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Evaluation of Analytical Chemical Methods for Detection of Estrogens in the Environment

Flemming Ingerslev and Bent Halling-Sørensen
Bent Halling-Sørensen APS

Danish Environmental Protection Agency
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Preface

Effluents from municipal and industrial wastewater treatment plants, and agricultural run-off and drainage add numerous exogenous compounds to the aquatic system. Recent studies in a number of countries have shown that the aquatic environment can possess estrogenic activity capable of influencing the fauna. Research has shown that the main substances causing these effects are the natural compounds estrone (E1), 17β-estradiol (E2) and estriol (E3) and the synthetic estrogen, 17α-ethinylestradiol (EE2). A few other steroid estrogens may also be reason for concern. The primary source of these substances in the environment has hitherto been attributed to human release through sewage treatment however, the question of whether waste from farm animals (cattle and pigs) (17 α-estradiol (E2-17α) is a significant source for the observed effects remains unanswered.

Answering this question requires finding simple, sensitive and specific chemical analytical methods for analysis and screening of samples from wastewater and surface waters for their estrogenic activity. This report evaluates the existing chemical methods, i.e., GC-MS, GC-MS-MS, LC-MS, LC-MS-MS, for determination of estrogenic activity in various environmental matrices. Furthermore, the use of immunochemical methods has been assessed and compared to the chemical analytical methods. The existing knowledge concerning the potential and limitations of these methods is described with the aim of detailing the limitations and draw-backs of available methods for monitoring wastewater and surface water. Furthermore, chemical analytical methods for monitoring agricultural drain water and animal manure slurry have been assessed. A parallel report has been produced assessing existing simple, sensitive and specific ***in vitro*** tests for rapid screening of samples from wastewater and surface waters for their estrogenic activity. This report evaluates of the existing ***in vitro*** methods for determination of estrogenic activity in various environmental matrices.

This report was written during July and August 2003 to address a scarcity of available information in the published literature on chemical analysis of steroid estrogens in environmental samples. The work was followed by a steering group with the following members:

- Inge Vibeke Hansen, Miljøstyrelsen (chairman)
- Jørgen Larsen, Miljøstyrelsen
- Line Hollesen, Miljøstyrelsen
- Nis Hansen, Eurofins
- Flemming Ingerslev, Bent Halling-Sørensen Aps
- Bent Halling-Sørensen, Bent Halling-Sørensen Aps

The report was written by Flemming Ingerslev and Bent Halling-Sørensen, The Danish University of Pharmaceutical Sciences. Henrik Rasmus Andersen and Kristine Andersen Krogh, The Danish University of Pharmaceutical Sciences has commented on draft versions of the report. This document has been reviewed for English grammar by James Carter of Arroyo Engineering Consultants, Inc., Las Vegas, USA.

Summary

This study presents some suggestions and recommendations related to the analysis of steroid estrogens (primarily E1, E2, EE2) in environmental matrices such as influent and effluent waste water, surface water, sludge, manure, and soil. An analytical chemical method consists of a number of individual steps; sampling, storage, sample preparation and analysis/detection. The storage of samples is important for the final result as the estrogen stability is limited in most environmental matrices, e.g., effluent waste water. Sample preparation is often very cumbersome and the quality of sample clean-up is important in the selection of analytical detection methods. Steroid estrogens are to some extent excreted as conjugates by both humans and animals. Deconjugation techniques have been developed that make it possible to detect the entire estrogen concentration level followed by GC-MS analysis. LC-MS based methods are also available for direct analysis of conjugates. Several analytical detection methods have been suggested in the literature for analysing estrogens in the environment. UV, GC-FID and HPLC are not generally recommended due to low sensitivity and reduced selectivity. GC or LC hyphenated with single MS (GC-MS or LC-MS) or the use of immunochemical techniques are the minimum necessary to provide sufficiently high quality results. But both single GC-MS and LC-MS can be used even for very complicated matrices if certain identified quality criteria are fulfilled (LOD = 0.3 -5 ng/L). The effective use of single MS therefore requires verification of the selectivity of the method following selected criteria. If single MS methods are applied without such additional criteria LODs will be at the level of 20 ng/L or more.

The more advanced methods; LC-MS-MS or GC-MS-MS are found most suitable because these techniques provide the highest sensitivity (lowest LOD or LOQ) (LOD = 0.1 ng/L) and selectivity. Methods with an LOD of less 0.1 ng/L estrogen are not available on a commercial level. Such methods need further research in 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 analysing waste water and STP effluents, but the selectivity is poor compared with the triple quadrupole instruments. Highly polluted samples should generally be avoided when using immunochemical methods due to adsorption to binding sites. Immunochemical methods are subject to problems with low selectivity and false positive samples. Variability is also often a severe problem with this technique. Because of these limitations, immunochemical methods are not recommended as a stand-alone “analytical tool”. 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.

The next few years will no doubt see the general application of these advanced techniques, integrated into completely automated, on-line systems. These integrated systems will improve analytical performance (analyse traceability, reliability, and repeatability), increase sample throughput, and reduce operating costs and contamination risks. 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 currently under development, can be expected in the near future. These advances promise to greatly simplify the detection and measurement of these important environmental pollutants in environmental matrices. The introduction of biosensors, most of which are still in the prototype phase, will provide another promising alternative to traditional methods for the field monitoring of estrogenic compounds.

Dansk sammenfatning

Nærværende rapport giver et overblik over kemiske analysemetoder til analyse af steroidøstrogener i (primært 17β-estradiol (E1), Østron (E2), og ethinylestradiol (EE2)) i miljømatricer, såsom spildevand fra renseanlæggets indløb og udløb, overflade vand, grundvand, slam, gylle, drænvand og jord. Rapporten foreslår endvidere en række overvejelser og rekommandationer i forbindelse med valg af analysemetode til en specifik matrice. Endelig er også nogle økonomiske overvejelser berørt.

En analysemetode består af flere individuelle trin; prøveudtagning, prøveopbevaring, prøveforberedelse samt analyse/detektion. Alle disse trin har indflydelse på den endelige analysekvalitet. På grund af steroidøstrogenernes begrænsede stabilitet er prøveopbevaring en vigtig faktor for at opnå et tilfredsstillende resultat. Prøveforberedelsestrinnet kan tit være meget tidskrævende og omstændigt. Kvaliteten af den endelige prøveoprensning er samtidigt afgørende for det videre valg af analyseudstyr til den egentlige detektion eller kvantifikation af analytten.

Steroid østrogener udskilles i nogen grad som konjugater med urin eller fæces. Både mennesker og dyr udskiller disse stoffer. Koncentrationsniveauet i relevante miljøer er ofte i størrelsesorden 0,01 ng/l til 500 ng/l, afhængig af dels det pågældende steroidøstrogen og dels typen af miljømatrice. Der findes som regel relativt høje koncentrationsniveauer i indløbsvand til renseanlægget sammenlignet med behandlet spildevand, grundvand og overfladevand. Steroidkonjugaterne er mere vandopløselige end steroiderne selv, derfor kan de kun analyseres ved hjælp af LC-MS. Således er en række LC-MS baserede analysemetoder udviklet til at analysere både de konjugerede og ikke konjugerede østrogener samtidigt. På grund af konjugaternes lave damptryk, er det ikke muligt at analysere disse direkte ved hjælp af GC-teknikker. Indirekte analyse kan dog udføres ved hjælp de enzymatiske dekonjugeringsteknikker der udviklet for miljømatricer. Ved anvendelse af disse er det muligt at analysere det totale østrogen niveau i en prøve både ved hjælp af GC-MS.

I litteraturen foreslås forskellige detektionsmetoder til analyse af steroid østrogener. GC eller HPLC med traditionelle detektionsprincipper (HPLC-DAD, HPLC-UV, GC-FID) findes ikke anvendelig til dette formål på grund af metodernes lave sensitivitet og dårlige selektivitet. Anvendelse af GC eller LC i kombination med MS og helst MS-MS er at foretrække, fordi der herved opnås analysemetoder med en højere sensitivitet og bedre selektivitet. Med immunokemiske teknikker opnås ligeledes tilfredsstillende sensitivitet, men deres anvendelse er af flere grunde problematisk og de kan kun benyttes hvis nedenstående overvejelser tages i betragtning. Både single GC-MS og LC-MS kan benyttes, selv til komplicerede matricer såsom slam, gylle og jord hvis analysemetoden benytter sig af en tilfredsstillende valideringsmetode indeholdende en række kvalitetskriterier. Hvis disse krav overholdes vil der være muligt at opnå detektionsgrænser (LOD) på ca. 0,3 til 2 ng/l med GC-MS i overfladevand eller behandlet spildevand ellers vil LOD ligge på ca. 20 ng/l. Lidt højere med LC-MS baserede metoder.

De mere avancerede LC-MS-MS eller GC-MS-MS metoder er de mest følsomme og har den højeste selektivitet og foreslås derfor benyttet specielt hvis østrogener skal måles i urene matricer (slam, gylle, eller jord) eller lave koncentrationer (overfladevand, spildevandsudløb). For disse teknikker vil LOD ligge på 0,1 til 0,5 ng/l for både GC og LC baserede metoder.

Metoder med en LOD på under 0,1 ng/l er ikke kommercielt tilgængelige og kræver yderligere forskning, hvad angår både prøveforberedelse og benyttelse af sensitive triple quadrupole instrumenter. Anvendelse af immunokemiske teknikker er også meget følsomme (LOD er rapporteret fra 0,05 ng/l og opefter) for i hvert fald spildevandsprøver med behandlet spildevand. Men metodernes selektivitet er dårlig sammenlignet med LC-MS-MS og GC-MS-MS. Specielt skal man være påpasselig med ikke at analysere prøver hvor ikke prøveoprensningen er i top. Falsk positive resultater og stor variabilitet er problemer der generelt ses ved benyttelse af de immunokemiske metoder. De foreslås derfor ikke benyttet som eneste analyseteknik, men kan pga. deres høje følsomhed og lave pris indgå i kombination med MS teknikkerne i en samlet større analysestrategi. Sådanne analysestrategier er dog i dag ikke udviklet.

Uden tvivl vil man, også på dette område, i løbet af få år se en langt større anvendelse af de mere avancerede MS-MS teknikker, der langt hen af vejen også vil blive integreret i automatiserede on-line systemer hvorved mange prøver kan analyseres på kort tid og til en fornuftig pris. Flere nye ekstraktionsteknikker, der dog endnu ikke kommercielt tilgængelige, er også på vej. Her skal nævnes molekylære imprinting materialer og immunoaffinitets kolonner, der vil simplificere prøveforberedelsen og gøre analysemetoderne både mere sensitive og mere selektive. Anvendelsen af biosensorer er en anden teknologi, der også i nær fremtid vil være et alternativ til de kromatografiske metoder i analysen af steroid østrogener i miljøet.

1 Introduction

Many chemical substances display estrogenic activity and may be suspected of causing adverse effects in humans and/or environmental organisms (***1***). At present only a few examples provide evidence that the presence of chemicals released to the environment by human activities is causing adverse effects on environmental organisms.
Recently evidence was presented showing that steroid estrogens released from humans are the main causal agents for the feminisation of fish in an aquatic environment impacted by sewage (***2;3***). The chemicals causing these effects are the natural compounds estrone (E1), 17β-estradiol (E2) and estriol (E3) and the synthetic estrogen, 17α-ethinylestradiol (EE2). A few other steroid estrogens are also reason for concern. The occurrence of these substances in the environment has hitherto mainly been considered as due to human release through sewage treatment, however, the question of whether release of farm animal waste (cattle and pigs) (17 α-estradiol (E2-17α) is a significant source for the observed effects remains unanswered.

Research focusing on assessing the extent, consequences and methods for solving the resulting problems is strongly dependent on reliable methods for detecting the substances and their metabolites in the environment. It has been shown that steroid estrogens are causing adverse effects in fish at concentrations as low as 0.1 ng/L (***3***). The lack of reliable analytical methods for analysing for steroid estrogens at such low concentrations (preferably lower) is a major impediment to the solution of this environmental problem. Even though a range of methods have been developed, the descriptions of these methods are scattered in the literature and an overview of these methods should be helpful. The objective of this report is to review the existing methods for detecting estrogens in relevant environmental matrices. This will be done with specific focus on the sensitivity of the methods, but as quality assurance and validation of the methods are very important, other parameters will also be considered in the assessment of the analytical methods.

For this purpose a number of delimitations are needed:

- Methods that are not published in the scientific peer review literature will generally not be evaluated.
- The diversity of the environment is enormous and therefore, a myriad of different environmental matrices exists. The matrices that will be covered in this report are:
    - wastewater related matrices (sewage influent, effluent, and sludge)
    - surface water and sediment
    - manure, soil, and groundwater
- Many of the papers cited in the current report will touch issues regarding pollution with estrogens and their environmental chemistry. As the current report is intended to address only analytical chemical information, it is outside the scope to comment on such issues.

The second chapter in this report discusses the background information needed to develop analytical methods for steroid estrogens. This includes

physical and chemical data for the steroid estrogens. Furthermore, a brief overview of exposure routes will be given in order to identify the environmental matrices that are relevant for the current report. For these matrices the expected concentrations will be discussed together with the potential environmental impact. The third chapter describes the different techniques used for analysis of steroid estrogens. The purpose of this chapter is to give an overview of the analytical chemistry methods used in analysing for steroid estrogens. The fourth chapter describes the specific problems that can be encountered when analysing each relevant matrix. The best available methods will be identified for each matrix state. In chapter five the various analytical methods are compared for effectiveness with regard to sensitivity, variability, selectivity and costs. In the sixth chapter recommendations and perspectives are presented, and some conclusions are drawn. Appendices 1 to 4 give overviews of the various analytical methods considered in this study.

2 Environmental and chemical properties of estrogens with relevance for analysis

This section describes the properties of steroid estrogens that are important in analysing for these substances. A limited number of steroid estrogens are important environmental pollutants and this report will cover only these compounds. Therefore, in this report the terms steroid estrogens and estrogens will be used for the natural estrogens, E1, E2, E2-17α (excreted by animals) and E3 and the synthetic analogs, EE2 and MeEE2. These substances are listed in Table 2.1 along with their full names and physicochemical data. All the substances are released from humans and animals as the structures shown in Table 2.1 or in various conjugated forms (see section 2.2.1 for more details). The primary conjugates are glucuronic acids and sulphate and these will therefore be included in this report. Other types of conjugates will not be mentioned in this report.

2.1 Physicochemical properties

The water solubility of steroid estrogens is low and range from 0.3 to approximately 13 mg/L with the natural steroids having the highest solubility.The synthetic steroids have the highest octanol-water partitioning coefficients (log K_{ow}). All steroids have very low vapour pressures and relatively high pK_a-values (above 10). From these data it can be seen that the estrogens are non-volatile, highly lipophilic substances that can be expected to adsorb to solids in environmental matrices. The low water solubilities of the substances, can cause problems when preparing solutions for analytical purposes, e.g., standards. As the compounds readily dissolve in organic solvents (e.g, methanol), using these solvents will solve this problem.

When estrogens are excreted from mammals, the primary route is via formation of glucuronic acid or sulphate conjugates (for more details see section 2.2.1). Conjugated steroids are a factor 10 to 50 more water soluble that the parent estrogens.

2.2 Release of estrogens from humans and animals and transport to the environment

The purpose of section 2.2 is to identify the matrices and analytes that are relevant when estrogens pollute the environment.

2.2.1 Metabolism and excretion of estrogens

The mechanisms and kinetics of de-conjugation of estrogens are important factors to determine, in order to assess and predict the estrogenic potency of surface waters. To facilitate the excretion with urine the female body primarily excretes estrogens in a biological inactive form as sulphate- and glucoronide conjugates. Such conjugates may, depending on different factors, easily be cleaved, resulting in a re-activation of the estrogens to an active form (***4***) (see Figure 2.1). This re-formation or de-conjugation of estrogens depends

partially on the acid-base properties of the environmental matrix and on possible bacterial processes within the matrix. Knowledge of these factors allows one to predict whether there are sufficiently high concentrations of the free and active compounds to elicit an estrogenic response in an exposed environmental organism.

Different glucuronid conjugates of estrogens are known (***5***). Conjugation of E2 and EE2 can occur in the C_3 position, in the C_{17} position and in both the C_3 and C_{17} positions. Estriol conjugation occurs in all the previous positions and can occur in the C_{18} position, as well. Sulphatation can also be expected in all the previously cited positions on the molecule. Conjugates possessing both glucuronidation and sulphatation also exist. Because the estrogen receptor is an unspecific receptor, a response will depend only on de-conjugation in the C_3 position. In Figure 2.1 de-conjugation pathways are exemplified by E2 and the biological activity is given. Similar tentative pathways could be identified for the other estrogens.

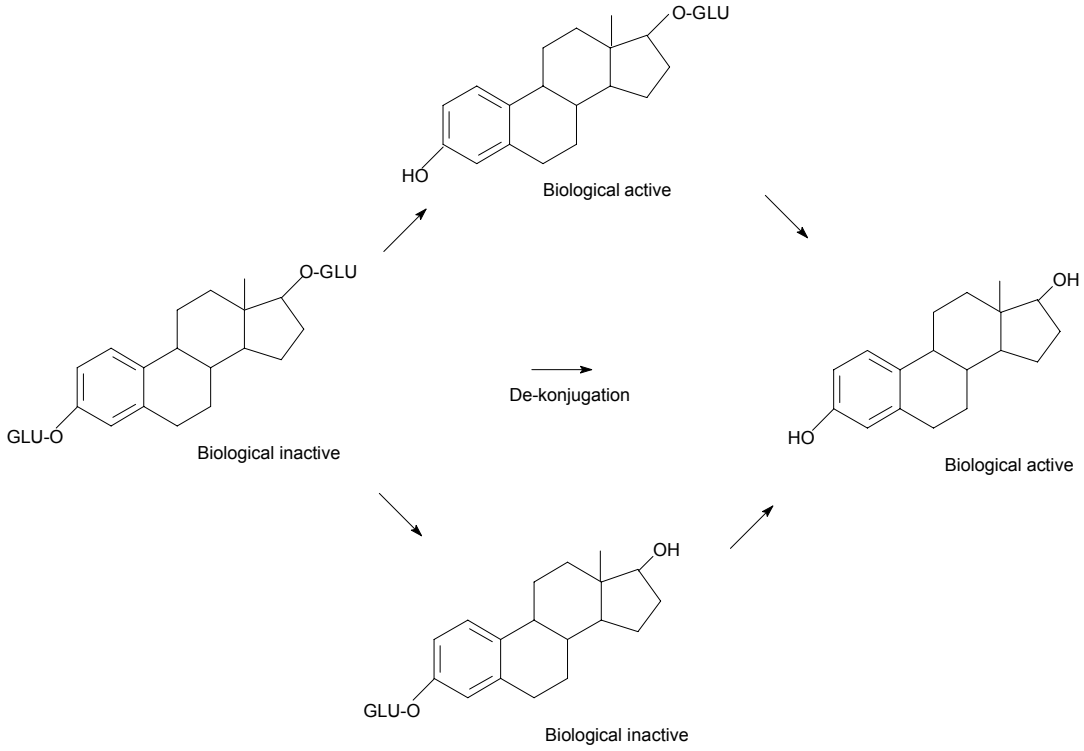


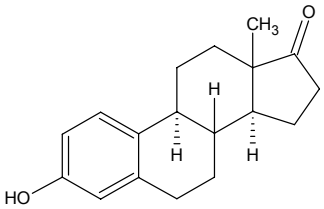
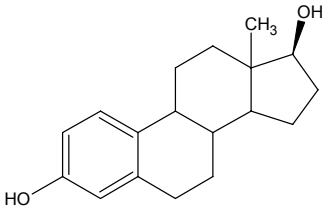
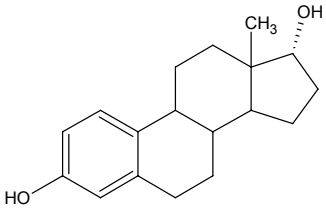
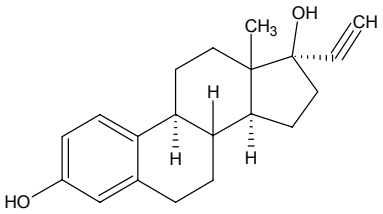
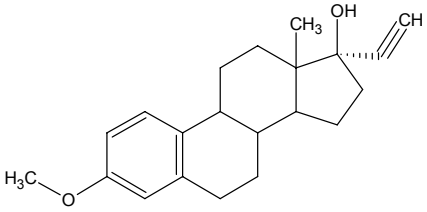
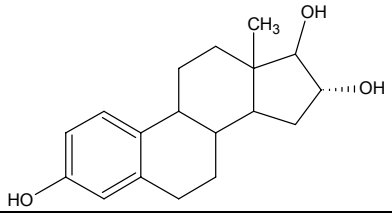
Figure 2.1:
De-conjugation of 17β-estradiol (E2) into biological active and inactive compounds.

