ELSEVIER

Journal of Chromatography A, 807 (1998) 229–239

JOURNAL OF CHROMATOGRAPHY A

Improved method for preparation and use of 9-anthryldiazomethane for derivatization of hydroxycarboxylic acids Application to diarrhetic shellfish poisoning toxins[1]

Michael A. Quilliam[a,*], Ana Gago-Martínez[b], José A. Rodríguez-Vázquez[b]

[a]*Institute for Marine Biosciences, National Research Council of Canada, 1411 Oxford Street, Halifax, Nova Scotia B3H 3Z1, Canada*
[b]*Universidad de Vigo, Facultad de Ciencias, Dpto. Química Analítica y Alimentaria, Aptdo. 874, 36200 Vigo, Spain*

Received 20 January 1997; received in revised form 23 January 1998; accepted 23 January 1998

Abstract

Application of a method for the ''in situ'' generation of 9-anthryldiazomethane (ADAM) to the derivatization of the carboxyl function in diarrhetic shellfish poisoning (DSP) toxins revealed the formation of artifact products. Using liquid chromatography–mass spectrometry, it was determined that these artifacts were due to base-catalyzed reactions between the solvent, ethyl acetate, and the hydroxyl groups of the analyte to produce *O*-acetylated ADAM derivatives. Using a new formulation, with tetrahydrofuran as solvent, it was possible to eliminate these artifact reactions. Various reaction parameters have also been re-optimized to ensure quantitative derivatizations. An assessment method was developed that was useful not only for optimizing reaction parameters, but also for evaluating the reagent potency before use on important samples. Finally, application of the method to the determination of DSP toxins in plankton and mussel tissue was demonstrated. Published by Elsevier Science B.V.

Keywords: Diarrhetic shellfish poisoning; Derivatization, LC; Anthryldiazomethane; Hydroxycarboxylic acids; Carboxylic acids; Toxins; Okadaic acid; Octanoic acid

1. Introduction

9-Anthryldiazomethane (ADAM) (Fig. 1) is a useful derivatization reagent for the analysis of carboxylic acids by liquid chromatography (LC) with fluorescence detection. It has been used for a wide range of compounds, including fatty acids [1–3], prostaglandins [4], acidic herbicides [5], polyether antibiotics [6], and diarrhetic shellfish poisoning (DSP) toxins (Fig. 2) [7–11]. ADAM provides significant advantages over many other reagents, such as selective derivatization of the carboxyl function, rapid and quantitative reaction under mild conditions and a highly fluorescent chromophore. Unfortunately, because it is very unstable, the reagent must be stored at low temperature (e.g., −70°C) and used promptly after the solution is prepared. Decomposition of the reagent may result in incomplete derivatization, as well as side products that can interfere in the analysis. Several commercial sources are available but the reagent is quite expensive. In some countries it is difficult to find a reliable supplier or means of delivery. Although ADAM can be synthesized by oxidation of 9-anthraldehyde hydrazone with mercuric oxide [12] or activated

*Corresponding author.
[1]NRCC No. 39740.

0021-9673/98/$19.00 Published by Elsevier Science B.V.
PII: S0021-9673(98)00069-7

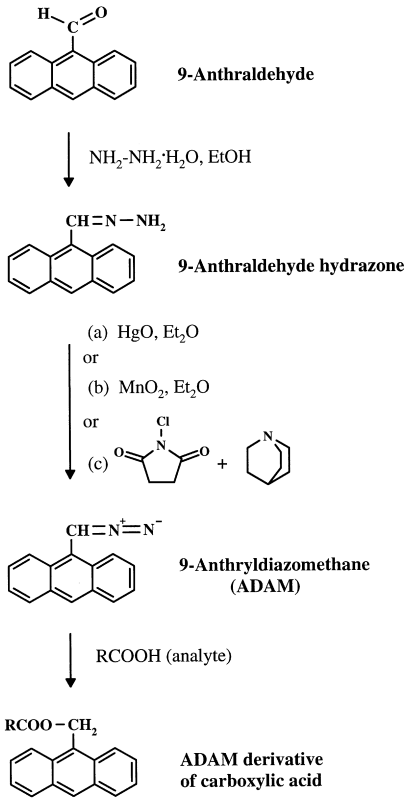
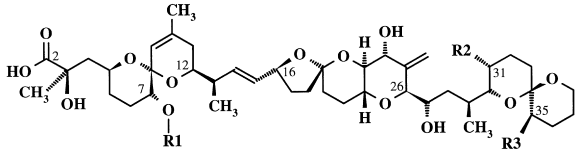


Fig. 1. Synthesis of 9-anthryldiazomethane (ADAM) and its reaction with carboxylic acids.



R1	R2	R3	
H	Me	H	Okadaic acid (OA)
H	Me	Me	DTX1
H	H	Me	DTX2
CH_3CO	Me	H	7-O-Acetyl-OA

Fig. 2. Structures of diarrhetic shellfish poisoning (DSP) toxins and the 7-*O*-acetyl derivative of okadaic acid.

manganese dioxide [2], these reactions give relatively low yields and are difficult to perform.

