1

PM-1 3 1/2" x 4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT

PRECISIONSM RESOLUTION TARGETS

Novel Approaches to the Oxidation of Paralytic Shellfish Poisoning Toxins for Analysis by High-Performance Liquid Chromatography

by Barbara Wong

**A thesis submitted to the
Faculty of Graduate Studies and Research
in partial fulfilment of the degree of
Doctor of Philosophy**

Department of Chemistry

**Carleton University
Ottawa, Ontario
July 18, 1996**

**© copyright 1996
Barbara Wong**

National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395, Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Exemple - sans référence

Exemple - avec référence

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-612-19394-2

Name Barbala Wong

Dissertation Abstracts International is arranged by broad, general subject categories. Please select the one subject which most nearly describes the content of your dissertation. Enter the corresponding four-digit code in the spaces provided.

ANALYTICAL CHEMISTRY

SUBJECT TERM

0486

U·M·I

SUBJECT CODE

Subject Categories

THE HUMANITIES AND SOCIAL SCIENCES

COMMUNICATIONS AND THE ARTS

Architecture 0729
Art History 0377
Cinema 0900
Dance 0378
Fine Arts 0357
Information Science 0723
Journalism 0391
Library Science 0399
Mass Communications 0708
Music 0413
Speech Communication 0459
Theater 0465

EDUCATION

General 0515
Administration 0514
Adult and Continuing 0516
Agricultural 0517
Art 0273
Bilingual and Multicultural 0282
Business 0688
Community College 0275
Curriculum and Instruction 0727
Early Childhood 0518
Elementary 0524
Finance 0277
Guidance and Counseling 0519
Health 0680
Higher 0745
History of 0520
Home Economics 0278
Industrial 0521
Language and Literature 0279
Mathematics 0280
Music 0522
Philosophy of 0998
Physical 0523

Psychology 0525
Reading 0535
Religious 0527
Sciences 0714
Secondary 0533
Social Sciences 0534
Sociology of 0340
Special 0529
Teacher Training 0530
Technology 0710
Tests and Measurements 0288
Vocational 0747

LANGUAGE, LITERATURE AND LINGUISTICS

Language 0679
General 0289
Ancient 0290
Linguistics 0291
Modern 0291
Literature 0401
General 0294
Classical 0295
Comparative 0297
Medieval 0298
Modern 0316
African 0591
American 0305
Asian 0352
Canadian (English) 0355
Canadian (French) 0593
English 0311
Germanic 0312
Latin American 0315
Middle Eastern 0313
Romance 0314
Slavic and East European

PHILOSOPHY, RELIGION AND THEOLOGY

Philosophy 0422
Religion 0318
General 0321
Biblical Studies 0319
Clergy 0320
History of 0322
Philosophy of 0469
Theology

SOCIAL SCIENCES

American Studies 0323
Anthropology 0324
Archaeology 0326
Cultural 0327
Physical 0310
Business Administration 0272
General 0770
Accounting 0454
Banking 0338
Management 0385
Marketing 0501
Canadian Studies 0503
Economics 0505
General 0508
Agricultural 0509
Commerce Business 0510
Finance 0511
History 0358
Labor 0366
Theory 0351
Folklore 0366
Geography 0351
Gerontology 0578
History 0578
General

Ancient 0579
Medieval 0581
Modern 0582
Black 0328
African 0331
Asia, Australia and Oceania 0332
Canadian 0334
European 0335
Latin American 0336
Middle Eastern 0331
United States 0331
History of Science 0585
Law 0398
Political Science 0615
General 0616
International Law and 0617
Relations 0814
Public Administration 0452
Recreation 0626
Social Work 0938
Sociology 0631
General 0628
Criminology and Penology 0629
Demography 0630
Ethnic and Racial Studies 0700
Individual and Family 0144
Studies 0709
Industrial and Labor 0455
Relations 0537
Public and Social Welfare 0543
Social Structure and 0544
Development 0545
Theory and Methods 0546
Transportation 0547
Urban and Regional Planning 0548
Women's Studies

THE SCIENCES AND ENGINEERING

BIOLOGICAL SCIENCES

Agriculture 0473
General 0285
Agronomy 0475
Animal Culture and 0476
Nutrition 0359
Animal Pathology 0478
Food Science and 0479
Technology 0480
Forestry and Wildlife 0817
Plant Culture 0777
Plant Pathology 0746
Plant Physiology 0306
Range Management 0287
Wood Technology 0308
Biology 0309
General 0329
Anatomy 0353
Biostatistics 0369
Botany 0793
Cell 0410
Ecology 0307
Entomology 0317
Genetics 0416
Limnology 0433
Microbiology 0821
Molecular 0778
Neuroscience 0472
Oceanography 0786
Physiology 0760
Radiation 0425
Veterinary Science 0996
Zoology

EARTH SCIENCES

Biogeochemistry 0425
Geochemistry 0996

Geodesy 0370
Geology 0372
Geophysics 0373
Hydrology 0388
Mineralogy 0411
Paleobotany 0345
Paleoecology 0426
Paleontology 0418
Paleozoology 0985
Palynology 0427
Physical Geography 0368
Physical Oceanography 0415

HEALTH AND ENVIRONMENTAL SCIENCES

Environmental Sciences 0768
Health Sciences 0566
General 0300
Audiology 0992
Chemotherapy 0567
Dentistry 0350
Education 0769
Hospital Management 0758
Human Development 0982
Immunology 0564
Medicine and Surgery 0347
Mental Health 0569
Nursing 0570
Nutrition 0380
Obstetrics and Gynecology 0354
Occupational Health and 0381
Therapy 0571
Ophthalmology 0419
Pathology 0572
Pharmacology 0382
Pharmacy 0573
Physical Therapy 0574
Public Health 0575
Radiology 0575
Recreation

Speech Pathology 0460
Toxicology 0383
Home Economics 0386

PHYSICAL SCIENCES

Pure Sciences 0485
Chemistry 0749
General 0486
Agricultural 0467
Analytical 0488
Biochemistry 0738
Inorganic 0490
Nuclear 0491
Organic 0494
Pharmaceutical 0495
Physical 0754
Polymer 0405
Radiation 0605
Mathematics 0986
Physics 0606
General 0608
Acoustics 0748
Astronomy and 0607
Astrophysics 0798
Atmospheric Science 0759
Atomic 0609
Electronics and Electricity 0610
Elementary Particles and 0752
High Energy 0756
Fluid and Plasma 0611
Molecular 0463
Nuclear 0346
Optics 0984
Radiation 0984
Solid State 0984
Statistics

Applied Sciences

Applied Mechanics 0346
Computer Science 0984

Engineering 0537
General 0538
Aerospace 0539
Agricultural 0540
Automotive 0541
Biomedical 0542
Chemical 0543
Civil 0544
Electronics and Electrical 0348
Heat and Thermodynamics 0545
Hydraulic 0546
Industrial 0547
Marine 0794
Materials Science 0548
Mechanical 0743
Metallurgy 0551
Mining 0552
Nuclear 0549
Packaging 0765
Petroleum 0554
Sanitary and Municipal 0798
System Science 0428
Geotechnology 0796
Operations Research 0795
Plastics Technology 0994
Textile Technology

PSYCHOLOGY

General 0621
Behavioral 0384
Clinical 0622
Developmental 0620
Experimental 0623
Industrial 0624
Personality 0625
Physiological 0989
Psychobiology 0349
Psychometrics 0632
Social 0451

The undersigned hereby recommend to
the Faculty of Graduate Studies and Research
acceptance of the thesis

Novel Approaches to the Oxidation of Paralytic Shellfish Poisoning Toxins
for Analysis by High-Performance Liquid Chromatography

submitted by
Barbara Wong

in partial fulfilment of the requirements for
the degree of Doctor of Philosophy

Chair, Department of Chemistry

Thesis Supervisor

Thesis Supervisor

External Examiner

Carleton University
August 15, 1996

Abstract

Two new high-performance liquid chromatographic (HPLC) methods were developed for the determination of paralytic shellfish poisoning (PSP) toxins, a group of structurally similar neurotoxins. The methods were based on two different post-column reactions, using either an anode or manganese dioxide to oxidize these toxins to fluorescent purine derivatives which were quantitated using a fluorescence detector. Both oxidative processes involved heterogeneous reactions at a solution-surface interface, which offered the advantage of pumpless reagent delivery.

In studying the post-column electrochemical reactor, the effect of certain reaction conditions on fluorescent product yield was examined. These experimental parameters included voltage, pH, temperature, and mobile phase flow rate. Other experiments were conducted to improve the chromatography of two of the more highly-retained compounds. The products of the electrochemical reaction were identified using a second HPLC method. After the conditions of oxidation and chromatography were optimized, the post-column electrochemical reaction method was applied to analyze shellfish and plankton samples, yielding results which were in agreement with accepted analytical methods.

The manganese dioxide oxidation of PSP toxins was studied in two parts. The first set of experiments involved batch reactions and the analysis of oxidation products. Detailed studies were conducted on reaction conditions, such as temperature, pH, time, amount of oxidant, and concentration of PSP toxin. Rate constants and activation energies were calculated for the manganese dioxide oxidation of two PSP analogues, saxitoxin and neosaxitoxin. A preliminary HPLC study, employing a UV detector to monitor the oxidation products, was conducted.

The information gathered in the batch experiments was used to develop a solid-phase post-column reactor based on oxidation by manganese dioxide. Experiments were conducted to optimize conditions, such as pH, flow rate, reactor dimensions, and manganese dioxide particle size. The contribution of the reactor to peak-broadening was studied, and the stability of the solid-phase reactor was examined. Under the chosen conditions, the post-column manganese dioxide reactor was successfully used to analyze shellfish and plankton samples.

Acknowledgements

As my years of study at Carleton come to an end, there are many people whom I would like to thank. Without them, my academic achievement would have been much more difficult, if not impossible.

To my supervisor, Jim Lawrence, I send a heartfelt thank-you. You have been a wonderful teacher – you always provided just the right amount of guidance while giving me enough room to learn on my own.

I am also grateful to Peter Buist, my co-supervisor and pusher of electrons. Thanks for taking interest in a field entirely different from your own.

A big thank-you and a batch of double chocolate chip cookies go out to Bob Burk, my unofficial second co-supervisor, for all the well-timed and much-needed encouragement and for helping me design my MnO_2 experiments and interpret the results.

For sharing my first experience in scientific research and for being a wonderful role model and friend, I thank Ton VanderNoot. I really miss having you around.

Thanks to Sharon Brewer and Mark O'Neill for being there when things got rough and for nagging me to get my comps out of the way.

Robert Kehle always provided me with the "stuff" I needed and made me laugh. Thanks for being my friend after everybody else had graduated.

My friends at Bodyworks deserve credit for keeping me healthy, mentally and physically. It was wonderful to be able to leave the frustrations of research in the lab at the end of each day.

For the all technical help and for being such terrific company during my long HPLC runs, thanks to my lab-mates and lab-neighbours, past and present: Ray Klassen,

Karen Pepper, Chantal Clroux, and Cathie Mnard. Also, thank-you to all the others at the Food Research Division for making the past four years so enjoyable.

I would like to thank my family for all their love and support throughout the years. My parents were always interested in what I was doing, even though I couldn't always explain it in plain English. And to my sister, Betty, thanks for sharing the undergrad and grad school experience with me. You always understood what I was going through.

And finally, to Mike Porter, my best friend and computer guru, thanks for keeping me on track and for believing in me. There were times when I thought I'd never make it this far, but you always knew I could.

Publications and Presentations

J.F. Lawrence and B. Wong (1996) "Development of a Manganese Dioxide Solid Phase Reactor for Oxidation of Toxins Associated with Paralytic Shellfish Poisoning" accepted for publication in *Journal of Chromatography A*

J.F. Lawrence, B. Wong, and C. Ménard (1996) "The Determination of Decarbamoyl Saxitoxin Analogues in Shellfish Using Prechromatographic Oxidation and HPLC with Fluorescence Detection" accepted for publication in *Journal of the Association of Official Analytical Chemists International*

J.F. Lawrence and B. Wong (1995) "Evaluation of a Post-Column Electrochemical Reactor for Oxidation of Paralytic Shellfish Poison Toxins" in *Journal of the Association of Official Analytical Chemists International*, **78**, 698.

B. Wong and J.F. Lawrence, "Post-Column Solid-Phase Reactor for HPLC Analysis of Paralytic Shellfish Poisoning Toxins," oral presentation at EnviroAnalysis '96: The Biennial International Conference on Chemical Measurement and Monitoring of the Environment, Ottawa, Ontario, May 1996

B. Wong and J.F. Lawrence, "Evaluation of a Post-Column Electrochemical Reactor for Oxidation of Paralytic Shellfish Poison Toxins," presented as a poster at the 24th International Symposium on Environmental Analytical Chemistry, Ottawa, Ontario, May 1994

Table of Contents

Abstract	iii
Acknowledgements	v
Publications and Presentations	vii
Table of Contents	viii
List of Tables	xii
List of Figures	xiii

Chapter 1

Introduction	1
1. Paralytic Shellfish Poisoning: Real World Problems	1
1.1 Outbreaks of PSP	1
1.2 Sources of PSP Toxins	2
1.3 Need for Analytical Methods for PSP Toxins	4
2. Chemical Properties of PSP Toxins	4
2.1 Structures and Chemistry	4
2.1.1 Effects of pH	6
2.1.2 pK_a Values of Saxitoxin and Neosaxitoxin	8
2.1.3 The Ketone-Gemdiol Equilibrium	8
2.1.4 Epimerization of the Hydroxysulfate Group in the GTXs	11
2.2 Mechanism of PSP Toxicity	12
3. Analytical Methods for PSP Toxins	12
3.1 PSP Assays	14
3.1.1 Bioassays	14
3.1.1.1 Mouse Bioassay	14
3.1.1.2 Fly Bioassay	16
3.1.2 Immunoassays	16
3.1.3 Pharmacological Assays	17
3.1.4 Chemical Assays	18
3.2 PSP Analyses	18
3.2.1 Historical Aspects	20
3.2.2 Capillary Electrophoresis	20
3.2.3 HPLC Methods	21

3.2.3.1 Post-column Periodate Reactor	21
3.2.3.2 Pre-chromatographic Oxidation	23
4. Goal of Thesis Research	25
5. Analytical Considerations	25
5.1 Reaction Detection in Liquid Chromatography	26
5.1.1 Pre-chromatographic vs. Post-column	26
5.1.2 Types of Post-column Reactors	27
5.1.2.1. Solution-phase Reactors	27
5.1.2.2 Solid-Phase Reactors	27
5.1.2.3 Electrochemical Reactors	28
5.1.2.4 Photochemical Reactors	29
5.2 Oxidants for Dehydrogenative Aromatization	30
 Chapter 2	
Experimental	31
1. General Description of Materials and Apparatus	31
1.1 Chemicals and Standards	31
1.2 Sample Extracts	31
1.3 HPLC Equipment	32
2. Post-Column Electrochemical Oxidation	32
2.1 Chromatographic Conditions	32
2.2 Electrochemical Reactor	34
2.3 Post-column Pumps for pH Adjustment	34
2.4 Identification of Oxidation Products	35
3. Study of Oxidation Products	35
3.1 HPLC Method	35
3.2 Pre-chromatographic Periodate Reaction	35
4. Batch Reactions with Manganese Dioxide	36
5. Post-Column Oxidation by Manganese Dioxide	36
5.1 Chromatographic Conditions	36
5.2 Manganese Dioxide Reactor	38
 Chapter 3	
Results & Discussion	39
1. Pre- and Post-Column Periodate Oxidation	39

2. Proposed Reaction Pathway for PSP Oxidation	39
3. Electrochemical Reactor	43
3.1 pH Studies	45
3.2 Voltage Studies	47
3.3 Temperature Studies	52
3.4 Study of Reaction Time	54
3.5 Identification of Electrochemical Oxidation Products	56
3.6 Improving the Chromatography of STX and NEO	58
3.6.1 Increasing the Heptanesulfonate Concentration	60
3.6.2 Heating the Column	62
3.7 Evaluation of the Analytical Method	66
3.7.1 Analytical Performance	66
3.7.2 Hydroxylated vs. Non-Hydroxylated Toxins	70
3.7.3 Confirmation of the NEO Peak	72
3.7.4 Application of Method to Real Samples	74
4. Oxidation by Manganese Dioxide	77
4.1 Batch Experiments	78
4.1.1 Preliminary Temperature Studies	78
4.1.2 pH Studies	81
4.1.3 Effect of Organic Solvent on the Reaction Yield	84
4.1.4 Changing the Amount of Oxidant	85
4.1.5 Differences between the STX and NEO Reactions	87
4.1.5.1 Reaction Rates	87
4.1.5.2 Changing the Mass of Manganese Dioxide	87
4.1.5.3 Monitoring of NEO Products by UV Detection	92
4.1.5.4 Changing the Concentration of PSP Standard	94
4.1.5.5 Calculation of Rate Constants & Activation Energies	94
4.1.5.5.1 Calculations for STX	96
4.1.5.5.2 Calculations for NEO	98
4.1.6 Oxidation of Sample Extracts by Manganese Dioxide	101
4.2 Post-Column Experiments	102
4.2.1 Optimization of pH	104
4.2.2 Optimization of Reaction Time	106
4.2.2.1 Changing the Dimensions of the Solid-Phase Reactor	106

4.2.2.2 Adjusting the Mobile Phase Flow Rate	106
4.2.3 Manganese Dioxide Particle Size	109
4.2.3.1 Quantifying the Peak-Broadening Effect	110
4.2.4 Stability of the Solid-Phase Reactor	110
4.2.5 Evaluation of the Analytical Method	114
4.2.5.1 Analytical Performance	114
4.2.5.2 Application of Method to Real Samples	116
5. Future Work	120
 Chapter 4	
Conclusions	122
1. Electrochemical Oxidation	122
2. Batch Reactions with Manganese Dioxide	123
3. Post-Column Manganese Dioxide Reactor	124
Bibliography	126

List of Tables

Table 1.	Net charge of various PSP toxins at different pH values	9
Table 2.	Gradient program used with the post-column electrochemical reactor	33
Table 3.	Gradient programs used with the post-column MnO ₂ reactor	37
Table 4.	Concentrations of PSP standards used to obtain calibration curve for electrochemical oxidation method	69
Table 5.	Estimated limits of detection for PSP toxins and minimum detectable concentrations for electrochemical oxidation method	69
Table 6.	Ratios of limits of detection (LOD) of OH-toxins to that of their non-OH analogues	72
Table 7.	Concentration of PSP toxins found in a mussel extract using post-column electrochemical reactor compared to results obtained using other HPLC methods	74
Table 8.	Data and derived values obtained for STX from Figure 39 used for Arrhenius plot	97
Table 9.	Data and derived values obtained for STX from Figure 42 used for Arrhenius plot	99
Table 10.	Data and derived values obtained for NEO from Figure 38 used for Arrhenius plot	100
Table 11.	Concentrations of PSP standards used to obtain calibration curve for MnO ₂ oxidation method	117
Table 12.	Estimated limits of detection for PSP toxins for post-column MnO ₂ oxidation method	118
Table 13.	Comparison of post-column reaction systems for PSP analysis	118
Table 14.	Concentration of various PSP toxins found in clam sample using MnO ₂ reactor and other HPLC methods	120

List of Figures

Figure 1.	World map showing locations of PSP outbreaks in 1970 and 1990	3
Figure 2.	Structures of PSP analogues	5
Figure 3.	Relative toxicity values for individual PSP analogues	7
Figure 4.	pK_a values for STX and NEO	9
Figure 5.	Ketone-gemdiol equilibrium for STX	10
Figure 6.	Epimerization of the gonyautoxins	11
Figure 7.	Hypothetical saxitoxin-receptor models	13
Figure 8.	Oxidation of STX to a fluorescent purine derivative	19
Figure 9.	Apparatus for the Sullivan post-column periodate reactor	22
Figure 10.	Oxidation products of periodate reaction identified by HPLC-mass spectrometry	24
Figure 11.	Schematic diagram of HPLC system with post-column electrochemical reactor	33
Figure 12.	Schematic diagram of HPLC system with post-column manganese dioxide reactor	37
Figure 13.	Chromatogram of PSP toxin standards obtained using the post-column periodate reaction method	40
Figure 14.	a) Chromatograms obtained for periodate-oxidized PSP standards b) Structures of oxidation products identified by LC-MS	41
Figure 15.	A possible reaction mechanism for oxidation of STX	42
Figure 16.	a) Electrodes in an amperometric detector b) Various configurations of HPLC amperometric detectors	44
Figure 17.	GTX3 peak height as a function of reaction pH for post-column electrochemical oxidation	47
Figure 18.	Peak height as a function of oxidation potential at pH 11	49
Figure 19.	Detector response as a function of oxidation potential for individual toxins at pH 7	50

