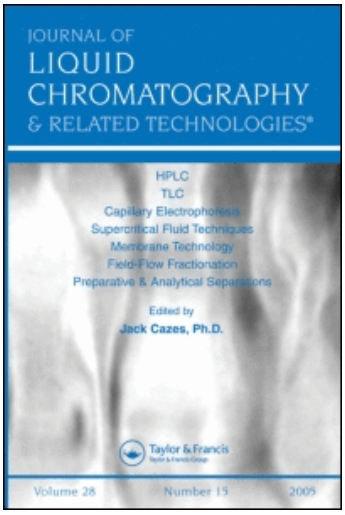

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Determination of Okadaic Acid and Related Toxins in Greek Mussels by HPLC with Fluorimetric Detection

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ABSTRACT

The investigation for the presence of diarrhetic shellfish poisoning (DSP) toxins, okadaic acid (OA), and dinophysistoxin-1 (DTX-1), in mussels (*Mytilus galloprovincialis*) harvested in Thermaikos Gulf (northern Aegean Sea) is reported. The digestive glands of mussels were homogenized with methanol–water (80 : 20 v/v), and OA was extracted from the homogenate with hexane–chloroform (50 : 50 v/v). OA was reacted

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with 9-anthryldiazomethane (ADAM) in methanol for 1 hr at 35°C. The product was purified by passing through a silica solid phase extraction cartridge before being determined by reversed-phase liquid chromatography with fluorimetric detection (HPLC/FLD). Linear calibration curves ($r = 0.9993$), in the typical analytical range of 1–10 ng OA-injected and detection limit of 0.14 ng OA-injected (S/N 3 : 1) were obtained. Recovery of the toxin from mussels ranged from 100.4% to 110.4%. Analysis of samples during a DSP episode in 2002 indicated the presence of OA at levels above the regulatory limit, while DTX-1 was detected in only one sample.

Key Words: Okadaic acid; Diarrhetic shellfish poisoning; Mussels; Liquid chromatography.

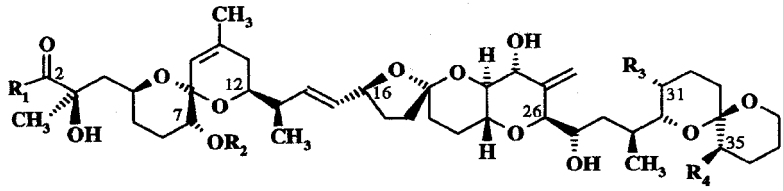
INTRODUCTION

Diarrhetic shellfish poisoning (DSP) is a gastro-intestinal syndrome that occurs in humans after consumption of contaminated bivalve mollusks. DSP has been recognized as a worldwide public health problem and of shellfish industry.[1] Contaminated shellfish contain toxins originally produced by marine dinoflagellates which, when ingested by the filter-feeding shellfish, are accumulated in their digestive glands. DSP toxins fall into three groups according to their carbon skeleton: the okadaic acid (OA) group involving OA and dinophysistoxins (DTXs) shown in Fig. 1, the pectenotoxin group, and the yessotoxin group.

The OA group toxins are produced by dinoflagellates belonging to the genera *Dinophysis* and *Prorocentrum.* OA and its methylated analogue dinophysistoxin-1 (DTX-1) have been identified as being responsible for most DSP outbreaks.[2–4] However, DTX-2 was the major toxin in Irish mussels during 1991–1994 DSP episodes.[5] Some other toxins like DTX-4[6] and DTX-3 are less toxic, while several OA ester derivatives were isolated as minor DSP toxin compounds from cultures of the *Prorocentrum* species.[7]

The official method for the detection of DSP toxins is the mouse bioassay developed first by Yasumoto et al.,[8] which, however, is time-consuming, non-specific, and has the problem of low sensitivity and matrix interferences from free fatty acids. Sensitive immunoassays have been developed for OA,[9] but they cannot be used for accurate quantification of toxin mixtures because of differences in the cross-reactivities of individual toxins with antibodies.

