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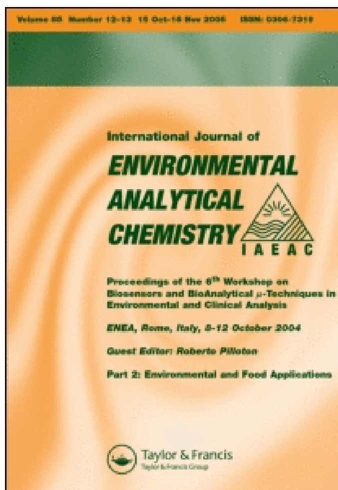

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# CONFIRMATION OF DOMOIC ACID IN SEAFOOD USING REVERSE PHASE LIQUID CHROMATOGRAPHY WITH NINHYDRIN POST-COLUMN DERIVATIZATION

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A confirmatory and quantitative technique is developed for domoic acid (DA) in seafood samples which is based on methanol-water extraction, separation by reverse phase liquid chromatography, a ninhydrin post-column derivatization of the acid, and UV detection at 402 nm. Tissue samples containing as low as 0.3 µg DA/g analyzed by a direct method using 242 nm can be confirmed by this technique. The linear range is excellent (1 ng to 4 µg) with a linear correlation coefficient of 0.994. Spike recovery of DA was 93% at a tissue concentration of 10 µg DA/g. The method was applied successfully to mussels, crabs, oysters, razor clams, and anchovies.

**KEYWORDS:** Domoic acid, seafood, high performance liquid chromatography, diode array detection, derivatization, ninhydrin

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## INTRODUCTION

Domoic acid (DA) is a naturally occurring neurotoxic amino acid which has been associated with amnesic shellfish poisoning (ASP)[1]. Several HPLC methods have been published which involve reverse phase liquid chromatography and UV detection or pre-column derivatization, followed by separation and detection by UV absorption or fluorescence[2–6]. UV detection of underivatized DA lacks specificity because interferents can exhibit UV spectra similar to that of DA. The pre-column derivatization methods have stringent requirements for pH control, excess reagent removal, multistep manipulation and are subject to interference from salts, buffers and proteinaceous materials[7].

The determination of DA by post-column derivatization has been done earlier by using ion exchange chromatography[8]. Post-column reactions do not change the chemistry of analytes for chromatographic separation, and do not produce any hydrolysis products. This procedure allows the use of two detectors in series so that spectra can be obtained both before and after the derivative is formed. Ninhydrin (1,2,3-triketohydrindene hydrate) reacts at a

high temperature in about 1 minute with secondary amino acids to yield a yellow-colored derivative. DA is a secondary amino acid which reacts with ninhydrin (NH) to form a derivative with an absorbance maximum at 402 nm.

Conventional UV detection at 242 nm is normally used for routine analysis of DA at a level of >0.5 μg/g of tissue. We describe here a post-column, ninhydrin derivatization HPLC method which can confirm as little as 0.3 μg/g of DA in tissue samples without extensive sample cleanup or pre-concentration. The reproducibility, sensitivity, linearity, accuracy and its application to seafood also are reported.

## EXPERIMENTAL

### *Liquid chromatography*

A Hewlett-Packard (HP) Model 1090 liquid chromatograph, equipped with an HP diode array detector (DAD), PV5 solvent delivery system, autoinjector and an HP 79994A 'Chem Station' for data acquisition were utilized. The pre-column, a Brownlee cyano cartridge (10 cm × 4.6 mm × 5 μm) and a Vydac analytical column (25 cm × 2.1 mm × 5 μm) and Spherisorb (25 cm × 4.6 mm × 5 μm) widebore column were obtained from Rainin Instrument Co., Inc., Emeryville, California. A 0.45 μm frit from HP was used ahead of the pre-column. The centrifuge was a Model CU-5000, Damon International Equipment Co. A Tekmar homogenizer was supplied by Tekmar Co., Cincinnati, Ohio. The post-column derivatization apparatus consisting of a Milton Roy mini pump, reaction coil (CRX 390 post-column reactor), Valco mixing tees, flow conditioner, pressure gauge, pressure relief valve, and backpressure regulator was supplied by Pickering Laboratories, Inc., Mountain View, California.

