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Determination of okadaic acid, dinophysistoxin-1 and related esters in Greek mussels using HPLC with fluorometric detection, LC-MS/MS and mouse bioassay

Artemis P. Louppis [a], Anastasia V. Badeka [a], Panagiota Katikou [b], Evangelos K. Paleologos [c], Michael G. Kontominas [a,*]

[a] Laboratory of Food Chemistry, Department of Chemistry, University of Ioannina, 45110 Ioannina, Greece
[b] National Reference Laboratory on Marine Biotoxins, Institute of Food Hygiene, Ministry of Rural Development and Food, Limnou 3A, 54627 Thessaloniki, Greece
[c] General Chemical State Laboratory, Ioannina Division, Dompoli 30, 45332 Ioannina, Greece

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ABSTRACT

An approach involving both chemical and biological methods was undertaken for the detection and quantification of the marine toxins okadaic acid (OA), dinophysistoxin-1 (DTX-1) and their respective esters in mussels from different sampling sites in Greece during the period 2006–2007. Samples were analyzed by means of a) high performance liquid chromatography with fluorometric detection (HPLC-FLD), using 9-athryldiazomethane (ADAM), as a pre-column derivatization reagent, b) liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and c) the mouse bioassay. Free OA and DTX-1 were determined by both HPLC-FLD and LC-MS/MS, while their respective esters were determined only by LC-MS/MS after alkaline hydrolysis of the samples. The detection limit (L.O.D.) and quantification limit (L.O.Q.) of the HPLC-FLD method were 0.015 μg/g HP and 0.050 μg/g HP, respectively, for OA. The detection limit (L.O.D.) and quantification limit (L.O.Q.) of the LC-MS/MS method were 0.045 μg/g HP and 0.135 μg/g HP, respectively, for OA. Comparison of results between the two analytical methods showed excellent agreement (100%), while both HPLC-FLD and LC-MS/MS methods showed an agreement of 97.1% compared to the mouse bioassay.

1. Introduction

Different clinical types of algae-related poisoning have attracted scientific attention among which diarrhetic shellfish poisoning (DSP), paralytic shellfish poisoning (PSP) and amnesic shellfish poisoning (ASP) are the most frequently encountered (Christian and Luckas, 2008). DSP is a gastrointestinal disease caused by ingestion of shellfish contaminated by okadaic acid (OA) and/or dinophysistoxins (DTX's) produced by marine dinoflagellates belonging to the genera *Dinophysis* and *Prorocentrum*. Shellfish, such as mussels, filter approximately 20 L/h of water. During algal blooms water may contain up to several million algae per liter. Although not all algae cells produce toxins, it is estimated that a significant accumulation of toxins will occur in mussels (Christian and Luckas, 2008). OA and to a lesser extent, its methylated analogue dinophysistoxin-1 (DTX-1) have been identified as being responsible for most DSP outbreaks in Greece (Mouratidou et al., 2004; Prassopoulou et al., 2009). Besides OA and DTX-1, DTX-2 has been implicated as an important DSP toxin in Irish (Carmody et al., 1996), Galician (Gago-Martinez et al., 1996) and Portuguese (Vale and Sampayo, 2002) shellfish. Additionally, DTX-3, a complex mixture of 7-O-acyl derivatives of DTX-1 ranging from tetradecanoic acid (14:0) to docosahexaenoic acid ($C_{22:6}$ – ω_3), was the main diarrhetic toxin

* Corresponding author. Tel.: +30 2651008342; fax: +30 2651008795.
E-mail address: mkontomi@cc.uoi.gr (M.G. Kontominas).

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found in scallops in Japan (Yasumoto et al., 1985). Human consumption of shellfish containing DSP toxins exceeding certain levels results in diarrhea, nausea, vomiting and abdominal pain. Symptoms begin 30 min to a few hours after ingestion while complete recovery occurs within 3 days (Yasumoto et al., 1978). The minimum doses of OA and DTX-1 necessary to induce above symptoms in adults have been estimated to be 40 and 36 μg respectively (Hamano et al., 1986). According to relevant data of the National Reference Laboratory on Marine Biotoxins of Greece (Thessaloniki, Greece), toxins of the OA group have been identified as being responsible for the majority of DSP outbreaks during the past 10 years in Greece (Prassopoulou et al., 2009).

At present, the mouse bioassay (MBA) still remains the reference method for the detection of lipophilic algal toxins (EU Regulation, 2074/2005) and all analytical methods used for this purpose have to be evaluated against bioassays. The MBA was first developed by Yasumoto et al. (1978). Toxicity in the MBA method is expressed in mouse units (MU). One MU is defined as the minimum quantity of toxin capable of killing a mouse of 20 g in weight within 24 h after intraperitoneal injection (Yasumoto et al., 1980). This is equivalent to approximately 4 μg of OA for the ddY mouse strain in Japan but may vary somewhat with the strain (Fernandez et al., 2003).

