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Review article

Analytical methods for the endocrine disruptor compounds determination in environmental water samples

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ABSTRACT

The potential risk of exposure to different xenobiotics, which can modulate the endocrine system and represent a treat for the wellness of an increasing number of people, has recently drawn the attention of international environmental and health agencies. Several agents, characterized by structural diversity, may interfer with the normal endocrine functions that regulate cell growth, homeostasis and development. Substances such as pesticides, herbicides, plasticizers, metals, etc. having endocrine activity (EDCs) are used in agriculture and industry and are also used as drugs for humans and animals. A difficulty in the analytical determination of these substances is the complexity of the matrix in which they are present. In fact, the samples most frequently analyzed consist of groundwater and surface water, including influent and effluent of wastewater treatment plants and drinking water.

In this review, several sample pretreatment protocols, assays and different instrumental techniques recently used in the EDCs determination have been considered. This review concludes with a paragraph in which the most recent hyphenated-instrument techniques are treated, highlighting their sensitivity and selectivity for the analyses of environmental water samples.

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1. Introduction

In the last 40 years, the possible consequences of exposure to xenobiotics, that can modulate the human and animal endocrine system, have drawn the international agencies attention including the European Commission, the European Parliament, the U.S. Environmental Protection Agency, the Organization for Economic Cooperation and Development, the WHO International Program on Chemical Safety, several non-governmental organizations and the chemical industry (OECD, 198 9; CSTEE, 1999). Only in 1996, the European Commission gave the first definition of endocrine disruption: "exogenous substance or mixture that alters the function of the endocrine system, causing adverse effects on the health of an organism, or its progeny, or (sub) population (European workshop on the Impact of Endocrine Disrupters on Human Health and Wildlife, Weybridge 2-4/12/1996)".

There are many issues in this research field that can range from human epidemiology difficulties to the many thousands of chemicals that could act as endocrine disruptors. Many natural and synthetic (or semi-synthetic) agents, characterized by different chemical structure, have the potential to destroy the normal endocrine processes that regulate cell growth, homeostasis and development. This Endocrine Disruptor Compounds (EDCs) interferes with the synthesis, storage, release, metabolism and transport by removing or binding the endogenous hormone-receptors [1].

EDCs are a group of persistent and bio-accumulative environmental contaminants (see Supplementary material section S.1 for contamination sources) that are found within several classes of chemicals, whose effects on the endocrine systems (see Supplementary material section S.2 for effects of exposure to EDCs) were ignored till a short time ago. Regarding the association between EDC and human health problems, early studies were carried out on aircraft operators that spayed DDT recordering a decline in sperm counts [2]. Scientific studies carried out for years about the presence of compounds with estrogenic activity in streams have demonstrated the presence of many natural or synthetic chemical compounds able to mimic or to interfere with the normal endocrine processes in animals and humans [3–5]. Over the years there have been recorded numerous environmental phenomena caused by these compounds, such as changes of the reproductive system, reduced eggs hatch and low youthful survival of sea turtles and alligators in Lake Apopka in Florida, occurring after the release of an insecticide containing metabolites of DDT [6,7].

Quantifying and cataloging the EDCs is an extremely difficult and time consuming task that required a lot of efforts by researchers. This type of operation is complicated because there are numerous compounds with this kind of activity and every year the list of compounds known as EDCs grows. Substances endowed with endocrine activity, such as pesticides, herbicides, plasticizers (such as bisphenol A, Table 1), metals, excreted estrogens (Fig. 1), etc., are used in agriculture and industry, or derived by anthrophic activities (e.g. medicines for humans and animals).

A further complication in the determination of these substances is the complexity of the matrix in which they are present, in fact, the samples analyzed often belong to groundwater, surface water, influent and effluent of wastewater treatment plants, but, also, to drinking water. In literature the determinations in aquatic samples, due to an environmental contamination (Fig. 2, and Table 2) of these substances were performed using several conventional analytical methods, such as chromatography techniques or biological approaches (see Supplementary material section S.3 for biological approaches). To this purpose, high sensitivity and selectivity of the methods are mandatory as these compounds exhibit their endocrine activity even at very low levels. Up to now, the methods that have provided the best results were gas and liquid chromatography, coupled to high efficiency tandem mass spectrometers achieving detection limits of 0.1 ng/L showing high selectivity.

In this review, several sample pretreatment protocols and assays and the different instrumental analyses able to the EDCs determination are illustrated.

Table 1
Sources of exposure to bisphenol A.

Bisphenol A
Varnishes and glaze
Baby bottle
Nail varnish
Bottles for water in polycarbonate
Flame retardant
Jars coating
Plates for microwave
Adhesives
Artificial teeth
Returnable containers
Dental sealant for children

Table 2
Examples of environmental contaminants that interfere with the endocrine system.

Insecticide	Surfactants
DDT	Nonylphenol
Organophosphates	Nonylphenol acetate
Herbicides	Plasticizers
Atrazine	Bisphenol A
Fungicides	Phytoestrogens
Mancozeb	Cumestrol
Tributyltin	Genistein
Heavy metals	Chemical industrial products
Cadmium	PCB And PDB
Organictin	Solar shielding
Lead	Musk fragranceDioxins and furans

2. EDCs analysis

The final analysis on the presence of compounds with hormonal activity in samples of water, wastewater, influent and effluent, ground and surface water is commonly performed by chromatographic methods such as gas chromatography (GC) and high performance liquid chromatography (HPLC). These separation methods are highly efficient and selective when coupled to high sensitivity detectors, such as mass spectrometer (MS) or tandem mass spectrometer (MS/MS) instead diode array detector (DAD). Undoubtly, the most used, flexible and efficient detector is the mass spectrometer in different operating modes. Among the problems that may complicate the determination of EDCs, the most obvious are the concentrations below the ng/L, the different chemical structures and the physical-chemical characteristics of the analyzed compounds. Among the most reliable and sensitive methods for the analysis of steroid hormones in water samples, recently, GC–MS, HPLC–MS and HPLC–MS/MS are recognized as the best analytical tools in environmental sciences. Chromatographic techniques allow simultaneous screening of steroids and their conjugates, and is not limited by factors such as non-volatility and high molecular

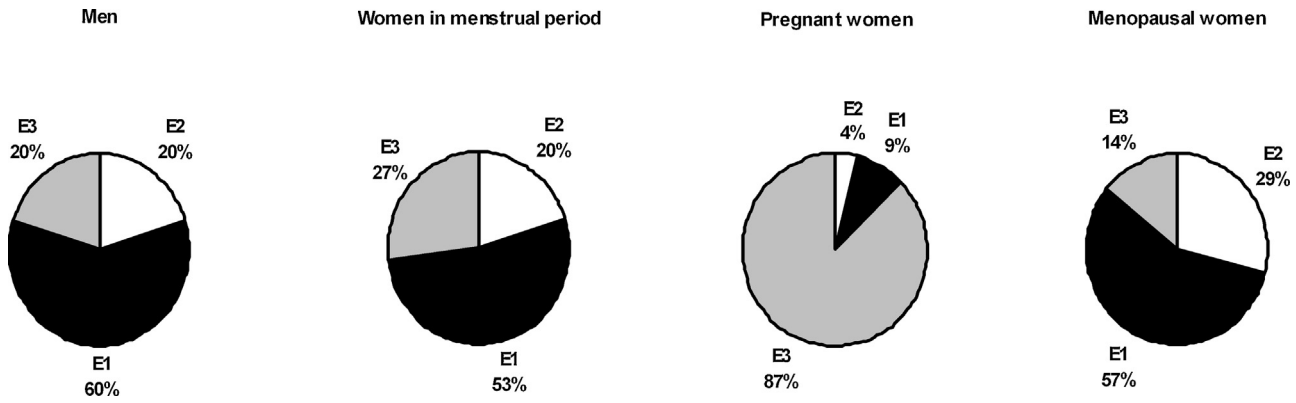

Fig. 1. Daily excretion of estrogen (as percentage) [8].

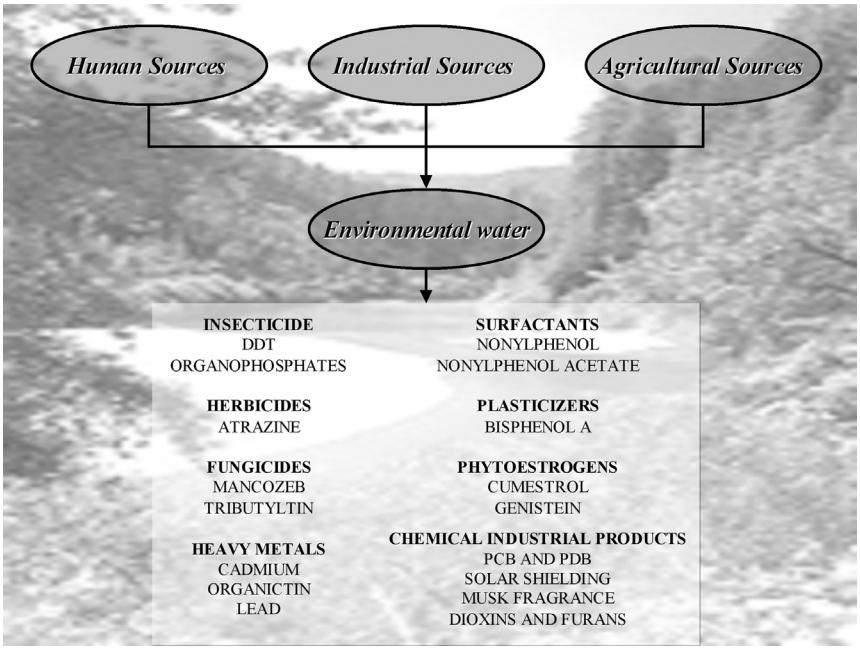

Fig. 2. Environmental contamination and principal compounds that interfere with the endocrine system.

weight of the analytes, bringing to the determination of conjugated and not conjugated estrogens without the need of derivatization [9,10].

