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# Development and validation of a high-performance liquid chromatographic method using fluorimetric detection for the determination of the diarrhetic shellfish poisoning toxin okadaic acid without chlorinated solvents

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**Abstract**

A modification of the high-performance liquid chromatographic method with fluorimetric detection method for the determination of diarrhetic shellfish poisoning toxins was developed to completely avoid the use of dangerous chlorinated solvents. The method was validated for the toxin okadaic acid (OA) over a period of 6 months where 12 calibrations were performed and 72 samples were analyzed. Analysis of toxic and non-toxic mussels, clams and scallops demonstrated its selectivity. Linearity was observed in the tested range of interest for monitoring purposes of edible shellfish, from the limit of detection (0.3 μg OA/g hepatopancreas) to 13 μg OA/g hepatopancreas. Intra-assay precision of the method was 7% RSD at the quantification limit (0.97 μg OA/g hepatopancreas at $S/N=10$). Accuracy was tested in triplicate recovery experiments from OA-spiked shellfish where recovery ranged from 92 to 106% in the concentration range of 0.8 to 3.6 μg OA/g hepatopancreas. Useful information on critical factors affecting calibration and reproducibility is also reported. Good correlation ($R=0.87$) was observed between the results of the method and those of the method of Lee, after the analysis of 45 samples of mussels from the galician rias. 

*Keywords:* Shellfish poisoning; Okadaic acid; Non-chlorinated solvents

## 1. Introduction

Diarrhetic shellfish poisoning (DSP) is a human syndrome, caused by the following liposoluble polyethers obtained from marine dinoflagellates: okadaic acid (OA), dinophysistoxin-1 (DTX-1), dinophysistoxin-2 (DTX-2) and dinophysistoxin-3 (DTX-3) [1,2]. These diarrhogenic and tumor promoting [3,4] substances can accumulate in filtering shellfish and reach the human consumer. DSP has become a worldwide public-health problem that severely affects the shellfish industry. The fluorimetric detection (FLD) by high-performance liquid

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chromatography (HPLC) of fluorescent derivatives of the toxins [5] has allowed worldwide the identification and quantification of the toxins. This technique became an essential complement of the official mouse bioassay in the regulatory tasks concerning the extraction of shellfish in growing areas, due to economic advantages over HPLC–mass spectrometry (MS) [6] and to analytical advantages over immunoassay-based methods [7,8] or phosphatase inhibition assays [9,10].

Sample preparation for the HPLC–FLD determination of these relatively polar liposoluble toxins usually requires use of dichloromethane or chloroform. These are dangerous chlorinated solvents that must be eliminated as soon as possible from ordinary analytical protocols. Toxicological studies of these solvents revealed several toxic activities: neurotoxicity [11,12], carcinogenesis [13], hepatotoxicity [14] and carboxyhemoglobinemia [15]. Toxicity is developed after their biotransformation through two independent pathways yielding different toxic metabolites, mainly carbon monoxide (via oxidative dehalogenation by the cytochrome P450-dependent mixed function oxidase system) and formaldehyde (via the glutathione-*S*-transferase pathway).

Several analytical protocols recently reported, avoided these kinds of solvents in the sample clean-up but, when compared with the original method [5], the selectivity decreased due to chromatographic interferences [16,17] and more complexity was required either in sample processing [18] or instrumental equipment [19]. Furthermore, some of them still required chlorinated solvents in the purification of the crude extract by liquid–liquid extraction.

In this paper we present a modification of the original method for the HPLC–FLD analysis of DSP toxins where chloroform was substituted by a mixture of non halogenated solvents. Furthermore, toxins were derivatized with 1-bromoacetylpyrene (BAP) to overcome the drawbacks of the 9-anthryldiazomethane (ADAM) reagent. The method was developed with the aim of introducing minimal modifications to assure its reliability and facilitate its implementation.

The method was submitted to validation for OA, the main toxin in Europe that is occasionally detected along with its close isomer DTX-2. Nevertheless, DTX-2 is not commercially available and DTX-1, the methylated derivative of OA, only exceptionally has affected shellfish in Northern Europe. Although similar results could be expected for the OA relatives DTX-1 and DTX-2, validation for these important toxins should be addressed later in this or other laboratories where these toxins currently occur.

