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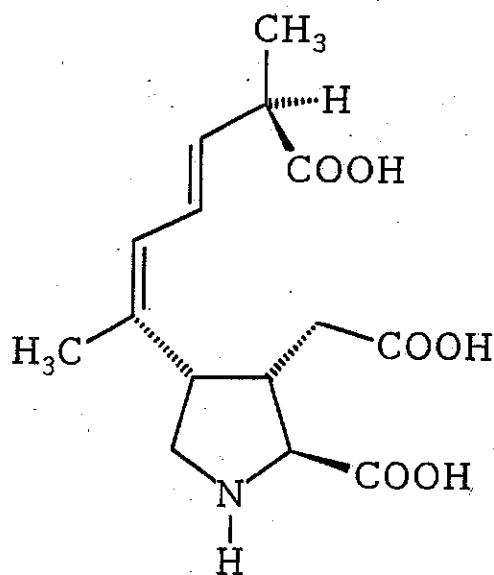
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A Rapid Extraction and Clean-up Procedure for the Determination of Domoic Acid in Tissue Samples[*]

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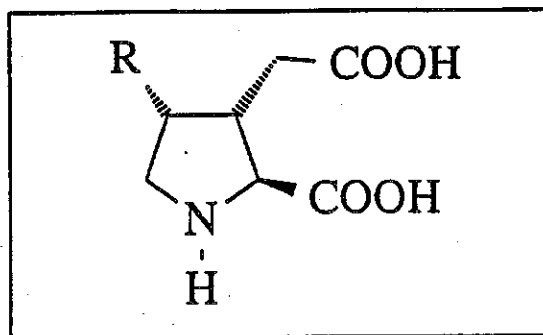[*]NRCC No. 33001

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A. INTRODUCTION

Amnesic Shellfish Poisoning

In late 1987, 153 people suffered from acute intoxication after eating cultured blue mussels (*Mytilus edulis*) harvested from a localized area in eastern Prince Edward Island, Canada (Quilliam *et al.*, 1989b; Perl *et al.*, 1990). Symptoms included nausea and diarrhea, which in some cases were followed by confusion, disorientation, loss of memory and even coma. Three elderly people died and some individuals still have persistent neurological symptoms. The term Amnesic Shellfish Poisoning (ASP) has been proposed for this syndrome. The causative toxic agent was isolated in this laboratory (Bird *et al.*, 1988; Wright *et al.*, 1989) and identified as domoic acid (**1**) (Figure 1), a rare naturally-occurring amino acid (Ohfune *et al.*, 1982). This compound is a member of a group of potent neurotoxic amino acids that act as agonists to glutamate, a neurotransmitter in the central nervous system (Tryphonas *et al.*, 1990a). The phytoplankton species *Nitzschia pungens* f. *multiseries* was subsequently identified as the primary source of **1** in the affected area (Subba Rao *et al.*, 1988; Bates *et al.*, 1989).

In addition to **1**, a number of closely-related compounds have since been detected in toxic mussel samples from the above incident and in laboratory-grown cultures of *N. pungens* (Quilliam *et al.*, 1989a). Three of these compounds, cis-trans isomers of **1**, have been identified as isodomoic acids D, E and F (**5**, **6** and **7**, Figure 1) (Wright *et al.*, 1990a), and a fourth compound, **8**, has been identified as a diastereomer of **1** (Wright, 1991). Other compounds closely-related to **1** have been reported previously in association with **1** in the red alga *Chondria armata* (Maeda *et al.*, 1986, 1987a, 1987b); these include the isodomoic acids A, B and C (**2**, **3** and **4**), nordomoic acid (**9**) and the domoilactones (**10** and **11**) (Figure 1). Toxicity assays have shown that compounds **2** to **8** are also neurotoxins, although of lower potency than **1** (Wright *et al.*, 1990b; Maeda *et al.*, 1986). Therefore, the compounds shown in Figure 1 are members of a family of compounds that can be referred to as ASP toxins, by analogy with the well-known paralytic shellfish poisoning (PSP) toxins.

The widespread occurrence of *Nitzschia spp.* suggests that ASP could be a world-wide problem. Indeed, two recent incidents on the west coast of the United States now support this claim. The first of these (September 1991) involved numerous deaths of pelicans and cormorants near Santa Cruz, California. Through the joint efforts of this laboratory and of the

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Figure 1: Structures of domoic acid and related compounds.

R =

1 Domoic acid

2 Isodomoic A

3 Isodomoic B

4 Isodomoic C
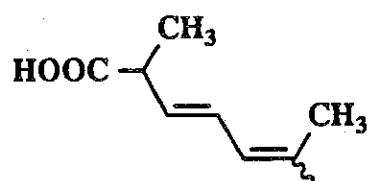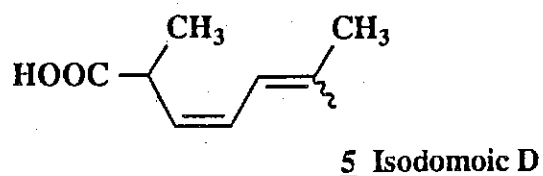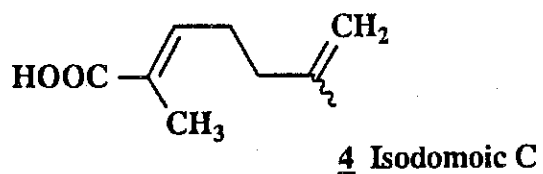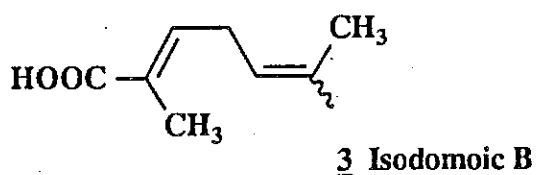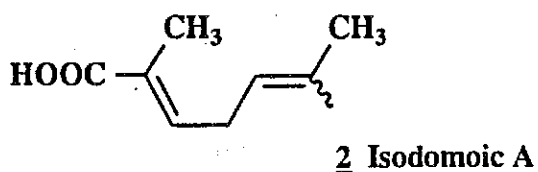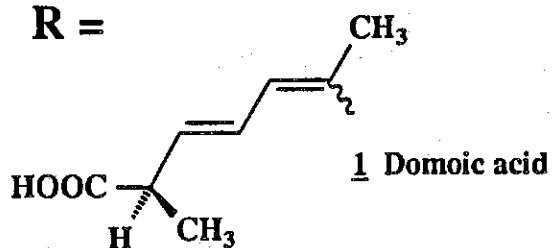
5 Isodomoic D

6 Isodomoic acid E

7 Isodomoic acid F

8 Domoic acid diastereomer

9 Nordomoic acid

10 Domoilactone A

11 Domoilactone B
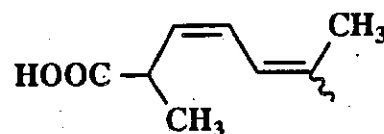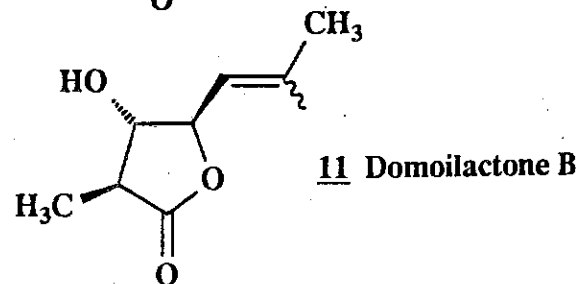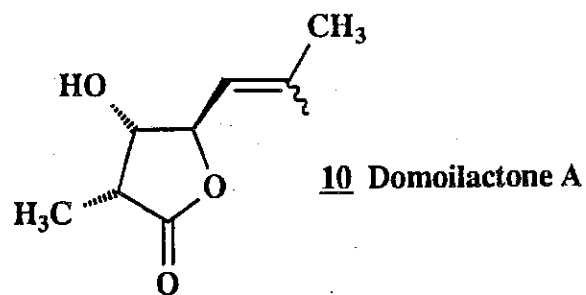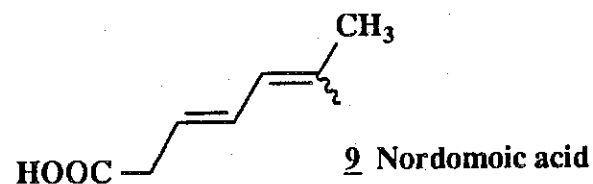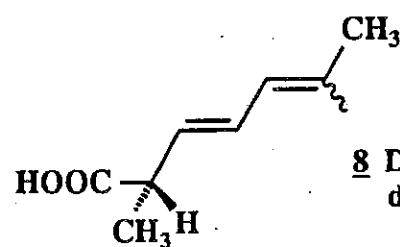
California Department of Fish and Game (Work *et al.*, 1992), the deaths were shown to be due to domoic acid produced by a bloom of the diatom *Nitzschia pseudoseriata*, with anchovies as the intermediate vector. The contamination of herbivorous fin-fish such as anchovies poses a serious potential human health hazard. The second, more recent incident (November 1991), in the states of Washington and Oregon, resulted in several people being affected by ASP after eating razor clams contaminated with domoic acid (Wiskerchen, 1991). After that incident, crabs in the same area were also found to be contaminated (Wiskerchen, 1991). The source of the toxin in these cases has not yet been established.

Analytical Methods

It is clear therefore that reliable analytical methods for the ASP toxins are vitally important for protection of the public. Since the AOAC mouse bioassay, currently used for routine monitoring of shellfish for PSP toxins (AOAC, 1980; Adams *et al.*, 1980), does not provide sufficient sensitivity for detection at the current regulatory level in Canada (20 μg $\underline{1}$ per gram of edible tissue), ASP toxins must be monitored by instrumental methods of analysis.

High-performance liquid chromatography with ultraviolet absorption detection (LC-UVD) is now the preferred analytical technique for the determination of domoic acid in shellfish (Quilliam *et al.*, 1988, 1989a; Lawrence *et al.*, 1989a, 1991) and has been used since 1987 by Canadian regulatory agencies to prevent other incidents of shellfish poisoning. The limit of detection (LOD) of domoic acid in an extract is 60 ng/mL (about 0.3 ng mass detection limit with a 5 μL injection on a 2.1 mm ID column). The LOD in tissue is dependent upon the method of extraction and cleanup, but is only practical at approximately 1 μg/g tissue with all the published sample preparation methods. If regulatory levels are lowered in the future, more sensitive analytical methods will be required. To lower the detection limits appreciably, either extensive pre-concentration methods or more sensitive instrumental methods will be required.

A very sensitive procedure based on FMOC derivatization and LC analysis with fluorescence detection has been developed for monitoring of domoic acid in other marine matrices such as seawater and phytoplankton (Pocklington *et al.*, 1990). This procedure has only recently been adapted to shellfish tissue extracts (Quilliam *et al.*, in preparation).

Confirmation Methods

Despite the implementation of these routine analytical methods in regulatory laboratories there is still a need for confirmatory assays, especially in situations involving legal action. Confirmation is most easily accomplished by acquiring the ultraviolet spectrum of the toxin, either after collection from the HPLC or through the use of a diode array detector (Quilliam *et al.*, 1989a). The UV spectrum of domoic acid has a characteristic 242 nm absorption maximum, due to its conjugated diene system (Falk *et al.*, 1989). It has been shown that tryptophan and some of its derivatives have similar retention times and can interfere in the HPLC-UVD analysis of ASP toxins; however, the UV spectra of tryptophan derivatives are very different from those of ASP toxins, so it is easy to differentiate the compounds using a diode array detector (Quilliam *et al.*, 1989a). Chemical derivatization for HPLC has also been proposed as a method for confirmation of domoic acid (Pocklington *et al.*, 1990; Lawrence *et al.*, 1989b).

During the initial study of the toxin, fast atom bombardment (FAB) mass spectrometry using a direct probe inlet was found to be useful for the qualitative confirmation of domoic acid in concentrated HPLC isolates (Wright *et al.*, 1989; Thibault *et al.*, 1989). We have since reported the development of an analytical procedure based on combined gas chromatography-mass spectrometry for the analysis of ASP toxins in shellfish tissue samples (Pleasance *et al.*, 1990). Although this method is applicable to concentrations of domoic acid in contaminated shellfish ranging from 1 to 500 µg/g wet tissue, its implementation required the development of an extensive clean-up procedure to facilitate derivatization to the *N*-trifluoroacetyl-*O*-silyl derivatives. Since LC-UVD is the method most commonly used for routine regulatory purposes, a combined liquid chromatography-mass spectrometry (LC-MS) method would be most desirable for confirmation of peak identity. We have investigated various LC-MS interfaces for the analysis of ASP toxins (Quilliam *et al.*, in preparation); these include continuous-flow FAB, thermospray, and ion-spray interfaces. In a preliminary communication (Quilliam *et al.*, 1989c), it was shown that ion-spray LC-MS is a very promising method for the analysis of ASP toxins, as well as of other marine toxins. Ion-spray LC-MS gives an LOD similar to that of HPLC-UVD.

Calibration Standards and Reference Materials

An important concern in any analytical method is the availability of accurate calibration standards and reference materials. Since domoic acid is very expensive and difficult to acquire in high purity, the NRC Marine Analytical Chemistry Standards Program (MACSP) developed a calibration standard (DACS-1) with a certified level of domoic acid (89 µg/mL) in acetonitrile/water (1:9) (Hardstaff *et al.*, 1990). Figure 2 shows the HPLC-UVD analysis of this standard. MACSP has also developed a mussel tissue reference material (MUS-1) which was made by blending contaminated mussel tissue with non-toxic tissues and with an equal volume of water, to give an homogenate containing ca. 100 µg/g of domoic acid (Hardstaff *et al.*, 1990). Figures 3 and 4 show the isocratic and gradient elution HPLC-UVD chromatograms, respectively, for the aqueous extract (*vide infra*) of this material. It is useful to note at this time that this material contains several ASP toxins besides domoic acid. Tryptophan (T) is also present in this extract and interferes with the determination of one of the ASP toxins (6) in the isocratic analysis.

