



A single-step pesticide extraction and clean-up multi-residue analytical method by selective pressurized liquid extraction followed by on-line solid phase extraction and ultra-high-performance liquid chromatography-tandem mass spectrometry for complex matrices



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ABSTRACT

Pesticides, a group of compounds linked to human activity, may, when in toxic levels, have a profound effect on water quality, and hence result in adverse consequences to aquatic life and ultimately to human health. Analytical challenges arise when successfully trying to determine these levels in environmental complex matrices. Therefore, fast, simple, sensitive and selective analytical methodologies for multi-residue determination of pesticides (atrazine, azoxystrobin, bentazon, λ-cyhalothrin, penoxsulam and terbuthylazine) in sediment, macrophytes (algae and aquatic plants) and aquatic animals were developed and validated. The established methods were matrix-dependent and were based on Selective Pressurized Liquid Extraction (SPLE) followed by on-line Solid Phase Extraction and Ultra Performance Liquid Chromatography-tandem Mass Spectrometry (on-line SPE-UPLC-ESI-MS/MS). This cutting-edge research methodology uses a small amount of sample, is time saving and reduces the use of organic solvents in compliance with Green Chemistry principles. The analytical features were adequate for all compounds in all studied matrices. The established methodology was applied on real marine samples and no pesticide concentrations above their respective method quantification limits were measured in sediments or aquatic plants. However, terbuthylazine was found in the macroalgae *Ulva* spp. (108 ng g⁻¹ dw) and all the prospected pesticides were measured above their respective method quantification limits in the bivalve *Scrobicularia plana* (atrazine: 48 ng g⁻¹ dw, azoxystrobin: 64 ng g⁻¹ dw, bentazon: 33 ng g⁻¹ dw, λ-cyhalothrin: 2531 ng g⁻¹ dw, penoxsulam: 50 ng g⁻¹ dw, and terbuthylazine: 44 ng g⁻¹ dw).

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1. Introduction

The use of pesticides for crop protection may result in the presence of toxic levels of residues in aquatic matrices due to pesticide mobility through air during application or through soil percolation during irrigation. In the aquatic environment, pesticides might freely dissolve in water or bind to suspended matter

and to sediments, and could be transferred to organism tissues during bioaccumulation processes, likely resulting in adverse consequences to aquatic life and ultimately to human health. However, the occurrence of pesticides in these ecosystems is generally documented for specific compartments such as water, and studies on their ultimate fate are missing in the scientific literature [1].

The determination of pesticides in environmental matrices is crucial for the assessment of their environmental risk, as risk is typically characterized in the risk assessment framework as the ratio between exposure concentrations and critical effect concentrations [2]. Also, in ecological quality programmes, the integration of chemical data and biological responses is reliably recommended in order to characterize the effects of pollutants, and bivalves have been

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successfully used as indicators of environmental contamination by pesticides [3]. However, environmental matrices are a chemical analytical challenge because of their high complexity and the presence of low concentrations of the target pesticides. Moreover, a wide spectrum of pesticides with a large range of physico-chemical properties can be found in these matrices.

Biological samples are analytically difficult also due to the irreversible adsorption of proteins in the stationary phase of chromatographic techniques, resulting in a loss of column efficiency and an increase in backpressure [4]. Hence, effective sample preparation, as the extraction and clean-up steps prior to chromatographic analysis, which allows high recoveries of the analytes while minimizing the presence of matrix interferences, is essential. Moreover, the successful determination of multi-residues in complex matrices requires that the sample preparation procedure be followed by an analytical method that should be sensitive and selective enough to quantify several compounds at trace levels. In addition, currently, the development of modern analytical methodologies only makes sense if the principles of Green Chemistry are complied with, since these principles also encompass laboratory practices.

Several sample preparation techniques are usually used for solid and semi-solid matrices, such as Microwave Assisted Extraction (MAE), Ultrasonic Solvent Extraction (USE) and Liquid–Liquid Extraction (LLE). However, Pressurized Liquid Extraction (PLE) is currently considered an advanced technique since it offers important benefits such as shorter extraction time, decreased solvent consumption and decreased sample handling, when compared to the traditional Soxhlet extraction procedure of biological samples [5–8]. Moreover, this technique is in line with the green aspects of sample preparation [9]. For the clean-up step, different solid-phase extraction sorbents such as Florisil® or alumina might be used, which could be placed directly in the extraction cell of the PLE technique (called Selective Pressurized Liquid Extraction (SPLE) technique). For instance, SPLE was successfully applied to the analysis of alkylphenols and bisphenol A in sediments [10] and mussels [11]. Another alternative for the clean-up step is the on-line Solid Phase Extraction (on-line SPE) technique, with main advantages, namely high sample throughput, minimal solvent utilization, fast sample preparation and the small sample volume required (5–40 mL) [12]. This technique has been widely applied to environmental analysis, clearly exhibiting better performance than off-line schemes [6]. For instance, the on-line SPE was successfully applied to the analysis of polycyclic aromatic hydrocarbons (PAHs) in sediments by Ericsson and Colmsjö [13]. No other studies based on SPLE or on-line SPE for the analysis of pesticides in aquatic complex matrices were found in the literature.

