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SEAFOOD TOXIN SYMPOSIUM

Rapid Extraction and Cleanup for Liquid Chromatographic Determination of Domoic Acid in Unsalted Seafood

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Domoic acid is the toxin responsible for incidents of amnesic shellfish poisoning. A rapid extraction and cleanup for the liquid chromatographic determination of domoic acid in unsalted seafood is reported. The method uses a single-step extraction with 50% aqueous methanol and a selective cleanup and preconcentration with strong-anion exchange, solid-phase extraction. Determination is performed by liquid chromatography with ultraviolet absorbance detection. The detection limit was 20–30 ng/g. Recoveries of 93% were achieved from 0.2 to 20 μg/g in mussel tissues. The method gave a precision of less than 3% for concentrations greater than 2 μg/g and less than 6% at 0.2 μg/g. A linear dynamic range of 10^4 can be achieved. The method was successfully applied to a variety of seafood products, including mussels, razor clams, crabs, and anchovies.

A serious outbreak of amnesic shellfish poisoning (ASP) occurred in late 1987 following the ingestion of cultured blue mussels (*Mytilus edulis*) originating from eastern Prince Edward Island, Canada (1, 2). The causative toxic agent was identified (3) as domoic acid (Scheme 1), a rare naturally occurring amino acid (4). This compound is a member of a group of potent neurotoxic amino acids that act as agonists to glutamate, a neurotransmitter in the central nervous system (5). The phytoplankton species *Pseudonitzschia pungens* f. *multiseries* was subsequently identified as the primary source of domoic acid in the affected area (6, 7).

The widespread occurrence of *Nitzschia* spp. suggests that ASP could be a worldwide problem. Indeed, 2 recent incidents in California and Washington support this claim. The California incident involving numerous deaths of pelicans and cormorants was caused by domoic acid produced by a bloom of the diatom *Pseudonitzschia australis* (8–10). Anchovies served as the intermediate vector. The Washington incident resulted in several people being affected by ASP after eating razor clams contaminated with domoic acid (11). Subsequently, domoic acid has been detected in viscera of Dungeness crabs from the same areas (11). The source of domoic acid in the clams and crabs has not yet been established.

Reliable methods for the analysis of domoic acid in seafood products are vitally important for protection of the public. Because the AOAC mouse bioassay for paralytic shellfish poisoning (PSP) toxins (12, 13) does not provide sufficient sensitivity for detection of domoic acid at the current regulatory level (20 μg/g in edible tissue), liquid chromatography with UV absorbance detection (LC–UVD) is the preferred analytical technique for determination of domoic acid in shellfish (14–15). The limit of detection (LOD) of the toxin in an extract solution is 10–80 ng/mL and depends on the sensitivity of the UV detector. The LOD in the original tissue sample is dependent on the method of extraction and cleanup; however, when using published sample preparation methods, practical LOD values are approximately 1 μg/g tissue. If regulatory levels are lowered in the future, more sensitive analytical methods will be required. To lower the detection limits appreciably, either extensive preconcentration methods or more sensitive instrumental methods will be required. A sensitive procedure based on fluorenylmethoxycarbonyl derivatization and LC analysis with fluorescence detection was developed for monitoring domoic acid in seawater and phytoplankton (16), but the published procedure could not be used with shellfish tissue extracts because of interference by coextractives. Previously published solid-phase extraction (SPE) procedures (14, 17) do not provide sufficient cleanup.

The extraction method adopted for official first action by AOAC is the same as that used for the PSP mouse bioassay (12) but with slight modifications (18, 19). This method involves boiling drained shellfish tissue with an equal volume of 0.1N HCl and then filtering a portion of the supernatant for LC–UVD. The procedure was adopted because it has the advantage of preparing a common extract for screening of both domoic acid and PSP toxins. The following problems exist, however: partial decomposition of domoic acid during and after the extraction, especially at trace levels (14); difficulty with certain matrixes (e.g., anchovy tissue tends to form a gel); a long sample preparation time; inability to store the extract due to rapid decomposition of domoic acid; and short LC column lifetime due to lack of a cleanup step.

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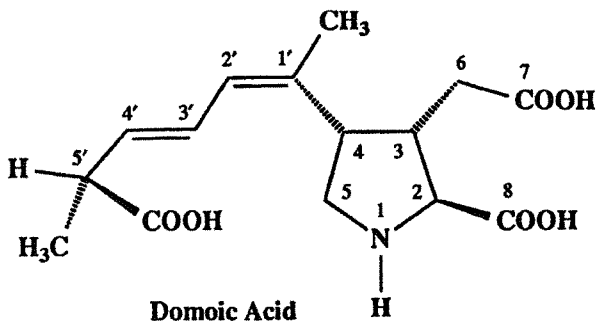

Domoic Acid

Scheme 1. Domoic acid structure.

Alternative extraction solvents, such as aqueous methanol and boiling water, were investigated by Quilliam et al. (14). Even though both solvents gave reproducible, high recoveries, the boiling water procedure was recommended at that time because it was compatible with a C_{18} SPE cleanup (14). A more extensive cleanup and preconcentration procedure for aqueous extracts was based on a combination of C_{18} and strong-cation exchange SPE cartridges and was developed for the confirmatory analysis of domoic acid by derivatization and gas chromatography–mass spectrometry (GC–MS) (17).

Because all the above methods are time-consuming, they do not lend themselves well to routine analysis in a regulatory setting. An aqueous methanol extraction procedure has some distinct advantages in this regard: It provides good recovery and has the potential to be very fast. The extract is cleaner than aqueous extracts, but it must be diluted 5-fold with water before LC to avoid appreciable peak broadening due to the "solvent wash-out" effect. Nevertheless, some laboratories have used this method routinely for regulatory monitoring purposes (M. Gilgan, personal communication). Because of the mild nature of the extraction, the method has good potential for trace analyses of domoic acid and related compounds such as labile metabolites. However, this potential will only be realized if the methanol extraction can be coupled with a suitable cleanup procedure that will allow preconcentration into a final extract with a low percent of organic solvent that is compatible with LC. Aqueous methanol could also be used to extract both domoic acid and the lipid-soluble diarrhetic shellfish poisoning toxins simultaneously.

The objectives of this study were to further investigate aqueous methanol extraction procedures with different seafood matrixes, and to develop a cleanup based on strong-anion exchange (SAX) that is compatible with the aqueous methanol extract and that provides substantial enrichment for trace level analysis. A preliminary report of the methodology was published (20).

Method Development and Evaluation

Optimization of Sample Preparation

(a) *Blended tissue homogenates.*—Uncontaminated mussel tissues (200 g) were homogenized with an equal volume of water to give a control homogenate. A 10.1 g portion of MUS-1 mussel tissue reference material (99 μg/g) (21) was mixed with 89.9 g control homogenate, and the slurry was homogenized thoroughly with a Brinkman Polytron to give a final blend containing 10.0 μg/g domoic acid. Further serial dilutions (1 + 9, w/w) with control tissue gave 1.0 and 0.10 μg/g blends. The amount of water used in the extraction solvent was adjusted because these blends already contain 50% extra water necessary to allow good homogenization.

(b) *Spiked tissues.*—Control mussel tissues were spiked with accurate volumes of domoic acid calibration solution (DACS-1B) (21) immediately before extraction.