2.1. Sampling and storage

The sampled volume can range from 50 mL to over 20 L depending on the sensitivity of the method, however, a volume exceeding 5 L should be avoided because it leads to an increase of the concentration of humic acid in the sample and therefore to an increase of the matrix effect [11]. The volume of the treated sample depends mainly on the sensitivity and detection ranges of the technique used for the final analysis. It usually ranges from volumes extracted and analyzed by radioimmunoassays [12] as low as 50 mL up to 20 L [13,14] or even 80 L, usually extracted using a liquid-liquid partition and analyzed by gas-liquid chromatography [15]. The optimum volume of a sample intended for analysis by HPLC–MS/MS and GC–MS/MS ranges from 250 mL and 1 L [16,17]. Except for rare cases, sampling and extraction occur simultaneously [18], in fact one of the most popular storage strategy consists of passing the sample through an SPE cartridge, as Carbograph-4, washing with methanol and then storing it at −18 °C. The use of this process does not show any significant loss of estrogen after 60 days of storage. An alternative to this procedure is to store the samples at a temperature of 4 °C in amber bottles previously cleaned with formaldehyde (1%, *v:v*), which also serves to inhibit the growth of microorganisms [9]. Storage of samples in the bottles, when preserved, does not lead to significant loss of estrogen after 24 days, contrary to the case of not preserved samples that instead suffers of huge losses [19].

Some studies, however, have reported retention periods of 1–5 days [8] and up to a week for samples preserved at 4 °C and freezed at −20 °C [20,21]. Instead, for the preservation of the sample, several authors [22] have added chemicals such as formaldehyde (1%, *v:v*) [23], methanol [13], sulfuric acid [24], and mercuric chloride [25] to prevent bacterial activity and/or store the samples on a solid support for the extraction [13,21]. In recent times was presented the '*passive sampler*', a device for sample collection based on the direct extraction of estrogenic compounds on a permeable membrane used as adsorbent support.

The main instrument of passive sampling is known as Polar Organic Chemical Integrative Sampler (*POCIS*) that is left in water for a period of time ranging from one up to ten weeks: the estrogenic compounds are extracted directly on the membrane by the addition of 100 mL of dichloromethane of analytical grade to each

900 mL of water sample by mixing in a clean bottle glass [26–30]. As adsorbent material two different membranes have been used; the first based on polyethersulfone, which presents greater adsorbent capacity, and the other based on polysulfone. The absorption kinetics of the compounds collected in laboratory conditions, were linear for 10 days and the results obtained using the *POCIS* for real samples were in good agreement with those obtained with the sampling spot.

2.1.1. Filtration

The wastewater samples for analysis usually contain a high amount both of organic material and suspended particles that could cause many problems during the subsequent stages of analysis. The filtration can be performed simultaneously with the collection of the sample, the extraction step or in separate steps, depending on the extend of coupling of these stages, by using different kinds of filters, having different diameter, filter supports and physical form.

Glass fiber filters are the most often used in the various studies and usually have a pore size ranging between 0.22 and 1.20 μm [26–29]. In the past, there have been several discussions regarding the retention of estrogens and xenoestrogens in the filter material [13,26] and many of them contributed to shed light on this dilemma confirming estrogenic activity [13] of filter material. Actually the analysts usually wash the filtration system with 3–10 mL of methanol to remove the analyte retained on filter [8,22,31]. Some studies report that, in addition [25] or alternatively [12] to filtration is possible proceed with the samples centrifugation, avoiding any analyte loss due to the filtration process. The main drawback can be, in this case the possible analytes coprecipitation with suspended particles, but in this case the precipitate can be washed with appropriate solvent in order to improve the overall recovery.

2.2. Extraction

2.2.1. Extraction methods

2.2.1.1. Solid phase extraction. In the past, the analytical methods used for the determination of organic contaminants in the water employed almost exclusively continuous liquid-liquid extraction (CLLE) [32]. This extraction technique has been replaced by solid phase extraction (SPE) using specific organic solvents [33] as elution solvent.

The United States Geological Survey (USGS) and the National Water Quality Laboratory (NWQL) have recommended use of GC–MS system after the SPE to analyze 67 compounds, including EDCs, in domestic and industrial wastewater [34]. This extraction technique is commonly used even before analysis with other systems such as HPLC [35]. The most important parameters in the application of the method SPE are the choice of a suitable solid adsorbent for the analyte of interest and of solvents for washing and elution [36]. Moreover, to prevent the phenomenon of adsorption breakthrough, the capacity of the cartridges or disks used in the SPE should be checked prior to their application. The typical fluxes, either for a cartridge or a disc, are of 3–10 mL/min and 10–100 mL/min [33], respectively. Depending on the choice of the type of adsorbent, the analytical results for a given compound show considerable differences [37]. For the SPE of estrogens and xenoestrogens both disks and cartridges can be used, even if the use of disks actually is more frequent, since their clog with suspended particles is less extended and, in addition, show a contact area with the adsorbent matrix relatively larger than that of the cartridges. These advantages result in better levels of extraction; moreover, the use of the discs allows a reduction of the contamination during elution of the sample due to leaching of plasticisers present in the material of the cartridge holder. However, the use of the cartridges should not be considered flawed because a simple filtration through a filter with pores of 0.22–0.45 μm prevents clogging of the cartridges and because adequate cleaning of the cartridge, before conditioning and the loading of the sample, with the solvents used for elution should reduce or even eliminate the leaching from plastic media cartridges. Furthermore the cartridges, unlike discs, have the advantage of being applied in automated systems for washing, conditioning, loading of the sample, the further washing, drying and the eventual final elution for numerous samples. The use of the discs, however, requires a large amount of solvent, which involves a high expenditure of time in the reconcentration of the eluate [38].

The most widely used adsorbent, both for the discs [9,25] and for the cartridges [13,21,39] is the octadecylsilane (C_{18}), although in several studies it has been reported the use of black carbon, graphite and the styrenedivinylbenzene (SDB). These are available respectively as cartridges, Carbograph-4 and ISOLUTE ENV+, Oasis HLB (terpolymer of polystyrene-divinylbenzene-*N*-vinylprolidone) and SDB-XC disks. Amberlite XAD 2, a hydrophobic polymeric adsorbent, used successfully for many years for the determination of organic compounds such as polycyclic aromatic hydrocarbons (PAHs) in water, has the advantage of presenting both a low affinity for the humic acid substances and a high volume capacity. This adsorbent proved to be inadequate for the preconcentration of estrogens from water; in fact comparing the efficiencies of Amberlite XAD 2 and a mixture of LiChrolut EN Bondesil and C_{18} for the extraction of cholesterol acetate, used as a surrogate standard in the determination of endogenous estrogens and exogenous recovery, the rates obtained with these adsorbents ranged, respectively, between 8 and 30% and between 61 and 94% [40]. The cartridges Carbograph-4 are known for their adsorbing power, especially towards the polar compounds, based on anion-exchange and hydrophobic properties [41]. The Oasis HLB, one of the most important classes of cartridges used for the extraction of estrogenic compounds, are characterized, however, by the ability to retain a large number of basic, neutral and acid compounds [42–45]. These cartridges are designed, in fact, to retain effectively both hydrophilic compounds and hydrophobic compounds by means of Van der Waals interactions and hydrogen bonds. Moreover, contrary to other cartridges, the hydro-lipophilic balance of HLB Oasis cartridges allows them to dry during the treatment of the sample. Other types of cartridges as the Envi+ or C_{18} gave adequate results for the analysis of compounds with hormonal activity [46–48], but are less selective in relation with the Oasis HLB.

A study by Laganà et al. [49], concerning the determination of natural female hormones (17β-estradiol, estriol, estrone), their synthetic derivatives (17β-ethinylestradiol), alkylphenols (4-nonylphenol and bisphenol A), isoflavonoids (ginestein and Biochanin A) and micoestrogen (zearalenone) in water samples reported the use of the cartridges HLB and Carbograph-4. These cartridges were conditioned sequentially; before the treatment of the sample with 10 mL of dichloromethane-methanol (50:50, *v:v*), 5 mL of methanol and 10 mL water and, after addition of the sample, washed with 10 mL of water and 0.4 mL of methanol. The retained compounds were then eluted with 7 mL of a solution of dichloromethane-methanol (50:50, *v:v*) and analyzed by using HPLC–MS/MS.