Instrumental methods offer the possibility for precise, sensitive, and automated analyses for both monitoring and research purposes. A commonly used technique for determining OA group toxins in shellfish is liquid



R_1	R_2	R_3	R_4	name
OH	H	Me	H	Okadaic acid (OA)
Me or Et or -C7-9 diols	H	Me	H	OA esters
OH	H	Me	Me	Dinophysistoxin-1 (DTX-1)
OH	H	H	Me	Dinophysistoxin-2 (DTX-2)
OH	acyl	H or Me	H or Me	"Dinophysistoxin-3"(DTX-3)
*	H	H	H	Dinophysistoxin-4 (DTX-4)

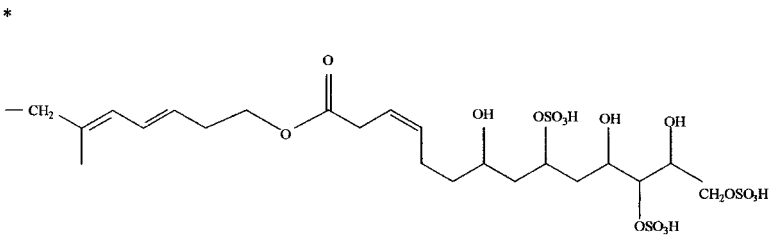


Figure 1. Molecular structure of OA and related DSP toxins.[1]

chromatography, after conversion of toxins to fluorescent products. 9-Anthryldiazomethane (ADAM) is a derivatization reagent, which provides the best reaction selectivity among several other reagents.[10] Fluorimetric determination of OA and related toxins, based on the method originally developed by Lee et al.,[11] has been implemented in many laboratories. However, the poor stability of the ADAM reagent and the presence of numerous other reactive coextractives in shellfish tissues, made necessary the application of minor modifications in order to improve efficiency and reliability of the method,[12–15] LC combined with ion-spray mass spectrometry (LC-MS) is another powerful method that is particularly well suited to the identification even of new toxins.[7,16,17]

The last few years, toxic dinoflagellates of the genus *Dinophysis* cf. *acuminata* were detected in Thermaikos Gulf, northern Greece,[18] an area with significant shellfish production (about 40,000 tones/year). The occurrence of this dinoflagellate species, which is an OA producer,[19] coincided with DSP toxicities detected in Greek shellfish (unpublished data). Because of the risk to human health, the competent authorities proceeded in closures of the production areas. Shellfish containing more than 0.16 μg OA equivalents per gram of mussel flesh (or 0.8 μg OA equivalents per gram of digestive glands), is considered unsuitable for human consumption.[a] Therefore, a reliable method for OA analysis, which had not before established in Greece, was urgently needed for the protection of human health and local economies. This paper presents the performance characteristics of the ADAM derivatization/HPLC analysis of OA in mussels from Thermaikos Gulf, Greece.

EXPERIMENTAL

Materials

Certified standard solutions of OA (CRM-OA-b, 24.1 ± 0.8 μg/mL in methanol) was purchased from the Institute of Marine Biosciences, National Research Council of Canada (Halifax, Nova Scotia, Canada) and stored at −20°C. Standard solutions of DTX-1 was a generous gift from the European Reference Laboratory in Vigo, Spain. The derivatization reagent, ADAM was obtained from Serva (Heidleberg, Germany), and kept deep frozen (−70°C) when not in use. Acetonitrile was of HPLC grade, while all the other solvents used were of analytical grade. Water used in HPLC was deionized, double-distilled, and filtrated through a membrane filter (0.45 μm pore size).

Mussel samples (*Mytilus galloprovincialis*) were harvested from cultures of Lefkoudi, Thermaikos Gulf (northwest Aegean Sea), over a period of 5 months, when a DSP episode occurred. Eight samples were collected, coming from mussels hung on ropes in 1 to 4 m depth. Control samples were also collected from other non-affected shellfish production areas of northern Aegean Sea, which had previously been found to be negative for DSP toxins, both by mouse bioassay[8] and by HPLC analysis.