Figure 20.	Hydrodynamic voltammograms of STX and NEO obtained using HPLC with electrochemical detection	51
Figure 21.	Peak area as a function of reaction temperature for electrochemical oxidation of STX and NEO	53
Figure 22.	GTX3 peak area as a function of effluent flow rate through electrochemical reactor	55
Figure 23.	Forces acting on an analyte molecule as it passes through a channel of the porous carbon electrode	57
Figure 24.	Structures of oxidation products obtained by electrochemical oxidation	59
Figure 25.	a) Retention time as a function of the concentration of ion-pair agent b) Peak width as a function of the concentration of ion-pair agent c) Peak height as a function of the concentration of ion-pair agent	61
Figure 26.	Models for interaction between ion-pair reagent and analyte	63
Figure 27.	a) Peak height and peak area as a function of column temperature b) Retention time as a function of column temperature	64
Figure 28.	Typical chromatograms of mixtures of PSP standards obtained using post-column electrochemical oxidation system	67
Figure 29.	Calibration curves for PSP standards obtained using post-column electrochemical method	68
Figure 30.	a) Proposed pathway for oxidation of NEO to nitrone compound b) Proposed pathway for oxidation of NEO to fluorescent purine	71
Figure 31.	Verification of the NEO peak by changing the oxidation potential	73
Figure 32.	Typical chromatograms of sample extracts containing PSP toxins using post-column electrochemical oxidation system	75
Figure 33.	Products formed from PSP toxins by reaction with manganese dioxide at various temperatures	79
Figure 34.	Chromatogram of products obtained from the oxidation of GTX2/3 by manganese dioxide at 90°C for 20 minutes	80
Figure 35.	pH profiles of reaction products of MnO ₂ oxidation of STX, NEO, and GTX2/3	82
Figure 36.	Chromatogram and chemical structures of products formed in the manganese dioxide oxidation of STX at pH 12	83

Figure 37.	Reaction yield for manganese dioxide oxidation of STX as a function of percent acetonitrile in the reaction mixture	86
Figure 38.	Product yield for manganese dioxide oxidation of STX and NEO as a function of reaction time	88
Figure 39.	Product yield as a function of amount of oxidant for the manganese dioxide oxidation of STX and NEO	89
Figure 40.	Possible complex formed on the surface of MnO_2 particle between tautomer of doubly deprotonated NEO and Mn (IV)	91
Figure 41.	Chromatograms (UV absorbance and fluorescence detection) of products obtained by periodate oxidation of NEO	93
Figure 42.	Reaction yield as a function of concentration of STX and NEO.	95
Figure 43.	Arrhenius plot for reaction of STX with manganese dioxide using data from Table 8	97
Figure 44.	Arrhenius plot for reaction of STX with manganese dioxide using data from Table 9	99
Figure 45.	Arrhenius plot for reaction of NEO with manganese dioxide using data from Table 10	100
Figure 46.	Chromatograms of sample extracts oxidized in batch reactions with manganese dioxide	103
Figure 47.	Detector response for individual PSP toxins as a function of pH using the post-column manganese dioxide reaction system	105
Figure 48.	Combined peak heights of oxidation products of individual PSP toxins as a function of pH for batch reaction by manganese dioxide	105
Figure 49.	Change in response for various PSP toxins as the size of the manganese dioxide reactor is changed	107
Figure 50.	Chromatograms depicting the change in STX peak size and shape with change in reactor volume	108
Figure 51.	Chromatograms of a previously oxidized mixture of GTX2/3 and STX used to quantify the peak-broadening effect of the manganese dioxide reactor	111
Figure 52.	Graph of response for individual PSP toxins as a function of number of days of reactor use	112
Figure 53.	Graph of response for individual PSP toxins as a function of days of reactor use after the periodate rinse	112

Figure 54. Chromatograms of PSP standards obtained using post-column manganese dioxide reactor	115
Figure 55. Calibration curve for post-column MnO₂ reaction system	117
Figure 56. Chromatograms of sample extracts obtained using post-column MnO₂ reaction system	119

Chapter 1

Introduction

1. Paralytic Shellfish Poisoning: Real World Problems

Paralytic shellfish poisoning (PSP) is a potentially fatal illness which occurs when humans consume contaminated shellfish such as mussels, clams, and other filter-feeding mollusks. The potent neurotoxins which cause this ailment originate from dinoflagellates, such as those belonging to the genus *Alexandrium*, which often produce highly coloured blooms, also known as “red tides” [1]. The toxic algae does not appear to harm the shellfish which feed on them, but mollusks which have accumulated PSP toxins pose a health threat to humans who eat them. The symptoms of PSP intoxication range from numbness of the lips and extremities to death due to respiratory paralysis [2]. With cases of PSP being reported all over the world, there is much interest in developing analytical methods for these toxins for monitoring shellfish harvests and for basic research.

1.1 Outbreaks of PSP

The phenomenon of red tides, possibly accompanied by paralytic shellfish poisoning, has been around for many centuries, as indicated by the following biblical reference from the time of Moses (ca. 1500 B.C.): “And all the waters that were in the river were turned to blood” [3]. One of the first documented cases of PSP illness was recorded by Captain George Vancouver in 1793 during his exploration of the Pacific Northwest [4]. Several of his men became sick, and one died after eating shellfish. The native people on the west coast of North America knew of the danger of PSP and stationed guards to watch for red tides that served as indicators of toxic shellfish [5]. During the 1800’s and the early part of this century, stories of PSP were recorded in

ships' logs, passed on by word of mouth, and became part of the lore in areas such as the Fundy coast of Nova Scotia, where PSP outbreaks occurred regularly. The 1927 outbreak of PSP in the San Francisco Bay area, in which over 100 people were seriously ill and six died, led to the beginning of modern PSP research [6, 7].

In recent years, PSP has become a global problem. As indicated in Figure 1, PSP outbreaks affected more than twice as many areas in 1990 as in 1970 [8]. Areas in which PSP incidence is endemic include the coastal regions of North America, western Europe, Japan, and southeast Asia [2, 9-14]. Some experts believe that human activities have caused the spread of PSP-producing species. Coastal pollution provides favourable conditions for the formation of phytoplankton blooms [15], while the shipping practice of transporting and dumping ballast water, which often contains PSP-producing algae in their hardy cystic stage, has facilitated the spread of these species [16]. It may also be argued that the perceived increase in PSP outbreaks is due to a better understanding and more vigilant monitoring, but this explanation alone cannot account for the species spreading and the new bloom formation currently being observed. The exact number of cases of PSP illness is not known, but it is estimated that 1600 cases occur each year, resulting in approximately 300 deaths [2].

1.2 Sources of PSP Toxins

Certain species of marine algae have been pinpointed as sources of PSP toxins, but it is still uncertain whether the toxins are actually produced by these algal species or by bacteria living on or inside them [1]. The biosynthetic pathways of PSP toxins have been studied but are not completely understood [17]. The toxins are not proteins and are believed to be synthesized in a series of steps requiring multiple genes. Researchers do not know why these toxins are produced, but some believe that the toxins serve as protection against grazing by predators such as zooplankton [8]. The study of the

Figure 1. World map showing locations of PSP outbreaks in 1970 and 1990 [8]

biosynthesis of PSP toxins is an active area of research and requires sensitive analytical methods which are capable of determining low levels of toxins in algae samples.

1.3 Need for Analytical Methods for PSP Toxins

It is difficult to predict the formation of toxic blooms, but they usually occur between the months of May and October in the Northern Hemisphere [3]. The appearance of these blooms does not necessarily lead to toxic shellfish, nor are contaminated shellfish always preceded by a visible bloom. Thus, it is not sufficient to monitor the water for toxic phytoplankton as an indicator for PSP-contaminated shellfish. In order to protect public health and to maintain the viability of the fishing industry, many countries routinely monitor shellfish harvests for PSP toxins. Analytical methods must be capable of determining sub-ppm concentrations of the toxins in shellfish tissue. The maximum allowable limit in Canada and many other countries is currently 80 μg of saxitoxin or "saxitoxin-equivalents" (a measure of relative toxicity of the analogues in the PSP family) in 100 g of shellfish tissue. These low detection limits, combined with complex sample matrices, pose a challenge to chemists in this field.

2. Chemical Properties of PSP Toxins

2.1 Structures and Chemistry

The family of compounds responsible for PSP is based on a perhydropurine skeleton with an additional five-membered ring fused at an angular position. As shown in Figure 2, various toxins are formed when hydroxyl, hydroxysulfate, and other functional groups are substituted for specific hydrogens in the parent compound, saxitoxin (STX). This substitution leads to compounds with very different chemical properties and toxicities than those of STX. The toxicity of the various analogues has been measured by intraperitoneal injection into mice. The relative toxicities of the PSP

R ₁	R ₂	R ₃	Carbamate Toxins	Sulfocarbamoyl Toxins	Decarbamoyl Toxins
H	H	H	STX	B1	dc-STX
OH	H	H	NEO	B2	dc-NEO
OH	H	OSO ₃ ⁻	GTX1	C3	dc-GTX1
H	H	OSO ₃ ⁻	GTX2	C1	dc-GTX2
H	OSO ₃ ⁻	H	GTX3	C2	dc-GTX3
OH	OSO ₃ ⁻	H	GTX4	C4	dc-GTX4

R₄:

Figure 2. Structures of PSP analogues. STX = saxitoxin, NEO = neosaxitoxin, GTX = gonyautoxin, dc = decarbamoyl

analogues are presented in Figure 3 [18]. Toxicity is measured in mouse units, defined as the amount of toxin required to kill a 20-gram mouse in 15 minutes. The chemistry and biochemistry of PSP toxins have been the subject of recent reviews [1, 19, 20].

2.1.1 Effects of pH

PSP toxins, particularly STX, are stable at mildly acidic and neutral pH. This has been demonstrated by an acidified STX standard which has been stored in our laboratory refrigerator for decades. The concentration of this standard has not changed over the years, and no degradation products have been detected. In the pH range of 3-7, the toxins can withstand high temperatures (e.g. boiling water used in cooking shellfish) and are routinely extracted from shellfish samples by boiling homogenized tissue in 0.1 M HCl [21].

At $\text{pH} < 2$, however, the C-21 sulfo group is hydrolyzed from the sulfocarbamate toxins to yield their carbamate analogues, a reaction which is accelerated by heat. There is concern that this hydrolysis might occur in the human stomach. The relatively non-toxic B1 might be converted to STX, its much more toxic carbamate counterpart, rendering “safe” shellfish toxic after consumption. Fortunately, experiments conducted using artificial and intact rat gastric juice and on rats given oral doses of B1 indicate that this hydrolysis would not occur to any significant extent in the stomach [22]. Another reaction which occurs at low pH is the cleavage of the carbamate group. When STX is treated with concentrated HCl, the decarbamoyl (dc-) analogue is produced [23].

At $\text{pH} > 7$, PSP toxins will degrade and lose their characteristic toxicity, probably due to their susceptibility to autoxidation under these conditions [18]. This oxidative reaction is employed in analytical methods (which will be discussed later) and is made possible by the deprotonation of the electron-withdrawing guanidinium groups at higher pH levels.

Figure 3. Relative toxicity values for individual PSP analogues. The unit of measurement is the mouse unit (MU) which is defined as the amount of toxin required to kill a 20-gram mouse in 15 minutes [18].

2.1.2 pK_a Values of Saxitoxin and Neosaxitoxin

Of all the toxins in the PSP family, STX and its N-1 hydroxy analogue, neosaxitoxin (NEO), have been studied the most thoroughly. The pK_a values for STX were found, by titration, to be 8.22 and 11.28 as illustrated in Figure 4a [24]. The first deprotonation, which occurs at an abnormally low pH for a guanidinium group, has been attributed to the C-8-centered group based on ¹H- and ¹³C-NMR measurements [19, 24]. This is due to insufficient participation of N-9 in the guanidinium resonance, which is probably caused by the stereochemical strain of introducing a double bond into the five-membered ring [18]. The substitution of a hydroxyl group for the hydrogen at the N-1 position leads to dramatically different chemical properties. Titrations of NEO reveal proton dissociations at 6.75 and 8.65 (see Figure 4b) [25]. The lower pK_a value is attributed to the guanidinium group centered on C-2 because the electron-withdrawing N-1 hydroxyl group destabilizes the positively-charged guanidinium group. The deprotonation of the C-8-centered group occurs at about the same pH as for STX. It is expected that the NEO molecule would have a net positive charge of approximately half of that of STX at physiological pH. This has been confirmed by an electrophoretic study [26]. Net charges of PSP analogues at various pH values is given in Table 1 [18].

2.1.3 The Ketone-Gemdiol Equilibrium

The functional group at C-12, which is common to all PSP toxins, is a hydrated ketone, a carbonyl group with which a water molecule has formed a reversible covalent bond (Figure 5). This gemdiol, C(OH)₂, is stabilized by the two electron-withdrawing guanidinium groups in the molecule and is in equilibrium with its ketone tautomer. NMR studies of STX have shown that the ketone-to-gemdiol ratio increases as the pH increases and that only deprotonated molecules are able to take the keto form in solution [27]. The ketone has the ability to enolize which allows the protons at C-11 to

Figure 4. pK_a values for (a) STX and (b) NEO. These values are lower than the pK_a of typical guanidinium groups (e.g. pK_a of arginine is 12.48).

Table 1. Net charge of various PSP toxins at different pH values (adapted from reference 18).

Toxin	Net Charge		
	pH < 6	pH 7-8 ^a	pH > 9 ^b
STX	+2	+2	+1
NEO	+2	+1	0
GTX1/4	+1	0	-1
GTX2/3	+1	+1	0
C1/2	0	0	-1
C3/4	0	-1	-2
B1	+1	+1	0
B2	+1	0	-1
dc-STX	+2	+2	+1
dc-NEO	+2	+1	0
dc-GTX 1/4	+1	0	-1
dc-GTX 2/3	+1	+1	0

^a Change in charge due to deprotonation of either N-13 [19] or N-1 hydroxyl [20]

^b Change in charge due to deprotonation of C-8 guanidinium group

Figure 5. Ketone-gemdiol equilibrium for STX

be exchanged rapidly and explains the epimerization of the gonyautoxins (GTXs) described below.

2.1.4 Epimerization of the Hydroxysulfate Group in the GTXs

The GTXs always occur in epimer pairs. GTX 2 and 3 (written 2/3) are alpha and beta forms, respectively, of the C-11 hydroxysulfate analogues of STX, while GTX1/4 are the N-1 hydroxylated equivalents of GTX2/3 (see Figure 6). The equilibrium ratio of GTX2 to GTX3 is about 7:3 at neutral or higher pH, but the thermodynamically less stable GTX3 is actually the original form existing in some live specimens [19]. In preparing certified standard solutions of GTX2/3 and GTX1/4, the two solutions of the GTX toxins are incubated in a tris-HCl buffer (pH 7.5) for 2 h at 70°C to yield a molar ratio of 4.0 for GTX2/GTX3 and 2.4 for GTX1/GTX4 [28]. These values were found to be constant for at least three months when sealed ampoules of standard solutions were stored at 4°C.

Figure 6. Epimerization of the gonyautoxins

2.2 Mechanism of PSP Toxicity

The PSP family effects its toxicity at the cellular level by selectively blocking Na^+ channels in the excitable membrane [29]. When an occluder, such as a PSP toxin, binds to the Na^+ channel, the cell is unable to maintain the proper potential across its membrane and, thus, cannot perform its normal functions. Several models of toxin-receptor interaction are illustrated in Figure 7 [19]. These models take into consideration that another highly-specific Na^+ channel blocker, tetrodotoxin (TTX), contains functional groups similar to those found in PSP toxins. The binding is believed to occur through the $=\text{NH}_2^+$ (or $=\text{NH}$) and the $-\text{OH}$ functional groups. Both STX and TTX have become invaluable tools as Na^+ channel blockers in pharmacology and will likely be very important in trying to understand the mechanism of the excitable membrane itself.

3. Analytical Methods for PSP Toxins

For convenience, analytical methods for PSP toxins can be separated into two classes – assays and analyses [18]. Assays are those methods which provide a single response to the sample. Analyses, on the other hand, involve a separation step prior to quantitation; that is, the compounds of interest in the sample are resolved, and a response is obtained separately for each.

In order to interpret the results from an analytical method, it is usually necessary to multiply the response by a response factor to obtain a more meaningful number. For example, the signal from a fluorescence detector would have to be multiplied by a concentration response factor (f_c) to get the concentration of a particular toxin. (This would be done individually for each component by constructing a calibration curve.) To calculate the toxicity of a sample component, the response would be multiplied by a toxicity response factor, f_t . Each analogue will have different f_c and f_t values, so each

Figure 7. Hypothetical saxitoxin-receptor models [19]

component must be treated separately. With assay methods, only a single result is obtained, so only one response factor can be used. Selecting a single response factor, f_t , which reflects the wide range of toxicities of the components which may or may not be present in the sample is often the major limitation of assays. Finally, since assays do not resolve sample components, they are not generally useful for research purposes (e.g. studying the metabolism of PSP toxins in shellfish species).

3.1 PSP Assays

3.1.1 Bioassays

The use of live organisms in assays generally provides a good correlation between assay response and sample toxicity. In order for assay results to be valid, the test organism must respond in a similar manner as humans to each of the toxins. The method of sample introduction into the test organism must be considered as well. For example, the use of intraperitoneal injections does not account for the processes which occur in the gastrointestinal tract and may distort the actual toxicity of the sample [30].

3.1.1.1 Mouse Bioassay

The mouse bioassay is by far the most widely used procedure in PSP monitoring programs. Sommer and Meyer conducted the first experiments in this area in the 1930's [31]. This work revealed the nature of PSP and determined the characteristics of the mouse bioassay. The procedure was later standardized and subjected to an inter-laboratory study by the Association of Official Analytical Chemists (AOAC). The mouse bioassay was approved as an official method and is still the only procedure recognized by the AOAC [21].

In brief, the mouse bioassay involves the extraction of homogenized shellfish tissue by boiling with 0.1 N HCl and the intraperitoneal injection of this extract into

each of three standardized (same strain and body weight) mice. The time between injection and death is measured and then converted to toxicity equivalents. A limitation of this method is the narrow range over which the results are quantitative. Sample extracts must be diluted, at times by trial and error, until a death time between 5 and 7 minutes is achieved. At times above or below this range, there is substantial variation in the results. The useful range of death times corresponds to an extract concentration for STX of between 0.26 and 0.36 ppm. Because testing must be repeated until a proper death time is achieved, ten or more mice may be used for one shellfish sample, especially if the toxicity is high.

Other problems with the mouse bioassay include high variability ($\pm 20\%$) from the true value, interference by either high salt concentration in the extract [32] or excessive zinc in the shellfish tissue [33], maintaining a sufficient supply of mice of similar size and strain, and growing public opposition to the use of mammals in bioassays. As well, the mouse test, which measures total sample toxicity, is not useful for research purposes since it does not indicate which PSP toxins were present in the sample. These difficulties have led to the search for a replacement for the mouse bioassay.

Despite the numerous drawbacks to this method, it is the benchmark against which all new analytical techniques are measured [30]. When there is poor correlation between the mouse bioassay and a new method, it is often assumed that the problem lies with the newer technique when the bioassay may actually be producing erroneous results. The inaccuracies of both procedures must be considered when examining results from comparative studies.

3.1.1.2 Fly Bioassay

The fly bioassay [34, 35] is similar to the mouse test, except that the common housefly replaces the mouse as the test organism. This method was designed primarily to reduce the costs associated with the mouse bioassay and to address the public's concerns over the killing of mice in the original procedure. Results indicate that the fly bioassay is slightly more sensitive than the mouse test, is not affected by high salt concentrations, and is much less expensive in terms of supplies. There are, however, some drawbacks to the procedure including a limited dynamic range, which leads to a trial-and-error dilution step. As well, the logistics of injecting such small organisms may limit the use of the fly bioassay.

3.1.2 Immunoassays

Immunochemical tests are ideal for on-site testing of shellfish for PSP toxins because they are sensitive, fast, and simple to use. Different approaches to immunoassay design, including enzyme-linked immunosorbent assay and radioimmunoassay, have been reviewed [18, 23, 30].

