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LIQUID CHROMATOGRAPHIC DETERMINATION OF DOMOIC ACID IN SHELLFISH PRODUCTS USING THE PARALYTIC SHELLFISH POISON EXTRACTION PROCEDURE OF THE ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS

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SUMMARY

Domoic acid, the recently discovered toxic substance found in contaminated mussels from an area in eastern Prince Edward Island (Canada) was extracted from mussel tissue using the procedure of the Association of Official Analytical Chemists for paralytic shellfish poisons. This involved a 5-min boiling of the sample with 0.1 *M* hydrochloric acid then cooling and centrifuging. An aliquot of the supernatant was diluted ten to one-hundred times with water, filtered and analysed by reversed-phase liquid chromatography with a mobile phase consisting of acetonitrile–water (12:88) at pH 2.5 and an absorption wavelength of 242 nm. The detection limit was about 0.5 mg/kg domoic acid in seafood samples. The technique was successfully applied to a variety of commercially purchased shellfish and shellfish products.

INTRODUCTION

Domoic acid (Fig. 1) was recently isolated and identified as the toxic substance found in contaminated blue mussels (*Mytilus edulis*) from eastern Prince Edward

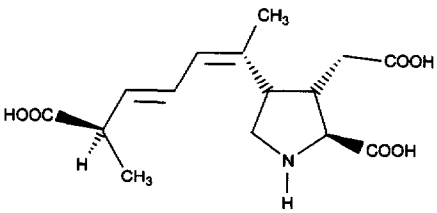

Fig. 1. Structure of domoic acid.

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Island (Canada)[1]. This neurotoxic amino acid is a naturally-occurring metabolite first isolated from the red alga, *Chondria armata*, by Japanese workers[2,3]. The substance has been synthesized[4] and evaluated for its insecticidal properties[5,6]. The source of the domoic acid found in the mussels is still under investigation, but results to date indicate that the marine pennate diatom *Nitzschia pungens*, is the most likely candidate[7].

The Association of Official Analytical Chemists (AOAC) mouse bioassay method for paralytic shellfish poisons (PSP) was found to be applicable to the detection of domoic acid[8]. About 40 mg/kg in wet mussel tissue may induce some characteristic symptoms of domoic acid intoxication while levels near 150 mg/kg produce repeatable time-to-death values. The method proved to be very useful since both types of shellfish toxins could be monitored by a single bioassay method, rather than by specific methods for each. However, domoic acid positive samples must be confirmed by an independent technique capable of accurately quantitating the substance. Trace analytical methodology for domoic acid in mussels was first developed employing a boiling water extraction followed by liquid chromatography (LC) with ultraviolet absorption detection as the determinative step[9]. This approach was simple and could detect less than 1 mg/kg domoic acid in mussel samples. The purpose of the work described in this report is to evaluate the application of LC to the detection and quantitation of domoic acid in shellfish using the AOAC PSP extraction procedure employed for the mouse bioassay. In this way both analytical and biological tests can be performed on the same extract.

EXPERIMENTAL

Reagents

Domoic acid was isolated from contaminated mussel tissue, purified (>95% purity) and characterized as described elsewhere[1]. Water was twice deionized (Milli-Q, Millipore, Bedford, U.S.A.), acetonitrile was HPLC-grade. All other solvents and chemicals were analytical-reagent grade materials. Standard solutions of domoic acid were prepared in water and diluted as required. All domoic acid standard and sample solutions were refrigerated when not in use.

Liquid chromatography

The system consisted of a Model 110B pump (Beckman), a 20-μl loop injector (Beckman), a Supelcosil LC-18 column (15 cm × 4.6 mm I.D., 5 μm), a variable-wavelength UV detector (Micromeritics) set to 242 nm (wavelength maximum for domoic acid) and 0.02 absorbance units full scale (a.u.f.s.), and a Varian 4270 integrating recorder. The mobile phase was acetonitrile–water (12:88, v/v) adjusted to pH 2.5 with 2% (v/v) orthophosphoric acid, degassed and filtered before use. The flow-rate was 1.0 ml/min.