Yoshida et al. [13] reported a convenient method for the ''in situ'' generation of ADAM by oxidation of the stable 9-anthraldehyde hydrazone in ethyl acetate, using an organic oxidant such as *N*-chlorosuccinimide in the presence of a base such as quinuclidine (see Fig. 1c). The hydrazone is available commercially or may be produced by reaction of 9-anthraldehyde with hydrazine in ethanol [12], as shown in Fig. 1. The problem of ADAM decomposition in storage can thus be solved by preparing a fresh solution whenever it is required. We have found the application of this reagent system to DSP toxins to be feasible [8–10], but we have occasionally observed extra peaks in chromatograms [8,9]. We have now determined that these peaks are due to artifacts arising from reactions between the reagent and hydroxyl functions in analytes, and report here on the identity and origin of the artifacts, as well as elimination of the problem with a new reagent formulation.

2. Experimental

2.1. Chemicals

HPLC-grade methanol, acetonitrile, ethyl acetate, tetrahydrofuran (THF) and *n*-hexane were obtained from BDH (Toronto, Canada). Chloroform (ACS grade, with 0.75% ethanol as stabilizer) was also purchased from BDH. HPLC-grade water was prepared by passing glass-distilled water through a Milli-Q water purification system equipped with ion-exchange and carbon filters (Millipore, Bedford, MA, USA). Quinuclidine, *N*-chlorosuccinimide, 9-anthraldehyde, hydrazine monohydrate and *N*,*N*-dimethylformamide were obtained from Aldrich (Milwaukee, WI, USA). Octanoic acid was purchased from Sigma (St. Louis, MO, USA). ADAM, delivered on dry ice from the supplier (Molecular Probes, Eugene, OR, USA), was immediately divided into 2.5 mg portions and stored in amber vials at −70°C.

The hydrazone of 9-anthraldehyde was prepared according to Nakaya et al. [12] by reaction of 9-anthraldehyde and hydrazine monohydrate in etha-

nol. It may also be purchased from Lancaster Synthesis (Windham, NH, USA). 7-*O*-Acetyl-okadaic acid was prepared as described previously [11] by reaction of okadaic acid with acetic anhydride in pyridine.

OACS-1, a certified calibration solution of okadaic acid (25 μg/ml), and MUS-2, a certified mussel tissue reference material, were provided by the Marine Analytical Chemistry Standards Program (National Research Council, Halifax, Canada). Plankton samples were kindly provided by J. McLachlan (National Research Council).

2.2. Reagent preparation

The reagent based on commercial ADAM was prepared daily by dissolving 2.5 mg of ADAM in 1 ml of THF. This was used as soon as possible and handled under yellow or subdued lighting.

Prior to preparation of the in situ ADAM reagent, the following solutions were prepared: (A) 9-anthraldehyde hydrazone in THF (35 m*M*, 7.70 mg/ml); (B) *N*-chlorosuccinimide in THF (35 m*M*, 4.7 mg/ml); and (C) quinuclidine in THF (70 m*M*, 7.8 mg/ml). These solutions could be stored for several days in the freezer but were always warmed to room temperature before use. The reagent was then prepared by mixing 500 μl aliquots of each solution: A, B and C. The solution was vortex mixed for 1 min. Any precipitate of quinuclidine hydrochloride was allowed to settle out. This does not appear to interfere with subsequent derivatization steps. The reagent mixture was allowed to stand at room temperature in the dark for 60 min and then used directly for the derivatization.

2.3. Reagent assessment

Reagent potency was assessed by a method similar to that proposed by Tuinstra et al. [14]. First, a solution of octanoic acid in THF (11.7 m*M*, 1.69 mg/ml) was prepared. Reaction 1 (excess ADAM, 10:1 molar ratio to octanoic acid) was prepared by mixing 200 μl ADAM reagent (either commercial or in situ type, in THF), 20 μl octanoic acid solution and 50 μl methanol. Reaction 2 (excess octanoic acid, 10:1 molar ratio to ADAM) was prepared by mixing 20 μl ADAM reagent solution, 200 μl octanoic acid solution and 50 μl methanol. Both reaction vials were capped tightly and left for 2 h at 37°C in the dark. After cooling, both were analyzed by LC with either UV or fluorescence detection. The potency of the ADAM reagent was calculated from the ADAM–octanoate peak area ratio (reaction-2/reaction-1) multiplied by 100. As discussed in Section 3.2, this is simply a measure of potency and does not translate directly into reagent purity. Usually only 30–40% is obtained due to decomposition of ADAM during the derivatization reaction.

2.4. Sample preparation and derivatization

Details of sample extraction procedures have been published previously [11]. Briefly, digestive glands from shellfish samples were extracted with methanol–water (4:1). Crude extracts were washed with hexane and partitioned with chloroform. Residues from the chloroform extract were dissolved in methanol to give a final extract containing 1 g tissue equivalent per ml of solution.

Derivatization was accomplished by first transferring 35.0-μl aliquots of either sample extracts or calibration solutions (OACS-1 or dilutions thereof) into 1.5-ml amber vials and then adding 100-μl aliquots of ADAM reagent to each vial. All work was done under yellow or subdued lighting. After sealing tightly with a PTFE-lined screw cap, the solutions were sonicated for 10 min in warm water (37°C) and then heated at 37°C for 2.5 h in the dark. Finally, a vacuum centrifuge (SVC-100H SpeedVac, Savant Instruments, Farmingdale, NY, USA) was used to evaporate all reaction solutions to dryness.

