FOOD CHEMICAL CONTAMINANTS

Quantitative Determination of Paralytic Shellfish Poisoning Toxins in Shellfish by Using Prechromatographic Oxidation and Liquid Chromatography with Fluorescence Detection

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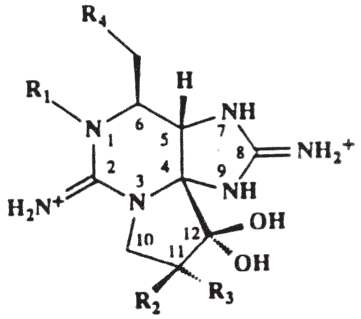
The prechromatographic oxidation LC method developed by Lawrence [*J. Assoc. Off. Anal. Chem.* 74, 404–409(1991)] for the determination of paralytic shellfish poisoning (PSP) toxins has been tested for the quantitative determination of PSP toxins in shellfish. All aspects of the method were studied and modified as necessary to improve its performance for routine regulatory purposes. The chromatographic conditions were changed to shorten analysis time. The oxidation reaction was tested for repeatability and the influence of the sample matrix on quantitation. An important part of the study was to quantitatively evaluate an ion exchange (-COOH) cleanup step using disposable solid-phase extraction cartridges that separated the PSP toxins into 3 distinct groups for quantitation, namely the C toxins, the GTX toxins, and the saxitoxin group. The cleanup step was very simple and used increasing concentrations of aqueous NaCl for elution of the toxins. The C toxins were not retained by the cartridges and thus were eluted unretained with water. The GTX toxins (GTX1 to GTX6 as well as dcGTX2 and dcGTX3) eluted from the cartridges with 0.05M NaCl while the saxitoxin group (saxitoxin, neosaxitoxin, and dcsaxitoxin) required 0.3M NaCl for elution. Each fraction was analyzed by LC after oxidation with periodate or peroxide. All of the compounds could be separated and quantitatively determined in spiked samples of mussels, clams, and oysters. The nonhydroxylated toxins could be quantitated at concentrations as low as about 0.02 μg/g (2 μg/100 g) of tissue while the hydroxylated toxins could be quantitated at concentrations as low as about 0.1 μg/g (10 μg/100 g). Average recoveries of the toxins through the complete cleanup procedure were 85% or greater for spiked extracts of oysters and clams and greater than 73% for mussels.

Received May 23, 2000. Accepted by AP January 10, 2001.

The most commonly used method to monitor paralytic shellfish poisoning (PSP) toxins (structures in Figure 1) in shellfish is the AOAC mouse bioassay (1). However, in the original collaborative study the method was never tested for accuracy (i.e., no spiked samples were included). In a recent interlaboratory evaluation of the method where spiked samples were included (2), it was shown that the mouse bioassay can sometimes underestimate the true concentration of PSP toxins in shellfish samples by as much as 3-fold. Because of this uncertainty, an alternative method would be desirable for regulatory purposes.

The prechromatographic oxidation LC method developed for the determination of PSP toxins (3–5) has been in use in various laboratories around the world for almost 10 years. Although the approach is relatively simple, sensitive, and particularly suited to rapid screening, it has not received much attention with regards to the quantitative determination of PSP toxins for regulatory purposes. The main drawback has been the inability of the method to easily distinguish between the sulfocarbamoyl and nonsulfocarbamoyl analogues of the N-1-hydroxylated toxins (such as B2 from neosaxitoxin and C3 and C4 from GTX1 and GTX4). This is an important limitation because the differences in toxicity between the sulfocarbamoyl and nonsulfocarbamoyl analogues are substantial and misidentification could lead to an inaccurate assessment of the toxicity of shellfish extracts. Some attempts to separate these groups from one another using ion exchange solid-phase extraction (SPE) cartridges have met with some success (4, 5). However, no quantitative evaluations nor repeatability studies were performed.

The aim of the present work was to critically evaluate and optimize the entire prechromatographic oxidation method for possible interlaboratory evaluation for the quantitative determination of selected PSP toxins in shellfish samples. Modifications have been made throughout the procedure to improve the quantitative aspects of the method. These include repeatability, ruggedness, recovery, influence of sample matrix, and a compaison with the mouse bioassay.



			Carbamate toxins	N-Sulfocarbamoyl toxins	Decarbamoyl toxins
		R_4:	H_2N—C(=O)—O---	$-O_3S$—NH—C(=O)—O---	H—O---
R_1	R_2	R_3			
H	H	H	STX[a]	B1	dcSTX
H	H	OSO_3^-	GTX2	C1	dcGTX2
H	OSO_3^-	H	GTX3	C2	dcGTX3
OH	H	H	NEO	B2	dcNEO
OH	H	OSO_3^-	GTX1	C3	dcGTX1
OH	OSO_3^-	H	GTX4	C4	dcGTX4

[a] STX = saxitoxin; NEO = neosaxitoxin; GTX = gonyautoxins.

Figure 1. Structures of PSP toxins.

Experimental

Liquid Chromatography

The LC system consisted of 2 Waters pumps (Models 510 and M-45), a Waters automated gradient controller, and manual injection port with a 50 μL loop (or Model U6K injector with a 2 mL; loop Mississauga, Canada). The column used was a Supelcosil (Oakville, Canada) LC-18 (15 cm × 4.6 mm id, 5 μm). For monitoring the LC effluent, a Shimadzu (Kyoto, Japan) Model RF-551 spectrofluorometric detector or a Jasco (Easton, MD) Model 820-FP dual monochromator detector (both with excitation set to 340 nm and emission to 390 nm), were used. A Varian (Walnut Creek, CA) Star Chromatography Workstation was used to analyze chromatograms and generate reports. The PSP oxidation products were eluted using a linear gradient of 2 mobile phases, A: 0.1M ammonium formate (6.31 g ammonium formate dissolved in 1 L water) and B: 0.1M ammonium formate in 5% acetonitrile–water (6.31 g ammonium formate dissolved in 950 mL water and 50 mL acetonitrile added), both adjusted to pH 6 by adding 6 mL 0.1M acetic acid (572 μL glacial acetic acid to 100 mL with water). The gradient was as follows: 0 to 20% mobile phase B in the first 7.5 min, 20 to 80% B for the next 3.5 min, and back to 100% A over the last 2 min. The flow rate was 2 mL/min.

