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DETECTION AND THERMAL DEGRADATION OF PARALYTIC SHELLFISH POISONING TOXINS

by

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A Thesis Submitted to the Faculty of Engineering in Partial Fulfilment of the Requirements for the Degree of

Doctor of Philosophy

Major Subject: Food Science and Technology

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LIST OF SYMBOLS AND ABBREVIATIONS

PSP	Paralytic shellfish poison
LD_{50}	Lethal dose
STX	Saxitoxin
GTX	Gonyautoxin
NEO	Neosaxitoxin
dc-STX	Decarbamoyl saxitoxin
dc-NEO	Decarbamoyl neosaxitoxin
dc-GTX	Decarbamoyl gonyautoxin
do-STX	13-deoxy decarbamoyl saxitoxin
do-GTX	13-deoxy decarbamoyl gonyautoxin
ELISA	Enzyme-linked immunosorbent assay
CE-UV	Capillary electrophoresis-Ultra violet
CZE	Capillary zone electrophoresis
CITP	Capillary isotachophoresis
HPLC	High performance liquid chromatography
LC/MS	Liquid chromatography with mass spectrometry
ESMS	Electroscopy mass spectrometry
FTID	Flame thermionic detection
FID	Flame ionization detection
MWD	Minimum peak width of detection
TTP	Tangent triggering percentage
MNL	Maximum noise level
TDT	Thermal death time

ACKNOWLEDGEMENTS

At the very outset, I would like to express my deepest appreciation and many thanks to Dr. Tom Gill for his valuable guidance, encouragement, generous support and supervision. His patience and confidence in me throughout this work is gratefully acknowledged. I also would like to express my sincere thanks to Dr. R. G. Ackman, Dr. A. T. Paulson and Dr. J. E. Stewart (Bedford Institute of Oceanography, Dartmouth) for serving in my guiding committee, providing valuable suggestions and making time for me in their busy schedule. Dr. Stewart provided some toxic algal cells which were used in part of this study.

My sincere gratitude and thanks are also due to Dr. M. V. Laycock, National Research Council of Canada, Halifax for helpful discussions and generously providing me with some PSP standards and partially purified toxins used in this whole study. I wouldn't have done much work without them. Valuable suggestions from Dr. M. Quilliam and R. Richards, NRC, Halifax are also appreciated. Thanks are due to Dr. J. F. Jellette, Jellette Biotech, Dartmouth for helping me with the neuroblastoma cell bioassay.

Many thanks go to J. W. Thompson and A. Timmins for their technical support throughout my lab work, and thanks are also due to D. Singer, R. Norton and C. Hotton for their support at some points. I am also very grateful to Dr. L. R. Correia, Department of Biological Engineering for allowing me to use the computerized oil bath for heating experiments. I would like to thank Prof. N. Ferguson, Dr. P. Adams, Dr. R. Gilkie for their support and encouragement. My heartfelt thanks go to all graduate students and staff in the Department of Food Science and Technology during my studies for their moral support and making the lab environment a safe and pleasant one.

I wish to thank my loving wife Swarna for her support, patience and encouragement towards the completion of the thesis. Deepest appreciation and love for my little sons Vinod and Dilshan who missed most of my time for them. Last, but not least, I would like to thank my family for their understanding, encouragement and support.

This work was supported in part by the Natural Sciences and Engineering Research Council of Canada through a Strategic Grant awarded to Dr. Gill.

ABSTRACT

The entire research work in the present study has been divided into 5 parts, and presented as 5 chapters with different objectives and an abstract in each chapter. In the first part, a rapid qualitative method was developed for the fractionation of paralytic shellfish poisoning toxins. Periodic acid, t-butyl hydroperoxide and hydrogen peroxide were tested as oxidants, and hydrogen peroxide was found to be the most convenient and efficient oxidant for the fluorometric detection of paralytic shellfish poisoning toxins. The fluorescence could be detected after incubation of toxins at 100°C for 3-5 min. This method was more efficient than the previously published peroxidation methods which involved lengthy incubation periods or time consuming pH adjustment. Individual toxins were detected in pico molar levels.

The second part involves the use of thin layer chromatography on Chromarods-SIII with the Iatroscan (Mk 5) and a flame thermionic detector (FTID) to develop a rapid method for the detection of PSP toxins and separate major compounds. Air and hydrogen flow, detector current and scan time were found to affect the FTID response of all PSP compounds. Quantities of toxin standards as small as 1-6 ng could be detected. Among numerous solvent systems tested, a mixture consisting of chloroform: methanol : water : acetic acid (30:50:8:2) could separate C toxins from gonyautoxins (GTX), which eluted ahead of neosaxitoxin (NEO) and saxitoxin (STX), and STX did not migrate.

The kinetics of thermal degradation of PSP toxins in scallop digestive glands were studied in part 3. Scallop homogenates were heated at different pH levels (3-7) for different times at 90, 100, 110, 120 and 130°C. All toxins were sensitive to higher temperatures and higher pH values, and the kinetics of thermal destruction were qualitatively similar to the thermal destruction of microorganisms. The levels of individual toxins in the homogenate and those generated during heating could be reduced significantly by heating at 130°C at pH 6-7. Studies on the thermal degradation of PSP toxins were continued in the

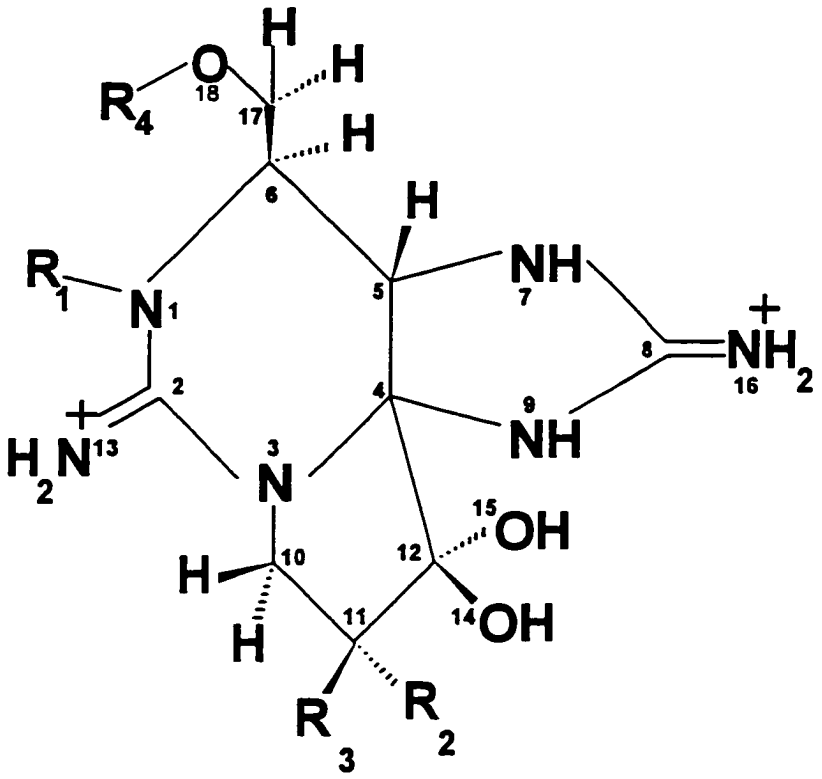
same manner using a mixture of purified and partially purified toxins, instead of homogenate, in the fourth part. Some toxin inter-conversions as well as degradation were noticed in both situations.

The fifth part of the study was focused mainly on the storage stability of PSP toxins under different conditions. Homogenates and purified toxin mixtures heated at 120°C for 1 h as well as unheated samples were stored for 1 year and 4 months, respectively, at three temperatures (-35, 5 and 25°C). There was no significant change in any toxin type when stored at -35°, regardless of pH, whereas C toxins and GTX 1/4 decreased after 4 months at 25°C. NEO and STX levels remained unchanged at all storage temperatures at low pH levels. Purified toxins used as primary analytical standards as well as partially purified toxins and toxins in homogenized matrix, sometimes used as secondary standards, can be stored safely at pH 3 and at ≤-35°C.

CHAPTER 1
INTRODUCTION

Paralytic shellfish poisoning (PSP) is a neurological disorder resulting from the consumption of shellfish contaminated with dangerous levels of one of the most toxic groups of neurotoxins known as saxitoxin (STX) and its derivatives (Fig.1.1). The STX family consists of 18 or more chemically related derivatives (Sullivan *et al.*, 1988; Hall *et al.*, 1990) which act by blocking the sodium channel in excitable membranes (Strichartz and Castle, 1990). Although the accumulation of the toxins in shellfish results from their feeding on toxin-producing dinoflagellates, they are also produced by freshwater cyanobacteria (Carmichael *et al.*, 1990), a calcareous red algae (Kotaki *et al.*, 1983) and some marine bacteria (Ogata *et al.*, 1989; Kodama *et al.*, 1990b). When bivalve shellfish such as clams, cockles, mussels, oysters and scallops ingest these toxic organisms through filter feeding, the toxins accumulate mostly in the digestive glands without causing apparent harm to the shellfish. These are some of the most potent biological poisons known to man (LD50 of STX is 10 μg/kg) (Vieytes *et al.*, 1993), and the consumption of such contaminated shellfish results in a variety of neurological symptoms that can even lead to death. In addition, the economy of fishing communities has been affected by PSP, causing a serious damage to the fisheries industry in most parts of the world.

In an attempt to protect the public from PSP incidents, many countries with shellfish fisheries have adopted monitoring programmes to detect and follow sporadic increases in toxicity, and if necessary, prohibit harvesting when the toxicity exceeds regulatory levels. The conventional mouse bioassay has been the main tool of these monitoring programmes in which the toxicity is expressed as μg STX equivalents/100g tissue. The regulatory level is 80 μg STX eq/100 g tissue, and shellfish harvesting is not permitted in Canada, Japan and many other countries when the total toxicity reaches or exceeds this regulatory level. This assay is costly, time-consuming and above all, inhumane. These factors have driven the efforts to develop simple, fast, and more socially acceptable screening assays for the detection of PSP toxins. Many alternatives including



	Molecular weight	R1	R2	R3	R4
STX	301.31	H	H	H	$CONH_2$
B1 (GTX5)	380.36	H	H	H	$CONHSO^-_3$
GTX 2	396.36	H	OSO^-_3	H	$CONH_2$
C1 (epi GTX8)	475.41	H	OSO^-_3	H	$CONHSO^-_3$
GTX 3	396.36	H	H	OSO^-_3	$CONH_2$
C2 (GTX8)	475.41	H	H	OSO^-_3	$CONHSO^-_3$
NEO	317.31	OH	H	H	$CONH_2$
B2 (GTX6)	396.36	OH	H	H	$CONHSO^-_3$
GTX 1	412.36	OH	OSO^-_3	H	$CONH_2$
C3	491.41	OH	OSO^-_3	H	$CONHSO^-_3$
GTX 4	412.36	OH	H	OSO^-_3	$CONH_2$
C4	491.41	OH	H	OSO^-_3	$CONHSO^-_3$
dc-STX	258.28	H	H	H	H
dc-GTX 2	353.33	H	OSO^-_3	H	H
dc-GTX 3	353.33	H	H	OSO^-_3	H
dc-NEO	274.28	OH	H	H	H
dc-GTX 1	369.33	OH	OSO^-_3	H	H
dc-GTX 4	369.33	OH	H	OSO^-_3	H

Fig. 1.1 Structure of PSP toxins

bioassays (housefly, fish, chick embryo, artemia, cell bioassay), immunoassays such as radioimmunoassay (RIA) and enzyme-linked immunosorbant assays (ELISA) and various chemical methods have been suggested. Immunological assays are promising, but are limited due to lack of cross reactivity of the available antibodies among the derivatives of STX whereas the most recent mouse neuroblastoma cell bioassay of Jellett *et al.* (1992) is rapidly becoming popular. Although utilitarian, most of these methods suffer from one or more disadvantages which prevented their widespread implementation, especially for regulatory work. Most of the chemical methods are more sensitive, but are time consuming and expensive. The most commonly used chemical tool for the detection of PSP toxins, high performance liquid chromatography (HPLC), involves on-line pre- or post column derivatization followed by fluorescence detection. HPLC is sensitive and reliable, but the equipment is expensive, the extraction and cleanup steps are complicated, and require skilled operations. In addition, there is a global shortage of analytical standards for the individual compounds in the STX family.

The development of quick assay methods for PSP toxins utilizing their chemical reactions and/or characteristics have centred around the development of coloured or fluorescent derivatives, the latter being the most common. Colourimetric assays based on the reaction of STX with picric acid (MaFarren *et al.*, 1958) or 2,3-butanedione (Gershey *et al.*, 1976), were subject to interferences and involved lengthy procedures. Thus, more sensitive detection methods focussed on fluorescence reactions based on toxin degradation with oxidants such as hydrogen peroxide to a pyrimidopurine (Wong *et al.*, 1971). The assay technique developed later using this mechanism was very sensitive (<1μg STX/100g tissue), and a number of investigations have been conducted over the years to utilize this method in routine shellfish toxicity monitoring. Thin layer chromatographic (TLC) methods extensively used to characterize PSP toxins sometimes also use fluorescence detection. Although this method provides only semiquantative results, it has been found to be useful for profiling the toxins produced by various strains of dinoflagellates. Various electrophoretic methods developed later based on the charge of toxin molecules also utilized the fluorescence oxidation method for the detection. Most of these chemical

detection methods involve expensive equipment, demand high levels of skill, are time consuming and some have poor sensitivities for N-1-hydroxy derivatives of STX. Thus, the first part of this study is an attempt to develop a rapid screening method for the fractionation of PSP toxins.

Structurally, PSP toxins can be divided into 4 main groups such as:

1. Toxins without sulfate conjugation (STX, NEO)
2. Toxins with single sulfate conjugation in the 11-hydroxyl group (GTX 1-4)
3. Toxins with sulfate conjugation in the side chain carbamoyl group (GTX 5-8,C3-4)
4. Toxins in which the carbamate moiety is hydrolysed undergoing decarbamoylation (decarbamoyl toxins).

According to the specific toxicities, the toxins can be divided into groups, the least toxic sulfocarbamoyl toxins (C 1-4, B1-B2), the most toxic carbamate toxins (STX, NEO, GTX 1-4), and decarbamoyl toxins (dc-GTX 1-4, dc-STX, dc-NEO) with intermediate toxicities. GTX 2 and 3 are α and β epimers, respectively, of saxitoxin 11-sulfate, and GTX 1 and GTX 4 are the α and β epimers, respectively, of neosaxitoxin 11-sulfate. The decarbamoyl toxins originally believed to be exclusively catabolic byproducts of toxin digestion in shellfish now include the recently discovered 13-deoxy-decarbamoyl derivatives (do-STX, do-GTX 2 and do-GTX 3) (Cembella, 1998), and can occur in low relative abundance in certain marine dinoflagellates (Oshima *et al.*, 1993). Although toxic algae contain most of these toxins in different quantities, mainly C toxins, the composition of the toxin profile may undergo different structural changes, perhaps converting the least toxic compounds into highly toxic compounds, in the shellfish tissues by bioconversions, after ingestion (Bricelj *et al.*, 1991). Even the toxins in fresh shellfish homogenates may undergo similar bioconversion, enzymatically, under optimum conditions such as pH and temperature (Shimizu and Yoshioka, 1981). Similarly, non-enzymatic, chemical conversions can take place by heating in mild acidic conditions, converting the least toxic sulfocarbamoyl toxins into more toxic carbamate toxins through a phenomenon known as "Proctor enhancement" (Hall *et al.*, 1980). The changes in total toxicities at different temperatures have been observed to different degrees during the past

few decades. The question arises as to how these toxins behave under such conditions as commercial canning and pasteurization.

Shellfish such as oysters, mussel, clams and cockles are pasteurized, canned and even smoked commercially to a great extent, globally. The quahog or hard clam, *Venus mercenaria*, and the soft shell clam, *Mya arenaria* are commercially canned in the East coast of Canada and America whereas the razor clam, *Machaera patula* is principally canned in the West coast. The meat of the razor clam is usually canned as minced meat. In the case of canning whole clams, without mincing, the process times vary considerably due to the fact that some clams are very tough (Lopez, 1975). Most of the bivalve species around the world are known to accumulate PSP toxins to different extents. Large amounts of toxins are accumulated by the butter clam, *Saxidomus giganteus* which was the original species used in the study of saxitoxin chemistry. Toxicities of some commercial species in the Bay of Fundy sometimes exceeds 150,000 MU/100g meat (Jamieson and Chandler, 1983).

Citric and phosphoric acids are commonly used as acidulants in the canning industry, and citric acid is used in the canning of a variety of seafoods including mussels, clams, scallops and oysters to inhibit discolouration, and the development of off-flavours and odours (Lopez, 1987). The second part of this work focussed mainly on the nature of PSP toxins during heating at different pH and temperature levels. Prakash *et al.* (1971) reported that commercial canning at pH 6.8 could reduce the toxicity by over 75%, and there may be a further decrease during storage after canning.

The entire work in the present study has been divided into 4 main sections each with its own objectives. The main objective of the first part was to develop a rapid detection method for the screening of large numbers of column fractions in a relatively short period of time. It was also desirable that the detection method would require a minimal sample volume.

The second part is also an attempt to develop a novel method for the detection of PSP toxins, and the objectives of this part are to examine the feasibility of using thin layer chromatography with the Iatroscan (Mark 5) flame thermionic detector (FTID) for the

detection of PSP toxins. There was also an attempt to identify the major PSP compounds by thin layer chromatography (TLC) in combination with FTID.

Thirdly, the thermal degradation of PSP toxins in scallop homogenates and in buffers (without a matrix) was studied with the objective of gaining knowledge on the effect of a wide range of pH levels (3-7) on the kinetics of thermal destruction of individual PSP components in scallop homogenate.

Finally, in the fourth section, a storage study was carried out using heated and unheated scallop homogenates, as well as mixtures of partially purified toxins. Toxins were stored at 3 different temperatures and the changes in these individual compounds were monitored chromatographically.

CHAPTER 2
LITERATURE REVIEW AND OBJECTIVES

2.1 Toxin Producing Organisms

STX was first isolated from the Alaskan butter clam, *Saxidomus giganteus* which showed year-round toxicity (Schantz *et al.*, 1957). Toxins with the same structure and chemical nature were later isolated from mussels exposed to red tide caused by *Gonyaulax catenella* blooms and the cultured cells of *G. catenella* (Schantz *et al.*, 1966). STX was believed to be the sole toxin involved in paralytic shellfish poisoning for almost two decades although Schantz (1960) observed that these toxins could not be isolated from scallops on the Canadian East Coast. Shimizu *et al.* (1975) isolated four new toxins called Gonyautoxins, from soft-shell clams exposed to a red tide caused by *G. tamarensis*. Buckley *et al.* (1976) indicated the presence of two new toxins in a similar sample. The toxins were later known to be produced by a completely different dinoflagellate, *Pyrodinium bahamanse* var. *compressa* (Kamiya and Hashimoto, 1978; Harada *et al.*, 1982a,b). These organisms were considered responsible for PSP in South Pacific waters (Kamya and Hashimoto, 1978; MacLean, 1979). Jackim and Gentile (1968) reported that the toxin extracted from the fresh water blue green algae, *Aphanizomenon flos. aquae*, resembles STX. "Aphantoxins", the toxins extracted from these blue greens were later identified as saxitoxin analogs (Shimizu *et al.*, 1977; Alam *et al.*, 1978, Ikawa, *et al.*, 1982).

Later, strains of different algae in the waters of New Hampshire were found to produce NEO and STX (Ikawa *et al.*, 1982. Shimizu *et al.*, 1984c, Shimizu, 1988). Calcareous macro red algae belonging to *Jania sp.* were found to contain GTXs and STX (Kotaki *et al.*, 1983) (Table 2.1).

The random and sporadic occurrence of these specific toxins among distantly related organisms has been a puzzling question to many scientists and recent systematic studies indicate that the toxigenicity of *Gonyaulax* is an intrinsic property of certain strains or subspecies. Oshima *et al.* (1993a) indicated that *Gymnodinium catenatum* isolates

Table 2.1 - PSP toxins producing organisms

Name	Occurrence	Toxin
Gonyaulax (=*Protogonyaulax*, *Alexandrium*)		
G. tamarensis (=*G.excavata*, *A.excavatum*		STX (Shimizu, 1988)
G. tamarensis var. *excavata*)	Worldwide	C's, GTX's, NEO (Shimizu, 1988)
G. catenella	Worldwide (especially West coast of North America)	
G. acatenella	N. America (West coast)	STX (Shimizu, 1988)
Protogonyaulax cohorticula	Thailand	GTX 1-4, NEO, STX (Kodama *et al.*, 1993)
A. minutum	Europe, New Zealand, Australia	NEO, STX (Chan *et al.*, 1997)
A. fundyense	Atlantic Canada	GTX's, NEO, STX, C's
A. fraterculus	Japan	
A. tamiyavanich, A. cohorticula	Thailand	GTX's, STX (Wisessang *et al.*, 1991)
A. ostenfeldii	Atlantic Canada, Spain	C1/2, B2, GTX 2/3, STX, NEO (Cembella, 1998)
Pyrodinium bahamanse var. *compressum*	South Pacific	NEO, STX, B1, B2, dc-STX, dc-NEO (Usup *et al.*, 1994)
Gymnodinium catenatum	Central and South America	B1, B2, C1-4 (Cembella, 1998)
Aphanizomenon flos-aquae	Fresh water	NEO, STX, Aphantoxin (Shimizu, 1988)
Lyngbia wollei	Freshwater	GTXs, dc-STX (Shimizu, 1996)
Oscillatoria morigeoti	Freshwater	GTX's, dc-STX (Shimizu, 1996)
Anabaena circinalis	Freshwater	C1/2, GTX 2/3, STX
Jania sp.	Tropical, sub-tropical red algae	STX, NEO, GTX's

collected from different countries have the same toxin profile which include mainly C toxins (C1-4) and GTXs 5 and 6. Studying the genetic differentiation among *Alexandrium* populations from Eastern Canada, Cembella and Destombe (1996) suggested that they are heterogeneous in their respective toxin profile. Also, there is a wide biogeographical variation in the toxin profile and toxicity (Cembella, 1998).

2.2 Intoxification of PSP Toxins

Paralytic shellfish poisoning was originally believed to occur in shellfish which accumulated toxins by filter feeding toxic plankton during blooms (Sommer and Meyer, 1937). Grazing on the toxic dinoflagellates, copepods accumulate the toxins and transfer them to other organisms in higher trophic levels through the marine food web (Teegarden and Cembella, 1996). These toxins are accumulated in the hepatopancreas of shellfish where toxicity is normally found. Bricelj *et al.* (1993) reported that there was no detrimental effect on the growth of the shell, but the tissue growth of shellfish was reduced after the ingestion of toxic dinoflagellates. However, some clams such as the Alaskan butter clam, *S. giganteus* were found to contain toxins even though there were no apparent plankton blooms. The toxicity which is mostly localized in the siphons, did not disappear even after a year in uncontaminated sea water (Schantz and Magnusson, 1964). PSP toxins were later found in non-filter feeding snails (Yashumoto and Kotaki, 1977; Kotaki *et al.*, 1981), crabs (Hashimoto *et al.*, 1967; Noguchi *et al.*, 1969; Shiomi *et al.*, 1982; Yashimoto *et al.*, 1981, 1983b; Daigo *et al.*, 1985), finfish (Anderson and White, 1989) and even in lobsters (Lawrence *et al.*, 1994; Watson-Wright *et al.*, 1991). Although toxic microalgae or the secondary transfer of the toxins through the food chain were originally suggested as the points of PSP origin, large amounts of the toxins have been found in the absence of toxic shellfish or plankton, and Shimizu (1988) indicated that there seemed to be a third mechanism of toxification of these organisms.

2.3 Food Web Transfer

It is now well known that PSP toxins are routinely taken up by a variety of marine organisms during blooms and transferred to others with massive kills of marine fish. Contamination of fish with these toxins has led to the death of seabirds (Anderson and White, 1989) and many types of fish. Studies with herring, menhaden, pollack, flounder and salmon show that these fish are sensitive to *Alexandrium* toxins and their oral and intraperitoneal dose responses to the toxins are similar to those for warm blooded animals (White, 1981). The oral LD_{50} for fish is 400-750 µg/kg whereas for terrestrial mammals (mice, rats, rabbits and cats) range from 200 to 600 µg/kg. Humans however, appear to be much more sensitive than this and rough estimates of the minimum lethal oral dose is found to be as low as 7 to 16 µg/kg (Schantz *et al.*, 1975) However, toxin levels as high as 150,000 µg toxins/100 g viscera have been reported from Bay of Fundy scallops in Eastern Canada (Jamieson and Chandler, 1983). Atlantic mackerel are apparently less sensitive and can accumulate levels as high as 40-209 µg/100 g toxins in the liver without any mortality. Consumption of migratory fish such as mackerel, herring, silver hake, capelin, butterfish as well as some squids containing PSP toxins in the viscera, resulted in the mortalities among dolphins, whales and seals (Anderson and White, 1989).

Chu *et al.* (1998) demonstrated the transmission of PSP toxins to the carnivorous edible gastropod, *Babylonia areolata* after eating the purple clam, *Hiatula dipos* which had ingested toxic dinoflagellate, *Alexandrium minutum*.

2.4 Toxin Biotransformation and Inter-conversions

The toxin profile in bivalve tissues can vary significantly from that of the toxic dinoflagellates ingested (Asakawa *et al.*, 1995). Changes in the toxin profile of shellfish tissues may arise from selective retention or elimination of individual toxins, epimerisation, or from a variety of biotransformation processes such as conversions in the presence of natural reductants, hydrolysis at low pH, or enzymatic conversions (Bricelj and Shumway, 1998a).

Clams (*Mercenaria mercenaria*) intoxicated with *A. tamarensis* GtL122, a strain rich in low potency C toxins showed a significant reduction of C1/2 toxin levels with a simultaneous increase in the proportion of GTX 2/3 and *de novo* appearance of STX (Bricelj *et al.*, 1991). Significant relative enrichment in STX also occurred in tissues of clams fed *A. fundyense* GtCA29, a species rich in carbamate toxins. Bivalves are extremely effective in converting the weakly toxic sulfocarbamoyl toxins (C1/2) present in the ingested dinoflagellates to high levels of dc-GTXs through biotransformation (Bricelj *et al.*, 1990, 1996 a and b; Lassus *et al.*, 1994; Bricelj and Shumway, 1998).

The metabolic transformations of PSP toxins in littleneck clams (*Protothaca staminea*), mussels (*Mytilus edulis*) and butter clam (*Saxidomus giganteus*) were studied by Sullivan *et al.* (1983) by incubating purified toxins in tissue homogenates at 20°C for up to 24 h. Enzymatic transformations of B1, C1 and C2 toxins into STX, GTX2 and 3 were observed.

The metabolic transformations occurring in the littleneck clam involved hydrolysis of the carbamate group from either the N-sulfocarbamoyl or carbamate toxins such as GTX and STX. Chemically, mild acid hydrolysis (0.2N HCl at 100°C) effectively removed the sulphur groups from C1 and B1 yielding the carbamate toxins. The authors confirmed that the metabolic transformations of the PSP toxins occurred in the littleneck clams involved enzymatic decarbamoylation. In addition, decarbamoyl derivatives of the PSP toxins can be prepared chemically by strong acid hydrolysis (with 7.5N HCl).

Harada *et al.* (1983) were the first to report the natural occurrence of dc-STX in tropical dinoflagellates, bivalves, coral reef crabs and calcareous alga, *Jania sp.* dc-STX was also found in the Atlantic surf clam, *Spisula solidissima* (Cembella *et al.*, 1993).

2.5 Anatomical Distribution of Toxins

The toxin levels in different parts of the same shellfish vary considerably among different species (Bricelj and Shumway, 1998). The anatomical distribution of toxins is of particular interest for those bivalves species of commercial interest. The scallop digestive gland, stomach complex and viscera contain by far the greatest proportion of the toxin,

whereas, contractile tissues (adductor muscle, pallial muscle, muscular foot) contain only a low proportion of the toxicity (Cembella *et al.*, 1994). The prominent foot of the surf clam *S. solidissima*, a sushi delicacy, also contains toxins. The toxicity of the adductor muscle in scallop which is consumed traditionally, contains far less PSP than the digestive gland (Shumway and Cembella, 1993), and rarely exceeds the regulatory levels, even during the bloom period.

2.6 Toxicity

2.6.1 Mechanism of Action

The molecular level mechanism of the toxic activity of STX was first described by Hille (1975) who proposed a model ("plug model") in which the two sodium channel-specific toxins, tetrodotoxin and STX, block the flow of sodium ions by plugging the channel.

All toxins produced by *Protogonyaulax* block the inward flow of Na^+ ions without affecting the resting membrane potential or K^+ channels. Neurotoxins that alter sodium channel function, act at several receptor sites (Catterall, 1985). However, the nature of the chemical interaction between a toxin molecule and the excitable membrane remains largely unknown. The primary assumption is based on the common presence of a cationic guanidium moiety and the hypothesis assumes that the guanidinium group enters into the sodium channel because of electrostatic attractions between its cationic charge and fixed anionic charges in the membrane (Kao and Walker, 1982). It was thought that the rest of the molecule was too bulky to pass through the sodium channel.

Shimizu (1982) who found structural problems associated with the plug model proposed a "lid model" in which 2 hydroxyl groups and a guanidinium group make a three-point binding in the vicinity of the channel (Fig. 2.1. a). Kao (1983) presented a model in which the toxins bind to the outside of the channel entrance (Fig. 2.1.b). In both cases, ionic interaction and hydrogen bonds were considered to hold the toxin molecule in position. Based on the studies of various toxins, Strichartz (1984) proposed covalent binding in a cavity (Fig. 2.1.c). Shimizu (1988) indicated that since most molecules

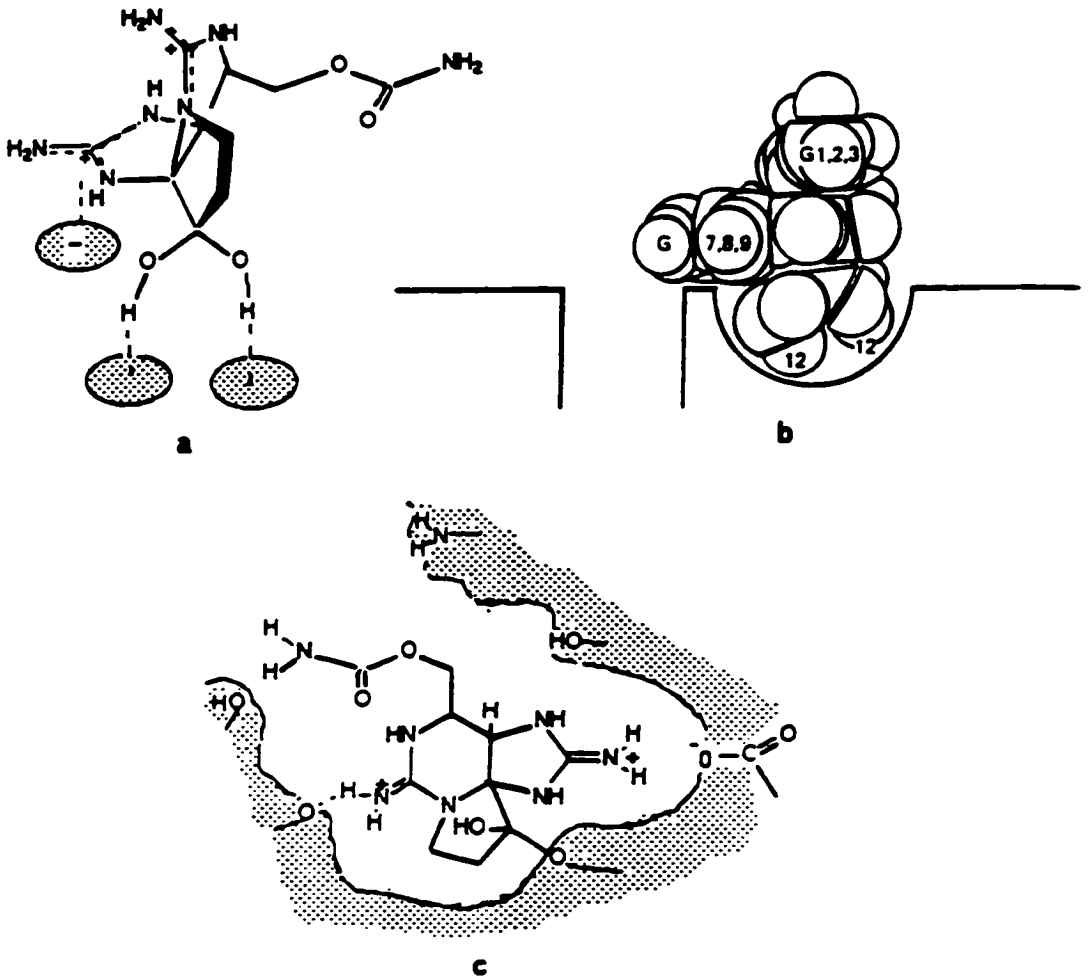


Fig. 2.1 Hypothetical saxitoxin-receptor interaction models: **(a)** Shimizu (1982) **(b)** Kao (1983) **(c)** Strichartz (1984)

possess potential ketal or acetal formation sites, such a model would be an attractive alternative for consideration.

Chimi *et al.* (1986) examined the mechanism of inhibition of acetylcholine release by GTXs in the longitudinal muscle strip preparation of guinea pig ileum, and found that GTXs might suppress the impulse conduction of the post-synaptic neuron by complete inhibition of the release of acetyl choline.

In the mouse bioassay, dc-STX was about 60% as toxic as STX (Ghazarrosian *et al.*, 1976) whereas Kao and Walker (1982) indicated that dc-STX was only 20% as toxic as STX. Studying the actions of dc-STX and dc-NEO on the frog skeletal muscle fibre, Yang *et al.* (1992) described the possible binding mechanism. Four site points have been identified in the STX/NEO binding site which probably anchor the toxin molecules (Fig. 2.2). Site (a) ion pairs with the 7,8,9 guanidinium site (b) and (c) form hydrogen bonds with the C12 hydroxyl groups (gem-diols) and site (d) hydrogen bonds with the N-1-OH of NEO.

Individual components of PSP have different toxicities. The toxicity of PSP toxins is traditionally expressed in mouse units (MU) where one mouse unit is defined as the amount of toxin required to kill a 20 g mouse within 15 min by interperiotoneal injection (AOAC, 1975). Saxitoxin, for instance, has a specific toxicity of 5500 MU/µg or 2045 MU/µmol, whereas the specific toxicities of other toxins vary depending upon the researchers mainly due to the differences in the assay conditions. Based on the toxicities reported by Genenah and Shimizu (1981), Wichmann *et al.* (1981) and Kohen *et al.* (1982), Sullivan *et al.* (1985) reported the toxicities for most of the individual PSP components (Table 2.2). The specific toxicity of NEO is still a matter of controversy and different authors have reported different values (2000 MU/ng, Genenah and Shimizu, 1981; 3900 MU/mg, Daigo *et al.*, 1985; 5900 MU/ng (as dihydrochloride), Yang *et al.*, 1992; 6708 MU/mg, Hall, 1982). B1 and B2 were found to have toxicities of 150 and 180 MU/µmol which, following hydrolysis increased to 2400 and 2900 MU/µmol (Koehn *et al.*, 1982)

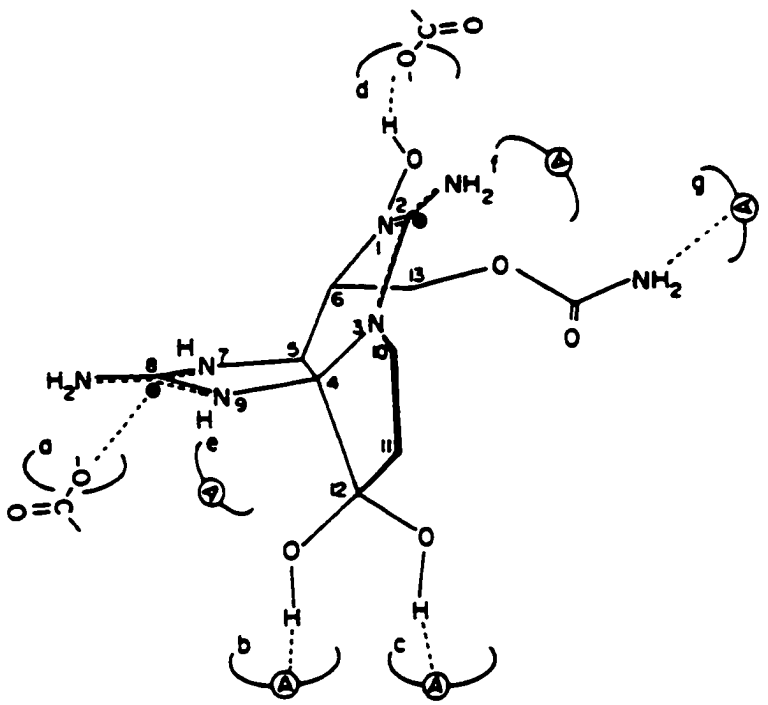


Fig. 2.2 Structure of NEO and deduced complementary site-points in receptor, which probably anchor toxin molecule to sodium channel protein in productive docking orientation. In the case of STX, there would be no bonding of N-1 group to site 'd' which is probably a deprotonated carboxylate function. Site 'a' forms an ion-pair with the cationic 7, 8, 9 guanidinium group; others are hydrogen acceptors, depicted as 'A' (Yang *et al.*, 1992)

Table 2.2 Specific toxicities of PSP toxins

Toxin	Toxicity (MU/μmole)	Toxin	Toxicity (MU/μmole)
STX	2100	dc-STX	900
NEO	2300	dc-NEO	900
GTX1	1900	dc-GTX1	950
GTX2	1000	dc-GTX2	380
GTX3	1600	dc-GTX3	380
GTX4	1900	dc-GTX4	950
GTX5	354		
B1	150		
B2	150		
C1	17		
C2	258		

2.6.2 Regulatory Levels

As of the 1st of January 1991, at least 21 countries have actual or proposed regulations for marine phycotoxins (Van-Egmond *et al.*, 1991). These regulations may involve environmental surveillance for toxic algal species and/or legal action to ensure that phycotoxin-contaminated fish and shellfish do not reach the consumer. Actual or proposed tolerance levels for PSP toxins in shellfish are typically: 400 MU/100g or 40-80 μg STX eq/100 g or 40-80 μg STX/100 g. Most countries including the U.S., Canada and Japan set the closure levels as 80μg STX eq/100 g whereas it is 40 μg STX eq/100 g for Germany (FDA, 1996; Jellett, 1993). It is also interesting to note that the minimum detectable limit for the mouse bioassay is 40 μg/100 g which would mean that this technique could not be used for regulatory purposes in Germany.

2.7 Isolation and Purification

Various isolation and purification procedures using column chromatography have been reported (Sommer *et al.*, 1948; Schantz *et al.*, 1957; Mold *et al.*, 1957). STX obtained from Alaskan butter clams were purified using weakly basic Amberlite IRC 50 and alumina chromatography (Schantz *et al.*, 1957). However, this isolation method was

not applicable to the other PSP toxins, which are not strongly basic. Shimizu *et al.* (1975) developed a procedure to separate toxins from other ingredients by selective adsorption on Bio-Gel P-2 or Sephadex G-15 and the toxin fractions were eluted in a dilute acetic acid solution followed by the application of these fractions to a column of a weak acidic carboxylic acid resin, Bio-Rex-70 in acid form. The pure toxins can be obtained in the reverse order of the net positive charge of the molecule, using acetic acid as eluant (Oshima *et al.*, 1977). However, the toxins with net negative charge could only be separated by either preparative thin layer chromatography or careful chromatography on Bio-Gel P-2. Individual PSP toxins purified by these methods were listed with synonyms in Table 2.3.

Alfonso *et al.* (1993) isolated and purified PSP toxins from the mussel, *Mytilus galloprovincialis*, in preparing of mixtures of PSP toxin standards. Simple and rapid isolation and purification procedures for the preparation of PSP toxins standards using toxic dinoflagellates, *A.excavatum*, a freshwater cyanobacterium, *Aphnizomenon fos-aquae* and scallop hepatopancrease were reported by Laycock *et al.* (1994). Compounds not commonly available were prepared by *in vitro* chemical interconversions of naturally occurring common compounds (Laycock *et al.*, 1995, 1997).

2.8 Structures and Chemical Properties

2.8.1 STX

The chemistry of PSP toxins has been studied by many authors and a tentative structure was presented by Wong *et al.* (1971). The STX molecule consists of perhydropurine skeleton with an additional five-membered ring fused at the angular position (Shimizu, 1988) (Fig.1.1). STX exists in an equilibrium of 3 molecular species: divalent cation, monovalent cation of the hydrated form, and monovalent cation in the keto form (Shimizu *et al.*, 1981). The preparation, spectral characterization, chromatographic and biological properties of several derivatives of STX including decarbamoylsaxitoxin and decarbamoylsaxitoxinol were reported by Koehn *et al.* (1981).

Table 2.3 Synonyms and abbreviations of PSP toxins commonly appearing in the literature

Compound name	Abbreviation	Semisystematic name
Saxitoxin	STX	saxitoxin
		11-hydroxyneosaxitoxin-11-O-sulfate (Scantz *et al.*, 1957)
Neosaxitoxin	NEO, neoSTX	N-1-hydroxysaxitoxin (White *et al.*, 1986; Indrasena and Gill, 1998)
Gonyautoxin 1	GTX 1, GTX_1, GTX-I	11α-hydroxyneosaxitoxin sulfate (White *et al.*, 1986)
Gonyautoxin 2	GTX 2, GTX_2, GTX-II	11α-hydroxysaxitoxin sulfate (White *et al.*, 1986)
		11-hydroxysaxitoxin (Shimizu *et al.*, 1975)
Gonyautoxin 3	GTX 3, GTX_3, GTX-III	11β-hydroxysaxitoxin sulfate (White *et al.*, 1986)
		11 hydroxysaxitoxin (Shimizu *et al.*, 1975, Buckley *et al.*, 1976)
Gonyautoxin 4	GTX 4, GTX_4, GTX-IV	11β-hydroxyneosaxitoxin sulfate (White *et al.*, 1986)
		11-hydroxyneosaxitoxin-11-O-sulfate (Shimizu *et al.*, 1975)
Gonyautoxin 5	GTX 5, GTX_5, GTX-V,B1	21-sulfosaxitoxin (White *et al.*, 1986)
		carbamoyl-N-sulfosaxitoxin (Oshima *et al.*, 1977)
Gonyautoxin 6	GTX 6, GTX_6, GTX-VI, B2	21-sulfoneosaxitoxin (White *et al.*, 1986)
		carbamoyl-N-sulfoneosaxitoxin (Oshima *et al.*, 1977)
Gonyautoxin 7	GTX 7, GTX_7, GTX-VII	decarbamoylneosaxitoxin (Hsu *et al.*, 1979)
Gonyautoxin 8	GTX 8, GTX_8, GTX-VIII, C2	21-sulfo-11β-hydroxysaxitoxin sulfate (White *et al.*, 1986)
		carbamoyl-N-sulfogonyautoxin-IV (Shimizu, 1980; Hall *et al.*, 1980)
Epigonyautoxin 8	C1, epi GTX-VIII	21-sulfo-11α-hydroxysaxitoxin sulfate (White *et al.*, 1986)
		carbamoyl-N-sulfogonyautoxin-II
C3		21-sulfo-11α-hydroxyneosaxitoxin sulfate (White *et al.*, 1986)
		carbamoyl-N-sulfogonyautoxin-I (Hall *et al.*, 1980)
C4		21-sulfo-11β-hydroxyneosaxitoxin sulfate (White *et al.*, 1986)
		carbamoyl-N-sulfogonyautoxin-IV (Hall *et al.*, 1980)

STX was found to be the most stable of all PSP compounds and can be stored in dilute HCl acid solution for years without loss of its potency. The hydrolysis of the carbonyl ester can occur in concentrated acid solutions such as 7.5N HCl at 100°C, whereas this toxin is extremely unstable under alkaline conditions, especially in the presence of oxygen. Biologically active dc-STX which has about 70% of the original toxicity can be prepared by the chemical modification of STX (Ghazarassian *et al.*, 1976). dc-STX was found to naturally occur in tropical dinoflagellates and bivalves (Harada *et al.*, 1983), whereas Lucas (1989) reported the occurrence of dc-STX in canned mussels.

2.8.2 NEO

Although this compound was first isolated from Alaska butter clam, it was later found to be a major component in most toxic shellfish, dinoflagellates, blue-green algae, and crabs (Shimizu, 1988). The imidazoline guanidine of NEO has a pK_a value similar to that of STX (8.65). However, the net positive charge of the molecule around physiological pH is reduced to about half of saxitoxin's (Fallon and Shimizu, 1977).

Although NEO was not the major toxic component in algae in the Atlantic region, it was proven to be a major component in most toxic shellfish, dinoflagellates, blue-green algae and crab samples. Spectroscopic and chemical evidence indicated that the structure of NEO would be the 1-N-hydroxysaxitoxin (Shimizu *et al.*, 1978b, Shimizu, 1988). The reduction of NEO with zinc and acetic acid resulted in the reductive cleavage of the N-hydroxyl group to give rise to STX and it was found to decompose in strong hydrochloric acid solutions.

2.8.3 GTX-1 and 4

Shimizu *et al.* (1975) first isolated these toxins from soft-shell clams exposed to *G. tamarensis* and later it was found to be present as a major component in many PSP samples. Reduction of GTX-1 gives a mixture of NEO and GTX-2 which can be further reduced to STX (Shimizu, 1988) and similar reductive bio-transformation was observed in scallop tissues (Shimizu and Yasumoto, 1981). GTX-4 is the 11-epimer of GTX-1.

2.8.4 GTX-2 and 3

The structure of GTX-2 was extensively studied by chemical degradation and spectroscopic methods (Shimizu *et al.*, 1976). On the basis of chemical and spectroscopic data, the structure of GTX-2 was initially proposed as 11α-hydroxysaxitoxin and its isomer, the 11β-epimer, GTX-3 epimerizes even near neutral pHs (Shimizu, 1984). At equilibrium, the ratio of GTX-2 to GTX-3 is 7:3 (in a solution of neutral or higher pH). These two compounds are always found concurrently, and acid hydrolysis resulted in STX by reductive elimination of the sulfonyloxy group.

2.8.5 GTX-5 (B1)

This toxin was found to be the carbamoyl-N-sulfate of neosaxitoxin and is the major toxin in the tropical dinoflagellate, *P. bahamese var. compressa* (Harada *et al.*, 1982a). This low toxic compound may be hydrolysed with a weak acid to produce highly toxic NEO.

2.8.6 GTX-6

This compound was found to be the carbamoyl-N-sulfate of STX and identical with B2 (Shimizu *et al.*, 1984b), and brief treatment of this toxin with dilute mineral acid liberates the STX by the cleavage of N-sulfate bond.

2.8.7 GTX-8 (C2)

This was found to be carbamoyl-N-sulfonyl-gonyautoxin-3 which can easily isomerize to epigonyautoxin-8 (C1) which is sulfonylgonyautoxin-2. GTX-8 can easily be converted to GTX-3 by mild acid treatment (Shimizu, 1988).

2.8.8 C3-C4

These two latent toxins initially found in *Gonyaulax* strains could be converted to more potent toxins by mild acid treatment (Shimizu, 1984). The structures of C3 and C4 were speculated to be sulfonyl derivatives of GTX1 and 4, respectively. Therefore, both

toxins easily release free GTX1 and 4 upon treatment with dilute acids.

2.9 Chemical Conversions

The chemistry of PSP toxins has been studied extensively, and many authors demonstrated the possibility of converting one compound to another under specific reaction conditions (Koehn *et al.*, 1981; Shimizu, 1988; Shimizu, 1984; Desbiens and Cembella, 1997; Nagashima *et al.*, 1991; Laycock *et al.*, 1993, 1995). Shimizu (1984) indicated that mild acid hydrolysis of C2 toxins resulted in the liberation of 1 mole of inorganic sulfate and GTX3. C2 easily isomerizes at slightly elevated pH and forms an equilibrium mixture of C2 and 11-α-isomer, C1 which could be converted to GTX2 under the same conditions (C2 = GTX8)

2.10 Detection of PSP Toxins

PSP toxins are detected by both assays as well as analyses. An analysis resolves the compounds of interest so that they can be quantified separately whereas an assay provides a single result which is the net effect of all substances present. There are biological and chemical assays as well as chemical analytical methods for the detection of PSP toxins.

2.10.1 Bioassays

2.10.1.1 Mouse Bioassay

Sommer and Meyer (1937) developed the mouse bioassay method for PSP toxins which became the official assay method globally (AOAC, 1980). Toxic tissue homogenates are heated with 0.1N HCl to convert low toxic compounds to their high toxic counterparts. Nagashima *et al.* (1990) indicated that the extraction with 0.1N HCl was not acidic enough to convert carbamoyl-N-sulfo component into corresponding carbamate counterparts and improved the extraction method by lowering the pH. In the mouse bioassay, mice weighing 20g are injected intraperitoneally each with 1 mL test solution of standardized pH and toxicity, and the time to death is measured. One mouse unit (MU) was defined as the amount of toxin needed to kill a 20g mouse in 15 min. Even

though this technique gave highly reproducible results, it did not give the amount of individual toxins and latent sulphate toxin of which the bioavailability, and *in vitro* toxicity may vary significantly. The method also required animals of uniform size and health which were difficult to obtain. Dose-death time curves in *ddy* strain male mice were established for STX, NEO, GTX5, a mixture of GTX1 and 4, a mixture of GTX 2 and 3, and a mixture of protogonyautoxin 1 (PX1), and PX2 (Nagashima *et al.*, 1991a). These curves clearly differed from the curves for STX derived from Sommer and Meyer (1937). Regression lines for these compounds were statistically similar to each other and were collectively expressed by an equation, Y=182x-0.22 in which Y represents the log of toxicity (MU) and x the reciprocal of death time in seconds. Thereby the toxicity (MU) would be calculated according to the following equation:

$MU = 10^{(182/t) - 0.22}$

where t = death time in seconds

Partially purified PSP toxins of scallop extracts consisting predominantly of GTX1-4 gave essentially the same regression line.

2.10.1.2 Fly Bioassay

Siger *et al.* (1984) discussed the response of the housefly to STX and contaminated shellfish as a replacement for the mouse bioassay. Ross *et al.* (1985) developed a fly bioassay method as an alternative to the mouse bioassay which involved the temporary immobilization of flies at low temperature. Flies were then injected with a minute dose of toxic test solution using a microsyringe. Although these techniques are more sensitive than the mouse bioassay, considerable technical skills are required for micro manipulations and micro injections. Moreover, this method has not been recognized officially.

2.10.1.3 Fish Bioassay

Wilson *et al.* (1975) reported on the routine use of the mosquito fish (*Gambosia affinis*) having a length of 1.5-3.0 cm to test the routine toxicity of intact and disrupted cell preparations of *Gymnodinium breve* and *G. monilata*. This method was ideally suited to detecting the presence of PSP toxins in bacterial culture supernant solutions or filtrates.

In 1992, Levin used the tropical fish *Notropis atherimoldes* for biological estimates of STX using the fish bioassay.

2.10.1.4 Tissue Culture Bioassay

Kogure *et al.* (1988) reported on the development of a tissue culture assay capable of detecting 3nM of STX or about 10^{-3} of a mouse unit. The assay uses mouse neuroblastoma cells, and is based on the observation that veratridine stimulates Na^+ influx of nerve cells and that ouabain is a specific inhibitor of Na^+-K^+ ATP-ase, which is the energy source for cellular exclusion of Na^+ ions. Jellett *et al.* (1992) modified this assay for convenience and speed by eliminating the need to count individual cells to determine the STX equivalents and instead employed a microplate reader for automated determination of absorbances of crystal violet from the surviving stained neuroblastoma cells. The lower detection limits were found to be approximately 10 ng STX equivalents per mL of extract (2 μg STX eq/100 g tissue). This assay was successfully used for the toxicological evaluation of STX, NEO, GTX 2/3 and dc-STX (Jellett *et al.*, 1995).

2.10.1.5 Competitive Displacement Assay

Davio and Fontela (1984) developed an assay to detect STX, by competitive displacement of [^{3}H]-STX from its receptor in rat brain membranes. The assay has a sensitivity of 0.15 ng/mL, and can be used to detect as little as 0.5 ng/mL in unextracted human plasma.

Sodium channels obtained from rat brain membrane preparations were coated on microfilter plates and used to develop a solid-phase radio-receptor binding assay by Vieytes *et al.* (1993). The tritiated sodium channel blocker saxitoxin [^{3}H]- saxitoxin; STX

was used to detect PSP by measuring the competitive displacement of other toxins. According to this method a total bound activity not lower than 70 pmol/mL was adequate to obtain a suitable signal.

2.10.1.6 Radio Immunoassays (RIA)

The immunological approaches to analyze PSP toxins were initiated by Johnson and Mulberry (1966). PSP toxins were conjugated to bovine serum albumen (BSA) and antibodies to PSP were prepared in rabbits. The antibodies were detected by positive hemaglutination and a mouse protection test (Wilson and Nakane, 1978; Chu and Fan, 1985). Monoclonal antibodies of saxitoxins were prepared by Davio *et al.* (1985).

Radio immunoassay and enzyme immunoassay techniques were developed later to access the PSP by immunological approaches (Carlson *et al.*, 1984); Cembella *et al.*, 1989). Carlson *et al.* (1984) developed a radio immunoassay for PSP using a rabbit anti-saxitoxinol antibody which is relatively specific for STX with little or no cross reactivity with NEO and other PSP toxins. Thus, this procedure has potential use for experimental studies on STX and where STX is the major shellfish toxicity problem.

Yang *et al.* (1987) also used radio immunoassay for the detection of PSP in clams and mussels. They indicated that the immunoassay developed by Carlson *et al.* (1984) had excellent potential for use as a screening method, but it could not be used reliably for shellfish containing significant levels of NEO, GTX-1 or 4.

2.10.1.7 Enzyme Immunoassays

Chu and Fan (1985) have developed a much improved immunoassay, the indirect enzyme-linked immuno-sorbent assay for STX in shellfish with high sensitivity for the parent toxins, but it cross-reacts extensively with other PSP toxins. Cembella *et al.* (1989) developed an absorption-inhibition enzyme-linked immunoassay procedure using rabbit anti-saxitoxin sensitized with STX-polyalamine (carrier) coupled with glutaraldehyde. This antibody cross-reacted with NEO, GTX-2,3 and mixtures of N-21-sulphocarbamoyl compounds. The sensitivities of ELISA methods were found to be about four orders of

magnitude more sensitive than HPLC where STX was the major toxin present. This procedure may be useful in the immunological assessment of PSP in shellfish due to broad specificity of the antibody for most if not all the PSP toxins.

Kitts *et al.* (1989) introduced a potential method for detecting paralytic shellfish poisons using antiserum against PSP contaminated shellfish and crabs. An ELISA method was developed using purified, high molecular weight protein which is referred to as saxitoxin-induced protein (SIP), obtained from crabs, *Hemigrapsus oregonesis* by Kitts *et al.* (1991). These ELISA results were confirmed by an immunoblotting procedure using anti-SIP antibody and the authors indicated that this method is fast and useful for the screening of antigen expressed in crabs as a consequence of PSP.

Usleber *et al.* (1991) developed a direct enzyme immunoassay method in a microtitration plate and test strip format for the detection of STX in shellfish. STX was coupled to horseradish peroxidase via a novel adaptation of the periodate reaction, and the detection of STX without instrumentation by visual evaluation of the test strip EIA was described. The detection limit for STX was 7 pg/mL (0.35 pg/assay) in the ELISA and 200 pg/mL in the test strip EIA using visual evaluation. The detection limits for STX in shellfish tissues by ELISA and the ELISA/test strip assay were 3 and 4 ng/g respectively.

Chu *et al.* (1992) produced and characterized antibodies against NEO. An indirect enzyme-linked immunosorbant assay in which either NEO-STX-BSA or NEO-STX-KLH (keyhole limpet haemocyanin) was coated to the micro plate, and used to monitor the antibody filter. As little as 0.5 ng/mL of either NEO or STX could be measured and the sensitivity of this ELISA method also compared favourably with that of the membrane assay in which 1 nmol (0.372 ng/mL) of PSP toxins would be measured (Davio and Fontelo, 1984). This new ELISA method was found to be more sensitive than the radio-immunoassay.

Usleber *et al.* (1996) described an approach to reducing the differences in toxin recognition with heterologous enzyme immunoassays for STX. By use of NEO-STX-HRP (horse radish peroxidase) or decarbamoyl STX-HRP as the labelled antigen instead of STX-HRP, the detection limits for NEO were improved to 100 pg/mL. However,

studying the results from 18 laboratories to analyze STX from shellfish extracts by different methods it was found that the ELISA method of Usleber *et al.* (1991) grossly overestimated the STX content in mussel extracts, probably due to cross reaction of antibodies with other PSP components.

Chu *et al.* (1996) found a method for screening of PSP toxins in naturally occurring samples with three different direct competitive enzyme-linked immunosorbant assays. A protocol using either anti-STX/STX-HRP or anti-NEO/STX-HRP was found to be most effective for screening PSP toxins in contaminated shellfish. An excellent agreement between the total PSP toxins (STX+NEO levels) was obtained when comparing the ELISA and mouse bioassays. Analysis of 15-40 naturally contaminated samples revealed that 211 (13.6.%) samples were positive by the ELISA as compared with 175 (11.3%) by the mouse bioassay.

STX was conjugated to BSA, KLH and egg albumin in the development of monoclonal antibodies of STX (Davio *et al.*, 1985). Some bioassay methods were reviewed by Levin (1992) and Hokama (1993). A novel approach to enzyme-linked immunosorbant assay screening for STX through immobilization of small molecules on solid matrices was described by Kralovec *et al.* (1995).

2.10.1.8 Microtiter Plate Assay

An isoform of the PSP toxin specific receptor saxiphilin, from the tropical centipede, *Ethmostigmus rubripes* was used as the basis for a radiometric, high-throughput, microtiter plate assay. Characterization of the assay revealed that it was able to detect several representatives from the various structural groups of PSP (Llewellyn *et al.*, 1998). The detection limits were 4 µg PSP/100 g tissue.

2.10.1.11 Channel Biosensor Assay

A tissue biosensor consisting of a Na^+ electrode covered with a frog bladder membrane integrated with a flow cell has been developed for the assay of PSP toxins (Cheun *et al.*, 1998), and the assay seems more sensitive than the mouse bioassay although

the detection limits were not given.

2.10.1.12 Other Bioassays

The chick embryo, brine shrimp (*Artemia salina*) and selected bacteria (*Staphylococcus epidermis, Micrococcus flavus,* and *Basillus subtilis*) were evaluated as alternative systems for the determination of PSP toxicity (Park *et al.*, 1986). Dose levels ranging from 0.045 to 0.3 µg STX were administered to the developing embryo through the air cell at either 0 or 96 h following incubation. Embryos dosed 96 h after incubation were the most sensitive with 100% motality at the 0.11 µg STX level. Brine shrimp larvae were sensitive to STX at a dose level of 5 µg. Limited growth inhibition was observed with the bacteria tested at concentrations of 0.0035 µg/well.

Daigo *et al.* (1989) developed a method for the detection of PSP using a lobster nerve-muscle based on the inhibitory action on the excitatory postsynaptic potential (EPSP) of a lobster nerve-muscle preparation. Smith *et al.* (1995) developed a method for the purification of STX-induced proteins (SIP). SIP are of interest because a system detecting the induction of SIP could be used to screen potentially contaminated shellfish extracts for the presence of PSP toxins, and the relative amount of SIP induced in the crab is related to the dose of STX administered.

Trainer *et al.* (1995) used reconstituted sodium channels for the detection of STX. Specific binding of the STX to the rat brain sodium channel was demonstrated using purified sodium channels reconstituted into phospholipid vesicles.

2.10.2 Chemical Assays

2.10.2.1 Colourimetric Methods

Several spectrophotometric procedures involving various colour reactions of the PSP toxins have been developed. McFarren *et al.* (1958) introduced the Jaffe test for toxin extracts. The assay is based on the reaction of STX with picric acid following cleanup of the shellfish extract on an ion-exchange resin. This test is not specific, and the lower detection limit was 100-150 ng/100g. After the modification of the Jaffe test, the

precision was much improved (McFarren, 1960).

Gershey *et al.* (1977) developed a colourimetric method for the determination of STX. This assay is based upon the reactions of 2,3-butanedione with guanidine or other compounds containing the guanidino group which are formed by the oxidation of the toxins with hydrogen peroxide under acidic conditions. This may be used for samples rich in STX and GTX2-3, but the detection limit is 0.5 μg/mL and interference may occur from arginine like compounds. In addition, the procedure was somewhat long.

2.10.2.2 Fluorometric Methods

Oxidation to a fluorescent product was first reported by Schantz (1961) who observed the formation of a fluorescent compound from STX under alkaline conditions in the presence of oxygen. This reaction was later studied by Wong *et al.* (1971) and the fluorescent compound formed by treatment of STX, with H_2O_2 in a NaOH solution was confirmed as an amino purinyl propionic acid derivative. Based on this reaction, Bates and Rapoport (1975) developed the first chemical assay method using the fluorescence of saxitoxin by alkaline peroxide (NaOH-H_2O_2). The technique involves alkaline peroxidation of STX to 8-amino-6-hydroxymethyl-2-imino purine-3 (2H)-propionic acid, the fluorescence of which is measured at pH 5. Shimizu *et al.* (1976) found that GTX-2/3, a major toxin in Atlantic PSP caused by *G. tamarensis*, afford similar fluorescent products. This assay was about 100 times more sensitive than the existing bioassay, and detection limits were about 0.30 mg STX/100 g shellfish. This method was later modified by other researchers to improve its sensitivity.

A continuous chromatographic method for the determination of individual PSP toxins using fluorescence detection was reported by Buckley *et al.* (1978). A toxin analyzer employing extraction with acidified ethanol, clean-up on Bio-Gel P2, and alkaline oxidation yielding fluorescent purine derivatives of STX and other PSP toxins was developed in this method. Shellfish with various PSP toxins were identified, however quantification of the identified toxins was not reported.

Bates *et al.* (1978) detected 0.4 µg STX/100 g shellfish tissues contaminated with PSP toxins by fluorescence oxidation with H_2O_2 after clean-up on a Bio-Rex 70 resin column. Using a strong cation exchange resin column for the study of PSP toxins, Ikawa *et al.* (1982) used a fluorescent oxidation method for the toxins chromatographed on Bio-Gel P2.

Oshima *et al.* (1984) developed a liquid chromatographic-fluorometric analysis of PSP toxins by alkaline peroxidation with t-butyl hydroperoxide which yielded highly fluorescent derivatives (Table 2.4).

A combination fluorescence assay and Folin-Ciocalteau phenol reagent assay was used by Mosley (1985) for the detection of PSP toxins. GTX 2,3 and STX were detected by H_2O_2 oxidation whereas NEO, GTX 1 and 4 were detected by Folin-Ciocalteau phenol reagent assay which involves addition of 0.375 mL Na_2CO_3 to sample aliquots of 2.5 mL, with subsequent vortexing and addition of 0.125 mL reagent followed by incubation at room temperature for 1 h. However, the results indicated that Folin-Ciocalteau reagent assay was only slightly more sensitive than mouse bioassay (0.4 MU/mL).

2.10.2.3 Thin Layer Chromatography

Buckley *et al.* (1976) used a thin-layer chromatographic-fluorometric method for the detection of *G. tamarensis* toxins isolated from soft shell clams (*Mya arenarea*). After the development of toxins in pyridine : ethyl acetate : water : acetic acid (75:25:30:15) for 1.5 h, the plates were sprayed with 1% H_2O_2 followed by heating at 100°C for 30 min, and the spots were detected by fluorometer. Although this method is sensitive, selective and works well on chromatographic fractions where considerable concentrations have been achieved, it could not be used in this form for the estimation of the residual toxins in crude shellfish extracts.

