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Improvement on sample clean-up for high-performance liquid chromatographic–fluorimetric determination of diarrhetic shellfish toxins using 1-bromoacetylpyrene

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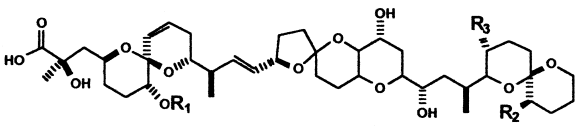
Abstract

Okadaic acid (OA) and dinophysistoxin-2, two of the main diarrhetic shellfish toxins, can be determined by high-performance liquid chromatography coupled to fluorimetry as pyrenacyl esters. Toxin fluorescent derivatives were obtained after quantitative derivatization with 1-bromoacetylpyrene in acetonitrile. An efficient improvement in the silica gel clean-up procedure of the pyrenacyl derivatives is reported. The clean-up cartridge is washed with hexane–dichloromethane (1:1, v/v), dichloromethane–ethyl acetate (8:2, v/v), and finally the pyrenacyl esters were eluted with dichloromethane–methanol (9:1, v/v). We compare this procedure with other methods already described. Good results were obtained with mussels, scallops and clams. The clean-up procedure showed good robustness when checked against silica and solvents activity. Using samples of mussel hepatopancreas with an OA concentration ranging from 0 to 2 μg OA/g hepatopancreas, the inter-assay relative standard deviation ranged from 5.5 to 12.6%. © 1998 Elsevier Science B.V.

Keywords: Diarrhetic shellfish poisoning; Food analysis; Toxins; Bromoacetylpyrene; Okadaic acid; Dinophysistoxins

1. Introduction

Diarrhetic shellfish poisoning (DSP) is a worldwide public-health problem with a great impact on the shellfish industry [1–3]. So far, more than ten fat-soluble polyethers are known to be DSP toxins, the most important toxins to cause diarrhetic symptoms being dinophysistoxin-1 (DTX-1), okadaic acid (OA) and dinophysistoxin-2 (DTX-2) (Fig. 1). Due



	R_1	R_2	R_3
Okadaic acid (OA)	H	H	Me
Dinophysistoxin-1 (DTX-1)	H	Me	Me
Dinophysistoxin-2 (DTX-2)	H	Me	H

Fig. 1. Structures of OA, DTX-1 and DTX-2.

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to the lack of a reliable instrumental method suitable to detect all DSP toxins, a mouse bioassay is currently being used to monitor their presence in shellfish. A commonly used analytical method to detect some of these toxins (OA, DTX-1 and DTX-2) is HPLC separation with fluorimetric detection (FLD) after conversion of the toxins to fluorescent products with 9-anthryldiazomethane (ADAM) [4]. This method provides field results that fit, within certain limits, with those obtained with the mouse bioassay [5]. The sample preparation step [solid-phase extraction (SPE) on silica gel] was improved later, rendering a better selectivity [6]. But the ADAM method has a high inter-laboratory variability [7] and the effect of the solvent activity on the key step, the silica gel clean-up, has been recently addressed [8,9] in order to assure the reproducibility and the robustness of the method.

Nevertheless, researchers are looking for other labeling reagents for FLD to avoid the disadvantages of the ADAM method [10–13], but none of the suggested reagents proved to surpass ADAM. Although some of them are more stable, cheaper or have a lower detection limit, they would require a complex post-derivatization clean-up, a double column high-performance liquid chromatography (HPLC) system with a valve-switching device, or longer time than ADAM to reach a stable quantitative derivatization.

Dickey et al. [14] have reported the conditions to esterify OA with 1-bromoacetylpyrene (BAP). Sample clean-up was according to the procedure previously reported for ADAM. Nevertheless, we observed that this clean-up method should be improved to reduce the extraneous peaks and ''chemical noise'' present in most chromatograms, mainly from scallops and stored frozen samples. Since BAP showed advantages in terms of stability, cost and esterification time, in this paper we report an easy and effective optimization on the SPE clean-up procedure for the pyrenacyl esters of DSP toxins. Standard solutions of OA/DTX-2 (so far, DTX-1 only exceptionally had affected shellfish in Europe [15]) and mussel, scallop and clam extracts are used to compare the results of this optimized rinsing procedure with those previously used for this pyrenacyl derivatives [14,16].

