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Reference Material Development for Paralytic Shellfish Poisoning Toxins and Associated Analytical Applications

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Reference Material Development for Paralytic Shellfish Poisoning Toxins and Associated Analytical Applications.

Stephen Burrell BSc (Hons)

Thesis submitted in fulfilment of requirement leading to the award of PhD

School of Chemical and Pharmaceutical Sciences

College of Sciences and Health

Dublin Institute of Technology

Kevin Street

Dublin 8

2015

Supervisors

Dr. Andrew D. Turner

Dr. Barry Foley

ABSTRACT

Food poisoning incidences relating to marine biotoxins are a global phenomenon and have the potential to severely impact the aquaculture industry. As a result, and as a legislative requirement in the European Union (EU), many countries have implemented monitoring programmes for these compounds but their success relies on the availability of certain quality assurance tools, two of which are reference materials (RMs) and proficiency testing. The limited amounts of RMs, in particular matrix certified reference materials (CRMs) for paralytic shellfish poisoning (PSP) toxins has been a limiting factor in the implementation of alternatives to the mouse bioassay for routine monitoring programmes. Various stabilisation procedures were investigated to ascertain the applicability of each for preparing RMs for various uses including internal QA/QC, proficiency testing and as candidates for certification. The beginning of these studies coincided with a large PSP toxic event in Icelandic waters. During that period mussels from two production sites on the north and north-west coasts of Iceland accumulated PSP toxins to levels many times over the EU regulatory limit. Mussels sampled during this period were characterised and presented along with phytoplankton data from the same period and presented as a first report of PSP toxins from Icelandic waters. Large quantities of naturally contaminated mussel tissues were harvested during this period for use in these studies.

Various stabilisation procedures were investigated, such as thermal treatment, the use of preserving additives, high pressure processing (HPP) and freeze drying, for their applicability in preparing RMs for PSP toxins. Extensive characterisation of the materials was performed through homogeneity and short and long-term stability studies using two LC-FLD methods to evaluate each technique in reducing levels of degradation, biotransformations or epimerization. Freeze drying proved the most effective technique evaluated and this

procedure was used to prepare RMs in two shellfish species, Pacific oyster (*Crassostrea gigas*) and blue mussels (*Mytilus edulis*). The technique improved the stability of all toxins assessed compared to untreated controls. A successful feasibility study was carried out in a *C. gigas* matrix which resulted in the production of a medium scale RM as a candidate CRM. Certification of this material was not carried out during the course of these studies however.

Although freeze drying proved the most effective in stabilising both the tissue matrix and the PSP toxins themselves, the labour intensive nature of the procedure as well as the significant per unit production costs led to alternative RM techniques being investigated. HPP of a *C. gigas* tissue matrix was performed and it provided an extremely effective technique for stabilising the toxins through a reduction of microbial activity. Stability studies showed the technique reduces or eliminates toxin degradation and epimerisation compared to untreated control materials with the technique potentially having applications in CRM development, although a full feasibility study was not conducted.

The use of heat treatment and preserving additives provided the simplest and most cost effective stabilisation procedure investigated with both techniques, particularly when combined, improving toxin and matrix stability compared to untreated controls. Each technique was evaluated separately and combined in one tissue matrix, *M. edulis* and applications for materials prepared by this procedure were examined. Combining the use of preserving additives with a thermal pre-treatment step provided sufficiently stable and homogenous RMs which were used as an internal QA/QC tool in the Irish National Monitoring Programme (NMP) and in proficiency testing (PT) schemes operated by QUASIMEME and VEREFIN.

Data from a RM prepared by the combined techniques above and used in the Irish NMP over a two year period provided evidence for the long-term stability of the material using a

classical stability model and was used to calculate an expanded uncertainty of measurement for the method used for official control purposes at the Marine Institute.

Materials prepared by these combined techniques were also used to develop the first commercially available PT scheme for PSP toxins. Data is presented from the first six years of this development exercise from participants using a wide array of methodologies. Data showed the improvement of participants over this period but found statistical differences in the datasets of both LC-FLD methods employed by some subscribers in determining dcSTX and GTX2,3. A material was also prepared for use in a separate PT scheme operated by VEREFIN which highlights the wide applicability of these RMs.

The techniques investigated during these studies have multiple applications in method development, as QA/QC tools, in CRM preparation and in proficiency testing schemes.

DECLARATION

I certify that this thesis which I now submit for examination for the award of PhD, is entirely my own work and has not been taken from the work of others, save and to the extent that such work has been cited and acknowledged within the text of my work.

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 another award in any Institute.

The work reported on in this thesis conforms to the principles and requirements of the Institute's guidelines for ethics in research.

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Signature _____ Date _____ Candidate

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ABBREVIATIONS

AOAC	Association of Analytical Communities
ASP	amnesic shellfish poisoning
AZP	azaspiracid poisoning
AZA	azaspiracid
CEFAS	Centre for Environment, Fisheries and Aquaculture Sciences
CRL	community reference laboratory
CRM	certified reference material
CWC	chemical weapons convention
DA	domoic acid
DAD	diode array detection
DI	deionised
DIT	Dublin Institute of Technology
DSP	Diarrhetic shellfish poisoning
DTX	dinophysistoxin
EFSA	European Food Standards Agency
ELISA	enzyme linked immuno sorbent assay
EU	European Union
FAO	Food and Agricultural Organisation
FLD	fluorescence detector
GTX	gonyautoxins
HABs	harmful algal blooms
HCl	hydrochloric acid
HNO_3	nitric acid
H_3PO_4	phosphoric acid
HILIC	hydrophilic interaction liquid chromatography
H_5IO_6	periodic acid
H_2O_2	hydrogen peroxide
HOAc	acetic acid
HP	hepatopancreas
HPLC	high performance liquid chromatography
HPP	high pressure processing
ILRM	interlaboratory reference material
IOC	Intergovernmental Oceanographic Commission
IPCS	International Programme on Chemical Safety
IRMM	Institute for Reference Materials and Measurement
ISO	International Standards Organisation
KFT	Karl Fischer titration
LC-FLD	liquid chromatography – fluorescence detection
LC-MS	liquid chromatography – mass spectrometry
LC-MS/MS	liquid chromatography tandem mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
LRM	laboratory reference material
MBA	mouse bioassay
MC	moisture content
MeOH	methanol
MI	Marine Institute
NaCl	sodium chloride

NaOH	sodium hydroxide
NEO	neosaxitoxin
NMP	national monitoring programme
NRCC	National Research Council Canada
NSP	neurotoxic shellfish poisoning
OA	okadaic acid
PCOX	post-column oxidation
preCOX	pre-column oxidation
PSP	paralytic shellfish poisoning
PST	paralytic shellfish toxin
PT	proficiency testing
PTX	pectenotoxin
QA	quality assurance
QC	quality control
QUASIMEME	Quality Assurance of Information for Marine Environmental Monitoring in Europe
QA	quality assurance
RM	reference material
SD	standard deviation
SOP	standard operating procedure
SPE	solid phase extraction
STX	saxitoxin
TEF	toxicity equivalency factor
UV	ultraviolet
VEREFIN	Finnish Institute for Verification of the Chemical Weapons Convention
WF	whole flesh
WHO	World Health Organisation
YTX	yessotoxin

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BACKGROUND AND OBJECTIVES

1.1 HARMFUL ALGAL BLOOMS, BIOTOXINS AND SHELLFISH POISONING

Phytoplankton are microscopic, single-celled plants that can grow in both marine and freshwater environments, providing a critical food supply for filter feeding bivalve shellfish such as mussels, oysters, clams and scallops. There are approximately 5000 known species of marine phytoplankton, a small proportion of which, less than 2%, can produce toxins that can find their way through the food chain to humans [1]. Dense concentrations of these cells can manifest as Harmful Algal Blooms (HABs), frequently referred to as "red tides".

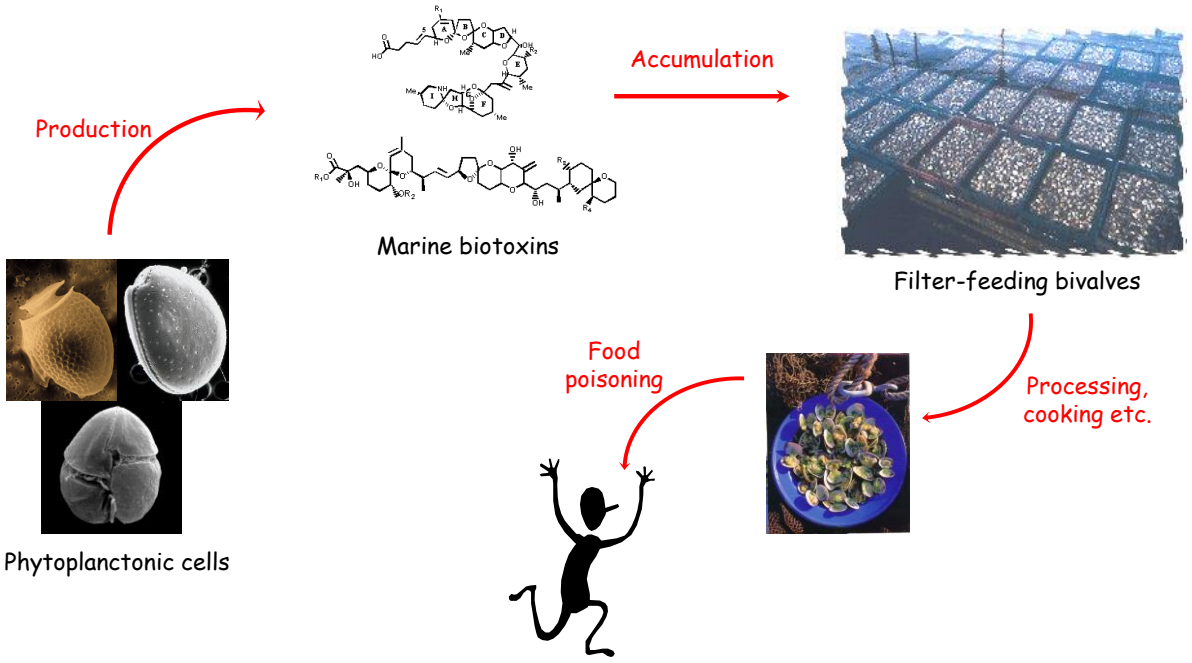


Figure 1-1: From phytoplankton to food poisoning.

Phytoplankton are a vital food source for filter feeding bivalve shellfish such as mussels, oysters, clams and scallops. Biotoxins can accumulate in the flesh of shellfish through filter feeding of toxin producing phytoplankton species, with the highest toxin concentrations generally found in the digestive gland (hepatopancreas) of the shellfish (Figure 1-2) [2].

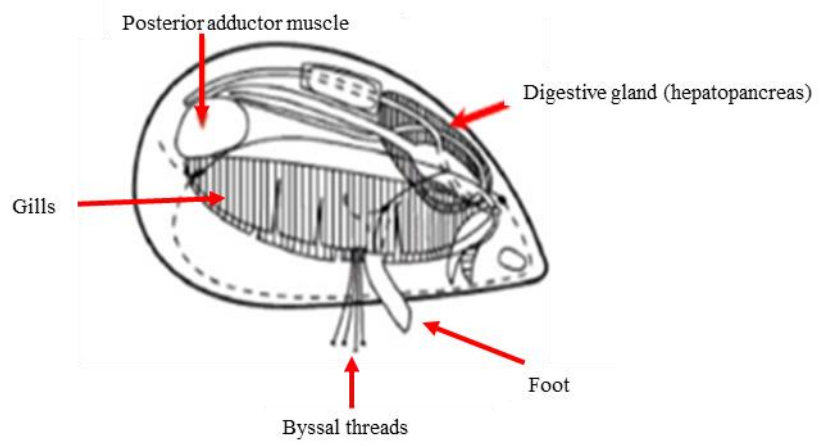


Figure 1-2: Internal anatomy of a mussel.

There are approximately 2000 cases of human poisoning reported each year through the consumption of fish and shellfish and results in an approximate 15% mortality rate. Impacts on public health and potential economic damage through reduced consumption and exportation can therefore be extensive [3]. Whilst the shellfish generally remain unaffected following accumulation of these phycotoxins, human consumption of contaminated seafood products can lead to a variety of symptoms experienced after as little as 30 minutes, and can include nausea, vomiting, abdominal pain, diarrhoea, memory loss, paralysis and in extreme cases death.

There are 5 internationally recognised syndromes of shellfish poisoning, namely:

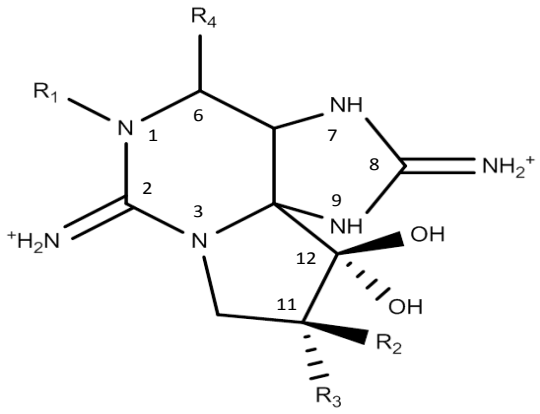
- Paralytic Shellfish Poisoning (PSP)
- Amnesiac Shellfish Poisoning (ASP)
- Diarrhetic Shellfish Poisoning (DSP)
- Azaspiracid Shellfish Poisoning (AZP) and
- Neurotoxic Shellfish Poisoning (NSP)

The research undertaken in these studies was on the PSP group of biotoxins.

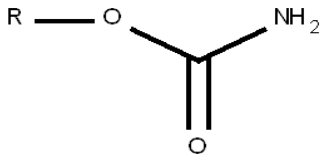
1.2 HYDROPHILIC BIOTOXINS

1.2.1 PARALYTIC SHELLFISH POISONING (PSP)

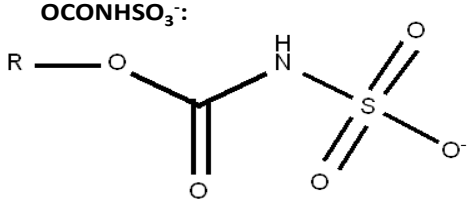
PSP is caused by a group of closely related compounds all based on a tetrahydropurine skeleton (Figure 1-3), with saxitoxin (STX) being the parent toxin and the first to be isolated in this group [4,5].



OCONH$_2$:



OCONHSO$_3^-$:



Analogue	R_1	R_2	R_3	R_4
STX	H	H	H	$OCONH_2$
NEO	OH	H	H	$OCONH_2$
GTX1	OH	OSO_3^-	H	$OCONH_2$
GTX2	H	OSO_3^-	H	$OCONH_2$
GTX3	H	H	OSO_3^-	$OCONH_2$
GTX4	OH	H	OSO_3^-	$OCONH_2$
GTX5 (B1)	H	H	H	$OCONHSO_3^-$
GTX6 (B2)	OH	H	H	$OCONHSO_3^-$
C1	H	OSO_3^-	H	$OCONHSO_3^-$
C2	H	H	OSO_3^-	$OCONHSO_3^-$
C3	OH	OSO_3^-	H	$OCONHSO_3^-$
C4	OH	H	OSO_3^-	$OCONHSO_3^-$
dcSTX	H	H	H	OH
dcNEO	OH	H	H	OH
dcGTX1	OH	OSO_3^-	H	OH
dcGTX2	H	OSO_3^-	H	OH
dcGTX3	H	H	OSO_3^-	OH
dcGTX4	OH	H	OSO_3^-	OH
doSTX	H	H	H	H
doGTX2	H	H	OSO_3^-	H
doGTX3	H	OSO_3^-	H	H
M1α	H	H	OH	$OCONHSO_3^-$
M1β	H	OH	H	$OCONHSO_3^-$
M2α	H	H	OH	$OCONH_2$
M2β	H	OH	H	$OCONH_2$
M3	H	OH	OH	$OCONHSO_3^-$
M4	H	OH	OH	$OCONH_2$

Figure 1-3: Structure of the principal PSP toxins.

These potent neurotoxins are mainly produced by marine dinoflagellates, in particular, *Alexandrium* spp. (Figure 1-4), *Gymnodinium catenatum* and *Pyrodinium bahamense* var. *compressum* [6] but have also been found to be produced by some freshwater cyanobacteria [7,8].

Saxitoxin and its congeners differ substantially in their toxicity (Table 1-1) and can be categorised into several subgroups based on their substituent sidechains, the most important of which are the carbamoyl, decarbamoyl and N-sulfo-carbamoyl groups (Figure 1-3). Saxitoxin is an inhibitory neurotoxin which has a high affinity for binding site 1 of the voltage-gated sodium ion channel in mammalian nerve cells, thus inhibiting conductance of signals along the neuron [9]. Depending on the dose, symptoms can include tingling sensation or numbness around the lips, face, neck and extremities, headache, dizziness, nausea, vomiting, diarrhoea, muscular paralysis, respiratory difficulties and in severe cases death through respiratory paralysis [10]. Shellfish contaminated with PSP toxins pose severe risks to human consumers and numerous accounts of intoxications leading to illness or death have been recorded from around the world [11–15].

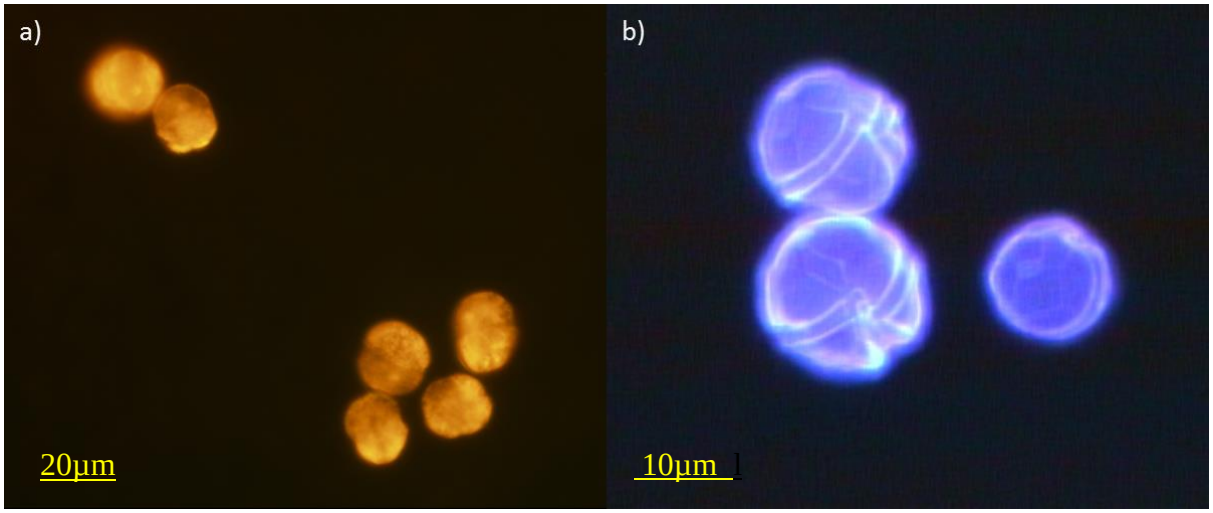


Figure 1-4: Alexandrium tamarense using a) fluorescence in situ hybridisation (FISH) probes and b) calcofluor white staining using a compound microscope with epi-flourescence microscopy (Olympus BX53, 100Mag).

STX comprises a 3,4-propinoperhydropurine tricyclic system together with two guanidino functional groups, which account for its high polarity and is soluble in water, methanol and ethanol but not in other organic solvents [4]. Paralytic Shellfish Toxins (PSTs) are prone to pH dependent conversions, are heat stable under mildly acidic conditions with the exception of the N-sulfo-carbamoyl toxins and are readily oxidised under alkaline conditions [16]. The N-sulfo-carbamoyl toxins such as GTX5 and C1,2 may be partially converted to the corresponding carbamate group toxins through hydrolysis if heated at low pH. These conversions can take place when PSTs are boiled with a strong acid such as HCl and can result in STX analogues with lower toxicity factors being chemically converted into ones with higher toxicities. Conversions do not take place in weak acids such as acetic acid even under boiling conditions [6].

Table 1-1: Toxicity equivalency factors (TEFs) of the principal PSP toxins.

Toxin	**Toxicity Equivalency Factors**	
	Oshima[a]	**EFSA[b]**
STX	1.00	1.00
GTX2	0.36	0.40
GTX3	0.64	0.60
NEO	0.92	1.00
GTX1	0.99	1.00
GTX4	0.73	0.70
GTX5	0.06	0.10
C1	0.01	-
C2	0.10	0.10
C3	0.01	-
C4	0.06	0.10
GTX6	0.06	0.10
dcSTX	0.51	1.00
dcGTX2	0.15	0.20
dcGTX3	0.38	0.40
dcNEO	-	0.40
11-hydroxy-STX	-	0.30

a TEFs calculated based on relative potency data by Oshima *et al.* [17].
b TEFs proposed by the CONTAM panel (EFSA) [6].

STX-group toxins, also referred to as PSTs are not detectable by either UV or fluorescence owing to the lack of a chromophore. They therefore require alkaline oxidation into fluorescent imino purine derivatives for detection [6].

The first recorded fatal incidence of shellfish poisoning occurred in 1793 during Captain George Vancouver's expedition into British Columbia. Upon landing in an area now known as Poison Cove, shellfish were collected by some of his crew for breakfast. This toxic banquet resulted in the death of one crew member five and a half hours after ingestion and severe PSP like symptoms experienced by four others; although they subsequently made a full recovery [18]. STX producing algae have been found to occur worldwide in both tropical and moderate climate zones [6], with detection of phytoplankton and contaminated shellfish tissues confirmed throughout regions of Europe, the Americas, Asia, Africa and Australasia.

The EU regulatory limit for PSTs is set at 800 micrograms per kilogram (800 µg/kg) [19] and the reference method is the mouse bioassay (MBA) [20]. A named alternative to the MBA, the so called "Lawrence method", was first cited in EU legislation in 2006 [21] which is based on high performance liquid chromatography with fluorescence detection (LC-FLD).

1.2.2 AMNESIC SHELLFISH POISONING (ASP)

Domoic acid (DA) (Figure 1-5), the amnesic shellfish poisoning toxin, belongs to a group of amino acids called the kainoids. They are classified as neuroexcitants or excitotoxins as they interfere with neurotransmission mechanisms in the brain [22].

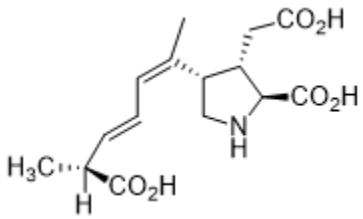


Figure 1-5: Structure of domoic acid.

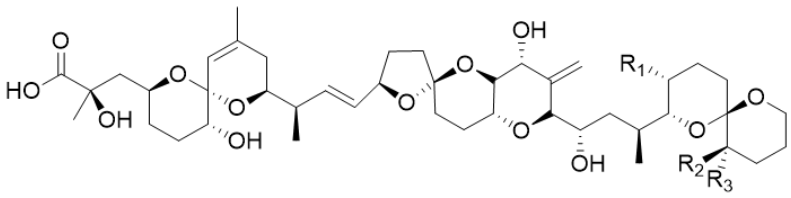
The toxin can be accumulated in shellfish feeding on a number of toxic *Pseudo-nitzschia* diatom species. Consumption of shellfish contaminated with ASP toxins can lead to intoxication with symptoms including, abdominal cramps, nausea, vomiting, diarrhoea, decreased reaction to deep pain, disorientation, hallucinations, confusion, short-term memory loss (amnesia) and seizures [23].

The EU regulatory limit is set at 20 milligrams of domoic acid per kilogram (20 mg/kg) [19] and the reference method [20] is based on that published by Quilliam *et. al.*, in 1995 for the quantitative determination of domoic acid in unsalted seafood [24].

1.3 LIPOPHILIC BIOTOXINS

1.3.1 DIARRHETIC SHELLFISH POISONING (DSP)

The class of toxins causing DSP includes okadaic acid (OA) and the dinophysistoxins DTX1, DTX2 and DTX3 (Figure 1-6). The first documented occurrence of DSP was in 1976 in Japan where numerous intoxications were reported [25].



Toxin	R_1	R_2	R_3
OA	CH_3	H	H
DTX1	CH_3	CH_3	H
DTX2	H	H	CH_3

Figure 1-6: Structure of the OA group toxins.

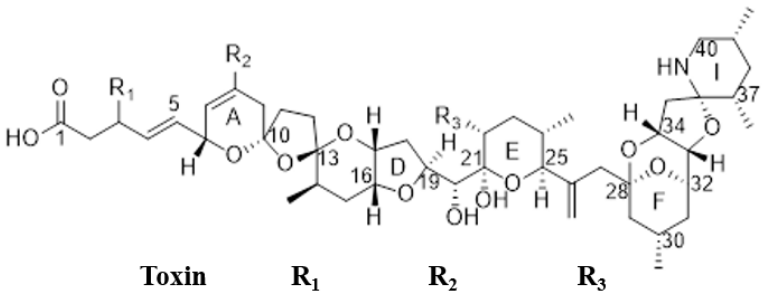
DSP is caused by certain *Dinophysis* and *Prorocentrum* species and has a worldwide geographical distribution [2]. No deaths have ever been attributed to DSP with patients

generally recovering within three days from symptoms which may include nausea, vomiting, diarrhoea and abdominal cramps [3]. In addition the polyether toxins OA and DTX1 may promote stomach tumours [26].

For many years, the EU reference method for the regulatory testing of DSP toxins was a qualitative rodent-based assay [19,20], usually implemented as a mouse bioassay (MBA). As of the 1st January 2015 the EU reference method was changed from the MBA to a liquid chromatography-mass spectrometry (LC-MS/MS) method as described by the EU- reference laboratory for marine biotoxins (EU-RLMB) [27]. The method must be capable of detecting at least OA, DTX1, DTX2, DTX3 and their esters and the EU regulatory limit is set at 160 micrograms of okadaic acid equivalents per kilogram (160 µg/kg) [19].

1.3.2 AZASPIRACID SHELLFISH POISONING (AZP)

Azaspiracids (AZAs)(Figure 1-7) are a recently discovered class of shellfish toxin, first implicated in human poisoning in 1995 in the Netherlands through consumption of mussels originating from Killary Harbour, Ireland [28]. Seven official poisoning incidents attributed to AZP have been reported to date with all originating from shellfish harvested in Ireland [29].



Toxin	R_1	R_2	R_3
AZA1	H	H	CH_3
AZA2	H	CH_3	CH_3
AZA3	H	H	H

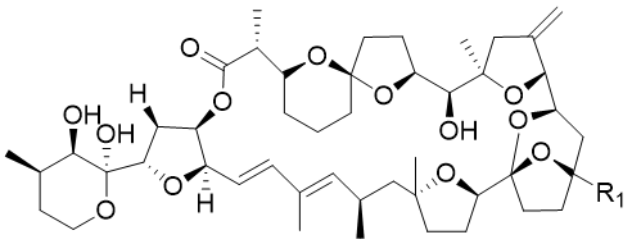
Figure 1-7: Structure of the AZA group toxins.

The first causative organism of AZP was only recently discovered in 2009 and named *Azadinium spinosum* [30], with many more *Azadinium* and related species being subsequently discovered [31]. Symptoms are similar to those described for DSP and include nausea, vomiting, abdominal cramps and diarrhoea and no deaths have been attributed to AZP [28].

As in the case of the DSP group toxins described above, the reference method in the EU for AZAs is the EU-RLMB LC-MS/MS method which must be capable of detecting at least AZA1, AZA2 and AZA3 [27]. The regulatory limit in the EU is 160 micrograms of azaspiracid equivalents per kilogram (160 µg/kg) [19].

1.3.3 YESSOTOXINS (YTXS) AND PECTENOTOXINS (PTXS)

Neither YTX nor PTX toxins have been implicated in shellfish poisoning in humans to date and there has been much debate on the toxicity of both groups with evidence showing PTXs (Figure 1-8) to be of no risk to humans [32] and the oral toxicity of YTXs (Figure 1-9) to be extremely low compared to its intraperitoneal toxicity [33].



Toxin	R_1
PTX1	CH_2OH
PTX2	CH_3

Figure 1-8: Structure of the PTX group toxins.

Both groups are named after the genus of scallop they were first isolated from, *Patinopecten yessoensis* [34,35] and the reference method in the EU for their detection is the EU-RLMB LC-MS/MS method described above [27].

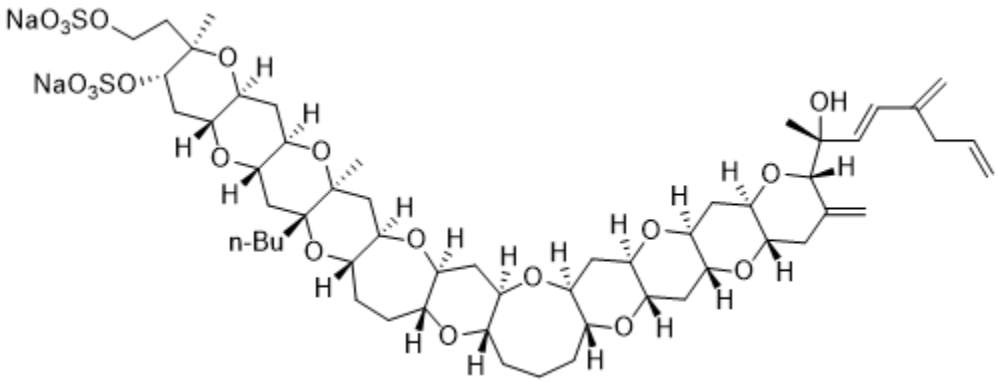


Figure 1-9: Structure of yessotoxin.

The regulatory limit in the EU is set at 160 micrograms of okadaic acid equivalents per kilogram for PTXs (160 µg/kg) [19]; owing to their general co-occurrence with DSP group toxins, and at 3.75 milligrams of YTX equivalents per kilogram (3.75 mg/kg) [36]. The causative organisms of PTXs are *Dinophysis* and *Protoperidinium* species and YTXs are caused by *Lingulodinium polyedrum* and *Protoceratium reticulatum* [34,35].

1.4 IRISH SHELLFISH INDUSTRY AND IMPACTS OF HABS

Azaspiracids are the most problematic toxin group in terms of farm closures for the Irish shellfish industry and they have been detected every year since the inception of the biotoxin monitoring programme in 2001 (Figure 1-10) [31]. Shellfish farm closures have occurred every year except 2004, due to AZP toxicity over the EU regulatory limit. Toxicity typically occurs in the mid to late summer months with those sites affected sometimes remaining closed for long periods due to the apparent slow depuration rates of these toxins from shellfish [37]. Ireland is the worst affected country for AZAs with Norway and the UK being the only other countries to report AZA levels over the EU regulatory limit. There have been seven official poisoning incidents associated with AZA toxins reported to date but numerous others have also been linked to this toxin group, with the source of all incidents traced back to shellfish harvested in Ireland [29].

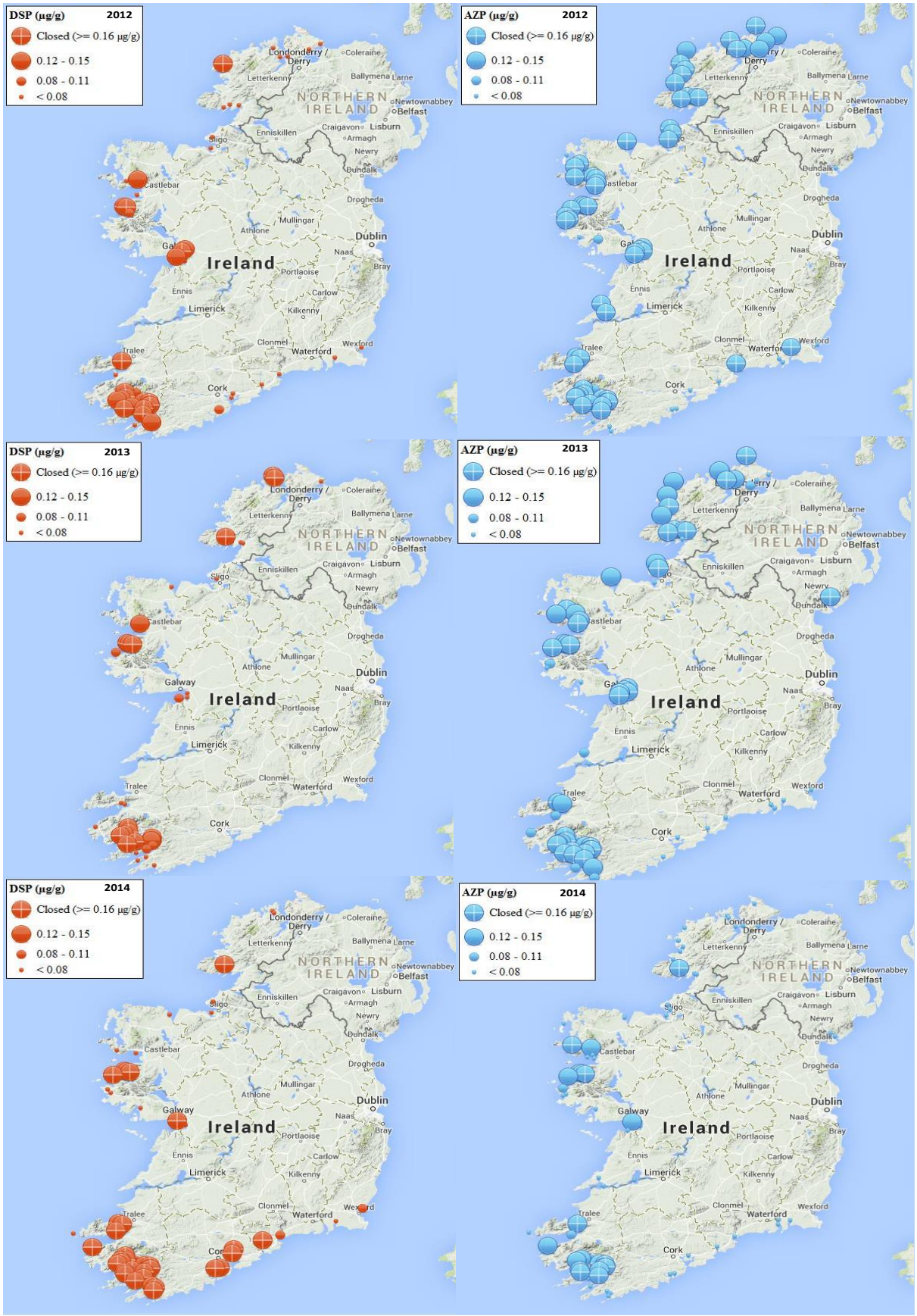


Figure 1-10: Shellfish farm closures from 2012-2014 caused by DSP and AZP toxins.

DSP is caused by the OA-group toxins and they represent the next most problematic for the Irish shellfish industry causing significant economic hardship to shellfish farmers due to toxin related farm closures. They have been detected in various shellfish species in Ireland since 2002. Toxicity typically occurs during the early to mid-summer months from *Dinophysis acuta* or *Dinophysis acuminata* blooms producing OA and DTX2 predominantly [38]. Depuration rates of OA-group toxins are faster than AZAs but both toxin groups regularly co-occur in Irish shellfish which compounds the problem for farmers [38]. PTXs have only recently been detected in Irish shellfish (since 2014), although regular monitoring of this toxin group has only been in effect since 2011. YTXs have never been detected in any Irish shellfish species to date.

Another problematic toxin group, although impacting far less is ASP caused by DA. Until recently scallops were the only species to be monitored year round for DA with levels in the main edible parts (posterior adductor muscle and gonad) rarely rising above the regulatory limit of 20 mg/kg, although it takes a long time for DA to depurate from scallops [39]. During large blooms of the causative organism, *Pseudo-nitzchias*, other species are regularly tested for this toxin group. Levels in these other species, mainly mussels can rise many times above the regulatory limit within a couple of weeks but fortunately toxicity quickly subsides due to apparently quick depuration rates.

PSP toxins have not been problematic for the Irish shellfish industry to date with only one location in the South of the country, Cork Harbour experiencing shellfish harvesting area closures as a result of PSTs over the EU regulatory limit of 800 µg/kg STX equivalents. Monitoring of PSP was carried out using the MBA [40] until 2011 at the Marine Institute (MI), after which a pre-column oxidation (preCOX) liquid chromatography with fluorescence detection (LC-FLD) method also called the “Lawrence method” resulting in AOAC Official Method (OM) 2005.06 was used [41].

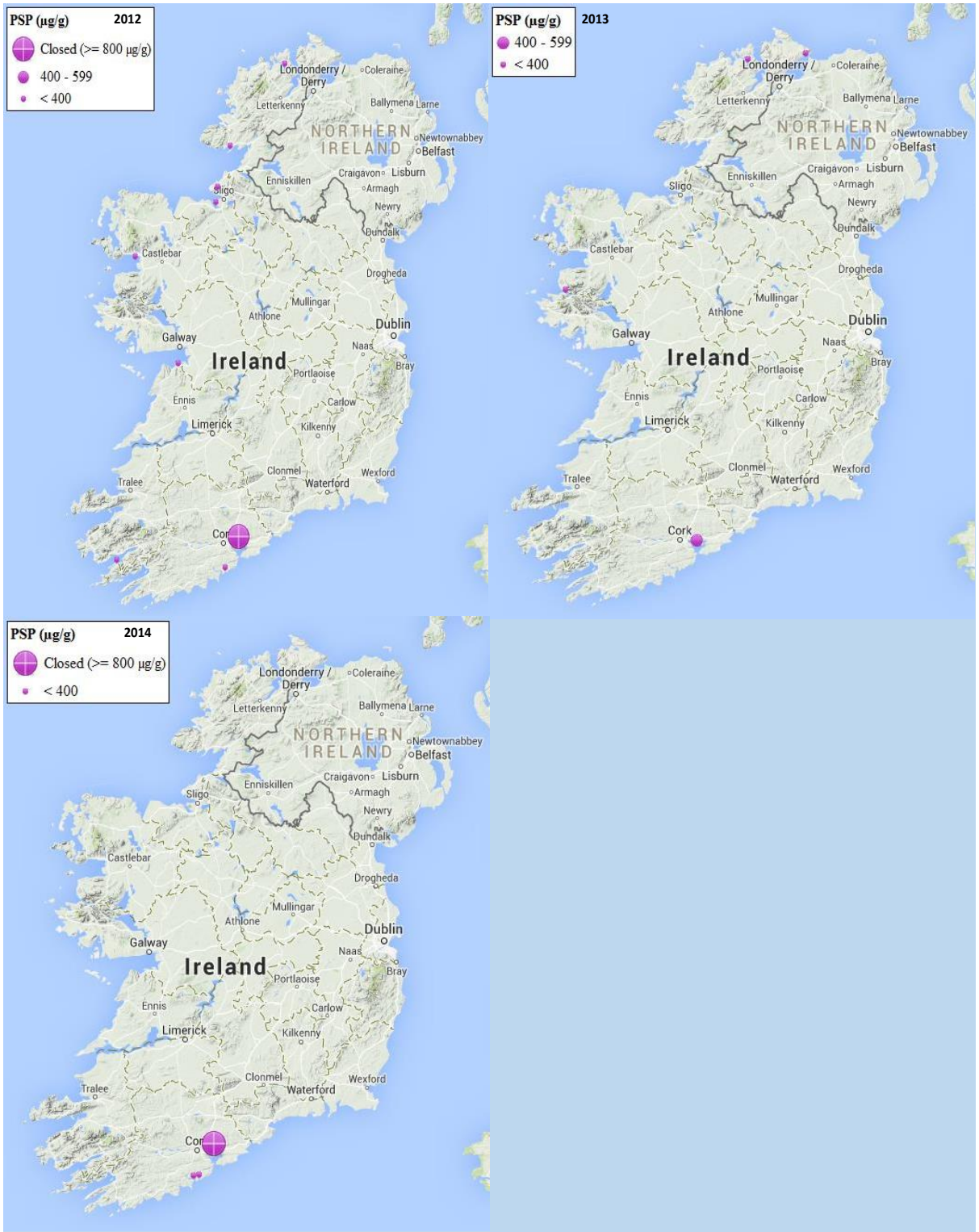


Figure 1-11: Locations where PSP toxins have been detected in shellfish 2012-2014.

Since the implementation of this method PSTs have been detected, albeit at low levels, in other locations along the west coast of Ireland (Figure 1-11). It is unclear whether the occurrence of these toxins in other locations is due to the spread of the toxin producing algal

species or, as is more likely, their detection is due to the improved sensitivity the preCOX LC-FLD method provides in comparison with the PSP MBA. The causative organism for PSP toxins in Irish waters are the *Alexandrium* species *tamarense* and *minutum*, which produce a toxin profile consisting predominantly of the gonyautoxins 2 & 3 (GTX2,3) as well as saxitoxin (STX). In samples close to or above the regulatory limit the gonyautoxins 1 & 4 (GTX1,4) are also typically detected. Toxin accumulation in shellfish from Cork Harbour is very predictable with the onset of toxin uptake typically taking place during the first two weeks of June each year. Toxin levels quickly rise to concentrations around the regulatory limit or above but drop within a couple of weeks, although very low levels of GTX2,3 can typically be detected for many months thereafter. Although *Alexandrium* species are detected in phytoplankton samples all along the West coast of Ireland, particularly during the summer months, toxin levels in shellfish remain low. This is thought to be because both toxic and non-toxic variants of *Alexandrium* exist in Irish waters with the non-toxic variant predominating outside Cork Harbour at present [42].

1.5 EU REGULATORY FRAMEWORK

There have been a number of EU Directives and Regulations relating to the production and placing on the market of live bivalve molluscs for human consumption. Two underlying Council Directives played an important role in setting out the goals to be achieved by a Member State (MS). First, Council Directive 91/492/EEC, last amended by Council Directive 97/79/EC, which laid down the health conditions for the production and the placing on the market of live bivalve molluscs and second, Council Directive 86/609/EEC which related to the protection of animals used for experimental or other scientific purposes [43–45].

In 2004 Council Regulations (EC) 853/2004 and 854/2004 were passed into law which brought together and replaced the existing hygiene regulations for the food sector [19,46]. Regulation (EC) No. 853/2004 lays down the maximum permissible levels of marine biotoxins in shellfish with PSP toxins for example set at a limit of 800 micrograms (µg) per kilogram (kg). Regulation (EC) No. 854/2004 gives the monitoring authorities in each MS the mandate to examine live molluscs for the presence of biotoxins.

Council Regulation (EC) No. 2074/2005 amended by Regulation (EC) No. 1664/2006 indicates the recognised testing methods for marine biotoxins for the purposes of Regulations (EC) No. 853/2004 and No. 854/2004 [20,21]. With regard to PSP toxins it follows previous directives in establishing the biological testing method as the reference method in the EU but for the first time lays down an alternative method that can be used for official control purposes. The so called "Lawrence Method", as published in the Journal of the AOAC as OM 2005.06 may also be used by MSs [41].

1.6 REFERENCE MATERIALS

The need for and use of reference materials (RMs) in the analysis of foodstuffs is necessitated by the quality assurance requirements of official control laboratories in order to demonstrate the adequacy of their testing methods. The definition of a RM as set down in the International Standards Organisation (ISO) 2008 guide on the definitions of reference materials and certified reference materials is stated as [47]:

"A material, sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process."

The nomenclature used in the area of RM production varies, with organisations and authors of literature using an assortment of different terms. Arguably the most important in terms of analytical capabilities are Certified Reference Materials (CRM). The characteristics of these materials allows the determination of the accuracy or trueness of a method providing valuable information on the performance of methods to both laboratories and accreditation bodies. Their effective use is highly dependent on the long-term stability of the material, so detailed feasibility studies on candidate CRMs must be undertaken to determine any potential degradation issues associated with the material through long-term or accelerated stability studies. The ISO 2008 guide further defines a CRM as [47]:

"Reference material characterised by a metrologically valid procedure for one or more specified properties, accompanied by a certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability."

1.6.1 Reference Material Types and Classification

RMs may be categorised in a number of different ways with two distinct types described as calibrant RMs and matrix RMs, the latter of which is dealt with in these studies [48,49]. The primary uses of these categories of RM are for analytical instrument calibration and the validation of entire analytical methods respectively, although they can be interchangeable. RMs can also be categorised based on their intended use and this will form the basis for how materials are defined herein.

These categories are described as follows [50]:

1. Certified Reference Material (CRM):

As detailed in the definition above, a CRM must be accompanied by or be traceable to a certificate or other piece of documentation issued by a certifying body. The material will

have one or more property values certified by a technically valid procedure together with uncertainty and traceability to the primary unit or standard [51].

Certification and the statistical principles involved in this process are detailed in various ISO documents.

2. Laboratory Reference Materials (LRMs):

These materials are generally used in the day-to-day quality control (QC) of official control sample testing. They are extracted and analysed alongside routine samples and thereby form one of the primary tools for the acceptance or rejection of a batch of samples. LRMs can also serve as a powerful and significantly cheaper tool, compared to the sole use of CRMs, in the in-house validation of a test method, particularly for parameters such as precision and ruggedness. As these materials are typically not transported to other laboratories, confirmation of stability under frozen conditions is usually sufficient for demonstration of fitness for purpose, in addition to homogeneity being adequately proven [50].

3. Interlaboratory Reference Materials (ILRMS):

The last category of matrix RMs were first defined by Hess *et* al. in 2007 [50] and are intended to check the comparability of testing methods through interlaboratory exercises, such as collaborative trials and proficiency testing. The stability of these materials during transportation internationally to participant laboratories can be demonstrated through stability studies using elevated temperatures to mimic adverse conditions potentially encountered during transit. This requirement typically necessitates the use of stabilising techniques in the preparation of these materials as well as adequate homogenisation techniques, a requirement of any RM production procedure.

1.6.2 PRODUCTION

The production of any RM requires a level of technical and organisational competence to ensure the key requirements of homogeneity and stability are adequately fulfilled [52]. Chapters 4 and 5 detail the various RM preparation techniques investigated in these studies but generically any RM procedure follows the basic key steps detailed in ISO guide 80:2014 and illustrated in Figure 1-12 [52]. This document gives guidance for the in-house preparation of what are termed quality control materials (QCMs), a category defined by ISO to encompass both LRMs and ILRMs.

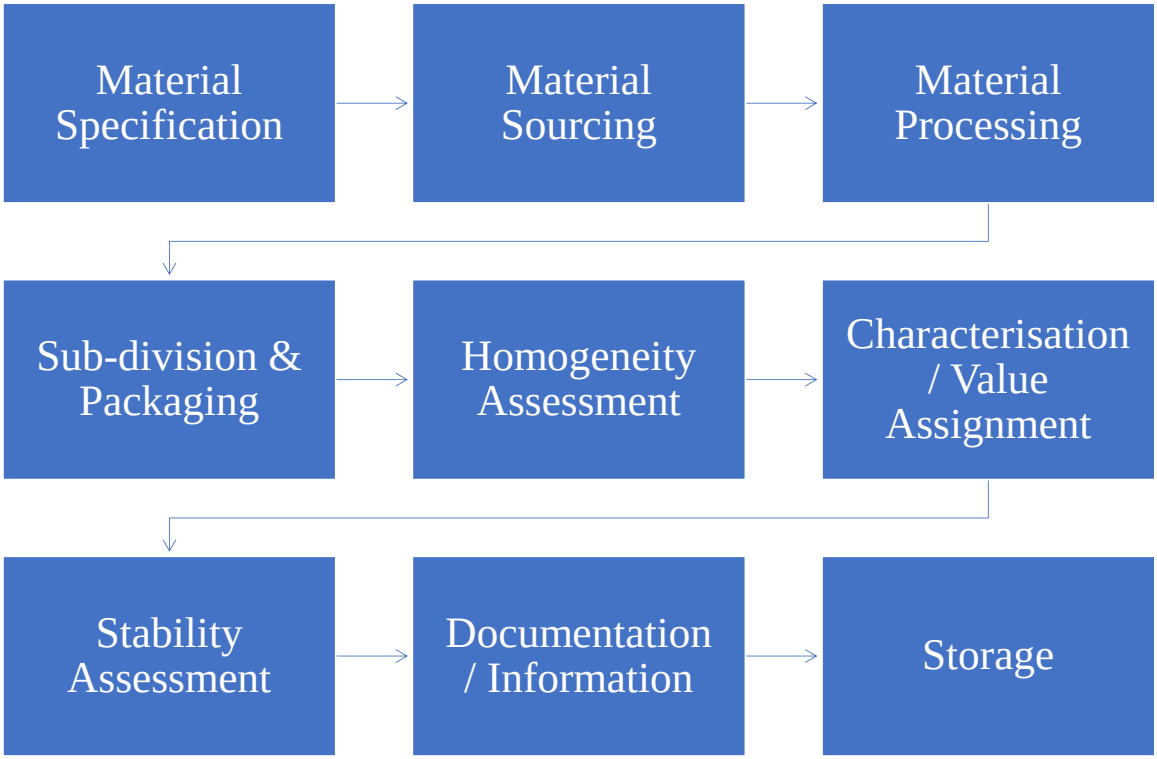


Figure 1-12: Key steps in the preparation of RMs (reproduced from ISO guide 80 [52].

The specification of the RM in general is that the materials prepared should be as close as possible to real life samples and be available in sufficient quantities. In the area of biotoxin monitoring the large variety of shellfish species covered in these programmes means, in general, choosing the species most frequently analysed; which in the case of the Irish NMP

blue mussels (*Mytilus edulis*) are. In the area of ILRM production (described in *Chapter 6*) a systematic approach was followed which sought to prepare materials from as many shellfish species as possible, although this was dictated by the authors' access to large enough quantities of naturally contaminated material. Other factors to consider in the material specification are the unit size required, an estimate of the total units required and the preparation yield [52]. Materials can then be sourced through various means including purchase from another organisation, collection of contaminated shellfish during toxic events, through toxin spiking or even shellfish feeding experiments [53].

One of the most important steps in this procedure is material processing which can involve a number of different steps aimed at ensuring appropriate homogeneity and stability for the final materials. Stabilising the material is discussed in detail in further chapters but the processing techniques for the wet tissue RMs, aimed at improving homogeneity, were developed and refined by McCarron in 2007 [54]. These techniques involved the use of various industrial blenders and grinders purchased during this period and further used during the course of these studies.

Sub-division and particularly packaging is another important factor to consider, with appropriate techniques having been shown to improve toxin stability. Sealing the final product under an inert gas, for example, prevents or minimises oxidative reactions from taking place as well as creating an environment inhospitable for microbial growth [54].

Once the material is prepared and dispensed, an assessment of its homogeneity and stability must be undertaken in addition to RM characterisation and value assignment. Homogeneity is determined by analysing a representative quantity of RMs from the entire production batch with a statistical evaluation of the data. For the RMs described in this thesis this involved calculating the coefficient of variation from the homogeneity data with comparison to

expected levels of method variability determined through validation of the test method, below which the RM was deemed sufficiently homogenous.

Depending on the final use of the materials, an assessment of stability in the short and/or long-term must also be undertaken. The stability studies contained in this thesis followed a reverse isochronous design which negates day-to-day instrumental variations through the analysis of all samples at the end of the study in one chromatographic sequence [55]. Material characterisation and value assignment are also important in establishing toxin profiles in the final product, as changes can occur during the processing steps. This is particularly important for ILRM preparation where PT organisers require the information to assess which toxins or toxin profiles are covered by their schemes and for the preparation of LRMs under private contract.

The last steps involve the preparation of accompanying documentation which generally include homogeneity and stability results, material characterisation and a description of the preparation techniques employed and the recommended storage conditions of the product determined through stability studies.

1.6.3 STABILISATION TECHNIQUES

In terms of the stability of biological matrices, microbial or biological activity is the most important influence to consider during preparation. Chemical activity and physical effects from, for example light and temperature are also important influences to consider, but microbial activity presents the biggest potential problem in RM preparation [56]. Various procedures to stabilise the matrix and therefore the analyte(s) have been described in the literature and the techniques used to prepare the RMs contained in this thesis are briefly described below.

1.6.3.1 PRESERVING ADDITIVES

The use of preserving additives to stabilise the biological matrix is a relatively easy and cost effective technique to employ in RM production. The use of the antioxidant ethoxyquin has been used previously in the preparation of CRMs for marine biotoxins [57–59] and its combined use with antibiotics in the preparation of marine biotoxin RMs [54]. Their combined use was shown to improve the stability of DA [60] and to a lesser extent AZA3 [54]. To date the combined use of these additives has not been reported for their stabilising effects on PSTs in shellfish matrices.

As in the case of most stabilisation techniques for biological matrices the use of preserving additives aims to reduce or eliminate biological activity and oxidative degradation from taking place in the final product. This is particularly important given the fact that processing and dispensing steps are not carried out aseptically in the procedures described in this thesis so any technique aimed at reducing or eliminating bacterially induced reactions in the final product may be beneficial.

1.6.3.2 THERMAL TREATMENT

Another relatively simple stabilisation technique is sterilization through heat treatment which aims to destroy bacteria present in the source tissues. As this step is usually performed prior to material processing and dispensing bacteria may still play a part in toxin stability due to the septic nature of further processing steps. The heat treatment step should however, significantly reduce bacteria colony numbers in the source tissues. Although the thermal stability of the analytes must be sufficient to withstand the high temperatures applied, to minimise degradation during this step [52].

1.6.3.3 FREEZE DRYING

Removal of water through drying is another technique to stabilise, in particular biological tissue matrices, improving both short and long-term stability as well as making the material easier to handle [52]. Freeze drying is a technique ideally suited to the preparation of thermally sensitive analytes owing to the relatively gentle freeze drying process. The process involves the removal of water from the matrix through a process called sublimation, which is the transition of a substance directly from the solid to the gas phase without going through the intermediate liquid phase [61]. This transition takes place at temperatures and pressures below the triple point of a substance, which is the temperature and pressure point where all three phases (solid, liquid and gas) exist in thermodynamic equilibrium [61]. The process involves reducing the pressure inside the freeze drying chamber while simultaneously increasing the temperature slowly through a series of ramp steps, typically 96 hours in the case of materials described herein. The removal of water reduces the likelihood of microbial growth formation [52].

This technique has been used for many years in the area of preservation in the food industry [56], as well as in the preparation of RMs for DA and some lipophilic toxins [62–65]. It has also been investigated for the preparation of a CRM containing two PSTs, STX and dcSTX [66–68]. This PST freeze dried CRM was not made commercially available and could not be distributed by the Institute for Reference Materials and Measurement (IRMM).

1.6.3.4 HIGH PRESSURE PROCESSING

High Pressure Processing (HPP) or High Hydrostatic Pressure Processing (HHP) is a non-thermal pasteurisation technique which has been used to produce food products free from microbial contamination for over a century [69]. The process is desirable not only for the

potential of decontaminating the food product but also because it minimises the impact on the nutritional and physical characteristics of the final product (Figure 1-13).

Figure 1-13: Picture of a strawberry after HPP treatment

The process generally involves subjecting the food product to pressures of between 150-600 MPa for times of 1-15 min [70]. Pressure is applied through water which surrounds the product and allows an even distribution of pressure throughout the product.

This process applied in the area of RM preparation has the potential advantage of leaving the final material and matrix practically intact and very close to real life samples generally analysed in a laboratory. However, prior to the work described in this thesis, such an approach has yet to be reported for RMs of any type.

1.6.4 HOMOGENEITY TESTING

Homogeneity is defined by ISO as the "uniformity of a specified property value throughout a defined portion of a reference material" [47]. The level of inhomogeneity of the material

should result in a smaller effect on the measurement result than the expected variation of the measurement process or should be below an established criterion value [52].

In general homogeneity testing requires the selection of a sufficient number of units, which are representative of the entire batch and the analysis of selected properties within those units. There are two aspects to homogeneity testing, between-unit and within-unit. Within-unit homogeneity is only a requirement where the RM has been dispensed with more than one portion, and it is essential that each portion taken yields similar results. Between-unit homogeneity seeks to determine if differences between the various units of a batch exist and if those differences are within acceptable limits. The RMs described in these studies were dispensed as single portions so within-unit homogeneity was not assessed.

The number of samples taken for homogeneity testing has recently been covered in ISO guide 80:2014 [52] and two sampling guidelines are described in the document. The first approach is to select samples from a stock comprising "n" individuals using the formula:

$$3(n)^{1/3}$$

This approach can be excessive in the number of samples chosen, particularly when dispensing large quantities of RMs (>1000) and therefore represents a significant analytical effort including the associated costs involved.

The second approach which is based on studies to determine the effects of reducing the number of units selected, concluded that in certain circumstances 10 units analysed in duplicate was sufficient in assessing homogeneity [51]. This approach, where ≥10 units are selected is deemed appropriate by ISO for materials used in proficiency testing schemes [71].

The use of either approach in selecting the number of units to be assessed is highly dependent upon the sampling approach taken, i.e. the selections are representative of the entire

production batch. The best way of ensuring representativeness is through stratified random sampling. This involves dividing the total population into smaller groups known as strata, from which simple random sampling or systematic sampling is applied to each stratum.

1.6.5 STABILITY TESTING

Stability is defined in ISO guide 30:2015 as the "characteristic of a reference material, when stored under specified conditions, to maintain a specified property value within specified limits for a specified period of time".

The stability and homogeneity of a RM are arguably the two most important factors to consider when preparing these materials. Depending on the final use of the material, different approaches to stability testing can be performed. For example if transportation of the RM is not required, such as an LRM for sole internal use at one site, then short-term stability testing would not be necessary and an assessment of its long-term stability would be sufficient. Where international dispatch of the materials may be required, such as the preparation of ILRMs, an assessment of the short-term stability is necessary as these materials need to be stable during potentially adverse transportation conditions. As RMs are often prepared in large enough quantities to enable their use over a number of years, a long-term stability assessment is necessary to determine storage conditions.

There are two main approaches in assessing the stability of the RM, classical and isochronous stability studies [55]. The "classical" model involves the analysis of materials, stored at various temperatures, and at pre-defined times during the course of the study. As analysis is carried out on different days, this model is highly dependent upon the long-term reproducibility of the method as well as its repeatability.

An alternative approach described by Lamberty *et al*. in 1998 [55] sought to negate these reproducibility issues through an isochronous experimental design, which can be used when the total duration of the study is known. This design can have two main formats, isochronous and reverse isochronous measurements. In the standard isochronous design the required amount of materials are transferred to the various storage conditions at the beginning of the study. After specific time periods during the study, materials are transferred from the storage conditions to a reference storage temperature where stability is assumed. This format relies on the assumption that the degradation process ceases once materials are removed from the temperature condition being investigated and stored at the reference temperature.

A reverse isochronous stability study involves transferring the required amount of materials to the reference temperature at the beginning of the study. At specific time periods during the study, materials are removed from the reference storage temperature and transferred to the storage conditions being investigated.

In the case of both the isochronous and reverse isochronous stability studies, all materials are removed from the storage conditions at the end of the study and analysed immediately. The long-term method reproducibility is therefore inconsequential following this approach and only method repeatability is a factor in the analysis.

1.6.6 CERTIFICATION

There are a number of technically valid approaches to certifying a reference material, including measurement by one or more methods involving one or many laboratories. The requirements for a CRM are that it is "characterised by a metrologically valid procedure for one or more specified properties, accompanied by a certificate that provides the value of the

specified property, its associated uncertainty, and a statement of metrological traceability" [51].

Accuracy and the uncertainty of the values determined are two important aspects in the certification process. The certified value(s) of the material presents the best estimate of the "true" value and is not expected to differ by more than the measurement uncertainty. The uncertainty of the measurement value should take into account errors associated with the measurement process, such as systematic or random errors. All components of the uncertainty should be evaluated and taken into account when calculating this value, with data generated through the measurement process, as well as homogeneity and stability study data being used to produce the final uncertainty of the CRM.

Measurement of the value can be carried out using a single definitive method, by two or more independent reference methods or by a network of qualified laboratories using one or more methods of demonstrated accuracy [51].

Certification of the RMs prepared during these studies was not carried out so the statistical approaches involved are not discussed further.

1.7 PROFICIENCY TESTING

The use of RMs is becoming increasingly important in the area of quality assurance (QA), particularly given the global nature of the shellfish industry. Implementing and running a robust quality system is of the upmost importance to any monitoring system in giving confidence to the analyst and industry as a whole. The use of proficiency testing (PT) as well as being a legislative requirement in the EU [72,73] is a powerful QA tool at the analyst's disposal enabling laboratories to evaluate their results against a designated consensus.

QUASIMEME was founded in 1992 and initially funded by the EU under a three year quality assurance project [74]. The aim was to establish a comprehensive network of laboratories producing demonstrably reliable chemical information while providing a holistic approach to quality assurance.

The determination of PSP toxins was added to the QUASIMEME scope in 2009 as a development exercise, and has been running each year since with one round organised annually. The wide array of methods available for determining PSP toxins gives choice to the analyst but does pose a problem to organisers of PT schemes in including as many of these methods as possible. The most widely used methods internationally for PSP analysis are the mouse bioassay (MBA) [40], liquid chromatography-fluorescence detection (LC-FLD) based on pre-column oxidation (preCOX) [41] and post-column oxidation (PCOX) [75], immuno-based assays (ELISA) and liquid chromatography with tandem mass spectrometry (LC-MS/MS).

The Finnish Institute for Verification of the Chemicals Weapons Convention (VEREFIN), located in the University of Helsinki was established in 1994 continuing a research project on Chemical Weapons (CW) which had been operating since 1973. VEREFIN supports CW disarmament, of which STX is listed in schedule 1 of the Chemical Weapons Convention (CWC), through the development of analytical methodologies used to identify Chemical Warfare Agents (CWA). Under the 7th EU Framework Programme for Research VEREFIN co-ordinated the EQuATox (Establishment of Quality Assurance for the Detection of Biological Toxins of Potential Bioterrorism Risk) project. As part of this project a PT scheme was organised which sought to evaluate existing methodologies for the determination of STX in real samples. A shellfish tissue RM prepared using one of the techniques described in *chapter 4* was used in this PT scheme.

1.8 EVOLUTION OF METHODOLOGIES FOR PSP DETERMINATION

As previously stated the MBA is the reference method in EU legislation for the determination of PSP toxins in Europe. It was first applied in the determination of PSP toxicity by Sommer and Meyer in 1937 [76]. Subsequently the procedure underwent standardisation, culminating in AOAC official method (OMA) 959.08 [40]. This is the reference method specified in EU legislation [21] and involves the extraction of the shellfish homogenate tissue in dilute hydrochloric acid followed by intraperitoneal injection of filtered extracts into replicate mice. The time taken from injection to mouse death is recorded and the toxicity determined from a conversion table developed by Sommer and Meyer [76]. Reported drawbacks of the method include underestimations of total toxicity caused by high salt concentrations or the presence of some metals in samples [10,77]. The presence of other metals, such as zinc, particularly evidenced in oyster tissues has been reported to increase the threat of false positives [78]. The method is also controversial in its use of large numbers of mice and in stipulating death as an endpoint. A further drawback of the MBA is that it only provides a total toxicity value and no information about the specific toxin profile of a sample. However, the method has been used globally for many years and has provided an excellent preventative method for the significant reduction of PSP intoxications worldwide.

Due to method performance concerns, ethical issues regarding animal welfare, and as a legislative requirement to reduce, refine or replace animal based assays, an alternative method was written into EU legislation in 2011 which is based on the oxidation of extracts before separation and determination by LC-FLD [21]. PSP toxins do not exhibit natural ultraviolet absorption or fluorescence and must therefore be oxidised into iminopurine derivatives before analysis using fluorescence detection [79]. The method cited in legislation was developed by Lawrence & Ménard, 1991 [80] and underwent international validation in 2004 before being approved as an AOAC official method in 2005 [41]. This method is based

on a two-step acetic acid extraction followed by solid phase extraction (SPE) cleanup using C_{18} reverse-phase cartridges to remove hydrophobic shellfish matrix interferences. Cleaned extracts are then oxidised using peroxide and/or periodate reagents before separation and analysis by LC-FLD (preCOX) to determine the qualitative or semi-quantitative presence of toxins. Full quantitation of samples containing N-hydroxylated toxins normally requires further sample extract clean-up using carboxylic acid ion-exchange SPE cartridges. This step separates the samples into three fractions each containing toxins in a different overall charge state, thereby enabling more accurate detection and quantitation of each toxin or epimeric pair [41].

The main drawbacks of the method relate to the complex chromatographic output as well as the lack of separation of epimeric pairs (GTX1 & GTX4, GTX2 & GTX3, C1 & C2, dcGTX2 & dcGTX3 and C3 & C4) characteristic of this toxin group. Users of this method calculate summed toxin concentrations for each epimeric pair. Sample toxicities are estimated from the sum of toxin concentrations, which leads to a slight overestimation as the more toxic analogue for each epimeric pair is used to calculate total toxicity. This is in spite of the fact that the epimer ratio in bivalve tissues typically reaches an equilibrium of 3:1 predominated by the less toxic α-epimers (GTX1, 2) compared to the more toxic β-epimers (GTX3, 4) [81]. Pre-column derivatization can produce one to three oxidation product peaks per toxin, leading to difficulties in determining toxin profiles and accurate quantitation. This is particularly pertinent for samples often used in PT schemes that have either a complex toxin profile or a profile not routinely seen by the participant's laboratory. Another drawback of using this method and all chemical based methods of analysis is the lack of certified reference standards for all the PSP toxins, most notably GTX6, C3, C4, dcGTX1 and dcGTX4. Although complex, the method has been refined and standardised since acceptance as a first action AOAC method, and in recent years has been implemented into routine official control

testing of shellfish in a number of countries including Ireland, UK, Portugal and New Zealand.

Methods based on post-column derivatization were first utilised for shellfish monitoring by Sullivan and Wekell in 1984 [82]. Further developments were described by Oshima *et al.* in 1989 [83] where the separation of the full suite of known PSP toxins was achieved using three separate chromatographic conditions. A method modified from those of Oshima, 1995 [17] and Thomas *et al.* 2006 [84] was first published by Rourke *et al*., 2008 [85]. This method underwent international validation under the protocol of the AOAC in 2010 and was subsequently accepted as AOAC official method 2011.02 [75]. The extraction technique is based on that of the MBA, specifically involving boiling in HCl. Partial hydrolysis of certain PSP toxins into more toxic analogues occurs with the PCOX/MBA extraction method which, some argue, mimics the process of digestion in the stomach thereby more accurately reflecting sample potential toxicity [86]. The milder conditions of the preCOX extraction method do not cause hydrolysis of the toxins, with toxin profile consequently remaining unaffected.

A distinct advantage the PCOX method has over the preCOX is the formers ability to separate the PSP epimers contained in this group [87]. One disadvantage with the PCOX methods are its inability to separate certain PSP toxins (dcNEO/NEO and GTX6/GTX4) unless a very long runtime is used [85]. OMA 2011.02 also requires two chromatographic runs in order to separate the full suite of PSP toxins. The method has recently been adopted into US legislation and has been implemented into official control monitoring programs within Canada, Norway and some US states.