Gonyautoxins and other guanidine components separated by TLC were detected by sprays and it was found that H_2O_2 was the best for their detection (Shoptaugh *et al.*, 1978). Toxins separated by TLC after development in *n*-butanol : acetic acid : water (2:1:1) were sprayed with H_2O_2 followed by heating for 30 min at 100°C to detect 0.2,

Table 2.4 Detection limits for PSP toxins by different chemical/biological methods

Method	**Detection limit**
Chemical assays	
1. Colourimetric methods	
a. Jaffe test with picric acid (McFarren *et al.*, 1958)	~0.3 - 0.5 µmol/100 g tissue
b. Colour with 2,3-butanedione (Gershey *et al.*, 1976)	~1662 pmol STX (0.5 µg/mL)
c. Folin-Ciocalteau phenol reagent assay (Mosley *et al.*, 1985)	0.05 MU/mL
2. Fluorescence oxidation methods	
a. Alkaline H_2O_2 oxidation (Bates and Rapoport, 1975)	~5 pmol (0.3 µg STX/100 g tissue)
b. Alkaline H_2O_2 oxidation (Bates *et al.*, 1978)	~1.3 nmol STX/100 g meat
c. t-butyl hydroperoxide oxidation (Oshima *et al.*, 1976)	0.16 nmol STX (0.33 MU), 2.3 nmol NEO (2.2 MU), 0.1 nmol GTX 2 (0.08 MU), 0.04 nmol GTX 3 (0.04 MU), 0.29 nmol GTX 1 (0.84 MU), 0.70 nmol GTX 4 (0.48 MU), 0.12 nmol GTX 5 (0.016 MU)
3. Separation and fluorescence detection by TLC	
a. Alkaline H_2O_2 on TLC plates (Buckley, 1976)	~133 pmol STX (10 µg/mL)
b. Fast blue reagent on TLC plates (Proctor *et al.*, 1975)	~33 pmol STX
c. Alkaline H_2O_2 on TLC plates (Shoptaugh *et al.*, 1978)	0.7 nmol STX, 0.1 nmol GTX 2, 0.1 nmol GTX 3
4. Separation and fluorescence detection by HPLC	
a. Fluorescence oxidation with o-pthalaldehyde (Onoue *et al.*, 1983)	0.1-1 nmol GTX 1-4, NEO, STX
b. Post column periodate oxidation with 2 mobile phases (Sullivan and Iwaoka, 1983)	1.7 pmol STX, GTX 2/3, 25 pmol NEO, GTX 1/4

Table 2.4 continued...

Method	**Detection limit**
c. Post column periodate oxidation with 2 mobile phases (Sullivan *et al.*, 1985)	0.04 µmol B1, 0.006 µmol C1,0.006 µmol C2, 0.004 µmol GTX1, 0.006 µmol GTX2, 0.006 µmol, GTX3 0.1 µmol GTX4, 0.065 µmol NEO, 0.014 µmol STX
d. Post column electrochemical reactor (Lawrence and Wong, 1995)	0.3 pmol STX, 2.6 pmol NEO, 5.1 pmol GTX 1, 0.2 pmol GTX 2, 0.1 pmol GTX 3, 1.7 pmol GTX 4, 1.3 pmol B1, 37.9 pmol B2
e. Post column periodate oxidation with 3 mobile phases (Oshima, 1995)	17-82 fmol C1-4, 17-110 fmol GTX 1-5, 52 fmol NEO, 25 fmol STX, 41 fmol dc-STX
f. Prechromatographic periodate oxidation (Lawrence *et al.*, 1991)	10-15 ng/g non-N-1 hydroxy compounds, 50-100 ng/g N-1-hydroxy analogues
5. Electrophoretic methods	
a. Capillary electrophoresis/UV (Thibault *et al.*, 1991)	56.8 fmol NEO, 49.8 fmol STX,
b. Capillary electrophoresis/Electrospray mass spectrometry (Locke and Thibault, 1994)	30 nmol GTXs, 16 nmol STX and NEO
Bioassays	
a. Mouse bioassay (Somer and Meyer, 1937)	116.3 nmol STX/100 g tissue
b. Mouse neuroblastoma cell bioassay (Jellett *et al.*, 1992)	6.6 nmol STX eq/100 g tissue

0.05 and 0.04 μg of STX, GTX2 and GTX3, respectively.

In the isolation and characterization of GTX-1 from the toxic scallop digestive glands, Noguchi *et al.* (1981) also used TLC and electrophoresis. The plates and cellulose acetate strips were sprayed with 1% H_2O_2 followed by heating at 110°C for 10 min for the detection of toxins.

Although the detection limits were not known, Noguchi *et al.* (1986) used TLC and electrophoresis to study the local differences in toxin composition of a xanthid crab *Atergaris floridus*. Electrophoresis was performed on cellulose acetate strips which were visualized under U.V. light (365 nm) before and after spraying with 10% KOH followed by heating for 10 min. A similar detection method was used on TLC plates on which the toxins were separated using a pyridine : ethyl acetate : acetic acid : water (15:5:3:4) solvent system. However, NEO was not completely separated from STX, and overlapped somewhat with GTX3. In studying the bacterial transformation of PSP toxins, Kotaki *et al.* (1985) also used TLC with a pyridine : ethyl acetate : acetic acid : water (75:35:15:30) solvent system and STX, dc-STX, GTX1.

2.10.2.4 Electrophoresis

Fallon and Shimizu (1977) used electrophoresis on cellulose acetate membrane strips, and the toxins were detected by spraying with 1% H_2O_2 followed by heating. Although the detection limits were not given, they were able to rank the seven toxins in order of cation strength at pH 8.7 as STX > GTX2 > NEO > GTX3 > GTX5 > GTX1 > GTX4.

More recently, capillary zone electrophoresis (CZE) methods combined with either UV (Thibault *et al.*, 1991) or mass spectral detection (Pleasance *et al.*, 1992) have been developed for the detection of PSP toxins. Thibault *et al.* (1991) developed a capillary zone electrophoresis (CZE) method for the analysis of PSP toxins. Capillary electrophoresis with UV detection was described for the separation and determination of non-derivatized toxins associated with PSP. Detection limits were 0.018, 0.015 ng for NEO and STX, respectively, and this offered better detection limits than Sullivan's HPLC-

FID method. However, owing to the limited sample volume (a few nanoliters) which can be loaded on the CE column, the CE-UV technique lacked the ability to provide good detection limits for samples of low concentration. Locke and Thibault (1994) improved the detection limits by applying on-column sample concentration with capillary isotachophoresis (CITP) and discontinuous buffer systems prior to CZE separation. This technique was found to be entirely compatible with electroscopy mass spectrometry (ESMS) and permitted the analysis of scallop extracts containing submicromolar levels of PSP toxins. CITP/CZE was combined with tandem mass spectrometry, in cases where higher levels of selectivity are required for unambiguous identification of individual toxins.

2.10.2.5 High Performance Liquid Chromatography (HPLC)

Rubinson (1982) used HPLC to separate STX from its other reaction products using a 25 cm Waters μ-Bondapak/C18 column, and a solvent system consisting of H_2O_2 with 25 nM formic acid and 3-4 nM pentane sulfonic acid.

Onoue *et al.* (1983) used o-phthalaldehyde for the fluorometric detection of PSP separated by HPLC. This reagent reacts with primary amines in the presence of 2-mercaptoethanol to give a fluorescent product, and the fluorescence intensity per MU differed greatly among the toxins. The fluorescence intensities per 1 nmol of toxins were highest for GTX2 and 3 followed by NEO, STX, GTX1 and 4 respectively, with the detection limits ranging from 0.1 to 1 nmol. However, since o-phthalaldehyde reacts with primary amines, the presence of α-amino compounds interferes with the determination of toxins.

Sullivan *et al.* (1983a) and, Sullivan and Iwaoka (1983) used HPLC for the separation and detection of PSP toxins on a bonded phase cyano column with post-column oxidation using alkaline periodate followed by the fluorescence detection of oxidized derivatives. This method was found applicable to separate and quantify STX, NEO, GTX 1,2,3,4 and their sulfocarbamoyl derivatives. Detection limits for STX, GTX2 and 3 were about 0.5 ng, and for NEO, GTX1 and 4 were about 8 ng.

Sullivan and Wekell (1984) also developed an HPLC procedure which involved preparation of an acid extract of the shellfish followed by ion-interaction chromatography on a polystyrene divinyl benzene resin column with post column oxidation and fluorescence detection. Sullivan *et al.* (1985) used this technique to compare its efficiency with the AOAC mouse bioassay method by testing 100 shellfish samples representing a variety of species. Among the advantages over the bioassay are significantly better sensitivity, greater sample throughput and the ability to determine the levels of each individual PSP toxins. Also, greater precision, less analytical time and the ability to determine any of the 18 PSP toxins individually.

Sullivan *et al.* (1985) subsequently improved the technique with higher sensitivity (Table 2.4), and adapted it to an autoanalyzer. Mass spectrometry combined with liquid chromatography has been commonly used to confirm separated compounds (White *et al.*, 1986; Quilliam *et al.*, 1989). Oshima *et al.* (1989) further improved the HPLC method for the separation of N-sulfocarbamoyl and decarbamoyl derivatives of STX, using C_8 bonded silica gel column using three different mobile phases with isocratic elution.

Prechromatographic oxidation and HPLC with fluorescence detection was used to study 10 toxins associated with PSP (Lawrence *et al.*, 1991). They were oxidized at room temperature under mild basic conditions with H_2O_2 and periodic acid, and it was found that N-1-hydroxylated toxins (NEO, B2, GTX1, C3) formed fluorescent products with periodic acid but not with H_2O_2. As little as 3-6 ng of each of the non-hydroxylated toxins and 7-12 ng of the hydroxylated compounds per g of shellfish could be detected (Lawrence and Ménard, 1991).

Automated pre-column oxidation and micro-column liquid chromatography with fluorescence detection was used by Janećek *et al.* (1993) for the analysis of PSP toxins. Periodate oxidation of the toxins yields fluorescent purines suitable for trace analysis by reversed-phase HPLC. The fluorescence oxidation products were characterized by liquid chromatography combined with mass spectrometry (LC/MS) (Quilliam *et al.*, 1993). The gradient elution on a microbore column with large volume injections and fluorescence detection permitted the detection of femtomole quantities of toxins (e.g., STX - 0.3

ng/mL). Janiszewski and Boyer (1993) studied the feasibility of electrochemical oxidation of PSP toxins in a post column reaction, and found that these toxins could indeed be oxidized electrochemically to fluorescent compounds. Evaluating this post column electrochemical reaction, Lawrence and Wong (1995) indicated that non-hydroxylated toxins improved the sensitivity with increasing pH and voltage. At optimum operating conditions, the sensitivity for STX, GTX 2 and 3 was an order of magnitude greater than that for NEO and B1 and two orders of magnitude greater than that for B2. The detection limit for STX was 0.1 ng (Table 2.4)

Stafford and Hines (1994) used solid phase extraction (SPE) and pre-chromatographic oxidation/HPLC with fluorescence detection to isolate, identify and quantify STX in rat urine. The lower limit of quantification with this method was 2 ng STX/mL of rat urine.

A liquid chromatographic method after post column derivatization with periodic acid was developed by Oshima (1995). The method involved 3 mobile phases. The first mobile phase separated C1-4 toxins, the second separated GTX 1-6 and the third mobile phase resolved STX, NEO and dc-STX. This seems the most effective HPLC method to date, not only because of its high sensitivity (20-110 fmol), but also for high resolution, especially for dc-STX. The various methods of quantitative separation and detection to date are summarized in Table 2.4.

2.11 Detoxification of PSP Toxins

2.11.1 Detoxification of Live Shellfish

No commercially practical method for eliminating or reducing toxicity has been developed. The method most often attempted involves transplanting toxic shellfish to non-toxic areas to allow depuration to proceed. However, the decreases in toxicity obtained are often too slow to be economically useful. This depends on the contaminating agent, species and tissue (Bricelj and Shumway, 1998). Bivalve shellfish have varying abilities to detoxify the accumulated toxins depending upon species. Blanco *et al.* (1997) introduced one- and two-compartment models by changing environmental variables for detoxification

kinetics of the mussel, *Mytilus galloprovincialis.* Bricelj and Shumway (1998) tabulated various environmental conditions used to detoxify a wide variety of live bivalves. Mussels and some clams (*M. mercenaria*) detoxify faster than many other bivalves when transferred to non-contaminated water. *Saxidomus giganteus, S. nuttall* and *Spisula solidissima* are considered as slow detoxifiers and some of them show year around toxicity. No effective method has as yet been developed to accelerate the detoxicification of live bivalves contaminated with PSP toxins. Ozonation, however, has been used to inactivate PSP toxins from crude extracts of toxic dinoflagellates or shellfish.

2.11.2 Post Harvest Technology and Commercial Canning of Shellfish

Various post-harvest processing methods such as steaming, boiling, pan frying, commercial canning etc. have been found to decrease the total toxicity at different levels with a considerable decrease by commercial canning techniques (Medcof *et al.*, 1947; Prakash *et al.*, 1971; Gill *et al.*, 1985; Berenguer *et al.*, 1993). The commercial canning process of Medcof *et al.* (1947) involved pre-cooking for 15-20 min (steam) at about 1 atmospheric pressure. The clams were then shucked, the siphons trimmed off, the meat washed in warm fresh water and packed in cans. A portion of the hot bouillon (pH 6.5) resulting from the steaming was used to fill the cans completely. The bouillon was usually seasoned with vinegar. The cans were sealed without being exhausted and retorted at 121.1°C (250°F) for 45 min. The toxicity decreased more than 90%. Prakash *et al.* (1971) indicated that the toxicity score was reduced further during storage, and substantial reduction in toxicity could be achieved by modifying the commercial method. Studies on different canning times suggest that the only variation in the normal commercial canning procedure that might be expected to help in reducing the toxicity of the final product would be extension of the retorting time (Medcof *et al.*, 1947).

In the process of conventional commercial canning of shellfish, the canned product undergoes 5 factory treatments that affect the toxicity score, before reaching consumers:

1. Steaming in closely covered containers for 20 min at atmospheric pressure and at 100°C,
2. Shucking and trimming of steamed meat,
3. Washing in fresh water, packing in cans, adding bouillon and sealing,
4. Retorting at 121.1°C for 45 min,
5. Storing in a warehouse for about 2 weeks, but sometimes extended to several months.

Clams are canned on both east and west coasts of Canada and the U.S. On the east coast the quahog or hard clam (*Venus mercenaria*) and the soft shell clam *(Mya arenaria)* are used, while on the west coast it is mainly the razor clam (*Machaera patula*) which is processed. Hard clams are usually processed at 115.6°C for 1 h. However, the canning procedures may vary widely not only according to the country or even among the plants in the same country, but also according to the species, type of tissue and the nature of the commodity of interest (whole or minced clam). The processes used in Atlantic Canada vary considerably not only due to the differences in the difficulty of sterilizing, but because some clams are very tough, requiring a long process for sterilizing and cooking (Lopez, 1975). Commercial processing of bivalve molluscs in Spain is also quite similar, except the processing time is only 45 min at 115°C (Berenguer *et al.*, 1993). PSP toxin content in naturally contaminated *Acanthocardia tuberculatum* (L) decreased by 70-80% after processing by this method at neutral pH.

Thermoplastic extrusion processing was applied to detoxify PSP toxins in scallops by Ohta *et al.* (1992). Scallop viscera lost most of its initial toxicity of 224.6 MU/g after extrusion processing. The reduction of toxicity ranged from 81.6-82.2% at 130°C and 85.6-97.8% at 170°C. The digestive glands contained GTX 1-4 and STX prior to processing while the extrudate processed at 170°C contained only STX which was present in considerable amounts. Noguchi *et al.* (1980) demonstrated the effectiveness of canning to reduce the PSP toxicity apparently at neutral pH. In all parts of the scallop body, much of the toxicity was lost with retorting and a steady reduction of the toxicity took place during storage of the canned product. After 30 days of storage, the toxicity scores of

digestive glands dropped near to or below the quarantine limit of 4 MU/g. Toxin interconversons resulting from heating under different conditions have been reported repeatedly in the literature. To date, the only comprehensive study of the kinetics of thermal destruction of PSP was published by Gill *et al.* (1985). In this study, the overall rates of thermal destruction were determined for total toxicity of soft shell clams over a wide range of processing temperatures. However, individual toxin levels were not determined and the effect of pH was not examined.

2.12 Thesis Objectives

Since most detection methods are time consuming, expensive and require highly developed skills, there is a need for rapid, simple and inexpensive methods for the detection of these toxins. The main objective of the first part of this work (Chapter 3) was to develop a rapid detection method for the screening of large numbers of column fractions in a relatively short period of time, using a minimal sample volume.

The objective of the second part (Chapter 4) was to attempt to develop a novel method for the detection of PSP toxins using automated thin layer chromatography with an Iatroscan (Mk 5) equipped with a flame thermionic detector. Identification of individual toxins based upon chromatographic mobility was also studied.

Since changes in specific PSP toxicities due to heating have been reported, the importance of a detailed study of this phenomenon is essential from the food safety perspective. There has been very little work done on the thermal degradation of PSP toxins, and the effect of pH on the degradation of individual toxins has not been reported in detail. Therefore, the thermal degradation of PSP toxins in scallop homogenates and in buffers (without matrix) was studied with the objective of gaining knowledge of the effect of a wide range of pH levels (3-7) on the kinetics of thermal destruction of individual PSP components (Chapters 5 and 6).

Conditions and time of storage are factors which are likely to affect the stability of PSP toxins. The long term stability or instability is important not only to the analyst who must store authentic toxin standards, but also to food regulatory agencies in the event that

specific toxicities could increase as a result of storage of shellfish with non detectable levels of these toxins. Although the toxicity may change during storage after heating (e.g., conventional commercial canning and pasteurization), there has not been an indication of the effect of pH and it is interesting to know the nature of individual toxins during storage at different pH levels. Therefore, heated and unheated PSP toxins were stored at different temperatures with the objective of determining the effect of a wide range of pH values on the change of individual toxins during storage (Chapter 7).

CHAPTER 3
FLUOROMETRIC DETECTION OF PARALYTIC SHELLFISH POISONING TOXINS

3.1 Abstract

A rapid qualitative screening method was developed for the fractionation of paralytic shellfish poisoning toxins. Periodic acid, t-butyl hydroperoxide and hydrogen peroxide were tested as oxidants for the fluorometric detection of paralytic shellfish toxins. Hydrogen peroxide was found to be the most convenient and efficient oxidant since the fluorescence can be detected after the incubation of toxins at 100°C for 3-5 minutes. In addition to the structure of the compound, the incubation temperature and time, the amount of acid and peroxide concentration affect the fluorescence reaction.

This method was more efficient than the previously published peroxidation methods which involved lengthy incubation periods or time consuming pH adjustment. Also, far greater sensitivity was achieved with the new method with levels of 0.027 pmol, 0.054 pmol, 0.023 pmol, 0.003 pmol, 0.0002 pmol and 0.0006 pmol being easily detected for saxitoxin (STX), neosaxitoxin (NEO), gonyautoxin 1 and 4 (GTX 1/4), gonyautoxin 2 and 3 (GTX 2/3), C toxins (C1 and C2) and B toxins (B1), respectively. The method is particularly valuable for the screening of fractions separated by column chromatography. This work has been published in Analytical Biochemistry (Indrasena and Gill, 1998).

3.2 Introduction

Paralytic shellfish poisons (PSP's) are responsible for acute and often fatal poisonings caused by the consumption of certain shellfish feeding on toxic dinoflagellates. The sporadic and unpredictable outbreaks usually cause serious health hazards and great losses to the seafood industry. The relationship between the occurrence of phytoplankton blooms and toxic shellfish was reported as early as 1937 by Sommer and Meyer, and the dinoflagellate, *Gonyaulax catenella* was identified as a causative organism. The best known paralytic shellfish toxin, saxitoxin, was first isolated from the Alaska butter clam

Saxidomus giganteus collected in certain areas in Alaska where the shellfish remain toxic year round (Schantz *et al.*, 1957).

It is apparent that the toxins possess a wide range of toxicities, with the sulfamate forms being the least toxic and the carbamate forms, the most toxic (Fig. 1.1). The decarbamoyl toxins for which data are available, appear to have toxicities slightly below those of the carbamate forms. Conversion of the toxin from one form to another (with a concomitant change in toxicity) can easily be accomplished. The sulfamate toxins can be converted to the carbamate form by acid hydrolysis. Although the net molar concentration of toxins in a sample may remain constant, the concentrations of the individual toxins and therefore the net toxicity will vary as a result of interconversions with time and may increase substantially.

Preparative separation of the toxins has been accomplished with a variety of techniques including gel filtration chromatography, ion exchange chromatography, thin layer chromatography and electrophoresis. These techniques have been reviewed by Sullivan *et al.* (1988). Detection of the toxins in chromatographic fractions has traditionally been accomplished by the mouse bioassay (Schantz *et al.*, 1957; Shimizu *et al.*, 1975; Hall *et al.*, 1980; Boyer *et al.*, 1980; Hall and Rechardt, 1984). However efforts have been directed towards the development of a suitable chemical assay for toxicity which would be more sensitive and reproducible.

Bates and Rapoport (1975) developed an alkaline oxidation method for the detection of PSP toxins, and recognized that saxitoxin and related compounds are generally unstable under alkaline conditions, especially in the presence of hydrogen peroxide, yielding fluorescent derivatives. Shoptaugh *et al.* (1981) further modified this method. A fluorescence method was reported to be capable of detecting as little as 0.04 μg STX, and was applied as a spray of 1% H_2O_2 for the detection of toxins separated on thin layer chromatographic plates (Buckley *et al.*, 1976).

Unfortunately, the alkaline peroxidation of PSP toxins was reported to lack sufficient sensitivity for the detection of N-1-hydroxy saxitoxins such as NEO, B2, GTX 1 and 4 as well as toxins C3 and C4 (Buckley *et al.*, 1978). Ikawa *et al.* (1982) separated

STX from GTX 2 and 3 using Bio Rad AG 50 strong cation exchange resin. Detection of toxic fractions was accomplished with the addition of a 1% H_2O_2 solution to 2 mL aliquots and the fluorescence at 380 nm determined after a 40 min incubation period. The method would not detect NEO and had poor sensitivity for the N-1-hydroxy gonyautoxins. Later, Mosely *et al.* (1985) found that the alkaline peroxidation technique, when coupled with a Folin-Ciocalteau reagent assay, improved the detection of the 11-hydroxy derivatives, but indicated that the Folin-Ciocalteau assay was only about twice as sensitive as the mouse bioassay.

The objective of the present research was to develop a method of PSP detection which would be at least as sensitive as the alkaline peroxidation method for STX but with similar sensitivity to the N-1-hydroxy derivatives. It was also desirable that the detection method would require a minimal sample volume, and be applicable to the screening of large numbers of column fractions in a relatively short time period.

3.3 Materials and Methods

Toxic samples of soft shell clams (*Mya arenaria*), blue mussels (*Mytilus edulis*), and scallop viscera (*Placopecten magellanicus*) were obtained from Fisheries and Oceans Canada (Inspection Branch), and homogenized in one volume 0.1M HCl in a Waring blendor and stirred overnight at 20°C. The homogenate was centrifuged at 20,000 x g for 30 min, and the supernatant fluid decanted and mixed with an equal volume of ethanol. The mixture was stirred overnight and held for 48 h at 5° C. The precipitated protein was removed by centrifugation and the supernatant fluid suction filtered through two layers of Whatman No. 1 filter paper using Celite as a filtering aid.

Ethanol was removed by rotary evaporation at 50° C. The aqueous PSP solution was further deproteinized by passing it through an Amicon PM-10 ultrafilter (molecular exclusion = 10,000 da). The clear liquid was then freeze dried, and the powder analysed by HPLC using the method of Sullivan and Wekell (1987). All of the HPLC equipment was purchased from Waters (Toronto ON) except for the Shimadzu fluorescence detector Model RF 535. All of the chromatographic conditions were identical to those described by

Sullivan and Wekell (1987).

3.3.1 Large Scale Fractionation of PSP Concentrate

The freeze-dried powder was fractionated on a Bio-Gel P2 (Bio Rad Laboratories) column (2.5 x 72 cm). Initially, a 0.5 g sample of powder was dissolved in 3 mL H_2O and applied to the column. A two step gradient consisting of one column volume H_2O followed by 0.1M acetic acid was used to elute the sample with a flow rate of 1 mL/min. Fifteen ml fractions were collected at room temperature, and tested for toxins using fluorescence oxidation and by the HPLC method of Sullivan and Wekell (1987). The fractions were subjected to oxidation with hydrogen peroxide according to Shoptaugh *et al.* (1981) (modified method of Bates and Rapoport, 1975), and the present peroxide method.

3.3.2 Standards

Two types of standards were used. Mixed standards of GTX 2 and 3, GTX 1 and 4, and C1 and 2 were obtained from the National Research Council (Institute of Marine Biosciences, Halifax, Nova Scotia). Individual standards including STX, NEO and B1 were also obtained from National Research Council. The mixed standards were used for calibration experiments (Table 3.1) and individual standards (GTX 1, GTX 2, GTX 3, GTX 4, STX, NEO) were used to determine the effect of peroxide level on fluorescence yield. In addition, pure STX standard was obtained from the U.S. Food and Drug Administration, Division of Microbiology (Cincinati OH) and used to compare the three oxidants, hydrogen peroxide, t-butylhydroperoxide and periodic acid.

3.3.3 Fluorescence of Oxidized Toxins

Standard STX samples (80 μg/mL) were oxidized separately with periodic acid, t-butyl hydroperoxide (Oshima *et al.*, 1984) and hydrogen peroxide (modified method of Shoptaugh *et al.*, 1981), and the fluorescence peak areas were compared in order to develop a rapid screening procedure for PSP toxins. The pre-scan facility of the luminescence spectrometer enabled the excitation at specific wavelengths while measuring

the total fluorescence yield in the emission spectrum. Thus, fluorescence readings represent total fluorescence of a wavelength scan around the emission wavelength optimum for each oxidized toxin. This approach was taken in order to obtain maximum sensitivity.

In the periodic acid method, 10 mL of 50 mM periodic acid and 20 mL of H_3PO_4 were dissolved in 60 mL distilled water and titrated with 5N NaOH to pH 7.8. Two hundred µL of this solution was used to oxidize 200 µL samples of serially diluted standard STX (containing 0.13 to 0.64 μg). Mixtures were incubated for 40 min at 25°C, and the fluorescence peak area was measured using excitation and emission wavelengths of 340 and 400 nm, respectively, using a Perkin Elmer Model LS 50 luminescence spectrometer equipped with a flow through cuvette and slit widths adjusted to 5 and 10 nm for excitation and emission, respectively.

The second method involved the addition of 200 µL of 0.4% t-butyl hydroperoxide solution in 40% ethanol to 200 µL samples of STX. The pH of the oxidant was adjusted to 10 with NaOH prior to diluting the oxidant solution to volume. The mixture of toxin and oxidant were vortexed, heated in a 100°C oven for 10 min and immediately cooled in ice water. The fluorescence was monitored at the excitation wavelength of 345 nm with 5 nm slit width and emission wavelength of 420 nm with 10 nm slit width.

The use of hydrogen peroxide as an oxidant was based on the method originally developed by Bates and Rapoport (1975) as modified by Shoptaugh *et al.* (1981) which used room temperature incubation for 40 min and excitation and emission wavelengths of 330 and 380 nm, respectively. Initially, 200 μL of 1N NaOH was added to a series of tubes containing 200 μL standard STX. The effect of H_2O_2 concentration on standard STX, NEO and GTX 1-4 was examined by adding 200 μL H_2O_2 solution ranging in concentration from 0.6 to 7.5%. After vortexing for 30 s, tubes were heated in a 100° C oven for 5 min, chilled immediately in ice and 1 drop of glacial acetic acid added to each tube. Tubes were vortexed for 30 s, and fluorescence read immediately at an excitation wavelength of 330 nm with 5 nm slit width and emission wavelengths of 380 nm with 10

nm slit width. The fluorescence peak area of appropriate blanks were subtracted from the samples. Different concentrations of NEO, GTX 2/3, GTX 1/4, B1 and C1/C2 were also oxidized in the same manner and the fluorescence was measured in order to determine the detection limits for each toxin component by the current peroxide method.

Detection limits for each toxin were determined by plotting fluorescence peak areas against toxin concentration for authentic standards, and regression equations for each plot were determined. Detection limit was defined as the toxin concentration at which the peak area diminished to zero.

The effect of incubation time in the presence of 200 µL 1.5% H_2O_2 was examined for samples incubated in the dark at 25°C according to Shoptaugh *et al.* (1981). At 15 min intervals, duplicate tubes were removed from the dark, treated with one drop of glacial acetic acid, vortexed, and the fluorescence measured immediately.

The effect of incubation time of these standard toxins in the presence of 200 µL 1.5% H_2O_2 was examined also for samples incubated in a 100°C oven. In this experiment, duplicate samples (and blanks) were removed, acidified, cooled and read at 5 min intervals.

The effect of acetic acid concentration was examined by oxidizing 200 µL samples with 200 µL 1.5% H_2O_2 at 100°C for 5 min and then quenching the alkaline oxidation with varying amounts (0.02 to 0.08 mL) of glacial acetic acid.

3.4 Results and Discussion

It has been observed that H_2O_2, periodic acid and t-butyl hydroperoxide can oxidize PSP toxins resulting in the production of fluorescent compounds with different intensities. However, under the present experimental conditions, periodic acid gave the lowest response for STX. Tertiary butyl hydroperoxide gave a better response than periodic acid, but was still lower than hydrogen peroxide (Fig. 3.1). Based on these results, using H_2O_2 as the oxidant, the fluorescence oxidation method of Shoptaugh *et al.* (1981) which includes room temperature incubation of samples in the dark for 40 min has been further improved.

Oshima *et al.* (1984) pointed out that alkaline oxidation with t-butyl hydroperoxide can convert all shellfish toxins, including those nonfluorescing by H_2O_2 oxidation, into fluorescent compounds. However, when the linearity of both oxidation methods was compared, the present hydrogen peroxide oxidation method gave superior response linearity with an r^2 of 0.96-0.98 for all toxins (Table 3.1) indicating also the possibility of using this method for quantification. Oshima *et al.* (1984) indicated that NEO gave lower fluorescence yields compared to other PSP toxins even when oxidized with t-butyl hydroperoxide. In the present study, the fluorescence response resulting from the H_2O_2 oxidation of NEO was lower than that of STX (50%) and even higher than that of GTX 1 and 4. The present peroxide oxidation method is rapid since reactions are completed in 5 min. This is slightly faster than the t-butylhydroperoxide method which utilizes an incubation time of 10 min. The periodic acid technique involves pH adjustment and a lengthy incubation time. The o-pthalaldehyde method described by Onoue *et al.* (1983a) seems less sensitive, and other previous peroxide oxidation methods involved 40 min incubation at room temperature. However, the present hydrogen peroxide method is far less complicated and better suited to analysing large numbers of samples than previously published procedures.

Sullivan *et al.* (1988) found STX to be extremely unstable under alkaline conditions in the presence of oxygen, a reaction leading to the formation of fluorescent degradation products which are amino purine derivatives (Figs. 3.2, 3.3). NEO gave a

Table 3.1 Calibration equations derived by simple linear regression of plots (peak area *vs.* amount of toxins) of different PSP components.**

Type of toxin	Amount of toxin (range, pmol/μL)	Calibration equation (x=amount of toxin, pmol/μL) (y=fluorescence peak area)	r^2 (n=18)
STX	0 to 1.03	y = 37.7x - 1.0	0.97
NEO	0 to 3.04	y = 22.4x - 1.2	0.98
C1/C2	0 to 0.56	y = 646.6x - 0.1	0.98
GTX 2/3	0 to 0.88	y = 32.1x - 0.1	0.96
GTX 1/4	0 to 0.73	y = 17.7x - 0.4	0.99
B1	0 to 0.01	y = 169.5x - 0.1	0.97

** Toxins were oxidized according to the present hydrogen peroxide oxidation method.

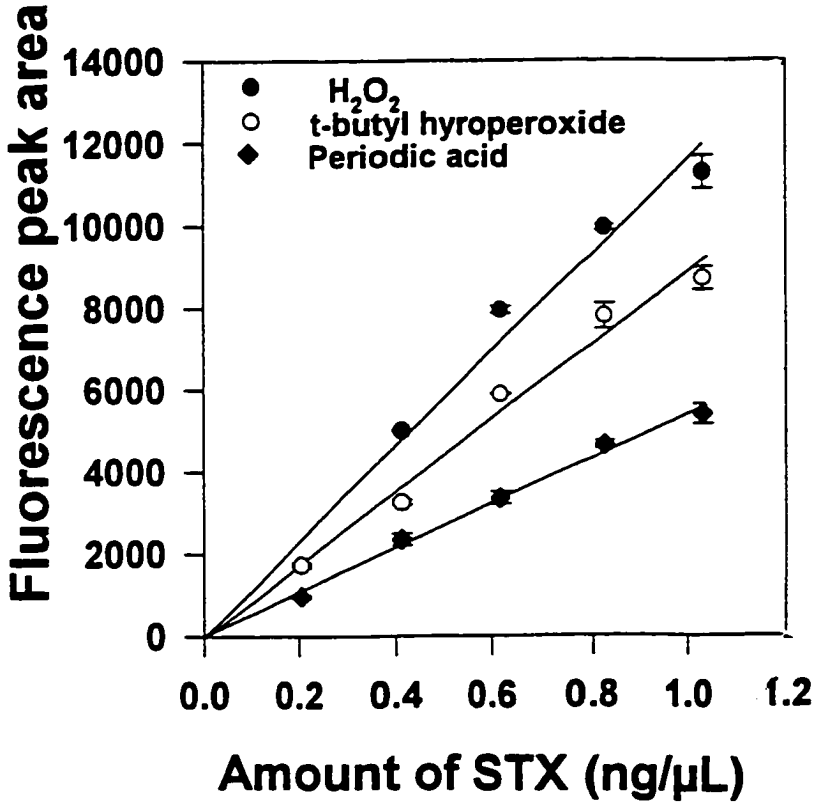


Fig. 3.1 Fluorescence oxidation of standard STX with periodic acid, t-butyl hydroperoxide and hydrogen peroxide (Error bars show the standard deviation of the mean, n = 3)

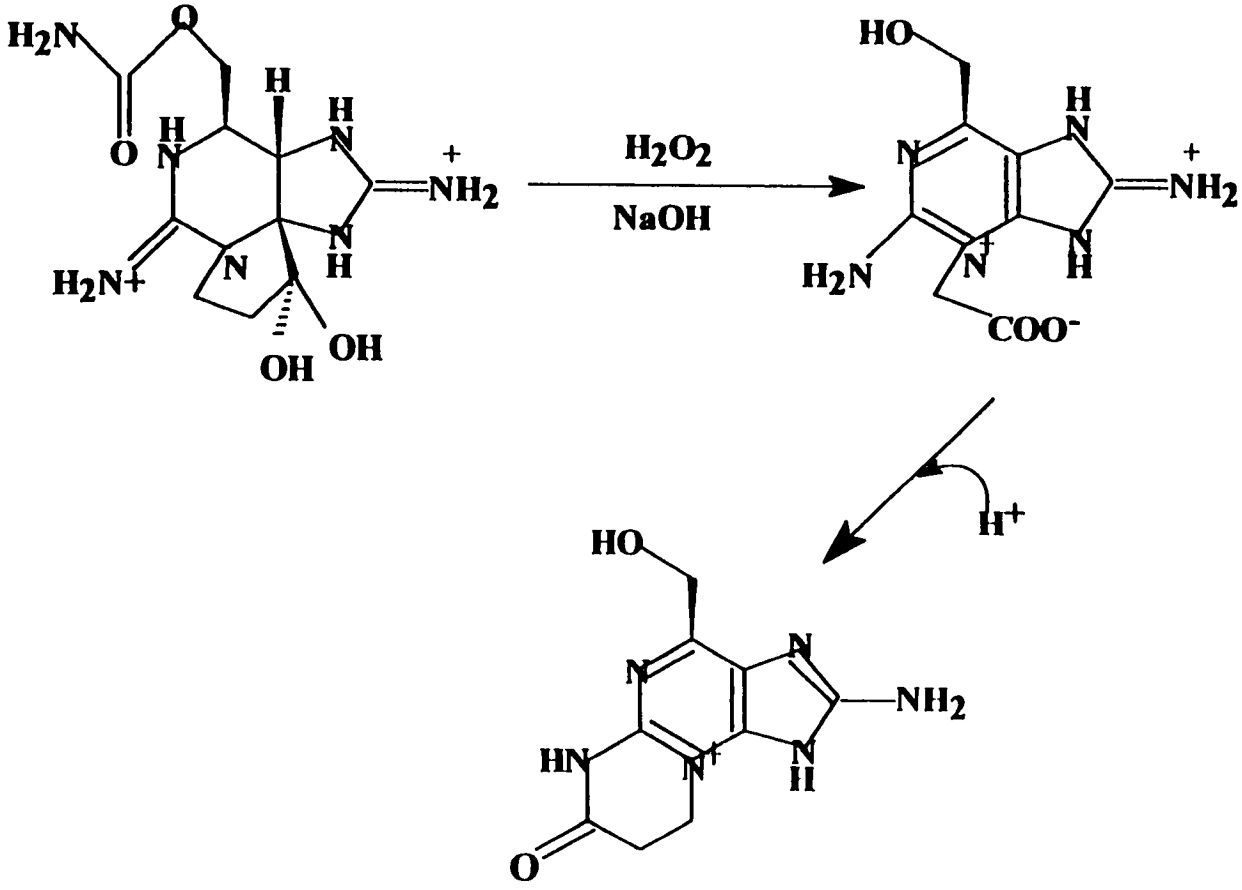


Fig. 3.2 Fluorescence oxidation of saxitoxin (adapted from Sullivan and Wekell, 1988)

lower fluorescence yield on alkaline oxidation (Fig. 3.4), possibly due to the presence of an additional hydroxyl group in the R_1 position which hinders the aromatization of the NEO structure. The results clearly show that by adjusting the H_2O_2 concentration to 1.5%, the sensitivity to NEO can be enhanced while both lower and higher levels result in loss of fluorescence. Others have reported that H_2O_2 is not a suitable oxidant for some of the toxins including NEO (Oshima *et al.*, 1984; Onoue *et al.*, 1983b). In the present study, optimal H_2O_2 concentrations were found to be approximately 1.5, 0.2, 1.5, 1.5, 1.5 and 1.5% for NEO, STX, GTX 1, GTX2, GTX 3 and GTX 4, respectively.

The fluorescence yields of PSP components vary widely with the hydrogen peroxide concentration, and it is interesting to note that STX gave a high fluorescence peak area even with very low concentrations of peroxide. The highest fluorescence peak area was obtained at concentrations between 0.5 - 1.5% (Fig. 3.3). Like NEO (Fig. 3.4), GTX 1 and GTX 4, GTX 2 and 3 gave their highest fluorescence peak areas when the peroxide concentration was 1.5% and declined rapidly after that (Fig. 3.3). It is interesting to note that all N-1- hydroxy toxins (NEO, GTX 1 and 4) had much lower fluorescence yields than the toxins with hydrogen atoms in the R_1 position (Fig. 1.1), above peroxide concentrations of 3%.

Acetic acid added to quench the alkaline peroxidation reaction had a considerable effect on the fluorescence reaction of PSP toxins (Fig. 3.5). The standards containing STX, NEO and GTX 2/3 gave the highest fluorescence yield when only 0.02mL of glacial acetic acid were added to the reaction system after the oxidation of toxins with hydrogen peroxide. Toxins oxidized with H_2O_2 without subsequent addition of acetic acid gave very small fluorescence yields which were indistinguishable from the blank, whereas the fluorescence was increased rapidly with the addition of acetic acid. In all cases the final acetic acid concentrations were 10-100 times the concentration of NaOH in the reaction mixtures. Two hundred µL of purified toxins composed of GTX 2/3, GTX 1/4, NEO, STX, C1/C2, B1 and B2 oxidized with 200µL alkaline H_2O_2 (1.5%) required only 1 drop of glacial acetic acid (0.02mL - 0.04 mL) to give the highest fluorescence yield.

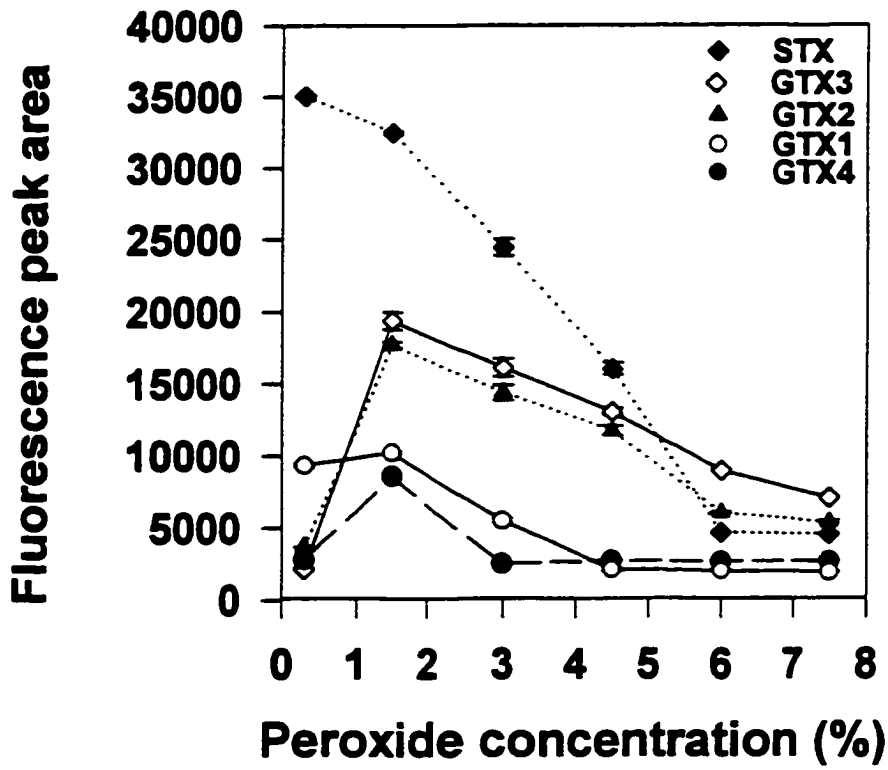


Fig. 3.3 Effect of hydrogen peroxide concentration on STX (93 pg/μL), GTX 1 and 4 (1.94 ng/μL), and GTX 2 and 3 (1.86ng/μL) (Error bars show the standard deviation of the mean, n = 3)

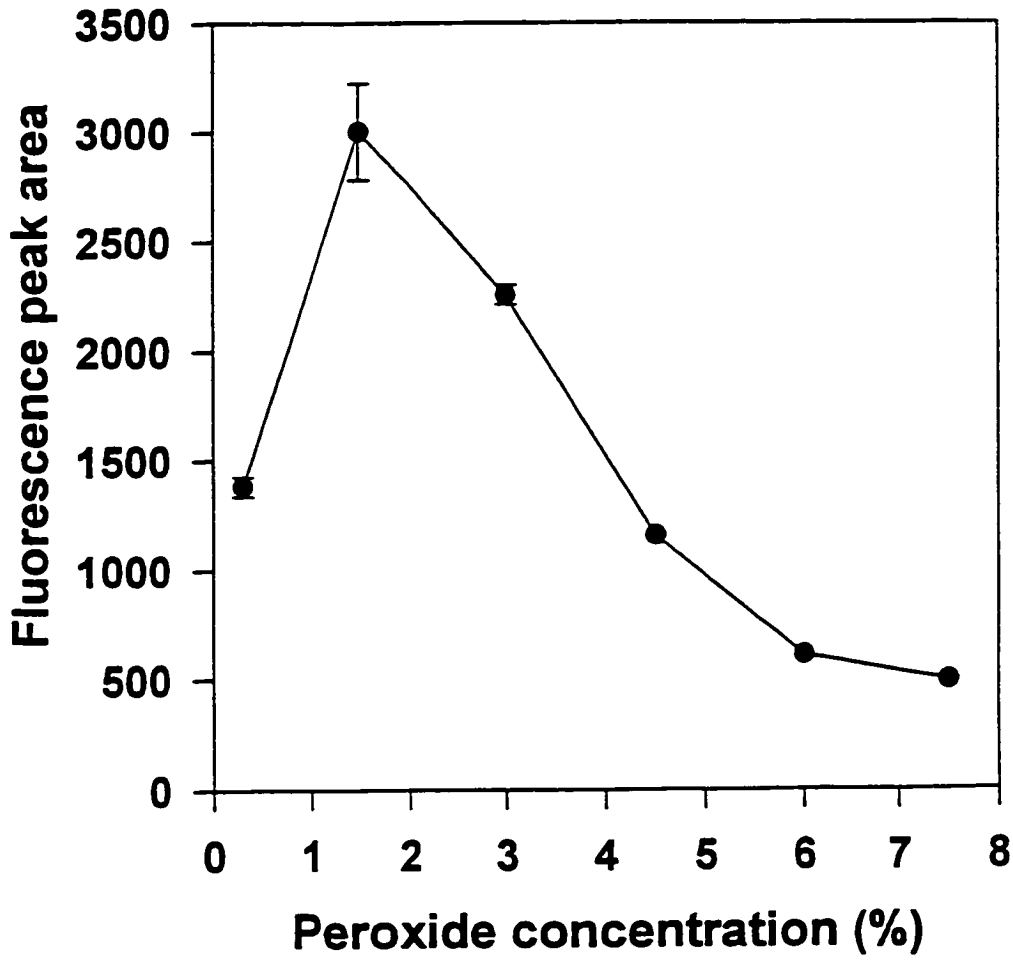


Fig. 3.4 Effect of hydrogen peroxide on fluorescence yield for NEO (0.49ng/μL) (Error bars show the standard deviation of the mean, n = 3)

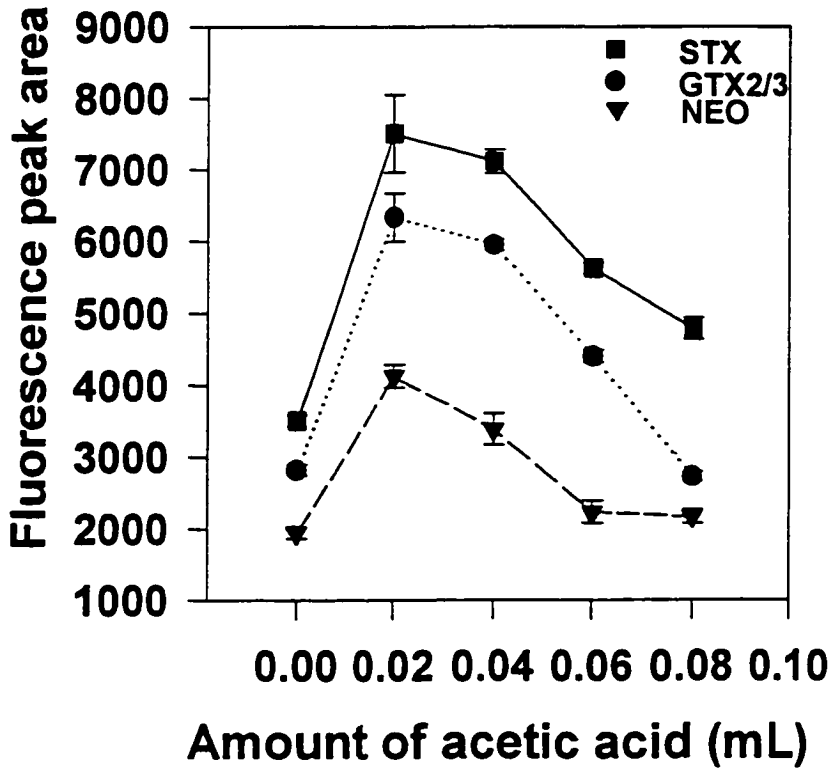


Fig. 3.5 Effect of acetic acid content on fluorescence yield for STX (1.5ng/μL), GTX 2/3 (1.7ng/μL) and NEO (1.01 ng/μL) (Error bars show the standard deviation of the mean, n = 3)

The temperature also had a considerable effect on the fluorescence oxidation reaction of PSP toxins. The conventional alkaline peroxide oxidation method involves the incubation of toxins at room temperature for 40 min with alkaline H_2O_2 (1%). In the present experiment, fluorescence peak areas of STX, GTX 2/3 and NEO were monitored for samples incubated at 25°C and 100°C. Highest fluorescence yields at 25°C were found after 45 min incubation for all four toxins and at 100°C, maximum yields were recorded at 3-5 min (Fig. 3.6). It was also observed that once acetic acid was added, the fluorescence emission remained constant for almost 24 h.

The following reaction conditions were selected as being optimal for the detection of PSP toxins: sample size = 200 μL; 1N NaOH = 200 μL; 1.5% H_2O_2 = 200 μL; incubation time at 100°C = 3-5 min; glacial acetic acid = 20-40 μL. A flow-through cell and peristaltic pump enabled rapid spectrofluorometric analysis of hundreds of samples collected from effluent from the chromatographic column used to fractionate PSP toxins.

In the present study STX could be easily detected even at 0.027 pmol whereas minimum detectable levels of NEO, GTX 1 and 4, GTX 2 and 3, C1 and C2, and B1, were 0.054 pmol, 0.023 pmol, 0.003 pmol, 0.0002 pmol and 0.0006 pmol, respectively. This compares favourably with the work of Buckley *et al.* (1976), Proctor *et al.* (1975), Bates and Rapoport (1975) and Gershey *et al.* (1976) who developed methods capable of detecting PSP levels of 133 pmol, 33 pmol, 5 pmol and 1662 pmol, respectively. The present method also compares favourably with the 1984 method of Oshima *et al.* which utilized t-butyl hydroperoxide to detect STX, NEO, GTX 2 and GTX 3 at levels of 0.16 nmol, 2.42 nmol, 0.1 nmol and 0.04 nmol, respectively. The present method is faster than any of the previously published screening techniques.

It is interesting to note that the sensitivity of this method for C1/C2 and B1 toxins seems relatively high. Although the method was slightly less sensitive for the detection of NEO as compared to STX, the other previously published methods yielded poor sensitivity for NEO. Shoptaugh *et al.* (1981) also indicated that the presence of B1 in the mixture tended to increase the fluorescence rapidly. However, the y-intercepts (Table 3.1) shows that all toxins tested required a certain amount in order to show detection above the

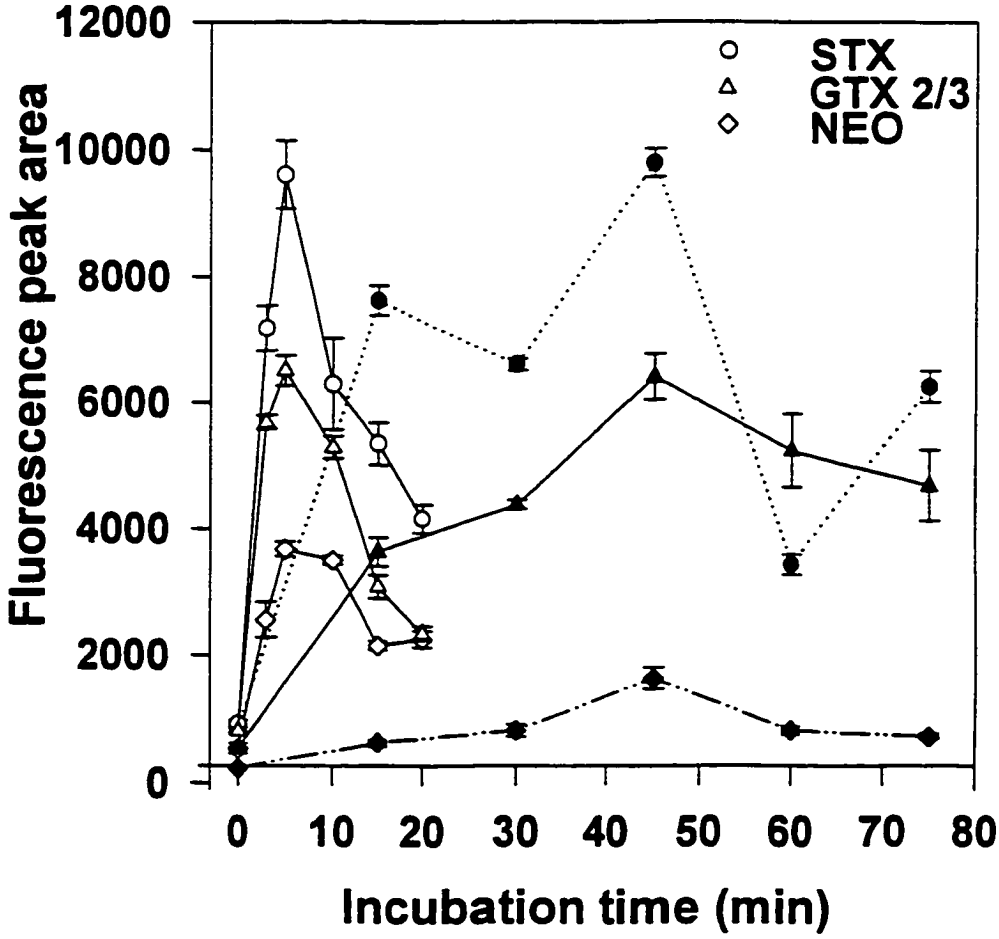


Fig. 3.6 Variation of fluorescence peak area with incubation at different temperatures (filled symbols = incubation at 25°C, open symbols = incubation at 100°C). (Error bars show the standard deviation of the mean, n = 3)

blank, and the threshold varied according to the compound. The fluorescence of the blanks of Bates and Rapoport (1975) and Shoptaugh *et al.* (1981) was equivalent to 56.4 pmol STX/g and 26.6 pmol STX/mL respectively.

Partially purified PSP toxins eluting from a Bio-gel P2 column were analysed spectrofluorometrically according to Shoptaugh *et al.* (1981), and by the current method. It was found that almost all fractions gave considerably higher fluorescence intensities by the current method (Fig. 3.7). The HPLC analysis clearly showed that most of the fractions contained at least one type of toxin but the fluorescence intensities varied according to the amount and type of toxins available in the fractions. The fractions containing only STX and/or GTX 2/3 had higher fluorescence intensities than those with any N-1-hydroxy compounds such as NEO and GTX1 and 4. However, it is interesting to note that fractions containing the N-1-hydroxy compounds gave higher fluorescence intensities when oxidized by the present method as compared to the method of Shoptaugh *et al.* (1981). Fraction number 3 had a higher fluorescence yields presumably since it contained mainly GTX 2 (Fig. 3.8) whereas fraction number 14 had mainly GTX 3 contaminated with small amounts of GTX 1/4 and NEO (Fig. 3.9). Fractions 4-13 contained small amounts of GTX 2 and 3 in varied proportions. Although fractions 15 - 17 mainly contained NEO and GTX 1 and 4 with or without small amounts of GTX 2 and 3, the current method was able to detect them with much higher sensitivity than by the Shoptaugh method. However, if optimum concentrations of H_2O_2 are used even GTX 1, 4 and NEO can be oxidized in 5 min at 100°C, and can be detected spectroflurometrically. A major advantage of the present method is that it is rapid and if using a spectrofluorometer equipped with a flow-through cell, can be used to rapidly screen hundreds of samples prior to further analysis with any of the more laborious and expensive chromatographic or electrophoretic methods. In this case, it is advisable to begin analysis of samples with highest fluorescence, and quantify by HPLC or other appropriate technique. When quantitative data indicate that fractions do not contain measurable amounts of toxins of interest, the rest of the fractions may be discarded.

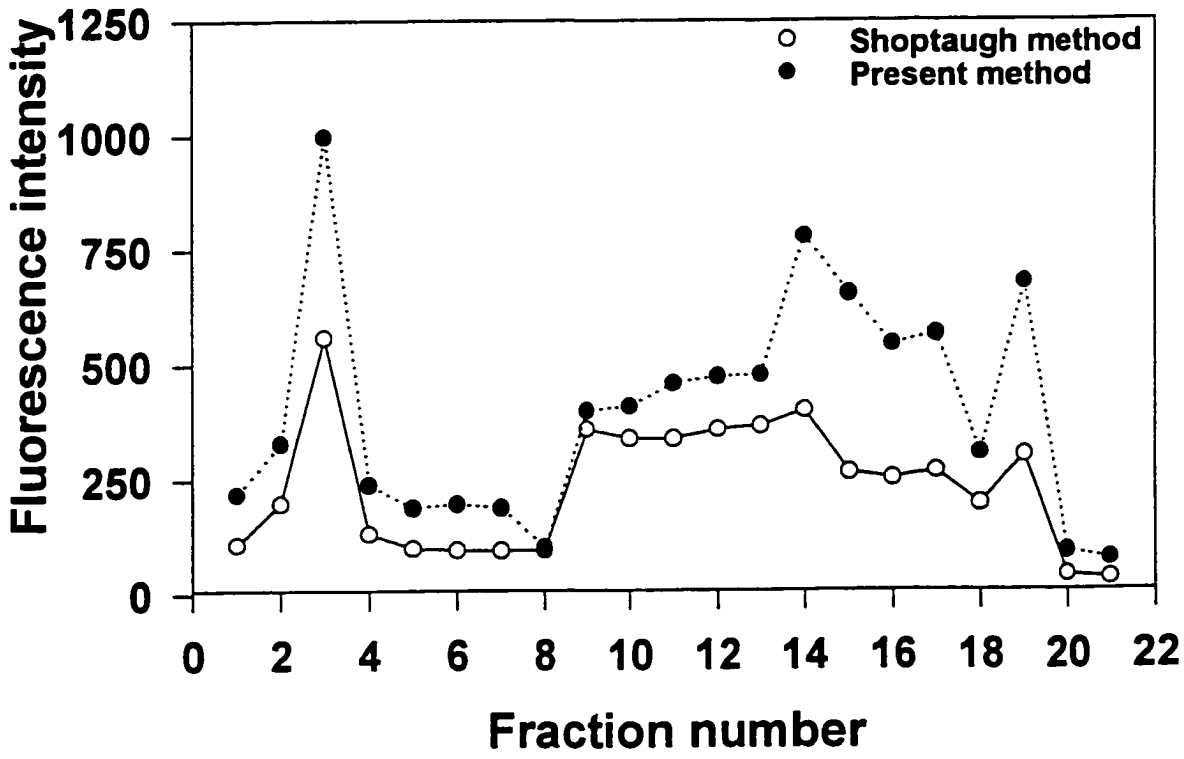


Fig. 3.7 Fluorescence of fractions of toxic clam, mussel and scallop viscera collected from Bio-Gel P2 column (○ = Shoptaugh *et al.* (1981), ● = present peroxide method).

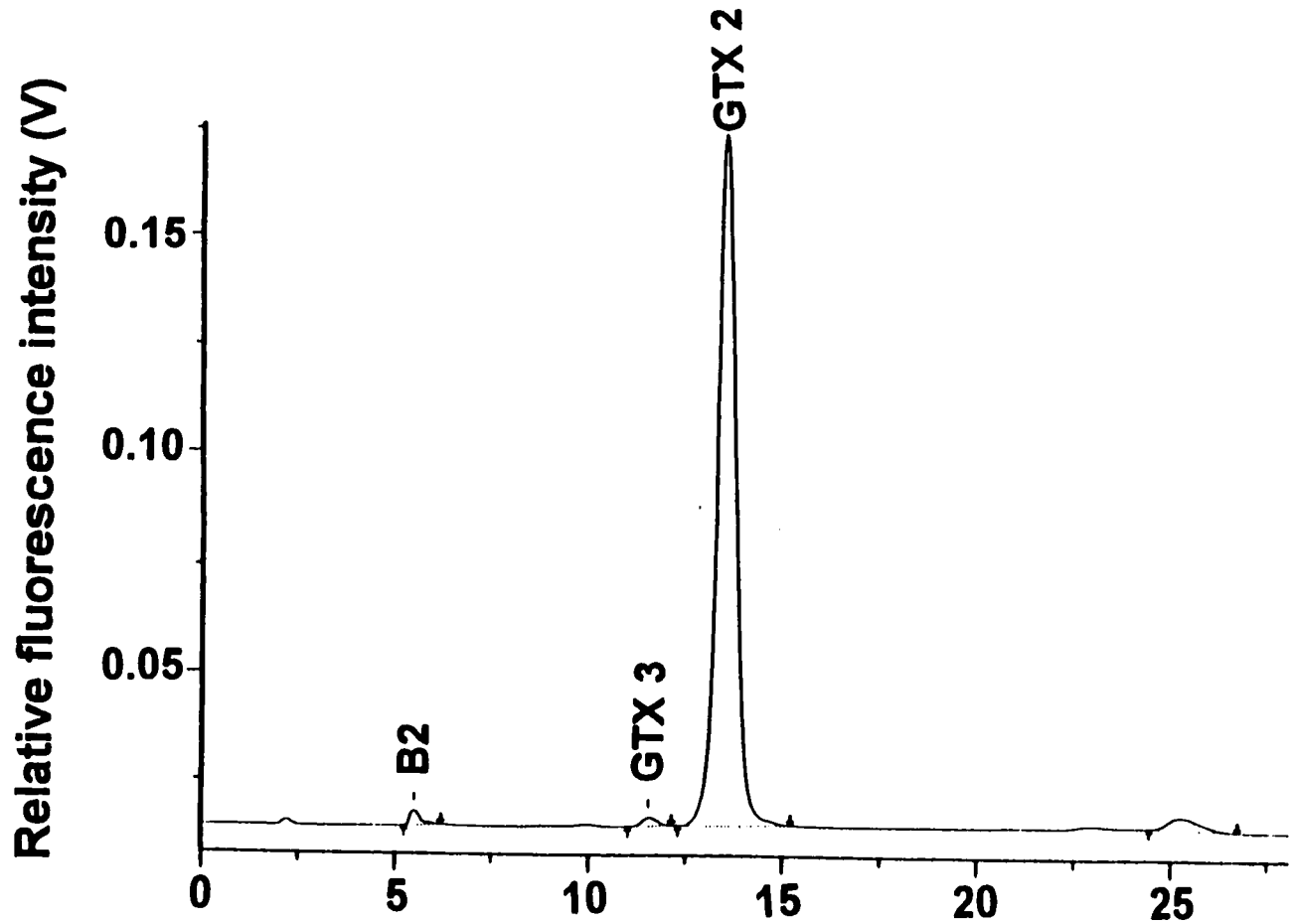


Fig. 3.8 HPLC chromatogram of fraction No. 3 (concentrated with GTX2) eluted from Bio-Gel P2 column

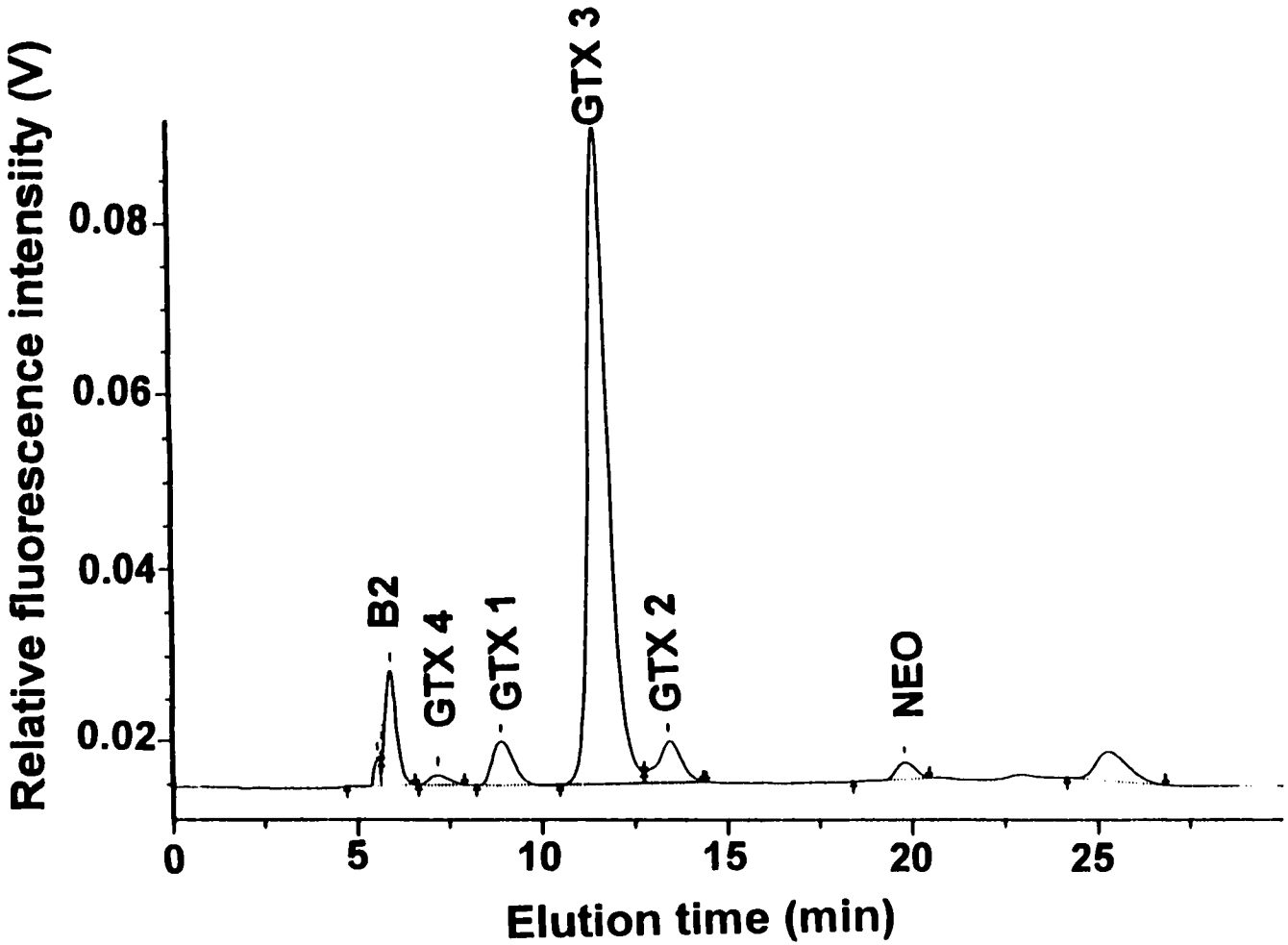


Fig. 3.9 HPLC chromatogram of fraction No. 14 (concentrated with GTX3) eluted from Bio-Gel P2 column

CHAPTER 4

SEPARATION OF PARALYTIC SHELLFISH POISONING TOXINS ON CHROMARODS-SIII BY THIN LAYER CHROMATOGRAPHY WITH THE IATROSCAN (MK 5) AND FLAME THERMIONIC DETECTION

4.1 Abstract

Thin layer chromatography (TLC) on Chromarods-SIII with an Iatroscan (Mk-5) equipped with a flame thermionic detector (FTID) was used to develop a rapid method for the detection of paralytic shellfish poisoning (PSP) toxins. The effect of variation in hydrogen (H_2) flow, air flow, scan time and detector current on the FTID peak response for both phosphatidylcholine (PC) and PSP were studied in order to define optimum detection conditions. A combination of hydrogen and air flow rates of 50 mL/min and 1.5-2.0 L/min, respectively, along with a scan time of 40 s/rod and a detector current of 3.0A (ampere) or above was found to yield the best results for the detection of PSP compounds. Increasing the detector current level to as high as 3.3A gave about 130 times more FTID response than did flame ionization detection (FID) for PSP components. Quantities of standards as small as 1 ng neosaxitoxin (NEO), 5 ng saxitoxin (STX), 5 ng B1- toxins (B1), 2 ng gonyautoxin (GTX) 2/3, 6 ng GTX 1/4 and 6 ng C-toxins (C1/C2) could be detected with the FTID. The method detection limits for toxic shellfish tissues using the FTID were 0.4, 2.1, 0.8 and 2.5 μg per g tissue for GTX 2/3, STX, NEO and C toxins, respectively. The FTID response increased with increasing detector current and with increasing scan time. Increasing hydrogen and air flow rates resulted in decreased sensitivity within defined limits.

