Analysis of 17-β-estradiol and 17-α-ethinylestradiol in biological and environmental matrices — A review☆

Luisa Barreiros [a,b,*], Joana F. Queiroz [a], Luís M. Magalhães [a], Adrián M.T. Silva [c], Marcela A. Segundo [a]

[a] UCIBIO, REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua de Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal
[b] Área Técnico-Científica de Farmácia, Núcleo de Investigação e Intervenção em Farmácia (NIIF), Centro de Investigação em Saúde e Ambiente (CISA), Escola Superior de Tecnologia da Saúde do Porto (ESTSP) – Instituto Politécnico do Porto (IPP), Rua Valente Perfeito 322, 4400-330 Vila Nova de Gaia, Portugal
[c] LCM – Laboratório de Catálise e Materiais - Laboratório Associado LSRE-LCM, Faculdade de Engenharia, Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

A B S T R A C T

The estrogens 17-β-estradiol (E2) and 17-α-ethinylestradiol (EE2) are reported as highly endocrine-disrupting agents, being recently included in an EU watch list regarding emerging aquatic pollutants. Therefore, the monitoring of these chemicals in the different environmental compartments assumes great importance. Moreover, due to the possible adverse effects on living beings, their occurrence on animal tissues and fluids must also be addressed. In recent years, a significant number of studies have described and proposed different analytical methodologies to detect and/or quantify E2 and EE2 mostly in environmental aqueous samples, including sludge and sediments and also in biological matrices such as plasma and tissues. Taking into account the complexity of real matrices and that both estrogens are generally present at trace levels, the development of accurate and reliable techniques for their determination can be quite a challenge. The present review aims at describing the main characteristics of the analytical methods recently used for E2 and EE2 determination in environmental and biological samples. The steps for sample preparation such as analytes extraction, preconcentration and clean-up are discussed and the instrumental based analytical techniques are compared. Furthermore, the application of biological tools to determine the total estrogenicity of environmental samples, as well as their potential combination with instrumental analyses, is highlighted.

Keywords: 17-β-estradiol (E2) 17-α-ethinylestradiol (EE2) Biological and environmental matrices Sample preparation Instrumental analysis

Abbreviations: AAE, aqueous alkali extraction; APCI, atmospheric pressure chemical ionization; APPI, atmospheric pressure photoionization; ASE, accelerated solvent extraction; BPA, bisphenol A; BSTFA, *N,O*-bis(trimethylsilyl)-trifluoroacetamide; cEEQ, estradiol equivalents calculated from concentration data; CI, chemical ionization; CLEIA, chemiluminescence enzyme immunoassay; CPRG, chlorophenol red-β-D-galactopyranoside; CV, cyclic voltammetry; DAD, diode array detector; DLLME, liquid–liquid microextraction; DLLME-SFO, dispersive liquid–liquid microextraction based on the solidification of a floating organic drop; DLLSME, dynamic liquid–liquid–solid microextraction; DNS-Cl, dansyl chloride; E1, estrone; E2, 17-β-estradiol; EE2, 17-α-ethinylestradiol; EDCs, endocrine-disrupting chemicals; EEQ, estradiol equivalents; EI, electron ionization; ELISA, enzyme-linked immunosorbent assay; ER-α, recombinant human estrogen related receptor alpha; ERE, estrogen-responsive sequences; ESI, electrospray ionization; FD, fluorescence detector; FPSE, fabric phase sorptive extraction; GC–MS, gas chromatography coupled to mass spectrometry; GC–MS/MS, gas chromatography coupled to tandem mass spectrometry; GPC, gel permeation chromatography; hER, human estrogen receptor; HF-LPME, hollow-fiber liquid-phase microextraction; HPLC, high performance liquid chromatography; HLB, hydrophilic–lipophilic balance; IS, internal standard; K_{ow}, octanol/water partition coefficient; LC–MS, liquid chromatography coupled to mass spectrometry; LC–MS/MS, liquid chromatography coupled to tandem mass spectrometry; LLE, liquid–liquid extraction; LOD, limit of detection; LOQ, limit of quantification; MAE, microwave accelerated extraction; MASE, membrane-assisted solvent extraction; MEKC, micellar electrokinetic chromatography; MIPs, molecularly imprinted polymers; MPs, magnetic particles; MRM, multiple reaction monitoring; MSPD, matrix solid-phase dispersion; MSTFA, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide; MTBE, methyl tert-butyl ether; MWCNTs/GCE, glassy carbon electrodes modified with multi-walled carbon nanotubes; NiTPPS, Ni(II)tetrakis(4-sulfonatophenyl) porphyrin; POCIS, polar organic chemical integrative samplers; PFBBr, pentafluorobenzyl bromide; PFBCl, pentafluorobenzoyl chloride; RAM, restricted access material; SIM, selected ion monitoring; SPE, solid-phase extraction; SPME, solid-phase microextraction; SRM, selected reaction monitoring; S_w, water solubility; SWV, square wave voltammetry; TBDMSCl, *tert*-butyldimethylsilyl chloride; TMCS, trimethylchlorosilane; TMSI, trimethylsilylimidazole; TWA, time-weighted average; UPLC, ultra-high performance liquid chromatography; WWTP, wastewater treatment plant; YES, Yeast Estrogen Screen.

☆ On the occasion of the 70th birthday of Prof. José Luís Costa Lima.

* Corresponding author at: Luisa Barreiros. Mailing address: UCIBIO, REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua de Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal. Tel.: +351 220428676. Fax: +351 226093483.
E-mail address: lbarreiros@ff.up.pt (L. Barreiros).

1. Introduction

Endocrine-disrupting chemicals (EDCs) constitute a group of organic pollutants with increasing importance due to their impact in the environment and human health. EDCs are generally defined as chemicals that may interfere with the function of the endocrine system in wildlife and humans by blocking or mimicking the normal effect of hormones, affecting their synthesis or metabolism, and altering hormone receptor levels [1]. The compounds exhibiting endocrine disrupting properties include a wide range of chemical groups, among which are steroid estrogens, both of natural and synthetic origin. This group of compounds, and particularly the natural hormone 17-β-estradiol (E2) and the synthetic estrogen 17-α-ethinylestradiol (EE2) (Fig. 1), is described as the EDCs with higher disrupting potency [2,3].

Estradiol (E2) is the major estrogen in vertebrates, being associated with the female reproductive system and maintenance of sexual characteristics [4]. Ethinylestradiol (EE2) is a synthetic hormone derived of the natural estrogen E2 which is mainly used as a component of oral contraceptives. Other applications of EE2 in human medicine include estrogen replacement therapy and suspension of breastfeeding [5]. Both estrogens (E2 and EE2) are largely excreted by human and animals in urine and feces as active free forms or inactive glucuronide and sulfate conjugates. The estrogen excretion varies as a function of gender, physiological and developmental state. The highest contributors are pregnant and menstrual women, excreting, respectively, 308 and 4.66 μg per day of E2 in urine and 202 and 0.2 μg per day of E2 in feces [6]. Except during these periods, woman and man present a similar E2 excretion, between 1.5–7 μg per day [7,8]. Assuming that approximately 17% of the total female population take the contraceptive pill regularly in western countries, it is estimated that 4.5 and 6 μg per day of EE2 are excreted per head in urine and feces, respectively [6,7]. These data were used to predict a total estrogen discharge of 4.4 kg per year per million inhabitants [8]. These estrogens eventually end up in the environment through discharge of wastewater treatment plant (WWTP) effluents and disposal of animal waste [2,8]. In fact, numerous studies have reported the presence of E2 and EE2 in waste and surface waters of various countries, in concentrations ranging from low ng L^{-1} to μg L^{-1} levels [7,9–14]. The two estrogens have also been detected in sludge and sediment samples [11,15–17], showing their potential to persist in the environment [18].

Solubility in water (S_w) and the octanol/water partition coefficient (K_{ow}) are considered crucial to assess the fate of chemicals in the environment [2]. In general, chemicals with K_{ow} values <10 are considered relatively hydrophilic, tending to have high S_w and low adsorption and bioconcentration factors. On the opposite, compounds with K_{ow} values $>10^4$ are considered very hydrophobic and have high sorption potential [2,19], such as E2 and EE2 with values log K_{ow} c.a. 4. Both E2 and EE2 also present low water solubility values (13 and 4.8 mg L^{-1}, respectively), reinforcing their hydrophobic nature and sorption potential.

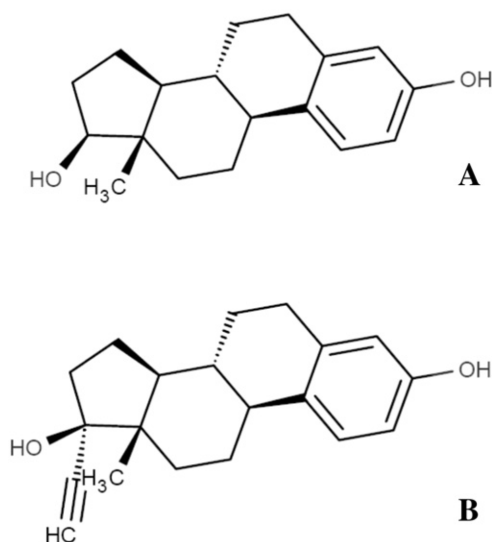

Fig. 1. Chemical structures of the estrogenic compounds 17-β-estradiol (E2) (A) and 17-α-ethinylestradiol (EE2) (B).

In a world with increasing population and resultant higher WWTP discharges, the presence of estrogens in the environment is an emerging problem due to the possible negative effects on ecosystems and living beings, even at low ng L^{-1} levels. This is particularly important in the case of E2 and EE2 as these estrogens have been identified as the major sources of estrogenic activity [2,4,8,13]. Indeed, the presence of E2 and EE2 in the environment has been associated with fish feminization (synthesis and secretion of vitellogenin), reproduction and behavior modifications, fertility reduction, increase of breast and testicular cancer in humans and promotion of abnormal reproductive processes [2,10,20,21]. Moreover, these chemicals have the potential to bioaccumulate and enter the food chain [22,23]. For all these reasons, the European Union has recently added E2 and EE2 to a new “watch list” of emerging aquatic pollutants included in the Water Framework Directive [24].

Having in mind the risks that steroid estrogens pose to humans and wildlife, the monitorization of these chemicals in the environment is of crucial importance and claim for development of valid and robust analytical methods. Therefore, the main purpose of the present paper is to provide a state of the art review of the current techniques applied to detect and quantify the estrogens E2 and EE2 in environmental and also in biological matrices. Due to the complexity of these matrices and the trace levels of estrogens normally detected, multi-step sample preparation is often needed to enrich analytes and reduce interferences [25]. Thus, this review targets recent methods describing the steps of sample extraction, clean-up and analytical determination, highlighting the most used techniques in each step for both types of matrix. It is based on information retrieved in a literature search performed on the *ISI Web of Knowledge* search engine for papers containing the words “estradiol” or “ethinylestradiol” and published in the last six years (2009–2014). A total of 114 papers were considered for this review.

2. Determination of E2 and EE2 in biological samples

In what concerns biological matrices, E2 and EE2 have been mainly determined in plasma and tissues of humans and other mammals, fish, invertebrates, and milk samples as summarized in Table 1 and a schematic representation about sample treatment strategies is presented in Fig. 2. The quantification of the estrogens E2 and EE2 in tissues and biofluids such as plasma or serum is essential to understand human biology and health [26,27], to determine the possible interaction of oral contraceptives (EE2) with co-administered drugs [28,29] or even to assess the possible impact of animal exposure to endocrine disruptive agents. Furthermore, one study has reported the development and validation of an analytical method for the simultaneous quantification of testosterone and E2 in a human cell line (H295R), in accordance with the requirements of the current EPA Steroidogenesis guideline [30]. Endogenous compounds such as E2 derivatives can potentially be used as growth promoters. The direct analysis of these chemicals in bovine hair has been proposed by Bichon et al. [31] as an efficient strategy for the detection of “natural” steroid abuse in cattle. Similarly, the determination of estrogens in milk and dairy products appears as a useful tool to monitor the introduction of natural and synthetic estrogens in the milk cycle by human action [32]. In order to assess the environmental fate of estrogens, several studies have focused on the analysis of fish tissues and plasma samples [33–35]. The main purpose of those studies has been to determine the uptake, elimination and bioaccumulation of estrogens, namely EE2, in fish or other aquatic organisms from natural ecosystems exposed to these compounds. The potential for transfer

into the terrestrial food chain through consumption of contaminated prey items has also been evaluated by the analysis of synthetic estrogens content in aerial insects (invertebrates) developing in areas adjacent to WWTPs [36].

2.1. Sample preparation

Plasma and serum samples are generally treated using liquid–liquid extraction (LLE), after addition of the adequate internal standards (IS). As most of the analytical techniques for estrogens determination use mass spectrometry detection, internal standards are often the target analyte isotopically labeled with deuterium or ^{13}C. Biological samples are supplemented in most cases with the deuterated standards E2-d_4 and EE2-d_4 but ^{13}C-E2 and ^{13}C-EE2 have also been added (Table 1). Several solvents or solvent mixtures have been applied for LLE of E2 and EE2, such as methyl tert-butyl ether (MTBE) [27], ethyl acetate [29], diethyl ether:hexane (70:30, *v*/*v*) [28], dichloromethane:methanol (2:1, *v*/*v*) [37] and hexane:ethyl acetate (75:25, *v*/*v*) [26]. In the work describing estrogens analysis in the human cell line H295R, the medium from cultured cells was also treated by LLE using dichloromethane containing the internal standard ^{13}C-E2 [30]. After extraction, organic and aqueous phases are generally separated by centrifugation.

