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# Analysis of trace levels of domoic acid in seawater and plankton by liquid chromatography without derivatization, using UV or mass spectrometry detection

Luiz L. Mafra Jr.[a,b], Claude Léger[c], Stephen S. Bates[c], Michael A. Quilliam[a,*]

[a] National Research Council, Institute for Marine Biosciences, 1411 Oxford St., Halifax, NS B3H 3Z1, Canada
[b] Dalhousie University, Department of Biology, Halifax, NS B3H 4J1, Canada
[c] Fisheries and Oceans Canada, Gulf Fisheries Centre, P.O. Box 5030, Moncton, NB E1C 9B6, Canada

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ABSTRACT

Quantitation of trace levels of domoic acid (DA) in seawater samples usually requires labour-intensive protocols involving chemical derivatization with 9-fluorenylmethylchloroformate and liquid chromatography with fluorescence detection (FMOC–LC–FLD). Procedures based on LC–MS have been published, but time-consuming and costly solid-phase extraction pre-concentration steps are required to achieve suitable detection limits. This paper describes an alternative, simple and inexpensive LC method with ultraviolet detection (LC–UVD) for the routine analysis of trace levels of DA in seawater without the use of sample pre-concentration or derivatization steps. Qualitative confirmation of DA identity in dubious samples can be achieved by mass spectrometry (LC–MS) using the same chromatographic conditions. Addition of an ion-pairing/acidifying agent (0.15% trifluoroacetic acid) to sample extracts and the use of a gradient elution permitted the direct analysis of large sample volumes (100 μl), resulting in both high selectivity and sensitivity (limit of detection = 42 pg ml$^{-1}$ by LC–UVD and 15 pg ml$^{-1}$ by LC–MS). Same-day precision varied between 0.4 and 5%, depending on the detection method and DA concentration. Mean recoveries of spiked DA in seawater by LC–UVD were 98.8% at 0.1–10 ng ml$^{-1}$ and 99.8% at 50–1000 ng ml$^{-1}$. LC–UVD exhibited strong correlation with FMOC–LC–FLD during inter-laboratory analysis of *Pseudo-nitzschia multiseries* cultures containing 60–2000 ng DA ml$^{-1}$ ($r^2 > 0.99$), but more variable results were obtained by LC–MS ($r^2 = 0.85$). This new technique was used to confirm the presence of trace DA levels in low-toxicity *Pseudo-nitzschia* spp. isolates (0.2–1.6 ng ml$^{-1}$) and in whole-water field samples (0.3–5.8 ng ml$^{-1}$), even in the absence of detectable *Pseudo-nitzschia* spp. cells in the water column.


## 1. Introduction

Domoic acid (DA), a neurotoxic tricarboxylic amino acid first isolated from the macroalga *Chondria armata* [1], was later identified as the causative agent of a human intoxication outbreak in Prince Edward Island, Canada, in 1987 [2]. During that episode, in which three people died and another 107 were hospitalized after ingesting contaminated shellfish [3], the diatom *Pseudo-nitzschia multiseries* was identified as the source of the toxin [4]. No other cases of human intoxication have been confirmed subsequent to that event, but DA has been detected in several regions worldwide, causing enormous economic losses to the aquaculture industry and massive death of marine fauna (e.g. [5–8]). Aquatic organisms, mainly bivalve molluscs, can accumulate high concentrations of the toxin upon suspension-feeding on planktonic assemblages containing toxic *Pseudo-nitzschia* spp. cells.

Liquid chromatography with ultraviolet detection (LC–UVD) was the first method developed to detect the toxin [9–11] and a protocol involving aqueous methanol extraction and strong anion-exchange (SAX) clean-up has been applied extensively to the analysis of DA in shellfish and fish tissues on a regulatory basis [12]. DA contains a characteristic conjugated diene chromophore with strong absorbance at 242 nm [13], which permits its detection by LC–UVD at concentrations as low as 4–80 ng ml$^{-1}$, depending on the sensitivity of the detector. However, DA concentrations in *Pseudo-nitzschia* cultures and phytoplankton field samples are often much lower, and therefore a more sensitive method of detection is required for research studies on algal production of DA and to investigate the presence of the toxin in seawater as an early alert for potential toxin accumulation in marine organisms.