2. Experimental

2.1. Reagents

Certified calibration solutions of OA were purchased from the Institute for Marine Biosciences, National Research Council of Canada, Halifax, Nova Scotia, Canada. A purified methanolic solution of DTX-2 was received as a generous gift from the European Community Reference Laboratory on Marine Biotoxins (Vigo, Spain). BAP (Aldrich, USA) was stored at −20°C, as were the acetonitrile solutions of BAP (0.2%, w/v), 10% diisopropylethylamine (DIPA) (Sigma, USA) and deoxycholic acid (DOCA) (0.005%, w/v) (Sigma). Light petroleum (b.p. 40–60°C), *n*-hexane, ethyl acetate and methanol were of analytical grade as were amylene-stabilized dichloromethane and chloroform (Panreac, Spain). Analytical-grade chloroform (stabilized with ethanol) was used for thin-layer chromatography. HPLC-grade acetonitrile (Panreac) and Milli-Q water (Millipore, Spain) were used in the HPLC mobile phase. We used bulk silica gel 60 (40–63 μm) for column chromatography (Merck, Germany), stored in normal laboratory environmental conditions, in the SPE clean-up of toxin derivatives before HPLC analysis. In the experiments carried out to test the activity of the silica in the SPE, we also used SPE silica cartridges provided by Whatman (USA) (500 mg) and Lida (USA) (600 mg). In the same set of experiments was used bulk silica gel (Merck, Spain), stored for 48 h under the following environmental conditions: (1) conditions of 100% water saturation, −25°C, (2) 80% relative humidity, 25°C, (3) normal environmental conditions (60–70% relative humidity), 25°C, and finally (4) silica taken to dryness after 48 h at 130°C. OA contaminated mussels were provided by the Asociación Nacional de Conservas de Pescados y Mariscos (ANFACO, Vigo, Spain) as were non-toxic mussels used in the recovery tests. Fresh non-toxic mussels, clams and frozen scallops were provided from a local market.

2.2. Sample preparation

Hepatopancreas carefully removed from whole body of shellfish was homogenized and a 2 g

subsample was extracted following the method of Lee et al. [4] but using amylene-stabilized dichloromethane instead chloroform. We did not find differences between chloroform and dichloromethane in the recovery of OA from methanolic extracts of mussels.

2.3. Derivatization

A 500 μl aliquot of the dichloromethane extract, equivalent to 0.025 g hepatopancreas, was placed on a polypropylene Eppendorf type microtube. The solvent was removed under a stream of nitrogen. Then, 80 μl of the 0.2% BAP solution and 20 μl of the 10% DIPA solution were added to the residue. After closing the microtube, the mixture was reacted for 20 min at 75°C in a water-bath.

2.4. Silica gel clean-up

Cartridge columns (8 ml) packed with 0.5 g of silica were used for the rinsing procedures. The sample is loaded in three portions of 0.5 ml of the first washing solution. The cartridge was washed with 5 ml of the first washing solution, and later with 5 ml of the second washing solution. The esters are finally eluted with 4 ml of the recovery solution.

The first washing solution was 5 ml hexane–dichloromethane (1:1, v/v), and the second washing solution was 5 ml dichloromethane–ethyl acetate (8:2, v/v). The recovery solution was dichloromethane–methanol (9:1, v/v). The dichloromethane used was stabilized with amylene (see Table 1).

2.5. HPLC

After removal of the solvent under a stream of nitrogen, the residue was dissolved in 100 μl of acetonitrile and the solution was protected from light with an aluminum foil. An aliquot of 20 μl was injected into the HPLC system.

The LC system consisted of a single HPLC pump (Kontron 422, Italy) set at a flow-rate of 1.1 ml/min; a variable-wavelength fluorescence detection system (Kontron SFM 25, Switzerland) set to the following conditions: excitation 356 nm, emission 440 nm, monocromator slits: 10 nm, high-voltage photomultiplier: 790 V; an injection port (Reodyne, USA) with a 20-μl loop. A Nucleosil-C_{18} (5 μm, 150×40 mm, Tracer Analytica, Spain) cartridge column eluted with acetonitrile–water (80:20, v/v) or a Hypersil-ODS (5 μm, 250×40 mm, Tracer Analytica) cartridge column eluted with acetonitrile–water (85:15,