A second UV detector (Waters 490E) connected in series with the HP DAD was utilized together with an HP 3390A integrator for data acquisition.

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### *Materials and reagents*

HPLC grade solvents and orthophosphoric acid were supplied by EM Service, Gibbstown, NJ. Reagent water was distilled water passed through an ion exchange column. DACS-1 standard (89 μg DA/mL) and MUS-1 (98 μg DA/g mussel tissue) were obtained from NRC Marine Analytical Chemistry Standards Program (MASP), Halifax, Nova Scotia, Canada (B34321). Ninhydrin was obtained from Sigma Chemical Co., St. Louis, MO. A 0.05M sodium borate buffer (prepared from boric acid, dissolved in deionized water and adjusted to pH 9.1 with sodium hydroxide) was supplied by Pickering Laboratories, Inc., Mountain View, CA. Ninhydrin reagent was prepared by dissolving 500 mg of the ninhydrin crystals in 10 mL methanol, and then mixing this solution with 200 mL of deoxygenated 0.05M sodium borate buffer (pH 9.1).

*Sample extraction*

The aqueous methanol extraction procedure described by Quilliam was used[9]. Briefly, 4.0 g of tissue sample are accurately weighed into a 50 mL centrifuge tube, 8 mL methanol are added and made to a total volume of 20 mL with distilled water. The mixture is then homogenized thoroughly using a Polytron high speed blender for 3 min at 70% of full scale voltage. After centrifugation at 2000 rpm for 10 min, approximately 2 mL of the top layer is filtered through a 0.45 μm Millex syringe filter. (Note: since 4 g of tissue with a density close to 1.0 is diluted to 20 mL, the solution concentration of DA in the extract is approximately one-fifth of that in the tissue sample). As detailed in an earlier publication[5], the extract is then used without further dilution. Aqueous methanol extracts of tissue samples were quite stable when filtered through 0.45 μm syringe filters and refrigerated; no losses of DA were detected after one week.

*HPLC analysis*

Using a cyano pre-column and Vydac microbore column, the gradient elution was programmed from 0.0% to 12.5% acetonitrile in water that was adjusted to pH 2.5 with orthophosphoric acid (230 μL/L), with a flow rate of 0.5 mL/min. The gradient was linear for twenty minutes beginning with sample injection. After an additional 10 min at fixed concentration, the eluent was programmed back to the initial conditions over a 7-min period. Column temperature employed was 50°C. With a Spherisorb column alone, gradient elution was programmed from 5% to 25% acetonitrile in water (pH 2.5) over 25 minutes, at a flow rate of 1.0 mL/min.

Working standard solutions of DA from 0.05 to 80 μg/mL, equivalent to tissue concentration of 0.25 to 400 μg/g, were prepared in 10% acetonitrile in water solution from a DACS-1 calibration solution. A 20 μL aliquot of the standards and sample extracts was injected by means of an autosampler.

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*Post-column derivatization*

The post-column derivatization system consisted of reagent pump, reaction coil, flow conditioner, Valco mixing tee, pressure gauge, pressure relief valve, and back pressure regulator as shown in Figure 1. The reagent pump was equipped with micrometer which controlled the flow rate of the ninhydrin reagent. The flow controller reduced the magnitude of pulses while the pressure relief valve protected the flow cell from damage. The back pressure regulator attached at the end of waste line suppressed bubble formation due to the high temperature of the mobile phase in the reaction coil. Electrical power to the reagent pump and reaction coil were controlled by the post-column interlock which switched them off as soon as the eluent pump pressure dropped below 500 psi. An optional DAD can be used to permit quantitation and confirmation based on underivatized DA.

