Journal of Chromatography, 594 (1992) 137–144
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 835

High-performance liquid chromatography of okadaic acid and free fatty acids in mussels

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(First received August 1st, 1991; revised manuscript received October 30th, 1991)

ABSTRACT

A high-performance liquid chromatographic method developed for the determination of both okadaic acid (OA) and free fatty acids (FFA) was used for analysing mussel samples collected in the Gulf of Trieste. OA and FFA extracted from mussel hepatopancreas were derivatized prior to their chromatographic separation and spectrofluorimetric detection. 9-Chloromethylanthracene (CA) was used as a fluorescent labelling agent. The presence of toxic fatty acids (*e.g.*, linolenic acid), which may interfere with the bioassay of diarrhoetic shellfish poisoning (DSP) toxins, in the lipidic fraction of the extract was observed, whereas no OA was detected in the analysed samples.

INTRODUCTION

Diarrhoetic shellfish poisoning (DSP) toxins were recognized for the first time as human pathogenic agents in Japanese seafood by Yasumoto *et al.* in 1978 [1]. Further research led to the elucidation of the structural formulae of the toxins, which are mainly constituted by okadaic acid (OA) and its derivatives [2–4], to the development of biological [1,5] and high-performance liquid chromatographic (HPLC) [6] methods of analysis and to the understanding of the toxicological and tumour-promoting activities of DSP toxins [7,8].

During 1989 and 1990, mussels farmed in the Adriatic Sea appeared to be contaminated (for long periods, related to algal blooms) by DSP toxins; as a consequence, the Italian hygiene authorities prohibited the sale of mussels, with severe economic consequences for the fishing industry [9].

In Italy, the official method of analysis for DSP toxins has been modified several times; currently a mouse bioassay test based on that developed by Yasumoto *et al.* [1], but with a modified threshold limit, is used [10]. The inter-laboratory repeatability of this biological method is very poor [11]. Further, in a previous paper, Lee *et al.* [6] pointed out the disadvantages of the mouse bioassay test and the possible interference (false-positive results) of free fatty acids (FFA). The toxicity of FFA in the biotest when mice are injected intraperitoneally (i.p.) with the liposoluble fraction extracted from mussels was also reported by Cassais and Perez [12], who listed the most toxic FFA in mussels.

As far as we know, previous papers on the HPLC analysis of DSP toxins refer to mussel samples collected in France, Spain, Norway and Japan [4,6,13,14], and the method has never been tested in Italy.

In this paper we describe the development and application of an HPLC method for OA, modified with respect to that formerly published by Lee *et al.* [6], and further integrated with the detection of the

0021-9673/92/$05.00

FFA present in the samples. This integration may possibly represent a key factor for understanding the discrepancies between the results given by the mouse bioassay and the HPLC test for DSP toxins.

EXPERIMENTAL

Apparatus

The chromatographic apparatus consisted of a pump module (Series 3 liquid chromatograph), a spectrofluorimetric detector (LS30) and a data processor (LCI-100) (all from Perkin-Elmer, Norwalk, CT, USA).

Reagents and samples

Pure (>97% by HPLC) okadaic acid (OA) [isolated from *Prorocentrum lima*, delivered in sealed vials, dissolved in dimethylformamide (DMF), 100 ± 5 μg/ml] was purchased from Moana BioProducts (Honolulu, HI, USA).

9-Anthryldiazomethane (ADAM) was obtained from Serva (Heidelberg, Germany) and used in methanol solution (0.1%, w/v); 9-chloromethylanthracene (CA), from Fluka (Buchs, Switzerland), was used in dimethylformamide solution (0.1%, w/v). Tetramethylammonium hydroxide (TMA) [25% (w/v) solution in methanol] was purchased from Aldrich (Steinheim, Germany) and was used diluted (1:500, v/v) in dimethylformamide. Acetone (analytical-reagent grade) and methanol, acetonitrile, chloroform and *n*-hexane (HPLC grade) were obtained from E. Merck (Darmstadt, Germany).

