High-sensitivity analysis of female-steroid hormones in environmental samples

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Steroid hormones are endocrine-disrupting compounds, which affect the endocrine system at very low concentrations, so interest in the sensitive determination of steroids in the environment has increased in recent years.

In this review, we discuss in detail how to enhance the sensitivity of analytical procedures for the determination of female-steroid hormones (estrogens and progestogens) in environmental matrices. Our objective is to help the reader choose the best analytical tool for sensitive, selective and fast determination of estrogens and progestogens. A number of steps in the analytical procedure, starting with the sample pre-treatment and ending with detection, could significantly contribute to enhancing sensitivity, so they need to be thoroughly optimized.

The best results in analysis of estrogens and progestogens have been achieved with liquid chromatography (LC), as separation method, and tandem mass spectrometry (MS), as detection method, but we also discuss analysis using gas chromatography coupled to MS. Sample preparation depends on the kind of sample. Its optimization is important in reducing matrix interferences and plays a significant role in enhancing sensitivity. Liquid samples were most frequently prepared with off-line solid-phase extraction, while solid samples were also extracted by liquid-liquid, pressurized-liquid, microwave and ultrasound extraction techniques. In several studies, derivatization improved the sensitivity of LC-MS detection.

Keywords: Derivatization; Endocrine-disrupting compound; Environmental analysis; Estrogen; Gas chromatography; Liquid chromatography (LC); Mass spectrometry (MS); Progestin; Progestogen; Sample preparation

Abbreviations: 2-OHE1, 2-hydroxyestrone; 2-OHE2, 2-hydroxyestradiol; 4-OHE1, 4-hydroxyestrone; 4-MeOE1, 4-methoxyestrone; 2-MeOE2, 2-methoxyestradiol; 4-MeOE2, 4-methoxyestradiol; 6KCST, 6-ketocholestanol; 7KCHOL, 7-ketocholesterol; 16α-OHE1, 16α-hydroxyestrone; αE2, 17α-estradiol; A, Androsterone; AA, Acetic acid; ACN, Acetonitrile; ACT, Acetone; AD, Androstenedione; AmAc, Ammonium acetate; AmF, Ammonium formate; AmOH, Ammonium hydroxide; AP, Acetoxyprogesterone; αZe, α-zearalanol; βZe, β-zearalanol; E2B, 17β-estradiol-3-benzoate; βS, β-sitosterol; BBP, Benzylbutylphthalate; BCA, Biochanin A; BET, Betamethasone; BME, *tert*-butylmethylether; BPA, Bisphenol A; BSA, *N,O*-bis-(trimethylsilyl)acetamide; BSTFA, *N,O*-bis-(trimethylsilyl)trifluoroacetamide; BUD, Budesonide; BZA, Bezafibric acid; CA, Clofibric acid; CHOL, Cholesterol; ClTA, Chlorotestosterone acetate; CM, Coumestrol; CMA, Chlormadinone acetate; C3O, Coprostan-3-one; CORT, Cortisol; CP, Caproxyprogesterone; CPN, Coprostanol; CST, Campesterol; DBP, Dibutyl phthalate; DCM, Dichloromethane; DCP, 2,4-dichlorophenol; DD, Daidzein; DE, Desonide; DES, Diethylstilbestrol; DEHP, bis/di-(2-ethylhexyl)phthalate; DEP, Diethyl phthalate; DF, Diclofenac; DHE, Di-*n*-hexylether; DHT, Dihydrotestosterone; DMA, Delmadidone acetate; DST, Desmosterol; E1, Estrone; E1-d_2, Estrone-d_2; E1-3G, Estrone-3-glucuronide; E1-3S, Estrone-3-sulfate; E1-3S-d_4, Estrone-3-sulfate-d_4; E2, 17β-estradiol; E2-d_3, Estradiol-d_3; E2-17Ac, Estradiol-17-acetate; E2-17G

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Estradiol-17-glucuronide; E2-3G, Estradiol-3-glucuronide; E2-3G17S, Estradiol-3-glucuronide-17-sulfate; E2-3S, Estradiol-3-sulfate; E2-3S-d_4, Estradiol-3-sulfate-d_4; E2-3S17G, Estradiol-3-sulfate-17-glucuronide; E2-3S17S, Estradiol-3,17-disulfate; E3, Estriol; E3-d_2, Estriol-d_2; E3-3G, Estriol-3-glucuronide; E3-3S, Estriol-3-sulfate; E3-16G, Estriol-16-glucuronide; ECL, Etiocholanolone; EDC, Endocrine-disrupting compound; EDD, Ethynodiol diacetate; EE2, 17α-ethynylestradiol/17α-ethinylestradiol; EE2-d_4, 17α-ethynylestradiol-d_4; EE2-3G, 17α-ethynylestradiol-3-glucuronide; EE2-17G, 17α-ethynylestradiol-17-glucuronide; EQ, Equilin; EQN, Equilenin; EST, Ergosterol; EtAc, Ethylacetate; EtOH, Ethanol; FBIBT, 12-(difluoro-1,3,5-triazinyl)-benz[f]isoindolo[1,2b][1,3]benzothiazolidine; FA, Formic acid; FLA, Fluocinolone acetonide; FMPTS, 2-fluoro-1-methylpyridinium *p*-toluenesulfonate; FMT, Fluoxymesterone; FST, Fucosterol; GEN, Genistein; GF, Gemfibrozil; HEP, Hepatonone; Hex, Hexestrol; HMP, 2-hydrazino-1-methylpyridine; HP, 17α-hydroxyprogesterone; IB, Ibuprofen; L, Levonorgestrel; MeBol, Methylboldenone; MeD, Methandriol; MeEE2, Mestranol; MegA, Megestrol acetate; MelA, Melengestrol acetate; MeOH, Methanol; MPA, Medroxyprogesterone acetate; MPG, Medroxyprogesterone; MSTFA, *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide; MT, Methyltestosterone; NE, Norethandrolone; NEA, Norethisterone acetate; NG, Norgestrel; NOR, Norandrosterone; NO, Norethindrone; NP, 4-n-nonylphenol; NX, Naproxen; NT, Nortestosterone; OP, 4-n-octylphenol; PA, Phenylalanine; PDMS, Polydimethylsiloxane; PFBBr, Pentafluorobenzyl bromide; PFPA, Pentafluoropropionic anhydride; PG, Progesterone; PGL, Prostaglandin; PNT, Nandrolone phenylpropionate; PREG, Pregnenolone; PS, Pinosylvin; PT, Testosterone propionate; RV, Resveratrol; SMT, Stigmastanol; SST, Stigmasterol; Stan, Stanozol; T, Testosterone; TB, Trenbolone; TBA, Trenbolone acetate; TEA, Triethylamine; Tm, Tamoxifen; TMS, Trimethylchlorosilane; TOPO, Tri-*n*-octylphosphine oxide; TRA, Triamcinolone acetonide; Z, Zeranol; Ze, Zearalenone; CPE, Cloud-point extraction; DLLME-SFO, Dispersive liquid-liquid microextraction with solidification of a floating organic drop; ECAPCI, electron-capture atmospheric pressure chemical ionization in negative mode; ECNI, Electron-capture negative ionization; HF-MMLLE, Hollow-fiber microporous membrane liquid-liquid extraction; LOV, Lab on valve; MAD, Microwave-accelerated derivatization; MAE, Microwave-assisted extraction; MASE, Microwave-assisted solvent extraction; SBSE, Stir-bar sorptive extraction; SBSEC, SBSE based on polydimethylsiloxane; SBSEM, SBSE based on poly(vinylpyridine-ethylene dimethacrylate) monolithic material; SEC, Size-exclusion chromatography; SPDE, Solid-phase disk extraction; STP, Sewage-treatment plant; TD, thermal desorption; TIS, Turbo ion-spray source; WWTP, Wastewater-treatment plant

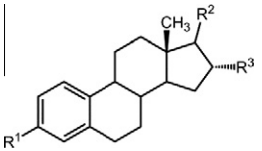
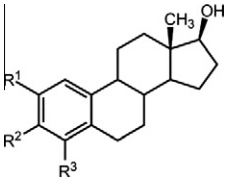
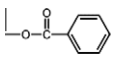
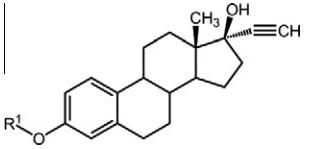
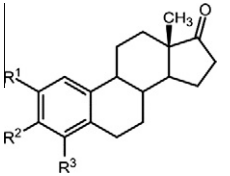
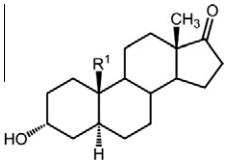
1. Introduction

Exposure to the natural and/or synthetic chemicals, which may interfere with the reproductive system and its development, is controversial in environmental science because of the potential risks to wildlife and humans. Due to their considerable effect on reproductive system in wildlife and humans, these chemicals are called ''endocrine-disrupting compounds'' (EDCs) [i.e. exogenous substances that interfere with the endocrine system (synthesis, secretion, transport, binding, action and elimination of natural hormones) and disrupt the physiologic function of hormones in the body], as reviewed by Miége et al. [1]. More detail about mechanisms of action and other effects of EDCs are available [2]. EDCs encompass not only estrogens, but also a wide range of chemicals, most of which are introduced into the environment by anthropogenic activities [3]. Estrogens and progestogens are the group of female-steroid hormones derived from cholesterol (CHOL) (Table 1). We can distinguish between endogenous and exogenous steroids. Endogenous estrogens, namely estradiol (E2), estriol (E3) and estrone (E1), and progestogens [e.g., progesterone (PG), 17α-hydroxyprogesterone (HP) and 10α-hydroxyprogesterone] are natural compounds in animals and humans. Exoestrogens include phytoestrogens (e.g., isoflavones), synthetic estrogens (e.g., diethylstilbestrol – DES), progestins (e.g., levonorgestrel – L), and industrial chemicals with suspected estrogenic activity (e.g., bisphenol A – BPA, and 4-nonylphenol – NP). Female steroids are widely used as contraceptives and also as medicaments for their protective function against various diseases. They have been administered in hormone-replacement therapy, helping in the treatment of hormonal disorders [4,5].

There is a considerable increase in the consumption of estrogens in human medicine (i.e. primarily contraception, management of menopausal and post-menopausal syndrome, physiological replacement therapy in deficiency states and treatment of prostate cancer) and in animal farming (i.e. growth promoters and developers of single-sex fish populations in aquaculture) [1,6]. Ethynylestradiol (EE2) is one of the two most common components contained in combined oral contraceptives (30–50 μg/tablet/day) with the other component being PG. The medication with exoestrogens has negative effects as well, one of the most serious being the development and evolution of breast cancer [4,5], so monitoring of steroid levels in urine during the hormone therapy is very important. Recent reviews about determination of steroid hormones in biological materials discussed this issue in detail [7,8].

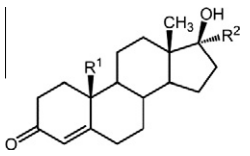
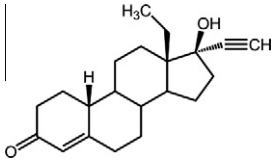
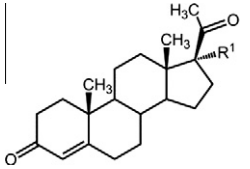
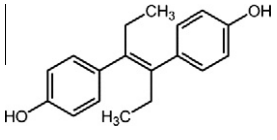
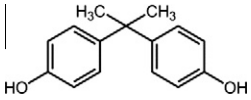
Another serious problem caused by steroid therapy involves pollution of the global environment. The occurrence of estrogenic substances in aquatic systems has already been described [9]. Estrogens are usually not entirely metabolized and they reach the aquatic environment mainly via effluents from wastewater-treatment plants (WWTPs) [1]. The natural hormone E2, its metabolites (E1 and E3) and conjugates (glucuronides and sulfates) are mainly excreted in the urine of mammals [10]. The main urinary excretion product is the sulfate of E1 [11].