Lower limits of detection (LODs) for DA in seawater can be achieved by reacting it with a fluorescent reagent, prior to LC with fluorescence detection (LC–FLD). The most common

* Corresponding author. Tel.: +1 902 426 9736; fax: +1 902 426 5426.
*E-mail address:* michael.quilliam@nrc-cnrc.gc.ca (M.A. Quilliam).

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method for the analysis of DA in seawater samples uses 9-fluorenylmethylchloroformate (FMOC–Cl) as the derivatizing agent, which results in a LOD ranging from 0.5 ng ml$^{-1}$ in isocratic elution down to 15 pg ml$^{-1}$ in gradient elution mode [14]. Despite of its high sensitivity, this procedure suffers from some problems: (a) labour-intensive operations; (b) poor selectivity due to the fact that many other compounds in the sample, such as other amino acids, can be derivatized and interfere with DA detection; (c) low precision when comparing multiple operators, probably due to differential handling during the derivatization step; and (d) gradual loss of detector sensitivity over time [14]. Other derivatization procedures have been published [15–17] but are no less labour-intensive, which is a serious obstacle for the use of LC–FLD on a large scale.

Several other methods have been developed to detect and quantify DA in seawater and tissue samples. They include approaches such as enzyme-linked immunosorbent assay (ELISA) [19–25], electrochemical ELISA [26], radioimmunoassay (RIA) [19,27], receptor binding assay [28], cytotoxicity assay [29–31], and surface plasmon resonance (SPR) [32–35]. Simple rapid assay techniques are very useful to screen a large number of samples for the presence of DA, provided that they are accurate and yield a low incidence of false negative results [18]. The primary benefit of these techniques is the ability to carry out measurements in the field. However, because of the occurrence of occasional false positives, a validated chemical analysis method is still necessary to confirm the presence of DA in positive samples, especially when regulatory closures of aquaculture operations are involved and when confirmation of a novel occurrence is required. Apart from LC–UVD and LC–FLD, other chemical methods available for the analysis of DA include thin-layer chromatography (TIC) [36], capillary electrophoresis (CE) [37–39], gas chromatography combined with mass spectrometry (GC–MS) [40,41], LC–MS [42–48] and rapid resolution LC coupled with tandem mass spectrometry (RRLC–MS/MS) [49]. LC–MS is generally regarded as the most important and legally accepted confirmatory tool, exhibiting good sensitivity, accuracy and extremely high selectivity. Among various interfaces investigated, electrospray ionization (ESI) exhibits the best results for the analysis of DA by LC–MS [18], providing a reproducible and unequivocal proof of the toxin presence in a sample.

Although these techniques are becoming more common in some laboratories, LC–UVD is often the only analytical tool available in many research institutes and regulatory agencies responsible for monitoring the occurrence of marine toxins. Furthermore, current chemical methods for detecting trace levels of DA in seawater make use of complicated and time-consuming procedures involving sample derivatization [14–17], sample clean-up [48,49] and/or pre-concentration [17,49] steps. The method described herein simplifies trace DA analysis and permits total automation, by employing a direct injection of large sample volumes into the LC system without any prior derivatization or clean-up, followed by UV detection and confirmation by MS detection, if required. Here, we evaluate the effectiveness and limitations of both detection methods. Our LC–UVD technique is just as sensitive as the FMOC–LC–FLD method, yet much less expensive and laborious, allowing up to ~300 chromatographic runs per week. Additionally, the present technique is accurate, precise and can reduce or even eliminate the problems of poor selectivity inherent of the FMOC–LC–FLD approach. Potential uses of this method include monitoring the presence of DA in seawater for early warning of toxic algal blooms and investigating DA production in cultures of *Pseudo-nitzschia* species, including putative or novel species. Here, we evaluate the method using seawater samples and phytoplankton cultures containing a broad DA concentration range (0.035–2000 ng ml$^{-1}$), and compare its results with those obtained by FMOC–LC–FLD in a different laboratory.