When an immunoassay is developed based on antibodies for a specific PSP toxin, it usually shows great sensitivity for that one toxin, but cross-reactivity of the antiserum with other PSP analogues is usually low. This effect has hindered the development of an immunoassay which gives a response consistently proportional to the toxicity of shellfish extracts. As well, immunochemical response factors of the toxins are not related to their primary biological activity or to the response factor in a bioassay. Thus, it may be difficult to convert immunoassay results into meaningful total toxicity measures [18]. As long as there is potential for underestimation of toxicity, immunoassay results cannot be used for monitoring purposes without a second method (e.g. mouse bioassay) to confirm those results.

Despite these problems, immunoassays are useful for pre-screening purposes, and research in this field remains active [36-38]. If antibodies could be developed for all the PSP analogues, then it may be possible to design a more accurate immunochemical test for PSP toxicity. Until now, one of the major obstacles in this field has been the lack of commercially available standards. Perhaps the recent introduction of certified standards of several PSP toxins by the Institute of Marine Biosciences of the National Research Council of Canada will facilitate more research in this area.

3.1.3 Pharmacological Assays

The pharmacological properties of PSP toxins have been used to develop several assay methods. The toxins bind to sodium channels in nerve membranes so that sodium cannot be transported into affected cells, causing membranes to become depolarized. Quantitation of the occurrence of one of these events in an assay provides a direct measure of sample toxicity. The correlation between assay results and human oral potency is excellent since Na^+ channel binding is, in fact, the mechanism of toxic action of PSP in the human body.

Several different approaches have been taken to pharmacological assays. A recently reported method employed microtiter plates coated with Na^+ channels in a competitive binding assay [39]. The procedure measures the displacement of ^3H -labelled STX by toxins in the sample extract. Another assay involves comparing the excitatory post-synaptic potential of nerve-muscle tissue from lobster before and after incubation with PSP-containing solution [40]. The decrease in potential is proportional to the level of PSP toxicity, presumably due to blockage of sodium channel conductance. Mouse neuroblastoma cell cultures were used to develop a PSP assay in which the addition of STX standards to the cultures prevented or limited the swelling and lysis of the cells after addition of ouabain and veratridine [41]. This approach

appears promising as a replacement for the mouse bioassay and is currently under review by the AOAC International [42]. The actual binding mechanism of PSP toxins to Na^+ channels was studied by placing voltage clamps across individual cells [43]. Although no quantitative work has been done in this area, single cell assays will likely be developed in the future.

3.1.4 Chemical Assays

Early PSP research included several colorimetric assays involving derivatization with either picric acid [44] or 2,3-butanedione [45]. These techniques suffered from matrix interferences and poor sensitivity. Bates and Rapoport developed another chemical assay [46, 47] based on the degradation of STX with hydrogen peroxide to a fluorescent pyrimidopurine. This reaction, shown in Figure 8, was first observed by Wong and coworkers [48] while working on the structural determination of STX. Although this assay suffered from problems such as recovery of only STX and NEO from samples and poor sensitivity for NEO, the oxidation of PSP toxins to fluorescent purines is the basis of most chromatographic methods used today.

3.2 PSP Analyses

The main differences between the assay methods in the previous section and the analytical methods in this section are: a) a separation step which allows each toxin to be quantitated individually, and b) the modern instrumentation employed for separation and detection. The choice of analytical methods is somewhat limited by the properties of the toxins. Because they are non-volatile and do not absorb strongly in the UV or visible range, common analytical methods such as gas chromatography and HPLC with UV/VIS detection cannot be used.

Figure 8. Oxidation of STX to a fluorescent purine derivative [46]

3.2.1 Historical Aspects

Early work in the area of PSP separation has been reviewed [18] and includes electrophoresis, thin-layer chromatography (TLC), and column chromatography. In the electrophoretic and TLC methods, visualization of the toxins was effected by reaction with alkaline peroxide solution and fluorescence detection. Ion-exchange column chromatography of PSP toxins was performed only as a clean-up step for the mouse bioassay. Fractions collected from porous polymer-packed columns were analyzed by the peroxide-fluorescence detection method. The information obtained from these early investigations would prove useful in the development of future HPLC methods.

3.2.2 Capillary Electrophoresis (CE)

With the advent of modern analytical instruments, more sophisticated separations could be achieved. A capillary electrophoretic method for the determination of STX has been reported [49]. The toxin was first derivatized using a fluorescent tag, o-phthaldialdehyde (OPA), and was detected by laser-induced fluorescence. Although extremely low mass detection limits were achieved, the method could not be applied to shellfish samples because the derivatization step, which is not selective for PSP toxins, would lead to interferences due to reaction of OPA with other primary amines in the sample matrix. The chemically unstable nature of OPA derivatives and the formation of multiple derivatives were other serious drawbacks to this method.

Thibault and coworkers reported a second CE method in which the underivatized toxins were separated and then detected by a high-sensitivity UV detector at 200 nm or by ion-spray mass spectrometry [50]. The method was used to determine STX and NEO in plankton and scallop extracts, and detection limits of ~20 pg (μ M range, due to extremely small injected volumes) were obtained. It is believed that sensitivity would be enhanced by combining the oxidation-fluorescence detection scheme with CE.

3.2.3 HPLC Methods

High-performance liquid chromatography (HPLC) is the most often used instrument-based technique for PSP analysis. Some earlier HPLC work included detection by post-column derivatization with OPA and fluorescent detection [51], but background interference was too high due to reaction of sample matrix components with OPA. Direct electrochemical detection has been used, but the sensitivity was found to be too poor for practical purposes [52]. The most commonly used detection scheme is based on oxidation by periodate (or peroxide) followed by fluorescence detection. The derivatization can be done either prior to the separation step or after the HPLC column using an on-line reactor. In the past, the main factor which prevented wide use of HPLC methods was the lack of commercially available PSP standards. Since certified standard solutions of the toxins are now available, the emphasis of PSP monitoring programs may shift from bioassays to instrumental analysis, especially since HPLC methods would likely be more economical for larger monitoring programs [53].

3.2.3.1 Post-column Periodate Reactor

The HPLC method for PSP toxins developed by Sullivan and coworkers is probably the most popular [54-56]. It involves the separation of the analogues by ion-pair chromatography, using heptanesulfonate as the ion-pair agent and a styrene-divinylbenzene polymer column. The separation is carried out at pH 7 so that all the carbamate toxins and most of the sulfocarbamates can be separated according to their formal positive charges (see Table 1, page 9). Due to their neutral or anionic nature, the C toxins are not retained and thus are not separated. As shown in Figure 9, the alkaline-buffered periodate reagent is introduced prior to the heated reaction coil, and dilute nitric acid is used to lower the pH to 7 before the fluorescence detector. The Sullivan method has the required sensitivity, selectivity, and reproducibility for monitoring and research

purposes, but it does suffer from some drawbacks from a practical point of view. The time required to assemble and dismantle the post-column reactor may limit its use, especially for analysis on an occasional basis. The reagent pump is prone to blockage due to precipitation of reagent and buffer salts, and the rinse procedure is time-consuming. Minor fluctuations in the reagent flow can affect the response noticeably.

Other post-column reaction procedures, similar to the Sullivan method, have been reported [57, 58]. These methods use three different sets of isocratic chromatographic conditions to separate the toxins in three groups: the STX group with a net charge of +2; the GTX group with a net charge of +1; and the neutral and anionic C toxins. Heptane- or octanesulfonate is used as the ion-pairing agent for the first two groups, and the tetrabutylammonium ion is used for the latter separation. One advantage of these methods over the original one is that the carbamate toxins can be separated from their decarbamoyl analogues. The use of a silica-based C-8 or C-18 column leads to more efficient chromatography as well. The major drawback to this approach is that few laboratories have three HPLC systems that can be committed to PSP analysis. This work does, however, represent an important accomplishment in PSP research since it allows for the separation of all the toxin analogues.

Figure 9. Apparatus for the Sullivan post-column periodate reactor [56]

3.2.3.2 Pre-chromatographic Oxidation

Lawrence and coworkers [59-62] reported another approach to the oxidation-fluorescence detection scheme in which the reaction is carried out in a small vial prior to chromatographic separation of the products. The reaction is performed at room temperature using either peroxide or periodate (in an alkaline buffer) as the oxidant and requires only several minutes to go to completion. Addition of acetic acid stops the reaction, after which the solution is ready for analysis. This method is more sensitive than the post-column approach because the reaction is allowed to go to completion. As well, the use of reverse-phase chromatography without ion-pairing agents offers more efficient and reproducible separation. Disadvantages to pre-chromatographic oxidation are: a) some of the analogues form the same derivatives (e.g. B2 and NEO) and thus are not separable by the method; and b) some the toxins react to form more than one product (multiple peaks on the chromatogram) which may complicate the quantitation process. Because of these drawbacks, the method may not be useful for research aimed at understanding the distribution of the analogues. For monitoring purposes, however, solid-phase extraction could be used to remove the relatively non-toxic sulfo carbamates from the more toxic analogues before the oxidation step.

Other aspects of the pre-chromatographic reaction method have been investigated. The products of periodate oxidation have been identified by HPLC-mass spectrometry [63] and are shown in Figure 10. These products are similar in structure to that identified by Bates and Rapoport [46]. The pre-chromatographic reaction method has also been automated using an HPLC autosampler for the oxidation step [64]. Automation made the method less labour-intensive and improved precision by better controlling reagent volumes and reaction times. In the same study, the authors investigated the use of a microbore column (1.0 mm i.d.) instead of a standard HPLC column (4.6 mm i.d.). It was found that detection limits could be greatly improved by

Products A, B, C

Product D

Products	R ₄	R _{2/3}	Precursors
A	H	H	STX, NEO, (B1), B2
B	H	SO ₃ H	GTX1-4, C1-4
C	SO ₃ H	H	B1, (B2)
D	—	—	dc-STX, NEO, B2, (STX), (B1)

() indicates minor product of periodate reaction

Figure 10. Oxidation products of periodate reaction identified by HPLC-mass spectrometry [63]

on-column trace enrichment. In this process, a large volume of sample dissolved in a solvent of low elutropic strength was injected, and the highly retained analyte became concentrated at the head of the column. A very high mass sensitivity was achieved, and the method was applied to analyze phytoplankton samples of fewer than 100 cells. Finally, chiral chromatography has been used to separate the oxidation products of GTX2/3 [61]. Unfortunately, the sensitivity of this method was poor due to the substantial background signal generated by the chiral reagent in the mobile phase. The development of new chiral stationary phases and mobile phase additives may lead to more research in this area.

4. Goal of Thesis Research

The aim of the research presented in this document was to investigate alternative methods of post-column PSP oxidation in order to overcome some of the difficulties encountered with the Sullivan method [56]. The hypothesis was that the oxidation of PSP toxins could be carried out using different reagents to generate the same fluorescent products generated by periodate. Choosing a different oxidant would allow the method of reagent delivery to be modified as well. Factors which had to be considered in developing a new analytical method were sensitivity, selectivity, ruggedness, and ease of use. As well, the fundamental chemistry behind PSP oxidation was to be examined in order to gain a better understanding of the reaction and to use this knowledge to optimize the reaction conditions within the analytical application.

5. Analytical Considerations

Background information in two key areas, along with knowledge of the chemistry PSP toxins (see Section 2 of this chapter), was required to design a novel reactor. A general knowledge of post-column derivatizations in HPLC facilitated the

choice of hardware configurations, and survey of the organic literature provided some insight as to what types of oxidants might work for this specific reaction. Only after reviewing these subject areas could a good analytical approach be designed.

5.1 Reaction Detection in Liquid Chromatography

Since there is no sensitive “universal” detector for HPLC, many analytes require special detection schemes which changes the analyte chemically to make it more readily detectable by conventional detectors. The wide range of approaches to derivatization in HPLC has been the subject of several recent books and chapters [65-67]. There are two different approaches to derivatization in chromatography, as had already been discussed earlier in this chapter: pre-chromatographic and post-column. Since there are advantages and drawbacks to both, the choice between the two is based on the requirements of the specific application.

5.1.1 Pre-chromatographic vs. Post-column

With pre-chromatographic derivatization, there are fewer restrictions on the reaction kinetics; reaction conditions such as time and pH can be easily manipulated; the reaction solvent does not have to be compatible with that of the HPLC; and excess reagent or undesirable side-products can be either be removed before injection or separated from the analytes on the analytical column. Disadvantages include the formation of side-products or degradation products which may interfere with the chromatography or the reproducibility of the reaction; the need for each sample to be handled individually; and the difficulty of separating products which may be more structurally similar after derivatization.

A post-column reaction does not need to proceed to completion, nor is it required to yield a single product. It is only necessary that the reaction be reproducible. Use of a

post-column system minimizes sample preparation and improves accuracy and reproducibility due to automation of the derivatization. (However, similar benefits may be attained for pre-chromatographic reactions by using an autosampler to perform the reactions.) Disadvantages of post-column derivatization include restricted control of reaction conditions, band broadening due to the volume of the reactor, and the need to change the HPLC instrumentation and mobile phases to accommodate the post-chromatographic reaction.

5.1.2 Types of Post-column Reactors

5.1.2.1. Solution-phase Reactors

The most common reactions employ solution phase chemistry and require a post-column pump to introduce the reagent and a mixing device (e.g. zero-volume T) to mix the effluent and the reagent. Sufficient time must be allowed for the reaction to occur, so a large void volume is usually added between the column and the detector. This may lead to band-broadening and thus poor peak separation unless measures, such as using coiled or knitted tubing to change the flow geometry, are taken decrease this effect [65]. As well, addition of post-column reagents dilute the sample stream, so the flow of reagents should be kept to a minimum.

5.1.2.2 Solid-Phase Reactors

Solid-phase reactor beds have been used for post-column reactions in HPLC. The reactor has a geometry similar to an HPLC column, and the reagent is usually either sorbed or bonded to a solid support. A solid reagent can be used, either alone or physically mixed with another material for support. The reaction occurs as the analyte plug contacts the reagent as it is swept through the reactor. The reactor can be placed either before or after the column.

Advantages of this approach over homogenous reactions include minimal band-broadening due to extra-column void volume (provided the reactor is packed properly) and decreased baseline noise due to pumpless delivery of reagent. As well, the integrity of the effluent is maintained since the reagent never leaves the reactor. However, certain points must be considered when using solid-phase reactors. The packing material must be compatible with the mobile phase, and any by-products of the reaction should not precipitate or be gaseous. Reaction products should not be retained to any significant extent by the packing material, and the reactor should have a long lifetime to reduce system down-time and cost of replacement parts.

5.1. 3 Electrochemical Reactors

Electrochemical detection is often used to monitor analytes which have an oxidizable or reducible moiety, but there have been some applications in which an electrochemical cell was used to convert the analyte to a more detectable species. This approach to derivatization is "reagentless" and thus pumpless, so there is no dilution of the effluent and no added baseline noise due to post-column pumping of reagents. An important point to consider is the compatibility of the mobile phase with the reactor (i.e. possible oxidation or reduction of mobile phase components). As well, electrochemical reactions are very specific and thus are suitable for relatively few classes of analytes.

Examples of electrochemical derivatization in a recent review [66] include the oxidation of catecholamines to strongly fluorescent products and the reduction of K vitamins to fluorescent compounds. More recently (approximately one year after the research presented in this document had begun), an investigation of a preliminary nature showed that on-line anodic oxidation of PSP toxins to fluorescent compounds was possible [68].

5.1.2.4 Photochemical Reactors

For many years, photochemistry has been studied by synthetic organic chemists, physical chemists, and others interested in the interaction of light with matter, but it was not until the mid-1970's when analytical chemists began to appreciate the potential of photochemical reactions for on-line derivatization. The products of photochemical reactions usually have the same or fewer chromophores as the starting material, which would render the products less detectable by UV-VIS than the parent compounds. This is not always the case. Some types of photochemical reactions (e.g. tautomerization, substitution, oxidation) have been used to form derivatives for UV-VIS or fluorescence detection [65]. Photochemical derivatizations have the advantage (or disadvantage) of being very selective and can be applied to relatively few analytes.

There are two configurations that have been used for on-line photochemical derivatization: flow-through and wrap-around. In the first design, the eluent passes through a flow cell with a small internal volume, similar to those used in UV-VIS detectors. The transparent windows in the cell allow the eluent to be exposed to UV radiation from an external lamp. A well-designed flow-through system will maximize irradiation of the analyte in the shortest time possible. The second set-up consists of a length of narrow-bore Teflon tubing wrapped around an elongated lamp. This arrangement introduces a significantly higher dead volume to the system, but the band-broadening effects can be minimized by knitting or weaving the tubing [65]. Wrap-around reactors generally provide more intense irradiation for greater lengths of time. For each derivatization, the reaction parameters (e.g. exposure time and intensity of light) must be optimized to attain maximum yield of the desired photoproduct but to minimize subsequent photolytic destruction of the product.

5.2 Oxidants for Dehydrogenative Aromatization

The wide range of oxidants used in organic reactions has been reviewed [69]. Some of these include air, oxygen, ozone, hydrogen peroxide, organic peroxy acids, and various inorganic substances. Besides the broad spectrum of reagents available, the types of oxidations which can be effected by these are many. The specific type of oxidation involved in the Bates and Rapoport oxidation [46] is dehydrogenative aromatization. Compounds containing six-membered rings are usually oxidized all the way to aromatic compounds because of the resonance energy liberated in the process: 36 kcal for benzene and 61 kcal for naphthalene [69]. This type of reaction is most likely to occur if there is already one double bond in the ring, but the tendency to aromatize is so strong that even elimination at a quaternary carbon centre will occur. Six-membered nitrogen-containing rings that already contain one or two double bonds in the ring are easily dehydrogenated to aromatic heterocyclic compounds by nitric acid, arsenic oxide, manganese dioxide, potassium ferricyanide, and others. Aromatization of cyclic compounds can also be effected by anodic oxidation [70].

Chapter 2

Experimental

1. General Description of Materials and Apparatus

Some aspects of the Experimental apply to all the experiments performed. The same HPLC equipment was used throughout, and the same, previously-quantitated sample extracts were chosen for analysis. The procedural details which were common for all the studies (electrochemical and manganese dioxide) are described in this section.

1.1 Chemicals and Standards

All solvents and reagents were analytical or LC grade materials, and deionized water (Milli-Q Water System, Millipore) was used. Certified standard solutions of the following PSP toxins were purchased from the Institute of Marine Biosciences of the National Research Council of Canada: STX, NEO, and GTX2/3. The GTX solution contained both epimers in a thermodynamically stable ratio. As well, a standard solution containing GTX1-4 and B1 was received as a gift from Dr. Y. Oshima, Tohoku University, Japan, and non-quantitative solutions of purified B1 and B2 toxins were received as gifts from Dr. S. Hall, Food and Drug Administration, Washington, DC. The standards were stored in a refrigerator at 4°C when not in use.

1.2 Sample Extracts

The shellfish extracts chosen for analysis had been prepared and analyzed as part of a previous study. The extraction and cleanup of shellfish samples were performed using the procedure for the mouse bioassay [21]. Briefly, 10 g of homogenized shellfish tissue were boiled with 10 mL 0.1 N HCl for 5 minutes. The mixture was cooled and centrifuged. A 1-mL aliquot of the supernatant was passed through a C-18 solid phase

extraction cartridge and the effluent collected. Following this, 2 mL water were added and the effluent combined with the first. An aliquot of this solution was diluted as required for analysis. The dilution factor was 7-10 times for samples contaminated near the regulatory guideline level of 0.8 $\mu\text{g/g}$ PSP toxins.

A plankton extract which contained mostly GTX3 with traces of GTX2 and STX was received as a gift from Dr. P. Wangersky, formerly of Dalhousie University, Halifax, NS, and was used for qualitative purposes only.

1.3 HPLC Equipment

The HPLC system (Beckman) consisted of two solvent pumps (model 110B), a gradient controller (model 431A), and an injection port equipped with a 50- μL loop. A dual monochromator fluorescence detector (Jasco 820-FP), set at λ_{ex} 330 nm and λ_{em} 400 nm, was used to monitor the effluent. Chromatograms were acquired using an integrator (BioRad 3392A), and peak heights were used for quantitation purposes, except where noted (e.g. when two flow rates were being compared). All sample and standard solutions were injected at least twice, and the average peak height was reported.

