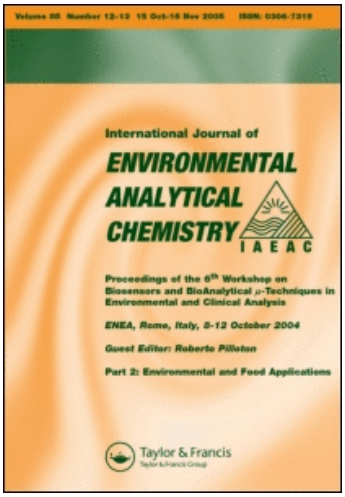

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

An Improved HPLC Procedure for Domoic Acid Analysis in Seafood

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To cite this Article Dhoot, J. S. , Rosario, A. R. Del , Appel, B. R. and Tamplin, B. R.(1993) 'An Improved HPLC Procedure for Domoic Acid Analysis in Seafood', International Journal of Environmental Analytical Chemistry, 53: 4, 261 — 268
To link to this Article: DOI: 10.1080/03067319308044430
URL: http://dx.doi.org/10.1080/03067319308044430

Intern. J. Environ. Anal. Chem., Vol. 53, pp. 261-268
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AN IMPROVED HPLC PROCEDURE FOR DOMOIC ACID ANALYSIS IN SEAFOOD

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(Received, 29 April 1992; in final form, 6 August 1992)

A high performance liquid chromatographic (HPLC) procedure was developed for the determination of domoic acid (DA) in seafood, including on-line sample purification. Unpurified aqueous methanol extracts of tissue samples were partially purified with a cyano precolumn cartridge. Separation was performed on a reverse-phase C-18 column with gradient elution, and UV detection. UV spectra were acquired for confirmation. Analyte recoveries from 89% to 100% were obtained for a variety of tissues. The detection limit with confirmation by UV spectrum was 0.1 μg/g. The method eliminates the use of a manual sample cleanup step. In contrast to other published procedures, the method is insensitive to NaCl, which may be present at high concentrations in some seafood samples. Further, tryptophan does not interfere.

KEY WORDS: Domoic acid, seafood, high-performance liquid chromatography, interferents, diode array detection.

INTRODUCTION

Since the 1987 discovery of Amnesic Shellfish Poisoning (ASP)[1,2], the isolation of the causative toxic agent, domoic acid (DA)[3,4] (Figure 1), and the identification of one or more phytoplankton species as the DA source[5,6], analytical methodology for DA has been the subject of numerous investigations. A recent report[7] described analytical alternatives, and a procedure for rapid extraction, cleanup, and HPLC analysis of DA. We shall refer to the method in that report[7] as the "Quilliam method". In addition to DA, structural isomers of DA have been identified[8] but will not be dealt with in the present study.

DA has a molar absorptivity of 2.63×10^4 at 242 nm, which enables detection in most marine species at levels of ≤ 0.1 μg/g of wet tissue. Since DA is water soluble (7.6 g/l) and methanol soluble (0.66 g/l), it is easily extracted[9].

Extraction of DA in 0.1 N HCl, i.e., the interim first action AOAC method[10,11], yields low recoveries and allows decomposition of DA at trace levels during storage[12]. Extraction by boiling water gives higher recoveries, but is time consuming. Aqueous methanol extraction by homogenizing, at room temperature, with a high speed blender gives recoveries usually above 90%[7], and is faster than the first two methods. Accordingly, it has become the extraction method of choice.