Numerous solvent systems were tested, and a solvent consisting of chloroform : methanol : water : acetic acid (30:50:8:2) could separate C toxins from GTX, which eluted ahead of NEO and STX. Accordingly, TLC with the Iatroscan (Mk-5) and FTID seems to be a promising, relatively inexpensive and rapid method of screening plant and animal tissues for PSP toxins. This work has been published in Journal of Chromatography (Indrasena *et al.*, 1999).

4.2 Introduction

Paralytic shellfish poisoning (PSP) is a neurological illness resulting from the consumption of shellfish contaminated with saxitoxin and/or its derivatives (Fig. 1.1) which are formed intracellularly from marine dinoflagellate algal cells such as *Alexandrium* (formerly *Gonyaulax* or *Protogonyaulax*) *tamarensis, A. catanella* and *Gymnodinium catenatum* or from fresh water cyanobacterium *Aphanizomenone flos-aquae* (Mahmood and Carmichael, 1986). Toxic dinoflagellates and their toxins can accumulate in filter feeding shellfish such as oysters, mussels, clams, cockles and scallops resulting in illness or death to birds, fish, marine mammals and humans. Records of human illness from consumption of shellfish dates back to the 1600's (Steidinger and Baden, 1975) and PSP has been reported to be a public health problem since 1793 (Prakash *et al.*, 1971). There are no known antidotes to the toxins and chemical detection is difficult.

Several detection methods including chemical, biochemical and bioassays have been developed since the toxins were first diagnosed as a primary cause of shellfish poisoning. The mouse bioassay (Sommer and Meyer, 1937) that established the basis for the standardized assay has been used world-wide for the detection of PSP in shellfish. Although this method provides adequate sensitivity for the detection of total PSP toxin in shellfish extracts (Boyer *et al.*, 1979), its narrow dynamic range, inherent variability and the social pressure to ban animal bioassays are currently increasing the demand for alternative methods for analysis.

Several spectrophotometric methods involving various colour reactions of the PSP toxins have been reported (Mcfarren, 1980; Gershey *et al.*, 1977) and fluorometric oxidation methods (Schantz *et al.*, 1961; Wong *et al.*, 1971; Bates *et al.*, 1978; Buckley *et al.*, 1978; Shoptaugh *et al.*, 1981; Ikawa *et al.*, 1982; Oshima *et al.*, 1984; Mosley *et al.*, 1985) have also been developed for the detection of PSP in shellfish tissues. These tests are nonspecific in nature and suffer from inadequacies in terms of sensitivity and specificity.

HPLC was used to separate saxitoxin from its other reaction products (Rubinson, 1982), and this technique was further improved by post-column derivatization (Onoue *et*

al., 1983; Sullivan and Iwaoka, 1983) and by pre-chromatographic oxidation (Slater *et al.*, 1989; Lawrence and Ménard, 1991) for the separation of a wide array of PSP toxins. Even though the HPLC techniques separate most of the PSP derivatives and are relatively sensitive, they are time consuming, require rather expensive instrumentation and rely on relatively difficult pre- or post-column derivatization procedures.

Various expensive electrophoretic (Fallon and Shimizu, 1977; Pleasance *et al.*, 1992; Locke and Thibault, 1994) and time consuming TLC methods in combination with peroxide oxidation (Shoptaugh *et al.*, 1981; Noguchi *et al.*, 1986) have been used to detect PSP toxins, although C toxins which are common in Atlantic shellfish, were not recorded using any of these TLC methods.

TLC with Iatroscan flame ionization detection (FID) has been commonly used for the analysis of lipid classes (Ackman, 1981; Parrish *et al.*, 1988), vitamins, amino acids and a variety of other lipophilic compounds (Ranny, 1987). The TLC/FID technique has also been used for the detection of tetrodotoxins in biological fluids (Ikebuchi *et al.*, 1988), although this technique has never been used for the detection of PSP toxins. Iatroscan analyses have several advantages over classical planar thin-layer techniques including simple, rapid and more accurate quantitative analysis, thus making it a natural choice for the analysis of PSP's even though they are usually present in very low concentrations. It should be noted that the core element of PSP toxins includes nitrogen atoms, and some may be found in the various groups that create the different toxins. The recent development of a flame thermionic detector (FTID) for the Iatroscan enables the detection of nitrogenous compounds with much higher sensitivity than previously possible with the FID (Parrish, 1988). The presence of up to 7 nitrogen atoms in the various PSP toxins suggests that FTID detection may provide improved sensitivity as compared to existing methods of PSP detection.

The objectives of this study were to examine the feasibility of using the Iatroscan with FTID to detect PSP toxins, and to attempt to identify at least the major toxin compounds by TLC/FTID.

4.3 Materials and Methods

4.3.1 Detection by FTID

The Iatroscan (TH-10) Mk-5 (Iatron Laboratories, Inc., Tokyo, Japan) with a FTID (DETector Engineering & Technology, Inc., Walnut Creek, CA) was used to detect PSP toxins and the peak response was determined by integrating the peak area (Appendix A.I). Operational conditions for the FTID were determined in preliminary studies using phosphatidylcholine (PC) which is non-volatile and contains one nitrogen atom per molecule. Minimum peak width of detection (MWD), tangent triggering percentage (TTP) and maximum noise level (MNL) were varied to improve the baseline, and the effect of variation in H_2 and air flow rates, detector current and scan speed were studied for PC prior to testing with STX, neosaxitoxin (NEO), gonyautoxin 2 and 3 (GTX 2/3), and C toxins (C1/C2).

Typically, about 60 - 300 ng/μL of individual PSP compounds (STX, NEO, GTX 2/3 and C1/C2) and PC were spotted on silica gel coated rods (Chromarods-SIII) with a Hamilton syringe and after oven drying for about 10 min, the rods were scanned initially with constant air flow rate of 1.5 L/min, varying the H_2 flow rate from 50 to 100 mL/min and the detector current from 2.6 to 3.3A. FTID and FID responses were obtained in the same manner keeping the air flow rate constant at 1.5 and 2.0 L/min while varying the H_2 flow rate and detector current from 50 to 100 mL/min and 2.6 to 3.3A, respectively. Once the optimum H_2 flow, air flow and detector current levels were obtained, the effect of scan time on the FTID response was studied while varying the detector current from 2.6 to 3.3A at constant H_2 and air flow rates of 50 mL/min and 1.5 L/min, respectively.

4.3.2 Standards and Toxin Samples

Commercial standards of PSP toxins (STX, NEO, GTX 2/3 and GTX 1/4) and purified B1 and C1/C2 toxins were obtained from the National Research Council (NRC), Halifax, Nova Scotia. Toxins were also extracted from cells of *Alexandrium excavatum* and scallop digestive glands. Concentrated *A. excavatum* cells were ultrasonicated with an equal amount of water for 30 min, and 16 g of scallop homogenates were homogenized

for 10 min at high speed settings using a Polytron homogenizer. The extracts were centrifuged at 6000 r.p.m for 30 min, the supernatants were defatted and the separated aqueous layers were filtered through 1000 da MW cutoff membranes prior to examination by TLC/FTID. Partially purified toxins rich in NEO and STX extracted from a strain of *Alexandrium tamarensis*, were also obtained from NRC and used for TLC/FTID. Toxin profiles of each extract were obtained by HPLC (Sullivan and Wekell, 1987).

4.3.3 Separation of Toxins on Chromarods-SIII

A series of 35 different solvent systems, including the pyridine-ethyl acetate-based systems conventionally used in planar TLC for the separation of PSP compounds, were tested for individual toxins. A chloroform-methanol-water based system was finally chosen to elute individual components of PSP. Initially about 0.2 μL of each compound (commercial standards) were individually spotted on the pre-scanned Chromarods-SIII and developed in chloroform : methanol : water : acetic acid (30:50:8:2) for 55 min. The rods were then oven dried at 105°C for about 10 min, and scanned at 40 s/rod using a H_2 flow rate of 50 mL/min, an air flow rate of 1.5 L/min and a detector current of 3.0A. Two microlitres of a mixture of toxin standards consisting of 4 ng STX, 12 ng NEO, 22 ng GTX 2/3, 13 ng C1/C2 were applied to each rod for scanning. In addition, 1.6 μL of an *A. excavatum* cell extract consisting of 22 ng C toxins, 3 ng GTX 2/3, 3 ng STX were also spotted and scanned. Two microlitres of NEO and STX-rich *A. tamarensis* extract (16 ng NEO and 14 ng STX) were also applied, developed and scanned in the same manner. Two microlitres of scallop extract consisting of 26 ng GTX 2/3, 5 ng STX, 5 ng NEO and 12 ng C toxins, were initially developed in acetone (100%) for 30 min, dried at 105°C for about 10 min and partially scanned under similar conditions. Chromarods were then developed in chloroform : methanol : water : acetic acid (30:50:8:2) for 55 min, dried and fully scanned as above.

4.3.4 Calibration

Different solution volumes (0.2 - 1.2 μL) of STX (850 μg/mL), NEO (100 μg/mL), GTX 2/3 (6.34 μg/mL), GTX 1/ 4 (113 μg/mL), B1 (0.15 mg/mL) and C toxins (C1 /C2, 0.1 mg/mL) were individually spotted on pre-scanned rods, developed in chloroform : methanol : water : acetic acid (30:50:8:2) for 55 min, oven-dried for about 10 min, and scanned at 40 s/rod, a detector current of 3.3A and H_2 and air flow rates of 50 mL/min and 1.5 L/min, respectively. Calibration curves were compared by analysis of covariance (ANCOVA) via multiple regression using indicator variables in MINITAB version 11.21 (APPENDIX A.c).

4.4 Results and Discussion

The FTID/FID detection system for the Iatroscan (Mk-5), consists of a combination of FID and FTID detectors in series. The FID provides a universal response to virtually all organic compounds, whereas the FTID provides specific responses primarily only to compounds containing nitrogen and/or halogen atoms (Patterson, 1988). In the present study, PC and all PSP individual standards had significantly higher FTID responses ($p<0.01$) than the FID responses, especially at any given level of detector current and scan time, and low levels of hydrogen and air flow. Under optimum conditions, the sensitivity of the FTID for pure toxin standards was 1, 5, 5, 2, 6 and 6 ng for NEO, STX, B1, GTX 2/3, GTX 1/4 and C1/C2, respectively. However, the method detection limits for toxic shellfish tissues using the current extraction protocol, gave significantly poorer sensitivity. In comparison with the quarantine limit of 0.8 µg STX equivalent per g edible tissue, the method detection limits for NEO, STX, GTX 2/3 and C1/C2 were 0.8, 2.1, 0.4 and 2.5 µg/g tissue, respectively. Although the sensitivity is perhaps not as high as that reported using other techniques, the findings reported here should be considered as preliminary and no doubt may be improved by manipulation of a number of parameters such as detector current, scan time, hydrogen and air flow rates and the solvent system.

4.4.1 Baseline

The factors such as flow rates of H_2 and air, detector current, amplitude, age of the rods, cleanliness of the rod, and the sample load affected the baseline. High H_2 (120 mL/min) and air flow (2.8 L/min) levels recommended by the operational instruction manual resulted in high noise levels. It became necessary to alter levels recommended by the manufacturer for MWD, TTP and MNL to 1%, 1.2% and 2 mm in order to achieve a baseline with minimum noise when scanning PC or PSP. These settings were often different from those recommended by the manufacturer.

4.4.2 Detector Current and H_2/Air Flow

The major parameters which control the magnitude of the FID/FTID signal are the H_2 and air flow, and the scan rate of the Chromarod (Ranny, 1987). The variations in H_2 flow change the thermochemistry of the flame - Chromarod interaction, and this affects both the vaporization and ionization efficiencies of the sample on the rod.

The FTID responses to individual toxins were also influenced by the variation in H_2 and air flow rates as well as detector current levels. The FTID responses to all toxins tested were similar with regard to the effects of detector current and H_2 flow for any fixed air flow rate (see Fig. 4.1, for example). When the detector current and H_2 flow were changed from 2.8 to 3.3A and 100 to 50 mL/min respectively, while the air flow rate was maintained at 1.5 L/min, the FTID response to STX increased exponentially from 2.1 to 182 mV.mm (Appendix A, Fig.4.I-III). STX was not detectable at detector currents below 2.8A due to a noisy baseline. However, when the air flow rates were increased from 1.5 to 3.0 L/min, the exponential relationship became more linear (Appendix A, Figs. VII-VIII). The maximum FTID response recorded for STX using the air flows of 2.0 and 3.0 L/min (detector current of 3.3A and H_2 flow rate of 50 mL/min) were 125 and 86 mV.mm, respectively. It is interesting to note that the peak response increased rapidly, at any air flow rate (from 1.5 to 3.0 L/min), when the detector current was increased from 2.6 to 3.3A with the simultaneous decrease of H_2 flow from 100 to 50 mL/min.

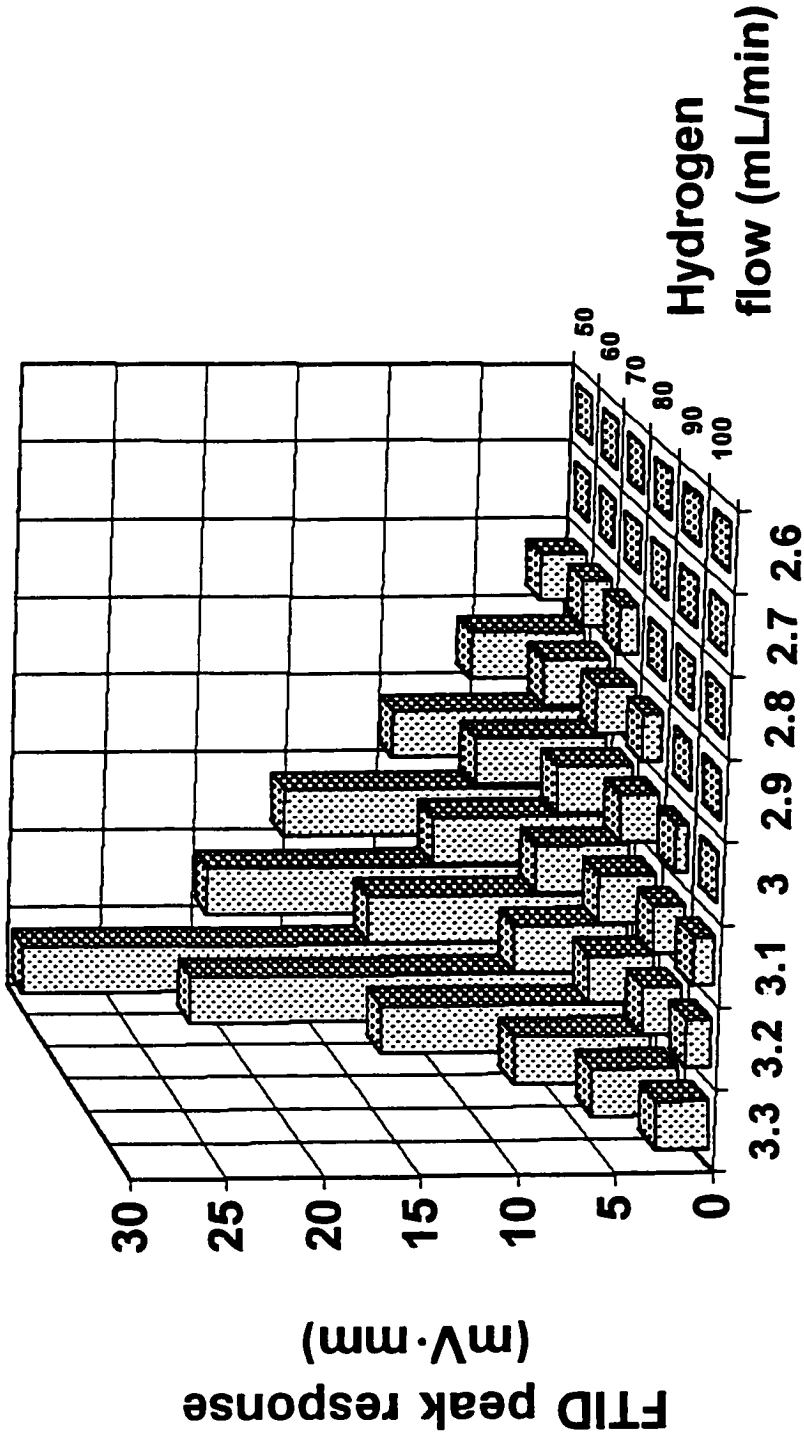


Fig. 4.1 Effect of H_2 flow and detector current on the FTID peak response to NEO (air flow = 1.5 L/min)

The FTID response to NEO, GTX 2/3 and C1/C2 followed the same general pattern with regard to detector current (Appendix A, Figs. 4.IV-XI), H_2 and air flow rates except that the response was 4-6 times lower for NEO than for STX. NEO, GTX 2/3 and C1/C2 also could be detected only at detector current settings ≥ 2.8A due to excessive noise, and H_2 flow rates above 70 mL/min had to be maintained. Air flow rates ≥3.0 L/min further limited the sensitivity of the detector.

4.4.3 Scan Time

Varying the scan times for the rods mainly affects the efficiency of vaporization of the sample. Once the optimum operational conditions of the Iatroscan Mark 5 FTID were established, the effect of scan time on sensitivity was studied using H_2 and air flow rates of 50 mL/min and 1.5 L/min, respectively, while varying the current from 2.6 to 3.3A.

STX was detectable with a detector current as low as 2.6A when the scan time was increased from 25 to 60 s/rod. Increasing scan times improved detector response up to a point. For STX, increasing scan times dramatically improved FTID sensitivity and this effect was most apparent at high detector current levels (2.9 - 3.2A, Fig. 4.2). However, if the scan time was extended too long (>50 s/rod) at high detector current settings, there was a reduction of the FTID response which was probably due to rapid disappearance of combustion products from the flame. High temperature resulting from high current (3.3A) and short scan times (50 - 60 s/rod) reduced the efficiency of rods in subsequent scannings. Therefore, moderate scan times such as 40 s/rod should be selected for higher responses in routine Iatroscan use.

4.4.4 Separation of PSP Toxins/Solvent Systems

By examining the development of individual PSP toxin standards in different solvent systems, the chloroform : methanol : water : acetic acid (30:50:8:2) system was found to be the best to separate at least the major PSP components (Table 4.1). Although pyridine, ethyl acetate, acetic acid and water in varied proportions were used to separate

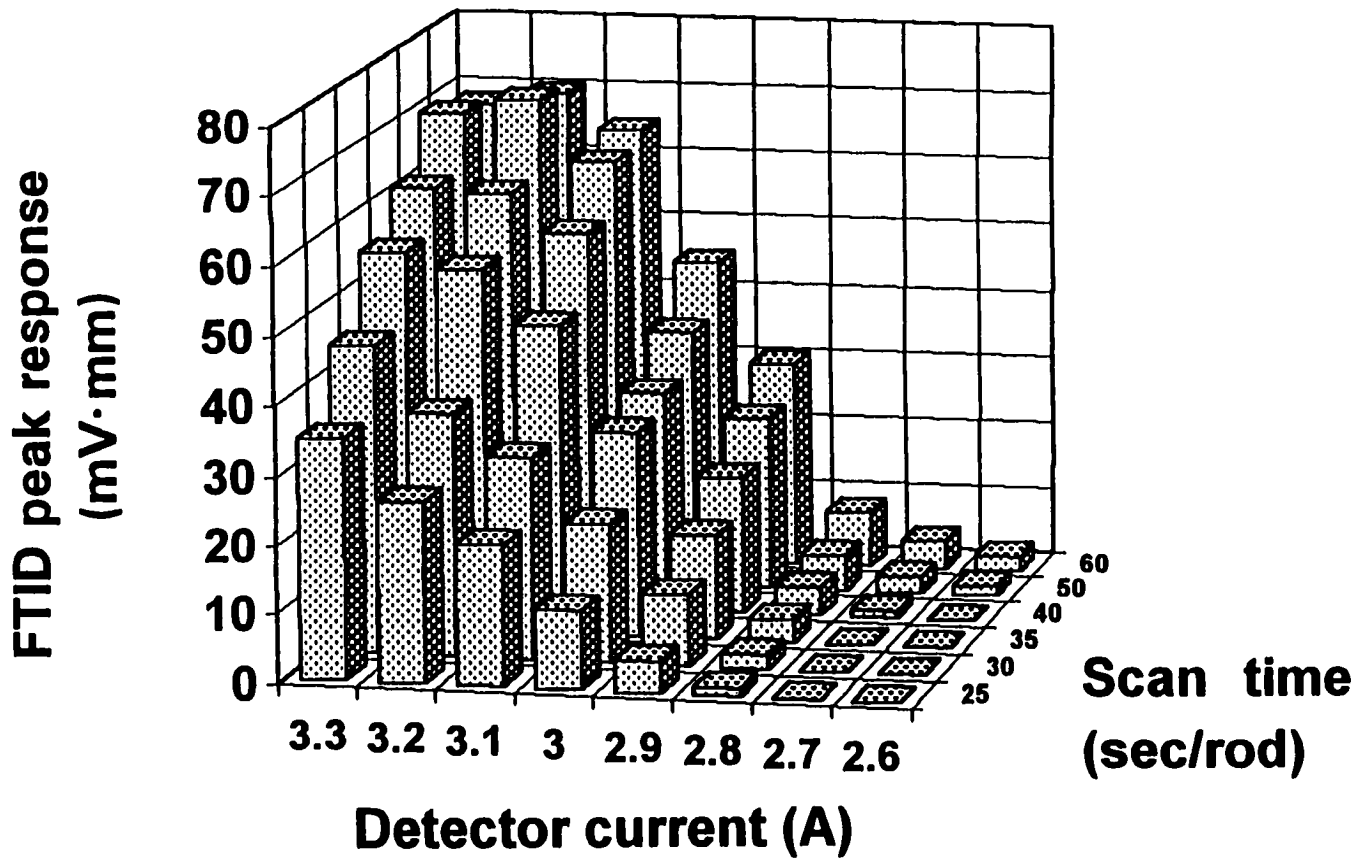


Fig. 4.2 Effect of scan time and detector current on STX response
(air flow = 1.5 L/min, H_2 flow = 50 mL/min)

Table 4.1 Relative mobilities of PSP individual components on Chromarods-SIII, developed in different solvent systems

Solvent system	Relative mobilities
Pyridine : ethyl acetate : water (45:15:18:12)*, Pyridine : ethyl acetate : water : acetic acid (45:15:10:5)*	All toxins co-eluted
Methanol : water : chloroform : acetic acid (45:45:10:5)	All toxins co-eluted
Acetic acid : water (40:40)	All toxins co-eluted
Ethyl acetate : water : formic acid (40:40:5)*	All toxins co-eluted
Methanol : ethyl acetate : water (35:35:10)	All moved partially from NEO and STX. NEO and STX did not move
Methanol : ethyl acetate : water (40:20:20), Methanol : ethyl acetate : water:formic acid (35:35:10:2), Methanol : ethyl acetate : water (35:40:5)	All toxins co-eluted
Methanol : ethyl acetate : water (35:45:10)	None moved
Butanol : methanol : water : ammonium hydroxide (40:20:20:2) or (35:45:10:10)	Very noisy base line and none moved. Double development didn't improve.
Chloroform : methanol : water : isopropanol (35:45:8:2)*	GTX's and C's eluted together and partially moved from NEO and STX
Chloroform : methanol : water (70:30:5)	None moved
Chloroform : methanol : water (40:40:20)	All toxins co-eluted
Chloroform : methanol : water (35:45:8)	GTX's separated from NEO and STX. NEO and STX didn't move

Chloroform : methanol : water : acetic acid (30:50:8:2)*	C's separated from GTX's and GTX's completely separated from NEO and STX. NEO partially separated from STX. STX didn't move
Pyridine : water : ethyl acetate : acetic acid (70:30:25:15)*	All toxins co-eluted
Water*	Only C's separated and GTXs, NEO, STX moved together
Methanol : water (50:50)*	All toxins co-eluted
Chloroform : methanol : water : acetic acid (40:40:8:2)*	C's partially separated from GTX's and GTX's completely separated from NEO and STX. NEO partially separated from STX. STX didn't move

* Same solvent system was used on Chromarods-A, but the resolution was not improved, in most cases even worse than Chromarods-SIII.

STX from other toxins by TLC (Noguchi *et al.*, 1986), a complete separation of all 6 toxins on Chromarods could not be achieved when the development was attempted in pyridine : water : ethyl acetate : acetic acid (70:30:25:15) for 1.5 h.

When standard toxin mixtures were spotted and developed in a chloroform : methanol : water : acetic acid (30:50:8:2) solvent system for 1 h, C1/C2 eluted to the solvent front (Fig. 4.3) followed by GTX 2/3 but it was difficult to achieve a complete separation of NEO from STX. Although GTX 2/3 were eluted as one sharp peak followed by a small broad peak, these peaks could not be able to be distinguished individually. However, GTX 2/3 were well separated from NEO and STX, and NEO moved very close to STX which remained at the origin. It is also interesting to note that when these co-spotted samples were developed only in H_2O for 50 min, C1/C2 moved ahead of GTX 2/3 which co-eluted with NEO and STX.

C toxins extracted from *A. excavatum* eluted to the top of the solvent front (Fig. 4.4) when developed in chloroform : methanol : water : acetic acid (30:50:8:2) for 55 min as in the case of C1/C2 standards. NEO derived from *A. tamarensis* partially separated from STX which did not move in this solvent system (Fig. 4.5). Toxic scallop homogenates were first developed in acetone, and some unknown compounds, possibly hydrophilic phytopigment derivatives, were eluted (Fig. 4.6A). After the partial scanning of these compounds, rods were developed in chloroform : methanol : water : acetic acid (30:50:8:2) to separate C toxins from GTX 2/3 (Fig. 4.6B). NEO and STX were present in relatively small amounts in the scallop extracts. It was observed that the cleanliness of the rods and the sample is critical since the baseline noise can be increased with any contaminating nitrogenous or halogenated compounds.

4.4.5 Calibration

Curvilinear calibration curves relating peak areas to amounts of spotted authentic standards were obtained for PSP toxins, and the calibration equations were "best fitted" using quadratic models. Some typical calibration curves are shown in Fig. 4.7, and calibration equations for PSP compounds are given in Table 4.2. However, quantification

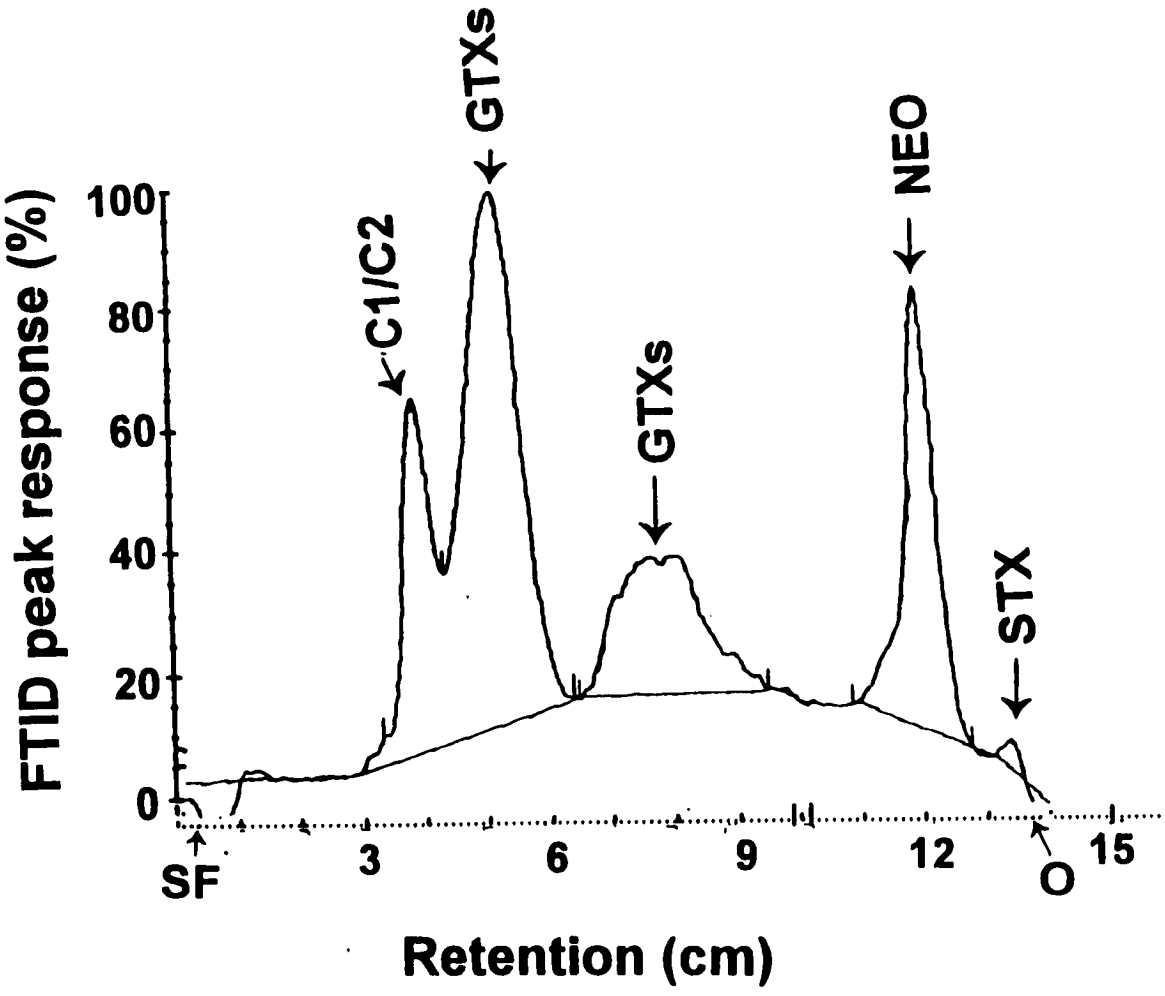


Fig.4.3 Chromatogram of a mixture of standard PSP toxins developed in chloroform : methanol : water : acetic acid (30:50:8:2) (air flow = 1.5 L/min, H_2 flow = 50 mL/min, detector current = 3.3 A, SF = solvent front, O = origin)

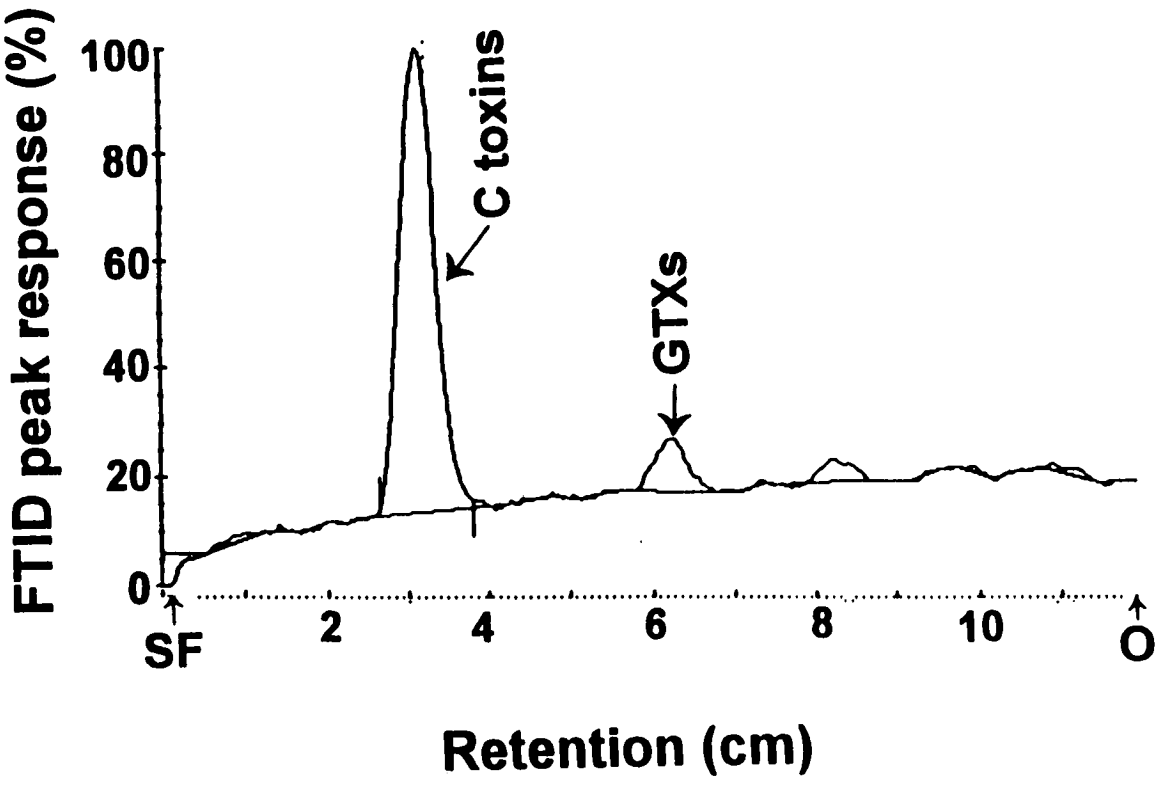


Fig. 4.4 Chromatogram of PSP toxins in *Alexandrium excavatum* cell extracts developed in chloroform : methanol : water : acetic acid (30:50:8:2) (same conditions as in Fig. 4.3) SF = solvent front, O = origin)

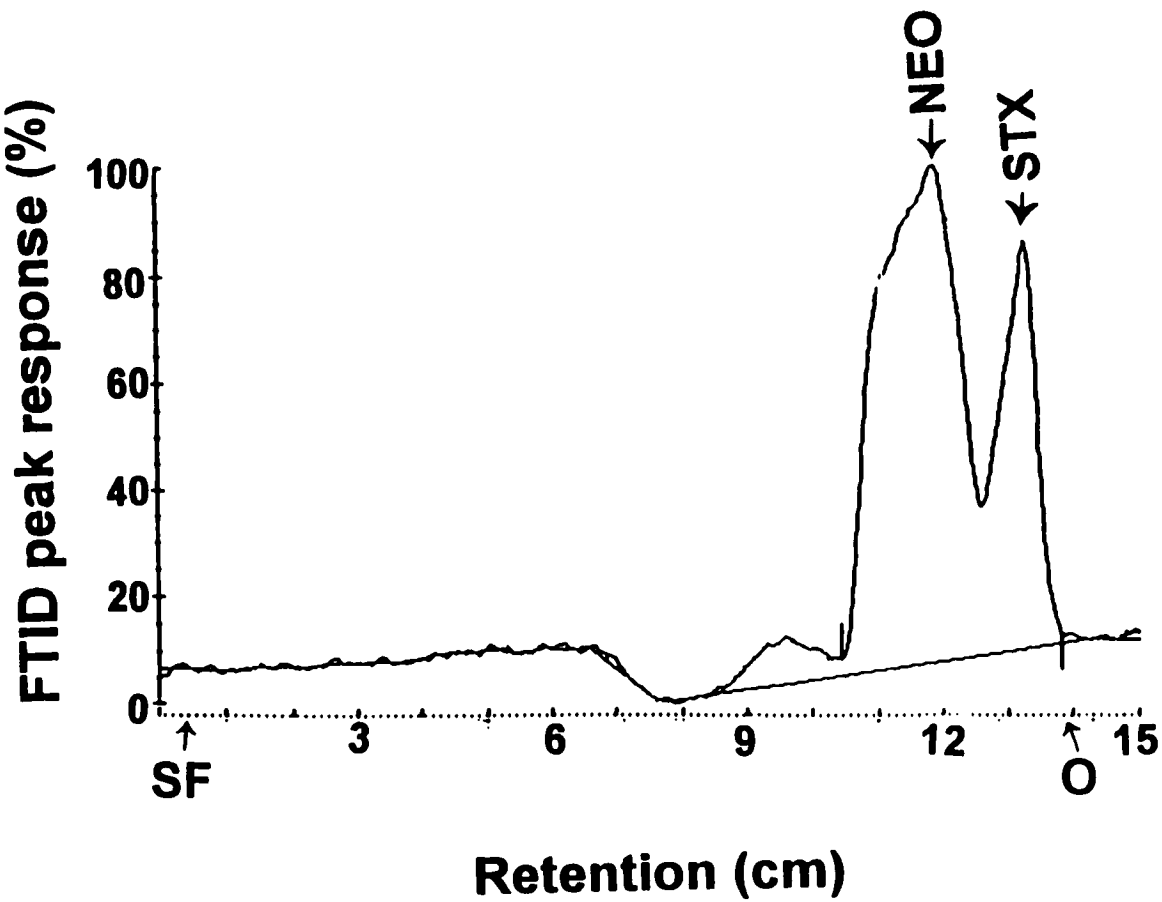


Fig. 4.5 Chromatogram of PSP toxins in partially purified mixture of STX and NEO extracted from *Alexandrium tamarensis* after development in chloroform : methanol : water : acetic acid (30:50:8:2) (same conditions as in Fig. 4.3 SF = solvent front, O = origin)

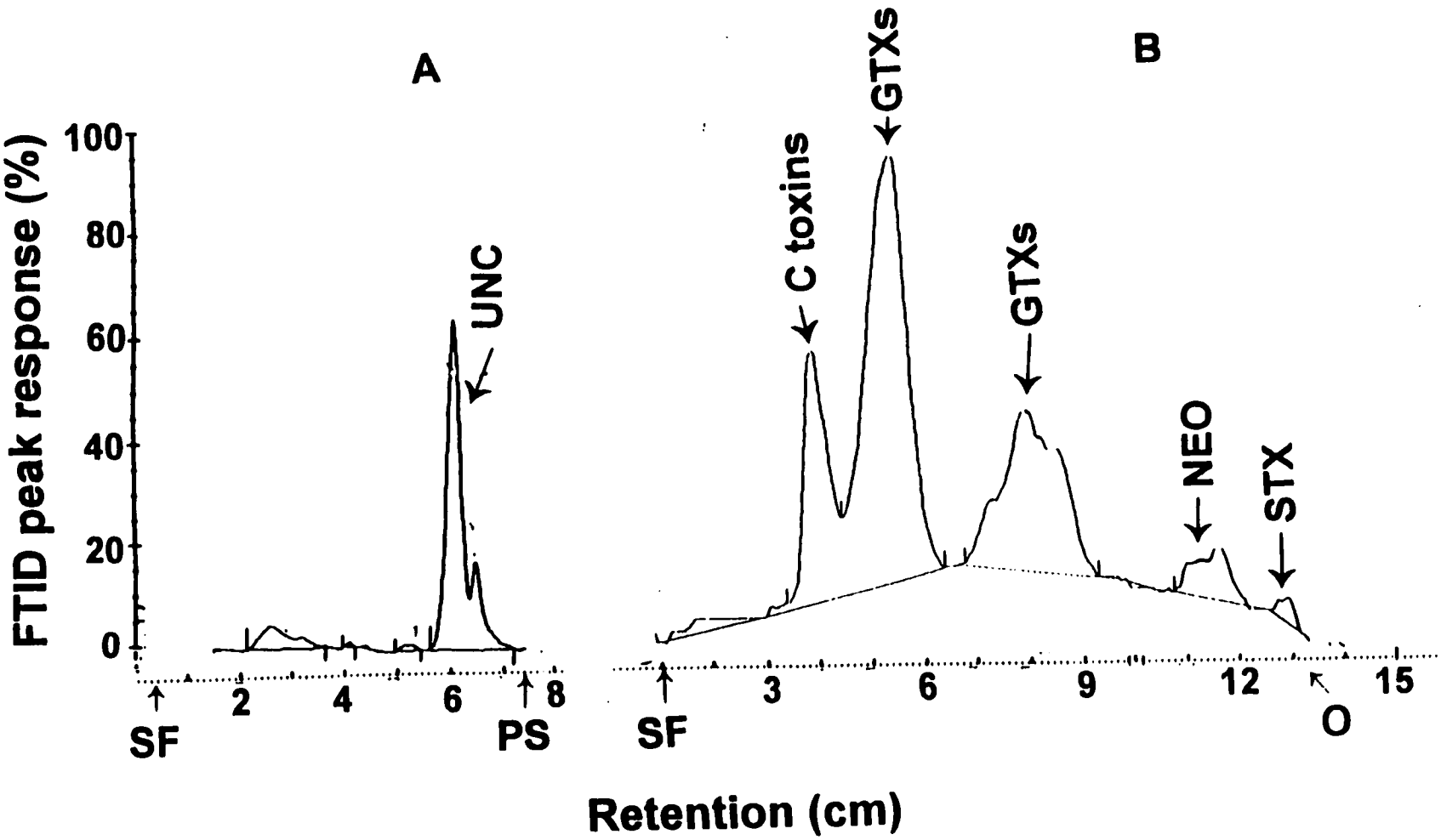


Fig. 4.6 Chromatogram of PSP toxins in scallop digestive glands **(A).** Partial scan (PS) after development in acetone **(B).** Full scan after development in chloroform : methanol : water : acetic acid (30:50:8:2) (same conditions as in Fig. 4.3, UNC = unknown compounds SF = solvent front, O = origin)

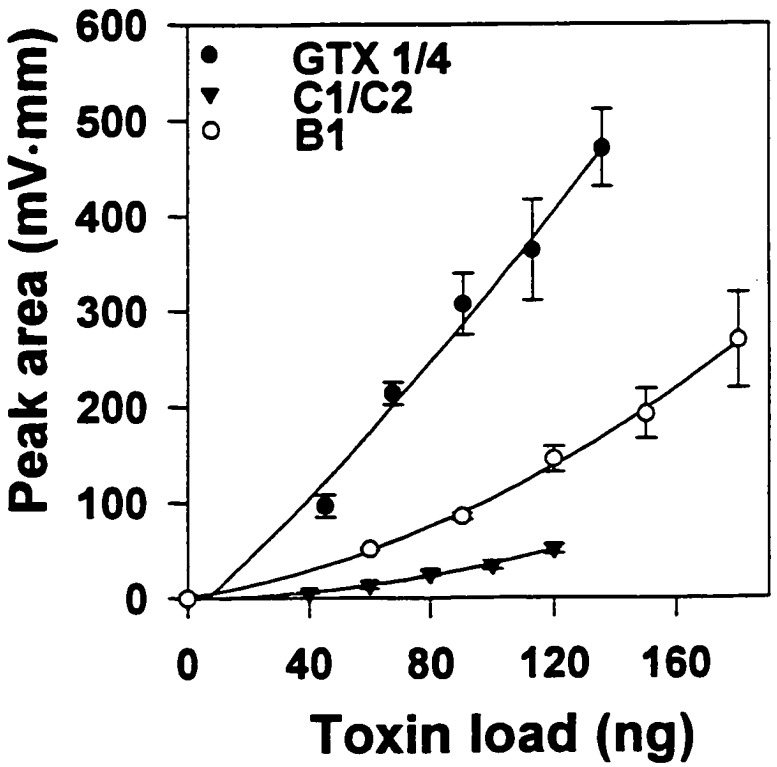


Fig.4.7 Calibration curves for GTX1/4 , C1/C2 and B1 based on the FTID peak response for different amounts of toxins (Vertical lines show upper and lower 95% confidence limits, n = 30)

Table 4.2 Calibration equations for PSP toxins

Type of toxin	Amount of toxin spotted (range, ng)	Regression equation (x=amount of toxin (ng), y=log FTID response (mV.mm))	R^2
B1	0 - 180	$y=0.7+0.5+0.01x^2$	0.94
C1/2	0 - 120	$y=0.8+0.6x+0.003x^2$	0.97
GTX 1/4	0 - 136	$y=-20+2.9x+0.01x^2$	0.95
GTX 2/3	0 - 7	$y=-0.2+0.6x+0.1x^2$	0.94
NEO	0 - 12	$y=-0.01+0.4x-0.2x^2$	0.95
STX	0 - 51	$y=-2.6+1.5x-0.01x^2$	0.98

of very low concentrations of any PSP component in a mixture of toxins may not be very accurate due to the nature of broad peaks and noise. The separation technique should be further improved to reduce background noise.

The slopes of linearized FTID responses of STX, NEO, GTX 2/3 and C1/C2 clearly indicate that the FTID response for NEO was higher than that of STX at any given current, hydrogen and air levels (Appendix A.III). To date, other chemical detection methods have suffered due to poor sensitivity for the N-1-hydroxy compounds such as NEO, C 3/4, B2 and GTX 1/4. However, with the present technique, the N-1-hydroxy PSP compounds gave responses similar to or greater than those of STX, GTX 2/3 and C1/2.

4.4.6 Inter and Intrarod Variability

The FTID response for the same amounts of individual PSP compounds may vary not only from one Chromarod to another (interrod) but also for the same rod (intrarod) at different times. The coefficient of variation of the FTID response, which is the standard

deviation expressed as percentage of the mean for each rod, was calculated at low and high loading levels for each PSP compound to check inter- and intrarod precision (Table 4.3). It was found that the coefficient of variation of FTID response for different rods as well as for the same rod for each toxin was higher at low amounts of spotting than high amounts, indicating better precision at higher loading levels.

Table 4.3 Mean coefficient of variation of FTID response for inter- and intrarod precision at two different loading levels of PSP toxins

Type of toxin	Interrod coefficient of variation (%)		Intrarod coefficient of variation (%)	
	Low load *	High load**	Low load*	High load**
STX	4.3	1.4	0.5	0.1
NEO	11.1	4.2	2.2	2.0
GTX 1/4	16.7	8.4	3.2	0.8
GTX 2/3	13.8	10.8	1.2	1.0
B1	9.9	2.6	1.0	0.8
C1/2	7.4	5.5	1.8	0.9

*Low load: STX - 17 ng, NEO - 4 ng, GTX 1/4 - 45.2 ng, GTX 2/3 - 2.3 ng, B1 - 60 ng, C1/2 - 40 ng

**High load: STX - 51 ng, NEO - 12 ng, GTX 1/4 - 135.6 ng, GTX 2/3 - 7.0 ng, B1 - 180 ng, C1/2 - 120 ng

4.4.7 Other Factors Affecting Sensitivity

The sensitivity of FTID decreased as the number of operational hours increased. Maximum sensitivity was always achieved with a new detector. Detector response data presented here was always obtained with a "new" detector. Sensitivity was also adversely affected by the continuous use of high current levels. Although FTID responses were

highest at high detector current settings, settings above 3.0A are not recommended because it decreases the ionization efficiency of the detector and results in poor sensitivity. Also high current settings lead to more frequent detector replacement and continuous use of high current deteriorates the physico-chemical nature of the Chromarods resulting in poor resolution. It is also important to note that no two FTID detectors gave identical response sensitivities even if both were of the same age.

4.5 Summary and Conclusions

The Iatroscan Mark 5 equipped with FTID is capable of detecting nitrogenous lipophilic compounds such as PC and hydrophilic organic compounds such as PSP toxins. H_2 and air flows, scan speed and detector current affect the FTID response. Hydrogen and air flow rates of 50 mL/min and 1.5 L/min coupled with a detector current of 3.3A and scan time of 40 s/rod gave the highest FTID responses and were 25 and 130 times higher for PC and PSP toxins than signals achieved by FID. This chemical detection technique involves relatively inexpensive equipment (Iatroscan system), is fairly rapid (detects a minimum of 60 samples in 1 h, and more if multiple development chambers are used), using sample sizes as small as 0.2 μL, and the solvent system used may also be used to purify PSP toxins by TLC. All PSP components tested can be detected with more or less similar sensitivity. Standard toxin amounts as small as 1 ng NEO, 5 ng STX and B1, 2 ng GTX 2/3, 6 ng GTX 1/4 and C1/C2 can be detected by TLC/FTID, however the method sensitivity for extracts from tissue requires improvement. These detection limits can perhaps be further improved by increasing the detector current level. Two major advantages of the Iatroscan-Chromarod analytical system are the handling of 10 Chromarods as a unit, facilitating rapid screening of multiple samples, and the flexibility offered by a partial scan and redevelopment in the same or a different solvent system.

CHAPTER 5

THERMAL DEGRADATION OF PARALYTIC SHELLFISH POISONING TOXINS IN SCALLOP DIGESTIVE GLANDS

5.1 Abstract

Digestive glands containing paralytic shellfish poisoning (PSP) toxins were isolated from toxic scallops. Citrate/phosphate buffers with pH values ranging from 3 to 7 were added to achieve predetermined pH levels. The samples were heated at 90, 100, 110, 120 and 130°C using a computer controlled oil bath, and 3 tubes at each pH level were transferred into an ice bath immediately after predetermined heating times for up to 120 min. Both heated and unheated homogenates were analyzed for toxins qualitatively and quantitatively by high performance liquid chromatography (HPLC).

Gonyautoxin (GTX) 2 and 3, saxitoxin (STX), neosaxitoxin (NEO) and C toxins were identified by HPLC. All toxins were most sensitive to higher temperatures and higher pH values. However, under gentle heating conditions and low pH, GTX 2 and 3 increased slightly. One explanation for this could be the increased extraction efficiency by heating. However, the conversion of sulfocarbamate toxins to highly toxic carbamate toxins upon heating in the presence of acid known as "Proctor" enhancement, could be another possible explanation for the apparent conversion of C1 and C2 toxins to GTX 2/3. The increase in STX may possibly be due to the conversion of GTX 2/3 and NEO into STX. The kinetics of thermal destruction were qualitatively similar to the thermal destruction of microorganisms. That is, the log survival of heated toxins was inversely proportional to time of heating and log decimal reduction time inversely related to temperature of heating. Efficacy of thermal destruction was highly dependent on pH, with more rapid thermal destruction at higher pH levels. The levels of individual toxins in the homogenate and those generated during heating could be reduced significantly by heating at 130°C at pH 6-7. This work has been published in Food Research International (Indrasena and Gill, 1999a).

5.2 Introduction

Paralytic shellfish poisoning (PSP), a serious problem endemic to many of the temperate coastal areas of the world, results from the consumption of certain bivalves containing a group of structurally related neurotoxins of dinoflagellate origin. The sporadic and unpredictable outbreaks usually cause serious health hazards to humans and great economic losses to the seafood industry. Carbamate toxins such as saxitoxin, neosaxitoxin, gonyautoxins and the sulfocarbamate toxins such as C toxins and B toxins (Fig.1.1) are found in most of the toxic dinoflagellate types in varying amounts. These toxins have different toxicities, carbamate toxins being the most toxic whereas the sulfocarbamate toxins are the least neurotoxic (Schantz, 1986).

Various methods have been proposed for the elimination of PSP toxins from toxic bivalves. Medcof *et al.* (1947) and Quayle (1969) have reported a decrease in toxicity of molluscs after being subjected to the normal processes of home cooking such as boiling or frying. Prakash *et al.* (1971) studied the effect of three different home cooking methods for soft-shell clams and found that steaming and frying reduced toxicity more than boiling. However, the total toxicity after cooking for 40 min was reduced by only 70% whereas heat treatment such as canning decreased the total toxicity of scallops by 90% although the kinetics of thermal destruction were not reported. Blogoslawski and Stewart (1978) reported that an ozone treatment enhanced the elimination of PSP from contaminated bivalves. Noguchi *et al.* (1980 a and b) also found a reduction of almost 90% in canned scallops when the pH of the heating medium was 6.5-7.0. Asakawa and Takagi (1983) studied the degradation of PSP at 70-110°C and found an 87% reduction in toxicity at pH 8 after heating at 110°C for 30 min. Gill *et al.* (1985) were the first to report on the kinetics of thermal destruction of PSP over a wide range of heating temperatures, although only one pH (pH 6.8) was used for the thermal treatment of soft shell clams. Kinetics were first order, and 90% reduction of toxicity could be achieved by heating the toxic clams for 43 min at 132.2°C or 193 min at 104. 4°C (e.g., the '$D_{121.1°C}$' and 'z' values were 71.4 min and 42C° respectively).

When heating toxic scallops under pressure, Asakawa *et al.* (1986) found a conversion of toxins along with detoxification. Chang *et al.* (1988) studied the total toxicity in mussels, *Mytilus edulis* over a pH range of 1-6 but at restricted heating temperatures and times (100°C and 121°C). They found that the toxins were more stable at low pH values. The thermal degradation patterns of individual PSP toxins in water were studied by Nagashima *et al.* (1991b) who found that the thermal degradation of PSP components progressed as a first order reaction, at different rates, depending upon the component and temperature. Applying extrusion processing to detoxify PSP infested scallops, Ohta *et al.* (1992) found 81.6-82.2% reduction of toxicity in digestive glands at 130°C and 85.6-97.8% reduction at 170°C.

Studying the effect of commercial processing on the PSP content of naturally - contaminated *Acanthocardia tuberculatum*, Berenguer *et al.* (1993) indicated that the total toxicity of the raw material decreased by about 95% after canning in salt water (40g/L) at 115°C for about 45 min. They also indicated that salt content may play a role in the initial detoxification. When toxic lobster hepatopancreas contaminated with PSP was cooked (boiling and steaming), the total toxicity was reduced by 65% overall. The STX decreased by 60% whereas GTX 2/3 (combined) decreased by 90-100% (Lawrence *et al.*, 1994).

In North America, scallop adductor muscle is the major portion of commercial interest. However, in other parts of the world, it is not uncommon to consume scallop viscera and roe. PSP accumulates largely in the digestive gland of bivalves such as scallops and usually the adductor muscle contains the least amount of toxins (Maruyama *et al.*, 1983). Repeated freezing and thawing can result in the migration of PSP from a highly toxic to barely toxic tissue (Noguchi *et al.*, 1984). Shimizu (1984) showed that the least toxic sulfocarbamate toxins such as C toxins, may be converted to highly toxic gonyautoxins which may further be converted to even more toxic compounds such as saxitoxin and neosaxitoxin by heating in mild acid. Most Atlantic shellfish are rich in C toxins and the consumption of steamed mussels sprinkled with lime or lemon juice is a common culinary practice. In conventional canning of food it is common to use acid as an

additional "hurdle" in addition to heat in order to prevent the outgrowth of the bacterial pathogen, *Clostridium botulimum*. However, for acidified products, the presence of even small quantities of the least toxic PSP compounds in the raw material may pose a threat to consumers.

The present study was carried out to study the effect of a wide range of pH values (pH 3, 4, 5, 6 and 7) on the kinetics of thermal destruction for individual PSP toxins in scallop homogenate.

5.3 Materials and Methods

5.3.1 Preparation of Homogenates

Toxic scallops were transported in ice from Digby and Yarmouth, Nova Scotia, Canada to the laboratory in Halifax, and were frozen immediately at -35°C. They were thawed at 5°C prior to homogenization. Pieces of shells, sand and other debris were removed and the tissue was washed gently. The digestive glands were carefully removed from the rest of the tissues and were collected in a container surrounded by ice. They were homogenized for about 15 min at room temperature (25°C) in a Commitrol 3600 flake cutter (Urshel Corp., Valparaiso, Indiana), to produce a finely-divided paste. The homogenate was thoroughly mixed so that all sub-samples taken from the homogenate would contain identical amounts of toxins. The homogenate was stored as small portions in several polyethylene bags at -35°C until used for heating experiments.

5.3.2 Composition of the Homogenate

The proximate composition, amino acid composition, mineral composition, pH and salinity of the homogenate were determined as additional information (Appendix B).

5.3.3 Buffering and Heating

Six-mL homogenate samples were mixed with 6 mL aliquots of 1.2 M citrate/phosphate buffer adjusted to pH values ranging from 3 to 7, and were heated under nitrogen in a thermostatically controlled oil bath. Temperatures were monitored using

thermocouples inserted through the screw caps. Control samples were heated in the presence of distilled water. The oil bath was heated to the required temperature (90, 100, 110, 120 and 130°C), and three tubes of each pH were removed after 10, 20, 40, 60, 80, 100 and 120 min, and immediately transferred into an ice bath. Heated/cooled homogenates were removed for the extraction and analysis as quickly as possible.

5.3.4 Extraction of Toxins

The homogenates were stirred well for about 2 min and the contents transferred into a 25 mL beaker. The tubes were rinsed with double distilled, de-ionized water and contents to the beaker and macerated with a Polytron homogenizer for 10 min at the high speed setting (8). The macerated homogenates were centrifuged in plastic tubes for 30 minutes at 4,266 x g. Two hundred and fifty μL clear supernatant were transferred to 5 mL vials and stored at -35°C for subsequent toxicity measurements using the cell bio-assay test. The remaining supernatant from each tube was transferred to a 150 mL separatory funnel and de-fatted with dichloromethane:water (2:1). The aqueous layer of each funnel was carefully transferred into Teflon tubes, centrifuged at 4,266 x g and frozen at -35°C until used for subsequent toxin analyses by HPLC.

5.3.5 Analysis of Toxins by High Performance Liquid Chromatography

All samples were ultrafiltered through 1000 da MW cutoff membranes (MSI, MICRON Separation Inc., West Bord, MA 01581) to remove proteins, prior to HPLC injection. PSP's were determined with a Waters high performance liquid chromatograph equipped with two Model 510 pumps, a WISP auto injection system controlled via a system interface module and a Shimadzu Model Rf 535 fluorescence detector (338 nm excitation, 400 nm emission). Samples were run in the HPLC using a Whatman PRP-1 column (15cm x 4.1mm) packed with 10µm beads using a binary elution gradient (mobile phase A: water with 1mM hexane and heptane sulphonic acid, mobile phase B: acetonitrile with hexane and heptane sulphonic acid) according to Sullivan and Wekell (1987). The HPLC was equipped with a dual reagent post column reaction system. Post-column

derivatization was carried out by sequential mixing with nitric acid (0.75M) and periodic oxidant (5N sodium hydroxide, 5mM periodic acid and 0.5M ammonium hydroxide) prior to the fluorescent detection. Individual toxins were identified by running a standard mixture of PSP toxins and were quantified using authentic PSP toxin standards whose concentrations were given as weight of free base in 0.1N acetic acid (μg toxins/mL) (batch No. 1), obtained from National Research Council, Halifax, Nova Scotia, Canada. Theoretical specific toxicities of individual toxins quantified by HPLC were calculated according to Schantz (1986) on the basis of specific toxicities using the following conversion factors:1 mg STX = 5500 mouse units (MU), 1 mg NEO = 5000 MU, 1 mg GTX2 = 2500 MU, 1 mg GTX3 = 2000 MU.

5.3.6 Kinetics of Thermal Destruction

All analyses were performed in triplicate and the decimal reduction times and z values for each toxin component were calculated according to Stumbo (1973). The best fitted mathematical model for the destruction of each PSP component was determined using MINITAB7 Version 11.21 for Windows in the Dalhousie University (DalTech) main frame computer. The destruction of toxins at different time intervals was compared statistically by analysis of variance (ANOVA) using the SAS statistical package.

5.3.7 Cell Bio-assay for Toxicity

The toxicities of the homogenates heated at 130°C for 10, 20, 40, 60, 80, 100 and 120 min at pH values 3, 4, 5, 6, 7 and non-buffered (NB) homogenate (pH = 5.8) were measured by automated end point determination using *in vitro* tissue cultured mouse neuroblastosoma cells according to Jellet *et al.* (1992) at Jellet Biotek Ltd. (Dartmouth, Nova Scotia, Canada). These assays were performed to confirm the accuracy of the HPLC method of toxin quantification. One sample from each pH was used for the bioassay and a simple linear regression was performed to obtain the r^2 of the relationship between the two methods (n=48).

5.4 Results and Discussion

The toxic scallop homogenate contained 71.12% (±2.34) water, 3.68% (±0.82) lipids, 10.52% (±2.42) ash, 8.82% (±1.23) protein (n=3), and glutamic acid, alanine and leucine were the most predominant amino acids; cysteine and glycine were present only in small amounts (Appendix B-Table 5.I). Triacylglycerols were the main lipid component in the homogenate followed by free fatty acids and other unknown oxidized compounds (Appendix B-Table 5.II). It also contained a wide array of minerals including iron (200 mg/kg), aluminum (84 mg/kg) and cadmium (54 mg/kg) (Appendix B-Table 5.III). The major toxins present in the scallop homogenate were identified chromatographically using authentic standards for comparison. The major toxins present in the unheated material included C toxins, GTX 2/3, NEO and STX at levels calculated as 144 (±6), 1247 (±24), 180 (±8) and 200 (±5) μg/100g (n=3), respectively.