Nevertheless, prior to extraction, protein rich samples such as milk should be submitted to protein precipitation and separation [32,43]. Subsequently, the supernatant can be simply extracted using LLE with methanol and dichloromethane [38] or miniaturized techniques such as hollow-fiber liquid-phase microextraction (HF-LPME) using 1-octanol as extraction solvent [32]. In HF-LPME, extraction normally takes place from an aqueous sample containing analytes (donor phase) into a small amount of a water-immiscible solvent (acceptor phase) that is in the pores and inside the lumen of a hollow-fiber [39]. Moreover, Zhong et al. [40] have proposed a dynamic liquid–liquid–solid microextraction (DLLSME) strategy using silica filaments coated with molecularly imprinted polymers (MIPs) as solid phase to retain/separate estrogens from milk and urine in on-line mode. In this study, proteins from biological samples were previously precipitated with acetonitrile and the resultant liquid phase and precipitate collected separately. Ultrasound extraction with acetone was subsequently used to treat the precipitate. This organic extract was concentrated by acetone vacuum distillation and dissolved in the liquid phase (from which acetonitrile was meanwhile removed), and further submitted to DLLSME as an aqueous sample.

Biological solid samples such as human and animal tissues (including invertebrates) are complex matrices that, prior to extraction, require homogenization with organic solvents [35,41] or buffer solutions [27]. After this step, the resultant homogenates are often freeze-dried, as this procedure has been associated with higher steroidal estrogens recoveries [34,35]. Homogenates extraction may then be performed using different techniques. As described for liquid samples, homogenates have been spiked with internal standards before extraction. Al-Ansari et al. [34,35] reported that the accelerated solvent extraction (ASE) with dichloromethane at elevated temperature and pressure (70 °C and 2000 psi, respectively) permitted to efficiently remove EE2 from whole fish tissue homogenates after extraction for 15 min. LLE, already referred for liquid biological samples, has also been applied for estrogens extraction from fish liver and endometrium tissue homogenates [26,39]. Furthermore, there is one study [25] reporting matrix solid-phase dispersion (MSPD) with C8 adsorbent and elution with methanol and acetone as a viable alternative for the extraction of feminizing chemicals from fish and clams. MSPD combines extraction and concentration in one step thus reducing potential loss or contamination of analytes. Firstly, biological tissues are mixed with an adsorbent (C8 in this case) which destructs tissues and releases the analytes. This mixture is subsequently packed into a cartridge and eluted with a reduced solvent volume, as performed in solid-phase extraction (SPE) [25]. On the other hand, E2 derivatives have been extracted from bovine hair through sonication with methanol [31].

Extracts from both liquid and solid samples can be further purified and concentrated using SPE. This procedure can be performed with various sorbents and eluents. As E2 and EE2 are hydrophobic compounds, they can be purified through reversed phase sorbents. On the other hand, these compounds present high pK_a values (~10.5), indicating their basic nature and possible clean-up using ion exchange SPE. Wheaton et al. [26] described SPE clean-up of human plasma extracts applying Oasis MCX μElution 96-well plate, which is a mixed-mode cation exchange sorbent for bases containing ionized sulfonic groups, and eluted with 5% NH_4OH in acetonitrile:2-propanol (90:10, *v*/*v*). Due to their hydrophobicity, both estrogens and plasma interferents were retained in the Oasis MCX SPE sorbent. However, as plasma interferents, namely phospholipids, are preferentially soluble in more protic solvents such as alcohols, the elution with low percentages of these solvents prevented desorption of interfering species and thus permitted the purification of estrogens extracts. Borges et al. [28] proposed C18 cartridges and elution with methanol:water (75:25, *v*/*v*) for on-line SPE clean-up of the same type of samples. In this case, a reversed phase separation based on non-polar – non-polar interactions was performed.

Innovative solid phases have also been proposed such as cellulose fabric as substrate for a sol–gel poly-THF coating [42]. Moreover, molecular recognition has been proposed for selective extraction, using MIPs immobilized on silica filaments [40], retained through electromagnetic interactions in solid-phase microextraction (SPME) fibers [43], in SPE mode [44], in MSPD format [45], and as hollow shells [46]. In all reports, MIPs targeting estrogens were synthesized, using E2 as template.

Fish and clams tissue extracts have been purified by SPE, using acidic alumina cartridges and elution with methanol/acetone [25], or a Bakerbond octadecyl C18 cartridge followed by an Oasis HLB (hydrophilic-lipophilic-balanced copolymer; polymer of N-vinylpyrrolidone and divinylbenzene) cartridge eluted with acetonitrile:water (70:30, *v*/*v*) and methanol, respectively [41]. The application of acidic alumina, a normal phase sorbent, permitted the adsorption of more polar interferents while the non-polar analytes of interest E2 and EE2 passed through unretained. Kwon et al. [41] verified that, for the determination of EE2 in fish liver by LC–MS/MS, ion suppression could be significantly reduced from 88% to 23% using a two-step clean-up procedure through C18 and Oasis HLB cartridges. The first sorbent removed the polar contaminants whereas the latter one had the ability to retain both non-polar and polar compounds. Other combinations of sorbents may be applied such as the successive clean-up through aminopropyl (NH_2) and silica C18 SPE cartridges described by Bichon et al. [31] for bovine hair extracts purification. Aminopropyl bonded silica sorbents, because of their polar functional groups, are much more hydrophilic relatively to the C18 bonded reversed phase silicas. Therefore, aminopropyl bearing sorbents have been shown to be effective for removing polar interferents from non-polar target analytes like E2 and EE2 in organic solvent extracts.

Besides SPE, biological solid matrices are often purified with other clean-up techniques. Gel permeation chromatography (GPC) has been applied to remove excess lipids from fish tissues and invertebrate extracts [34–36]. Moreover, an acetonitrile precipitation step can be introduced for cholesterol removal [34,35]. Two-dimensional liquid chromatography has been referred in the work of Chen et al. [25] for on-line clean-up of fish and clams tissue extracts. For this, extracts were loaded to a RAM (restricted access material) pre-column, which was operated in tandem with a C18 column. RAM was also applied for protein removal in cerebrospinal fluid samples [47].

2.2. Quantification of E2 and EE2

Several methods have been described for the identification and determination of estrogens in biological matrices. During many years, immunoassays have been the golden standard for estrogens quantification in biofluids such as serum or plasma [28,33]. However, such techniques

Table 1
Analytical methods for E2 and EE2 determination in biological samples.

Sample	Steps for sample preparation		Derivatization	Analytical technique	Standard	Recovery rate	LOQ	Detected values	Reference
Human plasma	LLE, diethyl ether: hexane (70:30, *v*/*v*)	SPE, C18, methanol: water (75:25, *v*/*v*)	–	LC–MS/MS, APPI+	EE2-d_4	69.1–79.0%	5 pg mL^{-1} (EE2)	75–85 ng mL^{-1} (EE2)	[28]
Human plasma	LLE, hexane:ethyl acetate (75:25, *v*/*v*); centrifugation (4000 rpm/5 min)	SPE, Oasis MCX μElution plate, 5% NH_4OH in acetonitrile: 2-propanol (90:10, *v*/*v*)	DNS-Cl, 60 °C/10 min	LC–MS/MS, ESI+	E2-d_4	90.7–112.4%	n.a.[a]	0.001–1 ng mL^{-1} (EE2)	[26]
Human serum	LLE, MTBE		–	LC–MS/MS, ESI+/−, APCI+/−, APPI+/−	E2-d_4	n.a.	0.05–2.5 nM (E2)	81 pg mL^{-1} (E2)	[27]
Serum	Precipitation with acetonitrile; centrifugation	SPE, MIP, methanol	–	Capillary electrophoresis, UV detection (210 nm)	E2	100%	n.a.[a]	0.1–200 μg mL^{-1} (spiked E2)	[44]
Human cerebrospinal fluid	Centrifugation, 4 °C/10 min	On-line RAM trap column	NH_4OH, DNS-Cl, 60 °C/15 min	LC–MS/MS, ESI+, APCI+	E2-d_3	n.a.	50 pg mL^{-1} (E2)	>13 pg mL^{-1} and <LOQ (E2)	[47]
Human cell line culture medium	LLE, dichloromethane		DNS-Cl, 60 °C/8 min	LC–MS/MS, APPI+	^{13}C-E2	98.9–128.0%	10 pg mL^{-1} (E2)	22.7–793 pg mL^{-1} (E2)	[30]
Fish plasma	Centrifugation (20,000 g/10 min) after derivatization		DNS-Cl, 60 °C/10 min	LC–MS/MS, APPI+	E2-d_3 and EE2-d_4	83.5–115.4%	1 ng mL^{-1} (E2 and EE2)	<LOQ-2.6 ng mL^{-1} (E2), <LOQ-5.9 ng mL^{-1} (EE2)	[33]
Rat plasma	LLE, ethyl acetate; centrifugation (2000 g/10 min)	LLE, diethyl ether:hexane (2:1, *v*/*v*); centrifugation (2000 g/10 min)	DNS-Cl, 60 °C/6 min	LC–MS/MS, ESI+	Norgestrel	65.5%	0.196 ng mL^{-1} (EE2)	0.192–59.4 ng mL^{-1} (spiked EE2)	[29]
Human urine	Centrifugation (4000 rpm/5–10 min); Filtration (nylon, 0.45 μm); Ultrasonic degassing	FPSE, sol–gel poly-THF coated extraction media, elution/back-extraction with methanol; Centrifugation (5 min); Filtration (0.22 μm)	–	HPLC-FD	E2 and EE2	90.7–90.9% (E2), 91.0–91.4% (EE2)	20 pg mL^{-1} (E2), 36 pg mL^{-1} (EE2)	1–10 ng mL^{-1} (spiked E2 and EE2)	[42]
Human urine	Dilution in PBS	Aptamer immobilization on vanadium disulfide nanoflowers and Au nanoparticles on modified glassy carbon electrode; exposition to E2 in diluted sample	–	Electrochemical detection, DPV	E2	92.0–105.2% (E2)	1.0 pM (E2)	0.21–5.47 nM (spiked E2)	[48]
Human urine	Urine expressed from diapers using $CaCl_2$; pH adjustment to 4.5	LLE, hexane; centrifugation (3000 rpm/15 min)	DNS-Cl, 50 °C/30 min	LC–MS/MS, ESI+	E2-d_4	92.5–119% (E2), 90.4–122% (EE2)	0.08 μg L^{-1} (E2), 0.02 μg L^{-1} (EE2)	0.34–1.06 ng mL^{-1} (E2), 0.25–1.30 ng mL^{-1} (EE2)	[53]
Milk	LLE, methanol and dichloromethane; centrifugation (2500 g/30 min)		DNS-Cl, 60 °C/5 min	LC–MS/MS, ESI+	E2-d_4	n.a.	0.2 pg mL^{-1} (E2)	1.0–2.4 pg mL^{-1}(E2)	[38]