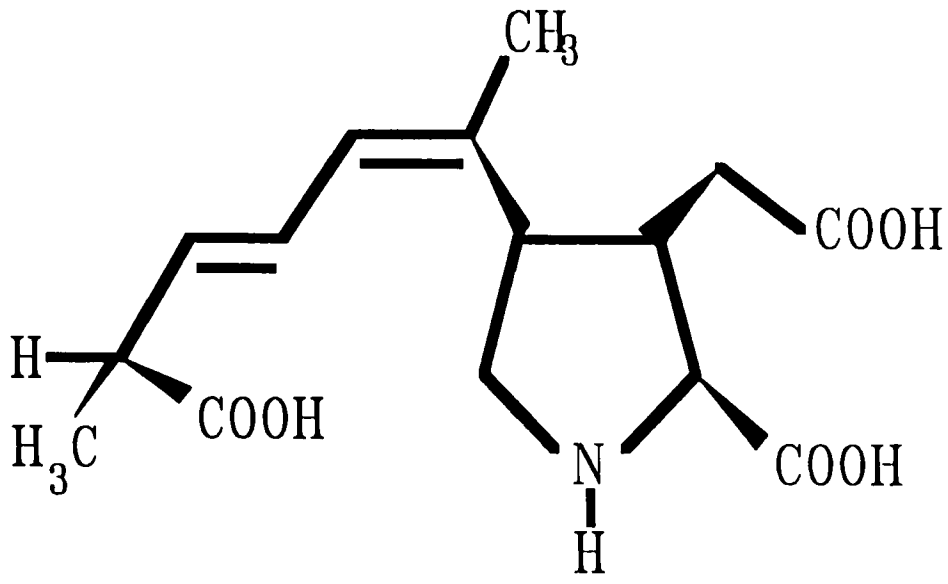

Figure 1 Chemical structure of domoic acid.

In the Quilliam method, following extraction, the extract was purified before HPLC injection. Purification is done by eluting the extract through a strong-anion exchange resin-filled cartridge. This multi-step, procedure is laborious, and, in our laboratory, caused losses of analyte ranging from 5 to 15%. Furthermore, the presence of high NaCl concentrations, such as those found in salted seafood samples, prevents use of this purification technique.

The present paper describes a novel approach to DA analysis in aqueous methanol extracts, which avoids the need for sample cleanup prior to HPLC analysis, and which retains its effectiveness in matrices containing elevated salt levels. In the method, a column switching technique is utilized which provides a clean-up procedure for removal of matrix interferences[13] and which protects the analytical column from rapid deterioration. This technique uses a precolumn, a switching valve and an analytical column, in series. After venting to waste most of the sample matrix, DA is transferred to the analytical column for further chromatographic separation and quantitation. We report here the chromatographic efficiency, analytical precision, accuracy, detection limit, the influence of potential interferents, and speed of analysis for the new procedure.