Reagents and Chemicals

All solvents and reagents were analytical grade. Acetonitrile and methanol were the only organic solvents used in the procedure, and in very limited amounts. Water was doubly deionized. Analytical standards of STX, NEO and mixtures of GTX1 and 4 and GTX2 and 3 were purchased from the National Research Council of Canada, Halifax, Canada, in glass ampoules as solutions in 0.1M acetic acid. Stock solutions were prepared by diluting (25 times) the contents of the ampoules in water. This yielded solutions of the toxins containing between 4–6 μg/mL each. These acidic solutions were found to be stable for ca 1 year when stored in the dark in a refrigerator (4°C) when not in use. Analytical working standard solutions were prepared by dilution of the stock solutions to yield concentrations in the range of 6–16 ng/mL, depending on the toxin. Some toxins (NEO in particular) in these dilute solutions showed slight (ca 5%) decomposition after 1 day. Thus, for quantitative purposes, small volumes of analytical working standard solutions were prepared daily as needed. It was found that acidification of these working solutions to ca pH 4.5 substantially increased the stability of the toxins, enabling use of the solutions for ca 1 week. However, too much acid in the standard solutions can negatively affect the periodate oxidation. If acid is used to stabilize the working solutions, then it is important to verify that it has no impact on the oxidation reaction. Other toxins used in the study, C1, C2, C3, B1(GTX5), and B2 (GTX6), were obtained as a gift from the U.S. Food and Drug Administration (S. Hall) but were not

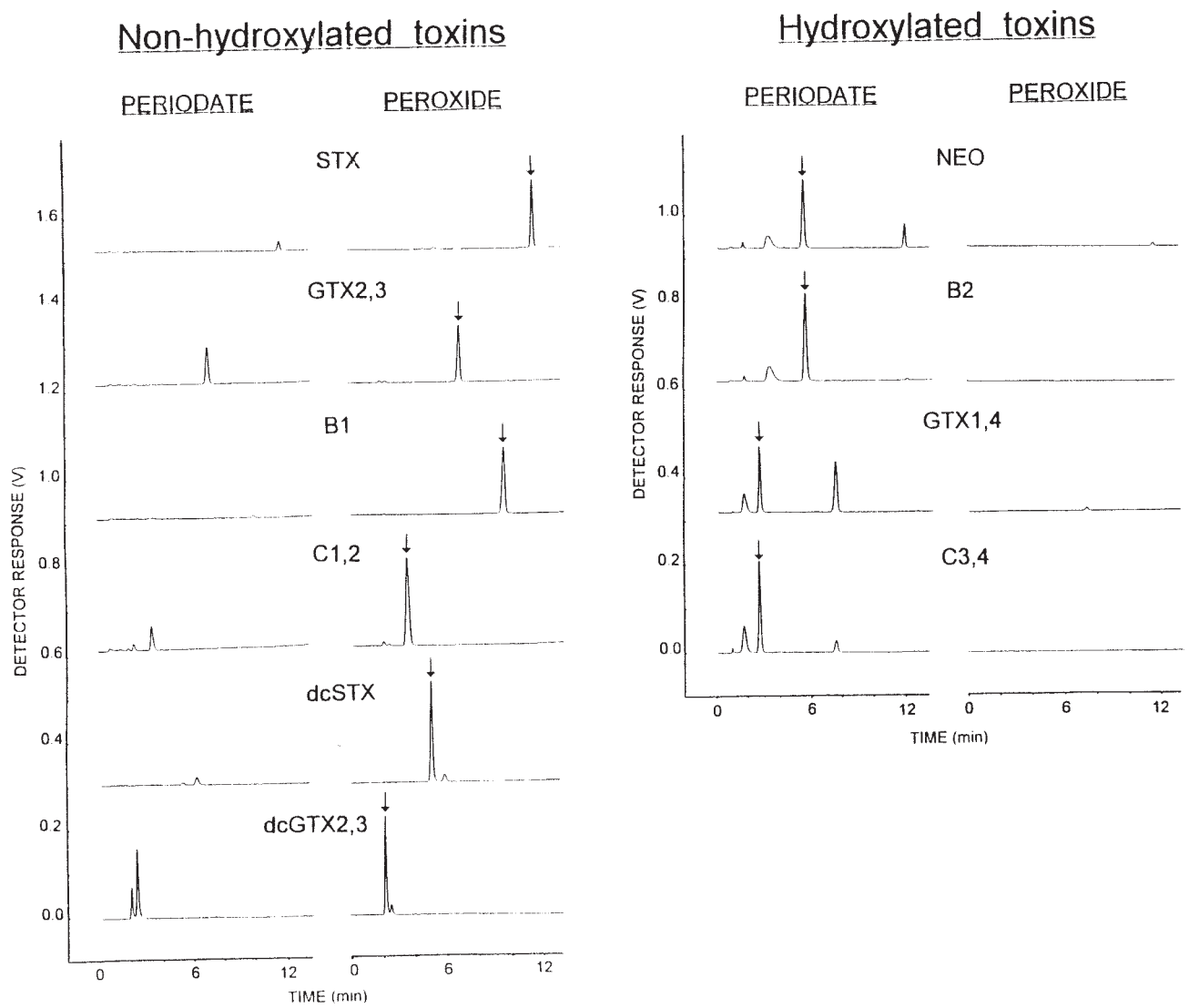


Figure 2. Chromatographic patterns showing the oxidation products formed after periodate and peroxide oxidations of the toxins included in this study. The same quantity of each toxin was used for each oxidation reaction. Arrows indicate peaks used for quantitation.

analytical standards. An analytical standard solution (not certified) of dcSTX was obtained as a gift from the National Research Council of Canada.

