A ROUTINE HPLC FLUORESCENCE METHOD FOR THE DETERMINATION OF THE DIARRHETIC SHELLFISH TOXINS OKADAIC ACID AND DTX-1 IN SHELLFISH

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Inspection Branch
Department of Fisheries and Oceans
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An improved HPLC fluorescence (Ex. 249 nm Em. 407 nm) procedure based on the method of Lee (1) is described for the routine determination of diarrhetic shellfish toxins (DSP). Okadaic acid (OA) and dinophysistoxin-1 (DTX-1) are extracted into methanol from the digestive glands of mussels, clams or scallops, extracted with hexane, partitioned into chloroform and taken to dryness under nitrogen. A portion of the residue is reacted with commercial 9-anthryldiazomethane (ADAM) reagent and the ADAM derivatives cleaned up on a silica cartridge. The derivatives are chromatographed on a Superspher 100 RP-18 (3u, 12.5 cm x 2 mm) column with an acetonitrile:water (70:30) to acetonitrile gradient flowing at 0.5 mL/min.. Standard curves were linear over the range of concentrations tested. The limits of detection are 0.15 ng or 0.03 µg/g in the digestive glands. Recoveries of samples spiked with 1.3 µg/g OA and 0.17 µg/g DTX-1 gave recoveries of 114% and 99 % respectively. Comparisons of silica cleanup columns from various manufactures showed a variation in recoveries of OA from 49.4 % to 104 %. A test to establish the reactivity of the prepared ADAM reagent is also presented.

RESUME

J.M. van de Riet, B.G. Burns, and M.W. Gilgan. 1995. A Routine HPLC- Fluorescence Method for the Determination of the Diarrhetic Shellfish Toxins Okadaic Acid and DTX-1 in Shellfish. Can. Tech. Rep. Fish. Aquat. Sci. 1985: vii+ 26 p.

On décrit une méthode améliorée de CLHP avec fluorescence (Ex. 249 nm, Em. 407 nm) basée sur la méthode de Lee (1) pour le dosage de routine des toxines diarrhéiques de coquillages. L'acide okadaïque (AO) et la dinophysistoxine-1 (DTX-1) sont extraits dans le méthanol à partir des glandes digestives des moules, des palourdes ou des pétoncles, puis extraits avec de l'hexane, partagés dans du chloroforme et amenés à siccité sous atmosphère d'azote. On fait réagir une partie des résidus avec du 9-anthryldiazométhane (ADAM) commercial et les dérivés ADAM sont épurés sur une cartouche de silice. Le dosage des dérivés se fait par chromatographie sur une colonne Superspher 100 RP-18 (3µ, 12,5 cm x 2 mm) avec un gradient acétonitrile:eau (70;30) à acétonitrile, à raison de 0,5 mL/min. Les courbes étalons se sont révélées linéaires pour l'intervalle des concentrations testées. Le seuil de détection est de 0,15 ng ou 0,03 µg/g dans les glandes digestives. Des échantillons dopés avec 1,3 µg AO/g et 0,17 µg DTX-1/g

ont donné respectivement des taux de récupération de 114 % et de 99 %. En comparant des colonnes d'épuration sur silice provenant de divers fabricants, on a constaté que le taux de récupération de l'AO variait de 49,4 % à 104 %. On présente également un test pour déterminer la réactivité du réactif ADAM préparé.

1. INTRODUCTION

The dinophysis toxins are close analogues of okadaic acid (OA) or OA-esters and, with OA, are what are usually considered to be the active agents of Diarrhetic Shellfish Poison (DSP). Okadaic acid is a lipid-soluble polyether fatty acid produced by the several sources, including the dinoflagellates *Dinophysis fortii* and *Prorocentrum lima* (2). The symptoms of DSP poisoning appear in individuals after consumption of shellfish which have been feeding on phytoplankton containing the diarrhetic toxins. The symptoms usually appear between four and twelve hours after consumption and include diarrhea, nausea, vomiting, abdominal pain and chills. The symptoms are usually gone within three days. However, the dinophysis toxins have been shown to be potent tumour promoters (3,4) hence even minor outbreaks are thus of substantial health concern.

Okadaic Acid and dinophysis toxin-1 (DTX-1), are the most common of the diarrhea causing toxins. They represent a serious threat to human health as well as posing a significant economic hardship to shellfish growers and harvesters, as harvesting of shellfish is banned during the intoxication period. Incidents of DSP poisonings have been noted worldwide since their first identification in 1978 (5) but are most prevalent in Europe and Japan where there is extensive shellfish culturing.

The Canadian Department of Fisheries and Oceans has been monitoring shellfish from coastal regions for many years for the presence of PSP using the mouse bioassay as the main detection and measurement method. This monitoring was increased in intensity and scope in 1988 to include monitoring for domoic acid determined by HPLC/UV. Concerns that DSP toxins were afflicting consumers without detection lead to an experimental program where concentrated organic extracts of shellfish digestive glands in aqueous suspension were subjected to the mouse bioassay. This program lead to the detection of a DTX-1 intoxication of mussels which was subsequently confirmed by LC/MS (6). No okadaic acid was detected during this intoxication. Since the presence of DSP was established in Atlantic Canada, the almost universally used Lee *et al.* (1) procedure for the HPLC/fluorescence estimation of the dinophysis toxins was implemented. This report is basically a description of the modifications made to that procedure to improve its reliability and utility in a routine monitoring laboratory.

Several biological and chemical methods have been used to detect and quantify OA and DTX-1. The mouse bioassay, using the procedures similar for PSP assay, has been used to detect the presence of DSP toxins (5,7,8), but is most effective when used as a screening procedure since it lacks specificity. It also suffers from insensitivity, is laborious in the extract preparation and is slow, requiring 24 hrs. for the full response. Large amounts of sample and solvents are required for estimations of the DSP toxins. In addition, the use of live animals in routine testing is generally to be avoided if possible.

A more specific and sensitive bioassay involves the use of ligated loops from rat intestines (9). Its advantage is that it uses the main symptom of the human toxicity as

the detection mechanism; the destruction of the intestinal mucosa. However, it requires the use of surgery and anaethesia, not common analytical laboratory skills, as well as live animals. The short term (<24 hrs.) cytotoxicity of mussel extracts to cell cultures (10) has been promoted as a viable alternative to the mouse bioassay. This technique requires the capability to maintain and manipulate cell cultures reliably, as well as being comparatively slow. DSP toxins may also be evaluated through the use of commercial immunoassay kits that are sensitive to OA and to a lesser extent DTX-1 (11,12). The primary advantages of the immunoassay are the simplicity of sample preparation, high specificity and speed with modest quantitation precision. The drawbacks of these kits are their very high cost for routine use and the lack of ability to detect DTX-1 as effectively as OA, when DTX-1 in the main toxin of concern in this area. The DSP toxins can be detected by their ability to enhance some phosphatase activities. The primary disadvantage of this technique is that it involves the maintenance of several enzyme systems and their assays which would again involve techniques not often found in a routine laboratory. The most desirable method, but also by far the most costly and technically most complicated, is the use of a LC/MS with an ion-spray, atmospheric pressure interface to detect and quantify OA and DTX-1 (6, 13). However, many routine laboratories lack the technical resource to run and maintain such as system.