Table 1. Steroid hormones with their molecular weights, p*Ka* and logP values, which were obtained from the SciFinder scholar database.

Abbreviation	Name	R1	R2	R3	MW	p*Ka*	K_{OW} (log P)	Structure
E3	Estriol	-OH	-β-OH	-OH	288.38	10.25	2.527	
E3-3S	Estriol-3-sulphate	$-OSO_3H$	-β-OH	-OH	368.44	-3.82	1.713	
αE2/ E2	(17α-)/(17β-)estradiol	-OH	-α-/β-OH	-H	272.38	10.27	4.146	
E2-17G	Estradiol-17-glucuronide	-OH	-β-O-glucuronic acid	-H	448.51	2.82	3.807	
16-oxoE2	16-oxoestradiol	-OH	-β-OH	=O	286.37	10.25	2.55	
E2-17Ac	Estradiol-17-acetate	-OH	$-\beta\text{-}OCOCH_3$	-H	314.42	10.26	5.027	
2-MeOE2	2-methoxyestradiol	$-OCH_3$	-OH	-H	302.41	10.29	3.842	
4-MeOE2	4-methoxyestradiol	-H	-OH	$-OCH_3$	302.41	10.29	3.929	
2-OHE2	2-hydroxyestradiol	-OH	-OH	-H	288.38	10.12	3.338	
E2-3S	17β-estradiol-3-sulphate	-H	$-OSO_3H$	-H	352.45	-3.82	3.331	
E2B	17β-estradiol-3-benzoate	-H		-H	376.49	15.06	5.095	
EE2/ αEE2	(17α-)ethynylestradiol	-H	-	-	296.4	10.24	4.106	
MeEE2	Mestranol (Me)	$-CH_3$	-	-	310.43	13.10	4.938	
E1	Estrone	-H	-OH	-H	270.37	10.25	3.624	
E1-3G	Estrone-3-glucuronide	-H	-O-glucuronic acid	-H	446.49	2.80	1.144	
E1-3S	Estrone-3-sulphate	-H	$-OSO_3H$	-H	350.43	-3.84	2.810	
16α-OHE1	16α-hydroxyestrone	-H	-OH	-H C16: α-OH	270.37	13.07	2.863	
4-OHE1	4-hydroxyestrone	-H	-OH	-OH	286.37	10.06	2.713	
2-OHE1	2-hydroxyestrone	-OH	-OH	-H	286.37	10.10	2.817	
4-MeOE1	4-methoxyestrone	-H	-OH	$-OCH_3$	300.39	10.27	3.407	
2-MeOE1	2-methoxyestrone	$-OCH_3$	-OH	-H	300.39	10.27	3.321	
A	Androsterone	$-CH_3$	-	-	290.44	15.14	3.932	
NOR	19-norandrosterone	-H	-	-	276.41	15.13	3.651	

(*continued on next page*)

Table 1. (*continued*)

Abbreviation	Name	R1	R2	R3	MW	p*Ka*	K_{OW} (log P)	Structure
T	Testosterone	$-CH_3$	-H	-	288.42	15.06	3.179	
MT	17α-methyltestosterone	$-CH_3$	$-CH_3$	-	302.45	15.13	3.559	
NT	19-nortestosterone	-H	-H	-	274.4	15.06	2.898	
NG	(*D*)-norgestrel	-	-	-	312.45	13.09	3.368	
L	(l)-norgestrel, levonorgestrel	-	-	-	312.45	13.09	3.368	
PG	Progesterone	-H	-	-	314.46	-	3.827	
AP	17α-acetoxyprogesterone	$-OCOCH_3$	-	-	372.50	-	3.638	
HP	17α-hydroxyprogesterone	-OH	-	-	330.46	13.03	3.040	
DES	Diethylstilbestrol	-	-	-	268.29	10.18	5.330	
BPA	Bisphenol A	-	-	-	228.29	10.29	3.641	

The compounds contained in contraceptives (EE2 and mestranol - MeEE2) also have a high endocrine potential and they are also excreted in urine by women medicated with these drugs [10]. The conjugates can be degraded in sewage-treatment plants (STPs), resulting in the release of active parent compound [12]. Large amounts of animal liquids and biosolids applied on agricultural fields might flow into nearby bodies of water or infiltrate through the soil into groundwater [1]. Cattle and poultry manure have been reported as a source of the environmental loadings of E2 [1]. Due to the activity of β-glucuronidase, glucuronides are decomposed before reaching STPs, but concentrations of the estrone-3-sulfate (E1-3S), may be important when considering total load reaching STPs [11].

Exposure of freshwater estuarine or fish living in estuarine to EDCs may alter their sexual function (feminization of fish, reproductive and developmental effects), and have some toxicological effects, particularly in ecosystems receiving high levels of poorly diluted WWTP effluents [1,9]. Since the sources of estrogens cannot be eliminated, a number of specific treatment processes in STPs have been optimized and discussed [9], so it is also important to determine the fate and the distribution of steroids and their conjugates in the environment.

Steroids are compounds with a potent activity at low concentrations (<ng/L) in target tissues, so effective analysis of steroids in liquid and solid environmental samples is necessary. For trace-level determination of steroids with similar structures contained in complex sample matrices, sensitive and selective methods are required [12]. This comparative overview of the literature published between 2004 and 2011 describes environmental analysis of female-steroid hormones at low concentrations. We address all individual steps involved in the analytical procedure, which may positively influence particularly the sensitivity of the analysis. We discuss chromatographic conditions, detection and sample preparation and their influence on separation efficiency, selectivity, and method sensitivity. Our aim was to find a method with the best characteristics (i.e. fast, selective and with the high sensitivity).

2. Sample preparation

Steroid hormones possess non-polar and non-ionic characteristics (see Table 1) that enable use of reversed-phase purification and separation strategies. The sample-preparation process is one of the most important and time-consuming parts of the analytical method. Its optimization plays an important role in the enhancement of sensitivity and the reduction of matrix interferences in wastewater, sludge and biological samples. The application of sample preparation with high pre-concentration is necessary to achieve limits of detection (LODs) at the ng/g level in solid samples and the ng/L level in liquid samples. Most analytical methods include a solid-phase extraction (SPE) step alongside a more time-consuming purification step (e.g., gel-permeation chromatography, normal-phase preparative chromatography or immunoaffinity clean-up) [9]. Normally, further clean-up steps are unnecessary. However, Kumar et al. [13] attached a Sep-Pak Plus NH2 cartridge (silica-based polar bonded phase with basic character) below the dried Oasis HLB cartridge (hydrophilic-lipophilic balanced copolymer) as a sample clean-up step, and improved gradient elution to eliminate matrix interferences. The use of the Sep-Pak Plus NH2 cartridge significantly reduced the amount of co-extracted acidic interferences [13].

2.1. Aqueous samples

Aqueous samples are usually processed with filtration followed by SPE, as outlined in the review by Streck [14]. This method is preferred for its experimental simplicity, easy automation, smaller consumption of non-aqueous solvents, and the availability of many sorbents with different selectivity [15].

2.1.1. Solid-phase extraction. SPE involves optimization of a number of steps, including conditioning, elution, sample volume, and type of SPE sorbent (Table 2). The hydrophobicity of each compound investigated can be estimated by its log P (log Kow), which is very important when choosing suitable conditions for the extraction (see Table 1).

C18, HLB, amine and STRATA X sorbents (copolymer adsorbents) were very often utilized for aqueous samples [13,14,16] (Table 2). Oasis HLB sorbent has enormous potential for the extraction of compounds with high polarity [14,17,18]. This sorbent was favored from other sorbents utilized for extraction in several comparison studies [16,19,20]. It provided high recoveries and excellent capture capabilities for acidic and neutral analytes across a wide polarity range.

By contrast, the results of Kuster et al. [21] demonstrated better performance of reversed-phase C18 cartridges (LiChrolut RP-18) for phytoestrogens (resveratrol, daidzein, coumestrol and genistein) at pH 7. For the analysis of selected compounds, Vega-Morales et al. [22] chose Sep-Pak Vac C18 from five solid-phase materials, including Sep-Pak Vac C18, Oasis HLB, Bond Elut-ENV, BondElut Plexa, and LiChrolut EN. In Laganà et al. [6], STRATA X-AW provided better results than Oasis HLB. Table 2 shows other types of SPE sorbents used for steroid extraction with high efficiency.

The use of polymeric cartridges (Oasis HLB and PLRP – polymeric reversed phase), selective tailor-made sorbent materials (e.g., molecularly-imprinted polymers – MIPs, immobilized receptors or antibodies – immunosorbents,

Table 2. Sample preparation in analysis of estrogens and progestogens

Substance	Matrix/Sample volume	Sample preparation	Clean up cartridges	Conditioning	Elution	Flow rate (mL/min)	Derivatization	Method	LOD (ng/L)	Recovery (%)	Ref.
E1, αE2, E2, E3, EE2, E1-3S, E2-3S, E3-3S, E1-3G, E2-17G, E2-3G, E3-3G, E2-3S17G, E2-3G17S	Environment. waters, 1 L	SPE	Autoprep EDS-1	10 mL MeOH 10 mL H_2O	6 mL EtAc (free estrogens) 10 mL 5 mM TEA/MeOH (conjug. estrogens)	20	–	LC-MS^2	Method detection limits (MDLs): 0.1–3.1	70–120	[29] (2003)
E1, αE2, E2, E3, EE2	Water samples, 250 mL	SPE	Oasis HLB	10 mL MeOH 10 mL H_2O	10 mL EtAc	10	On-line derivatiz. FBIBT in DMSO	LC-MS^2	0.067–0.29	75–88	[27]
BPA, E1, E2, E3, NP, OP	Surface waters; 1 L	SPE	Oasis HLB	6 mL MeOH 6 mL H_2O	2 × 3 mL MeOH	10	–	LC-MS^2	0.04–1.0	72–140	[35]
Estrogens, androgens and progestogens	WWTP and river Infl.: 70 mL Effl.: 200 mL River: 2 L	SPE	Oasis HLB followed by silica cartridges	6 mL EtAc 6 mL ACN 12 mL H_2O 4 mL EtAc 3 mL hexane:EtAc (90:10; v/v) 12 mL H_2O	15 mL EtAc 3 mL hexane:EtAc (38:62; v/v)	5–10 5–10	–	LC-MS^2	0.02–40	78–100	[24]
E1, E2, E3, E1-3G, E2G, E3-3G, E3-16G, E1-3S, E2-3S, E3-3S	River water, 500 mL	SPE	Oasis HLB	6 mL EtAc 6 mL MeOH 6 mL H_2O	6 mL EtAc (free estrogens) 6 mL MeOH with 2% AmOH (conj. estrogens)	6–8	Dansyl chloride	LC-MS^2 (Column switching)	0.049–8.9	68–105	[31]
E3-3S, E3-16G, E2-17G, E1-3G, EE2S, E3, E1-3S, E2, EE2, E1, DES, DD, RV, CM, G, NO, BCA, L, PG	Environment. waters, 500 mL	SPE	LiChrolut RP-18	5 mL H_2O 5 mL MeOH	5 mL MeOH 5 mL MeOH with 2% AmOH	0.2	–	LC-MS^2	0.04–2.01	81–153	[21]
E1, E2, E3, EE2, E1-3G, E2-17G, E3-3G, E1-3S, E2-3S, E3-3S, E2, E1-d_2, E2-d_3, E3-d_2, EE2-d_4, E1-3S-d_4, E2-3S-d_4	Wastewater from a WWTP and river water, 1000 mL	SPE and cleanup	SPE: Oasis HLB Cleanup: Sep-Pak Plus NH2 cartridge	6 mL MeOH 6 mL H_2O 5 mL H_2O	8 mL MeOH (free estrogens) 6 mL 0.5% AmOH in MeOH	10	–	LC-MS^2	0.2–0.8	63–127	[13]
E1, E2, EE2	River water, 2 L	SPE	Oasis HLB	5 mL EtAc 5 mL MeOH 3 × 5 mL H_2O	10 mL MeOH	Sample Loading: 10 Elution: 1	BSTFA	GC-MS^2	0.3	72–119	[34]
E1, E2, EE2, sulfonamides, tetracyclines and analgesics	Surface and wastewater; 1 L	SPE	Oasis HLB	5 mL MeOH 5 mL H_2O (pH 4)	2 × 5 mL MeOH	10	–	LC-MS^2	0.3–2.0	70–94	[36]