The use of mass spectrometry (MS) as a detection system for determining PSP toxins is desirable, based on the high sensitivity and selectivity this technique potentially gives the

user. Early LC-MS methods required the use of ion-pairing reagents in the mobile phase in order to ensure adequate retention of the charged PSP species [88,89]. This, along with the aqueous mobile phase these methods employed, led to poor ionization and signal suppression. Dell'Aversano *et al.,* 2005 [90] developed an LC-MS/MS method based on hydrophilic interaction liquid chromatography (HILIC) which increased detection sensitivity compared to previous LC-MSMS methods. Despite comparable sensitivities for the majority of toxins to the other methods described above, the use of LC-MS/MS methods in routine monitoring and therefore PT schemes is limited, mainly due to matrix effects problems. Recent advances in this area were made by Boundy *et al.* 2015 [91] where the application of solid phase extraction using graphitised carbon prior to LC-MS/MS analysis eliminates or significantly reduces these matrix related issues.

A number of functional and immunological assays have been developed for PSP toxins over the last 20 years although their use for routine monitoring purposes has been limited. This is mainly due to the lack of information these methods provide on the toxin profile of samples, the use and handling of radioisotopes or cross reactivity issues which may lead to false positives or false negatives.

A receptor binding assay (RBA) was developed by Vieytes *et al*., in the early 90's [92] and was further optimised by Doucette *et al*. [93]. This binding competition assay uses sodium channels isolated from rat brain membranes coated on to microtitre plates. Tritiated saxitoxin [^{3}H]STX competes with unlabelled STX and its derivatives for binding to the available sodium channel receptor sites. Unbound tritiated STX is removed by filtration and bound triated STX is quantified by liquid scintillation counting. This method has undergone extensive single laboratory validation (SLV) and a collaborative study [94,95] and has been accepted as an official AOAC method of analysis (OMA AOAC 2011.27) [96].

Several cytotoxicity assays have been developed and are based on the combined effect they exert in the presence of veratridine and ouabain on neuroblastoma cells [6]. The method, further developed by Jellet *et al*. in 1992 [97] yielded unsatisfactory results in an AOAC International collaborative study in 1999 however. A radioligand binding assay using a STX specific receptor, saxiphilin was developed by Llewellyn *et al*., in the late 90's but has not been subjected to formal SLV or collaborative studies to date [98].

Immunological assays use antibodies to detect the toxin of concern which are raised either in animal or cell cultures. Two formats of immunological assays have been developed for PSPs, ELISA (Enzyme-Linked ImmunoSorbent Assays) and LFA (Lateral Flow Assays). These methods are based on the antibodies ability to recognise and bind the toxin of interest. The most common immunoassay technique in use for PSP testing is that of ELISA. A number of commercial testing kits are available with one utilised within the Quasimeme PT rounds sold under the name Ridascreen™ (R-Biopharm, Darmstadt, Germany). The high number of STX analogues has however posed problems for developers of this and other similar ELISA methods, with NEO and GTX-1,4 in particular exhibiting poor cross reactivity with the Ridascreen assay [99].

The development of biosensors using biological components, such as antibodies to recognise and bind the toxins, has advanced over the last number of years. These sensors use antibodies as the bio-recognition component and surface plasmon resonance (SPR) as the detection component. These methods involve the immobilisation of the toxin onto a chip. Samples are then mixed with the bio-recognition component and are injected over the surface of the chip. Toxins present in the sample compete with toxins on the chip for binding to the antibody which if present change the resonance angle of the light [100]. An SPR based immunoassay developed for PSP toxins was subjected to various validation studies but, as in the case of the ELISA above, showed poor cross reactivity for some analogues, in particular GTX1,4 and

dcNEO [101–104]. A multiplex SPR method has also been developed for the ASP, DSP and PSP groups which has the advantage of detecting three toxin groups in a single test, although their development is still at an early stage [105].

Alternative methods for PSP determination include capillary electrophoresis coupled to ultraviolet (UV) or MS detection, although limited research has been carried out in this area [106–111].

1.9 RESEARCH AIMS AND OBJECTIVES

The primary aims and objectives of this research were to investigate techniques aimed at stabilising PSTs in various shellfish tissue matrices. Each technique was evaluated for its applicability in preparing RMs for different uses including official control monitoring in the Irish NMP, for PT schemes operated by QUASIMEME and VEREFIN and feasibility studies into the production of a CRM.

A further aim of this research was to characterise and present a first report of PSP toxins from shellfish harvested in Iceland. This toxic event occurred during the initial search for PSP contaminated materials for use in these studies.

Using one of the techniques developed during these studies, multiple materials were prepared and used in the first commercially available PSP PT scheme operated by Quasimeme and in a separate PT exercise organised by VEREFIN.

The major aims and objectives can be outlined as follows:

- Characterise materials harvested during a large PSP toxin event in Iceland during the summer of 2009 and present a first report including toxin profile determination together with associated phytoplankton data.
- Collect and characterise PSP contaminated material for use in these development exercises.
- Investigate various stabilisation techniques and their effects on PSTs and shellfish tissue matrices including their applicability for RM production for various uses. The techniques investigated included:
    - Heat treatment
    - Use of preserving additives (antioxidant and antibiotics)
    - High Pressure Processing (HPP)

- Freeze drying
- Combinations of the above

- Assess data generated over six years of PT exercises operated by QUASIMEME to investigate potential method dependency issues and if they relate to the analysis of particular PSP analogues.

Chapter 2

MATERIALS AND METHODS

2.1 SOURCE OF TISSUES

With the levels of PSP toxins in Irish shellfish rarely rising above the EU regulatory limit (MI unpublished data), coupled with a relatively simple toxin profile, it was necessary to source contaminated materials from collaborators of the MI for use in the RM investigations described in this thesis.

Table 2-1: Description of whole flesh (WF) source tissues used in the preparation of materials for the studies contained in this thesis, including country of origin, species and toxins present as determined by preCOX LC-FLD (AOAC 2005.06).

Country	Species	Predominant Toxin(s) Present	Trace Toxin(s) Present
Spain	Mussel (*Mytilus galloprovancialis*)	dcSTX	GTX5 + STX
Portugal	Clam (*Spisula solida*)	dcGTX2,3, dcSTX + dcNEO	
UK	Oyster (*Crassostrea gigas*)	GTX2,3, STX, GTX-1,4, NEO, C1,2	
Canada	Mussel (*Mytilus edulis*)	GTX2,3, STX, GTX1,4 + NEO	
Ireland	Mussel (*Mytilus edulis*)	GTX2,3 + STX	GTX1,4
Iceland	Mussel (*Mytilus edulis*)	GTX2,3, STX + GTX-1,4	
Norway	Mussel (*Mytilus edulis*)	GTX2,3, STX, GTX1,4 + NEO	

The beginning of this research coincided with a large PSP event in Iceland, as detailed in *Chapter 3*, and a large quantity of contaminated blue mussels (*Mytilus edulis)* were harvested and transported on ice to the MI. Large amounts of Mediterranean mussels (*Mytilus galloprovancialis*) were harvested in Spain in 2009, after which it was discovered that PST levels were above the EU regulatory limit. A sub-sample of the tissue material was also sent to the MI on ice.

The Norwegian Veterinary Institute (NVI), Canadian Food Inspection Agency (CFIA), Centre for Environment Fisheries and Aquaculture Science (CEFAS), UK also all provided PST contaminated shellfish with various toxin profiles as detailed in Table 2-1.

2.2 CONSUMABLES

2.2.1 CHEMICALS

All chemicals and solvents used were of analytical or HPLC grade. The deionised (DI) water was supplied from a reverse osmosis system (Barnstead Int., Dubuque, IA, USA). Acetic acid (HOAc), hydrochloric acid (HCl), nitric acid (HNO_3), ortho-phosphoric acid (H_3PO_4) ammonium formate, ammonium acetate, sodium chloride (NaCl), sodium hydroxide (NaOH), ammonium hydroxide (NH_4OH), hydrogen peroxide (H_2O_2), disodium hydrogenphosphate, 1M tetrabutyl ammonium phosphate, erythromycin, ethoxyquin, trichloroacetic acid (TCA) and periodic acid (H_5IO_6) were purchased from Sigma-Aldrich (Steinheim, Germany). Ampicillin and oxytetracycline were purchased from Fisher Scientific (Waltham, MA, USA). Methanol (MeOH) and acetonitrile (MeCN) were purchased from Labscan (Stillorgan, Ireland).

2.2.2 STANDARDS

Certified reference toxins: gonyautoxin 1 and 4 (GTX1,4), neosaxitoxin (NEO), decarbamoylsaxitoxin (dcSTX), gonyautoxin 2 and 3 (GTX2,3), gonyautoxin 5 (GTX5), N-sulfocarbamoyl-gonyautoxin 2 and 3 (C1,2), decarbamoylneosaxitoxin (dcNEO), decarbamoylgonyautoxin 2 and 3 (dcGTX2,3) and saxitoxin (STX) were obtained from the Institute of Biotoxin Metrology, National Research Council Canada (IMB, NRCC, Halifax, Nova Scotia, Canada).

For preCOX analysis, the CRMs were first diluted in water (adjusted to pH 4.0 ± 0.1 with 0.1M HOAc) to prepare primary stock solutions. Further dilutions were performed in 0.1 mM HOAc to prepare working calibration solutions. Primary and working standards were stored following NRCC recommendations [112]. Standards were prepared in three separate mixes as the oxidation products of some toxins co-elute. The concentration ranges and toxins contained in each mix are detailed in Table 2-2.

For PCOX analysis, the working standards were prepared as dilutions of NRC-CRMs, in 0.003M HCl for the GTX/STX toxins and in de-ionised water (pH 5.0 ± 0.1) for the C-toxins as described in AOAC OM 2011.02 [75]. The concentration ranges used in PCOX analysis are detailed in Table 2-3.

2.3 METHODS OF ANALYSIS

2.3.1 AOAC OFFICIAL METHOD 2005.06

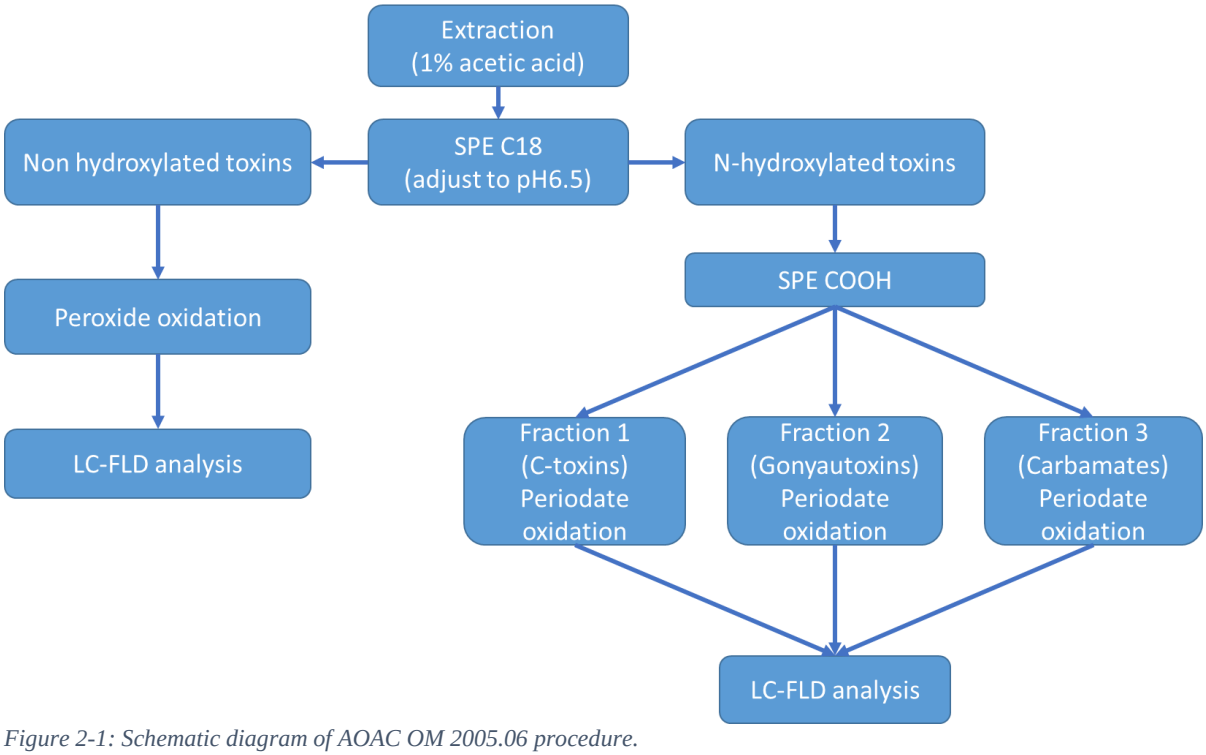


Figure 2-1: Schematic diagram of AOAC OM 2005.06 procedure.

EXTRACTION

Tissue samples (5.0 g ± 0.1 g) were extracted twice in 50 mL polypropylene (PP) centrifuge tubes with 3.0 mL volumes of 1% HOAc. The first extraction step was performed using a multi-tube vortex mixer (V400 Alpha Labs, UK) for 1 min on high power, before being placed into a boiling water bath (Grant SBB Aqua 26 plus) for 5 min. Sample tubes were removed from the boiling water bath and placed into a basin of cold water for 5 min. Sample tubes were then centrifuged (CR4-22 Jouan, Thermo Electron Corp., CA, USA) at 4500 rpm for 10 min, collecting the resultant supernatant in 15 mL PP test tubes. The remaining pellet was re-extracted using a multi-tube vortex mixer, as described above, before re-centrifugation at 4500rpm for 10 min. The supernatants from both extraction steps were combined in the graduated 15 mL PP test tubes and made up to 10 mL with de-ionised water.

SOLID PHASE EXTRACTION (SPE) C_{18}

Sample clean-up was performed using Supelclean (Supelcosil, Bellefonte, PA, USA) C-18 cartridges (500 mg/3 mL), following the method described by [41]. The cartridges were conditioned with 6.0 mL MeOH before being washed with 6.0 mL DI water. 1.0 mL of the crude extract was loaded onto the cartridge and eluted with 2.0 mL de-ionised water into 4.0 mL PP collection tubes. The extract was then adjusted to pH 6.5 ± 0.1 (Orion pH meter, Thermo Scientific Inc., Waltham, MA, USA) using 1M NaOH (3.99 g ± 0.01 g made up to 100 mL with DI water) before the total volume was made up to 4 mL with de-ionised water. Aliquots were then taken for oxidation with either peroxide or periodate reagent depending on the toxins present. The clean-up procedure was performed on an automated SPE unit (Gilson ASPEC XL4, UK), which was previously in-house validated (data not shown) unless otherwise stated in the analysis sections of each chapter.

SOLID PHASE EXTRACTION (SPE) CARBOXYLIC ACID

Ion exchange clean-up was performed to fractionate the C_{18}-cleaned extracts using Bakerbond (J.T. Baker, Phillipsburg, NJ, USA) COOH cartridges (500 mg/3 mL) [41]. The COOH SPE cartridges were conditioned with 10.0 mL 0.01M ammonium acetate (0.77 g ± 0.01 g ammonium acetate made up to 1 L with DI water) before loading a 2.0 mL aliquot of the C_{18} SPE cleaned extract. Fraction 1 containing the neutrally charged C toxins was eluted with 4.0 mL water and collected in a 12 mL PP tube, adjusting the final volume to 6.0 mL with DI water. 4 mL of 0.05M NaCl (0.29 g ± 0.01 g NaCl made up to 100 mL with DI water) were then passed through the cartridge, collecting the eluant containing the singly-charged gonyautoxins in a 4 mL PP tube, adjusting the final volume to 4 mL with DI water. Fraction 3, containing the double-charged carbamates, was collected by eluting 5 mL of 0.3M NaCl (1.75 g ± 0.01 g NaCl made up to 100 mL with DI water) through the cartridge and collecting in a 12 mL PP tube, ensuring the final volume was 5 mL through adjustment with DI water. The clean-up procedure was performed manually using a SPE manifold and pump from Sigma-Aldrich (Steinheim, Germany).

MATRIX MODIFIER

The matrix modifier, used in the periodate oxidation of standard and sample extracts as described in *section 2.3.1* was prepared weekly. An oyster (*Crassostrea gigas*) WF tissue, confirmed to be <LOD for all EU regulated biotoxins by LC-MS/MS (for lipophilic toxins), LC-UV (for DA and epi-DA) and LC-FLD OM AOAC 2005.06 (for PSTs), was dispensed in 5 g aliquots into PP tubes before being hermetically sealed and stored at -20°C. An aliquot was taken weekly, defrosted before being extracted, SPE C_{18} cleaned and adjusted to pH 6.5 according to *section 2.3.1*. This solution was stored at +4°C and used in the periodate oxidations described below.

OXIDATION

PSP toxins are not naturally fluorescent and must therefore be oxidised into a fluorescent form prior to analysis. Two oxidation reactions could be performed on the cleaned extracts depending on the toxin profile present in the tissue. Non-hydroxylated PSP toxins were oxidised using H_2O_2 while the N-hydroxylated toxins were oxidised using a periodate reagent. Oxidations were performed in 1.5 mL PP HPLC vials (Fisher Scientific, Waltham, MA, USA) using calibrated pipettes and multichannel timers (Fisher Scientific, Waltham, MA, USA).

PEROXIDE OXIDATION

250 µL of 1M NaOH was added to 25 µL 10% H_2O_2 in a 1.5 mL PP HPLC vial and vortex mixed. 100 µL of the standard solution or test extract, after C_{18} or COOH SPE clean-up, were added to the vial, mixed, and allowed to react at room temperature for 2 min. The reaction was stopped by adding 20 µL glacial HOAc. 50 µL of the oxidised solution were injected onto the HPLC system.

PERIODATE OXIDATION

The periodate oxidant was prepared daily by combining equal quantities of 0.03M H_5IO_6 (0.68 g ± 0.01 g H_5IO_6 made up to 100 mL with DI water), 0.3M ammonium formate (1.89 g ± 0.01 g ammonium formate made up to 100 mL with DI water) and 0.3M disodium hydrogen phosphate (4.26 ± 0.01 g disodium hydrogen phosphate) made up to 100 mL with DI water) and adjusted to pH 8.2 with 0.2M NaOH (0.79 g ± 0.01 g NaOH made up to 100 mL with DI water).

100 µL of matrix modifier (blank toxin free C-18 cleaned oyster tissue extract) were added to 100 µL of standard or test extract, after C_{18} or COOH SPE clean-up, in a 1.5 mL PP HPLC vial. 500 µL of periodate oxidant was added to the vial, mixed and allowed to react at room temperature for 1 min. The reaction was stopped through the addition of 5 µL glacial HOAc. 50 µL of the oxidised solution was injected onto the HPLC system.

LC-FLD Analysis

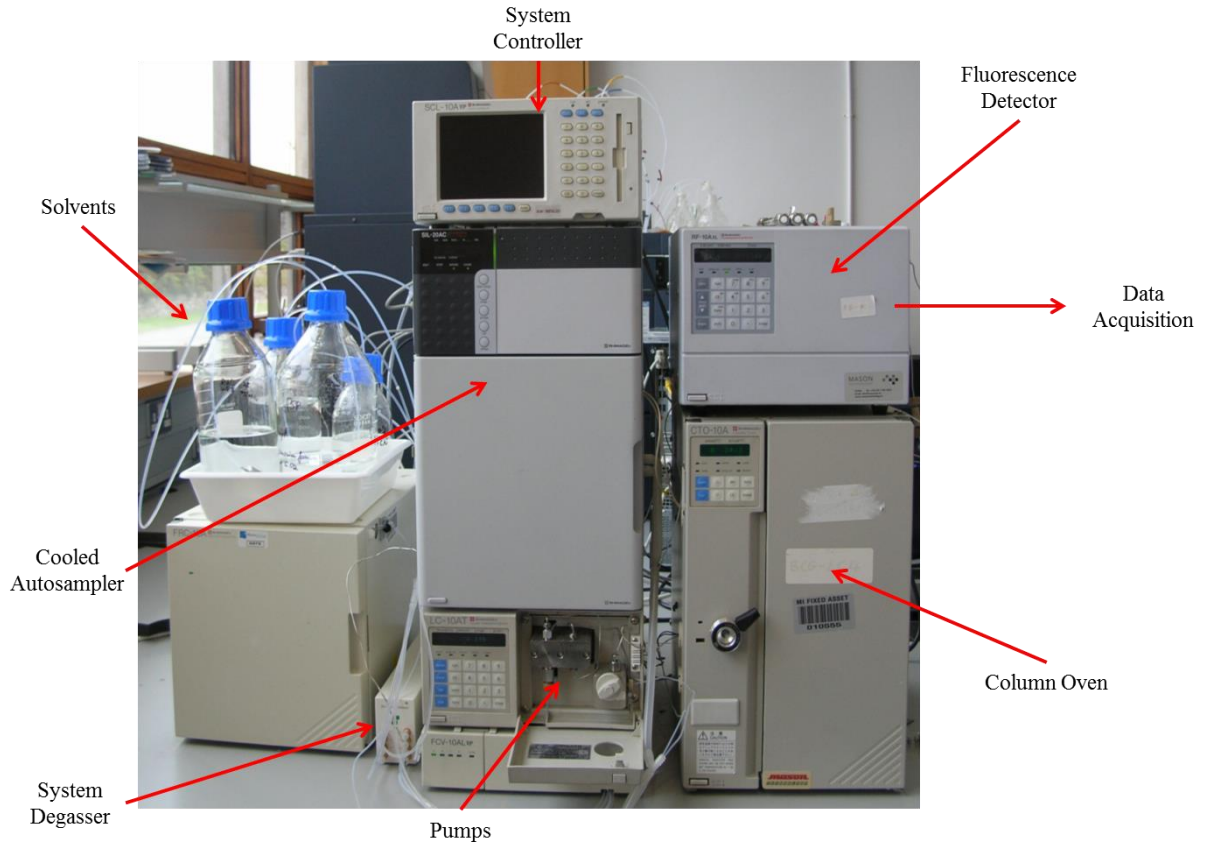


Figure 2-2: LC-FLD setup used for OMA AOAC 2005.06.

A Shimadzu (Kyoto, Japan) HPLC system with a fluorescence (FLD) detector (ex 340 nm, em 395 nm) (Shimadzu RF-10AXL), cooled autosampler set to +4°C (Shimadzu SIL-20A) and a degasser was used. The HPLC column was a reverse phase C-18 Supelcosil (150 mm x 4.6 mm, 5 µm) fitted with a C-18 Supelguard cartridge (20 mm) as recommended in the

official method [41]. The HPLC programme followed was a slightly modified gradient elution based on that published in AOAC 2005.06 [41] using a flow rate of 1.5 mL/min. MP A was prepared by dissolving ammonium formate (12.62 g ± 0.01 g) in DI water before making up to the mark with DI water in a 2L volumetric flask. MP B was prepared by dissolving ammonium formate (6.31 g ± 0.01 g) in DI water, adding 100 mL MeCN before making up to the mark with DI water in a 1 L volumetric flask. Both MPA and B were adjusted to pH6.0 ± 0.1 with 0.1M HOAc (572 µL glacial HOAc made up to 100 mL with DI water) before filtering through a 0.45 µm nylon filter.

The gradient followed was 0 - 5% MP B over 5 min, 5 - 70% B over the next 4 min, back to 0% B over 2 min, then keeping at this condition for 7 min before the next injection. PST concentrations in sample extracts were quantified against a five-point calibration for each toxin and were expressed in µmol/kg. The concentration range of the standards used during these studies are contained in Table 2-2.

Table 2-2: Concentration range of standards used for OMA AOAC 2005.06 analysis.

Mix	Toxin	Concentration Range (µmol/L)		
Mix 1	**STX**	0.005	→	0.417
	dcSTX	0.003	→	0.399
	GTX2,3	0.003	→	0.973
	GTX5	0.003	→	0.295
	dcGTX2,3	0.008	→	0.819
	C1,2	0.013	→	0.859
Mix 2	**NEO**	0.009	→	0.349
	GTX1,4	0.027	→	0.423
Mix 3	**dcNEO**	0.009	→	0.334

CALCULATIONS

PSTs produce between 1 – 3 oxidation products after peroxide or periodate oxidation, some of which co-elute. Identifying toxins present in samples and therefore quantification is complex, particularly when certain toxins are present in samples. The toxin dcSTX co-elutes

with both dcNEO and NEO and dcGTX2,3 co-elutes with the toxins GTX1,4 so a back calculation is required when these toxins are present in a sample together. Materials described in these studies only contained a combination of dcSTX and dcNEO so only this back calculation is described below.

The toxin dcSTX produces two oxidation products after both peroxide and periodate oxidation, with the first peak after peroxide oxidation used for quantification. The toxin dcNEO produces two oxidation products after periodate oxidation with the first peak used for quantification. In a sample containing both toxins the first dcNEO peak also has a contribution of dcSTX present in the sample. A set of dcSTX standards are periodate oxidised and a calibration curve generated from the area of the first peak of dcSTX versus dcSTX concentration. The amount of dcSTX present in the sample is known from the peroxide oxidation of the SPE C_{18} cleaned extract. This value can then be used to estimate the area of the dcSTX peak to be subtracted from the first dcNEO peak. The net area of dcNEO is then used to calculate its concentration.

PST concentrations in samples, as described in the following chapters were calculated as follows:

- Response (toxin) / slope = µmol/L
- Concentration (µmol/L) / Tissue weight x 10 (Final volume of extract) x Dilution Factor SPE = µmol/kg

  Dilution Factors: C_{18} cleaned extracts - 4

  COOH fraction 1 – 12

  COOH fraction 2 – 8

  COOH fraction 3 – 10
- µmol/kg x 372.2 g/mol (STX Mol. Wt. HCl form) x TEF = µgSTXdiHCl-eq/kg

The total toxicity of a sample was calculated by combining individual analogue concentrations and is expressed in µgSTXdiHCl-eq/kg.

UNCERTAINTY OF MEASUREMENT

An expanded uncertainty of measurement (U) was calculated for OMA AOAC 2005.06 during validation studies. Data was generated through the use of an LRM (internal MI code LRM-09-02) using OMA AOAC 2005.06 as part of the Irish National Monitoring Programme (NMP) for marine biotoxins. An aliquot of LRM-09-02 was extracted and analysed per batch over a two year period and the mean (996 µgSTXdiHCl-eq/kg) and relative standard deviation (8.7%) of the total toxicity data was generated.

The uncertainty of measurement was calculated as follows:

$$UCM = k \times C \times RSD$$

K = Coverage Factor = 2 (@ 95% confidence assuming a normal distribution)

C = 996 µgSTXdiHCl-eq/kg

RSD = 8.7%

$$UCM = 2 \times 996 \text{ µgSTXdiHCl-eq/kg} \times 0.087$$

$$UCM = 173 \text{ µgSTXdiHCl-eq/kg}$$

UCM at 996 µgSTXdiHCl-eq/kg ± 173 µgSTXdiHCl-eq/kg

UCM at 996 µgSTXdiHCl-eq/kg ± 17.4%

2.3.2 AOAC Official Method 2011.02

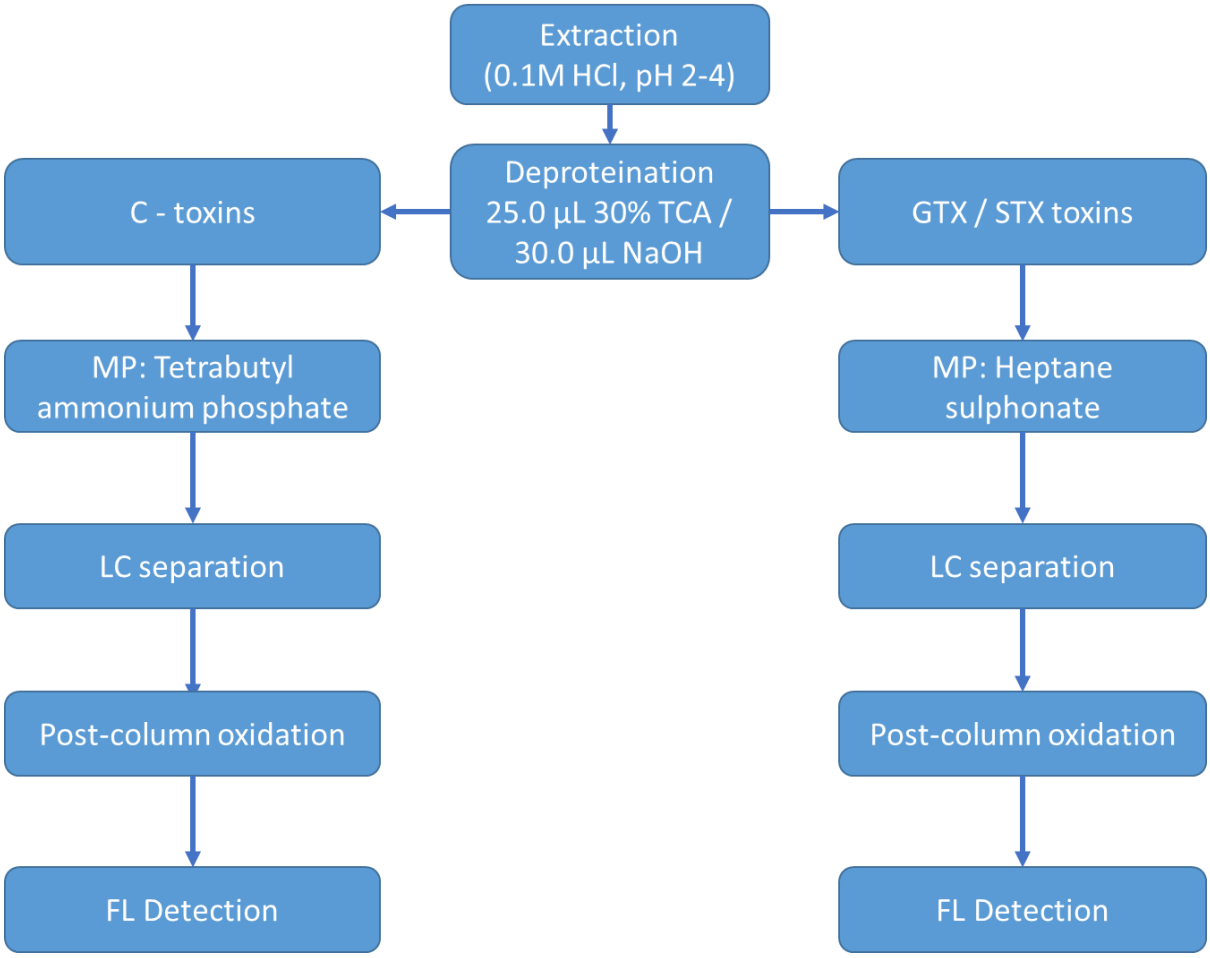


Figure 2-3: Schematic diagram of AOAC OM 2011.02 procedure.

Extraction

Tissue samples (5.0 ± 0.1 g) were extracted in 50 mL PP centrifuge tubes with 5 mL of 0.1M HCl (40.0 mL of 5M HCl made up to 2.0 L with DI water. 5M HCl prepared as 413.2 mL HCl made up to 1.0 L with DI water). The mixture was vortex mixed for 1.0 min ± 0.1 min and the pH adjusted to pH 2-4 if necessary. The mixture was placed in a boiling water bath for 5.0 ± 0.1 min, before being cooled in a basin of cold water for 5.0 ± 0.1 min, rechecking the pH and adjusting if necessary. The tubes containing the material were then centrifuged at 4500 rpm for 10 min. 500 µL of the resultant supernatant were transferred to a micro centrifuge tube, adding 25 µL 30% TCA (15.00 g ± 0.01 g TCA made up to 50.0 mL with DI

water) to deproteinate the extract, mixed well before centrifuging at 16000 x g for 5 min. Protein precipitation is induced by adding TCA which exposes more of the proteins hydrophobic structure, resulting in increased precipitation. This step is necessary as proteins could precipitate out in the reaction coil of the oxidation unit causing blockages. 20.0 ± 0.1 µL 1M NaOH were added to the supernatant, mixed well and centrifuged at 16000 x g for 5 min, before filtering through a 0.2 µm nylon disc filter into a PP HPLC vial.

LC-FLD Analysis

The same LC-FLD system described in *section 2.3.1* above was used for the PCOX analysis with fluorescence detector set to ex 330 nm, em 390 nm and fitted with an automated post-column derivatisation unit (Pickering Laboratories Inc., CA, USA) (Figure 2-4).

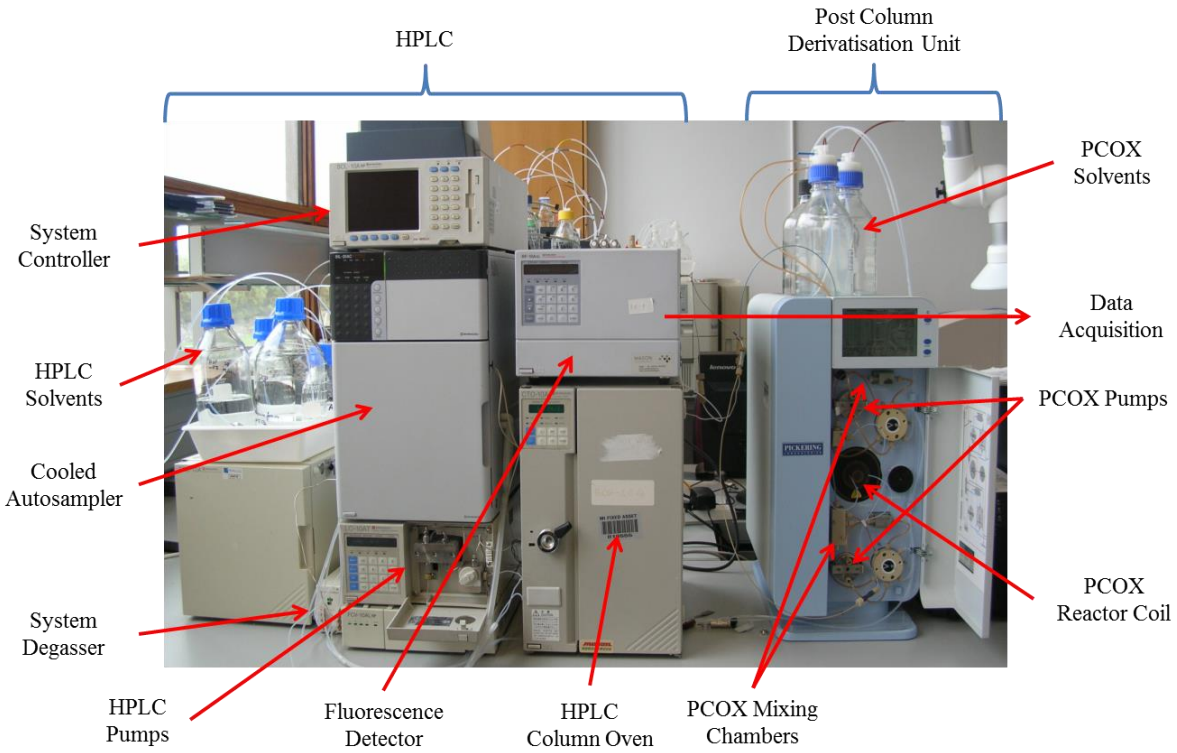


Figure 2-4: LC-FLD and post column derivatisation unit setup for OMA AOAC 2011.02.

The system was setup by connecting the LC tubing, just after the analytical column, into the derivatisation unit. Samples pass through the unit via two mixing chambers, the first used to oxidise the sample and the second to stop the reaction. The reactor coil (5 metres in length) is situated between both mixing chambers and this heats the sample to 85°C. After the reaction is stopped in mixing chamber 2 the LC tubing is plumbed back to the fluorescence detector.

The materials were analysed using two chromatographic runs as per [75], the first to determine GTX/STX toxins and the second for the C-toxins. The HPLC columns used were a Zorbax Bonus RP (150mm x 4.6mm, 3.5µm) fitted with a Zorbax guard cartridge (20mm) for the GTX/STX separation and a Thermo Betabasic 8 (250mm x 4.6mm, 5µm), fitted with a Thermo Betabasic guard cartridge (20mm) for the C-toxins. The HPLC programmes followed were as published in AOAC Official Method 2011.02 [75].

GTX/STX analysis: MP A was prepared by adding 44.0 mL 0.5M heptane sulphonate (11.01 g ± 0.01 g heptane sulphonate made up to 100 mL with DI water) to ~ 1.8 L DI water. This solution was mixed before adding 22.0 mL 0.5M H_3PO_4 (33.9 mL H_3PO_4 made up to 1.0 L with DI water), adjusting the pH of the combined solution to pH7.1 ± 0.1 with concentrated NH_4OH. The solution was transferred to a 2.0 L volumetric flask, made up to the mark with DI water before filtering through a 0.45 µm nylon filter. MP B was prepared by adding 22.0 mL 0.5M heptane sulphonate to ~ 0.8 L DI water. This solution was mixed before adding 33.0 mL 0.5M H_3PO_4, adjusting the pH of the combined solution to pH7.1 ± 0.1 with concentrated NH_4OH. 115 mL MeCN was added before transferring the solution to a 1.0 L volumetric flask, making the final volume up with DI water before filtering through a 0.45 µm nylon filter.

C-toxin analysis: MP A was prepared by adding 4.0 mL 1.0M tetrabutyl ammonium phosphate to ~ 1.8 L DI water. The pH of the solution was adjusted to pH5.8 ± 0.1 through

the addition of 1% NH_4OH before transferring to a 2.0 L volumetric flask, making up to the mark with DI water before filtering through a 0.45 µm nylon filter.

For the GTX/STX run a flow rate of 0.8 mL/min was used and the step gradient followed was 0 – 7.9 min, 0% MP B, 8.0 – 18.5 min, 100% MP B and back to 0% MP B for 5.4 min to re-equilibrate the column.

For the C-toxin analysis, a flow rate of 0.8 mL/min was used and the system was operated in isocratic mode.

POST-COLUMN DERIVITISATION

The PCOX unit was operated at 0.4 mL/min for both the oxidant and acid solutions with the reactor coil set to 85°C (reaction coil 5m x 0.5mm id). The oxidant solution was prepared by adding 400.0 mL 0.5M H_3PO_4 to ~1.2 L DI water. 200 mL 0.05M H_5IO_6 (11.40 g ± 0.01 g H_5IO_6 made up to 1.0 L with DI water) was added before adjusting to pH 7.8 ± 0.1 with 5M NaOH (20.0 g ± 0.01 g NaOH made up to 100 mL with DI water). The combined solution was transferred to a 2.0 L volumetric flask making up to volume with DI water, before filtering through a 0.45 µm nylon filter.

The acid solution was prepared by making 101.2 mL HNO_3 up to 2.0 L with DI water and filtering through a 0.45 µm nylon filter.

PST concentrations in sample extracts were quantified against a four-point calibration for each toxin and were expressed in µmol/kg. Total saxitoxin equivalents were calculated for each sample as an estimation of total toxicity using the guidance described in the AOAC official method [75].

The concentration range of the standards used during these studies are contained in Table 2-3.

Table 2-3: Concentration range of standards used for OMA AOAC 2011.02 analysis.

Toxin	Concentration Range (µmol/L)		
STX	0.019	→	0.195
dcSTX	0.032	→	0.390
GTX2	0.029	→	0.503
GTX3	0.011	→	0.191
GTX5	0.104	→	0.262
dcGTX2	0.061	→	1.713
dcGTX3	0.014	→	0.386
C1	0.031	→	2.706
C2	0.009	→	0.831
NEO	0.052	→	0.291
GTX1	0.142	→	0.733
GTX4	0.046	→	0.239

2.3.3 AOAC Official Method 959.08

The method involves the acidic aqueous extraction of shellfish tissue in 0.1M HCl. Aliquots (1 mL) were injected intraperitoneally into male albino CD1 strain mice in triplicate and toxicity (µgSTXdiHCl-eq/kg) was calculated from median death times using Sommer's tables [76]. The method was standardised using an STX certified reference standard obtained from the Institute of Marine Biosciences, National Research Council Canada (IMB, NRCC, Halifax, Nova Scotia, Canada).

2.4 Moisture Content Determination

2.4.1 Rotary Vacuum Method

Empty 50 mL PP centrifuge tubes were weighed using a 4-place balance before adding a 1.0 ± 0.1 g tissue sample into the pre-weighed tube, noting the weight of the tube and tissue combined. The tubes were then placed into a rotary drier (Jouan, Saint Herblain, France), dried under vacuum for 950 min on #4 heat setting. After completion of the run, the weights

of the dried centrifuge tubes were taken and the moisture content determined by the loss through drying as follows:

$$\textbf{\% Moisture Content} = \left(\frac{\boldsymbol{Loss\ through\ drying}}{\boldsymbol{Aliquot\ weight}}\right)\boldsymbol{x100}$$

2.4.2 KARL FISCHER TITRATION (KFT)

Volumetric KFT measurements were carried out using an Aquamax KF Volumetric (GR Scientific, Bedford, UK) on some of the freeze dried materials described in *Chapter 5*. The method was standardised using a Fluka hydranal water standard (Sigma Aldrich, Steinheim, Germany) with a certified water content of 10.02 mg/g ± 0.03 mg/g. The solid matrix samples were directly added to the vessel after standardisation with Hi-Dry KF Methanol (Sigma Aldrich, Steinheim, Germany) and titrated with a Romia one-component pyridine free KF reagent VC5 (Sigma Aldrich, Steinheim, Germany). The solvent was changed after each triplicate measurement.

2.5 QUASIMEME

All materials were prepared by the author and designed to test participants over a range of concentration levels and different complexities in toxin profile composition. The materials used were naturally contaminated with a range of PSTs and prepared following in-house procedures to ensure homogeneity. Stabilisation of the toxins and matrices was achieved using a combination of heat treatment and the addition of antibiotics and an antioxidant [113] with short-term stability and homogeneity studies performed on all materials before distribution to participants.

Table 2-4: Materials used in QUASIMEME rounds 2009-2014 with assigned codes, predominant toxins present and matrices.

Tissue	Rounds Used	Year	Codes Used	Predominant Toxins Present	Matrix
A	57	2009	QST075BT	dcSTX, STX, GTX5	*Mytilus galloprovincialis*
	61	2010	QST093BT		
	69	2012	QST132BT		
	2014-1	2014	Sample 1		
B	57	2009	QST076BT	dcSTX, GTX2,3, GTX1,4, STX, GTX5 & C1,2	*Mytilus edulis & Mytilus galloprovincialis*
	61	2010	QST095BT		
	69	2012	QST133BT		
C	61	2010	QST094BT	dcGTX2,3, dcSTX & dcNEO	*Spisula solida*
	65	2011	QST111BT		
	69	2012	QST134BT		
	2014-1	2014	Sample 2		
D	65	2011	QST113BT	GTX2,3 & STX	*Mytilus edulis*
	72	2013	QST152BT		
	2014-1	2014	Sample 3		
E	65	2011	QST114BT	GTX2,3, STX & dcSTX	*Mytilus edulis*
	72	2013	QST154BT		
F	69	2012	QST135BT	GTX2,3, STX, GTX1,4 & NEO	*Crassostrea gigas*
G	72	2013	QST155BT	GTX2,3, STX, GTX1,4 & dcSTX	*Mytilus edulis*
H	2014-1	2014	Sample 4	GTX2,3, STX, GTX1,4 & dcSTX	*Crassostrea gigas*

A total of six materials were used over the six exercises 2009-2014, with Tissues A & C being used in four rounds (R) apiece, Tissues B & D being used in three rounds, Tissue E used in two rounds and Tissues F, G & H used in one round only. Table 2-4 lists each of the materials used over the 6 year period.

No standardised method protocol was provided by QUASIMEME, apart from requesting TEFs to be used in total toxicity calculations, so participants were allowed to use either the analytical method routinely employed at their laboratories or any other method applicable to PST testing. In 2009 (R57) and 2010 (R61), the TEFs included in the protocol for use by participants in calculating total toxicity were those determined by Oshima *et al*. [17], while in

subsequent rounds 2011 (R65), 2012 (R69), 2013 (R72) and 2014 (R2014-1) TEFs recommended by the EFSA [6] were prescribed in the protocol (see Table 1-1).

Data submitted by participants depended on which method was employed at their laboratory, with all submitting a total toxicity result such that all methods could be assessed together (Table 2-5). Participants using either the preCOX, PCOX or LC-MS/MS methods of analysis, where individual analogue concentrations can be determined, could additionally submit these results, such that a data assessment and therefore z-scores could be calculated in addition to the total toxicity results. This allowed participants receiving less than satisfactory z-scores to pinpoint potential causes if they related to the determination of a particular analogue.

Table 2-5: Breakdown of methods used by participants in each of the rounds 2009-2014.

Year	Round	Methods Used					Total Number of Participants
		preCOX	PCOX	MBA	ELISA	LC-MS/MS	
2009	**57**	8	2	4	-	-	14
2010	**61**	9	5	2	-	-	16
2011	**65**	8	5	4	2	-	19
2012	**69**	9	5	2	-	-	16
2013	**72**	13	6	2	-	1	22
2014	**2014-1**	13	5	2	-	3	23

2.6 ICELANDIC SAMPLING

2.6.1 PHYTOPLANKTON SAMPLING

Samples were taken from two sites in Iceland: Eyjafjordur on the north coast and Breidafjordur on the west coast (Figure 3-1). There were two sampling sites in Breidafjordur: Flatey in the north of the fjord and Stykkisholmur in the south and one location in Eyjafjordur, Hrisey Island, located in the middle of the fjord. Phytoplankton sampling was carried out weekly from spring to autumn 2009. Toxic species were screened by net sampling

using a 20 µm mesh. The net was hauled from a depth of 5 metres to the surface several times. All samples were fixed in hexamine buffered formalin and examined under a microscope. If toxic species were detected in these net samples then 50 mL water samples were allowed to settle in a sediment chamber for 24 hours according to the Utermöhl method [114] and examined in an inverted microscope where toxic species were identified and counted [115]. Phytoplankton sampling, identification and counting was carried out by Dr. Karl Gunnarsson and his team at the Icelandic Research Institute who supplied results described in Chapter 3 (Table 3-2).

2.6.2 SHELLFISH SAMPLES

Samples were collected from two sites: Eyjafjordur (Hrisey Isalnd) on the north coast and Breidafjordur (Stykkisholmur) on the west coast between June and August 2009. Mussels at both harvesting locations are grown in mesh sleeves attached to suspended long lines. Samples were stored in their shells at <-20°C until frozen samples were dispatched in one batch to the Marine Institute on ice.

The samples were thawed and prepared by dissecting and removing the whole flesh from the shell, removing byssus threads and any fragments of shell before being homogenised using a Waring™ blender (Hartford, CT, USA). Aliquots of the homogenised tissues were then extracted and analysed, with the bulk tissues stored in PP containers frozen at -20°C.

Chapter 3

FIRST REPORT AND CHARACTERISATION OF PSP TOXINS FROM ICELAND

This chapter details work described by Burrell *et al*. 2013.

S. Burrell, T. Gunnarsson, K. Gunnarsson, D. Clarke, & A.D. Turner, (2013). First detection of paralytic shellfish poisoning (PSP) toxins in Icelandic mussels (*Mytilus edulis*): Links to causative phytoplankton species. *J. Food Con.* 31: 295-301

ACKNOWLEDGEMENT OF COLLABORATION

The work described in this chapter was carried out in collaboration with Dr. Thor Gunnarsson and Dr. Karl Gunnarsson and their teams at the Icelandic Food and Veterinary Authority and the Icelandic Research Institute respectively, as well as Mr. Dave Clarke at the MI, Ireland. From the work described in this chapter all phytoplankton sampling, identification and cell counting as well as shellfish sampling was carried out by the collaborators at their laboratories in Iceland. All shellfish samples were dissected, processed, extracted for both preCOX LC-FLD and MBA and analysed by the author at the MI laboratory. Intraperitoneal injections for MBA analysis were conducted by Mr. Dave Clarke at MI laboratories.

3.1 BACKGROUND AND AIMS

The marine sector is hugely important to the Icelandic economy. In 2009 alone marine products accounted for 42% of Iceland's total export value with the industry employing approximately 7300 people, representing nearly 4% of the overall Icelandic workforce [116].

Shellfish have been harvested commercially in Iceland over the last 40 years with Icelandic scallop (*Clamys islandica*) and ocean quahog (*Artica islandica*) being the main species harvested. Mussel farming is relatively new however, with investigations into its feasibility being carried out in 1973 and later in 1985-87 [117]. Since these initial investigations blue mussels (*Mytilus edulis*) have been grown experimentally around the coast of Iceland with harvesting figures contained in Table 3-1.

Table 3-1: Quantities of blue mussel harvested from Icelandic waters since 2009 (Results provided by the Icelandic Food and Veterinary Authority).

Year	Harvest Figures (tonnes)
2009	12
2010	32
2011	94
2012	63
2013	163
2014	48

With nearly 5000km of coastline, the Icelandic aquaculture industry has huge growth potential, making the implementation of an effective biotoxin monitoring program a necessity if the European and world shellfish markets are to be exploited.

HABs are a variable yet worldwide phenomenon and can pose severe economic risks especially to fledgling shellfish markets such as Iceland's. For human protection and as a statutory requirement, Iceland is obliged to conduct routine analysis of shellfish for regulated shellfish toxins from these harvesting sites. Due to the lack of biotoxin testing facilities in Iceland, shellfish samples have been transported to Ireland for biotoxin testing over the last

decade. Since 2005 toxic species of phytoplankton have been monitored in three fjords around the coast of Iceland, Eyjafjordur on the central north coast, Breidafjordur on the northwest coast and Hvalfjordur on the southwest coast (Figure 3-1) [115]. Phytoplankton sampling is carried out weekly from spring to autumn and closure of these sites for harvesting shellfish is recommended when cell numbers exceed 500 cells/L of *Alexandrium spp*.

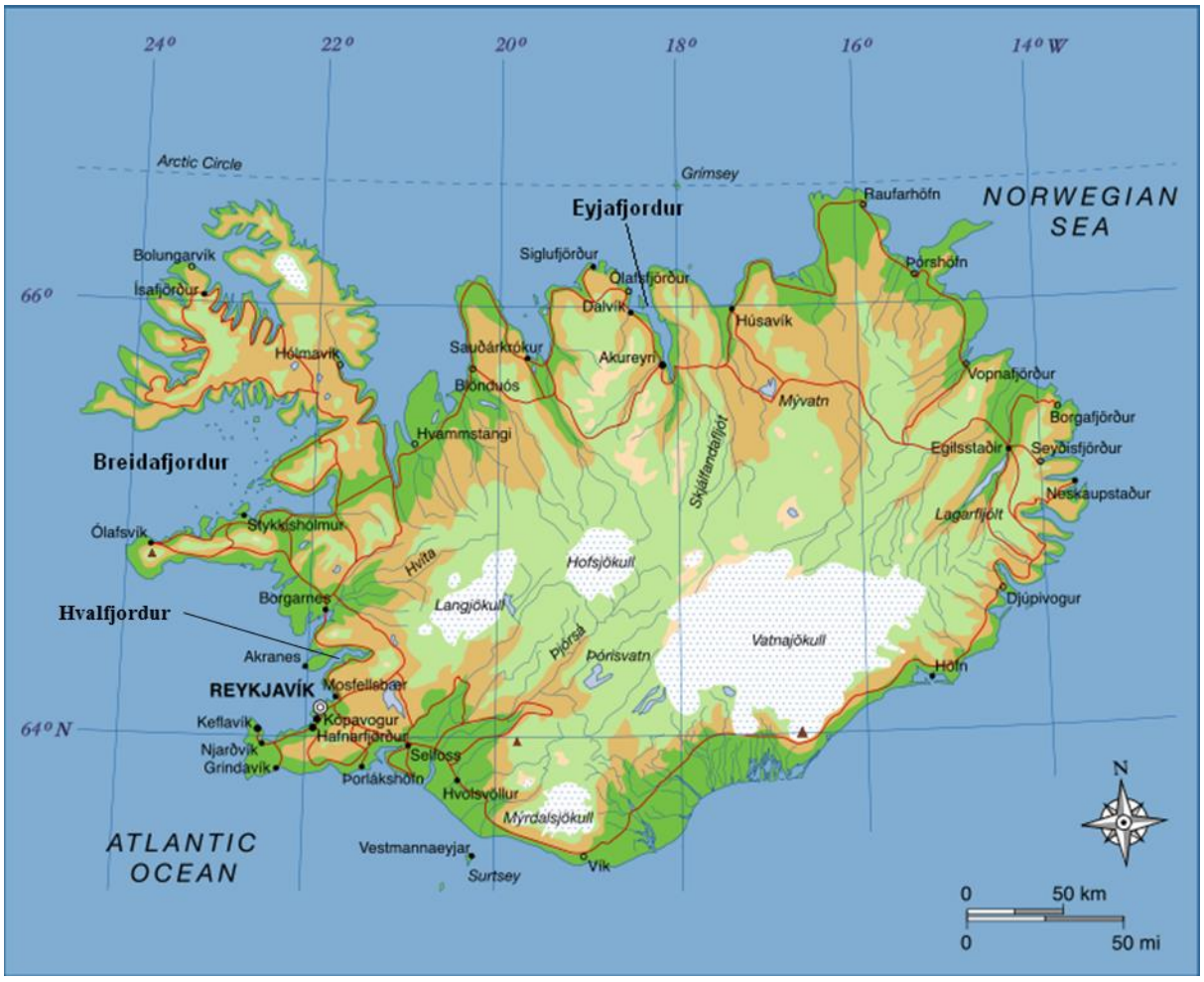


Figure 3-1: Map of Iceland showing shellfish production sites Hvalfjordur, Breidafjordur and Eyjafjordur.

In order to prepare the RMs for the studies described in this thesis, it was necessary to source relatively large quantities of shellfish tissues containing high levels of PSTs. The lack of facilities at the MI for conducting shellfish feeding experiments coupled to the fact that PSP toxins rarely rise above the EU regulatory limit in Irish samples necessitated sourcing these

toxins outside of Ireland. Shortly after commencing the research for this thesis in 2009, routine PSP testing by MBA confirmed very high PST levels never seen before in Icelandic samples.

The research contained in this chapter therefore, had two main aims. Firstly to collect and characterise *M. edulis* samples from fjords commercially producing this species and present the data as a first time report of PST profiles in Icelandic waters. Secondly to obtain and characterise large quantities of naturally contaminated shellfish tissues for the development work described in later chapters.

Data is presented from the analysis of whole flesh *M. edulis* samples collected from two of these fjords Breidafjordur located on the west coast and Eyjafjordur on the north coast during a bloom of *Alexandrium* spp. in 2009. Samples were analysed for PSP toxicity by MBA with additional confirmatory analysis carried out by LC-FLD to determine toxin profiles and total saxitoxin equivalents.

During this period a request was submitted to the Icelandic Food and Veterinary Authority by the author to harvest *M. edulis* samples in order to build-up stocks of contaminated shellfish at the MI. Consequently approximately 30 kg of shellfish meat in the shell was harvested and sent to the author under frozen conditions.

The contamination of blue mussels with PSTs in Iceland in 2009 represents a new and unique geographical location for the occurrence of these toxins, and one which may potentially result in a serious impact upon the livelihood of Icelandic shellfish producers and exporters.

3.2 RESULTS

3.2.1 TOXIC PHYTOPLANKTON SPECIES

Results obtained from the Icelandic phytoplankton monitoring program have shown variable levels of toxic species present since 2005.

3.2.1.1 BREIDAFJORDUR

Between the years 2005-2007, in Breidafjordur (Flatey), no *Alexandrium* spp. were found in any samples taken. In 2008 cell numbers exceeded 500 cells/L only once in late May of that year [115] but in June and July 2009 however, cell numbers of over 3500 cells/L were recorded at this site (data not shown).

Alexandrium spp. from the other sampling location in Breidafjordur, Stykkisholmur, have been found infrequently and in very low numbers in the years 2005-2008. In 2009 however high densities of cells were found, starting in late June and persisting until the middle of July, peaking at over 16,000 cells/L (Table 3-2).

3.2.1.2 EYJAFJORDUR

At Hrisey Island in Eyjafjordur, *Alexandrium* spp. have been observed each year from 2005 – 2008 with cell densities >6000cells/L found in 2005 (data not shown). In 2009 *Alexandrium* spp. peaked twice, firstly at over 8,000 cells/L in June and secondly at over 10,000 cells/L in July (Table 3-2).The *Alexandrium* populations detected in phytoplankton samples from both fjords were mainly composed of *A. tamarense* with small numbers of *A. ostenfeldii* being found in samples with very high cell counts overall.

Table 3-2: Phytoplankton cell counts taken during May to September 2009 from Eyjafjordur and Breidafjordur, Iceland.

Sample	Sampling Date	Cell Counts (*Alexandrium spp.)* cells/L	
		Eyjafjordur	Breidafjordur
1	25/05/2009	0	-
2	02/06/2009	0	-
3	08/06/2009	620	-
4	14/06/2009	1,000	-
5	15/06/2009	-	260
6	18/06/2009	1,300	-
7	21/06/2009	1,520	-
8	25/06/2009	2,200	-
9	26/06/2009	-	4,208
10	28/06/2009	8,750	-
11	30/06/2009	-	16,680
12	09/07/2009	360	6,500
13	13/07/2009	1,540	-
14	17/07/2009	-	1,880
15	20/07/2009	2,160	-
16	23/07/2009	10,920	-
17	28/07/2009	6,400	-
18	31/07/2009	-	160
19	05/08/2009	80	-
20	08/08/2009	20	-
21	10/08/2009	-	0
22	12/08/2009	120	-
23	18/08/2009	40	-
24	23/08/2009	60	-
25	26/08/2009	-	0
26	31/08/2009	20	-
27	06/09/2009	0	-
28	13/09/2009	0	-

3.2.2 MBA AND LC-FLD TOXICITY DATA

3.2.2.1 BREIDAFJORDUR

The MBA and LC-FLD toxicity data from Breidafjordur is presented in Table 3-3. The first mussel sample was collected on the 30/06/09 when toxicity was already over three times the regulatory limit. The toxicity rose to over 4 times this limit by the second sample taken on the

10/07/09 before dropping over the next 4 weeks to levels below this regulatory action level. The highest total toxicity result was observed in sample 2, with an MBA result of over 4500 µgSTXdiHCl-eq/kg.

Table 3-3: MBA and preCOX LC-FLD data of mussel (M.edulis) samples harvested from Breidafjordur, Iceland.

Sample	Sampling Date	Concentration (µmol/kg)			Total Toxicity µgSTXdiHCl-eq./kg	
		GTX-2,3	STX	GTX-1,4	HPLC-FLD	MBA
1	30/06/2009	6.06	2.25	1.24	2652	3800
2	10/07/2009	6.6	3.18	1.39	3175	4694
3	16/07/2009	2.55	1.44	0.47	1280	1141
4	01/08/2009	0.41	0.24	n.d	181	<LOQ
5	13/08/2009	0.13	0.12	n.d	74	<LOQ
6	26/08/2009	0.07	0.1	n.d	53	<LOQ

n.d. Toxin not detected
LOQ for MBA 280 µgSTXdiHCl-eq./kg

3.2.2.2 EYJAFJORDUR

The MBA and LC-FLD toxicity data generated from the analysis of the Eyjafjordur mussel samples collected during 2009 is presented in Table 3-4. Toxicity was found to be below but close to the regulatory action limit of 800µgSTXdiHCl-eq/kg in early June, seen in sample 1, but was found to rise quickly to nearly 10 times the limit in sample 2 within the subsequent two weeks.

Table 3-4: MBA and preCOX LC-FLD data of mussel (M.edulis) samples harvested from Eyjafjordur, Iceland.

Sample	Sampling Date	Concentration (µmol/kg)			Total Toxicity µgSTXdiHCl-eq./kg	
		GTX-2,3	STX	GTX-1,4	HPLC-FLD	MBA
1	08/06/2009	0.02	1.44	n.d	540	720
2	21/06/2009	9.05	6.18	3.39	5583	7460
3	28/06/2009	12.4	9.97	5.6	8564	8510
4	08/08/2009	2.63	0.86	0.48	1086	1050
5	23/08/2009	0.45	0.7	n.d	361	550
6	31/08/2009	0.28	0.54	n.d	264	440

n.d. Toxin not detected
LOQ for MBA 280 µgSTXdiHCl-eq./kg

This emphasises the speed with which these toxins can accumulate in shellfish tissue during toxic phytoplankton blooms and the importance of conducting regular routine flesh

monitoring. Toxicity levels remained high for a further 6-8 weeks and did not drop below regulatory limits until the end of August, sample 5. The highest total toxicity result was observed in sample 3, with results by LC-FLD and MBA of over 8500µgSTXdiHCl-eq/kg.

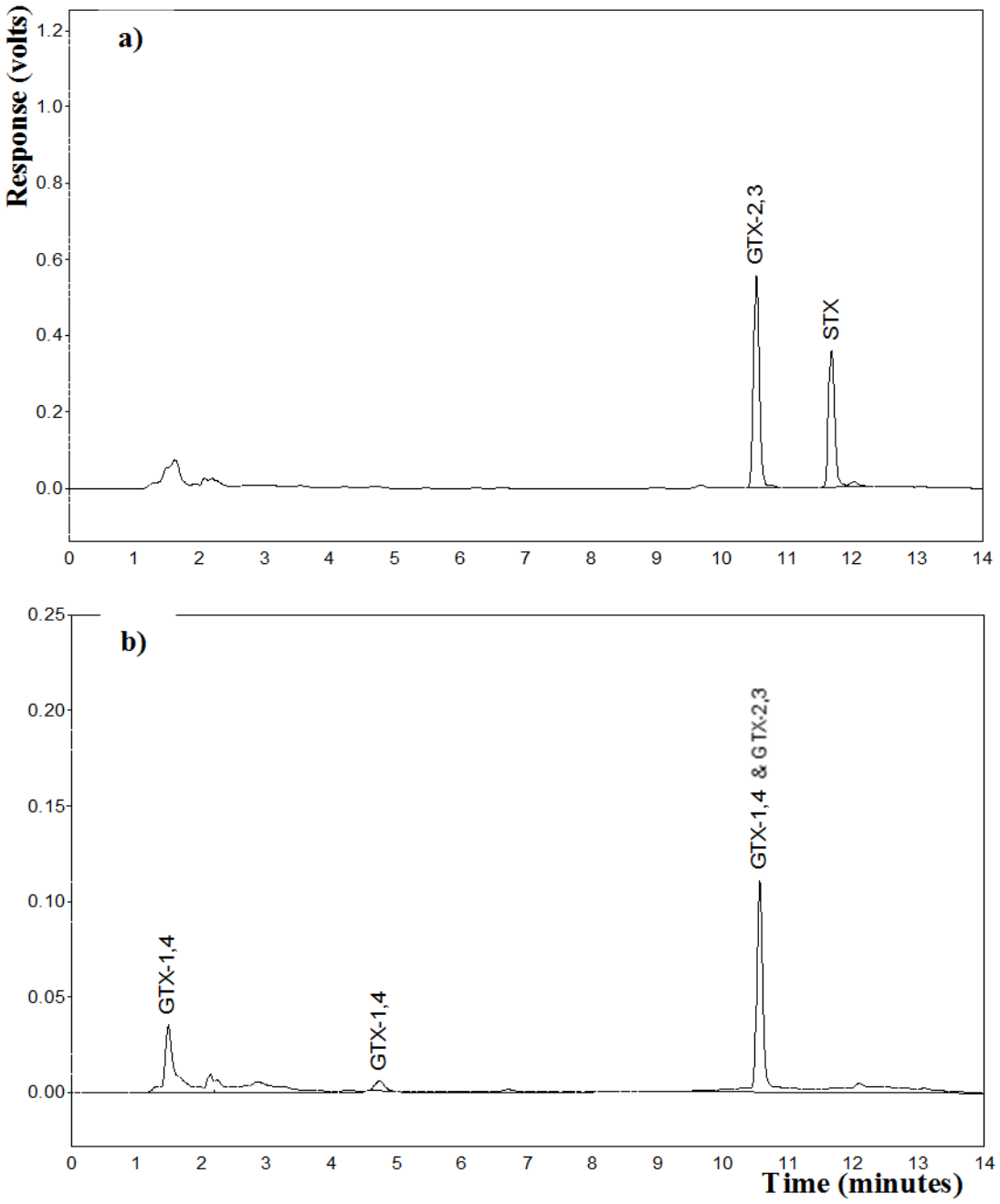


Figure 3-2: preCOX LC-FLD separation of PSP toxins present in an Icelandic sample from Breidafjordur in 2009 a) after SPE-C_{18} cleanup and peroxide oxidation and b) after SPE-ion exchange cleanup and periodate oxidation of fraction 2.

Chromatograms taken after a) peroxide and b) periodate oxidation of a sample from Breidafjordur are presented in Figure 3-2. These showed the clear presence of STX, GTX2,3 and GTX1,4 toxins. Results from both fjords showed the absence of any chromatographic peaks which may relate either to other PSP toxins or metabolic products. Analysis of unoxidised extracts of the samples revealed no interfering matrix co-extractives (data not shown) which may have interfered with the qualitative identification of the PSP toxins and subsequently compromised toxin quantitation.

3.3 DISCUSSION

Conditions within both fjords during the sampling periods were favourable for phytoplankton growth as confirmed through the data presented in Table 3-2, where cell counts of *Alexandrium* spp. reached record levels in both Eyjafjordur and Breidafjordur.

The exact causes of the high cell numbers observed is unknown and could be due to a number of factors. Temperature and salinity increases along the west and north coasts have been observed over the last decade due to a stronger inflow of Atlantic waters into these grounds [115]. It is unclear from results obtained to date whether these trends are related in any way to the effects of climate change or, as is more probable, relate to natural cyclic variations such as oscillations to the North Atlantic subpolar gyre [118,119]. Warmer more saline subtropical waters can spread north and westwards when this gyre weakens, as it controls the flow trajectory of the North Atlantic Current. A weakening of this gyre has been observed over the last decade which could explain the temperature and salinity increases observed by Gudfinnsson *et al*. [115].

A comparison of the results obtained from both the algal cell counts and the toxicity tests are illustrated in Figure 3-3. A clear correlation is evident between the high cell counts recorded

and flesh samples containing higher concentrations of PSTs. Notably, the data from Breidafjordur indicates a time delay between the highest concentrations of algae and toxin levels recorded in the flesh.