## 2. Experimental

### 2.1. Chemicals

Analytical grade acetonitrile (MeCN), methanol, trifluoroacetic acid (TFA), and formic acid (FA) were purchased from Caledon (Georgetown, Canada). The DA certified reference material, CRM-DA-e (99.4 μg ml$^{-1}$), was provided by the National Research Council (Halifax, Canada) and tryptophan was purchased from Valeant Pharmaceuticals (Montreal, Canada). Water was distilled and further purified using a Milli-Q purification system (Millipore, Billerica, MA, USA) and seawater (salinity = 30) was filtered using 0.22 μm cartridge filters (Harmsco, North Palm Beach, FL, USA).

### 2.2. Sample collection and preparation

A non-toxic (CLNN-13) and two toxic (CLN-47 and CLN-50) *P. multiseries* clones were obtained in the laboratory as offspring from the mating of other *P. multiseries* clones isolated from eastern Canada, as described in Davidovich and Bates [50]. They were batch-cultured in 1.5 l glass Fernbach flasks with f/2 medium [51] in autoclaved filtered seawater (FSW) at 16 °C, 30 salinity, 140 μmol quanta m$^{-2}$ s$^{-1}$ light intensity and a 14:10 h (light:dark) photoperiod. Toxic cultures were sampled over the entire growth cycle, representative of a wide range of DA concentrations, from 6 to ~2000 ng ml$^{-1}$. The non-toxic clone was used as a blank matrix to test the recovery of spiked amounts of DA. Culture samples (15 ml) were gently passed through glass microfiber filters (25 mm diameter, 0.7 μm particle retention; Whatman, Florham Park, NJ, USA) and both the filtrate and the particulate fractions were analysed, after disruption of the filters using a Vibra cell VC375 sonic dismembrator (Sonics & Materials, Danburry, CT, USA) for 3 min at 50% duty cycle, and re-filtration on Ultrafree-MC centrifugal filters (Durapore PVDF, 0.45 μm particle retention; Millipore, Bedford, MA, USA) at 10,000 × *g* for 30 s.

Field samples were collected from various locations in eastern Canada. Blank field samples (i.e., no detectable DA-producing *Pseudo-nitzschia* cells in the water) were collected near Herring Cove, Halifax on 5 March 2007. In addition, net tow and whole-water samples taken during a toxic *Pseudo-nitzschia* spp. bloom in Passamaquoddy Bay (Bay of Fundy) on 2 and 5 September, 2008, were kindly provided by Murielle LeGresley (St. Andrews Biological Station, Fisheries and Oceans Canada, St. Andrews, Canada). Finally, three-year-old frozen whole-water and net samples from Ship Harbour, Nova Scotia were also analysed. Field samples were sonicated as described above to disrupt the cells and the debris was subsequently removed on glass microfiber filters.

To test selectivity in the presence of DA isomers, FSW was mixed (9:1, v:v) with extracts from oysters, *Crassostrea virginica*, toxified in the laboratory by suspension-feeding on toxic *P. multiseries* cultures (Mafra et al. in preparation). Oyster tissues were extracted in 50% aqueous MeOH (4:1 v/v), as described in Quilliam et al. [12], and filtered in centrifugal filters prior to LC analysis, without any clean-up step.

Calibration solutions of DA were prepared from serial dilutions of the reference standard in either distilled/deionized water (DIW) or FSW, using a Microlab 500 series dispenser (Hamilton, Reno, NV, USA). Final DA concentrations, after correction for the Microlab dilution factor, ranged from 0.004 to 950 ng ml$^{-1}$. Negative blanks consisted of both DIW and FSW. In addition, a solution of tryptophan (~1 μg ml$^{-1}$), a potential chromatographic interference, was prepared in 10% MeCN to test its separation from DA in our chromatographic analysis.