Journal of Chromatography, 644 (1993) 321–331
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 248

Analysis of paralytic shellfish poisoning toxins by automated pre-column oxidation and microcolumn liquid chromatography with fluorescence detection☆

Mojmir Janeček☆☆ and Michael A. Quilliam*

Institute for Marine Biosciences, National Research Council of Canada, 1411 Oxford Street, Halifax, Nova Scotia B3H 3Z1 (Canada)

James F. Lawrence

Health Protection Branch, Food Research Division, Bureau of Chemical Safety, Food Directorate, Ottawa, Ontario, K1A 0L2 (Canada)

(Received April 19th, 1993)

ABSTRACT

Periodate oxidation of the toxins responsible for paralytic shellfish poisoning (PSP) yields fluorescent purines suitable for trace analysis by reversed-phase LC. Mobile phases containing perfluorinated acids, such as heptafluorobutyric acid, as ion-pair agents were found to provide high capacity factors for the oxidized products. Gradient elution on a microbore column with large volume injections and fluorescence detection permitted the detection of femtomole quantities of PSP toxins. A fully automated pre-column oxidation procedure was developed for an LC autosampler system in order to improve precision and allow unattended analyses. The complete method was applied successfully to various samples, including shellfish and toxic phytoplankton.

INTRODUCTION

Paralytic shellfish poisoning (PSP) is a worldwide problem caused by consumption of shellfish that have accumulated potent neurotoxins produced by toxigenic dinoflagellates, such as those belonging to the genus *Alexandrium* [1]. The PSP toxins include saxitoxin (STX) and several of its derivatives formed by addition of sulfo, hydroxysulfate and N-1-hydroxyl groups (Fig. 1).

The AOAC mouse bioassay is used routinely by regulatory laboratories for the determination of PSP toxins [2]. Although this method has the advantage of being non-selective and therefore well suited for protection of the public, it is recognized that bioassay suffers from considerable variability [3] and gives little information on toxin composition. In addition, there is pressure from animal rights groups to discontinue such tests; some European countries have banned them already.

The most commonly used chemical method for the analysis of PSP toxins is the combination of liquid chromatography (LC) with on-line post-column oxidation and fluorescence detection [4,5]. This approach evolved from earlier work by Bates and Rapoport [6] which showed that STX could be oxidized to a fluorescent purine by

* Corresponding author.
☆ NRCC No. 34869.
☆☆ On leave from Institute of Analytical Chemistry, Czech Academy of Sciences, Brno, Czech Republic.

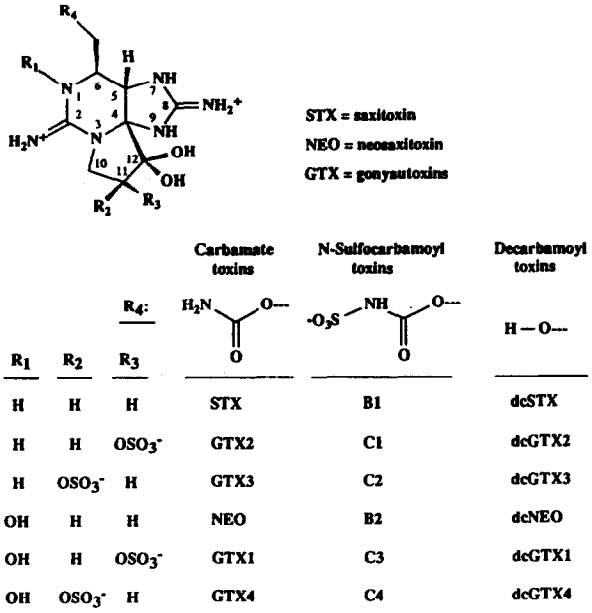


Fig. 1. Structures of the principal toxins responsible for paralytic shellfish poisoning (PSP).

hydrogen peroxide under alkaline conditions. Since this original procedure proved unsuitable for the N-1-hydroxylated PSP toxins, better oxidizing agents such as *tert.*-butyl hydroperoxide [4] or periodate [5] are used in post-column oxidation systems in order to detect all PSP toxins. Unfortunately, the set up and operation of such equipment is quite complex and requires considerable daily maintenance.

Recently, an alternative LC technique was reported [7,8]. The method involves a pre-column oxidation of the toxins followed by reversed-phase gradient elution and fluorescence detection of the oxidized products. Since the products are more amenable to reversed-phase LC than the precursor toxins, an octadecylsilica (C_{18}) column may be used to obtain better separation efficiencies than those possible using the PRP-1 column recommended in the most commonly used post-column method [5]. A mobile phase containing aqueous ammonium formate was found to provide good peak shape and reproducibility. In addition, the composition of the reaction mixture for pre-column periodate oxidation was optimized to improve fluorescent product yield and a solid-phase extraction clean-up was established to reduce interference from co-extractives and to perform group separations of the toxins [8].

The objectives of the work described here were as follows: (a) to investigate additional mobile phases for the reversed-phase chromatography of oxidized PSP toxins; (b) to improve the sensitivity and reproducibility of the analysis by using a microbore column, large volume injection, and fully automated pre-column oxidation reaction; and (c) to apply the method to shellfish and phytoplankton samples.