Sample extraction

The sample preparation and extraction were carried out exactly as described earlier[10]. Briefly, 100 g of homogenized shellfish tissue was mixed thoroughly with 100 ml of 0.1 *M* hydrocloric acid in a 500-m beaker. The contents were heated with stirring on a hot plate and allowed to boil gently for a period of 5 min. The mixture was then removed and permitted to cool in a refrigerator (4°C) for 30 min. The contents were

then quantitatively transferred to a graduated cylinder and diluted to exactly 200 ml. The contents were returned to the beaker, stirred and an aliquot of about 50 ml was removed and centrifuged for 5min at *ca.* 3000 rpm (700 *g*). A 0.5-ml portion of the clear supernatant was diluted to 25 ml with water in a volumetric flask and mixed thoroughly. About 2 ml of the solution were filtered (Millex HV, 0.45 μm, Millipore) for analyis by LC.

For comparison purposes, the above procedure was repeated using water instead of 0.1 *M* hydrochloric acid for the extraction.

RESULTS AND DISCUSSION

Chromatography

Fig. 2 shows typical chromatograms obtained for domoic acid in a mussel sample. A number of C_{18} reversed-phase columns (including, Ultrasphere 5-μm, Spherisorb 5-μm, μBondapak 10-μm, Vydac 5-μm, Lichrosorb 5-μm) were evaluated and all functioned well for the determinations. The only change necessary was an adjustment of the acetonitrile concentration (usually 12–18%) in the mobile phase to produce an acceptable retention time for domoic acid We have found that 6–10 min was optimal with the columns studied. At acetonitrile concentrations greater than 18% in the mobile phase, domoic acid was not completely resolved from other co-extractives in the samples. This was particularly a problem at low concentrations (<20 mg/kg) of domoic acid in the tissue where more concentrated sample extracts had to be injected. Ion-exchange chromatography employing a Vydac 302 IC column with

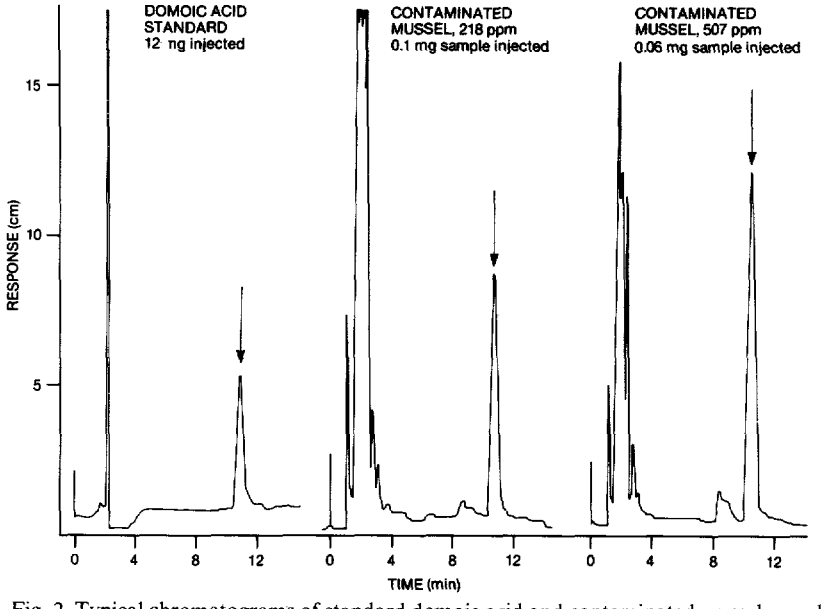

Fig. 2. Typical chromatograms of standard domoic acid and contaminated mussel samples. Mobile phase, acetonitrile–water (12:88) (pH 2.5). Supelcosil LC-18 (15 cm × 4.6 mm I.D.) column. UV detection at 242 nm and 0.02 a.u.f.s. Arrow indicates domoic acid retention time. Quantity of sample injected is equivalent to 0.1 and 0.06 mg, as indicated (ppm = mg/kg).

