Journal of Chromatography, 550 (1991) 595–601
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2316

Confirmation of domoic acid in shellfish using butyl isothiocyanate and reversed-phase liquid chromatography

JAMES F. LAWRENCE* and CATHIE MÉNARD
Food Research Division, Bureau of Chemical Safety, Food Directorate, Health Protection Branch, Ottawa, Ontario K1A 0L2 (Canada)

ABSTRACT

A simple chemical confirmatory technique has been developed for domoic acid, a neurotoxic amino acid of marine origin. After extraction with water–methanol, the domoic acid-containing extract is analysed directly by reversed-phase liquid chromatography with UV absorption detection at 242 nm. For confirmation of positive results an aliquot of the extract is evaporated to dryness and reacted with butyl isothiocyanate to form a thiourea derivative which elutes later than underivatized domoic acid. No additional sample cleanup is required in order to carry out the derivatization for confirmation of domoic acid at the Canadian 20 μg/g guideline level in shellfish. In mussel extract, domoic acid was converted to the thiourea derivative with a yield of 86–91% compared to a pure standard carried through the same reaction. The detection limit for the derivative was about 5–10 μg/g of equivalent domoic acid in extracts of mussels, clams or oysters.

INTRODUCTION

Domoic acid is a neurotoxic amino acid which was found to be the cause of a large-scale human poisoning in Canada in 1987 resulting from the consumption of contaminated mussels [1–3]. Following this incident several high-performance liquid chromatographic (HPLC) methods were developed and reported in the literature [4–6]. In addition, several chemical confirmation methods for domoic acid have been reported [7,8]. They involved the use of UV-absorbing or fluorescent reagents to form derivatives of domoic acid followed by their separation and detection by liquid chromatography (LC). Because these reagents also reacted with proteinaceous coextractives that were present in the shellfish extracts, additional sample purification was required before the confirmatory tests could be carried out.

Domoic acid contains a chromophoric group which absorbs strongly enough at 242 nm to enable its direct detection in shellfish tissue at levels of 1 μg/g or less [5]. This is well below the guideline safety level of 20 μg/g suggested by Health and Welfare Canada. Thus, for regulatory purposes a confirmatory technique need only be as sensitive as the direct method. However, a useful feature of a confirmatory test for domoic acid would be to be able to carry out the derivatization reaction on the same extract without additional sample cleanup and using the same LC system with

0021-9673/91/$03.50

only a mobile phase change. None of the above confirmation reactions achieve both these requirements.

We report here a confirmatory method for domoic acid in shellfish tissue which involves reaction of the N–H moiety of domoic acid with butyl isothiocyanate to form a derivative which elutes later than domoic acid. Additional sample cleanup was found to be unnecessary at levels at or above the guideline level of 20 μg/g.

EXPERIMENTAL

Reagents

Solutions of domoic acid (National Research Council, ARL, Halifax, Canada) were prepared in twice deionized water (Milli-Q, Millipore, Bedford, MA, USA). Butyl isothiocyanate, 99% (Aldrich, USA) and triethylamine 99+% (Aldrich) were used as received. All other solvents and chemicals were HPLC- or analytical-grade materials. All solutions of standards, samples and reagents were refrigerated when not in use.

Apparatus

The LC system consisted of two pumps (Beckman Model 114M) with a gradient controller (Model 421A) and an injection port (Altex, Model 210) with a 20-μl loop. Separations were achieved with a Supelcosil LC-18 (15 cm × 4.6 mm I.D., 5 μm) reversed-phased column. Domoic acid and its butyl isothiocyanate (BITC) derivative were detected with a diode array UV absorption detector (Hewlett-Packard Model 1040 A) using 242 nm as the monitoring wavelength. The mobile phase consisted of a linear gradient of 15–80% (v/v) acetonitrile in water (adjusted to pH 2.5) over 20 min at a flow-rate of 1.0 ml/min. The column was then washed with 100% acetonitrile for 5 min then reequilibriated with 15% acetonitrile in water (pH 2.5) before the next injection.