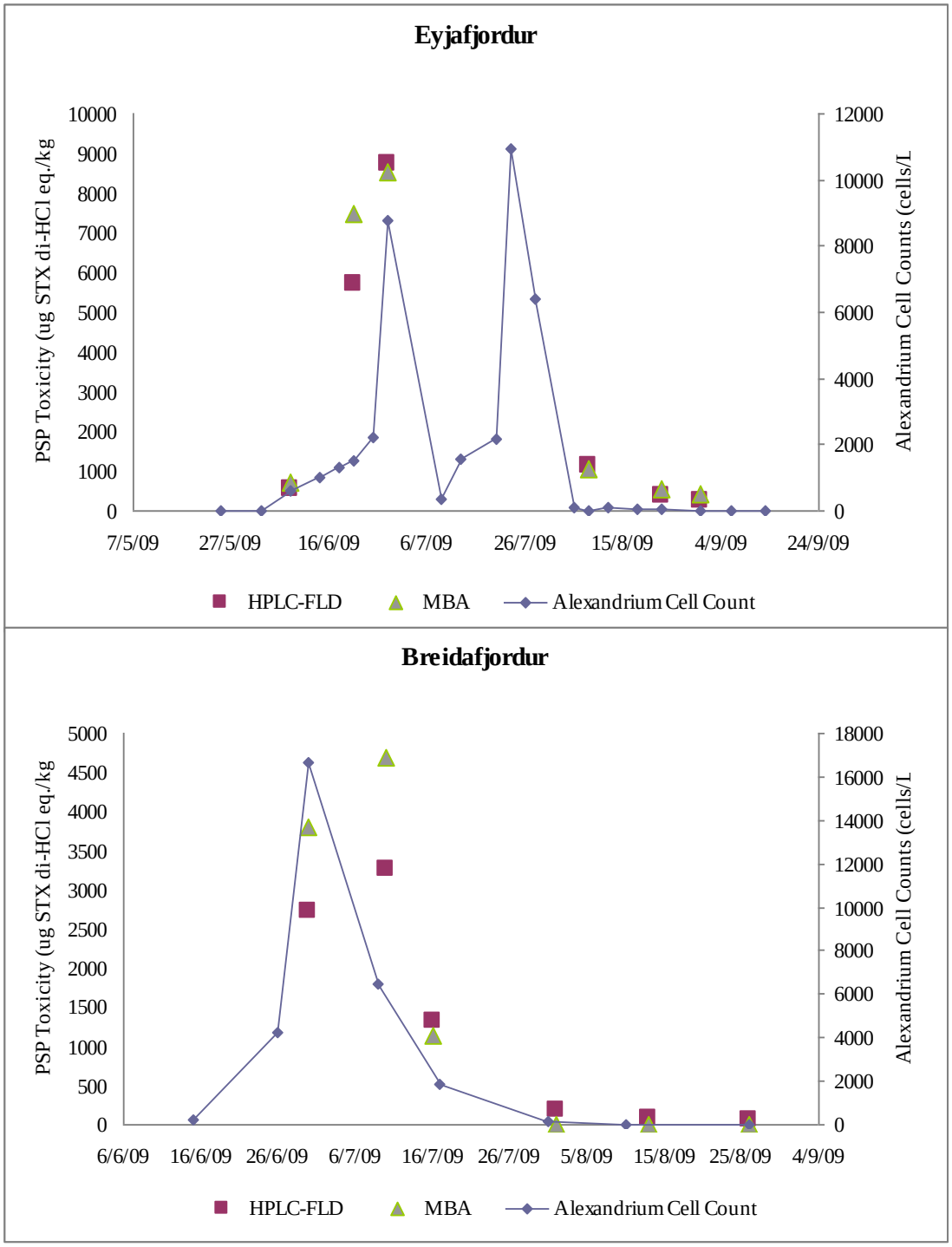


Figure 3-3: Comparison of Alexandrium Cell Counts in the water (cells/L) and total sample toxicity of the harvested mussels (µg STX di-HCl eq./kg) returned by both LC-FLD and MBA in a) Eyjafjordur and b) Breidafjordur.

There is also a clear relationship between the reduction of algal cells and the total toxicity determined in the flesh samples. Unfortunately, an absence of flesh samples collected from Eyjafjordur in July 2009 prevents an actual comparison between the toxicity of the flesh and the Alexandrium cell count during the second algal bloom at this location.

3.3.1 TOXIN PROFILE DETERMINATION

The preCOX LC-FLD method [41] has been proven as a valuable tool in the qualitative and quantitative determination of PSP toxins in shellfish [120]. The epimeric pairs (e.g. GTX2 and GTX3, GTX1 and GTX4, C1 and C2 and dcGTX2 and dcGTX3) are not separated analytically using this LC-FLD method and are therefore presented as a combined sum using the higher toxicity factor of the two co-eluted epimers to calculate total toxicity. Through analysis using this method the toxin profile was determined and found to be similar in both fjords with samples predominated by the carbamate toxins GTX2,3. STX was the next most abundant toxin present with GTX1,4 observed in half the samples analysed (Figure 3-4).

The toxin profiles determined in these samples are similar to those found in other areas where *Alexandrium spp*. predominates such as the UK [121] where the toxins GTX2,3 and STX predominate with lower levels of GTX1,4, NEO and GTX5 also being found, or in Ireland where GTX2,3 has been found to predominate [122] with lower relative concentrations of STX and GTX1,4 being determined (MI internal NMP data).

Interestingly, there is no indication of the presence of any of the N-sulfocarbamoyl toxins such as C1,2, which have been found to occur in mussels containing PSP toxins in some UK waters since 2008 [121] and which are associated with a number of different strains of *Alexandrium spp*. The Norwegian PSP toxin profile typically observed is slightly different to that observed in Iceland, being predominated by GTX1,4, with both NEO and STX being

found at lower relative concentrations [123]. The differences between profiles in the region and Iceland's is mainly the absence of the toxins NEO and C1,2 from samples analysed.

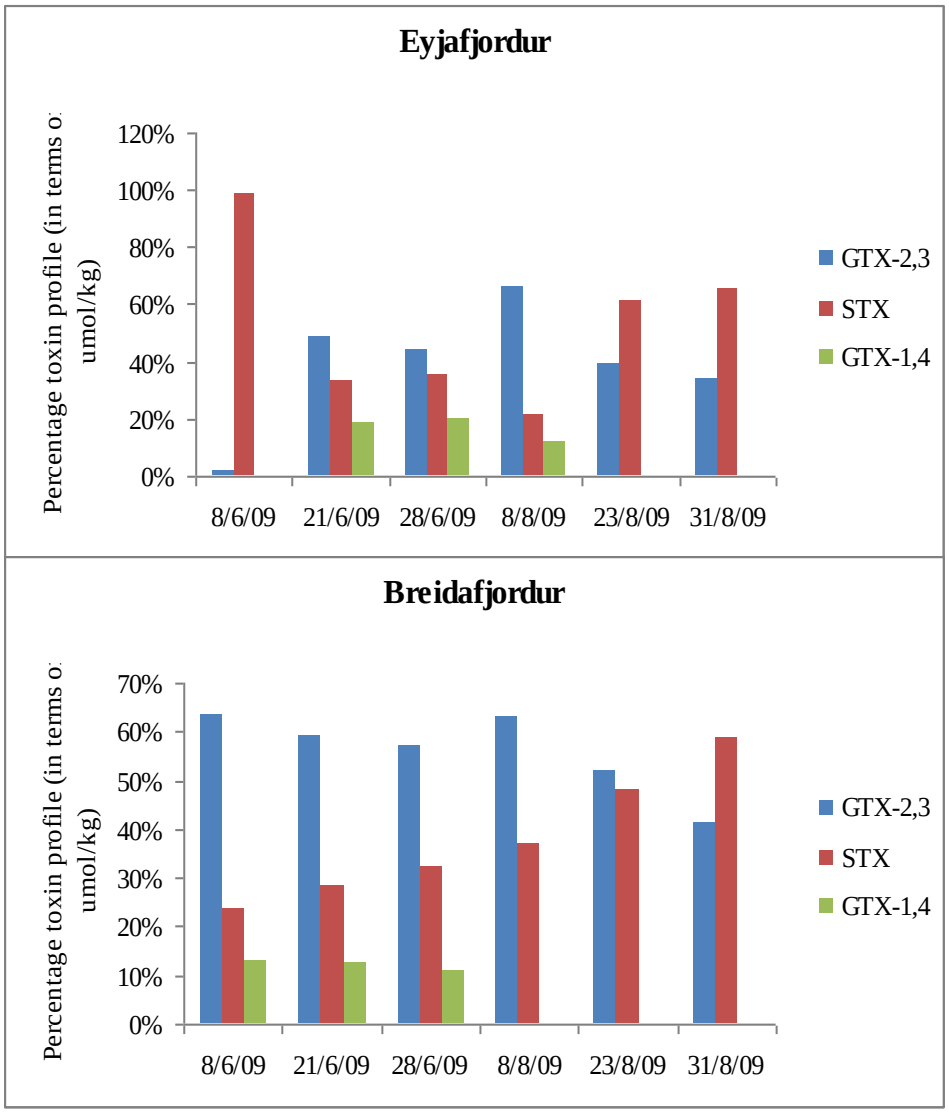


Figure 3-4: Percentage PST profiles determined by LC-FLD (in terms of µmol/Kg) from mussel samples collected from a) Eyjafjordur and b) Breidafjordur.

Profiles of *A. tamarense* mainly consist of the N-sulfocarbamoyl toxins, C1,2 and the high potency carbamate toxins GTX1-4, NEO and STX [124,125]. Profiles of A. ostenfeldii can contain the spirolides as well as the PSTs GTX6, C1,2 and GTX2,3 [126,127] The absence of the N-sulfocarbamoyl toxins C1,2 from mussel samples taken from both harvesting areas, if

not relating to the toxin profile in the source algae, could instead be due to the metabolic conversion of these toxins in shellfish to GTX2,3 via desulfonation and epimerization [128].

This hypothesis could explain the high concentrations of GTX2,3 found in samples as evidenced in Figure 3-4. The percentage toxin profile presented in this figure shows similarities between both fjords with GTX2,3 being the predominant toxins present in early samples taken in June and early August, although a discrepancy is noted in the data set with STX being the predominant toxin found in the Eyjafjordur sample from the 08/06/09. The ratio of GTX2,3 to STX changes by late August with STX becoming the predominant toxin present. Again this could relate either to changes in the toxin ratios present within the algal food source or alternatively relate to the potential toxin transformation of GTX2,3 to STX via desulfonation [129]. However it is noted that these in vitro experiments by Fast *et al.* were only carried out on clam tissues.

It is interesting to note that although the *Alexandrium* cell counts found in Breidafjordur (Figure 3-3) were considerably higher than those found in Eyjafjordur, the same ratio was not evident in the toxicity results of the mussel samples. The total PSP toxicity found in mussels from Eyjafjordur was nearly twice that found in mussels from Breidafjordur. This may relate to slower uptake of PSTs by mussels found in Breidafjordur compared to Eyjafjordur or a greater composition of non-toxin producing *Alexandrium* species present in Breidafjordur.

The absence of GTX1,4 in samples taken in early June and late August from Eyjafjordur and early August onwards from Breidafjordur is likely due to the low overall toxicity of these samples and the lower relative sensitivity of the N-hydroxylated toxins to their non-hydroxylated counterparts when analysed using OMA AOAC 2005.06 [120].

It is imperative therefore to have adequate knowledge of specific toxin profiles for the analysis and risk management of this group of potent neurotoxins due to the range of relative

toxicities exhibited by the various analogues. These results highlight the presence in Iceland of some of the most toxic PSP toxins as well as levels of toxicity which may provide a serious risk to the human consumer.

3.3.2 CHEMICAL AND BIOLOGICAL METHOD ANALYSIS

Toxicity results returned by both the reference MBA method and the preCOX LC-FLD method appear to correlate reasonably well for these samples (Figure 3-3), as observed previously in this species for mussels sampled from within UK waters [120]. Overall the MBA method gave slightly higher values compared to the preCOX LC-FLD as evidenced in Table 3-3 and Table 3-4, although a variability in this ratio is noted.

It is also clear from the results generated from samples 4-6 from Breidafjordur (Table 3-3), that the preCOX LC-FLD method provides useful data on the toxicity of samples containing levels of PSTs lower than the MBAs limit of quantitation. This again shows the usefulness of the preCOX LC-FLD method for the early warning of toxicity, especially important given the rapid increases in PSP toxin levels observed in these areas (Table 3-4). These results therefore clearly demonstrate the importance of a regular effective toxicity monitoring regime, without which there would be a clear potential risk to human consumers to toxic bloom events.

The level of observed time delay between the peaks in phytoplankton cell presence found in the water and the maximum levels of toxicity found in shellfish (Figure 3-3) is also of interest. At Breidafjordur, the peak in toxicity appears approximately two weeks after the measured maximum of *Alexandrium* cells. This observation is consistent with those observed previously from water and flesh samples collected in the St. Lawrence region, Canada [130]

or from Busta Voe Lee North, Scotland [131] where time delays of over 7 days have been found.

3.4 CONCLUSIONS

These novel findings represent the first report of PSP toxins in shellfish from Iceland and furthermore indicate the potential increase in the presence of the toxins and causative phytoplankton over the past few years. It is difficult to ascertain however, if this increase is due to the application of phytoplankton monitoring in Icelandic waters or truly represents an increase in the incidence of these toxic dinoflagellates. With the increasing economic importance placed upon the shellfish industry in Iceland, this highlights the importance of continued monitoring of both shellfish toxicity and their causative organisms. A full and thorough risk assessment can then be produced, for the occurrence of PSP in Icelandic waters so as to provide the necessary information to ensure an appropriate biotoxin monitoring programme is continued. Ongoing work will continue with the analysis of both water and flesh samples from both current and developing shellfish harvesting beds and over time build up more data on the timing and intensity of the algal blooms and the subsequent shellfish toxin accumulation. Further data will allow the ongoing assessment of the presence and variability of PSP toxicity and toxin profiles, ultimately providing an essential resource to ensure the continued development of the Icelandic shellfish production program.

Chapter 4

STABILISATION TECHNIQUES

4.1 BACKGROUND AND AIMS

As discussed in *Chapter 1*, homogeneity and stability are two of the most important parameters to consider in the preparation of RMs. In order for a material to be considered fit for purpose, the biological matrix and the PSTs themselves must be stable during transportation and throughout the materials use as well as ensuring uniformity across the entire production lot. Previous studies have proven the role of bacteria present in the digestive glands of shellfish in the biotransformation of PSTs [132], a process which may result in unstable PST concentration over time. Techniques investigated in this chapter, aimed at reducing or eliminating the bacterial effects, lowering the rates of toxin degradation, epimerization and biotransformations, should improve PST and matrix stability for RM production. Although the processing and dispensing procedures for all materials prepared in this study were not carried out aseptically, reducing or eliminating bacteria present in the source tissues, through the techniques investigated, significantly improves the stability of biological matrix RMs [52].

The stabilisation procedures investigated in this chapter include the effects of preserving additives, specifically antibiotics and an antioxidant spiked into tissue during preparation, thermal treatment of source tissues and the novel application of HPP. The use of preserving additives and the application of a thermal treatment step to sterilise source tissues has previously been shown to improve the stability of certain phycotoxins [54,60] but, along with HPP have not been investigated for the production of PST RMs. Due to the intended use of some of the materials prepared in this chapter, specifically in Quasimeme PT schemes where participants may use MBA, it was also necessary to determine if the preserving additives

would have any adverse effects on the animal assay, specifically inducing PSP like symptoms which could affect method interpretation.

The study was broken down into distinct sections which investigated the following:

- Spiking tissues with an antioxidant (ethoxyquin)
- Spiking tissues with antibiotics (ampicillin, erythromycin and oxytetracycline)
- Combined use of the preserving additives described above
- Thermal treatment
- Combined thermal treatment and spiking with the preserving additives described above
- HPP
- Untreated control materials

4.2 PRESERVING ADDITIVES

This section of the study investigated the separate use of an antioxidant, ethoxyquin and three antibiotics, ampicillin, erythromycin and oxytetracycline and their effects on biological matrix stability and the PSTs present (Figure 4-1). These preserving additives have previously been shown to improve the stability of domoic acid and AZA3 however they provided no additional stability improvements for the other toxins covered in that study, AZA1, -2, OA or DTX2 [54,60]. To the author's knowledge this is the first time these preserving additives have been investigated and compared directly to untreated control materials for their stabilisation effects on PSTs.

4.2.1 MATERIALS AND METHODS

4.2.1.1 SOURCE TISSUES

The Canadian, Norwegian, Spanish, Icelandic and blank toxin free Irish tissues as listed in Table 2-1 were used to prepare the RMs used in this section of the study. The tissues were naturally contaminated with GTX2,3, STX, GTX1,4, NEO and dcSTX and were diluted with a blank toxin free mussel tissue due to the high levels of PSTs present in the contaminated tissues. It was necessary to combine different source tissues in order to gain a more comprehensive toxin profile than a single tissue alone would provide, therefore the PST NEO, although present in sufficient quantities in the source tissue, was diluted to levels below the limit of detection (<LOD) of the preCOX and PCOX LC-FLD methods of analysis. For this reason stability and homogeneity of this toxin was not investigated.

An initial bulk tissue (>2.0 kg), containing the quantities detailed in Table 4-1 was prepared and subdivided into four separate lots, sealed in PP containers and stored at -20°C until further use. The four lots were then used to investigate the separate use of three antibiotics

combined (Tissue 1), an antioxidant (Tissue 2), and an untreated control material (Tissue 3). The fourth lot was prepared for use in freeze drying experiments as described in *Chapter 5, Section 5.5*.

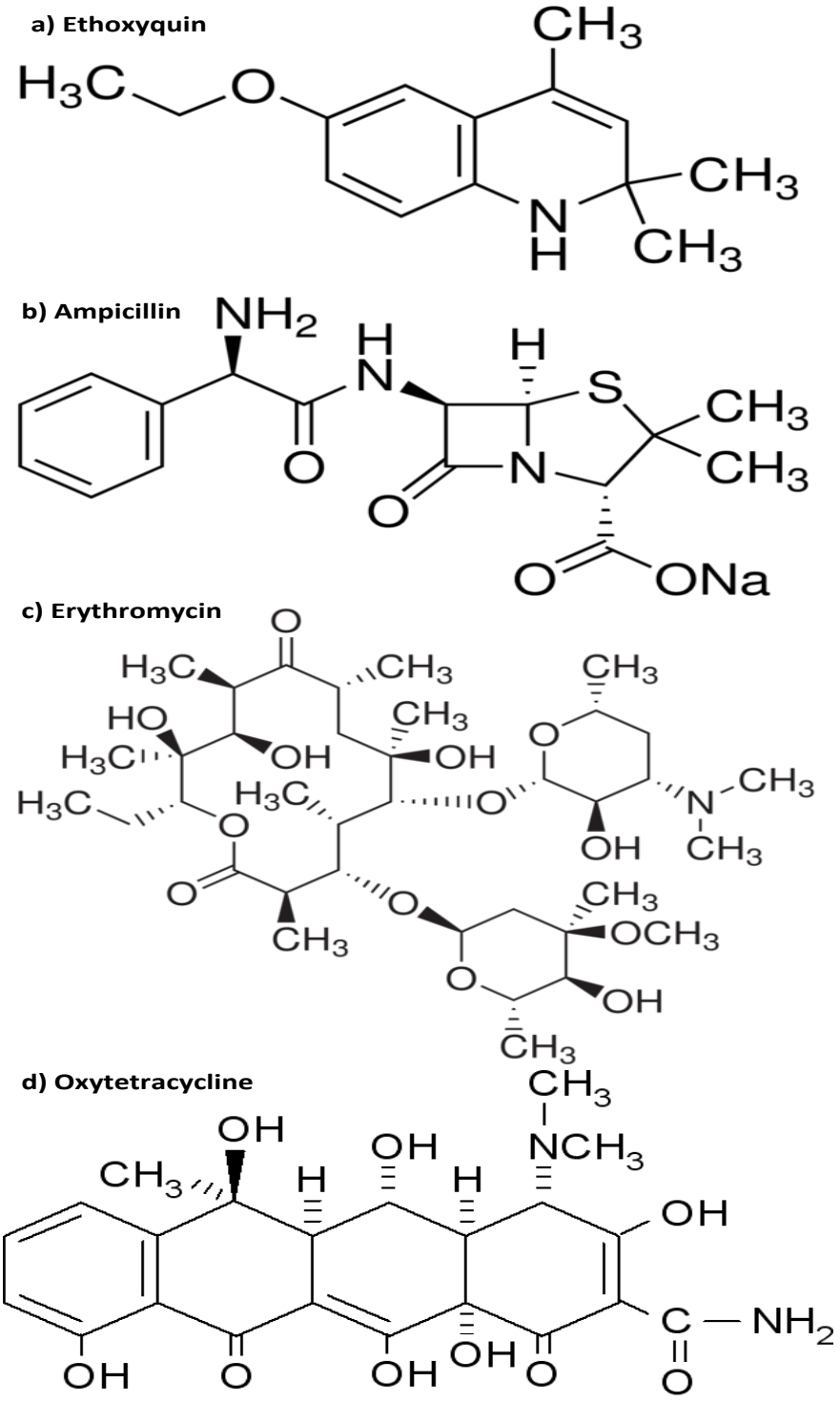


Figure 4-1: Chemical structures of the four additives used to stabilise the PSTs.

The bulk tissue was prepared by combining the proportions of tissues detailed in Table 4-1 in a 5 L Waring™ goblet. To ensure a good degree of homogeneity of the initial bulk tissue, in-house RM procedures (Standard Operating Procedure (SOP) No. BCT-058) were followed which have previously been shown to produce sufficiently homogeneous RMs [54]. This specifically involved homogenising the tissues on medium power for 5.0 min, scrapping down the insides of the goblet intermittently to ensure thorough mixing.

Table 4-1: Quantities of source wet frozen tissues used to prepare the bulk homogenate for the additives and freeze drying studies.

Country	Species	Quantity (kg)
Canada	*Mytilus edulis*	0.01
Norway	*Mytilus edulis*	0.18
Iceland	*Mytilus edulis*	0.38
Spain	*Mytilus galloprovancialis*	0.95
Ireland	*Mytilus edulis*	0.63

Aliquots were taken for MC determination (n=3) using the rotary vacuum method and analysis by preCOX LC-FLD (n=1) as described in the materials and methods *sections 2.4.1* and *2.3.1* respectively, to give approximate PST concentrations in the bulk tissue. The bulk homogenate was immediately subdivided into 3 x ~0.5 kg (Tissues 1-3) and 1 x ~0.7 kg (freeze drying experiment in *Chapter 5*) sub-samples by pouring the required amount of tissue into PP containers, sealing with lids and parafilm before storing at -20°C until required for use. The PST levels and MC results from this bulk homogenate are presented in Table 4-2.

Table 4-2: Toxin profile, concentrations and moisture content of bulk tissue used to prepare Tissues 1-3.

Material	Concentration (µmol / kg)						Total Toxicity (µg STX diHCl-eq / kg)	% Moisture Content
	dcSTX	GTX2,3	GTX5	STX	GTX1,4	neoSTX		
Bulk homogenate	0.65	3.5	0.05	1.99	1.9	0.71	2737	80.6

4.2.1.2 ADDITIVES MATERIAL PREPARATION

Both the antibiotics (Tissue 1) and antioxidant (Tissue 2) spiked materials were prepared similarly as follows. The frozen bulk homogenate described in *section 4.2.1.1* was removed from the freezer and allowed to defrost overnight in a fridge at +4°C. The preserving additives were prepared by dissolving in ethanol (1% w/w) and adding to the tissue homogenates to give 0.02% w/w as described previously by McCarron *et al.,* for domoic acid in shellfish [60]. For the antibiotics spike, 0.12 ± 0.01 g each of ampicillin, oxytetracycline and erythromycin were weighed into a 50 mL PP centrifuge tube using a calibrated 3-place balance. 6.0 ± 0.1 g ethanol was added to the centrifuge tube, capped and vortex mixed (Vortex Genie-2, Scientific Industries, NY, USA) for 5.0 min. The antioxidant spike was prepared in the same way, weighing 0.12 ± 0.01 g ethoxyquin into a 50 mL PP centrifuge tube with 6.0 ± 0.1 g ethanol and vortex mixing for 5.0 min.

The defrosted tissues were further mixed by hand using a spatula before transferring 464 ± 1 g into a Waring™ goblet. The additives/ethanol solution was then spiked into the tissue, washing out the centrifuge tube with a small volume of DI water before manually mixing with a spatula. The antibiotics/ethanol solution was spiked into tissue A and the antioxidant/ethanol solution spiked into tissue B. The final weights of both tissue A and B were made up to 600 ± 1 g with DI water in order to obtain a final tissue with a MC of ~85% to better reflect that found in a natural mussel matrix.

The tissues were homogenised using a Waring™ blender on medium power for 5.0 min, scrapping down the vessel walls intermittently to ensure thorough homogenisation. The tissues were transferred to PP beakers and further homogenised using a Polytron™ mixer for 30.0 min using a coarse head attachment and for 20.0 min using a fine head attachment. The Polytron™ homogenisation steps were carried out with the PP beaker placed in an ice-bath to

prevent localised heating through friction of the apparatus during homogenisation which could result in evaporation and an altered final MC.

The materials were dispensed as 5.3 ± 0.1 g aliquots using a calibrated peristaltic pump (Bernant, IL, USA) into 5 mL PP vials (Teklab Ltd., Durham, UK) to ensure minimum retrieval amounts of 5.0 g, sufficient for preCOX method extraction. The tubes were hermetically sealed with aluminium lids under a stream of argon using a manual heat sealer (MK 1, Seal-it-Systems, Lancashire, UK), before being fitted with wadded (polyethylene disc) screw caps. A total of 110 aliquots of Tissue 1 and Tissue 2 were dispensed providing a sufficient quantity for short (n=45) and long-term (n=45) stability studies as well as homogeneity (n=14) and MC (n=3) determinations. All materials were stored at -80°C until required.

4.2.1.3 UNTREATED CONTROL MATERIAL PREPARATION

The untreated control material (Tissue 3) was prepared by defrosting the bulk homogenate described in *section 4.2.1.1* overnight in a fridge at +4°C. The defrosted tissue was manually mixed using a spatula before transferring 464 ± 1 g into a Waring™ goblet. The final weight of the tissue was made up to 600 ± 1 g with DI water in order to obtain a final tissue with a MC of ~85% to better reflect that found in a natural mussel matrix.

The tissue was homogenised using a Waring™ blender on medium power for 5.0 min, scrapping down the vessel walls intermittently to ensure thorough homogenisation. The tissue was transferred to a PP beaker and further homogenised using a Polytron™ mixer for 30.0 min using a coarse head attachment and for 20.0 min using a fine head attachment. The Polytron™ homogenisation steps were carried out with the PP beaker placed in an ice-bath.

The material was dispensed as described in *section 4.2.1.2* above. A total of 110 aliquots of Tissue 3 were dispensed providing a sufficient quantity for short (n=45) and long-term (n=45) stability studies as well as homogeneity (n=14) and MC (n=3) determinations. All materials were stored at -80°C until required.

4.2.1.4 STUDIES AND ANALYSIS

The between-bottle homogeneity of the materials prepared in *sections 4.2.1.2* and *4.2.1.3* above were assessed through the intra-batch analysis of 14 ($3n^{1/3}$) aliquots selected through stratified random sampling of the entire fill series. This involved the division of the population into smaller groups known as strata, from which simple random sampling or systematic sampling is applied to each stratum.

All materials were stability tested over a short term (ca. 32 day) and long term (ca. 12 month) period following a reverse isochronous experimental design [55] to negate day-to-day instrumental variations. The short term study was conducted with triplicate samples consisting of five time points (0, 3, 8, 16 and 32 days for Tissue 1; 0, 4, 8, 17 and 32 days for Tissue 2 and 0, 4, 6, 17 and 31 days for Tissue 3) and three temperature conditions (-20°C, +4°C and +40°C). The time points used in the study of each material were slightly different to each other as it was necessary to alter the time models slightly to ensure analysis was completed within the required time frame. The long term study was conducted with triplicate samples consisting of five time points (0, 3, 6, 9 and 12 month) and three temperature conditions (-20°C, +4°C and +20°C). The reference temperature used in both studies was -80°C. A total of 9 aliquots were stored at this temperature for the duration of the study before being removed and extracted with the other stability study samples. In the case of all the stability studies aliquots of tissue (n=3), were removed from -80°C storage on the prescribed

day and transferred to the storage conditions being investigated. Table 4-3 is an example of a stability study data table and can be used to describe the process. On the first day of the study (Day 30, 12/06/11) 9 aliquots of tissue were removed from the -80°C freezer, transferring 3 tissue aliquots into each of -20°C, +4°C and +40°C storage conditions and recording the aliquot numbers of each in the table below. Two weeks later on the 26/06/11 another 9 tissue aliquots were removed from -80°C storage and placed in each of the three storage temperatures being investigated, recording their aliquot numbers in the same table below. This process was repeated on the 04/07/11 and the 08/07/11.

Table 4-3: Table used to input data for a short-term stability study.

		Time point & Date				
		30	**16**	**8**	**4**	**0**
	Sample	**12/06/11 Sunday**	**26/06/11 Sunday**	**04/07/11 Monday**	**08/07/11 Friday**	**12/07/11 Tuesday**
-20°C	#1 #2 #3					
+4°C	#1 #2 #3					
+40°C	#1 #2 #3					

On the final day of the study, 12/07/11 the 9 remaining tissue aliquots were removed from -80°C storage and their aliquot numbers recorded in the column labelled 0 in Table 4-3. At the same time all the other tissues were removed from the -20°C, +4°C and +40°C storage conditions, allowing them to equilibrate to room temperature before being extracted and analysed by preCOX LC-FLD closely following OMA AOAC 2005.06 [41] as detailed in *section 2.3.1*. PCOX LC-FLD [75] analysis was additionally performed on all samples to specifically investigate the extent of epimerisation, if any, of GTX2,3 in each material. The epimerisation of GTX1,4 was not determined as the concentration of these toxins in each

tissue was below the limit of quantification (<LOQ) of the PCOX LC-FLD method. GTX2 and GTX3 are good indicator toxins however for rates of epimerization in tissues as evidenced from previous studies [133–135]. For PCOX LC-FLD analysis, the crude acetic acid extracts prepared according to *section 2.3.1* were deproteinated and analysed following AOAC 2011.02 as described in *section 2.3.2* [75].

PST concentrations in sample extracts were quantified against a 4 - 5-point calibration for each toxin and are expressed as µmol/kg with total saxitoxin equivalents calculated as an estimation of total toxicity.

The MC of each of the Tissues 1-3 was determined using the rotary vacuum method as described in the materials and methods *section 2.4.1*. Aliquots (n=3) were selected from the beginning, middle and end of the fill series in order to determine if any evaporation had taken place during dispensing.

The organoleptic properties of each of the tissues was also assessed, specifically the odour of the tissues stored at the elevated temperature conditions was noted during extraction of the stability study samples.

4.2.2 RESULTS AND DISCUSSION

4.2.2.1 HOMOGENEITY AND MOISTURE CONTENT

All the materials prepared in this section of the study were suitably homogenous for all the PSTs detected, as evidenced through the coefficient of variances contained in Table 4-4.

A target %CV of below 8% for each analyte including total toxicity was set. This value is below expected levels of variability associated with intra-batch repeatability, determined through in-house validation of the preCOX LC-FLD method. The intra-batch repeatability of the preCOX LC-FLD method was determined to be >9% for all toxins determined during validation studies. The homogeneity techniques employed were therefore sufficient and the materials suitable for the study.

Table 4-4: Homogeneity results for the antibiotic spiked (Tissue 1), antioxidant spiked (Tissue 2) and untreated control (Tissue 3) materials showing mean toxin concentrations determined by preCOX LC-FLD (n=14).

	Material	dcSTX	GTX2,3	GTX5	STX	GTX1,4	Total Toxicity
		µg STX diHCl-eq. / kg					
Average	Tissue 1	205.4	606.0	2.7	705.8	490.5	2010
	Tissue 2	214.5	830.3	1.9	690.1	366.4	2103
	Tissue 3	194.0	641.8	2.1	545.8	304.1	1688
Stdev	Tissue 1	15.2	35.7	0.1	46.3	29.9	152.8
	Tissue 2	7.9	54.7	0.1	17.6	26.6	75.7
	Tissue 3	9.0	44.5	0.1	19.2	14.1	84.0
%CV	Tissue 1	7.4	5.9	3.0	6.6	6.1	7.3
	Tissue 2	3.7	6.6	3.9	2.6	7.3	3.5
	Tissue 3	4.7	6.9	5.9	3.5	4.7	5.0

Each of the three materials were prepared from a pooled homogenate and adjusted to the same target value for moisture content of 85%. Despite this fact, a statistical difference in total toxicity means ($P < 0.05$; one-way analysis of variance [ANOVA]) was evident between the three tissues which can be seen from the data contained in Table 4-4. The cause or causes of these differences remain unclear and may be attributed to day-to-day instrument variations

as the homogeneity determinations were carried out on separate days. Despite the statistical differences between the means, the processing techniques employed to make the materials were suitable to prepare sufficiently homogenous materials for use in these studies.

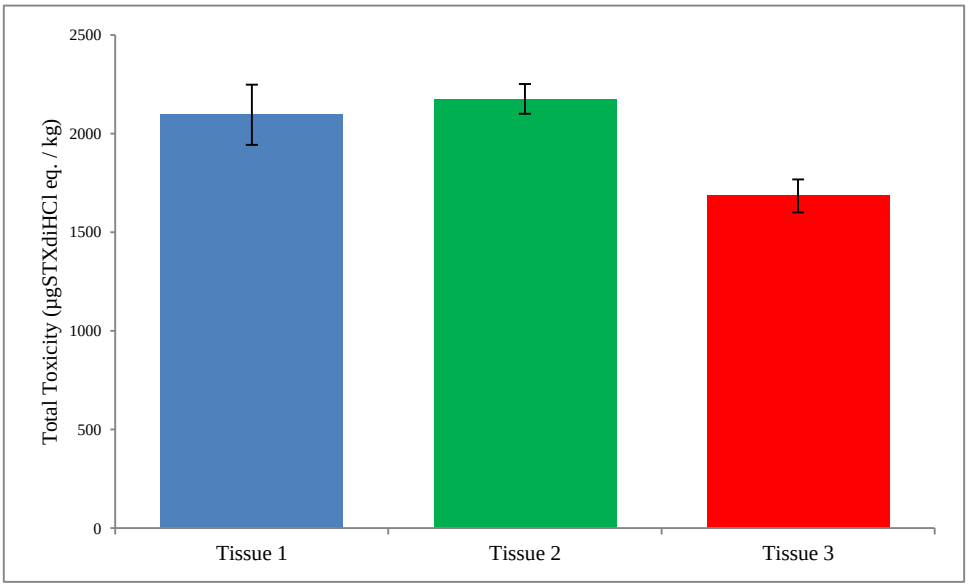


Figure 4-2: visual comparison of total toxicity homogeneity results for Tissues 1-3 (n=14).

The moisture content of each of the *Tissues 1-3* gave reproducible results for the three materials and is slightly higher than the desired value of 85% (Table 4-5). The coefficient of variances for each *Tissue 1-3* was low which indicates that evaporation during dispensing was minimal. Each tissue took approximately 1 ½ hours to dispense in total so evaporation during this short timeframe was not expected, particularly given the precautions that were put in place such as the use of an ice bath to cool the tissue during dispensing. RMs prepared at the MI for use in the NMP where >1000 aliquots are generally dispensed can take up to 5-6 hours to complete. Moisture content determinations of these large batches of RMs give coefficients of variance similar to those found in Table 4-5 (data not shown) which indicate the steps taken to minimise evaporation are sufficient even for dispensing large numbers of aliquots. The critical number of aliquots dispensed after which evaporation does present a problem is unknown but is >1000 if precautions are put in place.

Table 4-5: Moisture content of Tissues1-3 determined by the rotary vacuum method (n=5).

Material	Moisture Content	%CV
Tissue 1	86.0	0.05
Tissue 2	85.9	0.23
Tissue 3	85.6	0.07

4.2.2.2 SHORT-TERM STABILITY

PRECOX LC-FLD

Figure 4-3 a-e represents the short-term stability results for the three materials at three different storage temperatures, -20°C, +4°C and +40°C, determined through preCOX LC-FLD analysis. Data for dcSTX, GTX1,4, GTX2,3, GTX5 and STX were generated and results were normalised to the average of replicate analysis (n=9) of samples stored at the reference temperature of -80°C for the duration of the study. The limits of stability from Figure 4-3 were generated from two times the standard deviation of the -80°C replicate analyses.

Overall toxin stability was excellent in each of the Tissues 1-3 when stored at -20°C and additionally for Tissues 1 and 2 when stored at +4°C for the duration of the short-term study. Tissue 3 exhibited a slight increase in GTX2,3 concentration after 17 days at +40°C and after 31 days at +4°C which showed the improvement additives spiking specifically had on the stability of these toxins. However the stability of the other toxins at +40°C differed between each stabilisation technique and also the control material.

The stability of all the PSTs were improved in Tissue 2 compared to Tissue 3 when stored at +40°C which highlights the benefits of using an antioxidant in RM preparation for PSTs. The use of the antibiotics in Tissue 1 showed varying levels of stability for the PSTs with improvements made to GTX2,3 and GTX1,4 stability at +40°C compared to Tissue 3.

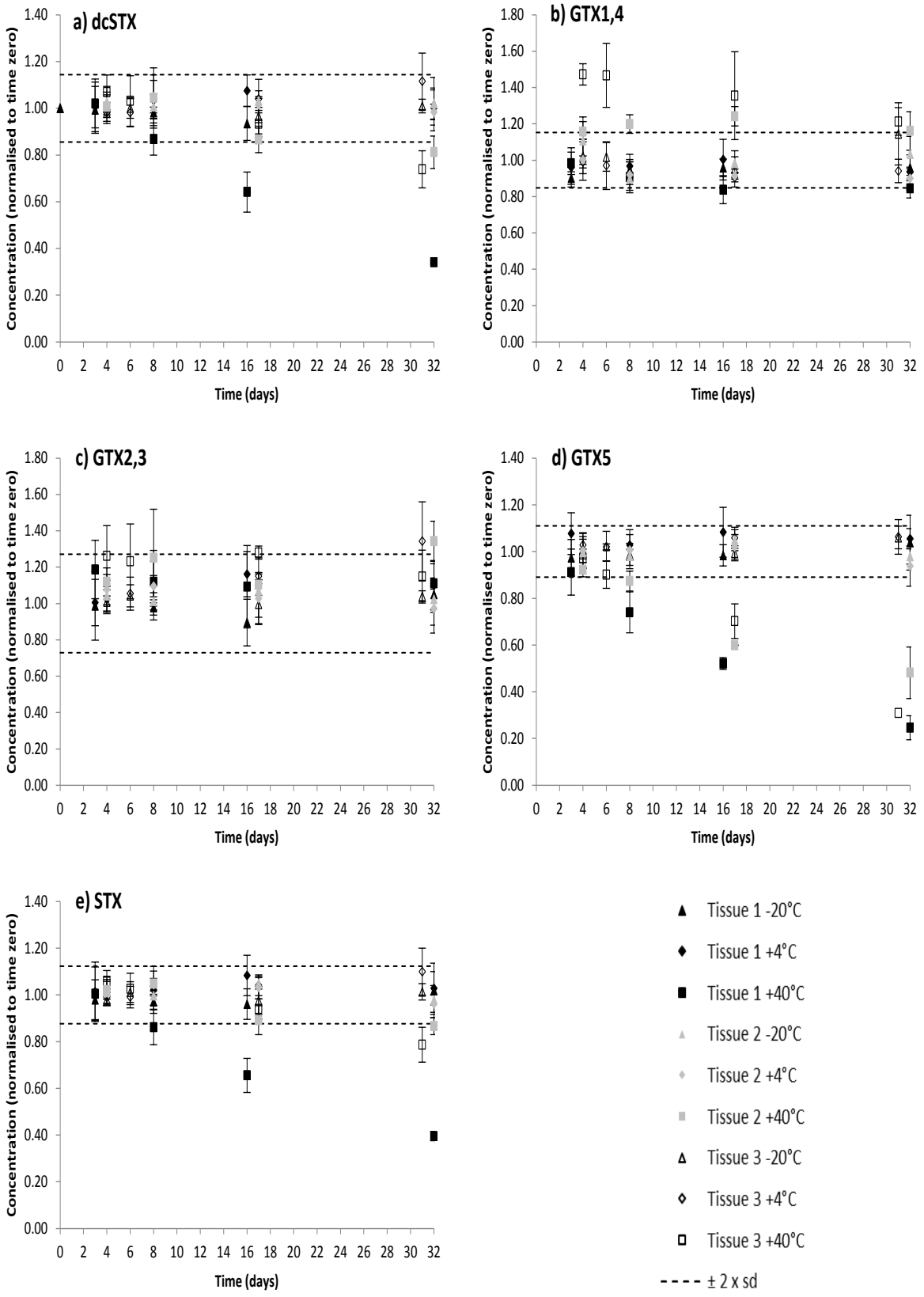


Figure 4-3: Short-term stability graphs for the PSTs present in Tissues 1, 2 and 3 determined by preCOX LC-FLD. Results are normalised to the time zero reference temperature of -80°C (error bars represent ± 2 s.d.).

Interestingly Tissue 1 appears to show instability issues for dcSTX and STX which are more pronounced than those observed in either Tissue 2 or 3 materials stored at +40°C, the exact causes of which remain unclear. Similar levels of instability were observed for GTX5 in all tissues when stored at +40°C which showed neither stability enhancement nor deterioration through the use of preserving additives for this toxin.

An increase in GTX2,3 concentration was observed in the antioxidant material after 32 days storage at +40°C and in the control material after 17 and 31 days storage at +40°C and +4°C respectively. The cause of this increase is unknown but could be due to biotransformations taking place in the tissue [132].

The use of the antioxidant ethoxyquin improved the stability of the PSTs overall compared to the control which proves its inclusion for PST RM production to be beneficial. The use of the antibiotics however gave different results with their inclusion improving the stability of GTX1,4 compared to the control. The stability of the other PSTs investigated was apparently worsened, albeit only at the higher temperature of +40°C; similar levels of stability were evident at the lower temperatures studied for all three tissues.

PCOX LC-FLD

Further analysis was conducted on the short-term stability study extracts for each of the stabilisation techniques by PCOX LC-FLD and results are presented in Figure 4-4 a-b. The PCOX LC-FLD method was used specifically to determine the rate of epimerization of GTX2 and GTX3, if any, in the materials prepared by each technique compared to those found in the untreated control. Limits of stability and normalised results were calculated as per the preCOX LC-FLD analysis above.

No epimerization or degradation was observed in any materials when stored at -20°C for the duration of the study with results falling within the limits of stability set. Tissue 1 showed some slight signs of epimerization at +4°C after 32 days storage. At +40°C epimerization of GTX2,3 in Tissue 1 was evident after 3 days storage which highlights the speed this reaction can occur when samples are stored at an elevated temperature.

Tissue 2, while similarly stable at -20°C as in the case of Tissue 1, showed slightly more instability issues at the higher temperatures investigated. Epimerization was evident after 4 days storage at +40°C and after 17 days storage at +4°C in the Tissue 2 samples.

The untreated control, Tissue 3 showed the epimerization of GTX2,3 at temperatures above -20°C. This was evident after 4 days storage at +40°C and after 17 days storage at +4°C. Further instability issues of GTX3 were evident in the Tissue 3 samples, not seen in either of the other tissues. The total reduction of GTX3 in Tissue 3 at +40°C as seen in Figure 4-4 a could not be attributed solely to epimerization as the increase in GTX2 concentration (Figure 4-4 b) does not occur to the same level. GTX3 concentration in Tissue 3 therefore reduces by ~50% most likely through a combination of epimerization to GTX2 and some degradation of the toxin itself. The reduction of GTX3 in Tissues 1 and 2 however is only ~20-25% and is accompanied by an increase in GTX2 levels of the same magnitude ~20-25%, which indicates epimerization is the predominant reaction taking place and very little if any toxin degradation is occurring in these tissues. Although spiking with additives does not significantly reduce epimerization rates compared to untreated materials, inclusion of antibiotics or an antioxidant does improve the stability of GTX3 by approximately 25% which highlights their advantage in RM preparation, particularly for PCOX LC-FLD analysis where individual epimers can be quantified.

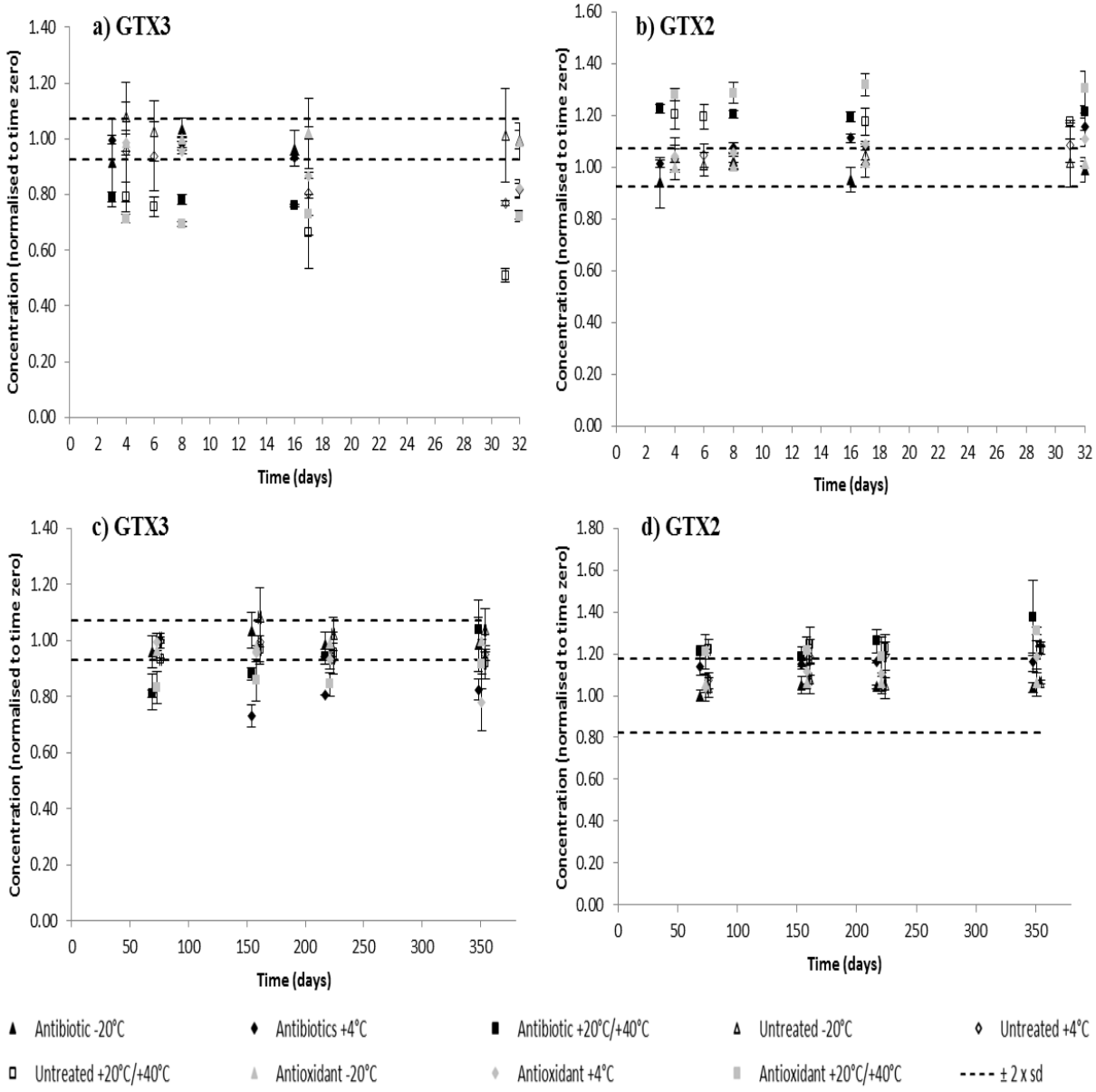


Figure 4-4: Stability graphs for the PST epimers GTX2,3 determined by PCOX LC-FLD for the short-term data (a-b) and the long-term data (c-d) Results are normalised to the time zero reference temperature of -80°C (error bars represent ± 2 s.d.).

The results from both the preCOX and PCOX analysis of the short-term stability samples provide useful information on the conditions required for transportation of materials stabilised using both techniques. Although transportation of matrix RMs ideally should be made under frozen conditions, results indicate materials would also be stable at +4°C for up to 32 days if spiked with antibiotics and up to 17 days if spiked with the antioxidant,

ethoxyquin. These findings indicate that if adverse conditions were experienced during transportation, PSTs would remain stable up to temperatures of +4°C for over two weeks.

4.2.2.3 LONG-TERM STABILITY

PRECOX LC-FLD

Figure 4-5 a-e represents the long-term stability results for the three materials at three different storage temperatures, -20°C, +4°C and +20°C, determined through preCOX LC-FLD analysis. Data for dcSTX, GTX1,4, GTX2,3, GTX5 and STX were generated and results were normalised to the average of replicate analysis (n=9) of samples stored at the reference temperature of -80°C for the duration of the study. The limits of stability from Figure 4-5 were generated from two times the standard deviation of the -80°C replicate analyses.

All three Tissues 1-3 exhibited excellent stability for all the PSTs throughout the duration of the yearlong study when stored at -20°C. Differing levels of stability were exhibited under the other storage conditions however.

At the elevated storage temperatures of +4°C and +20°C the use of preserving additives in Tissues 1 and 2 did not significantly improve PST stability compared to Tissue 3. GTX5 showed the greatest differences in stability across the three materials with Tissues 2 and 3 exhibiting apparent increases in toxin concentration at +20°C (Tissues 2 and 3) and +4°C (Tissue 3), the causes of which are unclear. Tissue 1 showed no increase in GTX5 concentration at this storage condition, in fact this tissue showed no signs of degradation until after 9 months of the study at +20°C and remained stable at +4°C over the course of the 12 months.

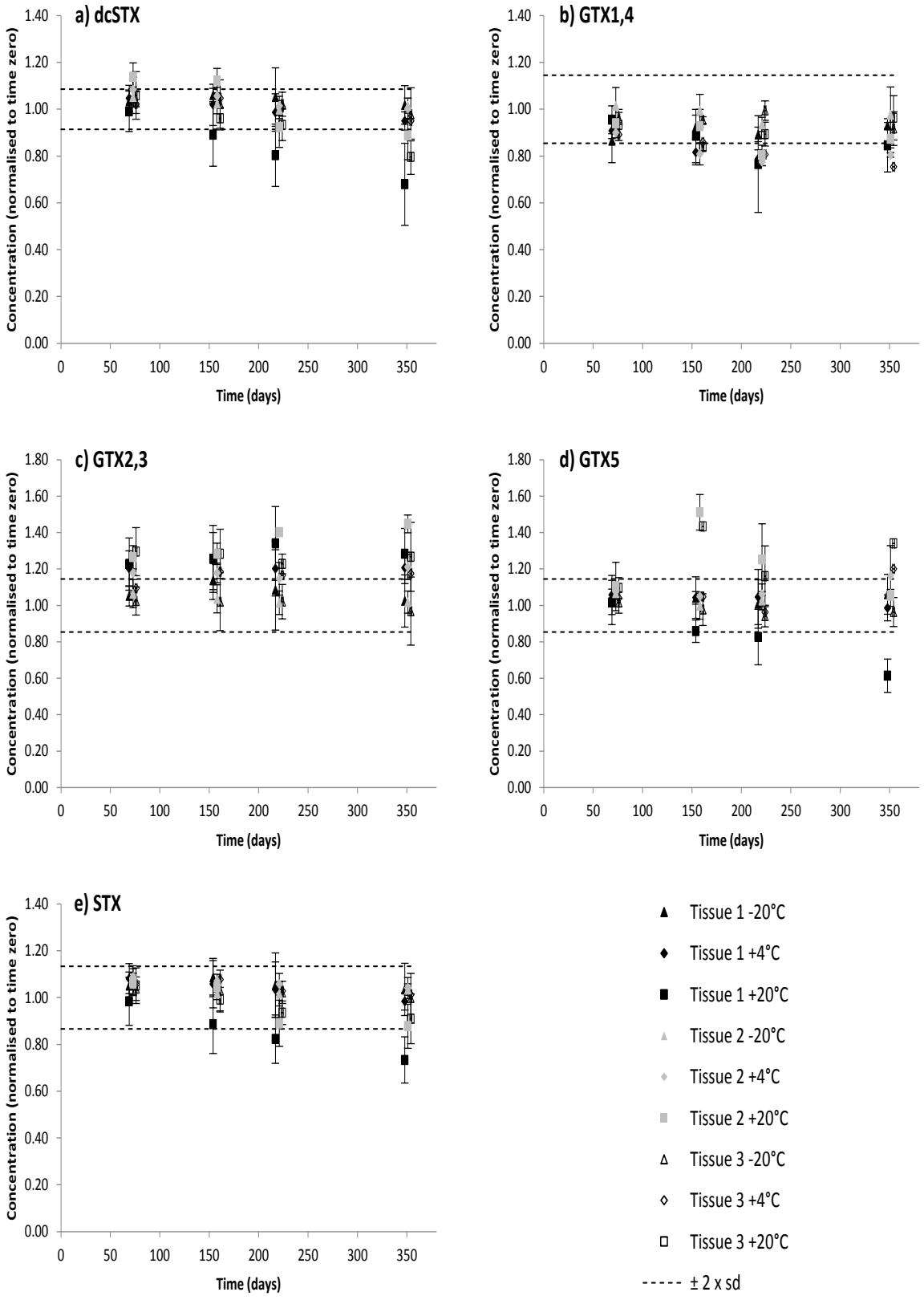


Figure 4-5: Long-term stability graphs for the PSTs present in Tissues 1, 2 and 3 determined by preCOX LC-FLD. Results are normalised to the time zero reference temperature of -80°C (error bars represent ± 2 s.d.).

As in the case of the short-term stability studies, but significantly more pronounced, were increases in GTX2,3 concentrations observed at both +20°C and +4°C storage. Similar increased GTX2,3 concentrations were observed in all three tissues at +4°C but Tissue 2 exhibited a greater increase at +40°C compared to either Tissues 1 or 3 which exhibited increases of a similar level.

The results prove that whilst some benefits were obtained following additive pre-treatment, long-term storage conditions for the materials stabilised with either antibiotics or the antioxidant should still be at -20°C or below.

PCOX LC-FLD

Further analysis was conducted by PCOX LC-FLD on the long-term stability study extracts of each of the materials and results are presented in Figure 4-4 c-d. Limits of stability and normalised results were calculated as per the preCOX LC-FLD analysis above. Data for the toxins GTX1 and GTX4 were not generated as the concentration of these toxins in each material was <LOD of the PCOX LC-FLD method.

No epimerization or degradation was observed in any materials when stored at -20°C for the duration of the study with results falling within the limits of stability set. Tissue 1 showed no visual evidence of epimerization at +4°C, although a low amount was observed after 32 days in the short-term study (Figure 4-4 a-b). GTX2 in Tissue 1 remained stable throughout the yearlong study at +4°C while GTX3 showed signs of degradation after 6 months storage with ~20% of this toxin degraded by the end of the study, which suggests a more selective degradation of the β-epimer occurs. At +20°C epimerization of GTX2,3 in Tissue 1 was evident after 3 months storage.

Tissue 2 while similarly stable at -20°C as in the case of Tissues 1 and 3 showed epimerization of GTX2,3 after 12 months storage at +4°C and after 3 months at +20°C. Epimerization was also observed to take place in Tissue 3 control materials but the process occurred earlier than either Tissue 1 or 2 at +4°C. At this temperature epimerization was evident in Tissue 3 material after 6 months storage compared to 12 months in the case of Tissue 2 and no epimerization was observed in Tissue 1. At +20°C epimerization was observed to take place after 3 months storage which was similar to both Tissues 1 and 2, showing no clear advantage the preserving additives provided.

The long-term stability study samples as determined by PCOX LC-FLD show no significant improvement in reducing epimerization rates through the inclusion of preserving additives. The rates of epimerization of all three tissues were similar at +20°C; however the use of antibiotics in Tissue 1 did lower epimerization rates in samples stored at +4°C (Figure 4-4 c-d).

Epimerization of these toxins followed predicted patterns with the β-epimer GTX3 being epimerized into the more stable α-form GTX2 [136], which was evident from both the short and long-term stability data generated through the PCOX LC-FLD analysis.

CHROMATOGRAPHIC AND MATRIX STABILITY

Another important factor to consider when choosing an RM preparation technique is ensuring good chromatographic stability is achieved. Figure 4-6 a-h displays chromatograms generated by preCOX LC-FLD for Tissues 1-3 by both peroxide and periodate oxidation after 12 months storage at +20°C. Chromatograms of Tissue 3 stored at -80°C for 12 months are also shown in this figure as a visual comparison.

The chromatograms generated following peroxide oxidation (Figure 4-6 a-c) for Tissues 1-3 show some differences in chromatographic peaks inferring either differing levels of toxin stability and/or the generation of naturally-flourescent matrix co-extractives over time. Chromatograms of Tissues 1 and 2 materials showing no formation of additional chromatographic peaks through the inclusion of either of the preserving additives compared to the control material stored at -80°C. Tissue 3 in comparison did show the additional formation of chromatographic peaks between 5.0 – 6.5 mins which could affect accurate quantitation of the C-toxins as their retention times are similar, although these toxins were not present in these tissues.

The periodate oxidised extracts used to quantify the N-hydroxylated toxins GTX1,4 are contained in Figure 4-6 e-h. Good chromatographic stability was observed in all tissues up to a retention time of ~9.0 mins which would not interfere with accurate quantitation of GTX1,4. A large matrix peak, not present in the control material stored at -80°C was evident in all three tissues at a retention time of ~9.2 mins however which indicates some matrix instability. The presence of this additional peak would not affect accurate quantitation of GTX1,4 however.

The retention time differences observed between the peroxide and periodate oxidised samples is due to both sets of analysis been carried out on different days. Retention time checks were carried out each day however, through comparison to standards.

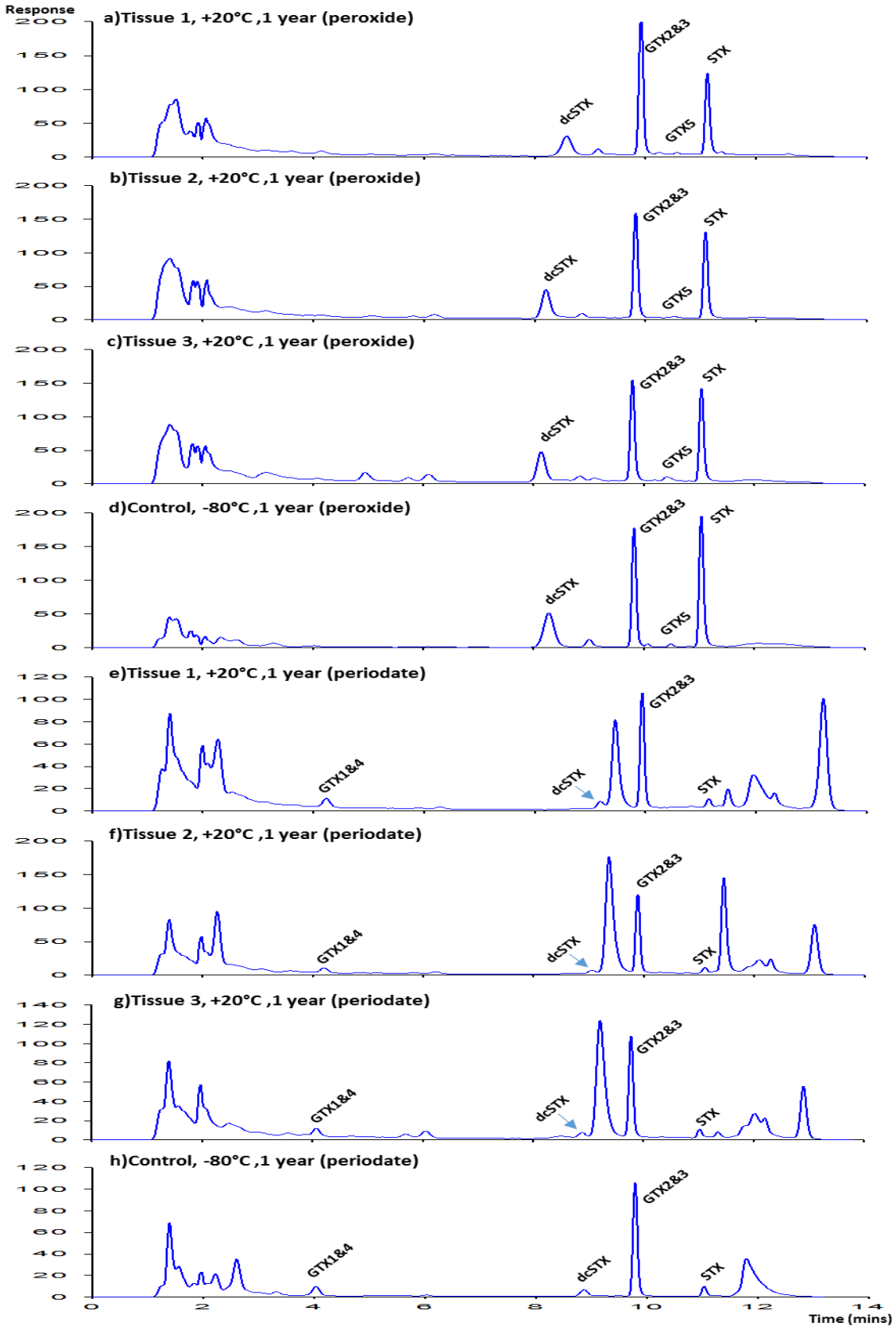


Figure 4-6: PreCOX LC-FLD chromatograms following peroxide (5a-d) and periodate (5e-h) oxidation of Tissues 1, 2, 3 and a control material stored for 12 months.

The organoleptic properties of each of the materials prepared are another indicator of the advantages using preserving additives have, particularly on matrix stability. Specifically the odour of the untreated control materials stored at +4°C and +40°C was significantly worse than either Tissue 1 or 2 materials stored at the same temperatures. The malodour indicated a high degree of rancidity of the mussel matrix in Tissue 3 which was reduced in the other two tissues.

4.2.3 CONCLUSIONS

All the materials used in this study were found to be homogeneous for the PSTs present as evidenced through the coefficients of variation determined for toxin concentrations quantified in selected tissue samples. This highlights the applicability of the in-house RM homogenisation techniques in preparing suitable materials for the study, as proven in previous studies at the MI [54].

The use of preserving additives improved the stability of the PSTs investigated in this section of the study which was particularly evident in the short-term samples at the higher temperature storage condition of +40°C. The use of the antibiotics (Tissue 1) improved the stability of the toxins GTX2,3 and GTX1,4 and the antioxidant (Tissue 2) improved the stability of dcSTX and STX when compared to the untreated control (Tissue 3) in the short-term. Both Tissues 1 and 2 showed excellent stability at +4°C for all the toxins investigated, whereas Tissue 3 showed some instability issues for GTX2,3 under this storage condition. The results indicate that the use of either an antioxidant or antibiotics in stabilising PSTs depends on the toxin profile present. Their combined use however may provide better overall stability for a range of PSTs. These short-term stability observations have particular importance in determining transportation conditions required for shipment of materials and in

the case of either Tissue 1 or 2, this could be carried out at temperatures of up to +4°C for as long as 32 days without adverse effects to toxin stability.

The long-term stability studies did not definitively prove the benefits of using preserving additives in RM preparation. Some minor improvements were observed in the case of GTX1,4 using either the antibiotics or the antioxidant but none were significant. Long-term storage of biological RMs should be at temperatures of -20°C or below.

Tissues 1 and 2 showed good chromatographic stability in peroxide oxidised samples with no formation of additional peaks evidenced in the chromatograms. Conversely Tissue 3 showed the formation of extra chromatographic peaks although these would not necessarily affect accurate quantitation of any of the PSTs investigated in this study but which could affect quantitation of the toxins C1,2 if they were present. This highlights another advantage of using preserving additives in RM preparation to not only improve toxin stability but to improve matrix and therefore chromatographic stability.

The results of these experiments indicate the applicability of using preserving additives in LRM and ILRM preparation but not for CRM production. The antibiotics and antioxidant spikes improve short-term stability, important for example in delivery of PT materials but the long-term stability of these materials was not significantly improved enough to warrant feasibility studies being undertaken for CRM production.

4.3 PRESERVING ADDITIVES AND THERMAL TREATMENT

This section of *Chapter 4* investigates the combined use of an antioxidant, ethoxyquin and three antibiotics, ampicillin, erythromycin and oxytetracycline which were separately evaluated in *section 4.2*. This section also evaluates the effects thermal treatment through autoclaving had on matrix stability and the PSTs present. The two stabilisation techniques were, as in the case of the *section 4.2* materials, investigated in parallel with an untreated control material.

Short and long-term stability experiments as well as homogeneity determinations were conducted on materials prepared by both techniques in comparison with an untreated control using two LC-FLD methods.

This section of Chapter 4 details work described in Burrell *et al.* 2015 [113].

Stephen Burrell, Valentin Clion, Virginie Auroy, Barry Foley & Andrew D. Turner, 2015. Heat treatment and the use of additives to improve the stability of paralytic shellfish poisoning toxins in shellfish tissue reference materials for internal quality control and proficiency testing, *Toxicon*. 99:80–88.

4.3.1 MATERIALS AND METHODS

4.3.1.1 SOURCE TISSUES

The Icelandic, Spanish and blank toxin free Irish tissues from Table 2-1 were used to prepare the RM used in this section of the study. The tissues were naturally contaminated with GTX2,3, STX, GTX1,4, and dcSTX and were diluted with a blank toxin free mussel tissue due to the high levels of PSTs present in the other tissues.

A bulk tissue (>4.0 kg) was prepared for use in this section of *Chapter 4* and subdivided into four separate lots, sealed in PP containers and stored at -20°C until further use. The bulk tissue was prepared by combining the proportions of tissues detailed in Table 4-6 into a 5 L Waring™ goblet. The bulk tissue was homogenised as described in *section 4.2.1.1*.

Table 4-6: Quantities of source tissues used to prepare bulk homogenate for additives, thermal treatment and freeze drying studies.

Country	Species	Tissue	Quantity (kg)
Iceland	*Mytilus edulis*	WF	1.26
Norway	*Mytilus edulis*	WF	1.00
Spain	*Mytilus galloprovancialis*	WF	1.00
Ireland	*Mytilus edulis*	WF	0.74

Aliquots were taken for MC determination (n=3) using the rotary vacuum method and analysis by preCOX LC-FLD (n=1) as described in the materials and methods *section 2.4.1* and *2.3.1* respectively, to give approximate PST concentrations in the bulk tissue. The bulk homogenate was immediately subdivided into 3 x ~0.8 kg (Tissues A-C) and 1 x ~0.8 kg (freeze drying in *Chapter 5*, *section 5.4*) aliquots by pouring the required amount of tissue into PP containers, sealing with lids and parafilm before storing at -20°C until required for use. The PST levels and MC results from this bulk homogenate are presented in Table 4-7.

Table 4-7: Toxin profile, concentrations and moisture content of bulk tissue used to prepare Tissues A-C and a freeze dried material in chapter 5.

Material	Concentration (µmol / kg)					Total Toxicity (µg STX diHCl-eq / kg)	% Moisture Content
	dcSTX	GTX-2,3	GTX-5	STX	GTX-1,4		
Bulk homogenate	0.65	0.94	0.06	0.62	0.22	685	81.6

4.3.1.2 Additives and Thermal Treatment Material Preparation

Both the additives spiked (Tissue A) and thermally treated (Tissue B) materials were prepared similarly as follows. The frozen bulk homogenate described in *section 4.3.1.1* above were removed from the freezer and allowed to defrost overnight in a fridge at +4°C. The preserving additives were prepared at a concentration of 0.02% w/w by dissolving in ethanol (1% w/w). For the additives spike, 0.18 ± 0.01 g each of ampicillin, oxytetracycline, erythromycin and ethoxyquin were weighed into a 50 mL PP centrifuge tube using a calibrated 3-place balance. 9.0 ± 0.1 g ethanol was added to the centrifuge tube, capped and vortex mixed for 5.0 min.

