 3-9: Separation of the lipophilic toxins on the BDS Hypersil C8 column (50×2mm, 3µm; no guard column) under MI gradient conditions, at 0.2ml/min

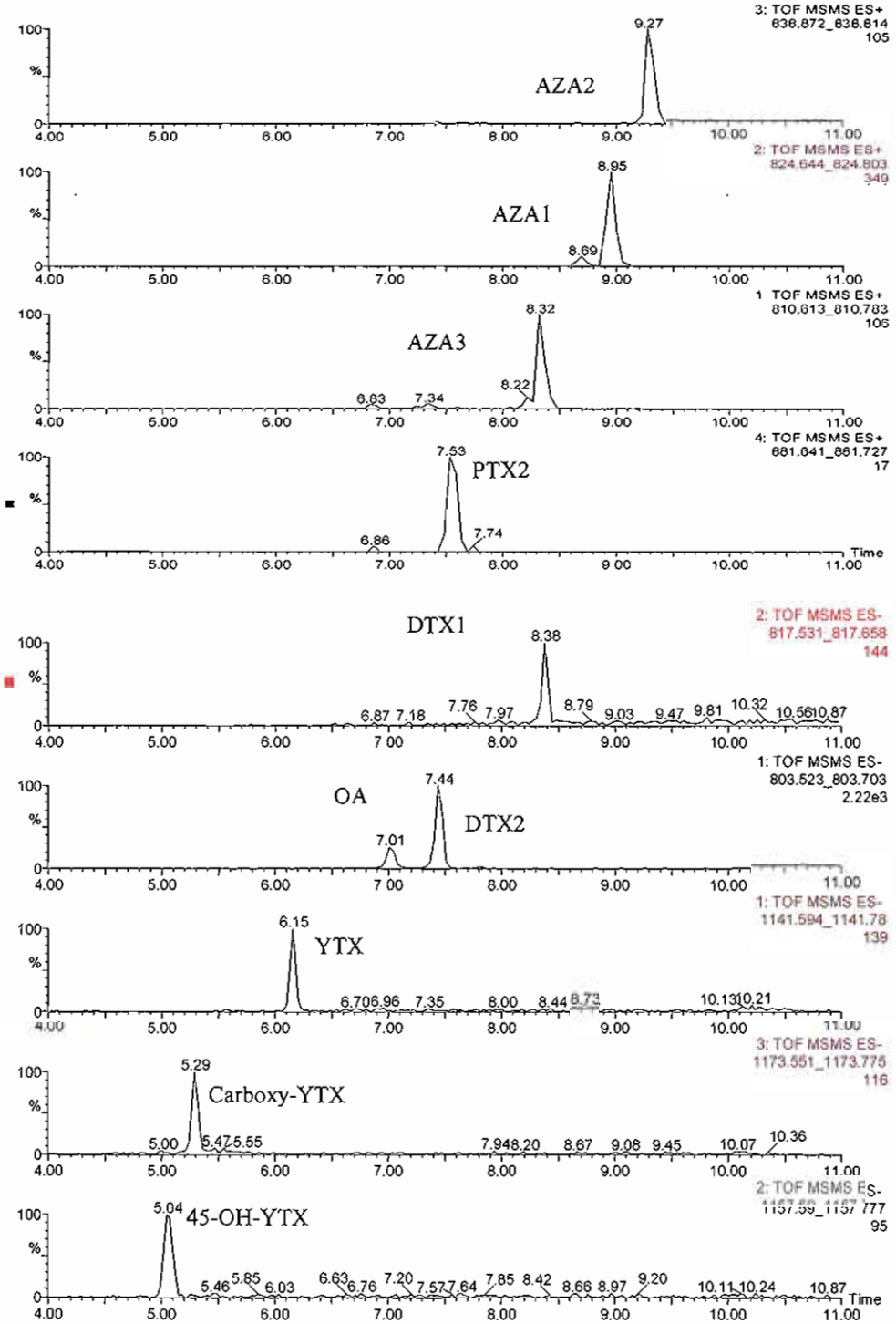


Figure 3-10: Separation of the lipophilic toxins on the BDS Hypersil C8 column (50×2mm, 3µm; no guard column) under NSVS gradient conditions, at 0.2ml/min.

3.2.1.5. Discussion

A series of columns with different dimensions were put forward for testing. The columns were differentiated using two factors, the resolution between components of the mixture and the packing efficiency of the columns (calculated by the number of plates (N) and the plate height (H)). In positive mode ionisation the BDS Hypersil C8 and Luna C18 columns represented the most efficient columns for the separation of AZAs. The best resolution between the AZAs (approx 5.22) is given achieved with the BDS Hypersil C8 column however the Luna C18 column has the highest plate number (N=6053) and lowest corresponding plate height (H=0.01).

In negative mode ionisation the column that gave the best separation (R=5.90) between OA and DTX2 and also resulted in the highest plate number (N=10221) was the Luna C18 column however this column could not be evaluated for the detection of DTX1 as there was a mistake with the MS acquisition file, which limited this column. The RP amide C16 column emerged as a good column choice for the analysis of the OA group toxins; good resolution was observed between OA/DTX2 (R=1.69) and DTX2/DTX1 (R=7.68), the plate number (N) and corresponding height was 2239 and 0.07 respectively.

The column, which was selected as a result of thee tests and is used currently at the Marine Institute and has been reported previously for the separation of marine toxins, is the BDS Hypersil C8 column (Quilliam *et al*, (2001); Hess *et al*, (2003); Suzuki *et al*, (2005); Stobo *et al*, (2005)). For this reason, this column was used in the gradient trials. In particular Stobo *et al*, 2005 has developed and validated the LCMS method according to IUPAC single laboratory validation requirements. The method used linear gradient conditions with a run time of 24 minutes and all toxins eluted in 12.5minutes.

For the gradient trials, the same elution order was found using the three proposed gradient conditions. Identical patterns of elution (retention time and elution order) were obtained between the gradient patterns of the MI and RIKILT and these conditions also represented the best separation. These experiments were conducted using LC-QToF-MS, which is not capable of polarity switching; therefore analysis was carried out in two separate sequences, however if detection was carried out using an MS system capable of positive-negative switching (such as the LC-TSQ-MS) then co-elution (PTX2/DTX-2; DTX1/AZA3) may be a problem. These experiments were also carried out in the absence of a guard column; this

would prevent the co-elution occurring for DTX1/AZA3 by increasing the retention of the AZA toxins.

3.2.2. BIOTOX LC method (B)

Through the use of the BIOTOX LC method (A) various drawbacks were noted such as, the difficulty that the BDS Hypersil C8 column has with the separation and detection of the YTX, a toxin that must be regarded during the LCMS method development. In addition some co elution was observed with (PTX2/DTX2; DTX1/AZA3), there are also concerns regarding the equilibration time at the end of the gradient being insufficient in eliminating matrix carryover. This carryover may lead to variability in retention times for certain toxins. These drawbacks highlighted the need for the development of alternative methods.

Waters have developed a new range of analytical columns, which can withstand a wide range of pHs and backpressures, the XBridge column is one such column. This allowed the use of a basic mobile phase to improve the retention and subsequent detection of YTX.

3.2.2.1. Preparation of materials

3.2.2.1.1. Toxins standards and reference materials

A multi-toxin standard was prepared in MeOH containing OA/PTX2 (40ng/ml), AZA1 (20ng/ml) and YTX (100ng/ml); the standards were obtained from the NRCC (NRC CRM OA, NRC CRM PTX2, NRC CRM YTX), the AZA1 used in the standards was described in section 2.2.2.

An LRM prepared at the MI was also used which contained OA, DTX1 and-2, esters, AZA1, -2, -3 and PTX2.

3.2.2.1.2. Extraction procedure

Toxins were extracted from the LRM using the extraction method as described in section 2.4.1

3.2.2.2. LCMS method development

The Xbridge column (C18, 5µm, 3.0 x 150mm) was tested using the acidic mobile phase as described in section 2.5.1 and 2.5.2. A gradient elution pattern was set up, which started at 30% B (95% acetonitrile) at time zero linearly rising to 90% B at 8 minutes. Then, 90% B was held for 2 minutes, decreased to 30% B over 1 minute which was held again for 2 minutes until the next run. The mobile phase was operated at 0.2mL/min with a sample injection volume of 10uL.

The Xbridge column was then tested using a basic mobile phase consisting of ammonium hydroxide. Mobile phase A= 100% aqueous with 0.05% NH_4OH. Mobile phase B = 95% acetonitrile with 0.05% NH_4OH. A binary gradient was created and started at 10% B and increased linearly to 100% B in 10min. This was held at 100% B for 3 min. The gradient was then linearly decreased to 10% B over 1 min and the system was left to equilibrate for another 4 min before the next injection. The mobile phase operated at a higher flow rate of 0.4 mL/min using an injection volume of 10uL.

3.2.2.3. Results

Below is a chromatogram illustrating the separation achieved with the Xbridge column using the universal mobile phase upon injection of the multi toxin standard (Figure 3-11); there was no resolution between the OA and YTX peak. When the Xbridge column was used with a basic mobile phase the separation and sensitivity of YTX improved significantly (Figure 3-12).

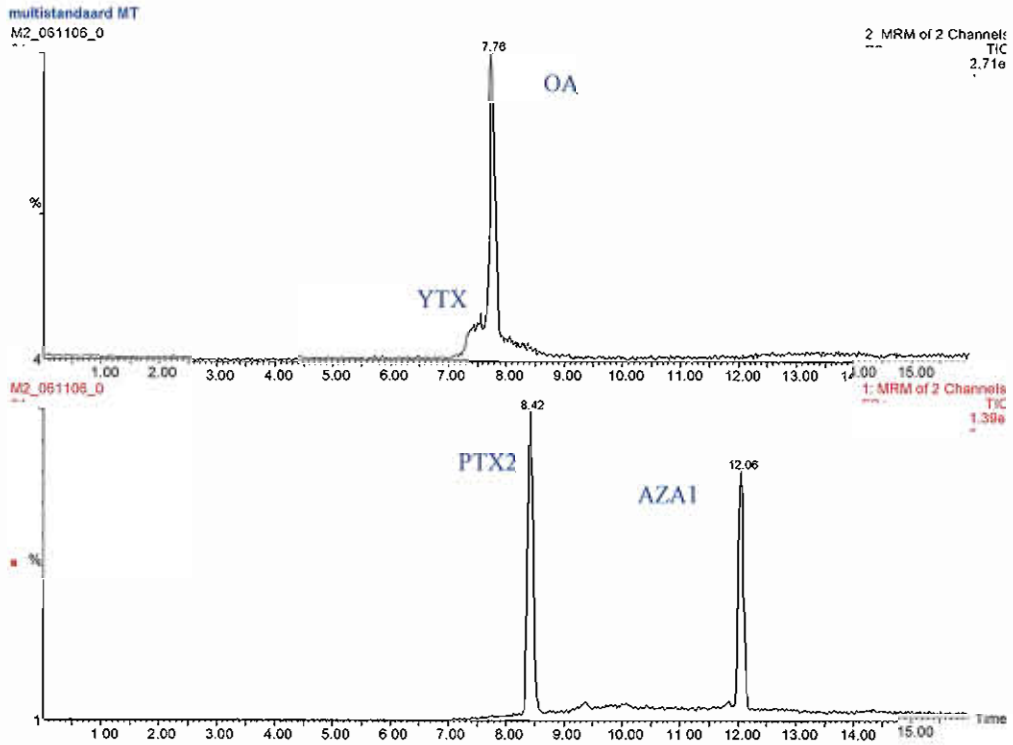


Figure 3-11: Separation of YTX, OA, PTX2 and AZA1 on the Xbridge column (150 x 2mm, 5µm) in gradient conditions using the universal mobile phase

An injection of the multi-toxin standard gave the following results (Figure 3-12) which was a significant improvement for the resolution of YTX. The peaks were well separated; OA eluted first (6.87mins), followed by YTX (7.35mins), AZA1 (9.12mins) and PTX2 (11.33). The peaks were sharp and there was no co-elution of toxins.

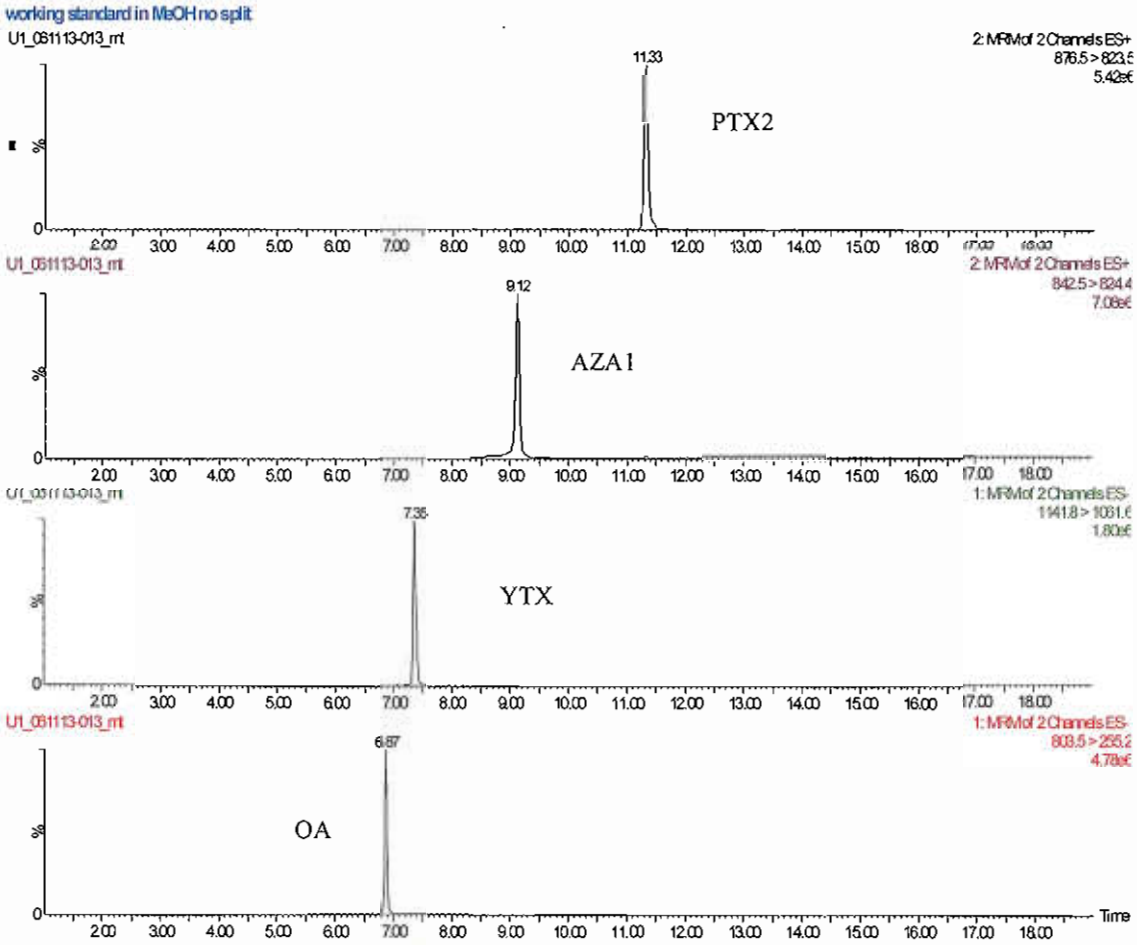


Figure 3-12: Separation of YTX, OA, PTX2 and AZA1 on the Xbridge column (150 x 2mm, 5µm) in gradient conditions using a binary gradient of mobile phases A (100% aqueous + 0.05% NH_4OH) and B (95% acetonitrile + 0.05% NH_4OH) analysed using LC-TSQ-MS

The separation achieved using an LRM material is shown below (Figure 3-13); all of the toxins eluted in 2.5 mins, which was a very narrow retention window for the elution of such a large number of toxins (except for PTX2 for which elutes 1.73 mins after AZA2). The resolution values (Table 3-4) reflected this short retention time frame, with R-values less than 1.5 between OA/DTX2, DTX2/YTX, YTX/DTX1 and AZA1/ AZA2. However, the separation of all toxins (except AZA1/AZA2) was sufficient (R>1).

The peak shape for the AZA toxins was poor (peak fronting), especially for AZA3 where the peak was almost half a minute wide.

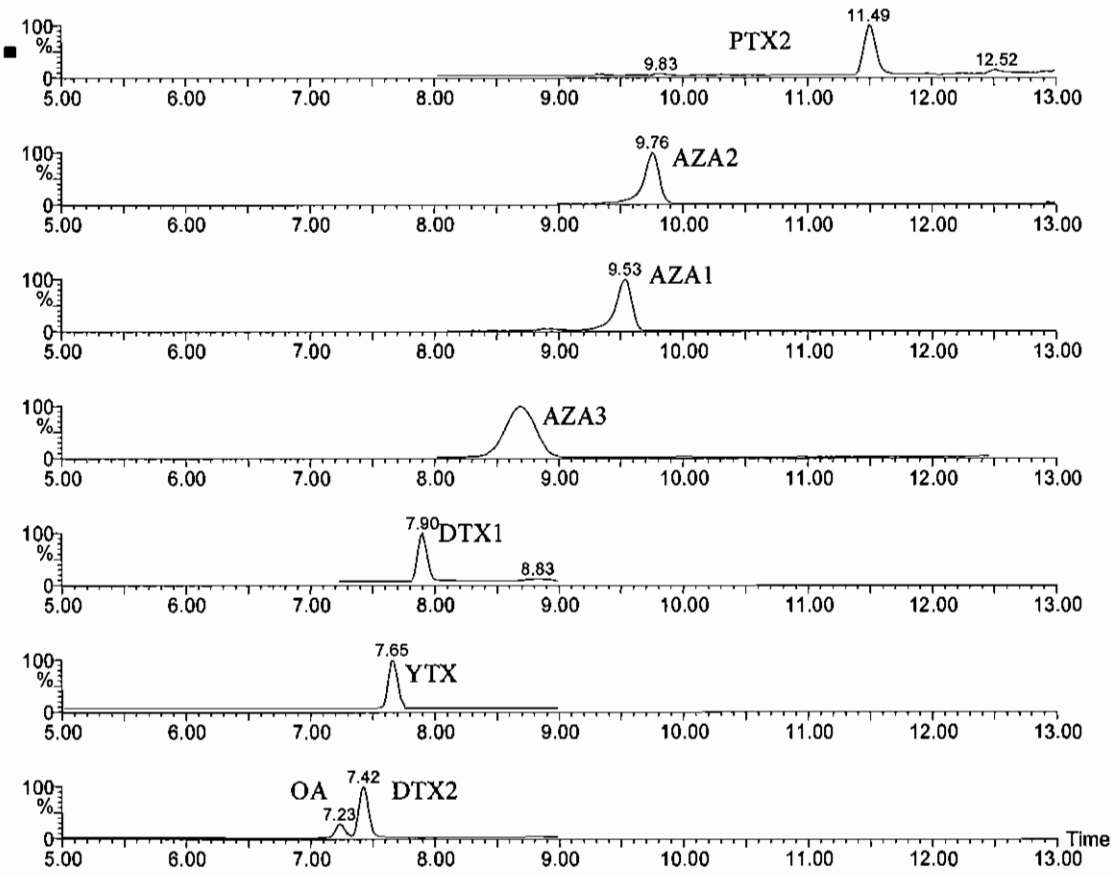


Figure 3-13: Separation of the lipophilic marine toxins on the Xbridge column (150 × 3mm, 5µm) using a binary gradient of mobile phases A (100% aqueous + 0.05% NH_4OH) and B (95% acetonitrile + 0.05% NH_4OH) analysed using LC-TSQ-MS

Table 3-4: Resolution of the lipophilic marine toxins on the Xbridge column (150 × 3mm, 5µm) under basic gradient conditions

	Compound 1		Compound 2		
	RT (min)	Width (min)	RT (min)	Width (min)	Resolution
OA / DTX2	7.23	0.15	7.42	0.21	1.06
DTX2 / YTX	7.42	0.21	7.65	0.19	1.16
YTX / DTX1	7.65	0.19	7.90	0.21	1.25
DTX1 / AZA3	7.90	0.21	8.69	0.59	1.98
AZA3 / AZA1	8.69	0.59	9.53	0.41	1.68
AZA1 / AZA2	9.53	0.41	9.76	0.48	0.51
AZA2 / PTX2	9.76	0.48	11.49	0.25	4.73

3.2.2.4. BIOTOX LC method (B) discussion

The need for alternative LC method/s arose following a preliminary validation study of BIOTOX method (A) including 18 laboratories. The pre-validation round highlighted the difficulties in reproducing results between laboratories when a specific set of LC and MS conditions were issued and where the prescribed method may not have been in regular use.

Further drawbacks were also experienced through use of the BIOTOX method (A) with the reproducibility of the separation and detection of the sulfated YTX. The pH (approximately 2.5) of the acidic mobile phase used in this method was not high enough to stabilise the negatively charged sulfate group on the YTX and therefore the compound was not being retained on the column. The inclusion of YTX during various other aspects of method development (sample clean-up) was an important requirement and therefore initiated the development of alternative methods to incorporate YTX.

The Xbridge column offers the separation of all of the regulated lipophilic marine toxins and this was achieved using a simple basic mobile phase (pH of approximately 10). With a higher pH the YTX was no longer in ionised form and was being retained on the column for a longer period. The resolution between the majorities of the toxins was greater than 1 (except for AZA1/AZA2), which was an indication that the separation is sufficient.

3.3. Transition study

The lack of calibration standards and reference materials is a significant problem in the development of LCMS methods for the analysis of marine toxins. In the case of the OA toxin group, there is currently only a commercial standard available for OA, which is also used in the quantification of DTX1 and DTX2. It may be the case that OA, DTX1 and DTX2 have different ratios of parent to fragment ion. Such differences in ion ratios would result in different quantitative results when using OA as the only calibrant to also quantify DTX1 and –2. The same situation is relevant to the AZA group where there is an AZA1 standard available, which is used for quantification of AZA2 and -3. To investigate these difficulties, a study was organised involving a number of partners involved in the BIOTOX project using a number of different mass analysers. The study examined a number of parameters:

1. Different ionisation modes (positive versus negative)
2. Different acquisition modes (single versus double transition monitoring)
3. Different transitions for a given compound

OA, DTX1 and DTX2 were analysed in both positive and negative mode; differences in repeatability and between lab comparability were examined. PTX2 was analysed in both modes and the same parameters were examined. AZA1, -2 and -3 were analysed in positive mode only. The results for the MI only are shown below, the conclusions arising from the study will also be discussed.

3.3.1. Experimental conditions

3.3.1.1. Preparation of materials

Each participating laboratory was supplied with two extracts of a laboratory reference material (LRM), which contains all of the relevant toxins included in the study (OA, DTX1, -2 PTX2, AZA1, -2 and -3).

Two matrix strengths (solvent to sample ratios (SSR)) were prepared for the study:

1. 4g extracted in 20ml (SSR = 5)
2. 2g extracted in 20ml (SSR = 10)

The LRM portions were extracted as described in section 2.4.2. Five LRM portions per matrix strength were prepared and combined to ensure a bulk homogenous solution and dispensed into glass vials for transport to the various institutes for analysis.

3.3.1.2. LCMS analysis

The toxins were separated using the BIOTOX conditions as previously outlined in section 2.5.2. Each lab was also provided with a set of calibrants prepared at the Marine Institute, containing OA, AZA1 and PTX2. The concentration ranges are indicated below:

Table 3-5: Concentration ranges of calibrants provided (by the MI) for the transition study

Toxins	Concentration range (ng/ml)
OA	7.9 – 13.6 – 26.9 – 80.2 – 134.7
AZA1	2.2 – 3.9 –7.6 – 22.8 – 38.4
PTX2	2.8 – 4.8 – 9.6 –28.6 – 48.0

The participants of the study each used different MS analysers; this is represented in Table 3-6, which shows the MS analysers available and the corresponding acquisition modes possible from such instruments. Using LC-TSQ-MS three different acquisition modes were

possible; parent monitoring mode, single transition monitoring, and double transition monitoring. The parent ions and transitions to be monitored are outlined in Table 3-7.

Each matrix strength sample was analysed in triplicate, with random positions in the run sequence; a standard was used throughout the run sequence to ensure that response of the MS did not drift significantly throughout the sequence (a minimum of one check standard, the second highest level of the standard curve was advised as the check standard).

Table 3-6: MS experiments possible according to the instrument available to the participants of the transition study

	Parent ion monitoring		SingleTransitions [2]		Double Transitions	
MS analyser	+ MODE	- MODE	+ MODE	- MODE	+ MODE	- MODE
SQ	1.OA, DTX'S, AZA 2. PTX-2 (1)	1. OA, DTX'S 2. PTX-2 (1)				
TSQ	1.OA, DTX'S, AZA 2. PTX-2 (1)	1. OA, DTX'S 2. PTX-2 (1)	1. OA, DTX'S, AZA 2. PTX-2 (1)	1. OA, DTX'S 2. PTX-2 (1)	1.OA, DTX'S, AZA 2. PTX-2 (1)	1. OA, DTX'S 2. PTX-2 (1)
Ion Trap	1.OA, DTX'S, AZA 2. PTX-2 (1)	1.OA, DTX'S 2. PTX-2 (1)			Trapping + selecting ion	Trapping + selecting ion

Note 1: PTX-2 was analysed separately due to co-elution with DTX-2; if sensitivity permitted, this was analysed in parallel

Note 2: Only 2 participants were asked to investigate the single transition mode

Table 3-7: Parent ions and transitions monitored for each compound in transition study

Toxins	Positive ion mode $[M + H]^+$, $[M + Na]^+$, $[M + NH_4]^+$, $[M - 2H_2O + H]^+$	Negative ion mode $[M - H]^-$
OA, DTX2	**827.5** 827.5>723.5 769.5>751.5	**803.5** 803.5>255.5 803.5>113.5
DTX1	**841.5** 841.5>737.5 783.5>765.5	**817.5** 817.5>255.5 817.5>113.5
PTX2	**876.5** 876.5 > 823.5 876.5 > 213.1	**857.5** 857.5> 627.4 857.5>137.1
AZA1	**842.5** 842.5>672.4 842.5>654.4	n/a
AZA2	**856.5** 856.5>672.4 856.5>654.4	n/a
AZA3	**828.5** 828.5>658.4 828.5>640.4	n/a

For the acquisition of the parent ion, there was only one channel acquired, hence quantification was carried out using this trace. For the acquisition using 2 transitions, calculations of concentrations were carried out separately for each transition.

The samples were analysed in triplicate and the results, expressed in ng/ml, were reported individually (not averaged).

To assess the importance of the matrix effects, the results expected for the SSR 5 extract (higher matrix strength) were calculated by doubling the results of the low matrix strength extract. Both sets of results (expected and found concentrations in SSR 5 extract) were compared by means of their % difference calculated according to the equation;

$$\% \text{ diff} = \frac{C_{SSR\,5\ expected} - C_{SSR\,5\,found}}{(C_{SSR\,5\,found} + C_{SSR\,5\,expected}) \div 2}$$

Equation 3-4 : Percentage difference experienced between expected and found results obtained from the higher matrix strength

Where $C_{SSR\,5\,found}$ *is the toxin concentration found in the SSR 5 extract*

And $C_{SSR\,5\,expected}$ *is twice the toxin concentration of the SSR 10 extract*

3.3.2. Results

The results obtained from the MI are presented below (Figure 3-14 to Figure 3-16). The toxins of the OA group (OA, DTX1 and -2) were analysed in positive and negative ionisation using parent ion monitoring (due to low sensitivity using MS/MS).

The AZA toxins and PTX2 were analysed in positive mode only using all three-acquisition modes. The LRM extracts used in the analysis were prepared at two different matrix strengths (SSR 5 and 10) to assess the role of matrix strength in MS detection.

The results for the OA toxins (Figure 3-14) indicated a significant difference between ionisation modes in parent ion monitoring. The results obtained from positive mode ionisation were significantly higher (up to 75%), compared to parent ion monitoring in negative mode.

In negative ionisation mode, the results were comparable regardless of the acquisition mode (parent ion monitoring, single or double transition monitoring). For double transition monitoring the variability between replicate injections was low (RSD of 6, 3 and 4% for OA, DTX2 and DTX1 respectively). The variability increased at higher matrix strength although the same trends were observed (RSD 7, 26 and 11% for OA, DTX2 and DTX1 respectively).

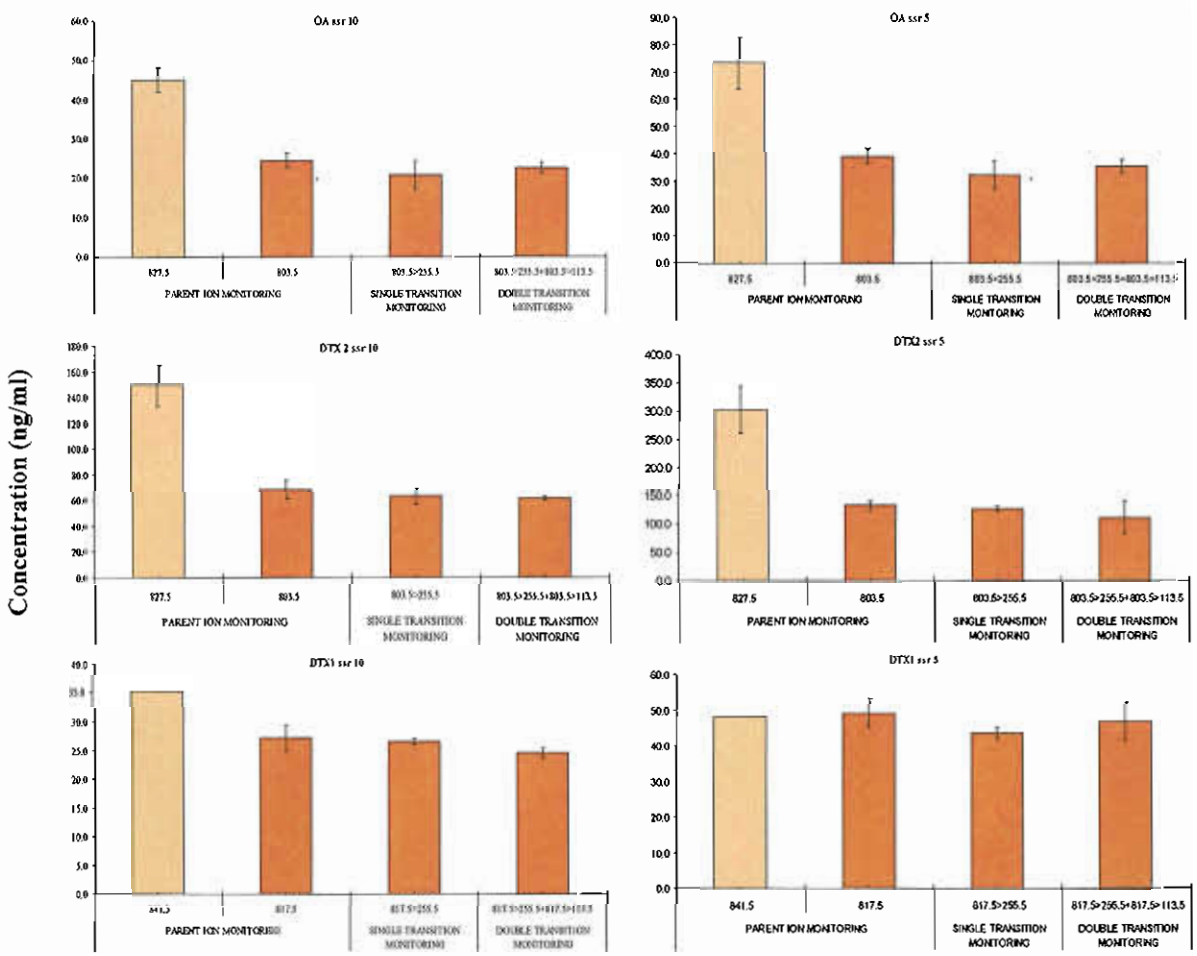


Figure 3-14: Concentrations of OA group toxins (OA, DTX1 and -2) in LRM material at two different matrix strengths (SSR 10 and 5) analysed using three different acquisition modes (parent ion monitoring (positive mode: light orange and negative mode: dark orange), single transition monitoring and double transition monitoring). Analysis conducted using LC-TSQ-MS. Error bars represent SD ± 1, n=3

The AZA toxins were analysed in positive mode ionisation only (Figure 3-15). In general, the concentrations found using parent ion monitoring were higher; concentrations were up to twofold higher (except for AZA2 (SSR 10), where the concentrations from single transition monitoring were the highest, and AZA3 (SSR5) where there was no significant difference between acquisition modes).

For AZA1 there was no significant difference between single and double transition monitoring; this was independent of matrix strength. As in the previous results for OA the variability between replicate injections was greater at higher matrix strength (% RSD ranging from 8-18%).

For AZA2 and -3 (SSR10) differences were observed between single and double transition monitoring. The concentrations obtained from double transition monitoring were considerably lower (approx 4 fold for AZA2). This same trend was not observed at a higher matrix strength where the trends were in agreement with what was observed for AZA1.

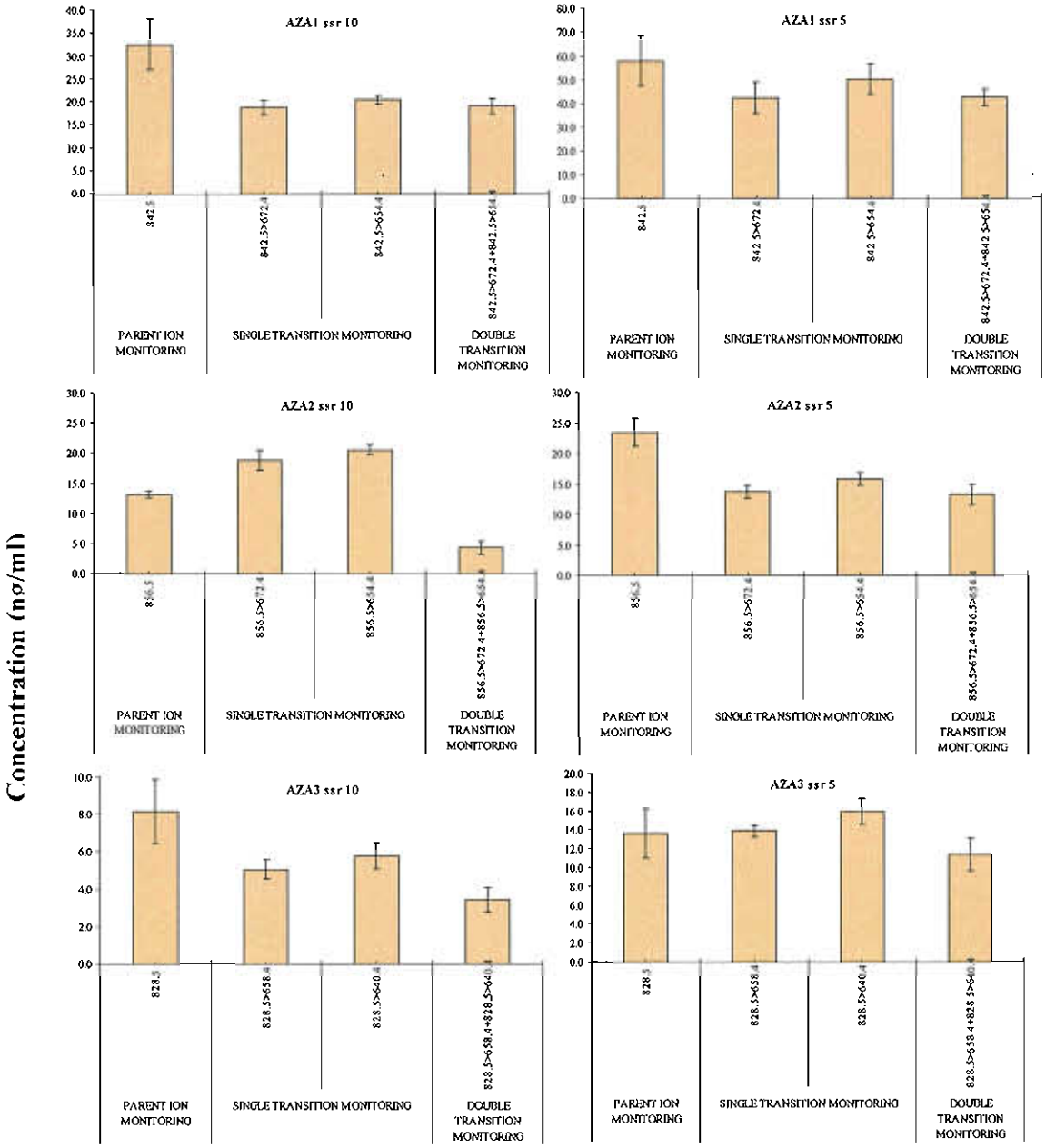


Figure 3-15: Concentrations of AZA group toxins (AZA1, -2 and -3) in LRM material at two different matrix strengths (SSR 10 and 5) analysed using three different acquisition modes (parent ion monitoring, single transition monitoring and double transition monitoring). Analysis conducted using LC-TSQ-MS. Error bars represent SD ± 1, n=3

For PTX2 (Figure 3-17)) there was a significant difference observed between parent ion monitoring and the two other acquisition modes used (single and double transition monitoring). At a lower matrix strength (SSR=10) the difference was related to a lower concentration in parent ion monitoring. At a higher matrix strength (SSR=5) this difference was related to a high variability associated with the replicate analysis in parent ion monitoring. There were no significant differences observed between single and double transition although the variability between replicate injections was lowest when using double transition monitoring (3.5 and 3% for SSR=10 and SSR=5 respectively).

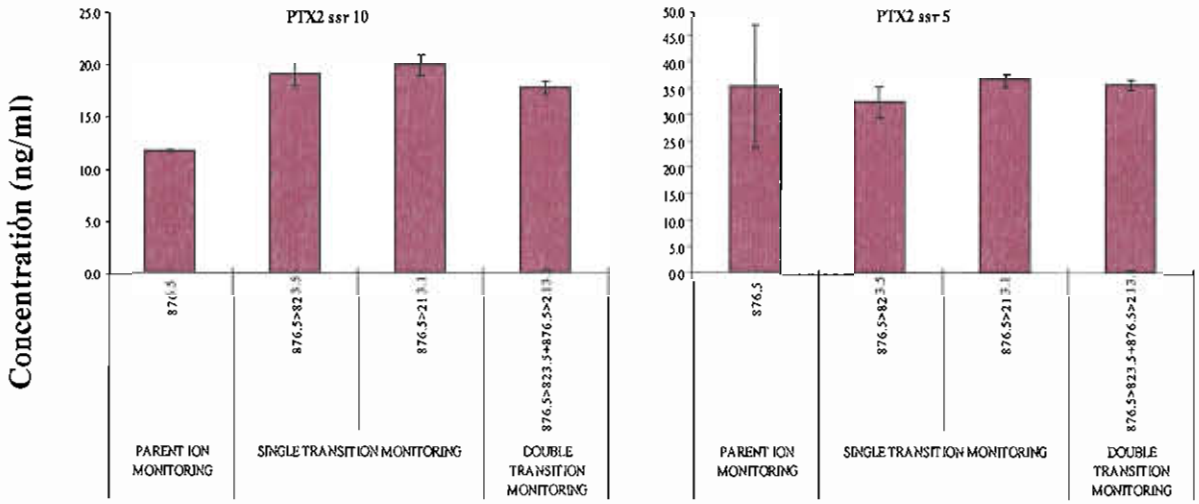


Figure 3-16: Concentrations of PTX2 in LRM material at two different matrix strengths (SSR 10 and 5) analysed using three different acquisition modes (parent ion monitoring, single transition monitoring and double transition monitoring). Analysis conducted using LC-TSQ-MS. Error bars represent SD ± 1, n=3

3.3.3. Discussion

This study included a number of laboratories with different MS detectors to assess the role that the MS conditions (ionisation mode, acquisition modes and choice of transition/s) play in the results obtained. The overall results of the transition study are presented in Appendix II and will be discussed as a function of the MI results here.

The OA toxins were the only group for which results for both ionisation modes were reported at the MI for sensitivity reasons; it was found that the ionisation mode significantly affected the results; in parent monitoring mode, the OA and DTX2 results reported by the participants were consistently and significantly higher in positive mode, except for participant 5 who found much higher OA and DTX2 concentrations in the negative ionisation mode (higher by a factor of 1.4 on average, observed at both matrix strengths). In the case of DTX1, all the participants reported higher results in negative mode. Between-laboratory variability was always greater in positive mode than in negative mode. This tendency is suggesting that matrix effects (ion enhancement) may play a more important role in positive acquisition mode for this group of compounds.

The results obtained from negative mode ionisation showed no significant differences between acquisition modes in all three toxins (OA, DTX1 and -2). Other laboratories, which reported results for single and double transition monitoring of the OA toxins in positive mode (769>751 and 827>723 for OA/DTX2 and 783>765 and 841>737 for DTX1) found significant differences between acquisition modes. These results raise some important concerns about the quantification of DTXs against OA, as OA and DTX2 behave differently from DTX1 depending on the conditions used.

For the AZA and PTX2 toxins (analysed in positive mode ionisation only) significant differences were observed between acquisition modes. For the AZAs, the results found in parent ion monitoring were higher than in single and double transition monitoring modes. In double transition monitoring no significant difference was observed for AZA1 within participants, even though there could be differences between participants. A similar trend was observed for AZA3 with good comparability of the data within labs except for a particular participant. For AZA2, differences were observed within participants.

Significant differences could be observed within and between participants when comparing single versus double transition monitoring.

For PTX2, the results in parent ion monitoring were variable when compared to single and double transition monitoring. At a lower matrix strength (SSR=10); the concentrations found were lower than the corresponding results in single and double transition monitoring, whereas at a higher matrix strength (SSR=5) the results were comparable but more variable than those of the other acquisition modes. Other participants in the study confirmed this. For PTX2 this higher variability may be due to an interference, with the same molecular weight as PTX2 as when the molecule is fragmented in single and double transition monitoring the variability decreases. Depending on the transition monitored in positive mode significant differences could be observed between single and double transition monitoring. In double transition monitoring the participants reported a good comparability of the results within and between modes. Other participants in this study were capable of analysing PTX2 in both ionisation modes; it was reported that the choice of the ionisation and acquisition mode also affects the analysis of PTX2 but not to the same extent as it does for the OA group.

3.3.4. Conclusion

This study found that the choice of ionisation mode, acquisition mode and transitions is important in MS analysis as it affects the results for the different toxins. Differences between negative and positive ionisation mode in parent ion monitoring were assessed for the OA group and PTX2 (not at MI). For these toxins the results between ionisation modes were significantly different, the difference was more critical in the case of the OA group. OA, DTX2 and PTX2 results were generally higher in positive mode, whereas DTX1 levels were higher in negative mode.

In double transition monitoring, no significant difference was observed within labs for OA/DTXs in negative mode and for AZA1 in positive mode. A good comparability was observed for PTX2 data within and between ionisation modes.

Two participants were asked to compare single and double transition monitoring (using Triple Stage Quadrupole (TSQ) detectors) and in some cases not all the data could be reported because of sensitivity issues; therefore the observations made may not be generalised. For these 2 laboratories, no significant difference was observed between both acquisition modes for the 803>255 and 817>255 transitions of OA/DTX2 and DTX1 respectively. However, in the case of PTX2 and AZAs the reported results could be different between acquisition modes depending on the transition monitored.

This study was brought about following concerns about the quantification of certain toxins against another toxin of the same group; this is necessary due to the lack of commercially available calibrants. This study raises some concerns about the quantification of DTXs against OA, as OA and DTX2 behave differently from DTX1 depending on the conditions used.

From the whole range of conditions assessed, it turned out that negative ionisation would be better suited for OA/DTXs, at least in the interest of minimising the between laboratory differences. Positive ionisation only has been used for AZAs to date. Either ionisation mode could be chosen for PTX2; negative ionisation would have the advantage of avoiding positive/negative switching at the retention time of DTX2 and PTX2 as these toxins co-elute in the BIOTOX chromatographic conditions. However these conditions would need to be optimised further.

3.4. Conclusion for LCMS method development and validation

The objective of this work was to evaluate critical parameters of the LC separation and MS detection, to identify the areas, which could lead to variability within and between laboratories in validation of the method. The separation and detection of the lipophilic toxins present a challenge due to the large number of compounds. Not every laboratory involved in the analysis of marine toxins uses the same MS system, and will therefore have different detection capabilities. The compromise is to suggest a method/s, which can provide the most repeatable results between laboratories.

The choice of column and gradient conditions were identified as critical parameters for the LC separation. The columns were differentiated upon their efficiency in the separation of toxins, using the plate number (N), plate height (H) and resolution (R). The BDS Hypersil C8 column, used currently at the Marine Institute and reported previously for the separation of marine toxins (Quilliam *et al*, 2001; Hess *et al*, 2003; Suzuki *et al*, 2005; Stobo *et al*, 2005) emerged as a viable column. Three gradient conditions were proposed; the most successful one achieved good resolution between toxins (MI gradient). The method was taken into a validation round, large variability was found within and between laboratories. The study highlighted the difficulties in proposing a specific set of LCMS conditions and concluded that the method was not fit for standardisation. Arising from this validation study and further difficulties with the separation and detection of YTX an additional method was developed. Using an Xbridge C18 column and a basic mobile phase, separation of all of the toxins was achieved with sufficient resolution and detection.

The role that the MS conditions have on the results obtained was examined; it was found that the choice of ionisation mode, acquisition mode and transitions is important in MS analysis as it can affect the results. For a laboratory involved in the analysis of marine toxins these MS conditions should be assessed for the particular MS analyser. From the different conditions assessed, it emerged that negative ionisation would be better suited for OA/DTXs, at least in the interest of minimising the between laboratory differences. Only positive ionisation has been shown for AZAs so far. Either ionisation mode could be chosen for PTX2; negative ionisation would have the advantage of avoiding positive/negative switching

at the retention time of DTX2 and PTX2 elute at the same time using the optimised BIOTOX chromatographic conditions.

