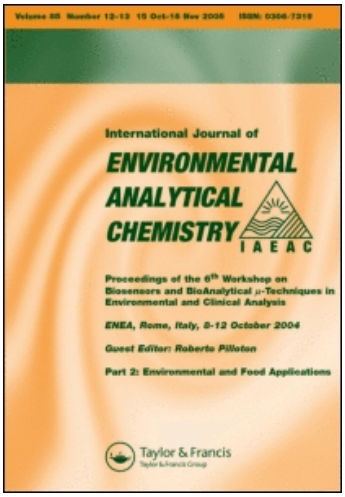

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF DOMOIC ACID, A MARINE NEUROTOXIN, WITH APPLICATION TO SHELLFISH AND PLANKTON*

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A recent outbreak of poisoning resulting from the consumption of cultured blue mussels (*Mytilus edulis* L.) from a localized area in Eastern Canada has been attributed to the presence of domoic acid (**1**), a relatively rare neurotoxic amino acid, previously found only in some algae of the family Rhodomelaceae. Studies on aqueous extracts of shellfish tissue indicated that the toxin and several of its isomers could be separated (and isolated in sufficient amounts for subsequent structural identification) by reversed-phase high-performance liquid chromatography (HPLC) with ultraviolet (UV) diode array detection (DAD). Aqueous acetonitrile containing 0.1% v/v trifluoroacetic acid was used as mobile phase. As the retention time and characteristic UV absorption spectrum of **1** ($\lambda_{max} = 242$ nm) permit unequivocal identification, the HPLC-DAD procedure was refined with a microbore column to provide a rapid (5 min), sensitive (0.3 ng detection limit) and reproducible assay method for the determination of **1** in shellfish tissue. Extraction was accomplished by boiling homogenized shellfish tissue for 5 min with distilled water. Extracts were taken through an octadecylsilica solid phase extraction clean-up prior to HPLC. This method has been applied to a variety of shellfish and phytoplankton samples.

BRIEF
Reversed-phase HPLC with ultraviolet diode array detection was used to analyze shellfish tissue and phytoplankton extracts for domoic acid. A rapid (5 min) and sensitive (0.3 ng detection limit) assay is presented.

KEY WORDS: Domoic acid, neurotoxin, shellfish, high-performance liquid chromatography, ultraviolet diode array detector.

C.A. Registry Numbers: Domoic acid 14277-97-5.

INTRODUCTION

From November 11 to December 12, 1987, 153 people suffered from acute intoxication after eating cultured blue mussels (*Mytilus edulis* L.) harvested from a localized area in eastern Prince Edward Island (P.E.I.). Symptoms included nausea and diarrhea which in some cases were followed by confusion, disorientation, loss

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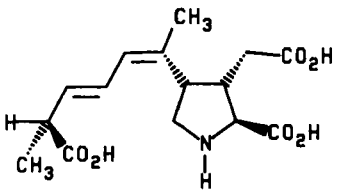

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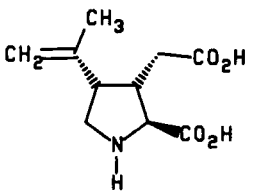

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of memory and even coma. Three elderly people died. In the other most severely affected cases neurological symptoms still persist.[1] The term Amnesic Shellfish Poison (ASP) has been proposed for this new shellfish toxin. The toxic agent was first detected in mussel digestive glands using the Association of Official Analytical Chemists (AOAC) mouse bioassay for paralytic shellfish poison (PSP).[2] Extracts of contaminated mussels injected intraperitoneally into laboratory mice produced an unusual scratching syndrome and eventual death. An intense investigation[3] using bioassay-directed analysis led to identification of the toxin as domoic acid (**1**), a relatively rare naturally-occurring secondary amino acid that was first isolated in 1958 from the red alga *Chondria armata* Okamura (Rhodomelaceae family).[4,5] The potent neurological activity of **1** and the structurally related compound kainic acid (**2**) has attracted a great deal of interest in recent years in pharmacological studies.[6-10] A total synthesis approach[11] in 1982 revealed the structure of the natural product to be that of (−)-domoic acid (**1**) with the 1′Z, 3′E, 5′R stereochemistry shown.