5.4.1 Effect of pH

Most of the individual toxin levels gradually decreased with heating time at neutral pH. The C toxin levels decreased rapidly (Fig. 5.1B) initially upon heating while the gonyautoxin levels increased somewhat at low pH, indicating the possible conversion of some C1/2 toxins into more highly toxic carbamate toxins such as GTX 2/3. The conversion of these least toxic sulfocarbamate toxins to highly toxic carbamate toxins by mild acid hydrolysis is known as "Proctor" enhancement (Hall *et al.*, 1980). Although the complete conversion of pure compounds is expected at pHs around 2, some conversions of C1/C2 toxins to GTX 2/3 at pHs 3-4, as observed in this study, is possible. Laycock *et al.* (1995) also indicated that there was a rapid increase in the conversion of C1/2 toxins to GTX 2/3 when the pH of the mussel homogenate was decreased from 5.8 to 2 with a considerable amount of conversion at pH 3-4 and the highest conversion at pH 1-2. Chang *et al.* (1988) also noticed that the total toxicity of mussel hepatopancreas autoclaved at 121°C gradually increased when heated at pH 1 to 3 with the highest toxin content being developed at pH 3. It is perhaps tempting to suggest that another possible reason for the apparent increase in some toxin levels under certain conditions could be the release of

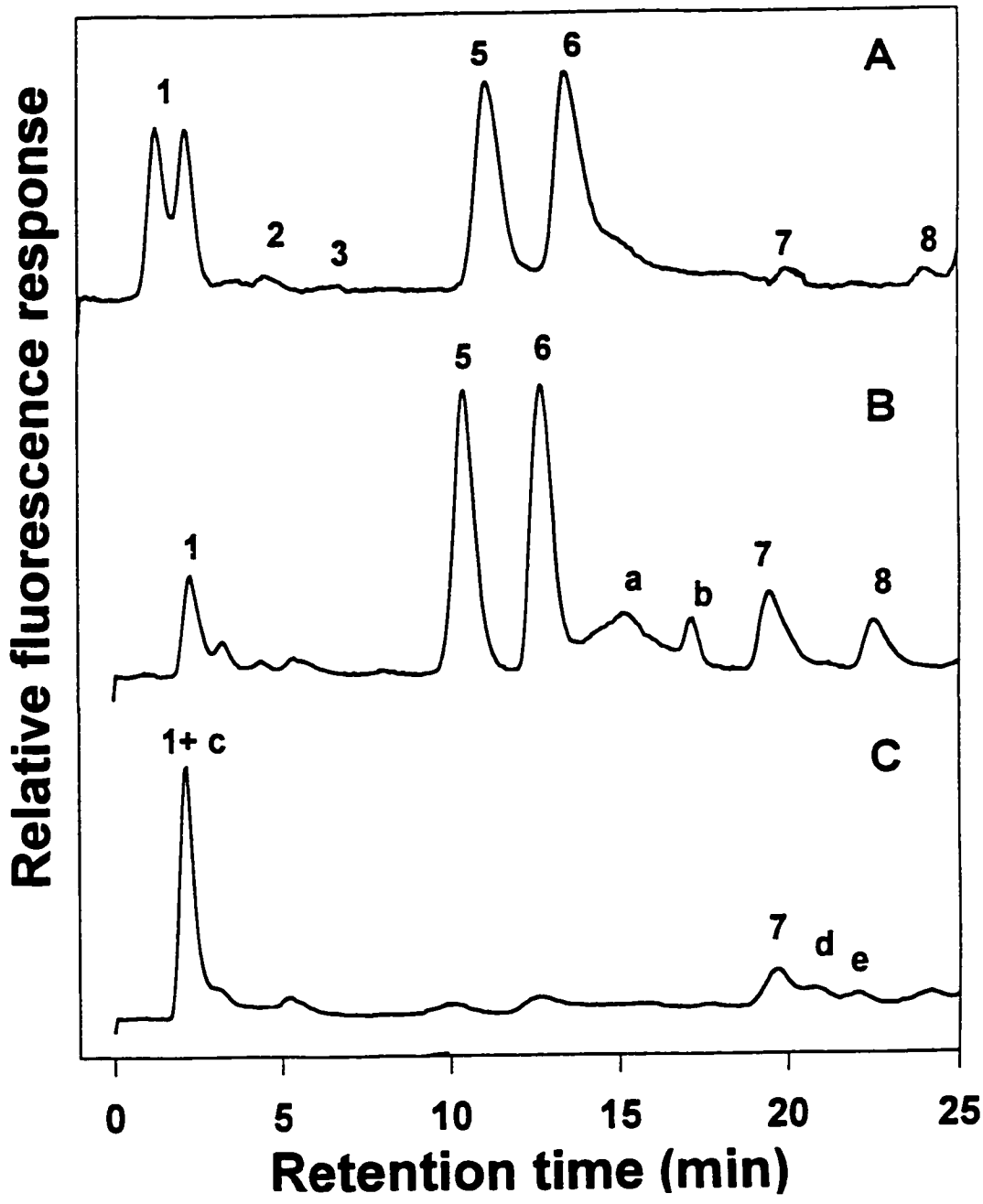


Fig 5.1 HPLC chromatograms of scallop homogenates with and without heating **A.** Initial homogenate prior to heating. **B.** Homogenate heated at 110°C at pH 3 for 40 min. **C.** Homogenate heated at 130°C at pH 7 for 60 min. (1 = C toxins, 2 = B2 toxin, 3 = GTX 4, 5 = GTX 3, 6 = GTX 2, 7 = NEO, 8 = STX, a-d = unknown compounds)

tissue-bound toxins as a result of heating. However, all of the PSP toxins have similar chemical structures, and therefore, one may expect that all of the toxins would be released from the tissue particles in the homogenates at similar rates. This was indeed not the case and the initial increases in toxin levels were only observed for the GTX 2/3, NEO and STX while the C toxins decreased under identical conditions.

Shimizu (1984) indicated that C1/2 and C 3/4 toxins may be converted to more potent toxins such as GTX 2/3 and GTX 1/4 by releasing the sulfonyl group. However, the C toxins apparently increased with prolonged heating at pH 7 (Fig. 5.1C), possibly due to the co-elution of some thermally degraded derivatives of carbamate toxins with the C toxins or conversion of degraded derivatives into C toxins. Many authors have shown that less-toxic, sulfocarbamoyl PSP's such as GTX 5 and 6 are easily converted into their highly-toxic carbamate counterparts such as STX and NEO on mild acid hydrolysis, resulting in an increase in toxicity (Kobayashi and Shimizu, 1981; Harada *et al.*, 1982; Nishio *et al.*, 1982; Hall and Reichardt, 1984).

When the homogenates were heated at 90°C and pH levels 3-4, GTX 2 and 3 levels slightly increased whereas at pH 5 and non-buffered homogenate (pH=5.8), they initially increased and then either decreased or remained at about the same levels as the unheated controls (Fig. 5.2A). This was in contrast to the data for pH 7 samples in which levels of GTX 2/3 were reduced by about 20% after heating for 2 hours. From a practical perspective, these data clearly indicate that heating at 90°C is not an effective means of detoxification of PSP regardless of the pH used.

The initial increase of GTX 2 and 3 was also observed within the first 10 min of heating at 110°C at pH values 3-5 (Fig. 5.2B), and the amount declined with time of heating. Again, the thermal destruction was more pronounced at higher pH levels. When the temperature was increased to 130°C (Fig. 5.2C), GTX 2/3 declined rapidly with temperature over the complete range of pH values (3 to 7) with the most rapid rates of thermal destruction being observed at higher pH levels (Appendix C- Figs. 5.I.A-B and Appendix D).

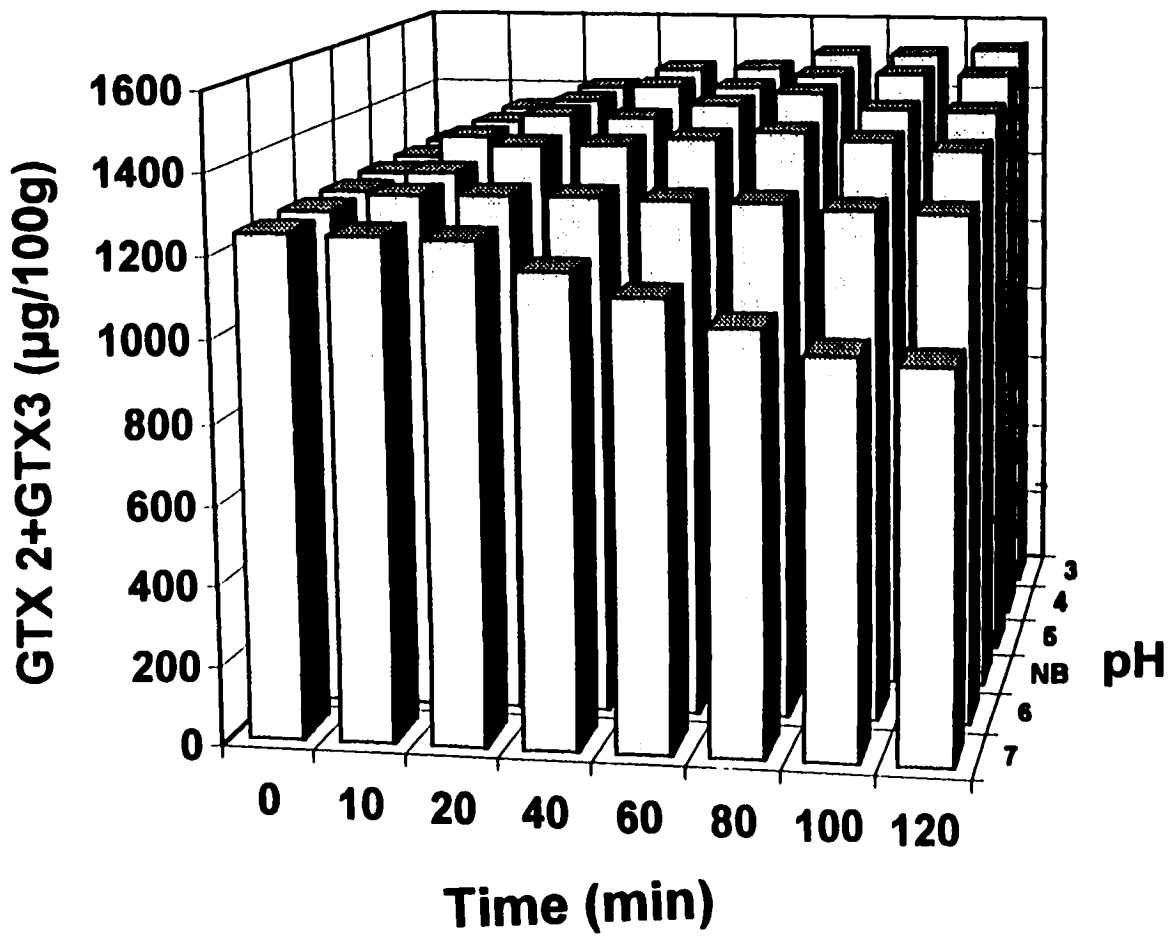


Fig. 5.2 A. Thermal degradation of GTX 2/3 in scallop homogenate heated at 90°C for different times and pH levels (each data point is an average of 3 analyses)

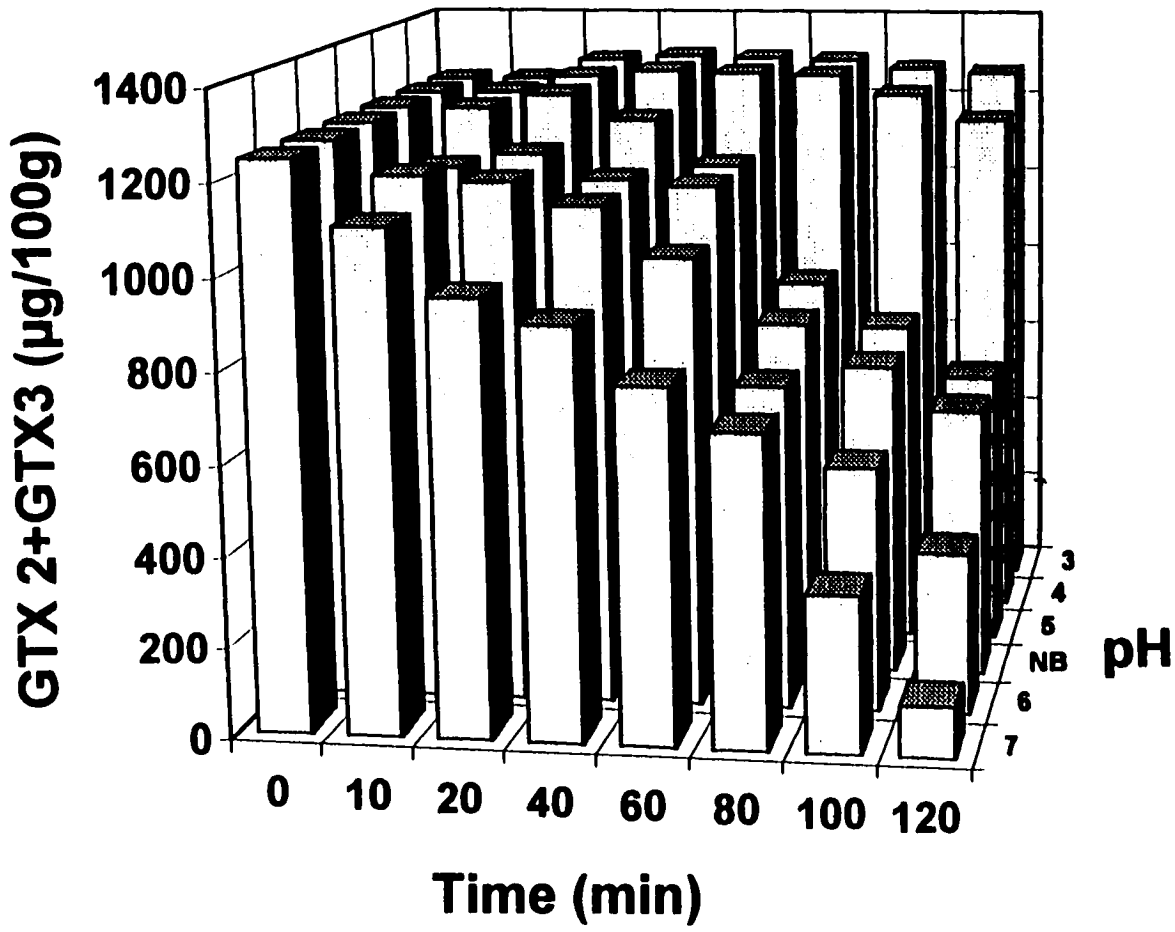


Fig. 5.2 B. Thermal degradation of GTX 2/3 in scallop homogenate heated at 110°C for different times and pH levels (each data point is an average of 3 analyses).

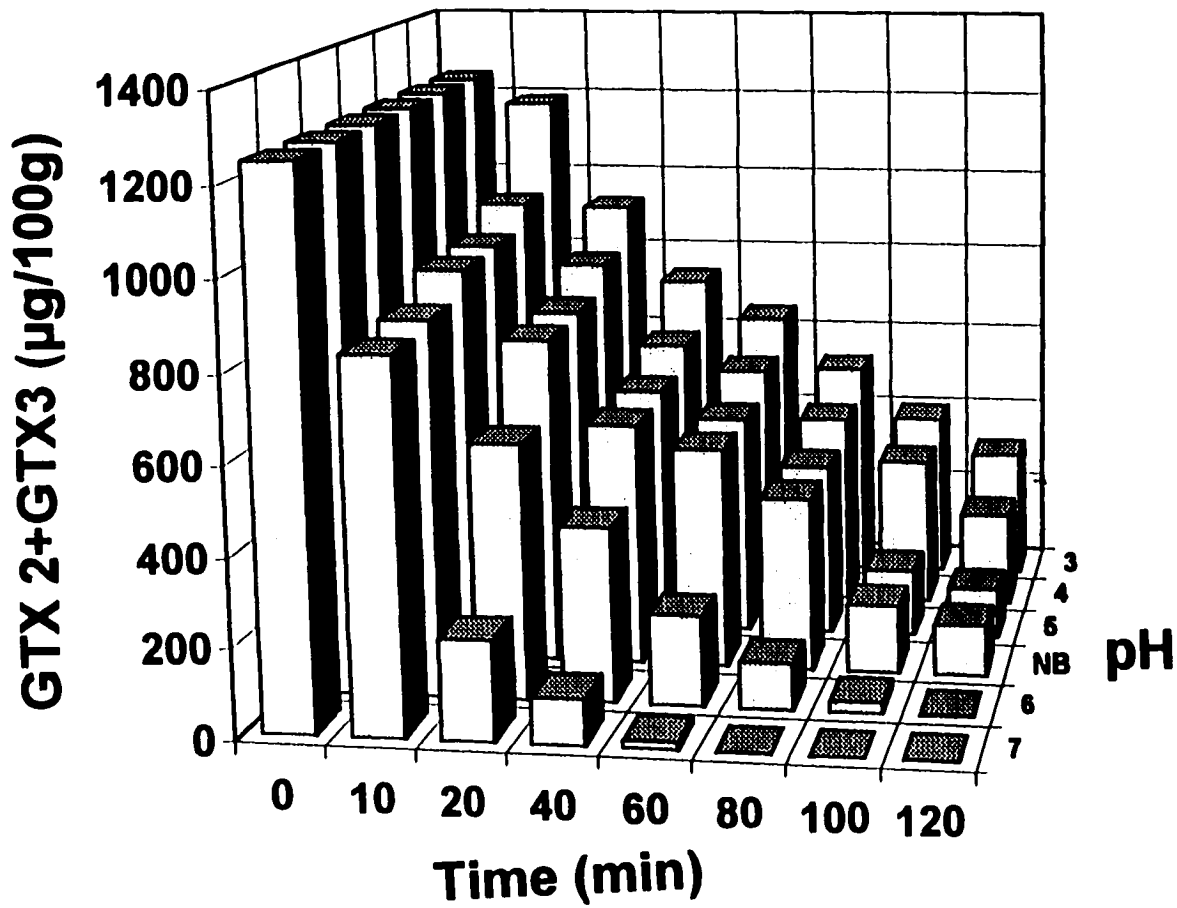


Fig. 5.2 C. Thermal degradation of GTX 2/3 in scallop homogenate heated at 130°C for different times and pH levels (each data point is an average of 3 analyses).

NEO and STX were found to be the most thermally stable toxins at all temperatures especially at low pH values (Appendix C, Figs.5.II.A-5.III.E). The amount of STX gradually increased at pH values 3-5 when the homogenates were heated at 90°C, whereas at pH 6, there was a slight decline followed by an initial increase which gradually declined through heating up to 120 min (Appendix C, Fig.5.III.A). This initial increase of STX may possibly result from the conversion of GTX 2/3 and NEO to STX. Even at 110°C, there was a slight initial increase of STX, especially at pH values 3-4 when heated for 40 to 60 min, and then levels declined with further heating. There was virtually no degradation of STX at pH values 3-4 at 90°C, even when heated for 120 min. Figure 5.3 illustrates that at 120°C, thermal destruction of STX and NEO is highly dependent on the pH, with higher rates of destruction at neutral pH. Also evident from Fig. 5.3 was the fact that the individual PSP toxins were destroyed semi-logarithmically (first order kinetics) as observed by Gill *et al.* (1985).

Shimizu (1988) indicated that STX is very stable in acidic solutions, and the hydrolysis of the carbamoyl ester can only occur in concentrated acid solutions, such as 7.5N hydrochloric acid at 100°C whereas NEO is not as stable as STX in acidic solutions and tends to decompose by reductive cleavage of the N-hydroxyl group to give STX. The data collected in this study also suggest that STX is more stable to heat and acid than NEO. Some unidentified peaks (Fig. 5.1) which eluted with C toxins, between gonyautoxin 2 and neosaxitoxin as well as after saxitoxin, may be due to the thermal destruction of major toxin derivatives in the homogenate. Nagashima *et al.* (1991) also found these extraneous peaks during the heating of PSP compounds. Since the homogenate contains a mixture of complex compounds including nucleotides, amines, amino acids, pigments and a variety of other biochemically active groups, many possibilities exist for their interaction with individual PSP compounds for the production of fluorescent derivatives during the HPLC analysis or during heating. Asakawa and Takagi (1983) also indicated that the rate of thermal degradation of PSP toxins may be affected by other compounds in the medium. The pH of the buffered samples did not change during heating, whereas unbuffered blank samples had changed the pH from 5.8 to

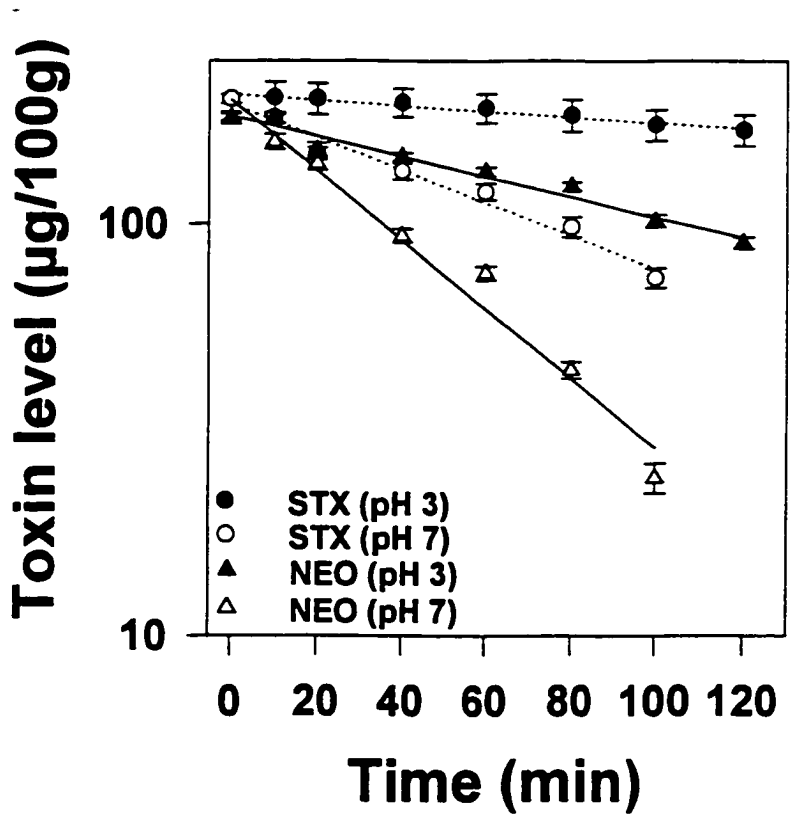


Fig 5.3 Thermal degradation of NEO and STX in scallop homogenate heated at 120°C for different times at pH 3 and 7 (each data point is an average of 3 analyses).

6.2 possibly due to the generation of amines during heating. However, this pH increase appeared to have little effect on the degradation patterns of individual toxins.

5.4.2 Kinetics of Thermal Destruction

Decimal reduction time (D) can be defined as the time (min) required to destroy 90% of a substance or organism at a given temperature, provided that the destruction is semi-logarithmic in nature. The D-value is not constant and varies with the physico-chemical nature of the components in the medium, temperature and various other factors (Banwart, 1989). A similar concept has been widely used in the thermal destruction of a variety of compounds important in the food industry. Gill *et al.* (1985) used this concept to study the toxicities of heated soft shell clam homogenates (pH = 6.8), and found that the kinetics of thermal destruction for PSP was first order as typically observed for most microorganisms and food components. In the present study, the decimal reduction time was calculated for individual toxins heated at all temperature levels and pH values. The r^2 values calculated from linear regression equations for homogenates at pH 7 and some at pH 6 were highly significant ($P \leq 0.01$), whereas at low pH the thermal destruction was negligible. The D values of NEO and STX (Table 5.1) were much higher at most temperatures and pH levels than those of GTX 2 and 3.

When the D values at different temperatures are plotted on a logarithmic scale, with the corresponding temperatures on a linear scale, a "phantom" TDT (thermal death time) curve is obtained, and the inverse slope of the curve is denoted as 'z' which indicates the temperature range necessary to bring about a tenfold change in the TDT or D value (Fig. 5.4). The z value varies widely for a variety of food components and food-related microorganisms (Banwart, 1989). A large z value and D value suggest thermal stability and so it is perhaps logical to note that the z values for individual PSPs were highest at low pH values (Table 5.2). Also, the z values for STX and NEO were higher than those for GTX's. According to the TDT curves in Fig. 5.4, at a conventional canning temperature (121.1°C), the scallop homogenate at pH 5 should be heated for at least 200 min to achieve 90% reduction of GTX 2/3 whereas the same homogenate requires only 58

Table 5.1 Decimal reduction time (min) for GTX 2/3, NEO and STX destruction in scallop homogenates heated at different temperature and pH levels

pH Temp.	3	4	5	6	7	NB
110°C						
GTX2/3	NS*	NS	402.6 (0.94)	231.1 (0.90)	140.0 (0.87)	360.1 (0.85)
NEO	NS	NS	430.8 (0.89)	226.2 (087)	168.1 (0.89)	332.4 (0.91)
STX	NS	NS	432.2 (0.58)	230.1 (0.96)	177.1 (0.68)	351.4 (0.83)
120°C						
GTX2/3	269.4 (0.98)	217.2 (0.98)	195.9 (0.97)	116.3 (0.98)	50.8 (0.98)	158.2 (0.97)
NEO	409.1 (0.97)	335.9 (0.96)	265.2 (0.97)	118.4 (0.99)	73.7 (0.93)	228.9 (0.98)
STX	N.S.	404.8 (0.58)	279.6 (0.64)	122.7 (0.85)	76.7 (0.94)	235.7 (0.71)
130°C						
GTX2/3	138.8 (0.99)	128.5 (0.98)	125.1 (0.94)	49.0 (0.96)	32.1 (0.94)	64.4 (0.94)
NEO	228.9 (0.98)	183.8 (0.96)	154.7 (0.98)	72.7 (0.99)	48.1 (0.96)	102.1 (0.97)
STX	279.9 (0.85)	197.6 (0.85)	159.1 (0.85)	78.6 (0.88)	48.6 (0.92)	120.9 (0.64)
Specific toxicity (MU/ 100 g)	262.0 (0.94)	200.6 (0.92)	154.4 (0.98)	78.9 (0.95)	40.0 (0.97)	120.7 (0.98)

Note: r^2 values are given in parenthesis. D values at 90° and 100°C were not included in the table since most r^2 values were not significant ($P \geq 0.05$).

NS* : not significant ($P \geq 0.05$).

NB: Non-buffered (control)

Table 5.2 z values (C°) of GTX 2/3, NEO and STX in scallop homogenate heated at different temperature and pH levels

pH / Toxin	3	4	5	6	7	NB
GTX 2/3	NS*	NS	$r^2 = 0.98$ $z = 39.5$	$r^2 = 0.99$ $z = 33.4$	$r^2 = 0.94$ $z = 28.3$	$r^2 = 0.96$ $z = 33.0$
NEO	NS	NS	$r^2 = 0.99$ $z = 45.4$	$r^2 = 0.98$ $z = 34.6$	$r^2 = 0.98$ $z = 33.0$	$r^2 = 0.96$ $z = 39.0$
STX	NS	NS	$r^2 = 0.99$ $z = 46.1$	$r^2 = 0.98$ $z = 35.5$	$r^2 = 0.98$ $z = 35.0$	$r^2 = 0.98$ $z = 48.1$

NB: Non-buffered (control)

NS* : not significant ($P \geq 0.05$).

z values for pH 3 and 4 were not included in the table since most r^2 values were not significant ($P \geq 0.05$).

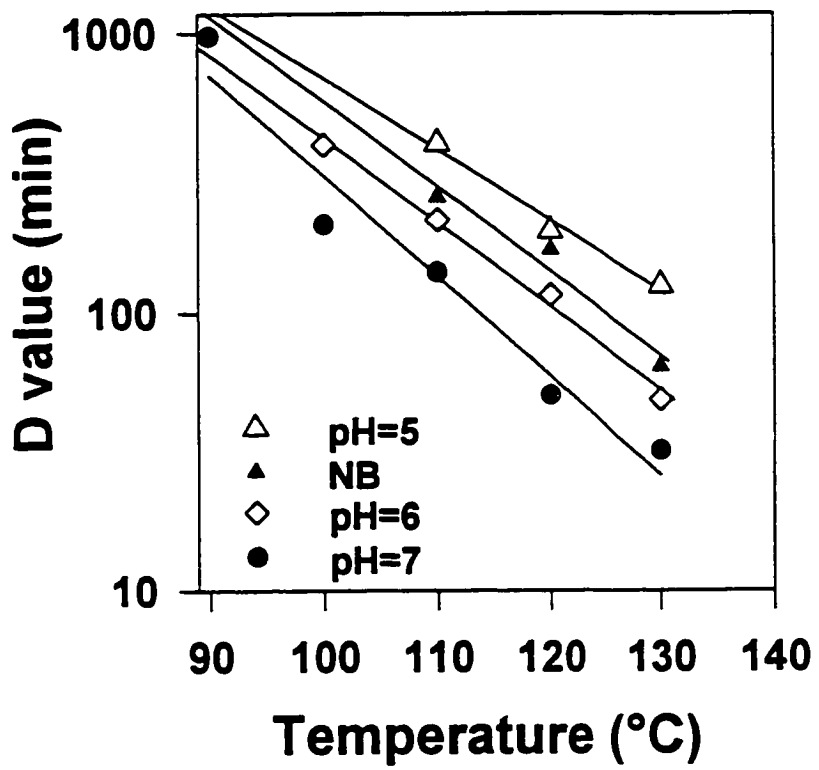


Fig. 5.4 TDT curves of GTX 2/3 in buffered and unbuffered (NB) scallop homogenates

min to obtain the same result at pH 7. Similar calculations may be used to determine times for decimal reduction of PSPs at any temperature using the thermal death time equation:

$$t = D (\log a - \log b),$$

where t = time of heating, D = time required to destroy 90% of toxins, a = initial amount of toxin, b = amount of toxin after heating time t.

5.4.3 Comparison of HPLC Data with Bioassay Data

A lot of work has been published on PSP toxin analysis by either chemical or biological methods, but little information is available on the comparison of both. In addition to the Sullivan and Wekell (1987) HPLC method for the analysis of individual amounts of PSP toxins, these levels were converted to integrated total toxicities and compared with data determined using a mouse neuroblastosoma bioassay procedure (Jellet *et al.*, 1992) in order to verify chromatographic data on thermal destruction. The variation in toxicity as determined by the bioassay procedure as a function of pH and heating time at 130°C is shown in Figure 5.5. The nature of the change in specific toxicities was similar to the degradation patterns of GTXs, NEO and STX determined by HPLC at the same temperature. The toxicity rapidly decreased with increasing pH and heating time. The theoretical specific toxicities of individual toxins quantified by HPLC were calculated according to Schantz (1986), and it is interesting to note that these values were significantly correlated ($P \leq 0.01$) with specific toxicities of the same samples measured by the neuroblastosoma cell bioassay (Fig. 5.6). D values calculated from the cell bioassay toxicities in Fig. 5.7 indicate that 90% reduction in total toxicity can be achieved by heating the pH 7 homogenate for 40 min at 130°C.

The most appropriate statistical models for the degradation of individual PSP toxins in scallop homogenates at different temperatures and pH values are shown in Table 5.3. The nature of the normal probability plot and the residual plot of each model were also checked in addition to the F-ratio and P-value, prior to determining the best fit. It is worth mentioning that the complexity of most models derived, is due to the scattered nature of the thermal data. A significant ($P \leq 0.01$) improvement of the linear regression

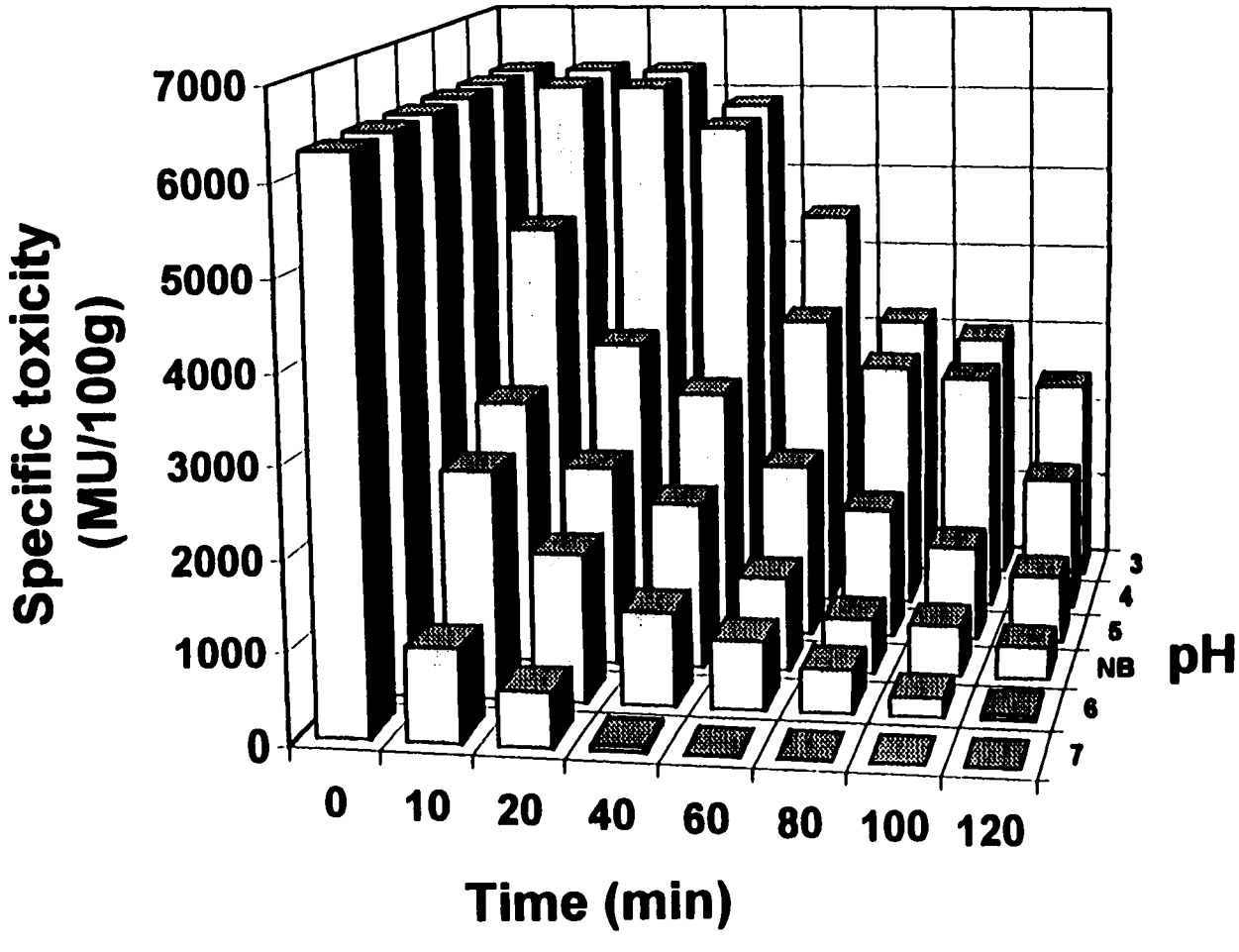


Fig. 5.5 Specific toxicities (mouse units) of PSP toxins in scallop homogenate heated at 130°C, as determined by the mouse neuroblastoma cell bioassay

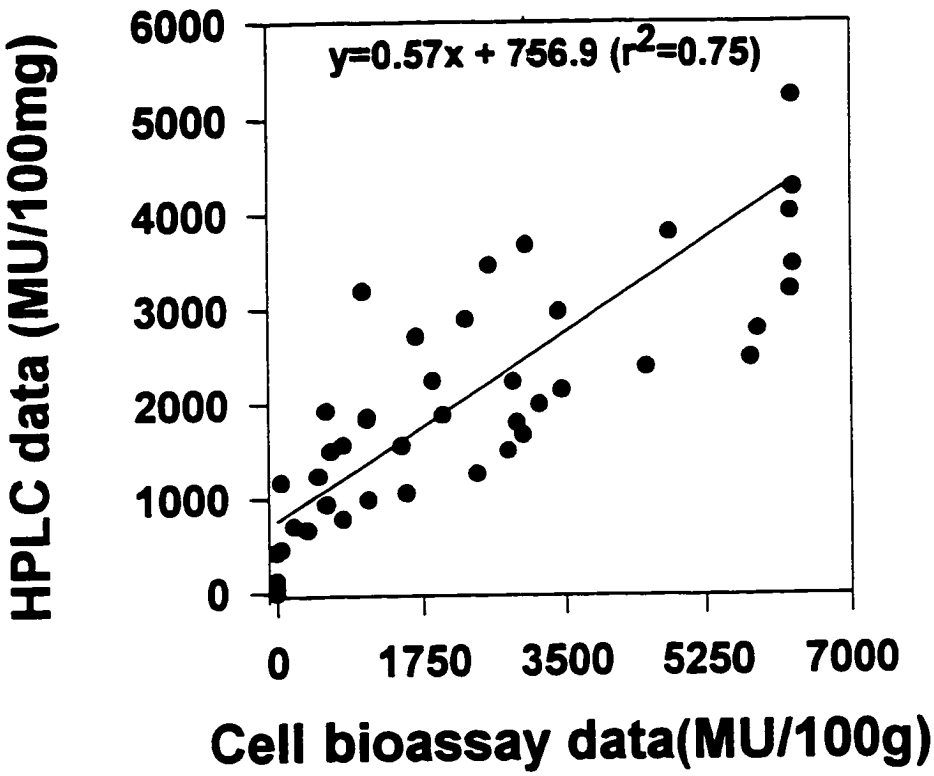


Fig. 5.6 Relationship between theoretical HPLC toxicities (mouse units) and cell bioassay toxicities (mouse units) of scallop homogenate heated at 130°C (n=48)

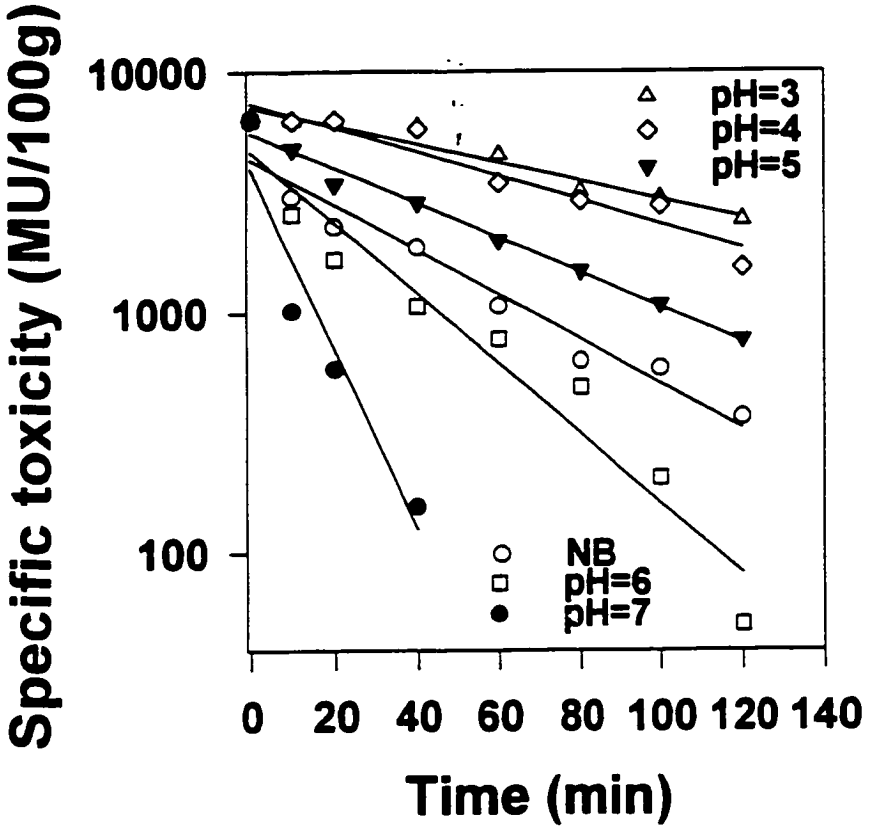


Fig. 5.7 Log-specific toxicities (mouse units) of PSP toxins in scallop homogenate with different pHs heated at 130°C as determined by cell bioassay.

Table 5.3 Statistical models for the degradation of PSP toxins in scallop homogenate

Model	R^2	F-ratio	P-value
GTX 2/3* = $-522+0.000001(t^3 \cdot T^3 \cdot pH^3)-0.001(T^3)-0.922(pH^3)-0.12(t \cdot T \cdot pH)$ $+12.2(t \cdot pH)-0.007(t^2 \cdot pH^2)+28(T)$	0.93	336.5	0
NEO* = $-93+1.49(T)+0.001(pH^2 \cdot t^2)+1.55(t \cdot pH)-0.015(t \cdot T \cdot pH)$ $-0.058(pH)^3-0.0001(T)^3$	0.90	243.7	0
STX* = $-247+0.112(t \cdot pH)+0.004(t^2)-3.62(pH^2)+11.8(T)-0.0028(t \cdot T \cdot pH)$ $-0.0719(T^2)+0.206(T \cdot pH)$	0.87	140.7	0

[* = toxin content in μg/100 g, t = time (min), T = temperature (°C), GTX = gonyautoxin, NEO = neosaxitoxin, STX = saxitoxin]

models of GTX's, NEO and STX by the addition of an interaction term (pH x time) clearly indicates that both pH and heating time together, rather than individually, have significant effects on the degradation of PSP components at each temperature level.

Addition of acidulants to some food products is a common practice in canning (Ocio *et al.*, 1994; Scanchez *et al.*, 1995). However, strict control of pH must be considered to be a critical control point during conventional canning or even pasteurization of such shellfish products, since thermal processing of non-toxic samples in acidified brines may present significant risk due to the thermally induced conversion of less toxic sulfocarbamate toxins to the highly toxic carbamate toxins particularly at acid pH.

5.4.4 Conclusions

Most of the individual toxins degraded more rapidly when heated at higher temperatures and pH levels for longer times. PSP toxicity decreased rapidly at 130°C at pH levels as high as 6 and 7. Conventional canning of acidified shellfish or even pasteurization of such products may actually produce toxicity levels of 'safe' raw materials to levels which are unsafe (>80 μg STX/100g edible tissue) in the finished product. There was a highly significant correlation between the mouse bioassay results and the theoretical PSP toxicities calculated from chromatographic data derived from the HPLC method of Sullivan and Wekell (1987).

CHAPTER 6

THERMAL DEGRADATION OF PARTIALLY PURIFIED PARALYTIC SHELLFISH POISONING TOXINS AT DIFFERENT TIMES, TEMPERATURES AND pH LEVELS

6.1 Abstract

Mixtures of purified and partially purified PSP toxins including C toxins (C1 and C2), B toxins (B1), gonyautoxins 1-4 (GTX), neosaxitoxin (NEO) and saxitoxin (STX) were heated at different temperatures, heating times and pH levels to study the effect of these parameters on the degradation of individual toxins. Citrate/phosphate buffers were used to achieve pH values ranging from 3 to 7. The samples were heated at 90, 100, 110, 120 and 130°C using a computer controlled oil bath. Both heated and unheated toxin samples were analyzed qualitatively and quantitatively by HPLC using post-column derivatization and fluorescence detection.

C toxin levels declined rapidly at low pH levels even at low temperatures, and co-elution of unknown compounds (presumably thermal degradation products) was noticed at higher pH levels and higher temperatures. GTX 1/4 toxins behaved differently with thermal degradation at higher pH levels. GTX 2/3 levels increased initially at low pH and then declined with subsequent heating, whereas saxitoxin (STX) levels increased consistently at pH 3-4. All gonyautoxins, STX, neosaxitoxin (NEO) and integrated total specific toxicity declined at high pH levels (6-7). The kinetics of thermal destruction were first order and qualitatively similar to the thermal destruction of microorganisms and comparable to the degradation patterns of PSP toxins in the scallop homogenate heated under similar conditions, i.e., the log survival of heated toxins was inversely proportional to time of heating at any particular temperature. Also, the log decimal reduction time was inversely related to temperature of heating. Efficacy of thermal destruction was highly dependent on pH, with more rapid thermal destruction of carbamate compounds at higher pH levels. However, heating at low pH resulted in conversion of the least toxic compounds to highly toxic compounds, suggesting that care must be taken when acidifying shellfish prior to

canning. This work has been submitted for publication in Journal of Food Science (Indrasena and Gill, 1999b).

6.2 Introduction

STX and its analogues produced by some dinoflagellates and cyanobacteria are among the most toxic low molecular weight compounds known (Laycock *et al.*, 1997). The organisms that feed on them can accumulate the toxins in high concentrations and transmit them to higher trophic levels through the food web. These toxins block the sodium channels of the central nervous system causing paralytic shellfish poisoning (PSP). PSP poses the most serious threat to public health due to the extreme toxicities of the toxins involved (Chen and Chou, 1998) with no known antidotes. Carbamate toxins such as saxitoxin, neosaxitoxin, gonyautoxins and the N-sulfocarbamyl toxins such as C toxins and B toxins are found in most of the toxic dinoflagellate types in varying amounts. These toxins have different toxicities, carbamate toxins being the most toxic whereas the sulfamate toxins are the least neurotoxic to humans.

Natural detoxification of contaminated shellfish has been a difficult task because different shellfish have different rates of toxin release. Although the toxicity can be reduced slowly by the normal process of home cooking such as boiling or frying (Medcof *et al.*, 1947, Quayle, 1969), the total toxicity was reported to be reduced only by 70% after cooking for 40 min. Prakash *et al.* (1971) indicated that steaming and frying reduced the toxicity more than by boiling, whereas retorting at 121.1°C instead of 104.4°C reduced the final toxin score by 25-50%. Lawrence *et al.* (1994) indicated that the total toxicity of the toxic lobster hepatopancrease could be reduced by 65% during normal cooking (boiling and steaming). The STX and GTX 2/3 (combined) levels could be decreased by 60 and 90-100% respectively. However, Desbiens and Cembella (1997) indicated that one third of the samples became more toxic due to toxin inter-conversions after the lobsters were cooked in hot steam for 20 min.

Noguchi *et al.* (1980a and b) reported that the toxicity of PSP infested scallops can be reduced by 90% by canning at 122°C for 22 min. Studying the effect of heating (70-

110°C) and pH (6-8) on the toxicity of scallops, Asakawa and Takagi (1983) found that the PSP toxicity was decreased by 87% after heating at 110°C and at pH 8 for 30 min. Asakawa *et al.* (1986) reported conversions of GTX's into STX along with detoxification during canning.

The total toxicity in mussels, *Mytilus edulis* over a pH range of 1-6, but over restricted heating temperatures and times (100 and 121°C) was studied by Chang *et al.* (1988), and it was found that these toxins were more stable at low pH values. Berenguer *et al.* (1993) reported that the total PSP toxicity of the raw material of naturally contaminated *Acanthocadia tuberculatum* was decreased by about 95% by canning them in salt water (40g/L) at 115°C for 45 min.

Ohta *et al.* (1992) applied extrusion processing to detoxify PSP toxins in scallops, and found that there were reductions of 81.6-82.2 and 85.6-97.8% at 130 and 170°C respectively. Gill *et al.* (1985) were the first to report the kinetics of the thermal destruction of PSP toxins, indicating that the kinetics were first order, and 90% reduction of total toxicity in soft shell clams (pH=6.8) could be achieved by heating for 43 min at 132.2°C or 193 min at 104.4°C (e.g. the '$D_{121.1°C}$' and 'z' values were 71.4 min and 42C° respectively). The kinetics of the thermal degradation of some individual PSP toxins were studied by Nagashima *et al.* (1991b) who reported that the kinetics were first order although the toxicity increased by 50% because of toxin inter-conversions occurring during heating at 100°C for 30-60 min.

A more systematic and detailed study on the combined effects of pH, heating temperature and time on the thermal degradation of individual PSP components in scallop digestive glands was done by Indrasena and Gill (1999) who reported that the kinetics of the thermal degradation of STX, NEO and GTX 2/3 toxins were first order. The highest D and z values were reported for STX and lowest values for GTX 2/3. The degradation kinetics were not reported for GTX 1 and 4 nor for the C toxins. In addition, the degradation rates may change according to the composition of the matrix or medium (Asakawa and Takagi, 1983). Thus, the present study has been carried out to gather more information on the kinetics of partially purified standard PSP toxins in buffer (pH 3-7),

especially GTX 1/4 and C toxins, in a medium without a matrix.

6.3 Materials and Methods

6.3.1 Buffering and Heating

Unknown amounts of purified and partially purified PSP toxins including STX, NEO, GTX 1-4, C toxins (C1 and C2) and B toxins (B1) were mixed together and diluted with 0.01N acetic acid. The toxins were obtained from the National Research Council (Institute of Marine Biosciences), Halifax, Nova Scotia. One millilitre aliquots of the mixture were transferred into 6 cm screw-cap culture tubes and 84 such tubes were prepared for each temperature level. One millilitre of citrate/phosphate (1.2M) buffer with pH values of 3, 4, 5, 6 and 7 were added to each tube. Another 14 tubes were prepared using 1 mL of double distilled, deionized water instead of buffer (NB), for each temperature level. The sample tubes were prepared in the same manner for all temperature levels. The tubes were thoroughly mixed, flushed with nitrogen, screw capped tightly and sealed.

The tubes were heated in a thermostatically controlled oil bath and temperatures were monitored using thermocouples inserted through the screw caps. The oil bath was heated to the required temperature, and two tubes of each pH were removed after 10, 20, 40, 60, 80, 100 and 120 min, and immediately transferred into an ice bath. Heated/cooled samples were removed for analysis as quickly as possible.

6.3.2 Analysis of Toxins by High Performance Liquid Chromatography

PSP toxins were determined with a Waters high performance liquid chromatograph equipped with 2 Model 510 pumps with auto injection system controlled via a system interface module, and a Shimadzu Model Rf 535 fluorescence detector (338 nm excitation, 400 nm emission). Samples were run in the HPLC using a Whatman PRP-1 column (15 cm x 4.1 mm) packed with 10 µm beads using a binary elution gradient (mobile phase A: water with 1mM hexane and heptane sulphonic acid, mobile phase B: acetonitrile with hexane and heptane sulphonic acid) according to Sullivan and Wekell (1987). The HPLC was equipped

with a dual reagent post column reaction system set up to mix the effluent stream with nitric acid (0.75M) and periodic oxidant (5N sodium hydroxide, 5mM periodic acid and 0.5M ammonium hydroxide) was used prior to the fluorescent detection. Individual toxins were identified by running a standard mixture of PSP toxins and were quantified using authentic PSP toxins obtained from National Research Council, Halifax, Nova Scotia. The toxin levels analyzed by the HPLC were converted to integrated total specific toxicities (Schantz, 1986; Sullivan, 1985).

6.3.3 Kinetics of Thermal Destruction

Decimal reduction times and z values for each toxin component were calculated according to Stumbo (1973). The best fitted mathematical model for the destruction of each PSP component was determined by the MINITAB Version 12.2 statistical passage for Windows in the Dalhousie University (DalTech) main frame computer. The destruction of toxins at different time intervals was statistically compared by analysis of variance (ANOVA) via multiple regression using indicator variables.

6.4 Results and Discussion

The standard toxin mixture contained C1 and 2, B1, GTX 1- 4, NEO and STX (Fig. 1.1). The kinetics of the degradation of most of the toxins were found to be first order, and comparable with the degradation patterns of individual toxins in the scallop homogenate reported in Chapter 5 and in a previous study (Indrasena and Gill, 1999a). Gill *et al.* (1985) and Nagashima *et al.* (1991) also reported that the kinetics of the thermal degradation of PSP toxins were first order. However, the rate of degradation depends not only on the type of toxins but also on the heating temperature, time and pH of the heating medium.

6.4.1 Effect of pH

With gentle heating at 90°C, the amount of C1/C2 toxins declined gradually and significantly ($P \leq 0.01$) over an initial time period of 2 h. Unlike other individual toxins in the mixture, C toxins declined rapidly at low pH levels with the fastest decrease at pH 3 during heating for 2 h followed by pH 4, NB (control sample with no buffer), 5, 6 and 7 (Fig. 6.1A-C, Appendix E- Fig. 6.I). The rapid reduction of C toxins at low pH levels may possibly be due to their conversion into more toxic carbamate counterparts such as GTX 2 and 3, rather than degradation (Fig. 6.2). Mild acid hydrolysis of C2 toxins liberates inorganic sulfate and GTX 3, whereas, C1 toxins which are epimers of the11 α-isomer of C2, can be converted to GTX 2 by the reductive cleavage of the O-sulfate group (Shimizu, 1984). In the preparation of commercial standards by chemical conversions in acid (HCl), Laycock *et al.* (1995) also observed rapid increase in the conversion of C1/2 toxins to GTX 2/3 at 100°C when the pH was decreased from 5.8 to 3, with a considerable amount of conversion at pH 3. The decline of these toxins at pH 7 may possibly be due to degradation rather than conversion. The rate of conversion or destruction was much faster at higher temperatures.

When the temperature was increased from 90°C through 120°C, the level of C toxins at low pH levels (3-4) declined faster than that at higher pH levels (pH 6-7), whereas pH 5 and NB samples had similar reduction rates. However, at pH 6, C toxins did not

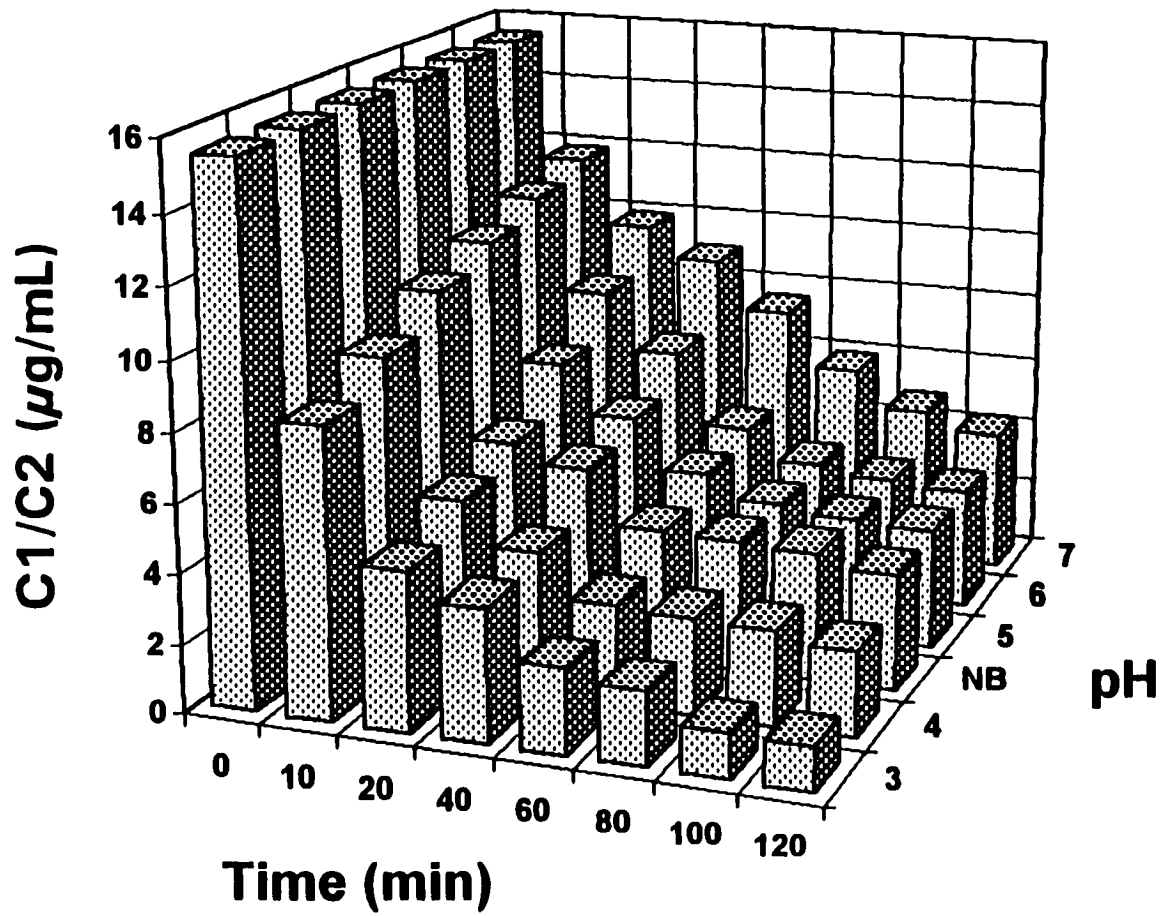


Fig. 6.1 A. Thermal degradation of C1/C2 in the standard toxin mixture heated at 90°C for different times and pH levels

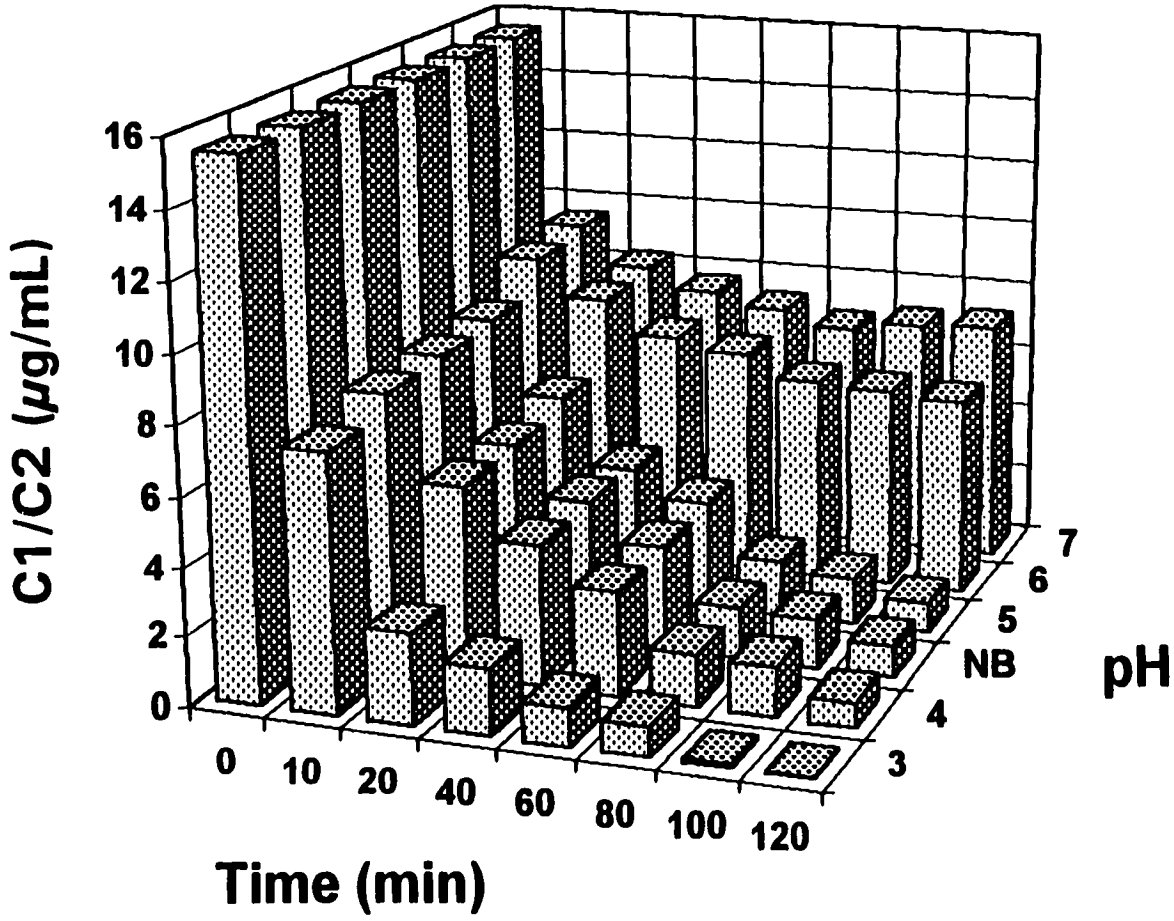


Fig. 6.1 B. Thermal degradation of C1/C2 in the standard toxin mixture heated at 110°C for different times and pH levels

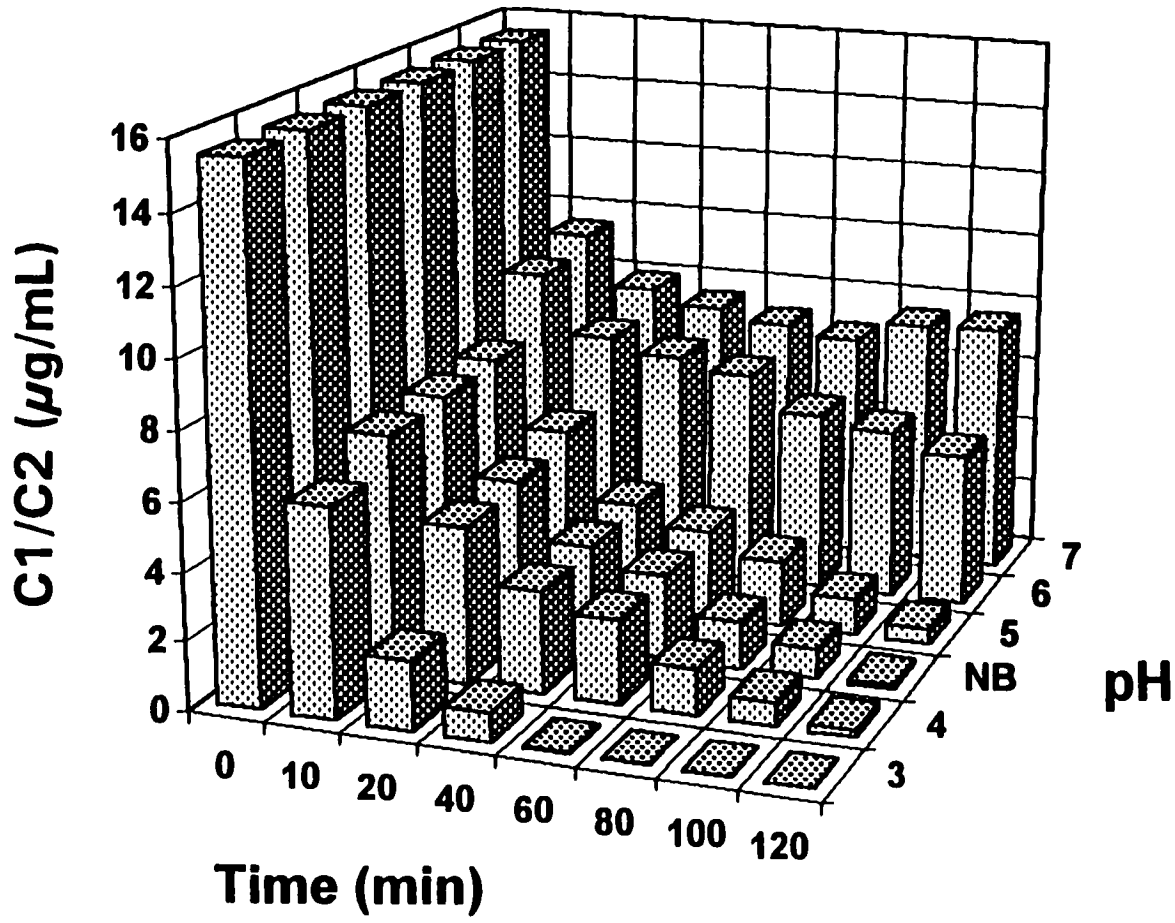
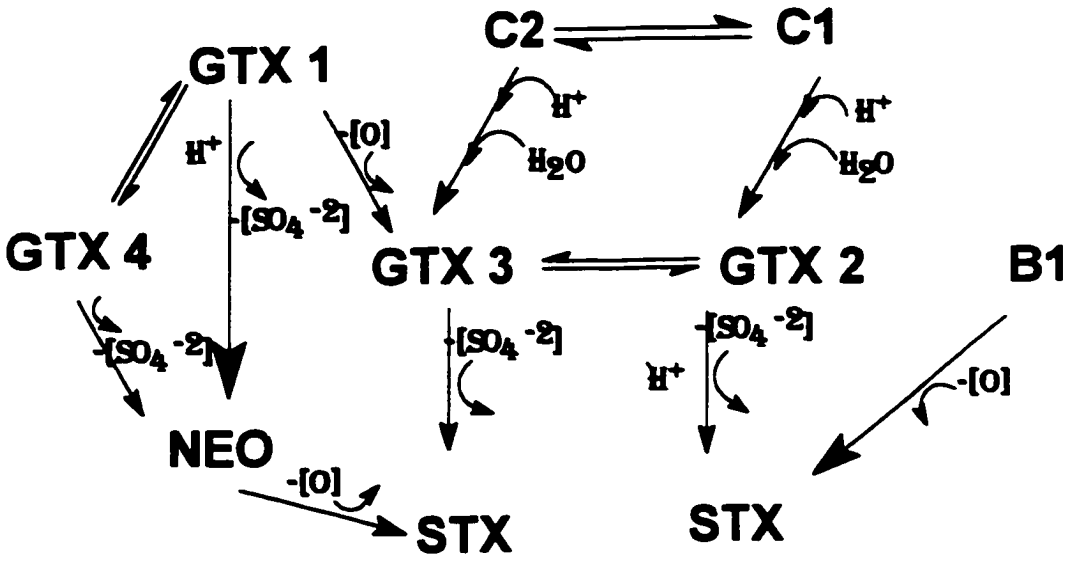


Fig. 6.1 C. Thermal degradation of C1/C2 in the standard toxin mixture heated at 120°C for different times and pH levels



C1 = 11- α isomer of C2 (sulfonyl gonyautoxin 2)
C2 = Carbamoyl-N-sulfonyl gonyautoxin 3
B1 = Carbamoyl-N-sulfate of NEO
GTX 1 = 11-α-hydroxy neosaxitoxin sulfate
GTX 4 = 11-β-hydroxy neosaxitoxin sulfate
GTX 2 = 11-α-hydroxy saxitoxin sulfate
GTX 3 = 11-β-hydroxy saxitoxin sulfate
NEO = 1-hydroxy saxitoxin
STX = Saxitoxin

Fig. 6.2 Possible conversions of PSP toxins during heating (Shimizu and Yoshioka, 1981)

change much when heated at 110-120°C for longer periods (>2 h). At pH 7, these toxins actually tended to increase gradually after 80 min of heating at 110 and 120°C. This observation may also be due to the co-elution of some thermally denatured PSP toxins in the reaction mixture. The accumulation of thermally degraded compounds was more pronounced at 130°C throughout the whole range of pH (3 to 7). The C toxin peak in scallop homogenates also increased gradually with increasing temperature, as indicated in Chapter 5, especially at neutral pH levels, possibly by the co-elution of thermally degraded compounds. C1/C2 toxins were found to be the most heat labile compounds in the standard toxin mixture, and their sensitivity to heating was significantly affected not only by the heating temperature and time but also by the pH.