(c) *Extraction.*—Different extraction solvents were tested for domoic acid recovery by using either the "dispersive" single-step procedure (*see* Method section) or the following "exhaustive" procedure. An 8 g tissue–water (1 + 1) homogenate (as prepared in [a]) was weighed into a graduated centrifuge tube. A 6 mL portion of extraction solvent (aqueous methanol or acetonitrile) was added and the sample was homogenized for 3 min at 10 000 rpm. The homogenate that remained on the Polytron probe was rinsed into a second tube with another 6 mL extraction solvent. This rinse was saved. The homogenized sample was centrifuged at 4000 × *g* for 10 min, and the supernatant was decanted into a 25 mL volumetric flask. The solution used to rinse the probe was added to the tissue residues and the sample was mixed on a Vortex mixer. After centrifugation, this supernatant was combined with the first supernatant. A third extraction of the tissue residues was performed with another 6 mL extraction solvent. The combined supernatants were made up to 25.0 mL with extraction solvent. A portion of this extract (10–15 mL) was filtered through a dry methanol-compatible 0.45 μm filter into a screw-capped storage vessel and sealed tightly.

Optimization of SAX Cleanup

(a) *Breakthrough studies.*—Extracts of MUS-1 reference material in various extraction solvents were passed slowly through conditioned SAX cartridges (*see* Method section), and 1 mL fractions were collected. Each fraction was analyzed by LC to determine domoic acid content.

(b) *Elution studies.*—After loading 1 g tissue equivalents of a MUS-1 extract onto conditioned SAX cartridges and performing the wash step (*see* Method section), various elution solvents were applied to the cartridges. Fractions (1 mL) were collected as soon as the eluting solvent was placed into the cartridge. In later tests, after the dead volume of the cartridge was determined (with a chromate solution) to be 0.5 mL, the first fraction collected was 0.5 mL, and subsequent fractions were 1 mL.

LC–MS Experiments

Combined LC–MS was performed using an HP1090 liquid chromatograph coupled to an API-III triple-quadrupole mass spectrometer (SCIEX, Thornhill, ON, Canada) equipped with an atmospheric pressure ionization (API) source and an ionspray interface. Air was used as the nebulizing gas, and a potential of 5000 V was applied to the interface needle. The LC

column and mobile phase were the same as those described in the *Method* section. However, gradient elution experiments were performed in which the acetonitrile was programmed from 5 to 25% over 25 min. Effluent from the column was split to provide a 30 µL/min flow to the ion-spray interface.

METHOD

Apparatus

(a) *Blender.*—Model 33BL73 commercial blender (Waring Products Division, Dynamics Corporation of America, New Hartford, CT).

(b) *Homogenizer.*—Model PT3000 Polytron equipped with a PT-DA-3012/2T generator (Brinkmann Instruments, Inc., Westbury, NY).

(c) *Centrifuge.*—Model MP4R with an 804S fixed-angle rotor (International Equipment Co., Needham Heights, MA).

(d) *Plastic centrifuge tube.*—Falcon 50 mL conical (No. 4-2099-5, Beckton Dickinson, Lincoln Park, NJ).

(e) *SPE manifold.*—Visiprep DL (No. 5-7044, Supelco, Inc., Supelco Park, Bellefonte, PA).

(f) *SAX cartridges.*—3 mL capacity, containing 500 mg silica derivatized with quaternary ammonium silane (LC-SAX, No. 5-7017, Supelco, Inc.).

(g) *Octadecylsilica cartridges.*—3 mL capacity, containing 500 mg octadecylsilica (LC-18, No. 5-7012, Supelco, Inc.).

(h) *Pipets.*—Adjustable volume (0–5 mL) digital pipet (Gilson Pipetman, No. GF23603, Mandel Scientific, Rockwood, ON, Canada).

(i) *Filters.*—Autovial syringeless filters, 0.45 µm Nylon-66 with glass microfiber prefilter (No. AV125UNAO, Whatman, Inc., Clifton, NJ).

(j) *Volumetric tubes.*—2 mL KIMAX (No. S-34865-B, Sargent-Welch Scientific Co., Skokie, IL).

(k) *Liquid chromatographs.*—*(1) System 1.*—Model 112 pump (Beckman Instruments, Inc., San Ramon, CA), Model HP1050 autosampler (Hewlett-Packard Co., Palo Alto, CA), Model CH30 column heater and Model TC50 temperature controller (Fiatron Laboratory Systems, Oconomowoc, WI), Model SF770 variable wavelength UV detector set to 242 nm (Schoeffel Instruments, Division of Kratos, Inc., Westwood, NJ), and Model HP3396 recording integrator (Hewlett-Packard). Cardinal data from the integrator were sent via the RS232C serial port to an 80386 MS-DOS personal computer running the Chromperfect 2 software (Justice Innovations, Inc., Palo Alto, CA). *(2) System 2.*—Same as System 1, except a Model HP1050 variable wavelength UV detector (Hewlett-Packard) was used. *(3) System 3.*—Model HP1090M system with a ternary DR5 pumping system, variable volume injector and autosampler, built-in HP1040 diode array detector (DAD), and HP79994 data system (Hewlett-Packard). The DAD was operated with sample wavelength at 242 nm and 10 nm bandwidth, reference wavelength at 350 nm and 100 nm bandwidth, and peak-triggered UV spectral acquisition.

(l) *LC column.*—Stainless steel, 25 cm × 4.6 mm id packed with 5 µm Vydac 201TP octadecylsilica, and equipped with a 201GCC54T guard column (The Separations Group, Hesperia, CA). Operating conditions: column temperature, 40°C; mobile phase flow rate, 1.0 mL/min; injection volume, 20 µL. Separations may be performed at ambient temperature but analysis time is increased by 20%, retention time is not as reproducible, and higher back-pressure occurs. If the LC is millibore compatible, a 25 cm × 2.1 mm id column packed with the same stationary phase can be used with a 5 µL injection volume and a mobile phase flow rate of 0.2–0.3 mL/min.

Reagents

(a) *Water.*—Distilled and passed through a Milli-Q water purification system equipped with ion-exchange and carbon filters (Millipore Ltd., Bedford, MA).

(b) *Solvents.*—Distilled-in-glass grade acetonitrile and methanol (BDH, Inc., Toronto, ON, Canada). Analytical grade formic acid and trifluoroacetic acid (TFA) (BDH, Inc.). Reagent grade concentrated ammonium hydroxide (Anachemia Ltd., Champlain, NY).

(c) *LC mobile phase.*—Mix 100 mL acetonitrile with ca 400 mL water, add 1 mL TFA, and dilute to 1 L with water. Degas with ultrasonication and gentle vacuum.

(d) *Extraction solvent.*—Mix equal volumes of methanol and water.

(e) *Cartridge wash solution and injection diluent (CH_3CN–H_2O, 1 + 9).*—Mix 1 volume acetonitrile with 9 volumes water.

(f) *Citric acid.*—Analytical grade citric acid monohydrate (BDH, Inc.).

(g) *Triammonium citrate.*—Analytical grade triammonium citrate (BDH, Inc.).

(h) *Citrate buffer eluent (0.5M, pH 3.2).*—Dissolve 40.4 g citric acid monohydrate and 14.0 g triammonium citrate in 400 mL distilled water, add 50 mL acetonitrile, and dilute to 500 mL with distilled water. (Trisodium citrate dihydrate [17.0 g] can be substituted for the ammonium salt. This buffer can also be prepared by dissolving 52.55 g citric acid monohydrate in 400 mL distilled water, adjusting pH to 3.2 with concentrated ammonium hydroxide [ca 13 mL], adding 50 mL acetonitrile, and diluting to 500 mL with distilled water.)