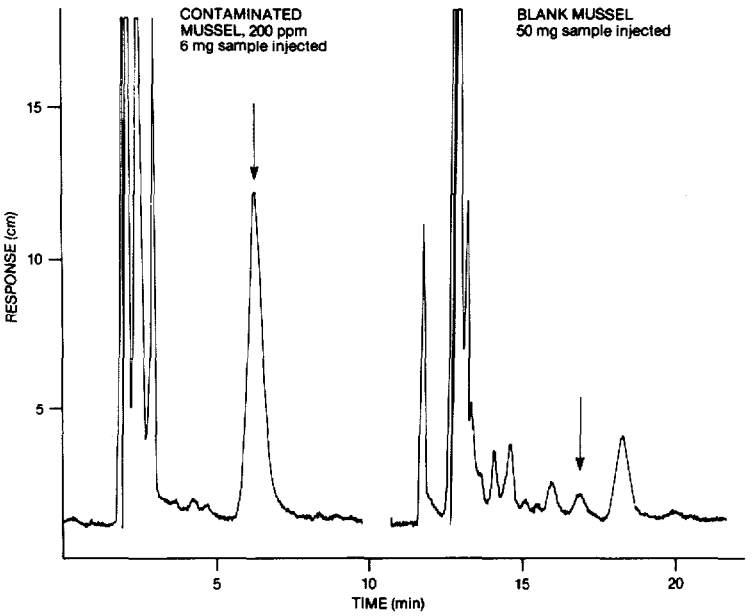

Fig. 3. Chromatograms of contaminated and blank mussel samples. Vydac 302 IC column. Mobile phase, 5% (v/v) acetonitrile in 0.008 *M* KH_2PO_4 (pH 6.9) at 2.0 ml/min. UV detection at 242 nm and 0.02 a.u.f.s. Arrow indicates domoic acid retention time. Quantity of sample injected is equivalent to 6 and 50 mg, as indicated (ppm = mg/kg).

a mobile phase of 5% acetonitrile in 0.008 *M* potassium dihydrogenphosphate (pH 6.9) at 2.0 ml/min was also successful in quantitating domoic acid in mussel extracts. Fig. 3 shows typical chromatograms obtained with the system. The peak corresponding to about 1 mg/kg in the blank sample was an interfering substance and not domoic acid since no peak was observed for domoic acid in this sample when reversed-phase chromatography was used. The ion-exchange system is useful for confirmation of reversed-phase results for domoic acid at high levels (*e.g.* >10 mg/kg) in shellfish.

From earlier work[9] it was observed that a mobile phase pH of 2.5 gave a symmetrical peak for domoic acid. We found that changing the pH to 2.0 or 3.0 caused a slight shift in retention time (more acidic, shorter retention) but peak symmetry and efficiencty remained essentially the same. A pH of 2.5 was selected for routine work.

Sample analysis

The AOAC PSP extraction procedure[10] was compared to the water extraction method reported earlier[9]. It was found that the PSP procedure consistently yielded lower domoic acid values for both spiked and naturally contaminated mussel tissue. In order to study this in more detail, boiling time studies were carried out with both acid and water extraction procedures. It was found that at 200 mg/kg in mussel tissue, domoic acid steadily decreased with increased boiling time resulting in a 7% decrease after 10 min and a 16% decrease after 20 min compared to the 5-min value.

TABLE I

COMPARISON OF PSP AND WATER EXTRACTION PROCEDURES FOR DOMOIC ACID IN MUSSELS

*Sample**	*Domoic acid found (mg/kg)*		*Ratio PSP:water (%)*
	PSP extraction	*Water extraction*	
Blank mussel	<1	<1	–
Blank mussel +19 mg/kg	14.3	16.3	88
Contaminated mussel 1	103	112	92
Contaminated mussel 2	202	231	87
Contaminated mussel 3	417	576	72
Contaminated mussel 4	156**	198**	79
Contaminated mussel 4 (refrigerated after heating and before centrifugation)	171**	193**	89

* Samples cooled at room temperature for 30 min after heating and before centrifugation; boiling time, 5 min.

** Average of duplicates.

For the water extraction no change was observed up to 10 min while at 20 min a 12% decrease was observed. Although the actual fate of domoic acid during the heating is not known, the boiling time was kept at exactly 5 min, in accordance with the AOAC collaboratively studied PSP procedure.