E1, E2, EE2	WWTP	On-line SPE	Oasis HLB	–	–	0.3–1	Dansyl chloride	LC-MS^2	0.4–0.7	79.7–95	[25]
BPA, E1, αE2, E2, E3, EE2	Water samples	On-line SPE	PLRP-s	4 mL ACN 4 mL H_2O	–	1.5	–	LC-MS^2	0.5–1	68–134	[38]
E1, E2, E3, EE2 and progestins	Environment. waters, 100 mL	SPDE	ENVI-18 SPE disk	10 mL MeOH 10 mL H_2O	3 × 10 mL ACN	150	–	LC-MS^2	0.5–3.4	76.7–97.3	[55]
			C-8 disk	10 mL MeOH 10 mL H_2O	5 mL ACN				–	23.9–78.9	
		SPE	C-18 cartridge	3 mL MeOH 3 mL H_2O	3 × 10 mL ACN	10				74–99.3	
BPA, E1, E2, E3, EE2	Sewage samples, 2.5 L	SPE	Sep-Pak Vac C18	3 × 5 mL MeOH 3 × 5 mL H_2O	2 mL MeOH	1–10	–	LC-MS^2	0.5–6	60–104	[22]
E1, E2, E3,EE2, E2-3S, E2-17Ac, E1-3S, 16α-OHE1, MeEE2	WWTP, 1 L	SPE followed by SEC	SPE: DVB-phobic Speedisk Cleanup: SEC	15 mL MeOH 15 mL H_2O	15 mL BME 15 mL MeOH	–	–	LC-MS^2	0.6–1.8	58–112	[10]
E1, E2, E3, EE2, LEV, NOR, MPG, PG	Environment. waters, 3 mL	On-line SPE	Hypersil GOLD C18	0.1% FA in H_2O	–	0.2	–	LC-MS^2	0.6–25	–	[26]
E1, E2, E3	Tap and sewage water	HF-MMLLE	Accurel PP (polypropylene) HF-membrane	DHE containing 10% (w/v) TOPO	H_2O	–	MSTFA	GC-MS	1.6–10	–	[44]
DES, E1, αE2, E2, E3, EE2, glucuronide, acetates and sulfates	Environment. waters, 500 mL river, 100 mL infl., 250 mL effl.	SPE	Oasis HLB	5 mL MeOH 5 mL H_2O	5 mL MeOH with 5% ACN	–	–	LC-MS	1–70	23–87	[16]
E1, E2, E3, EE2, NP, BPA	Activated sludge, 400 mL	SPE: water samples	SPE: Oasis HLB	5 mL MTBE 5 mL MeOH 5 mL H_2O	–	4–5	BSTFA + 1% TMCS	GC-MS	0.2–30.3	87–129	[17]
			Cleanup: Sep-Pak Plus silica cartridge	5 mL DCM:ACT (7:3; v/v)	10 mL DCM:ACT (7:3; v/v)	1–2					
BPA, E2, E3, EE2	Environment. waters	In-tube SPME	poly(AA-VP-bis) monolith	–	–	0.2		LC-FD/DAD	6–100	88–116	[41]
E1, E2, E3, E2-17Ac, EE2	Wastewater recycling systems, 1 L	SPE	STRATA X	–	–	–	–	LC-UV	40–70	66–118	[33]
DES, NT, MT, PG, PNT, PT, T	Wastewater	SBSEM-LD	Commercial stir bars coated PDMS	–	–	–	–	LC-DAD	140–410	48–110	[43]
E1, αE2, E2, EE2, E3	River waters, WWTPs Infl.: 100 mL Effl: 250 mL	Enzymatic hydrolysis, SPE	Oasis HLB followed by Florisil	6 mL MeOH 6 mL H_2O -	4 mL EtAc:MeOH (70:30; v/v); 5 mL ACT:heptane (75:25; v/v)	10	–	LC-MS^2	LOQ: 150–700	82–115	[1]

(*continued on next page*)

Table 2. (*continued*)

Substance	Matrix/Sample volume	Sample preparation	Clean up cartridges	Conditioning	Elution	Flow rate (mL/min)	Derivatization	Method	LOD (ng/L)	Recovery (%)	Ref.
E1, E2, E3	Water	CPE	–	Non-ionic surfactant TritonX-114 and 0.4 M Na_2SO_4		–	–	LC-UV	230–5000	81–99.5	[45]
E1, E2, E3, EE2	Water samples	DLLME-SFO	–	Extraction solvent: 1-dodecanol and 1-undecanol Dispersive solvent: MeOH		–	–	LC-PDA	800–3100	87–116	[46]
E1, αE2, E2, E3, E2-17Ac, EE2, E1-3G, E2-17G, E1-3S, E2-3S	Sewage sludge, 10 g	Lyophylization and homogenization and PLE	Sludge mixed with Al_2O_3	Extraction solvents: MeOH:ACT (1:1; v/v) and H_2O:MeOH (1:1; v/v)		–	–	$LC\text{-}MS^2$	0.15–175 ng/g	81–100	[48]
A, BET, BUD, DE, E1, FLA, L, NOR, PG, T, TRA	Soil, 5 g	PLE followed by SPE	Oasis HLB	5 mL ACN 5 mL ACN:AmOH (95:5; v/v), 5 mL ACN:H_2O (10:90; v/v)	5 mL ACN: AmOH (95:5; v/v)	2	–	$LC\text{-}MS^2$	0.08–2.84 ng/g	>80	[19]
E1, E2,EE2	River sediments, 1 g	MASE followed by SPE followed by silica cartridge	MASE SPE: STRATA X-AW	5 mL MeOH as extraction solvent SPE: 5 mL EtAc 5 mL MeOH 5 mL H_2O	SPE: 7 mL EtAc	–	–	$LC\text{-}MS^2$	0.015–0.04 ng/g	82–98	[9]
			Cleanup: silica cartridge	4 mL cyclohexane:EtAc (6:4, v/v), 4 mL cyclohexane	6 mL cyclohexane:EtAc (6:4, v/v)						
E1, E2, E3, EE2, NP, BPA	Activated sludge, 1 g	ULE followed by SPE and cleanup	ULE: 3 × 5 mL MeOH:ACT (1:1; v/v)			–	BSTFA + 1% TMCS	GC-MS	LOQ: 1.2–188.7 ng/g	71–124	[17]
			SPE: Oasis HLB	–	10 mL DCM:ACT (7:3; v/v)						
			Cleanup: Al_2O_3/silica gel column	15 mL MeOH:ACT (1:1; v/v) and 5 mL hexane	10 mL hexane, 10 mL hexane:ACT (2:1; v/v), 20 mL MeOH:ACT (1:1; v/v)	2					
αE2, E2, E2-d_4, E3, EE2	Sediments	MAE	–	Extraction with 10 mL MeOH:H_2O (95:5; v/v)	After evaporation dissolved in 0.2 mL MeOH:H_2O (1:1; v/v)	–	–	$LC\text{-}MS^2$	90–250 ng/g	98.8–107	[49]

and restricted access materials – RAMs) was typical for on-line extraction [14,23].

Sample volume varied according to type of matrix (from several mL to 4 L) and to type of extraction method [24]. The matrix of the sample can strongly affect the recoveries and the sensitivity in sewage-water samples depending on the sample volume [16]. Low volume of the sample speeds up sample preparation, but it can lead to lower pre-concentration. The sample volume of 1 L (commonly used) provided a 2000 times concentrated sample when reconstituted in 0.5 mL of proper solvent. Vega-Morales et al. [22] tested various sample volumes and observed equal signal intensities of sample volumes in the range 100–1000 mL for αE2, E2, BPA and other studied analytes [22]. In the on-line SPE configurations, small volumes (about 1 mL) were sufficient to obtain adequate sensitivity [23,25,26].

Elution was typically accomplished by methanol (MeOH) [4,5,13,15,21,26] and ethyl acetate (EtAc) (very often with connection to derivatization) [10,27,28] or in combination (Table 2). For the elution of estrogen-conjugates triethylamine (TEA) [10,29,30] as an ion-pair reagent and 2% ammonium hydroxide (AmOH) [21,31] were added into MeOH, while free estrogens were eluted with the help of EtAc or MeOH [13,29–31]. Moreover, the addition of 5% acetonitrile (ACN) into MeOH improved the recoveries of real samples [16]. In general, more hydrophobic EDCs (with a higher log P value) were eluted faster by MeOH with acetone (ACT) from the clean-up column [17]. Several authors divided the elution into two or three steps, and dried the sorbent between elution steps without changing elution solvent [4,6,13,15,19,30,31].

In most cases, SPE was performed off-line [1,6,13,16,21,22,24,27–37]. Only in a few studies SPE extraction was applied on-line [23,25,26,38]. With on-line SPE, it is possible to decrease the sample-preparation time, increase the sample throughput, and improve the sensitivity, especially in combination with liquid chromatography coupled to tandem mass spectrometry (LC-MS^2) [23,39]. This technique was successfully used for estrogen determination in influent/effluent WWTPs with the combination of derivatization with dansyl chloride [25]. Matrix effects, less flexibility, limited portability, expensive equipment and absence of extracts for further analysis or verification are among the disadvantages of on-line SPE [23].

Miniaturization of extraction methods has become a very pronounced trend in analytical chemistry. Some examples of miniaturization in sample preparation of steroid hormones are solid-phase microextraction (SPME) [40,41], and stir-bar sorptive extraction (SBSE) [42,43].

Yang et al. [40] revealed that both traditional methods (SPE and SPME) were comparable. The SPME technique was very often applied for sample preparation of anabolic steroids. Conventional SPME fibers have problems with fragility, low sorption capacity, and bleeding of thick-film coatings, which resulted in development of in-tube SPME. Wen et al. [41] compared monolith (polyether ether ketone – PEEK) in-tube and fused-silica capillary columns. The results indicated that the monolithic material showed great extraction capacity for the EDCs studied and high sensitivity within a short period of time (20 min and 16 min). The total time of the analysis, involving extraction, desorption and chromatography, was less than 34 min [41]. The in-tube SPME showed 50–89-fold higher sensitivity and the capillary column extraction 7–14-fold higher sensitivity than the direct-injection method (20 μL) [41]. Despite of these new methods, SPE still remains the most widely used.

