OCCURRENCE AND IMPLICATIONS OF ENDOCRINE DISRUPTING COMPOUNDS IN THE ENVIRONMENT

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Endocrine disrupting compounds (EDCs) have taken part in environmental and toxicological studies in the last years. Alkylphenols and bisphenol A stand out among all of them because of their widely use and their high estrogenic activity (adverse effects at low levels, µg/L). These compounds enter into the environment through industrial and wastewater treatment plants effluents and therefore, aquatic system is the most affected. Consequently, monitoring programs that control the occurrence of these pollutants are needed in order to preserve the aquatic environment and protect human health.

In this context, the International PhD (November, 2015) entitled **"Occurrence and implications of endocrine disrupting compounds in the environment"** was focused on: 1) the development and validation of novel and improved analytical methods for the determination of target EDCs in different matrices, 2) the presentation of new data about the distribution and behaviour of different EDCs (mainly alkylphenols and bisphenol A) in aquatic environment, 3) the evaluation of the risk that these compounds pose to the environment and to human health.

The research presented in this PhD implicates relevant contributions to Analytical Chemistry, but also to Environmental Sciences, Bioanalysis, Toxicology and Food Chemistry. A detailed explanation about all these contributions is shown in the following lines.

1. Development and validation of analytical methods

The Thesis describes different analytical methods to determine alkylphenols and bisphenol A in water, sediment and biota samples at trace levels in the aquatic ecosystem. The advantages of these methods are sensitivity, selectivity and compliance with the Green Chemistry principles, improving the previous methods found in the literature. Furthermore, two short-stays were carried out, based on the development of new method for the analysis of EDCs and emerging compounds in atmospheric particulate matter and seafood.

In 2010, the published analytical methodologies for the determination of alkylphenols (APs) and bisphenol A (BPA) in aquatic environmental matrices widely employed gas chromatography-mass spectrometry (GC-MS) as instrumental determination technique [1], being necessary a derivatization step to obtain adequate peaks. To solve

the limitations of GC-MS, liquid chromatography followed by tandem mass spectrometry (LC-MS/MS) was selected as alternative technique in this PhD because of its sensitivity and selectivity, avoiding the derivatization step. An appropriate separation of five target compounds and the two adequate internal standards was achieved in less than 12 min [2].

Regarding the extraction techniques for water analysis previously published, the most common were conventional ones (i.e. LLE, SPE), which required higher analysis time and reagent consumption and did not achieve the low limit concentrations set by Water Directive 2008/105/EC. Thus, two novel analytical methods were developed and validated for the determination of APs and BPA in different types of water samples. The first one was based on a dispersive liquid-liquid microextraction (DLLME-LC-MS/MS) [2] and the second one on a miniaturized membrane assisted solvent extraction (MASE), showed in Figure 1 [3]. Simplicity, fastness, selectivity, sensitivity are some advantages of these methods. The lower volume of sample needed facilitates the sampling, transport and their storage. Moreover, these methods are environmental friendly (low reagent consumption, low analysis time and no waste generation) according with Green Chemistry principles. Finally, both methodologies allow the determination of these EDCs in seawater samples, which can contribute to the understanding of their behaviour in this ecosystem, where they are scarcely studied.

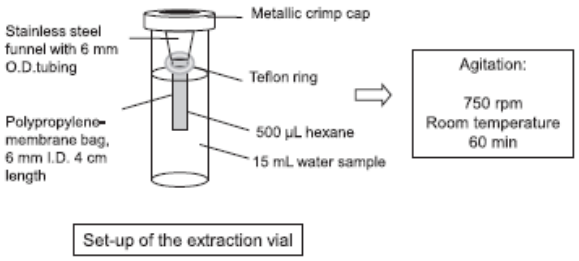

Figure 1. A scheme of miniaturized MASE system [3]

Another unresolved matter in the analysis of these EDCs was the establishment of a QA/QC protocol. The previous published methodologies did not consider aspects like Method Quantitation Limits (MQL) and uncertainty estimations or identification and quantitation criteria. In all the proposed analytical methods, a QA/QC protocol was included. It is worth mentioning that blank contamination problems are a common issue in the determination of these compounds due to their use as plastic additives and detergents manufacture. An exhaustive study about blanks was carried out in order to know the possible sources of contamination and to minimize them [4]. Solvents used as mobile phases seemed to be one of the major contributors to blank contamination. Target EDCs were found at ultratrace levels in Milli-Q water and methanol HPLC grade. However, using Milli-Q water obtained from a different purification system (which minimizes the contact with plastic reservoirs) and methanol LC-MS grade, a decrease of more than 80% in procedure blanks was achieved, as it is shown in Figure 2. Furthermore, a specific clean-up procedure of glass material and sample pre-treatment considerations were proposed [4].