EXPERIMENTAL

Chemicals

Trifluoroacetic acid and acetonitrile were purchased from BDH (Poole, UK), heptafluorobutyric acid (HFBA) and periodic acid from Sigma (St. Louis, MO, USA), and tridecafluoroheptanoic acid from Aldrich (Milwaukee, WI, USA). Distilled water was further purified by passage through a Milli-Q (Millipore, Bedford, MA, USA) water purification system equipped with ion-exchange and carbon filters. All other reagents were of analytical-reagent grade. Purified standards of PSP toxins were kindly provided by Dr. M.V. Laycock (Institute for Marine Biosciences, Halifax, Canada).

Equipment

Chromatography was performed using an HP1090M liquid chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a ternary DR5 solvent delivery system, variable volume injector/autosampler, and automated pre-column sample preparation system. The system was controlled by an HP7994A Pascal ChemStation, which enables the user to customize and automate various sample manipulations including pre-column derivatizations at ambient and elevated temperatures. The LC effluent was monitored with an HP1046A dual monochromator fluorescence detector fitted with the standard 4.5-μl flow cell. Stainless steel capillaries with internal diameter of 0.12 mm served as connection tubing.

A digital PMH63 pH meter (Radiometer, Copenhagen, Denmark) was used for the measurement of pH of mobile phases and oxidation mixtures. A Model 5415 microcentrifuge (Brinkmann, Westbury, NY, USA) was used for sample preparation. A vacuum manifold system was used for solid-phase extraction clean-up (Supelco, Bellefonte, PA, USA).

LC columns

Stainless-steel columns (25 cm × 1.0 mm I.D. and 10 cm × 2.1 mm I.D.) were packed by the slurry technique using an ethanol–glycerol (50:50, v/v) suspension under a pressure of 50 MPa. LiChrospher-100 RP-18 (Merck, Darmstadt, Germany) with particle diameter of 5 μm was used as a sorbent. Packing solvent (ethanol) was delivered by a single piston air-driven pump (Shandon, Cheshire, UK). Packed columns were tested on an apparatus consisting of an MPLC Micropump (Applied Biosystems, Santa Clara, CA, USA), a manual injector fitted with 0.5-μl loop (Valco, Houston, TX, USA) and a μLC-10 UV spectrophotometer with 0.5-μl flow cell (ISCO, Lincoln, NB). Anthracene was used for measurement of column efficiency, with a mobile phase of aqueous 80% acetonitrile at a flow-rate of 50 or 200 μl/min (for 1.0 or 2.1 mm I.D. columns, respectively) and detection at 260 nm.

Sample extraction

Shellfish tissue samples were extracted in 0.1 *M* HCl according to the AOAC mouse bioassay procedure [2]. Extracts were cleaned using a LC-18 octadecylsilica solid phase extraction cartridge (Supelco) [7] or by ultracentrifugation through a 10 000 NMWL filter (Millipore, Bedford, MA, USA) at 10 000 *g* for 20 min. Toxic phytoplankton extracts were kindly provided by Dr. A.D. Cembella (Institute for Marine Biosciences, Halifax, Canada). Cells of the marine dinoflagellate *Alexandrium* in unialgal culture (isolate Gt429, CCMP Collection, Bigelow Labs., Boothbay Harbour, ME, USA) and in natural mixed phytoplankton assemblages (from Gaspé, Québec, Canada) were sonicated in 0.03 *M* acetic acid followed by centrifugation (10 000 *g*, 10 min) and filtration (0.22-μm Millex filter, Millipore).

Pre-column oxidation and LC analysis

Sample derivatization was based on a periodate oxidation which converts all PSP toxins to fluorescent derivatives. The composition of the oxidation mixture and procedures for manual reaction were the same as described earlier [8]. The oxidation mixture was prepared daily. Automated reactions and analyses were carried out with reagents and samples placed in individual crimp-top plastic vials in the HP1090 autosampler. The latter were controlled through the "Injector Program" which is part of the standard HP7994A ChemStation software. The details of

TABLE I

INJECTOR PROGRAM USED FOR AUTOMATED PRE-COLUMN OXIDATION REACTION AND INJECTION

LC conditions: 25 cm × 1.0 mm I.D. column packed with 5 μm LiChrospher-100 RP18 (N = 11 000); mobile phase: A = 10 mmol/l HFBA in water adjusted to pH 4.2 with NH_4OH, B = acetonitrile, gradient from 0 to 20% B over 20 min; 100 μl/min flow-rate; fluorescence detection (335 nm excitation, 400 nm emission). A 12-min column equilibration time was used between analyses. Vial 0 = Periodate oxidation reagent (1:1:1 mixture of 0.03 *M* periodic acid, 0.3 *M* Na_2HPO_4 and 0.3 *M* ammonium formate adjusted to pH 9.0 with 1 *M* NaOH; prepared daily); vial 1 = water for needle rinse; vial 2 = acetic acid (1:1 mixture of water and glacial acetic acid).