For Tissue A the defrosted tissues were manually mixed using a spatula before transferring 734 ± 1 g into a Waring™ goblet. The additives/ethanol solution was then spiked into the tissue, washing out the centrifuge tube with a small volume of DI water before manually mixing with a spatula. The final weight of the tissue was made up to 900 ± 1 g with DI water in order to obtain a final tissue with a MC of ~85%.

For Tissue B the defrosted tissues were manually mixed using a spatula before being transferred to a 3L PP beaker and autoclaved (Systec VE-100, Focus Scientific Solutions, Meath, Ireland) at 121°C for 15.0 ± 0.1 min. The tissues were allowed to cool to room temperature before being transferred, including any liquid to a Waring™ goblet and homogenised on medium power for 5.0 ± 0.1 min. Aliquots (n=3) were taken for MC

determination which was determined to be ~82%. 670 ± 1 g of the tissue was gravimetrically transferred into a Waring™ goblet. The final weight of the tissue was made up to 800 ± 1 g with DI water in order to obtain a final tissue with a MC of ~85%.

The final homogenisation steps of Tissue A and B using both Waring™ and Polytron™ blenders, including the dispensing of both was carried out as described in *section 4.2.1.2.* A total of 150 aliquots of Tissue A and 120 aliquots of Tissue B were dispensed providing a sufficient quantity for short (n=45) and long-term (n=45) stability studies as well as homogeneity (n=16) and MC (n=3) determinations. All materials were stored at -80°C until further use.

4.3.1.3 UNTREATED CONTROL MATERIAL PREPARATION

The untreated control material (Tissue C) was prepared by defrosting the bulk homogenate described in *section 4.2.1.1* above overnight in a fridge at +4°C. The defrosted tissue was manually mixed using a spatula before transferring 734 ± 1 g into a Waring™ goblet. The final weight of the tissue was made up to 900 ± 1 g with DI water in order to obtain a final tissue with a MC of ~85%.

Tissue C was homogenised and dispensed as described in *section 4.2.1.3.* A total of 150 aliquots of Tissue C were dispensed providing a sufficient quantity for short (n=45) and long-term (n=45) stability studies as well as homogeneity (n=16) and MC (n=3) determinations. All materials were stored at -80°C until required.

4.3.1.4 STUDIES AND ANALYSIS

The between-bottle homogeneity of the materials prepared in *sections 4.3.1.2* and *4.3.1.3* above was assessed through the intra-batch analysis of 16 aliquots ($3n^{1/3}$) selected through stratified random sampling of the entire fill series.

All materials were stability tested over a short term (ca. 30 day) and long term (ca. 12 month) period following a reverse isochronous experimental design [55]. The short term study was conducted with triplicate samples consisting of five time points (0, 4, 6, 16 and 30 day for Tissue A, 0, 4, 8, 15 and 28 days for Tissue B and 0, 4, 8, 15 and 32 days for Tissue C) and three temperature conditions (-20°C, +4°C and +40°C). The time points used in the study of each material were again slightly different as it was necessary to alter the time models slightly to ensure analysis was completed within the required time frame. The long term study was conducted with triplicate samples consisting of five time points (0, 3, 6, 9 and 12 month) and three temperature conditions (-20°C, +4°C and +20°C). The reference temperature used in both studies was -80°C. The stability studies were set up and carried out as described in *section 4.2.1.4*.

At the end time point all samples were removed from storage, allowed to equilibrate to room temperature before being extracted and analysed by preCOX LC-FLD closely following OMA AOAC 2005.06 [41] as detailed in *section 2.3.1*. PCOX LC-FLD [75] analysis was additionally performed on all samples to specifically investigate the extent of epimerisation, if any, of GTX2,3 in each material. For PCOX LC-FLD analysis, the crude acetic acid extracts prepared according to *section 2.3.1* were deproteinated and analysed following AOAC 2011.02 [75].

PST concentrations in sample extracts were quantified against a 4 - 5-point calibration for each toxin and are expressed in µmol/kg with total saxitoxin equivalents calculated as an estimation of total toxicity.

The MC of each of the Tissues A-C was determined using the rotary vacuum method as described in the materials and methods *section 2.4.1*, selecting aliquots from the beginning, middle and end of the fill series.

4.3.2 RESULTS AND DISCUSSION

4.3.2.1 HOMOGENEITY

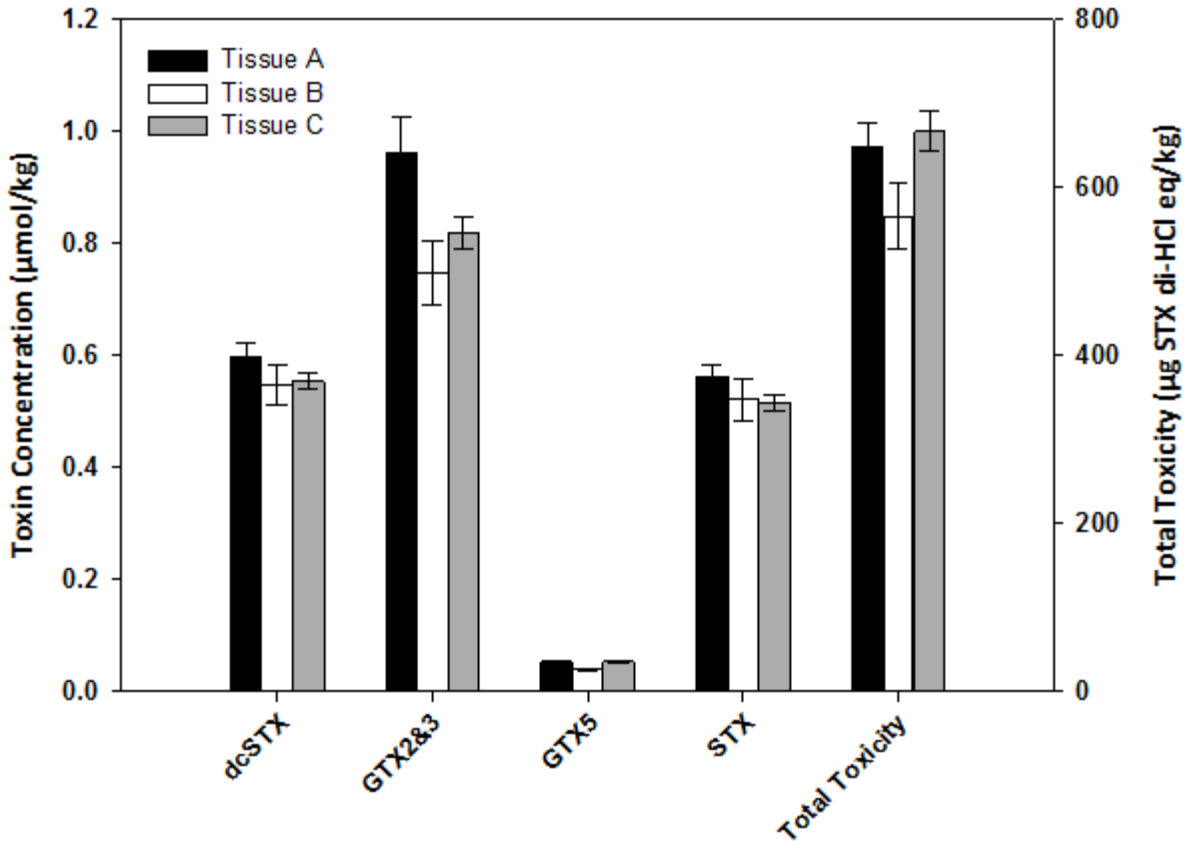


Figure 4-7: Homogeneity results for the additives spiked (Tissue A), heat treated (Tissue B) and untreated control (Tissue C) materials showing mean toxin concentrations (± 1 s.d) determined by preCOX LC-FLD (n=16).

Figure 4-7 illustrates the homogeneity results for the three tissues, showing mean PST concentrations and standard deviations (sd) associated with the triplicate analysis. This allows for a visual representation of the effects each treatment had on the toxin content, specifically if any toxin degradation occurred during the processing steps. The homogeneity results for the individual analogues and total toxicity from the three materials showed a statistical difference ($P < 0.05$; one-way analysis of variance [ANOVA]) between the three means, showing there were some effects on toxin concentrations following treated sample preparation. The quantity of toxin degraded during the preparation of materials and the acceptability of these losses depends on the needs of the RM producer and whether the improvements to toxin stability these techniques provide outweigh the toxin losses incurred through each processing technique. This was particularly important in the case of the heat treated materials, which showed the relative thermal stability of the toxins during exposure to high temperatures, even though a 15% reduction in total toxicity was determined, compared to the control. The toxin profiles in each tissue remained consistent, as evidenced from Figure 4-7, which showed no visual evidence for the degradation or biotransformation of toxins through any of the stability techniques.

The homogeneity results for each material were acceptable with percentage relative standard deviations (RSD%; n=16) of <8 % for total toxicity (mean = 5.0 %) and individual analogues (mean = 4.9 %), which were within expected levels of variability associated with intra-batch repeatability as determined through previous validation studies [120].

4.3.2.2 SHORT-TERM STABILITY

PRECOX LC-FLD

Figure 4-8 a-e represents the short-term stability results for the three materials at three different storage temperatures, -20°C, +4°C and +40°C, determined through preCOX LC-FLD analysis. Data for dcSTX, GTX1,4, GTX2,3, GTX5 and STX were generated and results were normalised to the average of replicate analysis (n=9) of samples stored at the reference temperature of -80°C for the duration of the study. The limits of stability from Figure 4-8 were generated from two times the standard deviation of the -80°C replicate analyses.

Overall toxin stability was excellent in each of the materials, including the untreated tissues when stored at -20°C or +4°C for the duration of the short-term study. The stability of the toxins at +40°C differed between each stabilisation technique and the control materials with the thermally treated tissues in particular showing significant stability improvements for all toxins present when compared to the untreated materials. From these results, heat treatment proved the most effective technique investigated with dcSTX, GTX1,4, GTX2,3 and STX showing excellent stability, even at the highest temperature of +40°C in addition to the two other temperature conditions investigated. Mean concentrations of the N-sulfocarbamoyl toxin GTX5 were however found to fall well below the standard deviation limits which may indicate some stability issues relating to this toxin. The level of degradation of GTX5 (ca.50%), in the +40°C materials was very similar for the two stabilisation techniques as well as the untreated materials however which showed neither an improvement nor deterioration in GTX5 stability through the use of either technique compared to the control.

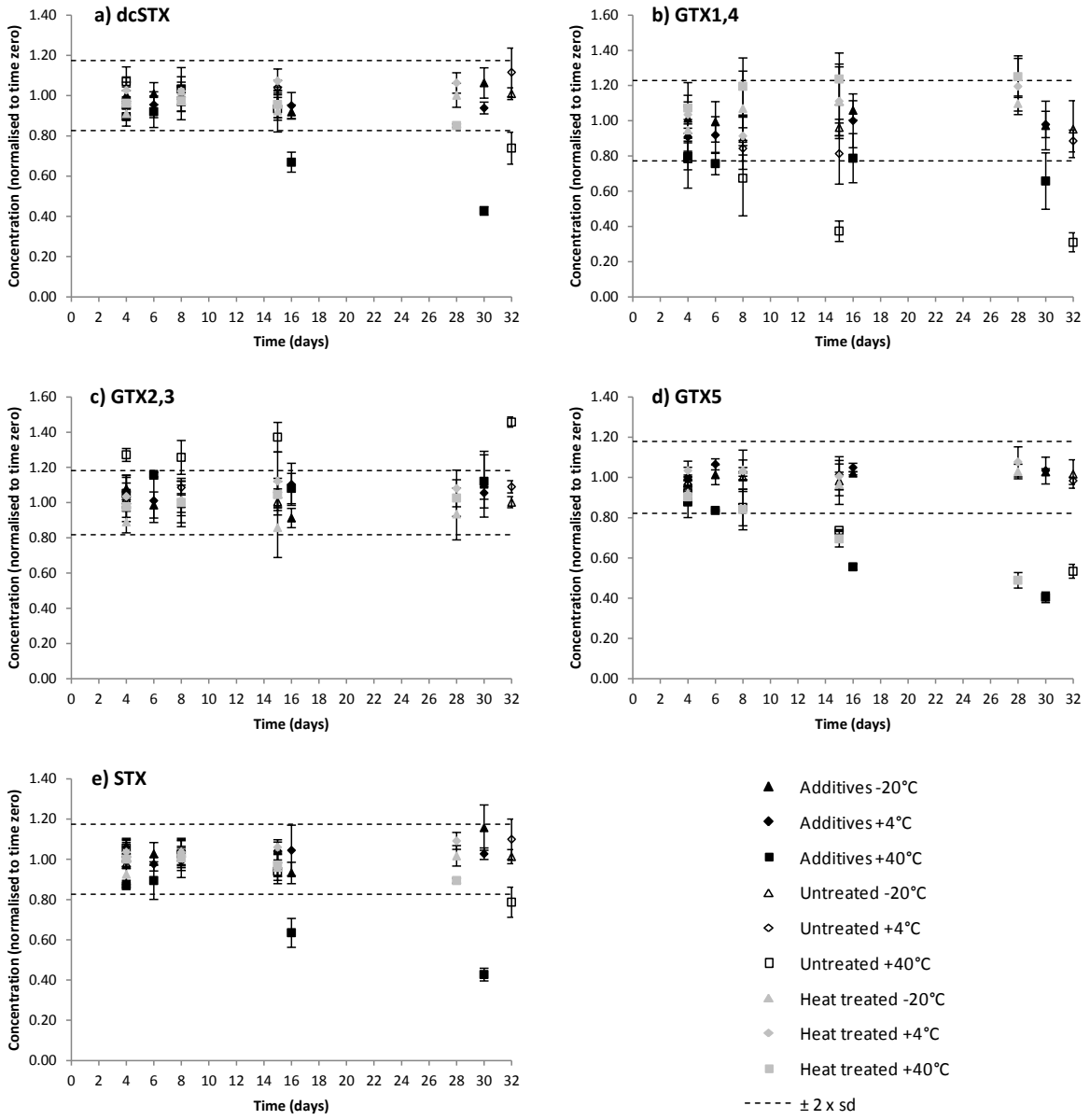


Figure 4-8: Short-term stability graphs for the PSTs present in Tissues A, B and C determined by preCOX LC-FLD. Results are normalised to the time zero reference temperature of -80°C (error bars represent ± 2 s.d.).

Similar stability issues for dcSTX, GTX1,4, GTX5 and STX were evident in both the additives spiked and control materials at +40°C indicating that no clear advantage is provided in the additive treated tissues in the case of short-term toxin stability. Spiking tissues with these additives did however significantly improve the stability of GTX2,3 with no stability issues evident from Figure 4-8 c under any of the temperature conditions studied. Conversely stability issues could be observed for these toxins after 4 days in the control materials stored

at +40°C where an apparent increase in concentration could be observed which culminated in an approximate 40% increase by the end of the study.

The preCOX analysis of these samples therefore clearly indicated that thermally treating materials provides a significant stability improvement to all the toxins investigated, except GTX5, compared to both the additives spiked and control materials. The inclusion of additives such as those used in this study only appear to improve the stability of the toxins GTX2,3 in the short-term compared to the control with no significant improvement made in stabilising the other toxins present, although no deterioration was evident either.

Previous studies carried out by Smith *et al*., proved the role of bacteria, present in the digestive glands of shellfish species, in the biotransformation of PSTs [132]. They concluded that, in the presence of bacterial isolates taken from mussel digestive glands, GTX1,4 reductively transformed with concomitant production of GTX2,3. A decrease in GTX1,4 concentration under the +40°C storage condition was observed in both the additives and control materials, which culminated in a total decrease of approximately 40% (additives) and 80% (control) by the end of the study. The increase in GTX2,3 concentration observed in Tissue C is therefore most probably due to the reductive transformation of GTX-1,4 present in the tissue. This transformation is thought to take place by the reductive elimination of the C-11 hydroxysulfate (OHSO3) and N-1 hydroxyl (OH) moiety (Figure 1-3) by bacteria as previously described by Kotaki *et al*, 1985 [137]. This reaction was observed to take place when these toxins were present with *Vibrio* and *Pseudomonas spp*. of bacteria present in blue mussels.

Further evidence for this hypothesis can be found in the fact that the slight increase in GTX-2,3 levels and the decrease in GTX-1,4 concentration observed in the additives material was significantly less than that observed in the control. If the changes in toxin concentration are

indeed due to bacterially induced biotransformations, then the inclusion of additives would limit the role these bacteria have in toxin transformation, through lowering the cell counts in the tissue, resulting in the smaller changes observed in the additives material.

Thermally treating the material also provided excellent stability with dcSTX, STX and GTX-2,3 stable under all storage conditions for the duration of the study. GTX-5 showed some degradation under the elevated storage condition however. This technique, while maintaining the same level of stability for dcSTX and STX, improves the stability of the toxins GTX-2,3 when compared to the control material.

PCOX LC-FLD

Further analysis was conducted on the short-term stability study extracts for each of the techniques by PCOX LC-FLD and results are presented in Figure 4-9 a-b. The PCOX LC-FLD method was used specifically to determine the rate of epimerization of GTX2 and GTX3, if any, in the materials prepared by each technique compared to those found in the controls. Limits of stability and normalised results were calculated as per the preCOX analysis above.

No epimerization or degradation was observed in any materials when stored at -20°C for the duration of the study with results falling within the limits of stability set. The materials spiked with additives also showed no evidence for epimerization of GTX2 and GTX3 at +4°C, although some slight degradation of GTX3 was observed after day 30 of the study under this temperature condition, with GTX2 remaining stable throughout. However epimerization of these toxins was observed in the additives spiked samples stored at +40°C which was evident after day 4 of the study.

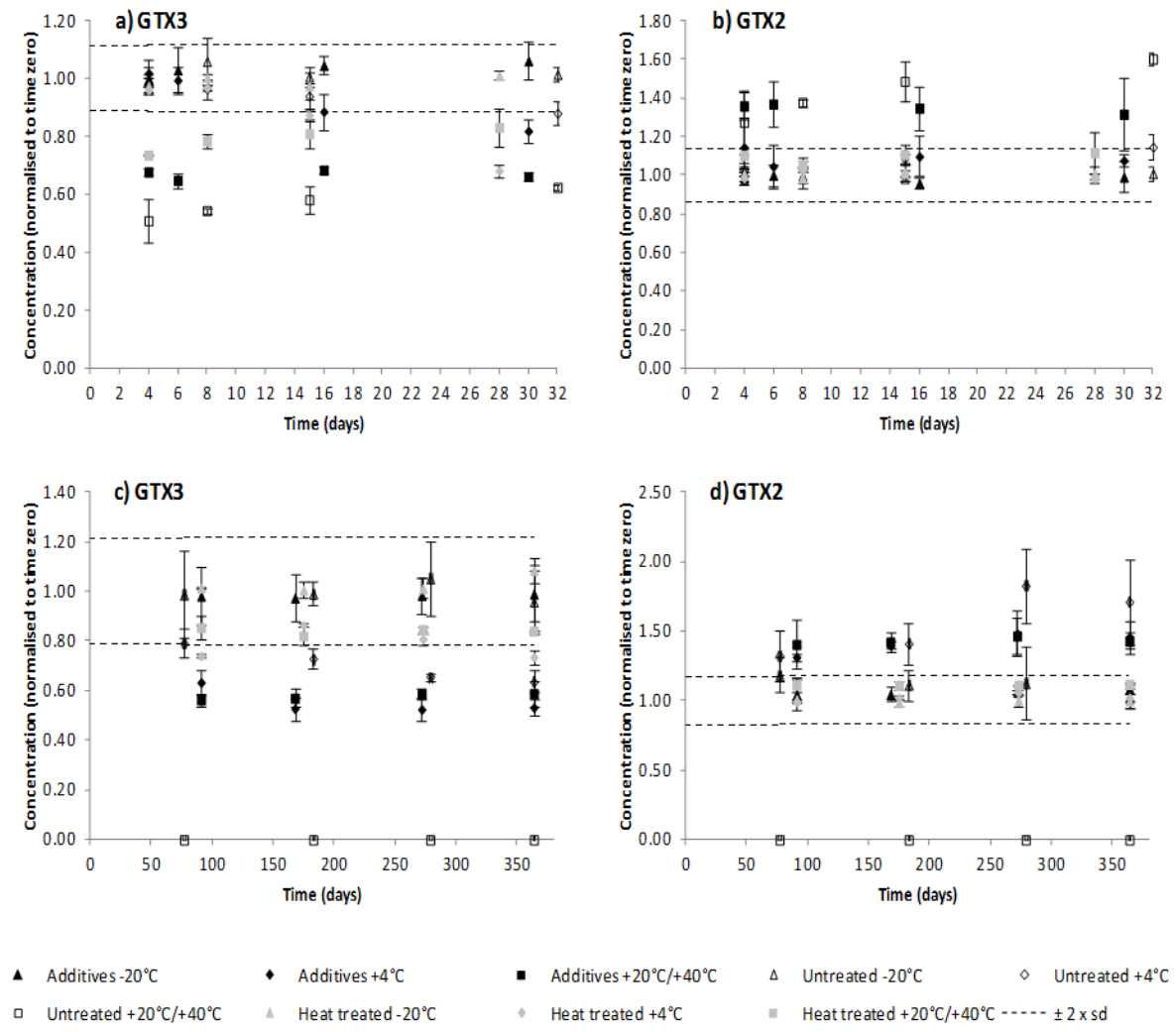


Figure 4-9: Stability graphs for the PST epimers GTX2,3 determined by PCOX LC-FLD for the short-term data (3a-b) and the long-term data (3c-d) Results are normalised to the time zero reference temperature of -80°C (error bars represent ± 2 s.d.).

No epimerization was observed in materials stabilised through thermal treatment under any of the temperature storage conditions, -20°C, +4°C or +40°C which showed stability improvements over both the additives and untreated materials. GTX2 remained stable throughout the study under all temperature conditions but there was some degradation of the toxin GTX3 evidenced after 15 days at +4°C and after 4 days at +40°C.

The control material while stable at -20°C showed signs of epimerization under the other two storage conditions investigated. This was evident after 32 days of the study in samples stored at +4°C and after 4 days in samples stored at +40°C. The rates of epimerization were greater

in the control materials compared to either the thermally treated or additives spiked materials which was particularly apparent under the +40°C storage condition. This highlights the significant improvements these techniques make to toxin stability and epimerization rates compared to the control materials in the short-term.

The results from both the preCOX and PCOX analysis provide useful information on the conditions required for transportation of materials stabilised using both techniques. Although transportation of matrix RMs ideally should be made under frozen conditions, results indicate materials would also be stable at +4°C for up to 15 days which would be indicative of any adverse conditions potentially experienced during transit which is particularly important for producers of RMs or PT providers.

4.3.2.3 LONG-TERM STABILITY

PRECOX LC-FLD

Figure 4-10 a-e represents the long-term stability results for the three materials at three different storage temperatures, -20°C, +4°C and +20°C, determined through preCOX LC-FLD analysis. Data for dcSTX, GTX1,4, GTX2,3, GTX5 and STX were generated and results were normalised to the average of replicate analysis (n=9) of samples stored at the reference temperature of -80°C for the duration of the study. The limits of stability from Figure 4-10 were generated from two times the standard deviation of the -80°C replicate analyses.

All three tissues exhibited excellent stability for all the toxins throughout the duration of the yearlong study when stored at -20°C. Differing levels of stability were exhibited under the

other storage conditions however with both the heat treated and additives spiked materials showing significant improvements to toxin stability compared to the control materials.

Heat treating the tissues provided better stability for the non N-hydroxylated toxins; dcSTX, GTX2,3 and STX including the N-sulfocarbamoyl toxin GTX5, compared to both the additives spiked and control materials. In comparison, the N-hydroxylated toxins GTX1,4 exhibited better stability in the additives-spiked materials compared to the control and thermally treated tissues.

Excellent stability was evident in the heat treated materials stored at +4°C in addition to the -20°C results. The toxins dcSTX and STX also showed excellent stability when stored at +20°C throughout the study, although some slight degradation of GTX5 was evidenced after 9 months at this higher temperature. GTX1,4 showed signs of degradation after 3 months at +20°C with these toxins totally degraded by month 9 in the heat treated materials, while an increase in GTX2,3 concentration was observed in samples after 6 months storage at this temperature.

The inclusion of combined additives to the tissues significantly improved the stability of the toxins compared to the control materials. This was particularly evident for the toxins GTX1,4 which showed no signs of degradation or increases in toxin content throughout the study under any of the storage conditions used even though these toxins were present at relatively low concentrations in the tissues. Some slight degradation of dcSTX, GTX5 and STX was evident after 12 months, 9 months and 9 months respectively when stored at +20°C, with these toxins exhibiting no stability issues under the +4°C storage condition with results falling between the limits of stability set. An increase in GTX2,3 concentration was observed in the additives spiked samples after 3 months under both the +4°C and +20°C storage temperatures.

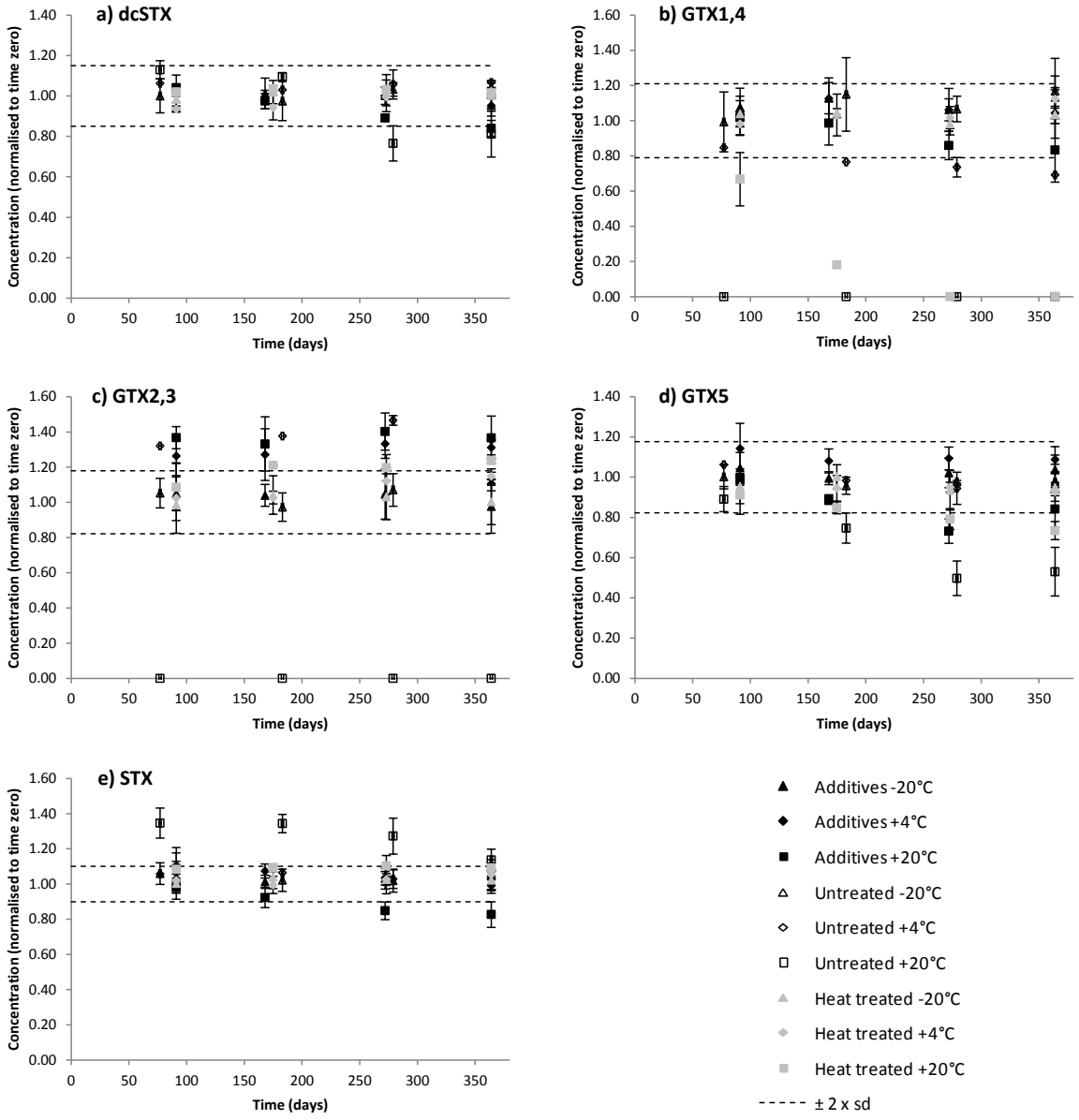


Figure 4-10: Long-term stability graphs for the PSTs present in Tissues A, B and C determined by preCOX LC-FLD. Results are normalised to the time zero reference temperature of -80°C (error bars represent ± 2 s.d.).

Stability issues were evident for all the toxins studied in the control materials most notably with GTX1,4 and GTX2,3 becoming completely degraded after 3 months storage at +20°C and GTX5 showing significant degradation (>50%) after 9 months storage at the same temperature. At +4°C GTX1,4 showed some signs of degradation after 6 months storage, conversely an increase in GTX2,3 concentration was observed after 3 months storage at the same temperature. GTX5 showed no stability issues at this temperature. An increase in STX

concentration was observed after 3 months storage at +20°C which was not evidenced in samples prepared by the two stabilisation techniques and this toxin was found to be stable at +4°C in the untreated materials. dcSTX showed some signs of degradation after 9 months storage at +20°C, although no dcSTX stability issues were observed in samples stored at the other two temperatures. These results highlight the benefits of both stabilisation techniques and specifically the level of stability provided to each toxin while emphasizing the significant improvement each procedure makes in comparison to the untreated materials.

PCOX LC-FLD

Further analysis was conducted by PCOX LC-FLD on the long-term stability study extracts of each of the materials and results are presented in Figure 4-9 c-d. Limits of stability and normalised results were calculated as per the preCOX analysis. Data for the toxins GTX1 and GTX4 were not generated as the concentration of these toxins in each material was <LOD of the PCOX LC-FLD method.

No epimerization or degradation was observed in any materials when stored at -20°C for the duration of the long-term study with results falling within the limits of stability set. As in the case of the short-term study, no epimerization was observed in the heat treated materials under any temperature condition, with the only stability issue evident in these materials being a slight degradation of GTX3 observed after 3 months at +4°C.

Epimerization was observed in the additives spiked materials under both the +4°C and +20°C storage temperatures after 3 months of the study, although this was a significant improvement compared to the control materials where epimerization of the toxins was evident in the +4°C samples but the GTX2,3 content was completely degraded by month 3 at +20°C.

The thermal treatment of materials again proved to be the most effective in minimising or eliminating the amount of epimerization of GTX2,3 with the additives spiked materials also significantly reducing these rates compared to the control tissues.

Epimerization of these toxins followed predicted patterns with the β-epimer GTX3 being epimerized into the more stable α-form GTX2 [136] which was evident from both the short and long-term stability data generated through the PCOX LC-FLD analysis.

CHROMATOGRAPHIC STABILITY

The chromatographic stability of each of the preparation techniques were compared through visual comparison. Figure 4-11 a-h displays chromatograms generated by preCOX LC-FLD for the three materials by both peroxide and periodate oxidation after 12 months storage at different temperatures. Chromatograms of the control material stored at -80°C for 12 months are also shown in this figure as a visual comparison. The chromatograms generated through peroxide oxidation (Figure 4-11 a-c) show differing levels of stability, with the control material showing the total degradation of the toxins GTX2,3 and the formation of an additional fluorescent peak close to that of GTX2,3 in the additives material (Tissue A). Good chromatographic stability was observed in the heat treated materials (Tissue B) with no visual evidence for the formation of additional chromatographic peaks by this processing technique after peroxide oxidation when compared to the control material stored at -80°C. The retention time differences observed in Figure 4-11 are due to the analysis of the tissues being carried out on separate days using different HPLC columns. Retention time QC checks through comparison to standards was however carried out each day.

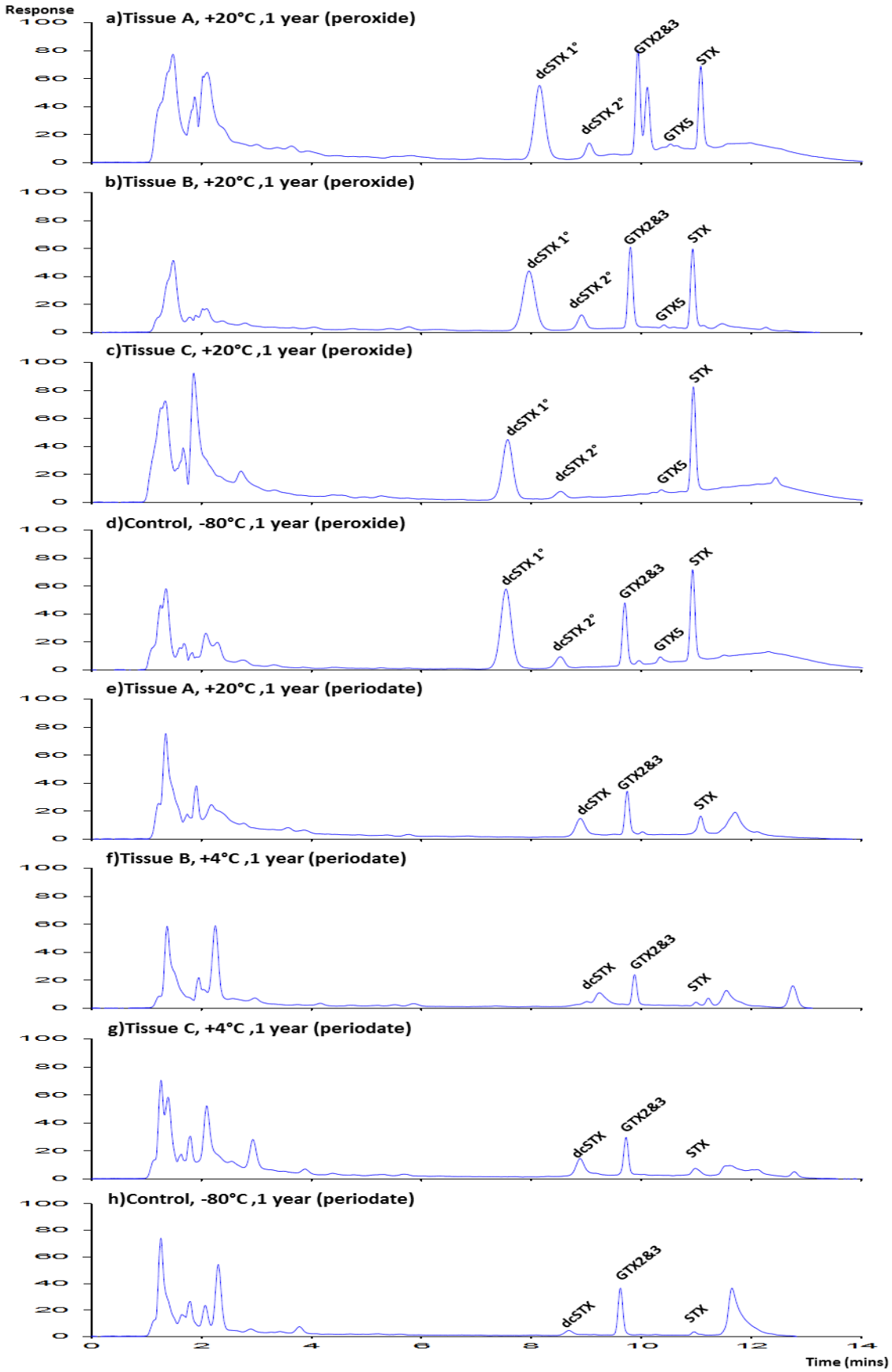


Figure 4-11: PreCOX LC-FLD chromatograms following peroxide (5a-d) and periodate (5e-h) oxidation of Tissues A, B, C and a temperature control material stored for 12 months.

The periodate oxidised extracts used to quantify the N-hydroxylated toxins GTX1,4 are illustrated in Figure 4-11 e-h. In the case of the control and heat treated materials, chromatograms from the +4°C storage condition are shown while a chromatogram for an additives spiked material stored at +20°C for 12 months is shown. This is due to the fact that the total GTX1,4 content in the heat treated and control materials was degraded under the +20°C storage condition. Good chromatographic stability was observed in the samples shown (Figure 4-11 e-h) with no visual evidence for the additional formation of chromatographic peaks from any of the processing techniques after periodate oxidation.

4.3.3 CONCLUSIONS

The techniques investigated in this study were found to significantly improve the stability of PSP toxins in *M. edulis* tissue matrices. The application of a heat treatment step or the inclusion of additives during material processing provided a more stable matrix that was less prone to degradation when compared to an untreated control material. As with previous studies showing the role of bacteria in biotransformations of PSTs [132], techniques such as those investigated in this study, aimed at reducing or eliminating the bacterial effects will lower the rates of toxin degradation, epimerization or biotransformations. Although the material processing and dispensing steps are not carried out aseptically, reducing or eliminating bacteria present in the source tissues through these techniques significantly improves the stability of matrix RMs [52].

As evidenced in both the short and long-term stability studies, frozen sample storage improved toxin stability for all three tissues. Of particular interest however is stability under elevated temperature conditions, which are used to mimic adverse transportation conditions. This is particularly important for RM producers and PT providers to know, as well as

ascertaining ideal long-term storage parameters. Under higher temperature storage conditions, both stabilisation procedures significantly improved toxin stability compared to the untreated control materials, although both techniques varied in the extent to which they enhanced stability. Specifically, the thermal treatment provided better stability for the non-N-hydroxylated toxins GTX2,3, GTX5, STX and dcSTX while the additives spiked materials provided better stability for the N-hydroxylated toxins GTX1,4.

This observation was particularly evident from the long-term study where the total GTX1,4 content in the thermally treated materials were completely degraded by month 9 with these toxins remaining stable throughout the study in the additives spiked material (Figure 4-10 b). The stability of STX and dcSTX in the thermally treated materials was significantly improved compared to the additives spiked with both toxins remaining stable throughout the 12 months under all temperature conditions, whereas some degradation was observed at +20°C in the additives spiked material. Thermally treating the material also eliminated the epimerization of GTX2,3 in contrast to both the additives spiked and control materials, although the inclusion of additives significantly improved the stability and lowered the epimerization rates compared to the control materials.

Overall both material preparation techniques proved effective in stabilising both the tissue matrix and the PSP toxins present as compared to the control material. Each technique provided a greater degree of stability for certain toxins which indicate that combining the two techniques for preparing RMs would result in a well stabilised material for both the non-N-hydroxylated and N-hydroxylated toxins. A combined technique of thermally treating the source tissues prior to spiking with additives is described in the following *section 4.4*.

These techniques also provide a cost effective means of improving PSP toxin stability and prove the applicability of these materials for a range of different uses including method

development and validation as well as use in the general QC of routine monitoring methods and PT schemes as detailed in the following section.

4.4 COMBINED TECHNIQUES, QUALITY CONTROL AND QUASIMEME

This section of *Chapter 4* presents data generated from a RM prepared by combining the techniques described in *section 4.3*, specifically thermally treating the source tissues prior to spiking with preserving additives. This optimised preparation technique is detailed and data is presented from its use over a two year period in the Irish NMP (internal MI code LRM-09-02) and in a development exercise as part of a proficiency testing scheme operated by QUASIMEME since 2011 (PT code Tissue B in *Chapter 6*).

This material's use in QUASIMEME PT schemes is detailed more thoroughly in *Chapter 6* but is highlighted here because a separate issue became apparent during the planning stages of the PT development exercise. This relates to methods used by some participants in QUASIMEME exercises, specifically the use of the MBA and the potential effects which the preserving additives/ethanol solution may have had on this assay. Although in this study each additive is spiked into tissues at levels well below their individual LD_{50} values no data exists in literature on their combined effects, if any. PST free *M. edulis* tissues as determined by both LC-FLD and MBA were spiked with these additives at various levels, extracted following the MBA protocol [40] and injected into mice to observe if there were any effects, particularly PSP-like symptoms which could be misinterpreted. The extraction procedure as described in the materials and methods *section 2.3.3* for the MBA was scaled down to incorporate the smaller aliquot size of 5 grams which was used for QUASIMEME samples. This aliquot size was chosen due to the large amount of tissue which would have been required to make samples for MBA at the prescribed quantity described in the official method [40].

Short-term stability as well as homogeneity were determined experimentally on materials prepared by the optimised preparation technique. Long-term stability was not assessed in this

material due to its primary use in PT schemes and internal QC/QA where stability during transportation is more of a requirement and which can be adequately determined through short-term stability studies.

4.4.1 MATERIALS AND METHODS

4.4.1.1 SOURCE TISSUES

The Spanish and UK (Busta Voe) tissues from Table 2-1 were used to prepare the RM used in this section of the study. The tissues were naturally contaminated with the carbamates GTX2,3 and STX, the decarbamoyl toxin dcSTX as well as the N-sulfocarbamoyl toxins C1,2 and GTX5.

The quantities detailed in Table 4-8 were combined in a 5 L Waring™ goblet. The bulk tissues were homogenised as described in *section 4.2.1.1*.

Table 4-8: Quantities of source wet frozen tissues used to prepare RM with combined techniques of spiking preserving additives with initial thermal pre-treatment..

Country	Species	Quantity (kg)
UK (Busta Voe)	*Mytilus edulis*	1.4
Spain	*Mytilus galloprovancialis*	1.4

Aliquots were taken for MC determination (n=3) using the rotary vacuum method and analysis by preCOX LC-FLD (n=1) as described in the materials and methods *section 2.4.1* and *2.3.1* respectively, to give approximate PST concentrations which are detailed in Table 4-9.

Table 4-9: Concentrations for combined source tissues used to prepare RM.

Material	Concentration (µmol / kg)						Total Toxicity (µg STX diHCl-eq / kg)
	dcSTX	GTX2,3	GTX5	STX	C1,2	GTX1,4	
Bulk homogenate	1.45	2.12	0.13	2.36	-	2.9	2975

4.4.1.2 MATERIAL PREPARATION

The preserving additives solution was prepared at a concentration of 0.02% w/w by dissolving in ethanol (1% w/w). For the additives spike, 0.6 ± 0.01 g each of ampicillin, oxytetracycline, erythromycin and ethoxyquin were weighed into a 50 mL PP centrifuge tube using a calibrated 3-place balance. 30 ± 0.1 g ethanol were added to the centrifuge tube, capped and vortex mixed for 5.0 min.

The tissues were autoclaved in 2 x 3L PP beakers at 121°C for 15.0 min. Subsequently the tissues were allowed to cool to room temperature before being transferred, including any liquid, into a Waring™ goblet and homogenised on medium power for 5.0 min. Aliquots (n=3) were taken for MC determination which was determined to be 76.9% with a %RSD of 0.31%. 2215 ± 1 g of the tissue were gravimetrically transferred into a Waring™ goblet. The additives /ethanol solution was spiked into the tissue washing out the centrifuge tube with a small volume of DI water before manually mixing with a spatula. The final weight of the tissue was made up to 3000 ± 1 g with DI water in order to obtain a final tissue with a MC of ~83%.

The final homogenisation and dispensing steps followed were as described in *section 4.2.1.2.* A total of 582 aliquots were dispensed which provided sufficient quantities for short-term stability studies, homogeneity determinations as well as its intended use as a QC material for

the Irish NMP and as a material in the first PSP development exercise organised by QUASIMEME. All materials were stored at -80°C until further use.

4.4.1.3 MBA MATERIALS

An *M. edulis* tissue was sourced from an Irish NMP sample originating from Carlingford Lough, Co. Louth, Ireland and shown to be free from all EU regulated marine biotoxins by fully accredited MI methods preCOX LC-FLD and MBA for PSP toxins, LC-MS/MS for the lipophilic toxins and LC-UV for domoic and epi-domoic acid (MI Irish National Accreditation Board No. 130T). The tissue was homogenised in a 3 L Waring™ goblet before being subdivided into four separate lots. Three tissues were prepared with additive spikes at 0.01, 0.02 and 0.05% w/w by dissolving antibiotic and antioxidant chemicals in ethanol at a volume of 1% of the total tissue weight.

The 0.01% (100 mg/kg) solution was prepared by weighing 0.012 ± 0.001 g each of ethoxyquin, ampicillin, erythromycin and oxytetracycline into a 50 mL PP centrifuge tube with 1.20 ± 0.01 g ethanol. This solution was vortex mixed (Vortex Genie-2, Scientific Industries, NY, USA) for 5.0 min before being spiked into 120 ± 1 g of the *M.edulis* tissue described above in a 1 L Waring™ goblet, stirred with a spatula and homogenised on medium power for 5.0 min.

The 0.02% and 0.05% additives solutions were prepared identically as above spiking 0.024 ± 0.001g and 0.060 ± 0.001 g of each additive respectively into 1.2 ± 0.1 g ethanol in a PP centrifuge tube. Each additive solution was spiked separately into 120 ± 1 g *M.edulis* tissue described above in a 1 L Waring™ goblet, stirred with a spatula and homogenised on medium power for 5.0 min. The fourth tissue remained untreated and contained no additives or ethanol spike.

4.4.1.4 STUDIES AND ANALYSIS

The between-bottle homogeneity of the material was assessed through the intra-batch analysis of 10 aliquots selected through stratified random sampling of the entire fill series. The number of aliquots chosen for homogeneity determination was based on the recommendations of ISO for PT materials [71].

The material was stability tested over the short term following a reverse isochronous experimental design [55] as conducted previously for other tissues. The short term study was conducted with triplicate samples consisting of five time points (0, 2, 8, 16 and 30 day) and three temperature conditions (-20°C, +4°C and +40°C). The reference temperature used was -80°C. The stability studies were carried out as described in *section 4.2.1.4.*

At the end time point all samples were removed from storage, allowed to equilibrate to room temperature before being extracted and analysed by preCOX LC-FLD closely following OMA AOAC 2005.06 [41] as detailed in *section 2.3.1*. PCOX LC-FLD analysis was not performed on this RM due to its primary use as an internal QC/QA material for the preCOX LC-FLD method routinely used at the MI for PST analysis.

PST concentrations in sample extracts were quantified against a 5-point calibration for each toxin and are expressed in µmol/kg with total saxitoxin equivalents calculated as an estimation of total toxicity as described in *section 2.3.1*.

The MC was determined using the rotary vacuum method as described in the materials and methods *section 2.4.1*, selecting aliquots from the beginning, middle and end of the fill series.

For the additives spiking MBA experiments, each material was extracted according to the AOAC official method 959.08 [40] using a scaled down procedure to incorporate the 5.0 g

aliquot size. Aliquots (1mL) were injected intraperitoneally into male albino CD1 strain mice in triplicate.

4.4.2 RESULTS AND DISCUSSION

4.4.2.1 ADDITIVES SPIKING MBA EXPERIMENTS

Mice were observed for 60 min after intraperitoneal injection with the extracted additives materials. No PSP-like or other symptoms were observed during this time which could be misinterpreted as a PSP positive sample. The additives should therefore have no adverse effect on PSP determination using the MBA.

4.4.2.2 HOMOGENEITY AND MOISTURE CONTENT

As evidenced from Table 4-10 the homogeneity of the PST-positive material was sufficient and was suitable for its intended purposes as a QC/QA and PT material. The target %CV of 8% was achieved for each analyte so again below expected levels of intra-batch repeatability. The homogeneity of the toxins C1,2 were not determined due to the lack of a CRM calibration standard available at the time, although homogeneity of the other toxins should be indicative of overall homogeneity in this tissue.

Table 4-10: Homogeneity results for LRM-09-02 (n=25)

	Concentration (µmol / kg)						Total Toxicity (µg STX diHCl-eq / kg)	% Moisture Content
	dcSTX	**GTX2,3**	**GTX5**	**STX**	**C1,2**	**GTX1,4**		
Average	0.77	1.44	0.12	0.40	-	0.34	888	84.8
Stdev	0.03	0.08	0.01	0.02	-	6.6	47	0.2
%CV	3.8	5.6	6.3	4.7	-	5.2	4.7	0.7

4.4.2.3 SHORT-TERM STABILITY

Figure 4-12 a-e represents the short-term stability results for the material prepared using the combined techniques at three different storage temperatures, -20°C, +4°C and +40°C, determined following preCOX LC-FLD analysis. Data for dcSTX, GTX1,4, GTX2,3, GTX5 and STX were generated and results were normalised to the average of replicate analysis (n=9) of samples stored at the reference temperature of -80°C for the duration of the study. As with previous experiments the limits of stability illustrated in Figure 4-12 were generated from two times the standard deviation of the -80°C replicate analyses.

Overall toxin stability was excellent in the materials stored at -20°C and +4°C for the duration of the short-term study. The stability of the toxins at +40°C differed however with GTX2,3 being the only toxins stable at this elevated storage temperature. Degradation was evident after 8 days in the case of dcSTX and after 16 days in the case of the toxins GTX1,4, GTX5 and STX. GTX5 showed the most signs of degradation with ~80% of this toxins total content in the tissue destroyed by the end of the study compared to ~60% of dcSTX and 40% of GTX1,4 and STX.

The results from the short-term stability studies provide useful information on the conditions required for transportation which was particularly important for this materials use in a PT scheme. Although transportation of matrix RMs ideally should be made under frozen conditions, results indicate materials would also be stable at +4°C for up to 30 days and at +40°C for as long as 8 days. This would mimic any adverse conditions potentially experienced during transit to participants, particularly to countries where delivery is logistically difficult.

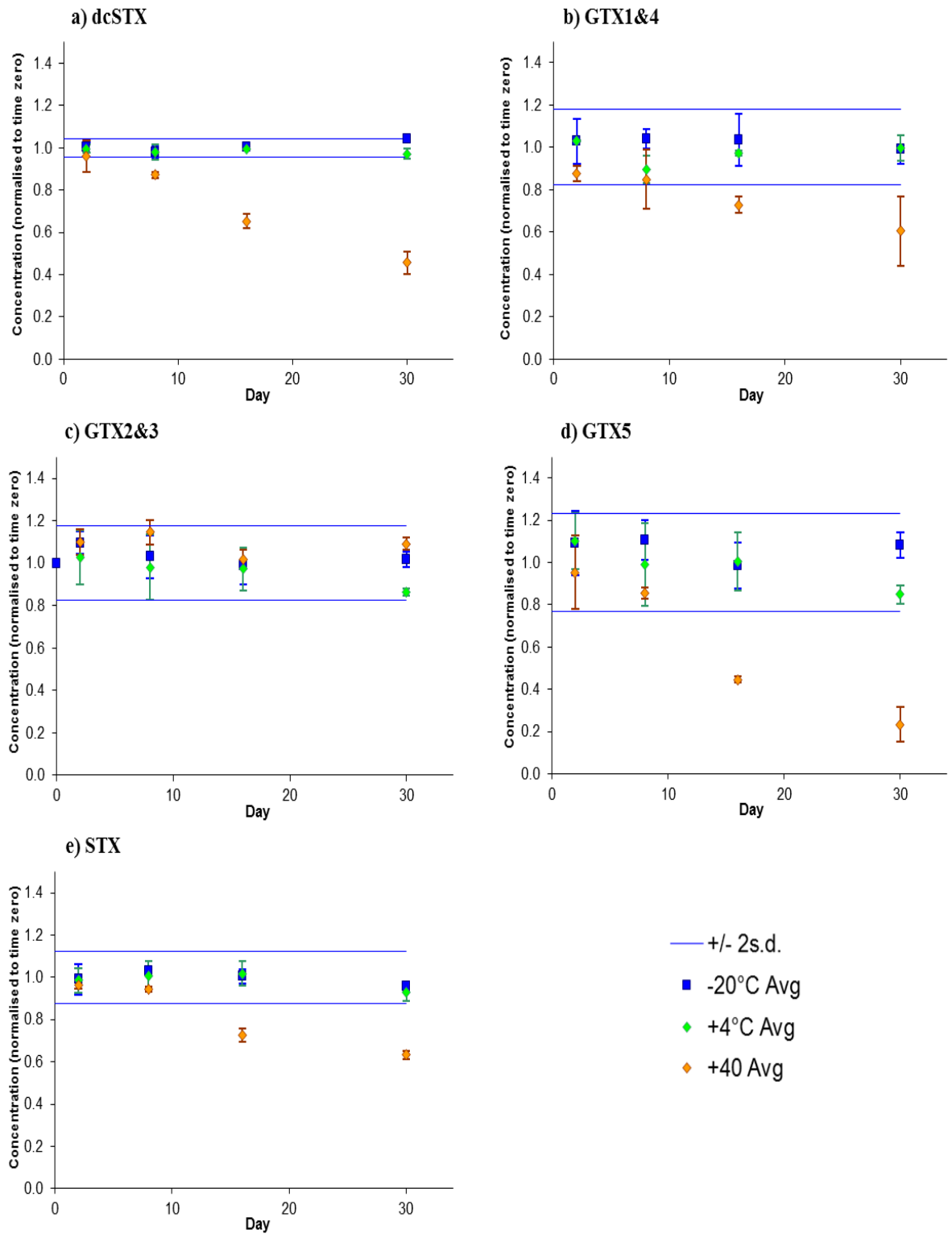


Figure 4-12: Short-term stability graphs for LRM-09-02.

4.4.2.4 INTERNAL QC AND PT SCHEMES

Total toxicity data generated through the use of this RM is presented in the Shewhart chart in Figure 4-13. This RM was used over a two year period from January 2011 to December 2012 as part of the Irish NMP for marine biotoxins and the data was generated using the preCOX LC-FLD method AOAC2005.06 [41]. This data was used to estimate the long-term method reproducibility as an RSD of 8.7%, which was used to calculate an expanded uncertainty of measurement (U) of ~17% at a concentration level just above the EU regulatory limit of 800µg STX diHCl eq./kg based on a coverage factor of 2. The data generated through this materials use and in validation and optimisation experiments was instrumental in the MI ceasing its use of the MBA and moving to PSP analysis by preCOX LC-FLD which was accredited by the Irish National Accreditation Board (INAB) in 2010 (130T).

The within lab reproducibility, expressed as an RSD of 8.7% (Total Toxicity) correlates well with data generated through previous validation studies [120,138,139]. In a single laboratory validation carried out on mussel tissues and reported by Turner *et al*., [120] long-term method reproducibility (> 2 months) was estimated through repeat analysis of an internal LRM. The RSD (%) was determined to be between 11-69% for the individual analogues with total toxicity giving a result of 17%. The low RSD (%) value determined at the MI is indicative of both long-term method reproducibility as well as the long-term stability of the LRM being used.

The use of such materials for internal QC can give valuable information on trends or drifts associated with the method. Figure 4-13 also demonstrates the within-laboratory reproducibility of this method which has been fully in-house validated. Changes to the system made over the two year period this material was in use had no noticeable effects on the overall performance of the method and therefore this RM use. Such changes included new

personnel being trained on and incorporated into running the method, analytical column and guard cartridge replacements, changes in suppliers of chemicals and consumables such as solid phase extraction cartridges, preparation of new standards on a monthly basis and the general and annual maintenance carried out on the instrument. The use of this RM in the Irish NMP also demonstrates the long-term stability of the material using a classical stability model.

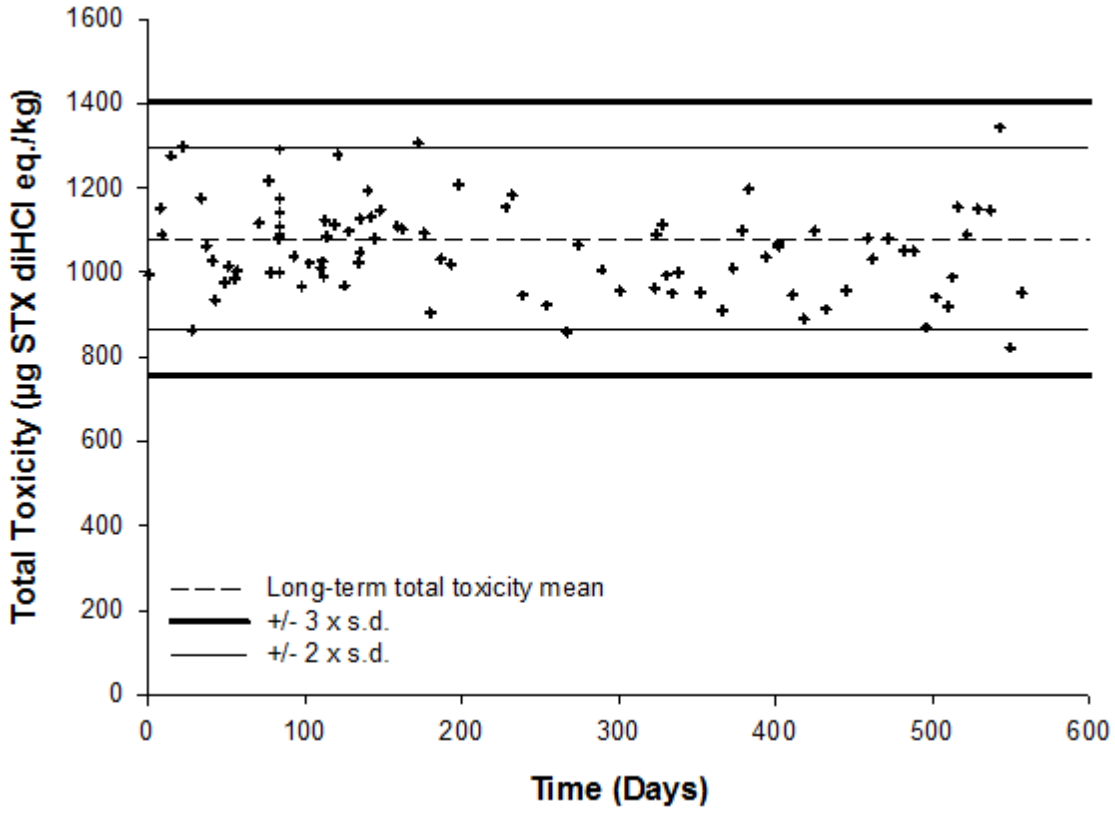


Figure 4-13: Shewhart chart representing total toxicity data collected over a two year period at the Marine Institute in the analysis of PSP toxins by preCOX LC-FLD (AOAC2005.06) as part of the Irish NMP.

The same RM has been used in three PSP development exercises of the QUASIMEME PT scheme over a four year period. These exercises gave further evidence for long-term stability and reproducibility, evidenced through consistent assigned values of 977, 950 and 986 µg STX diHCl eq./kg determined by participants and the associated between laboratory RSD values of 15, 16 and 13% respectively determined from participants receiving satisfactory z-

scores. The assigned values were determined from participants using a range of different methods based on preCOX and PCOX LC-FLD and MBA which demonstrates the applicability of these materials for a range of different methods. This PT data is further discussed in chapter 6.

A material prepared using one of these techniques was used in another PT scheme operated by VEREFIN in 2013. This PT scheme was organised to assess the performance of designated EU laboratories in analysing STX due to its inclusion on the schedule I list of UN chemical weapons. The details of these PT schemes are not discussed in this thesis but are described in Harju *et al.* 2015a and 2015b [140,141].

4.4.3 CONCLUSIONS

From the results contained in this section the combined techniques of thermally treating the source tissues prior to spiking with additives produced a stable RM for the toxins studied.

The results were indicative of both short-term stability under transportation conditions and the long-term stability of the material as evidenced through consistent assigned values in the case of the proficiency testing scheme and a low relative standard deviation of 10.5% for total toxicity data generated over 24 months as part of the Irish NMP.

Materials prepared by these techniques were used successfully for a number of different purposes including method development, optimisation and validation studies, as a QC tool in the Irish NMP for PSP toxins and as PT materials used by QUASIMEME and VEREFIN. The relatively simple techniques described in this chapter so far produce materials sufficiently stable and homogenous for the toxins studied.

4.5 HIGH PRESSURE PROCESSING

The research described in this section of *Chapter 4* details work described in Turner *et al.* 2014 [135].

Andrew D. Turner, Andy L. Powell and **Stephen Burrell.** 2014. Novel application of High Pressure Processing for the production of shellfish toxin matrix reference materials. *Toxicon. 90:1-14*

ACKNOWLEDGEMENT OF COLLABORATION

This work was carried out in collaboration with Dr. Andrew Turner and his team in CEFAS, Weymouth, UK. From the work described in this section the initial trial materials, batch 1 & 2 (HPP 1-6) were prepared by Dr. Turner. This included the extraction and analysis of these initial optimisation materials. Microbiological determination was conducted by Andrew Powell at CEFAS. The materials were HPP treated at AFBI facilities in Belfast. The optimised HPP tissue was transferred on ice to MI facilities where it was processed and dispensed by the author. The homogeneity and stability experiments were designed and conducted by the author at the MI including all toxin analysis by preCOX and PCOX LC-FLD. The control material was prepared by Dr. Turner at CEFAS, including all toxin analysis, and results were provided to the author for comparison purposes.

High Hydrostatic Pressure Processing (HHP) or High Pressure Processing (HPP) is a pasteurisation technique which has been employed for over a hundred years as a non-thermal method of preserving and sterilising food [69]. The process has been applied to high water content foodstuffs including seafood [142] and, owing to the minimal impact the process has on the physical and nutritional characteristics of the product coupled to its sterilising properties, is of interest to RM producers. The pressure is evenly distributed throughout the foodstuff by using water as the transmission medium. Pressures typically in the range of 150MPa to 600 MPa are applied to the product for 1 – 15 min. [70]. Application of HPP in the area of shellfish safety has garnered significant interest, particularly for bivalve molluscs such as oysters. This species, typically eaten raw may have significant microbial activity owing to the filter feeding activities of the animal, with HPP removing total viable counts as well as specific microbiological contaminants including Escherichia coli, Vibrio and norovirus [70,143–146].

The novel application of HPP was therefore investigated for the removal of biological activity from homogenised oyster tissues, specifically in producing stable and homogeneous matrix reference materials. The effects of various HPP parameters on biological activity and PSP toxin concentrations was assessed.

Short and long-term stability experiments as well as homogeneity determinations were conducted on materials prepared by both techniques in comparison with an untreated control using two LC-FLD methods which enabled the quantitation of individual PSP toxins and toxin epimeric pairs. The within-batch repeatability of the process was assessed through the treatment of replicate samples under different processing conditions and the between-batch repeatability examined through the treatment of a second larger volume of tissue also subsequently subjected to analysis to assess material homogeneity and stability.

4.5.1 MATERIALS AND METHODS

4.5.1.1 INITIAL STUDIES (CARRIED OUT BY THE COLLABORATOR AT CEFAS AND AFBI)

CONTAMINATED OYSTER PREPARATION

Pacific oysters (*Crassostrea gigas*) fed mass cultured toxic *Alexandrium* algae as described previously [147], were used to produce two batches of PSP positive material. 350L of an *Alexandrium fundyense* culture (strain CCMP 1719) was produced using a 12 tubular bag photobioreactor set to a 14:10 hour light:dark cycle at 17°C. The culture was subsequently fed to the Pacific oysters over a 5 day period using aerated re-circulating tanks set to 17°C. The oysters were removed from the tank after this period, shucked, homogenised and placed in polythene bags and stored at -20°C until further use. The first batch was diluted with PSP-free Pacific oyster tissue to reduce the total PSP levels to close to the regulatory action limit. The second batch remained undiluted.

HIGH PRESSURE PROCESSING

Following homogenisation, 700 g of the oyster tissue was divided into six sub-samples, with each further sub-divided into three sub-samples. These triplicate sub-samples were labelled and bagged together. In order to successfully transmit pressure through the material, it was necessary to suitably package the tissues to ensure the integrity of the seal was maintained. This involved further sealing the tissues in separate vacuum-pack bags which were placed into additional polythene bags, grouping replicates together. Samples were then sent to the Food Microbiology Branch of Agri-Food and Bioscience Institute (AFBI) of Northern Ireland together with travel control materials which were to remain unprocessed during the

procedure. The packaged samples were allowed to equilibrate to room temperature before the processing conditions detailed in Table 4-11 were applied.

Table 4-11: Experimental conditions used for HPP treatment of oyster tissues (batch 1 and 2).

Sample	Pressure (bar)	Temperature (°C)	Time (min)
HPP1	2000	5	5
HPP2	3500	5	5
HPP3	7000	5	5
HPP4	2000	35	5
HPP5	3500	35	5
HPP6	7000	35	5

A processing error resulted in samples being treated for 1.0 min only, with the remaining 4.0 min treatment applied the next day. Six months after this initial treatment, a second batch of materials, in singe 300 g samples was subjected to HPP using the same processing conditions but administered in single 5 min treatments. After both treatments, samples were shipped back to CEFAS under temperature controlled conditions alongside the untreated travel controls. On receipt at CEFAS, samples were opened and processed immediately.

BACTERIAL LEVEL TESTING

Pouches containing the HPP-treated as well as the untreated control samples were opened and tested for microbial activity using a total viable count (TVC) method. Each tissue sample was tested by taking duplicate 100µL aliquots of serial log10 dilutions spread onto the surface of plate count agar (Oxoid CM0325) plates, with dilutions performed using 0.1% peptone water depending on the microbial activity of the sample. Plates were incubated at 25 ± 1°C for a total of 7 days before being visually assessed, with plates yielding up to 300 colony forming units (CFU)/plate recorded for each dilution test. The mean of each duplicate pair was used

and results were expressed as CFU/g LRM. The nominal limit of detection of the TVC method was 5CFU/g.

TOXIN ANALYSIS

Aliquots of the HPP treated tissues as well as the untreated travel controls were removed from their packaging and allowed to equilibrate to room temperature. After thorough mixing aliquots were taken for toxin analysis with the remaining material stored at -20°C for future stability testing.

Sample extraction, SPE cleanup and oxidation was carried out at CEFAS flowing OMA AOAC 2005.06 [41]. LC-FLD analysis was performed using an Agilent (Stockport, UK) 1200 LC-FLD system with a Gemini C_{18} reversed-phase column (150 mm x 4.6 mm, 5 µm; Phenomenex, Manchester, UK) (set to 35 °C). Mobile phases and LC gradient conditions used were those described previously [147].

Samples were extracted and analysed at the MI by preCOX LC-FLD closely following OMA AOAC 2005.06 [41] as detailed in *section 2.3.1*. PCOX LC-FLD [75] analysis conducted at the MI was additionally performed on all samples to specifically investigate the extent of epimerisation. For PCOX LC-FLD analysis, the crude acetic acid extracts prepared according to *section 2.3.1* were deproteinated and analysed following AOAC 2011.02 [75].

Toxin concentrations were quantified against 5-6-point calibration standards with both individual PST concentrations and total saxitoxin equivalents determined. For the homogeneity and stability experiments, the extraction was scaled down to enable the testing of a suitable number of aliquots, specifically performing extractions on 2.0 g tissue and diluting to a total of 5.0 mL prior to C_{18} clean-up.