Light petroleum (b.p. 30–40°C) (AnalaR; BDH, Poole, UK) and anhydrous sodium sulphate (Carlo Erba, Milan, Italy) were also used.

Mussels were provided by a mussel farm in the Gulf of Trieste, and were all collected at the same site (approximately latitude 45°46′N, longitude 13°36′E) every 10 days during the winter and spring, 1990–91. The hepatopancreas (HP) of the mussels were excised from the fresh shellfish, homogenized and extracted (see Fig. 1).

Disposable cartridge column chromatography for sample purification was accomplished by using Sep-Pak Classic Cartridges (Waters-Millipore, Milford, MA, USA), packed either with silica (690 mg per cartridge) or C_{18} material (360 mg per cartridge).

Methods

Three different methods of sample preparation were tested. A schematic diagram of the three methods, for comparison, is reported in Fig. 1.

Method 1. The method described by Lee *et al.* [6] was followed.

Method 2. The Italian official mouse bioassay [10] was followed up to the final evaporation of the extract, which was redissolved in DMF for purification and subsequent chromatographic injection instead of being dissolved in Tween as required for i.p. injections into mice.

Method 3. This method uses two parallel routes of sample extraction and purification for either OA (method 3a) or FFA (method 3b) analysis.

For OA analysis (method 3a), steps similar to those in method 1 were followed, but for the derivatization of the extract CA was used instead of ADAM. An aliquot of 1 ml (from the total extracted volume of 10 ml) was evaporated under nitrogen and 200 μl of CA plus 100 μl of TMA solutions were added to the residue; the mixture was left to react, in a capped vial, in a thermostated water-bath at 75°C for 30 min, following a procedure similar to that used by Kaneda *et al.* [15]. After re-evaporation, the residue was submitted to a modified clean-up procedure similar to that suggested by Stabell *et al.* [16]. As our silica cartridges were packed with 0.69 g of phase instead of the 0.1 g used by Stabell *et al.*, the solvent volumes used in the clean-up procedure were proportionally modified.

For FFA analysis (method 3b), steps similar to those in method 2 were intitially followed; the volumes of the extraction solvents (acetone and diethyl ether) were reduced proportionally to those used in method 2 (as 1 g instead of 20 g of mussel hepatopancreas was extracted). The evaporated extract was redissolved in 2 ml of DMF, and an aliquot of 0.5 ml was derivatized by adding 1 ml of CA and 0.5 ml of TMA solutions [15]. A 500-μl volume of the derivatized mixture was loaded on a C_{18} Sep-Pak column (conditioned with DMF) and eluted using 10 ml of acetone. After evaporation, the volume was again adjusted to 500 μl using DMF and the solution was used for chromatographic injections (injection volume 4 μl).

According to method 1, an injection of 10 μl corresponds to 1/400th of the extract from 1 g of mussel hepatopancreas [6]. Method 3a uses an