2.1.2. Liquid-liquid extraction. Due to high recoveries and adequate selectivity, liquid-liquid extraction (LLE) has traditionally been used for the analysis of steroids but the main drawbacks were the long time for the procedure, the higher sample volume and the formation of emulsion [5]. This method was demonstrated to be time consuming in several studies, so new miniaturized LLEs (e.g., microextraction or green techniques) have been developed [44–47]. Cloud-point extraction (CPE), one of the green LLEs, was successfully applied to pre-concentration and extraction of estrogens in the study by Wang et al. [45]. This method had low LODs (0.23–5.0 ng/L) and high recoveries (81–99.5%) [45]. Another very fast, easy extraction technique used in analysis of steroids was dispersive liquid-liquid microextraction based on the solidification of a floating organic drop (DLLME-SFO) [46]. The time of the extraction was shortened from 48 h to a few minutes, recoveries were 87–116% in river water and 89–101% in tap water, and the LOD achieved by LC with ultraviolet detection (LC-UV) was 1.08 μg/L [46]. A hollow-fiber microporous membrane LLE (HF-MMLLE) developed for determination of estrogens in sewage-water samples is also one of the green techniques (20 μL of organic solvent and 100 mL of sample) [44]. This method coupled with gas chromatography-MS^2 (GC-MS^2) showed low LODs (1.6–10 ng/L) with high enrichment factors and extraction efficiency, but long processing time (180 min) [44].

2.2. Solid samples

Steroids and phenolic compounds tend to accumulate a great deal in solid matrices due to their lipophilicity expressed by their higher log P values [3]. Sediment may thus adsorb considerable amounts of phenolic EDCs and estrogens [3], so precise quantification of sex hormones and phenolic EDCs in sediment, especially in areas with higher accumulation, is necessary.

Solid sample pretreatment of steroid hormones includes more steps because of the complexity of the matrices, which can significantly affect the recovery

efficiencies of EDCs studied [17]. Comparison of the recovery efficiencies of BPA, E1 and E2 in solid and liquid samples indicated a decrease in the case of BPA [17]. Gomes et al. [47] also reported low recovery (57–58%) of E3 in their review analyzing estrogens in river-sediment samples. E3, with the smallest log P value (i.e. 2.527), had the highest polarity (three hydroxy groups) among the estrogens studied. It bonded more easily to the sample matrix, so it was more difficult to recover.

The most frequently used extraction techniques in the solid-sample pretreatment were SPE [17,19,23,24,47], pressurized-liquid extraction (PLE) [19,48] and microwave-assisted (solvent) extraction (MAE/MASE) [9,49]. Peng et al. [3] used column chromatographic fractionation for purification. This extraction method was time consuming with low LODs (0.6 ng/g) [3].

3. Analytical methods

A great number of studies describing steroid analysis have been published, but some problems still persist. The concentration of steroid hormones in real samples is extremely low (usually at the level of sub-ng/L to ng/L). The great variety of steroid compounds enhances the difficulty of their detection. Not only is effective clean-up pretreatment needed, but also a selective separation method and sensitive detection.

Nowadays, the most important methods used for steroid determination are LC-MS, LC-UV/FD (fluorescence detection), GC-MS, and immunoassays (IAs) (Tables 3–5). Previously, IAs were extensively applied in the field of steroid determination in biological matrices, but they have been replaced because of the problem with the cross-reactivity of polyreactive antibodies, analysis of only one analyte at a time, and no structural validation of the analyte [50].

Both LC and GC are considered to be the primary methods for the separation of steroids. Various types of detection for determination of estrogens have been used: MS^2>MS>FD>UV (ordered by decreasing sensitivity) [10,12,23,25,37,41,45,47]. It is difficult to achieve trace analysis with conventional detectors (UV and FD). Table 4 confirms this premise. Considering the low LODs (of the order of ng/L or ng/g), GC-MS/GC-MS^2 and especially LC-MS/LC-MS^2 are the methods of choice in the steroid-hormone analysis (Tables 3 and 5).

3.1. Liquid chromatography coupled to mass spectrometry

Currently, LC-MS and LC-MS^2 have become widely used tools for determination of estrogens in environmental samples because of their sensitivity and specificity. Unlike GC-MS, LC-MS is not limited by the non-volatility and the high molecular weight of steroids, and enables the determination of both conjugated and non-conjugated estrogens without a derivatization step or hydrolysis [9,51].

Nevertheless, the use of LC-MS as an analytical tool is not without difficulties [11]. Matrix effects occurring in LC-MS can result in ion suppression or enhancement of the signal of target analytes [39]. They are caused by the coelution of the peaks of interest with the matrix components [39]. Electrospray ionization (ESI) is known to be particularly predisposed to matrix suppression and isobaric interference when analyzing estrogens [11]. High efficiency of chromatographic separation as well as extensive clean-up steps to remove interferences arising from the matrix are necessary [9]. Comparison of ESI and atmospheric pressure chemical ionization (APCI) in terms of matrix effects in LC-MS^2 analysis has revealed a reduction of matrix effects in APCI. However, the sensitivity obtained in standard solutions was lower in APCI [10].

3.1.1. Chromatographic conditions. The choice of mobile phase is very important for not only better separation but also enhancement of ionization in MS. The most frequently used mobile phase was a combination of water and ACN [1,9,12,13,29,51–55], then water with MeOH [10,11,24,32,55–57]. In many studies [19,21,25,27,32,49,58,59], formic acid (FA) was another component of the mobile phase.

Sun et al. [55] tested six mobile phases for the analysis of E2, and the best response was obtained by MeOH-water, which also provided better efficiency in separating the matrix components, higher responses, and better peak shapes in other studies [49,58]. In ESI, MeOH-water as mobile phase gave higher ionization ratios (2–3 times) [10] and increased MS^2 signals (40–45%) [27]. However, in the work of Matějíček and Kubáň [27], unresolved peaks of αE2 and E2 after using MeOH were observed, and the highest selectivities were accomplished using ACN and 0.1% FA (v/v) [27]. Better resolution was also attained using acetic acid (AA) but the intensities of MS^2 signals decreased (in the range 13–18%) [27]. Improved response of the progestogens and detection of the phytoestrogens (in the same chromatographic run) were observed after addition of FA to the mobile phase comprising ACN-water [21].

Conventional LC (HPLC) is relatively time-consuming, so recently UHPLC has taken over increasingly in the analysis of female steroids [13,32,54,55,58]. UHPLC offers enhanced resolution, sensitivity, speed, and efficiency [30,39,54]. High sensitivity and specificity was also achieved with a two-dimensional (2D) chromatographic separation [39,49]. Lien et al. [58] reported that UHPLC provided sharper peaks (at width < 0.06 min) than 2D-LC with peak shapes at width < 0.3 min (Fig. 1).

In almost all studies, gradient elution was carried out. More detail about chromatographic conditions (type of

Table 3. LC-MS^2 methods for measurement of steroids

Substance	Matrix, sample preparation	Method	Column	Mobile phase/flow rate	Detector	Analysis time (min.)	Derivat.	LOD (S/N = 3) (ng/L)	Ref.
E1, αE2, E2, E3, EE2	Water samples SPE	LC-MS^2	Luna Phenyl-hexyl 50 × 2.0 mm; 3 µm	Linear gradient 0.25 mL/min A: 0.1% FA B: ACN	ESI(+)-IT SRM	15	FBIBT in DMSO	0.067–0.29	[27]
αEE2, E2, E1	Wastewater Immunoextraction	LC-MS^2	Betasil C_{18} 150 × 2.1 mm; 3 µm	Linear gradient 0.2 mL/min A: ACN B: H_2O	ESI(−)-QqQ	16	–	0.07–0.18	[52]
BPA, E1, E2, E3, NP, OP	Surface waters SPE	LC-MS^2	Zorbax SB-C18 30 × 2.1 mm; 3,5 µm	Gradient elution 0.3 mL/min A: 0.1% AmOH B: 0.1% AmOH in MeOH	ESI(−)-QqQ SRM	10	–	0.04–1.0	[35]
BCA, CM, DAI, DES, E1, E2, E3, E1-3G, E2-17G, E3-16G, E1-3S, E3-3S, EE2, EE2S, GEN, L, NOR, PG, RV	Environment. waters SPE	LC-MS^2	Guard column 4 × 4 mm; 5 µm STAR RP-18 125 × 2 mm; 5 µm	Gradient elution 0.2 mL/min A: H_2O (for estrogens); 0.1% FA (for phytoestrogens and progestogens) B: ACN	estrogens: ESI(−)-QqQ SRM progestogens and phytoestrogens: ESI(+)-QqQ SRM	45	–	0.04–2.0	[21]
Estrogens, androgens and progestogens	WWTP and river waters SPE	LC-MS^2	BEH C18 100 × 2.1 mm; 1.7 µm	Gradient elution 0.3 mL/min Estrogens: A: 0.1% AA B: ACN Progestogens and androgens: A: 0.1% FA B: MeOH	ESI(+)-QqQSRM	5.6 4.6	–	0.02–40	[24]
E1, E2, E3, E1-3G, E2-17G, E3-3G,E3-16G, E1-3S, E2-3S, E3-3S	River water	LC-MS^2 (Column switching)	Luna C18 100 × 2.0 mm; 3 µm ZIC-pHILIC 100 × 2.1 mm; 5 µm	Gradient elution 150 µl/min A: ACN: 5 mM AmAc (pH 6.8), 95:5 (v/v) B: ACN: 5 mM AmAc (pH 6.8), 75:25 (v/v)	Free and conjugated: TIS(−)-Q-IT Derivatized: TIS(+)-Q-IT SRM	25	Dansyl chloride	0.049–8.9	[31]
EE2, E2, E3, E1-3S	Wastewater SPE	LC-MS^2	Gemini C18 100 × 2 mm; 3 µm	Gradient elution 10 µL/min A: 0.1% AmOH B: MeOH with 0.1% AmOH	ESI(−)-QqQ SRM	18	–	0.1–0.2	[11]
E1, E2, 16α-OHE1, HEP, 2-MeOE1, PA, PGL	Mixture of standards	LC-MS^2	YMCbasic 150 × 4.6 mm; 5 µm	Linear gradient 1 mL/min A: H_2O B: MeOH	ENCI-QqQ ECAPCI(−)-QqQ SRM	13	PFBBr –	0.14–0.74 amol (ENCI) 7.02–62 fmol (APCI(−))	[56] (2000)

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Table 3. (*continued*)

