Review Article

Analytical Challenges and Recent Advances in the Determination of Estrogens in Water Environments

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Abstract

Estrogens have been shown to be present in the water compartment, mainly due to the inefficient removal in wastewater treatment plants (WWTP). The concentrations of these compounds, although very low (low ng/L), are sufficient to induce estrogenic responses and alter the normal reproduction and development of wildlife organisms. The compounds have been determined, by a variety of analytical procedures, in the influents and effluents of WWTP, fresh waters, rivers, and even drinking waters. Determination of natural and synthetic estrogens and progestogens in natural water is, however, a difficult analytical task, because of the very low detection limits required and the complexity of the matrix. Thus, in general, complicated, time-consuming extraction and purification processes, usually based on the application of solid–liquid extraction, are performed before final determination by immunoassay, high-performance liquid chromatography, or gas chromatography, very often coupled with mass spectrometry. This paper reviews the analytical methods so far described for the analysis of estrogens, which are currently important environmental pollutants presented in natural and wastewaters. Discuss of the main steps, from sampling up to analysis, and the techniques most commonly used in the determination is presented.

Introduction

The natural steroid hormones are generally synthesized from cholesterol in the gonads and adrenal glands (1). A precise idea about what an endocrine-disrupting compound means should be established in order to facilitate the identification of active compounds and to fulfill correct regulatory control rules. On this goal, the International Programme on Chemical Safety and the US EPA's Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) in 1998 (EDSTAC, 1998), updated in 2000 (EDSTAC, 2000), proposed the following definition: "endocrine disrupter is an exogenous substance or mixture that alters theory function the endocrine system causes adverse effects at the level of the organism, its progeny, populations or suprapopulations of organisms, based on scientific principles, data, weight-of-evidence, and the precautionary principle" (2). A variety of natural compounds and anthropogenic chemicals are known or predicted to influence the endocrine system, such as natural estrogens (e.g., 17β-estradiol, estrone), natural androgens (e.g., testosterone), phytosteroids (e.g., 17β-sitosterol), isoflavonoids (e.g., daidzeine), synthetic estrogens (e.g., 17β-ethinylestradiol), pesticides (e.g., atrazine), phthalates, alkylphenol ethoxylate surfactants, etc.

Steroid hormones are naturally occurring hormones like estrone (E_1), estradiol (E_2), and estriol (E_3) and synthetically prepared ones: ethynilestradiol (EE_2). They are a family of polycyclic ring structure chemicals containing a common carbon molecular framework. All sexual hormones have at the base a steroidal structure (Figure 1A). The most important physicochemical characteristics of these compounds are presented in Table I (3).

Natural steroid hormones are released into the environment almost all the time by the urine and excrement of all species, sexes, and types of farm animals. We can divide sources and release of naturally produced steroid hormones into few groups: produced by humans, produced by livestock, produced by wildlife, produced from treated sewage waste (4).

Data concerning non-domestic animals connected with the release of steroid estrogen hormones are very poor. However, it was affirmed that they are released into the water bodies by fish. This phenomenon was observed mainly before and during reproduction periods. In raw sewage, the level of hormones varies due to the source and amount of rainfall (5).

According to water circulation systems, estrogens may be present in all water bodies. Even after special cleaning treatment in municipal wastewater plants, trace amounts may penetrate into drinking water. During recent years, the trace-level presence of these compounds, especially with estrogenic properties, in the water of the environment has become a worldwide concern. Society has become agitated because of the potential risk to human life and

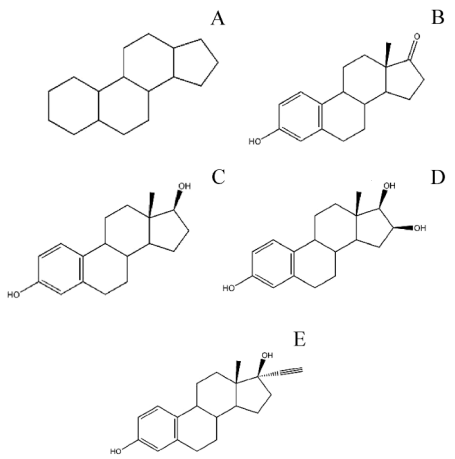

Figure 1. Estrogenic hormone structures: steroid structure (A); estrone (B); estradiol (C); estriol (D); ethinylestradiol (E).

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wildlife due to exposure to both natural and synthetic chemicals, which may interfere with reproduction and development.

The endocrine disruptors may be released directly or indirectly into the aquatic environment. The estrogenic chemicals end up in the aquatic environment through excretions of humans, farm animals, and wildlife (6,7). In urinary excretion there is a presence of natural estrogens such as estrone, estradiol, and estriol, which have medicine or veterinary applications. One of the major sources of contamination of the aquatic environment are wastewater treatment plants (WWTP) because several estrogens and related compounds are not totally removed or degraded by biological treatments. According to this, steroid hormones are found in rivers with a high charge of domestic and industrial wastewaters (8,9). In waterways, they may adsorb to solid particles such as bed sediment or soil, where estrogens may persist for quite a long period (10,11).

Endocrine-disrupting compounds (EDCs), especially estrogens, are chemicals of an increasing public concern, because the exposure of EDCs has caused adverse health impacts on wildlife (12,13). Many studies have suggested that estrogens from treated wastewater are responsible for male fish feminization and sexual disruption in some aquatic organisms (14–16). They have been detected in surface waters and wastewaters at concentration levels of ng/L. Most notable, estrogens such as 17β-estradiol, estrone, and ethinylestradiol have been implicated in feminization of male fish at concentrations as low as ng/L.

The entire world is paying attention to the research for finding the best method of changing the impact of EDCs on human and animal health. It is essential to establish some new controllable methods that allow complete elimination of estrogens from water. Due to an incomplete elimination from WWTPs, both estrogens and residues of pharmaceutical products are found in ground and surface waters (17–21).

The introduction of sewage treatment plant (STP) effluents, which contain various classes of contaminants, into aquifers and sediments creates the potential for groundwater contamination. The amount of EDCs in water can be estimated by analyzing aquifer materials that are characterized by a great absorbing capacity. Therefore, depending on the environmental conditions, the concentration of the estrogens can be determined by correlating the EDC amount in aquifers with real concentrations in water (22).

Within the last 10–15 years, the increasing use of liquid chromatography–mass spectrometry (LC–MS) has led to a revolution of environmental analysis, providing a new analytical tool that enables the identification of highly polar organic pollutants without derivatization, down to nanogram-per-liter levels in all kinds of water bodies (wastewater, surface water, groundwater, and drinking water). The major innovation that enabled this involved the development of the appropriate ionization interfaces to couple LC with MS. Currently, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are the most commonly used LC–MS interfaces. Further innovations have been made in rapid on-line extraction, microextraction, and on-line derivatization techniques in combination with gas chromatography (GC)–MS or GC–tandem MS detection (23,24).