For periodate oxidation, a matrix modifier consisting of a solution prepared from an oyster extract was added as a reagent to the oxidation mixture. It was found that the presence of the matrix modifier increased the yield of oxidation products of the hydroxylated toxins (B2, GTX1,4, and NEO) as well as the recovery when working with spiked shellfish samples. The matrix modifier was prepared from blank (PSP free) oysters, which were extracted and cleaned up on an SPE C_{18} cartridge as described below. The extract was adjusted to pH 6.5 with 1M NaOH and filtered using a 0.45 μm Acrodisc filter.

Sample Collection and Preparation

Shellfish samples consisting of oysters, clams, and mussels used for this study were purchased locally. The shellfish tissue was processed using a food chopper and then frozen for further analysis. They were analyzed for the presence of PSP toxins using the method described herein, and found to be negative for all toxins studied. These samples were used as blanks for method optimization purposes.

Sample Extraction

The standard mouse bioassay procedure for extraction of PSP toxins from shellfish samples was slightly modified to make the extracts more suitable for ion-exchange cleanup, that is by reducing the quantity of chloride ion present in the extracts. A 10 g portion of shellfish sample was extracted by boiling for 5 min with 10 mL 0.1M HCl (8.6 mL of ca 38.0% HCl to 1 L with water) in a 50 mL beaker. The beaker was covered with a watchglass during boiling and the mixture was stirred occasionally using a glass rod to avoid splashing. The beaker was then placed in a refrigerator for 5 min to cool. The mixture was transferred to a 50 mL teflon centrifuge tube and centrifuged for 10 min at 4500 rpm (3600

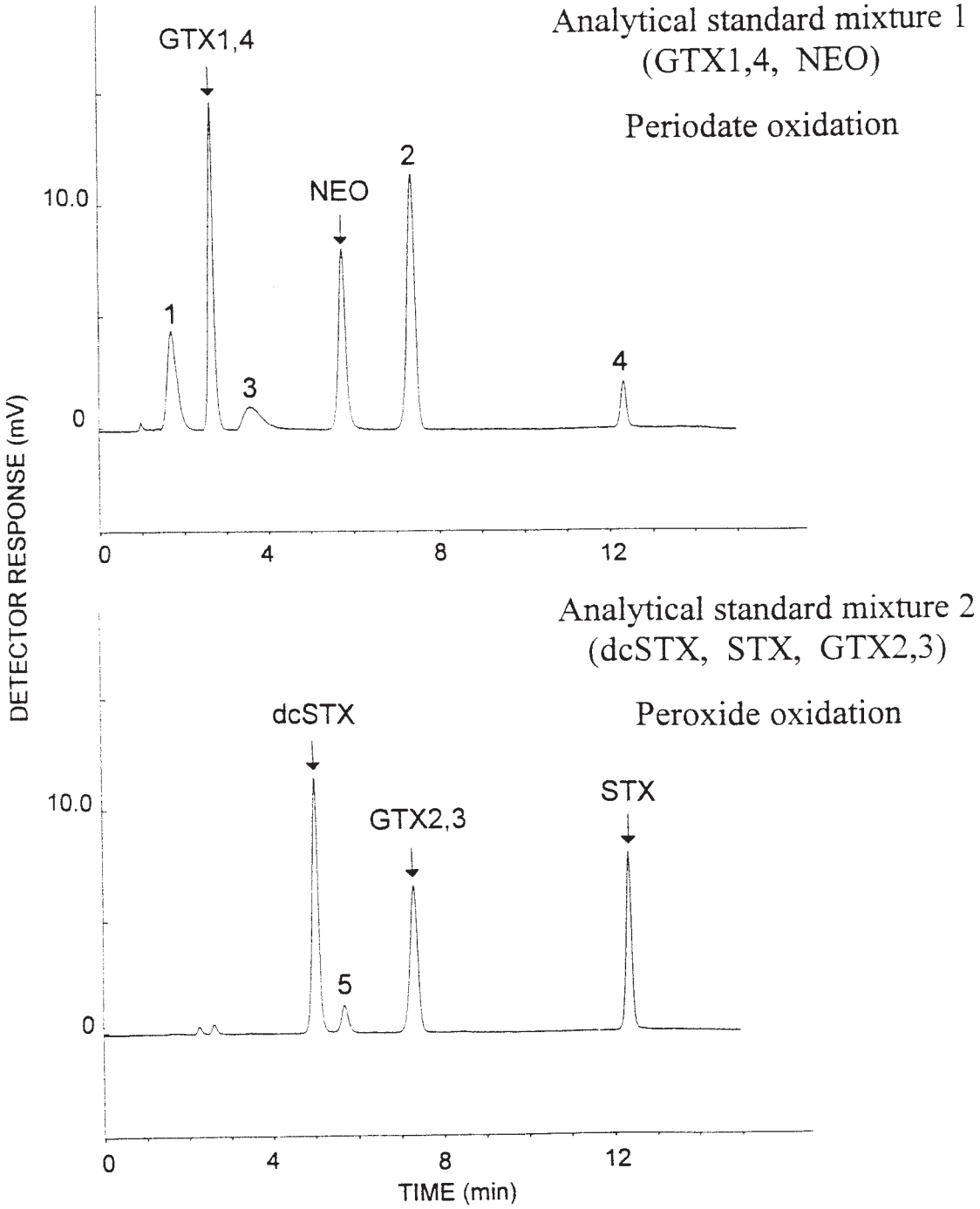


Figure 3. Typical chromatographic patterns obtained with 2 mixtures of analytical standards of PSP toxins. The hydroxylated toxins are oxidized with periodate and the nonhydroxylated toxins by peroxide. The arrows indicate the peaks used for quantitation. Peaks 1 and 2 are secondary oxidation products of GTX1,4 and peaks 3 and 4 are secondary oxidation products of NEO. Peak 5 is a secondary oxidation of dcSTX.

$\times g$). The supernatant was poured into a 50 mL graduated cylinder. The beaker was rinsed with 7 mL water and the rinse was poured into the teflon centrifuge tube, mixed well with the solid material remaining at the bottom, and centrifuged again for 10 min at 4500 rpm (3600 $\times g$). The supernatant was collected into the same graduated cylinder containing the first portion of extract. The volume of extract was made up to 20 mL with water.

