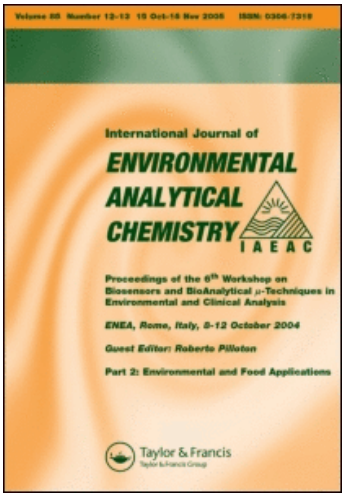

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Analysis of steroidal estrogen residues in food and environmental samples

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Analysis of steroidal estrogen residues in food and environmental samples

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A comprehensive review of analytical methods for estrogen residues in food and the environment is presented. Both humans and animals can excrete estrogen, which can be detected in the environment. Furthermore, replacement therapy and livestock manure also make the situation worse, as the low concentration of estrogens has endocrine-disruptive effects such as the feminization of male fish. In this review, we review the existing analytical methods, including high-performance liquid chromatography (HPLC), gas chromatography (GC), HPLC and GC with mass spectrometry (LC/MS and GC/MS), enzyme-linked immunosorbent assay, and various bioassays. These analytical methods for estrogens are compared with each other, and the advantages and disadvantages of each of these methods are highlighted. Specific aspects of these analytes, sample handling, chromatographic conditions, and detection methods are also discussed.

Keywords: Estrogen residues; Analytical methods; Food; Environmental samples

1. Introduction

Estrogens play a major role in the sexual differentiation, sex-gland development, and oocyte growth of most oviparous organisms. They also stimulate vitellogenesis, which is the formation of high-density glycolipophosphoprotein, and can serve as an energy source for the embryo development [1]. In 1977, it was noted that breast-cancer patients have significantly higher levels of estrogens in their urine than healthy postmenopausal women. Castagnetta *et al.* [2] then suggested that estrogens may be implicated as initiators, not just as promoters, in the carcinogenic process. Studies in rodents have demonstrated that estrogens or their catechol metabolites are carcinogens in various tissues, including the kidneys, liver, uterus, and mammary glands [3].

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Furthermore, effluents from UK wastewater-treatment works were first reported to be estrogenic to fish in 1994 [4]. Moreover, it has been suggested that estrogens may result in a decrease in human sperm count and quality [5].

Among the wide range of endocrine-disrupting compounds (EDCs), natural and synthetic estrogens are of particular interest due to their high estrogenic potency [6]. EDCs are environmental contaminants that disturb normal endocrine function and cause male reproductive dysfunction in humans and wildlife; there has also been an increase in these kinds of reports on EDCs [7–9].

Generally, natural estrogens were synthesized and excreted by the ovary, and their concentration in the body influences female fertility. Estrone (E1), estradiol (E2), and oestriol (E3) are crucial natural estrogens, and they always have a tetracyclic molecular framework and an aromatic A-ring as a distinctive part [10]. The key structural difference is the D-ring structure. Estrogens are metabolized to more polar compounds, which are easier to eliminate. The most frequently occurring reactions are hydrogenation of the double bonds, hydroxylation predominantly at the C_2, C_6, and C_{16} positions, and conjugation of the oxygenated functional groups mainly with glucuronic or sulfuric acid. Their backbone and the structure of the estrogens described in this review are shown in figure 1, and their physicochemical properties are given in table 1.

The primary natural estrogens are 17α-estradiol (17α-E2) and E1. 17β-Estradiol (17β-E2) is less active than the α-epimer. E3 is a weak estrogenic metabolite, principally formed and released during pregnancy, but may lead to estrogenic effects with constant exposure [11].

Synthetical estrogens in the environment have tremendous endocrine-disrupting effects under a very low concentration. For example, feminization of male fish occurs when the concentration of E2 is 1–10 ng L^{-1} [12] and the synthetic estrogens is 0.1 ng L^{-1} [13]. Many scholars consider the real reason of the hermaphroditic fish is the release of prophylactic and ethynilestradiol (EE2) [14, 15].

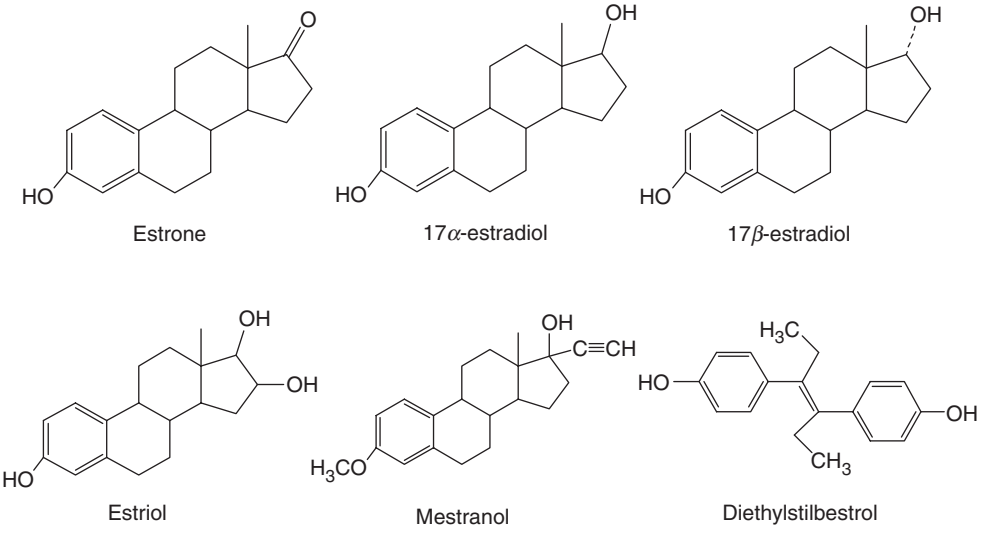

Figure 1. Structures of the estrogens described in the text.

Table 1. Selected physicochemical properties of estrogens[a].

Property	Estradiol	Estrone	Estriol	Reference
Formula	$C_{18}H_{24}O_2$	$C_{18}H_{22}O_2$	$C_{18}H_{24}O_3$	
MW ($g\,mol^{-1}$)	272.4	270.4	288.4	[46]
S_w ($mg\,L^{-1}$)	3.9–13.3	0.8–12.4	3.2–13.3	[45]
VP (Pa)	3×10^{-8}	3×10^{-8}	9×10^{-13}	[46]
log K_{ow}	3.1–4.0	3.1–3.4	2.6–2.8	[46]
pK_a	10.5–10.7	10.3–10.8	10.4	[46]

[a]MW: molecular weight; S_w: solubility in water; VP: vapour pressure; K_{ow} octanol–water partition coefficient; K_a: acid ionization constant.