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[a]Council of the European Communities. Commission Decision of 15 March 2002 laying down detailed rules for the implementation of Council Directive 91/492/EEC as regards the maximum levels and the methods of analysis of certain marine biotoxins in bivalve molluscs, echinoderms, tunicates, and marine gastropods (Text with EEA relevance) (notified under document number C (2002) 1001).

Analytical Procedure

The method applied for the determination of OA and DTX-1 in mussels was based on the procedure described by Lee et al.,[11] with minor modifications focused on the purification and derivatization steps described below.

Extraction and Purification of OA from Digestive Glands

OA is mainly accumulated in the digestive glands, also called hepatopancreas (HP), which on average, accounts for 20% of the mussel weight. The HP of the mussel samples examined in the present study, was dissected and homogenized in an Ultra-Turrax (IKA, Staufen, Germany) until a pourable homogenate was obtained. One gram of the homogenate was vortex extracted with 4 mL water–methanol (1 : 4) for 1 min, and centrifuged at 4000 rpm for 5 min. A 2.5 mL supernatant aliquot was rinsed twice with 2 mL of hexane, then 0.5 mL of water was added and partitioned twice with 2 mL of chloroform. The combined chloroform layers filled up to 10 mL.

In the method presented here, hexane was used to remove non-polar lipids instead of petroleum ether proposed in the early work of Lee et al.[12] Hexane has the advantage that no residues remain in the aqueous layer and that the amount of OA lost in the combined hexane layers is small ($<1\%$).[12]

Derivatization and Clean-up Procedure

For OA derivatization, an aliquot (0.5 mL) of either sample extract (in chloroform) or calibration standard, was transferred into 1.5-mL plastic amber vials and dried under N_2. The residues were esterified in 200 μL of 0.2% ADAM solution for 1 hr, in the dark, at 35°C. The ADAM solution was prepared daily, by dissolving ADAM (5 mg) in acetone (100 μL), volume adjusted to 2.5 mL with methanol, and filtration through a 0.45-μm membrane for immediate use. All work was done under yellow lighting.

The reaction conditions for the derivatization of OA were chosen based on relevant studies, and on their applicability on a routine basis. Lee et al.[11] reacted 100 μL 0.1% (w/v) ADAM in methanol for 1 hr at 25°C. A study conducted by Quilliam et al.,[20] revealed that for the quantitative conversion of analytes in real-world sample extracts, the excess of ADAM is necessary, and that the reaction rate is catalyzed with the use of methanol as a solvent of the derivatization reagent.[20] In the same study, it was found that temperature affects the stability of ADAM and, consequently, reaction yield, and that a 2- to 2.5-hr reaction time is adequate for quantitative derivatization.

After evaporating the solvent, the reaction products were redissolved in 1 mL of hexane–chloroform mixture (1 : 1) in three portions, and loaded to a solid phase extraction silica cartridge (650 mg, Alltech), preconditioned with 6 mL chloroform followed by 3 mL hexane–chloroform mixture (1 : 1). The sample passed slowly (1 drop/sec) through the clean up column, which was then washed with 5 mL of the same solvent followed by 5 mL of chloroform. The ADAM-OA esters were then eluted with 5 mL of chloroform–methanol mixture (95 : 5) and evaporated to dryness under N_2. The residue was reconstituted in methanol (0.2 mL) for HPLC analysis.

HPLC Analysis

HPLC analyses were performed on a Waters HPLC system (model alliance 2690 Separations module), equipped with in-line degasser, pump, autosampler, oven, and a variable wavelength fluorescence detector (model 474) set at 365 nm excitation and 415 nm emission wavelength. Data collection was performed by the Waters Millenium[32] Chromatography Manager software. Separation of the ADAM-OA derivative was carried out according to the chromatographic conditions shown in Table 1.

Identity of ADAM-OA and ADAM-DTX-1 peaks was confirmed by matching the retention time (RT) with standard solutions of OA and DTX-1, respectively. Quantification was based on five-point matrix matched calibration curves, constructed by spiking toxin-free sample extracts with OA standard solution. The five points of the calibration curve corresponded to 0.4, 0.8, 1.6, 2, and 4 μg OA/g HP, respectively. The OA calibration curves were also used for the calibration of DTX-1 measurements, since under the isocratic conditions applied, the relative molar responses for the two toxins are identical considering that the fluorescence signal is derived only from the anthracenyl moiety.