The Lee procedure (1) employing HPLC separation and fluorometric detection of the 9-anthryldiazomethane (ADAM) derivatives has been used with modifications by a number of laboratories for analytical testing (6,14,15,16). This method would appear to offer many advantages such as good sensitivity, small sample requirement, rapid turnaround time and the use of equipment generally available in most analytical laboratories, while obviating the need for live animal testing. With various modifications to the original Lee procedure to improve toxin recovery, to permit better control over the synthesis of the fluorescent derivative and the HPLC of the derivatives, we have developed a method for routine monitoring of shellfish for OA and DTX-1. The method, while still fairly complicated, appears to be reliable and overcomes many of the original pitfalls of the method such as poor reproducibility and separation, and the instability of the ADAM reagent.

2. MATERIALS AND APPARATUS

2.1 EQUIPMENT

2.1.1 HPLC System:

Pump system: Must be able to generate reliable, rapid binary gradients. This was achieved by a HP Model 1050 Ti series quaternary pump system controlled through a Dell 433/l personal computer (PC) with HP software.

Sample Injection: Must be able to accurately inject 1-10 µL of sample. This was achieved by a HP Model 1050 Ti series autosampler controlled by the PC.

Column Oven: HP air bath column oven Model 1050 Ti series stabilized at 35°C.

Monitor: Shimadzu Model RF551 fluorescence HPLC monitor with the excitation wavelength set at 249 nm and the emission wavelength set at 407 nm. Sensitivity was set at low with the gain at 1X.

Data Processing: The system should be able to use either peak height or peak area for integration. The system used a HP 35900 interface coupled to the Dell PC and a HP Deskjet 500 printer. The system also included HP data storage and manipulation systems and a monitor.

Column: Superspher 100 RP-18, 3u, 12.5 cm x 2 mm ID used without a guard column.

Alternate column #1:
Lichrospher 100 RP-18, 5u, 25 cm x 4 mm, used without a guard column.

Alternate column #2:
Vydac 210 TP54, 25 cm x 4.6 mm ID, C18, 5u, used without a guard column.

2.1.2 Homogenizers and Mixers:

Brinkmann Polytron with PT10 and PT07 generators.
Vortex Genie mixer.

2.1.3 Fluorimeter:

Turner model 111

2.1.4 Centrifuge:

International model HN-S with six arm swing head rotor set at approximately 1500 RPM.

2.1.5 Glassware:

16 x 100 mm screw capped disposable tubes
15 mL graduated centrifuge tubes
1.5 mL amber screw topped autosampler vials
500 µL amber crimp capped tapered microvials

2.1.6 Vacuum Manifold:

Supelco Visiprep DL 12 port

2.1.7 Silica Cartridges:

Waters Sep-Pak Plus

2.1.8 Silica columns:

J&W Scientific
Chromo-Sep
Supelco
J.T. Baker Ltd.
Waters/Millipore

2.1.9 DSP Check Kit

Sceti Co. Ltd.
Minami-Aoyama, Minato-Ku, Tokoyo 107
Akasaka P.O. Box No. 24
Tokyo, Japan

2.2 REAGENTS:

2.2.1 Solvents:

Water glass distilled, acetonitrile and methanol HPLC grade, all other solvents ACS grade.

2.2.2 Okadaic acid certified reference standard OACS-1:

National Research Council, Halifax, N.S..

2.2.3 Okadaic Acid (OA):

Diagnostic Chemicals Ltd., Charlottetown, P.E.I.

2.2.3.1 Stock standard:

25 µg of standard diluted to 250 µL with methanol.

2.2.3.2 Working standard (5 ng/µL):

10 µL of stock diluted to 200 µL with methanol.

2.2.4 Hyodeoxycholic Acid (HDC):

Internal standard, B-D Laboratories Inc., New York, NY.

2.2.4.1 Stock standard (0.1 mg/mL0.0):

10 g dissolved in 95% ethanol and diluted to the mark in a 100 mL volumetric.

2.2.4.2 Working standard (5 ng/µL):

0.5 mL of stock was diluted to 10 mL with 95% ethanol.

2.2.5 Prorocentrum lima Extract Stock Solution (3500 µg OA/mL, 520 µg DTX1/mL):

The solution was a concentrated extract from laboratory cultured Prorocentrum lima provided to us in 95% ethanol by T. deFrietas of the Institute of Marine Biology/ National Research Council. The solution contained both OA and DTX-1 and was standardized for OA against the certified reference standard OACS-1 . DTX-1, for the purposes of quantitation is considered to generate an equivalent response to OA (M. Quilliam, personal communication). Therefore, response factors generated for OA were also used for DTX-1.

2.2.5.1 Prorocentrum lima Extract Working Solution (7.61 µg OA/mL):

10 µL of Prorocentrum lima stock solution was diluted to 4.6 mL with methanol.

2.2.6 9-Anthryldiazomethane (ADAM):

Molecular Probes, Eugene, Oregon.

2.2.6.1 ADAM Stock:

1 mg portions of ADAM were placed into brown vials and stored frozen in a desiccator.

2.2.6.2 ADAM Working Reagent (1 mg/mL):

The required number of vials are removed from the desiccator and methanol added to each.

2.2.7 Chloroform/Hexane (1:1):

Equal volumes of chloroform and hexane are mixed.

2.2.8 Chloroform/Methanol (95:5):

10 mL of methanol were added to 190 mL of chloroform.

2.2.9 Mobile Phases:

2.2.9.1 Superspher column:

Solvent A: Acetonitrile:water (70:30)
Solvent B: Acetonitrile

2.2.9.2 Lichrospher column:

Solvent A: Acetonitrile
Solvent B: Water

2.2.9.3 Vydac column:

70% acetonitrile in water.

3. METHOD

3.1 SAMPLE PREPARATION:

The soft tissue of the shellfish was excised, drained briefly, and the digestive glands separated out. In order to obtain a representative sample 20 g or more of digestive glands were accumulated. The glands were blended thoroughly with a Polytron homogenizer and stored in a sealable plastic cup. If the sample was not to be processed immediately, it was stored in a freezer at -20°C.

3.2 SAMPLE EXTRACTION:

One gram of the digestive gland puree was measured into a 16 x 100 mL glass culture tube with a Teflon lined screw cap. The sample was blended with 4.0 mL of methanol with a Polytron homogenizer (ca. 30 sec at 1/2 to 3/4 max rpm with the PT07 generator) and centrifuged (ca. 5 min. at 3/4 max. in an International HN-S).

An aliquot (2.5 mL) of the methanollic extract was measured into a 15 mL graduated centrifuge tube. Water (0.5 mL glass distilled) was added. The mixture was extracted with hexane (2 times with 2.5 mL) mixing the phases thoroughly with a vortex mixer (ca. 20 passes) and centrifuging to clear the phases. The hexane was discarded.

Additional water (1.0 mL glass distilled) and chloroform (4.0 mL) was added to the above methanollic phase, mixed thoroughly with the vortex mixer and centrifuged to clear the phases as above. The chloroform phase was transferred to a 16 x 100 mm glass culture tube with a Teflon lined screw cap and the extraction repeated (without further addition of water). The combined chloroform layers were evaporated to dryness and then the residue was redissolved in methanol (0.5 mL).

3.3 FLUORESCENCE REACTION:

3.3.1 Preparation of ADAM solution (0.1%):

The storage desiccator containing the 1 mg samples of ADAM reagent was removed from the freezer and allowed to come to room temperature before being opened. One vial (containing 1 mg) was required for every 4 reactions to be done (including standards, blanks and samples). The required number of vials were removed and the desiccator was returned to the freezer. Methanol (1.0 mL) was added to each of the reagent vials and the vial mixed. The diluted reagent was made fresh before each batch of reactions.