Many estrogens have been detected in the rivers, reservoirs, lakes, and other waters. Because of the use of estrogen-replacement therapy and livestock manure, there have been many natural and synthetical estrogens released into the environment [16, 17]. Women excrete 10–100 μg of E2, EE2, E1, and E3 daily during the menstrual cycle. Depending on the phase of the menstrual cycle, pregnant women may secrete up to 30 mg of estrogen (mainly E3) daily [18–21]. Animals also excrete natural estrogens. An estimated 10 million cows and 43 million pigs excrete a daily mix of 10–30 kg of 17α-E2 and 80 kg of 17β-E2 in the US [22]. Both the synthetic estrogens and other chemical compounds that act as estrogenic active compounds and natural steroidal estrogens excreted by livestock manure may contaminate the surface and groundwater resources. The pollution caused by estrogen has also increased due to the overuse of synthesized estrogen in order to promote animal growth [23]. Anabolic steroid estrogens have been used as growth promoters to fatten cattle. This treatment may result in residues in the meat, which could be harmful to the consumer [24]. Steroid hormones have also been found in fish [25] and poultry [26, 27]. They have been found in other animal-derived food such as eggs [25] and milk [28].

The log K_{ow} of 3.1–4.7 indicates that estrogens are rather lipophilic and should appreciably adsorb into sediment and sludge [29, 30]. This assumption is underscored by the detection of high concentrations of estrogens in water released by dewatering sewage sludge. Therefore, a potential contamination of soil with estrogens may be caused by the application of digested sludge from municipal STP onto agricultural fields [31–33]. Further, it seems likely that estrogens are present in sediments, and because of their extremely high estrogenic potency, the possible threat to sediment biota cannot be ruled out.

The existence of estrogenic compounds which have the capacity to act as EDCs in environment and food may consequently affect both ecosystems and the human population. Public concern has been raised about their occurrence. Therefore, sensitive and specific methods are needed to detect EDCs in the complexity of matrixes [34].

The choice of method for determination of EDCs depends on the required outcome. Biological methodologies ascertain the endocrine-disrupting activity exhibited by a chemical or sample, while chemical techniques identify known chemicals and quantify the concentration within that sample [35]. Enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) can be used for multianalyte screening of a series of analytes [36, 37]. Instrumental analysis methods used for screening and confirmation, such as gas chromatography (GC) and high-performance liquid

chromatography (HPLC) followed by mass spectrometry are used most frequently [38–40]. Otherwise, there is currently a need to develop rapid methods to evaluate the overall estrogenic potential of different types of environmental waters and develop risk-assessment schemes [41, 42]. The use of bioassays to rapidly screen the exposure and demonstrate potential effects of estrogens can provide an ideal way to resolve this problem [43]. As a first estimate or screen to indicate the potential estrogenic activity of the chemicals, bioassays are cost-effective and useful for large numbers of environmental samples, although they are expensive and ambiguous due to a wide variety of mechanisms of action [44].

2. Analytical methods

Owing to concerns about residues of estrogens in food products of animal origin and drinking water, a number of techniques have been developed for their detection. The analysis of residual estrogens generally involves extraction from the samples with an appropriate solvent followed by one or more clean-up processes and then quantitative determination. High-performance liquid chromatography and gas chromatography coupled with mass spectrometry (HPLC/MS and GC/MS), LC tandem mass spectrometry (LC/MS/MS), and GC tandem mass spectrometry (GC/MS/MS) have usually been used for detection. These analytical methods have a good sensitivity and the capacity to detect many similar compounds together even in highly complicated matrices. However, they are expensive and thus not available for many laboratories. Some analytical methods for water and effluents just used simple GC or HPLC with ultraviolet–visible absorption (UV), fluorimetric, or electrochemical detection. Other methods, for example immunoassay, and bioassay methods have been used for the determination of very low concentrations of estrogens in the environment.

Tables 2–4 summarize the relevant information about the analytical methods (HPLC, LC/MS, GC/MS, and GC/MS/MS). Tables 2 and 3 detail the HPLC and LC/MS methods, including sample treatment of extraction and clean-up steps and the separation and detection techniques, even columns, mobile phases in HPLC, and limit of detection (LOD) of the method. Table 4 summarizes the sample treatment, columns and derivation used before detection, and LOD of the GC/MS and GC/MS/MS methods.

2.1 *Extraction methods*

Traditionally, the extraction of estrogens from food, such as egg, meat, fish, and cosmetics has been conducted with organic solvents. Such methods are laborious and time-consuming. Unconjugated estrogens have a low aqueous solubility ($0.8–13.3\,mg\,L^{-1}$) [45] and are easier to dissolve in organic solvents. Extraction is generally conducted with acetonitrile [63], methanol or ethanol [64], acetone [56], hexane [65], methylene chloride, and so on. Some organic solvents also denature the sample protein, resulting in a cleaner extraction and helping to release drug residues bound to proteins. Liquid–liquid extraction (LLE) can be used alone but is often

Table 2. HPLC methods for the determination of estrogens.

Analytes[a]	Sample matrix	Sample preparation	Determination technique	Comments	Reference
E1, E2	Topical estrogen HBF gel	Liquid–liquid extraction	HPLC-UV, with a Supelco Discovery C18 column (5 µm, 250 mm × 3.0 mm i.d.); detection at a wavelength of 225 nm; mobile phase: acetonitrile : methanol : water (23 : 24 : 53 v/v)	LOD (mg mL^{-1}) 4.2×10^{-5} for E2, 1.2×10^{-3} for E1; LOQ (mg mL^{-1}) 1.4×10^{-4} for E2, 4.0×10^{-3} for E1; recovery: 97.19% for E2, 97, 95% for E1	[47]
E1, 17α-E2	Pomegranate seed fruit juice and commercial preparations	Liquid–liquid extraction	HPLC/photodiode-array (PDA) with a Capcellpak C18 column (5 µm, 250 mm × 4 mm i.d.); detection at a wavelength of 225 nm; mobile phase: acetonitrile : water (35 : 65 v/v)	No relative information	[48]
17β-E2, 17β-E2-3-acet	Silicone intravaginal rings	Liquid–liquid extraction	HPLC/UV with a Spheredone C18 bonded reverse-phase column (5 µm, 150 × 4.6 mm i.d.); detection at a wavelength of 281 nm; mobile phase: acetonitrile : water (50 : 50 v/v)	No relative information	[49]
E1, 17β-E2, E3, EE2, mestranol	Waste water	Filtered through 0.2-µm filter, and no other preparation	HPLC-UV/DAD-FLD with a Hypurity C18 column (3 µm 150 mm × 2.1 mm i.d.); 210 nm for UV/DAD and 220/315 for FLD; mobile phase : acetonitrile : water: gradient elution	LOQ (mg L^{-1}) 0.01for E1 and Mestranol, and 0.005 for EE3 EE2	[50]

[a]E1: estrone; E2: estradiol; E3: oestriol; EE2: ethynyl estradiol; 17α-E2: 17α-estradiol; 17β-E2: 17β-estradiol; 17β-E2–3-acet: 17β-estradiol-acetate.

Table 3. LC/MS and LC/MS/MS methods for the determination of estrogens.