Line	Function			
1	Draw:	7.0 μl	from:	Vial: 0
2	Draw:	0.0 μl	from:	Vial: 1
3	Draw:	4.0 μl	from:	Sample
4	Draw:	0.0 μl	from:	Vial: 1
5	Draw:	7.0 μl	from:	Vial: 0
6	Mix:	7.0 μl	cycles:	3
7	Wait:	1.7 min		
8	Draw:	0.0 μl	from:	Vial: 1
9	Draw:	2.0 μl	from:	Vial: 2
10	Mix:	5.0 μl	cycles:	2
11	Inject			

Total injection volume: 20.0 μl

the entire method, including reagent compositions and LC conditions, are given in Table I.

RESULTS AND DISCUSSION

LC conditions

The previously published pre-column oxidation method [8] used a mobile phase of aqueous ammonium formate (100 mmol/l, pH 6) with a gradient from 0% to 5% acetonitrile and a reversed-phase Supelcosil LC-18 column. Although this system provided excellent performance for the oxidized PSP toxins, alternative mobile and stationary phases have been investigated in this work in an attempt to increase the retention of the analytes. This was desirable in order to facilitate high sensitivity analyses through the use of large injection volumes (see below).

In our past experience, we have found that 0.1% (v/v) (9 mmol/l) trifluoroacetic acid (TFA) in aqueous acetonitrile is an excellent mobile phase for the analysis of basic compounds [9]. The acidic conditions (pH 2.1) suppress interactions of the analytes with active silanol sites in the column resulting in symmetrical peak shapes, while the TFA anion acts as an effective ion-pair agent resulting in increased retention. Preliminary experiments, carried out on short, narrow bore (10 cm × 2.1 mm I.D.) columns, showed that most of the PSP oxidation products behaved well with such a mobile phase in combination with several different stationary phases (Zorbax Rx-C18, Vydac 201TP, LiChrospher RP-18, etc.). LiChrospher RP-18 (5 μm particle size) was selected as the sorbent for the remainder of the project because it is commercially available in bulk and proved most suitable in our hands for the preparation of high efficiency microbore columns (see below). Under isocratic conditions, with 10% acetonitrile in the mobile phase, it was observed that the retention times of the analytes increased with increasing concentration of TFA (up to 9 mmol/l). Since pH varies with the concentration of TFA, experiments were conducted to study the effect of pH, independent of TFA concentration. The mobile phase pH was controlled by the addition of NH_4OH. With a constant TFA concentration of 10 mmol/l and the pH adjusted over the range 2 to 8, the greatest retention was achieved at low pH. Fig. 2 shows the effect of pH on the retention times of oxidized NEO and STX.

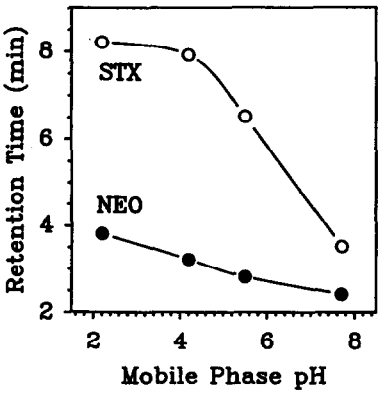


Fig. 2. Effect of mobile phase pH on the retention of the oxidized PSP toxins, saxitoxin (STX) and neosaxitoxin (NEO), in isocratic reversed-phase LC. The mobile phase contained 10 mmol/l trifluoroacetic acid (TFA) adjusted to the specified pH with NH_4OH. Other conditions as in Table II.

These results can be attributed to the formation of strong hydrophobic ion pairs between the oxidized toxins and the TFA. The toxins lose their positive charge as the solution pH approaches their dissociation constants (for unoxidized STX, $pK_a = 8.1$), and consequently ion pairs would not be created effectively at high pH. Peak shape and separation selectivity appeared

TABLE II

EFFECT OF ADDITION OF VARIOUS ACIDS TO THE MOBILE PHASE ON THE RETENTION OF OXIDIZED NEOSAXITOXIN (NEO) AND SAXITOXIN (STX)

Column: LiChrospher-100 RP-18 (10 cm × 2.1 mm I.D.) at ambient temperature; mobile phase: isocratic 10% acetonitrile, 10 mmol/l acid, adjusted to pH 4.2 with 1 *M* NH_4OH; flow-rate: 200 μl/min.

Ion-pair agent (at 10 mmol/l)	Retention time (min)	
	NEO	STX
Formic acid [HCOOH]	2.5	4.4
Acetic acid [CH_3COOH]	2.6	4.5
TFA [CF_3COOH]	3.6	7.9
HFBA [C_3F_7COOH]	13.1	23.6
TDFHA [$C_6F_{13}COOH$]	41.5	72.3

best at pH 4.2, so this was used for all subsequent experiments.