4. Description and evaluation of matrix effects

4.1. Introduction

In the development of any new LCMS based method, a study of matrix effects must be undertaken to gather information on the effects and trends to be expected in shellfish matrices. If matrix effects can be identified and characterised it will become easier to subsequently design and implement an appropriate sample pre-treatment scheme to remove or at least minimise them. Three experiments were designed to evaluate the matrix effects experienced in the LCMS analysis of lipophilic toxins prior to the development of a clean up step scheme.

Matrix matched standards were prepared to provide information on the difference in signal assumingly as a result of matrix effects. Matrix strength standards (MSS) were used to evaluate the role of matrix strength in matrix effects.

The role of lipid content in matrix effects was also examined, to determine their effect on the analysis of the lipophilic toxins. All of these studies were carried out on oyster, mussel and scallop tissue.

4.2. Matrix matched standards

The aim of this experiment was to determine whether any matrix effects occur in the detection of AZA1 and OA and PTX2. Matrix-matched standards were prepared by spiking uncontaminated oyster and scallop extract with known amounts of toxins across a range of concentrations. The concentration range was chosen to reflect the current regulatory limits and are represented in Table 4-1. The signal obtained was compared to the same level of toxins spiked into pure MeOH. The degree of matrix effects was calculated using the slope of the calibration curve obtained for the standards in matrix divided by the slope of the standards in MeOH. A percentage wass calculated which represents the degree of matrix effects (Equation 4-1).

$$\left(\frac{\text{Slope matrix}}{\text{Slope methanol}}\right)\times 100 = (\%)\text{ME}$$

Equation 4-1: Calculation of degree of matrix effects using the slope of the curve

4.2.1. Experimental

4.2.1.1. Preparation of stock standard solution

A stock solution containing OA, AZA1 and PTX2 (400ng/ml for OA and PTX2 and 1000ng/ml for AZA1) was prepared using standards as described in section 2.2.2.

4.2.1.2. Preparation of shellfish tissue

Oyster and scallop tissues (approx. 1kg of each) were separately homogenised to ensure a bulk representative sample. Four extracts of each tissue were prepared using the BIOTOX extraction procedure (2.4.2). The extracts were combined to give a homogenous solution of each matrix.

4.2.1.3. Preparation of matrix matched standards

The reference standards were prepared by the addition of defined volumes of stock standard solution (0, 100, 200, 400, 600 and 800 µL) into 10mL volumetric flasks and completing to the mark each time using MeOH.

For the standards in matrix, the same volumes of stock standard were added into 10mL volumetric flasks however, in this case shellfish extract was used to complete to the 10mL mark. To ensure consistent matrix strength in each standard a constant volume of shellfish extract was used in each level and the volume was completed with MeOH.

All the dilutions were carried out volumetrically and checked by weight. Therefore, all glassware (e.g. volumetric flasks) was pre-weighed.

Table 4-1: Concentration range of matrix matched standards

Toxins	Concentration range (ng/mL)
OA/PTX2	0– 4.0 – 8.0 – 16.0 – 24.0 – 32.0
AZA1	0– 10.0 – 20.0 – 40 – 60 – 80

4.2.1.4. LCMS analyses

LCMS analysis was conducted using both LCMS systems as described in 2.3.1 and 2.3.2 using the LCMS method as described in 2.5.1.

4.2.2. Results

The calibration graphs obtained from each instrument are shown below (Figure 4-1 and 4-2). Using LC-TSQ-MS (Figure 4-1) no significant matrix effects were observed in either oyster or scallop matrix in the analysis of OA. For AZA1 and PTX2 matrix effects were observed to varying degrees. PTX2 experienced the greatest degree of matrix effects (ion enhancement in both oyster (44%) and scallop (55%)). For AZA1, ion suppression (15%) was observed in the oyster-matched standards only.

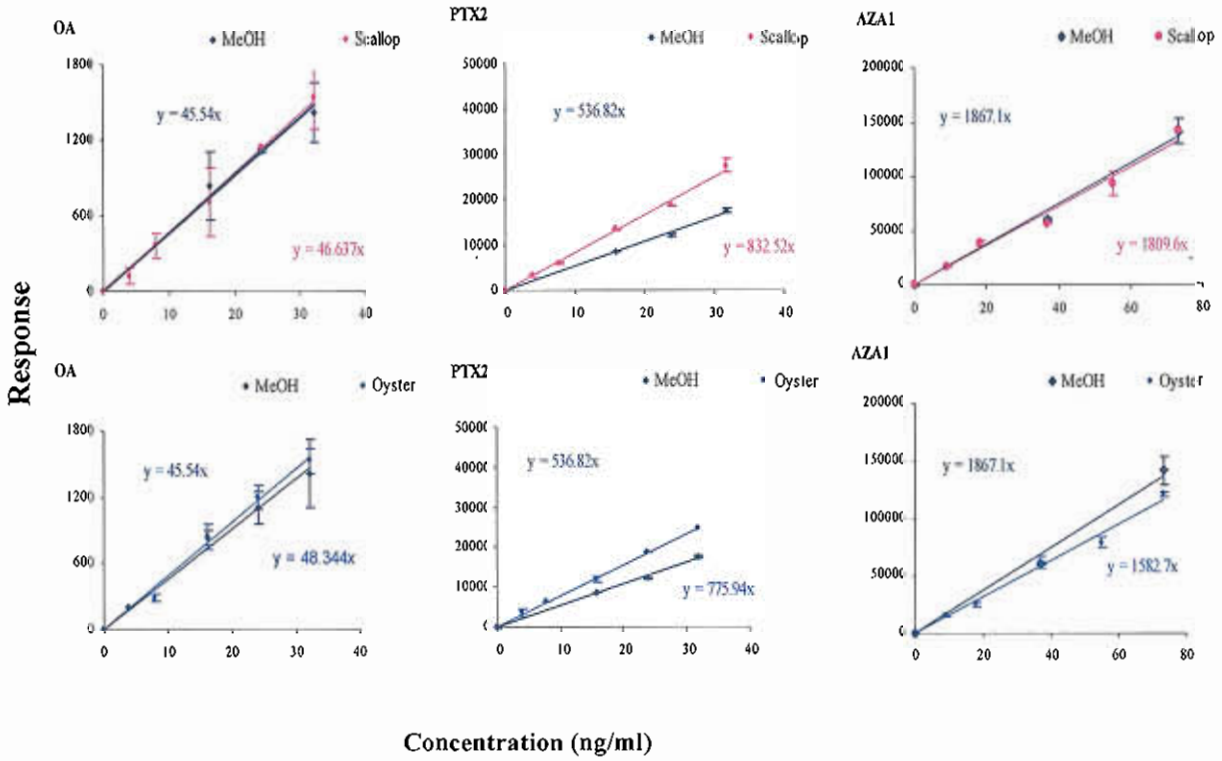


Figure 4-1: Signal obtained from the analysis of matrix matched standards prepared in oyster and scallop extract versus standards prepared in pure solvent for AZA1, OA and PTX2 analysed using LC-TSQ-MS, error bars represent SD±1

The results obtained using the LC-QToF-MS are shown below (Figure 4-2). Ion enhancement was observed for OA in scallop (30%) and oyster (40%), although there was a large variability associated with the signal, previously in LC-TSQ-MS no matrix effects were observed in the analysis of OA. Ion suppression (8%) was observed for AZA1 in scallop matrix only. Severe enhancement effects were experienced in the analysis of PTX2 in scallop and oyster (100%) using LC-QToF-MS, although for the scallop standards the highest point of the calibration curve drove the large slope difference, if this point were to be removed the slope difference would decrease as too would the degree of matrix effects.

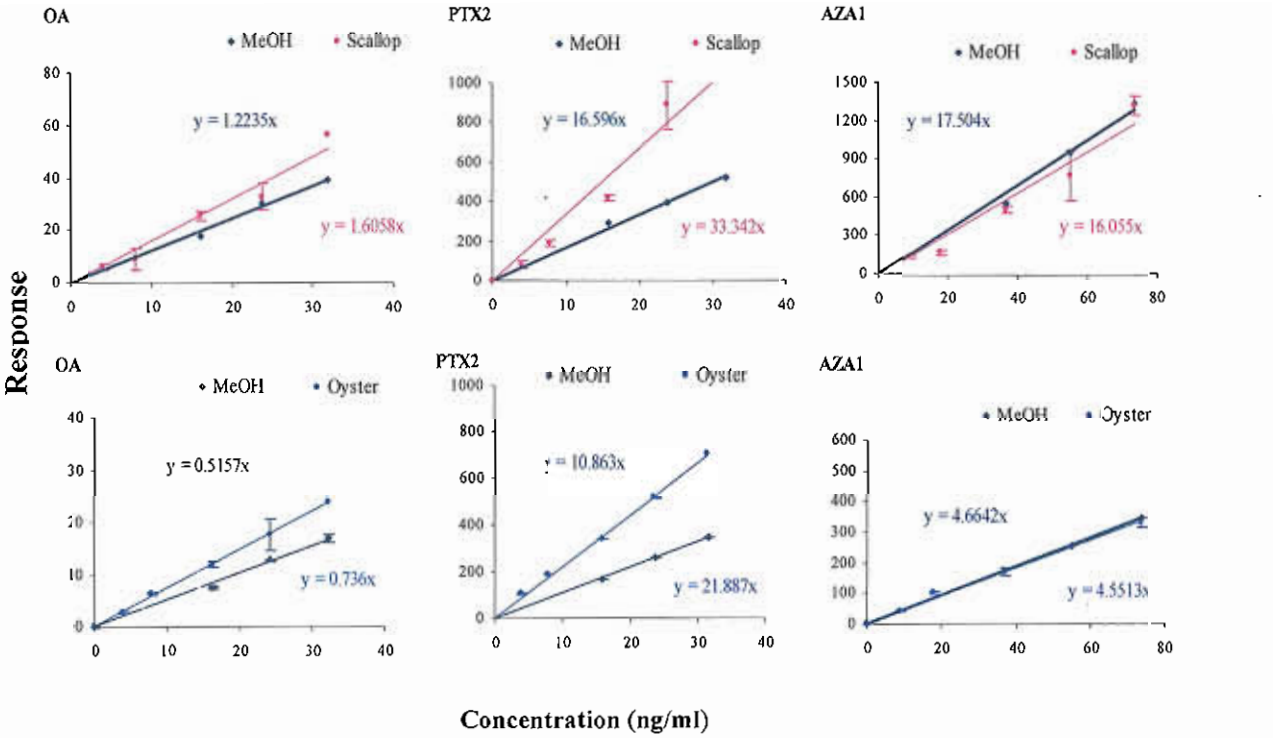


Figure 4-2: Signal obtained from the analysis of matrix matched standards prepared in oyster and scallop extract versus standards prepared in pure solvent for AZA1, OA and PTX2 analysed using LC-QToF-MS, error bars represent SD±1

Table 4-2: Degree of matrix effects (%) in shellfish extracts (oyster and scallop) determined from slope of line by LC-QToF-MS and LC-TSQ-MS analysis

	AZA1		OA		PTX2	
	TSQ	**QToF**	**TSQ**	**QToF**	**TSQ**	**QToF**
Oyster	-15	No effect	No effect	+40	+45	+100
Scallop	No effect	-8	No effect	+30	+55	+100

(+): Signal enhancement

(-): Signal suppression

Figure 4-3 represents the variability that can occur when analysing the same standards on separate days using LC-TSQ-MS, most likely related to the state of cleanliness of the ionisation source. In the first analysis (DAY1:OA) there were no significant matrix effects occurring however when the samples were analysed in a separate analysis sequence (DAY 2: OA) ion enhancement (20%) can be observed.

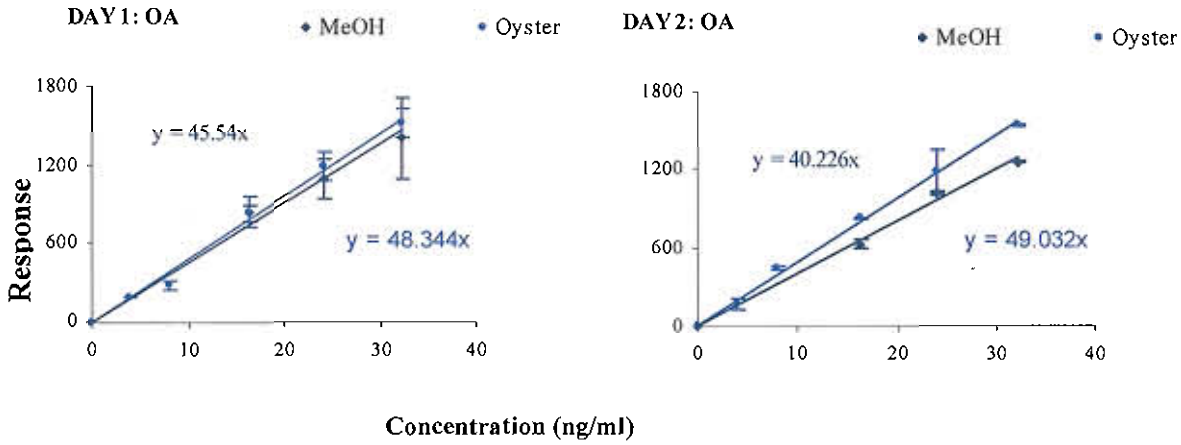


Figure 4-3: Signal obtained from the analysis of matrix-matched standards (OA) prepared in oyster, analysed using LC-TSQ-MS, error bars represent SD±1

Table 4-3: Degree of matrix effects (%) in matrix-matched standards (OA), prepared in oyster, calculated from slope of line by LC-TSQ-MS on two separate days

	Day 1	Day 2
Oyster	No effect	+20

(+): Signal enhancement

(-): Signal suppression

4.2.3. Discussion

The effects of interfering compounds on the response of LCMS in the analysis of marine toxins have been reported (Ito *et al*, 2002; Stobo *et al*, 2005; McNabb *et al*, 2005)and have been shown to be difficult to predict and subsequently control.

The aim of this experiment was to evaluate the matrix effects that occur in the analysis of lipophilic marine toxins through the use of matrix matched standards. The role of sample type (oyster or scallop), analyte (OA, AZA1 and PTX2) and instrument type (TSQ and QToF) in matrix effects were investigated.

Matrix effects have been shown to be analyte dependent, PTX2 was the compound that was most susceptible to matrix effects, and could be enhanced up to approximately 100% in some cases (in oyster and scallop matrix analysed by LC-QToF-MS-MS), the same effects were observed to a lesser degree when the standards were analysed using the LC-TSQ-MS-MS (average 50% enhancement). Such severe effects inhibit the accurate detection and quantification of PTX2. AZA1 suffered the least from matrix effects; an average of 15% suppression was encountered in oysters analysed by LC-TSQ-MS and 8% suppression in scallops analysed by LC-QToF-MS.

Matrix effects have also demonstrated a dependence on matrix type in some cases: OA was enhanced by almost 10% more in the oyster than the scallop-matched standards analysed using LC-QToF-MS. The opposite was observed in the analysis of PTX2 using LC-TSQ-MS where the scallop standards exhibited 10% more enhancement.

The matrix effects have shown similar trends on both instruments; AZA1 was signal suppressed on both instruments to varying degrees, OA and PTX2 demonstrated signal enhancement. OA was free from any matrix effect when analysed on the LC-TSQ-MS whereas the same sample could be enhanced by approximately 35% when analysed using the LC-QToF-MS, however there was a large variability associated with the measurement (represented using the error bars).

The results presented for the matrix-matched standards on both instruments may give an impression that using LC-TSQ-MS reduced the occurrence of matrix effects (for OA and PTX2), however this was not necessarily the case. Both instruments were of the same manufacturer and were equipped with identical ionisation sources; the difference between the two instruments was in the associated detection system (Triple Stage Quadrupole and Time

of Flight). As matrix effects originate during the ionisation process the trends of matrix effects should be the same between the two instruments (as they have the same ion source). The variability of matrix effects between the two instruments may have been related to the condition of the source (the source block of the instrument is dismantled frequently and cleaned with a mixture of solvents). Cleaning is necessary due to the crude nature of the samples being injected into the source at the time of analysis. Other factors such as the position of the probe (which delivers the sample into the MS) during the analysis may affect the ionisation process and therefore the matrix effects. This is represented in Figure 4-3 where the same matrix matched standards were analysed on two separate days using LC-TSQ-MS and the degrees of matrix effects differed significantly.

Through the use of matrix matched standards some information regarding the matrix effects arising from the analysis of lipophilic marine toxins was assembled.

- Ion suppression can be observed in the analysis of AZA1
- Ion enhancement can be observed in the analysis of OA and PTX2
- Matrix effects of the lipophilic toxins of interest can be sample and analyte dependent.
- Both instruments demonstrated similar trends of matrix effects

This study gave a better understanding of the matrix effects currently being experienced for the lipophilic toxins and is an important step for the subsequent efforts to include a method for removing and/ or minimising them.

4.3. Matrix strength standards (MSS)

The use of matrix strength standards was another approach in the evaluation of matrix effects. It is similar in principle to the previously described matrix matched standards in that the signal obtained from spiking into pure MeOH is compared to the same level of toxin spiked into matrix. However, in matrix strength standards the toxin level remains constant and the matrix strength (SSR) varies across a given range. The standards were prepared by a series of dilutions performed on an initial strong matrix (10g of wet tissue/10ml of 100%MeOH). Each dilution was spiked close to the regulatory limit for that toxin. The aim was to determine the impact that matrix strength has on the detection of the toxins.

The degree of matrix effects was calculated from the difference in signal from the matrix compared to that of the standard in MeOH. The matrices chosen for this experiment were oyster and scallop.

4.3.1. Experimental

4.3.1.1. Preparation of stock standard solution

A stock solution containing OA, AZA1 and PTX2 (400ng/ml) was prepared using calibration solutions as described in section 2.2.2.

4.3.1.2. Preparation of shellfish tissue

The material used was oyster and scallop tissue (same as in previous matrix matched standards), which were deemed to be less than the limit of detection (LOD) by the regulatory monitoring programme.

4.3.1.3. Extraction procedure

A single extraction was carried out at a very high sample to solvent ratio (1:1). A sub- sample of pooled tissue (10 ± 1.0 g) was weighed into polypropylene tubes and extracted with MeOH (10ml). The sample was vortexed using a multi-tube vortex mixer (V400 Alpha Labs, UK) for 2 minutes at full power. Further homogenisation was carried out using an Ultra-turrax ™ homogeniser (IKA -Werke, Staufen, Germany) at 11,000 rpm for one minute. After a centrifugation step at 4500rpm for 15 minutes (CR4-22 Jouan, Thermo Electron Corp., CA,

USA) the supernatant was transferred to a clean polypropylene tube. From this extract various properties were required for the preparation of the standards: density dry residue and moisture content. This dense solution was then used in a series of dilutions.

4.3.1.4. Density calculation

The weight of 4 aliquots (5ml) of the solution was taken and used in the calculation of density, according to the following equation:

$$Density = \left(\frac{Mass}{Volume}\right)$$

Equation 4-2: Calculation of the density of an extract solution

4.3.1.5. Dry residue determination

The dry residue was determined by recording the weight of 3 aliquots (5ml) of the extract after drying in an oven at 105 °C for 17 hours (or such a time when the extract is completely dehydrated).

4.3.1.6. Moisture content determination

The moisture content was determined by recording the weight loss of 5 aliquots (5g) of the matrix tissue after drying in an oven (for approx 17 hours or until all of the water has evaporated). The following calculation was applied:

$$Moisture\ content\% = \frac{(Mwet - Mdry)}{Mwet} \times 100$$

Equation 4-3: Calculation of moisture content of tissue used in the preparation of MSS

4.3.1.7. Preparation of matrix strength standards

The standards were prepared using the dilution scheme illustrated below (Table 4-4) using oyster, scallop and MeOH.

Table 4-4: Dilution scheme for the preparation of the Matrix Strength Standards (MSS)

Standard	**1**	**2**	**3**	**4**	**5**	**6**	7
Volume of stock (mL)	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Volume of matrix (mL)	9.6	7.6	5.6	3.6	2.6	1.6	0.6
Volume of MeOH (mL)	0	2	4	6	7	8	9

4.3.1.8. LCMS analysis

LCMS analysis was conducted using LC-TSQ-MS-MS (2.3.2) using the method as described in 2.5.1. An MRM transition was inserted for the analysis of PTX2, the transitions monitored were 876.5>823.5 and 876.5>876.5 with a cone voltage and collision energy of 30V and 30eV respectively.

4.3.2. Results

Various properties of the matrix were characterised prior to the preparation of the standards. The standards were prepared volumetrically and checked by weight; therefore the density of each extract was required (Table 4-5). The average density obtained was higher for the oyster extract (0.932g/mL).

Table 4-5: Densities obtained from replicate weights of oyster and scallop extract (n=4)

Aliquot number	Oyster density (g/ml)	Scallop density (g/ml)
1	0.936	0.931
2	0.932	0.929
3	0.932	0.925
4	0.929	0.922
Average	**0.932**	**0.927**
Standard deviation	0.003	0.004
Relative standard deviation (%)	0.308	0.390

Each standard level was expressed as a weight of dried residue; the assumption was that the stronger matrices would correspond to a larger weight of residue. The dried residue was determined for the initial extract (10g extracted in 10 ml) and is represented below (Table 4-6). The dried residue for each standard level was calculated according to the dilution performed on the initial extract.

Table 4-6: Weight of dried residue (mg) obtained from oyster and scallop extract (n=3)

Aliquot number	Oyster dried residue (mg)	Scallop dried residue (mg)
1	45.32	42.62
2	44.08	41.08
3	43.76	42.42
Average	**44.39**	**42.04**
Standard deviation	0.82	0.84
Relative standard deviation (%)	1.86	1.99

The moisture content of the shellfish tissue was also determined as an additional reference; the oyster displayed the highest value (Table 4-7).

Table 4-7: Moistnre content (%) determined for oyster and scallop homogenate

Aliquot number	Oyster moisture content (%)	Scallop moisture content (%)
1	79.10	76.68
2	79.23	76.70
3	79.47	77.23
4	79.30	76.39
5	79.22	75.94
Average	**79.26**	**76.59**
Standard deviation	0.13	0.47
Relative standard deviation (%)	0.17	0.62

Figure 4-4 illustrates the expression of matrix effects using the matrix strength standards. Dried residue values were plotted on the x-axis against the percentage recovery, which was plotted on the y-axis of the graph. The dried residue values were calculated for each standard level according to the dilution that was performed on the initial extract. The dried residue values were determined for these extracts and are represented in Table 4-6 (approx 44 mg and 42 mg for oyster and scallop respectively).

The red lines represent limits calculated from the signal of toxin in pure MeOH (SD ± 1). Any signal from a standard in matrix, which lies between these limits, was deemed to be free from matrix effects. A signal, which exceeds the upper limit, was enhanced and one, which falls below the limit, was suppressed. The error bars represent the precision of each measurement.

A1 and B1 of Figure 4-3 represents OA in oyster and scallop respectively; little matrix effects were observed across the range of standards. There was a large imprecision in the dilutions 1, 5 and 6, this may be related to the injection of such a large number of crude samples into the mass spectrometer as the values were based on the average of 3 replicate injections which were distributed randomly throughout the run sequence.

In the case of AZA1 (A2 and B2 of Figure 4-3) there was substantial ion suppression observed ranging from approximately 20-40% in oyster at the higher matrix strengths (dilutions 1, 2 and 3). These dilutions corresponded to the higher dried residue values. Between dilution 4 and 7 there were no significant matrix effects observed. The same suppression was not observed in scallops illustrated in B2

PTX2 shown in A3 and B3 of Figure 4-3 suffered the highest degree of matrix effects. In oyster matrix the signal was enhanced by approximately 40% around the currently employed matrix strength used in the extraction methods described in section 2.4.1 and 2.4.2, which corresponds to a dry residue of approx 8mg. The enhancement effects decreased with increasing matrix strength. In scallop the matrix effects were less prominent at the lower matrix strengths, which is what was expected, the enhancement increased with increasing matrix strength (approximately 15% enhancement at worst).

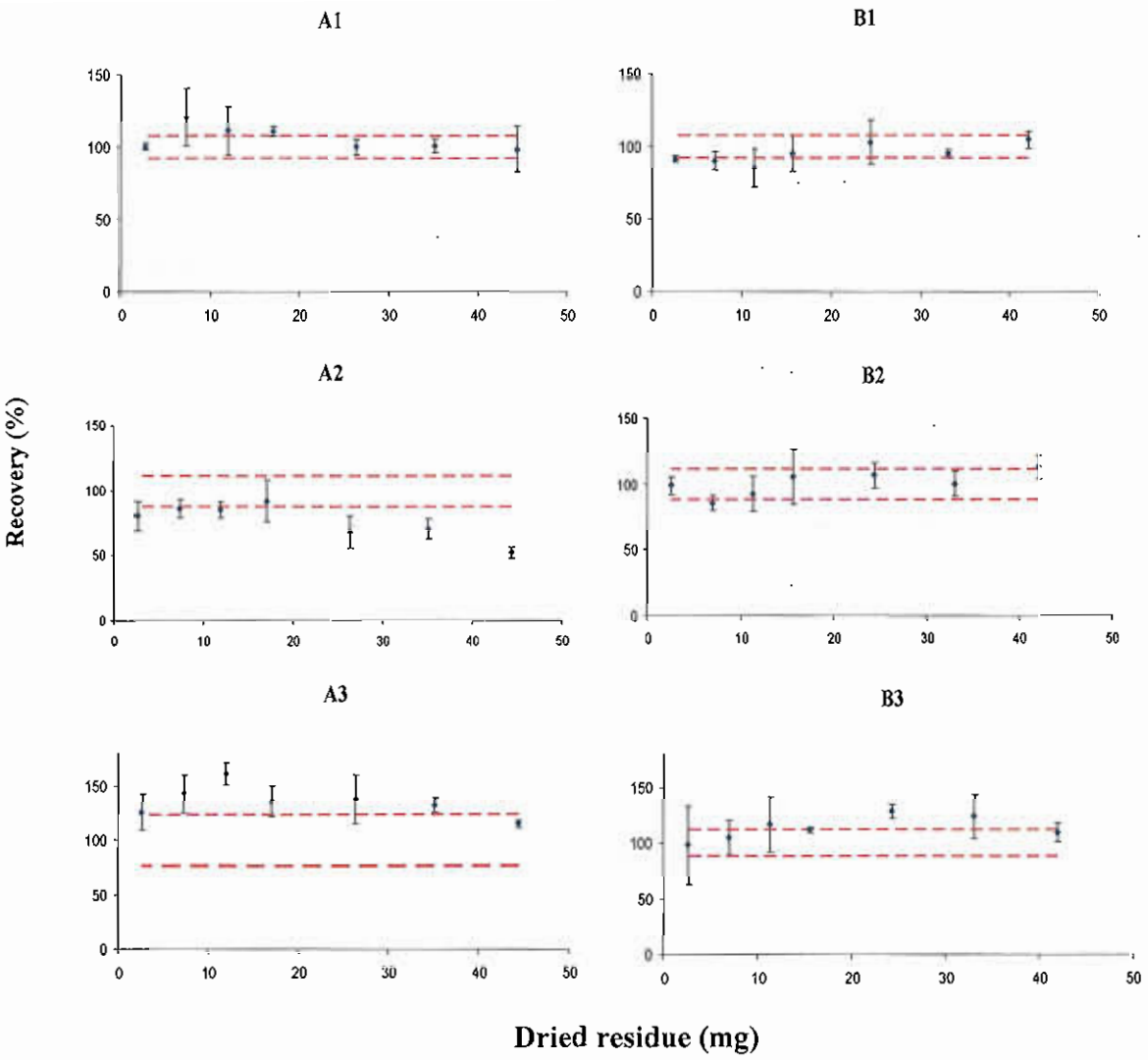


Figure 4-4: Graph of (1) OA, (2). AZA1 and (3) PTX2 in matrix strength standards in (A) Oyster and (B) Scallop (SD ± 1, n=3), analysis performed using LC-TSQ-MS

4.3.3. Discussion

MSS were taken as another approach for examining the matrix effects that occur as a function of matrix strength. The value for the matrix strength was expressed as a dry residue weight. The principle is that performing a dilution of the matrix will in turn dilute any matrix effects. From the current extraction method for lipophilic marine toxins (2.4.1) the dry residue of the matrix is approximately 8mg, which corresponds to between dilution 6 and 7 in the MSS standards, so in the currently used LCMS methods this particular standard strength are of most relevance. The only analyte, which suffered matrix effects at this matrix strength, was PTX2, which could be enhanced by up to 40% in oyster tissue.

Previous matrix matched standards experiments carried out at the routine matrix strength (approximately 8mg) indicated that there can be ion enhancement effects in the analysis of OA and PTX2 and ion suppression effects for AZA1 although this was dependent on a number of factors including analyte and the condition (cleanliness) of the ionisation source.

MSS has shown that OA suffered the least from matrix effects, regardless of matrix strength or sample type. Although it must be highlighted that at the matrix strength corresponding to the currently used extraction procedure there was a large imprecision on the average signal obtained this allowed the result to fall into the limits. Without the error bars the average signal was approximately 12% enhanced. AZA1 displays ion suppression (20-40%) but this was dependent on sample type and matrix strength. In the oyster tissue as the matrix strength got stronger so too did the suppression of the signal. This was only evident in oyster material (A3); in the scallop material (B3) no significant effects were observed.

As in the matrix-matched standards, PTX2 suffered the most from ion enhancement (approx 40%), these effects were more important at the current matrix strength employed in the oyster tissue (A3), in the scallop material the enhancement effects were less pronounced (15%).

In general, more matrix effects were observed in the oyster material, which was shown to have the highest dry residue and moisture content. This information may suggest that the more matrix is present, the more matrix effects that are experienced.

The total lipid content will be determined for both the oyster and scallop material to determine whether the amount of lipid present in the material is related to the degree of matrix effects observed.

4.4. Lipid content in shellfish matrices as a factor in matrix effects

Total Lipid content in wet tissue was determined for oyster, mussel and scallop matrices. Two methods for extracting total lipids were employed, the Bligh and Dyer (B&D) method (1959) and the later adapted Smedes method (1999). The aim of these experiments was twofold; firstly to extract the lipids using the aforementioned extraction methods and in doing so comparing the methods in terms of precision and repeatabability. No certified reference materials (CRMs) are commercially available for the lipid content in fish tissues, however QUASIMEME (Quality Assurance in measurement in the marine environment) produce tissues of a number of marine species as part of inter-laboratory proficiency schemes for the analysis of lipid content. Tissues from these schemes have a lipid content percentage as determined by a number of expert international laboratories. QUASIMEME recommend certain method quality parameters which state that results should be within certain Z scores (95% of results within $|Z| < 2$ and 99% within $|Z| < 3$). Therefore these tissues could be used as reference materials to express the proficiency of each extraction method.

The second aim was to examine the role of lipid content in matrix effects by examining the correlation between lipid content and degree of matrix effects experienced. This could be achieved as the material used in the lipid extractions was that as used in the preparation of the matrix strength standards and for which signal suppression/enhancement data had been generated. The total lipid content was determined for oyster, mussel and scallop tissue and also in the primary methanolic extract (after extraction of lipophilic toxins from the wet tissue). This was carried out to investigate the amount of lipids that are subsequently being injected into the MS to evaluate the contribution of these lipids to the matrix effects as characterised previously using matrix-matched and matrix strength standards.

4.4.1. Extraction of lipids from shellfish

4.4.1.1. Bligh and Dyer (1959)

The Bligh and Dyer (B+D) procedure used a mixture of chloroform/MeOH to extract the lipids from tissue. The extraction procedure was designed to destroy the association between the lipids and other cell constituents by firstly dissolving the lipids in a monophasic system of chloroform, water and MeOH and then adding further aliquots of water and chloroform producing a biphasic system. The biphasic system consists of a bottom layer consisting of 100% chloroform containing the lipids and a top layer of water and MeOH containing other non-lipid extractables.

The materials used were mussel, scallop and oyster and the reference QUASIMEME material. The procedure was adapted from the original B+D (Bligh and Dyer, 1959). A sub sample of tissue (4g ± 1.0g) was weighed into a polypropylene tube, chloroform (4ml), and MeOH (8ml) was added. The sample was homogenised using a multi-tube vortex mixer (V400 Alpha Labs, UK) for 2 minutes at full power. A further aliquot of chloroform (4ml) was added and the vortexing procedure was repeated. Deionised water (4ml) was added and vortex mixed again for two minutes. After a centrifugation step of 4500rpm for 5 minutes (CR4-22 Jouan, Thermo Electron Corp., CA, USA) the upper phase was discarded and the lower chloroform phase along with the pellet was filtered into a pre-weighed glass vial. A further addition of chloroform (4ml) was added to the pellet and filter paper to extract any remaining lipids; the solution was mixed on the vortex mixer for 1 minute and underwent another centrifugation step (4500rpm for 5 minutes). The resulting supernatant was transferred into the glass vial, which was evaporated to dryness (approx. 60 minutes) using a turbovap (Zymark, Caliper Life Sciences, MA).

4.4.1.2. Foppe Smedes (1999)

The B+D method has in the past been the internationally recognised method for the extraction of lipids. The use of chloroform however has both environmental and toxicological disadvantages that have resulted in the development of other techniques. The Smedes method is now internationally accepted by most laboratories as being more efficient than the B+D method. Additionally the use of non-chlorinated solvents results in comparable lipid determinations being obtained between laboratories that carry out the technique

The same material as described above in the B+D procedure was used in this extraction method. A sub sample of each tissue (4g ± 1.0 g) was weighed into a polypropylene tube (except in the case of the QUASIMEME where the whole extraction process was scaled down to half quantities). Isopropylacetate (8ml) and cyclohexane (10ml) was added and the samples were homogenised using an ultra turrax for two minutes. Deionised water (9ml) was added and the samples were homogenised again for one minute. A spatula tip of salt was added to prevent the formation of emulsions. The upper phase was removed using a Pasteur pipette and filtered through approximately 2cm of cyclohexane pre-washed glass wool placed in a funnel into a pre weighed glass vial. 10ml of 13% isopropylacetate in cyclohexane was added to the sample and ultra turraxed for a further 1minute, re-centrifuged using the same programme and again the upper phase was removed and filtered through the glass wool into the same glass vial. The combined supernatants were evaporated to dryness under nitrogen in a turbovap until two consecutive weights fell within 1%. The percentage lipid is calculated based on the weight of dried lipid in the glass vial divided by the initial sample intake as described below.

Weight of glass vial(mg) + Dried lipid(mg) – Weight of initial vial(mg) = weight of lipid extracted(mg)

Equation 4-4: Calculation of weight of extractable lipid (mg)

$$\left(\frac{\text{Weight of dried lipid} \,/\, 1000}{\text{Initial sample intake}} \right) \times 100 = \% \text{ Lipid in wet tissue}$$

Equation 4-5: Calculation of percentage lipid from the wet tissue

4.4.1.3. Extraction of lipid from solution

This method is an adaptation of the B+D method as previously described (4.4.1.1) designed to extract lipids from solutions. The objective was to determine the amount of lipids being extracted along with the lipophilic toxins using the current extraction methods as described in 2.4.1 and 2.4.2 and subsequently being introduced into the electrospray ionisation source (ESI).

The oyster mussel and scallop tissue were extracted using the BIOTOX extraction method (2.4.2). The methanolic extract was diluted with deionised water to a 40% aqueous solution. It was important to minimise the amount of MeOH in the extract so that lipids only partition into the chloroform phase; too much means that the chloroform layer would contain MeOH which in turn allow non-lipids to partition into the chloroform. An aliquot of this aqueous solution (3.5ml) was taken, 13.5ml of a 1:2 (v/v) solution of chloroform: MeOH was added. The solution was homogenised using a multi vortex instrument for one minute. The solution underwent further mixing following the additions of chloroform (4.4ml) and deionised water (4.4ml). After a centrifugation step (4,500 rpm for 5 minutes) the bottom phase was recovered using a pasteur pipette and transferred into a pre-weighed glass vial. The remaining phase was re-extracted with a second aliquot of chloroform (4.38ml) and carried through the same centrifugation step; the bottom phase was recovered and combined with the previous phase into the same glass vial. The sample was evaporated to dryness under nitrogen in the turbovap (at 25 °C for approximately 50 minutes).

It was possible to calculate the percentage of lipid in solution using the corresponding amount of wet tissue in the aliquot of extract taken for the lipid extraction. The BIOTOX extraction method uses 2g of wet tissue and is extracted to a final volume of 20ml with MeOH (100%) (2.4.2). This corresponds to a solvent to sample ratio (SSR) of 10. This method used 2.1ml of the methanolic extract (diluted to 3.5ml with deionised water) to carry out the lipid determination; 2.1 ml of extract corresponds to 210mg of wet tissue. This figure was used to calculate the % lipid in solution using the following equation:

$$\left(\frac{Weight\ of\ extracted\ lipid\ from\ solution\ (mg)}{Known\ lipid\ (\%)\ in\ wet\ tissue}\right) \times 100$$

Equation 4-6: Calculation of percentage lipid in a solution

4.4.1.3.1. Calculation of dry residue

The dry residue was calculated for each solution by weighing a 2ml aliquot of the solution and drying it at 100°C for approximately 20 hours. The weight of dried residue was then recorded.

4.4.2. Results

Both extraction methods displayed the same trend in that oyster tissue contained the highest average lipid content (2.1 ± 0.25%), followed by the mussel (1.5 ± 0.15 %) tissue and scallop (0.7 ± 0.15%).

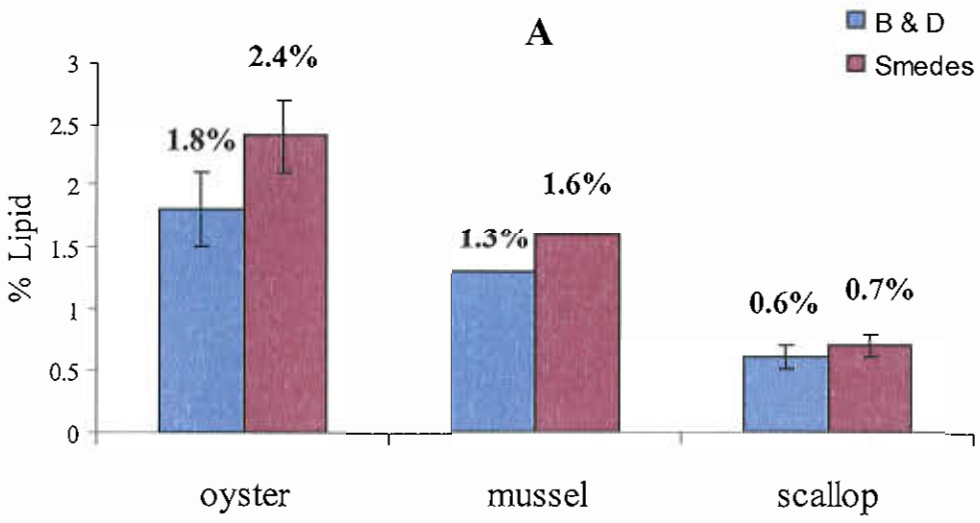


Figure 4-5: Total lipid content of oyster, mussel and scallop tissue extracted using adaptations of the methods of B&D and Smedes, SD ± 1, (n=3)

Overall, a similar trend of lipid content was found in the solutions as in the wet tissues (Table 4-8). It was possible to draw upon the data for the wet tissue and calculate the recovery of lipid in solution.

The total lipid content varied between matrix types (oyster, mussel and scallop); this was shown for both wet tissue and methanolic extracts of the tissue (Table 4-8), however the values of dried residues (were consistent regardless of the matrix (approx 20mg): mussel (21.2mg) > oyster (20.9mg)> scallop (19.2mg).

Table 4-8: Comparison of total lipid content extracted from solution and wet tissue and recovery data for lipid extracted from solution (calculated from expected amounts calculated from wet tissue experiments)

Sample Type	B+D wet tissue (% Lipid)	Actual % lipid from solution	Recovery In solution (%)
QUASIMEME	4.5	3.5	77.8
Oyster	1.8	1.6	88.9
Mussel	1.7	1.0	62.5
Scallop	0.6	0.7	116.7

Table 4-9: Dried residue values (mg) obtained from the methanolic extracts used in the lipid determination from solution

Aliquot number	Quasimeme Residue (mg)	Oyster Residue (mg)	Mussel Residue (mg)	Scallop Residue (mg)
1	21.1	18.9	20.7	18.7
2	20.8	19.5	19.8	18.8
3	20.7	19.4	23.3	19.0
Average	20.9	19.2	21.2	18.9
Standard Deviation	0.2	0.3	1.8	0.1
RSD (%)	1.0	1.8	8.6	0.7

4.4.3. Discussion

The aim of these experiments was to gather further information on the profile of the relevant shellfish matrices; the total lipid content was determined using various extraction methods. Using QUASIMEME material it was possible to assess the performance of each of the extraction methods using Z scores. To evaluate the role of lipids in matrix effects it was necessary to determine the amount of lipids that are co extracted during the extraction procedure for the lipophilic toxins from wet tissue (2.4.1 and 2.4.2). It is from this methanolic extract that matrix effects will arise as they are injected directly onto the MS.

Both extraction methods (B&D and Smedes) showed the same trend, the highest lipid content was found in oyster (2.1 ± 0.25%), followed by the mussel (1.5 ± 0.15 %) and scallop tissue (0.7 ± 0.15%). In comparing the two extraction methods the B+D method was not as precise as the Smedes method, this was shown using Z scores for the QUASIMEME reference material. (B+D: -1.08, Smedes: -0.8). The B+D method is being used less frequently in laboratories nowadays due to the use of chlorinated solvents in the procedure; it has also been shown that very minor deviations from the procedure can have negative effects on the results obtained (Smedes *et al*, 1996). The Smedes method uses more environmentally favourable solvents, and also offers better precision and repeatability between laboratories.

To evaluate the role that lipids play in matrix effects the total lipid content was determined in the methanolic extract, which contains the toxins. This was achieved using an adaptation of the B+D method for extraction of lipids from a solution. Prior to the extraction of the lipids the dry residue weight was obtained for each extract in the same manner as described in section 4.3.1.4. The dry residue was calculated to determine the weight of material being extracted from the wet tissue to obtain a value for the amount of matrix being injected into the source. The dry residue values obtained were similar (approx. 20mg) regardless of the matrix. The previous wet tissue lipid extraction had shown significant differences in the weight of lipids present in different matrices and so this suggests that the extraction method extracts the same weight of matrix components and that there may be other contributing components to matrix effects arising from the matrix such as salts or other non volatile compounds.

The results showed that extracting lipids from methanolic solutions of the wet tissue gives similar results (Table 4-8) to those expected and to the overall trend observed in the previous wet tissue lipid extractions (Figure 4-6). The recoveries obtained in solution were lower than the expected value; this may be due to the fact that some of the lipids were not extracted from the wet tissue due to the nature of the methanolic extraction procedure, which was designed to quantitatively extract the lipophilic toxins. Additionally, there was some uncertainty associated with the very low weighable amounts of lipids (approx. 2mg).

The previous prepared matrix effects experiments (4.2 and 4.3) indicated that the oyster tissue displayed the highest degree of matrix effects (ion suppression and enhancement), it has been shown that the oyster matrix contains the highest percentage of lipids in both wet tissue and solution, this would suggest that the lipids may play a role in matrix effects. The importance of this role is unclear.

4.5. Conclusion for description and evaluation of matrix effects

Matrix effects were characterised for oyster and scallop material containing some of the lipophilic toxins (OA, AZA1 and PTX2). The effects observed were shown to be sample and analyte dependent. Similar trends of signal behaviour (enhancement/suppression) were observed when comparing the two MS instruments (TSQ and QToF).

The use of matrix matched standards allowed the evaluation of the matrix effects by comparing the signal obtained of a standard solution in MeOH to the same amount of standard in shellfish matrix. Calibration graphs were constructed which allowed the calculation of the degree of matrix effects occurring. The results showed that PTX2 is most susceptible to matrix effects, displaying high degrees of ion enhancement (up to 100%). OA and AZA1 can show ion enhancement and suppression effects respectively, this is dependent on sample type and more so on the general condition of the analyser rather than the type of instrument used.

The use of matrix strength standards (MSS) was another approach in the evaluation of matrix effects; here the matrix effects were expressed as a function of matrix strength (expressed as weight of dried residue). Once again, it was shown that PTX2 is the compound most susceptible to matrix effects, suffering from high degrees of ion enhancement (40%). OA showed to be free from any matrix effects regardless of matrix strength when analysed using the LC-TSQ-MS-MS. The signal obtained for AZA1 can be suppressed at higher matrix strengths, this however is only applicable to oyster tissue.

Lipid data was obtained for the shellfish matrices involved in the evaluation study of matrix effects and it was shown that oyster material has the highest total lipid content in both wet tissue and in solution. This suggests that there may be a correlation between lipids and matrix effects.

This information arising from this study is valuable for the following chapters in which sample clean up schemes are developed for the relevant toxins. This is a difficult task as it has been shown during the course of this chapter that matrix effects are highly variable.

5. Sample clean up

5.1. Introduction

Since the recent advances in analytical instrumentation more efforts are being exerted into sample preparation techniques to achieve higher sensitivity, selectivity, accuracy and precision. The occurrence of matrix effects in the analysis of marine toxins by LCMS has been reported (Ito and Tsukada, 2001; Goto *et al*, 2001; Mc Nabb *et al*, 2005;Goto *et al*, 2001) and can have implications in quantitative method development by affecting accuracy and precision. The application of a clean up step could potentially remove or at least minimise matrix effects. Goto (2001) reported that SPE effectively removed components that were responsible for signal suppression of 50%.