Milk	Protein precipitation with acetonitrile containing acetic acid; centrifugation (3000 g/15 min)	HF-LPME, 1-octanol	–	HPLC-DAD/FD (diode array and fluorescence detectors connected in series)	E2 and EE2	97–118% (E2), 91–118% (EE2)	1.67–2.50 μg L^{-1} (E2), 3.12–5.70 μg L^{-1} (EE2)	1.7–63 μg L^{-1} (spiked E2), 3.1–142 μg L^{-1} (spiked EE2)	[32]
Goat milk	MSPD, MIP, acetonitrile	Filtration (nylon, 0.45 μm)	–	MEKC–DAD	E2 and EE2	81–83% (E2), 85–93% (EE2)	3.6 μg mL^{-1} (E2), 5.7 μg mL^{-1} (EE2)	<LOQ	[45]
Urine and milk	Protein precipitation with acetonitrile; centrifugation; precipitate ultrasound extraction with acetone	DLLSME, liquid phase and precipitate extract previously mixed	–	HPLC-UV (230 nm)	E2 and EE2	89.2–98.3% (E2), 83.6–94.9% (EE2)	n.a.[a]	<LOD-0.59 μg L^{-1} (E2), <LOD (EE2)	[40]
Milk powder	Protein precipitation with acetone; centrifugation (10,000 rpm/10 min); Filtration (0.22 μm)	Automated SPME, magnetic MIP as fiber coating, 0.4 M acetic acid/methanol as desorption solvent	–	HPLC-UV (280 nm)	E2	81.5–93.3% (E2)	5.0 ng g^{-1} (E2)	20–250 ng g^{-1} (spiked E2)	[43]
Endometrium tissue	Homogenization with ice-cold PBS	LLE, MTBE	–	LC–MS/MS, ESI+/−, APCI+/−, APPI+/−	E2-d_4	n.a.	0.05–2.5 nM (E2)	158 pg mL^{-1} (E2)	[27]
Fish tissue and fish food	Freeze-drying; ASE, dichloromethane, 70 °C/2000 psi	GPC and acetonitrile precipitation	PFBCl	GC–MS, CI−	^{12}C-EE2 and ^{13}C-EE2	74.5–93.7%	n.a.[a]	1.35–8.43 ng g^{-1} (EE2, tissue), 231 ng g^{-1} (EE2, food)	[34,35]
Fish and clams tissues	MSPD, C8, methanol and acetone	SPE, acidic alumina, methanol and acetone; Filtration (PTFE, 0.2 μm) or on-line 2-D LC (RAM, C18)	–	LC–MS/MS, ESI−, APPI−	E2-d_4 and EE2-d_4	81.4–98.5% (E2), 81.1–96.8% (EE2)	0.18 ng g^{-1} (E2), 0.41 ng g^{-1} (EE2)	1.40 ng g^{-1} (E2), <LOQ (EE2)	[25]
Fish liver	Homogenization with water:methanol (1:4, *v*/*v*); LLE, hexane	SPE, C18, acetonitrile:water (70:30, *v*/*v*); SPE, Oasis HLB, methanol; filtration (0.45 μm, PTFE)	–	LC–MS/MS, ESI−	EE2	96–100%	12.3 ng g^{-1} (EE2)	<LOQ	[41]
Bovine hair	Ultrasound extraction with methanol	SPE, NH_2, ethyl acetate; SPE, silica, chloroform and chloroform:ethyl acetate (75:25, *v*/*v*)	DNS-Cl, 60 °C/40 min	LC–MS/MS, ESI+	E2-d_3 and EE2	70–100%	n.a.[a]	2.5–200 ng g^{-1} (estradiol-17-esters)	[31]
Aerial insects (invertebrates)	Homogenization of dried insects with ethyl acetate	GPC	BSTFA, 65 °C/20 min	GC–MS	Diethylstilbestrol	>95%	0.14–1.9 ng g^{-1}	12.6–140.3 ng g^{-1} (EE2)	[36]
Chicken litter	Freeze-drying; Ultrasound extraction with dichloromethane: methanol (2:1, *v*/*v*); Centrifugation (3000 rpm/5 min)	SPE, silica gel, acetonitrile	–	HPLC-FD	E2 and EE2	73% (E2), 57% (EE2)	4.0 μg kg^{-1} (E2), 2.6 μg kg^{-1} (EE2)	60–718.8 μg kg^{-1} (E2), <LOD (EE2)	[37]

LOQ, limit of quantification; LOD, limit of detection; n.a., not available.

[a] LOQ not available. LOD of 0.001 ng mL^{-1} (EE2) [26]; 0.25 μg L^{-1} (E2) and 0.43 μg L^{-1} (EE2) [40]; 0.67 ng g^{-1} (EE2) [34,35]; 1–50 ng g^{-1} (estradiol-17-esters) [31].

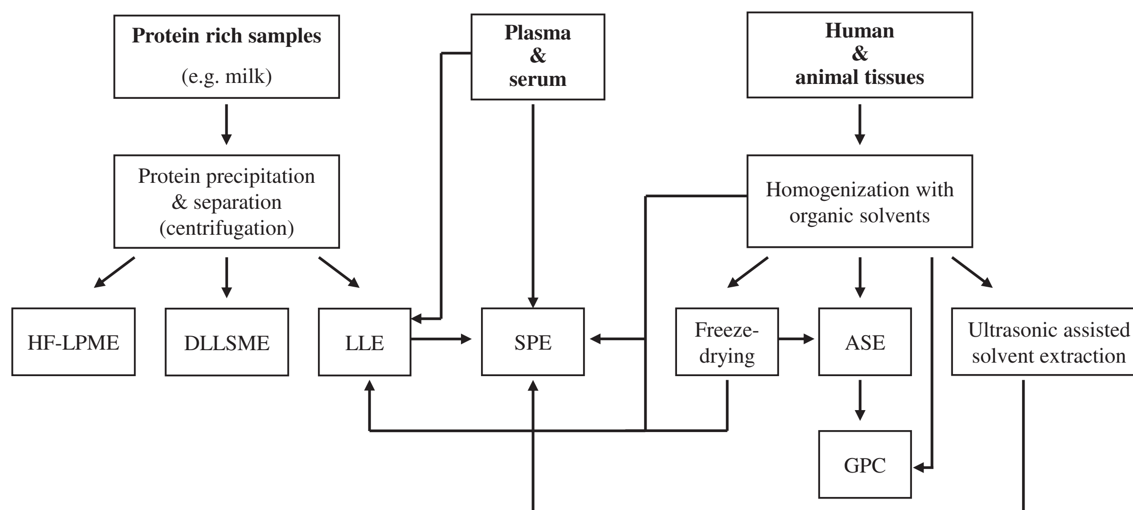

Fig. 2. Schematic representation about sample treatment strategies applied for biological matrices.

are time-consuming and prone to cross reactivity with other analytes which may result in low accuracy and limited sensitivity [28,33,35]. Adding to these drawbacks, the use of antibody-related methods often requires a separate assay for each biomarker, i.e., each estrogen has to be quantified individually. Therefore, more comprehensive methods, namely gas or liquid chromatography coupled to mass spectrometry (GC–MS or LC–MS, respectively), are increasingly becoming the methods of choice to determine estrogens in biological samples. GC–MS and LC–MS, also coupled to tandem mass spectrometry detection (MS/MS), present as major advantages a high specificity, a reduced or no cross reactivity and the possibility of simultaneous analysis of multiple compounds besides E2 and EE2 [30]. Other more recent methods reported included capillary electrophoresis [44], micellar electrokinetic chromatography (MEKC) [45], biosensors [48,49], HPLC with fluorescence detection (HPLC-FD) [37,42], modified electrochemical sensor [50], electrochemiluminescence [51], and chronoamperometry [52].

In fact, several biosensors have been proposed in the last year for application towards biological samples. Label-free aptamer sensor for E2 based on vanadium disulfide nanoflowers and Au nanoparticles and detection by differential pulse voltammetry was applied to spiked urine [48]. A similar approach based on thiol-capped E2 aptamers was implemented, using complementary DNA targeted to unbound E2 aptamer, providing a competitive scheme [51]. Using a recombinant human estrogenic receptor fragment, a biosensor was developed based on the competitive displacement of coumestrol, a fluorescent phytoestrogen [49].

Due to their low molecular weight and low volatility, gas chromatographic analyses of steroid estrogenic hormones often require previous derivatization to increase volatility of the target compounds and therefore improve the detection sensitivity and selectivity. Different derivatization procedures have been applied for the detection of both natural and synthetic estrogens in biological matrices by GC–MS. Al-Ansari et al. [34,35] developed an optimized method for the determination of estrogens in whole fish tissues which included a derivatization step with pentafluorobenzoyl chloride (PFBCl) following sample extraction and clean-up. On the other hand, derivatization with *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and heating at 65 °C for 20 min was proposed by Park et al. [36] for determination of estrogens in aerial invertebrates after homogenization in ethyl acetate and purification by GPC. Nevertheless, derivatization protocols are time consuming and may result in analyte loss [14]. Thus, in recent years, liquid chromatographic methods, which are not limited by analyte volatility and thermolability, have gone through major developments. Indeed, LC-based methods, namely LC–MS/MS, are increasingly becoming the most commonly employed analytical tool for estrogens detection and quantification in real matrices. This fact is depicted by the rising number of published papers reporting the use of these methods for E2 and EE2 analysis in biofluids and tissues [25–31,33,38,41,47,53]. Among the papers that were published between 2009 and 2014 describing the quantification of E2 and/or EE2 in biological samples by chromatography-based techniques, 86% employed LC whereas only 14% used GC analysis. Although mass spectrometry is the most used detection method, diode array (DAD) [32,40] and fluorescence (FD) [32] detectors have also been reported for these analytes.

GC–MS analysis of biological matrices has been generally conducted with chemical ionization (CI) in negative mode [34,35]. In the case of LC–MS techniques, E2 and EE2 can be ionized with electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI), in positive and negative polarity. When using the positive mode, derivatization with dansyl chloride (DNS-Cl) has been described to improve ionization efficiency [26,29–31,33,38,53]. In fact, a recent work combined both ionization modes to determine 12 endogenous estrogens and their intact conjugates in blood and urine, with detection of dansylated estrogens with positive ionization and their intact conjugates with negative ionization [54].

GC–MS methods to determine estrogens in biological matrices [34, 35] often employed the selected ion monitoring (SIM) mode, applied to the quantification ions *m*/*z* 490 for the pentafluorobenzoyl-derivative of EE2 and *m*/*z* 492 for the corresponding internal standard ^{13}C-EE2 derivative. In what concerns LC analysis with tandem MS detection, the precursor to product ion transitions commonly employed for the selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) of natural and synthetic estrogens include the *m*/*z* transitions 271.2 > 145.1 [25,27], 255.3 > 159.2 [27] and 271.1 > 183.0 [25] for E2 and the *m*/*z* transitions 275.4 > 147.0 and 259.3 > 161.2 [25,27] for the deuterated internal standard E2-d_4. For EE2, the *m*/*z* transitions 295.1 > 269.1 [28], 295.0 > 145.0 or 295.0 > 159.0 [25,41] have been applied, while the *m*/*z* transition 299.1 > 273.0 has been used for the correspondent internal standard EE2-d_4 [28]. The majority of these product ions are consistent with a fragmentation pathway that proceeds through ring cleavages and subsequent retrocyclization [23,55]. The precursor ion from E2 originates product ions at *m*/*z* 183, 159 and 145 corresponding to ring cleavages and losses of $C_5H_{12}O$, $C_7H_{12}O$ and $C_8H_{14}O$, respectively, whereas for EE2 the product ions at *m*/*z* 145 and 269 derive from, respectively, the loss of $C_8H_{14}O$ through ring fragmentation or the loss of an acetylene (C_2H_2) group [23,41,55]. Dansylated derivatives are generally monitored at the following ion transitions: *m*/*z* 506.4 > 171.0 for dansylated E2 [30,33,38]; *m*/*z* 509.4 > 171.1 for dansylated ^{13}C-E2 or E2-d_3 [30,33,38]; *m*/*z* 530.0 > 171.0 for dansylated

EE2 [29,31,33] and m/z 534.0 > 171.0 for dansylated EE2-d_4 [33]. The formation of the fragment ion at m/z 171 is consistent with the cleavage of the sulphonyl group from the dansyl moiety [29,31,33].

As estrogens are frequently measured at nanogram levels, it is desirable that the applied analytical methods permit the determination of such low concentrations and therefore provide low limits of detection (LOD) and quantification (LOQ). For solid biological matrices, the LOQs obtained by GC–MS and LC–MS/MS are generally in the ng g^{-1} level whereas for liquid counterparts LOQs of ng mL^{-1} or as low as pg mL^{-1} have been reported (Table 1). When UV or fluorescence detection were employed, higher LOQs were obtained but still in the ng mL^{-1} range.

Another critical point that must be addressed when measuring analytes in real samples is the matrix effect. This is especially important for mass spectrometry detection. The presence of matrix impurities can hamper ionization efficiency and, in co-elution with compounds, originates signal suppression or signal enhancement [11]. Sample clean-up steps and the use of isotopically labeled internal standards such as deuterated compounds normally permit to control the matrix effects. The reported recoveries of E2 and EE2 from biological matrices ranged from 70 to 130%, 90 to 120% and 75 to 100% for plasma, fish tissues and milk samples, respectively (Table 1), fostering their application to complex samples.

Indeed, the application of these methods permitted the assessment of E2 and EE2 concentrations in different biological matrices, sometimes at levels that raise concern for human health and wildlife (Table 1). EE2 was detected at quantities as low as 1 pg mL^{-1} up to 85 ng mL^{-1} in human plasma samples [26,28]. E2 was detected in female serum and endometrium tissue in levels as low as 81 and 158 pg mL^{-1}, respectively [27]. The plasma of fish exposed to estrogens presented E2 levels ranging from < 1 to 3 ng mL^{-1} while reported EE2 levels were slightly higher, ranging from < 1 to 6 ng mL^{-1} [33]. Furthermore, analysis of fish tissues revealed that E2 and EE2 were present at 1 and 1–8 ng g^{-1}, respectively [25,34,35]. According to the work of Park et al. [36], the aerial insects developing at sewage treatment works could present a median EE2 concentration as high as 42.4 ng g^{-1}. On the other hand, E2 was detected in milk samples at low concentrations (1–2.4 pg mL^{-1}) whereas EE2 was not detected [37,38]. Infant urine from diapers has been assessed ($n = 40$, reporting to ten individuals) and values ranging from 0.64–1.63 ng mL^{-1} and 0.21–0.93 ng mL^{-1} were found for E2 and EE2, respectively [53].