Table 1
Recovery of POA (8 ng OA) under different SPE conditions

Second washing solution (v/v); (ml)	Recovery solution (v/v); (ml)	POA recovery (%)	Relative cleaning effectiveness[a]
Dichloromethane [5]	Dichloromethane–methanol (95:5); [5]	84	1
Dichloromethane [7]	Dichloromethane–methanol (95:5); [5]	81	1
Acetone [3]	Dichloromethane–methanol (95:5); [5]	0	–
Dichloromethane–acetone (90:10); [1]	Dichloromethane–methanol (95:5); [5]	38	2
Dichloromethane [5]	Dichloromethane–acetonitrile (1:1); [5]	28	2
Dichloromethane–ethyl acetate (90:10); [5]	Dichloromethane–methanol (95:5); [5]	87	4
Dichloromethane–ethyl acetate (90:10); [5]	Dichloromethane–1-butanol (95:5); [5]	85	4
Dichloromethane–ethyl acetate (85:15); [5]	Dichloromethane–methanol (95:5); [5]	88	5
Dichloromethane–ethyl acetate (80:20); [5]	Dichloromethane–methanol (95:5); [5]	86	6
Dichloromethane–ethyl acetate (75:25); [5]	Dichloromethane–methanol (95:5); [5]	58	1
Dichloromethane–ethyl acetate (80:20); [5]	Dichloromethane–methanol (98:2); [5]	0	1
Dichloromethane–ethyl acetate (80:20); [5]	**Dichloromethane–methanol (90:10)**; [5]	**97**	**7**
Dichloromethane–ethyl acetate (80:20); [10]	Dichloromethane–methanol (90:10); [5]	93	7
Dichloromethane–ethyl acetate (80:20); [5]	Dichloromethane–methanol (80:20); [5]	102	5
Dichloromethane–ethyl acetate (80:20); [5]	Dichloromethane–methanol (60:40); [5]	40	1

[a] A relative parameter that expresses the ability of the rinsing procedure to remove extraneous peaks surrounding and overlapping POA from chromatograms.

v/v) was used. A PC-Integrator (Kontron 390) allowed data recording and peak integration.

3. Results

3.1. SPE improvement

Since the post-derivatization silica SPE is the key step in the HPLC–FLD determination of DSP toxins, we tried to improve it.

A general SPE strategy is, after loading the sample in the silica SPE cartridge, washing with solutions of increasing polarity to remove the less polar adsorbed substances, to finally release the analyte with a stronger solvent, including other compounds of similar polarity. So, we increased the polarity of the washing solutions, but ensured that toxin derivatives were not lost.

Firstly, chloroform (stabilized with amylene) was replaced with dichloromethane (also stabilized with amylene). Dealing with non-toxic mussel extracts spiked with OA, we compared the activity of both solvents in the clean-up and we found it to be very similar.

Since Lee et al.'s rinsing procedure did not present problems of delayed peaks, we did not modify the polarity of the first washing solution, i.e., hexane–dichloromethane (amylene-stabilized) (1:1, v/v).

In order to remove chromatographic interferences eluting at retention times close to that of the toxins (5 min$<t_R<$20 min) we increased the polarity of the second washing solution by adding other solvents to dichloromethane.

After substituting the chloroform with dichloromethane we tried to increase the polarity of the second washing solution by adding acetone to dichloromethane, but toxin loses were unacceptable. When adding a slightly less polar solvent, ethyl acetate (10%), all the extraneous peaks that appeared at retention times higher than OA were removed, and peaks neighboring OA were substantially decreased. By increasing the proportion of ethyl acetate to 20%, the background noise was notably reduced for 5 min $<t_R<$8 min. Further improvement at this of the chromatogram was obtained by increasing the polarity of the recovery solution using methanol. A higher methanol proportion in the recovery solution caused an increase in the toxin recovery. Hence, we established as optimal the solvent composition typed in bold in Table 1.

3.2. Control of the efficiency of the SPE

In order to test the purity of the peaks we collected the pyrenacyl okadaate (POA) and pyrenacyl-dinophysistoxins-2 peaks eluted from the HPLC column. After evaporating the solvent under nitrogen, the residues were dissolved in 100 μl of methanol and developed on aluminum silica gel plates (Merck Kieselgel 60 F_{254}, Spain) with chloroform–methanol (95:5). Plates were scanned in a TLC plate fluorescence reader set to 356 nm excitation and 440 nm emission (Perkin-Elmer LS 50 B, UK).