From production records, about 63 000 kg of harvested mussels contained domoic acid at levels as high as 900 μg/g of wet tissue. Very large quantities of domoic acid must have been produced in the affected area over a period of a few weeks. Since all candidate sources are natural and not unique to the area which was affected, it is probable that regulatory authorities and aquaculturists in many areas will have to monitor domoic acid levels in shellfish in the future. In view of this, the Atlantic Research Laboratory of the National Research Council of Canada has undertaken a major effort to further study the chemistry of this substance and methods for its analysis. The present work is devoted to the reversed-phase high-performance liquid chromatography (HPLC) of domoic acid and of several isomers also found in minor quantities in the contaminated mussels. HPLC played a key role in all stages of the investigation which led to the identification of domoic acid as the mussel toxin.[3] In the initial stages, HPLC with UV diode array detection (DAD) was used for "fingerprinting" extracts of toxic and control shellfish, and preparative HPLC was used for purification of the toxin for structural elucidation by spectroscopic techniques. Later, HPLC was used for accurate determination of domoic acid in mussel tissue in order to account for the observed toxicity and also for survey work on a wide variety of samples including

other shellfish and phytoplankton. Recently, it was shown that the pennate diatom *Nitzschia pungens* Grun. f. *multiseries* Hasle produces domoic acid and was likely the primary source of the toxin in the P.E.I. incident.[12,13]

The addition of domoic acid to shellfish monitoring programs will require a rapid, accurate, reproducible, and sensitive analytical method. The method must be applicable to a wide range of sample types and preferably provide at least two independent sources of evidence for identification purposes. Such a method, based on high speed HPLC-DAD, is presented below.

EXPERIMENTAL

Materials

All solvents used were HPLC-grade from Anachemia. Distilled water was further purified to HPLC-grade by passage through a Millipore water purification system (Milli-Q) equipped with ion-exchange and carbon filters. Trifluoroacetic acid (99.5% purity) was purchased from BDH.

High purity domoic acid (**1**) was isolated from an aqueous extract of contaminated mussels by ion exchange chromatography (details provided in ref. 3), followed by crystallization as a dihydrate (colorless needles) from deionized water; m.p. 215–216°, reported value 217°.[5] Drying the crystalline material *in vacuo* at 50° overnight afforded anhydrous domoic acid; $[\alpha]_D^{23} - 120.5$; elemental analysis found C = 57.54%, H = 6.98%, N = 4.16% (versus calculated values of C = 57.87%, H = 6.80%, N = 4.50% for $C_{15}H_{21}NO_6$). The proton chemical shift and spin-spin coupling data, derived from a spectrum recorded on an aqueous solution with a Bruker MSL-300 NMR spectrometer, were indistinguishable from literature values,[11] and the spectrum contained no signals due to impurities. Mass spectrometry[14] with fast atom bombardment ionization (glycerol/water, 1:1; $MH^+ = m/z$ 312) and electron ionization as the tris-*tert*-butyldimethylsilyl derivative ($M^+ = m/z$ 653; $[M\text{-}tBu]^+ = m/z$ 596) indicated no significant impurities. Similarly, the infrared spectrum showed no detectable impurities.[15] HPLC with UV absorbance detection as low as 200 nm also showed this material to be free of impurities. Samples of domoic acid of lower purity were also purchased from Sigma Chemicals, Cambridge Research Biochemicals and Regis Chemicals.

Sample Extraction

In a typical extraction, homogenized mussel tissue (100 g) was heated and boiled for 5 min (stirring) with distilled water (100 mL). The cooked mixture was centrifuged at 3500 rpm (5 min), the supernatant decanted and the residual pellet washed with water (50 mL) and recentrifuged. The combined supernatants were made up to 250.0 mL. For other extraction methods investigated, see Table 1.

Table 1 Methods examined for the extraction of domoic acid from mussel tissue

Method	*Solvent*	*Time*	*Temp.* (°C)	*Conditions*	*Estimated* % *Recovery*
A	0.1 N HCl[a]	5 min	100	Stirring	75–85
B	H_2O/CH_3OH (1:1)	24 h	25	Standing	85–90
C	H_2O/CH_3OH (1:1)	1 h	25	Shaking	80
		4 h			95
		20 h			100
D	H_2O/CH_3OH (1:1)	5 min	60–70	Stirring	100
E	H_2O	24 h	25	Shaking	100
F	H_2O	5 min	100	Stirring	100

[a]AOAC method for paralytic shellfish poison (2).

Phytoplankton and seaweed samples were extracted by sonication in a minimum volume of cold water, followed by filtration to remove debris.

Clean-up Procedure

After the pH of the crude extract had been adjusted to pH 6 to 7 (usually not necessary with aqueous extracts), an accurate volume of up to 2 ml was placed on a pre-rinsed (6 mL acetonitrile and 6 mL water) octadecylsilica LC-18 solid phase extraction cartridge (Supelco, Bellefonte, PA). The sample was eluted with 3 mL of 10% aqueous acetonitrile, with the eluate being collected in a 5-mL volumetric flask. The contents of the flask were made to volume with water. An aliquot was then passed through a dry 0.22 μm filter and used for HPLC analysis.