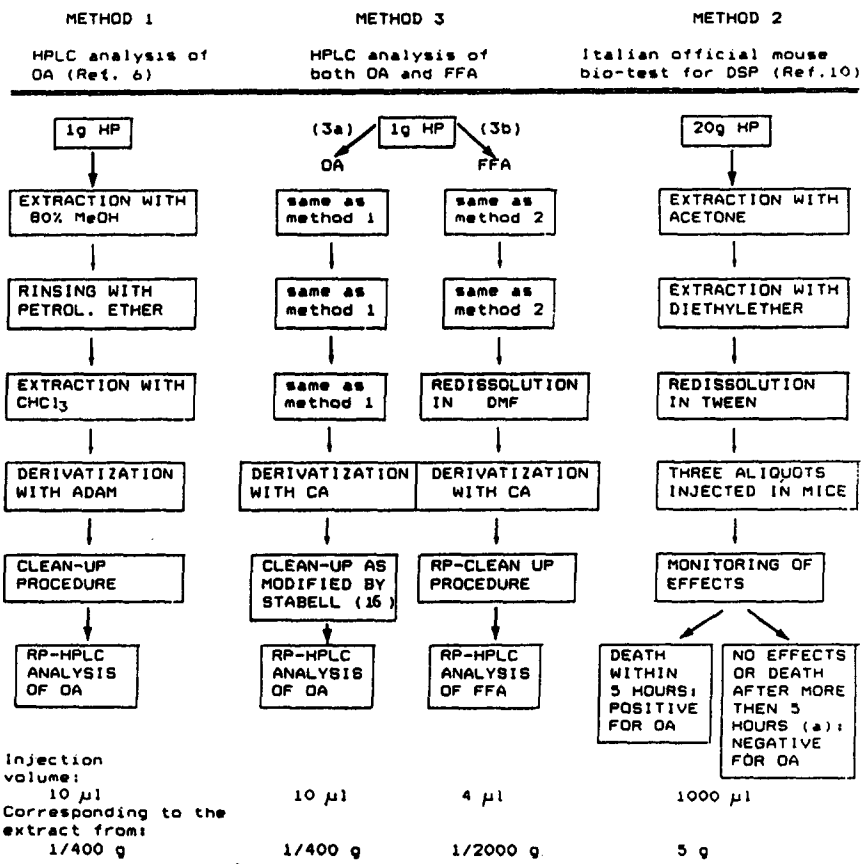

Fig. 1. Schematic diagram of analytical methods for DSP toxins. According to Italian law [10] the mouse biotest is positive when the average death time (three mice) is less than 5 h.

injection of the same volume (10 µl) and the same amount of extract, whereas in method 3b for FFA analysis the volume injected (4 µl) contains 1/2000th of the extract from 1 g of mussel hepatopancreas.

In method 2, as the weight of the dry extract from 5 g (corresponding to the dose used for injecting i.p. a 20-g mouse) was found to be about 100 mg, the dose used in the biotest is about 5000 mg/kg, which is very high.

Chromatographic conditions

The developed chromatographic conditions were as follows. Solvent A was acetone and solvent B was acetonitrile–water (50%, v/v), the solvent programme being 55% of A, isocratic for 6.5 min, then a linear gradient up to 95% of A in 26.5 min (total time), followed by 5 min of purging (98% of A) and 10 min of column re-equilibration. The solvent flow-rate was 1 ml/min. The column used was an Hibar LiChrosorb RP-8 (body porous, 5 µm) (25 × 0.4 cm I.D.) (E. Merck), equipped with a precolumn (4 × 0.4 cm I.D.) filled with a C_{18} phase (pellicular, 15 µm) (E. Merck). The chromatograms were recorded with the excitation and emission wavelengths of the spectrofluorimetric detector set at 366 and 404 nm, respectively.

RESULTS AND DISCUSSION

To enhance the detection sensitivity of very small amounts (ng) of OA, a derivatization reaction with ADAM as fluorescent labelling reagent was used [6]. A similar derivatization reaction occurs when CA is used instead of ADAM; recent applications of these

