

CHARLES UNIVERSITY IN PRAGUE  
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CHEMISTRY

**DEVELOPMENT OF NEW METHODS FOR  
DETERMINATION OF SELECTED  
PHARMACEUTICALS IN  
ENVIRONMENTAL SAMPLES**

Doctoral thesis  
2012

Mgr. Jana Aufartová

UNIVERZITA KARLOVA V PRAZE  
FARMACEUTICKÁ FAKULTA V HRADCI  
KRÁLOVÉ

KATEDRA ANALYTICKÉ CHEMIE

**VÝVOJ NOVÝCH METOD PRO  
STANOVENÍ VYBRANÝCH LÉČIV  
VE VZORCÍCH ŽIVOTNÍHO PROSTŘEDÍ**

Doctoral thesis  
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I declare that this thesis is my own work and all the sources of information used have been acknowledged.

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracovala samostatně. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány.

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## **Abstract**

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Titl of Doctor Thesis: Development of new methods for determination of selected pharmaceuticals in environmental samples

During last decade, the consumption of pharmaceuticals is increasing substantially. At the same time their occurrence in environment is increasing as well. Pharmaceuticals are released into environment by excretion, as conjugated or unchanged active compounds, as unused pharmaceuticals, which are not disposed according to the recommendations (e.g. thrown away into the rubbish or flushed down the toilet). Other possible sources of pharmaceuticals are in the agriculture, livestock and aquaculture. The current water treatment technologies do not remove all traces of pharmaceuticals in wastewaters. Therefore the monitoring of their occurrence in surface and wastewaters become more important.

Antibiotics and steroid hormones are a group of drugs used in human and veterinary medicine. The main problem with these groups of substances is the emergence of bacterial resistance, in the case of antibiotics, and the effect on the endocrine system, including the reproductive cycle, in the case of steroid hormones.

The theoretical part of the presented thesis is focused on the introduction dealing with the presence of antibiotics, steroid hormones and benzimidazole fungicides in the environment. Furthermore, there are several methods mentioned in this part used for the preparation of environmental samples such as solid phase extraction and other microextraction techniques. An important part of the presented work is an overview of methods used for the determination of steroid hormones and benzimidazole fungicides. These papers were published in international journals and as a book chapter (Chapter 5.2 and 5.3).

The practical part of this thesis is focused on the development of methods for determination of fluoroquinolone antibiotics in wastewater and its applications to samples of river water and wastewater. The newly developed sample preparation methodology and systematic development of analytical methods for fluoroquinolones was published in the international scientific journal (Chapter 5.1). Another scope of interest of the practical part of the thesis was a method development of sample preparation by microextraction techniques for steroid hormones from wastewater and sea water, followed by the determination using liquid chromatography with fluorescence and ultraviolet detection. This newly described methods were published or has been submitted in international scientific journals (Chapter 5.2).

All published papers are enclosed in a full version as supplements.

## **Abstrakt**

Univerzita Karlova v Praze, Farmaceutická Fakulta v Hradci Králové

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Kandidát: Mgr. Jana Aufartová

Školitel: Prof. RNDr. Petr Solich, CSc.

Název dizertační práce: Vývoj nových metod pro stanovení vybraných léčiv ve vzorcích životního prostředí

Neustálý nárůst spotřeby léčiv navyšuje také jejich výskyt v životním prostředí. Léčiva jsou do životního prostředí distribuována v podobě vyloučených metabolitů nebo nezměněných aktivních látek, jako nepoužité léčivé přípravky, které byly nesprávně zlikvidovány (např. spláchnutím do odpadu). Dalším zdrojem léčiv je živočišná výroba v zemědělství, chov dobytka a ryb. Současné technologie úpravy vody neumožňují odstranit veškeré zbytky léčiv ve vodách. Současně se zvyšováním množství těchto látek v odpadních a následně povrchových vodách narůstá význam jejich monitorování.

Antibiotika a steroidní hormony jsou velmi využívané skupiny léčiv jak v humánní, tak ve veterinární medicíně. Hlavním problémem těchto skupin látek je vznik bakteriální rezistence v případě antibiotik a ovlivnění endokrinního systému, včetně reprodukčního cyklu v případě steroidních hormonů.

Teoretická část předkládané dizertační práce je v nejprve zaměřena na problematiku výskytu antibiotik, steroidních hormonů a benzimidazolových fungicidů v životním prostředí. Následně jsou zmíněny metody pro přípravu vzorků životního prostředí jako je extrakce na tuhé fázi a další mikroextrakční techniky. Důležitou součástí této dizertační práce jsou vypracované rešeršní práce shrnující metody stanovení steroidních hormonů a benzimidazolových fungicidů. Tyto práce byly publikovány v mezinárodních odborných časopisech a jako kapitola knihy (kapitoly 5.2 a 5.3).

Praktická část této dizertační práce je zaměřena na vývoj metody stanovení fluorochinolonových antibiotik v odpadních vodách a její aplikaci na vzorcích říčních a odpadních vod. Nově vyvinutá metoda přípravy vzorků a systematický vývoj analytické metody stanovení fluorochinolonů byly publikovány v zahraničním impaktovaném časopise (kapitola 5.1). Dále je tato část práce zaměřena na steroidní hormony a vývoj mikroextrakčních metod přípravy vzorků odpadních a mořských vod následovanou stanovením pomocí kapalinové chromatografie s fluorescenční a ultrafialovou detekcí. Tyto nové metody jsou publikovány nebo zaslány k publikaci v zahraničním impaktovaném časopise (kapitola 5.2).

Všechny publikované práce jsou v plném znění uvedeny v přílohách.

## ***List of Abbreviations***

ACN	acetonitrile
APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photoionization
AS	activated sludge
BBD	box Benken design
BEH	bridge ethyl hybrid
BSTFA	N,O-bis(trimethyl)trifluoroacetamide
BTEX	benzene, toluene, ethyl benzene, xylene
CIPRO	ciprofloxacin
CPE	cloud point extraction
DDD	defined daily dose
DHPMM	dihydroxylated polymethylmethacrylate
DI-SDME	direct immersion a microdrop microextraction
DLLME	dispersive liquid liquid microextraction
DLLME-SFO	dispersive liquid-liquid microextraction based on the sonification of the floatation organic drop
DSDME	directly suspended droplet microextraction
DW	drinking water
E1	estrone
E2	estradiol
E3	estriol
EDCs	endocrine disruptor compounds
EDTA	ethylenediaminetetraacetic acid
EE2	ethynylestradiol
ENRO	enrofloxacin
ESI	electrospray ionization
FAO	Food and Agriculture Organisation
FD	fluorescence detection
FQs	fluoroquinolones
GC	gas chromatography
HF-LPME	hollow fiber liquid phase extraction
HPLC	high performance liquid chromatography
HS-SDME	headspace single drop microextraction
LC	liquid chromatography
LLE	liquid-liquid extraction
LLLME	liquid liquid liquid microextraction
LPE	liquid phase extraction
LPME	liquid phase microextraction
MAE	microwave assisted extraction
MALDI	matrix-assisted laser desorption/ionization
MeOH	methanol

MEPS	microextraction by packed sorbent
MIPs	molecular imprinted polymers
MRLs	maximum residue levels
MS	mass spectrometry
MSTFA	N-methyl-N-(trimethylsilyl)trifluoroacetamide
NOR	norfloxacin
OFLO	ofloxacin
PAHs	polycyclic aromatic hydrocarbons
PDMS	polydimethylsiloxane
PEEK	polyethyletherketone
PEFLO	pefloxacin
PLE	pressurized liquid extraction
QqQ	triple quadrupole
Q-TOF	hybrid quadrupole time of flight mass spectrometer
RRHT	Rapid Resolution High Through put
RW	river water
SBSE	stir bar sorptive extraction
SBSE-TD	stir bar sorptive extraction thermal desorption
SD-LPME	solid drop microextraction
SDME	single drop microextraction
SeaW	sea water
SPE	solid phase extraction
SPME	solid phase microextraction
STPs	sewage treatment plants
SW	surface water
UHPLC	ultra high performance liquid chromatography
UV	ultraviolet detection
WWTPs	wastewaters treatment plants

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# 1 **1 The aim of the work**

2           The main aim of the doctoral thesis was to develop new methods for determination  
3 of selected groups of pharmaceutical compounds in the environmental samples.

4           Theoretical part of thesis briefly introduce selected group of pharmaceuticals. The  
5 goal of theoretical part was to summarize an overview of scientific publications, which deal  
6 with the occurrence of emerging contaminants, such as fluoroquinolone antibiotics,  
7 endocrine disruptor compounds and benzimidazole fungicides in the environmental  
8 matrices.

9           The experimental work was mainly focused on the sample preparation. Several  
10 methods such as solid phase extraction (SPE) and dynamic or static in-tube solid phase  
11 microextraction (SPME) using experimental design were tested and developed. These  
12 methods were optimized in order to remove impurities, to reach high preconcentration factor  
13 and recovery for each target analyte. In all cases, sample preparations were followed by  
14 development of HPLC or UHPLC methods for determination of target compounds. The  
15 results (in the form of accepted publications) show the new possibilities for the  
16 determination of antibiotics and steroid hormones in the environment.

17

18

## 2 Introduction

Emerging contaminants enter to the environment mainly by wastewaters, agricultural farms and aquacultures. Hospital wastewaters contain a variety of toxic or persistent substances such as pharmaceuticals, radionuclides, solvents and disinfectants for medical purposes in a wide range. In many cases, these emerging contaminants correspond to unregulated pollutants, which may be candidates for a future regulation depending on the research on their potential health effects and the results of monitoring of their occurrence [1,2].

Pharmaceuticals are substances used in the diagnosis, treatment, alteration, or prevention of abnormal health or structural/functional conditions in the body, and there are a many compounds included in this group [3].

In the last few decades, the amount of pharmaceuticals released into the environment has increased considerably [4]. They have been increasingly detected in surface waters, ground waters and drinking water, since not all have been removed by conventional wastewater treatment plants (WWTPs) [5].

Surface water sampling programmes in the United Kingdom, continental Europe and North America have shown the presence of many different classes of pharmaceuticals. These compounds show a risk to the aquatic environment because of low concentrations combined with toxicity. Other pharmaceuticals such as natural and synthetic sex hormones or antibiotics are known to pose considerable risks of reproductive alternations or develop of resistance, respectively [6-8].

Pharmaceuticals are used also in animal raised on pastures. Therefore, their metabolites and conjugates are mainly excreted through the urine or faeces directly to the grassland [2] or used for the dispersion of manure on fields as fertilizer [6]. In addition, the pharmaceuticals used in aquaculture are released directly into the surface waters. However, the exposure of aquatic wildlife to human pharmaceuticals is most likely to occur from sewage treatment plants in low concentrations [9].

## 1 **2.1 Antibiotics**

2 Among all the pharmaceuticals, antibiotics, which are used to treat infections, have  
3 attracted special interest in studies of environmental waters. Some are used only in  
4 veterinary or human medicine, but most of them are used for both human and veterinary  
5 health purposes [3].

6 Besides the human and veterinary treatment of infections [10], they often apply in  
7 livestock farms preventively when the shed is changed. Furthermore, some antibiotics are  
8 used as growth promoters and thus are permanently added to the feed [9].

9 Antibiotic residues in the environment are suspected to induce bacteria resistance and  
10 some infections can no longer be treated with the presently known antidotes [9]. Also  
11 ground water can be exposed to antibiotic residues leaching from farmland fertilized with  
12 manure or through sewage disposal by spray and broad irrigation in agricultural areas. The  
13 polar antibiotics may not be eliminated effectively, as a large part of the elimination is  
14 achieved by absorption on activated sludge which is partly mediated through hydrophobic  
15 interactions [9]. In addition, antibiotics are extensively used in aquaculture. The main groups  
16 of pharmaceuticals used are tetracyclines, sulfonamides and chloramphenicol [11,12].

## 17 **2.2 Steroid hormones**

18 The exogenous substances that interfere with the endocrine system (e. g. with  
19 synthesis, secretion, transport, binding, action and elimination of natural hormones) and  
20 disrupt the physiologic function of hormones are called endocrine-disruptor compounds  
21 (EDCs). The effects of natural and synthetic EDCs found in the environment include  
22 decreasing sperm count in human males, increasing breast cancer in women and causing  
23 reproductive abnormalities in humans [13-15].

24 Steroid hormones are one of most potent active EDCs present in the environment,  
25 which are formed naturally by human beings and wildlife or produced synthetically. They  
26 can be generally divided in three groups: estrogens, gestagens and androgens [15-17].  
27 Extreme concentrations of sex hormones occur at specified times for normal  
28 physiologic functions; such periods include sexual differentiation, puberty,  
29 reproductive cycles, parturition, lactation and menopause [18].

30 Conventional WWTPs not remove some of EDCs efficiently thus they are detected  
31 in WWTPs effluents and they are releasing into rivers and lakes [15]. Among these, natural



1 and synthetic estrogens are already effective at a lower ng/l level, while most other  
2 chemicals having an estrogenic effect are biologically active at the µg/l level [15,19].

### 3 **2.3 Benzimidazole fungicides**

4 Other compounds dealing with environmental pollution are pesticides. Because of  
5 the widespread use of agricultural pesticides for different applications, the pesticide residues  
6 may present a main source of pollution, which poses risks to plant, animal and human health  
7 [20].

8 Benzimidazole fungicides are systemic pesticides widely used for prevention and  
9 treatment of parasitic infections in agriculture and aquaculture. They are efficient at low  
10 doses as well as they inhibit the development of a wide variety of fungi. Some of them are  
11 apply in agriculture for pre- and post- harvest protection of crops. These fungicides are  
12 either applied directly to the soil, or sprayed over crop fields [21].

13 Most of these compounds persist in the environment after their application, some of  
14 them even for many years. Therefore, some of the most detected pesticides during the  
15 monitoring programmes are crucial to assess consumers' exposure to those fungicides  
16 through foods [22].

### 17 **2.4 Recent development of analytical techniques**

18 Recently, a fast improvement of analytical techniques used for the determination of  
19 pharmaceuticals in environmental samples has been done.

20 In 2003 Agilent developed *100 Series high-throughput* LC/MS system as a pre-step  
21 of ultra high performance liquid chromatography (UHPLC). The main idea of this system  
22 was speed up the routine analysis and perform the system for thousands samples per day. In  
23 this case the pressure can reach almost 6 000 psi [23].

24 One year later Waters Corporation has performed *Waters Aquity* and made a UHPLC  
25 for real [24]. Since this company first constructed this type of system, they registered ultra  
26 performance liquid chromatography, UPLC, as a brand name of their systems. This system  
27 is able to achieve a pressure about 15 000 psi.

28 In 2006 Agilent introduced other system called *1200 Series Rapid* (1200 RRLC),  
29 which provides 60% better resolution than conventional HPLC [23]. In the same year  
30 Thermo Scientific performed *Accela* and Jasco *Xtreme-LC* [25, 26]. Till now, almost all the  
31 large companies, such as Shimadzu (*UFLCxr*, 2007; *Nexera*, 2010), Hitachi (*LaChromUltra*,

1 2007), Scientific Systems (*UltraHP*, 2007), Dionex (*RSLC*, 2008), Knauer (*Platin Blue*,  
2 2008), Perkin Elmer (*Flexar*, 2009), developed their UHPLC systems [27-31].

3         Since these systems are working with ultra high pressure, new additional devices,  
4 such columns with small particles or new construction of detection cells for UV and FD  
5 were needed. Some companies such as Waters or Shimadzu already introduced next  
6 generations of UHPLC, Aquity or Nexera, respectively [24, 27]. In general, companies who  
7 developed UHPLC also developed sub-2 $\mu$ m columns for various applications (Agilent, 1.8  
8  $\mu$ m RRHT; Waters, BEH Aquity; Shimadzu, Shim-pack XR-ODS; Knauer, UPLC columns,  
9 ect.).

10         The necessity of development of fast, cheap and sensitive methods without high  
11 financial initial input lead to further development in the area of separation columns.  
12 Therefore, company Advance Materials Technology developed a new column called *HALO*,  
13 which was firstly performed on Pittcon conference in 2007. These columns were based on  
14 the so called “Fused-Core particle technology“, which provided comparable results with  
15 sub- $\mu$ m particles column and half backpressure of them [32]. Additionally these columns  
16 could be used both in HPLC and UHPLC. Nowadays, there is a wide range of fused-core (or  
17 “porous shell”) column technology in the market.

18         UHPLC and the fused-core technology improved the sensitivity, which allowed  
19 faster detection and become employed very frequently for the environmental analysis.

20

### 3 Pharmaceuticals in the environment

One of the most important groups of the environmental pollutants is the pharmaceuticals, where antibiotics and steroid hormones are included.

Antibiotics are presented in the environment in a very low concentration and allow the development of the bacteria resistance [33].

Steroid hormones, which belong to the group called "endocrine disruptor compounds", can produce in low concentration different effects in organism and humans: disruption in the physiologic function of hormones and also can cause feminization of fishes, decreasing sperm count in human males, increasing the probability of breast cancer in women and causing reproductive abnormalities in humans [2, 7, 34].

Depending on the use of active pharmaceuticals and other active compounds there are several ways in which they can enter into the environment. Antibiotic are used in human and veterinary medicine, agriculture and aquaculture, industry (Figure 1.) and realised into the environment [7].

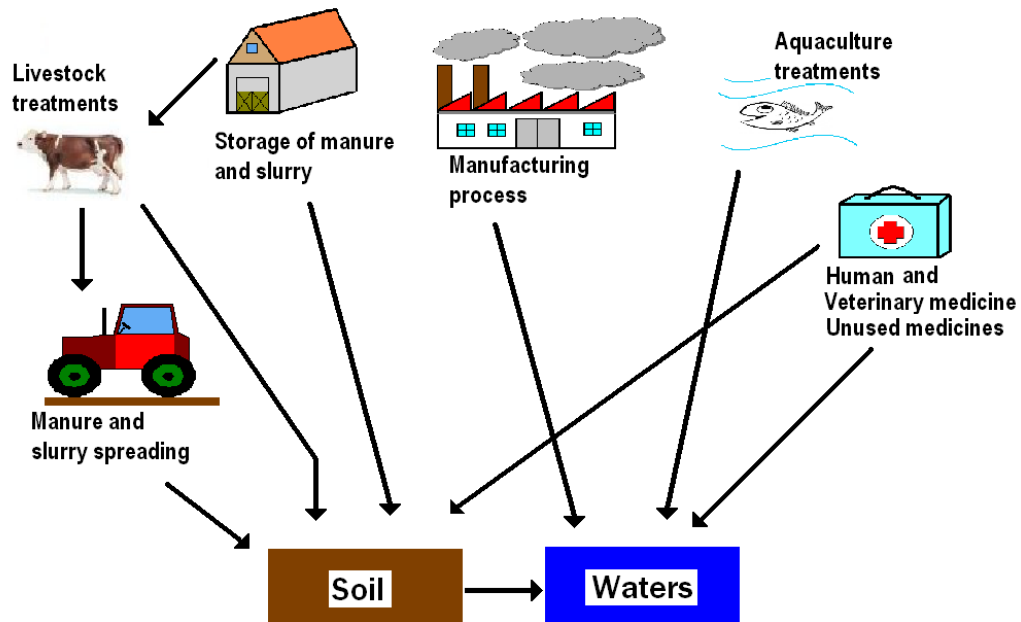


Figure 1: Source of most frequent environmental contaminants. Scheme was modified from [32].

Pharmaceuticals are presented in hospital wastewaters usually in a higher concentration than in the municipal sewages [35, 36]. In total, the flow in the concentration of pharmaceuticals are very low because of the lower share of effluents from hospitals to the

1 municipal effluent in developing countries. The dilution of hospital wastewater by municipal  
2 wastewater is more than 100 times [1, 37].

3 The pharmaceuticals are presented in sewage wastewater as well. It was found out,  
4 that more than 50% of the patients in the USA reported to store unused and expired  
5 medicines at their homes and more than half had flushed them down the toilet. The reasons  
6 for the use of medication are mostly due to a change of medication by the doctor (48.9%), or  
7 self-discontinuation (25.8%). Their most common method of disposal was to throw  
8 unwanted medicines in the rubbish (76.5%) or flush them down the toilet (11.2%). A  
9 significant role for the patient education for the correct disposal of unused and expired  
10 medications is very important in all countries [1, 38].

11 A typical concentrated animal feed operation (CAFO) confines a large number of  
12 animals (e.g., hundreds to thousands of cattle or pigs) into several large buildings and  
13 generates a tremendous amount of manure, which is often applied to nearby agriculture  
14 fields [39].

15 Elimination of organic compounds in the environment is the result of different  
16 processes. These processes can be divided on: biotic (i.e. biodegradation by bacteria and  
17 fungi) and non-biotic elimination processes (i.e. sorption, hydrolysis, photolysis, oxidation  
18 and reduction). It has to be noted that the results of bio- or photodegradation studies depend  
19 on conditions such as temperature, composition of matrix, latitude, etc. [7].

20 If a substance is light sensitive, the photo-decomposition may be of major significance  
21 in the elimination process. Data from the drug registration procedure may give guidance on  
22 some compounds where photo-decomposition can be expected to play a role. Photochemical  
23 decomposition can play an important role in surface waters as an additional elimination  
24 pathway or for effluent treatment.

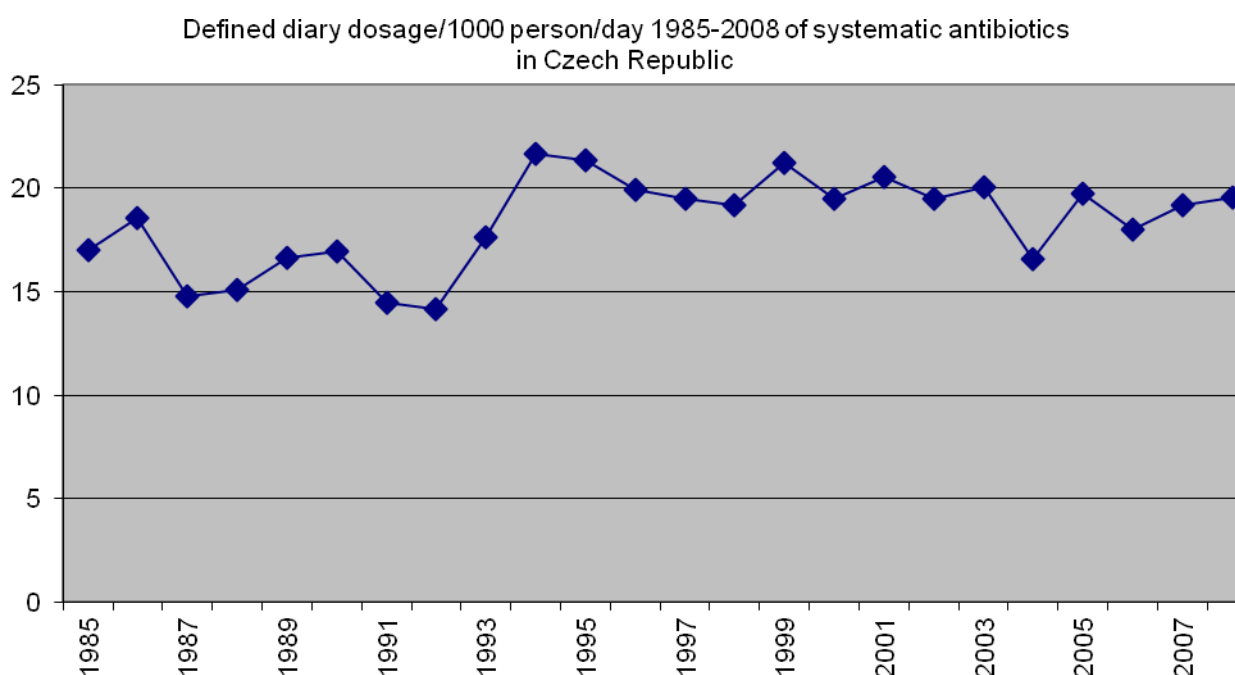
25 Another important pathway for the non-biotic elimination of organic substances in  
26 the environment is hydrolysis. However some pharmaceuticals are resistant to hydrolysis  
27 (e.g. sulphonamides and quinolones). Occurrence of antibiotics in WWTPs effluent is well  
28 known for the difficulty of their elimination by traditional bio-treatment methods. For  
29 advanced effluents, treatment oxidation processes are usually applied [7].

30

## 1 **3.1 Antibiotics**

### 2 **3.1.1 Sources of antibiotics**

3 The first antibiotics had a natural origin, e.g. penicillin was produced by fungi in the  
4 genus *Penicillium*, or streptomycin from bacteria of the genus *Streptomyces*. Nowadays, the  
5 antibiotics are acquired mainly by chemical synthesis or by chemical modification of some  
6 compounds of natural origin. Several soil bacteria (e.g. the group of *Actinomycetes* includes  
7 many soil bacteria such as *Streptomyces*) produce antibiotics such as  $\beta$ -lactams,  
8 streptomycins or aminoglycosides [7].



9

10 **Figure 2: Defined Diary Dosage per 1000 person per day in Czech Republic from 1985 to 2008. Data was**  
11 **taken from State institute for Drug Control of Czech Republic [40].**

12 Consumption of antibiotic compounds in human treatment varies from one country to  
13 another country. Data on the country-specific use for groups of antibiotics in different  
14 countries are available from different sources but mostly as DDD (Defined daily dose,  
15 according to WHO). Antibiotics use ranges from 8.6 to 36 in Europe [7]. Figure 2 shows  
16 information about consumption of antibiotic in Czech Republic from 1985 to 2008.  
17 Nowadays, In Czech Republic, a value is about 20 DDD/1000 persons/day. However, these  
18 data include some uncertainty and the consumption could be still higher [40].

19 Antibiotics are optimised with regard to their pharmacokinetics in the organisms;  
20 organic accumulation is similar to other pharmaceuticals and they are excreted as parent  
21 compounds or metabolites [41]. Excretion rates depend on the substance, the mode of

1 application and it has been shown that rates vary (e.g. tetracyclines and sulphonamides  
2 varying in range 40-90%) [42].

3 Antibiotic are frequently detected in effluents at levels from below 1 ng/L up to a  
4 few µg/L [37]. Several studies confirmed that in some Asian countries the concentrations are  
5 up to several mg/L. Antibiotic resistant bacteria are found in the aquatic environment, but  
6 the contribution of effluents to this development are not very clear [43].

7 Pharmaceutical WWTPs generates a high amount of pollution during the  
8 manufacturing process, housekeeping and maintenance operations [44]. Because of good  
9 manufacturing practice regulations (required for the manufacturing of pharmaceuticals) and  
10 the frequently high economic value of the active substances, the amount of emissions  
11 occurred during the manufacturing has been thought to be negligible. Indeed, such emissions  
12 are assumed to be low in Europe and North America [38].

13 Antibiotics are used to promote the growth of some animals in some countries where  
14 they are used at low doses in animal feeds and are considered to improve the quality of the  
15 product, with a lower percentage of fat and higher protein content in the meat [7]. Therapy  
16 includes almost all antibiotic treatment of animals showing frank clinical disease. Therefore,  
17 the disease decreases animal performance in livestock production, till the use of antibiotics  
18 started in the population.

19 Antibiotics have been used since the 1950s to control certain bacterial diseases of  
20 high-value fruit, vegetable and ornamental plants. Today, the antibiotic most commonly  
21 used on plants is streptomycin with oxytetracycline to a minor extent. Primary uses are on  
22 apples, pears and related ornamental trees for the control of blights [7].

23 The definition of aquaculture, according to Food and Agriculture Organisation  
24 (FAO), is “Aquaculture is the farming of aquatic organisms: fish, molluscs, crustaceans,  
25 aquatic plants, crocodiles, alligators, turtles, and amphibians. Farming implies some form of  
26 intervention in the rearing process to enhance production, such as regular stocking, feeding,  
27 protection from predators, etc.” [45]. In aquaculture, antibiotics have been used mainly for  
28 therapeutic purposes and as prophylactic agents.

29

### 3.1.2 Occurrence and fate of antibiotics in the environment

Widespread occurrence of antibiotic in the environment is well reported in the literature, and is recognized as an important emerging issue in the field of environmental chemistry [3] Antibiotics are not completely eliminated, as they are bioactive substances, acting effectively at low doses and excreted after a short time of residence [4].

Research has quite extensively studied the presence of antibiotics in the environment. After administration, antibiotics for human use or their metabolites are excreted into the effluent and reach the STPs. Active substances discharged with liquid manure can be washed off from the top soil after raining [7]. As for other pharmaceuticals, the concentrations of antibiotics measured in different countries have been found in the same range of concentrations in the sewage and surface water, respectively. In general, concentrations were in a higher 1 g/L range in hospital effluents, in a lower g/L range in the municipal waste water and in the higher and lower than g/L range in different surface waters such as ground water and sea water in harbours. Some antibiotics have been rarely found in drinking waters [7].

Some studies proved the potential of veterinary medicines to be taken up from soil by plants. Soil analyses indicated that, for selected substances, measurable residues of these are likely to occur in soils for at least some months following the application of manure containing these compounds. Some antibiotics are taken up by vegetables such as carrot roots (tubers), lettuce leaves and corn.

In intensive fish farming, infections are treated by feeding with antimicrobial agents putting them directly into the water. The substances used in fish go directly from the water without undergoing any kind of purification process. This phenomenon had already been investigated and the results demonstrated the presence of antibiotics applied extensively in fish farming in sediments beneath fish farms [7].

All antibiotics are degraded in natural ecosystems and some will disappear as a pollutant from natural ecosystems [10]. Several antibiotics (e.g.  $\beta$ -lactams, tetracycline) are non-biodegradable under aerobic conditions, only penicillin G was completely mineralized. Modelling antibiotic degradation provides some complications, for example the biodegradability of lincosamine in a sequence batch reactor was worse with municipal waste water than with synthetic waste water [7].

### 1 **3.1.3 Bacteria resistance and effect of antibiotics in the environment**

2 Several antibiotics are natural compounds that have been in contact with  
3 environmental microbiota and they are biodegradable, even they can be used as food  
4 resource for several microorganisms. Anyhow, since antibiotics are efficient inhibitors of  
5 bacterial growth produced by environmental microorganisms, it has been widely accepted  
6 that their role in the nature will be to inhibit microbial competitors.

7 The resistance that may develop in STPs is currently under discussion. In biofilms the  
8 bacterial density is very high, both in the aerobic and anaerobic septic tanks of STPs, in  
9 drinking water tubes and also in sediments. The sediment samples from antibiotic-polluted  
10 environments have higher antibiotic concentrations than the water samples from the same  
11 place [7].

12 The resistance of the bacteria to antibiotics have been found in the aquatic  
13 environment and in the soil [7]. A pre-requisite for a direct transfer of resistance is that the  
14 bacteria are able to survive, or at least that the genetic material is finally stable enough for  
15 the transfer to the new environment, e.g. from the human body to the surface water, where it  
16 is colder and poorer in nutrients.

17 Also antibiotic resistance genes can easily spread among bacterial species (or clones)  
18 that are closely phylogenetically related [7]. In general, the knowledge of subinhibitory  
19 concentrations and their effects in environmental bacteria is poor, especially with regard to  
20 resistance [33]. Concentrations that are under therapeutic levels could be important in the  
21 development of the resistance in some bacteria and their genetic transfer. The exposure of  
22 bacteria to sub-therapeutic antimicrobial concentrations is thought to increase the speed at  
23 which resistant strains of bacteria were developed. Resistance can be transferred to other  
24 bacteria, living in other environment places, for example ground water or drinking water [7].

25 Some effects of antibiotics on the growth of the plants have been reported, the main  
26 impact of these pollutants will be on the environmental microbiota. The effect of the  
27 antibiotics used for farming has mainly focused on foodborne pathogens. These bacteria  
28 (*Campylobacter jejuni*, *E. coli*, *Salmonella* or *Enterococcus faecium*) are present in animals  
29 and can infect humans. For those pathogens, both mutation-driven antibiotic resistance and  
30 the acquisition of antibiotic resistance genes are very important in concern for the human  
31 health, because the same strain can colonize animals and humans.



1 Antibiotic pollution can enrich the population of intrinsically resistant  
 2 microorganisms, and reduce the population of susceptible microbiota. For instance  
 3 Cyanobacteria (produce 1/3 of total free O<sub>2</sub> production and CO<sub>2</sub> fixation) are susceptible to  
 4 some antibiotics and the consequence of the antibiotic pollution might have for the  
 5 biosphere reinforced the idea that the release of antibiotics in natural environments will have  
 6 relevant consequences for the maintenance of the global activity in the microbiosphere [10].

### 7 3.1.4 Fluoroquinolones

8 Quinolones, derivatives of quinine, were discovered in the early 1960s, followed by  
 9 the development of the FQs in the 1970s and 1980s. The FQs were designed to extend the  
 10 spectrum of activity and improve pharmacokinetic properties compared with the quinolone  
 11 compounds. Table 1 shows of FQs structure, their substituents and specific functions of each  
 12 of them.

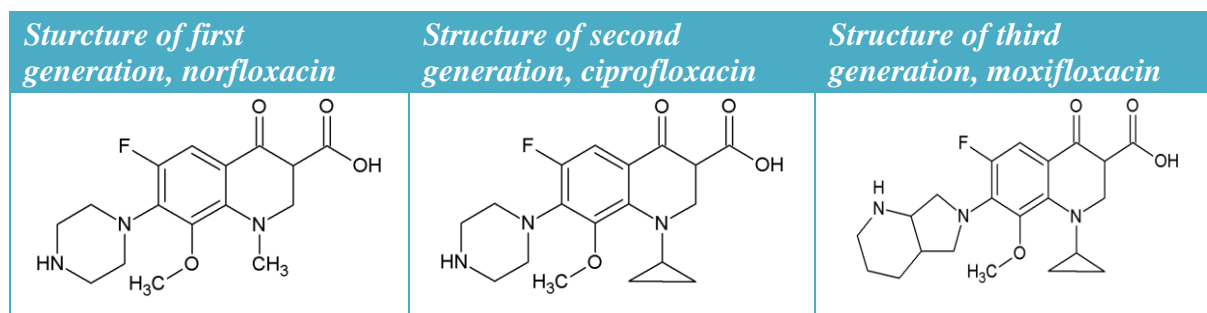
13 Chronologically, they were developed in "generations". First generation of FQs has  
 14 got a six member rings at the position 7. Second generation has got a cyclopropyl group at  
 15 the position 1 and the third generation has got five member rings or azabicyclo ring at the  
 16 position 7. Structures of these three generations are shown in Table 2. The antibacterial  
 17 activity of these compounds is also pH-dependent, because these drugs act by inhibition of  
 18 bacterial DNA gyrase, a process depends on pH [33, 36].

<i>Structure</i>	<i>Substituent</i>	<i>Function</i>
	-R1	Potency and affect pharmacokinetics
	-R2	Close to gyrase binding site
	-R3	Potency gram-positive activity
	-R4	Potency spectrum and affects pharmacokinetics
	-F	Gyrase and bacterial potency

19 **Table 1: Structure of fluoroquinolones, function of substituents [33].**

20

1



2

**Table 2: Generations of fluoroquinolones [32].**

3 The structure of FQs antibiotics has got some ionisable functional groups, the pKa of  
4 aniline (4.60) and pyridine (5.23) are very small, the nitrogen atoms at position 1, 8, and N-1  
5 of the piperazine ring can hardly have acid–base properties within pH ranges of  
6 environmental importance [33, 46].

7 Ofloxacin, norfloxacin, and enofloxacin, etc., have two relevant ionisable functional  
8 groups, the 3-carboxyl group and N-4 of the piperazine substituent. For 6-fluoro derivatives,  
9 for example norfloxacin and enofloxacin, C–F bond cleavage is less effective. The  
10 fragmentation of this C–F bond is a heterolytic process. This reaction is very interesting  
11 because there are very few examples of this cleavage for this strong bond [33, 47].

12 Studies that use test systems indicate that FQs remain active against the different  
13 groups of bacteria presented in waste water or soil. The selective toxicity of the FQs towards  
14 bacteria becomes clearly apparent. Table 3 shows some bacteria together with FQs, which  
15 effecting this bacteria.

<i>Organism</i>	<i>Compound</i>
<b>Sewage sludge bacteria</b>	ciprofloxacin
<b>Escherichia coli</b>	Nalixidix acid, norfloxacin, ciprofloxacin, ofloxacin
<b>Microcystis aeruginosa</b>	Flumequine, oxonilic acid
<b>Rhodomonas salina</b>	
<b>Selenastrum capricornutum</b>	
<b>Artemia salina</b>	Flumequine
<b>Vibrio fisheri</b>	Cinoxacin, Flumequine, lomefloxacin, nalixidic acid, norfloxacin, ofloxacin, oxonilic acid, pipemidic acid, piromidic acid

16

**Table 1: Organisms affected by quinolones [33].**

17 FQs are strongly absorbed and they tend to get accumulated in soil and sediments.  
18 Accumulation of FQs in soil mostly depends on its photostability, its binding and absorption

1 capabilities, its persistence and leaching by water. Most of FQs are very stable from the  
2 chemical point of view in relation with the hydrolysis and the high temperatures but are  
3 photolysed by UV light [33]. The most common photochemical reaction is defluorination.  
4 Field experiments were performed after the application of sludge to agricultural land and  
5 were confirmed the long-term persistence of the trace amounts of FQs in sludge-treated soils  
6 and they revealed their limited mobility into the subsoil. Ciprofloxacin and enrofloxacin  
7 have been determined quantitatively in effluents from urban wastewater treatment plants,  
8 and their behaviour during the mechanical and biological wastewater treatment has been  
9 studied by mass-flow analysis [33, 47]. These compounds are substantially eliminated in  
10 wastewater treatment (80–90%) by sorptive transfer to sewage sludge. It has been proved  
11 that ciprofloxacin which is in the river water samples was completely degraded after 3  
12 months, whereas only 20% of oxolinic acid present in these samples was degraded after five  
13 months. In experiments under aerobic conditions only 60% (kept in dark) and 87.5%  
14 (exposed to light) of initial ciprofloxacin potency was retained. In some plants studies, a  
15 decline in the plant growth was observed on the exposure to enrofloxacin. The findings are  
16 supported by previous laboratory in-vitro studies, in which the growth and development in a  
17 range of plants (e.g. *Phaseolus vulgaris*, *Glycine nax*, *Medicago sativa*, and *Zea mais*) were  
18 affected by veterinary medicines and in-vivo studies in which enrofloxacin at 5 mg/kg  
19 affected root and leaf growth [33]. The wastewater treatment process of quinolones-polluted  
20 waters removed efficiently these antibiotics using a process that included not only  
21 biodegradation, but also photodegradation [10].

22

## 1 **3.2 Steroid hormones**

### 2 **3.2.1 Sources of estrogens in the environment**

3 Estrogens have been identified like one of the most serious contributors to endocrine-  
4 disrupting effects observed in environments, since they are already effective at a lower ng/L  
5 level [49]. Most of the other chemicals having an estrogenic effect are biologically active at  
6 the µg/L level [19, 50]. Compounds related to these effects include a wide range of  
7 substances. A lot of compounds are classified as priority substances in the EU's Water  
8 Framework Directive (2000/60/EC). Due to the uncertainty in their impacts on terrestrial  
9 and aerial organisms as a result of lack of data, E1, E2 and EE2 are not yet included in the  
10 classification list of 146 substances with endocrine disruption, nevertheless, their  
11 femonisation effects in invertebrates and fish have been confirmed worldwide [1, 49].

12 The natural and synthetic estrogens cannot be removed completely in WWTPs and  
13 they are discharged into environmental waters, where they can reach high concentrations at  
14 the ng/L [51]. The majority of the studies reporting the occurrence of estrogens in surface  
15 waters are from the USA and European countries [1].

16 Natural occurrence of estrogens was reported. In Table 4 can be seen daily  
17 production of progesterone, testosterone and estrogens by humans. Estradiol (E2) and its  
18 main metabolites, estriol (E3) and estrone (E1), along with their conjugates (usually sulfates  
19 and glucuronides) are naturally presented in females at a higher level than in males.

20 It is known that steroid hormones also occur in tissues of non treated cattle,  
21 pigs and poultry. However, mammals and birds are not the only organisms that  
22 synthesize steroid hormones. The occurrence of steroid hormones in fish plasma has been  
23 frequently reported. Animal products such as milk and milk products contain steroid  
24 hormones as well. The presence of steroid hormones, especially of estrogens, in plants is  
25 controversial. Even single cell organisms like yeast are said to produce steroidal hormones,  
26 which commonly work as chemical messengers [52].

	<i>Progesterone</i> (µg/day)	<i>Testosterone</i> (µg/day)	<i>17β-estradiol and estrone</i> (µg/day)
<b>Men</b>	420	6480	140
<b>Women</b>	19600	240	630
<b>Prepubertal boy</b>	150	65	100
<b>Prepubertal girl</b>	250 µg/day	32	54

27 **Table 2: Daily production of progesterone, testosterone and estrogens in humans [52].**

1           Anyhow, recent publications suggest that steroids may be the main source of  
2           estrogenicity in many municipal STPs [53]. Effluents from STPs can be discharged into  
3           rivers estrogenic contaminants. Feminizing pollutants 17 $\alpha$ EE2, from oral contraceptives, and  
4           17E2, as well as their metabolites estrone (E1) and estriol (E3) have been detected at ng/L  
5           levels in natural water and WWTP influents/effluents [54]. The inactive hormones can be  
6           converted into their estrogenic active forms and therefore STPs can serve as a secondary  
7           source of environmental pollution.

8           Several studies demonstrated intersex fish downstream of STPs outfalls, therefore  
9           was the research focused on municipal effluents as sources of estrogenic chemicals.  
10          Subsequently the natural estrogens 17 $\beta$ E2, E1, E3 and the synthetic steroid estrogens  
11          17 $\alpha$ EE2 were identified as causative compounds [55]. Thus, it is important to evaluate  
12          whether these act as chemical reactors producing free estrogens, or if they act in a positive  
13          role removing free estrogens eventually formed upstream of STPs [16].

14          The high proportion of manure in compare to the land ratio often leads to the practice  
15          of applying the manure at disposal rates rather than agronomic rates (i.e., rates based on  
16          current soil nutrient levels and plant needs), further increasing the potential of hormone  
17          contamination in surface and ground waters.

18          Animals from farms can excrete large amounts of steroid hormones, e.g., estrogens  
19          (E2, E3, E1), androgens and progestagens (testosterone, progesterone) and some species can  
20          produce up to a few mg of hormone per animal per day [39] [55]. These natural hormones  
21          are concentrated in animal manure and may be released to the environment through  
22          overflows or leakages from storage structures or land applications, contaminating potentially  
23          the surface and ground waters [39].

24          For example, the estimated overall hormone excretion in the US is over 330 metric  
25          tons per year. In the State of New York, there are located farms where glacial outwash is  
26          abundant in streams and river valleys. The high permeability of the glacial outwash in these  
27          areas makes the groundwater vulnerable to the contamination. Many of the farms are also  
28          connected to headwater streams, which may be contaminated by animal steroid hormones  
29          [39].

30          Despite the fact that estrogens are relatively weakly sorbed, a number of field  
31          studies have demonstrated that sediments could act as a sink for these compounds in  
32          riverine, estuarine and marine environments, with concentrations up to 1000 times higher in

1 bed sediments than in the overlying water column. Furthermore, in case of sediment  
2 resuspension and estrogens remobilization, sediments may act as a secondary source of  
3 exposure to aquatic organisms living in the water column [56].

4 Since the sources of natural estrogens cannot be eliminated, a number of specific  
5 treatment processes in STPs have been optimized and discussed with regard to the estrogens  
6 removal [53]. Therefore, monitored concentrations of the conjugated as well as the un-  
7 conjugated estrogens are important [17, 49, 57]. In some countries, a large fraction of  
8 domestic wastewater is treated by activated sludge STPs.

### 9 **3.2.2 Occurrence and fate of estrogens in the environment**

10 Estrogenic contamination of surface waters is a concern of worldwide, with cases such  
11 as the feminization of male fish, reproductive abnormalities and skewed sex ratios attributed  
12 to the presence of steroid estrogens and xeno-estrogens [57]. Ethinylestradiol, the estrogens  
13 in many hormonal contraceptives, is at least in part responsible for the feminization of the  
14 fish in downstream from the sewage treatment plants [55].

15 Studies about estrogens in river waters revealed a lower concentration than in sewage  
16 waters [54]. For example, the research in some German rivers and streams showed that only  
17 estrone was presented and in other various Catalan studies, estrone and estrone-3-sulfate  
18 were detected [53]. In Table 5 are presented concentration of influent, effluent and removal  
19 rete in various countries in the world [55]. WWTPs appear to be less effective and more  
20 variable when remove E1 and EE2 [58].

21 Servos et al. [59] reported the presence of E1 and  $17\beta$ E2 in eighteen Canadian  
22 WWTPs; the last one was removed by 75–98% in the treated effluent, while the former was  
23 removed up to 98% although in several plants its concentration in the effluent was higher  
24 than in the influent. Similar monitoring campaigns were for example kept in Germany,  
25 Canada, Brazil, Italy and Japan [55].

26 Prior to excretion, estrogens are inactivated via sulfonation or glucuronidation. For  
27 wastewaters containing freshly excreted manure or urine, measurement of free estrogens  
28 alone may underestimate the total load of steroid estrogens entering the environment. The  
29 analysis of three dairy wastewater samples showed conjugated estrogens at similar  
30 concentrations to that of free estrogens [55].

31

1

Country	Influent concentration			Effluent concentration			Removal rate		
	(ng/L)			(ng/L)			(%)		
	E1	E2	EE2	E1	E2	EE2	E1	E2	EE2
Switzerland	24	7.6	4.3	2.4	<0.5	<0.5	90	>93	>88
	7.3	4.9	0.7	8.6	1.0	<0.5	-	80	>29
Germany	74.9	10.9	5.2	<1.0	<1.0	<1.0	>99	>91	>81
	27	15	2.4	25	5.4	2.4	10	64	0
Japan	113	20.6	-	18.2	3.19	-	83.9	84.5	-
	197	25.8	-	30.8	2.54	-	84.3	90.1	-
	28.7	22.9	-	2.8	0.49	-	90.3	97.9	-
Netherlands	11	-	0.5	2.7	-	-	75		
	42	14	<1.4	15	1.1	<1.4	64	92	-
	18	-	<0.2	<0.4	-	<0.2	98	-	-
	100	31	<1.4	6.3	0.7	<1.8	94	98	-
	87	9	8.8	2.1	<0.6	<0.2	98	94	98
	140	48	1.3	47	12	<0.3	66	75	77
Brazil	40	21	4.2	6.8	<0.4	0.9	83	99	78
Australia	54.8	22.0	<5.0	8.1	0.95	<0.1	85	96	-
Italy	71	16.1	3.9	9.6	1.5	0.4	86	88.9	87.2
	50.4	9.3	2.3	7.7	0.8	0.4	83.8	91.5	85.8
	67	9.2	3.4	4.1	0.9	0.5	93.9	87.6	84.3
	36.8	11.5	3.0	13.9	1.0	0.4	64.3	91.7	87.1
	35.2	8.6	2.9	30.3	1.9	0.5	18.8	76.2	82.9
	50.6	14.7	2.5	44.6	2.4	0.5	22.1	83.6	84.9
Sweden	14.5	3.2	<10	3	<1.6	<10	79.3	>50	-

2

**Table 3: Influent and effluent concentrations of WWTP in different countries [55]**

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9

The glucuronide metabolites have low estrogenic activity compared with free forms [55]. These inactive hormones can be converted into their estrogenic active forms mainly by  $\beta$ -glucuronidase and arylsulfatase enzymes of bacteria during the waste treatment processes. A number of searchers suggested that de-conjugation occurs during the STP process [55]. *Escherichia coli*, which is eliminated in large quantities in the faeces, it is able to synthesize large amounts of the  $\beta$ -glucuronidase enzyme, which has been suggested to be the responsible for the above transformation [17].

10

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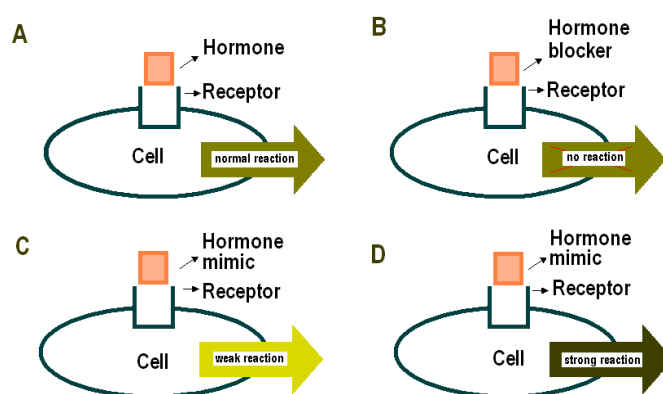
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Several recent studies have confirmed the ability of processes like electrochemical oxidation, ultrasound irradiation, ozonation,  $\text{TiO}_2$  photocatalysis and  $\text{H}_2\text{O}_2$ -promoted photolysis to degrade estrogens like E1, E2 and EE2 [60].

1 Major elimination of estrogens would take place when they arrive at WWTPs.  
2 Although estrogens removal in WWTPs is a complicated process including cleavage of  
3 conjugates, sorption to activated sludge (AS) and biodegradation by AS, the decrease of  
4 estrogens concentration through the AS treatment is primarily due to the biodegradation.  
5 The removal due to the sorption onto excess AS was found to be insignificant, less than a  
6 few percentages [58].

### 7 3.2.3 Effect of estrogens

8 EDCs are divided in four main categories, namely natural estrogens, synthetic  
9 estrogens, phyto-estrogens and various industrial chemicals (i.e. pesticides, persistent  
10 organochlorines, organohalogenes, alkyl phenols, heavy metals). Some of the various  
11 categories, natural and synthetic estrogens exhibit much stronger estrogenic activity than  
12 phyto- and xeno-estrogens [51].



13

14 **Figure 3: Effect of estrogens, possible receptor interactions. Figure was modified from [54].**

15 The endocrine system regulates the reproduction, the metabolism, the growth and  
16 development, the natural defences to the stress, as well as water, electrolyte and the  
17 nutritional balance of the blood. This system is an integrative system that controls the cell  
18 function and activities by communicating through chemical messengers, the hormones [4].  
19 As messengers are used hormones. Normal, strong, weak or even no reactions are provided  
20 in dependence on the hormones or mimic hormones. The mechanisms of interactions are  
21 shown on Figure 3.

22 The term xenoestrogen is often used for compounds that possess estrogenic activity,  
23 whereas the term synthetic estrogens refer to medical drugs that are mainly used for  
24 contraception and treatment of various diseases. Synthetic estrogens, such as the potent  
25 estrogen ethynyl estradiol (EE2), are used extensively for contraceptive and therapeutic



1 purposes (management of menopausal syndrome and in a wide range of cancers, mainly  
2 prostate and breast cancer) [61, 62].

3 Estrogens at very low concentrations can adversely affect to the production of  
4 vitellogenin in male fish and to the sex ratio of fish populations [34, 63, 64]. Several studies  
5 have shown that also birds, reptiles and mammals in polluted areas undergo alterations of  
6 the endocrine–reproductive system. Vitellogenin is a classic steroid-inducible protein; it is  
7 synthesized in the liver under the control of estrogens. Sullivan et al. described a screen that  
8 defines estrogens as something that induces vitellogenesis. The exposure to environmental  
9 estrogens, single or in combination, may be easily assessed in male fish, reptiles or birds and  
10 can be used as sentinels by measuring their vitellogenin plasma levels. However, this marker  
11 cannot be used in reproductively active females since ovarian estrogens would induce  
12 vitellogenin and proceed to obscure the contribution of xenoestrogens to vitellogenin plasma  
13 levels [18, 65].

14

### 1 **3.3 Benzimidazole fungicides**

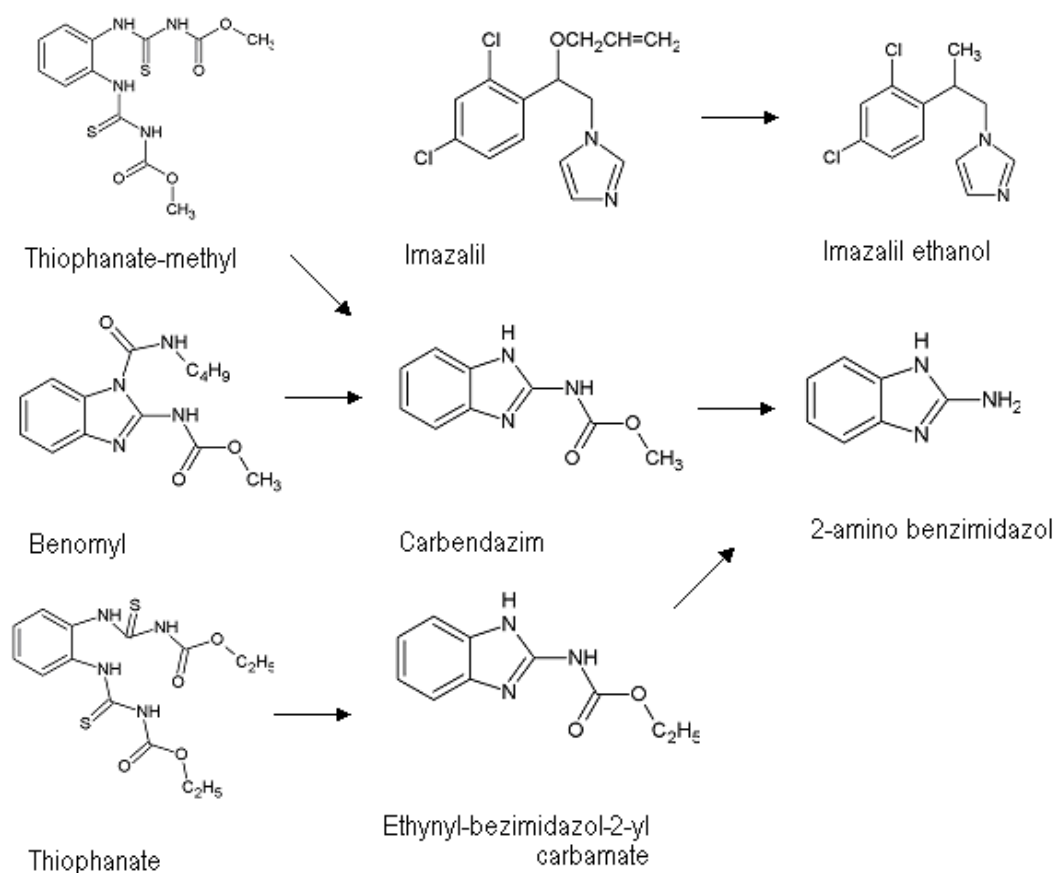
2 Being conscious that the amount of pesticides employed per year is increasing, the  
3 amount of fungicides released to the environment increases at the same rate [65]. Fungicides  
4 are very important in environment and agriculture; they are used to kill or inhibit fungi or  
5 fungal spores. They can be classified according to different criteria but the most usually  
6 employed are mode of action and chemical group [49].

7 Benzimidazoles are anthelmintic agents widely used in the treatment of parasitic  
8 infections in a wide range of species and as fungicidal agents in the control of spoilage of  
9 crops during storage and transport. Some benzimidazoles have also found applications as  
10 pre- or post-harvest fungicides for the control of a wide range of fungi affecting field crops,  
11 stored fruit and vegetables [65].

12 Agricultural applications of pesticides lead to the contamination of surface waters or  
13 sewage system through point (filling of sprayers, cleaning of spraying equipment, disposing  
14 of packing material, etc.) and non-point (runoff) sources. Uses of pesticides in urban areas  
15 may cause an input of pesticides into the WWTPs (e.g., washing of spraying equipment,  
16 disposal of unused products, etc.). Occurrence and fate of fungicides during wastewater  
17 treatment processes are largely unknown and very few studies are available [66]. The main  
18 area of concern with benzimidazole fungicides involves their negative effects in non-target  
19 plant crops [67].

20 Benomyl has been widely used as a systematic fungicide for a variety of food crops  
21 and ornament plants [68]. Besides its carcinogenic activity, it has been known for several  
22 years that chronic, subchronic and acute administration of benomyl to rats and mice resulted  
23 in male reproduction damage [69, 70]. Benomyl and its main metabolite carbendazim are  
24 systemic benzimidazole fungicides that play a very important role in plant disease control.  
25 These two chemicals are widely used to combat a broad range of diseases on many arable  
26 plants, including cereals and oilseed rape, as well as on fruits, vegetables and ornamentals.  
27 They are not only used during pre- and/or post-harvest treatments of plants, but have also  
28 extensive applications in post-harvest food storage. Benomyl and carbendazim are strongly  
29 absorbed on soil organic matter, but they can also be absorbed [68]. Thiophanatemethyl is  
30 used in crop protection and animal health, while benomyl is used in crop protection only. It  
31 is well established that carbendazim, the common stable metabolite of benomyl and  
32 thiophanatemethyl, is considered as the major fungitoxic principle of the benzimidazole

1 precursor fungicides. Some benzimidazole fungicides and the related metabolites and  
 2 derivatives mentioned are illustrated in Figure 4 [64, 67, 71].