In an attempt to further increase retention and to test the ion-pair hypothesis, other acid modifiers were investigated. As shown in Table II, formic acid and acetic acid gave similar low retention times for oxidized NEO and STX at pH 4.2, but retention times were increased dramatically with longer chain perfluorinated acids. Retention with HFBA was approximately triple that of TFA, and tridecafluoroheptanoic acid (TDFHA) tripled that of HFBA. The longer-chain perfluorinated acids presumably form ion pairs with greater hydrophobicity, resulting in a stronger retention of analyte on the octadecysilica stationary phase. This means that a higher percentage of acetonitrile can be used with HFBA or TDFHA to give similar retention values to those with TFA. It also means that a larger volume of aqueous sample may be injected in a gradient elution experiment starting from an initial 0% organic. As discussed below, this feature was used to improve sensitivity and to facilitate a fully automatic system. Since TDFHA (a solid at room temperature) proved difficult to dissolve, caused considerable foaming of the mobile phase due to its surfactant nature and is also quite expensive, HFBA was selected as the ion-pair agent for subsequent work.

Initial gradient elution experiments were conducted on the 2.1 mm I.D. LiChrospher RP-18 column. Using aqueous HFBA (adjusted to pH 4.2 with NH_4OH) as solvent A and acetonitrile as solvent B, the mobile phase was programmed from 0 to 20% B over 10 min. This resulted in a slight change in buffer concentration through the run but this did not seem to cause any problems. Fig. 3 presents some representative chromatograms for six individual toxin standards that were available in high purity. The chromatogram for GTX1 + 4 (data not shown) was very similar to that of GTX2 + 3. C3 + 4 was not available to us as a standard but a chromatogram for a mixture of C1 to C4 indicated that the products of C3 + 4 are the same as those for C1 + 2. Recently, we have reported the use of combined liquid chromatography–mass spectrometry (LC–MS) to characterize the oxidation products of the PSP toxins [10]. Fig. 4 presents the proposed structures of the major products. As can be seen in

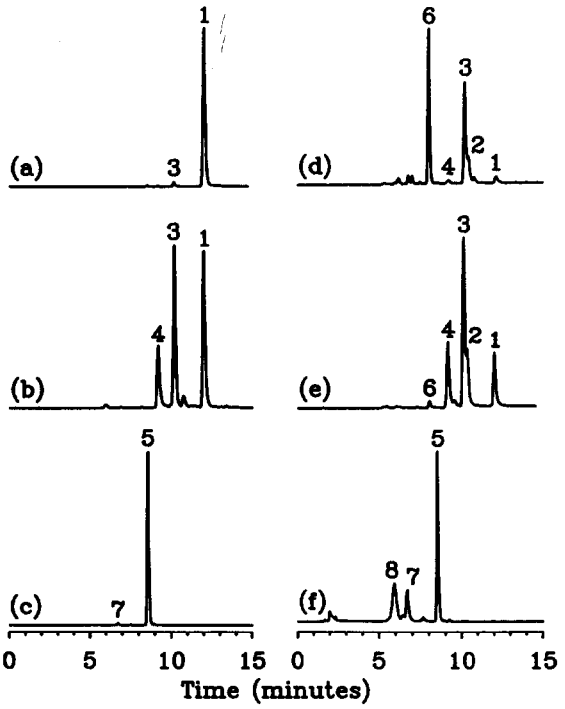
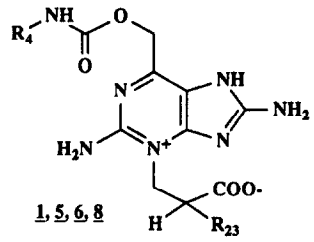


Fig. 3. Gradient elution LC analysis of oxidized toxin standards: (a) STX; (b) NEO; (c) GTX2 + 3; (d) B1; (e) B2; (f) C1 + 2. GTX2 + 3 and C1 + 2 are equilibrium mixtures of the epimeric toxins GTX2 and GTX3 and C1 and C2, respectively. GTX1 + 4 gives a similar result to that of GTX2 + 3, with a slightly larger peak 7. Oxidation reactions were performed manually; toxin concentrations before the oxidation reaction were approximately 100 μg/ml. LC conditions: gradient from 0 to 20% (over 10 min) acetonitrile in aqueous 10 mmol/l HFBA (adjusted to pH 4.2 with NH_4OH); 10 cm × 2.1 mm LiChrospher RP-18 column; 200 μl/min flow-rate; 1 μl injection volume; fluorescence detector gain set at 2^9. For peak Nos., see Fig. 4.

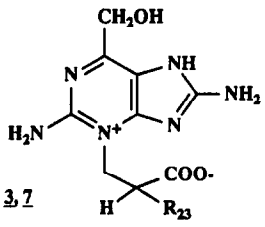
Figs. 3 and 4, STX and the GTX and C toxins yielded primarily single products (1 and 5, respectively) while NEO and the B toxins yielded several products (1, 2, 3, 4 and 6) under the oxidation conditions employed. Excellent peak shapes were observed for all toxins and this encouraged us to proceed with development of a microcolumn (1 mm I.D.) LC method.