Many efforts have been undertaken for the accurate analysis of the estrogens in water down to nanograms-per-liter and even subnanogram-per-liter concentrations. The first methods used primarily GC–MS or GC–ion trap-MS–MS detection at the end of the 1990s. Today, an increasing number of methods use LC–MS and LC–MS–MS (11,25,26). The main benefit of LC–MS–MS, in comparison to GC–MS, is that LC–MS–MS shows lower statistical errors and unnecessary derivatization. The lower sensitivity of the first-generation of LC–MS–MS methods is no longer a disadvantage, because the new LC–MS–MS systems are competitive with GC–MS sensitivities. Only when resolution is mandatory to separate isomers or congeners (such as for polychlorinated biphenyls, dioxins, or brominated flame retardants), GC–MS–MS systems are still the method of the choice (11,17–32).

In this paper, a discussion of the main steps, from sampling up to analysis, and the techniques most commonly used in the determination of estrogens is presented.

Sample Preparation

The estrogens have been frequently investigated in environmental water; thus, several analytical approaches have been reported in the literature. They usually comprise sample preparation steps (starting from sampling and sample storage; the most critical step is the extraction followed by the clean-up) and analysis (off-line and on-line solid-phase extraction [SPE] followed by chromatographic separation with selective detection—MS).

Sample collection

The assessment of the estrogenic activity in environmental water samples begins with the sample collection and some sort of storage until analysis. The sample collection must be significant for an entire site. The best sample storage strategy consists in passing the field sample through the SPE cartridge, washing the cartridge with methanol, and storing it at –18°C. Under these conditions, which facilitate the storage of many samples for extensive monitoring, no significant loss of the estrogens was observed after storage for 60 days. An alternative to this procedure is to store the samples at 4ºC in amber bottles, previously preserved with 1% formaldehyde solution. The addition of formaldehyde solution inhibits micro-bacterial growth (33). The storage of the water in bottles led to no significant losses of the estrogens after 24 days when the samples were preserved, but led to severe losses when the sample was not preserved (34).

The volume of the sample can vary from 50 mL to 20 L or even more depending on method sensitivity. A volume higher then 5 L should be avoided, because it leads to an increase of the humic acid concentration in the samples, and it creates high matrix effects (5). A sample volume between 250 mL and 1 L is considered optimum for high-performance liquid chromatography (HPLC)–MS–MS or GC–MS–MS analysis (20,35).

Within recent years, a new approach for sample collection has been presented. It involves the extraction of estrogenic compounds directly on an adsorption medium, which is a permeable membrane. The device used is the passive-sampler. The main passive sampling tool is known as Polar Organic Chemical Integrative Sampler (POCIS). The absorption

Table I. Physicochemical Characterization of Steroidal Hormone

Substance	Molecular weight (g/mol)	Melting point (°C)	H_2O solubility (g/L)	Half-life (h)	Hydrophobicity
Estrone	270.36	254–256	3	19	4.6
Estradiol	272.38	173–179	3.6	36	3.5
Estriol	288.39	282	NA*	NA	2.3
Ethynilestradiol	296.40	183	0.1	36 ± 13	3.7

* NA = Not available.

medium is sandwiched between two disc-shaped semi-permeable plastic membranes held in place by two metal compression rings, which are in turn mounted inside a protective perforated stainless steel cylinder. This device is left in the water for a period of time (1 to 10 weeks), and the estrogenic compounds are extracted directly on the membrane. The analytes were extracted by adding 100 mL of analytical-grade dichloromethane to each 900-mL sample of water, and mixed in a clean glass bottle. After this procedure, the next analytical steps can be performed (36–41).

Concerning the POCIS validation, Zhang and Hibberd (42) compared passive sampling with spot sampling for some EDCs, including the estrogenic hormones. As adsorbtion materials, there were two different membranes: polyethersulfone and polysulfone. The first one presents a greater adsorbtion capacity. The kinetics of compound uptake, under laboratory conditions, were linear for 10 days. The results obtained by POCIS for the real samples were in good agreement with those from the spot sampling.

Filtration

The filtration is usually the first step of the sample preparation. This step is particularly necessary when the subsequent extraction of the sample is based on the SPE, because the suspended solids could easily clog the adsorbent bed; and when the analysis is performed by immunochemical assay, to avoid undesired adsorption on to antibodies.

The filtration step can be performed simultaneously with the sample collection and/or extraction, or as a separate step. This will determine, in part, the type of the filter regarding to the physical form (pad, filter aid powder, glass wool, etc.), diameter, and the filter holder. Most of the studies reviewed employed the glass fiber filters with pore size between 0.22 and 1.20 µm (36–40).

Extraction

Extraction of the steroid sex hormones and the related synthetic compounds from the surface and wastewaters is usually performed by SPE. The liquid–liquid extraction (LLE) has rarely been reported (43–46).

For the extraction of the steroidal hormones, many SPE procedures are reported. Laganá et al. (47) determined female hormones: 17β-estradiol (17βE_2), estrone, estriol, and the synthetic contraceptive additive 17β-ethinylestradiol (17β-EE_2) that have a potency similar to natural hormones; some alkylphenols, such as 4-nonylphenol and bisphenol A, the isoflavonoids daidzein, genistein and biochanin A, and mycoestrogens, zearalenone, whose estrogenic properties were well-recognized. The water samples were extracted (1 L) using HLB and Carbograph-4 cartridge. They were conditioned before processing the sample. The cartridges were sequentially conditioned with 10 mL of dichloromethane–methanol (50:50, v/v), 5 mL of methanol, and 10 mL of water. After that, the sample was passed through the cartridge, followed by washing with 10 mL of water and 0.4 µL of methanol. The retained compounds were eluted with 7 mL of a dichloromethane–methanol (50:50, v/v) solution. Chromatographic analyses were done using LC–MS–MS, which reduces the sample preparation to a minimum. Therefore, the sample preparation step is a simple and inexpensive extraction system based on SPE. Two of the often-used SPE sorbents are: Oasis HLB (polystyrene-divinylbenzene-*N*-vinylpyrolidone terpolymer) and Carbograph-4 (graphitized carbon black). The Carbograph-4 cartridges were chosen because of their well-known adsorption power, based on anion-exchange and hydrophobic properties, especially toward polar compounds (48), while Oasis HLB were chosen for their ability to retain a large number of compounds; basic, neutral, and acidic (49). It is designed, in fact, for retaining both hydrophilic and hydrophobic compounds with high capacity by means of both van der Waals and H-donor-H acceptor interactions. Contrary to other cartridges, the hydrophilic-lipophilic balance of the Oasis HLB cartridges allows them to become dry during the sample manipulation, the sorbent's wet-ability being constant (50).

The Oasis HLB is one of the most important types of cartridge for the extraction of estrogenic compounds (51–53). Other types, such as Envi+ or C18, gave adequate results for EDCs analysis (54–57), but are less sensitive in comparison with the Oasis HLB. By combining the two cartridges, the selectivity can be increased (58). An excellent comparison of the various types of SPE sorbents, protocols, and devices has been made by Lopez de Alda and Barcelo (59). In this paper various procedures for the determination of several estrogens (estriol, estradiol, ethynyl estradiol, estrone, and diethylstilbestrol) and progestogens (progesterone, norethindrone, and levonorgestrel) in the environmental matrices, including water and river sediment, are described. In all procedures, final analysis of the target compounds is performed by the reversed-phase LC–diode array detection-MS, whereas the sample preparation always includes an SPE step (Table II).