3

4

**Figure 4: Structures of benzimidazoles and metabolites [72]**

5

Regulatory limits for these fungicides are generally all expressed as carbendazim, the  
 6 single measurement marker for the food. On account of their extensive use, residual  
 7 environmental impact and toxic effects at low levels, the regulations have set maximum  
 8 residue levels (MRLs) for benzimidazoles and their metabolites. The environmental impact  
 9 of the total benzimidazole-containing residues should be in a range of 0.01–10 mg/kg,  
 10 depending on the fungicide–commodity combination. In particular, for the majority of  
 11 benzimidazoles, the marker residue tolerance has recently been defined as the sum of a  
 12 parent drug and/or its related metabolites (sum-MRL substances) instead of single  
 13 compounds [72].

14

## 4 Sample preparation in environmental analysis

### 4.1 Methodology of sample preparation

#### 4.1.1 Liquid phase extraction

The liquid-liquid extraction (LLE) is based on the transfer of analytes from aqueous samples to a water immiscible solvent and is widely employed for sample preparations. Some shortcoming (e.g., emulsion formation and use of large sample volumes and toxic organic solvents) makes LLE more expensive, time consuming and environmentally an unfriendly method [73].

In LLE the sample is agitated in the presence of an extracting solvent that is not miscible with the sample. When the sample/solvent mixture has settled after agitation, two layers of liquids are visible, one of them will contain most of the compound that we are extracting. The shaking action has ensured that all parts of the sample come into contact with the extracting solvent. The compounds from the sample may pass into this extracting solvent and given time, and the equilibrium will be established between the two liquid layers. The equilibrium is described by the partition coefficient for the analyte, which is simply the ratio of concentrations for the analyte in the two liquids [74].

#### 4.1.2 Liquid phase microextraction method

To reduce costs and simplify the extraction procedure, new techniques have been developed, which are an alternative to miniaturized sample preparation (e.g. liquid-phase microextraction, LPME). Liquid-phase microextraction technique was developed in the 1990s, which is a miniaturized format of LLE. LPME is a solvent-minimized samplepretreatment procedure of LLE, in which only several mL of solvent are required to concentrate analytes from various samples rather than hundreds of mL needed in traditional LLE [75]. The research for this technique began by using small droplets of organic solvents suspended from the tip of a microsyringe needle. Table 6 shows classification of various microextraction techniques based on liquid phase microextraction [73].

27  
28

<i>Abbreviation</i>	<i>LPME</i>	<i>Modifications</i>
<b>SDME</b>	Single drop microextraction	Direct immersion (DI-SDME)
		Directly suspended droplet microextraction (DSDME)
		Dispersive liquid-liquid microextraction (DLLME)
		Hollow fibre (HF-LPME)
		Continuous flow microextraction (CFME)
<b>HF-LPME</b>	Hollow fibre microextraction	
<b>SDLPME</b>	Solid drop microextraction	
<b>DSDME</b>	Directly suspended droplet microextraction	
<b>DLLME</b>	Dispersive liquid-liquid microextraction	

2

**Table 6: Classification of LPME approaches with abbreviations. Modified from [73]**

3

Single-drop microextraction (SDME) is an LPME technique in which the extraction medium is in the form of a single drop of an immiscible extracting solvent suspended from a syringe into the liquid or gaseous sample medium. After the extraction, the organic drop is retracted back into the microsyringe and injected into the chromatographic system. This technique is not exhaustive, and only a small fraction of analyte is extracted and preconcentrated for the analysis. Different modes of SDME (e.g. DI-SDME, LLLME, CFME, HS-SDME) have been developed for various analytical applications [73, 76]. Figure 5A shows schemes of process of extraction by DI-SDME, HS-SDME and CFME.

11

*Direct immersion (DI-SDME)*, a microdrop of a water-immiscible organic solvent, is either immersed into a large flowing aqueous drop or held at the end of a teflon rod and suspend in a stirred aqueous sample solution to complete the extraction process [77]. Jeannot and Cantwell used a microsyringe containing the organic solvent in their study [78]. The droplet of the organic solvent is at the tip of the syringe needle. This needle is immersed in a stirred aqueous sample. After the extraction, the organic phase is drawn back into the microsyringe, which is used directly for the determination of the analytes. This approach is better suited for the separation and enrichment of non-polar or moderately polar analytes from relatively clean matrices. However, problems of stability of the drop at high stirring rates and temperatures were observed [73].

21

*Liquid-liquid-liquid microextraction (LLLME)* is a three-phase mode extraction suitable for basic and acidic analytes. The first name of this extraction was “solvent microextraction with simultaneous back-extraction” given by Ma and Cantwell in 1999. An ionizable solute is first extracted into an organic layer (lower density than water is required),

1 followed by the extraction, and then trapping into a second aqueous layer, whose pH  
2 resulted in the ionization of the solute. A recent modification of LLLME reported a large  
3 aqueous droplet, freely suspended at the top-centre position of a layer of immiscible organic  
4 solvent [79].

5 *Continuous flow microextraction* (CFME) makes a drop of solvent fully and  
6 continuously makes contact with fresh and flowing sample solution. The drop can be held at  
7 the tip of PEEK tubing, which is immersed in a continuously flowing sample in the  
8 extraction chamber. However additional equipment is required (e.g., a microinfusion  
9 pumps). There is a limitation to the extraction of non-polar or slightly polar semi-volatiles.

10 *Headspace* (HS-SDME) was introduced in 2001 by Theis et al.. In this technique the  
11 drop remains at the tip of the microsyringe throughout the extraction period and then is  
12 retracted back into the microsyringe. The analytes are distributed among three phases: the  
13 water sample; the headspace; and the organic drop [73, 80].

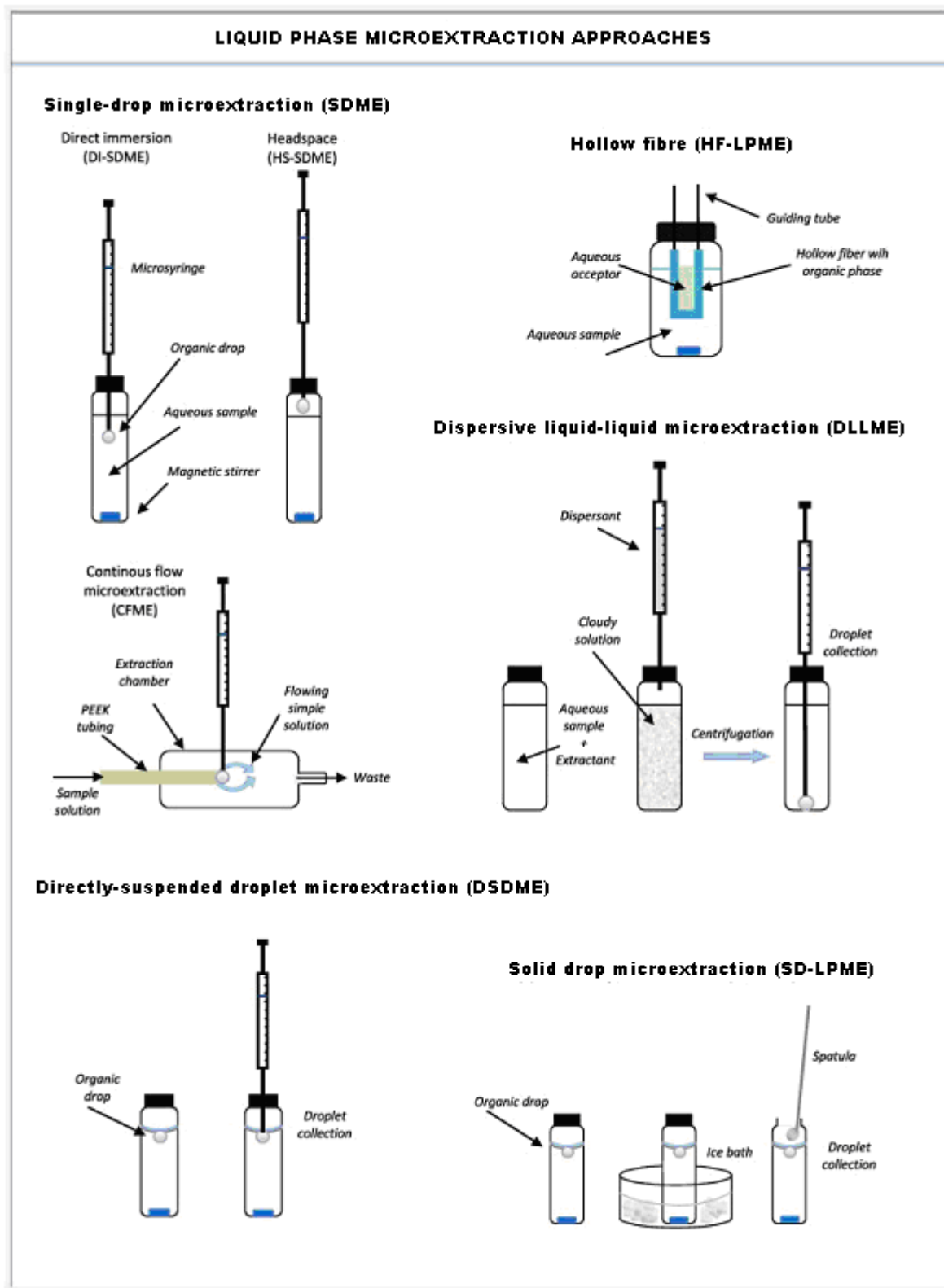
14 *Hollow-fibre liquid-phase microextraction* (HF-LPME) was developed in 1999 to  
15 improve the stability and the reliability of LPME by Pedersen-Bjergaard and Rasmussen  
16 [81]. In this case, the extracting phase is placed inside the lumen of a porous hydrophobic  
17 HF (the microextractant solvent is not in direct contact with the sample solution), which is  
18 inserted into a sample vial filled with the aqueous sample of interest. The analytes are  
19 extracted from the aqueous sample through the organic phase in the pores of the HF and  
20 then into an acceptor solution inside the lumen [82]. Therefore remarkable clean-up  
21 efficiency can be presented, since high molecular mass compounds cannot pass through the  
22 membrane barrier. This technique is suitable for inorganic and organic analytes over a wide  
23 range of polarity [72]. In Figure 5 is presented schema of this microextraction.

24 *Dispersive liquid-liquid microextraction* (DLLME) was published by Rezaee et al. in  
25 2006. Solvents used in this method must have high miscibility with both the extractant and  
26 the aqueous phase [73]. When the mixture of the extractant phase and the disperser is  
27 rapidly injected into the sample, turbulence is high and small droplets are created. After the  
28 formation of a cloudy solution, the surface area between the extracting solvent and the  
29 aqueous sample becomes very large, and the equilibrium is reached very quickly, decreasing  
30 the extraction time. After centrifuging the cloudy solution, the sedimented phase at the  
31 bottom of a conical tube is recovered and analyzed with an appropriate technique [83]. The

1 main disadvantage of DLLME is that it is limited to a small number of extractants due to the  
2 required conditions, and it is difficult to automate [73]. Figure 5 shows schema of DLLME.

3 In the same year as DLLME was developed also directly-suspended droplet  
4 microextraction (DSDME) by Lu and co-workers. When small volumes of an immiscible  
5 organic solvent is added to the surface of the aqueous solution, the vortex results in the  
6 formation of a single droplet near the centre of the rotation. The main disadvantage of the  
7 method is the difficulty of taking out the small amount of suspended droplet from the  
8 solution [73, 75]. Figure 5 demonstrate DSDME process.

9 One year later, 2007, to get over the problem of removing a tiny amount of the  
10 suspended droplet in DSDME was the method modified into solid-drop liquid-phase  
11 microextraction (SD-LPME) method by Khalili-Zanjani et al. [73]. In this method a suitable  
12 organic solvent (less than 20  $\mu\text{L}$ ) is delivered to the surface of the aqueous solution located  
13 in a glass vial. The organic solvent must have a melting point near to the room temperature.  
14 The aqueous phase is stirred for a set time and then the sample vial is transferred into an ice  
15 bath. After a short period of time, the organic solvent is solidified and can be removed using  
16 a small spatula. The solid drop melts quickly at the room temperature, and is retracted by a  
17 microsyringe and injected into an analytical instrument for analysis [84]. Figure 5 presents  
18 schema of SD-LPME method.



1  
2  
3

Figure 5: Scheme of microextraction methods [72].



### 1 **4.1.3 Solid phase extraction**

2 Solid phase extraction, as a scientific technique was developed in the 1970s. The  
3 course of development as a sample preparation technique progressed from initial latency  
4 (prior to 1968) through three subsequent phases (1968- 1977, 1977- 1989, 1989-present).  
5 SPE was practiced for at least two decades before 1968, when applications using synthetic  
6 polymers (such as styrene-divinylbenzene resins) were first published in the literature [74].

7 The introduction of prepackaged, disposable cartridges containing bonded silica  
8 sorbents, in 1977, certainly made the procedure more convenient and initiated another phase  
9 of development. Also that same year the first article using SPE on a bonded phase silica was  
10 published, which described the use of a Sep Pak™ C18 "cell" for the clean-up of histamines  
11 from wines. In 1989, SPE discs (also called "disks" or "membranes") were introduced,  
12 initiating another phase in the development of this method. Lately, designs for housing SPE  
13 sorbents have ranged from "pipette tip" styles to plastic or glass minicolumns with polymer  
14 or steel or teflon frits.

15 Some SPE devices are designed for the sample to be "pushed" through the sorbent  
16 while others are designed for a vacuum use, enabling the sample to be "pulled" through.  
17 Since the late 1980s, however, the extractions of membranes or discs, and "mega" columns  
18 containing several grams of sorbent have grown in popularity [74].

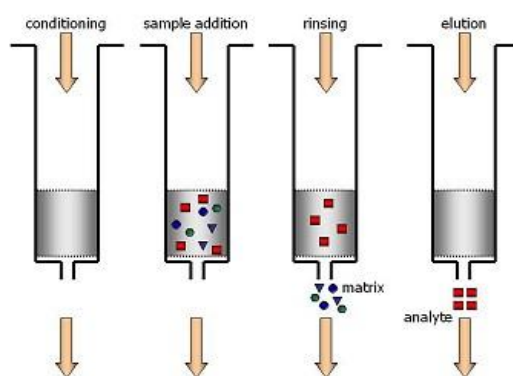
19 Although SPE in the column mode is very effective, it has also some drawbacks,  
20 such as channeling, limited flow rates, insufficient equilibration time for quantitative uptake,  
21 incomplete elution, and memory effects from previous extractions. An SPE device consists  
22 in a resin bed packed into a small extraction tube, usually made of plastic. The resin is  
23 packed between two frits to hold the resin bed securely in place. A liquid sample is passed  
24 through the resin bed by applying either a positive pressure or vacuum the column [85].

25 When the compound distributed between the liquid sample and the solid surface,  
26 either by simple adsorption to the surface or through penetration of the outer layer of  
27 molecules on that surface, equilibrium is set up. SPE simply requires a liquid sample to be  
28 passed through a bed containing sorbent particles onto which the analytes will retain [74].

29 It is possible to pass a large volume of sample through the smallest bed of sorbent  
30 that will completely retain all of compound of interest and elute compounds of interest in the  
31 smallest volume of solvent. There are components of the sample that mask the analyte  
32 during the analysis. The cleaned-up extract gives clearly identifiable signals from the

1 extracted components in the sample. Clean-up step may be achieved either by retaining the  
2 analyte on a solid phase sorbent or washing out interferences, or by retaining the  
3 interferences and washing out the analyte.

4 A significant advantage of SPE over LLE is that solvents that are miscible with the  
5 sample matrix may be used to elute the analytes. This eluent can be injected directly into the  
6 reversed phase HPLC system [74].



7  
8 **Figure 6: Scheme of solid phase extraction method [86].**

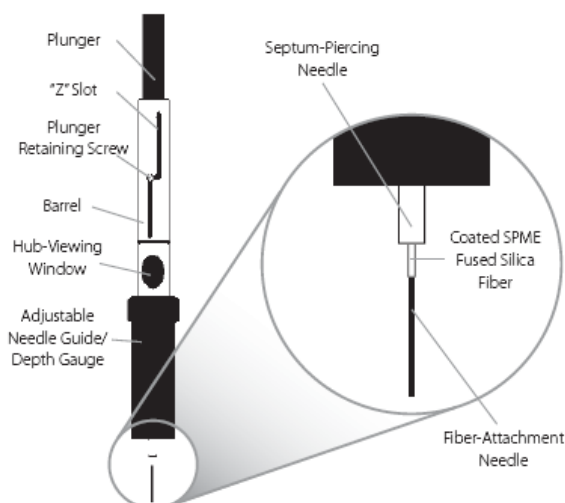
9 The mechanisms involved in the solid phase extraction are [85]:

- 10 **1.** Normal phase chromatography whose procedures typically involves a polar analyte,  
11 a mid- to nonpolar matrix and a polar stationary phase. Polar-functionalized bonded silicas  
12 (e.g. LC-CN, LC-NH<sub>2</sub>, and LC-Diol), and polar adsorption media (LC-Si, LCFlorisil,  
13 ENVI-Florisil, and LC-Alumina) are typically used under normal phase conditions. The  
14 retention of an analyte under normal phase conditions is primarily due to the interactions  
15 between the polar functional groups of the analyte and the polar groups on the sorbent  
16 surface.
- 17 **2.** The reversed phase chromatography whose procedure involves a polar or moderately  
18 polar sample matrix and a nonpolar stationary phase. The analyte of interest is typically mid-  
19 to nonpolar. Several SPE materials, such as alkyl- or aryl-bonded silicas (LC-18, ENVI-18,  
20 LC-8, ENVI-8, LC-4, and LC-Ph) are in the reversed phase category.
- 21 **3.** The Ion exchange chromatography that can be used for compounds which are  
22 charged in a solution. Anionic compounds can be isolated on LC-SAX or LC-NH<sub>2</sub> bonded  
23 silica cartridges. Cationic compounds are isolated by using LC-SCX or LC-WCX bonded  
24 silica cartridges. The primary retention mechanism of the compound is based mainly on the  
25 electrostatic attraction of the charged functional group on the compound to the charged  
26 group that is bonded to the silica surface.

1 Factors affecting ion-exchange selectivity are pH, ionic strength, organic solvent and flow  
2 rate [85].

### 3 **4.1.4 Solid phase microextraction methods**

4 Solid-phase microextraction (SPME) is a relatively recent technique, which was  
5 introduced in the early 1990s by Pawliszyn and Lord. Extraction SPME was commercially  
6 first introduced in 1993 by Supelco (Figure 7) [87].



7  
8 **Figure 7: First commercial solid phase microextraction introduced by Supelco [88].**

9 SPME was developed from the sample preparation process analogous to the standard  
10 solvent extraction procedures. The fibre tip was coated with the sample by dipping one end  
11 of the optical fibre in the solvent extract and then the volatile solvents were removing  
12 through the evaporation. The fibre tip was prepared for inserting into the injector of a GC  
13 system, and the analytes were volatilized onto the front of the GC column by a laser pulse.  
14 However, the original purpose of these coatings was simply to protect the fibres from  
15 breakage. Because of that the thin films used (10-100 $\mu$ m) [87].

16 In the initial work on SPME, sections of fused-silica optical fibres, both uncoated  
17 and coated with liquid and solid polymeric phases, were dipped into an aqueous sample  
18 containing test analytes and then placed in a GC injector. Those early experiments provided  
19 very important preliminary data that confirmed the usefulness of this simple approach, since  
20 both polar and non-polar chemical species were extracted rapidly and reproducibly, from  
21 aqueous samples [87].

22 The development of the technique accelerated rapidly with the implementation of  
23 coated fibres incorporated into a microsyringe, resulting in the first SPME device [89]. The

1 transport of analytes from the matrix into the coating begins as soon as the coated fibre has  
2 been placed in contact with the sample. Typically, SPME extraction is considered to be  
3 completed when the analyte concentration has reached a distribution balance between the  
4 sample matrix and the fibre coating. In practice, this means that once the equilibrium is  
5 reached, the extracted amount is constant within the limits of the experimental error and it is  
6 independent of further increases of extraction time [90, 91].

7 An *in-tube solid phase microextraction* concept (in-tube SPME) has also been  
8 expanded to facilitate the automation of sample preparation for HPLC (Figure 9). In that  
9 approach the sample components are extracted by the coating located on the inner surface of  
10 the hollow tubing and after the extraction is completed the analytes are washed into the  
11 HPLC column using the mobile phase or solvent. In SPME a small amount of extracting  
12 phase associated with a solid support is placed in contact with the sample matrix for a  
13 predetermined amount of time. When the equilibrium conditions are reached, then exposing  
14 the fibre for a longer time does not accumulate more analytes [90].

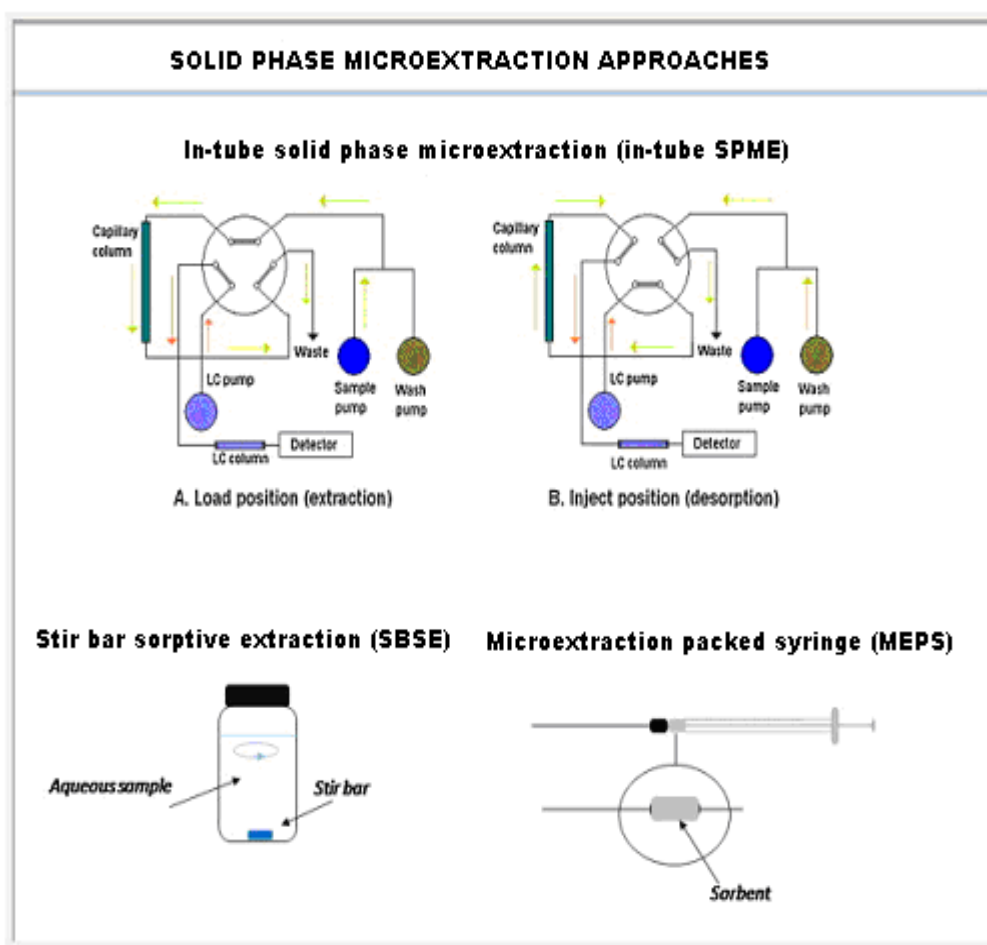


16 **Figure 8: Solid phase microextraction technique 1) an outer coated surface of fibre; 2) coated on internal**  
17 **capillary surface [88].**

18 The tube design can use very similar arrangements as SPE, however the primary  
19 difference, in addition to the volume of the extracting phase, is that the objective of SPME is  
20 never an exhaustive extraction. This substantially simplifies the design of the  
21 systems, because it concerns about breakthrough is not relevant since the exhaustive  
22 extraction is not an objective. In fact the objective of the experiment is producing full  
23 breakthrough as soon as possible, since this indicates that the equilibrium extraction has  
24 been reached [91, 92].

25 An outer coating of SPME and internal coated capillary surface of in-tube SPME  
26 technique are presented in Figure 8.

1 The stationary phase of in-tube SPME can be either prepared by sol–gel technology,  
 2 alternative chemical coating procedures or selected out of the range of commercially  
 3 available GC capillary columns. Examples for the first case are the synthesis of a zirconia-  
 4 based hybrid organic–inorganic sol–gel coating, which has been applied to the determination  
 5 of PAHs in combination with GC, or a  $\beta$ -cyclodextrin coating, used for the determination of  
 6 non-steroidal anti-inflammatory drugs. Another hybrid material to be used as a sorbent in  
 7 ITE is  $\text{TiO}_2$ -PDMS, a capillary coated with this technique was applied to the determination  
 8 of PAHs, ketones and alkylbenzene in aqueous samples [87].



9  
 10 **Figure 9: Scheme of solid phase microextraction techniques [4].**

11 *Stir bar sorptive extraction* (SBSE) was first introduced by Baltussen et al. in 1999  
 12 as a new and improved sample preparation technique. SBSE and SPME are solventless  
 13 sample preparation techniques based on sorptive extraction [93]. These stir bars, called  
 14 twisters® (GERSTEL GmbH & Co.), are coated with a polydimethylsiloxane (PDMS) layer,  
 15 which is the most widely used sorptive extraction phase. At present, only PDMS-coated stir  
 16 bars are commercially available, which is one of the main drawbacks of SBSE, because

1 polar compounds are poorly extracted due to the non-polarity of the PDMS polymer.  
2 Although the basic principles of SPME and SBSE are identical and the extraction phase is  
3 generally the same, the amount of PDMS is 50–250 times larger in SBSE. This feature  
4 allows the preconcentration efficiency to be improved compared to SPME, which is its main  
5 advantage [87]. Scheme of the method is presented in Figure 9.

6 In general, SBSE is considered to be superior to SPME in terms of sensitivity and  
7 accuracy for determinations of trace levels in complicated matrices, for example  
8 organophosphorous pesticides in honey. Here, like in most of the other applications, a  
9 commercially available 500  $\mu\text{m}$ –1 mm PDMS-coated stir bars was used. A simple PDMS  
10 rubber tubing mounted onto a glass rod was equally found to be suitable for automated  
11 sampling/desorption of a standard mixture of 44 organic compounds, including PAHs,  
12 phthalates, substituted benzenes and other. A similar approach, but including chemical  
13 bonding of a 500  $\mu\text{m}$  PDMS film onto a titanium tube, was used for an herbicide extraction.  
14 A normal polytetrafluoroethylene-coated stir bar was evaluated for sorptive extraction using  
15 phenanthrene as a model analyte [87].

16 Microextraction by packed sorbent (MEPS) is a recently developed technique that  
17 was introduced by Abdel-Rehim in the field of sample preparations. MEPS combines the  
18 sample processing, extraction and injection steps into a fully automated process as an at-  
19 line sampling/injecting device to GC or LC [93]. In MEPS, approximately 2 mg of the  
20 sorbent is thermo-packed inside a syringe (100–250  $\mu\text{L}$ ) as a plug or between the barrel and  
21 the needle as a cartridge. Sample extraction and enrichment take place on the sorbent bed.  
22 MEPS is a miniaturized format of SPE that is able to handle sample volumes as small as 10  
23  $\mu\text{L}$ , and it is also a technique that integrates the sorbent directly into the syringe, not in a  
24 separate column as in commercial SPE [94]. Different types of sorbents are commercially  
25 available, such as reversed (C18, C8, and C2), normal (silica) or ion-exchange stationary  
26 phases. The sorbent can be used several times with an adapter washing and reconditioning it  
27 to avoid the carry-over and to keep the adsorption power of the phase [73]. Scheme of  
28 MEPS is presented in Figure 9.

29 The molecularly imprinted polymers (MIPs) is a highly stable polymers with matrix  
30 that are adapted to the three-dimensional shape and functionalities of target analyte.  
31 Traditional techniques can improve selectivity using novel coating materials. Design  
32 variables include the choice of a monomer (which complexes with the target analyte with a  
33 high binding affinity), cross-linker (the length of which may determine the cavity size) and

1 polymerisation method (which dictates the nature of interactions between the polymer  
2 matrix and the template to dictate the ultimate extraction efficiency). Once the polymer is  
3 formed, the template is removed with an appropriate solvent, leaving a cavity that  
4 corresponds to the specific target analyte [4].

5

## 1 **4.2 Experimental design in sample preparation**

2 It is possible to find out which factors have an effect by changing them one at a time.  
3 This method could be also considered as in-efficient and can give misleading results.

4 The “one-factor-at-a-time” approach provides some disadvantages, such as more  
5 experiments are necessary, no additional information can be obtained in the experiments, it  
6 is not sure that the influence of a given factor will be the same in various values of the other  
7 factors. Additionally, if the results of these experiments are wrong, all conclusions will be  
8 wrong [95].

9 The word chemometrics, invented about 30 years ago, summarize concept of  
10 measurement in chemistry. Chemometrics is the application of mathematical and statistical  
11 methods and the principles of good science of measurement to efficiently extract useful  
12 information from chemical data. This included all processes that transform data and  
13 analytical signals into complex information. The original methods used are mathematical  
14 and statistical [96].

15 The aim of the use of statistical techniques, a factorial designs or response surface  
16 methodology, is a minimized effort, for evaluating the factor effects at several levels of the  
17 other factors. The interaction between factors can be evaluated, which is not possible in a  
18 classical experiment, including one-factor-at-a-time experiments [95, 97]. In recent years,  
19 multivariate applications of statistical techniques increased in the optimization of analytical  
20 methods. Wide spread range of information can be provided by a minimum number of  
21 experimental trials.

22 The experimental design can be defined as the strategy for setting up experiments in  
23 such manner that the information required is obtained as efficiently and precisely as  
24 possible. Experimentation is carried out to determine the relationship (usually in the form of  
25 mathematical model) between factors acting on the system and the response or properties of  
26 the system (the system being a process or a product, or both) and established experimental  
27 design for future optimization [97, 98].

28 It is very important to identify and list all factors which can affect process and  
29 surface response, although it supposes to have only small influence. Additionally, range of  
30 each factor has to be defined for future experiments. Once there are selected few most  
31 important factors, the next step is usually to study quantitatively effect on the response and  
32 their interactions. For this purpose can be used full factorial designs at two levels  $2^k$  [99].



1 In environmental screening studies are often focused to detect outliers in the huge  
2 amount of samples taken from the studied area. Usually, it is expected that most of the  
3 samples will exhibit the “normal” (unpolluted) state while a few (or none) of them will  
4 surpass permissible limits [100].

5 In general, more effective and time saving is using experimental design procedures,  
6 and especially to the so called Response Surface Methodology. However, as a preliminary  
7 step, this methodology requires some screening experiments to be carried out in order to  
8 establish the significant experimental factors, and also to determine the upper and lower  
9 levels for these factors, in an attempt to reach values near to the optimal response [95, 100].

10 In “all factor together” experiment, no information may be obtained by comparing the  
11 results of any 2 experiments. To find the effect of changing anyone of the factors we will  
12 need to use the results of all of the 8 experiments in the design (in case of 2 levels  
13 experiment).

14 The influence of every factor on the yield provides a higher precision and the standard  
15 error of estimation is halved. Additionally, the result of each experiment enters equally into  
16 the calculation of the effects of every factor and possible errors will probably not influence  
17 the general conclusions. Finally, the number of the experiments is the same as for the one-  
18 factor-at-a-time method [95].

### 19 **4.3 Experimental design used in this thesis**

20 Recently, the  $2^k$  design (k, factor number) is particularly useful in the early stages of  
21 an experimental work, and it provides the smallest number of runs with which k factors can  
22 be studied in a complete factorial design.

23 A  $2^3$  two-level factorial design can be preliminarily used to evaluate the significance  
24 of the main and interaction effects of the parameters investigated. The final regression  
25 models were calculated using the central composite design (CCD), which was obtained from  
26 the full factorial design and the star design.

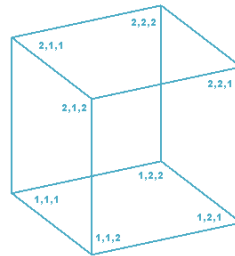
27

1

<i>Number of experiment</i>	<i>Parameter A (range 1-2)</i>	<i>Parameter B (range 1-2)</i>	<i>Parameter C (range 1-2)</i>
<b>1</b>	<i>1</i>	<i>1</i>	<i>1</i>
<b>2</b>	<i>1</i>	<i>1</i>	<i>2</i>
<b>3</b>	<i>1</i>	<i>2</i>	<i>1</i>
<b>4</b>	<i>1</i>	<i>2</i>	<i>2</i>
<b>5</b>	<i>2</i>	<i>1</i>	<i>1</i>
<b>6</b>	<i>2</i>	<i>1</i>	<i>2</i>
<b>7</b>	<i>2</i>	<i>2</i>	<i>1</i>
<b>8</b>	<i>2</i>	<i>2</i>	<i>2</i>

2

**Table 7: Example of experimental design**



3

4

**Figure 10: Experimental design  $2^3$  displayed geometrically**

5

In this work, we have chosen a  $2^3$  factorial design. It provides eight treatment combinations (as shows Table 7) can be also displayed geometrically as a cube (as shown in Figure 10). Because there are only two levels for each factor (usually extreme values), the response is approximately linear in the range of chosen extremes of the factor levels. Afterwards, the results are analyzed by statistical programs (SPSS, Statgraphic). Results can be evaluated by bivariable and partial correlations and give pilot information about possible influences of different parameters. The range of results is usually between 0 and 1, 0 means independent on the other value and 1 means dependent. When parameters of interest are chosen, experiments usually continue in other experimental designs.

14

A number of RSM procedures are available, Box–Wilson Central Composite Design was chosen due to its widespread use and versatility in some studies. This type of design consists of a factorial design together with centre and star points. That allows the evaluation of the relationship between experimental factors and observed results [95,101].

18

In other study, an orthogonal array experiment (Taguchi experiment) with variation of eight experimental parameters in 18 experiments was constructed. For the same research,

19

1 residuals in fruits, an array experiment with variation of four experimental parameters was  
2 also constructed [102].

3 In other published work, experimental design based on Taguchi's method was  
4 employed to screen the SPME conditions for the HF-SPME method extraction and the  
5 determination of the BTEX compounds [103].

6 The Box–Behnken design (BBD), which is a three-level factorial design, can be  
7 introduced as a combination of a two-level factorial design with an incomplete block design.  
8 This design is an efficient option in which the experimental points are located on the  
9 midpoints of the edges of a cube and at the centre (central points) [104].

<i>Number of experiment</i>	<i>Parameter A (range 1-2-3)</i>	<i>Parameter B (range 1-2-3)</i>
<b>1</b>	<b>1</b>	<b>1</b>
<b>2</b>	<b>1</b>	<b>2</b>
<b>3</b>	<b>1</b>	<b>3</b>
<b>4</b>	<b>2</b>	<b>1</b>
<b>5</b>	<b>2</b>	<b>2</b>
<b>6</b>	<b>2</b>	<b>3</b>
<b>7</b>	<b>3</b>	<b>1</b>
<b>8</b>	<b>3</b>	<b>2</b>
<b>9</b>	<b>3</b>	<b>3</b>

10 **Table 8: Example of response surface methodology.**

11

## 1 **5 Results and discussion**

2 This doctoral thesis deals with environmental samples, mainly water samples containing  
3 pharmaceuticals (fluoroquinolones and steroid hormones) and fungicides. Since an amount of  
4 pollutants in the environment is increasing it is necessary to find trustworthy, cheap and  
5 robust determination methods. Recently published methods are summarized and new sample  
6 extraction methods and analytical determination were developed in this thesis.

7

## 1 **5.1 Fluoroquinolones**

2 The occurrence of fluoroquinolones in environmental waters was confirmed by several  
3 studies and some studies using solid phase extraction followed by liquid chromatography  
4 with mass spectrometry determination of these compounds are presented.

5 Fluoroquinolones have two values of pKa for carboxylic group in a range of 5.7 to 6.3,  
6 whereas those for protonated amino group are higher (7.6–8.3). The intermediate form of  
7 FQs is a zwitterions [46].

8 The behaviour of quinolones during solid phase extraction was studied and acidic  
9 quinolones at acidic pH were present as uncharged species in the solution and they were  
10 less retained on C<sub>18</sub> cartridge. The piperazinyl quinolones present in cationic form at acidic  
11 pH were retained pretty well. Therefore, most of samples were adjusted to very acidic  
12 values of pH, in order to ensure that they will be in the desired chemical form. However,  
13 very acidic pH may not be optimum for the preconcentration purposes.

14 An another possibility is the use of cation-exchange mechanism to retain the  
15 piperazinyl quinolones over a wide range of pH values. FQs can be extracted using cation-  
16 exchange sorbents when they are in cationic form which means that the pH of the sample  
17 has to be below to the pKa constants [105].

18 Samples of quinolones were usually adjusted to pH in the acidic range in sample  
19 preparation by SPE. The best value to assure that FQs are in cationic form should be two  
20 units below the pKa constants [46]. A possible following step in the sample preparation of  
21 quinolones is an addition of EDTA. FQs can be bound to divalent cations and could not be  
22 effectively retained on SPE cartridges and determined. One study reported a tandem system  
23 [105]. The anion-exchange column was used for pre-purification since humic acids and  
24 others impurities were retained. Less impurity got onto Oasis HLB cartridge improving the  
25 clean-up procedure. As a result, a better clean-up step with less interference during the  
26 following analysis was achieved. Anyhow, the addition of other SPE cartridges increases  
27 financial demands of this method. In Table 9 are presented published studies, which used  
28 SPE as sample preparation method. Most commonly used cartridge was Oasis HLB and  
29 recovery was ranged in 61% - 161%. In all cases pH of sample was adjusted on value 4 or  
30 lower.

31

<i>Compounds</i>	<i>Matrix</i>	<i>SPE sorbent</i>	<i>pH of sample at SPE</i>	<i>Recovery (%)</i>	<i>Analytical method</i>	<i>Ref.</i>
CIPRO	SW (Surface water)	Oasis MCX	2.5	61 – 82	UHPLC-MS/MS	[106]
CIPRO, NOR, ENRO, SARA,	SW and DW (drinking water)	Oasis HLB	3.0	91 – 161	LC-MS/MS	[107]
CIPRO, ENRO, NOR, OFLO	WWTP	Anion-exchange and Oasis HLB in tandem	2.5	37 – 129	LC-MS	[108]
CIPRO, ENRO, NOR, OFLO	WWTP	Oasis HLB	3.0	72 – 99	LC-MS/MS	[109]
OFLO	WWTP, GW (ground waters)	Oasis HLB	-	30 – 116	LC-MS/MS	[110]
CIPRO, OFLO	SW	Oasis HLB	4.0	62 – 106	LC-MS/MS	[111]
CIPRO, ENRO	SW, WW (wastewater), WWTP	Oasis HLB	2.8-3.0	71 – 117	LC-MS/MS	[112]
NOR	SeaW (seawaters)	Oasis HLB	3.0	99 – 116	LC-MS/MS	[113]
CIPRO, ENRO	RW (River water), WWTP	Oasis HLB	2.8	88 - 112	LC-MS	[114]
OFLO	RW, WWTP	Oasis HLB	-	-	UHPLC-MS/MS	[115]
CIPRO, NOR	SW	Oasis HLB	3.0	86 – 103	LC-MS/MS	[116]
CIPRO, DIFL, ENRO, LOME, NOR, OFLO, PIP, SARA, TOS	WWTP	SPE	3.0		LC-MS	[117]
PIP, FLE, OFLO, PEFLO, ENO, NOR, CIPRO, DANO, ENRO, LOME, DIFL, SARA, GATI, SPAR, MOXI, CINO, OXO, NAL, FLU, PIRO	WW, RW	SPE	3.0		LC-MS	[118]
CIPRO, ENRO, FLE, FLU, LOME, MOXI, NOR, OFLO, OXO	SW, WW, WWTP sewage sludge	SPE	4.2		LC-MS	[119]

Table 9: Determination methods of fluoroquinolones

In this work the systematic method development was compared with a conventional method approach. Most of the presented methods were kept under acidic conditions, about pH 3 and lower. Since conventional approach began by searching in scientific literature, low pH was used.

First step of the determination of five fluoroquinolone antibiotics: pefloxacin, ofloxacin, ciprofloxacin, norfloxacin and enrofloxacin were carried on C<sub>18</sub> column HPLC-FD. Anyhow, due to the co-elution of the target compounds, sufficient separation was not achieved. Due to this reason, the separation was moved to UHPLC-MS/MS. As mobile phase, MeOH and buffers at acidic pH and various additives were investigated. Additives were for example formic acid, acetic acid or ammonium formate. Unfortunately, even after the optimization the method was not repeatable and the peak shapes were not either satisfactory.

For the systematic development approach, was employed a prearranged method development recommended by Waters Corporation. During this development we can identify four different columns, ACQ BEH C18, ACQ BEH Phenyl, ACQ BEH Shield RP 18 and ACQ BEH HSS T3 columns (50 x 2.1 mm, 1.7 μm or 1.8 μm). Two modifier (MeOH and ACN) and two buffers (pH 3 and pH 9) in gradient elution (95% of buffer to 5% of buffer, 5 min) are tested on each column at flow rate 0.6 mL/min at 30°C. This method development provides all combination in less than 6 hours, except HSS T3 at basic pH, because of instability of silica-based column in these conditions (all combination are shown in Table 9). The columns were switched automatically [46].

<i>Column</i>	<i>Modifier</i>	<i>Buffer</i>
<b>BEH C18</b>	MeOH / ACN	pH 3 / pH 9
<b>BEH Phenyl</b>		
<b>BEH Shield RP 18</b>		
<b>BEH HSS T3</b>		pH 3

**Table 10: Combinations of systematic approach**

After this set of experiments, the optimization continued based on the evaluated results. First, the mobile phase was chosen. Methanol as an organic modifier provided better results than ACN. Values of pH were a very important factor, since fluoroquinolones are zwitterions as mentioned above. The separation of norfloxacin and ciprofloxacin was not

sufficient and surprisingly a better separation was achieved with basic pH in tested columns (HSS T3 excluded), in contrast of published literature.

The order of the elution of compounds was changed in BEH Phenyl columns; at pH 3 there were ofloxacin (OFLO), pefloxacin (PEFLO), norfloxacin (NOR), ciprofloxacin (CIPRO) and enrofloxacin (ENRO) and at pH 9 NOR, CIPRO, OFLO, PEFLO and ENRO. To improve the peak shape and the separation the pH 10.5 was tested. In this pH, the separation of NOR and CIPRO was improved and tailing was not observed. Anyhow, the next switching of order has occurred and OFLO and PEFLO position on chromatogram has changed.

The final conditions of separation of target analytes were using ACQ BEH Phenyl (50 x 2.1 mm, 1.7  $\mu$ m) at a flow rate of 0.45 mL/min and 35°C. Mobile phase was composed from MeOH and 10.5 ammonium acetate buffer. Gradient elution initiated with 95% of buffer and kept for 1 min, then linearly decreased to 25% in 3 min and finally next 4 min increased buffer to 55%. Maxima of excitation and emission wavelengths were found at  $\lambda_{exc}=310$  and  $\lambda_{em}=415$  at basic pH. Some stability studies with XBridges columns were published and reported problems with stability of phenyl analytical column over pH 7. In our case no changes were observed (over 2000 injections). After the optimization the developed method was transferred to UPLC-MS/MS.

Fluoroquinolones provided the highest signal in electrospray positive mass spectra. The gradient elution was used in different conditions to the optimal conditions of systematic method development in order to reduce the analysis time. The flow rate was decreased due to the ESI connection at 0.35 mL/min and also to improve the ionisation of some additives which were tested. Ammonium acetate (0.5mM, 1mM and 2mM) could not be used due to a signal depression of target analytes. All parameters of MS detector were optimized in order to achieve the best sensitivity of precursor ions for all analytes. Afterwards the cone voltage was set up for every compound and was optimized the conditions for SRM transitions. An internal standard, deuterium-labelled NOR-d<sub>5</sub> was added for quantitation.

In order to a better compatibility with the chromatographic conditions, the SPE method was optimized at pH 10.5. For the extraction was chosen HLB cartridges were chosen due to the possibility of using high pH. The washing step was optimized for a different percentage of methanol (5-30% of MeOH in mQ-water). The best results were achieved at the percentage of 10% but some matrix effects were observed and therefore 0.5%, 1% and 2% of acetic acid were tested. Finally, the 2% of acetic acid was added to the washing solution.



After washing the solution mQ-water was applied again in order to avoid the influence of pH in the elution. For the elution was tested ACN and MeOH, the second one provided a better elution efficiency. The eluent was evaporated and the dry extract was dissolved in the mobile phase and kept in an ultrasonic bath for 30 min followed by vortex for 1 min.

The developed method was used for the determination of antibiotics in river waters and WWTPs. There were no antibiotics detected in river waters and in WWTPs were detected norfloxacin, ciprofloxacin and ofloxacin. The highest concentration was detected for ciprofloxacin. In cases where it was possible to evaluate the efficiency of the elimination process, the ratio for norfloxacin was 32% and for ciprofloxacin was 98%.

The results of this work were published in the Journal of Separation Science (Supplement I, Chapter 6.4, page 77).

## **5.2 Endocrine compounds in environmental waters**

Among all compounds in the environment, the hormone residues have become a cause for the possibility to affect the endocrine system of non-target organisms. The influence on the sexual development of fish in UK rivers was reported many years ago. Anyhow not only fish are supposed to be affected, endocrine disruptors (EDCs) can cause reproductive abnormalities in human beings and also a decreasing sperm count.

Steroid hormones can be classified as endogenous and exogenous, by their structure and by the pharmacological effects or in general in estrogens, gestagens and androgens. Because of the anabolic effects, estrogens have been used in animal fattening and androgens that control the development of masculine and have been used as grow promoters and to improve athletic performance.

The occurrence of steroid hormones and their conjugates was reported in wastewater, surface and ground waters and drinking water. The occurrence in the environment is explained by not sufficient remove in WWTPs. Commonly, natural estrogenic steroids (such E1, E2 and E3) are quite often detected in water samples, in contrast conjugated and synthetic forms are not detected very often. Commonly the concentration in surface and wastewaters does not exceed a few ng/L. In the river and the marine sediment it is about pg/g to ng/g. The concentration is usually higher than E2 and E3. Some hormones show relatively low polarity and therefore the sorption is expected. Hormones have the same way of entrance into the environment as other pharmaceutical drugs; via wastewater, sewage sludge or by manure spread into agricultural fields [4].

The environmental analysis provides a wide range of matrices, from aqueous to solid samples. The methods have to be sensitive and allow the identification of unknown metabolites or compounds. Gas chromatography with mass spectrometry was used with derivatization reagents; nowadays liquid chromatography is used more often since a derivatization step is not required for this determination and the limits of detection are under ng/L or ng/g. The sensitivity is a very important parameter, therefore just several studies used a diode-array or a fluorescence detector.

Due to the low concentrations it is necessary to obtain high recoveries, pre-concentration and to minimize interferences during the sample preparation. Solid phase extraction (SPE) is very frequently used to extract steroid hormones from aqueous samples; mainly Oasis HLB is the most commonly used cartridges. Pressurised liquid extraction (PLE) and solvent extraction assisted by ultrasonication and microwave-assisted extraction (MAE) are used for solid environmental samples.

To reduce the solvents and avoid the extraction by toxic organic solvents, microextractions techniques were employed. Additionally on-line coupling of microextraction methods is easier and provides a better sensitivity. Steroid hormones provide non-polar and non-ionic characters that facilitate the use of reversed phase purification and separation strategies. Solid samples cannot be applied directly to the microextraction techniques and the previous step is necessary [4].

EU banned the use of substances with hormonal action growth promotions and fattening purposes since 1981. Veterinary drugs (including hormones) are regulated by the establishment of maximum residue limits (MRLs) in foodstuff of animal origin as well. The analytical control of food produced by animals (and their primary products as well) were regulated by Council Directive 96/23/EC and 2002/657/EC. The Water Framework Directive tried to establish some programs to monitor the quality of the water for Member States and Associated States. Therefore, it was necessary to have a list of emerging contaminants and other possible candidates. Contaminant Candidate List (CCL-3) published in 2009 by U.S. Environmental Protection Agency (EPA) contains only 8 hormones. The European Commission (EC) studies the relevance of some organic micro-pollutants in these matrices to include them in a Sludge Directive. There is also a problem of illegal use of anabolic androgenic steroids in sports, such androstenedione, testosterone and methyltestosterone [120, 121].

### 5.2.1 Analytical methods

Recently the most important methods used for the determination of steroid hormones are LC-MS, LC-FD and GC-MS or immunoassays. However, several types of detection are used: MS<sup>2</sup>>MS>FD>UV (ordered by sensitivity) [121].

#### *Liquid chromatography coupled with mass spectrometry*

The main advantage of LC is the determination without derivatization and no limitation by the non-volatility and molecular weight of steroid compounds. Due to these reasons liquid chromatography is more used.

However, LC coupled with mass spectrometry detection is a sensitive method with the possibility of a matrix effect, as the suppression or the enhancement of the signal. Electrospray ionization (ESI) is known to be sensitive to the matrix suppression and isobaric interferences in comparison with the atmospheric pressure and the chemical ionization. Accordingly, ESI provided better detection limits in a standard solution.

The selection of the mobile phase is involved by the enhancement of ionization in MS. The most frequently used mobile phase was water with additives (e.g. formic acid) and ACN or MeOH. The best response for E2 was provided in the mobile phase composed of water and methanol due to better separation efficiency, with a higher response and a better peak shape in many studies. However in other study MeOH caused unresolved peaks of  $\alpha$ E2 and  $\beta$ E2 and the highest selectivity was accomplished by ACN and 0.1% of formic acid (or acetic acid with lower intensity of MS<sup>2</sup>). Almost all studies reported gradient elution and other several studies determined steroid hormones on UHPLC [4].

#### *Mass spectrometry detection*

Most often used ionization techniques were ESI (mainly negative mode), APCI and APPI. Triple quadrupole (QqQ) was mostly applied and it was achieved better results in comparison with the Q-TOF analyzer and the ion trap. Steroid hormones have a poor sensitivity in ESI, since they have non-polar character compared to the polar and ionisable organic compounds. Therefore some studies added chemical derivatization to the determination of the target compounds. The signals of some steroids and bisphenol A (BPA) were better with ESI than other techniques such APPI, APCI/APPI.

Dansyl derivatives measured by UHPLC coupled with ESI achieved the best performance. Also better responses were presented by Nieto et al. for sulphates and glucuronides with ESI. Post column addition of ammonium hydroxide improved the

sensitivity for ESI, but decreased for APCI. Tetrabutylammonium increased the sensitivity and ammonium acetate and formate caused the decreasing of response of the target analytes. Isobe et al. reported that the absolute abundance decreased with acetate buffer.

APCI provided similar or a lower signal compared to ESI for E1, E2, E3, EE2 and phytoestrogens. Anyhow, one study reported three times greater signal for E1 than using ESI. The signal intensity increased one or twice for the estrogens in complex matrices compared to ESI.

APPI reached the ion signal of neutral steroid compounds with comparable ionization for native and derivatized steroids. This technique provided a higher selectivity of some steroids compared to ESI and APCI. A mobile phase of 0.1% of formic acid in MeOH and 0.1% ammonium hydroxide in 2-propanol showed higher intensities in APPI(-) and APPI(+), respectively. Toluene directly infused into the APPI(-) improved the efficiency for the positive mode and in a little higher flow for the negative mode.

LC-QqQ-MS<sup>2</sup> reached better limits of detection and selectivity than LC-TOF-MS, in one study even 13 times [122].

### ***Derivatization***

The ionization of estrogens by ESI and APCI was less efficient than other polar compounds. Chemical derivatization could improve ionization and enhance signals in LC-MS, but can change the chromatographic behaviour of the target compounds. Derivatization can improve the sensitivity about 100 times or even 1000 times.

In general, dansyl chloride is mostly used for estrogens in water samples. The highest signal reached dansyl-estrogens 111 higher than the underivatized compounds. A better sensitivity of dansyl chloride and pentafluorobenzyl bromide was demonstrated in other studies. Online derivatization and sample preparation was performed in two studies, with 100 fold improvement of sensitivity of estrogens determination.

Girard reagents T and P were used in ESI (+) mode for the neutral steroid hormones and sub pg were measured by this technique. Girard reagent P was successfully used for MALDI-MS with characterization of 50-pg levels. However in comparison with 2-hydrazino-1-methylpyridine, girard reagent P provided lower sensitivity and not so good chromatographic behaviour.

Derivatization reagents such 1-(2,4-dinitro-5-fluorophenyl)-4-methylpiperazine and 4-(4-methyl-1-piperazyl)-3-nitrobenzoyl azide reached limits of detection about femtomolar range. The proton-affinitive derivatizing reagents is generally effective for improving of APCI(+) sensitivity. Acetylation of hydroxyl groups is the easiest derivatization method for APCI(+), but methyloxime derivatization allows enhancing sensitivity 40-60 fold. The 2-nitro-4-trifluoromethylphenyl hydrazones of pregnenole and progesterone increase ions intense 20 and 30 times in APCI(-) mode. Derivatization on phenolic groups of estrogens, such dansyl chloride (ESI+), 2-fluoro-1-methylpyridinium *p*-toluenesulfate (ESI-) and pentafluorobenzyl bromide (APCI+) was successfully used for environmental samples.

In GC determination, derivatization is a very important step, because of the increasing volatility, separation and stabiliztion of the thermolabile substances. The most widely derivatization agents are N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) and BSTFA with 1% trimethylchlorosilane (TMCS), which provided the highest trimethylsilylation. Nie et al. reported that aliphatic hydroxyl groups were more difficult to derivatize than the aromatic ones. However, after the ultrasonication, a complete silylation of both, aliphatic and aromatic groups was accomplished.

Bowden et al. reported some guidelines for the derivatization followed by the GC-MS analysis [122].

## 5.2.2 Sample preparation

### *Liquid phase extraction and microextraction techniques*

Liquid phase extraction (LPE) with high recoveries has been traditionally used for the determination of steroid hormones in environmental samples. Because of the time consuming process and the expensive cost, miniaturized techniques have replaced LLE in environmental analysis.

Dispersive liquid-liquid microextraction based on the solidification of the floatign organic drop (DLLME-SFO) was successfully used for the determination of steroid hormones. DLLME has some advantages of LPME, but there are some problems such as high density or necessity of centrifugation.

A dihydroxylated polymethylmethacrylate (DHPMM) coating on hollow-fibre liquid phase microextraction was used for the determination of estrogens. This method was combined with GC-MS and recoveries were between 86% and 110%. The next study

developed a method based on this technique for the extraction of several steroid hormones. Samples were determined by HF-LPME-GC-MS with enrichment factor over 1400 after derivatization. Liu et al. determined three estrogens in wastewater by HF-LPME followed by GC-MS. Recoveries were the same as in other studies and the limits of detection were higher. This technique was used for the determination of progesterone in human serum samples. Recently, molecularly imprinted polymer-coated polypropylene hollow fiber tube was used for the determination of several EDCs and as template was used diethylstilbestrol with a recovery of 83.7% – 90.6% in milk samples. HF-LPME has a very good stability for organic solvents and tolerance to higher temperatures and sampling times, on the other hand membrane pre-conditioning and the possibility of memory effects when membranes are reused are drawbacks.

Chang et al. extracted estrogens from rivers and tap waters by *solid-drop microextraction* (SD-LPME) coupled by ultra high performance liquid chromatography with photodiode-array detection and achieved enrichment factors 121- and 260-fold with good relative recoveries.

The reduction of organic solvent volume leads to development of single microdrop used in SDME technique. Unfortunately there was instability of the drop due to the limited drop surface and other problems such as carry over etc. These disadvantages can be overcome by SDME, which need proper solvent for the freezing.

Other technique such as the cloud point extraction (CPE) based on the use of micellar solutions, which a small amount allows the preconcentration and the analyte extraction to occur in just one step. Wang et al. applied this methodology for steroid hormones in WWTPs. The extraction was carried out by Triton X-114, in this case. The micellar solution (non-ionic surfactants) was successfully used in LPME for the extraction of some different organic compounds from different matrices, but as far as it is known, they have not been used for the extraction of steroid hormones.