## 2. Experimental

### *2.1. Reagents*

Certified calibration solutions of OA purchased from the Institute for Marine Biosciences, National Research Council of Canada, Halifax, Canada (reference OACS-1) were used for HPLC calibration. OA with a purity ≈98% (Alexis, Switzerland) was used in the recovery tests. Stock solutions of OA were stored at −80°C and diluted working solutions were stored at −20°C. BAP (Aldrich, USA) was stored at −20°C, as were the acetonitrile solutions of BAP (0.2%, w/v) and 10% diisopropylethylamine (DIPA) (Sigma, USA). Petroleum spirit (40–60°C), *n*-hexane, ethyl acetate and methanol were of analytical grade as were amylene-stabilized dichloromethane and chloroform (Panreac, Spain). HPLC-grade acetonitrile (Panreac) and Milli-Q water (Millipore, Spain) were used in the HPLC mobile phase. Bulk silica gel 60 (40–63 μm) for column chromatography (Merck, Germany), stored under room conditions, was used in the solid-phase extraction (SPE) clean-up of toxin derivatives before HPLC analysis.

### *2.2. Extraction*

#### *2.2.1. (A) Extraction of Lee*

Hepatopancreas carefully removed from whole body of shellfish was homogenized and 2-g subsamples were extracted following the method of Lee et al. [5].

#### *2.2.2. (B) Alternative extraction*

Initial solid–liquid extraction with 8 ml of 80% methanol per 2 g of shellfish hepatopancreas homogenate is carried out in the same way as in the usual procedure of Lee. The extraction of Lee constitutes a dispersive non-exhaustive extraction whose concentration values on shellfish show an

acceptable accuracy [20]. An aliquot (2 ml) of the aqueous–methanolic crude extract was transferred to a test tube fitted with a screw-cap and washed with *n*-hexane, 2×2 ml, by vortex-mixing for 30 s and centrifuged (1500 *g*/1 min). The upper layer was discarded each time, and water (2.8 ml) and ethyl acetate (3 ml) were added to the residual solution and vortex-mixed for 2 min. After centrifugation (1500 *g*/1 min) the upper ethyl acetate layer was transferred to a test tube. The ethyl acetate extraction was repeated once and both ethyl acetate extracts were combined and made up to 10 ml. OA was not stable in ethyl acetate extracts for longer than 1–2 days; hence extracts should be analyzed on the day.

Diethyl ether extracts tested during method development were obtained in the same way as ethyl acetate extracts.

### 2.3. Derivatization

A 500-μl aliquot of the final extract was placed in a polypropylene microcentrifuge microtube (reference 000-MICR-050 Elkay, Ireland). Ethyl acetate was removed under a stream of nitrogen. After that, 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 [21]. The sample was cleaned-up through SPE on the same day.

### 2.4. Silica gel solid-phase extraction

#### 2.4.1. (A) Reference method [21].

The reference method is a dichloromethane-based procedure that provides a high selectivity. Chromatograms were used as reference in method development.

#### 2.4.2. (B) Ethyl acetate-based method proposed in this paper

Cartridge columns (6 ml) packed with 200 mg of silica were used. The cartridges were reused by refilling with silica. Pre-conditioning was not necessary. Three subsequent times, a portion of 0.5 ml of hexane–ethyl acetate (78:22, v/v) was added to the sample and then transferred to the cartridge. Then, the cartridge was washed with 10 ml of hexane–ethyl acetate (78:22, v/v) and the esters were finally eluted with 5 ml of ethyl acetate–methanol (90:10, v/v).

#### 2.4.3. (C) Tetrahydrofuran-based method tested in this paper

Cartridge columns (6 ml) packed with 200 mg of silica were used. The sample was loaded in three portions of 0.5 ml of hexane–tetrahydrofuran (80:20, v/v). The cartridge was washed with 10 ml of hexane–tetrahydrofuran (80:20, v/v) and the esters were finally eluted with 5 ml of hexane–tetrahydrofuran–methanol (50:30:20, v/v/v).

### 2.5. HPLC

After removal of ethyl acetate solvent under a stream of nitrogen, the residue was dissolved in 100 μl of acetonitrile and the solution transferred to a capped glass vial and protected from light with an aluminum foil. Stability of the sterified toxin was observed at least for 4 days at room temperature, thus allowing batch analysis. An aliquot of 20 μl was injected into the HPLC system.