3. Determination of E2 and EE2 in environmental matrices

The excretion of steroidal hormones from human populations and also animals is, as already referred, a main contributor to the presence of EDCs in the environment. The majority of produced and released estrogens converge in wastewater treatment plants (WWTP) where their removal is a function of the physicochemical properties of the hormones, the type of treatment/disposal process, the physicochemical parameters prevailing in the system and the microbial activity [8]. Therefore, variable estrogen elimination rates can be achieved (0–90%) [8]. In fact, the conventional wastewater treatment processes may not be able to remove steroid hormones completely, which potentiates the release of these chemicals into the aquatic environment. In recent years, the presence of these compounds in the environment, namely E2 and EE2, has gathered much attention due to their adverse effects on living organisms, even at very low concentrations such as ng L^{-1} [8,44]. In order to monitor the environmental contamination with the estrogens E2 and EE2 and also the potential risk to biota, their occurrence and partitioning in different compartments such as wastewater influents and effluents, receiving surface waters, activated sludge and sediments must be assessed. Most of the published studies report estrogens determination in aqueous samples (wastewaters and surface waters) (Table 2). On the contrary, the presence of estrogens in sludge or sediments, possibly due to matrix complexity, has been largely overlooked and fewer works are described (Table 3) [5,11]. In fact, these two species are accumulated in the particulate matter section of river and waste waters [56]. Furthermore, a schematic representation about sample treatment strategies applied to environmental matrices is summarized in Fig. 3, where the most used techniques are highlighted.

3.1. Sample preparation

3.1.1. Water samples

After collection, and prior to extraction, wastewater and surface water samples are generally acidified and filtered. In most cases, acidification to pH 2–4 is achieved through the addition of HCl or H_2SO_4 solutions [9,16,57,58]. The pH decrease prevents microbial activity hence permitting sample preservation. Sample biodegradation may be also minimized through the addition of sodium azide [22]. In alternative, methanol can be used as conservation agent [14,59]. On the other hand, sample filtration is necessary to remove suspended particles that may interfere with subsequent analytical procedures. Aqueous matrices can be filtered through different materials. Glass fiber is described as the most reliable filter material for the elimination of suspended matter while minimizing analyte losses due to sorption [14] and therefore is referred in the majority of published studies. Different pore-sized glass fiber filters can be used to clean environmental water samples being 0.45, 0.7, 1 and 1.2 μm filters the most frequently described (e.g. [58, 60–63]). Few studies refer water filtration through other materials such as nylon [57,64,65] and cellulose nitrate [20]. Furthermore, consecutive filtrations and the addition of diatomaceous earth are suggested by some authors to avoid filter clogging [13].

Following pre-treatment procedures, the acidified and filtered aqueous samples are spiked with internal standards and may then be extracted by LLE [66] but mostly by SPE, either on cartridges or disks. The internal standards added to environmental samples are similar to those already described for biological matrices (see Section 2.1.). Besides the isotopically labeled E2 and EE2 molecules, other deuterated compounds were applied as internal standard such as the EDC bisphenol A-d_{16} (Table 2), targeting a multianalyte method.

SPE extraction can be performed using different sorbents. In 50 studies reporting estrogens quantification in aqueous matrices, 46% of them have applied Oasis HLB as reversed-phase sorbent (Table 2). In fact, Oasis HLB has been described by several authors as the sorbent that permits the achievement of higher analyte recoveries due to the combination of the hydrophilic N-vinylpyrrolidone and the lipophilic divinylbenzene that permits to retain acidic, basic and neutral compounds, whether polar or non-polar such as E2 and EE2 [67,68]. Ethyl acetate and methanol are the most commonly applied for elution when SPE is performed with this type of sorbent as these solvents are associated with higher E2 and EE2 recoveries [20,22,60,62–65,69–77]. Nevertheless, other solvents and solvent mixtures can be employed such as acetone [78,79], dichloromethane:acetone (70:30, v/v) [58,68], dichloromethane:methanol (50:50, v/v) [12] and methanol:tert-butyl methyl ether (10:90, v/v) [80]. Alternatively, SPE extraction of estrogens has been performed through Strata-X [46,49,56] and styrene divinylbenzene cartridges [81,82], which are also reversed phase polymeric sorbents, and octadecylsilane C18 cartridges [13,34,57,83–88], which are silica-based reversed phase sorbents that permit the strong retention of hydrophobic target analytes. In the latter case, sorbent immobilized in disk format has also been applied [89–91]. Moreover, Chen et al. [25] proposed E2 and EE2 extraction from river water using C8 instead of C18 disks. Disk-type sorbents can be successfully applied in SPE when analyzing feminizing chemicals in water as they allow a flow rate up to 100 mL per minute and reduce sample loading time [25,92]. Elution is mainly performed with the solvents mentioned before for Oasis HLB cartridges. Additionally, hexane [84,86] and acetonitrile [90] may also be applied. Furthermore, one recent study developed an on-line SPE method for the determination of eight selected hormones, including E2 and EE2, in urban wastewaters [14]. The on-

Table 2
Analytical methods for E2 and EE2 determination in environmental aqueous samples using instrumental analysis.

Sample	Steps for sample preparation			Derivatization	Analytical technique	Standard	Recovery rate	LOD/LOQ	Detected values	Reference
Wastewater influent, effluent and river	Acidification	LLE, dichloromethane, hexane and acetone	SPE, Na_2SO_4: florisil: Na_2SO_4 (1:2:1), hexane	MSTFA, 60 °C/15 min	GC–MS	BPA-d_{16}	77.91% (E2), 89.43% (EE2)	0.5 ng L^{-1} (E2), 5 ng L^{-1} (EE2)	0.1–18.6 ng L^{-1} (E2); <LOD-28.6 ng L^{-1} (EE2)	[66]
Wastewater influent and effluent	Filtration (glass fiber, 1.2 μm; nylon, 0.45 μm)	SPE, Oasis HLB, ethyl acetate	SPE, silica gel, ethyl acetate/acetone (2%, *v*/*v*)	MSTFA and pyridine, 60 °C/30 min	GC–MS, EI	BPA-d_{16}	91% (E2), 87% (EE2)	0.4 ng L^{-1} (E2), 2.0 ng L^{-1} (EE2)	1.4–12.7 ng L^{-1} (E2), <2.0 ng L^{-1} (EE2) [64]; 1.5–10.1 ng L^{-1} (E2), <2.0 ng L^{-1} (EE2) [65]	[64,65]
Wastewater	Filtration (glass fiber, 0.7 μm)	SPE, Oasis HLB, methanol		–	LC–MS/MS, APCI	isotope-labeled E2 and EE2	n.a.	0.2 ng L^{-1} (E2 and EE2)	<LOD	[60]
Wastewater influent and effluent	Filtration (glass fiber, 0.7 μm); Acidification (pH 2–3)	SPE, Oasis HLB, ethyl acetate	SPE, Sep-Pak silica, dichloromethane:acetone (70:30, *v*/*v*)	BSTFA (1% TMCS) and pyridine, 70 °C/60 min	GC–MS, EI	E2-d_4 and BPA-d_{16}	76.9–104.6%	1.5 ng L^{-1} (E2), 2.5 ng L^{-1} (EE2)	1.9–32.7 ng L^{-1} (E2), <2.5–44.6 ng L^{-1} (EE2)	[63]
Liquid phase of activated sludge and wastewater	Acidification (pH 3), Filtration (glass fiber, 1 μm)	SPE, Oasis HLB connected to Sep-Pak silica, dichloromethane: acetone (70:30, *v*/*v*)		BSTFA (1% TMCS) and pyridine, 60–70 °C/30 min, under ultrasonication	GC–MS, EI	E2-d_2 and BPA-d_{16}	70–120%	0.8 ng L^{-1} (E2), 4.0 ng L^{-1} (EE2); 1.0 ng L^{-1} (E2), 2.3 ng L^{-1} (EE2)	<LOD (E2); 6.3–6.7 ng L^{-1} (EE2); <LOQ-21.3 ng L^{-1} (E2); <LOQ-125.9 ng L^{-1} (EE2)	[58,68]
Wastewater	Filtration (glass fiber, 0.7 μm); Acidification (pH < 3)	SPE, Oasis HLB, ethyl acetate		BSTFA (1% TMCS) and pyridine, 70 °C/60 min	GC–MS, EI	BPA-d_{16}	>70%	0.08–1.9 μg L^{-1}	5.9–93.0 ng L^{-1} (E2), <LOD-11.53 ng L^{-1} (EE2)	[74]
Coastal and sea water	Filtration (glass fiber, 0.7 μm)	SPE, Oasis HLB, acetone		BSTFA, 70 °C/60 min	GC–MS, EI	E2-d_2	80–96% (E2), 43–57% (EE2)	2 ng L^{-1} (E2), 7 ng L^{-1} (EE2)	<LOD	[78,79]
River water	Addition of sodium azide; Filtration (glass fiber, 0.7 μm)	SPE, Oasis HLB, methanol		BSTFA and pyridine, 60–70 °C/30 min (GC)	GC–MS, EI; GC–MS/MS, EI+; LC–MS/MS, ESI−	E2-d_2, E2-d_4 and EE2-d_4	72–119%	GC–MS: 1.4 ng L^{-1} (E2), 0.8 ng L^{-1} (EE2); GC–MS/MS: 0.3 ng L^{-1} (E2 and EE2); LC–MS/MS: 1.2 ng L^{-1} (E2), 0.4 ng L^{-1} (EE2)	<LOD–3 ng L^{-1} (E2 and EE2)	[71]
Estuarine water	Filtration (glass fiber, 1.2 μm); Acidification (pH 2)	SPE, Oasis HLB, dichloromethane: methanol (50:50, *v*/*v*)	SPE, Sep-Pak silica, dichloromethane:methanol (50:50, *v*/*v*)	MSTFA (GC)	HPLC-DAD; GC–MS, EI	E2 and EE2	87% (E2), 108% (EE2) (HPLC)	7.0 ng L^{-1} (E2); 18.0 ng L^{-1} (EE2) (HPLC)	<LOD (E2); <LOD-101.9 ng L^{-1} (EE2) (HPLC)	[12]
Surface water	Addition of methanol; Acidification (pH 3) Filtration (glass fiber, 0.7 μm)	SPE, Oasis HLB, methanol and dichloromethane	Filtration (0.45 μm)	PFBCl and pyridine, 30 min	GC–MS, CI−	BPA-d_{16} and E1-d_4	135–163% [59]; 75–145% [16]	0.3 ng L^{-1} (E2); 0.21 ng L^{-1} (EE2)	<0.1–7.5 ng L^{-1} (E2); <0.7 ng L^{-1} (EE2)	[16,59]
Wastewater and surface water	Filtration (glass fiber, 0.45 μm); Acidification (pH 3–4)	SPE, Oasis HLB, methanol:MTBE (10:90, *v*/*v*)	SPE, silica gel and aluminum, methanol:acetone (50:50, *v*/*v*)	BSTFA:TMCS:TMSI (99:1:0.5, *v*/*v*/*v*), 60 °C/30 min	GC–MS, EI	E2-d_3 and pyrene-d_{16}	66.6–121.1%	0.02–0.48 ng L^{-1}	<LOD-67.4 ng L^{-1} (E2), <LOD-4100 ng L^{-1} (EE2)	[80]
Estuarine water	Filtration (cellulose nitrate, 0.45 μm)	SPE, Oasis HLB, ethyl acetate		BSTFA (1% TMCS) and pyridine, ultrasound-assisted	GC–MS, EI	E2-d_3	96–112%	0.35 ng L^{-1} (E2), 1.00 ng L^{-1} (EE2)	~1 μg/L (spiked E2 and EE2)	[20]
River and ocean coast water	Filtration (glass fiber, 0.45 μm); Acidification (pH 5)	SPE, Oasis HLB, ethyl acetate		BSTFA (1% TMCS) and pyridine, 65 °C/30 min	GC–MS	E2-d_2 and BPA-d_{16}	n.a.	0.6–0.8 ng L^{-1} (E2), 0.6 ng L^{-1} (EE2)	3.3–5.9 ng L^{-1} (E2), 2.1–4.4 ng L^{-1} (EE2)	[62]
Wastewater effluent and river	Filtration (glass fiber, 0.7 μm)	SPE, Oasis HLB, ethyl acetate		BSTFA (1% TMCS) and pyridine, 60–70 °C/30 min	GC–MS, EI	E2-d_2 and BPA-d_{16}	77.5–94.2% (E2), 72.8–91.4% (EE2)	0.7 ng L^{-1} (E2), 0.5 ng L^{-1} (EE2)	<0.7–22 ng L^{-1} (E2), <0.5–5 ng L^{-1} (EE2)	[75]
Estuarine water	Addition of sodium azide; Filtration (glass fiber, 0.7 μm)	SPE, Oasis HLB, ethyl acetate		BSTFA (1% TMCS) and pyridine, 60–70 °C/30 min	GC–MS/MS, EI+	E2-d_2	75–106%	0.28 ng L^{-1} (E2), 0.27 ng L^{-1} (EE2)	3.1–21.4 ng L^{-1} (E2), 1.5 ng L^{-1} (EE2)	[22]
Wastewater influent and effluent	Filtration (glass fiber); Acidification (pH 3–4)	SPE, Oasis HLB, methanol		BSTFA (1% TMCS), 65 °C/60 min	GC×GC–MS, EI	Anthracene	90–94% (E2), 88–94% (EE2)	1.7–2.0 ng L^{-1} (E2), 6.7–8.6 ng L^{-1} (EE2)	<LOD (E2 and EE2)	[76]

