Appendix

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

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

19th 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 AUTOMATICE METHOD DEVELOPMENT VERSUS CONVENTIONAL APPROACH – OPTIMIZATION AND DEVELOPMENT OF ANALYTICAL METHODOLOGY FOR THE DETERMINATION OF FLUOROCHINOLONE ANTIBIOTIC

12th 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

34th 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 DETERMINTATION OF STEROIDS FROM ESTROGEN AND PROGESTIN GROUPS

18th 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 DETERMNINATION OF ANTIBIOTIC IN ENVIRONMENTAL WATER

25th 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

70th 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

36th 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

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

36th 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

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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. Cromatogr. 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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Introduction

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 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

1. Ouzang, 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)

Acknowledgements

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

Instrumentation and chromatographic conditions

The chromatografic system was a Varian (autosampler 410) with DAD. Chromatographic separation was performed on NovaPack C_{18} 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 in15 min. After that, until 20 min, separation was in isocratic mode. Flow rate was 1.0 mL/min. The temperature into termostated column compartment was set at 30°C.

	Analyte	Abbreviation	t_R (min)	λ (nm)
1	Estriol	E3	5.3	224
2	Bisphenol A	BPA	9.8	224
3	Norethisterone	NORE	14.2	244
4	Ethynylestradiol	EE2	14.9	224
5	D-norgestrel	NORG	17.9	244

Table 1. Target compounds, retention times (t_R) and detection wavelengths (λ)

Experimental procedure

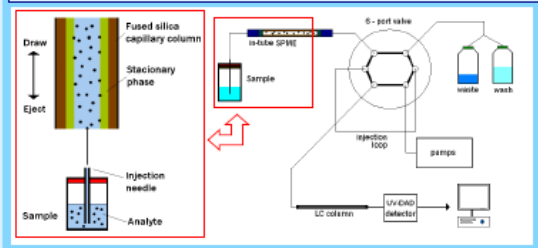
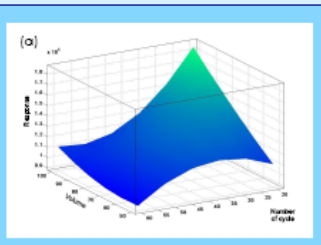


Figure 1. Scheme in-tube SPME-HPLC-DAD

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



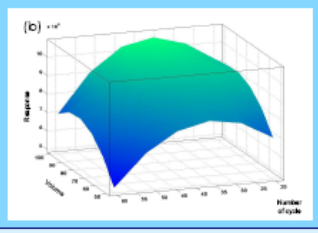


Figure 2.
Response of surface using a design matrix 2^3 (number of cycles (20, 40, 60) draw/eject and volume (50, 75, 100 µL) of each cycle) of BPA using:

(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)

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	6.6	5 – 1000	0.63	4.6	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	0.46	6.4	6.2	2 – 1000	0.22	6.2	4.4
NORG	2 – 1000	0.05	9.6	7.6	1 – 1000	0.05	5.8	2.7

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

Carboxen								
Analyte	Added (ng/mL)	mQ-water		sea water		WWTP		
		Preconcentration factor	RSD %	Recovery %	RSD %	Recovery %	RSD %
E3	200	12.2	2.8	111.4	5.5	79.9	8.4
BPA	100	13.3	3.1	98.4	2.3	96.4	7.0
NORE	100	17.1	5.8	83.0	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

Supel-Q								
Analyte	Added (ng/mL)	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

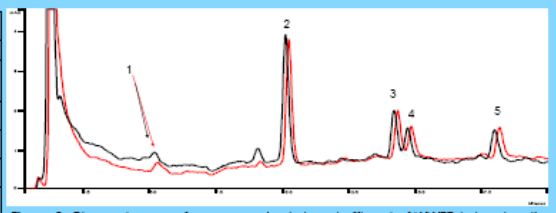


Figure 3. Chromatograms from sea water (■) and effluent of WWTP (■); extraction with Carboxen

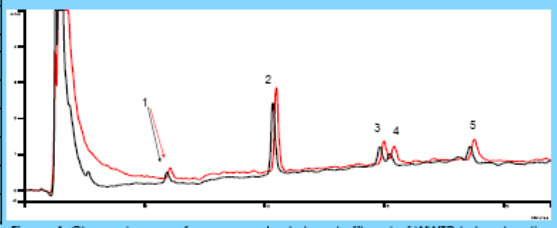


Figure 4. Chromatograms from sea water (■) and effluent of WWTP (■); extraction with Supel-Q

Conclusions

We have optimised in-tube SPME-HPLC-DAD for the simultaneous determination of estriol, bisphenol, norethisterone, ethynylestradiol 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.