(i) *Domoic acid calibration solutions.*—DACS-1B (100 µg/mL, Marine Analytical Chemistry Standards Program, National Research Council Canada, Halifax, NS, Canada) and accurate dilutions of DACS-1B in injection diluent (e) to give 1.0, 2.5, 10.0, and 25.0 µg/mL.

(j) *Mussel tissue reference material.*—MUS-1 (99 µg/g, Marine Analytical Chemistry Standards Program).

Preparation of Samples

(a) *Tissue preparation.*—After removal from shellfish, drain tissues to remove saltwater. For representative sampling, 100 g pooled tissue should be homogenized in a blender. Subsamples can be taken from this homogenate immediately after blending, when the sample is still well-mixed, or after subsequent mixing. If an analysis must be performed on a limited amount of sample, a portion of chopped or ground tissue can be weighed directly into the extraction tube.

(**b**) *Extraction.*—Accurately weigh 4 g tissue homogenate into a graduated centrifuge tube. Add 16.0 mL extraction solvent (methanol–water, 1 + 1) and homogenize the sample extensively (3 min at 10 000 rpm). Do not try to recover all the tissue remaining on the homogenizer probe but do wash the probe thoroughly after homogenization to prevent contamination of the next sample.

If a blender is to be used, weigh the homogenate into a tared stainless-steel microblender cup, add 16.0 mL extraction solvent, and blend at medium speed for 4 min. Pour ca 15 mL of the resulting slurry into a centrifuge tube.

Centrifuge at 3000 × *g* or higher for 10 min. Filter 10–15 mL supernatant through a dry, methanol-compatible, 0.45 μm filter into a screw-capped storage vessel and seal tightly. Samples should be analyzed as soon as possible; crude extracts and tissue homogenates should be tightly sealed and stored at −10°C or lower.

(**c**) *Diluted crude extract.*—For screening samples for a high level of contamination and for salted samples, deliver 1.0 mL filtered supernatant from step (**b**) to a 5 mL volumetric flask or graduated cylinder, dilute to 5.0 mL with water, mix, and analyze without the SAX cartridge cleanup (omitting steps [**d**] and [**e**]).

(**d**) *SAX cartridge conditioning.*—Pass 6 mL methanol, then 3 mL water, and finally 3 mL extraction solvent (methanol–water, 1 + 1) through the SAX cartridges before use or testing. Do not allow the cartridges to go dry at any point in the procedure.

(**e**) *SAX cartridge cleanup.*—Load 5.0 mL filtered supernatant from (**b**) onto the cartridge and let supernatant flow slowly (ca 1 drop/s). Stop flow just as the sample meniscus reaches the top of the cartridge packing. Discard the effluent. Wash the cartridge (at ca 1 drop/s) with 5 mL of the cartridge wash solution. Stop the flow just as the solvent meniscus reaches the top of the cartridge packing. Discard the effluent. Add 0.5 mL citrate buffer eluent and carefully allow to flow just until the solvent meniscus reaches the top of the cartridge packing. Discard the effluent. Place a 2 mL volumetric tube or flask under the cartridge. Elute the domoic acid with 2 mL citrate buffer eluent (1 drop/s) just until the mark is reached on the volumetric tube. Mix the solution before withdrawing an aliquot for LC analysis. Samples should be analyzed as soon as possible; crude extracts should be tightly sealed in a screw-capped glass vial and stored in the refrigerator. Extracts should not be frozen because domoic acid decomposes when acidic solutions are frozen.

LC Determination

Inject domoic acid calibration solutions over the range of 1 to 100 μg/mL. If good linearity of response and a zero intercept are evident, single-point calibration (e.g., 10 μg/mL) may then be used routinely. Replicate injections should have coefficients of variation <5%. Inject sample extracts (diluted and filtered methanol–water extracts from [**c**] or SAX-cleaned extract from [**e**] in *Preparation of Samples*) in duplicate. Avoid carry-over between injections of different samples by washing the injector loop. Average peak areas for each sample. Repeat single injections of domoic acid calibration solution every 2 h and duplicate injections every 8 h. In calculations, average the peak areas of standards immediately following and preceding a series of samples.

Calculations

Calculate the concentration of domoic acid (μg/g) in each sample according to the following formula:

$$\text{domoic acid (}\mu\text{g/g)} = \left(\frac{A_S}{A_C}\right) \times \left(\frac{C_C}{W_T}\right) \times F$$

where A_S is the average peak area for the sample, A_C is the average area for the calibration standard bracketing the sample, C_C is the concentration (μg/mL) of the calibration standard, W_T is the weight (g) of tissue homogenate extracted (ca 4 g), and F is a dilution factor (F = 8 for the SAX-cleaned extract; F = 100 for the diluted crude methanol–water extract).

Blanks and Recoveries

(**a**) *Extraction blank.*—Perform step (**b**) (*Preparation of Samples*), except substitute 4 g water in place of sample tissue. Analyze a portion of the diluted and filtered methanol–water extract from step (**c**), and then carry the remaining methanol–water extract through steps (**d**) and (**e**) and analyze again (*see* Preparation of Samples). Chromatograms should be free of peaks eluting near domoic acid or causing excessive baseline slope. As needed, replace methanol and water, modify between-sample rinsing procedures, and use alternative SAX column source.

(**b**) *SAX column recoveries.*—Perform an LC determination in duplicate of a filtered solution containing 10–30 μg/mL domoic acid in extraction solvent (ideally this should be an extract prepared from the tissue of interest, either naturally contaminated or spiked with domoic acid). Using 3 cartridges from the lot to be used, take the same solution through the SAX cleanup (steps [**d**] and [**e**] in Preparation of Samples). Perform the determination in duplicate for each of the 3 eluates. Calculate the percent recovery for each eluate using the appropriate dilution factors and the average domoic acid level determined in the crude extract. All 3 recoveries should be 85–115%, and recovery should average >90%. If recoveries do not satisfy these criteria, try another source of SAX cartridges.

Safety

Domoic acid is a neurotoxin and must be handled with caution. Acetonitrile and methanol are toxic, volatile solvents. Trifluoroacetic acid is toxic, volatile, and corrosive and should only be handled in a fume hood. All these substances are harmful if swallowed, inhaled, or absorbed through the skin.

Results and Discussion

Optimization of Sample Extraction

Aqueous 50% methanol gave high extraction yields for domoic acid in naturally-incurred mussel tissues (14). However,

