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In vitro bioaccessibility of the marine biotoxins okadaic acid, dinophysistoxin-2 and their 7-*O*-acyl fatty acid ester derivatives in raw and steamed shellfish

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ABSTRACT

Okadaic acid (OA), Dinophysistoxins (DTX1 and DTX2) and their acyl-derivatives (DTX3) are marine toxins responsible for the human diarrhetic shellfish poisoning. To date the amount of toxins ingested from consumption of shellfish has been considered equal to the amount of toxins available for uptake by the human body. The aim of this study is to assess the OA, DTX2 and DTX3 fractions released from raw and steamed mussels and cockles into the digestive fluids (bioaccessibility) using a static *in vitro* digestion model. Higher bioaccessibility was found in mussels (86 ± 4%) than in cockles (59 ± 9%). A significant reduction of ester derivatives of OA and an increase of OA were observed in the bioaccessible fraction of mussel samples, suggesting that DTX3 undergo conversion into their more toxic parent compounds during human digestion. However, similar increase of DTX2 and reduction of the respective acyl derivatives was not observed. Steaming lead to significant reduction of OA and analogues bioaccessibility in both species even though increased concentrations of toxins are obtained after this treatment. Risk assessment based solely on DSP toxins occurrence in seafood can conduct to an overestimation of the exposure and lead to more conservative regulatory measures.

1. Introduction

Okadaic acid (OA) and its analogues Dinophysistoxins (DTX1 and DTX2) are lipophilic marine toxins responsible for the human food borne diarrhetic shellfish poisoning (DSP). These toxins can be accumulated in shellfish during blooms of *Dinophysis* dinoflagellates (Hallegraeff et al., 1995). To prevent acute intoxications characterized by gastrointestinal disorders (e.g. diarrhea, nausea, abdominal pain and vomiting), shellfish cannot be harvested and marketed whenever toxins concentration exceed the Regulatory Limit of 160 μg OA equivalents per kg of shellfish meat (Regulation (CE) No 853/2004).

OA group toxins (Fig. 1) are commonly found in shellfish throughout Europe, Asia and South America (Hallegraeff et al., 1995). In Portugal, this toxin is commonly detected from spring to late autumn, being associated with *Dinophysis acuminata* blooms. DTX2 typically occurs in late summer during bloom events of *D. acuta* (Moita et al., 2016). In addition to these two isomeric compounds, a multiple and complex mixture of 7-*O*-acyl fatty acid derivatives of OA and DTX1-2 can be found in shellfish as a result of biotransformation (Marr et al., 1992; Suzuki et al., 1999; Vale and Sampayo, 2002a). The fatty acid ester derivatives of OA and DTX1-2 are collectively designated as DTX3. For monitoring purposes and shellfisheries management, each toxin detected and quantified in shellfish matrix is multiplied by a toxicity equivalent factor (TEF) (EURLMB, 2015). If their sum results in values above the Regulatory Limit, the sample is considered unsafe for human consumption. According to the European Food Safety Authority (EFSA)

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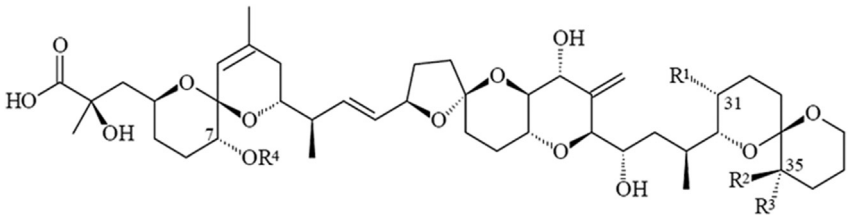

	R1	R2	R3	R4
OA	CH_3	H	H	H
DTX1	CH_3	CH_3	H	H
DTX2	H	H	CH_3	H
DTX3	(H or CH_3)	(H or CH_3)	(H or CH_3)	Fatty acid

Fig. 1. Structures of okadaic acid and dinophysistoxin 1, 2 and 3 (adapted from Larsen et al., 2007).

and based on the median lethal dose (LD_{50}) experiments following intraperitoneal (i.p.) injection in mice, the TEF for the OA-group toxins are: OA = 1, DTX1 = 1, DTX2 = 0.6. For 7-*O*-acyl fatty acid ester derivatives (DTX3) it is applied the TEF of the respective unesterified toxin (*i.e.* OA, DTX1 or DTX2) (EFSA, 2008).