Sample extraction

The extraction method employed has been described elsewhere [9]. A 5-g amount of homogenized shellfish tissue (Sorvall, USA) was mixed (vortex) with 5 ml of water in a 25-ml centrifuge tube for 1 min. Then 10 ml of methanol were added and the contents mixed (vortex) again for 1 min. The mixture was centrifuged and an aliquot of the clear supernatant was filtered through a 0.45-μm filter (Millex HV, Millipore) before injection into the LC system or derivatization. The PSP extraction procedure [6] was also employed for some samples and results compared to those from the water–methanol extraction method. Also, for comparison purposes, a 1-ml aliquot of the clear extract was cleaned up by employing a 1-ml phenylsulphonic acid strong cation-exchange solid-phase extraction (SPE) cartridge (Baker, USA) followed by a 1-ml octadecyl cartridge (Baker) exactly as described elsewhere [7]. Briefly, 1 ml of extract was added to a cation-exchange SPE cartridge which retained the domoic acid. The cartridge was washed with water then the domoic acid eluted with 0.5 *M* hydrochloric aicd. The acid extract was passed through a C_{18} SPE cartridge which retained the domoic acid. The C_{18} cartridge was then washed with water then the domoic acid eluted with 20% acetonitrile in water containing 1% acetic acid.

BITC Derivatization

The derivatization reagent was prepared fresh daily in a fume hood by mixing 200 μl of methanol with 50 μl triethylamine, 50 μl water and 20 μl butyl isothiocyanate in a small test-tube.

A 20-μl aliquot of sample extract was mixed with 20 μl of BITC reagent solution in a 1-ml reactivial. The contents were permitted to react for 20 min at room temperature with a gentle swirling of the tube after 10 min. The contents were then evaporated to dryness at 40°C with a stream of nitrogen for 15 min (to remove some of the excess reagent). The residue was dissolved in 0.5 ml of acetonitrile–water (15:85, v/v) and analysed by LC.

RESULTS AND DISCUSSION

Fig. 1 shows results for a standard solution of domoic acid before and after BITC derivatization. After reaction the domoic acid peak disappears and a derivative

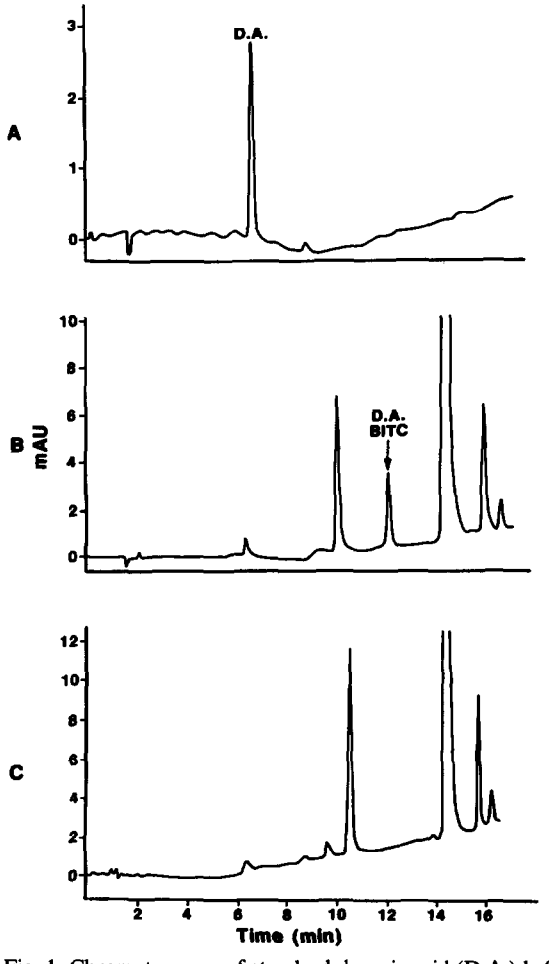

Fig. 1. Chromatograms of standard domoic acid (D.A.) before and after BITC derivatization: (A) 10 ng injected; (B) 10 ng equivalent domoic acid injected; and (C) blank reaction. Gradient conditions described in the text.

peak appears at a later retention time. The derivative peak was about 50% larger in surface area than underivatized domoic acid owing to its increased absorption due to the addition of the thiourea moiety to the molecule which also shifted the UV absorbance maximum to 246 from 242 nm for the underivatized domoic acid.