Ninhydrin reagent was delivered on line by pumping the solution into the mobile phase via a Valco mixing tee and passing it through a 1 mL reaction coil of teflon tubing (0.28 mm

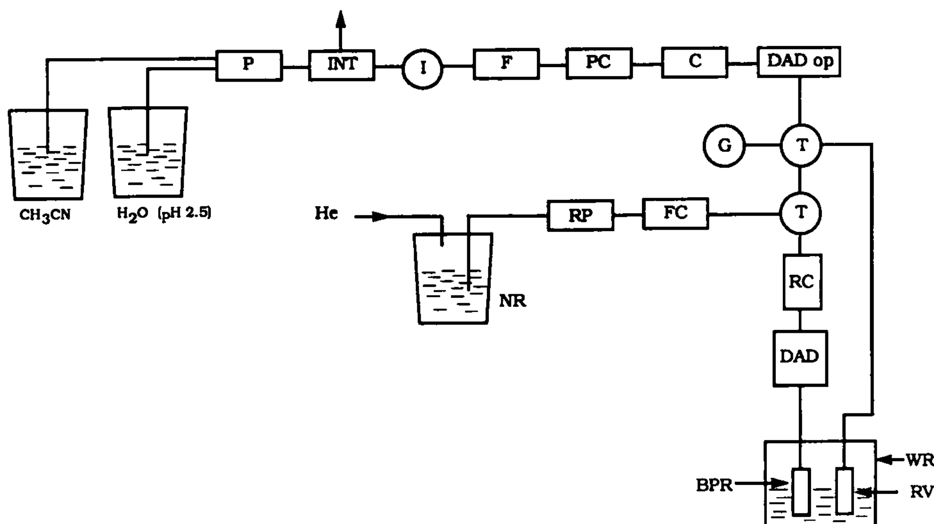


**Figure 1** Schematic representation of analysis train. P, INT, I, F, PC, C, DAD op, T, G, FC, RP, NR, RC, DAD, RV, BPR, WR denote the eluent pump, post-column interlock, injector, filter, precolumn, column, optional diode array detector or UV detector, Valco mixing tee, pressure gauge, flow conditioner, reagent pump, ninhydrin reagent, reagent coil, diode array detector or UV detector, pressure relief valve, back pressure regulator, and waste reservoir, respectively.

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i.d. × 16 m) thermostated at 145°C. The reaction coil was plumbed to the DAD. The flow rate of the ninhydrin reagent was adjusted by maximizing the UV absorbance signal of the ninhydrin derivative (DA-NH). It was determined to be 0.055 mL/min with the microbore column with a mobile phase flow rate of 0.5 mL/min. The Spherisorb widebore column, with a mobile phase flow rate of 1.0 mL/min, required a 0.118 mL/min ninhydrin reagent flow rate for maximum sensitivity. The ninhydrin reagent showed a small degree of deterioration over a period of one week and should be kept under pressure with helium or nitrogen gas at 3 psig in an amber-colored bottle. During the shutdown procedure, the reaction coil must be cooled down by passing the mobile phase through it for at least one hour.

### *UV detection*

Absorbance for quantitation of DA-NH was measured at 402 nm with a 10 nm bandwidth, while the UV spectrum for confirmation of the peak identification was scanned from 300 to 600 nm. The absorbance of DA itself, was measured at 242 nm.

## RESULTS AND DISCUSSION

### *Ninhydrin reaction*

The conditions used for the ninhydrin reaction with DA (Figure 2) are similar to those normally used with secondary amino acids[10], except that reverse phase chromatography replaces ion exchange chromatography. The phosphate buffer (pH 2.5) in the mobile phase for HPLC separation posed no problem with ninhydrin. The flow rate of ninhydrin reagent and the temperature of reaction coil (Figure 3 a,b) were optimized in order to obtain maximum sensitivity for DA derivative. This was achieved at pH 5.8 of the effluent which resulted from mixing the phosphate buffer (pH 2.5) and the borate buffer (pH 9.1). The ninhydrin reagent itself showed negligible absorbance at 402 nm wavelength which was found to be 0.4 mAU as compared to 0.2 mAU of mobile phase alone.

### *Spectroscopy*

Figure 4 shows the UV absorption spectra of DA and its derivative acquired using the HPLC and DAD. Domoic acid has a spectrum with a strong absorption maximum at 242 nm while the spectrum of the DA-NH exhibits a strong absorption maximum at 402 nm. These spectra are useful for the confirmation of peak identity. The minimum amounts of DA required to yield acceptable UV spectra for DA and DA-NH are 3 and 2 ng, respectively.