A proper extraction method was reported by Filali-Meknassi et al. (60). The estrogens: E_1, βE_2, αE_2, EE_2, and E_3 were concentrated using 200–1000 mL aqueous matrix of interest. To extract the studied compounds, two types of cartridges (C18 and NH2) were used, connected in series. The C18 cartridge was applied as the first one and was rinsed with deionized water (2 mL), methanol (2 mL), and dichloromethane (2 mL) prior to the extraction. The samples were passed through C18 cartridge followed by washing with 5 mL deionized water, drying, and finally washing with 5-mL hexane. The elution of compounds was done with 5 mL of dichloromethane. The solvent must be evaporated under nitrogen steam. Finally, the dry sample must be reconstituted with 3 mL of methanol and passed through NH_2 cartridge, which was first rinsed with 5 mL of methanol. The estrogens were eluted from the NH_2 cartridges with 5 mL methanol and evaporated under nitrogen steam to approximately 100 µL and reconstituted with 900 µL methanol. The sample must be kept at –25°C until the analysis. The recoveries of the analytes by this method were greater then 90% in all cases. The method has been shown to be accurate and precise.

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Table II. Summarization of SPE Possibilities for Estrogenic Compounds from Water

SPE sorbent	Matrix	Final determination	Recovery (%)	Refs.
Oasis HLB	Natural waters; sewage treatment plant	LC–MS–MS	> 91	47
C18; NH_2 silica bonded	Surface water; wastewater treatment plant; influent & effluent	LC–MS	> 81	60
C18; NH_2 silica bonded	Wastewater and purified water	LC–MS	> 91	61
C18	Natural waters	LC–UV	> 85	62
EN; RP-18e	STP; influent and effluent water; river water; drinking water	LC–MS	> 83	63
RP-18e	River water	LC–MS	> 90	64

dodecyl suplfate on alumina and cetyltrimethyl ammonium bromide on silica can be compared with Oasis HLB for concentration and purification of estrogens from natural water samples. The interactions between absorbents and estrogens are hydrophobic combined with cation-π. The recovery rate of the estrogens, investigated by HPLC–DAD, ranged between 85% and 105%, with a standard deviation ranging from 3% to 8% (65).

Another new technique uses SPE discs. It appears to be a convenient means of extraction of the estrogens from large volumes of water. Kelly reported good results for the extraction of estrone, estradiol, and ethinyl estradiol applying polytetrafluoroethylene SPE discs impregnated with C particles (66). A glass fiber filter paper and glass beads were placed on the top of the extraction discs to prevent clogging with the particulate matter. The SPE discs impregnated with polymeric particles seemed to provide a convenient procedure for the extraction of large volumes of river water samples, giving good recoveries of the major estrogens: estrone, estradiol, estriol, and ethinylestradiol, on the ng/L level. However, recoveries adapting discs were low for the sewage effluents, possibly due to overloading by the large amount of the matrix with the presence of the organic material. For these samples, the extraction applies large volume C cartridges and provides an alternative procedure (56). The river water and the WWTP effluents were tested for the presence of the pollutants E_1, E_3, $17\beta E_2$, and $17\beta EE_2$ using a new methodology that involves high-flow SPE and LC–MS–MS (67). Without adjusting the pH, they extracted it from an 1-L sample with PolarPlus C18 Speedisks under a flow rate exceeding 100 mL/min, in which six samples could be done simultaneously using an extraction station. The method was validated on spiked upstream river water; precisions were mostly within 10% of the tested concentrations (10–100 ng/L), with a relative standard deviation (RSD) lower than 10%. The limit of detection (LOD) of the environmental matrixes was 0.78–7.65 ng/L. A pre-filtration step before SPE may significantly influence the measurement of E_1 and EE_2 concentrations; disk overloading by water matrix may also impact the analyte recoveries, along with ion suppression.

A simple, rapid, and sensitive method for the determination of five estrogens: estrone, 17β-estradiol, estriol, ethinyl estradiol, and diethylstilbestrol, was developed by using a fully automated method consisting of in-tube solid-phase microextraction (SPME) coupled with LC–MS–MS (68). The optimum in-tube SPME conditions were 20 draw/eject cycles of 40 µL of sample using a supel-q plot capillary column as an extraction device. The extracted compounds were easily desorbed from the capillary by passage of the mobile phase, and no carryover was observed. Applying the in-tube SPME-LC–MS–MS method, the limits of detection of the five estrogens ranged from 2.7 to 11.7 pg/mL. The in-tube SPME method showed 34–90-fold higher sensitivity than the direct injection method. This method was applied successfully to the analysis of environmental water samples without any other pretreatment interference peaks. The details of the in-tube SPME technique and its applications have also been summarized in a number of reviews (69–74).

Off-line SPE is the most frequently used method of extraction. However, during recent years, a new on-line extraction method was developed. Rodriguez-Mozaz and co. (75) presented the advantages and disadvantages of this method. A drawback of the off-line SPE procedures is that they can be time consuming and cumbersome to perform, often requiring many steps before reaching an extract concentration suitable for instrumental analysis, of which only a small portion is actually injected onto the chromatographic column. The on-line SPE techniques have made it possible to develop a faster method by reducing the sample preparation time and thus increasing the sample throughput. The conditioning, washing, and elution steps can be performed automatically, and some systems also permit one sample to be extracted, while another is being analyzed by LC (76). The on-line procedures are particularly attractive in situations where, for example, large number of samples and/or sample series have to be analyzed routinely with high sensitivity, or when hazardous or highly infectious materials have to be processed; while the off-line procedures are favorable for their applicability to on-site sampling and for the opportunity to inject the same extract several times. In the following sections, an insight on the on-line configurations and sorbents applied for the environmental analysis is provided, and the multi-residue methods using on-line SPE to extract selected emerging contaminants from water are reviewed (77,78).

So far, the on-line configuration most widely used for the analytical determination of the emerging contaminants in environmental water samples has been SPE coupled with LC–MS and LC–MS–MS using quadrupole instruments. In such a configuration, the inherent advantages of on-line SPE and LC–MS are put together in a single methodology with the following main features: automation, sensitivity, selectivity, accuracy, reliability, high throughput, and minimal sample manipulation. However, the most monitored methods, including those which rely on the use of the on-line methodologies, are usually restricted to a few preselected compounds and very often are not sufficient to assess the quality of the water, and there are still many unknown micro-contaminants present that might be a threat to the environment and human health (79,80).

A novel, fully automated method is based on on-line SPE-LC–ESI-MS–MS, which allows the unequivocal identification and quantification of the most environmentally relevant estrogens in natural and treated water at the level well below those of concern has been presented recently by Rodriguez-Mozaz and co. (81). The RSD are between 1.43% and 3.89%, while recovery percentage is higher than 74%, which suggests that the method is highly precise and accurate. This method was used to investigate the presence of estrogenic compounds in waterworks before and after treatment process.

