FOOD CHEMICAL CONTAMINANTS

Rapid Postcolumn Methodology for Determination of Paralytic Shellfish Toxins in Shellfish Tissue

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A rapid liquid chromatographic (LC) method with postcolumn oxidation and fluorescence detection (excitation 330 nm, emission 390 nm) for the determination of paralytic shellfish toxins (PSTs) in shellfish tissue has been developed. Extracts prepared for mouse bioassay (MBA) were treated with trichloroacetic acid to precipitate protein, centrifuged, and pH-adjusted for LC analysis. Saxitoxin (STX), neoSTX (NEO), decarbamoylSTX (dcSTX), and the gonyautoxins, GTX1, GTX2, GTX3, GTX4, GTX5, dcGTX2, and dcGTX3, were separated on a polar-linked alkyl reversed-phase column using a step gradient elution; the *N*-sulfocarbamoyl GTXs, C1, C2, C3, and C4, were determined on a C-8 reversed-phase column in the isocratic mode. Relative toxicities were used to determine STX-dihydrochloride salt (diHCl) equivalents (STXeq). Calibration graphs were linear for all toxins studied with STX showing a correlation coefficient of 0.999 and linearity between 0.18 and 5.9 ng STX-diHCl injected (equivalent to 3.9–128 μg STXeq/100 g in tissue). Detection limits for individual toxins ranged from 0.07 μg STXeq/100 g for C1 and C3 to 4.1 μg STXeq/100 g for GTX1. Spike recoveries ranged from 76 to 112% in mussel tissue. The relative standard deviation (RSD) of repeated injections of GTX and STX working standard solutions was <4%. Uncertainty of measurement at a level of 195 μg STXeq/100 g was 9%, and within-laboratory reproducibility expressed as RSD was 4.6% using the same material. Repeatability of a 65 μg STXeq/100 g sample was 3.0% RSD. Seventy-three samples were analyzed by the new postcolumn method and both AOAC Official Methods for PST determination: the MBA ($y = 1.22x + 13.99$, $r^2 = 0.86$) and the precolumn LC oxidation method of Lawrence ($y = 2.06x + 12.21$, $r^2 = 0.82$).

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Testing shellfish for the group of potent neurotoxins responsible for paralytic shellfish poisoning is critical for consumers and for the shellfish industry in general. Paralytic shellfish toxins (PSTs) accumulate in shellfish, and consumption of these shellfish can lead to serious illness and death. Monitoring programs are needed to determine when it is safe to harvest and consume shellfish. The PST group comprises more than 20 different naturally occurring analogs of saxitoxin (STX). The toxins can be subgrouped into 4 categories: the most toxic, carbamate group, which includes STX and neosaxitoxin (NEO); the decarbamoyl group; the deoxy-decarbamoyl group; and the least toxic, *N*-sulfocarbamoyl group. The individual toxin levels of these analogs are usually expressed as STX equivalents (STXeq) so that an overall toxicity of a sample may be calculated (1, 2) when chemical or biological tests other than the mouse bioassay (MBA) are used. The dihydrochloride salt of STX (STX-diHCl) is used as the standard for the MBA; therefore, the regulatory limit is actually 80 μg STX-diHCl equivalents per 100 g of whole tissue. Ensure that the proper units are used when comparing chemical test results to MBA results. All references to STXeq in this paper refer to the diHCl salt.

The MBA has been the regulatory method for over 50 years and is an Official Method of AOAC INTERNATIONAL (3). The MBA method currently serves as the reference method in the European Union (EU) with the EU council directive 91/492/EEC (4) stating that the total PST content must not exceed 80 μg STXeq/100 g tissue. The time from exposure to death is used in the MBA to estimate the amount of toxin present in shellfish, with a detection limit for the method at 40 μg STXeq/100 g. Although the MBA method has proved to be very reliable, there is international pressure to reduce or eliminate testing involving animals (5, 6). The MBA provides little toxin profile information, but has the advantage of reporting the total toxicity of the sample. This method also is subject to considerable variability (7). Alternative methods that could reduce or completely eliminate MBA testing for PSTs in a regulatory environment are becoming very desirable.

A number of different approaches have been investigated to replace the MBA as a regulatory tool, including biological assays (8–11), electrophoresis (12), chemosensors (13), and immunoassays (14, 15). The most common chemical method

Table 1. Relative PST toxicities and concentrations of reference, stock, and working standard solutions

Toxin	Mouse units (MU)/μmole	CRM, μM[a]	Stock standard solution, μM	Working standard solution, μM
GTX4	1803	35	8.3	0.66
GTX1	2468	106	25.2	2.0
dcGTX3	935	32	7.9	0.32
dcGTX2	382	114	28.1	1.1
GTX5	160	65	17.5	1.4
GTX3	1584	39	10.3	0.41
GTX2	892	118	31.0	1.2
NEO	2295	65	16.6	1.3
dcSTX	1274	62	16.1	0.64
STX	2483	65	15.9	0.64
C1	15	114	31.2	2.5
C2	239	35	9.5	0.76
C3	33	34	2.5	0.34
C4	143	27	0.76	0.27

[a] CRM = Certified Reference Material.

uses a combination of liquid chromatography (LC) with either pre- or postcolumn oxidation followed by fluorescence detection (FLD; 16–19). This instrumental technology can screen samples while providing detailed toxin profile information, now that a variety of calibration solutions are available (20). The LC-FLD method of Lawrence et al. has been the subject of a successful interlaboratory study (2) and collaborative study (21) and has been accepted by AOAC as the first analytical alternative to the MBA (22). Although it meets the major safety criteria of equivalency to the MBA, the Lawrence method suffers from several drawbacks when applied in a regulatory environment. The major impediment to widespread use of the Lawrence method is the amount of time required to process samples containing significant amounts of PSTs (23). The Lawrence method also cannot distinguish isomeric toxins that may exhibit significantly different toxicities. This study describes the modification of a postcolumn approach previously reported by Oshima (18) and Thomas et al. (19) to address these shortcomings.