### *HPLC separation and quantitation*

Figure 5 shows the relative sensitivities of underivatized and derivatized DA. The absorbance signal of the derivative at 402 nm is about 80% of that for DA at 242 nm. The chromatogram peak for the derivative is sharp.

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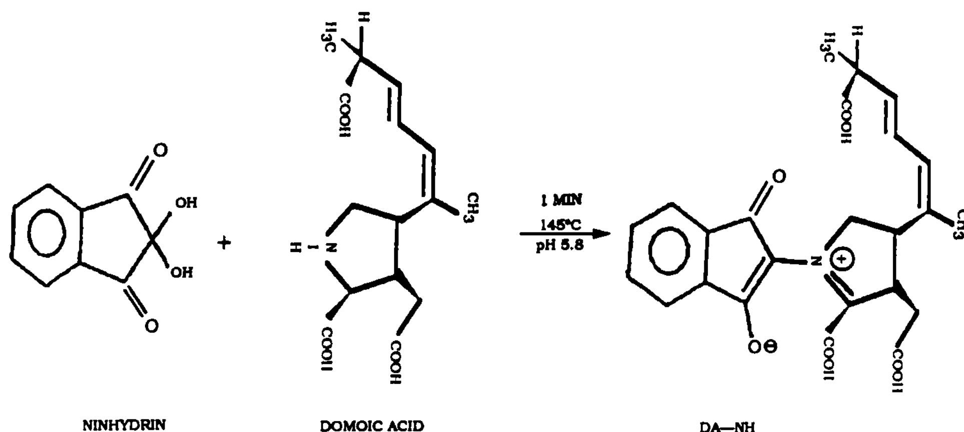