Table 1. Chromatographic conditions applied for the separation of the ADAM-(OA) derivative.

Stationary phase	RP C-18, Symmetry, Waters Co. (250 mm × 4.6 mm, 5 μm)
Mobile phase	Acetonitrile : water (80 : 20 v/v)
Flow rate	1 mL/min
Temperature	35°C
Loop volume	20 μL
Detection	Fluorescence, λ_{ex} 365 nm, λ_{em} 415 nm

RESULTS AND DISCUSSION

HPLC Separation

The procedure employed here, was found to be selective in the detection of OA and DTX-1, which after derivatization under the same conditions as OA, gave a peak far after the ADAM-OA ester (relative RT 1.48) (Fig. 2a and b, respectively). Analysis of reagent blanks showed negligible chemical background at the RT of ADAM-OA (Fig. 2c).

Performance Characteristics

The linearity of the fluorescence intensity with the amount of the injected OA ester was examined. The mean peak area ($n = 6$) was plotted against the injected amount of OA, and the resulting calibration curve revealed good linearity at the range of 1–10 ng OA on-column ($r = 0.9993$). At the same concentration range, mussel homogenates spiked with a standard solution of OA was also found to be linear ($r = 0.9989$) (Fig. 3). Comparing the slopes of the two curves using the *t*-test, a significant difference between them was found ($p = 0.05$), and for this reason quantification was based on matrix matched calibration curves.

The detection limit of OA was calculated according to the IUPAC criterion (signal-to-noise ratio of 3). In standard solutions, the detection limit was 0.14 ng/20-μL injection volume. In shellfish tissue, the minimum detectable level was 0.4 μg OA/g HP and the limit of quantification (signal-to noise ratio of 10) was 0.7 μg OA/g HP.

Recovery experiments were carried out on 1 g sub-samples of homogenized mussel HP, free of OA, spiked with 0.8, 2.0 and 4.0 μg OA/g HP. As seen in Table 2, recovery was satisfactory at both low and high concentration values, ranging between 100.4% and 110.4%.

For evaluating the precision of the method, repetitive analyses were performed within 1 day, and on six separate days, on a natural contaminated sample. Within-day reproducibilities of OA measurements in standard methanolic solution and mussel extracts were, on average, 3.1% and 8.8%, respectively. Between-day reproducibilities were also lower than 10%. Materials used in the silica cleanup step would account for the lower precision scores in the between-day reproducibility.[21] Quilliam showed that silica used in commercial SPE cartridges, as well as ethanol content in chloroform used as washing and eluting solvent, varied considerably, even among different production lots of the same manufacturer.[12]