### ***Sorbent extraction and microextraction techniques***

*Solid phase extraction* is one of the most used extraction methods for the determination of steroid hormones. C18, HLB, STRATA X and amine sorbents are widely used for aqueous samples. SPE off-line was reported in most cases. SPE was also successfully used with derivatization with dansyl chloride. Oasis HLB and the other polymeric cartridges

together with selective sorbent materials such as molecularly imprinted polymers or restricted access materials were typical materials for the online extraction.

Oasis HLB cartridge shows itself as one of the most potential sorbent. This sorbent was in combination with used Sep-Pak Plus NH<sub>2</sub> cartridge (silica-based polar phase with basic character) and improved the elimination of matrix interferences. In other study compared STRATA X and Oasis HLB and the first one mentioned achieved the best results.

SPE include several steps, such as conditioning, sample applying, clean-up and elution.

Sample volumes vary depending on the matrix and the type of method (online or offline), from just some mL (mainly online) to 4 L. Lower sample volumes decrease not only the matrix effect, but also the possibilities of preconcentration. However, small volumes as 1 mL were sufficient for the required sensitivity.

Elution is another very important step in SPE and was performed typically by methanol, ethylacetate, or in combination of both. Triethylamine and 2% of ammonium hydroxide were added into methanol for the elution of estrogen-conjugates. The improvement of the recoveries for real samples was presented also by adding 5% of acetonitrile into methanol. In several studies were reported that drying during the elution step was without changing the eluent.

Several miniaturized methods, such as the solid phase microextraction and the stir-bar sorptive extraction (SPME and SBSE) were reported.

In the study of Qiu et al. was developed a selective *solid phase microextraction* (SPME) fiber for anabolic steroids from a testosterone-imprinted polymer. This type of fiber was used for the extraction of anabolic steroids followed GC-MS. Androstane, stanolone, androstenedione and methyltestosterone were determined by a fiber placed in a homemade syringe and inserted into a GC-MS injection port. Molecular imprinted polymers (MIPs) technique was used to determine estrogenic compounds and being compared with non-imprinted polymer coated SPME. A higher sensitivity was obtained in this technique; however MIPs did not allow the separation of a wide range of analytes.

*Stir-bar sorptive extraction* with thermal desorption followed by GC-MS was used to determine estrogens, gestagens and androgens. This method included in situ derivatization with ethyl chloroformate and acetic acid anhydride. The same method was applied to the determination of estrogens compounds in river waters. In the extraction method, first was

performed in situ acylation and then the thermal desorption with quartz-wool-assisted in-tube silylation followed by GC-MS. A similar procedure was used for the determination of estrone and 17 $\beta$ -estradiol. The combination of SBSE method and thermal desorption followed by GC-MS was presented for the determination of hormones in soil and sediment samples. Tan et al. reported recoveries for the target hormones between 44% and 128%.

The combination of SBSE-LC was reported also for the determination of steroid sex hormones, but the recovery was in a range from 11.1% to 100.2%. PDMS is only commercially available coating for SBSE. For a better extraction efficiency, Hu et al. used a PDMS and  $\beta$ -cyclodextrin prepared by a sol-gel technique. The recovery was improved from 85% to 124%. The next modification of SBSE technique was reported by Huang et al.. This modification was based on the monolithic material, which was obtained in situ by copolymerisation of methacrylic acid stearyl ester and ethylene dimethacrylate. The same authors used a different monolithic material obtained by the in situ copolymerisation of vinylpyrrolidone and divinylbenzene to extract polar hormones without derivatization.

The next microextraction technique employed for the determination of mestranol, 17 $\alpha$ -EE2 in water samples was the *microextraction in packed sorbent* (MEPS) with a recovery higher than 75%. Lower recoveries (above 60%) were obtained by Anizan et al. for five steroid metabolites with a repeability under 11%.

The results of this work were published in *Analytica Chimica Acta* and *Trends of Analytical Chemistry* (Supplement II, Chapter 6.5, page 93 and Supplement III, Chapter 6.6, page 108).

### **5.2.3 In-tube SPME method for EDCs**

One of the most friendly used microextraction methods based on SPME is the in-tube SPME, which is possible to connect online to HPLC. This online method coupled with LC-FD and was optimized for the determination of EDCs in several studies. As a sorbent, it was used a poly(acrylamide-vinylpyridine-N,N'-methylene bisacrylamide) monolith. Low limits of detection were achieved in tap water, lake water and sewage water samples. The same results were published by Mitani et al. for in-tube SPME with LC-UV-MS for surface waters. The same column sorbent with different film thicknesses was applied by Saito et al. for nandrolone, testosterone, methyltestosterone and some other EDCs. The results were comparable with other studies [123].



The in-tube SPME was used twice in this work, using different modes; first the dynamic and then the static mode. In the first method, the dynamic mode consisted in the capillary was connected directly behind the injection needle and in front of the injection valve of the autosampler. In this connection no mobile phase flowed through the capillary. Connections were facilitated by the use of a 4cm x 1/6 in sleeve and polyethyletherketone (PEEK) tubing and were placed at each end of the capillary column. Stainless steel nuts, ferrules and connectors completed the connections. In the second, the static method, the capillary replaced the injection loop and it was connected the same way as in the case of the dynamic mode [123]. Desorbent was moved to the column by mobile phase, which pass through the capillary.

### ***Dynamic mode in-tube SPME***

There are several parameters to be optimized in the in-tube SPME method, such as the capillary column, the sample volume and the number of draw/eject cycle.

Due to the optimization in other studies 60cm of capillary length was selected and in preliminary working conditions we tested three different columns; Supel-Q(porous polymer), Carboxen (porous carbon molecular sieve) and CP-Sil 19CD (polysiloxane backbone), respectively. The target EDCs provided not suitable results for the extraction efficiency for CP-Sil and this column was removed from the next experiments.

Supel-Q and Carboxen capillary were tested with an addition of salt, which theoretically can improve the extraction. Samples without salt and with addition 15% of NaCl do not provide any differences.

The next step for the optimization was to do a change in pH, but the selected EDCs had  $pK_a$  about 10.0, and in lower values are in neutral form. Therefore it wasn't added any salt and the pH of samples was not adjusted (pH 5.5) [123].

The number of draw/eject cycles (one cycle takes less than 1.5 min) and sample volume were optimized by experimental design ( $3^2$  factorial design, with duplicate central points). Optimal conditions were tested in a range from 20 to 60 numbers of cycles and from 50 to 100  $\mu$ L for sample volume.

For Supel-Q was selected as an optimal condition 20 draw/eject cycles and 75  $\mu$ L of sample volume. Only Bishpenol A provided 60 draw/eject and 100  $\mu$ L on Supel-Q column, the others compounds showed the highest signal under the finally selected conditions. E3 showed almost the same signal for 20 draw/eject cycles and 100  $\mu$ L.

Optimal conditions for Carboxen were 40 draw/eject cycles and 100  $\mu\text{L}$  samples, which were reached as optimal for all the compounds except Bisphenol A and estriol. The optimal conditions for Bisphenol A was 20 draw/eject cycle, 75 $\mu\text{L}$  of sample volume and for estriol 40 draw/eject cycles and 50  $\mu\text{L}$  of sample volume, respectively.

Under optimal conditions of both capillary columns were constructed the calibration curves, the limits of detection and the limits of quantification. The results were compared with the optimised SPE method and the results were quite similar. Spiked and non-spiked real samples were determined by using in-tube SPME-HPLC-FLD with both capillary under optimal conditions. The recoveries obtained in all samples (including sea water and wastewater samples) were better than 80%. Samples of WWTP reached recoveries between 85 – 102% [123].

The results of this work were published in International Journal of Environmental Analytical Chemistry (Supplement 6.7, page 136).

#### ***Static mode in-tube SPME***

In-tube SPME is typically performed using a piece of a fused-silica capillary with a stationary phase coated on its inner surface (e.g., a short piece of a GC column). Supel-Q-PLOT capillary was found more efficient for the analysis of estrogens by Kataoka et al. [124]. This column was used by Mitani et al. [125-128] in length of 60 cm for the online in-tube SPME of estrogens. Therefore, we performed one cycle with a Supel-Q capillary with a length of 60 cm and used methanol as the elution reagent for the initial optimization.

The first stage of the in-tube SPME parameter optimization study consisted of a  $2^4$  factorial design, which the design consisted of 16 runs. The variables under consideration included the extraction time (10 and 30 min), desorption time (10 and 30 min), standard solution volume (25-50  $\mu\text{L}$ ) and desorption solution volume (25-50  $\mu\text{L}$ ). Partial and bivariate correlations between dependent and independent variables were investigated. An extraction time of 10 minutes, the desorption time of 10 minutes, the sample volume of 50  $\mu\text{L}$  and the desorption volume of 25  $\mu\text{L}$  were used in subsequent studies.

The optimal length of a capillary column ranges from 20 to 100 cm; we studied capillary lengths of 40, 60 and 80 cm. The results showed that a capillary length of 40 cm presented some technical problems, like a capillary break, that led to a short lifetime. Capillary lengths of 60 and 80 cm were tested, and significant differences were not observed. However, a capillary

length of 80 cm permitted a greater sample volume, which improved the results and it did not affect to the desorption efficiency.

After optimizing the capillary length, a  $3^2$  experimental design was used and the extraction time (2, 6, and 10 min) and sample/standard solution volume (35, 50 and 65  $\mu\text{L}$ ) were evaluated using a surface response methodology. All the data was obtained in a single cycle. The experiments were performed at a desorption time of 10 min, and 25  $\mu\text{L}$  of methanol was used as the desorbent. In all cases, the best results were obtained with 65  $\mu\text{L}$  of sample/standard solution at an extraction time of 2 min.

The desorption time (1, 2, and 10 min) was studied under the optimal extraction conditions. Therefore, the optimal in-tube SPME conditions for a single cycle were as follows: capillary column length of 80 cm, extraction time of 2 min, sample volume of 65  $\mu\text{L}$  and desorption time of 1 min.

In-tube SPME methods for EDCs in environmental samples were performed using sample volumes between 0.4 mL and 4 mL in draw/eject cycles [126, 128, 129]. After performing the single cycle optimization of in-tube SPME using methanol as the desorbent agent, the number of cycles in the extraction step was increased to improve the analytical signal.

Experiments were performed for 1, 2, 4, 8, 12 and 15 cycles, and 65  $\mu\text{L}$  of sample was used in each cycle. The number of cycles increased, the analyte signal also increased. The best signals were obtained when 12 or more cycles were conducted. However, the improvement in the results obtained with 15 cycles compared with 12 cycles was not sufficient to compensate for the increased extraction time. Thus, in subsequent experiments, 12 cycles were performed.

Micellar media can be used as an alternative to organic solvents for the desorption and has been used as desorbents in previous studies [130-132]. Under the optimal conditions for the proposed conventional in-tube SPME method, we replaced methanol with more environmentally friendly eluents in the desorption step. Based on the results of preliminary studies, two non-ionic surfactants were selected for the present study, including POLE (polyoxyethylene 10 lauryl ether) and Genapol (oligoethylene glycol monoalkyl ether). The desorption was performed under non-optimal conditions for comparison. The experiments were conducted under similar conditions as optimal conditions for methanol desorption. Both of the surfactants were used at a concentration of 1% (v/v). Although comparable results were obtained with Genapol and POLE, Genapol provided lower relative deviations for all of the target compounds. Therefore, Genapol was used in a further research.

Variables that affected the desorption step were studied using a surface response methodology and a  $3^2$  factorial design. The surfactant percentage (0.1, 0.55, and 1% v/v) and desorption volume (10, 15, and 20  $\mu\text{L}$ ) were selected as variables. In general, the highest surface response was achieved when the lowest concentration of Genapol (0.1%, v/v) and a desorption volume of 20  $\mu\text{L}$  were employed.

The optimal conditions for in-tube SPME with micellar desorption with Genapol were as follows: capillary column length of 80 cm, 12 cycles, sample/standard solution volume (for one cycle) of 65  $\mu\text{L}$ , extraction time of 2 min, desorption time of 1 min, 20  $\mu\text{L}$  of 0.1% (v/v) of Genapol as the desorption reagent.

For the study of the matrix effects in the optimized in-tube SPME method, the proposed procedure was used to extract and determine the selected EDCs in spiked seawater and WWTP effluent samples. The overall recoveries were 93.0-103.4 % in seawater samples and 93.0-101.2% in WWTP effluent samples. Using Genapol as the desorbent, the overall recoveries were 93.7-104.4 % in seawater samples and 90.5–106.9 % in WWTP effluent. Similar results were obtained with a FD.

The results of this work were submitted to *Chromatographia* (Supplement V, Chapter 6.8, page 154).

### **5.3 Benzimidazole fungicides**

Very important environmental pollutants are pesticides, which also provide a risk assessment. The target of this chapter is to summarize the extraction and determination methods of benzimidazole fungicides.

Pesticides are widespread used in agriculture; their residues may persist in the environment or can be transported into growing plants and fruits. They are applied directly to the soil or sprayed over the crops, therefore they avoid WWTP and they are released directly into the environment. The using of pesticides boosts the effectively of planting and inhibit the variety fungi and prevent parazitic infections. One of the most spread groups of pesticides are benzimidazole fungicides, containing an imidazole ring and acidic and basic nitrogen atoms. These compounds are used very often and the European Water Framework Directive (Directive 2006/11/CE4) established the maximum residue levels.

The first compound introduced from benzimidazole fungicides was thiabendazole (TBZ), quickly followed by parbendazole, cambendazole, mebendazole etc. Some used fungicides, such as netobimin or febantel that had higher bioavability due to their grater water solubility. Most of these compounds have  $K_{ow}$  in a range of 0.8 - 3.3. The molecules can be protonated ( $pK_{a1}$  5-6) or deprotonated ( $pK_{a2}$  12). Several others compounds with similar structure were found as fungicidal agents. Anyhow some modifications in the benzimidazoles results slowed the rates of elimination [20].

#### ***Liquid sample preparation***

Sample preparation is a crucial step in the analysis and includes the isolation of target analytes, reducing interferences originally presented in the sample and pre-concentration.

Liquid-liquid extraction (LLE), which was used as the first sample preparation method, was followed by the solid phase extraction (SPE), on-line supported liquid membrane (SLME, three-phase aqueous-organic solvent-aqueous system), microporous membrane liquid-liquid extraction (MMLLE, two-phase aqueous-organic solvent system), cloud point extraction (CPE) and solid phase microextraction (SPME) have been used for sample preparation of benzimidazole fungicides from liquid environmental samples.

Recently, SPE was used more often with a good recovery achieved by materials such as C18, polystyrenedivinybenzene, ethylvinylbenzene-divinybenzene, polystyrene and active carbon. Next sample preparation related to new sorbent materials is the molecularly imprinted polymers (MIPs). This technique was successfully applied for the benzimidazole extraction.

SPE technique is often replaced by other more environmental friendly microextraction techniques which are frequently an alternative for the sample preparation with not only benzimidazoles.

Different technique configurations have been developed such as static LPME, dynamic LPME, single-drop LPME, hollow fiber-based LPME, dispersive LLME and etc. However some problems in these methods are still persisting, e.g. instability of liquid drop in single-drop LPME or bubbles formation in HF-LPME. Benzimidazoles were extracted by CPE method with non-ionic surfactant such as POLE and Genapol. This method obtained comparable results with other reported ones and allowed an environmental friendly extraction. There was reported the use of surfactant-coated mineral oxide columns for SPE and demonstrated that these new materials are a promising tool for organic compounds [19].

### ***Solid samples extraction***

The most frequently used methods for the extraction of organic compounds from soils are Soxhlet or ultrasonic extraction. The newer extraction techniques, such as supercritical fluid extraction (SFE), pressurized liquid extraction (PLE) and microwave-assisted extraction (MAE), are faster, use much smaller amounts of solvents and are environmentally friendly techniques.

In the last few years, there has been an increase in the number of procedures using microwave energy to extract organic compounds from environmental matrices. Microwave assisted extraction (MAE) is a process of using microwave energy to heat solvent in contact with a sample in order to obtain partition of analytes from the sample matrix into the solvent. In comparison with other conventional methodologies, such as Soxhlet extraction, MAE requires less energy, shorter analysis periods and the use of smaller solvent volume of analysis.

The use of micellar media as alternative to conventional organic solvents in the MAE procedure could offer important advantages such as safety, simplicity, lower toxicity, Loir cost and greater compatibility with the aqueous organic mobile phase in the LC separation process. This combination, called microwave-assisted micellar extraction (MAME) has been applied to the extraction of different compounds from solid matrices. Among the advantages of the MAME are reduced volumes of extractant, low cost and reduced toxicity in comparison to organic solvents. Moreover, MAME reduce analysis time compared with conventional Soxhlet extraction. This extraction method was reported for benomyl, carbendazim, thiabendazole and fuberidazole in soil samples [20].

### *Analytical methods*

Numerous analytical methods have been reported for quantitation of benzimidazole fungicides in different matrices based on spectrophotometry, fluorimetry, phosphorimetry, electrochemical, enzyme-linked immuno-sorbent assay, gas chromatography-mass spectrometry, although is routinely carried out by high performance liquid chromatography coupled with ultraviolet, fluorescence, and mass spectrometry detectors.

In environmental samples, flow injection analysis (FIA) coupled with chemiluminescence (CL) is a well established technique for the ultra-trace analysis of a variety of compounds in diverse matrices using various CL reagents including benzimidazole fungicides (Fletcher et al. 2001). FIA-CL method for the determination of carbendazim has been reported (Liao and Xie 2006), which is based on the enhancement of CL reaction of luminol and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) by a carbendazim in sodium hydroxide–sodium dihydrogen phosphate medium (pH 12.6).

LC–MS and LC-MS/MS have found more widespread application in environmental analysis offering more sensitive detection and increased confidence in reporting results. It should be desirable the development of specific methods for benzimidazole fungicides in environmental samples using LC–MS/MS combined with new sample treatments to apply to real environmental samples. Alternatively, HPLC coupled to UV and fluorescence detection in series may offer a low cost to LC–MS/MS and may be particularly effective in the optimization of sample treatments [20].

This work was published as a chapter of book Fungicides (Supplement VI, Chapter 6.9, page 180).

## 6 Appendix

### 6.1 List of publications included in this doctoral thesis

#### 1. Supplement I (page 77)

M. Seifrtová, J. Aufartová, J. Vytlačilová, A. Pena, P. Solich, L. Nováková

**Determination of fluoroquinolone antibiotics in wastewater using ultra high-performance liquid chromatography with mass spectrometry and fluorescence detection**

Journal of Separation Science 33 (2010) 2094-2108.

(IF: 2.551)

#### 2. Supplement II (page 93)

J. Aufartová, C. Mahugo-Santana, Z. Sosa-Ferrera, J. J. Santana-Rodríguez, L. Nováková, P. Solich

**Determination of steroid hormones in biological and environmental samples using green microextraction techniques: An overview**

Analytica Chimica Acta 704 (2011) 33– 46

(IF: 4.310)

#### 3. Supplement III (page 108)

H. Tomšíková, J. Aufartová, P. Solich, L. Nováková, Z. Sosa-Ferrera, J. J. Santana-Rodríguez

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

Trends in Analytical Chemistry 34 (2012) 35-58

(IF: 6.623)



#### **4. Supplement IV (page 136)**

J. Aufartová, M.E. Torres-Padrón, Z. Sosa-Ferrera, P.Solich, J.J. Santana-Rodríguez

**Optimisation of an in-tube solid phase microextraction method coupled with HPLC for determination of some oestrogens in environmental liquid samples using different capillary columns**

International Journal of Environmental Analytical Chemistry 92 (2011) 1–15

(IF: 1.169)

#### **5. Supplement V (page 154)**

Jana Aufartová, María Esther Torres-Padrón, Zoraida Sosa-Ferrera , Lucie Nováková, Petr Solich, José Juan Santana-Rodríguez

**Development of a novel in-tube solid phase microextraction based on micellar desorption followed by LC-DAD-FD for the determination of some endocrine disruptor compounds (EDCs) in environmental liquid samples**

Chromatographia – sent

(IF: 1.169)

#### **6. Supplement VI (page 180)**

M. E. Torres-Padrón, J. Aufartová, Z. Sosa-Ferrera, J. J. Santana-Rodríguez

**Benzimidazole Fungicides in Environmental Samples: Extraction and Determination Procedures**

Fungicides, ISBN 978-953-307-266-1

Edited by: Odile Carisse, Publisher: InTech, 2010

## **6.2 Poster presentation at international scientific conferences**

- I. A. Pena, J. Aufartová, M. Seifrtová, C. Lino, P. Solich

**DETERMINATION OF FLUOROCHINOLONE ANTIBIOTIC IN  
DIFFERENT TYPE OF SOIL SAMPLES**

19<sup>th</sup> International Symposium on Pharmaceutical and Biomedical analysis, 8. – 12.  
June 2008, Gdansk, Poland

- II. M. Seifertová, J. Aufartová, L. Nováková, A. Pena, P. Solich

**UPLC AUTOMATIC METHOD DEVELOPMENT VERSUS  
CONVENTIONAL APPROACH – OPTIMIZATION AND DEVELOPMENT  
OF ANALYTICAL METHODOLOGY FOR THE DETERMINATION OF  
FLUOROCHINOLONE ANTIBIOTIC**

12<sup>th</sup> EuCheMS International Conference of Chemistry and the Environment, 14. – 17.  
June 2009, Stockholm (Sweden)

- III. J. Aufartová, M. Seifrtová, L. Nováková, P. Solich

**COMPARASION OF HPLC AND UPLC METHOD FOR DETERMINATION  
OF FLUOROQUINOLONES AUTOMATIC DEVELOPMENT AND  
CLASSICAL APPROACH**

34<sup>th</sup> International Symposium on High-Performance Liquid Phase Separations and  
Related Techniques, HPLC 2009, 28. June – 2. July 2009, Dresden, Germany

- IV. L. Nováková, M. Rabatinová, J. Aufartová, P. Solich

**THE DEVELOPMENT OF NEW UPLC-MS/MS METHOD FOR THE  
DETERMINATION OF STEROIDS FROM ESTROGEN AND PROGESTIN  
GROUPS**

18<sup>th</sup> IMCS 2009, 30. August – 4. September 2009, Bremen, Germany

V. M. Seifrtová, J. Aufartová, A. Pena, L. Nováková, P. Solich

**DEVELOPMENT OF NEW UHPLC-MS/MS METHOD FOR DETERMINATION OF ANTIBIOTIC IN ENVIRONMENTAL WATER**

25<sup>th</sup> International Symposium of Microscale Bioseparations MSB, 21. – 25. March 2010, Prague, Czech Republic

VI. L. Nováková, M. Seifrtová, H. Vlčková, J. Aufartová, P. Solich

**SYSTEMATIC METHOD DEVELOPMENT IS CRUCIAL IN MODERN PHARMACEUTICAL ANALYSIS**

70<sup>th</sup> FIP Congress of Pharmacy/Pharmaceutical Sciences, 28. August – 2. September 2010, Lisboa, Portugal

VII. J. Aufartová, L. Nováková, J.J. Santana Rodríguez, P. Solich, Z. Sosa Ferrera, M. E. Torres Padrón

**COMPARISON OF DIFFERENT CAPILLARY COLUMNS IN THE OPTIMIZATION OF IN-TUBE SPME OF SOME ENDOCRINE DISRUPTORS IN THE ENVIRONMENTAL LIQUID SAMPLES**

36<sup>th</sup> International Symposium on Environmental Chemistry, 5. – 10. October 2010, Rome, Italy

VIII. A. Betancor-Abreu, M.E. Torres-Padrón, Z. Sosa-Ferrera, J.J. Santana-Rodríguez, J. Aufartová

**DETERMINATION OF DIFFERENT STEROID HORMONES BY IN-TUBE SOLID PHASE MICROEXTRACTION AND LIQUID CHROMATOGRAMY IN WASTEWATERS**

EUROanalysis 16, European Conference on Analytical Chemistry, Challenges in Modern Analytical Chemistry, 11. – 15. September 2011, Belgrade, Serbia

- IX. J. Aufartová, M. E. Torres-Padrón, Z. Sosa Ferrera, L. Nováková, P. Solich, J.J. Santana-Rodríguez

**OPTIMIZATION OF A METHODOLOGY FOR THE DETERMINATION OF ENDOCRINE DISRUPTORS COMPOUNDS (EDCS) IN WATERS USING IN-TUBE SOLID PHASE MICROEXTRACTION COUPLED WITH MICELLAR DESORPTION**

EUROanalysis 16, European Conference on Analytical Chemistry, Challenges in Modern Analytical Chemistry, 11. – 15. September 2011, Belgrade, Serbia

- X. J. Nežádalová, J. Aufartová, M. Rabatinová, Z. Sosa-Ferrera, J. J. Santana-Rodríguez, P. Solich, L. Nováková

**DETERMINATION OF STEROID HORMONES IN SURFACE WATERS BY ULTRA HIGH LIQUID CHROMATOGRAPHY; COMPARISON OF FUSED-CORE SORBENT AND UHPLC AND BEH COLUMN**

EUROanalysis 16, European Conference on Analytical Chemistry, Challenges in Modern Analytical Chemistry, 11. – 15. September 2011, Belgrade, Serbia

### **6.3 Poster award**

The best poster presentation at the conference has been honoured with the Roland.W.

Frei Award:

*Comparison of different capillary columns in the optimization of in-tube SPME extraction of endocrine disruptors in environmental liquid samples*

36<sup>th</sup> International Symposium on Environmental Chemistry, 5. – 10.October 2010, Rome, Italy

**P52- COMPARISON OF DIFFERENT CAPILLARY COLUMNS IN THE OPTIMIZATION OF IN-TUBE SPME EXTRACTION OF ENDOCRINE DISRUPTORS IN ENVIRONMENTAL LIQUID SAMPLES**

J. Aufartova<sup>1</sup>, L. Nováková<sup>1</sup>, J. J. Santana-Rodríguez<sup>2</sup>, P. Solich<sup>1</sup>,  
Z. Sosa-Ferrera<sup>2</sup>, M. E. Torres-Padrón<sup>2</sup>

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Endocrine disrupting compounds (EDCs), such as bisphenol A, ethynylestradiol, levonorgestrel, norethisterone and estriol, are a class of emerging contaminants that are extensively and increasingly used in human medicine. They have become a major issue in the field of environmental science due to their ability to interfere with endocrine system in animal and humans. Trace level concentrations and their wide diversity are some of their characteristics for that their identification and quantification are necessary to understand their behaviour in the environment. Due to low concentration levels of these compounds in environmental waters, is necessary to apply an extraction and preconcentration process to improve their determination. In-tube solid phase microextraction has proved to be an appropriate method of extraction and preconcentration of different types of pollutants.

In this work we present the optimization of the chromatographic separation with two different analytical columns (phenyl and C18) and different capillary columns CP-SIL 19CB, Supel-Q porous layer open tubular (PLOT) and Carboxen 1006 PLOT for optimizing in-tube solid-phase microextraction of endocrine disruptors in environmental liquid samples coupled to high performance liquid chromatography with DAD detection (in-tube SPME-HPLC) [1-3]. Results obtained were evaluated and compared in function of different chromatographic parameters: time, peak shape and resolution for different extraction sorbents.

[1] Ouyang, G., Pawliszyn, J., *TrAC*, 25, 692-703 (2006)

[2] Mitani, K., Narimatsu, S., Izushi, F., Kataoka, H., *J. Chromatogr. A* 32, 469-478 (2003)

[3] Kataoka, H., Ishizaki, A., Nonaka, Y., Saito, K., *Anal Chim. Acta*, 1-2, 8-29 (2009)



# Comparison of different capillary columns in the optimization of in-tube SPME of some endocrine disruptors in environmental liquid samples

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## Instrumentation and chromatographic conditions

The chromatographic system was a Varian (autosampler 410) with DAD. Chromatographic separation was performed on NovaPack C<sub>18</sub> column (3.9 x 150mm, 4 μm, Waters) with column guard with the same sorbent (Waters).

Initial mobile phase was water-methanol (55:45 v/v) up to 40:60 in 15 min. After that, until 20 min, separation was in isocratic mode. Flow rate was 1.0 mL/min. The temperature into thermostated column compartment was set at 30°C.

Analyte	Abbreviation	t <sub>R</sub> (min)	λ (nm)	
1	Estrilol	E3	5.3	224
2	Bisphenol A	BPA	9.8	224
3	Norethisterone	NORE	14.2	244
4	Ethinylestradiol	EE2	14.9	224
5	D-norgestrel	NORG	17.9	244

Table 1. Target compounds, retention times (t<sub>R</sub>) and detection wavelengths (λ).

## Experimental procedure

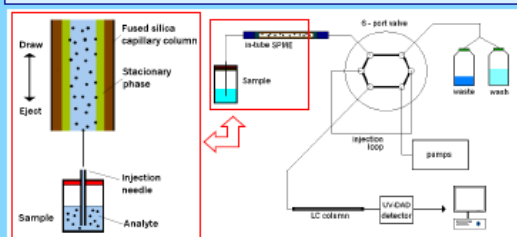


Figure 1. Scheme in-tube SPME-HPLC-DAD

Flow of draw* eject *	0.31 mL/min 3.40 mL/min
Length of capillary*	60 cm
Desorption*	50 μL of methanol
Volume of each cycle**	Carboxen: 100 μL Supel-Q: 75 μL
Number of draw/eject cycles**	Carboxen: 40 Supel-Q: 20

Table 2. Extraction conditions  
\* fixed conditions  
\*\* optimised conditions

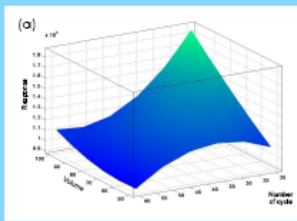
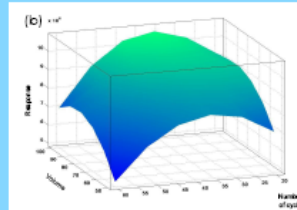


Figure 2.

Response of surface using a design matrix 2<sup>3</sup> (number of cycles (20, 40, 60) draw/eject and volume (50, 75, 100 μL) of each cycle) of BPA using:

(a) Carboxen [optimum conditions: 40 cycles and a volume of 100 μL in each cycle]



(b) Supel-Q [optimum conditions: 20 cycles and a volume of 75 μL in each cycle]

## Introduction

Endocrine disrupting compounds (EDCs), such as bisphenol A, ethinylestradiol, levonorgestrel, norethisterone and estrilol, are a class of emerging contaminants that are extensively and increasingly used in human medicine. They have become a major issue in the field of environmental science due to their ability to interfere with endocrine system in animals and humans. Trace level concentrations and their wide diversity are some of their characteristics.

For their identification and quantification is necessary to understand their behavior in the environment. Due to low concentration levels of these compounds in environmental waters, it is necessary to apply a extraction and preconcentration process to improve their determination.

In-tube solid phase microextraction (in-tube-SPME) has proved to be an appropriate method to extract and preconcentrate different types of pollutants [1-3].

In this work, different capillary columns, Supel-Q porous layer open tubular (PLOT) and Carboxen 1006 PLOT were selected for optimization in-tube SPME of endocrine disruptors in environmental liquid samples coupled to high performance liquid chromatography with DAD detection (in-tube SPME-HPLC-DAD). This method was applied for analysis of environmental aqueous samples collected in Gran Canaria island (Canary Islands, Spain).

## References

- Ouzang, G., Pawliszyn, J., TrAC, 25, 692-703 (2006)
- Mitani, K., Narimatsu, S., Izushi, F., Kataoka, H., J. Chromatogr. A 32, 469-478 (2003)
- Kataoka, H., Ishizaki, A., Nonaka, Y., Saito, K., Anal. Chim. Acta, 1-2, 8-29 (2009)

## Acknowledgements

Authors acknowledge the financial support of the Grant Agency of the Academy of Sciences of the Czech Republic (KJB 601100901).

## Results and application to water samples

Analyte	Carboxen				Supel-Q			
	Linear range (ng/mL)	LOD (ng/mL)	in-day precision (%)	intra-day precision (%)	Linear range (ng/mL)	LOD (ng/mL)	in-day precision (%)	intra-day precision (%)
E3	5-1000	0.21	6.3	6.5	5-1000	0.63	4.5	4.9
BPA	1-1000	0.04	5.1	5.3	1-1000	0.04	3.5	2.0
NORE	2-1000	0.04	5.3	2.8	1-1000	0.05	3.1	11.5
EE2	2-1000	0.45	6.4	6.2	2-1000	0.22	6.2	4.4
NORG	2-1000	0.05	9.5	7.5	1-1000	0.05	5.8	2.7

Table 3. Analytical characteristics of the proposed in-tube SPME-HPLC-DAD method

Analyte	Added (ng/mL)	Carboxen					
		mQ-water		sea water		WWTP	
		Preconcentration factor	RSD %	Recovery %	RSD %	Recovery %	RSD %
E3	200	12.2	2.6	111.4	5.5	79.9	8.4
BPA	100	13.3	3.1	98.4	2.3	95.4	7.0
NORE	100	17.1	5.8	89.0	4.1	94.5	5.8
EE2	100	10.2	6.0	77.1	2.0	85.7	6.3
NORG	100	23.1	4.5	84.5	5.0	82.5	4.6

Analyte	Added (ng/mL)	Supel-Q					
		mQ-water		sea water		WWTP	
		Preconcentration factor	RSD %	Recovery %	RSD %	Recovery %	RSD %
E3	300	5.0	6.0	71.7	8.1	57.1	2.7
BPA	100	15.8	2.2	84.5	9.3	93.8	2.2
NORE	100	28.4	3.3	95.4	2.2	88.7	7.8
EE2	100	33.0	6.4	95.4	9.9	106.5	6.7
NORG	100	39.4	5.1	94.9	8.3	89.1	6.4

Table 4. In-tube-SPME applications in liquid samples

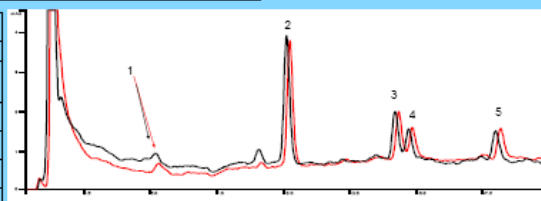


Figure 3. Chromatograms from sea water (■) and effluent of WWTP (■); extraction with Carboxen

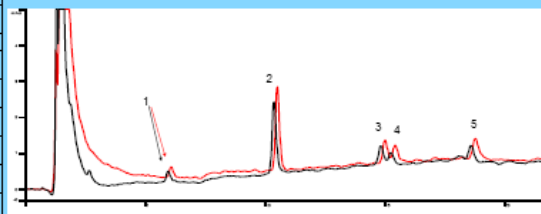


Figure 4. Chromatograms from sea water (■) and effluent of WWTP (■); extraction with Supel-Q

## Conclusions

We have optimised in-tube SPME-HPLC-DAD for the simultaneous determination of estrilol, bisphenol, norethisterone, ethinylestradiol and D-norgestrel using Supel-Q and Carboxen capillary columns. This method permits the on-line enrichment of the analytes with the advantages of minimum sample manipulation, low cost and high speed. Under the proposed conditions, no matrix effects were observed.



*36<sup>th</sup> International Symposium on Environmental  
Analytical Chemistry (ISEAC 36)  
October 05-09, 2010, Rome, Italy*

## POSTER AWARD



The International Association of Environmental  
Analytical Chemistry (IAEAC)

confers the *Roland W. Frei Award*

to:

Jana Aufartova

Department of Analytical Chemistry, Faculty of Pharmacy, Charles University,  
Heyrovského 1203, 500 05 Hradec Králové (Czech Republic)

for the best poster presentation by a young scientist.

Poster entitled:

*Comparison of different capillary columns  
in the optimization of in-tube SPME extraction  
of endocrine disruptors in environmental liquid samples*

Rome, October 09, 2010

A handwritten signature in black ink, appearing to read 'R. Pilloton'.

Dr Roberto Pilloton  
(Chairman)

A handwritten signature in black ink, appearing to read 'D. Klockow'.

Prof. Dr D. Klockow  
(President, IAEAC)



## **6.4 Supplement I**

Marcela Seifrtová, Jana Aufartová, Jitka Vytlačilová, Angelina Pena, Petr Solich, Lucie Nováková

**Determination of fluoroquinolone antibiotics in wastewater using ultra high-performance liquid chromatography with mass spectrometry and fluorescence detection**

Journal of Separation Science 33 (2010) 2094-2108.

Marcela Seifrtová<sup>1</sup>  
 Jana Aufartová<sup>1</sup>  
 Jitka Vytlačilová<sup>2,3</sup>  
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## Research Article

# Determination of fluoroquinolone antibiotics in wastewater using ultra high-performance liquid chromatography with mass spectrometry and fluorescence detection

A new ultra HPLC (UHPLC) method using both MS and fluorescence detection (FD) was developed for the determination of five fluoroquinolones in wastewaters. Systematic method development approach was compared with a conventional one. During the systematic approach, a possibility of automatic switching among four independent analytical columns of different chemistries has been used. Acidic as well as basic pH using ACN and methanol as organic modifiers was tested. The best separation of fluoroquinolones was obtained on phenyl analytical column at pH 10.5, which is a completely novel approach for separation of fluoroquinolones. Further, a new SPE procedure was developed for the sample preparation using basic pH as well. The sensitivity and selectivity of FD and MS detection were compared. FD at basic pH 10.5 demonstrated lower sensitivity than at acidic pH, which is conventionally performed. At basic pH, UHPLC-MS/MS was found about two orders of magnitude more sensitive than FD. Both methods were validated and subsequently UHPLC-FD method was used for the evaluation of stability of fluoroquinolones. UHPLC-MS/MS method was used for the analysis of wastewater samples. Norfloxacin and ciprofloxacin were detected in samples of influent and effluent from wastewater treatment plant. Ofloxacin was detected only in influent from wastewater treatment plant.

**Keywords:** Fluoroquinolones / Systematic method development / Ultra HPLC / Wastewaters

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## 1 Introduction

Consumption of antibiotics grows up significantly every year as they are a group of pharmaceuticals widely used in treatment of various bacterial infections. Fluoroquinolones (FQs) are an important group of broad-spectrum synthetic antibacterial agents used for the treatment of gram-negative

bacterial infections in both human and veterinary medicine [1]. FQs, as other pharmaceuticals, commonly enter into the environment mainly via urine and faeces, hospital wastewater and from veterinary use [2]. Several investigations have shown some evidence that pharmaceuticals are often not eliminated during wastewater treatment process in wastewater treatment plant (WWTP) [3]. The mechanism of their elimination is sorption to sewage sludge [2] and it leads to removal rate approximately between 80 and 90% [4, 5]. Another very important source of contamination with large variety of pharmaceuticals is hospital wastewater [5]. Antibiotics occur there at higher concentrations ( $\mu\text{g/L}$ ) [5] than in municipal wastewaters ( $\text{ng/L}$ ) [3, 6]. There are two reasons for this fact: their high usage in hospitals and high dilution of municipal wastewaters. FQs can persist in the environment as they have been shown to bind to solid particles [7]. Consequently, FQs may contaminate agricultural fields, disturb natural balance and accumulate in crops and vegetables [8]. Even very small amounts of antibiotics in everyday food may generate the strains of resistant bacteria in human and animal bodies, induce allergy and affect the liver [9]. The main risk for the public health is nevertheless the ability of FQs to induce the development of bacterial resistance.

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**Abbreviations:** AmAc, ammonium acetate; AmF, ammonium formate; AQC, Acquity; CIPRO, ciprofloxacin; DANO, danofloxacin; DIFL, difloxacin; ENO, enoxacin; ENRO, enrofloxacin; FAC, formic acid; FD, fluorescence detection; FLU, flumequine; FQ, fluoroquinolone; LOME, lomefloxacin; MeOH, methanol; NAL, nalidixic acid; NOR, norfloxacin; OFLO, ofloxacin; OXO, oxolinic acid; PEFL, pefloxacin; SARA, sarafloxacin; SRM, selected reaction monitoring; SST, system suitability test; UHPLC, ultra HPLC; WWTP, wastewater treatment plant

Despite lots of studies with positive detection of antibiotics and other pharmaceuticals in soils and environmental waters and despite of their negative effects on human health, there is no defined limit value for the occurrence of these pollutants in soils or natural waters. European Union requires maximum residue limits for the sum of enrofloxacin (ENRO) and its metabolite ciprofloxacin (CIPRO) for all food producing animals. For example, in cattle the maximum residue limits are 300 µg/kg in liver and 200 µg/kg in kidney [10].

FQs are characterized by fluorine atom at position 6 of the quinolone naphthyridine or benzoxazine ring (Fig. 1) [11]. They have two relevant ionizable functional groups, the 3-carboxyl group and *N*-4 of the piperazine substituent. Therefore, FQs have two  $pK_a$  values and their acid–base behavior will be significantly affected by physicochemical properties of the solvent [2]. Reported values of  $pK_a$  for carboxylic group range from 5.7 to 6.3, whereas those for protonated amino group are higher (7.6–8.3). The intermediate form of FQs is a zwitterion [12].

Several methods for the determination of FQs in environmental matrices including surface waters, wastewaters, WWTPs effluents and soils have been recently published (Table 1). LC with fluorescence detection (FD) or MS detection was mostly employed in environmental applications. Only in one study, capillary electrophoresis was used for the separation of FQs in biological and environmental samples [13]. FQs are naturally highly fluorescent compounds and thus FD is suitable for their detection. However, in comparison with MS, FD was found to be less sensitive and some impurities or other fluorescent compounds could interfere with their sensitive and specific detection [14]. This problem may occur especially in complex environmental matrices such as wastewaters where many other interfering compounds are typically present. Therefore, a coupling of LC-MS or LC-MS/MS is a method of choice for selective determination of FQs and also other pharmaceuticals in wastewaters.

SPE is the crucial step in environmental analysis as it is necessary to preconcentrate analytes because of very low concentrations typically found in environmental waters and due to the complexity of this matrix. Sample cleanup influences further analysis especially when MS detection is employed as ESI source is highly susceptible to matrix

interference. SPE is the most widely used procedure for the sample pretreatment of FQs in environmental matrices (Table 1). Only one study used solid-phase microextraction [15]. The most widely used SPE cartridges were polymeric Oasis HLB cartridges because their lipophilic divinylbenzene and the hydrophilic *N*-vinylpyrrolidone composition allows the extraction of polar and nonpolar analytes and the cartridges might be used within a wide pH range.

For the separation of FQs, C18 or C8 analytical columns were used (Table 1). In two studies, monolithic columns were employed [4, 16], whereas other methods applied conventional particulate columns. In all cases, the separation was done with mobile phase of acidic pH below the first  $pK_a$  value. Ferdig *et al.* [17] reported the problem of separation of norfloxacin (NOR) and CIPRO. The separation of these two antibiotics is challenging as they differ only by a small side group (ethyl and cyclopropyl (Fig. 1)). Toussaint *et al.* [18] compared C8 and C18 analytical columns and C8 stationary phase was chosen in order to reduce the retention time of acidic FQs and further to improve the symmetry of the chromatographic peaks. Only one study referred using phenyl analytical column for the separation of FQs together with tetracycline antibiotics by HPLC-FD [19]. The analyses were performed at pH 6.5 on the basis of already published article. However, the analysis took more than 25 min and some analytes were not baseline separated although it was sufficient for successful quantitation. Low sensitivity of FD at this pH was another drawback of this method. At this pH, FQs do not demonstrate sufficient fluorescence compared to the pH range of 3–4 [19] which might be the reason why almost all authors choose lower pH for FD. However, the selection of pH 6.5 was made as a compromise to ensure sufficient fluorescence for both classes of antibiotics studied.

Some of the developed HPLC methods [3, 12, 17] were highly time consuming (up to 40 min) and high amounts of organic solvents were used which is contrary to current trends in analytical chemistry that are miniaturizing equipment, lowering solvent consumption and of course decreasing the time of analysis. Ultra HPLC (UHPLC) allowed high separation efficiency and resolution, high sensitivity and much lower solvent consumption using sub-2-µm particles. This led to fast and high-resolution analysis compared to HPLC with conventional particle size sorbents. There was only one study employing UHPLC with MS

Compound	R1	R2	R3	General structure
NOR	-H	-H	-C <sub>2</sub> H <sub>5</sub>	
CIPRO	-H	-H	-Cyclopropyl	
PEFLO	-CH <sub>3</sub>	-H	-C <sub>2</sub> H <sub>5</sub>	
OFLO	-CH <sub>3</sub>	-H	-CH <sub>2</sub> -CH <sub>2</sub> -CH <sub>3</sub>	
ENRO	-C <sub>2</sub> H <sub>5</sub>	-H	-Cyclopropyl	

Figure 1. Chemical structures of selected fluoroquinolones

Table 1. An overview of methods for the determination of FOs in environmental waters<sup>a)</sup>

Substances determined	Matrix sample preparation	Stationary phase analytical column	Mobile phase	Detection	Analysis time (min)	Validation data	Ref.
9FOs: CIPRO, DIFL, ENRO, LOME, NOR, OFLO, PIP, SARA, TDS	WWTP effluents, surface water (river and lake water) SPE (150–500 mL, pH 3)	YMC ODS-AQ S-3 (50 × 4.0 mm)	A: water (pH 3.0) + ACN (98:2) B: ACN 5:95, gradient	ESI-MS [M+H] <sup>+</sup> FD Exc: 278 nm Em: 445–500 nm	35	$\bar{r}^2 > 0.9992$ LODMS = 8.6–49 ng/L	[3]
4FOs: OFLO, NOR, CIPRO, ENRO	Wastewater SPE (pH 4.5)	Chromolith Performance RP-18e (100 × 4.6 mm)	0.025 M H <sub>3</sub> PO <sub>4</sub> (pH 3.0 by TBA): MeOH/ACN 92:70:10, isocratic	FD Exc: 278 nm Em: 460 nm	12	$\bar{r}^2 > 0.997$ LOD = 0.5–85 ng/L LOQ = 25–250 ng/L	[4]
20FOs: PIP, FLE, OFLO, PEFO, ENO, NOR, CIPRO, DANO, ENRO, LOME, DIFL, SARA, GATI, SPAR, MOXI, CINO, OXO, NAL, FLU, PIRO IS = NOR-d <sub>5</sub>	Wastewaters, river water SPE (200–800 mL, pH 3)	ACO UPLC BEH C18 (100 × 2.1 mm, 1.7 μm)	MeOH/0.1% FAC 10:90, gradient	ESI-MS/MS [M+H] <sup>+</sup>	12	$\bar{r}^2 > 0.996$ LOD = 0.6–50 ng/L	[6]
10FOs: CIPRO, DANO, DIFL, ENRO, FLU, MARBO, NAL, NOR, OXO, SARA	Surface water (seawater, well water) SPE (250–500 mL, pH 5.5)	Inertsil C8 (250 × 4.6 mm, 5 μm)	A: 10 mM oxalic acid buffer (pH 4) + ACN (89:11, v/v) B: ACN, gradient	FD Exc: 248–297 nm Em: 361–507 nm	35	LOD = 0.05–1 μg/L	[12]
3FOs: OFLO, NOR, CIPRO IS = CIPRO- <sup>13</sup> C <sub>3</sub> <sup>15</sup> N	WWTP effluents SPE (250 mL, pH 3)	Zorbax SB-C8 (150 × 2.1 mm, 3.5 μm)	ACN/MeOH/FAC/water 6:12:0.5:81.5, isocratic	FD Exc: 278 nm Em: 460 nm ESI-MS ESI-MS/MS [M+H] <sup>+</sup>	20	$\bar{r}^2 > 0.999$ RSD < 6% LOQ = 2–10 ng/L	[14]
5FOs: ENO, OFLO, CIPRO, NOR, LOME	Surface water, wastewater SPME	CAPCELL PAK C8 (100 × 2.1 mm, 5 μm)	5 mM AmF (pH 3)/ACN 85:15, isocratic	ESI-MS/MS [M+H] <sup>+</sup>	7	$\bar{r}^2 > 0.997$ RSD = 0.5–9.7% LOD = 7–29 ng/L	[15]
3FOs: NOR, CIPRO, ENRO	Surface water (river water) SPE (1000 mL, pH 4)	Chromolith Performance RP-18e (100 × 4.6 mm)	0.025 M H <sub>3</sub> PO <sub>4</sub> (pH 3.0 by TBA)/MeOH 96:4, isocratic	FD Exc: 278 nm Em: 460 nm	16	$\bar{r}^2 > 0.994$ LOQ = 25 ng/L	[16]
9FOs: CIPRO, ENRO, FLE, FLU, LOME, MOXI, NOR, OFLO, OXO	Surface water, municipal wastewater, WWTP effluent, sewage sludge,	ID YMC-Pack Pro C18 (250 × 4.6 mm, 3 μm)	50 mM FAC/MeOH 78.5:21.5, gradient	FD Exc: 278–320 nm Em: 365–500 nm ESI-MS	40	$\bar{r}^2 > 0.999$ RSD < 5% LOQFD = 11–60 ng/L LOGMSMS = 0.3–7.0 ng/L	[17]

**Table 1.** Continued.

Substances determined	Matrix sample preparation	Stationary phase analytical column	Mobile phase	Detection	Analysis time (min)	Validation data	Ref.
	sediment SPE (500 mL, pH 4.2)			ESI-MS/MS [M+H] <sup>+</sup>			
9FQs: CIPRO, DANO, ENO, ENRO, NOR, QINO, FLU, NAL, OXO	Surface water (lake and river water) SPE (250 mL, pH 4)	Polarity $\mu$ C18 (150 × 3 mm, 3 $\mu$ m)	FAc (pH 2.5)/ACN 96:4, gradient	UV 275 or 255 nm	25	$r^2 > 0.99$ LOD = 8–20 ng/L	[25]

a) CINO, cinoxacin; FLE, flexoxacin; GATL, gatifloxacin; MARBO, marbofloxacin; MOXI, moxifloxacin; PIP, pipemidic acid; PIRO, piroimidic acid; SPAR, sparfloxacin; TOS, tosuflouxacin.

detection for the separation of 20 FQs within 12 min [6]. The separation was performed on BEH C18 analytical column at acidic pH. Despite very fast separation of 20 analytes, the disadvantage of this method was spending a lot of time for sample percolation during SPE step. Study of Tamtam *et al.* employed UHPLC-MS/MS for the determination of 17 antibiotics from different groups within 10 min [20]. However, no internal standard has been used for the quantification which is fully recommended for the analysis of environmental samples. Another study for the determination of 21 antibiotics from seven different classes by UHPLC-MS/MS at acidic pH was developed [21]. The analysis was performed within 10 min; however, authors spent about 80 min during SPE procedure.

Recently, Waters Corporation has designed a new column manager with the possibility of connection of four independent analytical columns into UHPLC system simultaneously. These columns can be switched automatically by means of a switching valve, enabling the possibility to perform fully automated systematic method development approach in one sequence. In our study, four analytical columns of different chemistries (reverse-phase C18 column BEH C18, C18 column BEH Shield RP18 with embedded polar phase, phenyl-hexyl analytical column BEH Phenyl, and silica-based analytical column HSS T3 C18) were tested. Two organic modifiers (methanol (MeOH) and ACN) and two buffers (acidic and basic) (e.g. pH 3 and 9) could be tested in gradient mode of elution. Using generic gradient within 5 h, 14 different chromatograms including all variables (column, buffer and organic modifier) were generated. Prearranged setting of “systematic method development” was developed and recommended by Waters Corporation as universal technique. Of course, any step (analysis conditions, wash step) and any input (column, buffer and organic modifier) can be modified upon request of the developed method. After series of measurement, the software is able to interpret results into reports with criteria set by the user (e.g. injection score report, peak capacity report, total peak with resolution higher than 1.5 report), allowing the final consideration for the selection of analytical column, organic modifier and pH of analysis. For example, total peak number report shows the number of integrated peaks in each separation (Fig. 2). Such preliminary results for all variables facilitate further method development and fine tuning.

In this study, conventional approach and systematic method development for the determination of five commonly used FQs were compared. Initially, the separation of FQs was performed at acidic pH according to the previously published results [4]. On the other hand, the separation of FQs at basic pH at values higher than  $pK_{a2}$  has never been performed probably because of low pH stability of conventionally used silica-based analytical columns. Performance and sensitivity of UHPLC method using both FD and MS detection were compared. The developed UHPLC-FD method was used for the evaluation of stability of FQ solutions, whereas developed UHPLC-MS/MS method was applied for the analysis of wastewater samples.

## 2 Materials and methods

### 2.1 Reagents and materials

Reference standards of FQ antibiotics (ofloxacin (OFLO), pefloxacin (PEFLO), CIPRO, NOR, ENRO and NOR d<sub>5</sub> – isotopically labelled) were obtained from Sigma-Aldrich (Prague, Czech Republic). All pharmaceuticals were of analytical grade (purity ≥98%). The ammonium acetate (AmAc), ammonium formate (AmF), formic acid (FAc), the ammonia, all reagent grade, ACN, HPLC-gradient grade and MeOH, LC-MS grade, were purchased from Sigma-Aldrich. HPLC-grade water was prepared by Milli-Q reverse osmosis Millipore (Belford, MA, USA) and it meets European Pharmacopoeia requirements.

### 2.2 Sample collection

Briefly, 24-h composite samples of influent and effluent after chlorination were collected from WWTP in the University Hospital in Hradec Králové in September 2009. The WWTP is a mechanical–biological sewage treatment plant with complete aerobic sludge stabilization (hydraulic

retention time is approximately 8 h). The maximum daily influx of the mechanical and biological part is 250 m<sup>3</sup>/day and it depends on the activity of the individual hospital departments. The main task of this wastewater plant is to remove the dangerous property of the wastewater infectivity. Treated wastewater flows directly to the municipal WWTP for the whole city, Hradec Králové. River samples were collected from the river Elbe in Hradec Králové. The samples were stored in 2-L glass bottles in fridge at 4°C and analyzed as soon as possible.

### 2.3 Preparation of standards, fortified samples and blank samples

Stock solutions of FQs were prepared at a concentration of 0.1 mg/mL in MeOH and they were stored in a glass vial at 4°C in dark. Working standard solutions were prepared in a mixture of AmAc, pH 10.5, and MeOH (9:1, v/v) by appropriate dilution of stock solution.

The water samples were filtrated through 0.2-µm filters to remove solid particles. To obtain fortified samples, 20 mL of water samples were spiked with antibiotic standard mixture to obtain final low-, medium- and high-concentra-

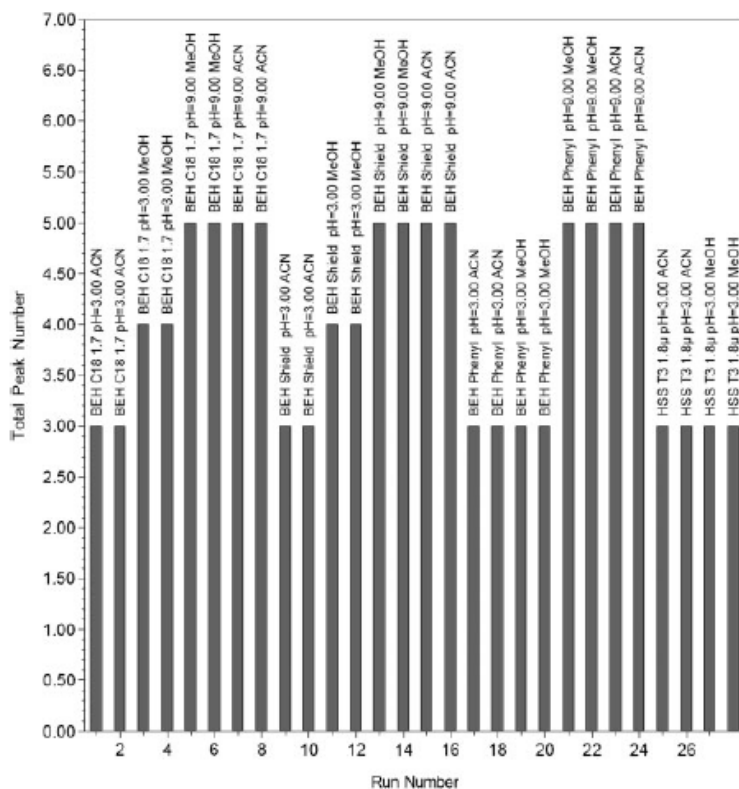


Figure 2. An example of report generated by Empower software. Total peak number report shows the number of separated peaks in each chromatogram.

tion level. Internal standard NOR-d<sub>5</sub> was added to each sample before sample preparation and to the mixture of standards of FQs. All samples (spiked samples and blank samples) were adjusted to pH 10.5 with ammonia and 0.02 g of EDTA was added to avoid antibiotics to form complexes with divalent ions and metals and to avoid possible sorption to, e.g. glass surface.