Clean, dry glass SPE tubes (7 ml capacity) equipped with PTFE frits were placed on an SPE vacuum manifold and packed with 500 mg of activated silica. The columns were conditioned with 6 ml chloroform, followed by 3 ml chloroform–hexane (1:1). The flow was stopped when the meniscus reached the top of the packing; the columns were not allowed to go dry thereafter. Residues from the evaporated ADAM reactions were re-dissolved and transferred to the columns using three 300-μl aliquots of chloroform–hexane (1:1) and passed slowly (1 drop/s) through to waste. The columns were washed with 5 ml chloroform–hexane (1:1), followed by 5 ml chloroform adjusted to

contain 1.15% ethanol [11]. After placing glass tubes under each column, 5 ml methanol–chloroform (1:9) was used to elute the ADAM derivatives. Eluates were evaporated to dryness under a stream of nitrogen and the residues were dissolved in exactly 500 μl methanol. These solutions were transferred to amber crimp-top vials for LC analysis.

2.5. Instrumentation

LC analyses were performed using a Hewlett-Packard (Palo Alto, CA, USA) HP1090L liquid chromatograph equipped with a ternary DR5 pumping system, variable volume injector, refrigerated autosampler, HP1040 diode array detector, HP1046A fluorescence detector and HP79994A data system. The LC column was stainless steel, 25 cm×4.0 mm I.D., packed with 5 mm LiChrospher-100 RP18 octadecylsilica (Merck, Darmstadt, Germany) and was maintained at 40°C. The mobile phase was aqueous 80% or 90% acetonitrile, the flow-rate was 1.0 ml/min and the injection volume was 10 μl. The fluorescence detector was operated with 254 nm excitation, 412 nm emission protected by a 280 nm cut-off filter, and xenon lamp pulse frequency of 55 Hz. The detector gain setting was adjusted to suit the concentration range of samples.

LC–MS analyses were performed using an HP1090L LC coupled to an API-III+ triple quadrupole mass spectrometer (SCIEX, Concord, Canada) equipped with an atmospheric pressure ionization (API) source and ion-spray interface. The same LC conditions as above were used except that 0.1% trifluoroacetic acid was added to the mobile phase in order to promote ionization, and the column was operated at ambient temperature.

3. Results and discussion

3.1. Identification of artifacts

We have found the method of Yoshida et al. [13] for in situ formation of the ADAM reagent to be very useful for the analysis of DSP toxins [8–10]. Occasionally, however, we have observed extra peaks in chromatograms [8,9]. A small peak at a relative retention time (RRT) of 1.26 (relative to the ADAM derivative of okadaic acid) appeared consistently with calibration solutions and sometimes with sample extracts. Occasionally, we have observed much stronger intensities of this peak and the appearance of two others at RRTs of 1.36 and 1.68 min, as shown in Fig. 3. The appearance of these peaks was at the expense of the ADAM–okadaate peak area. It was also noticed that the appearance of high levels of the artifact peaks generally correlated with the use of older solutions of quinuclidine in ethyl acetate. From these observations, it was apparent that artifact reactions were occurring and leading to side products due to additional modifications of either the reagent moiety or the okadaic acid structure itself. The

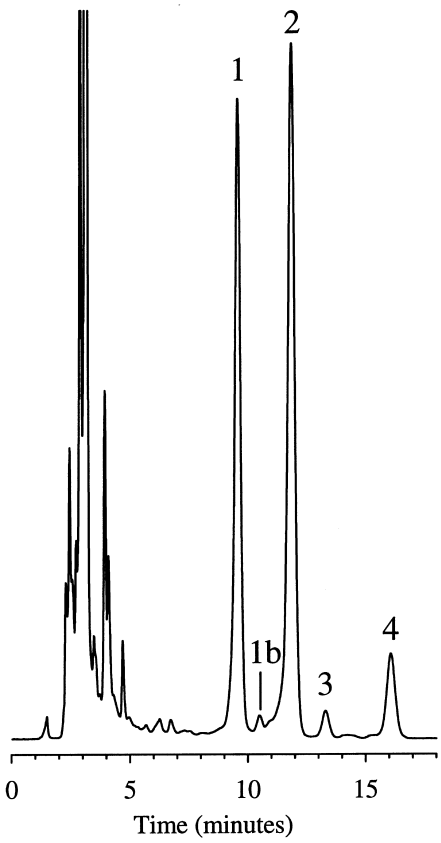


Fig. 3. LC–fluorescence chromatogram for reaction of Yoshida et al. [13] in situ ADAM reagent system with an okadaic acid standard (4 μg). Peak 1 is due to ADAM–okadaate and peak 1b is due to the derivative of an isomeric impurity in the standard. Peaks 2 to 4 are due to artifacts, as discussed in Section 3.1. Conditions: as in Section 2.5 with an aqueous 90% acetonitrile mobile phase.

reduced level of artifact formation with extracts of tissue samples suggested that co-extracted compounds inhibited the side reactions.

Examination of the excitation and emission fluorescence spectra for peaks 1 to 4 (Fig. 3) showed that they were all identical. This indicated that the structural modifications in the artifacts were remote from the ADAM moiety. The same sample that gave the chromatogram in Fig. 3 was subsequently analyzed by LC–MS with ion-spray ionization [11,15] and the results are presented in Fig. 4. The total ion current chromatogram (Fig. 4a) showed four peaks, whose relative retention times correlated with those in Fig. 3. The absolute retention times were different due to the use of a lower column temperature in the LC–MS analysis. The mass spectrum recorded at the apex of peak 1 (Fig. 4b) confirmed this component to be ADAM–okadaate. An $[M+H]^+$ ion was observed at m/z 995.6 and fragment ions due to sequential losses of water were observed at m/z 977.6 and 959.6. An additional ion observed at m/z 1012.6 appears to be $[M+NH_4]^+$, which could have been due in part to an ammonium salt contamination of the LC pumping system. The spectrum for peak 2 (Fig. 4c) showed a similar pattern of ions, but shifted higher by 42 u. Peak 3 gave a spectrum identical to