Extraction Methods

The extraction method that has been adopted for official first action by the AOAC is the method used for mouse bioassay of PSP toxins (AOAC, 1980), with only slight modifications (Lawrence *et al.*, 1989a, 1991). This procedure involves boiling 100 g of wet shellfish tissue with 100 mL 0.1 N HCl, adjusting the volume to 200 mL and the pH to 3, and then filtering off a portion of the supernatant for HPLC-UVD analysis. This procedure was adopted because it has the advantage of preparing a common extract for screening of both PSP and ASP toxins. One of the major problems with this acidic extraction method for ASP toxin analysis is that it is not suitable for accurate quantitation (e.g., in reference material work) because it is non-volumetric (solids form a substantial part of the final extract) and ASP toxins are partially decomposed during and after the extraction (Quilliam *et al.*, 1989a). Other problems include difficulty with certain matrices (e.g., anchovy tissue tends to form a gel), a long sample preparation time, inability to store the extract (due to rapid decomposition of ASP toxins), and difficulty in the analysis of trace levels of ASP toxins. The AOAC extraction results in an extract with 0.5 g equiv. tissue per mL. Since the extract is aqueous and acidic, it presents no problem with peak broadening due to direct injection into the HPLC (e.g., 20 µL on 4.6 mm ID columns); however,

Figure 2: HPLC-UVD chromatogram resulting from an injection of 4 μL of the DACS-1 domoic acid calibration standard (89 μg/mL; 356 ng injected). Conditions: 250 x 4.6 mm I.D. Vydac 201TP54 column; 1 mL/min 10% acetonitrile and 0.1% TFA in water; column temperature at 40°C; UV detection at 242 nm.
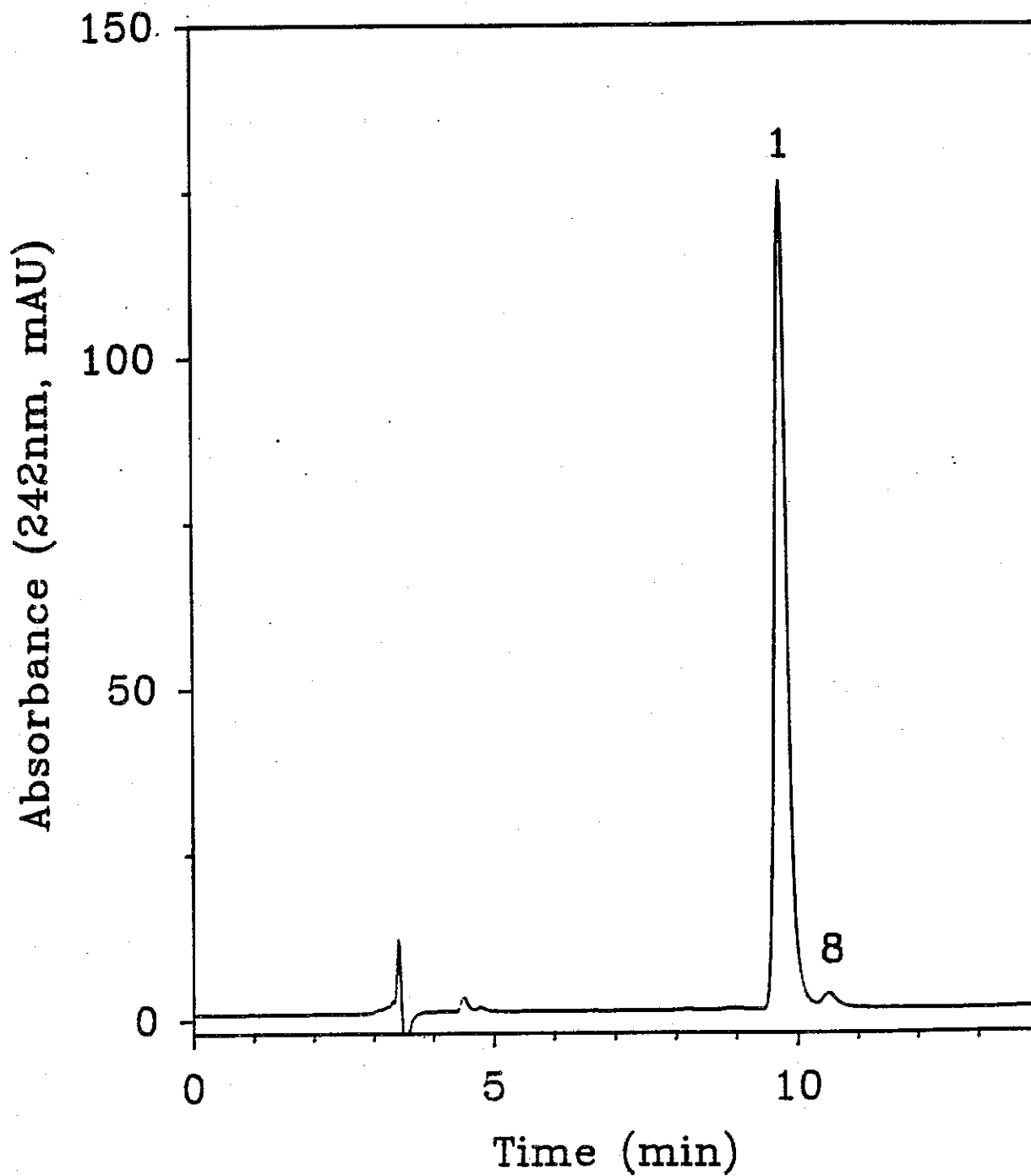
Figure 3: HPLC-UVD chromatogram of the MUS-1 mussel tissue reference material using the standard aqueous extraction and C18-SPE clean-up procedure. Conditions: 250 x 4.6 mm I.D. Vydac 201TP54 column; 1 mL/min 10% acetonitrile and 0.1% TFA in water; column temperature at 40°C; UV detection at 242 nm; 20 μL injection volume.
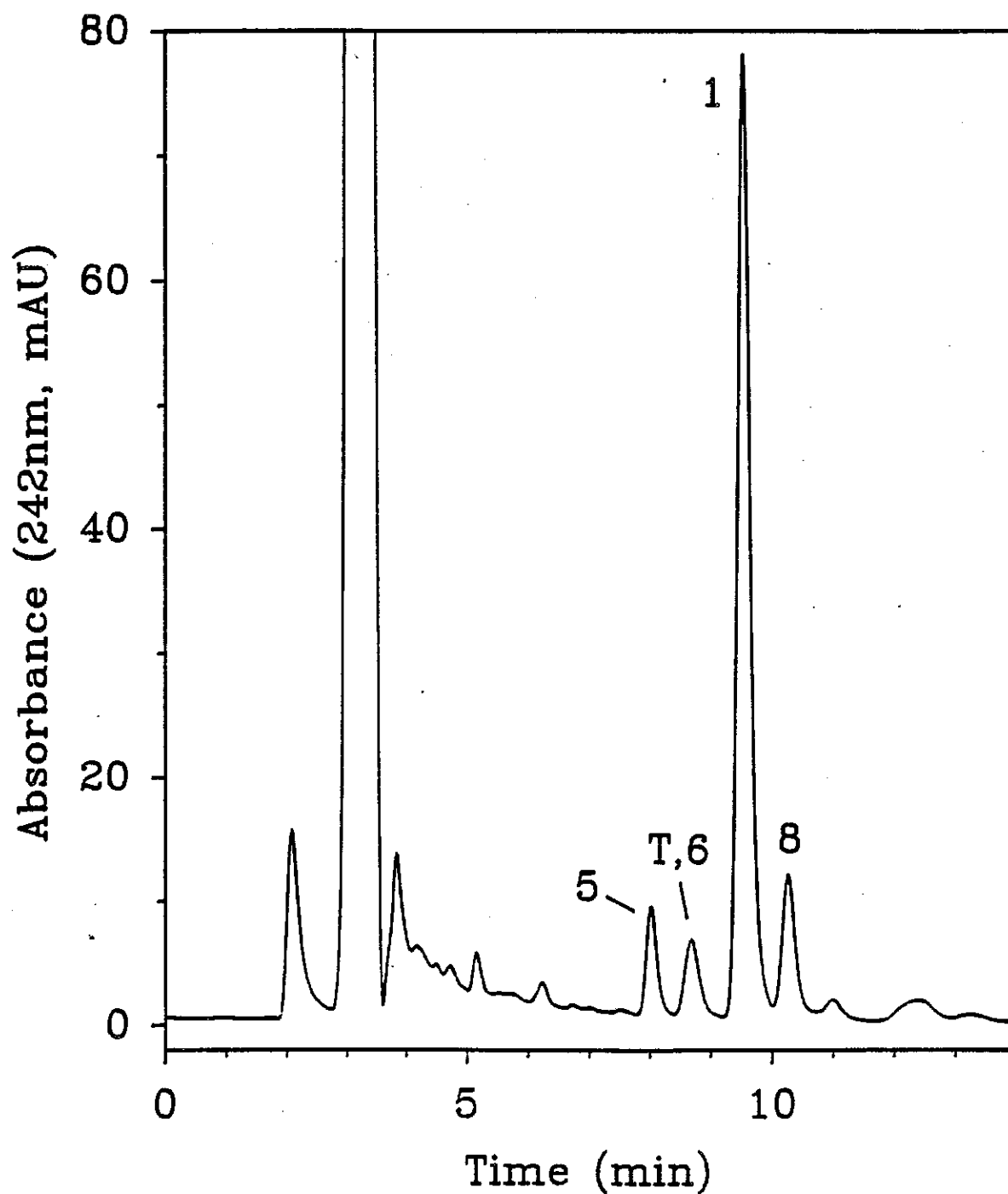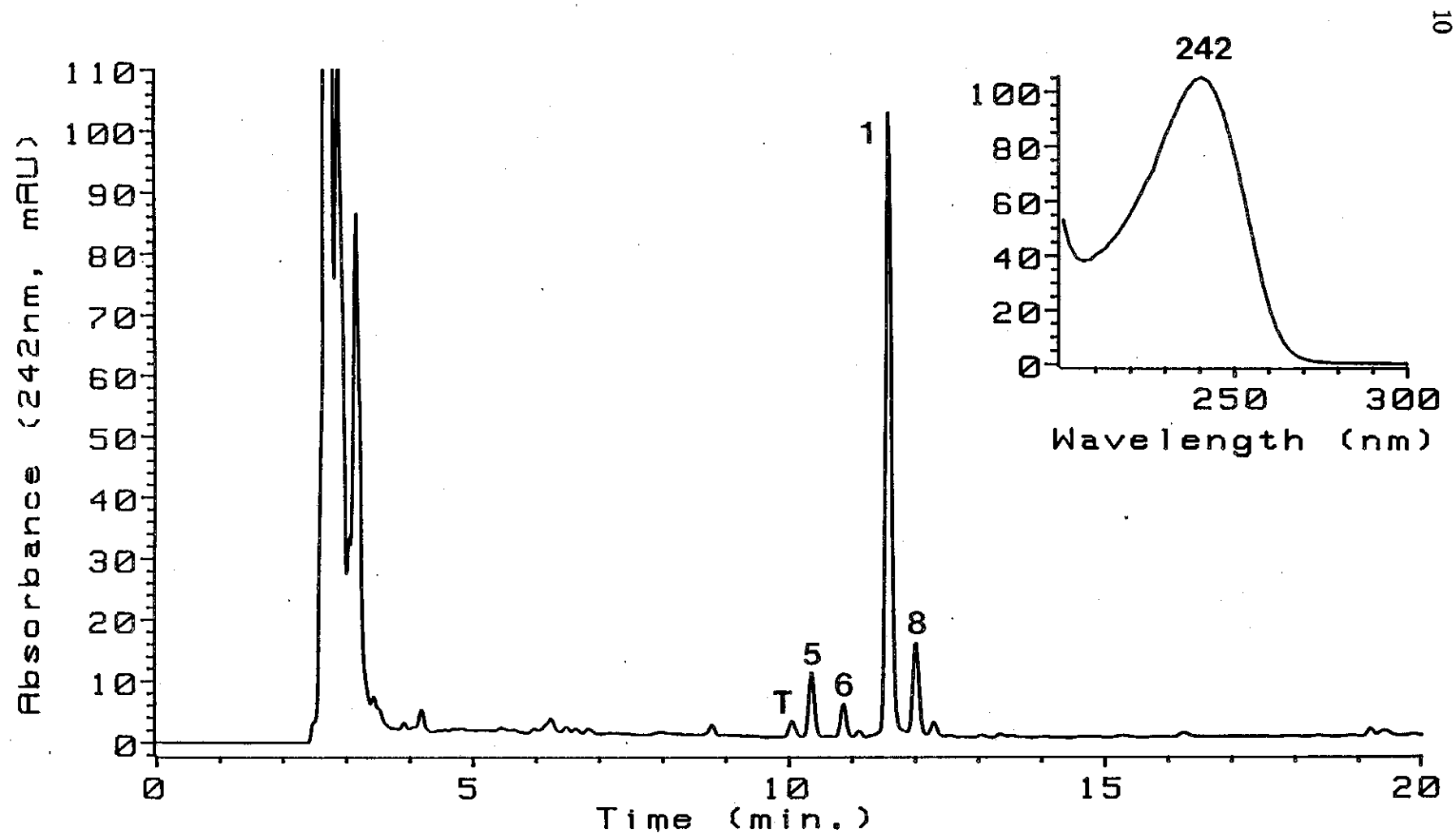
Figure 4: Gradient elution HPLC-DAD chromatogram of the MUS-1 mussel tissue reference material using the standard aqueous extraction and C18-SPE clean-up procedure. The inset shows the UV spectrum of domoic acid aquired by the diode array detector (DAD). Conditions: 250 x 4.6 mm I.D. Vydac 201TP54 column; 1 mL/min acetonitrile/water with 0.1% TFA, programmed from 5% to 25% acetonitrile over 25 min; column temperature at 40°C; UV detection at 242 nm; 20 μL injection volume.

a short column lifetime is very likely due to the lack of clean-up. The limit of detection (LOD) of this method when used with HPLC-UVD is in theory about 0.1 µg **1** per gram of wet tissue, but this is not really practical because the decomposition problem becomes much more severe at trace levels.

An improved extraction procedure based on a boiling aqueous extraction method was developed at IMB (Quilliam *et al.*, 1989a). This method was the one used for the determination of **1** in the MUS-1 reference material as it was found to give the most reproducible and highest recoveries of ASP toxins from the tissues. It is based on a volumetric, exhaustive extraction procedure. However, it is not the most convenient procedure for processing large numbers of samples -- it requires a time-consuming boiling water extraction to help precipitate protein. The procedure also calls for a clean-up by C18 solid phase extraction (SPE) (*vide infra*) prior to HPLC analysis to protect the HPLC column. The procedure extracts 100 g tissue into 250 mL of water (although scale-down is possible), resulting in a crude extract with 0.4 g equiv. tissue per mL of extract. The current C18 SPE method, however, contributes a further dilution factor of 2.5 to the extract instead of concentrating, resulting in a final cleaned extract with 0.16 g equiv. tissue per mL. This results in an LOD of 0.4 µg **1** per gram of wet tissue. The LOD could be much lower if a higher pre-concentration factor could be applied before analysis. However, the boiling water extraction also leads to some losses of ASP toxins at trace levels (Quilliam *et al.*, 1989a).

A third extraction procedure, on which this report focuses, is the aqueous methanol extraction method. This method was first used at IMB during the original 1987 toxic episode (Wright *et al.*, 1989) and was reported in early analytical investigations (Quilliam *et al.*, 1988, 1989a). This procedure has the advantage of being very fast, requires no heating of sample (only a Polytron homogenization), provides good recovery of ASP toxins, and gives a cleaner extract that can be used for direct injection on the HPLC. However, it is not possible to inject very much of this extract on the column without causing appreciable peak broadening (see example later in this report). Also, direct injections of this crude extract will still lead to a reduction in column life. The C18 SPE cleanup (*vide infra*) is not compatible with the extract due to the high percentage of organic solvent.

The extraction is best performed with 4 volumes of 80% aqueous methanol blended with 1 volume of wet tissue, resulting in an extract with 0.2 g equiv tissue/mL. With only 4 µL

injections possible on 4.6 mm ID columns (1 μL with 2.1 mm ID), the LOD is only 0.6 μg/g of tissue. Due to the mild nature of the extraction, the method does have good potential for trace analysis work, but only if it could be coupled with a suitable cleanup procedure that would allow a pre-concentration into a final extract with a low percentage of organic solvent for compatibility with reversed-phase HPLC. The main feature of this report is the development of such a clean-up procedure.

Clean-up Methods

The cleanup method presently used with the aqueous extraction procedure is that developed by Quilliam *et al.* (1989a). An aliquot (2.0 mL) of crude sample extract (pH 6-7, maximum of 10% organic) is loaded onto a C18 SPE cartridge pre-conditioned with 6 mL acetonitrile and 6 mL water. Collection of the eluate begins immediately after application of the sample; 3 mL of 10% acetonitrile/water is used to elute the sample. The final volume is made up to 5.0 mL. There is no attempt to remove the very polar components (including salts) from the extract, as this clean-up is intended only to remove the non-polar components that would elute late in the HPLC run. This method results in a 2.5-fold dilution of the raw extract, but still gives adequate sensitivity for regulatory work. Used with the extraction method described above, the final fraction contains 0.16 g equiv. tissue/mL.

We have been able to operate the C18 cartridge under acidic conditions where domoic acid (but not the more polar metabolites of the toxin) can be enriched on the cartridge and then eluted in a small volume of high % organic solvent. We have found that this is very risky as the breakthrough volumes are quite low (k´ for $\underline{1}$ at pH 2 in 10% ACN is only 3 to 4). In addition, the high % organic solvent in the resulting fraction does not allow a large volume injection on the HPLC.

A more extensive clean-up procedure for ASP toxins was developed for GC-MS work involving chemical derivatization (Pleasance *et al.*, 1990). An aliquot (1.0 mL) of crude aqueous extract is loaded onto a C18 cartridge previously conditioned with 6 mL methanol and 6 mL water. Collection of the eluate begins immediately after application of the sample; 4 mL of 10% methanol/water is used to elute the sample. The 5 mL of C18 eluate is then loaded onto an SCX (strong cation exchange) cartridge previously conditioned with 3 mL aliquots of

methanol, water and 0.1 M formic acid. After washing the cartridge with 5 mL of 0.1 M formic acid, the fraction containing ASP toxins is eluted with 5 mL of 0.1 M ammonium formate into a 5 mL volumetric and adjusted to volume. An aliquot (1 mL) of this solution is evaporated to dryness under vacuum with a SpeedVac concentrator before derivatization. This method also allows the preparation of concentrated extracts for LC-UVD or CE-UVD by simply dissolving the residue into a small volume of water (e.g., 50 µL to get up to a 4-fold pre-concentration factor over the raw extract: 1.6 g equiv. tissue/mL). Since the method is quite time-consuming, it does not lend itself well to routine analysis in a regulatory setting.