Nowadays, the Hemimicelles Admicelles and sodium dodecyl sulfate on alumina and cetyltrimethylammonium bromide on silica may also be used. These can be compared with Oasis HLB for the concentration and purification of estrogens from water samples. The recovery levels of estrogen, studied by HPLC-DAD, usually are between 85% and 105%, with a standard deviation ranging from 3 to 8% [50]. The combination of two cartridges may increase the selectivity. The association of C_{18} with the SDB reported in different studies was acceptable for the analytes studied [40,51]. The application of two different cartridges, C_{18} and NH_2 put in series, has been reported in a study [52] on the determination of estrogen E1, βE2, αE2, EE2 and E3 in the aqueous matrix. This method has

proved to be accurate with recoveries of analytes more than 90% in all cases.

The use of SPE disks seems to be very suitable also for the extraction of estrogen from large volumes of water. It has been reported, in fact, a study showing the use of disks impregnated with polytetrafluoroethylene particles C for the extraction of estrone, estradiol and 17β-ethinylestradiol [53]. These discs for the SPE impregnated with polymer particles, on which was placed a filter paper, glass fiber and glass spheres to avoid occlusion with the suspended material, gave good recoveries of estrogens at the level of ng/L from the extraction of samples with a high volume of river water. On the contrary, these extraction discs were found to be not appropriate for samples of wastewater effluent and water from sewage plants probably due to the overhead due to the rich matrix of organic material. For this type of sample was appreciated a new methodology that uses the SPE high flux with discs Polar Plus C_{18} Speedisks at flow rates greater than 100 mL/min [54]. In Table below a comparison of various types of adsorbents used in the SPE is showed for the determination of significant estrogenic compounds, according to the type of procedure and the matrix amount of sample (Table 3).

For the extraction of the wastewater samples of steroidal sex hormones and related synthetic compounds, the *off-line* solid phase extraction (SPE) method usually is used, whereas the use of *on-line* SPE [62] and LLE [15,23] methods was rarely reported. The *off-line* SPE, however, has some disadvantages, which in recent years led to the development of an efficient alternative: the *on-line* extraction system. The *on-line* procedures are particularly useful in high-sensitivity routine analysis of a large number of samples and/or series of samples, or in the analysis of hazardous or highly infectious materials. *Off-line* procedures are appreciated for their 'directly *in-situ* applicability' to the sample, and for the possibility to inject the same extract several times. The *on-line* configuration most widely used for the analytical determination of emerging contaminants in environmental samples of water was the SPE coupled with HPLC–MS and HPLC–MS/MS quadrupole. With this configuration, the inherent advantages of *on-line* SPE and HPLC–MS (Fig. 3) have been incorporated into a single methodology, which shows interesting features like automation, sensitivity, accuracy, reliability, high efficiency and minimal sample handling.

A new fully automated method, used for the detection of estrogenic compounds in water systems before and after the treatment, is based on the SPE *on-line* coupled to HPLC–ESI–MS/MS. This method allows an unambiguous identification and quantification of the more ecologically important estrogens and in treated waters at alarm levels much lower than those recently presented [69]. RSD is found to be between 1.43% and 3.89%, while the recovery percentages are greater than 74%. These results suggest that the method is highly accurate. Recent reviews concerning *on-line* SPE coupled to HPLC–MS [70] have shown that, when *on-line* configuration coupled with SPE–HPLC–MS/MS is considered, the SPE sorbents that can be used are both those traditional, such as silica or alkyl groups attached to polymers, and new materials such as molecularly imprinted polymers (MIPs) [71–74] and immobilized antibodies or receptors (immunoadsorbents). These techniques can be performed to further analyze other environmental contaminants such as alkylphenols, pesticides and their derivatives, products of disinfestation, mycotoxins, and other.

Another analysis that made use of the *on-line* SPE, coupled to GC–MS through a column, presented a LOD less than μg/L [75]; in fact the SPE followed by GC–MS, as before mentioned, it is recommended by the USGS and NWQL as an official method for the analysis of known and suspected EDCs [76]. Unfortunately, all methods, including those based on the use of *on-line* procedures, are typically limited to a few pre-selected compounds and very often are not sufficient to assess water quality, since many unrelated microcontaminants may be a threat to the environment and human health [77,78].

2.2.1.2. Solid phase micro extraction. SPME uses a modified syringe that has a needle in which are present coated fiber of adsorbent material and the amount of analyte that is adsorbed is directly related to its concentration in the sample [79] and it does not depend on the volume of the sample. This characteristic is very useful for high-volume environmental samples of water but with a very low concentration of analyte, which is the case of the EDCs.

In the *in-tube* SPME (Fig. 4), the sample pass through a capillary, which has been repeatedly internally coated with the adsorbent material, until the extracted analytes are eluted into the analytical instrument, such as an HPLC. This process can be fully automated in order to reduce the analysis time and to provide greater accuracy and sensitivity. This method, coupled with the HPLC–MS/MS method [80], was used for the determination of five estrogens: estrone, 17β-estradiol, estriol, ethinyl estradiol and diethylstilbestrol. The optimum conditions in the *in-tube* SPME were 20 extractions/injections in cycles of 40 μL of the sample using a capillary column as the extraction apparatus. The extracted compounds were easily desorbed using mobile phase and no carryover was observed. The limit of detection of the five estrogen ranged from 2.7 to 11.7 pg/mL showing a sensitivity 34–90 times higher than that reported for direct injection methods and has been successfully applied to the analysis of environmental water samples without giving any interference peak. Occasionally, it may be added a derivatizing agent, especially in the analysis of EDCs, whose choice may be able to improve the performance of the instruments. Besides, the choice of derivatizing agent, can affect the analysis of the EDCs. The process of in situ derivatization is the most commonly used although it has the disadvantage that only a few derivatizing agents do not suffer from matrix effects. A study of determination of alkylphenol and bisphenol A (BPA) in water samples employed an extraction with fibers coated with the derivatizing polymer N, *O*-bis (trimethylsilyl) trifluoroacetamide (BSTFA) before GC–MS analysis [81,82]. The detection limit of the method ranged from 0.07 to 2.34 ng/L, while the calibration range was linear between 0.01 and 15 μg/L.

It is also reported a determination of nonylphenol, nonylphenol mono and diethyl oxilate and their metabolites [83,84] with a SPME headspace-GC–MS method in which was instead used a derivatization of the sample. To validate this method, the experimental results were compared with other different situations, including the derivatization after the direct SPME, derivatization in the sample with various derivatizing agents, followed by derivatization in headspace SPME using fibers clothed with different materials. Colloids and natural organic matter present in the water samples to be analyzed can affect the recovery of EDCs in SPME [85,86]. Among the advantages of SPME, compared to other methods, we find a reduced use of solvents, which can make the technique easily automated [87] and the possibility of reuse of the fiber for other analysis after treatment.

2.2.1.3. Liquid phase micro extraction. The method of liquid–liquid extraction (LLE), typically used for the analysis of organic solutes in the water samples, is less and less used. This technique, in fact, requires a high amount of organic solvents, thereby producing a large amount of toxic waste, as well as an additional step of concentration. LLE is being replaced by liquid phase microextraction methods (LPME) that seem to solve these problems and at the same time reduce the analysis time [88,89]. These methods can be divided into three main categories: single drop microextraction (SDME), hollow fiber microextraction (HF-LPME) and dispersive liquid–liquid microextraction (DLLME).

Table 3
Examples of sample preparation for different EDCs as a function of matrix and the volume of sample.

Analyte	MATRIx	Vol (L)	Sample prep.	RECovery (%)	Ref.
E1, E2, EE2, E3	Natural water, purification plants	0.1 influent	SPE	80	[49]
		0.25 effluent			
		1 river water			
E1, E2, EE2, E3	Surface water; treatment plants sewage; influent and effluents	1	SPE	81	[52]
E1, E2, EE2, E3	Effluents and purified	1	SPE	91	[55]
E1, E2, EE2, E3, DES, PROG, NOR, LEV	Natural water	0.05	On-line SPE	85	[56]
E1, E2, E3, DES	Sewage treatment plants, influents and effluents water; drinking water	0.5	SPE	83	[24]
E1, E2, EE2, E3, DES, PROG, NOR, LEV	River water	–	SPE	90	[57]
17β-EE, E1	Influents	20	SPE, Fractionation, HPLC, LLE	–	[58]
E2, E3	Effluents	15	SPE, LLE, GPC, hydrolysis	–	[20]
17α-EE	Effluents	20	SPE LLE	35–95	[13]
PROG	Effluents	20	SPE, silica gel 60 Derivatization	–	[23]
MES	Effluents	25	SPE, Fractionation, HPLC	–	[40]
NOR, LEV	Effluents	2	SPE	–	[59]
DES	Effluents	7	SPE	78–83 75	[25]
	Effluents	1	SPE, Hydrolysis, SPE, Fractionation, HPLC	88–98	[60]
	Influents	1	SPE, Silica Gel, Derivatization	41–90	[14]
	Effluents	5	SPE	92–100	[8]
	Effluents	0.3–2	SPE, Hydrolysis	–	[9]
	Surface water	2–6	Fractionation HPLC		
	Effluents	2.5	SPE	92–99	[53]
	Effluents	2	SPE	79–108	[61]
	Effluents	0.2	SPE on line	96–111	[62]
	Effluents	0.03	SBSE-LD	11–100	[63]
	Effluents	0.5	SPE	44–112	[24]
	Effluents	2	SPE	90–119	[47]
	Natural and drinking water treatment plants	0.5	SPE	87–94	[64]
	Effluents	0.15–0.4	SPE	86–91	[31]
	Influents	0.5	SPE	88–97	[8]
	Effluents	1			
	Effluents	1	SPE	85–120	[65]
	Taipei water and drinking water treatment plants	2	SPE	–	[66]
	Effluents	2	SPE	72–78	[67]
Natural and synthetic steroids	Influent and effluent water	20–80	LLE, Hydrolysis, TLC	–	[15]
E2	Influent, effluent water and other types of samples	0.05–0.1	SPE	97	[68]
E2, EE, E1	Effluent water	20	SPE, Fractionation HPLC, LLE		[13]
E2, EE, E1, 17β-EE	Effluent and other types of samples	1	SPE, Hydrolysis, SPE Fractionation HPLC	88–98	[14]
E2, EE, E1	Effluent water	15	SPE, LLE, LLE, GPC Hydrolysis, LLE		[20]
E2, EE	Effluent water	5	SPE Fractionation HPLC	72–78 (EE)	[18]
E2, E1, MES, 17α-E2, E2-17-valer., 16α-OHE1, E2-17-acet.	Influent and effluent water	1	SPE, silica gel derivatization	41–90	[51]
E2, EE, E1, 17α- E2 (SS)	Effluent and other kinds of samples	1	LLE	35 (E2) 68 (EE) 60 (E1) 95 (17α- E2)	[23]
E3, E2, EE, E1	Influent and effluent and other kinds of samples	0.15 0.4	SPE	89–91	[22]
E3, E2, EE, E1 (Roman)	Influent and effluent water	0.5 1	SPE	88–97	[8]
E2, EE, E1	Effluent water	2.5	SPE	92–100	[53]
E3, E2, EE, E1, MES, LEV, NOR acetatE	Effluent water	20	SPE Silica Gel 60 Derivatization	–	[40]