This chapter describes the evaluation of two sample preparation techniques for shellfish tissues: LLE and SPE. The effectiveness of each technique in the removal of matrix effects will be considered in the following chapter 6.

5.2. Liquid-liquid extraction (LLE)

Some preliminary experiments were carried out to decide on an effective partitioning solvent using dichloromethane (DCM) and isopropyl acetate (IPA).

A study was undertaken using the optimised conditions with mussel and oyster matrices to examine the recoveries at each step of the partitioning procedure.

The evaporation and reconstitution steps were also examined to ascertain if they contributed to low recoveries being experienced during the procedure.

5.2.1. Preliminary liquid-liquid extraction trials

Two partitioning solvents, DCM and IPA were assessed for recovery of toxin from the extract.

5.2.1.1. Experimental conditions

5.2.1.1.1. Preparation of material

An (LRM) prepared at the Marine Institute containing OA, DTX2, DTX1, PTX2, AZA1, -2 and -3 was extracted as described in section 2.4.2. The primary methanolic extract (20mL) was combined with an equal volume of de-ionised water into 50ml polypropylene centrifuge tube and mixed thoroughly using a vortex mixer.

5.2.1.1.2. Hexane partitioning

The entire volume of the diluted extract (40mL) was mixed with an equal volume of n-hexane into a polypropylene centrifuge tube and vortex-mixed (V400 Alpha Labs, UK) thoroughly for one minute. Hexane will remove non-polar lipids from the extract (DTX3 may also be extracted due to its low polarity). The n-hexane layer was removed and the procedure was repeated with a fresh volume of n-hexane. The n-hexane layers were discarded.

5.2.1.1.3. Organic solvent partitioning

An aliquot (6ml) of the n-hexane washed extract was extracted with 6ml of DCM or IPA by vortex mixing for one minute in a 50 ml polypropylene centrifuge tube. The organic phase was recovered using a pasteur pipette and transferred to a fresh tube. The aqueous phase was re-extracted using a fresh volume (6mL) of solvent. After the aqueous phase was discarded (this contains any hydrophilic toxins and unwanted salts) the combined organic layers were evaporated to dryness using a rotary speedvac drier (Jouan RC 10.22); the residue was re-suspended in MeOH (3ml) prior to LCMS analysis.

5.2.1.1.4. LCMS analyses

LCMS analysis was conducted using LC-Q-ToF-MS (2.3.1) using the method as described in 2.5.1.

5.2.1.2. Results

The recoveries obtained for the partitioning solvent trials were variable; this was independent of the solvents used (Figure 5-1). The recoveries ranged from 55%-130% for IPA and 82%-134% for DCM.

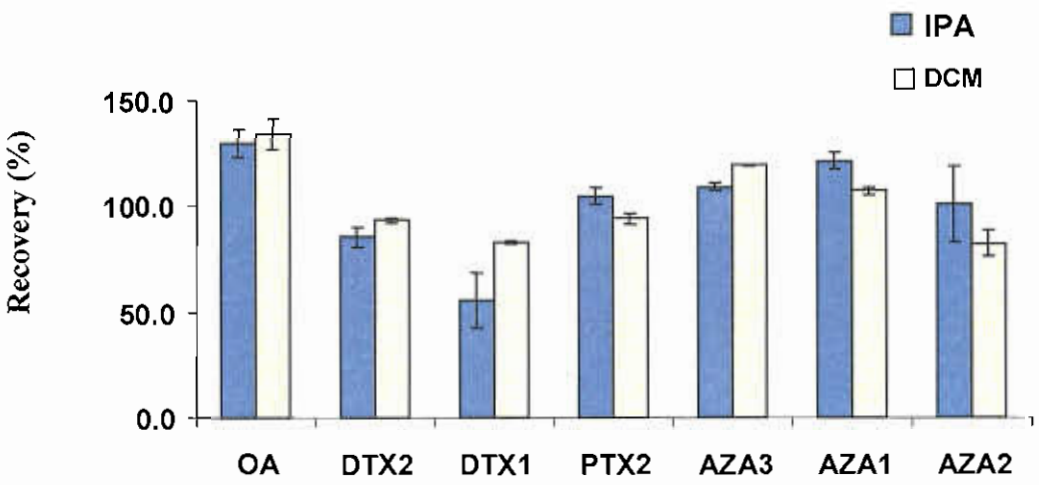


Figure 5-1: Recoveries obtained from partitioning solvent trial using IPA and DCM, error bars represent ± 1 SD (n=3)

The solvents were compared based on recovery of toxin (Table 5-1). Significant differences between the solvents were observed for the recovery of four compounds, DCM gave higher recoveries for DTX1 and AZA3 (82% and 119% respectively) compared to IPA (55% and 109%) whereas IPA gave higher recoveries for PTX2 and AZA1 (105% and 121%) compared to DCM (94% and 107%). The major differences observed were in the precision of the recovery values obtained. The precision (1 to 14%) for DCM was notably improved from the IPA precision values (15 to 38%).

Table 5-1: Recovery and precision (%) data of lipophilic toxins from LRM material (n=3) using LC-Q-ToF-MS

Toxin	Recovery		Precision	
	IPA	DCM	IPA	DCM
OA	130	134	25	5
DTX2	85	93	25	1
DTX1	55	82	33	12
PTX2	105	94	15	6
AZA1	121	107	34	14
AZA2	101	82	38	8
AZA3	109	119	34	14

5.2.1.3. Discussion

Most of the reported LLE procedures are based on the method as descried by Lee *et al*, 1987, where a partitioning step using hexane was used to remove fatty acids from the extract prior to a partitioning step using chloroform to isolate the toxins of interest. The use of chloroform in laboratories is now being reduced due to its toxic effects and being replaced with less environmentally detrimental solvents. The objective of these initial experiments was to investigate the recovery using both DCM and IPA as candidate partitioning solvents for the isolation of the lipophilic toxins after an initial hexane-washing step. Initially ethyl acetate (EtOAc) was envisaged as a candidate partitioning solvent; this was discarded due to the miscibility of the aqueous MeOH extract with EtOAc.

The recoveries obtained using IPA and DCM were variable (Figure 5-1), the solvents were examined using the recovery and precision values found. The recovery (82-134%) and precision (1-14%) values for the DCM partitioned extracts were significantly improved from the IPA cleaned extracts (55-130% and 15-38% for recovery and precision respectively).

5.2.2. LLE study using optimised conditions

A large-scale study was conducted to evaluate the LLE procedure as a viable clean up technique for the lipophilic marine toxins. Blank mussel and oyster matrix was used and spiked with OA and AZA1 close to the regulatory limit (160µg/kg). Each step of the partitioning procedure was evaluated in terms of recovery of toxins.

5.2.2.1. Experimental conditions

5.2.2.1.1. Preparation of materials

Non-contaminated whole flesh (WF) and hepatopancreas (HP) of mussels and oysters were spiked at the regulatory limit with OA/AZA1 (160µg/kg) standards as described in 2.2.2 prior to being extracted using the extraction method as described previously in 2.4.2.

5.2.2.1.2. Hexane partitioning

An aliquot of the extract (2.5ml) was mixed thoroughly with an equal volume of n-hexane in a 50mL polypropylene centrifuge tube. The mixture was vortex mixed for one minute and the hexane layer was removed, the procedure was repeated using a fresh volume of n-hexane. The MeOH phase was either evaporated to dryness and re-suspended in MeOH (1ml) (to examine the efficiency of hexane partitioning only) or taken further for an additional clean up step. All of the clean up steps were carried out in triplicate using the four matrices (mussel and oyster both WF and HP).

5.2.2.1.3. DCM partitioning

The hexane washed extract (2.5ml) was mixed with an equal volume of water in a 50mL polypropylene centrifuge tube. A 5ml aliquot of dichloromethane was added to the aqueous methanolic extract and vortex mixed for one minute. The DCM layer was recovered and transferred to a clean tube and the procedure was repeated with a fresh aliquot of DCM. The combined organic layers were evaporated to dryness using the rotary speedvac drier. The dried residue was re-suspended in 1ml of MeOH. A schematic presentation of the experimental is given in Table 5-2. Additionally a crude aliquot (2.5mL) of the sample was evaporated to dryness and re-suspended in 1mL of MeOH.

Table 5-2: Schematic of LLE experiments as conducted using optimised conditions

Sample	Matrix	Sample pre-treatment/s
WHOLE FLESH	Mussel Oyster	- Extraction - Hexane partitioning - DCM partitioning
HEPATOPANCREAS	Mussel Oyster	- Extraction - Hexane partitioning - DCM partitioning
WHOLE FLESH	Mussel Oyster	- Extraction - Hexane partitioning
HEPATOPANCREAS	Mussel Oyster	- Extraction - Hexane partitioning
WHOLE FLESH	Mussel Oyster	- Extraction - Evaporation to dryness - Reconstitution in 1mL
HEPATOPANCREAS	Mussel Oyster	- Extraction - Evaporation to dryness - Reconstitution in 1mL

5.2.2.1.4. LCMS analyses

LCMS analysis was conducted using LC-TSQ-MS-MS (2.3.2) using the LCMS method described in 2.5.1.

5.2.2.2. Results

The results presented in Figure 5-2 demonstrated that the addition of clean up steps lead to toxin losses for both AZA1 and OA.

For AZA the recoveries presented for the samples without any clean up range from 75% to 92% with no significant differences between the types of matrix used. The additional partitioning steps lead to a decrease of approximately 25% and 40% respectively in the average recovery obtained. The repeatability of the hexane and DCM (RSD of approx 5%, except for mussel HP which was at 18.4%) clean up was better than the hexane only (RSD of approx 23.4% for all matrices).

With OA, the patterns were similar to that of AZA1 in that with each additional clean up step there were recovery losses incurred. The average response obtained in the crude extract was higher in the WF tissues (116.7% and 123% for mussel and oyster respectively) than in the HP (79.8% and 73.8% for mussel and oyster respectively). Losses were incurred upon the application of hexane and hexane plus DCM clean-up steps approx (45% and 35% respectively).

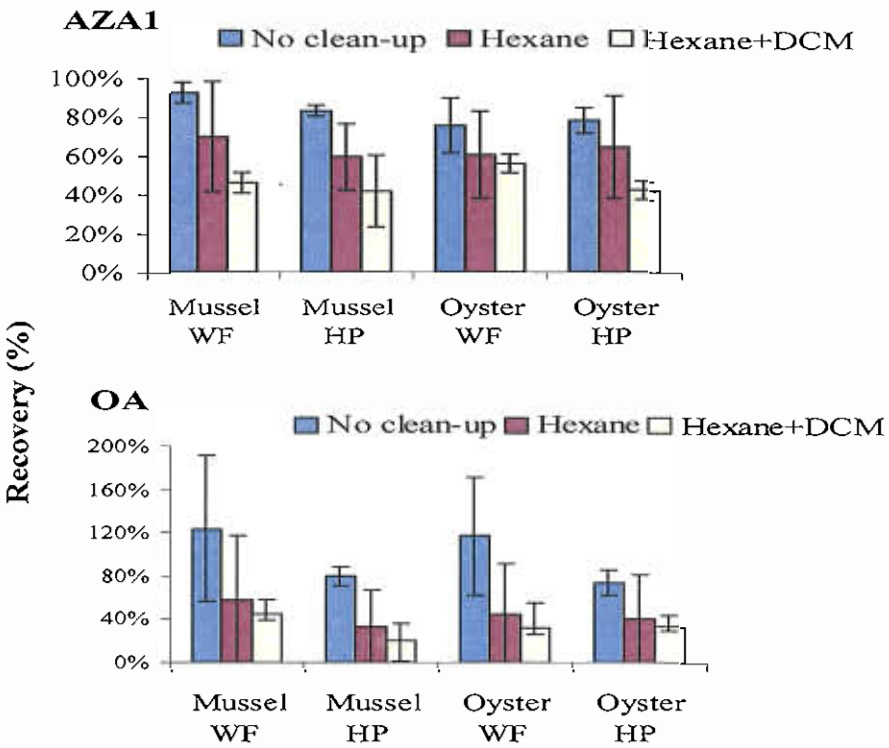


Figure 5-2:Comparison of (A) OA and (B) AZA1 recovery after different clean-up treatments of mussel and oyster WF and HP extracts. Error bars represent ± 1 SD (n=3)

5.2.2.3. Discussion

The purpose of this study was to assess the optimised LLE procedure in terms of toxin recovery, prior to the assessment of LLE as a possible technique for removing matrix effects. The collection of the cleaned fractions allowed the examination of the recovery of toxins at each stage of the LLE procedure, to ascertain where losses may have occurred.

For AZA1 the average recovery obtained in the crude sample was 83.4% with no significant differences in the tissue type (WF or HP), this lower recovery could be an expression of matrix effects as AZA1 has been reported to be a subject of ion suppression (Stobo *et al*, 2005). The high recoveries obtained for OA in the WF of oyster and mussel would be in agreement of the occurrence of ion enhancement, shown to occur for OA (Stobo *et al*, 2005). The same high recoveries for OA were not observed for in the HP, this may be due to the presence of more interfering components in the WF of the mussel and oyster extracts that were contributing to matrix effects.

The addition of clean up steps was shown to cause losses in recovery for OA (approx 2.5 fold when using hexane only and three fold when using hexane followed by DCM); this was independent of matrix type. Significant losses were also observed for AZA1 independent of matrix type also (approximately 1.5 fold for hexane only and twofold for hexane and DCM) although the losses were not to the same degree as those experienced for OA.

The variability between replicate samples was higher during the hexane only step; this was independent of toxin or matrix type. This may be due to the uncertainty of the procedure or may also be as a result of the evaporation of hexane and reconstitution steps that were required at the end of the procedure to prepare the sample for LCMS analysis.

The speed vac drier uses harsh drying conditions (centrifugal force, heat and vacuum) to evaporate the solvent. This results in a dried residue (ranging from dark brown to black in colour), which was difficult to re-suspend in only 1ml of MeOH. It was apparent following the evaporation step that the dried residue required more than 1mL of solvent to fully dissolve the residue and recover the toxins.

The results have shown that the described LLE method is not a viable sample pre-treatment technique for the marine toxins. There were significant toxin losses during the procedure. It

was thought that these losses were related to the evaporation and reconstitution procedures due to the difficulties encountered. The recoveries found in the crude extracts suggested that matrix effects were occurring in agreement with previous reports, high recoveries for OA (approx 120% in WF) suggest ion enhancement and lower recoveries for AZA1 (approx 80%) suggest ion suppression effects. For LLE to succeed in the removal of matrix effects it would be expected for AZA1 that the recoveries would increase as a result of the removal of any suppression effects, which would compensate (partially or fully) for the losses related to the clean up process. For OA, the results were more difficult to interpret; the recoveries decreased with each additional partitioning step in line with the trend expected for the removal of ion enhancement effects however, the losses in recovery were so severe they were more likely to be attributed with losses due to the LLE procedure.

To evaluate the efficiency of the LLE method in the removal of matrix effects, these recovery losses must be accounted for. This can be achieved by spiking blank LLE cleaned extracts prior to analysis and comparing this to the signal obtained from spiking into the crude sample. This was considered during chapter 6 (section 6.2).

5.2.3. Evaporation and re suspension trials

Following on from the results obtained in section 5.2.2.2 it was deemed necessary to investigate the low recoveries obtained further. The objective was to find the parameter of the evaporation/reconstitution procedure to which the poor recoveries could be attributed. Three factors were considered; (1) the container used for drying; (2) the reconstitution volume and (3) the drying instrument used.

5.2.3.1. Experimental conditions

5.2.3.1.1. Preparation of materials

The materials used were non-contaminated oysters spiked with OA and AZA1 standards at the regulatory limit (160µg/kg) and an LRM (MI) material which is prepared using mussel material. The tissues were extracted according to the BIOTOX extraction procedure (2.4.2).

5.2.3.1.2. Experimental design

The first part of the study is represented in Figure 5-3. The first two factors are considered, the container used for drying the extract and the volume used in the reconstitution step.
The second part of the study examined the drying technique using only the LRM material. All of the evaporation steps described in Figure 5-3 were carried out using the speedvac evaporator so to investigate another drying technique six 2.5ml aliquots of the LRM extract were evaporated to dryness using glass tubes in a Turbovap evaporator (Zymark TurboVap II). Three replicates of the dried residue were re-suspended in 1ml of 100% MeOH, the remaining three were re-suspended in 2.5ml of MeOH to further investigate the reconstitution volume.

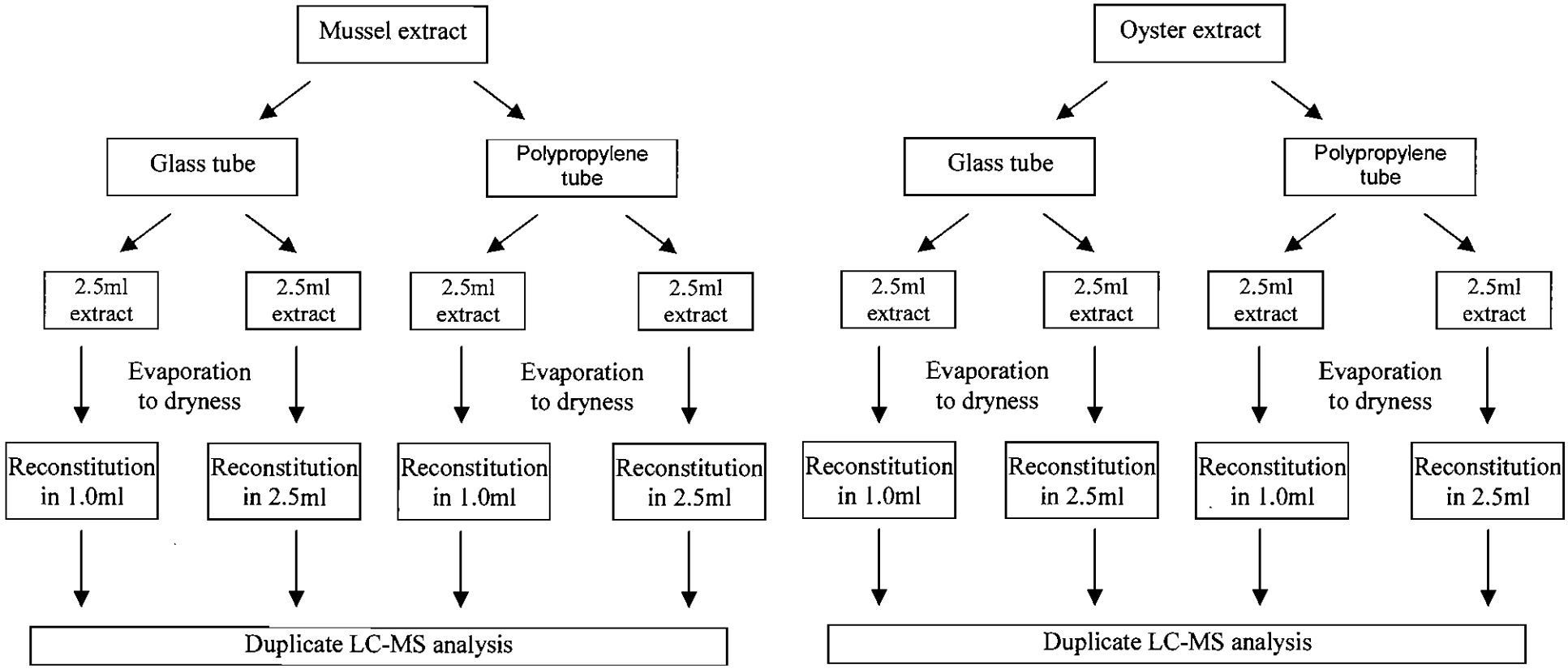


Figure 5-3: Schematic diagram of the protocol for the evaporation/reconstitution studies in which the reconstitution volume and drying container were examined, all evaporation was carried out using the speedvac drier

5.2.3.1.3. LCMS analyses

LCMS analysis was conducted using LC-TSQ-MS-MS (2.3.2) using the conditions as described in section 2.5.1.

5.2.3.2. Results

The first part of the experiment evaluated the effect that the reconstitution volume (1 and 2.5ml) and drying container (glass or polyproplylene) had on the recovery of OA and AZA1 from mussel and oyster material

For the mussel material (LRM) the results demonstrated a significant improvement in recovery when using 2.5ml instead of 1ml as the final reconstitution volume. For AZA1 the average recovery increased from 42% (1mL) to 86% (2.5mL) when using glass containers; for OA the recovery increased from 40 to 102%. The same trend was observed in the oyster material using glass only. For AZA1 the recovery increased from 55 to 93% and for OA the recovery increased from 58 to 107%.

The error bars (standard deviation) further represent the precision associated with the reconstitution in different volumes. In the LRM material a 2.5ml reconstitution volume corresponded to a standard deviation of approx 7.8% compared to approx 40% for 1 ml independently of the drying container (glass or polyproplylene) and toxin type (OA/AZA1). Approximately the same values were obtained for the spiked oyster material however this was only evaluated for the glass container, as the recovery of OA in the polypropylene was negligible when using 1ml.

No significant differences were observed between the drying containers using a 2.5ml reconstitution volume however the recoveries obtained from oyster material using 1ml were not detectable.

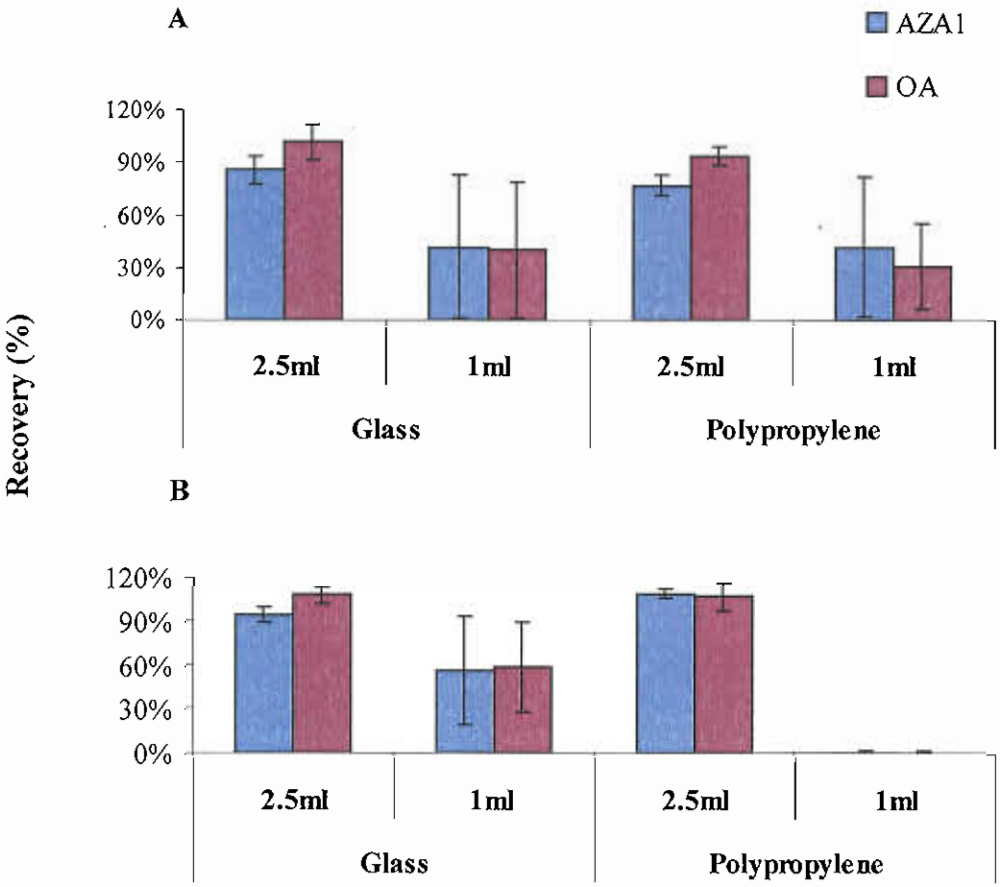


Figure 5-4: Comparison of the OA/AZA-1 recovery using different containers for evaporation in A: Mussel (LRM) and B:Spiked oysters. Error bars represent ± 1 SD (n=3)

The second part to the study examined the drying instrument and reconstitution volume. The same mussel (LRM) material was evaporated (in a glass tube) using the Turbovap drier.

The results (Figure 5-5) demonstrated more significant losses in recovery when a 1ml reconstitution volume was used (the recoveries for AZA1 and OA were reduced by a factor of 10 and 8 respectively), this was a similar loss as was observed using the speedvac. The recoveries for AZA1 in 2.5ml of MeOH were not affected by the use of a different drying instrument, for OA the recovery decreased from 102% using the speed-vac to 80% using the turbovap.

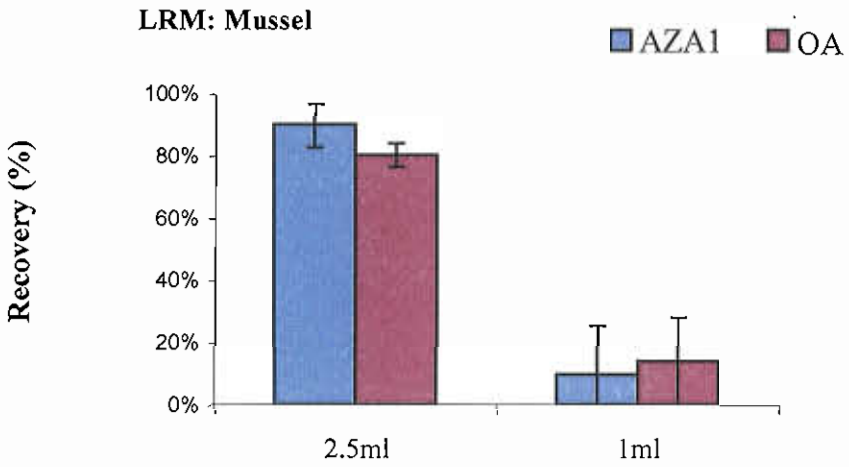


Figure 5-5: AZA1 and OA recovery after drying down the LRM extract in glass tubes using a turbovap and re-suspending the residue in 1 or 2.5ml of MeOH, error bars represent ± 1 SD (n=3)

5.2.3.3. Discussion

The aim of the evaporation and reconstitution studies arose from previous experiments where severe losses in recovery were being experienced with following partitioning steps. This was thought to be as a result of the evaporation and reconstitution step required to prepare the LLE cleaned extract for LCMS analysis. Complete solvation of the dried residue was not achievable when using only 1ml of solvent and this was leading to low recovery of toxins, which remained in the residue. To fully investigate this, the evaporation/reconstitution procedure was separated into three factors and each factor was evaluated to establish the origin of the poor recoveries:

1. The container used for drying.
2. The reconstitution volume
3. The drying instrument used

For the drying container used there were no significant differences observed when using glass or polypropylene for the drying steps, except for the oyster material when using polypropylene tubes for the procedure and a reconstitution volume of 1ml as this resulted in non detectable amounts of toxin.

The reconstitution volume was highlighted as the most critical factor in the recovery of toxin, the previous experiment had highlighted that 1ml of solvent was not a sufficient volume to fully re-suspend the dried residue and subsequently recover the toxins. Significant improvements (approx 50%) in recovery were found when using 2.5ml compared to 1ml. This was independent of toxin, drying container or matrix type (except for oyster material, where the drying container was a factor for reconstitution in 1ml).

The last part of the study compared two drying instruments (speedvac and tubovap) using the same two reconstitution volumes as before (1 and 2.5ml). Both drying instruments offer different mechanisms of solvent evaporation: the turbovap is a gentler method for drying the samples, a stream of nitrogen is delivered to the samples, which are held securely in a water bath at 40°C whereas the speed-vac uses centrifugal force, heat and vacuum to evaporate the solvent. There were no significant differences observed in the recovery of AZA1 between both instruments for a re suspension volume of 2.5ml in the same conditions, however there

was a decrease in the recovery obtained for OA. The difference between reconstitution volumes was greater than previously found using the speedvac. Losses of appox 80% were experienced when using 1ml.

The results indicated that the evaporation and reconstitution step may be responsible for the low recoveries experienced in the previous study. The factor which contributed most to the losses, was the reconstitution volume. A larger volume was required to sufficiently recover the toxins from the dried residue.

5.2.4. Conclusion for LLE

Liquid-liquid partitioning is a well-established clean up technique and can be a useful tool in the isolation of toxins from biological matrices. At preparative scale, LLE has been reported to give recoveries greater than 90% (Hess *et al*, 2007b). However, LLE poses difficulties on an analytical scale due to the use of much smaller volumes of matrix involved, which results in toxin losses with following partitioning steps.

The influence of the drying container and drying instrument were less significant roles in the evaporation/reconstitution than the reconstitution volume. The reconstitution step was required to prepare the extract for analysis and may also be necessary to concentrate the toxin. If there were adequate sensitivity on the MS system and a concentration step was therefore not required then it would be recommended to use a larger volume of solvent to ensure complete recovery of toxin.

Even with the improvements that can be achieved with the increased reconstitution volume the procedure is quite laboursome and requires the use of large amounts of glassware and solvents. Quilliam (1995) reported that even after a LLE clean up performed on mussel extract that the tissues were still dirty and this can be seen in the colour of the extract, which is an indicator of the degree of purification. A further SPE clean up was required to protect instrument life, reduce interferences and improve detection limits. McNabb *et al*, (2005) described a quantitative LCMS method for six key marine toxins, which used a hexane only partitioning, step to remove non-polar lipid components.

So whilst LLE has been shown to be a useful tool, it is a method, which has many associated drawbacks in modern analytical methods where reproducibility and recoveries must be adequate. The effectiveness of LLE in the removal of matrix effects was considered in chapter 6.

5.3. Solid phase extraction

In collaboration with RIKILT a series of SPE experiments were conducted. The aim of the collaboration was to develop a suitable clean up for four marine toxins belonging to the 4 regulated groups of lipophilic marine toxins (AZA1, PTX2, OA and YTX) and which could effectively remove (or at least minimise) the matrix effects attributed to the marine toxins.
Various SPE methods have been reported for the OA and AZA toxin groups (1.7.1.2) but to date a universal method has not been reported. The initial task was to find a suitable sorbent type, which could achieve the best recovery and reproducibility for the toxins, included in the studies. A wide range of cartridge manufacturers was evaluated during the course of the experiments. The initial investigations were focused on finding a sorbent and optimising the recovery of all of the toxins is described in this chapter. The work that followed on from this (carried out by RIKILT) will be discussed at the end of the chapter where the optimised procedure will be described.
Some use was made of an automated SPE system (Symbiosis) for some confirmation experiments upon the implementation of an offline SPE procedure.

5.3.1. Automated SPE (Symbiosis)

The Symbiosis Pharma is shown in Figure 5-6 (Spark Holland, Emmen, The Netherlands) consists of a temperature-controlled stacker, a temperature controlled autosampler (Reliance), a high-pressure dispenser (HPD single), a high-pressure dispenser mix with a solvent selection manifold, a gradient pump set and an automatic cartridge exchanger (ACE). The Symbiosis Pharma was coupled to a Quattro micro LC-MS/MS system (Waters corporation).

5.3.1.1. Materials and methods

An LRM material (MI) was extracted as described in section 2.4.2. The methanolic extract was diluted with water prior to the SPE (60/40, v/v MeOH: H2O). Using the integrated symbiosis software different wash steps were programmed starting at 0% MeOH increasing up to 90%; this was carried out under neutral, basic and acidic conditions using the general purpose (GP) cartridge, which contains a polymer similar to Oasis HLB/Strata X.

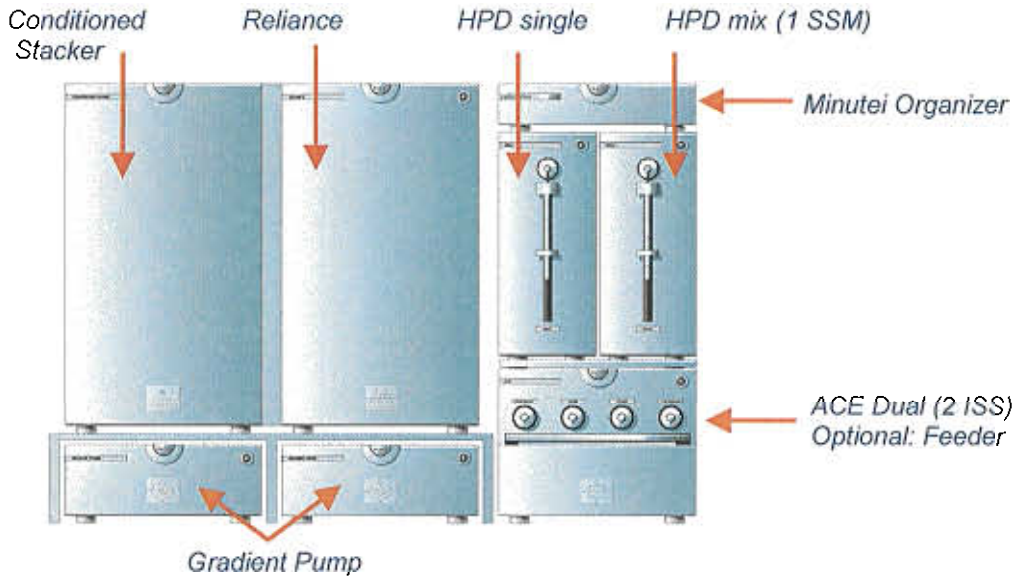


Figure 5-6: Symbiosis Pharma

5.3.1.2. Results and Discussion

The results obtained from the symbiosis indicated that were no significant losses in recovery until a wash composition of 70% organic is applied. This threshold was even higher for PTX2 where losses only started to occur at 90% organic. Without the use of error bars it is difficult to interpret the differences in recovery as a result of the pH of the washing solution, however the basic washing solution gave high recoveries for AZA1, PTX2 and YTX whereas a neutral washing solution is producing highest recoveries for OA.

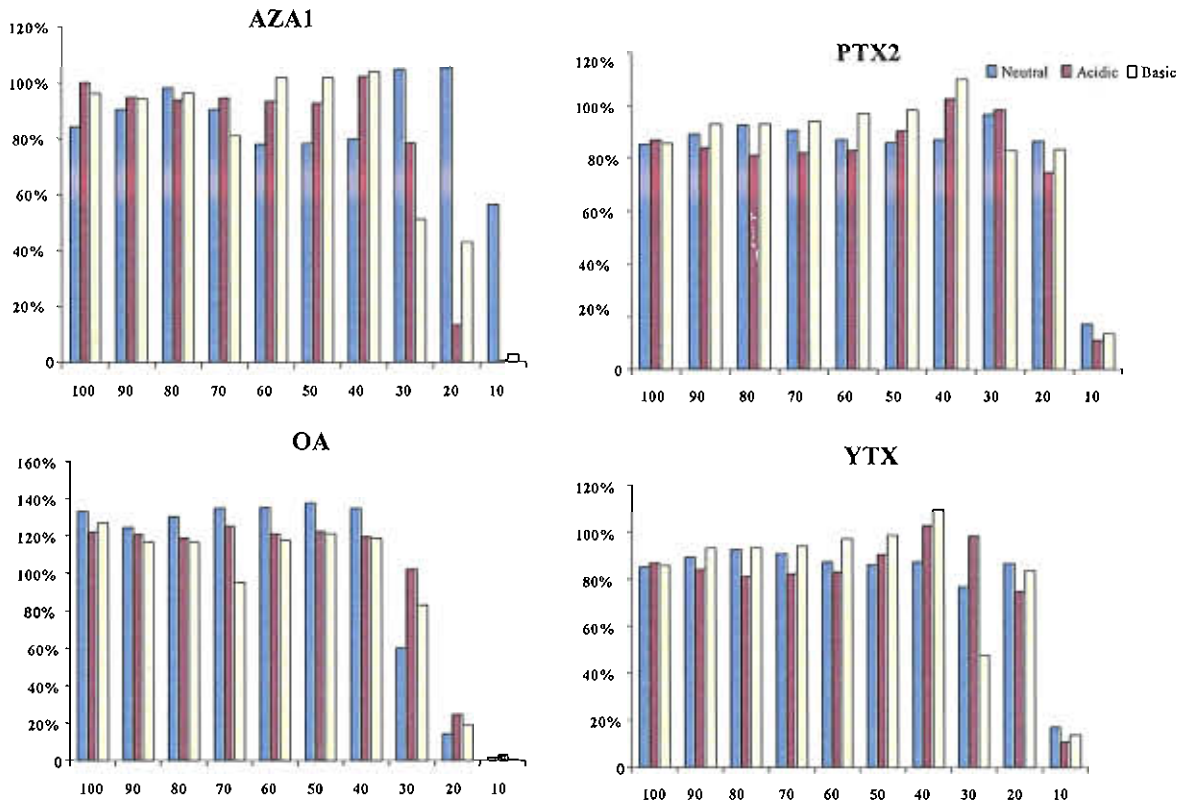


Figure 5-7: Optimisation of washing conditions using on-line SPE (Symbiosos Pharma) of LRM material under neutral, acidic and basic conditions, fractions were analysed using Quattro micro LCMS system (Waters Micromass)

5.3.2. Development of an offline solid phase extraction method

The aim of this study was to develop a universal offline SPE method for OA, AZA1, PTX2 and YTX which to date has not been reported. The initial development was based on finding an appropriate sorbent which could sufficiently recover the toxins included in the study.

Recent years have seen the development of new SPE sorbent chemistries, which has allowed flexibility in method development. The SPE methods reported to date have typically made use of silica-based sorbents. New sorbent chemistries consisting of co-polymers present new possibilities in retaining and eluting the marine toxins under various rigorous washing/eluting conditions where traditional silica based sorbents have struggled. These new sorbent chemistries allow more interactions to occur which can allow the introduction of a wider range of compounds into one clean up scheme.

5.3.2.1. Mechanism of SPE

For the purpose of this method development the SPE procedure was considered as five individual steps:

1. Conditioning of the sorbent
2. Equilibration of the sorbent
3. Loading of the sample onto the sorbent
4. Washing off of impurities from sample
5. Elution of the analyte from the sorbent

The steps that required the most optimisation were steps 3, 4 and 5. The conditioning and equilibration steps are steps that did not require extensive optimisation. Step 1 (conditioning) was required to activate the sorbent and this was achieved using MeOH. Step 2 (equilibration) prepares the sorbent for interaction with the analyte and used a solvent similar in composition to the sample to be loaded in the subsequent step.

The first series of experiments examined steps 3 and 4, the load and wash parameters of the procedure. For the loading step, the loading composition (volume and organic composition) was examined to optimise the amount of sample that could be loaded onto the sorbent without any breakthrough (toxins passing directly through the sorbent bed and not being retained).

For the wash step, the washing solution was examined using different buffers such as formic acid and ammonium hydroxide to act as pH modifiers and change the chemistry of the toxins therefore affecting their retention.

The elution step was generally carried out at the highest possible organic strength to ensure complete elution of the toxins. The elution step was examined at different pHs in various combinations with the washing step.

In the development of a new SPE method it is important to understand the passage of the toxins throughout the procedure. This can be achieved by collection and analysis of the various fractions (wash and elute). This measure can be useful in the manipulation of the various steps of the procedure to ensure the toxins are all present in one fraction.

5.3.2.2. Optimisation of the load and wash steps

5.3.2.2.1. Experimental conditions

Preparation of materials

An LRM (MI) was extracted as described in section 2.4.2. Two different loading compositions of the extract were prepared according to Table 5-3. The difference between the loading compositions was the amount of de-ionised water added to the extract.

Table 5-3: Dilution of LRM prior to SPE

Load composition	LRM (mL)	MeOH (mL)	De-ionised water (mL)
1: (50/50)	4	16	20
2: (25/75)	4	16	60

A multi-toxin stock standard was also prepared in MeOH containing OA/PTX2 (400 ng/ml), AZA1 (200 ng/ml) and YTX (1000 ng/ml); the standards were as described in section 2.2.2. Two loading compositions of this stock were prepared according to Table 5-4.

Table 5-4: Dilution of stock standard solution prior to SPE

Load composition	Stock standard (mL)	MeOH (mL)	De-ionised water (mL)
1: (50/50)	1.5	6	7.5
2: (25/75)	1.5	6	22.5

SPE solutions

Solutions of varying pHs (neutral, acidic, basic) and organic strengths (25, 50 and 70% aqueous methanol) were prepared for the optimisation of the wash steps.

Three neutral wash solutions (25, 50 and 75% aqueous MeOH) were prepared by the addition of 5, 10 or 15mL of MeOH to a 20mL volumetric flask and completing to the mark with deionised water

Three acidic wash solutions (1% formic acid) at different organic strengths (25, 50 and 75% aqueous MeOH) were prepared for the wash step by the addition of 0.2mL of formic acid into a 20mL volumetric flask with 5, 10 or 15mL of MeOH and completing each solution to the mark with deionised water.

Three basic wash solutions (1% ammonium hydroxide) with different organic strengths (25, 50 and 75% aqueous MeOH) were prepared for the wash step by the addition of 0.2mL of ammonium hydroxide into a 20mL volumetric flask with 5, 10 or 15mL of MeOH and completing each solution to the mark with deionised water.

SPE Equipment

The cartridge applied for this experiment was an Oasis ™ HLB (hydrophilic-lipophilic balance) (60mg/3ml); the sorbent consists of a water wettable copolymer, which gives the sorbent a retention capacity for both polar and non-polar compounds. The lipophilic polymer is divinylbenzene (DVB); the hydrophilic component is N-vinylpyrrolidone.

SPE procedure

The SPE procedure was carried out at two different loading compositions (25/75 and 50/50) of the LRM and standard solution.

Seven different wash procedures were evaluated. Using neutral, acidic and basic conditions each wash step was carried out at two different organic strengths, one that was at a similar organic composition to the loaded sample and another, which was 25% higher than the loading composition.

All treatments using the LRM were carried out in duplicate and once only when using the standard solution (preservation of standards). The wash fractions were also collected for analysis to investigate breakthrough of toxin.

An evaporation and reconstitution step was also included to evaluate the need for a concentration step in the procedure (using one of the replicate elutes). After the elution step of 2mL for each replicate procedure, 1 ml of solution was carried forward for direct LCMS analysis and a further 1mL was evaporated to dryness using a turbovap instrument and re-suspended in 0.5mL of MeOH for analysis.

The schematic of the experimental is illustrated below in Figure 5-8.

LCMS analysis

LCMS analysis was carried out using the BIOTOX method B (2.5.3).

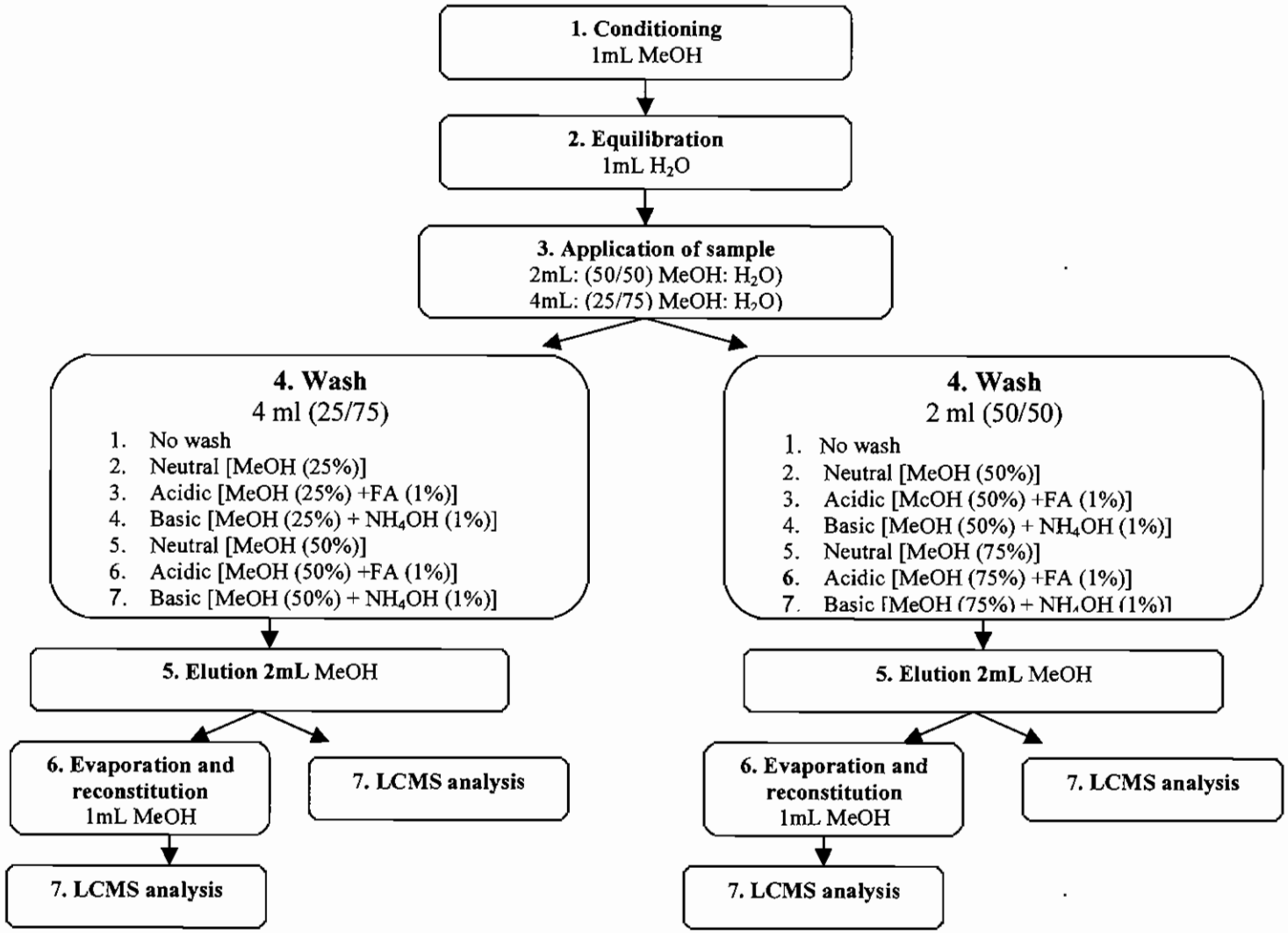


Figure 5-8: Schematic of SPE experiment to optimise load and wash composition, using two different loading compositions of LRM and standard material. SPE conducted using Oasis ™ HLB (60mg/1mL). LCMS analysis was conducted using LC-TSQ-MS

5.3.2.2.2. Results

SPE purification of multi-toxin standard

The recoveries obtained from purification by SPE of the multi-toxin standard in MeOH are presented (Figure 5-9). This analysis gives a value for the recovery of toxin without any effects arising from the matrix. It was also an opportunity to examine the loading composition of the extract (25/75 and 50/50). Replicate analyses were not performed and therefore without the presence of error bars it was difficult to say if the loading composition played a significant role in the recovery of AZA1, OA, YTX and PTX2.