The LC system consisted of a single HPLC pump (Kontron 420, Italy) set at a flow-rate of 1.1 ml/min, a variable-wavelength fluorescence detector (Shimadzu RF-535, Japan) set to 356 nm excitation and 440 nm emission and an HPLC autosampler with a loop of 20 μl (20 μl were injected) (Kontron 360, Italy). A Hypersil-ODS (5 μm, 250×4 mm, Tracer Analytica, Spain) cartridge column eluted with acetonitrile–water (85:15, v/v) was used. Data collection and analysis were done using the Kontron chromatographic data system 450-MT2-V3.0. The final value of OA concentration in shellfish is obtained from the equation: μg OA/g hepatopancreas=ng OA on-column×0.25.

### 2.6. Calibration

Calibrations were performed from duplicate determinations of five different amounts of OA (1–12 ng OA on-column equivalent to 0.25 to 3 μg OA/g hepatopancreas). Every calibration was completely run on the same day. Methanolic certified calibration solutions of OA (25.3 ng OA/μl) were used for HPLC calibration. Intermediate diluted solutions of 0.2 ng OA/μl were carefully prepared for every

calibration from which different volumes (25 to 300 μl) were transferred to polypropylene microtubes to be derivatized.

### 2.7. Samples

Throughout a period of 6 months, different lots of non-toxic shellfish were provided by a local market and analyzed: five lots of fresh mussels (*Mytilus galloprovincialis*), three lots of fresh clams (*Dosinia excelsa*) and two lots of scallops (*Pecten maximus*). Recovery experiments were carried out by spiking with OA hepatopancreas homogenates (2 g); spiked samples were equilibrated by stirring the hepatopancreas homogenate and the added methanolic solution of OA for 3 min.

### 2.8. Intercomparison

Nine lots (78 samples) of mussel samples collected weekly in the galician rias in Spring 1999, were homogenized and an aliquot of 2 g of hepatopancreas homogenate was simultaneously analyzed with the original method of Lee et al. [5] (Laboratory of ANFACO-CECOPESCA) and the proposed method without chlorinated solvents (Laboratory of the Department of Pharmacology, University of Santiago de Compostela). In every laboratory, the homogenate was extracted and the extract was determined in duplicate.

### 2.9. Statistics

Statistical analysis was carried out with the software package Statistica 5.0 (StatSoft, USA), except for the quality control chart that was obtained with Microcal Origin 4.00 (Microcal Software, USA).

The quality control chart of the daily mean fluorometric response (peak height/ng OA) corresponding to the 12 daily calibrations performed over a period of 6 months comprises an X chart (average of the peak height/ng OA ratios) and a range chart (difference between the highest and the lowest daily value of the peak height/ng OA ratios). Plots for every calibration were obtained from 10 standard samples. Upper and lower control limits (UCL and LCL) are set at $\pm 3\sigma$ from average response and average range (AVE).

Response linearity in the calibration was checked through a linearity plot where the response factor (peak height divided by the amount of OA on-column) is plotted versus the amount of OA on-column [22].

## 3. Results

### 3.1. Method development

Sample preparation prior to HPLC–FLD determination of BAP-derivatives of DSP toxins comprise three stages: extraction, derivatization and clean-up of the fluorescent derivatives through SPE. Chloroform [5] or dichloromethane [21] are currently used as much in extraction as in clean-up. Hence, we separately addressed both stages with the aim of finding new solvents that were able to replace dichloromethane and/or chloroform.

#### 3.1.1. Extraction

The common extraction procedure of Lee et al. [5] begins with the homogenization of the digestive glands and after liquid–liquid extraction, toxins are transferred to chloroform.

Our work intended to simply replace the final chlorosolvent by another solvent with a solubility strength (estimated by means of the experimental parameter Rohrscheneider polarity) close to chloroform. This solvent should be also immiscible with water to allow liquid–liquid extraction. Two solvents that fulfilled these requirements were tested: ethyl acetate and diethyl ether.