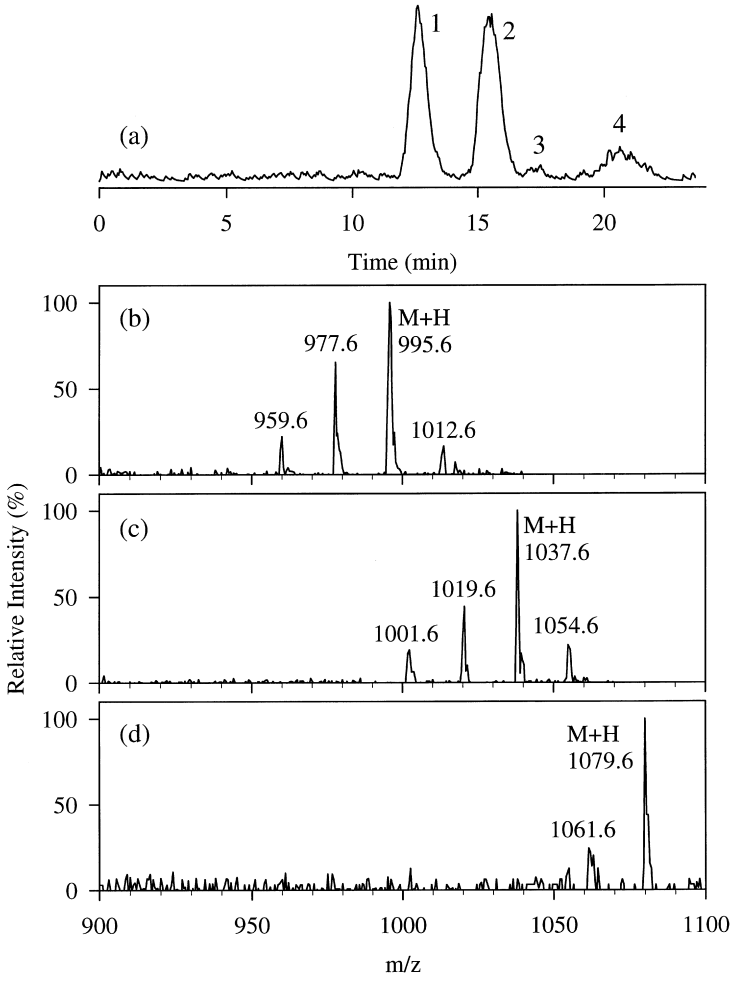


Fig. 4. LC–MS analysis of the same in situ ADAM reaction of okadaic acid shown in Fig. 3. The total ion current chromatogram is presented in (a), while the ion-spray mass spectra acquired for peaks 1, 2 and 4 are given in (b), (c) and (d), respectively. Conditions: full scan acquisition; chromatography as in Fig. 3, except column at room temperature.

that of peak 2 (data not shown), while peak 4 gave a spectrum with ions shifted higher by another 42 u (Fig. 4d). These data suggested the substitution of acetyl (CH_3CO) groups for hydrogens, presumably on one or two of the four hydroxyls present in okadaic acid.

Treatment of okadaic acid with an equimolar concentration of acetic anhydride, followed by derivatization with commercial ADAM, resulted in three new peaks in the LC–fluorescence chromatogram, at exactly the same retention times as peaks 2, 3 and 4 in Fig. 3. The fluorescence and mass spectra of these acetylation products were found to be identical to those of the compounds giving the artifact peaks. Coincidentally, the 7-*O*-acetyl derivative of okadaic acid had already been synthesized in one of our laboratories and used as an internal standard for DSP toxin analysis [11]. The ADAM derivative of this compound gave a retention time identical to that of artifact peak 2. It should be noted that the 7-hydroxy function of okadaic acid is particularly reactive and is readily acetylated [11]. Thus, we conclude that the primary artifact (peak 2) was due to the ADAM derivative of 7-*O*-acetyl-okadaic acid, while peak 3 was due to an isomer with acetyl substitution at a different hydroxyl and peak 4 was due to a doubly-acetylated product.

It is clear that the only possible source of the acetyl function in the ADAM derivatization procedure is ethyl acetate, the solvent recommended by Yoshida et al. for the in situ reaction [13]. We have not explored the mechanism of this acetylation any further. At this point, we can only speculate that quinuclidine catalyzes or is directly involved in the formation of an active intermediate from ethyl acetate that can then acetylate hydroxyl functions. There is a precedent in the literature for the formation of reactive acylating agents from esters and nucleophilic tertiary amines. As discussed in detail for imidazole [16], this phenomenon is known as nucleophilic catalysis and proceeds through intermediates with a $CH_3CO–N^+R_3$ structure (i.e., *N*-acetylquinuclidine in this case).

3.2. Reagent optimization

The obvious solution to this problem was to eliminate the ethyl acetate from the reagent system. Therefore we investigated alternative solvents, including *N*,*N*-dimethylformamide, acetonitrile and THF. All three solvents were found to eliminate the artifact formation entirely. THF proved to be the most suitable solvent because it gave a cleaner blank reaction and it was a good solvent for all reagents. Therefore THF was substituted for ethyl acetate in the in situ method.