There seems to be the greatest difference between loading compositions in the case of AZA1. In general the 50/50 loading composition gave higher recoveries ranging from 117 to 139% (except for a basic wash step at 75% MeOH where recovery is low (60%). The highest recoveries were achieved when the sample loading composition was 50/50 using a basic wash (50% MeOH), the recoveries decreased when a higher organic strength was applied in the wash (by up to 50%). Analysis of the wash fractions highlighted any losses that occurred as a result of the washing conditions. For AZA1 breakthrough (9%) into the wash fraction was only observed for the 50/50 loading composition under acidic conditions at 75% organic strength.

For OA, YTX and PTX2 there was generally less of a difference observed between load compositions, except for the final basic wash condition at a higher organic strength where the 50/50 loading composition suffered significant losses (recovery of approx 5% for OA under these conditions). Breakthrough into the washing fractions was found for OA and YTX. For OA recoveries ranging from 16 to 32% were found in the wash fractions corresponding to the 50/50 loading compositions at the 75% MeOH, under all conditions, this does not totally account for the poor recoveries. For YTX recoveries of 14 and 43% were found in the wash fractions of the 25/75 and 50/50 loading compositions respectively (under neutral conditions). In addition, a recovery of approx 20% YTX was found in the wash fraction, under basic conditions using the 50/50 loading composition.

In general the optimum conditions correspond to a basic wash at the same organic strength as the loading composition.

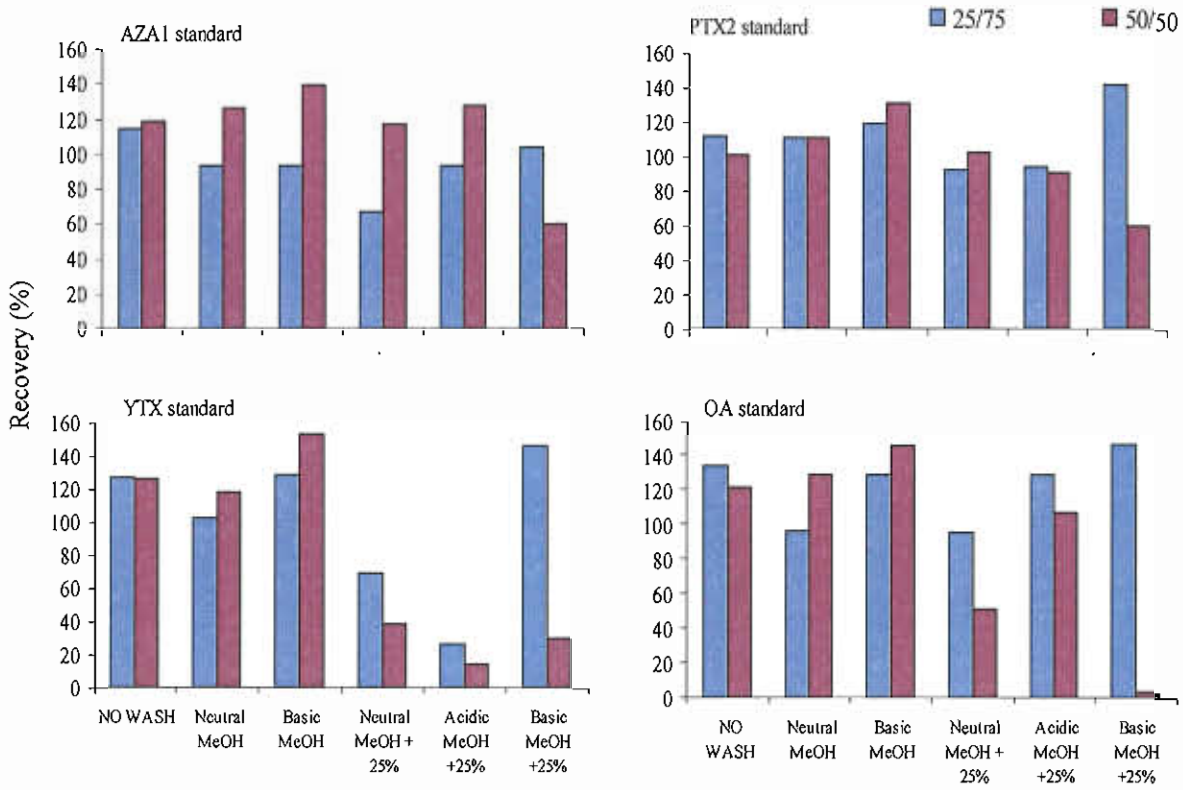


Figure 5-9: Recoveries obtained from standard solution (AZA1, PTX2, YTX and OA) purified by SPE using Oasis ™ HLB (60mg) under two loading compositions (25/75 and 50/50) and different washing conditions

SPE purification of the LRM material

The recoveries of the AZA toxins from the LRM material (Figure 5-10) were more variable in the 50/50 loading compositions. The AZA3 recoveries were very high (ranging from 100-160%) whereas the AZA1 recoveries ranged from 73 -103% and the AZA2 recoveries ranged from 93-130%. Losses were observed when the washing solution contained a stronger organic content (75%). Collection and analysis of the wash fractions indicated that for all of the AZAs approx 10% recovery was found in the acidic and basic washing fractions only.

The recoveries obtained from the 25/75 dilutions were less variable within and across the series of washing conditions and toxins. Recoveries of 100% were achieved with all conditions, the optimum recoveries result from a basic wash step at a high organic strength (50%), although there was higher variability associated with these recoveries. No breakthrough into the washing fractions was observed for the 25/75 loading composition.

The recoveries were reduced significantly upon evaporation and reconstitution for the 50/50 and 25/75 dilutions (approximately 35% and 25% respectively). For the 50/50 loading composition the higher recoveries previously observed for AZA3 were not experienced.

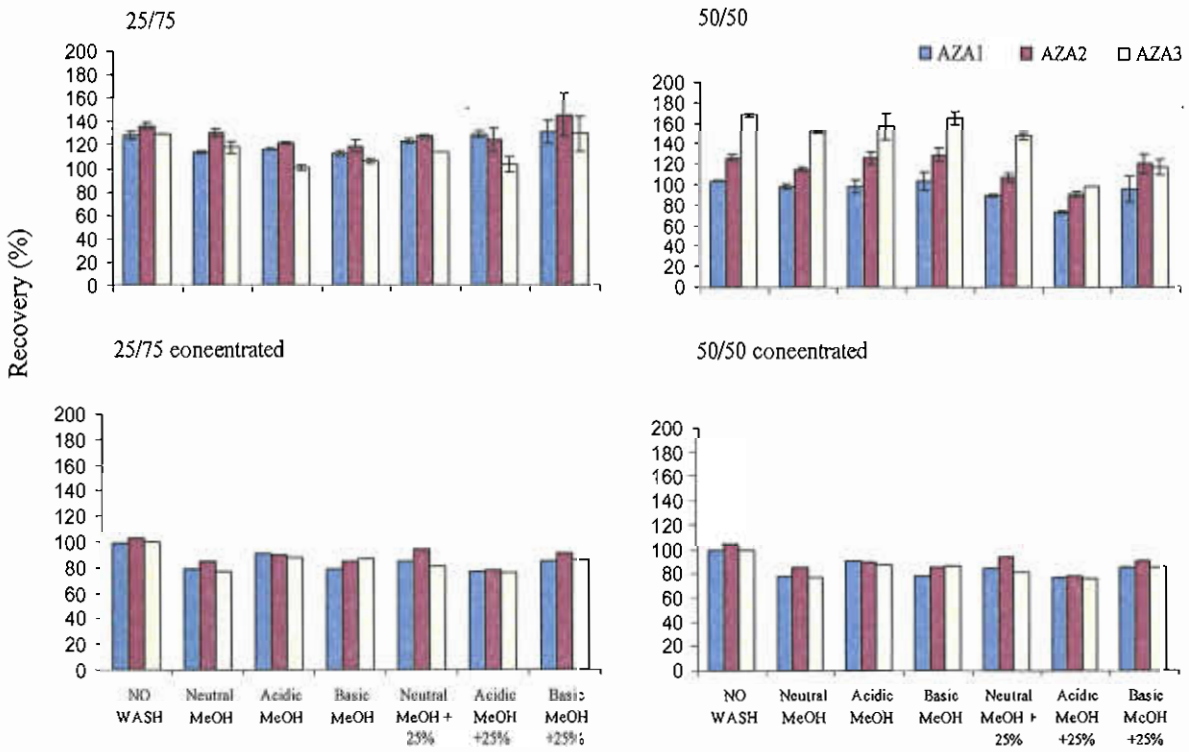


Figure 5-10: Recoveries obtained for AZA toxin group following SPE (Oasis ™ HLB, 60mg) of LRM material using two loading compositions (25/75 and 50/50) and seven washing conditions (error bars represent SD ± 1, n=2) plus subsequent concentration steps (based on a single treatment and analysis), all fractions analysed using LC-TSQ-MS

For OA, DTX1 and DTX2 (Figure 5-11) the same trend was observed as previously for the AZA toxins, a loading composition of 25/75 led to less variability across the series of washings, especially when washing solutions contained a higher organic content. When using a basic wash at a high organic composition (50%), the highest recoveries were obtained (125, 164 and 205% for OA, DTX1 and DTX2 respectively).

Significant losses in recovery were incurred in the 50/50 dilutions upon the application of a 25% higher organic strength under all washing conditions (losses up to approx 100%). Some of these losses can be accounted for through the analysis of the washing fractions, where average recoveries obtained were approx 40, 16 and 70% for the neutral, acidic and basic washes respectively.

Only one replicate of the concentration step was carried out and so without error bars it was difficult to confirm the significance of any differences in recovery that were observed. For the 25/75 dilution the results showed that for OA after evaporation the recoveries were variable: under neutral (25%), basic (25%) and acidic (50%) washing conditions increases in recovery (from 20-30 %) were found, under neutral (50%) and Basic (50%) losses of approx 30% were observed. For DTX1 and -2 losses of up to 50% were experienced. For the 50/50 concentrated losses were found for OA and DTX1 (approximately 25%). The recoveries for DTX2 showed no significant change from the concentration step. Despite any changes in recovery, the trends across the different washing conditions were the same for all 3 toxins and for both loading compositions.

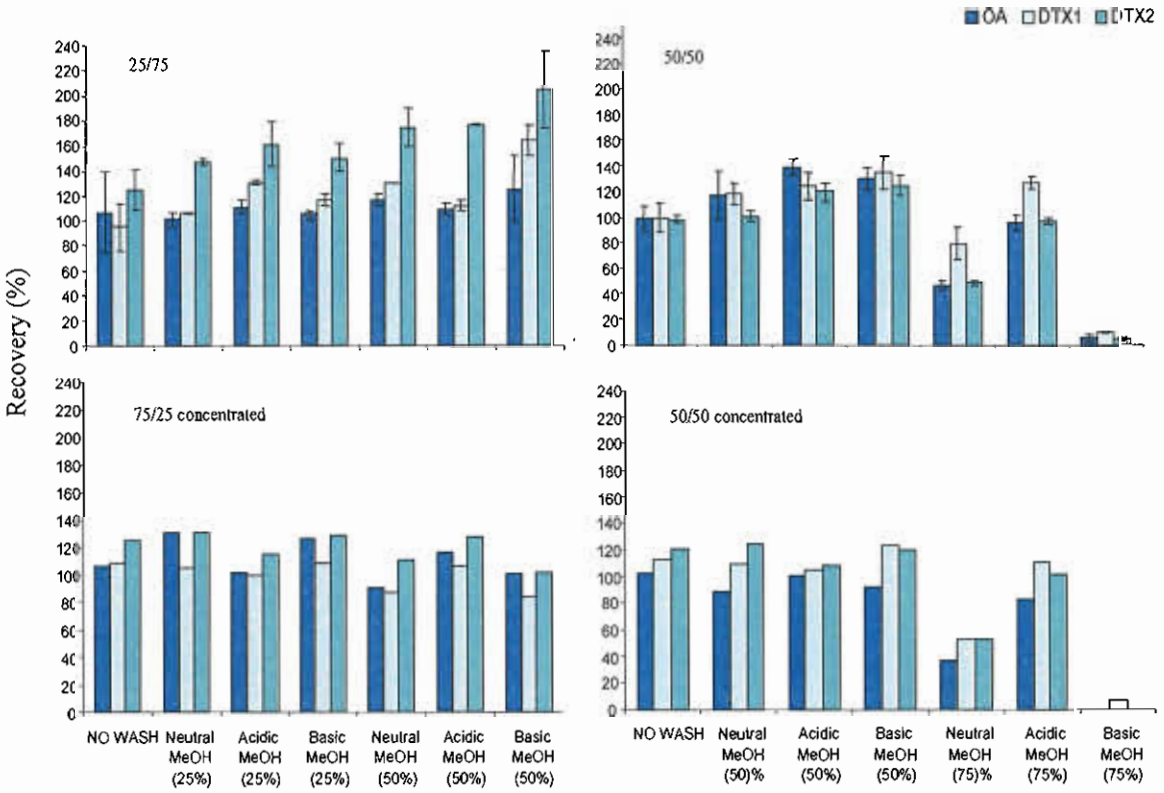


Figure 5-11: Recoveries obtained for OA toxin group following SPE (Oasis ™ HLB, 60mg) of LRM material using two loading compositions (25/75 and 50/50) and seven washing conditions (error bars represent SD ± 1, n=2) plus subsequent concentration steps (based on a single treatment and analysis), all fractions analysed using LC-TSQ-MS

The recoveries for YTX are presented in Figure 5-12. For the 25/75 loading composition of YTX the highest recovery (125% ± 3%) was found using a basic washing step with an organic strength of 50% MeOH. Using a 50/50 loading composition the highest recovery (127% ± 12%) was obtained with a basic washing step with an organic strength of 50% MeOH but with less precision. As in all toxin groups, increasing the organic strength when using the 50/50 loading composition caused severe recovery losses; for the neutral washing step approx 43% of the toxin recovery was found in the wash fraction.

Regardless of the loading composition the use of an acidic wash solution caused significant recovery losses (all recoveries less than 35%), these low recoveries could not be accounted for in the washing fractions. The trend of recovery was the same across the range of washing conditions when a concentration step was applied. For the 25/75 concentrated loading compositions, recovery losses were observed (30-40%) except in the acidic washes where previously severe losses had been observed the recovery in the 25/75 dilution was improved by up to 70%. This was not the same for the 50/50 loading where the recoveries in acidic conditions were all below 20% regardless of the inclusion of a concentration step. Recovery losses (ranging from 20-40%) were observed in the other washing conditions.

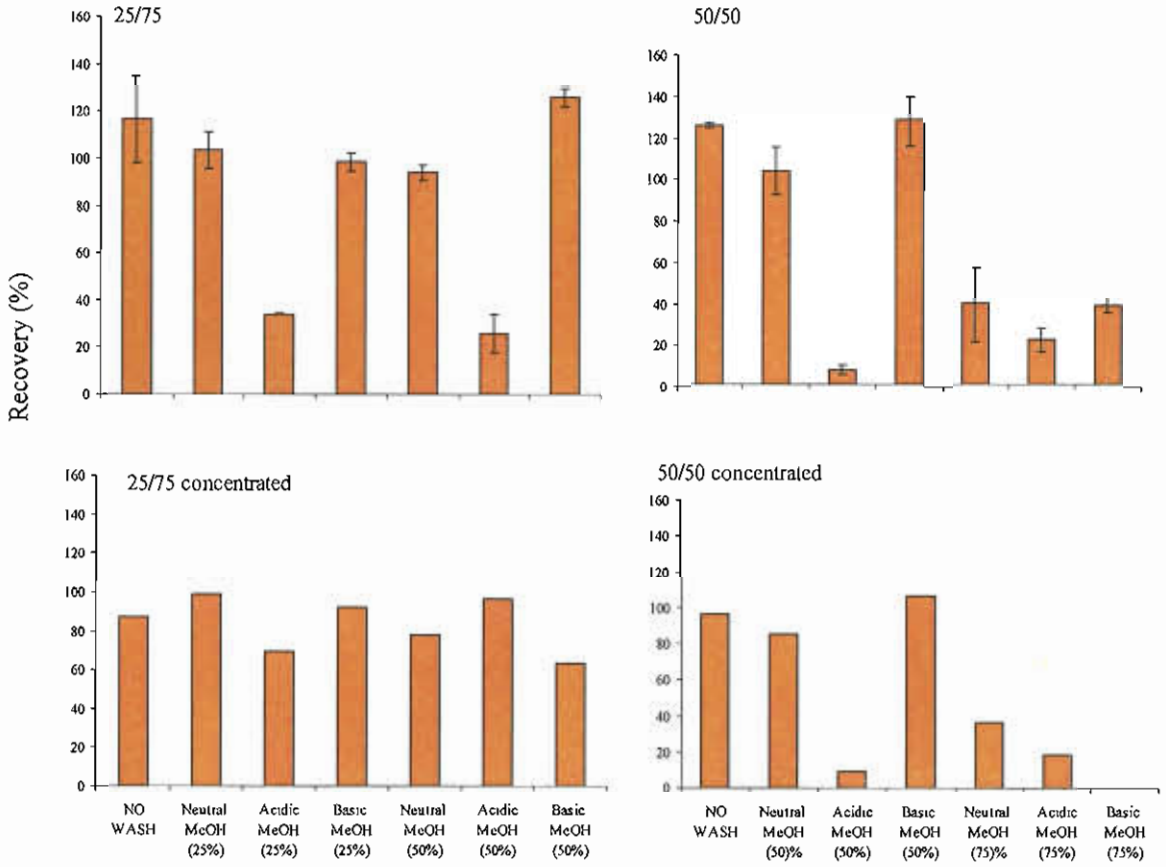


Figure 5-12: Recoveries obtained for YTX following SPE (Oasis ™ HLB, 60mg) of LRM material using two loading compositions (25/75 and 50/50) and seven washing conditions (error bars represent SD ± 1, n=2) plus subsequent concentration steps (based on a single treatment and analysis), all fractions analysed using LC-TSQ-MS

The results obtained for PTX2 are presented in Figure 5-13. The optimum recovery for PTX2 (94%) was achieved when the sample was loaded in a 25/75 dilution and washed with a basic solution of 50% MeOH. For the 50/50 load composition the optimum recoveries were also achieved using a basic wash at 50 and 75% MeOH (75% and 71% recoveries respectively). The concentration step reduced these recoveries by approximately 20%. The precision (error bars) associated with the acidic washes was generally worse (±2 and 4% for 25/75 and 50/50 respectively).

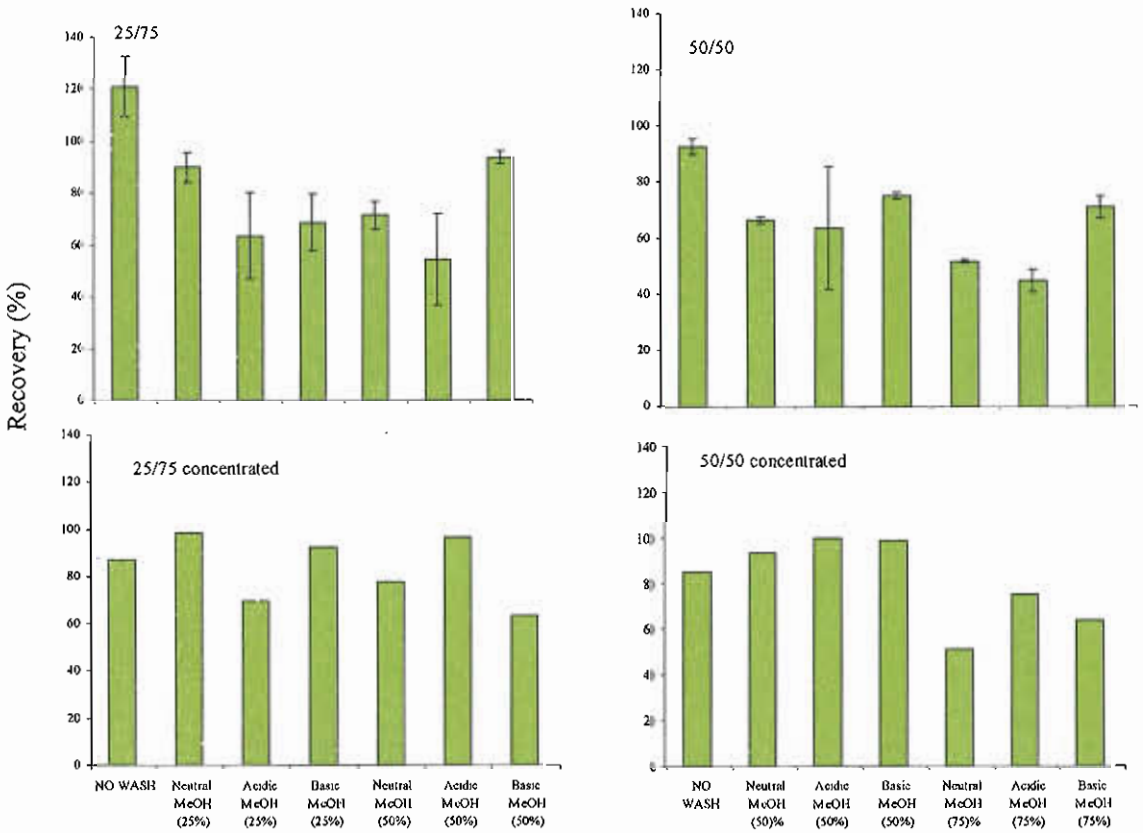


Figure 5-13: Recoveries obtained for PTX2 following SPE (Oasis ™ HLB, 60mg) of LRM material using two loading compositions (25/75 and 50/50) and seven washing conditions (error bars represent SD ± 1, n=2) plus subsequent concentration steps (based on a single treatment and analysis), all fractions analysed using LC-TSQ-MS

5.3.2.2.3. Discussion

The objective of this experiment was to focus on the recovery of toxin during SPE. Two parameters of the SPE procedure (loading and washing steps) were considered as a function of recovery.

By performing the SPE on the multi-toxin standard it was possible to calculate the recovery of toxin free from any effects arising from the matrix. However, the matrix can offer protection to the toxins and so losses due to adsorption may be occurring during the SPE of standard in pure solvent; therefore it was necessary to also perform the same procedure on shellfish matrix (LRM).

The results from SPE carried out on the standard indicated that there were no significant differences observed between the loading compositions for OA, YTX and PTX2, except at a 50/50 loading composition using a basic (75%) washing step where recovery losses were observed. The same recovery losses were found for AZA1 under these conditions, however for AZA1 (unlike OA, YTX and PTX2), there were significant differences observed between loading compositions, the 50/50 dilutions gave significantly improved recoveries (up to 50%) compared to the 25/75 dilutions.

The losses experienced at the higher organic strengths for all of the toxins could only be partially accounted for in OA and YTX group where breakthrough into the wash fraction was found. The optimum washing conditions regardless of loading composition was a basic wash (50% MeOH). For the 25/75 dilutions this corresponded to using a stronger wash and for the 50/50 dilutions the wash was at the same strength as the loaded sample.

For the LRM material the loading composition was more critical than previously observed in the standard material. Promisingly, the optimum recoveries were achieved using the same conditions for all of the toxins, using a loading composition of 25/75 with a basic washing step (50% MeOH).

The same amount of matrix was applied onto the sorbent in each load step (2ml of 50/50, 4mL of 25/75); the difference was in the amount of de-ionised water used in the preparation of the sample prior to SPE. Overall, the best recoveries resulted from the loaded sample with a higher component (75%). No breakthrough into the wash was detected for the 25/75 loading composition for the OA, PTX2 and AZA toxins suggesting that a high aqueous component in the load step can serve to improve the retention during the washing step without inhibiting the subsequent elution of analyte.

Where low recoveries were found analysis of the wash fractions indicated whether some/all of the toxins may have been lost as a result of breakthrough When breakthrough into the wash fractions was detected this was an indication that the washing conditions were too strong and when no breakthrough was detected this suggested that the toxin was still retained on the sorbent and could be eluted in the next step (which was the aim of the SPE). Another reason for the low recoveries may be due to the analytes being retained on the sorbent (incomplete elution), breakthrough of the toxins straight through the sorbent bed upon loading or the degradation of toxins under harsh SPE conditions.

Significant amounts of breakthrough were experienced for the 50/50 load step for the OA group and YTX although not in consistent amounts; for the OA group (at washes using a higher organic percentage) breakthrough was detected in all washing fractions: 40%, 16% and 70% for the neutral, acid and basic washes respectively. This accounted for most of the low recoveries.

For YTX, breakthrough into the wash (43%) was detected in the neutral washing fraction only but this does not fully account for the low recoveries or explain the low recoveries using acidic conditions. Smaller amounts of breakthrough were detected in the AZA group (approx 10%) in acidic wash (75% MeOH).

The inclusion of a concentration step after SPE was also investigated. A concentration step is important when dealing with lower detectable amounts of analyte and where the MS sensitivity may be low. These experiments have shown that the use of a concentration step gives rise to variability in the values found.

The purpose of the SPE procedure was to purify the sample prior to LCMS analysis, to remove any interference that may contribute to matrix effects. The washing step is a critical factor; the washing serves to remove any interfering compounds without causing any breakthrough of analyte into the washing fraction. This experiment has shown that the optimum recoveries were achieved using 25/75 and a basic washing (50% MeOH), comparable recoveries can be achieved using a 50/50 load step with a basic wash step (50% MeOH), however if the washing solution contains 75% MeOH losses may occur.

5.3.2.3. Further optimisation of wash step

The previous experiment highlighted the possibilities that SPE holds in the clean up of shellfish extracts containing lipophilic marine toxins using one sorbent type. The washing and loading compositions were examined for one particular cartridge type; this experiment introduces more cartridge manufacturers including the Oasis ^{TM} HLB (with a larger amount of sorbent bed due to the larger volumes of wash solvents to be applied).

5.3.2.3.1. Experimental conditions

Preparation of materials

For this experiment the LRM (MI) only was used. An LRM was extracted as described in section 2.4.2. A load composition of 40:60 (MeOH: Deionised water) was chosen due to the nature of the rigorous washes to be applied and the larger sorbent bed (200mg), a washing composition of 50% methanol was chosen as the Symbiosis results (5.3.1.2) had shown that losses were not likely to occur at this organic strength. This was prepared by the combination of 20mL of LRM extract, 60mL of MeOH and 120mL of deionised water.

Preparation of SPE solutions

Three wash solutions were prepared to be applied either individually, by pair or by application in series of all three (acidic, neutral and basic). All of the wash solutions were prepared at a composition of 50% MeOH.

A neutral solution (200ml) was prepared by the addition of 100ml of MeOH to 100ml of deionised water.

An acidic solution (1% formic acid) was prepared by adding 2ml of formic acid and 100ml of MeOH into a 200ml volumetric and completing the volume with de-ionised water.

A basic wash solution (1% ammonium hydroxide) was prepared by adding 2ml of ammonium hydroxide and 100mL of MeOH into a 200ml volumetric and making up to the complete volume with deionised water.

SPE Equipment

Along with the Oasis ^{TM} HLB (200mg) some other cartridges were also included:

- Isolute ENV+ (200mg). A co-polymer consisting of a hyper cross linked hydroxylated polystyrene divinylbenzene
- Bond Elut LRC Certify (300mg) (Varian). A silica based mixed mode sorbent based on non polar and strong cation exchange mechanism
- Strata SDB-L (200mg) (Phenomenex). A polymeric sorbent based on the conventional styrene-divinylbenzene polymer.

SPE procedure

All of the possible variations of the wash solutions were used of which there were nine. All of the wash fractions were collected for LCMS analysis. The protocol for this SPE experiment is as follows:

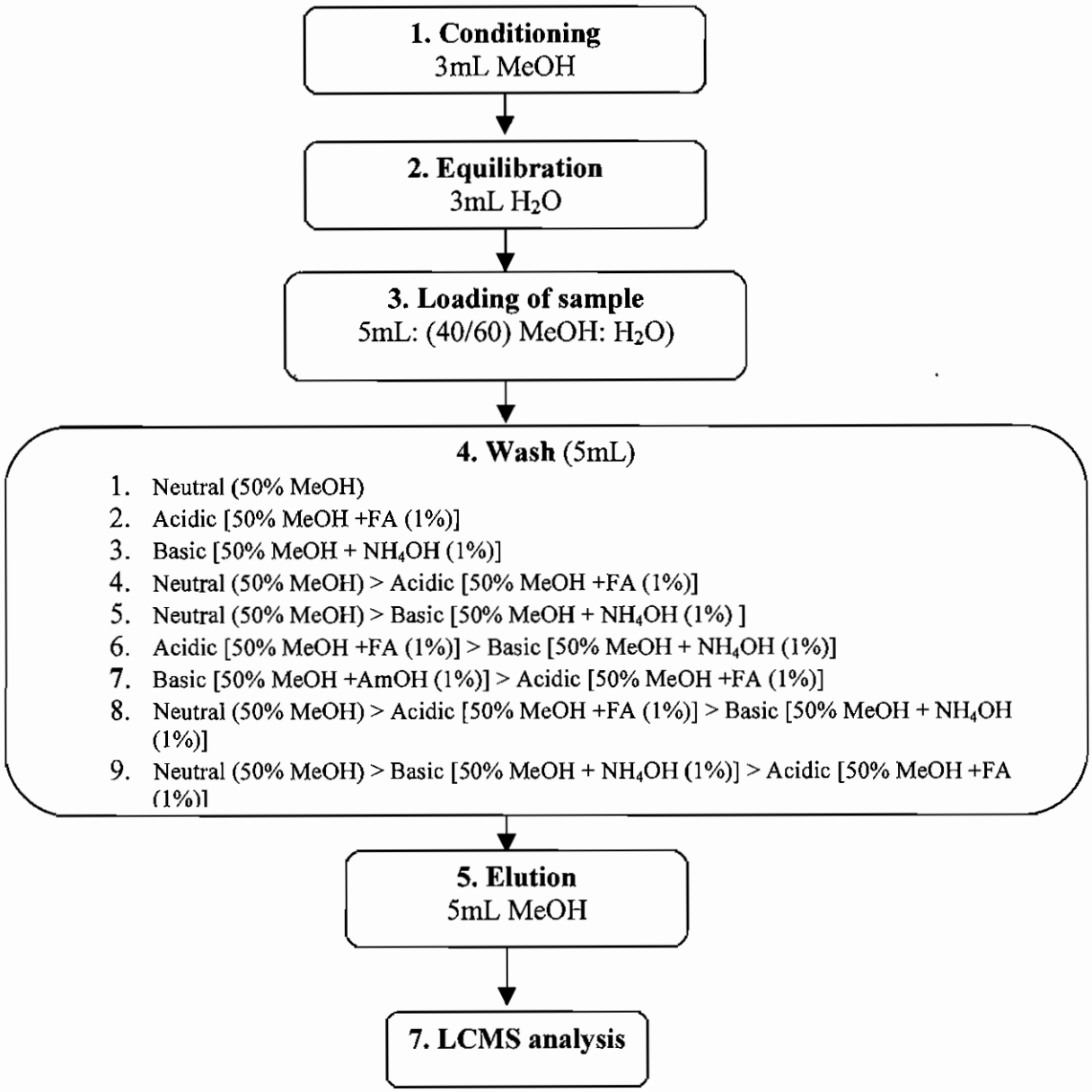


Figure 5-14: Schematic of SPE experiment to further optimise washing step using LRM material using different cartridge types (Oasis ™ HLB (200mg); Strata SDB-L (200mg); Isolute ENV+ (200mg); Bond Elut LRC Certify (300mg)). LCMS analysis was conducted using LC-TSQ-MS

LCMS analysis

LCMS analysis was carried out using the XBridge method as described in section (2.5.3).

5.3.2.3.2. Results

The results are presented according to the ionisation mode in which they are analysed. However, this does not suggest that ionisation mode has an effect on the recovery. The highest recoveries for all of the toxins were obtained using the HLB and Strata cartridges. For the HLB firstly (Figure 5-15), in positive ionisation mode the recoveries for the AZAs and PTX2 were high for all the wash conditions (ranging from 90 to 140%) with no detectable breakthrough. In negative mode ionisation (YTX and OA/DTX) the situation was slightly more complex. Substantial break-through of YTX occurred depending on the wash protocol applied, resulting in low or even non-detectable recovery. With exception to YTX, the highest recoveries of toxins resulted with a single acidic wash (ranging from 120 to 139%), with little break-through.

The optimum recovery (134%) for YTX (with no breakthrough) was achieved with single basic wash step. A combined acidic and basic wash also gave good recovery (118%), but also some breakthrough (20%) indicating that this condition was less favourable. For the OA group breakthrough posed a problem when more than one wash step was applied.

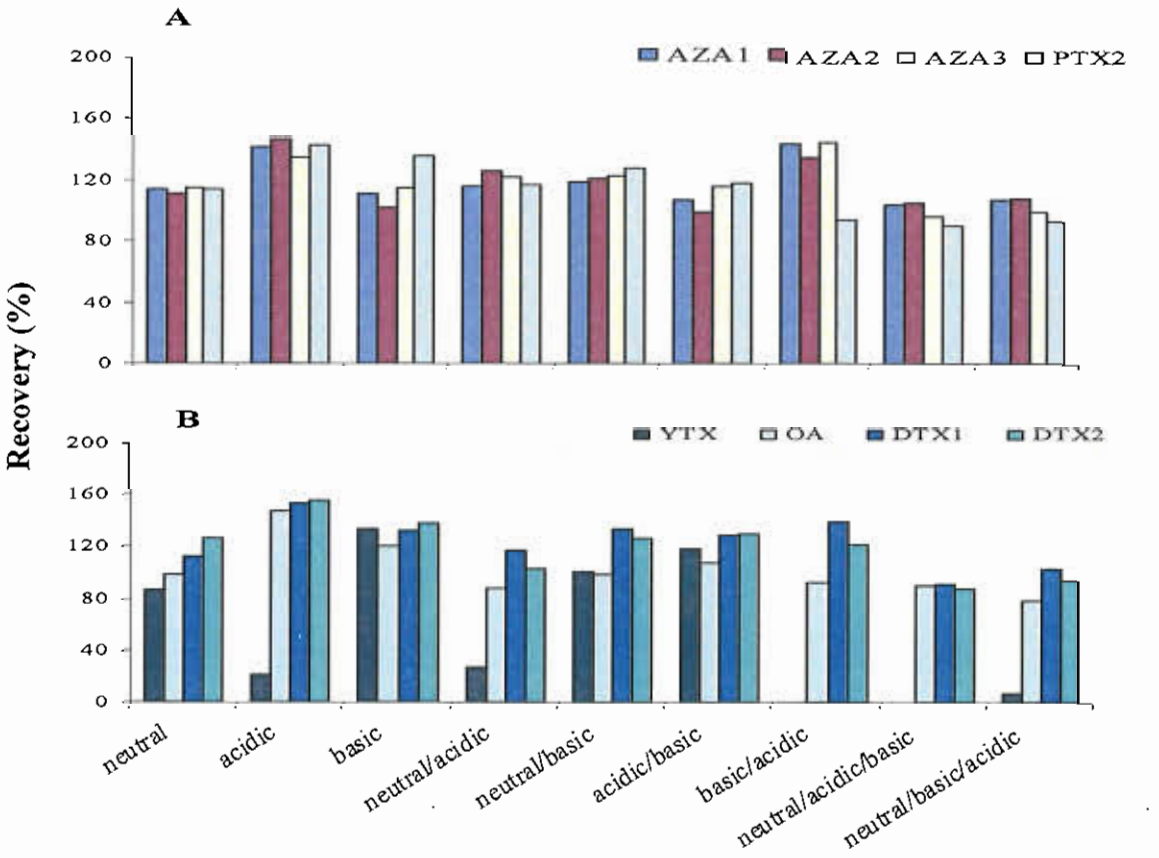


Figure 5-15: Recoveries obtained from SPE procedure carried out using LRM on Oasis ™ HLB (200mg/6mL) cartridge under various washing conditions analysed by LCMS in (A) Positive and (B) negative mode ionisation

In the case of Strata SDB-L (Figure 5-16) recoveries for the AZAs were more variable. Low recoveries were found when only neutral (approx 17%) or acidic (approx 50%) washing steps were applied. No break-through occurred, indicating that the elution of the AZAs under these conditions is perhaps not complete. Good recoveries were obtained when a basic washing step was incorporated into the procedure. Recoveries that exceed 100% may indicate that factors causing ion suppression in the crude LRM extract are selectively removed by the SPE protocol. Break-through is observed for YTX and OA/DTXs on the Strata cartridge as previously found using the Oasis ™ HLB, the amounts are generally smaller for the corresponding washing condition when compared to Oasis ™ HLB.

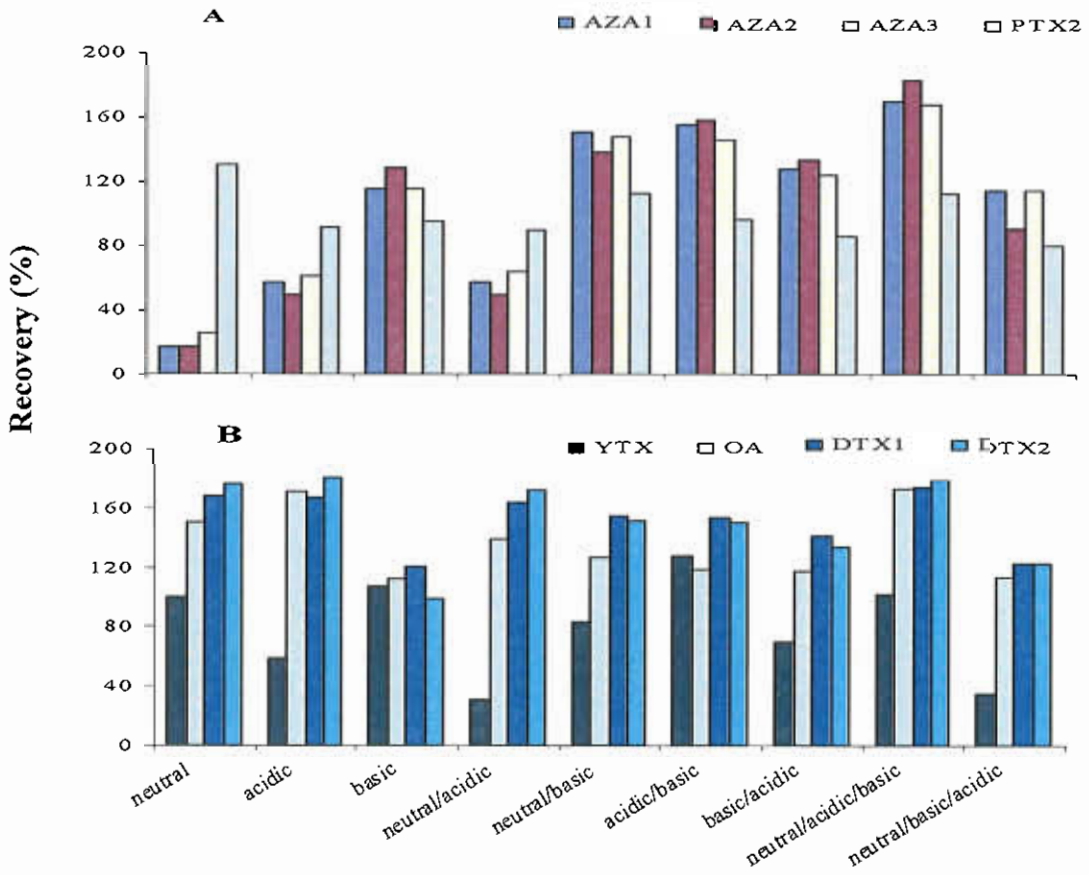


Figure 5-16: Recoveries obtained from SPE procedure carried out using and LRM from Strata SDB-L (200mg/6mL) cartridge under various washing conditions analysed by LCMS in (A) Positive mode ionisation and (B) negative mode ionisation

The results obtained from the Bond Elut Certify and Isolute ENV+ cartridges are presented below (Figure 5-17 and Figure 5-18).

Like the Strata and Oasis brand cartridges the Bond Elut LRC Certify cartridge was compound specific and requires specific washing conditions for successful SPE. In negative mode ionisation the OA toxins were successfully recovered using neutral, acidic and a combination of neutral/acidic. YTX was only recovered (100%) using a single acidic wash, however upon the introduction of a basic step the recoveries were no longer detectable.

PTX2 was recovered under all conditions with recoveries from 80-140%. The AZA group required a basic wash step to achieve sufficient recoveries. Successful retention and elution of all compounds was possible using this cartridge however different washing conditions were required for the different toxin groups.

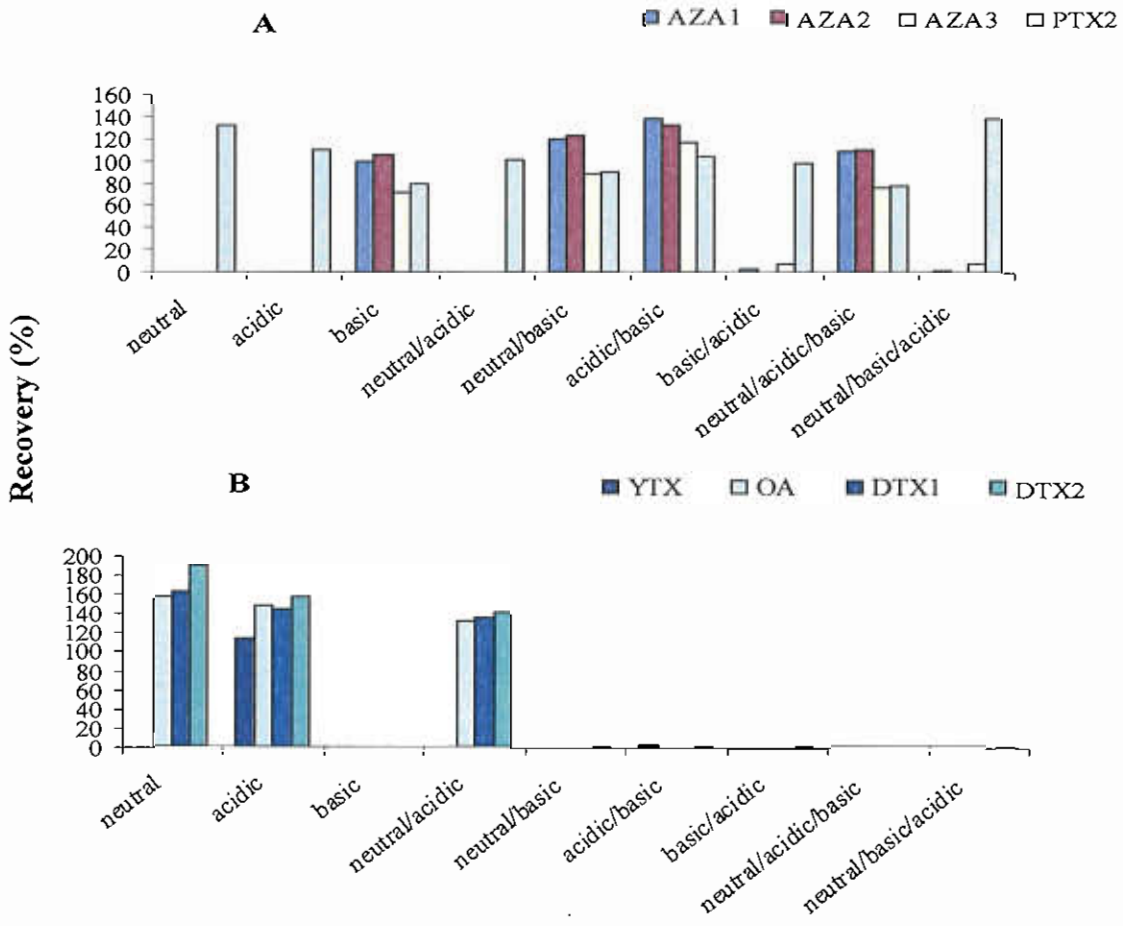


Figure 5-17: Recoveries obtained from SPE procedure carried out using LRM on Bond Elut LRC Certify (300mg/10mL) cartridge under various washing conditions analysed by LCMS in (A) Positive and (B) negative mode ionisation

The Isolute ENV+ could retain and elute OA and its DTX analogues (approx 120% recovery) under acidic conditions only. The remaining wash conditions resulted in very low recoveries (less than 60%). For the toxins analysed in positive mode ionisation, PTX2 (95%) was recovered using a single acidic step. For the AZA toxins a basic step gave increased recoveries to approximately 50% (this trend is similar to the Strata and Oasis cartridges). Once again this cartridge requires different washing conditions to successfully recover the different toxin groups due to the chemistry of the sorbent.