The levels of GTX 1 and 4 gradually declined during heating at 90°C for 2 h (Fig. 6.3A). Unlike C toxins, GTX 1/4 degraded most at pH 7 and the effect was greatest at higher temperatures. The gradual reduction in the GTX 1/4 levels at low pH levels may possibly also be due to their conversion into more toxic NEO (Fig. 6.2). Since GTX 1 and 4 are epimeric 11-hydroxyneosaxitoxin sulfates, they can be converted to NEO by the reductive cleavage of the O-sulfate groups when heated in mild acid. However, when heated from 90 through 130°C, the level of GTX 1/4 declined rapidly and significantly ($P \leq 0.01$) with the highest rates observed at pH 6-7 (Figs 6.3 B-C, Appendix F- Figs. 6.II.A-C and Appendix F).

Unlike GTX 1/4, GTX 2 and 3 increased steadily ($P \leq 0.05$) at low pH levels (3-5) when heated at 90°C for 40 min (Appendix E: Fig. 6.III.A) whereas at pH 7 there was no increase. GTX 2/3 gradually increased at low pH levels when heated at 100-110°C and then declined (Appendix E-Figs. 6.III. B-C). This gradual increase may be due to the conversion of C1/C2 to GTX 2/3 by the removal of sulfo groups by carbamoylation. The decrease of the GTX 2/3 levels at low pH may be due to thermal degradation as well as possible conversion into more toxic STX (Fig. 6.2). However, at higher pH levels, the reduction may also be due to thermal destruction rather than conversion. The amount of GTX 2/3 increased slightly within the first 20 min of heating at 120°C at low pH levels (3-4 and control) and then declined slowly, whereas the sample at pH 5 did not change very much

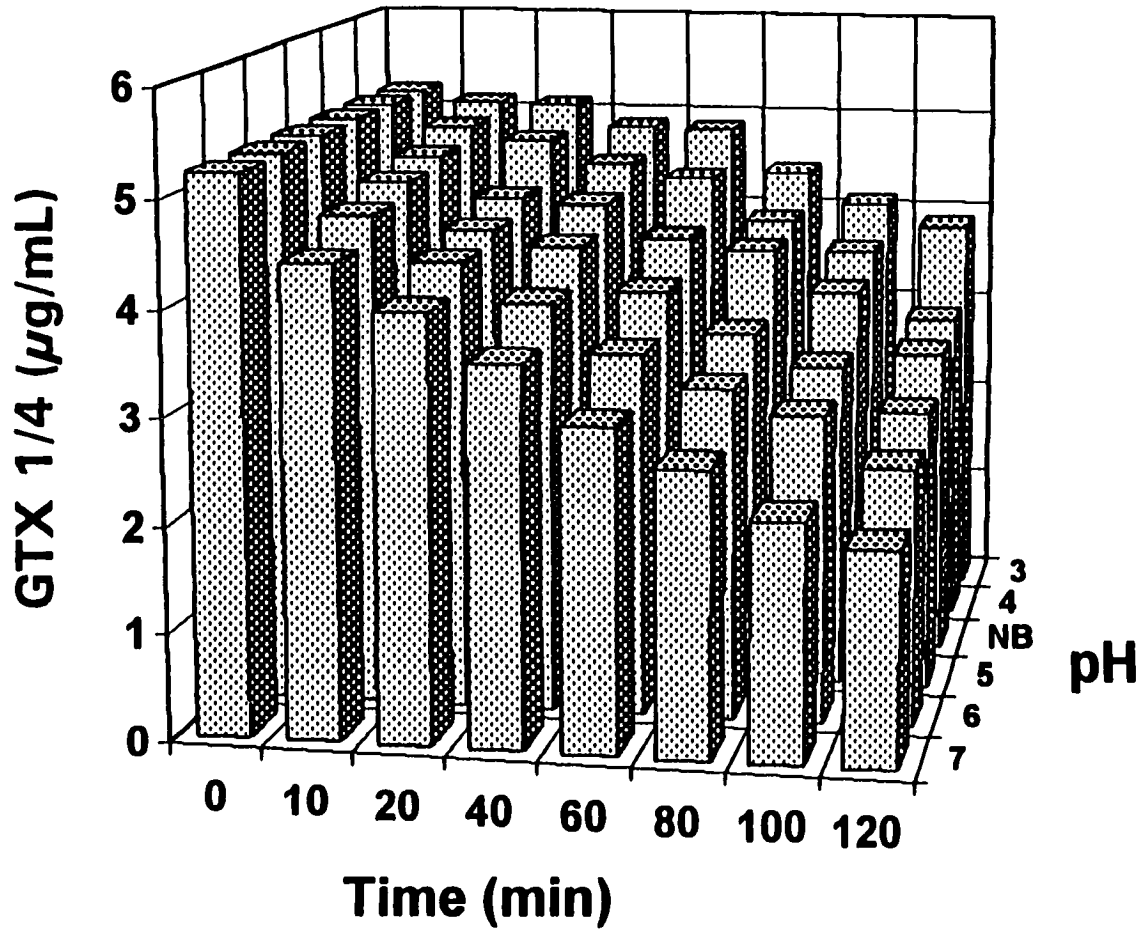


Fig. 6.3 A. Thermal degradation of GTX 1/4 in the standard toxin mixture heated at 90°C for different times and pH levels

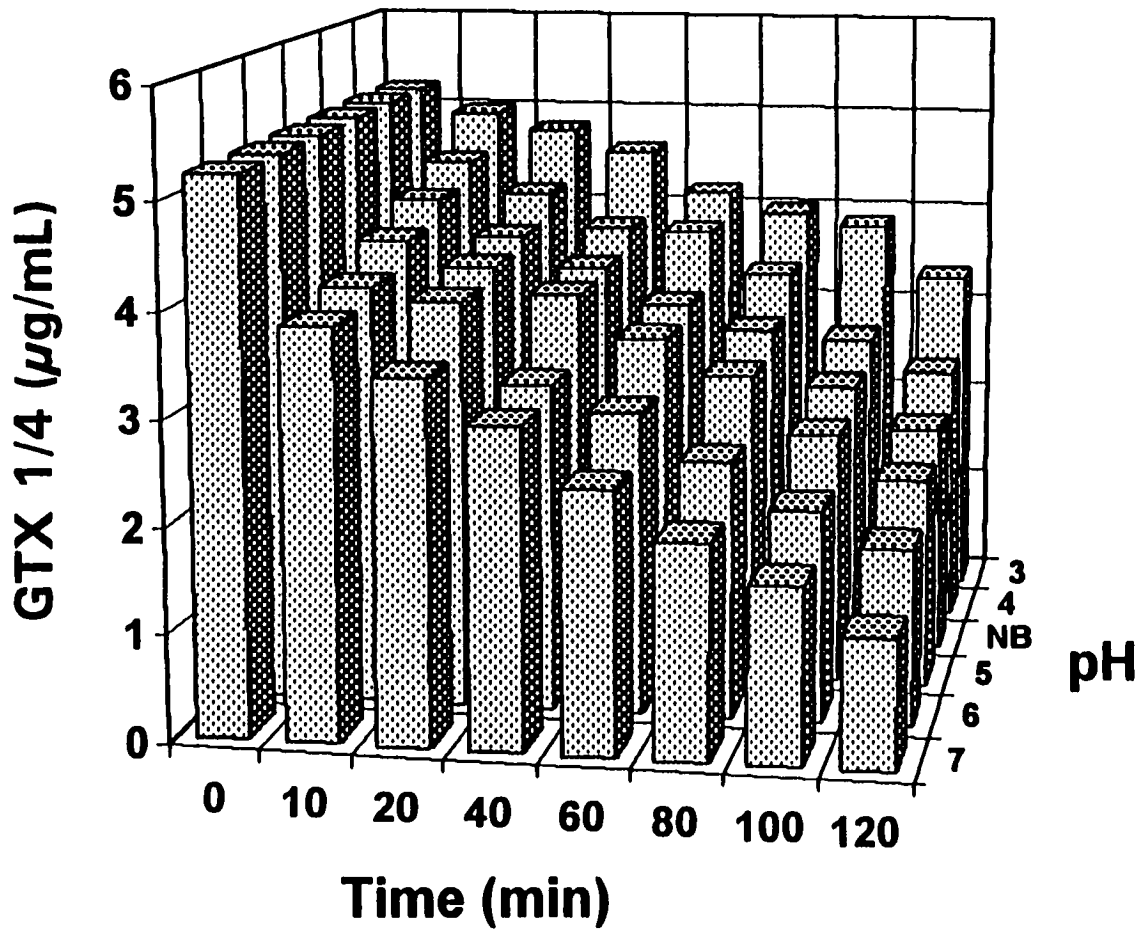


Fig. 6.3 B. Thermal degradation of GTX 1/4 in the standard toxin mixture heated at 110°C for different times and pH levels

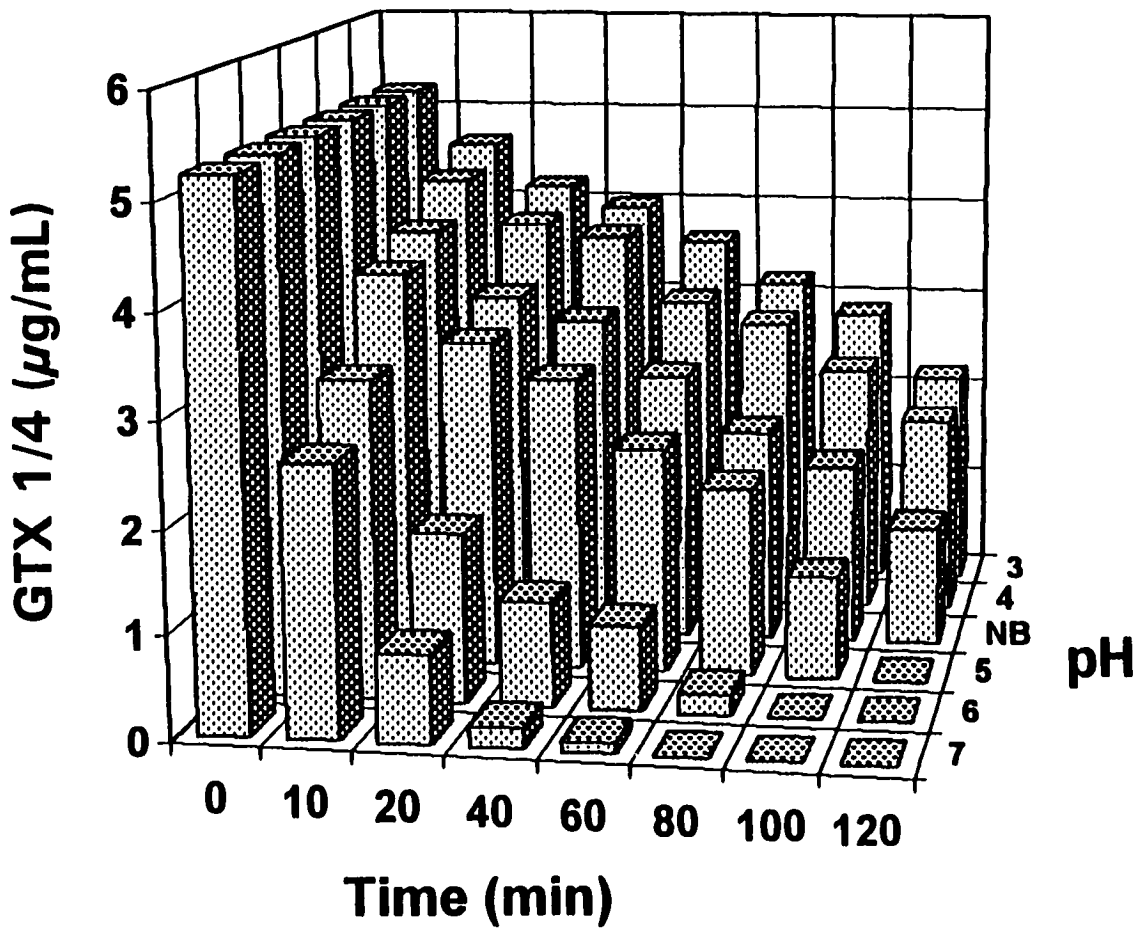


Fig. 6.3 C. Thermal degradation of GTX 1/4 in the standard toxin mixture heated at 130°C for different times and pH levels

(Appendix E: Fig. 6.III.D). These toxins gradually decreased at pH 6-7 and it was more pronounced when heated at 130°C. However, it is difficult to determine the fate of toxins during heating at moderately high temperatures (100-110°C) due to possible conversions and destruction simultaneously.

The changes in B1 toxin were difficult to quantify during heating due to the small quantities present in the samples. It is possible that these toxins may be converted to STX at low pH levels (Fig. 6.2). An extraneous peak was observed and eluted between B1 and GTX 3. This may be one of the thermally degraded components of PSP or decarbamoyl counter parts of GTXs. The degradation patterns of NEO at different temperatures were quite similar to those of STX and highest degradation rates were noticed at higher pH levels (Appendix E-Figs. 6.IV.A-6.V.E).

The amount of STX increased slowly and gradually after 40 min of heating at 90°C at pH 3-4 whereas at pH 6-7 there was no noticeable increase or decrease. A similar pattern was noticed when toxins were heated at 90 through 110°C except the STX content started to decline after 20 min at pH 6-7 (Appendix E: Figs. 6.V.A-C). When the temperature was further increased to 120°C, the amount of STX started to decline after 10 and 40 min at high (6-7) and low pH levels (3-4), respectively, (Appendix E: Fig. 6.V.D) whereas at 130°C, STX content declined at all pH levels with fastest rates at high pH levels (Appendix E: Fig. 6.V.E). Indrasena and Gill (1999a) observed the similar degradation pattern of STX in a heated toxic scallop homogenate under similar conditions as reported in Chapter 5. However, STX in the standard PSP mixture degraded much slower than that in the scallop homogenate. In addition to this inherent resistance to heat, STX may be generated through heating by conversion of GTX 2/3, B1 toxin and NEO during mild heating at low pH levels. This may in part explain the slower degradation rate of STX in the standard toxin mixture. GTX 2 and 3 can be converted to STX by heating in the acid by reductive elimination of the sulfonyl group (Shimizu, 1988). NEO in the medium also can be converted to STX by reductive cleavage of N-hydroxyl group.

6.4.2 Integrated Specific Toxicity

The theoretical integrated specific toxicities may be determined as mouse units (MU/mL) using the conversion factors reported by Schantz (1986) and Sullivan (1985). Figs. 6.4A-C show the change in the total integrated specific toxicities at 90, 110 and 130°C. The total toxicity increased slightly at pH 3-4 at 90°C whereas there was a gradual decline at pH 6-7. However, the total toxicity gradually declined with increasing heating times and temperature with the maximum decrease at neutral pH levels (6-7). It was also noticed that the total toxicity did not change much at low pH levels (pH 3-4), even at higher temperatures (Appendix E-Figs. 6.VI.A-B). Chang *et al.* (1988) also indicated that the total toxicity increased at pH 3 followed by gradual decrease with increasing pH. Gill *et al.* (1985) and Asakawa and Takagi (1983) reported the gradual decrease in total toxicity during heating around neutral pH levels.

6.4.3 Reaction Kinetics

Decimal reduction time (D value) which is defined as the time (min) required to destroy 90% of a substance or organism at a given temperature was calculated for each toxin type at each pH and temperature level as the negative slope of the survivor curves. This concept has been widely used in the thermal destruction of a variety of compounds important in the food industry, and the D value can vary widely with the physico-chemical nature of the compounds in the medium, temperature and various other factors (Banwart, 1989). Gill *et al.* (1985) indicated that the kinetics of thermal destruction of PSP in heated soft shell clam homogenates (pH=6.8) was first order and the D values decreased semi-logarithmically with increasing temperature. D values for some individual toxins in scallop homogenate heated at different temperature and pH levels (pH 3-7) are reported in Chapter 5.

TDT (thermal death time) curves for C1/2 and GTX 1/4 toxins are shown in Figs. 6.5 and 6.6, respectively, and the D values calculated for C1/2 and GTX 1/4 toxins from the linear regression equations of the survivor curves are shown in Tables 6.1 and 6.2. The D values of C toxins were smaller at low pH levels than those at higher pH levels (Table

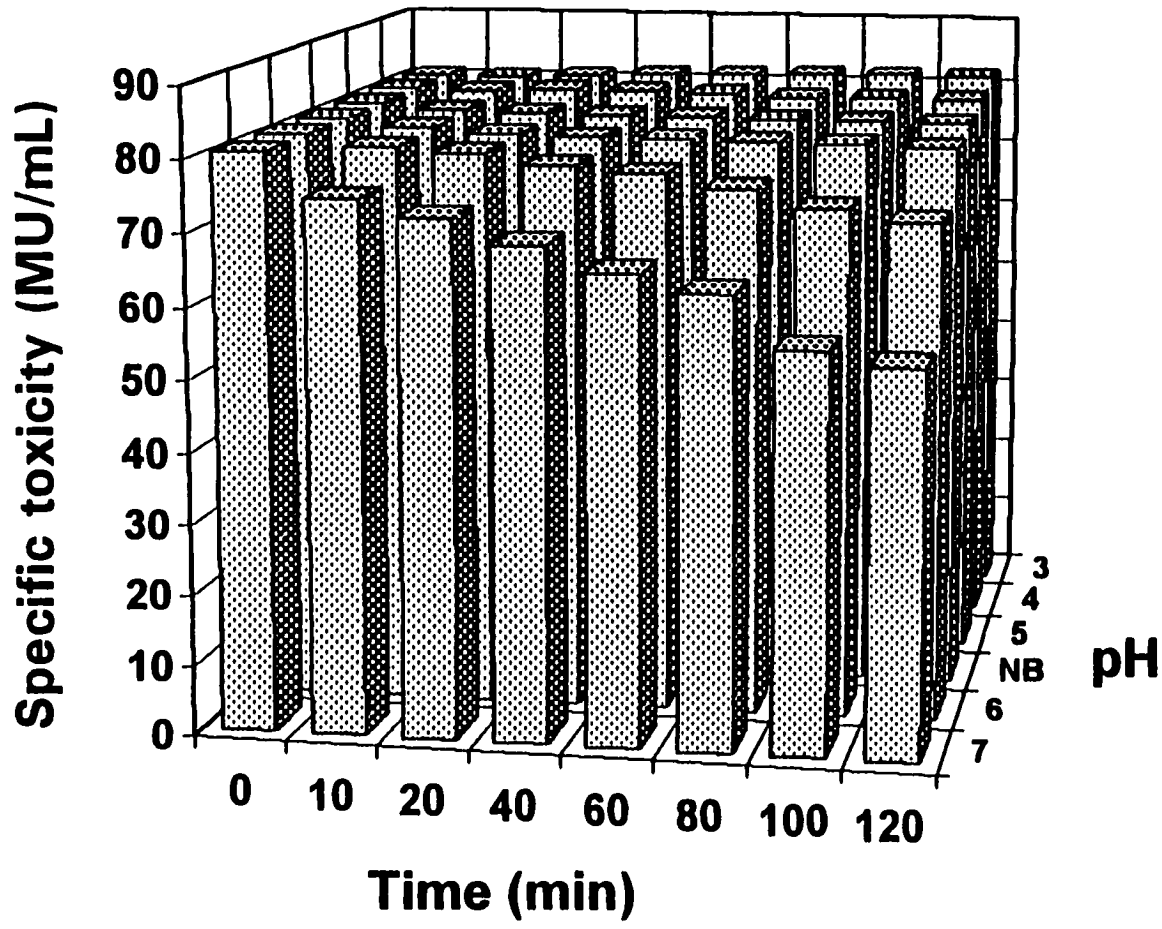


Fig. 6.4 A. Variation of specific toxicities of the standard toxin mixture heated at 90°C for different times and pH levels

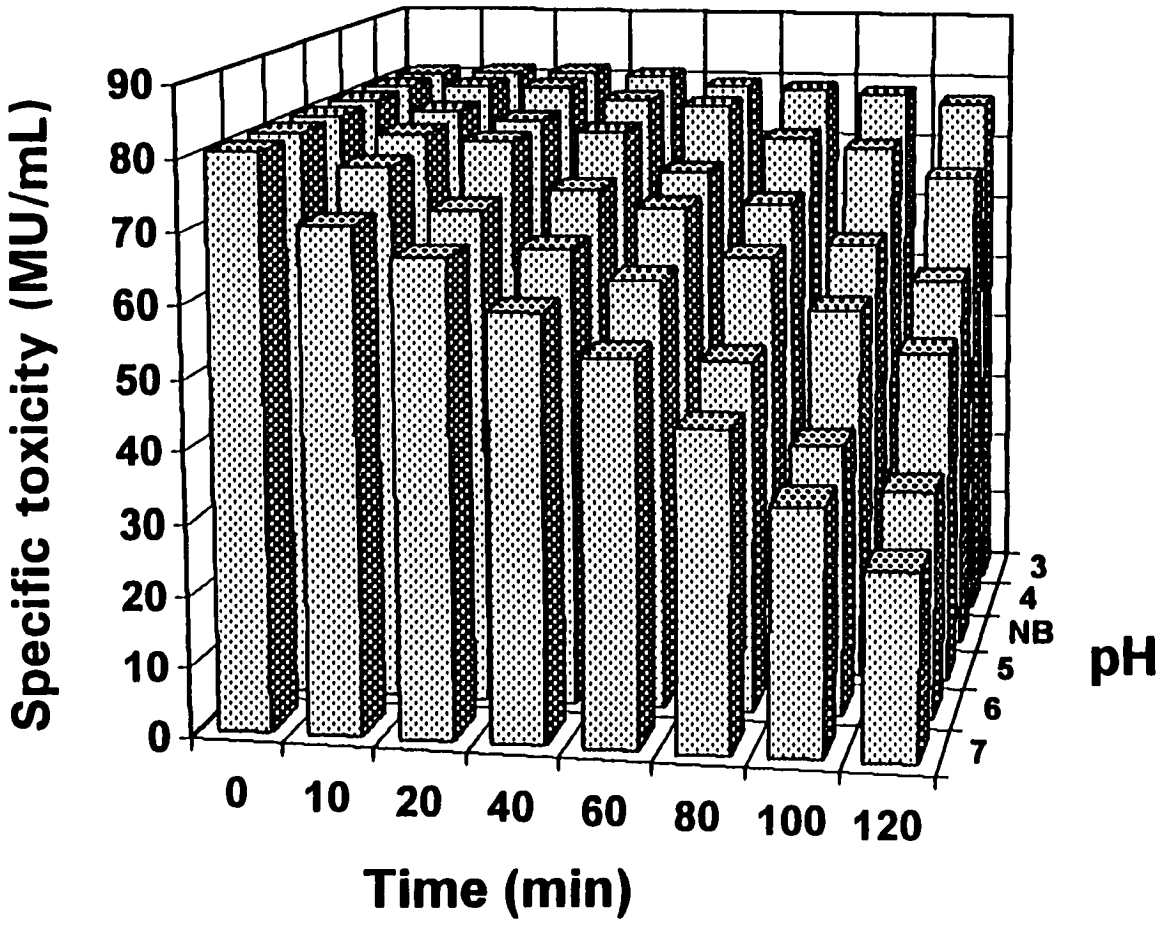


Fig. 6.4 B. Variation of specific toxicities of the standard toxin mixture heated at 110°C for different times and pH levels

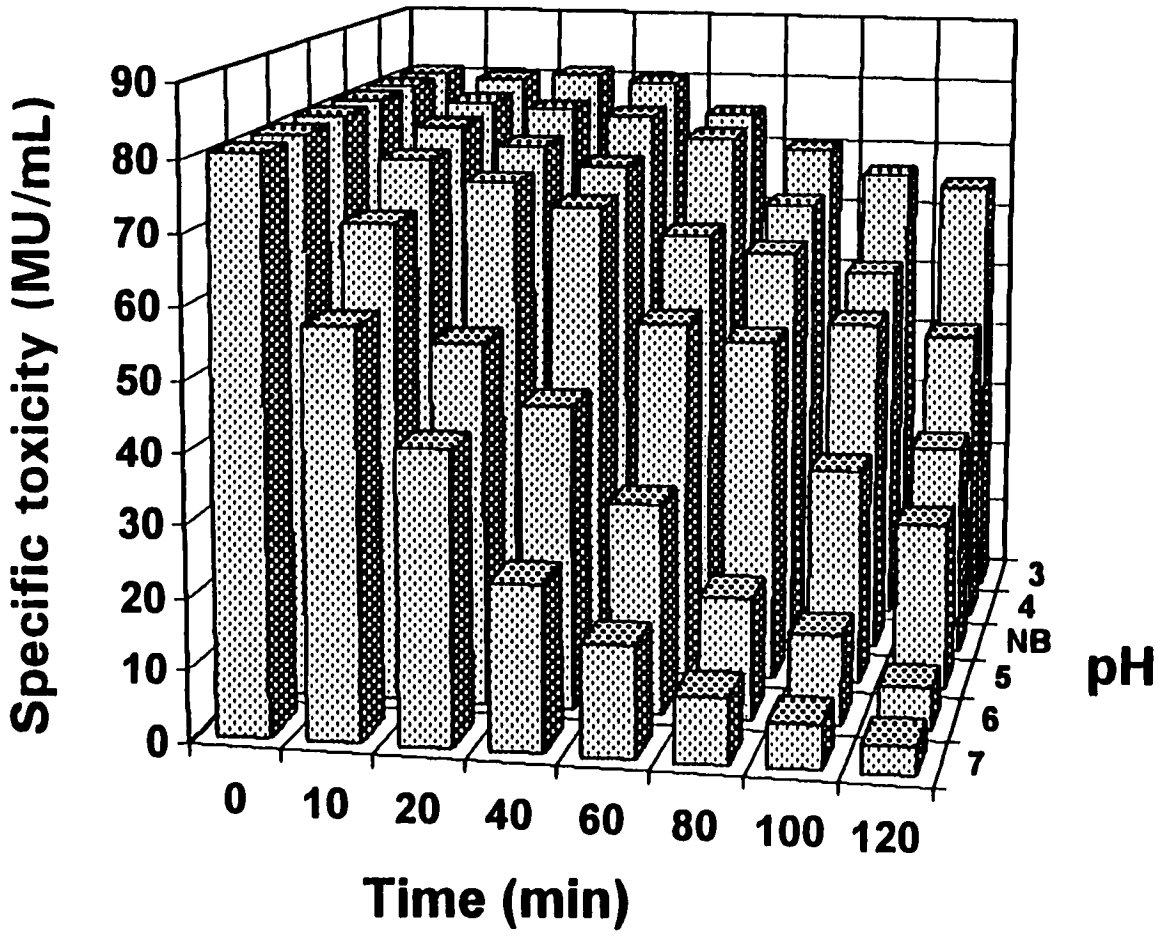


Fig. 6.4 C. Variation of specific toxicities of the standard toxin mixture heated at 130°C for different times and pH levels.

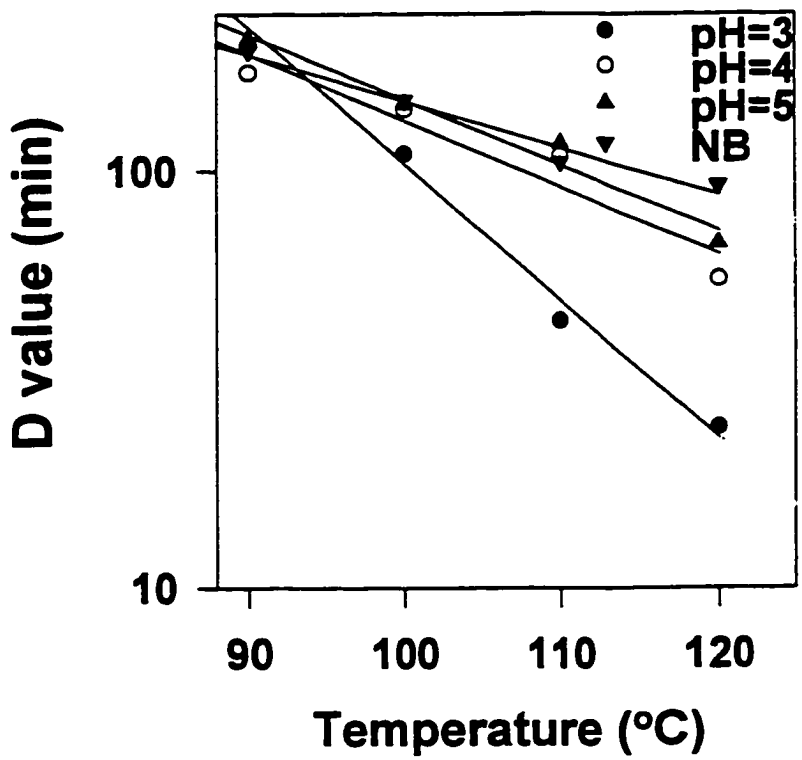


Fig. 6.5 TDT curves for C1/C2 toxins in the standard toxin mixture

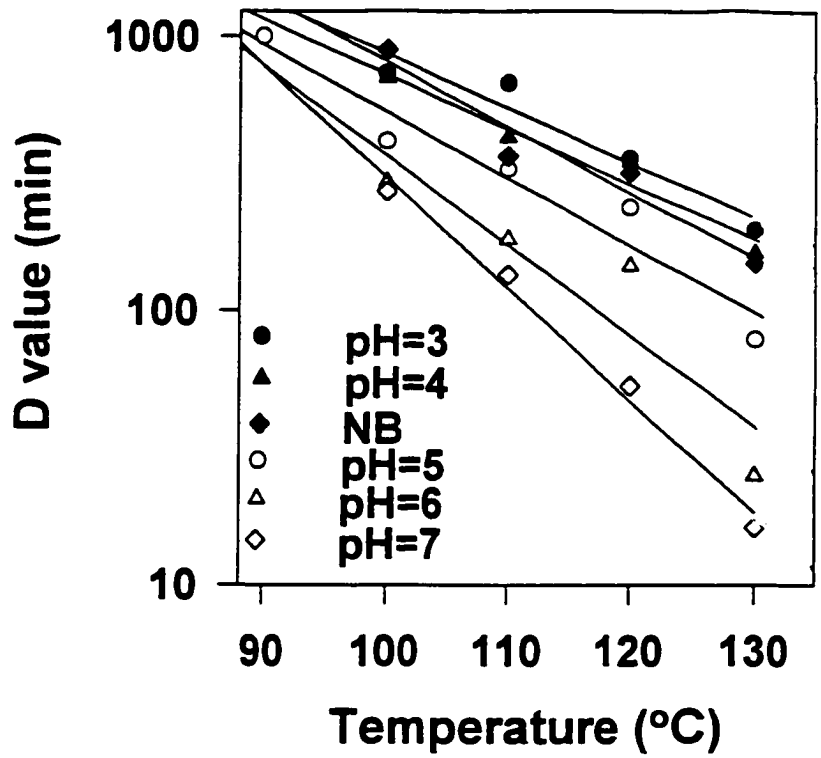


Fig. 6.6 TDT curves for GTX 1/4 toxins in the standard toxin mixture

Table 6.1 Decimal reduction time (D, min) for C toxins (C1/C2)

pH Temp.	3	4	5	6	7	NB
90°C	$r^2 = 0.91$ D=117.25	$r^2 = 0.78$ D = 171.9	r^2=0. 86 D = 194.1	$r^2 = 0.91$ D = 187.4	$r^2 = 0.598$ D = 226.6	$r^2 = 0.77$ D =206.8
100°C	$r^2 = 0.89$ D = 109.7	$r^2 = 0.85$ D = 140.1	r^2=0.96 D = 147.5	$r^2 = 0.91$ D = 189.6	NS	$r^2 = 0.77$ D = 144.7
110°C	$r^2 = 0.91$ D = 43.6	$r^2 = 0.96$ D = 108.8	$r^2 = 0.96$ D=104.9	$r^2 = 0.69$ D=374.19	NS	r^2= 0.96 D=116.5
120°C	$r^2 = 0.95$ D = 22.3	$r^2 = 0.95$ D = 55.07	r^2=0.94 D = 92.3	$r^2 = 0.66$ D = 296.2	NS	$r^2 = 0.89$ D = 67.1
z value (C°)	$r^2 = 0.91$ z = 28.9	$r^2 = 0.94$ z = 62.7	r^2=0.97 z = 89.5	NS	NS	r^2=0.97 z = 63.9

NS: r^2 values are not significant ($P \geq 0.05$)

6.1). Thus, 90% reduction of C1/C2 could be obtained by heating at 120°C for 22.3 min at pH 3 or for 67.1 min at pH 7. The decimal reduction times clearly indicate that GTX 1 and 4 were more heat stable than C toxins (Table 6.2), and 90% reduction of GTX 1/4 could be achieved by heating at 120°C for 56.8 min at pH 7 or for 360 min at pH 3. D values for GTX 2/3 in the standard toxin mixture also decreased with increasing pH and temperature, and ranged from 53.7 to 378 min (pH 7-3) at 120°C. It is also interesting to note that STX had the highest D values, especially at low pH levels, suggesting that STX is the most heat stable toxin examined. However, D values at 120°C for NEO and STX varied from 89.7 to 380 min and 259 to 997 min, respectively, for the same pH range. The extremely high D values of STX clearly indicate the thermal resistance of STX, especially at low pH levels. TDT curves for the theoretical integrated specific toxicities calculated from HPLC data are shown in Fig. 6.7, and it is clear that D values decrease with increasing temperature and pH, with the values ranging from 117.6 to 842 min at 120°C for the pH range of 7-3. The D values for total toxin levels were much higher at low pH levels and temperatures. At all temperatures, the lowest D values for all carbamate toxins were obtained at pH 7 whereas the highest values were obtained at pH 3.

z values were calculated as the negative slope of the "phantom" TDT curves obtained by plotting D values at different temperatures on a logarithmic scale with the corresponding temperatures on a linear scale. z values indicate the temperature range necessary to bring about a tenfold change in the TDT or D value, and may also vary according to the type of food components and food related microorganisms (Banwart, 1989). z values calculated for the individual PSP toxins in the standard mixture are shown in Table 6.3. STX had the highest z values, indicating low temperature sensitivity.

Thus, it is clear that both pH and heating time together, rather than individually, affect the degradation of any PSP component in the mixture without a matrix, as also reported in Chapter 5, for toxins in a matrix (scallop homogenate). Linear regression models derived for the destruction of individual toxins could be improved by the addition of an interaction term (pH x time) and the best fitted models for the degradation of individual standard toxins as well as for the specific toxicity are shown in Table 6.4. The

nature of the normal probability plot, and the residual plot of each model were also checked in addition to the F-ratio and P-value, to determine the best fit. In conclusion, the pH, heating time and temperature together, rather than individually, have significant affect ($P \leq 0.05$) on the degradation of PSP toxins. C toxins were found to be the most heat labile followed by GTX 1/4, GTX 2/3, NEO and STX, respectively.

Table 6.2 Decimal reduction times (D, min) for GTX 1/4

pH Temp.	3	4	5	6	7	NB
90°C	NS	NS	r^2=0. 41 D =1007.1	r^2 = 0.78 D = 385.9	r^2 = 0.88 D = 366.4	NS
100°C	r^2 = 0.86 D = 744.1	r^2 = 0.92 D = 712.1	r^2 =0.93 D = 419.4	r^2 = 0.97 D = 295.4	r^2 = 0.97 D = 273.6	r^2 = 0.43 D = 889.9
110°C	r^2 = 0.89 D = 681.1	r^2 = 0.91 D = 431.4	r^2 = 0.94 D = 329.1	r^2 = 0.94 D = 182.9	r^2 = 0.92 D = 135.1	r^2 = 0.93 D = 368.7
120°C	r^2 = 0.92 D = 360.3	r^2 = 0.94 D =351.9	r^2 =0.96 D = 239.4	r^2 = 0.98 D = 145.9	r^2 = 0.98 D = 56.9	r^2 = 0.79 D = 320.5
130°C	r^2 = 0.94 D = 197.2	r^2 = 0.97 D = 162.4	r^2 = 0.91 D = 78.5	r^2 = 0.94 D = 25.3	r^2 = 0.99 D = 16.2	r^2 = 0.97 D = 148.7
z value (C°)	r^2 = 0.93 z= 49.8	r^2 = 0.95 z= 49.6	r^2 = 0.93 z= 40.6	r^2 = 0.84 z= 30.3	r^2 = 0.99 z= 24.5	r^2 = 0.94 z= 41.6

NS: r^2 values for survivor curves are not significant ($P \geq 0.05$)

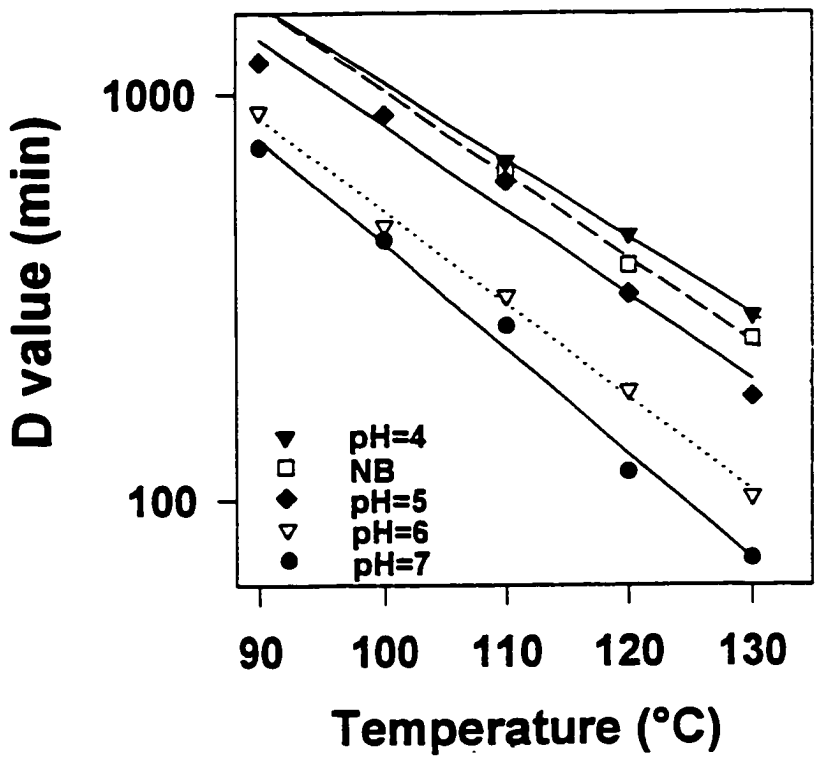


Fig. 6.7 TDT curves for total specific toxicities of the standard toxin mixture

Table 6.3 z values (C°) for standard PSP toxins

pH Toxin	3	4	5	6	7	NB
C1/2	$r^2 = 0.91$ z = 28.9	$r^2 = 0.94$ z = 62.7	$r^2 = 0.97$ z = 89.5	NS	NS	$r^2 = 0.97$ z = 63.9
GTX 1/4	$r^2 = 0.93$ z= 49.8	$r^2 = 0.95$ z= 49.6	$r^2 = 0.93$ z= 40.6	$r^2 = 0.84$ z= 30.3	$r^2 = 0.99$ z= 24.5	$r^2 = 0.94$ z= 41.6
GTX 2/3	NS	$r^2 = 0.87$ z = 60.3	$r^2 = 0.99$ z = 39.8	$r^2 = 0.98$ z = 34.3	$r^2 = 0.94$ z = 27.9	$r^2 = 0.94$ z = 42.9
NEO	NS	NS	$r^2 = 0.99$ z= 56.1	$r^2 = 0.99$ z= 38.7	$r^2 = 0.99$ z= 35.2	$r^2 = 0.99$ z= 54.7
STX	NS	NS	$r^2 = 0.98$ z = 59.9	$r^2 = 0.98$ z = 38.9	$r^2 = 0.98$ z = 35.3	NS
Specific Toxicity (MU/mL)	NS	$r^2 = 0.97$ z= 45.4	$r^2 = 0.98$ z= 44.9	$r^2 = 0.99$ z= 41.8	$r^2 = 0.99$ z= 38.5	$r^2 = 0.98$ z= 44.4

** r^2 values for TDT curves are not significant ($P \geq 0.05$)

Table 6.4 Statistical models for the degradation of PSP toxins in the standard PSP toxin mixture

Model	R^2	F-ratio	P-value
$C12 = 12.7 - 0.00788 (pH \cdot T) - 0.00263 (T \cdot t) - 0.0499 (pH \cdot t) + 0.000544 (pH \cdot T \cdot t) + 0.157(pH^2) + 0.00137(t^2)$	0.85	170.79	0
$GTX\ 14 = 97.5 + 0.595\ pH - 2.66\ (T) - 0.00747\ (pH \cdot T) - 0.000076\ (pH \cdot T \cdot t) + 0.0254\ (T^2) - 0.000079\ (T^3) - 0.0000001\ (pH^3 \cdot t^3 \cdot T^3)$	0.95	324.28	0
$GTX\ 2/3 = -12.1 + 0.000001(t^3 \cdot T^3 \cdot pH^3) - 0.002(T^3) - 0.872(pH^3) - 0.109(t \cdot T \cdot pH) + 16.4(t \cdot pH) - 0.005(t^2 \cdot pH^2) + 34(T)$	0.84	201.96	0
$NEO = 0.378 - 0.00422\ (T) + 0.00467\ (t) + 0.00101\ (pH \cdot t) - 0.000020\ (pH \cdot T \cdot t) + 0.000122\ (T^2) - 0.000039\ (t^2) - 0.000001\ (T^3)$	0.94	331.03	0
$STX = - 6.12 + 3.46\ (pH) - 0.0205\ (T) + 0.0200\ (t) - 0.00784\ (pH \cdot T) + 0.00758\ (pH \cdot t) - 0.000122\ (pH \cdot T \cdot t) - 0.521\ (pH^2) + 0.00223\ (T^2) + 0.0321\ (pH^3) - 0.000012\ (T^3)$	0.94	381.57	0
Specific toxicity (MU/mL) $= 14 + 0.609\ (T) + 0.711(t) + 25.7\ (pH) - 0.121\ (T \cdot pH) + 0.125(t \cdot pH) - 0.00287(T \cdot t \cdot pH) - 0.00227(T \cdot t) - 1.34(pH^2) + 0.0000001(t \cdot T \cdot pH)^2$	0.95	507.1	0

[t = time (min), T = temperature (°C)]

CHAPTER 7

STORAGE STABILITY OF PARALYTIC SHELLFISH POISONING TOXINS

7.1 Abstract

Variations in the paralytic shellfish poisoning (PSP) toxin profiles of scallop digestive glands and a mixture of purified PSP toxins were studied. Changes in C toxins (C1- 2), GTX (gonyautoxin) 1- 4, STX (saxitoxin) and NEO (neosaxitoxin) were studied during long term storage at -35°C, 5°C and 25°C and at different pH levels. Citrate/phosphate buffers with pH values ranging from 3 to 7 were added to scallop homogenate and the purified toxin mixture to achieve predetermined pH levels. Homogenates and purified toxin mixtures were stored for one year and 4 months, respectively, at -35°C, 5°C and 25°C. Homogenates and purified toxin mixtures were also heated at 120°C for one hour, and stored in the same manner. PSP toxins in the homogenates and in the purified mixtures were analyzed every 4 months and every month, respectively, by HPLC.

C toxins levels decreased rapidly during storage at 25°C with a significant decrease after the first 2 months. Although the GTX 2/3 levels in the unheated samples did not change initially at pH 3-4, they decreased at pH 6-7 with the fastest rate at 25°C followed by 5°C. There was no significant change in any toxin type when stored at -35°C regardless of pH. GTX 1/4 in the toxin mixture decreased significantly after 4 months at 25°C. Toxin levels in heated samples decreased during storage at room temperature. NEO and STX levels remained unchanged at all storage temperatures at low pH (pH 3), whereas NEO levels continued to decrease at higher pH levels (pH 6-7) at 25°C. The results clearly demonstrated that all toxins were stable at low pH (pH 3-4) and -35°C. Thus, it is recommended that purified toxins used as primary analytical standards as well as partially purified toxins and toxins in a homogenized matrix, sometimes used as secondary standards, can be stored safely at pH 3 and at ≤-35°C. Also, thermal processing of shellfish at neutral pH levels (pH 7) is safer than at acid pH levels, since the toxicity decreased most after heating at neutral pH, and decreased further during storage at room temperature. This

work has been submitted for publication in Food Chemistry (Indrasena and Gill,1999c).

7.2 Introduction

Paralytic shellfish poisoning toxins are imidazoline derivatives which are accumulated by shellfish *via* the food chain and cause sporadic food poisoning in humans in many parts of the world. These toxins have historically been a problem for the shellfish industry on both the Pacific and Atlantic coasts of Canada and the U.S. (Lawrence *et al.*, 1994). Several species of the dinoflagellate genus, *Protogonyaulax (Alexandrium)*, produce PSP toxins (Fig. 1.1) which are all related to STX. Structurally, they can be divided into carbamate, sulfomate and decarbamoyl toxins. Due to the nature of these differences in charge and the substitutional groups to the basic saxitoxin structure, they bind to site 1 of the sodium channel (Catterall *et al.*, 1988) with different affinities resulting in different toxicities. The stability of each toxin derivative may also change under different environmental conditions and storage.

Thermal stabilities of these toxins have been studied by many authors (Medcof *et al.* 1947; Prakash *et al.*; 1971; Noguchi *et al.*, 1980; Asakawa and Takagi, 1983; Gill *et al.*, 1985; Asakawa *et al.*, 1986; Chang *et al.*, 1988; Ohta *et al.*, 1992; Berenguer *et al.*, 1993; Lawrence *et al.*, 1994; Indrasena and Gill, 1999) and some indicated that most of the PSP compounds are thermally stable at normal cooking temperatures (Gill *et al.*, 1985; Nagashima *et al.*, 1991; Indrasena and Gill, 1999). However, some can undergo structural changes especially at low pH values, converting the less-toxic carbamoyl-N-sulfo PSP compounds such as B1 and B2 into highly toxic carbamoyl counterparts such as STX and NEO (Kobayashi and Shimizu, 1981; Harada *et al.*, 1982; Nishio *et al.*, 1982; Hall and Reichardt, 1984; Alfonso *et al*, 1993; Indrasena and Gill, 1999a and b). Commercial canning can reduce the PSP content when shellfish were processed in brine water (Berenguer *et al.*, 1993). Conventional canning around neutral pH reduced total toxicity by about 90-95% and the toxicity further decreased by another 36-65% during storage at room temperature for one year (Prakash *et al.*, 1971).

Louzao *et al.*, (1994a) reported that lyophilization may render PSP toxins unstable during storage. They also indicated that lyophilized C toxins and GTXs lost toxicity during storage for 2 months at -80, -20, 4 and 35°C with the fastest rate at 35°C. Alfonso *et al.* (1994) demonstrated that STX is more stable than NEO in acidic solutions and the toxins in solution were more stable than in the lyophilized form. They also suggested that STX could be stored at temperatures below 5°C in dilute acidic solutions for up to 2 years without loss of toxicity. Shimizu (1988) also indicated that STX can be stored in dilute hydrochloric acid for years without loss of potency and the hydrolysis of the carbamoyl ester can only occur in concentrated acid solutions such as 7.5N hydrochloric acid at 100°C. According to Louzao *et al.* (1994b), B2 was the most unstable toxin under any condition and at temperatures above 0°C whereas GTX 2 and 3 were the most stable in acidic solutions, especially at 4°C.

However, a detailed study of the nature of changes in PSP toxicity during storage at different pH levels has not been published to date. The objective of this study was to examine the stability of purified PSP toxins and PSP compounds in toxic scallop digestive glands during storage at different pH values and temperatures.

7.3 Materials and Methods

7.3.1 Preparation of Homogenates

Toxic scallops provided by Fisheries and Oceans Canada Inspection Branch (Digby and Yarmouth), were transported in ice to the laboratory in Halifax and were frozen immediately at -35°C. They were thawed at 5°C prior to homogenization. Pieces of shells, sand and other debris were removed and tissue was washed gently. The digestive glands were carefully removed from the rest of the tissues and were collected in a container surrounded by ice. They were homogenized in a Commitrol 3600 flake cutter (Urshel Corp., Valparaiso, Indiana) to produce a finely-divided paste. The homogenate was thoroughly mixed so that all sub-samples taken from the homogenate would contain identical amounts of toxins.

The proximate analysis, amino acid composition, mineral composition, pH and salinity of the homogenate were determined as additional information.

7.3.2 Buffering, Heating and Storage

Six-mL homogenate samples were mixed with 6 mL aliquots of 1.2 M citrate/phosphate buffer adjusted to pH's ranging from 3 to 7 and stored under nitrogen in sealed screw-cap tubes at 5, 25 and -35°C for one year. Samples prepared in the same manner were heated under nitrogen for 60 min in screw cap culture tubes in a 120°C thermostatically controlled oil bath and temperatures were monitored using thermocouples inserted through the screw caps. Control samples (with no buffer, NB) were heated under nitrogen in the presence of distilled water. After heating, all tubes were immediately transferred into an ice bath. Heated/cooled homogenates were also stored under similar conditions. Three samples of both heated and unheated homogenate from each pH and temperature combination were removed for the extraction and analysis every four months. Homogenates stored at 25°C were extracted only once after one month.

Mixtures of partially purified toxins containing STX, NEO, GTX 1, GTX 2, GTX 3, GTX 4, C1, C2, and B1 with the pH levels ranging from 3 to 7 were also prepared and heated in the same manner. Both heated and unheated samples were stored under nitrogen at 5, 25 and -35°C for four months and 2-3 samples from each pH and temperature were analyzed monthly for four months.

7.3.3 Extraction of Toxins

The homogenates were stirred well for about 2 min and the contents transferred into a 25 mL beaker. The tubes were rinsed with double distilled, de-ionized water and added to the beaker, macerated with a Polytron homogenizer for 10 min at the high speed setting (8). The macerated homogenates were centrifuged in plastic tubes for 30 min at 6000 r.p.m (4266 g), the supernatant from each tube was transferred into a 150 mL separatory funnel and de-fatted with dichloromethane:water (2:1). The aqueous layer of each funnel was carefully transferred into Teflon tubes, centrifuged and frozen at -35°C

until used for subsequent toxin analyses by HPLC.

7.3.4 Analysis of Toxins by HPLC

All samples were ultrafiltered through 1000 da MW cutoff membranes (MSI, MICRON Separation Inc., West Bord, MA) to remove proteins, prior to HPLC injection. PSP's were determined with a Waters high performance liquid chromatograph equipped with two Model 510 pumps, a WISP auto injection system controlled via a system interface module and a Shimadzu Model Rf 535 fluorescence detector (338 nm excitation, 400 nm emission). Samples were run in the HPLC using a Whatman PRP-1 column (15 cm x 4.1 mm) packed with 10 µm beads using a binary elution gradient (Mobile phase A: water with 1mM hexane and heptane sulphonic acid, Mobile phase B: acetonitrile with hexane and heptane sulphonic acid) according to Sullivan and Wekell (1987). The HPLC was equipped with a dual reagent post column reaction system. Post-column derivatization was carried out by sequential mixing with nitric acid (0.75M) and periodic oxidant (5N sodium hydroxide, 5mM periodic acid and 0.5M ammonium hydroxide) prior to the fluorescence detection. Individual toxins were identified by running a standard mixture of PSP toxins and were externally quantified using authentic PSP toxins obtained from the National Research Council of Canada, Institute for Marine Biosciences, Halifax, Nova Scotia. Theoretical specific toxicities for HPLC data obtained for scallop homogenates were calculated using toxicity factors for individual compounds according to Hall and Reichardt (1984).

7.3.5 Statistical Analysis

The toxin levels ($\log_{10}$) at each pH and time interval were compared by Analysis of Variance (ANOVA) with general linear model *via* multiple regression using indicator variables for log transformed data, and in some cases the rates of change in the amounts of individual toxin components were compared by analysis of covariance (ANCOVA) *via* multiple regression using indicator variables, in MINITAB Version 12 on the Dalhousie University main frame computer.

7.4 Results and Discussion

The toxic scallop homogenate contained 71.12% (±2.34) water, 3.68% (±0.82) lipids, 10.52% (±2.42) ash, 8.82% (±1.23) protein, and glutamic acid, alanine and leucine were the most predominant amino acids whereas cysteine and glycine were present only in small amounts. Triacylglycerols were the main lipid component in the homogenate followed by free fatty acids and other unknown oxidized compounds. It also contained a wide array of minerals including iron (200 mg/kg), aluminum (84 mg/kg) and cadmium (54 mg/kg).

Prakash *et al.* (1971) indicated that conventional canning reduces the level of toxins by a considerable extent, and there was a significant further decrease during storage at room temperature after canning. Findings in Chapters 5 and 6 clearly indicate that although there is a significant decrease in toxicity after heating at neutral pH levels and at commercial sterilization temperatures, most of the carbamate toxins remained unchanged when heated at low pH levels. During the storage of such heated samples, in the present study, the toxin levels varied widely depending on the pH and the storage temperature. Both heated and unheated homogenates containing mainly GTX 2-3, NEO, STX and C toxins as well as the partially purified toxin mixture consisting of C1-2, GTX 1-4, NEO and STX (Fig. 7.1A-B) changed at different rates during the storage at all temperatures.

Specific toxicities determined using the mouse neuroblastosoma cell bioassay (Jellet *et al.*, 1992) were positively correlated (r^2=0.75, P≤ 0.05) with the theoretical integrated toxicities calculated from data obtained from Sullivan and Wekell (1987) HPLC method (Chapter 5; Indrasena and Gill, 1999a). Theoretical specific toxicities calculated for HPLC data obtained for some homogenate samples stored at different pH levels and temperatures in the present study are shown in Fig. 7.2. These toxicities did not change significantly during storage at -35°C at any pH or at 5°C at pH 3, whereas there was a slight decrease at 5°C at pH 7. It is clear from Fig. 7.2 that heating has a far greater effect than storage with regard to total PSP destruction. The pronounced effect of pH is also demonstrated. For example, heating toxic homogenates at 120°C for 60 min resulted in a nearly 2-fold reduction in total toxicity at pH 7 compared with that at pH 3, although subsequent storage for 12 months at 5°C showed no comparable pH effect.

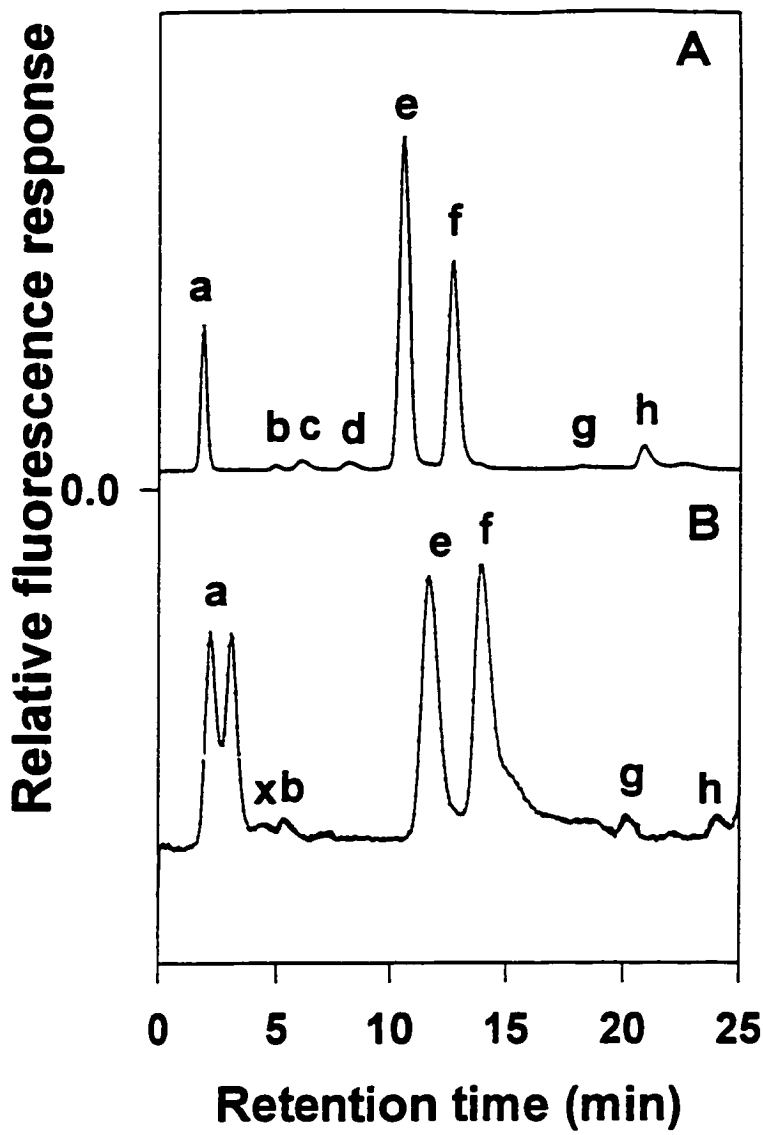


Fig. 7.1 A. HPLC chromatogram of initial mixture of purified PSP toxin mixture (a = C1-2, b = GTX 4, c = GTX 1, d = B1, e = GTX 3, f = GTX 2, g = NEO, h = STX)

B. HPLC chromatogram of initial scallop homogenate Purified toxin mixture (a = C1-2, x = B2, b = GTX 4, e = GTX 3, f = GTX 2, g = NEO, h = STX)

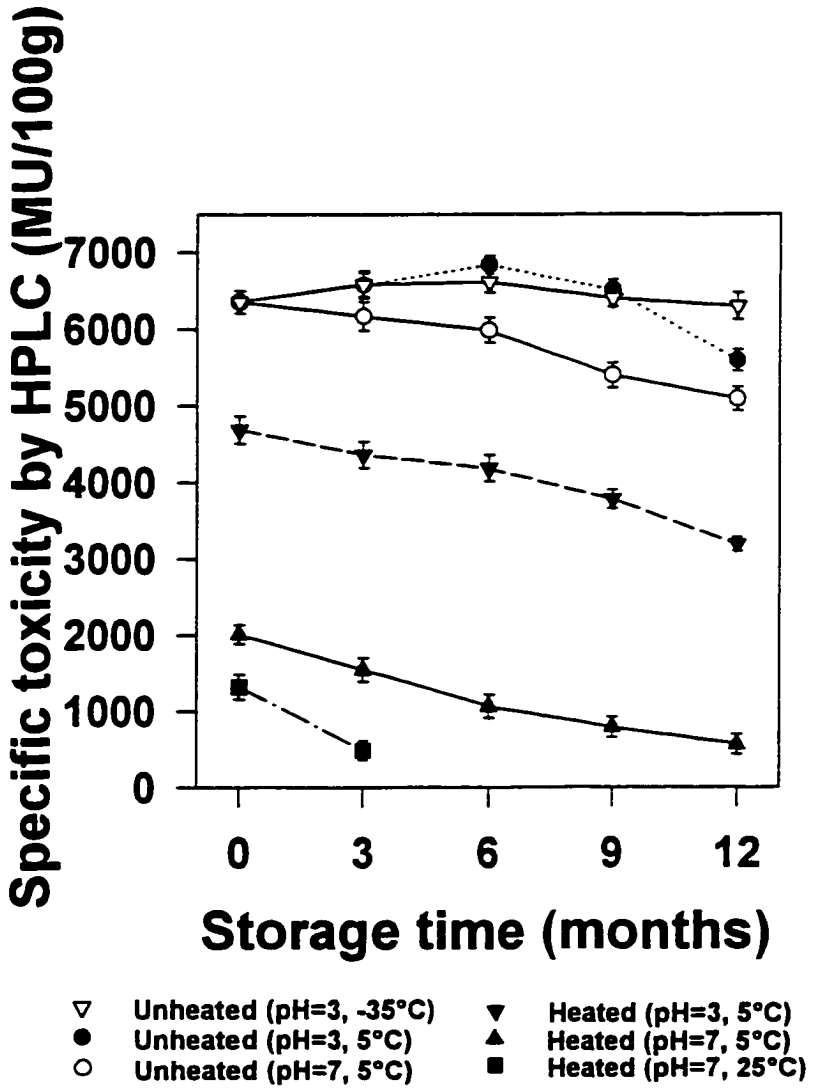


Fig. 7.2 Variation in the calculated specific toxicities of scallop homogenate during storage.

C toxins, having relatively low toxicity can be readily converted into more highly toxic carbamate toxins such as GTX 2 and 3 under certain conditions. It was mainly for this reason that the C toxins were monitored in some detail. However, the C toxin levels in the purified toxin mixture did not change significantly ($P \geq 0.05$) during storage at -35°C for 4 months. Although they did not change significantly at 5°C at low pH values, there was a significant decrease ($P \leq 0.05$) at pH 7 after 2 months (Fig. 7.3A-B). The levels of these toxins also decreased significantly after one month of storage at pH 6-7 and 25°C. There was about a 25% decrease at pH 5 at 5°C and about 47% decrease at 25°C after 4 months. The C toxin level decrease at low pH values may possibly be due to the conversion of C1 and C 2 toxins into GTX 2 and 3. However, it is difficult to conclude that this conversion is indeed significant since levels of GTX 2/3 may also be increasing or decreasing by other mechanisms, simultaneously. Although there was about a 40% decrease at pH 7 at 25°C after 2 months, the C toxin levels in the pH 6-7 toxin mixture increased after 3 months. This apparent increase may be either due to co-elution of some degraded compounds or to the conversion of some other toxin counterparts to C toxins or by both mechanisms. However at low pH, the toxin degradation was less severe. C toxin levels in the scallop homogenate began to decrease significantly at all pH levels after one month at 25°C, however at -35°C, it remained almost unchanged for 12 months.