Microcolumn LC

One of the advantages of gradient elution is the possibility of improving concentration detection limits through the on-column trace enrichment effect [11]. This effect allows the use of large injection volumes, provided that the sam-



1, 5, 6, 8



3, 7

Prod. No.[a]	R_4	R_{23}[b]	Precursors
1	H	H	STX, NEO, (B1), (B2)
5	H	OSO_3H	GTX1-4, C1-4
6	SO_3H	H	B1, B2
8	SO_3H	OSO_3H	(C1-4)

Prod. No.[a]	R_{23}	Precursors
3	H	dSTX, NEO, B1, B2, (STX)
7	OSO_3H	(C1-4), (GTX1-4)
2, 4	unknowns	(dSTX), (NEO), (B1), (B2)

[a] Oxidation product number (peak no. in Figures).

[b] R_{23} = either R_2 or R_3 (see Figure 1).

( ) = minor product from these toxins.

Fig. 4. Proposed structures for the oxidation products of the PSP toxins. These were based on fluorescence and mass spectra, as well as relative retention times [10]. Table III presents the relative yields and retention times of the products from the different toxins.

ple is dissolved in a solvent with a low eluotropic strength and that analytes have high retention factors. From preliminary experiments, it was found that up to 90 μl of oxidized reaction mixture could be injected on the 2.1 mm I.D. column using the HFBA mobile phase and gradient elution described above, without substantial band-broadening. On a 4.6 mm I.D. column, this translates to a 420-μl injection volume. Although our LC autoinjection system could have been reconfigured to allow such large volumes, it was more convenient to keep the system in the standard configuration (25 μl maximum) required for other routine analyses. Therefore, to fully utilize the trace enrichment effect, we continued our work on 1.0 mm I.D. microbore columns. Although such an approach is more difficult to implement on conventional LC systems, it has the advantage that less sample is consumed to achieve the same concentration detection limit (*i.e.*, a lower mass detection limit). As indicated later, this is very useful for certain applications such as the analysis of plankton samples.

LiChrospher RP-18 microcolumns (25 cm × 1.0 mm I.D.), prepared in our laboratory using the slurry packing technique, gave moderately high efficiencies ($N = 11\,000$, measured using a micro-LC-UV detector equipped with a 0.5-μl flow cell). When the columns were used on the HP1090 LC system, a relatively high flow-rate of 100 μl/min was used to both decrease analysis time and ensure compatibility with the pumping system and the size of the flow cell (4.5 μl) in the fluorescence detector. The large size of the flow cell concerned us a great deal before starting the experiment, since a 0.5–1-μl flow cell size is normally used for microbore work. However, we were surprised to find that column efficiency was deteriorated by only 20% over that measured on the micro-LC system. This was deemed quite acceptable, since the detection sensitivity associated with a large flow cell compensated for the extra band broadening. Our observations are supported by a recent report [12] which showed that it is the design of the flow cell and connecting tubing rather than the absolute flow cell volume that is of primary importance, and that it is possible to use certain commercially available LC detectors fitted with

conventionally sized flow cells in microbore column work. The HP1046A fluorescence detector used in our study seems to have a design suitable for microbore work.

Using gradient elution with the HFBA–aqueous acetonitrile mobile phase, it was possible to inject 20 μl of oxidation reaction solution without significant band broadening. This represents a 20-fold increase over the normal 1-μl injection volume for a 1.0 mm I.D. column. Fig. 5 demonstrates clearly the high-efficiency separations that are possible with such a system. Fig. 5a shows the chromatogram from a 20-μl injection of a mixture of GTX2 + 3 and NEO, oxidized by a manual reaction according to the earlier method [8]. Similarly, Fig. 5c shows the chromatogram resulting from a mixture of STX and B2. In

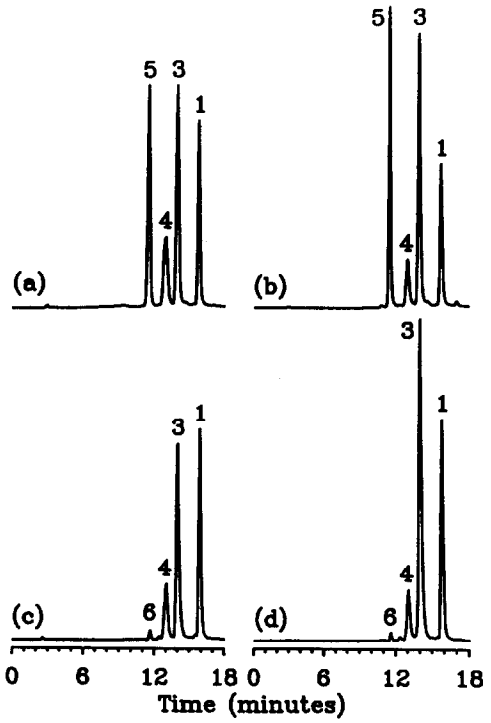


Fig. 5. Analyses of 2 standard PSP toxin mixtures by LC using manual (a, c) and automated (b, d) pre-column oxidation. Mixture 1 (a, b) contained GTX2 + 3 and NEO at 1.2 and 4.4 μg/ml, respectively. Mixture 2 (c, d) contained B2 and STX at 3.3 and 5.0 μg/ml, respectively. Peak 1 in (c, d) is due to STX mainly, but there is a small contribution from B2, especially in the manual reaction. LC conditions: see Table I; 20 μl injection volume for both manual and automated reactions; fluorescence detector gain set at 2^{11}.

general, with this system, we have obtained better separation efficiency, peak shape and baseline stability than with the previous ammonium formate mobile phase [8]. Some changes in the order of elution of analytes were observed, however. Table III provides a listing of the retention times of the oxidized PSP toxins on the microcolumn system.