Another attempt was proposed by Brossa et al. (82). Their method is based on on-line SPE coupled with GC–MS through an on-column interface. The LOD for the method was at a level under µg/L.

The most recent review concerning on-line SPE coupled with LC–MS has been published by Rodriguez-Mozaz and Lopez de Alda (75). They have described advanced technologies that have found increasing application in the analysis of environmental contaminants, although their application for the determination of emerging contaminants such as steroidal hormones (previously unknown or unrecognized pollutants) has still been limited. So far, they indicate that when using the on-line configuration of SPE coupled with LC–MS–MS, the SPE sorbents can be used in both traditional (alkyl-bonded silicas and polymers) and novel restricted access materials, molecularly imprinted synthetic polymers (MIPs), and immobilized receptors or antibodies (immunosorbents) as materials. This technique can be used to investigate other contaminants such as: alkylphenols, pesticides, and their derivatives, dezinfection by-products, mycotoxins, and so on. The on-line SPE-LC–MS procedures in terms of accuracy, reproductibility, reliability (confirmation) of results, and capacity for the multi-analyte determination are highly superior of those off-line and even compared with the other techniques, such as biosensors.

Derivatization

To improve the stability of the compound and also the precision and the sensitivity of the GC analysis, the sample extract is usually derivatized (83–87). The derivatization is carried out in the case of thermolabile, polar, and low volatile compounds, such as estro-

gens, to avoid thermal decomposition and to improve chromatographic separation and the sensitivity of the analysis. Unfortunately, there is sometimes a loss of the sample during the additional manipulation. LC–MS and LC–MS–MS have some benefits over GC–MS analysis of the estrogens in the environmental water. These methods may be coupled with on-line devices for sample preparation and pre-concentration techniques, such as SPE, and estrogens can be analyzed without derivatization (88). In Table III, common derivatization agents used for the analyses of estrogen has been summarized.

Sometimes derivatization can be processed during SPE by modifying sorbent with a derivatization agent. Such a method was developed by Salvador et al. (97), which used dansyl chloride for derivatization. An Oasis HLB column was used for the sample clean-up and derivatization support. The reaction between hydroxyl groups and reagent takes place in the on-line SPE column. Dansylated estrogens are eluted further back and analyzed with HPLC–MS–MS. The method allows determinations at 1 ng/L level in savage influent and effluent samples. Similar, Okeyo et al. (98) indicate that SPME can be used for both derivatization and extraction. In this case, the extraction fiber has a derivatization bis(trimethylsilyl) trifluoroacetamide headspace. The detection limit can be decreased in this way to low ng/L levels.

For the determination of estrogens, it was shown that derivatization with pentafluorobenzyl (PFB) bromide is useful for more sensitive determinations with GC–negative ion chemical ionization (NI-CI)-MS (99) or LC–NI-APCI-MS (100). PFB derivatives were used as electron-capturing derivatives to produce an intense (M–PFB) ion in both ionization systems (101). A highly sensitive and accurate method can be obtained based on the derivatization of EE with different agents.

The sensitivity may be improved by using appropriate derivatization reagents [dansyl chloride, 2-fluoro-1-methylpyridinium *p*-toluene sulfonate (FMPTS), pentafluorobenzoyl bromide (PFBBr)] to modify the structure of estrogens so that their ionization efficiency is increased, making them more detectable by the LC–MS. Lin and co. (102) tested how environmental matrices influence the detectability of the estrogens. Both qualitative and semi-quantitative comparisons of the derivatization method were made. It was found that dansyl chloride derivatives created signal intensities one or two orders of magnitude greater than those normally found in the underived estrogen standards. The signal derived by FMPTS were analyte-dependent. The PFBBr derivatives produced signals that were as much as 5.8 times those found in the underivatized estrogens. The results indicate that PFBBr is better to be used for estrogen analysis in complex samples such WWTP or STP influent or effluent, while dansyl chloride should be used mainly for clean water analysis.

Table III. Derivatization Agents and Procedures Used for Estrogen Analyses

Derivatization agent	Detection limit (ng/L)	Final determination	Refs.
Pentafluorobenzoyl	0.2–17	GC–NCI-MS*	89
bromide	2–70	GC–NCI-MS	90
Pentafluorobenzoil trimetylsilyl	0.2–0.6	GC–NI-CI-MS	91
p-Nitrobenzoyl chloride	2.7–8.3†	HPLC–FD‡	92
MSTFA§	1–100	GC–MS	93
Dansyl chloride	0.0025	LC–MS–MS	94
	1	HPLC–ESI-MS–MS	95
	0.005–20	HPLC–ESI-MS–MS	96

* NCI = negative chemical ionization.
† µg/L range.
‡ HPLC–FD = high-performance liquid chromatography with fluorescence detection.
§ MSTFA = *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide.

Chromatographic Analysis

Up to now, the sensitive and uncomplicated methods for steroidal hormone analysis in the water samples were developed using GC–MS, LC–MS, and MS–MS. These techniques are recognized as the most sensitive and reliable instruments in environmental science. Biological methods like immunoassay are among the most sensitive analytical methods, but they are very limited, on the one hand. On the other hand, chromatographic techniques, which are not as sensitive as biological ones, enable simultaneous screening of both steroids and conjugates, and the other compounds. Finally, LC is not limited by the factors like non-volatility and high molecular weight, and enables the determination of both conjugated and unconjugated estrogens without the need of the derivatization (33,103).

GC and HPLC are commonly used for separation and determination of procedures, but the former is applicable only to volatile estrogens and not to non-volatile ones, such as conjugates. GC–MS is also widely used for the determination of the estrogen hormone, but LC–MS is recently considered to be the most promising analytical method for the determination of steroids, including conjugated steroids due to its sensitivity, specificity, and versatility. Chromatographic analyses of steroidal hormone have been reviewed by Wolthers and Kraan (104).

The ability to provide timely, accurate, and reliable data is very important for any method to perform an analysis of the chemicals. Therefore the method validation should be an integrated part of the process of developing analytical methods. It is important to define the intended purpose of an analytical method, and the method validation only needs to prove that the method is acceptable for this purpose. In environmental analysis, this is particularly important, as the need for high precision may be limited. For steroid estrogens specifically, the purpose of analytical methods can often be limited to reliable detection of whether the substances are present above certain levels or not. Thus, in this particular case, only limited method validation is needed. However, false positive or negative results should be avoided because of the impact that can be created. Some researchers consider that using an MS system in selected ion monitoring (SIM) mode, or MS–MS, is enough for confirmation, but if there are interfering ions or only one transition is monitored, the results cannot be considered valid. Moreover, there are great differences between MS interfaces [e.g. atmospheric pressure ionization (API) spectra are more limited than electron ionization (EI) spectra]. Nowadays, the use of high accuracy mass spectrometers, such as time of flight (TOF) and hybrid quadrupole (Q-TOF) allows result confirmation (105–107).