Fig. 2 compares results obtained for an extract of naturally contaminated mussels containing 538 μg/g domoic acid. The derivatized extract contains many more peaks due to reaction of proteinaceous coextractives with BITC to form products with increased UV absorption. However, the domoic acid derivative is separated and can be identified and quantitated in the extract. This was impossible to do using phenyl isothiocyanate (PITC) as a derivatization reagent [7] since the PITC derivatives of the coextractives completely prevented the detection of domoic acid even at the 500 μg/g level. Fig. 3 compares partial chromatograms of a reagent blank, an uncontaminated (blank) mussel and a mussel sample containing 92 μg/g and derivatized with BITC. The majority of peaks appearing in the chromatograms result from sample coextractives. No peak corresponding to derivatized domoic acid was found in the reagent blank nor the uncontaminated mussel sample.

For comparison purposes we applied the BITC derivatization reaction to extracts cleaned up using the two-cartridge SPE cleanup described earlier [7]. Fig. 4 shows results obtained with the same extract of a naturally contaminated mussel

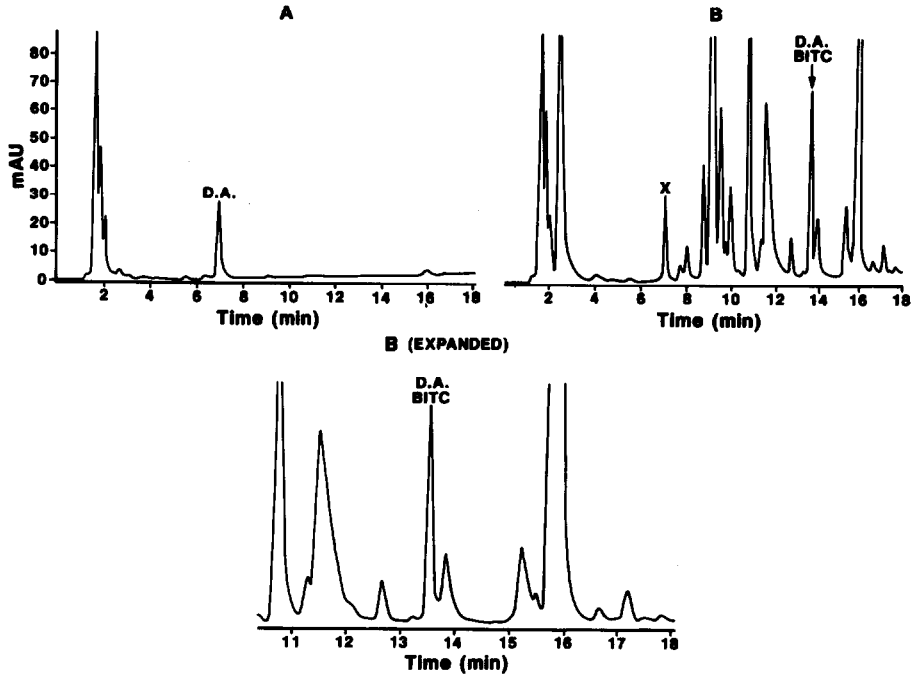

Fig. 2. Chromatograms of domoic acid in naturally contaminated mussel extract (538 μg/g), (A) before and (B) after BITC derivatization (lower chromatogram B expanded between 10–18 min for clarity). Conditions as in Fig. 1 except for a slight decrease in gradient rate. In both chromatograms, 0.2 mg of equivalent sample injected. X = Reagent peak.