HPLC

Analyses were performed on a Hewlett–Packard model 1090M HPLC equipped with a DR5 solvent delivery system, variable volume (1 to 25 μL) injector and autosampler, built-in HP1040 DAD and HP79994 data system. Columns (25 cm × 4.6 mm I.D. or 2.1 mm I.D.) packed with 5 μm Vydac 201TP (Separations Group, Hesperia, CA) were used at 40 °C. The mobile phase was aqueous acetonitrile with 0.1% v/v trifluoroacetic acid. High resolution separations were performed with the 4.6 mm I.D. column using a 20 μL injection and linear gradient elution from 5% to 25% acetonitrile over 25 min at 1 mL/min. High speed analyses used the 2.1 mm column with a 5 μL injection volume and isocratic elution with 10% acetonitrile at 0.5 mL/min. Detection was effected by monitoring absorption at 242 nm with a 10 nm bandwidth. UV spectral acquisition was either triggered by peaks or continuous at 640 msec intervals. Quantification was

accomplished by comparing the areas of peaks from unknowns with those from standard solutions prepared from pure domoic acid.

RESULTS AND DISCUSSION

Reversed-Phase HPLC-DAD

The selection of HPLC conditions for domoic acid was essentially completed in the early stages of the investigation leading to the identification of the toxin.[3] Following the early observation that the toxin was water soluble, a number of HPLC columns and mobile phases were investigated for the "fingerprinting" of toxic and control mussel extracts. The method that proved successful was selected on the premise that the unknown toxin was an ionizable compound such as a peptide, since toxic peptides have been reported in cyanobacteria.[16] Reversed-phase HPLC of peptides is often accomplished with wide-pore fully endcapped or polymeric octadecylsilica (ODS) stationary phases and with a mobile phase of aqueous acetonitrile containing 0.1% trifluoroacetic acid (TFA) as an ion-suppression/ion-pairing agent.[17] TFA is useful because it is transparent to 210 nm and volatile for preparative work. Vydac 201TP ODS stationary phase (5μm spherical particles) used with this mobile phase proved very effective for the toxin identification and subsequent quantification. It is important to note that for bioassay-directed isolation of the toxin, it was necessary to substitute acetic acid (1% v/v) for TFA since trifluoroacetate salts are rodenticides and give false positives in toxicity determinations by mouse bioassay.

Once the toxin was identified as domoic acid, the successful HPLC conditions could be rationalized. The compound is polyfunctional with three carboxyl groups and one secondary amino group, the pK_a's of which have been reported[5] to be 2.10, 3.72, 4.97 and 9.82. Therefore a mobile phase pH of 2 to 3 is required to effectively suppress ionization, in order to prevent tailing due to adsorption on column active sites and to increase the capacity factor (k') relative to salts and other endogenous compounds in the sample. Indeed, measurements have shown that the k' of domoic acid decreases as pH increases and at a pH greater than 4 serious band broadening occurs for trace levels. A 0.1% TFA concentration is recommended since loss of resolution was observed for several samples when only 0.01% TFA (pH 3) was used. Isocratic separations with 10% acetonitrile gave a k' of approximately 3 for domoic acid (*vide infra*).

Figure 1 presents the gradient elution chromatogram generated for an extract of the digestive glands of toxic mussels. A diode array detector (DAD) allowed the acquisition of UV absorption spectra. One inset in Figure 1 shows the UV spectrum for domoic acid, which has an absorption maximum (λ_{max}) at 242 nm. This spectrum proved very useful in the identification of the toxin, since it suggested a conjugated diene system as a possible substructure, and facilitated routine confirmation of peak identity in survey work (*vide infra*).

The other inset in Figure 1 shows a number of minor peaks that elute close to domoic acid. The relative retention times and tentative identities of some of these

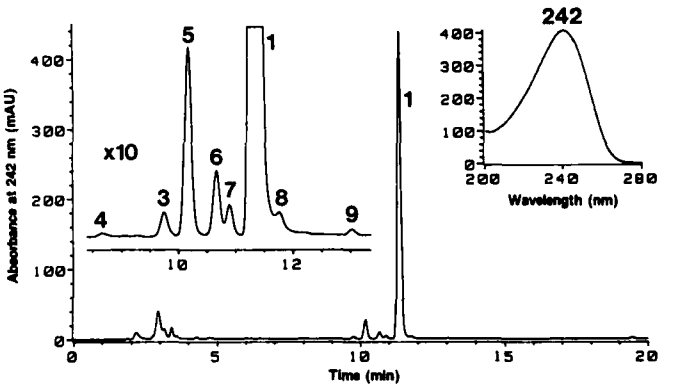


Figure 1 Gradient reversed-phase HPLC-DAD analysis of an aqueous extract of mussel digestive glands containing high levels (800 μg/g wet tissue) of domoic acid (**1**). The inset at the right shows the UV spectrum of **1** and the one at the left is an expansion of the chromatogram showing the presence of tryptophan (**3**) and compounds **4** to **9** (see Table 2 for peak identities). Conditions: 25 cm × 4.6 mm Vydac 201TP column with 1.0 mL/min CH_3CN/H_2O/TFA; gradient elution from 5.0:94.9:0.1 to 25.0:74.1:0.1 over 25 min.