The new postcolumn method performance was compared with the "gold standard" MBA as well as the Lawrence precolumn oxidation method. Fourteen of the most toxic and most commonly occurring PSTs were chosen for the study, including STX; NEO; decarbamoylsaxitoxin (dcSTX); gonyautoxin (GTX)-1,2,3,4,5; decarbamoylgonyautoxin (dcGTX)-2,3; and *N*-sulfocarbamoyl gonyautoxin (C)-1,2,3,4 to ensure that the majority of the toxin profiles could be addressed. This method was evaluated against a number of criteria essential to meeting the needs of a regulatory environment, including the practicality for regulatory work, equivalency of results to the MBA and/or the Lawrence method results, applicability to a variety of toxin profiles, reliability on a daily basis, cost, and ease of use. Instrument and analyst time were also considered as factors. The most important consideration in method acceptance for regulatory use was and continues to be the safety of the consumer. The method was applied to a variety of shellfish matrixes, containing numerous toxin profiles, collected throughout eastern Canada.

METHOD

Apparatus

(**a**) *LC system*.—Agilent 1200 quaternary solvent delivery system, autosampler equipped with 0.1–100 μL variable volume injector, column oven, column-switching valve, and data-handling module (Agilent Technologies, Kirkland, QU, Canada).

(**b**) *Postcolumn reaction system*.—Waters postcolumn reaction module capable of maintaining temperature at 85°C with reagents delivered by Waters Reagent Manager pumps (Waters, Milford, MA).

(**c**) *Reaction coil*.—Supelco knitted teflon tube with total volume of 1.0 mL (Sigma-Aldrich Canada, Oakville, ON, Canada).

(**d**) *Fluorescence detector*.—Agilent 1200 FLD operated at an excitation wavelength of 330 nm and an emission wavelength of 390 nm.

(**e**) *LC columns*.—(*1*) Agilent Zorbax Bonus RP, 4.6 × 150 mm, 3.5 μm; (*2*) Thermo BetaBasic 8, 4.6 × 250 mm, 5 μm (Fisher Scientific, Nepean, ON, Canada).

(**f**) *Centrifuge*.—Eppendorf 5415C equipped with F-45-18-11 rotor; maximum 16 000 × *g*.

Reagents

All solvents and reagents were analytical or LC grade materials. All mobile phase and postcolumn reagents were filtered through a 0.2 μm membrane before use.

(**a**) *Water*.—Glass-distilled or deionized (DIW).

(**b**) *DIW (pH 5.0)*.—Acidify DIW to pH 5.0 by dropwise addition of 10% acetic acid (HOAc).

Table 2. Postcolumn LC system suitability conditions

Toxins	Conditions
GTXs and STXs	Artifact peak must be at least 70% baseline-resolved between GTX3 and GTX2
	GTX5 must be at least 40% baseline-resolved between dcGTX3 and dcGTX2
	dcSTX and STX must be at least 70% baseline-resolved
	GTX4 retention time must be between 5 and 7 min
	STX retention time must be between 17 and 23 min
C toxins	C2 must be at least 70% baseline-resolved between C1 and C3
	C1 retention time must be between 4 and 7 min