Although sensitive LC–MS–MS methods using electrospray ionization (108) and APCI (53) are available, both of these methods can be susceptible to response of a loss due to ion suppression caused by matrix effects present in the complex samples (109–111). When ion suppression is present, an additional preparative step including clean up or utilization of LC–MS methods may be required. The use of MS–MS provides added specificity, which is necessary when analyzing the samples of an increased matrix complexity. To aid in determining whether GC–MS–MS or LC–MS–MS analysis should be done on a sample, preliminary screening of the groundwater samples for the presence of estrogens can be done using enzyme-linked immunosorbent assay (ELISA) or other assays.

Many other researchers investigated steroidal hormone in different matrices by HPLC–MS–MS (112–120) and by GC–MS–MS

(114–119,121,122). A good possibility to determine EDCs in water is by determining the contaminants indirectly from fauna existing in the site of interest (120,123–127).

GC methods

GC–MS is the most widely applied technique for the determination of estrogens and progestogens in water. Generally, the detection of steroids by GC techniques including MS was reviewed in 1999 (104). GC–MS is the most popular of all complex techniques for GC. GC–MS–MS is the hyphenated technique combining GC with tandem MS. MS–MS is any general method involving at least two stages of the mass analysis either in conjugation with a dissociation process or a chemical reaction that causes a change in mass or an ion. Although various ionization methods are available, EI, and chemical ionization (CI) are the most common for GC–MS analysis.

In the most common MS–MS, the first analyzer is used to isolate a precursor ion, which then undergoes of a fragmentation, either spontaneously or by some activation, to yield product ions and neutral fragments. A second spectrometer analyzes the product ions. By use of MS–MS instruments, the selectivity of the analysis is increased not only by a specific mass quantitation, but this specific mass can be related to a specific fragmentation of the product ions. The detection limits achieved by the different methods employing GC–MS or GC–MS–MS as final analytical techniques were in the range 0.5–74 ng/L and 0.1–2.4 ng/L, respectively. An overview of GC–MS and GC–MS–MS determination of estrogens is presented in Table IV.

GC was the first chromatographic method used for the determination of steroidal hormone. Kuch et al. (135) analyzed a series of compounds with estrogenic activity (E_1, 17α-E_2, 17β-E_2, 17β-EE_2, and other EDCs) in surface and drinking water from southern Germany by GC–CI-MS. During this analysis, the sample were extracted by SPE followed by derivatization of the phenolic compounds. The recoveries of the steroids lay in the range between 71% and 79%, with the exception of the estradiols (between 56% and 67%). The RSD varied from 9% to 15% and indicated a satisfactory reproducibility and precision of the whole analytical protocol. Endogenous steroids such as E_1, αE_2, and βE_2, and the exogenous estrogen EE_2 were determined almost unexceptionally in the lowest ng/L range.

In the drinking water, E_1 and EE_2 were found at an average of 400 and 350 pg/L, respectively; αE_2 could only be determined in one sample at 300 pg/L, while βE_2 was found at an average of 700 pg/L. The investigated steroids were found in the lower ng/L range with mean concentrations of 2.5 ng/L E_1, 1 ng/L E_2 and E_2, and 1.5 ng/L EE_2. This correlates widely with the results of previous investigations (136,137). The differences in the concentrations up to a factor of 10–20 for E_1, βE_2, or EE_2 may be caused by different weather conditions (sunshine and dryness, dilution by rain, temperature) in the sampling period, differences in the length of time through the treatment process, state of the art sewage treatment plants, and composition of the influent water.

Soliman and co. (138) presented a rapid GC–MS method for routine measurement of steroidal hormone and some other human pharmaceuticals in water. A short (12 m) column and steep temperature programming ramp (18°C/min) allowed a rapid GC separation followed by sensitive detection by MS in the SIM mode. Method detection limits between 8 and 85 ng/L were achieved without sample clean-up or derivatization. A 40,000-fold concentration factor was achieved by on-line continuous (O-C) LLE of the water samples with dichloromethane. Obviously, the GC–MS method could be applied to the SPE and the SPME isolation procedures.

Chlorinated derivatives of E_2 were reported to be produced by aqueous chlorination (139,140). Nakamura and co. (141) determined estrone and its chlorinated estrones in drinking water. After the water disinfection process with chloride, it is possible to change nontoxic compounds to toxic ones, like these chlorinated estrones: 2-chloroestrone, 4-chloroestrone, and 2,4-dichloroestrone. These estrones were determined by GC–EI-MS in SIM mode. They estimated the risk of these chlorinated E1 and related derivatives for human health and for aquatic wildlife; we need more detailed quantification in the various water samples, including drinking water and studies of biological activities, including estrogenicity of these compounds.

Like the rest of the world, New Zealand is affected by the presence of steroidal hormone as contaminants in water (142). GC–MS analysis had

Table IV. Survey of GC–MS and GC–MS–MS Methods for Quantitative Determination of Estrogens and Related Synthetic Compounds in Aquatic Environmental Samples

Compound	Matrix	Sample preparation	Detection method	LOD (ng/L)	Refs.
E_1, E_2 ,E_3, EE_2 + other steroidal hormone	River water STP	Filtration/SPE (Oasis HLB + NH_2)/ Derivatization (MSTFA)	ESI-MS	0.3–9.0	128
E_1, 17β-E_2 ,E_3 17αEE_2 and related compounds	STP effluents WWTP	Filtration/SPE (C18; Oasis HLB) /Derivatization (BSTFA)	ESI-MS ESI-MS–MS	< 100	129
E_1, 17βE_2 ,E_3 17αEE_2 and related compounds	River water STP effluents	Filtration/SDB-XC extraction disc; C18/Derivatization (PFBO)	Quadrupole-MS	0.03–0.50	89
E_1, 17αE_2, 17βE_2, E_3	Wastewater	SPE (Carbograph)/ N2 Evaporation (70°C)/ SPE (C18)/Derivatization (BSTFA)	ESI-MS	75	130
Steroidal estrogens	Groundwater	Filtration/SPE (StrataX)/Derivatization (BSTFA)	ESI-MS-MS	2–4	131
E_1, 17αE_2 17βE_2, E_3 EE_2	Pearl River Delta	Filtration/SPE (ENVI 18) Derivatization (BSTFA; MSTFA)	ESI-MS	0.1–5	132
17β-Oestradiol Oestrone, 17α-Ethinyloestradiol	River water	Extraction (C18-discs)/ N_2 evaporation/ Derivatization (MTBSTFA*)	ESI-MS MS–MS	1	133
E_1, 17αE_2 17βE_2, E_3	Tama River Japan	Filtration/SPE (ENV/124) /Derivatization (PFBBr)	APCI-MS	0.1–0.28	134
E_1, 17βE_2 17βE_2, E_3	Groundwater	Filtration/SPE (Oasis HLB) Derivatization (PFBBr, TMSI†)	APCI-MS	0.2–0.6	91
E_1, 17βE_2	Elbe River Germany	SPE (SDB-1, Chromabond HR-P and EASY, Nexus Isolute ENV+, LiChrolute EN, Oasis HLB)	ESI-MS	1	92

* MTBSTFA = *N*-methyl-*N*-tert.-butyldimethylsilyltrifluoroacetamide.
† TMSI = *N*-trimethylsilylimidasole.

shown that farm effluent samples contain high level of estradiol (19–1360 ng/L) and its breakdown product estrone (41–3123 ng/L) compared with piggery or goat farm effluents. The combined load for these estrogens (excluding h epimer) varied from 60 to > 4000 ng/L. The piggery effluent provided the lowest total estrogen load (46 ng/L), with estrone accounting for nearly 60% of the measured estrogens in this sample, while the synthetic analogue, 17β-ethynylestradiol was detected only in one WWTP sample at trace level. An estrogen receptor competitive binding assay was used to test the biological activity of the samples and confirmed that most agricultural waste samples contain high levels of estrogenic compounds. The potential of these wastes to cause endocrine disruption in the receiving ecosystem is unknown at the present. This study showed that animal wastewaters are an emerging source of estrogenic contaminants for natural waters.