Preliminary experiments have shown that about 80 % of 17β-estradiol glucoronide conjugates are detected as E2 and E1 in environmental matrices after 20-30 hours, and after 50 hours 10-20 % of the E1 and E2 was still not degraded (***6***). In other studies slower degradation of E2 and the primary degradation products E1 were observed. Here 88 % was degraded to E1 after 24 hours, and 95 % of the E1 was degraded after 14 days (***7***). In corresponding studies under anaerobic conditions E2 degraded considerably slower (50 % after 7 days), while E1 accumulated. Concerning the synthetic estrogen EE2 only 20 % was degraded after 24 hours under aerobic conditions.

2.2.2 Human release of estrogens

Estrogens excreted by humans may occur in a range of matrices where analytical methods should be available. Human waste is released to the sewer system which leads to the sewage treatment plants (STPs) where the estrogens can either be removed by degradation or adsorption to the sludge. If no removal occurs the estrogens are released to the receiving waters (streams, rivers or coastal waters) where they can adsorb to sediment and other solids. The estrogens adsorbed to sewage sludge may re-enter the aqueous phase when the sludge is dewatered otherwise they may reach the agricultural fields if the sewage sludge is used for fertilizing the soil. An overview of the different compartments that determine how estrogens from humans may enter the environment is shown in Figure 2.2.

Table 2.1:
Structures and physicochemical data for estrogens (data from (2)).

Substance	Structure	Molecular weight	Water solubility (mg/L at 20 °C)	Vapour pressure (mm Hg)	Log K_{ow}
Estrone (E1)		270.4	13	$2.3 \cdot 10^{-10}$	3.43
17β-estradiol (E2)		272.4	13	$2.3 \cdot 10^{-10}$	3.94
17α-estradiol (E2-17 α)		272.4	-	-	4.01
17α-ethinylestradiol (EE2)		296.4	4.8	$4.5 \cdot 10^{-11}$	4.15
Mestranol (MeEE2)		310.4	0.3	$7.5 \cdot 10^{-10}$	4.67
Estriol (E3)		288.4	13	$6.7 \cdot 10^{-15}$	2.81

- Data not available.

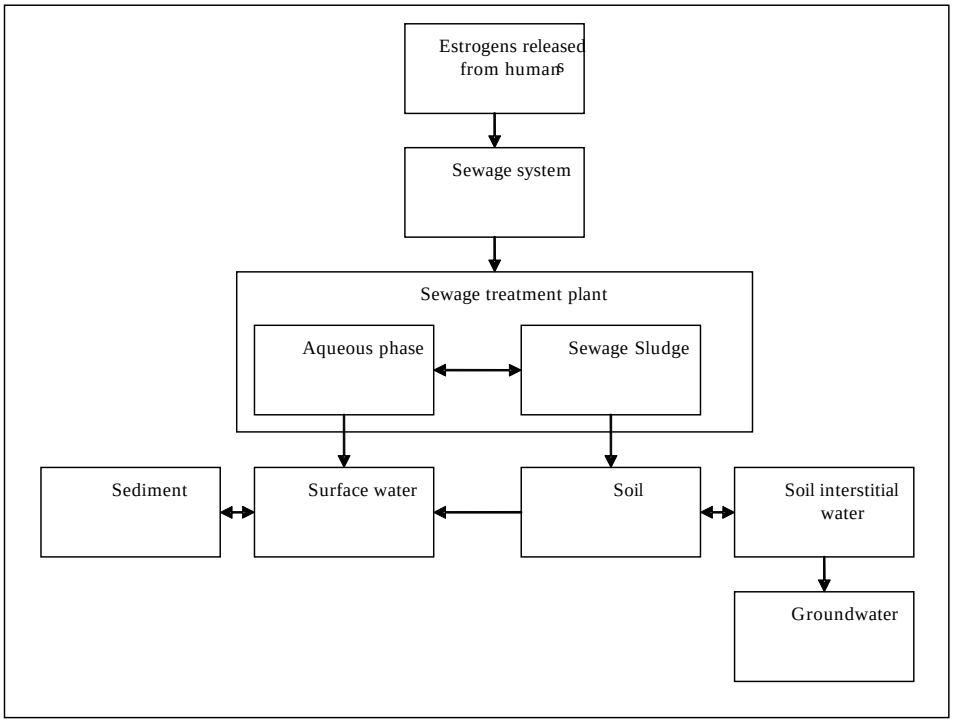


Figure 2.2:
Conceptual diagram illustrating the compartments where estrogens from humans may occur and where chemical analysis is needed.

The understanding of the processes determining the exposure routes depicted in Figure 2.2 is complicated further by the fact that estrogens are excreted in conjugated forms. It is well known that microorganisms can re-activate estrogens by breaking down the conjugated substances (***4;6;8***). As the un-conjugated estrogens bind more strongly to solids (e.g. sewage sludge) than the relatively hydrophilic conjugated forms, it is important to evaluate whether the STPs act as chemical reactors producing free estrogens, or if they act in a positive role in removing free estrogens. Therefore monitored concentrations of the conjugated as well as the un-conjugated estrogens are crucial in order to understand the processes involved in the removal of estrogens in STPs.

Several authors specify the amounts of estrogens released from humans (***9-11***). Generally the endogenous excretion of hormones by healthy pre-menopausal women is reported to range from 10 to 100 µg estrogens per day. The amount of E1 excreted is typically twice as high as of E2 and E3. After menopause, women only excrete between 5 to 10 µg estrogens daily. The values for normal men average from 2 to 25 µg per day (***12***). Pregnant women may excrete up to 30 mg per day, but average values are around 250 µg/day (***11***). Women using contraception-pills are assumed to excrete the whole daily dose of 25-50 µg.

Based on data on the daily excretion of estrogens from humans, influent and effluent concentrations of steroid estrogens in sewage treatment plants (STPs) can be estimated using simple assumptions about dilution in sewage water, sorption to sewage sludge, dilution of sewage effluent, etc. Using such methods, the expected environmental concentrations have been calculated for a range of relevant compartments (***10;13;14***). With regard to the analyte concentrations for methods for detecting estrogens, these estimates are

important as they represent maximum or worst-case levels of the concentration of estrogens in the analytical matrices. More realistic concentration levels of estrogen released from humans can be obtained from monitoring data reported in numerous publications (see e.g., (***15-19***)). Table 2.2 lists representative concentrations of estrogens in a range of relevant matrices. Both estimated concentrations and measured data from literature are shown. The purpose of presenting the data is to indicate the concentration levels of estrogens to be expected in the environment; therefore an extensive presentation of all references with measured environmental data as presented in several reviews is omitted (***3;19***).

Table 2.2:
Representative selection of expected and measured environmental concentrations in matrices relevant for estrogens released from humans (<LOD and >LOQ indicate that estrogens were detected at concentrations below limits of detection or quantification).

		E1	E2	E3	EE2	MeEE2	Refe-rences
Estimated	Sewage influent (ng/L)	12-102	5-44	49-115	1.1-5.1	N.D.[b)]	(*14;20*)
	Sewage effluent (ng/L)[a)]	0.6-51	0.3-22	2.5-58	0.06-2.6	N.D.[b)]	(*14;20*)
	Sewage sludge (ng/g)	2.7-25	>1-5.1	N.D.[b)]	N.D.[b)]	N.D.[b)]	(*21*)
	Surface water (ng/L)	0.22-2	0.27-2.5	N.D.[b)]	0.02-0.24	N.D.[b)]	(*22*)
	Sediment (ng/kg)	0.71-16	1.5-33	N.D.[b)]	0.51-9.8	N.D.[b)]	(*22*)
Measured	Sewage influent (ng/L)	44-490	11-180	<LOD-263	<LOD-120	5.3-120	(*23-25*)
	Sewage effluent (ng/L)	<LOD-82	<LOD-21	<LOD-28	<LOD-62	N.D.[b)]	(*3*)
	Sewage sludge (ng/g)	<LOQ-37	<LOQ-49	N.D.[b)]	<LOQ-17	<LOQ	(*26*)
	Surface water (ng/L)	<LOD-17	<LOD-8.8	<LOD-3.1	<LOD-5.1	N.D.[b)]	(*3;19*)
	Sediment (ng/g)	<LOQ-2	<LOQ-1.5	N.D.[b)]	<LOQ-0.9	<LOQ	(*26*)

a) Concentrations in sewage effluent are estimated assuming 50-95% removal.
b) N.D. indicates that no data are available in literature.

2.2.3 Release of estrogens from farm animals

The emission of natural estrogens from farm animals (cattle, pigs, chicken, etc.) is potentially a major source of estrogen pollution in the environment. The major components are E1, E2, 17α-estradiol (E2-17α) and their conjugates. In contrast to humans, the release of E2-17α is significant (up to 56% (***27***)). After storage in the manure-tank, estrogens excreted from stabled animals may be released to the soil environment when manure is used for fertilization of soil. Alternatively the compounds may reach the soil, directly in urine and faeces from grazing animals. Sorption of the estrogens is a significant removal process in both the manure and in soil and the release to the aqueous environment (groundwater and surface water) may therefore be minimal. An overview of the different compartments that determines how estrogens from animals may enter the environment is shown in Figure 2.3.

Quantities of estrogens vary among other things with animal species and with the types of animal production. Therefore, estimates of the total amounts released are encumbered with greater uncertainties than similar data for humans and further, the regional differences in animal farming practices are important issues to consider. Okkerman and co-workers (***27***) estimated the release of estrogens from animals in the Netherlands. According to their literature-study, the concentration of estrogens in faeces and urine from non-

pregnant cows is around 30 µg/kg and 15 µg/L respectively. The average excretion during pregnancy is reported to be 1.3 mg/day/animal. Similarly the average excretion of estrogens in manure is 1.13 mg/kg from pregnant pigs. The release from non-pregnant pigs is estimated to be 100-200 times lower. The assumption that 3% of manure applied on fields in the Netherlands is leached to surface water results in estimated concentrations of approximately 1.3 µg/L in surface water (***27***).

Due to the difficult analytical matrix, very few investigations report concentrations in manure and soil of estrogens of animal origin. In fact it is still a question whether the estrogens from animals reach surface waters. As a consequence it is not possible to give a general picture of the concentrations measured in manure; therefore Table 2.3 lists only examples from the few existing studies.

Table 2.3:
Overview of concentrations of steroid estrogens detected in manure and in manure impacted water.

Sample description	E1	E2	E2-17α	References
Manure (ng/g dw)[a)]	28-72	46-50	120-190	(*28*)
Manure (ng/g dw) [a)]	51.9-640	14.8-1229.1	N.D.	(*29*)
Run-off samples from soil applied with poultry litter (ng/L)		90-2520		(*30*)
Springwater with groundwater from a karst formation under agricultural influence (ng/L)		6-66		(*31*)

a) dw = dry weight, no data on dw-content in the manure samples was available, the dw in manure varies between 4 and 10 % and depends of the animal species (*32*)
b) N.D. indicates that data are available.

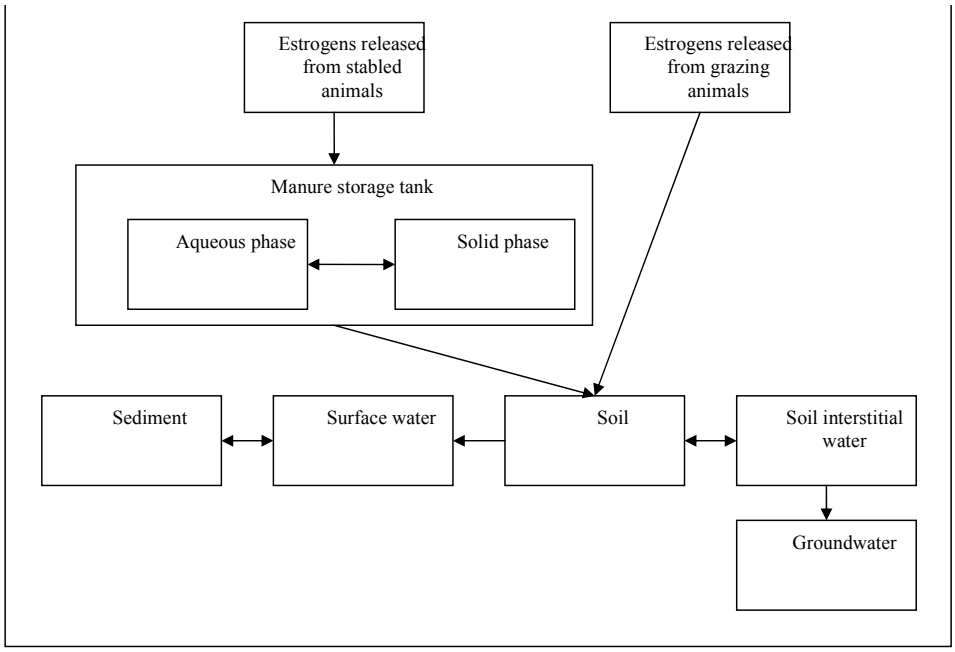


Figure 2.3:
Conceptual diagram illustrating the compartments where estrogens from animals may occur and where chemical analysis is needed.

2.3 Effect concentrations in the environment

A detailed overview of the effects that estrogens may cause in the environment is not the purpose of the current report. However, from an analytical perspective the effect concentrations expressed as NOEC (No Observed Effect Level) or LOEC (Lowest Observed Effect Level) are of interest because such data expresses the sensitivity needed for analytical methods. The vast amount of data on adverse effects on environmental organisms due to estrogens have hitherto been reported for various fish (***3;33;34***) species. A comparison of the LOEC's obtained in different studies is presented in Table 2.4. Based on these data and considering that the occurrence of a pollutant in the environment is considered safe only if the concentration is orders of magnitude below the LOEC's obtained in laboratory experiments (***35***) it is obvious that extremely sensitive analytical techniques (at least below 0.05 ng/L) are needed to detect estrogens at sub-effect concentrations in surface water. When sewage effluent is entering surface water the dilution obviously reduces the concentration of estrogens. It is common praxis to operate with a 10-fold dilution when the risk of the release of sewage is assessed. Thus, a concentration of estrogens in sewage effluent leading to LOEC's in surface water is 10 times higher than the values indicated in Table 2.4.

Table 2.4:
Lowest observed effect concentrations of estrogens in fish studies from literature (from (*36*)).

Substance and fish species	LOEC	Reference
Estrone Medaka *(Oryzias latipes)*	8 ng/L	(*37*)
17β-estradiol Fathead Minnow *(Pimephales promales)*	0.5 ng/L	(*38*)
17α-ethinylestradiol Medaka (Oryzias latipes)	0.03	(*37*)

The release of estrogens when manure is used for fertilizing agricultural soil, may lead to effects on soil organisms. Very few studies report such data. One example is a study showing that the growth of alfalfa (***Medicago sativa***) was significantly stimulated by concentrations of steroid estrogens of 10 ng/L (***39***). Due to the limited data available it is not possible to establish lowest observed effect concentrations for steroid estrogens in the soil environment.

3 Analytical methodologies

This chapter describes the different techniques used for analysis of estrogens. The methods are described from a technical and chemical perspective. Giving an overview of the publications describing the methods is also an objective of this section.

3.1 Conceptual description of analytical methods

It is important to realise that an analytical chemical method consists of a number of individual steps that can be divided in four groups as shown as in Table 3.1. This table also give examples of problems that should be considered under each step. An optimal chemical analysis is only achieved if the behaviour of the substances to be analysed (the analytes) is well understood in each of these steps. It is important to realise that an error made in one of these steps (e.g., during sampling) may have consequences for the whole analytical method.

Table 3.1:
Steps that are included in an entire analytical method. Under each step are listed problems that should be considered when performing the analysis.

Sampling	Storage	Sample Preparation	Analysis/detection
Homogeneity of sample, Variations of samples taken at different times, Problems (e.g., degradation, sorption, etc.) caused by the sampling equipment	Unwanted reactions of the analyte with the storage container Instability of the analytes to improper storage	Extraction of analyte Removal of interfering substances (cleanup) Pre-concentration	Intrumentation should be chosen so that the most cost effective analysis can be made

3.2 Validation of methods for analysis of estrogens

The ability to provide timely, accurate, and reliable data is central to any method for analysis of chemicals. Therefore method validation should be an integrated part of the process of developing analytical methods. A number of authorities and organisations have published detailed guidelines stating how method validation should be performed (see e.g., (***40***)), for an overview). In the current context it is important to note that these descriptions cover many fields of analytical chemistry and contain many details. However, all the various guidelines state that it is important to define the intended purpose of an analytical method and that method validation only needs to prove that the method is acceptable for this purpose. In environmental analysis this is particularly important, as the need of high precision may be limited. For steroid estrogens specifically, the purpose of analytical methods often can be limited to reliably detecting whether the substances are present above a certain concentration level or not. Thus in this particular case, only limited method validation is needed.

A number of validation parameters are defined in order to describe the quality of the entire analytical process. These parameters are determined on the basis of a validation procedure, including the analysis of a range of samples in matrix. It is important to stress that the validation should cover all the steps of the analytical procedure (including sampling, storage and preparation). An overview of the validation parameters is given in Table 3.2.

Table 3.2:
Overview of method validation parameters.