OA and DTX1 are potent serine/threonine protein phosphates inhibitors, in particular for protein phosphatase 2A (PP2A), while DTX2 has less inhibitory activity (Bialojan and Takai, 1988; Takai et al., 1992; Aune et al., 2007). OA and DTX1 are also considered as potent tumor promoters (Suganuma et al., 1988; Fujiki and Suganuma, 1993). The toxicity of the 7-*O*-acyl fatty esters derivatives of OA-group toxins (DTX3) is still unclear. Earlier studies indicated no significant toxicity of 7-*O*-acyl fatty ester derivatives in the mouse bioassay (Yasumoto et al., 1985), whereas other studies revealed a degree of toxicity though lower than OA. 7-*O*-acyl fatty esters of OA-group toxins are considered weak inhibitors of serine/threonine protein phosphates with reduced lethality in mice and moderate cytotoxicity (Yanagi et al., 1992; Takai et al., 1992).

The presence of esterified forms of OA and DTX1-2 toxins in shellfish depends on shellfish species ability to biotransform these toxins via acylation with fatty acid esters. While certain species, such as mussels and donax clams may contain a toxin profile dominated by free toxins, other species such as cockles and razor clams may only contain 7-*O*-acyl fatty esters of OA and DTX1-2 (Vale and Sampayo, 2002a; Rossignoli et al., 2011). Despite the toxicity of 7-*O*-acyl fatty esters derivatives is considered low, human poisoning incidents have been reported in Portugal, Norway and Chile after the consumption of shellfish contaminated with esterified derivatives (Vale and Sampayo, 2002b; Torgersen et al., 2005; García et al., 2005). Human poisoning events associated with the ingestion of less toxic fatty acid ester derivatives may be related with an eventual conversion into potent parental compounds after hydrolysis in the gastrointestinal tract (Doucet et al., 2007; Braga et al., 2016).

Limited research has focused on the human toxicological potential of OA group toxins. Most studies have used i.p. injection of OA in mice, whereas few have focused on mice oral exposure. Mice orally exposed to OA revealed only 59% of the toxin in the gastrointestinal tract (Matias et al., 1999). To date, the amount of toxins ingested in food has been considered in risk food assessment as the amount of toxins available for uptake by the human body leading to a significant overestimation of exposure risks to consumers. Assessing bioaccessibility is a useful tool to infer about the potential differences between the amount of toxins ingested and the portion available for assimilation. Bioaccessibility corresponds to the amount of a specific food component that is released from the food matrix during the digestive process, thus being available to cross the intestinal epithelium and enter in the systemic circulation (Metian et al., 2009; Versantvoort et al., 2005; Guerra et al., 2012). Recently Braga et al. (2016) investigated the bioaccessibility of OA (free and esterified) in mussels (*Mytilus galloprovincialis*) and donax clams (*Donax* spp) using a static *in vitro* digestion model that simulates the human digestion. Bioaccessibility percentages of 88 and 75% were observed for mussels and donax clams, respectively. Despite most seafood is only consumed after culinary treatment, bioaccessibility studies are generally based in raw products, representing a potential bottleneck in risk assessment and in the definition of public health safety guidelines (Maulvault et al., 2011). In this context, the aim of the present study is to assess the bioaccessibility of OA, DTX2 and their 7-*O*-acyl fatty acid ester derivatives in two types of shellfish species: species with low ability to biotransform OA-group toxins (mussels) and species with high ability to esterify OA-group toxins (cockles). The effect of cooking processes (steaming) on the bioaccessibility of toxins is also evaluated.

2. Materials and methods

2.1. Collection of shellfish and sample preparation

Six samples of naturally contaminated mussels (*Mytilus galloprovincialis*) and five samples of cockles (*Cerastoderma edule*) were collected from shellfish producing areas of the Portuguese west coast between August and December 2015. Each sample was composed by at least 30 individuals. Samples were obtained through the Portuguese Monitoring Program for Marine Biotoxins carried out by the Portuguese Institute of the Sea and Atmosphere (IPMA). Bivalves were opened, removed from the shell, washed with running water to remove any residue (e.g. sand) and properly drained. Each sample was divided into two portions, one used for raw assessment and another to investigate the effect of culinary treatment in biotoxins bioaccessibility (steaming at 105 °C during 7.5 min, wrapped in aluminum foil). Raw and steamed samples were homogenized with a blender and stored at −20 °C until further analysis.

2.2. In vitro human digestion model

2.2.1. Digestion protocol

OA bioaccessibility was assessed using a static *in vitro* human