3.3.1.1 ADAM Reagent Verification:

An aliquot (50 µL) of ADAM reagent was added to a solution of chloroform-5% glacial acetic acid (5 mL). The mixture was allowed to react for 10 minutes. An aliquot (50 µL) was diluted with chloroform (5 mL), the solution vortexed and allowed to sit for two minutes. Blanks were prepared by adding ADAM reagent (50 µL) to chloroform (5 mL), allowing them to react for ten minutes and then by diluting as above. The diluted solutions were read on the filter Fluorimeter using the 1X scale and a 7-60 excitation filter and a emission filter combination of 2-A and 47-B. (excitation approx. 350 nm: emission approx. 440 nm).

3.3.2 Reaction of standards:

An aliquot (10 µL) of the P. lima extract working standard was added to an amber (1.5 mL) autosampler vial containing internal standard (10 mL HDC working standard) and evaporated to dryness under a steam of nitrogen in a N-evaporator with a water bath temperature of less than 40°C. ADAM reagent (200 µL)was added, the vial capped tightly, mixed and allowed to react in the dark for one hour at room temperature.

3.3.3 Reaction of samples:

An aliquot of extract (0.1 g equivalent of digestive glands) was transferred into each of two amber vials as above. Internal standard (10 µL) was added to both and P. lima extract working standard (10 µL) was added to one vial as a spike. The samples were evaporated to dryness and reacted with ADAM reagent as above. Reagent blanks containing only HDC were carried with each set of samples.

3.4 CLEANUP OF DERIVATIZED SAMPLES:

The silica Sep-Pak Plus cartridges were conditioned on the vacuum manifold by washing with methanol (4 mL), chloroform (4 mL), and chloroform/hexane (1:1, 2 mL). The flow rates through the cartridges was set between 5 and 10 mL per minute. The methanol was evaporated from the standards, blanks, samples and samples plus spikes with a nitrogen jet, as above, after the reaction has been completed. The residue was quickly transferred with chloroform/hexane (1:1, 2 x 0.5 mL) to a conditioned cartridge. The cartridge was washed with chloroform/hexane (1:1, 5 mL) and chloroform (5 mL) and the esters eluted from the column with chloroform/methanol (95:5, 5 mL) into tapered centrifuge tubes (15 mL). The eluate was evaporated to dryness with nitrogen as above and redissolved in methanol (100 µL). The sample was transferred to an amber, 500 µL, tapered microvials and the vial capped with a Teflon lined crimp cap.

3.5 CHROMATOGRAPHIC CONDITIONS:

3.5.1 Routine HPLC (Superspher):

The HPLC system was stabilized at 35°C with 100% solvent A flowing through the column at 0.5 mL/min.. The initial conditions were maintained for one minute after injection then a gradient was started from 100% solvent A to 66.7% solvent B over 6 minutes, held at 66.7% for 2.9 minutes. A gradient to 100% B over 0.4 minutes was begun while increasing the flow to 0.8 mL/min. over the same time period. These conditions were held for 8.7 minutes then returned to 100% A and 0.5 mL/min. flow over 2 minutes, ending the run at 21 minutes.

3.5.1.1 Analysis:

The system was standardized for peak areas and retention times by at least two repeat injections (5 µL) of P. lima extract working standard. Standardization at other levels was required if different columns , conditions or equipment was used.

Aliquots (5 µL) of each sample, spiked sample or blank were injected onto the HPLC column. Peaks were identified by comparison of retention times with the most recently run standards, directly or through the use of relative retention times using the internal standard, HDC.

3.5.1.2 Standard curve:

Graded volumes (1, 5, 10, and 20 µL) of P. lima extract or OA working standard were used to prepare a standard curve. Repeat injections (5 µL) appeared to be acceptable for a single point standardization.

3.5.2 HPLC Alternate Column #1: Lichrospher

3.5.2.1 Isocratic Conditions:

The column was stabilized at 35°C with 80% acetonitrile flowing through the column at 1.1 mL/min.. The run time was 45 minutes.

3.5.2.2 Gradient Conditions:

The column was stabilized at 35°C with 75% acetonitrile flowing through the column at 1.1 mL/min.. These conditions were held for 10 minutes after injection, then increased to 90% acetonitrile at 30 minutes, held for six minutes then returned back to 75% acetonitrile over two minutes. The column was restabilized over seven minutes for a run time of forty five minutes.

For analysis aliquots (10 µL) of each standard, sample, spiked sample, and blank were injected. The other conditions were as above.

3.5.3 HPLC Alternate Column #2: Vydac

3.5.3.1 Isocratic Conditions :

The column was stabilized at 35°C with 70% acetonitrile at 1.1 mL/min.. Run time was forty five minutes.

The analytical conditions were as for the Lichrospher.

3.6 CALCULATIONS:

To determine the total μg of DTX-1 or OA in the sample:

$$\frac{PA}{PAS} \times D = \text{total } \mu g \text{ DTX-1 or OA in the sample}$$

Where PA = peak area/5 μL injection
PAS = peak area per μg DTX-1 or OA standard
D = dilution factor

The concentration of DTX-1 or OA is normally expressed on a wet weight basis, such as μg/g. To obtain this value simply divide the total DTX-1 or OA by the weight of the original tissue extracted (W) in grams. When 5 μL of the final sample extract is injected the dilution factor 'D' above is 200.

$$\mu g \text{ DTX-1 or OA/g} = \frac{PA \times 200}{PAS \times W}$$

The value obtained is corrected for recovery using the HDC internal standard.

3.7 CONFIRMATION WITH OA ANTIBODIES

Samples that have been found to contain a significant level of DTX-1 or Okadaic Acid by HPLC fluorescence were qualitatively and, crudely quantitatively, confirmed using the UBE DSP Check Kit. Since the DSP- Check Kit antibody has only about 1/5th the affinity for DTX-1 as for OA (17), for which the kit was designed, confirmation of samples which apparently only contain DTX-1 must be performed both with a concentrated tissue extract as well as with the tissue extract prescribed by the kit procedure. The concentrated extract used normally has been at least 6 times as concentrated as the normal kit extract.

The kit function is based on a competitive binding of an OA-specific monoclonal antibody, tagged with horse-radish peroxidase, for OA-BSA bound to the test wells and for OA or DTX-1 analyte in the solution. The degree of binding of the tagged antibody

to the OA-BSA in the wells, and hence the relative lack of analyte in solutions, is indicated by the intensity of the colour formed by the action of the horse-radish peroxidase; the more bindable analyte present, the less colour formed.

A sample of the suspect digestive-gland puree was extracted according to the kit procedure (1g / 6 mL 90% methanol /water and subsequently diluted with an equal volume of water). In addition an aliquot of the final extract fraction from the analytical procedure was transferred to a disposable screw-capped culture tube and evaporated to dryness. The residue was dissolved in sufficient methanol/water (45% v/v) to make 1.0 mL of the solution equivalent to 1.00 g of digestive gland. This concentrated extract as well as the extract prepared according to the UBE Kit procedure was then used for the test.

To perform the test, samples of the analyte extracts in 45% methanol/water are mixed with enzyme-tagged antibody. After a period of time the solutions were removed from the wells and the wells rinsed. The color was then developed by the addition of a chromogen solution to each well and the intensity of the colour reaction compared with authentic OA standards and blanks; the more intense the colour the lower the amount of OA (or DTX-1) present. The whole procedure requires 30-45 minutes if the extract concentrate is already available.