With a change in reaction solvent, it became important to check all reaction parameters. One of the first concerns was to optimize reagent yield in the reagent preparation step. Yoshida et al. [13] used a reaction with excess lauric acid to monitor reagent yield from the in situ reaction. Tuinstra et al. [14] took this approach further and developed what they claimed to be a comprehensive method for determination of ADAM purity. In this method, a calibration curve was generated first by analyzing reactions of excess ADAM with known amounts of heptanoic acid. The slope of the curve gave the molar response for the ADAM derivative. An excess of heptanoic acid was then reacted with a known mass of ADAM. It was suggested that the results would allow a calculation of actual ADAM concentration in the reagent solution.

We have tested a similar procedure for ADAM reagent assessment. Octanoic acid was selected as the test substrate since it gave a good retention time (11.5 min) under the LC conditions used for DSP toxin determinations. Fig. 5 shows the LC analysis of the reaction of octanoic acid with an excess of ADAM. Both UV and fluorescence detection were used in this experiment to demonstrate that either detector may be used for the experiment. The UV detector proved very useful because of the long-term reproducibility of absorbance measurements. Once it had been established that the LC gave a linear response for ADAM–octanoate and that the detector signal was not saturated ($<$1.2 AU), we found it acceptable to perform just two reactions as detailed in the Section 2.3 (i.e., ADAM–octanoate ratios of 10:1 and 1:10) to derive a value for ADAM ‘‘potency’’. Whether this potency value represents actual yield, as claimed by Tuinstra et al. [14], will be discussed further below.

One of our first concerns was the overall concentration of ADAM, which determines the reagent to analyte ratio. In past experiments, we have found

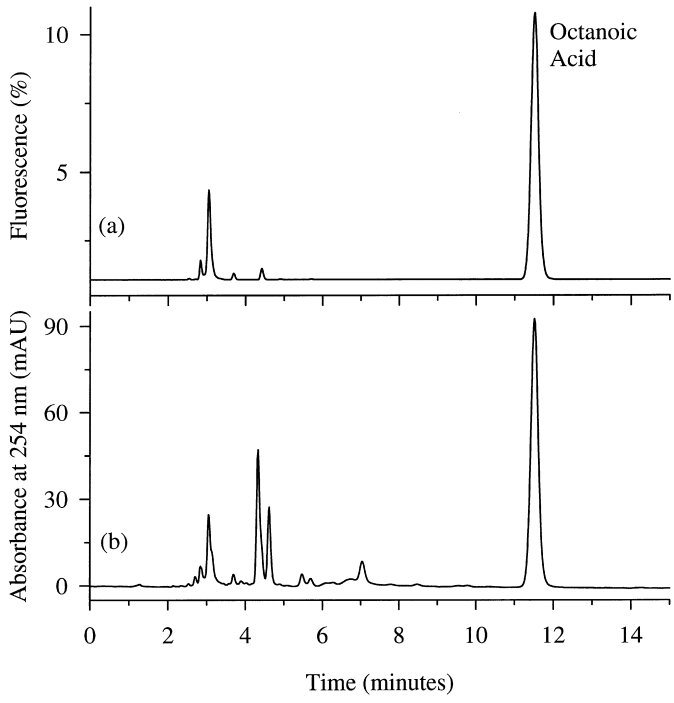


Fig. 5. LC chromatograms for a reaction of excess octanoic acid with the new formulation of in situ ADAM. Detection was provided by both fluorescence (a) and UV absorbance (b).

it necessary to increase ADAM concentration from the 0.1% (w/v) level used by Lee et al. [7] to 0.2% (w/v) or 9.6 m*M* in order to achieve quantitative conversion of analytes in real-world sample extracts [11]. The Yoshida in situ procedure [13] called for the following solutions: (A) 6.9 m*M* 9-anthraldehyde hydrazone; (B) 6.9 m*M* *N*-chlorosuccinimide; and (C) 69 m*M* quinuclidine. After mixing equal portions, a 2.3 m*M* ADAM concentration would result if we assume a 100% reaction yield. Therefore, in our experiments, the 9-anthraldehyde hydrazone concentration was increased five-fold to 35 m*M* (solution A), which resulted in an 11.7 m*M* ADAM concentration assuming a 100% conversion of the hydrazone.

Variation of the *N*-chlorosuccinimide concentration in solution B over the range of 20 to 60 m*M* revealed that 35 m*M* was the optimum for reagent yield (i.e., equimolar with the hydrazone). Increased quinuclidine concentration, over that recommended by Yoshida et al., did not show increased yields. Therefore, 70 m*M* quinuclidine was selected for solution C.

At this stage, it appeared that reagent concentrations were optimized. However, whenever reagent potency was measured, as described above and in Section 2.3, it was never greater than 30–40%. Was it possible that the percentage conversion of hydrazone to ADAM was so low, or had reactions with octanoic acid not gone to completion? When several samples of commercial ADAM were tested, it was found that potency values ranged from 40% for fresh material down to 20% for some older materials. It was difficult to believe that purity of the commercial material could be so poor. In order to better understand what was happening and to better control the reagent assessment procedure, we performed a series of experiments to study the kinetics of ADAM production, its reaction with octanoic acid and its decomposition under different conditions.