Surface and ground water	Filtration (glass fiber, 0.45 μm); Acidification (pH 3)	SPE, Oasis HLB, dichloromethane/-acetone and methanol	SPE, Sep-Pak silica	–	UPLC–MS/MS, ESI−	E2 and EE2	n.a.	0.01–0.11 ng L^{-1} (E2), 0.01–0.18 ng L^{-1} (EE2)	0.04–1.58 ng L^{-1} (E2), 0.07–0.60 ng L^{-1} (EE2)	[9]
Surface water and wastewater	Filtration (glass fiber, 3 μm; cellulose acetate, 0.45 μm); Acidification (pH 4)	SPE, Oasis HLB, methanol		–	LC–MS/MS, ESI−	E2 and EE2	70–94%	1.0 ng L^{-1} (E2), 2.0 ng L^{-1} (EE2)	6–102 ng L^{-1} (E2), <LOQ-24 ng L^{-1} (EE2)	[73]
Wastewater and river	Filtration (glass fiber, 1 μm)	SPE, Oasis HLB connected to Sep-Pak NH$_2$, methanol (free estrogens) and 0.5% NH$_4$OH in methanol (conjugated estrogens)		–	UPLC–MS/MS, ESI−	E2-d$_3$ and EE2-d$_4$	63–127%	0.5 ng L^{-1} (E2 and EE2)	1.4–77.2 ng L^{-1} (E2), <LOD (EE2)	[72]
Wastewater effluent	Filtration (glass fiber, 0.7 μm)	SPE, Oasis HLB, methanol		–	LC–MS/MS, ESI−	E2-d$_4$ and EE2-d$_4$	89–106%	1.2 ng L^{-1} (E2), 0.4 ng L^{-1} (EE2)	1.3 to 4.6 ng L^{-1} (E2), <0.4 to 1.3 ng L^{-1} (EE2)	[69]
Wastewater effluent and river water	Addition of sodium azide; Filtration (glass fiber, 0.7 μm)	SPE, Oasis HLB, methanol		–	LC–MS/MS, ESI−	E2-d$_2$	n.a.	1.2 ng L^{-1} (E2), 0.4 ng L^{-1} (EE2)	<1.2–7.6 ng L^{-1} (E2) <0.4–1.9 ng L^{-1} (EE2)	[70]
Wastewater	Filtration (glass fiber, 0.7 μm; celite)	SPE, ENVI-18, acetone and methanol		PFBCl	GC–MS, CI−	^{13}C-E2 and ^{13}C- EE2	n.a.	24.3 ng L^{-1} (E2), 0.5 ng L^{-1} (EE2)	24.7–66.9 ng L^{-1} (E2), 0.5–9.8 ng L^{-1} (EE2)	[13]
Water from tanks with goldfish	Filtration (glass fiber, 1.2 μm)	SPE, ENVI-18, methanol		PFBCl	GC–MS, CI−	^{13}C-EE2	99%	0.36 ng L^{-1} (EE2)	134.9–145.1 ng L^{-1} (EE2)	[34]
Wastewater	Acidification (pH < 3); Filtration (filter paper, 0.65 μm; membrane filter, 0.45 μm)	SPE, C18, methanol		–	LC–MS/MS, ESI−	E2 and EE2	89–91% (E2), 79–96% (EE2)	1.2–3.3 ng L^{-1} (E2), 0.9–2.8 ng L^{-1} (EE2)	3.5–16.3 ng L^{-1} (E2), <LOD-9.3 ng L^{-1} (EE2)	[88]
Estuarine water	Filtration (cellulose acetate, 24.8 μm; glass fiber, 1.2 μm); Acidification (pH 3)	SPE, C18, ethyl acetate		–	LC–MS, ESI−	E2 and EE2	94.7% (E2), 92.1% (EE2)	3.6 ng L^{-1} (E2), 4.7 ng L^{-1} (EE2)	<LOD-62.6 ng L^{-1} (E2), 5.6–63.8 ng L^{-1} (EE2)	[87]
Water from Drinking Water Treatment Plant	Filtration (glass fiber)	SPE, C18, methanol: ethyl acetate (1:5, *v*/*v*)	SPE, florisil, dichloromethane:acetone (90:10, *v*/*v*)	–	LC–MS/MS, ESI−	EE2-d$_4$	n.a.	n.a.	15–7380 ng L^{-1} (spiked EE2)	[85]
Lake water	Filtration (glass fiber, 0.45 μm)	SPE, C18, methanol, diethyl ether, hexane		BSTFA (1% TMCS) and pyridine, 70 °C/30 min	GC–MS	E2 and EE2	70.3–104.7%	0.13 ng L^{-1} (E2 and EE2)	0.96–4.20 ng L^{-1} (E2), 1.93–16.37 ng L^{-1} (EE2)	[86]
Surface water	Filtration (nylon, 0.45 μm); Acidification (pH 5)	SPE, LiChrolut RP-18, methanol and 2% NH$_4$OH in methanol		–	LC–MS/MS, ESI−	E2 and EE2	>80%	1.22 ng L^{-1} (E2), 1.51 ng L^{-1} (EE2)	<LOD (E2 and EE2)	[57]
Kraft pulp mill effluent	Filtration (glass fiber, 0.4 μm)	SPE, C18, methanol, ethyl acetate and n-hexane		–	GC–MS	n.a.	n.a.	n.a.	0.020–0.080 μg L^{-1} (sterol and phenol compounds)	[84]
Wastewater effluent and river water	Filtration (PVDF, 0.45 μm)	SPE, C18, methanol:dichloromethane (50:50, *v*/*v*)	Filtration (PTFE, 0.2 μm)	DNS-Cl or PFBBr 60 °C/30 min	UPLC–MS/MS, ESI+/−, APCI+/−, APPI+/−, APCI/APPI+/−	E2-d$_4$ and EE2-d$_4$	75–80%	0.45–0.81 ng L^{-1} (E2), 0.52–0.91 ng L^{-1} (EE2)	1.8 ng L^{-1} (E2), 2.8 ng L^{-1} (EE2) (mean values)	[89]
Underground well, tap water and lake	Acidification (pH 6)	SPE, C18 disk, acetonitrile		–	Electrochemical detection with carbon nanotube electrodes, CV	EE2	94–105%	120 nM (EE2)	1–50 μM (spiked EE2)	[90]
Drinking water and wastewater	Addition of 1% formaldehyde; Centrifugation (9400 rpm/30 min, 4 °C)	SPE, C18 disk or cartridges, acetonitrile		–	HPLC-DAD; HPLC-FD	E2 and EE2	81–93% (E2), 82–96% (EE2)	DAD: 6.3–9.0 ng L^{-1} (E2), 5.3–7.3 ng L^{-1} (EE2); FD: 2.1–7.5 ng L^{-1} (E2), 1.0–6.3 ng L^{-1} (EE2)	DAD: 16–200 ng L^{-1} (E2 and EE2); FD: 3–200 ng L^{-1} (E2 and EE2),	[114]
				–	HPLC–MS/MS, negative ionization mode	E2-d$_4$ and EE2-d$_4$	n.a.	0.16–0.21 ng L^{-1} (E2), 0.16–0.17 ng L^{-1} (EE2)	0.48–200 ng L^{-1} (E2 and EE2),	
Surface water	Acidification	SPE, fiber filter/C18/SDB-XC disks, methanol, acetone and	Extracts drying by Na$_2$SO$_4$; SPE, silica, dichloromethane	BSTFA 80 °C/30 min	GC–MS, EI	4,4′-difluorobiphenyl and decachlorobiphenyl	85.2% (E2), 83.6% (EE2)	0.28 ng L^{-1} (E2), 0.47 ng L^{-1} (EE2)	0.35–3.76 ng L^{-1} (E2), 2.10–2.43 ng L^{-1} (EE2)	[91]

(*continued on next page*)

Table 2 (*continued*)

Sample	Steps for sample preparation			Derivatization	Analytical technique	Standard	Recovery rate	LOD/LOQ	Detected values	Reference
		dichloromethane								
River water	Filtration (PVDF, 0.45 μm)	SPE, C8, methanol and acetone	Filtration (PTFE, 0.2 μm)	–	UHPLC–MS/MS, ESI−, APPI−	E2-d_4 and EE2-d_4	78.6–105% (E2), 65.9–74.7% (EE2)	1.27 ng L^{-1} (E2), 3.70 ng L^{-1} (EE2)	<LOD	[25]
Wastewater influent and effluent	Filtration (glass fiber, 0.3 μm)	On-line SPE, two Hypersil Gold C18 columns in tandem, methanol and 0.1% formic acid in water		–	LC–MS/MS, APCI+	^{13}C-E2	71–95%	21–24 ng L^{-1} (E2), 18–21 ng L^{-1} (EE2) (complete SPE–LC–MS/MS method)	<LOD-74 ng L^{-1} (E2), <LOD (EE2)	[14]
Wastewater effluent and surface water	Filtration (glass fiber, 1 μm)	SPE, styrene divinyl benzene, dichloromethane	GPC	–	LC–MS/MS, ESI−	E2-d_4 and EE2-d_4	n.a.	0.05 ng L^{-1} (E2 and EE2)	0.08–2.32 ng L^{-1} (E2), 0.06–1.61 ng L^{-1} (EE2)	[82]
Wastewater	Filtration (cellulose, 125, 11 and 0.45 μm)	MASE, polyethylene membranes, chloroform	SPE, florisil, dichloromethane:ethyl acetate:methanol (40:40:20, *v*/*v*); Filtration (PTFE, 0.2 μm)	–	LC–MS/MS, ESI−	E2-d_3	96–115% (E2), 58–123% (EE2)	3 ng L^{-1} (E2), 100 ng L^{-1} (EE2)	<LOD-84 ng L^{-1} (E2), <LOD (EE2)	[102]
Water from Drinking Water Treatment Plant	POCIS, 80:20 (*w*/*w*) Isolute ENV+ and Ambersorb 1500 carbon dispersed on S-X3 Bio Beads, tetrahydrofuran: methanol: acetone (40:30:30, *v*/*v*/*v*)			–	LC–MS/MS, ESI−	BPA-d_{16}	n.a.	0.3 ng mL^{-1} (E2), 0.4 ng mL^{-1} (EE2)	<LOD (E2 and EE2)	[23]
Wastewater effluent and river water	POCIS, 80:20 (*w*/*w*) Isolute ENV+ and Ambersorb 572 carbon dispersed on S-X3 Bio Beads, methanol:toluene: dichloromethane (10:10:80, *v*/*v*/*v*) and dichloromethane			TBDMSCl, 60 °C/30 min	GC-MS, EI	E2-d_3	96–99%	0.5 pg mL^{-1} (E2), 10 pg mL^{-1} (EE2)	13–36 ng L^{-1} (E2), <LOD (EE2)	[93]
River water	Filtration (PVDF, 0.45 μm)	On-column SPE, MIP, methanol:acetic acid (90:10, *v*/*v*)		-	HPLC-DAD	EE2	75% (EE2)	8 μg L^{-1} (EE2)	8–200 μg L^{-1} (spiked EE2)	[95]
River and tap water	Filtration (0.45 μm)	DLLME-SFO, 1-undecanol (extraction solvent), methanol (dispersive solvent)		-	UPLC-DAD	E2 and EE2	87–116%	0.8 to 2.7 μg L^{-1} (river water), 1.4 to 3.1 μg L^{-1} (spiked tap water)	3–5 μg L^{-1} (spiked E2), 4–6 μg L^{-1} (spiked EE2)	[96]
Real water		DLLME-SFO		*p*-nitrobenzoyl chloride, 35 °C/20 min	HPLC-FD	E2 and EE2	89.1–129.4% (E2), 72.8–125.9% (EE2)	0.005–0.5 μg L^{-1}	n.a.	[97]
Real water		SPE-DLLME, carbon nanotubes, methanol		Derivatizing reagent, 40 °C/25 min	HPLC-FD		83.13–122.39%	0.13–6.33 ng L^{-1}	<LOQ	[100]
Mineral water, run-off and wastewater	Filtration (polyester, 0.2 μm); Acidification (pH 3); NaCl addition (30%, *w*/*v*)	DLLME, chloroform (extraction solvent), acetonitrile (dispersive solvent)		–	MEKC-MS, ESI−	E2 and EE2	56–91% (E2), 57–79% (EE2)	0.55–0.92 μg L^{-1} (E2), 0.41–0.91 μg L^{-1} (EE2)	<LOD	[98]
Wastewater, ground water, drinking water and river water	Filtration (filter paper, 11 μm; nylon, 0.45 μm); Ultrasonic degassing	FPSE, sol–gel poly-THF coated extraction media, elution/back-extraction with methanol	Centrifugation (5 min); Filtration (0.22 μm)	–	HPLC-FD	E2 and EE2	89.4–97.4% (E2), 89.0–98.0% (EE2)	20 pg mL^{-1} (E2), 36 pg mL^{-1} (EE2)	1–10 ng mL^{-1} (spiked E2 and EE2)	[42]
River water	Filtration (filter paper; glass fiber, 1.5 μm);	EE2 extraction with anti-EE2 antibodies		–	Electrochemical detection with	EE2	96–105% (EE2)	0.01 ng L^{-1} (EE2)	2.07–12.07 ng L^{-1} (EE2)	[21]

	pH adjustment to 7	immobilized on MPs		carbon nanotube electrodes, cyclic voltammetry, SWV					
Mineral water, lake water, wastewater	Filtration (0.45 μm)	Synthesis and immobilization of modified estrogens on MPs; competition of free and immobilized estrogens for antibodies in solution	–	Electrochemical immunosensor-s-screen-printed electrodes, SWV	E2 and EE2	80–140%	1 ng L^{-1} (E2), 10 ng L^{-1} (EE2)	0.7–74 ng L^{-1} (E2), 15.5–18.8 ng L^{-1} (EE2)	[104]
Tap and sea water	Filtration (0.22 μm)	Piezoelectric biosensor: thiol-labeled BPA immobilization on sensor surface modified with Au nanoparticles; sample incubation with human estrogen receptor (ER-α); competitive binding assay (ER-α binds to thiol-BPA)	–	Piezoelectric biosensor: Frequency change monitoring before and after the immune--reaction (unbounded ER-α detected by the biosensor)	E2	94.7–107.8% (E2)	2.6 nM (0.71 ng mL^{-1}) (E2)	4.73–5.49 nM (spiked E2)	[119]

LOQ, limit of quantification; LOD, limit of detection; n.a., not available.

line SPE system consisted of a sample delivery system and a dual switching-column array. The sample delivery system was an autosampler used for in-loop sample injection and a quaternary pump used to load the SPE column with the content of the sample loop. Online SPE was performed with two Hypersil Gold aQ C18 columns (20 × 2 mm, 12 μm particle size) in tandem. This on-line approach was coupled to a LC–MS/MS system permitting to quantify the target compounds with reduced analysis time per sample (<15 min), good precision and low limits of detection (ng L^{-1} levels) [14].

