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Multimycotoxin analysis in water and fish plasma by liquid chromatography-tandem mass spectrometry

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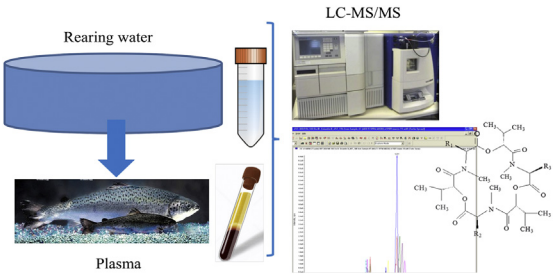
HIGHLIGHTS

- DLLME with lower-density extractant solvent to mycotoxin analysis in water and fish plasma.
- The method presents high sensitivity and specificity.
- This method allows to rapid identification and quantitation.
- The extraction method is environmentally friendly and can reduce extraction time.
- The method validated is adequate to be applied in samples from toxicity studies.

GRAPHICAL ABSTRACT



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ABSTRACT

High performance liquid chromatography-mass spectrometry was used for the determination of 15 mycotoxins in water and fish plasma samples, including aflatoxins, fumonisins, ochratoxin A, sterigmatocistin, fusarenon-X and emerging *Fusarium* mycotoxins. In this work, dispersive liquid–liquid microextraction (DLLME) was assessed as a sample treatment for the simultaneous extraction of mycotoxins. Results showed differences in recovery assays when different extraction solvents were employed. Ethyl acetate showed better recoveries for the major part of mycotoxins analyzed, except for aflatoxins B_2, G_1 and G_2, which showed better recoveries when employing chloroform as extractant solvent. Fumonisins and beauvericin exhibited low recoveries in both water and plasma. This method was validated according to guidelines established by European Commission and has shown to be suitable to be applied in dietary and/or toxicokinetic studies in fish where is necessary to check mycotoxin contents in rearing water and fish plasma.

1. Introduction

Mycotoxins are naturally occurring secondary metabolites of fungi colonizing a wide variety of food and feed. However, organic soil material and aqueous natural samples can be contaminated. In this sense, previous research has documented mycotoxins as environmental contaminants in aqueous natural samples (Kolpin et al., 2014; Maragos, 2012; Schenzel et al., 2010; Wettstein and Bucheli, 2010).

Since the first dispersive liquid–liquid microextraction (DLLME) method was reported in 2006 by Rezaee et al. to analyze organic pollutants in water samples, several different options have been

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reported. DLLME technique is based on a ternary component solvent system where the dispersion of the fine droplets of the extraction solvent is carried out in the aqueous phase. The principal advantage of DLLME is that the surface area between extraction solvent and aqueous sample is infinitely large; thus, the equilibrium state is achieved quickly and the extraction time is very short (Karami-Osboo et al., 2013). First DLLME methods employed a mixture of a high-density extraction solvent, a water-miscible solvent, and a polar disperser solvent. On one hand, the extraction solvent must be able to extract analytes, must be soluble in the disperser solvent and insoluble in aqueous samples, and normaly presents a density higher than that of water, while, on the other hand, the disperser solvent has to be soluble in both water and extraction solvent (Rezaee et al., 2006). However, nowadays, newly developed techniques have been described by several authors. In this way, depending on the extraction solvent used, DLLME method may be classified in two broad categories: those that use a lower-density extraction solvent (Taherimaslak et al., 2015), and those that use a higher-density extraction solvent (Campone et al., 2011a).

Although DLLME for mycotoxin extraction is still scarce, some DLLME studies about mycotoxin determination in different matrices are reported. However, in these studies, normaly, only one mycotoxin or a reduced group of them are analyzed, for example zearalenone (ZEN) in beer (Antep and Merdivan, 2012), ochratoxin A (OTA) in wines (Arroyo-Manzanares et al., 2012; Campone et al., 2011b), deoxynivalenol (DON) in wheat flour (Karami-Osboo et al., 2013), patulin (PAT) in apple juice (Víctor-Ortega et al., 2013), aflatoxins in pistachio nuts (Taherimaslak et al., 2015), aflatoxin M_1 in milk (Campone et al., 2013), among others.

Recent studies have reported the presence of mycotoxins in feed for aquacultured fish (Nácher-Mestre et al., 2013; Pietsch et al., 2013; Tolosa et al., 2014), which indicates that European aquaculture is commonly exposed to feed-borne *Fusarium* mycotoxins. As fish is fed in water, the contact between contaminated feed and water can lead to contamination of the rearing water. In this sense, results reported by Woźny et al. (2013) indicated that animal feed may be a possible source of ZEN contamination in water. In fact, some toxicity studies on fish, reported that mycotoxins were also present in the rearing water (Nomura et al., 2011).