Table I shows results obtained comparing acid and water extractions with boiling times of 5 min for samples containing different levels of domoic acid. It can be seen that acid extraction produced 72–92% of the water extraction values when extracts were permitted to cool on the bench at room temperature. This is not attributed to a poorer extraction efficiency but to a degradation of domoic acid during the extraction and subsequent cooling. If the extracts were refrigerated for 30 min immediately after heating to cool them to room temperature before centrifugation, the amount of domoic acid recovered increased from 79 to 89% relative to the water extraction (see Table I, contaminated mussel 4). Refrigeration of the acid extracts was found to markedly improve the long term stablity of domoic acid. Mussel extracts that were permitted to sit at room temperature for five days showed a 30–50% decrease in domoic acid content, whereas when refrigerated (4°C), there was no significant change after three weeks. As a result of these studies, all sample extracts were refrigerated immediately after heating as well as during storage. Stability can also be improved by passing the extracts trough a reversed-phase C_{18} solid phase extraction cartridge as demonstrated earlier[9].

The repeatability of the method using the above conditions was quite acceptable. Coefficients of variation for replicate determinations ranged from 2.9 to 6.8% for mussel samples containing 4.0–500 mg/kg domoic acid. The detection limit was estimated to be about 0.5 mg/kg (signal-to-noise ratio of 3:1) depending upon quantity and type of sample injected. Fig. 4 shows a typical result for an extract of a blank

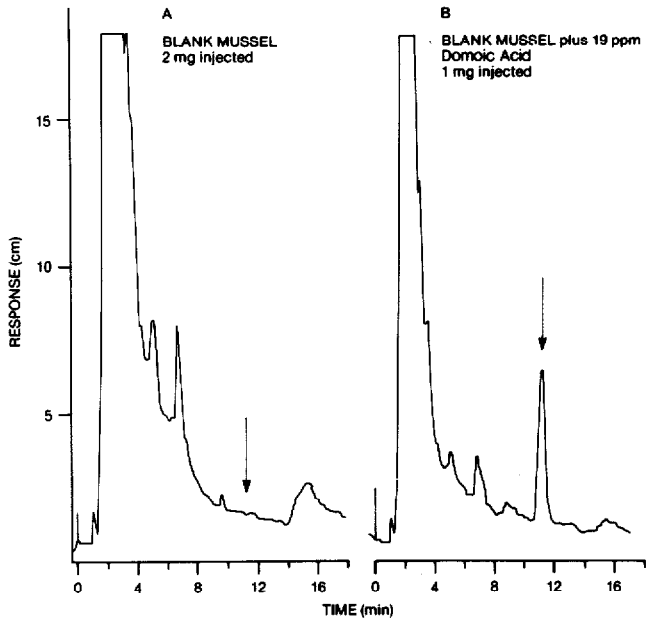

Fig. 4. Chromatograms of (A) blank mussel and (B) blank mussel plus 19 mg/kg domoic acid. Conditions as in Fig. 1. Arrow indicates domoic acid retention time. Quantity of sample injected is equivalent to 2 and 1 mg, as indicated (ppm = mg/kg).