The majority of published studies on estrogens monitoring in environmental waters are based on the collection of discrete samples at a specific time point [11,93]. In alternative, passive sampling strategies involving the continuous collection of analytes *in situ* have been designed. These approaches permit the determination of time-weighted average (TWA) concentration of pollutants over extended sampling periods and their pre-concentration thus increasing the ability to detect analyte trace amounts [23]. Among passive samplers, polar organic chemical integrative samplers (POCIS), originally developed to sequester and concentrate polar organic chemicals, have been successfully applied to extract E2 and EE2 from water treatment plants samples and river water [23,93]. POCIS samplers are in general constituted by a sorbent phase sandwiched between two microporous polyethersulfone membranes. In the two referred studies, a triphasic adsorbent comprising a 80:20 (*w*/*w*) mixture of the sorbents Isolute ENV+ and Ambersorb dispersed on S-X3 Bio-Beads was employed. Isolute ENV+ is a hydroxylated polystyrene–divinylbenzene resin able to extract organic compounds based on non-polar interactions whereas Ambersorb is a carbonaceous matrix used for adsorption extraction of both polar and non-polar analytes. S-X3 Bio-Beads are porous styrene–divinylbenzene copolymer beads commonly applied for size exclusion separation [23,93,94]. The application of POCIS samplers to water analysis has permitted the extraction of E2 and EE2 with high recoveries (96 and 99%, respectively) [93].

The selectivity of solid phase extraction techniques may be potentiated through the development of sorbents based on molecularly imprinted polymers (MIPs). Bravo et al. [95] developed a MIP imprinted with EE2 for the extraction of this estrogen from river water samples. This was accomplished using methacrylic acid as functional monomer, ethyleneglycol dimethacrylate as crosslinker, EE2 as template and acetonitrile as the solvent. The synthesized MIPs were subsequently applied for on-column solid phase extraction of EE2 with methanol:acetic acid (90:10, *v*/*v*) elution. The developed on-column MIP procedure permitted to successfully extract EE2 from river water with a pre-concentration/enrichment factor of 30-fold.

Dispersive liquid–liquid microextraction based on the solidification of a floating organic drop (DLLME-SFO) has also been proposed for E2 and EE2 analysis in real water samples [96,97]. The method described by Chang and Huang [96] for water samples utilizes 1-undecanol and methanol as extraction and dispersive solvents, respectively, while chloroform/acetonitrile [98] and chlorobenzene/acetone [99] have been applied for similar purpose. Furthermore, solid-phase extraction can be combined with DLLME for the selective determination of estrogens in complex matrix samples as reported for the analysis of E2 and EE2 from aqueous matrices [100]. Online μLLE has been implemented using a supramolecular solvent that was later trapped in an inline filter, from where it was eluted and directed to HPLC [101]. Membrane-assisted solvent extraction has also been proposed as a technique providing low matrix effect for multiresidue analysis, including E2 and EE2 [102].

Magnetic particles (MPs) can be employed as a support for the capture and pre-concentration of analytes and have been attracting great interest in the scientific community. The surface of these particles is modified with different biological materials such as antibodies which, due to the specificity of the antigen-antibody binding, permit their application as a bioseparation tool [21]. Xin et al. [103] used an anti-E2 antibody and MPs to determine E2 in river, waste, and tap waters whereas Martínez et al. [21] developed an analytical method for EE2 extraction from river water samples using anti-EE2 antibodies previously immobilized on MPs. More recently, Kanso et al. [104] described the synthesis and immobilization of modified estrogens on MPs for the detection of E2 and EE2 in wastewaters and surface water samples.

3.1.2. Sludge and sediments

Activated sludge and sediments are highly complex solid matrices which represents an increased difficulty for estrogens determination. Highly efficient pre-treatment procedures are therefore mandatory to minimize matrix interferences and concentrate the analyte trace amounts (ng g^{-1}) generally present. In most cases, sediment and sludge samples are freeze-dried and sieved before extraction [15,22,63,68,79,105]. Nevertheless, sediment samples can be simply air dried [91] (Table 3).

After the addition of the adequate internal standards, the dried samples can be extracted through different techniques. The majority of available extraction methods were already referred for biological samples (see Section 2.1.). Accelerated solvent extraction (ASE) and ultrasonic assisted solvent extraction are the procedures most commonly applied. Several authors have reported an efficient extraction of estrogens from environmental matrices using ASE with a solvent mixture of methanol and acetone (1:1, *v*/*v*), at 75–80 °C and 1500 psi [15,63,105]. Two other studies also used ASE for estrogens concentration but with different solvent mixtures and/or temperature and pressure conditions such as dichloromethane:acetone (1:1, *v*/*v*) [91] or acetone:n-heptane (1:1, *v*/*v*) at 120 °C and 118 bar/1700 psi [17].

Ultrasonic liquid extraction of sediments and sludge is often performed with the same solvent mixtures described for ASE, namely methanol:acetone (1:1, *v*/*v*) [68] and dichloromethane:acetone (1:1, *v*/*v*) [106]. Ethyl acetate is also a possible option for ultrasonic assisted solvent extraction of sediment matrices [16]. Moreover, one study reported the sequential ultrasonic extraction of suspended particulate matter and sediments using first ethyl acetate and then a mixture of methanol:acetone (1:1, *v*/*v*) [79]. Ultrasonication times may vary from 15 to 20 min and are generally followed by a centrifugation step to remove suspended particles from the extract before purification [11]. Other extraction techniques described to extract and enrich E2 and EE2 include microwave accelerated extraction (MAE) using methanol, 110 °C and 200 psi [22]. In comparison with classic methods described for extraction of EDCs from sediments such as acid digestion, liquid extraction and Soxhlet extraction, the major benefits of MAE are low solvent volume, reduced extraction time, complete decomposition of organic matter and possibility of multiple sample extractions [22]. Matrix solid-phase dispersion (MSPD) with C8 adsorbent and elution with methanol and acetone has also been proposed by Chen et al. [25] to extract and concentrate feminizing chemicals from sediments (see Section 2.1.).

After ASE, ultrasonication or MAE, the obtained organic solution can be evaporated, diluted in ultrapure water and further extracted by SPE through Oasis HLB cartridges [22,63,68,105]. The analytes are eluted using solvent or solvent mixtures previously outlined for aqueous samples (Table 3).

As already mentioned for biological samples (see Section 2.1.), sample extracts must be in most cases further purified before analytical determination. Sediment and sludge extracts have to be necessarily cleaned up due to the high loading of organic matter such as humic substances and pigments that, if not removed, can cause severe interferences in compound identification and quantification [22]. Likewise, regarding aqueous samples, purification is especially important in wastewaters where the higher dissolved organic matter content may limit analyte recoveries.

Among the available approaches, SPE is the most widely applied for extracts clean-up, both for liquid and solid matrices. Some authors [66,91] have proposed an initial clean-up step after analytes extraction and prior to SPE through silica gel or florisil cartridges that consists on the

Table 3
Analytical methods for E2 and EE2 determination in environmental solid samples using instrumental analysis.

Sample	Steps for sample preparation			Derivatization	Analytical technique	Standard	Recovery rate	LOD/LOQ	Detected values	Reference
Sludge from three WWTPs	Freeze-drying and sieving	Mix with Na_2SO_4; ASE, methanol:acetone (1:1, *v*/*v*), 75 °C/1500 psi; Filtration (glass fiber, 0.7 μm); SPE, Oasis HLB, methanol	SPE, NH_2, ethyl acetate:methanol (4:1, *v*/*v*)	MSTFA, 65 °C/30–40 min	GC-MS, EI	E2-d_4 and EE2-d_4	79–132%	1–2 ng g^{-1} (E2), 1–3 ng g^{-1} (EE2)	10–13 ng g^{-1} (E2), <**3**–5 ng g^{-1} (EE2)	[105]
Sludge from WWTPs	Freeze-drying and sieving	ASE, methanol:acetone (1:1, *v*/*v*), 75 °C/1500 psi; SPE, Oasis HLB, ethyl acetate	SPE, silica and alumina, methanol: acetone (1:1, *v*/*v*)	BSTFA (1% TMCS) and pyridine, 70 °C/60 min	GC-MS, EI	E2-d_4 and BPA-d_{16}	77.2–118.3%	0.3–2.0 ng g^{-1}	2.3–8.2 ng g^{-1} (E2), 11.8–61.0 ng g^{-1} (EE2)	[63]
Activated sludge and sediment	Freeze-drying	ASE, methanol:acetone (1:1, *v*/*v*), 80 °C/1500 psi	4 clean-up steps: LLE, florisil clean-up, aqueous alkali extraction and HLB enrichment	–	LC–MS/MS, ESI−	E2 and EE2	88–97% (sludge), 75–100% (sediment)	0.05 ng g^{-1} (E2), 0.1 ng g^{-1} (EE2)	0.12–7.1 ng g^{-1} (E2),<LOD-0.7 ng g^{-1} (EE2)	[15]
Sediment	Air-drying	ASE, dichloromethane: acetone (1:1, *v*/*v*)	Extracts drying by Na_2SO_4; SPE, silica, dichloromethane	BSTFA 80 °C/30 min	GC-MS, EI	4,4′-difluorobiphenyl and decachlorobiphenyl	71.5% (E2), 68.7% (EE2)	0.05 μg kg^{-1} (E2), 0.16 μg kg^{-1} (E2)	0.05–2.63 ng g^{-1} (E2), 0.15–0.45 ng g^{-1} (E2)	[91]
Sediment		ASE, acetone:*n*-heptane (1:1, *v*/*v*), 120 °C/118 bar		–	LC–MS, APCI	EE2	n.a.	1 ng mL^{-1}	0.48–1.48 μg kg^{-1} (E2)	[17]
Sediment	Freeze-drying and sieving	MAE, methanol, 110 °C/200 psi	SPE, Oasis HLB, ethyl acetate	BSTFA (1% TMCS) and pyridine, 60–70 °C/30 min	GC-MS/MS, EI+	E2-d_2	86–102%	0.06 ng g^{-1} (E2), 0.14 ng g^{-1} (EE2)	<LOD-11.2 ng g^{-1} (E2), <LOD (EE2)	[22]
Coastal sediment	Ultrasonic assisted solvent extraction, dichloromethane: acetone (1:1, *v*/*v*)	SPE, silica, methanol		BSTFA (5% TMCS and pyridine 1:1, *v*/*v*), 60 °C/30 min	GC-MS, EI	E2 and EE2	84%	n.a.	0.06–16.81 ng g^{-1} (E2), 4.18–48.14 ng g^{-1} (E2)	[106]
Sediment	Addition of sodium azide; Freeze-drying and sieving	Ultrasonic assisted solvent extraction, ethyl acetate	SPE, silica, ethyl acetate	PFBCl and pyridine, 30 min	GC-MS, CI−	BPA-d_{16} and E1-d_4	75–106%	1 ng g^{-1} (E2), 0.75 ng g^{-1} (EE2)	<3.5 ng g^{-1} (E2), <LOD (EE2)	[16]
Sediment and suspended particulate matter (marine environment)	Freeze-drying	Ultrasonic assisted solvent extraction, ethyl acetate and methanol:acetone (1:1, *v*/*v*)	SPE, florisil, ethyl acetate or acetone	BSTFA, 70 °C/60 min	GC-MS, EI	E2-d_2	60–127%	5 ng g^{-1} (E2 and EE2)	<LOD	[79]
Solid phase of activated sludge	Freeze-drying	Ultrasonic assisted solvent extraction, methanol:acetone (1:1, *v*/*v*); Acidification (pH 3) and filtration (glass fiber, 1 μm); SPE, Oasis HLB, dichloromethane: acetone (70:30, *v*/*v*)	SPE, neutral Al_2O_3/silica gel, methanol:acetone (1:1, *v*/*v*)	BSTFA (1% TMCS) and pyridine, 60–70 °C/30 min, under ultrasonication	GC–MS, EI	E2-d_2 and BPA-d_{16}	71.3–123.5%	1.2 ng g^{-1} (E2), 10.0 ng g^{-1} (EE2)	<LOD	[68]
Sediment	MSPD, C8, methanol and acetone	SPE, acidic alumina, methanol and acetone; Filtration (0.2 μm, PTFE) or on-line 2-D LC (RAM, C18)		–	LC–MS/MS, ESI−, APPI−	E2-d_4 and EE2-d_4	89.2–91.8% (E2), 86.3–90.4% (EE2)	0.096 ng g^{-1} (E2), 0.11 ng g^{-1} (EE2)	<LOD	[25]

LOQ, limit of quantification; LOD, limit of detection; n.a., not available.