Table 3 (*Continued*)

Analyte	MATRIx	Vol (L)	Sample prep.	RECovery (%)	Ref.
E3, E2, E1, EE,	Influent and effluent water	0.5	SPE	57–112	[38]
E3, E2, EE, DES, E1, NOR, LEV, pROG	Effluent water	1	SPE	87–94	[31]
E2, EE, E1	Effluent water	2	SPE Fractionation HPLC	–	[21]
E2	Influent and effluent water (PSP)		SPE	–	[18]
EE	Effluent water	7.08	SPE	–	[25]
E2, EE, E2 gluc.	Effluents	0.3–2	SPE, Hydrolysis, Fractionation HPLC	65–79	[9]
E2 solf.	Surface water	2-6			
E3, E2, EE, DES, E1, NOR, LEV, PROG	Effluent water	0.2	SPE on-line	96–112	[62]

E1: estrone; E2: estradiol; E3: estriol, EE: ethinyl estradiol, E2-17-valer.: Estradiol-17-valerate, 16α-OH-E1: 16α-hydroxy estrone, E2-17.acet: estradiol 17-acetate; E2-gluc: estradiol glucuronide; solf-E2: estradiol sulfate; SS: surrogate standard; PROG: progesterone; MES: mestranol; NOR: norethindrone; NOR-acetate: norethindrone acetate; LEV: levonorgestrel; DES diethylstilbestrol; SPE Extraction solid phase TLC: thin layer chromatography; GPC: gel permeation chromatography; PSP: pilot-scale plant; LLE: liquid–liquid extraction; HPLC: high performance liquid chromatography; GLC: gas–liquid chromatography; IA: immunoassay; NI: negative ionization; PI: positive ionization; ELISA Assay Immuno-Absorbent linked to an enzyme; DAD diode array detector; GC: gas chromatography; MS: mass spectrometer; LC: liquid chromatography; UV: Ultraviolet; RIA: radioimmunoassay; FL: fluorescence.

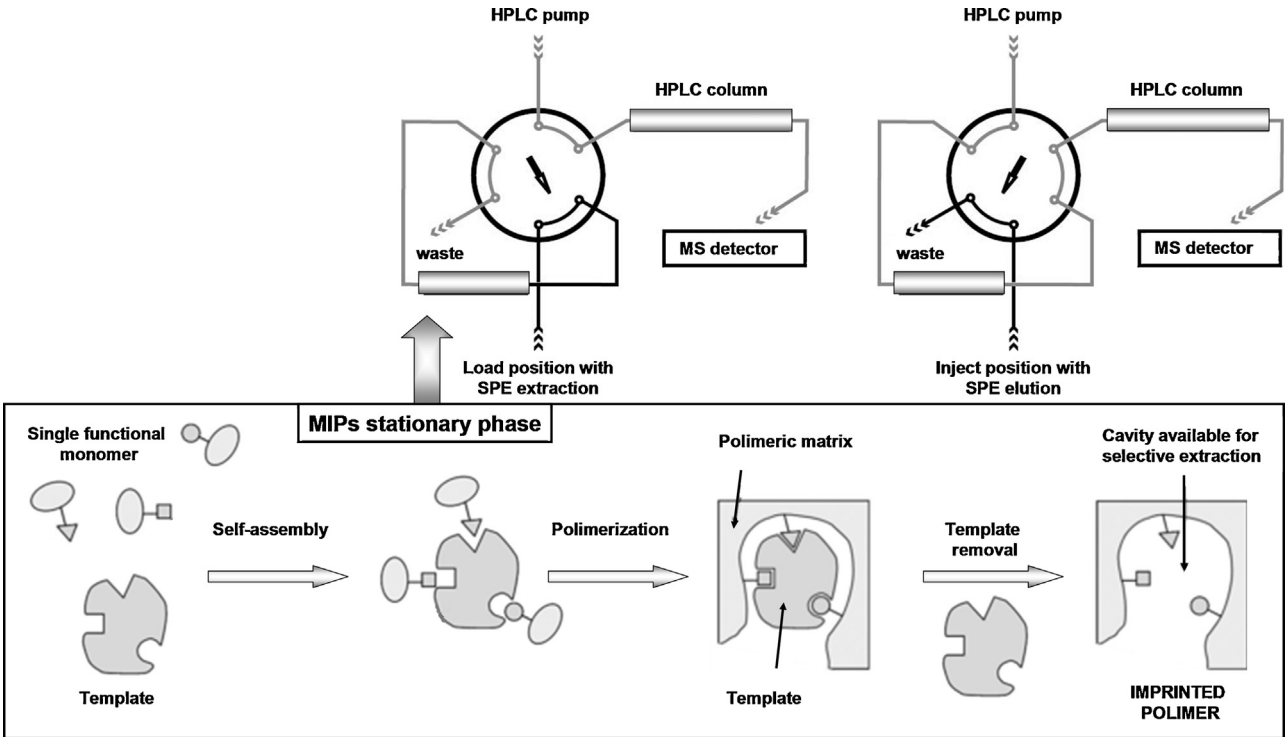

Fig. 3. On-line SPE-HPLC–MS instrument configuration, also with MIPs sorbent (into the box the general synthetic procedure for MIPs production).

In a study, in order to be able to analyze BPA in river water samples, it has been used the SDME method [90]. The droplet of solvent was subsequently withdrawn, derivatized and injected in the GC–MS system. Several operating parameters including the choice of solvent, the exposure time, the mixing speed can be selected by comparing the peak areas in the different conditions, in order to improve the analytical procedure. Using toluene as solvent, the limit of detection and quantification were, respectively, 2 and 10 ng/L. One of the major problems that may be encountered in the SDME method regards the retention of the droplet of solvent into the sample container that is agitated during the period of exposure. In fact, the size of the droplet, in some cases, is reduced with the time of exposure; this can be caused both by the strength of agitation or by the dispersion of the solvent in the aqueous sample.

A better solution seems to be the HF-LPME, in which the use of hollow fibers with porous polypropylene can manage to protect the droplets in the extracting solvent, by containing them directly and exposing to the aqueous sample [91]. Even in this case, the solvent exposed to the sample and the BSTFA derivatizing agent are inserted simultaneously into the injector of the GC–MS system for the process of derivatization. The limits of detection and quantification of this method were found to be in the range of 0.005–0.015 and 0.012 and 0.026 g/L respectively, thus comparable to the SPME headspace and LLE. From the results, HF-LPME technique was considered a simple and rapid alternative to the more common method of extraction for EDCs.