Parameter	Meaning	Determination
Accuracy	Is the closeness of the measured result to the true value	Is usually determined by using reference material of well defined concentration
Precision	Is the closeness of the agreement among repeated measurements	Is determined as the standard deviation between repeated measurements
Reproducibility	Is the closeness of the agreement between measurements when repeated under changed macroscopic conditions (e.g. different instruments)	Is determined as the standard deviation between repeated measurements
Selectivity	Is the extent to which the analyte may be determined without interference from other components.	Is determined by measuring the analyte concentration in presence of other compounds
Robustness	Is the sensitivity of the method towards external influences	Usually not determined in formal ways
Limits of detection	Is the lowest concentration the analyte can be detected	See comments in text
Limits of quantification	Is the lowest concentration the analyte can be quantified	See comments in text
Measuring range	Is the concentration range where the analyte can be quantified with acceptable accuracy and precision	Is concentration where it is documented that accuracy and precision is satisfactory
Sensitivity	Is the smallest detectable difference in analyte concentration	Determined from the slope of a linear regression line

The accuracy, precision, and reproducibility are all expressing the confidence one may attach to an analytical result. For several reasons, these parameters are of considerable importance in the analysis of estrogens in the environment. Particularly, when samples of different origin are analysed. Therefore, it is important to stress that these parameters, such as the standard deviation, confidence intervals or similar quantifiers, should be specified in the reporting and assessment of any analytical result.

As estrogens belong to the group of steroids, which are all very similar chemical compounds, the selectivity problems encountered are often due to interference from other steroids in the matrix. In the current context, the selectivity problems are considered severe making it necessary to document the selectivity of analytical methods. Due to limited selectivity, several authors who published monitoring results are suspected of over-estimating the concentrations of steroid estrogens in the environment. An important example is the work by Kolpin and co-workers (***41***) who reported concentrations that were higher than worst-case estimates could substantiate (***42;43***).

The selection of methods for analysis of steroid estrogens in environmental samples is most often dictated by the limits of detection and quantification since extreme sensitivity is required in most cases. The determination of the limits of quantification and detection (LOQ and LOD) can be made in several ways and has therefore been subject for discussion (***44***). All approaches however, depend on an estimate of precision at or near zero concentration. In practice LOD and LOQ are expressed as the concentration where the relative standard deviations of replicate samples is below a certain level (typically within 95% confidence limits). Alternatively LOD/LOQ is defined on basis of a comparison of the strength of the analytical signal with the strength of the background signal (signal to noise ratio).

3.3 Sample handling

3.3.1 Sample collection, preservation and handling

Very few articles in the literature address sample collection, preservation and handling of natural estrogens in environmental matrices.

Existing records reveal that both discrete and composite samples of waste water influents, effluents and occasionally, partially treated STP water are collected, analysed and reported. Sampling periods ranging from 6 hours to 5 days have been used to collect composite samples. In many instances discrete samples have been colleted. Grab (discrete) samples do not, in general, seem appropriate for assessing the presence of estrogens and xeno-estrogens in influents and effluents, particularly if the aim of the study is to evaluate the performance of the STP plant. Despite this many of the studies in the literature were performed with grab samples of effluents alone, and no samples of influents were collected at all. Some papers have justified the use of discrete samples in preference to bulked composite samples by remarking that the stability of estrogen compounds in waste water was, at that time, unknown.

One study by Baronti et al. (***25***) has investigated the stability of estrogens in treated and untreated water samples. Baronti and co-workers showed in their study that the best storage strategy passed the field samples through the extraction cartridge (C18 or Carbograph-4), washed the cartridge with a methanolic water solution (10%), and stored the cartridge at -18°C. Under these conditions, which provide a practical way to store many samples in extensive monitoring programs, no significant loss of estrogens was observed after storage for 60 days. An alternative but less secure procedure is to store the water in bottles preserved with 1 % aldehyde at 4°C. In the same paper Baronti and co-workers reported that there were no significant losses of the estrogens after 28 days when the samples were preserved, but severe losses occurred when the samples were not preserved. Other studies have used methanol, sulphuric acid or mercuric chloride to preserve the samples, however, methanol should under no circumstances be selected as the preservation chemical as this may influence the deconjugation af steroids in the sample. Methanol will always increase the degradation of the estrogens in the matrix in question.
Data indicative of the potential degradation of estrogens in unpreserved water for storage periods shorter than 7 days, for instance 24-48 h, were, unfortunately, not available. Freezing of unpreserved samples at -20 °C has been used in a few studies. Unfortunatly these studies have not investigated the loss of estrogens during storage. From a preliminary study of the estrogen

concentration in samples of effluents from 20 Danish STPs, our experience indicates that the estrogen concentration decreased significantly in unpreserved water samples within 48 hours (***45***).

The volume of sample processed depended mainly on the sensitivity of the technique used for the final analysis and varied from 50 mL extracted and analysed by radioimmunoassay by Shore et al (***46***) to 20 L (***8***) or even 80 L, extracted by liquid-liquid partition and analysed by gas-liquid chromatography by Tabak et al (***47***). It is not advisable to extract more than approximately 5 L with existing sample preparation methods because greater volumes only create other problems, e.g., extracts with a high load of humic acid.

The literature (e.g., Ternes et al. (***26***)) provides only a few examples of how to sample soil, manure or sludge (solid phase of sludge). Care should be taken to investigate the loss of analyte in these matrices by performing recovery studies on the effects of time, handling methods and sample preservation techniques.

3.3.2 Sample preparation

Section 3.3.2 primarily discusses sample preparation methods presented in the papers referred to in appendices 1 to 4.

3.3.2.1 Sample prep and cleanup for chemical analyses and bioassays

Filtration

Because waste water usually contains a high load of organic material and suspended particles, filtration is usually the first step of sample preparation. The filtration step is particularly necessary when subsequent extraction of the sample is based on the use of solid-phase extraction (SPE), because suspended solids could easily clog the absorbent bed. Similarly, when the analysis is performed by immunochemical assay, filtration helps avoid undesired adsorption on to antibodies. The filtration step has been performed in numerous ways, but most of the studies reviewed employed glass filters with a pore size between 0.22 and 1.2 µm. The question of whether the estrogens and xeno-estrogens are retained by the filter material has been investigated by several studies and retention was found to be negligible (***17;48***).

Despite these findings, analysts often wash the filtration system with methanol after filtration of the waste-water samples to remove any analyte adsorbed on the particles in the filter. A few studies also use centrifugation of the samples in addition to filtration for removing suspended matter.

Extraction

Extraction of steroid sex hormones and related synthetic compounds from waste water is usually performed by off-line solid-phase extraction (SPE). On-line SPE and Liquid-liquid extraction (LLE) have rarely been reported. Both disks and, most often, cartridges have been employed for the SPE of estrogens and xeno-estrogens from waste water. Both disks and cartridges have advantages and disadvantages. Disks are not clogged by suspended matter present in the samples as easily as cartridges. Disks also have a comparatively larger surface area for adsorbent-matrix contact, which results in higher extraction rates, and finally disk samples are free of contamination, whereas, cartridge samples can be contaminated by plasticizers leached from the cartridge support material during elution. Cartridges have the advantage

of being amenable to system automation, because devices are available for automated washing, conditioning, sample loading, drying and elution of a large number of samples.

SPE cartridges

Octadecyl (C18) bonded silica has been the most widely employed SPE adsorbent in both the cartridge and disk formats. Other adsorbents reported in the literature (see appendices 1 to 4) include graphitized carbon black, packed in cartridges, and styrenedivinylbenzene, which is commercially available in Isolut ENV+ cartridges and SDB-XC disks. The SDB proved to be unsuitable whereas the other cartridges were somewhat effective.
In contrast Amberlite XAD2, a polymeric adsorbent used successfully to determine organic water pollutants was inadequate for preconcentration of estrogens.
In most studies samples were extracted from raw waste water, with neither pH adjustment of the samples nor the addition of modifiers. A few papers reported the addition of 1-2% (v/v) methanol to the sample to facilitate SPE.

Sample-loading flow rates varied greatly among applications but were usually between 0.5 and 70 mL/min. Subsequent drying of the cartridge with either nitrogen or air is a common practise with no reported analyte loss.
The solvent, volume and number of steps used for elution depend mainly on the type of adsorbent and format used (cartridge or disk). Elution of the compounds retained by C18 is usually performed with pure or aqueous (80-85%) methanol, in two steps with total elution volumes varying between 10 and 20 mL for cartridges and between 15 and 60 mL for disks. Graphitized carbon black adsorbents which are also often used for the extraction of estrogens behave both as non-specific adsorbents and anionic exchangers (***49***). Therefore stepwise desorption with water, acidified methanol, and methanol, before elution of the analytes of interest with dichloromethane-methanol (80:20 or 60:40) are required to achieve neutral-acid class fractionation and to furnish cleaner final extracts.

Purification

Sample extraction from heavily contaminated samples (manure, soil and to some extent waste water) often requires further clean-up before analysis. This has been achieved by very different means, including liquid-liquid extraction, solid-phase purification on C18/NH2 columns, silica gel columns chromatography, gel permeation on BioBeads SX-3 columns, high-performance liquid chromatography (HPLC) fractionation, or combinations of all these. Preparative gel permeation chromatography (GPC) has been used successfully to remove components with high molecular masses from sludge extracts before Silica Gel Clean-up. Details of the GPC method and evaluation of the GPC procedure are reported in the references (***50***) and (***26***). In some later work the GPC extract was used after Silica Gel Clean-up to reduce the load on the GPC. Some of the purification procedures were developed to isolate the estrogenic active fractions from the waste water extract for further identification of the compounds responsible for such activity, rather than for simple clean-up of the extract. Irrespective of the purpose of the analysis, however, thorough purification, very often consisting of several steps, is usually required for the accurate determination of these compounds at their active concentrations. Good detection limits without purification have only been reported by studies using biological techniques for analysis or graphitized carbon black adsorbents as SPE. If GC-MS or LC-MS is used for analysis purification may be especially important for reducing matrix interference.

Evaporation
To achieve sufficient overall method sensitivity or for solvent exchange extracts must be concentrated, and, very often a concentration step is performed several times throughout the complete analytical procedure. The volume reduction techniques used in the different methods reviewed were rotary evaporation and nitrogen evaporation, the choice depended mainly on the volume of extract to be concentrated. The concentration step is critical and can result in losses. A few precautions may reduce this risk. These include control of flow rate and temperature during nitrogen evaporation, protection of sample solution from light, and ensuring that the extract is not left completely dry for extended periods of time.

3.3.2.2 Deconjugation techniques using enzymatic hydrolysis

In many cases non-conjugated as well as conjugated estrogens are present in environmental samples. For several reasons detection and measurement of these constituents may be unnecessary. In such cases, deconjugation of all the estrogens in the sample has been suggested.

Estrogen conjugates in waste water can be quantified by including an hydrolysis step which converts the conjugated forms into the active hormones during the sample preparation process. This step is necessary if final analysis is performed with GC-MS or GC-MS-MS. Both LC-MS and Immunoassay techniques can be used for direct analysis of conjugates although the methods have the same drawbacks as for the unconjugated compounds. Comparing the results of thes two methods allows one to simultaneously determine the concentration of both free and conjugated forms.
Glucuronidase enzymes have been used for hydrolysis of glucuronide and sulphate conjugates. The concentrations of the conjugated enzymes have been determined from differences between the results for hydrolyzed and unhydrolyzed samples. It should, however be noted that whereas the deconjugation of glucoronide is 100%, only 30% of the sulphate conjugate is cleaved resulting in an underestimate of this conjugate form. Adler and co-workers (***17***) suggest that sulphate conjugates may be cleaved by use of Arylsulphatase. They incubated samples for deconjugation over night at 37 °C adding 100 µl Glucuronidase/ Arylsulfatase-suspension Type HP-2 (Sigma Chemie Nr. G7017) in 10 ml Natrium-acetat buffer (pH=5) achieving a nearly 100% deconjugation.
An alternative deconjugation technique is acid hydrolysis that has been obtained by adding hydrochloric acid (1M) to the freshly collected samples and then incubating at 80 °C for 20 minutes (***51***). This technique is problematic as the steroid estrogens might decompose during this process.

The LC-MS method makes a direct measurement of conjugates and avoids the hydrolysis step. This is a clear advantage.

3.3.2.3 The use of standard and internal standards
In order to obtain accurate values for the estrogen concentrations, the standard used should be as pure as possible (near 100%). In any case, the amount of estrogen present in a certain quantity of the standard material should be known exactly. Therefore, certified reference material should be used if available.
The choice of the internal standard, the labelled counterpart of the estrogen to be analysed, is very important. During the analytical procedure, its identity should be fully preserved, i.e., none of its isotopic labels is permitted to exchange for some part.

^{14}C-label has often been employed (located in the steroid skeleton) as an internal standard, which has the advantage that no label exchange occurs, but a major disadvantage is that its radioactivity makes it a health hazard risk. Another disadvantage of ^{14}C- labelled steroids is the general inclusion of appreciable amounts of unlabelled material. Therefore at present most researchers employ isotopically labelled steroids in which some (at least two) hydrogen atoms are replaced by deuterium atoms at free exchange positions or alternatively, at least two ^{12}C atoms are replaced by ^{13}C. Besides being radioactively stable these internal standards contain only a few percent unlabelled compounds.

Precise measurement of the amount of the unlabelled and labelled steroids is of crucial importance, as this eventually determines the accuracy and precision of the end result. The analytical balance and pipettes used should be carefully calibrated and their tolerance known. In all relevant papers much attention has been paid to this subject. In the hands of experienced personnel this stage in the analytical procedure can be carried out in such a way that it contributes no more than 0.1-0.2 % of the total error in the final result.

The internal standard should be added as the first analytical step, mostly as an alcoholic solution; it is important that good equilibrium is reached before extraction and that the amount of alcoholic solution added does not result in precipitation of other constituents.

3.3.2.4 Comparison of sample prep methods use for bioassays and for chemical analysis

Sample preparation for bioassays and chemical analysis is in principal identical. The more clean-up of the sample the less probability that many false-positive results will occur. High loads of suspended solids will result in undesired adsorption on to antibodies. The highest level of sample prep and cleanup is especially important when using bioassays. For some of the most sophisticated GC-MS-MS methods less clean-up may be acceptable.

3.4 Analytical detection methods

Estrogens are detected using several different detection methods. The purpose of the current section is to describe methods published in the scientific literature. It is important to stress that the techniques used for detection of estrogens are used in other contexts than environmental analysis and therefore a vast amount of literature related to other fields (see e.g., (***52***)) exists, but these will not be covered here.

Currently, most of the work related to the analysis of steroid estrogens in the environment has been made on surface water or sewage influent or effluent. Therefore, only for these matrices have analytical methods with satisfactory method validation for detection of steroid estrogens been documented. This will be reflected in the current section that primarily deals with analytical methods for such matrices.

3.4.1 Methods based on gas chromatography (GC-MS and GC-MS-MS)

3.4.1.1 General information

In gas chromatography (GC) the compounds to be analysed are vaporised and eluted by a stream of gas as a mobile phase through the column. The

mobile phase is used only as a carrier gas so that interactions of the mobile phase with the analyte are of no significance. The analyte is normally dissolved in a liquid and GC is normally used primarily for volatile organic compounds. The predominant separation principle is then the partition of substances between the liquid stationary phase and the gaseous mobile phase.

GC-MS is the most popular of all hyphenated techniques for gas chromatography, and the combination of a powerful separation technique with the high degree of structural information provided by the mass spectrometry (MS) has made GC-MS the workhorse of trace analytical laboratories. This combination gives the possibility of combining an automated separation on the GC with structural information (masses) on the MS. The most popular mass analyser is the quadrupole mass filters that allow high scanning speeds up to a transmission range of m/z equal 2000.

GC-MS-MS is the hyphenated technique combining the GC with a tandem mass spectrometry (MS-MS) (triple quadrupole). MS-MS is any general method involving at least two stages of mass analysis either in conjugation with a dissociation process or a chemical reaction that causes a change in the mass or charge of an ion (see below). In the most common MS-MS a first analyser is used to isolate a precursor ion, which then undergoes a fragmentation, either spontaneously or by some activation, to yield product ions and neutral fragments. A second spectrometer analyses the product ions. By using a MS-MS instrument the selectivity of the analysis is increased as not only is a specific mass used for quantification, but this specific mass can be related to a specific fragmentation of product ions. If the analyte is part of a complicated matrix this will reduce the matrix interference. If single GC-MS is used for analysing compounds in complicated matrices, such as treated or un-treated waste water, rules for identification must be set up. These rules might include matching of retention time, presence of molecular ion of target compound, presence of at least two additional qualifier ions and matching of ion ratios within 50% for the two qualifier ions.

A special version of the MS-MS instruments is the ion-trap MS-MS. An ion trap can be imagined as a quadrupole bent on itself in order to form a closed loop. This allows the instrument to trap a large number of molecules for ionization, thereby increasing the sensitivity of the instrument. These instruments have regularly been applied in this field.

For more information on MS detectors see the chapter on liquid chromatography based techniques.

3.4.1.2 Ionisation methods

Although various ionization methods are available, electron impact (EI) and chemical ionization (CI) are the most common for general use in GC-MS analysis. Of these two techniques EI is by far the most widely used. EI ionization produces fragment rich mass spectra that may provide structural information. In EI sample molecules entering the ion source from the gas chromatographic column, are ionized by thermal electrons emitted from a tungsten or rhenium filament (the cathode) and accelerated towards the anode. The electrons collide with the sample molecules, transferring part of the kinetic energy of the electrons to the molecules. This causes excitation, fragmentation, and ionisation. As the distribution of the internal energy directly affects the appearance of the mass spectra, and is strongly dependent on the electron beam energy, (Eel), it is usually fixed at a standard value of 70

eV. Spectra are compiled in libraries and used for identification of compounds via a search procedure.
In chemical ionization (CI), the analytes are ionized by gas-phase ion-molecule reactions. To achieve this, the reagent gas (e.g. methane, iso-butane, ammonia or water) is introduced into the ion source at comparatively high pressure (0.01-2 Torr). Reagent gas ions are generated by electron impact ionisation. The analyte molecules are then ionized indirectly via a series of collisions with the reagent gas ions. These collisions involve only a small amount of energy, with a narrow distribution, and the process is often referred to as "soft" ionization technique. Soft ionization leads to less fragmentation and thus more abundant molecular ions are obtained in CI compared to EI. While the low degree of fragmentation increases the sensitivity of CI-MS, it yields only limited structural information. A valuable feature of CI, however, is that its selectivity can be tuned by the choice of reagent gas. Depending on the reagent gas, positive (PCI) or negative ions (NCI) give the most sensitive ionisation.

3.4.1.3 The use of GC for analysing natural estrogens

The analytical determination of natural estrogens in environmental matrices has been dominated by the use of GC-MS and GC-MS-MS. Both conventional MS and MS-MS (ion trap and triple quad.) detection are accomplished in the EI mode of ionization. The use of NCI and PCI has also been reported, however, it is important to note that only GC combined with MS and tandem MS, respectively, provide sufficient selectivity and inherent sensitivity to analyse for natural estrogens in complicated matrices such as treated waste water, sludge, manure or soil.
The major difference between single MS and MS-MS is in the selectivity of the analysis (see example in section 5). Interference from the matrix may be a major problem with single MS. This problem is especially acute for EE2, where measured concentrations are sometimes higher than anticipated suggesting that this difference may be due to interference by natural organic matter. Using MS-MS this interference may be reduced by using one or more daughter ions for quantification. In most studies the instrument has been operated at 70 eV, in the selected ion monitoring (SIM) mode. The analysis is conducted after sample derivatization (see below). The use of derivatization agents in sample preparation for GC analysis is one of the major drawbacks of using GC for analysing natural steroids (see below). An overview of recent publications presenting GC detection based methods is given in Appendix 3.