Analytes[a]	Sample matrix	Sample preparation	Determination technique	Comments	Ref.
E1, E2, EE2, E2-gluc, E1-sulf, E3, DES, E2-acet	Waste water	Samples were filtered through0.45 μm HVLP filters (Millipore, Bedford, MA); stored at 4°C in the dark, and then the samples was performed using an automated on-line SPE sample processor Prospekt-2tm (Spark Holland, Emmen, The Netherlands)	LC/ESI/MS/MS with a Purospher STAR-RP-18e analytical column (5 μm, 125 cm × 2 mm i.d.); mobile phase: acetonitrile–water, gradient elation; MS/MS detection was performed in the selected reaction monitoring (SRM) mode using ESI in the NI mode	LOD (ng L^{-1}) E2-glue: 0.38; E1-sulf: 0.01; E3: 0.38; E2: 0.22; EE2: 0.31; E1; 0.07; DES: 0.07; E2-acet: 0.08	[51]
E1, E2, E3, EE2	Treated sewage and river water	Analytes were extracted from 150 mL of raw sewages, 400 mL of treated sewages and 4 L of river water. Analytes were filtered through 1.5 μm pore size Whatman GF/C glass fibre pad (Maidstone, UK), then analytes were extracted by SPE cartridges filled with 0.5 g of Carbograph 4 (Lara, Rome, Italy)	LC/ESI/MS/MS with a Alltech C18 reversed phase column (5 μm, 250 mm × 4.6 mm i.d.); mobile phase: acetonitrile-water, gradient elation. A PE Sciex API 2000 tandem triple-quadrupole mass spectrometer (Perkin-Elmer) equipped with a turbo ion spray source operated in the NI mode was used	LOQ (ng L^{-1}) STP influent / STP effluent / River water E3: 0.6 / 0.2 / 0.02 E2: 0.6 / 0.2 / 0.02 EE2: 0.9 / 0.3 / 0.03 E1: 0.2 / 0.08 / 0.008	[52]
17β-E2, 17α-E2, EE2, E1, DES	Influent STP water and river water	Samples were filtered through a 0.45-μm filter (MSI, Westboro, MA), and then were extracted using 200-mg LiChrolut EN cartridges (Merck, Darmstadt, Germany)	LC/ESI/MS with a Lichro CART RP-18 (5 μm, 250 mm × 4 mm i.d.); mobile phase: 0.5% (v/v) acetic acid Millio-water as A acetonitrile as B, gradient profile; ESI was performed in negative mode. Chromatograms were recorded under full-scan acquisition mode (m/z 60–300) and under SIM acquisition mode.	LOD (μg L^{-1}) Influent STP water / River water 17β-E2: 0.4 / 0.03 17α-E2: 0.6 / 0.06 EE2: 0.3 / 0.03 E1: 0.02 / 0.002 DES: 0.01 / 0.001	[53]

17β-E2, E1, EE2,	Effluent	Samples were vacuum filtered with baked (450°C) glass-fibre filters (<0.45 µm type A/E, Gelman Sciences), then extracted by layered-bed Lichrolut EN (0.2 g) and C18 (0.5 g, Varian BondElut) solid-phase extraction cartridges	LC/ESI/MS with a Keystone Scientific C18 column (3 µm, 150 mm × 2.1 mm i.d.); mobile phase: acetonitrile-water, gradient elation. Selected ion monitoring of (M-H)-estrogen ions in negative polarity electrospray ionization mode was performed for maximum sensitivity	LOD (µg L^{-1}) 0.18 for 17β-E2, 0.07 for E1, and no relative information for EE2	[54]
E3, E2, EE2, E1, DES	Waste water	Samples were filtered with a 0.45 µm membrane filter, and the experiment was performed using an automated sampler processor ASPEC XL (Automated Sample Preparation with Extraction Columns) from Gilson (Villiers-le-Bel, France). The extraction procedure was based on the use of SPE with an octadecylsilica C18 sorbent known as Lichrolut RP-18 (500 mg, 6 mL) from Merck.	LC/APCI/MS with a Lichrospher 100RP-18 column (5 µm, 250 mm × 4 mm i.d.), 50% acetonitrile, 50% methanol as A, and water as B, they are both acidified with 0.5% acetic acid, gradient elution.In full scan mode the m/z range was 100 to 400 in negative ion (NI) mode and from 70 to 1000 in positive ion (PI) mode of ionization. LC-DAD/MS with a Lichrospher 100 RP-18 column (5 µm, 250 mm × 4 mm); acetonitrile/water was used as the mobile phase, gradient elution. MS detection was performed by using two different interfaces:both ESP and APCI, are in the positive ion mode of operation. For the group of estrogens ESP was in the negative ionmodein when these compounds were not detected with the APCI interfacez	LOD (ng L^{-1}) E3 DAD 50 ESP 50 APCI 5000 E2 50 250 3000 EE2 50 500 3000 E1 50 100 3000 DES 50 25 2000	[55]

[a]E1: estrone; E2: estradiol; E3: oestriol; EE2: ethynyl estradiol; 17-α-E2: 17α-estradiol; 17β-E2: 17β-estradiol; E2-acet: estradiol-17-acetate; E1-sulf: estrone-3-sulfate; E2-glue: estradiol-17-glucuronide; DES: diethylstibestrol

Table 4. GC, GC/MS, and GC/MS/MS methods for the determination of estrogens.

Analytes[a]	Sample matrix	Sample preparation	Determination technique	Comments	Derivation	Ref.
E_1, 17β-E2, EE2 mestranol	Sludge sediment	Samples were freeze-dried, extracted with methanol and acetone, and for each extraction step ultrasonicated and then centrifuged. Also, gel-permeation chromatography silica (GPC) column was used; Silica-gel column; SPE (RP-C18 was filled into glass catridges); HPLC for cleanup	GC/MS/MS with a capillary column (Restek, Bad Soden, Germany; XTI-5; 30 m × 0.25 mm, i.d. 0.25 μm); GC temperatures: 50°C isothermal for 3.5 min, 20°C min^{-1} to 240°C, 2°C min^{-1} to 290°C, and 290°C isothermal for 10 min. MS parameters: temperature of transfer line (direct interface), 280°C; ion source, EI mode; electron energy, 70 eV; ion trap temperature, 250°C; collision-induced dissociation (CID) RF, 120 V; and window for MS/MS, 3 *m/z*	LOD (ng g^{-1}) E1: sediment 0.2 sluge 2; 17β-E2: sediment 0.2 sluge 2; EE2: sediment 0.4 sluge 4; mestranol: sediment 0.4 sluge 2	*N*-Methyl-*N*-(trimethylsilyl-trifluoroacetamide (MSTFA)/trimethylsily-limidazole (TMSI)/ dithioerytrol (DTE), (1000:2 : 2; v/v/w)	[56]
17β-E2	Mineral salt medium containing 17β-E2 degradated by activated sludge	Sample was extracted with methyl *tert*-butyl ether (MTBE, 5 mL × 3), and the MTBE extract was dried through anhydrous sodium sulfate and evaporated to dryness	GC/MS with a HP-5-MS fused silica column (30 m × 0.25 mm, i.d. 0.25 μm); Full scan runs from *m/z* 50 to 600	No relative information	Pentafluoropropionic acid anhydride (PFPA)	[57]
E1, 17β-E2, E3, EE2	Surface water	Samples from July 1996: Extracted with toluene, dried with sodium sulfate and concentrated with a rotary evaporator. Samples from 1998/1999: SPE (200 mg cross-linked styrene-divinylbenzene copolymer phase ENV+)	HRGC/LRMS with a DB-XLB fused silica capillary column (J&W Scientific Products, Köln, Germany; 30 m × 0.25 mm, i.d. 0.25 μm); MS: ion-monitoring (SIM) mode	LOD: between 0.1 and 1.7 ng L^{-1}, for the samples from January 1999: 1.5–4.7 ng L^{-1}	3 : 2 : 3-mixture of *N-O*-bis-(trimethylsilyl)-trifluorogcetamide (BSTFA), trimethyl chorosilane (TMCS) and *N*-trimethylsily limidazole (TMSI)	[58]