Substance	Matrix, sample preparation	Method	Column	Mobile phase/flow rate	Detector	Analysis time (min.)	Derivat.	LOD (S/N = 3) (ng/L)	Ref.
E1, αE2, E2, E3, EE2, E1-3S, E2-3S, E3-3S, E1-3G, E2-17G, E2-3G, E3-3G, E2-3S17S, E2-3S17G, E2-3G17S	Environment. waters SPE	LC-MS^2	Zorbax Extend-C18 150 × 1 mm; 3.5 μm	Gradient elution 40 μL/min A: ACN B: H_2O C: 100 mM TEA (pH 12.2)	ESI(−)-QqQ SRM	29	–	0.1–3.1	[29] (2003)
BPA, BCA, DD, E1, E3, E2,EE2, G, NP, Ze, αZe, βZe	STP influent, effluent and river water SPE	LC-MS^2	Supelguard 20 × 4.6 mm LC-18 packing Alltima 250 × 4.6 mm ; 5 μm	Different gradient and mobile phase composition depending on the group of analyzed compounds, 1 ml/min	ESI, APCI (+/-)-QqQ SRM	5–30	–	0.1–8.4 except NP	[6]
E1, E2, E3, EE2, and their glucuronides and sulfates	WWTP and river water SPE	LC-MS^2	BEH C18 100/ 50 × 2.1 mm; 1.7 μm	Gradient elution 0.2 mL/min A: H_2O B: ACN	ESI(−)-QqQ SRM	10–12	–	0.2–0.8	[13]
E1, E2, EE2	WWTP influent and effluent On-line SPE	LC-MS^2	SunFire C18 150 × 2.1 mm; 5 μm	Isocratic elution 0.3 mL/min H_2O:ACN (20:80, v/v) with 0.1% FA	TIS(+)-QqQ SRM	17	Dansyl chloride	0.4–0.7	[25]
BPA, E1, αE2, E2, E3, EE2	Water samples On-line SPE	LC-MS^2	Guard column 4 × 3.0 mm RP-Intersil ODS-3 100 × 4 mm; 3 μm	Gradient elution 1 mL/minA: H_2O B: ACN:MeOH (70:30; v/v)	ESI(−)-QqQ SRM	16	–	0.5–1	[38]
E1, E2, EE2	Surface water SPE	LC-MS^2	NUCLEODUR C18 ISIS 125 × 2 mm; 3 μm	Gradient elution 0.25 mL/min A: H_2O with 10 mM AmAc B: ACN with 10 mM AmAc	ESI(−)-QqQ	25	–	0.3–2.0	[36]
E1, E2, E3,EE2, E2-3S, E2-17Ac, E1-3S, 16α-OHE1, MeEE2	STP influents and effluents SPE and SEC	LC-MS^2	SecurityGuard column Synergi RP-MAX 150 × 2 mm; 4 μm	Gradient elution 0.2 mL/min A: H_2O B: MeOH	ESI(−)-QqQ APCI(+)-QqQ SRM	35	–	0.6–1.8	[10]
BPA, E1, E2, E3, EE2	Sewage samples SPE	LC-MS^2	Pursuit XRs Ultra-C18 50 × 2 mm; 2.8 μm	Gradient elution 0.2 mL/min A: H_2O B: MeOH with 0.1% AA and 15 mM AmAc	ESI(+/−)-QqQ SRM	29	–	0.5–6	[22]
E1, E2, E3, EE2 and progestins	Environment. waters SPDE	LC-MS^2	Guard column 4 × 4 mm; 1.7 μm BEH RP-C18 50 × 2.1 mm; 1.7 μm	Gradient elution 0.1 mL/min Group 1 A: MeOH B: H_2O Group 2 A: ACN B: H_2O	Group 1 ESI(−)-QqQ Group 2 ESI(+)-QqQ	10	–	0.5–3.4	[55]

BCA, DD, E1, EE2, E3, GEN	Water samples SPE	LC-MS^2	Guard column 4 × 4 mm; 5 μm STAR-RP-18e 125 × 2 mm; 5 μm	Gradient elution 0.2 mL/min A: ACN B: H_2O	Only estrogens ESI(−)-QqQ SRM	45	–	0.4–1	[54]
	Water samples SPE	LC-MS^2	AQUITY BEH C_{18} 50 × 2.1 mm; 1.7 μm	Gradient elution 0.4 mL/min A: H_2O B: ACN	Estrogens ESI(−)-Q-TOF Isoflavones ESI(+)-Q-TOF	16	–	5–30	
E1, E3, EE2, αE2, E2	Aqueous samples SPE	LC-MS^2	Guard column Xbridge BEH300 C18 150 × 2.1 mm; 3.5 μm	Gradient elution 0.2 mL/min A: ACN B: H_2O	ESI(−)-QqQ SRM	15	–	0.12–0.9	[1]
E1, E2, E3, EE2	Water samples SPE	LC-MS^2	AQUITY BEH C_{18} 100 × 2.1 mm; 1.7 μm	Gradient elution 0.5 mL/min A: 10 mM FA (pH 2.9) B: ACN	ESI(+)-QqQ SRM	3.2	Dansyl chloride	Waters:0.23–0.91 On-column LODs: 0.05–0.2 pg	[58]
		LC-MS			ESI(+)-QqQ SIM			Effluents: 1.03–1.75 On-column LODs: 0.44–1.48 pg	
DES, E1, αE2, E2, E3,EE2, glucuronides, acetates and sulfates	Environment. aqueous samples SPE	LC-MS^2	Kromasil 100 C_{18} 250 × 4.6 mm; 5 μm	Gradient elution 1 mL/min A: acidic H_2O (pH 2.8) B: ACN	ESI(−)-QqQ SRM	35	–	1–70	[16]
E2, E1, EE2	WWTPs SPE and LLE	LC-MS^2	Guard column 2 × 2.1 mm, 3 μm Hypersil BDS C18 250 × 2.1 mm, 3 μm	Linear gradient 0.2 mL/min A: H_2O B: ACN	ESI, APCI (+/−)-IT	30	–	1.5–3	[53] (2003)
DES, E1, E2, E3, EDD, EE2, L, NO, MeEE2, PO	Water samples SPE	LC-DAD/MS	Guard column 4 × 4 mm; 5 μm LiChrospher 100 RP 250 × 4 mm; 5 μm	Gradient elution 1 mL/min A: ACN B: H_2O	Estrogens: ESI(−) Progestogens: ESI (+)/APCI(+) DAD: λ = 197, 225 and 242 nm	42	–	ESI:2–500 APCI:20–5000 DAD:50–500	[51] (2000)
E1, E2,EE2	River sediments MASE and SPE and cleanup	LC-MS^2	C18 Symmetry 150 × 2.1 mm; 3.5 μm	Gradient elution 0.25 mL/min A: H_2O B: ACN	ESI(−)-QqQ SRM ESI(−)-TOF	19	–	0.015–0.04 ng/g 0.2–0.5 ng/g	[9]

(*continued on next page*)

Table 3. (*continued*)

Substance	Matrix, sample preparation	Method	Column	Mobile phase/flow rate	Detector	Analysis time (min.)	Derivat.	LOD (S/N = 3) (ng/L)	Ref.
E1, αE2, E2, EE2	River sediments	On-line 2D-LC-MS^2	Column 1: Luna Phenyl-hexyl 50 × 2.0 mm; 3 μm Column 2: XBridge Shield RP18 150 × 2.1 mm; 3,5 μm	Gradient elution 0.2–0.75 mL/min A: 0.1% FA B: MeOH Gradient elution 0.3 mL/min A: 0.1% FA B: MeOH	APPI(+)-IT	18	–	0.09–0.25 ng/g	[49]
A, BET, BUD, DE, E1, FLA, L, NOR, PG, T, TRA	Soil PLE and SPE	LC-MS^2	Zorbax Eclipse XDB C18 (with precolumn) 100 × 2.1 mm; 3.5 μm	Gradient elution 0.2 mL/min A: 0.2% FA B: ACN + 0.2% FA	ESI(+)-QqQ SRM	17	–	0.08–2.84 ng/g	[19]
E3, EE2S, EE2G, E1-3G, E1-3S, αE2, E2,EE2, E1, DES, E2-17Ac	Sewage sludge PLE	LC-MS^2	Kromasil 100 C18 250 × 4.6 mm; 5 μm	Gradient elution 1 mL/min A: acidic H_2O (pH 3) B: ACN	ESI(−)-QqQ SRM	37	–	26–175 ng/g	[48]
AP, Bol, ClTA, CMA, CP, DES, DE, DMA, E1, E3, E2, EE2, Ed, FMT, Hex, HP, TB, MeBol, MeD, MegA, MelA, MT, MPA, NE, NG, NT, PG, T, TBA, Stan, βZ	Kidney fat matrices LLE	LC-MS^2	Guard column 7.5 × 4.6 mm; 5 μm Alltima C18 250 × 4.6 mm; 5 μm	Gradient elution 1 mL/min A: MeOH B: H_2O, acidified with 0.1% CF_3COOH	APCI(+)-QqQ SRM	>20	–	> 2 ppb	[60] (1999)

Table 4. LC-FD/DAD/UV and other methods used in determination of estrogens and progestogens

Substance	Matrix, sample preparation	Method	Column	Mobile phase/flow rate	Detector setting	Analysis time (min)	Derivat.	LOD (ng/mL)	Ref.
E2	Water samples SPE	Enzyme-linked immunosorbent assay	–	–	Photometric Analyzer λ = 450 nm	142	–	0.0025	[54]
E2, E3, BPA, EE2	WWTP SPME	LC-DAD/FD	Hypersil ODS 200 × 4.6 mm; 5 μm	Isocratic elution 1 mL/min ACN: 0.02 M phosphate solution (45:55; v/v; pH 4.5)	DAD: λ = 280 nm FLD: λ_{ex} = 227 nm λ_{em} = 315 nm	12	–	0.006–0.1	[41]
DES, NT, MT, PG, PNT, PT, T	Wastewater SBSEM-LD	LC-DAD	Supelcosil LC-18 250 × 2.0 mm; 5 μm	Gradient elution 1 mL/min A: H_2O B: ACN	λ = 240 nm	22	–	0.14–0.41	[43]
E3, E2, E1, PG	Sediment CPE	LC-UV	Inertsil ODS-C18 250 × 4.6 mm; 5 μm	Gradient elution 1 mL/min A: ACN B: H_2O	Estrogens: λ = 200 nm PG: λ = 240 nm	25.5	–	0.23–5.0	[45]
E1, E2, E3, EE2	River and tap water DLLME-SFO	LC-PDA	BEH Phenyl column 100 × 2.1 mm; 1.7 μm	Gradient elution 0.4 mL/min A: H_2O B: ACN	λ = 280 nm	7	–	0.8–3.1	[46]

column, length, diameter, particle size, mobile phase etc.) can be seen in Table 3.

3.1.2. Mass-spectrometry detection

ESI, APCI and atmospheric pressure photoionization (APPI) have been ionization techniques used in LC-MS for the determination of steroid hormones. In spite of low proton affinities of estrogens, they were in most cases ionized in ESI negative-ion mode (−) [1,4,6,9–11,13,16,21,22,31,36,38,51,52,54,55], with progestogens and phytoestrogens in ESI positive-ion mode (+) [19,21,24,51,52]. Among other analyzers (ion trap – IT, time-of-flight – TOF), triple quadrupole (QqQ) was most often applied and achieved results superior to the Q-TOF analyzer [9,54]. The composition of mobile phase and other modifiers added directly into the mobile phase or post-column had important effects on the MS detection. It is difficult to decide which organic modifier is the best due to dependence on substance, type of ionization and mass analyzer. The advantages of MS detection are clearly seen in Table 3.

3.1.2.1. ESI mode. Generally the best electrospray ESI sensitivity is achieved with analytes already existing in ionized forms in solution [12]. Steroids with non-polar character, devoid of ionizable functional groups (amine or carboxylic acid groups), have poor sensitivity in ESI, compared to polar and ionizable organic compounds [12]. Therefore, chemical derivatization (both on-line and off-line) has been reported as a step to increase sensitivity [12,27,60]. Nevertheless, derivatization was not that widely employed. Only a limited number of studies were presented [12,25,27,31,56,58,60], of which some [12,27,31,58,61] compared the sensitivity and found it improved.

A comparison study of Lien et al. [58] showed that signals of native steroids (E1, E2, E3, EE2) and BPA were better with ESI(−) that those with APPI(−), APCI(−), and APCI/APPI(−). Dansyl derivatives measured by UHPLC coupled with ESI-MS provided the best performance regarding the sensitivity and matrix effects (see sub-section 4.1) [58]. Nieto et al. [48] reported higher responses for sulfates and glucuronides in ESI.