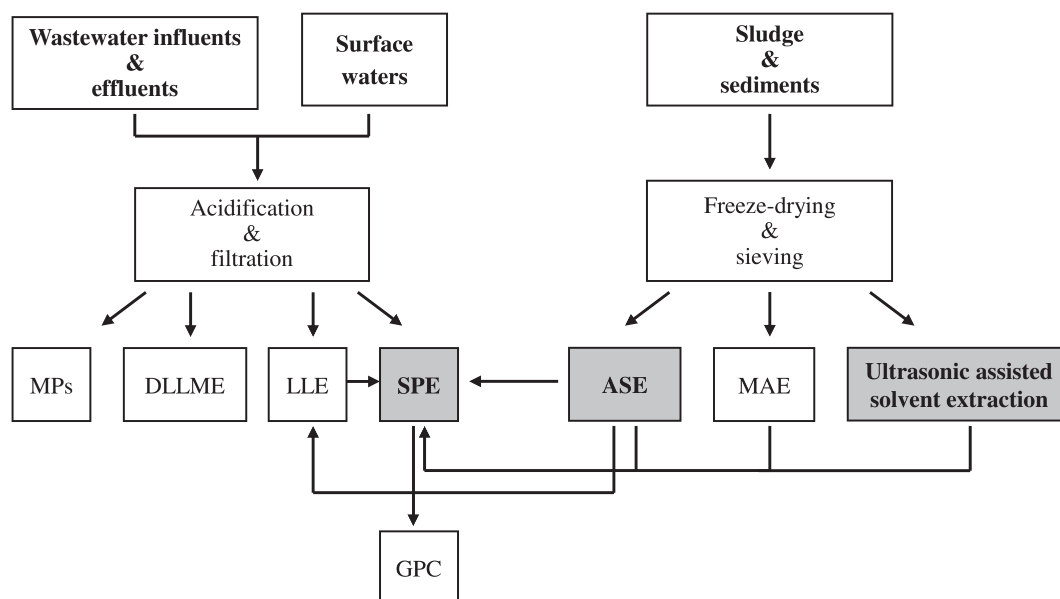

Fig. 3. Schematic representation about sample treatment strategies for environmental matrices. The most used techniques are highlighted in gray.

addition of Na_2SO_4 to dry the organic extracts. SPE purification has been performed in most cases through silica gel sorbent [9,12,16,63–65,91], a normal phase sorbent that permits the clean-up of E2 and EE2 organic extracts by adsorption and subsequent discard of unwanted polar compounds. Moreover, silica has been employed in combination with aminopropyl residue [72,105], which results in a stationary phase with basic character, and neutral [63,68,80] or acidic alumina [25]. The polar functionalized aminopropyl bonded silicas, being more hydrophilic than silica, increase the sorbent potential to adsorb polar interferents from the non-polar E2 and EE2 in extracts. Likewise, neutral and acidic alumina are polar adsorption media containing aluminum oxide used for normal phase separations but can also be used as ion exchangers to separate impurities from E2 and EE2 in organic extracts. Other SPE sorbents such as the extremely polar magnesium-silicate based florisil [66,79,85] and the reversed phase C18 [103] have also been employed in environmental extracts purification. Silica gel or bonded silica cartridges have been applied independently or connected to the SPE cartridge used for analytes extraction [58,68,72]. Different solvents and solvent mixtures have been used for elution of E2 and EE2 from clean-up cartridges (Tables 2 and 3). Ethyl acetate [16,79], methanol [72], dichloromethane [91], acetone [79] and mixtures containing these organic solvents [12,58,63–65,68,80,106] were described for the elution of silica gel sorbents whereas alumina cartridges were generally eluted with methanol:acetone (50:50, *v*/*v*) [25,63,68,80]. The three studies describing extracts clean-up through florisil cartridges applied hexane [66], ethyl acetate [79], acetone [79] or dichloromethane:acetone (90:10, *v*/*v*) [85] as eluents. When C18 bonded reversed phase silica was employed, E2 was eluted with the mixture ethyl acetate:methanol (5:1, *v*/*v*) [103].

Recently, Chen et al. [15] designed a multi-clean up procedure for estrogens determination in samples possessing high matrix interferences, namely sludge and sediments. The developed procedure was composed of four clean-up steps: liquid–liquid extraction (LLE), clean-up using florisil, aqueous alkali extraction (AAE) and hydrophilic–lipophilic balance (HLB) enrichment. The AAE clean-up step permitted to remove the organic interferents that, due to the similar polarity, were not separated from the target analytes by LLE and florisil SPE. After optimization, and in comparison with samples that were not purified, this multi-step strategy decreased the matrix interference effects on E2 and EE2 from > 80% to 0.5–7.5% and from 60% to absent in sludge and sediments, respectively. In consequence, it was possible to achieve higher estrogens recoveries: 88–97% in activated sludge samples and 75–100% in sediment samples.

Despite the wide application of SPE, other techniques can be applied for E2 and EE2 extracts purification. GPC has been used by Williams et al. [82] to clean the organic extracts of sewage treatment plant effluents and receiving waters samples before analysis. This clean-up procedure removed the matrix impurities with higher molecular weight [11]. On the other hand, the on-line two-dimensional liquid chromatography proposed by Chen et al. [25] for the clean-up of fish and clams tissue extracts (see Section 2.1.) has also been applied with success to sediment matrices.

3.2. *Quantification of E2 and EE2*

3.2.1. *Instrumental methods*

The detection and quantification of estrogens in environmental samples rely on either instrumental or biological methods. Until now, numerous methods have been developed and subsequently optimized for E2 and EE2 determination in this type of matrix, and most of them are chromatography based methodologies. As already observed for biological matrices (Section 2.2.), both gas and liquid chromatography coupled to mass spectrometry were preferably applied (75%). Gas chromatography (GC) coupled with mass spectrometry (MS) and tandem MS was the first to be developed and is still widely used, a fact easily confirmed by the higher number (43%) of published studies reporting the application of GC–MS in environmental samples monitoring (Tables 2 and 3). In most cases, GC–MS analysis of both aqueous and solid (sludge or sediment) matrices has been performed with electron ionization (EI) and SIM mode for quantification. Chemical ionization (CI) can also be employed for estrogens determination in the environment and has been reported by some authors [13,16,34,59]. As previously referred, GC-based techniques usually demand for sample derivatization to suit the chromatographic behavior of the target analytes. Different procedures have been proposed for estrogens derivatization in environmental extracts. Derivatization with the addition of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% of trimethylchlorosilane (TMCS) and pyridine and heating at 60–80 °C for 30–60 min was the procedure selected in most studies (Tables 2 and 3). When compared with other protocols, these derivatizing

conditions have been associated with higher silylation power and more satisfactory sensitivity and selectivity [20,68].

Despite this, other reagents were used for estrogens derivatization in environmental samples such as *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) [12,64-66,105], pentafluorobenzoyl chloride (PFBCl) [13,16,34,59] and *tert*-butyldimethylsilyl chloride (TBDMSCl) [93]. As derivatization procedures are frequently time consuming, Vallejo et al. [20] proposed their optimization through the use of ultrasonication in cup horn boosters (miniaturized ultrasound baths) that permitted to reduce the reaction time from 30–60 min to values as low as 1 or 10 min. Moreover, the application of ultrasonication to derivatization has been associated with increased recovery and sensitivity of the developed analytical method [58,68]. The LODs of E2 and EE2 obtained with the ultrasound assisted derivatization and GC–MS method developed by Vallejo et al. (0.35–6.1 ng L^{-1} [20]) were comparable or even with high analyte recoveries, ranging from 96 to 112%. In other study, Nie et al. [68] observed that ultrasonication increased significantly the analytical signal of EE2 derivatives by 44%.

Out of the numerous studies analyzing estrogenic compounds in environmental matrices by GC, only two employed tandem MS detection, operated with positive electron ionization and in multiple reaction monitoring mode for quantitative analyses [22,71]. Both studies described sample extracts derivatization with BSTFA + pyridine and reconstitution in hexane followed by 1 μL sample injections in splitless mode for GC–MS/MS analysis. The molecular ions of E2, EE2 or corresponding derivatives have been frequently selected as the quantification ions for GC–MS analysis of environmental matrices. The ditrimethylsilyl derivative of E2, originated through silylation of both aliphatic and aromatic hydroxyl groups, has been quantified using the molecular ion at *m/z* 416, while the ion at *m/z* 418 has been applied for the corresponding internal standard E2-d_2 derivative [63,66,68]. The fragmentation of the parent ion at *m/z* 416 gives origin to the daughter ion at *m/z* 285 that has also been used for E2 quantification [63,66,71]. In fact, the *m/z* transition 416 > 285 has been selected for E2 quantification by GC coupled with tandem MS detection [22,71]. In the case of EE2, the *m/z* transition 425 > 193 [22,71] or the ions at *m/z* 196 [66] or 285 [63,68,71] have been commonly used for quantification by GC–MS/MS or GC–MS analysis, respectively. According to Ternes et al. [3], both EE2 and co-eluting matrix compounds such as humic substances, exhibited the *m/z* values 440, consistent with the molecular weight of silylated EE2, and 425, consistent with the loss of one methyl group. In order to minimize this interference, the ion at *m/z* 285 has been preferably selected in some cases for EE2 quantification [63,68,71]. On the other hand, when derivatization with PFBCl was performed, the resultant derivatives of E2 and EE2 have been generally quantified using the ions at *m/z* 660 and *m/z* 490, respectively [16,59]. The fragment ion at *m/z* 660 is consistent with the E2 pentafluorobenzoyl-derivative with both hydroxyl groups derivatized completely whereas the fragment ion at *m/z* 490 corresponds to the EE2 derivative with only one hydroxyl group derivatized.

In fact, overestimation of EE2 concentrations with GC–MS has been reported [107], probably due to a co-eluant substance, supposed to be tetracosanic acid because of its similar behavior to EE2 in GC–MS with respect to retention time and mass spectra. Besides the application of tandem MS, the approach proposed by Gunatilake at al. [76], resorting to two-dimension GC, is suitable to circumvent this drawback presented by single quadrupole MS detection.

LC–MS/MS is the second most applied method (30%) for analytical determination of E2 and EE2 in water, sludge and sediment samples. Indeed, when looking at publication date, the most recent studies use LC–MS/MS, suggesting the increasing importance of this technique. As mentioned in Section 2.2., LC-based methods do not require previous derivatization but this step can be performed to obtain higher sensitivity. While analyzing estrogenic chemicals in river water and effluents from a sewage treatment plant by LC–MS/MS, Lien et al. [89] reported that previous chemical derivatization with dansyl chloride or pentafluorobenzyl bromide (PFBBr) added on moieties that improved ionization and enhanced analytical signals. Furthermore, other derivatizing compounds such as *p*-nitrobenzoyl chloride have been applied to HPLC analysis with fluorescence detection by introducing a fluorescent fluorophore into the target estrogens molecules as it reacts with hydroxyl and phenolic hydroxyl groups of E2 and EE2, originating fluorescent derivatives [97,108]. LODs attained by this methodology (5 ng L^{-1}) reached values that are comparable with those obtained by LC–MS/MS. HPLC-FD without derivatization has also been proposed, with LODs from 20 to 42 pg mL^{-1}, encompassing advanced sample treatment before analysis [42] or DLLME using an ionic liquid (1,3-dipenthylimidazolium hexafluorophosphate) [109]. Recently, MEKC coupled to electrospray ion trap MS has been described as a suitable technique, employing matrix-matched calibration [98].

In comparison to other chromatography techniques coupled with MS detection, the LC–MS/MS method is more susceptible to matrix interferences, resulting in a reduction of the signal-to-noise ratio and a subsequent reduction in reliability and stability [68,71,89]. To minimize the matrix effects, several strategies have been adopted such as selective extraction, additional clean-up, efficient LC separation or modification of mobile phase composition [89,102,110]. Moreover, while comparing different LC systems and ion sources, Lien et al. [89] verified that it was inconclusive to determine which system was least susceptible to matrix effects but observed that derivatized analytes were less prone to matrix interferences than underivatized ones.