C_{18} Cleanup

A 1 mL aliquot (0.5 g shellfish equivalent) of the above crude extract was passed through a 3 mL SPE C_{18} cartridge (Supelco) previously conditioned with 6 mL methanol followed by 6 mL water. The effluent was collected into a 5 mL graduated centrifuge tube. The cartridge was washed with

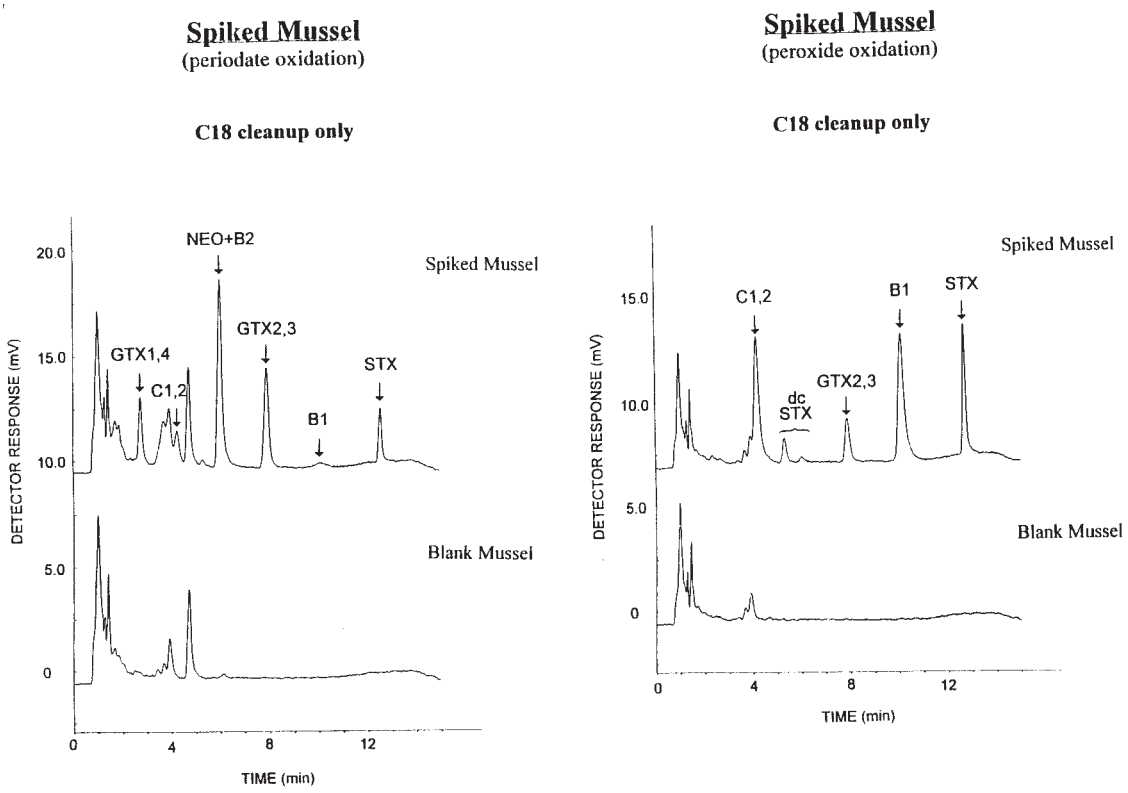


Figure 4. Chromatograms of blank and spiked mussel extracts after SPE C_{18} cleanup. Spiking levels tested were 0.18–1.6 μg/g per toxin.

2 mL water which was combined with first effluent. The volume was made up to 4 mL with water. (To perform oxidations at this stage, aliquots of the extracts were adjusted to pH 7.0 before mixing with the oxidation reagents [6].)

Ion-Exchange Separation of the Toxins

A 3 mL SPE carboxylic acid (COOH) ion-exchange cartridge (Bakerbond, J.T. Baker, Toronto, Canada) was conditioned with 10 mL 0.01M ammonium acetate (0.77 g to 100 mL with water then 10-fold diluted to obtain 0.01M). A 1 mL aliquot (0.125 g shellfish tissue equivalent) of shellfish extract from the SPE C_{18} cleanup step was diluted to 5 mL with water and the pH adjusted to 6.0 (±0.2) using pH indicator paper (calibrated against a pH meter), with 1% NH_4OH. The adjusted solution was passed through the SPE-COOH cartridge and the effluent collected as fraction 1A. Then 4 mL water was passed through the cartridge and collected as fraction 1B. Fractions 1A and 1B contained the C toxins. The toxins, GTX1,4, GTX2,3, B1, B2, and dcGTX2,3 were recovered with 4 mL 0.05M NaCl solution (0.29 g to 100 mL with water) as fraction 2. The cartridge was then eluted with 5 mL 0.3M NaCl solution (1.75 g to 100 mL with water) and collected as fraction 3. This fraction contained STX, NEO, and dcSTX. All fractions were analyzed by LC after periodate and peroxide oxidations as described below. In order to increase the sensitivity of the method (if necessary), each fraction can be concentrated down to 1 mL under a stream of nitrogen at 65°C before the oxidation reactions without affecting the analysis.

Periodate Oxidation

All reagents and solutions used in the oxidation reactions were dispensed using autopipets (Eppendorf) with disposable plastic tips. The periodate reagent was prepared daily by mixing 5 mL each of 0.03M periodic acid (1.71 g to 250 mL with water), 0.3M ammonium formate (4.73 g to 250 mL with water), and 0.3M Na_2HPO_4 (20.1 g to 250 mL with water), and adjusting with 0.2M NaOH to pH 8.2 using a pH meter. A 100 μL volume of standard solution or sample extract after ion-exchange cleanup was added to 100 μL matrix modifier solution (prepared as described in the *Reagents and Chemicals* section, above) in a 1.5 mL microcentrifuge tube. Then 500 μL periodate reagent was added and mixed well on a Vortex mixer. The solution was permitted to react for 1 min at room temperature, then 5 μL concentrated acetic acid was added and mixed. The contents were allowed to stand for 10 min at room temperature before injecting 50 μL (or 100 μL) into the LC system.