3.4.1.4 GC-methods with non-MS detectors

The use of GC for analysis of natural estrogens in complicated environmental matrices without the hyphenation of an MS instrument is not recommended and therefore not treated further in this work.

3.4.1.5 Detection limits

The detection limits achieved with the different methods employing GC-MS or GC-MS-MS as final analytical techniques were in the range of 0.5 – 74 ng/L and 0.1 – 24 ng/L, respectively.

3.4.1.6 Capillary Columns

GC separation is performed with a variety of capillary columns using helium as carrier gas, with temperature programs from approximately 45 to 300 °C. Sample volumes of 1 to 4 µL extracts are injected in the splitless mode.

3.4.1.7 Derivatization agents

In order to improve the stability of the compounds and the sensitivity and precision of the GC-MS or GC-MS-MS analysis derivatization agents are always used. Several derivatization agents such as bis-(trimethylsilyl)-triflouroacetamide, N-methyl-N-(tert.)-butyl-dimethylsilyl-triflouroacetamide (MTBSTFA) and heptaflouro-butyric anhydride, have been used depending on the choice of ionisation technique. The analytes are usually derivatized in the –OH groups of the steroid ring. The ion masses selected for quantification in each case vary depending on the derivatization reaction performed. Table 3.3 gives an overview of some of the derivatization agents used. First, in the MS ionisation mode chosen, one or more fragments ions in the mass spectrum should be present with m/z values of 400 or greater and in abundant numbers allowing precise mass fragmentographic measurement in the lower pictogram range. Second, after selection of a pair of fragments for steroid and internal standard, best results in terms of accuracy and precision are obtained when the unlabelled steroid does not contribute considerably to the mass fragment chosen for the labelled steroid (two or three mass units higher than that of the unlabelled).

Table 3.3:
Overview of derivatization agents used.

Derivatisation agents	Reference
Bis-(trimethylsilyl)triflouroacetamide contaning 10% trimethylchlorosilane	(*53*)
N-methyl-N-(tert-butyldimethylsilyltriflouroacetmaide (MTBSTFA) contaning 1% *tert*-butyldimethylchlorosilane (TBDMCS)	(*54;55*)
N-methyl-N-(trimethylsilyl)triflouroacetamide (MSTFA)-trimethylsilylimidazole (TMSI)-dithioerytrol (DTE), 1000:2:2 (v/v/w)	(*6;56*)
Heptaflourobutyric anhydride	(*48*)
Pentafluoropropionic acid	(*56;57*)
N-methyl-N-trimethylsilyl-2,2,2-triflouroacetamide (MSTFA)-triethylsilyliode (TMSI)-dithioerythritol (DTE)	(*58*)
Acetic anhydride (extractive acylation)	(*59*)
Triflouroacetyl, heptaflourobutyl, pentadecaflourooctanoyl, and perflourotolyl derivates (GC-NCI-MS mode)	(*60*)
Comparion of performance of derivates both for PCI and NCI	(*61*)

Kelly (***54***) and Mol et al. (***55***) report that the tert-butyldimethylsilyl derivatives are formed more quickly and are much less sensitive to hydrolysis than many silyl derivates e.g. trimethylsilyl derivates. Only the mono-substituted derivates are formed (with the hydroxyl group of the unsaturated ring), however, and steric hindrance of other active sites, may result in marginal improvement of the sensitivity of GC-MS analysis. Therefore, these derivatives are not considered useful in the context of estrogen analysis.

Nakamura et al. 2001 reported that NCI-MS provides high sensitivity for the PFB-TMS derivates of the estrogens. Other derivatives that run nicely in the negative chemical ionisation mode are the perfluorobenzoyl derivates (***60***). Peak tailing due to the presence of water, and difficulty in re-dissolving the derivatives in the solvent commonly used for GC injection have been reported in some studies.
A comprehensive comparison of different derivatisation agents for EI, PCI and NCI was published a few months ago by Lerch and Zinn (***61***). This paper is a good starting point for assessing the usefulness of different agents when developing new methods.

3.4.2 Methods based on high performance liquid chromatography (HPLC)

High performance liquid chromatography systems are used less for environmental analysis than gas chromatographic (GC) methods. Recent instrumental improvements have however increased the sensitivity of these systems and more extensive use of this technique should be encouraged. The main advantage of applying the LC based methods for environmental analysis of estrogens is that glucuronic and sulphuric metabolites can be detected while the derivatisation of the analytes needed in GC-systems is un-necessary. An overview of recent publications presenting detection methods using HPLC is presented in Appendices 1 and 2. It is evident that the majority of methods presented are aimed for analysis of sewage effluent or surface water. The number of papers presenting methods for analysis in other environmental matrices is sparse.

3.4.2.1 HPLC-separation

Analysis of chemicals in high performance liquid chromatography (HPLC) systems is performed in a separation unit (the chromatographic column) and a detection unit. The purpose of the chromatographic unit is to separate the analytes to such an extent that selective detection of each substance can be made in the detection unit. It is important to note that if the detection unit used is able to unambiguously identify the analytes, a complete chromatographic separation is not necessary.

It is generally accepted that if selective detection systems, such as tandem mass spectrometers, are used to detect steroid estrogens and the relevant metabolites in environmental samples, the separation does not need to be complete (***62;63***). In this case the total time for separation of the analytes is in the range of 12-15 minutes. If less specific detection methods are used and baseline separation therefore is needed, the time for complete separation of all analytes is around 30 minutes (***64***). The usual means of achieving separation is in columns with octadecyl silica based stationary phases.The mobile phases consist of water:acetonitrile or water:methanole mixtures with gradient elutions from 20-50% to 100% organic phase (see references in appendices 1 and 2). Examples are available from the literature of using ammonium-acetate (***63***) or triethylamine (***62***) for buffering the mobile phase, but in most cases no further addition is made. A pH-adjustment of the eluent is considered unimportant with the exception of the reported post-column addition of ammonia (***25***) or triethylamine (***14***).

3.4.2.2 HPLC combined with non-mass spectrometry detectors

Due to the limited sensitivity it is not surprising that only a few reports exist on methods for environmental analysis of estrogens using detectors other than mass spectrometers. Snyder et al. (***65***) used fluorescence detection of E2 and EE2 and a range of synthetic phenolic endocrine disruptors. Ying et al. (***66***) recently presented a similar method with similar limits of detection. The sensitivity of the fluorescence methods is low, this technique is rarely used because of severe problems with interference from the matrix and is obviously not recommended.

The use of spectrophotometric techniques including diode array detectors (DAD) is common in HPLC systems. This technique is also widely used, e.g., in biomedical analysis (***52***). For environmental analysis, the sensitivity is generally too low and interference from other substances in the sample is a further drawback. The technique can be used only in combination with sample preparation techniques which pre-concentrate the sample extensively.

One such method has been presented using a combination of a fully automated sample preparation and HPLC-DAD system. The limits of detection for this method of detecting steroid estrogens in surface water was 15 ng/L (***67***). In a study using the less laborious solid phase micro extraction (SPME) technique, sample preparation limits of detection of 300-700 ng/L were obtained. With UV-detection and using a more sensitive electrochemical detection the sensitivity was increased by approximately 10 times (***68***).

3.4.2.3 Liquid chromatography combined with mass spectrometry

The development of techniques for coupling mass spectrometers to liquid chromatography holds promise for the analysis of steroidal estrogens in environmental samples. Furthermore, the ongoing development of more sensitive mass spectrometers with the possibility of analysing for estrogens without derivatization would provide such advantages that HPLC coupled to mass spectrometry may in the future replace gas chromatography coupled to mass spectrometry as the preferred analytical technique for many types of samples.

In the LC-analysis of steroid estrogens in environmental samples, the highest sensitivity is achieved by coupling the mass analyser and the HPLC by using electrospray ionisation (ESI) in negative ionisation mode (***25;62;69;70***). Similar sensitivity was achieved by the use of atmospheric pressure chemical ionisation (APCI) (***57;63***). However, as the use of the latter technique is only reported once, ESI is considered the best choice. Only recently, a new ionisation method, atmospheric pressure photo ionisation (APPI), was developed (***71***). This technique purports to improve the sensitivity in the analysis of steroid estrogens by 1-2 orders of magnitude (***72;73***) when compared to APCI. At present, no reports have been published on the use of this technique on environmental samples, therefore, the question of matrix-related problems in using the technique remains unanswered. At present the instrumentation is becoming commercially available making further investigation of the use of this promising technique possible.

A range of mass detectors are available for coupling with HPLC. Due to the high sensitivity, quadrupole instruments have been used almost exclusively (to the knowledge of the authors, the only LC-method published using other mass detectors is a method using ion-trap mass detection (***74***)).

Although the sensitivity is lower for single MS-instruments (see e.g. (***74***)), methods have been developed with detection limits below 1 ng/L (***70;75***). In these methods selected ion monitoring of the $[M-H]^-$ ions was used. Due to the reduced specificity of single MS systems, the treatment of the sample before it enters the mass-detector must be selective. This has been achieved either by using a selective sample preparation procedure (such as immunoaffinity extraction (***70***)) or by using a chromatographic procedure with very good separation.

The use of triple quadrupole MS-MS instruments has increased selectivity and sensitivity substantially. In the LC-ESI-MS-MS analysis of environmentally relevant estrogens, the highest sensitivity is achieved when recording in the MRM-mode (multiple reaction mode), which is an MS-MS experiment where one or more specific products of a selected precursor ion is monitored.
Table 3.4 lists the pairs of precursor and product ions for estrogens that have been used in analyses. It is important to note that in positive ionization mode

the precursor ion for E2, EE2 and E3 differs from the molecular weight of the analytes due to fragmentation of the molecules in the ion-source.

Recently developed new generations of triple quadrupole and other types of MS-MS instruments are purported to have substantially increased sensitivity, but no proof for this is yet found in literature.

Table 3.4:
Overview of precursor in product ions used in the detection of estogens in LC-MS-MS-instruments.

Substance	Ionization mode	Monoisotopic molecular weight	Precurser ion (m/z)	Product ion (m/z)	Examples of references
E1	-	270	269	143	(*14;25;49;69*)
				145	(*14;25;49;62;69*)
E1	+		271	133	(*57;63*)
				157	(*63*)
E2	-	272	271	183	(*14;25;49;69*)
				145	(*14;25;49;62;69*)
E2	+		255	133	(*57;63*)
				159	(*63*)
EE2	-	296	295	159	(*14;25*)
				145	(*14;25;62*)
EE2	+		279	133	(*57*)
				149	(*63*)
				205	(*63*)
E3	-	288	287	171	(*14;25;49;62;69*)
				145	(*14;25;49;69*)
E3	+		271	133	(*57;63*)
				159	(*63*)

3.4.3 Methods based on Immunochemical techniques (immunoassay)

In the field of environmental analysis, immunochemical techniques are getting more and more attention because of their high sensitivity, ease of use, short analysis time, cost-effectiveness and several other advantages (***76-78***). In the health care sector, immunochemical methods are widely used (***79***) including methods for the detection of estrogens (***80***). Therefore it is not surprising that immunoassays provide an alternative for the detection of steroid estrogens also in environmental samples.

3.4.3.1 Principles of immunoassay

The basis of immunochemical analytical detection methods is the capability of antibodies to specifically recognise and form stable complexes with antigens. Antibodies are proteins that specifically bind to chemical molecules in non-covalent bindings. Polyclonal antibodies are extracted from serum from live animals (typically mice or rabbits) vaccinated with the antigen and monoclonal antibodies are produced using in vitro cell assays. The monoclonal antibodies provide higher specificity and sensitivity but their production-price is much higher. Immunoassays employ antibodies as analytical reagents. The assays are based on the observation that in a system containing the analyte and a specific antibody, the distribution of the analyte between the bound and the free form is quantitatively related to the total analyte concentration.

The wide use of immunochemical analytical methods is due to the different techniques for applying a label on the antibody and thereby improving the sensitivity for the detection of the antigen-antibody complex. The detection of the antigen-antibody complex can be made in one of two configurations. In

non-competitive assays the complex measured is formed when the analyte itself is introduced in the test system. In competitive assays the complex with the analyte is formed by replacement of the antigen in the labelled antigene-antibody complex with the non-labelled analyte (competitive assay). The latter technique provides a higher sensitivity and is therefore widely used for environmental analysis.

A suite of different labelling techniques are used, all with the purpose of making the detection of the label possible using classical chemical techniques. The radio immunoassay (RIA) utilizing radioactive isotopes, as label was discovered first, but several other types of labels have been developed including enzyme-linked immunosorbent assays (ELISA), enzyme immunoassay (EIA), fluorescence (FIA), electrochemical immunoassay and several other techniques. The common design of these techniques is in microtiter-plates, but the diversity of the instrumentation in the designs is extensive. The techniques differ by several means and they vary in sensitivity, ease of use, cost-effectiveness, and several other factors. In many cases, however, the choice of method is determined by the possible labelling.

Some advantages and disadvantages of environmental immunoassays are listed in Table 3.5 (***77;78***). The rapidity, the cost-effectiveness and the ease of use combined with the sensitivity favours immunoassays for a role as tools for screening of environmental pollutants. However, the major drawback is the absence of a threshold below which samples can be considered as negative. For the same reason, as a part of the method validation, immunoassays should always be confirmed by specific chemical analysis, e.g., GC-MS-MS or similar techniques.

Table 3.5:
Advantages and disadvantages of environmental immunoassays.

Advantages	Disadvantages
Sensitive	*Not 100% specific, vulnerable to cross reactivity*
Rapid	*Requires independent confirmation (e.g. HPLC-MS-MS or GC-MS-MS)*
Cost-effective	*Not suitable for small sample loads*
Small sample volume	*Syntesis of antibody can be difficult and expensive*
Easy to use	*Only one substance can be analysed at a time*
Wide applicability	
Reduced sample preparation	
Simultaneous analysis of multiple samples	
Easily automated, ideal for large samples load	
Suited for field use	

3.4.3.2 Immunoassays for analysis of steroid estrogens in the environment

Immunoassays were the first methods applied for detection of environmental estrogens (***46;81;82***). The analytical validity of these and other early works are generally considered insufficient when compared to the level of more recent

publications. This may explain why the immunoassays are less used than classical analytical techniques for detection of steroid estrogens.

An overview of immunochemical detection methods for estrogens in environmental samples published in peer-reviewed papers is shown in appendix 4. It should be stressed that the extensive number of papers reporting analytical methods for the clinical laboratory using immunoassays is not mentioned in this report.
In the current context three classes of immunochemical methods for environmental samples can be defined. The first is methods using existing systems for clinical analysis. The second is methods developed for environmental analysis and third, (a mix of these two), is the commercial test kits that are offered by several companies.

The combination of a specially designed sample preparation procedure in combination with an immunoassay detection system for clinical analysis (typically RIA) (***65;82***) provides the advantages of using a highly validated detection system. Clinical laboratories provide immunoassay methods (typically RIA and ELISA) for steroid estrogens that are well documented, with limits of detection of 10-100 ng/L (***65;83***). These assays have a high throughput of samples at cost efficient price levels. Unfortunately, the problems related to variability and accuracy are severe (***84***). The clinical immunoassays are designed for analysis of serum extracts, etc., which are matrices very different from extracts of environmental samples. Therefore, attention should be paid to the risk of cross-reactions and other effects from the matrix. It is important to stress that analysis confirmation using GC-MS-MS or HPLC-MS-MS should be made in order to validate the method. Furthermore, an extensive use of spiked control-samples is recommended.

Commercial test kits (ELISA-assays) for detection of E1, E2, EE2 or the sum of the three substances are available from several companies. The majority of these kits are intended for analysis of blood, serum and other samples, and yet these assays have been successfully applied by several authors (***48;85***). A few companies offer ELISA-kits especially for detection of E1, E2 or EE2 in sewage effluent and freshwater samples (***86;87***). Limits of detection are usually at the level of 0.01 to 0.05 µg/L depending on the substance. But much higher LOD have been reported in the literature. The assays are delivered without the equipment needed for the sample preparation (SPE-columns and various solvents), but with detailed description of sample preparation procedures, which should allow detection of sub ng/L concentrations. Analysis of such concentrations is documented and confirmed with LC-MS-MS detection. Comparison of results shows that the results obtained with this technique may differ up to two-fold from LC-MS-MS. The assays are reported to cross-react insignificantly (up to 16%) with a few metabolites of steroid estrogens, but a weakness is that no such data are provided regarding other chemicals. Therefore, whole sample cross-reactivity should be considered when such assays are used.

Appendix 4 gives an overview of the immunoassays especially for environmental analysis. It can be seen that the techniques are relatively sensitive in comparison with other techniques; furthermore, the demand for high sample purity is limited so the sample preparation needed for these techniques is limited. At present the development of immunoassays for environmental analysis is in its early stage, therefore, a comparison of advantages and disadvantages of the different techniques is irrelevant.

As previously stated the immunoassay techniques do have several limitations (see Table 3.5). The major problem is the false-positive reactions obtained in most assays. A good illustration of this problem is given by Huang and Sedlak (***48***). Sewage effluent was analysed for E2 fractions made by preparative HPLC and it was found that ELISA-signals from interfering compounds corresponded to up to 3 ng/L at retention times where E2 was absent. The problem of false positives is particularly important in relation to the issue of estrogens in the environment because the presence of the substances at concentrations close to or below detection limits are still capable of causing adverse environmental effects.

In the current context, new studies demonstrating the use of the separative power of the immunochemical techniques in sample preparation or chromatography in analytical chemistry should be mentioned (***88;89***). Such techniques have also been presented for the analysis of steroid estrogens in environmental samples (***70;90***). Particularly the two step sample preparation procedure (solid phase extraction – Immunoaffinity extraction) presented by Ferguson et al. (***70***) is promising as it was demonstrated that detection limits in sewage effluents was 0.07 and 0.18 ng/L for E1 and E2 respectively. It is remarkably that these low concentrations were detected on a single-MS system where the sensitivity is relatively low.

3.4.4 Other techniques

In closed experimental systems for investigation of biological and environmental chemical properties of the estrogens e.g. biodegradation or mass balance studies, the use of radio labelled chemicals is an important alternative to the methods described above. The precision and sensitivity of the techniques for analysis of e.g., ^{14}C is at the same level or better than the best GC-MS-MS and LC-MS-MS methods mentioned above. In addition, the need of sample preparation is limited and the workload using these techniques is limited. Therefore the isotope-labelled substances have been used to study the persistence of steroid estrogens in soil (***91;92***) and wastewater (***93***).

The major disadvantage in the use of these techniques is that specific laboratory facilities are needed and that the price of radio labelled chemicals is high.

4 Application of analytical methods for steroid estrogens

This section describes the properties of the different environmental matrices of relevance in this report. The main objective is to identify the problems that should be considered in the analysis of steroid estrogens in different types of environmental samples. A further objective is to recommend which methods should be used for the different purposes.