2.2.2. Derivatization

The derivatization procedures can be carried out even during the SPE, by modifying the adsorbent with a derivatizing agent, as reported in a study [92] which used an OASIS HLB for the sample cleaning and as a support of derivatization. Estrogens, derivatized

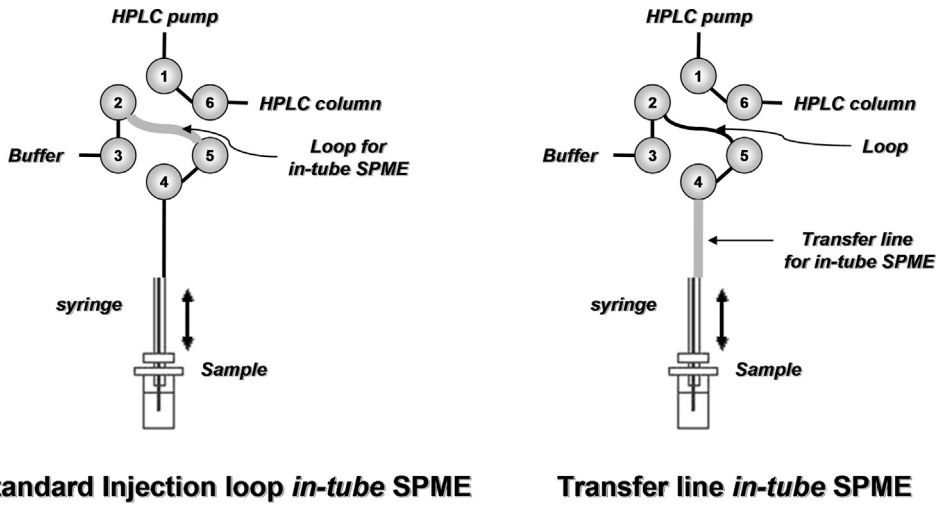

Fig. 4. In-tube SPME.

with dansyl chloride and analyzed with HPLC–MS/MS, were determined at levels of 1 ng/L in influent and effluent water samples. Moreover, were reported analyses in which the SPME was used both for extraction and for derivatization. Fiber extraction present bis-(trimethylsilyl) trifluoroacetamide as the derivatizing agent, and the limit of detection by this procedure is reduced at ng/L or lower. The derivatization with pentafluorobenzile bromide (PFB) seemed useful for sensitive determination with GC-negative ion chemical ionization (NI-CI)–MS [93] or HPLC–NI-APCI Atmospheric Pressure Chemical Ionization-MS [94,95].

One study [67] has shown that the environmental matrices may influence the choice of derivatization agent. Indeed dansyl chloride gave a signal intensity of one or two orders of magnitude greater than that normally induced by the standard estrogen not derivatized. Even estrogens derivatized with pentafluorobenzoil bromide (PFBBr) produce signals greater of 5.8 times compared to not derivatized estrogens, while the signal from 2-fluoro-1-methylpyridinium p-toluene sulfonate (FMPTS) is found to be rather dependent on the analyte. The results, therefore, show that is preferable the use of PFBBr for the analysis of estrogens in complex matrices such as influent samples, effluent and treatment plants water, in contrast to the dansyl chloride which should only be used for the analysis of clean water. In Table 4 are reported the derivatizing agents actually used in the determination of EDCs.

2.2.3. Concentration

The concentration step, as reported in literature [22], can be critical and can result in losses of more volatile compounds when the sample is completely dried. In a study was concluded that the losses occurred during the blow-down are minimal and that the concentration step is not so critical in the analysis of estrogens and progestins [27]. Despite this conclusion, during the concentration step, however, some precautions were taken to minimize possible analytes losses, such as controlling the flow and temperature during evaporation with nitrogen, the protection of the sample from light to prevent photolytic degradation and not leave the extract completely dry for a long time.

2.2.4. Purification

The extracts from highly contaminated wastewater often require additional cleaning before analytical determination. This can be obtained by very different methods, including liquid-liquid extraction, purification of the solid phase on C_{18} and NH_2 columns [8], chromatographic column on silica gel [51], gel permeation on columns of Bio-Beads SX 3, fractionation on HPLC [18] or combinations of all these [14]. Some of the purification procedures were developed not only to purify the extracts, but also to isolate the active fractions from the extract of wastewater for further identification of the compounds responsible for this activity [13]. However, good detection limits, without the purification step, in the nanogram levels or sub-nanogram per liter were reported by a research group using as adsorbent graphitized carbon black for the SPE before analysis by HPLC–tandem MS [22].

Table 5 reports the general characteristic of the extraction procedures.

2.2.5. Enzymatic hydrolysis

Analysis of conjugated estrogens have received little attention due to their low power compared to estrogen related compounds unconjugated or because they are decomposed inside the plants for purification of wastewater through the treatment procedures [20,22,51]. However, some authors studied the presence of conjugated estrogens in wastewater and concluded that addition of a capable step of hydrolysis to convert the conjugated forms in the active hormones, during the process of preparation of the sample, appears to be necessary in the case in which the final analysis is done by immunoassay and instead convenient in the case of GC–MS ones.

Glucuronidase enzyme was used to conduct the hydrolysis of glucuronide and estrogen sulfates [14,20,27]. By measuring the concentration differences between the hydrolyzed samples of hormones conjugates and not conjugates, it was demonstrated that this enzyme converts quantitatively estradiol glucuronide to free estradiol, while only 30% of estradiol sulfate is split. This result leads to the conclusion that the levels of sulfated hormone may be underestimated, but the GC–MS, which allows the simultaneous determination of both the free and the conjugated forms, can avoid this risk.

2.3. HPLC for determination of EDCs

2.3.1. HPLC vs GC

In literature the use of high-performance liquid chromatography (HPLC) for the separation and the final analysis of estrogenic compounds and progestin extracts from matrices of waste water is not very present, although recent instrumental progress in the coupling of this chromatographic technique with mass spectrometry (MS) encourages greater use [8,18,22,24,31,62]. Indeed, the determination of sex steroids in environmental samples is usually carried out by GC; however, the use of HPLC for this purpose

Table 4
Derivatizing agents used in the determination of EDCs.

Derivatizing agent	Ref.
Bromide pentafluorobenzoyl (PFBBr)	[76]
	[96]
Trimensilil pentafluorobenzoyl	[97]
P-nitrobenzoyl chloride	[98
N-methyl-*N*-(trimethylsilyl) trifluoro acetamide (MSTFA)	[99]
Dansyl chloride	[100]
	[101]
	[102]
Bis-(trimethylsilyl) trifluoro acetamide with 10% of trimethylchlorosilane	[23]
n-methyl-*n*-(tert-butyl dimethyl silyl) trifluoro acetamide (MTBSTFA) with 1% tert-butyl dimethyl chlorosilane (TBDMCS)	[53]
	[103]
n-methyl-*n*-(trimethylsilyl) trifluoro acetamide (MSTFA)-trimethylsilyl imidazole (TMSI)-ditioeritrolo (DTE), 1000:2:2 (v/v/w)	[51]
Heptafluoro butyric anhydride	[27]
Penta fluoride propionic acid	[31]
n-methyl-N-trimethylsilyl-2, 2,2-trifluoro acetamide (MSTFA)-trimethylsilyl iodide (TMSI)-dithioerythritol (DTE)	[40]
Acetic anhydride (extractive acetylation)	[20]
N-methyl-*N*-(tert butyl methyl silyl) trifluor acetamide (MTBSTFA)	[103]

has increased in recent years because it provides more flexibility. In fact, contrary to GC–MS techniques, HPLC–MS is not limited to volatile or low molecular weight compounds and allows the detection of various forms of estrogen (eg, conjugated) without the need of a previous derivatization phase.

Moreover, the advantage of the GC–MS compared to the HPLC–MS consists of the availability of data from the mass spectrum, which are useful for the identification of unknown peaks in active estrogenic fractions. The recent introduction of a MS/MS detector has essentially increased the performance of chromatographic methods reducing the limits of detection and supporting the identification of the analyte. In fact, in terms of accuracy and repeatability, HPLC–MS, and GC-MS/MS or HPLC–MS/MS are all generally satisfactory.

2.3.2. Detectors

Due to limited sensitivity, only a few studies have used methods employing HPLC detectors other than MS for the analysis of environmental estrogens. It has been reported in the literature the use of fluorescence detector (FLD) or diode array detector (DAD), for the determination of estrogens (natural and synthetic) and progestogens because they possess chromophore groups that allow their determination to specific wavelengths (197 nm for estrogen and 242 nm for progestins). Nevertheless, neither the sensitivity nor the selectivity of this detection method allows the determination of these compounds in complex matrices, such as wastewater, at very low levels (ng/L) in which they are normally present [24]. Except some authors [18,62], generally used instrument configuration is the separation of estrogen and/or progesterone receptors using HPLC coupled to MS detector. In fact, before the advent of HPLC–MS, many of these polar compounds were difficult and impossible to determine [104] without preliminary derivatization step. In addition to the determination of sex hormones from environmental samples, HPLC–MS/MS is widely used for the same purpose in biological samples [105,106]. In fact, the massive presence of steroid hormones being detected in the farmland [107–109], was also in addition determined in the leaves of plants irrigated with underground water contaminated by these compounds through the rains [110].

2.3.3. Operating conditions

Many studies regarding separations of estrogens and progestins performed by HPLC were carried out with the octadecyl silica as stationary phase (250 mm × 4.6 mm id, 5 μm particle size).

Generally, in these studies, were employed mixtures of water-acetonitrile as the mobile phase and gradient elution from 20 to 50% up to 100% of organic solvent. In addition, regarding the change in the mobile phase to improve the detection sensitivity by means of MS, when performed, was always utilized post-column employing ammonia in methanol [22] and triethylamine [8]. These modifiers were added to promote the deprotonation of weakly acidic estrogen and increase the response of the mass spectrometer operating in the negative ionization mode (negative ion) interfaced via electrospray.