Moreover, the distribution of female hormones, 17β-estradiol and estrone, was determined in effluents of 18 selected municipal treatment plants across Canada by Servos and co. (143). Estrogens (E_1, E_2, E_3, and EE_2) have been often detected at a level of ng/L in STP influent and effluent water (144–146). Those estrogens were also reported to be found at a similar level (ng/L) in coastal surface water (147). Zuo et al. (148) reported that high concentrations, up to 4.7 ng/L, of EE_2 were detected in estuary seawater, where EE_2 may affect lobster and fish abundance in the coastal seawater. Furthermore, E_1, E_2, and EE_2 were detected at a level of pg/L, even in drinking water (135). Therefore, considering the growing population and the concentrations of such estrogens in the effluent from STPs, they could play significant roles as endocrine disruptors in aquatic wildlife.

In order to address the Austrian situation concerning endocrine disrupting compounds, a consortium called Austrian Research Cooperation on Endocrine Modulators (ARCEM) was established in 1999 (149). Among several other issues that were investigated, ARCEM monitored more than 400 ground- and surface-water samples for selected estrogenic hormones and industrial chemicals. Appropriate analytical methods were established using GC–MS for the detection of hormones. Since analytical results were forwarded for toxicological assessments within the program, quantification limits below 0.1 ng/L (ethinyl estradiol) and 10 ng/L (industrial chemicals) were required depending on the individual compound. In this program, Hohenblum and co. (150) determined steroidal hormones by means of an isotope dilution technique and GC with high-resolution MS.

All data gathered in the ARCEM program demonstrate that the concentrations are comparable to the concentrations that were measured in Germany, Switzerland, and other countries (151–153). The results indicate that both hormones occur in the selected ground- and surface-water sites with detectable concentrations.

LC methods

Due to limited sensitivity, it is not surprising that only a few reports exist on methods for environmental analysis of estrogens by LC using detectors other than MSs. The use of spectrophotometric techniques, including diode array detectors (DAD), is common in HPLC systems, but a high sensitivity determination in a very low concentration range (ng/L) such as in the environmental samples has not emerged. The adequate techniques of choice for analysis of the described groups of emerging pollutants are LC–MS and LC–MS–MS. Before the advent of LC–MS, many of these polar compounds were difficult and sometimes impossible to measure (154).

In the last decades, LC–MS has experienced impressive progress, both in terms of technology development and application. The interface designs have changed considerably and have become much more sophisticated and efficient. Various forms of MS need to be considered for estrogen analysis, because MS is a family of techniques. High accuracy is a well-known attribute of MS because it is a very specific technique, and because it uses stable isotope internal standards. Nevertheless, MS is not always accurate even when such standards are employed (155). The high specificity overall in an MS-based method is achieved in one of the three ways: including a chromatographic, electrophoretic, immunoextraction, or another resolving technique prior to MS detection; using a high resolution form of MS such as a dual-sector, TOF, or ion cyclotron resonance instrument; relying on one of several forms of tandem MS. These high-specificity options can be combined for ultrasensitive estrogen detection.

Recent advances in MS for the measurement of estrogens and other EDCs in aquatic environmental samples have been reviewed (156).

Today, the interfaces most widely used for the LC–MS analysis of steroids in the aquatic environment are ESI and APCI. LC–MS and LC–MS–MS have been mostly applied in the SIM mode. LC–MS–MS offers very good sensitivity and selectivity of the trace analysis of environmental pollutants (109,157). LC–MS–MS is used widely to determine sexual hormones not only from environmental samples, but also from biological (158–161). Therefore, in agricultural soil, a high concentration of estrogenic compounds, which due to rainfall are going to contaminate groundwater, have been detected (162–167). The presence of a steroidal hormone was detected in the plant leaves as well. The steroidal hormones were filling the plants due to irrigation water (166).

The estrogen analysis by ESI-MS encounters three general problems (168). The first one, which is a major shortcoming for trace ESI-MS, is that its response is often analyte-dependent. The second problem is related to the first: the condi-

Table V. Survey of LC–MS and LC–MS–MS Methods for Quantitative Determination of Steroid Sex Hormones and Related Synthetic Compounds in Aquatic Environmental Samples

Compound	Matrix	Sample preparation	LOD (ng/L)	Refs.
E_1, E_2, E_3, 17α-EE_2 and related compounds	Baltic Sea	Filtration/SPE (Oasis HLB)	0.1–3	170
E_1, E_2, E_3, 17α-EE_2	STP	Filtration/SPE	0.07–0.18	171
E_1, E_2, E_3, EE_2, and sulfonated derivatives	Tamagawa River, Japan	Filtration/ SPE (Shodex EDS-I)/Fluorisil pufication	0.2–34	172
E_1, E_2, E_3, EE_2, and sulfonated/ glucuronated derivatives	STP and river waters	SPE (Carbograph4)	0.003–15	173
E_1, E_2, E_3, and EE_2	STP	SPE (Oasis HLB)	0.04–0.24	174
E_1, E_2, E_3, and EE_2	River water	SPE (Superclean Envi+)	5.1–6.4	175
E_1, E_2, E_3, and EE_2	River water	Filtration/SPE	2.3–10.6	176
E_1, E_2, E_3, and EE_2	STP	Filtration/SPE	2–200	177
E_1, E_2, E_3, EE_2, E_1, and 3S	WWTP	SPE (C18)/evaporation	0.1–0.2	178
E_1, E_2, and EE_2	New York waters	Extraction (SDB-XC discs)	4	179

tions in the overall system for highest sensitivity of each analyte may be different. However, this problem can be minimized by instantly changing some of the conditions during the analysis. Third, response can be very dependent on analyte purity (e.g., as an HPLC peak), which gets worse with high throughput (fast HPLC). This is due to increased matrix effects that suppress or enhance analyte signals. This general problem for ESI-MS has been recently studied in more detail for drug analysis, and it was found that APCI can also have this problem (169). Zhang and Henion (88) reported the analysis of three endogenous estrogen sulfates, E_1-3S, E_3-3S, and E_2-3S, along with two synthetic estrogens and a stable isotope internal standard, by LC–ESI-MS–MS.