3.3. Recovery of OA from spiked samples

The whole recovery experiment was repeated on three different days. Each day, three 2 g subsamples of homogenized mussel hepatopancreas which did not contain OA, were spiked with 0.5, 1.0 and 2.0 μg OA/g homogenate.

Daily calibration curves were obtained with the same certified calibration solution in the range 1–12 ng OA injected. Each calibration curve was obtained, at least, from nine sample injections. Triplicate experiments were made from the methanol–water extract including liquid–liquid extraction, derivatization, clean-up and HPLC determination. We also used a different ampoule of certified OA calibration solution each day.

3.4. Stability of the POA

An OA standard sample was derivatized and cleaned up. The final acetonitrile solution (300 μl) was stored in a polypropylene freezing vial (0.5 ml) with screw closure. The vial was protected from light with an aluminum foil and maintained at room temperature. The sample was periodically injected. The results show the stability of the derivatized samples at least for 4 days.

3.5. Robustness of the silica gel clean-up

In order to test the influence of the silica provider in the clean-up, silica from three different commercial providers was used, and identical results were found in the recovery and cleaning effectiveness of derivatized mussel extracts. Another factor suggested to affect the reproducibility of the clean-up of the anthryl derivatives is the activity of the silica due to the adsorption of water from the air on its surface. Activating the silica at 130°C for 24 h was recommended [8]. Some experiments were made to test the sensibility of the method described in this paper against this factor: four 5 g lots of bulk silica were packed into a folded filter paper and stored for 48 h in different environmental conditions of relative humidity. OA standard solutions and OA spiked mussel extracts with a final toxin content equivalent to 3.6 μg OA/g hepatopancreas, were derivatized and cleaned up in the different lots of silica. Chromatograms were similar for dry silica, silica stored at ambient conditions and silica stored at 80% relative humidity. Nevertheless, the clean-up was unsuccessful when using the wet silica that had been stored at 100% relative humidity, due to a extremely low recovery of the POA. With respect to the solvents, it is worth mentioning the importance of never using ethanol stabilized dichloromethane, since sometimes it dramatically increased the losses of POA due to the high polarity of the ethanol.

3.6. Evaluation of the rinsing procedures

Most of the samples provided chromatograms with extraneous peaks surrounding the peak of POA, and at t_R close to PDTX-2. These interferences sometimes overlapped the OA peak, causing an overestimation of the toxin content. This effect being more important with low toxic sample levels. The following four rinsing procedures with silica gel were compared in the clean-up of a chloroform mussel extract spiked with OA: (1) Dickey et al.'s rinsing procedure [14]. (2) Kelly et al.'s rinsing procedure [16] (results not shown). (3) Substituting chloroform with dichloromethane in procedure 1. (4) Optimized procedure proposed in this paper.

It was observed that chloroform and dichloromethane show a similar activity in the silica gel clean-up of pyrenacyl derivatives. Finally, it is clear the greater cleaning ability of the rinsing procedure reported in this paper: all the extraneous peaks surrounding POA were removed during the SPE (Fig. 2).

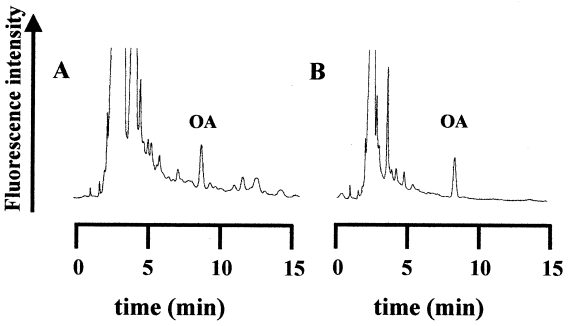

Fig. 2. OA spiked mussel extract submitted to different clean-up procedures after derivatization with BAP. The final toxic level was equivalent to 3.6 μg OA/g hepatopancreas: (A) procedure of Dickey et al. [14]. (B) Optimized clean-up proposed in this paper. The column used was Hypersil-ODS, 5 μm, 250×40 mm.