2. Post-Column Electrochemical Oxidation

2.1 Chromatographic Conditions

The HPLC system was assembled as shown in Figure 11. Using conditions similar to those outlined in the Sullivan post-column periodate method [56], ion-pair chromatography was used to separate the toxins. The analytical column was a Hamilton PRP-1 (15 cm x 4.1 mm id, 10 μm) which was heated to 45°C by a column heater (BioRad). The mobile phase composition and gradient program are given in Table 2. Mobile phases were degassed by filtration and sonication and refrigerated when not in use. Fresh mobile phase was prepared from stock solutions at least every second day.

Figure 11. Schematic diagram of HPLC system with post-column electrochemical oxidation. The post-column buffer and acid pumps were only used to study basic reaction pH levels and were removed when pH 7 was chosen.

Table 2. Gradient program used with the post-column electrochemical reactor.

Mobile Phases	<p>A 3.0 mM heptanesulfonate 1.5 mM ammonium phosphate, pH 7 1% (v/v) acetonitrile</p> <p>B 3.0 mM heptanesulfonate 6.25 mM ammonium phosphate, pH 7 25% (v/v) acetonitrile</p>
Gradient Program	<p>flow: 1.0 mL/min</p> <p>%B 0-28 in first 10.5 min 28-100 in next 7.5 min</p>

2.2 Electrochemical Reactor

An electrochemical HPLC detector from Coulochem, consisting of a controller (model 5100A) and an analytical cell (model 5010), served as the electrochemical reactor. The cell was comprised of two porous carbon electrodes operating in coulometric mode so that, in theory, all the analyte passing through the cell would react. The reactor was operated at various voltages, and an equilibration period of 30 minutes was allowed every time the voltage was changed. The two electrodes operated independently, so that one could be switched off to study the effect of electrode surface area. Otherwise, both electrodes were set to the same potential for maximum yield of oxidation products. In order to study the reaction temperature, the housing of the electrochemical cell was dismantled, and the lead alloy-encased electrode assembly was wrapped in plastic and placed in a water bath.

2.3 Post-column Pumps for pH Adjustment

To attain alkaline pH conditions in the electrochemical reactor, two post-column pumps (Altex 110A) were installed as shown in Figure 11 using zero-volume mixing tees (Valco). The pumps were equipped with pulse-dampeners and old PRP-1 columns to reduce the baseline noise due to pump pulsations. A buffer solution (0.1 M Na_2HPO_4 , adjusted to pH 12 with KOH solution) was used to increase the pH of the eluent prior to the reactor. Dilute nitric acid (0.1 M) was introduced between the reactor and the detector to lower the pH of the sample stream to 7, the optimum pH for the fluorescence of the derivatized PSP toxins. Both post-column pumps were operated at 0.3 mL/min. The reaction pH, which was adjusted by changing the amount of KOH added to the buffer, was measured by turning the acid pump off and analyzing a small volume of the effluent.

2.4 Identification of Oxidation Products

The products of electrochemical oxidation at pH 7 and a voltage of 0.75 V were studied by collecting the appropriate fractions of effluent at the outlet of the fluorescence detector. These fractions were analyzed using the HPLC method for oxidation products of PSP toxins [60]. Peaks were identified by comparison of retention times to those of periodate oxidation products, whose structures have been identified by LC-MS [63]. A detailed description of the pre-chromatographic periodate reaction and the HPLC conditions is provided in the following section.

3. Study of Oxidation Products

3.1 HPLC Method

The oxidation products were analyzed as outlined in the pre-chromatographic oxidation method [60]. Both mobile phases were comprised of 0.1 M ammonium formate, adjusted to pH 6.0 with acetic acid. Mobile phase B contained 10% acetonitrile (v/v) while mobile phase A contained none. A two-part linear gradient program was used to increase %B from 0 to 5.3 in the first 8 minutes and then to 40 in the next 7 minutes. The column was a Supelcosil LC-18 (Supelco, 5 μ m, 15 cm x 4.6 mm i.d.). In one of the MnO₂ experiments, a variable wavelength UV detector (BioRad 1305A, set at 330 nm) was installed between the column and the fluorescence detector to check for UV-absorbing, non-fluorescent oxidation products.

3.2 Pre-chromatographic Periodate Reaction

The periodate reactions were carried out in plastic 0.5-mL centrifuge tubes as described in reference 60. The buffered periodate reagent, prepared each day from stock solutions, contained 0.1 M ammonium formate, 0.1 M dibasic sodium phosphate, and 0.01 M periodic acid. The pH of the solution was adjusted to 8.0 by addition of an

appropriate amount of 1 M NaOH. Reactions were performed by combining 20 μ L of PSP standard (or sample extract) and 100 μ L of buffered periodate in a vial. The contents of vial were shaken for several seconds and allowed to react for 3 minutes. A 1- μ L aliquot of glacial acetic acid was added to stop the reaction, and the sample was analyzed by HPLC after 10 minutes.

4. Batch Reactions with Manganese Dioxide

The batch reactions were carried out under various conditions, but typical experimental parameters are given in parentheses. A 1.5-mL plastic microcentrifuge vial containing 200 μ L 0.1 M phosphate buffer (pH 8) and 200 μ L standard solution of a PSP toxin (0.1 ng/ μ L) was pre-heated in a water bath (60°C) for one minute. A known mass of MnO₂ (0.01 g) was added, and the vial was shaken and returned to the water bath. The mixture was shaken briefly every two minutes for the duration of the reaction. At the end of the desired reaction time (10 minutes), 3 μ L of acetic acid and 600 μ L of water were added. The mixture was transferred using a pasteur pipette to a syringe fitted with a 0.45- μ m syringe filter (Acrodisc, Gelman Sciences). After filtration, the solution was allowed to stand for 10 minutes and then analyzed by the HPLC method described in section 3.1 of this chapter.

5. Post-Column Oxidation by Manganese Dioxide

5.1 Chromatographic Conditions

A schematic representation of the post-column system is provided in Figure 12. A Hamilton PRP-1 (10 μ m, 15 cm x 4.1 mm i.d.) was used as the analytical column and was maintained at 45°C with a column heater (BioRad). Chromatographic conditions were similar to those used in the post-column periodate reaction system [56] and are outlined in Table 3. The gradient program had to be modified towards the end of this

Figure 12. Schematic diagram of the HPLC system with the post-column manganese dioxide reactor

Table 3. Gradient programs used with the post-column MnO₂ reactor. Gradient I was used for most of the experiments but had to be modified in order to separate the earlier-eluting toxins.

	Gradient I	Gradient II
Mobile Phases	<p>A 3.0 mM heptanesulfonate 1.5 mM ammonium phosphate adjusted to pH 7 1% (v/v) acetonitrile</p> <p>B 3.0 mM heptanesulfonate 6.25 mM ammonium phosphate adjusted to pH 7 25% (v/v) acetonitrile</p>	<p>A 3.0 mM heptanesulfonate 1.5 mM ammonium phosphate adjusted to pH 7</p> <p>B 3.0 mM heptanesulfonate 6.25 mM ammonium phosphate adjusted to pH 7 25% (v/v) acetonitrile</p>
Gradient Program	<p>flow: 0.8 mL/min</p> <p>%B 3-23 in first 7.5 min 23-100 in next 8 min</p>	<p>flow: 0.8 mL/min</p> <p>%B 0-19 in first 8 min 19-100 in next 8 min</p>

study in order to separate the earlier-eluting compounds (see gradient II, Table 3).

Mobile phases were degassed and stored as described previously and prepared at least every second day.

5.2 Manganese Dioxide Reactor

The solid-phase reactor was assembled using an empty HPLC column housing (7.5 cm x 4.6 mm i.d.). The column was dry-packed by the tap-and-fill method with MnO_2 (Anachemia) that had been ground and sieved to a particle size range 45-75 μm . The packing was then compressed by flushing with mobile phase A, and the void volume at the head of the column was filled with additional MnO_2 . This step was repeated until the formation of void volumes ceased. The reactor was heated to 85°C by a second column heater. Two post-column pumps (Altex 110B), each equipped with a pulse-dampener and an old PRP-1 column to reduce baseline noise, were used to introduce pH-adjusting solutions before and after the reactor. The first one delivered an alkaline buffer solution (0.1 M Na_2HPO_4 , adjusted to pH 11.5 with KOH) at a rate of 0.3 mL/min, while the second pumped 0.08 M nitric acid, also at 0.3 mL/min. To maintain the integrity of the reactor, the mobile phases and alkaline buffer solution were purged continuously with helium. When not in use, the entire HPLC system was left in mobile phase A at 0.01 mL/min.

Chapter 3

Results & Discussion

1. Pre- and Post-Column Periodate Oxidation

Since the periodate oxidation of PSP toxins has been well studied, it has and will continue to provide a benchmark for comparison of new HPLC methods. For that reason, some of those results are presented here. A typical chromatogram of a mixture of PSP toxins obtained using the Sullivan post-column reaction method is shown in Figure 13 [56]. Any post-column method that uses ion-pair chromatography will show a similar elution pattern since the reaction occurs after the separation step and does not affect the chromatography. Results of the pre-chromatographic oxidation method are shown in Figure 14 [63]. The periodate reaction was performed in the same manner as outlined earlier in the Experimental section, but different mobile phases were chosen for their compatibility with the ion-spray mass spectrometer used for identification of the products. Despite the different mobile phases, the elution patterns remained the same. In all our studies of the oxidation products, a pre-column periodate reaction was used as a reference point. Peaks were identified by comparison of retention times vs. the pure reference standard.

2. Proposed Reaction Pathway for PSP Oxidation

A possible reaction mechanism for the oxidation of STX is outlined in Figure 15. An understanding of reaction pathways is important because it helps explain some of the trends observed in our experiments. The proposed mechanism is based on two dehydrogenation steps, each requiring the transfer of two electrons. In order for the reaction to proceed, the guanidinium group must be rendered labile via deprotonation. For STX and the other non-hydroxylated toxins, the C-8 centered guanidinium group

Figure 13. Chromatogram of PSP toxin standards obtained using the post-column periodate reaction method [56]

Figure 14. Top: Chromatograms obtained for periodate-oxidized PSP standards. Bottom: Structures of oxidation products identified by LC-MS [63]

Figure 15. A possible reaction mechanism for the oxidation of STX. The final product shown has retained the carbamate group but is otherwise the identical the product identified by Bates and Rapoport [46]. (R = carbamate group)

would provide the initial point of attack since it has the lower pK_a . The reaction mechanism would be similar for NEO and the other N-1-OH toxins. The electron acceptor can be either periodate or, in the case of our experiments, an anode or manganese dioxide.

3. Electrochemical Reactor

Electrochemical processes are oxidation-reduction reactions which are either initiated by electricity or can be used to generate electricity. In the first case, the flow of electrons brings about electron-transfer between the electrode and the analyte, and the atoms or molecules undergo subsequent changes in oxidation state or structure.

Chromatographers have been able to apply this phenomenon in the form of HPLC amperometric detectors. These flow-through cells are generally equipped with three electrodes: the working, auxiliary, and reference electrodes as shown schematically in Figure 16a. Electron transfer occurs between the working electrode and the analyte, while the auxiliary electrode acts as the counter-electrode. A current is generated by a DC potentiostat in order to maintain a constant cell potential. The working electrode potential is measured versus the current-free reference electrode by a high-impedance amplifier. The analyte is detected by measuring the current at the working electrode. In most amperometric detectors (e.g. thin-layer or wall-jet type), the cell geometry allows for the conversion of only a small percentage of the analyte. Coulometric detectors are a special type of amperometric detector that will oxidize or reduce nearly 100% of the analyte by using a high-surface area porous working electrode. The various configurations for HPLC amperometric detectors are illustrated in Figure 16b. The electrochemical detector chosen for our experiments was a coulometric detector equipped with two reticulated glassy carbon electrodes in series. In most cases, both

Figure 16. (a) Schematic representation of the electrodes in an amperometric detector. A current is generated by a DC source to maintain a constant cell potential.

- (b) Configurations of HPLC amperometric detectors:
 1. thin-layer 2. wall-jet 3. coulometric
 (W.E. = working electrode)

electrodes were operated at the same voltage to maximize the surface area available for reaction.

As mentioned in the Theory section, there have been applications in which electrochemical detectors have been used as reactors. In these cases, the signal from the electrochemical detector was not important since the products of the oxidative or reductive process obtained at the working electrode were quantitated further downstream by a second HPLC detector. In the case of PSP toxins, the method of detection is fluorimetry, which provides detection limits several orders of magnitude better than those obtained by electrochemical detection alone [52]. Although electrochemical detection can be very sensitive, it was found not to be very useful for PSP determination. There was a high background signal generated by the mobile phase, and the baseline was noisy due to pulsations from the post-column pump which was required to adjust the effluent pH.

For analytical purposes, fluorescence detection is adequate in terms of sensitivity and selectivity, but fundamental research into the actual electrochemical reaction itself is hindered because only the fluorescent products of the oxidative reaction are quantitated. If any other products were formed, they would not be taken into account. Attempts were made to record the signals from both the electrochemical and fluorescence detectors simultaneously, but difficulties were encountered because of the poor sensitivity of the electrochemical detector. When solutions of sufficiently high PSP concentration were used, a reasonable signal was observed on the electrochemical detector, but the fluorescence detector would be overloaded.

3.1 pH Studies

In the first set of experiments, the effect of pH on the oxidation efficiency was examined. The reaction pH was adjusted with the use of post-column pumps. Using a

shellfish extract as the test sample, the results shown in Figure 17 were obtained for GTX3. Similar trends were observed for GTX2 and STX. These results are consistent with the proposed reaction pathway because higher pH levels (between 8.5 and 11) lead to a greater degree of deprotonation of the toxins, and only the deprotonated species are susceptible to oxidation. At $\text{pH} > 11$, the peak height seems to either plateau or decrease. The decreased yield might be caused by the increase in buffer pH. The high-pH buffer led to a high background current in the electrochemical detector, perhaps causing the detector to function improperly. Since the signal from the electrochemical detector was not recorded, it is uncertain whether the electrodes continued to function properly when the signal overload light switched on. It could be that an overload would cause a decrease in the efficiency of the electrode.

At this point of the research, a paper which outlined some preliminary work in the same area appeared in the literature [68]. These researchers had successfully performed electrochemical oxidation of PSP toxins at $\text{pH} 7$. It would be advantageous to forego the use of the pH-adjusting solutions since they caused many technical difficulties. These included baseline noise due to pump pulsations and dilution of the sample stream. For example, no data was obtained for NEO as it could not be detected at $\text{pH} \geq 8$, even when quantities as high as 100 ng (in a 50- μL injection) were analyzed.

Evaluation of our electrochemical reactor at $\text{pH} 7$ revealed that all the PSP analogues studied were more readily detected than at $\text{pH} 11$. It was originally thought that the reaction yield would have been better at $\text{pH} 11$ because a higher percentage of the toxin is deprotonated and, thus, more easily oxidized. Extrapolation of the graph in Figure 17 would seem to indicate better sensitivity at high pH as well. In practical terms, however, the pump-free reactor (at $\text{pH} 7$) was a more favourable choice because of the elimination of both the baseline noise associated with the post-column pumps and the high background current in the electrochemical reactor due to the conductivity of

Figure 17. Peak height as a function of reaction pH for post-column electrochemical oxidation of GTX3

added buffer solution. Several studies were conducted at pH 11 but were not repeated at pH 7. It is expected that many of the trends observed would be similar at basic and neutral pH levels. Except where noted, all remaining experiments were conducted at reaction pH 7.

3.2 Voltage Studies

The effect of applied voltage on the yield of fluorescent product was studied at both pH 7 and 11. The upper limit of the voltage range studied was determined by the background current of the electrochemical detector. The background current was higher when the alkaline buffer solution was used to adjust the pH due to higher conductivity of the effluent. Consequently, the voltage could not be set higher than 0.65 V at pH 11. For the non-N-1-hydroxylated toxins, higher potentials led to better reaction yields. As can be seen in Figures 18 and 19, this trend was observed at both basic and neutral pH levels. The hydroxylated toxins, NEO and B2, exhibited different behaviour with local maxima observed at ~0.7 volts applied at pH 7. A possible explanation for this difference might be found in the hydrodynamic voltammograms (see Figure 20) obtained by HPLC with electrochemical detection [68]. It is uncertain whether the two curves represent samples of equi-molar concentrations, but the qualitative difference in their shapes is marked. The plateau in the NEO curve, observed between 0.40 and 0.75 V, suggests that there are two distinct steps in the oxidation of the NEO [68]. It is likely that the products obtained after the second oxidation step (i.e. on the second steep portion of the hydrodynamic voltammogram) are not fluorescent. This would correspond to the drop in detection of fluorescent products for NEO at voltages greater than 0.70 V. The sulfocarbamoyl analogue of NEO, B2, seems to exhibit similar behaviour except that the change occurs at a slightly higher potential. To check for the presence of non-fluorescent products, the fraction of the effluent containing the NEO

Figure 18. Peak height as a function of oxidation potential at pH 11. Detector response has not been standardized. Amounts injected per 50 μ L: STX = 31.5 ng, GTX2 = 0.90 ng, GTX3 = 0.46 ng. (The large amount of STX needed was due to poor chromatography.)

Figure 19. Fluorescence detector response as a function of oxidation potential for individual toxins at pH 7. The results have been normalized to cm/ng for all toxins except B2 (cm/10 ng). The graphs have been plotted on three different scales because of the different sensitivities for the various analogues.

Figure 20. Hydrodynamic voltammograms of STX and NEO obtained using HPLC with electrochemical detection [68]

peak would have to be collected and analyzed by the HPLC method for product identification (see Experimental section). To detect the non-fluorescent compounds, a UV-VIS detector would have to be used instead of the fluorimeter. This experiment would have required the use of a highly concentrated NEO standard because UV-VIS detectors are inherently less sensitive than fluorescence detectors. Unfortunately, due to the limited supply of PSP standards, this experiment was not performed. Using the information provided by Figure 19, 0.75 V was chosen as the optimum operating potential for mixtures of PSP toxins.

3.3 Temperature Studies

To study the effect of temperature on the reaction, the "analytical cell" portion of the electrochemical detector was dismantled, and the solid lead alloy cylinder which encased the electrodes was wrapped in plastic and heated in a water bath. The manufacturer of the electrochemical detector recommended the use of higher temperatures when attempting to improve detector sensitivity since the addition of heat will help speed up most reactions [71]. This experiment was conducted at pH 7 with 0.70 V applied and using isocratic elution. Results of the temperature study are shown in Figure 21. When the temperature was elevated slightly above ambient conditions, the yield of fluorescent products increased for both STX and NEO. As the temperature rose beyond 40°C, the peak areas for both analogues decreased. It is possible that the addition of heat caused the oxidation to proceed further than desired, perhaps breaking one of the purine rings to form a product which did not fluoresce. As was the case for the voltage studies, the detection of possible non-fluorescent products could not be carried out because of limited supply of PSP standards. Another possible explanation of the reduced reaction yields might be the use of excessively high temperatures which caused the electrochemical detector not to function properly. Besides lowering the peak

Figure 21. Peak area as a function of reaction temperature for electrochemical oxidation of STX and NEO

area for STX and NEO, heating the reactor also caused more baseline noise in the fluorescence signal which might have been caused by uneven cooling of the effluent after the reactor. This could have led to bubble formation or irregular flow patterns in the detector cell. The remainder of experiments were performed without heating the electrochemical reactor.

3.4 Study of Reaction Time

In order to study the effect of reaction time on the electrochemical oxidation of PSP toxins, the flow rate of the effluent through the cell was varied. This study was conducted at pH 11, but the results should hold true for other pH values, as well. The flow of the mobile phases was varied while the post-column pumps were each maintained at 0.3 mL/min. The peak area for GTX3 is plotted against the total flow rate (of mobile phase and buffer) through the cell in Figure 22. Since the flow rate is essentially the linear velocity of the sample through the reactor, reaction time is inversely proportional to the flow rate (time = distance/velocity). Assuming that product yield (peak area) is proportional to reaction time and, thus, inversely proportional to flow, a plot of peak area as a function of flow rate would level off asymptotically as the curve in Figure 22 appears to do. The negative slope of the plot at lower flow rates indicates that the reaction is probably not near completion under any of the conditions studied in this experiment. It is expected that the curve would also level off at lower flow rates as the reaction yield approaches 100%.