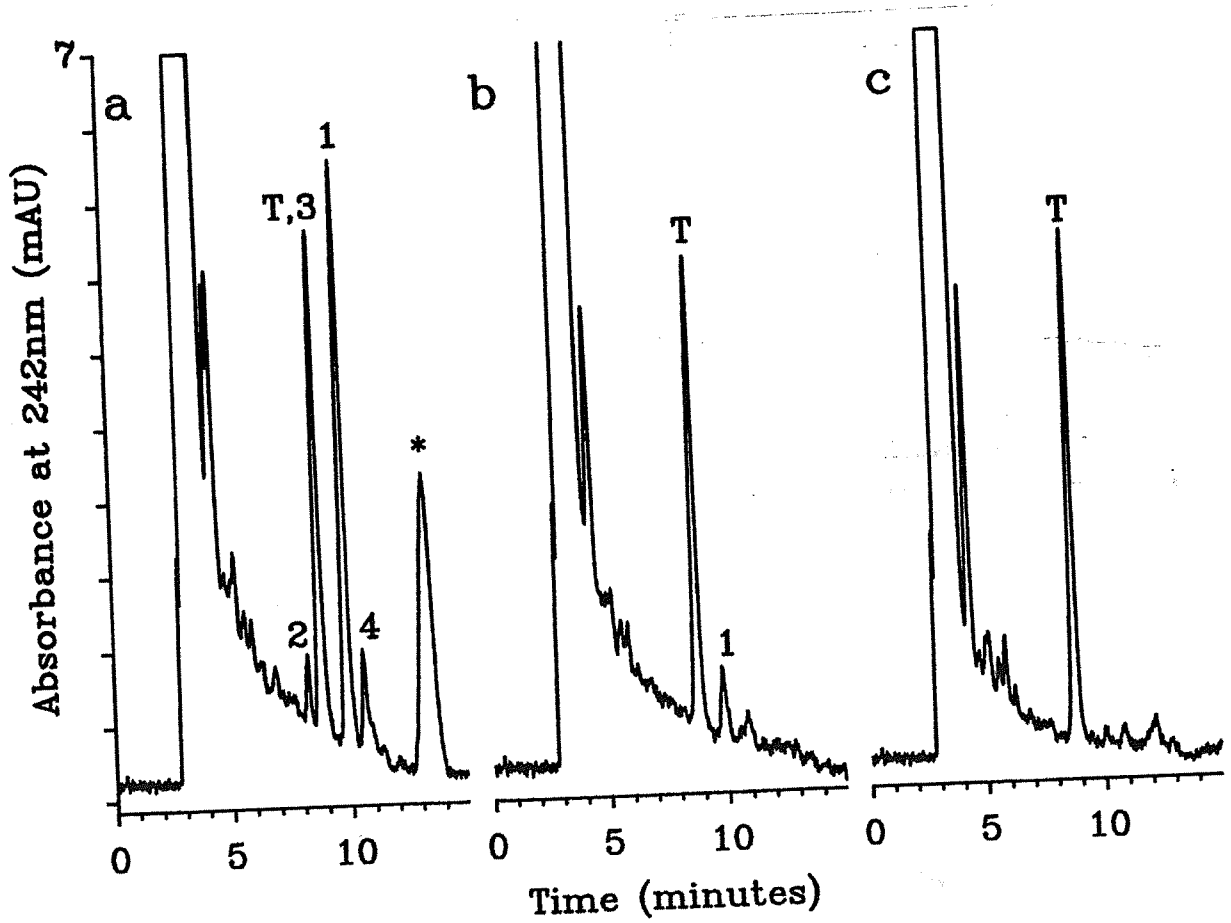

Figure 1. Liquid chromatograms of crude aqueous methanol extracts (filtered and diluted as in step [c], *Preparation of Samples*) of blended mussel tissue homogenates with domoic acid concentrations of 20 μg/g (a) and 2 μg/g (b) and of a control mussel tissue (c). Peak identities: 1 = domoic acid; 2, 3, and 4 = isomers of domoic acid; T = tryptophan. The asterisk indicates a late eluting peak from a previous injection, a typical problem with the analysis of crude extracts. Analyses performed using LC system 1.

the procedures used were cumbersome and not suitable for routine analysis. Therefore, a rapid extraction procedure using a Polytron homogenizer to disrupt and blend tissues with aqueous methanol was investigated. Acetonitrile was also investigated as a possible solvent. Preliminary experiments determined that an organic solvent concentration of 50% or higher was required for effective precipitation of protein and extraction of domoic acid. Because of the high cost of pure domoic acid, many spike experiments could not be performed during the development of the method. Therefore, mussel tissue homogenates were prepared by blending the MUS-1 reference material (21) with control tissue. The resulting domoic acid concentrations in these homogenates were 10.0, 1.0, and 0.10 μg/g. Because the homogenates were prepared with 50% water added (to ensure good homogeneity and precise subsampling), these values represented levels of 20.0, 2.0, and 0.20 μg/g domoic acid in the original tissue.

Crude extracts were analyzed by direct injection into the LC system. Extracts had to be filtered and diluted 5-fold in water before injection (or injection volume had to be reduced 5-fold) to avoid extensive broadening of the domoic acid peak associated with injecting a solvent of stronger elution power than the mobile phase ("solvent wash-out" effect). Figure 1 shows the chromatograms resulting from analyses of 50% aqueous methanolic extracts of the homogenates with 20 and 2 μg/g domoic acid and the control tissue. Domoic acid at the 2 μg/g level was barely detectable using the crude extract. One of the problems frequently encountered in analysis of crude extracts is shown in Figure 1a. A late-eluting peak from a previous injection has appeared in the chromatogram. Although such peaks can be recognized from their widths, analyses must be repeated if such a peak coincides with the domoic acid peak. Also observed in these chromatograms are other peaks due to tryptophan and some isomers of domoic acid (14, 22–24). The conditions used to obtain Figure 1 caused tryptophan to coelute with one of the domoic acid isomers. Because such isomers are also toxic (25), although to a lesser extent than domoic acid, their determination should also be considered important. Because coelution of tryptophan and domoic acid with other mobile and stationary phases is possible, analysts should be care-

ful to test their systems for separation. Oxidized tryptophans may also interfere, as was shown previously for a rancid scallop sample (14).

Blended homogenates were extracted by different methods and by different solvents. An "exhaustive" extraction procedure, in which tissue solids were extracted 3 times and the final extract made to volume, was tested against a "dispersive" single-step procedure. Technically, an exhaustive extraction is a more volumetric procedure, whereas a dispersive extraction introduces a positive bias due to the volume of undissolved solids. However, with a 4:1 volume ratio of extracting solvent to tissue (tissue is about 75% water already), only a 5% positive bias should be introduced. As shown in a following section, this systematic error appears to be cancelled fortuitously by another systematic error of a slightly poorer extraction yield, and concentration values of acceptable accuracy result.

Direct analyses of crude extracts of the 20 μg/g homogenates showed that the best extraction yield (almost 100%) was provided by an exhaustive 50% aqueous methanol extraction (Table 1). Lower concentration blends (2 and 0.2 μg/g) could not be measured by direct injection of crude extracts, but analyses performed after SAX cleanup showed that the extraction yield was constant down to the 0.2 μg/g level. This finding is in contrast to the AOAC boiling acid extraction procedure (18, 19) which shows poorer recoveries at trace levels due to increased decomposition of domoic acid.

A single-step dispersive extraction with 50% aqueous methanol provided excellent recovery (almost 98%) and was much faster for routine regulatory work. Extraction with a blender rather than a Polytron gave a slightly lower but acceptable yield (96%). A dispersive extraction with 75% aqueous methanol provided a much lower recovery of domoic acid (85%), although an exhaustive 75% methanol extraction was found to give good recovery (data not shown). Therefore, a "universal" extraction procedure for domoic acid and DSP toxins should be feasible. Our experiments to develop such a procedure will be reported elsewhere. We hoped that acetonitrile might be a suitable extraction solvent, because it can provide better precipitation of protein and less extraction of lipid materials. However, substitution of acetonitrile for methanol gave much lower recoveries. These observations are reasonable considering the solubility of domoic acid in different solvents: 7.6 g/L in water, 0.66 g/L in methanol, and 0.0011 g/L in acetonitrile (26).