First, it was important to study the reaction of excess octanoic acid with commercial ADAM (not

the in situ reagent) to see how long that took to proceed to completion. Two reactions with a 10:1 molar excess of octanoic acid over ADAM were conducted, one with no methanol present (only THF) and the other with 20% methanol in THF. These solutions were placed in the LC autosampler, maintained at 37°C and analyzed by LC–UV at timed intervals. From the resulting ADAM–octanoate peak area data (not shown), it was apparent that methanol had a dramatic effect on reaction rate. When methanol was present, no further increase in the peak area occurred after 24 min; with THF only, the peak area was still increasing after 2 h and had still not reached the level achieved with 20% methanol present. Such observations on the catalytic effect of alcohols have been reported previously for diazomethane [17] and even for ADAM [6]. Since the mechanism for reaction of a diazo compound with a carboxylic acid involves a first step of proton transfer from oxygen to carbon to give a diazonium ion ($RCH_2N_2^+$) and a carboxylate anion [18], it is no surprise that the nature of the solvent plays a significant role. It is a concern, however, that some reported ADAM methods are based upon the use of nonpolar solvents entirely [2,4]. It is clear that samples should be dissolved in methanol to improve reaction kinetics. A reaction time of 30 min appeared suitable for assessment experiments in which octanoic acid is in excess.

Most importantly, when the results of this experiment were calibrated using a molar response factor for ADAM–octanoate (see Section 2.3), it was found that the maximum conversion of hydrazone to ADAM–octanoate was only 37%. This agreed with the earlier observations and suggested that either the hydrazone had not been fully converted to ADAM or the ADAM was decomposing during the course of the derivatization reaction with octanoic acid.

The next experiment was a fairly complicated one designed to determine the kinetics of ADAM formation for the in situ reaction, as well as the effects of heat and methanol on the stability of the generated ADAM. After mixing the three reagent solutions (A, B and C), the ADAM reagent mixture was first allowed to react at room temperature. Aliquots were withdrawn at timed intervals, mixed with excess octanoic acid and analyzed by LC to quantify the resulting ADAM–octanoate after 30 min reactions at 37°C. At the 60 min point, the reagent mixture was split into three portions. One portion was mixed with a 20% volume of methanol and placed in a heater block at 37°C. The other two portions were simply diluted with an additional 20% volume of THF. One of these latter was maintained at 37°C, while the other was left at room temperature. Aliquots were again withdrawn from each of these three reactions at timed intervals, mixed with excess octanoic acid and analyzed by LC after 30 min reactions at 37°C. Peak areas were corrected for the dilution caused by addition of extra solvent.

Fig. 6 shows the results of this experiment. If we assume that the ADAM–octanoate peak area is proportional to ADAM concentration, the ADAM concentration in the reagent mixture increased rapidly over the first 10 min and approached a maximum at 60 min. With only THF present and a temperature of 20°C, the ADAM concentration remained essentially constant for up to 4 h. These results indicated that the reagent should be used between 1 and 4 h after preparation, although it is likely that it could be stored in the freezer for a considerable time, as reported by Yoshida et al. [13]. With a temperature of 37°C, the THF-only mixture showed a 20% decomposition of ADAM after 2 h, indicating a significant thermal instability. When methanol was present, along with heating at 37°C, there was an even more significant decomposition: about 50% after 2 h heating. This is most likely due to an additional side-reaction of the diazonium ion with methanol to form 9-anthracenylmethanol [18]. These results indicate that an excess of ADAM is required for derivatization experiments, not only to deal with the consumption of reagent by sample co-extractives, but also to deal with thermolytic and solvent-induced decomposition during the reaction. This is true for both commercial ADAM and in situ generated reagent.

The results of both of these experiments indicate that it will not be possible to measure quantitatively the purity of ADAM or its yield in the in situ reaction by using the Tuinstra et al. [14] approach. Nevertheless, the procedure is useful for the routine assessment of reagent potency on a relative basis. To properly calibrate the method, it would be necessary to have a reference sample of ADAM of known purity. This could probably be best achieved by

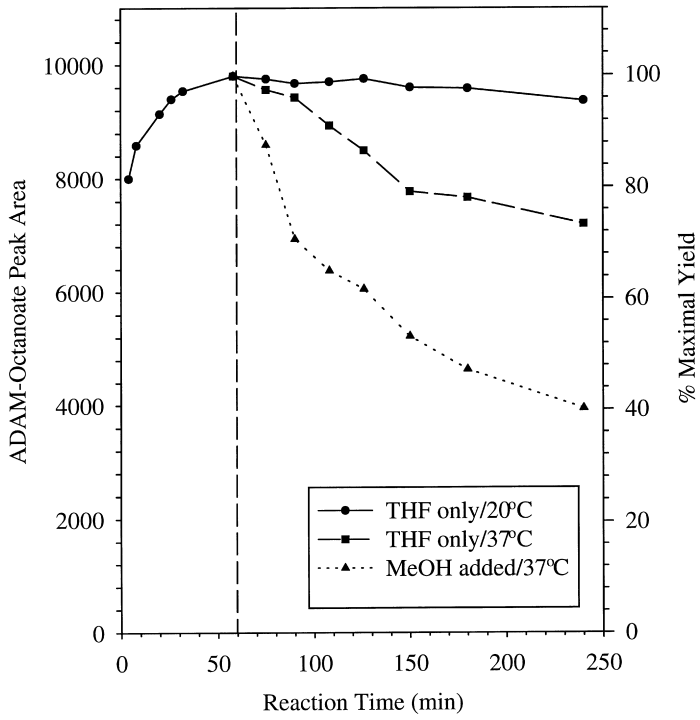


Fig. 6. The kinetics of ADAM formation in the in situ reaction, and the effect of heat and methanol on the stability of the generated ADAM. After mixing solutions A, B and C (see Section 2.2) to produce the reagent mixture, the reaction was allowed to proceed at room temperature. At 60 min the reagent mixture was split into three portions. One portion was mixed with a 20% volume of methanol and heated at 37°C (triangles). The other two portions were simply mixed with an additional 20% volume of THF. One of these was maintained at 37°C (squares), while the other was left at room temperature (circles). The assessment procedure, based on reaction of solution aliquots with excess octanoic acid (see Section 2.3), was used to measure ADAM yield at timed intervals.

performing quantitative NMR on a sample of commercial ADAM. However, this was deemed outside the scope of the present study.