Peroxide Oxidation

A 25 μL volume of 10% (w/v) aqueous H_2O_2 was added to 250 μL 1M NaOH (4 g to 100 mL with water) in a 1.5 mL plastic microcentrifuge tube. A 100 μL volume of standard solution or sample extract after ion-exchange cleanup was then added, mixed and permitted to react for 2 min at room temper-

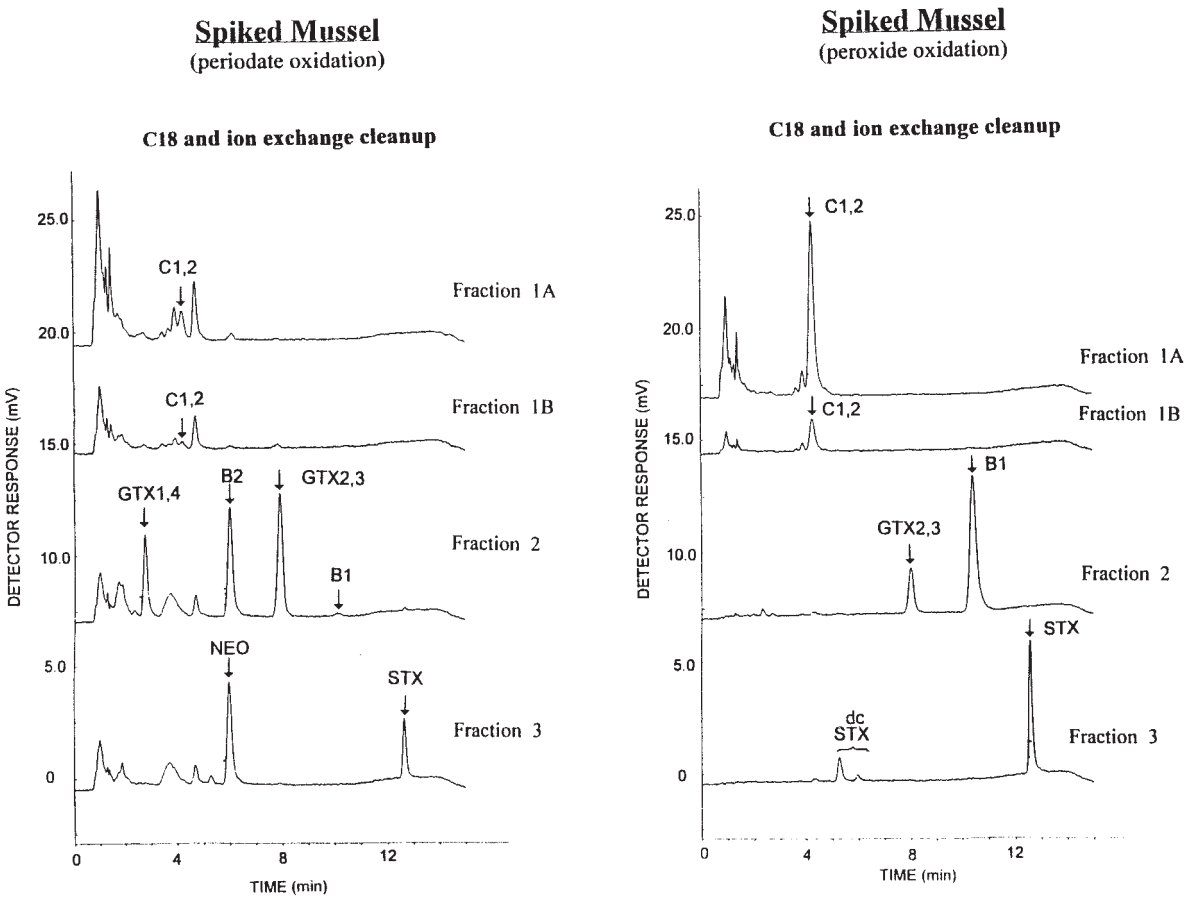


Figure 5. Chromatograms of blank and spiked mussel extracts after cleanup using SPE C_{18} and ion exchange. Spiking levels are the same as in Figure 4.

ature. A 20 μL volume of glacial acetic acid was then added and mixed with the reactant solution. A 25 μL aliquot of this solution was analyzed by LC. (*Note*: Injecting more than 25 μL may cause peak broadening.)

Quantitation

Saxitoxin, dcSTX, NEO, GTX2,3 (together), and GTX1,4 (together) were the only toxins that were accurately quantitated in this study because they were the only compounds for which analytical standards were available. However, they represent the most toxic and most commonly found PSP toxins in shellfish. The detector response (peak area) was determined from a series of calibration standards carried through the oxidation reactions and the linear range established. Each toxin was quantitatively determined in shellfish tissue by direct comparison with analytical standards at similar concentrations as anticipated in the sample. For convenience, 2 analytical standard mixtures were used for quantitating the toxins: One consisting of saxitoxin, dcSTX, and GTX2,3 and the second consisting of the hydroxylated toxins, NEO and GTX1,4 (used only for periodate oxidation).

STX and GTX2,3 produced single oxidation products with both oxidation reactions (4, 5) while dcSTX produced 2 oxidation products with both reactions (7). However, NEO and GTX1,4 each produced 3 peaks after periodate oxidation (4, 5) but only the second eluting peaks were used for quantitation. Figure 2 shows typical chromatographic patterns obtained after oxidation for all of the toxins studied. Because some PSP toxins (NEO and B2; GTX1,4 and C3,4) give the same oxidation products, their quantitative determination was only done after separation by ion-exchange chromatography as described in the *Experimental* section.

Results and Discussion

Method Modifications

(**a**) *Chromatography/detection.*—To reduce analysis time, the gradient and flow conditions were reassessed. The conditions described in the experimental decreased the chromatography time by half and still maintained separation efficiency. Smaller id (2.1 mm) columns were evaluated and while they could provide rapid analysis times, they were less robust for routine analyses of shellfish extracts.