## 2.4 SPE

A completely new extraction procedure for the preconcentration of FQs and cleanup at basic pH 10.5 was developed. Water samples were extracted through Oasis HLB cartridges (6cc/200 mg, Waters, Prague, Czech Republic). The cartridges were activated with 3 mL of MeOH and conditioned with 3 mL of water, pH 10.5. After sample percolation, the SPE cartridges were washed with 3 mL of 10% MeOH in water, 3 mL of 2% acetic acid and 12 mL of water. After being washed, HLB cartridges were dried and subsequently analytes were eluted with 2 mL of 1% ammonia in MeOH. The eluate was evaporated to dryness under a gentle stream of nitrogen and redissolved in 0.5 mL of mixture of 10 mM AmAc, pH 10.5, and MeOH (9:1). The sample was further sonicated for 30 min and vortexed for 1 min. Finally, the sample was filtered through 0.2 µm PTFE filter to remove possible particles and injected onto UHPLC system. An achieved enrichment factor was 40.

Cleanup efficiencies were studied in order to monitor the effect of pH on retention of FQs. The effects of the type and pH of solvents used during conditioning and washing step and elution were evaluated.

## 2.5 LC and MS

### 2.5.1 LC

Two UHPLC Acquity (ACQ) chromatographic systems were used in this study (Waters). UHPLC for systematic method development and for UHPLC-FD measurements consisted of ACQ-binary solvent manager, ACQ-sample manager, ACQ-column manager, ACQ-PDA detector and ACQ-FLR detector. During the systematic method development ACQ BEH C18, ACQ BEH Shield RP 18, ACQ BEH Phenyl and ACQ HSS T3 C18 columns all in 50 × 2.1 mm, 1.7 or 1.8 µm dimensions were used. They were all obtained from Waters. The columns were maintained at 30°C at a flow rate 0.6 mL/min. The injection volume was 2 µL. The gradient elution was used for the separation. MeOH or ACN was used as the organic modifier (solvent A) and 10 mM AmF buffer (pH 3.0) or 10 mM AcAc buffer (pH 9) was used as aqueous component of mobile phase (solvent B). The gradient conditions were initiated with 5% A followed by a linear increase to 95% A in 5 min and by re-equilibration for 2 min. The design of systematic method development was as follows: First, each column was equilibrated for 2 min.

Subsequently, one blank (initial mobile phase) and two samples (mixture of FQs) were injected at four different gradients using ACN/buffer, pH 3, MeOH/buffer, pH 3, MeOH/buffer, pH 9, and ACN/buffer, pH 9, always after 2 min of equilibration. After a set of experiments, each column was washed with ACN for 2 min and ACQ-CM automatically switched to the next column. This process was repeated on BEH C18, BEH Shield RP 18 and BEH Phenyl, whereas on HSS T3 C18 only two gradients using ACN/buffer, pH 3, and MeOH/buffer, pH 3, could be performed because of limited stability of silica-based column. The results showed better separation of FQs at pH 9. Thus, in second set of experiments, AmAc solution at pH 10.5 and widely used 0.1% FAc (solvent B) were tested.

The measurements of fluorescence spectra at acidic pH 3.0 and basic pH 10.5 were performed in order to check the optimal excitation and emission wavelengths. Moreover, the comparison of FD sensitivity at commonly used  $\lambda_{\text{exc}} = 278$  nm and  $\lambda_{\text{em}} = 450$  nm and the optimal excitation and emission wavelengths found at acidic and basic pH were accomplished.

The gradient separation at basic conditions resulting from systematic approach was further optimized. The separation of FQs using UHPLC-FD was finally enabled using ACQ BEH Phenyl (50 × 2.1 mm, 1.7 µm). The column was maintained at 35°C at a flow rate 0.45 mL/min and the injection volume was 2 µL. MeOH (solvent A) and 10 mM AmAc pH 10.5 (solvent B) were used as mobile phases. The gradient conditions were initiated with 5% A kept for 1 min followed by linear increase to 75% A in 3 min. During next 4 min, the concentration of solvent A was decreased to 45% and then followed re-equilibration to initial conditions. The fluorescence detector was operated at 278 nm excitation and 450 nm emission wavelengths based on our previously published method [4] and the sensitivity was compared with the measurement at  $\lambda_{\text{exc}} = 310$  nm and  $\lambda_{\text{em}} = 415$  nm, which were fluorescence maxima found at basic pH.

The comparison of sensitivity at acidic pH was performed as well. Separation at acidic pH was achieved based on our previous experience using ACQ BEH C18 (50 × 2.1 mm, 1.7 µm) at 30°C under isocratic condition at a flow rate of 0.3 mL/min. The mobile phase was a mixture of 10 mM AmF (pH 3) and MeOH (81:19, v/v). The measurements were carried out in two series because of impossibility to separate all five FQs sufficiently. In first set, OFLO, NOR and ENRO were measured. PEFLO and CIPRO were measured in second set of experiments. The detector was operated at 278 nm excitation and 450 nm emission wavelengths. For the comparison of FD sensitivity, the detection was performed at excitation 310 nm and emission wavelengths 447 nm, which were optimal values at acidic pH.

Second UHPLC system consisted of ACQ-binary solvent manager, ACQ-sample manager and ACQ-TUV detector. It was coupled to Quattro Micro triple quadrupole mass spectrometer (Micromass, Manchester, GB, UK) equipped



with a multi-mode ionization source (ESCI). Separation of FQs was achieved by an ACQ BEH Phenyl analytical column (50 × 2.1 mm, 1.7 μm). The column was maintained at 35°C at a flow rate 0.35 mL/min and the injection volume was 2 μL. MeOH (solvent A) and 0.5 mM AmAc pH 10.5 (solvent B) were used as a mobile phase. The gradient conditions were initiated with 2% A followed by linear increase to 45% in 2 min. During next 0.5 min, the concentration of solvent A was further increased up to 70% following by re-equilibration for 2 min to initial conditions.

### 2.5.2 MS

ESI positive mode was used for analysis. Ion source was set up as follows: capillary voltage: 1000 V, ion source temperature: 130°C, extractor: 2.0 V and RF lens: 0.2 V. The desolvation gas was nitrogen at a flow rate of 500 L/h and the temperature 450°C. Cone voltage was set up individually for each analyte (Table 2). Nitrogen was also used as a cone gas (100 L/h) to prevent the contamination of sample cone. Quantitation of all analytes was performed using selected reaction monitoring (SRM) experiment. Two specific SRM transitions were optimized for each analyte in order to increase selectivity of the method. Argon was used as a collision gas and collision energy was optimized for each analyte individually as summarized in Table 2. The MassLynx 4.1 software was used for MS control and data gathering. QuanLynx software was used for data processing and quantification–regression analysis of calibration curves and calculation of concentrations.

### 2.6 System suitability test and validation

Identification of the target FQs was accomplished by a comparison of retention times (UHPLC-FD) and using the two SRM ion transitions for each compound (UHPLC-MS/MS). The first SRM transition was utilized for quantitation and the second for confirmation. Moreover, secondary ion ratio of the two SRM ion transitions was compared with

those of standards for unequivocal confirmation. Difference within 20% was considered as an agreement. In order to compensate for the loss of target analytes during the extraction procedure and account for the matrix effects, an internal standard NOR-d<sub>5</sub> was spiked to the samples prior to extraction.

An important part of method validation is the system suitability test (SST), details of which are usually given in Pharmacopoeias [22]. The SST was performed under optimized chromatographic conditions. Resolution, asymmetry factor, repeatability of retention time and peak area were tested in UHPLC-FD method. UHPLC-MS method was checked only for the repeatability of retention times and peak area.

Calibration curves of all analytes in the concentration range of 0.01–5 μg/mL for UHPLC-FD were measured. The concentration range used for UHPLC-MS/MS method was from 0.5 to 500 ng/mL, corresponding to concentrations in water samples ranging between 0.0125 and 12.5 μg/L taking into account the 40-fold concentration factor of the SPE. Calibration curves were generated by linear regression of peak areas of the standard solutions against their respective concentrations. LODs and LOQs were established based on *S/N*. LOD was expressed as *S/N* = 3 and LOQ was expressed as *S/N* = 10.

Method precision and accuracy were established to evaluate the SPE procedure. For the precision, spiked wastewaters at three different concentration levels were measured in three replicates to calculate RSD, which describes the closeness of agreement between series of measurements. Accuracy was determined as a method recovery using spiked wastewaters, again at three different concentration levels in three replicates in order to establish the closeness of agreement between the true and the measured value as it corresponds to International Conference on Harmonization requirements [23]. Recoveries were calculated by comparison of the peak areas obtained from spiked samples with the peak areas from the same samples without standard solution addition (blanks), finally, with the areas obtained by direct injection of a standard solution at

**Table 2.** SRM transitions for all analytes and MS/MS parameters

Compound	Precursor	Fragment		Dwell time	Cone V	Collision E	SRM ratio	t <sub>R</sub>	
NOR	319.9	[M+H] <sup>+</sup>	301.9	[M+H-H <sub>2</sub> O] <sup>+</sup>	0.05	30	20	3.4	1.25
			276.2	[M+H-CO <sub>2</sub> ] <sup>+</sup>	0.05	30	15		
NOR-D5	324.9	[M+H] <sup>+</sup>	307.0	[M+H-H <sub>2</sub> O] <sup>+</sup>	0.05	30	20	4.0	1.24
			281.1	[M+H-CO <sub>2</sub> ] <sup>+</sup>	0.05	30	15		
CIPRO	331.9	[M+H] <sup>+</sup>	314.0	[M+H-H <sub>2</sub> O] <sup>+</sup>	0.05	25	20	1.1	1.37
			230.5	[M+H-H <sub>2</sub> O-C <sub>2</sub> H <sub>5</sub> N-C <sub>3</sub> H <sub>4</sub> ] <sup>+</sup>	0.05	30	35		
PEFLO	333.9	[M+H] <sup>+</sup>	289.9	[M+H-CO <sub>2</sub> ] <sup>+</sup>	0.05	30	15	1.5	1.62
			316.3	[M+H-H <sub>2</sub> O] <sup>+</sup>	0.05	30	20		
OFLO	362.0	[M+H] <sup>+</sup>	261.2	[M+H-H <sub>2</sub> O] <sup>+</sup>	0.05	30	25	2.6	1.71
			318.0	[M+H-CO <sub>2</sub> ] <sup>+</sup>	0.05	30	20		
ENRO	359.9	[M+H] <sup>+</sup>	316.1	[M+H-CO <sub>2</sub> ] <sup>+</sup>	0.05	30	20	3.5	2.00
			341.9	[M+H-H <sub>2</sub> O] <sup>+</sup>	0.05	30	20		



the concentration level expected after sample treatment. The method was considered accurate if recoveries were in the range 80–120% and precision was satisfactory if RSD was lower than 20%.

### 3 Results and discussion

#### 3.1 Conventional method development

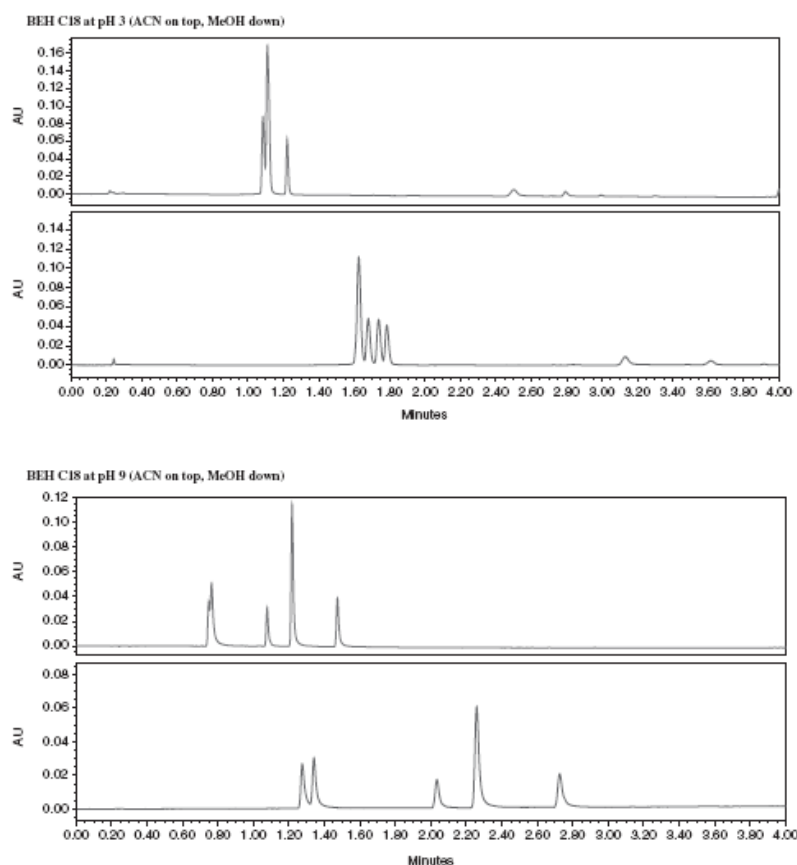
The analysis of four FQs including NOR, CIPRO, OFLO and ENRO using HPLC-FD performed on monolithic column was previously published by our group [4]. Further, PEFLO was added into analytical method as it is used for the treatment in Czech Republic as well. This became a challenge, because the separation of OFLO and PEFLO at low pH on C18 was difficult even impossible because of similar retention on analytical column and consequently the elution at same retention times. Although various gradient elutions employing different additives were used, the separation with sufficient resolution of those analytes has

not been achieved. Thus the coupling of HPLC with fluorescence detector could not be used for separation of all five FQs anymore.

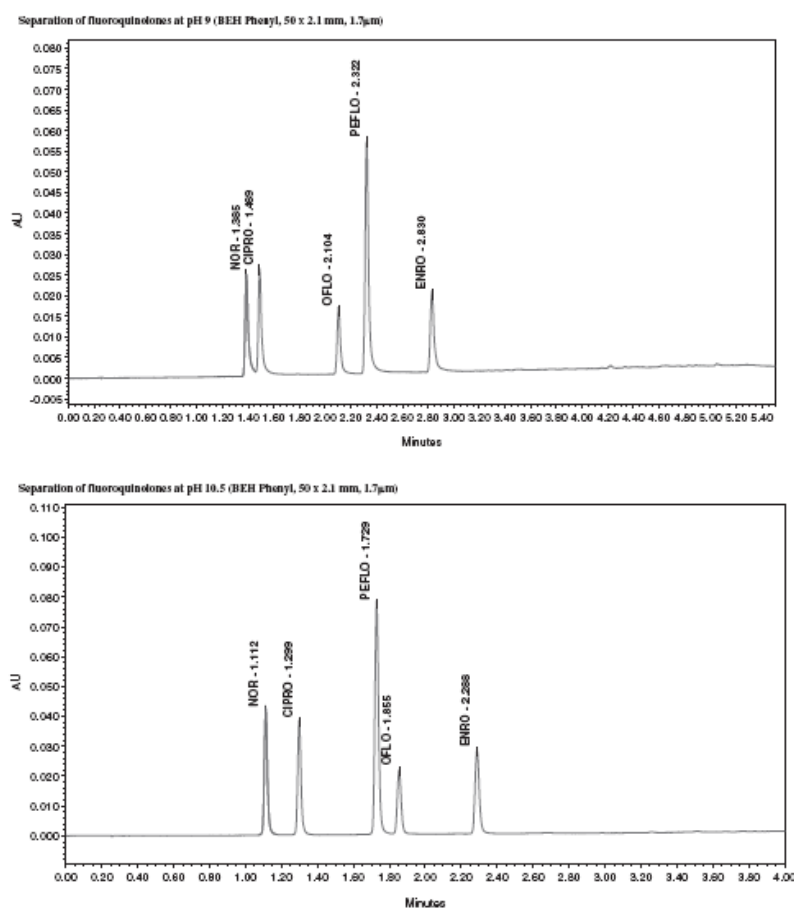
Consequently, further experiments were performed on UHPLC system. A new method was developed on UHPLC-MS/MS using BEH C18 analytical column. Mobile phase employed MeOH in combination with volatile additives including FAC, AmAc or AmF buffers at acidic pH, which is in accordance with the previously published results dealing with the analysis of FQs (Table 1). However, the results obtained were not repeatable. The peak shapes at low pH were also not satisfactory, probably due to low concentrations of additives. Therefore, systematic method development was employed in order to re-optimize the method properly.

#### 3.2 Systematic method development

For the systematic method development, the prearranged settings developed and recommended by Waters Corpora-



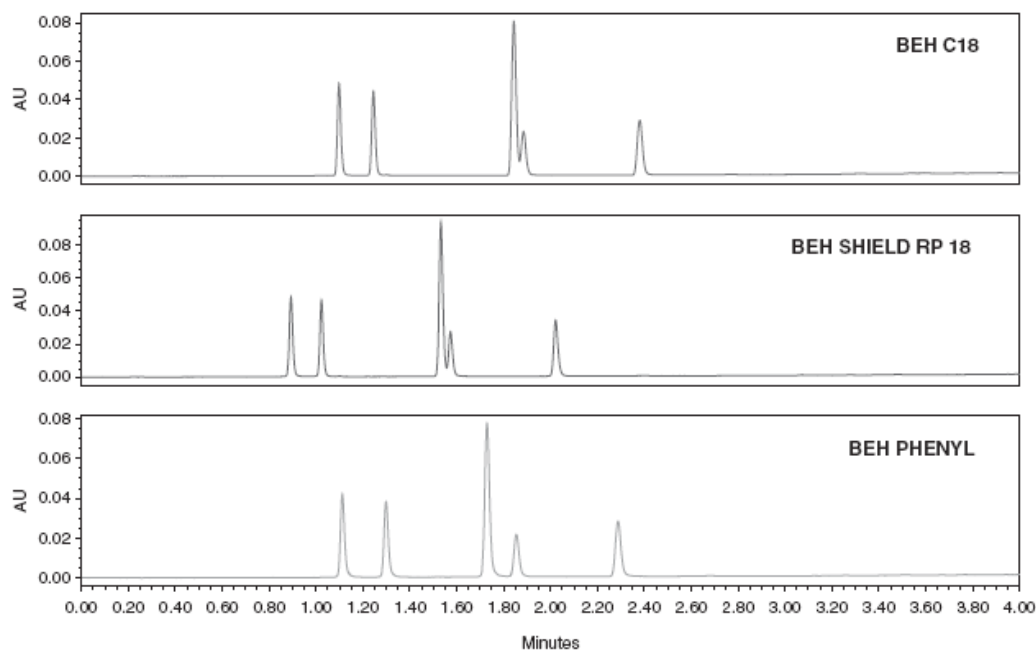
**Figure 3.** The influence of organic modifier and pH on separation of FQs on C18 analytical columns, conventionally used in FQs analysis.



**Figure 4.** Chromatogram of standard solution at pH 9 (upper) and at pH 10.5 (lower) obtained on UHPLC system. The peak order switch is demonstrated in dependence on relatively small change of pH.

tion, described in Section 2.5, were used. C18 analytical column seems to be a standard in the analysis of FQs. Therefore, it was chosen for the first judgment of organic modifier and pH choice. As shown in Fig. 3, MeOH was substantially more efficient in the separation of FQs than ACN and it was used for further method development. Second, pH was found to be a crucial factor for FQs separation as they are zwitterions and their ionization is pH dependent. At pH 3, which is the pH usually used in FQs analysis [4, 15], the separation of two peaks (NOR and CIPRO) was insufficient. Contrary to all the published results of separations performed at acidic pH, much better separation was obtained at pH 9 on all tested columns BEH C18, BEH Phenyl and BEH Shield RP 18 (chromatographic data not shown, see report in Fig. 2). HSS C18 must have been excluded from pH 9 experiments as it is silica-based column and thus its pH range is limited. Moreover, the elution order of FQs at pH 9 was changed as follows: NOR, CIPRO, OFLO, PEFLO, ENRO in contrast to elution

order at acidic pH which was OFLO, PEFLO, NOR, CIPRO and ENRO. The best resolution of the first two peaks – NOR and CIPRO – was observed on BEH Phenyl column. However, slight peak tailing was still observed. In order to improve peak shape and the resolution of the first two peaks, higher pH of 10.5 was tested in further experiments. Surprisingly, such relatively small change in pH (about 1.5 unit) was again crucial in FQs separation. As it was expected, the peak shape improved and there was no more tailing observed. In addition, the resolution between the peaks of NOR and CIPRO was substantially improved (Fig. 4). Moreover, pH 10.5 also caused the switching of the order of OFLO and PEFLO and final order of separated peaks was as follows: NOR, CIPRO, PEFLO, OFLO and ENRO. Subsequently, BEH C18 and BEH Shield RP18 analytical columns were no more suitable for the analysis of tested FQs as the peaks of OFLO and PEFLO were not baseline separated any more (Fig. 5). BEH Phenyl showed the best separation of critical pairs of peaks NOR/CIPRO



**Figure 5.** Chromatograms obtained during systematic method development on different stationary phases. Mobile phase composition: MeOH and AmAc pH 10.5.

and OFLO/PEFLO. Therefore, it was used in further study for the development of UHPLC-FD as well as for UHPLC-MS/MS method.

Using systematic method development revealed that FQs can be successfully separated at pH 10.5 on BEH Phenyl ( $50 \times 2.1$  mm,  $1.7 \mu\text{m}$ ) analytical column. Recently, there was a published study dealing with the stability of analytical columns [24]. They reported the instability of XBridge phenyl analytical column (Waters) over pH 7. However, in this study, we have not observed any changes of peak shapes or retention times during usage of BEH phenyl analytical column at basic pH 10.5 even after 3000 injections (approx. 2000 injections at basic pH).

### 3.3 Optimization of UHPLC analysis with FD and MS detection

#### 3.3.1 UHPLC-FD

The gradient profile and mobile phase flow rate used in the systematic method development were further optimized in order to keep baseline separation of all FQs and to shorten the analytical run. The separation of critical pairs NOR/CIPRO and PEFLO/OFLO in one mixture is an analytical challenge. The previously published studies using FD typically did not include combination of all these four analytes, especially PEFLO has not been included in analysis.

Most of the studies utilized low pH, as fluorescent properties of FQs are more advantageous (Table 1). In our study, commonly used excitation and emission wavelengths, 278 and 450 nm, were used for the detection at acidic pH [3, 4, 14, 15]. However, fluorescence spectra measurements were performed in order to compare optimum excitation and emission wavelengths at acidic pH 3.0 and basic pH 10.5. The difference of fluorescence spectra could be observed in dependence on pH of mobile phase. CIPRO, PEFLO and ENRO showed the same fluorescence spectra. At acidic pH, they showed the maxima of  $\lambda_{\text{exc}} = 310$  nm and  $\lambda_{\text{em}} = 447$  nm, whereas at basic pH the optimal  $\lambda_{\text{exc}}$  and  $\lambda_{\text{em}}$  wavelengths were 310 and 415 nm, respectively. NOR revealed similar spectra; however, small differences from CIPRO, PEFLO and ENRO could be observed. The optimal  $\lambda_{\text{exc}}$  and  $\lambda_{\text{em}}$  at acidic pH were 311 and 447 nm, whereas at basic pH these optimal  $\lambda_{\text{exc}}$  and  $\lambda_{\text{em}}$  were 308 and 415 nm, respectively. OFLO showed completely different spectra from the other FQs. At acidic,  $\lambda_{\text{exc}} = 298$  nm and  $\lambda_{\text{em}} = 490$  nm, whereas at basic pH the optimal  $\lambda_{\text{exc}}$  and  $\lambda_{\text{em}}$  wavelengths were 295 and 464 nm, respectively. Consequently, further assay comparing the sensitivity of FD of FQs at commonly used  $\lambda_{\text{exc}} = 278$  nm and  $\lambda_{\text{em}} = 450$  nm and the optimal wavelengths was carried out and the results revealed no significant difference of sensitivity at the same pH. However, 10- to 50-fold higher sensitivity of FD at acidic pH 3.0 than at basic pH 10.5 was observed (Table 3). Our results were in agreement with the results of Schneider *et al.*

Table 3. SST and validation data for UHPLC-FD method and UHPLC-MS/MS method at basic pH 10.5<sup>a)</sup>

SST	UHPLC-FD at basic pH						UHPLC-MS/MS								
	NOR	CIPRO	PERLO	OFLO	ENRO	NOR	CIPRO	PERLO	OFLO	ENRO	NOR	CIPRO	PERLO	OFLO	ENRO
Resolution	–	1.71	5.52	2.86	6.61										
Asymmetry factor	1.08	1.04	1.02	0.97	1.05										
$f_R$	2.49	3.07	3.89	4.31	5.23						1.25	1.37	1.62	1.71	2.00
Repeatability $f_R$ (% RSD)	0.14	0.13	0.10	0.10	0.04						0.00	0.00	0.20	0.00	0.26
Repeatability A (% RSD)	0.52	1.00	0.26	1.10	0.81						3.19	4.23	4.55	4.04	4.56
<b>Validation</b>															
Linearity ( $r^2$ )	0.9998	0.9995	0.9990	0.9991	0.9999	0.9990	0.9989	0.9986	0.9984	0.9989	0.9990	0.9989	0.9986	0.9984	0.9989
Linearity-range (ng/mL)	150.00–5000.00	100.00–5000.00	50.00–5000.00	50.00–5000.00	100.00–5000.00	1.00–5000.00	1.00–5000.00	5.00–500.00	0.50–500.00	0.50–500.00	1.00–5000.00	1.00–5000.00	5.00–500.00	0.50–500.00	0.50–500.00
IDL (pg injected into column)															
LOD (ng/mL)	50.00	25.00	25.00	10.00	25.00	50.00	50.00	1.00	1.00	0.10	0.50	0.50	1.00	0.10	0.10
LOQ (ng/mL)	150.00	100.00	50.00	50.00	100.00	50.00	50.00	5.00	5.00	1.00	1.00	5.00	5.00	0.50	0.50
Tested levels (L1, L2 and L3) (ng/mL)	128.95	606.57	79.11	88.97	88.99	250, 1000, 2500	250, 1000, 2500	50, 100, 250	50, 100, 250	50, 100, 250	50, 100, 250	50, 100, 250	50, 100, 250	50, 100, 250	10, 50, 100
Recovery L1 (%)	108.65	292.00	63.56	75.21	74.66						100	102	92	100	102
Recovery L2 (%)	80.63	37.76	83.39	92.37	96.18						99	100	119	101	102
Recovery L3 (%)	0.22	1.54	1.71	1.22	1.92						98	90	85	90	100
Intra-day precision L1 (%)	4.21	9.95	2.08	1.47	0.42						5.00	3.17	4.02	2.10	4.29
Intra-day precision L2 (%)	4.99	9.45	1.59	1.13	1.32						3.00	2.58	0.96	1.92	2.27
Intra-day precision L3 (%)											2.98	1.49	3.84	0.62	2.07
Inter-day precision L1 (%)											7.61	16.63	18.69	14.08	14.34
Inter-day precision L2 (%)											11.85	9.72	19.46	12.50	16.55
Inter-day precision L3 (%)											18.71	19.70	18.69	10.24	13.07
<b>UHPLC-FD at acidic pH</b>															
Linearity ( $r^2$ )	0.9998	0.9986	0.9983	0.9999	0.9996										
Linearity-range (ng/mL)	1.00–500.00	2.50–500.00	1.00–500.00	2.50–1000.00	2.50–500.00										
LOD (ng/mL)	0.50	1.00	0.50	1.00	1.00										
LOQ (ng/mL)	2.50	2.50	1.00	2.50	2.50										
Increase of sensitivity	75 ×	300 ×	300 ×	60 ×	150 ×										

a) Second part of the table shows the sensitivity of FD detection at acidic pH 3.0.

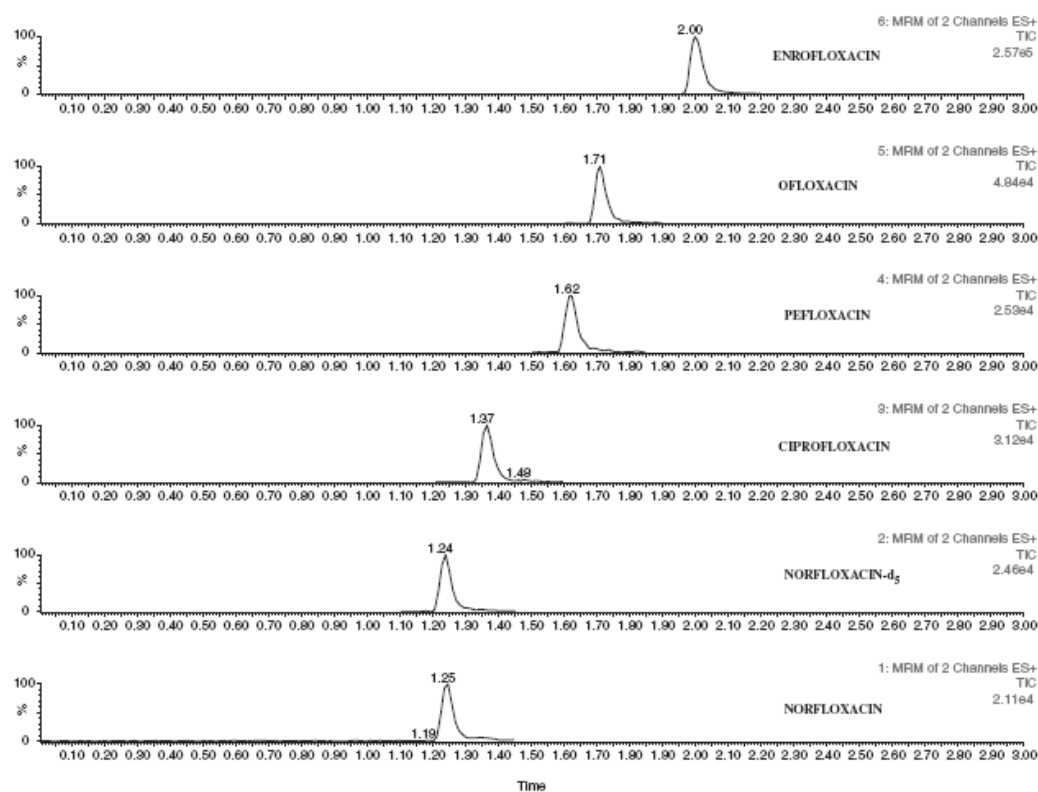


Figure 6. Chromatogram of standard mixture of FQs.

[19] who pointed out lower sensitivity of FD detection at high pH.

This newly developed UHPLC-FD method was applied only for the evaluation of stability of FQ in standard stock solution at basic pH, as FQs have never been analyzed at such conditions. The FQs were stable for 2 weeks (peak area did not decrease under 95% of initial peak area for all five FQs tested) and after this time interference near to retention time of CIPRO occurred avoiding the accurate quantitation.

### 3.3.2 UHPLC-MS/MS

UHPLC-MS/MS was employed for more specific determination of FQs in wastewater samples. For all FQs, protonated molecule  $[M+H]^+$  gave the highest intensity signal in electrospray positive mass spectra, and therefore it was chosen as precursor ion (Table 2). The gradient used for the separation of FQs was slightly different from the generic one used during the systematic method development. The flow rate of mobile phase was decreased to 0.35 mL/min as high flow rate is not suitable for connection with ESI and the gradient elution was adjusted in order to reduce analysis time. Moreover, the optimization of mobile phase additive was performed. Several

lower concentrations of AmAc pH 10.5 were tested (0.5, 1 and 2 mM) as the concentration 10 mM AmAc employed for UHPLC-FD could not be used in UHPLC-MS since signal suppression occurred. Finally, concentration 0.5 mM AmAc was used for further experiments.

Subsequently, all the parameters of mass spectrometer were tuned in order to obtain appropriate sensitivity of precursor ion for all analytes (Section 2.5). Cone voltage was set up individually for each analyte (Table 2). Finally, at optimized conditions product ions for SRM transitions were chosen according to the fragmentation pathways in Product ion scan mode. Collision energy was optimized for each analyte and for each of its two transitions individually in order to get high sensitivity (Table 2). Deuterium-labeled NOR-d<sub>5</sub> was used as internal standard for quantitation. At these conditions, five FQs were eluted within 2.5 min (Fig. 6).

### 3.4 SPE and matrix effects

As the chromatographic analysis has been performed at basic pH 10.5 in this study, a new extraction procedure had to be

developed in order to be well compatible with this pH and to achieve better compatibility of method. This new extraction procedure is contrary to all published results, where SPE was usually performed at acidic pH 3 or 4 (Table 1). Oasis HLB cartridges were chosen for the SPE because of their properties and possibility of usage at high pH.

The matrix interference is a very important problem in the environmental area. Therefore, different solvents for SPE washing step were tested in order to obtain clean extract and minimal matrix effects. First, the concentration of MeOH in milli-Q water was optimized (5, 10, 15, 20 and 30% MeOH in milli-Q water). The best recoveries were achieved with the 10% MeOH concentration. Ultra-pure water was used as a second washing solvent. However, it did not prevent the occurrence of interferences. Therefore, several acidic solvents have been subsequently tested (0.5, 1 and 2% acetic acid). The best recoveries were obtained after the percolation of 3 mL of 2% acetic acid followed by washing the cartridge with 12 mL of ultra-pure water because lower amount led to decrease of recoveries of FQs. Thus final washing step was percolation of 3 mL of 10% MeOH, 3 mL of 2% acetic acid and 12 mL of ultra-pure water.

MeOH and ACN were tested as elution solvents and MeOH showed better elution efficiency than ACN. Finally, only 2 mL of elution solvent has been found to be satisfactory. After elution, samples were evaporated to dryness and dissolved in a mixture of 10 mM AmAc pH 10.5 and MeOH (9:1). The dissolution time in order to dissolve dry extract sufficiently was another crucial issue. The best results were obtained after 30 min in ultrasonic bath followed by use of vortex for 1 min.

### 3.5 SST and method validation

The SST was performed by ten subsequent injections of standard mixture of FQs at a concentration of 100 ng/mL for UHPLC-MS and 1 µg/mL for UHPLC-FD. The repeatability of the injection of standard solution was established (retention times and peak areas were checked, the repeatability was expressed as RSD in %). SST results are summarized in Table 3. For both UHPLC methods, the repeatability for retention times was within 0.26% RSD. Repeatability for peak area was within 5% for UHPLC-MS and within 1.1% for UHPLC-FD method, which is fully acceptable. For UHPLC-FD further parameters including resolution and asymmetry factor were evaluated (Table 3). They were both within the limits of acceptance given by Pharmacopoeia [22].

#### 3.5.1 Linearity–calibration range

Calibration curves of all analytes were measured in the concentration range of 0.5–500 ng/mL for UHPLC-MS to define method sensitivity. Sensitivity of UHPLC-FD was compared at acidic and basic pH and the linearity was

measured in the concentration range of 0.001–0.5 and 0.05–5 µg/mL for acidic and basic pH, respectively. Correlation coefficients for all FQs were higher than 0.9990 for both methods, indicating good linearity in the tested concentration ranges (Table 3).

#### 3.5.2 Accuracy and precision

Accuracy and precision were established by spiking wastewater samples at three concentration levels (Table 3) using SPE step described in Section 2.4. Method precision was determined as intra-day and inter-day variability of three determinations at three different levels expressed as % RSD. For UHPLC-MS intra-day precision was generally within 5% RSD, whereas inter-day precision within 20% RSD, which is fully acceptable taking into account the complexity of environmental samples. Method accuracy was determined as percentage of recovery using wastewater samples spiked with standard solutions treated by SPE at three concentration levels – results are summarized in Table 3. Recoveries related to NOR-d<sub>3</sub> for UHPLC-MS typically ranged from 84 to 103%.

Concerning UHPLC-FD, spiked river water treated by SPE was first evaluated. Method accuracy was expressed as percentage of recovery. The results were between 95 and 101% (data not shown). Fortification of wastewater samples with standard solutions at three concentration levels treated by SPE showed the unsuitability of UHPLC-FD for the analysis of wastewaters at developed conditions. First, the interferences with peak of CIPRO were observed as summarized in Table 3. The values of recovery were 606, 292 and 37% at three fortification levels, respectively. Only intra-day precision was established as the results of accuracy were not satisfactory. Intra-day precision was within 10% RSD. This is in agreement with study of Lee *et al.* [14] who pointed out the unsuitability of fluorescence detector for the analysis of wastewaters. Thus wastewater samples were analyzed only by UHPLC-MS/MS.

In order to verify the absence of potential interferences around the retention time of FQs and to assess the specificity of the method, the determination of matrix effects was performed. Wastewater sample treated by SPE was injected into the UHPLC system and standard solution was injected using direct infusion. At the retention times of FQs, neither negative nor positive peaks signifying the matrix effects were observed.

LODs and LOQs were calculated based on *S/N*. They were established first using standard solutions in mobile phase by the injection of the smallest amounts which provide *S/N* = 3. The results for UHPLC-FD and UHPLC-MS/MS methods excluding concentration factor are summarized in Table 3. As UHPLC-FD was not found convenient for the evaluation of wastewater samples at basic pH, only UHPLC-MS/MS was further used for their determination. UHPLC-MS/MS method had appropriate sensitivity to perform the determination of FQs in wastewater samples reaching LOQ

12.5–125 ng/L with a sample volume of only 20 mL. Such low volume needed for sample preparation is not typical, as previously published studies used volumes ranging typically from 250 to 1000 mL (Table 1). Large volumes of samples could be inconvenient for routine sample preparation, because the filtration and percolation of such high volume on SPE cartridges is very time consuming as the reported percolation flow rates are usually of about 3–5 mL/min. In study of Li *et al.* they developed very sensitive UHPLC-MS/MS method; however, they spent more than 80 min for percolation of 250 mL of sample during SPE procedure [21]. Another previously published UHPLC-MS/MS method [6], which developed ultra-fast separation, however, spent also up to 80 min on SPE using sample volumes of 200, 400 or 800 mL of environmental waters achieving the same detection limits as our method which used only 20 mL of wastewater samples. Using high volume of sample can lead to sensitive method if the concentration factor is high; however, spending lot of time on SPE is not suitable. The LODs of antibiotics in study of Tamtam *et al.* [20] are comparable with ours even though they used higher volume of sample (100 mL) and spent more than 30 min during the SPE procedure. For the purpose of analysis of wastewaters, such preconcentration using 20 mL of sample was sufficient, achieving similar LODs as other methods using high volumes of water samples. Moreover, comparing the instrumental LODs expressed as amount of picogram injected into analytical column, this newly developed UHPLC-MS/MS method at basic pH is more sensitive than the others that have already been published [20, 21].

The comparison of sensitivity of MS and FD detection was done. Developed UHPLC-MS/MS method was about two orders of magnitude more sensitive than UHPLC-FD at basic pH. The sensitivity of UHPLC-MS/MS was comparable to UHPLC-FD at acidic pH which is in agreement with the study of Lee *et al.* [14].

### 3.6 Applicability of the method to real samples

The river water samples and samples from WWTP were analyzed by UHPLC-MS/MS with SPE at pH 10.5. No antibiotics were detected in river water samples. A total of 18 samples of influent and effluent from WWTP were analyzed and NOR, CIPRO and OFLO were detected in influent sample in average concentrations of 38.11, 2468.70 and 20.27 ng/L, respectively, and only NOR and CIPRO in effluent sample in concentration levels of 25.69 and 38.06 ng/L, respectively. The CIPRO concentration detected in influent was very high compared with other studies where usually OFLO was the dominant antibiotic [6, 14].

Comparing the concentrations in influent with those in effluent, we evaluated the efficiency of treatment process. OFLO was detected only in influent sample and thus efficiency of treatment process could not be evaluated. However, from the low concentration of 20.27 ng/L detected

in influent and no detection in effluent sample, its elimination can be concluded during treatment process. The elimination ratios for NOR and CIPRO were 32 and 98%, respectively. These results are consistent with those reported earlier [14, 21].

## 4 Concluding remarks

A new fast UHPLC method for the determination of five FQ antibiotics was developed. Conventional and systematic method development approaches were compared. During the systematic approach, a possibility of automatic switching among four independent analytical columns of different chemistries (BEH C18, BEH Shield RP18, BEH Phenyl and HSS T3 C18) has been used. ACN and MeOH as well as buffers of acidic and basic pH were tested in gradient mode of elution. Surprisingly and contrary to all the previously published results, the best separation of FQs was obtained on BEH Phenyl analytical column at basic pH (10.5), showing that pH of mobile phase is crucial for further analysis of FQs.

MS and FD at basic pH were compared from the point of view of sensitivity and applicability to the analysis of real wastewater samples. MS showed two orders of magnitude higher sensitivity and convenience for the analysis of wastewaters at basic pH than FD which showed some interferences avoiding accurate quantification of analytes. A new SPE procedure was successfully developed at basic pH using volume of only 20 mL of wastewater sample. The method was validated in terms of linearity, precision and recovery using spiked wastewater samples. Calculated recoveries ranged from 84 to 103% in wastewaters, which is fully acceptable considering the complexity of matrix. The LOQs were found to be low enough to determine FQ antibiotics in hospital wastewaters (LOQ = 12.5–125 ng/L). Therefore, the method could be applied to analyze samples from WWTP.

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*The authors have declared no conflict of interest.*

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## **6.5 Supplement II**

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**Determination of steroid hormones in biological and environmental samples using green microextraction techniques: An overview**

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## Review

## Determination of steroid hormones in biological and environmental samples using green microextraction techniques: An overview

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Water samples

## ABSTRACT

Residues of steroid hormones have become a cause for concern because they can affect the biological activity of non-target organisms. Steroid hormones are a potential risk for wildlife and humans through the consumption of contaminated food or water. Their determination requires extraction and clean-up steps, prior to detection, to reach low concentration levels. In recent years, a great effort has been made to develop new analytical methodologies, such as microextraction techniques, that reduce environmental pollution. Researchers have modified old methods to incorporate procedures that use less-hazardous chemicals or that use smaller amounts of them. They are able to do direct analysis using miniaturised equipment and reduced amounts of solvents and wastes. These accomplishments are the main objectives of green analytical chemistry. In this overview, we focus on microextraction techniques for the determination of steroid hormones in biological (e.g., human urine, human serum, fish, shrimp and prawn tissue and milk) and environmental (e.g., wastewaters, surface waters, tap waters, river waters, sewage sludges, marine sediments and river sediments) samples. We comment on the most recent applications in sorptive-microextraction modes, such as solid phase microextraction (SPME) with molecularly imprinted polymers (MIPs), in-tube solid-phase microextraction (IT-SPME), stir-bar sorptive extraction (SBSE) and microextraction in packed sorbent (MEPS). We also describe liquid-phase microextraction (LPME) approaches reported in the literature that are applied to the determination of steroid hormones.

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## 1. Introduction

### 1.1. Background

In the last few decades, the amount of chemicals released into the environment has increased considerably. Among these compounds, hormone residues have become a cause for concern because they can affect the biological activity of non-target organisms. They are a potential risk for wildlife and humans through the consumption of contaminated food or water. The occurrence of chemical compounds influencing the sexual development of fish in

English rivers was reported 15 years ago [1]. These exogenous substances that interfere with the endocrine system and disrupt the physiologic function of hormones are called endocrine-disruptor compounds (EDCs). The effects of natural and synthetic EDCs found in the environment include decreasing sperm count in human males, increasing breast cancer in women and causing reproductive abnormalities in humans [2,3]. The most potent active EDCs present in the environment belong to the chemical class of steroids, which are formed naturally by humans and wildlife or produced synthetically. Steroid hormones that are biosynthetically present in the body are called endogenous hormones, and exogenous steroids are foreign compounds, either naturally or synthetically produced. In addition to this classification, they can also be classified by their chemical structure and their pharmacological effects. Using these criteria, steroid hormones can be generally divided in three groups: estrogens, gestagens and androgens [4,5].

Regarding chemical structure, steroids are comprised of a skeleton of three cyclohexal carbon rings and one pentagonal carbon ring, which are generally arranged in a 6-6-6-5 structure to which various functional groups and side chains are attached. All steroids can be derived from cholesterol. Table 1 shows some examples of steroid hormones and their parent compound, cholesterol. The three main natural estrogens, estrone (E1), estradiol (E2) and estril (E3), are C<sub>18</sub> steroids that differ in the oxidation state of their rings. These C<sub>18</sub> steroids stimulate the development of female reproductive structures and secondary sexual characteristics. Synthetic estrogens, such as ethinylestradiol (EE2) or mestranol (MES), are derived from E2. Because of their anabolic effects, estrogens have been used in animal fattening. Gestagens, also called progestagens, are derived from the C<sub>21</sub>-steroid pregnane such as progesterone (P). Androgens are C<sub>19</sub> steroids that stimulate or control the development of masculine characteristics. The most well-known androgen is testosterone (T). Natural and synthetic androgens, like all groups of steroids, have been used as growth promoters and in human and veterinary therapies. Because of their myotrophic action, anabolic androgenic steroids, including testosterone, have been widely used by athletes to improve athletic performance.

The occurrence of hormone residues has been increasingly reported in wastewater [6–9], surface waters and groundwaters [10–13] and even drinking water [14–16]. The occurrence is especially significant in places near influents and effluents of wastewater treatment plants (WWTPs). WWTPs are considered to be one of the principal sources of hormone contamination because they do not completely remove these compounds [17,18]. In general, the natural estrogenic steroids E1, E2 and E3 are often detected in water samples, while their conjugated forms or synthetic steroids, EE2 and MES, are detected only sporadically. Concentrations of estrogens in treated wastewater normally do not exceed a few ngL<sup>-1</sup>, but values of 51 ngL<sup>-1</sup> for E1 have been reported [19]. The concentration of E1 in urine is approximately twice that of E2 or E3 [20]. This fact, combined with the biodegradation of E2 to E1 by oxidation (e.g., in WWTPs [21]), means that greater amounts of E1 can be expected in wastewater and surface water. The levels of gestagens in surface water, wastewater and sediments are in the same range as those of estrogenic steroids. Androgenic steroids in the aquatic environment originate from WWTPs effluents from paper mills and livestock-breeding operations. The androgenic steroids that are typically identified in WWTPs are testosterone and its metabolites or precursors of male and female hormones [22–24]. Influent of WWTPs can exhibit high levels of androgen steroids, but levels in effluents and surface water are usually several orders of magnitude lower.

Some of these compounds exhibit relatively low polarity; therefore, sorption to a solid phase is the expected behaviour. Human medications, including synthetic hormones, can enter the soil mainly through sewage sludge and spread into agricultural fields

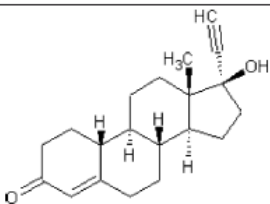
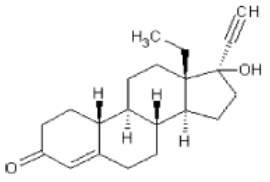
**Table 1**  
Chemical structures and properties of most common hormones.

Name (Abbr.)	Systematic name	Synthetic/ natural (S)/(N)	Group	pK <sub>a</sub> log Kow	Summary structure	Structure
Estrone (E1)	Estra-1,3,5(10)-trien-17-one	N	C <sub>18</sub>	10.77 3.4	C <sub>18</sub> H <sub>22</sub> O <sub>2</sub>	
Estradiol (E2)	Estra-1,3,5(10)-triene-3,17-diol	N	C <sub>18</sub>	10.08 4.0	C <sub>18</sub> H <sub>24</sub> O <sub>2</sub>	
Estriol (E3)	(16-alpha,17-beta)-estra-1,3,5(10)-triene-3,16,17-triol	N	C <sub>18</sub>	10.4 2.4	C <sub>18</sub> H <sub>24</sub> O <sub>3</sub>	
Ethynylestradiol (EE2)	19-norpregna-1,3,5(10)-triene-20-yn-3,17-diol	S	C <sub>18</sub>	10.4 3.7	C <sub>20</sub> H <sub>24</sub> O <sub>2</sub>	
Mestranol (MES)	19-norpregna-1,3,5(10)-triene-20-yn-17-ol	S	C <sub>21</sub>	13.1 4.7	C <sub>21</sub> H <sub>26</sub> O <sub>2</sub>	
Progesterone (P)	Pregn-4-ene-3,20-dione	N	C <sub>21</sub>	n.a. 3.9	C <sub>21</sub> H <sub>30</sub> O <sub>2</sub>	
Testosterone (T)	Androst-4-en-3-one	N	C <sub>19</sub>	9.74 3.3	C <sub>19</sub> H <sub>28</sub> O <sub>2</sub>	

Table 1 (Continued)

Name (Abbr.)	Systematic name	Synthetic/ natural (S)/(N)	Group	p <i>K</i> <sub>a</sub> log K <sub>ow</sub>	Summary structure	Structure
Androsterone (ADR)	Androstan-17-one	S	C <sub>19</sub>	15.14 3.7	C <sub>19</sub> H <sub>30</sub> O <sub>2</sub>	
Boldenone (BOLD)	Androsta-1,4-dien-3-one	S	C <sub>19</sub>	15.05 3.1	C <sub>19</sub> H <sub>26</sub> O <sub>2</sub>	
Nandrolone (NDL)	Estr-4-en-3-one	S	C <sub>18</sub>	15.06 2.6	C <sub>18</sub> H <sub>26</sub> O <sub>2</sub>	
Methyltestosterone (MeT)	Androst-4-en-3-one	S	C <sub>19</sub>	15.13 3.4	C <sub>20</sub> H <sub>30</sub> O <sub>2</sub>	
Epiandrosterone (EADR)	Androst-2-[3,2-c]pyrazol- 17-ol	N	C <sub>19</sub>	15.14 3.8	C <sub>19</sub> H <sub>30</sub> O <sub>2</sub>	
Stanozolol (SZL)	Androst-2-eno[3,2- c]pyrazol	S	C <sub>21</sub>	3.84; 15.15 5.5	C <sub>21</sub> H <sub>32</sub> N <sub>2</sub> O	
Androstenedione (ADD)	Androst-4-ene-3,17-dione	N	C <sub>19</sub>	n.a. 2.8	C <sub>19</sub> H <sub>26</sub> O <sub>2</sub>	

Table 1 (Continued)

Name (Abbr.)	Systematic name	Synthetic/ natural (S)/(N)	Group	pK <sub>a</sub> log Kow	Summary structure	Structure
19-Norethisterone (19-No)	19-Norpregn-4-en-20-yn-3-one	S	C <sub>21</sub>	13.9 3.0	C <sub>20</sub> H <sub>26</sub> O <sub>2</sub>	
Norgestrel (Ng)	13-ethyl-17-ethynyl-19-nortestosterone	S	C <sub>21</sub>	13.09 3.5	C <sub>21</sub> H <sub>28</sub> O <sub>2</sub>	

[25–27]. Natural hormones also come from use of animal manure and organic fertilisers [28]. Concentrations of estrogens in riverine and marine sediment are in the range of several  $\text{pg g}^{-1}$  to a few  $\text{ng g}^{-1}$ . The levels in these environmental compartments seem to depend mostly on the proximity of the sediments to potential sources, such as WWTPs [29,30], and on the type of treatment the wastewater receives. Usually, concentrations of E1 in sediments are slightly higher than those of E2 and E3 and range from  $\text{sub-ng g}^{-1}$  to  $11.9 \text{ ng g}^{-1}$  [29,31,32].

The molecular structures and properties of the most frequently studied steroid hormones are shown in Table 1.

### 1.2. Legislation

It has been shown that, at low concentrations, steroid hormones produce alterations in the endocrine system leading to growth, developmental, or reproductive alterations in exposed animals. These changes may be expressed later in the life cycle or even in future generations [33]. For this reason, the EU banned the use of substances with a hormonal action for growth promotion or fattening purposes of farm animals through several directives since 1981 [34–36]. Similarly, the EU regulates the use of veterinary drugs, including both synthetic and natural hormones, by describing a procedure for the establishment of maximum residue limits (MRLs) in foodstuffs of animal origin [37]. Another EU directive [38] states that the administration of substances with estrogenic, androgenic or gestagenic action in husbandry and aquaculture is forbidden, as is the importation of meat from treated animals from other countries. The control of residue from steroid hormones in food-producing animals and their primary products (meat, eggs and honey) is regulated by Council Directive 96/23/EC [39]. Directive 2002/657/EC [40] regulates the implementation of the analytical methods and the interpretation of the results by providing performance criteria and instructions for validation. This directive establishes common criteria for the interpretation of test results and introduces a system of identification points (IPs) to interpret the obtained data. This system is based on the number and the ratio of the ions in the obtained MS spectrum. For the confirmation of banned substances such as steroid hormones, a minimum of four IPs are required. The parameters that need to be evaluated during the validation procedure are selectivity, specificity, linearity, trueness, recovery, applicability, ruggedness, stability, repeatability, reproducibility and decision limit ( $\text{CC}\alpha$ ) and detection limit ( $\text{CC}\beta$ ).

The Water Framework Directive (WFD) sets the EU strategy against pollution of water by dangerous substances. The WFD provisions will require the Member States and Associated States to establish programs to monitor the quality of water, which implies a review of human activity on the pollutants and an economic analysis of water use. In this context, there is an urgent need for a list of emerging contaminants as possible candidates for introduction into the WFD list of priority substances. This can be amended every four years with revisions and additions of new contaminants [41]. In the same way, the U.S. Environmental Protection Agency (EPA) published the final Contaminant Candidate List (CCL-3) in September 2009, which is a drinking water priority contaminant list for regulatory decision-making and information collection. The listed contaminants are either known or anticipated to exist in drinking water systems and will be considered for potential regulation. This final CCL-3 contains 104 chemicals and 12 microbial contaminants, and it includes three pharmaceuticals, eight hormones, several disinfectant by-products and industrial additives [42].

These substances tend to accumulate in sewage sludge during wastewater treatment due to their physico-chemical properties. Therefore, they should be analysed to detect the presence of pollutants before re-use in agricultural activities. Although current legislation only regulates the concentration of toxic heavy metals and nutrients for the agricultural use of sewage sludge, the European Commission (EC) is studying the relevance of some of the organic micro-pollutants in these matrices to include them in a future Sludge Directive [4].

Due to the illegal use of anabolic androgenic steroids in some sports, the Medical Commission of the International Olympic Committee (IOC) has prohibited them. Illegal steroid use constitutes unfair competition and can be harmful to the physical and mental health of those under aberrant use. The list of prohibited anabolic agents includes androstenedione (ADD), fluoxymesterone, metandienone, methyltestosterone (MeT), testosterone and many other related substances [43].

### 1.3. Analysis

The presence of steroid hormones in the environment covers a wide range of physical matrices, from aqueous to solid samples. Moreover, they are present in edible matrices, such as muscle, organ tissue, fat and milk, with different features [44–46]. It is important to note that ultratrace concentrations of steroid



hormones can produce endocrine disruption [2,47]. Their determination, therefore, requires high-sensitivity analytical methods. These methods must be sensitive enough to cover not only the legislative limits but also to allow the determination of banned, forbidden or unknown compounds. Gas chromatography with mass spectrometry (GC-MS) [48,49] has been commonly used with different derivatisation reagents to improve sensitivity and selectivity. However, methods based on high-performance liquid chromatography-mass spectrometry (LC-MS) techniques have the advantage of direct analysis of the samples without need for a derivatisation step [50–53]. Liquid chromatography tandem mass spectrometry (LC-MS-MS) provides the lowest limits of detection (LODs), which are in the sub-ng L<sup>-1</sup> or sub-ng g<sup>-1</sup> range [54,55]. Moreover, this technique is superior in terms of selectivity, helping to avoid false positives when analysing complex matrices. Due to their relatively high LODs and low selectivity, diode-array (DAD) or fluorescence detectors (FD) are rarely used.

To obtain high recoveries and minimise interference, the determination of steroid hormones requires extraction and clean-up steps prior to detection. Solid phase extraction (SPE) is frequently used to extract these compounds from aqueous samples, and Oasis HLB is the most commonly used cartridge type. The classical procedure for the extraction of solid matrices, Soxhlet extraction, has been replaced by methods such as pressurised liquid extraction (PLE) and solvent extraction assisted by ultrasonication and microwave-assisted extraction (MAE). After the extraction of soils, sludge or sediments, SPE has been the clean-up method of choice in most studies. Oasis HLB has achieved the best recoveries in addition to good repeatability.

The demand to reduce the solvent volumes and avoid the use of toxic organic solvents has led to substantial efforts to adapt existing sample preparation methods to the development of new approaches. Researchers have modified old methods to incorporate procedures that use less-hazardous chemicals or that use smaller amounts of them. These analytical technologies can be used for direct analysis through the use of miniaturised equipment and reduced amounts of solvents and wastes, which are the main objectives of green analytical chemistry. In this overview, we focus on microextraction techniques for the determination of steroid hormones in biological and environmental samples. In the following sections, we comment on the most recent applications in sorptive-microextraction modes, such as SPME with molecularly imprinted polymers (MIPs), in-tube solid-phase microextraction (IT-SPME), stir-bar sorptive extraction (SBSE) and microextraction in packed syringe (MEPS). We also describe LPME approaches reported in the literature that have been applied to the determination of steroid hormones.