During the last 10 years, many methods for the determination of estrogenic compounds in water have been developed. Various LC–ESI-MS methods used for the determination of estrogenic compounds are presented in Table V.

MS detection shows significant selectivity when the target compound exists in a complex matrix. In other words, MS detection is a reliable way for identifying the target compound, and HPLC is the adequate tool for optimizing resolution before the MS detection. In order to overcome the potential contamination problems associated with E_2 determination, manual procedures should be excluded as much as possible; therefore, an on-line autopretreatment system would be an effective alternative.

Respecting these considerations, Watabe and co. (180) determined 17β-estradiol in the river water using a fully automated LC–MS method. They prepared a column of surface modified (SM) MIPs for 17β-estradiol, utilizing 6-ketoecradiol as a pseudo template. Target compound (E_2) is retained while the interfering matrix constituents, with no affinity for MIPs, pass through the void volume into waste.

MIPs for E_2 were synthesized from 4-vinyl pyridine and ethylene dimethacrylate as a functional monomer and cross-linking agent, respectively. MIPs selectively retain E_2 and provide excellent chromatographic resolution from interfering of inherent compounds in the river water sample matrices. Therefore, freshly prepared MIPs were applied to quantitative MS (ESI^-) detection of low levels of E_2 in the river water sample. In order to pre-concentrate the target compound for HPLC analysis, column switching was coupled with a pretreatment column packed with the MIPs. The repeatability of the actual determinations of the river water sample, in which background E_2 was not detected, spiked with 50 ng/L of E_2, was 2.2% RSD with detection and quantitation limits of 1.8 and 5.4 ng/L, respectively. The surface modification of MIP particles packed in the pretreatment column provided selective affinity and the on-line concentration of low levels of E_2, while simultaneously eliminating the sample matrix interference, resulting in a significant increase of sensitivity and reproducibility for the LC–MS analysis of E_2 in the riverwater samples.

However, it is quite difficult to make a direct determination. To avoid a derivatization step, which can be annoying and difficult, a new LC–MS–MS method was developed. By using HPLC–ESI-MS–MS, Reddy and co. (174) presented a sensitive method (LOD ranged from 0.04 to 0.28 ng/L) to measure steroid conjugates (glucuronide and sulphate) in the matrix-rich sewage influents and effluents. The analyzed compounds were the glucuronide and sulfate conjugates of estrone (i.e., estrone-3-sulfate and estrone-3-glucuronide) and βestradiol (i.e., β-estradiol-3-glucuronide, β-estradiol-17-glucuronide, β-estradiol-3-sulfate, and βestradiol-17-sulfate). The MS was operated in the negative ESI mode using MRM. The precision of the method was good as determined by the RSD of analyses of the three separate samples. The RSD was < 10% for the most of the detected steroid sulfates; glucuronides had slightly higher variability.

A highly sensitive and uncomplicated method of analyzing steroidal hormones in river and the estuarine water samples was developed by Yamamoto and co. (175) using LC–MS–MS equipped with an ESI source and an atmospheric pressure photoionization (APPI) source. Steroidal hormones included not only estrogen but also androgen and conjugate forms of these two. APPI displayed greater sensitivity than ESI for most of the examined unconjugated steroids, with very high sensitivity for testosterone and 4-androstene-3, 17-dione in particular. Contrarily, ESI was more effective for conjugated hormones. The developed method was applied to the determination of hormones in the rivers of Osaka City and their estuaries, where the detected hormones were affected by the effluent from municipal WWTPs, and the hormone concentration values were comparable to those reported in previous studies of such effluent. Because of the two-way flow and stagnancy of the streams and the watercourses, the continuous input of steroidal hormones from WWTPs seems to bring about local accumulation. The levels of androgen were 1 order of magnitude lower than those of estrogen. Estrone, estrone 3-sulfate, and 4-androstene-3,17-dione were detected in almost all water samples, with maxima of 51, 5.1, and 6.4 ng/L, respectively.

A method, which employs SPE and LC–MS–MS, using ESI in both positive and negative modes, has been developed for the trace analysis of 15 pharmaceuticals, four metabolites of pharmaceuticals, three potential endocrine disruptors, and one personal care product in various waters by Vanderford and co. (181). Unlike many previous LC–MS–MS methods, which suffer from matrix suppression, this method uses isotope dilution for each compound to correct the matrix suppression, as well as SPE losses and instrument variability (182–186). The method was tested on five matrices, and the results indicate that the method is very robust. The matrix spike recoveries for all the compounds were between 88% and 106% in wastewater influent, 85% and 108% in wastewater effluent, 72% and 105% in surface water impacted by wastewater, 96% and 113% in surface-water, and 91% and 116% in drinking water. The method reports limits for all compounds between 0.25 and 1.0 ng/L, based on 500 mL of the extracted samples and a final extract volume of 500 µL.

In terms of accuracy and repeatability, LC–MS, GC–MS–MS, and LC–MS–MS are generally satisfactory, although the derivatization step used prior to GC, in addition to being time-consuming, can constitute a source of inaccuracy. An advantage of GC–MS compared to LC–MS is the availability of the data of mass spectra, which are useful for the identification of unknown peaks in estrogenically active fractions. The recent introduction of MS–MS detection has essentially improved the performance of chromatographic methods by reducing detection limits and supporting analyte identification. In the future, the development of the equipment favors the choice of LC-based methods as a strategy for analyzing estrogens. Also, some new ionization techniques such as APPI and others have been developed for several types of highly sensitive triple quadrupole instruments. These developments may mean that LC–MS–MS will become the first choice of analytical methods due to increased sensitivity and higher selectivity.

Comparison with other determination methods

Different combinations of chemical and biological methods have been used for assessing EDCs in environmental matrices. Generally, aqueous samples are concentrated via LLE or SPE to enhance the detection of the target compounds, prior to bioassays and chemical detection by GC–MS or LC–MS.

Experiments determining sexual hormone can be performed by bioassay techniques, both in vivo and in vitro. In vivo experiments are avoided because they are often time-consuming and expensive, and thus sophisticated analytical techniques for the measurement

and assessment of EDCs are highly valued. The low environmental concentrations, complicated sample matrices, and the diversity of target compounds have generated a need for different robust in vitro bioassays. In vitro yeast estrogen screening assays have successfully been used to assess estrogenic activity in environmental samples (167), as they respond to all substances with receptor-mediated estrogenic activity regardless of the chemical structure. Natural and synthetic estrogens seem to be the most potent inducers of activity in in vitro studies (187). To identify the individual estrogenic compounds in environmental samples, the results from biological assays must be combined with GC or LC coupled with MS analyses. The results are highly sensitive and sometimes enough to avoid bioassay techniques. GC or LC connected with single MS or the use of the immunochemical techniques is the minimum necessity for providing sufficiently high quality results.