Table 1. Effect of extraction solvent and method on recoveries of domoic acid from a blended mussel tissue homogenate (20.0 μg/g level)

Solvent ratio (MeOH:H_2O)	Extraction method	Recovery ± SD, %[a]	RSD, %[b]
50:50	Exhaustive, Polytron	99.9 ± 2.4	2.4
50:50	Dispersive, Polytron	97.7 ± 2.0	2.1
50:50	Dispersive, blender	96.1 (n = 1)	—
75:25	Dispersive, Polytron	85.4 ± 1.6	1.8

The stability of domoic acid in crude extracts was also examined. No significant decomposition of domoic acid in mussel tissue extracts was observed over a period of 1 week if the sample was stored in a freezer. No significant levels of methyl esters of domoic acid were observed in neutral aqueous methanol solutions. However, the formation of esters in acidic methanol solutions could be detected using LC–MS analysis. The stability of domoic acid in methanolic extracts contrasts sharply with that in AOAC acid extracts. Domoic acid decomposed rapidly in acidic extracts (up to 50% in 1 week) (14).

In summary, 50% aqueous methanol was the best extraction solvent. A dispersive extraction allowed rapid analyses, but an exhaustive extraction was preferred for the highest level of accuracy (e.g., reference material work). The analysis of crude extracts may be useful for screening shellfish for high levels of domoic acid (e.g., near or above the current regulatory limit of 20 μg/g), but quantitative reliability may be poor because of the low signal strengths and possible interferences.

Optimization of Sample Cleanup

The main challenge in this project was to develop a cleanup procedure that would be compatible with aqueous methanol extracts, provide a high degree of cleanup, and yield a high preconcentration factor. Several types of SPE cartridges were tested until a silica-based SAX material gave spectacular performance in sample cleanup. Initial experiments were performed on a 50% aqueous methanol extract of the MUS-1 reference material (100 μg domoic acid/g homogenate, or 200 μg/g tissue). We found that 5 mL of extract representing 1 g equivalent of tissue could be loaded onto the SAX cartridge without significant breakthrough of domoic acid. Also, domoic acid could be eluted from the cartridge quantitatively with 2 mL aqueous 10% acetonitrile containing 2% (about 0.5M) formic acid to provide direct compatibility with LC analysis. Even more interesting was the observation that chromatograms of the resulting extract were very clean and showed only peaks due to the solvent, domoic acid, and its isomers. In particular, all the tryptophan was eliminated from the extract (it was observed in the loading and washing fractions). The SAX cartridge appeared to show a high degree of chemical selectivity toward the very acidic domoic acid when samples were loaded in aqueous methanol solutions. SAX was used effectively for the cleanup of other acidic analytes such as the fumonisin mycotoxins (27).

To determine the loading capacity of the SAX cartridge, a breakthrough experiment was conducted by passing MUS-1 extracts slowly through cartridges while collecting 1 mL fractions. Analysis of the fractions revealed when the column capacity was exceeded. MUS-1 was used because such an experiment should be performed with a high level of domoic acid in the presence of real sample matrix. Data are presented in Figure 2 for different types of extracts: aqueous, 50% methanol, and 75% methanol. The concentration of domoic acid in the fractions is expressed relative to the concentration in the crude extract loaded onto the cartridge. The data show that, for all the extracts, more than 1 g equivalent of tissue can be loaded onto the cartridge without significant breakthrough. About twice as

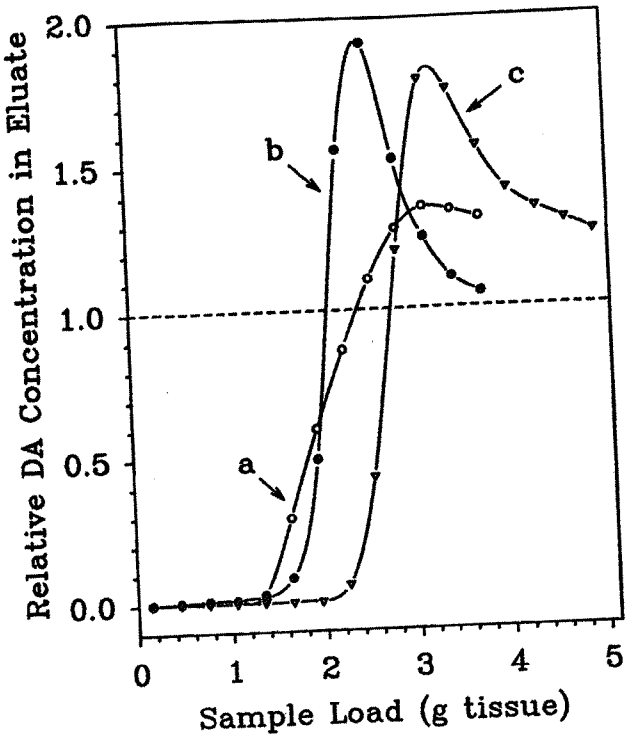

Figure 2. Measurement of the breakthrough of domoic acid from SAX cartridges as a function of the gram equivalents of tissue loaded from different extracts: a, 100% aqueous; b, 50% methanol; and c, 75% methanol. Extracts of MUS-1 reference material were passed through a preconditioned cartridge and individual fractions were collected and analyzed directly by LC. The concentration of domoic acid in the fractions is expressed relative to the concentration in the crude extract loaded onto the cartridge.

much can be loaded with the 75% methanol extract. This difference should allow a higher preconcentration factor in the analysis, but the factor is partially offset by the lower extraction efficiency with methanol. An interesting maximum, at a concentration greater than unity, is observed in all the curves. This maximum is probably due to other components in the sample matrix (e.g., salts) that force accumulated domoic acid off the cartridge. Similar breakthrough curves were observed for 50% methanol extracts of razor clam, crab, and anchovy tissues. However, one batch of anchovy that turned rancid showed a breakthrough of domoic acid at about 0.6 g equivalent of tissue loaded. Salted samples, or tissues containing a lot of undrained saltwater, also present breakthrough problems.

The elution of an anionic analyte from a SAX cartridge may be accomplished in 3 ways: by reducing the pH with an acidic eluent to disrupt the ionic bonding, by displacing the analyte with a more strongly-bound anion, and by using a high ionic strength buffer. The efficiency of different solvents for the elution of domoic acid was tested by preloading several SAX cartridges with MUS-1 extract and collecting 1 mL fractions as the eluting solvent was passed through the cartridge. The first experiments used 0.5M formic acid (in acetonitrile–water, 1 + 9). As shown in Table 2, domoic acid eluted very quickly, mostly in the first 2 mL. However, domoic acid decomposes at the low pH of the formic acid. For some trace level samples, the decomposition was found to be quite rapid, and losses were up to 5% per day when stored at room temperature. This decomposition is not a problem if samples are analyzed immediately, but it does cause some concern if extracts must be archived for a period of time.