B. DEVELOPMENT OF A NEW METHOD

Summary

The objective of this research was to develop and evaluate an extraction and clean-up procedure for the rapid determination of domoic acid in a variety of tissue sample types, particularly at trace levels. We set as a goal that the method should allow a detection limit of below 100 ng/g tissue with HPLC-UVD analysis as the final analytical step.

After examining the different extraction methods that could be used, it was concluded that the fastest and mildest extraction method is the aqueous methanol procedure. This had already been shown to give acceptable extraction yields in earlier experiments (Quilliam *et al.*, 1989a). As discussed below, experiments were performed to determine the optimum percentage of methanol, as well as to determine whether acetonitrile would be a suitable solvent in place of methanol. It quickly became clear that 50% aqueous methanol is the best extracting solvent. Experiments were also designed to determine whether an "exhaustive" extraction was required, or whether a "dispersive" method (single extraction step) could be used. As shown below, it was found that the "dispersive" method is adequate for routine monitoring, but the exhautive method is preferred for the highest level of accuracy.

The main challenge in this project was to develop a clean-up procedure that would be compatible with the aqueous methanol extraction method. After testing different types of solid phase extraction (SPE) cartridges, it was found that a strong anion exchange (SAX) cartridge

gives spectacular performance in sample clean-up. With this technique, a high pre-concentration factor is possible, leading to a detection limit well below 100 ng/g tissue. Interestingly, the SAX cartridge also provides excellent chemical selectivity towards the ASP toxins. The possibilities of interferences by other compounds such as tryptophan derivatives is therefore greatly reduced.

Preparation of Samples

We have found that the Polytron homogenizer is a very effective tool for extraction of tissue samples. However, it is best if the tissues are blended prior to the extraction, in order to break down the large lumps and sinewy tissues. Since it is advisable to sample a fairly large amount of shellfish tissue for representative sampling, we recommend preparing an homogenate of up to 100 g tissue blended with an equal volume of water. Sub-samples can then be withdrawn for replicate extractions. If only small amounts of tissue are available, the extraction can still proceed if the tissue is finely chopped first.

Extraction Solvent

The efficiency of different solvents for the extraction of domoic acid at different levels was tested by preparing homogenates of mussel tissue containing blends of the MUS-1 reference material and control tissue. The blended homogenates contained domoic acid at 10.0, 1.0 and 0.10 µg/g. Since the homogenates were prepared with 50% water, these values represent levels of 20.0, 2.0 and 0.20 µg/g in the original tissue. Table 1 presents the results of analyses of these homogenates with different methods. Raw extracts were analyzed by direct injection into the HPLC. As shown in Figure 5 it was important to keep the injection volume for methanolic extracts at 4 µL to avoid extensive band broadening. Figure 6 shows the analyses of the blend samples with 20 and 2 µg/g domoic acid, as well as the control tissue. Domoic acid at the 2 µg/g level is barely detectable with the raw extract. One of the problems frequently encountered in analysis of raw extracts is shown in Figure 6a. A late-eluting peak from a previous injection has appeared in the chromatogram. Although it is possible to recognize such peaks from their width, analyses must be repeated if such a peak coincides with ASP toxin peaks.

Table 1: Recoveries of domoic acid from blended tissue homogenates.[*]

Domoic Acid Concentration:		20.0 µg/g		2.0 µg/g		0.20 µg/g	
		Result (µg/g) ± s.d.	% Recovery	Result (µg/g) ± s.d.	% Recovery	Result (µg/g) ± s.d.	% Recovery
50% Methanol Exhaustive	Before SAX	19.97 ± 0.48	99.9	-		-	
	After SAX[#]	19.02 ± 0.32	95.1	1.891 ± 0.024	94.6	-	
50% Methanol Dispersive	Before SAX	19.54 ± 0.40	97.7	-		-	
	After SAX[#]	18.64 ± 0.24	93.2	1.858 ± 0.040	92.9	0.186 ± 0.010	93.0
75% Methanol Dispersive	Before SAX	17.07 ± 0.31	85.4	-		-	
	After SAX[#]	15.52 ± 0.60	77.6	1.522 ± 0.044	76.1	-	
50% Methanol Dispersive, with blender	Before SAX	19.21 (n=1)	96.1	-		-	
	After SAX[#]	18.60 (n=1)	93.0	-		-	

[*] Values reported are means and standard deviations (s.d.) of 6 replicate measurements; concentrations are reported in terms of original tissue (without additional water added to homogenate).

[#] SAX procedure: 5.0 mL extract on cartridge; eluted with 2.0 mL citrate buffer.

Figure 5: HPLC-UVD chromatograms resulting from (a) 4 μL and (b) 20 μL injections of a raw methanolic extract of razor clam tissue contaminated with domoic acid at 40 μg/g. Same conditions as in Figure 2.
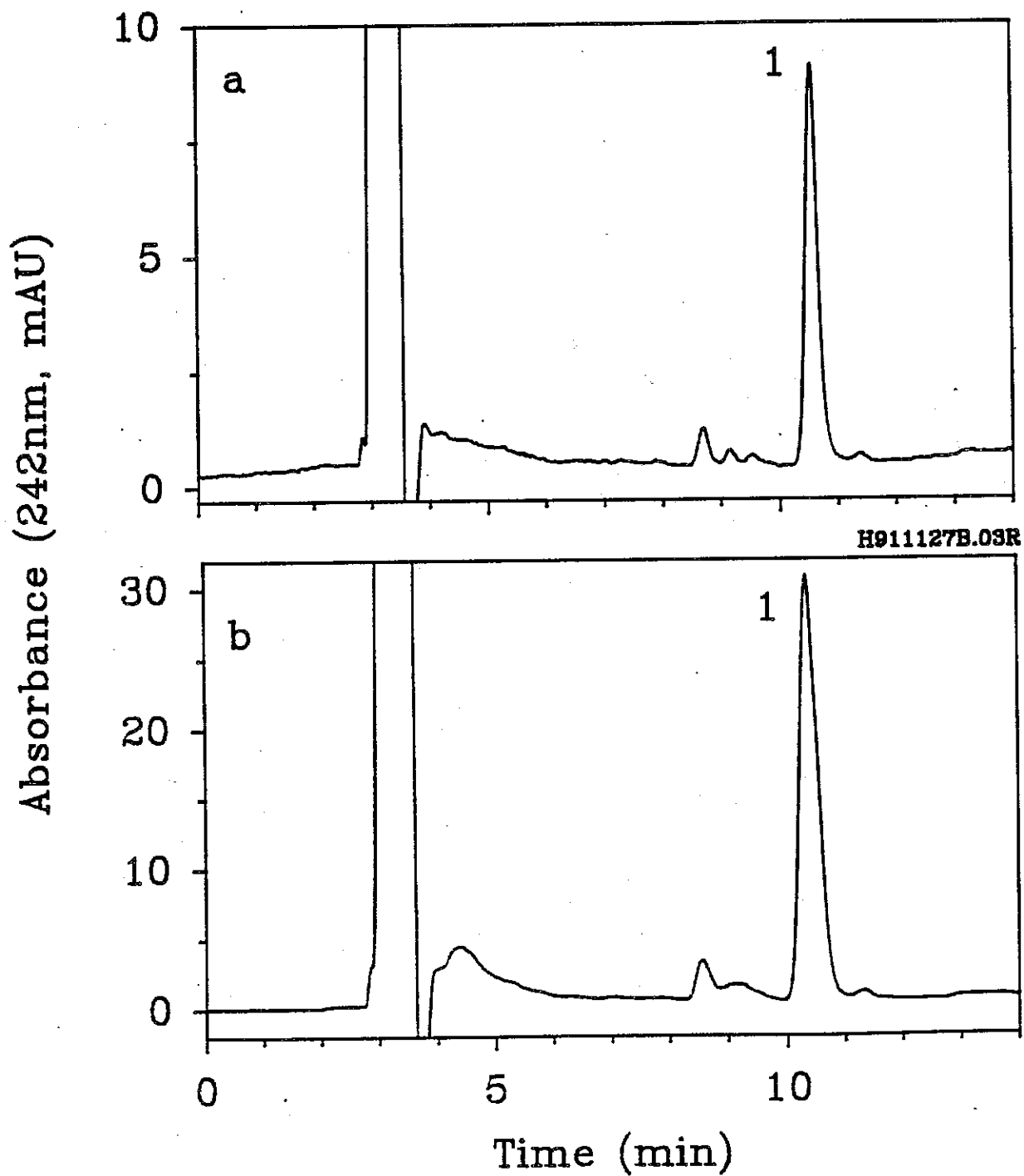
Figure 6: HPLC-UVD chromatograms resulting from 4 µL injections of raw methanolic extract of blended MUS-1 and control tissues with domoic acid at 20 µg/g (a) and 2 µg/g (b), and control tissue only (c). Same conditions as in Figure 2. The asterisk indicates a late eluting peak from a previous injection; this is a typical problem with the analysis of raw extracts.
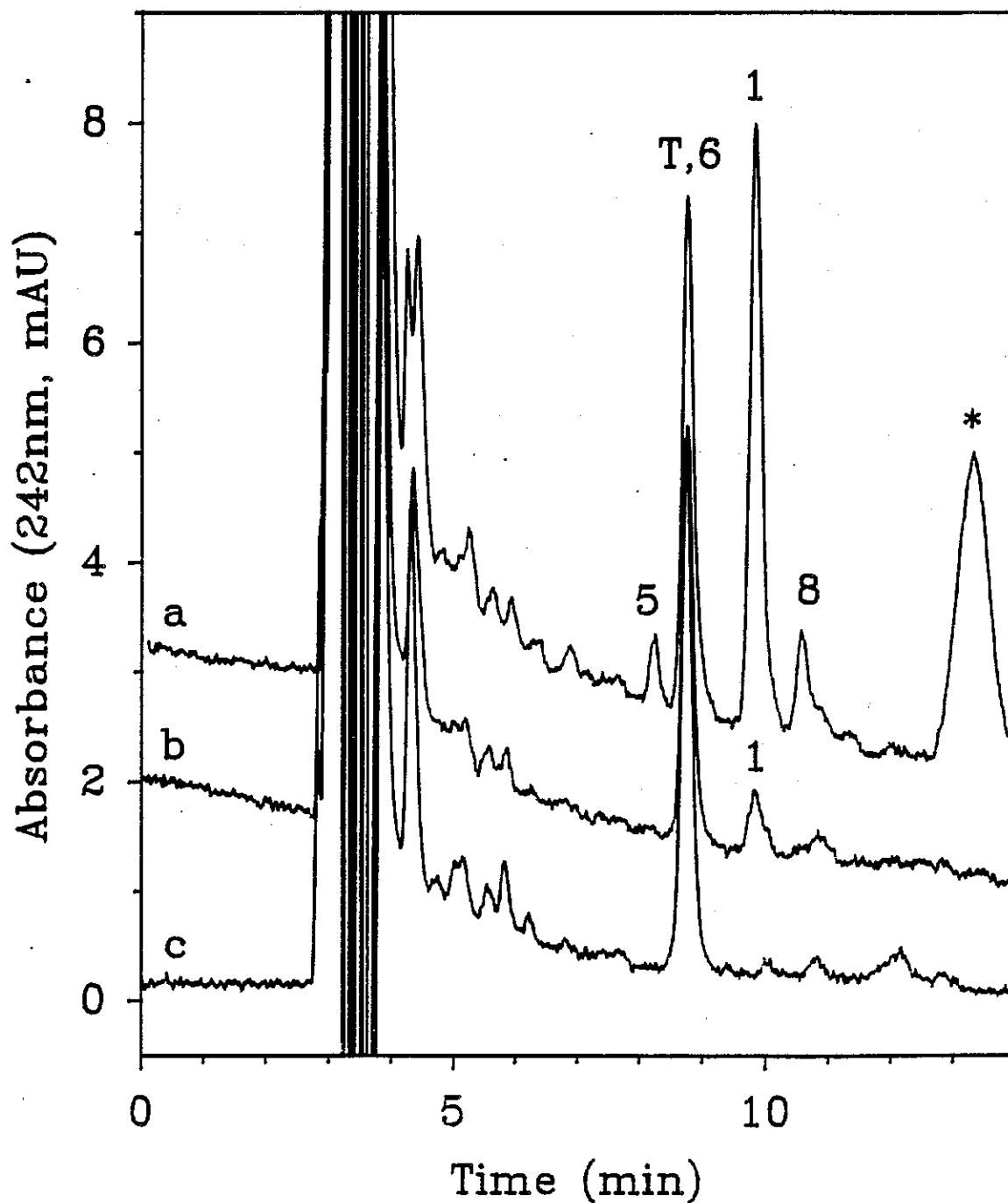
As shown in Table 1, direct analyses of raw extracts of the 20 µg/g blends showed that the best extraction yield (almost 100%) is provided by an exhaustive 50% aqueous methanol extraction, in which the tissue solids are extracted three times and the final extract is made to volume. Lower concentration blends (2 and 0.2 µg/g) could not be measured by direct injection of the raw extracts, but analyses performed after SAX clean-up (discussed further below) showed that the extraction yield appears to be constant down to the 0.2 µg/g level. This is in contrast to the AOAC boiling acid extraction procedure, which shows poorer recoveries at trace levels, probably due to increased decomposition.

A single step "dispersive" extraction with 50% aqueous methanol was found to provide excellent recovery (almost 98%), however, and is much faster for routine regulatory work. Technically, an exhaustive extraction is a more volumetric procedure, while a dispersive extraction introduces a positive bias in the results due to the volume of undissolved solids. However, with a 4:1 volume ratio of extracting solvent to tissue (which is about 75% water already), there should be only a 5% positive bias introduced. This means that the single step dispersive extraction is in fact only extracting about 93% of the domoic acid, but the two systematic errors (lower recovery and positive bias) fortuitously cancel each other resulting in quite accurate concentration values. Extraction in a blender rather than a Polytron gave a slightly lower yield.

A dispersive extraction with 75% aqueous methanol (CH_3OH/H_2O, 3:1) provided a much lower recovery of domoic acid; it should be noted, however, that we have found that an exhaustive 75% methanol extraction gave good recovery (data not shown). It was hoped that acetonitrile might be a suitable extraction solvent, since it can provide better precipitation of protein and less extraction of lipid materials. However, substitution of acetonitrile for methanol gave much lower recoveries. The results are reasonable considering the solubility of domoic acid in different solvents: 7.6 g/L in water, 0.66 g/L in methanol, and 0.0011 g/L in acetonitrile (Falk *et al.*, 1991).

The stability of domoic acid in raw extracts has been examined. No significant decomposition of mussel tissue extracts was observed over a period of 1 week if stored in a freezer. No significant levels of methyl esters of domoic acid have been observed in neutral aqueous methanol solutions. However, we have observed the formation of esters in acidic methanol solutions. It is important to ensure that storage vessels are tightly sealed, as solutions

can evaporate quite rapidly in a freezer if vial caps are loose. The stability of domoic acid in methanolic extracts contrasts sharply with that in AOAC acid extracts. Domoic acid has been observed to decompose rapidly in acidic extracts -- up to 50% in 1 week (Quilliam *et al.*, 1989a).

It was decided that a 50% aqueous methanol dispersive extraction would be used in the final recommended procedure for regulatory work (see section D). For studies requiring the highest possible recovery (e.g., reference material work), it is advisable to use an exhaustive extraction procedure with the same solvent.