In a third experiment, the rate of reaction of excess (10:1) ADAM with octanoic acid was studied. Two reactions were initiated, one with 20% methanol present and the other with only THF, and maintained at 37°C in the LC autosampler. Repeated analyses at timed intervals generated the data presented in Fig. 7. The results again show that methanol increases the rate of reaction for ADAM and is essential for quantitative conversion of the analyte to the derivative. Even after 3 h reaction, the THF-only reaction is less than 50% complete. The reaction with methanol present, however, is complete after 2.5 h. Therefore, 2.5 h was selected as the reaction time for sample derivatization. It should be noted that reaction kinetics with the DSP toxin, okadaic acid, (in actual mussel tissue extracts) have also been tested and it was determined that a 2- to 2.5-h reaction was adequate for quantitative derivatization.

3.3. Application

The optimized in situ ADAM derivatization procedure, detailed in Section 2.4, has been used successfully in our laboratories for two years now. Fig. 8 shows some representative results for the analysis of DSP toxins. The first chromatogram (Fig. 8a) shows the results for an okadaic acid standard and it is clear that none of the acetyl artifacts (compare to Fig. 3) have been formed. The other chromatograms in Fig. 8 show the analyses of extracts of a *Prorocentrum lima* sample (Fig. 8b), MUS-2 mussel tissue certified reference material (Fig. 8c), and a control (non-toxic) mussel tissue

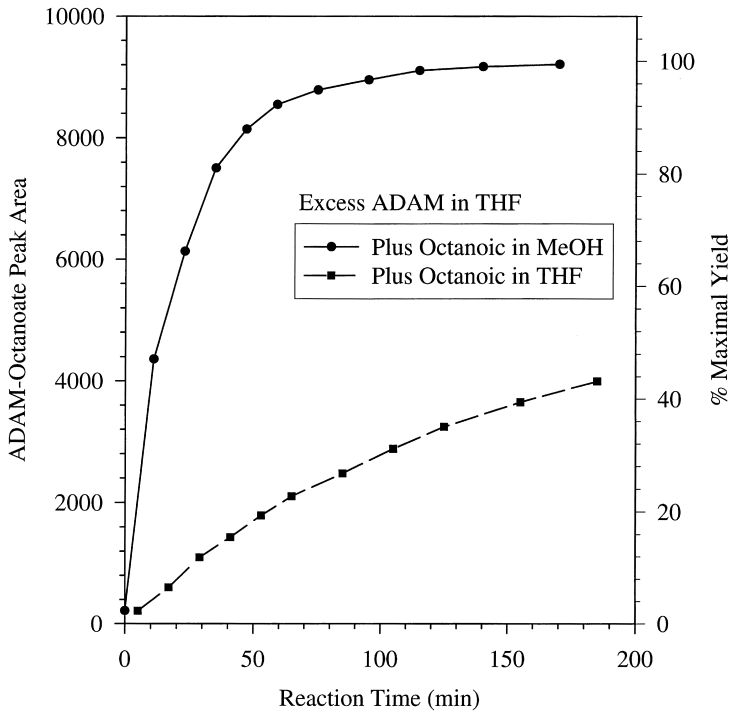


Fig. 7. The kinetics of conversion of ADAM to ADAM–octanoate as a function of reaction time for reactions with an excess of octanoic acid (10:1 molar ratio), in the presence (20%, v/v, circles) or absence (squares) of methanol. These solutions were placed in the LC autosampler, maintained at 37°C and analyzed by LC–UV at timed intervals.

sample (Fig. 8d). MUS-2 is an homogenized slurry of mussel digestive glands certified to contain 11.0±0.3 μg/g okadaic acid and 0.96±0.081 μg/g DTX1. A quantitative trial using the in situ reagent and external calibration on three replicate samples gave 11.7±0.6 μg/g okadaic acid and 0.97±0.09 μg/g DTX1, close matches with the certified values. Assuming that digestive glands represent approximately 20% of the whole animal, the DTX1 concentration in MUS-2 is equivalent to 200 ng/g in whole tissue, the legal tolerance level in most countries.

4. Conclusions

Using a new formulation of the in situ ADAM reagent system previously proposed by Yoshida et al. [13], it has been possible to eliminate artifact reactions caused by acetylation of hydroxyl functions in analytes. It was established that ethyl acetate is the source of the acetyl group and that substitution by THF solves the problem. Various parameters have been re-optimized to ensure quantitative derivatizations. An assessment method based on the reaction of ADAM with excess octanoic acid was found to be useful not only for optimizing reaction parameters, but also for evaluating the reagent potency before use on important samples. However, it has been determined that, due to decomposition of ADAM during the course of any derivatization reaction, it is not possible to use this procedure for the quantitative measurement of ADAM purity as suggested previously by Tuinstra et al. [14].