Five other eluting solvents were examined (*see* Table 2): 0.5M ammonium formate (pH 6.5), 0.5M ammonium formate buffer (pH 3.8), 0.2M formate buffer (pH 3.8), 0.5M ammonium citrate buffer (pH 4.5), and 0.5M ammonium citrate buffer (pH 3.2). Ammonium formate was examined because it is useful for other analytical procedures where the fraction must

Table 2. Efficiency of different solvents for the elution of domoic acid from SAX cartridges preloaded with MUS-1 tissue extract (1 g tissue equivalent, 50% aqueous methanol)[a]

Elution solvent[b]	Domic acid in successive fractions, %				
	0–1, mL	1–2, mL	2–3, mL	3–4, mL	4–5, mL
0.5M Formic acid, pH 2.2	67.6	31.9	0.37	0.10	0.03
0.5M Ammonium formate, pH 6.5	18.4	75.2	6.0	0.37	0.07
0.5M Formate buffer, pH 3.8	18.1	80.4	1.2	0.24	0.05
0.2M Formate buffer, pH 3.8	ND[c]	69.1	29.4	1.3	0.23
0.5M Citrate buffer, pH 4.5	2.4	94.6	2.4	0.43	0.20
	0–0.5, mL	0.5–1.5, mL	1.5–2.5, mL	2.5–3.5, mL	3.5–4.5, mL
0.5M Citrate buffer, pH 4.5	ND	89.0	9.7	1.1	0.20
0.5M Citrate buffer, pH 3.2	ND	93.1	6.1	0.9	ND

[a] One milliliter fractions were collected as the test solvent was passed through the cartridge. The precision of the experiment was not adequate to determine overall recoveries.
[b] All elution solvents have 10% acetonitrile added.
[c] ND, not detected.

Table 3. Recoveries of domoic acid from blended mussel tissue homogenates using different extraction procedures combined with the SAX cleanup[a]

Solvent ratio (MeOH:H_2O)	Extraction method	Spike level, µg/g	Recovery ± SD, %[b]	RSD, %[c]
50:50	Exhaustive, Polytron	20.0	95.1 ± 1.6	1.7
50:50	Exhaustive, Polytron	2.00	94.6 ± 1.2	1.3
50:50	Dispersive, Polytron	20.0	93.2 ± 1.2	1.3
50:50	Dispersive, Polytron	2.00	92.9 ± 2.0	2.2
50:50	Dispersive, Polytron	0.20	93.0 ± 5.0	5.4
50:50	Dispersive, blender	20.0	93.0 (n = 1)	—
75:25	Dispersive, Polytron	20.0	77.6 ± 3.0	3.9
75:25	Dispersive, Polytron	2.00	76.1 ± 2.2	2.9

[a] SAX procedure consisted of 5.0 mL extract loaded on cartridge and elution with 2.0 mL citrate buffer (0.5M, pH 4.5).
[b] Values reported are means and standard deviations (SD) of 6 replicate extractions and measurements; concentrations are reported in terms of original tissue (without the additional water added to make the homogenate).
[c] RSD, relative standard deviation.

be evaporated to dryness (e.g., GC–MS with chemical derivatization [17]). Direct injection of this solution into the LC system is not recommended, however, because peak splitting and broadening occur due to the higher pH of the solution versus that of the LC mobile phase. The 0.5M formate buffer with a pH of 3.8 gave good peak shapes in chromatograms, and solutions of domoic acid were found to be stable on storage. However, the elution profile from the SAX column was not as good as that obtained using ammonium citrate buffers (Table 2). The citrate anion is well-known as a very strong eluting agent in anion exchange chromatography.

Because the dead volume of the SAX cartridge is 0.5 mL, a procedure was tested in which 0.5 mL citrate buffer was allowed to flow into the cartridge before collection was started. The results of this experiment are shown in Table 2. No domoic acid could be detected in the first 0.5 mL, and over 99% of the domoic acid eluted in the next 2 mL. We decided that this procedure would be used for the final recommended method. However, studies where the highest sensitivity is not required (e.g., routine monitoring at the current regulatory level of 20 µg/g), a simpler procedure would be to load 3 mL sample extract and elute with 3 mL citrate buffer (including the first 0.5 mL). This procedure gives slightly better recovery and greater ease of operation.

The pH 4.5 citrate buffer was found to be quite compatible with our LC system and gave only slightly broader peaks than those with the 0.5M formic acid. However, under high-speed LC conditions, appreciable peak broadening was observed (R. Bailey, personal communication). Therefore, the pH 3.2 citrate buffer was tested and found to eliminate peak broadening. Cleaned up samples were found to be very stable in this buffer. Less than 1% decomposed per week for extracts of samples with domoic acid levels greater than 1 µg/g, and less than 1% decomposed per day for extracts of trace level samples (0.1 µg/g). The citrate buffer concentration is not very critical; concentrations from 0.25 to 1M are effective for elution of domoic acid. A 0.5M citrate buffer at pH 3.2 provides the best compromise between efficiency of elution, stability of the collected fraction, and good performance during LC.

Although several brands of SAX cartridges were tested and found to be effective, some manufacturers' cartridges did not extract domoic acid. Packing materials prepared by reacting silica with *N*-trimethoxysilylpropyl-*N,N,N*-trimethylammonium chloride, and supplied in the chloride form, are most effective. We recommend that all new lots of SAX cartridges be tested for recovery.

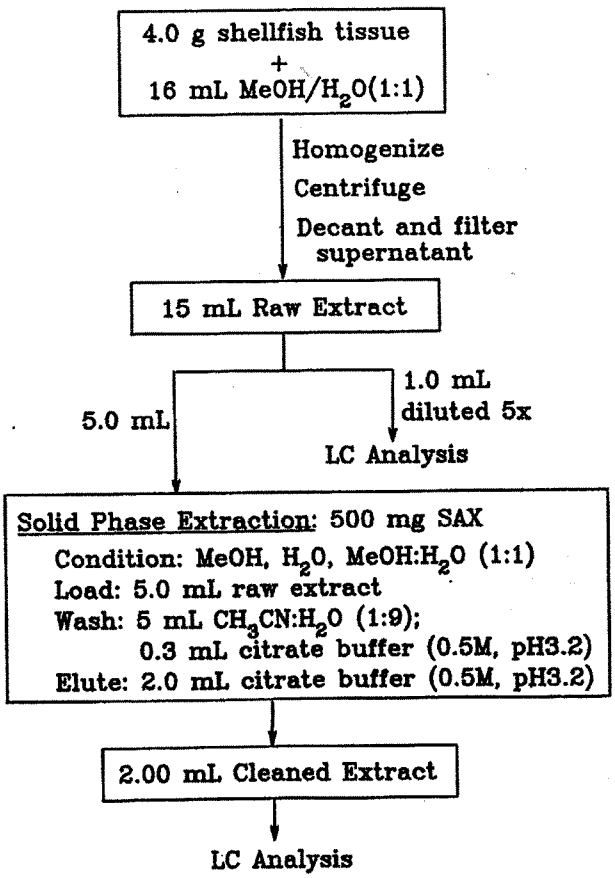

Figure 3. Schematic summary of the final extraction and cleanup procedure.

A modification of the cleanup procedure in which cartridges were taken to near dryness between each different solvent was also examined. This modification was done partly to test the robustness of the procedure, but also to see how compatible the procedure would be with certain robotic systems that use an "air-push" step between each solvent type. Only slightly lower recoveries were observed and the procedure proved quite convenient. However, with some samples, the cartridge seemed to plug more easily. The reason for this was not explored.

Regeneration and reuse of the SAX cartridges was investigated using the procedures described by Sydenham et al. (27). Passage of 5 mL 0.1N HCl followed by 8 mL water regenerated the cartridges. Although less than 0.2% cross-over contamination was observed when appropriate care was taken, we recommend that cartridges be used only once. The risk of generating false positives is too great if regenerated cartridges are used on a routine basis.

Although crude, unfiltered extracts of mussel tissues could be applied to the SAX cartridges, prefiltration appears to be important with some samples, such as razor clams, to prevent clogging of the cartridge. Filtering is especially important if a high-speed centrifuge is not used in the extraction procedure.