Strong Anion Exchange Clean-up

Initial experiments on the strong anion exchange clean-up were performed on 50% aqueous methanol extracts of the MUS-1 reference material (200 μg **1** per g of tissue). It was found that 5 mL of extract representing 1 g equiv. of tissue could be loaded onto an SAX cartridge with negligible breakthrough. It was also found that ASP toxins could be eluted from the cartridge quantitatively with 2% formic acid. Figure 7 shows the isocratic HPLC-UVD analysis of a MUS-1 extract taken through such a clean-up, with elution from the cartridge using 5 mL water/acetonitrile (9:1) with 2% formic acid. This chromatogram should be compared with that in Figure 3, which is from an analysis of the same tissue material using a boiling water extraction and C18-SPE clean-up Quilliam *et al.*, 1989a). It can be seen that the SAX extract appears cleaner and that it is about 3-fold more concentrated. The same extract was also analyzed by gradient HPLC-DAD and these data are shown in Figure 8. Comparing this to Figure 4, it can be seen that tryptophan (T) is absent from the SAX extract. Analyses of the fractions eluting from the SAX cartridge during loading of the sample extract revealed that tryptophan is not retained by the SAX. It appears that the cartridge is very selective to domoic acid; this is a real advantage for the present clean-up procedure because it will add extra chemical selectivity to the analysis. As indicated earlier, tryptophan and its derivatives are the major interferences thus far observed for HPLC-UVD analysis of ASP toxins.

In order to determine the loading capacity of the SAX cartridge, a "breakthrough" experiment was conducted by passing MUS-1 extracts slowly through cartridges while collecting 1 mL fractions. Analysis of the fractions revealed when the column capacity was

Figure 7: HPLC-UVD analysis of MUS-1 tissue, after extraction with 50% methanol and clean-up through an SAX-SPE cartridge (using 2% formic acid for elution from the cartridge). Same conditions as in Figure 3.
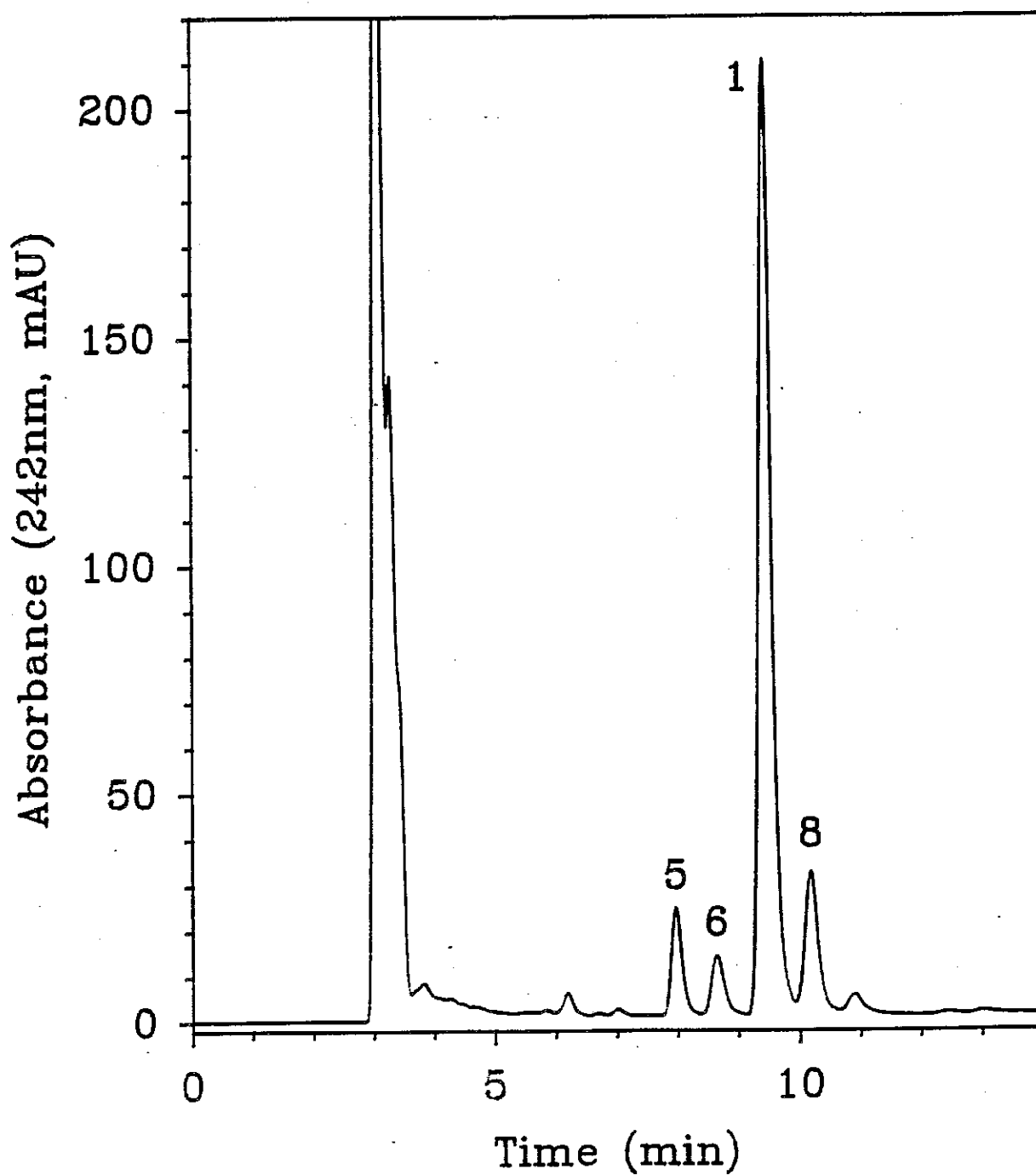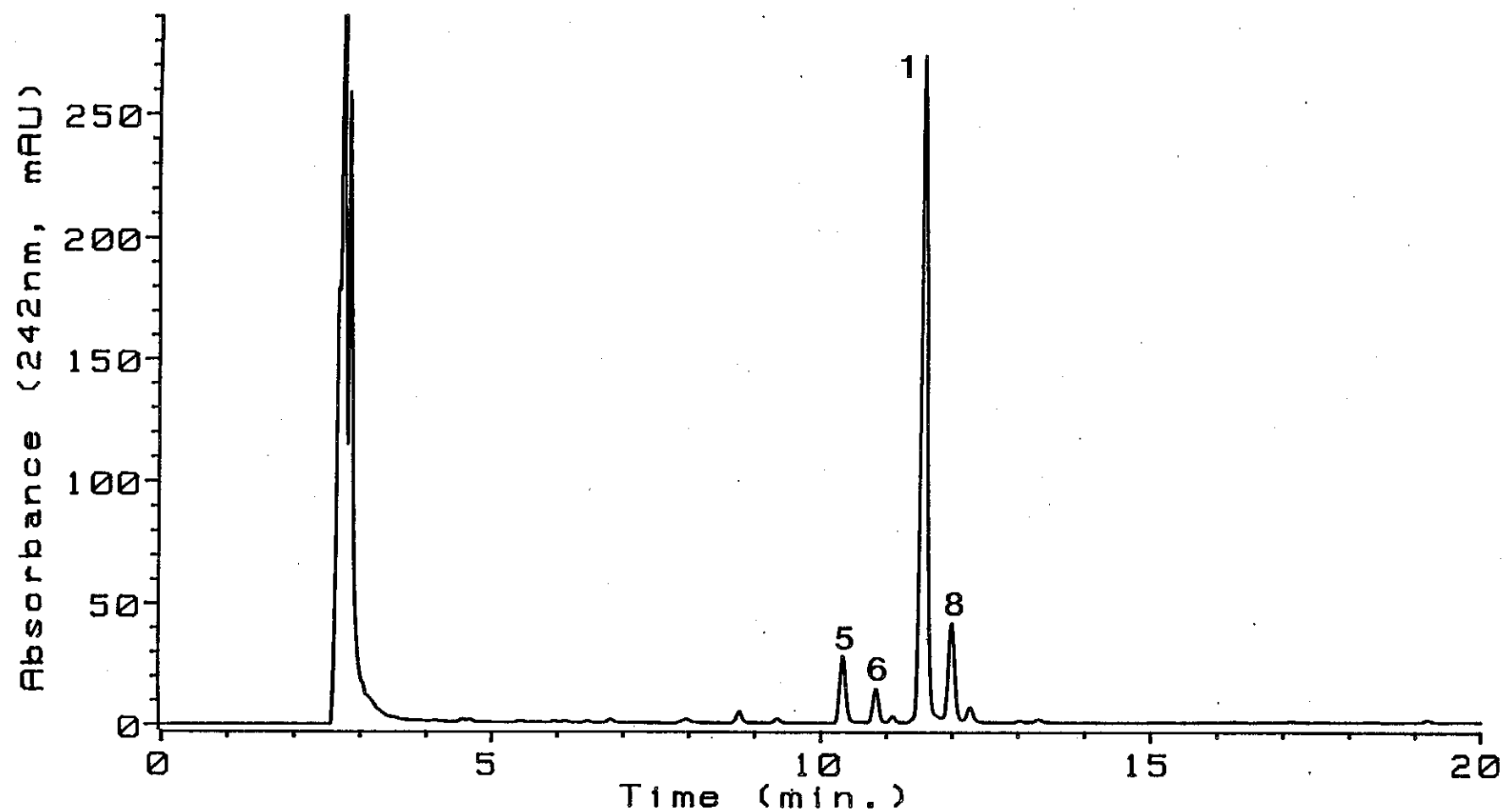
Figure 8: Gradient elution HPLC-DAD chromatogram of the same MUS-1 tissue extract (50% methanol/SAX clean-up) analyzed in Figure 7. Same conditions as in Figure 4.
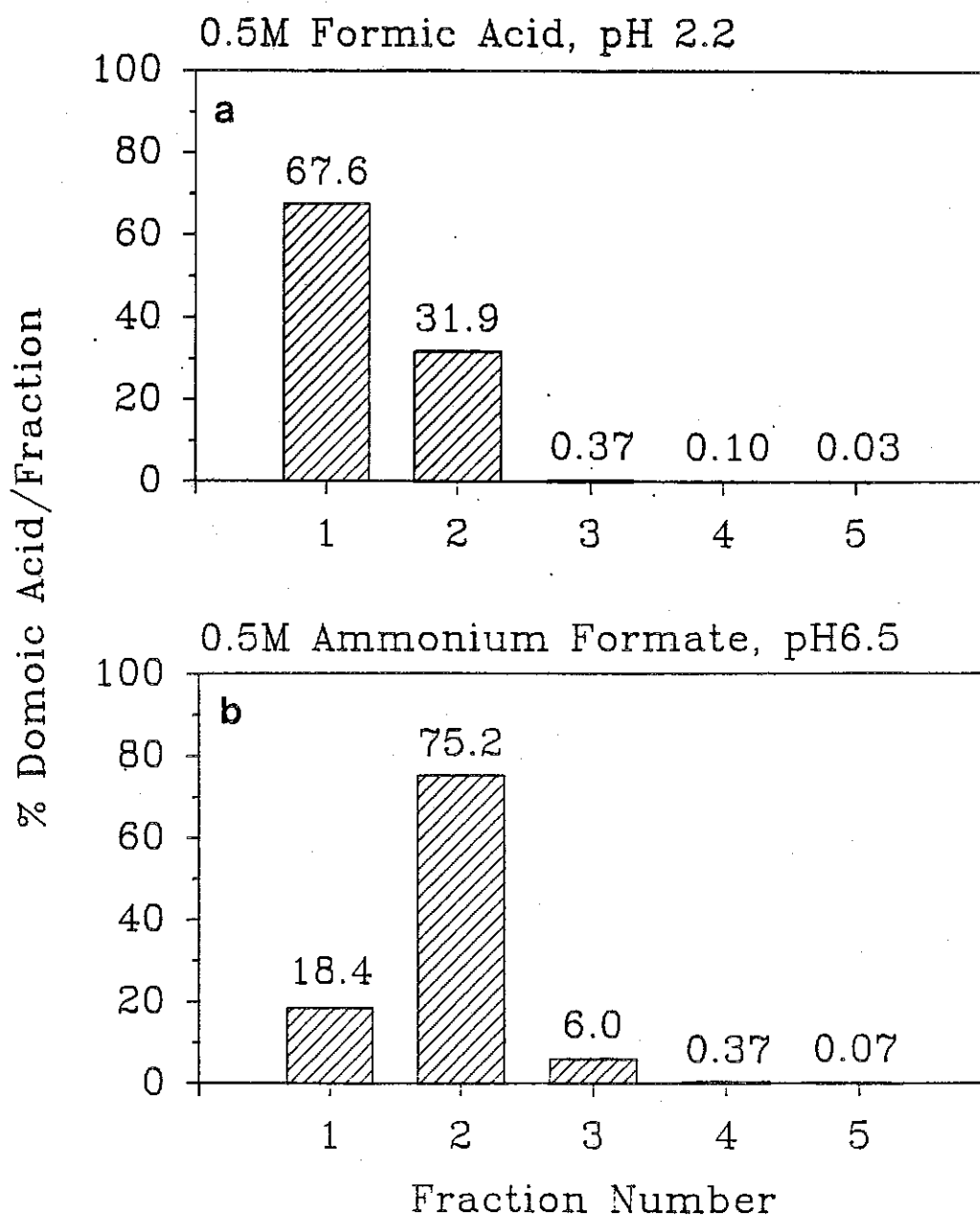
exceeded. MUS-1 was used because it is important to perform such an experiment with a high level of domoic acid in the presence of real sample matrix. Data are presented in Figure 9 for different types of extracts of MUS-1: aqueous, 50% aqueous methanol, and 75% aqueous methanol. The concentration of domoic acid in the fractions is expressed relative to the concentration in the raw extract being loaded onto the cartridge. The data show that, for all the extracts, up to 1 g equiv. of tissue can be loaded onto the cartridge without significant breakthrough. About twice as much can be loaded with the 75% methanol extract. This should allow a higher pre-concentration factor in the analysis, but is partially offset by the lower extraction efficiency with this solvent. An interesting maximum, at a concentration greater than unity, is observed in the curves. This is probably due to sample matrix (salts, etc.) forcing accumulated domoic acid off the cartridge. A similar breakthrough curve was observed with razor clam extracts, and no breakthrough was detected with anchovy extracts loaded at the 1 g tissue level.

The elution of an anionic analyte from an SAX cartridge may be accomplished in two ways: (a) by reducing the pH with an acidic eluent in order to disrupt the ionic bonding; (b) by replacing the analyte with a more strongly-bound anion. Our first experiments used acidic eluents: (a) 1% trifluoroacetic acid (TFA), and (b) 2% formic acid (both in acetonitrile/water, 1:9). Both eluted the domoic acid very quickly; as shown in Figure 10a, most of it is eluted in the first 2 mL with 2% formic acid. The problem with both of these eluting solvents is the low pH, which does lead to decomposition of ASP toxins. The decomposition in 1% TFA was quite rapid for some low level extracts. Formic acid solutions seemed quite stable for all but a few samples, where losses of up to 5% in a day were observed. If samples are analyzed immediately, this is not a problem, but does cause some concern if samples need to archived for a period of time. Therefore, we have examined several other types of eluents. Figure 10 shows the results of experiments designed to test the efficiency of five different solvent systems: (a) 0.5M (ca. 2%) formic acid; (b) 0.5M ammonium formate (pH 6.5); (c) 0.5M formate buffer (pH 3.8); (d) 0.2M formate buffer (pH 3.8); and (e) 0.5M citrate buffer (pH 4.5). Ammonium formate was examined because it is of interest for other analytical procedures where the fraction has to be evaporated to dryness (e.g., GC-MS with chemical derivatization). Direct injection of this solution into HPLC is not recommended, however, because peak splitting and broadening occurs due to the higher pH of the solution versus that of the HPLC mobile phase (see Figure 11). A 0.5M formate buffer with a pH of 3.8 gives good peak shapes in chromatograms and ASP toxins appeared quite stable. However, the elution profile was not as good as that obtained