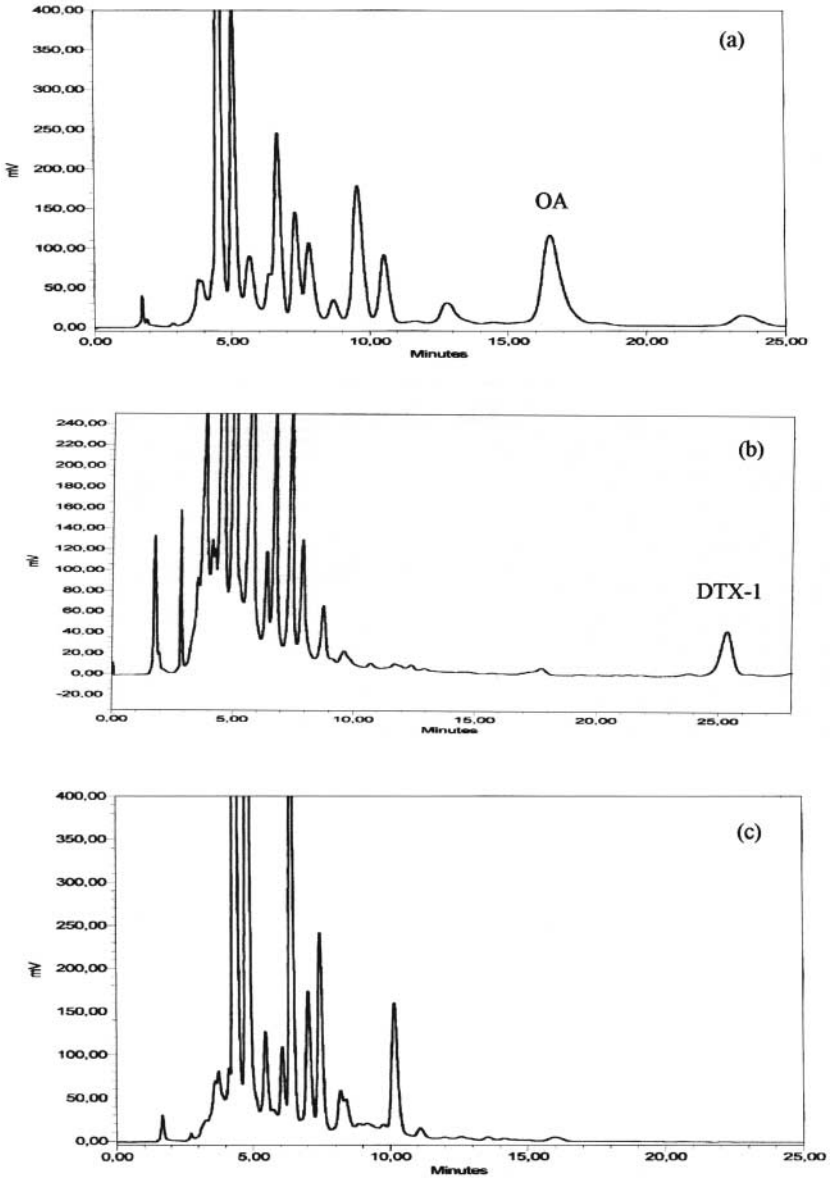

Figure 2. Typical HPLC chromatograms of: (a) standard solution of OA (10 ng/20 μL injection volume); (b) standard solution of DTX-1 (4 ng/20 μL injection volume); and (c) blank sample. LC conditions are described in Table 1.