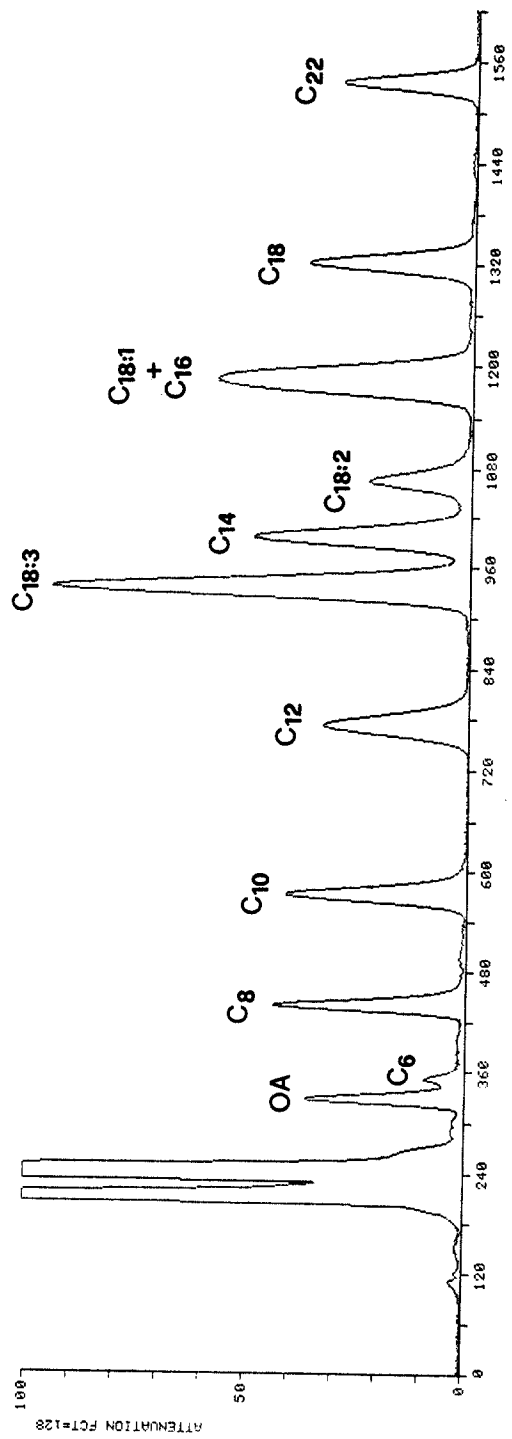

Fig. 2. Chromatogram of a mixture of OA and FFA derivatized with CA and injected without purification. Spectrofluorimetric detection with λ_{ex} 366 nm and λ_{em} 404 nm. Injection volume, 10 μl. The mixture shown was not prepared for quantitative analysis; the peaks correspond to about 80 ng of OA and to various amounts (from 20 to 100 ng) of FA.

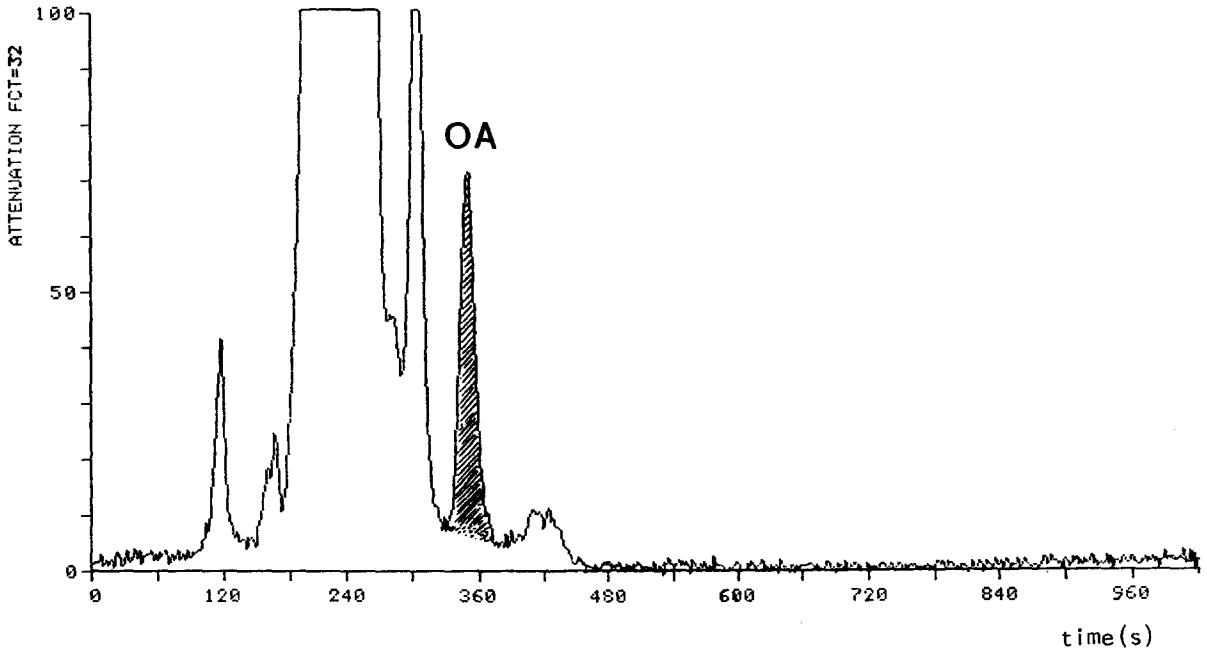

Fig. 3. Chromatogram of a mussel sample spiked with OA to obtain the recovery graph. The sample injected (10 μl) was prepared using method 3a and the OA peak corresponds to 40 ng.