## 2. Microextraction approaches

The analytical process comprises four main steps: sample collection, sample preparation, final analysis and evaluation of the results. Though each step can affect the environment, they each make different contributions towards pollution. However, the sample preparation step is considered to be the most polluting step of the entire analytical procedure [56]. In the samples, the analytes may exist in 1000-fold smaller quantities than bulk constituents. Therefore, the use of organic solvents is required to enrich the target compounds making the analytes more suitable for separation and detection. This step also removes potentially interfering matter.

To make this step “greener”, sample preparation techniques that minimise solvent consumption, such as solid phase microextraction (SPME), stir-bar sorptive extraction (SBSE) and liquid-phase microextraction (LPME) approaches, have replaced the more solvent-consuming techniques, such as liquid-liquid extraction

(LLE) or solid-phase extraction (SPE). These techniques miniaturise sample preparation and therefore, reduce organic solvent consumption. In addition, the on-line coupling of extraction and analysis results in a higher sensitivity, a reduced potential for analyte loss and a reduction of the sample amount needed for analysis. Moreover, especially in environmental and biological samples, the low concentration of target compounds, such as hormones, in the sample makes it essential to have a preconcentration step. In this sense, these microextraction techniques allow both objectives to be met: obtaining low concentrations and minimising solvent consumption.

Solid samples, such as sediments or tissue samples, cannot be submitted to the microextraction techniques directly. Therefore, a previous step is required. For example, in the case of tissue samples, they generally are wrung and stored at -18 °C before analysis. After the samples are spiked with the desired level of concentration, they are mixed and homogenized in an organic solvent, such as acetonitrile, sonicated and centrifuged. Then, a volume of the supernatant is subjected to the microextraction procedure [57,58].

Fig. 1 shows a scheme of microextraction approaches described in this overview.

### 2.1. Sorbent microextraction

The introduction of solid-phase microextraction (SPME) by Pawliszyn and co-worker [59] initiated the interest in microextraction techniques in analytical chemistry. Using the SPME technique, target analytes are extracted from aqueous or gaseous samples onto a solid polymeric fiber [60]. This solvent-free technique allows the integration of sampling, isolation and enrichment into one step. SPME is portable, simple to use, relatively fast and can be automated and coupled online to analytical instrumentation. Thus, this methodology has been used to determine steroid hormones in several samples [61–63]. However, the coated fibers are generally expensive and have a limited lifetime. In this sense, new versions of this technique have been developed to overcome the problems related to the use of conventional fiber SPME.

#### 2.1.1. Molecularly imprinted polymers

Molecular imprinting [64,65] is a technique for the preparation of synthetic polymers with a predetermined selectivity for desired template molecules, and the molecularly imprinted polymer (MIP) is a highly stable polymer that possesses recognition sites within the polymer matrix that are adapted to the three-dimensional shape and functionalities of an analyte of interest [66]. The fiber coating is the key factor of the SPME technique; therefore, its selectivity can be enhanced using novel coating materials, such as molecularly imprinted polymers (MIPs) (Fig. 1A). Design variables include the choice of a monomer (which complexes with the target analyte with a high binding affinity) [67], cross-linker (the length of which may determine the cavity size) and polymerisation method (which dictates the nature of interactions between the polymer matrix and the template to dictate the ultimate extraction efficiency). Once the polymer is formed, the template is removed with an appropriate solvent, leaving a cavity that corresponds to the specific target analyte [68].

Regarding this process, Qiu et al. [69] have developed a type of selective SPME fiber for anabolic steroids from testosterone-imprinted polymer. The fibers were subsequently used for the extraction of anabolic steroids and then analysed with GC-MS. Methacrylic-acid (MAA) functional monomers, trimethylolpropanetriacrylate (TRIM) crosslinkers and testosterone template molecules were used to prepare the SPME fibers through a thermal radical copolymerisation procedure. The fibers were placed in a homemade syringe and inserted into a GC and/or GC-MS injection port. The proposed method was applied for

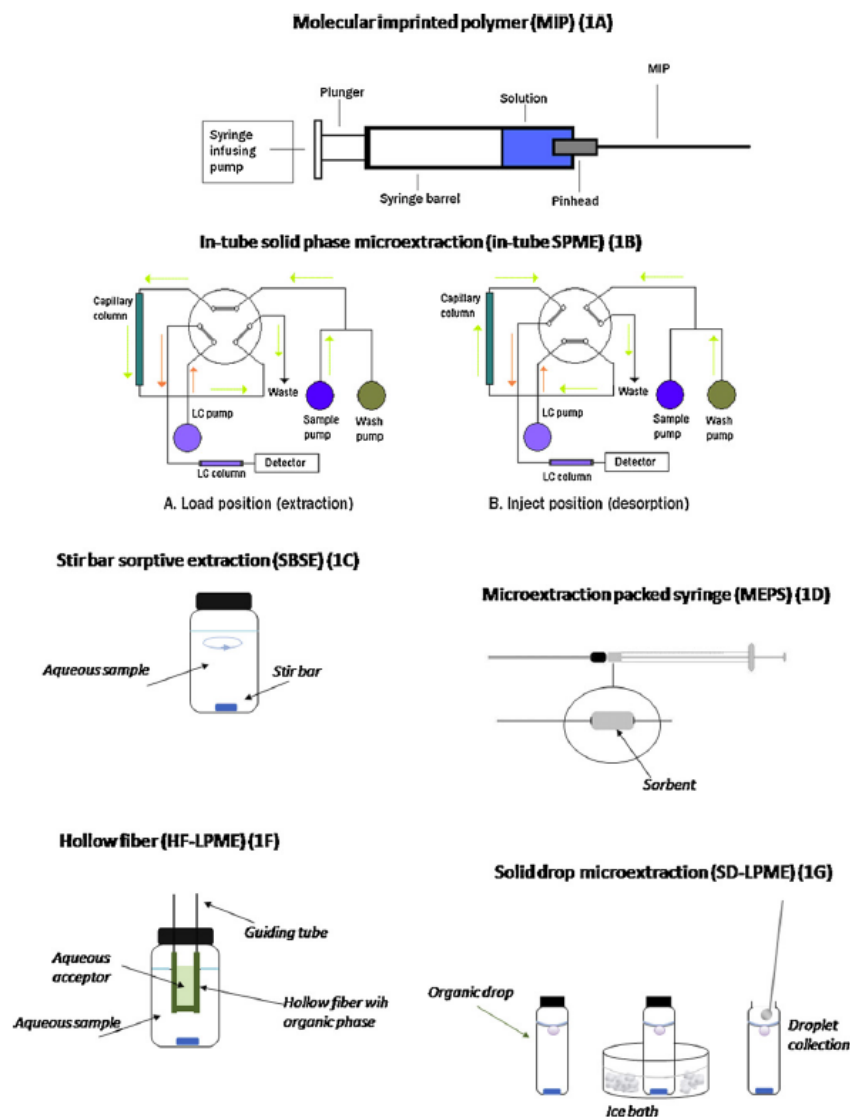


Fig. 1. Microextraction approaches used in the determination of hormones in environmental samples.

the determination of androsterone (ADR), stanolone (STAN), androstenedione and methyltestosterone in spiked human urine. The obtained LODs were in the range of  $0.2\text{--}0.8\ \mu\text{g L}^{-1}$ , whereas the recoveries were 80.1–108.4% after 20 min of extraction time.

The MIPs technique has been also used to determine estrogenic compounds, such as estriol, estrone and  $17\beta$ -ethynylestradiol, in fishery samples [57]. An MIP-coated SPME fiber with  $17\beta$ -estradiol as a template was prepared by an improved multiple copolymerisation method. In this paper, the characteristics of the MIP-coated SPME, extraction capability and selectivity as compared to the commercial fiber and the non-imprinted polymer (NIP)-coated SPME fiber were investigated. The MIP-coated SPME fiber was coupled directly with LC-UV for simultaneous multi-residue monitoring of the four residual estrogens in fish and shrimp tissue samples. The

LODs were in the range of  $0.98\text{--}2.39\ \mu\text{g L}^{-1}$ , and the recoveries were 80.0–83.6% and 85.0–94.1% for fish and shrimp tissue samples, respectively. The extraction procedure was performed in 55 min.

Higher LODs of 0.023 and  $0.076\ \text{mg L}^{-1}$  were obtained by Jiang et al. [58] for the determination of estrogenic compounds ( $17\beta$ -estradiol, estriol, and diethylstilbestrol) in fish and prawn tissue samples using the same technique. In this case, the MIP was synthesised by a thermo-polymerisation method using methacrylic acid (MAA) as the functional monomer, ethylene glycol dimethacrylate (EGDMA) as the cross-linker, acetonitrile as the porogenic solvent, and  $17\beta$ -estradiol as the template.

MIP is an artificial receptor with the function of selective desorption of molecules with similar molecular structures. Consequently, MIP could be an appropriate tool to remove selective compounds



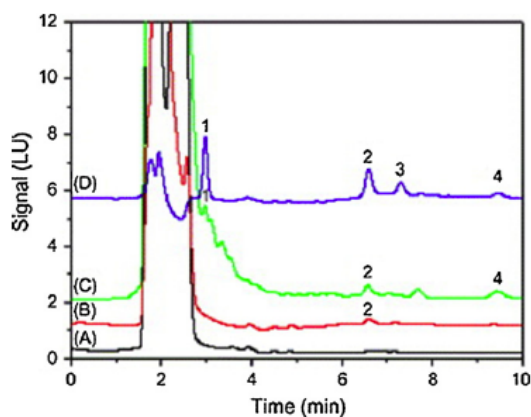


Fig. 2. Analysis of endocrine-disruptor compounds using in-tube SPME-HPLC. Tap water sample (A), lake water sample (B), sewage water sample (C) and standard sample at  $2 \text{ ng mL}^{-1}$  level (D). The PEEK tube extraction column was applied to the microextraction device. Peaks: 1: estriol, 2: bisphenol A, 3:  $17\beta$ -estradiol, and 4:  $17\alpha$ -ethinylestradiol. Chromatogram is taken from [71].

because, in comparison to other adsorbents such as natural antibodies and receptors, it is much more stable in terms of the chemical and mechanical properties of adsorbents. However, MIPs also present some problems, such the need of new MIPs that allow the separation of a wide range of analytes and improving the preparation procedure and its robustness. This improvement will probably enhance both their ease of handling and general applications.

#### 2.1.2. In-tube solid-phase microextraction

In-tube SPME is an automated version of SPME in which an open tubular fused-silica capillary with an inner surface coating is used as the extraction device [70]. Analytes in liquid samples are directly extracted and concentrated into the stationary phase by repeated draw/eject cycles or static sorption of sample solution (Fig. 1B). This technique can overcome problems related to the use of conventional fiber SPME, such as fragility, low sorption capacity, and bleeding of thick-film coatings of the fibers. In addition, in-tube SPME requires lower sample volumes, is easy to automate and is versatile, according a wide range of available coatings.

Wen et al. [71] have developed an on-line method for the simultaneous determination of four endocrine disruptors ( $17\beta$ -estradiol, estriol, bisphenol A and  $17\alpha$ -ethinylestradiol) in environmental waters by coupling in-tube SPME to LC with fluorescence detection. A poly(acrylamide-vinylpyridine-*N,N'*-methylene bisacrylamide) monolith was selected as the extraction medium. Low detection and quantification limits were achieved in the range of  $0.006$ – $0.10 \mu\text{g mL}^{-1}$  and  $0.02$ – $0.35 \mu\text{g mL}^{-1}$ , respectively, for spiked lake waters. Fig. 2 shows the chromatogram obtained for these compounds in tap water sample, lake water sample, sewage water sample and standard sample.

Similar compounds (estrone, estradiol, estriol, ethinylestradiol, diethylstilbestrol) were studied by Mitani et al. [72] using in-tube SPME with LC-UV and MS detection in surface water samples. Four different GC columns were used in this study, and optimal results were achieved with 20 draw/eject cycles of  $40 \mu\text{L}$  of the sample using a Supel-Q PLOT capillary column as the extraction device. The LODs obtained under optimal conditions were between  $0.0027$  and  $0.0117 \mu\text{g L}^{-1}$ . Saito et al. applied the same sorbent of the GC column with the same length and different film thicknesses for in-tube SPME-LC-MS extraction of boldenone (BOLD), nandrolone

(NDL), testosterone, methyltestosterone, epiandrosterone (EADR) and stanozolol from human urine samples [73]. The optimum in-tube SPME conditions were also 20 draw/eject cycles with a sample size of  $40 \mu\text{L}$ . However, the steroid LODs were higher, in the range of  $0.009$ – $0.182 \mu\text{g L}^{-1}$ . In comparison with direct injection, in-tube SPME has a 20–33-fold better LOD than direct injection, and the results are comparable with other studies involving in-tube SPME.

The main advantage of the in-tube SPME technique is that it enables the automatization of the analytical process, allowing extraction, desorption and injection to be performed continuously. In addition, the use of a GC commercial column increases the number of stationary phases and allows a wide range of applications. The main disadvantage of this technique is its requirement of very clean samples, which is due to the capillary being easily blocked.

#### 2.1.3. Stir-bar sorptive extraction

SBSE was first introduced by Baltussen et al. [74] in 1999 as a new and improved sample preparation technique. SBSE and SPME are solventless sample preparation techniques based on sorptive extraction. In SBSE, the extraction phase is placed on a magnetic bar that captures the analytes (usually by partition) during stirring (Fig. 1C). These stir bars, called twistlers® (GERSTEL GmbH & Co.), are coated with a polydimethylsiloxane (PDMS) layer, which is the most widely used sorptive extraction phase. At present, only PDMS-coated stir bars are commercially available, which is one of the main drawbacks of SBSE, because polar compounds are poorly extracted due to the non-polarity of the PDMS polymer. Although the basic principles of SPME and SBSE are identical and the extraction phase is generally the same, the amount of PDMS is 50–250 times larger in SBSE. This feature allows the preconcentration efficiency to be improved compared to SPME, which is its main advantage [75].

Tienpont et al. [76] used SBSE to determine hormones, including estrogens, gestagens and androgens, in human urine samples. After sampling the stir bar, coated with PDMS, it was placed in a thermal desorption unit coupled on-line to capillary gas chromatography mass spectrometer (SBSE-TD-CGC-MS). *In situ* derivatisation with ethyl chloroformate and acetic acid anhydride enhanced both the recovery into the PDMS layer and chromatographic analysis. The limits of detection were at the  $0.1$ – $1 \mu\text{g L}^{-1}$  sample level in ion extraction mode and extended to the  $\text{ng L}^{-1}$  and sub- $\text{ng L}^{-1}$  level in the selected-ion-monitoring mode. Kawaguchi et al. [77] applied the same methodology to determine natural and synthetic estrogens, such as estrone,  $17\beta$ -estradiol and  $17\alpha$ -ethinylestradiol, in river water samples by derivatisation with acetic acid anhydride. The detection limits were also in the  $\text{ng L}^{-1}$  range. Later, the same authors [78] developed a “dual derivatisation method” to determine  $17\beta$ -estradiol in river-water samples. This method involved SBSE with *in situ* acylation (first derivatisation) and thermal desorption (TD) with quartz-wool-assisted (QWA) in-tube silylation (second derivatisation) followed by gas chromatography-mass spectrometry (GC-MS).  $17\beta$ -Estradiol possesses both an aromatic and an aliphatic hydroxyl group. In this study, the aromatic hydroxyl group of E2 was derivatised by SBSE with *in situ* acylation, thereby increasing the percentage recovery of E2. Next, the aliphatic hydroxyl group was derivatised by TD with QWA in-tube silylation, thereby improving the volatility of E2 in GC-MS and the sensitivity of the analysis. However, the LODs obtained were similar to their previous work. A variation of this procedure was developed by Stopforth et al. [79] to determine estrone and  $17\beta$ -estradiol in human urine samples. The method was based on the *in situ* derivatisation of estrogens with acetic acid anhydride, extraction of the derivatives by aqueous SBSE and final exposure of the stir bars to acetic acid anhydride vapours (headspace acylation) before thermal desorption and GC-MS determination. LODs of  $0.02$  and  $0.03 \mu\text{g L}^{-1}$  were obtained.

Almeida and Nogueira [80] combined stir bar sorptive extraction and liquid desorption followed by high performance liquid chromatography with diode array detection (SBSE-LC-DAD) for the simultaneous determination of nine steroid sex hormones (estrone, 17 $\alpha$ -estradiol, 17 $\beta$ -estradiol, 17 $\alpha$ -ethynylestradiol, diethylstilbestrol, mestranol, progesterone, 19-norethisterone and norgestrel) in water and urine samples. For liquid desorption purposes, the stir bars were placed into a 2 mL glass vial filled with 1.5 mL of solvent (methanol and acetonitrile). Stir bars coated with 126  $\mu$ L of PDMS were used, yielding recoveries ranging from 11.1% (17 $\beta$ -estradiol) to 100.2% (mestranol) and limits of detection between 0.3 and 1.0  $\mu$ g L<sup>-1</sup>.

PDMS is the only commercialised coating for SBSE. Therefore, SBSE has been mainly applied to extract non-polar and weakly polar compounds and fails in the extraction of strongly polar compounds unless they have been previously derivatised. To obtain better selectivity for polar compounds and a higher extraction capacity, novel extraction phases have been developed. Hu et al. [81] used a stir bar coated with a composite composed of polydimethylsiloxane and  $\beta$ -cyclodextrin (PDMS/ $\beta$ -CD) prepared by a sol-gel technique. They used this SBSE and LC-FD to determine estrogens (estriol, estrone, 17 $\beta$ -estradiol and 17 $\alpha$ -ethynylestradiol) in environmental water samples. This technique allowed LODs within the range of 0.04–0.11  $\mu$ g L<sup>-1</sup> to be obtained for estrogens using UV detection with recoveries of 85–124%.

Another modified stir bar was used by Huang et al. [82] to determine sex hormones in urine samples by LC with diode-array detection. This stir bar was based on monolithic material (SBSEM), which was obtained by *in situ* copolymerisation of methacrylic acid stearyl ester and ethylene dimethacrylate (EDMA) in the presence of a porogen solvent containing 1-propanol and 1,4-butanediol. LODs and LOQs of the proposed method for the target compounds were achieved within the range of 0.062–0.38 and 0.20–1.20  $\mu$ g L<sup>-1</sup>, respectively. The same authors used a different monolithic material obtained by the *in situ* copolymerisation of vinylpyrrolidone and divinylbenzene in the presence of a porogen solvent containing cyclohexanol and 1-dodecanol with azobisisobutyronitrile as the initiator [83] to extract strongly polar hormones without derivatisation (nandrolone, testosterone, diethylstilbestrol, methyltestosterone, progesterone, testosterone propionate and nandrolone phenylpropionate). Although the LODs were satisfactory, the recoveries were not as high as expected. However, the proposed method reduced the long extraction times usually required for this technique from 2–4 h to 30–150 min. The same stir bar (SBSEM-LC-DAD) was used for the determination of seven steroid hormones in wastewater [84]. The recoveries of the spiked target compounds in real samples ranged from 48.2% to 110%. Fig. 3 shows the chromatograms obtained using SBSEM and with stir bar based on polydimethylsiloxane material (SBSEC).

The potential contamination of soil with hormones may be caused by the application of digested sludge from municipal WWTPs onto agricultural fields. Therefore, it is necessary to develop methodologies that can determine these analytes in soil and sediment samples. To this end, SBSE in combination with thermal desorption coupled to GC-MS has been used by Tan et al. [85] to determine estrone, 17 $\beta$ -estradiol and androsterone in solid and sludge samples. Recoveries for the target compounds using this analytical technique ranged from 44% to 128%. The LODs were 2.0 ng L<sup>-1</sup> for water samples and 0.02 ng g<sup>-1</sup> for solid samples, whereas the LOQs were 5.0 ng L<sup>-1</sup> for water samples and 0.06 ng g<sup>-1</sup> for solid samples.

In general, SBSE is considered advantageous in terms of sensitivity and accuracy for the determination of trace levels in difficult matrices. SBSE has a wide range of applications for non-polar and weakly polar compounds, but it fails in the extraction of polar compounds due to the non-polar character of PDMS. To overcome this

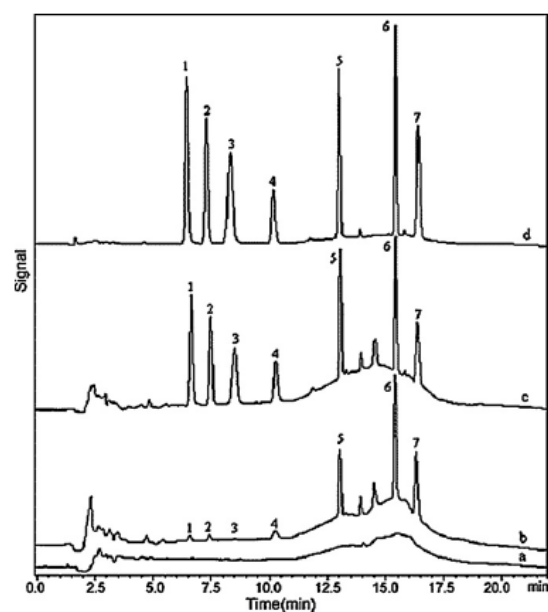


Fig. 3. Chromatograms of seven steroid hormones (SSHs). (A) Direct injection of spiked Milli-Q-purified water sample with each SSH at 50 ng mL<sup>-1</sup>. (B) Spiked Milli-Q-purified water sample with each SSH at 50 ng mL<sup>-1</sup> and treated with stir bar based on polydimethylsiloxane material (SBSEC). (C) Spiked Milli-Q-purified water sample with each SSH at 50 ng mL<sup>-1</sup> and treated with stir bar based on monolithic material (SBSEM). (D) Standard sample with each SSH at 5.0  $\mu$ g mL<sup>-1</sup>. Peaks: 1: nortestosterone, 2: testosterone, 3: diethylstilbestrol, 4: methyltestosterone, 5: progesterone, 6: testosterone propionate and 7: nandrolone phenylpropionate. Chromatogram is taken from [84].

limitation, novel extraction phases that have better affinity for polar compounds could be developed. However, a much longer equilibration time is required because the extraction time is controlled by the diffusion from the sample matrix through the boundary layer into the extraction phase. In addition, the process could not be fully automated when the analytes loaded on the coated stir bar cannot be desorbed by thermal desorption. The manual transfer of the stir bar to the desorption unit may cause the partial loss of analytes.

#### 2.14. Microextraction in packed sorbent

Microextraction by packed sorbent (MEPS) is a recently developed technique that was introduced by Abdel-Rehim [86–88] in the field of sample preparation. MEPS combines the sample processing, extraction and injection steps into a fully automated fashion as an at-line sampling/injecting device to GC or LC [89–93]. In MEPS, approximately 2 mg of the sorbent is thermo-packed inside a syringe (100–250  $\mu$ L) as a plug or between the barrel and the needle as a cartridge (Fig. 1D). Sample extraction and enrichment takes place on the sorbent bed. MEPS is a miniaturised format of SPE that is able to handle sample volumes as small as 10  $\mu$ L, and it is also a technique that integrates the sorbent directly into the syringe, not in a separate column as in commercial SPE. Different types of sorbents are commercially available, such as reversed (C<sub>18</sub>, C<sub>8</sub>, and C<sub>2</sub>), normal (silica) or ion-exchange stationary phases. The sorbent can be used several times with an adapted washing and reconditioning to avoid carry-over and to keep the adsorption power of the phase. Because of miniaturisation, the extraction time, sample size and solvent volumes are considerably reduced, and the elution extract is directly compatible with an on-line injection in LC, GC or CE (capillary electrophoresis). One of



the disadvantages of the MEPS technique is the unavailability of the many chemical sorbents, which is not the case for traditional SPE. Another disadvantage is the strong dependence of the analyte recovery on the continual movement of the plunger, which dictates the rate of the sample passing through the sorbent.

Prieto et al. [94] applied MEPS coupled to GC–MS for the simultaneous determination of a large variety of analytes, including mestranol and 17 $\alpha$ -ethynylestradiol, in water samples. The microextraction was carried out with a MEPS device made with a 100  $\mu$ L gas-tight syringe equipped with a small container incorporated into the needle. This assembly, called “barrel insert and needle” (BIN), was filled with 2 mg of a sorbent commonly used for reverse-phase chromatography or SPE, namely silica-gel sorbents modified with C<sub>18</sub>. Apart from the optimisation and evaluation of the MEPS methodology, this study investigated the influence of humic acids on the extraction efficiency of the target compounds and the sensitivity of their determination in real samples, such as snow and wastewater. The LODs for 800  $\mu$ L of sample were between 0.2 and 266 ng L<sup>-1</sup> and the corresponding recoveries were greater than 75%.

Lower recoveries and higher LODs were obtained by Anizan et al. [95] when they applied the MEPS technique to urine samples, which shows the difficulty of determining this type of analyte in biological samples such urine. The method was optimised using five model steroid metabolites (16 $\alpha$ -hydroxyandrosterone, 2 $\alpha$ -hydroxytestosterone, 11-keto,5 $\beta$ -androstanedione, 6 $\alpha$ -hydroxyestradiol and 7 $\beta$ -hydroxypregnenolone) and then applied to urine samples collected from control versus androstenedione-treated bovines. They used the BIN assembly with a C<sub>18</sub> sorbent. The target analytes were determined by GC–MS after derivatisation. This study also compared this methodology with SPME, and the obtained results showed a clear degradation of the SPME fiber, whereas no degradation was observed with the MEPS. Repeatability and recovery yields were found to be below 11% and above 60%, respectively, for all model compounds, whereas the LODs were in the 5–15  $\mu$ g L<sup>-1</sup> range depending on the compounds.

## 2.2. Liquid-phase microextraction

This microextraction technique, which emerged in the 1990s [96–98], is a miniaturised format of LLE and overcomes many of its disadvantages and some of those of SPME (e.g., non-dependence on a commercial supplier). It is simple to use, generally quick, and characterised by its affordability and reliance on widely available materials. Research on this technique began using small droplets of organic solvents suspended from the tip of a microsyringe needle. However, new approaches have been developed to analyse compounds of a different nature and to obtain large enrichment factors using relatively short extraction times [99].

### 2.2.1. Hollow-fiber liquid-phase microextraction (HF-LPME)

To improve the stability and reliability of LPME, Pedersen-Bjergaard and Rasmussen introduced HF-LPME in 1999 [100]. In this approach, the extracting phase is placed inside the lumen of a porous hydrophobic hollow fiber such that the microextractant solvent is not in direct contact with the sample solution (Fig. 1E). The organic solvent forms a thin layer within the wall of the hollow fiber. The hollow fiber is then placed into a sample vial filled with the aqueous sample of interest. The analytes are extracted from the aqueous sample through the organic phase in the pores of the hollow fiber and then into an acceptor solution inside the lumen. The major advantage of this technique is that the sample may be stirred or vibrated vigorously without any loss of the extracting liquid because it is mechanically protected [101–104].

Basheer et al. [105] used dihydroxylated polymethylmethacrylate (DHPMM) coated on hollow-fiber membrane as a sorbent

for the extraction of trace amounts of natural and synthetic estrogens, such as diethylstilbestrol, estrone, 17 $\beta$ -estradiol and 17 $\alpha$ -ethynylestradiol, in reservoir and potable water samples. This polymer, compared with SPME sorbent materials, has a high number of functional groups (–OH) that makes it more amenable for the extraction of polar compounds such as estrogens. The HF-LPME method in combination with GC–MS detection allow LODs between 0.03 and 0.8 ng L<sup>-1</sup> and recoveries between 87 and 108% for tap water and 86 and 110% for reservoir water.

Zorita et al. [106] developed a method based on this technique for the determination of steroid hormones (17 $\beta$ -estradiol, estrone and 17 $\alpha$ -ethynylestradiol) in tap and sewage water samples from Sweden. Sample preparation and analysis were performed by HF-LPME combined with GC–MS. In this approach, only the organic liquid in the lumen (10  $\mu$ L) of the hollow fiber membrane was utilised for depleting extraction. LODs of 1.6–10 ng L<sup>-1</sup> and enrichment factors over 1400 after derivatisation were obtained.

HF-LPME followed by LC–UV was used by Liu et al. [107] for the determination of three synthetic estrogens, namely diethylstilbestrol, dienestrol, and hexestrol, in wastewater. Although the recoveries, greater than 86%, obtained were similar to those obtained in other works, the LODs achieved for the estrogens were higher and ranged from 0.25 to 0.5  $\mu$ g L<sup>-1</sup>.

Another steroid hormone, progesterone, was determined in human serum sample using this microextraction technique coupled to GC–MS. The 10  $\mu$ L microsyringe used for this miniaturised HF-LPME procedure allowed an LOD of 0.5  $\mu$ g L<sup>-1</sup> and recoveries near 100% for samples spiked with different amounts of progesterone.

Recently, Liu et al. [108] developed a new method combined MIPs and HF-LPME. The molecularly imprinted polymer-coated polypropylene hollow fiber tube (MIP-HFT) was photoinitiated for the copolymerisation of diethylstilbestrol as a template molecule,  $\alpha$ -methacrylic acid was used as a functional monomer, and ethylene glycol dimethacrylate was used as a crosslinking agent. The characteristics and applications of the proposed method were investigated using dienestrol and hexestrol as the structural analogues of a diethylstilbestrol template, and phenol and methylbenzene were taken as reference compounds. The MIP-HFT was employed in the LC–UV analysis of spiked milk samples. The LODs were found to be in the range 2.5–3.3  $\mu$ g L<sup>-1</sup> and their average recoveries were 83.7–90.6% in spiked milk samples. Chromatograms of estrogen spiked solutions of milk samples and determination of three estrogens in spiked sample solutions with molecularly imprinted polymer-coated polypropylene hollow fiber tube (MIP-HFT) and the non-imprinted polymer hollow fiber tube (NIP-HFT) are shown in Fig. 4.

### 2.2.2. Solid-drop microextraction (SD-LPME)

In 2007, Khalili-Zanjani et al. [109] introduced a new extraction method based on solidifying the floating organic droplet. In this microextraction mode, an appropriate volume of a suitable organic solvent is delivered to the surface of an aqueous solution located in a glass vial. The aqueous phase is stirred for the desired time, and the sample vial is then transferred into an ice bath. After a short period of time, the organic solvent is solidified and can be removed by a small spatula (Fig. 1F). Its main drawback is the limited selection of extracting solvents because only a few organic solvents have melting points close to room temperature [110]. Chang and Huang [111] determined levels of 17 $\beta$ -estradiol, 17 $\beta$ -ethynylestradiol, estriol and estrone in river and tap water samples using this technique coupled to ultra-performance liquid chromatography with photodiode-array detection (UPLC–DAD). They used 1-undecanol as the extraction solvent and methanol as the dispersive solvent, which allowed enrichment factors of between 121- and 260-fold. The relative recoveries ranged from

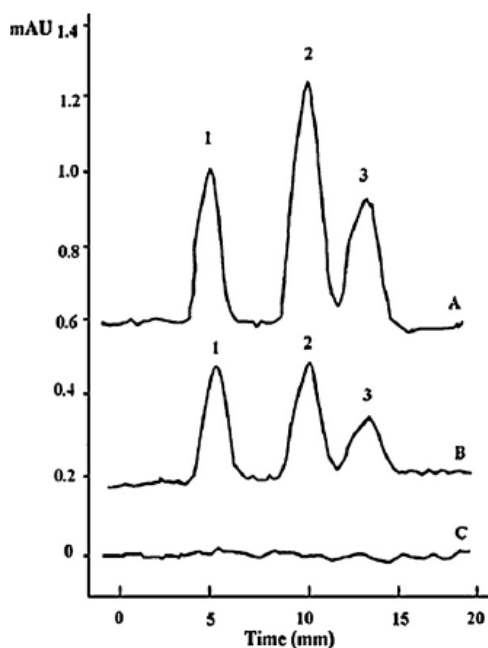


Fig. 4. Chromatograms of  $100\mu\text{gL}^{-1}$  estrogen spiked solutions of milk samples and determination of three estrogens in spiked sample solutions with molecularly imprinted polymer-coated polypropylene hollow fiber tube (MIP-HFT) and the non-imprinted polymer hollow fiber tube (NIP-HFT). (A) Spiked sample solution extracted with MIP-HFT, (B) spiked sample solution extracted with NIP-HFT, and (C) estrogens spiked sample solution without MIP-HFT and NIP-HFT. Peaks: 1: diethylstilbestrol, 2: dienestrol, and 3: hexestrol. Chromatogram is taken from [108].

87% to 116% for river water and from 89% to 102% for tap water, and the method LODs ranged from  $0.8$  to  $2.7\mu\text{gL}^{-1}$  for spiked river water and from  $1.4$  to  $3.1\mu\text{gL}^{-1}$  for spiked tap water.

The development of greener extraction methods has led to a significant reduction in organic solvent volume such that a single microdrop is sufficient for extraction using methods such as SDME. However, the disadvantages of these types of extraction are the instability of the drop, limited drop surface and, consequently, slow kinetics and the possibility of carry over because the extracting liquid is in contact with a surface. These disadvantages might be prevented by SD-LPME; however, the limitation in the choice of the proper solvent and the need for freezing the extract are their main drawbacks. HF-LPME has the advantages of an enhancement in the stability of organic solvents and tolerance of higher sampling times and temperatures. It has also high potential for automation and miniaturisation, but the need for membrane pre-conditioning and the possibility of memory effects when membranes are reused are drawbacks. Although there have not been applications of dispersive liquid–liquid microextraction (DLLME) to the determination of hormones, this technique has the same advantages of LPME, namely high recoveries and enrichment factors. Its drawbacks are that three solvents are needed, the extracting solvent must have high density, which limits the choice of a suitable solvent, and centrifugation must be applied. However, it is worth noting that the application of these microextraction techniques on biological samples or samples with a complex matrix requires a primary clean-up step.

Table 2 summarises the determination methods of the steroid hormones addressed in this overview. Table 3 gives a description of each technique and lists the advantages and disadvantages of them.

### 3. Future trends

Other methodologies have been developed to eliminate or minimise the use of organic solvents as extractants. One of these techniques, cloud point extraction (CPE), is based on the use of micellar solutions. These organized structures have a good capacity to solubilise solutes of different character and nature, which allow materials that are sparingly soluble or non-soluble in water to be solubilised in water because they bind to the micelles in solution. Another important property is that when the micellar solution is heated, it becomes turbid over a narrow temperature range, which is referred to as its cloud-point temperature. When the temperature rises above the cloud point, the solution is separated into two different phases: a surfactant-rich phase and an aqueous phase. The small volume of the surfactant-rich phase allows preconcentration and analyte extraction to occur in one step, prior to gas- or liquid-chromatographic analysis [112–114]. Wang et al. [115] applied this methodology to determine four estrogens, estriol, estradiol, estrone and progesterone, in effluent from wastewater treatment plants (WWTPs) by high-performance-liquid-chromatography separation and ultraviolet detection (LC-UV). A solution of 0.25% (w/v) of Triton X-114 was used as the extractant solvent, and  $\text{Na}_2\text{SO}_4$  was used to modify the solution ionic strength. Under optimum conditions, preconcentration factors from 86 to 152 were obtained using 10 mL of water sample. LODs in the range of  $0.23$ – $5.0\mu\text{gL}^{-1}$  and recoveries of 81.2–99.5% were obtained.

The use of micellar systems could also become an advantageous tool in LPME. Non-ionic surfactants have been widely used for the extraction of different organic substances from different types of matrices [116,117]. However, these extractants have been rarely used in LPME [118–120], and as far as we know, they have not been used to determine steroid hormones.

Regarding the use of new solvents, the use of ionic liquids to extract steroid hormones is expected, owing to their properties and compatibility with a variety of final determination techniques. Ionic liquids have many unique properties, such as the possibility to alter their polarity by selecting appropriate cations and anions such that they can be miscible with water and organic solvents. As a result, they are very versatile solvents. Originally, the use of ionic liquids in microextraction procedures was mainly limited to LC. However, some recent papers described several approaches that make ionic liquids compatible with GC. Moreover, ionic liquids can prove especially valuable for the extraction of strongly polar analytes, such as steroid hormones. Thus 1-hexyl-3-methylimidazolium hexafluorophosphate ([C6MIM][PF6]) and 1-octyl-3-methylimidazolium hexafluorophosphate ([C8MIM][PF6]) have been used to extract emergent pollutants by dispersive liquid–liquid microextraction (DLLME) [121] and HF-LPME [122] with satisfactory recoveries.

Microextraction techniques must be optimised by adjusting the parameters that affect the extraction procedure. Sample volume, organic solvent type and volume, agitation conditions, temperature, pH, extraction time and ionic strength must be optimised to maximise the extraction yield. Most procedures described in the literature use a step-by-step approach, in which one parameter is varied while all other parameters are kept constant. This approach does not permit the study of the interaction between the parameters that influence the process. Moreover, it requires a large number of experiments. The use of experimental designs to optimise these parameters should be used to reduce the number of experiments [123]. The real advantages of experimental design are in terms of reduced experimental effort and of increase quality of information. The optimisation by “one variable at a time” does not guarantee at all that the real optimum will be hit. This because this approach would be valid only if the variables to be optimised would be totally independent from each other. On the contrary, experimental design

**Table 2**  
Determination of hormones in biological and environmental samples.

Compounds	Matrix	Sample preparation	Analytical system	Recovery (%)	LOD	Ref.
ADR, STAN, ADD, MeT, T	Urine, water	SPME MIP	GC-MS	80.1–108.4	0.02–0.1 ng mL <sup>-1</sup>	[50]
E3, E1, 17β-E2, 17β-E2	Fish and shrimp tissue	SPME MIP	LC-UV	80.0–94.0	0.98–2.3 μg L <sup>-1</sup>	[51]
17β-E2, E3, DES	Fish and prawn tissue	MISPE	LC-UV/FD	78.3–84.5	0.023–0.76 mg L <sup>-1</sup>	[52]
17β-E2, E3, BPA, 17α-EE2	Surface waters	In-tube SPME	LC-FD	86–116	0.006–0.1 ng mL <sup>-1</sup>	[54]
E1, 17β-E2, E3, EE2, DES	Surface waters	In-tube SPME	LC-UV/MS/MS	86.1–106.8	2.7–11.7 pg mL <sup>-1</sup>	[55]
BOLD, NDL, T, MeT, EADR, ADR, STAN	Urine	In-tube SPME	LC-MS	85.7–117.3	9–182 pg mL <sup>-1</sup>	[56]
E1, 3α-H-5β-A-16, 3β-H-5,16-ADD, 3α-H-5α-A-16, 5α-A-16-e-3, 8,4,16-ADD-3, EC, iso-A, 5α-A-3,17-DD, 3α-H-3β-Pg-20, 3α,20α-DH-5α-Pg	Urine, blood	SBSE-TD	GC-MS	c.a. 40%	0.3 μg L <sup>-1</sup>	[59]
E1, 17β-E2, 17β-EE2	Water	SBSE-TD	GC-MS	90.3–105.7	0.2–1 pg mL <sup>-1</sup>	[60]
17β-E2	River water	SBSE-TD	GC-MS	93.1–98.4	0.5–100 pg mL <sup>-1</sup>	[61]
E1, 17α-E2, 17β-E2, 17α-EE2, DES, MES, P, 19-No, Ng	Water, urine	SBSE	LC-DAD	11.1–100.2	0.3–1 μg L <sup>-1</sup>	[63]
E3, 17β-E2, 17α-EE2, E1, BPA	Water	SBSE	LC-FD	84.6–124.1	0.04–0.11 μg L <sup>-1</sup>	[64]
T, P, MeT, DES, NDL phenylpropionate, T propionate	Urine	SBSE	LC-DAD	21.2–81.8	0.062–0.38 ng mL <sup>-1</sup>	[65]
NDL, T, DES, MeT, P, T propionate, NDL phenylpropionate	Water	SBSEM	LC-MS	n.a.	0.036–0.068 ng mL <sup>-1</sup>	[66]
NT, P, DES, T, MeT, T propionate, NDL phenylpropionate	Wastewater	SBSEM	LC-DAD	48.2–110	0.14–0.26 ng mL <sup>-1</sup>	[67]
ADR, E1, 17β-E2, and their acryl derivatives	Water, sludge	SBSE	GC-MS	73.8–100	0.2; 0.02 ng g <sup>-1</sup>	[68]
MES, 17α-EE2, BPA	Water	MEPS	LVI-GC-MS	97–113	48.5–292.6 ng L <sup>-1</sup>	[77]
16α-hydroxy-ADR, 2α-hydroxy-T, 11-keto,5β-ADD, 6α-hydroxy-E2,7β-hydroxy-Pg	Urine	SPME and MEPS	GC-MS	60–98	5–15 μg L <sup>-1</sup>	[78]
DES, E1, 17β-E2, 17α-EE2	Water	PC-HFME	GC-MS	86–110	0.03–0.8 ng L <sup>-1</sup>	[88]
17β-E2, E1, 17α-EE2	Sewage water	HF-MMLLE	GC-MS	45–98	1.6–10 ng L <sup>-1</sup>	[89]
DES, dienestrol, hexestrol	Wastewater	HF-LPME	LC-UV	86.0–95.5	0.25–0.5 ng L <sup>-1</sup>	[90]
DES, dienestrol, hexestrol	Milk	MIP-HFT	LC-UV	83.7–90.6	2.5–3.3 μg L <sup>-1</sup>	[91]
E1, E2, EE2, E3	River and tap water	DLIME-SFO	UPLC-PDA	87–116	0.8–2.7 μg L <sup>-1</sup>	[94]
E3, E2, E1, P	Water	CPE	LC-UV	81.2–99.5	0.23–5.0 ng mL <sup>-1</sup>	[98]

Estrone (E1), estradiol (E2), estriol (E3), ethynylestradiol (EE2), diethylstilbestrol (DES), androstosterone (ADR), epiandrostosterone (EADR), androstenedione (ADD), stanolone (STAN), boldenone (BOLD), nandrolone (NDL), progesterone (P), pregnolone (Pg), norethisterone (No), norgestrel (Ng), 17β-ethynylestradiol (17β-EE2), 17α-ethynylestradiol (17α-EE2), bisphenol A (BPA), diethylstilbestrol (DES), 17β-estradiol (17β-E2), stanozol (STAN), nortestosterone (NT), 16α-hydroxyandrostosterone (16α-hydroxy-ADR), 2α-hydroxytestosterone (2α-hydroxy-T), 11-keto,5β-androstenedione (11-keto,5β-ADD), 6α-hydroxyestradiol (6α-hydroxy-E2), 7β-hydroxypregnonolone (7β-hydroxy-Pg), 3α-hydroxy-5β-androst-16-ene (3α-H-5β-A-16), 3β-hydroxy-5,16-androstenedione (3β-H-5,16-ADD), 3α-hydroxy5α-androst-16-ene (3α-H-5α-A-16), 5α-androst-16-en-3-one (5α-A-16-e-3), 8,4,16-androstadien-3-one (8,4,16-ADD-3), 5α-androst-3,17-dione (5α-A-3,17-DD), etiocholanolone (EC), iso-androstosterone (iso-A), 3α-hydroxy-5β-pregnan-20-ene (3α-H-3β-Pg-20), and 3α,20α-dihydroxy-5α-pregnane (3α,20α-DH-5α-Pg).

Solid phase microextraction molecular imprinted polymer (SPME MIP), molecular imprinted solid phase extraction (MISPE), in-tube solid phase microextraction (in-tube SPME), stir bar sorptive extraction-thermal desorption (SBSE-TD), stir bar sorptive extraction-liquid desorption (SBSE-LD), stir bar sorptive extraction on monolith material (SBSEM), micro extraction packed syringe (MEPS), polymer coated hollow fiber microextraction (PC-HFME), hollow fiber microporous membrane liquid-liquid extraction (HF-MMLLE), molecular imprinted polymers hollow fiber tube (MIP-HFT), dispersive liquid-liquid microextraction based on solidification of floating organic drop (DLIME-SFO), cloud point extraction (CPE) gas chromatography-mass spectrometry detection (GC-MS), high performance liquid chromatography-ultraviolet detection (LC-UV), high performance liquid chromatography-fluorescence detection (LC-FD), large volume injector gas chromatography-mass spectrometry detection (LVI-GC-MS), high performance liquid chromatography-diode array detection (LC-DAD), high performance liquid chromatography-mass spectrometry detection (LC-MS), high performance liquid chromatography-photo diode array (LC-PDA), limit of detection (LOD), and not available (n.a.).

approaches consider some related variables at the same time, take into account variable interactions. Although this approach has been scarcely used in the determination of hormones by microextraction techniques, Prieto et al. used a Central Composite Design with two instrumental variables, fill and injection speed, when they deter-

mined mestranol and 17α-ethynylestradiol in water samples by MEPS [94].

To improve the performance of these microextraction methodologies, automation should be introduced into sample preparation. These techniques are highly effective in saving time and in

**Table 3**  
Comparison of microextraction techniques.

	MIP	In-tube SPME	SBSE	MEPS	HF-LPME	SD-LPME
Extraction time (min)	30–40	20–30	30–240	2–10	10–120	10–30
Enrichment factor	Moderate	Moderate	High	Moderate	High	High
Cost	Moderate	Low	Moderate	Low	Low	Low
Operation—Handling	Difficult	Easy	Easy	Easy	Easy	Easy
Potential for automation	Low	High	Moderate	High	Moderate	Low
Advantages	Selectivity	Low sample volumes Wide range of available coatings	Great efficiency in preconcentration	Different types of sorbents commercially available Sorbent can be used several times	Non-dependence on a commercial supplier Great stability of the organic phase due to their mechanical protection	Non-dependence on a commercial supplier
Disadvantages	Difficult in preparation procedure and its low robustness	Requirement of very clean samples	Fails in the extraction of polar compounds Long equilibration times	Strong dependence of analyte recoveries on the continual movement of the plunger	Need for membrane pre-conditioning and possibility of memory effects	Limitation in the choice of the proper solvent and the need for freezing

Molecular imprinted polymer extraction (MIP), in-tube solid phase microextraction (in-tube SPME), stir bar sorptive extraction (SBSE), microextraction packed syringe (MEPS), hollow fiber liquid phase microextraction (HF-LPME), and solid drop liquid phase microextraction (SD-LPME).



obtaining better reproducibility compared to manual methods. Automation of LPME seems to be very difficult but some advances have been made in this direction. Thus, Pawliszyn et al. have introduced fully automated HF-LPME, including filling the extraction solvent, sample-vial transfer and agitation, withdrawing the solvent to the syringe and introducing the extraction phase into injector [124]. Several automated procedures involving direct immersion and headspace SDME have been also developed [125,126]. An attempt of automation of DLLME was also made for the analysis of inorganic species [127].

#### 4. Conclusions

In this work, green analytical methodologies for the determination of steroid hormones reported in the scientific literature have been reviewed. Residues of these compounds are a potential risk for wildlife and humans through the consumption of contaminated food or water. Prior to detection, extraction and clean-up steps are required to obtain high recoveries and minimise the presence of interferences. Although SPE has been frequently used to extract these compounds, the need for new analytical methodologies that use less-hazardous chemicals has led to the development of new approaches. Among these approaches, different applications of SPME, such as MIPs, in-tube solid-phase microextraction, SBSE and MEPS, have been employed. DLLME and HFME, two approaches of LPME, have been also used to determine steroid hormones, mainly in water samples. Regarding sorbent microextraction, SBSE is the technique most widely used for the extraction and preconcentration of this type of pollutant, whereas HFME is the approach most used in LPME.

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## **6.6 Supplement III**

Helena Tomšíková, Jana Aufartová, Petr Solich, Lucie Nováková, Zoraida Sosa-Ferrera, José Juan Santana-Rodríguez

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

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

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

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

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

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

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

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

## 1. Introduction

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

and also as medicaments for their protective function against various diseases. They have been administered in hormone-replacement therapy, helping in the treatment of hormonal disorders [4, 5].

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

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

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

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

(continued on next page)

**Table 1. (continued)**

Abbreviation	Name	R1	R2	R3	MW	pKa	K <sub>ow</sub> (log P)	Structure
T	Testosterone	-CH <sub>3</sub>	-H	-	288.42	15.06	3.179	
MT	17 $\alpha$ -methyltestosterone	-CH <sub>3</sub>	-CH <sub>3</sub>	-	302.45	15.13	3.559	
NT	19-nortestosterone	-H	-H	-	274.4	15.06	2.898	
NG	(D)-norgestrel	-	-	-	312.45	13.09	3.368	
L	(l)-norgestrel, levonorgestrel	-	-	-	312.45	13.09	3.368	
PG	Progesterone	-H	-	-	314.46	-	3.827	
AP	17 $\alpha$ -acetoxyprogesterone	-OCOCH <sub>3</sub>	-	-	372.50	-	3.638	
HP	17 $\alpha$ -hydroxyprogesterone	-OH	-	-	330.46	13.03	3.040	
DES	Diethylstilbestrol	-	-	-	268.29	10.18	5.330	
BPA	Bisphenol A	-	-	-	228.29	10.29	3.641	

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

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

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

## 2. Sample preparation

Steroid hormones possess non-polar and non-ionic characteristics (see Table 1) that enable use of reversed-phase purification and separation strategies. The sample-preparation process is one of the most important and time-consuming parts of the analytical method. Its optimization plays an important role in the enhancement of sensitivity and the reduction of matrix interferences in wastewater, sludge and biological samples. The application of sample preparation with high pre-concentration is necessary to achieve limits of detection

(LODs) at the ng/g level in solid samples and the ng/L level in liquid samples. Most analytical methods include a solid-phase extraction (SPE) step alongside a more time-consuming purification step (e.g., gel-permeation chromatography, normal-phase preparative chromatography or immunoaffinity clean-up) [9]. Normally, further clean-up steps are unnecessary. However, Kumar et al. [13] attached a Sep-Pak Plus NH2 cartridge (silica-based polar bonded phase with basic character) below the dried Oasis HLB cartridge (hydrophilic-lipophilic balanced copolymer) as a sample clean-up step, and improved gradient elution to eliminate matrix interferences. The use of the Sep-Pak Plus NH2 cartridge significantly reduced the amount of co-extracted acidic interferences [13].

### 2.1. Aqueous samples

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

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

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

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

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

Table 2. Sample preparation in analysis of estrogens and progestogens

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

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

(continued on next page)



Table 2. (continued)

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

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

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

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

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

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

Yang et al. [40] revealed that both traditional methods (SPE and SPME) were comparable. The SPME technique was very often applied for sample preparation of anabolic

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

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

## 2.2. Solid samples

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

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



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

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

### 3. Analytical methods

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

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

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

#### 3.1. Liquid chromatography coupled to mass spectrometry

Currently, LC-MS and LC-MS<sup>2</sup> have become widely used tools for determination of estrogens in environmental samples because of their sensitivity and specificity. Unlike GC-MS, LC-MS is not limited by the non-volatility and the high molecular weight of steroids, and enables the determination of both conjugated and

non-conjugated estrogens without a derivatization step or hydrolysis [9,51].