Figure 9: Measurement of the breakthrough of domoic acid on SAX-SPE cartridges with different types of extracts of MUS-1 tissue as a function of the gram equivalents of tissue loaded on the cartridge. Extracts of MUS-1 were loaded continuously onto a pre-conditioned cartridge and individual fractions were collected and analyzed directly by HPLC-UVD. The concentration of domoic acid in the fractions is expressed relative to the concentration in the raw extract being loaded onto the cartridge.
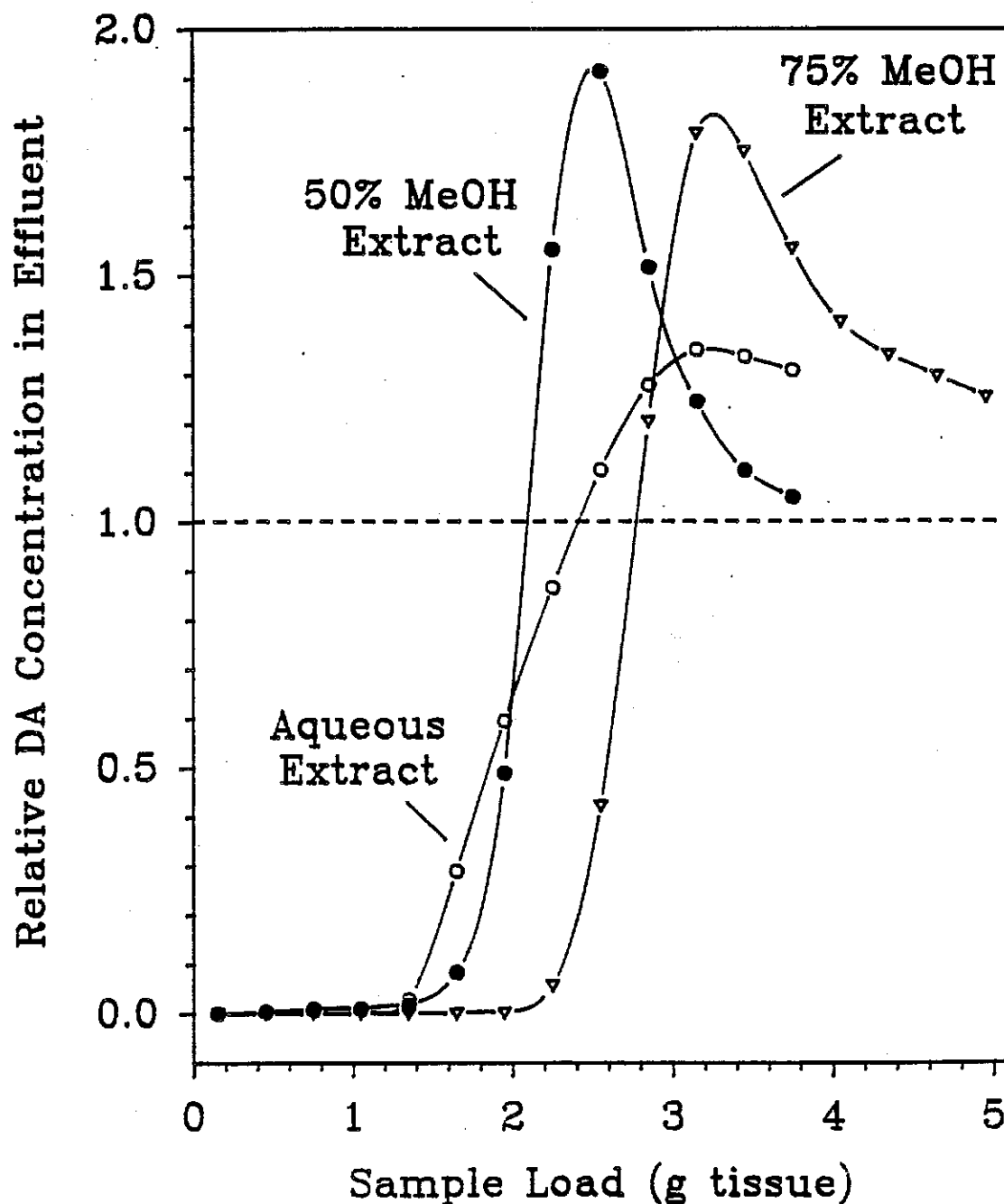
Figure 10: Measurement of the efficiency of different acids, salts or buffers (all in 10%
acetonitrile) for the elution of domoic acid from an SAX SPE cartridge pre-loaded with 1
g equiv. of MUS-1 tissue extract (50% methanol). Each fraction was 1 mL in volume,
with collection of fractions starting as soon as the eluting solvent was placed on the
cartridge. The amount of domoic acid in each fraction is expressed as a percentage of the
total domoic acid eluted from the cartridge. The precision of this experiment was not
adequate to determine overall recoveries of domoic acid, but in all cases it appeared to be
close to 100%. [continued on next page]

Figure 10 (continued) 25

Figure 10 (continued). Plots c, d, and e showing % Domoic Acid/Fraction versus Fraction Number for three different buffer conditions:

c. 0.5M Formate Buffer, pH 3.8
- Fraction 1: 18.1
- Fraction 2: 80.4
- Fraction 3: 1.2
- Fraction 4: 0.24
- Fraction 5: 0.05

d. 0.2M Formate Buffer, pH 3.8
- Fraction 1: nd
- Fraction 2: 69.1
- Fraction 3: 29.4
- Fraction 4: 1.3
- Fraction 5: 0.23

e. 0.5M Citrate Buffer, pH 4.5
- Fraction 1: 2.4
- Fraction 2: 94.6
- Fraction 3: 2.4
- Fraction 4: 0.43
- Fraction 5: 0.20

Figure 11: HPLC-UVD chromatograms of different mussel tissue extracts after clean-up
through the SAX SPE cartridge, using the following elution solvents: (a) 2% formic acid
in 10% acetonitrile; (b) 0.5 M ammonium formate in 10 % acetonitrile. The double-peak
effect for the latter is due to the presence of the ammonium formate and the high pH (6.5)
of the solution. Same conditions as in Figure 3.
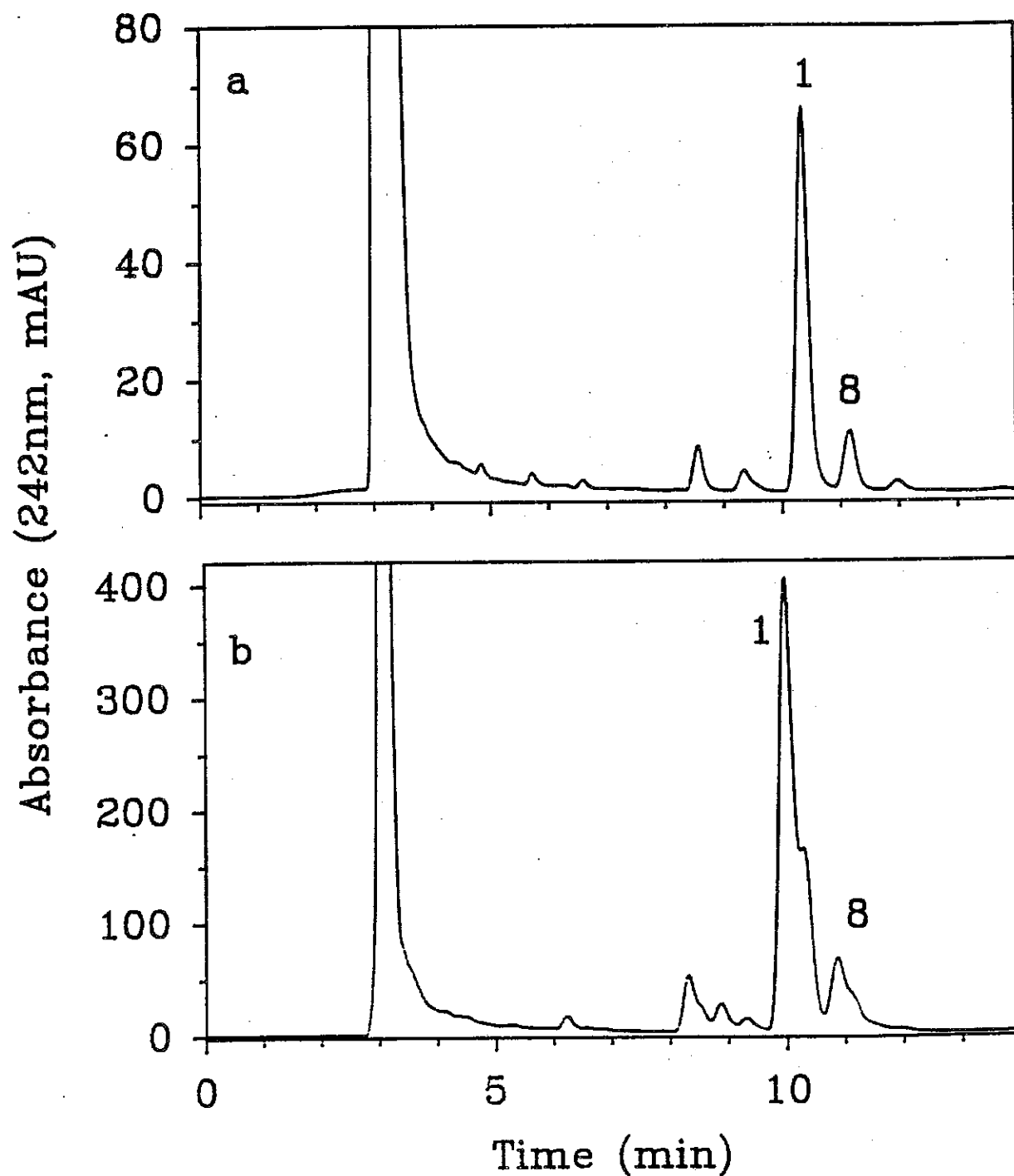
using citrate buffer.

The best compromise between efficiency of elution, stability of ASP toxins in the collected fraction, and good performance in HPLC, seems to be the 0.5M citrate buffer. Cleaned-up samples have been found to be very stable in this buffer, with less than 1% decomposition per day for all samples tested. Since the dead volume of the SAX cartridge is 0.5 mL, a procedure was tested in which 0.5 mL of citrate buffer was placed into the cartridge before collection was started. The results of this experiment are shown in Figure 12. No domoic acid could be detected in the first 0.5 mL, and over 99% of the domoic eluted in the next 2 mL. It was decided that this procedure would be used for the final recommended method (see section D). For studies where the highest sensitivity is not required, a simpler procedure would be to elute with 5 mL of citrate buffer (including the first 0.5 mL) into a 5 mL volumetric flask. This gives slightly better recovery and greater ease of operation.

Method Evaluation

A rigorous evaluation was performed on mussel tissue blends using the optimized method (see section D) (i.e., a 50% aqueous methanol extraction, 1 g equiv. tissue loading on the SAX cartridge, and elution with 2 mL of citrate buffer). The results of these experiments are presented in Table 1. The recovery of domoic acid is pretty constant at 93%, right down to the 200 ng/g level. The recovery with the optional 5 mL citrate buffer elution is slightly higher (data not shown), but due to the lower sensitivity it is not possible to make good measurements at low concentrations such as 200 ng/g. The precision of the determinations was excellent: 1.5% RSD at 20 μg/g, 2.2% RSD at 2 μg/g, and 5.4% RSD at 200 ng/g.

Chromatograms for the blended tissue extracts are presented in Figures 13-16. Figure 13 shows the analyses of the blended tissues at the 20 and 2 μg/g levels, in comparison with a control tissue extract. These results should be compared to Figure 6 which shows the analysis of the raw extracts. A 12.5-fold increase in sensitivity is achieved with the pre-concentration factor from the SAX cartridge and with the ability to inject larger volumes into the HPLC. The control extract is much cleaner after SAX than before: tryptophan and other interferences have been mostly eliminated. Figure 14 shows analyses of the 200 ng/g blend sample versus the control extract. At this level, some co-extractives with similar retention times are apparent. The

Figure 12: Measurement of the efficiency of the 0.5 M citrate buffer (in 10% acetonitrile) for the elution of domoic acid from an SAX SPE cartridge pre-loaded with 1 g equiv. of MUS-1 tissue extract (50% methanol). The first fraction collected was 0.5 mL, and subsequent fractions were 1 mL in volume. The amount of domoic acid in each fraction is expressed as a percentage of the total domoic acid eluted from the cartridge.