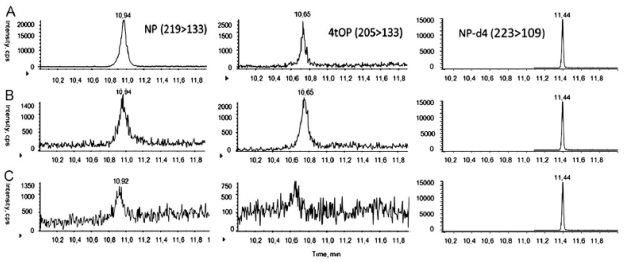

Figure 2. Chromatograms of methanol at initial conditions (A), using Milli-Q water purified in a different system (B) and using methanol LC-MS (C) as mobile phase.

In comparison with the number of analytical methods published for water analysis, few methods can be found for solid samples (i.e. sediments and biota species), although the physic-chemical properties of these EDCs show their capability to be associated to those matrices [5]. Furthermore, the proposed analytical methods are based on conventional extraction techniques (i.e. Soxhlet) followed by tedious clean-up step to minimize matrix interferences. In this context, 3 analytical methods to determine APs and BPA at trace levels in solid environmental samples were developed and validated. The extraction techniques employed in each method are based on the same principles of pressurized liquid extraction (PLE), but with some variations in the experimental procedure. One of them is the selective pressurized liquid extraction (SPLE) which allows the simultaneous extraction and clean-up of samples by adding a clean-up sorbent in the PLE cell (Figure 3). This simple, fast and semi-automatic procedure minimizes the analysis time and the reagents consumption. Moreover, due to its versatility, it can be applied to the analysis of sediment [6] and biota species [7], with small modifications.

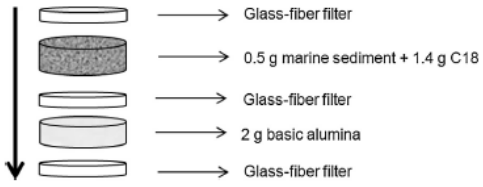

Figure 3. Scheme of a filled extraction cell for SPLE [6,7]

The other extraction technique is pressurized hot water extraction (PHWE) based on the use of water at subcritical conditions; in this way, medium polar and non polar analytes, as APs and BPA, can be extracted. This technique was applied to the determination of APs in sediment samples [8] followed by a miniaturized MASE in order to

concentrate the obtained aqueous extracts. The developed PHWE-MASE-LC-MS/MS method is simple and fast but the main advantage is, without doubts, the low consumption of reagents and waste generation which complies with Green Chemistry principles. Low MQL were obtained in all cases allowing the determination of target compounds at trace levels. Finally, the validation of these five analytical methods allowed to investigate the occurrence, distribution and behaviour of target compounds in the aquatic environment and to evaluate their risk for the environment and human health, as it will be commented in Sections 2 and 3, respectively.

As a complement of her formation, during her PhD Dr. Salgueiro-González carried out two short stays (both national and international). The first one was done in 2011 (12/09/2011-21/12/2011) at the Institute of Environmental Assessment and Water Research of the Spanish Council for Scientific Research (IDAEA-CSIC) under the supervision of Dr. Miren López de Alda (Dr. D. Barceló research group) and it was focused on the determination of EDCs in air particulate matter in order to evaluate their occurrence and risk for human health via inhalation (as a first approach). Up to then, only few investigations about this topic could be found in the literature [9]. To achieve this objective, a multi-residue methodology based on a pressurized liquid extraction (PLE) followed by LC-MS/MS was developed and validated [10], allowing the determination of 13 EDCs (including phthalates, sex homones, alkylphenols and bisphenol A) in less than 30 min.

An international short stay was carried out in 2015 (17/03/2017-27/07/2015), at the Water Institute Northern Region (IAREN) in Porto (Portugal) under the supervision of Dr. M.F. Alpendurada. Because of the great experience of this research centre in pesticides analysis, the topic was mainly focused on the environmental behaviour of selected pesticides (fungicides, herbicides and insecticides) in a Portuguese river. This work was done in collaboration with the University of Coimbra (Department of Life Science) with the aim of evaluating the occurrence, distribution and behaviour of these pesticides in the aquatic environment. Thus, different matrices (i.e. water, sediment, biota and algae samples) were collected and analyzed with the proposed analytical methods [11]. A total of 4 methods based on the on-line solid phase extraction (on line SPE) followed by UPLC- MS/MS were developed and validated for water analysis and PLE-on line SPE-LC-MS/MS methods were selected for solid samples. Satisfactory results were obtained and herbicides were the most common pesticides found in the studied area.