A second experimental parameter that could be changed to study reaction time was the electrode surface area. Since there were two identical electrodes in series in the analytical cell, the surface area and, thus, reactor residence time could be halved by switching one of the electrodes off. The effect of this was tested at pH 7 and 11 for STX and GTX2/3 and at pH 7 for NEO. Switching the second electrode off caused a 50%

Figure 22. GTX3 peak area as a function of effluent flow rate through electrochemical reactor

decrease in peak area in all cases. The results indicated that the peak area was proportional to the reaction time, confirming the trend observed in the flow experiments.

The results indicated that the product yield could be improved by either decreasing the flow rate or increasing the electrode surface area. Use of lower flow rates, however, would make the HPLC runs an unreasonable length as each sample already requires 25 minutes for separation and column re-equilibration. An increase the electrode surface area could have been achieved by connecting a second electrochemical detector on-line after the first, but additional equipment was not available. As well, the second electrochemical detector would have introduced additional void volume to the post-column system, thus increasing the degree of peak broadening.

The data collected did not provide sufficient information to determine which was the rate-limiting step: the sorption of the analyte onto the electrode surface or the transfer of electrons from the analyte to the anode. As shown in Figure 23, the analyte molecule is carried along the length of the reactor by the flowing sample stream. Diffusion of the analyte to the electrode surface (shown by the vertical arrow) is an essential step of the oxidation process. A longer residence time in the reactor (ie. a lower flow rate) provides greater opportunity for diffusion and, hence, oxidation to occur. Designing a reliable experiment to study reaction time and mechanism was made difficult by equipment constraints. Potentiostatic electrochemical studies conducted off-line might have provided some useful insight into the reaction mechanism, but this equipment was not available, nor was the quantity of pure PSP standard that would have been required to make these types of measurements.

3.5 Identification of Electrochemical Oxidation Products

The products of the anodic oxidation of PSP toxins were studied by collecting the fraction of the effluent that contained the peak of interest and analyzing it by reverse-

Figure 23. Schematic representation of the forces acting on an analyte molecule as it passes through a channel of the porous carbon electrode

phase HPLC. Product identification was accomplished by comparing the retention times to those of the products from pre-chromatographic periodate oxidation of a PSP toxin standard [56]. The results of this study are summarized in Figure 24. The products formed by electrochemical oxidation were similar to those obtained using periodate as the oxidant. The only difference was that the carbamate group was not hydrolyzed to a significant extent in the electrochemical process as it was in the periodate reaction. The electrochemical experiment was conducted at pH 7, but it is believed that the products would have been similar at alkaline pH levels. It is expected that the products obtained for NEO and B2, and perhaps even STX, under more basic reaction conditions would no longer have the (sulfo)carbamate group attached. Longer reaction times in the alkaline periodate reaction led to cleavage of the (sulfo)carbamate group for STX, B1, and B2 [63]. The same hydrolysis was also observed at high pH and at high temperature in the MnO_2 oxidation of PSP toxins (see sections 4.1.1 and 4.1.2 of this chapter).

3.6 Improving the Chromatography of STX and NEO

The chromatography of PSP toxins has been studied thoroughly by Sullivan and coworkers in developing the post-column reaction method [56]. The efficiency of polystyrene-divinylbenzene columns is known to be less than ideal [72], and the use of ion-pair reagents is associated with reproducibility problems caused by non-equilibrium conditions [73]. Although the chromatography was not the focus of our research, several experiments were performed attempting to improve the shape of the broad, tailing peaks obtained for the late-eluting toxins, STX and NEO.

It was postulated that the tailing was caused either by poor chromatography or by the adsorption of the oxidation product to the electrode surface. Flow-injection analysis experiments (without HPLC column) were conducted to check if the peak broadening was indeed occurring at the electrode. These studies gave ambiguous results since the

Products A, B, C

Product D

Toxin	Oxidation Products
STX	A
NEO	A, (D)*
GTX2	B
GTX3	B
B1	C, (D)
B2	C, (D)

$R_{2/3} = \text{H}$ for products A and C; $R_{2/3} = \text{OSO}_3\text{H}$ for product B
 $R_4 = \text{H}$ for products A and B; $R_4 = \text{NH}_2$ for product C

Figure 24. Structures of oxidation products obtained by electrochemical oxidation, identified by co-elution with periodate-oxidized PSP standards. The products obtained by the two reactions are similar. (Refer to Figure 14, page 41, for periodate oxidation products.)
 * () = minor product

toxins passed through the reactor with other materials present in the PSP standards. (When the column was reinstalled, the extraneous materials eluted at the solvent front.) Several different approaches were taken in trying to alleviate any possible desorption problems at the electrode, including heating the electrode, using more acetonitrile at the end of the gradient, and introducing an organic solvent (methanol or acetonitrile) into the stream before the electrode. None of these effected the desired change. The drawbacks associated with heating the electrode were discussed earlier in section 3.3 of this chapter. Increasing the amount of organic modifier (in the mobile phase or after the column) would shift the adsorption-desorption equilibrium towards desorption because the ion-paired analytes are more soluble in the less polar solution phase. Diminished contact between the analyte and the electrode would lead to a lower reaction yield.

3.6.1 Increasing the Heptanesulfonate Concentration

One experimental parameter that did affect the peak shapes of NEO and STX was the concentration of the ion-pairing agent, heptanesulfonate, in the mobile phase. The mobile phases were made up as outlined in the Experimental except that only 1 mM heptanesulfonate was used. To increase the concentration in the mobile phases, appropriate volumes of the 50 mM heptanesulfonate stock solution were added. The results of this study are shown in Figure 25. Increasing the concentration of ion-pairing agent led to greater peak heights and narrower peaks (peak area did not change) and, as expected, longer retention times. The longer retention times are due to a greater degree of interaction between the analyte and the column via the ion-pairing reagent; that is, the partitioning of the analyte between the mobile phase and the stationary phase is shifted towards the adsorbed state because there is more ion-pair reagent bound to the stationary phase.

a **b** **c**

Figure 25. (a) Retention time as a function of the concentration of ion-pair agent
(b) Peak half-width as a function of the concentration of ion-pair agent
(c) STX peak height as a function of the concentration of ion-pair agent

The improvement in peak shape was initially thought to be due to the increased competition between the oxidation products and the excess heptanesulfonate for surface sites on the electrode. This would have increased the desorption rate of the oxidation product from the electrode and decreased the contribution to peak tailing by the electrode. If this were true, then the extra heptanesulfonate could be added using a post-column pump before the reactor to achieve the same effect. The post-column ion-pair reagent pump did not change the peak width of NEO and STX, so it was concluded that the source of the tailing peaks was not the electrode but the PRP-1 column.

Two proposed mechanisms of retention for ion-pair chromatography are depicted in Figure 26 [73]. It is likely that the actual mechanism is a combination of the two, depending on the amount of organic modifier present in the mobile phase. The toxins form ion-pairs with the heptanesulfonate, either at the surface of the stationary phase or in the mobile phase. Ideally, retention of the analyte on the column would always involve formation of an ion-pair, but sometimes undesirable active sites are present on the stationary phase. Sorption at these sites causes mixed-mode interactions which leads to peak-broadening. Since STX and NEO are the most cationic of the PSP analogues, they would be most likely to sorb to any negatively-charged active sites on the column. Increasing the heptanesulfonate concentration would coat the stationary phase to a greater degree [73], leaving fewer of these sites available to interact with NEO and STX.

3.6.2 Heating the Column

In another attempt to reduce the tailing of the STX and NEO peaks, an HPLC column heater was installed. (The Sullivan method recommends heating the analytical column to improve the efficiency of the chromatography.) The results of the STX study, represented graphically in Figure 27a, reveal that the peak area remained constant, but

Figure 26. Models for interaction between ion-pair reagent and analyte (adapted from reference 73).

- A** The paired-ion model. An ion-pair is formed in the mobile phase prior to its partition into the stationary phase.
- B** The dynamic ion-exchange model. The lipophilic tails of the ion-pair reagent are adsorbed onto the stationary phase and form an ion-exchange-type surface. Sample ions then partition onto the stationary phase by a conventional ion-exchange process.

a **b**

Figure 27. (a) Peak height and peak area as a function of column temperature
(b) Retention time as a function of column temperature

that the peak height increased with higher column temperatures. As well, the retention times were reduced slightly at higher temperatures (see Figure 27b).

Higher temperatures led to increased thermal energy of the analyte. This would have two opposing effects: 1) increased thermal energy would translate into higher diffusion rates and more random motion of the analyte which might lead to peak broadening, and 2) the analyte would migrate faster, leaving less time for the first effect to occur. Even though the retention times decreased and the peaks became narrower, it is unlikely that a 3% decrease in residence time would lead to a 50% increase in peak height. Besides, the contribution of analyte diffusion to band broadening is usually minimal when high efficiency HPLC columns are used [74]. Instead, it is likely that the reduction of the peak-broadening effect was mainly due to more effective mass transfer between the stationary and mobile phases. As well, the analyte bound to the non-specific binding sites (described previously in section 3.6.1) would be thermally desorbed as the column temperature increased.

The results of the column temperature study revealed that peak area remained fairly constant and thus might be more reliable than peak height for quantitation of HPLC peaks. Generally, it is better to use peak area instead of peak height because changes in chromatographic conditions such as mobile phase composition or column efficiency affect the peak shape and thus peak height. However, the precision of the peak area measurements is not as good as that for the peak height, as shown by the error bars in Figure 27a. The integration equipment available for these experiments could not be programmed to provide reliable measurements of peak area. The use of peak height in all the studies presented in this thesis was validated by careful monitoring of chromatographic peak shapes. Quantitative comparison of results was made only when the chromatographic conditions were the same (i.e. samples analyzed on the same day or the next day, with system sensitivity checked by analysis of standard solution)

3.7 Evaluation of the Analytical Method

3.7.1 Analytical Performance

After optimization of the experimental parameters, mixtures of PSP toxins could readily be analyzed using the post-column electrochemical reactor. Typical chromatograms of mixtures of PSP standards are shown in Figure 28. All the analogues available for our study were baseline-separated by the gradient program outlined in the Experimental section. The upwards drift in the baseline from approximately 16 to 20 minutes is a result of the solvent gradient and is reproducible in all sample, standard, and blank injections. The broad peak which appears in the bottom chromatogram with an elution time of approximately 15 minutes may originate from a mixture of STX and dc-STX, two analogues which would not be resolved by this particular gradient program. These analogues, however, were not specified as being present and were not quantitated by the provider of this standard mixture.

Calibration curves, obtained by analyzing a mixture of PSP standards, are shown in Figure 29. The concentrations of the individual toxins in the mixture are given in Table 4, and this solution was diluted to give the relative concentrations shown on the graph. (The proportion of each toxin was chosen so that the peaks on the chromatogram would be approximately the same height.) The injection volume was 50 μL . The upper limit of the linear dynamic range (LDR) was reached for GTX3 and B1 (ca. 0.4 and 5 ng, respectively), while the calibration graphs for the other toxins were linear in the concentration range studied. The limits of detection (LODs) were estimated by analyzing the chromatograms of very dilute standard solutions of each toxin. Table 5 gives the estimated LODs which are based on a 3:1 signal-to-noise ratio. As well, the minimum detectable concentrations of each toxin in shellfish tissue are presented. The relative standard deviation of replicate injections is $\leq 10\%$, with variations caused mainly by an improperly re-equilibrated column or electrochemical reactor.

Figure 28. Typical chromatograms of mixtures of PSP standards obtained using post-column electrochemical oxidation system. Oxidation parameters were pH 7 and 0.75 V. Integrator attenuation = 7 for both chromatograms

Amounts injected (ng/50 μ L):

A B₂ = 90, B₁ = 11, GTX₃ = 0.97, GTX₂ = 4.0, NEO = 7.0, STX = 0.94

B GTX₄ = 9.0, GTX₁ = 26, B₁ = 11, GTX₃ = 2.5, GTX₂ = 7.5

Figure 29. Calibration curves for PSP standards obtained by analyzing a mixture of toxins using post-column electrochemical oxidation.

* The relative concentration is the dilution factor for the mixture, where the value for 1.0 is given on the following page in Table 4.

Table 4. Concentration of PSP toxins in the mixture used to obtain calibration curves in Figure 29. The injection volume was 50 μL .

Toxin —	Concentration (ng/ μL)
STX	0.017
NEO	0.084
GTX2	0.054
GTX3	0.012
B1	0.138
B2	1.02

Table 5. Estimated limits of detection for PSP toxins (based on 3:1 signal to noise ratio) and minimum detectable concentrations (based on total dilution factor of 30 for samples contaminated near the regulatory limit of 0.8 ppm) for the post-column electrochemical oxidation method.

Toxin —	Limit of Detection (ng/50- μL injection)	Minimum Detectable Concentration ($\mu\text{g/g}$)
STX	0.10	0.06
NEO	0.84	0.50
GTX1	2.10	1.26
GTX2	0.09	0.05
GTX3	0.05	0.03
GTX4	3.70	0.42
B1	0.50	0.30
B2	15.00	9.00

For GTX3 and B1, the LDR spans an order of magnitude. The flattening of these calibration curves is not likely due to column-overload since an injection of 51 ng of B2 fell in the linear portion of that calibration curve. (B1 and B2 have approximately the same molecular mass.) The limited linear dynamic response is likely related to the reaction rate at the electrode surface and the rate of mass transfer between the solution and the surface. No further experiments were conducted in this area since this would have required more precise control of the reaction time and a means of detecting any non-fluorescent products of the electrochemical reaction.

3.7.2 Hydroxylated vs. Non-Hydroxylated Toxins

The sensitivity of the electrochemical oxidation method is always greater for the non-hydroxylated toxins than for their hydroxylated counterparts. Examination of the ratios of the LODs of the OH-toxins to those of the non-OH analogues (see Table 6, page 72) reveals that the non-OH toxins are about 10-30x more detectable; that is the N-1-OH group renders the molecule less susceptible to oxidation at pH 7 and at 0.75 V. A possible explanation for this can be found in the proposed reaction mechanisms for NEO, shown in Figure 30. Because the C-2-centered guanidinium group has a lower pK_a than the C-8-centered group, the six-membered ring would be targeted in the first steps of the reaction. The presence of the electron-withdrawing OH group would make the loss of an electron from N-1 very difficult. As shown in Figure 30a, the product which would likely be generated if this proposed pathway were followed is not the fluorescent purine derivative but a nitron compound. The N-O group at position 1 in the nitron product would either quench the fluorescence of this compound or change the maximum λ_{ex} and λ_{em} significantly from that of the purine derivative, rendering it undetectable in this detection scheme.

Figure 30. (a) Proposed pathway for oxidation of NEO to nitron compound
 (b) Proposed pathway for oxidation of NEO to fluorescent purine derivative

Table 6. Ratios of limits of detection (LOD) of OH-toxins to that of their non-OH analogues

OH/non-OH	Ratio of LOD
NEO / STX	8
GTX1 / GTX2	23
GTX4 / GTX3	14
B2 / B1	30

The reaction scheme in Figure 30b (on previous page) shows a possible pathway to the purine compound yielded by STX. The NEO molecule would have to be doubly deprotonated before the reaction could be initiated. The ring containing C-8 would be aromatized first, beginning with the removal of an electron from the C-8-centered guanidinium group. The N-1-OH moiety would be lost in a dehydration step. (Although hydroxide is a poor leaving group, the energetically favourable process of aromatization would likely overcome this difficulty.) Since only a small fraction of NEO is doubly deprotonated at pH 7, the oxidation of NEO would be slower than the STX reaction. Thus, under similar reaction conditions, a smaller percentage of NEO than STX is converted to the fluorescent derivative, making NEO ~10x less detectable than STX.

3.7.3 Confirmation of the NEO Peak

The unusual behavior of the hydroxylated toxins with increasing voltage (refer to Figure 19) can be used to confirm their presence in a sample. Figure 31 shows two chromatograms of the same mixture of PSP standards obtained at different oxidation potentials. Because 0.80 V is beyond the optimum voltage for the oxidation of NEO, the NEO peak is smaller than the one obtained at the lower voltage. The non-hydroxylated

Figure 31. Verification of the NEO peak by changing the oxidation potential. As the voltage increased, the peak height for the hydroxylated toxins (STX and GTX_{2/3}) increased, while the peak height for NEO decreased.

toxins, however, produce larger peaks at higher potentials. (The broader peaks for NEO and STX in the upper chromatogram are not related to the oxidation potential but are probably due to non-equilibrium column conditions at the time of injection.)

3.7.4 Application of Method to Real Samples

Although an extensive study was not carried out, the usefulness of the post-column electrochemical reactor was tested by analyzing some shellfish and plankton extracts. Table 7 gives the concentrations of PSP toxins in a mussel sample found by several HPLC methods. Although the results do not match exactly, these values would be considered to be in agreement. An inter-laboratory study of the various HPLC methods [75] has shown that there is often a wide distribution in results obtained for the concentration of PSP toxins in shellfish. In this study, the overall relative standard deviation for 7 pre-chromatographic and 13 post-column periodate determinations of STX was 52%. Typical chromatograms of various sample extracts obtained using the post-column electrochemical method are shown in Figure 32.

Table 7. Concentration (in $\mu\text{g/g}$) of PSP toxins found in a mussel extract using post-column electrochemical reactor. The uncertainty is reported as the standard deviation of four separate determinations.

Method	GTX3	GTX2	NEO	STX
Electrochemical	2.30 ± 0.02	6.36 ± 0.06	5.76 ± 0.19	5.78 ± 0.04
Pre-col. periodate ¹	— 17.79 —		4.64^3	2.78
Post-col. periodate ^{1,2}	— 11.03 —		5.28^3	2.90

¹ From a previous study, 1990

² GTX2.3 and NEO B2 were determined separately but were reported as a single figure

³ Includes concentration of B2

Figure 32. Typical chromatograms of sample extracts containing PSP toxins. The mussel sample had a total concentration of $1 \mu\text{g}$ PSP per gram of tissue, while the clam was contaminated at a level of $25 \mu\text{g/g}$ total PSP. The plankton sample was analyzed for qualitative purposes only. Integrator attenuation = 8 for top chromatogram and 5 for two others.

The two main factors which contributed to inaccurate determinations were inadequate re-equilibration of the column and irreproducible reactions at the electrode surface. It was found that rinsing the electrode with 0.1 M nitric acid every week removed substances that may have adsorbed onto the surface and kept the electrode in good working order. As well, the importance of allowing the column and electrode to re-equilibrate with mobile phase A between HPLC runs was observed. Otherwise, the chromatography became less reproducible as illustrated by the shape of STX peaks in Figure 31. The overall performance of the electrochemical reactor was good, and the system required much less maintenance than the benchmark post-column periodate reaction system.

4. Oxidation by Manganese Dioxide

Manganese dioxide is a black powdery substance that is insoluble in water, nitric acid, and cold sulfuric acid. It has been used as an oxidizing reagent in many different organic reactions, including catalytic oxidations [69] as well as stoichiometric reactions. In the latter, MnO_2 is reduced to MnO [76]. The activity of manganese dioxide as a reagent can be altered by changing the particle size, by using different drying temperatures in the preparation (synthesis) of the reagent, and by after-treatment with 15% nitric acid and drying at 220-250°C [69]. Commercially prepared MnO_2 usually contains traces of Mn_3O_4 [77] and about 5% water of hydration [78]. The MnO_2 reagent used in our experiments, purchased from Anachemia, was ground and sieved to obtain a smaller, more uniform particle size (usually 45-75 μm).

The studies of the oxidation of PSP toxins with MnO_2 were done in two parts 1) batch reactions in which the oxidation was performed in a small vial and the oxidation products were separated and quantitated by HPLC, and 2) post-column reactions in which the oxidation of the toxins was performed on-line after the separation of the parent compounds. The batch experiments allowed for a detailed study of the reaction itself since the reaction conditions could be easily controlled and changed. The knowledge gained in the batch experiments was used to develop the post-column MnO_2 reactor which could then be applied to analyze real samples.

The use of metal oxides in on-line solid-phase reactors is not uncommon. Several examples have appeared in the literature recently. Lead (IV) dioxide was used in an HPLC system for the oxidation of malachite green, a triphenylmethane dye which is used to treat fish for fungal and protozoan infections [79]. MnO_2 has been employed in an on-line reactor in an atomic absorption method for the oxidation of isoniazid, tuberculosis drug [80]. As well, a reactor bed packed with MnO_2 was incorporated in a flow-injection system for the spectrophotometric determination of N-substituted

phenothiazines, a group of pharmaceuticals which include antihistamines and drugs to control Parkinson's Disease [81].