0.5M Citrate Buffer, pH 4.5
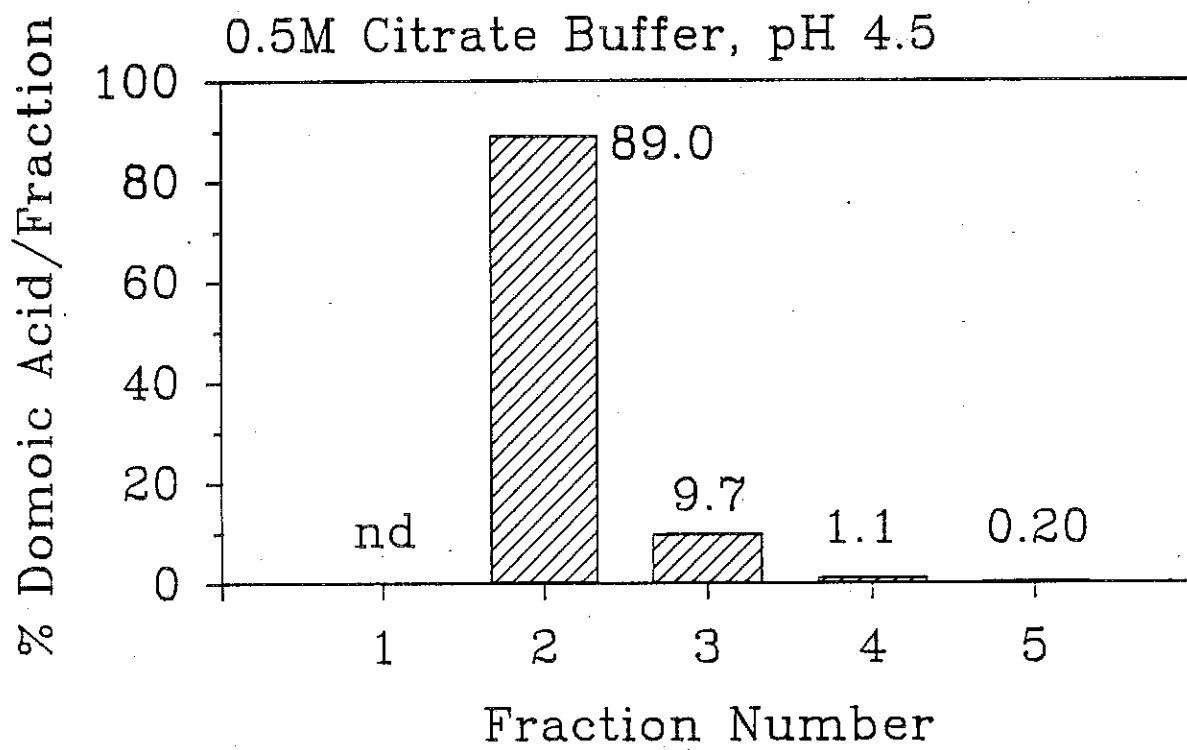
Figure 13: HPLC-UVD analyses of blended MUS-1/control tissues with domoic acid at 20 μg/g (a) and 2 μg/g (b), and control tissue (c) using the optimized extraction/clean-up procedure (i.e., 50% methanol extraction (4 g tissue extracted into 20 mL); 1 g equiv. tissue loaded onto the SAX SPE; and elution with 2 mL citrate buffer). Conditions as in Figure 3.
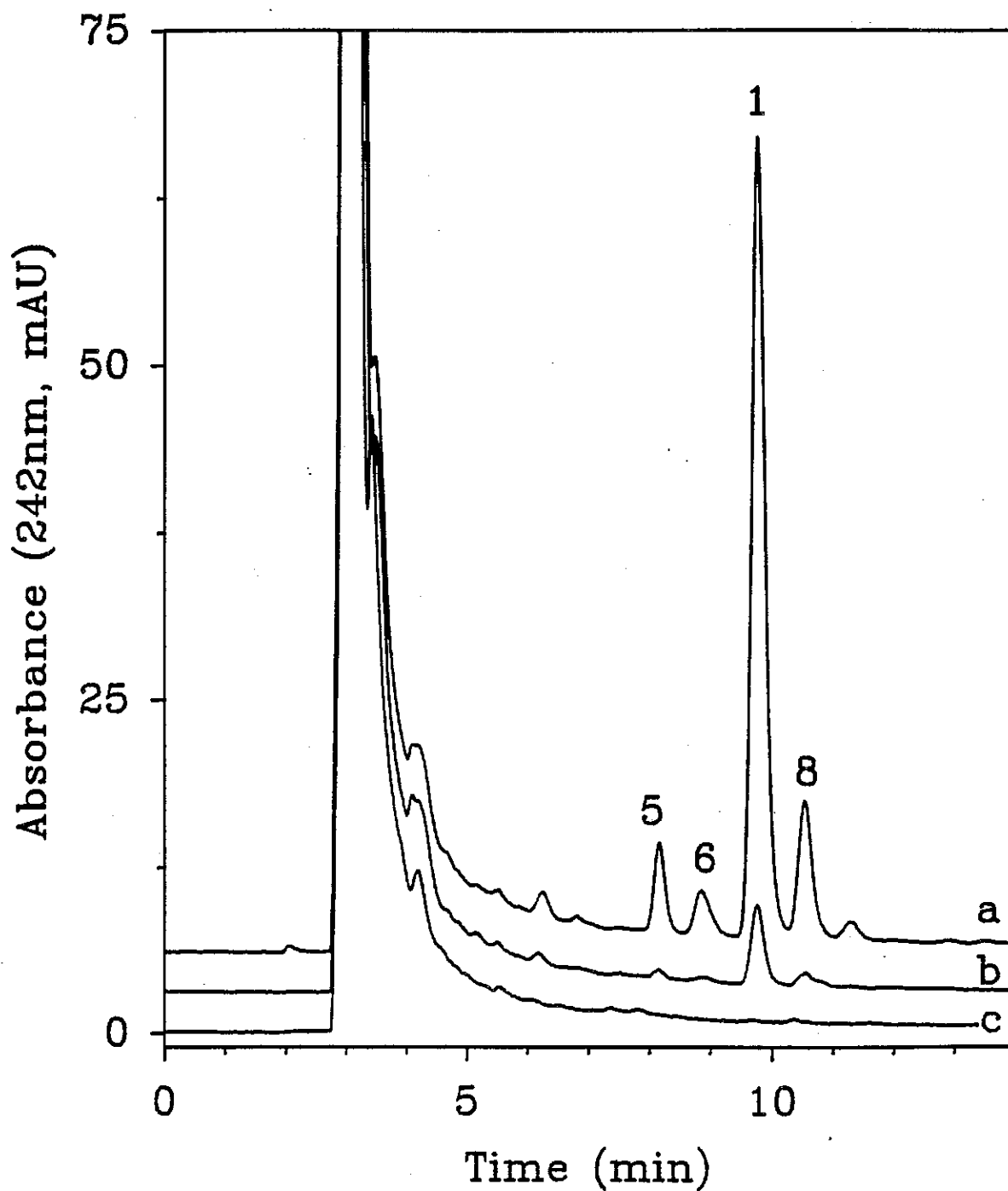
Figure 14: HPLC-UVD analyses of blended MUS-1/control tissue with domoic acid at 200 ng/g (a), and control tissue (b) using the optimized extraction/clean-up procedure (i.e., 50% methanol extraction (4 g tissue extracted into 20 mL); 1 g equiv. tissue loaded onto the SAX SPE; and elution with 2 mL citrate buffer). Conditions as in Figure 3.
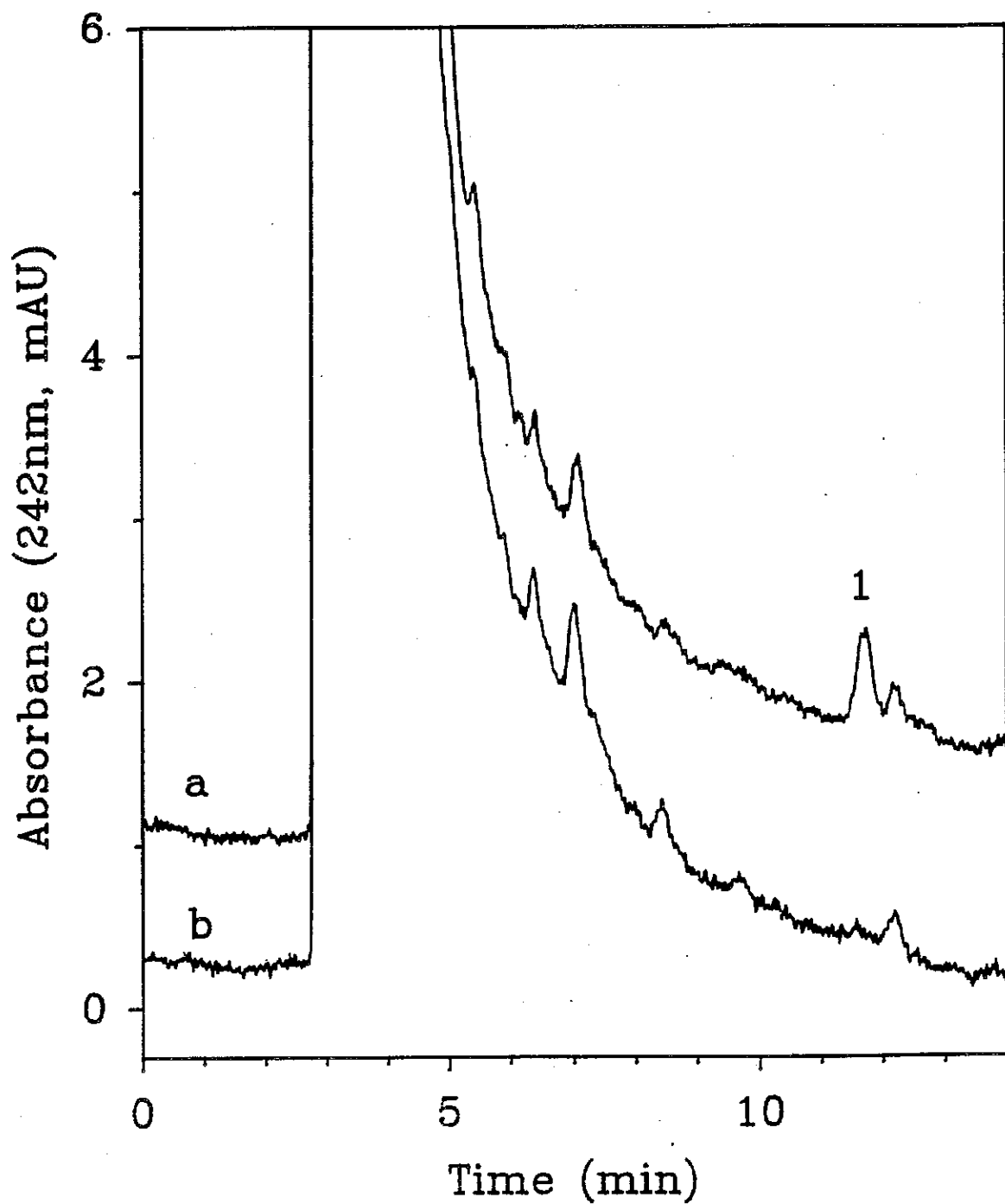
Figure 15: HPLC-DAD analyses of blended MUS-1/control tissue samples with domoic acid at 20 µg/g (a) and 2 µg/g (b) using the optimized extraction/clean-up procedure (i.e., 50% methanol extraction (4 g tissue extracted into 20 mL); 1 g equiv. tissue loaded onto the SAX SPE; and elution with 2 mL citrate buffer). Conditions as in Figure 3, except that an HP1090 HPLC-DAD system was used. The inset shows the UV spectrum acquired for domoic acid in the extract of the 2 µg/g sample.
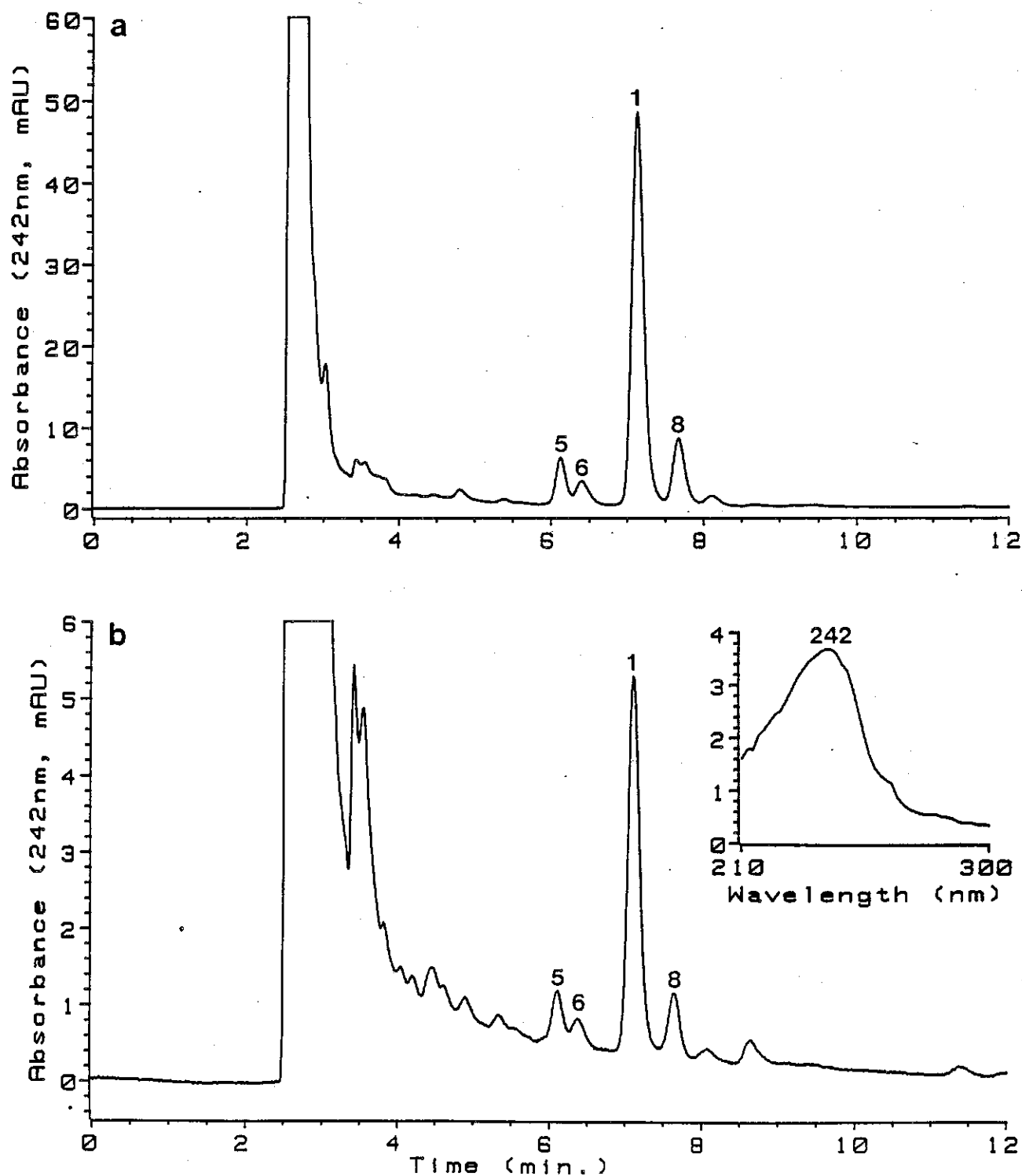
Figure 16: HPLC-DAD analyses of blended MUS-1/control tissue sample with domoic acid at 200 ng/g (a), and the control tissue (b) using the optimized extraction/clean-up procedure (i.e., 50% methanol extraction (4 g tissue extracted into 20 mL); 1 g equiv. tissue loaded onto the SAX SPE; and 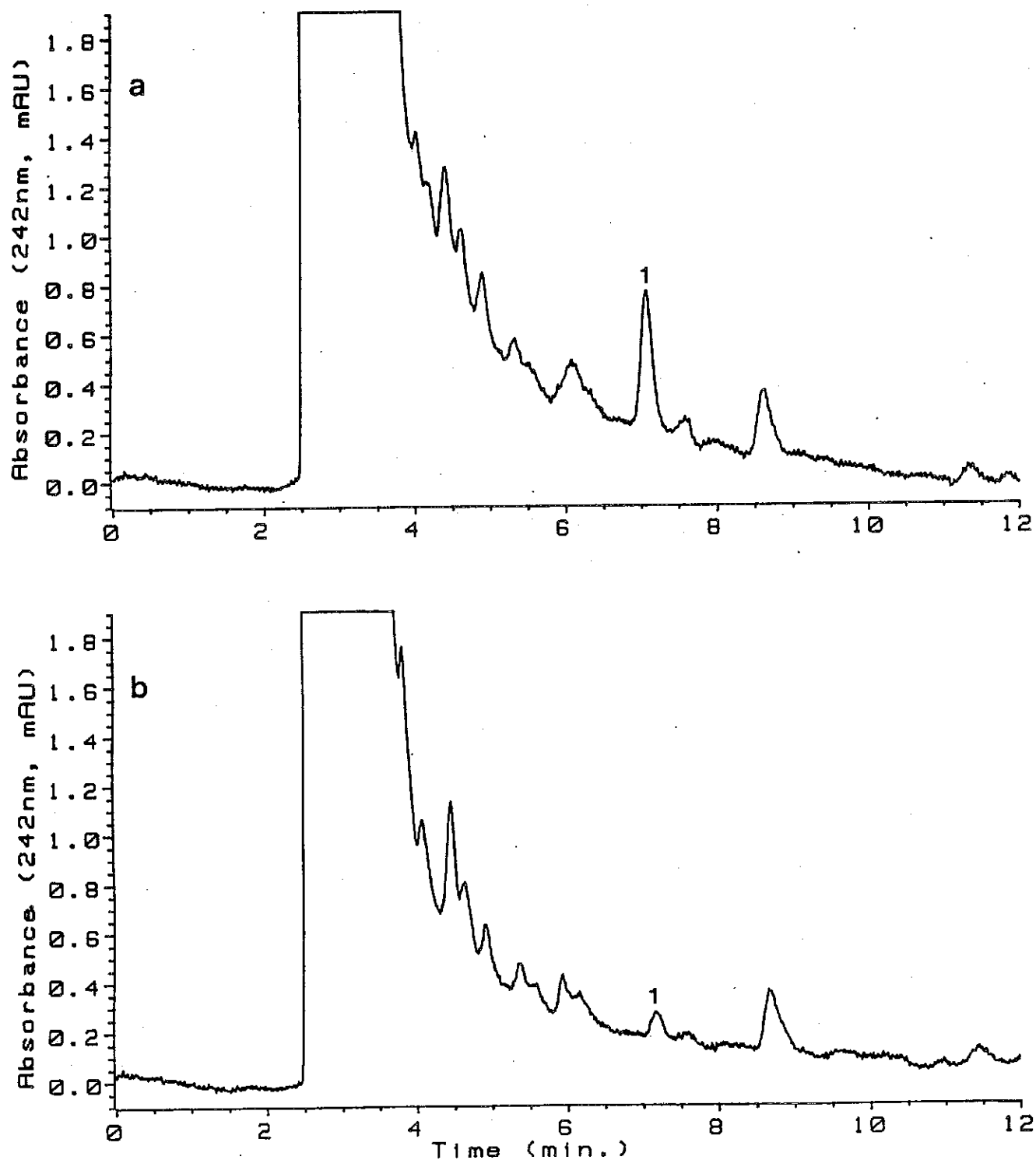elution with 2 mL citrate buffer). Conditions as in Figure 3, except that an HP1090 HPLC-DAD system was used. The small peak marked 1 in the control tissue chromatogram (b) is believed to be domoic acid and is probably due to lab contamination.

analyses in Figures 13 and 14 were performed on a relatively inexpensive HPLC system, with a 12 year old UV detector. The limit of detection of the method with this HPLC-UVD system was estimated to be 100 ng/g (S/N = 3).

Figures 15 and 16 show the analyses of the same extracts on the more sensitive HP1090M HPLC-DAD system. Good UV spectra can be acquired by the DAD at the 2 µg/g level (see Figure 15b). This is quite important for confirmation of peak identity for both research and regulatory work. Some domoic acid was observed in this particular control extract at about the 35 ng/g level (see Figure 16b). We believe that this was due to laboratory contamination, which becomes a serious problem at the trace level. As previously noted with fluorescence derivatization techniques for domoic acid (Pocklington *et al.*, 1990), it is important to use disposable supplies to avoid cross-contamination. The detection limit with the HP1090 HPLC-DAD was estimated to be about 30 ng/g (S/N = 3).