2. Occurrence and distribution of target compounds in the environment

This Thesis investigates the fate and occurrence of 4-alkylphenols and bisphenol A in the aquatic environment. Environmental studies in Galicia were carried out applying the proposed methods, and the spatial-temporal distribution of these compounds and their behaviour into the same ecosystem (water-sediment-biota partition) were also evaluated. As far as we know, no previous works related to the presence of the target EDCs in this area (NW Spain) can be found in the literature.

Different environmental studies were carried out to evaluate the occurrence and behaviour of APs and BPA in the aquatic environment. Marine ecosystem was also considered even though the lack of data in the literature about the distribution of these EDCs. Taking into account their magnitude and the relevance of results, three studies are briefly commented in the following lines. It is important to highlight that the obtained results were part of different research projects carried out in collaboration with several organisms and institutions (i.e. Instituto Español de Oceanografía (C.O. Vigo and C.O. Murcia), University of Coimbra-Department of Life Sciences).

The first one was focused on the analysis of seawater samples from the North East Atlantic Ocean (Iberian coast and Biscay Gulf) [12], in the frame of a regional research project (Xunta de Galicia, 2012). The occurrence and spatial distribution of target compounds were considered, identifying the main sources of contamination. As an example, Figure 4 shows the fate of these EDCs in one of the estuaries studied.

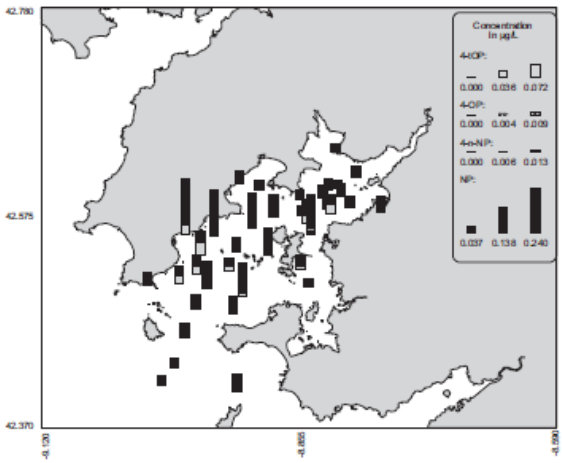

Figure 4. Levels (µg/L) and spatial distribution of alkylphenols and bisphenol A in Ría de Arousa (Galicia, NW Spain) [12]

The second investigation was focused on the occurrence and distribution of APs and BPA in the Miño river [13], which stands as a frontier between Spain and Portugal in its last 75 km. This study was part of an international project TEAM MINHO (POCTEC 2007-2013) which aim was to implement the Water Framework Directive in this estuary, taking into account both biological and chemical investigations. Water, sediment and biota species (Asiatic clams) were analyzed with the aforementioned methodologies. Occurrence, spatial and seasonal

distribution, as well as partitioning of target EDCs were evaluated.

The last study was focused on the fate of APs and BPA in mussel samples collected in 24 sampling points from Spanish Atlantic coast and Bay of Biscay [17]. The occurrence and spatial distribution of pollutants were investigated and moderate bioaccumulation of some APs was found.

In all of them, the results highlighted the importance of bisphenol A and 4-*n*-alkylphenols (linear isomers) as environmental pollutants, even though their impact is still under debate by the scientific community.

3. Evaluation of risks for the environment and human health

As a first approach, this Thesis applies risk assessment to evaluate the risk of EDCs for the aquatic environment and human health, in a last term. Moreover, the effects that these EDCs cause in some marine organisms were also evaluated.

The obtained data concerning concentrations and distribution of target EDCs were employed to evaluate the risk they pose to the environment and human health. As a first approach, *Risk quotient* (RQ), *daily intake* (DI) and *estrogenic activity* (EEQ) estimations were employed [12,13,14], showing a low-moderate risk for the environment and no risk for human health.

Finally, some of the analytical methodologies were also applied in ecotoxicological studies in collaboration with the Department of Ecology and Animal Biology (Universidade de Vigo). One of them studied the bioaccumulation of 4-*n*-nonylphenol in mussels and its effects in three enzymes [15]. Results showed a moderate bioaccumulation of this compound and an inhibition and induction effects of some enzymes, demonstrating its disruption capability. This collaboration is maintained right now, and different studies including other compounds (i.e. BPA) and other species (i.e. sea urchin larvae) are being carried out.

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