STUDY DESIGN

The first batch of HPP-treated tissues, including untreated travel controls were analysed for PSP toxin concentration as well as being assessed for biological activity. Triplicate samples from each of the processing conditions HPP1-6 were analysed to determine the within batch variability of each condition as well as determining optimum HPP parameters. The second batch of HPP treated materials was assessed as these materials were subjected to a single 5 min HPP treatment while also generating a sufficient volume of tissue for stability testing. Extracted tissues were analysed by LC-FLD in triplicate with samples spread evenly throughout the instrumental sequence. Selected HPP-treated tissues were re-homogenised and assessed for homogeneity and stability as described below.

4.5.1.2 ANALYSIS AND STUDY DESIGN (CARRIED OUT BY THE AUTHOR AT THE MI)

HOMOGENEITY

The selected HPP-treated bulk tissues were combined, mixed thoroughly before weighing 2.0 g aliquots into pre-labelled plastic vials and hermetically sealed with foil caps under a stream of nitrogen. Aliquots were stored at -20°C until required for analysis. The homogeneity of the HPP-treated oyster tissue was assessed through the intra-batch analysis of samples selected through stratified random sampling. Ten aliquots were selected, extracted and analysed according to *section 2.3.1*.

STABILITY STUDIES

All materials were stability tested over a short term (ca. 14 day) and long term (ca. 6 month) period following a reverse isochronous experimental design [55]. The short term study was conducted with triplicate samples consisting of five time points (0, 2, 5, 10 and 14 day) and

four temperature conditions (-20°C, +4°C, +20°C and +40°C). The long term study was conducted with triplicate samples consisting of five time points (0, 1, 2, 4 and 6 month) and three temperature conditions (-20°C, +4°C and +20°C). The reference temperature used in both studies was -80°C. In the case of all the stability studies; aliquots (n=3) were removed from -80°C storage on the prescribed day and transferred to the storage conditions being investigated.

At the end time point in both studies samples were removed from the various storage conditions, allowed to equilibrate to room temperature before being extracted and analysed by preCOX LC-FLD closely following OMA AOAC 2005.06 [41] as detailed in *section 2.3.1*. PCOX LC-FLD [75] analysis was additionally performed on all samples to investigate the extent of epimerisation, if any. For PCOX LC-FLD analysis, the crude acetic acid extracts prepared according to *section 2.3.1* were deproteinated and analysed following AOAC 2011.02 [75].

PST concentrations in sample extracts were quantified against a 4 - 5-point calibration for each toxin and are expressed in µmol/kg with total saxitoxin equivalents calculated as an estimation of total toxicity.

The stability studies for the untreated oyster tissue were carried out at a different time and using a different model to the HPP described above so the time points graphically represented in both the short and long-term studies differ slightly (Figure 4-16 & Figure 4-18).

4.5.2 RESULTS

PROCESSING CONDITIONS

The processing conditions recorded during the HPP treatment of the batch 1 and 2 materials are detailed in Table 4-12. The pressures recorded during the HPP treatment were all within 1% of the set values. Adiabatic heating during the process could not be avoided so the processing temperatures recorded during the treatment are different to the set values.

Table 4-12: Actual HPP conditions recorded during the two processing batches.

Sample	Batch 1			Batch 2		
	Maximum temperature	Minimum pressure (bar)	Maximum pressure (bar)	Maximum temperature	Minimum pressure (bar)	Maximum pressure (bar)
HPP1	15°C	1998	2020	15°C	1998	2017
HPP2	18°C	3999	4030	17°C	3996	4019
HPP3	21°C	5989	6023	22°C	5961	6023
HPP4	42°C	1997	2019	39°C	1988	2011
HPP5	45°C	3977	4027	45°C	3977	4019
HPP6	50°C	5959	6021	49°C	5961	6029

BIOLOGICAL ACTIVITY

The TVC testing results of both HPP treated batches of oyster tissue and the untreated travel controls are contained in Table 4-13. The data clearly demonstrates the advantages HPP has on removing biological activity with each treatment HPP 1-6 significantly reducing microbial activity compared to the travel controls, although the lower values in replicate C, batch 1 were noted. The most intensive treatment HPP 6 provided the greatest success in terms of microbial removal.

Table 4-13: Results from duplicate microbial testing of HPP-treated Pacific oyster tissues plus travel controls from two batches of processing, after incubation for 7 days at 25°C (units CFU/g; LOD = 5 CFU/g).

	Batch 1						Batch 2	
Sample	Replicate A		Replicate B		Replicate C			
HPP 1	<LOD	20	20	<LOD	40	40	<LOD	<LOD
HPP 2	<LOD	20	10	<LOD	20	50	300	<LOD
HPP 3	<LOD	30	50	20	30	<LOD	200	<LOD
HPP 4	10	40	20	<LOD	70	<LOD	800	<LOD
HPP 5	20	<LOD	<LOD	30	40	<LOD	1100	300
HPP 6	<LOD	20	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Travel	160000	120000	340000	160000	10000	10000	6000000	6000000

4.5.2.1 PreCOX LC-FLD

BATCH 1

Mean toxin concentrations including total toxicity for each of the HPP treatments and untreated control materials are detailed in Table 4-14. Toxin concentrations were found to be similar in each of the HPP treatments including the travel controls, apart from HPP 1 where a lower concentration of GTX1,4 was determined which resulted in a lower STX-eq/kg value (Figure 4-14).

Table 4-14: Results from PreCOX LC-FLD analysis of HPP-treated tissues (batch 1) and travel controls, showing toxin concentrations and total saxitoxin in STX di-HCl eq/kg (± 1 s.d; n=3).

	HPP1	HPP2	HPP3	HPP4	HPP5	HPP6	Controls
GTX1,4	83 ± 7	102 ± 7	102 ± 6	109 ± 7	103 ± 5	103 ± 5	100 ± 7
NEO	46 ± 4	55 ± 4	55 ± 3	58 ± 3	54 ± 2	56 ± 3	58 ± 7
C1,2	3.7 ± 0.4	4.6 ± 0.3	4.4 ± 0.3	3.9 ± 0.2	4.2 ± 0.4	4.5 ± 0.7	4.2 ± 0.4
GTX2,3	5.8 ± 0.7	6.8 ± 0.6	6.6 ± 1	6.1 ± 0.6	7.2 ± 0.8	6.9 ± 0.8	7.3 ± 0.8
STX	4.4 ± 0.5	5.2 ± 0.3	5.0 ± 0.4	5.2 ± 0.3	5.4 ± 0.2	5.1 ± 0.2	5.2 ± 0.3
Total	143 ± 12	174 ± 11	173 ± 10	182 ± 10	174 ± 7	175 ± 7	175 ± 15

The percentage relative standard deviations (%RSD) as seen in Table 4-14 were below those expected for within-batch repeatability determined for this method during validation studies [120,148]. There was a statistical difference found in the HPP 1 (GTX1,4, NEO, GTX2,3 and

total STX-eq.) and HPP 4 materials (GTX2,3 only) using two-tailed t-tests using pooled standard deviations (two samples assuming equal variance). Two-tailed t-tests showed no statistical difference between the means of the other materials however.

Chromatograms of selected HPP treated and travel controls after periodate and peroxide oxidation are illustrated in Figure 4-15. They show no visual evidence for the generation of additional chromatographic peaks in comparison to the control materials which may relate to the formation of degradation or other naturally fluorescent products.

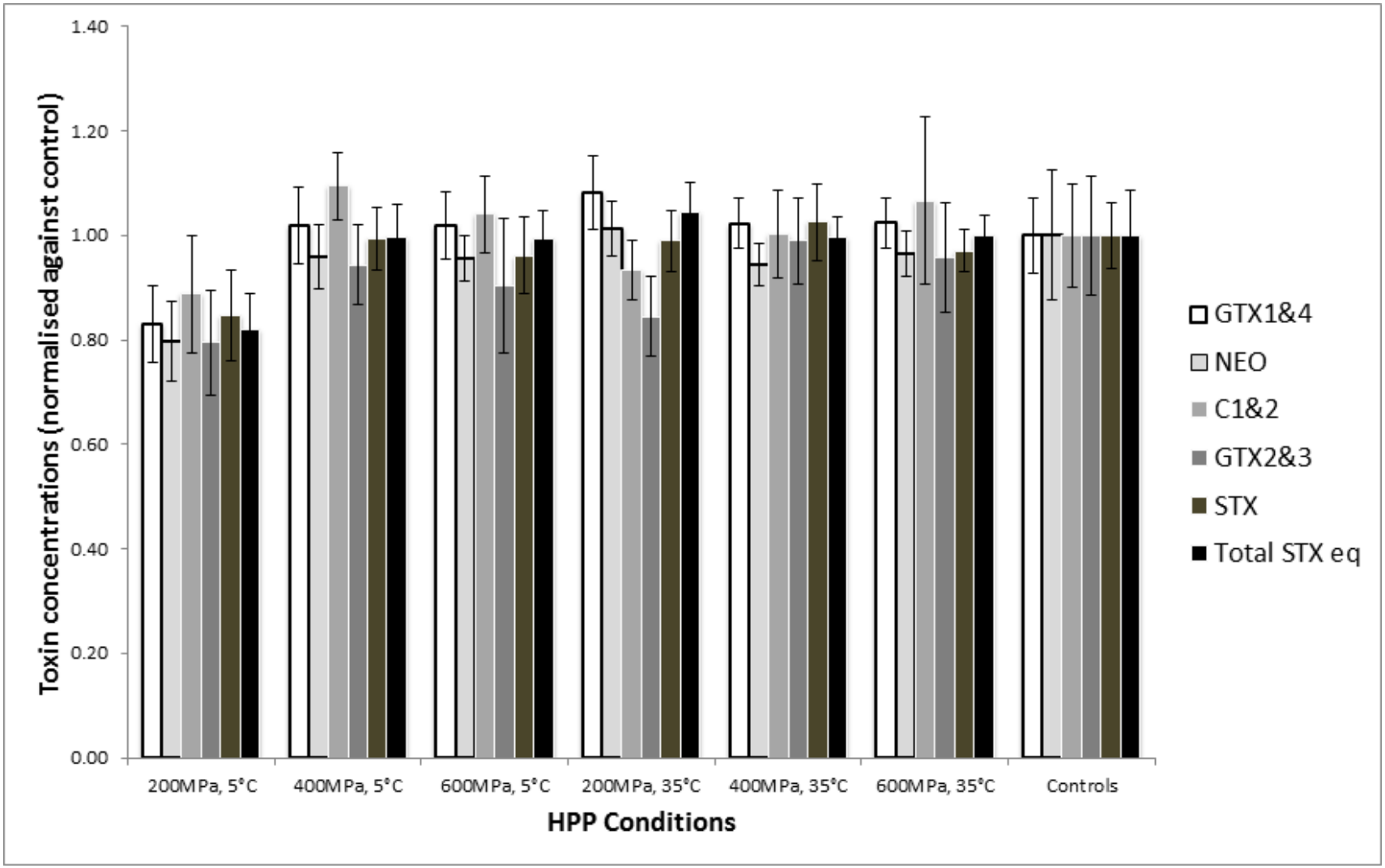


Figure 4-14: Results from PreCOX LC-FLD analysis of HPP-treated oyster tissues and travel controls showing mean toxin concentrations ± 1 s.d. (n=3).

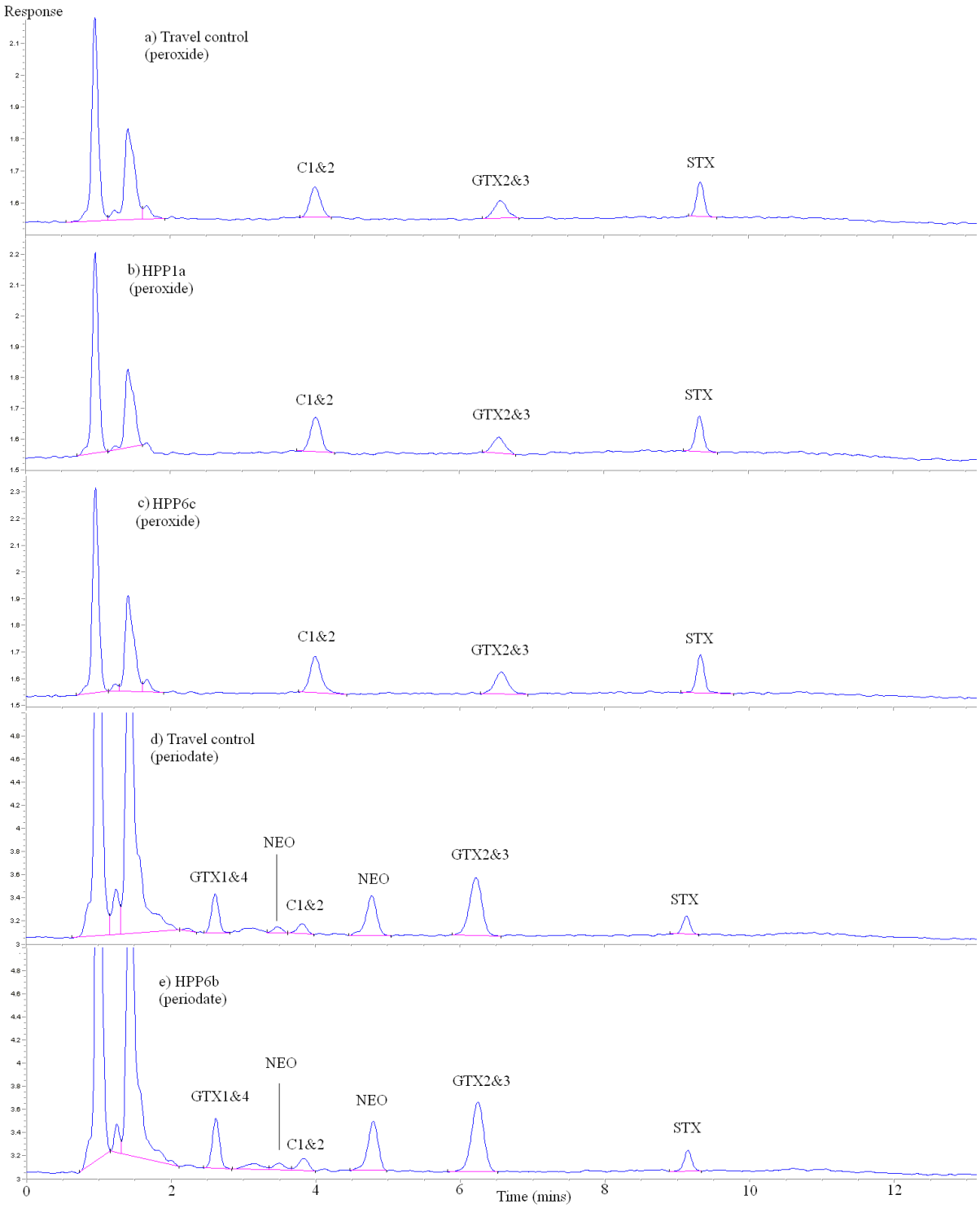


Figure 4-15: Chromatograms obtained from the PreCox LC-FLD analysis following periodate and peroxide oxidation of travel controls and HPP-treated oyster tissues.

BATCH 2

Mean toxin concentrations including total toxicity for each of the HPP treatments (batch 2) and untreated control materials are detailed in Table 4-15 where tissues were HPP treated for a single 5 min step. Although STX-eq values between HPP 1-6 and the travel controls were similar, some differences between individual toxin concentrations were noted across the various treatments. The HPP 1-3 samples processed at the lower temperature of 5°C showed no visual differences in toxin concentrations compared to the travel controls. Conversely the HPP 4-6 samples processed at the higher temperature of 35°C showed some evidence for a reduction of GTX2,3 and STX concentrations and an increase in GTX1,4 and NEO concentrations, compared to the travel controls. A statistical difference was found for the lower GTX2,3 and STX concentrations but not the GTX1,4 or NEO increases after a two-tailed t-test.

Table 4-15: Results from PreCOX LC-FLD analysis of HPP-treated tissues (batch 2) and travel controls, showing toxin concentrations and total saxitoxin in STX di-HCl eq/kg (± 1 s.d; n=3).

	HPP1	HPP2	HPP3	HPP4	HPP5	HPP6	Controls
GTX1,4	284 ± 61	282 ± 86	269 ± 3	357 ± 37	329 ± 70	317 ± 8	296 ± 34
NEO	197 ± 35	187 ± 50	206 ± 31	231 ± 23	227 ± 57	217 ± 26	194 ± 27
C1,2	20 ± 1.4	21 ± 0.7	20 ± 1.7	20 ± 0.6	18 ± 0.3	19 ± 1.0	21 ± 0.3
GTX2,3	45 ± 6	45 ± 3	40 ± 3	36 ± 1	33 ± 0.7	35 ± 2	51 ± 4
STX	62 ± 8	63 ± 7	51 ± 3	46 ± 1	43 ± 1	43 ± 1	65 ± 4
Total	609 ± 110	600 ± 125	587 ± 41	692 ± 61	651 ± 128	632 ± 37	628 ± 70

BULK SAMPLE TESTING

Bulk materials were prepared for homogeneity and stability testing from HPP 5 and HPP 6, batch 2 materials only, which were treated at 6000 bar and 50°C for 5 min.

4.5.2.2 HOMOGENEITY

From the coefficients of variances contained in Table 4-16, all materials were suitably homogeneous for all the PSP toxins present. A target %CV of below 8% for each analyte,

including total toxicity was set which is below expected levels of variability associated with intra-batch repeatability, determined through in-house validation of the LC-FLD method at the MI. The homogeneity techniques employed were sufficient and the materials were suitable for the analytical study.

Table 4-16: Mean concentrations (STX di-HCl eq/kg), standard deviations (sd) and percentage relative standard deviations (%RSD) of toxins in HPP-treated tissues as determined by PreCOX LC-FLD (n=10).

	GTX-2,3	STX	C-1,2	GTX-1,4	NEO	Total
Mean	77	55	34	307	486	960
sd	5	1	2	9	26	35
%RSD	6.2	2.5	4.5	2.9	5.3	3.6

4.5.2.3 SHORT-TERM STABILITY

PRECOX LC-FLD

Figure 4-16 displays the stability results generated by preCOX LC-FLD analysis over 14 days for the HPP oyster tissue, and 16 days for the untreated controls, at four different temperature conditions. Data were generated for all toxins present in the materials as well as total toxicity (Figure 4-16a-f) and results were normalised to the average of replicate analyses of samples stored at -80°C for the duration of the study (time zero). Limits of stability were generated from two times the standard deviation of the -80°C time zero average.

Similar stability between the HPP treated and control materials was observed at storage temperatures of -20°C and +4°C for the duration of the 14 and 16 day studies respectively. Significant improvements could be observed between both materials at the higher temperatures studies however, particularly for the toxins C1,2, GTX2,3 and NEO.

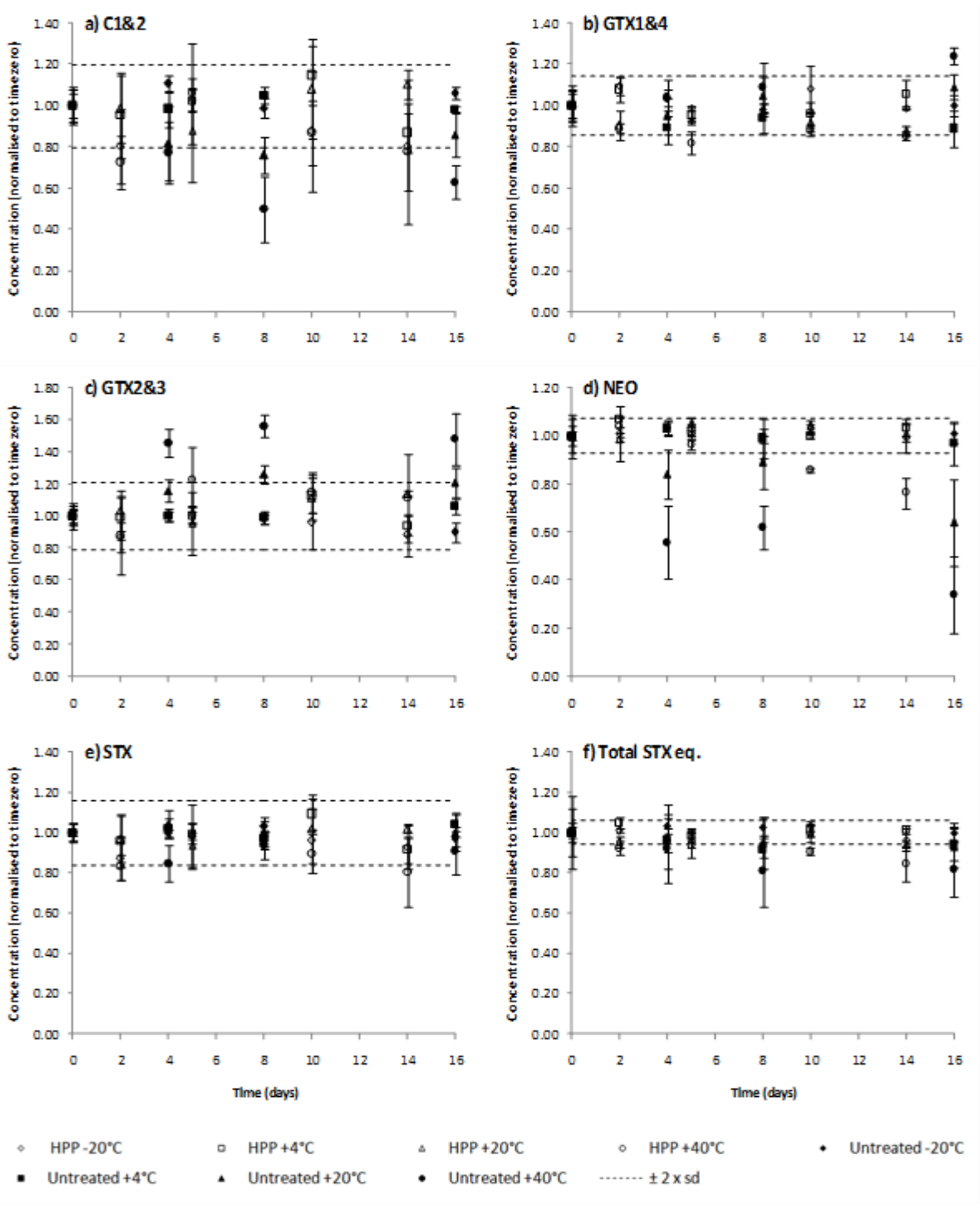


Figure 4-16: Stability charts of PSTs and total saxitoxin equivalents in HPP and untreated tissues over 14 days (HPP) and 16 days (untreated) storage respectively, following preCOX LC-FLD analysis. Concentrations are normalised to time zero (error bars represent ± 2 sd).

Excellent stability was exhibited for all toxins stored at +20°C in the HPP materials with GTX2,3 also found to be stable at +40°C for the duration of the study. Some signs of degradation of the other toxins were observed under the +40°C storage temperature but not to the extent as that observed in the untreated controls. In the case of NEO ~70% of this toxin was degraded by the end of the study in the untreated control compared to ~25% in the HPP materials. An apparent increase in GTX2,3 concentration was observed in the untreated controls at +20°C and +40°C after 8 and 4 days respectively. No stability issues were noted for these toxins in the HPP treated materials however.

PCOX LC-FLD

PCOX LC-FLD analysis was conducted on the HPP tissues stored under all four storage conditions (-20°C, +4°C, +20°C and +40°C) and results are presented in Figure 4-17. The same analysis was conducted on the untreated tissues, with only three storage conditions (-20°C, +4°C and +22°C) studied however. The stability results of the PSP epimers C1,2, GTX1,4 and GTX2,3 in both tissues are presented in Figure 4-17a-f with limits of stability and normalised results calculated as per the preCOX analysis above.

Excellent stability was observed in both the HPP and untreated tissues at -20°C for all the epimers over the course of the short-term study. In addition GTX1,4 were found to be stable at +4°C in both tissues with no signs of epimerisation evident from Figure 4-17 c+d. Epimerisation was observed to take place in the untreated material at +22°C for these toxins, while some degradation of GTX4 was evident in the HPP tissue at +20°C and +40°C with GTX1 showing good stability under the same temperature conditions.

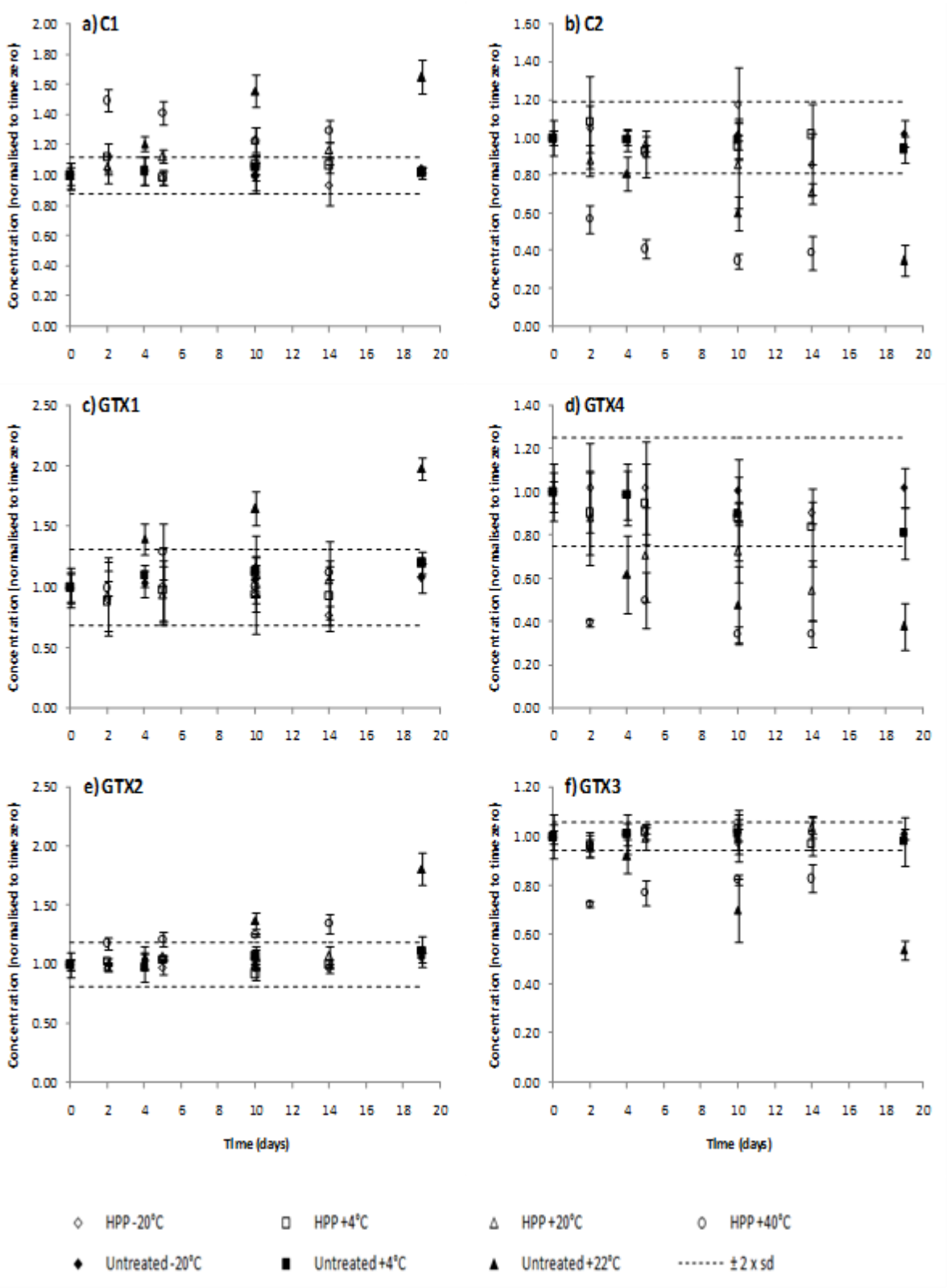


Figure 4-17: Stability charts of the PST epimers C1,2, GTX1,4 and GTX2,3 in HPP and untreated tissues over 14 days (HPP) and 19 days (untreated) storage respectively, following PCOX LC-FLD analysis. Concentrations are normalised to time zero (error bars represent ± 2 sd).

GTX2,3 showed good stability in both tissues when stored frozen at -20°C but epimerisation was observed to take place in tissues stored above this temperature. No epimerisation of C1,2 was observed to take place at +4°C in either tissue but was evident in samples stored at the higher temperatures. The rate of epimerisation was far greater in the untreated tissues stored at +22°C in comparison to HPP samples stored at either +20°C or +40°C however, which demonstrates the significant improvements that this stability technique has over untreated samples.

4.5.2.4 LONG-TERM STABILITY

PRECOX LC-FLD

Figure 4-18 shows the long-term stability results over 6 months duration for all toxins, including total toxicity in both the HPP and untreated oyster tissues generated by preCOX LC-FLD. Three temperature conditions were studied in both tissues with -20°C and +4°C common to both and the higher temperature conditions of +20°C and +40°C studied in the HPP and untreated tissues respectively. This was due to the stability studies of both tissues being carried out at different times and using a different model. Normalised concentrations and limits of stability were calculated as above.

Excellent stability was exhibited throughout the 6 month study for all toxins stored at -20°C, however differing levels of stability were observed at the other temperature conditions. In addition to the -20°C results, HPP treated materials also exhibited excellent stability at +4°C with the exception of GTX1,4 where some degradation was observed after 2 months at this temperature. As in the case of the HPP materials, GTX1,4 stability issues were evident at

+4°C in the untreated controls but additionally stability problems were observed after 6 months for GTX2,3.

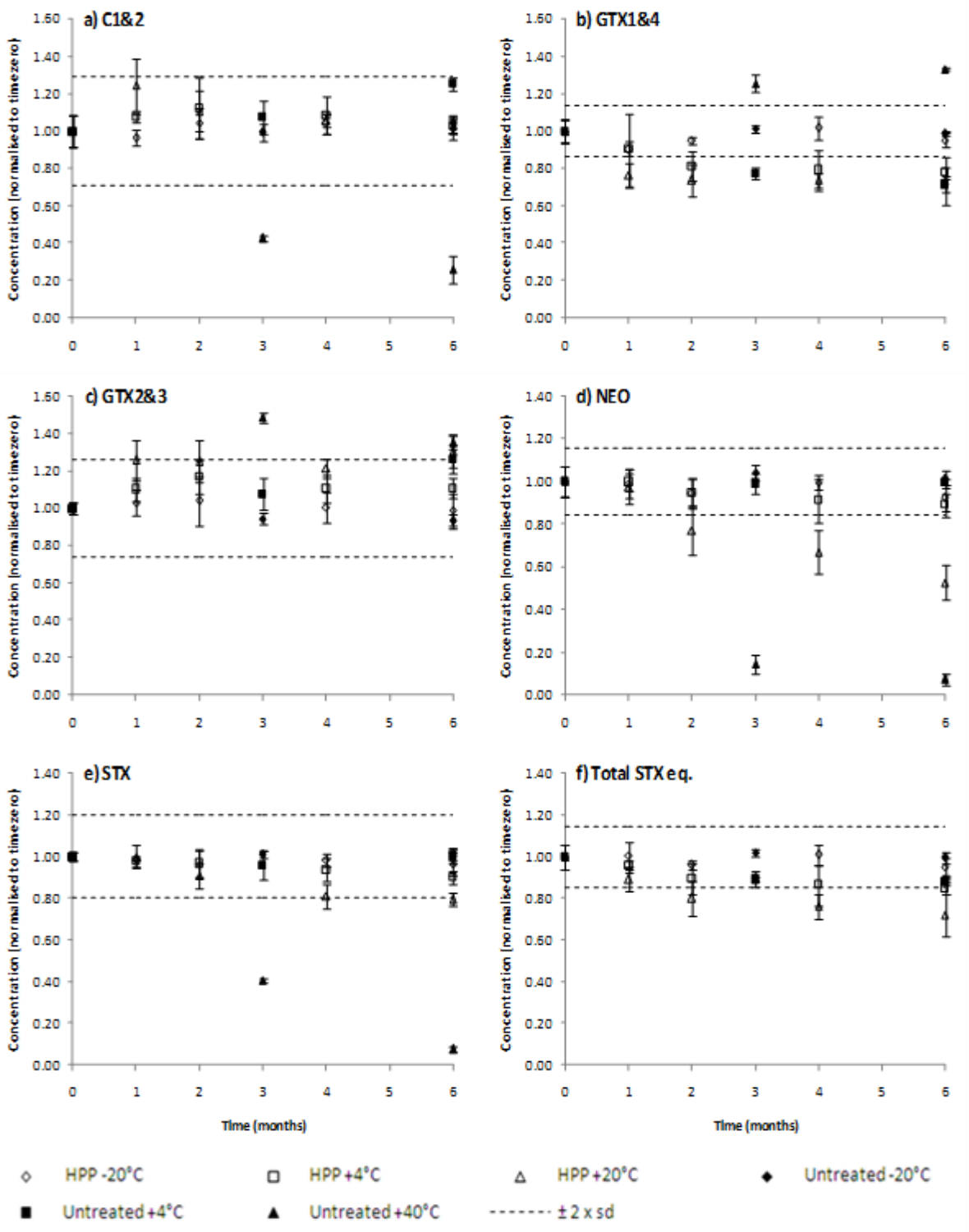


Figure 4-18: Stability charts of PSTs and total saxitoxin equivalents in HPP and untreated tissues over 6 months storage, following preCOX LC-FLD analysis. Concentrations are normalised to time zero (error bars represent ± 2 sd).

In the case of GTX1,4, some degradation was observed in the HPP material at both the +4°C and +20°C storage conditions after 2 months and 1 month respectively. The same level of degradation was observed in the untreated material at +4°C, but an increase in GTX1,4 concentration was observed at +40°C, both evident after 3months.

In materials stored at higher temperatures, C1,2 and STX stability issues were evident in the untreated controls with ~80% and 90% of the toxins degraded by the end of the study. These toxins were found to be stable in the HPP materials, although the different temperature conditions in both studies are noted.

The total toxicity, expressed in STX equivalency, was found to be stable in the HPP material at -20°C and +4°C but some slight degradation was observed at +20°C, while the untreated material gave stable results under all temperature conditions over the 6 months.

PCOX LC-FLD

Long-term stability results of both HPP treated and control materials generated by PCOX LC-FLD analysis are presented in Figure 4-19. Temperature conditions of -20°C and +4°C were assessed in both tissues with an additional +20°C storage temperature studied in the HPP tissue only. The limits of stability and normalised results contained in Figure 4-19a-f were calculated as above.

Excellent stability in both the HPP and untreated controls were exhibited over the 6 month study when stored frozen at -20°C, with no signs of epimerisation or toxin degradation observed. In addition the untreated material also showed excellent stability at +4°C for all three pairs of epimers studied.

Epimerisation of C1,2 and GTX2,3 was observed to take place in the HPP treated material, particularly at +20°C. GTX1 was found to be stable in both the HPP and untreated tissues under all storage conditions over the 6 month study. GTX4 showed signs of degradation after 1 month at +20°C and after 6 months at +4°C in the HPP tissue while no degradation was observed in the untreated tissues for this toxin.

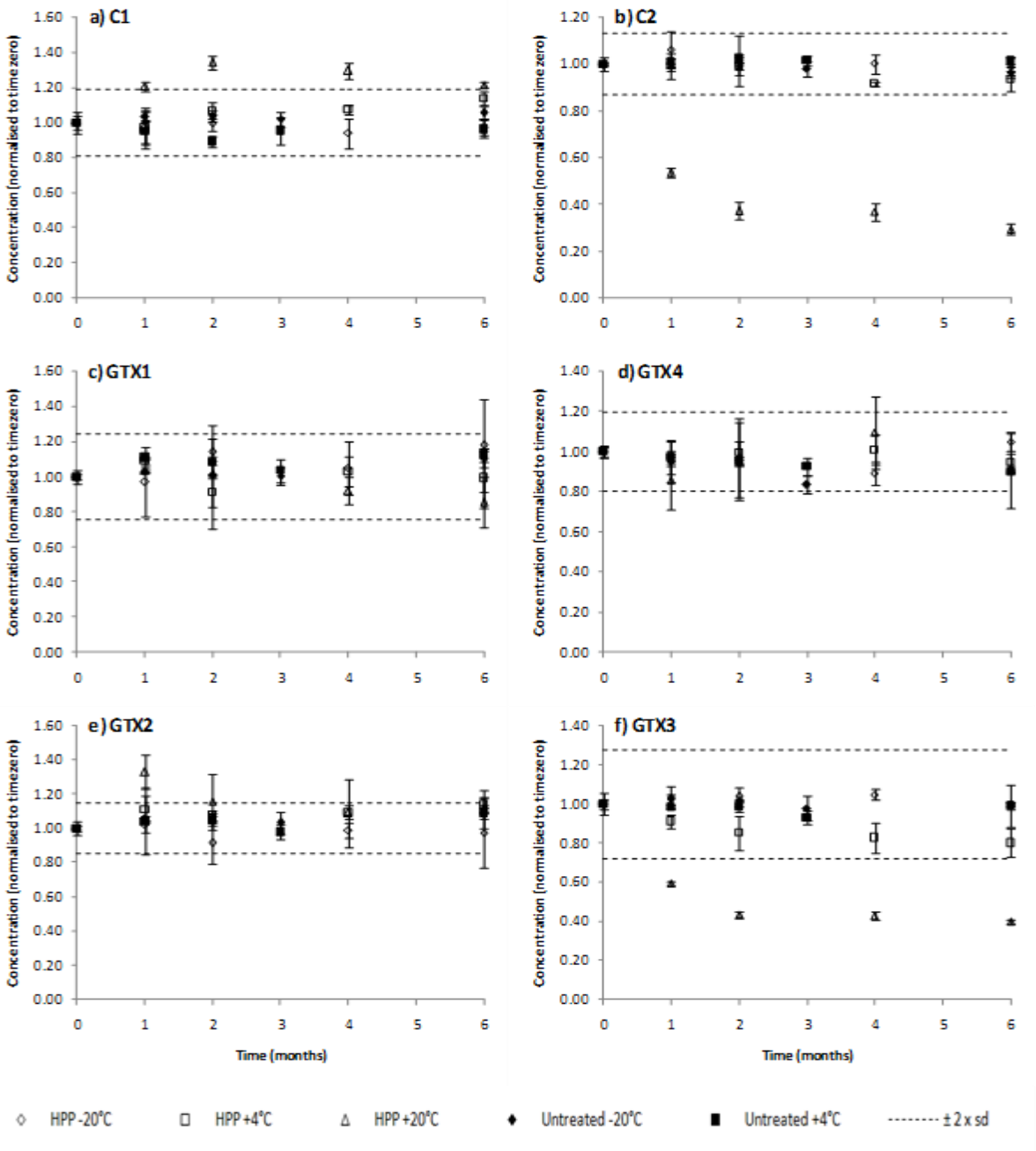


Figure 4-19: Stability charts of the PST epimers C1,2, GTX1,4 and GTX2,3 in HPP and untreated tissues over 6 months storage, following PCOX LC-FLD analysis. Concentrations are normalised to time zero (error bars represent ± 2 sd).

4.5.3 DISCUSSION

The treatment of Pacific oyster tissues by High Pressure Processing under different conditions was shown to successfully remove biological activity from the materials. Analysis by preCOX LC-FLD showed that HPP under these conditions showed no significant changes to toxin content compared to untreated controls. In addition there was no visual chromatographic differences between the HPP treated and control materials either.

A reduction in GTX1,4 concentrations was noted in one set of samples (HPP 1, batch 1), the causes of which are unknown. The second batch of HPP treated materials, processed in a single 5 min step showed statistically different GTX2,3 and STX results in samples treated at higher temperatures compared to the untreated controls. This may have been caused by the single 5 min processing step, although it is not clear if these differences are repeatable or not.

The stability of the PSP toxins was significantly improved in HPP treated materials both in the short and long-term compared to control samples where no pre-treatment was carried out. Figure 4-16 - Figure 4-19 which were generated by different analytical methods employed to investigate potential degradation, conversion or epimerisation issues associated with this stabilisation technique graphically present these findings.

Under frozen storage conditions both the HPP and untreated materials showed excellent stability. At storage temperatures above freezing, toxins in HPP treated materials showed significant stability improvements over untreated particularly in the short-term. Figure 4-16a-f clearly illustrates this with all toxins, in particular GTX2,3 and NEO stability considerably improved when compared to the controls. Transportation of HPP treated materials should be carried out under frozen conditions but stability results prove that even at temperatures as high as +4°C the toxins would be stable for up to 5 days. This has particular relevance for

RM producers or PT providers where the stability of the toxins during transportation is of critical importance.

No toxin degradation was observed in the HPP tissue for any toxins in the short-term 14 day study up to temperatures of +20°C, while some toxins, namely GTX2,3 and STX were also stable at temperatures as high as +40°C. Conversely untreated materials showed stability issues at temperatures above +4°C, particularly GTX2,3 and NEO. Epimerisation of the toxins GTX2,3 observed in HPP tissue in the short-term stability study would require materials to be transported at temperatures below +4°C.

The results generated through these studies clearly show the improvements HPP treatment gives to PSP toxin stability and highlight the advantages this processing technique gives.

4.6 OVERALL CONCLUSIONS

The main aims of the research undertaken as part of these studies were to improve the understanding of the mechanisms of PST degradation, biotransformation, and epimerization taking place in various shellfish tissue matrices. The chapter has detailed various techniques investigated for their potential effects on reducing or eliminating these toxin instability issues, and through the findings, shortlist applicable methods for the production of RMs for various uses. As discussed in the introduction, RMs have multiple uses and the techniques investigated were evaluated for their potential feasibility in CRM, ILRM or simple LRM production. Each technique was evaluated not only for its influence on PST stability, which is of critical importance, but also for its accessibility and ease of use by laboratories. Investment in the equipment used in producing these RMs to both stabilise and ensure homogeneity can be substantial and therefore accessibility may be an issue for monitoring and research laboratories particularly those under fiscal constraints. This chapter presents techniques which are polar opposite in terms of equipment costs with HPP providing arguably the best stabilisation method investigated but also representing the costliest in terms of equipment required. Conversely simpler techniques such as the use of preserving additives or thermal treatment represent a cost effective means of producing high quality RMs. These techniques may not be feasible in CRM production owing to the superior stability offered by alternatives such as HPP or freeze drying (*Chapter 5*), but their use as internal LRMs and for use as PT materials has been adequately demonstrated.

Table 4-17 lists the RMs prepared in this chapter and summarises the stability observations determined through the short and long-term studies. As expected no issues were observed in any of the tissues when stored at -20°C including the untreated control materials which shows degradation, epimerization and biotransformations of toxins are minimised under this storage condition.

Table 4-17: Summary of RMs prepared in Chapter 4 with short and long-term stability details.

Material	Matrix	Stabilisation Technique	Analyte	Stability							
				-20°C		+4°C		+20°C		+40°C	
				Degradation	Epimerization	Degradation	Epimerization	Degradation	Epimerization	Degradation	Epimerization
Tissue 1	**Mussel**	**Antibiotic Spike**	dcSTX	348 days	n/a	348 days	n/a	69 days	n/a	8 days	n/a
			GTX2,3	348 days	32 days	32 days	16 days	0 days	0 days	32 days	0 days
			GTX5	348 days	n/a	348 days	n/a	154 days	n/a	3 days	n/a
			STX	348 days	n/a	348 days	n/a	154 days	n/a	3 days	n/a
			GTX1,4	348 days	n/a	69 days	n/a	154 days	n/a	8 days	n/a
Tissue 2	**Mussel**	**Antioxidant Spike**	dcSTX	351 days	n/a	351 days	n/a	224 days	n/a	8 days	n/a
			GTX2,3	351 days	32 days	32 days	8 days	0 days	0 days	17 days	0 days
			GTX5	351 days	n/a	221 days	n/a	73 days	n/a	4 days	n/a
			STX	351 days	n/a	351 days	n/a	351 days	n/a	17 days	n/a
			GTX1,4	351 days	n/a	73 days	n/a	158 days	n/a	4 days	n/a
Tissue 3	**Mussel**	**Untreated Control**	dcSTX	354 days	n/a	354 days	n/a	221 days	n/a	17 days	n/a
			GTX2,3	354 days	32 days	17 days	6 days	0 days	0 days	6 days	0 days
			GTX5	354 days	n/a	224 days	n/a	73 days	n/a	6 days	n/a
			STX	354 days	n/a	354 days	n/a	354 days	n/a	17 days	n/a
			GTX1,4	354 days	n/a	76 days	n/a	76 days	n/a	0 days	n/a
Tissue A	**Mussel**	**Additives Spike**	dcSTX	364 days	n/a	364 days	n/a	168 days	n/a	6 days	n/a
			GTX2,3	364 days	364 days	30 days	16 days	0 days	0 days	30 days	0 days
			GTX5	364 days	n/a	364 days	n/a	91 days	n/a	6 days	n/a
			STX	364 days	n/a	364 days	n/a	168 days	n/a	6 days	n/a
			GTX1,4	364 days	n/a	364 days	n/a	364 days	n/a	4 days	n/a
Tissue B	**Mussel**	**Thermal Treatment**	dcSTX	364 days	n/a	364 days	n/a	175 days	n/a	28 days	n/a
			GTX2,3	364 days	364 days	364 days	364 days	91 days	364 days	28 days	0 days
			GTX5	364 days	n/a	364 days	n/a	175 days	n/a	8 days	n/a
			STX	364 days	n/a	364 days	n/a	364 days	n/a	28 days	n/a
			GTX1,4	364 days	n/a	364 days	n/a	0 days	n/a	8 days	n/a
Tissue C	**Mussel**	**Untreated Control**	dcSTX	364 days	n/a	364 days	n/a	183 days	n/a	15 days	n/a
			GTX2,3	364 days	364 days	32 days	0 days	0 days	0 days	0 days	0 days
			GTX5	364 days	n/a	364 days	n/a	77 days	n/a	8 days	n/a
			STX	364 days	n/a	364 days	n/a	0 days	n/a	15 days	n/a
			GTX1,4	364 days	n/a	77 days	n/a	0 days	n/a	4 days	n/a
LRM-09-02	**Mussel**	**Additives Spike & Thermal Treatment**	dcSTX	30 days	n/a	30 days	n/a	n/a	n/a	2 days	n/a
			GTX2,3	30 days	n/a	30 days	n/a	n/a	n/a	30 days	n/a
			GTX5	30 days	n/a	30 days	n/a	n/a	n/a	8 days	n/a
			STX	30 days	n/a	30 days	n/a	n/a	n/a	8 days	n/a
			GTX1,4	30 days	n/a	30 days	n/a	n/a	n/a	8 days	n/a
HPP Tissue	**Oyster**	**HPP**	GTX2,3	180 days	180 days	180 days	180 days	120 days	14 days	2 days	2 days
			STX	180 days	n/a	180 days	n/a	120 days	n/a	14 days	n/a
			GTX1,4	180 days	180 days	30 days	180 days	14 days	180 days	8 days	14 days
			C1,2	180 days	180 days	180 days	180 days	180 days	10 days	0 days	0 days
			NEO	180 days	n/a	180 days	n/a	30 days	n/a	5 days	n/a

N.B. All time points listed in columns titled degradation indicate stability was observed up to and including that stated; after which degradation or instability issues were observed.
Time points listed in columns titled epimerization indicate this was not observed to occur in materials up to and including this point; after which epimerization was observed.
A time point of 0 indicates degradation or epimerization was observed to occur before the first time point in the study.
n/a analysis not carried out.
*degradation or epimerization observed before the first time point of 3 months.

Thermally treating materials significantly improved toxin stability with tissues stabilised in this way showing no instability issues when stored at +4°C over a one year period in addition to the -20°C observations. This treatment was the only one observed to have no stability issues at +4°C. This treatment also eliminated or minimised epimerization of the toxins GTX2,3 over the same time period at -20°C, +4°C and +20°C. This relatively simple technique of autoclaving source tissues prior to being dispensed provides considerable benefits in RM preparation and could be used as a stabilisation pre-step to any of the other techniques investigated in combining these procedures.

The use of antibiotics in RM preparation gave more stability to the PSTs when compared to the use of an antioxidant alone. This was particularly evident in the +20°C and +40°C samples where instability issues were observed earlier in Tissue B, apart from STX which was found to be more stable in this material. The stability enhancements observed in the antibiotics spiked material (Tissue 1) were also observed in the combined additives spiked material (Tissue A) which indicated these improvements have come mostly from the effects of antibiotics. The use of the antioxidant would still be recommended however because of the stability improvements this additive provides for STX compared to antibiotics.

HPP also proved an excellent stabilisation technique significantly decreasing instability issues compared to untreated controls. Arguably this technique provided no significant stability improvements compared to the thermal treatment of tissues with the latter technique being substantially more cost effective. However, both studies were carried out in different matrices so a study of HPP treatment of *M. edulis* tissues would be required to definitively prove this conclusion.

An interesting observation concerns the toxins GTX2,3 in the untreated control materials, Tissue 1 and Tissue C which can be seen in the long-term stability graphs in Figure 4-5 and

Figure 4-10. Figure 4-5 shows GTX2,3 increases at +40°C by the first time point after 3 months storage and this increase remains relatively consistent over the yearlong study with no obvious degradation of these toxins. Conversely Figure 4-10 shows the complete degradation of GTX2,3 at the same temperature after 3 months storage. The source of GTX2,3 in both tissues is slightly different but the preparation techniques in both cases were identical so the source of the instability issues could come from the alternative tissues used in both or potential contamination during the process as steps are not carried out aseptically. Bacteria may have been introduced into the tissue during its preparation which has led to the instability issues observed or this issue could relate to different bacterial colonies being present in the source tissues.

Another interesting observation relates to dcSTX and the instability issues observed in the antibiotics material (Tissue 1) compared to the untreated control (Tissue 3) where stability up to 221 days storage at +20°C was evident for this toxin. This deterioration in stability of dcSTX could be due to bacteria being introduced during the processing steps as these are not carried out aseptically; although spiking with antibiotics should minimise these effects.

Microorganisms, such as bacteria present in the shellfish can spoil the tissue leading to the degradation of the material. Toxin biotransformations are also known to take place in tissues when certain bacteria are present [132], so any process aimed at reducing the numbers of these microorganisms, destroying them completely or stopping them from multiplying will aid in the stabilisation of the tissue.

All the techniques investigated in these studies improved the stability of the PSP toxins overall. The applicability of each technique in preparing RMs depends on their final use however.

The relatively simple techniques of thermal pre-treatment or the inclusion of additives improves the stability of the material without adding significant costs. These techniques could be employed by most laboratories and would lead to a significant improvement in the standard of RMs being produced. Bacteria play a major role in the stability of both the matrix and the toxins themselves, as evidenced through the stability studies, and biotransformations have been observed to take place in these tissues.

The techniques detailed in this chapter were used to prepare materials which have been used in PT schemes globally. Multiple materials were prepared by additives spiking with an initial heat treatment step and used in QUASIMEME PT schemes as detailed in Chapter 6. Another material, stabilised in the same way was prepared and used in a PT scheme organised by VERIFIN, one of eighteen laboratories globally which has been assigned "designated status" by the OPCW (Organisation for Prohibition of Chemical Weapons). This scheme was organised to assess EU MSs proficiency in determining PSTs, in particular STX which is a Schedule 1 substance under the CWC (Chemical Weapons Convention).

Based on the information generated in this chapter, recommendations for the preparation of stable and homogeneous reference materials would be:

- The homogenisation techniques employed in these studies, such as the use of Waring™ and Polytron™ blenders, were sufficient to prepare materials fit for purpose.
- HPP was the most effective in stabilising PSTs in a Pacific oyster tissue matrix although access to the relevant equipment may be a limiting factor in using this technique for other purposes than CRM preparation.

- The thermal treatment of tissues significantly improves matrix and PST stability and presents a cheap and simple technique in preparing RMs. Furthermore thermal treatment could be carried out in combination with other stabilisation techniques.
- Materials prepared through the combined use of preserving additives coupled with a thermal pre-treatment step significantly improve PST stability. Materials prepared in this way were shown to have a wide range of applications including PT, internal QC/QA or for validation studies.

Chapter 5

FREEZE DRYING

5.1 BACKGROUND AND AIMS

The presence of water in biological matrices is an essential ingredient for microbial growth. The previous chapter dealt with stabilisation techniques in the preparation of wet tissue RMs where water was not removed but increased in most cases to alter the final MC of the material to reflect natural shellfish matrices. The biological matrix, unless stabilised through, for example one of the techniques investigated in the previous chapter is prone to bacterially induced degradation, biotransformations or epimerization as evidenced from the instability issues of untreated control materials. Removal of water from the matrix should therefore improve stability through alteration of the environment necessary for bacterial colonies to flourish.

The techniques investigated in the previous chapter proved effective in stabilising PSTs in mussel and oyster tissue matrices but their feasibility in the production of CRMs was questionable mainly due to epimerization issues observed at storage conditions above freezing. An alternative technique of freeze drying was investigated in order to ascertain if stability, particularly epimerization rates, could be minimised or eliminated. Freeze drying was previously investigated in the preparation of RMs for PSTs [67,68,149] where homogeneity and stability were adequately demonstrated in mussel tissues. The stability experiments carried out in the study by van Egmond *et al.,* in 1998 [149] was not assessed in comparison with wet tissue homogenates however, nor were the details provided on the freeze drying processes involved, parameters which are critically important in assuring homogeneity with consistent recoveries [150]. More recently this technique was used to

successfully prepare a candidate CRM for multiple phycotoxins [63–65], although this material did not contain PSTs.

The aim of this chapter was to investigate this stabilisation technique by developing a freeze drying method using instrumentation at the MI, incorporating specialised processing and dispensing techniques, to ensure homogeneity and minimise water uptake by the hygroscopic material. Feasibility of producing an oyster CRM was assessed and a large scale RM was produced using the optimised freeze drying, processing and dispensing conditions. The ultimate aim was to prepare a candidate RM for certification.

5.2 FEASIBILITY STUDY

The research described in this section of *Chapter 5* appears as described in Turner *et al.* 2013 [133].

Turner A, Lewis A, Hatfield R, Higman W, **Burrell S** (2013) A feasibility study into the production of a freeze-dried oyster reference material for paralytic shellfish poisoning toxins. Anal Bioanal Chem 405:8621–8632.

ACKNOWLEDGEMENT OF COLLABORATION

The work in this section was carried out in collaboration with Dr. Andrew Turner and his team at CEFAS. The RM was freeze dried, processed and dispensed by the author and collaborator at MI facilities. The finished product was shipped back to CEFAS facilities on ice and stability studies were set up and conducted by the collaborator at his facilities. Toxin analysis was carried out by the author and collaborator during a site visit by the author to CEFAS facilities.

Figure 5-1: Site visit made by the author to CEFAS facilities during the freeze drying feasibility studies.

Here, the objective was to develop procedures using modern freeze-drying processes for the generation of a freeze dried shellfish tissue and to conduct experiments to assess the characteristics of the material in relation to its suitability as a candidate RM. Oyster tissue was selected as the matrix of preference for two main reasons. Firstly, there is a known issue in this species with the comparability of method performance between the MBA and non-animal testing methods [77]. Availability of a suitable CRM in this matrix would provide a useful tool for ongoing method assessment purposes. Secondly, it was the authors understanding that a mussel reference material was to be made available by the NRCC in the near future, so concentrating on a different shellfish species was likely to be of greater interest. This section of work was a collaborative study involving CEFAS and the MI. Paralytic Shellfish Toxin (PST) contaminated oyster tissues generated following shellfish feeding experiments at CEFAS were shipped to the MI where a suitable freeze-drying process had been developed, optimised and applied. Preliminary materials were assessed at both laboratories before tailored tissue materials were shipped back to CEFAS for subsequent investigations to assess the RM characteristics, including the comparison of toxin profiles with the wet tissues and assessing the stability and homogeneity between tissue sub-samples. In particular the stability of the tissues was examined over both short term and long term storage and over a range of temperature conditions. The data generated was subsequently used to determine the feasibility in producing a freeze dried oyster tissue for PSP toxins with the future aim of producing a large volume of certified RM. The flow chart contained in Figure 5-2 details the steps involved in preparing the feasibility study materials.

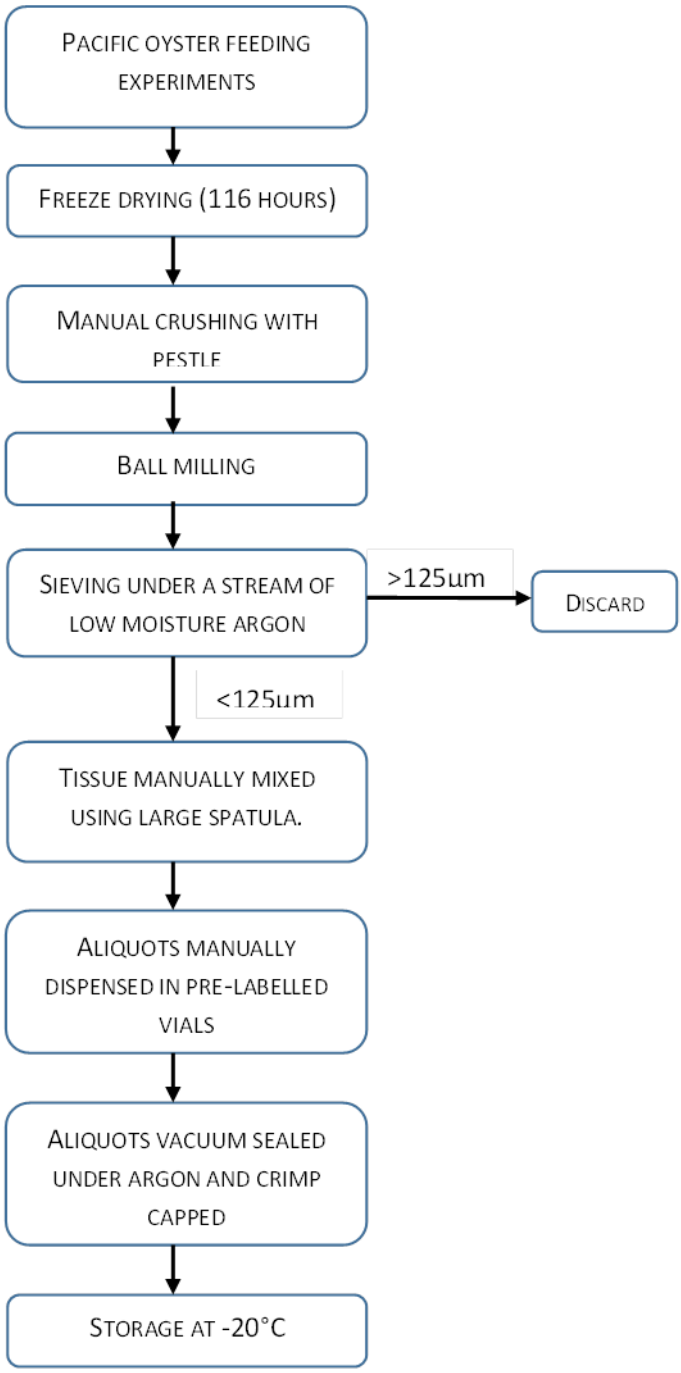


Figure 5-2: Flow chart of process used to prepare freeze dried feasibility study tissues.

5.2.1 SOURCE TISSUE

Pacific oyster (*Crassostrea gigas*) tissue contaminated with PSTs was prepared at CEFAS through mass culturing of toxic *Alexandrium fundyense* [147] and feeding experiments as described in *section 4.5.1.1*. Once complete, the oysters were shucked and homogenised as one batch of ~4 kg of whole flesh tissue. The bulk tissue was further sub-divided in two, half of which was used to prepare a wet tissue RM and the other half used to prepare the freeze dried RM. The wet tissue RM was prepared at CEFAS through the addition of DI water, to adjust the MC to ~85%, and adjustment to pH 4.0 to stabilise the toxins present in the tissue. The tissue was dispensed as 6.0 g aliquots into PP vials and hermetically sealed under a stream of nitrogen. Aliquots were stored at -20°C until further use.

The MC of the sub-sample for freeze drying was determined gravimetrically following replicate baking steps at CEFAS before being adjusted to ~85% MC through the addition of DI water. The tissue was then frozen at -20°C before dispatch to the MI using temperature controlled transport containers. Upon receipt, the material was stored at -20°C until further use.

5.2.2 FREEZE DRYING

All equipment used to prepare the freeze dried materials were acid washed, rinsed thoroughly with DI water before being placed in a desiccator containing activated silica beads. The oyster tissue was removed from the freezer and allowed to defrost to room temperature overnight before being homogenised further through the use of Waring™ and Polytron™ blenders. The tissue was transferred into two stainless steel freeze drying trays, covered in tinfoil and frozen overnight at -20°C.

The trays were removed from the freezer and immediately transferred to the freeze dryer (VirTis, Virtual 50XL) which were then subjected to a 116 hour freeze drying programme. The freeze drying programme was similar to that described in Table 5-5, FD-1 but 20 hours longer in duration. The process involved increasing the temperature in the chamber over a 116 hour period from -35°C to +20°C through a series of ramping steps. Simultaneously the pressure inside the chamber was reduced from atmospheric pressure to ~1 Pa.

Upon completion of the programme the trays were removed from the chamber and the freeze dried cakes of oyster tissue were manually ground using a pestle to break up large conglomerates. The tissue was transferred to stainless steel bowls containing 25 x 20 mm steel balls, in ~100 g lots. The tissue was milled using a planetary ball mill (Retsch PM100) for 5.0 min at 400 rpm. The milled material was sieved through a 2mm sieve to remove large pieces of material, followed by a 125 µm sieve to remove any material with particle sizes >125 µm for 30 min. This was due to issues with the homogeneity of powder materials with particle sizes above 125µm. To minimise water uptake by the hygroscopic freeze dried material, the sieving unit was covered with a polyethylene sheet and a steam of low moisture content argon was constantly flushed over the unit [62]. The sieved material was manually mixed using a large spatula before dispensing 2 x 2.2 g aliquots into 5 mL amber glass ampoules, sealed under a vacuum of low moisture content argon. The remaining material was transferred to PP bags, vacuum heat sealed and stored at -20°C until further use.

5.2.3 DISPENSING

The optimum aliquot size to dispense these materials was determined through weighing variable masses (0.1, 0.2, 0.3, 0.5 and 1.0 g) of the 2.2 g aliquot, dispensed in the previous section, into 50 mL PP centrifuge tubes. The tissues were reconstituted to 5.0 g through the

addition of DI water and vortex mixing for 5.0 min. The five samples were then extracted and analysed according to OMA AOAC 2005.06 as described in *section 2.3.1*.

The remaining freeze dried material was removed from the freezer and allowed to equilibrate to room temperature overnight. The vacuum sealed bag was opened and the freeze dried material transferred to a PP beaker. The material was mixed using a large spatula before being dispensed as ~0.53 g aliquots into pre-labelled 5 mL amber glass ampoules. The dispensing steps were carried out in a modified glove box purged with low moisture content argon which was kept at a low constant flow throughout the process. After dispensing, the aliquots were removed from the glove box and transferred to the freeze dryer where they were vacuum sealed under an atmosphere of low moisture content argon. 300 aliquots were dispensed and crimp capped before being stored at -20°C until further use.

5.2.4 RECONSTITUTION, EXTRACTION AND TOXIN ANALYSIS

The freeze dried material was re-constituted by weighing 0.50 ± 0.01g into 50 mL PP centrifuge tubes. 4.50 g of DI water was added, making a total sample mass of 5.0 ± 0.01g. Re-constituted freeze dried tissues were then extracted and analysed closely following OMA AOAC 2005.06 [41] as detailed in *section 2.3.1*. PCOX LC-FLD [75] analysis was additionally performed on all samples. Crude acetic acid extracts prepared according to *section 2.3.1* were deproteinated and analysed following AOAC 2011.02 [75]. The instrumentation used in this study was the same as that described in *section 4.5.1.1* (Toxin Analysis), with additional PCOX analysis performed at CEFAS using a standard Agilent 1200 LC-FLD instrument including a quaternary LC pump module, two Agilent isocratic pumps and an external column oven containing the post-column reaction coil.

PST concentrations in sample extracts were quantified against a 6-point calibration for each toxin with PST concentrations and total saxitoxin equivalents determined.

5.2.5 TOXIN PROFILE AND HOMOGENEITY STUDIES

The toxin profiles of the untreated wet tissue RM and the freeze dried RM were compared by extracting aliquots (n=3) of each material. The freeze dried RM was initially re-constituted, as described above before both untreated and freeze dried aliquots were extracted and analysed following OMA AOAC 2005.06 as described in *section 2.3.1*. Analysis of both materials was performed in the same chromatographic sequence.

The homogeneity of both the untreated and freeze dried materials was assessed through the intra-batch analysis of 14 aliquots. Tissues were re-constituted, extracted and analysed as described above.

5.2.6 STABILITY STUDIES

Short and long-term stability studies were conducted on both the untreated and freeze dried materials following a reverse-isochronous experimental design [55]. The short term study was conducted with triplicate samples consisting of five time points (0, 4, 10, 19 and 33 days) and four temperature conditions (-20°C, +4°C, +22°C and +40°C). The long term study was conducted with triplicate samples consisting of five time points (0, 3, 6, 9 and 12 month) and three temperature conditions (-20°C, +4°C and +40°C). The reference temperature used in both studies was -80°C.

At the end time point all samples were removed from storage, allowed to equilibrate to room temperature before being re-constituted as described above, and extracted and analysed by

preCOX LC-FLD closely following OMA AOAC 2005.06 [41] as detailed in *section 2.3.1*. PCOX LC-FLD [75] analysis was additionally performed on all samples to specifically investigate the extent of epimerisation of GTX1,4, GTX2,3 and C1,2. For PCOX LC-FLD analysis, the crude acetic acid extracts prepared according to *section 2.3.1* were deproteinated and analysed following AOAC 2011.02 [75].

5.2.7 RESULTS AND DISCUSSION

5.2.7.1 CHARACTERISATION

PreCOX LC-FLD analysis results from variable masses of re-constituted freeze dried material are contained in Table 5-1. Total saxitoxin equivalents values ranged from 140 – 2060 µgSTXdiHCl-eq/kg for the powder masses of 0.1 – 1.0 g, although the N-hydroxylated PSTs were quantified directly from SPE C_{18} cleaned extracts so these values may be overestimated [151]. The signal to noise ratios of the main quantitation peaks are also summarised in Table 5-1.

Table 5-1: PST concentrations (µg STX di-HCl eq/kg) determined by preCOX LC-FLD in freeze-dried oyster tissues following reconstitution to a total of 5.0g with variable masses (0.1 to 1.0g) of powder (signal to noise ratios of quantitation peaks shown in brackets).