Automated reaction system

Most pre-column derivatization methods suffer from the disadvantage of being labor-intensive and relatively imprecise due to potentially poor volumetric and reaction time control. The latter is quite important for the pre-column oxidation procedure since both reaction time and the time between completion of the reaction and LC analysis can affect relative proportions and overall yields of oxidation products. A fully automated reaction could improve precision and allow unattended analyses.

Several commercially-available LC autosampler systems permit automated derivatizations to be performed prior to LC analysis. The HP1090 LC system used in this study can perform such reactions through the HP ChemStation's "Injector Program". User-defined volumes of reagents and sample may be drawn into a reaction capillary where they are mixed, allowed to react, and then injected on the column. An injector program that allows the current pre-column oxidation procedure is listed in Table I. It uses a 4.0-μl aliquot of sample mixed first with 14 μl of oxidation reagent and then with 2.0 μl of concentrated acetic acid. The entire injection sequence including the 1.7-min reaction time takes 3 min. It is important to note that the oxidation reaction is pH-dependent. It was reported previously [8] that the optimum pH for the oxidation reagent was 8 for the manual reaction, and that samples should be adjusted to pH 8 before reaction to avoid buffer effects from the sample matrix. For the automated reaction it was observed that the fluorescence response for STX and GTX2 + 3 reached a maximum at pH 9; the response for NEO decreased gradually with increased pH over the range of 8 to 10.5. An oxidation reagent pH of 9 was selected as a

TABLE III

RETENTION TIMES AND RELATIVE YIELDS OF OXIDATION PRODUCTS FOR VARIOUS PSP TOXINS USING THE FULLY AUTOMATED OXIDATION PROCEDURE

Product code[a]	Retention time (min)[b]	Relative yield (%)[c]							
		STX	dcSTX	NEO	B1	B2	GTX2 + 3	GTX1 + 4	C1 + 2[e]
1	15.9	99		30	2	3			
2	14.3		25	1	1	1			
3	14.0	1	75	60	30	80			
4	13.0[d]			10	2	12			
5	11.2						99	96	75
6	11.2				65	2			
7	4.8						1	4	10
8	3.8[d]								15

[a] Oxidation product code numbers refer to structures in Fig. 4 and are used to identify peaks in chromatograms.
[b] Conditions: as in Table I.
[c] Approximate relative yield of products from each toxin estimated from relative peak heights; insufficient standards to give relative sensitivities between toxins.
[d] Broad peak.
[e] Expected products for C3 + 4 also, but no individual standards were available.

compromise. A pH meter was used to adjust pH to ensure the best reproducibility.

Fig. 5b and d shows the chromatograms resulting from the automated pre-column oxidation of the same standard solutions analyzed after manual reaction in Fig. 5a and c, respectively. While there is no deterioration in the separations due to the automated reaction, there are some slight changes in relative responses for the toxins. For the automated reaction of NEO, there is an increase of product 3 relative to products 1 and 4 (Fig. 5b *vs.* 5a). The same observations are true for B2 (Fig. 5d *vs.* 5c), which is converted to essentially the same array of products as NEO in addition to compound 6. In comparing Fig. 5c and d, it is important to keep in mind that B2 and STX contribute to peak 1, with B2 contributing somewhat less in Fig. 5d due to its increased conversion to 3. The reasons for the different profiles with the automated system are not completely understood at this time; the profiles are highly reproducible, however.

Table III presents the retention times and relative proportions of the oxidation products observed for the automated analyses of all the toxins that were available to us. Because toxin standards with accurate concentrations were not available, the relative sensitivities for each toxin can not be presented at this time. Accurate calibration will be very important for the future implementation of this method, as the relative molar response factors of the different toxins appear to vary considerably. Of course, this has also been a major problem with the post-column oxidation LC method [4,5]. Since completion of this project, accurate calibration solutions for STX, NEO and GTX2 + 3 have become publicly available from the NRC Marine Analytical Chemistry Standards Program.

The reproducibilities of both the automated and manual methods were compared by performing replicate analyses. The results indicated that the automated method is much more reproducible than the manual method. The relative standard deviations (R.S.D., $n = 6$) for peak areas of STX, NEO and GTX2 + 3 standards were as follows: (a) 11, 18 and 18%, respectively, for manual reaction; (b) 3.5, 3.4 and 2.2%, respectively, for automated reaction. Retention times for the gradient elution procedure were also found to be very reproducible (0.1 to 0.2% R.S.D.). It should be mentioned, however, that

since the column was left at ambient temperature in our experiments, there were some variations from day to day when the room temperature changed. It is recommended that the column temperature be thermostatically controlled for the best long-term retention time reproducibility. Good linearity of response and zero intercepts were observed for calibration curves generated by the automated analysis of serially diluted solutions of STX, NEO and GTX2 + 3.