Such bioassays, however, reveal only the total toxicity of a sample providing no indication of each individual algal toxin involved in a given outbreak. A further important argument against bioassays is the growing ethical concerns against the use of laboratory animals (Fernandez et al., 2003). In addition, the MBA is time consuming and may give false positive results because of interferences by other toxins, such as saxitoxin (Fernandez et al., 2003), Gymnodimine (GYM) and Spirolides (SPXs) (Suzuki et al., 2005) or by fatty acids (Takagi et al., 1984; Suzuki et al., 1996). These problems have led to the introduction of HPLC (Lee et al., 1987; Vale and Sampayo, 1999; Mouratidou et al., 2004; Prassopoulou et al., 2009) and more recently to LC-MS based methods (Pleasance et al., 1992; Suzuki and Yasumoto, 2000; Vale and Sampayo, 2002; Fux et al., 2007; Christian and Luckas, 2008; Gerssen et al., 2009) for both the identification and quantitative determination of algal toxins. Such analytical methods have been used by several research and monitoring laboratories following in-house validation. However, sample preparation procedures still differ largely between laboratories (Aasen et al., 2005; Suzuki et al., 2005).

Although OA and its methylated analogues dinophysistoxin-1 (DTX-1) and dinophysistoxin-2 (DTX-2) have been identified as being responsible for most DSP outbreaks (Luckas, 1992; Lawrence and Scoot, 1993; Van Egmond et al., 1993; Carmody et al., 1996; Vale and Sampayo, 1999), much less attention has been paid to the acyl derivatives of DSP toxins, also referred to as dinophysistoxin-3 (DTX-3). Due to their high molecular weight and lipophilicity, they cannot be directly detected with adequate sensitivity both by the fluorometric procedure of Lee et al. (1987) and by LC-MS/MS, but an alkaline hydrolysis reaction to release fatty acids from the parent toxins may contribute to a better estimation of their abundance. On the other hand, the lack of standards for these substances poses a further problem in their analytical determination (Vale and Sampayo, 2002).

In the present study, a) an HPLC method, using ADAM as fluorescent derivatizing reagent, b) an LC-MS/MS method and c) the reference mouse bioassay have been used to detect and/or quantify OA, DTX-1 and its acyl derivatives in mussels. Correlation of results of all three methods was also attempted. All samples analyzed originated from the DSP toxic episodes of years 2006 and 2007 in Greece.

2. Materials and methods

2.1. Samples

Mussels (*Mytillus galloprovincialis*) were collected during the DSP episodes of 2006 and 2007 from several sampling stations belonging to three different production areas of Greece: the Gulf of Thermaikos in northern Greece (Thessaloniki, Pieria, Imathia), the Gulf of Maliakos in central Greece (Fthiotida), and the Gulf of Saronikos in southern Greece (Megara) (Fig. 1). A small number of market samples were also tested at the same time periods (Table 1). A total of 103 samples, originating from the Greek National Monitoring Program for Marine Biotoxins (National Reference Laboratory on Marine Biotoxins of Greece), were tested upon arrival to the laboratory by the MBA method. Selected samples, both positive and negative according to the MBA, were stored at −70 °C until chemical analyses. The hepatopancreas (HP) of mussels was used in all methods employed, as the lipophilic DSP toxins mainly accumulate in this organ (Murata et al., 1982). All samples were analyzsed in duplicate.

2.2. Reagents

Solvents used for the MBA were Tween-60 grade "for synthesis" (Sigma, Sigma–Aldrich, St. Louis, MO, USA) and acetone (Merck, Darmstadt, Germany), analytical grade.

Solvents used for the HPLC-FLD and LC-MS/MS analyses were LC grade methanol, chloroform, acetone, water, acetonitrile, *n*-hexane (Merck, Darmstadt, Germany). Okadaic acid (OA) standard solution (NRC CRM-OA-b, Institute for Marine Biosciences, Canada) was used for the preparation of the calibration curve for both in HPLC-FLD and LC-MS/MS methodology. A certified Reference Material Mussel Tissue with a certified value of 10.1 μg OA/g and 1.3 μg DTX-1/g (NRC CRM-DSP-Mus-b, Institute for Marine Biosciences, National Research Council of Canada, Halifax, Canada) was used for recovery determination both in HPLC-FLD and LC-MS/MS methods. 9-anthryldiazomethane (ADAM) from Serva (Heidelberg, Germany) was used for derivatization of both standard solutions and samples. All standards, reference materials and the derivatization reagent were stored at −20 °C. Solid Phase Extraction (SPE) cartridges packed with silica were purchased from Alltech (Deerfield, USA).

2.3. Mouse bioassay

The MBA for the determination of DSP toxicity in samples was performed as described by Yasumoto et al.