	Weight of freeze-dried powder				
	0.1g	**0.2g**	**0.3g**	**0.5g**	**1.0g**
GTX1,4	71 (2.7)	117 (7.0)	333 (10)	493 (15)	1143 (21)
NEO	34 (2.6)	112 (6.8)	180 (8.1)	254 (15)	614 (20)
C1,2	4.8 (1.8)	13 (2.9)	18 (5.1)	26 (6.8)	56 (8.5)
GTX2,3	18 (1.3)	30 (2.9)	48 (5.1)	70 (6.8)	139 (8.5)
STX	12 (2.7)	31 (8.4)	35 (12)	55 (14)	107 (20)
Total	140	302	613	898	2060

The aliquot size of 0.50 g in Table 5-1 was chosen and the materials were dispensed at this mass. This aliquot size ensured that after re-constitution, extraction and analysis that the

material had a concentration close to the EU regulatory limit. This size was also chosen as at this level the least concentrated toxins in the material still produced a signal to noise of >5.0 after preCOX LC-FLD.

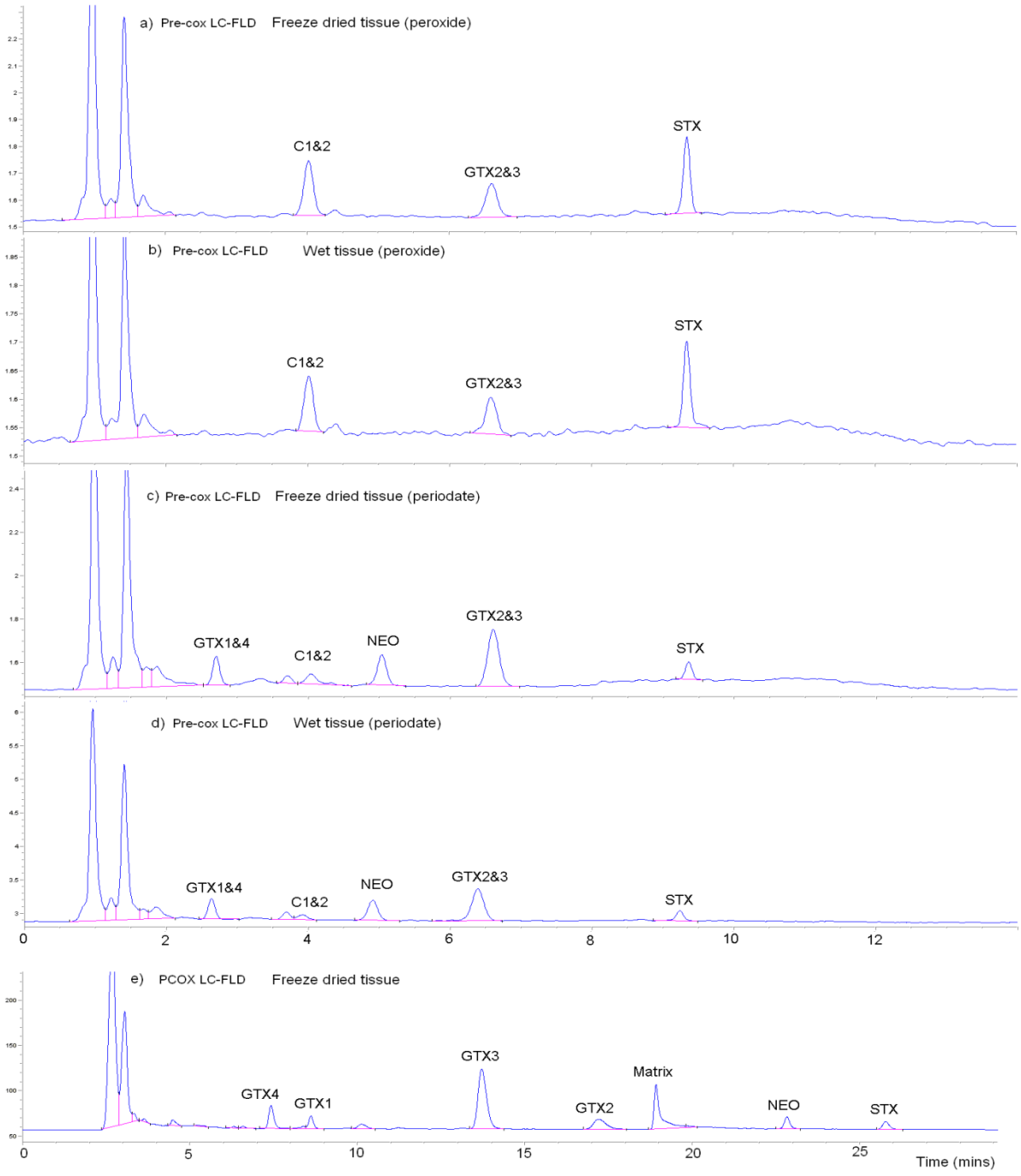


Figure 5-3: PreCOX and PCOX LC-FLD chromatograms of freeze-dried and unprocessed wet oyster tissue.

Figure 5-3 a-d contains chromatograms of both freeze dried and untreated wet oyster tissues after periodate and peroxide oxidation and analysis by preCOX LC-FLD. Chromatograms of both tissues were visually similar and showed the presence of the non-N-hydroxylated toxins C1,2, GTX2,3 and STX and the N-hydroxylated toxins GTX1,4 and NEO. There was evidence for the formation of additional chromatographic peaks which may have been caused by the freeze drying process. The PCOX LC-FLD chromatogram of the freeze dried tissue is contained Figure 5-3 e, which also shows no additional fluorescent peaks from the freeze drying process.

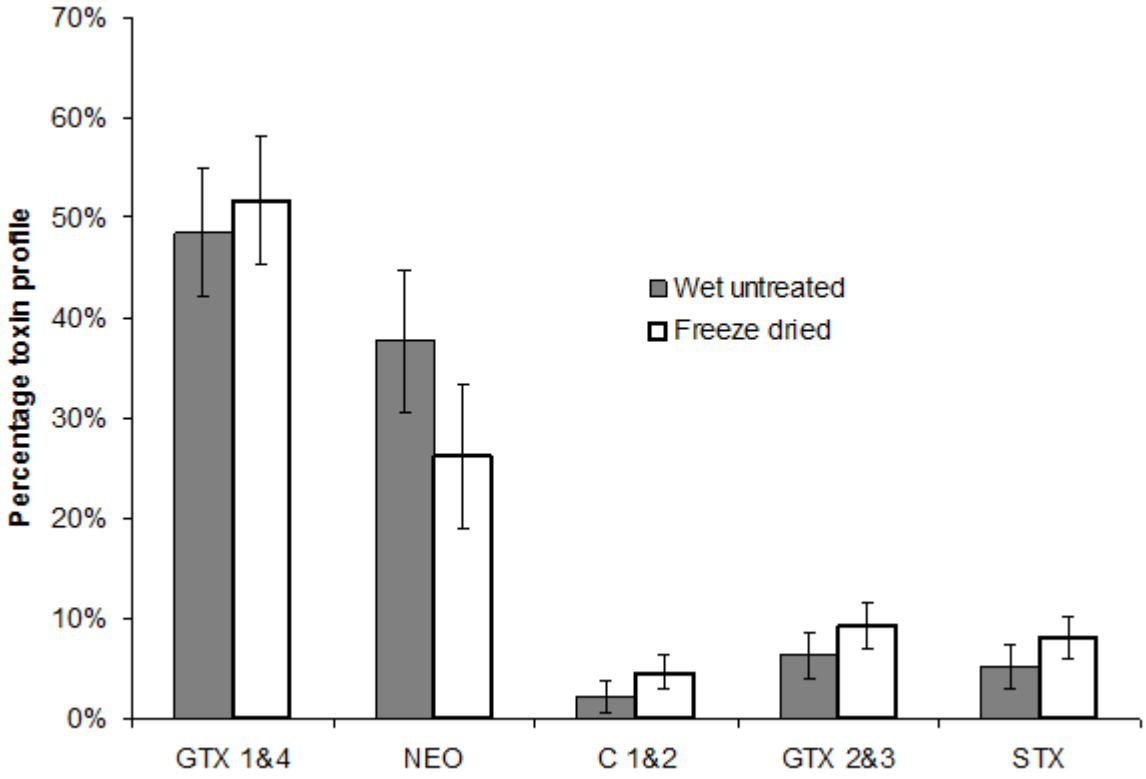


Figure 5-4: Comparison of PST profiles in a) wet untreated and b) freeze-dried oyster tissues (error bars represent ± 1 s.d. of triplicate samples).

Toxin profiles of both the untreated wet tissue RM and the freeze dried RM are contained in Figure 5-4 and are expressed as a percentage of total saxitoxin equivalents. The PST profiles

in both tissues had similar relative proportions, although a potential loss in concentration of NEO was noted but were still within repeatability limits. There was no visual evidence for any degradation or conversion of the PSTs through the freeze drying process.

5.2.7.2 HOMOGENEITY

Both the freeze dried and untreated wet tissue RMs were suitably homogenous for all the PSTs detected, as evidenced through the coefficient of variances contained in Table 5-2.

A target %CV of below 8% for each analyte including total toxicity was set. This value is below expected levels of variability associated with intra-batch repeatability, determined through in-house validation of the preCOX LC-FLD method [148]. The homogeneity techniques employed were therefore sufficient and the materials suitable for the study.

Table 5-2: Mean concentrations (µg STX di-HCl eq/kg), standard deviations (sd) and percentage relative standard deviations (%RSD) of toxins in reconstituted freeze-dried and untreated wet frozen oyster tissues as determined by preCOX LC-FLD (n=14).

Sample		GTX1,4	NEO	C1,2	GTX2,3	STX	Total
Freeze-dried	**Mean**	519	320	26	70	59	993
	sd	32	17	2	5	3	48
	%RSD	6.1%	5.3%	6.9%	7.8%	4.3%	4.9%
Wet tissue	**Mean**	718	770	17	34	48	1586
	sd	30	51	1	3	2	65
	%RSD	4.1%	6.7%	6.1%	7.7%	3.4%	4.1%

The data from both materials was similar and showed there were no major effects on the distribution of toxins in the freeze dried powder from sample processing, re-constitution or analysis.

5.2.7.3 SHORT-TERM STABILITY

PRECOX LC-FLD

Figure 5-5 a-j represents the short-term stability results for the untreated wet tissue RM and the freeze dried RM at four different storage temperatures, -20°C, +4°C, +22°C and +40°C, determined through preCOX LC-FLD analysis. Data for GTX1,4, GTX2,3, NEO, C1,2 and STX were generated and results were normalised to the average of replicate analysis (n=9) of samples stored at the reference temperature of -80°C for the duration of the study. The limits of stability from Figure 5-5 were generated from two times the standard deviation of the -80°C replicate analyses.

Overall toxin stability was excellent in both materials when stored at -20°C for the duration of the study with the freeze dried materials additionally showing excellent toxin stability at +4°C. The stability of the toxins at the higher temperatures studied showed variability however. Some degradation of GTX1,4 and NEO in the untreated RM was observed after 14 days storage at +4°C, while the other toxins present in the material remained stable at this temperature. At the higher temperatures of +20°C and +40°C degradation of NEO, C1,2 and STX was evident after as little as 4 days of the study with ~80% of the total toxin content in the case of NEO degraded by day 33. Apparent increases in GTX1,4 and GTX2,3 concentrations were evident in materials stored at these higher temperatures with ~30% and ~60% increases respectively noted at +40°C (Figure 5-5 a-e).

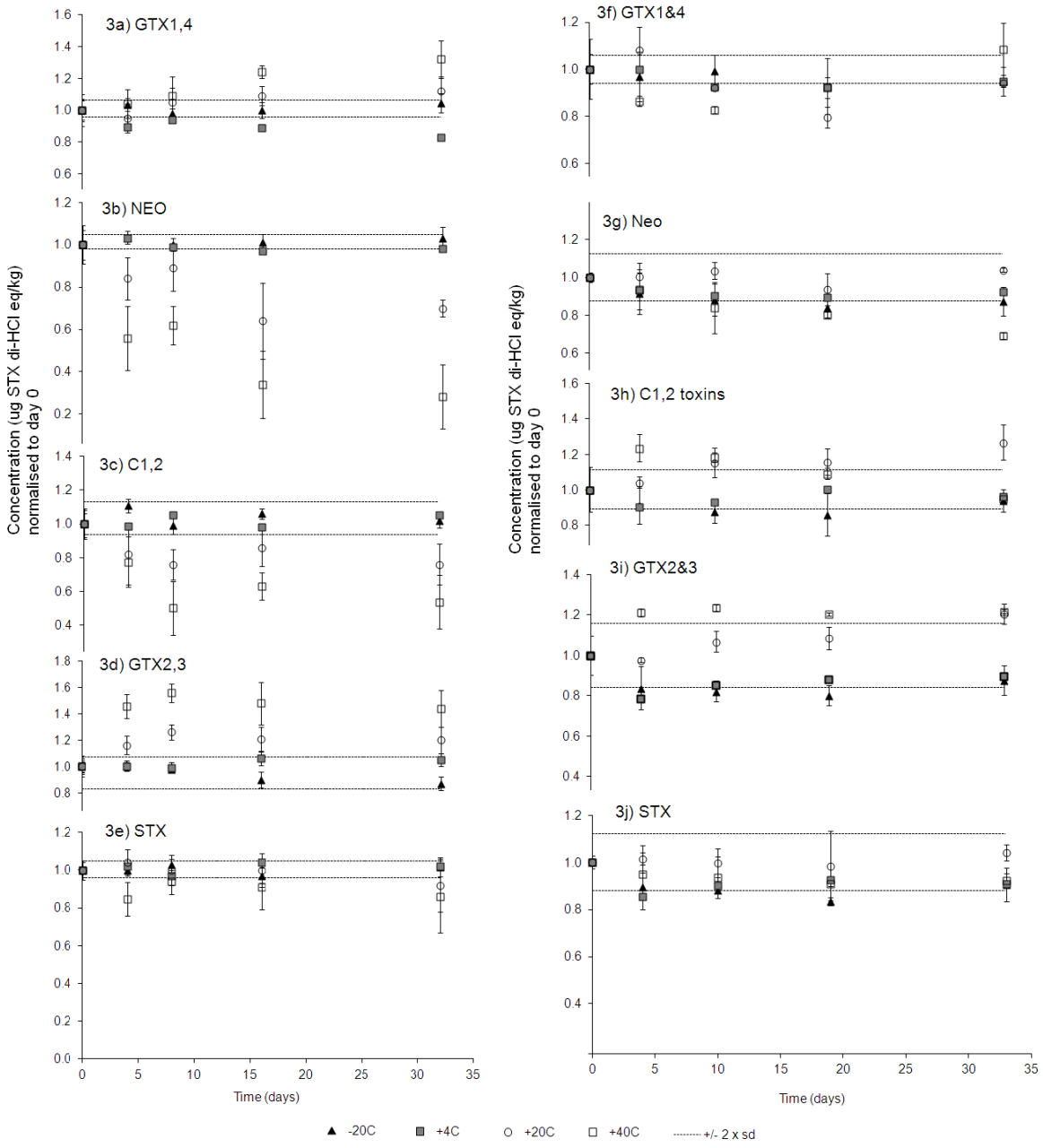


Figure 5-5: PreCOX LC-FLD results showing short term stability of PST concentrations normalised to the -80°C reference samples in untreated wet frozen (3a-3e) and freeze-dried (3f-3j) oyster tissues over 1 month (error bars represent ±1 sd of triplicate samples).

Conversely the freeze dried materials showed no evidence for the degradation or enhancement of any toxins when stored at -20°C or +4°C. At +20°C STX and NEO also showed excellent stability but there was evidence for the degradation of GTX1,4 after 9 days

and increases in C1,2 and GTX2,3 concentrations after 19 and 33 days respectively at this temperature. At +40°C STX also showed excellent stability during the study but degradation of GTX1,4 and NEO was evident after 9 and 4 days respectively. Under this temperature condition apparent increases in C1,2 and GTX2,3 concentrations after 4 days were noted.

These results prove conclusively the significant stability enhancement freeze drying gives PSTs compared to untreated wet tissue RMs in the short-term. This has particular importance for transporting materials where temperatures could potentially rise above freezing.

PCOX LC-FLD

Further analysis was conducted on the short-term stability study extracts for both the untreated wet tissue RM and the freeze dried RM by PCOX LC-FLD and results are presented in Figure 5-6 a-l. Limits of stability and normalised results were calculated as per the preCOX LC-FLD analysis above. No PCOX LC-FLD stability data for the tissues stored at +40°C is presented due to instability issues in both wet and freeze-dried tissues stored at this temperature.

Excellent stability was exhibited in both the untreated and freeze dried materials stored at -20°C throughout the study with no evidence for epimerisation taking place under frozen conditions. In addition the freeze dried materials also showed no evidence for epimerisation of any of the three epimeric pairs at +4°C over the 33 day study. At the higher temperature storage condition of +20°C epimerisation was observed to take place in the freeze dried materials with increases in relative concentrations of GTX1, GTX2 and C1, coupled with decreases in GTX4, GTX3 and C2 respectively.

Conversely epimerisation was observed to take place at +4°C and +20°C in the untreated wet tissue RM. At +4°C GTX1,4 and GTX2,3 showed signs of epimerisation after 33 days but C1,2 remained stable at this storage condition. At +20°C epimerisation was clearly evident after 4 days of the study for all three epimeric pairs with an approximate 80% toxin conversion observed by the end of the study.

These results again highlight the advantages freeze drying materials have on the stability of PSTs, particularly epimerisation which was significantly reduced. These results confirm the transport of freeze dried materials containing these toxins could be carried out at temperatures of up to +4°C.

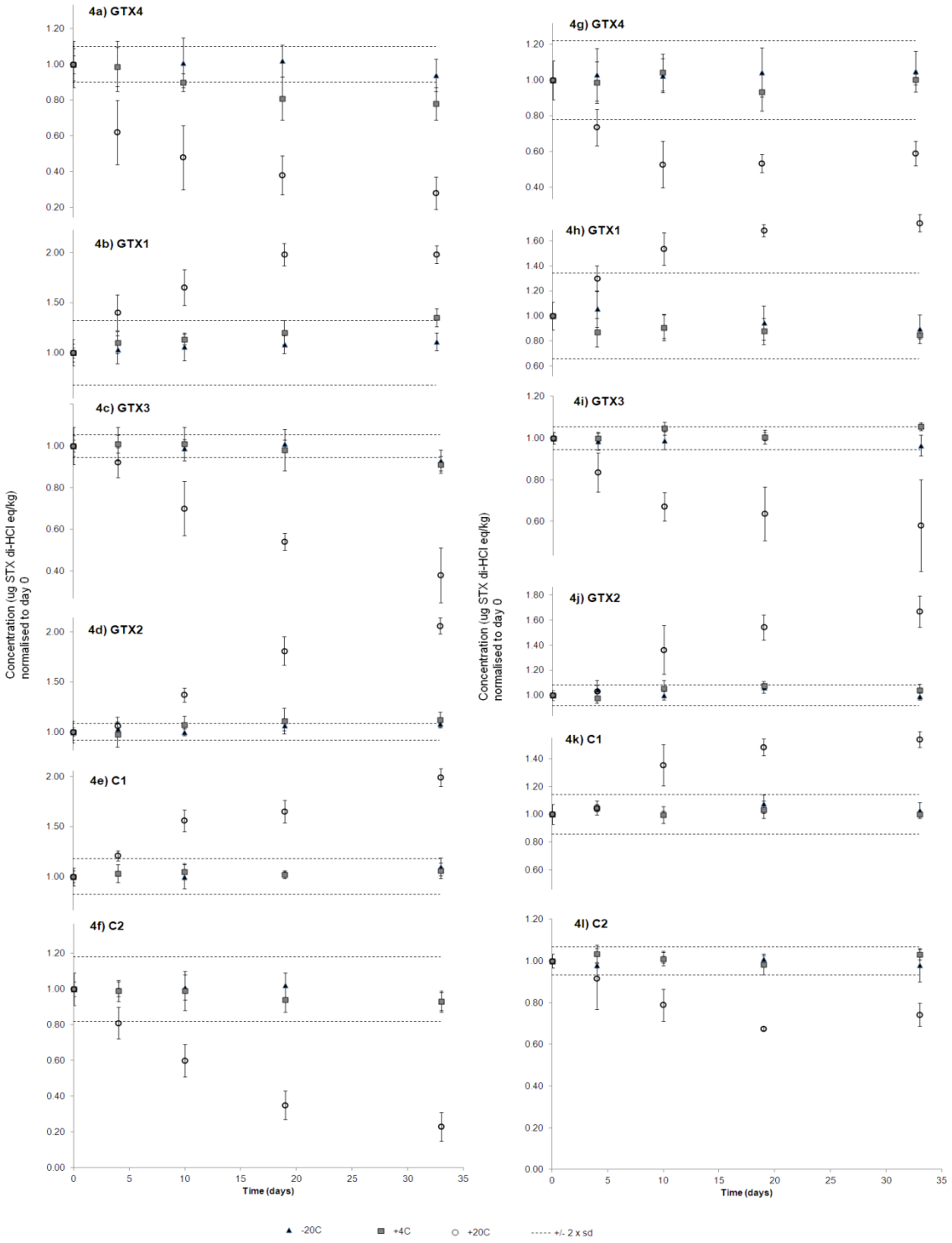


Figure 5-6: PCOX LC-FLD results showing short term stability of PST concentrations normalised to the -80oC reference samples in untreated wet frozen (4a-4f) and freeze-dried (4g-4l) oyster tissues over a 1 month assessment period (error bars represent ±1 sd of triplicate samples).

5.2.7.4 LONG-TERM STABILITY

PRECOX LC-FLD

Figure 5-7 a-e represents the long-term stability results for both materials at two different storage temperatures, -20°C and +4°C, determined through preCOX LC-FLD analysis. Results were normalised and limits of stability set as described above. Data was not generated for materials stored at +40°C due to the instability of the toxins at this temperature.

Overall excellent stability was observed in both materials when stored at -20°C throughout the 12 month study with freeze dried materials also showing excellent stability at +4°C with no signs of toxin degradation or enhancement during this period. The wet tissue RM did show stability issues however, with degradation of GTX1,4 noted by month 3 and increases in C1,2 and GTX2,3 concentrations after 6 and 3 months respectively.

This study highlights the long-term stability of PSTs in freeze dried materials with excellent stability observed under frozen or refrigerated storage conditions. The data confirms freeze drying materials to be advantageous in stabilising these toxins compared to untreated wet tissues.

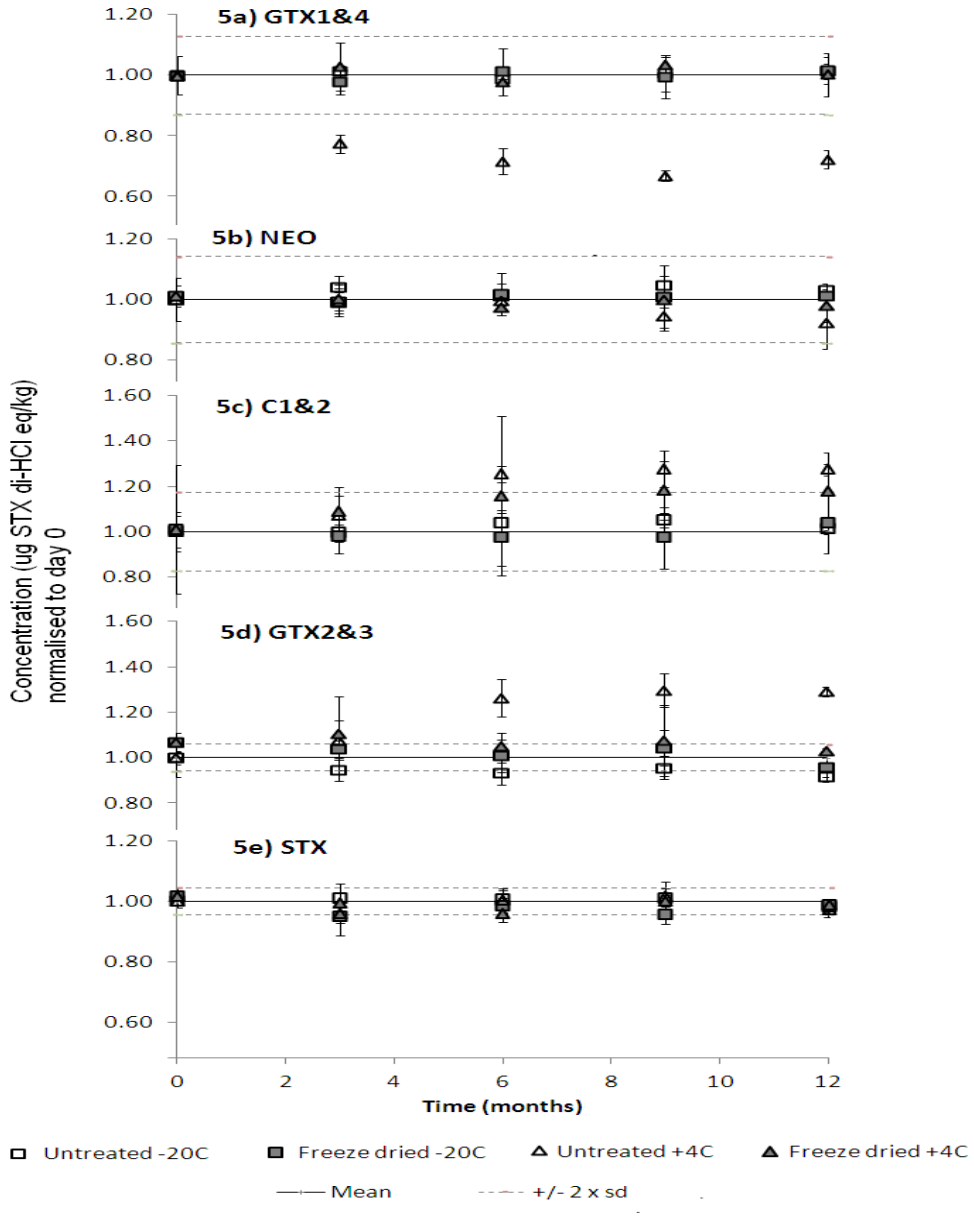


Figure 5-7: PreCOX LC-FLD results showing normalised long term stability data of a) GTX1,4 b) NEO c) C1,2 d) GTX2,3 e) STX in untreated wet frozen and freeze-dried oyster tissues held at -20oC and +4oC over 1 year (error bars represent sd of triplicate samples).

PCOX LC-FLD

Further analysis was conducted by PCOX LC-FLD on the long-term stability study extracts of both materials and results are presented in Figure 5-8 a-f. Limits of stability and normalised results were calculated as above. PCOX LC-FLD analysis was only conducted on

samples stored at -20°C and +4°C due to stability issues in materials stored above these temperatures.

No epimerization or degradation was observed in the freeze dried materials stored at both -20°C and +4°C, with any variability throughout the 12 months being within the limits specified by within-batch repeatability.

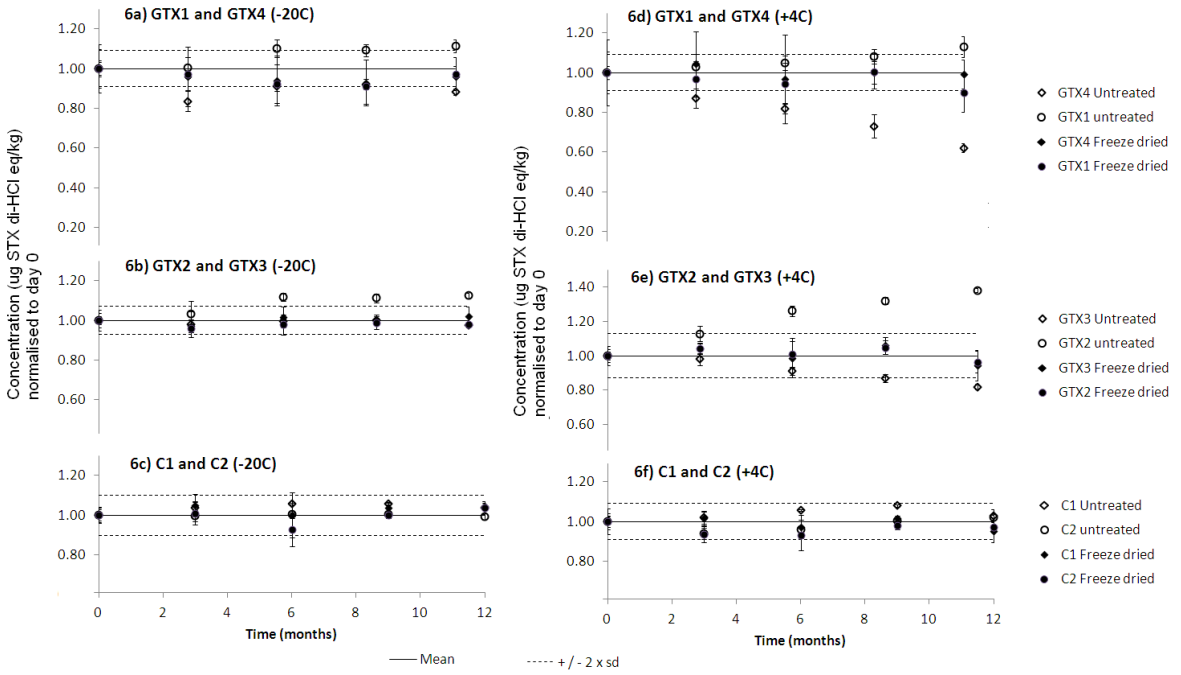


Figure 5-8: PCOX LC-FLD results showing long term stability of PST concentrations normalised to the -80oC reference samples in untreated wet frozen and freeze-dried oyster tissues held at -20oC (6a-c) and +4oC (6d-f) over a 12 month assessment period (error bars represent ±1 sd of triplicate samples).

The untreated wet tissue RMs showed signs of epimerisation and degradation at both these storage conditions however. Epimerisation of GTX4 to GTX1 was observed in these materials at both temperature conditions studied with signs of additional degradation of GTX4 in samples stored at +4°C (Figure 5-8 a+d). Epimerisation of GTX2,3 was also observed in the untreated tissues stored at +4°C which was evident after 9 months storage. An increase in GTX2 concentration was noted in frozen samples, although epimerisation is

not assumed as this increase did not coincide with any decreases in GTX3 concentration. No epimerisation of C1,2 was observed to take place in the wet tissue RM at either of the temperatures studied.

Overall the long-term stability study gave further evidence for the improvements freeze drying provides in the production of RMs, with no epimerisation observed in frozen or refrigerated tissues over a 12 month period.

5.2.7.5 USE AS CANDIDATE REFERENCE MATERIAL

The work detailed in this section of the study has shown the feasibility in producing a freeze dried oyster tissue as a candidate RM for PSP toxins. The technique, including all associated processing steps, were sufficient to produce homogeneous materials with significant PST stability enhancements made when compared with untreated wet tissue RMs. The freeze dried tissues showed excellent short and long-term stability, the data of which can be used to determine optimum transportation and long-term storage conditions.

The results confirm freeze drying as an excellent technique to prepare PST RMs with scope to produce a larger, production scale batch of materials that could be released as a commercial product.

5.2.8 CONCLUSIONS

Through feeding experiments of Pacific oysters, a naturally contaminated tissue was used to prepare a wet tissue and a freeze dried RM. Initial characterisation of the materials showed no visual evidence for changes to the toxin profile of the material from the freeze drying process.

Analysis of replicate aliquots of both the untreated and freeze dried materials showed the materials to be homogeneous, which proved the freeze drying processing techniques developed during these studies were sufficient. Short and long-term stability, determined using both the preCOX and PCOX methods proved conclusively the advantages this technique gives compared to untreated wet tissue RMs. The study provides good evidence for the applicability of the freeze drying process for the preparation of homogenous and stable PST reference materials, with excellent scope for producing CRMs.

5.3 OPTIMISATION STUDIES

5.3.1 AIMS AND OBJECTIVES

The feasibility study carried out in the previous section demonstrated the improved stability this technique provides for PSTs and also indicated that the facilities at the MI are suitable for producing high quality freeze dried RMs. This section focused on optimising the freeze drying programme including the processing and dispensing steps.

Freeze drying programmes of 2, 3 and 4 days duration were compared and contrasted to observe the effects on PST content and MC. The hygroscopic nature of freeze dried materials coupled to the fact that these materials were not always kept under low moisture conditions for example during transfer to different apparatus led to a re-freeze drying study being examined. This process was investigated with tissues freeze dried, processed and dispensed before being re-freeze dried in their containers using a short time programme before sealing.

The capacity of the freeze drier and processing techniques were also determined as this would be a factor in producing any large scale RMs using this method. The dispensing of the freeze dried materials was also optimised using a semi-automated powder dispenser (Zinsser-Analytic, Berkshire, UK) purchased and used instead of manually dispensing each aliquot using a spatula and bench top balance, as was carried out in the feasibility study in the previous section.

5.3.2 SOURCE TISSUES

The Spanish tissue from Table 2-1 was used to prepare the optimisation study material which contained, dcSTX, GTX5 and STX. An initial bulk tissue was prepared by transferring ~3.0 kg of the Spanish tissue into a 5 L Waring™ goblet with 0.300 ± 0.001 L DI water to aid mixing. The tissue was homogenised on medium power for 5.0 min before aliquots were taken for MC determination (n=3) using the rotary vacuum method and analysis by preCOX LC-FLD (n=1) as described in the materials and methods *section 2.4.1* and *2.3.1* respectively, to give approximate PST concentrations in the bulk tissue. The bulk homogenate was immediately subdivided into 5 aliquots by pouring ~ 0.6 kg of tissue into PP containers, sealing with lids and parafilm before storing at -20°C until required for use. The PST levels and MC results from this bulk homogenate are presented in Table 5-3.

Table 5-3: Toxin profiles, concentrations and moisture content of bulk tissue used in freeze drying optimisation studies.

Material	Concentration (µmol / kg)			Total Toxicity (µg STX diHCl-eq / kg)	% Moisture Content
	dcSTX	GTX5	STX		
Bulk homogenate	1.31	0.13	0.04	507	82.9

5.3.3 FREEZE DRYING

Table 5-4 gives details of the various freeze drying programs evaluated in the optimisation studies. As discussed previously five freeze drying optimisation experiments were conducted using various lengths of time, depth of tissue in each tray and one process where the material was re-freeze dried after being dispensed.

Each experiment, FD-I - FD-V was conducted on separate days but each study followed similar processes. A container was removed from the freezer and allowed to defrost overnight at +4°C. 526.0 ± 0.1 g of each tissue was transferred to a 1 L Waring™ goblet, made up to

600.0 ± 0.1g with DI water to adjust the MC to ~85% and homogenised on medium power for 5.0 min.

Table 5-4: Details of the five freeze drying conditions used in optimisation studies.

Material	FD Times (hours)	Tissue depth on tray (mm)	Secondary Freeze Drying
FD-I	96	~5	No
FD-II	72	~5	No
FD-III	72	~10	No
FD-IV	72	~10	Yes
FD-V	48	~10	No

The tissue was then transferred to two freeze drying trays in the case of FD-I and FD-II experiments and transferred into one tray in the case of the FD-III, FD-IV and FD-V experiments. This was to ensure the depth of tissue in each tray was roughly as stated in Table 5-4.

Table 5-5: Details of freeze drying programmes used to prepare materials contained in chapter 5.

Process	FD-I	FD-II/III/IV	FD-V	Secondary 2nd Step	Temperature (°C)	Pressure (mbar)	Hold/Ramp
	Time (h)						
Thermal Treatment	0	0	0	0	20	1013	-
	2	1.5	1	1	-35	1013	R
Primary Drying	2.1	1.6	1.1	0.6	-35	1013	H
	5.7	4.3	2.8	1.4	-35	1.03	R
	7.7	5.8	3.8	1.9	-35	1.03	H
	8.7	6.5	4.3	2.2	-25	0.21	R
	28.7	21.5	14.3	7.2	-25	0.21	H
	29.7	22.3	14.8	7.4	-20	0.13	R
	48.7	36.5	24.3	12.2	-20	0.13	H
	49.7	37.3	24.8	12.4	-10	0.13	R
	56.7	42.5	28.3	14.2	-10	0.13	H
	57.7	43.3	28.8	14.4	0	0.13	R
	71.7	53.8	35.8	17.9	0	0.13	H
Secondary Drying	72.7	54.5	36.3	18.2	20	0.01	R
	74.7	56	37.3	18.7	20	0.01	H
	78.7	59	39.3	19.7	20	0.01	R
	94	70.5	47	23.5	20	0.01	H
Secondary Set Point	96	72	48	24	20	0.01	H
Condenser Condition	-70°C						

The trays were then placed into the freeze drier, the door was sealed and the pre-programmed freeze dry sequence as described in Table 5-5 was started.

5.3.4 MATERIAL PROCESSING AND DISPENSING

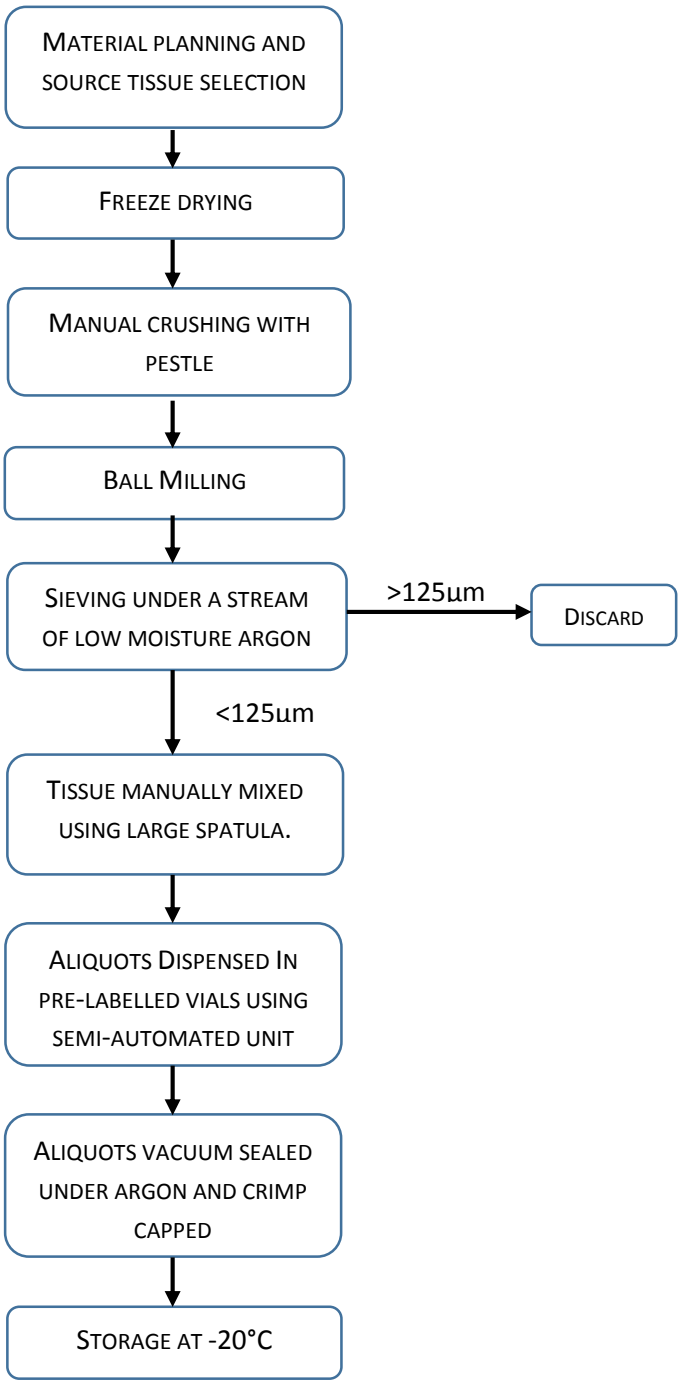


Figure 5-9: Flow chart depicting steps in the preparation of the freeze dried materials contained in section 5.3.

All equipment used to process and dispense the freeze dried material was first washed in a 2% Decon-90™ solution followed by thorough rinsing in DI water. The equipment was then

placed in an oven at +40°C for ~5.0 hours before being transferred to a large desiccator containing activated silica beads. These steps were carried out in order to reduce the potential for bacterial contamination as much as possible through the use of Decon-90™ and to keep the equipment sufficiently dry to process the hygroscopic freeze dried materials.

The flowchart contained in Figure 5-9 illustrates the freeze drying process followed. Upon completion of the freeze drying programme, the vacuum was released and the door was opened. The tray was removed and the cake formed by the freeze dried material was manually ground to break up large conglomerates using a large mortar. Approximately 90.0 g freeze dried material remained after the process and in each case this was transferred equally to three grinding bowls containing 25 x 15 mm stainless steel balls. The lid was attached and the material was ground for 5.0 ± 0.1 min at 400 rpm using a planetary ball mill (Retsch PM 100, Verder Group, The Netherlands).

The material was transferred to a 125 µm sieve fitted with a base plate and attached to a motorised sieve shaker (Impact Test Equipment Ltd., U.K.). The sieve shaker was covered with a large plastic sheet and sealed with brown tape. A hose was inserted through an opening made at the top of the sheet and low moisture content argon was flushed over the sieve plates during the shaking sequence of 30.0 ± 0.1 min. A small hole was made at the bottom of the sheet to allow pressure release during this process.

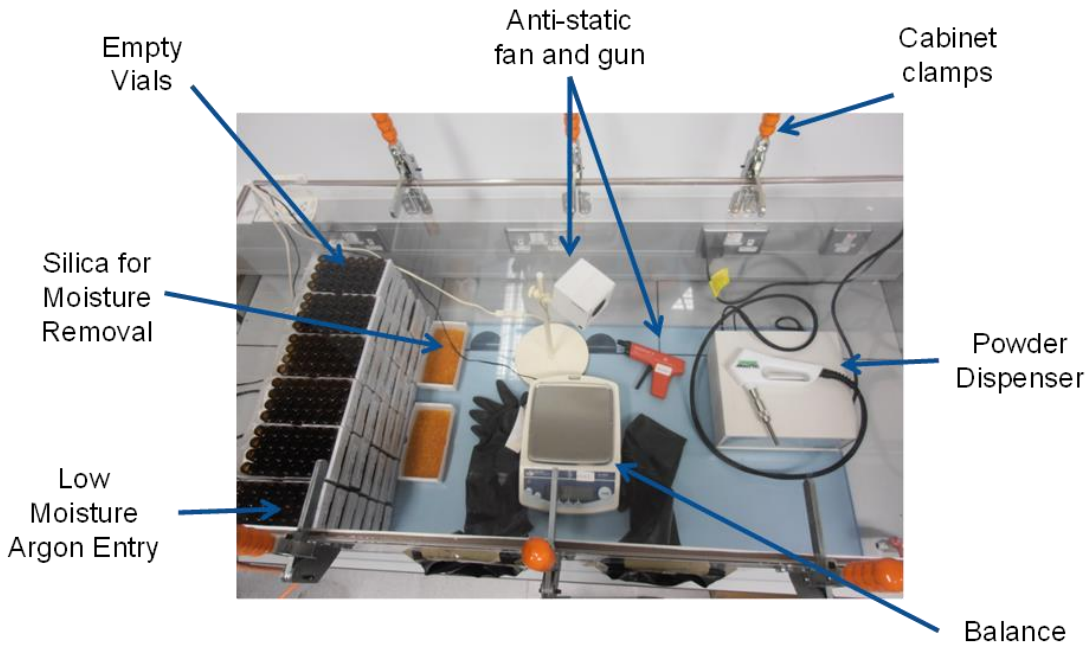


Figure 5-10: Photo of modified glove box constructed to dispense the freeze dried materials described in this chapter showing the setup for dispensing the RMs.

The base plate containing the material (~80.0 g) passed through the 125 µm sieve was transferred to a 100 mL PP beaker and placed immediately into the modified glove box constructed for dispensing the materials in this section. The glove box also contained all the dispensing equipment and the setup is illustrated in Figure 5-10.

The glove box chamber was purged with low moisture content argon for ~5.0 min with the exhaust valve fully open. The argon flow rate was lowered before dispensing the freeze dried material as 0.76 g aliquots using a calibrated powder dispenser (Zinnsser Analytical, Germany) into 5 mL amber serum vials (Wheaton, USA). The freeze dried material was continually mixed with a spatula throughout the dispensing process.

An antistatic fan was switched on throughout the dispensing process and an antistatic gun discharged periodically to prevent issues with dispensing powders and static. After being fully dispensed the FD-I – FD-IV vials were removed from the glove box, placed on a freeze

dry tray, and lyophilisation stoppers inserted half way. In the case of the FD-V materials lyophilisation stoppers were not initially inserted and the vials were placed back into the freeze drier and subjected to the 2nd step freeze drying program as detailed in Table 5-5. Once this 2nd freeze drying step was complete, lyophilisation stoppers were inserted halfway in the vials.

All materials, FD-I – FD-V, were placed back into the freeze drier, the door was sealed and the vacuum applied, removing air from the chamber. A cylinder of low moisture content argon was attached to the exhaust vent of the freeze drier before releasing half the vacuum in the chamber to argon. The shelves were contracted, pushing the lyophilisation stoppers fully in and sealing the vials, before releasing the chamber to atmospheric pressure. This created a small vacuum inside each of the vials which held the lyophilisation stoppers in place. The vials were removed from the freeze drier, crimp capped before being stored at -80°C until further use. Only 50 aliquots of each material FD-I – FD-V were dispensed and any remaining freeze dried material discarded as this number was sufficient to carry out the studies contained in this section.

Figure 5-11 illustrates the processes involved in dispensing the freeze dried materials.

Figure 5-11: Pictures showing the various steps in the freeze drying process, I) Virtis 50XL instrument, II) dispensing freeze dried materials inside modified glove box, III) dispensed materials in freeze drier with shelves contracted to push lyophilisation stoppers into place IV) sealed aliquots of freeze dried materials and V) various stages of materials being dispensed with empty containers (A) to sealed and crimp capped aliquots (F).

5.3.5 RECONSTITUTION, EXTRACTION AND TOXIN ANALYSIS

A total of 10 aliquots was randomly selected from the entire fill series and all extracted on the same day. All materials FD – I -V were first reconstituted with DI water by weighing 0.75 ± 0.01 g into a PP centrifuge tube and making the final weight up to 5.0 ± 0.1 g with DI water. The centrifuge tubes containing the tissue were then vortex mixed for 1.0 ± 0.1 min, sonicated for 1.0 ± 0.1 min followed by vortex mixing for 1.0 ± 0.1 min. The reconstituted tissue was then extracted according to OMA AOAC 2005.06 as detailed in *Section 2.3.1*. Toxin analysis was only carried out using preCOX LC-FLD as stability and specifically epimerization was not being assessed in this study. The moisture content of the freeze dried tissues was determined using a volumetric Karl Fischer method as described in *Section 2.4.2*.

5.3.6 RESULTS AND DISCUSSION

The homogeneity results are detailed in Table 5-6 and graphically represented in Figure 5-12 to give a visual comparison. The FD-I and FD-II materials showed the least amount of toxin degradation from 3 and 4 day freeze drying when compared to the source tissue concentrations contained in Table 5-3.

Table 5-6: Homogeneity results for freeze dried tissues prepared in optimisation studies (n=10).

Material	Concentration (µgSTX diHCleq/kg)											
	dcSTX			GTX-5			STX			Total Toxicity		
	Average	Stdev	%CV	Average	Stdev	%CV	Average	Stdev	%CV	Average	Stdev	%CV
FD-I	441	14	3.2	4.8	0.4	7.8	17	1	6.5	463	15	3.3
FD-II	452	19	4.3	5.2	0.2	3.9	18	1	4.6	475	20	4.2
FD-III	401	17	4.5	4.5	0.2	5.2	15	1	5.2	422	19	4.4
FD-IV	372	13	3.6	4.3	0.2	3.7	15	1	4.7	392	14	3.6
FD-V	391	16	4.3	4.9	0.3	5.2	16	1	3.5	412	17	4.2

The results suggest the use of longer, less aggressive freeze drying programmes of 96 or 72 hours in duration coupled with tissue depths of ~50 mm are ideal for these materials. Under these conditions PST degradation from the freeze drying process is minimised, leaving total toxin content very similar to that found in the original source tissues.

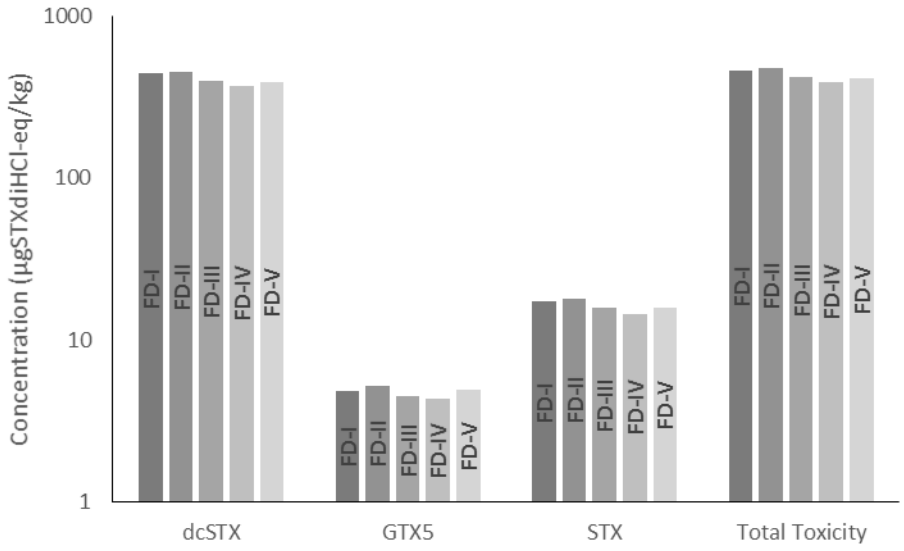


Figure 5-12: Graphical representation of homogeneity data generated for freeze drying optimisation studies using a logarithmic scale.

There was a statistical difference ($P < 0.05$; one-way analysis of variance [ANOVA]) found between the means of the five materials which showed some effects on toxin concentration following each of the freeze drying treatments. FD-IV materials showed the greatest level of toxin degradation which was most likely caused by the re-freeze drying of these aliquots. The more aggressive freeze drying programme of 2 days (FD-V) demonstrated the benefits of using a longer programme as toxin instability was caused by the more rapid increases in temperature and pressure required for this programme.

The moisture content of these tissues was assessed using a volumetric Karl Fischer method and the results are contained in Table 5-7. Moisture content of these freeze dried tissues is important to minimise the risk of bacterial growth and ideally this level should be below ~5%

[56]. The conditions investigated in this section gave varying results with 4 and 3 day freeze drying programmes producing MC levels acceptable for materials and close to the targeted value of 5%. Materials freeze dried only once and with a tissue depth of 100 mm (FD-III & FD-V) gave the highest MC determined which indicates that tissue depth in each tray should be below 10 mm and ideally ~5 mm in order to obtain a sufficiently low MC.

Table 5-7: Moisture content of freeze dried tissues.

Material	Average	Stdev	%CV
FD-I	5.5	0.3	6.1%
FD-II	5.8	0.4	6.9%
FD-III	7.2	0.4	5.7%
FD-IV	2.7	0.1	4.2%
FD-V	8.9	0.5	7.9%

The FD-IV materials gave the lowest MC of any determined which although in theory this provides the most inhospitable conditions for microbial growth it also produces the most hygroscopic material which when opened would absorb water relatively quickly affecting toxin concentration and compromising efforts to accurately certify the material [62,63].

From these results the ideal conditions were determined to be either 3 or 4 day freeze drying programmes with ~ 5 mm tissue depth in each tray. The results indicate the freeze drying of tissues can be adequately carried out at the MI using in-house apparatus and the various processing steps are sufficient in dealing with hygroscopic materials.

The optimised FD process used in this study is represented on the phase diagram of water graphically represented in Figure 5-13. The red trace corresponds to the freeze drying programme applied and shows the process of water sublimation from the tissue, passing straight from the solid to the gas phase.

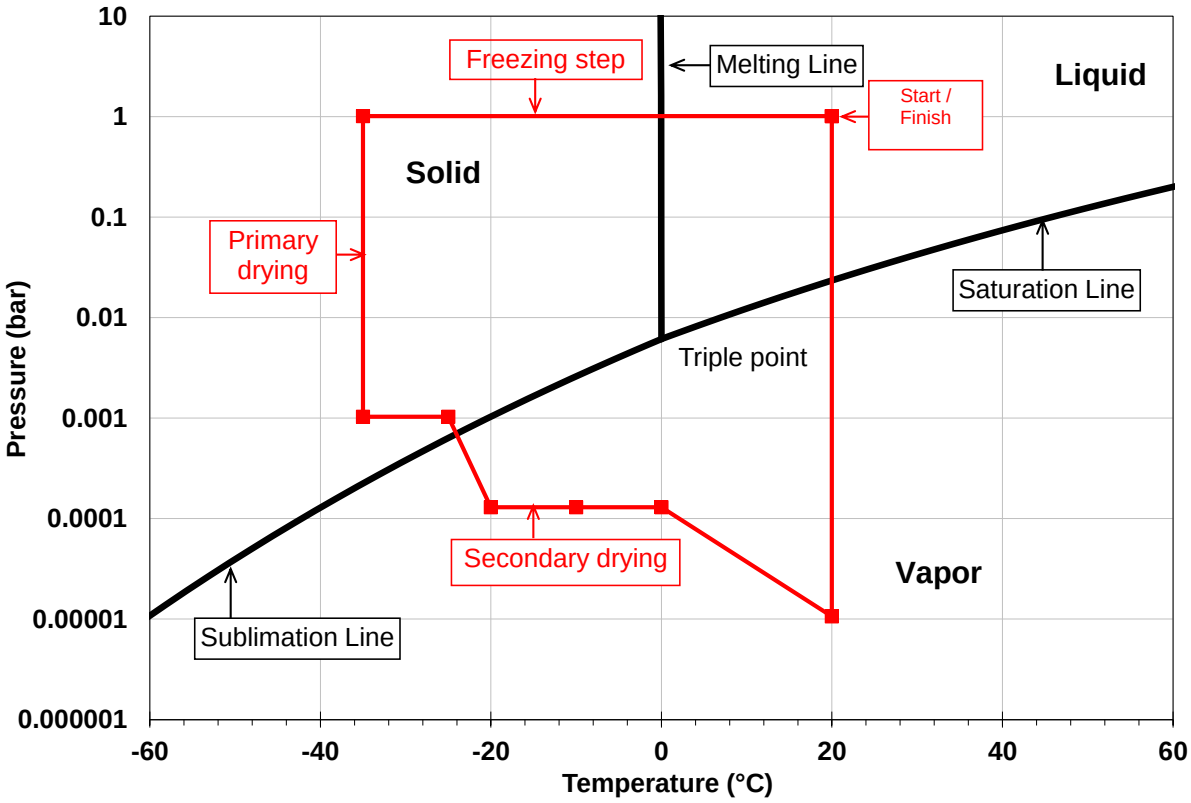


Figure 5-13: Phase diagram of water showing optimised freeze drying program.

Figure 5-14 below shows the variation of temperature and pressure applied during the FD process as a function of time and illustrates the conditions used in the 96 hour freeze drying programmes.

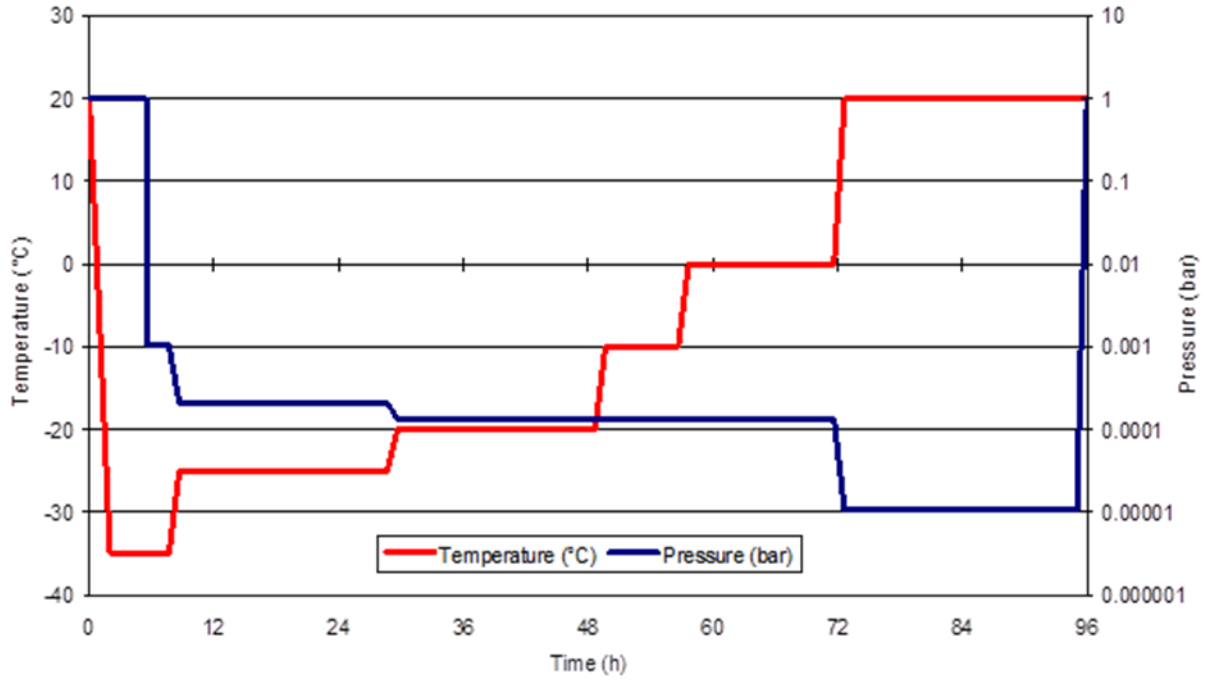


Figure 5-14: Graphical representation of temperature and pressure changes applied to the tissues during the 4-day optimised freeze drying process.

5.4 FREEZE DRYING USING OPTIMISED PROGRAM

The previous section sought to optimise the conditions for freeze drying materials at the MI and also the processing conditions necessary to produce small and relatively large scale freeze dried materials. The optimised techniques show that the processing of hygroscopic materials using the equipment either constructed or purchased as part of these studies, to be suitable for producing freeze dried materials with the required MCs and sufficient homogeneity.

Once the optimised conditions had been determined further studies were conducted to assess the stability of a range of PSTs in both short and long-term studies.

5.4.1 SOURCE TISSUES

The material used in this section was prepared as part of the additives study in chapter 4 as described in *Section 4.3.1.1*. The bulk homogenate was removed from the freezer and allowed to defrost overnight in a fridge at +4°C. The tissue was manually mixed with a spatula before transferring 734 ± 1 g into a Waring™ goblet. The final weight was made up to 900 ± 1 g with DI water to alter the final MC to ~85% as the freeze drying programme was optimised with tissues at this level. The slurry was poured into the freeze drying trays with a tissue depth of ~50 mm, and placed into a freezer overnight at -20°C.

5.4.2 FREEZE DRYING AND MATERIAL PROCESSING

The trays were removed from the freezer and placed into the freeze drier for the duration of the 96 hour programme as detailed in Table 5-5. Upon completion of the freeze drying programme the material was removed from the instrument and was processed and dispensed

as described in *Section 5.3.4*. Dispensed aliquots were placed into -80°C storage until further use. 130 aliquots of this material were dispensed which gave sufficient numbers for short and long-term stability studies as well as homogeneity determination. The remaining freeze dried material not dispensed was transferred to a vacuum bag, sealed and stored at -80°C.

5.4.3 STABILITY AND HOMOGENEITY STUDIES

The between-bottle homogeneity of the materials was assessed through the intra-batch analysis of 16 aliquots ($3n^{1/3}$) selected through stratified random sampling of the entire fill series.

The material was stability tested over a short term and long term period following a reverse isochronous experimental design [55] as in previous experiments. The short term study was conducted with triplicate samples consisting of five time points (0, 4, 7, 17 and 31 day) and three temperature conditions (-20°C, +4°C and +40°C). The long term study was conducted with triplicate samples consisting of five time points (0, 3, 6, 9 and 12 month) and three temperature conditions (-20°C, +4°C and +20°C). The reference temperature used in both studies was -80°C. In the case of all the stability studies; aliquots (n=3) were removed from -80°C storage on the prescribed day and transferred to the storage conditions being investigated.

At the end time point all samples were removed from storage, allowed to equilibrate to room temperature before removing the crimp seal and lyophilisation stopper. Immediately 0.75 ± 0.01 g of each tissue was weighed out to minimise moisture uptake of the hygroscopic material. The tissues were reconstituted and extracted as described in *Section 5.3.5*.

Analysis was carried out by preCOX LC-FLD closely following OMA AOAC 2005.06 [41] as detailed in *Section 2.3.1*. PCOX LC-FLD [75] analysis was additionally performed on all

samples to specifically investigate the extent of epimerisation, if any, of GTX2,3 in each material. For PCOX LC-FLD analysis, the crude acetic acid extracts prepared according to *Section 2.3.1* were deproteinated and analysed following AOAC 2011.02 [75].

PST concentrations in sample extracts were quantified against a 4 - 5-point calibration for each toxin and are expressed in µmol/kg with total saxitoxin equivalents calculated as an estimation of total toxicity.

The MC of these freeze dried tissues was not determined due to technical issues with the Karl Fischer apparatus which could not be rectified during the course of these studies. The 96 hour freeze drying programme, including material processing and dispensing techniques as detailed in *Section 5.3* has previously demonstrated that materials can be produced with a MC of ~5% however.

5.4.4 RESULTS AND DISCUSSION

All the materials prepared in this section of the study were suitably homogenous for all the PSP toxins quantified, as evidenced through the coefficient of variances contained in Table 5-8. The homogeneity techniques employed were therefore sufficient and the materials were suitable for the analytical study.

Table 5-8: Homogeneity results for freeze dried material prepared using optimised 96 hour program (n=16).

	dcSTX	GTX-2,3	GTX-5	STX	Total Toxicity
	µg STX diHCl-eq. / kg				
Average	237.9	237.4	2.7	263.2	738.5
Stdev	8.4	15.8	0.1	8.3	32
%CV	3.5	6.7	4.4	3.2	4.3

5.4.4.1 SHORT-TERM STABILITY

PRECOX LC-FLD

Short-term stability graphs for dcSTX, STX, GTX-2,3, GTX1,4 and GTX-5 are presented in Figure 5-15. Results are normalised to the -80°C freezer mean on day zero and are presented with error bars of the standard deviation from the triplicate analyses.

Excellent stability was demonstrated at -20°C and +4°C for all the toxins studied in this section with all data points falling within the limits of stability set. The PSTs dcSTX, STX, GTX1,4 and GTX5 also showed excellent stability when stored at +40°C in addition to the other conditions. Some slight instability issues were evident for GTX2,3 at this temperature condition with a slight increase in concentration observed after 17 days storage. This GTX2,3 increase was not as pronounced as that observed in the control materials (Figure 4-8) which highlights the stability improvements this technique provides.

The results provide evidence that the toxins would be stable during transportation, even at elevated temperatures of up to +40°C for 17 days. This has particular relevance for PT providers or suppliers of RMs where stability during transportation to participants or customers is critical.

As discussed in the previous chapter, the role of bacteria could explain the slight increases in GTX2,3 concentration observed in freeze dried samples stored at +40°C through biotransformations [132]. GTX2,3 increases were also observed in the untreated control (Tissue C) materials as evidenced in *Section 4.3.2.2*.

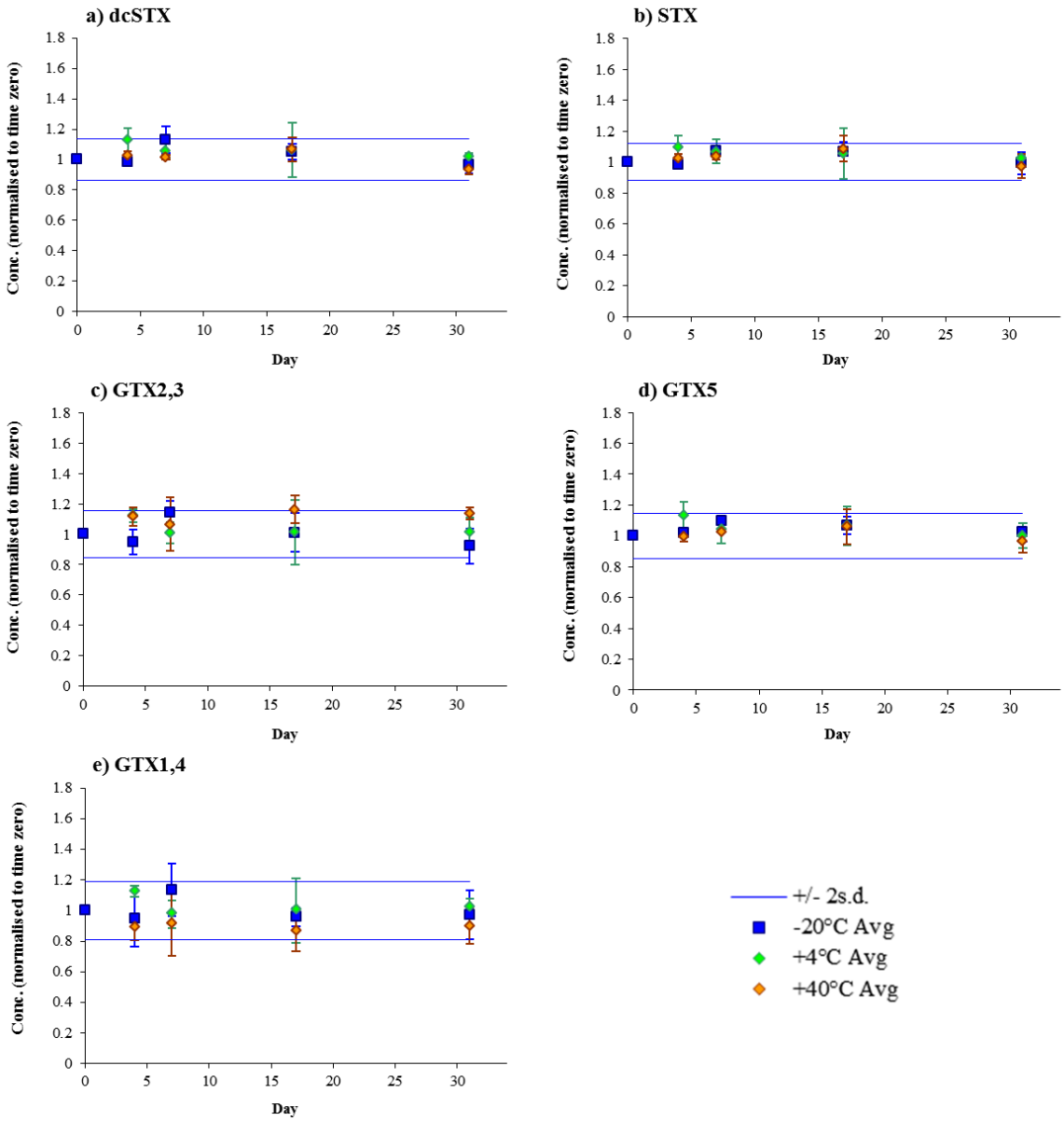


Figure 5-15: Short-term stability graphs by preCOX LC-FLD for freeze dried material using optimised method.