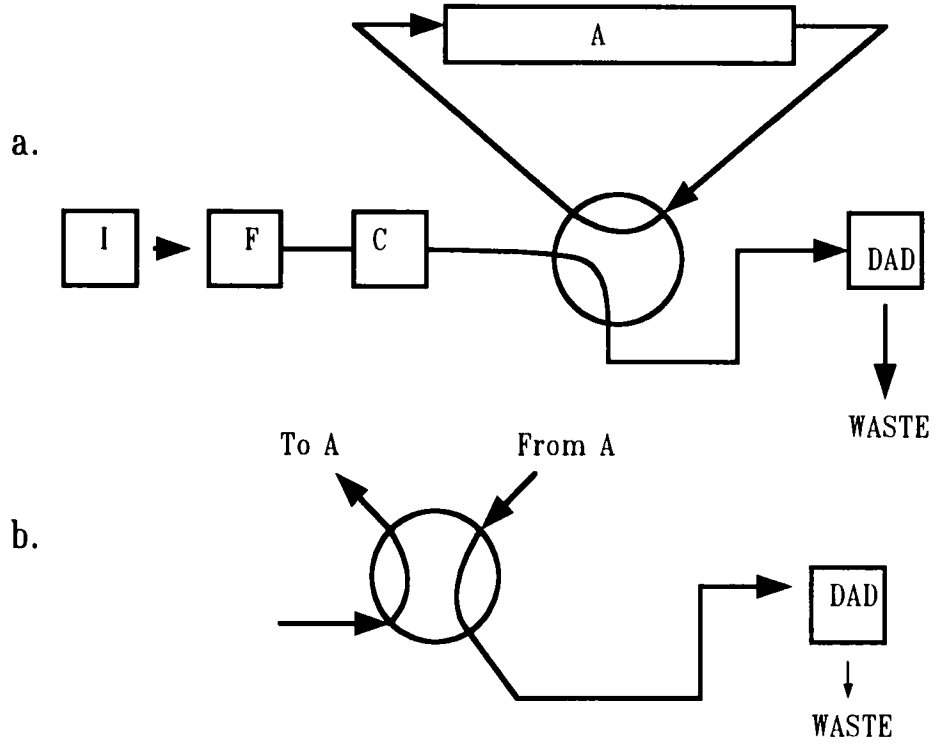

Figure 2 Schematic representation of analysis train, (a) valve in the bypass position for sample loading, and (b) valve directs sample through A. I, F, C, A, and DAD denote injector, filter, pre-column cartridge, analytical column, and diode array detector, respectively.

EXPERIMENTAL

Equipment

A Hewlett-Packard (H-P) Model 1090 Liquid Chromatograph, equipped with an H-P diode array detector, PV5 solvent delivery system, autoinjector and an H-P 79994 A "Chem Station" for data acquisition are utilized. The precolumn, a Brownlee cyano cartridge (10 cm × 4.6 mm × 5 μm), and analytical column, Spherisorb ODS2 (25 cm × 4.6 mm × 5 μm) were obtained from Rainin Instrument Co., Inc., Emeryville, CA. The on-line apparatus (Figure 2) consists of a 0.45 μm frit from H-P and a Rheodyne Model 7000, high pressure, 2-position, 6-port switching valve.

The centrifuge is a Model Cu-5000, Damon International Equipment Co., and we use a Tekmar homogenizer, Tekmar Company, Cincinnati, Ohio.

Materials

All solvents are HPLC-grade, and, together with the 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) as well as MUS-1 mussel tissue reference material (98 μg DA/g tissue) were obtained from NRC Marine Analytical Chemistry Standards Program (MASP), Halifax, Nova Scotia, Canada (B34321).

Sample extraction

The aqueous methanol extraction described by Quilliam[7] is used. Briefly, 4.0 g of tissue sample are accurately weighed into a 50 ml centrifuge tube, 8 ml methanol added and made to a total volume of 20 ml with distilled water. The mixture is homogenized thoroughly using a high speed blender for 3 minutes at 70% of full scale voltage. After centrifugation at 2000 rpm for ten min, approximately 2 ml of the top layer is filtered through a 0.45 μm Millex syringe filter. Since 4 g of tissue, with a density close to 1.0, are diluted to 20 ml, the solution concentration of DA in the extract is approximately one fifth of that in the tissue sample. The work described here employed the extract without further dilution. However, preliminary trials suggest that a factor of two to three dilution before analysis may be beneficial when analyzing tissues containing elevated salt and/or fat concentrations (e.g., anchovies). Aqueous methanol extracts of tissue samples, when filtered through 0.45 μm syringe filters, were quite stable and no losses were found over one week when refrigerated.

HPLC analysis

The gradient elution is programmed from 0.0% to 12.5% acetonitrile in water adjusted to pH 2.5 with orthophosphoric acid (230 μl/l) at a flow rate of 1.0 ml/min. The gradient is linear for 20 minutes, beginning with sample injection. After an additional 20 minutes at fixed concentration, the eluent is programmed back to the initial conditions over a 5 minute period, thus resulting in a 45-minute cycle. Absorbance for quantitation is acquired at 242 nm with a 10 nm bandwidth. The UV spectrum data for confirmation of peak identification is scanned from 220 to 300 nm. Column temperature is 30°C.

Working standard solutions of DA from 0.04 to 80 μg/ml, equivalent to tissue concentrations of 0.2 to 400 μg/g, were prepared in 10% acetonitrile in water solution from a DACS-1 calibration solution. A 50 μl aliquot of the extract was injected by means of an autosampler, with the valve switched to bypass the analytical column. Three minutes after injection, the analytical column was placed on-line by switching the valve.