C. APPLICATION TO DIFFERENT SAMPLES

The new extraction/clean-up procedure has proved valuable for a number of different sample types that have passed through our lab recently. Figure 17 shows a chromatogram from an analysis of anchovies collected from Monterey Bay during the September 1991 incident in which numerous pelicans and cormorants died (Work *et al.*, 1991). A high level of domoic acid (75 µg/g), as well as some isomers (5 and 6), was easily measured. Oily fish samples such as these posed no problem for the procedure. For comparison, a boiling aqueous extract taken through the C18-SPE clean-up (Quilliam *et al.*, 1989a) is shown in Figure 18. The extraction proved more difficult, giving a much lower result (75% of the value by the new procedure). It can also be seen that there is a very high level of tryptophan (T) present in the extract; the SAX-SPE procedure eliminated the tryptophan completely. Figure 19 shows the analysis of the stomach contents of a dead pelican from the Californian incident. Anchovies were the major constituents of the stomach contents. Again, an excellent result was observed.

Figure 20 shows the analysis of a razor clam sample sent to us by the US-FDA after the Washington ASP incident in late 1991. This sample contained 40 µg/g domoic acid. The only problem observed with this sample was the appearance of a precipitate in the raw 50% methanol extract after standing awhile. It was necessary to filter off this material prior to SAX clean-up.

Figure 17: HPLC-UVD chromatogram of anchovy tissue contaminated with 75 μg/g domoic acid using the optimized extraction/clean-up procedure (i.e., 50% methanol extraction (4 g tissue extracted into 20 mL); 1 g equiv. tissue loaded onto the SAX SPE; and elution with 2 mL citrate buffer). Conditions as in Figure 3.
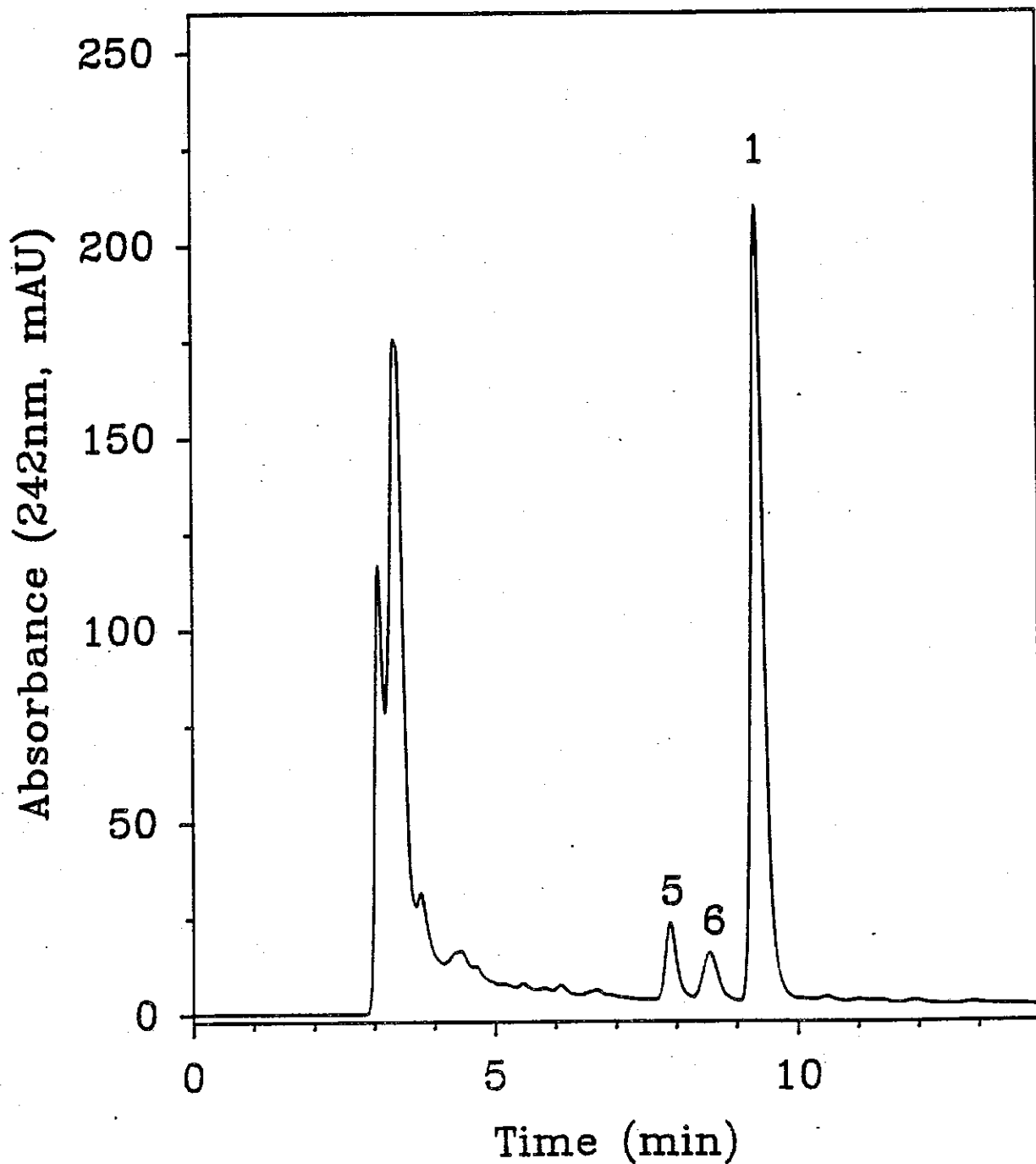
Figure 18: HPLC-UVD chromatogram of the same anchovy tissue as in Figure 17, but using a boiling water extraction and C18-SPE clean-up procedure. A high level of tryptophan (T) is present in this extract, but not in that analyzed in Figure 17. Conditions as in Figure 3.
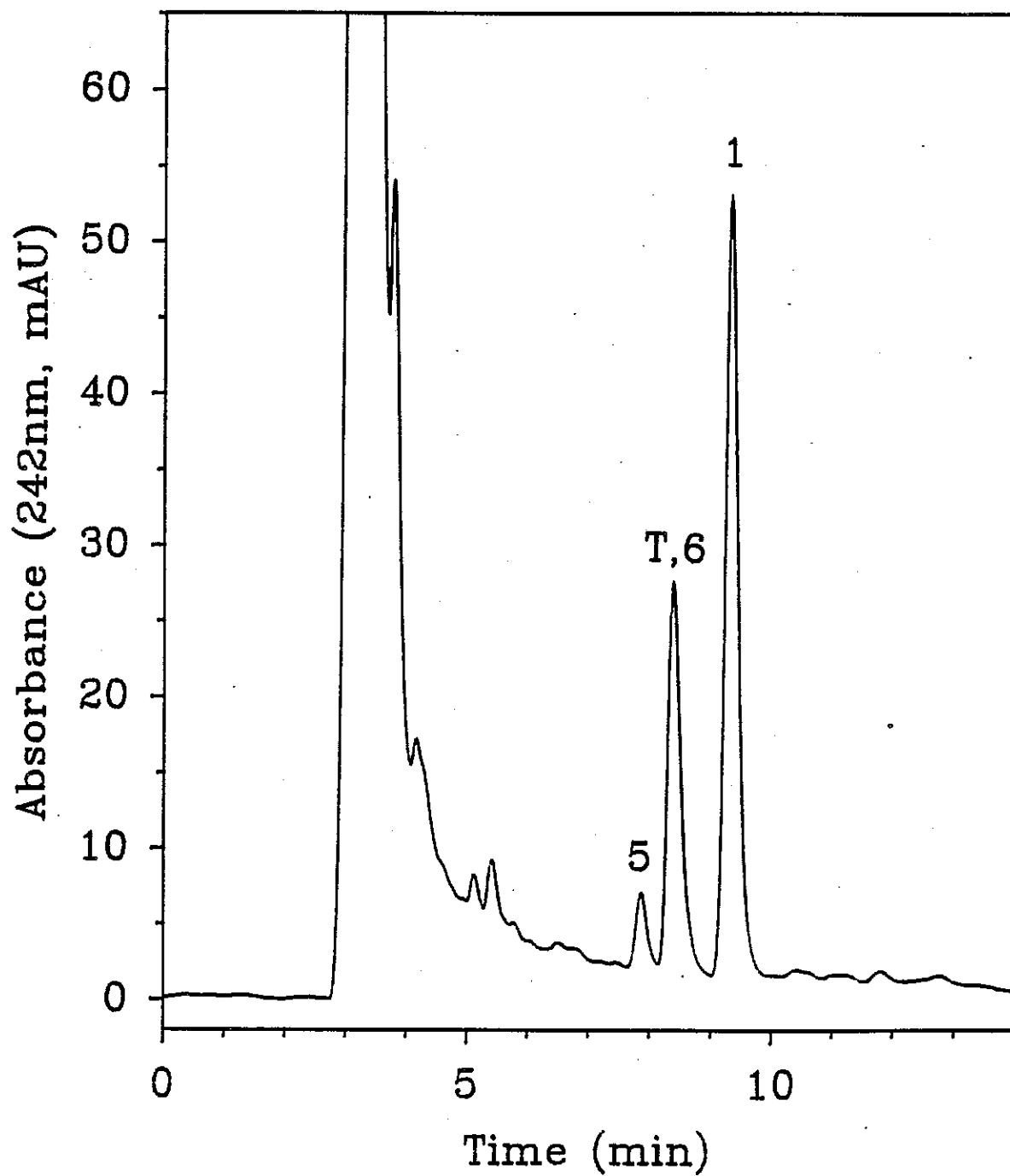
Figure 19: HPLC-UVD analysis of the stomach contents of a pelican that died from domoic acid poisoning using the optimized extraction/clean-up procedure (i.e., 50% methanol extraction (4 g tissue extracted into 20 mL); 1 g equiv. tissue loaded onto the SAX SPE; and elution with 2 mL citrate buffer). Conditions as in Figure 3.
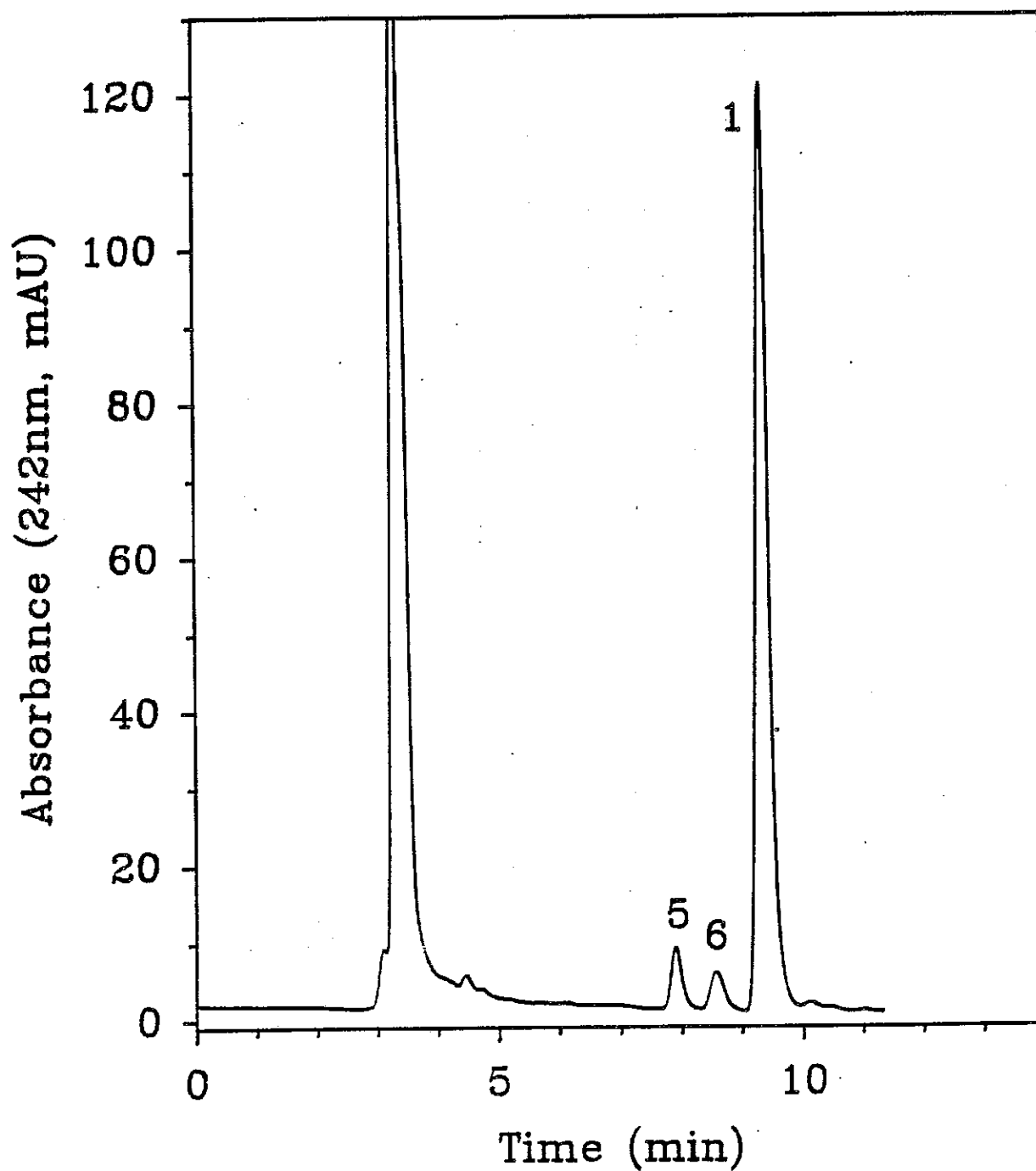
Figure 20: HPLC-UVD chromatogram of razor clam tissue contaminated with 40 μg/g domoic acid using the optimized extraction/clean-up procedure (i.e., 50% methanol extraction (4 g tissue extracted into 20 mL); 1 g equiv. tissue loaded onto the SAX SPE; and elution with 2 mL citrate buffer). Conditions as in Figure 3.
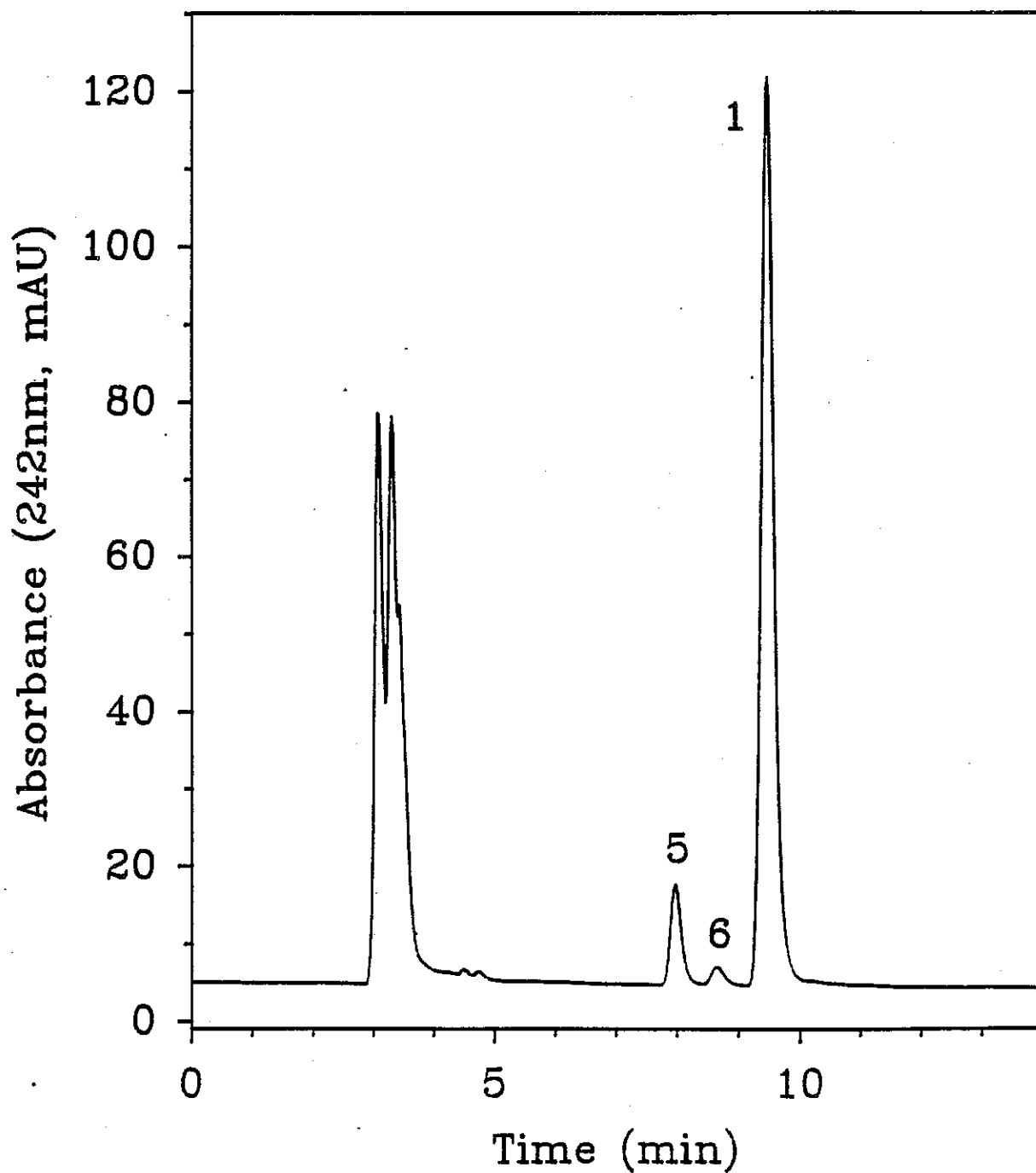
Figure 21: Ion-spray HPLC-MS analysis of the same razor clam extract analyzed in Figure 20. The selected ion mass chromatogram (m/z 312) is shown in (a), while the mass spectrum acquired in a separate run is shown in (b). Conditions: 250 x 1 mm I.D. Vydac 201TP54 column; 50 µL/min 10% acetonitrile and 0.1% TFA in water; column temperature at ambient.
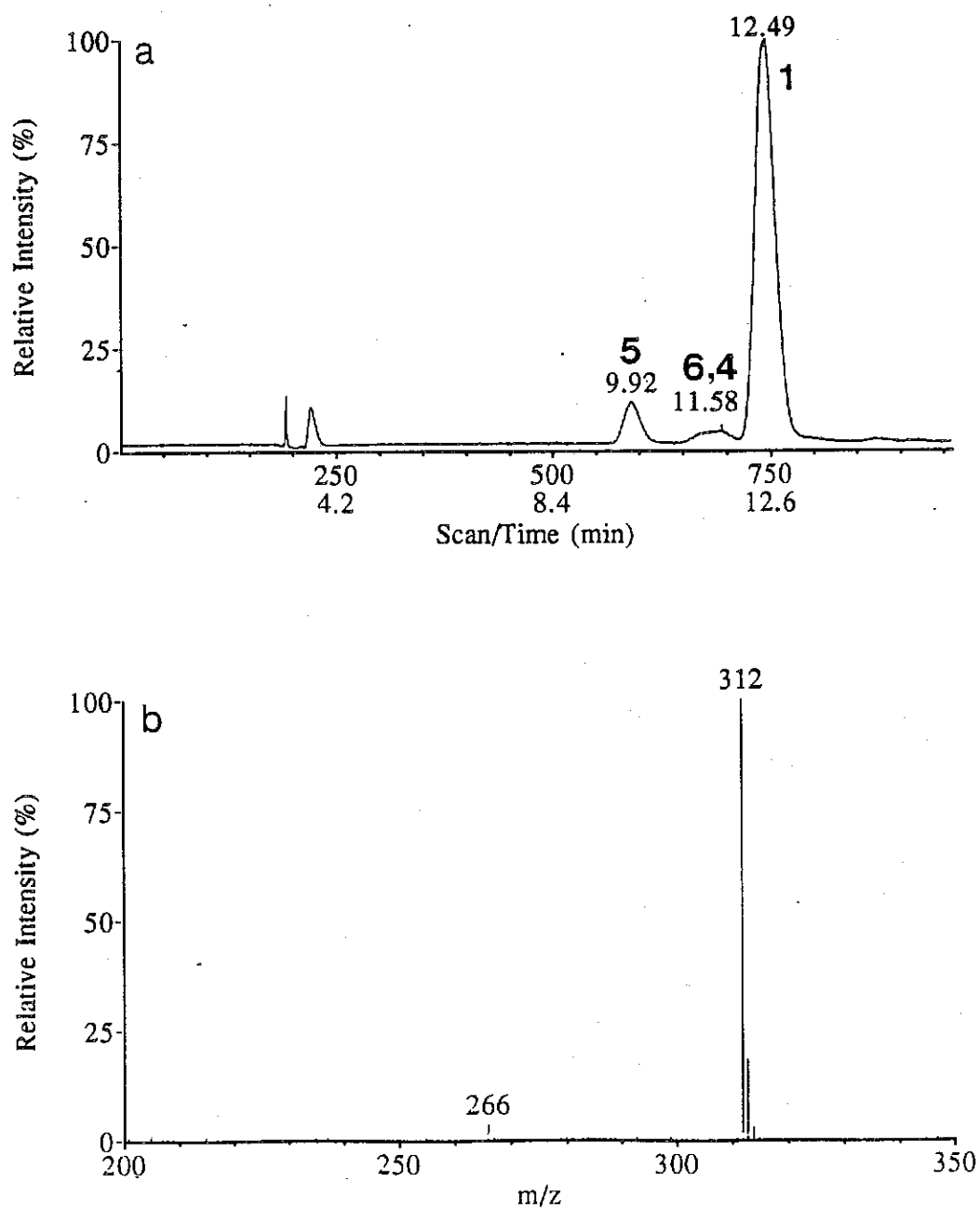
A 75% methanol extraction did not give such a problem and still gave a good recovery of the domoic acid.