The combination of the large volume injection with microbore LC makes this one of the most sensitive methods for the measurement of PSP toxins. An example of the trace analysis of a diluted toxin standard mixture is shown in Fig. 6, along with that of a blank reaction. Estimated values for the detection limits of STX, NEO and GTX2 + 3, listed in Table IV, are in the low femtomole range. These are about 80 times more sensitive than the Sullivan and Wekell post-column oxidation method [5] based on mass detection limits. Concentration detection limits are as low as 0.35 pmol/l for GTX2 + 3 and 1 pmol/l for STX. Accurate estimates for the detection limits of other toxins can not be provided until reliable standards are available, although from the present work they all appear to be of the same order of magnitude.

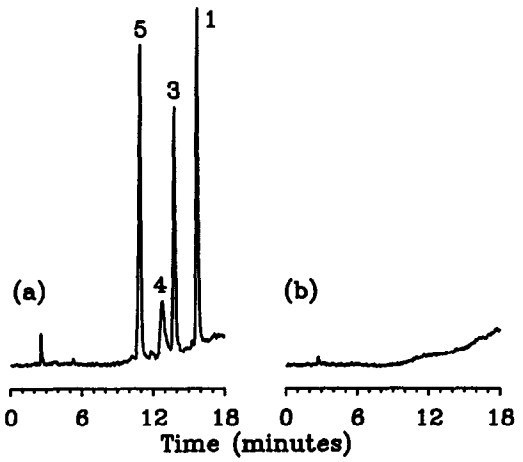


Fig. 6. Automated pre-column oxidation LC analyses of: (a) a low-level PSP toxin standard mixture, containing GTX2 + 3, NEO and STX at 16, 50 and 33 ng/ml, respectively; and (b) a blank reaction. LC conditions: see Table I; fluorescence detector gain set at 2^{18}.

TABLE IV

ESTIMATED DETECTION LIMITS ACHIEVED IN THE FULLY AUTOMATED ANALYSIS PROCEDURE

LC conditions as in Fig. 5 and Table I, detector gain = 2^{18}, 4 μl sample reacted (20 μl reaction mixture injected). MDQ = Minimum detectable quantity injected on-column (estimate for $S/N = 3$); MDC = minimum detectable concentration in the sample extract (estimate for $S/N = 3$).

Analyte	MDQ		MDC	
	pg	fmol	ng/ml	pmol/l
STX	1.2	4.0	0.30	1.0
NEO[a]	2.2	7.0	0.56	1.8
GTX2 + 3	0.6	1.5	0.14	0.35

[a] Based on the principal product (3) for NEO.

Application to samples

Some practical applications of the automated pre-column oxidation/microcolumn method are illustrated in in Figs. 7 and 8. An extract of a contaminated scallop liver currently being examined as a candidate reference material for PSP toxins gave the chromatogram in Fig. 7a, after a simple cleanup through a C_{18} solid-phase extraction cartridge [8]. Two outstanding peaks were observed corresponding to products from GTX2 + 3 and STX; one very weak peak was observed corresponding to the product from NEO. Concentrations were estimated to be 23, 3.6 and 19 μg/g in the original tissue for GTX2 + 3, NEO and STX, respectively; these results are in good agreement with those from analyses using the post-column oxidation system [5]. A similar chromatogram was acquired from raw scallop liver extract after centrifugation through a molecular mass 10 000 filter. The chromatogram from an extract of mussel tissue contaminated at 1 μg/g total toxin (as determined by mouse bioassay) is given in Fig. 7b. The presence of C's, GTX's, NEO and STX is indicated. This was confirmed using the post-column oxidation system.

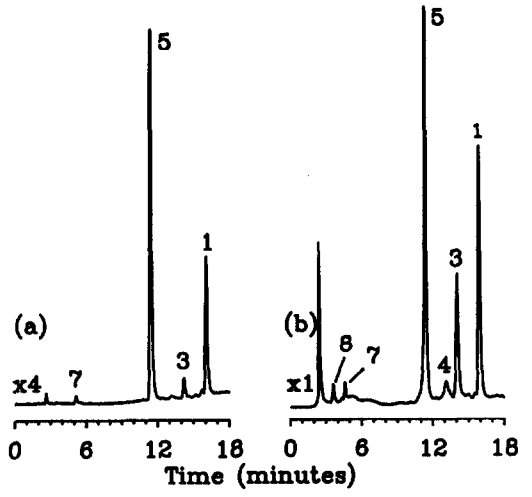


Fig. 7. Analyses of extracts of shellfish contaminated with PSP toxins using the automated pre-column oxidation procedure: (a) scallop liver (candidate reference material); (b) mussel tissue with 1 μg/g STX equivalent by mouse bioassay. LC conditions: see Table I; fluorescence detector gain set at 2^{14} for (a) and 2^{17} for (b); 250 μg tissue equivalent injected for each.