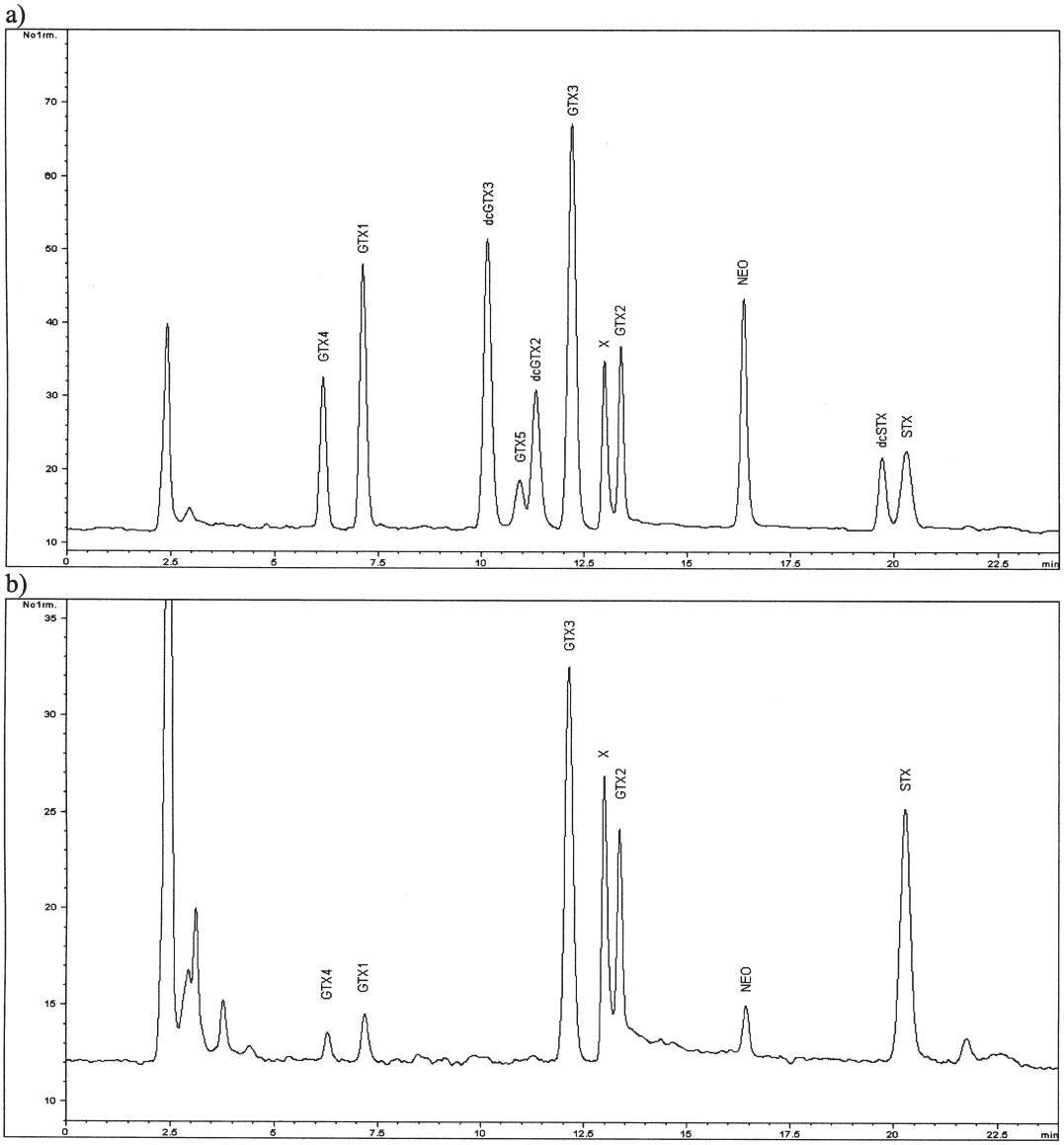

Figure 1. Chromatograms of (a) GTX and STX matrix-matched mixed working solution (10 μL) on an Agilent Zorbax Bonus RP (4.6 × 150 mm, 3.5 μm). Mobile phase: (A) 5.5 mM H_3PO_4, 11 mM heptane sulfonate, pH 7.1. (B) 16.5 mM H_3PO_4, 11 mM heptane sulfonate, pH 7.1 containing 11.5% MeCN. Gradient: 100% mobile phase A for 7.9 min; step to 100% mobile phase B at 8 min; hold for 10.5 min, step to 100% mobile phase A at 18.6 min, 0.8 mL/min. Ox = 5 mM H_5IO_6, 100 mM H_3PO_4, pH 7.8, 0.4 mL/min; H+ = 0.75 M HNO_3, 0.4 mL/min. (b) Mussel sample containing 119 μg STXeq/100 g GTX and STX toxins, conditions as above. In both chromatograms, the artifact peak is labeled "X."

(**c**) *LC mobile phases (GTXs and STXs).—Solvent A.*—11 mM heptane sulfonate, 5.5 mM phosphoric acid (H_3PO_4) aqueous solution adjusted to pH 7.1 with ammonium hydroxide (NH_4OH). *Solvent B.*—11 mM heptane sulfonate, 16.5 mM H_3PO_4, 11.5% acetonitrile (MeCN) aqueous solution adjusted to pH 7.1 with NH_4OH.

(**d**) *LC mobile phase (C toxins).*—2 mM tetrabutyl ammonium phosphate aqueous solution adjusted to pH 5.8 using 10% HOAc if too basic or 1% NH_4OH if too acidic. The pH must only be adjusted in one direction, and if the pH is overshot the solution must be remade.

(**e**) *Postcolumn oxidant.*—100 mM H_3PO_4, 5 mM periodic acid (H_5IO_6) aqueous solution adjusted to pH 7.8 with 5 M sodium hydroxide (NaOH).

(**f**) *Postcolumn acid.*—0.75 M nitric acid (HNO_3).

(**g**) *Primary standards.*—National Research Council Canada (NRC) Certified Reference Materials (CRMs) for C1, C2, dcGTX2, dcGTX3, dcSTX, GTX1, GTX2, GTX3, GTX4, GTX5, NEO, and STX; NRC in-house reference materials for C3 and C4 (NRC Institute for Marine Biosciences, Halifax, NS, Canada). These CRMs were used as supplied by the NRC. The lack of a specific salt does not imply the free-base form of