HPLC–MS and HPLC–MS/MS (which offers a more than satisfactory sensitivity and selectivity in trace analysis of environmental contaminants [111,112]) techniques were mostly used in the mode SIM (selected ion monitoring) and the most widely used interfaces for HPLC–MS analysis of steroids in the aquatic environment were the ESI and APCI [113,114].

A research group, by using ESI in negative ionization mode as the interface for the determination of estrogen extracted from wastewater samples, has been able to obtain high sensitivity [115], which demonstrates the superiority of the ESI interface as compared to the APCI [22,24]. However, a recent study [31] on the analysis of traces of estrogen in effluent samples, using the HPLC–MS/MS, showed that even APCI interface in positive ionization mode can provide a good sensitivity, although in analytical conditions similar to a previous study.

Various studies also showed that the use of triple quadrupole mass spectrometers in HPLC–MS/MS system [8,22,24] has led to an increase in selectivity and sensitivity, with detection limits much better than those obtained with a single quadrupole MS [24].

In different methods describing HPLC–MS/MS for the determination of estrogen in effluent, the confirmation criteria considered for the identification of the analyte were that the HPLC retention times of the analytes should be within 2% of those of the standard and that the relative abundances of at least 2 absolute ionic precursor ion → product ion transitions had to be within 20% of those obtained with the standard. In Fig. 5 were reported MS/MS fragment chemical structures observed for the main EDCs generally analysed.

A highly sensitive method and not complicated analysis of steroid hormones in samples of river water and estuary was developed [116] by using MS/MS detector equipped with an electrospray source (ESI) and atmospheric pressure photoionization (APPI). The analyzed steroids include not only estrogens but also androgens and their conjugates. The APPI showed greater sensitivity than ESI for most of the non-conjugated steroids examined, with a very high sensitivity in particular for testosterone and 4-androstene-3,17-dione. On the contrary, ESI was more efficient for conjugated hormones.

In order to overcome the potential problems of contamination during the EE2 determination, manual procedures should be

Table 5
Procedures for EDCs extraction and purification.

Analyte	Matrix	Volume (L)	Extraction	Elution solvent		Derivatization	Ref.
E1, E2, EE2, E3	Natural water, purification plants	0.1influent 0.25 effluent 1 river water	SPE	7 mL	Dichloromethane: Methanol (50:50, v:v)	–	[49]
E1, E2, EE2, E3	Surface water; treatment plants sewage; influent and effluents	1	SPE	–	–	–	[52]
E1, E2, EE2, E3	Effluents and purified	1	SPE	–	–	–	[55]
E1, E2, EE2, E3, DES, PROG, NOR, LEV	Natural water	0.05	On-line SPE	Mobile phase	Methanol and water (gradient elution)	–	[56]
E1, E2, E3, DES	Sewage treatment plants, influents and effluents water; drinking water	0.5	SPE	2 x 4 mL	Acetonitrile	–	[24]
E1, E2, EE2, E3, DES, PROG, NOR, LEV	River water	–	SPE	–	–	–	[57]
17β-EE, E1	Influents	20	SPE, LLE	–	–	–	[58]
E2, E3	Effluents	15	SPE, LLE, GPC	Not reported	Ethyl acetate (SPE and GPC) Cyclohexane (LLE)	Acetic anhydride	[20]
17α-EE	Effluents	20	SPE LLE	5 (or 2.5) mL 3 x 50 mL	Methanol: water at different percentages Dichloromethane	–	[13]
PROG	Effluents	20	SPE, silica gel 60	70 mL	methylene chloride	–	[23]
MES	Effluents	25	SPE	30 mL	Acetone and Methanol	n-methyl-N-trimethylsilyl-2, 2,2 - trifluoro acetamide (MSTFA)- trimethylsilyl iodide (TMSI)- dithioerythritol (DTE)	[40]
NOR, LEV	Effluents	2	SPE	10 mL + 10 mL	Ethyl acetate + dichloromethane	–	[59]
DES	Effluents	7.08	SPE	10 mL	Methanol	–	[25]
	Effluents	1	SPE, Hydrolysis, SPE	40 mL	Methanol	–	[60]
	Influents	1	SPE, Silica Gel	3 x 5 mL	Methanol	–	[14]
	Effluents	5	SPE	–	–	–	[8]
	Effluents Surface water	0.3–2 2-6	SPE, Hydrolysis	15–30 mL	Methanol	–	[9]
	Effluents	2.5	SPE	2 x 30 mL	Methanol: water (85: 15, v:v)	*n*-methyl-*n*-(tert-butyl dimethyl silyl) trifluoro acetamide (MTBSTFA) with 1% tert-butyl dimethyl chlorosilane (TBDMCS)	[53]
	Effluents	2	SPE	3 mL	Ethyl acetate	*N*-methyl-*N*-(trimethylsilyl) trifluoro acetamide (MSTFA)	[61]
	Effluents	0.2	SPE on line	Mobile phase	Acetonitrile and water (gradient elution)	–	[62]
	Effluents	0.03	SBSE-LD	1.5 mL	Acetonitrile	–	[63]
	Effluents	0.5	SPE	10 mL	Acetonitrile	–	[24]
	Effluents	2	SPE	2 x 10 mL	Ethanol	–	[47]
	Natural and drinking water treatment plants	0.5	SPE	8 mL	Methanol	–	[64]
	Effluents	0.15–0.4	SPE	10 mL	Dichloromethane: MeOH (60:40, v:v)	Penta fluoride propionic acid	[31]
	Influents	0.5	SPE	10 mL	Dichloromethane: MeOH (60:40, v:v)	–	[8]
	Effluents	1					
	Effluents	1	SPE	6 mL	Ethyl acetate: methanol (5:1, v:v)	–	[65]
	Taipei water and drinking water treatment plants	2	SPE	15 mL	Methanol: Dichloromethane (50:50, v:v)	–	[66]

	Effluents	2	SPE	15 mL	Methanol: Dichloromethane (50:50, v:v)	–	[67]
Natural and synthetic steroids	Influent and effluent water	20–80	LLE, Hydrolysis, TLC	–	–	–	[15]
E2	Influent, effluent water and other types of samples	0.05-0.1	SPE	–	–	–	[68]
E2, EE, E1	Effluent water	20	SPE, Fractionation HPLC, LLE	5 mL	Diethyl ether: hexane, at different percentages	–	[13]
E2, EE, E1, 17β-EE	Effluent and other types of samples	1	SPE, Hydrolysis, SPE	3 x 5 mL	Methanol	–	[14]
E2, EE, E1	Effluent water	15	SPE, LLE, LLE, GPC Hydrolysis, LLE	Not reported	Ethyl acetate (SPE and GPC) Cyclohexane (LLE)	Acetic anhydride	[20]
E2, EE	Effluent water	5	SPE	15 mL 25 mL 10 mL	Acetone Dichloromethane Hexane	–	[18]
E2, E1, MES, 17α-E2, E2-17-valer., 16α-OHE1, E2-17-acet.	Influent and effluent water	1	SPE, silica gel derivatization	4 x 1 mL	Acetone	*n*-methyl-*n*-(trimethylsilyl) trifluoro acetamide (MSTFA)-trimethylsilyl imidazole (TMSI)- ditioeritrolo (DTE), 1000:2:2 (v/v/w)	[51]
E2, EE, E1, 17α- E2 (SS)	Effluent and other kinds of samples	1	LLE	70 mL	Methylene chloride	Bis-(trimethylsilyl) trifluoro acetamide with 10% of trimethylchlorosilane	[23]
E3, E2, EE, E1	Influent and effluent and other kinds of samples	0.15 0.4	SPE	12 mL	Methylene chloride : methanol (80:20, v :v),	–	[22]
E3, E2, EE, E1	Influent and effluent water	0.5	SPE	10 mL	Dichloromethane: MeOH (60:40, v:v)	–	[8]
(Roman)		1					
E2, EE, E1	Effluent water	2.5	SPE	2 x 30 mL	Methanol: water (85:15, v:v)	*n*-methyl-*n*-(tert-butyl dimethyl silyl) trifluoro acetamide (MTBSTFA) with 1% tert-butyl dimethyl chlorosilane (TBDMCS)	[53]
E3, E2, EE, E1, MES, LEV, NOR acetatE	Effluent water	20	SPE	30 mL	Acetone and Methanol	*n*-methyl-*N*-trimethylsilyl-2, 2,2-trifluoro acetamide (MSTFA)-trimethylsilyl iodide (TMSI)-dithioerythritol (DTE)	[40]
E3, E2, E1, EE,	Influent and effluent water	0.5	SPE	2 x 4 mL	Acetonitrile	–	[38]
E3, E2, EE, DES, E1, NOR, LEV, pROG	Effluent water	1	SPE	10 mL	Dichloromethane: MeOH (60:40, v:v)	Penta fluoride propionic acid	[31]
E2, EE, E1	Effluent water	2	SPE	2 x 10 mL	Dichloromethane	–	[21]
E2	Influent and effluent water (PSP)	5	SPE	15 mL 25 mL 10 mL	Acetone Dichloromethane Hexane	–	[18]
EE	Effluent water	7.08	SPE	10 mL	Methanol	–	[25]
E2, EE, E2 gluc.	Effluents	0.3–2	SPE	15–30 mL	Methanol	–	[9]
E2 solf.	Surface water	2-6					
E3, E2, EE, DES, E1, NOR, LEV, PROG	Effluent water	0.2	SPE on-line	Mobile phase	Acetonitrile and water (gradient elution)	–	[62]

E1: estrone; E2: estradiol; E3: estriol, EE: ethinyl estradiol, E2-17-valer.: Estradiol-17-valerate, 16α-OH-E1: 16α-hydroxy estrone, E2-17.acet: estradiol 17-acetate; E2-gluc: estradiol glucuronide; solf-E2: estradiol sulfate; SS: surrogate standard; PROG: progesterone; MES: mestranol; NOR: norethindrone; NOR-acetate: norethindrone acetate; LEV: levonorgestrel; DES diethylstilbestrol; SPE Extraction solid phase TLC: thin layer chromatography; GPC: gel permeation chromatography; PSP: pilot-scale plant; LLE: liquid–liquid extraction; HPLC: high performance liquid chromatography.

