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Ultra-high performance liquid chromatography with fluorescence detection following salting-out assisted liquid–liquid extraction for the analysis of benzimidazole residues in farm fish samples☆

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ABSTRACT

Ultra-high performance liquid chromatography (UHPLC) coupled with fluorescence detection (FL) has been proposed for the first time to determine thirteen benzimidazoles (BZs) in farmed fish samples. In order to optimize the chromatographic separation, parameters such as mobile phase composition and flow rate were carefully studied, establishing a gradient mode with a mobile phase consisted of water (solvent A) and acetonitrile (solvent B) at a flow rate of 0.4 mL/min. The separation was performed on a Zorbax Eclipse Plus RRHD C_{18} column (50 × 2.1 mm, 1.8 μm), involving a total analysis time lower than 12 min. Salting-out assisted liquid-liquid extraction (SALLE) was applied as sample treatment to different types of farmed fish (trout, sea bream and sea bass). To obtain satisfactory extraction efficiencies for the studied analytes, several parameters affecting the SALLE procedure were optimized including the amount of sample, type and volume of the extraction solvent, and the nature and amount of the salt used. Characterization of the method in terms of performance characteristics was carried out, obtaining satisfactory results for the linearity ($R^2 \geq 0.997$), repeatability (RSD ≤ 6.1%), reproducibility (RSD ≤ 10.8%) and recoveries (R ≥ 79%; RSD ≤ 7.8%). Detection limits between 0.04–29.9 μg kg^{-1} were obtained, demonstrating the applicability of this fast, simple and environmentally friendly method.

1. Introduction

Aquaculture is defined by FAO as the farming of aquatic organisms, in both coastal and inland areas, including fish, mollusks, crustaceans and aquatic plants, involving interventions in the rearing process to enhance production [1]. Most of the aquaculture systems in the world are based on crash cultivation methods, which are characterized by high stock density and volume, and the use of formulated feeds containing antibiotic, anthelmintic, antifungal, and pesticides among other substances. Their widespread use can cause serious health problems in consumers, such as allergic reactions in hypersensitive individuals, congenic malformations, and bacterial resistance [2,3].

Benzimidazoles (BZs) are veterinary drugs widely used as antiparasitic agents in domestic animals and as fungicidal agents to control a wide range of fungi affecting field crops, stored fruit and vegetables. They can reach the aquatic ecosystem through agricultural runoff and recycled wastewater, from farmed fish feed and, finally they can be employed directly in aquaculture practices for prevention and treatment of tape-worm infections [4,5].

The European Union (EU) has set maximum residue limits (MRLs) for BZs and their metabolites in animal products [6] although for fish, the MRL is not regulated yet. However, in the absence of real data, several studies have demonstrated the presence of some BZs and their metabolites in different fish samples after an oral dose. Sørensen et al. [7] detected fenbendazole (FBZ), fenbendazole sulfoxide (FBZ-SO) and fenbendazole sulfone (FBZ-SO_2) shortly after administration, in muscle and skin tissues of trout given feed containing FBZ in an aquaculture pilot plant. Shaikh et al. [8] determined albendazole (ABZ) and its three major metabolites in muscle tissue of three fish species sampled in the first day after the administration period.Thus, the presence of BZ residues in this type of samples is one of the key issues for food safety and raises much public concern. For this reason, the development of analytical methods to determine the presence of these compounds is needed.

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In recent years, several analytical approaches have been reported for the determination of BZs in different type of samples, using mainly liquid chromatography (LC) [4] coupled with ultraviolet absorption (UV) [9–11]. Lately, LC coupled with mass spectrometry (MS) has also played a relevant role in the monitoring of BZs and other contaminant residues due to the high selectivity, sensitivity and the capacity for unambiguous identification provided by this technique [12–16]. Nevertheless, the main drawback of LC–MS methods is the high instrumental cost. In addition, the analytical performances of these methods could be affected by matrix effect when electrospray ionization is used [17]. Another option for the determination of BZs in food samples after a LC separation could be the use of FL detection, which can be highly selective and much more sensitive than UV and less expensive than LC–MS. Thus, BZs have a common structure containing a bicyclic ring formed by the fusion of benzene and imidazole and two amino groups which can be protonated or deprotonated under different conditions. Some, such as albendazole (ABZ) and its metabolites, thiabendazole (TBZ) and fuberidazole (FUB), present native fluorescence [4]. There are few studies dealing with the determination of BZ compounds by high performance liquid chromatography (HPLC) with FL detection, mainly applied in clinical samples [18–20] and environmental water [21–23]. To the best of our knowledge only one application of LC-FL has been found for the monitoring of BZ residues in food derived from animals, related to the determination of ABZ and its metabolites in muscle tissues of different fishes [8]. However, the Achilles heel of these methods is the low number of compounds analyzed (4) and the use of two isocratic modes of analysis, one for parent ABZ and the other for its metabolites, because they could not find an optimal situation in one single run. Consequently the total analysis time increased to almost 40 min. These limitations could be overcome by the use of ultra high performance liquid chromatography (UHPLC), due to its advantages associated with the use of stationary phase particles smaller than 2.0 μm, allowing an increased efficiency with a shortened analysis time [24], and it is more environmentally friendly than a HPLC system.