Post-column addition of 40 mM AmOH improved the sensitivity of estrogens by 122–146% on the previous signal of ESI(−), due to a better deprotonation of phenolic groups of E1 and E3 [6]. In APCI mode, the same additive decreased signal in most cases (for six of nine substances studied) [6]. Increased sensitivity for LC-ESI(−)-MS was found when the mobile phase was alkalized by TEA [29]. PG in the presence of FA in the mobile phase was positively enhanced in ESI(+) because acidic conditions helped to minimize sodium-adduct formation [19]. Schlüsener and Bester [10] found that the addition of buffers (ammonium acetate – AmAc, ammonium formate – AmF or AmOH at varying

Table 5. GC-MS methods in determination of estrogens and progestogens. Total ion current chromatogram (TIC)

Substance	Matrix/sample preparation	Method	Analytical column	Mobile phase/ flow rate	Detector setting	Analysis time (min)	Derivatiz.	LOD (ng/L)	Ref.
BPA, BZA, CA, DF,EE2, GF, IB, NX, NP	Water SPE	GC-MS	Retention gap 2.5 m × 0.32 mm HP-5MS 30 m × 0.25 mm; 0.25 μm film thickness	Thermal gradient helium;1 mL/min	CI(−) SIM	36	PFBBr	0.01–0.06	[72]
E1, E2, E3, EE2, MeEE2	Water SPE	GC-MS	DB-5 silica column 30 m × 0.32 mm; 0.25 μm film thickness	Thermal gradient helium	EI	17.8	BSTFA + 1% TMCS	0.02–0.1	[70]
E2	Water sample SBSE and TD	GC-MS	DS-5MS 30 m × 0.25 mm; 0.5 μm film thickness	Thermal gradient	EI SIM	22.5	Acetic anhydride and BSTFA	0.5–2	[42]
DEP, NP (technical grade), CP, DBP, NP, BBP, BPA, DEHP, ECL, A, Tm, E1, E2	WWTP SPE SBSE	GC-MS	DB-5MS 30 m × 0.25 mm; 0.5 μm film thickness	Thermal gradient helium, 1.2 mL/ min	SIM	40.05	BSTFA	1–5	[67]
BPA, DCP, DEP, DBP, DEHP, E1, E2, EE2, OP, NP, TB	WWTP SBSE	GC-MS	HP-5MS FSOT column (5% diphenyl, 95% dimethylsiloxane) 30 m × 0.25 mm; 0.25 μm film thickness	Thermal gradient helium	EI	31.85	BSTFA	–	[68]
E1, E2, E3, EE2, NP, BPA	Activated sludge in STPs SPE, ULE	GC-MS	HP-5MS 30 m × 0.25 mm; 0.25 μm film thickness	Thermal gradient helium; 1.5 mL/ min	EI TIC, SIM	30.5	BSTFA + 1% TMCS	LOQ Liquid: 2.0–30.3 LOQ Solid: 1.2–188.7 ng/g	[17]
DEHP, BPA, CST, CHOL, CPN, C3O, DST, αE2, E2, E1, E3, E2B,EE2, EST, EQ, EQL, FST, 6KCST, 7KCHOL, MeEE2, NG, NOR, PS, βS, SMT, SST, T, αZe	Wastewaters SPE	GC-MS	1 m polysiloxane guard column DB5-HT 15 m × 0.25 mm;	Thermal gradient helium, 0.9 mL/ min	Full scan	34.25	BSTFA + 10% TMCS	MDLs: 1–500	[37]
E1, E2, αEE2, E3,	Sewage sludge LLE and SPE	GC-MS	BPX5 capillary column 30 m × 0.22 mm; 0.25 μm	Thermal gradient helium; 1 mL/min	EI SIM	48	BSTFA + 1% TMS	–	[47]

AD, CORT, CHOL, DHT, DES, E2, E1, EE2, MT, PG, PREG, T	Liquid and solid sludge SPE	GC-MS	Zebron ZB-5 30 m × 0.25 mm; 0.25 μm film thickness	Thermal gradient helium; 1 mL/min	EI SIM	25.5	MSTFA	–	[71]
BPA, E1, E2, αE2, E3, NP, MeEE2	River sediment Sonication, Soxhlet and mechanical extraction	GC-MS	HP-5MS 30 m × 0.25 mm; 0.25 μm film thickness	Thermal gradient helium, 1 mL/min	SIM	34	PFPA	MDLs: 0.1–0.6 ng/g	[3]
E1, E2, E3, EE2, E2G, E2-3S, MeEE2, NO, NG, PG	River water SPE	GC-MS	HP5–MS column 30 m × 0.25 mm; 0.25 μm film thickness	Thermal gradient helium; 1 mL/min	EI SIM	32.5	MSTFA	0.4–1.9 ng/g	[30]

concentrations) to the mobile phase decreased the responses of the analytes, due to lower ionization ratios of steroid hormones [10]. Isobe et al. [29] also reported the absolute abundance decreased when acetate buffer was used as a mobile phase.

3.1.2.2. APCI mode. Supposedly, APCI should give better sensitivity for non-polar compounds. However, there was no sensitivity improvement for the group of estrogens compared with ESI(+); differences were most pronounced for E1 and E3 [6]. The percentage of relative intensity of E1 was 100% with ESI(+) and 34% with APCI(+) with a similar mobile phase. For phytoestrogens, only very slight response was obtained using APCI(+), unlike the increase in sensitivity with ESI [6]. There were different findings for APCI(−) when native estrogens in various water matrices (river, drinking and wastewater) measured in APCI(−) and ESI(−) were compared [62]. There was no significant difference between signal intensities of E2, E3 and EE2 detected in ESI(−) or APCI(−) in drinking water, while the signal intensity of E1 was 3 times greater in APCI(−). Moreover, in complex matrices, signal intensities of native estrogens in APCI(−) increased by 1–2 orders of magnitude compared to ESI(−) [62]. Estrogens detected under ESI(−) were predisposed to matrix effects and the signals were more suppressed in river and STP effluents.

3.1.2.3. APPI mode. APPI is relatively young ionization technique, which offers an alternative means of ionization for neutral (non-polar) compounds. Mobile-phase composition is critical to APPI sensitivity, and it is also compound-dependent [58]. APPI enhanced the ion signal of neutral steroids and provided comparable ionization for both native and derivatized steroid compounds [63]. More effective ionization of nine steroids leading to cleaner chromatograms and higher selectivity was incurred with APPI (compared to ESI or APCI) [64].

Matějíček [49] studied the influences of the mobile-phase composition and dopants on the ionization efficiency of analytes studied in APPI. A mobile phase comprising 0.1% FA (v/v) in MeOH showed the highest abundances in APPI(+), and 0.1% AmOH (v/v) in 2-propanol showed the highest intensities in APPI(−). Propanol provided excellent results in APPI(−) as well, but it caused a high column back-pressure [49]. A significant decrease in signal intensity was observed when AmF or AmOH were used in APPI(+). In APPI(−) mode, FA, AA or AmF had negative effects as mobile phase modifiers. Toluene, directly infused into the APPI(−) source as a dopant improved the ionization efficiency at the flow rate of 0.030 mL/min for the positive-ion mode, and at the flow rate of 0.035 mL/min for negative-ion mode [49].

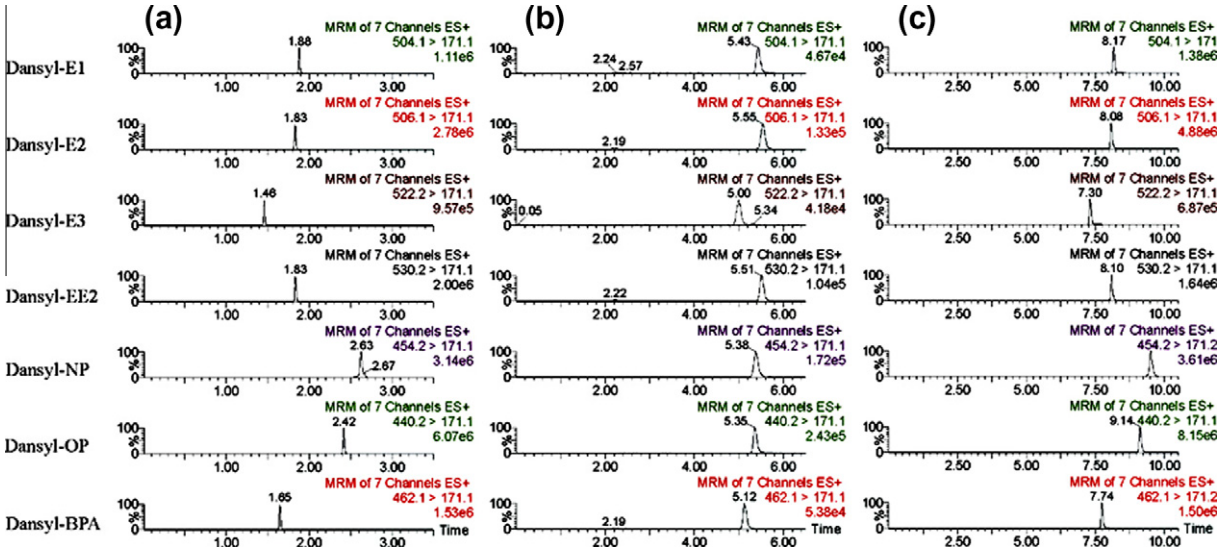

Figure 1. LC-MS chromatograms of dansylated compounds in ESI(+) mode using (a) UHPLC, (b) the mixed-mode column, and (c) 2D-LC (Reprinted from [58] with permission).

3.1.2.4. Mass analyzers. More details about the MS conditions, including mass-to-charge ratio, precursor ions, product ions, polarity and collision energy of selected compounds are given in Table 6.

Labadie and Hill [9] compared the suitability and the performances of LC-TOF-MS and LC-MS^2 (QqQ) for determination of estrogens in river sediment. LC-MS^2 achieved 13 times lower LODs than LC-TOF-MS, and also showed better selectivity [9]. Farré et al. [54] also compared UHPLC-Q-TOF-MS with HPLC-MS^2 (QqQ). The sensitivity of HPLC-MS^2 (selective reaction monitoring mode – SRM) was up to one order of magnitude better than using the UHPLC-Q-TOF-MS method (scan mode). In terms of selectivity, both HPLC-MS^2 (QqQ) and UHPLC-Q-TOF-MS showed excellent performance, but UHPLC achieved shorter analysis times [54]. The use of QqQ together with the SRM (Fig. 2) will enhance the sensitivity, and reach low LODs and limits of quantification (LOQs) as well as low background noise [4,13].

3.2. Gas chromatography coupled to mass spectrometry

Despite high resolution, lower operation cost and reduced waste of solvent, GC was used less in analysis of steroids than LC, mainly due to the difficulty of sample preparation, as mentioned above. Derivatization was applied in all studies with GC-MS determination (see subsection 4.3. below).

4. Derivatization in chromatographic methods

4.1. Liquid chromatography coupled to mass spectrometry

Despite the specificity and the versatility of LC-MS, ionization efficiency varied between different ionization techniques and compounds [61]. Ionization of estrogens and phenolic xenoestrogens by ESI and APCI were less efficient than that of more polar compounds [58]. Chemical derivatization using appropriate derivatizing reagents could improve ionization and enhance signals in LC-MS [12,27,31,58,61].

The topic of derivatizing reagents in steroid analysis has already been reviewed [61,65]. Derivatization also changes the chromatographic behavior of the analyte, so it can also enhance the specificity [65]. The sensitivity of steroid detection by ESI and APCI is increased by introducing permanently-charged moieties or moieties easily ionizable with protons or electrons [61]. Derivatization improved the sensitivity of derivatized estrogens 100 times [27] and 1000 times [31]. Dansyl chloride was the most commonly used derivatizing agent for the LC-MS analysis of estrogens in water samples [12,25,31,58,59].

Girard reagents T (GirT) and P (GirP) were used for neutral steroids in ESI(+) mode [61,65]. Their derivatives of the oxosteroids could be detected and identified at sub-picogram (sub-pg) levels by ESI-MS. However, the reactivity of oxo groups varies in different positions [65]. GirP derivatives were also suitable for analysis with matrix-assisted laser desorption/ionization coupled to MS (MALDI-MS) and were characterized at the 50-pg level [61].