As occurs in water samples, studies in plasma only analyze one mycotoxin or a reduced group of them mainly in terrestrial animals (Corcuera et al., 2011; Devreese et al., 2012) and also their metabolites (Ivanova et al., 2014; Saengtienchai et al., 2014; Winkler et al., 2014).

In this context, the aim of this study is to develop a method for the simultaneous detection of fifteen mycotoxins in water and fish plasma samples using a modified DLLME method and HPLC-MS/MS for the analysis. The final method should be applicable for evaluation of rearing water and fish plasma from *in vivo* toxicity assays.

2. Materials and methods

2.1. Chemicals and reagents

All solvents (acetonitrile, methanol, chloroform and ethyl acetate) were purchased from Merck (Darmstadt, Germany). Deionized water (>18 MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore Corporation, Bedford, MA, USA). Ammonium formate (HCO_2NH_4, 97%) and formic acid were supplied by Sigma–Aldrich (St. Louis, USA). All solvents were passed through a 0.22 μm cellulose filter from Membrane Solutions (Texas, USA).

The stock standard of mycotoxins (AFB_1, AFB_2, AFG_1, AFG_2, OTA, FUS-X, STG, FB_1, FB_2 and FB_3) were purchased from Sigma–Aldrich. Enniatins (ENs) and beauvericin (BEA) toxin solutions were provided by Biopure (Tulln, Austria). Individual stock solutions (1 mg) were diluted in 1 mL of methanol (concentration of 1000 mg/L). Then, intermediate solutions with decreasing concentration were prepared by dilution from the stock (from 1000 mg/L to 100 mg/L, from 100 mg/L to 10 mg/L, and from 10 mg/L to 1 mg/L). They were stored in glass-stoppered bottles and darkness in security conditions at −20 °C. Working standard solutions (ranged from 0.01 to 100 μg/L) were prepared by the suitable dilution from 1 mg/L solution and were kept in darkness at 4 °C.

Water and plasma samples were obtained from a group of jundiás (*Rhamdia quelen*) maintained in tanks of 300 L at 18–20 °C and 12 h/day of light/darkness.

2.2. Procedures

2.2.1. Sample preparation

The method of addition of standards to water and plasma samples were based in the addition of a known amount of analytes to be detected (fortified samples). These fortified samples were spiked with a working mixture of the mycotoxins (the concentration added corresponded to 10 and 100 times the limit of quantification (LOQ) for each mycotoxin analyzed). The concentration range for each analyte was as follows: For aflatoxins, recovery assays were performed with concentrations of 20 μg/L, and 200 μg/L; for emerging *Fusarium* mycotoxins, 10 μg/L, and 100 μg/L; and for fumonisins, FUS-X, STG and OTA, 100 μg/L, and 1000 μg/L.

2.2.2. Extraction procedure in water

A solution of 5 mL of water and 1 g of NaCl was placed in a 15 mL tube with conical bottom and mixed in a vortex for 1 min. Then, a mixture of the disperser solvent (900 μL of MeCN) and the extraction solvent (700 μL of EtOAc) was added and mixed in a vortex for 1 min. A cloudy dispersion consisting of water, disperser and extraction solvents appeared. Then, the mixture was centrifuged at 4500 rpm for 5 min and 5 °C for phase separation. After this procedure, two different phases appeared. Since ethyl acetate is lighter than water (density, $d = 0.897$ g/mL), the extraction phase floated on the surface of the aqueous solution, so the upper layer containing the mycotoxins was collected using a syringe and placed into a 15 mL tube, which was evaporated until dryness under a gentle stream of N_2. Finally, samples were reconstituted with 1 mL of MeOH:H_2O (50:50, v/v), filtered (0.22 μm) and injected into the LC–MS/MS system.

2.2.3. Extraction procedure in plasma

For plasma samples, an additional step before DLLME was carried out in order to precipitate proteins. To do this, 750 μL of MeCN were added to 250 μL of plasma and this mixture was centrifuged at 4500 rpm 4 °C for 5 min. The supernatant was placed in a clean eppendorf for DLLME extraction and 0.2 g NaCl were added. Then, a mixture of the disperser solvent (475 μL of MeCN) and the extraction solvent (350 μL of EtOAc) was added and mixed in a vortex for 1 min, followed by a centrifuged step at 4500 rpm for 10 min and 4 °C for phase separation. After this procedure, two different phases appeared. The organic upper phase containing the analytes was removed and evaporated until dryness under a gentle stream of N_2. Finally, samples were reconstituted with 1 mL of MeOH:H_2O (50:50, v/v), filtered (0.22 μm) and injected into the LC–MS/MS system.

2.3. Method validation

The method was validated according to the guidelines established by the EU Commission Decision 2002/657/EC. The