The new procedure is also very useful for other types of analyses. Figure 21 shows the ion-spray LC-MS analysis of the razor clam extract. A very strong signal in the m/z 312 ($[M+H]^+$ of $\underline{1}$) mass chromatogram at the correct retention time for domoic acid confirms the presence of the toxin. Further proof is provided by a full mass spectrum (Figure 21b) acquired at 12.49 min. It is also possible to confirm the presence of isomers $\underline{4}$, $\underline{5}$ and $\underline{6}$ in the sample. We are investigating the suitability of the SAX clean-up for capillary electrophoresis (CE) and GC-MS analyses.

D. RECOMMENDED PROCEDURE

This section details the final recommended procedure designed for rapid screening of domoic acid in small portions (2 to 8 g) of tissue samples. The extraction may be scaled up, but it is important to avoid overload of the clean-up cartridge as instructed below.

Preparation of Tissue Homogenate

Tissue samples should first be blended with an equal weight of distilled water in a blender. For representative sampling of shellfish it is generally advisable to use 100 g of tissue. Subsampling from this homogenate can be done immediately after blending while still well-mixed, or later after mixing again very well.

If an analysis must be performed on a limited amount of sample, a portion of chopped or ground tissue (at least 2 g) can be weighed directly into the centrifuge tube used below, followed by an equal weight of distilled water.

Extraction:

NOTE: All extraction and centrifugation is performed in centrifuge tubes, either graduated or marked for the final mixture volume. Disposable tubes and other supplies are recommended to avoid cross-contamination between samples.

1. Weigh accurately W_H grams of homogenate as prepared above into a graduated centrifuge tube. A mimimum of 4 g homogenate (2 g tissue) must be used for the procedure described below.

    [*Recommended:* 8 g homogenate representing 4 g tissue]

2. Add W_H mL methanol to the tube.

    [*Recommended:* 8 mL methanol]

3. Add about 0.5 x W_H mL distilled water to adjust total volume to 2.5 x W_H mL.

   [*Recommended:* about 4 mL water to make to 20.0 mL total]

4. Homogenize the sample extensively using a Polytron for 3 min at 10,000 rpm (do not try to recover all tissue from the probe, but do wash it thoroughly afterwards to prevent contamination of the next sample).

5. Centrifuge for 10 min at 4000 rpm (about x2500 g) or higher, if possible.

6. Remove supernatant (at least 5 mL); if it is not clear, filter through a syringe filter.

   NOTE: We have found that domoic acid is quite stable in mussel tissue extracts for several weeks if stored in the freezer. The vessel must be sealed well to avoid evaporation.

SPE Clean-up:

NOTES:

a. Our solid phase extraction (SPE) experiments were performed on a Supelco SPE manifold system (12 SPE tubes can be used simultaneously).

b. Do not let the cartridges go dry at any point in the procedure.

c. Do not try to increase the loading on the cartridge or breakthrough will occur; the present level is set for a 1 g tissue equivalent loading (5 mL of extract as prepared above).

1. Condition a fresh 3 mL LC-SAX SPE cartridge (Supelco, strong anion exchange, 500 mg packing) with the following:

   a) 6 mL methanol
   b) 3 mL distilled water

c) 6 mL 0.01 N sodium hydroxide in distilled water

d) 3 mL methanol/water (1:1, v/v)

2. Load 5.0 mL sample extract (as prepared above) onto the cartridge and allow to flow slowly (about 1 drop per second). Stop the flow just as the sample meniscus reaches the top of the cartridge packing. Discard the effluent.

3. Wash the cartridge with 5 mL acetonitrile/water (1:9, v/v). Stop the flow just as the solvent meniscus reaches the top of the cartridge packing. Discard the effluent.

4. Add 0.5 mL of Citrate Buffer onto the cartridge. Carefully allow this to flow slowly until the meniscus falls to the top of the packing and then stop the flow. Discard the effluent.

   [Citrate Buffer = 0.25 M citric acid and 0.25 M ammonium citrate in acetonitrile/water (1:9, v/v), pH 4.5]

5. Place 2-mL volumetric tube or flask under the cartridge.

   NOTE: if a high pre-concentration factor is not required for the highest sensitivity, a 5-mL volumetric can be used instead for better precision and slightly higher recovery; in this case, there is no need to perform step 4.

6. Elute the domoic acid with Citrate Buffer slowly (about 1 drop per second) just until the mark is reached on the volumetric tube. Mix the solution before withdrawing an aliquot for LC analysis.

   NOTE: Domoic acid has been found to be quite stable in the Citrate Buffer: less than 1% loss per day for trace level samples (<10 µg/g) if kept in the refrigerator in a well-sealed vial. We have not completed evaluation of the effects of freezing such solutions. It is important to note that it has been observed previously that freezing of 0.1% TFA solutions leads to decomposition of domoic acid (Quilliam *et al.*, 1989a).

HPLC Analysis:

Reference: Quilliam *et al.* (1989a), Int. J. Environ. Anal. Chem. **36**, 139.

Column: 25cm x 4.6 mm ID, Vydac 201TP5 or Supelco LC-PAH

Column Temperature: 40°C

Mobile Phase: 1 mL/min acetonitrile/water (1:9, v/v) with 0.1% trifluoroacetic acid (TFA)

Detector: 242 nm UVD

Injection volume: 20 μL

NOTES:

a. Other C18 stationary phases may be used, but we have found that the Vydac 201TP phase gives the best selectivity for separation of domoic acid and its isomers.

b. The flow rate can be increased to 1.5 to 2 mL/min for high speed analyses; alternatively, a 2.1 mm ID column can be used with up to 0.5 mL/min flow rates and 5 μL injection volume.

c. Separations may be performed at ambient temperature, but analysis time increases by about 20% and retention time reproducibility is not as good. If higher flow rates are used, a heated column is important to reduce solvent viscosity and pressure.

d. Formic acid (0.2%) may be used in place of TFA; this gives a slightly better separation of tryptophan and its derivatives from domoic acid, but results in poorer separation of the domoic acid isomers.

e. Calibration should be performed using accurate solutions of domoic acid in acetonitrile/water (1:9). DACS-1 (89 μg/mL domoic acid; available from NRC's

Marine Analytical Chemistry Standards Program, 1411 Oxford St., Halifax, NS, B3H 3Z1, 902-426-8281) and dilutions thereof may be used to prepare a calibration curve. If good linearity and zero intercept are observed, a single point calibration may then be used routinely.

f. Calculations should be performed using the following equation:

$$C_S \text{ (ug/g tissue)} = \frac{A_S \times C_C}{A_C \times W_H} \times \frac{V_F \times V_T \times 2}{V_A}$$

where:

C_S = domoic acid concentration in tissue sample (not the homogenate) (µg per gram of tissue)

A_S = domoic acid peak area for sample solution

A_C = domoic acid peak area for calibrant solution

C_C = domoic acid concentration in calibration solution (µg/mL)

W_H = weight of homogenate extracted (50% of this is tissue; if sample has been weighed directly into extraction tube, substitute 2 x W_S, where W_S is the weight of tissue) (g)

V_F = final volume of eluate from SPE (either 2.0 mL or 5.0 mL)

V_T = total volume of homogenate and extracting solvent (e.g., 20 mL)

V_A = volume of raw extract applied to SPE cartridge (usually 5.0 mL)

The factor of 2 accounts for the equal weight of distilled water added to the tissue; see "Preparation of Tissue Homogenate", p. 39.

E. EXPERIMENTAL

Materials

All solvents used were HPLC-grade from Anachemia. Distilled water was further purified to HPLC-grade by passage through a Milli-Q (Millipore, Bedford, Mass.) water purification system equipped with ion-exchange and carbon filters. Trifluoroacetic acid (99.5% purity), citric acid, ammonium citrate, formic acid, and ammonium formate were purchased from BDH. Domoic acid calibration standard (DACS-1) and mussel tissue reference material (MUS-1) were provided by the NRC Marine Analytical Chemistry Standards Program (Halifax, NS). Blended tissues with lower levels of domoic acid were prepared by thoroughly mixing MUS-1 with the appropriate weight of control (non-toxic) mussel tissue and an equal weight of distilled water. Subsampling for further dilution with control tissue or for analysis was performed while maintaining mixing of the homogenates.

Extraction Procedures

Tissue samples (usually 100 g) were first blended with an equal weight of distilled water using a Waring commercial blender (Model 33BL73). Aqueous methanol dispersive extractions were performed as detailed in Section D using a Brinkman PT3000 Polytron equipped with a PT-DA-3012/2T generator. Centrifugation was performed on an IEC Centra MP4R centrifuge equipped with an 804S fixed angle rotor operated at 7000 rpm. Boiling aqueous extractions were performed as described previously (Quilliam *et al.*, 1989a).

Clean-up Procedures

The strong anion exchange (SAX) SPE clean-up was performed as detailed in Section D using 3 mL LC-SAX (500 mg) cartridges from Supelco (Bellefonte, PA). C18 SPE clean-up was performed as described previously (Quilliam *et al.*, 1989a), with the following clarification: collection of the eluate from the cartridge begins immediately after the sample is placed on top of the cartridge.

HPLC

Most isocratic HPLC-UVD analyses were performed on a simple modular system made from the following components: Hewlett-Packard HP1050 autosampler, Beckman 112 pump, Fiatron CH30 column heater and TC50 temperature controller, Schoeffel SF770 variable wavelength UV detector, and Hewlett-Packard HP3396 recording integrator. Cardinal data from the integrator was sent via the RS232C serial port to an 80386 MS-DOS personal computer running the Chromperfect 2 software (Justice Innovations Inc., Palo Alto). Sigmaplot 4.0 software (Jandel Scientific) was used for plotting chromatograms and other data.

HPLC-DAD analyses were performed on an Hewlett-Packard HP1090M system with a ternary DR5 pumping system, variable volume injector and autosampler, built-in HP1040 DAD and HP79994 data system. The DAD was operated with measurement of absorption at 242 nm with a 10 nm bandwidth, and with peak-triggered UV spectral acquisition.

All HPLC-UVD data presented in this report were acquired using a 5 μm Vydac 201TP column (25 cm x 4.6 mm I.D.) (Separations Group, Hesperia, CA) equipped with a guard column and maintained at 40°C. The mobile phase was aqueous acetonitrile with 0.1% (v/v) trifluoroacetic acid pumped at a flow rate of 1 mL/min. Isocratic analyses were performed with 10% acetonitrile, while gradient analyses used a linear program from 5% to 25% acetonitrile over 25 min. A 20 μL injection volume was used for extracts taken through the clean-up; only 4 μL were used for raw aqueous methanol extracts.

LC-MS

LC-MS experiments were conducted on a SCIEX (Thornhill, Ont.) API-*III* tandem mass spectrometer equipped with an IonSpray™ interface. Separations were performed as above, except that a 25 cm x 1.0 mm I.D. column (packed with 5 μm Vydac 201TP) was used at ambient temperature with a flow rate of 50 μL/min and an injection volume of 1 μL.

F. ACKNOWLEDGMENTS

G. REFERENCES

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