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

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

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

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

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

Table 3. LC-MS<sup>2</sup> methods for measurement of steroids

Substance	Matrix, sample preparation	Method	Column	Mobile phase/flow rate	Detector	Analysis time (min.)	Derivat.	LOD (S/N = 3) (ng/L)	Ref.
E1, $\alpha$ E2, E2, E3, EE2	Water samples SPE	LC-MS <sup>2</sup>	Luna Phenyl-hexyl 50 $\times$ 2.0 mm; 3 $\mu$ m	Linear gradient 0.25 mL/min A: 0.1% FA B: ACN	ESI(+)-IT SRM	15	FBIBT in DMSO	0.067–0.29	[27]
$\alpha$ EE2, E2, E1	Wastewater Immunoextraction	LC-MS <sup>2</sup>	Betasil C <sub>18</sub> 150 $\times$ 2.1 mm; 3 $\mu$ m	Linear gradient 0.2 mL/min A: ACN B: H <sub>2</sub> O	ESI(-)-QqQ	16	-	0.07–0.18	[52]
BPA, E1, E2, E3, NP, OP	Surface waters SPE	LC-MS <sup>2</sup>	Zorbax SB-C18 30 $\times$ 2.1 mm; 3.5 $\mu$ m	Gradient elution 0.3 mL/min A: 0.1% AmOH B: 0.1% AmOH in MeOH	ESI(-)-QqQ SRM	10	-	0.04–1.0	[35]
BCA, CM, DAI, DES, E1, E2, E3, E1-3G, E2-17G, E3-16G, E1-3S, E3-3S, EE2, EE2S, GEN, L, NOR, PG, RV	Environment. waters SPE	LC-MS <sup>2</sup>	Guard column 4 $\times$ 4 mm; 5 $\mu$ m STAR RP-18 125 $\times$ 2 mm; 5 $\mu$ m	Gradient elution 0.2 mL/min A: H <sub>2</sub> O (for estrogens); 0.1% FA (for phytoestrogens and progestogens) B: ACN	estrogens: ESI(-)-QqQ SRM progestogens and phytoestrogens: ESI(+)-QqQ SRM	45	-	0.04–2.0	[21]
Estrogens, androgens and progestogens	WWTP and river waters SPE	LC-MS <sup>2</sup>	BEH C18 100 $\times$ 2.1 mm; 1.7 $\mu$ m	Gradient elution 0.3 mL/min Estrogens: A: 0.1% AA B: ACN Progestogens and androgens: A: 0.1% FA B: MeOH	ESI(+)-QqQSRM	5.6	-	0.02–40	[24]
E1, E2, E3, E1-3G, E2-17G, E3-3G, E3-16G, E1-3S, E2-3S, E3-3S	River water	LC-MS <sup>2</sup> (Column switching)	Luna C18 100 $\times$ 2.0 mm; 3 $\mu$ m ZIC-pHILIC 100 $\times$ 2.1 mm; 5 $\mu$ m	Gradient elution 150 $\mu$ L/min A: ACN: 5 mM AmAc (pH 6.8), 95:5 (v/v) B: ACN: 5 mM AmAc (pH 6.8), 75:25 (v/v)	Free and conjugated: TIS(-)-Q-IT Derivatized: TIS(+)-Q-IT SRM	25	Dansyl chloride	0.049–8.9	[31]
EE2, E2, E3, E1-3S	Wastewater SPE	LC-MS <sup>2</sup>	Gemini C18 100 $\times$ 2 mm; 3 $\mu$ m	Gradient elution 10 $\mu$ L/min A: 0.1% AmOH B: MeOH with 0.1% AmOH	ESI(-)-QqQ SRM	18	-	0.1–0.2	[11]
E1, E2, 16 $\alpha$ -OHE1, HEP, 2-MeOE1, PA, PGL	Mixture of standards	LC-MS <sup>2</sup>	YM/basic 150 $\times$ 4.6 mm; 5 $\mu$ m	Linear gradient 1 mL/min A: H <sub>2</sub> O B: MeOH	ENCI-QqQ SRM ECAPCI(-)-QqQ SRM	13	PFBBr	0.14–0.74 amol (ENCI) 7.02–62 fmol (APCI(-))	[56] (2000)

(continued on next page)

Table 3. (continued)

Substance	Matrix, sample preparation	Method	Column	Mobile phase/flow rate	Detector	Analysis time (min.)	Derivat.	LOD (S/N = 3) (ng/L)	Ref.
E1, $\alpha$ E2, E2, E3, EE2, E1-3S, E2-3S, E3-3S, E1-3G, E2-17G, E2-3G, E3-3G, E2-3S17S, E2-3S17C, E2-3G17S	Environment. waters SPE	LC-MS <sup>2</sup>	Zorbax Extend-C18 150 × 1 mm; 3.5 $\mu$ m	Gradient elution 40 $\mu$ L/min A: ACN B: H <sub>2</sub> O C: 100 mM TEA (pH 12.2)	ESI(-)-QqQ SRM	29	-	0.1–3.1	[29] (2003)
BPA, BCA, DD, E1, E3, E2, EE2, G, NP, Ze, $\alpha$ Ze, $\beta$ Ze	STP influent, effluent and river water SPE	LC-MS <sup>2</sup>	Supelguard 20 × 4.6 mm LC-18 packing Altima 250 × 4.6 mm; 5 $\mu$ m	Different gradient and mobile phase composition depending on the group of analyzed compounds, 1 ml/min	ESI, APCI (+/-)-QqQ SRM	5–30	-	0.1–8.4 except NP	[6]
E1, E2, E3, EE2, and their glucuronides and sulfates	WWTP and river water SPE	LC-MS <sup>2</sup>	BEH C18 100' 50 × 2.1 mm; 1.7 $\mu$ m	Gradient elution 0.2 mL/min A: H <sub>2</sub> O B: ACN	ESI(-)-QqQ SRM	10–12	-	0.2–0.8	[13]
E1, E2, EE2	WWTP influent and effluent	LC-MS <sup>2</sup>	SunFire C18 150 × 2.1 mm; 5 $\mu$ m	Isocratic elution 0.3 mL/min H <sub>2</sub> O:ACN (20:80, v/v) with 0.1% FA	TIS(+)-QqQ SRM	17	Dansyl chloride	0.4–0.7	[25]
BPA, E1, $\alpha$ E2, E2, E3, EE2	On-line SPE Water samples On-line SPE	LC-MS <sup>2</sup>	Guard column 4 × 3.0 mm RP-Intersil ODS-3 100 × 4 mm; 3 $\mu$ m	Gradient elution 1 mL/minA: H <sub>2</sub> O B: ACN:MeOH (70:30; v/v)	ESI(-)-QqQ SRM	16	-	0.5–1	[38]
E1, E2, EE2	Surface water SPE	LC-MS <sup>2</sup>	NUCLEODUR C18 ISIS 125 × 2 mm; 3 $\mu$ m	Gradient elution 0.25 mL/min A: H <sub>2</sub> O with 10 mM AmAc B: ACN with 10 mM AmAc	ESI(-)-QqQ	25	-	0.3–2.0	[36]
E1, E2, E3, EE2, E2-3S, E2-17Ac, E1-3S, T6 $\alpha$ -OHE1, MeEE2	STP influents and effluents SPE and SEC	LC-MS <sup>2</sup>	SecurityGuard column Synergi RP-MAX 150 × 2 mm; 4 $\mu$ m	Gradient elution 0.2 mL/min A: H <sub>2</sub> O B: MeOH	ESI(-)-QqQ APCI(+)-QqQ SRM	35	-	0.6–1.8	[10]
BPA, E1, E2, E3, EE2	Sewage samples SPE	LC-MS <sup>2</sup>	Pursuit XRs Ultra-C18 50 × 2 mm; 2.8 $\mu$ m	Gradient elution 0.2 mL/min A: H <sub>2</sub> O B: MeOH with 0.1% AA and 15 mM AmAc	ESI(+/-)-QqQ SRM	29	-	0.5–6	[22]
E1, E2, E3, EE2 and progestins	Environment. waters SPDE	LC-MS <sup>2</sup>	Guard column 4 × 4 mm; 1.7 $\mu$ m BEH RP-C18 50 × 2.1 mm; 1.7 $\mu$ m	Gradient elution 0.1 mL/min Group 1 A: MeOH B: H <sub>2</sub> O Group 2 A: ACN B: H <sub>2</sub> O	Group 1 ESI(-)-QqQ Group 2 ESI(+)-QqQ	10	-	0.5–3.4	[55]

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

(continued on next page)

Table 3. (continued)

Substance	Matrix, sample preparation	Method	Column	Mobile phase/flow rate	Detector	Analysis time (min.)	Derivat.	LOD (S/N = 3) (ng/L)	Ref.
E1, $\alpha$ E2, E2, EE2	River sediments	On-line 2D-LC-MS <sup>2</sup>	Column 1: Luna Phenyl-hexyl 50 $\times$ 2.0 mm; 3 $\mu$ m Column 2: XBridge Shield RPI8 150 $\times$ 2.1 mm; 3.5 $\mu$ m	Gradient elution 0.2–0.75 mL/min A: 0.1% FA B: MeOH Gradient elution 0.3 mL/min A: 0.1% FA B: MeOH	APPI(+)-IT	18	–	0.09–0.25 ng/g	[49]
A, BET, BUD, DE, E1, FLA, L, NOR, PG, T, TRA	Soil PLE and SPE	LC-MS <sup>2</sup>	Zorbax Eclipse XDB C18 (with precolumn) 100 $\times$ 2.1 mm; 3.5 $\mu$ m	Gradient elution 0.2 mL/min A: 0.2% FA B: ACN + 0.2% FA	ESI(+)-QqQ SRM	17	–	0.08–2.84 ng/g	[19]
E3, EE2S, EE2G, E1-3G, E1-3S, $\alpha$ E2, E2, EE2, E1, DES, E2-17Ac	Sewage sludge PLE	LC-MS <sup>2</sup>	Kromasil 100 C18 250 $\times$ 4.6 mm; 5 $\mu$ m	Gradient elution 1 mL/min A: acidic H <sub>2</sub> O (pH 3) B: ACN	ESI(-)-QqQ SRM	37	–	26–175 ng/g	[48]
AP, Bol, CITA, CMA, CP, DES, DE, DMA, E1, E3, E2, EE2, Ed, FMT, Hex, HP, TB, MeBol, MeD, MegA, MeIA, MT, MPA, NE, NG, NT, PG, T, TBA, Stan, $\beta$ Z	Kidney fat matrices LLE	LC-MS <sup>2</sup>	Guard column 7.5 $\times$ 4.6 mm; 5 $\mu$ m Alltima C18 250 $\times$ 4.6 mm; 5 $\mu$ m	Gradient elution 1 mL/min A: MeOH B: H <sub>2</sub> O, acidified with 0.1% CF <sub>3</sub> COOH	APCI(+)-QqQ SRM	>20	–	> 2 ppb	[60] (1999)





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

Substance	Matrix, sample preparation	Method	Column	Mobile phase/flow rate	Detector setting	Analysis time (min)	Derivat.	LOD (ng/mL)	Ref.
E2	Water samples SPE	Enzyme-linked immunosorbent assay	–	–	Photometric Analyzer $\lambda = 450$ nm	142	–	0.0025	[54]
E2, E3, BPA, EE2	WWTP SPME	LC-DAD/FD	Hypersil ODS 200 $\times$ 4.6 mm; 5 $\mu$ m	Isocratic elution 1 mL/min ACN: 0.02 M phosphate solution (45:55; v/v; pH 4.5)	DAD: $\lambda = 280$ nm FLD: $\lambda_{exc} = 227$ nm $\lambda_{em} = 315$ nm	12	–	0.006–0.1	[41]
DES, NT, MT, PG, PNT, PT, T	Wastewater SBSEM-LD	LC-DAD	Supelcosil LC-18 250 $\times$ 2.0 mm; 5 $\mu$ m	Gradient elution 1 mL/min A: H <sub>2</sub> O B: ACN	$\lambda = 240$ nm	22	–	0.14–0.41	[43]
E3, E2, E1, PG	Sediment CPE	LC-UV	Inertsil ODS-C18 250 $\times$ 4.6 mm; 5 $\mu$ m	Gradient elution 1 mL/min A: ACN B: H <sub>2</sub> O	Estrogens: $\lambda = 200$ nm PG: $\lambda = 240$ nm	25.5	–	0.23–5.0	[45]
E1, E2, E3, EE2	River and tap water DLLME-SFO	LC-PDA	BEH Phenyl column 100 $\times$ 2.1 mm; 1.7 $\mu$ m	Gradient elution 0.4 mL/min A: H <sub>2</sub> O B: ACN	$\lambda = 280$ nm	7	–	0.8–3.1	[46]

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

### 3.1.2. Mass-spectrometry detection

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

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

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

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

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

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





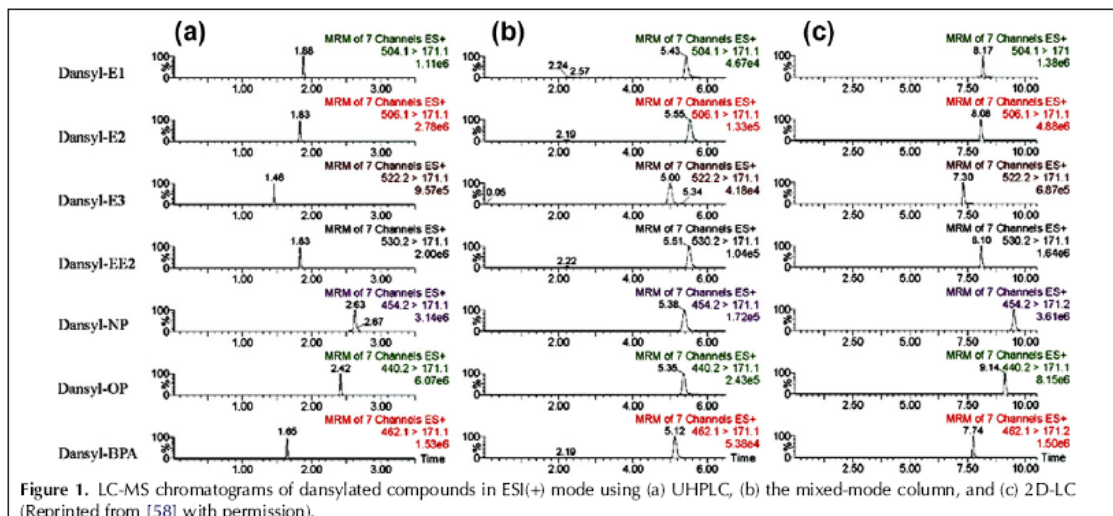
AD, CORT, CHOL, DHT, DES, E2, E1, EE2, MT, PG, PREG, T	Liquid and solid sludge SPE	GC-MS	Zebtron ZB-5 30 m × 0.25 mm; 0.25 µm film thickness	Thermal gradient helium; 1 mL/min	EI SIM	25.5	MSTFA	-	[71]
BPA, E1, E2, αE2, E3, NP, MeEE2	River sediment Sonation, Soxhlet and mechanical extraction	GC-MS	HP-5MS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

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

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

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

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



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

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

### 3.2. Gas chromatography coupled to mass spectrometry

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

## 4. Derivatization in chromatographic methods

### 4.1. Liquid chromatography coupled to mass spectrometry

Despite the specificity and the versatility of LC-MS, ionization efficiency varied between different ionization

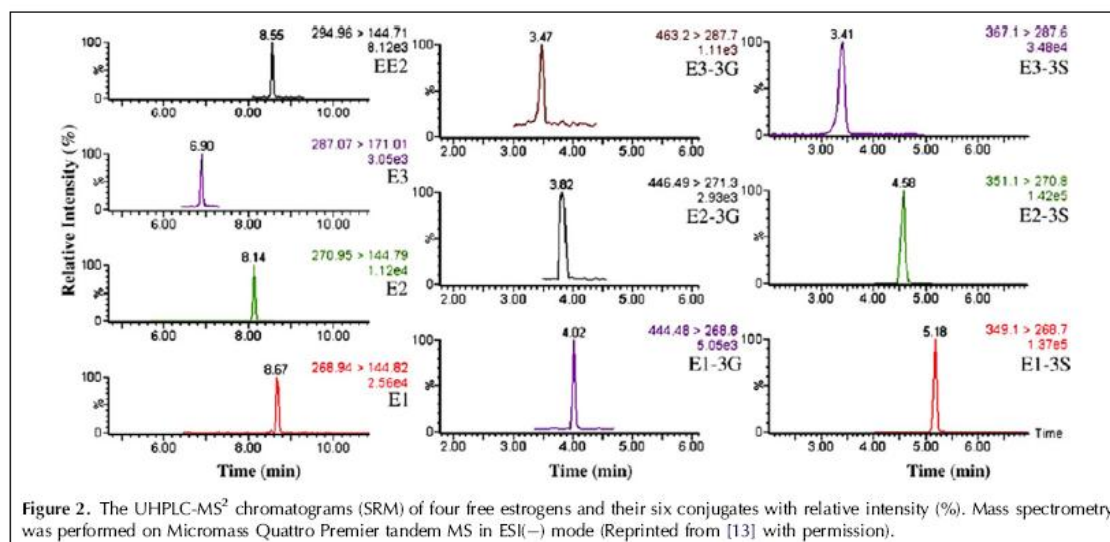
techniques and compounds [61]. Ionization of estrogens and phenolic xenoestrogens by ESI and APCI were less efficient than that of more polar compounds [58]. Chemical derivatization using appropriate derivatizing reagents could improve ionization and enhance signals in LC-MS [12,27,31,58,61].

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

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

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

Table 6. SRM conditions used for LC-MS <sup>2</sup>					
Compound	Collision energy (eV)	Precursor ion (m/z)	Product ions (m/z)	Polarity	Ref.
DES	30, 55, 35, 45	267	222, 237, 131	[M-H] <sup>-</sup>	[4,16,21,48]
DHT	31	273	123	[M+H] <sup>+</sup>	[31]
DHT-d3	31	276	123	[M+H] <sup>+</sup>	[31]
E1	47,45, 57, 40, 55	269	145, 143, 159, 253, 183	[M-H] <sup>-</sup>	[4,6,9,10,16,21,31,48]
	29, 11, 21, 27	271	159, 133, 253	[M+H] <sup>+</sup>	[10,26]
E1-d4	51, 45	273	147, 161, 145	[M-H] <sup>-</sup>	[9-11]
E1-3G	45, 25, 30, 20	445	269, 113	[M-H] <sup>-</sup>	[16,21,31,48]
E1-3S	44, 100, 30, 55, 40, 65, 25, 30	349	269, 145, 113	[M-H] <sup>-</sup>	[10,12,16,21,31,48]
E1-3S-d4	45, 76, 65	353	273, 147	[M-H] <sup>-</sup>	[10,11]
E2	24	255	159, 133	[M+H] <sup>+</sup>	[10]
	54, 53, 30, 45, 57, 40, 50, 55	271	145, 183, 239, 159, 237, 401, 359	[M-H] <sup>-</sup>	[4,9-11,16,21,31,48]
E2-d3	65	290	147	[M-H] <sup>-</sup>	[11]
E2-d4	57, 45	275	147, 187	[M-H] <sup>-</sup>	[9]
E2-d5	50	276	147	[M-H] <sup>-</sup>	[11]
E2-17Ac	24	255	159, 133	[M+H] <sup>+</sup>	[10]
	38, 59, 30, 55	313	253, 145	[M-H] <sup>-</sup>	[10,16,48]
E2-3G	-	447	271, 113	[M-H] <sup>-</sup>	[31]
E2-17G	30, 20	447	271, 113	[M-H] <sup>-</sup>	[16,48]
	30	463	85, 287	[M+H] <sup>+</sup>	[21]
E2-3S	30, 45, 55	351	271, 145, 80	[M-H] <sup>-</sup>	[16,31,48]
E3	49, 58, 45, 11, 22,50, 55, 40, 35	287	171, 253, 133, 145, 159	[M-H] <sup>-</sup>	[4,6,10,11,16,21,26,31,48]
E3-3G	-	463	287, 113	[M-H] <sup>-</sup>	[31]
E3-16G	-	463	278, 113	[M-H] <sup>-</sup>	[31]
E3-3S	35, 30	367	287, 80	[M-H] <sup>-</sup>	[21,31]
EE2	60, 45, 30, 40, 47, 49	295	145, 159, 199	[M-H] <sup>-</sup>	[6,9-11,16,21,48]
	22, 21, 20, 27	297	133, 159	[M+H] <sup>+</sup>	[10,26]
EE2-3G	35, 30	351	271, 80	[M+H] <sup>+</sup>	[4,21]
EE2-17G	30, 50	447	113, 271	[M+H] <sup>+</sup>	[4,21]
EE2-d4	60, 50, 47	299	174, 147, 161	[M-H] <sup>-</sup>	[1,9-12,29]
EE2-d4	28, 31	283	135, 161	[M+H] <sup>+</sup>	[1,10]
L	20, 22, 44	313	245, 185, 91	[M+H] <sup>+</sup>	[21,26]
PG	20, 22, 26	315	273, 160, 109, 97	[M+H] <sup>+</sup>	[4,21,26]
PREG	20	317	256, 159, 109	[M+H] <sup>+</sup>	[4]
T	35	289	109	[M+H] <sup>+</sup>	[31]
T-d3	35	292	109	[M+H] <sup>+</sup>	[31]





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

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

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

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

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

best results for detection of dansyl derivatives (2–3 times higher sensitivity than 10 mMFA and MeOH). 10 mM FA and MeOH were optimal for APCI(+) and APCI/APPI(+). The best detection of PFBBBr derivatives was attained with water-MeOH in APCI(-) [58].

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

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

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

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

#### 4.3. Gas chromatography coupled to mass spectrometry

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

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

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

Substance	Time/temperature of incubation	Derivatization agent	Method	LOD (ng/L)	Ref.
E1, $\alpha$ E2, E2, E3, EE2	On-line pre-column derivatization 30 min/at room temperature	FBIBT in DMSO	LC-MS <sup>2</sup>	0.067-0.29	[27]
E1, E2, E3, EE2	1 min vortex-mixing, 30 min/50°C	Dansyl chloride	LC-MS <sup>2</sup>	0.05-0.5	[58]
E1, E2, E3	5 min/60°C	Dansyl chloride	LC-MS <sup>2</sup>	0.049-8.9	[31]
E1, E2, EE2	On-line derivatization: 3 min/40°C	Dansyl chloride	LC-MS <sup>2</sup>	0.4-0.7	[25]
EE2	30 min/45°C	Dansyl chloride	LC-MS <sup>2</sup>	<0.75	[12]
E1, $\alpha$ E2, E2, E3, EE2	30 min/60°C	PFBBr	LC-MS <sup>2</sup>	0.14-0.74 amol (ENCI)	[56] (2000)
BPA, BZA, CA, DF, EE2, GF, IB, NX, NP	60 min/60°C	PFBBr	GC-MS	7.02-62 fmol in APCL(-)	[72]
E1, E2, E3, EE2, MeEE2	MAD: 60 s/600 W; 30 min/60°C	BSTFA + 1% TMS	GC-MS	0.02-0.1	[70]
E1, E1-d <sub>4</sub> , E2, E2-d <sub>4</sub> , EE2, EE2-d <sub>4</sub>	30 min/60-70°C	BSTFA	GC-MS	0.7-1.4	[34]
E2	SBSE - in situ acylation: stirring at 1000 rpm for 120 min at room temperature	Acetic anhydride and BSTFA	GC-MS <sup>2</sup>	0.3	[42]
DEP, NP (technical grade), CP, DBP, NP, BBP, BPA, DEHP, ECL, AS, Tm, E1, E2, E1, E2, E3, EE2, NP, BPA	TD - in tube silylation 30 min/70°C	BSTFA	GC-MS	0.5-2	[67]
DEHP, BPA, CST, CHOL, CPN, C3O, DST, $\alpha$ E2, E2, E1, E3, E2B, EE2, EST, EQ, EQL, FST, 6KCST, 7KCHOL, MeEE2, NG, NOR, PS, $\beta$ S, SMT, SST, T, $\alpha$ Ze	Ultrasound-assisted derivatization 30 min/60-70°C 180 min/90°C	BSTFA + 1% TMS BSTFA + 10% TMS	GC-MS GC-MS	1-5	[17]
DHT, E1, $\alpha$ E2, NT, T	Headspace derivatization: 60 min/60°C	BSTFA	GC-MS	LOQ liquid: 2.0-30.3 LOQ solid: 1.2-188.7 ng/g	[69] (1998)
BPA, E1, E2, $\alpha$ E2, E3, NP, MeEE2	120 min/60°C	PFPA	GC-MS	MDLs: 0.1-0.6 ng/g	[3]
E1, E2, E3, EE2, E2-17G, E2-3S, MeEE2, NO, NG, PG	30-40 min/65°C	MSTFA	GC-MS	0.4-1.9 ng/g	[30]
BPA, DCP, DBP, DEP, DEHP, E1, E2, EE2, NP, OP, TB	SBSE: 60 min under 900 rpm	BSTFA	GC-MS	-	[68]
E1, E2, E3, EE2	60 min/70°C	BSTFA + 1% TMS	GC-MS	-	[47]
AD, E2, CORT, CHOL, DHT, DES, E1, EE2, 17-MT, PG, PREG, T	MAD: 1 min/900 W MSTFA: 30 min/55°C	MSTFA	GC-MS	-	[71]

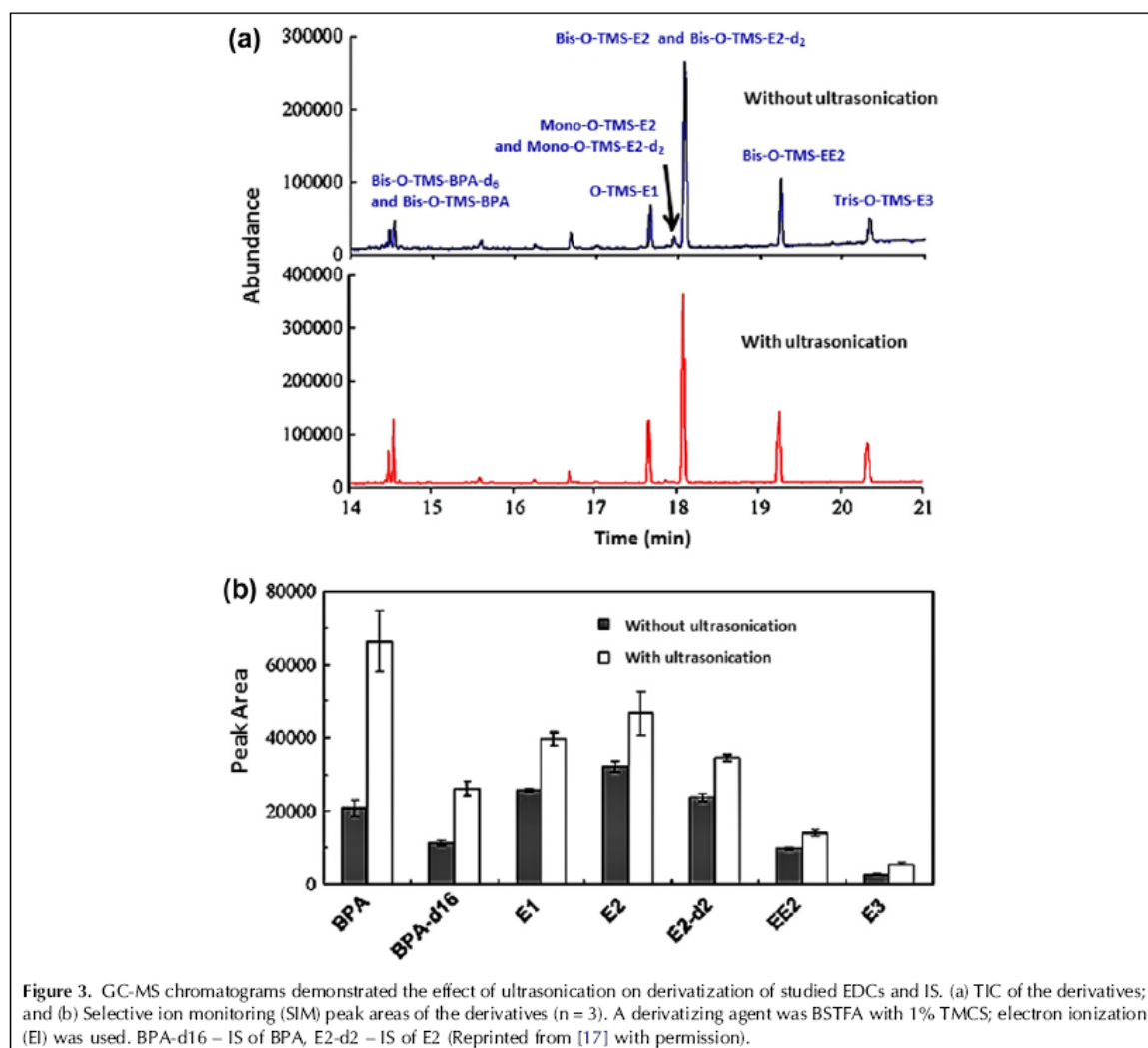
natural estrogens, phenolic compounds had comparable results when derivatized by each of these three reagents.

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

Nie et al. [17] investigated the effect of ultrasonication on the derivatization efficiency of EDCs analyzed. Their study indicated that aliphatic hydroxy groups were more difficult to derivatize (only about 8% of E2 and E2-d<sub>2</sub> were transformed to mono-*O*-TMS forms) than aromatic ones. After ultrasonication, a complete silylation of both

aliphatic and aromatic hydroxy groups was accomplished. The peak area of the EDC derivatives increased after ultrasonication from 44% (for EE2) to 219% (for BPA). Ultrasonication could also considerably enhance the recovery (70–130%) and the sensitivity of the detection method (Fig. 3) [17].

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



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

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

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

## 5. Conclusions

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

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

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

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1 **6.7 Supplement IV**

2

3 Jana Aupartová, Maria Ester Torres-Padrón, Zoraida Sosa-Ferrera, Petr Solich, José Juan Santana-  
4 Rodríguez

5 **Optimisation of an in-tube solid phase microextraction method coupled with HPLC for**  
6 **determination of some oestrogens in environmental liquid samples using different capillary**  
7 **columns**

8 International Journal of Environmental Analytical Chemistry, 92 (2011) 1–15

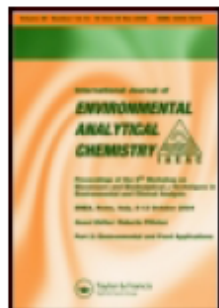
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## Optimisation of an in-tube solid phase microextraction method coupled with HPLC for determination of some oestrogens in environmental liquid samples using different capillary columns

J. Aupartová<sup>ab</sup>, M.E. Torres-Padrón<sup>a</sup>, Z. Sosa-Ferrera<sup>a</sup>, P. Solich<sup>b</sup>  
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A simple on-line method for simultaneous determination of some oestrogens including oestriol (E3), norethisterone (NORE), ethynylestradiol (EE2), D-norgestrel (NORG) and bisphenol A (BPA), in environmental liquid samples was developed by coupling in-tube solid phase microextraction (in-tube SPME) to high-performance liquid chromatography with diode array (DAD) and fluorescence (FLD) detectors. Two capillary chromatographic columns (Supel-Q<sup>TM</sup> and Carboxen<sup>TM</sup> 1006 porous layer open tubular) were selected to develop this method. To achieve optimum extraction performance, several parameters were investigated including number of draw/eject cycles and the sample volume for each of the columns. Reproducibility was satisfactory for inter- and intra-day precision, yielding % RSDs of less than 10% and 7.6%, respectively. Limits of detection (LODs) and quantification (LOQs) for the proposed method using a DAD detector were achieved in the ranges of 0.04–0.63 ng mL<sup>-1</sup> and 0.12–1.9 ng mL<sup>-1</sup>, depending of the capillary column used. Fluorescence detection improved these parameters for E3, BPA and EE2, obtaining LODs of 0.005–0.03 ng mL<sup>-1</sup> and LOQs of 0.015–0.08 ng mL<sup>-1</sup> using Supel-Q and LODs of 0.01–0.015 ng mL<sup>-1</sup> and LOQs of 0.025–0.04 ng mL<sup>-1</sup> using Carboxen. The proposed method was successfully applied to spiked environmental waters obtaining recoveries greater than 80%.

**Keywords:** oestrogens; endocrine disruptor chemicals (EDCs); in-tube SPME; sea water; effluent of wastewater treatment plant; liquid chromatography

### 1. Introduction

Endocrine-disrupting chemicals (EDCs) are a class of emerging environmental contaminants that are extensively and increasingly used in human and veterinary medicine. Natural and synthetic oestrogens such as ethynylestradiol, norethisterone and D-norgestrel, display the strongest oestrogenic effects, although their concentrations are very low in the environment. Their presence in the environment is largely attributable to their presence in wastewater treatment plants (WWTPs). Many of these oestrogens pass through wastewater treatment systems, and they are discharged continuously into

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environmental waters, where they can reach concentrations at the level of nanogram per litre [1–5].

These compounds have been reported to affect ecosystems, e.g., feminisation of wild fish populations living downstream of wastewater effluent. Thus, a rapid, efficient and simple method to determine levels of oestrogenic compounds in environmental water samples is important for monitoring these compounds [6].

In general, environmental waters cannot be analysed without sample pretreatment because analytes in these samples are too diluted or matrix is at trace level too complex. A sample preparation step is necessary to extract organic compounds from the aqueous medium and to bring the concentration of analytes to a suitable level before chromatographic analysis.

Liquid–liquid extraction (LLE) has been used to extract EDCs from environmental waters [7]. However, this method requires a relatively large volume of organic solvents, so it produces larger amounts of toxic wastes. Other pretreatment methods, such as solid-phase extraction (SPE) [8–10] have been widely applied to the extraction of EDCs from this kind of samples. This method still requires time-consuming multi-step sample preparation processes that usually include extraction, elution, evaporation and sample reconstitution steps. High-throughput applications and automated instrumentation are becoming more and more important. Stir bar sorptive extraction (SBSE) [11–13] and in-tube solid phase microextraction (SPME) [14,15] have been developed to advance the analysis method in this direction and increase analysis sensitivity [16].

The in-tube solid phase microextraction technique (in-tube SPME) is a relatively new, solvent free and fast extraction and pre-concentration technique, which allows convenient automation of the extraction process. Automation not only reduces the analysis time, but also provides better accuracy, precision and sensitivity than off-line manual techniques [17]. After 1993, when commercial version of SPME fibre (Supelco) came onto the market, the new innovations inspired by this method were published. Eisert and Pawlitzyn published the first approach to develop an automated in-tube SPME coupled to HPLC [18]. In 1999, Kataoka, Lord and Pawlitzyn reported the automation of in-tube SPME for ranitidine [19]. In-tube SPME was developed for the determination of target compounds in liquid samples and attempts to overcome problems posed by SPME, such as fragility of the capillary, long extraction time and the necessity of additional equipment [20,21]. In-tube SPME requires less complex instrumentation than regular SPME and higher sensitivity can be obtained. In general, in-tube SPME provides larger surface area for extraction by comparison to fibre SPME. In this approach, the analytes are aspirated and directly extracted from the sample into the coated stationary phase of a capillary column operated in the dynamic (draw/eject) or static (kept in capillary) mode and then the analytes are desorbed by introducing a stream of mobile phase (or other desorption reagent) or static desorption solvent when the analytes are more strongly adsorbed onto the capillary coating. This technique is suitable for automation which is a great advantage in the coupling of SPME to an HPLC system. Automated in-tube SPME-HPLC facilitates shortening of the total analysis time, better accuracy and higher precision than manual techniques. The analytical portion of sample determination is usually conducted by gas chromatography (GC) or liquid chromatography (LC) using different detectors including mass spectrometry or tandem mass spectrometry, fluorescence and UV-DAD [14–17,21]. In-tube SPME has been applied in different kind of samples: urine and serum samples [22], herbal medicines [23], food [24] and environmental samples [25,26].



In the present study, we have developed an automated on-line extraction and preconcentration method to achieve high throughput analysis for the determination of some EDCs in environmental water samples because the monitoring of these compounds in the aquatic medium is important in order to establish the adverse biological effects in wildlife, fish and humans. Target oestrogens included natural oestrogens (such as oestriol (E3)), semisynthetic oestrogens (such as 17 $\alpha$ -ethynylestradiol (EE2)) and synthetic oestrogens (such as 19-norethisterone (NORE), D-norgestrel and (levonorgestrel; NORG)) and bisphenol A (BPA). Table 1 shows the structures and properties of the selected EDCs in the present study. Considerable effort has been expended to develop reliable analytical methodologies for analysing them. Developed in-tube SPME is a friendly environmental method that uses small amounts of organic solvents. For this reason, we have evaluated the variables that affect to the process. In this sense, three capillary columns were tested and two of them were used for comparison and evaluation of the most suitable sorbent for extraction of the target compounds. Other parameters that affecting the efficiency of the in-tube SPME extraction were optimised. Based on the obtained results, this method was applied to spiked sea water and wastewater samples from an effluent of WWTPs, employing HPLC with DAD and FL detection to examine matrix effects.

## 2. Experimental

### 2.1 Instrumentation

The HPLC system consisted of a Varian pump fitted with a Varian Autosampler 410 with a volume selector, a Column Valve Module with an internal oven and a Varian photodiode array (PDA) and fluorescence (FL) detectors. The system and the data management were controlled by Star software from Varian (Varian Inc., Madrid, Spain).

### 2.2 Reagents and solutions

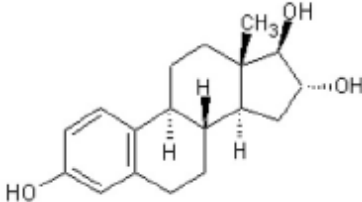
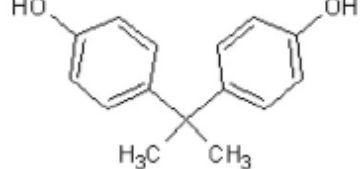
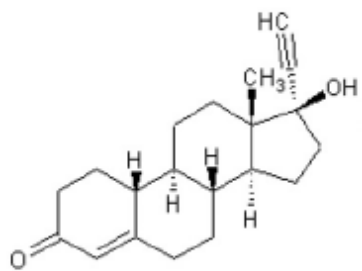
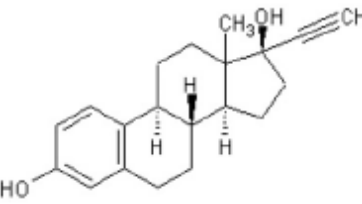
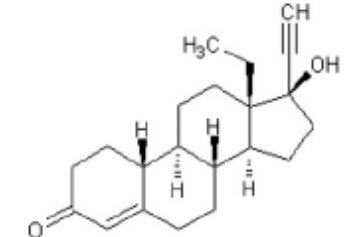
The EDCs selected in the present study, which includes: oestriol (E3), bisphenol A (BPA), 19-norethisterone (NORE), 17 $\alpha$ -ethynylestradiol (EE2) and D-norgestrel (NORG) were purchased from Sigma-Aldrich (Steinheim, Germany). All pharmaceutical standards were 98–99% pure. Individual standard solution of these compounds were prepared in methanol at a concentration of 100  $\mu\text{g mL}^{-1}$  and stored in the dark at 4°C prior to use. Working solutions for experiments were prepared by dilution of the stock solutions with water to a concentration of 1  $\mu\text{g mL}^{-1}$ .

HPLC-grade methanol was obtained from Panreac Quimica S.A. (Barcelona, Spain) and glacial acetic acid glacial was obtained from Scharlau Chemie S.A. (Barcelona, Spain). Ultra-high-quality water obtained from a Milli-Q water purification system (Millipore, USA) was used for all experiments and solutions.

### 2.3 Columns, mobile phase and chromatographic conditions

A NovaPack C<sub>18</sub> (3.9 mm  $\times$  150 mm, 4  $\mu\text{m}$  particle diameter, Waters) column with a column guard of the same sorbent (Waters) was used for separations of the analytes. The analytical column was inserted into the column module at 30  $\pm$  0.2°C.

Table 1. Structure, abbreviations, retention times ( $t_R$ ) and detection wavelength ( $\lambda$ ) used for monitoring oestrogens included in this study.

N°	Structure	Analyte	Abbreviation	$t_R$ (min)	Log $K_{ow}$	$pK_a$	$\lambda$ (nm)
1		Oestriol	E3	5.3	2.5	10.4	224
2		Bisphenol A	BPA	9.8	3.3	9.7	224
3		Norethisterone	NORE	14.2	3.3	13.1	244
4		Ethynylestradiol	EE2	14.9	3.7	10.4	224
5		D-norgestrel	NORG	17.9	3.5	13.1	244



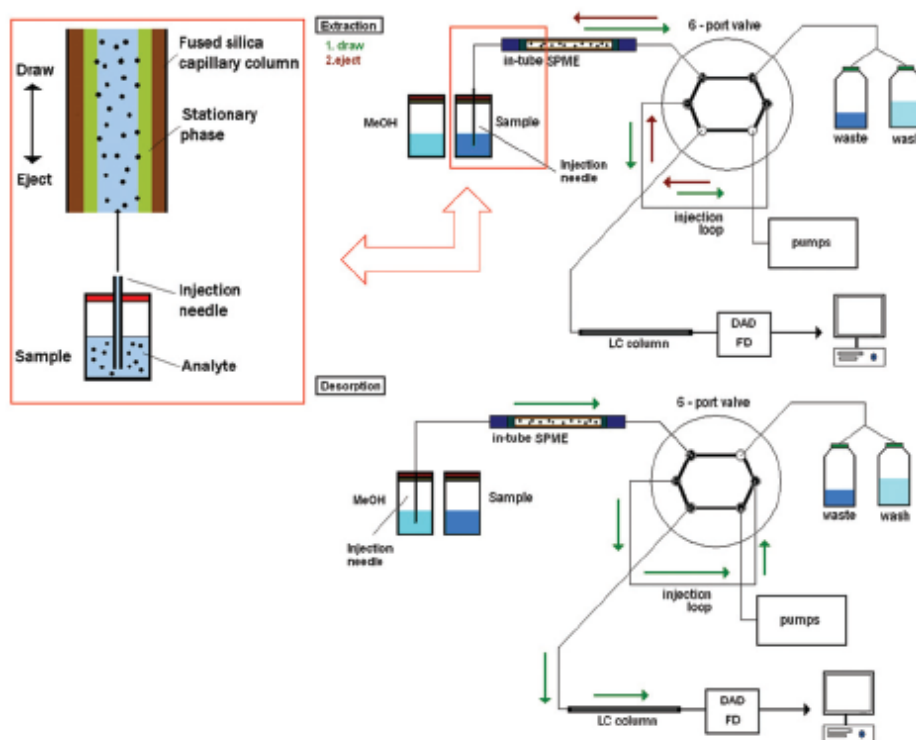


Figure 1. Schematic diagram of the automated in-tube SPME-HPLC system.

To carry out the in-tube SPME, three different capillary columns were used: Supel-Q™ PLOT fused silica capillary column (30 m × 0.32 mm i.d., Supelco, Boston, USA), Carboxen™ 1006 PLOT fused silica capillary column (30 m × 0.32 mm i.d., Supelco, Boston, USA) and a WCOT fused silica CP-Sil 19 CB capillary coating (25 m × 0.32 mm i.d., Varian, Madrid, Spain).

Chromatographic separations of the studied EDCs were conducted using high performance liquid chromatography with UV and fluorescence detectors. Extract samples volumes of 50 µL were injected into the chromatographic system. The mobile phase composition was optimised to achieve the best separation. The eluent was used at a flow rate of 1.0 mL min<sup>-1</sup>; water-methanol at a ratio of 55:45 (v/v) reached 40:60 (v/v) at a time of 15 min, and the eluent was then isocratic up to 20 min. A good separation of all of the analytes was obtained in 20 min of elution time. A wavelength of 224 nm was monitored for E3, BPA and EE2 and a wavelength of 244 nm was monitored for NORE and NORG. The fluorescence detector was operated at an excitation wavelength of 228 nm and an emission wavelength of 315 nm to detect E3, BPA and EE2. The retention times of the analytes are presented in Table 1.

#### 2.4 In-tube solid-phase microextraction

The configuration used for in-tube SPME is shown in Figure 1. The capillary column was placed directly behind the injection needle and in front of the injection valve of the

autosampler. Capillary connections were facilitated by the use of a 4 cm × 1/16 in sleeve. Polyetheretherketone (PEEK) tubing was placed at each end of the capillary. Stainless steel nuts, ferrules and connectors were used to complete the connections.

The autosampler software was programmed to control the in-tube SPME extraction, desorption, and injection. Vials (2 mL) were filled with 1 mL of sample for extraction. Additional vials (2 mL) containing 1.5 mL of methanol were used for desorption of the target compounds. The extraction of the five oestrogens onto the capillary coating (by aspirating of the sample into the injection loop) was performed by 20 or 40 repeated draw/eject cycles of 75 µL or 100 µL of the samples for the Supel-Q or Carboxen columns, respectively, at a flow rate of 0.31 mL/min. The wash step was performed with 30 µL of Milli-Q-water after each draw/eject cycle to remove impurities. The extracted compounds were desorbed from the capillary coating by 50 µL of methanol, transported directly to the LC column and detected by PDA and fluorescence detectors. The capillary column and injection needle were washed in one step and conditioned by five repeated draw/eject cycles (450 µL in total volume) of methanol between each sample extraction. In all cases, a section of 60 cm of each column was used.

### 2.5 Spiking of samples

Sea water samples from a submarine outfall were collected at 70 m from the Jinámar coast (Gran Canaria island, Spain) and stored in pre-cleaned amber glass bottles in the dark at 4°C until analysis. Samples were filtered through a 0.45 µm filter. Samples from a wastewater treatment plant in Las Palmas de Gran Canaria were collected, acidified to a pH < 3, stored at 4°C in 2.5 L glass bottles until extraction. One millilitre of each sample was spiked with the standards prepared in methanol to obtain the final working concentration.

### 2.6 Statistical analysis

Experimental design was performed using Statgraphic Plus Software, version 5.1 (Manugistic, Rozeville, MO, USA). Studies of the partial and bivariate correlations were conducted using SPSS 17.0 (Chicago, Illinois, USA). Profiles of surface response for the extraction of the target compounds were evaluated in Matlab 6.5 (Mathworks, Inc., Natick, Massachusetts, USA).

## 3. Results and discussion

### 3.1 Optimisation of in-tube SPME conditions

The development of the in-tube SPME procedure for target analyte extractions required the testing of several parameters such as the stationary phase of the in-tube SPME capillary column, the sample volume in each draw/eject cycle, the number of draw/eject cycles and the injection volume. In the present study, in tube SPME conditions were optimised with standard solution (1 µg mL<sup>-1</sup> of each target analyte) using an HPLC-DAD.

Previous studies [17,20] recommended an optimal length of capillary column of 50–60 cm to minimise peak broadening in the process. For this reason, we have adopted a length of 60 cm of different commercial columns for our experiments. Moreover, we have selected the best configuration adapted to our equipment because it is not possible

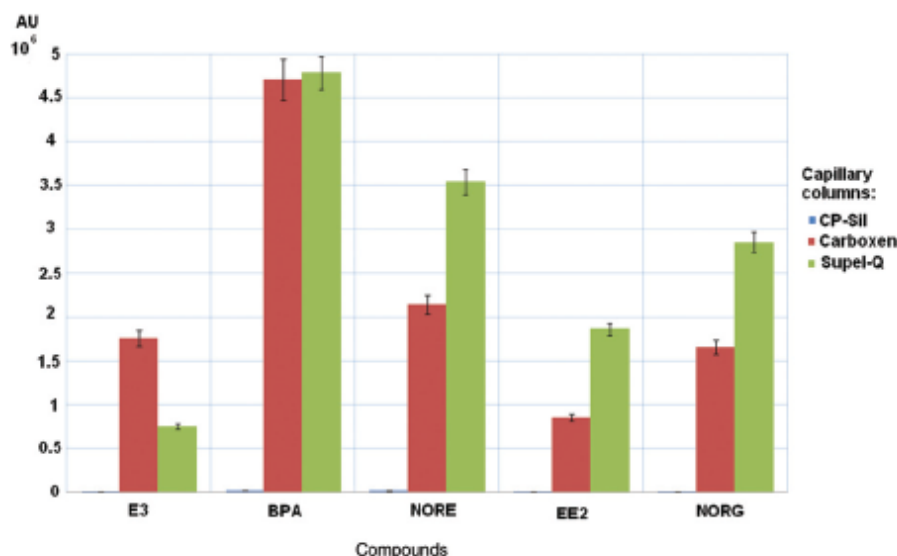


Figure 2. Influence of different capillary coatings in the extraction efficiency of the selected EDCs. Each compound was initially extracted by 14 draw/eject cycles of 90  $\mu\text{L}$  of standard solution ( $1\mu\text{g mL}^{-1}$  of each) at a flow of  $0.31\text{ mL min}^{-1}$  and desorbed with 50  $\mu\text{L}$  of methanol.

to connect the capillary column as described by other authors owing to the system characteristics. With our connections, mobile phase does not pass through the capillary column. This fact increases the life-time of capillary and it does not affect the desorption step.

The preliminary working conditions were established, including a capillary length of 60 cm, a volume of 90  $\mu\text{L}$  per cycle, 14 draw/eject cycles and 50  $\mu\text{L}$  of methanol as desorbent. The draw/eject cycle flow rates was 0.31 mL/min. Using these conditions, three different capillary columns (Supel-Q<sup>TM</sup> PLOT, Carboxen<sup>TM</sup> 1006 PLOT, CP-Sil 19 CB) were evaluated for extraction efficiency. As is shown in Figure 2, the CP-Sil 19 CB capillary column (with a liquid stationary phase of Cyanopropil phenyl-(14%) and methylpolysiloxane (86%)) showed low affinity for the target analytes and it was not suitable for extracting them. This column was removed from further consideration in this study. The Carboxen 1006 (porous carbon molecular sieve) column yielded acceptable results although the Supel-Q PLOT (porous polymer-type capillary) column was the best candidate for extraction of the target analytes, except for E3, because they have a large adsorption surface area. Obtained results with Supel-Q are in agreement with other published papers [17]. Both of these capillary columns were used for the following optimisation steps.

Generally, the extraction efficiency of the analytes into a stationary phase in SPME can be increased by changing the pH and salt concentration of the sample solution. Acidic and basic compounds can be effectively extracted from acidic and alkaline sample solutions, respectively. However, the stability of each compound at the pH of the sample solution must be determined beforehand. EDCs have  $\text{pK}_{\text{a}}$ s around 10.0 and pH conditions of less than this value ensure that all compounds are in the neutral form, which are optimal for their extraction.

Table 2. Design matrix of  $3^2$  factorial with duplicated central points established to optimisation of this study.

Run number	Number draw/ eject cycles	Sample volume (mL)
1	20	50
2	20	75
3	20	100
4	40	50
5	40	75
6	40	75
7	40	75
8	40	100
9	60	50
10	60	75
11	60	100

Theoretically, the addition of salt to aqueous samples increases the coating-matrix distribution constant of neutral organic molecules. This so-called ‘salting-out’ effect in which the increase of salt reduces the water concentration because the water molecules form hydration spheres around the ionic salt molecules and thus, causes the hydrophobicity of the chemicals. However, the salt deposits can clog the column during in-tube SPME. The salt content was studied in the range of 0% to 15% NaCl (w/v) of the sample matrix. The extraction efficiency was not significantly affected by salt addition. Thus, extractions were made at the sample pH (pH 5.5) and no additional NaCl was used.

The extraction time, including the number of draw/eject cycles and the sample volume were evaluated using a  $3^2$  factorial design with duplicate central points (Table 2), in which both variables are represented against response. The number of draw/eject cycles was varied from 20 to 60 cycles and the sample volume was varied from 50 to 100  $\mu\text{L}$ . Figure 3 shows the response surface for (a) Supel-Q and (b) Carboxen capillary columns for EE2. The best responses were obtained with 20 draw/eject cycles and 75  $\mu\text{L}$  of sample volume for Supel-Q and 40 draw/eject cycles and 100  $\mu\text{L}$  of sample volume for Carboxen. Similar results were obtained for the rest of the target analytes, except for BPA and E3. Best responses for BPA were obtained with 60 draw/eject cycles and 100  $\mu\text{L}$  of sample volume for Supel-Q and 20 draw/eject cycles and 75  $\mu\text{L}$  of sample volume for Carboxen. Best results for E3 were obtained with 20 draw/eject cycles and 100  $\mu\text{L}$  of sample volume for Supel-Q and 40 draw/eject cycles and 50  $\mu\text{L}$  of sample volume for Carboxen.

The final optimal conditions for the selected EDCs were 20 draw/eject cycles with 75  $\mu\text{L}$  of sample volume for Supel-Q column and 40 draw/eject cycles with 100  $\mu\text{L}$  of sample volume for Carboxen column.

The typical chromatograms of the five selected EDCs obtained by in-tube SPME-HPLC-DAD under the above optimised conditions and direct injection into the LC column are shown in Figure 4. Preconcentration factors were obtained from peak areas provided by absolute amounts injected. Depending on the compound and considering that the vial contained 1 mL of sample and the volume of each draw/eject cycle was 50  $\mu\text{L}$ , the



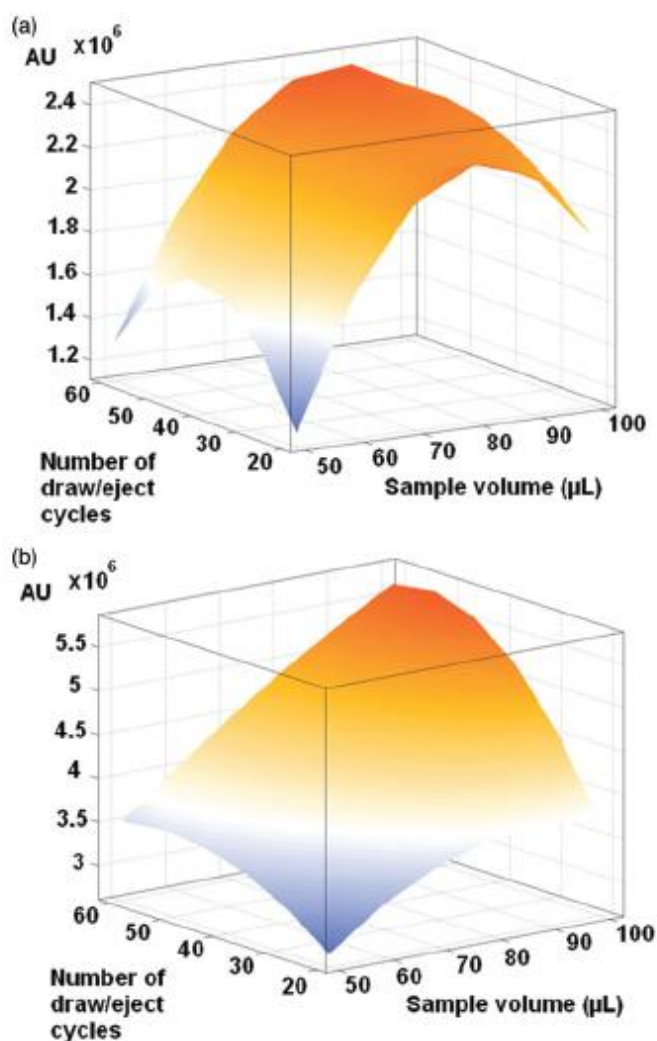


Figure 3. Response surface for optimal conditions of EE2: (a) Supel-Q column and (b) Carboxen column. Standard solution contained  $1\mu\text{g mL}^{-1}$  of each oestrogen at a flow of  $0.3\text{ mL min}^{-1}$  and desorbed with  $50\mu\text{L}$  of methanol.

in-tube SPME method using the Supel-Q column presented a preconcentration factor between 5 and 39 and a preconcentration factor between 10 and 23 was obtained for the Carboxen capillary column.

### 3.2 Analytical performance characteristics

Calibration curves were constructed using different concentration levels ranging from  $1\text{--}1000\text{ ng mL}^{-1}$  (HPLC-DAD) and  $0.1\text{--}100\text{ ng mL}^{-1}$  (HPLC-FLD). The linearity of the method was evaluated using the correlation coefficients of determination ( $R^2$ ). In all cases, the  $R^2$  values were higher than 0.9970.

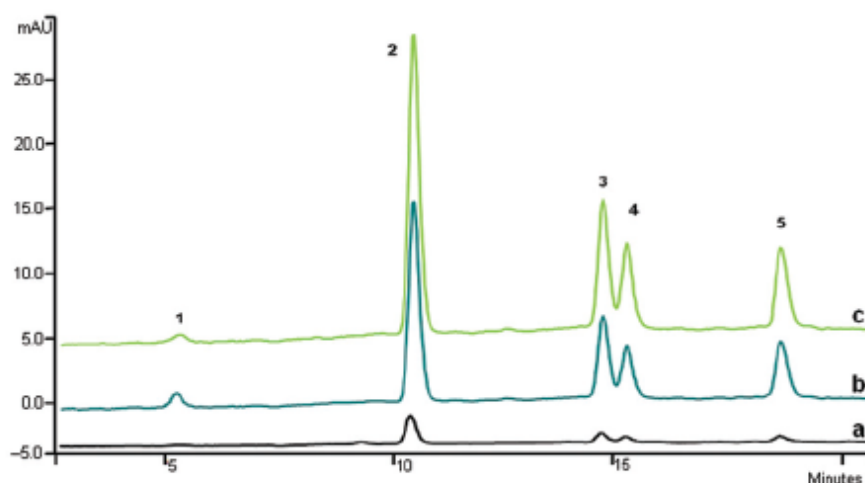


Figure 4. Chromatograms of the selected EDCs obtained by in-tube SPME-HPLC-DAD with direct injection (a) using Carboxen column (b) and using Supel-Q column (c), in optimal conditions. Standard solution contained  $500 \text{ ng mL}^{-1}$  of each target analyte in Milli-Q water. The numbering refers to Table 1.

The reproducibility of this method was determined by inter-day and intra-day precision. As shown in Table 3, excellent method reproducibility was found for both capillary columns: intra-day % RSDs of less than 7.6% and 11.5% and inter-day % RSDs of less than 9.6% and 6.2% were obtained for the Carboxen and Supel-Q columns, respectively.

Limits of detection (LODs) and quantification (LOQs) were determined according Taverniers *et al.* [27]. LODs and LOQs signals were obtained using ten independent sample blanks and calculated as signal to noise ratio of 3 or 10, respectively. The data are displayed in Table 3. LODs and LOQs for the proposed method using a DAD detector were achieved in the range of  $0.04\text{--}0.63 \text{ ng mL}^{-1}$  and  $0.12\text{--}1.9 \text{ ng mL}^{-1}$  using Supel-Q and  $0.04\text{--}0.46 \text{ ng mL}^{-1}$  and  $0.12\text{--}1.4 \text{ ng mL}^{-1}$  using Carboxen. Fluorescence detection improved these parameters for E3, BPA and EE2: LODs of  $0.005\text{--}0.03 \text{ ng mL}^{-1}$  and LOQs of  $0.015\text{--}0.08 \text{ ng mL}^{-1}$  were obtained using the Supel-Q; LODs of  $0.01 \text{ ng mL}^{-1}$  for BPA and EE2, LODs of  $0.015 \text{ ng mL}^{-1}$  for E3 and LOQs of  $0.025\text{--}0.04 \text{ ng mL}^{-1}$  were obtained using Carboxen. The results that were obtained with in-tube SPME and fluorescence detection were similar to those obtained by other authors with similar methods [28] or with mass spectrometry detection for E3, BPA and EE2 [17] in environmental waters. Other methodologies have been optimised for some oestrogens in water samples and the results obtained were similar to those found in this work [29]. Only better results were obtained using GC-MS but with derivatisation [30,31].

The results obtained using the optimised method was compared with those obtained using a SPE extraction procedure [32]. Table 4 shows the average recoveries and standard deviations obtained over two independent extractions applying both methodologies. It was observed that the results are similar.

Table 3. Analytical parameters obtained for the selected oestrogens by in-tube SPME using different detection techniques.

Analyte	Carboxen											
	UV-DAD				Fluorescence				Precision			
	LOD <sup>a</sup>	LOQ <sup>b</sup>	LOD <sup>a</sup>	LOQ <sup>b</sup>	LOD <sup>a</sup>	LOQ <sup>b</sup>	In-day <sup>c</sup>	Intra-day <sup>d</sup>	LOD <sup>a</sup>	LOQ <sup>b</sup>	LOD <sup>a</sup>	LOQ <sup>b</sup>
E3	0.21	0.63	0.015	0.04	0.63	1.89	6.3	6.6	0.03	0.08	4.6	4.9
BPA	0.04	0.12	0.01	0.025	0.04	0.12	5.1	5.3	0.008	0.02	3.5	2.0
NORE	0.04	0.12	nd	nd	0.05	0.15	5.3	2.8	nd	nd	3.1	11.5
EE2	0.46	1.38	0.01	0.03	0.22	0.66	6.4	6.2	0.005	0.015	6.2	4.4
NORG	0.05	0.15	nd	nd	0.05	0.15	9.6	7.6	nd	nd	5.8	2.7

<sup>a</sup>Detection limits are calculated as signal to noise ratio of 3 (in ng mL<sup>-1</sup>); <sup>b</sup>Quantification limits are calculated as signal to noise ratio of 10 (in ng mL<sup>-1</sup>); <sup>c</sup>In-day precision, %RSD (*n* = 6; 200 ng mL<sup>-1</sup> for E3 and 100 ng mL<sup>-1</sup> for the rest); <sup>d</sup>Intra-day precision, %RSD (*n* = 6; 200 ng mL<sup>-1</sup> for E3 and 100 ng mL<sup>-1</sup> for the rest); <sup>nd</sup>Non detected in working conditions ( $\lambda_{em}$  = 228 nm;  $\lambda_{exc}$  = 315 nm).

Table 4. Comparison of in-tube SPME and SPE procedures to a Milli-Q water solution containing 50 ng mL<sup>-1</sup> of target analytes.

Analyte	% Recovery*		
	In-tube SPME		SPE [32]
	Carboxen	Supel-Q	
E3	99.7 ± 4.4	84.2 ± 1.9	96.7 ± 6.5
BPA	100.6 ± 7.0	98.8 ± 4.5	94.8 ± 2.8
NORE	90.5 ± 8.6	81.4 ± 2.8	103.9 ± 0.8
EE2	81.6 ± 1.6	80.9 ± 1.4	83.9 ± 2.4
NORG	90.0 ± 5.4	80.0 ± 3.4	98.2 ± 0.2

\*n = 2.

### 3.3 Application to the analysis of oestrogens in environmental waters

To study matrix effects of the optimised in-tube SPME, this method was used to extract and determine the selected EDCs in several spiked water samples from sea water and WWTP origins to confirm its applicability and feasibility. Non-spiked real samples were run using in-tube SPME-HPLC-FLD with both capillary columns as described. EDCs were not detected at the retention time of the compounds under study. Matrix effects were studied comparing the response of the analytes in net solution to the response of the analytes spiked into a blank matrix sample that was carried through in-tube SPME process. Known amounts of five EDCs were spiked into each water sample and their recoveries were calculated. Extraction efficiency was not significantly affected by salt addition and matrix effects were not observed. Recoveries obtained in all samples, including sea water samples, were better than 80%.