E1, 17α-E2, 17β-E2, EE2	River water	Extraction of the water samples was performed using Bakerbond Speedisk octadecyl-bonded silica (C18XF), 50 mm (JT Baker, Deventer, The Netherlands); cleanup by silica cartridge (Si, 500 mg, 10 mL, Sopachem, The Netherlands)	GC/MS/MS with a BPX-5 fused silica capillary column (SGE, Austin, TX; 25 m $\times$ 0.22 mm, i.d. 0.25 μm); the temperature programme was: initial temperature 100°C, directly ramped at 17 min^{-1} to 250°C, second ramp at 2 min^{-1} to 268°C, and finally ramped at 30 min^{-1} to 300°C	LOD: 0.25 ng L^{-1}	Mixture of MSTFA (*N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide), NH4I, and thanethiol	[40]
E1, 17α-E2, 17β-E2, EE2	Surface water, Drinking water	SPE: 100 mg of the ethinylbenzene-divinylbenzene copolymer LiChrolut EN (Merck, Darmstadt, Germany) was filled into specially designed glass cartridges (10 mm i.d.)	HRGC/(CH4-NCI) MS with a DB-5MS (J&W Scientific, Folsom, CA; 60 m $\times$ 0.32 mm, i.d. 0.25 μm); (80°C, 3 min), 10°C min^{-1} (200°C, 1 min), 30°C min^{-1} (245°C, 10 min), 0.45°C min^{-1} (260°C, 1 min), 30°C min^{-1} (300°C, 10 min); MS: ion monitoring (SIM) mode, HRGC-ECD with a RTX 1701 (Restek, Bad Soden, Germany; 30 m $\times$ 0.32 mm, i.d. 0.25 μm); ECD temperature: 300°C; (80°C, 3 min), 10°C min^{-1} (200°C, 1 min), 30°C min^{-1} (245°C, 5 min), 0.5°C min^{-1} (260°C, 5 min) 30°C min^{-1} (280°C, 10 min)	LOD (ng L^{-1}): STW effluents: E1, 0.10; 17α-E2, 0.15; 17β-E2, 0.15; EE2, 0.10, River Water: E1, 0.10; 17α-E2, 0.15; 17β-E2, 0.15; EE2, 0.10, Drinking water: E1, 0.05; 17α-E2, 0.10; 17β-E2, 0.10; EE2, 0.05	10% pentafluorobenzoyl chloride (PFBCl) in toluene	[59]
E1, E2, E3, EE2, mestranol	River water	Samples were extracted from the aqueous phase by Dichloromethane (DCM), evaporated to dryness with nitrogen, and dissolved in hexane	BPX-5 fused silica capillary column (SGE, Milton Keynes, UK; 30 m $\times$ 0.33 mm i.d. 0.25 μm); GC temperature: 50°C isothermal 3.5 min; 20°C min^{-1} to 240°C; 2°C min^{-1} to 290°C for 10 min; MS: EI mode	LOD: 5 ng mL^{-1}	Mixture of *N*-methyl-*N*-(trimethylsily) trifluoroacetamide: trimethylsilylimidazde: dithioerythritol (1000 : 2 : 2 v/v/V)	[60]
E1, 17β-E2, EE2, DES	Tap water and reservoir water	Samples were extracted through PC-HFME procedure and SPME procedure separately	DB-5 fused silica capillary column (J&W Scientific, Folsom, CA, USA; 30 m $\times$ 0.32 mm i.d. 0.25 μm,); the GC temperature: 50°C (2 min); 20°C min^{-1} to 100°C; 10°C min^{-1} to 200°C; 20°C min^{-1} to 300°C (2 min); MS: SIM mode	PC -HFME / SPME / MSTFA E1 2.3 8.0 17α-E2 2.7 7.0 EE2 0.3 11.0 DES 0.1 18.3		[61]

(*continued*)

Table 4. Continued.

Analytes[a]	Sample matrix	Sample preparation	Determination technique	Comments	Derivation	Ref.
E1, 17β-E2	Beef and veal; liver; milk and milk products; pork and meat products; poultry and eggs; fish, plant, yeast and fermented alcoholic beverages	After different preparations, the samples were extracted by methanol, water, hexane, dichloromethane, and purified on an Amberlite XAD-2 column followed by a fractionation in phenolic and neutral steroids through a Celite/KOH column coupled to an $A1_20_3$ column	GC/MS with a DB-5 MS column (30 m $\times$ 0.25 mm, 0.25 μm); MS: selected ion monitoring	The determination limit of this GC-MS method was about 0.01–0.3 $\mu g\,kg^{-1}$ depending on the hormone and matrix.	*N*-methyl-*N*-trimethylsilytrifluoroacctamide/trimethyliodosilane/dithioerythritol (1000 : 2 : 2)	[25]
E1 17α-E2 17β-E2 E3	Muscle tissu of beef cattle	Samples were homogenized immediately before analysis, extracted by methanol and heaed in a water bath at 60°C and then centrifuged; used C8-SPE and Si SPE for cleanup	GC/MS witj a BPX-5 fused silica LOD: 0.02–0.1 mg/kg capillary column (SGE, Weiterstadt, Germany; 25 m$\times$0.22 mm i.d. 0.25μm); MS: selected ion monitoring	*N*-Methyl-*N*-(trimethylsilyl)-triflu oroacetamide (MSTFA)-trimethyliodosilane (TMIS)-DTE(1000:2:2, v/v/v)	[62]	

[a] E1: estrone; E2: estradiol; 17α-E2: 17α-estradiol; 17β-E2: 17β-estradiol; E3: oestriol; EE2: ethynyl estradiol; DES: diethylstibestrol.

followed by solid-phase extraction (SPE), solid-phase micro-extraction (SPME), and supercritical fluid extraction (SFE) [66]. Nowadays, SPE is a well-established technique owing to its low cost and easy operation, and because it can be used for the analysis of numerous different classes of compounds in a variety of matrices [67]. Some methods handle water samples with SPE for all of extraction, concentration, and purification without any additional procedures.

On-line and off-line SPE techniques have been used for extraction and concentration of estrogens in environmental water samples [68–71]. The on-line SPE methodology, which results in minimum sample preparation requirements and minimum manipulation by the analyst, has a smaller margin of error and numerous advantages compared with off-line methods. However, most of these techniques require large sample volumes. Therefore, an in-tube SPME technique [72, 73], using an open tubular fused-silica capillary with an inner surface coating as the SPME device, was developed to resolve this problem, and this can be easily coupled with HPLC and LC/MS.