derivatization reactions have been published relating to the HPLC determination of FFA in human serum (derivatized with ADAM) [17] and in beer (derivatized with CA) [15].

In the extract of mussel hepatopancreas, OA and FFA may both be present, and we developed a chromatographic system that allows for their simultaneous separation in an artificial standard mixture (Fig. 2). This separation cannot be obtained with mussel extracts, owing to the separate methods (3a and 3b) of sample preparation.

In our hands, method 1 failed to yield reproducible results; similar problems, with large inter-laboratory variations, were recently reported [16]. The method was therefore modified, changing the derivatization agent from ADAM to CA, and adopting the sample clean-up procedure suggested by Stabell *et al.* [16]. CA was chosen instead of ADAM for labelling the analytes because of the instability of ADAM. Fig. 3 shows the chromatogram obtained from a mussel sample spiked with OA, extracted, derivatized and purified according to method 3a.

The calibration (c) and recovery (r) graphs of OA, obtained from standard solutions and from spiked samples, respectively, are described by the following equations:

(c) $y = 0.400 \cdot 10^6 + 1.410 \cdot 10^6 x$ ($R^2 = 0.99994$)

(r) $y = -0.200 \cdot 10^6 + 1.313 \cdot 10^6 x$ ($R^2 = 0.99798$)

The accuracy of the method relates to the recovery of OA from spiked samples; the recovery, calculated as the ratio between the slope of the recovery graph and that of the calibration graph, was 93.1 ± 3.2% ($n = 5$). The resulting precision (relative standard deviation) was 3.4%. These values are close to those reported by Edebo *et al.* [13].

Japanese law sets the maximum level for DSP in shellfish hepatopancreas (HP) at 0.5 MU/g HP (MU = mouse units), that is, 2 μg OA/g HP [6]; in Norway the maximum tolerable level was set at 24 μg OA per 100 g of shellfish meat [16]. These concentrations are almost equivalents. As far as the Italian law is concerned, the tolerable level of OA (which must be determined using the mouse biotest with a recently modified threshold limit) can hardly be expressed as a weight concentration value. It may anyway be assumed that the tolerated concentration