Chromatograms from samples difficult to clean are shown in Fig. 3: hepatopancreas extracts from commercial frozen scallops cleaned up as per Dickey et al. [14] or with the optimized clean-up proposed in

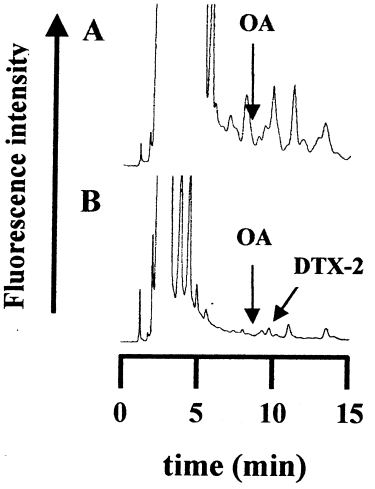

Fig. 3. Comparison on the rinsing procedure of Dickey et al. [14] (A) and the optimized rinsing procedure (B). Chromatograms correspond to an extract of scallops containing 0.40 μg DTX-2/g hepatopancreas. The column used was Hypersil-ODS, 5 μm, 250×40 mm.

this paper (Fig. 3A,B). All the interferences obtained with the clean-up of proposed by Dickey et al. could led to a misidentification of this substances as OA or DTX-2 or an incorrect determination of the toxins content. However, when the same sample is submitted to our improved clean-up it can be seen that the shellfish was free of OA and had a low concentration of DTX-2 (0.4 μg/g hepatopancreas).

3.7. Quantitation, reproducibility and recovery

We evaluated clean-up losses caused by directly comparing heights of the peaks before and after the SPE (Fig. 4). The losses were 3% for POA and 7% for pyrenacyldeoxycholate (PDOCA) ($n=3$). Since we had a extremely low amount of DTX-2, only one experiment was made to check its loses during SPE, but they were also negligible (4%) ($n=1$). DTX-1 was not used due to the low incidence of this toxin in Europe. However, we used DOCA with the aim of checking the losses of substances eluting at higher retention times by HPLC on C_{18} columns. Nevertheless, DOCA is not used as an internal standard due to significant variations on t_R and peak height [8].

Fig. 4 shows the chromatograms corresponding to a methanolic solution of OA and DOCA, before (A) and after (B) SPE. Fig. 4C corresponds to a methanolic extract of DTX-2 after silica gel clean-up.

Linearity of the peak heights of POA was tested in the range 1.0 ng OA to 40 ng OA ($r=0.999$). The detection limit for POA with fluorescence detection (HPLC with a Nucleosil-C_{18} column) was 0.5 ng at a signal-to-noise ratio of 3. This detection limit was reduced to 0.25 ng with the Hypersil ODS column. These detection limits are equivalent to 0.1 μg OA/g and 0.05 μg OA/g hepatopancreas, respectively.

Table 2 gives the recovery data corresponding to homogenates of non-toxic mussel hepatopancreas that were spiked each with 0.5, 1.0 and 2.0 μg OA per 1 g of the digestive glands. The relative standard deviation (R.S.D.) of the three determinations made

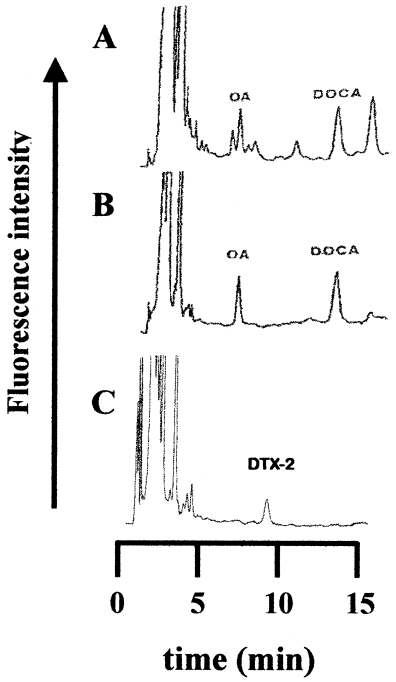

Fig. 4. Chromatograms corresponding to a methanolic solution of OA (12 ng) and DOCA (16 ng), (A) before the silica gel clean-up and (B) after the silicagel clean-up. (C) Typical chromatogram of a DTX-2 (5.7 ng) methanolic solution obtained with the new silica gel clean-up. The column used was Nucleosil-C_{18}, 5 μm, 150×40 mm.