For the optimization of an SPE, the most important parameters to evaluate are the type of sorbent, sample volume, flow rate used for sample loading, and sample pH [74]. Selection of the sorbent often depends on the nature of the matrix and the physicochemical properties of the target analytes. The selection of the sample volume relies on the characteristics of both the sorbent and the analytes, and by method sensitivity and throughput requirements. In principle, if the SPE sorbent has a smaller particle size, the capacity and efficiency will be enhanced; also, the increase in the sorbent surface area will lead to better interactions between the analytes in the water sample and the surface of the sorbent. Efficient contact between the sample analytes and the sorbent surface will improve the recovery. The acidification of the water sample was shown to have no influence on recovery, whereas a pH higher than 9 leads to significant losses of diethylstilbestrol (DES) and estradiol-17-acetate [53, 75].

Presently, SPE has been used for extraction and concentration of estrogens in environmental water samples most frequently, and the developments are given in formats, phases, automation, new types of experimental procedures. Emphasis is placed on the large choice of sorbents for trapping analytes over a wide range of polarities, such as highly cross-linked copolymers, functionalized copolymers, graphitized carbons, or certain specific *n*-alkylsilicas. New selective phases such as mixed-mode and restricted-access matrix sorbents, immunosorbents, and molecularly imprinted polymers are introduced also. New formats have also been introduced, for example the 96-well SPE plates, fast-flow on-line extraction, and microfibres for SPME [76–79]. The principles and application details of automated 96-well SPE and fast-flow on-line extraction have been described in a number of publications [80–84]. The trend has been to simplify the procedure for biological samples with the introduction of non-conditioned solid-phase extraction (NC-SPE) technology.

2.2 *Clean-up procedures*

Because of the complexity of the sample matrices, a clean-up step is required prior to the chromatographic determination. The disposal progress is generally very complex for organic samples but simple for water samples.

Recently, there has been a tendency for more rapid sample preparation methods, which can be coupled with the on-line separation technique. In particular, one-step sample-pretreatment procedures with an automation capability are needed. Immunoaffinity extraction, which provides unique and powerful techniques and enables selective extraction and concentration of individual compounds or classes of compounds from liquid matrices in one step, can achieve this purpose. The molecular imprinting technique is also developed because of its analytical applications of molecularly imprinted polymers (MIPs) for screening bioactive compounds with high selectivity.

2.2.1 Immunoaffinity extraction. For selectively isolating and concentrating minor components of interest from a complex mixture, a highly selective sample pre-treatment is necessary. Immunoaffinity extraction (IAE) is a powerful technique [85–89], which immobilizes antibodies raised against the target analytes on a solid support and packed into a pre-column for the selective SPE of the analytes. An antibody can also bind one or more analytes with a structure similar to the analyte that has induced the immune response, and this is the so-called cross-reactivity of antibodies [90]. This negative feature for immunoassay is utilized in extraction [91]. Full automation of such analytical systems can be achieved by coupling the immuno-precolumn to column liquid chromatography (LC) with a column-switching system [90, 92].

Nearly all of the environmental applications of immunosorbent extraction published to date have used polyclonal antibodies, which are generally less pure and less reproducible on a batch-to-batch basis than monoclonal antibodies produced from a single cultured cell line [93]. The use of highly selective immunosorbents in sample preparation prior to analysis allows the removal of interfering sample matrix compounds present in the wastewater extracts that would otherwise cause severe ionization suppression of the estrogens during the electrospray process. IAE removes much of the isobaric noise from the selected ion-monitoring chromatograms, increasing the signal-to-noise ratios for analytes, and contributing to the low detection limits (0.18 and 0.07 $ng\,L^{-1}$ for E2 and E1, respectively) achieved by the current method [18].

2.2.2 Molecular imprinting technique. The molecular imprinting technique (MIT) is a valuable method for preparing polymeric materials with specific binding properties that have potential applications such as chemical sensors, micro-reactor mimicking enzymes, stationary phases for HPLC, catalysts, and membranes for separating toxic chemicals [94–99]. The model outlined in figure 2 is used mostly to explain the formation of molecularly imprinted binding sites. Functional monomers are first pre-organized around a template molecule and 'locked' into place by copolymerization with a cross-linking monomer. Then, the removal of the template affords a polymer with specific binding sites for the template. This technique allows extraction, trace enrichment, and cleanup to be achieved in one step, owing to the selectivity of the antigen–antibody interaction. In addition, most MIPs are remarkably stable against mechanical stress and high temperatures and pressures, resistant against treatment with acid, base, or metal ions, and stable in a wide range of solvents [100]. However, it should be noted that because MIPs are typically made in organic solvents, their optimal binding conditions usually require an organic-based medium. Furthermore, the

polymers can be used repeatedly—well in excess of 100 times over several years without any loss in the 'memory effect'. They are currently used as chromatographic and SPE media, and offer tremendous potential as the recognition element for immunoassays and sensors [101, 102].

Molecularly imprinted materials can be prepared in different ways, and two basic approaches may be distinguished: one is a self-assembly approach, where the prearrangement between the print molecule and the functional monomers is formed by non-covalent or metal coordination interactions; and the other is a preorganized approach, where the aggregates in solution prior to polymerization are maintained by reversible covalent bonds. By using a high percentage of cross-linker, polymers of substantial rigidity and complete insolubility can be obtained.

Several studies have shown that the major factors governing the recognition ability of an imprinted polymer are the shape of the cavities and the orientation of the functional groups situated inside them, with the latter considered being predominant. For this reason, multipoint binding imprinted polymers perform better than one-point binding polymers.

Meng *et al*. [103] prepared a synthetic MIP sorbent for estrogenic compounds using a non-covalent imprinting technique. MIP microspheres 1 to 2 μm in size were synthesized in acetonitrile by using 17β-E2 as the template, acrylamide as the functional monomer, and trimethylpropanol trimethacrylate as the cross-linker. Sibrian-Vazquez and Spivak [104] has investigated a new strategy for monomer design that combines interactive monomer functionality with a cross-linking format, giving non-covalent MIPs with an improved performance. Umpleby II *et al*. [105] eliminated the low-affinity binding sites by esterification with diazomethane or phenyldiazomethane. Selectivity in the esterification reaction was achieved using a guest molecule as an *in situ* protecting group that preferentially shields the high-affinity sites and leaves the low-affinity sites exposed to reaction. Ki *et al*. [106] used a thermally reversible bond for the preparation of the silica monomer–template complex, which allowed the researchers to remove the template by simple thermal reaction and to simultaneously introduce various functional groups into the cavity. The monomer–template complex was prepared by the reaction of 3-(triethoxysilyl) propyl isocyanate with E1 in the presence of dibutyltin dilaurate. Yang *et al*. [107] also used the same method to make a MIP.

A polymer imprinted with 17β-E2 was used for the determination of 17β-E2 in HPLC based on a fluorescence sensing system [108]. When imprinted and non-imprinted polymers were compared, an imprinting factor of 8.83 was calculated, and 17β-E2 could be detected in the range of 0.1–4 $\mu mol\,L^{-1}$. Ye *et al*. [109] reported an interesting approach, generated monodisperse microspheres imprinted with 17β-E2, and investigated the polymers in competitive radioassays, which demonstrated the high specificity of the MIP toward the template. In immunoassays, it could be shown that the recognition properties of the MIP toward the template were comparable with those of antibodies, or even better.