4.1 Sewage and sewage sludge

In samples of influent, effluent, and sludge from sewage treatment plants (STPs), the concentrations of steroid estrogens are so low that the most sensitive analytical detection techniques are needed. Additionally, complicated sample clean up techniques must be used especially in raw sewage and sewage sludge. Although sewage treatment plants are the main source of pollution with steroid estrogens, they also provide an important process for reduction of the problem. The key for solving the problem is a detailed understanding of the processes which determine the fate of the steroid estrogens in the sewage treatment plant. To achieve this understanding, analytical data of high precision and accuracy are needed. Although many methods exist for analysing sewage-related samples, the combined requirements of high sensitivity, high precision/accuracy and sophisticated sample cleanup techniques necessitate the development of suitable methods for analysing STP products.

Detection of steroid estrogens in samples from sewage effluent has been successfully accomplished by using both GC-MS, GC-MS-MS, and LC-MS-MS (see appendices 1 to 3). As previously mentioned, false positive signals may be detected using these techniques. The combination of LC with MS-MS is the only method suitable for analysing the conjugated metabolites of the steroid estrogens. Cleanup and pre-concentration of the samples can be made using conventional C18 SPE-columns, but often an additional cleaning step using silica gel, etc., is needed in order to remove ionic substances that may interfere with the analysis (e.g., humic acids, etc.). The use of GC based techniques necessitates derivatisation of the analyte due to the low vapour pressure of the steroid estrogens.

Sewage influent has a higher content of particulate matter and other substances which may interfere with the detection of steroid estrogens. Therefore, sample preparation of sewage influent is more cumbersome than preparation of sewage effluent. Generally, the methods presented for sewage influent are the same as for sewage effluent but the number of times the sample can be pre-concentrated is lower due to problems of clogging in the SPE-cartridges. While the presence of conjugated steroid estrogens is low in the sewage effluent due to de-conjugation in the STP, conjugates are expected to occur in sewage influent making the use of LC-MS-MS–methods more appropriate in this case.

Analysis of estrogens in sewage sludge is obviously extremely complicated. Hitherto, only one study has presented a viable analytical method (***26***). In this

case, the sludge is freeze dried and extracted using methanol. The extracts are subsequently cleaned up using gel permeation chromatography and silica gel. Finally, the steroid estrogens are silylated using MSTFA before analysis using GC-MS-MS.

4.2 Surface water and sediment

Surface water typically contains enough particulate matter that filtration of the sample prior to pre-concentration is necessary. The low concentrations of steroid estrogens in surface water (rivers, lakes, sea-water) require sample pre-concentration of a thousand-fold or more prior to analysis which preferably should be made on tandem MS-systems (LC or GC). The pre-concentration is generally achieved using C18 cartridges but graphitized carbon is also an alternative. After pre-concentration, further clean-up procedures using silica gel, etc., may be recommended, but many methods which avoid this step have been presented.

A range of methods has been presented using other detection techniques (UV-detection, Fluorescence, GC- or LC-MS) but these are less sensitive and less reproducible.

Analysis of steroid estrogens in seawater has only been performed in a few cases. Chemically, the major difference between seawater and freshwater is the content of various inorganic salts. Steroid estrogens are expected to occur at lower concentrations in seawater than in freshwater due to greater dilution in seawater. Consequently, the methods used for seawater do not differ from methods used for freshwater.

A few examples of analytical methods for sediments of freshwaters (***26;94-96***) and seawater (***97***) have been presented. All methods use liquid extraction followed by various methods for cleaning up the extract. In the methods for freshwater, the sediment has been freeze dried prior to extraction. Due to the low concentrations, a pre-concentration step is needed before analysis. Both GC and LC coupled to mass spectrometry have been used to detect the steroid estrogens. Although LC-MS has been demonstrated to work, MS-MS is preferred because of the high concentrations of potentially interfering substances that may be present in the sample.

4.3 Manure and soil

Animal excreta come in different forms depending on the animal species, the production methods on the farm and other similar factors. In the current context a division between liquid (urine, water, etc.) and solids (faeces, straw, soil, etc.) is sufficient. Animal excreta contain numerous substances and matrix problems are severe. Although the animal excreta often occur as slurry, the solid phase constitutes a major obstacle for analytical chemists. This problem is predominant for steroid estrogens as a significant portion of the substances are sorbed to the solid phase. Thus, in order to overcome the challenge of analysing steroid estrogens in animal excreta, an efficient method for cleanup and extraction is essential.

The number of reports presenting data on the occurrence of estrogens in manure is limited (***27-29***), and when data are presented, the documentation is sparse. The lack of published data and documented methodology indicates that further development of methods for the detection of steroid estrogens in manure is needed. A number of authors have published methods for the

analysis of antibiotics from manure using liquid-liquid extraction (***98;99***) or lyophilization followed by accelerated solvent extraction (***100***). Although antibiotics are much more hydrophilic, similar methods may be used for steroid estrogens. Despite the difficulties foreseen with regard to extraction and cleanup of the samples, steroid estrogens may occur in these matrices at such high concentrations that less advanced techniques can be used for their detection.

At present, no methods have been presented for the analysis of steroid estrogens in soil. The difficulties that can be foreseen resemble those of analysing sediment and to some extent manure. Therefore it is believed that soil can be analyzed using methods similar to those used for sediment.

4.4 Other matrices

4.4.1 Groundwater

Groundwater is considered to be a clean matrix; therefore, the major problem encountered in analysing for steroid estrogens in groundwater is the low concentrations that are expected. Analytically, the difference from surface water analysis is insignificant. Therefore, the most sensitive methods should be used, implying the need for thorough pre-concentration and sophisticated instruments (MS-MS-techniques).

4.4.2 Bank filtration, septic tanks etc.

The presence of estrogens in drainage water and water influenced by release from scattered settlements have not been reported, but exposure routes shown in Figures 2.2 and 2.3 imply that estrogens (both E1, E2 and EE2) may be present in samples from such sources. Existing analytical methods provide an adequate starting point for the development of methods for analysing these matrices.

5 Recommendations, Perspectives and Conclusions

Analysis of steroid estrogens in environmental samples is a major challenge for analytical chemists and several aspects of this field are still considered unsolved. At present a myriad of instruments and techniques is available for the analysis of steroid estrogens. The selection of the instrument which is optimal for the purpose is problematic. It is important to realize that the selection of an optimal method is highly dependent on the type of matrix that is analyzed. Therefore, in selecting the correct method, a number of criteria should be considered.
The origin of the sample is crucial in determining which of these criteria are most critical. In most cases, however, sensitivity is the limiting factor in the development of these analytical methods. While little discussion is needed regarding the selection of sample preparation techniques for improving sensitivity, the many alternatives for the choice of analytical detection methods makes the overview more complicated.

In a typical case the anticipated concentration level of estrogens in a given sample will force one to use a specific type of instrument and analytical method simply because of a required LOD, or LOQ. The choice of instruments and methods will again force one to define a number of specific validation criteria in order to validate the applied analytical method. Recall that in chapter 2 we gave an overview of the estrogen concentration level that can be expected in the different types of environmental matrices. In Table 5.1 we repeat the major figures.

Table 5.1:
Overview of typical concentration levels given in table 2.2 together with most applied extraction techniques and clean-up procedures proposed in the literature for the different matrices.

Matrix	Typical levels of estrogens in samples (ng/L or ng/g dwt)	Often applied extraction technique	Clean – up procedure
Surface water (river water, reservoir, ground water etc.), (ng/L)	0.01 – 5	Filtering, SPE	C18 or graphitized carbon black adsorbents
Sediment (ng/kg)	0.7 – 40	Freeze drying, PLE, SPE, GPC	C18 or graphitized carbon black adsorbents
STP (ng/L)		Filtering, SPE, GPC or HPLC fractionation	C18 or graphitized carbon black adsorbents
effluent	– 80		
influent	0.1 – 500		
Sludge (ng/L)	<LOD to 50	Freeze drying, PLE, SPE, GPC or HPLC fractionation	C18 or graphitized carbon black adsorbents
Run off samples from soil (ng/L)	<LOD to 2600	Freeze drying, PLE, SPE, GPC or HPLC fractionation	C18 or graphitized carbon black adsorbents
Manure (ng/g dwt)	<LOD to 1300	Freeze drying, PLE, SPE, GPC or HPLC fractionation	C18 or graphitized carbon black adsorbents

SPE = solids phase extraction, PLE = pressurized liquid extraction, GPC = gel permeation chromatography.

5.1 Sample preservation, extraction and clean-up

An effective sample clean-up procedure and pre-concentration technique may allow the use of less sensitive analytical methods and equipment. Table 5.1 outlines the different primary extraction techniques and clean-up procedures presented in literature for the discussed matrices. Matrices such as sludge, soil, manure and influent waste water require more effort in sample clean-up due to the possibility of matrix interference than do surface water or ground water. A comprehensive clean-up may thus allow the use of less sensitive detection methods if the criteria for variability and selectivity of the method are fulfilled (see below). Conversely, a reduction in sample preparation effort will require the use of analytical methods having a high selectivity. The choice of methods depends highly on the available expertise in a particular laboratory because sample extraction and clean-up is often expert work.
Appropriate sample preservation is highly important. The best storage suggested in the current literature consists of passing the field samples through the extraction cartridge, washing the cartridge with methanol, and storing the cartridge at -18°C. Under these conditions no loss was observed after storage for 60 days. Preservation with methanol is not recommended.

5.2 Analytical equipment

Several different analytical detection methods have been suggested in the literature for analysing estrogens in the environment. Table 5.2 gives some recommendations and limitations when using the different methods. UV, GC-FID and HPLC are not included in Table 5.2 as we generally do not recommend the use of these methods due to low sensitivity and selectivity. GC or LC hyphenated with single MS or the use of immunochemical techniques is the minimum necessary for providing sufficiently high quality results.
Table 5.2 furthermore shows, (which may be a little bit confusing to understand), that nearly all methods may be used for analysing the estrogens in the different matrices. As seen from the table, LC-MS-MS or GC-MS-MS are the analytical techniques that provide the highest sensitivity (lowest LOD or LOQ). But both GC-MS and LC-MS may also be used even for very complicated matrices, if certain identified quality criteria are fulfilled. The use of single MS requires verification of the selectivity of the method following selected criteria. Combined criteria such as: a) matching of retention time within 0.02 min. of values obtained from analysis of certified standards, b) presence of the molecular ion of the target compound, c) presence of at least two additional qualifiers ions (at least one of which was a fragment of the parent compound structure) and, d) matching of ion ratios within 50% for the two qualifier ions, may increase the selectivity of the method.Increased selectivity combined with proper sample preparation could potentially match the performance of the triple quadrupole systems in terms of variability and sensitivity. Deuterated surrogate standards should always be used as internal standards and added before derivatisation. The above procedure is consistent with that used by laboratories at the National Institute for Drug Abuse.

Table 5.2:
Ranking of analytical techniques after sensitivity, variability and sensitivity applicable for the different matrices assumed that the optimal sample preparation have been made.

Matrix	GC-MS	GC-MS-MS	LC-MS	LC-MS-MS	Immuno-techniques
Levels of LOD reported in the literature (ng/L)*	0.3-2	0.1 – 2	1-5	0.1 – 0.5	0.05-850
Surface water (river water, recevoir, ground water etc.)	(+)	+	(+)	+	(++)
STP	(+)	+	(+)	+	(++)
effluent					
influent					
Sludge	(+)	+	(+)	+	(++)
Soil	(+)	+	(+)	+	(++)
Manure	(+)	+	(+)	+	(++)

+ = may be applied if LOD, LOQ criteria are fulfilled
(+) = may be applied if selectivity problems is solved by definition of criteria and if LOD, LOQ criteria is fulfilled.
(++) = may be applied if selectivity problems are solved by definition of criteria and awareness of falls positive.
UV, HPLC, GC-FID etc are irrelevant in this contends due to low selectivity and improper selectivity.
*Data reported in appendices 1 to 4.

5.2.1 GC-MS or LC-MS versus GC-MS-MS or LC-MS-MS methods

As shown in Table 5.2 both the single MS techniques and triple quadrupole and ion trap MS may be used if appropriate validation is performed. An often asked question is therefore, what is gained, other than enhanced sensitivity, by using the MS-MS based techniques in place of single MS. MS-MS techniques also improve the selectivity. Ternes et al. (***18***) presented EI spectra (Figure 5.1) of EE2 and an unknown impurity illustrating the problem. Both compounds exhibited exactly the same retention time and both EI spectra showed the m/z values of 440 (molecular weight of silylated 17α-ethinylestradiol) and 425 (Mw minus CH_3), however with a different ratio. Using MS-MS –detection (two lowest chromatograms on the figure) of the target ion m/z 425, a confirmation with regard to identification and quantification of EE2 can be carried out. Due to the fact that the MS-MS-spectra of the contraceptive and the unknown impurity are different, a precise quantification is possible using the product ions m/z 193 and m/z 231 of the precursor ion m/z 425. For the unknown compound these two precursor ions were not formed showing that this unknown compound was not 17α-ethinylestradiol, but an impurity that co-eluted. Using single MS detection it would not have been possible to distinguish between the two compounds.

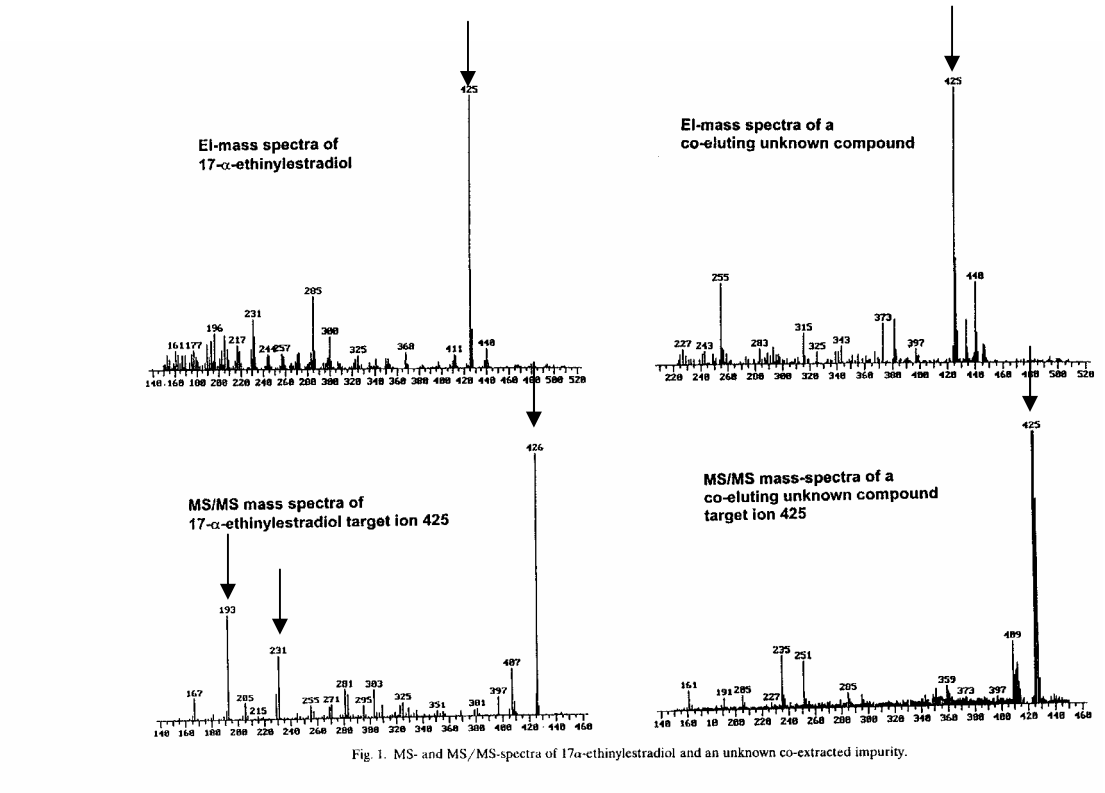


Fig. 1. MS- and MS/MS-spectra of 17α-ethinylestradiol and an unknown co-extracted impurity.

Figure 5.1:
MS – and MS-MS spectra of 17 α-ethinylestradiol and an unknown co-extracted impurity (figure reproduced after Figure 1 in reference (*18*)).

5.2.2 Comparison of LC or GC methods

In terms of accuracy and repeatability, LC-MS, GC-MS-MS and LC-MS-MS are in general satisfactory, although the derivatisation step used prior to gas chromatography, in addition to being time-consuming, can constitute a source of inaccuracy. An advantage of GC-MS compared with LC-MS, is the availability of external libraries of mass spectra which are useful for the identification of unknown peaks in estrogenically active fractions. The recent introduction of the tandem mass spectrometry detection, has substantially improved the performance of chromatographic methods by reducing detection limits and aiding analyte identification.

Future development of equipment favours the choice of LC based methods as a strategy for analysing estrogens. The same situation that occurred in pesticide analysis may be repeated in this field. Five years ago many pesticide analyses were performed with GC based techniques, but the LC-MS-MS are now so sensitive that they rival the GC based techniques in selectivity and sensitivity. Also, new ionisation techniques such as the APPI and others have been developed for several types of highly sensitive triple quadrupole instruments. These developments may cause the LC-MS-MS to become the first choice analytical method due to increased sensitivity and higher selectivity.

5.2.3 Immunochemical techniques

Immunochemical methods provide very sensitive methods, especially for waste water and STP effluents, but the selectivity is poor compared with the triple quadrupole instruments. Highly polluted samples should generally be avoided due to adsorption to binding sites. These methods are also subject to

problems with low selectivity and false positive samples. Variability is also a severe problem. Methods for the conjugated estrogens are also on the market, but suffer from the same problems as the methods available for parent estrogens. Immunochemical methods are not recommended as a stand alone "analytical tool". Immunochemical methods have the potential to provide useful data when used in conjunction with chemical analysis. Today such strategies are not developed and research is needed to develop an appropriate strategy combining such methods with LC-MS-MS or GC-MS-MS or single MS.

5.2.4 Comparison of methods

Figure 5.2 A to D gives an overview that ranks the analytical methods according to the four most important parameters (A:sensitivity, B:variability, C:selectivity and D:investment costs for the purchase of analytical instruments) to be used in selecting adequate methods for analysing estrogens in matrices. These rankings anticipate that the highest possible clean-up is performed so matrix interference is reduced as much as possible.