RESULTS AND DISCUSSION

Separation

Figure 3A shows the resolution of a tissue sample using only the precolumn, under the HPLC conditions described. Domoic acid is seen to elute at 5 minutes, following most of the matrix

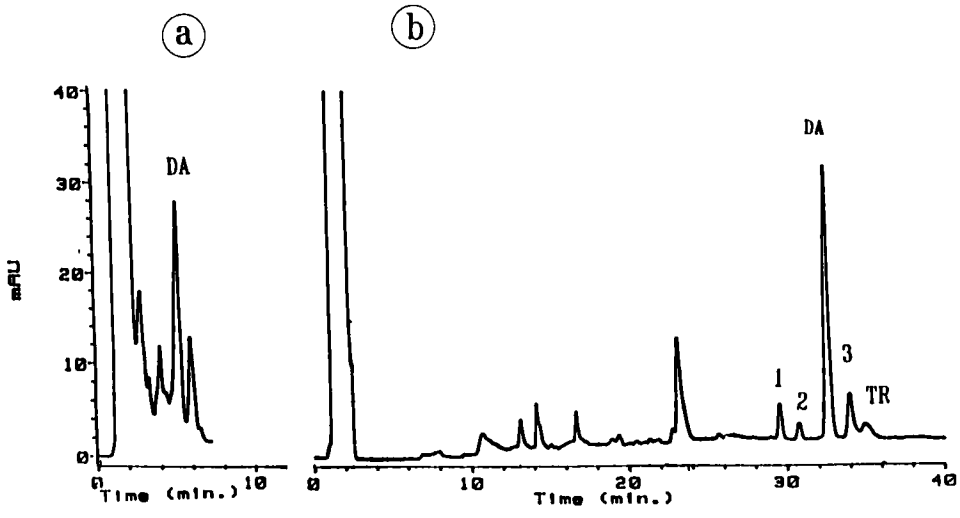

Figure 3 Chromatograms obtained using HPLC separation and UV detection of domoic acid (DA), its isomers, and tryptophan (TR) in extract from mussel tissue containing 20 μg/g DA (a) using cyano cartridge only; (b) using cyano cartridge plus analytical column. DA isomers 1,2,3 represent isodomoic D, isodomoic acid E, and domoic acid diastereomer, respectively.

showing absorbance at 242 nm. Figure 3B shows the resolution when a 50 μL injection of the same extract was analyzed using the precolumn and switching the eluent from waste to the analytical column 3 minutes after injection. Resolution of DA and its isomers from one another and from the matrix was greatly improved, resulting in sharp and symmetric peaks. Tryptophan (TR) and/or its related oxidation products, which can elute close to or simultaneously with DA on some columns, elutes about 2 minutes after DA.

Peak confirmation

Figure 4 is a chromatogram of a crab tissue extract with a DA concentration of 0.5 μg/g. The insert shows the UV spectrum of DA and TR in the extract. Confirmation was based on retention time, spectral match and peak purity. By making the determination of the UV spectrum of DA from 200 to 300 nm a routine part of the analysis, confusion with TR is made very unlikely[12].

Method evaluation

The calibration curve, i.e., peak area vs. amount of DA, was linear over four orders of magnitude ranging from 0.5 ng/injection to 20 μg/injection, with a linear correlation coefficient of 0.996. Results of precision and recovery determinations are presented in Table 1. The recoveries of DA from tissue extracts blended with extracts from MUS-1 to provide concentrations corresponding to tissues concentrations between 0.2 and 98 μg DA/g ranged from 89% to 100%. The precision, obtained from 6 replicate injections of each extract was