3.8 EVALUATION OF SILICA COLUMNS

To test the suitability of various Silica columns from different commercial sources, reacted samples of the *P. lima* extract standard were loaded onto the various columns (see above and Table 3), The conditioning, wash and elution steps remained the same as those for the Sep-Pak cartridges, except for the fact that the washing solvents were both collected, evaporated and analysed by HPLC. The elution solvent was collected in fractions (2 mL) to determine if the elution profile of the columns were different from that of the Sep-Pak. Each 2 mL fraction of the eluate was collected, evaporated and analysed by HPLC as above.

4. RESULTS AND DISCUSSION

OA and DTX-1 are easily eluted and completely separated from interfering materials in less than 10 minutes from the Superspher column (Figure 1). The solvent system provides adequate resolution and minor interferences can be removed by slight changes in the gradient. Retention times are very stable, typically varying less than 0.5% during an overnight run with the detection limit being approximately 0.15 ng (equivalent to 0.03 μg/g in the digestive gland). Lee *et al.* (1) reported the lowest measurable concentrations for OA and DTX-1 in digestive glands to be 0.4 μg/g. The ten-fold increase in sensitivity with the present method may be partially explained by our use of the excitation wavelength of 249 nm and the emission wavelength of 407 nm, as suggested by M.A. Quilliam (17), and to the use of the shorter, narrow bore Superspher column. The Superspher column has proved to be quite stable and has shown little change in response over a several-month period of heavy usage. Similar results have been obtained with both the Vydac and Lichrospher columns using isocratic elution systems (Figures 2 and 3), but with lower resolutions and more interferences in difficult samples.

The measurement of peak areas for OA and DTX-1 quantitation was quite reproducible. The percent relative standard deviations of OA and DTX-1 results over a range of standard and sample concentrations as determined on the HPLC by five replicate injections is shown in Table 1. Standard OA varied less than one percent over an order of magnitude while DTX-1 varied from 1.5 to 7.7%. These higher variations can be expected as the system approaches its detection limit. Variations in actual samples of mussel digestive gland extracts that contained DTX-1 at close to the alert level of 1.0 μg DTX-1/g of digestive gland (interim level accepted by Inspection and Health Canada) were quite small, ranging from 1.3 to 1.0% for DTX-1 levels of 0.47 and 0.92 μg/g, respectively. Replicate analysis of mussel digestive gland tissues over a range of DTX-1 concentrations are shown in Table 2. Levels obtained during the first analysis (A), compared with the second (B), gave ratios (A/B) which were or were very close to 1.0. Ratios (A/B) varied from 0.8 to 1.1 for DTX-1 values ranging from 0.04 to 4.7 μg/g . The ratio of 0.8 was observed near the detection limit of 0.03 μg/g while ratios in the more heavily contaminated samples were 0.9 and 1.1 for samples in the range of 4.7 to 2.6 μg DTX-1/g respectively (Table 2). Quantitation using peak heights should also be quite acceptable.

The results of a typical standard curve are shown in Figure 4, with linear responses noted over the range of concentrations tested.

The Superspher column was chosen for routine analysis because it allowed increased sensitivity (0.15 ng (Superspher) *vs* 0.30 ng (Licrospher) and 1.0 mg (Vydac) detection limits), more rapid turnaround time (25 min. *vs* 45 min.) and a substantial reduction in solvent use (0.5 mL/min vs 1.1 mL/min) over both the Lichrospher and Vydac columns. The alternate columns, however, may still prove to be quite useful for confirmations of critical samples or can be used if the small column is not available.

The chromatogram of a mussel digestive gland extract containing no OA and DTX-1 and the same sample spiked with *P. lima* extract at a level of 0.76 µg/g OA and 0.10 µg/g DTX-1 is shown in Figure 5. There were no major contaminating compounds present that interfered with the quantitation of the OA or DTX-1. Similar results were noted for digestive gland extracts of clams and scallops. Some care however, must be taken, on all of the HPLC systems, to ensure interfering materials do not co-chromatograph with the OA or DTX-1. Adjustments to the column oven temperature or solvent conditions may be required to ensure proper chromatography.

Recoveries of OA and DTX-1 from mussel digestive glands, spiked with 1.3 µg/g of OA and 0.17 µg/g DTX-1, using the recommended HPLC method gave values of 114 and 99% respectively after a single methanol extraction. Recoveries of OA and DTX-1 by the Lee Procedure in our hands gave values of 85 and 79% respectively. All of these values compare quite favourably with those reported by Lee *et al.* (1) where recoveries of OA from mussel digestive glands spiked at the 1.0 µg/g level gave a recovery of 96%.

Recoveries of HDC, OA and DTX-1 eluted from columns packed with silica adsorbent as supplied by various manufacturers, compared with the Waters Sep-Pak Plus cartridge are shown in Table 3. Elution conditions closely mimicked those used for the Sep-Pak cartridges. As can be seen there is a considerable variation in the activity of the silica produced by the various manufacturers, with recoveries ranging from a low of 49.4% for OA on the Supelco column to a high of 140% for HDC on the Chromo-Sep column. The Chromo-Sep column appeared to mimic the Sep-Paks most closely, while the Waters/Millipore column, which would have been expected to be very similar, showed the most variation. Care should therefore be exercised when substituting columns or cartridges from different manufacturers or when substituting different products supplied by the same manufacturer. Changes to the elution conditions may be required to obtain the desired results.

One of the most critical stages in the recommended procedure is the preparation and use of the ADAM reagent. The availability of good quality commercial ADAM reagent has removed much of the difficulties experienced in the past with the initial quality of the reagent. However, at the startup of the procedure it is difficult to determine if the reagent is functioning adequately. If stored reagent solutions are used an indicator of the remaining activity is important. For these reasons the test procedure was developed. The results of a test to check the reactivity of freshly prepared 0.1% ADAM reagent over a 24 hr period are shown in Figure 6. The ADAM reagent has the most activity within two hours of preparation, then quickly drops off in activity over the next five hours to approximately one half its original activity. The reagent subsequently appears to remain relatively stable over the next seventeen hours. Lee *et al.* (1) reported that a 0.1% solution of ADAM reagent remained active for one week. It is important to remember, however, that if maximum activity is required that the solution should be prepared fresh just before use.

Overall the results indicate that the OA and DTX-1 extractions and measurements made using the recommended method agree quite favourably with those reported by other researchers (1, 6, 14, 15). It has been demonstrated that OA and DTX-1 can be successfully measured in representative members of the

invertebrate marine species. Although the procedure is relatively lengthy and complicated it provides more than adequate sensitivity for the intended use.

The principal problems experienced in this laboratory with the various versions of the method have mostly been caused by interfering substances. As can be appreciated after examining one of the example chromatograms, the elution patterns are complex and crowded, therefore considerable care and caution must be exercised to avoid misidentifying the toxins. To avoid this we routinely use the UBE DSP check-kit to confirm the presence and approximate quantity of the measured toxin. While the kit is much too costly for routine use, it has proven very valuable as a confirmatory check. Only a few instances of the detection of DTX-1 by HPLC/fluorescence confirmed by the antibody reaction proved to not be confirmable by HPLC/MS. It is suspected that the failures were due to the long time interval between preparing the trace level samples and the eventual HPLC/MS examination. Whenever practical, the results are confirmed though the use of LC/MS.

Prior to the implementation of the HPLC -fluorescence analytical procedure the UBE Kit was adopted to validate the positive results obtained from the mouse bioassay procedure for the detection of the DSP toxins. The bioassay procedure is non-specific and there was need for assurance that the resulting deaths were due to the presence of the DSP toxins and not some other unrelated material.