Regarding the sample treatment to be applied for the control of BZs in aquaculture products, the more relevant points, which have to be taken into account, are the analyte extraction efficiency and the sample throughput. Different extraction procedures have been published for the determination of BZs in fish samples, the most common one being solid-phase extraction (SPE), using LC as separation technique. Some examples are the determination of FBZ, FBZ-SO and FBZ-SO_2 in trout [7], FBZ, FBZ-SO and FBZ-SO_2 in trout and eel tissues [25] or mebendazole (MBZ) in eel [26] following muscle tissue samples extraction with ethyl acetate or acetonitrile. To a lesser degree, other extraction procedures have also been developed such as ultrasound assisted extraction followed by liquid-liquid extraction (LLE) for the determination of ABZ, albendazole sulfone (ABZ-SO_2), febantel, FBZ, flubendazole, MBZ, FBZ-SO, TBZ, triclabendazole (TCB) and other veterinary drugs in gilthead sea bream and sea bass [27] and the determination of ABZ and their metabolites in salmon, tilapia and rainbow trout [8]. Liquid-liquid extraction (LLE), presents some advantages, including low cost, simplicity, high extraction efficiency and low time consumption but the main drawback is the environmental impact of the organic solvents employed. Salting-out assisted liquid-liquid extraction (SALLE) is a LLE technique based on the addition of an appropriate amount of a salt to a mixture of aqueous sample and water–miscible organic solvent causing the formation of a two-phase system through a "salt-induced phase separation" phenomenon, simultaneously extracting the target analytes into the organic phase [28]. This technique is fast, simple, cheap and environmentally safe. It must be noted that, to the best of our knowledge, just one method has been reported for the determination of 4 BZs used as fungicides in salinity samples using a SALLE procedure [29]. In other study a related sample treatment named QuEChERS has been proposed for determination of ABZ, FBZ, MBZ, FBZ-SO and TBZ and other veterinarian drugs in gilthead sea bream [30].

In this work, UHPLC coupled with FL detection is proposed for the first time for the monitoring of 13 BZs in fish samples (trout, sea bream and sea bass) with a total analysis time of 12 min. A SALLE procedure has been introduced because of its simplicity, rapid partition equilibrium, low environmental impact (only 2 mL of organic solvent employed) and compatibility with subsequent chromatographic conditions.

2. Experimental

2.1. Reagents and materials

Acetonitrile (MeCN) (99.9%), methanol (MeOH) (99.9%), isopropanol (IPA) (99.9%), tetrahydrofuran (THF) (≥99.9%), and acetone (ACO) (100%), (LC–MS HiPerSolv grade) were supplied from VWR (Radnor, PA, USA). Ammonium acetate and ethanol (EtOH) (99.9%), were supplied from Merck (Darmstadt, Germany). Ultrapure water (Milli-Q plus system, Millipore, Bedford, MA, USA) was used throughout the work.

Salts used for aqueous and organic-phase partitioning were purchased from different suppliers: ammonium sulfate ($(NH_4)_2SO_4$) (99.5%) was obtained from VWR (Radnor, PA, USA); magnesium sulfate anhydrous ($MgSO_4$) (96%) and sodium chloride (NaCl) (>99%) were obtained from Panreac (Barcelona, Spain).

Analytical standards of albendazole (ABZ) (99.0%), albendazole sulfone (ABZ-SO_2) (99.3%), albendazole sulfoxide (ABZ-SO) (98.0%), benomyl (BEN) (99.0%), carbendazim (CBZ) (99.0%), fuberidazole (FUB) (99.5%), oxibendazole (OXI) (99.9%), thiabendazole (TBZ) (99.8%), 5-hidroxy thiabendazole (5-OH-TBZ) (99.9%), triclabendazole (TCB) (99.7%), triclabendazole sulfone (TCB-SO_2) (99.4%) and triclabendazole sulfoxide (TCB-SO) (99.8%) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Albendazole-2-aminosulfone (ABZ-NH_2-SO_2) (99.0%) was supplied by Dr. Ehrenstorfer (Augsburg, Germany).

Individual stock standard solutions of 1000 mg L^{-1} (ABZ-SO, ABZ-NH_2-SO_2, FUB, TBZ, 5-OH-TBZ, TCB-SO_2, TCB-SO), 500 mg L^{-1} (ABZ, ABZ-SO_2, BEN, TCB) and 250 mg L^{-1} (OXI, CBZ) were prepared by dissolving accurately weighed amounts of each compound in MeOH. They were stored in dark glass bottles at −20 °C, being stable for at least 6 months. Intermediate stock standard solutions for UV detection containing 10 mg L^{-1} of each BZ were obtained by mixing individual stock standard solutions and subsequent dilution with water. Intermediate stock standard solutions for FL detection containing 10 mg L^{-1} (5-OH-TBZ, TCB, OXI), 5 mg L^{-1} (TCB-SO, TCB-SO_2), 2.5 mg L^{-1} (ABZ-SO), 1.25 mg L^{-1} (CBZ, BEN, ABZ, TBZ), 625 μg L^{-1} (ABZ-NH_2-SO_2), 150 μg L^{-1} (ABZ-SO_2) and 40 μg L^{-1} (FUB) were obtained by mixing individual stock standard solutions and subsequent dilution with water. It must be noted that different concentrations of BZs have been used due to the different fluorescence quantum yield of these compunds.

They were also stored in dark glass bottles at 4 °C. Standard working solutions containing all the BZs were prepared by the proper dilution of the intermediate stock standard solutions with water at the required concentration.

Teflon (PTFE) syringe filters (0.2 μm × 13 mm) and fiberglass syringe filters (0.72 μm × 13 mm) from (VWR, Radnor, PA, USA) and nylon syringe filters, 0.2 μm × 13 mm (Bonna-Agela Technologies Inc, Wilmington, USA) were used for filtration of sample extracts prior to their injection into the UHPLC system.

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