**Figure 2** Schematic of the reaction of domoic acid with ninhydrin reagent.

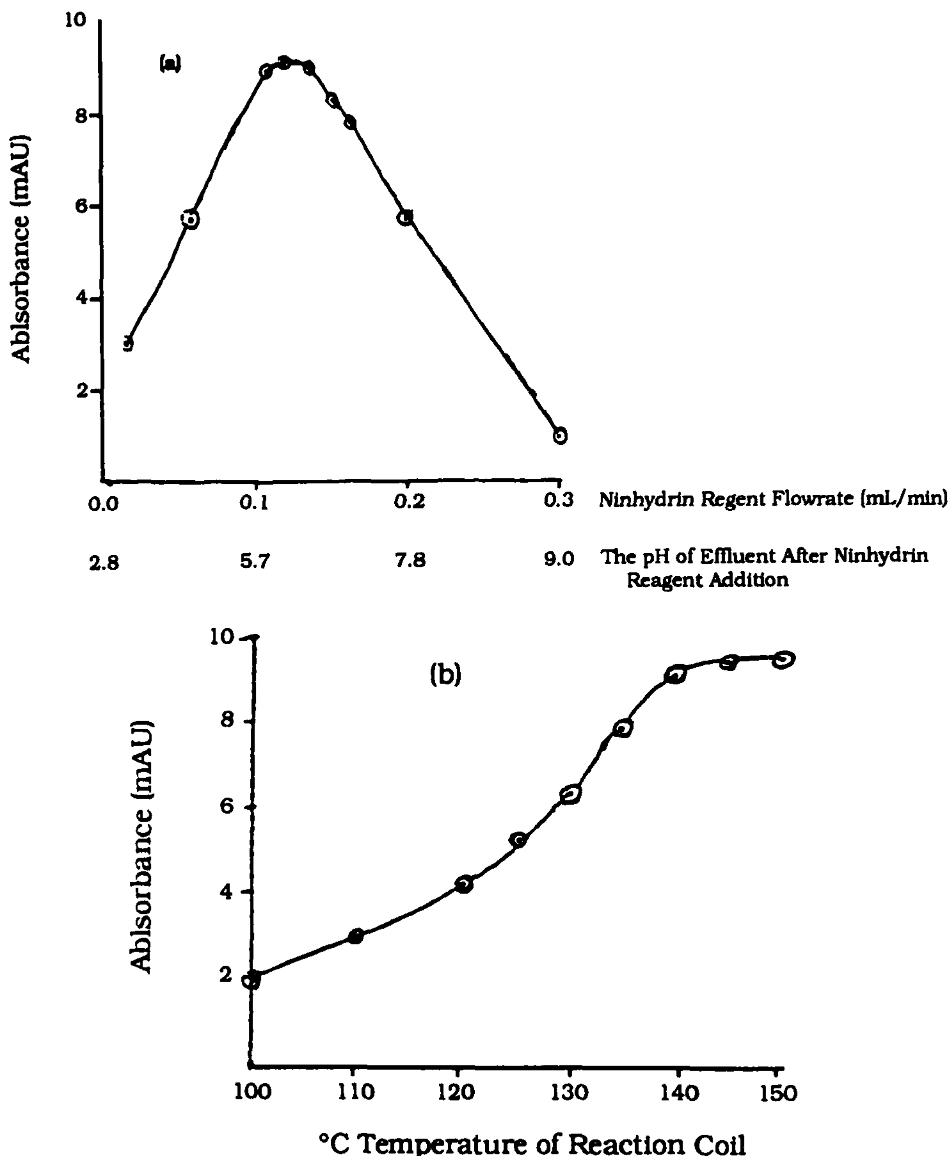


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**Figure 3** Absorbance (402 nm) of DA derivative at 4.0 μg DA/mL concentration in relation to a) flow rate of ninhydrin reagent b) temperature of reaction coil. Conditions: Spherisorb (25 cm × 4.6 mm × 5 μm); gradient elution 5% to 25% $CH_3CN$ in water (pH 2.5) in 25 min; mobile phase flow rate 1.0 mL/min; ninhydrin reagent flow rate 0.118 mL/min; column temperature 50°C; injection volume 20 uL.

Figure 6(a) shows a chromatogram generated after derivatization of DA in mussel tissue blended with reference material MUS-1 with a DA concentration of 10 μg/g of tissue. The NH derivative of DA and its isomers, peaks 1, 2, and 3, are clearly separated and resolved from one another and DA recovery was 93%. Figure 6(b) shows the chromatogram of a