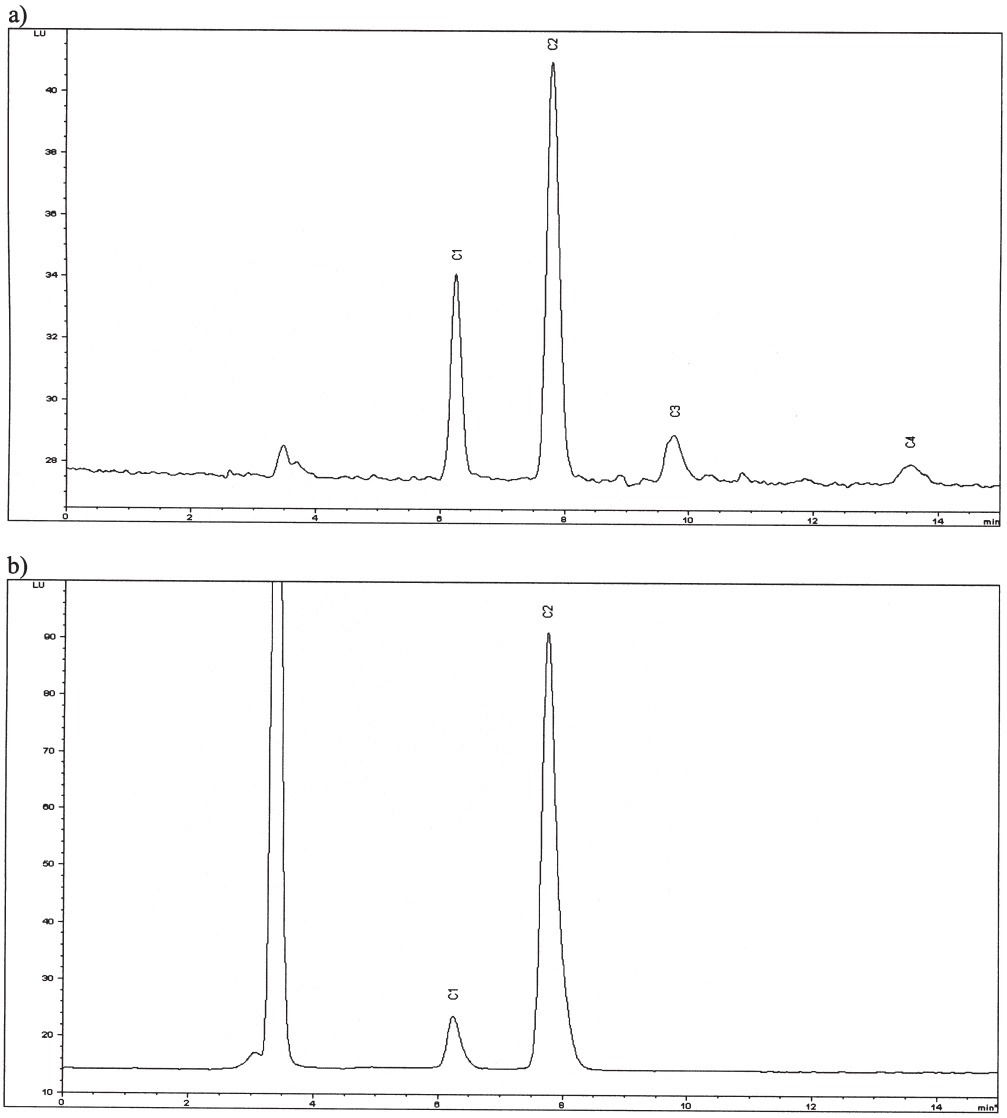

Figure 2. Chromatograms of (a) C toxin mixed working solution (5 μL) on a Thermo BetaBasic 8 (4.6 × 250 mm, 5 μm). Mobile phase (isocratic): 2 mM tetrabutyl ammonium phosphate, pH 5.8, at 0.8 mL/min. Ox = 5 mM H_5IO_6, 100 mM H_3PO_4, pH 7.8, 0.4 mL/min; H+ = 0.75 M HNO_3, 0.4 mL/min. (b) Mussel sample containing 51.4 μg STXeq/100 g C toxins, conditions as above.

toxins in the preceding list, but is simply a list of the toxins used. The NRC has only one form of each toxin available. STX for standardization of MBA was obtained from the U.S. Food and Drug Administration (FDA).

(**h**) *Stock solutions (0.76–31 μM; see Table 1)*.—Prepare individual stock standards gravimetrically as per NRC instructions (24). Perform dilutions with 0.003 M HCl for the GTXs and STXs and DIW (pH 5.0) for C toxins.

(**i**) *Neat mixed working solutions (0.269–2.496 μM; see Table 1)*.—Prepare 2 solutions, the first containing dcGTX2, dcGTX3, dcSTX, GTX1, GTX2, GTX3, GTX4, GTX5, NEO, and STX, and the second containing C1, C2, C3, and C4 (Table 1). Perform dilutions with 0.003 M HCl for the GTXs and STXs and DIW (pH 5.0) for C toxins.

(**j**) *Matrix-matched mixed working solutions*.—Follow the instructions for the neat mixed working solutions but dilute matrix-matched working solutions using a toxin-free mussel extract as the diluent.

Sampling

Samples of shellstock collected during the summer of 2005 as part of the toxin monitoring program of the Canadian Food Inspection Agency, Dartmouth, NS, Canada, were used in this study. The majority of the samples were collected from coastal regions of New Brunswick, Canada; Nova Scotia, Canada; and Prince Edward Island, Canada but also included offshore and imported products. Samples consisted mainly of mussels (*Mytilus edilus*) and clams (*Mya arenaria*) but included a small number of other species such as scallops and oysters. Samples were shucked and analyzed by MBA on receipt. AOAC MBA extracts were stored at 4°C prior to postcolumn LC analysis, and

Table 3. Method performance statistics for the new postcolumn method and the Lawrence method as applied in the authors' laboratory

Toxins	Lawrence method LOD, μg STXeq/100 g	New postcolumn method LOD, μg STXeq/100 g	New postcolumn method spike recovery, % ± SD[a,b]
GTX4	2.8	1.6	99 ± 13
GTX1	2.8	4.1	112 ± 7
dcGTX3	0.98	0.25	101 ± 8
dcGTX2	0.98	0.67	100 ± 4
GTX5	1.5	0.90	98 ± 5
GTX3	0.80	0.38	102 ± 2
GTX2	0.80	1.5	76 ± 5
NEO	2.8	2.3	106 ± 6
dcSTX	2.0	2.1	102 ± 2
STX	3.0	3.9	100 ± 3
C1	0.002	0.07	100 ± 2
C2	0.002	0.15	95 ± 3
C3	0.05	0.07	NA
C4	0.05	0.41	NA

[a] Average of 5 replicate analyses.

[b] Spiked at approximately 3 × LOD for each toxin.

tissue homogenate was stored at –20°C prior to precolumn LC analysis.

Sample Extraction and Cleanup

Thoroughly clean the outside of the shellfish with fresh water. Shuck the samples onto a No. 10 sieve and drain for 5 min. Homogenize the soft tissue in a standard household blender in preparation for extraction. Prepare a sufficient amount of tissue for MBA, LC-FLD precolumn and postcolumn analyses.

Postcolumn LC-FLD and MBA.—Extract 100 g samples of homogenized shellfish tissue according to the AOAC MBA method (3) using 0.1 M HCl. Store aliquots of the extract in scintillation vials for later injections into mice or for further cleanup and LC postcolumn analysis. Deproteinate samples destined for postcolumn FLD analysis by adding 25 μL 30% (w/v) trichloroacetic acid (TCA) to 500 μL shellfish extract in a microcentrifuge tube. Mix in a Vortex mixer and centrifuge at 16 000 × *g* for 5 min. Add 20 μL 1.0 M NaOH, mix, and centrifuge at 16 000 × *g* for 5 min. Filter through 0.2 μm syringe filter into an autosampler vial in preparation for LC analysis.

Precolumn oxidation LC-FLD.—Extract 5 g homogenized shellfish tissue with 1% HOAc, boil for 5 min, and clean up using C18 solid-phase extraction (SPE) and COOH SPE cartridges according to the method of Lawrence (22) in preparation for LC analysis. Apply the method for "Application of the Method for Routine Analysis" as described by Lawrence (Lawrence screen; 22). If toxins are detected, continue with the full Lawrence method.