A comparison between ELISA, GC, and HPLC methods for the determination of estrogens is presented by Li et al. (103). The results show that the ELISA method gave good correlation with GC and HPLC results, although there was a slight tendency for ELISA values to be a little bit higher than values of the GC and HPLC. It is possible that the clean-up and/or derivatization processes required for GC and HPLC cause some loss of the target compounds, resulting in lower estimated values. However, it seems that compared to LC or GC, ELISA gave better results but had some disadvantages [e.g., it is not 100% specific, vulnerable to cross reactivity, requires independent confirmation (e.g., HPLC–MS–MS or GC–MS–MS), which are not suitable for small sample loads, synthesis of antibody can be difficult and expensive].

Another comparison was made by Sawaya and co. (188). It was shown that for the determination of two xenobiotic estrogenic compounds, namely diethylstilbestrol (DES), and ethinylestradiol, both methods, ELISA and GC–MS, respectively, are proper to be used. The obtained data showed that the level of DES and ethinylestradiol ranged from not-detected to 1.2 and not-detected to 0.90 ppb, respectively. In view of the results obtained by ELISA, the employment of a cut-off value of 0.30 ppb would make it reasonable to obtain low false-positive results, thus indicating that such a technique provides a fast and reliable method for the detection and screening of the anabolic samples. All samples (both negative and positive) were subjected to GC–MS analysis for confirmatory purposes. The results obtained from the GC–MS analysis were found to be negative. These results show that the activity seen and reported above was due to the matrix of the samples, but not due to the active estrogenic compounds. Data on extraction recovery and coefficients of variation are also reported. These results and some other similar indicate that GC–MS is a good alternative to ELISA (189–193).

The risk of increasing the concentration of EDC compounds in the environment, especially an aquatic one, is very high. A screening of estrogenic activity on coastal surface water in the Baltic Sea has been made already (194). As an analytical method for this purpose, LC–MS–MS has been selected. The monitored compounds were E_1, E_2, E_3, and EE_2. The LC system was coupled with a triplestage quadrupole MS (API 4000, Applied Biosystems/MDS Sciex; Foster City, CA) in MRM mode. By this way, it was shown that the response in the yeast estrogen screen (YES) was expressed as measured estradiol equivalents, which was in the range of 0.01 to 0.82 ng/L. Samples from stations located in inner coastal waters showed higher estrogenic activities than those from outer located stations. A comparison of measured estrogenicity (YES) and calculated estrogenicity (chemical analysis) showed significant differences, probably due to the presence of anti-estrogenic compounds and/or the estrogenic activity of unknown, not identified contaminants. The main contributors to the overall estrogenic activity were synthetic and natural hormones.

Nowadays, the problem is to find an adequate method to remove the estrogenic hormones from aqueous environment. The tendency is to use polypore mushrooms. The method efficiency can be verified through both chromatographic and immunoassay methods. Such experiments were realized by Auriol et al. (61), which indicated that both methods of determination are suitable and sensitive to notice the estrogen concentration changes.

Future Trends

The analysis of steroid hormones in the environment constitutes a difficult task, firstly because of the complexity of the environmental matrices, and secondly because of their very low, physiologically active environmental concentrations. Thus, to achieve the sensitivity and selectivity for their analysis at physiologically active concentrations (pg-ng/L in water), quite laborious and time-consuming procedures are required. A typical analytical procedure includes, within the sample preparation, various steps, such as filtration, extraction, purification, hydrolysis, derivatization, and evaporation. For many years, the environmental determination of steroid sex hormones has been dominated by the use of the biological techniques, such as immunoassays, and GC–MS. However, recently the applications of LC–MS have experienced rapid growth, due to instrumental developments. The introduction of the LC–MS–MS has largely improved the performance of the technique by reducing the detection and quantitation limits and enhancing analyte identification.

GC–MS is an appropriate method for the separation of the noncomplex samples of estrogenic compounds. In order to separate and determine a high number of steroidal hormones, with a better resolution, new chromatographic techniques were developed by coupling GC and LC with two mass spectrometers. Sensitivity, selectivity, and variability are only a few parameters which are increased by using GC and LC tandem MS.

The next few years will show the general application of these advanced techniques, integrated into completely automated, on-line systems. This will improve analytical performance, increase sample throughput, and reduce operating costs and also contamination risk. Further advances in the form of new extraction techniques, such as those based on the use, on-line or off-line, of molecular-imprinting materials and immunoaffinity cartridges, which are yet under development, and can be expected in the near future. These advances promise to simplify the detection and measurements of steroidal hormones in environmental matrices.

Conclusions

As we saw previously, triple quadrupole instruments such as GC–MS–MS and LC–MS–MS are superior to all other techniques regarding sensitivity. Moreover, new modes of ionization and sample handling may improve the sensitivity even more. One example is the APPI technique, but other ionization techniques such as ESI and APCI are more widely used. The research in sample preparation has shown promising new tools that may be tried in this field. But under all circumstances, if an LOD of less than 0.1 ng/L is needed, then further research is required. LC–MS–MS seems superior to other methods. Single LC–MS and GC–MS methods are of limited value without extraordinary sample clean-up efforts, as LODs are often below the sensitivity

level recommended for both effluent sewage and surface water analysis.

The advantage of using LC is that the enzymatic hydrolysis step required for the immunoassay analysis of both conjugated and unconjugated estrogens, and derivatization step that normally precedes a subsequent GC–MS analysis, can be obviated. Deconjugation techniques have been developed that make it possible to detect the entire estrogen concentration level followed by GC–MS analysis. Methods based on LC–MS are also available for direct analysis of conjugates. UV, GC–FID, and HPLC are not generally recommended due to low sensitivity and reduced selectivity. GC or LC connected with single MS or the use of immunochemical techniques are the minimum necessary to provide sufficiently high quality results. Both single LC–MS and GC–MS can be used even for very complicated matrices if certain identified quality criteria are fulfilled. The more advanced methods like LC–MS–MS or GC–MS–MS are found most suitable for analysis, because these techniques provide the highest sensitivity (LOD = 0.1 ng/L) and selectivity. Methods with an LOD of less than 0.1 ng/L of estrogen are not available on a commercial level. Such methods need further research for sample preparation combined with the application of highly sensitive triple quadrupole instruments. Immunochemical methods are also very sensitive (LOD = 0.05–850 ng/L), at least for analyzing wastewater and STP effluents, but the selectivity is poor compared with the triple quadrupole instruments. Immunochemical methods have the potential to provide useful data when used in connection with chemical analysis, but today such strategies are not developed and research is needed to develop an appropriate strategy combining immunochemical methods with LC–MS–MS or GC–MS–MS or single MS.

In spite of 100 years of chromatography, during which thousands of articles have been published on steroid analysis including estrogens, further advances are still needed for the analysis of endogenous estrogens in different aqueous samples.

Acknowledgments

Rodica Domnica Briciu acknowledges Assoc. Prof. Costel Sarbu for ERASMUS scholarship and Technical University of Gdansk, Poland. This work has been funded by the Ministry of Science and Higher Education (Project KBN 2475/B/P01/2007/33 and N N404 027535).

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Manuscript received July 16, 2008;
Revision received September 8, 2008.