36th 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 comlumns in the optimization of in-tube SPME extraction of endocrine disruptors in environmental liquid samples

Rome, October 09, 2010

Dr Roberto Pilloton
(Chairman)

Prof. Dr D. Klockow
(President, IAEAC)

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.

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Accepted April 29, 2010

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
DOI 10.1002/jssc.201000215

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 (μg/L) [5] than in municipal wastewaters (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; **PEFLO**, 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

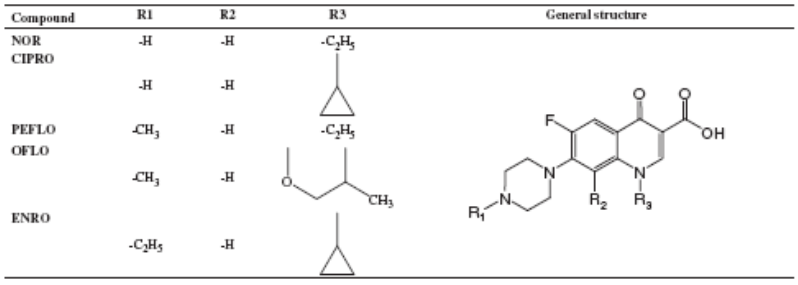

Figure 1. Chemical structures of selected fluoroquinolones

Table 1. An overview of methods for the determination of FQs in environmental waters[a)]

Substances determined	Matrix sample preparation	Stationary phase analytical column	Mobile phase	Detection	Analysis time (min)	Validation data	Ref.
9FQs: CIPRO, DIFL, ENRO, LOME, NOR, OFLO, PIP, SARA, TOS	WWTP effluents, surface water (river and lake water) SPE (150–500 mL, pH 3)	YMC ODS-AQ S-3 (50 × 4.0 mm) Discovery RP-Amide C16 (50 × 4.0 mm)	A: water (pH 3.0)+ACN (98:2) B: ACN 5:95, gradient	ESI-MS $[M+H]^+$ FD Exc: 278 nm Em: 445–500 nm	35	r^2>0.9892 LODMS = 8.6–49 ng/L	[3]
4FQs: OFLO, NOR, CIPRO, ENRO	Wastewater SPE (pH 4.5)	Chromolith Performance RP-18e (100 × 4.6 mm)	0.025 M H3PO4 (pH 3.0 by TBA): MeOH/ACN 920:70:10, isocratic	FD Exc: 278 nm Em: 450 nm	12	r^2>0.997 LOD = 8.5–85 ng/L LOQ = 25–250 ng/L	[4]
20FQs: PIP, FLE, OFLO, PEFLO, ENO, NOR, CIPRO, DANO, ENRO, LOME, DIFL, SARA, GATI, SPAR, MOXI, CINO, OXO, NAL, FLU, PIRO IS = NOR-d_5	Wastewaters, river water SPE (200–800 mL, pH 3)	ACQ UPLC BEH C18 (100 × 2.1 mm, 1.7 μm)	MeOH/0.1% FAc 10:90, gradient	ESI-MS/MS $[M+H]^+$	12	r^2>0.996 LOD = 0.6–50 ng/L	[6]
10FQs: 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]
3FQs: OFLO, NOR, CIPRO IS – CIPRO-$^{13}C^{15}N$	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 Em: 450 nm ESI-MS ESI-MS/MS $[M+H]^+$	20	r^2>0.999 RSD < 6% LOQ = 2–10 ng/L	[14]
5FQs: 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 $[M+H]^+$	7	r^2>0.997 RSD = 0.5–9.7% LOD = 7–29 ng/L	[15]
3FQs: NOR, CIPRO, ENRO	Surface water (river water) SPE (1000 mL, pH 4)	Chromolith Performance RP-18e (100 × 4.6 mm)	0.025 M H_3PO_4 (pH 3.0 by TBAI/MeOH 960:40, isocratic	FD Exc: 278 nm Em: 450 nm	16	r^2>0.994 LOQ = 25 ng/L	[16]
9FQs: 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	r^2>0.9889 RSD < 5% LOQFD = 11–60 ng/L LOQMS/MS = 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 $[M+H]^+$			
9FQs: CIPRO, DANO, ENO, ENRO, NOR, CINO, FLU, NAL, OXO	Surface water (lake and river water) SPE (250 mL, pH 4)	Polarity dC18 (150 × 3 mm, 3 μm)	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; GATI, gatifloxacin; MARBO, marbofloxacin; MOXI, moxifloxacin; PIP, pipemidic acid; PIRO, piromidic acid; SPAR, sparfloxacin; TOS, tosufloxacin.