LC Postcolumn Determinations

GTX and STX toxins.—Equilibrate the LC system for ≥20 min at a column oven temperature of 40°C with 100% solvent A flowing at 0.8 mL/min. Construct a step gradient as follows: 100% solvent A for 7.9 min; step to 100% solvent B at 8 min; hold for 10.5 min; step to 100% A at 18.6 min; equilibrate for 5.4 min.

C toxins.—Equilibrate the LC system for ≥20 min at a column oven temperature of 20°C with mobile phase flowing at 0.8 mL/min. Operate the system in the isocratic mode.

Postcolumn reaction module.—Oxidant flow rate, 0.4 mL/min; acid flow rate, 0.4 mL/min; reaction oven temperature, 85°C; reaction coil, 5 m × 0.50 mm id.

Inject mixed working solutions (10 μL for GTX and STX toxins and 5 μL for C toxins) to ensure that system suitability conditions (Table 2) are met, and construct a linear regression curve of peak area vs concentration in μM. Inject 10 μL sample extracts, blanks, and spikes for GTX and STX toxins, and 5 μL sample extracts, blanks, and spikes for the C toxins. Calculate the μmoles of STXeq for each toxin in the sample extracts using the linear regression of the calibration graph and the specific relative toxicities of each individual PST (Table 1). For comparison to MBA results, use the following equation to calculate the toxicity in the traditional units of "μg STXeq per 100 g tissue" in the specific case of 0.1 kg tissue being extracted with 0.1 L solvent in a single-step dispersive extraction (final volume = 0.2 L):

Table 4. Percent relative standard deviation (% RSD) of retention time (RT) and instrument response of repeated injections of PST standard solutions determined by this method

Toxin (μg STXeq/100 g)	RT (% RSD)	Peak area (% RSD)
GTX4 (20.69)	0.11[a]	2.2[a]
GTX1 (85.79)	0.16[a]	1.2[a]
dcGTX3 (4.98)	0.23[a]	3.1[a]
dcGTX2 (7.25)	0.18[a]	2.8[a]
GTX5 (3.45)	0.19[a]	2.4[a]
GTX3 (9.37)	0.07[a]	1.5[a]
GTX2 (15.97)	0.03[a]	1.5[a]
NEO (47.79)	0.24[a]	2.8[a]
dcSTX (13.07)	0.45[a]	1.8[a]
STX (25.19)	0.49[a]	1.8[a]
C1 (0.56)	0.46[b]	7.3[b]
C2 (2.72)	0.85[b]	4.5[b]
C3 (0.17)	1.8[b]	15[b]
C4 (0.58)	2.4[b]	11[b]

[a] Average of five 10 μL injections.

[b] Average of five 5 μL injections.

$$\text{Sample toxicity (μg STXeq/100 g)} = \sum_{i=1}^{n} C_i \times T_i \times \frac{0.20}{0.10} \times \frac{372.2}{2483} \times F \times 0.1$$

This is simplified to:

$$\text{Sample toxicity (μg STXeq/100 g)} = \sum_{i=1}^{n} C_i \times T_i \times F \times 0.03$$

where C_i = concentration of each toxin "i" in micromoles per liter (μM); T_i = specific toxicity of each toxin "i" in mouse units per micromole (MU/μmole); F = 1.16 for MBA data calibrated against the FDA STX solution (if the MBA was calibrated against the NRC standard, a value of F = 1.00 would be used); 372.2 = molecular weight of STXdiHCl (g/mole).

This F factor of 1.16 must be applied when comparing data calibrated against the NRC STX CRM with the MBA data, which has been calibrated against the FDA STX standard (100 μg/mL stated concentration). A concentration of 86 μg STX-diHCl/mL is observed for the FDA STX standard when calibrated using the NRC STX CRM (1).

LC Precolumn Determinations

Inject 50 μL cleaned-up extract and the periodate oxidation of the cleaned-up extract onto a Supelcosil LC-18-DB, 4.6 × 15 cm, 5 μm column as described by Lawrence (22). If toxins are detected, inject periodate and/or peroxide oxidations of required fractions according to Lawrence (22). Quantify each toxin by direct comparison to analytical standards. Calculate the amount of PSTs present as μg STXeq/100 g sample using the PST relative toxicity values as described by Lawrence (22) in order to compare to the MBA. Calculate total toxicity by summing the individual toxin contributions. Apply factor of 1.16 as in postcolumn determinations for comparison with MBA data.

MBA Determinations

Inject 17–23 g mice intraperitoneally with 1 mL HCl extract according to the AOAC Official Method **959.08** (3) and record death times. Calculate the amount of PSTs present as μg STXeq/100 g sample using Sommer's Table (3).

Results and Discussion

A new postcolumn method for the determination of PSTs was developed and compared to AOAC Official Methods for PST determination. Oshima's postcolumn method (18) required 3 injections to quantify the 14 toxins included in this study. The number of injections was decreased to 2 by Thomas et al. (19), but the separation of GTXs and STXs took 60 min, and used a trinary mobile phase system. The GTX and STX toxin method was improved by consolidating the trinary mobile phase system into a binary step gradient, which allowed a decreased run time of 24 min. All GTX and STX toxins studied were baseline-resolved with the exception of GTX5, which was 50% baseline-resolved (Figure 1). The C toxins were baseline-resolved and quantified in <15 min (Figure 2) in an isocratic system very similar to that described by Oshima (18). Differences between the new postcolumn method for C toxin determination and Oshima's method (18) include a different cleanup procedure, a different concentration of tetrabutyl ammonium phosphate, a different LC column, and different oxidation conditions. This study included 14 currently available commercial standards. An additional standard, decarbamoylneosaxitoxin (dcNEO), was not included at this time due to co-elution with NEO under the rapid separation system. It is possible to resolve dcNEO and NEO with a 75 min trinary step gradient (19). The oxidation products of dcNEO co-elute with the oxidation products of both dcSTX and STX when the Lawrence method is used (B. Niedzwiadek, Health Canada, Ottawa, ON, Canada, personal communication, 2006). From a regulatory perspective, this was not a major issue in the postcolumn method, as the relative toxicity of dcNEO is less than that of NEO. The worst case scenario would be a slight overestimation of total toxicity, further protecting the consumer. Gonyautoxin-6 (GTX6) was not included in this study due to the lack of standard availability, but elutes