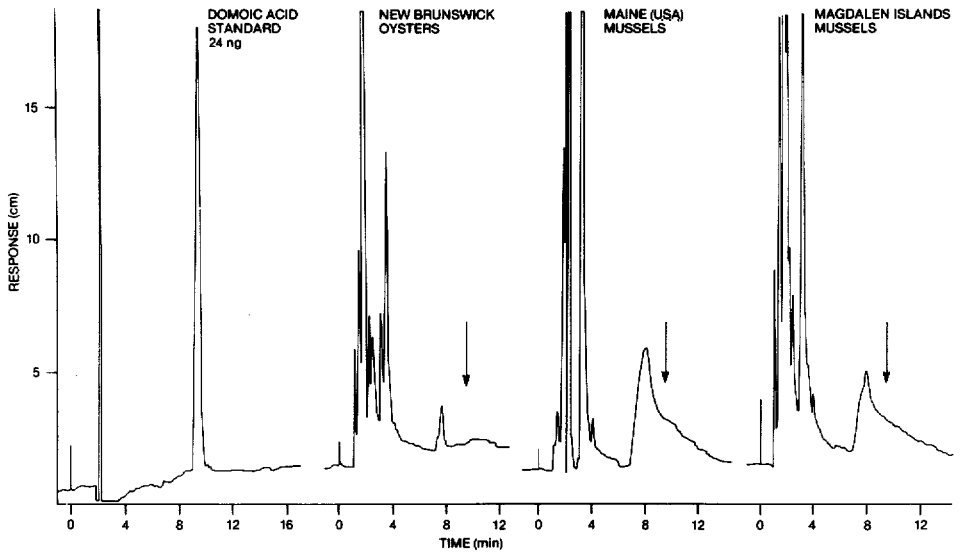

Fig 5. Chromatograms of oyster and mussel samples from the Atlantic region of Canada and a mussel sample from Maine, U.S.A.; 0.2 mg of equivalent sample injected. Conditions as in Fig. 1 except that the mobile phase contained 12.5% acetonitrile. Arrow indicates domoic acid retention time.

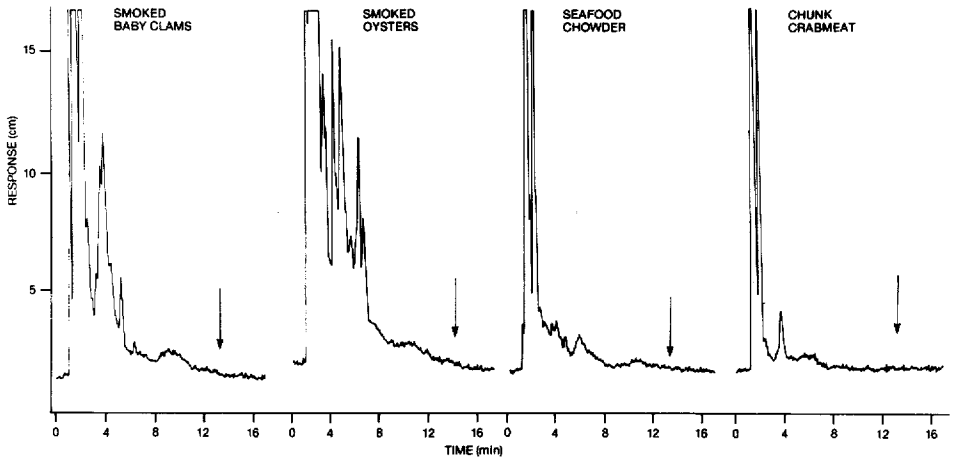

Fig. 6. Chromatograms of different seafood products; 0.5 mg of equivalent sample injected. Conditions as in Fig. 1 except that a Spherisorb ODS 15 cm × 4.6 mm I.D. column was employed. Arrow indicates domoic acid retention time.

mussel which was obtained from the Atlantic coast of Nova Scotia. When spiked with 19 mg/kg domoic acid before acid extraction, good recovery was observed (Table I).

Fig. 5 shows results obtained with oysters and mussels from the Atlantic coast outside the area of eastern Prince Edward Island. No domoic acid was found in any of the samples. The broad tailing peak in the mussels from Maine, U.S.A., and the Magdalen Islands in the Gulf of St. Lawrence was not observed in mussels from Prince Edward Island nor Nova Scotia (blank, Fig. 3).

Fig. 6 shows chromatograms of various seafood products obtained using the acid extraction procedure. The method worked well for all shellfish products tested including oysters, mussels and clams (canned, pickled and smoked), as well as shrimp, crab, lobser and seafood chowders. Recoveries were verified by regularly including spike samples in the analysis scheme. No domoic acid was found in any of 44 different retail products analysed.

CONCLUSION

The PSP extraction procedure employing 0.1 *M* hydrochloric acid was found to provide reproducible results with sufficient sensitivity to detect domoic acid in shellfish down to less than 1 mg/kg. This level is far more than adequate to confirm positive results employing the mouse bioassay procedure. Although the recoveries and stability of the acid extracts are not as good as extracts obtained using boiling water extraction, reliable results may be obtained with certain precautions such as keeping the boiling time to exactly 5 min and refrigerating the samples immediately after extraction and during long term storage. The advantage of the acid extraction is that both PSP toxins and domoic acid can be screened using the mouse bioassay on the same sample extract and confirmation of domoic acid by LC can also be done using the same extract.

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