5.2.4.1 Sensitivity

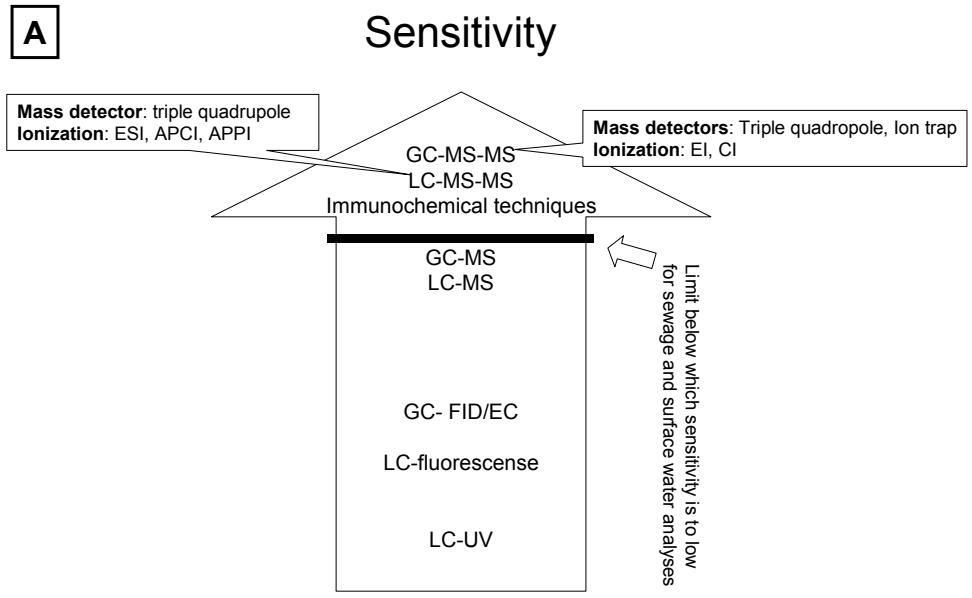
From a sensitivity point of view the triple quadrupole instruments such as GC-MS-MS and LC-MS-MS are superior to all other instruments. Furthermore, new modes of ionisation and sample handling which are on the way to market, may improve the sensitivity even more. One example is the newly developed APPI ionisation technique (see section 3.4.2.3). But other ionisation techniques such as both ESI and APCI are also adequate for use. 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 0.1 ng/L is required further research is needed. LC-APPI-MS-MS seems superior to other ionisation methods (e.g. ESI or APCI), but it is still not very well documented. Single GC-MS and LC-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. Immunochemical methods are very sensitive, but care must be taken to recognize false positive samples.

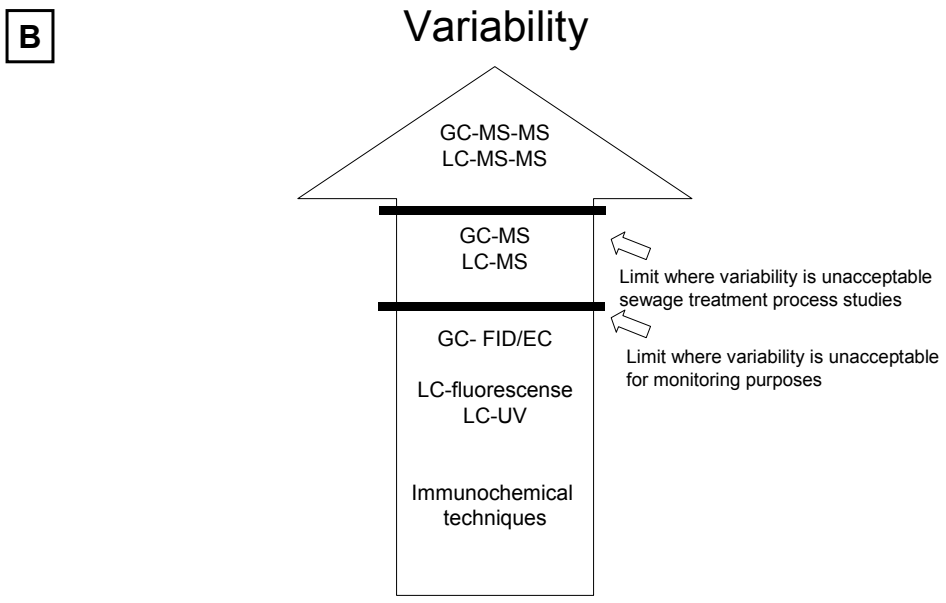
5.2.4.2 Variability

As with the parameter "sensitivity", "variability" is much improved when the triple quadrupole instruments are used. In fact, a cut-off line has been drawn below the GC-MS-MS and LC-MS-MS instruments that excludes the other methods from use in sewage treatment process studies (see Figure 5.2B). Both single GC-MS and LC-MS may be applied from a variability point of view for monitoring purposes. GC-FID/EC, LC-fluorescence, LC-UV and immunochemical techniques are all inadequate from a variability viewpoint.

5.2.4.3 Selectivity

Highest selectivity is obtained (see section 5.2.1) using the triple quadrupole Instruments, and these instruments are recommended for all analyses of estrogens in manure, soil and sludge. Figure 5.2C shows that single MS instruments are sufficiently selective if the previously discussed selective criteria are fulfilled for the applied method (see above). With single MS selectivity problems are severe but may be managed. Again, GC-FID/EC, LC-fluorescence, LC-UV and immunochemical techniques are inadequate if high selectivity is required. The immunochemical methods have selectivity problems that might not be solvable.


A
Sensitivity
Mass detector: triple quadrupole
Ionization: ESI, APCI, APPI
Mass detectors: Triple quadropole, Ion trap
Ionization: EI, CI
GC-MS-MS
LC-MS-MS
Immunochemical techniques
GC-MS
LC-MS
GC- FID/EC
LC-fluorescense
LC-UV
Limit below which sensitivity is to low for sewage and surface water analyses


B
Variability
GC-MS-MS
LC-MS-MS
GC-MS
LC-MS
Limit where variability is unacceptable sewage treatment process studies
GC- FID/EC
LC-fluorescense
LC-UV
Limit where variability is unacceptable for monitoring purposes
Immunochemical techniques

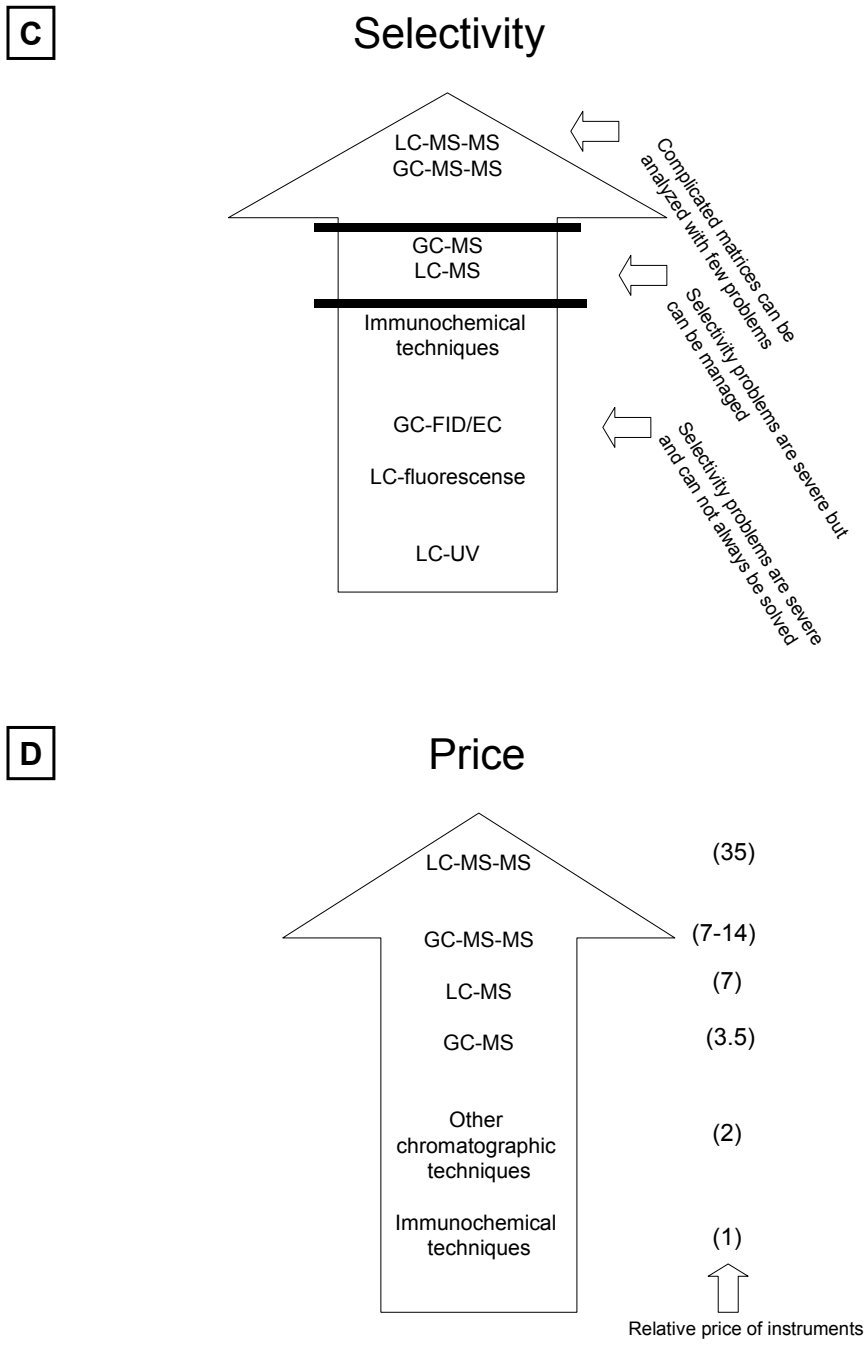


Figure 5.2 A to D:
Overview that ranks the analytical methods according to the four most important parameters (sensitivity, variability, selectivity and price) to be used in selecting adequate methods for analysing estrogens in matrices.

5.2.4.4 Costs

The purchase prices of the different instruments are ranked on Figure 5.2D. Price is often an important criterion when selecting an analysis strategy and must be taken into account when selecting the optimal method. One strategy is to place the costs on a high degree of sample clean-up; another is to purchase a sensitive, but often expensive instrument.

The immunochemical techniques are the lowest in cost and are 35 times less expensive than the most advanced LC-MS-MS. GC-MS-MS is less

expensive than LC-MS-MS. Single GC-MS is half the price of single LC-MS. Other chromatographic techniques which are often not recommend for use, are all lower in cost. Sample preparation is a crucial step and its cost must be considered. Sample preparation costs vary widely between the different matrices. The least expensive are surface water/ground water samples, whereas, sample preparation for STP influent, manure or soil will probably be a factor of 4 more expensive than the surface water/ground water samples. Sample preparation for effluents from STP will probably be a factor of 2 more expensive than the surface water/ground water samples.

Table 5.3 attempts to recommend preferred analytical methods for estrogen analysis of effluent waste water or surface water, depending on the expected concentration levels in the samples. These recommendations are made taking into account the above discussion of selectivity, sensitivity, variability and cost. If required LOD levels are below 0.1 ng/L estrogen (E1, E2 or EE2) (e.g. LOEC concentrations), no method functions today without research. LODs at the level of 0.1 to 5 ng/L estrogen may be reached with all triple quadrupole and ion trap instruments. As indicated above, proper sample preparation techniques can raise the selectivity and sensitivity of single MS methods, allowing these methods to be used at levels much lower than the 20 ng/L listed in Table 5.3. Without using extra validation criteria, however, single MS instruments should be used with great care below 20 ng/L.

Table 5.3:
Recommendation of selected methods for analysing treated STP effluent / River water depended on estrogen levels in the sample.

Estrogen conc. levels	Recommended methods
< 0.1 ng/L	LC-MS-MS plus research in sample preparation and clean-up. GC-MS-MS plus research in sample preparation and clean up.
0.1 – 5 ng/L	LC-MS-MS (trip quad)
	GC-MS-MS (trip quad or ion-trap)
≈ 20 ng/L	High amount of samples; Recommend a combination of GC-MS, LC-MS
	And Immunochemical methods

5.2.5 Future perspectives

The recent introduction of the tandem mass spectrometry detection instruments, LC-MS-MS or GC-MS-MS (first on the market and better documented), has substantially improved the performance of chromatographic methods by reducing detection limits and aiding analyte identification. The next few years will no doubt see the general application of these advanced techniques, integrated into completely automated, on-line systems. These integrated systems will improve analytical performance (analyse traceability, reliability, and repeatability), increase sample throughput, and reduce operating costs and contamination risks. 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 currently under development can be expected in the near future. These advances promise to greatly simplify the detection and measurement of these important environmental pollutants in environmental matrices. The introduction of biosensors, most of which are still in the prototype phase, will provide another promising alternative to traditional methods for the field monitoring of estrogenic compounds.

6 References

1. Tyler CR, Routledge EJ. Natural and anthropogenic environmental oestrogens: the scientific basis for risk assessment - Oestrogenic effects in fish in English rivers with evidence of their causation. Pure and Applied Chemistry 70:1795-1804 (1998).

2. Routledge EJ, Sheahan DA, Desbrow C, Brighty GC, Waldock M, Sumpter JP. Identification of Estrogenic Chemicals in STW Effluent. 2. In Vivo Responses in Trout and Roach. Environ Sci Technol 32:1559-1565 (1998).

3. Bachmann Christensen L, Winther-Nielsen M, Helweg C. Feminisation of fish - The effect of estrogenic compounds and their fate in sewage treatment plant and nature. 729: (2002).

4. Panter GH, Thompson RS, Beresford N, Sumpter JP. Transformation of a non-oestrogenic steroid metabolite to an oestrogenically active substance by minimal bacterial activity. Chemosphere 38:3579-3596 (1999).

5. Ritter JK. Roles of glucuronidation and UDP-glucuronosyltransferases in xenobiotic bioactivation reactions. Chemico-Biological Interactions 129:1-208 (2000).

6. Ternes TA, Kreckel P, Mueller J. Behaviour and occurrence of estrogens in municipal sewage treatment plants - II. Aerobic batch experiments with activated sludge. Sci Tot Env 225:91-99 (1999).

7. Lee HB, Liu D. Degradation of 17ß-Estradiol and its Metabolites by Sewage Bacteria. Wat , Air Soil Poll 134:351-366 (2002).

8. Belfroid AC, Horst AVd, Vethaak AD, Schäfer AJ, Rijs GBj, Wegener J, Cofino WP. Analysis and occurrence of Estrogenic hormones and their glucuronides in surface water and waste water in The Netherlands. Sci Tot Env 225:101-108 (1999).

9. Christensen FM. Pharmaceuticals in the Environment - A Human Risk. Reg Tox Pharm 28:212-221 (1998).

10. Shore LS, Shemesh M. Naturally Produced Steroid Hormones and their Release into the Environment. Pure and Applied Chemistry. (In Press).

11. Johnson AC, Williams RJ, Ulahannan T. Comment on "Identification of Estrogenic Chemicals in STW Effluent. 1. Chemical Fractionation and in Vitro Biological Screening". Environ Sci Technol 33:369-370 (1999).

12. Williams CL, Stancel GM. Estrogens and Progestins. **Eds.:**Hardman, J. G., Goodman Gilman, A., and Limbird, L. E. 9: (1996).

13. Arcand-Hoy LD, Nimrod AC, Benson WH. Endocrine-modulating Substances in the Environment: Estrogenic Effects of Pharmaceutical Products. International Journal of Toxicology 17:139-158 (1998).

14. Johnson AC, Belfroid AC, Corcia AD. Estimating steroid oestrogen inputs into activated sludge treatment works and observations on their removal from the effluent. Sci Tot Env 256:163-173 (2000).

15. Lopez de Alda MJ, Barcelo D. Review of analytical methods for the determination of estrogens and progestogens in waste waters. Fresenius J Anal Chem 371:437-447 (2001).

16. Spengler W, Körner W, Metzger M. Substances with estrogenic activity in effluents of sewage treatment plants in southwestern Germany. 1. Chemical analysis. Environ Toxicol Chem 20:2133-2141 (2001).

17. Desbrow C, Routledge EJ, Brighty GC, Sumpter JP, Waldock M. Identification of Estrogenic Chemicals in STW Effluent. 1. Chemical Fractionation and in Vitro Biological Screening. Environ Sci Technol 32:1549-1557 (1998).

18. Ternes TA, Stumpf M, Mueller J, Haberer K, Wilken RD, Servos M. Behaviour and occurrence of estrogens in municipal sewage treatment plants - I. Investigations in Germany, Canada and Brazil. Sci Tot Env 225:81-90 (1999).

19. Ying GG, Kookana RS, Ru YJ. Occurrence and fate of hormone steroids in the environment. Env Int 28:545-551 (2002).

20. Williams RJ, Johnson AC, Smith JJL, Kanda R. Steroid Estrogen Profiles along River Stretches Arising from Sewage Treatment Works Discharges. Environ Sci Technol 37:1744-1750 (2003).

21. Andersen HR, Siegrist H, Halling-Sorensen B, Ternes TA. Fate of Estrogens in a Municipal Sewage Treatment Plant. Environ Sci Technol. (In Press).

22. Williams RJ, Jürgens MD, Johnson AC. Initial Predictions of the Concentrations and Distribution of 17β-oestradiol, oestrone and ethinyl oestradiol in 3 English Rivers. Wat Res 33:1663-1671 (1999).

23. Busch K, Hegemann W, Spengler P, Metzger JW. Influence of Different Process Techniques in Sewage Plants to Eliminate Selected Endocrine Disruptors. (2002).

24. Solé M, López de Alda MJ, Castillo M, Porte C, Ladegaard-Pedersen K, Barceló D. Estrogenicity Determination in Sewage Treatment Plants and Surface Waters from the Catalonian Area (NE Spain). Environ Sci Technol 34:5076-5083 (2000).

25. Baronti C, Curini R, D'Ascenzo G, Corcia AD, Gentili A, Samperi R. Monitoring Natural and Synthetic Estrogens at Activated Sludge Sewage Treatment Plants and in a Receiving River Water. Environ Sci Technol 34:5059-5066 (2000).

26. Ternes TA, Andersen HR, Gilberg D, Bonerz M. Determination of estrogens in sludge and sediments by liquid extraction and GC/MS/MS. Anal Chem 74:3498-3505 (2002).

27. Okkerman PC, Groshart CP, Pijnenburg AMCM. Chemical study on estrogens. report no. 2001.028. National Institute for Coastal and Marine Management (RIKZ), The Hague, the Netherlands (2003).

28. Vethaak AD, Rijs GBj, Schrap SM, Ruiter H, Gerritsen A, Lahr J. Estrogens and xeno-estrogens in the aquatic environment of the Netherlands. report no 2002.001. Institute of Inland Water Management and Waste Water Management and Waster Water Treatment (RIZA) and the Dutch Institute for Coastal and Marine Management (RIKZ), Lelystad, the Netherlands (2002).

29. Wenzel A, Küchler T, Henschel K-P, Schnaak W, Diedrich M, Müller J. Konzentrationen östrogen wirkender Substanzen in Umweltmedien. Forschungsbericht 216 02 011/11: German Federal Environmental Agency, Berlin (1998).

30. Finlay-Moore O, Hartel PG, Cabrera ML. 17β-Estradiol and Testosterone in Soil and Runoff from Grasslands Amended with Broiler Litter. J Environ Qual 29:1604-1611 (2000).

31. Peterson EW, Davis RK, Orndorff HA. 17β-Estradiol as and Indicator of Animal Waste Contamination in Mantled Karst Aquifers. J Environ Qual 29:826-834 (2000).

32. Damgaard Poulsen H, Friis Kristensen V. Normtal for husdyrgødning, En revurdering af danske normtal for husdyrgødningens indhold af kvælstof, fosfor og kalium. Rapport no. 736. Danmarks Jordbrugsforskning, Ministeriet for Fødevarer, Landbrug og Fiskeri (1997).