GTX 2/3 levels in the purified toxin mixture did not change significantly ($P \geq 0.05$) at -35°C. However, GTX 2/3 decreased by about 20% at pH 7 after 4 months and were significantly ($P \leq 0.05$) reduced after 3 months at 25°C and pH 7 (Fig. 7.4A, Appendix G). Heated toxin mixtures followed a similar pattern with nearly complete GTX 2/3 destruction in unbuffered and pH 7 samples within a 4 month storage period at 5°C (Fig. 7.4B). Figure 7.5A illustrates that the GTX 2/3 levels in the unheated homogenates remained more or less constant at 5°C for approximately 3 months. Beyond this 3 month period, levels gradually decreased with storage time, with the greatest change occurring at the highest pH levels. Heating followed by 5°C storage resulted in more dramatic toxin reductions with the greatest degradation occurring at the higher pH levels (Fig. 7.5B). The consistent decrease of toxin levels may be due to chemical degradation or the conversion of GTX 2/3 into STX

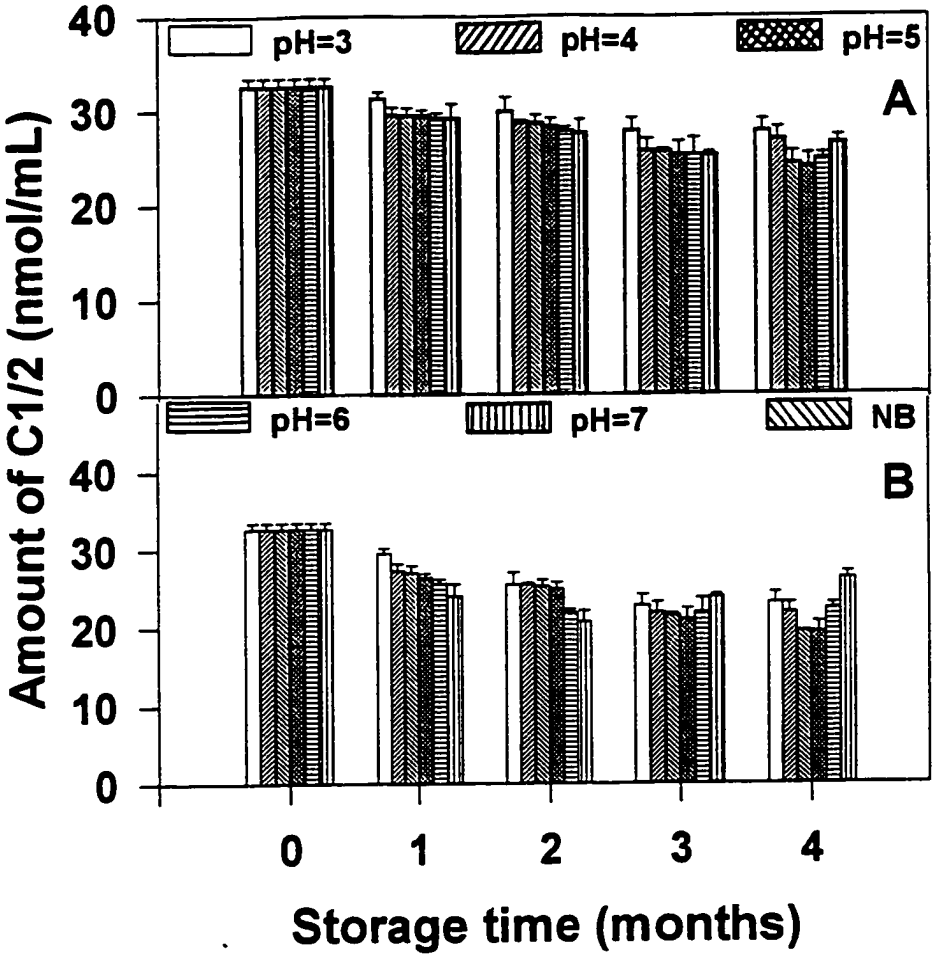


Fig. 7.3 Changes in C1/2 toxins in the unheated purified toxin mixture **A.** Storage 5°C **B.** Storage at 25°C (Error bars show the standard deviation of the mean, n=2)

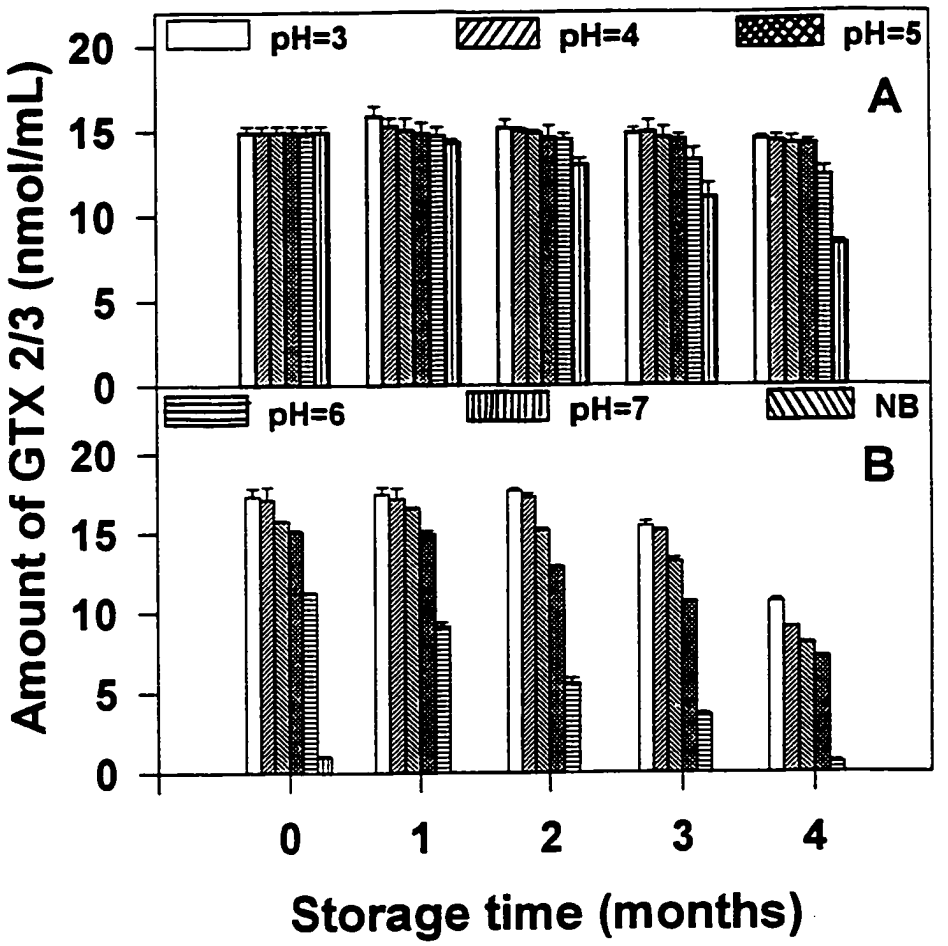


Fig. 7.4 Changes in GTX 2/3 toxins in the purified toxin mixture **A**. Unheated samples stored at 5°C **B**. Heated samples stored at 5°C (Error bars show the standard deviation of the mean, n=2)

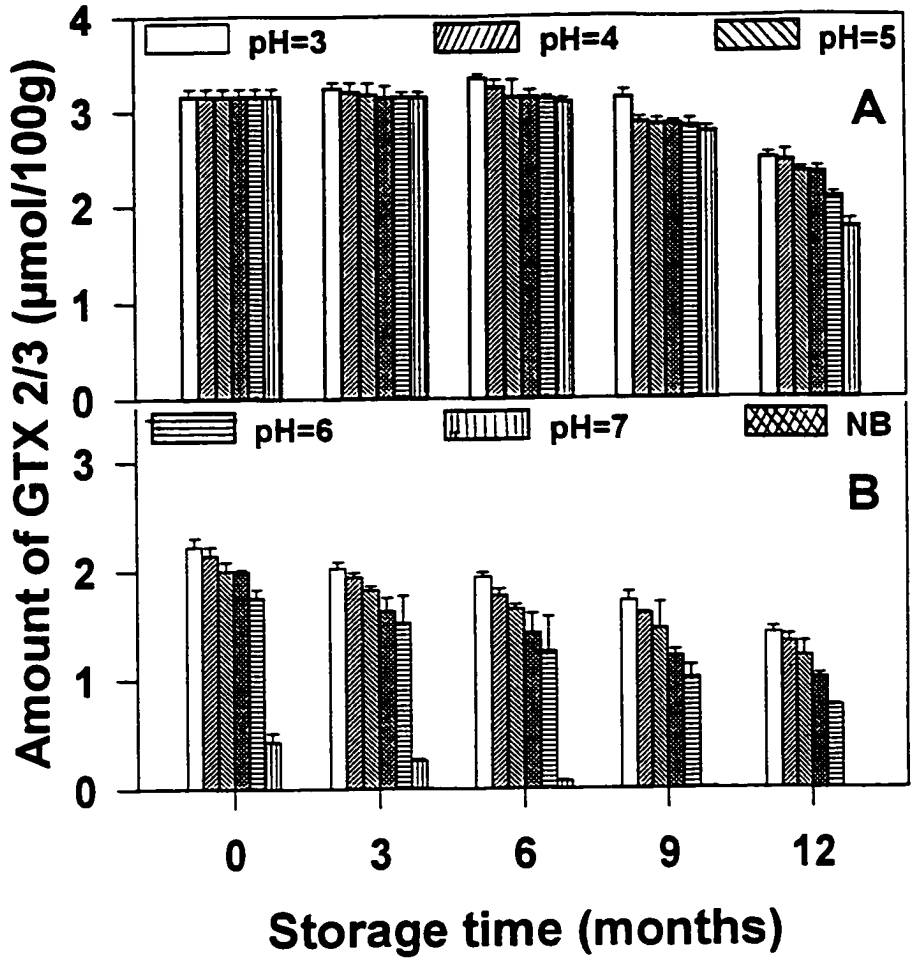


Fig. 7.5 Changes in GTX 2/3 toxins in scallop homogenate
A. Unheated samples stored at 5°C **B.** Heated samples stored at 5°C (Error bars show the standard deviation of the mean, n=3)

by reductive elimination of the sulfonyl group (Shimizu and Yoshioka, 1981) or both. Louzao *et al.* (1994a) indicated that GTX 2 and 3 in lyophilized samples containing C1-4 toxins increased initially, and decreased during storage at -80, -20, 4 and 37°C over a period of 2 years. Louzao *et al.* (1994b) also noticed that all GTXs were stable for almost 2 years when stored at -80 and -20°C in mild acidic solutions whereas the GTX 2 and 3 content increased at 4 and 37°C initially due to interconversions and then gradually declined. However, these changes were only studied under acid conditions (pH value not given); whether the toxin profiles were significantly changed from the initial samples, was difficult to judge since their data were not statistically analyzed. The current study clearly demonstrated that at -35°C and at pH 3-4, GTX 2/3 levels in homogenates as well as in the purified toxin mixture were stable throughout the study period.

The GTX 1/4 levels in the purified toxin mixture changed at all pH levels with time of storage and, as with the other toxins, the changes were more pronounced at neutral pH and at the highest storage temperature. GTX 1/4 levels decreased significantly ($P \leq 0.05$) at 25°C after 3 months with the maximum destruction of 88% at pH 7 after 4 months (Fig. 7.6A). However, these toxins remained almost unchanged at pH 3-4 and at 5°C. There was only a 17% decrease in GTX 1/4 at pH 7. GTX 1 and 4 are epimeric 11-hydroxyneosaxitoxin sulphates which can be converted to NEO by reductive cleavage of the O-sulphate group (Shimizu and Yoshioka, 1981) and it may be that the decrease of GTX 1/4 on storage may possibly be due to the conversion of these toxins into NEO. Samples stored at -35°C did not show any change for 4 months. The GTX 1/4 content in the heated toxin mixtures also had the same general pattern of change during storage (Fig. 7.6B) but the scallop homogenate did not contain measurable amounts of GTX 1/4.

The levels of NEO in both heated and unheated homogenate as well as in the purified toxin mixture did not change at any pH during the storage at -35°C. Although there was no significant change in the NEO level in the purified toxin mixture at 5°C at low pH levels (pH 3-5), it degraded slowly at pH 7 resulting in the maximum degradation of 27% after 4 months. NEO levels in unheated purified toxin mixtures decreased by about 33% at pH 7 after 4 months of storage at 25°C (Fig. 7.7A). It was apparent that the toxin

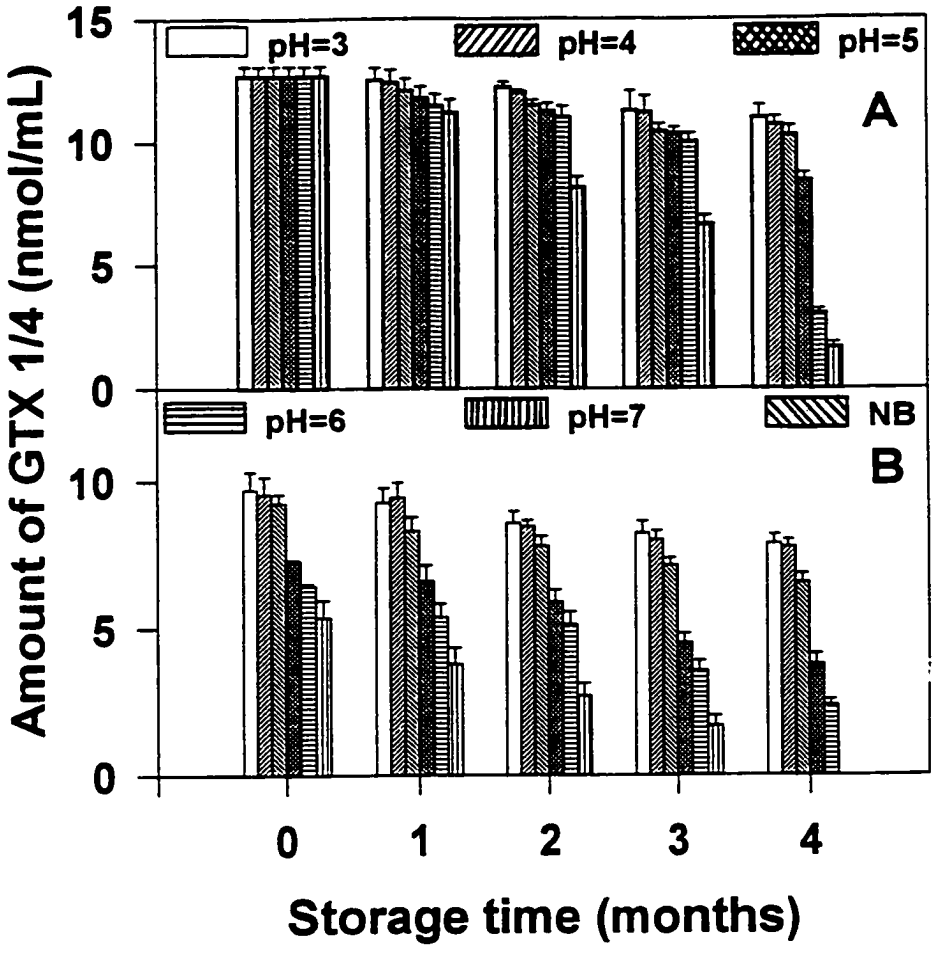


Fig. 7.6 Changes in GTX 1/4 toxins in the purified toxin mixture **A.** Unheated samples stored at 25°C **B.** Heated samples stored at 25°C (Error bars show the standard deviation of the mean, n=2)

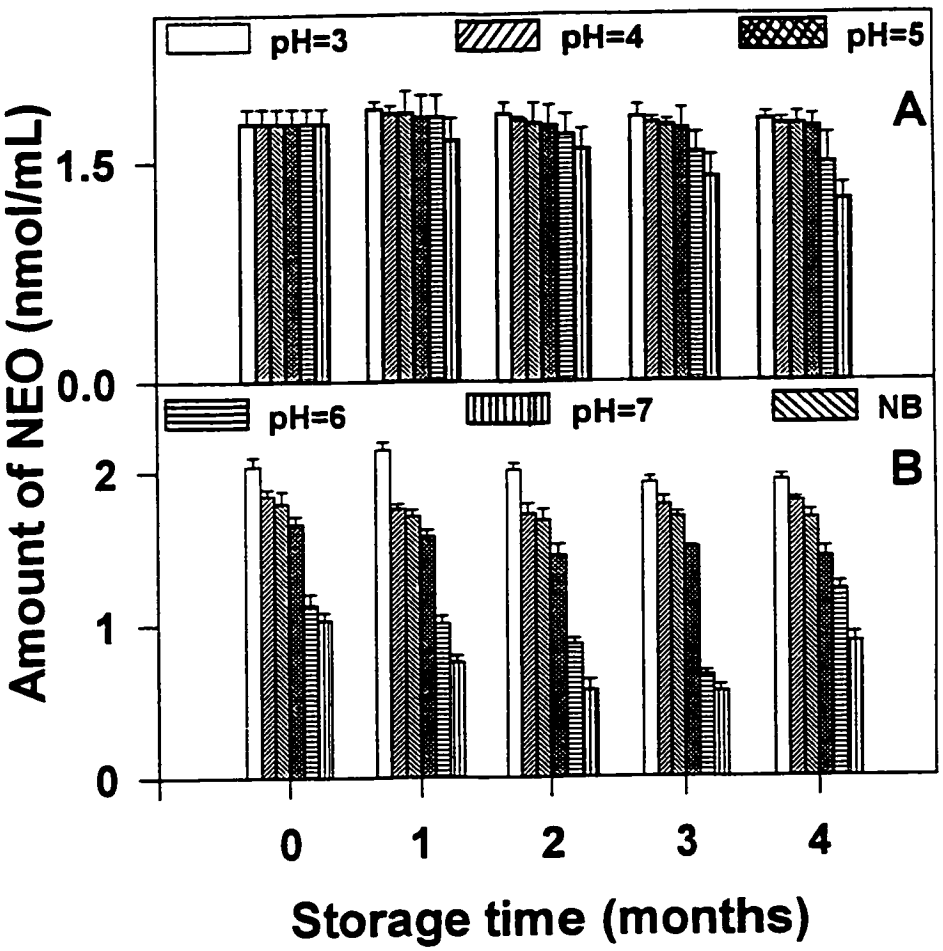


Fig. 7.7 Changes in NEO in the purified toxin mixture
A. Unheated samples stored at 25°C B. Heated samples stored at 25°C (Error bars show the standard deviation of the mean, n=2)

degradation observed in Fig. 7.7 was primarily due to heating. The degradation due to storage was small by comparison. Although the heated toxin mixture contained more NEO than in the unheated mixture (due to possible conversion of GTX 1/4 into NEO during heating), NEO levels in the heated toxin mixture also had the same general pattern with maximum decrease of 45% during the storage at 25°C (Fig. 7.7B). The amount of NEO in the scallop homogenate at 5°C and at pH 3-5 also did not change significantly throughout the study period, whereas at pH 7, the NEO levels declined with the maximum loss of 29% after 12 months (Fig. 7.8A). However, it is interesting to note that NEO degraded much faster at 25°C giving the maximum decrease of 25% at pH 7 after one month whereas a significant change was not observed at pH 3-5 and NB samples. Alfonso *et al.* (1994) indicated that lyophilized NEO as well as the amount of NEO in mild acidic solutions stored even at -80°C, decreased after 6 months and these toxins almost disappeared completely at -20°C after 1 year.

STX levels in the frozen (-35°C) homogenate and purified toxin mixture did not change during frozen storage at -35°C at any pH level. STX levels in both heated and unheated homogenates did not change significantly at pH 3-5 and NB samples for 12 months at 5°C whereas it decreased by 4.5% and 12% at pH 6 and 7, respectively (Fig. 7.8B). However at 25°C, STX content at pH 6 and 7 increased by 1.4 and 2.5% after one month although there was no significant change at low pH values. STX in the heated homogenate decreased with the maximum decrease of 61% after 12 months at 5°C and 41% after 1 month at 25°C. STX content in the purified toxin mixture at pH 3-5 at 5°C also remained unchanged whereas there was about a 13% decrease at pH 7 after 4 months. Even at 25°C, STX levels at pH 4-5 as well as the NB sample did not change significantly ($P \geq 0.05$) (Fig. 7.9A). It is also interesting to note that STX levels in the heated purified toxin mixture also followed the same general pattern as unheated samples with the maximum destruction at pH 6-7 at 25°C after 4 months (Fig. 7.9B).

The results in the present study clearly demonstrate that regardless of whether purified or in a shellfish matrix, PSP toxins are most stable at low pH levels. As reported in Chapters 5 and 6, these toxins remain unchanged or increase slightly during heating at low

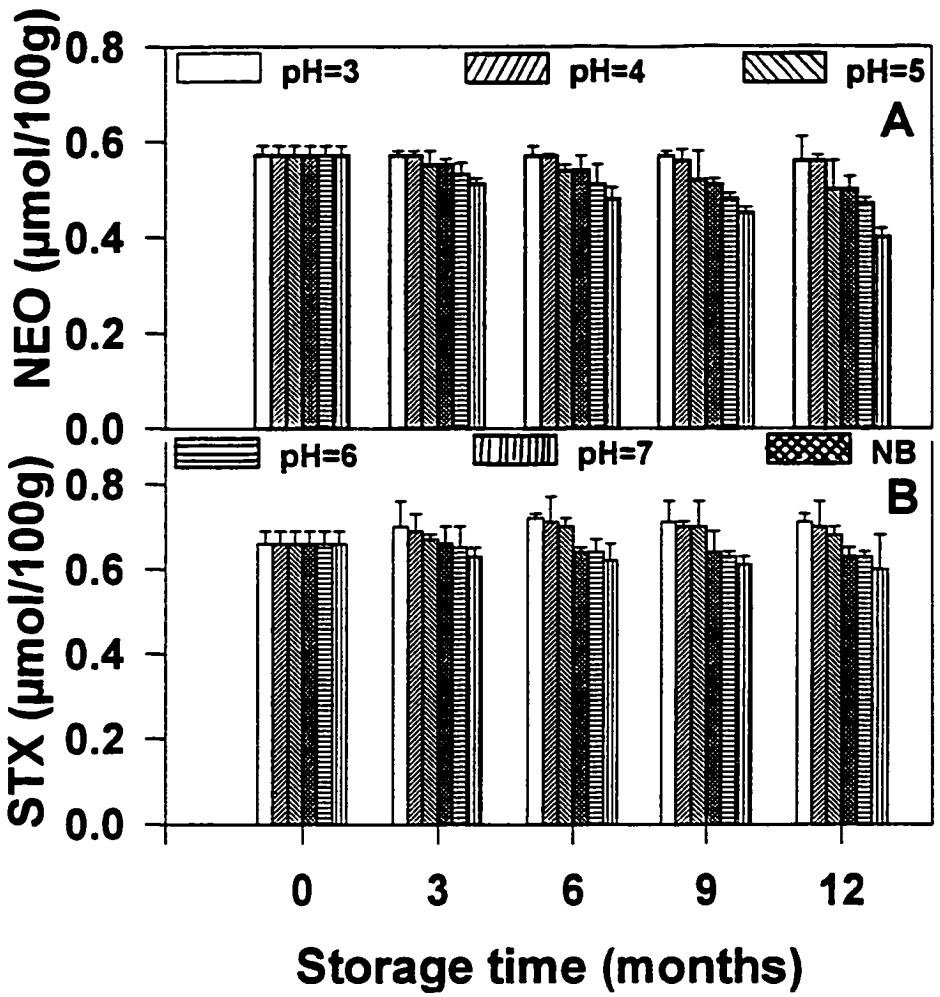


Fig. 7.8 A. Changes in NEO in scallop homogenate stored at 5°C
B. Changes of STX in scallop homogenate stored at 5°C
(Error bars show the standard deviation of the mean, n=3)

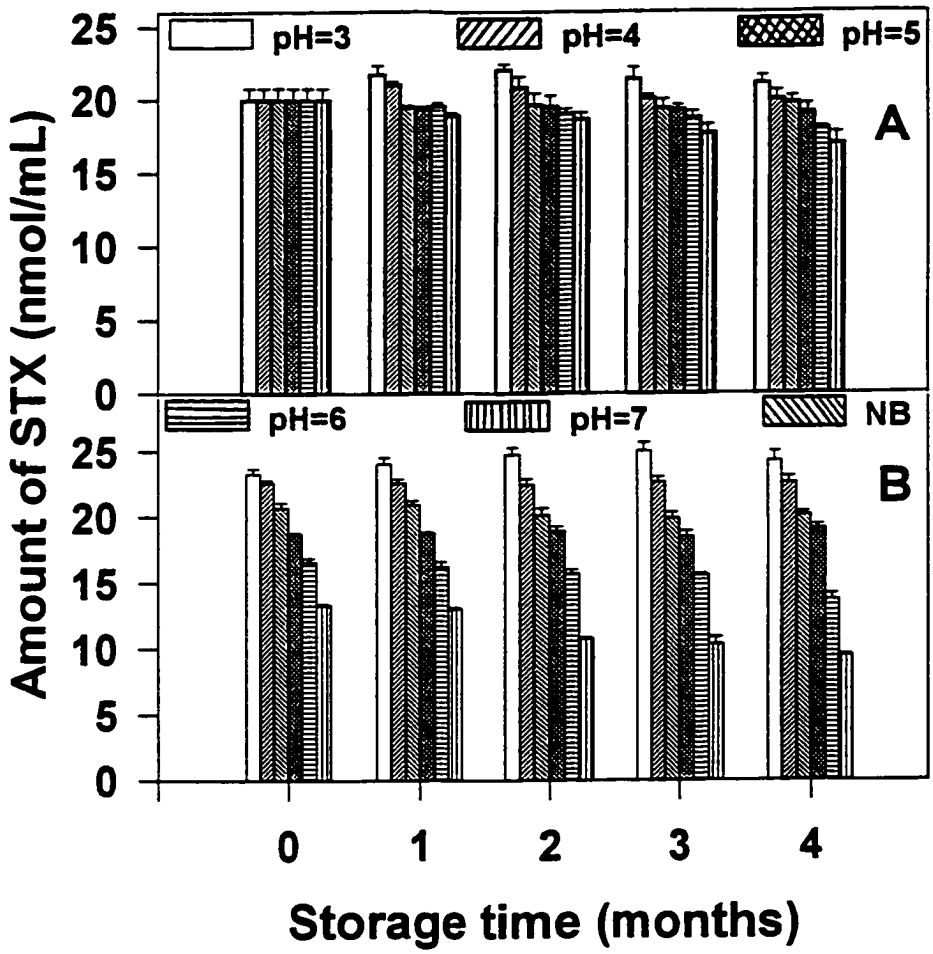


Fig. 7.9 **A.** Changes in STX in the unheated purified toxin mixture stored at 25°C **B.** Changes of STX in the heated purified toxin mixture stored at 25°C (Error bars show the standard deviation of the mean, n=2)

pH values, and the storage study confirms that toxin levels retain their potency under acidic conditions during storage even at room temperature for 4 months or 12 months at 5°C. However, the toxicity rapidly decreased at higher pH levels (pH 7) during storage at room temperature after heating at a conventional canning temperature, as also indicated by Prakash *et al.* (1971). Toxins stored in pure form as primary analytical standards or a somewhat less pure form as secondary standards, may change with time depending upon the conditions of storage. Thus, selection of proper storage conditions is important. Generally speaking, pH 3 was judged as the best pH and low temperature storage at ≤-35°C is recommended to prevent toxin interconversions and/or degradation.

7.5 Conclusions

- All toxins are most stable at -35°C and at pH 3-4.
- C toxins are the most sensitive followed by GTX 1/4 for changes at all pH values at 5 and 25°C.
- STX followed by NEO are the most stable at both -35 and 5°C, and especially at pH 3-4.
- Although previous reports indicate further rapid decrease in toxicity during storage subsequent to canning, these decreases during storage of toxic shellfish after canning are dependent upon the pH of the matrix in which the toxins are found.

CHAPTER 8

SUMMARY AND CONCLUSIONS

8.1 Detection of PSP Toxins

Paralytic shellfish poisoning poses a worldwide public health problem, and it is essential to have a rapid detection method for screening purposes. The most common method for determining the overall toxicity of shellfish extract is the mouse bioassay. Owing to ethical considerations and public outcry in the use of live animals as well as the marginal sensitivity of the assay, there have been many efforts to develop simple, rapid, reliable and cost-effective alternatives. These include bioassays, and various physico-chemical and immunological methods. Most of the chemical methods published to date involve the use of expensive instruments and highly trained staff, and are time consuming. Two different detection methods were attempted in the present study, and it was found that they are much faster than any other existing chemical detection methods.

Fluorescence detection is the most common chemical detection method, and H_2O_2, t-butylhydroperoxide and periodic acid have been reported as oxidants used in various detection techniques and analysis of PSP toxins. These three oxidants were used to develop a rapid, qualitative screening method for the fractionation of PSP toxins in the present fluorescent detection method, and H_2O_2 was found to be the most convenient and efficient oxidant since the fluorescence can be detected after the incubation of toxin samples at 100°C for 3-5 min. The main advantage of this method is that it is rapid, and a flowthrough cell and peristaltic pump enabled rapid screening of hundreds of samples collected from effluent from chromatographic columns used to fractionate PSP toxins. The fluorescence detection therefore facilitated further analysis with any of the more laborious and expensive chromatographic or electrophoretic methods. To accomplish this, samples with highest fluorescence were selected for subsequent detailed quantitative analysis using HPLC. Thus, only those fractions with toxins as determined by preliminary screening needed to be assayed by HPLC. Also, far greater sensitivity was achieved with pico molar levels (0.0002-0.054) for all individual toxins.

Thin layer chromatography on Chromarods SIII with the Iatroscan (Mark-5) flame thermionic detector was also used to develop a rapid method for the detection of PSP toxins. FTID response varied significantly according to the levels of hydrogen and air flow, detector current and scan speed. FTID peak response of STX, NEO, GTX 2/3 and C1/2 toxins significantly increased with increasing detector current, while decreasing the air flow, hydrogen flow and scan speed, simultaneously. A combination of hydrogen and air flow rates of 50 mL/min and 1.5-2.0 L/min, respectively, along with a scan speed of 40 sec/rod and detector current of 3.0 A or above were found to yield the best results for the detection of PSP compounds. In addition, factors such as age and cleanliness of the rods as well as sample load affected the baseline. Other parameters such as MWD, TTP and MNT levels recommended by the manufacturers were altered to 1%, 1.2% and 2 mm in order to achieve a baseline with minimum noise. Quantities of authentic standards as small as 1-6 ng could be detected by TLC/FTID. After testing numerous solvent systems, chloroform : methanol : water : acetic acid (30:50:8:2) was found to be the most suitable solvent system to separate major compounds in a standard toxin mixture consisting of NEO, STX, GTX 2/3 and C1/2 toxins and a sample of concentrated toxic scallop homogenate, prior to the detection by FTID. C toxins eluted to the top of the solvent front followed by GTX 2/3, NEO and STX, respectively. GTX 2/3 were well separated from NEO and STX, and NEO moved very close to STX which remained at the origin. The main obstacle of this work was lack of sufficient amounts of purified individual toxins to test different multiple solvent systems. Perhaps not only the resolution but also the FTID response may be improved by changing the solvent system. Although the purified individual compounds can be detected easily, the method detection limits for toxic shellfish extracts using the FTID ranged from 0.4 to 2.5 μg/g tissue for individual toxins. These detection limits also may be further improved by improving the solvent system, and of course by increasing the detector current. The main advantages of this method include the involvement of relatively inexpensive equipment (Iatroscan system), using sample sizes as small as 0.2 μL, and the solvent system used for TLC/FTID may also be used to purify PSP toxins by preparative TLC. This method is rapid since about 60 samples can be detected in 1h, after

development, and possibly more if multiple development chambers are used. The major deficiency of most of the previously published detection methods is relatively poor response to the N-1-hydroxy compounds such as GTX 1/4 and NEO. The TLC/FTID technique can detect all PSP compounds with more or less similar sensitivity. However, the detection sensitivity for extracts from toxic shellfish tissues requires improvement.

8.2 Stability of Toxins

The thermal stability of PSP toxins was studied using toxic scallop digestive glands and a partially purified toxin mixture. The homogenate prepared from toxic scallop digestive glands was heated at different pH levels (3-7) and temperatures (90-130°C) for 120 min. The kinetics of thermal degradation of individual toxins in the matrix (homogenate) were found to be first order. Decimal reduction times calculated from the thermal destruction curves decreased with increasing temperature and pH of the matrix. Significant destruction was not observed at 90-100°C, especially at low pH levels. An initial increase of GTX 2/3 levels at low pH levels under gentle heating may be due to conversion of C1 and 2 toxins into GTX 2 and 3, respectively, whereas the initial increase of STX may be due to possible conversion of GTX 2/3 and NEO into STX by 'Proctor' enhancement. STX had the highest D values at all temperature levels followed by NEO and GTX 2/3. The total PSP toxicity determined by mouse neuroblastosoma cell bioassay rapidly decreased at 130°C and at pH levels as high as 6 and 7. Cell bioassay data were correlated highly significantly ($P \leq 0.01$) with the theoretical PSP toxicities calculated from chromatographic data derived from HPLC. Although the addition of acidulants to some food products is a common practice in canning, conventional canning of acidified shellfish or even pasteurization of such products may actually produce increases in toxicity levels of 'safe' raw materials to levels which are unsafe (>80 μg STX equivalents/100 g edible tissue) in the finished product due to the thermally induced conversion of less toxic sulfocarbamate toxins to the highly toxic carbamate counterparts at acid pH levels. Therefore, strict control of pH must be considered to be a critical control point during conventional canning or even pasteurization of such shellfish products.

When a mixture of partially purified standard toxins consisting of C1/2, B1, GTX 1-4, NEO and STX was heated in buffers, without a matrix, the kinetics of the thermal degradation of individual toxins were found to be first order, and were comparable with the degradation patterns of individual PSP toxins in the scallop homogenate. Similarly, the rate of degradation was found to depend not only on the type of compound, but also on the heating temperature, time and pH of the heating medium. C1/2 levels significantly decreased ($P \leq 0.05$) with gently heating at pH 3-4 while GTX 2/3 levels increased by possible conversion of C1/2 to GTX 2/3. GTX 1/4 levels significantly decreased ($P \leq 0.05$) at all pH levels with increasing temperature by possible conversion of GTX 1/4 to NEO at low pH levels and mostly by degradation at higher pH levels. STX and NEO also increased initially at 90-100°C by possible toxin inter-conversions. As in the case of PSP toxins in a matrix (homogenate), STX was found to be the most heat stable compound followed by NEO and GTX 2/3, respectively, whereas C1/2 toxins were the most heat labile followed by GTX 1/4 and GTX 2/3, respectively. Also STX had the highest D values followed by NEO, GTX 2/3 and GTX 1/4, respectively. The theoretical integrated specific toxicities determined as mouse units using conversion factors for HPLC data, also increased slightly at pH 3-4 at 90-100°C. The toxicity decreased with increasing temperature and pH of the medium. Thermal degradation of toxins heated in a matrix (homogenate) or without a matrix (in buffer) depends on the combined effect of pH, heating time and temperature. Statistical models derived for the degradation of individual toxins at temperature level in both situations could improve significantly by adding interaction terms (pH x time).

Heated and unheated PSP toxins in a matrix (homogenate) and without a matrix (in buffer) were stored at -35, 5 and 25°C for different times. All individual toxins were most stable at -35°C and at pH 3-4. Some compounds were not stable at 25°C, especially at high pH levels (pH 6-7). C 1/2 toxins were the most sensitive followed by GTX 1/4 whereas STX and NEO were the most stable during the storage at 5 and 25°C, especially at pH 3-4. Although previous reports indicate further rapid decrease in toxicity during storage subsequent to canning, these decreases depend upon the pH of the matrix in which the toxins are found. Purified toxins used as analytical standards as well as partially purified

toxins and toxins in a homogenized matrix sometimes used as secondary standards can be safely stored at pH 3 and at ≤-35°C.

8.3 The Need for More Research

The changes in individual toxins on a molar basis in a mixture, with or without a matrix, were difficult to explain totally, due to simultaneous toxin inter-conversions and degradations at different rates. Similar heating or storage experiments under same conditions with individual compounds, rather than a few compounds in mixtures, would be a reasonable choice for a future research to fulfil the complete understanding of the nature of individual toxins during heating at different pH and temperature levels.

Another important area of toxin research which has been neglected to date is the effect of shellfish microflora on the degradation or apparent depuration of PSP toxins. Evidence exists for such degradation (Sugawara *et al.*, 1997) and perhaps bacteria could potentially be used for biological control of these naturally occurring toxins. Finally and perhaps, most important is the need for development of rapid, sensitive tests (perhaps in kit form) for PSP toxins. Although a test kit is available for the mouse neuroblatoma cell bioassy (Maritime Invitro Shellfish Test or MIST) which is not convenient for field use, the need for rapid analytical methods which can be used in non-skilled hands at aquaculture sites, processing plants and by individuals collecting their own shellfish for personal consumption, cannot be overstated.

REFERENCES

Ackman, R. G. (1981) Flame ionization detection applied to thin-layer chromatography on coated quartz rods. Methods in Enzymology, 72:205-252.

Alam, M., Shimizu, Y., Ikawa, M., and Sasner, J.J. Jr. (1978) Reinvestigation of the toxins from *Aphanizomenon flos-aquae* by high performance chromatographic methods. J. Environ. Sci. Health, A13:493-499.

Alfonso, A., Vieytes, M. R., Botana, A. M., Goenaga, X. and Botana, L. M. (1993) Preparation of mixtures of paralytic shellfish toxins (PSP) standards from mussel hepatopancrease. Fres. J. Anal. Chem., 345:212-216.

Alfonso, A., Louzao, M. C., Vieytes, M. R. and Botana, L. M. (1994) Comparative study of the stability of saxitoxin and neosaxitoxin in acidic solutions and lyophilized samples. Toxicon, 32:1593-1598.

Anderson, D.M. and White, A.W. (1989) Toxic dinoflagellate and marine mammal mortalities. *Proceedings of an expert consultation held at the Woods Hall Oceanographic Institution*. Technical Report. CRC-89-6, Coastal Research Center, 1-34.

Ares, J. A. and Dymsza, H. A. (1982) Development and use of a proficiency test specimen for paralytic shellfish poisoning. J. Food Sci., 47:1041-1044.

Asakawa, M. and Takagi, M. (1983) The effect of pH and heating on PSP, relating to boiling or canning process of toxic scallops. Bull. Fac. Fish., Hokkaido Univ., 34:260-263.

Asakawa, M., Iida, A. and Oishi, K. (1986) Studies on paralytic shellfish poison composition when heated under pressure as related to the canning process of toxic scallops. Bull. Fac. Fish., Hokkido Univ., 37:252-256.

Asakawa, M., Miyazawa, K., Takayama, H. and Noguchi, T. (1995) Dinoflagellate *Alexandrium tamarense* as the source of paralytic shellfish poison (PSP) contained in bivalves from Hiroshima Bay, Hiroshima Prefecture, Japan. Toxicon, 33(5):691-697.

Association of Official Analytical Chemists (1980) Methods of Analysis, 13th ed., Washington, D.C., Association of Official Analytical Chemists. Paralytic shellfish poison, biological method. In *Official Methods for Analysis*, 12th ed. (rev.), Washington, DC, Assoc. Office. Anal. Chem. 28:319- 326.

Banwart, G. J. (1989) Control of microorganisms by destruction. In *Basic Food Microbiology*, ed. G. J. Banwart, pp 651-685. New York: Van Nostrand Reinhold.

Bates, H. A. and Rapoport, H. (1975) A chemical assay for saxitoxin, the paralytic shellfish poison, J. Agric. Food Chem., 23:237-239.

Bates, H. A., Kostriken, R. and Rapoport, H. J. (1978) A chemical assay for saxitoxin, J. Agric. Food Chem., 26:252-254.

Berenguer, J. A., Gonzalez, L., Jimenez, I., Legarda, T. M., Olmedo, J. B. and Burdaspal, P. A. (1993) The effect of commercial processing on the paralytic shellfish poison (PSP) content of naturally-contaminated *Acanthocardia tuberculatum* L. Food Add. Contam., 10:217-230.

Blanco, J., Morono, A., Franco, J. and Reyero, M. I. (1997) PSP detoxification kinetics in the mussel *Mytilus galloprovincialis.* One- and two-compartment models and the effect of some environmental variables. Mar. Ecol. Prog. Ser., 158:165-175.

Blogoslawski, W. J. and Stewart, M. E. (1978) Paralytic Shellfish Poison in *Spisula solidissima*: Anatomical location and ozone detoxification. Mar. Biol., 45:261-264.

Boyer, G. L., Witchmann, C. F.,. Mosser, J., Schantz E. J. and Schnoes, H. K. (1979) In *Toxic Dinoflagellate Blooms*, eds. D. L. Taylor and H. H. Selinger, pp. 373-376., Elsevier, Amsterdam, The Netherlands.

Boyer, G. L. (1980) Chemical Investigations of the Toxins Produced by Marine Dinoflagellates, Ph.D. Thesis, University of Wisconsin, Madison.

Bricelj, V. M., Lee, J. H., Cembella, A. D., Anderson, D. M. (1990) Uptake kinetics of paralytic shellfish toxins from the dinoflagellate *Alexandrium fundyense* in the mussel *Mytilus edulis*. Mar. Ecol. Prog. Ser., 63:177-188.

Bricelj, V. M., Lee, J. H., Cembella, A. D. (1991) Influence of dinoflagellate toxicity on uptake and loss of paralytic shellfish toxins in the northern quahog, *Mercenaria mercenaria*, Mar. Ecol. Prog. Ser., 74:33-46.

Bricelj, M., Greene, M. and Cembella, A. D. (1993) Growth of the blue mussel *Mytilus edulis* on toxic *Alexandrium fundyense* and effects of gut passage on dinoflagellate cells. In *Toxic Phytoplankton Blooms in the Sea*, eds. T.J. Smyda and Y. Shimizu, pp 371-376 Elsevier, B. V .

Bricelj, M. and Cembella, A. D. (1995) Fate of gonyautoxins in surfclams, *Spisula solidissima*, grazing upon toxigenic *Alexandrium*, In *Harmful Marine Algal Blooms*, eds. P. Lassus, E. Erard, P. Gentien and C. Marcallou, pp 413-418, Technique et documentation, Lavosier, Intercept Ltd.

Bricelj, V. M., Cembella, A. D., Laby, D., Shumway, S. E. and Cucci, T. (1996) Comparative physiological and behavioural responses to PSP toxins in two bivalve molluscs, the soft shell clam, *Mya arenaria*, and surfclam, *Spisula solidissima*. In *Harmful and Toxic Algal Blooms*, eds. T. Yasumoto, Y. Oshima and Y. Fukuyo, pp 405-408 International Oceanographic Commission of UNESCO .

Bricelj, M. V., Ward, J. E., Cembella, A. D. and MacDonald B. A. (1998) Application of video-endoscopy to the study of bivalve feeding on toxic dinoflagellates. In *Harmful Algae*, eds. B. Reguera, J. Blanco, M. L Fernández and T. Wyatt, pp 453-456, Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO.

Bricelj, M. and Shumway, S. E. (1998a) An overview of the occurrence and transfer kinetics of paralytic shellfish toxins in bivalve molluscs. In *Harmful Algae*, eds. B. Reguera, J. Blanco, M. L. Fernández and T. Wyatt, pp 431-436, Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO.

Bricelj, M. and Shumway, S. E. (1998b) Paralytic shellfish toxins in bivalve molluscs: Occurrence, transfer kinetics, and biotransformation, Rev. Fish. Sci., 6(4):315-383.

Buckley, L. J., Ikawa, M. and Sasner, J. J. (1976) Isolation of *G. tamarensis* toxins from soft shell clams (*Mya arenaria*) and a thin layer chromatographic-fluorometric method for their detection. J. Agric. Food Chem., 24:107-111.

Buckley, L. J., Oshima, Y. and Shimizu, Y. (1978) Construction of paralytic shellfish analyser and its application. Anal. Biochem., 85:157-164.

Carlson, R. E., Lever, M. L., Lee, B. W. and Guire, P. E. (1984) Development of immunoassay for paralytic shellfish poisoning. In *Seafood Toxins*, ed. E. D. Regalis, ACS Symposium Series 262, pp 181-192, American Chemical Society, Washington, DC.

Carmichael, W. W., Mahamood, N. A. and Hyde, E. G. (1990) Natural toxins from cyanobacteria (blue-green algae). In *Marine Toxins: Origin, Structure and Molecular Pharmacology*, pp 87-106, eds. Hall, S. and Strichartz, G., Washington, American Chemical Society.

Catterall, W.A. (1985) The voltage sensitive sodium channel: A receptor for multiple neurotoxins, In *Toxic Dinoflagellates. Proceedings of the Third International Conference on Toxic Dinoflagellates*, St. Andrews, NB, Canada, eds. D.M. Anderson, W.W. White and D.G. Baden, pp 329-341, Elsevier Sci. Pub. Co., Inc., New York.

Catterall, W. A. (1988) Molecular properties of voltage-sensitive sodium and calcium channels. Braz. J. Med. Biol. Res., 21:1129-1144.

Cembella, A. D., Shumway, S. E. and Lewis, N. (1993) Anatomical distribution and spatio-temporal variation in paralytic shellfish toxin composition in two bivalve species from the Gulf of Maine. J. Shellfish Res., 12:389-403.

Cembella, A. D., Shumway, S. E. and Larocque, R. (1994) Sequestering and putative biotransformation of paralytic shellfish toxins by sea scallop *Placopecton magellanicus*: seasonal and spatial scales in natural populations. J. Exp. Mar. Biol. Ecol., 180:1-22.

Cembella, A. D. and Destombe, C. (1996) Genetic differentiation among *Alexandrium* populations from eastern Canada, In *Harmful and Toxic algal Blooms*, eds. T. Yasumoto, Y. Oshima and Y. Fukuyo, pp 447-450, Intergovernmental Oceanographic Commission of UNESCO.

Cembella, A. D. (1998) Ecophysiology and metabolism of paralytic shellfish toxins in marine micro algae. In *Physiological Ecology of Harmful algal Blooms*, eds: D. M. Anderson, A. D. Cembella and G. M. Hallegraeff, pp 381-403, NATO ASI series, Vol. G 41, Spriger-Verlag Berlin Heideberg.

Chang, D., Shin, D., Cho, H., Park, M., Pylon, J. And Park, Y. (1988) Studies on distribution, characterization and detoxification of paralytic shellfish poison in Korea. 2. Purification and characterization of PSP extracted from cultured sea mussel, *Mytilus edulis*. Bull. Korean Fish. Soc., 21: 161-168.

Chang, F. H., Anderson, D. M., Kulis, D. M. and Till, D. G. (1997) Toxin production of *Alexandrium minutum* (Dinophyceae) from the Bay of Plenty, New Zealand. Toxicon, 35:393-409.

Chen, C. and Chou, H. (1998) Transmission of the paralytic shellfish poisoning toxins, from dinoflagellate to gastropod. Toxicon, 34(3):515-522.

Cheun, B. S., Loughran, M., Hayashi, T., Nagashima, Y. and Watanabe, E. (1998) Use of channel biosensor for the assay of paralytic shellfish toxins. Toxicon, 36(10): 1371-1381.

Chimi, K., Taba, N., Tachibana, M., Katsuda, S. I. and Niiya, I. (1986) Effect of paralytic shellfish poison on acetylcholine release from the myenteric plexus of guinea pig. J. Food Hyg. Soc. Japan, 27(6):624-629.

Chu, F. S. and Fang, T. S. l. (1985) Indirect enzyme-linked immunosorbant assay for saxitoxin in shellfish. J Assoc. Off. Anal. Chem., 68:13-16.

Chu, F. S., Huang, X. and Hall, S. (1992) Production and characterization of antibodies against neosaxitoxin. J. Assoc. Off. Anal. Chem. Int., 75(2):341-345.

Chu, F. S., Hsu, K., Huang, X., Barrett, R. and Allison, C. (1996) Screening of paralytic shellfish poisoning toxins in naturally occurring samples with three different direct competitive enzyme-linked immunosorbant assays. J. Agric. Food Chem., 44:4043-4047.

Daigo, K., Uzu, A., Arakawa, O., Noguchi, T., Seto, H. and Hashimoto, K. (1985) Isolation and some properties of Neosaxitoxin from a xanthid crab *Zosimus aeneus*. Bull. Jpn. Soc. Sci. Fish., 51:309-313.

Daigo, K., Noguchi, T., Kawai, N., Miwa, A. and Hashimoto, K. (1989) Detection of paralytic shellfish poison by a lobster nerve-muscle. In *Red Tides: Biology, Environmental Science and Toxicology*, eds. T. Okaichi, D. M. Anderson and T. Nemoto, pp 387-390, Int. Symp. on Red Tides, Takamatsu, Japan, 10-14 November 1987,

Davio, S.R., Hewetson, J.F. and Beheler, J.E. (1985) Progress toward development of monoclonal antibodies of saxitoxins, antigen preparation and antibody detection. In *Toxic Dinoflagellates* Proceedings of the Third International Conference on Toxic Dinoflagellates, St. Andrews, NB, Canada. eds. D.M. Anderson, W.W. White and D.G. Baden, pp 343-348, Elsevier Sci. Pub. Co., Inc., New York.

Davio, S. R. and Fontelo, P. A. (1984) A competitive displacement assay to detect saxitoxin and tetrodotoxin. Anal. Biochem.,141:199-294.

Desbiens, M. and Cembella, A. D. (1997) Retention and possible transformation of paralytic shellfish toxins in lobster (*Homarus americanus*). Bull. Aquacul. Assoc. Canada, 2: 75-77.

FDA (1996) Fish and fishery products hazards and control guide, U. S. Food and Drug Administration, Center for Food Safety and Applied Nutrition., Part I, 19th June 1996, 6:4-20.

Fallon, W. E.and Shimizu, Y. (1977) Electrophoretic analysis of paralytic shellfish toxins. J. Environ. Sci. Health, A12(9): 455-464.

Flynn, K. and Flynn, K. J. (1996) An automated HPLC method for the rapid analysis of paralytic shellfish toxins from dinoflagellates and bacteria using precolumn oxidation at low temperature. J. Experm. Mar. Biol. Ecol., 197:145-157.

Genenah, A. A. and Shimizu, Y. (1981) Specific toxicity of paralytic shellfish poisons. J. Agric. Food Chem., 29:1289-1291.

Gershey, R.A., Neve, R. M., Musgrave, D. L. and Reichardt, P. B. (1977) A colorimetric method for determination of saxitoxin. J. Fish. Res. Bd. Canada, 34: 559-63.

Ghazarossian, V.E., Schantz, E.J., Schnoes, H.K. and Strong, F.M. (1976) A biologically active acid hydrolysis product of saxitoxin. Biochem. Biophys. Res. Commun., 68:776-780.

Gill, T. A. , Thompson, J. W. and Gould, S. (1985) Thermal resistance of paralytic shellfish poison in soft-shell clams. J. Food Prot., 48:659-662.

Hall, S., Reichardt, P. B. and Neve, R. A. (1980) Toxins extracted from an Alaskan isolate of *Protogonyaulax* Sp. Biochem. Biophys. Res. Com., 97:649-653.

Hall, S. (1982) Toxins and toxicity of Protogonyaulax from northern Pacific., Ph.D. dissertation, University of Alaska.

Hall, S. and Reichardt, P. B. (1984) Cryptic paralytic shellfish toxins. In *Seafood Toxins*, ed. E. P. Ragelis, pp 113-124. American Chemical Society, Washington, D. C.

Hall, S., Strichartz, G., Moczydlowski, E., Ravindran, A. and Rechardt, P. B. (1990) The saxitoxins: sources, chemistry and pharmacology. In *Marine Toxins:Origin, Structure and Molecular Pharmacology* pp 29-65, eds. S. Hall and G. Strichartz, American Chemical Society, Washington.

Harada, T., Oshima, Y., Kamiya, H., and Yasumoto, T. (1982a) Confirmation of paralytic poisoning toxins in the dinoflagellate *Pyrodinium bahamanse* var. *compressa* and bivalves in Palau. Bull. Jpn. Soc. Sci. Fish., 48:821-825.

Harada, T., Oshima, Y., and Yasumoto, T. (1982b). Structure of two paralytic shellfish toxins. Gonyautoxins-V and gonyautoxins-V1, isolated from a tropical dinoflagellate, *Pyrodinium bahamanse* var. *compressa*. Agric. Biol. Chem., 46:1861-1864.

Harada, T., Oshima, Y. and Yasumoto, T. (1983). Natural occurrence of decarbamoylsaxitoxins in tropical dinoflagellates and bivalves. Agric. Biol. Chem., 47(1):191-193.

Hashimoto, Y., Konoso, S., Yashumoto, T., Inoue, A. and Noguchi T., (1967). Occurrence of toxic crabs in Ryukyu and Amami Islands. Toxicon, 5:85-90.

Hokama, Y. (1993) Recent methods for detection of seafood toxins: recent immunological methods for ciguatoxins and related polyethers. Food Add. Contam., 10(1):71-82.

Ikawa, M,. Wegener, K., Foxall, T. L., Sasner, J. J. Jr., Noguchi, T. and Hashimoto, K. (1982) Use of strong cation exchange resin column for the study of PSP, J. Agric. Food Chem., 30: 526-528.

Ikawa, M., Wegener, K., Faxall, T.L. and Sasner, J.J., Jr. (1982) Comparison of the toxins of the *Gonyaulax* toxins. Toxicon, 20:747-752.

Ikebuchi, J., Suenaga, K., Kotoku, S., Yuasa, I. and Inagaki, O. (1988) Thin-layer chromatography with flame ionization detection for the determination of tetrodotoxin in biological fluids, J. Chromatogr., 432:401-406.

Indrasena, W. M. and Gill, T. A. (1998) Fluorometric detection of paralytic shellfish poisoning toxins. Anal. Biochem., 264:230-236.

Indrasena, W. M., Ackman, R. G. and Gill, T. A. (1999) Separation of paralytic shellfish poisoning toxins on Chromarods-SIII by thin-layer chromatography with the Iatroscan (Mark 5) and flame thermionic detection. J. Chromatogr. A, 855:657-668.

Indrasena, W. M. and Gill, T. A. (1999a) Thermal degradation of paralytic shellfish poisoning toxins in scallop digestive glands. Food Res. Int., 32(1):49-57.

Indrasena, W. M. and Gill, T. A. (1999b) Thermal degradation of partially purified paralytic shellfish poisoning toxins at different time, temperature and pH levels (submitted for publication in J. Food Sci.)

Indrasena, W. M. and Gill, T. A. (1999c) Storage stability of paralytic shellfish poisoning toxins. (Submitted for publication in Food Chemistry)

Jackim, E. and Gentile, J. (1968) Toxins of blue green alga: similarity to saxitoxin. Science, 162:915-916.

Jamilson, G. S. and Chandler, R. A. (1983) Paralytic shellfish poison in sea scallop (*Placopecton magellanicus*) in the west Atlantic, Can. J. Fish. Aqua. Sci., 40(3):313-318.

Janećek, M, Quilliam, M. A. and Lawrence, J. F. (1993) Analysis of paralytic shellfish poisoning toxins by automated pre-column and micro column liquid chromatography with fluorescence detection, J. Chromatogr., 644:321-331.

Janiszewski, J. and Boyer, G. L. (1993) In *Toxic Phytoplankton Blooms in the Sea*, eds. T. J. Smayda and Y. Shimizu, pp 889-894, Elsevier Science Publishers, New York, NY.

Jellett, J. F., Marks, J. L., Stewart, J. E., Dorey, M. L., Watson-Wright, W. and Lawrence, J. (1992) Paralytic shellfish poison (saxitoxin family) bioassay: Automated endpoint determination and standardization of the *in vitro* tissue culture bioassay, and comparison with the standard mouse bioassay. Toxicon, 30: 1143-1156.

Jellett, J. F. (1993) Phycotoxins and shellfish aquaculture. World Aquacul., 24(4):32-43.

Jellett, J. F., Stewart, J. E. and Laycock, M. V. (1995) Toxicological evaluation of saxitoxin, neosaxitoxin, gonyautoxin II, gonyautoxin II plus III and decarbamoylsaxitoxin with the mouse neuroblastoma cell bioassay, Toxic. invitro, 9(1):57-45.

Jellett, J. F., Wood, C. R., Belland, E. R. and Doucette, L. I. (1997) The MIST shippable cell bioassay kits for PSP: An alternative to the mouse bioassay. *Proceedings of the VIII International Conference on Harmful Algae* (abstracts), 25-29 June 1997,VIGO Spain pp 3.

Johnson, H. M. and Mulberry, G. (1966) Paralytic shellfish poison: Serological assay by passive haemagglutination and bentonite flocculation. Nature, 211:747-748.

Kamiya, H. and Hashimoto, Y. (1978) Occurrence of saxitoxin and related toxins in Palanan bivalves. Toxicon, 16:303-306.

Kao, C.Y. and Walker, S.E. (1982) Active groups of saxitoxin and tetrodotoxin as deduced from actions of saxitoxin analogues on frog muscle and squid axon. J. Physiol., 323:619-637.

Kitts, D. D., Townsley, P. M. and Smith, D. (1989) A potential method for detecting paralytic shellfish poisons, Northwest Environ., J., 5(1):149-151.

Kitts, D. D., Smith, D. S. and Owen, T. (1991) An enzyme-linked immunosorbant assay to detect the presence of paralytic shellfish poison using an induced crab protein marker. Food Agric. Immunol., 3(2):49-56.

Kobayashi, M. and Shimizu, Y. (1981) Gonyautoxin VIII, a cryptic precursor of paralytic shellfish poisons. J. Chem. Soc. Chem. Commun., 16: 827-830.

Koehn, F. E., Ghazarossian, V. E., Schantz, E. J., Schnoes, H. K. and Strong, F. M. (1981) Derivatives of saxitoxin. Bioorg. Chem., 10:412-428.

Kodama, M., Ogata, T., Sato, S., Honda, T. and Miwatani, T. (1990) Production of paralytic shellfish toxins by a bacterium, *Moraxella* sp. isolated from *Protogonyaulax tamarensis*, Toxicon, 28:707-714.

Kodama, M., Ogata, T. and Sato, S. (1988) Bacterial production of saxitoxin. Agric. Biol. Chem., 52(4):1075-1077.

Koehn, F.E., Hall, S., Wichmann, F. C., Schnoes, H.K. and Reichardt, P.B. (1982) Dinoflagellate neurotoxins related to saxitoxin: Structure and latent activity of toxins B1 and B2. Tetrahedron Let., 23(22):2247-2248.

Kogure, K., Tamplin, M. L., Simidu, U. and Colwell, R. R. (1988) A tissue culture assay for tetrodotoxin, saxitoxin and related toxins. Toxicon, 26:191-197.

Kotaki, Y., Oshima, Y. and Yashumoto, T. (1985) Bacterial transformation of paralytic shellfish toxins in coral reef crabs. Bull. Jap. Soc. Sci. Fish., 51(6):1009-1013.

Kotaki, Y., Tajiri, M., Oshima, Y. and Yashumoto, T. (1983) Identification of a calciferous red alga as the primary source of paralytic shellfish toxins in coral reef crabs and gastropods. Bull. Jpn. Soc. Sci. Fish., 49:283-286.

Kotaki, Y., Oshima, Y. and Yasumoto, T. (1981) Analysis of paralytic shellfish toxins in marine snail. Bull. Jpn. Soc. Sci. Fish., 47:943-946.

Kralovec, J. A., Laycock, M. V., Richards, R. and Usleber, E. (1996) Immobilization of small molecules on solid matrices: A novel approach to enzyme-linked imunosorbant assay screening for saxitoxin and evaluation of anti-saxitoxin antibodies. Toxicon, 34(10): 1127-1140.

Krog, P. (1979) Measurements of paralytic shellfish poisons. Netd. Vet. Med., 31:302-308.

Lassus, P., Ledoux, M., Bardouil, M., Bohec, M. and Erard, E. (1994) Kinetics of *Alexandrium minutum* Halim toxin accumulation in mussels and clams. Nat. Toxins, 2:329-333.

Lawrence, J. F. and Ménard, C. (1991) A study of ten toxins associated with paralytic shellfish poison using prechromatographic oxidation and liquid chromatography with fluorescence detection, J. Assoc. Off. Anal. Chem., 74(6):1006-1012.

Lawrence, J. F. and Ménard, C. (1991) Liquid chromatographic determination of paralytic shellfish poisons is shellfish after prechromatographic oxidation. J. Assoc. Off. Anal. Chem., 74(6):1006-1012.

Lawrence, J. F., Maher, M. and Watson-Wright, W. (1994) Effects of cooking on the concentration of toxins associated with paralytic shellfish poison in lobster hepatopancreas. Toxicon, 32:57-64.

Lawrence, J. F. and Wong, B. (1994) Evaluation of a postcolumn electrochemical reactor for oxidation of paralytic shellfish poison toxins. J. Assoc. Off. Anal. Chem. Int., 78(3):698-704.

Lawrence, J. F., Ménard, C. and Cleroux, C. (1995) Evaluation of prechromatographic oxidation for liquid chromatographic determination of paralytic shellfish poisons in shellfish. J. Assoc. Off. Anal. Chem. Int., 78(2): 514-520.

Laycock, M. V., Thibault, P., Ayer, S. W. and Walter, J. A. (1994) Isolation and purification procedures for the preparation of paralytic shellfish poisoning toxin standards. Nat. Toxins, 2:175-183.

Laycock, M. V., Kralovec, J. and Richards, R. (1995) Some *in vitro* chemical conversions of paralytic shellfish poisoning (PSP) toxins useful in the preparation of analytical standards. J. Mar. Biotech., 3:121 - 125.

Laycock, M., Kralovec, J. and Richards, R. (1997). A simple procedure for sulfation and 35S radiolabelling of paralytic shellfish poisoning (PSP) gonyautoxins. Nat. Tox., 5:36-42.

Levin, R. E. (1991) Paralytic shellfish toxins: Their origin, characteristics and methods of detection: A review. J. Food Biochem., 15:405-417.

Levin, R. E. (1992) Development of a tropical fish assay for saxitoxin. J. Food Safety, 13:1-5.

Llewellyn, L. E., Doyle, J. and Negri, A. P. (1998) A high-throughput, Microtiter plate assay for paralytic shellfish poisons using the saxitoxin-specific receptor, saxiphilin. Anal. Biochem., 261:51-56.

Locke, S. and Thibault, P. (1994) Improvement in detection limits for the determination of paralytic shellfish poisoning toxins in shellfish tissues using capillary electrophoresis/electro spray mass spectrometry and discontinuous buffer system, Anal. Chem., 66:3436-3446.

Lopez, A. (1975) Canning of marine products. In *A complete course in canning and related processes*. Book I - Basic information on canning., The Canning Trade inc., pp 547-549, Baltimore, Maryland, .

Lopez, A. (1987) In *A complete course in canning. and related processes*. Book II - Packaging; Aseptic processing; Ingredients. The Canning Trade inc., pp 306-307, Baltimore, Maryland.

Louzao, M. C., Alfonso, A., Botana, A. M., Goeonaga, X., Cabado, A. G., Vieytes, M. R. and Botana, L. M. (1994a) Effect of lyophilization on the stability of gonyautoxins obtained from contaminated mussels, Toxicon, 32: 807-817.

Louzao, M. C., Alfonso, A., Cabado, A. G., Botana, A. M., Goeonaga, X., Vieytes, M. R. and Botana, L. M. (1994b) Study of the stability of gonyautoxins in acidic solutions. Fres. J. Anal. Chem., 349:465-468.

Lucas, B. (1989) The occerrence of decarbamoyl-saxitoxin in canned mussels. Public health aspects odf Seafood-borne Zoonetic Diseases. *Proceedings of the WHO sympsium*, pp 89-97, ed. E. M. Bernath , Hannoven, FDA, .

MacLean, J.L. (1979) Indo-Pacific red tides. In *Toxic dinoflagellate bloom*, eds.D.L. Taylor and H.H. Seliger, pp173-178, Elsevier, North Holland, New York.

Mahmood, N. and Carmichael, W. M. (1986) Paralytic shelfish poison produced by the fresh water cyanobacterium *Aphanizomenon flos-aquae* NH-5, Toxicon, 24: 175-181.

Manger, R. L., Leja, L. S., Lee, S. Y., Hungerford, J. M., Hokama, Y., Dickey, R. W., Granade, H. R., Lewis, R., Yashumoto, T. and Wekell, M. M. (1995) Detection of sodium channel toxins: Directed cytotoxicity assays of purified ciguatoxins, brevetoxins, saxitoxins, and seafood extracts. J. Assoc. Off. Anal. Chem. Int., 78(2): 521-527.

Maruyama, J. , Noguchi, T., Onoue, Y., Ueda, Y., Hashimoto, K. and Kamimura,S. (1983) Anatomical distribution and properties of the toxins in highly PSP-infested scallops from Ofunato Bay during 1980-1981. Bull. Jpn. Soc. Sci. Fish., 49:233-235.

Maruyama, J. , Noguchi, T., Matsunaga, S. and Hashimoto, K. (1984) Fast atom bombardment and secondary ion mass spectrometry of paralytic shellfish poisons and tetrodotoxin. Agric. Biol. Chem., 48(11):2783-2788.

McFarren, E. F., Schantz, E. J., Campbell, J. E. and Lewis, K. H. (1958) Chemical determination of paralytic shellfish poison in clams. J. Assoc. Offic. Anal. Chem., 41:168-177.

McFarren, E. F. (1960) Collaborative studies of the chemical assay for paralytic shellfish poison. J. Assoc. Off. Anal. Chem., 43: 544-547.

Medcof, J. C., Leim, A. H., Needler, A. B., Needler, A. W. H., Gibbard, J. and Naubert, J. (1947) Paralytic shellfish poisoning on the Canadian Atlantic Coast. Bull. Fish. Res. Bd. Canada, 75: 1-32.

Mold, J. D., Bowden, J. P., Stanger, D. W., Maurer, J. E., Lynch, J. M., Wyler, R. S., Schantz, E. J. and Riegal, B. (1957) Paralytic shellfish poison. VII. Evidence for the purity of the poison isolated from toxic clams and mussels. J. Am. Chem. Soc., 79:5235-5238.

Mosley, S., Ikawa, M. and Sasner, J. J. (1985) A combination fluorescence assay and folin-cioclateau phenol reagent assay for the detection of paralytic shellfish poisons. Toxicon, 23: 375-381.

Nagashima, Y., Noguchi, T., Kawabata, T. and Hashimoto, K. (1990) Improved extraction method for paralytic shellfish poison to be used in mouse bioassay. Nippon Suisan Gakkaishi, 56(5):766-770.

Nagashima, Y., Noguchi, T., Kawabata, T. and Hashimoto, K. (1991a) Dose-death time curves of paralytic shellfish poisons in ddy strain mice. Nipon Suisan Gakkaishi, 57(4):699-704

Nagashima, Y., Noguchi, T., Tanaka, M. and Hashimoto, K. (1991b) Thermal degradation of paralytic shellfish poison. J. Food Sci., 56: 1572-1575.

Nishio, S., Noguchi, T., Onoue, Y., Maruyama, J., Hashimoto, K. and Seto, H.(1982) Isolation and properties of Gonyautoxin-5, an extremely low toxic component of paralytic shellfish poison. Bull. Jpn. Soc. Sci. Fish., 48: 959-961.

Noguchi, T., Konosu, S., and Hashimoto, Y. (1969) Identity of the crab toxin with saxitoxin. Toxicon, 7: 325-326.

Noguchi, T., Ueda, Y., Onoue, Y., Kono, M., Koyama, K., Hashimoto, K., Seno, Y. and Mishima, S. (1980a) Reduction of toxicity of highly PSP - infested scallops during canning process and storage. Bull. Jpn. Soc. Sci. Fish., 46:1273 - 1277.