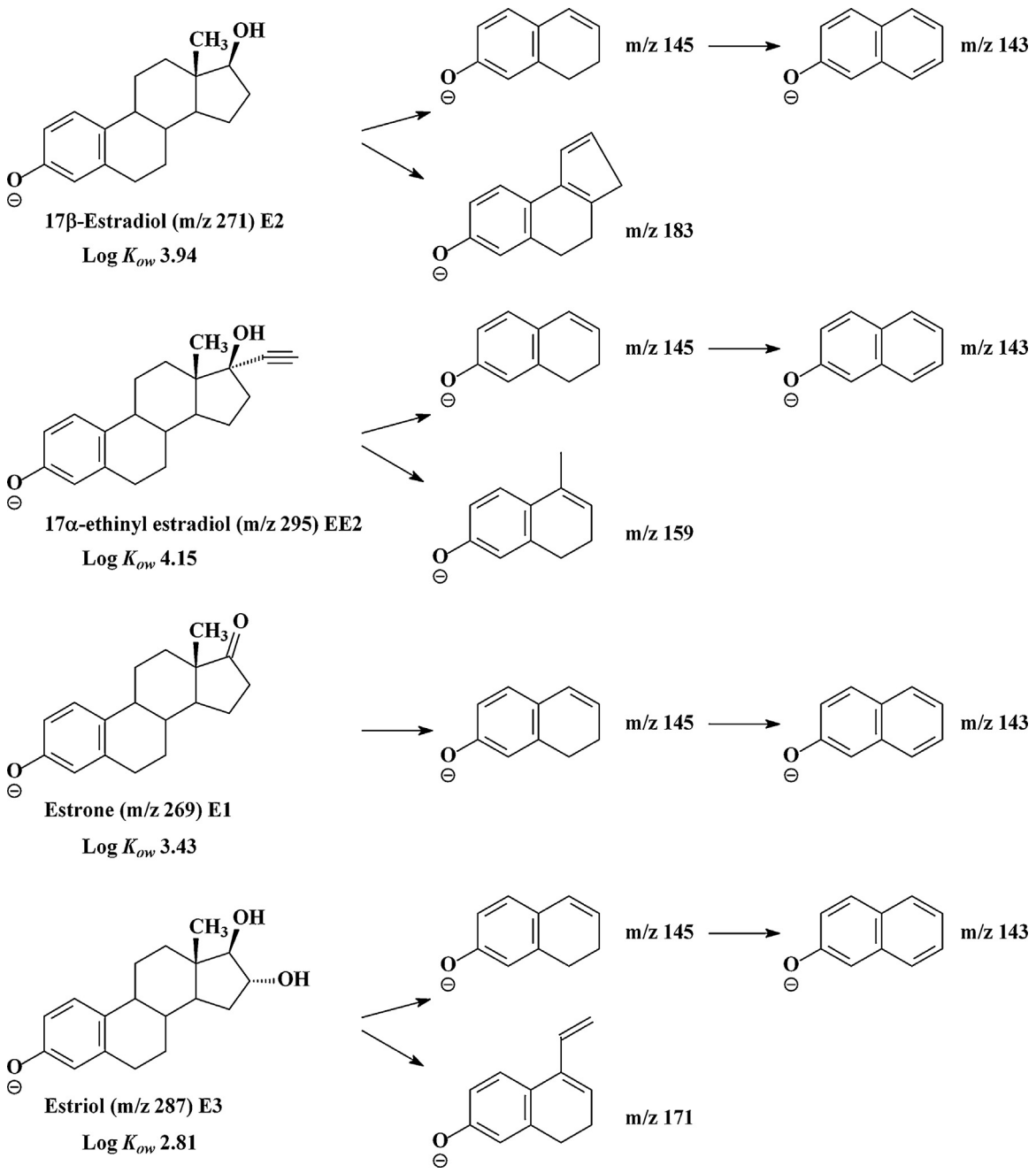

Fig. 5. MS/MS fragments chemical structures.

avoided as much as possible. In agreement with these considerations a study, preparing a column surface-modified (SM) with molecularly imprinted polymer (MIP) using the 6-ketoecradiolo as a template [117], determined 17β-estradiol in river water samples using a HPLC–MS method totally automated. MIP, used to restrain E2, were synthesized by fresh 4-vinylpyridine and ethylene glycol dimethacrylate, used respectively as functional monomer and a cross-linking agent. This column eliminates any interfering constituents of the matrix: in fact the researched compound E2 is retained selectively, while interferers, which have no affinity for MIP, pass through providing excellent chromatographic resolution. In order to preconcentrate target compounds for HPLC analysis, the switching of the column was coupled to a pretreatment column filled with MIPs. The surface modifications of MIP particles packed in the pretreatment column therefore can provide a selective affinity and *on-line* concentration of low levels of E2, eliminating interferences of the sample matrix, leading to a significant increase in sensitivity and reproducibility for the HPLC-MS analysis of E2 in the samples of river water. The future development of equipments it is expected to favor the choice of methods based on HPLC as a strategy for the analysis of estrogen. Even new ionization techniques such as APPI and other were developed for different types of highly sensitive triple quadrupole instruments.

These developments may mean that HPLC–MS/MS will become the first choice of the analytical methods due to the increased sensitivity and higher selectivity. Table 6 reports the studies on the EDCs determination by HPLC instrumentation.

2.3.4. GC analysis of EDCs

Gas chromatography coupled with mass spectrometry was the first chromatographic method used for the determination of steroid hormones and is undoubtedly the most used technique for the

Table 6
Studies on the determination of endocrine compounds by HPLC.

Analyte	Matrix	Sample preparation	Analysis	Ref.
E2, EE	Effluent water	SPE HPLC fractionantion	HPLC–FL	[18]
E3, E2, EE, E1	Influent, effluent water and other	SPE	HPLC–NI–ESI–MS/MS (Alltima)	[22]
E3, E2, EE, E1	Influent and effluent water, Rome	SPE	HPLC–NI–ESI–MS/MS	[8]
E3, E2, E1, EE	Influent ed effluent	SPE	HPLC–PI–APCI-MS/MS(Alltima)	[31]
E3, E2, EE, DES, E1, NOR, LEV, PROG	Effluent water	SPE	HPLC–DAD–NI–ESI–MS (LiChrospher 100 RP-18)	[24]
E3, E2, EE, DES, E1, NOR, LEV, PROG	Effluent water	SPE on-line	HPLC–DAD–NI–ESI–MS	[62]
E1, E2, E3, 17α-EE2 E Derivatives	Baltic sea	Filtration SPE	HPLC*	[118]
E1, E2, E3, 17α-EE2	Wastewater treatment plant	Filtration SPE	HPLC*	[119]
E1, E2, E3, EE2, and Sulphonated	Tamagawa river, Japan	Filtration SPE	HPLC*	[120]

E1: estrone; E2: estradiol; estriol E3; EE: ethinyl estradiol; E2-17-valer.: Estradiol-17-valerate; 16α-OH-E1: 16α-hydroxy estrone; E2-17.acet: oestradiol 17 acetate; E2-gluc: estradiol glucuronide; solf-E2: estradiol sulfate; SS: surrogate standard; PROG: progesterone; MES: mestranol; NOR: norethindrone; NOR-acetate norethindrone acetate; LEV: levonorgestrel; DES diethylstilbestrol; SPE: solid phase extraction; TLC: thin layer chromatography; GPC: gel permeation chromatography; PSP: pilot-scale plant; LLE: liquid–liquid extraction, HPLC: high performance liquid chromatography, GLC: gas-liquid chromatography; NI: negative ionization; PI: positive ionization; DAD diode array detector; GC: gas chromatography; MS: mass spectrometer; LC: liquid chromatography; UV: Ultraviolet; FL: fluorescence; *: HPLC–MS or HPLC-tandem MS

determination of estrogen and progestin in wastewater extracts. In many studies, the separation by means of GC was performed with a variety of capillary columns (Table 7) usually after injection of 1–4 μL of sample extracts in splitless mode, using helium as the carrier gas with temperature programs from about 45 to 300 °C. Although there are various methods of ionization, EI and CI surely result to be the most commonly used methods for GC-MS analysis. The analysis by conventional MS [13,20,21,23,25,40] and, to a lesser extent, by using MS/MS (ion trap) [8,27,51] were made at 70 eV, usually in the SIM mode, after derivatization of the sample. In terms of sensitivity, HPLC and GC–MS/MS are comparable with the latter showing a slightly higher value. In terms of accuracy and repeatability both techniques are, in general, satisfactory, although, as already mentioned, the derivatization step normally necessary for the subsequent analysis by GC–MS and GC–MS/MS, takes time and can be a source of inaccuracy [13]. An advantage of GC–MS, compared to HPLC–MS, is the availability of a large library of mass spectra useful for the identification of unknown peaks in estrogenic fractions [13,21].