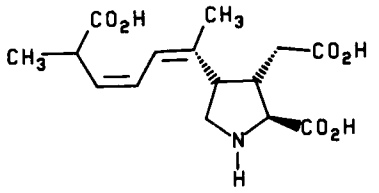
Table 2 *Compounds observed in the extract of toxic mussels (refer to Figures 1 and 2 for corresponding peaks)*

Cmpd.	*Rel. ret. time*		λ_{max} (*nm*)	*Identity*
	LC1[a]	*LC2*[b]		
1	1.000	1.000	242	Domoic acid[c]
3	0.860	0.863	220, 280	Tryptophan[d]
4	0.765	0.665	244	Not yet identified
5	0.898	0.854	244	*Cis-trans* isomer of **1**[e]
6	0.941	0.902	241	*Cis-trans* isomer of **1**[e]
7	0.961	0.912	220	Not yet identified
8	1.038	1.080	242	Diastereoisomer of **1**[e]
9	1.151	1.330	243	*Cis-trans* isomer of **1**[e]

[a]Gradient elution (see Figure 1 for conditions).
[b]Isocratic elution (see Figure 2 for conditions).
[c]Positively identified by retention time, optical rotation, and UV, NMR, FTIR and mass spectra (ref. 3).
[d]Positively identified by retention time plus UV and mass spectra.
[e]Consistent with NMR, FTIR and mass spectra (ref. 18).

compounds are presented in Table 2, along with the absorption maxima observed in their UV spectra. Compound **3** has been positively identified (by retention time plus UV and mass spectra) as the amino acid tryptophan. Some of the other compounds have been determined to be isomers of **1**. Compounds **5**, **6** and **9** give absorption maxima near 242 nm and have been shown by NMR, FTIR and mass spectroscopies to be geometrical (*cis-trans*) isomers of **1**.[18]

Compound **7** gives a UV absorption spectrum with a λ_{max} of 220 nm, which indicates that it does not have the conjugated diene system. This compound may be one of the previously identified isodomoic acids,[19] although this remains to be determined by spectroscopic studies. Compound **8**, which gives a UV spectrum



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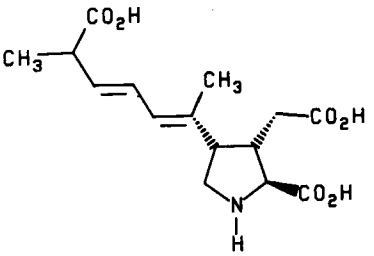

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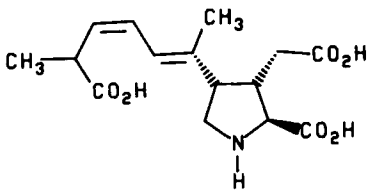

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identical to that of **1**, has been tentatively identified as a diastereoisomer of **1** since it also gives identical NMR, FTIR and mass spectra under the experimental conditions used.[18] The toxicological significance of these isomers has not yet been established.

The separation of domoic acid and its isomers varies between different stationary phases. Columns packed with Vydac 201TP, Supelcosil LC-PAH, Zorbax-ODS, Hypersil-ODS, and Vydac 214TP phases were tested and it was found that the best separation was provided by the first two packing materials, which gave similar selectivity. It is possible to vary the separation selectivity by changing the mobile phase composition (e.g., different organic modifiers such as methanol and buffers such as phosphate) but we have found the acetonitrile/water/TFA mobile phase to be very reliable and convenient to prepare. The high speed isocratic HPLC method presented below (see Figure 2) shows that there are significant differences in separation selectivity between gradient and isocratic conditions.

Extraction

Several methods were examined for the extraction of domoic acid from mussel tissue. Table 1 presents a summary of the results of experiments in which 100 g subsamples of a large homogenate of toxic mussel tissue with a relatively high level of domoic acid (approximately 250 μg/g wet tissue) were extracted using different solvents and conditions. Further extractions of tissue residues were performed with complementary methods to determine if all free domoic acid had been extracted by any single method. If no further domoic acid could be recovered, the first extraction was considered quantitative. It has not been

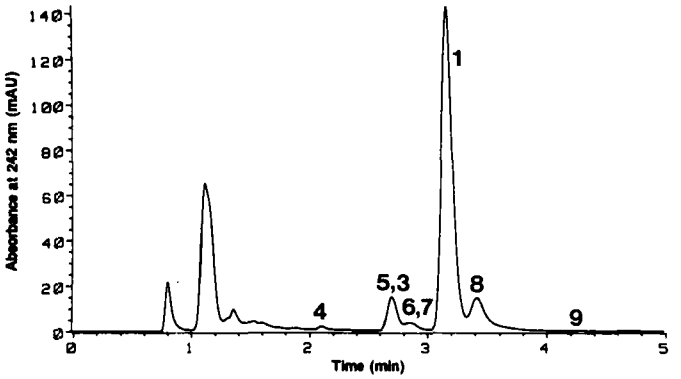

Figure 2 Isocratic high-speed HPLC analysis of an aqueous extract of autoclaved mussel tissue homogenate containing high levels (516 μg/g wet tissue) of domoic acid (**1**). Conditions: 25 cm × 2.1 mm Vydac 201TP column with 0.5 mL/min CH_3CN/H_2O/TFA (10.0:89.9:0.1).

determined yet if some domoic acid is sequestered in a covalently bound form not accessible to the extraction methods used.