The monitoring of phytoplankton is an important activity that can allow warnings to be issued to aquaculturists when plankton blooms

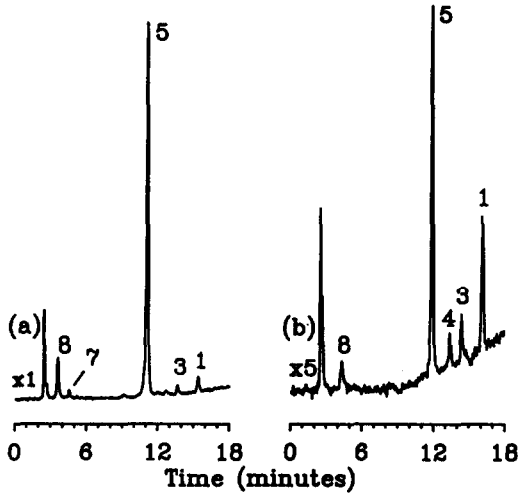


Fig. 8. Analyses of phytoplankton samples containing endogenous levels of PSP toxins using the automated pre-column oxidation procedure: (a) *Alexandrium tamarense* (Gt429), equivalent of 60 cells injected; (b) *Alexandrium excavatum* in a mixed phytoplankton sample, equivalent of 20 cells of *Alexandrium* injected. LC conditions: see Table I; fluorescence detector gain set at 2^{18}.

are starting or have occurred. A simple, rapid and sensitive assay for PSP toxins would be a useful tool for such monitoring programs to determine if a bloom is in fact toxic. We have tested a number of plankton samples and found that the present automated pre-column oxidation method should be very useful for screening plankton samples. Fig. 8a shows the results obtained for an extract of cultured *A. tamarense* (Gt429). The analysis was performed on the equivalent of only 60 cells. Similarly, an extract of *Alexandrium excavatum* cells in a natural phytoplankton assemblage (Gaspé) gave the chromatogram in Fig. 8b. This represents the analysis of the equivalent of only 20 *Alexandrium* cells. The main toxins observed in this sample were the GTX's, but small amounts of C's, NEO and STX were also present. Non-toxic plankton samples gave no significant peaks upon analysis.

CONCLUSIONS

Automated pre-column oxidation coupled with LC and fluorescence detection is a very sensitive and reproducible method for the routine screening analysis of PSP toxins. The method is more sensitive and far easier to perform on a routine basis than is the post-column oxidation method. However, due to the formation of identical oxidation products from some toxins and multiple products from others, the method is less useful than the post-column oxidation for research work directed at understanding the distribution of toxin structures.

The very high mass sensitivity provided by the microcolumn-based technique makes it very useful for the screening of phytoplankton samples for PSP toxins. Since an analysis may be achieved with fewer than 100 cells, it should be relatively easy to perform "cell-picking" experiments from mixed phytoplankton populations.

Although the microcolumns provide the highest mass sensitivity, it is important to keep in mind that an automated pre-column oxidation reaction is also applicable to conventional chromatographic systems. Indeed, we have had good success with both 2.1 and 4.6 mm I.D. columns

and we have been able to implement the method on other manufacturers' autosampler systems.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. M.V. Laycock for PSP toxin standards, Dr. A.D. Cembella for the plankton extracts, Dr. P.G. Sim for the scallop tissue extract and Mr. W.R. Hardstaff and Mr. R. Richards for technical assistance. Funding for this project was provided in part by Health and Welfare Canada.

REFERENCES

1 Y. Shimizu, in A.T. Tu (Editor), *Handbook of Natural Toxins: Marine Toxins and Venoms*, Marcel Dekker, New York, 1988, p. 63.
2 *Official Methods of Analysis*, Associations of Official Analytical Chemists, Arlington, VA, 14th ed., 1984, sections 18.086–18.092; 15th ed., 1990, section 959.08G.
3 D.L. Park, W.N. Adams, S.L. Graham and R.C. Jackson, *J. Assoc. Off. Anal. Chem.*, 69 (1986) 547.
4 Y. Oshima, M. Machida, K. Sasaki, Y. Tamaoki and T. Yasumoto, *Agric. Biol. Chem.*, 48 (1984) 1707.
5 J.J. Sullivan and M.M. Wekell, in D.E. Kramer and J. Liston (Editors), *Seafood Quality Determination*, Elsevier/North-Holland, New York, 1987, p. 357.
6 H.A. Bates and H. Rapoport, *J. Agric. Food Chem.*, 23 (1975) 237.
7 J.F. Lawrence, C. Menard, C.F. Charbonneau and S. Hall, *J. Assoc. Off. Anal. Chem.*, 74 (1991) 404.
8 J.F. Lawrence and C. Menard, *J. Assoc. Off. Anal. Chem.*, 74 (1991) 1006.
9 S. Pleasance, P. Blay and M.A. Quilliam, *J. Chromatogr.*, 558 (1991) 155.
10 M.A. Quilliam, M. Janecek and J.F. Lawrence, *Rapid Comm. Mass Spec.*, in press.
11 K. Slais, D. Kourilova and M. Krejci, *J. Chromatogr.*, 282 (1983) 363.
12 D.N. Mallett and B. Law, *J. Pharm. Biomed. Anal.*, 9 (1991) 53.