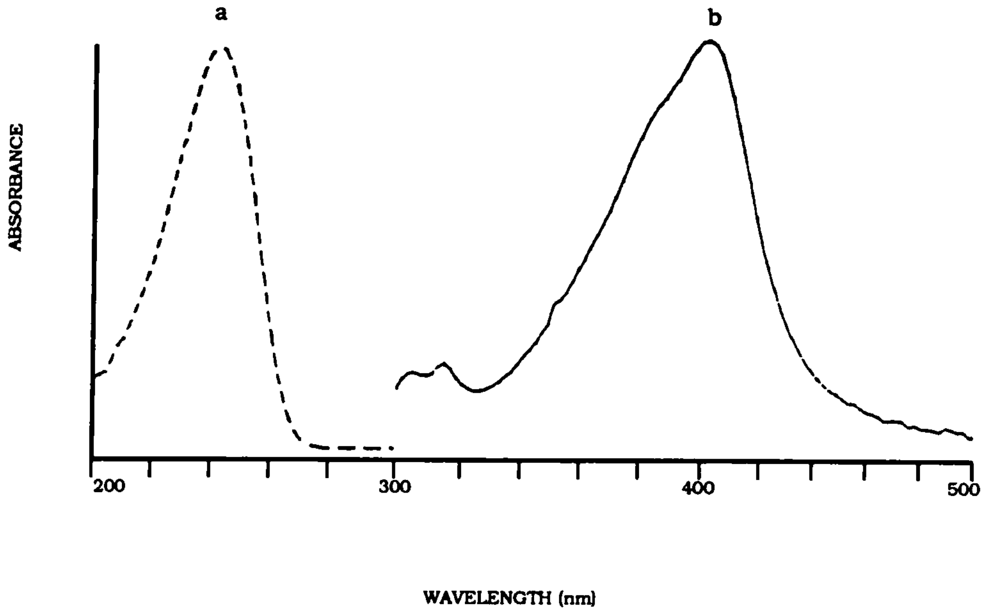


**Figure 4** Ultraviolet absorption spectra of a) domoic acid b) ninhydrin derivative of domoic acid.

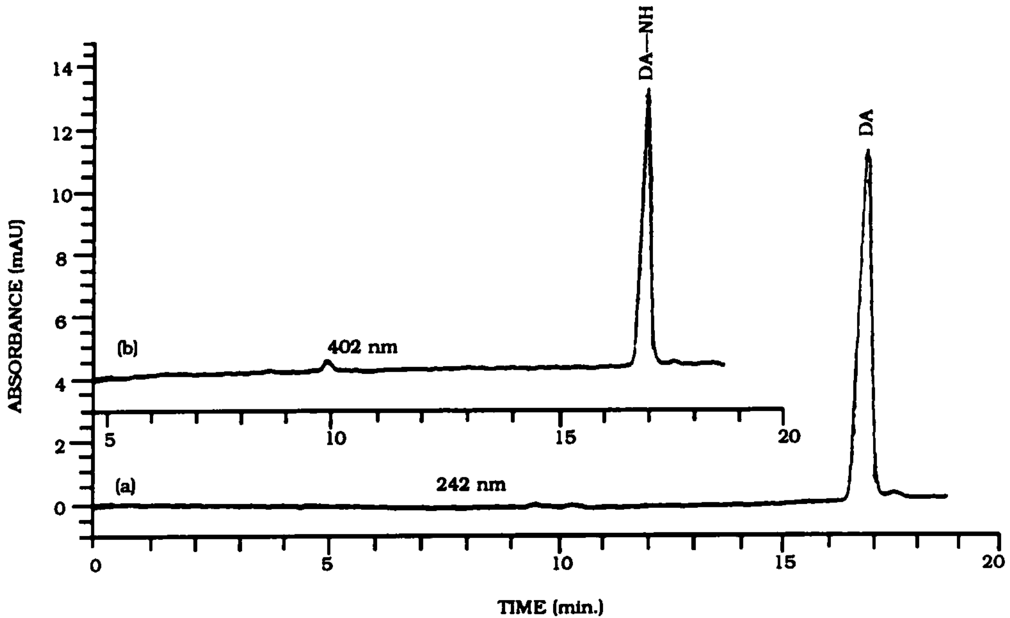


**Figure 5** Gradient reverse phase HPLC of domoic acid at 4.0 μg/mL concentration: (a) without derivatization; (b) with ninhydrin derivatization using the same HPLC and DAD system. DAD detection provided both the 242 nm and 402 nm UV absorbance. Conditions: as in Figure 3.