As evidenced from the short-term stability results above, freeze drying provides the best stability for both PSTs and the matrix itself compared to the other two techniques investigated or the untreated control (Figure 4-8) where stability issues at +40°C were evident.

PCOX LC-FLD

Further analysis was conducted by PCOX LC-FLD on the short-term stability study extracts of each of the materials and results are presented in Figure 5-16 a-b. Limits of stability and normalised results were calculated as per the preCOX analysis above. Data for the toxins GTX1 and GTX4 were not generated as the concentration of these toxins in each material was <LOD of the PCOX LC-FLD method.

No epimerization or degradation was observed in any materials when stored at -20°C or +4°C for the duration of the study with results falling within the limits of stability set. However epimerization was observed in materials stored at +40°C which became evident after 4 days of the study. As such, it would seem that tissue samples stabilised by freeze-drying only, would still need to be sent under temperature control conditions, if the end user required quantitation of individual epimers. These results were similar to those observed for Tissue A in *Section 4.3.2.2* (where additives were used to stabilise the matrix) which shows no significant improvement freeze drying makes to epimerization rates in the short-term compared to additives spiking. Freeze drying does, however, improve stability compared to the untreated control (Tissue C, *Section 4.3.2.2*) where epimerization was also observed at +4°C in addition to the +40°C samples.

Interestingly the thermally treated material (Tissue B) in *Section 4.3.2.2* did not show any epimerization of GTX2 and GTX3 under any of the temperature conditions studied. GTX2 remained stable throughout the study and GTX3 showed some slight degradation but epimerization of these toxins was not evidenced. This demonstrated the improvement that thermally treating materials has on epimerization rates and suggests this step could be performed in tandem with any other stabilisation technique being undertaken to combine the advantages of these various techniques.

Epimerization of these toxins followed predicted patterns with the β-epimer GTX3 being epimerized into the more stable α-form GTX2 [136] which was evident from both the short and long-term stability data generated through the PCOX LC-FLD analysis.

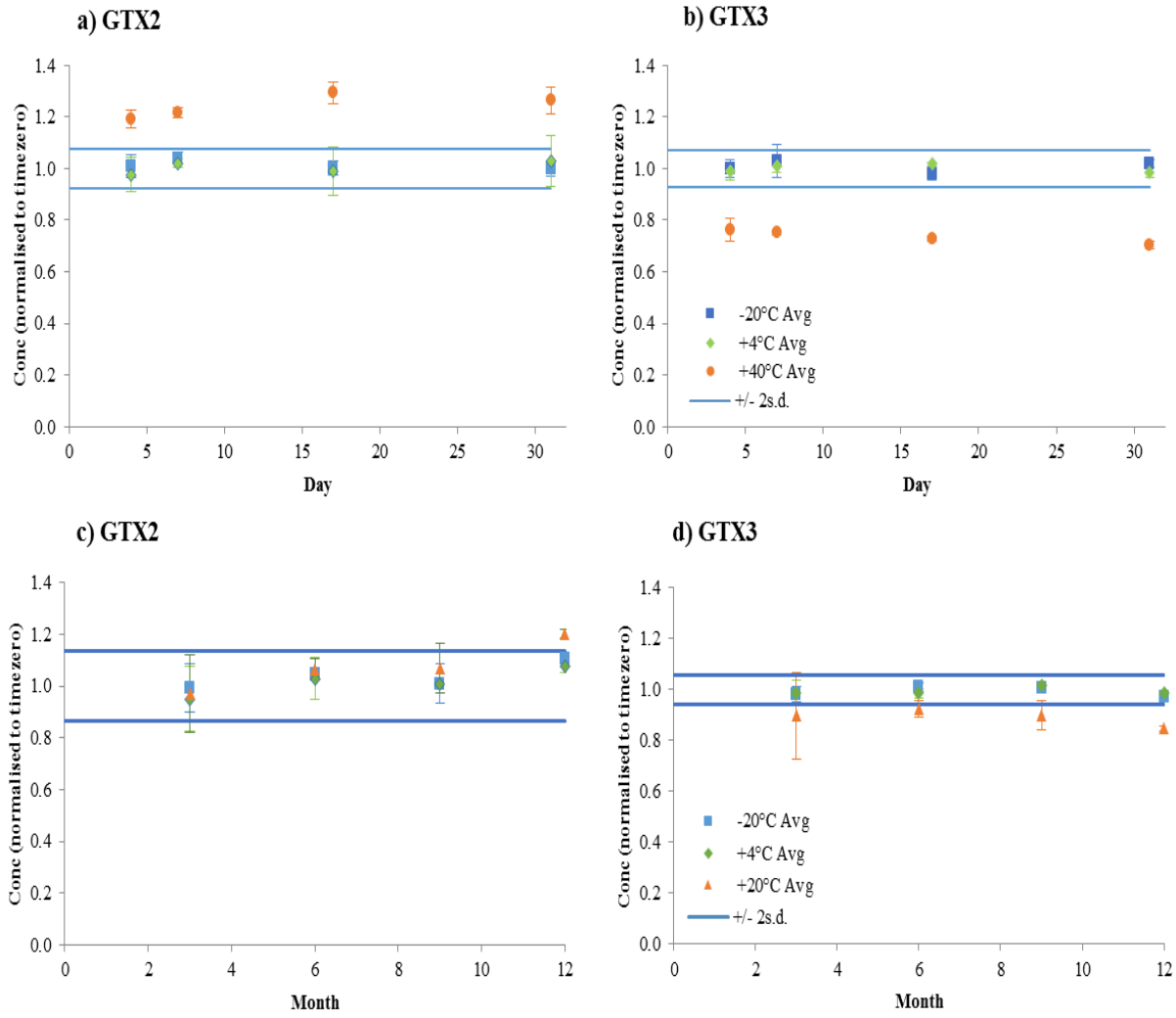


Figure 5-16: Short and long-term stability data by PCOX LC-FLD for freeze dried material using the optimised method.

5.4.4.2 LONG-TERM STABILITY

PRECOX LC-FLD

Figure 5-17 a-e represents the long-term stability results for the freeze dried material at three different storage temperatures, -20°C, +4°C and +20°C, determined through preCOX LC-FLD analysis. Data for dcSTX, GTX1,4, GTX2,3, GTX5 and STX were generated and results were normalised to the average of replicate analysis (n=9) of samples stored at the reference temperature of -80°C for the duration of the study. The limits of stability from Figure 5-17 were generated from two times the standard deviation of the -80°C replicate analyses.

The stability of all the toxins studied in this section was maintained for 9 months under all the temperature conditions, -20°C, +4°C and +20°C with no evidence for toxin biotransformations or degradation during this time. Interestingly by the last time point of the study (month 12) instability of dcSTX, STX and GTX5 was evident in samples under all storage conditions with data points falling outside the limits of stability set. GTX2,3 also showed the same drop in concentration in the month 12 samples under all temperature conditions although no data points fell outside the limits of stability. Conversely GTX1,4 exhibited excellent stability throughout the yearlong study under all temperature conditions.

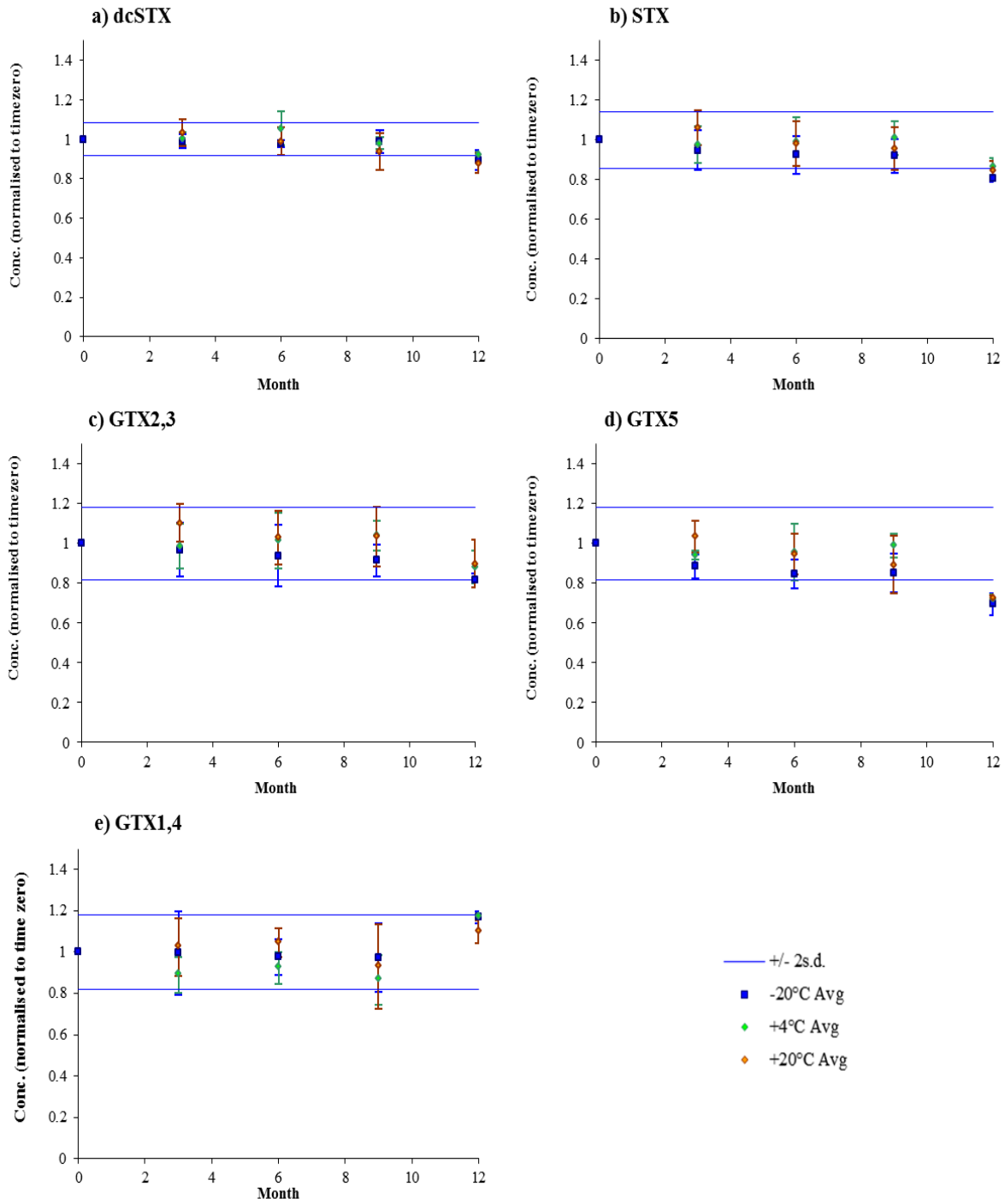


Figure 5-17: Long-term stability graphs by preCOX LC-FLD for freeze dried material using optimised method.

The cause or causes of these instability issues for the non N-hydroxylated toxins in the month 12 samples are unknown but may have been caused by an error in re-constituting the tissues prior to extraction. The concentrations of the N-hydroxylated toxins GTX1,4 after periodate

oxidation did not show the same decrease in concentration. The fact that all three temperature conditions degraded to approximately the same level in the case of each toxin is peculiar and could be indicative of a systematic error. Previous materials described in this thesis stabilised in multiple ways and indeed untreated control materials were all stable when stored at -20°C. The excellent stability exhibited by the freeze dried materials in the early part of the long-term study also makes this sudden instability drop difficult to explain. The analysis of all the long-term stability samples was repeated using fresh reagents but results confirmed the instability issues in the month 12 samples.

Freeze drying greatly improved toxin and matrix stability compared to the control material as can be seen from Figure 4-10. In particular the gonyautoxins 1-4 were completely degraded by month 3 of the study at +20°C in the control but remained stable in the freeze dried materials up to 9 months. Freeze drying also improved the stability of the other toxins present in these tissues compared to the untreated controls.

In addition this technique showed improvements over additives spiking and heat treatment (Figure 4-10), particularly GTX1,4 in the heat treated and GTX2,3 in the additives spiked materials. This has proven freeze drying to be the most effective in stabilising the PSTs compared to additives spiked, heat treated or untreated controls as described in *Section 4.3*.

PCOX LC-FLD

Further analysis was conducted by PCOX LC-FLD on the long-term stability study extracts and results are presented in Figure 5-16 c-d. Limits of stability and normalised results were calculated as described for the preCOX analysis above.

As in the case of the short-term study no epimerization or degradation was observed in the freeze dried materials stored at -20°C or +4°C for the duration of the study with results

falling within the limits of stability set. A small amount of epimerization was however observed in materials stored at +20°C which was evident after 12 months of the study. The only other instability issue observed at this temperature was a slight degradation of GTX3, evident after 3 months.

Freeze drying significantly improved the stability of the PSTs compared to the untreated control (Tissue C) evident in *Section 4.3.2.3* where these toxins were completely degraded by month 3 of the study at storage temperatures of +20°C. Improvements were also evident in samples stored at +4°C where epimerization of GTX2,3 was observed in Tissue C (Figure 4-9) but these toxins remained stable in the freeze dried tissues for 12 months. Freeze drying also showed improvements in PST stability compared to materials spiked with additives (Tissue A) where epimerization of GTX2,3 could be observed after 3 months storage at +4°C and +20°C.

Similar levels of stability were evident in the -20°C and +4°C freeze dried and heat treated (Tissue B) samples as described in *Section 4.3.2.3*. Although slight, the epimerization of GTX2,3 observed in the freeze dried materials after 12 months storage represented a minor deterioration in stability of toxin epimers compared to those heat treated where this phenomenon was not observed (Figure 4-9). Both stabilisation techniques did however show instability of GTX3 evident by the slight degradation of this toxin at +20°C (freeze dried) and +4°C (Heat treated) after 3 months.

CHROMATOGRAPHIC STABILITY

As in the case of the other techniques investigated, chromatographic stability of freeze dried materials, specifically those stored at +20°C was assessed in comparison to untreated control materials. Figure 5-18 a-f displays chromatograms generated by preCOX LC-FLD for the

freeze dried and untreated control (Tissue C) materials by both peroxide and periodate oxidation after 12 months storage at different temperatures. Chromatograms of the untreated control material stored at -80°C for 12 months are also shown in this figure as a visual comparison.

The chromatograms generated following peroxide oxidation (Figure 5-18 a-c) show differing levels of stability, with the control material showing the total degradation of the toxins GTX2,3 by month 12 of the study. Conversely excellent GTX2,3 stability was evident in the freeze dried tissues stored at +20°C for 12 months with the chromatogram visually identical to that from the untreated control material stored at -80°C for 12 months (Figure 5-18 a+c). Additionally there was no visual evidence for the formation of additional chromatographic peaks from the freeze drying process itself.

The chromatograms obtained following the analysis of periodate oxidised extracts used to quantify the N-hydroxylated toxins GTX1,4 are illustrated in Figure 5-18 d-f. Again freeze dried materials showed excellent chromatographic stability with the chromatogram identical to that observed in the untreated control stored at -80°C. The untreated control stored at +20°C for 12 months did not show good stability however with the chromatogram showing the total degradation of GTX1,4 (Figure 5-18 e). There was no visual evidence for the additional formation of any chromatographic peaks in the freeze dried chromatograms giving further evidence for the improvements this technique offers in stabilising the matrix.

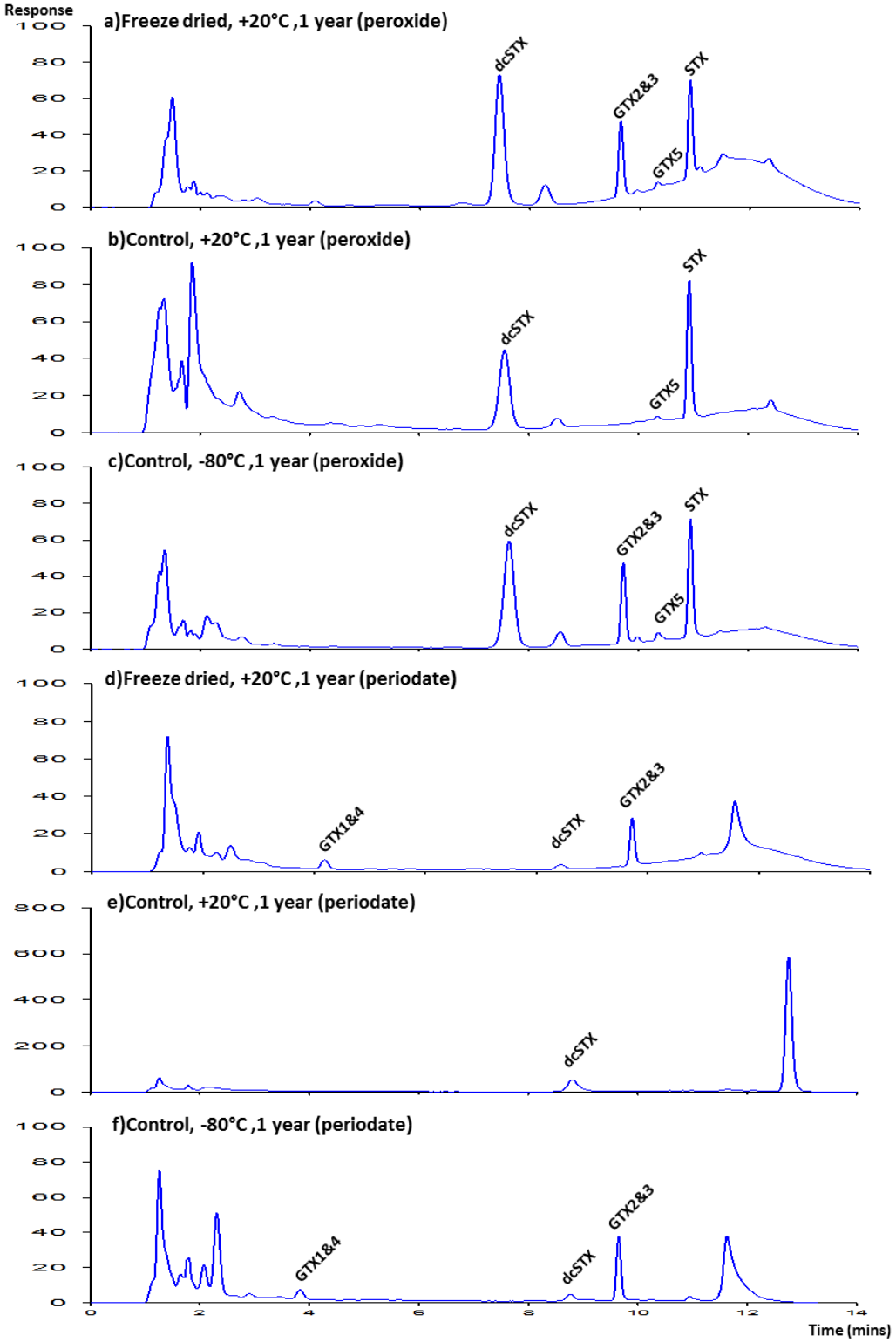


Figure 5-18: Chromatograms of freeze dried and control materials under different storage conditions after peroxide or periodate oxidation and preCOX LC-FLD analysis.

5.4.5 CONCLUSIONS

Similarly to the optimisation studies in *section 5.3* freeze drying and the associated processing techniques developed at the MI produced homogeneous RMs with significant stability improvements in comparison to an untreated control as well as heat treatment and the use of preserving additives. Freeze drying also provided a more stable matrix as demonstrated by the chromatographic stability of samples stored at high temperatures.

Whilst this study has shown compelling evidence for advantages with freeze drying, the technique does however require specialised equipment not readily accessible by most biotoxin laboratories. There is also the requirement for personnel with expertise in both the freeze drying process itself as well as the labour intensive processing techniques. The freeze drying process does also significantly alter the biological matrix compared with wet materials, so some researchers may debate whether such materials are ideally applicable for internal QC RM preparation. Materials treated by heat and chemical additives which were prepared from the same bulk homogenate, provide much more cost effective techniques albeit without providing materials with the same levels of toxin stability. Use of heat treatment or the preserving additives may be acceptable for the preparation of an internal QC material but freeze drying clearly provides a more appropriate tool for CRM production.

Excellent short-term stability was demonstrated in these materials even at higher storage temperatures which indicates transport of these materials could be carried out at temperatures above freezing. This would provide a very significant advantage to the RM producer if transporting these materials globally. Long-term stability was also demonstrated by preCOX LC-FLD, although a potential anomaly was seen in the month 12 samples under all storage conditions which could not be definitively explained.

PCOX LC-FLD analysis of short and long-term stability study samples showed evidence for instability issues of GTX2,3 in the +40°C and +20°C stored samples respectively. Epimerization of these toxins was observed particularly in the +40°C samples in the short-term studies with the rates of epimerization significantly reduced at +20°C in the long-term, thus demonstrating the significance of temperature on epimerization rates. The extent of epimerization of these toxins was less than that observed in the additives spiked material and untreated controls but interestingly heat treatment reduced these rates more than the freeze dried materials (Figure 4-9 a-d). This suggests that heat treating the materials prior to freeze drying may provide a more stable matrix less prone to epimerization issues. This experimental technique is described in the next section.

5.5 COMBINED FREEZE DRYING WITH THERMAL PRE-TREATMENT

An additional experiment described in this section coupled this freeze drying technique with a thermal pre-treatment step in an effort to combine the superior stability of the PSTs observed in the freeze dried materials with the reduced epimerization rates of heat treated materials, as seen in *Chapter 4*.

The material was prepared in combination with a study on the individual use of preserving additives together with an untreated control material as described in Chapter 4 (*Section 4.2*).

5.5.1 SOURCE TISSUES AND INITIAL TREATMENT

The source tissues and quantities used to prepare this material can be seen in Table 4-1 and the preparation of this initial material is described in *Section 4.2.1.1*.

The tissue was removed from the freezer, allowed to defrost in a fridge at +4°C overnight before being transferred to a 5L PP beaker. The beaker was covered with tin foil and sealed with autoclave tape, making small holes in the tinfoil lid to allow pressure release during the process. The tissue was autoclaved (K200E Touchclave, LTE, UK) at 121°C for 15 min, cooled in a fridge at +4°C before transferring 590 ± 1 g of the material to a 2L Waring™ goblet. The final weight was made up to 763 ± 1 g with DI water to adjust the final MC to ~85% as the four day freeze drying programme was optimised with materials containing this level of moisture. The tissue was homogenised on medium power for 5.0 ± 0.1 min before dividing the slurry equally into two freeze drying trays at a tissue depth of ~1.0 ± 0.1 cm. The trays were covered with tinfoil and placed in a freezer at -20°C overnight.

5.5.2 FREEZE DRYING AND MATERIAL PROCESSING

The trays were removed from the freezer and placed into the freeze drier where the 96 hour programme as detailed in Table 5-5 was started. Upon completion of the freeze drying programme the material was removed from the instrument, processed and dispensed as described in *Section 5.3.4*. A total of 120 aliquots of this material were dispensed as 0.78 ± 0.01 g portions (to ensure minimum retrieval of 0.75 g for extraction) and stored at -80°C until further use.

5.5.3 STABILITY AND HOMOGENEITY STUDIES

The between-bottle homogeneity of the materials was assessed through the intra-batch analysis of 15 ($3n^{1/3}$) aliquots selected through stratified random sampling of the entire fill series.

All materials were stability tested over a short term (31 day) and long term (12 month) period following a reverse isochronous experimental design [55] as described previously. The short term study was conducted with triplicate samples consisting of five time points (0, 4, 8, 16 and 31 day) and three temperature conditions (-20°C, +4°C and +40°C). The long term study was conducted with triplicate samples consisting of five time points (0, 3, 6, 9 and 12 month) and three temperature conditions (-20°C, +4°C and +20°C). The reference temperature used in both studies was -80°C. The stability studies were carried out as described in *section 4.2.1.4.*

At the end time point all samples were removed from storage, allowed to equilibrate to room temperature before removing the crimp seal and lyophilisation stopper. 0.75 ± 0.01 g of each tissue was weighed out immediately after the stopper was removed to minimise moisture uptake. The tissues were reconstituted and extracted as described in *Section 5.3.5*.

Analysis was carried out by preCOX LC-FLD as per OMA AOAC 2005.06 [41] (*Section 2.3.1*). PCOX LC-FLD [75] analysis was additionally performed on all samples to assess the presence of any epimerisation, if any, of GTX2,3 in each material. For PCOX LC-FLD analysis, the crude acetic acid extracts prepared according to *Section 2.3.1* were deproteinated and analysed following AOAC 2011.02 [75].

PST concentrations in sample extracts were quantified against a 4 - 5-point calibration for each toxin and are expressed in µmol/kg with total saxitoxin equivalents calculated as an estimation of total toxicity.

The MC of these freeze dried tissues was not determined due to technical issues with the Karl Fischer apparatus which could not be rectified during the course of these studies. As determined in the optimisation studies in *Section 5.3* however, the 96 hour freeze drying programme and associated processing and dispensing techniques produced materials with the required MC of ~5%.

5.5.4 RESULTS AND DISCUSSION

All the materials prepared in this section of the study were suitably homogenous for all the PSP toxins quantified, as evidenced through the coefficient of variances contained in Table 5-9, which were all below 8%. The homogeneity techniques employed were therefore sufficient and the materials deemed suitable for the consequent study.

Table 5-9: Homogeneity results from freeze dried material with thermal pre-treatment (n=15).

	dcSTX	GTX2,3	GTX5	STX	GTX1,4	Total Toxicity
	µg STX diHCl-eq. / kg					
Average	198.4	622.5	1.8	598.1	297.9	1718.7
Stdev	5.6	43.6	0.1	15.2	22.2	57.1
%CV	2.8	7.0	5.2	2.5	7.4	3.3

Homogeneity results of Tissues 1-3 summarised in Table 4-4 compare similarly to those above which shows the stability of the PSTs subjected to freeze drying and thermal treatment. The total toxicity figures above and those of the untreated control (Tissue 3) show no significant toxin degradation occurred during the stabilisation processes.

5.5.4.1 SHORT-TERM STABILITY

PRECOX LC-FLD

Short-term stability graphs for dcSTX, STX, GTX2,3, GTX5 and GTX1,4 are presented in Figure 5-19 for the material prepared. Results are normalised to the -80°C freezer mean on day zero and are presented with error bars of the standard deviation from the triplicate analyses.

Excellent stability was demonstrated at -20°C and +4°C for all the toxins studied in this section with all data points falling within the limits of stability set. There were some instability issues evidenced in samples stored at +40°C however, with dcSTX, STX, GTX2,3 and GTX5 degrading slightly by the end of the study, as evidenced by data points falling outside the limits of stability. However, the calculated limits of stability, particularly for the toxins dcSTX, STX, and GTX5, were very tight. This was due to the low %CVs determined for materials stored at -80°C for the duration of the study from which these figures are calculated (n=9). GTX5 is also present at a very low concentration (~ 400 times less than the EU reg. limit) in these materials which is just above the LOQ of the preCOX LC-FLD method as determined through in-house validation (data not shown). GTX1,4 remained stable throughout the short-term study even at the elevated storage temperature of +40°C. The results therefore indicate again that transportation of these heat treated and freeze dried materials could be carried out under freezing or cooled storage conditions, but could not be conducted at room temperature safely.

This combined technique also significantly improved the stability of the PSTs relative to an untreated control as well as antibiotic and anti-oxidant spiked materials as seen in Figure 4-3. These four materials were all prepared from the same bulk homogenate prepared in *Section*

4.2 so these materials can be compared for the levels of stability they provide. In terms of percentage difference of toxin degradation observed, this freeze drying technique with thermal pre-treatment provided better stability for dcSTX (9%), STX (8%) and GTX5 (48%) compared to the untreated control (Tissue 3) in samples stored at +40°C.

Under the same temperature conditions the material also showed significant improvements over the use of antibiotics (Tissue 1) for all the PSTs except GTX2,3 where degradation of these toxins was observed in the freeze dried material but remained stable in the antibiotics spiked tissues. Similar levels of stability were observed in the antioxidant spiked material (Tissue 2) for the toxins dcSTX and STX but stability improvements were observed in the freeze dried materials for the gonyautoxins 1-5.

The short-term stability results further emphasise the applicability of this technique in preparing RMs and the inclusion of a thermal pre-treatment step before freeze drying provides the same level of stability as freeze drying alone for most toxins, although GTX5 showed slightly more instability issues.

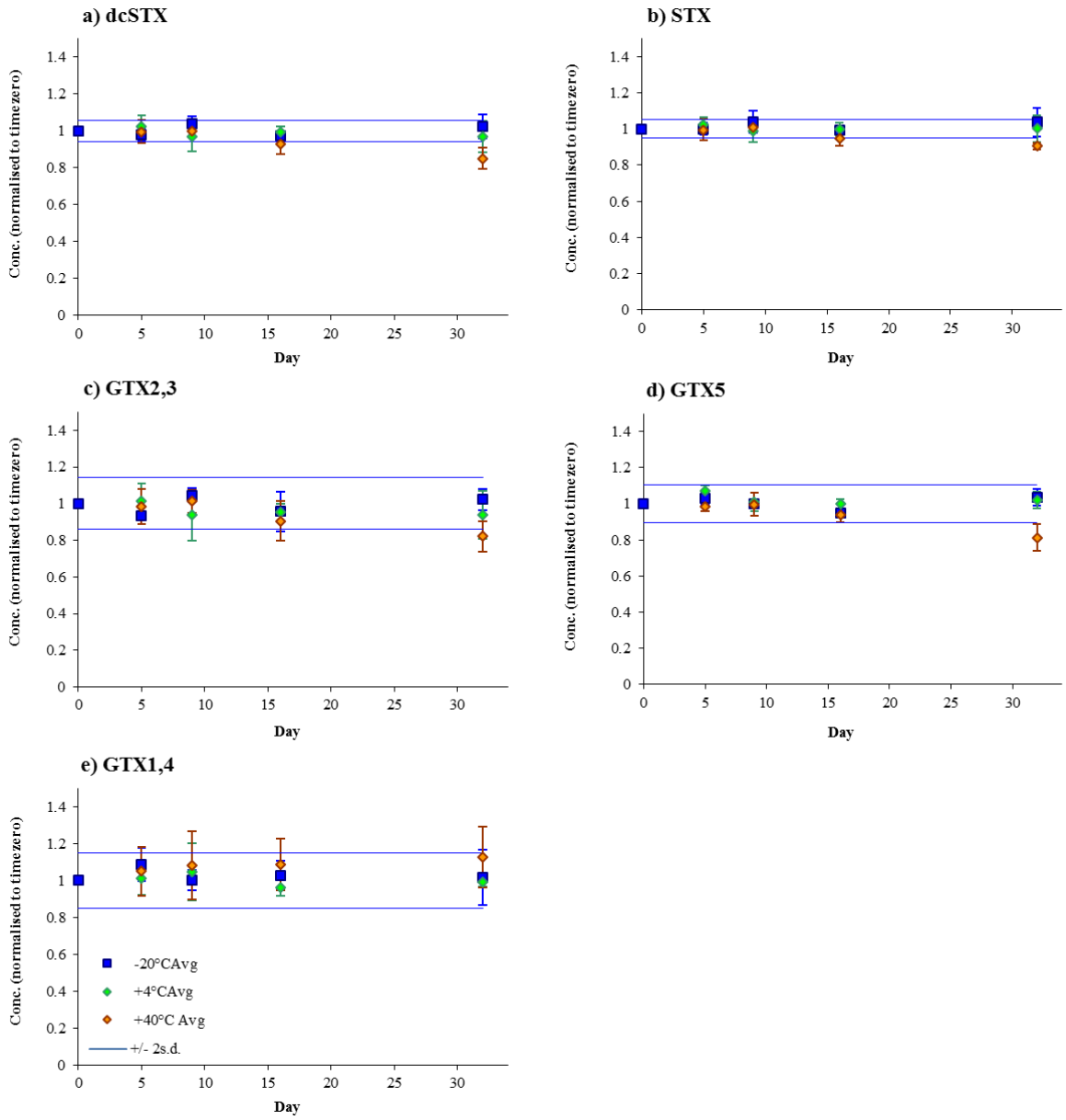


Figure 5-19: Short-term stability graphs for PSTs present in the freeze dried material with thermal pre-treatment.

PCOX LC-FLD

Further analysis was conducted by PCOX LC-FLD on the short-term stability study extracts of each of the materials and results are presented in Figure 5-20 a-b. Limits of stability and normalised results were calculated as per the preCOX analysis. Data for the toxins GTX1 and GTX4 were not generated as the concentration of these toxins in each material was <LOD of the PCOX LC-FLD method.

No epimerization or degradation was observed in any materials when stored at -20°C or +4°C for the duration of the study with results falling within the limits of stability set. Epimerization was observed however in materials stored at +40°C, evident after 4 days of the study, which was similar to the observations made in the *Section 5.4* for freeze drying alone. The epimerization rates were significantly reduced however in the thermally pre-treated materials which gives evidence for the benefits of combining the two techniques.

Significant improvements were made over the untreated control (Tissue 3) as observed in Figure 4-4, with rates of epimerization reduced by ~40% in samples stored at +40°C and eliminated in samples stored at +4°C. Similarly this technique reduced epimerization rates in the antibiotic (Tissue 1) and the antioxidant (Tissue 2) spiked materials in the +40°C samples by ~15% in both cases. Epimerization was evident in the Tissue 1 and 2 materials stored at +4°C but this had been eliminated in the freeze dried material which again shows the advantages this technique provides.

PCOX LC-FLD analysis of both the short and long-term stability study samples showed epimerisation of the β-epimer GTX3 into the more stable α-form GTX2 as expected [136].

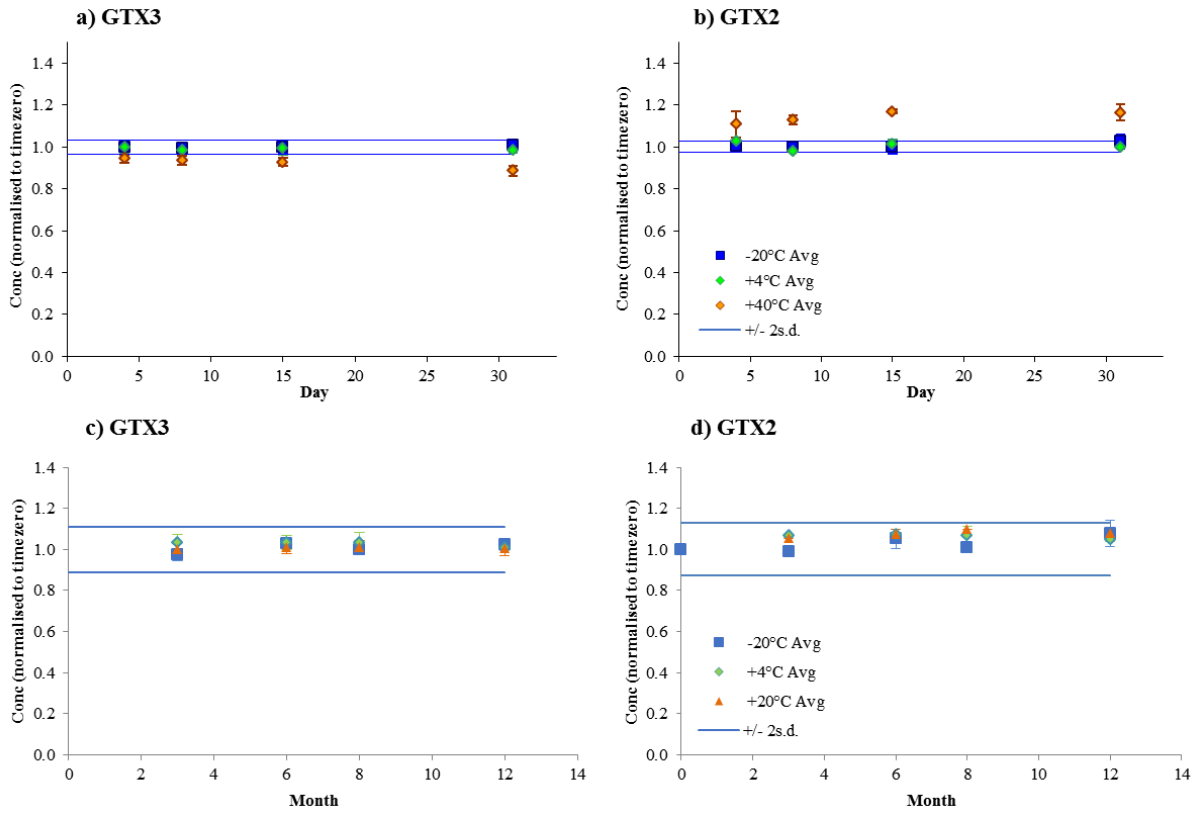


Figure 5-20: Short and long-term stability data by PCOX LC-FLD for freeze dried material with thermal pre-treatment step.

5.5.4.2 LONG-TERM STABILITY

PRECOX LC-FLD

Long-term stability graphs for dcSTX, STX, GTX2,3, GTX5 and GTX1,4 are presented in Figure 5-21 for the material prepared in this section. Results were normalised to the -80°C freezer mean on day zero and are presented with error bars of the standard deviation from the triplicate analyses.

Excellent toxin stability was exhibited in the freeze dried materials following the long-term study with none of the toxins investigated showing any signs of degradation even at the elevated storage temperature of +20°C. The data therefore provides evidence for the excellent long-term stability of these toxins in freeze dried materials which are thermally pre-treated. These materials could be stored at temperatures of up to +20°C without adversely affecting

PST content although long-term storage would always be recommended at temperatures below freezing.

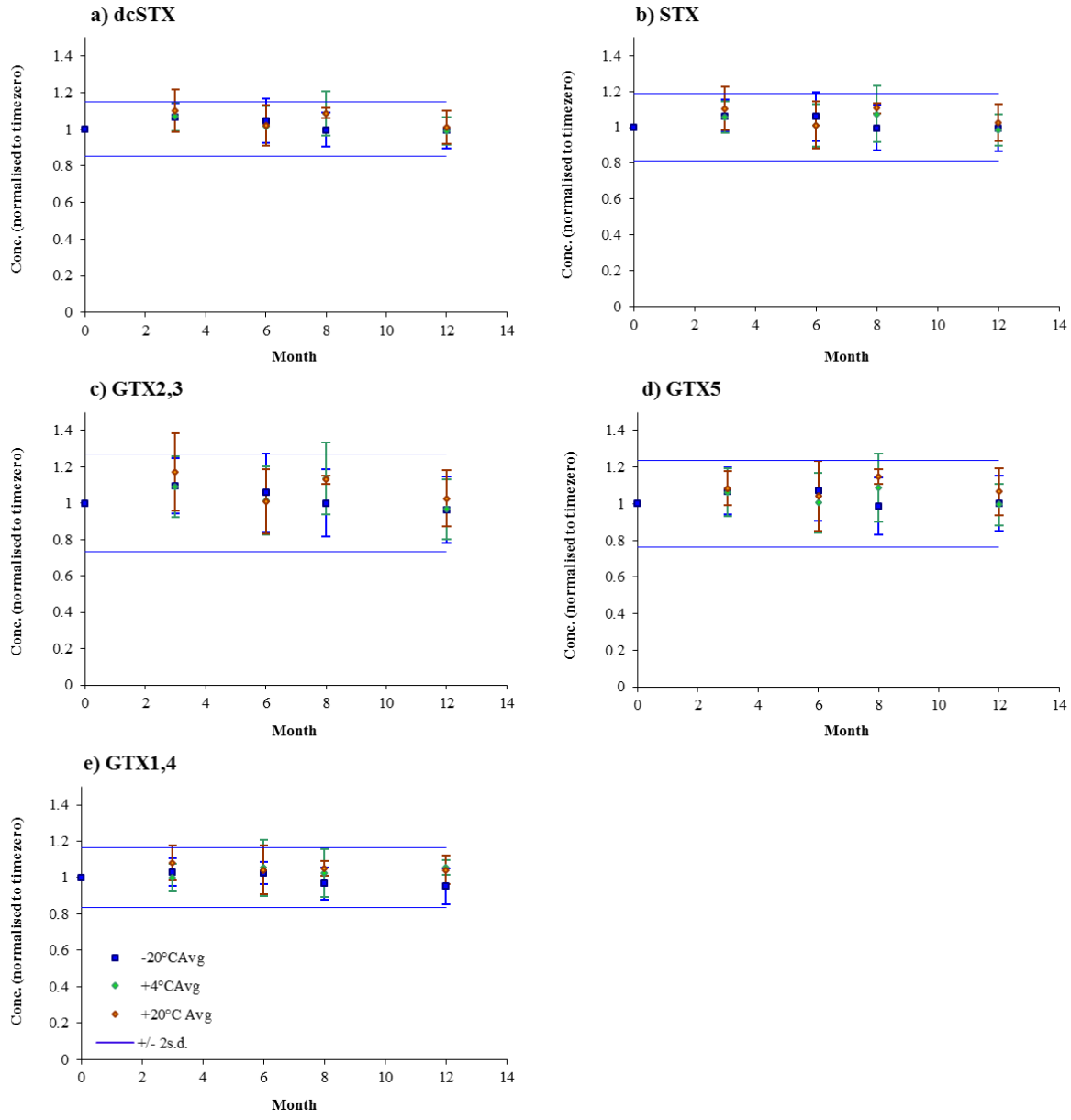


Figure 5-21: Long-term stability graphs for freeze dried material with thermal pre-treatment.

The degradation of the toxins observed in the month 12 freeze dried samples from the previous section (Figure 5-17) was not evident in these materials. This shows either the improved stability of this combined technique or, as is more likely, the same anomaly

observed in that dataset was not repeated in the preparation and analysis of these dual-treated materials.

The stability of the PSTs was greatly enhanced in these materials compared to the untreated control (*Tissue 3, Section 4-2*) apart from STX which displayed comparable stability to this material. This combined technique also showed better stability compared to the use of the antibiotics (Tissue 1) and antioxidant (Tissue 2) spiked materials from the same figure, particularly for the toxins GTX2,3 where significant increases (~30%) were observed in samples stored at +40°C.

PCOX LC-FLD

Further analysis was conducted by PCOX LC-FLD on the long-term stability study extracts of each of the materials and results are presented in Figure 5-20 c-d. Limits of stability and normalised results were calculated as per the preCOX analysis.

No epimerization or degradation was observed in any materials when stored at -20°C, +4°C or +20°C for the duration of the long-term study with results falling within the limits of stability set. These results show the significant improvements the thermal pre-treatment step makes in reducing epimerization rates over an extended time period compared to freeze drying alone (Figure 5-16). This was particularly evident in the case of GTX3 which showed signs of degradation after month 3 of the study in materials prepared by freeze drying alone (Figure 5-16) with this toxin remaining stable for 12 months in the materials prepared by the combined techniques.

Significant improvements were made over the untreated control (Tissue 3) as observed in Figure 4-4, with rates of epimerization reduced by ~20% in samples stored at both +4°C and +20°C. This combined technique also significantly reduced epimerization rates compared to the antibiotics (Tissue 1) and antioxidant (Tissue 2) spiked materials by as much as 40% in the +20°C samples.

CHROMATOGRAPHIC STABILITY

The chromatographic stability of the combined freeze dried and thermally pre-treated material was assessed against an untreated control. Figure 5-22 a-f displays chromatograms generated by preCOX LC-FLD for the material described in this section and an untreated control (*Tissue C, Section 4-2*) by both peroxide and periodate oxidation after 12 months storage at different temperatures. Chromatograms of the untreated control material stored at -80°C for 12 months are also shown in this figure as a visual comparison.

The chromatograms generated through peroxide oxidation (Figure 5-22 a-c) show the excellent chromatographic stability of the freeze dried and thermally treated material stored at +20°C (a) compared to the control (c) stored at -80°C. There was no visual evidence for the formation of extra fluorescent components from the freeze drying process itself or from storage at this elevated temperature for 12 months. The control material stored at +20°C for 12 months (b), while still maintaining the same toxin profile, did show evidence for the formation of extra chromatographic peaks between 2 – 7 min. Although this did not affect quantitation of any of the PSTs present in these materials, some of these peaks may be problematic in quantifying the C-toxins if they were present.

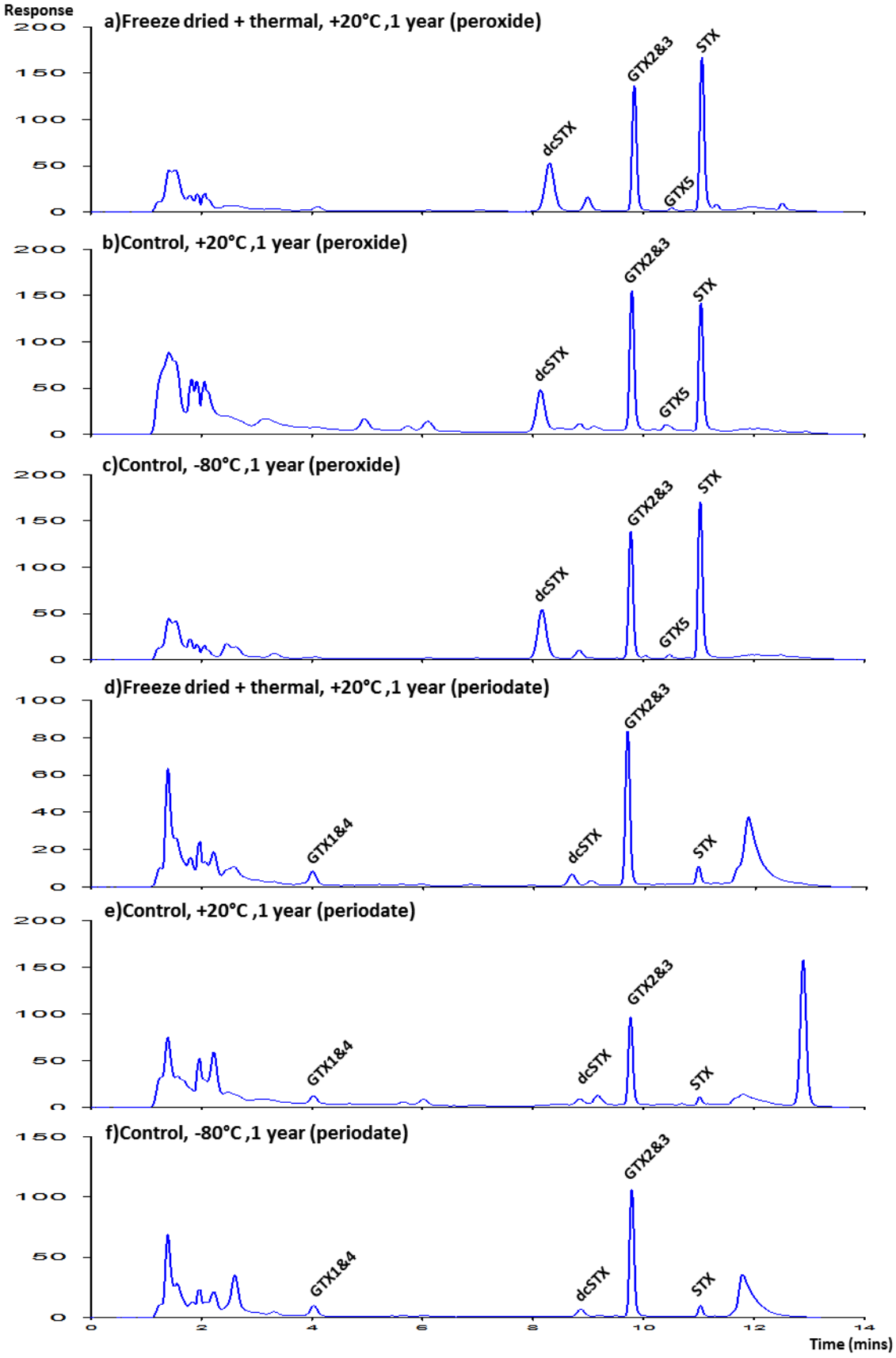


Figure 5-22: Chromatograms of freeze dried/thermally treated and control materials stored at different temperatures after peroxide and periodate oxidation and analysis by preCOX LC-FLD.

The periodate oxidised extract analysis used to quantify the N-hydroxylated toxins GTX1,4 are illustrated in Figure 5-22 d-f. Again the freeze dried and thermally treated material stored at +20°C (d) showed excellent chromatographic stability with the chromatogram identical to that observed in the untreated control (f) stored at -80°C. There was no visual evidence for the additional formation of any chromatographic peaks in the freeze dried chromatograms giving further evidence for the improvements this technique gives in stabilising the matrix. The untreated control stored at +20°C for 12 months (e) showed the formation of two extra peaks in the chromatogram at ~6 min, although the toxin profile remained intact.

5.5.5 CONCLUSIONS

As demonstrated within this chapter, freeze drying is an excellent technique for preparing homogeneous PST RMs. Excellent stability has been observed over both short and long-term timeframes over a range of different temperatures. The application of a thermal treatment step to the source tissues prior to being freeze dried was successful in further lowering the rates of epimerization observed in materials prepared by freeze drying alone (*Section 5-4*). Both techniques stabilise PSTs and the matrix in different ways that combined, result in a procedure suitable for RM preparation. The thermal treatment of the materials sterilizes and therefore destroys most if not all bacteria in the source tissues which alone would produce a very stable material. The processing and dispensing steps are not carried out aseptically however so bacterial contamination of the aliquots during these steps is inevitable. The freeze drying of tissues lowers the water content of the final product creating an environment unsuitable for microbial growth. These combined techniques therefore result in the same desired outcome, lowering rates of degradation, epimerization or biotransformations, through different routes, sterilization and water removal.

This procedure could have specific application in the production of CRMs and is arguably the most appropriate technique investigated in this thesis for these purposes owing to the superior stability exhibited in those materials.

5.6 PREPARATION OF CANDIDATE PSP CRM

The preparation of RMs, particularly those intended for internal QC, method development and validation use or for distribution to participants in PT exercises, can be performed adequately using one of the relatively simpler techniques described in Chapter 4. These techniques such as heat treatment or the use of preserving additives including all associated processing techniques have been shown successfully to produce homogenous materials, stable for their intended purposes. The preparation of these relatively small scale RMs does not therefore necessitate the use of freeze drying even with the advantages this technique affords the user.

This labour intensive and significantly more costly technique does have applicability in the production of CRMs however where long-term stability of the toxins and matrix is of primary importance. CRM production is also generally carried out through dispensing large quantities of materials thus lowering the overall unit cost.

The experiments conducted in this chapter so far have resulted in the development of an optimised freeze drying programme and processing technique that produces high quality homogeneous and stable materials. Using these optimised techniques a large scale freeze dried material was prepared in collaboration with CEFAS, UK as a candidate CRM.

5.6.1 SOURCE TISSUES AND INITIAL TREATMENT

The material was prepared from a naturally contaminated oyster tissue matrix which was produced through feeding experiments carried out in CEFAS facilities. The feeding experiments are detailed in *Section 5.2.1*.

Approximately 12 kg of homogenised oyster tissue in PP containers was sent to the MI in temperature controlled transport boxes. After receipt the PP containers were immediately transferred to a freezer at -20°C until further use.

The tissues were initially removed from the freezer and allowed to defrost at +4°C before being transferred equally to 4 x 5 L PP beakers. The beakers were covered with tin foil and sealed with autoclave tape, making small holes in the tinfoil lid to allow pressure release during the process. The tissues were autoclaved (K200E Touchclave, LTE, UK) at 121°C for 15 min, cooled in a fridge at +4°C before transferring to a 25 L PP bucket previously washed with Decon-90 (which contains a bactericide), followed by rinsing with DI water.

The material was then homogenised using a Silverson BX batch mixer (Silverson, MA, USA) set on medium power for 30.0 ± 0.1 min (Figure 5-23). The bucket was placed in an ice-bath during mixing to prevent localised heating of the tissues through friction.

Figure 5-23: Silverson BX batch mixer used to homogenise the oyster material prior to freeze drying.

Aliquots were taken for MC determination (n=3) using the rotary vacuum method and analysis by preCOX LC-FLD (n=1) as described in the materials and methods *Section 2.4.1* and *2.3.1* respectively, to give approximate PST concentrations in the bulk tissue. The bucket was sealed and stored in the fridge at +4°C until further use.

The concentrations and MC of this tissue are summarised in Table 5-10. The levels determined for this tissue were at concentrations that would ensure the final freeze dried material would have total toxicity slightly higher than the current EU regulatory limit. This analysis also confirmed the profile of the material as containing most of the relevant PSTs. The MC determined was higher than the 85% normally used but the capacity of the freeze drier was sufficient for this quantity of water removal.

Table 5-10: Concentrations of PSTs and MC of bulk tissue used to prepare candidate CRM.

Material	Concentration (µmol / kg)						Total Toxicity (µg STX diHCl-eq / kg)	% Moisture Content
	dcGTX2,3	GTX-2,3	STX	C1,2	GTX-1,4	NEO		
Oyster Tissue Homogenate	0.74	0.48	0.31	0.23	1.94	0.72	1331	92.6

5.6.2 FREEZE DRYING AND MATERIAL PROCESSING

The container of tissue was removed from the fridge and re-homogenised using the Silverson BX batch mixer (Silverson, MA, USA) set on medium power for 5.0 ± 0.1 min. The homogenised slurry was then poured into seven freeze drying trays at a tissue depth of ~5 mm, wrapped in tin foil and placed in a walk-in freezer at -20°C overnight.

The trays were subsequently removed from the freezer and placed into the freeze drier where the 96 hour programme detailed in Table 5-5 was started. Upon completion of the freeze drying programme the material was removed from the instrument and the freeze dried tissue in each tray was manually cut up and transferred into a bag, vacuum sealed and stored at +4°C. The quantities of tissues pre and post freeze drying are contained in Table 5-11.

Table 5-11: Weights of wet and freeze dried tissues in preparation of candidate CRM.

Freeze Drying Tray	Weight of wet tissue slurry before freeze drying (kg)	Weight of freeze dried material (kg)
1	1.451	0.127
2	1.388	0.123
3	1.469	0.129
4	1.396	0.126
5	1.544	0.138
6	1.472	0.132
7	1.458	0.132
Total	**10.178**	**0.907**

As described in previous sections all equipment used to process and dispense the freeze dried materials was first washed in Decon-90 followed by rinsing in DI water. The equipment was placed in an oven at +40°C for ~4 hours to dry before being placed in a desiccator until further use.

The freeze-dried material was processed in batches as follows:

1. Two vacuum sealed bags of freeze dried tissue were taken and placed in the modified glove box, along with three large steel bowls containing 25 x 20 mm stainless steel balls and the 125 µm sieve, base plate and lid.
2. The glove box lid was fitted and clamped and the exhaust valve opened. A cylinder of low moisture content argon was attached to the entry valve and the chamber was purged for ~5.0 ± 0.1 min before lowering the flow rate of argon.
3. The vacuum bags of freeze dried material were opened and poured equally into the three milling bowls before placing the lids on top.
4. The argon flow was stopped and the glove box lid removed. The milling bowls were taken and secured on the planetary ball mill (Retsch PM 100-Verder Group, The

Netherlands). The tissues were ground for 5.0 ± 0.1 min each before being placed back into the glove box.

5. The glove box lid was fitted and clamped and the exhaust valve opened. The entry valve was opened and the chamber was purged for ~5.0 ± 0.1 min before lowering the flow rate of argon. The lids of the milling bowls were then removed and the contents of each bowl transferred to the 125µm sieve plate. The sieve lid was attached before opening the glove box.
6. The material was placed on the motorised sieve shaker (Impact Test Equipment Ltd., UK) and covered with a plastic sheet, sealed with tape. A hose from a cylinder of low moisture argon was inserted into the sheet cover at the top of the sieve shaker, flushing the gas over the sieve plates. The freeze dried material was sieved for 30.0 ± 0.1 min before removing and placing back into the glove box.
7. The chamber was purged with low moisture argon as described above before removing the sieve lid and 125µm sieve plate. The contents of the base plate were transferred to a vacuum bag before removing the glove box lid and vacuum sealing the bag.

Steps 1 – 7 were repeated until all seven bags of freeze dried material had been processed which gave ~800 g of milled and sieved freeze dried tissue.

The material was dispensed by transferring the amber glass vials from the dessicator into the glove box along with the freeze dried material, semi-automated powder dispenser and all other associated equipment required. The setup of the glove box is as appears in Figure 5-10. The glove box lid was fitted and clamped before purging the chamber with low moisture argon. The antistatic fan was turned on and the antistatic gun discharged to aid in dispensing the powders. The antistatic gun was discharged periodically throughout the dispensing process.

The vacuum bags containing the freeze dried materials were opened and transferred into a large PP container and continually mixed with a large spatula for 5.0 ± 0.1 min. The powder dispenser was calibrated to ~0.77 g aliquot sizes before the material was dispensed into pre-labelled amber vials. After 588 aliquots had been dispensed, parafilm was placed over the beaker containing the remaining freeze dried material. The glove box lid was taken off and the amber vials were removed and placed on freeze drying trays. Lyophilisation stoppers were inserted halfway before placing the trays into the freeze drier. The door was sealed and the vacuum applied removing air from the chamber. A cylinder of low moisture argon was attached to the exhaust valve and a partial vacuum was released allowing argon to flow into the freeze drying chamber. The shelves were contracted which pushed the lyophilisation stoppers fully into the vials before releasing the remaining vacuum to atmosphere. The vials were removed and placed in boxes.

Simultaneously to the aliquots being sealed above, empty amber vials were transferred from a desiccator to the glove box. The lid was put on and clamped, purging the chamber of air with low moisture argon and removing static build-up through use of the gun. The remaining material was dispensed before sealing the vials with lyophilisation stoppers as described above. All vials were further crimp sealed manually.

A total of 1003 aliquots of this freeze dried material were dispensed and stored at -20°C. The vials were placed into boxes and transferred back to CEFAS facilities under cooled conditions.

5.6.3 ASSOCIATED STUDIES

The stability and homogeneity studies have not been carried out on this material to date due to time constraints at the collaborators laboratory and the completion of another candidate PSP CRM stabilised through gamma irradiation [151,152]. The material is stored at -80°C until required for homogeneity, stability and/or certification studies.

5.6.4 CONCLUSIONS

Although no stability or homogeneity studies have been carried out on this material to date, the process does highlight the fact that facilities and techniques developed at the MI during the course of these studies are applicable in the preparation of medium scale freeze dried materials.

5.7 SUMMARY OF FINDINGS AND CONCLUSIONS

This chapter has focused on the processing technique of freeze drying and its effects on PST and matrix stability for the preparation of RMs. Although this technique has previously been used to prepare a PST CRM it was never made commercially available. The previous study only covered one matrix, mussel, which contained two toxins, STX and dcSTX, and the freeze drying, processing and dispensing processes used were not detailed. Finally, the material was not prepared in parallel with an untreated control material to measure the effectiveness of the technique.

For these reasons the technique of freeze drying was evaluated here specifically to ascertain the following:

- The feasibility of preparing RMs by this method using MI facilities and evaluating multiple PSTs in a matrix other than mussel which had been previously studied.
- Once the feasibility of carrying out this technique at the MI had been established, to optimise the process, including the freeze drying programme, processing and dispensing techniques and to determine the capabilities of the MI in terms of capacity.
- Investigate issues arising from the initial feasibility study, specifically increased GTX2,3 concentrations observed in materials stored at higher temperatures.
- Using the techniques developed, to prepare a relatively large scale freeze dried material as a candidate CRM.

The initial feasibility study contained in *Section 5.2* details the first biotoxin RM to be prepared in MI facilities and was successful in demonstrating the capabilities of equipment, which had been recently purchased by the MI, in preparing materials by this technique. This initial study confirmed freeze drying as a very effective technique in PST RM preparation which was carried out in a new matrix, and which contained multiple toxins, some of which

had not been previously evaluated. These initial studies did however highlight some stability issues, specifically increasing toxin concentrations, which were evident in both the short and long-term stability studies affecting the gonyautoxins1-4 and the C-toxins. Significant rates of epimerization were also observed in this initial material which affected all three pairs of epimers.

The processing and dispensing techniques used to prepare these materials were then optimised in an effort to solve the issues highlighted above. Although the MC of this initial material was not determined as a KF apparatus was not available at the time of preparation, it seemed that moisture uptake by the highly hygroscopic material during the processing steps led to a final MC that was too high and therefore lead to the instability observed. This was due to various processing and dispensing steps being followed outside of moisture controlled conditions, i.e. outside of the glovebox purged with low moisture argon.

The optimisation experiments therefore sought to develop the freeze drying process in order to minimise moisture uptake during the various stages of production. A KF apparatus was acquired for these optimisation studies and the resulting data demonstrated the suitability of the processing and dispensing techniques, as well as the specific freeze drying time programmes, to produce materials with the desired MC of ~5%.

Two other freeze dried materials were prepared in tandem with studies on preserving additives and thermal treatment as detailed in *Chapter 4*. Full stability and homogeneity studies were carried out on these materials, specifically to determine if improvements were made from the feasibility studies, namely reduced rates of epimerization and a reduction or elimination of toxin biotransformations. The first material prepared by freeze drying alone improved the stability of the toxins, specifically increasing GTX2,3 and GTX1,4 concentrations observed in the feasibility study materials was significantly reduced. These

stability improvements could be due to a lower MC in these materials compared to the feasibility materials although this could not be substantiated as technical difficulties with the KF apparatus prevented their MC being determined. The stability improvements could also be due to both studies being carried out in mussel and oyster tissue matrices respectively and the different bacterial loads each naturally contains. Although overall toxin stability was improved, epimerization rates were observed to take place at similar levels and to the same extent.

For this reason a further technique was investigated which involved heat treating the materials prior to freeze drying. These techniques would combine the benefits of both, sterilization through heat treatment to destroy bacteria and freeze drying to lower the MC sufficiently to ensure conditions, for any remaining microbial activity, were as inhospitable as possible. The material prepared in this way showed improvements over materials previously prepared, specifically reducing epimerization rates over the long-term while providing the same level of overall toxin stability observed in materials prepared by freeze drying alone.

Finally this chapter described the preparation of a material on a relatively large scale as a candidate CRM using the optimised freeze drying, processing and dispensing techniques developed, including initially thermally treating the materials. This material has not been characterised, assessed for homogeneity or stability or been certified to date. This is due to a gamma irradiated PST CRM being made commercially available during this time coupled to the fact that the biotoxin CRM market is relatively small. It is likely that the material will be assessed as a full CRM within the next 12 months (CEFAS, personal communication).

From the studies contained in this chapter, freeze drying has proven to be the most effective technique for preparing PST RMs, providing superior stability to any of the other techniques investigated in the previous chapter. The applicability of the technique is, however, limited

solely to the preparation of CRMs and no other RM forms such as LRMs or ILRMs. This is due to the highly labour intensive process of freeze drying, processing and dispensing materials, as well as the large capital investment needed for all equipment which cost in excess of €250,000 in the case of the MI facilities. In terms of everyday usage in the case of general LRMs for internal QC/QA purposes, freeze drying would not represent a cost effective means of preparation and techniques such as the use of preserving additives or simple thermal treatment provide a better option for monitoring or research laboratories.

Chapter 6

MATERIAL USE IN PROFICIENCY TESTING SCHEMES – QUASIMEME

Chapter 6 details work described in Burrell *et al.* 2015, including extra data analysis not contained in the TrAC article.

Stephen Burrell, Steven Crum, Barry Foley & Andrew D. Turner, 2015. Proficiency Testing of Laboratories for Paralytic Shellfish Poisoning Toxins in Shellfish by QUASIMEME: A Review. *Trends in Analytical Chemistry. (Accepted Manuscript TRAC-D-15-00087)*

ACKNOWLEDGEMENT OF COLLABORATION

The work in this section was carried out in collaboration with Mr. Steven Crum and Ms. Ann-Marie Ryan at QUASIMEME. The RMs used in these exercises were all prepared by the author at MI facilities, including all associated homogeneity and stability studies. Materials were transported at -20°C to QUASIMEME facilities, Wageningen, The Netherlands where they were received by the collaborators and stored at -20°C until further use. All protocols and submission templates for results used by participants during these exercises were designed and written by the author. PT materials were packed and transported to participants by the collaborators. Participants submitted their results using an online share-point site operated by QUASIMEME after which the collaborators compiled all results for input into the Cofino software system. Participant data analysed using the Cofino model was then provided to the author for the analysis described in this chapter.

6.1 INTRODUCTION AND BACKGROUND

As discussed in Chapter 1, an essential component in any effective monitoring program is the proper application of a quality management system, incorporating both quality assurance and quality control [153]. Proficiency testing (PT) plays a vital role in the implementation of these programs, and participation in these schemes is a requirement both legislatively in the EU [72,73] as well as by most national accreditation bodies.

The stabilisation techniques described in Chapter 4, specifically the application of a thermal pre-treatment step prior to spiking with preserving additives was selected to prepare materials for use in these PT exercises. The techniques were chosen not only for the enhanced stability they provide the PSTs, especially during transport to participants laboratories, but also for the cost effectiveness of the preparation technique. This latter point was particularly important in the early rounds of this scheme in demonstrating the commercial viability of running this development exercise. For instance freeze drying would arguably provide superior PST stability but the unit cost to produce these materials in the quantities needed in these exercises would be too high to justify, which could jeopardise the sustainability of future exercises.

Six rounds of PSP exercises have been delivered by QUASIMEME since 2009 with a wide array of different methodologies used by participants.

6.1.1 METHODS OF ANALYSIS

There are few internationally recognised methods available for determining PSP toxins and these have been discussed in *Chapter 1*. Participants of QUASIMEME exercises over the last six years have used a range of these plus other developed methods and the breakdown, including total numbers of subscribers in each round are detailed in Table 6-1.

Table 6-1: Total number of participants in each round including breakdown of methods used.

Year	Round	Methods Used					Total Number of Participants
		preCOX	PCOX	MBA	ELISA	LC-MS/MS	
2009	**57**	8	2	4	-	-	14
2010	**61**	9	5	2	-	-	16
2011	**65**	8	5	4	2*	-	19
2012	**69**	9	5	2	-	-	16
2013	**72**	13	6	2	-	1	22
2014	**2014-1**	13	5	2	-	3	23

*Ridascreen ELISA used by participants

The methods used by QUASIMEME participants are described in *section 1.8,* including some of the major drawbacks and advantages of each method. The majority of QUASIMEME participants have used preCOX LC-FLD methods since these exercises began. Participation in these exercises is predominated by EU laboratories with approximately 60% of total subscriptions coming from this region. Two preCOX methods have been used to date with most participants using OMA AOAC 2005.06 [41]. The second closely-related method, used by one participant in 2014 is that described by the European Committee for Standardisation (CEN) and was recently published as European standard DIN EN 14526 [154]. Both methods involve the oxidation of toxins into iminopurine derivatives before separation and determination by LC-FLD. Participants using PCOX methods of analysis have increased since the start of this development exercise and numbers have remained steady in the intervening years. Most PCOX participants have used OMA AOAC 2011.02 [75] to determine the toxicity of QUASIMEME samples. One participant (Lab 5) used the original PCOX method developed by Oshima [17] for the first three years, 2009-2011. This lab did not submit results during the subsequent two years but submitted results again in 2014 using OMA AOAC 2011.02 (Table 6-4). The complexities in accurate identification of these toxins using either preCOX or PCOX methods are discussed in *Chapter 1*. To illustrate these complexities example chromatograms of a sample used in these exercises are contained in

Figure 6-1 a-d. Participants using mass spectrometry for determining PSP toxins have only recently submitted results using this technique in 2013 and 2014.