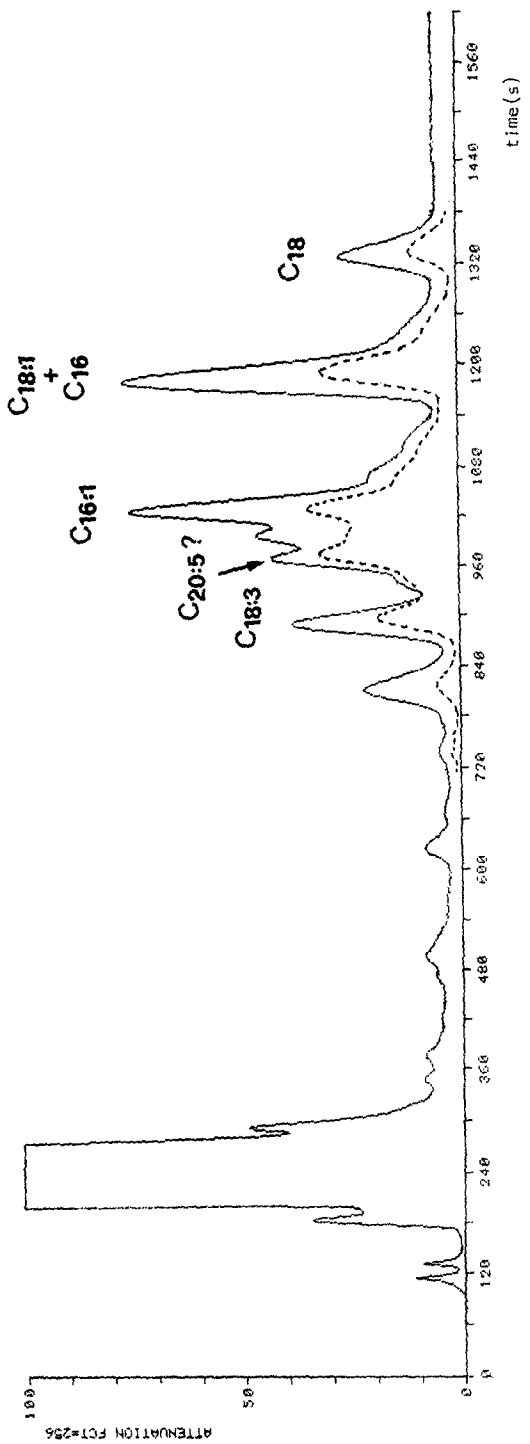

Fig. 4. Chromatograms of FFA of mussel hepatopancreas. The samples were prepared according to method 3b. The continuous and dashed lines refer to samples collected on March 18th and May 21st, 1991, respectively. Injection volume, 4 μl.

of OA is similar to or slightly higher than the above-cited values (2–2.5 μg OA/g HP).

The detection limit of OA using the HPLC method was reported to be 0.4 μg/g [6]; our detection limit, at a signal-to-noise ratio of 3, is higher, 1 μg/g, but good enough to detect OA levels well within the limits of the cited laws. In the mussel samples analysed so far to test the method, no OA was found, or its concentration was below the detection limit.

The analytical methods for DSP toxins which use the mouse biotest suffer from variability problems. As the toxicity of the FFA (when injected i.p. in the mouse biotest) present in the extract was well known [6,12], we developed a parallel route (method 3b) for determining the FFA present in the same sample as tested for OA. In this instance, the extraction was performed according to the Italian official biotest, to produce comparable samples.

Fig. 4 shows the chromatograms obtained from two different mussel samples following method 3b. The chromatogram drawn with the continuous line refers to a sample collected on March 18th 1991, whereas that drawn with the dashed line refers to a sample collected on May 21st 1991. The FFA distribution is similar, but the total amount is higher in the sample collected in March. The different concentrations of FFA found in mussel HP are linked to the biological cycle and reflect similar differences observed in mussel meat [18]. Comparing the peak retention times and by using the standard addition technique, some of the FFA (including C18:3) were identified, as listed. Our tentative assignment of one peak of the chromatogram, attributed to C20:5, was deduced from its expected elution order. The use of a more complex FFA standard mixture (including more polyunsaturated FA and possibly all of those listed [12] as the most toxic, *viz.*, linolenic, C18:3; araquidonic, C20:4; and eicosapentaenoic, C20:5) is planned for future studies.

A calibration and a recovery graph of palmitoleic acid (C16:1), used as a standard, was obtained. The same recovery value (95%) and the same fluorescence response were attributed to all FFA, and the area sum of all peaks was used to obtain the total amount of FFA in mussel HP. The FFA concentrations found in the two samples were 136 and 360 μg/g HP (these are only rough figures because different FA may give slightly different fluorescence responses). These concentrations are far from the dose (12 mg per mouse) reported as effective in causing death in mice [12], but the differences could explain the large variability of the results obtained by means of the mouse biotest. If and when future incidents of mussel contamination with DSP toxins occur, a systematic application of the present instrumental method, coupled with the official biomethod, should prove very useful for a more detailed interpretation of the results.

ACKNOWLEDGEMENTS

We acknowledge the courtesy of Professor T. Yasumoto, who sent us reprints of his papers on DSP toxins and provided valuable information about the availability of OA standard. We also thank Mr. M. Minca for providing mussel samples and M. Franco Orel for his skilled technical assistance.

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