2.3 *Measurement techniques*

For the determination of estrogens, gas chromatography/electrical conductivity detector (GC/ECD), GC/MS, GC/MS/MS, LC/UV, LC/fluorescence detection, liquid chromatography/photodiode-array (LC/PAD), LC/MS and LC/MS/MS have been

used. GC/MS and LC/MS are used more frequently. In recent years, many new methods have been developed, such as ELISA, bioassays, and MIT.

2.3.1 HPLC and LC/MS. For biological samples, HPLC is still a major analytical tool and is especially powerful when coupled with advanced detectors such as MS or nuclear magnetic resonance (NMR). A number of methods have been published, and tables 2 and 3 summarize the HPLC and LC/MS methods that are reviewed here.

Silica-based reversed-phased columns, such as C_{18}, have been chosen more often for separation [47, 50, 52]. The mobile phases mainly consist of acetonitrile–water mixtures, methanol–water mixtures, or ternary mixtures of acetonitrile–methanol–water. Sometimes, the mobile phases are acidified with 0.5% acetic acid [53, 55].

Several spectroscopic techniques, such as UV [47, 49], fluorescence detection [50], PDA [48, 55], and MS [110–113] have been used in LC analysis of estrogens.

In the past, UV was almost exclusively used, but recently LC/MS techniques have become more popular. LC/MS, unlike GC/MS, is not limited by such factors as non-volatility and high molecular weight, and enables the determination of both conjugated and non-conjugated estrogens without the need for derivatization or hydrolysis. LC/MS has often been carried out with an electrospray (ESI) interface operated in the negative ion mode (NI) or atmospheric pressure chemical ionization (APCI). Another benefit of LC/MS methods is the possibility of integrating sample preparation and enrichment online with the analysis. In recent years, many attempts have been made using LC/MS for the analysis of estrogens in water samples [113–116]. In addition, the emergence of LC/MS/MS has largely improved the performance of the technique by reducing the detection and quantification limits and enhancing analyte identification [117–121]. In order to achieve sufficient sensitivity, most of these methods are based on LC/MS and LC/MS/MS. However, they are not accessible in all laboratories for routine analysis due to the high cost of equipment and the requirement for an experienced operator. Thus, HPLC is a good choice, except for the limits of sensitivity and selectivity, and so a sample pretreatment step prior to chromatographic analysis is usually necessary.

2.3.2 GC and GC/MS. Researchers who have monitored estrogens in water samples have used a very sensitive and selective technique, such as GC/MS or GC/MS/MS [122–126], and the water samples are frequently pre-concentrated with SPE to achieve the object of trace analysis. The limit of quantification (LOQ) is in the low nanograms-per-litre range for such methods.

In order to decrease the polarity of the material and enhance the sensitivity and effect of the column, silylation and acetylation are often used before quantitative determination. Different agents have been used for silylation, and the *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) is used most frequently [40, 61, 75, 127]. Other agents were also used, such as *N*,*O*-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) [128–130], *N*-trimethylsilyl acetamide (TMSA) [131], and *N*-(tert-butyldimethylsilyl)-*N*-methyl-trifluoroacetamide (MTBSTFA) [132–134]. They can be used alone or in combination with a small proportion of different catalysers, such as *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide/trimethylchlorosilane (MSTFA/TMCS) [135, 136], (*N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA)/trimethylsilylimidazole (MSTFA/TMIS) [62, 63], bis-(trimethylsilyl)-trifluoroacetamide/trimethylchloro

silane/trimethylimidazole (BSTFA/TMCS/TMSI) [58], (*N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA)/trimethylsilylimidazole (TMSI)/dithioerytrol (DTE) [56], and pentafluorobenzoyl chloride (PFBCl) [137], which have been employed for the derivatization of the hydroxyl groups contained in the estrogen moiety. Acetylation with anhydrides, such as heptafluorobutyric anhydride [138] and pentafluoropropionic acid anhydride (PFPA) [57, 139], is the other frequently applied derivatization technique. More importantly, the humic and fulvic acids in the water samples may disturb the derivatization. Both increasing the quantity of agents for derivatization and prolonging the time of action can enhance the rate of product.

Method detection limits for GC/MS are typically above $1\,ng\,L^{-1}$, and this has resulted in a significant number of 'no detections' in the data reported using these methods. Methods based on GC/MS/MS are somewhat more sensitive, $0.1–1\,ng\,L^{-1}$ for treated effluent, yet still require the derivatization step prior to analysis [140–142]. GC/ECD has also been used. Pinnella [143] isolated the catecholestrogens from 17β-E2 by SPE, after derivatizing and subjecting them to solvent exchange prior to analysis. The LODs of the method are 0.8 and $1.3\,ng\,mg^{-1}$, and the LOQs are 2.6 and $4.3\,ng\,mg^{-1}$. The use of GC solely for the determination of estrogens is rare, and GC/MS and GC/MS/MS as well as LC/MS and LC/MS/MS are frequently used. Most of the development of this technique is concerned with the improvement of silylation and acetylation.

2.3.3 Enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA). ELISA for detection of estrogens has been developed and is especially suitable for screening large numbers of hormonally active substances with a great sensitivity [144]. Unlike an RIA, in which a radioactive isotope is used [145–149], ELISA does not entail serious problems in the disposal of samples, and it does not require the use of reagents that are harmful to human health.

Li *et al*. [150] developed an ELISA, which had an LOD of $0.14\,\mu g\,L^{-1}$ E1 in water. When it was combined with a SPE method to extract and preconcentrate E1 from water samples, the SPE-ELISA could detect E1 down to $1.25\,ng\,L^{-1}$ level in water, and the results correlated well with those obtained using GC and HPLC methods. Huang and Sedlak [142] reported a method for the detection of estrogenic hormones in wastewater using an ELISA technique with a sensitivity of $0.1\,ng\,L^{-1}$. This method was selective and could suffer from interferences caused by the presence of a complex extract matrix if a purification technique was not applied prior to analysis. Monteverdi and Di Giulio [151] developed a method using primary hepatocyte cultures from the channel catfish (*Ictalurus punctatus*) with an ELISA to detect and quantify the production of vitellogenin (VTG), a liver-derived estrogen-induced lipoprotein; the detection limit for this assay was typically $15–25\,ng\,VTG\,mL^{-1}$ medium. Nash *et al*. [152] developed a simple and rapid ELISA, which used acetylcholinesterase tracer to increase the sensitivity of assay so that reliable measurements of each steroid could be achieved with only 10 μL of plasma and the sensitivities taken as the concentration of E2 at $1.6\,pg\ well^{-1}$. Typical standard curves showed a workable range of $0.8–400\,pg\ well^{-1}$. A polyclonal antibody against the highest-molecular-weight band of putative VTG was generated in sheep, and an indirect antibody-capture competitive ELISA was developed. VTG was purified from the plasma of 17β-E2-injected male greenback flounder, *Rhombosolea tapirina*. The LOD of 17β-E2 in plasma was $0.3\,ng\,mL^{-1}$,

and a working range for the standard curve of 0.16–20 $\mu g\,mL^{-1}$ was chosen on the basis of the linear portion of the displacement curve [153].