4.1 Batch Experiments

Oxidation of PSP standards by manganese dioxide was carried out in small vials. A water bath was used to heat the vials, and the reaction mixture was shaken at regular intervals to ensure proper contact between the oxidant and analyte. The pH was controlled by using a buffer, and the MnO_2 was removed using a syringe filter at the end of the reaction. The chromatographic peaks were identified by comparison to a PSP standard that had been oxidized by the pre-chromatographic periodate method.

4.1.1 Preliminary Temperature Studies

A study was conducted to choose a suitable reaction temperature for each of the toxins. Twenty-minute reactions were carried out at 20, 40, and 90°C. Based on experience with the pre-chromatographic periodate reaction, a reaction pH of 8 was chosen as a starting point. The results of the preliminary temperature study are shown in Figure 33. Under the specified temperature and pH conditions, STX and NEO each yielded a single product which co-eluted with the carbamate product obtained in the STX-periodate reaction. The yield of this single product increased with higher temperatures for both STX and NEO.

The chiral products formed by GTX2/3 could not be separated under the chromatographic conditions used and were quantitated together. For the reactions at 20 and 40°C, a single chromatographic peak was observed (identical to that obtained for GTX2/3 in the periodate reaction), but Figure 34 shows that two peaks were obtained for the 90-degree reaction. The first peak (A) eluted was the one observed at lower temperatures, but the other product (B) eluted with the same retention time as the one

Temperature (°C)	GTX2/3	NLO	STX
20	A	B	B
40	A	B	B
90	A, B	B	B

Figure 33. Products formed from PSP toxins by reaction with manganese dioxide at various temperatures. Peak identification was performed by comparison of retention times with periodate-oxidized PSP standards

2 OF 2

PM-1 3 1/2" x 4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT

PRECISIONSM RESOLUTION TARGETS

Figure 34. Chromatogram of products obtained from the oxidation of GTX2/3 by manganese dioxide at 90°C for 20 minutes. Peak labels correspond to the letters in Figure 33.

that was produced by STX. (Refer to Figure 33 for structures.) It would seem that higher temperatures led to the cleavage of the 11-hydroxysulfate group from the product, but the loss of the hydroxysulfate group is a reductive step which would be unlikely to occur in an oxidizing medium. Structural identification (e.g. by LC-MS/MS) could not be carried out due to a lack of equipment.

A temperature of 40°C was chosen for the remaining GTX2/3 batch experiments so that a single peak could be used for quantification. For STX and NEO, a temperature of 90°C was selected because higher reaction yields were obtained.

4.1.2 pH Studies

The effect of reaction pH on the yield of fluorescent product was studied by changing the pH of the buffer solution. NEO and STX were oxidized at 90°C, and the GTX2/3 reactions were performed at 40°C. Results of the pH study are shown in Figure 35.

A similar pattern of product formation was seen for both STX and NEO. At lower pH levels, only the carbamate product was formed, but two additional products were obtained under more basic conditions. The chromatogram and the structures of the STX reaction products formed at pH 12 are shown in Figure 36. The first peak (A) was assigned to the decarbamoyl product. The next peak (B) has the same retention time as the deoxy-decarbamoyl product formed by decarbamoyl-STX (dc-STX) when it is oxidized by periodate or peroxide [62, 82]. The structure of the dc-STX product was elucidated by ^{13}C - and ^1H -NMR [82]. There is, however, some uncertainty regarding the structure of the compound responsible for peak B. The formation of the dc-STX product would have required a reductive step which would be improbable in the presence of an oxidizing agent such as MnO_2 . The final peak (C) in Figure 36 was assigned to the carbamate product.

a **b** **c**

Figure 35. pH profiles of reaction products of manganese dioxide oxidation of a) STX, b) NEO, and c) GTX2/3. The structures of the oxidation products of the STX and NEO products are given in Figure 36, and those of GTX2/3 are shown in Figure 33. (Legend: **carb** = carbamate, **decarb** = decarbamoyl, **deoxy** = deoxy-decarbamoyl, **11-hy** = 11-hydroxysulfate)

Peak A

Peak B

Peak C

Figure 36. Chromatogram and chemical structures of products formed in the manganese dioxide oxidation of STX at pH 12.

Raising the pH of the GTX2/3 oxidation had the same effect as increasing the temperature (see section 4.1.1 of this chapter). At low pH, the single peak obtained co-eluted with the GTX2/3-periodate product. Alkaline reaction conditions led to the formation of that same product, as well as a second product which had the same retention time as the carbamate product obtained for STX.

4.1.3 Effect of Organic Solvent on the Reaction Yield

The yield of the MnO_2 reaction was much lower than the yield of the pre-chromatographic periodate reaction (MnO_2 yield $\leq 20\%$ of periodate yield). Even after optimizing pH and temperature of the MnO_2 oxidation, low yields were obtained for all toxins studied. The 20-minute reaction time allowed for the MnO_2 oxidation was much longer than the 3 minutes required for the periodate reaction to go to completion. It was thought that some of the product was being lost in the procedure, either by adsorption to the solid-phase oxidant or to the filter membrane.

To study this further, the MnO_2 was separated from the solution by use of a centrifuge instead of a syringe filter. Analysis of the supernatant fluid gave a product concentration equal to that obtained from the filtered sample solution, which indicated that the use of filter was not responsible for the low yield. The possibility that some of the oxidation product could be adsorbed to the MnO_2 was studied by drawing the remainder of the supernatant liquid off and washing the solid oxidant, first with 1 mL of water and then with a 1-mL aliquot of a 1:1 mixture of acetonitrile and water. Both washings were analyzed for oxidation product which may have been removed from the MnO_2 surface. The water wash contained a small amount of product, probably remaining from the small volume of solution which could not be removed by the pipette the first time. The acetonitrile-water wash had a lower oxidation product concentration

than the water wash, indicating that the organic solvent had not removed any additional product from the oxidant surface.

The effect of adding an organic modifier to the reaction mixture was examined by mixing an appropriate volume of acetonitrile into the buffer solution. The results of the study are shown in Figure 37. Increasing the amount of acetonitrile increased the reaction yield for STX at 20°C but decreased it at 90°C. There are two effects which the organic solvent would have on the reaction mixture: 1) since the viscosity of acetonitrile at 25°C (0.35 centipoise) is lower than that of water (0.89 cp), the viscosity of the liquid in the reaction mixture would be lowered; and 2) the equilibrium between STX in solution and STX adsorbed to the MnO₂ surface would be shifted towards the solution side because STX is more soluble in the water-acetonitrile solvent than in water. The lower viscosity would allow STX to diffuse more readily through the solution to the surface of the oxidant, while the increased solubility of STX in the solution phase would decrease the oxidation yield by hindering the adsorption step. It appears that the first effect is greater than the second at 20°C, and that the opposite is true at 90°C.

4.1.4 Changing the Amount of Oxidant

A preliminary study was conducted to investigate the effect of changing the amount of MnO₂ used. The results of this investigation revealed another fundamental difference between STX and NEO. For STX and GTX2/3, the reaction yield increased by a significant amount (~100%) when the mass of MnO₂ was doubled from 0.05 to 0.10 g. The yield of NEO remained essentially unchanged (up by only 10%) under the same conditions. The marked difference in the behavior of the hydroxylated and the non-hydroxylated toxins warranted a more detailed study, which is described in the following section.

Figure 37. Reaction yield for manganese dioxide oxidation of STX as a function of percent acetonitrile in the reaction mixture.

4.1.5 Differences between the STX and NEO Reactions

4.1.5.1 Reaction Rates

The effect of reaction time on the yield of oxidation product was studied using STX and NEO standards. The results of this investigation are presented in Figure 38. The rate of formation of fluorescent products from STX is constant for times from 5 to 25 minutes at 69°C and at higher temperatures as well. (The other data was not included because, due to slightly different shaking conditions, it did not correlate quantitatively to the data at 69°.) The non-zero y-intercept of the STX graph in Figure 38a indicates that the initial reaction rate was different than those observed for reaction times ≥ 5 minutes. The different initial rate will be further discussed on page 101.

Figure 38b shows that, for NEO, the formation of fluorescent products levelled off after ~7 minutes at all reaction temperatures studied. The reason for the flattening of these curves was not that the reaction had gone to completion; the reaction yield at 70°C is still much lower ($\leq 20\%$) than that obtained by periodate oxidation. Since NEO differs structurally from STX only by the N-1-OH group, the difference in their oxidation must relate directly to that functional group. More experiments were conducted to attempt to elucidate the mechanistic differences in the oxidation of STX and NEO.

4.1.5.2 Changing the Mass of Manganese Dioxide

A detailed investigation was conducted to follow up on the trends observed in the preliminary study described earlier in section 4.1.4 of this chapter. The mass of MnO_2 used in the oxidation process was changed while keeping all other experimental parameters constant (for STX, pH 8.0 and 10 minutes; for NEO, pH 8.5 and 6 minutes). The experiment was carried out at several different temperatures, and the results are presented in Figure 39. The yield of fluorescent products from STX increased

a **b**

Figure 38. Product yield for manganese dioxide oxidation of (a) STX and (b) NEO as a function of reaction time

a

b

Figure 39. Product yield as a function of amount of oxidant for the manganese dioxide oxidation of (a) STX and (b) NEO. In (a), the particles MnO₂ particles were 20-45 μ m; in (b), 45-75 μ m. When graph (b) is superimposed on (a), it occupies the shaded area after correction for MnO₂ surface area.

proportionally with the mass of oxidant. For NEO, however, the reaction yield remained constant when the mass of MnO_2 exceeded 0.004 g. The differences between the two sets of results are actually more marked than these graphs indicate. For the STX reactions, the particle size range of the MnO_2 used was 20-45 μm . When the mass of small MnO_2 particles was varied in the NEO study, the same reaction yield was obtained, even in the sub-mg range. For practical reasons, larger particles (45-75 μm) had to be used in the NEO study to effectively decrease the amount of MnO_2 available for reaction. When the mass of MnO_2 was in the range of 0.01 g, it was observed that decreasing the particle diameter of the MnO_2 used (i.e. increasing the specific surface area available for reaction) led to increased reaction yield for STX, but not for NEO. Assuming that the particles are spherical and that the mean particle diameter is 32.5 μm for the 20-45- μm range and 60 μm for the 45-75- μm range, then the ratio of calculated surface areas of the small-to-large particles is 3.4. If the NEO graph were superimposed on the STX graph while accounting for the surface area difference, it would only occupy the shaded area in the STX graph.

The difference between the reaction yield vs. amount of oxidant graphs for STX and NEO suggests that there is a difference in reaction pathways, linked to the only structural difference between the two analogues, the N-1-OH group. The flat portion of the NEO curves might indicate that the reaction is diffusion limited, but the reaction mixture was shaken at regular intervals, and the yield did not change significantly when the mixture was shaken more frequently. It was then postulated that one of two things was happening: 1) the different reaction pathway for NEO was leading to other products which did not fluoresce; or 2) NEO and MnO_2 were forming a complex that was not susceptible to further reaction. This complex might be an oxide formed at the N-1 position as illustrated in Figure 40. The tautomer of doubly deprotonated NEO [83] possesses lone pairs on the oxygen atom which are available for bonding to Mn (IV)

Figure 40. Possible complex formed on the surface of manganese dioxide particle between tautomer of doubly deprotonated NEO (second $pK_a = 8.65$) and Mn (IV) site. (Drawing is schematic only and does not show rutile structure of MnO_2 .)

sites on the surface of the MnO_2 . There might be sites with Mn in even higher oxidation states, and NEO would form stronger bonds at these sites. This would explain why the total reaction yield increased with higher temperatures. NEO would be released from the more labile sites (with Mn in lower oxidation states) when heat was applied, but the sites with the higher oxidation states would have retained NEO in the bound state, preventing it from being oxidized to the fluorescent purine.

4.1.5.3 Monitoring of NEO Products by UV Detection

A UV detector, set at 330 nm, was installed on-line between the HPLC column and the fluorescence detector to monitor any non-fluorescent products which may have formed. Oxidation reactions of relatively concentrated ($\sim 1 \text{ ng}/\mu\text{L}$) NEO standards were carried out using either periodate or MnO_2 as the oxidant. The products were separated by reverse-phase HPLC and monitored by both detectors. The only products that would be detected using this method would probably have the same two-ring backbone as the known products; any other products would likely differ in chromatographic behavior and UV-absorbing properties. For the MnO_2 reaction of NEO, there were no peaks for UV-absorbing products, other than those of the fluorescent compounds (i.e. the chromatograms from the two detectors were essentially identical). The periodate oxidation of NEO, however, produced a peak in the UV trace which was not present in the fluorescence trace (see Figure 41). No additional UV peaks were seen for STX with either periodate or MnO_2 as the oxidant. The presence of the non-fluorescent NEO product helps account for the lower sensitivity of the periodate oxidation methods for NEO compared to STX, but it does not contribute any clues to the original question of the differences between the MnO_2 reactions of STX and NEO. It is possible that other products which do not absorb at 330 nm are formed from NEO, but this could not be verified due to equipment limitations and insufficient quantities of PSP standards.

Figure 41. Chromatograms of products obtained by periodate oxidation of NEO.
Top: UV detection at 330 nm; bottom: fluorescence detection at λ_{ex} 330 nm, λ_{em} 400 nm.
Peak assignment for bottom chromatogram: A – unidentified peak, B – decarbamoyl product, C – carbamate product.
(Refer to products 4, 3, and 1 in Figure 14 for chemical structures.)

4.1.5.4 Changing the Concentration of PSP Standard

In order for the MnO_2 oxidation to be a useful analytical tool, the reaction should produce an amount of fluorescent product which can easily be related back to the original concentration of toxin present in the reaction mixture. Ideally, the response would be linear; that is, the reaction should be first order with respect to each PSP analogue. To verify this, a series of reactions were carried out using various concentrations of STX and NEO. Different temperatures were tested for STX. All these oxidations were performed at pH 8, which led to the formation of only the carbamate product for both STX and NEO. The results of this study are shown in Figure 42. In the concentration range tested, the reaction yield increased proportionally with the amount of PSP standard used.

4.1.5.5 Calculation of Rate Constants & Activation Energies

The rate constant and the energy of activation was calculated for each reaction using the information presented in sections 4.1.5.2 and 4.1.5.4 of this chapter. In order to do this, several assumptions had to be made:

- 1) The fluorescent purine was the only product being produced so that the peak height on the chromatogram was proportional to the amount of STX or NEO consumed (ie. a measure of how far the reaction had proceeded).
- 2) The surface area of MnO_2 available for reaction was proportional to the mass of the oxidant.
- 3) The rate of the reaction was constant during the course of the time period allotted; that is, the product yield (peak height) is proportional to the rate of the reaction provided that total time was the same for each reaction.

Since only fluorescent products could be quantitated, the first assumption has to be made so that any calculations could be performed at all. The results presented earlier in this

a

b

Figure 42. Reaction yield as a function of relative concentration (* 1 = 0.093 ng/ μ L) for the manganese dioxide oxidation of (a) STX and (b) NEO.

Note: The y-axes of the two graphs are not drawn to the same scale.

chapter did not prove or disprove the first assumption. To increase the confidence level in the second assumption, the MnO_2 was sieved to obtain particles of a more uniform size. In the STX experiments, the masses of MnO_2 were large enough that the approximation held true, but the small amounts of larger particles used in the NEO reactions led to uncertainty in the second assumption. The third approximation would be true for STX for times between 5 and 25 minutes according to Figure 38. A reaction time of 10 minutes was selected to ensure that the product yield was sufficiently high and that the waiting period was not excessively long. Since the curves for NEO in Figure 38 indicate that the reaction rate was not constant as the reaction proceeded, the initial rates of reaction were used in the calculations.

4.1.5.5.1 Calculations for STX

According to Arrhenius, the temperature dependence on the rate constant can be predicted by the following equation:

$$k = A \cdot \exp(-E_a/RT)$$

$$\ln k = \ln A - E_a/RT \quad \text{but } \ln A = \text{constant} = A'$$

$$\ln k = A' - (E_a/R) \cdot (1/T)$$

where k is the rate constant, A is a pre-exponential factor (which is almost temperature-independent), E_a is the activation energy, R is the universal gas constant, and T is the temperature in K.

The object of this set of calculations was to find the activation energy from the slope of the $\ln k$ vs. $1/T$ graph. Information from the STX graph in Figure 39 was used to obtain the data which is presented in Table 8. The peak height at 0.015 g MnO_2 was read off the STX graphs were divided by the reaction time (10 minutes) to obtain the average reaction rate (in cm/min). This rate was then divided by [STX] (0.0311 ng/ μL , after dilution with buffer solution) and the mass of MnO_2 (0.015 g) to obtain the

Table 8. Data and derived values obtained for STX from Figure 39. The rates of reaction for the oxidation of STX are calculated by dividing the peak heights obtained using 0.015 g of MnO₂ by the reaction time. The uncertainty on the temperature measurements was $\pm 0.5^\circ\text{C}$, and the uncertainty on the rates are based on the reproducibility of chromatographic analyses.

Temperature, T	Rate	Rate Constant, k	ln k
(K)	(cm/min)	$10^9 \times (\text{cm} \cdot \mu\text{L}/\text{min} \cdot \text{g}^2)$	—
± 0.5	$\pm 5\%$	$\pm 5\%$	± 0.05
324.5	0.14	300	5.70
333	0.19	407	6.01
337	0.24	514	6.24
343	0.29	622	6.43

Figure 43. Arrhenius plot for reaction of STX with manganese dioxide using data from Table 8. The slope and uncertainty were found using least squares method.

normalized rate constant, k . The Arrhenius plot of this data is shown in Figure 43 (on previous page). The experimental activation energy was found to be (37 ± 5) kJ/mol.

This set of calculations was repeated using the data obtained by changing [STX] which was shown in Figure 42. The reaction rates were found by dividing the peak heights at 0.093 ng/ μ L by the 10-minute reaction time. The normalized rate constants were obtained by multiplying by 3 to account for the dilution of STX and dividing by the mass of MnO_2 (0.02 g). The resulting data and graph are shown in Table 9 and Figure 44, respectively. The activation energy calculated using data obtained from Figure 42 was (38 ± 5) kJ/mol and is in close agreement to the previously calculated value. The differences between the reaction rates in the two STX studies (and thus the derived values in Tables 8 and 9) can be attributed to two factors: slight fluctuations in the reaction temperature ($\pm 0.5^\circ\text{C}$) and variability in the HPLC determinations ($\pm 5\%$).

4.1.5.5.2 Calculations for NEO

Calculations for NEO were performed using the data presented in Figure 38b. The initial reaction rates were obtained directly from this graph by using the first several data points that fell in the linear portion of each curve. The data and graph used to calculate E_a for the NEO oxidation are shown in Table 10 and Figure 45. The activation energy was found to be (56 ± 1) kJ/mol, which is higher than that calculated for STX.

The calculated rate constants indicate that the initial reaction rates for NEO are faster than those for STX (at 10 minutes). For example, the rate constant at 60°C for the NEO reaction is (4.438×10^{12}) cm $\cdot\mu\text{L}\cdot\text{g}^{-2}\cdot\text{min}^{-1}$ while the average value for the oxidation of STX is (0.405×10^{12}) cm $\cdot\mu\text{L}\cdot\text{g}^{-2}\cdot\text{min}^{-1}$. The difference is even greater when the surface area of MnO_2 is used instead of the mass. (Recall that larger MnO_2 particles were used in the NEO experiments.) To account for this difference, the NEO

Table 9. Data and derived values obtained for STX from Figure 42. The rates of reaction for the oxidation of STX are calculated by dividing the peak heights obtained using $0.093 \text{ ng}/\mu\text{L}$ of STX by the reaction time. The uncertainty on the temperature measurements was $\pm 0.5^\circ\text{C}$, and the uncertainty on the rates are based on the reproducibility of chromatographic analyses.

Temperature, T	Rate	Rate Constant, k	ln k
(K)	(cm/min)	$10^9 \times (\text{cm} \cdot \mu\text{L}/\text{min} \cdot \text{g}^2)$	—
± 0.5	$\pm 5\%$	$\pm 5\%$	± 0.05
324.5	0.18	289	5.67
333	0.25	402	6.00
337	0.33	530	6.27
343	0.38	611	6.41

Figure 44. Arrhenius plot for reaction of STX with manganese dioxide using data from Table 9. The slope and uncertainty were found using least squares method.