Method A, in which tissue homogenate is boiled 5 min with 0.1 N HCl, is the AOAC method used routinely for monitoring shellfish by mouse bioassay.[2] It would be advantageous if the extract from one method could be split and used for both PSP and domoic acid assays. Unfortunately, it was found that the extraction yield was only 75% to 85%. In addition, the domoic acid concentration in the extract was observed to decrease with time of storage (up to 50% loss in one week at room temperature). Extracts taken through the clean-up procedure discussed below, however, have been found to be stable. The utility of this approach for routine monitoring programs has been investigated separately.[20]

Extraction with aqueous methanol (methods B to D) is a conventional natural products method and was used in the initial work to identify the toxin. Methods C and D gave good extraction yields, but required evaporation of the extract under reduced pressure prior to clean-up and HPLC analysis. Although this is time-consuming, these methods do give the best overall enrichment for trace determinations since the extract can be ultimately in a small volume.

The aqueous solubility of domoic acid (25 ± 2 mM at pH 3.2 and 20°) led us to investigate extractions in distilled water. Both methods E (shaking 24 h at 25 °C) and F (boiling 5 min) gave quantitative recoveries. Method F, detailed in the Experimental section is recommended for shellfish tissue extraction because it is fast, easy and reproducible (*vide infra*) and the boiling serves to denature and precipitate protein. The amount of tissue used in the extraction was typically 100 g with a final extract volume of 250 mL. Although smaller samples in the range of 1 to 20 g have been extracted successfully into 5 to 50 mL volumes, a larger sample size minimizes sampling error due to observed differences between individual animals. Experiments in which uncontaminated mussel tissue was spiked with pure domoic acid at the 3 μg/g of wet tissue level showed good recovery and precision

(mean = 70%; s.d. = 10%; $n = 6$). The stability of aqueous extracts was also investigated. If frozen within a day of extraction, the concentration of domoic acid was stable over several months. If left at room temperature, some decrease in concentration was observed, possibly due to bacterial growth. Extracts which have been taken through the clean-up procedure described below were observed to be stable for up to a week at room temperature. If other determinations may be done later, it is advisable that a portion of an aqueous extract be subjected to the clean-up as soon as possible and the remainder frozen. One word of precaution is necessary. Some solutions of pure domoic acid in water with 0.1% TFA were found to decompose when frozen. This has been attributed to a freeze-concentration effect in which both the TFA and domoic acid are concentrated into a small volume of liquid as the bulk of the solution freezes. Calibration solutions made with acetonitrile/water (1:9) were found to be very stable to both freezing and bacterial decomposition.

Clean-Up

The extracts of mussel tissue contain both particulate material and low polarity substances such as lipids that present problems for HPLC analysis, particularly high speed isocratic determinations. Partitioning the aqueous extract with dichloromethane and filtration of the aqueous layer was found to be acceptable, but was quite time-consuming. Therefore, a rapid clean-up procedure based on solid phase extraction (SPE) was developed and tested.

The clean-up, which is detailed in the Experimental section, involves eluting 1 to 2 mL of aqueous extract through a reversed-phase SPE cartridge with 10% aqueous acetonitrile into a 5-mL volumetric flask. This simple procedure gave excellent reproducibility and effectively removed substances that would have eluted at long retention times from the HPLC. Quantitative recovery of domoic acid from standard solutions and extracts was observed: six replicate determinations of a spiked control extract at the 0.5 μg/mL level gave a mean recovery of 97% (s.d. = 5%). It is important to ensure that the extract has a pH of 6 to 7, since poor recovery was observed for trace level extracts at low pH (e.g., extraction method A). Extraction methods B through F gave near-neutral extracts for most shellfish samples. As mentioned above, extracts taken through this clean-up appear to be quite stable. Several hundred samples were analyzed using this method before significant HPLC column deterioration was evident. The method could be automated easily.

While this procedure is suitable for extracts ranging from 0.5 to 500 μg domoic acid per mL of extract, trace level samples need to be further concentrated. Evaporation of the cleaned-up extract under reduced pressure and dissolution into volumes as low as 0.1 mL was found to be possible, but poor volumetric control results in lower precision. We are currently investigating an SPE clean-up based on a combination of reversed-phase and strong cation exchange cartridges that should allow extensive enrichment of domoic acid in the final extract.

