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A multiresidue approach for the simultaneous quantification of antibiotics in macroalgae by ultra-high performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

Together with fish, algae reared in aquaculture systems have gained importance in the last years, for many purposes. Besides their use as biofilters of effluents, macroalgae's rich nutritional profiles have increased their inclusion in human diets but also in animal feeds as sources of fatty acids, especially important for the fish industry. Nonetheless, algae are continuously exposed to environmental contaminants including antibiotics and possess the ability for bioaccumulation of such compounds. Therefore, the present paper describes the development and validation of an ultra-high performance liquid chromatography with tandem mass spectrometry (UPLC–MS/MS) method for the simultaneous quantification of antibiotics in the green macroalgae *Ulva lactuca*. This multi-residue method enables the determination of 38 compounds distributed between seven classes and was fully validated according to EU Decision 2002/657/EC.

1. Introduction

The current nutritional demands of populations worldwide have shifted favoring more diverse diets with higher energy inputs, higher protein content and lower carbohydrates percentage. Following this trend, the selection of food items rich in proteins has increased with special focus on meat, milk and fish. A portion of 150 g of fish provides between 50 and 60% of an adult's daily requirements, which is one of the reasons why fish consumption has intensified in the last decades jumping from 9 kg per capita in the 1960s to 19.2 kg in 2012 [1]. Therefore, to supply the markets and provide the consumers with their needs while avoiding the overexploitation of wild stocks, aquaculture has grown into an important economic activity with over 66 million tons of fish produced by 2012 [2].

Together with fish, algae have also gained importance over the years representing a total production of 23.8 million tons reared in the aquaculture industry [1]. There are several reasons why algae constitute such a relevant commodity. In many countries, algae are part of human diets, especially in East Asia where they have been consumed for centuries. Recently, their importance as foodstuff has increased not only in Asia but also in Western countries, since they grow easily in seawater (coastal areas, aquaculture ponds, estuaries) but also due to their properties. Analyses on their nutritional profiles reveal a high content in proteins, fibers, fatty acids, vitamins, minerals, essential amino acids and also antioxidant properties [3–5]. Apart from direct consumption algae are also used for fertilizers, cosmetics, sources for phycocolloids and gelling agents (alginates, carrageenans, agar) in the food industry and meals added to feeds for animal nutrition [5]. Marine algae are also rich in fatty acids including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are essential to fish and are included in feed formulation for farmed animals. In traditional feeds the source for these constituents is fishmeal and fish oils, obtained from fish. Therefore, the use of algae as additives to feed is a viable alternative to reduce the reliance on fish products [1,6]. Also, macroalgae with elevated production rates allied to high protein content are receiving more attention as novel feeds with potential benefits. Finally, another contributor for the high production rates registered for algae is their use as biofilters in

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integrated multi-trophic aquaculture systems [7]. These primary producers are reared in tanks where effluents present nutrient-enriched media that constitute optimal conditions for algae growth, which in turn will improve the quality of the discharges by decreasing nutrient concentrations through uptake and accumulation, acting as extractive organisms [7,8]. However, due to their particular characteristics, algae are also able to accumulate substances other than nutrients, including contaminants such as metals [9] and pharmaceuticals [10–13]. In aquaculture, the use of antibiotics for prophylaxis and therapeutic purposes is well described and necessary to control disease outbreaks and animal welfare. Depending on the route of administration, dosages required and length of treatment, such pharmaceuticals can be present in high concentrations in effluents [14–17]. It has also been established that macroalgae are able to accumulate these substances from the water column at different rates and concentrations depending on the antibiotic. Previous studies have demonstrated the bioaccumulation of furaltadone [11], chloramphenicol [12] and sulfathiazole [13] by the macroalgae *Ulva lactuca* and its ability as a bioindicator of contamination. Macroalgae are included in fish feeds as meals, that is, air or sun-dried algae are ground into powder, which is then mixed with other ingredients resulting in dry pellets. However, since algae are rarely submitted to pre-treatment before incorporation, it is expected that any substances that may have been accumulated previously will be integrated into feeds.

Therefore, to prevent unintentional contamination of feeds and subsequently fish and other organisms, and inadvertent intake of unwanted substances, algae should be routinely analyzed before incorporation in any products intended for human and animal consumption, both direct and indirect.