Table 10. Data and derived values obtained for NEC from Figure 38. The initial rates were calculated from the linear portions of the peak height vs. reaction time plots. The R^2 values for these rates range from 0.990-0.998. The uncertainty on the temperature measurements was $\pm 0.5^\circ\text{C}$, and the uncertainty on the rates are based on the reproducibility of chromatographic analyses.

Temperature, T	Rate	Rate Constant, k	ln k
(K)	(cm/min)	$10^9 \times (\text{cm } \mu\text{L}/\text{min g}^2)$	—
± 0.5	$\pm 5\%$	$\pm 5\%$	± 0.05
326	0.31	2507	7.83
333	0.55	4438	8.40
343	0.87	7056	8.86

Figure 45. Arrhenius plot for reaction of NEO with manganese dioxide using data from Table 10. The slope and uncertainty were found using least squares method.

rate constant would be multiplied by 3.4 which is the ratio of surface areas of the small-to-large MnO_2 particles (from section 4.1.5.2).

The difference in the rate constants of the STX and NEO oxidations might indicate that NEO is more reactive than STX, despite the fact that the sensitivity for NEO is lower than that for STX in most analytical methods [56, 60]. A more likely explanation for the discrepancy can be found in Figure 38. The STX graph shows that the reaction rate is fairly steady from 5-25 minutes, but the y-intercept of this straight line is not zero. This indicates that the initial reaction rate is faster (ie. the slope is steeper) than the average reaction rate after 10 minutes, which was the value used in the STX calculations. If the rate of the STX reaction at 69°C is assumed to be constant between 0 and 5 minutes, it would be approximately the same as the initial rate of the NEO reaction at 70°C (without accounting for the MnO_2 surface area ratio). It appears that the activation energies and the initial reaction rates of the STX and NEO oxidations are not significantly different, and thus their activation mechanisms may indeed be similar. Then, the differences in the trends observed in the STX and NEO experiments presented in this section would be due to rate-limiting steps, such as diffusion or desorption, which occur after the activation step.

4.1.6 Oxidation of Sample Extracts by Manganese Dioxide

The reaction between MnO_2 and PSP analogues was applied to analyze shellfish and plankton extracts for GTX2/3. These analogues were chosen because they were known to be present in the samples and, under the chosen reaction conditions (pH 10, 40°C , 20 minutes, and 0.1 g of MnO_2), GTX2/3 gave a single peak which no other PSP analogue would generate. STX and NEO were not used in this study because they both gave the same product and could not be quantitated separately. The chromatograms obtained for the products of MnO_2 oxidation of the sample extracts are shown in

Figure 46. The concentration of GTX2/3 for the plankton extract determined in this experiment agreed well with that obtained using the electrochemical and the pre-chromatographic periodate methods. The results for the two shellfish extracts, however, were 5-10 times lower than the concentrations found by the pre-chromatographic periodate method. The concentrations of GTX2/3 in the extracts were all within a range in which response was proportional to the concentration. It would appear that the reaction yield was somehow affected by the complex sample matrix of the shellfish. It was uncertain whether the diminished response is due to a phenomenon in the reaction mixture or in the transfer and filtration process. This matter was not subjected to further investigation, however, because it was decided that pre-chromatographic oxidation by MnO_2 would not be useful as an analytical tool. Besides possible matrix interferences, oxidation by MnO_2 led to multiple products for most toxin analogues, and the proportion of each product changed with pH and temperature. As well, some toxin analogues formed identical oxidation products. These factors would make the quantitation and identification processes very difficult.

4.2 Post-Column Experiments

Despite the problems encountered with analysis of sample extracts, the batch reaction studies indicated that MnO_2 had the potential to be a solid-phase oxidant for the post-column oxidation of PSP toxins. The advantage of the post-column mode is that multiple oxidation products need not be separated, identified, and quantitated. Instead, chromatographic separation is carried out on the parent compounds so that each individual PSP toxin elutes as a single peak. Each PSP analogue then passes through the reactor separately and undergoes oxidation to one or more products which are then quantitated together.

Figure 46. Chromatograms of sample extracts oxidized in batch reactions with manganese dioxide. The mussel sample had a total concentration $1 \mu\text{g}$ PSP per gram of tissue, while the clam extract contained $25 \mu\text{g/g}$ total PSP. The plankton chromatogram was qualitative only.

The solid-phase post-column reaction system for PSP toxins was assembled as described in section 5.2 of the Experimental chapter. The reactor consisted of an empty HPLC column packed with MnO_2 particles. Using the information obtained in the batch mode as a guide, various experimental parameters were evaluated.

4.2.1 Optimization of pH

The pH was changed by adjusting the pH of the buffer solution delivered by the first post-column pump. The reaction pH was measured by turning the acid pump off and sampling the effluent. The reactor was maintained at a temperature of 85°C with a column heater. The results of the pH study of STX, NEO, and GTX2/3 are shown in Figure 47. For the non-hydroxylated toxins, as the pH increased from 8-11, the yield of fluorescent products increased. At $\text{pH} > 11.5$, the sensitivity for STX and GTX2 decreased, but the sensitivity for GTX3 showed no signs of decreasing in the pH range studied. The NEO pH curve exhibited a slightly different behaviour as it reached a maximum at pH 10 and decreased for one pH unit and then began to rise again.

The effect of reaction pH on the sensitivity in the post-column reaction system is similar to the results obtained in the batch reactions. In order to compare the batch reactions to the post-column reactor, the yields of the various products for each toxin, taken from Figure 35, were added together to obtain the curves shown in Figure 48. STX and NEO were studied at approximately the same reaction temperatures in the batch and post-column experiments, so the curves shown in Figure 47 are almost identical to those in Figure 48. For GTX2/3, a lower temperature was used in the batch reactions than in the post-column reactor. Although the distribution of GTX2/3 oxidation products would not be the same at the two temperature, the total yield of fluorescent products exhibits a similar pattern.

Figure 47. Detector response for individual PSP toxins as a function of pH using the post-column manganese dioxide reaction system.

Figure 48. Combined peak heights of oxidation products of individual PSP toxins as a function of pH for batch reaction by manganese dioxide. This graph is the sum of the pH curves from Figure 35 for each analogue. Amounts injected: GTx2/3 (total 3.0 ng), NEO (1.3 ng), STX (1.1 ng)

4.2.2 Optimization of Reaction Time

Based on the findings of the batch experiments, the yield of fluorescent products will increase significantly for STX with longer reaction times, but the yield for NEO will remain essentially unchanged. While the reaction rate of the GTX2/3 pair was not studied extensively in the batch mode, results indicated that product yield increased with longer reaction times. Two different approaches were taken to change the reaction time in the post-column mode: 1) changing the volume of the reactor bed, and 2) changing the flow rate of the sample stream.

4.2.2.1 Changing the Dimensions of the Solid-Phase Reactor

HPLC columns of different lengths and internal diameters were packed with MnO_2 and tested in the post-column reaction system to determine the effect reactor geometry had on the yield of fluorescent products. It was observed that as the volume of the reactor increased, the reaction yield increased for STX and GTX2/3, but it did not change for NEO. Some results of this experiment are shown in Figure 49. These findings agree with those obtained in the batch reactions; STX continued to form fluorescent products with extended reaction times, while the NEO reaction proceeded to completion relatively quickly. Increasing the volume of the reactor had another effect on the HPLC results. The peaks became broader, as illustrated in the chromatograms of STX in Figure 50, due to the larger void volume in the reactor.

4.2.2.2 Adjusting the Mobile Phase Flow Rate

The first experiments with the post-column MnO_2 reactor were performed at a mobile phase flow rate of 1.0 mL/min. The trends observed in the first set of reaction time studies were confirmed by adjusting the flow rate of the mobile phase to 0.75 and then to 1.2 mL/min. For STX and GTX2/3, the longer reaction time generated larger

Figure 49. Change in response (cm²/ng) for various PSP toxins as the size of the post-column manganese dioxide reactor is changed

Figure 50. Chromatograms depicting the change in STX peak size and shape with change in reactor volume from 0.52 mL to 1.25 mL

chromatographic peaks, while the sensitivity for NEO remained unchanged. The drawback to lower mobile phase flow rates was the length of time required to elute the more polar toxins. A flow of 0.8 mL/min was chosen to balance out the advantage of higher reaction yields and the disadvantage of longer chromatographic runs.

4.2.3 Manganese Dioxide Particle Size

Initially, the reactor columns were packed with MnO_2 taken directly from the bottle. However, the degree of band-broadening in the system was worse than it had been for any of the electrochemical oxidation experiments. All the peaks during the chromatographic run, not just the late-eluting NEO and STX peaks, were broadened. As well, the peaks were not tailing, as they had in the electrochemical system, but were broadened equally at both the front and tail ends. It was deduced that the void volume in the post-column system (mostly from the reactor) was the cause of the peak-broadening.

To reduce the effects of this problem, the MnO_2 was ground by mortar and pestle to decrease the average size MnO_2 particles, allowing them to be packed into the reactor more densely. However, problems were encountered with obstruction of the end frit by very small MnO_2 particles. This difficulty was overcome by sieving the MnO_2 to particle size range of 45-74 μm after it had been ground. This process narrowed size distribution of the particles to a range which minimized the void volume and eliminated the blockage of the outlet frit.

The change in peak width was not the only benefit attained by grinding and sieving the MnO_2 . The peaks obtained for STX and GTX2/3 were larger due to the increase in MnO_2 surface area per unit volume in the reactor. The peak area for NEO did not change. These results agree with the results obtained in the batch experiments, in which the addition of more MnO_2 (ie. increased surface area available for reaction) led to higher reaction yields for STX and GTX2/3 but not for NEO.

4.2.3.1 Quantifying the Peak-Broadening Effect

Even after the changes described above were made, the peak shape was less than ideal. Further improvements could likely be made in the reactor packing efficiency but the required equipment was not available. In order to quantify the peak-broadening effect of the reactor, the HPLC system was set up to operate in the product-identification mode using a C-18 column, and the post-column hardware (pumps, mixing devices, and reactor) were left in place. A mixture of GTX2/3 and STX was oxidized by periodate in a vial, and the oxidation products were analyzed twice, once with the MnO_2 reactor on-line and the second time with a zero-volume union in place of the reactor. Since the pre-chromatographic periodate reaction gives nearly 100% yield of both STX and GTX2/3 oxidation products, the previously oxidized toxins did not react with the MnO_2 in the reactor. The chromatograms obtained in this study, shown in Figure 51, reveal that the peaks were twice as wide with the reactor in place as they were without the reactor. The peak areas in the two chromatograms are the same, indicating the assumption that the oxidation product would not react with the MnO_2 in the reactor was indeed valid.

4.2.4 Stability of the Solid-Phase Reactor

It was noticed that, after using a newly packed MnO_2 reactor for several days, the peak heights obtained for the non-hydroxylated toxin analogues decreased. A graph of the response vs. days of reactor use is shown in Figure 52. The reduction in sensitivity for STX was the greatest. (The GTX3 data was not included because the peak shape was poor and, thus, the peak heights obtained were not reproducible. Similar stability studies, performed previously with a different reactor, showed that the GTX3 response vs. time curve was very similar to that of GTX2.)

Figure 51. Chromatograms of a previously oxidized mixture of GTX2/3 and STX used to quantify the peak-broadening effect of the manganese dioxide reactor. The top chromatogram was obtained with the solid-phase reactor on-line. The reactor was replaced by a zero-dead volume union for the second chromatogram. (Peak identification: A – GTX2/3, B– STX)

Figure 52. Graph of response for individual PSP toxins as a function of days of use, illustrating the loss of reactivity of the manganese dioxide over time.

Figure 53. Graph of response for individual PSP toxins as a function of days of use after the periodate rinse.

It was originally thought that the decreased reactivity was due to the reduction of MnO_2 on the surface of the particles in the reactor. Attempts to regenerate the activity of the oxidant were made by flushing the reactor bed with a buffered periodate solution (0.01 M each periodate, ammonium formate, dibasic sodium phosphate, pH 8.0) for 2 hours at a rate of 0.5 mL/min. The reactor was then rinsed well with water and mobile phase prior to further use. The sensitivity of the method for the various toxins in the days after the regeneration attempt is shown in Figure 53 (on previous page). On the first day after the rinsing procedure, the sensitivity for STX and NEO was very poor, but the reaction yield for GTX2 (and GTX3, based on the assumption in the previous paragraph) was only reduced slightly from the pre-rinse eighth day value. Towards the end of the study, the sensitivity for STX and NEO seemed to be improving, but it began to decrease slightly for GTX2/3. It is uncertain whether the observed improvement in sensitivity for STX and NEO was due to increased reaction yield as a result of the periodate rinse. Since these improvements were not observed in a preliminary study of a similar nature (results not reported), there is a possibility that the changes resulted from an inadvertent change in chromatographic conditions. Since the regeneration process did not achieve the main goal of restoring the efficiency of the reactor for STX, no further studies were conducted in this area.

At the conclusion of this study, there was some doubt regarding the link between the decreased efficacy of the reactor and the consumption of the surface MnO_2 via reaction with PSP toxins. The drop in sensitivity was noticed, not so much during the course of the working day, but from day to day, after overnight storage in an acetonitrile-water mixture. It was then postulated that the drop in sensitivity was caused by reaction of the surface MnO_2 with a component of the storage solution. This theory was supported by an unintentional experiment in which a new MnO_2 reactor was packed but remained stored in degassed deionized water for a week. On its first day in use, this

reactor showed the same signs of deactivation as a reactor that had already been used for several days.

The stability problems were alleviated by continuous purging of the mobile phases and the alkaline buffer solution with helium to remove any dissolved gases. To decrease the chances of reaction of trace impurities in the storage solution with the MnO_2 , the reactor was stored in helium-purged mobile phase A (3 mM heptane sulfonate, 1.5 mM ammonium phosphate, pH 7), flowing at a rate of 0.01 mL/min. By following these new degassing and storage procedures, the reactivity of the MnO_2 column was preserved for at least 8 days of use over a 1.5 week period. Beyond this time, the reactor may lose its reactivity due to reaction with trace components of the mobile phase and native components of sample extracts. A slight reduction in sensitivity over a period of weeks should not pose a problem for monitoring purposes since PSP standards, analyzed daily, could be used to normalize the response. Another practical approach to the problem of decreased reactivity is to replace the reactor every week or two. The MnO_2 reactors are inexpensive and easily prepared.

4.2.5 Evaluation of the Analytical Method

4.2.5.1 Analytical Performance

After the experimental parameters had been optimized, the analytical performance of the post-column reactor was tested. Typical chromatograms of PSP standards, shown in Figure 54, reveal that the analogues studied were well separated. Standard A was obtained using Gradient I as outlined in the Experimental section. This gradient program was used for all the studies described in this section (except for those performed under isocratic conditions), but it had to be modified slightly (Gradient II) so that the earlier eluting toxins could be separated. Modification of the gradient caused a noticeable change in sensitivity for some toxins. This is probably due to the change in

Figure 54. Chromatograms of PSP standards obtained using post-column manganese dioxide reactor. Baseline drift is caused by the mobile phase gradient. Integrator attenuation = 5 for both chromatograms.

Standard A was separated using Gradient I. Amounts (ng) injected in 50 μ L: GTX3 (0.45), GTX2 (1.8), NEO (28), STX (0.70)

Standard B was separated using Gradient II. Amounts (ng) injected in 50 μ L: GTX4 (1.1), GTX1 (3.1), B1 (1.3), GTX3 (0.36), GTX2 (1.2)

composition of the mobile phase (percent acetonitrile) in which these toxins were eluted. In the batch experiments, it was shown that increased amounts of acetonitrile in the reaction mixture led to lower yields of fluorescent products for STX at elevated temperatures (see section 4.1.3 of this chapter).

Calibration curves for the various toxins are presented in Figure 55. (Note that the label on the x-axis is relative concentration of dilution factor, with the values of 1.0 given in Table 11.) The estimated LODs, based on a 3:1 signal-to-noise ratio, and the subsequent minimum detectable concentrations in shellfish tissue are given in Table 12. These numbers are comparable to those obtained in other post-column reactors, as illustrated by the comparison in Table 13. The LODs for the MnO_2 reaction system could probably be improved by decreasing the degree of peak broadening through the use of a more efficiently packed reactor column. This would likely lead to better reproducibility than the within-day repeatability of $\leq 10\%$ (relative standard deviation).

4.2.5.2 Application of Method to Real Samples

The post-column MnO_2 reaction system was used to analyze some extracts of shellfish and plankton which had previously been quantitated by other methods. The chromatograms of the extracts are shown in Figure 56. The calculated concentrations of PSP toxins in a contaminated clam sample, along with those obtained using other methods, are given in Table 14. Generally, the MnO_2 results are in good agreement with other HPLC results.

The greatest contribution to inaccurate and imprecise results in the post-column MnO_2 method would be the broad chromatographic peaks which may have led, at times, to poor correlation between peak height and toxin concentration. The day-to-day change in the efficiency of the reactor, as discussed in section 4.2.4 of this chapter, did not pose a problem since PSP standards were used to normalize the response daily. (The within-

Figure 55. Calibration curve for post-column manganese dioxide reaction system.

* The label on the x-axis is relative concentration (or dilution factor). The values for 1.0 are given below in Table 11.

Table 11. Concentrations of PSP standards used to obtain calibration curve in Figure 55

PSP Toxin	Concentration (ng/ μ L)
STX	0.014
NEO	0.56
GTX2	0.036
GTX3	0.009

Table 12. Estimated limits of detection for PSP toxins (based on 3:1 signal to noise ratio) and minimum detectable concentrations (based on total dilution factor of 30 for samples contaminated near the regulatory limit of 0.8 ppm) for the post-column manganese dioxide reaction method.

Toxin	Limit of Detection	Minimum Detectable Concentration
—	(ng/50- μ L injection)	(μ g/g)
STX	0.11	0.07
NEO	1.40	0.84
GTX1	1.00	0.60
GTX2	0.09	0.05
GTX3	0.22	0.13
GTX4	0.50	0.30
B1	0.65	0.39

Table 13. Comparison of post-column reaction systems for PSP analysis. The limits of detection are given in ng/injection for each method. (* n/a = not available)

Toxin	Periodate	Electrochemical	MnO ₂
STX	0.09	0.10	0.11
NEO	0.70	0.84	1.40
GTX1	1.15	2.10	1.00
GTX2	0.04	0.09	0.09
GTX3	0.04	0.05	0.22
GTX4	n/a*	0.70	0.50
B1	0.38	0.50	0.65
B2	n/a	15.0	n/a

Figure 56. Chromatograms of sample extracts obtained using post-column MnO_2 reaction system. The clam sample contained a total PSP concentration of $25 \mu\text{g/g}$, while the mussel tissue was contaminated near the regulatory limit of $0.8 \mu\text{g/g}$. The plankton sample was for qualitative purposes only, but the solution injected contained $0.02 \text{ ng}/\mu\text{L}$ GTX3. Integrator attenuation = 7 for the clam sample, 5 for the mussel sample, and 4.5 for the plankton sample.

day precision was not a problem.) Although the use of the MnO_2 reactor with shellfish samples has not been studied extensively, preliminary results indicate that it has the potential to be a useful analytical method for the determination of PSP toxins.

Table 14. Concentration (in $\mu\text{g/g}$) of various PSP toxins found in clam sample using MnO_2 reactor and other HPLC methods. The MnO_2 analyses were carried out over consecutive four days, with duplicate injections being performed each day.

Method	GTX3	GTX2	NEO	STX
Post-col. MnO_2	1.03 ± 0.07	1.98 ± 0.08	3.40 ± 0.17	4.76 ± 0.44
Electrochemical ¹	1.2	0.72	5.0	4.7
Pre-col. periodate ²	— 3.9 —		2.1 ⁵	5.8
Pre-col. periodate ³	— 1.8 —		3.2 ⁵	3.3
Post-col. periodate ⁴	— 5.6 —		2.8 ⁵	4.2

1. Standard deviations were not calculated because analysis was only done in duplicate
2. From a previous study, 1990
3. Analysis repeated for confirmation, 1995
4. GTX2/3 and NEO/B2 were determined separately but were quantitated together in report from 1990.
5. Includes concentration of B2

5. Future Work

Because there is so much about PSP toxins which is unknown, this field of research is a very active one. In the area of analytical chemistry, there is a need to study the separation of the toxins to find a replacement for the troublesome ion-pair chromatographic conditions used in post-column reaction systems. New packing materials for HPLC columns may prove helpful in the search for an improved method.