In this evaluation, 2 fluorescence detectors were tested. The Shimadzu that we used was about 5 times less sensitive than the Jasco. This had an impact on the detection limits of the method. For the less sensitive detector, the shellfish ex-

Table 1. Average recoveries (percent ± std. dev.) of PSP toxins from spiked shellfish extracts (quadruplicate analyses)[a]

		Oysters		Mussels		Clams	
Fraction	PSP	Periodate	Peroxide	Periodate	Peroxide	Periodate	Peroxide
1A + 1B	C1,2	—	98 ± 5	—	106 ± 8	—	75 ± 6
2	GTX1,4	91 ± 6	—	77 ± 4	—	97 ± 7	—
	GTX2,3	—	74 ± 5	—	58 ± 5	—	77 ± 5
	B1	—	90 ± 4	—	82 ± 5[b]	—	86 ± 2
	B2	96 ± 7	—	119 ± 6	—	88 ± 4	—
3	NEO[c]	108 ± 1	—	109 ± 2	—	114 ± 5	—
	STX	—	78 ± 3	—	70 ± 3	—	104 ± 7
	dc-STX[c]	—	111 ± 2	—	74 ± 2	—	113 ± 1

[a] Spiking levels for all samples: C1,2 (0.6 μg/g), B1 (0.6 μg/g approximate), B2 (0.6 μg/g approximate), GTX1,4 (0.51 μg/g), GTX2,3 (0.19 μg/g), STX (0.18 μg/g), NEO (0.45 μg/g), dc-STX (0.40 μg/g).

[b] Spiked in mussels at 6.1 μg/g.

[c] Duplicate analyses only.

tracts after cleanup had to be concentrated before oxidation so that enough toxin was oxidized in order to be detected. However, this could only be done within limitations, because too much sample extract in the oxidation mixture adversely affected the yield of oxidation products and thus the quantitation. For a regulatory method that must quantitate total PSP toxins at about 0.8 μg/g (80 μg/100 g, the regulatory guideline in Canada and many other countries), each individual toxin should be quantifiable at concentrations 5–10 times less than this. As the method is set up at present, a performance requirement of the fluorescence detector is that it must be capable of detecting saxitoxin, oxidized with peroxide according to the procedure described above, at 50 pg/injection with a signal to noise ratio (S/N) of at least 10:1.

(**b**) *Oxidation reactions*.—From most of the earlier work on prechromatographic oxidation of PSP toxins, it has been observed that the oxidation reactions are very susceptible to the oxidation environment. Slight changes in pH, reagent composition, and shellfish matrix can have an impact on the quantity and type of oxidation products produced. In some recent work (7), it has also been clearly shown that no single pH is optimum for all toxins. In fact, the optimum pHs vary considerably among the toxins particularly for the periodate oxidation. Thus compromise oxidation conditions must be used. The conditions described in the *Experimental* section represent the best conditions for detecting all the toxins that may be present in a shellfish extract and may not be the best for any single toxin. However, for best repeatability it is important that the reaction conditions be adhered to closely.

In addition to reagent composition and pH, it was found that certain unknown components of shellfish matrixes had a positive impact on the periodate oxidation of the hydroxylated toxins such as NEO and GTX1,4. Spiking shellfish extracts (cleaned up only with C_{18}) immediately before the periodate reaction led to recoveries of 130–150%. However, spiking the extracts after ion-exchange cleanup consistently led to recoveries of only about 40% compared with a standard carried through the same oxidation. Low recoveries were also observed when STX was spiked after ion-exchange cleanup, however, peroxide oxidation of the same extracts gave recoveries of 90–100%. After many attempts to obtain consistently high and uniform recoveries, we found that adding a small amount of a blank shellfish extract (such as oysters, mussels, or clams), improved the yield and repeatability for periodate oxidation of standard solutions and samples cleaned up by either C_{18} or ion-exchange. Thus this addition was incorporated into the procedure. The oysters extract was used in this study because it had the cleanest chromatographic background compared with mussels or clams. No investigations were performed to determine which particular components of the shellfish extracts were responsible for the improvement in the periodate oxidation.

(**c**) *Sample extraction*.—The standard mouse bioassay extraction procedure was used with minimal changes. It was found from earlier work that boiling with dilute HCl acid produced high yields of the toxins (5). The only modifications were in the rinsing steps where water was used and not 0.1M HCl for rinsing the beaker and the centrifuge tube as well as to make up the final volume of the extract if necessary. This was done to reduce the amount of chloride in the extracts because it had an influence on the elution of the toxins from the ion exchange cartridge.

(**d**) *SPE ion-exchange cleanup*.—The ion-exchange separation of the sulfocarbamoyl toxins from their carbamoyl counterparts was completely reevaluated in terms of recovery, repeatability, and ruggedness. This separation step is critical for separating these 2 groups of toxins in order to have an accurate estimation of shellfish toxicity. It was found that the SPE ion-exchange approaches reported earlier (4, 5) were not consistent enough in separating NEO from B2, and some of