Upon the institution of the analytical procedure concern was raised as to the possibility of obtaining false positive results due to the complexity of the normal chromatograms. For this reason it was felt that the immunoassay procedure would provide some level of reassurance in the validity of the results. Due to the high cost, the kit was normally utilized to confirm the first incident of detectable levels of DTX-1 or OA at a suspect site. The test kit was also used in cases where the level of toxin was at or near the acceptance level of 1 ug/g in the digestive gland.

Table 4 summarizes the results obtained from the use of the kit and compares them to the data obtained from the analytical procedure. It should be noted that as used the quantitative estimate from the immunoassay was relative to the visual comparison of the colour level of a sample compared to a blank and a low and or a high standard. It can be seen from the table that in most cases the combination of the analytical procedure and the immunotest kit provided suitable assurance that the results were indeed due to the presence of the DSP toxins. However, in the fall of 1994 substantial levels of DTX-1 were found in scallops at a site in Mahone Bay. The combined bioassay, immunoassay and HPLC-Fluorescence assay yielded a positive determination of the presence of DTX-1. Unfortunately, evaluations performed by HPLC/MS (17) determined that no DTX-1 was present. For this reason a confirmatory procedure, which will be reported separately, was developed utilizing an extra cleanup step.

The method has been successfully used to detect several occurrences of DTX-1 intoxication in Eastern Canada, including one serious occurrence which resulted in human poisonings in Newfoundland during the fall of 1993. So far we have detected no OA in this area, although there is anecdotal evidence that other researchers have.

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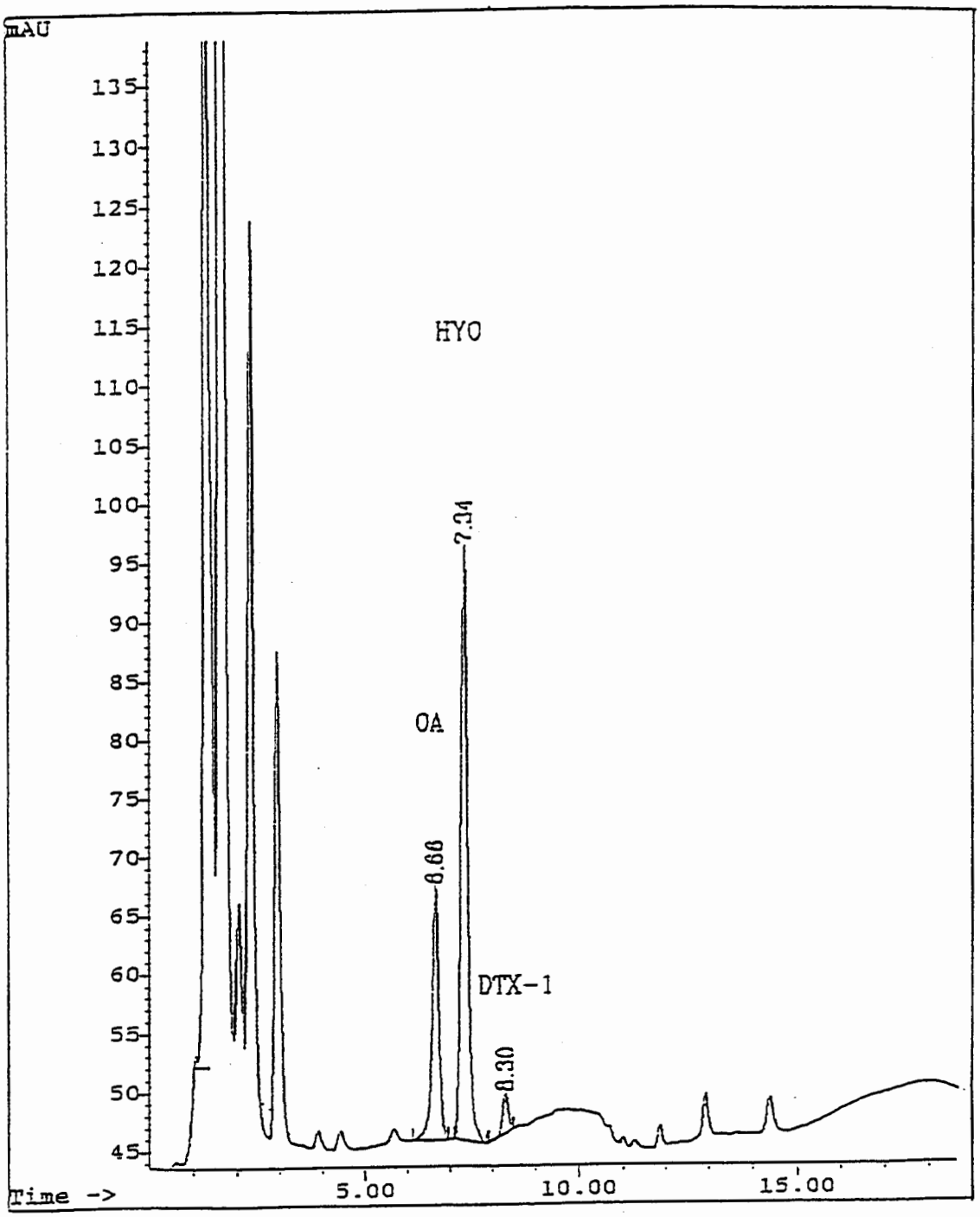

Figure 1. HPLC fluorescence trace (excitation 249 nm, emission 407 nm) of a P. lima working standard (5 µL) run on a Superspher 100 (12.5 cm x 2 mm ID) 3 µ, RP-18 column. Operating conditions , oven temperature 35°C, flow rate 0.5 mL/min.. Program: 70% acetonitrile (1.0 min.) to 90% acetonitrile (6.0 min.) and hold (2.9 min.). Begin a gradient to 100% acetonitrile (0.4 min.) while increasing the flow to 0.8 mL/min., hold (8.7 min), then return to initial conditions (1.0 min.).