Another aspect of the analytical chemistry which might be studied is the use of other oxidants for derivatization of PSP toxins. Since peroxide and periodate were used successfully, it may be prudent to try peroxy derivatives such as peroxyacetic acid and peroxybenzoic acid. As well, photochemical oxidation of the toxins could be studied.

This type of reactor has been employed for on-line reactions of a similar nature and would eliminate the problems associated with the addition of post-column reagents using pumps.

Further research directly relating to the thesis research might include identification of the products of electrochemical and MnO_2 oxidation by LC-MS/MS as was done for the periodate oxidation products [63]. It may also be possible to use the reactions studied to develop a post-column reactor for capillary electrophoresis (CE). There have been no reports of the use of the post-column oxidation-fluorescence detection scheme for PSP toxins in CE methods to date. Reducing the size of the reactors to make them suitable for use in CE would likely be very challenging, but on-line oxidation would lead to an improvement the detection limits of PSP toxins in CE methods, as it did in HPLC.

Chapter 4

Conclusions

1. Electrochemical Oxidation

A coulometric HPLC detector was successfully used as a post-column electrochemical reactor for oxidation of PSP toxins to fluorescent derivatives. The toxins were first separated by ion-pair chromatography, oxidized at the anode of the reactor, and then monitored by a fluorescence detector. Various reaction parameters were studied and optimized.

The pH of the solution in the reactor was adjusted by introducing an alkaline buffer solution into the effluent by use of a post-column pump. As the reaction pH increased, the yield of fluorescent products improved for the non-hydroxylated toxins (STX and GTX2/3). The hydroxylated toxins could not be detected under these conditions. When a reaction pH of 7 was selected, no post-column pumps were needed because the mobile phase had a pH of 7. Under these reaction conditions, the baseline noise was reduced, and all toxin analogues were detectable.

The potential of the electrochemical reactor was studied in the range of 0.60 to 0.85 V. For the non-hydroxylated toxins, the reaction yield increased with higher voltages. As the potential was increased in this range, the hydroxylated toxins initially showed an increase in yield, but a maximum was observed at approximately 0.70 V, after which the yield decreased.

The effect of higher reaction temperatures was studied by heating the coulometric detector. When the temperature was increased slightly above ambient temperature (30-35°C), higher reaction yields were obtained for STX and NEO. The product yield decreased as the temperature was raised beyond 40°C. This effect, however, may have been due to technical problems with the electrochemical reactor.

The reaction time was adjusted by changing the flow rate of the mobile phase or by turning off one of the two electrodes in series in the reactor. In both cases, the yield of fluorescent products increased with longer reaction times.

An improvement in the STX peak shape was achieved by heating the column and by increasing the concentration of ion-pair agent in the mobile phase. It was determined that the band-broadening occurred in the HPLC column and not in the post-column components of the system.

Identification of the products of electrochemical oxidation was carried out by comparison of retention times with those of periodate-oxidized PSP standards. The electrochemical oxidation products had structures similar to those products obtained in the periodate reaction.

The limits of detection for PSP toxins of the post-column electrochemical reaction system were in the same range as those reported for the post-column periodate reactor. The N-1-OH toxins were approximately an order of magnitude less detectable than their non-OH counterparts. The limit of detection for STX was 0.1 ng per injection. The system was successfully used to analyze shellfish and plankton sample extracts, yielding results which were in agreement with those obtained using established HPLC methods. These studies indicate that the on-line electrochemical reactor might be useful for routine use in a monitoring program.

2. Batch Reactions with MnO_2

The oxidation of PSP toxins to fluorescent purine derivatives was carried out using solid manganese dioxide. These reactions were performed in small vials, and the oxidation products were analyzed by reverse-phase HPLC. Chromatographic peaks, identified by co-elution with periodate-oxidized PSP standards, indicated that the products of MnO_2 oxidation were similar to those obtained in the periodate reaction.

The effect of various reaction parameters on the yield of fluorescent products was investigated. As the temperature increased, the yield of product increased for STX and NEO, but GTX2/3 generated multiple products at elevated temperatures. The effect of reaction pH on the yield of fluorescent product was studied in the range of 7-12. As the pH increased from 7-9, the yield of fluorescent product improved for all toxins studied. At pH levels higher than this, each analogue yielded multiple products.

It was observed that the STX reaction with MnO_2 was first order with respect to both MnO_2 (surface area available for reaction) and STX concentration. The reaction rate (measured as appearance of fluorescent product) was constant for times between 5 and 25 minutes. The initial reaction rate, however, would seem to be faster than that measured experimentally.

In contrast, the NEO reaction was not affected by the amount of MnO_2 in the reaction mixture. Another difference observed was that the production of fluorescent compounds from NEO ceased after approximately 5 minutes. It is possible, however, that the unusual reaction kinetics of STX at times < 5 minutes may be similar to that of NEO. The reaction was found to be first order in the concentration of NEO. Experimental rate constants and activation energies were calculated for the STX and NEO reactions.

3. Post-Column MnO_2 Reactor

A solid-phase reactor was constructed by packing an empty HPLC column with solid MnO_2 . Post-column pumps were used to introduce pH-adjusting solutions to the effluent. The effect of pH on the detectability of each PSP analogue was studied. As the pH increased, sensitivity for most of the toxins improved. At pH > 11, the sensitivity began to decrease for STX and GTX2. The detectability of NEO reached a local

maximum at pH 10, dropped for one pH unit, and began to rise again. The post-column pH results for all analogues follow the same trends observed in the batch experiments.

The reaction time was changed by adjusting the flow rate of the sample stream through the reactor bed and by using reactors of different volumes (changing the reactor residence time). Both sets of experiments generated similar results, which agreed with the findings of the batch studies. The yield of fluorescent derivatives increased with longer reaction times for STX and GTX2/3 but remained unchanged for NEO.

It was found that the effects of peak broadening could be reduced by grinding and sieving the solid oxidant before packing the reactor column. The use of smaller particles provided a second benefit – the sensitivity for STX and GTX2/3 improved because of the increase in specific surface area of the MnO_2 .

The reaction efficiency of the MnO_2 reactor was noticed to decrease with time. It was found that the stability and thus the lifetime of the reactor could be extended by constant purging of the mobile phases with helium and keeping the HPLC system (including the reactor) in mobile phase A at 0.01 mL/min when not in use.

The detection limits achieved using the post-column MnO_2 reactor were comparable to those obtained using the electrochemical and periodate reactors. As was the case with other HPLC methods, the N-1-OH toxins were about 10x less detectable than the non-OH analogues. The limit of detection for STX was 0.1 ng per injection. The MnO_2 reaction system was used to analyze PSP-contaminated plankton and shellfish samples, yielding results that were close to those produced by other methods. These preliminary studies show that the post-column MnO_2 reactor might be a useful method for routine monitoring purposes.

Bibliography

1. S. Hall, G. Strichartz, E. Moczydlowski, A. Ravindran, and P.B. Reichardt, in *Marine Toxins*, S. Hall and G. Strichartz, Eds., American Chemical Society, Washington, DC, 1990, pp. 29-65.
2. J. I. Smith, in *Handbook of Natural Toxins 7: Food Poisoning*, A.T. Tu, Ed., Marcel Dekker, New York, 1992, pp. 401-414.
3. E.V. Morse, *J. Am. Veterin. Med. Assoc.*, **171**, 1178 (1977).
4. G. Vancouver, *A Voyage of Discovery to the North Pacific Ocean and Round the World*, Vol. 2, Robinson, London, 1798, pp. 284-286.
5. T. Shibamoto and L.F. Bjeldanes, *Introduction to Food Toxicology*, Academic Press, San Diego, CA, 1993, pp. 54-58.
6. F.W. Tanner and L.P. Tanner, *Food-Borne Infections and Intoxications*, 2nd Ed., Barrard Press, Champaign, IL, 1953, pp. 126-129.
7. C.Y. Kao, in *Algal Toxins in Seafood and Drinking Water*, I.R. Falconer, Ed., Academic Press, London, 1993, pp. 75-86.
8. D.M. Anderson, *Sci. Am.*, **271**, 62-68 (1994).
9. B.D. Gessner and J.P. Middaugh, *Am. J. Epidemiol.*, **141**, 766-770 (1995).
10. A.W. White, J. Nassif, S.E. Shumway, and D.K. Whittaker, in *Toxic Phytoplankton Blooms in the Sea*, T.J. Smayda and Y. Shimizu, Eds., Elsevier, Amsterdam, 1993, pp. 435-440.
11. R.A. Corrales and E.D. Gomez, in *Toxic Marine Phytoplankton*, E. Granéli, B. Sunström, L. Edler, D.M. Anderson, Eds., Elsevier, New York, 1990, pp. 453-458.
12. Q. Adnan, in *Toxic Phytoplankton Blooms in the Sea*, T.J. Smayda and Y. Shimizu, Eds., Elsevier, Amsterdam, 1993, pp. 199-202.
13. A. La Barbera-Sánchez, S. Hall, and E. Ferraz-Reyes, in *Toxic Phytoplankton Blooms in the Sea*, T.J. Smayda and Y. Shimizu, Eds., Elsevier, Amsterdam, 1993, pp. 281-286.
14. E.C.D. Todd, T. Kuiper-Goodman, W. Watson-Wright, M.W. Gilgan, S. Stephen, J. Marr, S. Pleasance, M. A. Quilliam, H. Klix, H.A. Luu, and C.F. B. Holmes, in *Toxic Phytoplankton Blooms in the Sea*, T.J. Smayda and Y. Shimizu, Eds., Elsevier, Amsterdam, 1993, pp. 335-339.
15. T.J. Smayda, in *Toxic Marine Phytoplankton*, E. Granéli, B. Sunström, L. Edler, D.M. Anderson, Eds., Elsevier, New York, 1990, pp. 29-40.

16. G.R. Rigby, I.G. Steverson, C.J. Bolch, and G.M. Hallegraeff, in *Toxic Phytoplankton Blooms in the Sea*, T.J. Smayda and Y. Shimizu, Eds., Elsevier, Amsterdam, 1993, pp. 169-176.
17. Y. Shimizu, S. Gupta, and H.-N. Chou, in *Marine Toxins*, S. Hall and G. Strichartz, Eds., American Chemical Society, Washington, DC, 1990, pp. 21-28.
18. J.J. Sullivan, M.M. Wekell, and S. Hall, in *Handbook of Natural Toxins 3: Marine Toxins and Venoms*, A.T. Tu, Ed., Marcel Dekker, New York, 1988, pp. 87-106.
19. Y. Shimizu, in *Handbook of Natural Toxins 3: Marine Toxins and Venoms*, A.T. Tu, Ed., Marcel Dekker, New York, 1988, pp. 63-89.
20. Y. Shimizu in *Progress in the Chemistry of Natural Products*, Vol. 45, W. Herz, H. Grisebach, and G.W. Kirby, Eds., Springer-Verlag, New York, 1984, pp. 235-264.
21. Association of Official Analytical Chemists, *Official Methods of Analysis*, 16th Ed., Vol. II, AOAC International, Arlington, VA, 1995, Section 35.1.37.
22. T. Harada, Y. Oshima, and T. Yasumoto, *Toxicon*, **22**, 476 (1984).
23. J.M. Hungerford and M.M. Wekell, in *Handbook of Natural Toxins 7: Food Poisoning*, A.T. Tu, Ed., Marcel Dekker, New York, 1992, pp. 415-473.
24. R.S. Rogers and H.J. Rapoport, *J. Am. Chem. Soc.*, **102**, 7335 (1980).
25. Y. Shimizu, C.-P. Hsu, W.E. Fallon, Y. Oshima, I. Miura, and K. Nakanishi, *J. Am. Chem. Soc.*, **100**, 6791 (1978).
26. W.E. Fallon and Y. Shimizu, *J. Environ. Sci. Health A*, **12**, 455.
27. Y. Shimizu, C.P. Hsu, and A. Genenah, *J. Am. Chem. Soc.*, **103**, 605 (1981).
28. National Research Council of Canada, Marine Analytical Chemistry Standards Program, literature accompanying calibration solutions for paralytic shellfish poisoning toxins (PSP-1), 1994.
29. G. Strichartz and N. Castle, in *Marine Toxins*, S. Hall and G. Strichartz, Eds., American Chemical Society, Washington, DC, 1990, pp. 2-20.
30. J.J. Sullivan, in *Algal Toxins in Seafood and Drinking Water*, I.R. Falconer, Ed., Academic Press, London, 1993, pp. 29-48.
31. H. Sommer and K.F. Meyer, *Arch. Pathol.*, **24**, 560.
32. E.J. Schantz, E.F. McFarren, M.L. Schafer, and K.H. Lewis, *J. Assoc. Off. Anal. Chem.*, **41**, 160 (1958).

33. A.W. McCulloch, R.K. Boyd, A.S.W. deFreitas, R.A. Foxall, W.D. Jamieson, M.V. Laycock, M.A. Quilliam, J.L.C. Wright, V.J., Boyko, J.W. McLaren, M.R. Miedema, R. Pocklington, E. Arsenault, and D.J. A. Richard, *J. Assoc. Off. Anal. Chem.*, **72**, 384 (1989).
34. A. Siger, B.C. Abbott, and M. Ross, in *Seafood Toxins*, E.P. Ragelis, Ed., American Chemical Society, Washington, DC, 1984, pp. 193-196.
35. M. Ross, A. Siger, and B.C. Abbott, in *Toxic Dinoflagellates*, D.M. Anderson, A.W. White, and D.G. Baden, Eds., Elsevier, New York, 1985, pp. 433-438.
36. A. Cembella, Y. Parent, D. Jones, and G. Lamoureaux, in *Toxic Marine Phytoplankton*, E. Granéli, B. Sunström, L. Edler, and D.M. Anderson, Eds., Elsevier, New York, 1990, pp. 339-344.
37. E. Usleber, R. Dietrich, E. Märtlbauer, and G. Terplan, *Lett. Appl. Microbiol.*, **18**, 337 (1994).
38. E. Usleber, E. Schneider, G. Terplan, and M.V. Laycock, *Food Addit. Contam.*, **12**, 405 (1995).
39. M.R. Vieytes, A.G. Cabado, A. Alfonso, M.C. Louzosa, A.M. Botana, and L.M. Botana, *Anal. Biochem.*, **211**, 87 (1993).
40. K. Daigo, T. Nogichi, N. Kawai, A. Miwa, and K. Hashimoto, in *Red Tides*, T. Okaichi, D.M. Anderson, and T. Nemoto, Eds., Elsevier, New York, 1989, pp. 387-389.
41. J.F. Jellett, J.E. Stewart, M.V. Laycock, *Toxic. in Vitro*, **9**, 57 (1995).
42. M.A. Quilliam, personal communication.
43. T. Narahashi, M.L. Roy, and K.S. Ginsburg, *NeuroToxicology*, **15**, 545 (1994).
44. E.F. McFarren, E.J. Schantz, J.E. Campbell, and K.H. Lewis, *J. Assoc. Off. Anal. Chem.*, **41**, 168 (1958).
45. R.M. Gershey, R.A. Neve, D.L. Musgrave, and P.B. Reichardt, *J. Fish. Res. Board Canada*, **34**, 559 (1977).
46. H.A. Bates and H. Rapoport, *J. Agric. Food Chem.*, **23**, 237 (1975).
47. H.A. Bates, R. Kostriken, and H. Rapoport, *J. Agric. Food Chem.*, **28**, 252 (1978).
48. J.L. Wong, M.S. Brown, K. Matsumoto, R. Oesterlin, and H. Rapoport, *J. Am. Chem. Soc.*, **93**, 4633 (1971).
49. B.W. Wright, G.A. Ross, and R.D. Smith, *J. Microcolumn Sep.*, **1**, 85 (1989).
50. P. Thibault, S. Pleasance, and M.V. Laycock, *J. Chromatogr.*, **542**, 483 (1991).

51. Y. Onoue, T. Noguchi, Y. Nahashima, K. Hashimoto, S. Kanoh, M. Ito, K. Tsukada, *J. Chromatogr.*, **257**, 373 (1983).
52. J.F. Lawrence and C. Ménard, unpublished work.
53. J.J. Sullivan, M.M. Wekell, and J.E. Wiskerchen, in *2nd World Congress, Foodborne Infections and Intoxications. Proceedings*, Institute of Veterinary Medicine, Berlin, 1986.
54. J.J. Sullivan and W.T. Iwaoka, *J. Assoc. Off. Anal. Chem.*, **66**, 297 (1983).
55. J.J. Sullivan and M.M. Wekell, in *Seafood Quality Determination*, D.E. Kramer and J. Liston, Eds., Univ. of Alaska, Sea Grant College Program, Anchorage, 1987, pp. 357-371.
56. J.J. Sullivan, in *Marine Toxins*, S. Hall and G. Strichartz, Eds., American Chemical Society, Washington, DC, 1990, pp. 66-77.
57. Y. Oshima, *J. AOAC Int.*, **78**, 528 (1995).
58. J.M. Franco and P. Fernández-Vila, *Chromatographia*, **35**, 613 (1993).
59. J.F. Lawrence, C. Ménard, C. Charbonneau, and S. Hall, *J. Assoc. Off. Anal. Chem.*, **74**, 404 (1991).
60. J.F. Lawrence and C. Ménard, *J. Assoc. Off. Anal. Chem.*, **74**, 1006 (1991).
61. J.F. Lawrence, C. Ménard, and C. Cléroux, *J. AOAC Int.*, **78**, 514 (1995).
62. J.F. Lawrence, C. Ménard, and B. Wong, *J. AOAC Int.*, in press.
63. M.A. Quilliam, M. Janeček, and J.F. Lawrence, *Rapid. Commun. in Mass Spectrom.*, **7**, 482 (1993).
64. M. Janeček, M.A. Quilliam, and J.F. Lawrence, *J. Chromatogr.*, **644**, 321 (1993).
65. *Reaction Detection in Liquid Chromatography*, I.S. Krull, Ed., Marcel Dekker, New York, 1986.
66. *Detection-Oriented Derivatization Techniques in Liquid Chromatography*, H. Lingeman and W.J.M. Underberg, Eds., Marcel Dekker, New York, 1990.
67. E.J. Wolff in *A Practical Guide to HPLC Detection*, D. Parriott, Ed., Academic Press, San Diego, 1993, pp. 211-232.
68. J. Janiszewski and G.L. Boyer, in *Toxic Phytoplankton Blooms in the Sea*, T.J. Smayda and Y. Shimizu, Eds., Elsevier Science Publishers, New York, 1993, pp. 889-894.

69. M. Hudlický, *Oxidations in Organic Chemistry*, American Chemical Society, Washington, DC, 1990.
70. S.D. Ross, M. Finkelstein, and E.J. Rudd, *Anodic Oxidation*, Academic Press, New York, 1975.
71. *Instruction Manual*, Coulochem Detector (Model 5100A), ESA, Inc., Bedford, MA, 1987.
72. J. Li, L.M. Litwinson, and F.F. Cantwell, *J. Chromatogr. A*, **726**, 25 (1996).
73. B.A. Bidlingmeyer, *J. Chromatogr. Sci.*, **18**, 525 (1980).
74. V.R. Meyer, *Practical High-Performance Liquid Chromatography*, John Wiley and Sons, New York, 1994.
75. H.P. vanEgmond, H.J. van denTop, W.E. Paulsch, X. Goenaga, and M.R. Vieytes, *Food Adit. Contam.*, **11**, 39 (1994).
76. E.F. Pratt and J.F. Van deCastle, *J. Org. Chem.*, **26**, 2973 (1961).
77. *The Merck Index*, 10th Ed., M. Windoliz, S. Budavari, R.F. Blumetti, and E.S. Otterbein, Eds., Merck & Co., Inc., Rahway, NJ, 1983.
78. L.F. Fieser and M. Fieser, *Reagents for Organic Synthesis*, John Wiley and Sons, New York, 1967.
79. J.L. Allen, J.R. Meinertz, and J.E. Gofus, *J. AOAC Int.*, **75**, 646 (1992).
80. L.L. Zamora, J.V.G. Mateo, and J.M. Calatayud, *Anal. Chim. Acta*, **265**, 81 (1992).
81. A. Kojlo and J.M. Calatayud, *Talanta*, **42**, 909 (1995).
82. C.S. Blanco, Ph.D. Thesis, Universidad de Santiago, 1993.
83. W.D. Powrie and R.J. Bose, unpublished contract work (# OSZ 79-00267), submitted to Health & Welfare Canada, 1982.

END

1 6 10 6-9 7

FIN