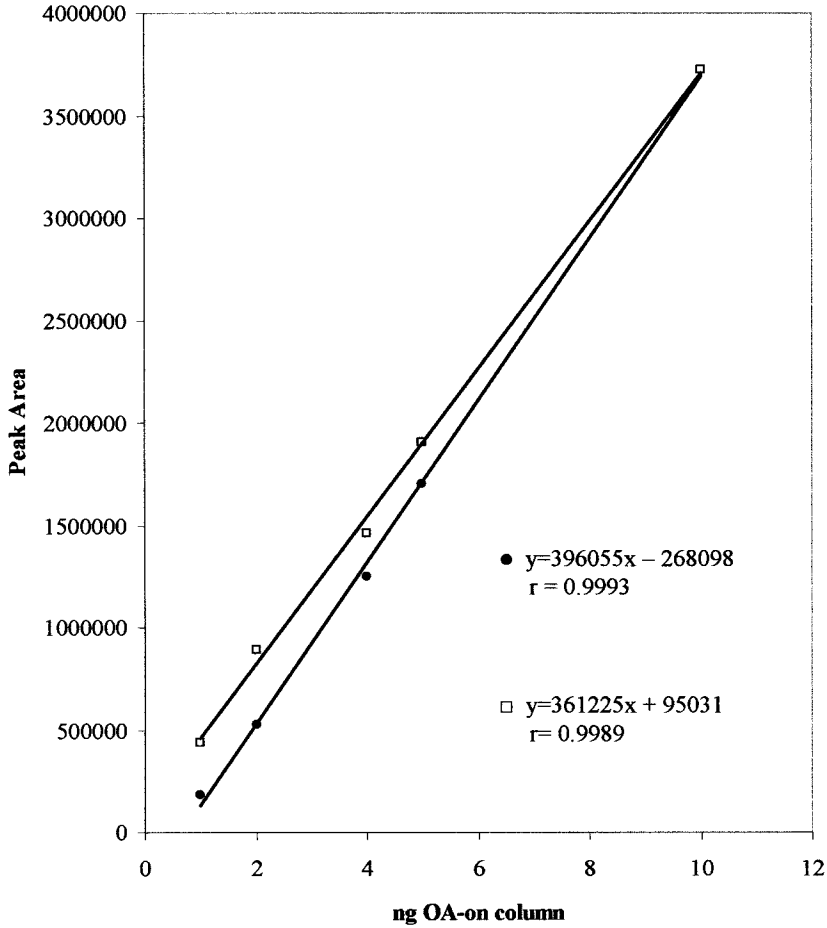

Figure 3. Calibration curves for OA in (dark circles) standard solutions in methanol and (open squares) standard solutions of OA spiked in muscle extract. Each point is the mean of six repetitive assays.

The stability of OA, spiked in mussel tissues after derivatization with ADAM and cleanup was studied, by storing the final methanolic solutions (in autovials with screw caps) for various storage periods (0–4 days), at various temperatures (−20°C, 4°C, and 22°C) in the dark. From the results of Table 3, it can be concluded that the derivative is stable for at least 4 days at all storage conditions tested. The ADAM-OA derivative coming

Table 2. Percent recovery of OA from mussels spiked with known amounts of the toxin (mean ± SD, $n = 6$).

OA added (μg/g HP)	OA found (μg/g HP)	Recovery (%)
0.8	0.88 ± 0.08	110.4
2.0	2.01 ± 0.27	100.4
4.0	4.02 ± 0.47	100.4

from standard OA solution was found to be stable at 22°C for at least 7 days ($p = 0.05$). These findings are in accordance with the findings of other investigators, who showed that the ADAM derivative is stable for 5 days at −20°C,[22] or for 7 days at 4°C.[23]

Application of the Method to Mussels from Thermaikos Gulf

In non-toxic mussels, some unknown peaks appeared in the area of OA elution, with RTs relative to RT of ADAM-OA 0.70, 0.77, 0.93, 1.17, and/or 1.29 (Fig. 4a), caused probably by artifact reactions.[12] In most chromatograms of real-world samples, especially those with high OA content, no other compound was co-eluted with ADAM-OA giving resolution higher than one. An HPLC chromatogram of a toxic mussel sample containing OA and DTX-1 is depicted in Fig. 2b.

The concentrations of OA determined in mussels from Thermaikos Gulf during the DSP episode of 2002, are presented in Table 4. In almost all samples, OA was detected at levels remarkably high (maximum value 36.06 μg/g HP), that exceeded, by far, the regulatory limit of 0.8 μg/g HP.

In only one sample collected in February 2002, DTX-1 was detected along with OA, at lower concentration levels (0.51 μg/g HP vs. 6.14 μg/g HP). It is

Table 3. Stability data of the ADAM-OA derivative (mean ± SD, $n = 5$).

Storage period (days)	Remaining OA (%)		
	−20°C	4°C	22°C
0	100.0 ± 0.0	100 ± 0.0	100 ± 0.0
1	97.0 ± 2.5	98.7 ± 3.5	106.3 ± 8.0
2	101.6 ± 1.6	95.5 ± 3.8	102.9 ± 4.6
4	98.6 ± 7.4	97.2 ± 2.3	109.8 ± 4.0