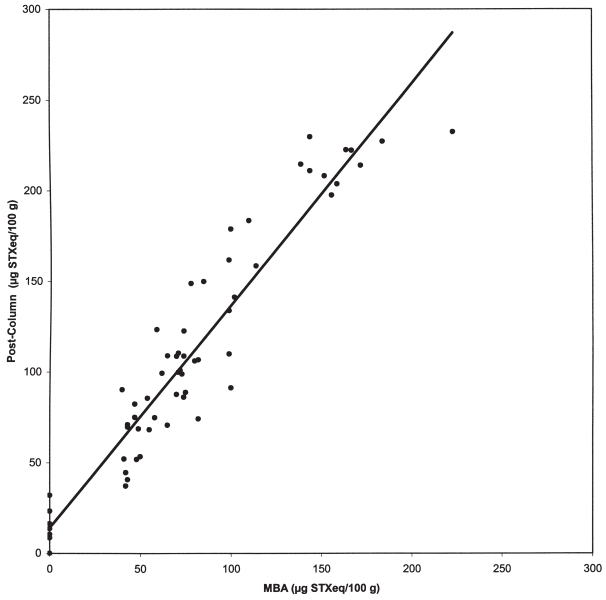

Figure 3. Correlation between results of the MBA method and the new postcolumn method for samples up to 250 µg STXeq/100 g; $y = 1.22x + 13.99$; $r^2 = 0.86$.

immediately before GTX4 under the described chromatographic conditions.

PSTs were extracted using the AOAC MBA method (3) for postcolumn LC analysis; therefore, the toxin profile quantified using the postcolumn method was very similar to that injected into the mouse. Protein remaining in the AOAC MBA extract can be trapped on column frits, leading to the rapid development of backpressure, an attendant decrease in column performance, and possible damage to LC pumping systems. TCA was used to remove protein from the AOAC MBA extract and, in so doing, extended column life. Some concern was expressed that the use of TCA might change the toxin profile even though the pH was returned to its original level quickly. No differences were observed in the toxin profiles following treatment with TCA. However, treatment with TCA increased column life so that approximately 600 samples could be analyzed before significant deterioration of the column was observed. Without TCA treatment, column deterioration is evident after approximately 100 samples have been analyzed.

The LC system performed reliably and was simply shut down at the end of each daily run. No problems were associated with start-up the next day. The postcolumn system (pumps and reaction coil) was flushed once a week with 0.75 M HNO_3 followed by DIW. As a precaution, the column was removed from the LC and the entire fluid path was flushed with 10% MeCN in DIW to prevent line blockage due to the precipitation of buffers. If the system is to be shut down for extended periods, it is recommended that the pumps are not left in the harsh acid or oxidant environment. Following these maintenance procedures, no problems were experienced other than the very minor difficulties that are typically encountered with modern LC pumping systems.

The maximum sample throughput of the new postcolumn method and the Lawrence method was compared because of its importance in a regulatory environment. A single LC system could analyze 31 samples per 24 h period with the postcolumn method, including attendant standards and quality assurance samples. In those situations where the Lawrence screen could be used, approximately 40 samples could be processed in a 24 h period. However, if positive samples are encountered, as is the case in our laboratory where approximately 30% of samples received are positive for PSTs, a combination of the Lawrence screen and full methodologies is required. Using a combination of full and screen methodologies allows only an average of 16 samples to be processed each day. In addition, results from those samples requiring the full method will be delayed up to a further 24 h while the COOH SPE fractions are prepared and oxidized prior to LC analysis. This is a major limitation of the Lawrence methodology in a regulatory environment (23).

Limits of detection (LODs) of the new postcolumn method and the Lawrence method are shown in Table 3. LODs ranged from 0.07 µg STXeq/100 g for C3 to 4.1 µg STXeq/100 g for GTX1 for the postcolumn method. This compared quite favorably with detection limits for the Lawrence method, which ranged from 0.002 µg STXeq/100 g for C1, C2 to 3.0 µg STXeq/100 g for STX as applied in our laboratory. Adequate detection capability for regulatory purposes was supplied by both LC methods. A spiking study near the limit of quantitation for individual toxins demonstrated that the new postcolumn method recovered between 76% (GTX2) and 112% (GTX1) of toxins (Table 3). No spiking data are currently available for C3 or C4 due to the limited supply of standards, but these recoveries are expected to fall within the range of recoveries for other toxins examined.

A calibration graph for STX was linear between 0.18 and 5.9 ng STX injected, which was equivalent to 3.9–128 µg STXeq/100 g in tissue. Calibration graphs for other toxins showed very similar results. The correlation coefficients of the calibration graphs for all toxins ranged from 0.999 to 1.00. Stock and working solutions of GTXs and STXs were stored at 4°C; stock and working solutions for C toxins were stored at ≤–20°C. Standard solutions have been stored for >12 months with no noticeable deterioration.