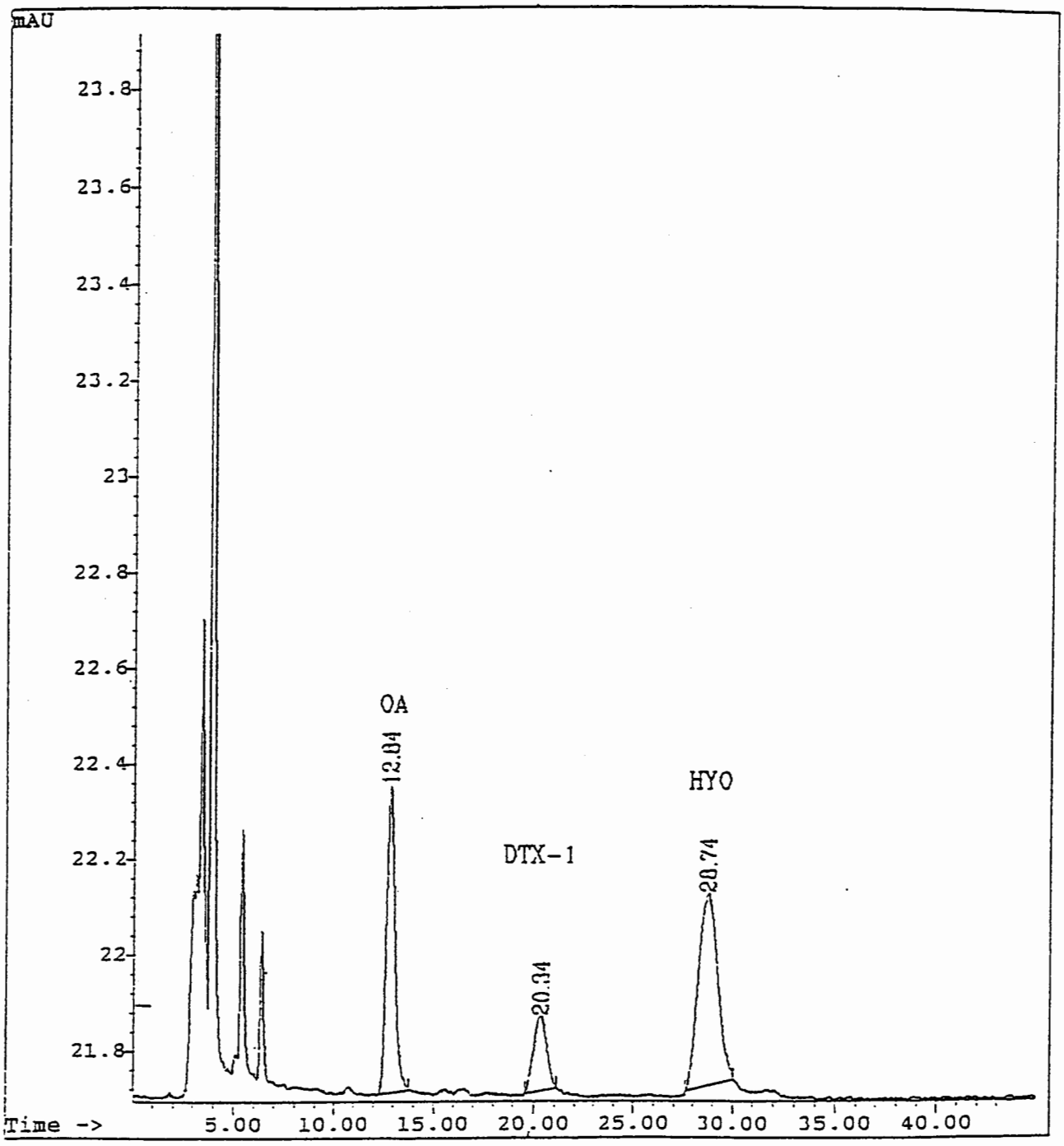

Figure 2. HPLC fluorescence trace (excitation 249 nm, emission 407 nm) of standard OA (10 ng), HDC (10 ng) and a qualitative DTX-1 standard (approximately 5 ng) run on a Vydac 210 TP (25 cm x 4.6 mm ID) 5 μ, reversed column. Operating conditions : flow rate 1.1 mL/minute, column oven 35°C, 70% acetonitrile run isocratically (45 min.).

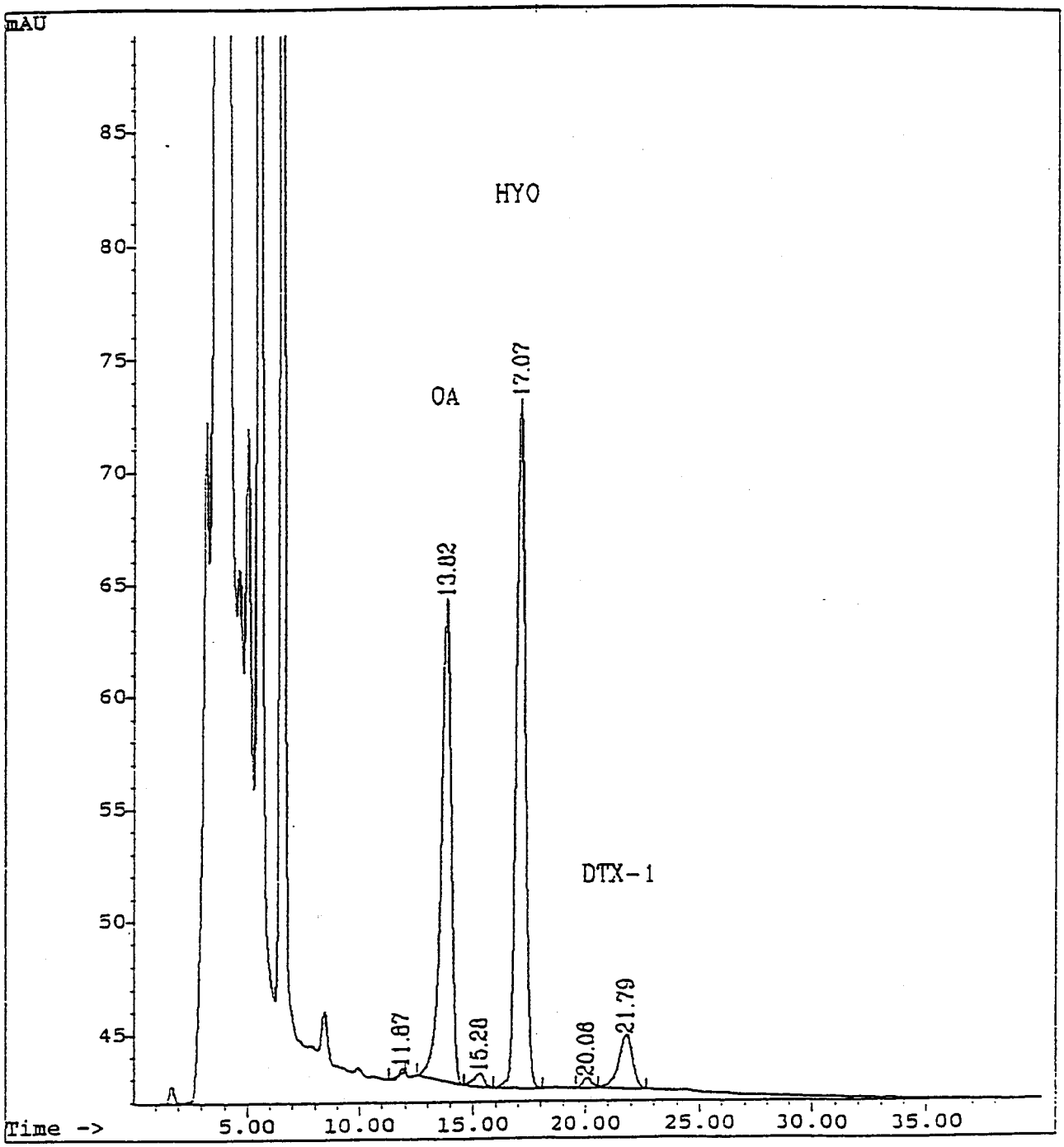

Figure 3 HPLC fluorescence trace (excitation 249 nm, emission 407 nm) of a P. lima standard (10 μL) run on a Lichrospher (25 cm x 4 mm ID) RP-18, 5 μ, reversed phase column. Operating conditions: oven temperature 35°C, flow rate 1.1 mL/min., 80% acetonitrile run isocratically (45 min.).