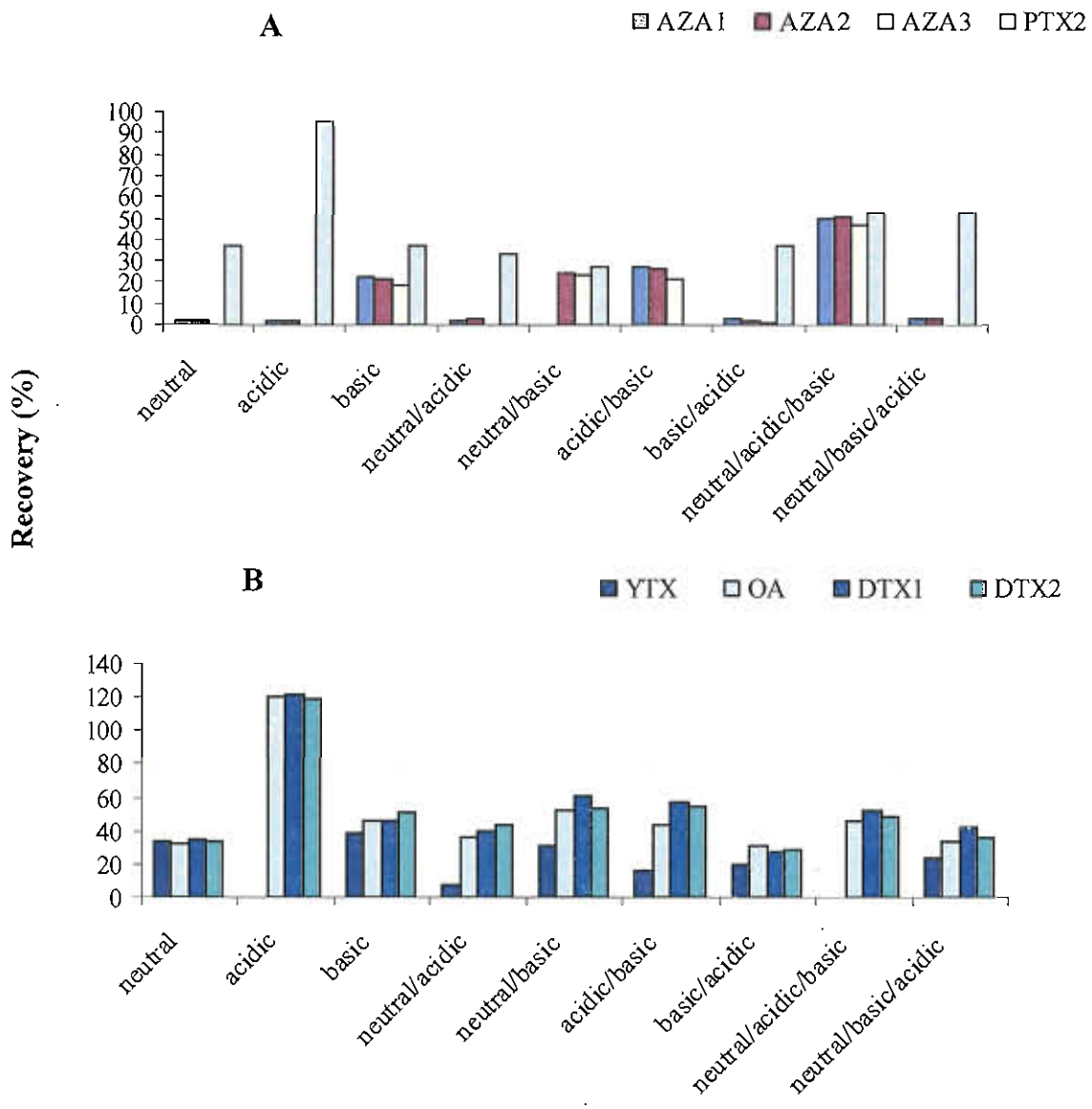


Figure 5-18: Recoveries obtained from SPE procedure carried out using LRM on Isolute ENV+ (200mg/6mL) cartridge under various washing conditions analysed by LC-TSQ- MS in (A) Positive and (B) negative mode ionisation

5.3.2.3.3. Discussion

The mechanism of SPE chosen for this work is to load the sample (diluted with water) onto the sorbent and to use the washing step to remove interferences before eluting the toxins from the sorbent. The previous experiment had shown that it was possible to retain and subsequently elute all of the relevant toxins using a single SPE procedure/cartridge, various parameters of the procedure were also examined (load and wash), however as this was optimised for a particular cartridge type (Oasis ™ HLB, 60mg) at a relatively early stage of method development these conditions were used as an approximation for the conditions to be used from that point.

The aim of this study was to examine the washing step further. Three wash solutions were prepared (acidic, neutral or basic) and applied either individually, by pair or by application in series of all three. This study provided information on the use of different wash conditions (different pHs) in one washing procedure in the removal of a wider range of interferences. The use of a series of washing conditions is only advantageous if no losses of toxins are experienced.

Of all of the cartridges included in the study the Oasis ™ HLB and Strata SDB-L cartridges were applied well across the range of toxins. It was necessary to interpret the results as a complete group and choose a condition that gives the optimum recoveries for all four toxins and in this respect the Strata SDB-L gave the highest recoveries for the whole group. This was more noticeable for the OA, DTX1, and -2 and YTX toxins where lower recoveries were attributed to breakthrough into the washing fractions during the wash step. This breakthrough was more severe on the Oasis ™ HLB. The same breakthrough was not observed for the AZA toxins and PTX2 (in either cartridge) indicating that the elution of the AZAs under these conditions may not be complete where the recoveries are particularly low. YTX was the compound, which displayed the most variability between washing conditions, therefore the recoveries were considered as a whole group incorporating YTX. The optimum recoveries for the whole group were using a single basic step or a combination of acidic /basic washing conditions.

Both the Bond Elut LRC certify and Isolute ENV+ cartridges showed that OA, DTX1 and -2 required an acidic wash step for sufficient recoveries and that the AZA group showed an

improved recovery upon the introduction of a basic step on the Bond Elut. This was not the case using the Oasis ™ HLB and Strata SDB-L cartridges where neither cartridge displayed such severe specificity.

In the development of a new SPE method, consideration must be given to transferability of the procedure between different laboratories where the procedure must be robust enough to be repeatable between laboratories. The cartridge manafacturer must not also be a limiting factor; the procedure must be described for a certain cartridge type more so than a specific company manufacturer. The Oasis ™ HLB and Strata SDB-L cartridges are comparable in terms of recovery of toxins and both of these cartridges are packed with co polymers and are making use of the same sorbent chemistry (hydrophilic-lipophilic phases).

5.3.2.4. Wash and elute combination

This experiment was designed to evaluate the use of the elution step in improving the recoveries. The previous experiments indicated that the use of basic conditions in the wash can significantly improve recoveries without any degradation of the toxins. A neutral elution step has been used to this point; this experiment examines the introduction a basic elution step to investigate the effect on recovery.

5.3.2.4.1. Experimental conditions

Preparation of materials

Four LRMs were extracted as described in section 2.4.2 and combined to give a bulk homogenous material. A load composition of approx 30/70 (MeOH: Deionised water) was prepared by the combination of 80mL of LRM material with 190mL of deionised water.

Preparation of SPE solutions

The wash solutions (50mL) were prepared at an organic strength of 10% MeOH.

A neutral wash solution was prepared by the addition of 5mL of MeOH to a 50mL volumetric flask and completing to the final volume with deionised water.

A basic wash solution (1% ammonium hydroxide) was prepared by adding 0.5mL of ammonium hydroxide and 5mL of MeOH into a 50mL volumetric and making up to the complete volume deionised water.

For the neutral elution solvent MeOH (100%) was used.

A basic elution solution (1% ammonium hydroxide) was prepared by the addition of 0.5mL ammonium hydroxide into a 50mL volumetric and making up to the complete volume with MeOH.

SPE Equipment

The Oasis TM HLB (200mg/3mL) and Strata-X cartridges (200mg/3mL) were used for this experiment. The functionalised Strata- X have numerous retention mechanisms, which can yield high recoveries and possess a larger surface area and replaces the previously described strata SDB-L (200mg/6ml) (5.3.2.3.1).

SPE procedure

The SPE procedure is illustrated below using LRM material diluted to a 30/70 MeOH: H2O. Four replicates of each treatment were carried out. There were four wash/elute combinations possible.

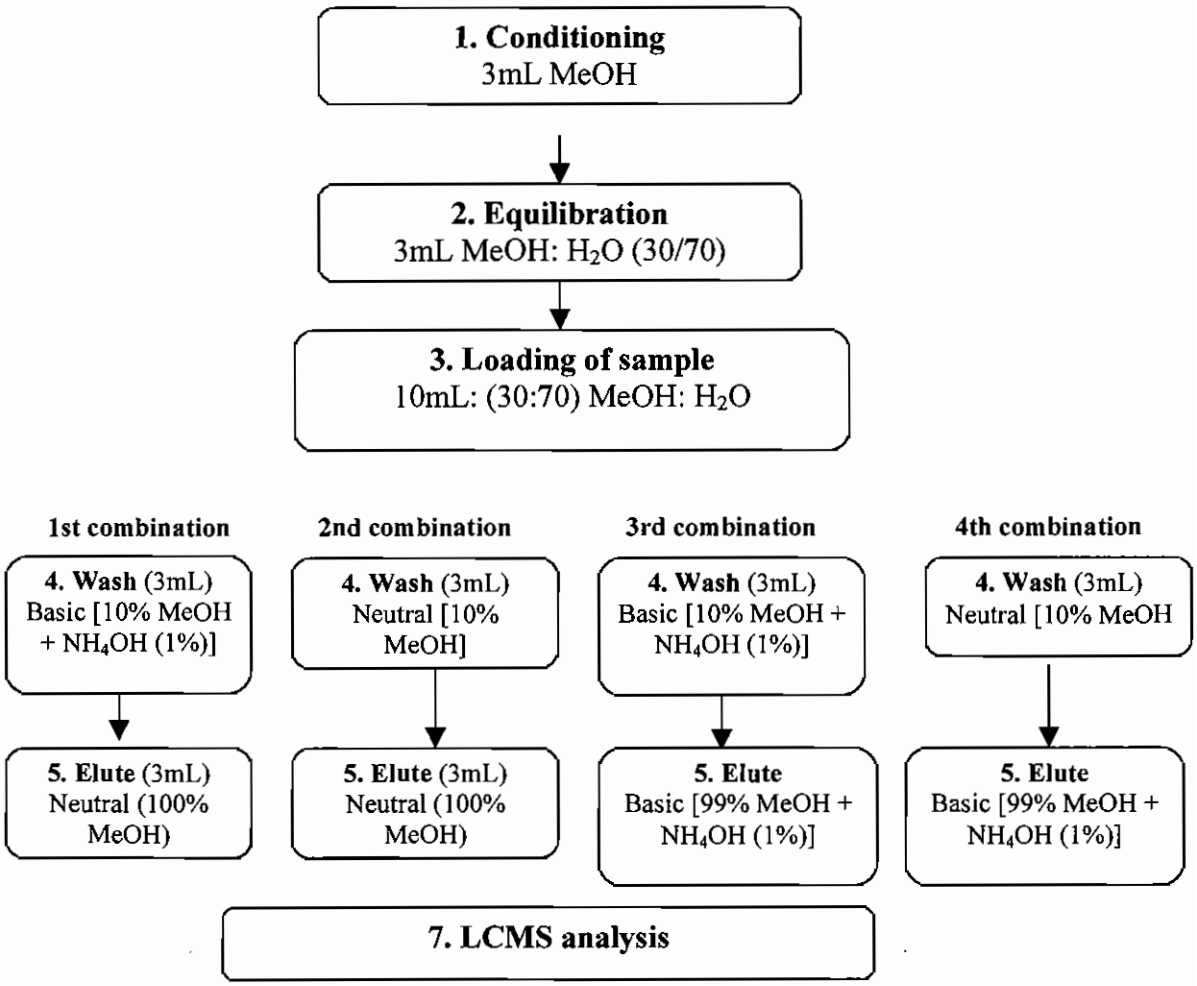


Figure 5-19: Schematic of SPE procedure conducted using Oasis ™ HLB (200mg/3mL) and Strata-X cartridges (200mg/3mL) for wash and elute combination experiments using LRM material

LCMS analysis was conducted using the conditions as described in section 2.5.1.

5.3.2.4.2. Results

The results below represent the recoveries obtained from both of the cartridges involved in the experiment under the various wash and elute combinations.

For the AZA group firstly, there were no significant differences observed between the two cartridges under the various combinations except in the neutral wash/neutral elution combination where the Oasis ™ HLB gave significantly better recoveries than the Strata X (approximately 50% for all of the toxins). The recoveries for AZA1 were significantly greater (approximately 70%) than AZA2 and -3 in both cartridges.

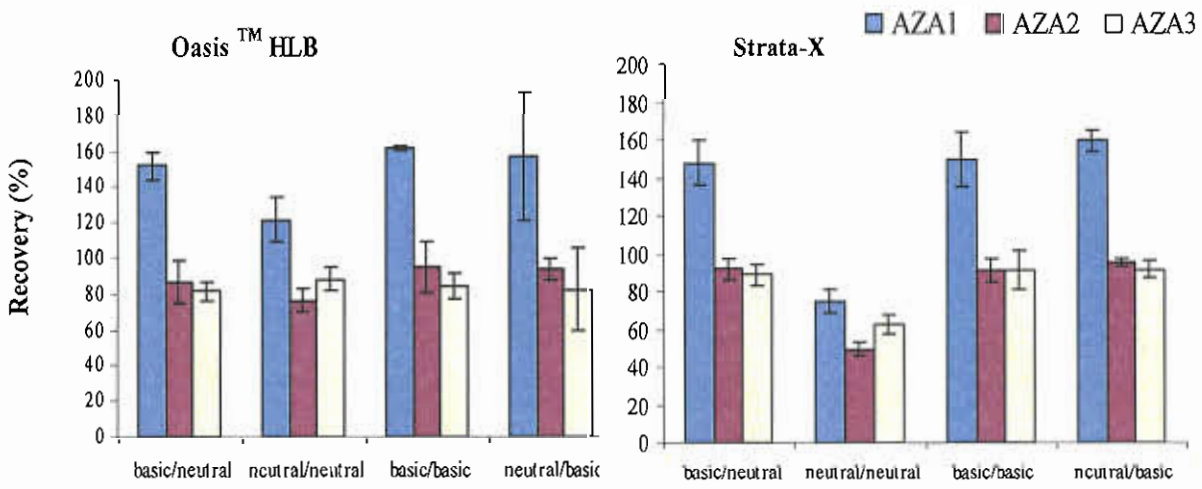


Figure 5-20: Recoveries of AZA group found from SPE of LRM material using various wash and elution combinations from Strata SDB-L and Oasis ™ HLB cartridges, cleaned fractions analysed by LC-TSQ-MS, error bars represent SD ± 1, (n= 4)

The recoveries of OA toxin group were less variable across the range of wash and elute combinations and cartridge types, except for the neutral/neutral combination using Oasis ™ HLB where recoveries were significantly higher (ranging from 132 to 144%). The basic wash/neutral elution combination resulted in the lowest recoveries for both cartridge types with recoveries ranging from approximately 92 to 110%. The precision was also better for the Strata-X when a basic component is used in the elution step.

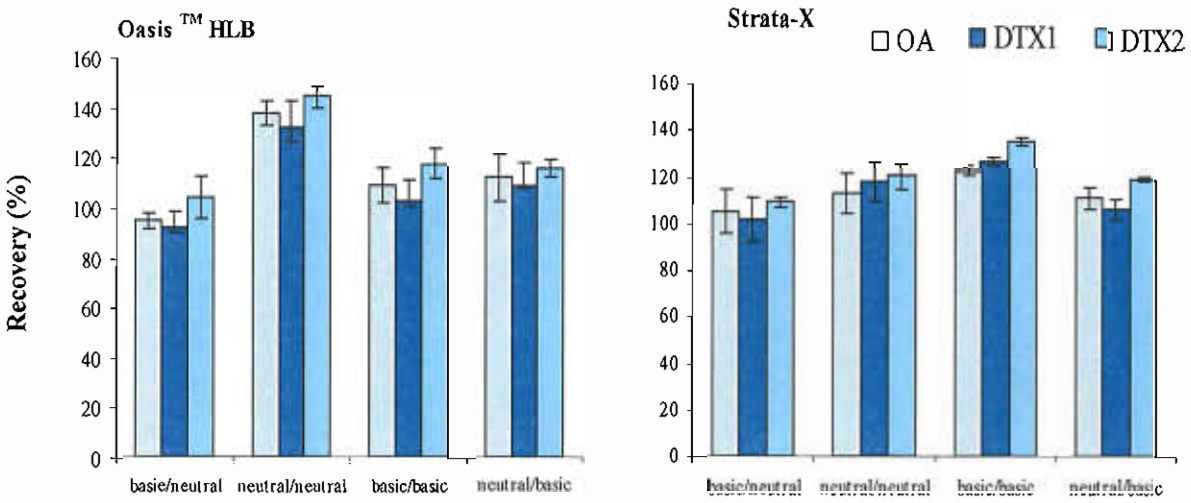


Figure 5-21: OA group recoveries found from SPE of LRM material using various wash and elution combinations from Strata SDB-L and Oasis ™ HLB cartridges, cleaned fractions analysed by LC-TSQ-MS, error bars represent SD ± 1, (n= 4)

5.3.2.4.3. Discussion

The previous experiments indicated that the introduction of a basic component into the washing step can improve the recoveries of YTX and PTX2, and although these toxins are not included in this study it has been shown that basic conditions do not have a detrimental effect in the recovery of the OA and AZA toxins.

Basic conditions could also be introduced into the elution step to improve recoveries. At this stage of the method development the emphasis is still centred on the recovery of the toxins.

Four wash/elute combinations were applied during this procedure, using the two cartridge materials that have provided high recoveries to date:

1. Basic/Neutral
2. Neutral/ Neutral
3. Basic/Basic
4. Neutral/Basic

The first notable observation was the very high recoveries obtained for AZA1 (up to 160%) compared to AZA2 and -3 (approximately 90%) on both cartridges.

The Neutral/Neutral combination gives the lowest recoveries for AZA1 and -2 on both cartridges with no significant differences observed between the other conditions.

For the OA toxins included in the study (OA, DTX1 and -2) the neutral/neutral combination gives the highest recoveries for all of the toxins using the HLB cartridge, which is not in agreement with the AZA toxins. These results represent the difficulties involved in obtaining the highest recoveries using one set of conditions. The Basic/Basic combination would be a good compromise for both cartridges and all toxins.

5.3.3. Conclusion for SPE

The main objective of this study was to develop a SPE method to include the key lipophilic toxin groups (OA, AZA, PTX2 and YTX).

The SPE procedure was considered as five steps:

1. Conditioning
2. Equilibration
3. Loading of sample
4. Washing of impurities from sample
5. Elution of the analyte

The steps of most importance during the developmental phase were the loading, washing and eluting steps. A literature search carried out on the current SPE methods for the lipophilic toxins revealed various methods for the individual groups, but to date a universal method for all of the lipophilic toxins has not been reported.

In the development of a new SPE method the emphasis is primarily based on the recovery of toxins. The sample is loaded onto the sorbent in such a composition as to effectively interact with the sorbent particles without any breakthrough. The loaded sample must have an adequate and methanolic content so as to wet the sorbent and also not allow the analyte to pass through the sorbent bed. The optimum loading composition was determined to be a dilution of the extract to 25/75 (MeOH:Water).

The purpose of the washing step was to remove any interfering components whilst at the same time not eluting the toxins. The symbiosis results indicated that losses were not experienced up to a washing composition of 70% MeOH. During the first experiment, the optimum recoveries of the LRM were found when the washing step was at a higher organic strength (50% MeOH) to that of the loaded sample (25% MeOH).

The elution step was required to effectively remove the analyte from the sorbent, this can be achieved using MeOH or adding a basic buffer.

The SPE method development was carried out in collaboration with another Institute (RIKILT), where further developments were carried out on the work as described. The loading step, washing conditions and elution volume were further investigated. For the loading step they found that the optimum loading composition was a 30/70 MeOH/H_2O dilution of the primary methanolic extract (similar to what was found in the preliminary experiments).

For the washing step, their investigations found that the optimum washing strength was found to be 20% MeOH. For complete elution of all of the toxins, a volume that was the same as the loaded sample was required (3mL).

The final protocol was decided upon by the partners and is shown below. The recommended cartridge is the Strata-X 60mg/3mL (Phenomenex), but the method also performs adequately on the HLB ™ Oasis .The procedure can also be scaled up or down.

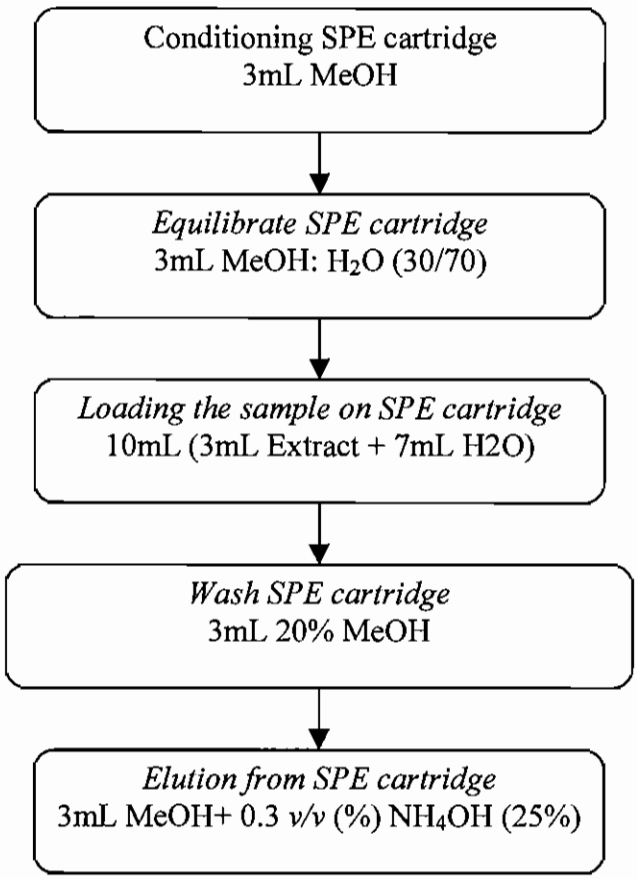


Figure 5-22: Finalised SPE conditions as decided by Marine Institute and RIKILT

6. Role of sample clean-up in the removal of matrix effects

6.1. Introduction

Chapter 4 involved the characterisation of matrix effects that are currently experienced in the LCMS analysis of lipophilic marine toxins (OA, AZA1 and PTX2). Chapter 5 described the development of two sample clean-up techniques (LLE and SPE), which were developed with the objective of removing matrix effects. This chapter examined the optimised clean up techniques to assess their relative effectiveness in the removal of matrix effects from shellfish extracts.

Mussel, oyster and scallop tissue were used for this study. The oyster and scallop material was that same material used previously in the preparation of matrix matched and matrix strength standards (4.2 and 4.3 respectively) and for which the total lipid content was calculated. The mussel material was a blank composite sample obtained from RIKILT.

Various parameters of each clean-up were determined; the matrix effect in the crude extract; the recovery of toxin throughout the clean-up; the removal of matrix effects upon LLE and a combination of recovery and removal of matrix effects. The parameters were investigated independently of each other. The following equations define each parameter that was evaluated:

$$\frac{\text{Area Pre Spiked WS}}{\text{Area Post Spiked WS}} \times 100\%$$

Equation 6-1:Calculation of recovery for working standard

$$\frac{\text{Area pre spiked}}{\text{Area post spiked}} \times 100\%$$

Equation 6-2: Calculation of toxin recovery from purified shellfish extract

$$\frac{\text{Area crude extract}}{\text{Area WS}} \times 100\%$$

Equation 6-3: Calculation of matrix effect in the crude shellfish extracts

$$\frac{\text{Area post spiked}}{\text{Area WS}} \times 100\%$$

Equation 6-4: Calculation of matrix effects in extract after clean up

$$\frac{\text{Area pre spiked}}{\text{Area WS}} \times 100\%$$

Equation 6-5: Combination of clean-up effect and recovery (clean up efficiency)

6.2. LLE in the removal of matrix effects

The initial method development for LLE focused on the recovery of toxin through various partitioning conditions, various drawbacks were encountered such as; losses in recovery with additional partitioning steps, losses were also attributed to the evaporation and reconstitution step required at the end of the procedure to prepare the extract for LCMS analysis. This study examined the recovery independently of the effectiveness of the clean up; this was achieved by spiking the toxin into the extract after LLE has been carried out. The combination of recovery and the removal of matrix effects gave a value, which takes the recovery into account in the overall effectiveness of the technique.

6.2.1. Experimental conditions

6.2.1.1. Preparation of materials

The extraction procedure was carried out similarly to the BIOTOX extraction procedure (2.4.2) (except that a SSR ratio of 5 was used instead). Three extractions were carried out per tissue.

An aliquot of shellfish tissue (4g ± 0.1g) (mussel, oyster and scallop) was weighed into a 50ml polypropylene centrifuge tube and extracted with 6mL of MeOH and vortexed on the multi-tube mixer (V400 Alpha Labs, UK), for 1 minute at full power. After centrifugation at 3,000 rpm ± 2,000 rpm for 5 min the supernatant was decanted into a 20mL volumetric flask. The pellet was re-extracted with 6mL of MeOH and vortexed for 1 min. The solution was centrifuged at 3,000 rpm ± 2,000 rpm for 5 min; the supernatant was decanted into the same volumetric flask

A third extraction of the pellet was carried out with 6mL of MeOH using an Ultra-turrax ™ homogeniser for 1 min at full power. After another centrifugation step (3,000 rpm ±

2,000 rpm for 5 min) the supernatant was decanted in the same 20mL volumetric flask. The solution in the volumetric flask was made up to the mark with MeOH.

6.2.1.2. Preparation of stock standard

A toxin standard (10ml) containing OA (320ng/ml) and AZA1 (200ng/ml) was prepared for spiking into the tissues. The standards used were as described in section 2.2.2. Each extract was spiked with a consistent volume of stock (50uL) to reflect the regulatory limit for that toxin; for OA spiking was carried out at the regulatory limit (16ng/ml) and for AZA1 the extracts were spiked close to the limit (10ng/ml).

6.2.1.3. Spiking of shellfish extracts (pre and post LLE)

Crude samples

Three aliquots of each extract and MeOH (1900uL) were spiked with 100uL of stock for direct injection onto the LCMS; these samples represented the crude extracts and were necessary to evaluate any matrix effects present.

Pre -LLE spiked samples

Five aliquots of each shellfish extract and MeOH (1900uL) were spiked with stock standard (100uL) prior to LLE clean up.

Post - LLE spiked samples

Five aliquots of each shellfish extract and MeOH (1900uL) were spiked with MeOH (100uL) prior to LLE clean up; the cleaned extracts were spiked with stock standard (100uL) after the LLE was carried out.

The clean up was evaluated after hexane partitioning only and hexane followed by DCM partitioning.

6.2.1.4. LLE Protocol

The LLE protocol is represented below (Figure 6-1):

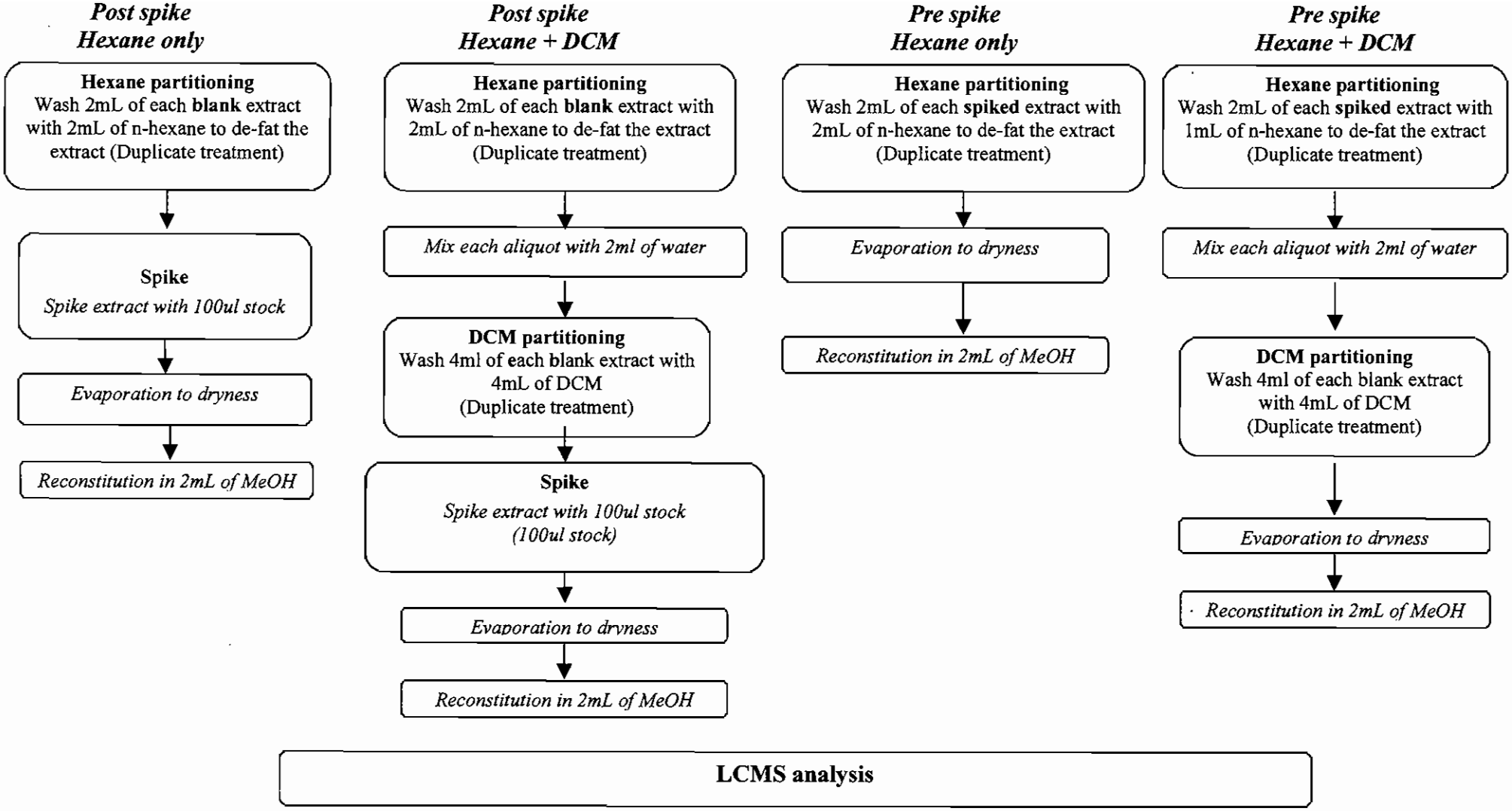


Figure 6-1: Protocol for the evaluation of effectiveness of LLE in the removal of matrix effects. LCMS analysis conducted using LC-TSQ-MS

6.2.1.5. LCMS analyses

LCMS analysis was conducted using the conditions as described in section in 2.5.1

6.2.2. Results

The recoveries are represented for OA and AZA1 (Figure 6-2). For OA, the recoveries in all matrices were approx 100% when a hexane step only is applied. For OA the recoveries after a double partitioning with hexane and DCM were variable, ranging from 17.8% in scallop to 135.1% in oyster. The variability (represented by the error bars) was higher for the hexane followed by DCM (RSD values ranging from 15.3 to 35.3%), compared to hexane only (5.9 to 16.7%). The same trend was observed for AZA1, the recoveries were less variable when using a hexane step only (ranging from 86% in scallop to 124% in MeOH), this was compared to recoveries obtained (ranging from 36% in scallop to 86% in oyster) when using hexane and DCM.

This difference in recovery between the different treatments was consistent for OA and AZA1 in MeOH, mussel and scallop (better recoveries using hexane only), however for the oyster material there was in fact an increase in the recovery of OA (by approx 40%) for the hexane and DCM treatment. For AZA1 the difference in recovery between the two clean-up treatments was difficult to interpret due to the large variability between replicates (represented by the error bars).

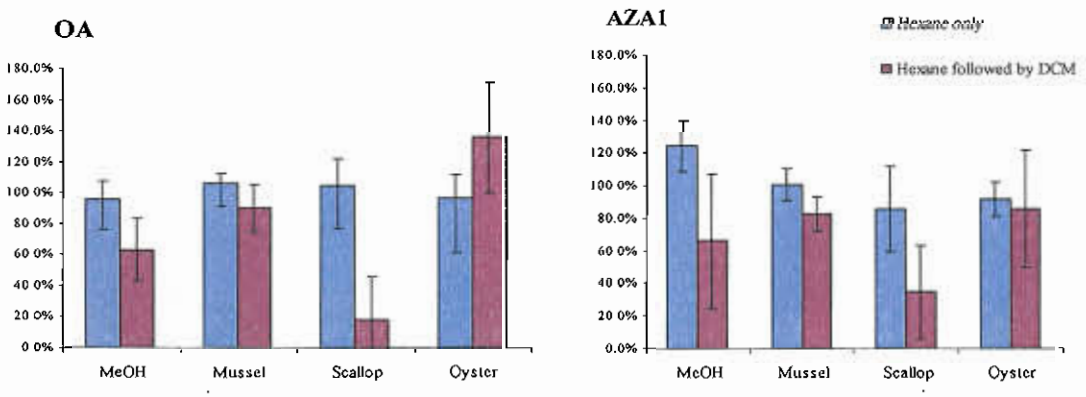


Figure 6-2: Recovery of OA and AZA1 after hexane only and hexane followed by DCM partitioning analysed using LC-TSQ-MS. Error bars represent RSD±1 (n=3)

The matrix effects in the crude extracts are represented below for OA and AZA1 (Figure 6-3). The LCMS analysis was carried out in two separate runs due to a long analysis time required for the complete series of experiments therefore it was necessary to calculate the matrix effects in each individual run. The trends of matrix effects were in line with previous results for matrix-matched and matrix-strength standards (4.2.2); ion suppression (approx 36%) and enhancement (approx 14%) were observed for AZA1 and OA respectively and were similar in both run sequences. The effects were more pronounced in scallop for AZA1 (45% suppression) and in mussel for OA (28% enhancement) although the error bars indicated that the difference may not be significant between shellfish matrices.

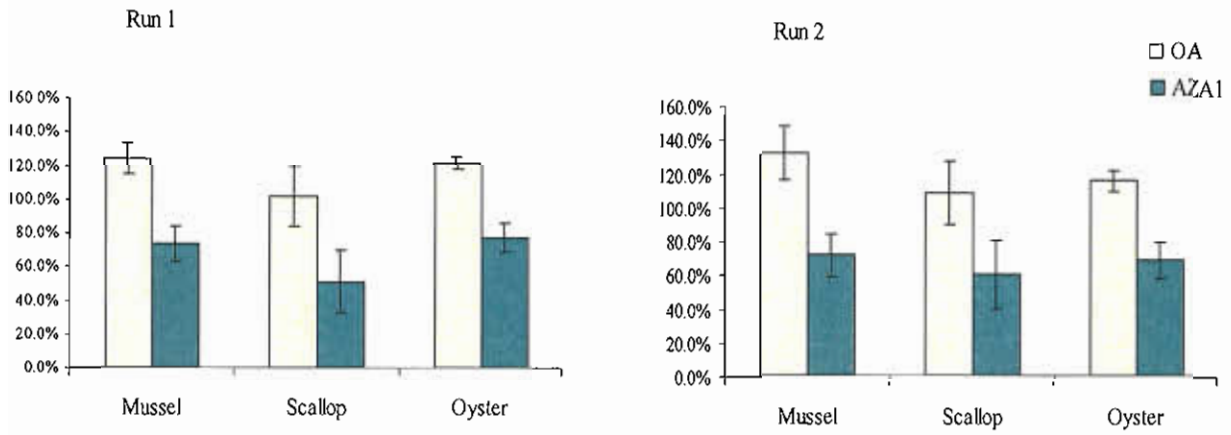


Figure 6-3: Matrix effects in crude extract s of mussel, scallop and oyster analysed using LC-TSQ-MS in two separate runs. Error bars represent RSD±1 (n=3)

The matrix effects after LLE clean up are represented below (Figure 6-4). For OA there was no clean up effect, the enhancement in the crude extracts (ranging from 5% in scallop to 28% in mussel) was in fact increased after hexane (ranging from 30% in scallop to 45% in mussel) and further increased after hexane and DCM (by up to 100% in scallop). The variability associated with the scallop extracts made it difficult to interpret the significance of the clean up effect for OA, as the matrix effects in the crude extracts were minimal (approx 6% enhancement).

For AZA1 the matrix effects observed in the crude mussel extracts (28% ion suppression) were removed, with no significant difference between clean-up treatments. There was also a significant effect for oyster; for a hexane only step the recovery increased from 68% to 108%, for hexane and DCM the recovery was improved from 77% in the crude extract to 123% in the cleaned extract. In scallops, for AZA1 the suppression effects in the crude sample (approx 45%) were reduced (to 17%) with no difference between the two treatments.

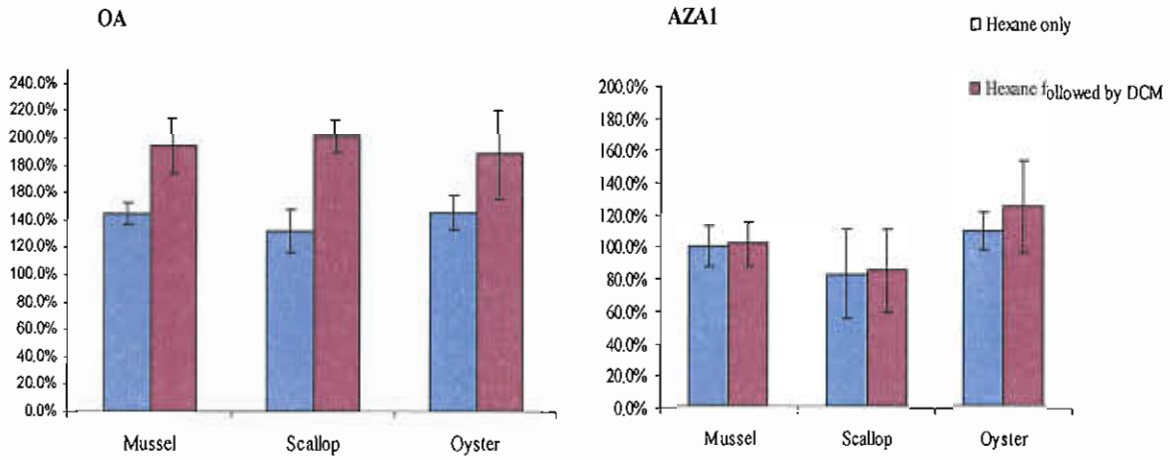


Figure 6-4: Matrix effects in mussel, scallop and oyster material for OA and AZA1 after clean-up (hexane only and hexane plus DCM partitioning) analysed using LC-TSQ-MS. Error bars represent RSD±1 (n=3)

Figure 6-5 represents the overall efficiency of the LLE and was calculated using a combination of the recovery of toxins and the removal of matrix effects. For OA firstly, using a hexane only treatment the overall efficiency of the clean up was not significantly different between matrix types, with values ranging from 138% in scallop to 153% in mussel. The values were more variable when a hexane and DCM step was applied (ranging from 36% for scallop to 254% for oyster). These high values reflect the presence of matrix effects and indicate that the clean up was not successful for OA and only served to increase the degree of matrix effects. The lower values of the scallop matrix were driven by the low recoveries obtained for scallop (presented in Figure 6-2).

There was less variability associated with AZA1; there were no significant differences observed between clean up treatments for mussel and oyster and the clean up efficiency was reflected in the values obtained for these matrices (approx 100%). For the scallop extracts the lower values obtained (70% and 30% for hexane and hexane and DCM) reflected the low recoveries obtained for scallop (especially when using hexane and DCM).

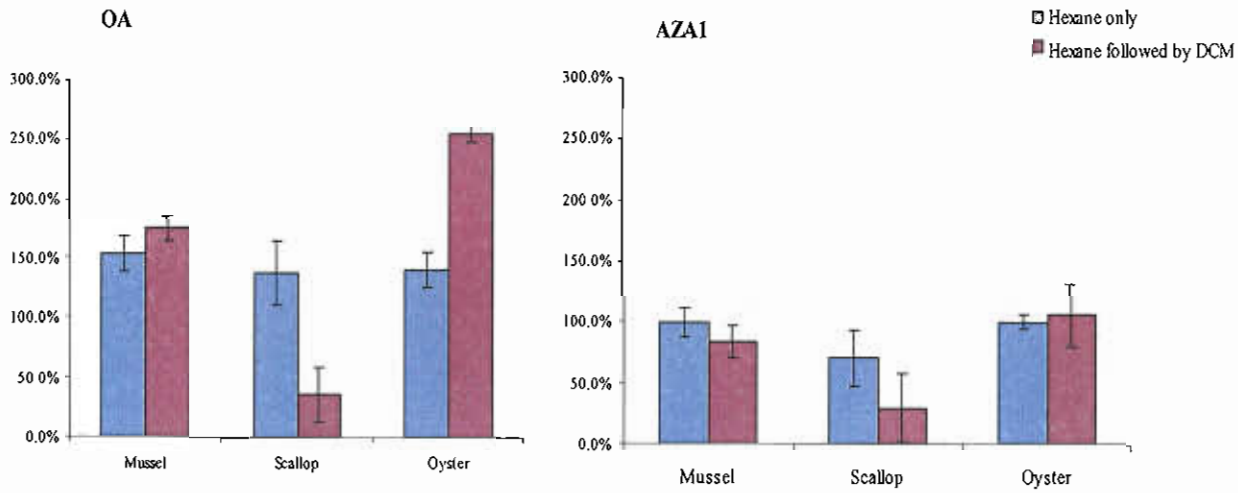


Figure 6-5: The combination of recovery and removal of matrix effects using LLE for mussel, scallop and oyster analysed using LC-TSQ-MS. Error bars represent RSD±1 (n=3)

6.2.3. Discussion

The purpose of this experiment was to evaluate the effectiveness of LLE in the removal of matrix effects. To date, methods have been reported which include LLE as a sample pre-treatment step (Quilliam, 1995; Ito and Tsukada K, 2001; Fernandez *et al*, 1996; McNabb *et al*, 2005) but they do not include quantitative descriptions of the effectiveness of LLE in the removal of matrix effects.

In the initial method development for LLE, recovery losses were a concern (losses were experienced with each additional partitioning step). Losses were also experienced during the evaporation and reconstitution steps. A reconstitution volume of 2.5ml compared to 1ml was required to fully dissolve the dried residue of primary methanolic extract used in the LLE (2.5ml). These results indicated that re suspension problems were likely to occur as a result of sample concentration. This study accounted for toxin losses occurring during the LLE procedure by spiking toxin into the extract after LLE has been performed to calculate the effectiveness of the clean up (the recovery of the procedure was calculated separately using working standards).

The recovery data obtained during this experiment was in agreement with the initial results in that losses were incurred with additional partitioning steps (except for oyster where the recovery was increased by approx 40%, this was coupled with large variability). The most severe losses were observed for scallop after a second partitioning step with DCM, this was noted during the experiment when a large emulsion of the scallop extract formed at the interface of the two solvents (aqueous MeOH and DCM) making it difficult to fully recover the lower DCM phase (containing the toxins).

The matrix effects in the crude samples were investigated, the trends of signal effects were in agreement with the previous matrix matched standards (4.2.2); ion enhancement (ranging from 5-28%) was observed for OA and ion suppression (27-44%) was observed for AZA1. This experiment used a lower SSR (5) than the matrix-matched standards (SSR=10) to increase the matrix strength of the extract and therefore increase the degree of matrix effects to be removed by LLE.

LLE showed no clean-up effect for OA; the enhancement effects were in fact increased for a hexane step and even further increased using a double partitioning with hexane and DCM, this was shown for all matrices. The ion suppression effects shown for AZA1 in mussel and oyster were effectively removed. For scallop, the suppression was significantly reduced. In

general the hexane only treatment gave better results than hexane followed by DCM treatment.

The overall efficiency of the procedure was expressed using a combination of recovery and removal of matrix effects. For AZA1 values of approx 100% were found in mussel and oysters, in scallops the lower efficiency (from 25-75%) values were driven by the poor recoveries calculated using the working standards, especially with the hexane and DCM treatment. Overall for AZA1, a significant clean up was observed in all matrices. The use of a hexane only clean up would be more beneficial overall due to the losses of AZA1 recovery in scallop when using hexane and DCM.

The overall efficiency of the procedure was not as favourable for OA as for AZA1. Using a hexane only step values for mussel and oyster ranged from 140 to 153%; these high values represent the presence of enhancement effects. The variability was greater when using a hexane and DCM treatment with the efficiency values ranging from 36% in scallops to 254% in oyster. For OA, LLE has shown only to compound the enhancement effects that were observed in the crude matrices and would have no beneficial effect.

This study has highlighted the difficulty in using a universal clean-up scheme, which has a positive clean-up effect for all of the toxin groups included. A more realistic option may be the development of separate clean-up treatments for specific toxins groups. The use of different clean up treatments for individual toxin groups may not be feasible in a statutory laboratory where sample turnaround is a priority and where the benefits of the clean up may be variable.

6.3. SPE in the removal of matrix effects

This study was undertaken to evaluate the efficiency of SPE in the removal of matrix effects; the clean-up effect was evaluated using the same parameters used for LLE. This study was undertaken in collaboration with two other institutes (RIKILT and IMARES) to evaluate the transferability of the SPE procedure between laboratories. The protocol (Figure 6-6) was designed in accordance with the method development that was undertaken previously (5.3). Sub samples of the same shellfish homogenates were provided to the partners involved in the study. There were some differences between the two studies (LLE and SPE). For SPE an SSR of 10 (compared to 5 for LLE) was used and PTX2 was also included into the study.