Noguchi, T., Ueda, Y., Onoue, Y., Kono, M., Koyama, K., Hashimoto, K., Takeuchi, T., Seno, Y. and Mishima, S. (1980b) Reduction of toxicity of highly PSP -infested scallops during canning process and storage. Bull. Jpn. Soc. Sci. Fish., 46:1339 - 1344.

Noguchi, T., Nagashima, Y., Maruyama, J., Kamimura, S. and Hashimoto, K.(1984) Toxicity of the adductor muscle of markedly PSP-infested scallop *Patinopecten yessoensis*. Bull. Jpn. Soc. Sci. Fish., 50:517-519.

Noguchi, T., Arakawa, O., Daigo, K. and Hashimoto, K. (1986) Local differences in toxin composition of a xanthid crab *Atergatis floridus* inhabiting Ishigaki Island, Okinawa, Toxicon, 24: 705-710.

Ocio, M. J., Scanchez, T., Fernandez, P. S., Rodrigo, M. and Martinez, A. (1994) Thermal resistance characteristics of PA 3679 in the temperature range of 110 - 121 degree as affected by pH, type of acidulant and substrate. Int. J. Food Microbiol., 22:239-247.

Ohta, T., Nomata, H., Takeda, T and Kaneko, H. (1992) Studies on shellfish poison of scallop *Patinopecton yessoensis*. I. Reduction in toxicity of PSP infested scallops during extrusion processing. Sci. Rep. Hokkaido Fish. Exper. Stan., 38: 23-30.

Onoue, Y., Noguchi, T., Maruyama, J., Hashimoto, K., Seto, H. (1983a) Separation of tetrodotoxin and paralytic shellfish poisons by HPLC with a fluorescence detection using o-pthalaldehyde. J. Chromatogr., 257: 373-379.

Onoue, Y., Noguchi, T., Maruyama, J., Hashimoto, K., Seto, H. (1983b) Properties of two toxins newly isolated from oysters. J. Agricul. Food Chem., 31:420-423.

Oshima, Y., Fallon, W. F., Shimizu, Y., Noguchi, T. and Hashimoto, Y. (1976) Toxins of the *Gonyaulax* sp. and infested bivalves in Owase Bay. Bull. Jpn. Soc. Sci. Fish., 42:851-856.

Oshima, Y., Buckley, L.J., Alam, M., Shimizu, Y. (1977) Heterogenity of paralytic shellfish poisons. Three new toxins from cultured *Gonyaulax tamarensis* cells (*Myaarenaria*, and *Saxidomus giganteus*. Comp. Biochem. Physiol., 57C:31-34.

Oshima, Y., Machida, M., Sakasi, K., Tamooki, Y. and Yasumoto, T. (1984) Liquid chromatographic fluorometric analysis of paralytic shellfish toxins, Agric. Biol. Chem., 48:1707-1711.

Oshima, Y., Sugino, K. and Yasumoto, T. (1989) Latest advance in HPLC analysis of paralytic shellfish toxins. In *Mycotoxins and Phycoytoxins* 88. Proc. 7th Internat. IUPAC Symposium on Mycotoxins and Phycoytoxins in 1988. eds. S. Natori, K. Hashimoto and Y. Ueno, pp 319-326, Elsevier Science Publishers, Amsterdam, The Netherlands.

Oshima, Y., Blackburn, S. I. and Hallegraeff (1993a) Comparative study on paralytic shellfish toxin profiles of the dinoflagellate *Gymnodinium catenatum* from three different countries. Mar. Biol., 116:471-476.

Oshima, Y., Itakura, H., Lee, K. L., Yasumoto, T., Blackburn, S. and Hallegraeff, G. (1993b) Toxin production by the dinoflagellate, *Gymnodinium catenatum*. In *Toxic Phytoplankton Blooms in the Sea*, eds. T. J. Smadya and Y. Shimizu, pp 907-912, Elsevier, New York..

Oshima, Y. (1995) Post column derivatization liquid chromatographic method for paralytic shellfish toxins. J. Assoc. Off. Anal. Chem. Int., 78(2):528-532.

Park, D. L., Agutrre-Flores, I., Scott, W. F. and Alterman, E. (1986) Evaluation of chicken embryo, brine shrimp, and bacterial bioassays for saxitoxin. J. Toxicol. Environ. Health, 18:589-594.

Parrish, C. C., Zhou X. and Herche, L. R. (1988) Flame ionization and flame thermionic detection of carbon and nitrogen in aquatic lipid and humic-type classes with an Iatroscan Mark IV, J. Chromatogr., 435:350-356.

Patterson, P. L. (1988) Flame vaporization of non-volatile samples with simultaneous FID and thermionic (FTID) detection. DET Report, DETector Engineering and Technology, CA, 14:1-14.

Pleasance, S., Ayer, S., Laycock, M. V. and Thibault, (1992) Ionspray mass spectrometry of marine toxins. III analysis of paralytic shellfish poisoning toxins by flow-injection analysis, liquid chromatography/mass spectrometry and capillary electrophoresis/ mass spectrometry, P. Rapid Commun. Mass. Spec., 6:14-24.

Prakash, A., Medcoff, J. C. and Tannant, A. D. (1971) Paralytic Shellfish Poisoning in Eastern Canada. Bull. Fish. Res. Bd. Canada, 177: 58-65.

Proctor, N. H., Chan, S. L and Trevor, A. J. (1975) Production of saxitoxin by cultures of *G. tamarensis*, Toxicon, 13: 1-9.

Quayle, D. B. (1969) Paralytic shellfish poisoning in British Colombia. Bull. Fish. Res. Bd. Canada, 168:1-69.

Quilliam, M. A., Thomson, B. A., Scott, G. J. and Siu, K. W. M. (1989) Ion-spray mass spectrometry of marine toxins. Rapid Commun. Mass. Spec., 3(5):145-150.

Quilliam, M. A. (1996) Liquid chromatography-mass spectrometry of seafood toxins. In *Application of LC-MS in Environmental Chemistry*, ed. D. Barceló , pp 415-444, Elsevier, Amsterdam, .

Quilliam, M. A, Janeček, M. and Lawrence, J. F. (1993) Characterization of the oxidation products of paralytic shellfish poisoning toxins by liquid chromatography/mass spectrometry. Rapid Commun. Mass. Spec., 7:482-487.

Ranny, M. (1987) In *Specialized Applications of Thin Layer Chromatography with Flame Ionization Detection*, ed. J. Janek, pp. 125-130, D. Reidel Publishing Company, Holland.

Ross, M. R., Siger, A. and Abbott, B. C. (1985) The house fly: an acceptable subject for paralytic shellfish toxin bioassay. In *Toxic Dinoflagellates*, Proc. Third Int. Conf. Toxic Dinoflagellates, pp 433-438, ed. D. M. Anderson, Elsevier, New York.

Rubinson, K. A. (1982) HPLC separation and comparative toxicity of saxitoxin and its reaction products, Biochem. Biophys. Acta., 687:315-320.

Salter, J. E., Timperi, R. J., Hennigan, L. J., Sefton, L. and Reece, H. (1989) Comparison evaluation of liquid chromatographic and bioassay methods of analysis for determination of paralytic shellfish poisons is shellfish tissues. J. Assoc. Off. Anal. Chem., 72(4):670-673.

Scanchez, T., Rodrigo, M., Ocio, M. J., Fernandez, P, and Martinez, A. (1995) Growth and heat resistance of *Clostridium sporogenes*, PA 3679 spores heated and recovered in acidified media. J. Food Prot., 58:656-660.

Schantz, E. J., Mold, J. D., Stagener, D. W., Shavel, J., Riels, F. J., Bowden, J. P., Lynch, J. M., Wyler, R. S., Reigel, B., and Sommer, H. (1957) Paralytic shellfish poisoning VI. A procedure for the insolation and purification of the poison from toxic clams and mussel tissues, J. Am. Chem. Soc., 79:5230-5235.

Schantz, E.J. (1960). Biochemical studies of paralytic shellfish poisons. Ann. N.Y. Acad. Sci., 90:843-855.

Schantz, E. J., Mold, J. D., Howard, W. L., Bowden, H. P., Stanger, D.W. , Lynch, J. M., Wintersteiner, J. D., Dutcher, J. D., Walters, D. R. and Reigel, B., (1961) Paralytic shellfish poison, VIII. Some chemical and physical properties of purified clam and mussels poisons, Can. J. Chem., 39: 2117-2123.

Schantz, E.J. and Magnusson, H.W. (1964) Observations on the origin of the paralytic poison in Alaska butter clam. J. Protozool., 11:239-242.

Schantz, E.J., Lynch, J.M., Vayvada, G., Matsumoto, K., and Rapoport, H. (1966) The purification and characterization of the poison produced by *Gonyalax catenella* in axenic culture. Biochem., 5: 1191-1195.

Schantz, E.J., Ghazarossian, V.E., Schnoes, H.K., Strong, F.M., Springer, J.P., Pezzanite, J.O. and Clardy, J. (1975) Paralytic poisons from marine dinoflagellates. In *First International Conference of Toxic Dinoflagellate Blooms*, ed. V.R. LoCicero, pp 267-274, Mass. Sci. Tech. Found., Wakefield, .

Schantz, E. J. (1986) Chemistry and biology of saxitoxin and related toxins. In *Tetrodotoxin, saxitoxin and the molecular biology of the sodium channel*, eds. C. Y. Rao and S. R. Levinson. pp 15-23, Annals N.Y. Acad. Sci., Vol. 479.

Shimizu, Y., Alam, M., Oshima, Y., and Fallon, W.E. (1975) Presence of four toxins in red-tide infested clams and cultured *Gonyaulax tamarensis* cells. Biochem. Biophys. Res. Commun., 66:731-737.

Shimizu, Y., Buckley, L.J., Alam. M., Oshima, Y., Fallon, W.E., Kasai, H., Miura, I., Gullo, V.P. and Nakanishi, K. (1976) Structure of Gonyautoxin II from the East Coast toxic dinoflagellate, *Gonyaulax tamarensis*. J. Am. Chem. Soc., 98:5414-5416.

Shimizu, Y., Alam., M., Oshima, Y., Buckley L.J., Fallon, W.E., Kasai, H., Miura, I., Gullo, V.P. and Nakanishin, K. (1977) Chemistry and distribution of deleterious dinoflagellate toxins. In *Marine Natural Products Chemistry*, eds. D.J. Faukner and W.H. Fenical, pp 12-128, Plenum, New York.

Shimizu, Y., Hsu, C.P., Fallon, W.E., Oshima, Y., Miura, I. and Nakanishi, K. (1978) The structure of neosaxitoxin. J. Am. Chem. Soc., 100:6791-6793.

Shimizu, Y. and Yoshioka, M (1981) Transformation of paralytic shellfish toxins as demonstrated in scallop homogenates. Science, 212:547-549.

Shimizu, Y., Hsu, C.P., and Genenah, A. (1981) Structure of saxitoxin in solution and sterochemistry of dehydrosaxitoxin. J. Am. Chem. Soc., 103:605-609.

Shimizu, Y. (1984) Paralytic shellfish poisons. In *Progress in the Chemistry of Organic Natural Products*. 45. eds. W. Herz, H. Grisebach and G. W. Kirby. pp 236 -264, Wien-Springer-Vertag New York.

Shimizu, Y., Norte, M., Hori, A., Genenah, A. and Kobayashi, M. (1984) Biosynthesis of saxitoxin analogues: The unexpected pathway. J. Am. Chem. Soc., 106:6433-6434.

Shimizu, Y. (1988) The chemistry of paralytic shellfish toxins. In *Handbook of Natural Toxins*, Vol. 3, Marine Toxins and Venoms, ed. A. T. Tu. pp 63-85, Marcel Dekker, Inc., New York.

Shimizu, Y. (1996) Micro algal metabolites: a new perspective, Ann. Rev. Microbiol., 50:431-465.

Shiomi, K., Inaoka, H., Yamanaka, H., and Kikuchi, T. (1982) Occurrence of a large amount of gonyautoxins in a xanthid crab *Atergatis floridus* from China. Bull. Jpn. Soc. Sci. Fish., 48:1407-1410.

Shoptaugh, N., Buckley, L. J., Ikawa, M. and Sasner, J. J. Jr. (1978) Detection of *Gonyaulax* toxins and other guanidine compounds on thin-layer silica gel chromatography. Toxicon, 16:511-513.

Shoptaugh, N., Corter, P. W., Foxall, T. L., Sasner, J. J. and Ikawa, M. (1981) Use of fluorometry for the determination of *G. tamarensis* var. *excavata* toxins in New England shellfish, J. Agric. Food Chem., 29:198-200.

Shumway, S. E. and Cembella, A. D. (1993) The impact of toxic algae on scallop culture and fisheries. Rev. Fish. Sci., 1(2):121-150.

Siger, A., Abbott, B. C. and Ross, M. (1984) Response of the house fly to saxitoxin and contaminated shellfish. In *Seafood Toxins*. ed. E. P. Ragelis, pp 193-196, American Chemical Society, Washington, D. C.

Slater, J. E., Timperi, R. J., Hennigan, L. J., Sefton, L. and Reece, H. J. (1989) Comparison evaluation of liquid chromatographic and bioassay methods of analysis for determination of paralytic shellfish poison in shellfish tissues, J. Assoc. Off. Anal. Chem., 72 (4): 670- 673.

Smith, D. S., Kitts, D. D., Fenske, B., Owen, T. G. and Shyng, S. (1995) The development of a purification procedure for saxitoxin-induced protein. Biotechnol. Appl. Biochem., 21:7-18.

Sommer, H. and Mayer, K. F. (1937) Paralytic shellfish poison, Arch. Pathol., 24 (5): 560-598.

Sommer, H, Monnier, R. P., Reigel, B., Stanger, D. W., Mold, J. D., Wikholm, D. M. and Kiralis, E. S. (1948) Paralytic shellfish poison. I. Occurrence and concentration by ion exchange. J. Am. Chem. Soc., 70:1015-1018.

Stafford, R. G. and Hines, H. B. (1994) Method for the identification of saxitoxin in rat urine, J. Chromatogr. B., 657:119-124.

Steidinger, K. and Baden, D. (1975) The economic halo of a red tide. In *Proceedings of the 1st International Conference on Toxic Dinoflagellate Blooms*, eds. V. R. Lociero, 1975, 507, p. 153, Mass. Sci. Technol. Found., Wakefield, MA.

Strichartz, G., Rando, T., Hall, S., Gitschier, J., Hall, L., Magnani, B. and Bay, C. H. (1986) On the mechanism by which saxitoxin binds to and blocks sodium channels. In *Tetrodotoxin, Saxitoxin and the Molecular Biology of the Sodium Channel*, eds. C. Y. Kao and S. R. Levinson, pp 97-112, Ann. New York Acad. Sci.,Vol. 479, New York,.

Strichartz, G. and Castle, N. (1990) Pharmacology of marine toxins: Effects on membrane channels. In *Marine Toxins:Origin, Structure and Molecular Pharmacology*, S. Hall and G. Strichartz, pp 2-20, Washington:American Chemical Society.

Stumbo, C. R. (1973) Organisms of greatest importance in the spoilage of canned foods. In *Thermal Bacteriology in Food Processing*, pp 10-80. New York Academic Press.

Sugawara, A., Imamura, T., Aso, S. and Ebitani, K. (1977) Change of a paralytic shellfish poison by the marine bacteria living in the intestine of the Japanese surf clam, *Pseudocardium sybillas*, and the brown sole, *Pleuronectes herzensteini*. Sci. Rep. Hokkaido Fish. Exp. Stn., 50:35-47.

Sullivan, J.J. and Iwaoka, W.T. (1983) High pressure liquid chromatographic determination of toxins associated with paralytic shellfish poisoning. J Assoc. Offic. Anal. Chem., 66:297-303.

Sullivan, J.J., Wayne, T., Iwaoka, W.T. and Liston, J. (1983) Enzymatic transformation of PSP toxins in the littleneck clam (*Protothaca staminea*). Biochem. Biophys. Res. Commun., 114(2):465-472.

Sullivan, J.J., Simon, M.G. and Iwaoka, W.T. (1983a). Comparison of HPLC and mouse bioassay methods for determining PSP toxins in shellfish. J. Food Sci., 48:1312-1314.

Sullivan, J. J. and Wekell, M. M. (1984) Determination of paralytic shellfish poisoning toxins by high pressure liquid chromatography. In *Seafood toxins*, ed. E. P. Ragelis, pp 275-280, American Chemical Society, Washington, D. C.

Sullivan, J. J., Wekell, M. M. and Kentala, L. L. (1985) Application of HPLC for the determination of PSP toxins in shellfish. J. Food Sci., 50:26-29.

Sullivan, J. J. and Wekell, M. M. (1987) The application of High Performance Liquid Chromatography in a paralytic shellfish poisoning monitoring program. In *Sea Food Quality Determination*, Proceedings of an International Symposium Coordinated by the University of Alaska. eds. D. E. Kramer and J. Liston.pp 357-371, Elsevier Amsterdam.

Sullivan, J. J., Wekell, M. M. and Hall, S. (1988) Detection of paralytic shellfish toxins. In *Handbook of natural toxins, Vol. 3, Marine toxins and venoms*, ed. A. T. Tu, pp 87-106, Marcel Dekker, INC. New York and Basel.

Sullivan, J. J. (1990) High performance liquid chromatographic method applied to paralytic shellfish poisoning research. In *Marine Toxins:Origin, Structure and Molecular Pharmacology*, eds. S. Hall and G. Strichartz, pp 66-77, Washington: American Chemical Society.

Teegarden, G. J. and Cembella, A. D. (1996) Grazing of toxic dinoflagellates, *Alexandrium* spp., by adult copepods of coastal Maine: Implications for the fate of paralytic shellfish toxins in marine food webs, J. Exper. Mar. Biol. Ecol.,196:145-176.

Teegarden, G. J. and Cembella, A. D. (1996) Grazing of toxic dinoflagellates, *(Alexandrium* spp) by estuarine copepods: Particle selection and PSP toxin in marine food webs. In *Harmful and Toxic algal Blooms*, eds. T. Yasumoto, Y. Oshima and Y. Fukuyo, pp 393-396, Intergovernmental Oceanographic Commission of UNESCO.

Thibault, P., Pleasance, S. and Laycock, M. V. (1991) Analysis of paralytic shellfish poisons by capillary electrophoresis. J. Chromatogr., 542:483-501.

Trainer, V. L., Baden, D. G. and Catterall, W. A. (1995) Detection of marine toxins using reconstitued sodium channels. J. Assoc. Off. Anal. Chem. Int., 78(2):570-573.

Usleber, E., Schneider, E. and Terpian, G. (1991) Direct enzyme immunoassay in microtitration plate and test strip format for the detection of saxitoxin in shellfish. Lett. Appl. Microbiol., 13:275-277.

Usleber, E., Dietrich, R., Märtlbauer, E. and Terpian, G. (1994) Effect of heterologous paralytic shellfish poisoning toxin-enzyme conjugates on the cross-reactivity of a saxitoxin enzyme immunoassay. Lett. Appl. Microbiol., 18:337-339.

Van-Egmond, H. P., Van-den-Top, H. J. and Speijers, G. L. A. (1991) Worldwide regulations for marine phycotoxins. Proceedings of Symposium on Marine Biotoxins, Paris, 30-31 January 1991, ed. J. M. Fremy, pp 167-172, Lab. Toxicol. Natl. Inst. Public Health and Environ. Prot., 3720 BA, Bilthoven, Netherlands.

Van-Egmond, H. P., Van-den-Top, H. J., Paulsch, W. E., Goenaga, X. and Vieytes, M. R. (1994) Paralytic shellfish poison reference materials: an intercomparison of methods for the determination of saxitoxin. Food Add. Contam., 11(1):39-56.

Vieytes, M. R., Cabado, A. G., Alfonso, A., Louzao, M. C., Botana, A. M. and Botana, L. M. (1993) Solid-phase radioreceptor assay. Anal. Biochem., 211:87-93.

Watson-Wright, W., Gillis, M., Smith, C., Trueman, S., McGuire, A., Moore, W., MacLachlan, D. and Sims, G. (1991) Monitoring of PSP in hepatopancreas of lobster from Atlantic Canada. Can. Tech. Rep. Fish. Aquat. Sci., 1799:21-26.

White, A.W. (1981) Sensitivity of marine fish to toxins from the red-tide dinoflagellate *Gonyaulax excavata* and implications for fish skills. Mar. Biol., 65:255 - 257.

White, A.W. (1984). Paralytic shellfish toxins and finfish. In *Seafood Toxins*, ed. E.P. Ragelis, ACS Symposium Series 262, American Chemical Society, Washington, DC, 171-180.

White, K. D., Siphon, J. A. and Hall, S. (1986) Fast atom bombardment mass spectrometry of 12 marine toxins isolated from *Protogogyaulax*. Anal. Chem., 58:562-565.

Wichmann, C.F., Boyer, G.L., Divan, C.L., Schantz, E.J. and Schnoes, H.K. (1981) Neurotoxins of *Gonyaulax excavata* and Bay of Fundy scallops. Tetrahedran Let., 22:1941- 1946.

Wilson, B. W. and Nakane, P. K. (1978) Recent development in the periodate method of conjugating HRP to antibodies. In *Immunofluorescence and related staining techniques*. ed. W. Knapp, pp 215-224, Elsevier, Amsterdam.

Wilson, B. W., Ray, S. M. and Aldrich, D. V. (1975) *Gymnodinium breve*: Population growth and development of toxicity in culture. *Proc. 1st Int. Conf. on Toxic Dinoflagellates*, 1974, V. R. Locicero Ed. pp 127-141, The Massachusetts Science and Technology Foundation, Wakefield MA.

Wisessang, S., Ogata, T., Kodama, M., Fukuyo, Y., Ishimaru, T., Saitanu, K., Yongvanich, T. and Piyakarnchana, T. (1991) Accumulation of paralytic shellfish toxins by green mussel *Perna viridis* by feeding on cultured cells of *Alexandrium cohorticula* isolated from the Gulf of Thailand. 57(1):127-131.

Wong, J. L., Brown, M. S., Matsumoto, K., Oesterton, R. and Rapoport, H. (1971) Degradation of saxitoxins to a pyrimido [2,1-6] purine. J. Amer. Chem. Soc., 93: 4633-4334.

Yang, G. C., Imagire, S. J., Yasaei, P., Ragelis, E. p., Park, D. l., Page, S. W., Carlson, R. E. and Guire, P. E. (1987) Radio immunoassay of paralytic shellfish toxins in clams and mussels. Bull. Environ. Contam. Toxicol., 39:264-271.

Yang, L., Kao. C.Y. and Oshima, Y. (1992) Actions of decarbamolysaxitoxin and decarbamoylneosaxitoxin on the frog skeletal muscle fibre. Toxicon, 30(5/6):645-652.

Yashumoto, T., Oshima, Y. and Konta, T. (1981) Analysis of paralytic shellfish toxins of xanthid crabs in Okinawa. Bull. Jpn. Soc. Sci. Fish., 47:957-959.

Yashumoto, T., Oshima, Y., Tajiri, M. and Kotaki, Y. (1983) Paralytic shellfish toxins in previous unrecorded species of coral reef crabs. Bull. Jpn. Soc. Sci. Fish., 49: 633-636.

Yashumoto, T. and Kotaki, Y. (1977) Occurrence of saxitoxin in a green turban shell. Bull. Jpn. Soc. Fish., 43:207-211.

Appendix A: I. Schematic illustration and principle of operation of the FID/FTID detector

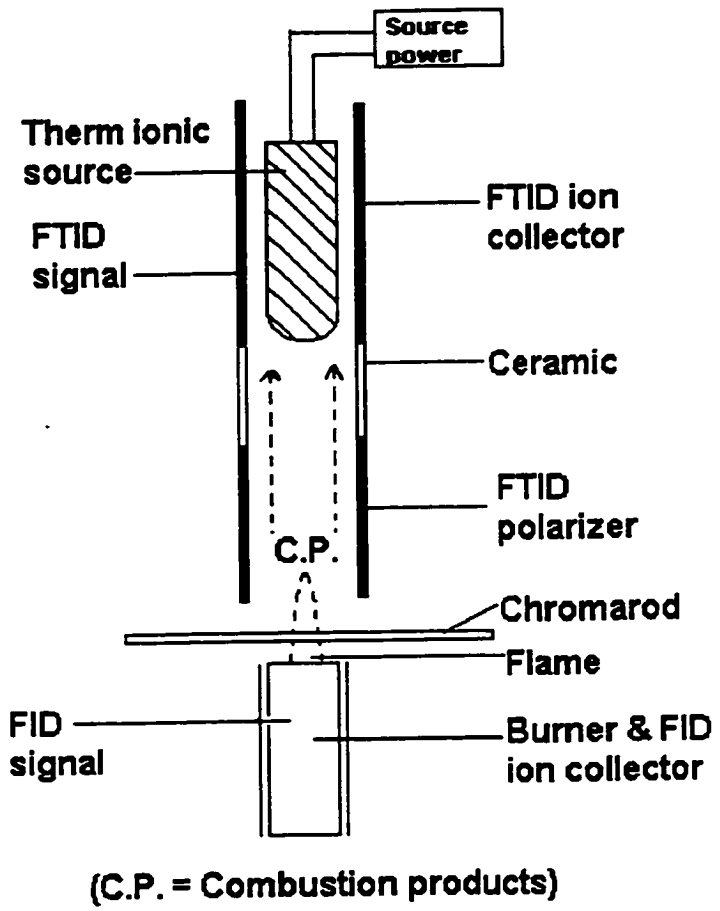


Fig. 4.I Schematic illustration of the FID/FTID system

Principle of Operation

The basic components of the FID/FTID Detction System are schematically illustrated in Fig. 4.I. The detector hardware is located in a tower-like structure that is mounted onto the top cover of the Iatroscan. The mounting of the FID/FTID tower structure has provisions for the horizontal and vertical alignment of the detector tower

with respect to the Iatroscan burner and Chromarods. When properly aligned, the detector tower provides a cylindrically-shaped chimney for the effluent of the Iatroscan flame. The bottom of the tower structure consists of a metal tube which serves as the FID polarizer electrode. This polarizer is connected *via* an insulating ceramic tube to a unique thermionic ionization transducer which is located further downstream of the Iatroscan flame. The function of the thermionic ionization transducer is to selectively re-ionize those neutral products of combustion which are electronegative in chemical nature. The collection of these re-ionized combustion products downstream of the flame constitutes the FTID signal. The FTID electronic module includes a negative ion electrometer for measuring this signal. A separately packed module provides the electronics required for operation of the FTID. This electronic module includes a power supply that provides an elctrical heating current and has a bias voltage for the thermionic source. In typical operation, the surface temperature of the thermionic source is in the range of 400 - 600°C. (Patterson, 1988).

APPENDIX A: II Effect of H_2 flow and detector current on the FTID peak response to PSP toxins

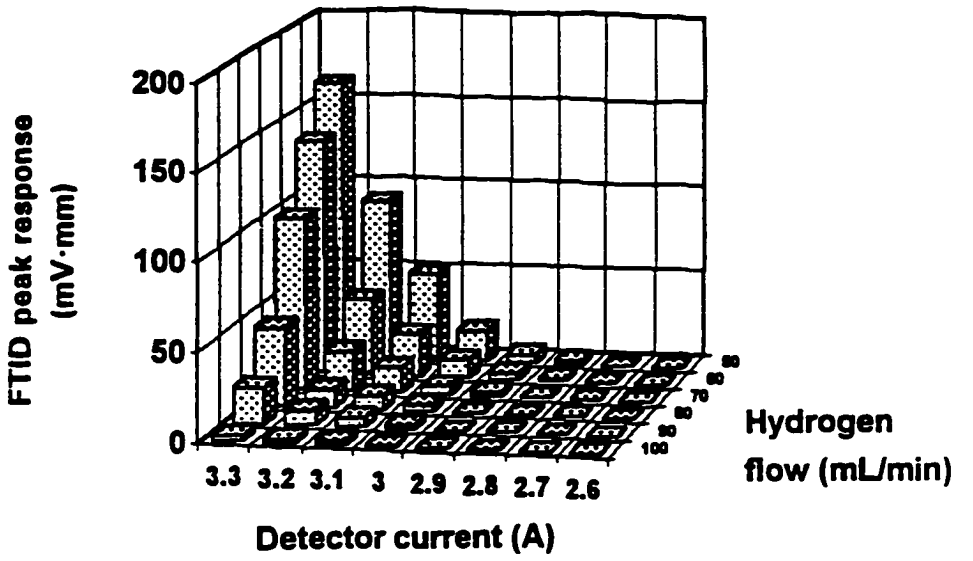


Fig. 4.II Effect of H_2 flow and detector current on the FTID peak response to STX (air flow = 1.5 L/min)

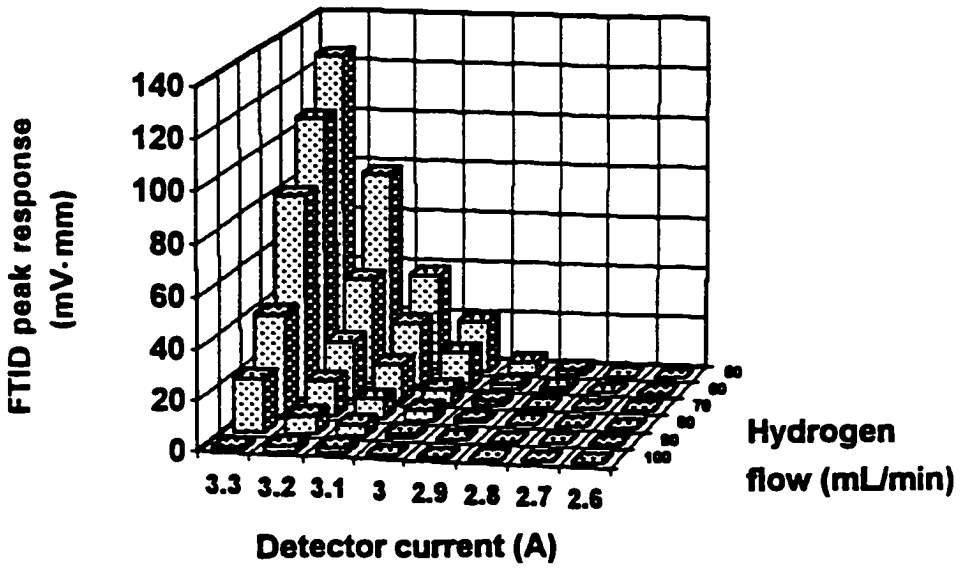


Fig. 4.III Effect of H_2 flow and detector current on the FTID peak response to STX (air flow = 2.0 L/min)

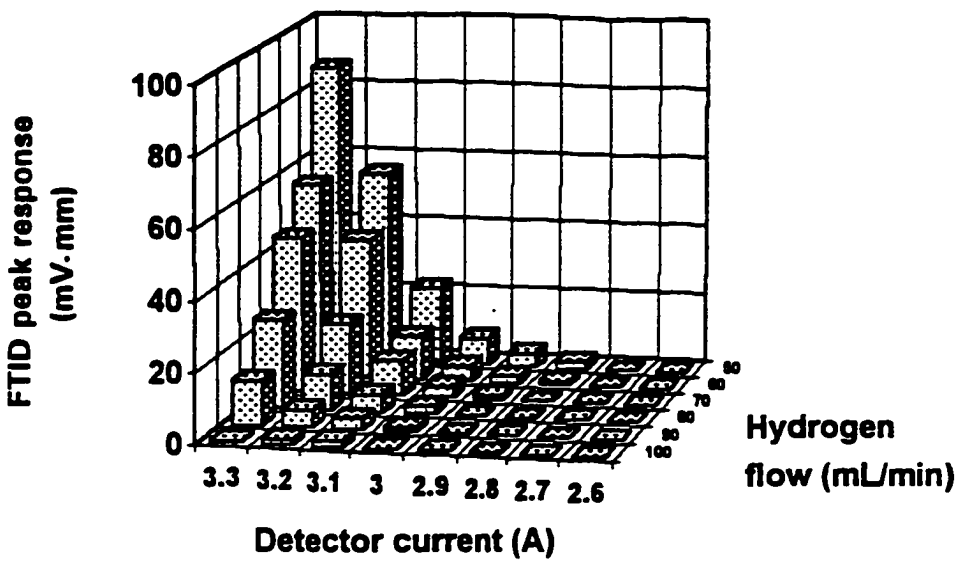


Fig. 4.IV Effect of H_2 flow and detector current on the FTID peak response to STX (air flow = 3.0 L/min)

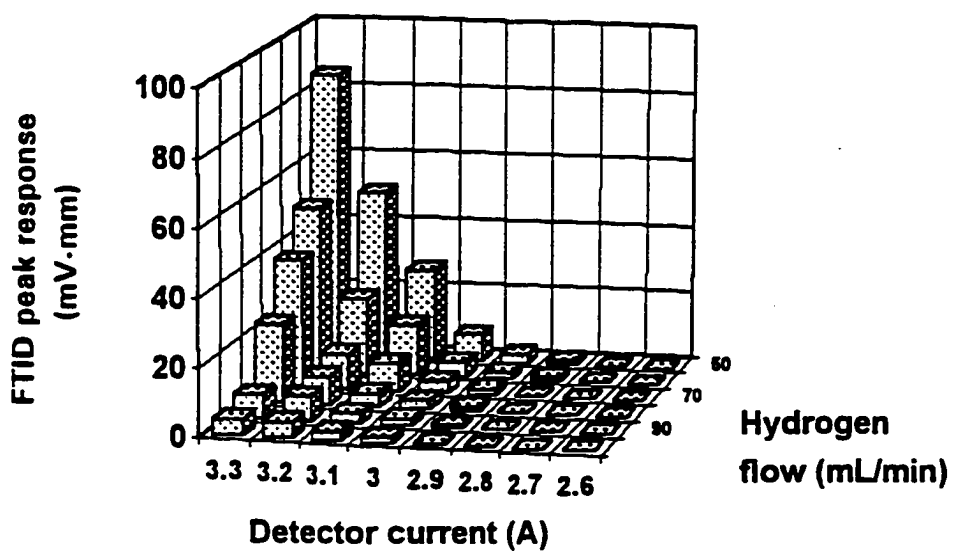


Fig. 4.V Effect of H_2 flow and detector current on the FTID peak response to C1/C2 (air flow = 1.5 L/min)

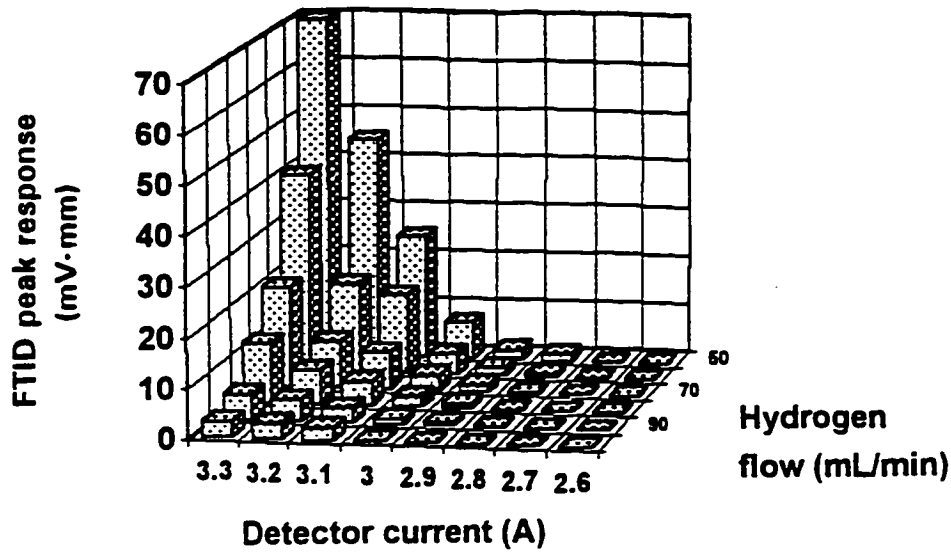


Fig. 4.VI Effect of H_2 flow and detector current on the FTID peak response to C1/C2 (air flow = 2.0 L/min)

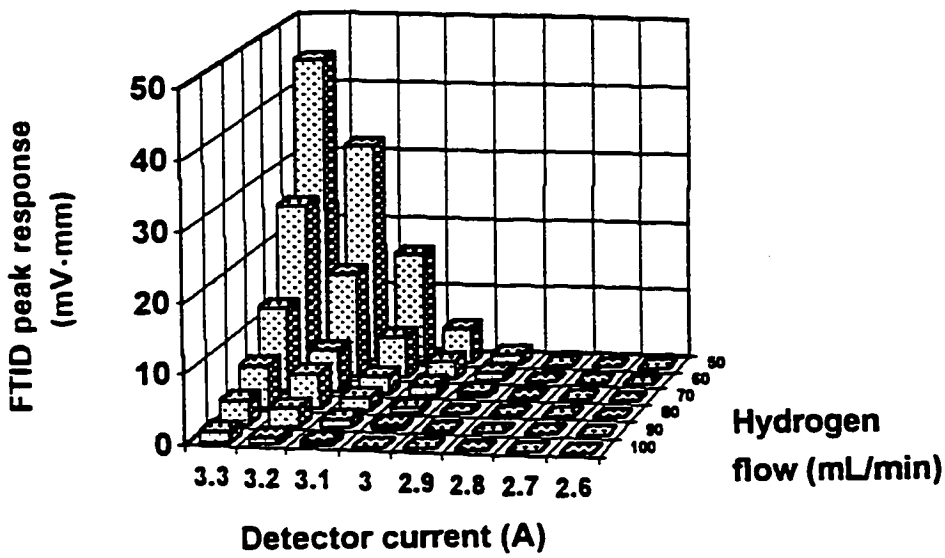


Fig. 4.VII Effect of H_2 flow and detector current on the FTID peak response to C1/C2 (air flow = 3.0 L/min)

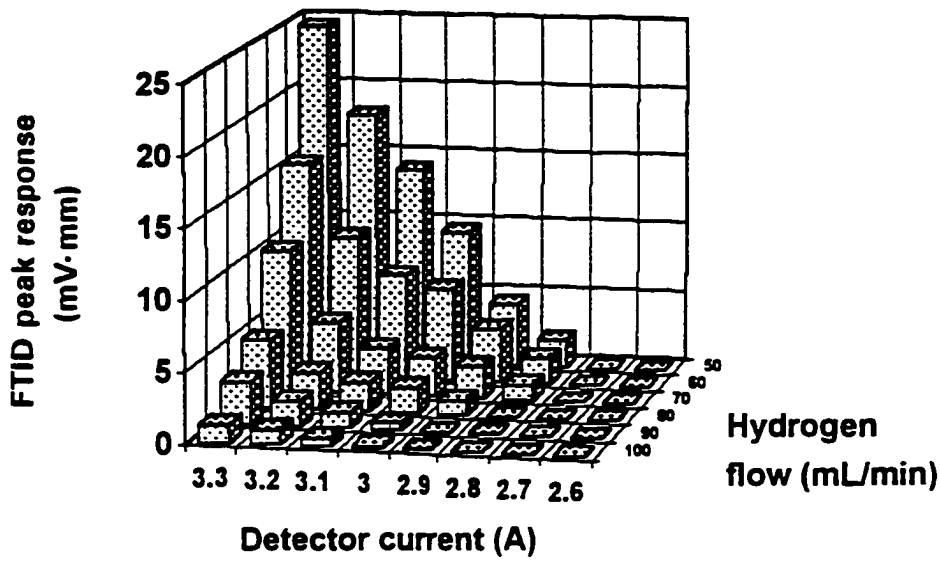


Fig. 4.VIII Effect of H_2 flow and detector current on the FTID peak response to NEO (air flow = 2.0 L/min)

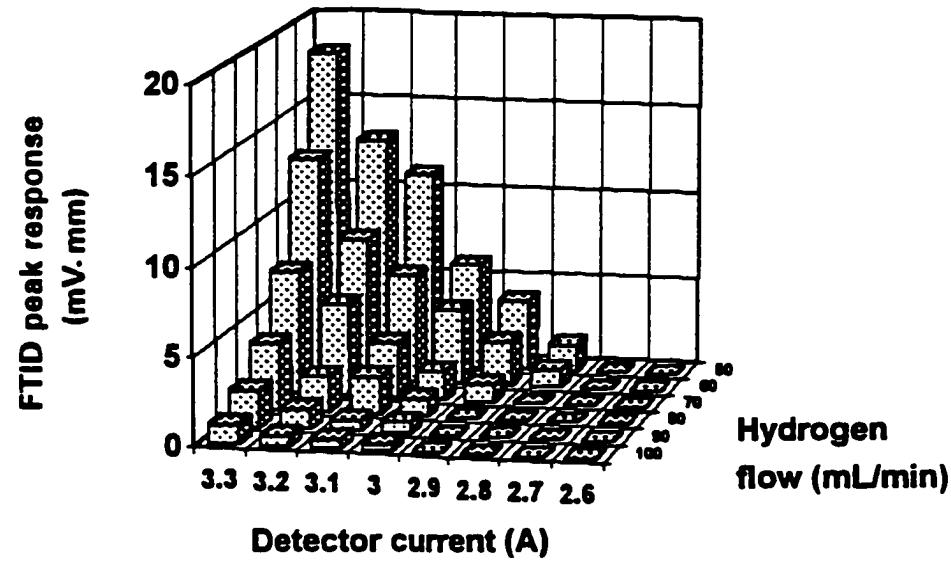


Fig. 4.IX Effect of H_2 flow and detector current on the FTID peak response to NEO (air flow = 3.0 L/min)

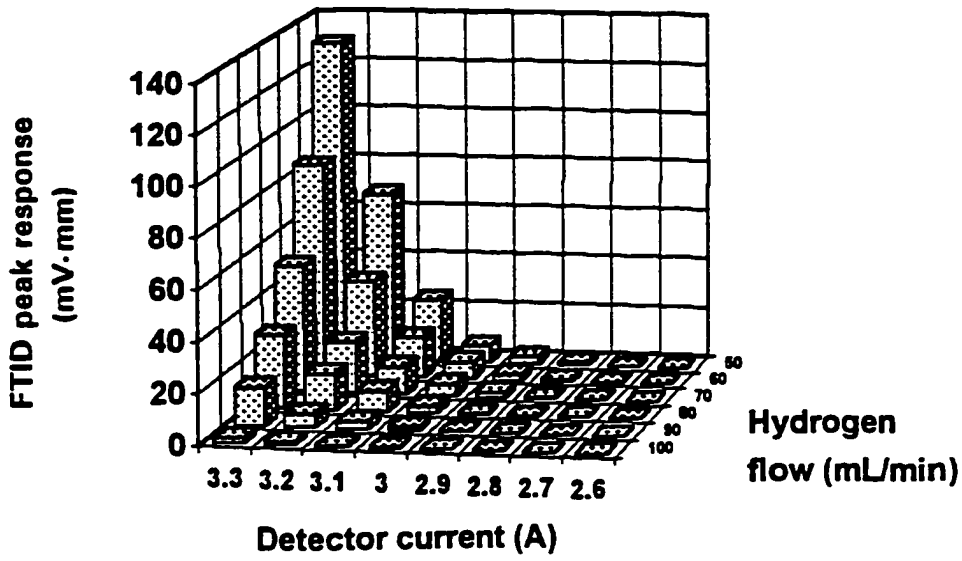


Fig. 4.X Effect of H_2 flow and detector current on the FTID peak response to GTX 2/3 (air flow = 1.5 L/min)

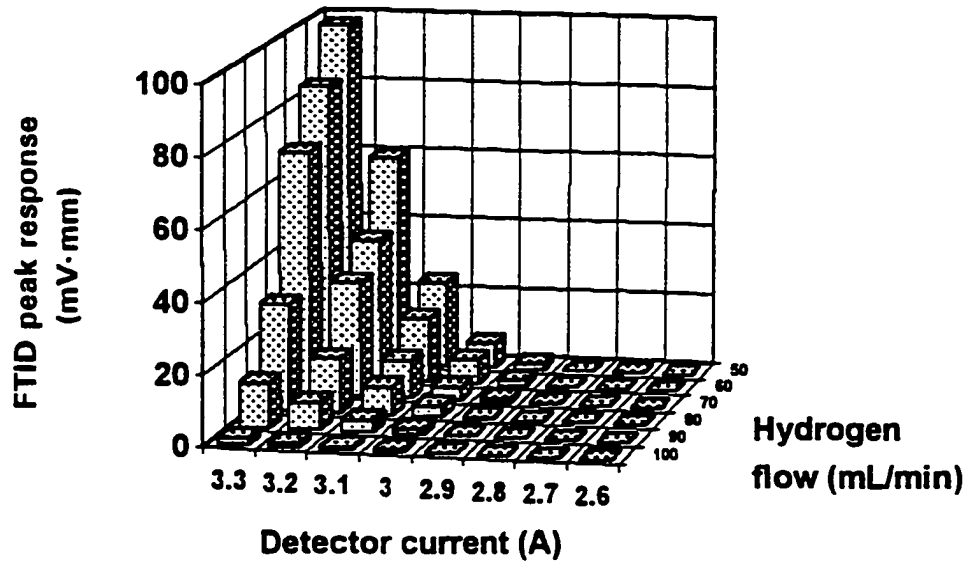


Fig. 4.XI Effect of H_2 flow and detector current on the FTID peak response to GTX 2/3 (air flow = 2.0 L/min)

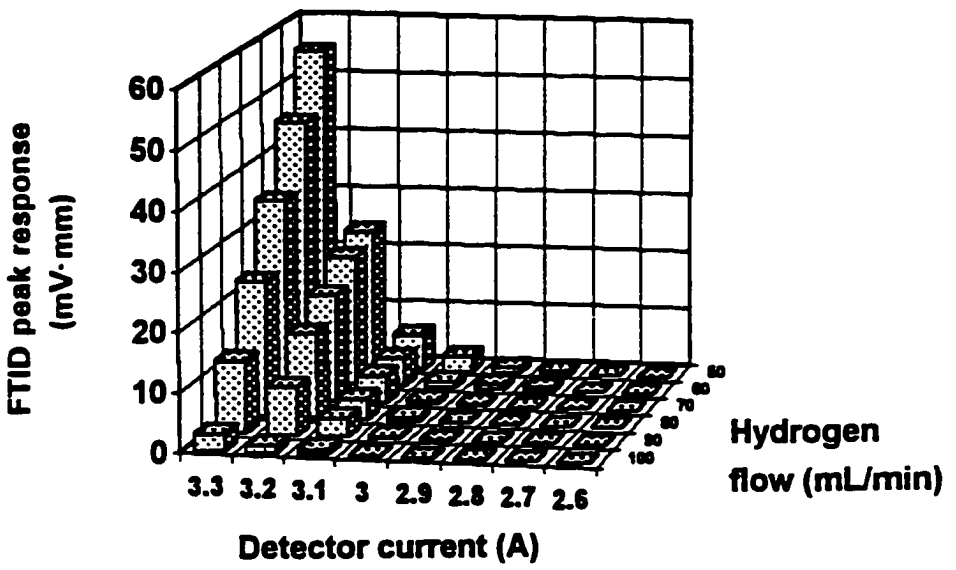


Fig. 4.XII Effect of H_2 flow and detector current on the FTID peak response to GTX 2/3 (air flow = 3.0 L/min)

APPENDIX A: III Analysis of covariance for FTID response of PSP toxins

Factor	Type	Levels	Values
C6	fixed	6	1 2 3 4 5 6

Analysis of Variance for STX, NEO, GTX 1/4, GTX 2/3, C1/2 and B1 using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
C5	1	707635	7219	7219	21.05	0.000
C6	5	483585	4135	827	2.41	0.043
C6*C5	5	114927	114927	22985	67.03	0.000
Error	84	28804	28804	343		
Total	95	1334952				

Term	Coef	StDev	T	P
Constant	-19.377	4.624	-4.19	0.000
C5	2.1135	0.4606	4.59	0.000
C5*C6				
STX	0.1643	0.5315	0.31	0.758
NEO	0.817	1.217	0.67	0.050
GTX 1/4	1.5878	0.4712	3.37	0.001
GTX 2/3	-0.445	1.984	-0.22	0.823
C 1/2	-1.6278	0.4742	-3.43	0.001

(C5 = covariates, C6 = indicator variables)

APPENDIX B: Composition of scallop homogenate

Table 5.I Amino acid composition of scallop homogenate**

Component name	Concentration (nmol/mg)
Taurine	0.00
Aspartic acid	79.59
Threonine	99.54
Serine	63.06
Glutamic acid	164.54
Proline	0.00
Glycine	7.45
Alanine	180.45
Cysteine	8.60
Valine	106.53
Methionine	47.99
Isoleucine	81.07
Leucine	131.65
Norleusine	0.00
Tyrosine	30.24
Phenylalanine	58.95
Histidine	27.49
Lysine	15.04
Ammonia	324.61
Arginine	84.55
Cysteic acid	0.00
Tryptophan	0.00

**The amino acid content in the scallop homogenate was analyzed qualitatively and quantitatively using amino acid analyzer at the Institute for Marine Biosciences, National Research Council, Halifax, Nova Scotia.

Table 5.II Lipid class composition of scallop homogenate**

Lipid class	Amount (%)
Triacylglycerols	36.4 (±2.4)
Free fatty acids	12.2 (±0.8)
Cholesterol	10.7 (±0.6)
Diacylglycerols	6.4 (±0.4)
Polar lipids (acetone mobile polar lipids, phospholipids)	34.3 (±1.2)

** Lipid class composition of the scallop homogenate was determined by thin layer chromatography with Iatroscan (Mk 3) flame ionization detection (TLC/FID).

Table 5.III Mineral composition of scallop homogenate analyzed by ICP-MS**

Inorganic analyte	Amount (mg/Kg)
Aluminum (Biota)	84.0
Antimony (Biota)	<0.5
Arsenic (Biota)	2.9
Barium (Biota)	4.1
Beryllium (Biota)	<1.5
Boron (Biota)	8.5
Cadmium (Biota)	54.0
Chromium (Biota)	2.1
Cobalt (Biota)	0.4
Copper (Biota)	5.5
Iron (Biota)	200.0
Lead (Biota)	0.24
Manganese (Biota)	8.3
Molybdenum (Biota)	2.8
Nickel (Biota)	1.1
Selenium (Biota)	2.9
Silver (Biota)	1.9
Strontium (Biota)	5.7
Thallium (Biota)	< 0.02
Tin (Biota)	<0.5
Uranium (Biota)	0.07
Vanadium (Biota)	2.4
Zinc (Biota)	26.0

** The mineral composition of the scallop homogenate was determined at Philip Analytical Services Corporation (MDS Environmental Laboratories), Fenwick Street, Halifax, Nova Scotia, using a Sciex/Perkin-Elmer Elan 5000 ICP-MS after acid digestion

APPENDIX C: Thermal degradation of PSP toxins in scallop homogenate

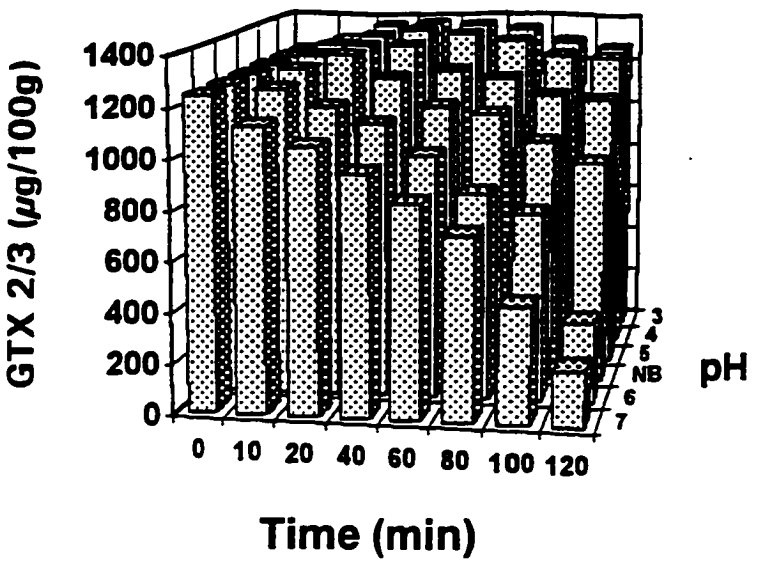


Fig. 5.I A Thermal degradation of GTX 2/3 in scallop homogenate heated at 100°C for different times and pH levels

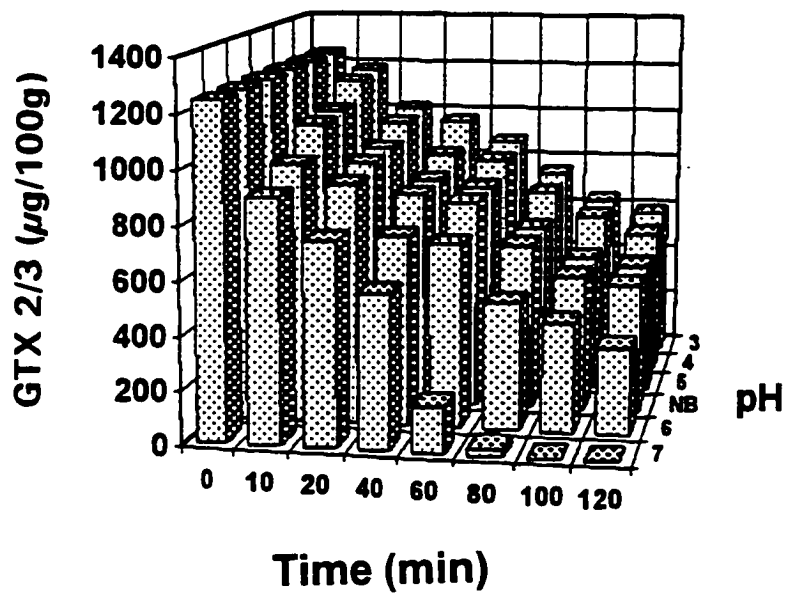


Fig. 5.I.B Thermal degradation of GTX 2/3 in scallop homogenate heated at 120°C for different times and pH levels

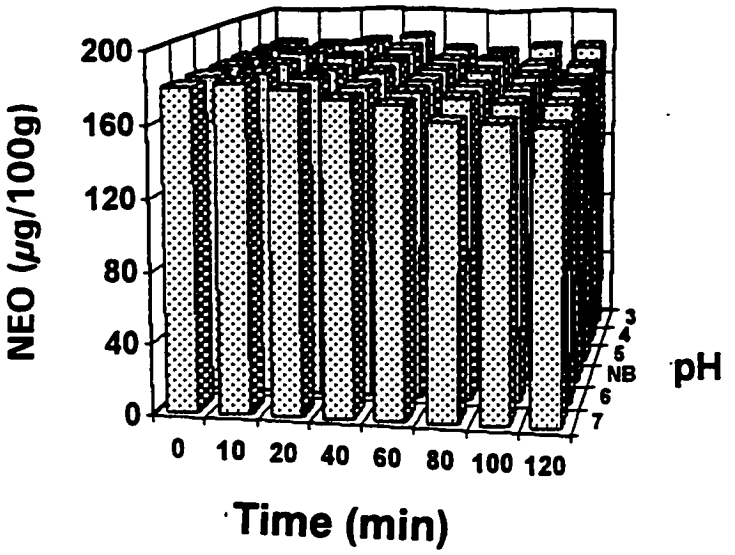


Fig. 5.II. A Thermal degradation of NEO in scallop homogenate heated at 90°C for different times and pH levels

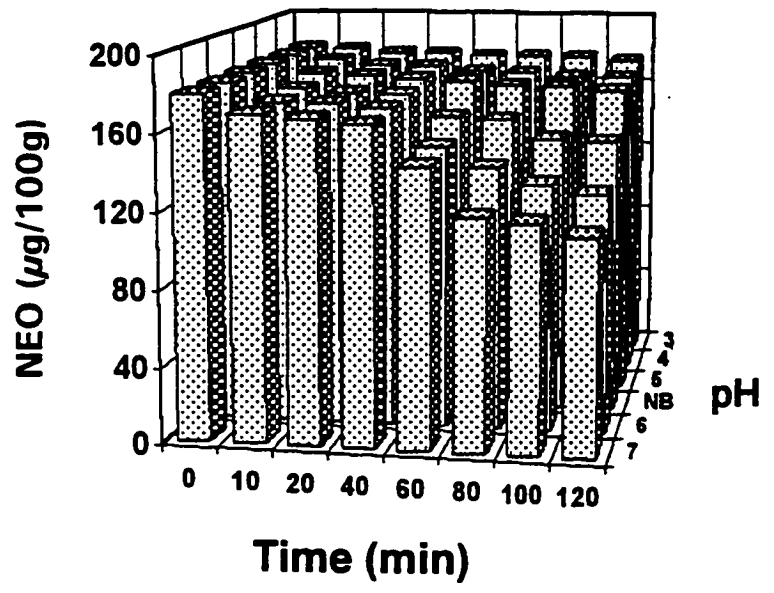


Fig. 5.II.B Thermal degradation of NEO in scallop homogenate heated at 100°C for different times and pH levels

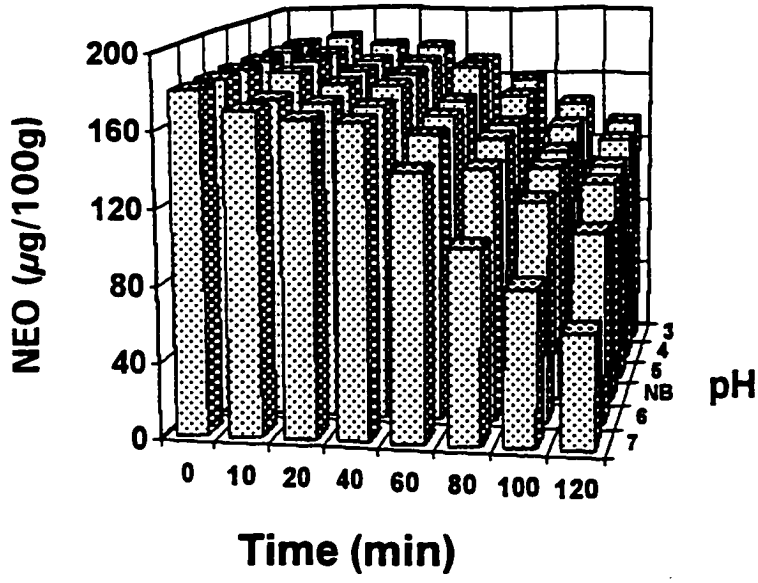


Fig. 5.II.C Thermal degradation of NEO in scallop homogenate heated at 110°C for different times and pH levels

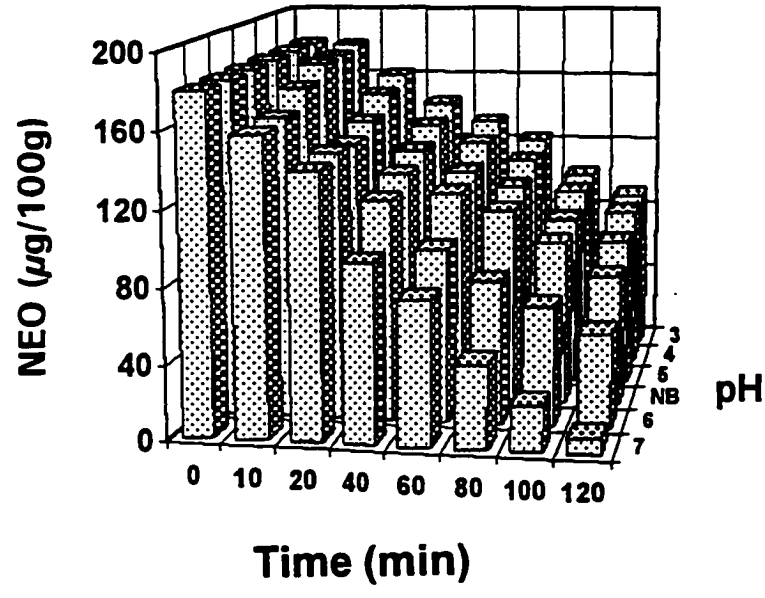


Fig. 5.II.D Thermal degradation of NEO in scallop homogenate heated at 120°C for different times and pH levels

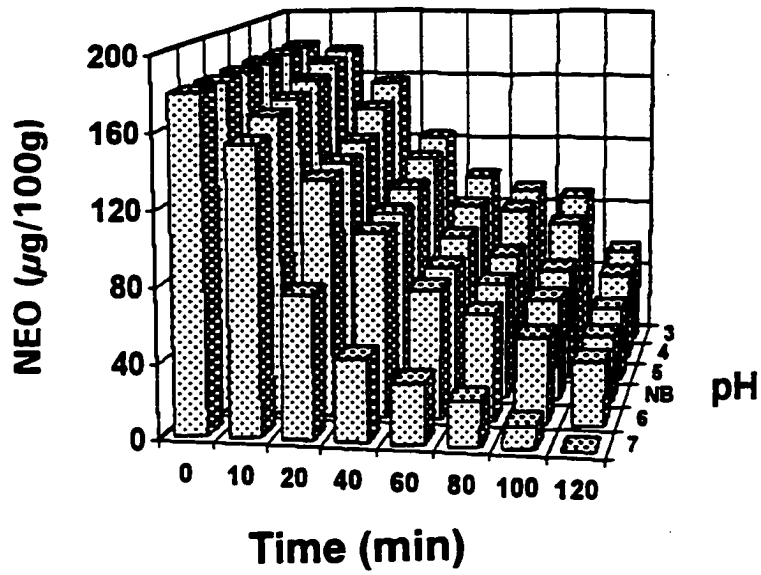


Fig. 5.II.E Thermal degradation of NEO in scallop homogenate heated at 130°C for different times and pH levels

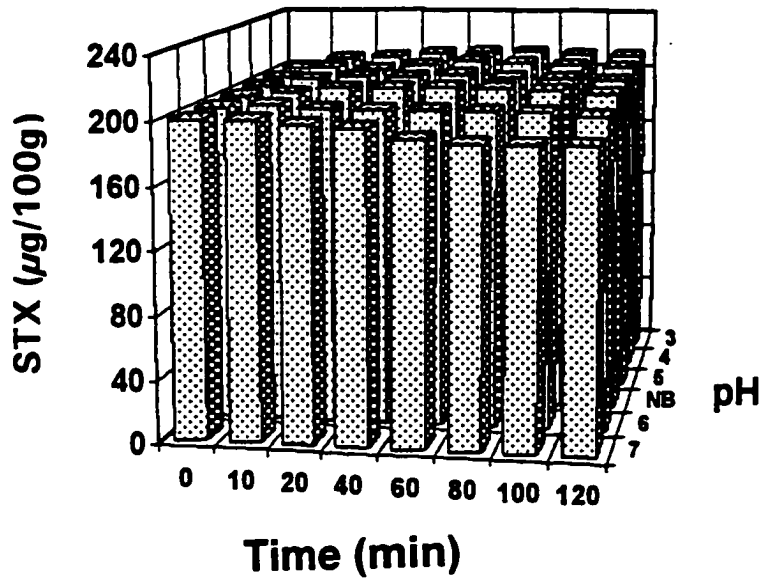


Fig. 5.III. A Thermal degradation of STX in scallop homogenate heated at 90°C for different times and pH levels

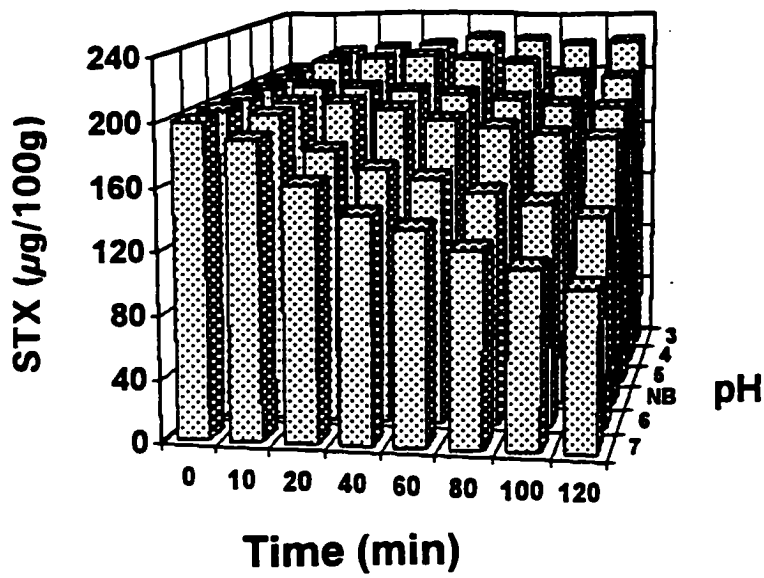


Fig. 5.III.B Thermal degradation of STX in scallop homogenate heated at 100°C for different times and pH levels