Another derivatization reagent used for carbonyl compounds in ESI(+) was 2-hydrazino-1-methylpyridine (HMP). The derivatives manifested not only higher sensitivity but also better chromatographic behavior compared to GirP [61,65]. Further derivatizing agents for steroids with the hydroxy group used in ESI(+) mode were 1-(2,4-dinitro-5-flourophenyl)-4- methylpiperazine (PPZ) and 4-(4-methyl-1-piperazyl)-3-nitrobenzoyl azide (APZ) [66]. These derivatives achieved LODs in the

Table 6. SRM conditions used for LC-MS^2

Compound	Collision energy (eV)	Precursor ion (*m/z*)	Product ions (*m/z*)	Polarity	Ref.
DES	30, 55, 35, 45	267	222, 237, 131	$[M-H]^-$	[4,16,21,48]
DHT	31	273	123	$[M+H]^+$	[31]
DHT-d3	31	276	123	$[M+H]^+$	[31]
E1	47,45, 57, 40, 55	269	145, 143, 159, 253, 183	$[M-H]^-$	[4,6,9,10,16,21,31,48]
	29, 11, 21, 27	271	159, 133, 253	$[M+H]^+$	[10,26]
E1-d4	51, 45	273	147, 161, 145	$[M-H]^-$	[9–11]
E1-3G	45, 25, 30, 20	445	269, 113	$[M-H]^-$	[16,21,31,48]
E1-3S	44, 100, 30, 55, 40, 65, 25, 30	349	269, 145, 113	$[M-H]^-$	[10,12,16,21,31,48]
E1-3S-d4	45, 76, 65	353	273, 147	$[M-H]^-$	[10,11]
E2	24	255	159, 133	$[M+H]^+$	[10]
	54, 53, 30, 45, 57, 40, 50, 55	271	145, 183, 239, 159, 237, 401, 359	$[M-H]^-$	[4,9–11,16,21,31,48]
E2-d3	65	290	147	$[M-H]^-$	[11]
E2-d4	57, 45	275	147, 187	$[M-H]^-$	[9]
E2-d5	50	276	147	$[M-H]^-$	[11]
E2-17Ac	24	255	159, 133	$[M+H]^+$	[10]
	38, 59, 30, 55	313	253, 145	$[M-H]^-$	[10,16,48]
E2-3G	-	447	271, 113	$[M-H]^-$	[31]
E2-17G	30, 20	447	271, 113	$[M-H]^-$	[16,48]
	30	463	85, 287	$[M+H]^+$	[21]
E2-3S	30, 45, 55	351	271, 145, 80	$[M-H]^-$	[16,31,48]
E3	49, 58, 45, 11, 22,50, 55, 40, 35	287	171, 253, 133, 145, 159	$[M-H]^-$	[4,6,10,11,16,21,26,31,48]
E3-3G	–	463	287, 113	$[M-H]^-$	[31]
E3-16G	–	463	278, 113	$[M-H]^-$	[31]
E3-3S	35, 30	367	287, 80	$[M-H]^-$	[21,31]
EE2	60, 45, 30, 40, 47, 49	295	145, 159, 199	$[M-H]^-$	[6,9–11,16,21,48]
	22, 21, 20, 27	297	133, 159	$[M+H]^+$	[10,26]
EE2-3G	35, 30	351	271, 80	$[M+H]^+$	[4,21]
EE2-17G	30, 50	447	113, 271	$[M+H]^+$	[4,21]
EE2-d4	60, 50, 47	299	174, 147, 161	$[M-H]^-$	[1,9–12,29]
EE2-d4	28, 31	283	135, 161	$[M+H]^+$	[1,10]
L	20, 22, 44	313	245, 185, 91	$[M+H]^+$	[21,26]
PG	20, 22, 26	315	273, 160, 109, 97	$[M+H]^+$	[4,21,26]
PREG	20	317	256, 159, 109	$[M+H]^+$	[4]
T	35	289	109	$[M+H]^+$	[31]
T-d3	35	292	109	$[M+H]^+$	[31]

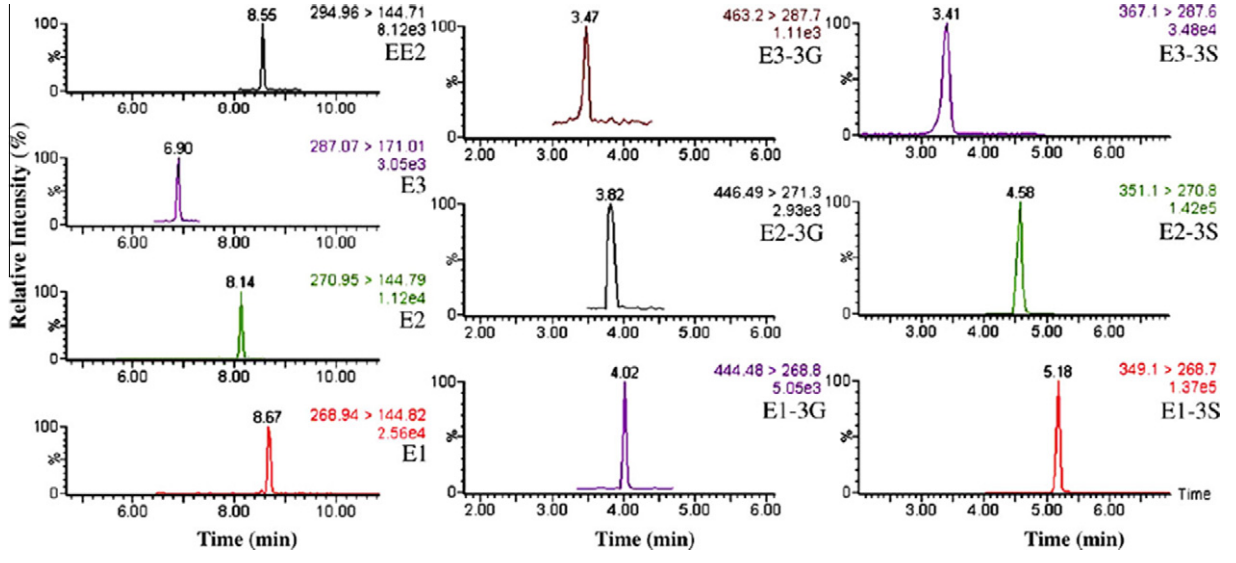

Figure 2. The UHPLC-MS^2 chromatograms (SRM) of four free estrogens and their six conjugates with relative intensity (%). Mass spectrometry was performed on Micromass Quattro Premier tandem MS in ESI(−) mode (Reprinted from [13] with permission).

femtomolar (fmol) range [66]. Derivatization for LC-ESI(+)-MS was also performed with trifluoromethanesulfonic acid as a catalyst and dansylhydrazine as a derivatizing reagent [57]. The time for the derivatization of oxosteroids (in pmol) runs to 25 min in this method [57]. The introduction of proton-affinitive derivatizing reagents to the analyte without increase of hydrophilicity is generally effective for increasing the sensitivity in APCI(+). Acetylation of the steroid hydroxy groups is one of the easiest derivatization methods used in APCI(+), but a more effective technique for enhancing sensitivity of steroids containing oxo groups (40–60 fold) was methyloxime derivatization [61].

The 2-nitro-4-trifluoromethylphenylhydrazones of pregnenolone and progesterone provided intense ions in APCI(−), which were 20 times and 30 times better than those obtained without derivatization. Higashi also described several derivatizing agents for electron-capture APCI (ECAPCI) based on the introduction of electron-affinitive groups [65].

In the comparative study of Alary et al. [63], APPI provided comparable ionization of both native steroids and pentafluorobenzene derivatives. Also using APPI-MS, a better LOD was obtainable because of low background noise [63]. In the APCI(−) mode, ultra-high sensitivity can be obtained by tagging neutral steroids with moieties with electron affinity [61,63].

Three derivatization agents have been used to react with phenolic groups of estrogens and compared with underivatized ones in various environmental matrices [62]. Dansyl chloride in ESI(+), 2-fluoro-1-methylpyridinum *p*-toluenesulfonate (FMPTS) in ESI(+), and pentafluorobenzyl bromide (PFBBr) in APCI(+) were applied as derivatizing agents. Native estrogens were detected in ESI(−) (reference values) and APCI(−). With simple matrices (e.g., drinking water), dansyl-estrogens produced the highest signal intensity (111 times higher than the reference values) and PFB-estrogens provided 3–9 times better intensity [62]. In river water and STP effluents, sensitivity was significantly enhanced in PFB-estrogens (11.5–264/9.6–32 times), dansyl-estrogens (3.3–11/2.1–20 times), FMP-estrogens (1.4–11.8/1–4 times) and native estrogens (13.2–139/3.7–14 times) under APCI(−) compared to the sensitivity of native estrogens in ESI(−) [62].

Other studies have also shown better sensitivity with dansyl chloride or PFBBr [25,58]. Signal intensities of dansyl derivatives in ESI(+), APCI(+) and APPI(+) mode, PFBBr derivatives in APCI(−) mode and underivatized analytes showed that both dansyl and PFBBr derivatives significantly improved the detection sensitivity compared to underivatized compounds (859–8460 times in ESI, 354–4030 times in APPI, 23–472 times in APCI, 21–344 times in APCI/APPI, and 5–41 times in ECAPCI [58]). The combination of 10 mM FA (pH 2.9) and ACN as the mobile phase for ESI(+) and APPI(+) provided the best results for detection of dansyl derivatives (2–3 times higher sensitivity than 10 mM FA and MeOH). 10 mM FA and MeOH were optimal for APCI(+) and APCI/APPI(+). The best detection of PFBBr derivates was attained with water-MeOH in APCI(−) [58].

Nevertheless, modern mass spectrometers also reach low LODs without derivatization [64]. A combination of QqQ with SRM mode facilitates the development of methods with low LODs and limits of quantification (LOQs), and great identification capability in complex samples [4]. The sensitivity of the method developed is influenced by the type and the age of the analyzer used.

Table 7 summarizes derivatization conditions and agents of female-steroid hormones.

4.2. On-line derivatization in liquid chromatography-mass spectrometry

On-line sample preparation allows combination of extraction and derivatization in one step. Automated derivatization techniques have been developed {e.g., on-line pre-column derivatization by 12-(difluoro-1,3,5-triazinyl)-benz[f]isoindolo[1,2b][1,3]benzothiazolidine (FBIBT) [27] and on-line SPE with on support immobilized dansyl chloride [25]}. Both techniques reached low LODs: 0.07–0.38 ng/L (LC-QqQ-MS), 0.5–2 ng/L (LC-IT-MS) [27]; and 0.4–0.7 ng/L (LC-QqQ-MS) [25]. Derivatization time of on-line SPE with on support immobilized dansyl chloride was 4 min. The main advantage of on-line pre-column derivatization by FBIBT was the more than 100-fold improvement in sensitivity of estrogen determination [27].

4.3. Gas chromatography coupled to mass spectrometry

A derivatization step, necessary for GC determination, was used in all studies. The aim of derivatization in GC is to increase the volatility and to improve the separation and the stabilization of thermolabile substances. GC-MS analysis without derivatization also leads to adsorption on the column and decreases sensitivity.

The most widely used derivatizing reagents in GC-MS analysis of steroids were *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) [28,34,46,60,67–69], BSTFA with 1% trimethylchlorosilane (TMCS) [10,17,70,71] and *N*-methyl-*N*-(trimethylsilyl)trifluor-acetamide (MSTFA). BSTFA with 1% TMCS showed the highest trimethylsilylation power and provided sufficient sensitivity and selectivity [17]. Some of the derivatization agents can be synthesized easily from commercially-available starting materials by a one-step reaction (e.g., FBIBT) [27].