High Speed HPLC Determination

Although the gradient elution separation given in Figure 1 is acceptable, the overall analysis is quite long when the time necessary for column re-equilibration is taken into consideration. A high speed isocratic determination was required for processing hundreds of samples and for use on less sophisticated equipment. Figure 2 presents a chromatogram from our implementation of such an analysis in which a 2.1 mm I.D. Vydac 201TP column is operated with 0.5 mL/min of a 10% aqueous acetonitrile mobile phase and with detection at 242 nm. A 40 °C column temperature helps to reduce column head pressure to approximately 200 bar. Domoic acid has a retention time of 3.2 min and the determination is complete in 5 min. The separation of the isomers and tryptophan is not as good under these conditions, but this was acceptable for the rapid determination of domoic acid.

In comparing Figures 1 and 2, it is interesting to note in the latter the increased level of compound **8** which has been attributed tentatively to a diastereoisomer of domoic acid. This sample was a mussel tissue homogenate that was packaged and autoclaved at 120°, for evaluation as a possible reference material for distribution under the Marine Analytical Chemistry Standards Program (MACSP). In separate experiments, it was found that domoic acid slowly rearranges to compounds **4** to **9** (particularly **8**) at high temperatures and/or under acidic conditions. Although the rearrangements do not appear to be significant under the extraction conditions described above, this observation should be taken into consideration in any analysis of cooked or acidified samples.

Confirmation of peak identity with a high degree of confidence is possible by matching the retention time and UV spectrum with that of authentic domoic acid. Additional confirmation is possible with fast atom bombardment (FAB) mass spectrometry or electron ionization mass spectrometry after chemical derivatization.[14] An amino acid analyzer (ion exchange column with ninhydrin reaction detection) can also be used for further chromatographic confirmation.[3]

The detector response was calibrated initially by using a domoic acid solution obtained by heart-cutting a peak from a semi-preparative scale HPLC separation. The concentration of the domoic acid was then determined by measuring the absorbance at 242 nm and using the literature value of 2.63×10^4 for the molar absorptivity.[4] Later, a primary calibration solution (70 μg/mL) was prepared from a known weight of highly purified anhydrous domoic acid. The latter solution gave a value of $(2.62 \pm 0.02) \times 10^4$ at pH 7 for the molar absorptivity. Instrument response was established to be sufficiently linear over the range of 1 ng to 1 μg injected (correlation coefficient of 0.99995) that a single-point external calibration was used. The reproducibility of peak areas for repeat injections of the calibration solution made over several days was of the order of 1% relative standard deviation (RSD). The overall reproducibility of the method was found to be excellent. For example, extractions and determinations on 12 subsamples of one tissue homogenate (514 μg domoic acid per gram of wet tissue) gave a 3.2% RSD. Other replicate measurements over the range of 10 to 900 μg/g indicated a 3 to 5% RSD. Trace level determinations at the 1 μg/g concentration gave a 10 to 15%

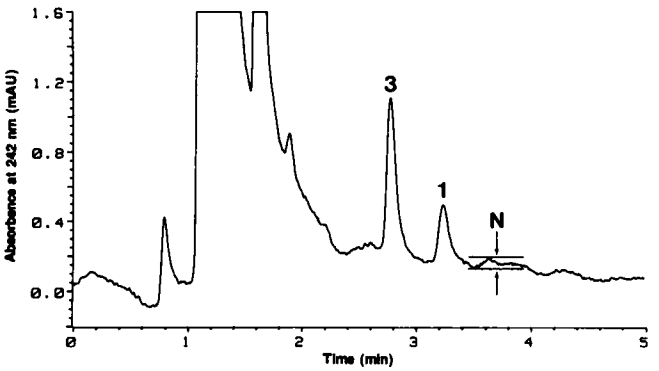

Figure 3 HPLC analysis of an aqueous extract of control mussel tissue spiked with domoic acid (1) at 0.8 μg/g wet tissue. The amount of domoic acid injected is 0.6 ng. The medium-term noise (N) was used to determine the detection limit as 0.3 ng injected (signal/noise = 3). Conditions: same as Figure 2.

RSD. We are investigating internal standards for working with low extract volumes, where the precision of the external calibration method would be less.

The high molar absorptivity of domoic acid at 242 nm and the use of a microbore column facilitated a sensitive assay. As shown in Figure 3 for a spiked control mussel extract, the detection limit was estimated to be 0.3 ng injected (signal to noise ratio of 3). With a 5 μL injection volume and the regular extraction and clean-up procedure presented in the Experimental section, this corresponds to a concentration of 0.4 μg/g wet tissue. The minimum identification limit, defined as the quantity of analyte required for the DAD to provide a UV spectrum of sufficient quality to permit identification, was estimated to be 3 ng injected. Uncontaminated mussel tissues were extracted regularly to check for laboratory contamination when the domoic acid content of the samples analyzed was near the detection limit.

Applications

A variety of shellfish samples (mussels, clams, scallops, oysters, and quahogs) from different sources have been taken through extraction and analyzed successfully, giving acceptable chromatograms, although not all of these contained domoic acid. No significant interfering peaks at the retention time of domoic acid were observed for the samples we have examined. The only modifications required for different sample types were minor ones related to the history of the sample. For example, during the analysis of pickled mussels, it was essential to analyze the juice in which they were preserved, since the preservation process extracts a significant portion of the domoic acid.