Acknowledgements

The technical assistance of Mr. W. Hardstaff is appreciated, as are helpful discussions with Dr. J. Pincock (Chemistry Department, Dalhousie University). AGM gratefully acknowledges the financial

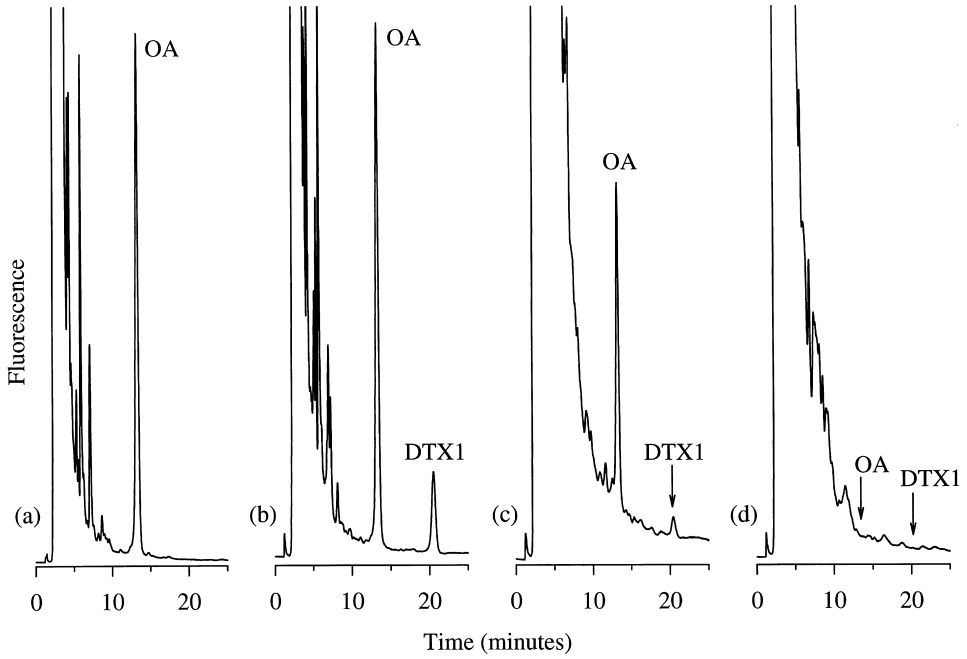


Fig. 8. Application of the optimized in situ ADAM reagent system to the LC analyses of (a) okadaic acid standard (250 ng derivatized, 5 ng injected) and extracts of: (b) a *Prorocentrum lima* sample (1 mg derivatized); (c) MUS-2 mussel tissue certified reference material containing 11.0 μg/g okadaic acid and 0.96 μg/g DTX1 (35 mg tissue derivatized); and (d) a control mussel tissue sample (35 mg tissue derivatized). Conditions: as in Section 2.5 with an aqueous 80% acetonitrile mobile phase.

support of the Conselleria de Educacion, Xunta de Galicia.

References

[1] N. Nimura, T. Kinoshita, Anal. Lett. 13 (1980) 191.
[2] S.A. Barker, J.A. Monti, S.T. Christian, F. Benington, R.D. Morin, Anal. Biochem. 107 (1980) 116.
[3] G. Kargas, T. Rudy, T. Spennetta, K. Takayama, N. Querishi, E. Shrago, J. Chromatogr. 526 (1990) 331.
[4] M. Hatsumi, S. Kimata, K. Hirosawa, J. Chromatogr. 253 (1982) 271.
[5] T. Suzuki, S. Watanabe, J. Chromatogr. 541 (1991) 359.
[6] K. Takatsuki, S. Suzuki, I. Ushizawa, J. AOAC Int. 69 (1986) 443.
[7] J.S. Lee, T. Yanagi, R. Kenma, T. Yasumoto, Agric. Biol. Chem. 51 (1987) 877.
[8] A. Gago-Martinez, Doctoral Thesis, Universidade de Vigo, Vigo, Spain, 1992.
[9] J.A. Rodriguez-Vazquez, A. Gago-Martinez, A. Ibanez-Paniello, P. Burdaspal-Perez and T. Legarda-Gomez, in T.J. Smayda and Y. Shimizu (Editors), Toxic Phytoplankton Blooms in the Sea, Elsevier, Amsterdam, 1993, p. 571.
[10] J.C. Marr, L.M. McDowell, M.A. Quilliam, Nat. Toxins 2 (1994) 302.
[11] M.A. Quilliam, J. AOAC Int. 78 (1995) 555.
[12] T. Nakaya, T. Tomomoto, M. Imoto, Bull. Chem. Soc. Japan 40 (1967) 691.
[13] T. Yoshida, A. Uetake, H. Yamaguchi, N. Nimura, T. Kinoshita, Anal. Biochem. 173 (1988) 70.
[14] L.G.M.Th. Tuinstra, A.H. Roos, J.M.P. van Trijp, M. Kockerols and R. Hartemink, in J.M. Fremy (Editor), Actes Du Colloque Sur Les Biotoxines Marines, Centre National d'Etudes Veterinaires et Alimentaires, Paris, 1991, p. 111.
[15] M.A. Quilliam, in D. Barcelo (Editor), Applications of LC–MS in Environmental Chemistry, Elsevier, Amsterdam, 1996, p. 415.
[16] W.P. Jencks, Catalysis in Chemistry and Enzymology, Dover Publications, New York, 1987, p. 67.
[17] H. Schlenk, J.L. Gellerman, Anal. Chem. 32 (1960) 1412.
[18] M. Regitz and G. Maas, Diazo Compounds: Properties and Synthesis, Academic Press, Orlando, FL, 1986, pp. 96–114.