In the study of Peng et al. [3], derivatization was performed by pentafluoropropionic anhydride (PFPA). PFPA was compared with BSTFA and BSTFA + 1% TMCS under the same conditions (60°C for 2 h). Natural estrogens showed better separation and higher sensitivity after using PFPA than the other two. In contrast to

Table 7. Derivatization in estrogen and progestogen analysis

Substance	Time/temperature of incubation	Derivatization agent	Method	LOD (ng/L)	Ref.
E1, αE2, E2, E3, EE2	On-line pre-column derivatization 30 min/at room temperature	FBIBT in DMSO	LC-MS^2	0.067–0.29	[27]
E1, E2, E3,EE2	1 min vortex-mixing, 30 min/50°C	Dansyl chloride	LC-MS^2	0.05–0.5	[58]
E1, E2, E3	5 min/60°C	Dansyl chloride	LC-MS^2	0.049–8.9	[31]
E1, E2, EE2	On-line derivatization: 3 min/40°C	Dansyl chloride	LC-MS^2	0.4–0.7	[25]
EE2	30 min/45°C	Dansyl chloride	LC-MS^2	<0.75	[12]
E1, αE2, E2, E3,EE2	30 min/60°C	PFBBr	LC-MS^2	0.14–0.74 amol (ENCI) 7.02–62 fmol in APCI(−)	[56] (2000)
BPA, BZA, CA, DF,EE2, GF, IB, NX, NP	60 min/60°C	PFBBr	GC-MS	0.01–0.06	[72]
E1, E2, E3,EE2, MeEE2	MAD: 60 s/800 W; 30 min/80°C	BSTFA + 1% TMS	GC-MS	0.02–0.1	[70]
E1, E1-d_4, E2, E2-d_4, EE2, EE2-d_4	30 min/60–70°C	BSTFA	GC-MS GC-MS^2	0.7–1.4 0.3	[34]
E2	SBSE – in situ acylation: stirring at 1000 rpm for 120 min at room temperature TD – in tube silylation	Acetic anhydride and BSTFA	GC-MS	0.5–2	[42]
DEP, NP (technical grade), CP,DBP, NP, BBP, BPA, DEHP, ECL, AS, Tm, E1, E2	30 min/70°C	BSTFA	GC-MS	1–5	[67]
E1, E2, E3, EE2, NP, BPA	Ultrasonic-assisted derivatization 30 min/60–70°C	BSTFA + 1% TMS	GC-MS	LOQ liquid: 2.0–30.3 LOQ solid: 1.2–188.7 ng/g	[17]
DEHP, BPA, CST, CHOL, CPN, C3O, DST, αE2, E2, E1, E3, E2B,EE2, EST, EQ, EQL, FST, 6KCST, 7KCHOL, MeEE2, NG, NOR, PS, βS, SMT, SST, T, αZe	180 min/90°C	BSTFA + 10% TMS	GC-MS	MDLs. 1–500	[37]
DHT, E1, αE2, NT, T	Headspace derivatization: 60 min/60°C	BSTFA	GC-MS	60	[69] (1998)
BPA, E1, E2, αE2, E3, NP, MeEE2	120 min/60°C	PFPA	GC-MS	MDLs: 0.1–0.6 ng/g	[3]
E1, E2, E3, EE2, E2-17G, E2-3S, MeEE2, NO, NG, PG	30–40 min/65°C	MSTFA	GC-MS	0.4–1.9 ng/g	[30]
BPA, DCP, DBP, DEP, DEHP, E1, E2, EE2, NP, OP, TB	SBSE: 60 min under 900 rpm	BSTFA	GC-MS	–	[68]
E1, E2, E3,EE2	60 min/70°C	BSTFA + 1% TMS	GC-MS	–	[47]
AD, E2, CORT, CHOL, DHT, DES, E1, EE2, 17-MT, PG, PREG, T	MAD: 1 min/900 W MSTFA: 30 min/55°C	MSTFA	GC-MS	–	[71]

natural estrogens, phenolic compounds had comparable results when derivatized by each of these three reagents.

Okeyo and Snow [69] reported headspace-BSTFA derivatization on an SPME fiber (60 min at 60°C). Published LODs were at the low-ng/mL range [69]. Low LODs (0.01–0.2 ng/L) were also achieved after application of PFBBr [72]. More details about derivatizing agents and other conditions are given in Tables 5 and 7.

Nie et al. [17] investigated the effect of ultrasonication on the derivatization efficiency of EDCs analyzed. Their study indicated that aliphatic hydroxy groups were more difficult to derivatize (only about 8% of E2 and E2-d_2 were transformed to mono-*O*-TMS forms) than aromatic ones. After ultrasonication, a complete silylation of both aliphatic and aromatic hydroxy groups was accomplished. The peak area of the EDC derivatives increased after ultrasonication from 44% (for EE2) to 219% (for BPA). Ultrasonication could also considerably enhance the recovery (70–130%) and the sensitivity of the detection method (Fig. 3) [17].

Long derivatization time (30–180 min) was the challenge for new derivatization techniques in GC-MS. One of them was microwave-accelerated derivatization (MAD) used for analysis of natural and synthetic estrogenic steroids [70,71]. Due to the small sample volume (100 μL), microwave radiation could penetrate the entire sample and thus heat the sample fluid, which completed the reaction [70]. LODs were 0.02–0.1 ng/L and the irradiation time was 60 s [70].

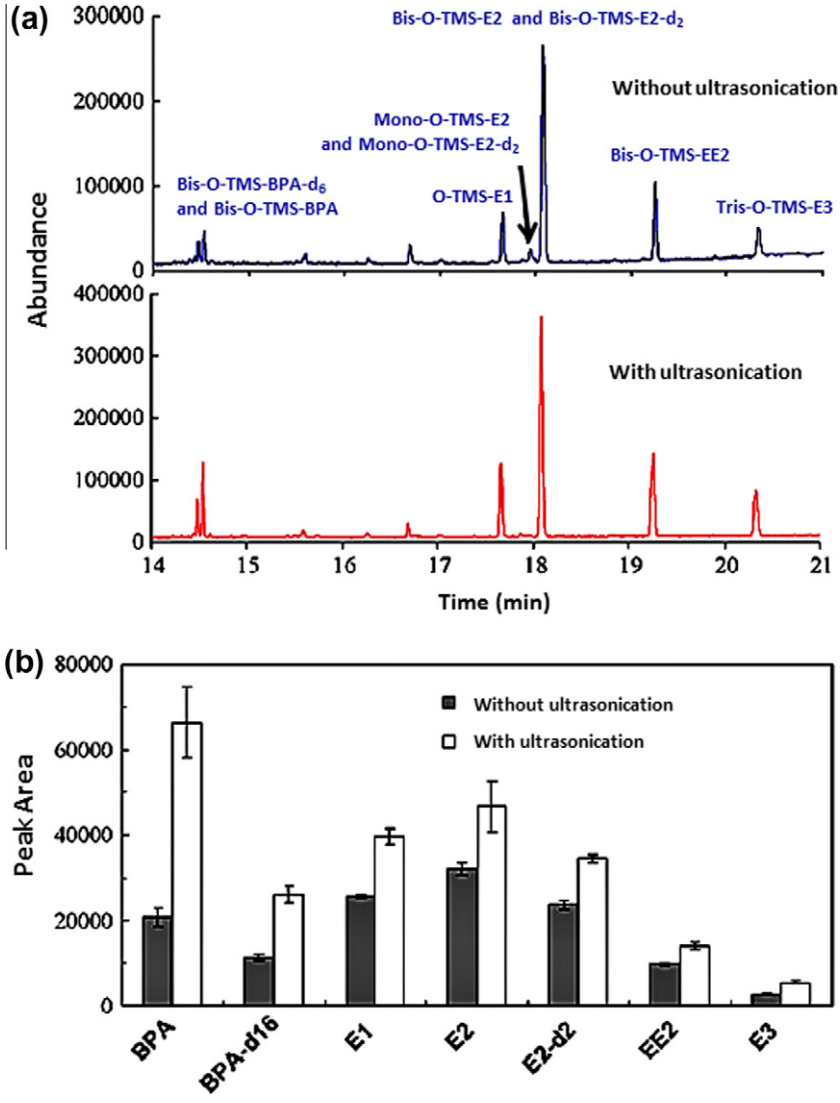

Figure 3. GC-MS chromatograms demonstrated the effect of ultrasonication on derivatization of studied EDCs and IS. (a) TIC of the derivatives; and (b) Selective ion monitoring (SIM) peak areas of the derivatives (n = 3). A derivatizing agent was BSTFA with 1% TMCS; electron ionization (EI) was used. BPA-d16 – IS of BPA, E2-d2 – IS of E2 (Reprinted from [17] with permission).

Bowden et al. [71] investigated three silyl derivatization reagents [i.e. BSTFA/TMCS, MSTFA and *N,O*-bis(trimethylsilyl)acetamide (BSA)] at various incubation times and temperatures using different derivatization techniques. Mono-hydroxylated steroid derivatization using BSTFA/TMCS with classical heating achieved the highest relative reduction factor (RRF) values at the temperature range of 55–70°C for 15–30 min [71]. MAD, sonication-assisted derivatization (SAD) and block heating were compared [71]. The application of microwave heating (at 900 W for 1 min) proved to be more efficient than the optimized traditional heating methods. Microwave heating increased RRF values for all the steroids derivatized with BSTFA/TMCS. In addition, the time of the derivatizing method (1–30 min) and amount of reagent needed per sample were reduced [71]. SAD highlighted the potential of water-bath heating, but no improvement of steroid derivatization was reported [71].

The study of Bowden et al. [71] put together guidelines for derivatization followed by GC-MS analysis, as follows.

(1) Reactions with BSTFA/TMCS often reach the highest RRF, but reactions with MSTFA have generally comparable RRF values with better reproducibility.
(2) BSTFA/TMCS was the best reagent selectively avoiding the derivatization of carbonyl functional groups.
(3) No improvement was reported by the temperature above 70°C or derivatization time over 60 min for BSTFA/TMCS and MSTFA.
(4) The temperature under 40°C was not efficient for any of the reagents they studied.
(5) The ideal conditions of each derivatizing agent (for comprehensive derivatization) were 55°C (for BSTFA/TMCS), 70°C (for MSTFA) and 90°C (for BSA) for 30 min.
(6) Compared to traditional thermal heating methods, the best results for microwave reaction were at 900 W for 1 min.
(7) In application of MAD, the sample must not evaporate under intense heating in the microwave.

5. Conclusions

In this review, we have shown that the sensitivity of analytical methods for determination of estrogens and progestogens can be increased by number of optimization steps in each part of the analytical process. The sample-preparation process is one of the most important and time-consuming parts of the analytical method. Preconcentration is very important, especially for environmental samples, where the concentrations of steroids are very low. Optimization plays an important role in enhancing sensitivity and reducing matrix effects.

Aqueous samples were most frequently processed with off-line SPE. With solid samples, SPE, LLE, PLE, MAE/MASE or ultrasonication techniques were frequently applied. In several studies, a derivatization step was carried out in order to improve the sensitivity in LC-MS detection. In GC, derivatization was always necessary. Extensive reviews about derivatization of neutral steroids for LC-MS have been published [61,65]. Improvement of sensitivity for derivatized compounds were published in not only these reviews but also other studies comparing derivatization to methods without derivatization (in LC-MS). On-line derivatization in one step with extraction was also developed [25].

HPLC and GC have become the main methods for the separation of estrogens, and various types of detection has been used: MS^2, MS, FD and UV (in order of decreasing sensitivity). LC-MS^2 is one of the most convenient techniques available in the analysis of steroid hormones. Two SRM transitions were enough for confirmation of compounds from environmental samples. In spite of the lower background noise in biological matrices with APCI, applications with ESI were still about five times greater. We recommend use of stable isotopically-labeled internal standards for reliable quantitation.

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