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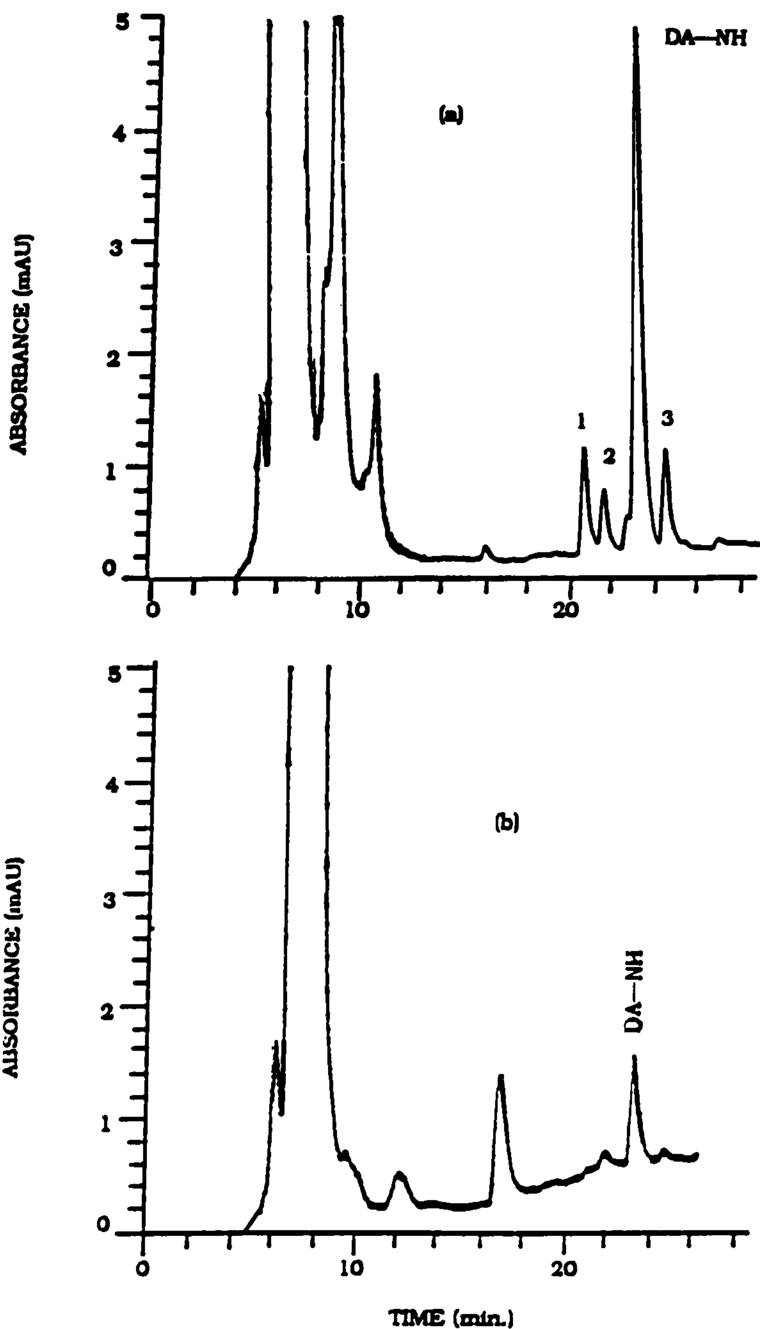


**Figure 6** Chromatograms of domoic acid after ninhydrin derivatization in: (a) mussel spiked with MUS-1 (10 µg/g); (b) naturally occurring contaminated anchovies extract (2.2 DA µg/g). Conditions: cyano pre-column (10 cm × 4.6 mm × 5 µm) and Vydac analytical column (25 cm × 2.1 mm × 5 µm); gradient elution 0.0% to 12.5% $CH_3CN$ in water (pH 2.5) in 20 min; hold 10 min mobile phase flow rate 0.5 mL/min; ninhydrin reagent flow rate 0.055 mL/min, column temperature 50°C; and injection volume 20 uL.