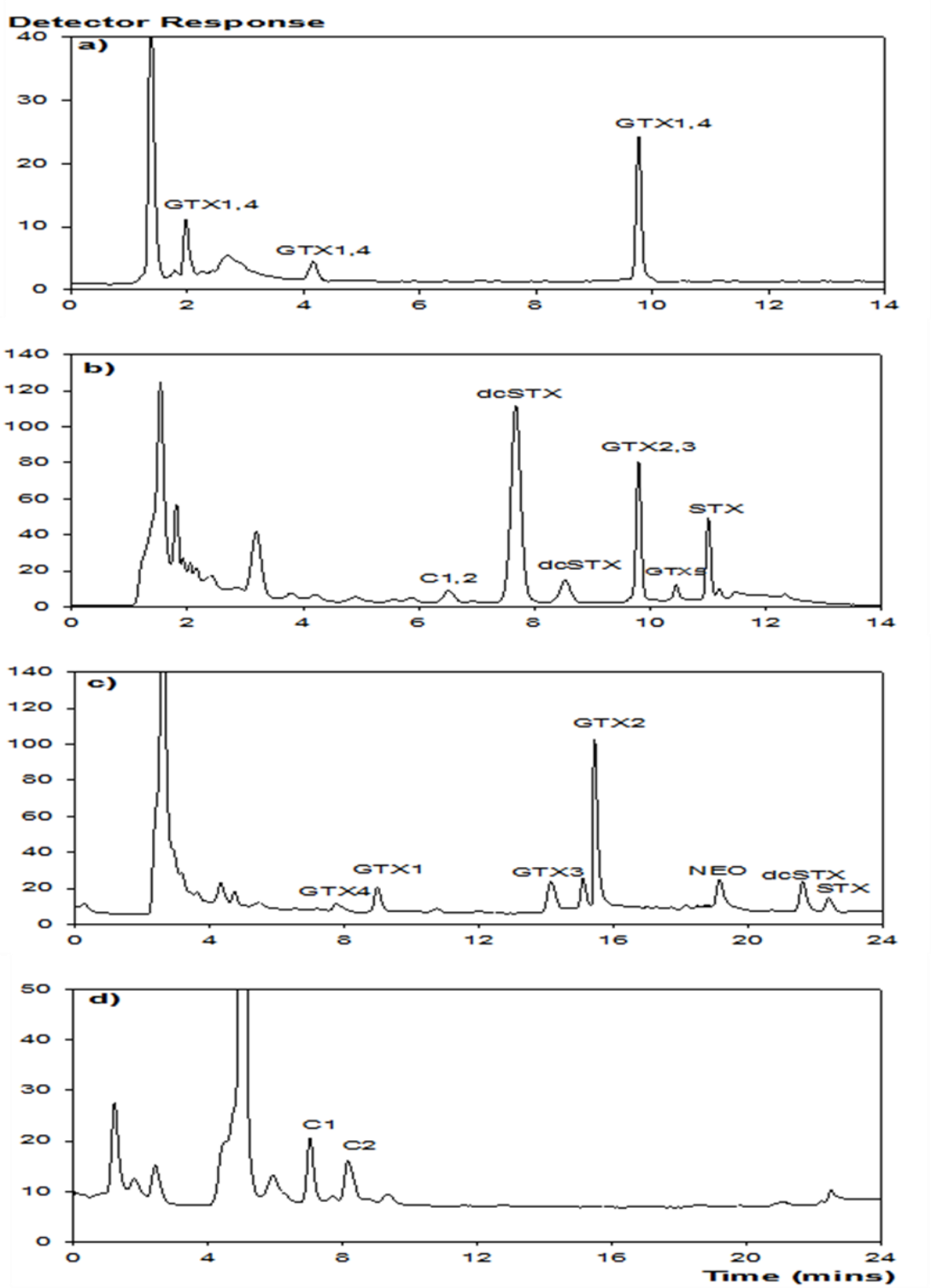


Figure 6-1: Chromatograms of Tissue B (QST076BT, 095BT & 133BT) obtained by a) preCOX LC-FLD analysis of fraction 2 obtained after carboxylic acid solid phase extraction (SPE), periodate oxidised, b) preCOX LC-FLD analysis of C_{18} SPE cleaned, peroxide oxidised, c) PCOX LC-FLD analysis of GTX/STX toxins and d) PCOX LC-FLD analysis of C-toxins.

6.1.2 TOXICITY EQUIVALENCY FACTORS (TEFS)

The PSP analogues differ substantially in their toxicity and their TEFs have been derived from MBA data. Table 1-1 lists the TEFs reported by Oshima et al. in 1995 [17] alongside values compiled by the Scientific Panel on Contaminants in the Food Chain (CONTAM) of the European Food Safety Authority (EFSA) in 2009 [6]. The two sets of data are similar with the exception of dcSTX where the TEF was increased to 1.0 from 0.5131 by the CONTAM panel. This change would significantly affect reported total toxicities of samples with dcSTX as the predominant toxin present which is the case in some of the samples supplied by QUASIMEME. The implications of these differences are discussed in detail below.

6.1.3 STATISTICAL METHODS FOR THE ASSESSMENT OF PROFICIENCY TESTING PERFORMANCE

The assessment of laboratory performance or PT data has historically been carried out using ISO guide 5725: Accuracy (trueness and precision) of measurement methods and results, to obtain an estimate of the mean and the uncertainty of the measurement [155]. The standard makes the assumption that values are normally distributed and there is an equal within-laboratory variance, and it was developed for PT studies where participants were required to use the same protocol. Due to the nature of PT schemes where different analytical methods are used, data is very often non-normally distributed and may be either positively or negatively skewed or bi- or multi-modal.

Laboratories in PT studies should, in principle only participate when their methods are fully validated and statistically controlled and under these circumstances datasets are then normally distributed. QUASIMEME exercises generally have world-wide participation and

therefore methodologies and standards may differ between countries which may give rise to systematic differences in the datasets.

In an effort to overcome issues associated with non-normally distributed data, robust statistics have been applied to data from PT schemes. Robust statistics are statistical methods which perform well for data from a wide range of probability distributions, particularly non-normally distributed datasets containing a small number of outliers. Extreme values are not discarded as outliers with this approach and are down weighted to minimise the effect on the entire dataset. This approach works well with datasets that have 7-10% of extreme values or even highly skewed datasets but the robust means may be affected in datasets containing a small number of values (< 10) or a larger proportion of extreme values [156]. This breakdown in the robust model is caused by the group of down weighted values forming their own cluster which influences the magnitude of both the robust mean and standard deviation. Extreme values can generally be traced back to gross reporting errors by participants or the use of incorrect units and these errors can generally be identified from the dataset or through contact with the laboratory. Removing these extreme values adds a subjective element into the evaluation which the robust model sought to avoid.

6.1.4 QUASIMEME'S COFINO MODEL FOR DATA ASSESSMENT

The data assessment carried out by QUASIMEME is based on ISO guide 13528 concerning the proficiency testing of analytical chemistry laboratories [157] with some slight modifications. The assigned value and z-scores are calculated using a model developed by Cofino *et al.* [158] which was specifically designed for use in the determination of population characteristics [159]. Robust statistics form the basis to the ISO13528 guide to data assessment but this model can be limited where a high percentage of extreme values are

submitted. The Cofino model is unique as it can be used directly with a whole range of datasets including tailing or skewed data, datasets containing extreme outliers and bimodal or multimodal distributions.

The model works by identifying clusters of data within a dataset exhibiting a high level of agreement. From this the mean, standard deviation (s.d.) and percentage of data associated with each cluster is calculated. A distinct advantage this model has over the standard robust model is that no preparation of the dataset is necessary, either by using subjective boundaries or outlier testing before entering it into the database for assessment.

The Cofino model uses the concept of wavefunctions from quantum mechanics and applies them utilising the power of matrix algebra. The model uses probability density functions for each observation weighting values centred around the mean more heavily than those further away from the mean. The mean and variance estimators derived from this model are less sensitive to asymmetric, tailing datasets. The detailed, graphical information provided by the model can be seen in Figure 6-2 and the derivation of each is described in the QUASIMEME handbook [159]. The graphical information includes:

-A plot of the population density functions (Figure 6-2a).

-Matrix overlap (Kilt) plot which is a colour density plot very sensitive to identifying the structure of data, especially modality (Figure 6-2 b).

-A ranked overview of the means and standard deviations of each data set (Figure 6-2 c).

-Z-score plot for reviewing performance against targets (Figure 6-2 d).

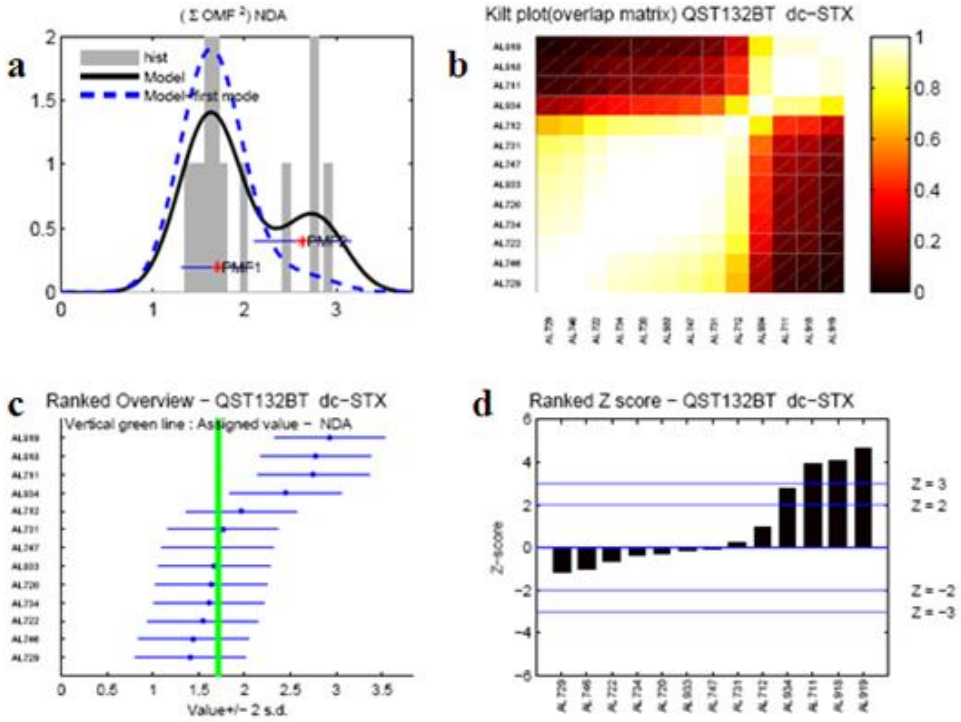


Figure 6-2: Data plots for the toxin dcSTX found in sample QST132BT in 2012. a) Summed probability density functions (PDFs) for all data (black line) and for the first mode, PMF1, (blue line) with histogram of individual measurements in grey. Each observation from a participant is described by a PDF and is not regarded as a value using the Cofino model. b) The Kilt plot (Overlap matrix) showing degree of overlap of each pair of data. Areas of the map coloured white indicate complete overlap (agreement) for the observations concerned while black indicates no overlap. c) Ranked overview of all data with error bars of ± 2 s.d. d) Ranked z-score plot for all data.

6.2 MATERIALS AND METHODS

All materials were prepared by the author and designed to test the performance of QUASIMEME participant methods with the analysis of a wide variety of analytes, whilst minimising the number of samples to be tested. Materials incorporated a range of toxin concentration levels and different complexities in toxin profile composition. The shellfish tissues used were naturally contaminated with a range of different PSP toxins and prepared following in-house procedures to ensure homogeneity. Stabilisation of the toxins and matrices was achieved using a combination of heat treatment and the addition of antibiotics and an antioxidant [60,113] with short-term stability and homogeneity studies performed on all materials before distribution to participants [51].

A total of eight materials have been used over the six exercises 2009-2014, with Tissues A & C being incorporated into four rounds each, Tissues B & D used in three rounds, Tissue E used in two rounds and Tissues F, G & H used in one round only. Table 6-2 lists each of the materials and the rounds in which they were used, the assigned codes, predominant toxins present, matrices studied and the homogeneity results determined. The coefficients of variation were calculated from the homogeneity data in Table 6-2 and compared to expected levels of method variability determined through validation of the test method, below which the RM was deemed sufficiently homogenous.

The exercises are true proficiency tests, meaning no standardised method protocol was provided by QUASIMEME and participants were requested to use the analytical method routinely employed at their laboratories. The only stipulation in the protocol was the use of the TEFs supplied and these were requested to be used in total toxicity calculations. In 2009 (R57) and 2010 (R61), the TEFs supplied in the protocol were those reported by Oshima [17], while in 2011 (R65), 2012 (R69), 2013 (R72) and 2014 (R 2014-1) TEFs recommended by EFSA [6] were prescribed in the protocol (Table 1-1).

Table 6-2: List of materials used in each exercise, assigned codes, predominant toxins present, matrices studied and homogeneity results.

Tissue	Rounds Used	Year	Codes Used	Predominant Toxins Present	Matrix	Homogeneity Results (n=15) µgSTXdiHCl eq. / kg
A	57	2009	QST075BT	dcSTX, STX, GTX-5	Mytilus galloprovincialis	533 ± 24
	61	2010	QST093BT			
	69	2012	QST132BT			
	2014-1	2014	Sample 1			
B	57	2009	QST076BT	dcSTX, GTX-2,3, GTX-1,4, STX, GTX-5 & C-1,2	Mytilus edulis & Mytilus galloprovincialis	1174 ± 45
	61	2010	QST095BT			
	69	2012	QST133BT			
C	61	2010	QST094BT	dcGTX-2,3, dcSTX & dcNEO	Spisula solida	2829 ± 62
	65	2011	QST111BT			
	69	2012	QST134BT			
	2014-1	2014	Sample 2			
D	65	2011	QST113BT	GTX-2,3 & STX	Mytilus edulis	1400 ± 44
	72	2013	QST152BT			
	2014-1	2014	Sample 3			
E	65	2011	QST114BT	GTX-2,3, STX & dcSTX	Mytilus edulis	1249 ± 55
	72	2013	QST154BT			
F	69	2012	QST135BT	GTX-2,3, STX, GTX-1,4 & NEO	Crassostrea gigas	803 ± 22
G	72	2013	QST155BT	GTX-2,3, STX, GTX-1,4 & dcSTX	Mytilus edulis	829 ± 29
H	2014-1	2014	Sample 4	GTX-2,3, STX, GTX-1,4 & dcSTX	Crassostrea gigas	1715 ± 51

The format of the data submitted by participants is dependent upon the method employed for material testing. All participants are required to submit a total toxicity result for each sample, enabling the assessment and comparison of all methodologies. Participants using either the preCOX, PCOX or LC-MS/MS methods were able to submit concentration data for individual analogues, thereby enabling the assessment of performance for each individual toxin, or epimeric pair, in addition to total toxicity. This allows participants receiving less than satisfactory z-scores to pinpoint potential causes of method failure or operator error, if they relate to the determination of a particular analogue.

6.3 INTERCOMPARISON RESULTS

Up to 23 laboratories submitted data over the six exercises and Table 6-3 summarises these results. The table contains assigned values for each determinand and/or total toxicity, the number of observations or participant submissions for each determinand as well as between-laboratory CV (%) and percentage of participants receiving satisfactory z-scores. The performance of laboratories was not assessed in cases where an assigned value could not be calculated. In these cases an indicative value was generated and no z-scores were calculated. Only materials where assigned values could be calculated are listed in Table 6-3. The criteria set out in calculating an assigned value and z-scores are contained in the QUASIMEME manual [159].

Table 6-3: Summary data 2009-2014

Year	Test Material and Code	STX	dcSTX	dcGTX-2	dcGTX-3	dcGTX-2,3	C-1,2	GTX-2	GTX-3	GTX-2,3	GTX-5	GTX-1	GTX-4	GTX-1,4	neoSTX	dcNEO	Total Toxicity
		µmol/kg															µgSTXdiHCleq./kg
2009	Mussel QST075BT																461, 14
	Tissue A																34.7, 50
	Mussel QST076BT																977, 14
	Tissue B																43.8, 50
2010	Mussel QST093BT	0.07, 6	1.69, 13								0.20, 10						400, 16
	Tissue A	31.4, 42	34.3, 85								39.2, 69						44.4, 50
	Clam QST094BT		3.47,14													1.37, 7	1886, 16
	Tissue C		41, 50													35.5, 57	45.4, 44
	Mussel QST095BT	0.37, 11	0.93, 13				0.48, 8			1.36, 11	0.13, 7			1.14, 8	0.17, 5		950, 15
	Tissue B	22.8, 77	28.4, 71				43.2, 50			13.1, 75	15.8, 46			21.5, 55	58.1, 33		36, 44
2011	Clam QST111BT		3.53, 12			9.32, 7										1.40, 8	2670, 19
	Tissue C		14.3, 83			29.2, 71										19.7, 63	19.4, 89
	Mussel QST113BT	2.56, 12						1.01, 5		1.15, 7							1139, 19
	Tissue D	15.3, 83						22.4, 80		29.4, 86							29.8, 58
	Mussel QST114BT	1.92, 12	0.58, 12							1.54, 7							1187, 19
	Tissue E	15.8, 75	27, 92							18.4, 71							27, 63
2012	Mussel QST132BT	0.08, 10	1.71, 13								0.21, 10						629, 15
	Tissue A	62.8, 69	22.7, 69								20.4, 75						18.3, 60
	Mussel QST133BT	0.35, 14	0.97, 13				0.35, 6			1.09, 8	0.12, 7	0.83, 5		0.90, 7	0.14, 6		986, 15
	Tissue B	23, 86	29.2, 69				26.1, 71			20.6, 75	3.77, 64	14, 80		19.3, 86	35.2, 56		31.7, 53
	Clam QST134BT		3.34, 13			9.00, 8					0.10, 4					1.32, 9	2734, 15
	Tissue C		17.8, 62			23.9, 63					43.6, 44					24.4, 67	19, 67
	Oyster QST135BT	0.60, 14								1.62, 7				0.69, 7	0.12, 7		789, 15
	Tissue F	27.8, 86								20.5, 71				39.3, 50	79.9, 50		38, 47
2013	Mussel QST152BT	2.59, 19						1.06, 6	0.45, 6	1.31, 12							1245, 18
	Tissue D	13.2, 68						5.16, 100	1.66, 100	27.2, 92							10.6, 72
	Mussel QST154BT	1.98, 19	0.56, 17					1.36, 6	0.54, 6	1.53, 12							1246, 18
	Tissue E	20.3, 68	25.3, 76					16.8, 100	4.67, 83	18.4, 83							16.9, 78
	Mussel QST155BT	0.32, 18	0.54, 17					0.84, 6	0.30, 6	0.95, 12			0.17, 5	0.54, 10			742, 17
	Tissue G	27.2, 78	26.3, 76					24.7, 100	6.76, 83	16.9, 83			48.1, 83	37.6, 64			18.6, 67
2014	Mussel Sample 1	0.10, 13	1.96, 21								0.20, 13						750, 22
	Tissue A	76.7, 65	30.5, 57								44.5, 75						32.5, 55
	Clam Sample 2		3.46, 20	11.20, 6	3.07, 6	10.20, 14										1.46, 13	3045, 22
	Tissue C		23.9, 65	4.4, 100	9.0, 67	36.6, 50										53.2, 46	22.1, 68
	Mussel Sample 3	2.61, 20						0.94, 6	0.42, 6	1.21, 14							1289, 21
	Tissue D	17.6, 80						9.33, 83	11.5, 100	32.1, 79							16.6, 71
	Mussel Sample 4	1.94, 21	0.09, 13					2.24, 7	0.74, 7	2.41, 14			0.17, 6	0.74, 9	0.24, 11		1569, 22
	Tissue H	8.5, 90	55.1, 63					8.3, 86	6.5, 100	25.0, 64			27.1, 86	44.7, 55	46.8, 57		15.3, 68

N.B. Codes used in table. Assigned Value, Numbers of Observations / Coefficient of Variation % (CV%), % z-scores satisfactory (|z| < 2).

Figure 6-3 and Figure 6-5 graphically represent z-scores generated over the last six exercises with calculated upper and lower z-score limits for satisfactory ($z \leq |2|$), questionable ($z > |2| \leq |3|$) and unsatisfactory ($z \geq |3|$) data.

A list of the methods used by participants since 2009, including references where possible, is detailed in Table 6-4. It should be noted that participants 3 and 8 changed from using the MBA as their method of analysis in R57 (2009) to using preCOX and PCOX methods respectively, when they next participated in 2010 and 2011.

6.3.1 TISSUE A

This tissue was prepared at approximately half the EU regulatory limit, which is just above the limit of detection for the MBA and was designed to test participants at a low concentration level with a relatively simple toxin profile containing 3 predominant analogues.

From the data contained in Table 6-3, 14-22 labs submitted total toxicity results in the four rounds this material has been used, R57, 61, 69 and 2014-1. Only total toxicity results were submitted in R57, while in R61, 69 and 2014-1 toxin concentrations were also requested. The assigned values for total toxicity showed some variability over the four rounds with concentrations of 461, 400, 629 and 750 µg STX diHCl-eq./kg being assigned respectively. This variability can mainly be attributed to changes made to the protocol which prescribed the use of Oshima TEFs in R57 and 61 and the use of EFSA recommended TEFs in R69 and 2014-1. DcSTX is the predominant toxin present in this tissue so the increased TEF value significantly affected total toxicity results submitted, which can be clearly seen from Figure 6-4. The precision of the participants, expressed by the between laboratory CV% has exhibited variability over the four rounds with values of 34.7, 44.4, 18.3 and 32.5% respectively.

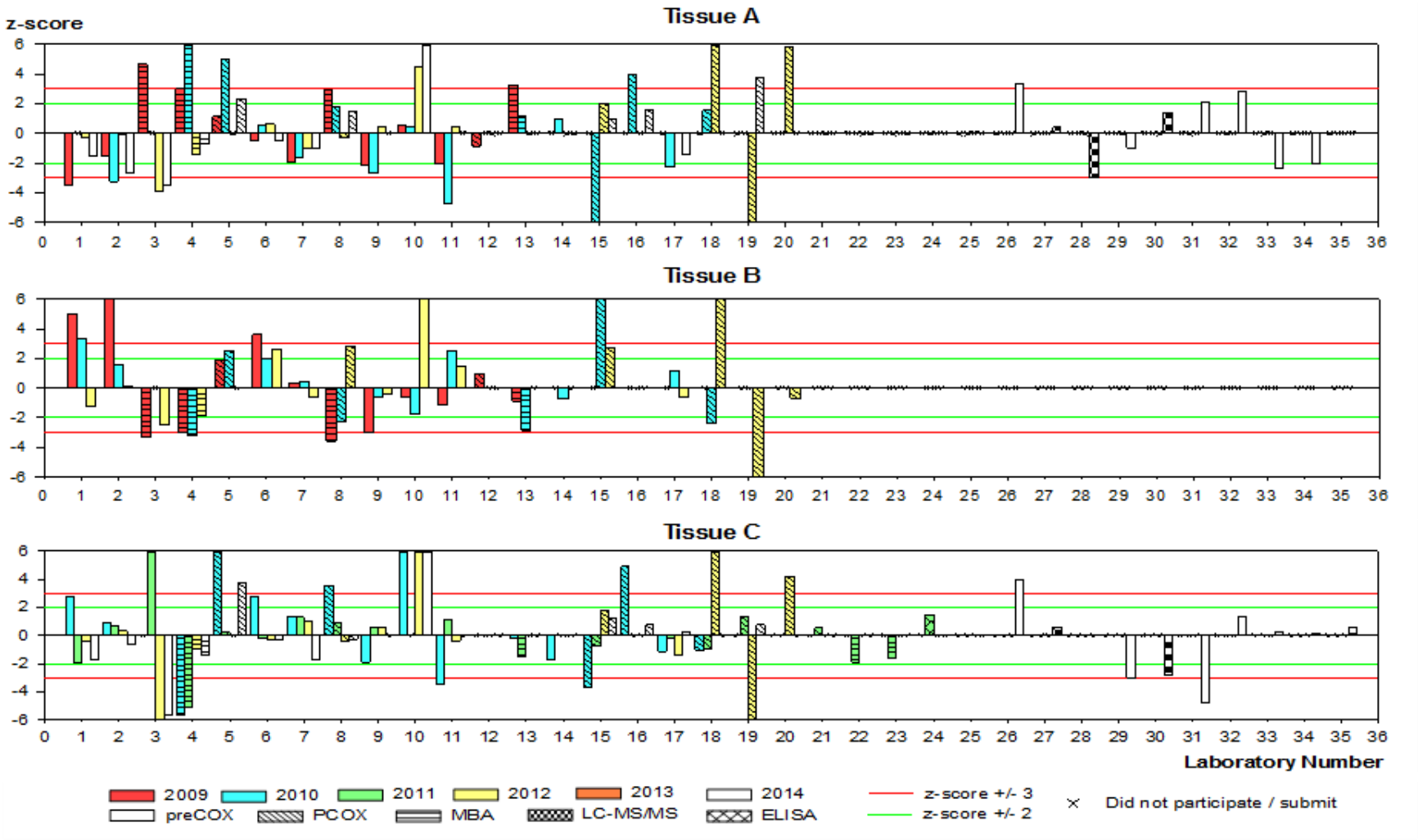


Figure 6-3: Distribution of participants' z-scores for Tissues A-C used in various rounds from 2009-2014.

Table 6-4: Overview of methods used from 2009-2014 with references where applicable.

Lab Number	Method Used					
	2009	2010	2011	2012	2013	2014
1	1	1	1	1	1	1
2	1	1	1	1	1	1
3	2	-	1	1	1	1
4	2	2	2	2	2	2
5	3	3	3	-	-	4
6	1	1	1	1	1	1
7	1	1	1	1	1	1
8	2	4	4	4	4	4
9	1	1	1	1	1	-
10	1	1	-	1	1	1
11	1	1	1	1	1	-
12	4	-	-	-	-	-
13	2	2	2	-	-	-
14	-	1	-	-	-	-
15	4	4	4	4	4	4
16	-	4	-	-	4	4
17	-	1	1	1	1	1
18	-	4	4	4	-	-
19	-	-	4	4	4	4
20	-	-	-	4	-	-
21	-	-	5	-	-	-
22	-	-	2	2	-	-
23	-	-	2	-	-	-
24	-	-	5	-	-	-
25	-	-	-	-	4	-
26	-	-	-	-	1	1
27	-	-	-	-	6	6
28	-	-	-	-	-	6
29	-	-	-	-	-	1
30	-	-	-	-	-	6
31	-	-	-	-	-	1
32	-	-	-	-	-	7
33	-	-	-	-	-	1
34	-	-	-	-	-	1
35	-	-	-	-	-	2

Codes:
1. OMA AOAC 2005.06
2. OMA AOAC 959.08
3. Oshima (PCOX)
4. OMA AOAC 2011.02
5. ELISA (Ridascreen)
6. LC-MS-MS Internal method
7. DIN EN 14526

6.3.2 TISSUE B

This material was prepared at approximately 1.2 times the EU regulatory limit and had a more complicated toxin profile than tissue A containing 9 predominant PSP analogues.

14-15 labs submitted total toxicity results for this material in R57, 61 and 69 with assigned values in each round of 977, 950 and 986 µgSTXdiHCl-eq./kg respectively. The assigned values for the three rounds were consistent, even with the change to the prescribed TEFs made for R69. This is due to dcSTX being present in much lower quantities in this material compared to Tissue A which therefore has a lower overall effect on the assigned value. The improvement of participants over the three rounds was evident from the laboratory precision values of 43.8, 36.0 and 31.7 CV% respectively.

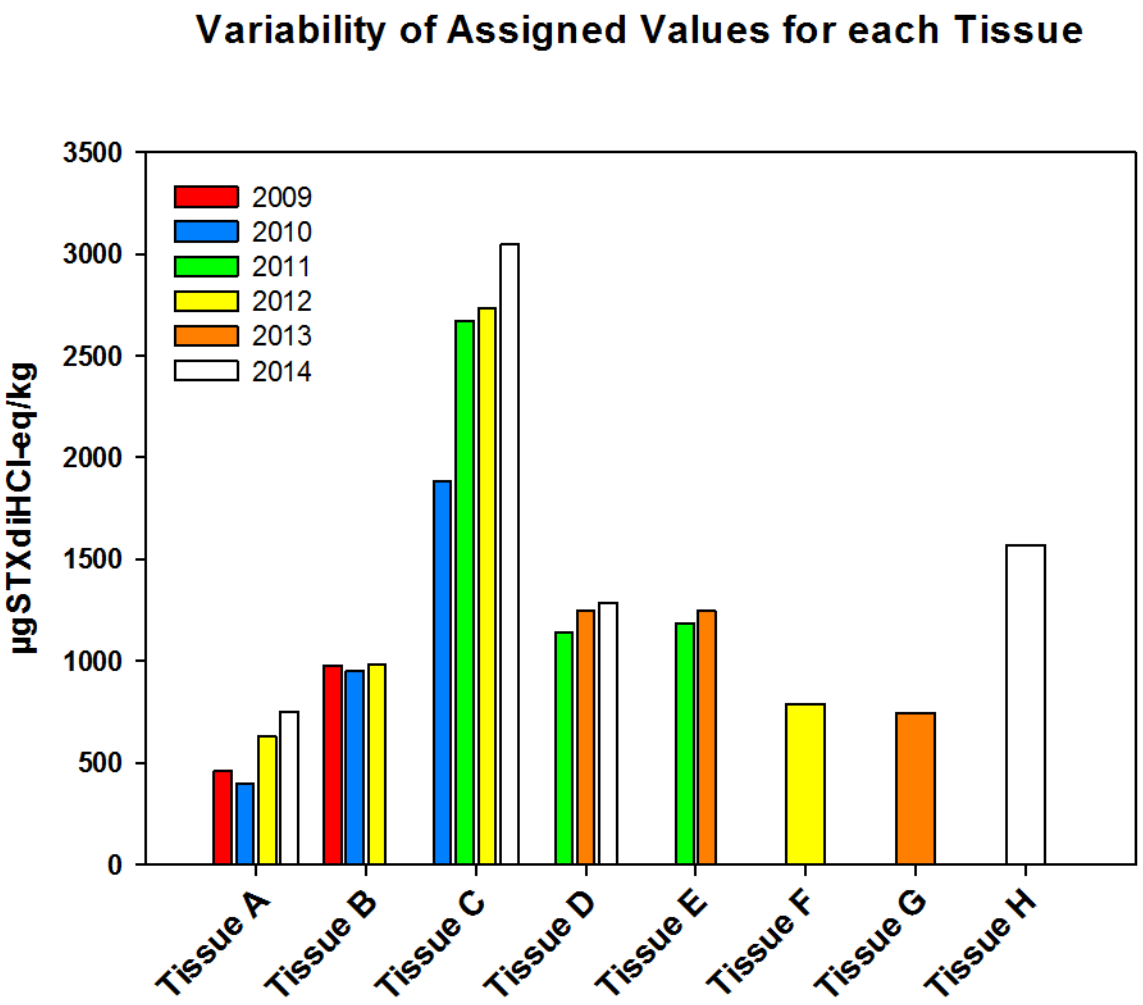


Figure 6-4: Assigned values determined in multiple rounds for Tissues A-E and in single rounds for Tissues F-H.

6.3.3 TISSUE C

This material was prepared at approximately 3 times the EU regulatory limit and although only containing 4 predominant analogues it represented a significantly more challenging toxin profile for participants using chemical based methods of analysis due to the presence of dcNEO and other decarbamoyl toxins.

Up to 22 labs submitted total toxicity results for this material in R61, 65, 69 and 2014-1 with assigned values of 1886, 2670, 2734 and 3045 µgSTXdiHCl-eq./kg respectively. The increase in the assigned value from R61 to those calculated in R65, 69 and 2014-1 can, as in

the case of Tissue A, be attributed to the change in TEF values prescribed in these later rounds. The between lab %CVs over these four rounds were 45.4, 19.4, 19.0 and 22.1% which showed significant improvements from this materials early use.

It is interesting to note that in R2014-1 (Table 6-3) the assigned values for dcGTX2 (11.20 µmol/kg) and dcGTX3 (3.07 µmol/kg), generated from PCOX users data produces a significantly higher result if summed together (14.27 µmol/kg) when compared to the assigned value for dcGTX2,3 together (10.20µmol/kg) which was generated from preCOX users only. This along with the data for GTX2 and GTX3 (see Tissues D, E, G & H) suggest that both LC-FLD methods are not comparable when determining these epimer pairs. Previous studies carried out by the EU-RLMB to extend the validation of AOAC 2005.06 to include dcGTX2,3 found low mean recoveries (53 – 59%) from spiked mussel and clam tissues using this method [139]. A single laboratory validation to refine and extend the preCOX AOAC 2005.06 method to include additional toxins also showed differences between both LC-FLD methods in determining dcGTX2,3 [120]. This may explain some of the differences found between the dcGTX2,3 results generated by both LC-FLD methods above. An interlaboratory ring trial organised by CEFAS in 2011/2012 showed statistically significant differences between both methods in determining GTX2,3 with results generated by PCOX LC-FLD ~50% higher than those determined by preCOX LC-FLD [151]. This study did not include the toxins dcGTX2,3.

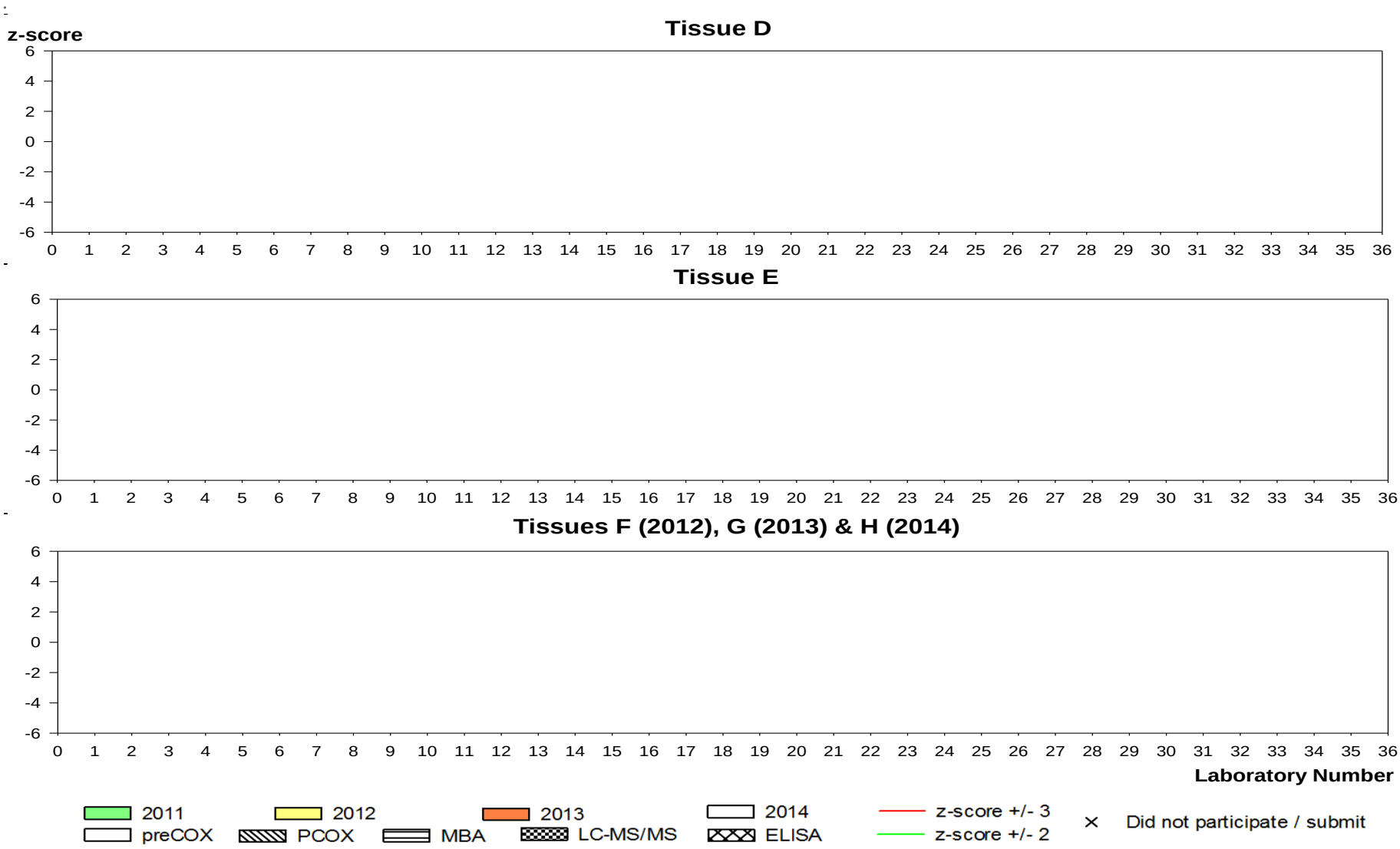


Figure 6-5: Distribution of participants' z-scores for Tissues D-F used in various rounds from 2011-2014.

6.3.4 TISSUE D

The material was prepared at approximately 1.5 times the EU regulatory limit and represented a simple toxin profile for the participants, containing just STX and GTX2,3.

Up to 21 labs submitted total toxicity results for this material in R65, 72 and 2014-1 with values of 1139, 1245 and 1289 µgSTXdiHCl-eq./kg assigned in each round respectively. The improvement in laboratory precision was again evidenced by the decrease in between laboratory CV% of 29.8, 10.6 and 16.6% observed over the three rounds.

As in the case of Tissue C the individual assigned values for GTX2 and GTX3 in 2013 and 2014 were significantly higher than those for GTX2,3 together submitted by preCOX users only.

6.3.5 TISSUE E

This material was prepared at approximately 1.5 times the EU regulatory limit and again represented a relatively simple toxin profile of only 4 predominant analogues (STX, dcSTX and GTX2,3).

Up to 19 labs submitted total toxicity results for this material in R65 and 72 with assigned values in each round of 1187 and 1246 µgSTXdiHCl-eq./kg respectively. Improvement in laboratory performance was again evidenced in the CV% decreasing from 27.0% to 16.9% over the two year period.

In 2013, where enough data was available to enable the generation of assigned values for GTX2 and GTX3 separately, these results were significantly higher than the total GTX2,3 concentrations generated by preCOX users.

6.3.6 TISSUES F, G & H

Tissues F and G were both prepared around the EU regulatory limit and Tissue H was prepared at approximately twice this level. All three tissues contained 6 predominant analogues (STX, dcSTX, GTX2,3, GTX4, NEO).

15 labs submitted data for Tissue F in R69, 17 labs submitted data for Tissue G in R72 and 22 labs submitted data for Tissue H in R2014-1. Values of 789, 742 and 1569 µgSTXdiHCl-eq./kg were assigned to each material respectively in the three rounds with between lab CV% of 38.0, 18.6 and 15.3%.

As previously seen in Tissue E the assigned values for GTX2 & GTX3 in Tissues G & H were significantly higher when summed together, than those determined as GTX2,3 together from participants reporting preCOX data.

6.4 DISCUSSION

6.4.1 IDENTIFICATION OF POOR PERFORMANCE

The source or sources of poor performance in PT schemes can be difficult to pinpoint for both the participant and organiser. While it is possible to see where an inappropriate method has been used or the application of the method has not been carried out correctly, for example ELISA determination of Tissues D and E significantly overestimating total toxicity (Figure 6-5), finding correlations between method choice and analytical performance is very difficult even with the large amount of data submitted by participants over the last six years. This is mainly due to the large number of variabilities potentially influencing PST analysis and results, for example changes to staff and associated training, reagents used, slight variations in the oxidation of toxins, and a range of other factors.

The majority of extreme z-scores ($z \geq |6|$) achieved by participants can be traced back to either gross calculation errors made during the conversion of concentration data into total sample toxicities (in some cases 2-3 orders of magnitude difference) or the misidentification of toxins present in chromatographic data. Adequate training of personnel, particularly new employees, in-house validation of methods and a comprehensive review process prior to submission of PT results is therefore of huge importance and would minimise, if not eliminate these extreme results. Experienced laboratories can also suffer from variable performance levels due to the recruitment of new staff requiring training or the implementation of new methodologies and instrumentation.

While the causes of extreme z-scores are in general easier for participants to trace and remedy, unsatisfactory z-scores ($z > |3| < |6|$) are a lot more difficult to account for. New participants to these schemes typically take two to three exercises before receiving satisfactory z-scores, the exact causes of which are unknown but are most likely because of their unfamiliarity with the submission process, reporting units requested, and analysing toxin profiles atypical of the participant's geographical location. This can be seen in Figure 6-3, particularly for laboratories 1, 2 and 4 where initial unsatisfactory z-scores received in the first round were improved in subsequent exercises. The opposite of this can also be the case however (see laboratory 5 in Figure 6-3) therefore participants have to be continually vigilant to ensure satisfactory performances are maintained.

6.4.2 Factors Affecting Poor Performance

Previous PT schemes, ring trials and interlaboratory studies for PSP toxins have highlighted issues related to each of the methods used [151,160–162]. The MBA is known to suffer from recovery issues caused by "salt" effects [160] and the presence of some metals, particularly

zinc can have a large suppressive effect on the bioassay [77] with underestimation of total toxicity particularly in samples close to the EU regulatory limit. These observations could explain some of the MBA results returned by participants in particular Tissues B-E where results were consistently below the assigned value (24 of 27 in total) with some results ~60% below the assigned value.

The choice of extraction method can also have a fundamental influence on results, with previous studies showing that under boiling HCl conditions (MBA & PCOX methods), partial hydrolysis of the N-sulfocarbamoyl toxins, GTX-5 and C-1,2 transformed them into their carbamoyl counterparts, STX and GTX-2,3 respectively [162]. It was noted however that the N-sulfocarbamoyl toxins were not present at concentrations high enough to significantly contribute to the variability associated with the dataset.

Other factors which may cause poor performance could include but are not limited to the use of inappropriate consumables, inter-batch performance of SPE cartridges or batch to batch issues with chromatographic columns which may cause retention and matrix issues.

6.4.3 CRMs

The lack of certified reference materials (CRMs), both solvent and matrix for all the PSP toxins is a problem consistently highlighted in the area, although a matrix CRM has recently been produced by CEFAS [152]. This lack of CRMs, particularly for the toxins GTX6 and C3,4, although not present in any QUASIMEME samples to date, has highlighted problems in the PT schemes delivered by the EU Reference Laboratory for Marine Biotoxins (EURL-MB) [163,164]. Participants using preCOX methods can perform a hydrolysis step converting GTX6 into NEO and C3,4 into GTX1,4 which allows an indirect means of quantification for

those toxins. Although the toxicity of GTX6 is relatively low it can still contribute significantly to the overall toxicity of some samples [87].

6.4.4 MISIDENTIFICATION OF TOXINS

The misidentification of toxins, particularly dcNEO, has been an issue for some participants and has been a source of poor performance in these exercises (see Tissue C in Figure 6-3). The dcNEO misidentification was particularly highlighted in the analysis of Tissue C. Users of PCOX methods of analysis identify this toxin as NEO due to the methods inability to separate both toxins unless a very long run time is utilised (>60mins). This is an accepted limitation of the PCOX method and presents no consumer risk in a real sample scenario owing to the 10-fold difference in TEF values between the two toxins. It can potentially lead to a significant over estimation of total toxicity owing to the differences in TEF however, and for the most part resulted in participants receiving unsatisfactory or extreme z-scores. This was not always the case however as some laboratories received satisfactory z-scores even with dcNEO misidentified, (see Lab 8 & 15 in 2011, 2012 and 2014 for Tissue C, Figure 6-3). The analysis of dcNEO can also pose problems to preCOX participants as this toxin co-elutes with dcSTX, requiring a back calculation in order to estimate toxicity. Only two preCOX participants (Lab 11 & 14) have failed to correctly identify dcNEO as being present in Tissue C. Lab 11 received an unsatisfactory z-score for this sample while Lab 14 received a satisfactory z-score as they significantly overestimated the dcSTX content in the sample, negating the fact that dcNEO was not quantified. In subsequent rounds Lab 11 correctly identified dcNEO in this sample and received satisfactory z-scores. Lab 14 did not participate in subsequent rounds. As no data is removed by QUASIMEME before assigned values and z-scores are calculated, the onus is on participants to have a comprehensive review process of

final reports and z-score results to ensure that correct identification of all toxins present in the materials has been achieved.

6.4.5 Recovery Correction Factors

The application of method recovery correction factors to results generated from preCOX analysis is an important issue and has been high on the agenda of the EURL-MB Working Group for PSP toxins over the last number of years. Only one QUASIMEME participant has submitted both non-recovery corrected (Lab 10) and recovery corrected (Lab 26) results in 2013 and 2014 (Figure 6-3 & Figure 6-4), with none of the recovery corrected results receiving satisfactory z-scores in either round. This observation is not surprising as the assigned values (from which z-scores are calculated) generated for each tissue are calculated from the group which is weighted more heavily by non-recovery correcting participants. Applying recovery correction factors prior to submission of results would need to be stipulated in future protocols in order to ensure all participants carry out this procedure.

6.4.6 Method Dependency and Individual Analogues

Finding correlations between proficiency test performance and method choice or between performance and the presence of specific analogues in test samples is difficult. For instance the plot of the population density functions in Figure 6-6 (left) clearly shows a bi-modal distribution of the data in the determination of dcSTX in sample QST132BT with the smaller mode PMF2 (circled red) resulting from PCOX users (3 Labs). The main mode of data (PMF1) was shown to arise from the remaining preCOX users, although one PCOX participant is also contained in this main mode of data. Another sample, QST133BT used in the same round also showed a bimodal distribution in the determination of dcSTX (data not

shown). It might be easy to deduce from this and the Kilt plot (Figure 6-6 right) that there is a method dependency issue between both LC-FLD methods relating to the analysis of dcSTX. This trend however, is not consistent, as the analysis of the same two samples in 2009 and 2010 showed no bi-modality for this toxin with the entire data set fitting a Gaussian distribution and results from PCOX users randomly spread throughout the entire series. Furthermore other samples used in these exercises that contained dcSTX showed no bi-modality in their distributions.

ELISA results showed variable performance levels with the only analysis of Tissue C (Lab 24, Figure 6-3) by this method producing a satisfactory z-score, whereas ELISA analysis carried out on Tissues D and E (Lab 21 & 24, Figure 6-5) produced all extreme z-scores (>6). Tissues D and E contain the toxins GTX2,3 and STX, with Tissue E also containing dcSTX. This profile is relatively simple and would represent a fairly standard North European profile so the ELISA results if not pertaining to analyst or submission errors should be further investigated.

Although LC-MS/MS has not been used extensively in PSP PT schemes to date, results generated by this method are encouraging with only one unsatisfactory result submitted to date with most receiving satisfactory z-scores. LC-MS/MS participants have also demonstrated the applicability of their methods to cover a variety of different toxin profiles with satisfactory results received from a range of different samples [165].

Although we have attempted to look for correlations between method choice and performance, no obvious or consistent patterns could be discerned from any of the PSP PT rounds completed to date.

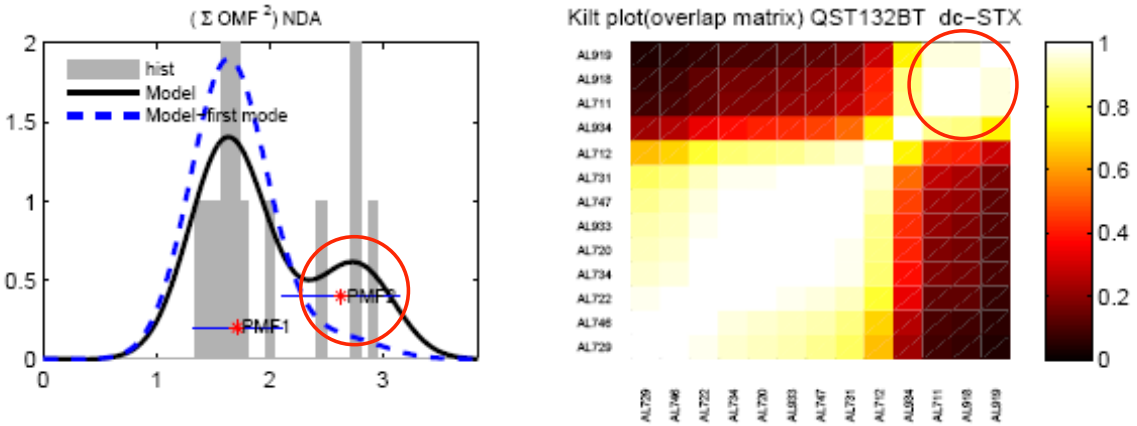


Figure 6-6: Graphical output from the Cofino model with population measurement function (left) and kilt plot (right) showing two modes of data in the analysis of dcSTX in 2012.

6.4.7 PERFORMANCE IMPROVEMENTS

Overall, the development of this exercise and the performance of participating laboratories have improved since its inception in 2009. The numbers of participants has increased each year (apart from 2012) which highlights the strengthening of the exercise and its economic sustainability going forward. The performance of laboratories has mostly improved and this is clearly evidenced from Figure 6-7 which displays the averages of both the coefficient of variation for all samples in each round and the percentage of participants receiving satisfactory z-scores. The percentage of participants receiving satisfactory z-scores has increased most years as laboratories gain experience in these exercises and in determining potentially atypical toxin profiles. The precision of the laboratories as expressed by the coefficient of variation has also showed signs of significant improvement from the early exercises in 2009 and 2010. The coefficients of variations are also comparable to other more established shellfish biotoxin exercises organised by QUASIMEME, such as exercise BT-11 for lipophilic marine biotoxins. Datasets in 2014 for BT-11 produced %CVs in the range 20 - 38%, which equated to between 45 – 80% of participants receiving satisfactory z-scores. In the same year, the PSP development exercise generated %CVs in the range 15 – 33% with 55 -71% of participants receiving satisfactory z-scores. Through adding PSP toxins to their

scope, QUASIMEME have established a comprehensive PT scheme for all EU regulated shellfish biotoxins.

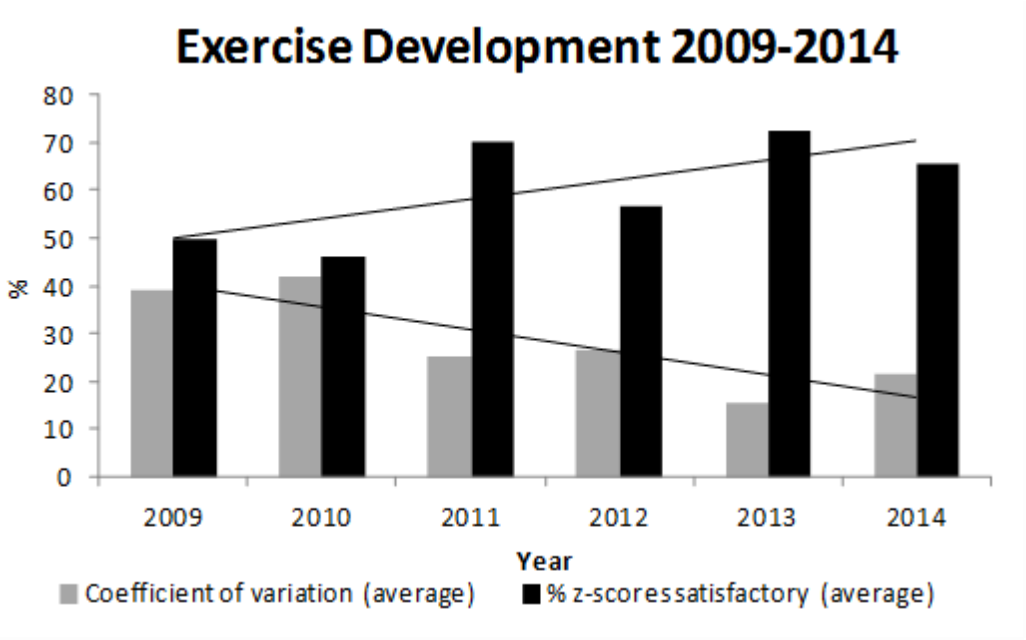


Figure 6-7: Improvements made over the duration of the PSP development exercise 2009-2014 with trend lines showing the average decrease in %CVs observed and the increase in the percentage of participants receiving satisfactory z-scores..

6.4.8 Summary Results for Individual Toxins

The graphs contained in Figure 6-8 present the data generated for the toxins STX, dcSTX and GTX2,3 and represent all results supplied for these toxins by participants. In R57, 2009, participants were requested to only submit total toxicity results so no concentrations of these analogues were available. The concentrations submitted by participants were normalised to the assigned value in that particular round such that all results could be presented in one figure.

Results for all determinants, where an assigned value was calculated, were normalised in this way and the data was further assessed as follows. The normalised data was divided into those participants using preCOX or PCOX methods of analysis and some non-valid data was

removed where participants made gross reporting errors in calculating concentration or during submission. To minimise the influence of extreme results in this further statistical evaluation, outliers were identified and removed prior to assessment (note that non-valid or extreme results are not removed in the general QUASIMEME assessment as the Cofino model is specifically designed to cope with these scenarios). The identification of outliers was carried out by applying a Dixon's test ($\alpha = 0.05$), with a maximum of one result removed from each data set.

In the case of the epimers, GTX2 & 3, PCOX participants submitted results for each individual analogue, while preCOX participants, where epimeric separation is not possible, submitted a result for the sum of the epimeric pair only. To compare the two methods, it was therefore necessary to sum the individual analogue concentrations from PCOX participants and normalise this concentration to the assigned value for the combined pair of epimers, generated from preCOX participant results only.

Comparison of both methods was only carried out on the toxins dcSTX, STX and GTX2,3 as there was insufficient data submitted by participants for other toxins present in the samples.

After the removal of non-valid data and outliers, the normalised mean and standard deviations were calculated for each method and all determinants. A paired t-test (two tailed, $\alpha=0.05$) was then performed on the data to observe if there was a statistical difference between the two LC-FLD methods in determining the concentrations of those toxins.

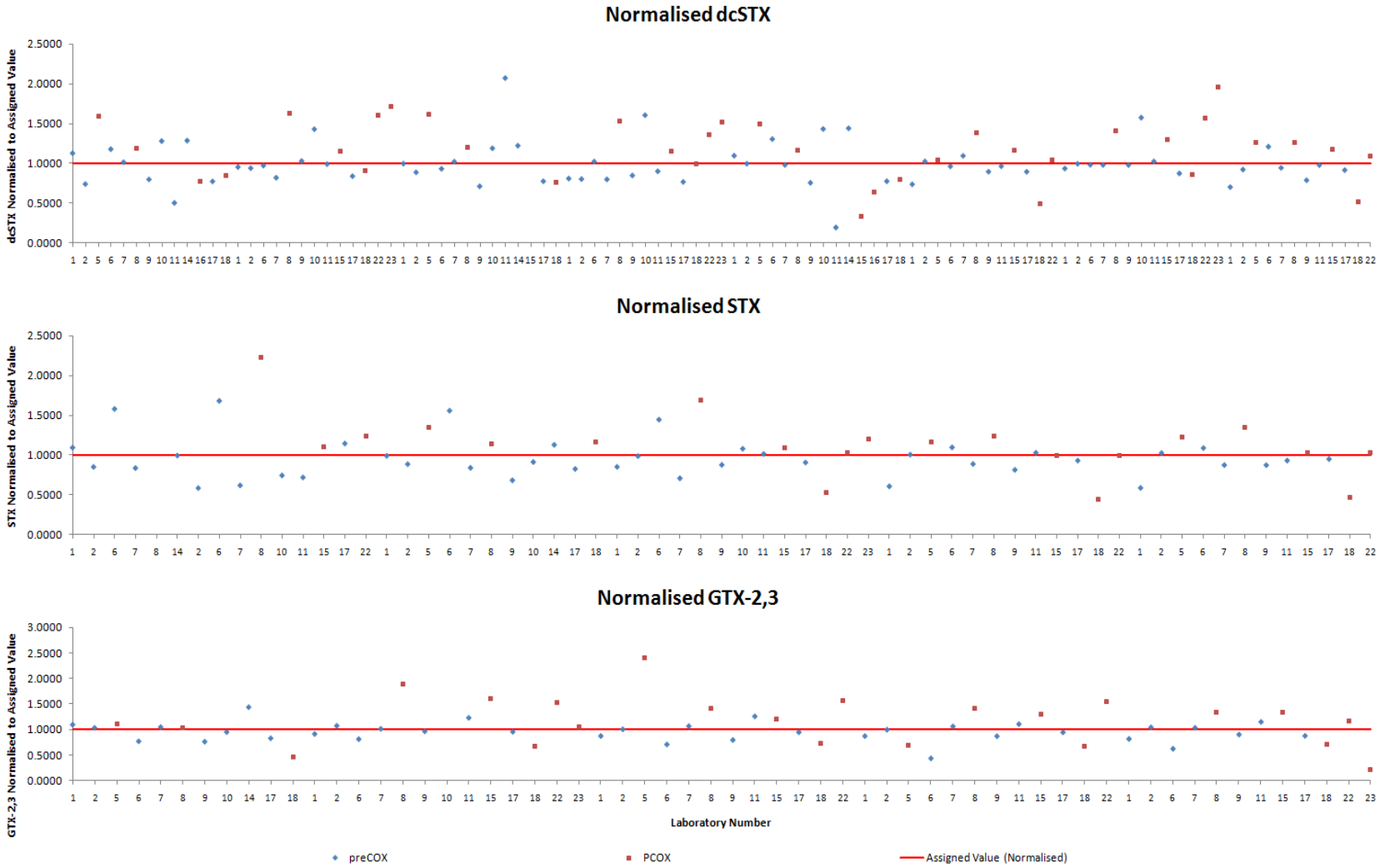


Figure 6-8: Summary data of all determinations for STX, dcSTX and GTX2,3 normalised to the assigned values in each round.

The results of the paired t-test showed there to be a statistical difference between both data sets in the case of dcSTX and GTX2,3 but not for STX. The statistical differences observed could be partially explained through the extraction techniques of both methods. As discussed earlier, partial hydrolysis of the N-sulfocarbamoyl toxins to their carbamoyl counterparts may explain the higher mean values observed from PCOX participants for the toxins GTX2,3, although C1,2 were not present in very high concentrations. If however, hydrolysis of the N-sulfocarbamoyl toxins statistically differentiates both methods for GTX2,3 determination then these differences would be expected for STX as well. Hydrolysis of GTX5 to STX would also be expected to take place, and although GTX5 was not present in high levels in these tissues, it was present at similar levels to the C-toxins.

Another major difference between the PCOX and preCOX extraction methods is the cleanup method employed. Samples extracted by the preCOX method are subjected to a minimum of SPE cleanup using C_{18} cartridges and depending on the toxin profile present may also require further SPE cleanup with carboxylic acid cartridges. This cleanup step is not carried out in the PCOX extraction method, where samples, after initial extraction, follow a deproteinisation step using trichloro-acetic acid and centrifugation. The application of the SPE cleanup steps in the preCOX method would, unless recovery factors are determined and applied, underestimate results generated using this method, owing to losses during the process. Differences between both of these methods have also been highlighted in results from previous studies by Turner *et al*. [151] and in PT schemes operated by the EU-RLMB [163,164,166].

6.5 CONCLUSIONS

Six development exercises for PSTs have been delivered since 2009 by QUASIMEME, with global participation from laboratories using a range of different methods of analysis based on

LC-FLD, LC-MS/MS, animal toxicity and immuno-based assays. Whilst no specific and consistent method dependency issues could be detected from the datasets, factors affecting poor performance were highlighted with suggestions made on how improvements could be made. Data is continually monitored by QUASIMEME through their Scientific Advisory Board (SAB) to determine issues contributing to poor performance and any method related issues. Personalised feedback to participants, particularly those with unsatisfactory performances is not carried out by QUASIMEME owing to the large scope of determinants within their remit and the practicalities of extensively reviewing so many datasets.

Chapter 7

CONCLUSIONS AND FURTHER WORK

7.1 CONCLUSIONS

The research detailed in this thesis describes investigations of various RM preparation techniques evaluated for their stabilising effects on PSTs and their applications in various internal and external QA/QC programmes.

The research began similarly to any planned RM preparation, by sourcing materials contaminated with PSTs necessary to complete the studies. Shortly after the commencement of these studies a large PSP event took place in Iceland. Large quantities of contaminated materials were harvested and used in the RM experiments but samples were also taken throughout the toxic period and analysed at the MI using various methods. As PSTs had not been previously reported from Icelandic waters the data was compiled, including phytoplankton data, and presented as a first report (*Chapter 3*). Shellfish tissues were also sourced from other MI collaborators which provided samples of various shellfish species containing an assortment of toxin profiles. Once adequate quantities of tissues had been sourced, various stabilisation techniques were evaluated to observe their effects on PST and matrix stability.

Freeze drying proved to be the most effective technique investigated, with the greatest level of PST stability demonstrated in materials prepared in this way (*Chapter 5*). Freeze drying had not previously been carried out within MI facilities so it was necessary to initially carry out a feasibility study on the technique to evaluate MI capabilities as well as confirming the stability advantages of the technique. Once the feasibility of carrying out this technique at the MI was demonstrated, all associated processing and dispensing steps were optimised. This was carried out through a series of experiments aimed firstly at gaining experience in

preparing materials in this way and secondly to confirm the equipment at the MI was suitable to prepare materials at MCs close to ~5%. Further experiments, using the optimised conditions, were then conducted, specifically to investigate issues arising from the feasibility study which related to toxin biotransformations and epimerization. Through sterilization of the source tissues prior to freeze drying these reactions were minimised. A final, relatively large scale material (>1000 aliquots), was then prepared using all the experimentally determined conditions to produce a material as a candidate CRM in collaboration with CEFAS. This material is awaiting certification and characterisation studies due to funding issues and the current availability of another PST CRM from CEFAS.

The use of preserving additives, both separately and combined were also evaluated for their stabilisation effects and both were proven to be effective in RM preparation for various PSTs, specifically GTX1,4 (*Chapter 4*). The heat treatment of tissues was also evaluated in parallel with the additives study and this technique provided excellent matrix and toxin stability for the remaining PSTs investigated in that section, namely dcSTX, STX, GTX5 and GTX2,3. The results from this study indicated that combining both techniques would produce a material with excellent stability for all the toxins studied. Materials were prepared in this way and used internally at the MI in the routine QC/QA of the NMP for PSP toxins and in a development exercise for international PT schemes operated by QUASIMEME and VEREFIN. The short-term stability, demonstrated sufficiently in these materials, was an important prerequisite for these PT materials.

The novel application of HPP in RM preparation was also investigated for the stabilising effects this technique provided (*Chapter 4*). The technique provided excellent matrix and toxin stability and was the only other technique, other than freeze drying, which could be considered for a feasibility study into CRM production. The HPP equipment used in this

section is expensive although it is available as a commercial service to paying customers. This may limit its' applicability in LRM or ILRM preparation as other techniques such as thermal treatment or the use of preserving additives provide more cost effective stabilisation techniques.

The final part of this research concerned a specific application of materials prepared through thermal treatment and spiking with preserving additives. The first commercially available PST PT scheme was operated by QUASIMEME using materials prepared using these techniques and results from these exercises were presented (*Chapter 6*). Participants used a range of different methodologies from animal based assays to instrument based methods of analysis. Participants' results were evaluated specifically to determine if trends existed in the datasets and if they related to specific methods or analogues being determined. Results suggested many factors influence participant performance and therefore z-scores so specific method or analogue dependency issues could not be definitively determined.

The studies contained in this thesis provided PST RM preparation techniques for multiple uses in LRM, ILRM and potential CRM production. Materials were used in the daily QC/QA of produce from Irish shellfish farmers and provided an excellent material for method development and validation studies. These studies were crucial in the movement away from the use of animal based assays to instrumental based methods of analysis in Ireland. The use of preserving additives or thermal treatment provides an extremely cost effective means for PST RM preparation and should be within the capacity of most monitoring or research laboratories.

7.2 FURTHER WORK

The research contained in this thesis focused on tools that can be used in statutory monitoring of PSP toxins in shellfish. The research sought to gain further understanding on the mechanisms of PST degradation, epimerization or biotransformations taking place in shellfish tissue matrices and to highlight the advantages of each technique for various RM uses. One use for these materials is in PT development exercises and participant data was evaluated over a 6 year period from schemes operated by QUASIMEME.

The findings from this research have raised some questions and have highlighted some areas that warrant further investigation such as the following.

- The continued monitoring of Icelandic shellfish for marine biotoxins including causative organisms in water samples. This is in order to produce a thorough risk assessment for the occurrence of not only PSP toxins but other toxin groups present in Icelandic waters. Data on the timing and intensity of these algal blooms will provide a more comprehensive picture on toxin accumulation in Icelandic shellfish which will aid in setting up an Icelandic monitoring programme.
- HPP treatment of different tissue matrices, particularly mussel, could be investigated to observe if the same level of stability as seen in oysters tissues is evident.
- The certification studies should be carried out on the large scale freeze dried material prepared at the end of *Chapter 5*. The stability and homogeneity of this material could be determined after which the material could be made commercially available. The certification, if not carried out using a multi method approach, could be achieved through an interlaboratory exercise (consensus of expert laboratories).
- Expand the QUASIMEME PSP exercise to include more shellfish species and toxin profiles in the programme. This will specifically involve sourcing various species

from third party suppliers or alternatively, shellfish could be fed with various species of phytoplankton to produce the different profiles required. The inclusion of toxins not covered so far in these exercises such as those predominating in shellfish from the South of Europe for example GTX6 and C3,4 should also be considered. The inclusion of these toxins could be problematic however as there are currently no CRMs available.

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BOOK CHAPTER

Stephen Burrell & Andrew D. Turner. Detection of paralytic shellfish poisoning toxins in molluscs. In: Richard Lewis and Yiu-Chung Wong (eds). Analysis of Food Toxins and Toxicants. Wiley, Oxford, UK.

CONFERENCE PRESENTATIONS

Joint WHO/FAO/WTO workshop (Action CA3A23-4), Mexico City, Mexico, April 11th – 15th, 2011. Multiple oral presentations (non-refereed).

2nd Joint Marine and Freshwater Toxins Analysis Symposium and AOAC Task Force Meeting, May 1st – 4th 2011, Baiona, Spain. Poster presentation (non-refereed).

4th Joint Marine and Freshwater Toxins Analysis Symposium and AOAC Task Force Meeting, May 5th – 9th 2013, Baiona, Spain. Oral presentation (non-refereed).

66th Irish Universities Chemistry Research Colloquium, Galway, Ireland. June 19th – 20th 2014. Oral presentation (non-refereed).

East China Sea Fisheries Research Institute, Chinese Academy of Fisheries Science, Shanghai, China, 9th Nov 2015. Oral presentation (non-referred).

Yellow Sea Fishery Research Institute, Chinese Academy of Fisheries Science, Qingdao, China, 12th Nov 2015, Oral presentation (non-referred).