Working standards were prepared using a mussel tissue extract to assist in the identification of toxins present in the samples, as matrixes caused a slight positive retention time shift for GTX4 and GTX1 in the new postcolumn method. Exact matrix matching of standards was not required for any matrixes studied, including various species of mussels, clams, scallops, and oysters. Matrix-matched standards assisted in resolving interfering peaks, as most samples have an artifact peak (Figure 1, peak X) corresponding to the step gradient solvent front. This artifact peak did not contain any toxins included in this study and was generally well resolved, but over time may co-elute with GTX3 or GTX2. It was found

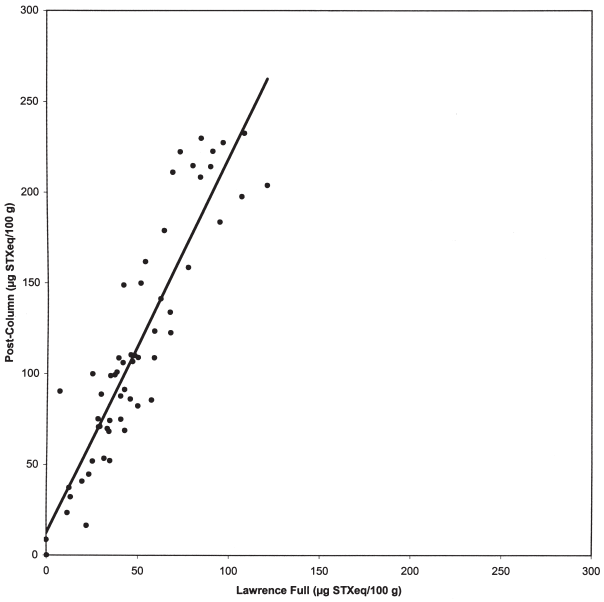

Figure 4. Correlation between results of the full Lawrence method and the new postcolumn method for samples up to 250 µg STXeq/100 g; $y = 2.06x + 12.21$; $r^2 = 0.82$.

that a temperature adjustment of <±5°C easily resolved all 3 peaks with no significant impact on overall run time or separation of other toxins. This adjustment may be effective for up to several weeks, depending on column usage. The first injection each day should contain GTX2, GTX3, and the artifact peak (matrix standard or check sample), and this injection will be used to adjust the column temperature to meet system suitability criteria. The elution conditions (gradient step time, column temperature) must be confirmed each time a new column is used, and after this only small changes to column temperature should be needed. No unresolvable interferences were observed in any of the mussel, clam, oyster, or scallop samples tested. Retention times were stable; the relative standard deviation (RSD) varied from 0.03 to 2.4% (Table 4). Replicate injections of standard and tissue extract solutions indicated good peak response repeatability over the range of concentrations studied with RSDs ranging from 1.2 to a maximum of 15% (Table 4). Quantification was based on peak areas. The method showed good within-laboratory reproducibility; a mussel tissue extract containing 195 µg STXeq/100 g analyzed over 21 days showed an RSD of only 4.6%. The uncertainty of measurement based on precision data for the same mussel tissue extract was 9%. Repeatability RSD of a 65 µg STXeq/100 g mussel tissue analyzed 5 times was 3.0%.

The MBA has a long successful history of preventing consumer illnesses and deaths. Therefore, equivalency to the MBA is essential. More than 50 positive shellfish samples with MBA results between 40 and 223 µg STXeq/100 g were analyzed by MBA, pre- and postcolumn methods. The MBA results were plotted against the postcolumn results in Figure 3; the slope was 1.22 and the correlation coefficient was 0.86. It was expected that the postcolumn results would be slightly higher than the MBA results. It has been reported widely that salt effects lead to an underestimation of the toxicity of shellfish especially with samples near the MBA detection limit (7, 18, 25). The vast majority of samples with MBA results near the regulatory limit show very similar postcolumn results.

The Lawrence method has been approved by AOAC as the first official LC method for PSTs (22). The comparison of MBA and Lawrence screen results exhibited a slope of 0.79 and a correlation coefficient of 0.36. Although the correlation was poor, samples with higher MBA values generally produced higher values in the Lawrence screen method. This points out the necessity of running the full Lawrence method when PSTs are detected if accurate results are to be obtained. The MBA is known to have a large variation (17, 26), due in large part to the fact that it uses a biological system. It was expected that the results from the pre- and postcolumn methods would be quite comparable since neither method uses a biological system. Figure 4 compares the full Lawrence method and the postcolumn method results. A slope of 2.06 indicates that the postcolumn results were approximately 50% higher than the results of the full Lawrence method but the correlation coefficient was good (0.82). Lawrence and Menard (27) initially noted this trend of postcolumn methods producing higher results than precolumn methods. Experiments carried out to determine where toxicity might be lost while using the Lawrence method highlighted 3 stages for potential toxin loss. Standard solutions and positive samples were extracted using the Lawrence method, and monitored at various stages using the new postcolumn LC system. In our laboratory, approximately 7% of the total toxicity was lost during C18 SPE cartridge cleanup, 11% was lost to the pH adjustment after the C18 SPE, and an additional 11% was lost during the COOH SPE cleanup. These losses totaled 29% of overall toxicity, resulting from the full Lawrence cleanup procedure. Correcting for these losses provided a simple solution and provided a corrected slope of 1.4 with the new postcolumn data. There is also an expected difference due to different extractant acids. The HOAc extraction used by the Lawrence method is milder than the HCl extraction used by the AOAC MBA method and is not subject to the Proctor enhancement, which converts *N*-sulfocarbamoyl toxins to the more toxic carbamate forms (28).

Both LC methods were compared in our laboratory to determine the pros and cons of each method in a regulatory environment. The positive aspects of the postcolumn method were easier interpretation of data, separation of all analytes tested, and faster turnaround times for positive samples (31 versus 16 samples/day/LC system assuming a 30% positive rate). The precolumn advantages were excellent chromatographic performance, faster turnaround time when most samples tested negative for PSTs, and no postcolumn system required. One concern with the Lawrence method is the possibility of a single sample accidentally not being

oxidized; a sample would be reported as a false negative if it was not oxidized. Caution must be exercised to ensure that the proper volumes and reagents have been added to each vial before LC injection. Although the postcolumn equipment has a few additional moving parts which may fail in day-to-day operation, postcolumn system failure is very obvious, as all standards, spikes, and control samples would also be affected. The total analysis cost (capital purchases and consumables) for the new postcolumn method was less than that of MBA analysis if capital costs are depreciated over 7 years. The Lawrence screen cost approximately the same as MBA analysis and the full Lawrence method was nearly triple the cost of MBA analysis, due to increased consumable costs (SPE cartridges, filters).