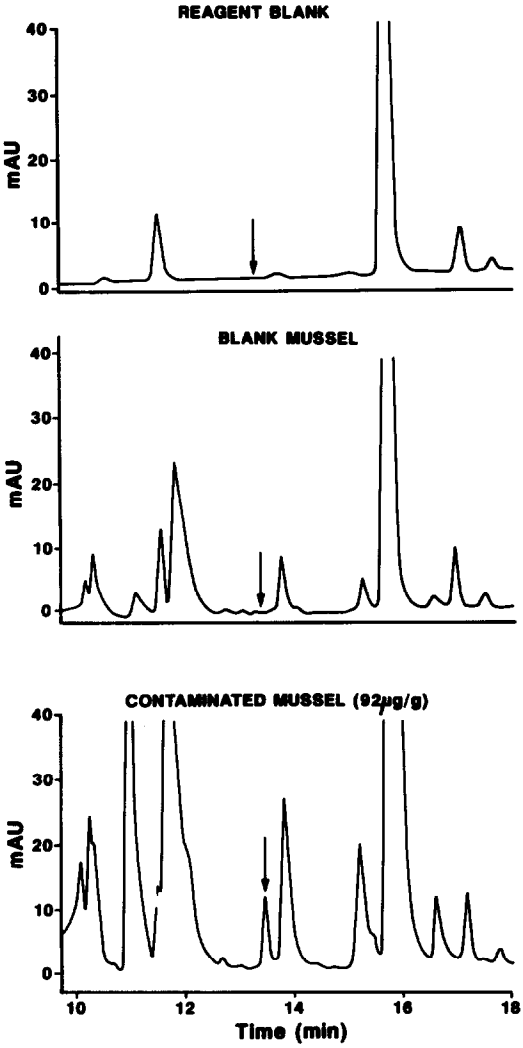

Fig. 3. Chromatograms obtained for a reagent blank, an uncontaminated (blank) mussel and a naturally contaminated mussel (92 µg/g) all after BITC derivatization. Equivalent of 0.2 mg of sample injected. Conditions as in Fig. 2.

sample (538 µg/g domoic acid) as illustrated in Fig. 1, before and after the SPE cleanup. The cleanup is particularly effective in removing coextractives resulting in a chromatogram which appears as clean as a derivatized domoic acid standard. Although the cleanup is rather selective for domoic acid, it is not really necessary for quantitation of BITC-domoic acid as is observed in the upper (A) chromatogram (uncleaned extract) of Fig. 4.

Fig. 5 compares chromatograms for an SPE cleaned-up and non-cleaned-up extract of uncontaminated mussel spiked with 22 µg/g domoic acid. Again the

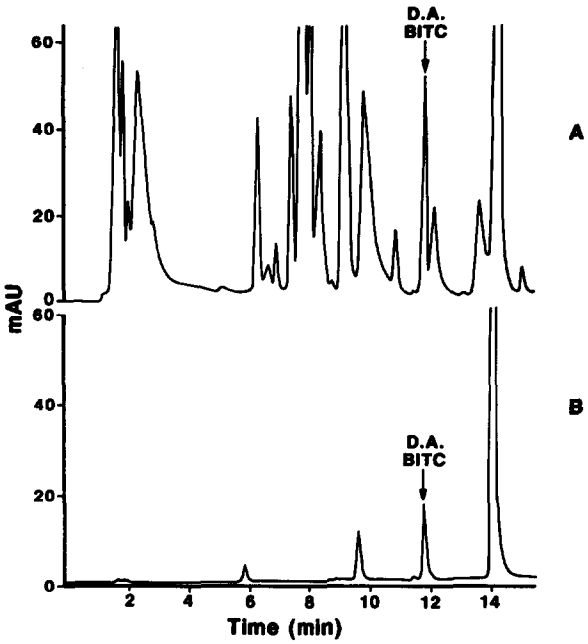

Fig. 4. Chromatograms of cleaned and uncleaned extracts of naturally contaminated mussels after BITC derivatization (538 μg/g). (A) Not cleaned, 0.2 mg of equivalent sample injected; (B) cleaned, 0.1 mg equivalent of sample injected. Conditions as in Fig. 1.

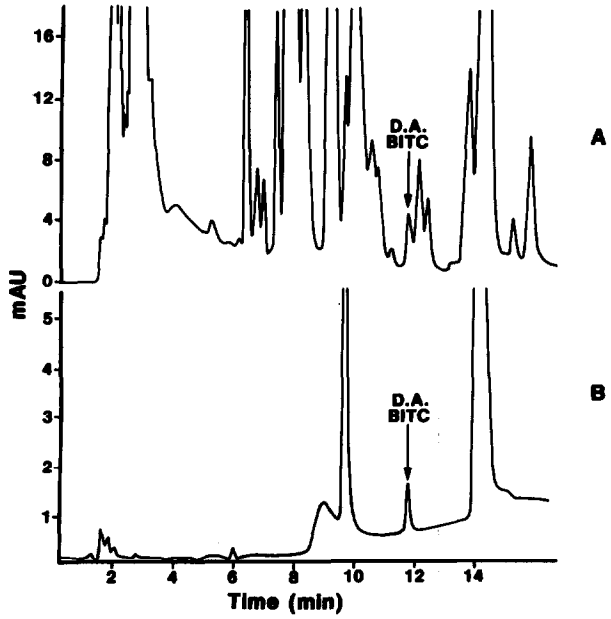

Fig. 5. Chromatograms of cleaned and uncleaned extracts of spiked mussels after BITC derivatization (22 μg/g). (A) Not cleaned, 0.2 mg of equivalent sample injected; (B) cleaned, 0.1 mg equivalent sample injected. Conditions as in Fig. 1.