The present study aims to develop and validate robust analytical methodologies for the determination of atrazine, which was excluded in 2004 as an active substance from Annex I of Directive 91/414/EEC [14], and other five pesticides: azoxystrobin, bentazon, λ -cyhalothrin, penoxsulam and terbuthylazine in marine complex matrices. This selection of pesticides corresponds to the most currently-used pesticides of the Mondego river valley (Lower Mondego, an area of intense agriculture practices in Portugal), with the exception of atrazine, which was widely used until 2007 and is considered as a persistent pesticide [15], thus possibly leaving a legacy. The SPLE technique was used to enable the simultaneous and automatic in-cell clean-up process, which is then followed by multi-residue quantification using on-line SPE clean-up and Ultra Performance Liquid Chromatography–tandem Mass Spectrometry (on-line SPE–UPLC–MS/MS). The methods developed by the present study were applied to real environmental marine samples, such as sediment, the macroalgae *Ulva* spp., the aquatic plant *Zostera noltii* and the bivalve *Scrobicularia plana*.

2. Material and methods

2.1. Standards and reagents

The following PESTANAL® analytical standards were used in the present study: atrazine 99.1% (Riedel-de Haen 45330), azoxystrobin 99.4% (Fluka 31697), bentazon 99.7% (Riedel-de Haen 45341), λ -cyhalothrin 97.8% (Fluka 31058), penoxsulam 96.9% (Fluka 32094) and terbuthylazine 98.8% (Fluka 45678). Standard individual and mixture stock solutions were prepared in methanol (Carlo Erba, HPLC-Plus) and stored at 4 ± 2 °C.

For the extraction procedures, methanol, acetonitrile and acetone were purchased from Sigma-Aldrich. The sorbents used in the clean-up process, such as neutral alumina, basic alumina, silica gel, Florisil® and primary-secondary amine (PSA), were obtained from Supelco. For the LC mobile phase preparation, the methanol Chromasolv® HPLC-grade and ammonium acetate used came from Sigma-Aldrich. Ultrapure water was purified in a Direct MilliQ water system.

2.2. Site description and sampling procedures

Samples were collected in 2014 in the Mondego estuary, which is a shallow warm-temperate intertidal system located on the west coast of Portugal. Due to the absence of certified reference materials of sediments, macrophytes (algae and aquatic plants) and aquatic animals for method optimization and validation, fresh material was collected during low tide in the most downstream area of intertidal flats exposed during low tide (40°07,815'N; 8°49,692'W). The collection of material was carried out a few days after the end of the rainy season and when the work for the productive season in the Lower Mondego was not yet started, which, in 2014, corresponded to the first days of March. During collection, samples were stored in aluminium extrusion containers and transported to the laboratory in 12 V car refrigerators. Sediment samples were frozen immediately upon arrival at the laboratory. Macrophytes (*Ulva* spp. and *Z. noltii*) were rinsed briefly in running tap water and gently scrubbed with paper towels to remove most surface microbial and epiphytic organisms, and then frozen. Aquatic animals were represented in the method optimization and validation process by aquatic worms (*Nereis diversicolor*) and bivalves (*S. plana*). Both organisms are predominantly surface deposit-feeders of sedimentary organic matter. Therefore, after collection, worms were maintained in aerated recirculating aquatic systems composed of glass tanks (25 × 20 × 9.5 cm) and appropriate life support systems in a 20 °C temperature-controlled room and under a natural light regime for gut clearing purposes. Each tank was filled with 2.0 L of reconstituted water at a salinity of 17 (Tropic Marin Salt, Tropical Marine Centre) according to the ASTM E729 guideline [16]. After 72 h the organisms were dried with paper towels and frozen. Concerning bivalves, *S. plana* were purchased alive in a local food shop and were maintained for depuration purposes under the same conditions as worms. After 96 h the organisms were dissected and frozen. In order to reduce physico-chemical pesticide degradation [17], the frozen macrophytes and animals were freeze-dried (UniEquip Unicryo MC-4L) and then grinded (Ika T18 basic or Ika MF10 basic), and, after homogenization, were saved in new amber glass vials (Supelco 27004 and 27182). A mixture (50:50) of *Ulva* spp. and *Z. noltii*, and a mixture (50:50) of *N. diversicolor* and *S. plana* homogenized tissues were used for macrophyte's and animal's method optimization and validation, respectively.

The material for confirmatory analyses (samples of sediment, *Ulva* spp., *Z. noltii* and *S. plana*) were collected during low tide in a Mondego estuary upstream sampling station (40°07,308'N; 8°50,515'W) in August 2014, which represents the highest probability of the presence of pesticide residues in the estuary. Sediment

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