Ethyl acetate was definitely selected for the liquid–liquid extraction because it provided cleaner chromatograms than diethyl ether, independently of the SPE clean-up applied. Later improvements were obtained substituting petroleum spirit with *n*-hexane in the intermediate liquid–liquid extraction, as is reflected in the Experimental section. Addition of anhydrous sodium sulfate sped up the evaporation of the ethyl acetate before derivatization by removing the water solubilized in ethyl acetate (up to 80 g water/l). The transfer rate of OA from the aqueous–methanolic solution to ethyl acetate allowed a quantitative transfer after two extractions with ethyl acetate and OA was not detected in subsequent extracts.

*3.1.2. Silicagel solid-phase extraction*

Recently we reported a highly efficient procedure of SPE on silicagel for the BAP-derivatives of DSP toxins, although this optimized procedure still required the use of dichloromethane [21]. In developing an alternative procedure to avoid dichloromethane, silicagel was maintained as solid adsorbent while looking for a solvent with an eluting strength similar to dichloromethane/chloroform. Solvents with values of Rohrscheneider polarity [23] and eluotropic strength on $Al_2O_3$ ($\epsilon^0$) [24] close to those of dichloromethane/chloroform were first selected, except for those with strong dipolar moments such as acetone or acetonitrile because they caused high losses of toxin BAP-derivatives during the washing steps of silicagel SPE [21].

Solvents selected under the above conditions were: diethyl ether, tetrahydrofuran and ethyl acetate. Clean-up efficiency was adjusted by means of the concentration of hexane in the washing solution, an apolar solvent which eluting strength on silicagel is close to zero. Total washing volume and the amount of adsorbent were adjusted to obtain optimal conditions. Analysis of mussel extracts (ethyl acetate and also chloroform extracts) showed that diethyl ether was unable to remove chromatographic interferences. On the other hand, as much the tetrahydrofuran as the ethyl acetate based methods provided a selective clean-up of the BAP-derivative of OA. Toxin losses were not detected and peak heights corresponding to a sample of OA standard were not statistically different before (10.6 ng OA on-column; $n=3$) and after ($n=3$) the clean-up (*t*-test: tetrahydrofuran-based method *P*-level=0.4702; ethyl acetate-based method *P*-level=0.8945).

We finally propose the ethyl acetate clean-up and the ethyl acetate liquid–liquid extraction, a combination which provided the best results: the modified extraction yields optimal toxin recoveries (accuracy) and the alternative SPE proposed, selectively cleans the sample up without significant losses.

*3.2. Method validation*

*3.2.1. Linearity*

Mean peak heights from double measurements (derivatization, clean-up and determination) corresponding to five amounts of OA (1, 2, 4, 8 and 12 ng OA on-column) prepared from the certified OA solution generated the least-squares regression curve. Twelve daily calibrations performed throughout a period of 6 months showed: (a) the *y*-intercept of the regression curves was not significantly different from zero in any of the daily calibrations (the lowest *P*-value in the *t*-test of the intercept was 0.2187). (b) Linearity of mean peak height versus OA on-column was observed in the tested calibration range of 1 to 12 ng. Linear correlation coefficient *R* ranged from 0.9970 to 0.9998 with a median value of 0.9994. Linearity plots obtained for every calibration (percentage response factor versus the amount of OA on-column) showed that the responses fell within 100±10% of the mean response in the range of 2 to 12 ng OA but occasionally increased to 100±20% in the 1–2 ng range, in accordance with a decrease in precision. The near-zero slope of the linearity plot (the slope value lay between −0.71 and 1.68) demonstrate that systematic deviations of the linearity over the calibration range are not present. A typical regression curve with the corresponding linearity plot is shown in Fig. 1; in addition to the standard solutions of calibration, 14 shellfish samples were analyzed in the same day and the OA retention time was 8.76 min with a relative standard deviation (RSD) of 0.59%. (c) Intra-assay standard deviation (SD) of peak heights increases proportionally to concentration (heterocedasticity) but regression curves were obtained with the non-weighed least-squares method, which showed that the regression curves were essentially identical to those obtained