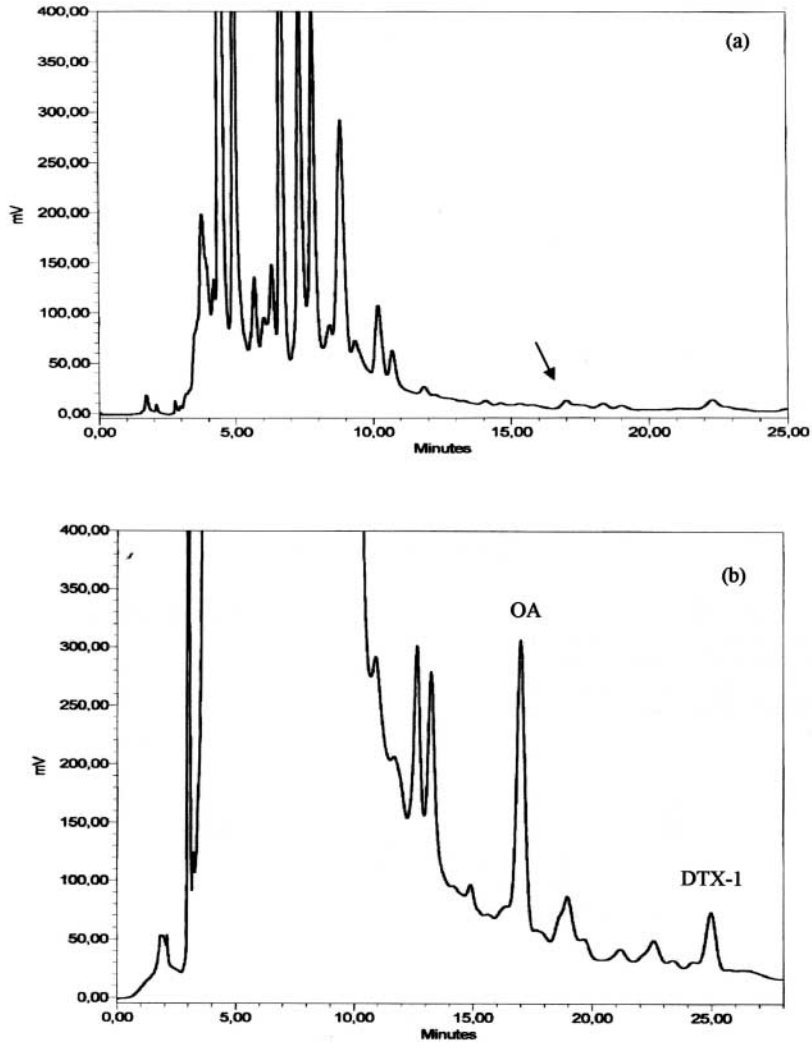

Figure 4. Typical HPLC chromatograms of: (a) HP extract from non-toxic mussels; (b) mussels containing 6.2 μg OA/g HP and 1.49 μg DTX-1/g HP. LC conditions are described in Table 1. The arrow shows the position at which ADAM-OA peak could be eluted.

concluded that the HPLC/FLD method can also be applied for the detection of some pectenotoxins eluting before OA (e.g., PTX-2sa with a relative RT of 0.86).[24] In the samples analyzed, no other peak eluted, except for those mentioned above.

Table 4. Levels of OA and DTX-1 detected in mussels originated from Lefkoudi, Thermaikos Gulf, during a DSP episode in 2002.

Sampling date	OA (μg/g HP)	DTX-1 (μg/g HP)
8 February	1.81	n.d.
21 February	6.14	0.51
15 March	8.15	n.d.
28 March	7.71	n.d.
10 April	36.06	n.d.
24 April	6.73	n.d.
10 May	3.98	n.d.
6 June	n.d.	n.d.

Note: n.d.: not detected.

CONCLUSIONS

In the last few years, DSP toxins have become increasingly frequent in Greek coastal waters. The present study has demonstrated that the HPLC/FLD method originally developed by Lee et al.[11] is sufficiently efficient and reproducible, allowing us to obtain accurate information on the concentrations of OA in Greek shellfish. OA and DTX-1 was confirmed in mussels originating from a production area of Thermaikos Gulf (NW Aegean Sea).

The derivatization reagent (ADAM) was found to be selective of the carboxylic group, giving highly fluorescent chromophore. Unfortunately, given the fact that it is unstable and at least two people were involved in its delivery to the laboratory, there was an occasional impact to the products quality, leading to the cost of the analyses. On the other hand, the lack of standard solution of other DSP toxins, which can be detected with the ADAM/HPLC method, did not allow us to have a broader estimation of the DSP toxicity in mussels. For this reason, the sanitary control is based on the mouse bioassay, which estimates the global DSP toxicity in a sample. HPLC/FD though, can be implemented in a complementary manner to overcome the lack of specificity of the mouse bioassay.

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