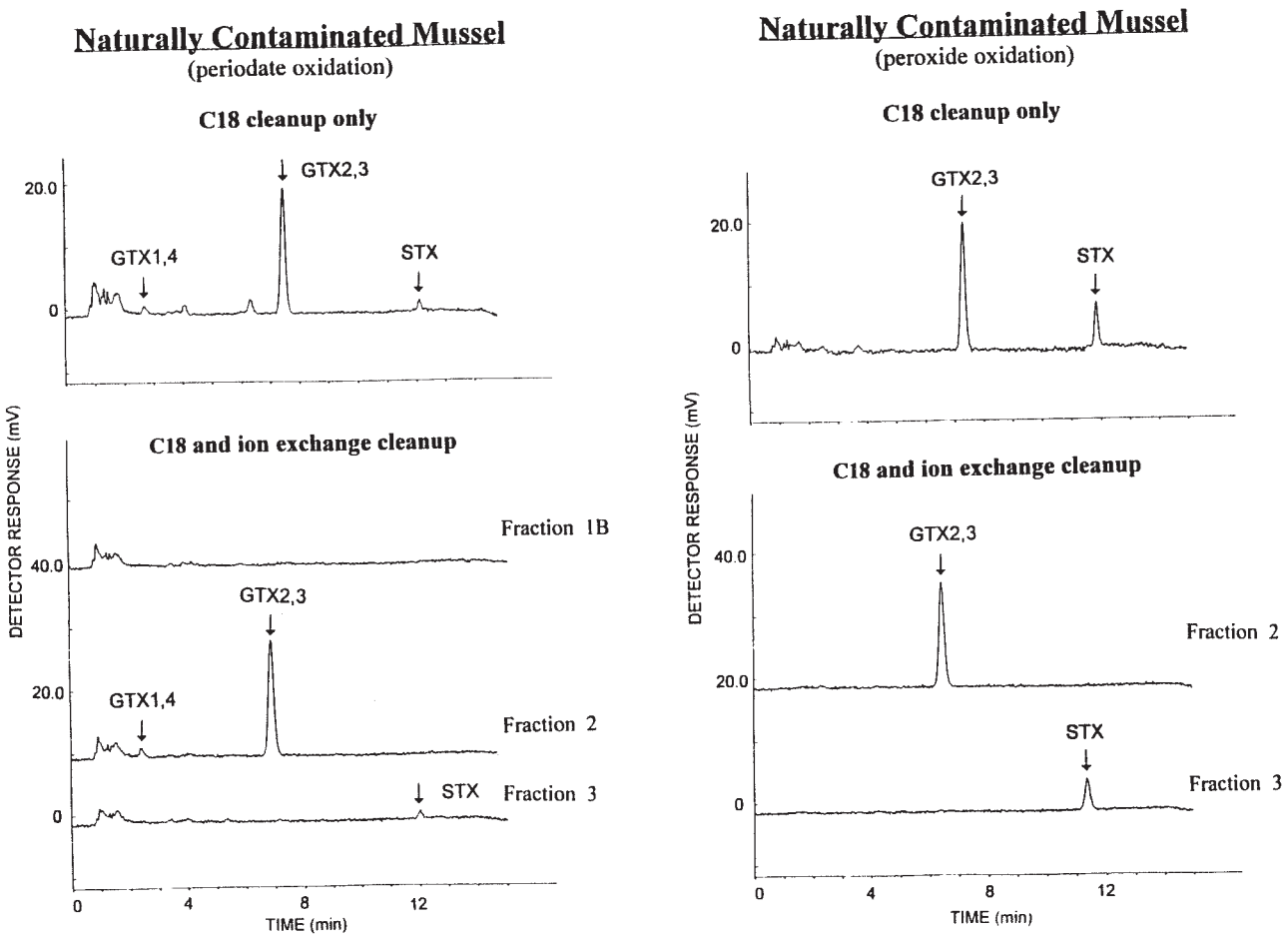


Figure 6. Chromatograms of extracts of naturally contaminated mussels after cleanup using SPE C_{18} and ion exchange.

the other toxins were not consistently separated into discreet fractions. After considerable effort, a repeatable elution pattern was obtained using weak cation exchange (-COOH) cartridges and different concentrations of NaCl as the elution agents. [For this work SPE COOH (Bakerbond) cartridges were used. Some tests with other -COOH cartridges indicated that the selected elution conditions were not applicable to other brands. Thus, optimum elution conditions must be determined for each brand of cartridge.] In the present evaluation, more than 100 cartridges from 3 different batches of Bakerbond product were used and found to provide consistent elution patterns for all the toxins studied.

The C toxins were unretained and eluted in the sample effluent and water wash (fractions 1A and 1B). These fractions were combined in the present work, but may be analyzed separately just to verify the elution pattern (especially to ensure that no GTX toxins elute prematurely). All GTX toxins (GTX1,4, dcGTX2,3, B1, and B2) eluted in the second fraction. The STX group of toxins (STX, NEO, and dcSTX) were much more strongly retained and required 0.3M NaCl for complete elution. The advantage of using NaCl for elution instead of acid (as in earlier work [refs. 4, 5]) is that it was much easier to handle and store than acids and that it was not necessary to adjust the pH before oxidation. The salt did not affect the oxidation reactions, even after the fractions were concentrated 4 or 5 times.

The quantity of sample extract that could be passed through the ion-exchange cartridge without affecting the toxin elution patterns was 0.125 g equivalent sample. Larger quantities affected the elution of the GTX group of toxins causing them to elute slightly sooner and to partially appear in the water wash (fraction 1B). The STX group was less affected and as much as 0.4 mg equivalent sample could be cleaned up without affecting the toxins in fraction 3. This effect is likely at least partially due to the chloride content of the extracts although this was not directly studied.

Method Validation

The modified procedure was tested by analyzing mainly spiked extracts of blank shellfish tissue that had been ex-

tracted by the mouse bioassay extraction procedure. The reason for this was that not enough analytical standards of the toxins were available to spike the material before the extraction. For example, to spike each toxin at 0.8 μg/g in 10 g sample would require 8 μg standard for each single recovery study and to do these in duplicate or triplicate would be too expensive or consume most of our standards (which were not commercially available and would be difficult to replace). However, a few spiking experiments were performed before extraction using GTX2,3 to ensure the efficiency of the mouse bioassay extraction.

Figure 2 shows typical individual chromatographic patterns after both periodate and peroxide oxidations for all the toxins studied in this work. The arrows indicate the peaks used for quantitation. The results for each set represent the same quantities of toxin taken for the oxidation reactions so that relative responses resulting from the 2 reactions can be visually compared. It can be observed that the peroxide oxidation is not useful for any of the hydroxylated toxins. However, it is the preferred reaction for the nonhydroxylated toxins. It can also be observed that GTX1,4 and C3,4 as well as NEO and B2 yield similar patterns and thus, if present together, could not be unequivocally identified and quantitated without prior separation. Figure 3 shows calibration standard mixtures of various toxins used for the routine quantitation of PSP toxins. The arrows indicate the peaks used for quantitation.

Figure 4 shows chromatograms of a blank mussel extract along with an aliquot of the same extract spiked with a mixture of 11 PSP toxins carried through the C_{18} cleanup step only. It can be observed that 6 peaks attributable to PSP toxins appear in the chromatogram of the periodate oxidation and 5 peaks appear in the chromatogram of the peroxide oxidation. The 5 peaks in the latter chromatogram can be easily quantitated against external calibration standards because no other PSP toxins yield these peaks under the peroxide oxidation conditions used. The periodate results are less clear. The main oxidation product of B2 elutes with that of NEO and from previous studies it is known that C3,4 elutes with GTX1,4 (*see* also Figure 2). C1,2 and B1 give a poor response after periodate oxidation and as can be observed, are best quantitated after peroxide oxidation. GTX2,3 and STX may be quantitated after periodate oxidation as long as there is no GTX1,4 or NEO present because these compounds respectively yield secondary product peaks at the retention times of GTX2,3 and STX (*see* Figure 2).