Facing the challenges related with macroalgae, the development of new methodologies for pharmaceutical monitoring in these matrices is paramount. The lack of methods encountered in the past for the quantification of antibiotics in such matrix has led to the development of three methodologies dedicated specifically for the determination of furaltadone [18], chloramphenicol [19] and sulfathiazole [20], based on liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). These methods have in common a simple solvent extraction without the need for a clean-up step (extracts were compared with and without SPE-Solid Phase Extraction), which significantly reduced the time and costs associated thus increasing the number of samples analyzed in the same period of time. This is especially important in control laboratories that handle a considerable amount of samples. Furthermore, given the large number of analytes that can be found in the environment and the proven ability of macroalgae to accumulate such compounds [11–13] the availability of multi-class and multi-residue methods is essential. In light of these concerns the present work describes the development and validation of a sensitive screening and confirmatory multi-residue analytical method based on UHPLC–MS/MS to be applied in routine analyses on macroalgae samples (*Ulva lactuca*). The method developed allows the simultaneous detection of an extended list of 38 antibiotics from tetracyclines, macrolides, sulfonamides, benzenoids, quinolones and penicillins. The compounds selected cover the most prescribed compounds in veterinary medicine but also banned substances such as chloramphenicol.

2. Experimental

2.1. Chemicals and reagents

With the exception of solvents used in the mobile phases, all reagents were of analytical grade. Ethyl acetate, acetonitrile and formic acid were acquired from Merck (Darmstadt, Germany) while ethylenediaminetetra acetic acid (EDTA) was supplied by Sigma-Aldrich (Madrid, Spain). Ultrapure water was obtained daily from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Nitrogen was generated in-house with a generator from Peak Scientific Instruments Ltd. (Chicago, IL, USA). Solvents and water were filtered through 0.45 μm Whatman nylon membrane filters (Whatman, Maidstone, USA) prior to degassing in ultrasonic bath.

The target analytes classified by classes together with structures and other relevant data are presented in Table 1. Standards for tetracyclines, macrolides, sulfonamides, quinolones, penicillins, trimethoprim and chloramphenicol were purchased from Sigma-Aldrich, with the highest purity grade available. Internal standards, acquired from the same supplier, were as follows: demethyltetracycline for tetracyclines, roxithromycin for macrolides, sulfameter for sulfonamides and trimethoprim, lomefloxacin for quinolones, penicillin V for penicillins and d5-chloramphenicol for chloramphenicol.

Individual stock solutions (1 mg mL^{-1}) were prepared by accurately weighing each standard and internal standards, adjusting for purity and salt forms and dilution in methanol. Solutions were vortex mixed and sonicated, when necessary to assure complete dissolution.

Spiking standard solutions were prepared by diluting each stock and corresponding internal standard solution in methanol to final concentrations of 100 μg L^{-1}, with the exception of chloramphenicol and respective isotope, which were diluted to 10 μg L^{-1}. These solutions were used throughout the validation process and sample analyses, depending on the selected validation levels (VL). The VL was chosen during compound and method optimization as the reasonably lower concentration possible to achieve and to validate, and only related to the sensitivity of the method since that are no legislated tolerance levels for antibiotics in macroalgae. For all antibiotics the VL is 20 μg kg^{-1} with the exception of chloramphenicol with 1 μg kg^{-1}. Based on previous knowledge on stability, solutions were kept for 1 month at −5 °C.

2.2. Sample preparation and extraction

Green macroalgae (*Ulva lactuca*) samples were collected at a local beach during low tide. After rinsing with seawater to remove epibionts and debris from the surface, samples were frozen immediately at −80 °C and kept until analyses.

The first step of sample preparation consisted on grinding the algae to produce homogeneous samples. Next, subsamples of 200 ± 0.01 mg were weighed into 20 mL glass centrifuge tubes and internal standard solutions were added in accordance to the VL of each class of compounds (20 μL of chloramphenicol-d5 of a 10 μg L^{-1} solution and 40 μL of each of the other internal standards with 100 μg L^{-1} concentration). Following vortex mixing, samples were left to stand for a minimum of 10 min at room temperature, protected from light. To optimize the method four different extracting solvents were tested, as follows: (a) 5 mL of ethyl acetate; (b) 5 mL of ethyl acetate and 1 mL of 0.1 M EDTA; (c) 5 mL of acetonitrile and (d) 5 mL of acetonitrile and 1 mL of 0.1 M EDTA.

For each combination, solvents were added to the samples, which were then placed in a Reax shaker for 20 min and centrifuged for 15 min at 3100 x *g*. The resulting supernatants were transferred to new tubes and evaporated to dryness under a gentle nitrogen stream at 40 °C. The resulting dry residues were redissolved with 400 μL of a solution of formic acid 0.1% in water (mobile phase A), filtered through 0.45 μm PVDF Mini-Uniprep™ vials and injected into the UHPLC–MS/MS under MRM-optimized conditions.