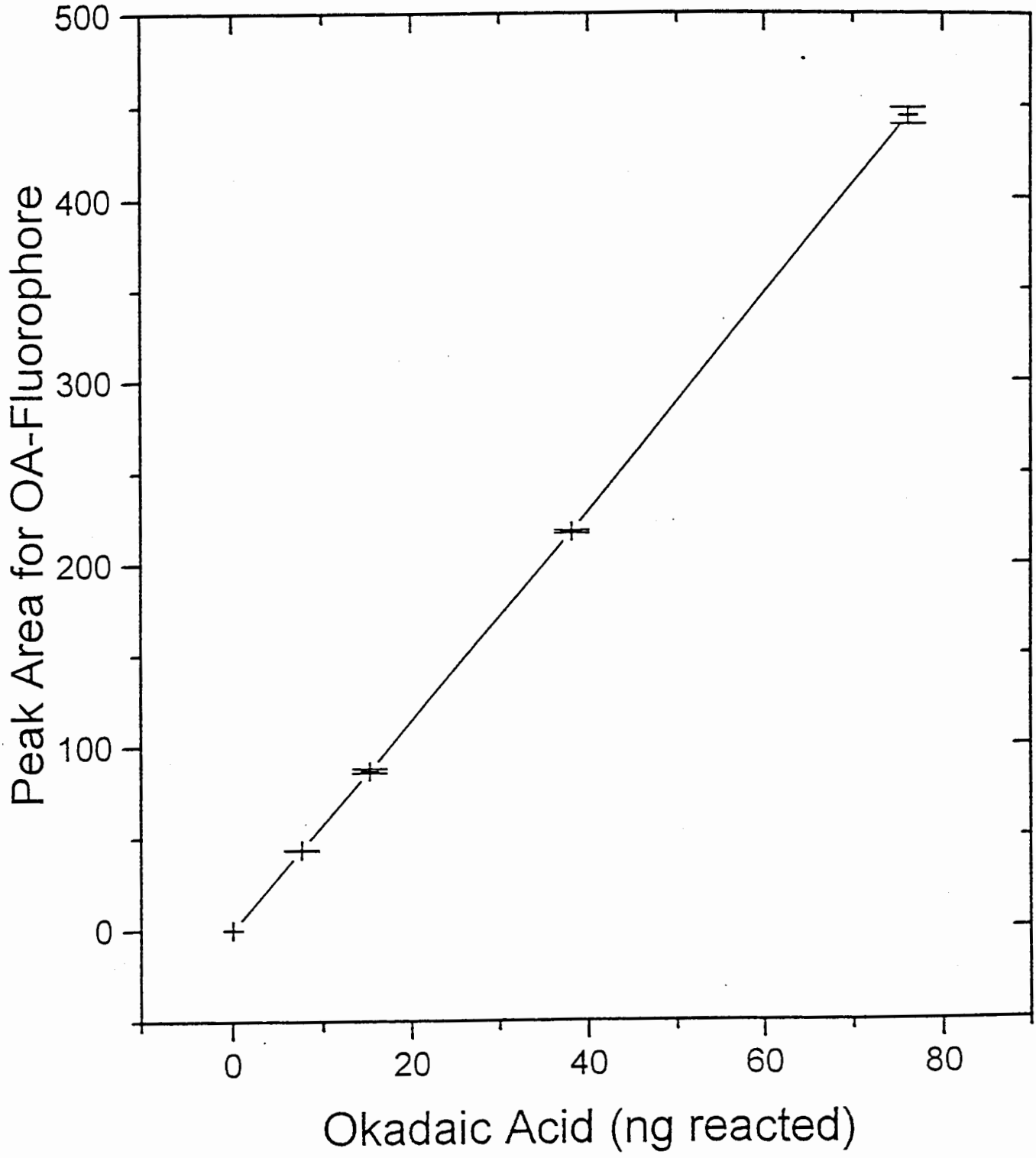

Figure 4: Calibration curve for okadaic acid and DTX-1 run on the Superspher column. Conditions as in Figure 1.

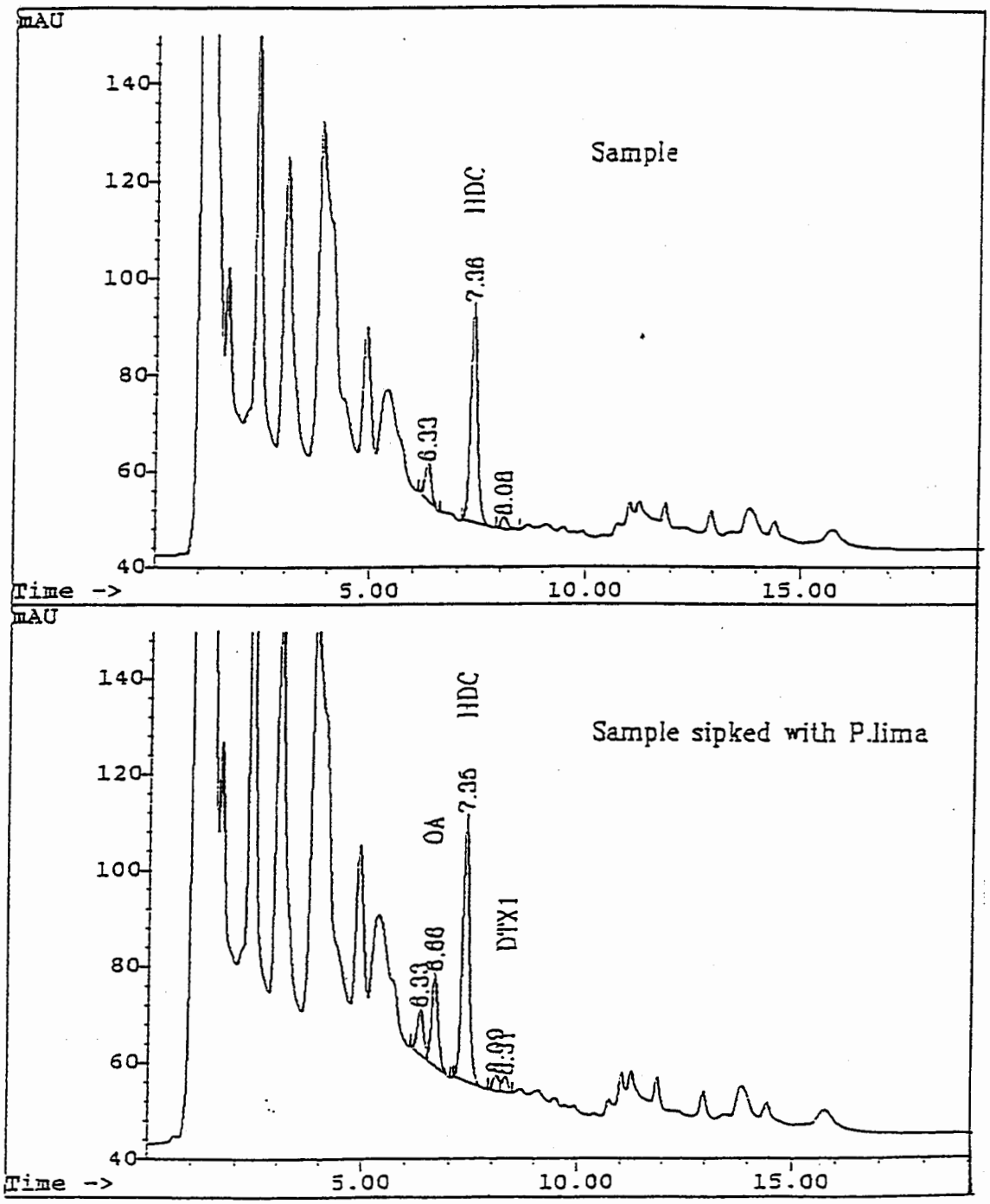

Figure 5: HPLC fluorescence trace of a mussel digestive gland extract containing only the internal standard HDC and the same mussel extract spiked at a level of 0.76 µg/g OA and 0.10 µg/g DTX-1 run on the Superspher 100, RP-18 column. Conditions were as in Figure 1.