A German study [121] reported the analyses of surface and drinking water samples in southern Germany, by means of SPE with subsequently derivatization of phenolic compounds and final instrumental analyses using GC–CI–MS, containing a series of compounds with estrogenic activity (E1, 17α-E2, 17 β-E2, 17β-EE2 and other EDC). The recoveries of the steroids were in the range between 71% and 79%, with the exception of the recoveries of estradiols, which were comprised between 56 and 67%. The RSD ranged from 9 to 15% indicating a satisfactory reproducibility and accuracy of the analytical protocol. The endogenous steroids such as E1, and αE2, βE2 and exogenous estrogen EE2 were determined almost routinely in the lowest range of ng/L.

In drinking water, in fact, E1 and EE2 were found respectively at an average of 400 and 350 pg/L; αE2 could only be determined in a sample at 300 pg/L, while βE2 has been found at an average of 700 pg/L. The sought steroids were found at levels of ng/L with an average concentration of 2.5 ng/L for E1, 1 ng/L for E2-α, β-E2, and 1.5 ng/L for EE2. This correlates largely with the results of previous investigations [14,40]. Probably, differences in concentrations up to factors of 10–20 for E1, βE2, or EE2 might be caused by weather conditions (sunshine, dryness, dilution with rain, temperature) in the sampling period, by duration of the treatment process, by state of the art for wastewater treatment and by composition of the tributary.

A rapid method [122] was developed for routine measurements in water of steroid hormones and certain pharmaceuticals for human use, which involves the use of short columns of 12 m with a temperature ramp programming of 18 °C/min. This method allowed a rapid separation by GC followed by a sensitive detection with MS in SIM mode, without sample cleaning or derivatization step, thanks to a concentration factor of 40,000 times reached using LLE in a continuous *on-line* procedure [123,124].

About chlorinated steroids and their effects in water, one research study [125] was able to determine estrone and its chlorinated in drinking water thanks to the GC–EI–MS in SIM mode. These studies showed that the process of disinfection of water with chloride can change non-toxic compounds in toxic ones, leading to the formation of chlorinated estrons such as chloro estrone-2,4-chloro estrone and 2,4-dichloro estrone. In order to understand and estimate the severity of the problem related to water contamination with EDCs, a consortium, called the cooperation of Austrian researchers on endocrine modulators (*ARCEM*) [126], was created in Austria in 1999. The analytical methods for the analysis and monitoring used by *ARCEM* for over 400 samples of groundwater and surface water of estrogenic hormones and industrial chemicals were based on GC–MS system. Since the analytical results were to be sent to a toxicology test, the program required quantitation limits lower than 0.1 ng/L for ethinyl estradiol and 10 ng/L for industrial chemicals, depending on the individual compound. For this program, the research team [127] determined steroid hormones by means of a technique of isotope dilution and GC–MS high resolution. All data collected by the program *ARCEM* have shown that the concentrations are comparable to those measured in Germany, Switzerland and other countries [128–130] and the results indicate that these compounds are found in selected sites of groundwater and surface water with a detectable concentration.

2.4. Recent trends using hyphenated techniques and on-line systems

The identification of emerging water contaminants requires new techniques and methods that are capable of determining large numbers of analytes in a single analysis. Most of EDC analysis determination is based on GC and/or HPLC methods thanks to their highly efficiency and selectivity that give the primary information about the physic-chemical properties to classify the sample composition [132–135], even if new approaches in this way must accompany the

Table 7
Determination of EDCs by gas chromatographic methods.

Analyte	Matrix	Sample preparation	Analysis	Ref.
EE	Influents	SPE	GC–MS	[58]
17α-E2	Effluents	LLE	GC–MS	[13]
MES	Effluents	SPE	GC–MS	[40]
NOR	Effluents	HPLC fractionation	GC–MS	[59]
LEV		SPE		
DES	Effluents	SPE	GC–MS	[25]
E1, E2, E3, EE2, Other hormones	River water, Wastewater treatment plant	Filtration SPE Derivatization (MSTFA)	GC–ESI–MS	[43]
E1, 17β-E2, E3, 17α-EE2 E Derivatives	Effluent water and Wastewater treatment plant	Filtration SPE Derivatization (BSTFA)	GC–ESI–MS GC–ESI–MS–MS	[131]
E1, 17β-E2, E3, 17α-EE2 E Derivatives	River, wastewater treatment plant, Effluent water	Filtration Extraction discs Derivatization (PFBO)	GC-quadrupole-MS	[76]

traditional techniques, supported by the fact that these compounds have their endocrine activity at very low levels. The detector widely used, due to his versatility and efficiency, is the mass spectrometer, applied in different operating modes and based on accurate mass measurements, which proved to be a useful tool in tentative identification.

Many authors have started to use these new analysis methods. The most common approaches have seen the use of these tools for the identification of EDCs either as a target of analysis or as a non-target [136]. Most of the compounds analysed are correctly identified at different sub-ppb levels. Identification at 0.1 μg/L is more problematic for some compounds, especially in complex-matrix samples. By contrast, many contaminants can be identified properly at level as low as 0.02 μg/L in cleaner matrices [137].

Several organic pollutants, usually drug degradation by-products not reported in official lists, are identified by liquid chromatography/time-of-flight/mass spectrometry, which led to highly selective information about elemental compositions and the determination empirical formula [138]. Some authors have noted that there is a correlation between the abundance of an isotopic peak and the presence of some pollutants in water samples. The presence of the isotopic peaks can reduce the possibility of errors in the pollutants identification. When the abundance revealed for an isotopic peak is between 60 and 200 counts, the error observed is below 20%, while when it is higher than 200 counts the error decreased to below 10%. GC-TOF system would be an ideal complementary tool for the determination of low polar pollutants in water samples. This approach based on the experimental isotopic abundance reduces the number of possible compositions and the number of the EDCs possible empirical formulas, giving a good compromise between the analysis time and information gathered [139]. Triple quadrupole mass spectrometer (QqQ) systems have been found very efficient and simple mass analysers to detect EDCs at very low concentration levels [140], even if in last years better limit of quantifications were obtained [141,142], also with different and more easily and routinely instrument configuration [143].

Recently several highly hyphenated instrument configurations, as automated and *on-line* systems, were described and reviewed in literature [144–150]. In these configurations the sample was directly processed by a fully-automated and *on-line*, system developed for preconcentration and analysis. Preconcentration step, in these cases, is generally achieved by solid phase extraction onto a different stationary phases (as reported in Section 2.2.1) using sequential injection analysis (SIA). In this configuration, as also reviewed by He and coworker [151], is not possible the use of SBSE method, even if it provides high extraction efficiency, better reproducibility, and no special skills are required.

For this reason, Van Hoeck et al. [152] used the multi-stir bar sorptive extraction-single desorption-capillary gas chromatography/mass spectrometry instrument configuration for the multiresidue screening of endocrine-disrupting chemicals and pharmaceuticals in aqueous samples.

Boix et al. use large volume direct injection method, coupled to ultra-high-performance liquid chromatography and triple quadrupole mass spectrometer (UHPLC–MS/MS QqQ) for the determination of 40 emerging compounds, including pharmaceuticals and drugs of abuse [153].

Another novelty is represented by the recent introduction of *in-situ* derivatization in order to obtain a sample that can be easily analyzed using GC-MS instrument configuration [154,155].

In conclusion, it can be said that all these new techniques are very effective when used in the detection of pharmaceuticals, endocrine disrupting compounds (EDCs) and pesticide degradation products [156]. To this regard, the possible limit that can be envisioned is the lack of a wide spectral library for mass spectra and the use of software not yet perfectly efficient and foir this reason often is necessary not only a single laboratory validation procedure, but a complete interlaboratory validation of an environmental monitoring method for the analysis of endocrine disrupting compounds, especially when trace amounts analysis are required [157].

3. Conclusions

Of the two systems of chromatographic separation, the GC was found to be the most widely used, thanks to the high compatibility with the mass spectrometer. However, the evolution of the techniques of HPLC coupled with MS, which led to the resolution of the problems encountered with GC analysis, rendered this technique the most popular method for the analysis of increasingly important environmentally aggressive compounds such as EDCs.

The combination of chromatographic systems with biological methods may improve the analysis of these compounds, but to date this strategy is under development. In the coming years it will be possible to use these methods integrated into fully automated online systems that should result in better test results reducing the possibility of contamination, leakage, improving the preparation of the sample and decreasing the interference of the matrix and decreasing cost of operations. The development and refinement of analytical techniques by the research comunity for the control of EDCs in water joined with the use of efficient wastewater treatment plants, are and will be necessary to help the regulatory bodies since the increasing use of these compounds, of the world population and the not complete knowledge of the effects of endocrine active substances on people will sinergistically exacerbate their detrimental effects on the environment.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2016.01.034.

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