Method Evaluation

A rigorous evaluation of the SAX cleanup method was performed on various extracts of the MUS-1–control mussel tissue blends. The results of the experiments are presented in Table 3. In these experiments, the pH 4.5 citrate buffer was used. The pH 3.2 citrate buffer was not used until later but gave very similar recoveries. The highest recoveries (95%) and precision were observed for the exhaustive extraction procedure. With the dispersive extraction procedure, the recovery of domoic acid was constant at 93% to the 0.2 μg/g level. The precision of the determinations was excellent: 1.3% relative standard deviation (RSD) at 20 μg/g, 2.2% RSD at 2 μg/g, and 5.4% RSD at 0.2 μg/g. Figure 3 provides a schematic diagram of the final extraction and cleanup procedure. A final test of the method (with the pH 3.2 citrate buffer) was conducted. Control mussel tissue was spiked with pure domoic acid at 25 μg/g. A recovery of 95.8 ± 1.6% ($n = 5$) was observed.

Chromatograms for the SAX cleaned extracts of the mussel tissue homogenates are presented in Figure 4. These results should be compared with Figure 1, which showed the chromatograms for the crude extracts before cleanup. A 12.5-fold increase in sensitivity was achieved because of the 2.5-fold pre-

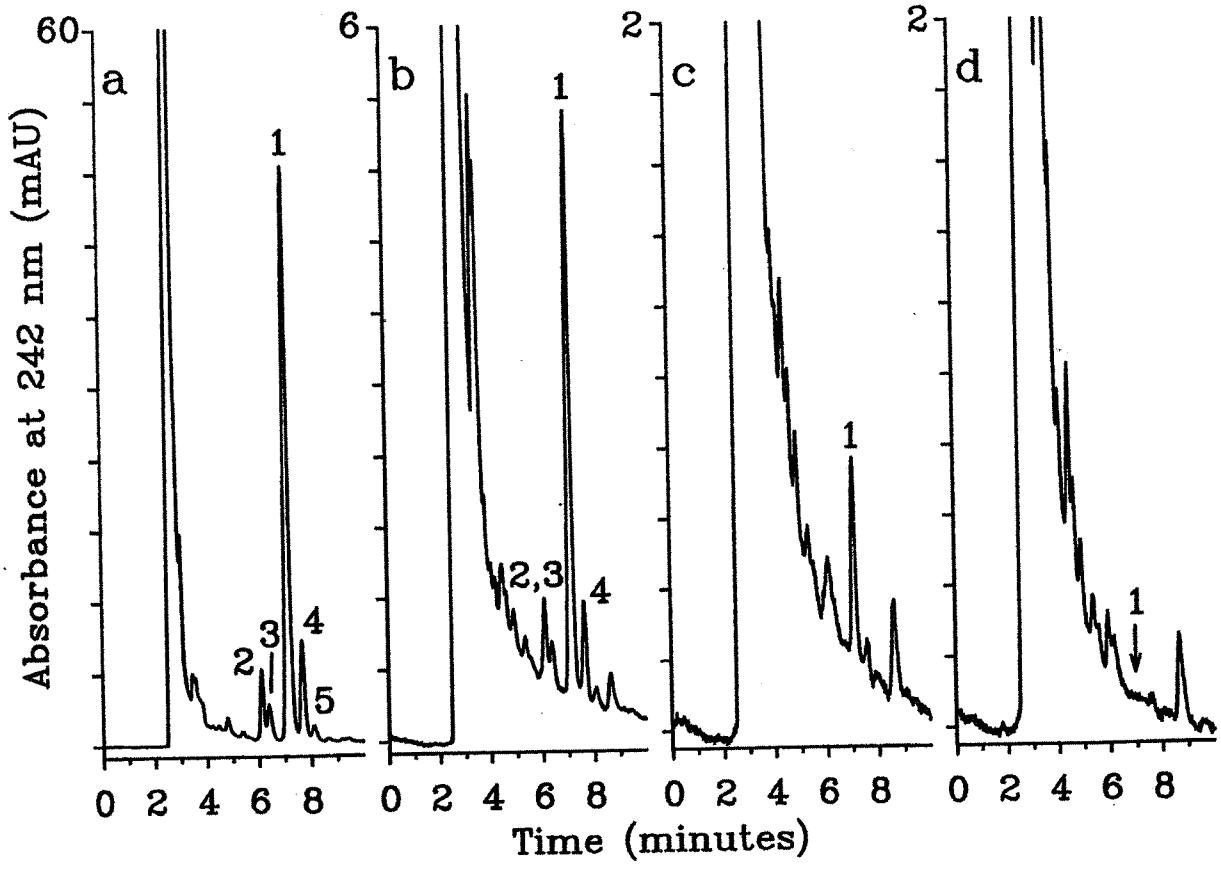

Figure 4. Liquid chromatograms of SAX-cleaned extracts (from step [e], *Preparation of Samples*) of blended mussel tissue homogenates with domoic acid concentrations of 20 μg/g (a), 2 μg/g (b), and 0.2 μg/g (c), and of a control mussel tissue (d). Peak identities: 1 = domoic acid; 2, 3, 4, and 5 = isomers of domoic acid. Analyses performed using LC system 3.

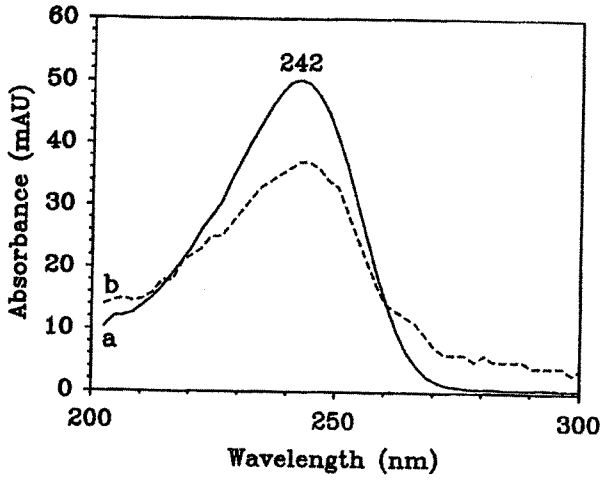

Figure 5. UV spectra of domoic acid acquired by the diode array detector at the apex of peak 1 in Figures 4a (curve a, 20 μg/g in tissue) and 4b (curve b, 2 μg/g in tissue).

concentration factor from the SAX cartridge combined with the ability to inject 4 times more extract into the LC system. The control extract was much cleaner after SAX; tryptophan and other interferences were eliminated. The detection limit for the method with this LC system (No. 3), which uses a diode array detector, was estimated to be about 30 ng/g (signal-to-noise ratio, 3). The more sensitive HP1050 detector used in LC system 2 gave a method detection limit of 20 ng/g. The linear dynamic range with these detectors was over 10^4. Good UV spectra of domoic acid were acquired by the diode array detector to the 2 μg/g level (Figure 5). This ability is quite important for confirmation of peak identity for research and regulatory work.

Application to Different Samples

The new extraction and cleanup procedure proved valuable for a number of different sample types that passed through our laboratory. Figure 6 shows chromatograms from the analyses of various seafood products: razor clams (40 μg/g), Dungeness crab (0.8 μg/g), and anchovies (75 μg/g). Anchovies were collected from Monterey Bay during the September 1991 incident in which numerous pelicans and cormorants died (9, 10). Oily fish samples such as these posed no problem for the procedure.