cleaned-up extract yields a chromatogram similar to a standard. However, the clean-up is not necessary to quantitate domoic acid even at this level although it is a useful tool for additional confirmation puposes because of its good ability to selectively isolate domoic acid from the sample extracts. The small peak near 6 min in the chromatograms of the cleaned-up extracts in Figs. 4 and 5 is unreacted domoic acid which is not observed in the chromatograms of the non-cleaned-up extracts due to a reagent interference. The recovery of domoic acid through the SPE cleanup was about 80%.

The water-methanol extraction was found to be simpler and yielded higher recoveries of domoic acid compared to the acid extraction method. Also, although domoic acid could be confirmed by direct BITC derivatization of the acid extracts, the water–methanol extracts yielded cleaner chromatograms and higher yields of BITC derivative (86–91% yield at 22 μg/g for the water–methanol extraction and 64% yield at 45 μg/g for the acid extraction).

The BITC derivative of domoic acid was stable at 4°C for up to four days in actual derivatized shellfish extracts. This enables batch derivatizations to be performed on one day with HPLC analysis on the next.

The detection limit (3:1, signal-to-noise) for BITC–domoic acid in shellfish extracts was estimated to be about 5–10 μg/g under the conditions employed. This could be improved by employing the SPE cleanup and injecting more material into the LC system. However, for regulatory purposes at the 20 μg/g level, this is unnecessary.

The repeatability coefficient of variation of replicate BITC reactions ($n = 3$) for a spiked mussel extract (22 μg/g domoic acid) was 12% while for a naturally contaminated sample (28 μg/g domoic acid) it was 6%. The derivatization reaction provided linear results in extracts over the range of domoic acid concentrations studied (22–538 μg/g).

The method was successfully applied to the confirmation of domoic acid in fresh mussels, oysters and clams as well as canned pickled mussels. No interfering materials were found in any of these products.

REFERENCES

1 J. L. C. Wright, R. R. Boyd, A. S. W. De Frietas, M. Falk, R. A. Foxall, W. D. Jamieson, M. V. Laycock, A. W. McCulloch, A. G. McInnes, P. Odense, V. P. Pathak, M. A. Quilliam, M. A. Ragan, P. G. Sim, P. Thibault, J. A. Walter, M. Gilgan, D. J. A. Richard and D. Dewar, *Can. J. Chem.*, 67 (1989) 481.
2 M. A. Quilliam and J. L. C. Wright, *Anal. Chem.*, 61 (1989) 1053A.
3 R. F. Addison and J. E. Stewart, *Aquaculture*, 77 (1989) 263.
4 M. A. Quilliam, P. G. Sim, A. W. McCulloch and A. G. McInnes, *Int. J. Environ. Anal. Chem.*, 36 (1989) 139.
5 J. F. Lawrence, C. F. Charbonneau, C. Ménard, M. A. Quilliam and P. G. Sim, *J. Chromatogr.*,, 462 (1989) 349.
6 J. F. Lawrence, C. F. Charbonneau and C. Ménard, *J. Assoc. Off. Anal. Chem.*, 74 (1991) 68.
7 J. F. Lawrence, C. F. Charbonneau, B. D. Page and G. M. A. Lacroix, *J. Chromatogr.*, 462 (1989) 419.
8 R. Pocklington, J. E. Milley, S. S. Bates, C. J. Bird, A. S. W. DeFreitas and M. A. Quilliam, *Int. J. Environ. Anal. Chem.*, 38 (1990) 351.
9 M. W. Gilgan, B. G. Burns and G. J. Landry, presented at the *104th Annual Meeting of the Association of Official Analytical Chemists, New Orleans, 1990*, poster No. 3108.