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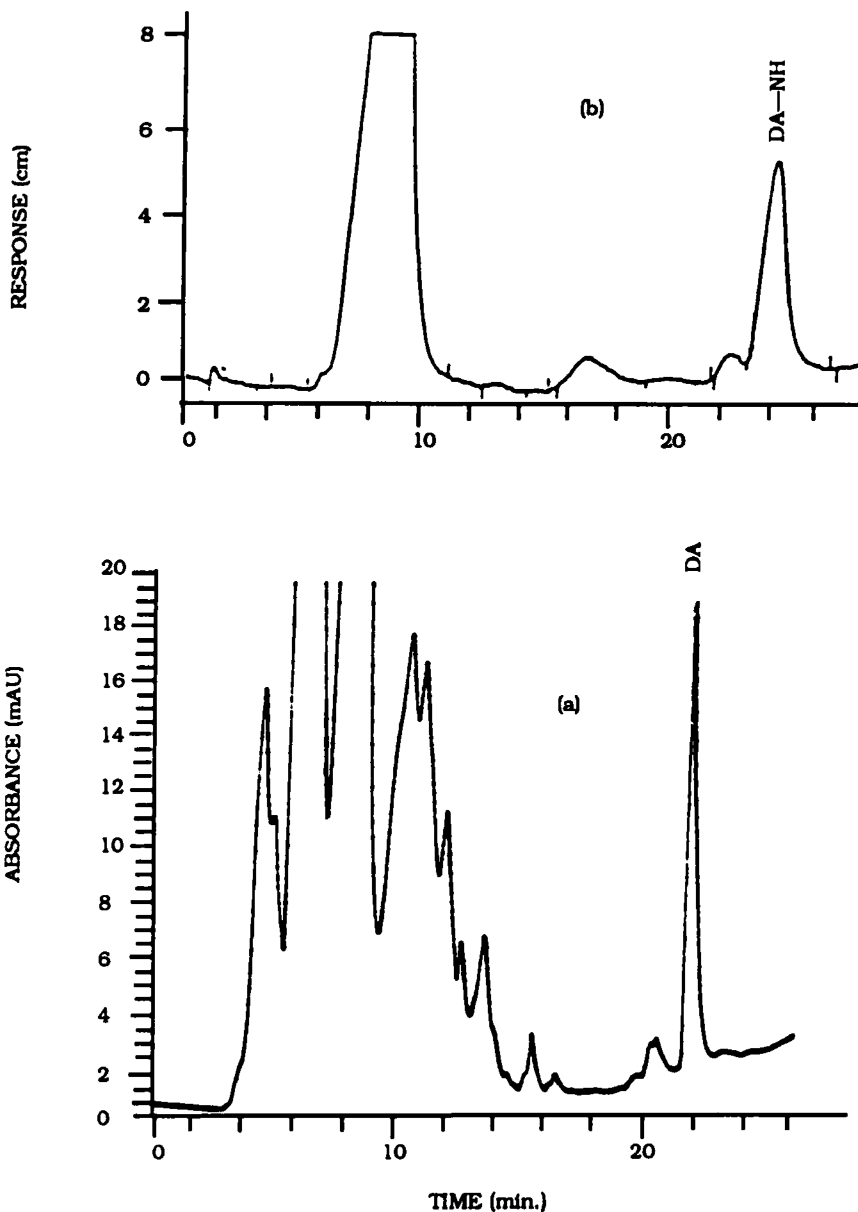


**Figure 7** Chromatograms obtained: before, (a) at 242 nm; and after, (b) ninhydrin derivatization at 402 nm, of razor clam naturally contaminated with DA concentration of 31.0 μg/g. Conditions: as in Figure 6.

naturally DA-contaminated anchovy sample containing 2.2 μg/g DA. Tryptophan, under some conditions coelutes with DA, but does not give a peak absorbing at 402 nm. Additional sample purification was found unnecessary, even at low concentration levels.

### *Quality assurance*

The lowest DA concentration level which was confirmed with this procedure was 0.3 μg/g mussel tissue using a 20 μL injection in both widebore and narrowbore column systems. The reproducibility (precision) of replicate analyses of a mussel extract spiked with 20 μg/g DA was found to be 3.4% (n = 4), while that of a naturally contaminated tissue sample of razor clam with 31 μg/g DA was 4.0% (n = 3). The calibration curve was linear over a range of 1 ng to 4 μg with a linear correlation coefficient of 0.994.

### *Applications*

This method was applied successfully to the measurement of DA in crabs, oysters, anchovies, razor clams, and mussels. No interfering materials were evident. Figure 7 shows chromatograms for DA analysis performed with two UV detectors connected in series; the first detector measures the UV absorbance at 242 nm for DA before derivatization and the second at 402 nm after derivatization. The only critical factor is the pressure, which should not exceed 600 psi in order to protect the flow cell and reaction coil from damage. A constant system pressure at 145 psi in the flow cell was maintained when obtaining these chromatograms.

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## CONCLUSION

Reverse phase HPLC followed by a ninhydrin post-column derivatization procedure provides good sensitivity, selectivity, reproducibility, and accuracy for the confirmation and quantitation of domoic acid in seafood. The procedure is especially advantageous when two UV detectors are used in series to measure DA concentration, as well as to obtain spectra before and after ninhydrin derivatization for sensitive confirmation of domoic acid.

### *Acknowledgement*

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