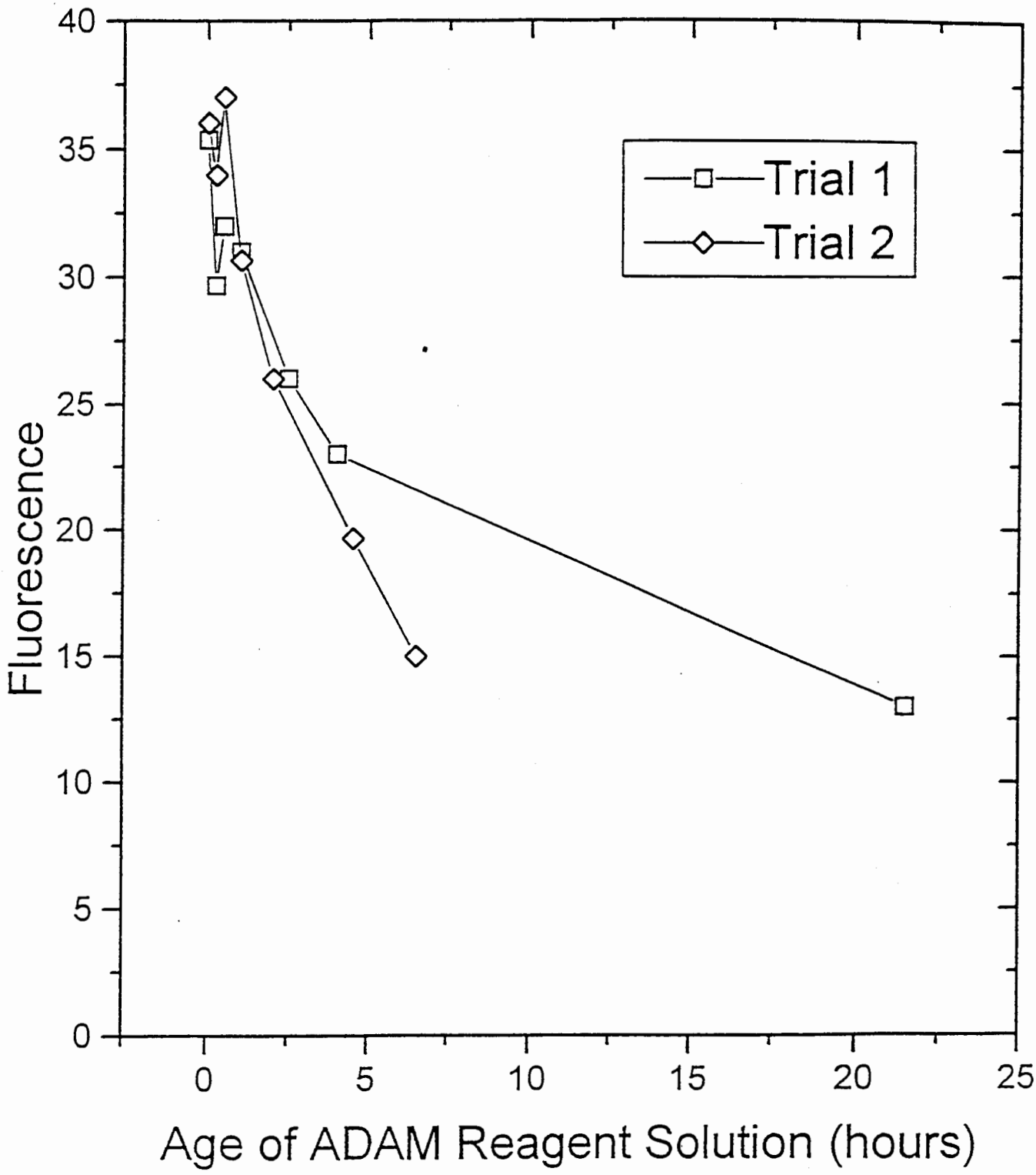

Figure 6. Fluorescence (excitation approximately 350 nm, emission approximately 440 nm) readings versus sampling time showing the activity of the ADAM reagent sampled over a 24 hr period after preparation.

Table 1: The OA and DTX-1 content of replicate injections of various standards and samples determined by the recommended method.

SAMPLE	ESTIMATE [µg/g[a] (%rsd)[b]]	
	OA	DTX-1
Standard #1	7.6 (1.0)	0.99 (7.7)
Standard #2	76.1 (1.0)	9.9 (1.5)
Mussel Digestive Glands #1		0.47 (1.3)
Mussel Digestive Glands #2		0.92 (1.0)

[a] Standard values are in ng/ 5 ul injection
[b] Average of five replicate injections

Table 2: Replicate OA and DTX-1 analysis of mussel digestive gland tissue analyzed by the recommended method over a range of concentrations.

Sample	Estimate (µg/g)				Ratio A/B
	Extract #1 (A)		Extract #2 (B)		
	OA	DTX-1	OA	DTX-1	
1	ND	0.14	ND	0.14	1
2	ND	0.68	ND	0.68	1
3	ND	2.9	ND	2.6	1.1
4	ND	0.09	ND	0.1	0.9
5	ND	4.2	ND	4.7	0.9
6	ND	0.06	ND	0.07	0.9
7	ND	0.04	ND	0.05	0.8

Table 3: Recoveries of OA and DTX-1 on SPE columns from various manufacturers versus the Waters Sep-Pak Plus cartridge.

COLUMN MANUFACTURER	RECOVERIES[a] [Percent (Sd)]		
	HDC	OA	DTX-1
J & W Scientific	100 (17.8)	79.8 (4.70)	78.2 (9.81)
Chromo-Sep	140 (6.08)	103 (16.0)	99.0 (8.21)
Supelco	103 (8.56)	49.4 (19)	56.1 (26.8)
J.T. Baker Ltd.	104[b]	104 (8.98)	83.5 (16.3)[c]
Waters/Millipore	55.8 (56.7)	65.5 (36.9)	88.4 (15.0)[c]

[a] three analyses except as noted
[b] one analysis only
[c] two analyses

Table 4 Comparison of immunotest kit results to the data obtained from the analytical procedure

Species	Date	Sample Site	UBE Kit Results	Analytical Results	
				OA ug/g	DTX-1 ug/g
Mussel	93/05/04	Ship Harbour	++	ND	ND
Mussel	93/05/06	Ship Harbour	-	ND	ND
Mussel	93/05/07	Ship Harbour	-	ND	ND
Mussel	93/05/07	Ship Harbour	-	ND	ND
Scallop	93/05/09	Country Harbour	-	ND	ND
Scallop	93/05/14	Country Harbour	-	ND	ND
Mussel	93/05/09	Country Harbour	-	ND	ND
Mussel	93/05/17	Ship Harbour	-	ND	ND
Scallop	93/06/15	Country Harbour	+	ND	ND
Mussel	93/06/16	Indian Point	+	ND	0.08
Mussel	93/07/06	Martins Point	3+	ND	0.19
Mussel	93/07/20	Ship Harbour	4+	ND	0.06
Clam	93/07/23	Weymouth	+	ND	0.09
Mussel	93/10/24	Alexander Bay	7+	ND	4
Scallop	94/10/03	Graves Shoal	2+	ND	(0.6)ND*
Scallop	94/10/06	Graves Shoal	2+	ND	(0.7)ND*

* The numbers in brackets were the HPLC-Fluorescence estimated of DTX-1 present in the samples by the usual procedure. The ND (not detected) was determined after the special cleanup and confirmation.