Figure 4 shows the HPLC analysis of the aqueous extract of soft-shell clams taken from eastern P.E.I. two months after the poisoning incident. A low level of domoic acid (2.6 μg/g wet tissue), confirmed with a correct UV spectrum, was still detected.

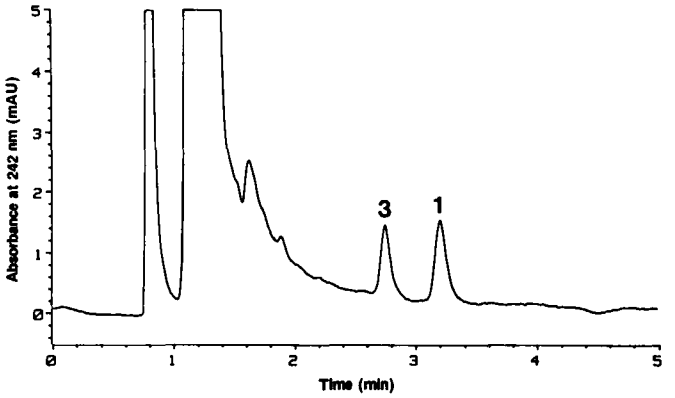

Figure 4 HPLC analysis of an aqueous extract of soft-shell clams with a low level of domoic acid (**1**) contamination (2.6 μg/g wet tissue). Conditions: same as Figure 2.

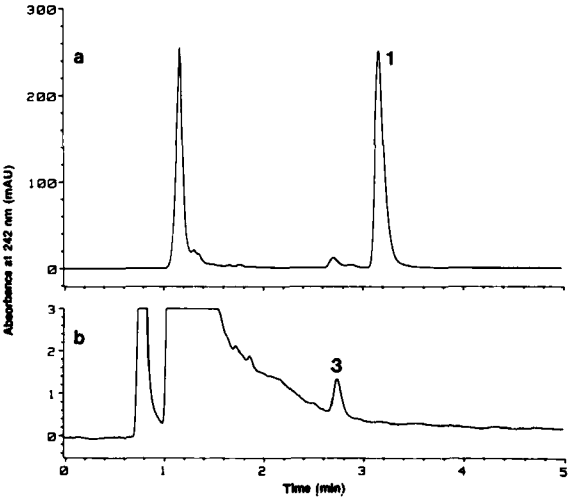

Figure 5 HPLC analysis of aqueous extracts of offshore scallop digestive glands (a) and edible tissue (b). Conditions: same as Figure 2.

An interesting observation is that domoic acid appears to be quite widely spread and is not unique to the P.E.I. region. Some scallop samples taken from various offshore locations had levels of domoic acid in the digestive glands as high as 700 μg/g. Figure 5 shows that edible adductor muscle tissue of the scallop is free of domoic acid, however, even when the digestive glands are laden with domoic acid.

Only one sample analyzed thus far has given a "false positive" in the HPLC chromatogram. Figure 6 shows the analysis of a scallop sample that had been left accidently at room temperature for some time and had turned rancid. Although the roe, digestive glands and edible tissue showed no trace of domoic acid, the juices associated with the tissues did give a peak in the chromatogram with a retention time matching that of domoic acd exactly. However, a check on the UV spectrum acquired by the DAD showed that it was not domoic acid. The spectrum