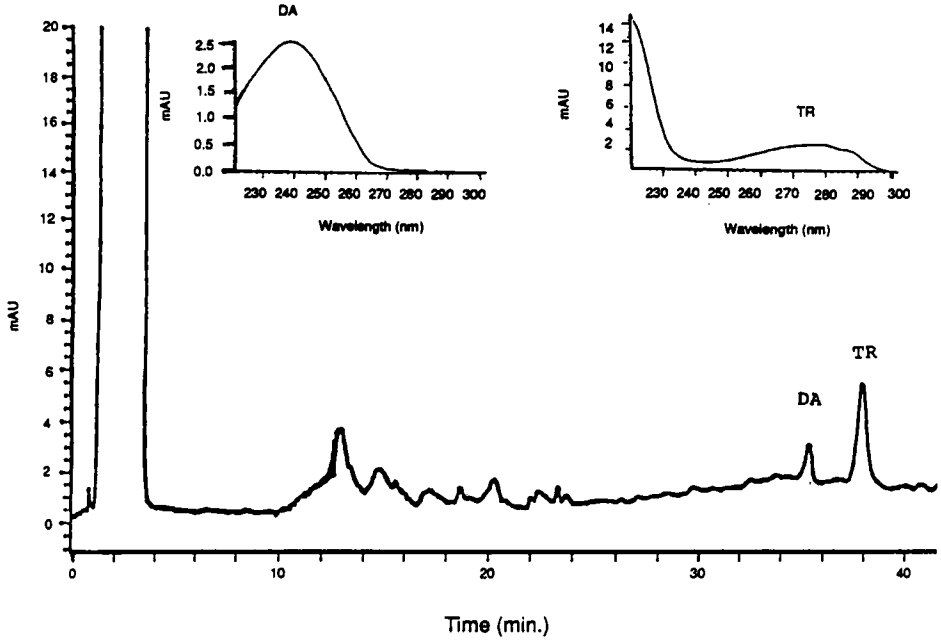

Figure 4 Chromatogram of extract from crab tissue containing 0.5 μg/g domoic acid, with UV spectra of domoic acid (DA) and tryptophan (TR).

excellent, with coefficients of variation 7.2% at 0.2 μg/g, 5.3% at 1.0 μg/g and 2.1% at 50.0 μg/g. Crabs, anchovies, razor clams and oyster extracts also gave recoveries of 93% to 97% at the 1.0 μg/g level. The detection limit, based on the 3:1 signal to noise ratio criterion, was 0.1 μg DA/g of tissue sample, or 1.0 ng/50 μl injection.

Effect of NaCl on DA recovery

Extraction of a 4 g sample of salted anchovies, composited from two lots, yielded an aqueous methanol solution which, when analyzed by flame photometry, yielded 0.75M Na^+ This was

Table 1 Recovery and precision results for HPLC analysis of domoic acid

Matrix	*Number of Injections*	*DA Concentration μg/g*	*Recovery (%)*	*Coefficient of Variation (%)*
Mussel	6	0.2	89	7.2
	6	1.0	92	5.3
	6	20	96	4.2
	6	50	97	2.1
Crab	4	1.0	96	3.1
Anchovies	6	1.0	97	5.2
Razor clam	7	1.0	93	4.9
Oyster	5	1.0	95	6.3
MUS-1	5	100	100	3.1

assumed to represent the concentration of NaCl. Therefore, concentrations of 0.375 M, 0.75 M, and 1.50 M NaCl were added to a standard solution of DA (20 μg/g), and subjected to DA analysis by the method described above. The measured DA concentration remained unchanged, i.e., with a recovery of 100 ± 4%. Similarly, the recovery of DA spiked at 20 μg/g into the salted anchovy sample extract was > 98%, Further, recovery of DA in the anchovy sample extract was unaffected even when the salt level was increased to 1.5M.

Application

We found that late eluting interferences in the methanol extracts of anchovies and crabs suppressed the analyte peak while using gradient elution without the column switching technique. No problem with late-eluting interferences was observed with the column switching technique. When automated, we expect that thirty seafood samples can be analyzed in a 24-hr period of unattended operation. Further, the precolumn cartridge and the analytical column showed no sign of degradation even after analyzing 100 seafood samples.

CONCLUSION

Aqueous methanol extraction of tissue samples, followed by HPLC analysis using a precolumn, switching valve, and analytical column, with UV detection, has been demonstrated to provide an accurate, precise, and sensitive procedure for the determination of DA in seafood. The procedure is especially advantageous when tissue samples contain elevated salt concentrations.

Acknowledgment

The authors wish to thank Debra Wadford for preparing the samples for this study, R. Danielson for drawing the figures, and M. G. Volz for his thoughtful review of the manuscript.

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