Both the pre- and postcolumn methods have demonstrated that they are viable alternatives to MBA analysis. These LC methods effectively measured the toxin content in shellfish tissue containing a variety of toxin profiles. The main advantages of the new postcolumn method in a regulatory setting were higher throughput and faster turnaround of positive samples. The speed of analysis provided by this method is essential in a regulatory environment where decisions are required on a timely basis.

Future work will concentrate on running the new postcolumn method in parallel with the MBA over one shellfish season to ensure that the method is robust, reliable, and accurate and can be counted upon to protect the health and safety of consumers. Approximately 1000 samples have been analyzed concurrently with no significant problems. Validation data for additional toxins will be generated when standards become available, and alternate extraction methods which may reduce turnaround time will be evaluated.

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References

(1) Quilliam, M.A. (2004) in *Supplemental Information for PSP CRMs,* NRC Certified Reference Materials Program, National Research Council, Institute for Marine Biosciences, Halifax, NS, Canada, pp 1–5

(2) Lawrence, J.F., Niedzwiadek, B., & Menard, C. (2004) *J. AOAC Int*. **87**, 83–100

(3) *Official Methods of Analysis* (2000) 17th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **959.08**

(4) Council of the European Community (1991) *Off. J. Eur. Commun*. **L268**, 1–14

(5) Balls, M.B., Goldburg, A.M., Fentem, J.H., Broadhead, C.L., Bursch, R.L., Festing, M.F.W., Frazier, J.M., Hendrickson, C.F.M., Jennings, M., van der Kamp, M.D.O., Morton, D.B., Rowan, A.N., Russell, C., Russel, W.M.S., Spielman, H., Stephens, M.L., Stokes, W.S., Straughan, D.W., Yager, J.D., Zurlo, J., & van Zutphen, B.F.M. (1995) *The Report and Recommendation of ECVAM Workshop 11, ATLA* **23**, 883–866

(6) Hess, P., Grune, B., Anderson, D.B., Aune, T., Botana, L.M., Caricato, P., van Egmond, H.P., Halder, M., Hall, S., Lawrence, J.F., Moffat, C., Poletti, R., Richmond, J., Rossini, G.P., Seamer, C., & Vilageliu, J.S. (2006) *The Report and Recommendations of a Joint ECVAM/DG SANCO Workshop (ECVAM Workshop 55), ATLA* **34**, 193–224

(7) Park, D.L., Adams, W.N., Graham, S.L., & Jackson, R.C. (1986) *J. AOAC Int*. **69**, 547–550

(8) Jellett, J.F., Doucette, L.I., & Belland, E.R. (1998) *J. Shellfish Res.* **17**, 1653–1655

(9) Louzao, M.C., Vieytes, M.R., Cabado, A.G., Baptista de Sousa, J.M.V., & Botana, L.M. (2003) *Chem. Res. Toxicol.* **16**, 434–437

(10) David, L.S., & Nicholson, R.A. (2004) *Chemosphere* **55**, 1315–1321

(11) Manger, R.L., Leja, L.S, Lee, S.Y., & Hungerford, J.M. (2003) *J. AOAC Int.* **86**, 540–543

(12) Thibault, P., Pleasance, S., & Laycock, M.V. (1991) *J. Chromatogr.* **542**, 483–501

(13) Gawley, R.E., Pinet, S., Cardona, C.M., Datta, P.K., Ren, T., Guida, W.C., Nydick, J., & LeBlanc, R.M. (2002) *J. Am. Chem. Soc.* **124**, 13448–13453

(14) Usleber, E., Dietrich, R., Burk, C., Schneider, E., & Martlbauer, E. (2001) *J. AOAC Int*. **84**, 1649–1656

(15) Jellett, J.F., Roberts, R.L., Laycock, M.V., Quilliam, M.A., & Barrett, R.E. (2002) *Toxicon* **40**, 1407–1425

(16) Lawrence, J.F., & Niedzwiadek, B. (2001) *J. AOAC Int.* **84**, 1099–1108

(17) Sullivan, J.J., Wekell, M.M., & Kentala, L.L. (1985) *J. Food Sci.* **50**, 26–29

(18) Oshima, Y. (1995) *J. AOAC Int.* **78**, 528–532

(19) Thomas, K., Chung, S., Ku, J., Reeves, K., & Quilliam, M.A. (2006) in *Molluscan Shellfish Safety*, K. Henshilwood, B. Deegan, T. McMahon, C. Cusack, S. Keaveney, J. Silke, M. O'Cinneide, D. Lyons, & P. Hess (Eds), The Marine Institute, Galway, Ireland, pp 132–138

(20) Quilliam, M.A. (2006) in *Molluscan Shellfish Safety*, K. Henshilwood, B. Deegan, T. McMahon, C. Cusack, S. Keaveney, J. Silke, M. O'Cinneide, D. Lyons, & P. Hess (Eds), The Marine Institute, Galway, Ireland, pp 139–144

(21) Lawrence, J.F., Niedzwiadek, B., & Menard, C. (2005) *J. AOAC Int.* **88**, 1714–1732

(22) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **2005.06**

(23) Ben-Gigirey, B., Rodriguez-Velasco, M.L., Villar-Gonzalez, A., & Botana, L.M. (2007) *J. Chromatogr. A* **1140**, 78–87

(24) *Certificate of Analysis* (2006) National Research Council, Institute for Marine Biosciences, Halifax, NS, Canada

(25) Asp, T.N., Larsen, S., & Aune, T. (2004) *Toxicon* **43**, 319–327

(26) Holtrop, G., Petrie, J., McElhiney, J., & Dennison, N. (2006) *Toxicon* **47**, 336–347

(27) Lawrence, J.F., & Menard, C. (1991) *J. AOAC Int.* **74**, 1006–1012

(28) Hall, S., Reichardt, P.B., & Neve, R.A. (1980) *Biochem. Biophys. Res. Commun.* **97**, 649–653