33. Brown RP, Greer RD, Mihaich EM, Guiney PD. A Critical Review of the Scientific Literature on Potential Endocrine-Mediated Effects in Fish and Wildlife. Ecotoxicol Environ Saf 49:17-25 (2001).

34. Sumpter JP, Jobling S. Vitellogenesis as a Biomarker for Estrogenic Contamination of the Aquatic Environment. Env Health Persp 103:173-178 (1995).

35. Technical Guidance Document in Support of the Commission Directive 93/67/EEC on Risk Assessment for New Notified Substances and the Commision Regulation (EC) 1488/94 on Risk Assessment for Existing Substances. The European Commision, Brussels, Belgium (1996).

36. Andersen HR, Ternes TA, Halling-Sørensen B. Steroidøstrogener i renset spildevand kan være årsag til effekter på danske fisk. Dansk Kemi 83:28-31 (2002).

37. Metcalfe CD, Metcalfe TL, Kiparissis Y, Koenig BG, Khan C, Hughes RJ, Croley TR, March RE, Potter T. Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by in vivo assays with Japanese medaka (Oryzias latipes). Environ Toxicol Chem 20:297-308 (2001).

38. Miles-Richardson SR, Kramer VJ, Fitzgerald SD, Render JA, Yamini B, Barbee SJ, Giesy JP. Effects of Waterborne exposure of 17 β-estradiol on secondary sex characteristics and gonads of fathead minnows (***Pimephales promelas***). Aquatic Toxicology 47:129-145 (1999).

39. Shore LS, Kapulnik Y, Ben-Dov B, Fridman Y, Wininger S, Shemesh M. Effects of estrone on 17β-estradiol on vegetative growth of ***Medicago sativa***. Physiologia Plantarium 84:217-222 (1992).

40. Thompson M, Ellison SLR, Wood R. Harmonized guidelines for single-laboratory validation of methods of analysis. Pure and Applied Chemistry 74:835-855 (2002).

41. Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, Buxton HT. Pharmaceuticals, Hormones, and Other Organic Wastewater Contaminants in U.S. Streams, 1999-2000: A National Reconnaissance. Environ Sci Technol 36:1202-1211 (2002).

42. Ericson JF, Laenge R, Sullivan DE. Comment on "Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: A national reconnaissance". Environmental Science & Technology 36:4005-4006 (2002).

43. Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, Buxton HT. Response to Comment on,"Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: A national reconnaissance". Environmental Science & Technology 36:4007-4008 (2002).

44. Geiss S, Einax JW. Comparison of detection limits in environmental analysis - is it possible? An approach on quality assurance in the lower working range by verification. Fresenius J Anal Chem 370:673-678 (2001).

45. Ingerslev F, Andersen HR, Hansen M, Jakobsen AM, Andersen Krogh K, Søeborg T, Halling-Sørensen B En præliminær kortlægning af steroidøstrogenudledningen fra danske rensningsanlæg. Dansk Kemi 84:46-49 (2003).

46. Shore LS, Gurevitz M, Shemesh M. Estrogen as an Environmental Pollutant. Bull Environ Contam Toxicol 51:361-366 (1993).

47. Tabak HR, Bloomhuff RN, Bunch RL. Steroid Hormones as Water Pollutants II. Studies on the Persistence and Stability of Natural Urinary and Synthetic Ovulation-Inhibiting Hormones in Untreated and Treated Wastewaters. Dev Ind Microbiol 22:497-519 (1981).

48. Huang C-H, Sedlak D. Analysis of Estrogenic Hormones in Municipal Wastewater Effluent and Surface Water Using Enzyme-linked Immunosorbent Assay and Gas Chromatography/Tandem Mass Spectrometry. Environ Toxicol Chem 20:133-139 (2001).

49. Gentili A, Perret D, Marchese S, Mastropasqua R, Curini R, Di Corsia A. Analysis of estrogens and their Conjugates in Sewage and River Waters by Solid-Phase Extraction then Liquid

Chromatography-Electronspray-Tandem Mass Spectrometry. Chromatographia 56:25-32 (2002).

50. Andersen HR, Siegrist H, Halling-Sorensen B, Ternes TA. Fate of Estrogens in a Municipal Sewage Treatment Plant. Environ Sci Techn. (In Press).

51. Majima K, Fukui T, Yuan J, Wang G, Matsumoto K. Quantitative Measurement of 17β-estradiol and Estriol in River Water by Time-Resolved Fluoroimmunoassay. Analytical Sciences 18:869-874 (2002).

52. Shimada K, Mitamura K, Higashi T. Gas chromatography and high-performance liquid chromatography of natural steroids. J Chromatogr A 935:141-172 (2001).

53. Barber LB, Brown GK, Zaugg SD. ACS symposium series 747:97-123 American Chemical Society, Washington (2000).

54. Kelly K. Analysis of Steroids in environmental water samples using solids-phase extraction and ion-trap gas chromatography-mass spectrometry and gas chromatography-tandem mass spectrometry. J Chromatogr A 872:309-314 (2000).

55. Mol HGJ, Sunarto S, Steijger OM. Determination of endocrine disruptors in water after derivatization with N-methyl-N(***tert.***-butyldimethyltrifuoroacetamide) using gas chromatography with mass spectrometric detection. J Chromatogr A 879:97-112 (2000).

56. Nakamura S, Sian TH, Daishima S. Determination of estrogens in river water by gas chromatography-negative-ion chemical-ionization mass spectrometry. J Chromatogr A 919:275-282 (2001).

57. Lagana A, Bacaloni A, Fago G, Marino A. Trace analysis of estrogenic chemicals in sewage effluent using liquid chromatography combined with tandem mass spectrometry. Rapid Communications in Mass Spectrometry 14:401-407 (2000).

58. Kuch HM, Ballschmiter K. Determination of endogenous and exogenous estrogens in effluents from sewage treatment plants at the ng/L-level. Fresenius J Anal Chem 366:392-395 (2000).

59. Larsson DGJ, Adolfsson-Erici M, Parkkonen J, Pettersson M, Berg AH, Olsson P-E, Förlin L. Ethinylestradiol - an undesired fish contraceptive? Aquatic Toxicology 45:91-97 (1999).

60. Xiao XY, McCalley D. Quantitative analysis of estrogens in human urine using gas chromatography/negative chemical ionisation mass spectrometry. Rapid Communications in Mass Spectrometry 14:1991-2001 (2000).

61. Lerch O, Zinn P. Derivatisation and gas chromatography-chemical ionisation mass spectrometry of selected synthetic and natural endocrine disruptive chemicals. J Chromatogr A 991:77-97 (2003).

62. Isobe T, Shiraishi H, Yasuda M, Shinoda A, Suzuki H, Morita M. Determination of estrogens and their conjugates in water using solid-

phase extraction followed by liquid chromatography-tandem mass spectrometry. J Chromatogr A 984:195-202 (2003).

63. Lagana A, Fago G, Marino A, Santarelli D. Liquid chromatography tandem mass spectrometry applied to the analysis of natural and synthetic steroids in environmental waters. Analytical Letters 34:913-926 (2001).

64. Lopez de Alda MJ, Gil A, Paz E, Barceló D. Determination of estrogens and progestogens in river sediments by liquid chromatography/mass spectrometry. The Analyst 127:1299-1304 (2002).

65. Snyder SA, Keith TL, Verbrugge DA, Snyder EM, Gross TS, Kannan K, Giesy JP. Analytical methods for Detection of Selected Estrogenic Compounds in Aqueous Mixtures. Environ Sci Technol 33:2814-2820 (1999).

66. Ying GG, Kookana RS, Chen J. On-Line Solid Phase Extraction and Fluorescence Detection of Selected Endocrine Disrupting Chemicals in Water By High-Performance Liquid Chromatography. Journal of environmental Science B37:225 (2003).

67. de Alda MJL, Barcelo D. Determination of steroid sex hormones and related synthetic compounds considered as endocrine disrupters in water by fully automated on-line solid-phase extraction-liquid chromatography-diode array detection. J Chromatogr A 911:203-210 (2001).

68. Penalver A, Pocurull E, Borrull F, Marce RM. Method based on solid-phase microextraction-high-performance liquid chromatography with UV and electrochemical detection to determine estrogenic compounds in water samples. J Chromatogr A 964:153-160 (2002).

69. D'Ascenzo G, Corcia AD, Gentili A, Mancini R, Mastropasqua R, Nazzari M, Samperi R. Fate of natural estrogen conjugates in municipal sewage transport and treatment facilities. Sci Tot Env 302:199-209 (2003).

70. Ferguson PL, Iden CR, McElroy AE, Brownawell BJ. Determination of steroid estrogens in wastewater by immunoaffinity extraction coupled with HPLC-electrospray-MS. Anal-Chem 73:3890-3895 (2001).

71. Robb DB, Covey TR, Bruins AP. Atmospheric pressure photoionisation: An ionization method for liquid chromatography-mass spectrometry. Anal Chem 72:3653-3659 (2000).

72. Alary J-F. Comparative Study: LC-MS/MS Analysis of Four Steroids Compounds Using a New Photoionization Source and a Conventional APCI Source. Proceedings of the 49th ASMS Conference on Mass Spectrometry and Allied Topics, May 27-31, 2001, Chicago, Illinois (2001).

73. Alary J-F, Berthemy A, Tuong A, Uzabiaga M-F. Comparative LC-MS/MS Analysis of Four Neurosteroids Compounds and their Acetyl-PentaBenzyl Derivatives Using a Photoionization Ion Source and a

Conventional Atmospheric-Pressure Chemical Ionization Source. Proceedings of the 49th ASMS Conference on Mass Spectrometry and Allied Topics, May 27-31, 2001, Chicago, Illinois (2001).

74. Benijts T, Dams R, Gunther W, Lambert W, De Leenheer A. Analysis of estrogenic contaminants in river water using liquid chromatography coupled to ion trap based mass spectrometry. Rapid Communications in Mass Spectrometry 16:1358-1364 (2002).

75. de Alda MJL, Barcelo D. Determination of steroid sex hormones and related synthetic compounds considered as endocrine disrupters in water by liquid chromatography-diode array detection-mass spectrometry. J Chromatogr A 892:391-406 (2000).

76. Emon JMV, Gerlach CL. Environmental monitoring and human exposure assessment using immunochemical techniques. Journal of Microbiological Methods 32:121-131 (1998).

77. Vanderlaan M, Watkins BE, Stanker L. Environmental Monitoring by immunoassay. Environ Sci Technol 22:247-254 (1988).

78. Sherry J. Environmental Immunoassays and Other Bioanalytical Methods: Overview and Update. Chemosphere 34:1011-1025 (1997).

79. Luppa PB, Sokoll LJ, Chan DW. Immunosensors-principles and applications to clinical chemistry. Clinica Chimica Acta 314:1-26 (2001).

80. Diamandis EP, Christopoulus TK. Immunoassay. Academic Press (2003).

81. Aherne GW, English J, Marks V. The role of Immunoassay in the Analysis of Microcontaminants in Water Samples. Ecotoxicol Environ Saf 9:79-83 (1985).

82. Aherne GW, Briggs R. The Relevance of the presence of certain synthetic steroids in the aquatic environment. J Pharm Pharmacol 41:735-736 (1989).

83. Svenstrup B. Personal communication. Statens Seruminstitut Copenhagen Denmark.

84. Giese RW. Measurement of endogenous estrogens: analytical challenges and recent advances. J Chromatogr A 1000:401-412 (2003).

85. Snyder SA, Villeneuve DL, Snyder EM, Giesy JP. Identification and quantification of Estrogen Receptor Agonists in Wastewater effluents. Environ Sci Technol 35:3620-3526 (2001).

86. Japan EnviroChemicals. Estrogen (E1/E2/E3) ELISA Kit; 17ß-Estradiol (E2) ELISA Kit. Japan EnviroChemicals, Ltd., 3-8 Doshomachi, 2-chome, Chuo-ku, Osaka City, Japan.

87. Biosense. Prod. no. E60000401, E61000401, E62000401, E63000401. Biosense Laboratories AS, Thormøhlensgt. 55, N-5008 Bergen, Norway.

88. Rhemrev-Boom MM, Yates M, Rudolph M, Raedts M. (Immuno)affinity chromatography: a versatile tool for fast and selective purification, concentration, isolation and analysis. Journal of Pharmaceutical and Biomedical Analysis 24:825-833 (2001).

89. Hage DS, Nelson MA. Chromatographic Immunoassays. Anal Chem 73:198A-205A (2001).

90. Nishiyama S, Goto A, Saito K, Sugita K, Tamada M, Sugo T, Funami T, Goda Y, Fujimoto S. Concentration of 17 beta-estradiol using an immunoaffinity porous hollow-fiber membrane. Anal Chem 74:4933-4936 (2002).

91. Colucci MS, Bork H, Topp E. Persistence of Estrogenic Hormones in Agricultural Soil: I. 17β-Estradiol and Estrone. J Environ Qual 30:2070-2076 (2001).

92. Colucci MS, Topp E. Persistence of Estrogenic Hormones in Agricultural Soil: II. 17α-Ethynylestradiol. J Environ Qual 30:2077-2080 (2001).

93. Layton AC, Gregory BW, Seward JR, Schultz TW, Sayler GS. Mineralization of Steroidal Hormones by Biosolids in Wastewater Treatment Systems in Tennessee U.S.A. Environ Sci Technol 34:3925-3931 (2000).

94. de Alda MJL, Barcelo D. Use of solid-phase extraction in various of its modalities for sample preparation in the determination of estrogens and progestogens in sediment and water. J Chromatogr A 938:145-153 (2001).

95. Lopez de Alda MJ, Gil A, Paz E, Barcelo D. Occurrence and analysis of estrogens and progestognes in river sediments by liquid chromatography-electrospray-mass spectrometry. Analyst 127:1299-1304 (2002).

96. Petrovic M, Tavazzi S, Barcelo D. Column-switching system with restricted access pre-column packing for an integrated sample cleanup and liquid chromatographic-mass spectrometric analysis of alkylphenolic compounds and steroid sex hormones in sediment. J Chromatogr A 971:37-45 (2002).

97. Thomas KV, Hurst MR, Matthiessen P, Waldock M. Characterization of estrogenic compounds in water samples collected from United Kingdom Estuaries. Environ Toxicol Chem 20:2165-2170 (2001).

98. Haller MY, Müller SR, McArdell CS, Alder AC, Suter MJF. Quantification of veterinary antibiotics (sulfonamides and trimethoprim) in animal manure by liquid chromatography-mass spectrometry. J Chromatogr A 952:111-120 (2002).

99. De Liguoro M, Cibin V, Capolongo F, Halling-Sorensen B, Montesissa C. Use of oxytetracycline and tylosin in intensive calf farming: evaluation of transfer to manure and soil. Chemosphere 52:203-212 (2003).

100. Søegaard Jensen B, Jacobsen AM, Loke ML, Halling-Sorensen B. Antibakterielle stoffer i svinegylle - en præliminær undersøgelse af nedbrydning i gylletanken. Dansk Kemi 84:28-31 (2003).

101. Lopez de Alda MJ, Barcelo D. Determination of steroid sex hormones and related synthetic compounds considered as endocrine disrupters in water by fully automated on-line solid-phase extraction-liquid chromatography-diode array detection. J Chromatogr A 911:203-210 (2000).

102. Ferguson PL, Iden CR, McElroy AE, Brownawell BJ. Determination of steroid estrogens in wastewater by immunnoaffinity extraction coupled with HPLC-Electrospray-MS. Anal Chem 73:3890-3895 (2001).

103. Croley TR, Hughes RJ, Koenig BG, Metcalfe CD, March RE. Mass spectrometry applied to the analysis of estrogens in the environment. Rapid Communications in Mass Spectrometry 14:1087-1093 (2000).

104. Rose J, Holbech H, Lindholst C, Nørum U, Povlsen A, Korsgaard B, Bjerregaard P. Vitellogenin induction by 17β-estradiol and 17α-ethinylestradiol in male zebrafish ***(Danio rerio)***. Comparative Biochemistry and Physiology Part C 131:531-539 (2002).

105. Isobe T, Shiraishi H, Yasuda M, Shinoda A, Suzuki H, Morita M. Determination of estrogens and their conjugates in water using solid-phase extraction followed by liquid chromatography-tandem mass spectrometry. J Chrom A 984:195-202 (2003).

106. D'Ascenzo G, Di Corcia A, Gentili A, Mancini R, Mastropasqua R, Nazzari M, Samperi. R. Fate of natural estrogen conjugates in municipal sewage transport and treatment facilities. Sci Total Environ 302:199-209 (2003).

107. Baronti C, Curini R, D'Ascenzo G, Di Corcia A, Gentili A, Samperi R. Monitoring Natural and Synthetic Estrogens at Activated Sludge Sewage Treatment Plants and in a Receiving River Water. Environ Sci Tech 34:5059-5066 (2000).

108. Gentili A, Perret D, Marchese S, Mastropasqua R, Curini R, Di Corcia A. Analysis of free estrogens and their conjugates in sewage and river waters by solid-phase extraction then liquid chromatography-electrospray-tandem mass spectrometry. Chromatographia 56:25-32 (2002).

109. Zhang H, Henion J. Quantitative and Qualitative Determination of Estrogen Sulfates in Human Urine by Liquid Chromatography/Tandem Mass Spectrometry Using 96-Well Technology. Analytical Chemistry 71:3955-3964 (1999).

110. van der Wal S, Huber JFK. Separation of steroid conjugates by high-performance liquid chromatography. J Chrom A 251:289-310 (1982).

111. Rodgers-Gray TP, Jobling S, Morris S, Kelly C, Kirby S, Janbakhsh A, Harries JE, Waldock M, Sumpter JP, Tyler CR. Long-Term Temporal Changes in the Estrogenic Composition of Treated Sewage

Effluent and Its Biological Effects on Fish. Environ Sci Technol 34:1521-1528 (2001).

112. Kuch HM, Ballschmiter K. Determination of endocrine-disrupting phenolic compounds and estrogens in surface and drinking water by HRGC-(NCI)-MS in the picogram per liter range. Environ Sci Technol 35:3201-3206 (2001).

113. Siegener R, Chen RF. ACS symposium series 747:125 American Chemical Society, Washington (2000).

114. Lee HB, Peart TE. Determination of 17 beta-estradiol and its metabolites in sewage effluent by solid-phase extraction and gas chromatography mass spectrometry. J AOAC Int 81:1209-1216 (1998).

115. Jahr D. Determination of Alkyl, Chloro and Mononitrophenols in Water by Sample-Acetylation and Automatic On-Line Solid Phase Extraction-Gas Chromatography-Mass Spectrometry. Chromatographia 47:49-56 (1998).

116. Coille I, Reder S, Bucher S, Gauglitz G. Comparison of two fluorescence immunoassay methods for the detection of endocrine disrupting chemicals in water. Biomolecular Engineering 18:273-280 (2002).

117. Valentini F, Compagnone D, Gentili A, Palleschi G. An Electrochemical ELISA procedure for the screening of 17β-estradiol in urban waste waters. Analyst 127:1333-1337 (2002).