6.3.1. Experimental conditions

6.3.1.1. Preparation of materials

The mussel, oyster and scallop material was extracted as per the BIOTOX extraction method (SSR=10); the extraction method is described fully in section 2.4.2.

6.3.1.2. Preparation of stock standard

A toxin standard (5mL) was prepared for spiking into the tissues containing OA, PTX2 (320ng/mL) and AZA1 (200ng/mL). The standard solutions used in the preparation of stock were as described in section 2.2.2. Each extract was spiked with a constant volume of stock (50uL) to reflect the regulatory limit for that toxin; for OA spiking was carried out at the regulatory limit (16ng/ml) and for AZA1 the extracts were spiked close to the limit (10ng/ml).

6.3.1.3. Spiking of shellfish extracts (pre and post SPE)

Crude samples

Five aliquots of each extract and MeOH (950uL) were spiked with stock (50uL) for direct injection onto the LCMS. These samples represented the crude extracts and were necessary to evaluate any matrix effects present.

Pre-SPE spiked samples

Five aliquots of each shellfish extract and MeOH (950uL) were spiked with stock standard (50uL) prior to SPE clean up.

Post-SPE spiked samples

Five aliquots of each shellfish extract and MeOH (950uL) were spiked with MeOH (50uL) prior to SPE clean up; the cleaned extract was spiked with stock standard (50uL) after the SPE was carried out.

6.3.1.4. Preparation of SPE solutions

An equilibration solvent was prepared (50ml) at an organic strength of 30% MeOH by mixing 15mL of MeOH with 35mL of de-ionised water into a 50ml volumetric flask.

A neutral wash solution (20% MeOH) was prepared by the addition of 10ml of MeOH to a 50ml volumetric flask and completing to the final volume with deionised water.

A basic elution solution (0.3% v/v NH4OH) was prepared by adding 0.15ml of NH_4OH into a 50mL volumetric and making up to the complete volume with MeOH.

6.3.1.5. SPE equipment

Strata-X cartridges (30mg/1mL) were obtained from phenomenex and used as the reference SPE cartridge during this experiment.

6.3.1.6. SPE procedure

The SPE procedure was as follows:

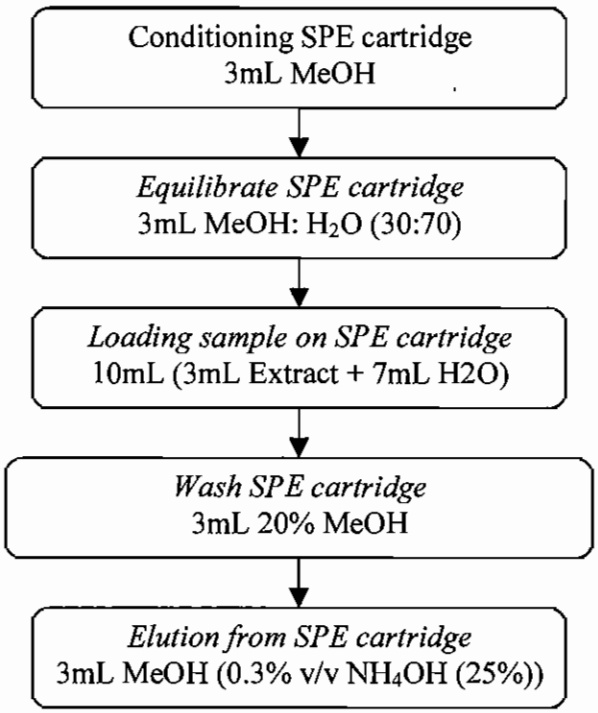


Figure 6-6: SPE procedure followed in the study investigating the effectives of SPE in removing matrix effects (transferability study)

6.3.1.7. LCMS analyses

LCMS analysis was conducted using the conditions as described in section 2.5.1.

6.3.2. Results

The recoveries for all three toxins (OA, AZA1 and PTX2) were comparable in MeOH, scallop and oyster (ranging from 69 to 93%). In mussel material the recovery values for OA and AZA1 were high (122 and 119% respectively), the recovery of PTX2 in mussel was comparable to the values in the other matrices. There was a large variability associated with replicate recovery values of all toxins in all matrices (relative standard deviations ranging from 9 to 26%). This variability was apparent for SPE carried out using MeOH; which was chosen to represent SPE without any effects arising from the nature of the matrix.

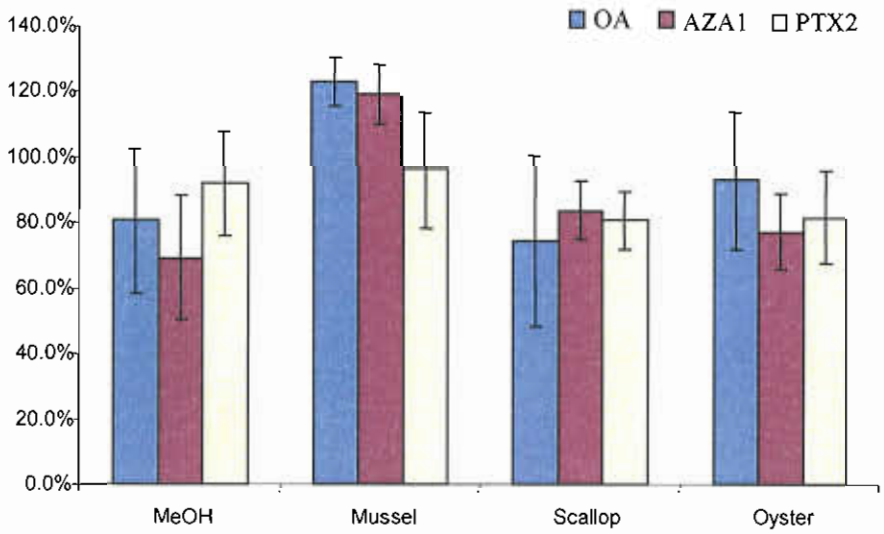


Figure 6-7: Recovery values obtained for OA, AZA1 and PTX2 in MeOH in mussel, scallop and oyster extract SPE (Strata-X cartridge (30mg/1mL). Fractions analysed using LC-TSQ-MS. Error bars represent RSD±1, (n=3)

The matrix effects in the crude extracts are represented below (Figure 6-8). No significant matrix effects were observed for OA and AZA1 in all three matrices. Ion enhancement effects were observed for PTX2 in all matrices (57, 79 and 97% for scallop, oyster and mussel respectively).

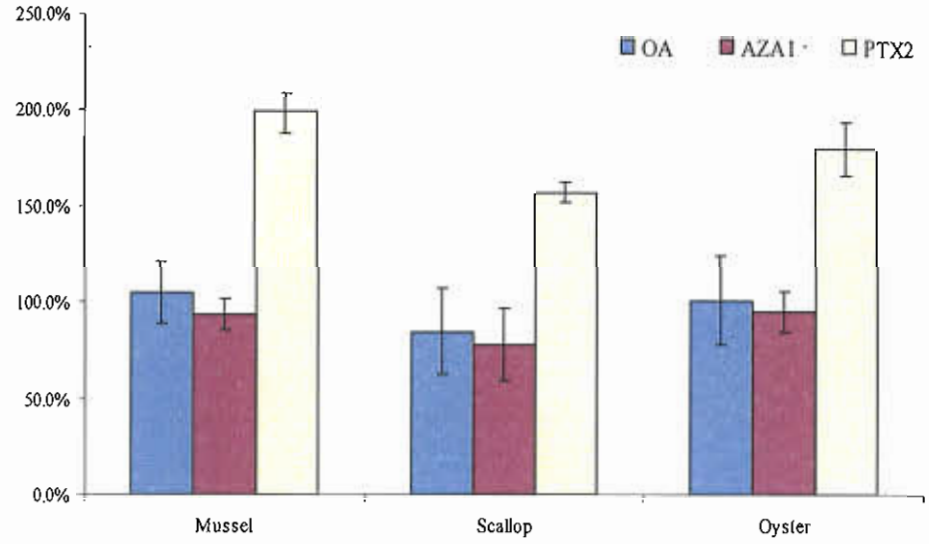


Figure 6-8: Matrix effects in crude extract s of mussel, scallop and oyster analysed using LC-TSQ-MS. Error bars represent RSD±1, (n=3)

The influence of SPE on matrix effects could only be assessed for PTX2 (Figure 6-9) as this was the only compound that demonstrated matrix effects (average of 77% ion enhancement in all matrices). For the scallop and oyster matrices there was no change in the enhancement effects after SPE. For mussel extracts the enhancement effects were increased by approx. 20% after SPE.

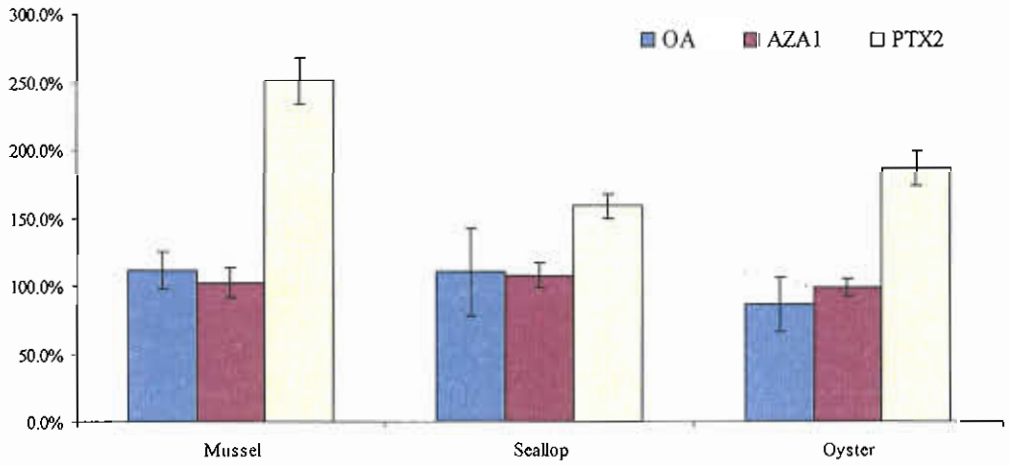


Figure 6-9: Matrix effects in mussel, scallop and oyster material for OA and AZA1 after SPE. (Strata-X (30mg/1mL)). Fractions analysed using LC-TSQ-MS. Error bars represent RSD±1, (n=3)

The combination of recovery and removal of matrix effect is represented below (Figure 6-10). Again, this parameter was only relevant for PTX2 and it was shown that the only effect of SPE was to increase the enhancement effect in mussel.

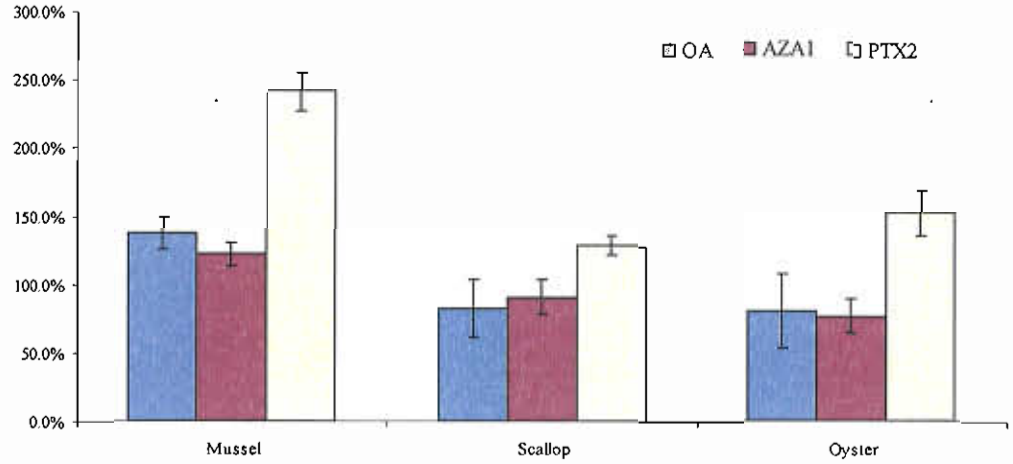


Figure 6-10: The combiuation of recovery and removal of matrix effects using SPE for mussel, scallop and oyster analysed using LC-TSQ-MS. Error bars represent (SD±3)

6.3.3. Discussion

A search of SPE methods published for marine toxins showed that some research had been carried out in this area (1.7.1.2) on specific toxin groups. Quilliam (1995) used an aminopropylsilica column for the clean up of OA toxins in mussels after an initial LLE step; the author evaluated the clean- up effects and used the colour of the extracts as an indication of the clean-up effect. There was a significant change in colour after the SPE (pale yellow) compared to the initial LLE cleaned extracts (dark brown in colour). Goto *et al*, (2001) reported that Silica SPE effectively removed compounds responsible for ion suppression for OA, DTX1, palOA, palDTX1, PTX6, PTX2SA and YTX in shellfish extracts.

The SPE procedure used for this study was optimised between two laboratories (MI and RIKILT); various parameters of the procedure were optimised including load, wash and elution conditions in terms of recovery of all of the toxins included in the study (OA, AZA1, PTX2 and YTX). At the early stage of method development, the focus was to find an appropriate sorbent and optimise a set of SPE conditions to achieve maximum recovery of all toxins. When this objective was achieved, the next step in the development was to test the effectiveness of the SPE procedure in the removal of matrix effects.

This study was carried out in three separate laboratories; the protocol agreed upon for this study used the current BIOTOX extraction procedure (2.4.2), which corresponds to a solvent to sample ratio of 10. This was chosen to reflect the matrix strength that is most relevant to laboratories using this SSR for extracting lipophilic marine toxins using 100% MeOH. The oyster and scallop material was retrieved from the routine monitoring program of the MI and was deemed to be less than LOQ for OA and AZA group toxins. The mussel sample was a blank composite sample obtained from RIKILT. The materials were distributed between participant labs to ensure that the same material was used to assess the clean-up treatments. The same toxin standards were also used to further minimise any differences.

Matrix effects were only experienced for PTX2 and therefore this was the only compound for which the clean- up could be evaluated. No clean-up effect was found for

PTX2 in the scallop and oyster matrices, for PTX2 in the mussel extract the enhancement effects were increased by approx. 20% after SPE.

Another partner (RIKILT) which used similar LCMS conditions to those used in this experiment (mobile phase, column, and MS detector) found matrix effects in the crude

extracts for all of the toxins; enhancement was observed in PTX2 (approx 40%) and OA (approx 20%) along with ion suppression for AZA1 (approx 30%). A clean-up effect was only observed for AZA1 where suppression was reduced to less than 20%. Another partner (IMARES), using different conditions, found matrix effects for OA and PTX2 but no clean-up effect was observed for these toxins. This highlights the highly variable nature of matrix effects and the subsequent difficulties in developing a universal sample clean up scheme.

Matrix-matched standards prepared at the Marine Institute (4.2.1.3) using the same oyster and scallop extracts and analysed using the same conditions, showed different degrees of matrix effects (ion suppression for AZA1 and less severe enhancement effects for PTX2).

From the results of this study it was clear that matrix effects can vary significantly between laboratories, this was also apparent when the same materials were used and in one partners case, where similar LCMS conditions were used. The highly variable nature of matrix effects between laboratories makes it difficult to assess the transferability of the SPE procedure. If the proposed SPE procedure holds no beneficial role in the removal of matrix effects, for a laboratory involved in a routine monitoring program and for which sample turnaround times are important an additional SPE step would be time consuming.

6.4. Conclusion for LLE and SPE clean – up in the removal of matrix effects

In the development of a new LCMS method a study must be undertaken into the occurrence of matrix effects. If matrix effects are being experienced to sufficiently high degrees they can affect the accurate quantification of the analyte of interest. A study of the matrix effects occurring in the analysis was undertaken in chapter 4. The effects were found to be analyte and matrix type dependent. Similar trends of matrix effects were observed between two LCMS instruments (both using ESI), to varying degrees. A search through literature on the topic also indicted the highly variable nature of matrix effects being experienced in the LCMS analysis of marine toxins.

The evaluation of and development of the clean up techniques was undertaken with the objective to develop a universal clean-up technique to be used as part of the sample pre-treatment scheme for a reference LCMS method.

For LLE a strong matrix strength (SSR 5) was applied to induce stronger matrix effects, this was according to the principle that the stronger the matrix strength, the stronger the matrix effects. LLE showed a clean-up effect for AZA1 in mussel, scallop and oyster. The optimum clean up effect was found using a hexane only partitioning. For OA the enhancement effects in the crude extracts were more pronounced after clean up with differences between clean-up treatments. LLE has shown to have a positive effect for AZA1 and could be considered for the clean up of AZA1 contaminated shellfish. In general a hexane only step was more favourable

For SPE the clean-up effect could not be fully investigated at the Marine Institute. For OA and AZA1, there were no significant matrix effects observed in the crude extracts. For PTX2, severe enhancement effects were observed in all matrices and SPE only served to increase the enhancement effects. The other partners involved in the study of the transferability of the SPE method found varying degrees/trends of matrix effects arising from the matrix (even when conditions were similar). Only one of the partners found that SPE had a significant clean-up effect.

The SPE method that was developed recovered all of the toxins sufficiently. The loading, washing and elution conditions were optimised as a function of recovery before the effects that these conditions had in the removal of matrix effects could be considered. One of the

partners of the study found clean-up effects using the SPE procedure. This is a measure of the potential that SPE holds for the removal of matrix effects. The study undertaken highlighted the difficulties that arise in testing the viability of a SPE between laboratories due to the highly variable nature of matrix effects occurring between laboratories.

The clean up studies have further highlighted the complex nature of shellfish matrices and the difficulties in removing interfering compounds from the matrix even after the initial extraction with MeOH (100%). All of the clean up studies carried out made use of the simple methanolic extraction (described fully in section 2.4) to prepare the wet tissue for clean up using MeOH. However, due to the high amounts of water in the tissue (moisture content of approx 80-85%) and the miscibility of water and MeOH other more hydrophilic components may be co-extracted (with water). This methanolic extraction is therefore presenting quite a complex matrix to the clean up. This is further complicated by the differences between shellfish species.

7. Overall findings

LCMS development and validation

Initial studies focused on the design and implementation of LC and MS conditions. The early development of LC was carried out in collaboration with two other laboratories; the conditions that were decided upon are fully described in 2.5.2 and were adapted from methods reported previously by Quilliam *et al*, (2001) and later by Hess *et al*, (2003). The mobile phase and the analytical column were the same as published by the previous authors, however modifications were applied. The analytical column was the same (a Thermo Electron BDS-Hypersil C_8 column (50 x 2mm, 3µm)) but did have a guard column fitted, the gradient elution differed slightly operating at a flow rate of 0.2ml/min.

A pre-validation study of this method was carried out to ensure that the method as described above would respond to the criteria of a reference method. The study highlighted some difficulties associated with the use of the method; large Z-scores and variations were observed, especially between laboratories that applied the same conditions. The conclusion arising from the study was that the LCMS method was not suitable for standardisation at this stage of development.

In light of the validation study, and further problems with the separation and detection of the YTX toxins, a further LC separation method was developed. This separation method used a basic mobile phase to help the retention of YTX and an analytical column (XBridge column (C18, 5µm, 3.0 x 150mm)) packed with hybrid particles (Ethylene-Bridged Hybrid particle, BEH Technology™). This multi-method allows the separation of all the regulated lipophilic toxins with a minimum overlap.

A second validation round is currently being undertaken, for this study the participants will be free to use the conditions, which are most appropriate to their instrument and conditions used in that particular laboratory. The first validation prescribed a set of LC and MS conditions, in some laboratories there was no previous experience with these conditions, this may have contributed to the variability. Allowing more freedom of conditions to the participant's means that the variability between laboratories may

decrease. The desired outcome of this study would be the identification of critical parameters in LCMS methods for use in the analysis of lipophilic marine toxins rather than prescribing a strict set of parameters to be implemented.

A study, which examined the impact that the MS conditions have on the results obtained, was designed and coordinated at the Marine Institute and included five other labs with a variety of MS detectors (Single Quadrupole (SQ), Triple stage Quadrupole (TSQ) and Ion trap (IT)). The MS parameters investigated were: ionisation mode (positive or negative), acquisition mode (parent ion monitoring, single transition monitoring or double transition monitoring) and choice of transitions (one transition only or a combination of two). The study concluded that the choice of ionisation mode, acquisition mode and transitions is important in MS analysis as it affects the analysis of the toxins. Differences between negative and positive ionisation mode in parent ion monitoring were assessed for the OA group and PTX2. For these toxins the results between ionisation modes were significantly different and the difference was more important in the case of the OA group. OA, DTX2 and PTX2 results were generally higher in positive mode, whereas DTX1 levels were higher in negative mode. From the whole range of conditions assessed, it turned out that negative ionisation would be better suited for OA/DTXs, at least in the interest of minimising the between laboratory differences. Only positive ionisation has been shown for AZAs so far. Either ionisation mode could be chosen for PTX2; negative ionisation would have the advantage of avoiding positive/negative switching at the retention time of DTX2 and PTX2 as these toxins co-elute in the BIOTOX chromatographic conditions

This study has shown that MS conditions play a significant role in the results obtained. Similar trends were found between laboratories; however it would still be recommended that each laboratory carry out these investigations with their own instrument, to ensure that the correct ion ratios are being obtained for those toxins that are being quantified using a different calibrant.

Assessment of matrix effects and evaluation of clean up techniques

With the development of a new LCMS method a study into the associated matrix effects must be undertaken. Scallop and oyster tissue were used as the matrices for these studies. Matrix matched standards were prepared using the scallop and oyster extracts. The degree of matrix effects was calculated for OA, AZA1 and PTX2 in both matrices using LC-QToF-MS and LC-TSQ-MS. Similar trends were observed in both instruments (although to varying degrees). The matrix effects were shown to be analyte and matrix dependent; ion suppression was observed in the analysis of AZA1 and ion enhancement effects were observed for OA and PTX2.

Matrix strength standards (MSS) were prepared to examine the role that matrix strength plays in matrix effects. The standards confirmed the trends of matrix effects that were found in the matrix-matched standards (ion suppression for AZA1 and ion enhancement for OA/PTX2). OA was shown to suffer the least from matrix effects independently of matrix strength. AZA1 demonstrated a higher degree of matrix effects at a stronger matrix strength (in oyster only). As for the matrix-matched standards, PTX2 suffered the most drastically from matrix effects, this was more prominent at the lower matrix strengths (more critical for oyster), which corresponds to the currently used extraction procedures for the extraction of wet tissue prior to LCMS analysis. The total lipid content was determined in the oyster and scallop material to investigate the role that lipids play in matrix effects. It was found by the extraction methods of Bligh and Dyer (1959) and Foppe Smedes (1999) that the oyster tissue contained the highest percentage of lipid (2.1 ± 0.25%). The oyster material had shown in general to be more susceptible to matrix effects and therefore this suggests that lipids may contribute to matrix effects. The trends of matrix effects that are occurring with this particular form of ionisation (ESI) are: ion suppression for AZA1 and ion enhancement for OA/PTX2, the occurrence and degree of these matrix effects may vary significantly depending on the nature of the tissue. This study clearly showed that different extraction solvents will lead to different lipid contents in the crude extract. As the type of lipid extracted may also impact on the matrix effect, it is recommended that future studies on matrix effects also take into account the lipid profile of extracts and evaluate the influence different lipid components may have on matrix effects for specific marine toxins.

In an effort to remove or at least minimise matrix effects two sample clean-up treatments were evaluated: LLE and SPE. The LLE technique used a hexane-partitioning step to remove the non-polar lipids from the extract followed by partitioning with dichloromethane to isolate the toxins of interest from any interfering compounds. An evaporation and reconstitution step was required to prepare the extract for LCMS analysis. Recovery losses were encountered with each subsequent partitioning step coupled with increased variability between replicate treatments. Further losses were experienced at least in part as a result of the evaporation and reconstitution steps. Initially a reconstitution volume of 1ml was used to re-suspend the dried residue of the original extract (2.5ml) that was used in the clean up. This volume was not sufficient to fully dissolve the residue, when a reconstitution volume of 2.5ml was applied the recoveries increased by up to 100% in some cases. The variability and labour consuming nature associated with the clean-up technique led to the development of alternative clean-up methods such as SPE.

The development of an SPE method was conducted in collaboration with another partner in the BIOTOX project (RIKILT). For SPE the main objective was to develop a method to include the lipophilic toxin groups (OA, AZA, PTX2 and YTX). The mechanism of SPE chosen was similar to the principles of reverse phase chromatography; the sample was loaded onto the cartridge, various washing conditions were used to remove interferences before the toxins were eluted from the cartridge. The clean up was evaluated firstly in terms of recovery of toxin throughout the procedure. Strata X and Oasis HLB ™ (Co polymer sorbents) gave the best recovery of all toxins included. The steps of most importance during the developmental phase were the loading, washing and eluting steps. The loaded sample must have an adequate aqueous and methanolic content so as to wet the sorbent and also not allow the analyte to pass through the sorbent bed. The optimum loading composition was determined to be 30:70 (MeOH/Water). The purpose of the washing step is to remove any interfering components whilst at the same time not eluting the toxins. The optimum washing strength and composition was a neutral composition of 20% MeOH. The elution step is required to effectively remove the analyte from the sorbent, this was achieved using MeOH or adding a buffer to further increase the recoveries.

The clean-up schemes were developed with a view to remove or at least minimise matrix effects. To evaluate each clean-up in terms of the removal of matrix effects experiments were undertaken which evaluated a number of parameters: the recovery of each toxin throughout the procedure, matrix effects in crude extracts, clean-up effects and combination of recovery and clean up effect. Both studies used the same material (mussel, oyster and scallop) and LCMS conditions; the difference in the LLE/SPE studies was in the matrix strength of the extract to be purified. Matrix strength can be identified as the ratio of solvent to extracted sample material (= SSR, solvent to sample ratio expressed in ml/g).

For LLE a strong matrix strength (SSR=5) was chosen to induce a matrix effect (for OA and AZA1) so that the clean-up effect could be examined effectively. The clean up was evaluated for a single partitioning only using a hexane step and also a double partitioning using hexane followed by DCM treatment to evaluate the effectiveness of each treatment in removing interferences. LLE showed a clean-up effect for AZA1 in mussel and scallop only; the ion suppression effects shown for AZA1 were effectively removed in mussel with no significant difference between clean-up treatments; for oyster the suppression effects are removed using a hexane only step, when a DCM partitioning is included there are enhancement effects experienced. For OA the enhancement effects in the crude extracts were more pronounced after clean up with differences between clean-up treatments.

The situation for SPE was more complex; the study was undertaken in collaboration with two other laboratories, using the protocol as optimised in the developmental stage (Chapter 5). The study prescribed a matrix strength (SSR=10), which corresponds to the currently used extraction procedures. At this matrix strength the matrix effects arising from the crude extracts were minimal (for OA and AZA1) and therefore the clean up effect could not be evaluated for these toxins. A strong ion enhancement was observed for PTX2, SPE only served to increase these effects. The other participants experienced variable degrees of matrix effects arising from the same materials, spiked in the same manner and using the same standard solutions. Only one participant observed a clean-up effect; where for OA, YTX and AZA1 matrix effects were reduced to less than 20%.

LLE is a simple clean up technique, which has shown to have a clean up effect for AZA1; this could be achieved using a hexane partitioning only. This indicates the potential that LLE holds under certain conditions for the removal of matrix effects.

The nature of matrix effects are highly variable between laboratories, this was found even when the materials and conditions used were similar. This makes it increasingly difficult to assess the clean up effect of SPE between laboratories. The optimised SPE has shown good recoveries of all of the toxins (84-120%), this is promising due to the different lipophilicities of the toxins themselves. This good recovery is a good starting point for developing alternative washing and elution series, which could remove or minimise interferences.

8. Conclusions

The aim of this project was the identification and evaluation of critical parameters within a multi-toxin LCMS method, to contribute the findings to a reference LCMS method. A large amount of the work included was carried out in collaboration with a number of laboratories involved in the EU funded BIOTOX project: a multi-disciplinary project focused on the development, validation and standardisation of reference methods (LCMS) and cost effective assays for the identification and quantification of lipophilic marine toxins. A lot of the research described during this thesis was driven by the deliverables outlined in the BIOTOX project (Anon, (2004C)).

This study has identified the extent and variability of matrix effects in the LCMS analysis of marine toxins between three major commercial shellfish species (mussel, oyster and scallop) and three toxin groups (OA, AZA, PTX and YTX) as well as some differences between laboratories.

Attempts to remove these matrix effects through clean up by either LLE or SPE proved difficult and were only partially successful.

The extent to which matrix effects differ between analytes, instruments, matrix strength and between laboratories in crude and LLE/SPE-cleaned extracts suggest that single laboratory validation may be a route that needs to be explored if LCMS methods are to be used for official control.

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Appendix I

Optimised LLE and SPE conditions

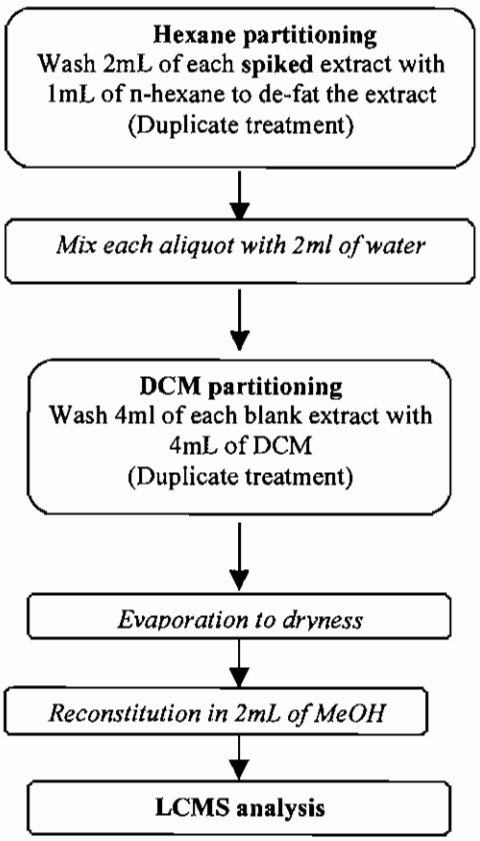

Figure AI-1: Optimised LLE procedure

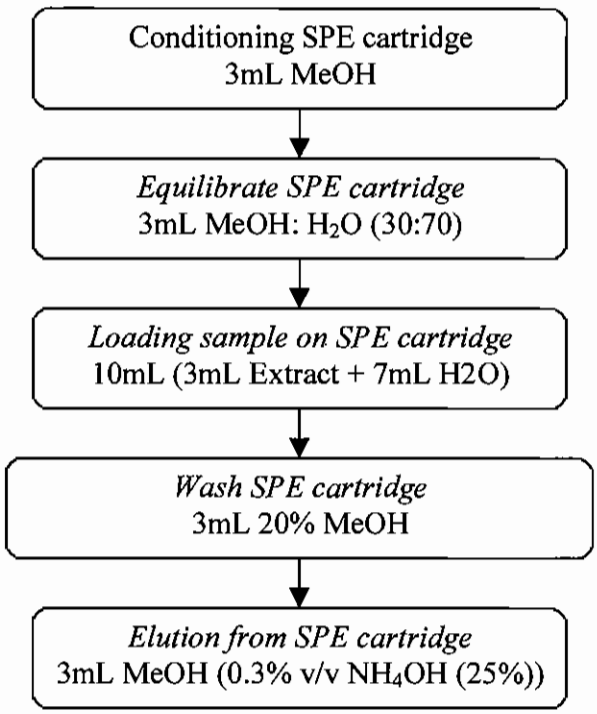

Figure AI-2: Optimised SPE procedure

Appendix II

Results section of the Transition Study report

Report prepared by Ronel Bire.

Results and discussion

3.1. OA group

3.1.1. OA

As shown in Table 4 and Figure 1, the ionisation mode significantly influences the results obtained for OA in both extracts, when monitoring the parent ion (m/z 827 and m/z 803 in positive and negative mode, respectively). Indeed, for the extract having the lower matrix strength (SSR 10) all the participants except participant 5 reported much higher results in positive mode, by a factor of 1.4 (participant 3; TSQ) to 3.2 (participant 1; SQ) on average. The participant 5 reported higher results in negative mode (factor of 1.4 on average). A similar trend was observed for the second extract (SSR 5).
The results acquired in negative were also found to be much tighter (overall CV of 23% and 19% for SSR 10 and 5 respectively).
A 2-way ANOVA could not be carried out to assess the significance of the differences observed between ionisation modes (positive versus negative) and participants because the data was not normally distributed. However, 1-way ANOVAs performed for each ionisation mode showed significant differences between certain sets of results. In positive mode, the results were statistically different between participants except for participants 2 / 5 for the SSR 10 extract and for participants 2 / 3 and 3 / 4 for the SSR 5 extract. In negative mode, the results were statistically different between participants except for participants 1 / 3 and 5 / 6 for the SSR 10 extract and for participants 2, 4 and 6 altogether for the SSR 5 extract.
Although the % difference values obtained for both ionisation modes are variable and somewhat difficult to interpret, it can be noticed that the average of the absolute values of the % difference was lower in negative (8.3%) than in positive mode (19.0%). However, no statistical difference (T-test) was found within ionisation mode between the expected and found OA concentrations in the SSR 5 extract.

In comparison to what was previously observed for the parent monitoring mode, the OA results presented in Table 5 show that the difference between positive and negative mode is not that important when monitoring specific double transitions. However, a closer look at the individual data shows that the results of the participant 5 are still higher in negative by a factor of 1.6, whereas participant 3 again reported higher results in positive mode. These results have to be interpreted cautiously as both participants only reported the results of 1 transition in positive mode (827>723) against 2 in negative mode.
The mean results observed in positive and negative for each transition are similar to those obtained in negative for the 803 trace (parent monitoring) (ca. 20 and 40 ng/ml for SSR 10 and 5 respectively). Furthermore the CV is somewhat comparable between modes when monitoring double transitions (more so for the SSR 10 extract).

The distribution of the OA results acquired in positive and negative double transition monitoring is presented in Figure 2 and Figure 3 respectively. These box plots show that the results obtained in negative for the 2 monitored transitions (803>113 and 803>255) are more comparable than in positive mode (769>761 and 827>723). This was partly confirmed by T-tests performed on the data. Indeed, the results reported by all participants for the SSR 10 extract for both transitions in positive mode are significantly different (t=-2.83; p=0.012), but are not in negative. However for the SSR 5 extract the results of both sets of transitions are not significantly different whatever the ionisation mode used.

Due to the limited amount of data it is difficult to compare the single and double transition monitoring modes (Table 6). Based on the results reported for the 803>255 transition in negative mode and for both extracts, there in no significant difference between single and double transition monitoring (conclusions drawn from a T-test).

3.1.2. DTX2

The distribution of the DTX2 results in parent monitoring mode (m/z 827 and 803 in positive and negative mode respectively) is shown in Figure 4 and confirms the trend previously observed for OA, in that there is a difference depending on the ionisation mode. Indeed, 5 of the 6 participants reported significantly higher results in positive compared to negative mode by a factor of 1.5 (participant 1: SQ) to 2.2 (participant 6: TSQ). Inversely, the participant 5 reported results 1.5 times higher in negative than in positive mode (Table 7).
The overall results are also tighter in negative (CV of 14% for both extracts) compared to positive mode (27% and 36% for the SSR 10 and 5 extracts respectively).
Participant 1 reported similar results in positive (m/z 827) and negative mode (m/z 803) for the SSR 5 extract, whereas a significant difference was observed at lower matrix strength. Here the positive results (m/z 827; SSR 5) are underestimated as they are twice lower than those reported by the other participants. The participant 5 also reported results twice lower in positive but for both extracts (in around 60 and 100 ng/ml for SSR 10 and 5 respectively).

The box plots presented in Figure 5 and Figure 6 as well as the Table 8 show that the DTX2 results in negative are similar whatever the transition used, whereas there is a higher discrepancy in the results obtained in positive for the monitored transitions (results statistically significantly different). The overall CVs show that the results in negative are also much tighter and they are similar to those obtained in parent monitoring mode.

Based on the results reported for the 803>255 transition in negative mode and for both extracts (Table 9), there in no significant difference between single and double transition monitoring (conclusions drawn from a T-test).

3.1.3. DTX1

The results reported in parent monitoring mode for DTX1 (m/z 841 and 817 in positive and negative mode respectively) confirm that there is a difference between the ionisation modes. Interestingly the DTX1 results are much higher in negative than in positive mode, by a factor of 1.4 (participant 1; SQ) to 2.9 (participant 5) for the SSR 10 extract (Table 10). The overall results are slightly tighter in negative (CV 16%) than in positive mode (19%).

In double transition monitoring mode the participants experienced problems with the 783>765 transition (positive mode); participant 2 is the only one who reported results. The results presented in Table 11, in Figure 8 and Figure 9 show a good comparability between the transitions monitored in negative mode (817>255 and 817>113) with better CVs compared to positive mode (841>737). Furthermore, the results obtained in negative mode are similar to those reported in negative parent monitoring mode.
Even in double transition monitoring mode the results are higher in negative than in positive ionisation.

Based on the results reported for the 803>255 transition in negative mode and for both extracts (Table 12), there in no significant difference between single and double transition monitoring (conclusions drawn from a T-test).

3.1.4. Conclusions

In parent monitoring mode, the OA and DTX2 results reported by the participants were consistently and significantly higher in positive mode, except for participant 5 who found much higher OA and DTX2 concentrations in the negative ionisation mode. In the case of DTX1, all the participants reported higher results in negative mode.
In double transition monitoring, the results reported in positive for both transitions (769>751 and 827>723 for OA/DTX2 and 783>765 and 841>737 for DTX1) were significantly different, whereas in negative mode similar results were obtained when monitoring the transitions 803>255 and 803>113 for OA/DTX2 and 817>255 and 817>113 for DTX1.
Due to the scarcity of the data available in single transition mode, the only results that were used for comparison purposes are those of 803>255 for OA/DTX2 and 817>255 for DTX1. No significant difference was observed between single and double transition monitoring.

3.2. PTX group

3.2.1. PTX2

In contrary to what was observed for OA and the DTXs, the choice of the ionisation mode does not affect the quantification of PTX2 to the same extent (Table 13). A 2-way ANOVA performed on the results reported by participants 2, 3 and 5 showed that there is a significant difference between positive and negative mode within participants 2 and 3 but not for participant 5. Anyhow, the difference between ionisation modes is not as important as in the case of the OA/DTXs.
The overall variability of the results in both ionisation modes and for both extracts is comprised between 20.4 and 27.7%.
The average % difference (absolute value) between the expected and found PTX2 concentrations in positive and negative mode is of 17.7 and 8.0% respectively. The T-test and the Mann-Whitney rank sum test performed on the data acquired in positive and negative respectively showed that there is no significant difference between the expected and the found PTX2 concentrations.

The situation in double transition monitoring mode is very similar to that in parent monitoring mode. As shown in Table 14 the results in both ionisation modes are similar; because the data is not normally distributed it was not possible to carry out statistical analysis (2-way ANOVA).
There seems to be a good comparability of the results obtained with the individual transitions in each mode (Figure 11 and Figure 12), and the results are similar to those reported in parent monitoring.
The % difference (absolute value) between the expected and found PTX2 concentration is lower in positive (5.8 and 7.1% for 876>223 and 876>213 respectively) than in negative (12.4 and 12.7% for 857>627 and 857>137 respectively). However, the T-tests or Mann-Whitney rank sum tests performed concluded that in fact there is no significant difference between found and expected PTX2 concentrations, whatever the ionisation mode and the transition monitored.

The data related to the single transition mode is presented in Table 15. Participant 4 reported a significant difference between single and double transition when monitoring the 876>823 transition; participant 6 did not concur. However for the 876>213 transition both participants reported significant differences between acquisition modes.

3.2.2. Conclusions

The choice of the ionisation and acquisition mode also affects the analysis of PTX2 but not to the same extent as it does for the OA group.
In parent monitoring mode the data reported for both ionisation modes was significantly different, with the PTX2 results acquired in positive mode being slightly higher.
In double transition monitoring the participants reported a good comparability of the results within and between modes.
Depending on the transition monitored in positive mode significant differences could be observed between single and double transition monitoring.

3.3. AZA group

3.3.1. AZA1

The AZA1 concentrations found in the extracts in parent monitoring mode are presented in Table 16 and the data distribution is shown in Figure 13. The overall variability is higher in the SSR 10 extract (%CV = 26%) compared to the SSR 5 (17%) and the average % difference (absolute value) between the expected and found AZA1 concentrations in the SSR 5 extract is of 18%. However, the T-test did not reveal any significant difference between the expected and the found AZA1 concentrations.
The statistical analysis of the values (1 way ANOVA using the Holm-Sidak method) showed that there is no significant difference between the values reported by participants 2, 5 and 6 for the SSR 10 extract. The values of the participants 3 and 4 are significantly higher than the others. As for the SSR 5 extract, the participants 2, 4 and 6 reported similar results, whereas those of participants 3 and 5 are significantly higher (but comparable between each other).

The results related to the double transition mode are presented in Table 17 and in Figure 14. For both extracts no significant difference was found within the participating labs when monitoring either transition (842>672 or 842>654) but all the reported sets of data were significantly different between participants, except between participants 4 and 6 for the SSR 5 extract only and the 842>672 transition.
In double transition mode, the average % difference (absolute value) between the expected and found AZA1 concentrations in the SSR 5 extract is comparable whatever the transition used (about 7%). However the difference between the expected and found AZA1 concentrations is not significant whatever the transition monitored.

When monitoring the 842>672 transition, the use of single versus double transition monitoring mode did not affect the results reported by participants 4 and 6 as no significant difference was observed in both extracts (within lab results) (Table 18). In the SSR 10 extracts the results between labs and within each acquisition mode were significantly different but in the SSR 5 the reported results were similar.
For the 842>654 transition a significant difference was reported by the participant 4 between both acquisition modes. Indeed, much higher results were found in single transition mode for both matrix strengths.

3.3.2. AZA2

The AZA2 concentrations reported for both extracts in parent monitoring mode are presented in Table 19 and the distribution of the data is shown in Figure 15. The Kruskal-Wallis ANOVA on ranks performed on the data related to the SSR 10 extract concluded that the difference in the medians was not significant, although differences could be noticed in the reported values. For instance the participant 5 values are about twice lower than those of the participants 3 and 6. In the case of the SSR 5 extract the statistical analysis (Kruskal-Wallis ANOVA on ranks) identified two groups: the first one is composed of the participants 3, 4 and 6 that reported similar higher values, in comparison to the second group (participants 1, 2 and 5). The overall variability observed in the reported data is similar whatever the extract (24 to 27%) and the average % difference (absolute value) between the expected and found

AZA2 concentrations in the SSR 5 extract is of 23%; a T-test showed that this difference is significant.

In double transition monitoring (Table 20 and Figure 16), the results reported by the participants 2 and 4 for both extracts using either transition (856>672 or 856>654) were similar within lab (2-way ANOVA). However, significant differences were noticed for the other participants (3, 5 and 6) depending on the transition monitored.
In double transition mode, the average % difference (absolute value) between the expected and found AZA2 concentrations in the SSR 5 extract is of 18% and 13% for the 856>672 and 856>654 transitions, respectively. The difference in the case of the 856>672 transition is significant (Mann-Whitney rank sum test).

The results related to single transition monitoring are presented in Table 21. In the case of the 856>672 transition, the results reported by participant 6 using a single transition or double transitions were significantly different. However no difference was observed for participant 4 in the same conditions. The picture is the total opposite when considering the 856>654 transition, as this time the only significant difference between acquisition mode was observed for participant 4. Once again this participant reported much higher values in single transition mode for this second transition (see AZA1 results).

3.3.3. AZA3

The AZA3 concentrations found in the extracts in parent monitoring mode are presented in Table 22 and the data distribution is shown in Figure 17. The Kruskal-Wallis ANOVA on ranks performed on the results reported for the SSR 10 extract concluded that there is no significant difference between participants, although participants 5 and 6 reported much lower results than participants 1 and 2. In the case of the SSR 5 extract, the statistical analysis of the data showed that participants 2, 5 and 6 reported similar results. Similarly, no significant difference was observed between participants 2 and 4. All the other sets of results were found to be significantly different when compared to each other.
The overall variability in the SSR 10 and SSR 5 extracts is of about 27 and 25% respectively. The average % difference (absolute value) between the expected and found AZA3 concentrations in the SSR 5 extract is of 18%. The difference between the expected and found AZA3 concentrations is not significant as determined by a Mann-Whitney rank sum test.

The results related to double transition monitoring are presented in Table 23 and Figure 18. Because the normality test performed on the results reported for both extracts failed, the 2-way ANOVA tests could not be carried out. A closer look at the data shows that the results reported for both transitions (828>658 and 828>640) are somehow comparable within lab and for both extracts, except for participant 3 who reported a 3-fold difference between the results (much higher for the 828>640 transition). The same trend was observed for the AZA2 results reported by participant 3, although to a lesser extent, but not for AZA1.
In double transition mode, the average % difference (absolute value) between the expected and found AZA3 concentrations in the SSR 5 extract is of 13.9% and 15.6% for the 828>658 and 828>640 transitions, respectively. In both cases, the difference observed between the expected and found AZA3 concentrations was not significant, as determined by a Mann-Whitney rank sum test and a T-test, respectively.

The results related to single transition monitoring are presented in Table-24. No significant difference was observed within lab (participants 4 and 6) when monitoring the 828>658 transition in either acquisition mode (single or double transition monitoring). Similarly to what was observed for AZA1 and AZA2, participant 4 reported much higher values for the 828>640 transition in single transition monitoring.

3.3.4. Conclusions

In double transition monitoring no significant difference was observed for AZA1 within participants, even though there could be differences between participants. A similar trend was observed for AZA3 with a good comparability of the data within lab except for participant 3. For AZA2, differences were observed within participants.
Significant differences could be observed within and between participants when comparing single versus double transition monitoring. Participant 4 consistently reported higher values in single transition monitoring for the transition 828>640, 842>654 and 856>654 corresponding to AZA3, -1 and -2 respectively.