Among the different ionization modes available for LC–MS and LC–MS/MS (see Section 2.2.), ESI in negative mode is the dominant technique for estrogens determination in environmental aqueous or solid extracts (Tables 2 and 3). Both SRM and MRM modes have been used for E2 and EE2 quantification purposes. The precursor to product ion transitions employed for E2 quantification include the *m/z* transitions 271.0 > 145.0 [71,73,89], 271.0 > 183.0 [71,89] and 255.0 > 159.0 [14], values previously referred for biological samples that correspond to fragments originated through ring cleavages (see Section 2.2.). The deuterated internal standards E2-d_4 and E2-d_2 are generally monitored at the *m/z* transitions 274.6 > 147.0 [89] and 273 > 186 [71], respectively. The ion transitions at *m/z* 295 > 145 [71,73,89], *m/z* 295 > 159 [89] and *m/z* 279 > 159 [14] have been used for EE2 quantification while the *m/z* transition 298.9 > 147 has been applied for the correspondent internal standard EE2-d_4 [89]. The fragment ions at *m/z* 145 and 159 are consistent with ring cleavages and losses of, respectively, $C_8H_{14}O$ and $C_9H_{12}O$ from the original molecular ion [23,55]. Dansyl derivatives have been generally monitored at ion transitions already referred for biological matrices (see Section 2.2.), i.e., *m/z* 506.1 > 171.1 for dansylated E2; *m/z* 510.1 > 171.0 for dansylated E2-d_4; *m/z* 530.2 > 171.1 for dansylated EE2 and *m/z* 534.2 > 171.0 for dansylated EE2-d_4 [89]. Moreover, the product ion at *m/z* 156, formed through loss of a methyl group from the dansyl moiety fragment at *m/z* 171, has also been used [31,89].

Some authors have evaluated the performance of different ionization techniques for the analysis of steroidal estrogens in the environment. Chen et al. [25] verified that both ESI and APPI were suitable for ionizing E2 and EE2 although the latter mode provided better signal intensities. The same study observed that ESI was applicable to a wider range of feminizing chemicals (phenols and estrogens). In turn, the work published by Lien et al. [89] on estrogenic chemicals analysis in water compared the performance of several combinations of LC systems and four ionization modes – ESI, APCI, APPI and APCI/APPI combo (dual-source ionization to expand the range of compounds simultaneously analyzed) – and concluded that dansylated compounds with ESI at UPLC conditions produced the most intense signals and presented less matrix effects.

Furthermore, an overall comparison of GC–MS, GC–MS/MS and LC–MS/MS for the measurement of the estrogens estrone, E2 and EE2 in environmental samples revealed that the three techniques are able to generate similar analyte concentrations [71]. GC–MS is the simplest to

operate but presents higher detection limits, which is relevant when measuring chemicals at trace levels. The tandem MS techniques, especially GC–MS/MS, are more selective thus preventing false positive identification and permitting to achieve lower limits of detection [22,71]. Nevertheless, when compared with LC–MS/MS, GC–MS/MS requires derivatization before analytical determination which can limit sample high-throughput. A recent inter-laboratory comparison of analytical methods reported that the best approach would be based on LC–MS/MS coupled with the calibration technique of isotope dilution for assessment of E2 [111]. Moreover, considering the two species E2 and EE2, GC/MS/MRM can be regarded as the method of choice [112,113].

Other analytical approaches such as electrochemical oxidation of estrogenic compounds on an electrode surface have attracted some interest due to low cost, simple operation, portability, high sensitivity and fast response. For instance, Liu et al. [90] constructed a composite electrode by electrodepositing a Ni(II)tetrakis(4-sulfonatophenyl) porphyrin (NiTPPS) compound on a carbon nanotube-coated glassy carbon electrode and used cyclic voltammetry (CV) to detect low concentrations of EE2 in water samples. NiTPPS and carbon nanotubes were employed to enhance the electrochemical oxidation signals of EDCs and to minimize electrode fouling. Martínez et al. [21] established a new electrochemical methodology through the combination of antibodies on magnetic particles for analytes separation and pre-concentration and glassy carbon electrodes modified with multi-walled carbon nanotubes (MWCNTs/GCE). Square wave voltammetry (SWV) was employed for detection. Moreover, Kanso et al. [104] used recently screen-printed electrodes and SWV to detect E2 and EE2 previously immobilized on magnetic beads. These two combined approaches were successfully applied to the determination of estrogens in both surface and wastewater samples.

The analytical strategies described for the assessment of environmental contamination with E2 and EE2 commonly present high recovery rates for aqueous matrices, with values ranging from 75 to 120% in the majority of published studies (Table 2) and, as expected due to matrix complexity, slightly lower recoveries for sediment and sludge samples, with values between 60 and 130% (Table 3). The limits of detection (LOD) obtained by chromatography techniques with mass spectrometry detection are generally in the ng L^{-1} level for liquid matrices and ng g^{-1} for solids, with values ranging in most cases from <1 and 7 ng L^{-1} and from <1 and 5 ng g^{-1}, respectively (Tables 2 and 3). As expected, when UV (DAD) or fluorescence detection was used, higher LODs in the μg L^{-1} range were obtained (Table 2) [114]. On the other hand, electrochemical detection approaches permit the achievement of detection limits as low as those reported for MS techniques, i.e., at ng L^{-1} levels.

The monitoring of the estrogenic compounds E2 and EE2 in the different environmental compartments by the above mentioned methodologies revealed that both analytes are frequently detected in wastewaters and surface waters and also sediments and sludge, suggesting the potential for dissemination of these compounds. The environmental concentrations determined by the studies included in this review vary from 1 to almost 150 ng L^{-1} in aqueous matrices (Table 2) and from 1 to approximately 60 ng g^{-1} in sludge and sediments (Table 3). The estrogens content is generally reduced by the wastewater treatment process, but to levels that still raise concern.

3.2.2. Biological based assays

Whereas instrumental analysis of environmental samples may permit the identification of target compounds and the quantification of their individual concentrations, biological techniques can be used to determine the total endocrine-disruptive activity [68]. In the case of estrogenic chemicals such as E2 and EE2, this activity is generally expressed as total estrogenicity. The results of chemical determination do not take into account potential interactions between compounds and/or the presence of compounds that although unknown may have effect on the overall estrogenic activity [65]. Therefore, in alternative, different *in vivo* and *in vitro* assays may be employed to measure the total estrogenicity of real environmental samples, without the necessity of knowing all compounds present that contribute to that activity. The *in vitro* assays permit the evaluation of integrated estrogenic activity based on the interaction between chemicals and estrogenic receptors. Commonly used *in vitro* bio-assays include the Yeast Estrogen Screen (YES assay) [115], and the cell-based assays E-Screen [116] and estrogen responsive chemically activated luciferase (ER-Calux®) [117]. The yeast-based assays are less susceptible to non-sterile conditions than those based on the use of mammalian or fish cell lines which makes that type of determination more suitable for complex environmental matrices [70]. In fact, the performed literature search revealed that the yeast-based YES screen was the assay most frequently applied (58%) for estrogenic activity measurement in both aqueous and solid environmental samples [17,69,70,74,81,84]. The YES assay was originally developed by Routledge and Sumpter [115] on a *Saccharomyces cerevisiae* yeast strain whose genome was modified with integration of the DNA sequence encoding the human estrogen receptor (hER). The integrated DNA also contained expression plasmids carrying estrogen-responsive sequences (ERE) and an appropriate reporter gene encoding the enzyme β-galactosidase (*lacZ*). In the presence of estrogens, the enzyme β-galactosidase is synthesized and released into the medium where it metabolizes the chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG) resulting in a color change from yellow to red.

Cell-based biological assays for estrogenic activity assessment have also been proposed. Avbersek et al. [65] employed the ER-Calux® assay to investigate the presence of natural and synthetic estrogenic compounds in natural and wastewaters whereas Atkinson et al. [13] developed a fish specific, estrogen receptor-dependent reporter gene assay to monitor the persistence of steroidal estrogens during wastewater treatment processes. The two referred bio-assays follow the same basic principle: transfection of a cell line with an expression vector containing the DNA sequence of an estrogen receptor and an estrogen-dependent luciferase reporter gene.

The total estrogenicity assessed by biological assays is generally calculated by comparison with the activity of the natural estrogen E2 and expressed as estradiol equivalents (EEQ). Reported detection limits (LOD) range from 0.02 to 0.68 ng of EEQ per L for liquid matrices and 0.04 ng of EEQ per g for sediments. Regarding the measured estrogenic activity, surface and wastewater samples presented EEQ values ranging from <0.2 to 80 ng L^{-1} whereas in sediments the interval 0.04–6 ng g^{-1} was observed. According to these estrogenicity values, wastewater treatment processes may not be as efficient as initially assumed in estrogens removal.

Immunoassays such as the quantitative enzyme-linked immunosorbent assay (ELISA) and chemiluminescence enzyme immunoassay (CLEIA) have also been applied to determine estrogens in environmental waters. Two studies [61,83] used commercially available ELISA kits to determine the spatial and temporal fluctuations of E2 and EE2 concentrations in estuarine and wastewater samples. On the other hand, Brander et al. [118] created a polyclonal antibody to the estrogen-responsive fish proteins chorion and choriogenin that was subsequently validated to measure the response to aqueous EE2 through an ELISA using fish body homogenates. Xin et al. [103] developed and tested the feasibility of a CLEIA assay for E2 determination in river, waste, and tap water samples. Immunoassays are cost effective and easy to use, exhibiting good potential for widespread application. A direct competitive ELISA method, based on polyclonal antibodies against E2 and EE2, haptens conjugated to horseradish peroxidase and tetramethylbenzidine as enzyme substrate was recently proposed and applied to wastewaters after DLLME [99]. However, as previously referred (see Section 2.2.), these techniques do not permit the simultaneous determination of multiple compounds and may be limited by cross-reactivity.

Biosensors have also been proposed for environmental samples. Recently, Hu et al. [119] developed a label-free piezoelectric-based

biosensor based on detection of estrogen receptor (ER-α). After incubation with sample containing estrogenic compounds, including E2 and EE2, the depletion of ER-α was assessed. A more complex scheme used antibodies immobilized in magnetic nanoparticles labeled with Pb^{2+} that interacted with E2 retained in the surface of a glassy carbon electrode modified with graphene sheets. The amount of E2 was proportional to the amount of lead, detected by anodic stripping voltammetry [120].

Combining instrumental and biological tools is valuable and desirable as it can provide complementary information to fully understand the impact of environmental contamination with estrogens. Increased application of combined instrumental analysis and biological assays is evident from the literature as the majority of studies employing bioassays use them in combination with instrumental methods, namely liquid or gas chromatography coupled with mass spectrometry detection [13,16,17,65,69,70,74,84]. The two types of analytical methods generate different but compatible information about steroid estrogens and estrogenicity that might be integrated to better assess causal links between effects observed in the environment and chemical analysis profile [16]. In order to compare the results of instrumental and biological assays, theoretical estradiol equivalents can be calculated from concentration data (cEEQ) and related to estradiol equivalents (EEQ) determined by the used estrogenicity assay [16,65]. There is generally a significant correlation between EEQ values from bio and instrumental analyses [16,70] and disagreements are attributed to the presence of unknown compounds or to synergistic and antagonistic effects [65,70].

4. Conclusions

The majority of analytical methods recently used for E2 and EE2 determination in biological and environmental matrices are based on gas or liquid chromatography with mass spectrometry detection (GC–MS or LC–MS, respectively). Detection by tandem mass spectrometry (MS/MS) has been increasing in the last years, probably due to the increase in availability of this type of detector in laboratories worldwide. Estrogens analysis by GC methods demands for sample derivatization, performed mainly with PFBCl and BSTFA. On the contrary, LC-based methods are not limited by analyte volatility and thermolability which potentiates their increasing utilization for estrogens detection and quantification. GC–MS or GC–MS/MS analyses have been performed with EI or CI ionization, whereas LC counterparts have been conducted with ESI, APCI or APPI modes.

Nevertheless, previous sample treatment is necessary. LLE has been the most frequently selected technique for estrogens extraction from liquid biosamples (e.g. plasma) or samples supernatant after precipitation of protein rich liquids (e.g. milk). In turn, environmental aqueous matrices, following acidification and filtration, have been mostly extracted by SPE. Biological solid matrices, such as animal tissues, and also sludge and sediments, have been generally freeze-dried and extracted by ASE or ultrasonication with organic solvents. The resultant extracts from both liquid and solid samples have been further cleaned-up and concentrated using SPE and also GPC.

The state of the art of the current techniques employed for E2 and EE2 determination presented in this paper evidenced the importance of maximizing method accuracy, precision and sensitivity. Automation through the development of on-line sample preparation techniques coupled with liquid chromatography might contribute to achieve these aims. In comparison with classical analytical methodologies, these hyphenated techniques present as main advantages improved sensitivity, reduced analysis time, reduced sample contamination and degradation, higher reproducibility, precision and accuracy, and reduced solvent and sample consumption.

E2 and EE2 monitoring in the environment is often limited by the trace amounts normally present. Indeed, there is a lack of screening methods that can be applied to such low expected concentration values. Moreover, the majority of quantification studies are based on specific time point samples. To overcome this limitation, field deployable methods should be developed and implemented for the continuous *in situ* monitoring of analytes in the different environmental compartments.

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