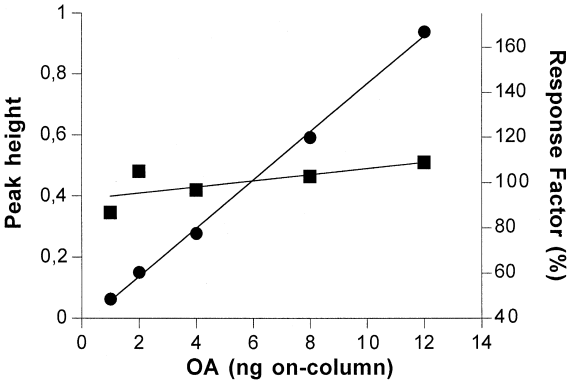


Fig. 1. Calibration with intermediate linear fitting. ● Regression curve ($R^2=0.9981$). ■ Corresponding linearity plot (slope of the linearity plot regression line=1.356); mean response factor of the five calibration points was set as the 100% response factor.

with the more rigorous weighed least-squares. (d) Optimal frequency of recalibration was established by studying the fluorescence/mass ratio or response factor over time; the RSD of the slope of the calibration curves was 7.1% although occasional deviations from statistical control of the mean fluorimetric response were observed over the 6 months in the corresponding X control chart (plot not shown). Critical factors affecting the signal-to-mass ratio by order of importance were, the accuracy in the composition of the washing solution in SPE, the accuracy in the composition of mobile phase and the accuracy of the calibration solution prepared from the certified OA. Therefore, the system should be recalibrated every time new SPE solutions and mobile phase are used, and their simultaneous preparation is recommended. Nevertheless, due to the long-term stability of the response, we consider a three-point calibration in later routine work feasible.

*3.2.2. Selectivity*

Chromatographic interferences were not observed at the retention time of OA in non-toxic shellfish (Fig. 2). Chromatographic resolution $R_s$ of the OA peak from shellfish extracts usually ranges from 3 to 7 although more complicated mussel samples were analyzed where $R_s$ values for OA decreased to 1.7 due to the presence of extraneous peaks related to seasonal variations; nevertheless, resolution was sufficient in all tested samples to achieve a selective determination.

*3.2.3. Precision*

*3.2.3.1. (a) Standard solutions of OA: calibration*

Intra-assay standard deviation of the fluorimetric signal from standard solutions ($n=24$) increased with the amount of toxin injected (SD=0.0044+0.0050 ng on-column $R=0.993$). Intra-assay variability explains most of the total variability and hence inter-calibration RSD was just slightly higher than intra-calibration RSD.

*3.2.3.2. (b) Shellfish samples*

Intra-assay standard deviation observed in shellfish samples did not show dependence on concentration ($n=29$). Inter-assay RSD over different days ranged from 17% to 8.5% in the concentration range 0.6 to 2.0 μg OA/g hepatopancreas (data not shown). Inter-assay results were obtained from five mussel samples analyzed over 3 days in triplicate with daily calibration.

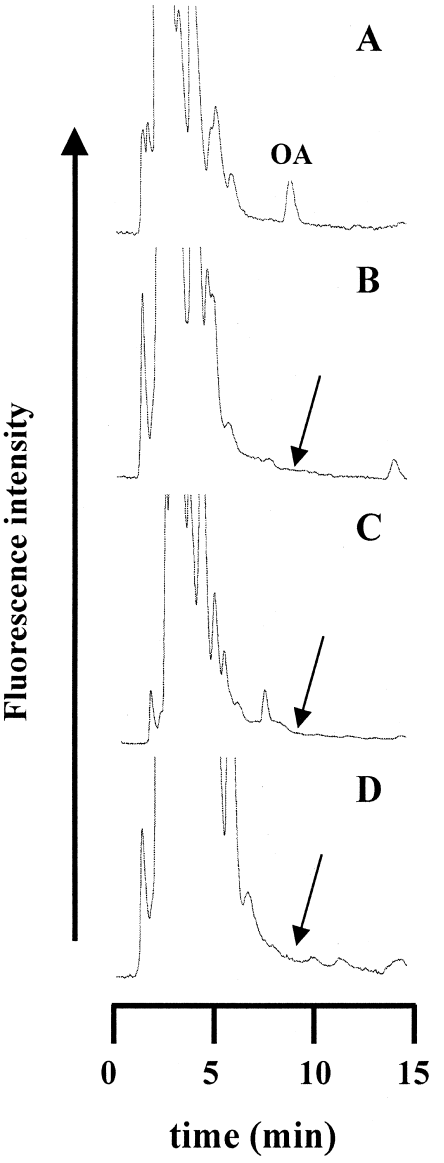


Fig. 2. Chromatograms corresponding to (a) mussels containing 2.9 μg OA/g hepatopancreas, (b) non-toxic clams, (c) non-toxic scallops and (d) non-toxic mussels. Arrows indicates the expected elution time of the DSP toxin OA.