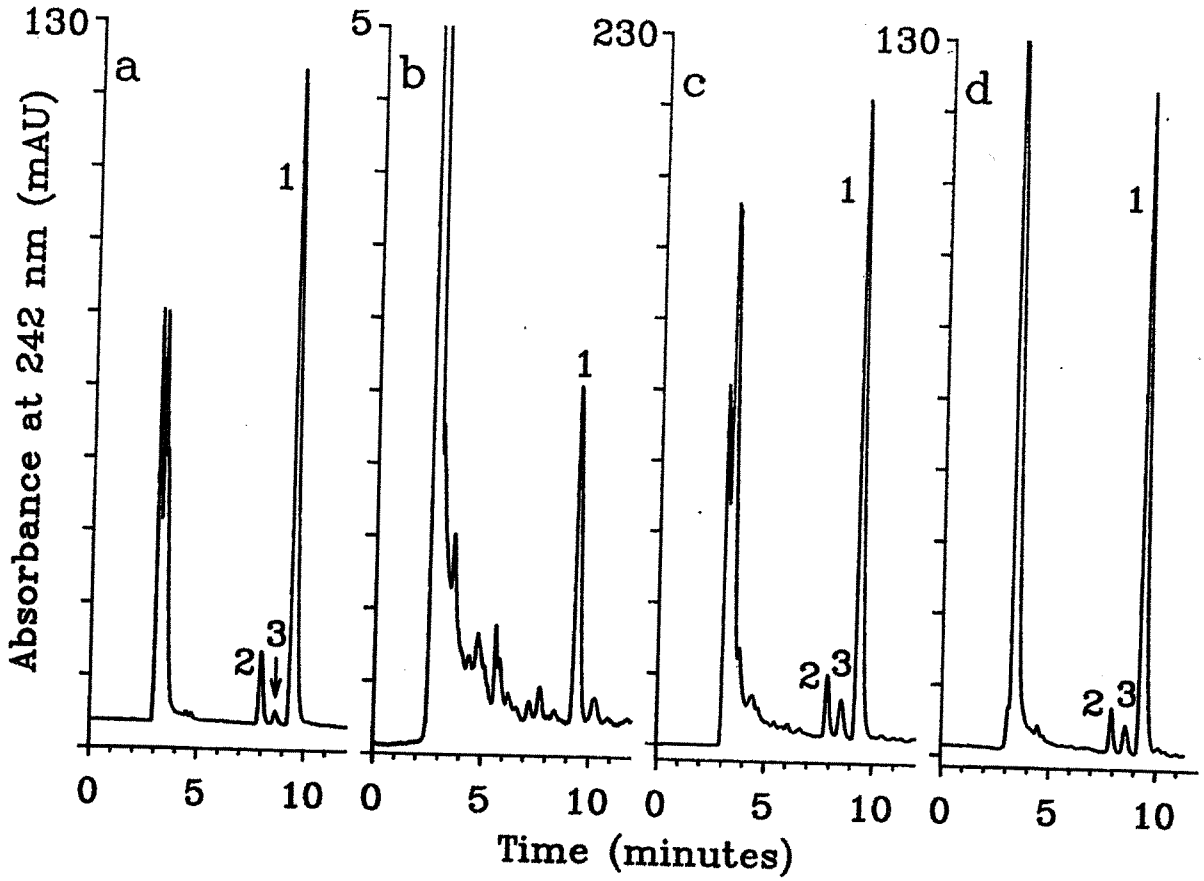

Figure 6. Liquid chromatograms of SAX-cleaned extracts (from step [e], *Preparation of Samples*) of the following (with domoic acid in μg/g): a, whole razor clam tissue (40 μg/g); b, Dungeness crab tissue (meat plus viscera, 0.8 μg/g); c, whole anchovies (75 μg/g); and d, the stomach contents of a dead pelican (42 μg/g). Peak identities: 1 = domoic acid; 2 and 3 = isomers of domoic acid. Analyses performed using LC system 2.

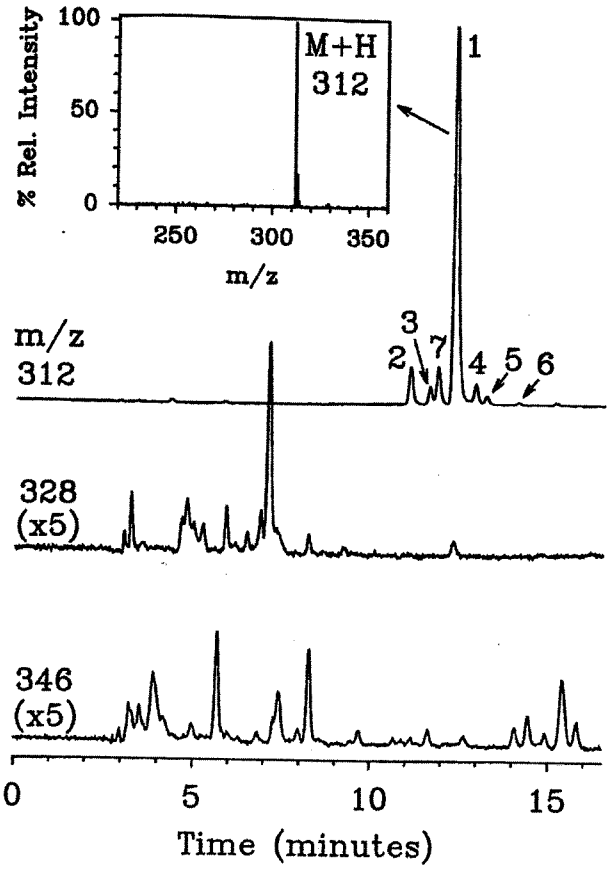

Figure 7. LC–MS analysis of SAX-cleaned extract of anchovies (75 μg/g domoic acid) using selected ion monitoring of *m/z* 312, 328, and 346. The inset shows the full mass spectrum of domoic acid (acquired in a separate analysis). Peak identities: 1 = domoic acid; 2, 3, 4, 5, 6, and 7 = isomers of domoic acid; other peaks are metabolites and degradation products of domoic acid. Gradient elution was used in this analysis (*see* Experimental).

For comparison, a boiling aqueous extract of the anchovy sample was taken through a C_{18} SPE cleanup (14). The extraction proved much more difficult, gave a lower result (75% of the value by the new procedure), and showed a very large tryptophan peak. An analysis of the stomach contents (mainly anchovies) of a pelican that died from ASP in the California incident is shown in Figure 6d.

The new procedure was also very useful for other types of analyses. We tested it with both capillary electrophoresis and ion-spray LC–MS (28) and found the resulting extracts to be completely compatible with each method. Results obtained using these techniques will be published separately. However, the ion-spray LC–MS analysis results for an anchovy extract (Figure 7) show very strong signal in the *m/z* 312 ($[M + H]^+$ of domoic acid) mass chromatogram at the correct retention time. This signal confirms the presence of the toxin. Further proof of identity is provided by a full mass spectrum (inset of Figure 7) acquired at the apex of the peak. A number of isomers of domoic acid (peaks 2–7) were also detected (14). More interesting is the appearance of other peaks in the *m/z* 328 and 346 mass chromatograms. These peaks are metabolism or degradation products of domoic acid in the anchovy. The peaks in the *m/z* 328 trace correspond to compounds with an oxygen atom added to domoic acid. Possible candidates for some of these compounds include the previously identified domoilactones (23). The peaks in the *m/z* 346 trace indicate further addition of a water molecule. Work is currently underway to characterize these compounds. The important point to be made here is that the SAX cartridge procedure is also effective at extracting these compounds.

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