HPLC chromatograms of WWTP samples are shown in Figure 5 and the analysis results are outlined in Table 5. As shown in this table, the recoveries of these compounds were 84–106% in sea water samples and 80–96% in WWTP effluent samples using the Carboxen capillary column with DAD detector. The recoveries of these compounds were 85–94% in sea water and 90–102% in WWTP effluent samples using the Supel-Q column and the same detector. Similar results were obtained with the fluorescence detector.

## 4. Conclusions

Three different capillary columns (Supel-Q<sup>TM</sup> PLOT, Carboxen<sup>TM</sup> 1006 PLOT, CP-Sil 19 CB) were evaluated to optimise an automated in-tube SPME method for extraction of some EDCs from aqueous samples. CP-Sil 19 CB capillary column (with a liquid stationary phase of Cyanopropil phenyl-(14%) and methylpolysiloxane (86%)) showed low affinity for the target analytes.

Carboxen and Supel-Q capillary columns presented the best results and they were used in an in-tube SPME-HPLC process to extract and determine oestriol (E3), bisphenol A (BPA), 19-norethisterone (NORE), 17 $\alpha$ -ethynylestradiol (EE2) and D-norgestrel (NORG) in aqueous samples using in-tube solid phase microextraction



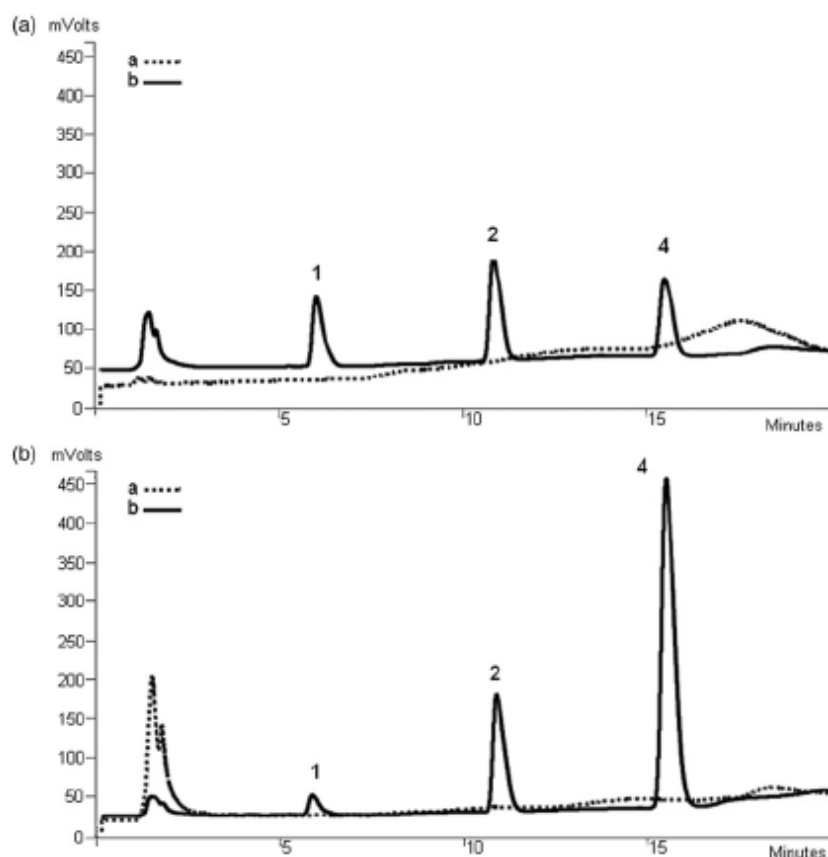


Figure 5. Chromatograms of the selected EDCs obtained by in-tube SPME-HPLC-FLD with (a) Carboxen column (b) Supel-Q column for (a) non-spiked and (b) spiked with  $50 \text{ ng mL}^{-1}$  of each target analyte real WWTPs sample. The numbering refers to Table 1.

Table 5. Recovery percentages ( $n=3$ ) obtained for sea water and WWTP effluent samples spiked with  $50 \text{ ng mL}^{-1}$  of selected oestrogens using Carboxen and Supel-Q capillary columns.

Analyte	UV-DAD		Fluorescence	
	Sea water	Effluent WWTP	Sea water	Effluent WWTP
<b>Carboxen</b>				
E3	$96.7 \pm 3.7$	$92.2 \pm 8.3$	$101.4 \pm 3.4$	$80.2 \pm 5.6$
BPA	$96.8 \pm 2.5$	$92.2 \pm 2.7$	$98.9 \pm 3.2$	$95.9 \pm 2.5$
NORE	$85.5 \pm 3.0$	$93.9 \pm 4.6$	nd	nd
EE2	$84.22 \pm 1.9$	$87.4 \pm 2.0$	$85.8 \pm 3.0$	$85.2 \pm 1.4$
NORG	$88.0 \pm 2.7$	$85.0 \pm 4.1$	nd	nd
<b>Supel-Q</b>				
E3	$86.2 \pm 9.2$	$88.0 \pm 2.5$	$75.3 \pm 2.3$	$59.4 \pm 6.0$
BPA	$85.2 \pm 2.0$	$96.0 \pm 2.9$	$87.1 \pm 3.1$	$91.1 \pm 1.6$
NORE	$92.5 \pm 4.0$	$90.5 \pm 2.4$	nd	nd
EE2	$93.6 \pm 2.2$	$102.5 \pm 4.9$	$91.8 \pm 2.7$	$99.4 \pm 4.4$
NORG	$92.5 \pm 3.8$	$90.2 \pm 1.9$	nd	nd

coupled to HPLC (in-tube SPME-HPLC). Different steps were completely automated and the proposed method used diode array (DAD) and fluorescence (FLD) detection, which allowed the identification of these compounds in environmental water samples. The proposed method is simple and precise and used conventional equipment. Moreover, this method is also environmentally friendly and inexpensive owing to its low solvent volume extraction technique. Therefore, the proposed method could be useful and practical in the screening and determination of these EDCs in environmental water samples.

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1 **6.8 Supplement V**

2

3 Jana Aufartová, María Esther Torres-Padrón, Zoraida Sosa-Ferrera , Lucie Nováková, Petr Solich,

4 José Juan Santana-Rodríguez

5 **Development of a novel in-tube solid phase microextraction based on micellar desorption**  
6 **followed by LC-DAD-FD for the determination of some endocrine disruptor compounds**  
7 **(EDCs) in environmental liquid samples**

8 Chromatographia – sent

9

## Chromatographia

# DEVELOPMENT OF A NOVEL IN-TUBE SOLID PHASE MICROEXTRACTION BASED ON MICELLAR DESORPTION FOLLOWED BY LC-DAD-FD FOR THE DETERMINATION OF SOME ENDOCRINE DISRUPTOR COMPOUNDS (EDCs) IN ENVIRONMENTAL LIQUID SAMPLES

--Manuscript Draft--

<b>Manuscript Number:</b>	
<b>Full Title:</b>	DEVELOPMENT OF A NOVEL IN-TUBE SOLID PHASE MICROEXTRACTION BASED ON MICELLAR DESORPTION FOLLOWED BY LC-DAD-FD FOR THE DETERMINATION OF SOME ENDOCRINE DISRUPTOR COMPOUNDS (EDCs) IN ENVIRONMENTAL LIQUID SAMPLES
<b>Article Type:</b>	Original
<b>Keywords:</b>	endocrine disruptor compounds (EDCs); in-tube solid phase microextraction; micellar desorption; environmental liquid samples.
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<b>Order of Authors Secondary Information:</b>	
<b>Abstract:</b>	An innovative methodology based on in-tube SPME using micellar desorption coupled to liquid chromatography combined with diode array (DAD) and fluorescence (FD) detection is proposed for the determination of five endocrine disruptors compounds (estriol, bisphenol A, norethisterone, 17 $\alpha$ -ethynylestradiol and D-norgestrel) in environmental samples. For the first time, in the present study, a micellar desorption was used with a GC Supel-QTM capillary column with a length of 80 cm to optimize the in-tube SPME method. To identify the optimal extraction conditions, several parameters, including the extraction time, desorption time, column length, desorption volume, sample volume and number of injections, were investigated. Under the optimal extraction conditions, micellar desorption was studied as an alternative to organic eluents. Using a DAD, the limits of detection (LODs) and quantification (LOQs) for the proposed method were 0.03-2.0 ng mL <sup>-1</sup> and 0.1-6 ng mL <sup>-1</sup> respectively. In contrast, using a FD detector, the LODs and LOQs were 0.02-0.14 ng mL <sup>-1</sup> and 0.06-0.4 ng mL <sup>-1</sup> for estriol, bisphenol A and 17 $\alpha$ -ethynylestradiol, respectively. The proposed method was successfully applied to environmental matrices (seawater and wastewater from a wastewater treatment plant) and relative recoveries greater than 86% were obtained in all cases.

1 DEVELOPMENT OF A NOVEL IN-TUBE SOLID PHASE MICROEXTRACTION  
2 BASED ON MICELLAR DESORPTION FOLLOWED BY LC-DAD-FD FOR THE  
3 DETERMINATION OF SOME ENDOCRINE DISRUPTOR COMPOUNDS (EDCs)  
4 IN ENVIRONMENTAL LIQUID SAMPLES

5

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15

16 **Abstract**

17 An innovative methodology based on in-tube SPME using micellar desorption coupled  
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19 detection is proposed for the determination of five endocrine disruptors compounds  
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33 all cases.

34

1 *Keywords:* endocrine disruptor compounds (EDCs), in-tube solid phase microextraction,  
2 micellar desorption, environmental liquid samples.

### 3 **1. Introduction**

4 The endocrine system is an integrated system that controls the cell function and  
5 activities of mammals, amphibians, birds, fish, and various invertebrates by  
6 communicating through chemical messengers. Pollutants called endocrine disruptor  
7 chemicals (EDCs) can present a mimetic behaviour as endogenous regulators and  
8 change the outcome of reproduction, growth and development.

9 EDCs enter in the natural environment by different ways, through municipal and  
10 industrial wastewater [1-3]. Laboratory studies have shown that these EDCs exert  
11 potent estrogenic effects at low concentrations in water. These compounds are  
12 composed of varieties of industrial and household chemicals such as chlorinated  
13 insecticides, polychlorinated biphenyls, bisphenol A (BPA), alkylphenols and their  
14 ethoxylates derivatives, and steroid oestrogens.

15 Especially, steroid hormones and contraceptives are of special concern due to  
16 their endocrine potency. Steroid hormones that are biosynthetically present in the  
17 body are called endogenous hormones, while exogenous steroids are foreign  
18 compounds, either naturally or synthetically produced [4]. Some of them, like estriol  
19 (E3) and 17 $\alpha$ -ethynylestradiol (EE2), enter in wastewater treatment plants (WWTPs)  
20 after their excretion in urine and they persist in water, due to their incomplete removal  
21 from wastewater treatment plants (WWTPs) [1]. The development of sensitive  
22 analytical methods to access the presence in water of these compounds is therefore  
23 required.

24 Current standard methods for EDCs analysis in water samples are primarily based  
25 on solid phase extraction (SPE) followed by liquid chromatography (LC) or gas  
26 chromatography (GC), which usually requires a prior derivatization [5-10]. However,  
27 the sample preparation process often miniaturised because solution must provide high  
28 performance, rapid analysis with low operating costs and limited environmental  
29 pollution [11]. Conventional extraction techniques such as an on-line continuous liquid-  
30 liquid extraction with dichloromethane coupled to a GC-MS [12] and a  
31 chemiluminescent recombinant yeast assay based method have been published [13].  
32 Liquid microextraction techniques have been used in environmental samples. In this



1 sense, there is a hollow-fiber liquid phase microextraction (HF-LPME) combined with  
2 GC-MS method for the determination of steroid hormones like 17 $\beta$ -estradiol in tap and  
3 sewage water samples [14] or followed by LC-UV for the determination of some  
4 synthetic estrogens like diethylstilbestrol [15]. Also, an automated solid-phase  
5 microextraction (SPME) method has been tested for steroid hormones analysis in  
6 environmental aqueous samples with simultaneous derivatisation and GC-MS analysis  
7 [16]. Some studies using stir bar sorptive extraction (SBSE) have been presented using  
8 HPLC-DAD [17] or GC-MS [18] in the final analysis. Another promising method to  
9 reduce solvent volume is in tube solid-phase microextraction (in tube SPME).

10 In-tube SPME is a simple microextraction technique which can be easily coupled to  
11 a LC system. This technique uses a coated open capillary column as SPME device and  
12 has received growing interest from many areas, such as the determination of  
13 environmental samples [19-21], food samples [22-25], and biological and  
14 pharmaceutical samples [26-27] because it integrates sample extraction, concentration and  
15 injection into one step.

16 Until now, to the best of our knowledge, in-tube SPME for the determination of  
17 EDCs has only been performed using desorption by organic solvents or a mobile phase  
18 [20,28,29]. An alternative to the use of organic solvents is the application of micellar  
19 media. Surfactants or surface-active agents are amphipathic substances that can  
20 associate to form molecular aggregates, known as micelles, in aqueous solution.

21 Special features of micellar systems like high capacity for solubilising compounds  
22 from different types of matrices, low cost and low toxicological effects have done that  
23 they are considered environmental friendly solvent for the extraction step [30].  
24 Specifically, non-ionic surfactants have been widely used to extract organic substances  
25 from different types of matrices, their potential as extractants has been demonstrated  
26 [31-33]. Moreover, non-ionic surfactants are compatible with aqueous-organic mobile  
27 phases in chromatographic analysis, which facilitates these applications.

28 In the present study, extraction parameters including the extraction time, sample  
29 volume and capillary column length were optimized. Variables that affect the desorption  
30 efficiency with micellar media (desorption time, desorption volume or concentration of  
31 desorbent) were also studied. In most practical studies, optimization is based on  
32 systematic studies, and one factor is investigated -at-a-time. With this type of  
33 optimization, possible interactions between factors are not considered. The experimental

1 design methodology is the best choice for evaluating a large number of parameters and  
2 optimizing experimental conditions. Experimental design methodology includes a set of  
3 experiments that allows all of the parameters to be varied at the same time, thereby  
4 minimizing the number of experiments and allowing all of the possible interactions to  
5 be considered [34-36].

6 The extracted samples were analysed using LC with DAD and FD detection.  
7 The optimal procedures were applied to the analysis of E3, BPA, NORE, EE2 and  
8 NORG in environmental water samples.

## 9 **2. Experimental**

### 10 **2.1. Instrumentation**

11 The LC system consisted of a Waters pump (model 510) fitted with a Rheodyne  
12 injector valve (model 7725i) with a 20 µl sample loop, a Waters 2996 photodiode array  
13 and a Waters 474 scanning fluorescence detector. The system and data management  
14 were controlled by Empower software from Waters.

### 15 **2.2. Reagents and solutions**

16 Endocrine disruptor compounds evaluated in the present study, including estriol  
17 (E3), bisphenol A (BPA), norethisterone (NORE), 17 $\alpha$ -ethynylestradiol (EE2) and D-  
18 norgestrel (NORG), were purchased from Sigma–Aldrich (Steinheim, Germany). All  
19 pharmaceutical standards were 98–99% pure. Individual standard solutions of the  
20 aforementioned compounds were prepared in methanol at a concentration of 100 µg·mL<sup>-1</sup>  
21 and were stored in the dark at 4 °C prior to use. Working solutions were prepared by  
22 diluting the stock solutions with water, and a concentration of 1 or 0.5 µg·mL<sup>-1</sup> was  
23 used for the experiments. Non-ionic surfactants, including polyoxyethylene 10 lauryl  
24 ether (POLE) and oligoethylene glycol monoalkyl ether (Genapol), were obtained from  
25 Sigma-Aldrich (Madrid, Spain) and were prepared in ultra-high-quality water. HPLC-  
26 grade methanol was obtained from Panreac Química S.A. (Barcelona, Spain). Ultra-  
27 high-quality water obtained from a Milli-Q water purification system (Millipore, USA)  
28 was used throughout the study.

### 2.3. Column, mobile phase and chromatographic conditions

A NovaPack C<sub>18</sub> (3.9 mm x 150 mm, 4 µm particle diameter, Waters) column and a guard column with the same sorbent (Waters) were used to separate the analytes. The analytical column was stored at room temperature.

The used mobile phase composition consisted of water and methanol (42:58) in isocratic mode for up to 30 minutes at a flow rate of 0.5 mL·min<sup>-1</sup>. Good separation of the analytes was obtained within 30 min. Table 1 shows the characteristics of detection of each selected compound for the present study.

#### *In-tube solid-phase microextraction procedure.*

An 80 cm GC Supel-Q<sup>TM</sup> fused silica capillary column was used as the extracting phase and injection loop. The capillary was placed in the high-pressure six-port valve and was used to replace the injection loop. The system was operated manually. Capillary connections were facilitated using 4 cm sleeve of 1/16 in. polyetheretherketone (PEEK) tubing at each end of the capillary. Stainless steel nuts, ferrules and connectors were used to complete the connection. Figure 1 shows a schematic diagram of the flow for the in-tube SPME preconcentration device and the connection to the analytical column.

In the load position of the valve, 65 µL of standard or sample solutions were manually injected into the capillary column and were retained in the column for 2 minutes. This process was repeated in 12 cycles. After extraction, desorption was achieved by adding 25 µL of methanol or 20 µL of Genapol (0.1%, v/v) to the load position of the injector. After 1 minute of desorption, the valve was rotated to the inject position, and the eluent was transported directly to the LC column by the mobile phase and was detected by DAD and FD. After each sample, the capillary column was washed and conditioned with 0.5 mL of methanol and 0.5 mL of MilliQ-water.

### 2.4. Spiking of the samples

Samples from a submarine outfall (seawater samples) were collected at a distance of 70 m from Jinámar Coast (Gran Canaria island, Spain) and were stored in pre-cleaned amber glass bottles in the dark at 4°C until analysis. Samples were filtered through a 0.45 µm filter. Samples from a wastewater treatment plant in Las Palmas de Gran Canaria (Spain) were collected, acidified to pH < 3, and stored at 4°C in 2.5 L glass bottles until extraction. Each sample was spiked with 100 ng mL<sup>-1</sup> of the standard in

1 methanol to obtain the final working concentration. Under these conditions, the samples  
2 were directly analyzed by in-tube SPME-LC.

### 3 **2.5. Experimental design and Statistical analysis**

4 The experimental design was performed using Statgraphics Plus graphic software,  
5 version 5.1 (Manugistic, Rockville, MD, USA). Partial and bivariate correlation studies  
6 were conducted using SPSS 17.0 (Chicago, Illinois, USA). The surface response profile  
7 for the extraction of target compounds was evaluated in Matlab 6.5. (Mathworks Inc.,  
8 Natick, Massachusetts, USA).

## 9 **3. Results and Discussion**

### 10 **3.1. Optimization of in-tube SPME conditions**

11 The in-tube SPME was developed to overcome problems related to the use of  
12 conventional fiber SPME, including fragility, low sorption capacity and bleeding from  
13 thick film coatings [28]. The development of in-tube SPME for target analyte extraction  
14 required the study of several parameters, including the extraction and desorption time,  
15 sample and desorbent volume, length of capillary, number of cycles and type of  
16 desorption reagent. In the present study, in-tube SPME conditions were optimized using  
17 standard solutions containing  $1 \mu\text{g}\cdot\text{mL}^{-1}$  of each target analyte.

18 In-tube SPME is typically performed using a piece of a fused-silica capillary with  
19 a stationary phase coated on its inner surface (e.g., a short piece of a GC column).  
20 Kataoka et al. [28] and Mitani et al. [25, 29, 37] found that a Supel-Q-PLOT capillary  
21 was more efficient for the analysis of steroid compounds. Therefore, we chose initially a  
22 Supel-Q capillary with a length of 60 cm and used methanol as the elution reagent in a  
23 single cycle for the initial optimization.

24 Different experimental design approaches can be used for the optimization of an  
25 analytical method. A screening process can be performed to study a large number of  
26 factors that affect the response signal, and the optimal level of factors can be determined  
27 to obtain the best response surface [26]. Thus, we planned different experimental  
28 designs to obtain the optimal conditions for the in-tube procedure.

#### 29 **3.1.1. Experiment design – screening**

30 A  $2^K$  design (K, factor number, two levels) is useful in the early stages of  
31 experimental work and provides the smallest number of runs with K factors studied in a

1 complete factorial design. Because there are only two levels for each factor, the  
2 response is approximately linear over the range of levels.

3         The first stage of the in-tube SPME parameter optimization study consisted of a  
4  $2^4$  factorial design (two levels and four variables), which the design consisted of 16  
5 runs. The variables under consideration included the extraction time (10 and 30 min),  
6 desorption time (10 and 30 min), standard solution volume (25-50  $\mu\text{L}$ ) and desorption  
7 solution volume (25-50  $\mu\text{L}$ ). By applying a factorial design, the influence of each  
8 variable on the analyte recovery rate could be determined. Partial and bivariate  
9 correlations between dependent and independent variables were investigated and are  
10 shown in Table 2. For all of the compounds, the strongest correlations were obtained for  
11 the sample volume. The E3 and EE2 desorption volume negatively affected the  
12 extraction; however, we believe that this result was caused in part by the shape of the  
13 peak because the corresponding recoveries were on the same level. For the other  
14 variables (extraction and desorption time), the results showed only a slight dependence.  
15 According to the aforementioned results, an extraction time of 10 minutes, a desorption  
16 time of 10 minutes, a sample volume of 50  $\mu\text{L}$  and a desorption volume of 25  $\mu\text{L}$  were  
17 used in subsequent studies.

### 18                 3.1.2. Selection of the capillary column length

19         The optimal length of a capillary column ranges from 20 to 100 cm and the  
20 internal diameter of a capillary used in combination with a LC varies between 0.25 and  
21 0.32 mm [28]. Under the conditions outlined in section 3.1.1. (extraction time of 10  
22 min, desorption time of 10 min, standard volume of 50  $\mu\text{L}$ , 25  $\mu\text{L}$  of methanol as the  
23 desorbent), we studied capillary lengths of 40, 60 and 80 cm. The results showed that a  
24 capillary length of 40 cm presented technical problems, like the capillary break, that led  
25 to a short lifetime. Capillary lengths of 60 and 80 cm were tested, and significant  
26 differences were not observed. However, a capillary length of 80 cm permitted a greater  
27 sample volume, which improved the results and it did not affect to the desorption  
28 efficiency. Therefore, a capillary column with a length of 80 cm was selected.

### 29                 3.1.3. Optimization of extraction time and sample volume

30         After optimizing the capillary length, a  $3^2$  experimental design (two variables  
31 and three levels) was performed, and the extraction time (2, 6, and 10 min) and  
32 sample/standard solution volume (35, 50 and 65  $\mu\text{L}$ ) were evaluated using surface  
33 response methodology. The experiments were performed at a desorption time of 10 min,

1 and 25  $\mu\text{L}$  of methanol was used as the desorbent. Figure 2 shows the responses  
2 obtained for NORE (A) and EE2 (B). In all cases, the best results were obtained with 65  
3  $\mu\text{L}$  of sample/standard solution at an extraction time of 2 min.

4 Then, the desorption time (1, 2, and 10 min) was studied under the optimal  
5 extraction conditions. The results obtained at a desorption time of 1 min were not  
6 significantly different from those obtained at a desorption time of 2 and 10 min.  
7 Therefore, the optimal in-tube SPME conditions for a single cycle were as follows:  
8 capillary column length of 80 cm, extraction time of 2 min, sample volume of 65  $\mu\text{L}$   
9 and desorption time of 1 min.

#### 10 3.1.4. Optimization of cycle number

11 After performing the single cycle optimization of in-tube SPME using methanol  
12 as the desorbent agent, the number of cycles in the extraction step was increased to  
13 improve the analytical signal.

14 Under the optimal conditions, experiments were performed in 1, 2, 4, 8, 12 and  
15 15 cycles, and 65  $\mu\text{L}$  of sample was used in each cycle. As shown in Figure 3, as the  
16 number of cycles increased, the analyte signal also increased. The best signals were  
17 obtained when 12 or more cycles were conducted. However, the improvement in the  
18 results obtained with 15 cycles compared with 12 cycles was not sufficient to  
19 compensate for the increased extraction time. Thus, in subsequent experiments, 12  
20 cycles were performed.

#### 21 3.1.5. Selection of micellar media as the desorbent agent

22 The selection of an appropriate desorption solvent is a very important for in tube  
23 SPME. Micellar media can be used as an alternative to organic solvents for desorption  
24 and have been used as desorbents in previous studies [31-33]. Under the optimal  
25 conditions for the proposed conventional in-tube SPME method, we replaced methanol  
26 with more environmentally friendly eluents in the desorption step. In this work, two  
27 non-ionic surfactants were selected instead of organic solvent, including POLE  
28 (polyoxyethylene 10 lauryl ether) and Genapol (oligoethylene glycol monoalkyl ether).  
29 The experiments were conducted under the following conditions: capillary column  
30 length of 80 cm, 12 cycles, standard solution volume of 65  $\mu\text{L}$ , extraction time of 2 min,  
31 desorbent agent volume of 25  $\mu\text{L}$  and a desorption time of 1 min. Both of the surfactants  
32 were used at a concentration of 1% (v/v). Although comparable results were obtained

1 with Genapol and POLE, Genapol provided lower relative deviations for all of the target  
2 compounds. Therefore, Genapol was used in further research.

### 3 3.1.6. Optimization of micellar desorption

4 Variables that affect the desorption step were studied using surface response  
5 methodology and a  $3^2$  factorial design. The surfactant percentage (0.1, 0.55, and 1% v/v)  
6 and desorption volume (10, 15, and 20  $\mu\text{L}$ ) were selected as variables. In general, the  
7 highest surface response was achieved when the lowest concentration of Genapol  
8 (0.1%, v/v) and a desorption volume of 20  $\mu\text{L}$  were employed. The surface response of  
9 NORE and EE2 is shown in Figure 4.

10 In agreement to the obtained results, the optimal conditions for in-tube SPME  
11 using micellar desorption with Genapol were as follows: capillary column length of 80  
12 cm, 12 cycles, sample standard solution volume of 65  $\mu\text{L}$  for each cycle, extraction time  
13 of 2 min, desorption time of 1 min, 20  $\mu\text{L}$  of 0.1% (v/v) of Genapol as the desorption  
14 reagent.

15 Figure 5 shows the chromatograms obtained from the direct injection of target  
16 analytes in a solution of methanol and injection with in-tube SPME using methanol and  
17 Genapol (0.1%, v/v) as desorption eluents. As shown in the figure, in-tube SPME with  
18 methanol and micellar desorption enabled the satisfactory separation and identification  
19 of the target compounds.

### 20 3.2. Analytical performance characteristics

21 Under the optimum extraction conditions, calibration curves were constructed  
22 using concentrations ranging from 1  $\text{ng}\cdot\text{mL}^{-1}$  to 1000  $\text{ng}\cdot\text{mL}^{-1}$  (LC-DAD) and 0.1  
23  $\text{ng}\cdot\text{mL}^{-1}$  to 1000  $\text{ng}\cdot\text{mL}^{-1}$  (LC-FD). In all cases, the correlation coefficients ( $R^2$ ) were  
24 greater than 0.99. Each level of concentration was prepared and injected three times.

25 The reproducibility of the method was evaluated by determining the inter-day  
26 and intra-day precision. As shown in Table 3, excellent reproducibility was obtained for  
27 both desorption reagents. Namely, intra-day % RSDs of less than 6.9 and 7.1 and inter-  
28 day % RSDs of less than 6.5 and 7.6 were obtained for methanol and Genapol in LC-  
29 DAD, respectively.

30 The limits of detection (LODs) and quantification (LOQs) were determined  
31 using six independent sample blanks and were calculated as a signal-to-noise ratio of  
32 3 and 10, respectively. The data are shown in Table 3. With a DAD detection, the



1 LODs and LOQs of the proposed method ranged from 0.03 to 1.04 ng·mL<sup>-1</sup> and 0.1 to  
2 3.13 ng mL<sup>-1</sup> when methanol was used and 0.03-1.95 ng·mL<sup>-1</sup> and 0.10-5.95 ng mL<sup>-1</sup>  
3 when Genapol (0.1%, v/v) was employed. Fluorescence detection improved these  
4 parameters for E3, BPA and EE2. Specifically, LODs of 0.02 - 0.15 ng mL<sup>-1</sup> and  
5 LOQs of 0.05-0.47 ng mL<sup>-1</sup> were obtained with methanol, and LODs of 0.02-0.14  
6 ng·mL<sup>-1</sup> and LOQs of 0.06-0.42 ng·mL<sup>-1</sup> were obtained using micellar desorption.

7 Previous works [15, 17] reported determination of steroid sex hormones in  
8 water and urine matrices with DAD detection with similar results to the obtained in  
9 this work.

10 The obtained LODs for the combination of in-tube SPME- MD-LC-DAD-FD  
11 approach could be improved using MS detection. However, it is demonstrated that the  
12 proposed methodology can be an alternative to the use of organic solvents which makes  
13 possible to use it like an easier and cheaper approach to analyse EDCs in another type  
14 of liquid matrices like facultative pond wetland systems.

### 15 3.3. Application to the analysis of environmental waters

16  
17 To study matrix effects in the optimized in-tube SPME method, the proposed  
18 procedure was used to extract and determine the selected EDCs in spiked seawater and  
19 WWTP effluent samples. Known amounts of the five EDCs were spiked into each water  
20 sample, and their recoveries were calculated. All samples were analysed in triplicate.  
21 Non-spiked real samples were analysed using in-tube SPME-LC-DAD-FD with  
22 methanol and Genapol, as described above. EDCs or interference were not detected at  
23 the retention times of the selected compounds. This fact was verified using a SPE  
24 procedure.

25 Chromatograms of seawater and WWTP samples are shown in Figure 6. Good peak  
26 shapes and resolution were achieved for all compounds with no interference from water  
27 matrix. The results are outlined in Table 4. As shown in the table, when methanol was  
28 used with a DAD, the overall recoveries were 93.0-103.4 % in seawater samples and  
29 93.0-101.2% in WWTP effluent samples. Using Genapol as the desorbent, the overall  
30 recoveries were 93.7-104.4 % in seawater samples and 90.5–106.9 % in WWTP  
31 effluent. Similar results were obtained with a FD detection. These results indicate that  
32 no appreciable difference is found when the extraction is carried out in different

1 matrices and therefore the applicability in environmental water samples is  
2 demonstrated.

#### 3 **4. Conclusions**

4 A novel in-tube SPME-MD methodology was developed for the determination of  
5 EDCs in environmental liquid samples. An innovative feature of the present work was  
6 the use of micellar media instead of organic solvent and the use of experimental design  
7 methodology to optimize the proposed method. The extraction efficiency obtained using  
8 Genapol (0.1%, v/v) was comparable to that of methanol, which is a conventional  
9 solvent. The manually handled in-tube SPME was connected to LC-DAD and LC-FD  
10 systems. The proposed extraction method displayed good reproducibility, and  
11 recoveries greater than 86% were obtained. Finally, the optimal procedure was  
12 successfully applied to the analysis of EDCs in two different environmental water  
13 matrices. The developed method is simple, efficient and precise, and conventional  
14 equipment can be employed. Moreover, the proposed method is environmentally  
15 friendly and inexpensive, due to its low solvent volumes. After a careful selection of the  
16 extraction and desorption conditions, it was demonstrated that the proposed method  
17 could be useful for the screening and determination of EDCs in some environmental  
18 matrices. The obtained results from the application of this methodology in  
19 environmental water samples suggest that this process can be a promising alternative to  
20 other conventional procedure like SPE.

21

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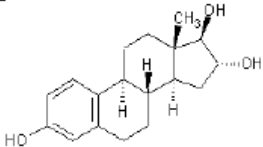
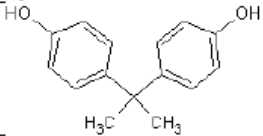
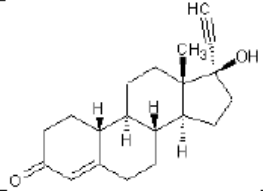
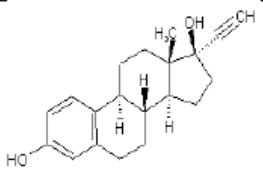
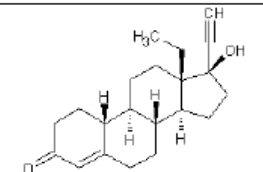
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**Table 1: Abbreviations, structure, CAS number, log  $K_{ow}$  and detection wavelength ( $\lambda$ ) used for monitoring the target analytes.**

	Name	Abbreviation	Structure	CAS number	log $K_{ow}$	DAD $\lambda$ (nm)	FD $\lambda_{exc}/\lambda_{em}$ (nm)
1	Estriol	E3		50-27-1	2.5	224	228/315
2	Bisphenol A	BPA		80-05-7	3.3	224	228/315
3	Norethisterone	NORE		68-22-4	3.3	244	-
4	Ethynyl estradiol	EE2		57-63-6	3.7	224	228/315
	Norgestrel	NORG		83150-76-9	3.5	244	-

**Table 2. Partial and bivariate correlations between selected variables (maxima correlation are +1 and -1).**

<b>Experimental design – screening</b>	<b>E3</b>	<b>BPA</b>	<b>NORE</b>	<b>EE2</b>	<b>NORG</b>
Sample volume	0.490	0.916	0.945	0.893	0.948
Desorption volume	-0.691	-0.094	-0.138	-0.369	-0.114
Extraction time	-0.063	-0.014	0.049	0.041	0.043
Desorption time	0.014	-0.073	-0.046	0.027	-0.010
Sample volume x Desorption volume	0.539	0.216	0.402	0.788	0.342
Sample volume x Extraction time	0.035	0.031	-0.140	0.081	-0.128
Sample volume x Desorption time	-0.078	0.167	0.132	-0.054	0.030
Desorption volume x Extraction time	-0.060	-0.001	0.007	0.016	0.005
Desorption volume x Desorption time	0.133	-0.007	-0.006	0.011	-0.001
Extraction time x Desorption time	0.009	-0.001	0.002	-0.001	0.000

**Table 3. Analytical parameters obtained for the selected compounds by in-tube SPME using different detection techniques and desorption agents.**

Analyte	MeOH								Genapol							
	DAD				FLUORESCENCE				DAD				FLUORESCENCE			
	LOD <sup>a</sup>	LOQ <sup>b</sup>	precision		LOD <sup>a</sup>	LOQ <sup>b</sup>	precision		LOD <sup>a</sup>	LOQ <sup>b</sup>	precision		LOD <sup>a</sup>	LOQ <sup>b</sup>	precision	
Inter-day <sup>c</sup>			intra-day <sup>d</sup>	inter-day <sup>c</sup>			intra-day <sup>d</sup>	inter-day <sup>c</sup>			intra-day <sup>d</sup>	inter-day <sup>c</sup>			intra-day <sup>d</sup>	
E3	1.04	3.13	5.1	6.2	0.15	0.47	4.5	4.2	1.95	5.95	7.6	6.5	0.14	0.42	4.9	4.3
BPA	0.03	0.10	4.7	4.1	0.02	0.05	5.9	5.7	0.03	0.93	5.2	4.8	0.02	0.06	6.5	7.2
NORE	0.08	0.25	3.8	3.8	n.d. <sup>e</sup>	n.d. <sup>e</sup>	n.d. <sup>e</sup>	n.d. <sup>e</sup>	0.10	0.33	5.0	5.4	n.d. <sup>e</sup>	n.d. <sup>e</sup>	n.d. <sup>e</sup>	n.d. <sup>e</sup>
EE2	0.14	0.43	4.6	6.9	0.06	0.17	5.3	5.2	0.18	0.55	2.9	2.7	0.1	0.29	3.7	6.0
NORG	0.08	0.16	6.5	5.2	n.d. <sup>e</sup>	n.d. <sup>e</sup>	n.d. <sup>e</sup>	n.d. <sup>e</sup>	0.07	0.19	6.7	7.1	n.d. <sup>e</sup>	n.d. <sup>e</sup>	n.d. <sup>e</sup>	n.d. <sup>e</sup>

<sup>a)</sup> Limits of detection are calculated as signal to noise ratio of 3 (in ng·mL<sup>-1</sup>)

<sup>b)</sup> Limits of quantification are calculated as signal to noise ratio of 10 (in ng·mL<sup>-1</sup>)

<sup>c)</sup> Inter-day precision, % RSD (n=6, 100 ng·mL<sup>-1</sup> for all compounds)

<sup>d)</sup> Intra-day precision, % RSD (n=6, 100 ng·mL<sup>-1</sup> for all compounds)

<sup>e)</sup> n.d.: not detected

**Table 4.: Recovery percentages (n=3) obtained for sea water and WWTP effluent samples spiked with 100 ng mL<sup>-1</sup> of selected compounds using methanol and Genapol (0.1%, v/v) as desorption agents.**

<b>MeOH</b>	<b>DAD</b>		<b>FLUORESCENCE</b>	
<b>Analyte</b>	<b>Sea water</b>	<b>Effluent of WWTP</b>	<b>Sea water</b>	<b>Effluent of WWTP</b>
<b>E3</b>	103.4±2.6	95.5±6.9	100.7±4.7	94.4±2.6
<b>BPA</b>	98.3±9.1	98.7±3.3	98.5±9.1	98.8±7.0
<b>NORE</b>	96.1±3.5	101.2±3.6	n.d. <sup>a</sup>	n.d. <sup>a</sup>
<b>EE2</b>	95.5±4.6	94.7±2.2	86.8±6.9	94.1±7.2
<b>NORG</b>	93.0±5.2	93.0±3.8	n.d. <sup>a</sup>	n.d. <sup>a</sup>
<b>Genapol</b>	<b>DAD</b>		<b>FLUORESCENCE</b>	
<b>Analyte</b>	<b>Sea water</b>	<b>Effluent of WWTP</b>	<b>Sea water</b>	<b>Effluent of WWTP</b>
<b>E3</b>	94.2±3.1	91.0±2.7	100.1±1.7	90.9±2.5
<b>BPA</b>	99.2±5.4	96.3±6.8	92.5±4.7	94.2±3.1
<b>NORE</b>	93.7±3.3	106.9±2.7	n.d. <sup>a</sup>	n.d. <sup>a</sup>
<b>EE2</b>	104.4±2.8	97.7±1.5	103.2±3.0	93.3±3.1
<b>NORG</b>	97.7±5.9	90.5±6.1	n.d. <sup>a</sup>	n.d. <sup>a</sup>

<sup>a)</sup> n.d.: not detected



**Figure 1.** Schematic diagram of the in-tube SPME connection (A) Load position (B) Inject position.

**Figure 2.** Surface response of sample volume and extraction time of NORE (A) and EE2 (B). Each compound was initially extracted with one cycle of 65 $\mu$ L of standard solution (1 $\mu$ g $\cdot$ mL<sup>-1</sup> of each compound). Desorption was provided during 10 min by 25  $\mu$ L of methanol.

**Figure 3.** Influence of number of cycles in the extraction process of the proposed method. Each compound was extracted with different number of cycles of 65 $\mu$ L of standard solution (1 $\mu$ g $\cdot$ mL<sup>-1</sup> of each compound). Desorption was provided during 10 min by 25  $\mu$ L of methanol.

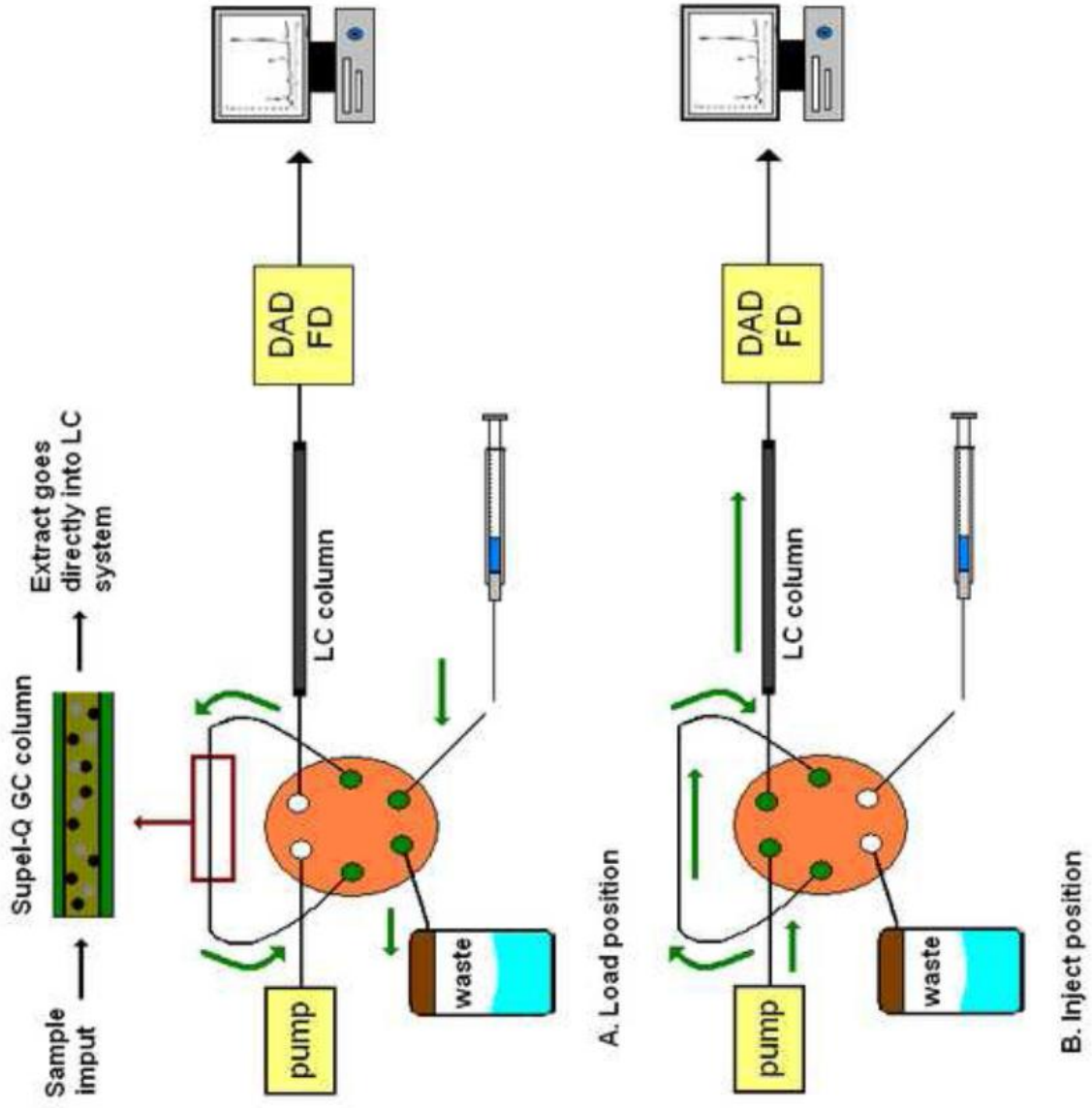
**Figure 4.** Surface response in the optimization of micellar desorption using Genapol of (A) NORE and (B) EE2. Standard solution (1 $\mu$ g $\cdot$ mL<sup>-1</sup> of each compound) was extracted with 12 cycles of 65 $\mu$ L during 2 minutes. Time of desorption: 1minute.

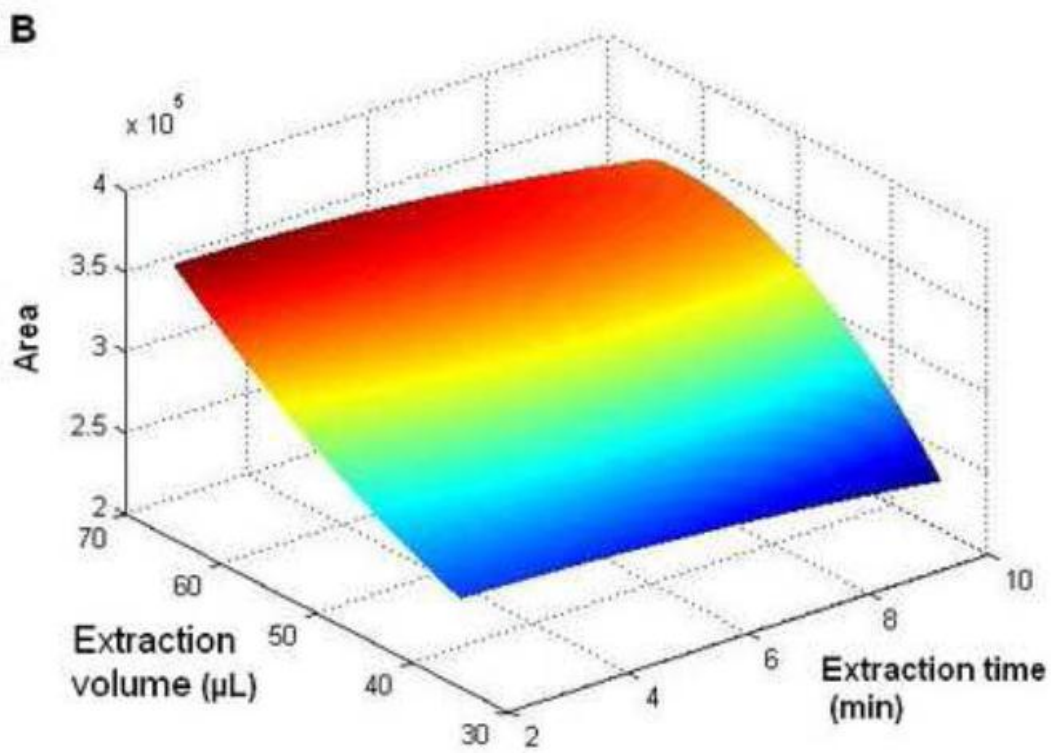
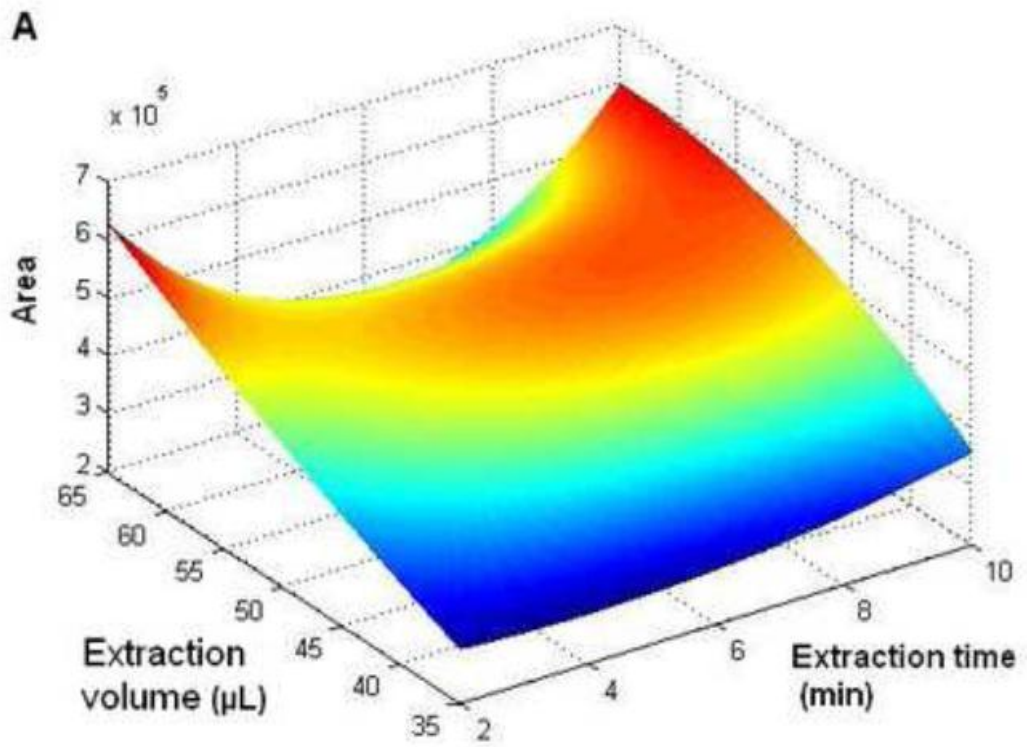
**Figure 5.** Chromatograms of the selected EDCs obtained by in-tube SPME LC-DAD at 280 nm wavelenght with (a) direct injection, (b) using Genapol and (c) methanol as desorption agent, in optimal conditions. Standard solution contained 1  $\mu$ g $\cdot$ mL<sup>-1</sup> of each target analyte. The numbering refers to Table 1.

**Figure 6.** Chromatograms of the selected EDCs obtained by in-tube SPME-LC-DAD: A) (a) non-spiked sea water and (b) spiked sea water with Genapol as desorbent (b), B) (a) non-spiked WWTP and (b) spiked WWTP with Genapol as desorbent. Samples were spiked with 100 ng $\cdot$ mL<sup>-1</sup> of each compound. The numbering refers to Table 1.