A rare earth ion chelate has a large Stokes shift, narrow emission bands, and long fluorescence decay time (over 10 000 times longer than general fluorescence). Therefore, time-resolved fluoroimmunoassay (TR-FIA), which uses a rare earth ion chelate for labelling and detection, is a highly sensitive measurement [154]. Ito *et al.* [154] have developed a simple and rapid TR-FIA for simultaneous determination of E3 using europium and samarium ion chelate. The measurable ranges for E3 were 0.39–100 $ng\,mL^{-1}$. Huo *et al.* [155] developed a competitive TR-FIA for the determination of DES residues in chicken liver. The LOD was determined to be 0.05 $ng\,g^{-1}$, and the LOQ was less than 0.18 $ng\,g^{-1}$. The results obtained by the TR-FIA and ELISA showed a good correlation, and TR-FIA was validated for the determination of incurred chicken liver and confirmed by LC/MS/MS. A direct TR-FIA system for measuring E2 in bovine plasma was developed by Takahashi *et al.* [156], the sample was detected without prior extraction and purification, and the minimum detectable concentration was 0.625 pg $well^{-1}$. A new fluorometric enzyme immunoassay for 17β-E2 using biotinylated estradiol (BE) as a probe ligand was described. In this method, 17β-E2 was detected indirectly by a solid-phase avidin-biotin-binding assay, in which the biotin was immobilized on a microtitre plate. The LOD and linear range for the determination of 17β-E2 were 0.12 $nmol\,L^{-1}$ and from 0.12 to 25 $nmol\,L^{-1}$, respectively [157].

Sometimes RIA can be used to measure very low levels of estrogens in samples [149]; however, the treatment of samples is slow and complex. RIA is suitable for large numbers of samples, but few published techniques provide reliable measurements when the sample volume is small [158]. Furthermore, just like other immunoassays, it also shows cross-reactivity with several natural estrogens [159]. Consequently, LLE [160, 161] and SPE [162] followed by HPLC or an immunoaffinity column (IAC) [163] had to be performed before the RIA analysis. Surface plasmon resonance (SPR)-based immunoassay is not as sensitive as traditional RIA and ELISA, but this method requires no separation and washing after addition of the antibody, steps which are relatively time-consuming [164, 165].

Water samples from municipal wastewater treatment plants were extracted *in situ* using SPE disks, and RIA was used for detection of E2 and EE2 following a procedure previously described for the measurement of these compounds in plasma and adapted for environmental samples [166]. The LOD for E2 and EE2 were 0.427 and 0.211 $\mu g\,L^{-1}$ [161]. Geisler *et al.* [149] described a new method for simultaneous measurement of the three main estrogen fractions, E1, E2, and estrone sulfate (E1S) in breast tumour tissue. HPLC was used to purify the individual estrogen fractions prior to RIA analysis. The detection limit of this method was 4.3 $fmol\,g^{-1}$ tissue for E2, 19.8 $fmol\,g^{-1}$ tissue for E1 and 11.9 $fmol\,g^{-1}$ E1S, respectively.

In vitro screening for estrogenic activity has often been performed using traditional receptor-binding assays with radiolabelled 17β-E2 [167]. Because the use of radio-isotopes has drawbacks, for example radiation hazards and the need for special facilities, several non-isotopic estrogen receptor-binding assays have been recently developed [168–170].

The homogeneous enzyme immunoassay (HEIA) is simpler and faster, and separation or washing steps are not required. Therefore, HEIA, based on measuring changes in enzyme activity caused by the formation of an immune complex in

a solution, is regarded as an excellent strategy for overcoming the disadvantages associated with ELISA [171–173].

2.3.4 In vivo and *in vitro* bioassay. Several *in vivo* and *in vitro* assays based on their ability to bind to an estrogen receptor (ER) have been applied to evaluating the total estrogenicity. The most common *in vitro* test systems are based on the use of yeast cells (YES assay) or MCF-7 breast-cancer cell proliferation assay (E-screen assay) or on binding to isolated ER (ELRA) or ER-mediated chemically activated luciferase gene expression (CALUX) assay [168, 174–176]. In the *in vivo* assay, VTG release by fish is detected as a biomarker to estimate the total estrogenicity in the environmental water samples.

The strain for the Yeast Estrogenicity Screening (YES) assay carries a LacZ reporter plasmid, and its promoter region has estrogen responsive elements (ERE). The use of recombination gene technology can make ER express in the cell of yeast and make ERE with the signal gene in series. More estrogens combine to ER, and more signal genes will be expressed [177].

Routledge and Sumpter [178] developed a reporter gene assay with a recombinant strain of yeast that expressed the human estrogen receptor. In this YES assay, the level of α-galactosidase activity indicated the level of ER signalling activated by ligand binding. Therefore, this bioassay system could be a powerful tool to evaluate total estrogenic activity in environmental samples. Kawanishi *et al*. [179] also used the same method to measure the estrogenic activities of 13 water samples from Lake Biwa-Yodo River. Müller *et al*. [180] introduced a YES as a biological detection method for high-performance thin-layer chromatography (HPTLC). Yeast cells were grown directly on HPTLC plates, and estrogenic substances there induced the production of the enzyme β-galactosidase. The minimal detectable amount for E2 was 2.75 pg. Heisterkamp *et al*. [181] reported a bioassay-directed chemical analysis (BDCA) scheme, which combined a yeast screen for estrogenic activity with LC/MS detection after LLE and fractionation by size-exclusion chromatography (SEC). Noguerol *et al*. [177] proposed a rapid method for ligand detection for different vertebrate receptors in yeast, based on the use of fluorogenic substrates for the widely used reporter β-galactosidase gene. In this method, β-galactosidase activity was calculated from kinetic data, rather than from end-point measurements, which increased accuracy and facilitated the statistical analysis of the data. Five different Austrian lager beers have been investigated for estrogenic activity by a yeast two-plasmid system harboring the human ER, after concentration by SPE. The total activity corresponded to an average of 43 ng of 17β-E2/L of beer [182].

Receptor assays can be used to detect all compounds with an affinity for a given receptor [183]. This is particularly relevant in screening methods for compounds for which no detailed information is present with regard to the identity. Jungbauer and Beck [184] described a yeast reporter bioassay for the rapid determination of estrogenic activity in environmental samples. Recently, a simpler yeast-based reporter gene bioassay for estrogens was developed, featuring direct measurement of yeast-enhanced green fluorescent protein (yEGFP) for detection of estrogen activity [185]. The only handling involved was the addition of the yeast suspension to the sample extract. After waiting 4 or 24 h, the fluorescence intensity of yEGFP was directly measured in a plate reader.

YES is currently widely used in monitoring estrogenic activity in environmental samples. However, the practical application of YES for environmental samples has not been consistently successful because of problems arising from sample interference. Pre-concentration is still necessary for the yeast screen and for LC/MS analysis. Undiluted total extracts showed acute toxic effects in the yeast screen, which was overcome by dilution or by SEC fractionation [185].