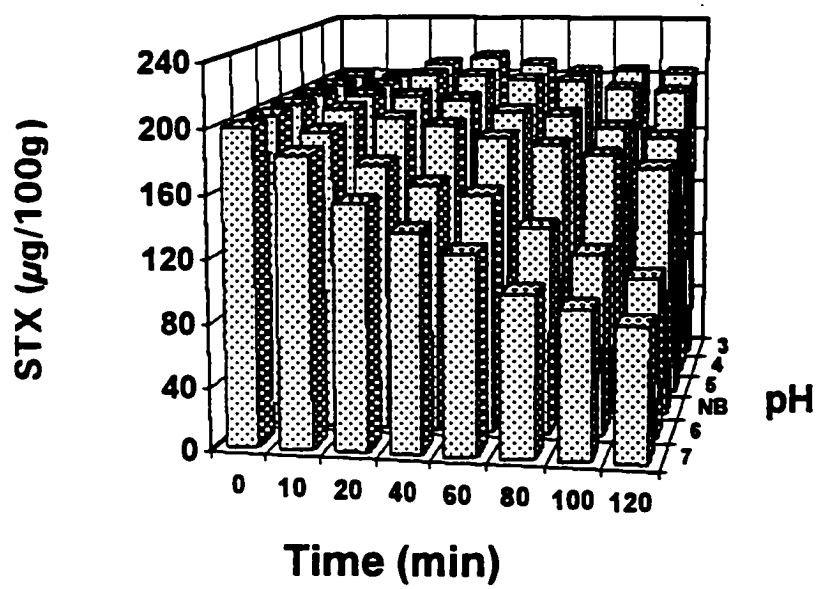


Fig. 5.III.C Thermal degradation of STX in scallop homogenate heated at 110°C for different times and pH levels

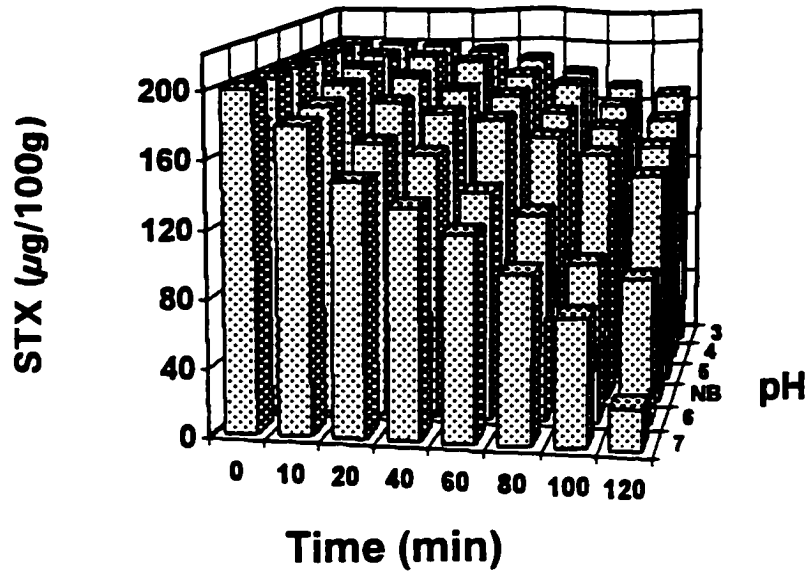


Fig. 5.III.D Thermal degradation of STX in scallop homogenate heated at 120°C for different times and pH levels

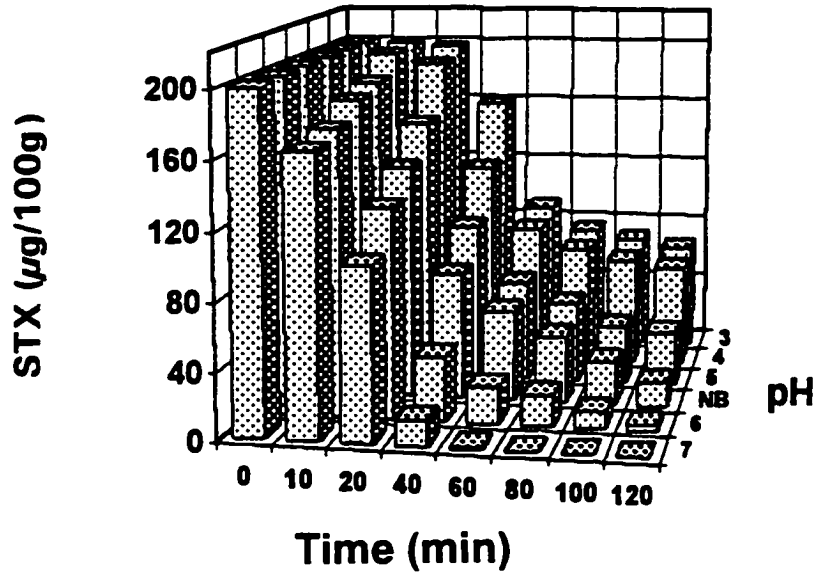


Fig. 5.III.E Thermal degradation of STX in scallop homogenate heated at 130°C for different times and pH levels

APPENDIX D: Analysis of variance for PSP toxins in scallop homogenate heated at different temperatures

Analysis of variance for GTX 2/3 in scallop homogenate (heated at 100°C) by Least Squares Means

Source	DF	Type III SS	Mean Square	F Value	P value
time	7	344.4624212	49.20888743	914.62	0.000
pH	5	166.2662122	33.253224224	671.25	0.000
time*pH	35	32.42142432	0.926326409	26.79	0.000

Significant pairs ($P \leq 0.05$) chosen from Least Squares Means for effect time*pH

pH =6, time = 0 subtracted from: pH 6*time 80 and above
pH =7, time = 0 subtracted from: pH 7*time 60 and above
NB, time = 0 subtracted from: pH 5.8*time120

Analysis of variance for GTX 2/3 in scallop homogenate (heated at 110°C) by Least Squares Means

Source	DF	Type III SS	Mean Square	F Value	P value
time	7	312.24626362	44.60660909	914.62	0.000
pH	5	163.68548595	32.73709719	671.25	0.000
time*pH	35	45.73314278	1.30666122	26.79	0.000

Significant pairs ($P \leq 0.05$) chosen from Least Squares Means for effect time*pH

pH =5, time = 0 subtracted from: pH 5*time 80 and above
pH =6, time = 0 subtracted from: pH 6*time 60 and above
pH =7, time = 0 subtracted from: pH 7*time 60 and above
NB, time = 0 subtracted from: pH 5.8*time 80

Analysis of variance for GTX 2/3 in scallop homogenate (heated at 120°C) by Least Squares Means

Source	DF	Type III SS	Mean Square	F Value	P value
time	7	262.62372958	37.51767565	3028.04	0.000
pH	5	373.73443726	74.74688745	6032.79	0.000
time*pH	35	78.64940463	2.24712585	181.36	0.000

Significant pairs (P ≤ 0.05) chosen from Least Squares Means for effect time*pH

pH =3, time = 0 subtracted from: pH 3*time 60 and above
pH =4, time = 0 subtracted from: pH 4*time 60 and above
pH =5, time = 0 subtracted from: pH 5*time 40 and above
pH =6, time = 0 subtracted from: pH 6*time 40 and above
pH =7, time = 0 subtracted from: pH 7*time 20 and above
NB, time = 0 subtracted from: pH 5.8*time 40 and above

Analysis of variance for GTX 2/3 in scallop homogenate (heated at 130°C) by Least Squares Means

Source	DF	Type III SS	Mean Square	F Value	P value
time	7	73.64665193	10.52095028	3722.13	0.000
pH	5	50.94412253	10.18882451	3604.63	0.000
time*pH	35	26.43270811	0.75522023	267.18	0.000

Significant pairs (P ≤ 0.05) chosen from Least Squares Means for effect time*pH

pH =3, time = 0 subtracted from: pH 3*time 40 and above
pH =4, time = 0 subtracted from: pH 4*time 40 and above
pH =5, time = 0 subtracted from: pH 5*time 40 and above
pH =6, time = 0 subtracted from: pH 6*time 20 and above
pH =7, time = 0 subtracted from: pH 7*time 20 and above
NB, time = 0 subtracted from: pH 5.8*time 40 and above

Analysis of variance for NEO in scallop homogenate (heated at 100°C) by Least Squares Means

Source	DF	Type III SS	Mean Square	F Value	P value
time	7	129.30371530	18.47195933	240.36	0.000
pH	5	192.99866278	38.59973256	502.27	0.000
time*pH	34	38.89340275	1.14392361	14.89	0.000

Significant pairs (P ≤ 0.05) chosen from Least Squares Means for effect time*pH

pH =6, time = 0 subtracted from: pH 6*time 120
pH =7, time = 0 subtracted from: pH 7*time 100 and above

Analysis of variance for NEO in scallop homogenate (heated at 110°C) by Least Squares Means

Source	DF	Type III SS	Mean Square	F Value	P value
time	7	171.67139585	24.52448512	893.58	0.000
pH	5	137.98762705	27.59752541	1005.55	0.000
time*pH	29	40.57098087	1.39899934	50.97	0.000

Significant pairs ($P \leq 0.05$) chosen from Least Squares Means for effect time*pH

pH =5, time = 0 subtracted from: pH 5*time 120
pH =6, time = 0 subtracted from: pH 6*time 100 and above
pH =7, time = 0 subtracted from: pH 7*time 80 and above
NB, time = 0 subtracted from: pH 5.8*time 120 and above

Analysis of variance for NEO in scallop homogenate (heated at 120°C) by Least Squares Means

Source	DF	Type III SS	Mean Square	F Value	P value
time	7	35.61943354	5.08849051	1985.95	0.000
pH	5	23.63629966	4.72725993	1844.97	0.000
time*pH	35	12.15166247	0.34719036	135.50	0.000

Significant pairs ($P \leq 0.05$) chosen from Least Squares Means for effect time*pH

pH =3, time = 0 subtracted from: pH 3*time 80 and above
pH =4, time = 0 subtracted from: pH 4*time 80 and above
pH =5, time = 0 subtracted from: pH 5*time 60 and above
pH =6, time = 0 subtracted from: pH 6*time 60 and above
pH =7, time = 0 subtracted from: pH 7*time 40 and above
NB, time = 0 subtracted from: pH 5.8*time 60 and above

Analysis of variance for NEO in scallop homogenate (heated at 130°C) by Least Squares Means

Source	DF	Type III SS	Mean Square	F Value	P value
time	7	137.50050021	19.64292860	2512.78	0.000
pH	5	76.89658312	15.37931662	1967.37	0.000
time*pH	35	46.37272592	1.32493503	169.49	0.000

Significant pairs ($P \leq 0.05$) chosen from Least Squares Means for effect time*pH

pH =3, time = 0 subtracted from: pH 3*time 60 and above
pH =4, time = 0 subtracted from: pH 4*time 60 and above
pH =5, time = 0 subtracted from: pH 5*time 40 and above
pH =6, time = 0 subtracted from: pH 6*time 40 and above
pH =7, time = 0 subtracted from: pH 7*time 20 and above
NB, time = 0 subtracted from: pH 5.8*time 40 and above

Analysis of variance for STX in scallop homogenate (heated at 100°C) by Least Squares Means

Source	DF	Type III SS	Mean Square	F Value	P value
time	7	46.8113268	6.6873324	80.66	0.000
pH	5	52.733439	10.2546688	123.68	0.000
time*pH	35	22.3369772	0.6381993	7.70	0.000

Significant pairs ($P \leq 0.05$) chosen from Least Squares Means for effect time*pH

pH =6, time = 0 subtracted from: pH 6*time 120
pH =7, time = 0 subtracted from: pH 7*time 100 and above

Analysis of variance for STX in scallop homogenate (heated at 110°C) by Least Squares Means

Source	DF	Type III SS	Mean Square	F Value	P value
time	7	30.51499906	4.35928558	451.99	0.000
pH	5	20.00310347	4.00062069	414.80	0.000
time*pH	35	9.14153912	0.26118683	27.08	0.000

Significant pairs ($P \leq 0.05$) chosen from Least Squares Means for effect time*pH

pH =6, time = 0 subtracted from: pH 6*time 100 and above
pH =7, time = 0 subtracted from: pH 7*time 80 and above

Analysis of variance for STX in scallop homogenate (heated at 120°C) by Least Squares Means

Source	DF	Type III SS	Mean Square	F Value	P value
time	7	29.510996539	4.215856648	620.05	0.000
pH	5	32.678056794	6.535611359	961.23	0.000
time*pH	35	6.567355032	1.87638715	27.60	0.000

Significant pairs ($P \leq 0.05$) chosen from Least Squares Means for effect time*pH

pH =6, time = 0 subtracted from: pH 6*time 80 and above
pH =7, time = 0 subtracted from: pH 7*time 60 and above
NB, time = 0, subtracted from: pH 5.8*time 120

Analysis of variance for STX in scallop homogenate (heated at 130°C) by Least Squares Means

Source	DF	Type III SS	Mean Square	F Value	P value
time	7	76.43203635	10.91886234	677.25	0.000
pH	5	16.87317099	3.37463420	209.32	0.000
time*pH	35	12.91455173	0.36898719	100.13	0.000

Significant pairs ($P \leq 0.05$) chosen from Least Squares Means for effect time*pH

pH =3, time = 0 subtracted from: pH 3*time 60 and above
pH =4, time = 0 subtracted from: pH 4*time 60 and above
pH =5, time = 0 subtracted from: pH 5*time 40 and above
pH =6, time = 0 subtracted from: pH 6*time 40 and above
pH =7, time = 0 subtracted from: pH 7*time 20 and above
NB, time = 0 subtracted from: pH 5.8*time 40 and above

APPENDIX E: Thermal degradation of PSP toxins in the standard toxin mixture heated at different temperatures

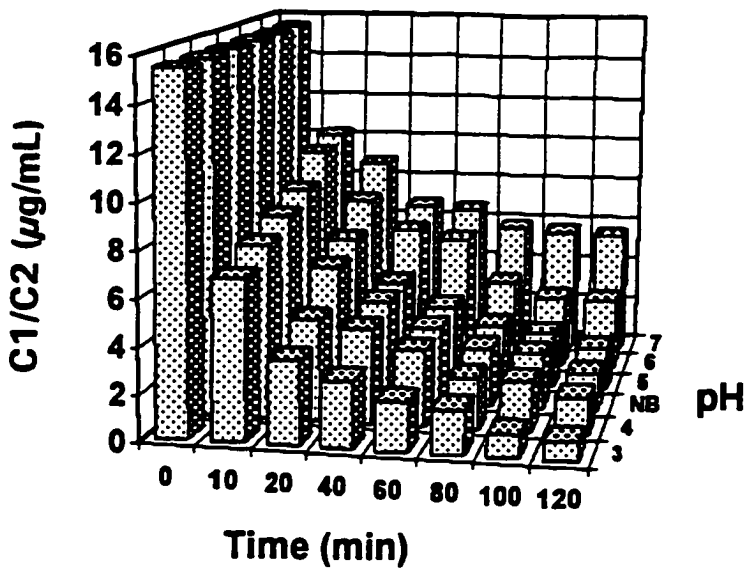


Fig. 6.I Thermal degradation of C1/C2 in the standard toxin mixture heated at 100°C for different times and pH levels

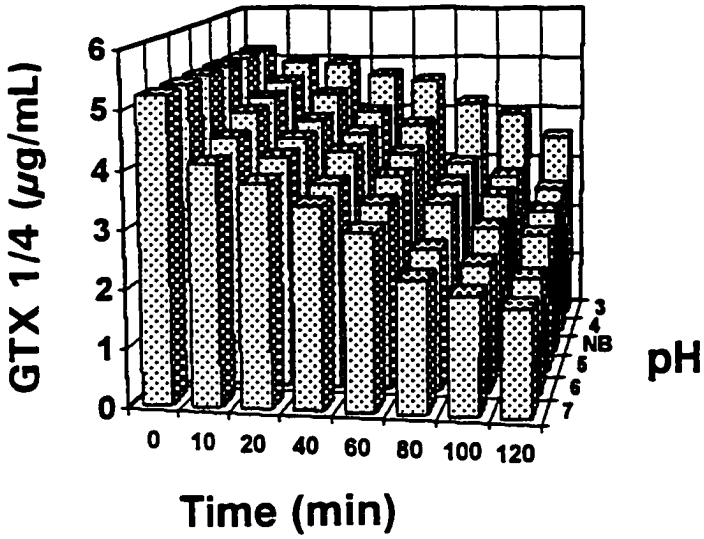


Fig. 6.II.A Thermal degradation of GTX 1/4 in the standard toxin mixture heated at 100°C for different times and pH levels

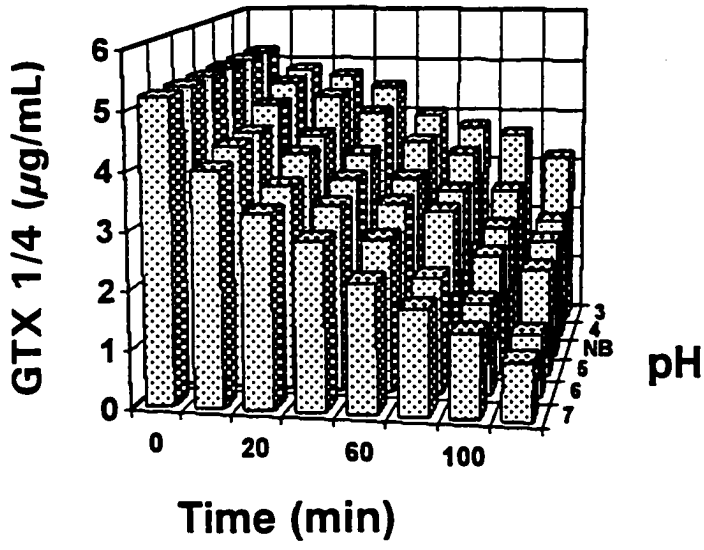


Fig. 6.II.B Thermal degradation of GTX 1/4 in the standard toxin mixture heated at 120°C for different times and pH levels

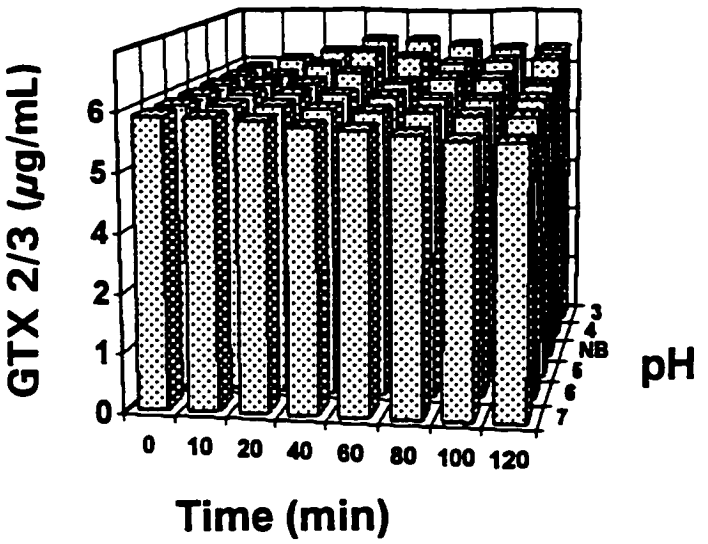


Fig. 6.III.A Thermal degradation of GTX 2/3 in the standard toxin mixture heated at 90°C for different times and pH levels

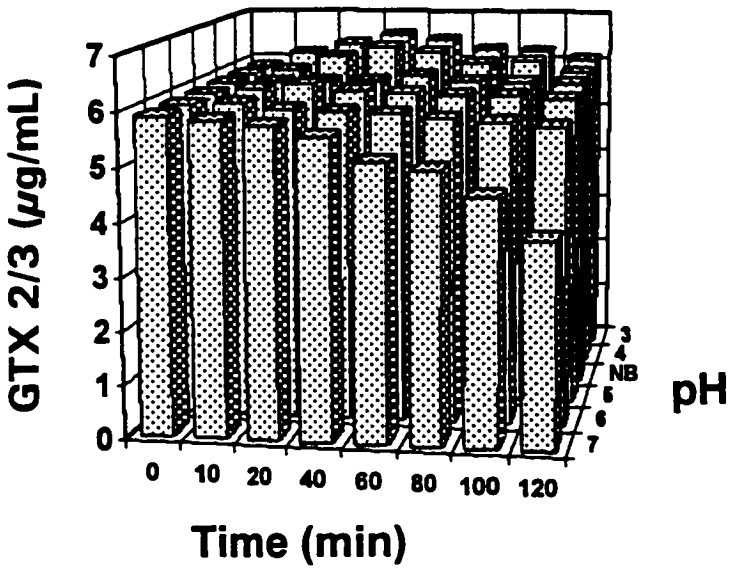


Fig. 6.III.B Thermal degradation of GTX 2/3 in the standard toxin mixture heated at 100°C for different times and pH levels

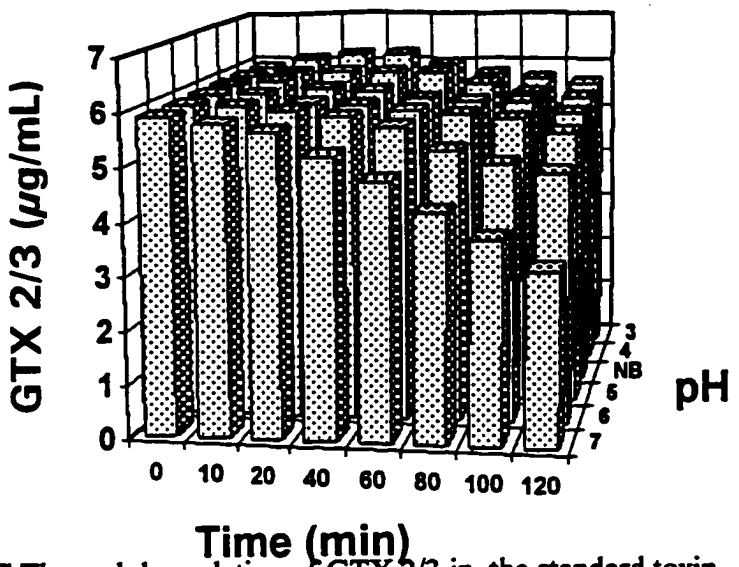


Fig. 6.III.C Thermal degradation of GTX 2/3 in the standard toxin mixture heated at 110°C for different times and pH levels

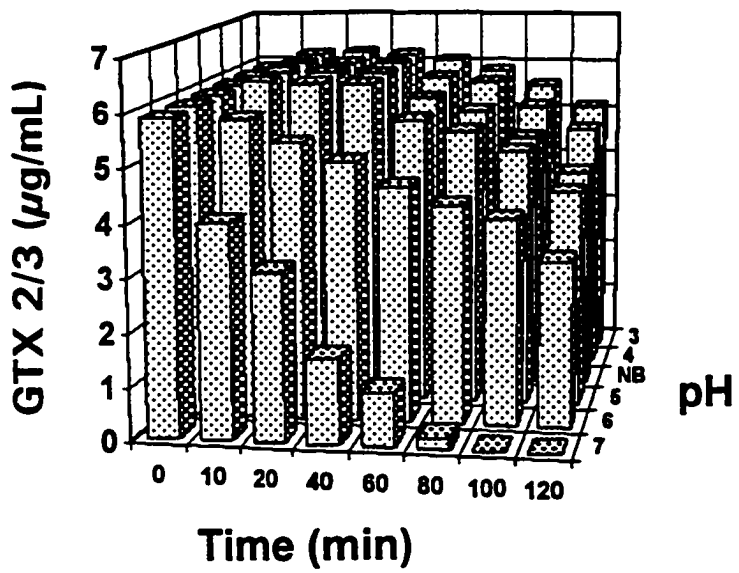


Fig. 6.III.D Thermal degradation of GTX 2/3 in the standard toxin mixture heated at 120°C for different times and pH levels

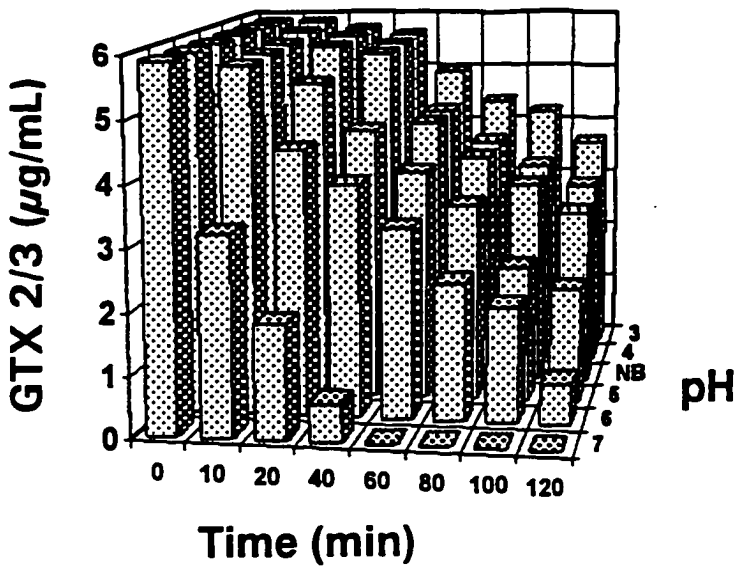


Fig. 6.III.E Thermal degradation of GTX 2/3 in the standard toxin mixture heated at 130°C for different times and pH levels

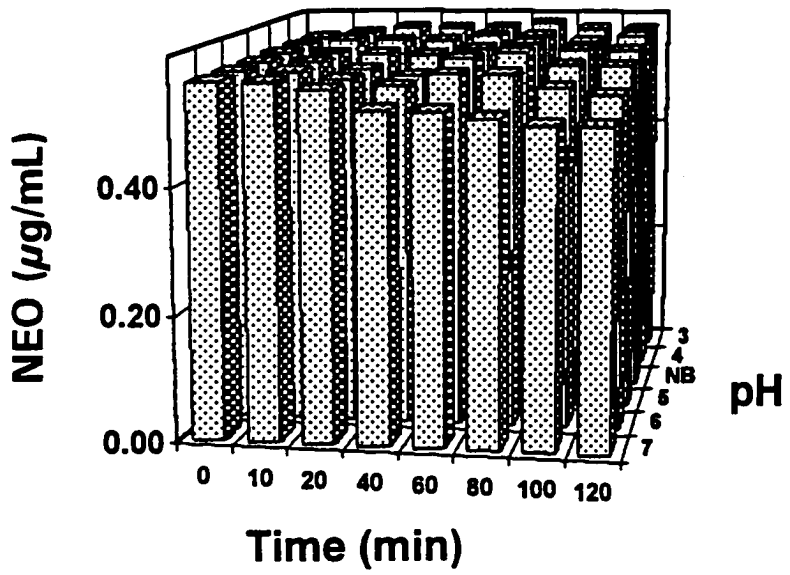


Fig. 6.IV.A Thermal degradation of NEO in the standard toxin mixture heated at 90°C for different times and pH levels

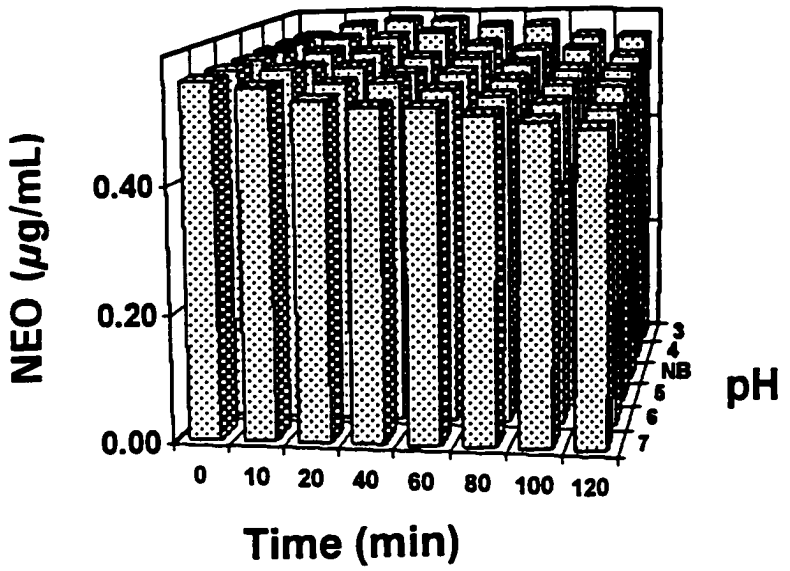


Fig. 6.IV.B Thermal degradation of NEO in the standard toxin mixture heated at 100°C for different times and pH levels

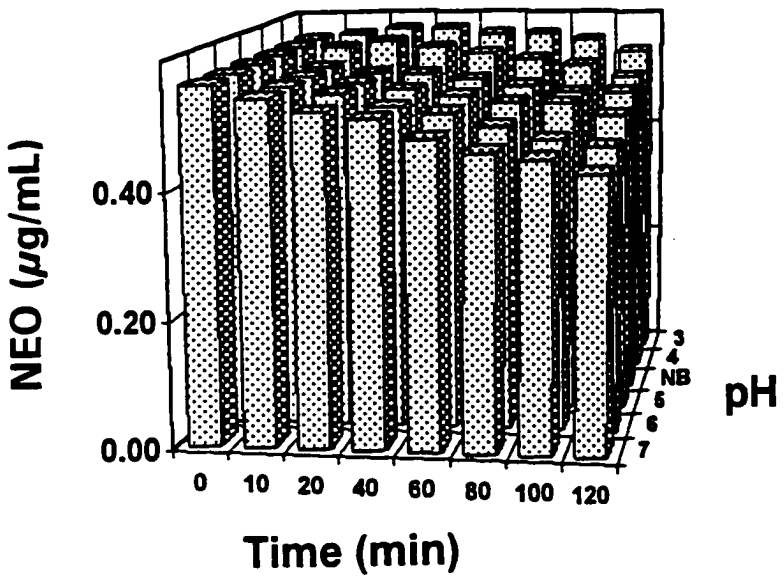


Fig. 6.IV.C Thermal degradation of NEO in the standard toxin mixture heated at 110°C for different times and pH levels

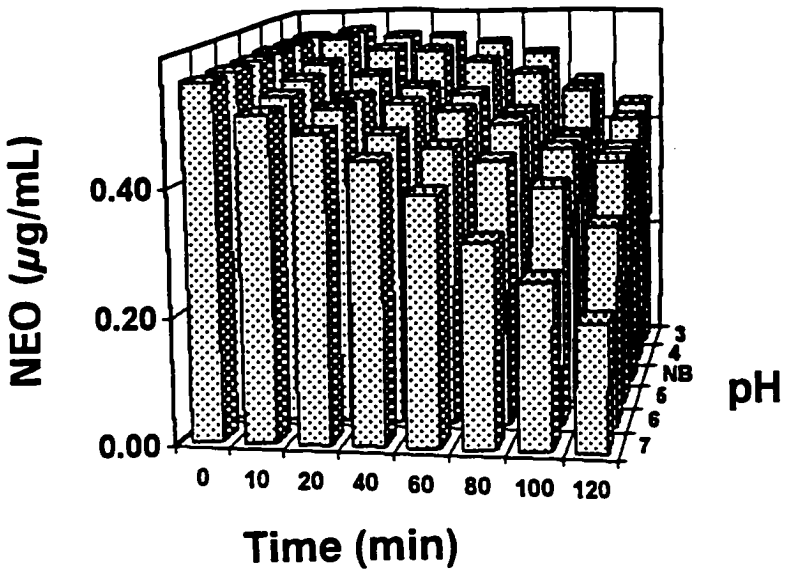


Fig. 6.IV.D Thermal degradation of NEO in the standard toxin mixture heated at 120°C for different times and pH levels

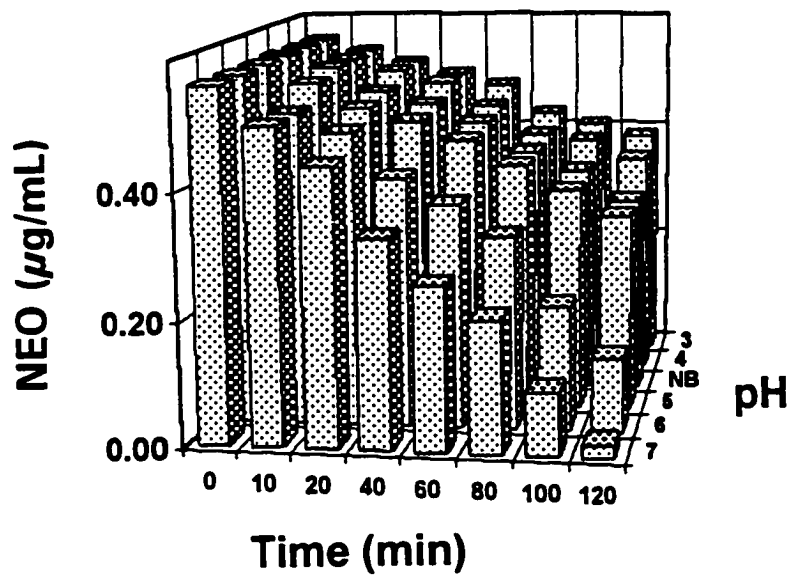


Fig. 6.IV.E Thermal degradation of NEO in the standard toxin mixture heated at 130°C for different times and pH levels

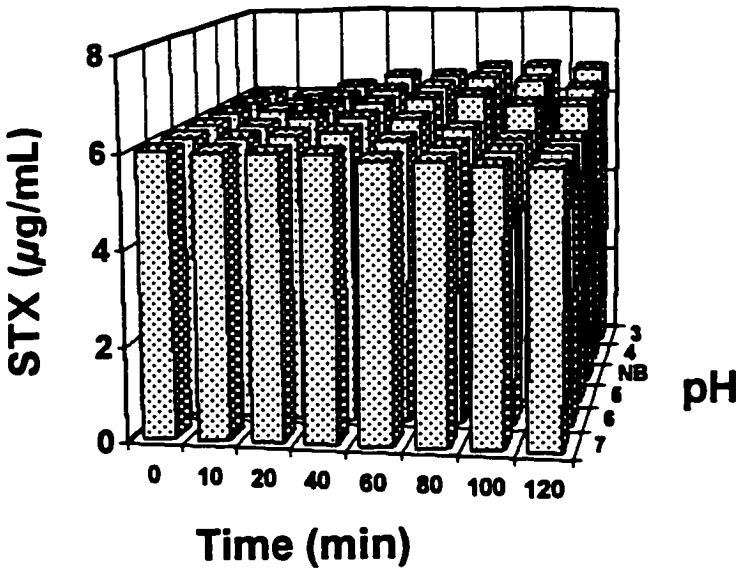


Fig. 6.V.A Thermal degradation of STX in the standard toxin mixture heated at 90°C for different times and pH levels

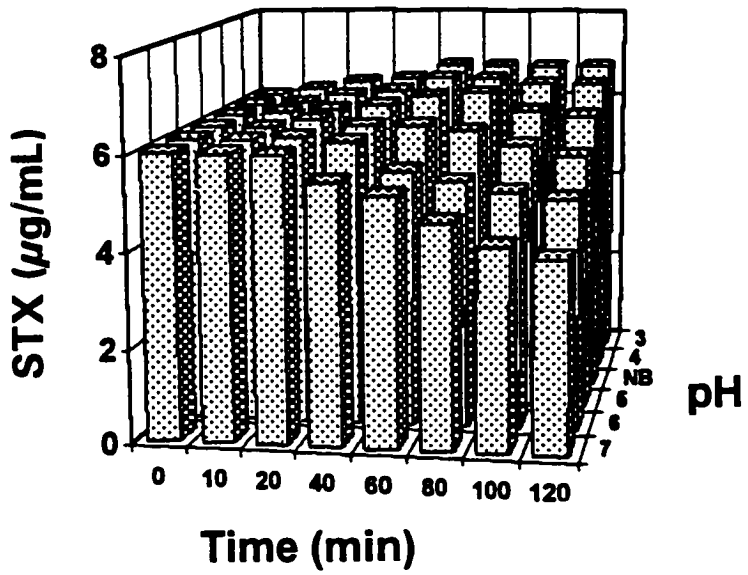


Fig. 6.V.B Thermal degradation of STX in the standard toxin mixture heated at 100°C for different times and pH levels

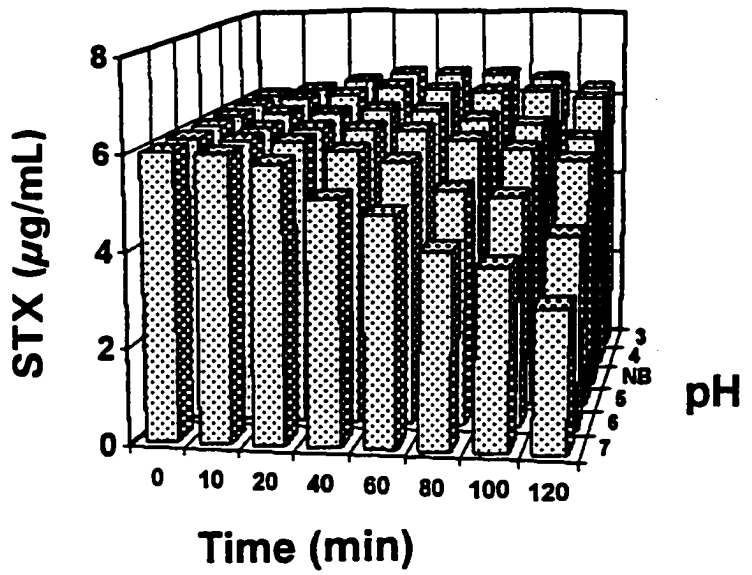


Fig. 6.V.C Thermal degradation of STX in the standard toxin mixture heated at 110°C for different times and pH levels

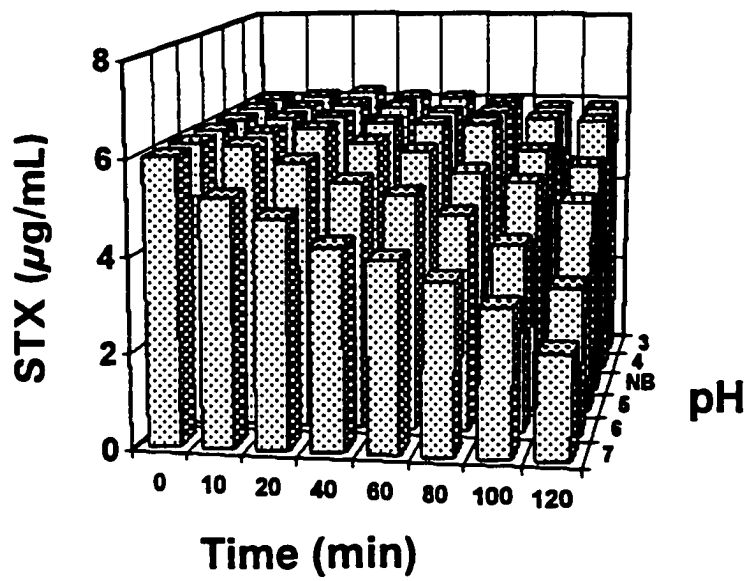


Fig. 6.V.D Thermal degradation of STX in the standard toxin mixture heated at 120°C for different times and pH levels

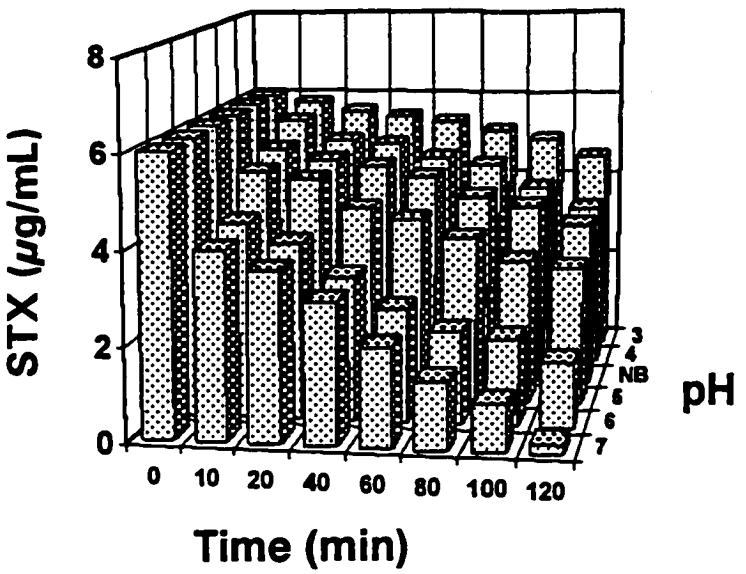


Fig. 6.V.E Thermal degradation of STX in the standard toxin mixture heated at 130°C for different times and pH levels

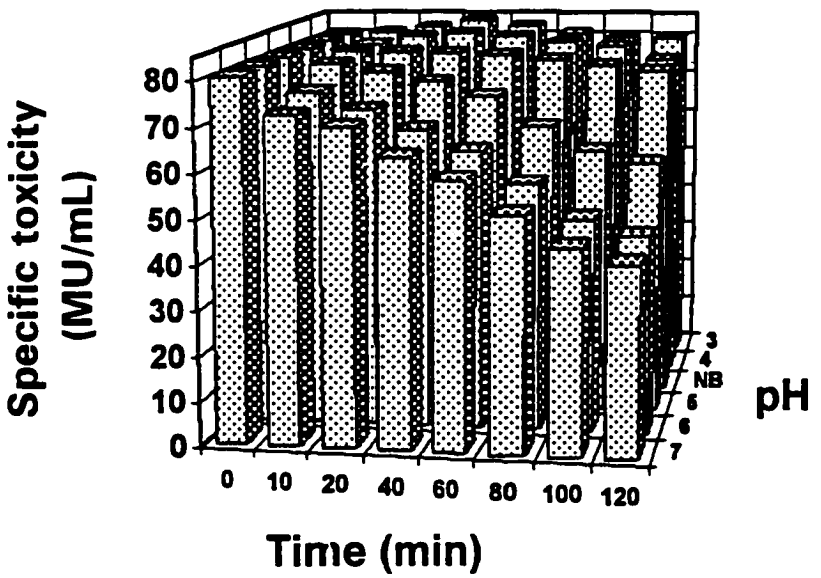


Fig. 6.VI.A Variation of specific toxicities of the standard toxin mixture heated at 100°C for different times and pH levels

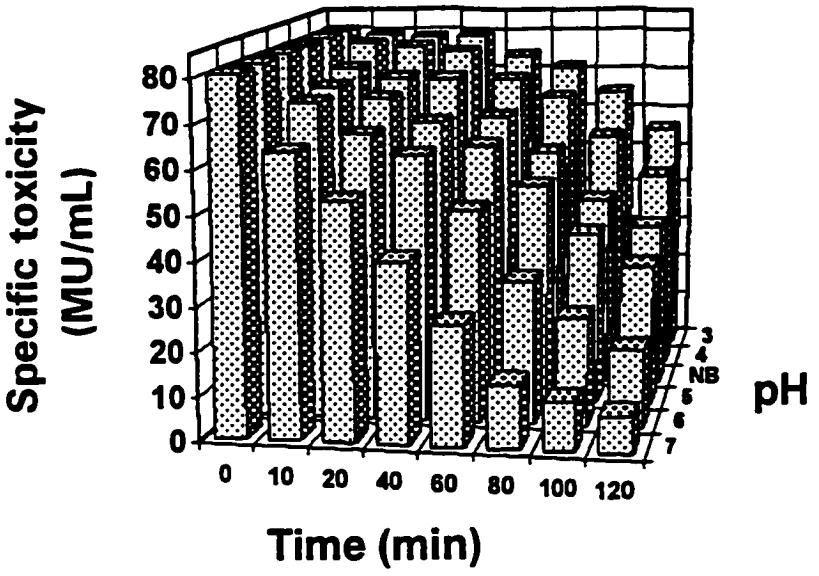


Fig. 6.VLB Variation of specific toxicities of the standard toxin mixture heated at 120°C for different times and pH levels

APPENDIX F: Analysis of variance for PSP toxins in the purified toxin mixture heated at different temperatures

Analysis of Variance for C1/2 toxins in the toxin mixture (heated at 90°C), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	5	6.43893	6.43893	1.28779	674.52	0.000
time	7	31.34035	31.34035	4.47719	2345.09	0.000
pH*time	35	2.27254	2.27254	0.06493	34.01	0.000
Error	48	0.09164	0.09164	0.00191		
Total	95	40.14346				

Significant pairs ($P \leq 0.05$) chosen after multiple comparison by Bonferroni Simultaneous Test and all pairwise comparisons among levels of pH*time
pH = 3, time = 0, subtracted from: pH 3*time 10 and above
pH = 4, time = 0, subtracted from: pH 4*time 10 and above
pH = 5, time = 0, subtracted from: pH 5*time 10 and above
pH = 6, time = 0, subtracted from: pH 6*time 10 and above
pH = 7, time = 0, subtracted from: pH 7*time 10 and above
NB, time = 0, subtracted from: pH 4.8*time 10 and above

Analysis of Variance for C1/2 toxins in the toxin mixture (heated at 100°C), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	5	10.0436	10.0436	2.0087	137.65	0.000
time	7	39.2891	39.2891	5.6127	384.62	0.000
pH*time	35	3.5778	3.5778	0.1022	7.00	0.000
Error	48	0.7005	0.7005	0.0146		
Total	95	53.6109				

Significant pairs ($P \leq 0.05$) chosen after multiple comparison by Bonferroni Simultaneous Test and all pairwise comparisons among levels of pH*time
pH = 3, time = 0, subtracted from: pH 3*time 10 and above
pH = 4, time = 0, subtracted from: pH 4*time 10 and above
pH = 5, time = 0, subtracted from: pH 5*time 10 and above
pH = 6, time = 0, subtracted from: pH 6*time 10 and above
pH = 7, time = 0, subtracted from: pH 7*time 10 and above
NB, time = 0, subtracted from: pH 4.8*time 10 and above

Analysis of Variance for C1/2 toxins in the toxin mixture (heated at 110°C), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	5	47.8408	47.8408	9.5682	257.87	0.000
time	7	77.5549	77.5549	11.0793	298.60	0.000
pH*time	35	42.8910	42.8910	1.2255	33.03	0.000
Error	48	1.7810	1.7810	0.0371		
Total	95	170.0678				

Significant pairs (P≤ 0.05) chosen after multiple comparison by Bonferroni Simultaneous Test and all pairwise comparisons among levels of pH*time

pH = 3, time = 0, subtracted from: pH 3*time 10 and above
pH = 4, time = 0, subtracted from: pH 4*time 10 and above
pH = 5, time = 0, subtracted from: pH 5*time 10 and above
pH = 6, time = 0, subtracted from: pH 6*time 10 and above
pH = 7, time = 0, subtracted from: pH 7*time 10 and above
NB, time = 0, subtracted from: pH 4.8*time 10 and above

Analysis of Variance for C1/2 toxins in the toxin mixture (heated at 120°C), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	5	310.703	310.703	62.141	7682.53	0.000
time	7	214.981	214.981	30.712	3796.91	0.000
pH*time	35	254.801	254.801	7.280	900.04	0.000
Error	48	0.388	0.388	0.008		
Total	95	780.873				

Significant pairs (P≤ 0.05) chosen after multiple comparison by Bonferroni Simultaneous Test and all pairwise comparisons among levels of pH*time

pH = 3, time = 0, subtracted from: pH 3*time 10 and above
pH = 4, time = 0, subtracted from: pH 4*time 10 and above
pH = 5, time = 0, subtracted from: pH 5*time 10 and above
pH = 6, time = 0, subtracted from: pH 6*time 10 and above
pH = 7, time = 0, subtracted from: pH 7*time 10 and above
NB, time = 0, subtracted from: pH 4.8*time 10 and above

Analysis of Variance for GTX 1/4 in the toxin mixture (heated at 90°C), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	5	1.870961	1.870961	0.374192	70.51	0.000
time	7	1.511105	1.511105	0.215872	140.68	0.000
pH*time	35	1.511105	1.511105	0.024411	4.60	0.000
Error	48	1.511105	1.511105	0.005307		
Total	95	4.491194				

Significant pairs ($P \leq 0.05$) chosen after multiple comparison by Bonferroni Simultaneous Test and all pairwise comparisons among levels of pH*time

pH = 5, time = 0, subtracted from: pH 5*time 120
pH = 6, time = 0, subtracted from: pH 6*time 60 and above
pH = 7, time = 0, subtracted from: pH 7*time 40 and above

Analysis of Variance for GTX 1/4 in the toxin mixture (heated at 100°C), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	5	1.84587	1.84587	0.36917	72.96	0.000
time	7	5.20725	5.20725	0.74389	147.01	0.000
pH*time	35	0.72547	0.72547	0.02073	4.10	0.000
Error	48	0.24288	0.24288	0.00506		
Total	95	8.02147				

Significant pairs ($P \leq 0.05$) chosen after multiple comparison by Bonferroni Simultaneous Test and all pairwise comparisons among levels of pH*time

pH = 3, time = 0, subtracted from: pH 3*time 100 and above
pH = 4, time = 0, subtracted from: pH 4*time 100 and above
pH = 5, time = 0, subtracted from: pH 5*time 80 and above
pH = 6, time = 0, subtracted from: pH 6*time 60 and above
pH = 7, time = 0, subtracted from: pH 7*time 40 and above
NB, time = 0, subtracted from: pH 4.8*time 40 and above

Analysis of Variance for GTX 1/4 in the toxin mixture (heated at 110°C), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	5	5.10593	5.10593	1.02119	87.92	0.000
time	7	11.68211	11.68211	1.66887	143.68	0.000
pH*time	35	4.67035	4.67035	0.13344	11.49	0.000
Error	48	0.55755	0.55755	0.01162		
Total	95	22.01594				

Significant pairs ($P \leq 0.05$) chosen after multiple comparison by Bonferroni Simultaneous Test and all pairwise comparisons among levels of pH*time

pH = 3, time = 0, subtracted from: pH 3*time 120
pH = 4, time = 0, subtracted from: pH 4*time 100 and above
pH = 5, time = 0, subtracted from: pH 5*time 80 and above
pH = 6, time = 0, subtracted from: pH 6*time 40 and above
pH = 7, time = 0, subtracted from: pH 7*time 40 and above
NB, time = 0, subtracted from: pH 4.8*time 60 and above

Analysis of Variance for GTX 1/4 in the toxin mixture (heated at 120°C), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	5	5.18692	5.18692	1.03738	142.44	0.000
time	7	12.41088	12.41088	1.77298	243.44	0.000
pH*time	35	4.06857	4.06857	0.11624	15.96	0.000
Error	48	0.34959	0.34959	0.00728		
Total	95	22.01597				

Significant pairs ($P \leq 0.05$) chosen after multiple comparison by Bonferroni Simultaneous Test and all pairwise comparisons among levels of pH*time

pH = 3, time = 0, subtracted from: pH 3*time 120 and above
pH = 4, time = 0, subtracted from: pH 4*time 80 and above
pH = 5, time = 0, subtracted from: pH 5*time 60 and above
pH = 6, time = 0, subtracted from: pH 6*time 40 and above
pH = 7, time = 0, subtracted from: pH 7*time 20 and above
NB, time = 0, subtracted from: pH 4.8*time 60 and above

Analysis of Variance for GTX 1/4 in the toxin mixture (heated at 130°C), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	5	660.015	660.015	132.003	2196.49	0.000
time	7	617.310	617.310	88.187	1467.40	0.000
pH*time	35	599.730	599.730	17.135	285.12	0.000
Error	48	2.885	2.885	0.060		
Total	95	1879.939				

Significant pairs ($P \leq 0.05$) chosen after multiple comparison by Bonferroni Simultaneous Test and all pairwise comparisons among levels of pH*time

pH = 3, time = 0, subtracted from: pH 3*time 100 and above
pH = 4, time = 0, subtracted from: pH 4*time 100 and above
pH = 5, time = 0, subtracted from: pH 5*time 60 and above
pH = 6, time = 0, subtracted from: pH 6*time 20 and above
pH = 7, time = 0, subtracted from: pH 7*time 20 and above
NB, time = 0, subtracted from: pH 4.8*time 60 and above

Analysis of Variance for GTX 2/3 in the toxin mixture (heated at 90°C), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	5	0.070726	0.070726	0.014145	9.96	0.000
time	7	0.048245	0.048245	0.006892	4.85	0.000
pH*time	35	0.189490	0.189490	0.005414	3.81	0.041
Error	48	0.068202	0.068202	0.001421		
Total	95	0.227446				

Significant pairs ($P \leq 0.05$) chosen after multiple comparison by Bonferroni Simultaneous Test and all pairwise comparisons among levels of pH*time

pH = 3, time = 0, subtracted from: pH 3*time 40 and 60

Analysis of Variance for GTX 2/3 in the toxin mixture (heated at 100°C), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	5	1.133841	1.133841	0.226768	24.83	0.000
time	7	0.703004	0.703004	0.100429	11.00	0.000
pH*time	35	1.24332	1.243320	0.035523	3.89	0.000
Error	48	0.438347	0.438347	0.009132		
Total	95	2.880422				

Significant pairs ($P \leq 0.05$) chosen after multiple comparison by Bonferroni Simultaneous Test and all pairwise comparisons among levels of pH*time

pH = 3, time = 0, subtracted from: pH 3*time 40
pH = 7, time = 0, subtracted from: pH 7*time 120

Analysis of Variance for GTX 2/3 in the toxin mixture (heated at 110°C), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	5	0.238565	0.238565	0.047713	34.66	0.000
time	7	0.119607	0.119607	0.017087	12.41	0.000
pH*time	35	0.239387	0.239387	0.006840	4.97	0.000
Error	48	0.066068	0.066068	0.001376		
Total	95	0.663627				

Significant pairs ($P \leq 0.05$) chosen after multiple comparison by Bonferroni Simultaneous Test and all pairwise comparisons among levels of pH*time

pH = 6, time = 0, subtracted from: pH 6*time 120
pH = 7, time = 0, subtracted from: pH 7*time 80 and above

Analysis of Variance for GTX 2/3 in the toxin mixture (heated at 120°C), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	5	320.447	320.447	64.089	61.67	0.000
time	7	99.115	99.115	14.159	13.63	0.000
pH*time	35	366.784	366.784	10.480	10.08	0.000
Error	48	49.881	49.881	1.039		
Total	95	836.228				

Significant pairs (P≤ 0.05) chosen after multiple comparison by Bonferroni Simultaneous Test and all pairwise comparisons among levels of pH*time

pH = 3, time = 0, subtracted from: pH 3*time 120
pH = 4, time = 0, subtracted from: pH 4*time 120
pH = 5, time = 0, subtracted from: pH 5*time 80 and above
pH = 6, time = 0, subtracted from: pH 6*time 60 and above
pH = 7, time = 0, subtracted from: pH 7*time 20 and above
NB, time = 0, subtracted from: pH 4.8*time 60 and above

Analysis of Variance for GTX 2/3 in the toxin mixture (heated at 130°C), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	5	578.233	578.233	115.647	86.53	0.000
time	7	218.757	218.757	31.251	23.38	0.000
pH*time	35	475.256	475.256	13.579	10.16	0.000
Error	48	64.149	64.149	1.336		
Total	95	1336.395				

Significant pairs (P≤ 0.05) chosen after multiple comparison by Bonferroni Simultaneous Test and all pairwise comparisons among levels of pH*time

pH = 3, time = 0, subtracted from: pH 3*time 120
pH = 4, time = 0, subtracted from: pH 4*time 100 and above
pH = 5, time = 0, subtracted from: pH 5*time 80 and above
pH = 6, time = 0, subtracted from: pH 6*time 60 and above
pH = 7, time = 0, subtracted from: pH 7*time 20 and above
NB, time = 0, subtracted from: pH 4.8*time 80 and above

Analysis of Variance for NEO in the toxin mixture (heated at 100°C), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	5	0.950806	0.950806	0.190161	139.71	0.000
time	7	0.665579	0.665579	0.095083	69.86	0.000
pH*time	35	0.807664	0.807664	0.023076	16.95	0.000
Error	48	0.065335	0.065335	0.001361		
Total	95	2.489384				

Significant pairs (P≤ 0.05) chosen after multiple comparison by Bonferroni Simultaneous Test and all pairwise comparisons among levels of pH*time

pH = 7, time = 0, subtracted from: pH 7*time 120

Analysis of Variance for NEO in the toxin mixture (heated at 110°C), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	5	1.67030	1.67030	0.33406	56.61	0.000
time	7	3.66968	3.66968	0.52424	88.83	0.000
pH*time	35	1.70092	1.70092	0.04860	8.23	0.000
Error	48	0.28327	0.28327	0.00590		
Total	95	7.32417				

Significant pairs (P≤ 0.05) chosen after multiple comparison by Bonferroni Simultaneous Test and all pairwise comparisons among levels of pH*time

pH = 6, time = 0, subtracted from: pH 6*time 120
pH = 7, time = 0, subtracted from: pH 7*time 100 and above

Analysis of Variance for NEO in the toxin mixture (heated at 120°C), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	5	5.12569	5.12569	1.02514	120.23	0.000
time	7	12.68168	12.68168	1.81167	212.48	0.000
pH*time	35	4.75743	4.75743	0.13593	15.94	0.000
Error	48	0.40926	0.40926	0.00853		
Total	95	22.97406				

Significant pairs (P≤ 0.05) chosen after multiple comparison by Bonferroni Simultaneous Test and all pairwise comparisons among levels of pH*time
pH = 3, time = 0, subtracted from: pH 3*time 120
pH = 4, time = 0, subtracted from: pH 4*time 120
pH = 5, time = 0, subtracted from: pH 5*time 80 and above
pH = 6, time = 0, subtracted from: pH 6*time 80 and above
pH = 7, time = 0, subtracted from: pH 7*time 60 and above
NB, time = 0, subtracted from: pH 4.8*time 100 and above

Analysis of Variance for NEO in the toxin mixture (heated at 130°C), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	5	41.1257	41.1257	8.2251	806.83	0.000
time	7	73.1441	73.1441	10.4492	1024.99	0.000
pH*time	35	130.0607	130.0607	3.7160	364.52	0.000
Error	48	0.4893	0.4893	0.0102		
Total	95	244.8199				

Significant pairs ($P \leq 0.05$) chosen after multiple comparison by Bonferroni Simultaneous Test and all pairwise comparisons among levels of pH*time

pH = 3, time = 0, subtracted from: pH 3*time 100 and above
pH = 4, time = 0, subtracted from: pH 4*time 100 and above
pH = 5, time = 0, subtracted from: pH 5*time 80 and above
pH = 6, time = 0, subtracted from: pH 6*time 60 and above
pH = 7, time = 0, subtracted from: pH 7*time 40 and above
NB, time = 0, subtracted from: pH 4.8*time 80 and above

Analysis of Variance for STX in the toxin mixture (heated at 100°C), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	5	0.950557	0.950557	0.190111	31.79	0.000
time	7	0.223775	0.223775	0.031968	5.35	0.000
pH*time	35	0.801368	0.801368	0.022896	3.83	0.000
Error	48	0.287008	0.287008	0.005979		
Total	95	2.262709				

Significant pairs ($P \leq 0.05$) chosen after multiple comparison by Bonferroni Simultaneous Test and all pairwise comparisons among levels of pH*time

pH = 7, time = 0, subtracted from: pH 7*time 120

Analysis of Variance for STX in the toxin mixture (heated at 110°C), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	5	1.81940	1.81940	0.36388	52.59	0.000
time	7	1.51934	1.51934	0.21705	31.37	0.000
pH*time	35	1.64328	1.64328	0.04695	6.79	0.000
Error	48	0.33210	0.33210	0.00692		
Total	95	5.31412				

Significant pairs ($P \leq 0.05$) chosen after multiple comparison by Bonferroni Simultaneous Test and all pairwise comparisons among levels of pH*time

pH = 6, time = 0, subtracted from: pH 6*time 120
pH = 7, time = 0, subtracted from: pH 7*time 80 and above

Analysis of Variance for STX in the toxin mixture (heated at 120°C), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	5	9.57337	9.57337	1.91467	395.78	0.000
time	7	9.47494	9.47494	1.35356	279.79	0.000
pH*time	35	6.63024	6.63024	0.18944	39.16	0.000
Error	48	0.23221	0.23221	0.00484		
Total	95	25.91077				

Significant pairs ($P \leq 0.05$) chosen after multiple comparison by Bonferroni Simultaneous Test and all pairwise comparisons among levels of pH*time

pH = 5, time = 0, subtracted from: pH 5*time 100 and above
pH = 6, time = 0, subtracted from: pH 6*time 60 and above
pH = 7, time = 0, subtracted from: pH 7*time 40 and above
NB, time = 0, subtracted from: pH 4.8*time 100 and above

Analysis of Variance for STX in the toxin mixture (heated at 130°C), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	5	62.8869	62.8869	12.5774	1792.68	0.000
time	7	99.8513	99.8513	14.2645	2033.14	0.000
pH*time	35	196.3771	196.3771	5.6108	799.71	0.000
Error	48	0.3368	0.3368	0.0070		
Total	95	359.4521				

Significant pairs ($P \leq 0.05$) chosen after multiple comparison by Bonferroni Simultaneous Test and all pairwise comparisons among levels of pH*time

pH = 3, time = 0, subtracted from: pH 3*time 100 and above
pH = 4, time = 0, subtracted from: pH 4*time 100 and above
pH = 5, time = 0, subtracted from: pH 5*time 80 and above
pH = 6, time = 0, subtracted from: pH 6*time 60 and above
pH = 7, time = 0, subtracted from: pH 7*time 40 and above
NB, time = 0, subtracted from: pH 4.8*time 80 and above

APPENDIX G: Analysis of variance for PSP toxins stored at different temperatures

Analysis of Variance for C1/2 toxins in the toxin mixture (storage at -5°C), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	4	1.34362	1.343626	0.335791	101.31	0.000
time	5	0.046089	0.046089	0.009218	2.78	0.000
pH*time	20	0.243750	0.243750	0.012187	3.68	0.000
Error	30	0.099438	0.099438	0.003315		
Total	59	1.732439				

Significant pairs ($P \leq 0.05$) chosen after multiple comparison by Bonferroni Simultaneous Test and all pairwise comparisons among levels of pH*time

pH = 7, time = 0, subtracted from: pH 7*time 2 and above

Analysis of Variance for C1/2 toxins in the toxin mixture (storage at 25°C), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	4	0.31046	0.31046	0.7762	41.15	0.000
time	5	6.26654	6.26654	1.253318	664.4	0.000
pH*time	20	0.75912	0.75912	0.03976	21.08	0.000
Error	30	0.05659	0.05659	0.00189		
Total	59	7.42871				

Significant pairs ($P \leq 0.05$) chosen after multiple comparison by Bonferroni Simultaneous Test and all pairwise comparisons among levels of pH*time

pH = 3, time = 0, subtracted from: pH 3*time 3 and above
pH = 4, time = 0, subtracted from: pH 4*time 3 and above
pH = 5, time = 0, subtracted from: pH 5*time 3 and above
pH = 6, time = 0, subtracted from: pH 6*time 1 and above
pH = 7, time = 0, subtracted from: pH 7*time 1 and above
NB, time = 0, subtracted from: pH 4.8*time 3 and above

Analysis of Variance for GTX 2/3 toxins in the toxin mixture (storage at 25°C), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	4	0.224631	0.224631	0.056158	23.24	0.000
time	5	0.356891	0.356891	0.070379	29.12	0.000
pH*time	20	0.356891	0.356891	0.015369	6.36	0.000
Error	30	0.072504	0.072504	0.002417		
Total	59	0.956408				

Significant pairs (P≤ 0.05) chosen after multiple comparison by Bonferroni Simultaneous Test and all pairwise comparisons among levels of pH*time

pH = 7, time = 0, subtracted from: pH 7*time 3 and above

Analysis of Variance for GTX 2/3 toxins in the unheated scallop homogenate (storage at -5°C), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	4	1.28598	1.28598	0.32150	40.76	0.000
time	5	7.87488	7.87488	0.1.57498	426.82	0.000
pH*time	20	1.11373	1.11373	0.006216	15.09	0.000
Error	60	0.22140	0.22140	0.00526		
Total	89	10.49599				

Significant pairs (P≤ 0.05) chosen after multiple comparison by Bonferroni Simultaneous Test and all pairwise comparisons among levels of pH*time

pH = 6, time = 0, subtracted from: pH 6*time 12
pH = 7, time = 0, subtracted from: pH 7*time 12

Analysis of Variance for GTX 1/4 toxins in the unheated toxin mixture (storage at 25°C), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	4	0.860208	0.860208	0.215052	40.76	0.000
time	5	0.135845	0.135845	0.0027169	5.16	0.000
pH*time	20	0.124318	0.124318	0.006216	4.72	0.000
Error	30	0.15783	0.15783	0.00526		
Total	59	2.956408				

Significant pairs ($P \leq 0.05$) chosen after multiple comparison by Bonferroni Simultaneous Test and all pairwise comparisons among levels of pH*time
pH = 6, time = 0, subtracted from: pH 4*time 3 and above
pH = 7, time = 0, subtracted from: pH 7*time 3 and above