To sum up, precision of the method is basically explained by the intra-assay variability as much in

shellfish samples as in toxin standard solutions. The different precision dependence on concentration of shellfish and standard samples was attributed to the critical effect of measuring the volume to derivatize: in shellfish samples a constant volume (constant error) of 0.5 ml of extract is taken while different volumes of the intermediate standard solution of 0.2 ng OA/μl are derivatized according to the desired amount of toxin on-column (non-constant error).

### 3.2.4. Detection limit

#### 3.2.4.1. (a) Standard solutions of OA

The least favorable estimation gave a limit of detection at $S/N=3$ (100% uncertainty) of 0.5 ng OA on-column (0.1 μg OA/g hepatopancreas): the highest estimation of standard deviation when OA→0) at a 95% level of confidence together with the lowest estimation of the slope of the calibration curve at the same confidence level was considered. The limit of quantification at $S/N=10$ (30% uncertainty) is 1.7 ng OA on-column (0.4 μg OA/g hepatopancreas).

#### 3.2.4.2. (b) Shellfish samples

Standard deviation in shellfish samples was approximately constant over concentration; the maximum standard error of 0.097 observed gives a limit of detection of 0.3 μg OA/g hepatopancreas at a $S/N=3$. The corresponding limit of quantitation at $S/N=10$ was 1.0 μg/g hepatopancreas.

### 3.2.5. Accuracy

In order to demonstrate the accuracy of the method, non-toxic hepatopancreas from mussel and clams were spiked with known amounts of OA and analyzed in duplicate at every toxic level from 0.8 to 3.6 μg OA/g hepatopancreas. Results shown in Table 1 demonstrate the accuracy of the method.

### 3.2.6. Critical factors

(a) The microtubes where the esterification reaction between OA and 1-bromoacetylpyrene is driven have been revealed as a noteworthy critical factor: microtubes made from plastics other than polypropylene caused extraneous chromatographic peaks or interfered with the nucleophilic reaction of esterification limiting the reaction yield. (b) Reproducibility of the SPE on silicagel depends on the accuracy of the composition of the washing solution. Therefore, variations in the fluorimetric response factor can be observed from set to set of washing solution prepared, requiring a new calibration. Nevertheless, the solution can be stored for several weeks in sealed bottles. (c) Precision is mainly determined by the precision of the measurement of volume derivatized, according to the fact that derivatization is the main dilution step.

### 3.2.7. Intercomparison

Correlation of dual results of the 45 samples with an OA content in the range 1 to 12 μg/g hepatopancreas is shown in the plot of Fig. 3. Samples with a toxin concentration below the limit of quantification were rejected for the correlation. Additional calibrations were performed to assess the linearity of the response at concentrations up to 13.5 μg OA/g hepatopancreas (equivalent to 50 ng OA on-column).

Table 1
Recovery and variability data from 2 g of shellfish hepatopancreas spiked with OA

| Sample | OA added (μg OA/g hepatopancreas) | OA found ($n=2$) (μg OA/g hepatopancreas) | Mean recovery (%) |
|---|---|---|---|
| OA+clam | 0 | Not detected | |
| | 0.90 | 0.9, 1.0 | 105 |
| | 1.80 | 1.9, 1.8 | 103 |
| | 3.61 | 3.6, 3.5 | 98 |
| OA+mussel | 0 | Not detected | |
| | 0.81 | 0.8, 0.8 | 99 |
| | 1.61 | 1.4, 1.7 | 96 |
| | 3.23 | 2.9, 3.0 | 91 |