ELRA is a newly developed *in vitro* bioassay for estrogens [186]. It was based on the biomolecular recognition of target compounds by the ER, and employs the same principles as competitive immunoassays based on ligand–protein interactions. For the estrogen ELRA, human estrogen receptors are used for binding estrogenic substances, and it measures the binding affinity of all EDCs with an agonistic and antagonistic impact on estrogen receptors [187]. In contrasting the sensitivity and cross-reactivity for known estrogens of the ELRA to the radio-receptor assay [186], YES [188, 189] and the E-Screen assay [190], a close correlation was obtained. Thus, it can be concluded that the ELRA can be used as a rapid and effective screening tool for environmental purposes, which have a high accuracy, fast speed, low costs, and low demands on laboratory equipment. However, because of the high variability of their results due to the heterogeneity of organisms and also the request of sensitivity, further instrument analyses may be needed to certify the results [191].

The E-screen assay measures the estrogen-induced increase in cell proliferation as a value of estrogenic property, and it is a very sensitive assay by which one can measure single as well as multiple chemicals at the same time [192, 193]. Although cell-proliferation assays may have their limitations [194], they can give quantitative estimates of test samples, so they can be quite applicable for environmental samples [195].

Unlike ELISAs, which are designed for their chemical specificity, the E-screen can detect activity in environmental samples without any information as to the chemical structure of compounds present [196]. The E-screen assay has been used in a large number of analyses of total estrogenic activity in tissue and environmental samples [197–200], but it may yield false-negative or -positive results when used in isolation [201, 202]. Thus, it is better to use it as a screening method for determining EDC activities in the aquatic environment.

The CALUX assay, which was initially developed for the detection of dioxins and dioxin-like PCBs [203], is a suitable choice for screening new and existing chemicals, as well as complex environmental samples for unexpected estrogenic activity that may be further identified by chemical analysis [30, 204–206]. In the CALUX assay, chemicals are added to sterile medium containing serum with lipids and proteins, which mediate the cellular availability and membrane transport of test chemicals. Exposure of cells to estrogens results in the uptake of chemicals through the cell membrane, binding to the endogenous ER, activation of the receptor, and consequently binding of the ligand–receptor complex to EREs present in the promoter region of the luciferase gene. Luciferase protein is then induced and is easily measured by the process of lysing the cells, adding luciferin substrate, and measuring light photon production [207].

The CALUX was more sensitive then the recombinant yeast screen [207] and the *in vivo* transgenic zebrafish assay [176] that may be explained by the lower actual target cell exposure in the transgenic fish. The high sensitivity of the CALUX assay minimizes the potential for false-negative results. However, false-positives may occur primarily

due to the fact that an *in vitro* assay may poorly predict the toxicokinetics of a substance *in vivo* [176].

VTG is a large, dimeric, highly modified egg yolk precursor protein belonging to a large family of lipoproteins [208]. It is normally synthesized in the liver of adult non-mammalian female vertebrates in response to estrogenic stimulation. The livers of both reproductively immature females and males are able to synthesize and secrete VTG in response to estrogen stimulation [209–211]. Thus, VTG has been used as a biomarker for xenobiotic exposure to estrogenic compounds in fishes, turtles and amphibians for which *in vivo* and *in vitro* assays have been developed [212–216].

To be effectively utilized as a biomarker, assays are required to accurately measure the concentration of VTG in plasma. The principal methods in use are ELISA or other types of immunoassays utilizing specific antibodies [217, 218]. ELISA techniques have proven to be a reliable and sensitive way of measuring VTG levels and have been developed to detect the concentration of VTG in the plasma for a number of fish species. Routledge *et al*. [12] kept adult male rainbow trout and adult roach exposed for 21 days in the water of environmentally relevant concentrations of 17β-E2 and E1. Plasma levels of VTG were determined using previously validated radioimmunoassays. The results indicate that environmentally relevant concentrations of natural steroidal estrogens are sufficient to account for the levels of VTG synthesis observed in caged male fish placed downstream of certain sewage-treatment plant (STP) effluent discharges in British rivers. Thorpe *et al*. [219] assessed the estrogenic activity of E2, E1, and EE2, and the combined effects of a mixture of E2 and EE2, using VTG induction in a 14-day *in vivo* juvenile rainbow trout screening assay. A competitive ELISA was developed to determine VTG in rare minnow (*Gobiocypris rarus*) based on the separation and purification of rare minnow VTG (r-VTG) as well as the production of polyclonal antibody against r-VTG in rabbits, and the results agree with GC/MS analysis [220]. Flammarion *et al*. [221] used chub as the experimental object and found that E2 exposure resulted in a significant and rapid increase in plasma VTG in both male and female chub. Plasma VTG was measured using a competitive ELISA for carp (*Cyprinus carpio*) according to Tyler *et al*. [222] with some modifications. Nilsen *et al*. [223] developed quantitative ELISAs for VTG in common carp/fathead minnow, zebrafish, and Japanese medaka. The assays were developed using a combination of monoclonal and polyclonal fish VTG antibodies in a sandwich format, using stabilized VTG from the test species as a standard. All of the VTG ELISAs for carp, fathead minnow, fathead minnow, zebrafish, and medaka have a low minimal detection limit of ng mL^{-1} level.

3. Conclusion

The occurrence of EDCs in the environment has led to a growing awareness that both animals and humans may be adversely affected. This may lead to cancer, reproductive-system disorders, decreases in sperm counts, and the disturbances in the balance of the ecosystem. Based on daily excretion of estrogens by humans and animals, dilution factor, and previous observations by other authors, ng L^{-1} levels of estrogens are expected to be present in aqueous environmental samples [25, 26, 30, 33, 34]. The analytical difficulties associated with the determination of such low estrogen

concentrations in complex aqueous matrices have limited extensive surveys on the occurrence of estrogens in the aquatic environment.

The analytical methods for residue monitoring should be specific, sensitive, simple, and economical in time and cost. The current estrogen-detection technologies are primarily based on chromatographic methods or bioassay methods. Methods such as HPLC and GC are specific and sensitive analytical methods which also have many disadvantages, such as complex pretreament processes, which are time-consuming and laborious, and toxic solvents such as acetonitrile and methanol are used as extraction solvents and/or HPLC mobile phases. These methods are suitable for confirmation but not suitable for screening large amounts of samples. Determination by GC/MS requires that the steroids be derivatized to more volatile forms prior to analysis, so LC/MS is utilized more frequently now. The sample treatment is still to be developed, such as on-line SPE, SMPE, and IAE.

ELISA is specific, sensitive, simple, and economical in time and cost. Like any other newly developed technique, immunoassays also have disadvantages, such as a significant development time, and are not suitable for multi-residue analysis. This may give erroneous results due to cross-reactions, poor accuracy, or reproducibility, and overestimation of trace amounts of estrogens caused by non-specific matrix effects [224–226]. Thus, a further confirmation analysis using instruments is needed to ascertain the results of ELISA.

The appearance of bioassay methods described above as a suitable alternative or as a complementary analytical tool can be used for screening samples, thus providing fast and specific data, but these approaches are not capable of identifying the chemical structure of environmental contaminants.

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