Table 2
Recovery and variability data from OA spiked mussels

Spiked sample (μg OA/g hep)	Day	Found ($n=3$) (mean±S.D.)	Recovery (%)	R.S.D. (%)
0.5	1	0.49±0.10	98	20
	2	0.45±0.06	90	10
	3	0.50±0.06	101	12
	[a]	0.48±0.03	96	5.5
1.0	1	0.85±0.06	85	7.4
	2	0.91±0.04	91	3.9
	3	1.08±0.10	108	9.1
	[a]	0.94±0.12	94	12
2.0	1	1.88±0.34	94	18
	2	1.73±0.02	86	0.9
	3	1.94±0.22	97	11
	[a]	1.85±0.11	92	6

[a] Rows marked with this symbol show the recoveries and deviations among the three days. R.S.D. allows an estimation of the inter-assay reproducibility of the method.

in the same day, allows an estimation of the repeatability of the method. We also carried out the whole experiment over three different days; R.S.D. of the OA determinations made over three different days allows an estimation of the reproducibility of the method between different days.

3.8. Applications

Several samples were analyzed according to this method. Fig. 5 shows the chromatograms obtained from boiled cooked samples that contained 6.3 μg OA/g hepatopancreas (A) and commercial non-toxic mussels (B) and clams (C). As can be seen, interferences were not observed at the retention times of DSP toxins in non-toxic shellfish.

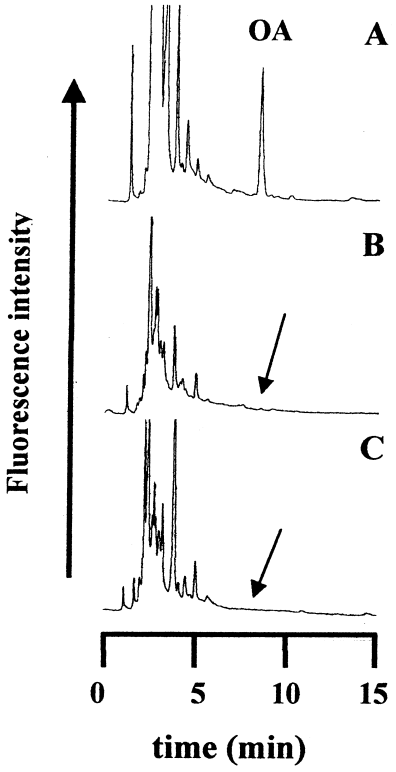

Fig. 5. Chromatograms corresponding to (A) boiled cooked mussels containing 6.3 μg OA/g hepatopancreas (equivalent to 31.5 ng OA injected), (B) non-toxic mussels and (C) non-toxic clams. Arrows mark the expected elution time of OA. The column used was Hypersyl-ODS, 5 μm, 250×40 mm.

4. Discussion

The BAP reagent yields pyrenacyl esters of OA, DTX-2 and DOCA. The clean-up procedure employed was SPE on silica gel and was the same for the clean-up of ADAM derivatives. Although that clean-up procedure allowed the determination of OA in standard solutions and in fresh samples of mussels, the peak of POA showed a shoulder corresponding to an interference that reduced the accuracy of the determination of OA. Furthermore, other interfering peaks were present on the chromatograms. Therefore, we report an improved clean-up that among other considerations, eliminates the possibility of overestimating OA peaks due to interferences in overlapping peaks.

This clean-up over silica gel has seemed very robust and reproducible, even with silica stored at room conditions up to 80% relative humidity. Neither the silica manufacturer nor the silica particle size had an appreciable effect on the clean-up since the BAP derivatives showed no mobility on the silica with any of the washing solutions (k' very high). The silica gel clean-up procedure is simple and inexpensive since it requires only one SPE refillable cartridge.

The application of BAP as a fluorescence labeling reagent for OA and its derivatives can overcome the whole properties of ADAM when this specific SPE clean-up is applied. This reagent is very stable and its acetonitrile solutions can be used for more than two weeks when stored protected from light at −20°C. Very fast derivatization (only 20 min) and toxin derivatives are more fluorescent than ADAM (we found that BAP derivatization yielded 1.5-times more fluorescence than ADAM in the OA peak), resulting both properties in a lower detection limit for a given HPLC system.

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