Overview of published HPLC-methods

Analytes	Matrix	Sample size	Sample preparation	Preconcentration (x times)	LC-column	Detection method	LOD (ng/L) [a)]	LOQ (ng/L)[a)]	RSD (%) (at which concentration.) [a)]	Recovery (%)[a)]	Levels (ng/L)	ref
E1, E2, EE2, E3	STP-in	0.15 L	SPE cartridge with 0.5 g graphitized carbon black	750x	Alltima 25x4.6 RP-C18 µm	ESI(-)-MS-MS (API PE Sciex 2000)	0.2-0.6		2.4-5.6			(*25*)
	STP-out	0.4 L		2000x			0.08-0.2		2.7-6.2			
	Surface water	4 L		20000x			0.008-0.02		3.1-4.8			
E1, E2, E3 + G-&S-conjugates	Urine	0.005 L	SPE cartridge with 0.5 g graphitized carbon black	25x	Alltima 25x4.6 RP-C18 µm	ESI(-)-MS-MS (API PE Sciex 2000)		16-70	3-7	76-98	n.d.-72	(*69*)
	Wastewater	0.05 L		250x				2-7				
	STP-in	0.100 L		500x				1-2				
	STP-out	0.250 L		2500x				0.3-1				
E1, E2, E3, MeEE2, EE2, DES, progestogens	STP-out and surfacte water	0.5 L	C18 SPE	1000x	Lichrospher 100 RP-18/Lichrospher 60 RP-select B	DAD-ESI(-)-MS	50-500		<18%	>83%	n.d.-263	(*24;75*)
E1, E2, E3, EE2 + G-&S-conjugates	STP-in	0.1 L	SPE cartridge with 0.5 g graphitized carbon black	500x	Alltima 25x4.6 RP-C18 µm	ESI(-)-MS-MS (API PE Sciex 2000)	0.4-0.85			73-100	<100	(*49*)
	STP-out	0.25 L		1250x			0.04-0.24			82-98		
	Surface water	2 L		10,000x			0.005-0.03			78-100		
E1, E2, EE2, E3, DES, progestagens	Surface water	0.2 L	PLRP-S cartridge	b)	Lichrospher 100 RP-18/Lichrospher 60 RP-select B	DAD	15		3	103-112		(*101*)
E!, E2, EE2, E3, E2-17α, G-&S-conjugates	STP-out Surface water	0.5 L	EDS-1 SPE cartridge + fluorisil cartridge	1000-10,000x	Waters Xterra MS C18/Zorbax, extend C18	ESI(-)-MS-MS	0.1-1.5		3-17	77-106	0.3-34	(*62*)
E1, E2, E2-17α EE2, E3, progestagens	Surface water, Groundwater	1 L	SPE cartridge with 0.5 g graphitized carbon black	5000x	Alltech, C18, 5 µm	APCI(+)-MS-MS (Sciex API 365)	0.5-1		3.8-7.0	82-91		(*63*)
E1, E2, EE2, E3	STP-in STP-out	1 L	ENVI-CARB cartridge	4000x	Alltima C18 column	APCI(+)-MS-MS (Sciex API 365)		0.5 (E_2, EE_2) 1 (E_1, E_3)	8-11			(*57*)
E1, E2	STP-out	1L	Lichrolut+C18 SPE cartridge +Immunosorbent cartridge	5000x	Betasil, C18, 3 µm, 15 cm	ESI(-)-MS		0.07-0.18	<5%	92-107		(*102*)

E2, EE2 and phenolic EDCs	STP-out, surface water	5 L	Empore® disk	5000x	Preparative normal phase chromatrography, polar fraction separated on phenomenex ODS C18 columns	Fluorescence 229 nm excitation, 310nm emission	3.2 [c)]	0.8 [c)]		72-78		(*65*)
E2, EE2, E3	STP-in	0.5 L	SPE cartridge with 0.5 g graphitized carbon black	2,500x		ESI(-)-MS-MS (API PE Sciex 2000)	0.4-0.5		<10%	88-97		(*14*)
	STP-out	1 L		5,000x			0.2-0.25					
E1, E2, EE2, E3	Surface water	0.05 L	SPE C18 cartridge	1000x	Poruspher ® C18 column	ESI(-)–MS ion trap	4.2-10.6	13.9-35	<16.1%	95.8-109.5		(*74*)
E1, E2, E2-17α EE2, E3, MeEE2	Suface water	3.5 mL	85 µm polyacrylate SPME fibers	d)	Lichrospher C18 column	UV-ED	70-80		<11% (intra-day) <15% (between days)			(*68*)
E2, E3, EE2 and various EDCs	Surface water	0.03 L	PRP-1 SPE cartridge	Nd	SGE-ODS column	Fluorescense 230 nm excitation, 290 nm emission	20-50		nd	≈100		(*66*)
E1, E2, EE2, E3	River water STP-out water	1 L	ENVI-18 cartridge (supelco)	1000x	Phenomenex Luna C-18 Column	ESI(-)-MS-MS (Micromass)	5		nd			(*103*)
E1, E2, EE2, E3, DES and other steroids	River sediment	5 g	Lyophilisation – ultrasonification extraction – C18SPE	55 [e)]	Lichrospher C18 column	DAD-ESI(-)-MS	0.05-1 ng/g		11-19	93-100	Nd<22.8 ng/L	(*95*)
E1, E2, EE2, E3, DES, other steroids, and EDC's	River sediment	5 g	Lyophilisation – pressurised liquid extraction	40 [e)]	LiChrospher ADS Lichrospher C18	RAM-LC-MS	1-5 ng/g		2.5-7.1	94-104		(*96*)
E2, EE2	Fish estrogenicity assay	0.05-1 L	Waters Sep-Pak C18 1000 mg cartridges	167-3333	Phenomenex prodigy C18	LC-APCI(+)-MS		0.6-1	13.3-13.8	78-83 (E2) 80-85 (EE2)	10-500 ng/L	(*104*)

a) Data only presented for the non-conjugated estrogens. LOQand LOD are reported for extracted and preconcentrated samples
b) Samples were eluted directly into the HPLC-column
c) Snyder et al. only presented LOD and LOQ for the instrument and not for the whole method. They claim that samples were preconcentrated 5000 times, however this is questionable.
d) As SPME was used, the number of times the sample was preconcentrated can not be reported.
e) As solid samples were analysed, the number refers to the preconcentration of the sediment-extract

Abreviations: DES=diethylstilbestrol; EDCs=endocrine disrupting chemicals; ESI=electrospray ionisation; UV=ultraviolet detection; UV-ED=ultraviolet –electrochemical detection, dw=dry weight

Overview of published HPLC methods for analysing conjugated estrogens

Compounds	Samples	Sample preparation	Analytical method	LOD/LOQ	Ref
E1-3S, E1-3G, E2-3S, E2-3G, E2-17S, E2-3S17G, E2-3G17S, E2-3,17DiS, E3-3S, E3-3G, E1, α-E2, β-E2, E3, EE2	River, lake water, effluent (STP) Detection: E1-3S: 0.3-2.2 ng/L E2-3S: 0.2-1 ng/L	Filtration (1L) pH-adjustment to 3.5-5 with acetic acid SPE: Autoprep EDS-1 Elution: Free: ethyl acetate; Conjugates: 5mM TEA in MeOH Florisil clean-up (free) Dissolved in 100 µL-1mL	LC-MS/MS, ESI (-) StP: Zorbax Extend-C_{18} column (150 mm x 1 mm I.D., 3.5 µm, Agilent) MP: A: Acetonitrile; B: H_2O, C: 100 mM TEA in H_2O (pH 12.2) Temp: 30 ºC, FR: 40µL/min; IV: 10 µL	MDL: Free: 0.1-1.5 ng/L; Conjugates: 0.1-3.1 ng/L	(*105*)
E1-3S, E1-3G, E2-3S, E2-3G, E2-17G, E3-3S, E3-3G,E3-16G E1, α-E2, β-E2, E3, EE2	Female urine, septic tank collecting domestic wastewater, influent and effluent (STP)	Preservation with formaldehyde (1%, v/v) Filtration (Whatman GF/C glass fiber, 1.5 µm), washing filter with 3 mL MeOH, added to the aqueous extract SPE (0.5 g Carbograph 4, GCB) washing After loading of the water samples, the cartridges were washed sequentially with: 50 mL of distilled water, 10 mL MeOH acidified with formic acid 50 mM and 5 mL MeOH Elution of the free: 12 mL methylene chloride:MeOH (80:20, v/v) After elution of the free estrogens from the SPE the cartridges were reversed(?), eluted with 20 mL methylene chloride/MeOH (60:40, v/v) containing 10 mM NaAcetate Reconstituted with 200 µL H_2O:MeOH (50:50, v/v) after solvent evaporation	LC-MS/MS, ESI (-) StP: Alltima C_{18} column (25x4.6 mm I.D., 3.5 µm) MP: A Acetonitril: B: Water both 10 mM formic acid FR: 1 mL/min; IV: 50 µL	LOQ (ng/L): Urine: Free: 16-40; conjugates: 20-600; Wastewater: F:2-4; C: 2-60; STP influent: F: 1-2; C: 0.8-30; STP effluent: F: 0.5-1; C: 0.3-12	(*106;107*)
E1-3S, E1-3G, E2-3S, E2-3G, E2-17G, E3-3S, E3-3G,E3-16G	Raw sewage, treated sewage, river water	Filtration (Whatman GF/C glass fiber, 1.5 µm), washing filter with 3 mL MeOH, added to the aqueous extract SPE (0.5 g Carbograph 4, GCB) cartridges	LC-MS/MS, ESI (-) StP: Alltima C_{18} column (250 x 4.6 mm I.D., 3.5 µm)	LOD (ng/L): STP influent (100 mL) F: 0.4-0.85, C: 0.2-15 STP effluent (250 mL)	(*108*)

E1, α-E2, β-E2, E3, EE2		washing with 10 mL dichloromethane:MeOH (80:20, v/v) containing 5mM TMACl (tetraethylammonium Chlorid), 5 mL MeOH, 20 mL water acidified (pH 2) with HCl, distilled water 5 mL Loading on the SPE After the loading the SPE were sequentially washed with 50 mL distilled water, 10 mL MeOH acidified with 50 mM formic acid and 10 mL MeOH Elution: F: 10 mL dichloromethane: MeOH (80:20, v/v) into glass vials with conical bottom; C: were back eluted from the SPE with 10 mL dichloromethane: MeOH (80:20, v/v) containing 5mM TMACl into glass vials. Extracts were evaporated at 30 ºC under N_2. Re-dissolved in 200 µL Water:MeOH (50:50; v/v)	MP: Free: A: Acetonitril: B: Water both 10 mM formic acid; Post column addition of MeOH containing 40 mM NH_3, at FR:0.1mL/min Conjugated: Acetonitril: B: Water both 10 mM formic acid; FR: 1 mL/min; IV: 50 µL	F: 0.04-024, C: 0,04-6 River water (2 L) F: 0.005-0.03, C: 0.05-0	
E1-3S,E32-3S, E3-3S, E2-17S, E2-3,17DiS d4-E2-3S (IS)	Human urine	Robotic SPE extraction in the 96.Well Disk Plate	LC-MS/MS, ESI (-) StP: Keystone Betasil C_{18} column (150 x 2 mm, 5 µm) MP: 5 mM ammonium sacetate, pH = 5.4/MeOH (50:50) FR: 200 µL/min; IV: 20 µL	LOQ: C: 0.2 ng/mL	(*109*)
	Human urine		HPLC-UV		(*110*)

FR: flow rate; MDL: method detection limit; IV: injection volume; MP: mobile phase; StP: stationary phase.

Overview of published GC methods

Survey of GC-MS and GC-MS-MS methods used for quantitative determination of natural and synthetic steroids.

Compounds	Matrix	Extraction	Clean-up	Derivatization	Separation and detection method	MS system	LOD (ng/l)	Reference
E2, E1, EE	STP effluent	SPE (C18 col)	-SPE (C18 col) -HPLC fraction. -LLE	-	GC-(EI)-MS	Finnigan MAT Magnum ion trap	0.2	(*17*)
E2, E1, EE	STP effluents	SPE (C18 col)	-HPLC fraction.	-	GC-MS	Not reported	0.5-1	(*111*)
E2, 17α-E2, E1, EE	Surface and drinking water	SPE (LiChrolut EN)		PFBCl	GC-(NCI)-MS	HP 5973 MSD (Hewlett-Packard)	0.05-0.15	(*112*)
E2, E1, EE	STP effluent	SPE (ENV+ col)	-LLE, GCP (Biobeds SX-3) -hydrolysis	Acetic anhydride	GC-(EI)-MS	Not reported	Not reported	(*59*)
E2, E1, EE, 17α-E2 (SS)	River water and STP effluent	Contineous LLE	-	Bis-(trimethylsilyl)triflouro-acetamide with 10% trimethylchlorosilane	GC-(EI)MS	HP 5970 MSD (Hewlett-Packard)	58 (only E2)	(*53*)
E2, E3, E1, EE	STP effluent	SPE (LiChrolut EN/Bondesil C18 col.)	Silicagel 60	MSTFA/TMSI/DTE (1000:4:2)	GC-(EI)MS	HP 5970B MSD (Hewlett-Packard)	1	(*58*)
EE	STP effluent	SPE (Empore C18 disk)	-	-	GC-(EI)MS	Finigan Voyager	74	(*113*)
E2, EE	Surface water	SPE (C18 col. Or disk or PS-DVB col.) or	-	MTBSTFA	GC-(EI)MS	Voyager (Interscience)	50-300	(*55*)

		LLE						
E2, E3, E1	STP effluent	SPE (C18 col.)	-	Pentafluoropropionic acid anhydride	GC-(EI)MS	HP 5890 MSD (Hewlett-Packard)	5-10	(*114*)
EE	Tap and river water	In sample acetylation on line SPE (PLRP)	-	Acetic anhydride	GC-(EI)MS	Finnigan MAT 44S	15	(*115*)
E2, E1, EE	STP influent and effluent	SPE (SBD-XC disk)	-SPE (C18/NH2 col.) -HPLC fraction.	-	GC-(EI)MS-MS	Not reported	0.1-1.8	(*14*)
E2, 17α-E2, E1, E2-17-acet, E2-17-valer.	Influent and effluent	SPE (C18/EN col.)	Silicagel	MSTFA/TMSI/DTE (1000:2:2)	GC-(EI)MS-MS	Varian Saturn 4	0.5-1	(*18*)
E2, EE, E2gluc, E2sulf	Surface water, STP effluent	SPE (C18 disk)	-Hydrolys. -HPLC fration.	Heptafluorobutyric anhydride	GC-(EI)MS-MS (only E2)	Finnigan GCQ ion trap (ThermoQuest)	0.2-0.4	(*48*)
E2, 17α-E2, E1, EE, glucuronides	Surface and waste water	SPE (SDB-XC disk)	- hydrol. -SPE (C18/NH2) -HPLC -fraction.	SIL A reagent	GC-(EI)MS-MS	Saturn IV ion trap (Varian)	0.1-2.4	(*8*)
E2, E1, EE	Reservoir and river water, SPE effluent	SPE (C18 disk)	-	MTBSTFA with 1% TBDMCS	GC-(EI)MS-MS	ThermoQuest GCQ ion trap	1	(*54*)

Abbreviations: PFBCI = Pentafluorobenzylbenzene; GPC = gel permeation chromatography; SS=surrogate standard; MSTFA= N-methyl-N-(trimethylsilyl)-trifluoroacetamide; TMSI = trimethylsilylimidazole; DTE= dithioerytrol; MTBSTFA= N-methyl-N-(tert)-butyldimethylsilyltrifluoroacetamide; E2-17-valer. = estradiol-17-valerate; E2-17-acet.=estradiol-17-acetate- E2gluc=estradiol glucuronide; E2sulf= estradiol sulphate; TBDMCS= tert.-butyldimethylchlorosilane.

Overview of published immunochemical methods

Analytes	Matrix	Sample volume	Sample preparation	Preconcentration (x times)	Assay –format	LOD (ng/L) [a)]	LOQ	RSD (%) (at which concentration.)	Cross reactivity	Recovery (%)	Levels (ng/L)	ref
E2	Surface water and STP-out	5 L	Empore disc	5000	RIA	0.107			11.2% for E1) 1.7% for E3 <1% for EE2 and others		nd-3.6	(*65*)
EE2						0.053			0.3% for E2 <0.1% for E1, E2 and others <0.01% for others	63-78	Nd-0.76	
E2, EE2	Surface water	2-6 L	Empore disc	3333-10000	HPLC-cleanup ELISA	0.05			b)	91-108 (E2) 54-89 (EE2)	0.05-0.8 (E2) <0.05-0.07 (EE2)	(*48*)
	STP-out	0.2-2 L		333-3333		0.1				49-117 (E2) 51-110 (EE2)	0.2-4.1 (E2) <0.1-2.4 (EE2)	
E2	STP-out	0.03 L	C18 column	6	RIA	ND			25% w E1		40-360	(*39*)
E1, E2, EE2	Synthetic wastewater	c)	c)	c)	TIRF	70-160			70-112			(*116*)
E1, E2, EE2	Synthetic wastewater	c)	c)	c)	ETIA	10-850			52-109			(*116*)
E2, E3	River water	0.02	d)	d)	TR-FIA	32 (E2) 5.5 (E3)			<1.1 (E1, EE2, and others)	97-119		(*51*)
E2	STP-out	0.02	Liquid-liquid exraction with diethyl ether	40	Electrochemical ELISA	0.125		3-5	<1% for E1 <0.3% for E2-17α <0.2% for testosterone and others	84-91	<2	(*117*)
E2					ELRA							

a) LOD and LOQ are reported after preconcentration and extraction of samples
b) The authors report a significant background signal (up to 3 ng/L) in the HPLC-fractions without E2 and EE2.
c) No sample preparation was made, samples were only tested on synthetic sewage
d) No sample preparation was made

Abreviation: RIA=radioimmunoassay; TIRF=Total internal reflection fluorescence; ETIA= Energy transfer immunoassay; TR-FIA=Time-resolved-fluoroimmunoassay

Appendix 5

List of abbreviations

APCI	Atmospheric Pressure Chemical Ionisation
APPI	Atmospheric Pressure Photo Ionisation
CI	Chemical ionisation
DAD	Diode Array Detector
E1	Estrone
E2	17β-estradiol
EE2	17α-ethinylestradiol
E2-17β	17β-estradiol
E3	Estriol
EI	Electron impact
ELISA	Enzyme-linked Immunosorbent Assay
ESI	Electrospray ionisation
GC	Gas Chromatography
GPC	Gel Permeation Chromatography
HPLC	High Pressureerformance Liquid Chromatography
LC	Liquid Chromatography
LLE	Liquid-liquid Extraction
LOEC	Lowest Observed Effect LevelConcentration
LOD	Level Limit Of Detection
LOQ	Level Limit Of Quantification
MeEE2	Mestranol
MS	Mass Spectrometry
MS-MS	Tandem Mass Spectrometry

NOEC	No Observed Effect Concentration
PLE	Pressurised Liquid Extraction
RIA	Radio Immunoassay
SPE	Solid-phase Extraction
STP	Sewage Treatment Plant