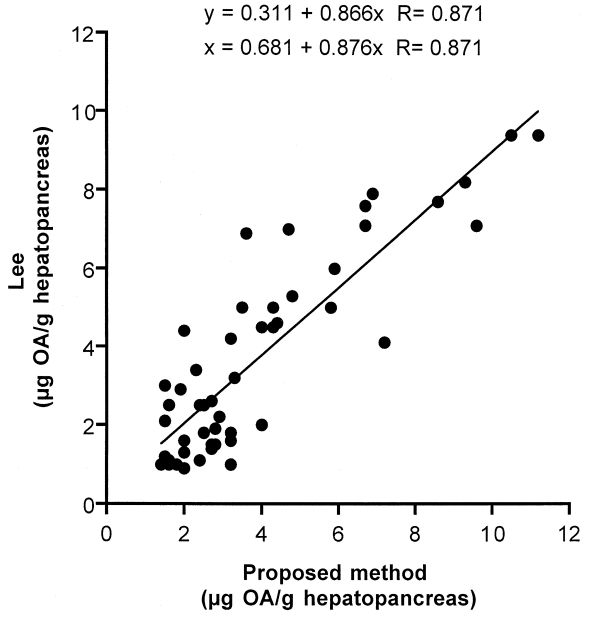


Fig. 3. Correlation of the results of the analysis of mussel samples with the method of Lee and with the proposed method. Samples with less than 1 μg OA/g hepatopancreas (the limits of quantification were not plotted).

## 4. Discussion

Due to its economic advantages, HPLC–FLD is the most widespread instrumental method of analysis of DSP toxins at the moment, as much for research purposes as for the regulation of seafood. Subsequent optimizations of the original method [5] were mainly centered on solving the analytical drawbacks of the original version. Nevertheless, the highly toxicological risks of the halogenated solvents currently used to handle these liposoluble toxins are a concern. Two recent papers proposed alternative procedures completely free of these highly toxic solvents [16,17], although there are other papers where the most solvent-consumer step, the extraction, still requires dichloromethane [18,19]. Nevertheless, none of these methods was submitted to a validation process and the presence of many extraneous peaks in the chromatograms published and the inadequacy in the accuracy of the data reported are a drawback to them becoming a serious alternative to the HPLC–FLD analysis of DSP toxins.

The method presented here was developed by changing as little as possible the original method of Lee. The strategy of just introducing slight modifications could make the implementation of the method easier in all the laboratories where DSP toxins are currently determined by HPLC–FLD and makes the method more reliable: extraction of the toxins from shellfish is unchanged, the SPE clean-up of the labeled toxins is also carried out on silicagel as a solid adsorbent, and the steps of the whole protocol are thoroughly parallel to the original method. One additional change was that the fluorescent labeling reagent of the toxins, ADAM, was substituted by BAP. Its advantages of lack of chromatographic interferences, stability, cost, esterification time and fluorescence yield are well known [21,25,26]. SPE procedures over silicagel are highly widespread in modern instrumental analysis. Its development and improvement can be greatly facilitated by considering the parameter ''eluotropic value over alumina'' ($\epsilon^0$) [24]. The results obtained in the development of the alternative SPE, described in this work, support the foreseen usefulness of this experimentally determined parameter. Taking into account the theoretical eluting strength of the washing solution (estimated by this parameter) and the loaded volume of silica in the SPE cartridge, similar washing performance could be expected for the ethyl acetate-based clean-up, the tetrahydrofuran-based clean-up and the dichloromethane-based clean-up. In fact, experimental results confirmed the ability of the three procedures to clean-up these samples. Nevertheless, the ability of the recovery solution to elute the labeled toxin afterwards, was not correlated with the eluting strength estimated through $\epsilon^0$, but the presence of the protic solvent methanol. Yet, the control of the eluting strength was very important to minimize chromatographic interferences due to substances left in the SPE cartridge.

The method was tested for a period of 6 months, allowing the identification of critical factors and the establishment of its analytical performance and limitations for the analysis of the DSP toxin OA. We think that the validation presented here clearly shows the viability of this modified protocol for the HPLC–FLD analysis of OA and presumably of its isomer DTX-2 and its methyl derivative DTX-1. The future validity of the method lies in the economic advantages of HPLC–FLD over other more powerful and

expensive methods, such as HPLC–MS, that cannot be considered a universal alternative at the moment. It is expected that HPLC–FLD will continue to be complementary of newly developed biological methods such as the promising protein phosphatase inhibition assay [9,10].

The quality of the data obtained with the instrumentation described, allows the determination of the toxins at the ppm level, the concentration range of interest for edible shellfish with a typical incertitude at this trace-level without dangerous chlorinated solvents.

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