Figure 5 shows chromatograms of the same spiked extract as shown in Figure 4, but including the ion-exchange cleanup step in addition to the SPE C_{18}. It can be clearly seen that C1,2 elutes in fractions 1A and 1B, the GTX toxins (GTX1,4, B2, GTX2,3, and B1) completely in fraction 2, while NEO and STX as well as a small quantity of dcSTX (that was present as a decomposition product in our B1 standard) appeared only in fraction 3. Under these conditions, the individual toxins can be quantitated by comparison with known calibration standards by using periodate oxidation for the hydroxylated toxins and peroxide for the nonhydroxylated analogues.

In the particular case where NEO and dcSTX are present together in the same sample, dcSTX is quantitated directly using peroxide oxidation as described above and NEO is quantitated by comparison with a standard after periodate oxidation but after subtracting the contribution of dcSTX to the NEO peak. This is done by measuring the peak height or area of the first peak of dcSTX (the small peak that elutes just before the NEO peak in Figure 5) and then calculating the peak height or area of the second dcSTX peak (that elutes with NEO) by using the peak ratios obtained from the standard dcSTX after periodate oxidation as shown in Figure 2. This situation was tested by mixing standards of NEO and dcSTX at mass ratios of (0.7:1, 1.5:1, and 3:1) in water at the equivalent 0.7–1.0 ppm NEO and calculating the NEO concentration after the oxidation reactions. The results for the 1.5:1 and 3:1 mixtures were 93 and 94%, respectively, of the expected values. Where dcSTX was in excess of NEO (0.7:1) the calculated concentration of NEO was 79% of the expected value. When a mixture of the 2 at a ratio of 1.5:1 was spiked into a blank mussel extract, the calculated NEO concentration was 92% of the expected value. These results indicate that calculating NEO in the presence of dcSTX by difference does provide acceptable results for quantitation. Alternatively, the first eluting peak of NEO (which is unique for NEO and B2) can be used for quantitation, although the detection limit using this peak is significantly higher than by using the second NEO peak for quantitation. A similar situation for quantitation of GTX1,4 would occur if dcGTX2,3 were also present in the same sample. The quantitation of NEO, dcSTX, and dcNEO (or similarly GTX1,4, dcGTX1,4, and dcGTX2,3) together in the same sample would be more complicated but could be accomplished. Attempts to verify this in the present work were not performed.

Quantitatively, Table 1 lists average recoveries and repeatabilities (±standard deviation) obtained from 4 replicates each of spiked crude extracts of oysters, clams, and mussels carried through the complete cleanup procedure with both periodate and peroxide oxidations. The overall recoveries and repeatabilities are acceptable at the spiking levels tested (0.18–0.6 μg/g per toxin, as well as B1 spiked in the mussels at 6.1 μg/g). Saxitoxin and GTX2,3 showed lower recoveries than the other toxins studied. These 2 toxins were spiked at the lowest levels (0.18 and 0.19 μg/g, respectively) which may have been a contributing factor. Subsequent studies on these toxins spiked at the 1.0 μg/g level produced recoveries of about 90% (peroxide).

The extraction step was tested by spiking triplicate portions of homogenized blank mussel tissue with GTX2,3 at 1.0 μg/g. The recoveries averaged 91% (peroxide), verifying that the extraction procedure yields high recovery of the toxins. In addition, 6 samples of naturally contaminated shellfish were analyzed and the results compared with the mouse bioassay. The following results were obtained (mouse bioassay, saxitoxin equivalents/LC, total PSP toxins): scallop (2.8 μg/g/2.9 μg/g), mussel A (0/0), mussel B (3.0/4.6), mussel C (14/19), clam A (0.8/0.4), and clam B (3.0/3.6). These samples contained GTX2,3 (predominant) and relatively small amounts of

GTX1,4 and STX. For most of the samples, the LC results were somewhat higher than those obtained by the bioassay. This is because the LC values were calculated by simply adding the individual results obtained for each toxin without converting the data to saxitoxin equivalents. Converting the LC data to saxitoxin equivalents would lower the results bringing them into closer agreement with the bioassay results. Figure 6 shows chromatograms before and after ion-exchange cleanup of the mussel B sample mentioned above. The chromatograms are relatively simple to interpret and quantitate.

The detection limits for calibration standards in ng per injection at 3:1 S/N using the Jasco detector were approximately: *for periodate*: C1,2 (350 pg, not quantitative), C3,4 (330 pg, not quantitative) GTX1,4 (70 pg), GTX2,3 (9 pg), B1 (6.4 ng, not quantitative), B2 (80 pg, not quantitative), STX (79 pg), NEO (30 pg), dcSTX (70 pg); *for peroxide*: C1,2 (60 pg, not quantitative), B1 (60 pg, not quantitative), GTX2,3 (20 pg), STX (12 pg), dcSTX (10 pg).

In terms of μg/g in the samples studied the detection limits were approximately: C1,2 (0.1 μg/g, not quantitative), C3,4 (0.1 μg/g, not quantitative), GTX1,4 (0.07 μg/g), GTX2,3 (0.02 μg/g), B1 (0.1 μg/g, not quantitative), B2 (0.13 μg/g, not quantitative), STX (0.02 μg/g), NEO (0.06 μg/g), dcSTX (0.02 μg/g).

In conclusion, the prechromatographic oxidation LC method has been modified to improve the quantitative and operational aspects. The aim of this work is to produce a method that is capable of quantitating individual toxins in shellfish based on comparisons with known analytical standards as is commonly done with most other food contaminants. The results obtained should be useful for regulatory enforcement purposes. It is planned to further validate this method by submitting it to an interlaboratory collaborative study.

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