Table AII-1: OA results in parent monitoring mode

		SSR 10		SSR 5		Expected results SSR 5			
		OA +	OA -	OA +	OA -	OA +		OA -	
Participant	MS intrument	827	803	827	803	827	% diff	803	% diff
1	SQ	53.8	17.0	84.5	35.1	107.6	24.0%	34.0	-3.2%
		53.7	18.7	91.4	33.1	107.4	16.1%	37.4	12.2%
		60.5	17.4	88.8	34.4	121.0	30.7%	34.8	1.2%
2	TSQ	19.4	13.4	51.5	26.5	38.8	-28.1%	26.8	1.1%
		19.5	13.3	46.0	26.4	39.0	-16.5%	26.6	0.8%
		18.1	11.6	43.6	25.5	36.2	-18.5%	23.2	-9.4%
3	TSQ	29.5	18.9	57.6	34.0	59.0	2.5%	37.8	10.8%
		28.9	18.8	51.1	41.8	57.7	12.3%	37.6	-10.8%
		28.1	20.9	53.9	42.1	56.2	4.1%	41.8	0.7%
4	TSQ	32.5	nd	58.0	41.8	65.0	11.4%		
		nd	nd	61.3	38.6				
		nd	nd	60.3	44.7				
5	TSQ (LIT)	16.1	24.4	26.8	48.5	32.2	18.3%	48.8	0.6%
		17.5	23.0	27.0	47.4	35.0	25.8%	46.0	-3.0%
		18.5	23.9	26.8	49.2	37.0	32.0%	47.8	-2.9%
6	TSQ	41.7	25.8	62.1	36.9	83.3	29.2%	51.5	33.2%
		46.0	25.5	77.1	41.9	91.9	17.5%	51.1	19.8%
		47.6	22.6	80.0	38.9	95.3	17.5%	45.2	14.9%
	Average	33.2	19.7	58.2	38.2		19.0%		8.3%
	stdev	15.2	4.6	20.3	7.3				
	%CV	45.7%	23.2%	34.9%	19.2%				

Table AII-2: OA results in double transition monitoring mode

		SSR 10				SSR 5				Expected results SSR 5							
		OA +		OA -		OA +		OA -		OA +				OA -			
Participant	MS	769>751	827>723	803>255	803>113	769>751	827>723	803>255	803>113	769>751	% diff	827>723	% diff	803>255	% diff	803>113	% diff
2	TSQ	17.2	19.6	22.4	23.2	37.3	57	48	48.6	34.4	-8.1%	39.2	-37.0%	44.8	-6.9%	46.4	-4.6%
		12.5	21.7	18.5	18.5	32.9	63.7	41.4	42.3	25.0	-27.3%	43.4	-37.9%	37	-11.2%	37	-13.4%
		18.8	30.8	19.1	16.6	38.1	61.5	54.2	47.5	37.6	-1.3%	61.6	0.2%	38.2	-34.6%	33.2	-35.4%
3	TSQ	nd	26.9	19.7	20.6	nd	61.9	34.4	34.8			53.9	-13.8%	39.3	13.3%	41.1	16.5%
		nd	23.2	16.7	18.6	nd	47.6	32.7	36.8			46.4	-2.5%	33.4	2.3%	37.2	1.3%
		nd	22.7	20.7	18.7	nd	48.0	37.8	38.9			45.5	-5.3%	41.3	8.9%	37.3	-4.2%
4	TSQ	16.4	20.7	17.1	10.7	39.5	39.8	34.3	37.5	32.8	-18.5%	41.4	3.9%	34.2	-0.3%	21.4	-54.7%
		13.8	18.8	18.1	19	39.9	43.1	37.6	29.4	27.6	-36.4%	37.6	-13.6%	36.2	-3.8%	38	25.5%
		15.7	21.6	22.1	18.4	35.2	41.6	40.9	43.6	31.4	-11.4%	43.2	3.8%	44.2	7.8%	36.8	-16.9%
5	TSQ (LIT)	nd	15.4	24.7	25.1	nd	27.0	46.3	47.7			30.8	13.1%	49.4	6.5%	50.2	5.1%
		nd	15.8	23.6	23.2	nd	26.1	45.7	44.9			31.6	19.1%	47.2	3.2%	46.4	3.3%
		nd	17.6	23.7	24.8	nd	25.7	47.3	45.3			35.2	31.2%	47.4	0.2%	49.6	9.1%
6	TSQ	nd	nd	20.9	nd	nd	nd	34.5	nd					41.9	19.4%		
		nd	nd	23.3	nd	nd	nd	33.0	nd					46.6	34.2%		
		nd	nd	22.0	nd	nd	nd	37.0	nd					44.0	17.1%		
	Average	15.7	21.2	20.8	19.8	37.2	45.2	40.3	41.4		17.2%		15.1%		11.3%		15.8%
	stdev	2.3	4.4	2.5	4.0	2.7	14.0	6.6	6.0								
	%CV	14.5%	20.9%	12.2%	20.3%	7.2%	31.0%	16.3%	14.4%								

Table AII-3: Comparison of the OA results obtained in single and double transition monitoring mode

		SSR 10				SSR 5				Expected results SSR 5							
		OA +		OA -		OA +		OA -		OA +				OA -			
		769>751	827>723	803>255	803>113	769>751	827>723	803>255	803>113	769>751	% diff	827>723	% diff	803>255	% diff	803>113	% diff
4	Single transition monitoring	16.9	29.6	15.3	13.6	39.3	48.9	37.4	30.5	33.8	-15.0%	59.2	19.1%	30.6	-20.0%	27.2	-11.4%
		18.0	28.5	20.1	17.1	35.8	54.4	41.6	28.5	36.0	0.6%	57	4.7%	40.2	-3.4%	34.2	18.2%
		16.7	30.6	18	14	35.6	54.5	49.5	28.9	33.4	-0.4%	61.2	11.6%	36	-31.6%	28	-3.2%
6		nd	nd	16.9	nd	nd	nd	36.4	nd					33.8	-7.4%		
		nd	nd	22.1	nd	nd	nd	26.7	nd					44.2	49.4%		
		nd	nd	23.6	nd	nd	nd	34.1	nd					47.2	32.2%		
4	Double transition monitoring	16.4	20.7	17.1	10.7	39.5	39.8	34.3	37.5	32.8	-18.5%	41.4	3.9%	34.2	-0.3%	21.4	-54.7%
		13.8	18.8	18.1	19	39.9	43.1	37.6	29.4	27.6	-36.4%	37.6	-13.6%	36.2	-3.8%	38	25.5%
		15.7	21.6	22.1	18.4	35.2	41.6	40.9	43.6	31.4	-11.1%	43.2	3.8%	44.2	7.8%	36.8	16.9%
6		nd	nd	20.9	nd	nd	nd	34.5	nd					41.9	19.4%		
		nd	nd	23.3	nd	nd	nd	33.0	nd					46.6	34.2%		
		nd	nd	22.0	nd	nd	nd	37.0	nd					44.0	17.1%		

Table AII-4: DTX2 results in parent monitoring mode

		SSR 10		SSR 5		Expected results SSR 5			
		DTX2 +	DTX2 -	DTX2 +	DTX2 -	DTX2 +		DTX2 -	
Participant	MS intrument	827	803	827	803	827	% diff	803	% diff
1	SQ	101.0	63.6	123.0	123.0	202.0	48.6%	127.2	3.4%
		99.4	65.7	115.0	123.0	198.8	53.4%	131.4	6.6%
		103.0	65.9	129.0	123.0	206.0	46.0%	131.8	6.9%
2	TSQ	120.5	65.1	253.2	150.8	241.0	-4.9%	130.2	-14.7%
		126.4	66.7	224.1	153.7	252.8	12.0%	133.4	-14.1%
		126.4	66.1	205.5	152.5	252.8	20.6%	132.2	-14.3%
3	TSQ	117.6	74.2	222.0	143.6	235.3	5.8%	148.4	3.3%
		114.3	71.2	207.7	173.8	228.6	9.6%	142.4	-19.9%
		118.6	84.6	222.0	175.5	237.2	6.6%	169.1	-3.7%
4	TSQ	129.8	nd	206.0	144.3	259.6	23.0%		
		nd	nd	202.4	156.6				
		nd	nd	205.2	159.3				
5	TSQ (LIT)	59.3	93.1	99.5	189.0	118.6	17.5%	186.2	-1.5%
		63.1	86.2	98.8	182.0	126.2	24.4%	172.4	-5.4%
		63.6	90.9	101.0	184.0	127.2	23.0%	181.8	-1.2%
6	TSQ	129.4	76.6	287.2	130.0	258.9	-10.4%	153.2	16.4%
		154.8	66.7	268.9	128.2	309.7	14.1%	133.4	4.0%
		161.2	62.9	347.8	142.8	322.4	-7.6%	125.8	-12.7%
	Average	111.8	73.3	195.5	152.0		20.5%		8.5%
	stdev	29.8	10.5	71.4	21.9				
	%CV	26.7%	14.3%	36.5%	14.4%				

Table AII-5: DTX2 results in double transition monitoring mode

		SSR 10				SSR 5				Expected results SSR 5							
		DTX2 +		DTX2 -		DTX2 +		DTX2 -		DTX2 +				DTX2 -			
Participant	MS	769>751	827>723	803>255	803>113	769>751	827>723	803>255	803>113	769>751	% diff	827>723	% diff	803>255	% diff	803>113	% diff
2	TSQ	55.5	92.1	68.7	68.9	127.6	218.7	163.5	148.1	111.0	-13.9%	184.2	-17.1%	137.4	-17.3%	137.8	-7.2%
		54.4	104.1	65.9	65.2	118.2	206.5	142.5	128.3	108.8	-8.3%	208.2	0.8%	131.8	-7.8%	130.4	1.6%
		66.3	130.4	64.6	66.9	116.1	202.6	158.9	158.3	132.6	13.3%	260.8	25.1%	129.2	-20.6%	133.8	-16.8%
3	TSQ	nd	127.3	79.0	77.1	nd	256.7	144.6	152.2			254.7	-0.8%	158.0	8.8%	154.3	1.4%
		nd	106.9	71.4	69.5	nd	204.3	152.9	139.3			213.8	4.5%	142.7	-6.9%	139.0	-0.3%
		nd	104.8	77.1	76.6	nd	206.4	174.1	156.8			209.6	1.5%	154.1	-12.2%	153.2	-2.3%
4	TSQ	36.1	78.4	70.5	73	74.5	135.8	138.7	144.7	72.2	-3.1%	156.8	14.4%	141.0	1.6%	146.0	0.9%
		34.3	74.4	73.2	80.6	73.2	123.8	141.7	148.3	68.6	-6.5%	148.8	18.3%	146.4	3.3%	161.2	8.3%
		35.6	81.7	83	83.2	74.4	127.2	152.9	157.2	71.2	-4.4%	163.4	24.9%	166.0	8.2%	166.4	5.7%
5	TSQ (LIT)	nd	61.7	95.1	94.9	nd	103.0	181.0	176.0			123.4	18.0%	190.2	5.0%	189.8	7.5%
		nd	58.1	89.3	90.4	nd	97.4	179.0	176.3			116.2	17.6%	178.6	-0.2%	180.8	2.5%
		nd	60.0	92.2	89.6	nd	101.0	174.0	173.0			120.0	17.2%	184.4	5.8%	179.2	3.5%
6	TSQ	nd	nd	62.3	nd	nd	nd	nd	nd					124.7			
		nd	nd	55.5	nd	nd	nd	78.6	nd					111.1	34.2%		
		nd	nd	61.5	nd	nd	nd	134.7	nd					123.0	-9.0%		
	Average	47.0	90.0	74.0	78.0	97.3	165.3	151.2	154.9		8.2%		13.4%		10.1%		4.8%
	stdev	13.5	25.0	11.8	9.9	25.8	55.7	26.1	14.8								
	%CV	28.7%	27.8%	16.0%	12.7%	26.5%	33.7%	17.2%	9.5%								

Table AII-6: Comparison of the DTX2 results obtained in single and double transition monitoring mode

		SSR 10				SSR 5				Expected results SSR 5							
		DTX2 +		DTX2 -		DTX2 +		DTX2 -		DTX2 +				DTX2 -			
		769>751	827>723	803>255	803>113	769>751	827>723	803>255	803>113	769>751	% diff	827>723	% diff	803>255	% diff	803>113	% diff
4	Single transition monitoring	42.8	118	86.3	57	80.4	187.6	158.1	112.5	85.6	6.3%	236	22.9%	172.6	8.8%	114	1.3%
		41.2	112.9	97	53.8	78.8	182.4	175.4	109.9	82.4	4.5%	225.8	21.3%	194	10.1%	107.6	-2.1%
		39.6	109	95.4	57.9	79.9	182.5	190.5	109.6	79.2	-0.9%	218	17.7%	190.8	0.2%	115.8	5.5%
6		nd	nd	56.6	nd	nd	nd	123.9	nd					113.2	-9.0%		
		nd	nd	65.8	nd	nd	nd	131.1	nd					131.6	0.4%		
		nd	nd	68.2	nd	nd	nd	127.8	nd					136.4	6.5%		
4	Double transition monitoring	36.1	78.4	70.5	73	74.5	135.8	138.7	144.7	72.2	-3.1%	156.8	14.4%	141.0	1.6%	146.0	0.9%
		34.3	74.4	73.2	80.6	73.2	123.8	141.7	148.3	68.6	-6.5%	148.8	18.3%	146.4	3.3%	161.2	8.3%
		35.6	81.7	83	83.2	74.4	127.2	152.9	157.2	71.2	-4.4%	163.4	24.9%	166.0	8.2%	166.4	5.7%
6		nd	nd	62.3	nd	nd	nd		nd					124.7			
		nd	nd	55.5	nd	nd	nd	78.6	nd					111.1	34.2%		
		nd	nd	61.5	nd	nd	nd	134.7	nd					123.0	-9.0%		

Table AII-7: DTX1 results in parent monitoring mode

						Expected results			
		SSR 10		SSR 5		SSR 5			
		DTX1 +	DTX1 -	DTX1 +	DTX1 -	DTX1 +		DTX1 -	
Participant	MS intrument	841	817	841	817	841	% diff	817	% diff
1	SQ	17.7	24.1	20.6	51.0	35.4	52.9%	48.2	-5.6%
		18.7	27.2	22.0	54.5	37.4	51.9%	54.4	-0.2%
		20.8	26.4	23.2	50.5	41.6	56.8%	52.8	4.5%
2	TSQ	17.8	25.8	24.3	62.6	35.6	37.7%	51.6	-19.3%
		16.8	29.8	20.1	62.0	33.6	50.3%	59.6	-3.9%
		15.8	29.1	20.7	63.5	31.6	41.7%	58.2	-8.7%
3	TSQ	nd	38.5	nd	71.6			77.1	7.3%
		nd	36.6	nd	74.2			73.3	-1.3%
		nd	37.3	nd	75.5			74.6	-1.3%
4	TSQ	nd	nd	18.9	63.7				
		nd	nd	15.0	75.6				
		nd	nd	14.6	70.7				
5	TSQ (LIT)	11.3	36.8	25.3	73.2	22.6	-11.3%	73.6	0.5%
		13.1	32.2	28.0	70.2	26.2	-6.5%	64.4	-8.6%
		12.4	35.5	26.3	72.1	24.7	-6.2%	70.9	-1.7%
6	TSQ	nd	29.5	nd	48.2			58.9	20.0%
		nd	25.2	nd	53.6			50.4	-6.2%
		nd	26.9	nd	46.0			53.8	15.5%
	Average	16.0	30.7	21.6	63.3		35.0%		7.0%
	stdev	3.2	5.0	4.2	10.2				
	%CV	19.8%	16.3%	19.3%	16.2%				

Table AII-8: DTX1 results in double transition monitoring mode

		SSR 10				SSR 5				Expected results SSR 5							
		DTX1 +		DTX1 -		DTX1 +		DTX1 -		DTX1 +				DTX1 -			
Participant	MS	783>765	841>737	817>255	817>113	783>765	841>737	817>255	817>113	783>765	% diff	841>737	% diff	817>255	% diff	817>113	% diff
2	TSQ	13.2	7.9	23.9	28	28.9	12.7	58.3	62.8	26.4	-9.0%	15.8	21.8%	47.8	-19.8%	56.0	-11.4%
		17.5	11.5	24.5	27	28	11.8	48	50.6	35.0	22.2%	23.0	64.4%	49.0	2.1%	54.0	6.5%
		18.6	11.3	24.9	27.7	27.5	11.4	56.5	60.9	37.2	30.0%	22.6	65.9%	49.8	-12.6%	55.4	-9.5%
3	TSQ	nd	48.9	33.2	31.3	nd	80.2	62.2	56.4			97.9	19.8%	66.4	6.5%	62.6	10.4%
		nd	44.6	33.4	34.0	nd	63.4	57.7	55.0			89.3	33.8%	66.8	14.6%	68.1	21.2%
		nd	39.3	35.0	35.1	nd	70.2	64.9	63.5			78.7	11.3%	69.9	7.4%	70.2	10.1%
4	TSQ	nd	nd	36.7	20.6	nd	nd	73.2	48.8					73.4	0.3%	41.2	-16.9%
		nd	nd	38.2	22.8	nd	nd	75.9	61.8					76.4	0.7%	45.6	-30.2%
		nd	nd	37.4	27.8	nd	nd	81.3	60.1					74.8	-8.3%	55.6	-7.8%
5	TSQ (LIT)	nd	18.6	33.3	28.7	nd	34.6	66.2	56.5			37.2	7.2%	66.6	0.6%	57.4	1.5%
		nd	20.9	34.4	29.1	nd	33.5	68.3	58.4			41.9	22.3%	68.7	0.6%	58.2	-0.3%
		nd	21.2	32.5	27.5	nd	32.7	65.5	57.9			42.4	25.9%	65.0	-0.7%	55.0	-5.1%
6	TSQ	nd	nd	24.1	nd	nd	nd	42.9	nd					48.2	11.6%		
		nd	nd	26.0	nd	nd	nd		nd					52.0			
		nd	nd	24.2	nd	nd	nd	50.6	nd					48.4	-4.4%		
	Average	16.4	24.9	30.8	28.3	28.1	39.0	62.3	57.7		20.4%		30.3%		6.4%		10.9%
	stdev	2.9	15.4	5.5	4.1	0.7	26.3	10.8	4.6								
	%CV	17.4%	61.8%	17.8%	14.4%	2.5%	67.6%	17.4%	8.0%								

Table AII-9: Comparison of the DTX1 results obtained in single and double transition monitoring mode

		SSR 10				SSR 5				Expected results SSR 5							
		DTX1 +		DTX1 -		DTX1 +		DTX1 -		DTX1 +				DTX1 -			
		783>765	841>737	817>255	817>113	783>765	841>737	817>255	817>113	783>765	% diff	841>737	% diff	817>255	% diff	817>113	% diff
4	Single transition monitoring	nd	nd	38.0	18.6	nd	nd	80.8	42.8					76	-6.1%	37.2	-14.0%
		nd	nd	41.4	20.0	nd	nd	79.2	38.9					82.8	4.4%	40	2.8%
		nd	nd	44.1	21.6	nd	nd	87.3	41.1					88.2	1.0%	43.2	5.0%
6		nd	nd	26.6	nd	nd	nd	42.6	nd					53.2	22.1%		
		nd	nd	25.9	nd	nd	nd	45.3	nd					51.8	13.4%		
		nd	nd	27.0	nd	nd	nd	43.1	nd					54	22.5%		
4	Double transition monitoring	nd	nd	36.7	20.6	nd	nd	73.2	48.8					73.4	0.3%	41.2	-16.9%
		nd	nd	38.2	22.8	nd	nd	75.9	61.8					76.4	0.7%	45.6	-30.2%
		nd	nd	37.4	27.8	nd	nd	81.3	60.1					74.8	-8.3%	55.6	-7.8%
6		nd	nd	24.1	nd	nd	nd	42.9	nd					48.2	11.6%		
		nd	nd	26.0	nd	nd	nd		nd					52.0			
		nd	nd	24.2	nd	nd	nd	50.6	nd					48.4	-4.4%		

Table AII-10: PTX2 results in parent monitoring mode

		SSR 10		SSR 5		Expected results SSR 5			
		PTX2 +	PTX2 -	PTX2 +	PTX2 -	PTX2 +		PTX2 -	
Participant	MS intrument	876	857	876	857	876	% diff	857	% diff
1	SQ	14.3	nd	27.1	nd	28.6	5.4%		
		16.0	nd	26.5	nd	32.0	18.8%		
		14.9	nd	26.9	nd	29.8	10.2%		
2	TSQ	16.1	13.6	32.8	25.9	32.2	-1.8%	27.2	4.9%
		17.0	14.0	34.4	28.5	34.0	-1.2%	28.0	-1.8%
		17.0	12.8	33.2	26.9	34.0	2.4%	25.6	-5.0%
3	TSQ	16.5	14.4	28.3	22.3	33.0	15.2%	28.7	25.1%
		16.5	11.8	27.4	24.2	33.0	18.3%	23.7	-2.2%
		16.5	14.4	30.2	24.4	33.0	8.9%	28.8	16.6%
4	TSQ	18.5	nd	34.2	28.9	37.0	7.9%		
		nd	nd	34.0	27.0				
		nd	nd	33.5	29.5				
5	TSQ (LIT)	10.0	8.0	15.7	15.9	20.0	24.1%	16.1	1.1%
		10.2	8.4	15.1	16.2	20.4	29.9%	16.7	3.0%
		10.2	8.2	15.8	14.4	20.4	25.4%	16.3	12.4%
6	TSQ	11.5	nd	46.0	nd	23.0	-66.7%		
		11.7	nd	37.3	nd	23.4	-45.8%		
		11.5	nd	22.8	nd	23.0	0.9%		
	Average	14.3	11.7	29.0	23.7		17.7%		8.0%
	stdev	2.9	2.8	8.0	5.4				
	%CV	20.4%	23.7%	27.7%	22.6%				

Table AII -11: PTX2 results in double transition monitoring mode

		SSR 10				SSR 5				Expected results SSR 5							
		PTX2 +		PTX2 -		PTX2 +		PTX2 -		PTX2 +				PTX2 -			
Participant	MS	876>823	876>213	857>627	857>137	876>823	876>213	857>627	857>137	876>823	% diff	876>213	% diff	857>627	% diff	857>137	% diff
2	TSQ	14.9	15.3	13.9	13.0	30.4	30.1	25.1	25.7	29.8	-2.0%	30.6	1.6%	27.8	10.2%	26.0	1.2%
		16.2	15.9	15.7	13.8	30.9	30.8	28.0	27.2	32.4	4.7%	31.8	3.2%	31.4	11.4%	27.6	1.5%
		16.5	16.4	11.2	13.7	31.5	31.2	30.4	25.3	33.0	4.7%	32.8	5.0%	22.4	-30.3%	27.4	8.0%
3	TSQ	17.0	16.1	15.5	13.5	32.0	32.4	23.4	24.6	34.0	6.3%	32.3	-0.3%	31.0	27.8%	27.1	9.7%
		13.8	12.3	12.2	14.5	24.5	26.3	23.1	25.1	27.6	11.9%	24.6	-6.4%	24.4	5.6%	29.1	14.8%
		12.7	11.7	15.0	14.8	25.2	27.0	26.2	26.8	25.3	0.3%	23.4	-14.4%	30.0	13.3%	29.7	10.2%
4	TSQ	15.5	17.2	nd	10.7	29.6	34.3	nd	19.5	31.0	4.6%	34.4	0.3%			21.4	9.3%
		15.0	15.9	nd	10.3	29.9	33.0	nd	17.6	30.0	0.3%	31.8	-3.7%			20.6	15.7%
		15.7	16.6	nd	11.5	30.1	32.3	nd	20.5	31.4	4.2%	33.2	2.7%			23.0	11.5%
5	TSQ (LIT)	7.6	10.0	8.7	10.8	14.7	18.2	14.2	17.5	15.2	2.9%	19.9	9.0%	17.4	20.2%	21.5	20.8%
		6.9	9.1	9.3	10.7	15.0	17.8	13.8	17.2	13.7	-9.0%	18.2	2.4%	18.6	29.3%	21.4	21.7%
		6.8	10.5	7.9	10.6	14.5	16.9	15.8	15.9	13.5	-6.5%	21.0	21.1%	15.8	0.1%	21.2	28.2%
6	TSQ	17.9	16.3	nd	nd	34.1	33.2	nd	nd	35.8	4.9%	32.7	-1.7%				
		19.8	15.4	nd	nd	34.2	34.9	nd	nd	39.5	14.4%	30.9	-12.1%				
		19.6	19.1	nd	nd	35.3	30.7	nd	nd	39.3	10.6%	38.2	21.8%				
	Average	14.4	14.5	12.2	12.3	27.5	28.6	22.2	21.9		5.8%		7.1%		12.4%		12.7%
	stdev	4.2	3.0	3.0	1.7	7.2	6.1	6.1	4.2								
	%CV	29.5%	20.7%	24.9%	14.0%	26.2%	21.5%	27.6%	19.4%								

Table AII -I2: Comparison of the PTX2 results obtained in single and double transition monitoring mode

		SSR 10				SSR 5				Expected results SSR 5							
		PTX2 +		PTX2 -		PTX2 +		PTX2 -		PTX2 +				PTX2 -			
		876>823	876>213	857>627	857>137	876>823	876>213	857>627	857>137	876>823	% diff	876>213	% diff	857>627	% diff	857>137	% diff
4	Single transition monitoring	20	19.8	16.4	11.8	36.1	35.8	28.2	20.4	40.0	10.2%	39.6	10.1%	32.8	15.1%	23.6	14.5%
		19.8	19.7	11.7	11.1	35.5	37.8	19.6	19.5	39.6	10.9%	39.4	4.1%	23.4	17.7%	22.2	12.9%
		20.2	20.5	11.1	9.4	36.6	36.4	22.5	18.9	40.4	9.9%	41	11.9%	22.2	-1.3%	18.8	-0.5%
6		17.8	20.8	nd	nd	35.2	36.7	nd	nd	35.6	1.1%	41.6	12.5%				
		18.9	18.6	nd	nd	29.3	38.1	nd	nd	37.8	25.3%	37.2	-2.4%				
		20.1	20.1	nd	nd	32.2	35.2	nd	nd	40.2	22.1%	40.2	13.3%				
4	Double transition monitoring	15.5	17.2	nd	10.7	29.6	34.3	nd	19.5	31.0	4.6%	34.4	0.3%			21.4	9.3%
		15.0	15.9	nd	10.3	29.9	33.0	nd	17.6	30.0	0.3%	31.8	-3.7%			20.6	15.7%
		15.7	16.6	nd	11.5	30.1	32.3	nd	20.5	31.4	4.2%	33.2	2.7%			23	11.5%
6		17.9	16.3	nd	nd	34.1	33.2	nd	nd	35.8	4.9%	32.7	-1.7%				
		19.8	15.4	nd	nd	34.2	34.9	nd	nd	39.5	14.4%	30.9	-12.1%				
		19.6	19.1	nd	nd	35.3	30.7	nd	nd	39.3	10.6%	38.2	21.8%				

Table AII -13: AZA1 results in parent monitoring mode

				Expected results	
		SSR 10	SSR 5	SSR 5	
		AZA1 +	AZA1 +	AZA1 +	
Participant	MS intrument	842	842	842	% diff
1	SQ	35.6	61.0	71.2	15.4%
		28.2	56.2	56.4	0.4%
		29.1	51.6	58.2	12.0%
2	TSQ	38.2	58.7	76.4	26.2%
		35.2	53.1	70.4	28.0%
		25.2	45.4	50.4	10.4%
3	TSQ	50.2	79.7	100.4	23.0%
		47.5	78.0	95.0	19.7%
		50.7	79.9	101.4	23.7%
4	TSQ	58.7	57.4	117.4	68.6%
		nd	68.9		
		nd	56.7		
5	TSQ (LIT)	33.3	70.4	66.6	-5.5%
		31.9	70.5	63.8	-10.0%
		32.8	71.4	65.6	-8.5%
6	TSQ	38.8	69.9	77.6	10.4%
		28.5	51.4	57.0	10.3%
		30.3	52.8	60.6	13.8%
	Average	37.1	62.9		17.9%
	stdev	9.7	10.8		
	%CV	26.1%	17.1%		

Table AII -14: AZA1 results in double transition monitoring mode

						Expected results			
		SSR 10		SSR 5		SSR 5			
		AZA1 +		AZA1 +		AZA1 +			
Participant	MS intrument	842>672	842>654	842>672	842>654	842>672	% diff	842>654	% diff
2	TSQ	32.0	31.6	59.1	57.9	64.0	8.0%	63.2	8.8%
		29.5	30.9	59.3	57.5	59.0	-0.5%	61.8	7.2%
		29.2	27.2	53.7	51.9	58.4	8.4%	54.4	4.7%
3	TSQ	33.7	33.5	65.6	65.0	67.5	2.8%	67.1	3.2%
		33.7	32.8	65.3	62.2	67.5	3.3%	65.6	5.3%
		34.7	34.3	69.0	67.8	69.3	0.5%	68.6	1.1%
4	TSQ	23.8	23.9	48.6	48.2	47.6	-2.1%	47.8	-0.8%
		24.4	25.2	44.5	44.6	48.8	9.2%	50.4	12.2%
		23.8	23.4	43.7	45.7	47.6	8.5%	46.8	2.4%
5	LIT	36.1	35.5	72.7	72.3	72.2	-0.7%	71.0	-1.8%
		37.7	36.5	71.0	71.1	75.4	6.0%	73.0	2.6%
		37.7	37.2	71.4	72.5	75.4	5.4%	74.4	2.6%
6	TSQ	18.7	18.6	45.5	45.5	37.3	-19.8%	37.2	-20.2%
		17.6	16.9	41.6	41.5	35.2	-16.8%	33.7	-20.7%
		22.9	19.2	40.5	35.2	45.8	12.2%	38.4	8.7%
	Average	29.0	28.4	56.8	55.9		6.9%		6.8%
	stdev	6.8	6.9	12.0	12.2				
	%CV	23.3%	24.2%	21.1%	21.9%				

Table AII -15: Comparison of the AZA1 results obtained in single and double transition monitoring mode

							Expected results			
			SSR 10		SSR 5		SSR 5			
			AZA1 +		AZA1 +		AZA1 +			
			842>672	842>654	842>672	842>654	842>672	% diff	842>654	% diff
4	TSQ	Single transition monitoring	26.1	50.7	45.9	97.2	52.2	12.8%	101.4	4.2%
			26.0	54.0	42.1	86.8	52.0	21.0%	108.0	21.8%
			24.6	49.8	41.1	87.6	49.2	17.9%	99.6	12.8%
6	TSQ		18.0	20.0	49.4	57.6	36.0	-31.4%	40.0	-36.1%
			17.8	20.1	42.2	48.3	35.6	-17.0%	40.2	-18.3%
			20.7	21.5	35.8	44.9	41.4	14.5%	43.0	-4.3%
4	TSQ	Double transition monitoring	23.8	23.9	48.6	48.2	47.6	-2.1%	47.8	-0.8%
			24.4	25.2	44.5	44.6	48.8	9.2%	50.4	12.2%
			23.8	23.4	43.7	45.7	47.6	8.5%	46.8	2.4%
6	TSQ		18.7	18.6	45.5	45.5	37.3	-19.8%	37.2	-20.2%
			17.6	16.9	41.6	41.5	35.2	-16.8%	33.7	-20.7%
			22.9	19.2	40.5	35.2	45.8	12.2%	38.4	8.7%

Table AII-16: AZA2 results in parent monitoring mode

				Expected results	
		SSR 10	SSR 5	SSR 5	
		AZA2 +	AZA2 +	AZA2 +	
Participant	MS intrument	856	856	856	% diff
1	SQ	11.3	17.1	22.6	27.7%
		10.9	16.1	21.8	30.1%
		9.9	16.5	19.8	18.2%
2	TSQ	16.5	14.2	33.0	79.7%
		8.8	12.2	17.6	36.2%
		6.7	10.3	13.4	26.2%
3	TSQ	13.1	21.7	26.2	18.8%
		14.8	20.1	29.5	37.8%
		13.3	21.6	26.6	20.9%
4	TSQ	nd	20.0		
		nd	20.2		
		nd	20.8		
5	LIT	7.8	13.5	15.6	14.2%
		7.9	13.3	15.9	17.3%
		7.4	13.8	14.8	6.9%
6	TSQ	13.7	25.8	27.4	6.0%
		12.7	21.4	25.4	17.1%
		13.1	23.2	26.2	12.1%
	Average	11.2	17.9		23.1%
	stdev	3.0	4.3		
	%CV	26.8%	24.3%		

Table AII -17: AZA2 results in double transition monitoring mode

						Expected results			
		SSR 10		SSR 5		SSR 5			
		AZA2 +		AZA2 +		AZA2 +			
Participant	MS intrument	856>672	856>654	856>672	856>654	856>672	% diff	856>654	% diff
2	TSQ	8.8	9.2	15.6	16.0	17.6	12.0%	18.4	14.0%
		8.6	8.8	15.6	15.9	17.2	9.8%	17.6	10.1%
		8.0	7.9	14.0	14.3	16.0	13.3%	15.8	10.0%
3	TSQ	8.0	12.1	14.6	22.9	16.0	9.4%	24.3	5.7%
		8.2	11.4	14.6	21.8	16.5	11.7%	22.7	4.0%
		8.7	12.1	15.5	23.5	17.3	11.0%	24.2	3.1%
4	TSQ	7.5	7.9	13.7	13.2	15.0	9.1%	15.8	17.9%
		7.5	8.0	13.0	13.3	15.0	14.3%	16.0	18.4%
		7.6	7.6	12.5	13.4	15.2	19.5%	15.2	12.6%
5	LIT	8.3	9.1	14.8	16.9	16.7	12.3%	18.2	7.4%
		7.9	8.8	15.2	17.4	15.8	4.2%	17.6	1.5%
		7.9	9.3	15.3	17.6	15.8	3.0%	18.6	5.2%
6	TSQ	2.1	4.5	9.8	14.8	4.3	-78.5%	9.0	-48.2%
		3.7	4.5	9.9	11.4	7.4	-29.0%	9.0	-23.8%
		4.2	5.8	12.2	13.8	8.3	-38.0%	11.5	-18.0%
	Average	7.1	8.5	13.7	16.4		18.3%		13.3%
	stdev	2.1	2.4	1.9	3.7				
	%CV	28.8%	27.8%	14.1%	22.6%				

Table AII-18: Comparison of the AZA2 results obtained in single and double transition monitoring mode

			SSR 10		SSR 5		Expected results SSR 5			
			AZA2 +		AZA2 +		AZA2 +			
			856>672	856>654	856>672	856>654	856>672	% diff	856>654	% diff
4	TSQ	Single transition monitoring	8.4	15.7	13.2	28.0	16.8	24.0%	31.4	11.4%
			8.1	15.2	12.2	24.8	16.2	28.2%	30.4	20.3%
			7.2	14.4	11.9	25.1	14.4	19.0%	28.8	13.7%
6	TSQ		4.9	6.6	14.8	16.1	9.8	-40.7%	13.1	-20.2%
			5.6	5.5	12.7	16.7	11.2	-12.6%	11.0	-41.2%
			5.1	5.3	13.7	14.7	10.2	-29.3%	10.6	-32.2%
4	TSQ	Double transition monitoring	7.5	7.9	13.7	13.2	15.0	9.1%	15.8	17.9%
			7.5	8.0	13.0	13.3	15.0	14.3%	16.0	18.4%
			7.6	7.6	12.5	13.4	15.2	19.5%	15.2	12.6%
6	TSQ		2.1	4.5	9.8	14.8	4.3	-78.5%	9.0	-48.2%
			3.7	4.5	9.9	11.4	7.4	-29.0%	9.0	-23.8%
			4.2	5.8	12.2	13.8	8.3	-38.0%	11.5	-18.0%

Table AII-19: AZA3 results in parent monitoring mode

				Expected results	
		SSR 10	SSR 5	SSR 5	
		AZA3 +	AZA3 +	AZA3 +	
Participant	MS intrument	828	828	842	% diff
1	SQ	13.9	22.2	27.8	22.4%
		11.6	24.2	23.2	-4.2%
		13.2	21.2	26.4	21.8%
2	TSQ	13.8	19.6	27.6	33.9%
		10.3	15.8	20.6	26.4%
		7.6	12.9	15.2	16.4%
3	TSQ	nd	nd		
		nd	nd		
		nd	nd		
4	TSQ	nd	21.6		
		nd	21.7		
		nd	22.8		
5	LIT	7.9	13.7	15.8	14.0%
		7.8	13.7	15.6	13.2%
		8.0	14.5	16.0	9.7%
6	TSQ	9.8	16.5	19.6	17.2%
		6.4	11.8	12.8	8.1%
		8.3	12.4	16.6	29.0%
	Average	9.9	17.6		18.0%
	stdev	2.7	4.4		
	%CV	26.8%	24.8%		

Table AII -20: AZA3 results in double transition monitoring mode

						Expected results			
		SSR 10		SSR 5		SSR 5			
		AZA3 +		AZA3 +		AZA3 +			
Participant	MS intrument	828>658	828>640	828>658	828>640	828>658	% diff	828>640	% diff
2	TSQ	13.4	12.6	25.0	24.0	26.8	6.9%	25.2	4.9%
		12.1	11.6	24.4	23.0	24.2	-0.8%	23.2	0.9%
		11.4	11.3	22.4	21.4	22.8	1.8%	22.6	5.5%
3	TSQ	3.1	8.5	5.0	14.4	6.2	21.2%	17.0	16.3%
		2.9	7.7	4.7	15.3	5.8	21.3%	15.3	-0.2%
		3.2	8.8	4.9	14.9	6.4	25.7%	17.7	17.1%
4	TSQ	10.2	9.3	19.8	17.8	20.4	3.0%	18.6	4.4%
		10.1	9.2	17.9	17.0	20.2	12.1%	18.4	7.9%
		9.6	9.2	17.9	16.5	19.2	7.0%	18.4	10.9%
5	LIT	9.9	10.4	19.6	17.9	19.8	1.1%	20.8	15.1%
		10.2	9.3	19.4	19.9	20.4	5.3%	18.6	-6.9%
		9.8	10.9	18.5	18.8	19.6	6.1%	21.7	14.5%
6	TSQ	5.4	4.2	16.6	14.4	10.9	-41.7%	8.3	-53.3%
		6.5	4.3	9.5	12.7	13.1	31.7%	8.6	-38.5%
		5.9	4.0	14.9	11.8	11.9	-22.1%	8.0	-38.0%
	Average	8.3	8.8	16.0	17.3		13.9%		15.6%
	stdev	3.4	2.7	6.9	3.6				
	%CV	41.7%	30.8%	42.8%	20.8%				

Table AII-21: Comparison of the AZA3 results obtained in single and double transition monitoring mode

							Expected results			
			SSR 10		SSR 5		SSR 5			
			AZA3 +		AZA3 +		AZA3 +			
			828>658	828>640	828>658	828>640	828>658	% diff	828>640	% diff
4	TSQ	Single transition monitoring	11.1	17.2	17.0	34.6	22.2	26.5%	34.4	-0.6%
			9.4	17.7	16.7	31.4	18.8	11.8%	35.4	12.0%
			9.0	17.5	16.9	32.5	18.0	6.3%	35.0	7.4%
6	TSQ		5.3	6.6	13.8	17.1	10.6	-26.2%	13.2	-25.7%
			4.5	5.5	14.4	16.2	9.0	-46.2%	11.0	-38.2%
			5.4	5.3	13.2	14.4	10.8	-20.0%	10.6	-30.4%
4	TSQ	Double transition monitoring	10.2	9.3	19.8	17.8	20.4	3.0%	18.6	4.4%
			10.1	9.2	17.9	17.0	20.2	12.1%	18.4	7.9%
			9.6	9.2	17.9	16.5	19.2	7.0%	18.4	10.9%
6	TSQ		5.4	4.2	16.6	14.4	10.9	-41.7%	8.3	-53.3%
			6.5	4.3	9.5	12.7	13.1	31.7%	8.6	-38.5%
			5.9	4.0	14.9	11.8	11.9	-22.1%	8.0	-38.0%

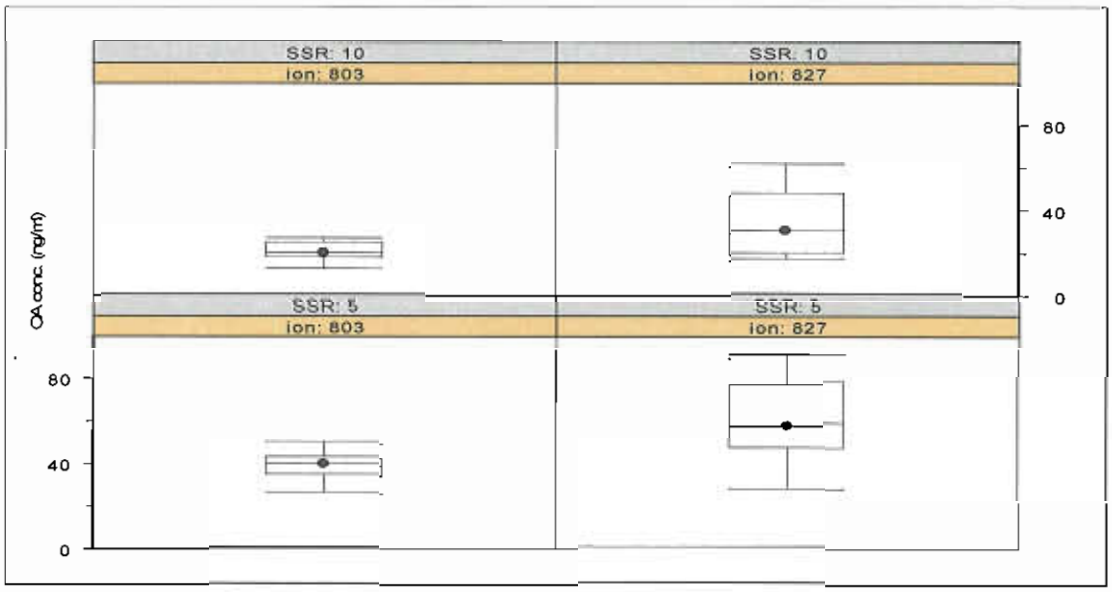

Figure AII-1: Distribution of the OA results acquired in parent monitoring mode

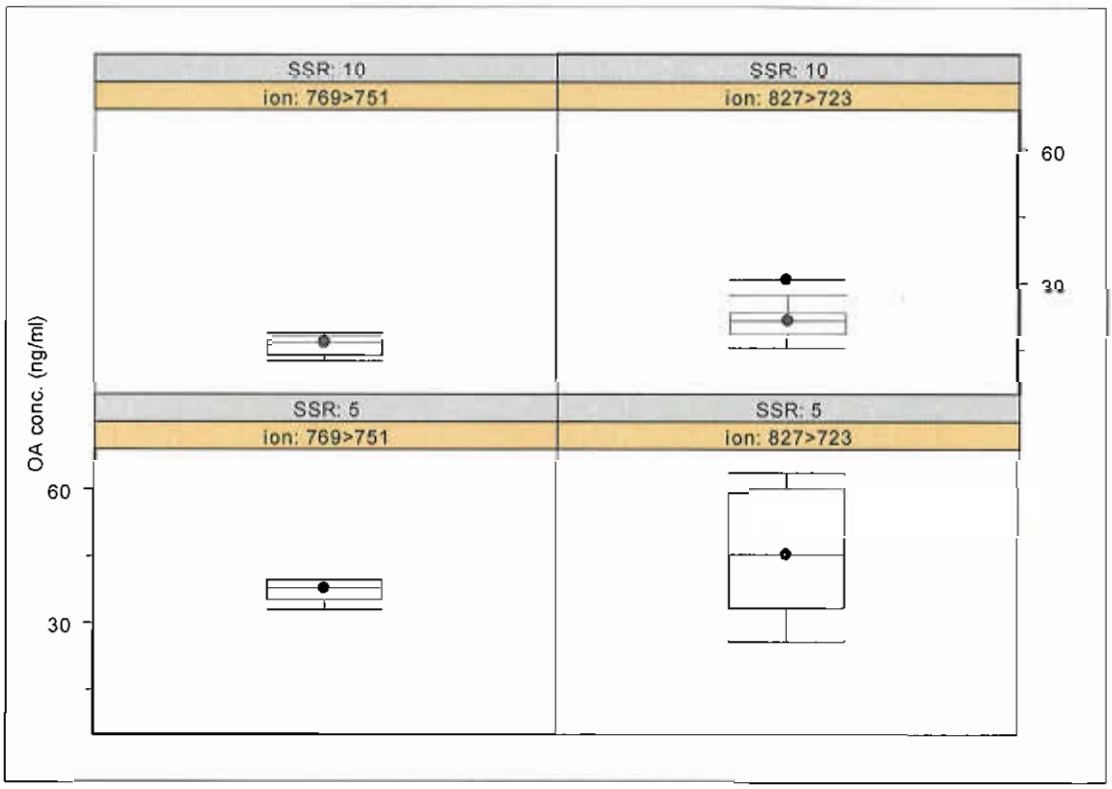

Figure AII-2: Distribution of the OA results acquired in positive double transition monitoring mode