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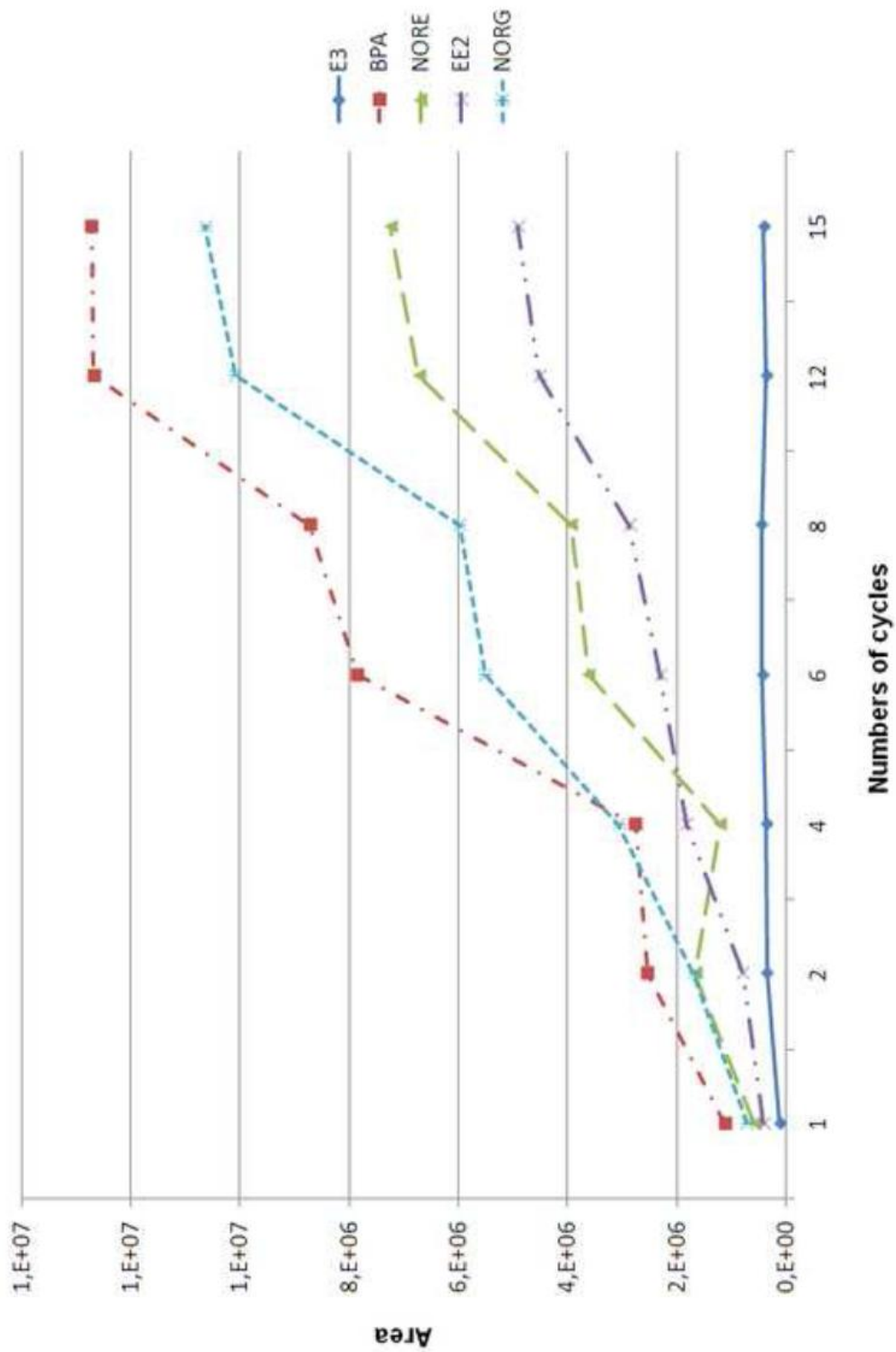
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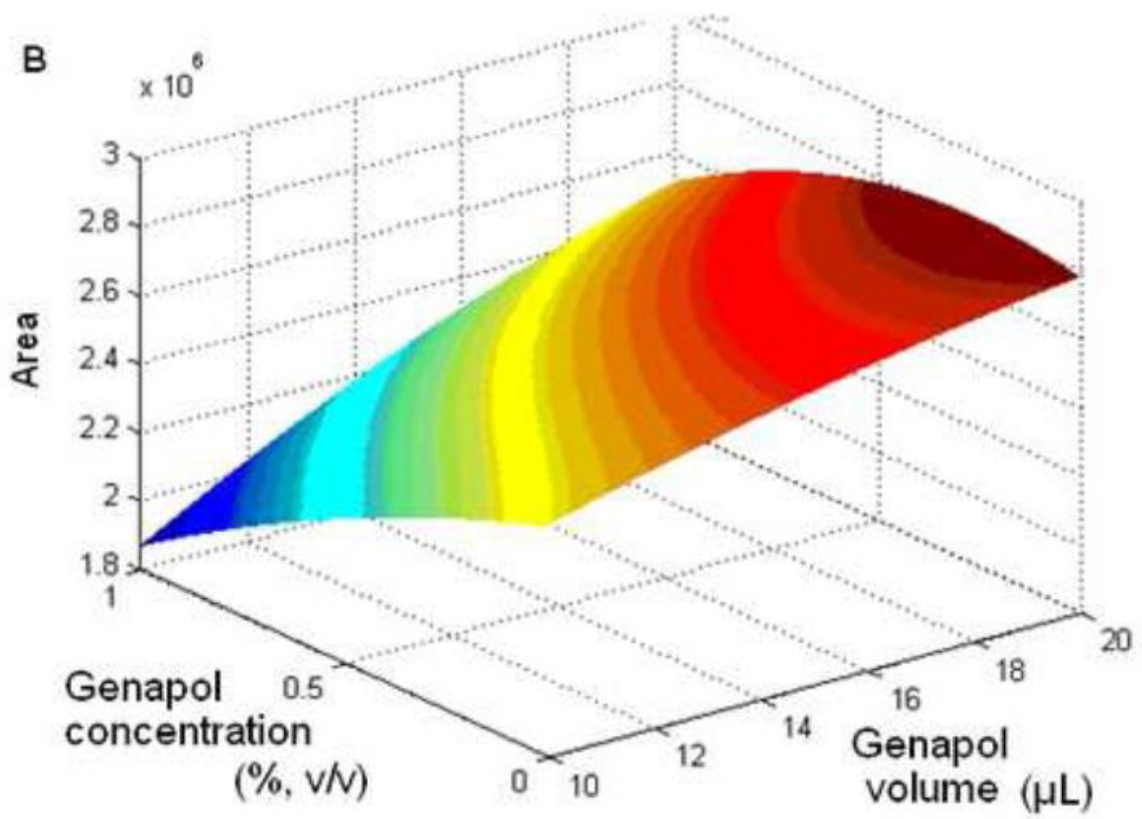
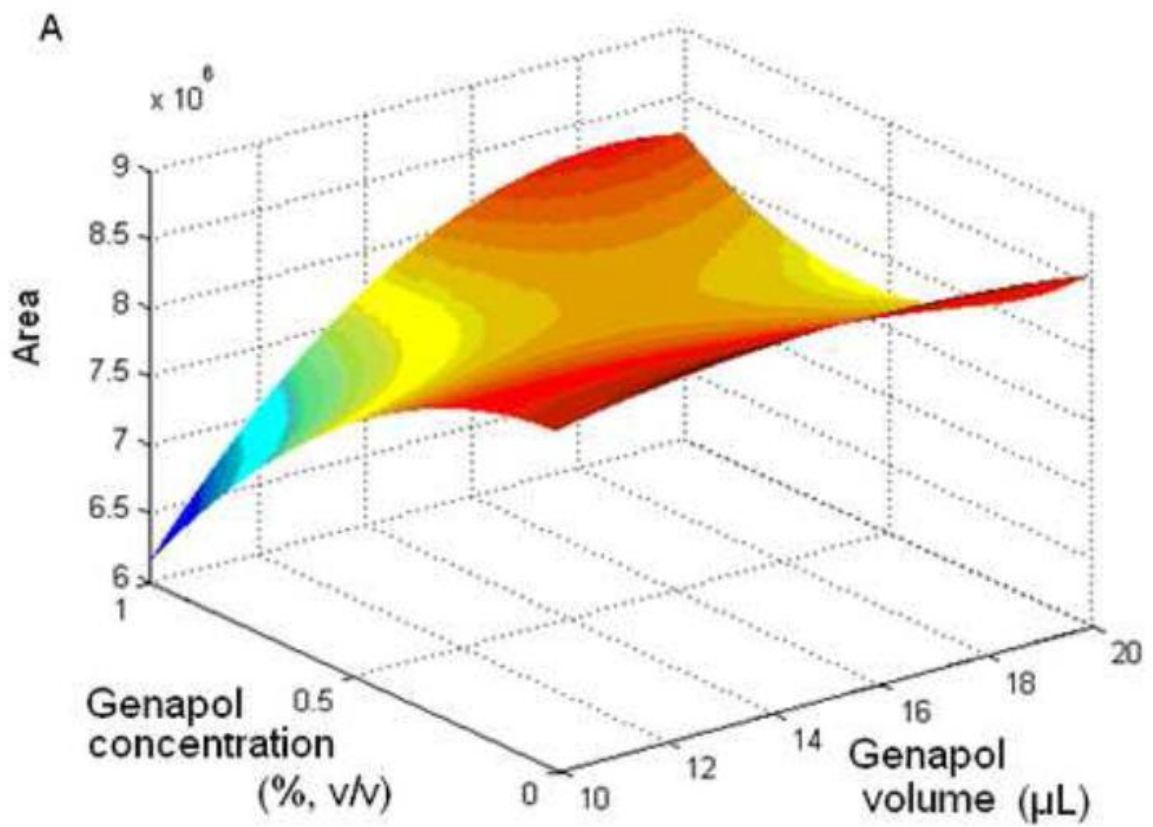


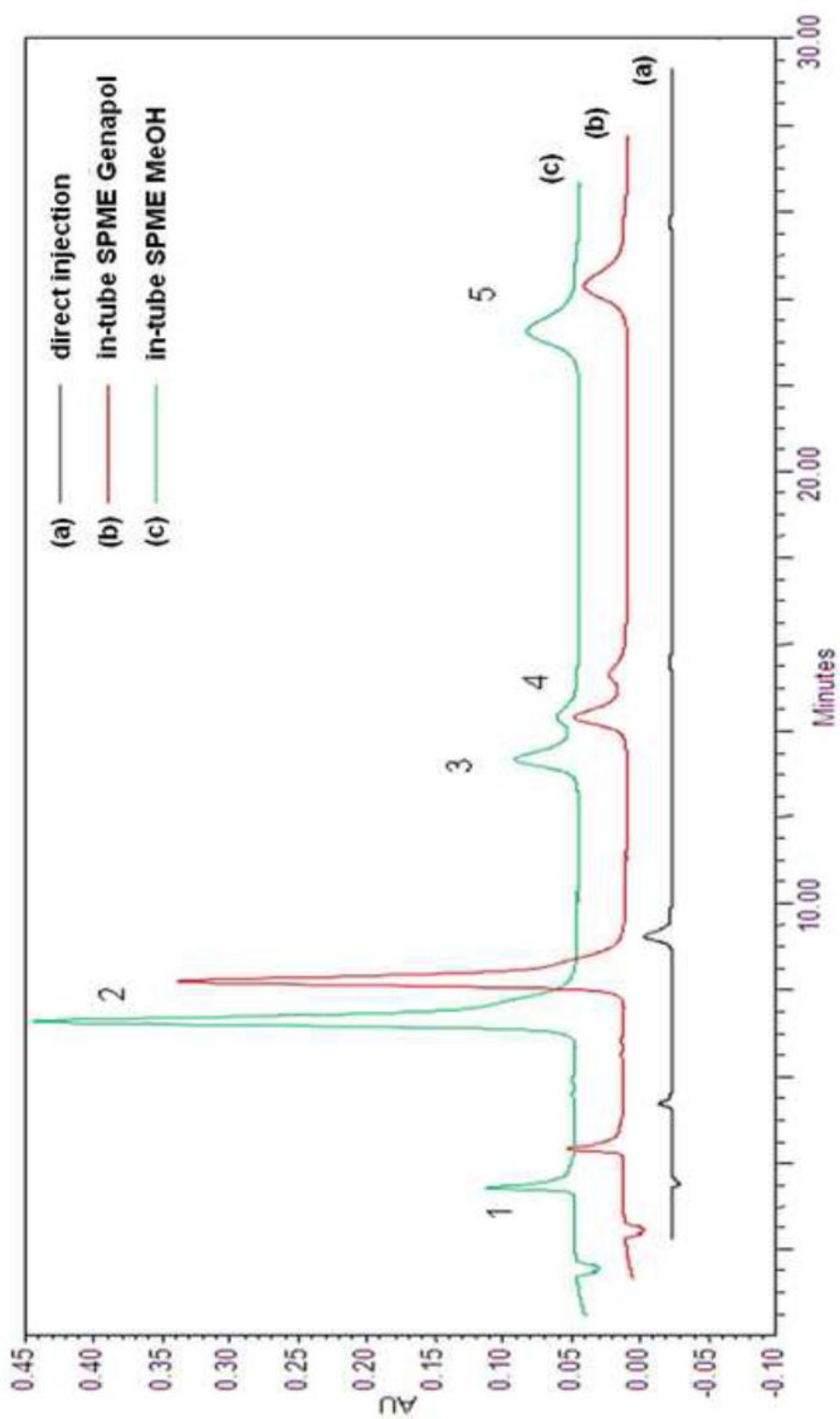


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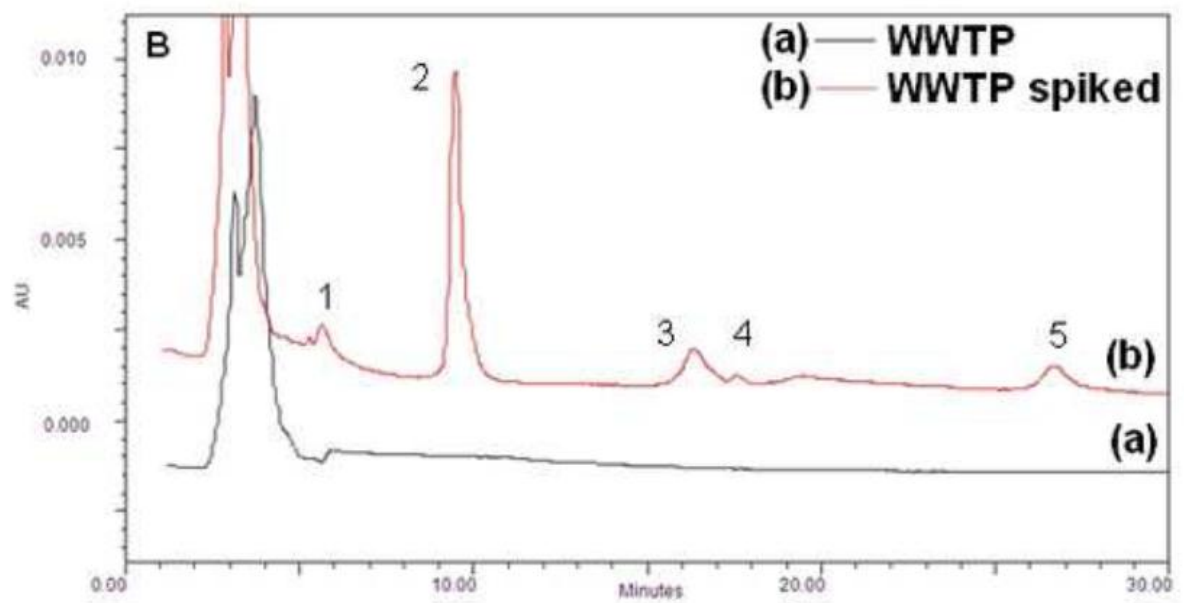
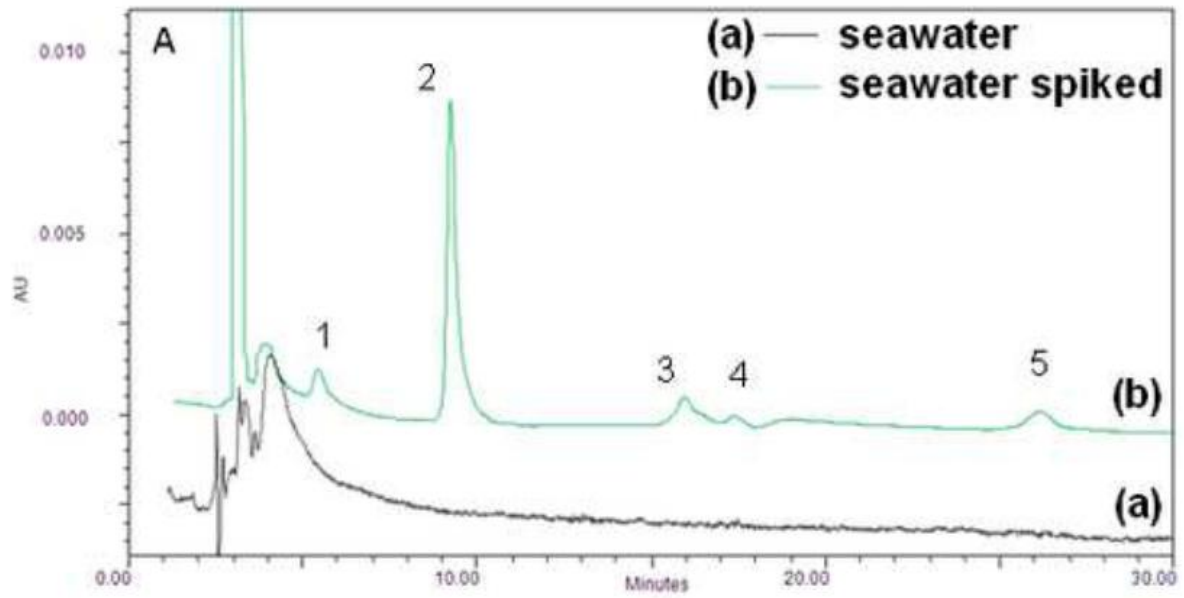






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1 **6.9 Supplement VI**

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3 Maria Esther Torres-Padrón, Jana Aufartová, Zoraida Sosa-Ferrera, José Juan Santana-Rodríguez

4 **Benzimidazole Fungicides in Environmental Samples: Extraction and Determination**

5 **Procedures**

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# Benzimidazole Fungicides in Environmental Samples: Extraction and Determination Procedures

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## 1. Introduction

Because of the widespread use of agricultural pesticides for different applications, the pesticide residues may present a main source of pollution, which poses risks to plant, animal and human health. Benzimidazole fungicides (BFs) are the largest chemical family that have an imidazole ring containing both acidic and basic nitrogen atoms. They are used for prevention and treatment of parasitic infections in agriculture and aquaculture and are efficient at low doses as well as they inhibit the development of a wide variety of fungi. Some benzimidazoles have also found applications as pre- or post-harvest fungicides for control of a wide range of pathogens. They are either applied directly to the soil, or sprayed over crop fields (Wu et al., 2009). Most of these compounds persist in the environment after their application, with some even remaining for many years. This group includes thiabendazole analogues and benzimidazole carbamates.

Thiabendazole (TBZ) was the first benzimidazole to be marketed. After its introduction, a number of alternative benzimidazoles offering similar activity came on the market, such as parbendazole (PAR), cambendazole (CAM), mebendazole (MBZ), fuberidazole (FDZ) and oxibendazole (OXI). BFs possessing sulphide and sulphoxide functional groups were subsequently introduced, offering a wider spectrum of activity and efficacy. Albendazole (ABZ), fenbendazole (FBZ), triclabendazole (TCB) and oxfendazole (OFZ) have been used in the treatment of different stages of gastrointestinal nematodes. Luxabendazole (LUX) is another benzimidazole-sulphide used but is not licensed for use in the European Union. Netobimin (NETO) and febantel (FEB), which are the pro-drugs of ABZ and FBZ, respectively, have greater water solubility resulting in improved absorption and increased bioavailability. Similar probenzimidazoles have found widespread use as fungicidal agents, including benomyl (BNM) and thiophanate-methyl (TPM), which are precursors of carbendazim (MBC). Such modifications have given rise to new BFs with much slower rates of elimination, higher potencies and broader activity spectra. It is well established that MBC, the common stable

metabolite of BNM and TPM, is considered as the major fungitoxic principle of the benzimidazole precursor fungicides. Accordingly, regulatory limits for these fungicides are generally all expressed as MBC, the single measurement marker for the food safety or environmental impact of the total benzimidazole-containing residues (Danaher et al., 2007).

The octanol-water partition coefficient,  $K_{ow}$ , is an important property because it can provide an indication of the solubility of these residues in different solvents and give an indication of the elution conditions necessary for liquid chromatography. Most of these molecules have similar  $K_{ow}$  values, which are generally in the range 0.8–3.3. Under suitable conditions, molecules may be protonated ( $pK_a \sim 5-6$ ) or deprotonated ( $pK_a \sim 12$ ). Few  $pK_a$  values have been published but a summary of the experimental  $pK_a$  values and calculated octanol-water partition coefficients ( $K_{ow}$ ) are listed in Table 1.

Although many public benefits have been realized by the use of benzimidazole compounds, their potential impact in both the environment and public health cannot be disregarded. Even if acute toxic effects of benzimidazole compounds are scarce due to their high lethal dose 50 values, several toxic effects have been associated to a chronic exposure to benzimidazole compounds, such as teratogenicity, congenic malformations, polyploidy, diarrhea, anemia, pulmonary edemas, or necrotic lymphadenopathy.

On account of their extensive use, residual environmental impact and toxic effects at low levels, regulations have set maximum residue levels (MRLs) for benzimidazoles and their metabolites to ensure consumer safety with the range of 0.01–10 mg kg<sup>-1</sup>, depending on the fungicide–commodity combination (Plant Protection-Pesticide Residues-Regulation (EC) No. 396, 2005). In particular, for most benzimidazoles, the marker residue tolerance has recently been defined as the sum of a parent drug and/or its related metabolites (sum-MRL substances) instead of single compounds (Danaher et al., 2007).

Their massive use in the last years has led into their accumulation in the environment, thus contaminating the water streams. European Water Framework Directive (Directive 2006/11/CE 4) has established a maximum concentration level (MCL) of 0.1 µg L<sup>-1</sup> for most benzimidazole compounds present in natural waters, and a total concentration of all pesticides of 0.5 µg L<sup>-1</sup>. The need to determine low concentrations of these substances as well as matrix complexity means that analytical methods with high sensitivity, selectivity and resolution have to be applied to soil, sediment, water and other environmental samples. Elaborate sample preparation involving analyte isolation and enrichment is generally necessary before the final analysis, which is usually performed using gas chromatography (GC) or high-performance liquid chromatography (HPLC).

The selection of an adequate sample treatment protocol allowing to carry out a multiresidue determination of benzimidazole compounds is currently a challenge, due to their chemical properties. The development of highly sensitive methods for the multiresidue determination of benzimidazole compounds in environmental samples is desirable, and for that purpose a preconcentration step and appropriate instrumental techniques are usually required. In this sense, extensive sample cleanup or preconcentration procedures might be applied to the determination of a wide variety of benzimidazole compounds, and the use of highly selective detection methods (i.e. tandem mass spectrometry) is usually required for multiresidue analysis.

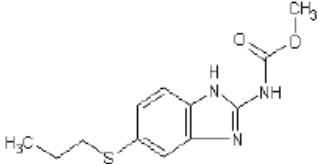
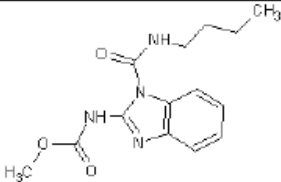
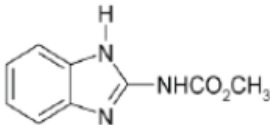
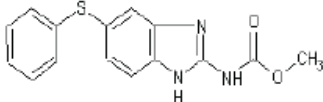
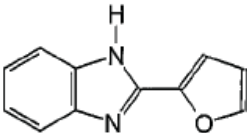
A large amount of effort has been invested in the past few decades to develop and validate analytical methodologies to quantify benzimidazole compounds and their metabolites in environmental samples at concentration levels below the legislated MCLs.

In this chapter some methodologies for the determination of BFs in environmental samples are presented. Its scope is the coverage of the main aspects which are involved in their determination in environmental matrices: sample handling, extraction/preconcentration and determination. We will conclude with a general conclusion and notes on future perspectives.

## 2. Environmental liquid samples preparation

Contamination of natural waters by pesticides is one of the main environmental problems around the world because of the common use, persistence, bioaccumulation and toxicity of these pollutants. Determining the degree of ground and surface water contamination by these compounds is one of the fundamental aims of environmental analytical laboratories. To reach the low limits of detection is necessary the use of sensitive multi-residue methods for detecting and identifying these compounds, with the fewest number of intermediate step.

Sample preparation is one of the most important steps in a whole analytical process. The objective of the sample preparation is not only to isolate the target analytes from the samples, thus reducing or even eliminating the interferences originally present in the sample, but also simultaneously to concentrate the analytes to facilitate their determinations at low levels. Sample preparation gradually becomes a major part of analysis, capable of taking up to 80% of the total time of a complete analysis process. Different steps in the process, extraction, clean-up and detection play a key factor in the safety and accuracy of analysis.

Name	Chemical structure	K <sub>oc</sub>	pK <sub>a1</sub>	pK <sub>a2</sub>
Albendazole (ABZ)		2.2-2.92	5.54	13.11
Benomyl (BNM)		1.4	4.48	
Carbendazim (MBC)		1.29-1.69	5.52	13.09
Fenbendazole (FBZ)		3.07-4.01	5.12	12.72
Fuberidazole (FDZ)		2.71	4.0	

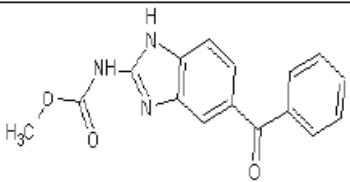
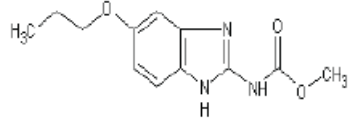
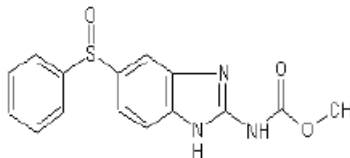
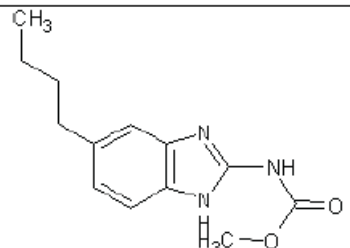
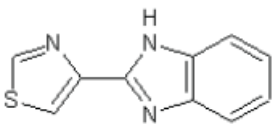
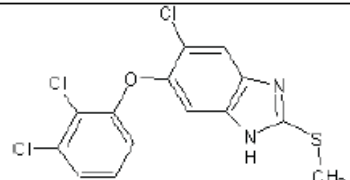
Name	Chemical structure	K <sub>oc</sub>	pK <sub>a1</sub>	pK <sub>a2</sub>
Mebendazole (MBZ)		2.44-2.52	4.13	11.79
Oxibendazole (OXI)		1.86-2.63	6.26	13.78
Oxfendazole (OFZ)		1.88-2.13	4.13	11.79
Parbendazole (PAR)		1.86-2.63	5.99	13.53
Thiabendazole (TBZ)		1.58-1.76	5.82	12.79
Triclabendazole (TCB)		4.90-6.66	5.31	12.91

Table 1. Chemical structures and calculated properties of some benzimidazole fungicides (BFs) (Danaher et al., 2007)

Because the low concentration levels in environmental waters, an extraction and preconcentration step is usually required. First extraction method was liquid-liquid extraction (LLE) with conventional organic solvent (Blanchflower et al. 1994; Fernández et al., 2001). However, LLE suffers from the disadvantages of being time-consuming, expensive and requiring large volumes of both samples and toxicity. Other methods, like solid phase extraction (SPE) (Picón et al., 2000; Garrido et al., 2003; Moral et al., 2006), on-line supported liquid membrane (SLME), microporous membrane liquid-liquid extraction (MMLLE) (Sandahl et al., 2000), cloud point extraction (CPE) (Halko et al., 2004) and solid phase microextraction (SPME) (López Monzón et al., 2007) have been proposed to extract and concentrate BFs from aquatic environments.

In recent years, solid phase extraction (SPE) has widely been applied for the analysis of different pesticides in water samples, owing to the high enrichment factors achievable by this methodology using the high breakthrough volumes of materials such as  $C_{18}$  (Picón et al., 2000; Zamora et al., 2003; Garrido Frenich et al., 2003), polystyrene-divinylbenzene (Guenu et al., 1996), ethylvinylbenzene-divinylbenzene (Junker-Buchheit, 1996), polystyrene (Shimamura et al., 1998) and active carbon (Jeannot et al., 2000). In this sense, Guenu et al. used precolumns packed with PS-DVB sorbents for the on-line determination of very polar pesticides, including carbendazim (Guenu et al., 1996). The evaluation of the PS-DVB sorbent was first carried out by measuring the recoveries using off-line extraction with a 20 mg PS-DVB cartridge and percolating drinking and river water both spiked with  $0.1 \mu\text{g L}^{-1}$  of each pesticide. Limits of detections (LODs) were at the  $0.05\text{--}0.3 \mu\text{g L}^{-1}$  level in surface waters for different polar pesticides.

Other authors used  $C_{18}$  extraction cartridges for extracting carbendazim, fuberidazole and thiabendazole from water samples obtaining LODs between  $0.001$  to  $0.125 \mu\text{g L}^{-1}$ . Satisfactory predictions ranging from 102 to 114% for carbendazim, 96 to 115% for fuberidazole and 90 to 107% for thiabendazole were sufficiently low to determine pesticide residues in water samples (Picón et al., 2000; Garrido Frenich et al., 2003). SPE requires large volumes of sample ( $>200\text{mL}$ ) and organic solvent ( $12\text{--}50 \text{mL}$ ) and BFs losses occur during the evaporation of the extracts (Guenu et al., 1996).

Recent developments in SPE field are mainly related to the use of new sorbent materials. Molecularly imprinted polymers (MIPs) have proven to be a very valuable technique for selective solid-phase extraction of the template molecule and structurally related compounds. The inherent selectivity of the molecular recognition of these materials allows a high degree of sample clean-up to be achieved (Baghianni et al., 2006; Pichón et al., 2006). Additionally, MIPs have also been used for sample enrichment for the determination of a wide range of analytes. MIPs have successfully been applied as highly selective sorbents for the extraction of benzimidazole compounds in an organic media (de Prada et al. 2007; Cacho et al., 2008; Turiel et al., 2005). However, coelution of the different benzimidazole compounds has been described when using these imprinted polymers as selective stationary phases (Cacho et al., 2009). For that, development of molecularly imprinted solid-phase extraction (MISPE) procedure permit the enrichment of benzimidazoles fungicides, based on an on-line sample enrichment of water samples by means of an imprinted polymer, synthesized by precipitation polymerization using thiabendazole as template molecule, methacrylic acid as functional monomer, and divinylbenzene as cross-linker. Initial experiments carried out by solid-phase extraction on cartridges demonstrated a clear imprint effect for thiabendazole, as well as the ability of the imprinted polymer to selectively rebind several benzimidazole compounds. The developed methodology has been applied to the quantification of thiabendazole, carbendazim, and benomyl in river, tap and well water samples within a single analytical run at concentration levels below the legislated maximum concentration levels. In this sense, obtained detection limits were of  $2.3\text{--}5.7 \text{ng L}^{-1}$  for the analysis of benzimidazole fungicides in different water matrices. Recoveries obtained for the determination of benzimidazole fungicides in spiked samples ranged from 87% to 95%, with relative standard deviations (RSD s) below 5% in all cases (Zamora et al., 2009).

López-Monzón et al. (2007) established a suitable and sensitive method for simultaneous determination of BFs (carbendazim, benomyl, fuberidazole and thiabendazole) in water samples using solid phase microextraction (SPME). Authors evaluated the efficiency of extraction of these compounds on different kinds of fibres and optimized several SPME

conditions: extraction time, ionic strength, extraction temperature and desorption time. The optimized SPME procedure was used for extraction and determination of these compounds in different environmental water samples (sea, sewage, and ground waters). A Carboxen-polydimethylsiloxane (CAR-PDMS) fibre was the optimum coating for extraction of these targets. Obtained recoveries ranged from 80.6 to 119.6 with RSDs below 9% and limits of detection between 0.03 and 1.3 ng mL<sup>-1</sup> for the different analytes. A chromatogram of this process is shown in Figure 1.

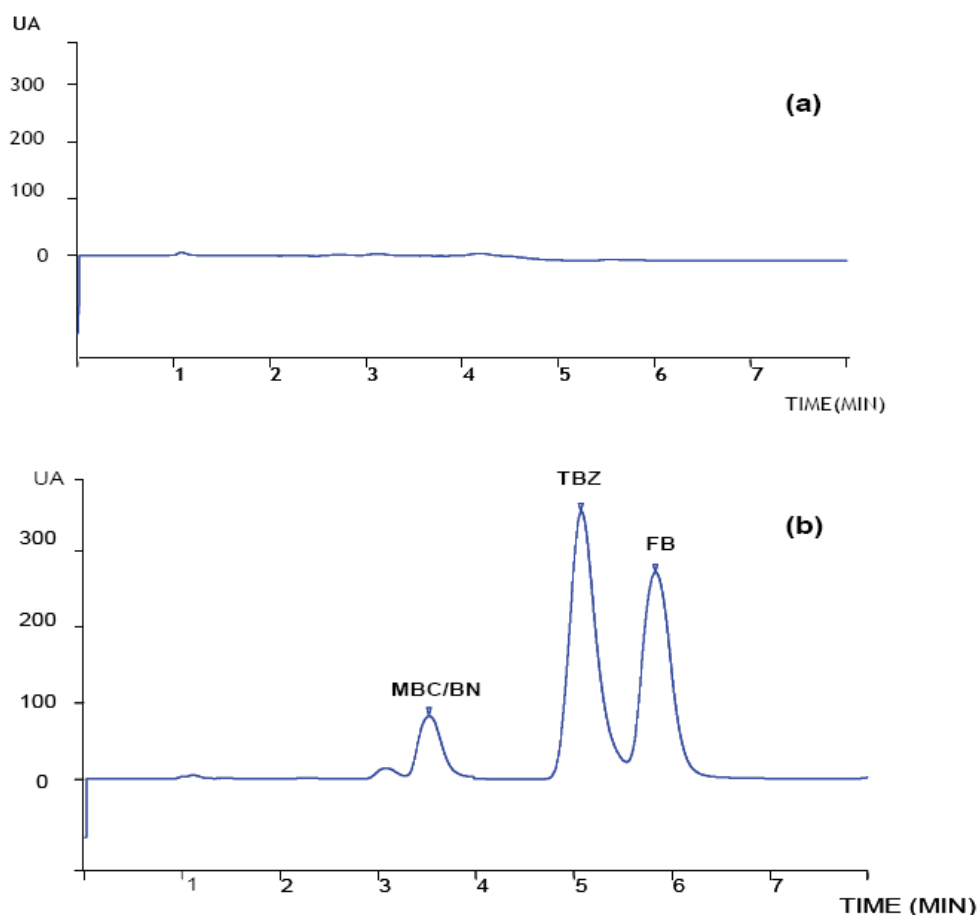


Fig. 1. Obtained chromatogram for a blank sample (a) and for the extract of benzimidazole fungicides from a spiked seawater sample (b) under optimum SPME extraction (López-Monzón et al., 2007).

Extraction process like microporous membrane liquid-liquid extraction (MMLLE) and supported liquid membrane extraction (SLME) have been demonstrated to be efficient for sample preparation. MMLLE is a two-phase aqueous-organic solvent system and SLM a three phase aqueous-organic solvent-aqueous system, which leads to more selective extraction. All of them require lower amount of sample (4-20 mL) and organic solvent. An inconvenient of these procedures is time-consuming although enrichment rates of 0.6 times min<sup>-1</sup> by SLME and 2.7 times min<sup>-1</sup> by MMLLE have been reported (Sandahl et al., 2000) and SPME takes more than 1 h to obtain a concentration factor of 80 (López Monzón et al., 2007).

Other pre-treatment method to extract and preconcentrate BFs in liquid samples is cloud-point extraction (CPE). The cloud point phenomenon occurs when micellar solutions of non-ionic or zwitterionic surfactants are heated above certain temperature, referred to as cloud point temperature, and they become turbid. At the cloud point, the surfactant solution undergoes phase separation into a surfactant-rich liquid phase and an almost micelle-free dilute solution whose concentration is equal to or lower than the critical micelle concentration. Obtained small volume of the surfactant-rich phase permitting extraction schemes to be designed allows us to preconcentrate and extract the analytes in one step, prior to liquid chromatographic analysis. CPE using non-ionic surfactant such as POLE and Genapol X-080 provides good extraction efficiency of different BFs in environmental liquid samples (Halko et al., 2004). The limit of detection (LOD) were  $6 \text{ ng mL}^{-1}$  for carbendazim,  $\text{ng mL}^{-1}$  benomyl,  $0.15 \text{ ng mL}^{-1}$  for thiabendazole and  $0.01 \text{ ng mL}^{-1}$  for fuberidazole in both surfactants. Obtained recoveries in spiked water samples ranged from 68% to 94% for Genapol and from 68% to 96% for POLE.

Other techniques like liquid phase microextraction, LPME (a small amount of a water-immiscible solvent and an aqueous phase containing the analytes of interest) have emerged as an attractive alternative for sample preparations because of its simplicity, effectiveness, low cost, minimum use of solvents and excellent sample cleanup ability. Different configurations of this technique have recently emerged, including static LPME, dynamic LPME, single-drop LPME and hollow fiber-based liquid-phase microextraction, HF-LPME (extracting phase is placed inside of a porous hydrophobic hollow fiber) (Psillakis et al., 2002; Psillakis et al., 2003; Rasmussen et al., 2004; Pedersen-Bjergaard et al., 2005). However, several disadvantages, such as the instability of liquid drop in single-drop LPME, air bubbles formation in HF-LPME, long analysis time and relatively low precisions, are often encountered for such techniques. Very recently, a novel microextraction technique, named dispersive liquid-liquid microextraction (DLLME), based on dispersion of tiny droplets of the extraction solvent within the aqueous solution has been developed by Assadi and co-workers (Rezaee et al., 2006; Berijani et al., 2006; Fattahi et al., 2007). DLLME is a miniaturized LLE that uses microliter volumes of the extraction solvent. This method was applied to extract carbendazim and thiabendazole in water and soil samples using  $80.0 \mu\text{L}$  of  $\text{CHCl}_3$  as extraction solvent (Wu et al., 2009). Obtained limits of detection for MBC and TBZ were  $0.5$  and  $1.0 \text{ ng mL}^{-1}$  for liquid samples and  $1.0$  and  $1.6 \text{ ng g}^{-1}$  in solid samples, respectively.

Studies on the use of surfactant-coated mineral oxides columns for SPE have demonstrated these new sorbent materials to be a promising tool for the extraction/preconcentration of organic compounds in a wide polarity range (Merino et al., 2004; Moral et al., 2005). Adsorption of ionic surfactants on mineral oxides is a cooperative process; firstly, a monolayer of surfactant (i.e. hemimicelles) is formed with the surfactant head-group facing towards the oxide surface and its hydrocarbon tail-groups protrude into solution, interacting laterally between them. After that, surfactant adsorption occurs through hydrophobic interactions between hydrocarbon tail-groups, which results in the formation of discrete surface aggregates termed admicelles. Because of the amphiphilic character of surfactants, admicelles are aggregates in which there are regions of different polarity, acidity, etc. This feature makes these aggregates extremely versatile extractants because of the number of interactions they can establish with analytes. This type of process have been used for carbendazim, thiabendazole and fuberidazole preconcentration in water samples (Moral et al., 2006). Recently, Moral et al. (2009) have used supramolecular solvents based

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on vesicles of decanoic acid as a good extractant of benzimidazolic fungicides from natural waters. The high concentration of decanoic acid in the extractant phase and the capability of solubilisation of the vesicles permitted the favourable partition of analytes from environmental water using a quite low volume of supramolecular solvent (100  $\mu\text{L}$ ). Actual concentration factors around 150–200 are easily obtained using a single-step extraction without the need of solvent evaporation. Equilibrium conditions are rapidly established, the whole extraction takes about 20 min and several samples can be simultaneously extracted. The proposed method provided detection limits for the determination of CBZ, TBZ and FBZ in natural waters of 32, 4 and 0.1  $\text{ng}\cdot\text{L}^{-1}$ , respectively, and a precision, expressed as relative standard deviation of 5.5% for CBZ, 4.0% for TBZ and 2.5% for FBZ.

### 3. Environmental solid samples preparation

Analytical methods for the determination of BFs in soil are scarce in the available scientific literature. Traditional methods employ large volumes of solvents under aggressive shaking and/or temperature conditions. The most frequently used methods for the extraction of organic compounds from soils are Soxhlet or ultrasonic extraction. The newer extraction techniques, such as supercritical fluid extraction (SFE) (Van der Velde et al., 1992; Snyder et al., 1993; Ling and Liao, 1996), pressurized liquid extraction (PLE) (Mogadati et al., 1999) and microwave-assisted extraction (MAE), are very attractive because they are faster, use much smaller amounts of solvents and are environmentally friendly techniques.

SFE uses a solvent in its supercritical state. This technique is attractive because it is fast, uses small amounts of solvent and commercially available SFE systems allow sample extraction in unattended operations. PLE is done in a closed-vessel at elevated temperatures and pressures. The higher temperature at which the extraction is conducted increases the capacity of the solvent to solubilise the analyte, and the higher pressure increases the diffusion rate into the pores of the matrix, thus facilitating the mass transfer of the analyte into the extracting solvent.

A method based on the sonication of soil samples placed in small columns (sonication-assisted extraction in small columns, SAESC) has been developed for the rapid and sensitive analysis of herbicides and insecticides in soil (Sánchez-Brunete et al., 1998; Castro et al., 2001). In 2002, Sánchez-Brunete et al. published a method for the simultaneous determination of fungicides in soil. The method is simple and uses low volumes of ethyl acetate as extracting solvent, reducing the human exposure to toxic solvents and the environmental impact of the analytical procedure although they did not include BFs in their work (Sánchez-Brunete et al., 2002).

In the last few years, there has been an increase in the number of procedures using microwave energy to extract organic compounds from environmental matrices. Microwave-assisted extraction (MAE) is a process of using microwave energy to heat solvent in contact with a sample in order to obtain partition of analytes from the sample matrix into the solvent (Shu et al., 2003; Ramil Criado et al., 2003). In comparison with other conventional methodologies, such as Soxhlet extraction, MAE requires less energy, shorter analysis periods and the use of smaller solvent volume of analysis. Coscollá et al. (2009) developed a confirmatory and rapid procedure for extraction of different pesticides, including thiabendazole and carbendazim, in PM 2.5 particles by MAE using ethyl acetate. Recoveries ranged from 72 to 109% and the limit of quantification (LOQ) of 6.5  $\text{pg}\cdot\text{m}^{-3}$  were obtained for this method.

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The use of micellar media as alternative to conventional organic solvents in the MAE procedure could offer important advantages such as safety, simplicity, lower toxicity, lower cost and greater compatibility with the aqueous organic mobile phase in the liquid chromatography (LC) separation process. This combination, called microwave-assisted micellar extraction (MAME) has been applied to the extraction of different compounds from solid matrices (Padrón-Sanz et al., 2002; Eiguren Fernández et al., 2001; Mahugo-Santana et al., 2004; Padrón Sanz et al., 2005). Among the advantages of the MAME are reduced volumes of extractant, low cost and reduced toxicity in comparison to organic solvents. Moreover, MAME reduce analysis time compared with conventional Soxhlet extraction.

In this sense, a MAME procedure for benomyl (BNM), carbendazim (MBC), thiabendazole (TBZ) and fuberidazole (FDZ) in soil samples was published by Halko et al. (2006). MAME extraction is influenced by different factors which must be controlled in order to obtain satisfactory results. The surfactant concentration, pH of solution, irradiation power and extraction time were studied and optimized using a factorial design. MAME using non-ionic surfactants, such as POLE and Genapol X-080 provides good extraction efficiency (between 71-105%) of the studied fungicides and LODs between 0.02-0.6  $\mu\text{g g}^{-1}$  for FDZ and TBZ and 25-30  $\mu\text{g g}^{-1}$  for MBC/BNM. MAME is an alternative not only to classical extraction such as Soxhlet extraction, but also to MAE.

#### 4. Detection and determination methods

Numerous analytical methods have been reported for quantitation of benzimidazole fungicides in different matrices based on spectrophotometry (Chiba, 1977), fluorimetry (Zamora et al. 2003; Cuesta et al. 2003), phosphorimetry (Salinas y et al. 2005), electrochemical (Huebra et al. 2000), enzyme-linked immuno-sorbent assay (Itak et al. 1993), gas chromatography-mass spectrometry (Lesueur et al. 2008), although is routinely carried out by high performance liquid chromatography coupled with ultraviolet (Melo et al. 2005; Singh et al. 2007; Prousalis et al. 2004), fluorescence (Moral et al. 2006; Hu et al. 2008), and mass spectrometry (Radisic et al. 2009) detectors. These methods have many advantages of high sensitivity and accuracy and some of them have been adopted by regulatory agencies. In environmental samples, flow injection analysis (FIA) coupled with chemiluminescence (CL) is a well established technique for the ultra-trace analysis of a variety of compounds in diverse matrices using various CL reagents including benzimidazole fungicides (Fletcher et al. 2001). FIA-CL method for the determination of carbendazim has been reported (Liao and Xie 2006), which is based on the enhancement of CL reaction of luminol and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) by a carbendazim in sodium hydroxide-sodium dihydrogen phosphate medium (pH 12.6). This method was applied to the determination of carbendazim in tap-water samples with a linear range of 0.02-2  $\text{mg mL}^{-1}$  and a limit of detection of 7.24  $\text{ng mL}^{-1}$ . Recently, a simple FIA-CL method for the determination of BFs (fuberidazole, benomyl, and carbendazim) was developed because CL emission was observed when the reaction of  $\text{Cu}^{+2}$  and  $\text{H}_2\text{O}_2$  was carried out in an alkaline  $\text{Na}_2\text{CO}_3$  solution without CL reagent. On the addition of a trace amount of BFs to a mixture of  $\text{Cu}^{+2}$ - $\text{H}_2\text{O}_2$ - $\text{Na}_2\text{CO}_3$ , CL emission was enhanced (Waseem et al. 2007, Waasem et al., 2010).

Gas chromatography (GC) determination of BFs is difficult because their thermal instability do not permit their analysis directly unless they are derived into thermally stable derivatives. However, TBZ and TCB are sufficiently volatile to allow their determination by GC without derivatisation. Some reseachers have found GC coupled to mass spectrometry

useful for confirmation of the presence of benzimidazole residues. However, GC-MS procedures usually require derivatisation of residues to induce volatility and allow the generation of suitable MS spectra for confirmatory analysis and, as a result, have been largely replaced by LC-MS/MS. Some quantitative GC methods have been developed by some researchers to determine the presence of benzimidazole fungicides in different matrices including soils (Castro et al., 2001; Leuseur et al., 2008).

#### 4.1 Liquid chromatography based detection systems

##### 4.1.1 Detection by UV and fluorescence.

Spectrometric methods are suitable for quantitation of high levels of benzimidazole residues in different matrices. However, when quantitation of benzimidazoles in the low  $\mu\text{g kg}^{-1}$  range and greater selectivity is required, chromatographic separation of residues prior to spectrometric detection is generally required.

To analyze the growing number of modern pesticides and their degradation products, which are generally thermolabile, polar and non-volatile, the analytical methods employing gas chromatography in combination with specific detectors or coupled with mass spectrometry (MS) are not reliable without a time-consuming derivatization step, which itself can generate interferences. Consequently, liquid chromatography (LC) combined with diode array detection (DAD) has been used increasingly in recent years as a complementary method for pesticide analysis.

Benzimidazole fungicides like albendazole (ABZ), benomyl (BNM), carbendazim (MBC), fenbendazole (FBZ), fuberidazole (FDZ) and thiabendazole (TBZ) have been used from Cacho et al. (2009) to optimise a MISPE method coupled to HPLC-UV. These compounds were monitored at 200 and 240 nm. LODs obtained were between 0.03-0.09  $\text{ng mL}^{-1}$  depending of the compound. The obtained detection limits were below the legislated MCL in all cases.

Zamora et al. (2009) coupled the molecularly imprinted solid-phase extraction (MISPE) procedure to a HPLC-UV system to determine benomyl (BNM), carbendazim (MBC) and thiabendazole (TBZ) in river, tap and well waters. LODs obtained were between 2.5-5.0  $\text{ng L}^{-1}$  depending of the studied compound.

A number of researchers have developed different methods for determination of benzimidazole residues using HPLC coupled to fluorescence detection. Fluorescence detection offer sensitivity and selectivity to methods, potentially reducing the need for extensive sample clean-up.

Moral et al. (2006) used supramolecular sorbents for the extraction/preconcentration of BFs: carbendazim (CBZ), thiabendazole (TBZ) and fuberidazole (FDZ), and to evaluate their applicability for the analysis of natural waters. Same authors optimized supramolecular solvent-based extraction and the fluorescence measurements were performed at 286/320 (time = 0-9 min) and 300/350 nm (time 9-15 min) excitation/emission wavelengths, respectively. These wavelengths were selected on the basis of the excitation and emission spectra obtained for each of the target analytes solubilized in the mobile phase. Obtained detection limits were 0.032  $\text{ng mL}^{-1}$  for CBZ, 0.004  $\text{ng mL}^{-1}$  for TBZ and 0.0001  $\text{ng mL}^{-1}$  for FBZ (Moral et al., 2009).

Wu et al. (2009) used a HPLC-fluorescence detection for the determination of carbendazim (MBC) and thiabendazole (TBZ) in water and soil samples obtaining good results. For water samples, LODs were 0.5  $\text{ng mL}^{-1}$  for MBC and 1.0  $\text{ng mL}^{-1}$  for TBZ. In the case of the soil samples, LODs were 1.0  $\text{ng g}^{-1}$  for MBC and 1.6  $\text{ng g}^{-1}$  for TBZ.

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LC-fluorescence could be applied as an alternative technique to allow determination and confirmation of selected benzimidazole residues. However, not all benzimidazole residues fluoresce naturally. For that, other methods like LC-MS for determination and confirmation of BFs will be reviewed for environmental samples.

#### 4.1.2 Detection by mass-spectrometry.

In the last years, liquid chromatography-mass spectrometry (LC-MS) techniques have advanced in their sensitivity, specificity and reliability. Progress is mostly due to development of hyphenated LC-tandem MS techniques, which are today the methods of choice for the determination of trace organic analytes in environmental samples. Swift growth in the use of LC-MS/MS for the analysis of organic contaminants in environmental matrices has been compelled by the need for high-quality data on their occurrence in the environment at very low concentration levels (Petrovic et al., 2010). Application of tandem mass spectrometry (MS/MS) has resulted in improved determination and confirmation of polar compounds in different environmental matrices.

LC should preferably be applied for the analysis of organic micropollutants only when using tandem MS because this combination is able to produce fragment ions that are necessary for the explicit identification of the analytes (Reddersen & Heberer 2003). LC-MS/MS allows separation and detection of compounds having the same molecular mass but different product ions, even if they co-elute. MS/MS detection is therefore preferred for increased analytical sensitivity and selectivity in complex water matrices (Díaz-Cruz & Barceló 2005). The most commonly used LC-MS/MS interfaces are atmospheric pressure ionization technologies, such as ESI and atmospheric pressure chemical ionization (APCI). ESI is well suited for the analysis of polar compounds, whereas APCI is very effective in the analysis of medium-polarity and low-polarity substances (Radjenovic et al. 2007). Optimization of MS parameters, including cone voltage and collision energy, is performed via flow injection analysis for each compound of interest (Gros et al. 2006; Baugros et al. 2008).

As discussed above, the majority of the analytical procedures published for determining benzimidazoles have been developed based on high performance liquid chromatography (HPLC) because of the polar nature (zwitterion) and thermal lability. In recent years, HPLC-based method combined with sensitive mass spectrometric detection (LC-MS) or versatile tandem mass spectrometry (LC-MS/MS) amenable to multiple reaction monitoring (MRM) has become the analytical tool of choice for simultaneous sensitive quantification and confirmation of a wide range of target fungicides and the structurally related metabolites in complex matrices (Wang et al., 2007; Economu et al., 2009).

Balitz (1999) described the determination of benzimidazole residues including TBZ, ABZ, MBZ, FDZ, OFZ and OXI using LC-MS and LC-MS/MS optimizing, initially, by direct infusion of a standard solution of each benzimidazole. After that, BFs residues were separated using ammonium acetate and acetic acid in the mobile phase to produce protonated molecular ions. Obtained mass spectra were simple and consisted of a few fragments of the protonated molecular ion  $[M+H]^+$ . Guo et al. (2010) included BNM, MBC and FDZ to develop their multi-residue procedure for effective extraction of BFs for analysis by LC-MS/MS. Table 2 shows the individual precursor to product ion transitions specific to different analytes including precursor ion ( $Q_1$ ) and product ion ( $Q_3$ ) obtained for these authors. These methods were applied in muscle, eggs and agricultural products. Balitz (1999) obtained detection limits between 3 and 20  $\mu\text{g kg}^{-1}$ . Gou et al. (2001) improved these

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LODs because they used a QuEChERS process (salting-out liquid-liquid partitioning extraction followed by dispersive solid phase extraction), obtaining values between 0.15-0.30 ng g<sup>-1</sup>.

Compound	Q <sub>1</sub>	Q <sub>3</sub>
ABZ	266	233
BNM	291	192
MBC	192	132
FBZ	300	268
FDZ	185	92
MBZ	296	264
OXI	250	176.1
OFZ	316.2	159.1
TCB	359	343
TBZ	202	131

Table 2. Typical ions of the BFs in LC-MS and LC-MS/MS (from Balizs (1999) and Gou et al. (2010)).

To our knowledge, there not papers exclusively related with determination of BFs in environmental samples using LC-MS/MS. However, there are some published multiresidue methods that include benzimidazole compounds. For example, Jeannot et al. (2000) used LC-MS for the multi-residue analysis of pesticides in surface waters among those included CBZ. Limit of detection for CBZ was 6 ng L<sup>-1</sup>.

It is important to remember that the analysis is complicated by the instability of several benzimidazoles during analytical processes. The accepted methods have been well documented for measuring BNM or/and tiophanate-methyl as MBC or other breakdown products (Di Mucio et al., 1995; Singh et al., 2007), which entails a complete or quantitative conversion of the parent molecule to its stable metabolite prior to analysis. The major disadvantage of such a total residue approach is the conversion into other transformation products, which might also be marker species already present as their natural occurrence in the sample (Danaher et al., 2007). To overcome this problem, successive efforts have been made in developing methodology for direct determination of BNM or other benzimidazoles like tiophanate-methyl in the intact form, by addition of decomposition retardants (Chiba et al., 1977), or by virtue of the enhanced stabilities in strongly acidic aqueous media or at low temperatures (García Reyes et al., 2004; Cacho et al., 2009).

#### 4.2 Other methods

Bioassays have been used to detect benzimidazole residues in food but are more routinely used to evaluate the potency of anthelmintic substances. In the other hand, immunochemical technologies offer a number of advantages in environmental analysis (e.g., reduced time of analysis, low limits of detection (LODs) with sensitivity in many cases comparable to conventional chromatographic techniques, high throughput of samples, cost-effective detection, and adaptability to field use). However, one of the most important advantages is their possible automation that can be adapted to work remotely, on-line or at-site (Farré et al., 2007).

Within the most representative immunochemical technologies applied to environmental analysis are enzyme-based immunoassays which usually use a change in color or emission

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of light to measure the concentration of the analyte. This technique offers numerous advantages over other immuno-techniques because their signal is amplified by forming a great number of product molecules and they are widely used, especially those based on heterogeneous conditions, such as enzyme-linked immunosorbent assays (ELISAs). Although there have been a number of significant applications of ELISA technology in the area of benzimidazole analysis in biological matrices, there not significant works related with environmental samples. However, Thomas et al. (1996) developed a method that incorporates many of the advantages of immunochemical techniques into a format more suitable for routine analytical laboratories. It is high-performance immunoaffinity chromatography (HPIAC). In HPIAC, the antibody is immobilized onto a high-performance liquid chromatographic support and used as an affinity ligand to extract the target analyte from an aqueous sample injected onto the HPIAC column. Any material not specifically recognized by the antibody passes through the column to waste, while the target analyte remains bound to the immobilized antibody until the mobile phase conditions are changed to disrupt the antibody analyte interaction. The analyte is subsequently eluted and detected. These authors described the application of HPIAC coupled on-line with either HPLC-DAD or HPLC-MS to determine the fungicide carbendazim in water samples and they compared the obtained results with the ELISA technique. LODs were better with HPIAC methods ( $0.025 \text{ ng mL}^{-1}$  for HPLC-MS and  $0.075 \text{ ng mL}^{-1}$  for HPLC-DAD) than ELISA method ( $0.10 \text{ ng mL}^{-1}$ ).

Table 3 presents a summary of the most representative methods to extract and determine BFs in terms of the sample matrix and analytical parameters.

Analytes	Samples	Extraction	Determination technique	Analytical Parameters	Ref.
CBZ	soil	-	HPLC-UV/Vis	RSDs: 4.5% LODs: $0.3 \mu\text{g mL}^{-1}$	Huebra et al. (2000)
CBZ	water	SLM, MMLLE	HPLC-UV	RSDs: < 5% LODs: $0.1 - 0.5 \mu\text{g L}^{-1}$	Sandahl et al. (2000)
CBZ, FDZ, TBZ	water	SPE	cross-section fluorimetry	Recovery: 90-120% RSDs: 1.5 - 4.8 % LODs: $0.017 - 0.29 \text{ ng mL}^{-1}$	Zamora et al. (2000)
CBZ, FDZ, TBZ	water	SPE	spectrofluorimetry	Recovery: 91-115% RSDs: 0.02-4.0 % LODs: $0.1-100 \mu\text{g L}^{-1}$	Garrido et al. (2002)
CBZ, FDZ, TBZ	water	SPE	luminescence spectrometer	Recovery: 97-109% RSDs: 3.3-6.4% LODs: $0.001-0.13 \mu\text{g L}^{-1}$	Zamora et al. (2002)
CBZ, BNM, FDZ, TBZ	water	CPE	HPLC-Fluorescence	Recovery: 70-96 % LODs: $0.01-6 \text{ ng mL}^{-1}$	Halko et al. (2004)

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Analytes	Samples	Extraction	Determination technique	Analytical Parameters	Ref.
FDZ	water	-	fluorescence spectrophosphorimeter	RSDs: 3.1% LODs: 95 ng L <sup>-1</sup>	Salinas et al. (2005)
CBZ, BNM, FDZ, TBZ	soil	MAME	HPLC-Fluorescence	Recovery: 70-105 % LODs: 0.02-0.06 µg g <sup>-1</sup> 25-30 µg g <sup>-1</sup> (MBC/BNM)	Halko et al. (2006)
CBZ	soil	ultrasonic solvent extraction, QuEChERS, PLE	GC-MS HPLC-MS/MS	RSDs: <20 % LODs: 0.01 ng g <sup>-1</sup>	Lesueur et al. (2007)
CBZ, BNM, FDZ, TBZ	water	SPME	HPLC-Fluorescence	RSDs: <9.0 % LODs: 0.03-1.3 ng mL <sup>-1</sup>	López Monzón et al. (2007)
ABZ, BNM, FBZ, FDZ, CBZ, TBZ	spiked river, tap and well water	MISPE	HPLC-DAD	Recovery: 89-105% RSDs: 2.1-6.7% LODs: 0.03-0.09 µg L <sup>-1</sup>	Cacho et al. (2009)
CBZ, TBZ	water, soil	LLME	HPLC-Fluorescence	Recovery: 50.8-70.9% RSDs: 3.5-6.8% LODs: 0.5-1.0 µg L <sup>-1</sup> ; 1.0-1.6 ng g <sup>-1</sup>	Wu et al. (2009)
CBZ, FDZ, TBZ	river, underground water	supramolecular solid-phase extraction	HPLC-Fluorescence	RSDs: 2-6 % LODs: 0.1-32 ng L <sup>-1</sup>	Moral et al. (2009)
BNM, CBZ, TBZ	water	MISPE online	HPLC-DAD	Recovery: 87-95% RSDs: <5% LODs: 2.3-5.7 ng L <sup>-1</sup>	Zamora et al. (2009)

**Abbreviations:** RSD: Relative standard deviation, LOD: limit of detection, LC: liquid chromatography, GC: Gas chromatography, HPLC: high performance liquid chromatography, UV: Ultraviolet visible detection, PDA: Photodiodearray detection, MS: mass spectrometry, APCI: atmospheric pressure chemical ionisation, MIP: molecularly imprinted polymer, MISPE: Molecularly imprinted solid-phase extraction, ELISA: enzyme linked immunoassays, SPE: solid phase extraction; LLME: liquid-liquid microextraction; SPME: solid phase microextraction; MAME: Micellar assisted microwave extraction; CPE: cloud point extraction.

Table 3. Most representative methods to determine benzimidazole fungicides (BFs) in environmental samples.

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## 5. Conclusions and future perspectives.

This chapter describes the most relevant aspects which are involved in the determination of benzimidazole fungicides (BFs) in environmental samples. It is well known that sample preparation is one of the most critical steps in the determination of trace pollutants in different environmental matrices. In order to improve the quality of the analytical methods used, sample preparation techniques should be improved for this proposal. On the other hand, although some of the techniques presented in this overview are known and have been applied (HPLC with UV and/or fluorescence detection) to determine BFs in environmental samples, trends in this field should develop multi-residue methodology that allows the determination of the complete range of benzimidazole fungicides (BFs) in different environmental matrices in a single analysis. In this sense, advanced analytical methods have been developed and optimized with the aim of improving precision and sensitivity. LC-MS and LC-MS/MS have found more widespread application in environmental analysis offering more sensitive detection and increased confidence in reporting results. It should be desirable the development of specific methods for BFs in environmental samples using LC-MS/MS combined with new sample treatments to apply to real environmental samples. Alternatively, HPLC coupled to UV and fluorescence detection in series may offer a low cost to LC-MS/MS and may be particularly effective in the optimization of sample treatments.

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## 1 **7 Conclusion**

2           The presented thesis is focus on the determination of pharmaceuticals in the environment,  
3 which become increasing concern due to their presence in the environment. Traditional extraction  
4 techniques are replaced by microextraction and greener methods, more suitable to environment.  
5 However, environmental matrices are complex and concentration of target analytes are very low,  
6 therefore solid phase extraction is still often used method for sample preparation. Recently, mass  
7 spectrometry is used more than the other detection techniques. This was confirmed by  
8 summarization of methods for sample preparation and determination of steroid hormones and  
9 benzimidazole fungicides.

10           The other part of the thesis is based on sample preparation of environmental waters samples  
11 followed by liquid chromatography. New methods for determination of fluoroquinolone antibiotic  
12 and steroid hormones were developed, validate and applied to the real environmental samples.

13           First study was focused on the determination of fluoroquinolones. For developed of  
14 analytical method systematic method development designed by Waters Corporation was used and  
15 compared by other known methods. Target analytes were extracted and detected in pH 10.5 in  
16 contrast to scientific literature. The optimized method was applied on samples from wastewater  
17 treatment plant of hospital in Hradec Králové.

18           The second and third study dealt with determination of endocrine disruptors, steroid  
19 hormones, respectively. Extraction method was performed by in-tube solid phase microextraction  
20 coupled with high performance liquid chromatography with ultraviolet and fluorescence detection.  
21 In dynamic mode in-tube SPME was automatized and whole process was operated by autosampler.  
22 In static mode, manual Reodyne, where injection loop was replaced, was used for extraction. Both  
23 methods were optimized by experimental design and applied to the sea water and wastewater,  
24 respectively.

25           The detection of pharmaceuticals in environment is important and it is necessary to continue  
26 in their monitoring to determine their fate and occurrence in the environment. Residues of  
27 pharmaceuticals can affect aquatic organism as well as human health. Additionally, monitoring of  
28 pharmaceuticals in the environment shows insufficiency of wastewater treatment plants and the  
29 important necessity of improvements of wastewater treatment process.

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