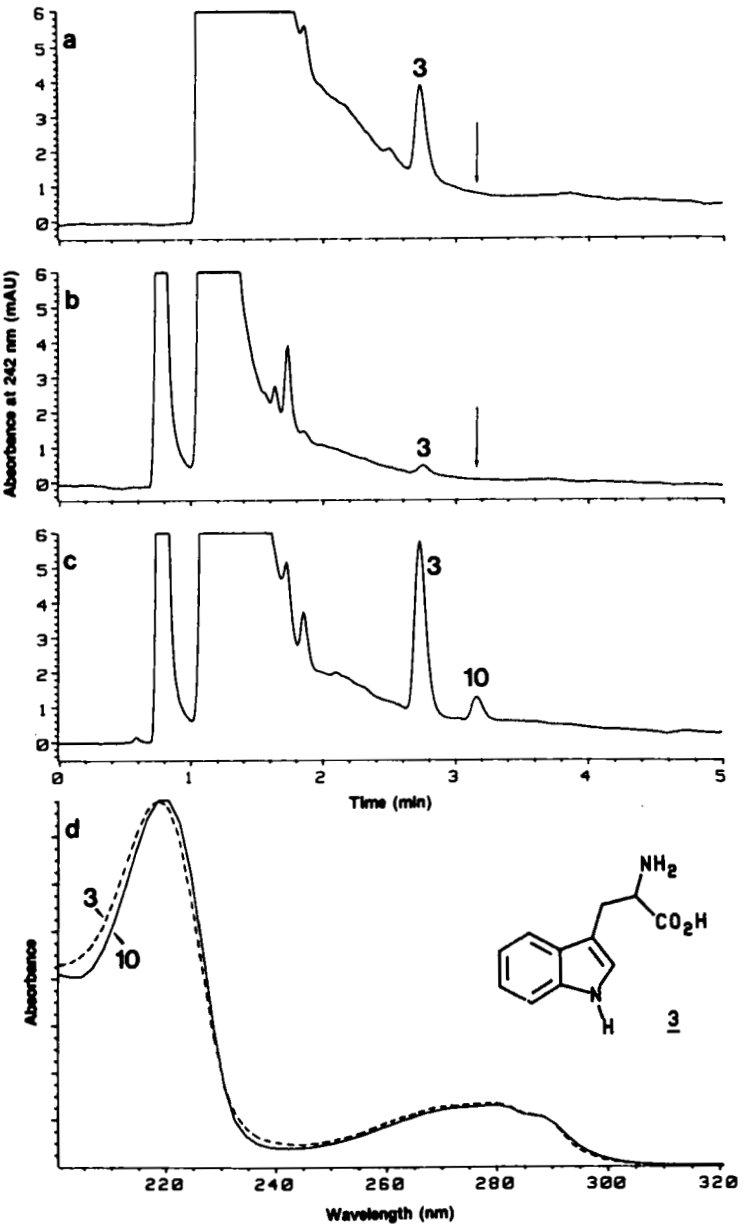

Figure 6 HPLC analysis of aqueous extracts of offshore scallop roe (a) and edible tissue (b). Chromatogram (c) shows an analysis of some rancid juices associated with the tissues. Peak **10** had a retention time identical with that of domoic acid, but the UV spectrum (d) acquired with the diode array detector indicates that it is not domoic acid. The spectrum of **10** suggests that this compound is related to tryptophan (**3**). Conditions: same as Figure 2.

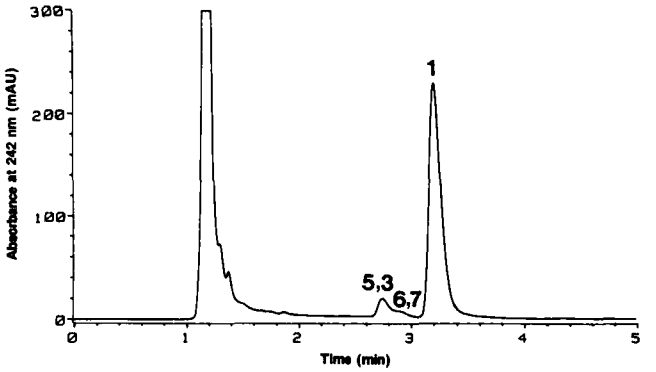

Figure 7 HPLC analysis of an aqueous extract of a plankton sample taken from the Cardigan River in December 1987. The predominant (>99%) species present in this sample was *Nitzshia pungens*. Conditions: same as Figure 2.

was very similar to that of tryptophan, suggesting that it may be an oxidation product of tryptophan.

A number of phytoplankton and seaweed samples have also been examined to determine the source of the domoic acid. While methods for the quantitative analysis of these types of samples are not the subject of this paper, it is important to note that irreproducible results were encountered, particularly with the seaweeds, where the domoic acid appears to be binding to other materials (perhaps carbohydrates) when a boiling water extraction is performed. More reliable extraction methods for these types of samples will be reported elsewhere. Figure 7 shows the analysis of a sample taken from a plankton bloom in the Cardigan River in eastern P.E.I. in December 1987. The predominant (>99%) species present in the sample was *Nitzschia pungens*, which has now been shown to produce domoic acid in culture.[12] The concentration of domoic acid was found to be over 1% by dry weight! Most of the domoic acid isomers are also present in this sample.

CONCLUSION

The HPLC method described herein has been in routine use in our lab for several months.[21] Other laboratories have been provided with details of the methodology, as well as calibration standards, and are routinely using either this method or slightly modified versions. The HPLC determination is fast, reliable and sensitive, and with the diode array detector, provides unambiguous confirmation of domoic acid. By comparison, the AOAC mouse bioassay[2] gives reliable time-to-death measurements at about 150 μg domoic acid per gram of tissue,[20] considerably higher than that afforded by the HPLC method. In the likely event that levels below that detectable by mouse bioassay are set by regulatory authorities as the acceptable level, the HPLC method should find widespread application in monitoring work.

Two other approaches to the determination of domoic acid and its isomers have been developed in this laboratory and will be published separately. One of these involves the use of fluorescence detection after derivatization with the 9-fluorenylmethylchloroformate reagent. The other approach uses methods based on mass spectral detection (GC-MS and LC-MS). Both of these approaches provide sensitive confirmatory assays for domoic acid.

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