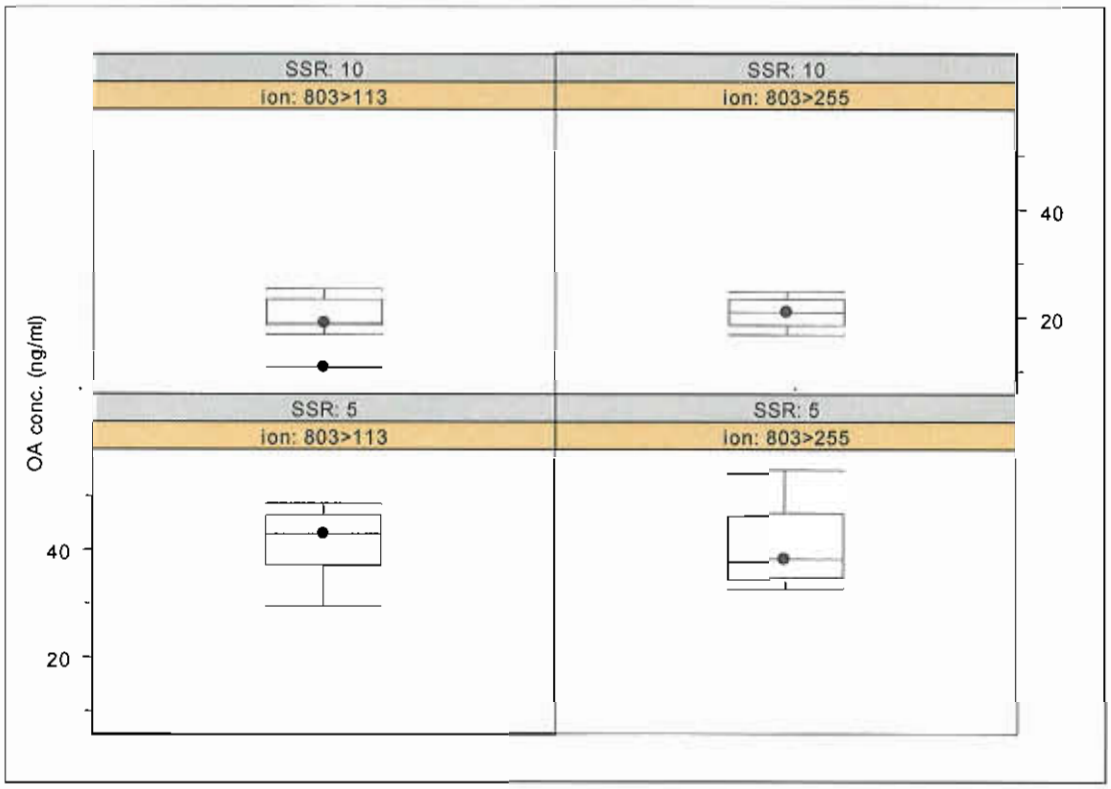

Figure AII-3: Distribution of the OA results acquired in negative double transition monitoring mode

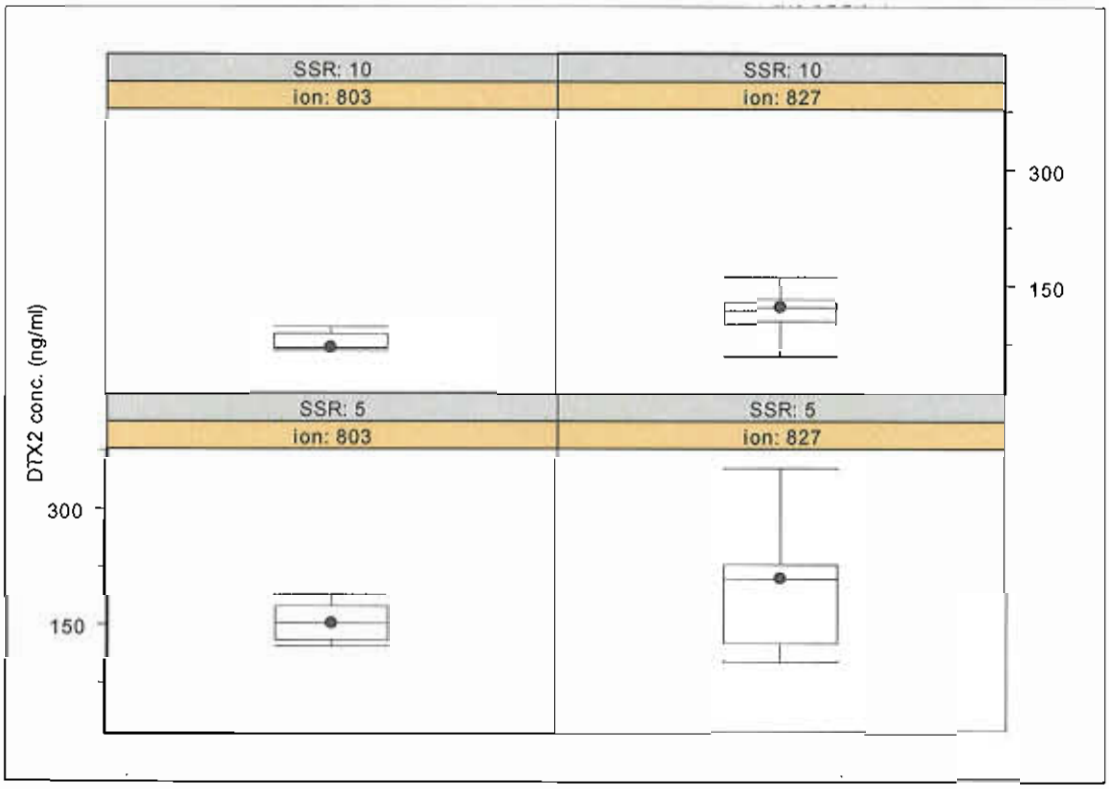

Figure AII-4: Distribution of the DTX2 results acquired in parent monitoring mode

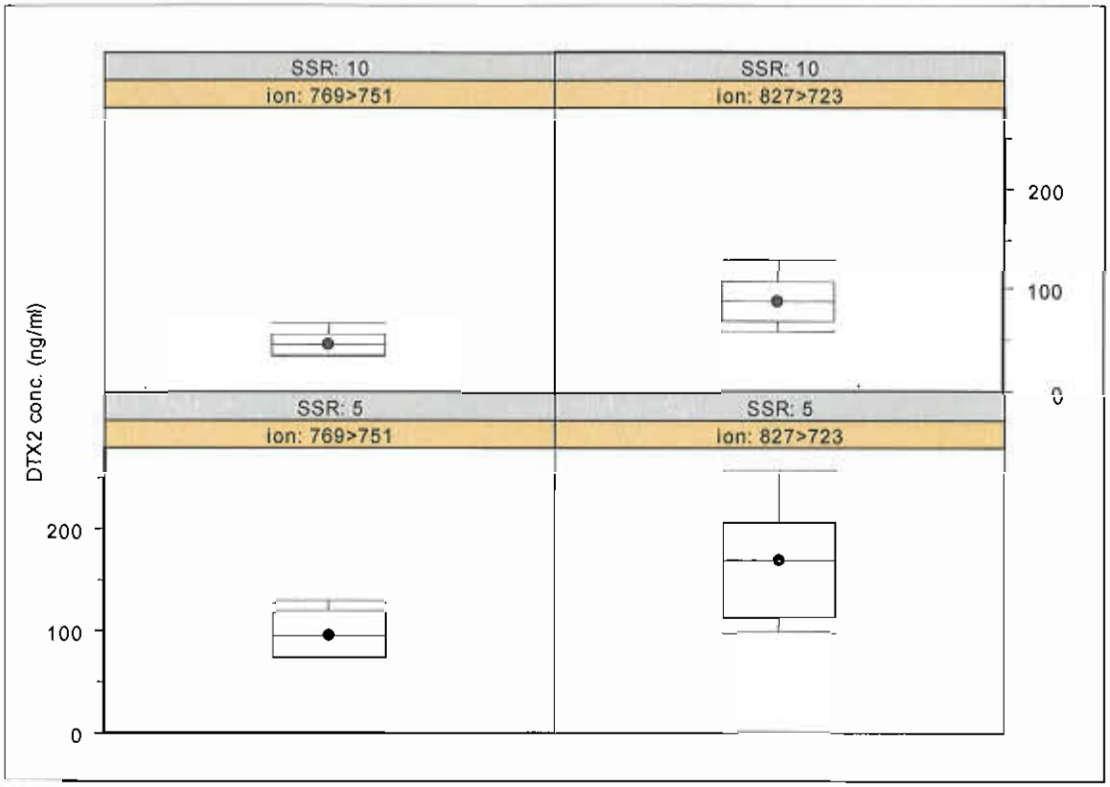

Figure AII-5: Distribution of the DTX2 results acquired in positive double transition monitoring mode

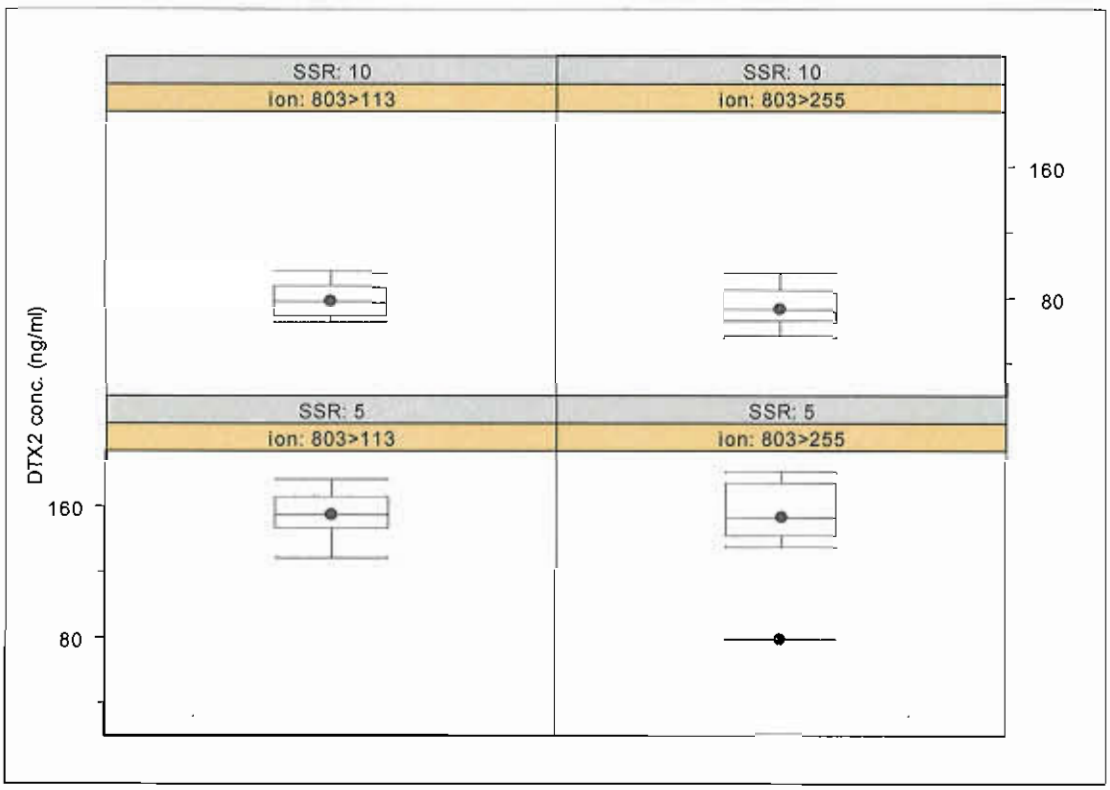

Figure AII-6: Distribution of the DTX2 results acquired in negative double transition monitoring mode

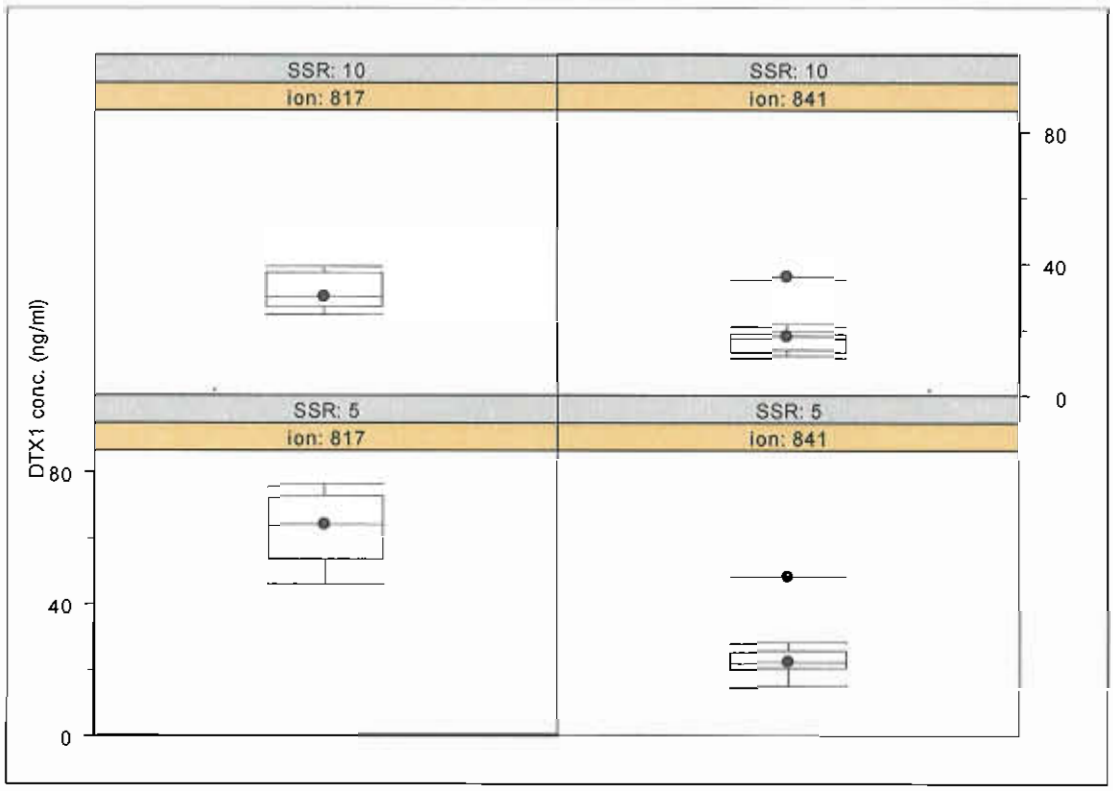

Figure AII-7: Distribution of the DTX1 results acquired in parent monitoring mode

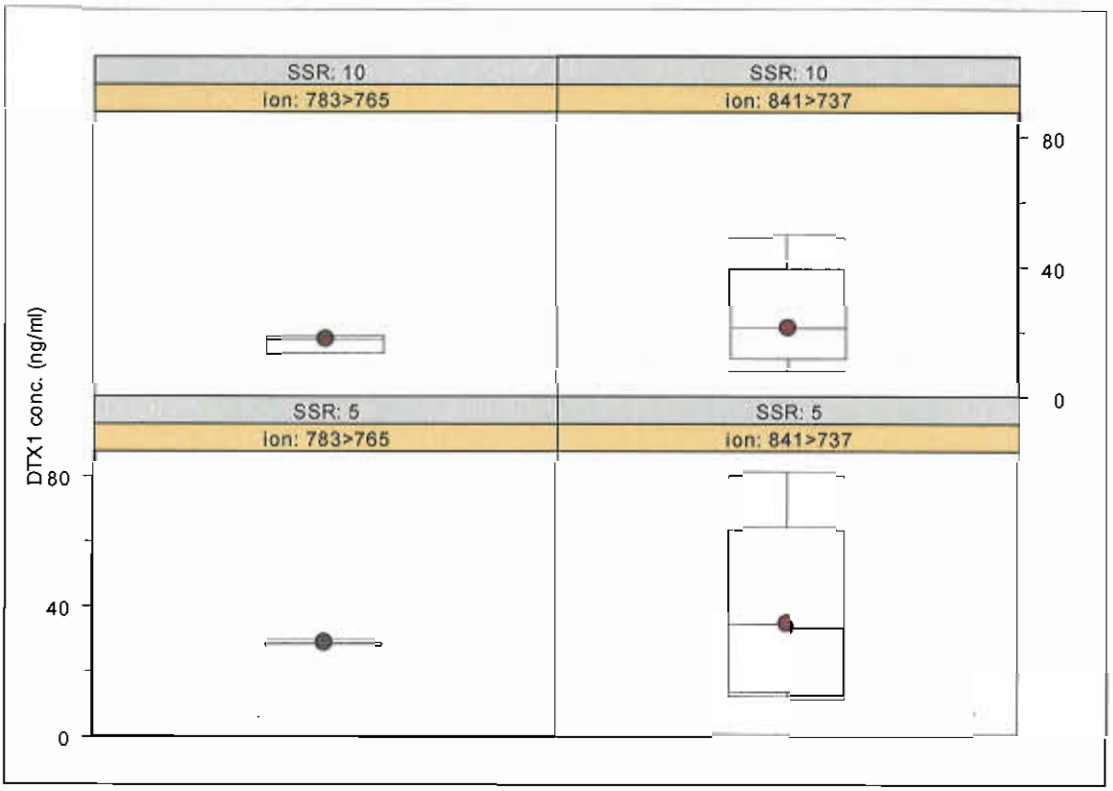

Figure AII-8: Distribution of the DTX1 results acquired in positive double transition monitoring mode

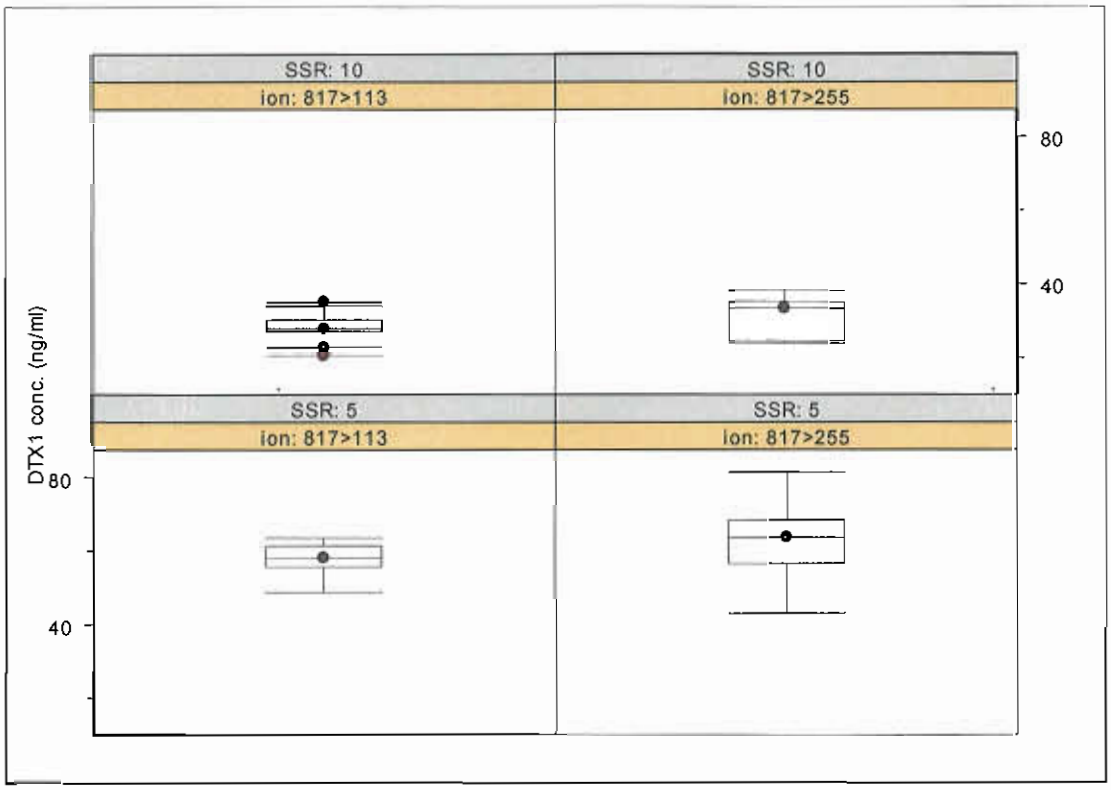

Figure AII-9: Distribution of the DTX1 results acquired in negative double transition monitoring mode

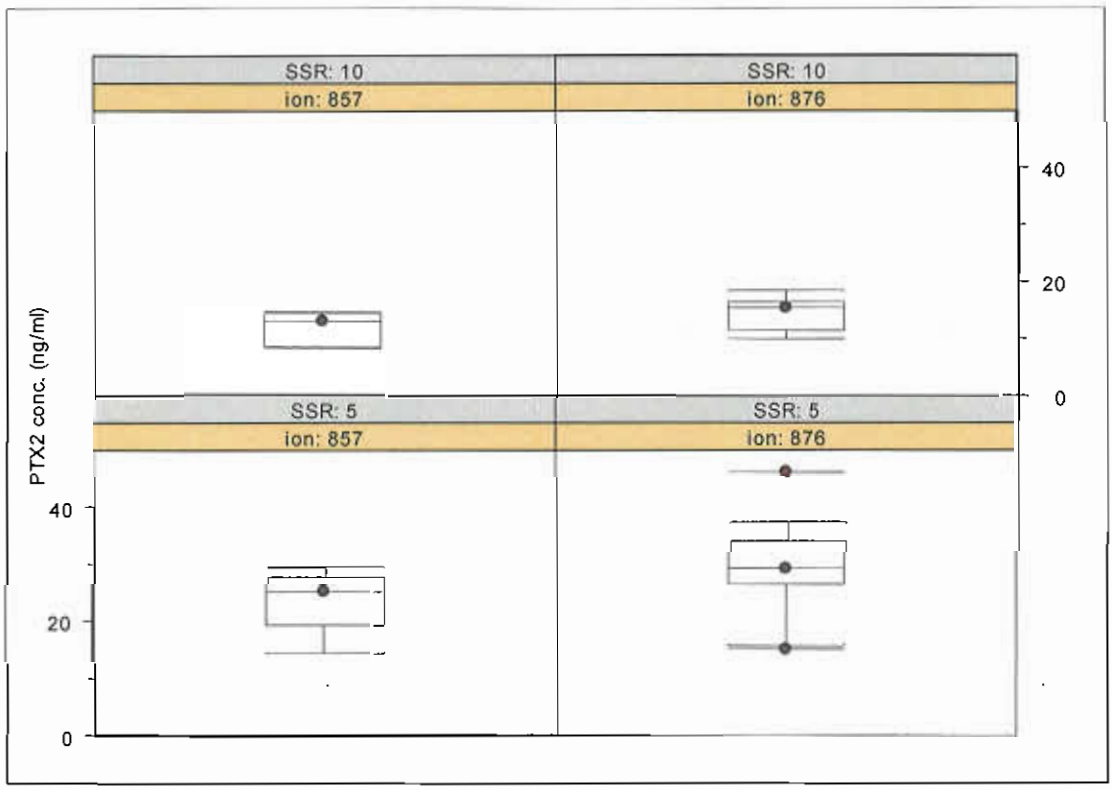

Figure AII-10: Distribution of the PTX2 results acquired in parent monitoring mode

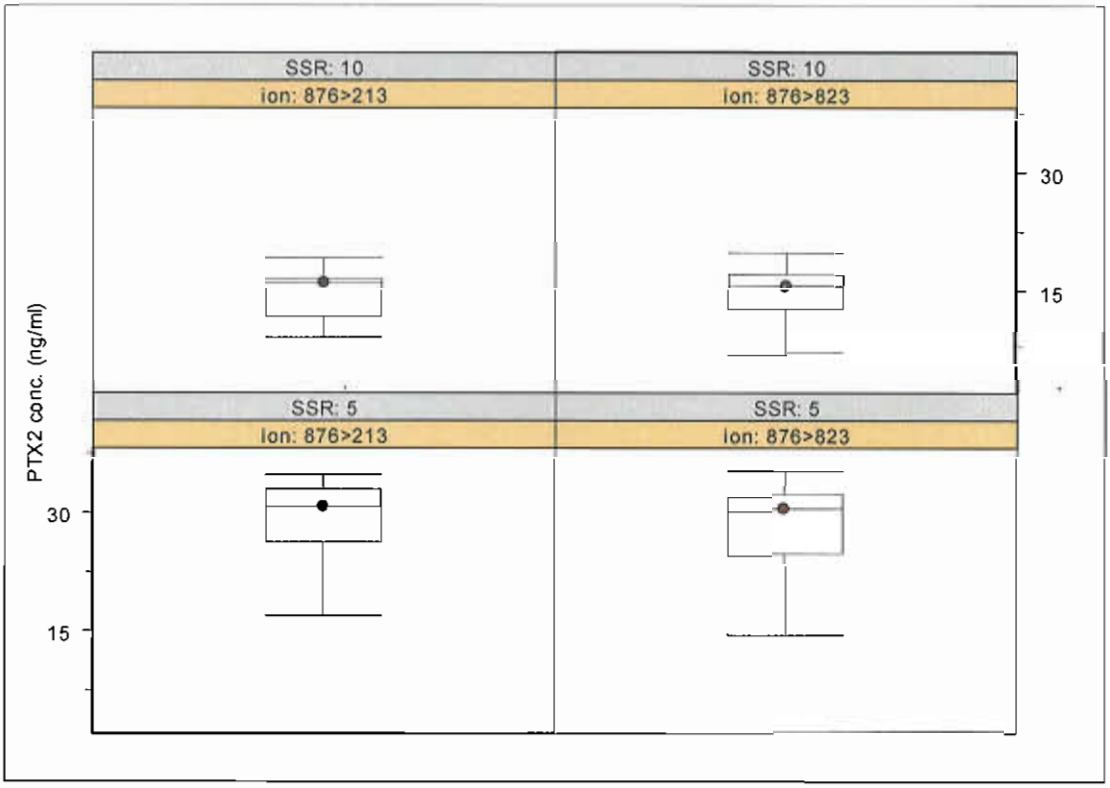

Figure A-11: Distribution of the PTX2 results acquired in positive double transition monitoring mode

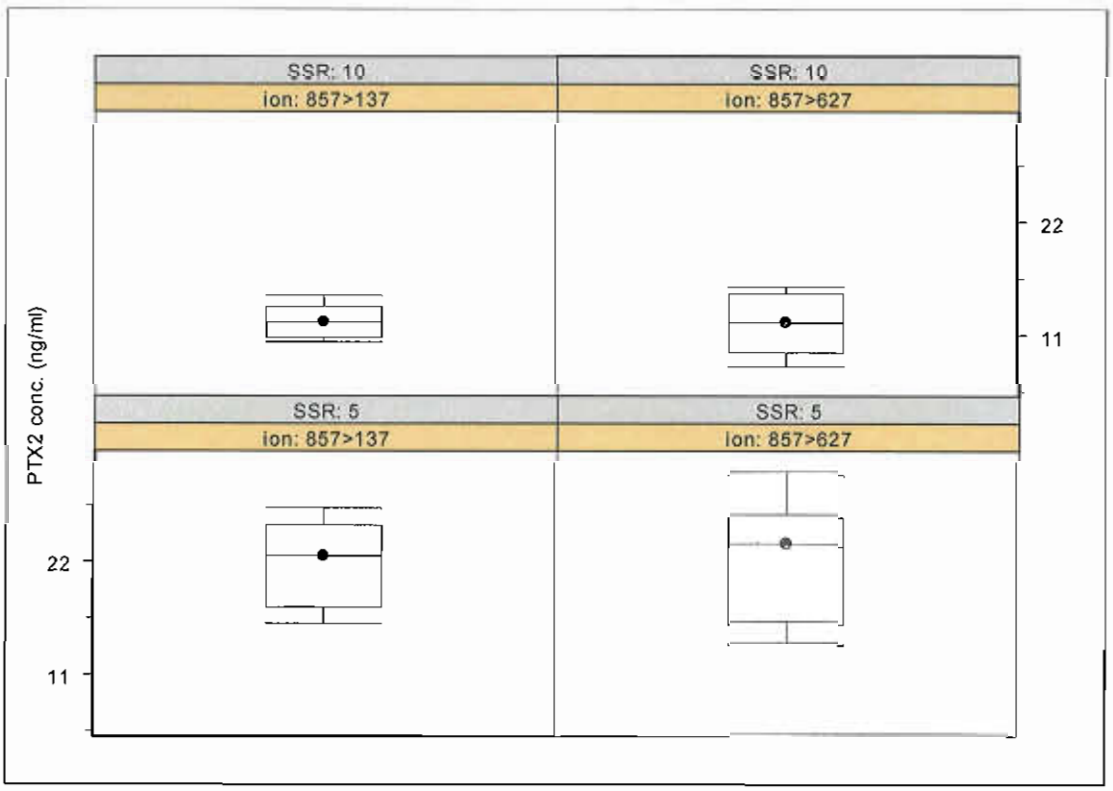

Figure A11-12: Distribution of the PTX2 results acquired in negative double transition monitoring mode

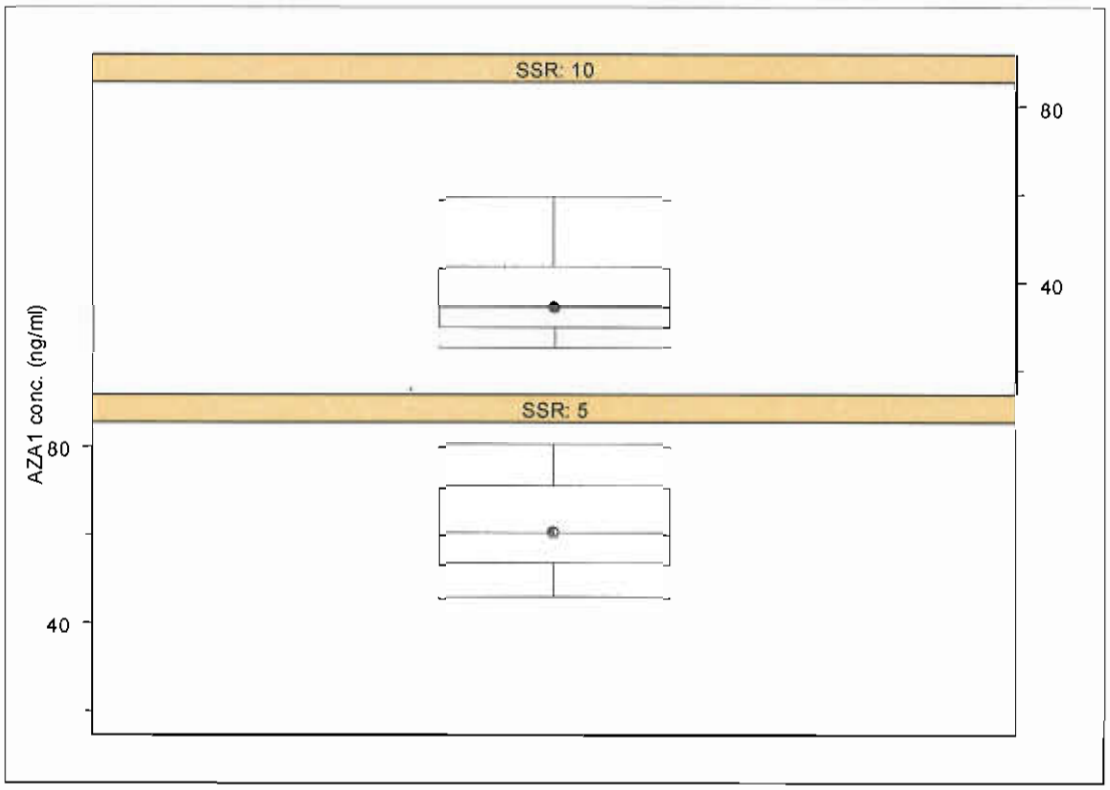

Figure AII-13: Distribution of the AZA1 results acquired in positive parent monitoring mode

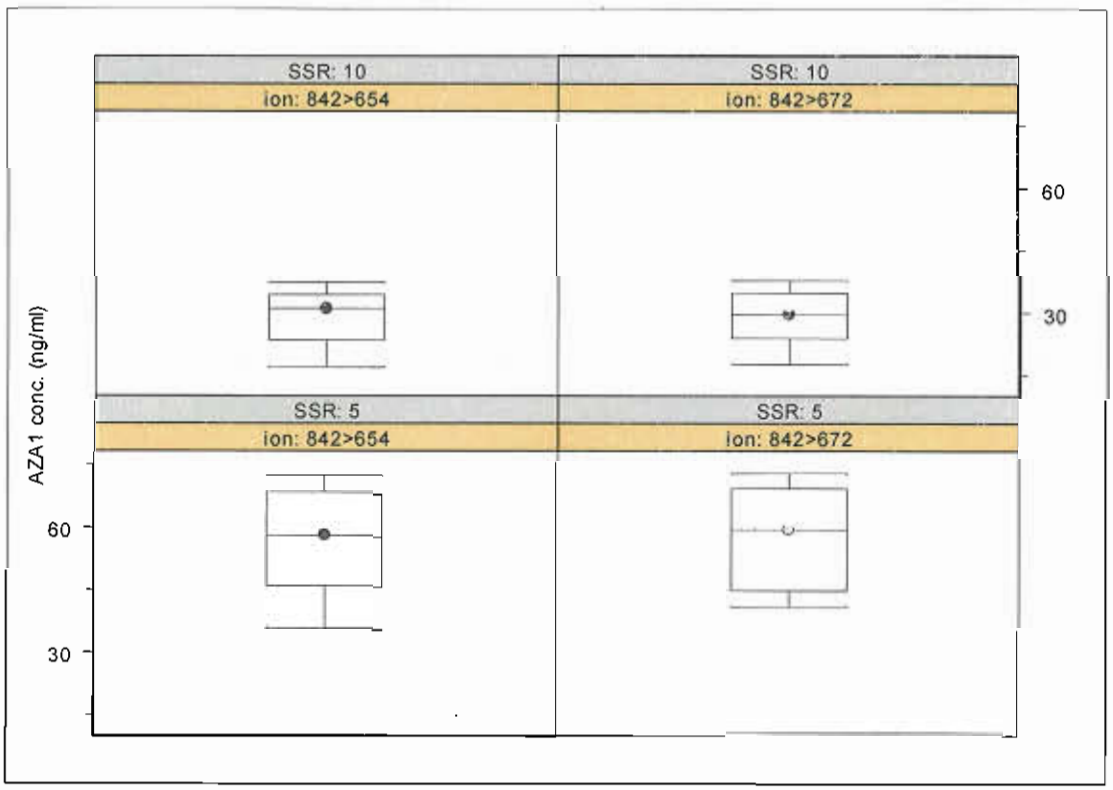

Figure AII-14: Distribution of the AZA1 results acquired in positive double transition monitoring mode

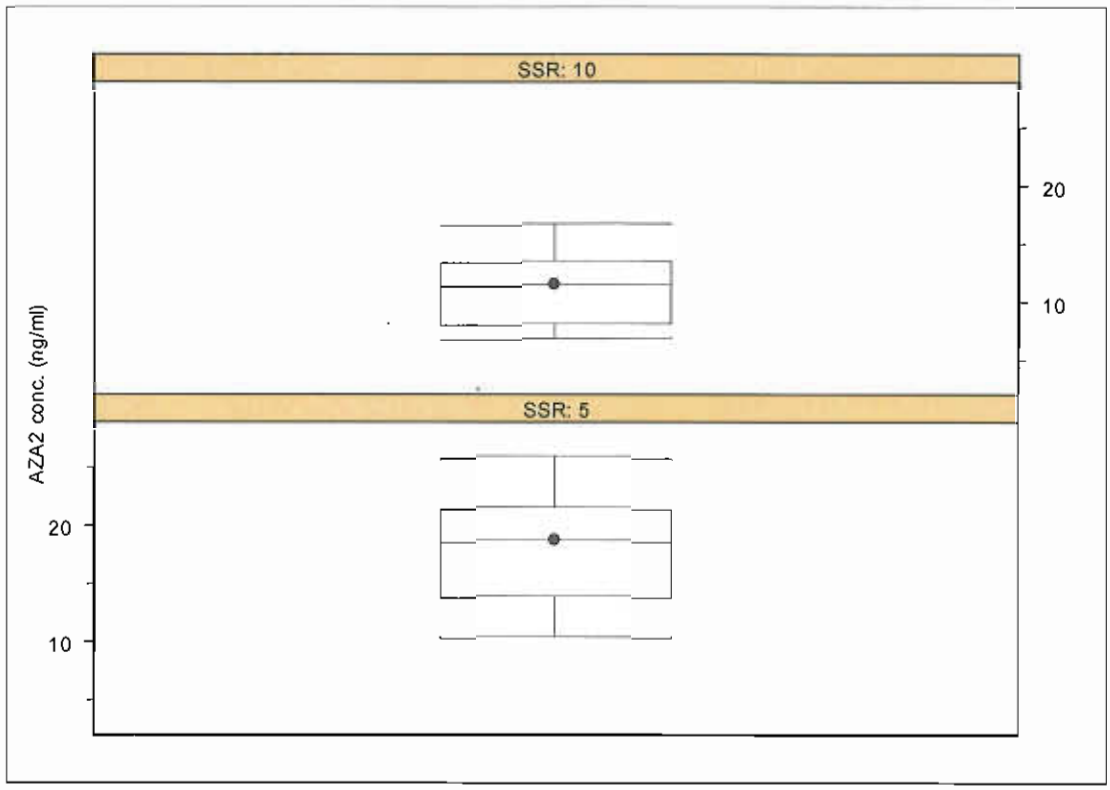

Figure AII-15: Distribution of the AZA2 results acquired in positive parent monitoring mode

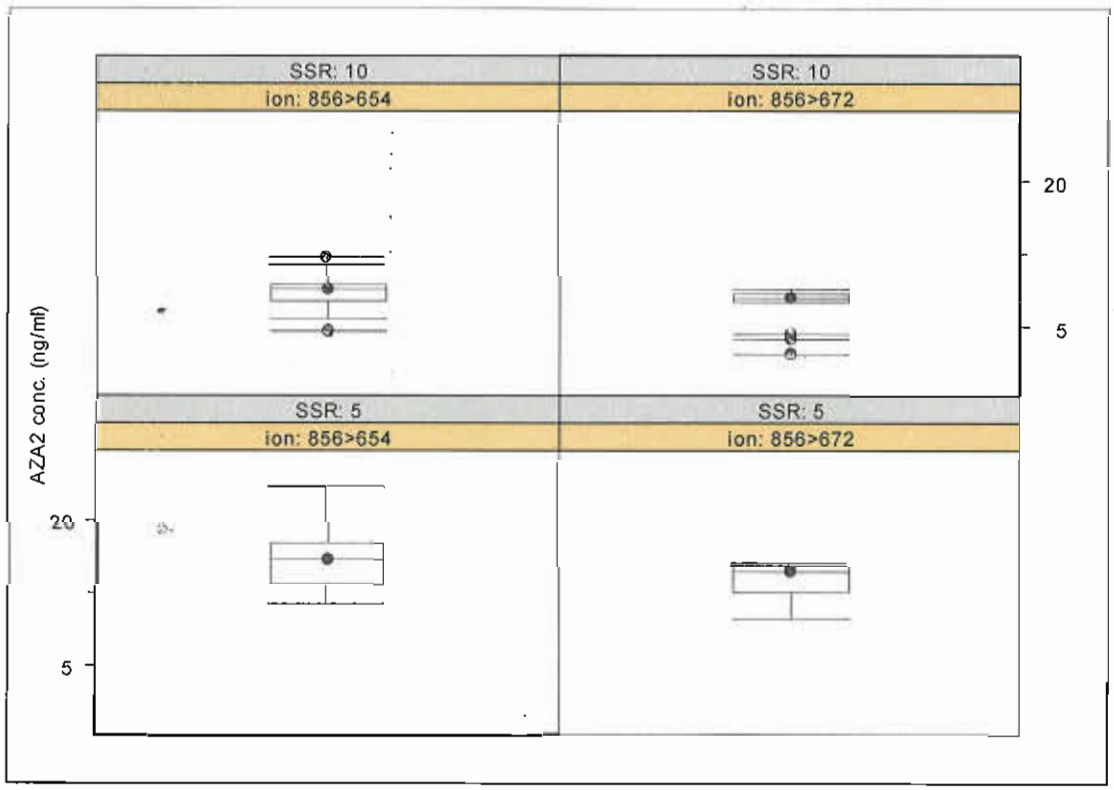

Figure AII-16: Distribution of the AZA2 results acquired in positive double transition monitoring mode

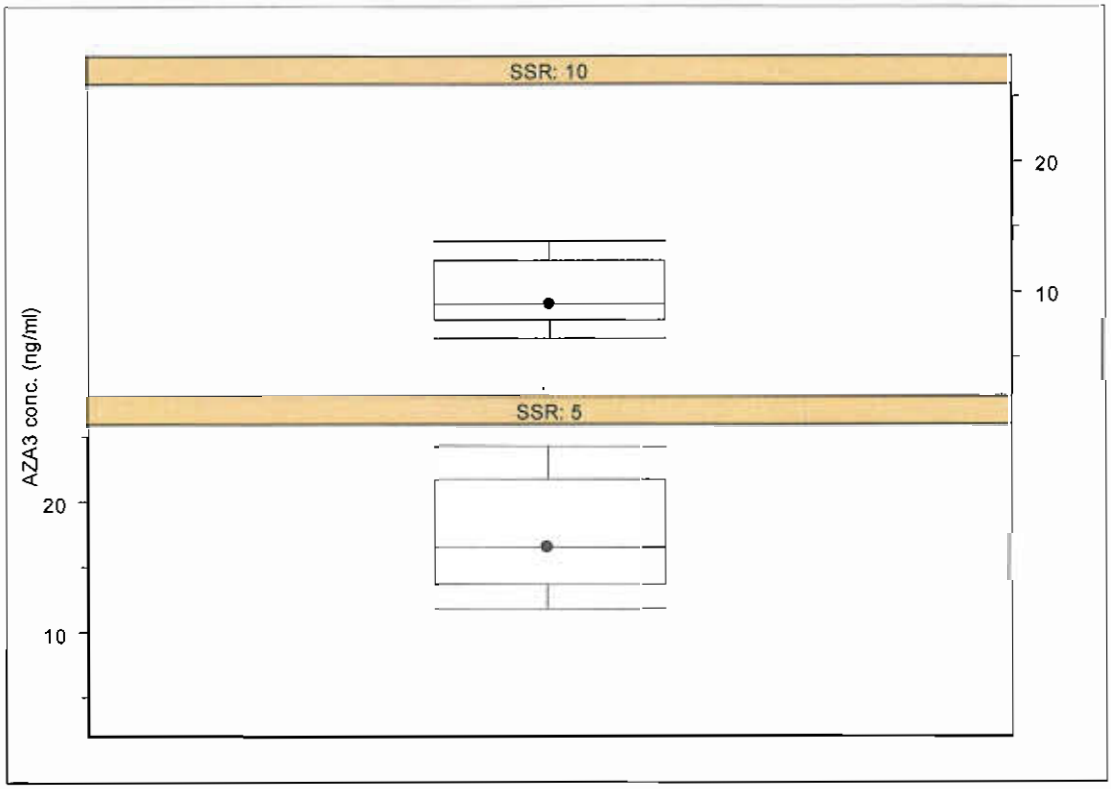

Figure AII-17: Distribution of the AZA3 results acquired in positive parent monitoring mode

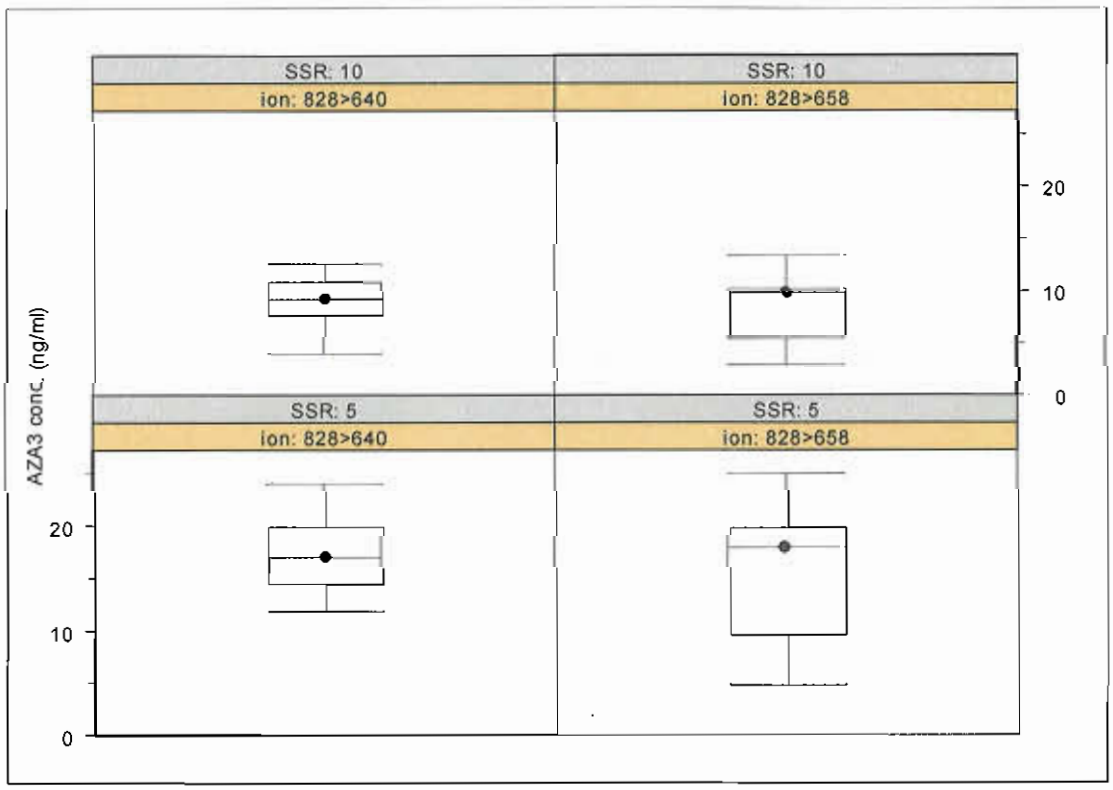

Figure AII-18: Distribution of the AZA3 results acquired in positive double transition monitoring mode