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_5 – 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^3/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-

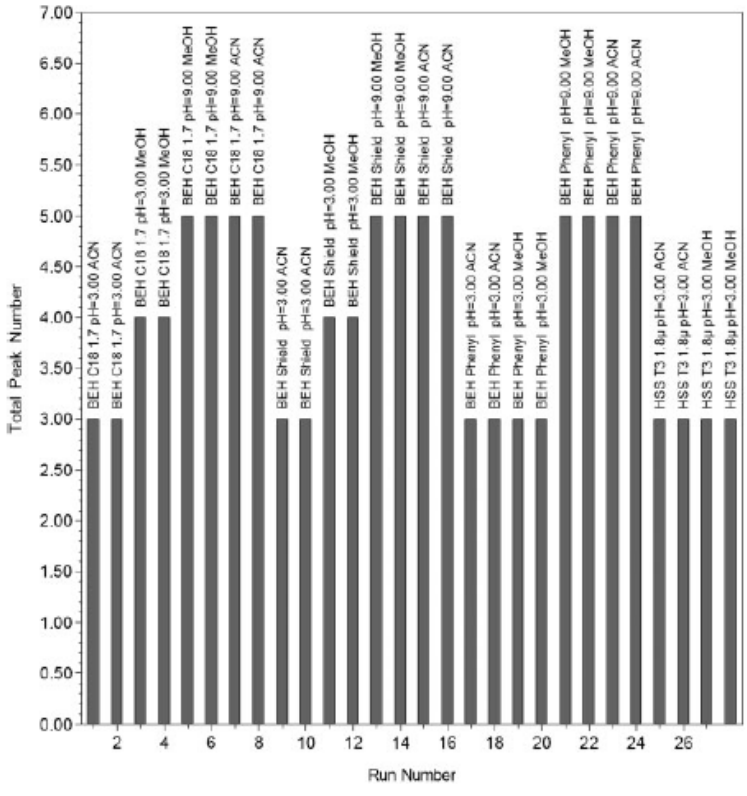

Figure 2. An example of report generated by Empower software. Total peak number report shows the number of separated peaks in each chromatogram.

tion level. Internal standard NOR-d_5 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 $\lambda_{exc} = 278$ nm and $\lambda_{em} = 450$ 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 $\lambda_{exc} = 310$ nm and $\lambda_{em} = 415$ 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 was 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_5 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_R
NOR	319.9	$[M+H]^+$	301.9	$[M+H-H_2O]^+$	0.05	30	20	3.4	1.25
			276.2	$[M+H-CO_2]^+$	0.05	30	15		
NOR-D5	324.9	$[M+H]^+$	307.0	$[M+H-H_2O]^+$	0.05	30	20	4.0	1.24
			281.1	$[M+H-CO_2]^+$	0.05	30	15		
CIPRO	331.9	$[M+H]^+$	314.0	$[M+H-H_2O]^+$	0.05	25	20	1.1	1.37
			230.5	$[M+H-H_2O-C_2H_5N-C_3H_4]^+$	0.05	30	35		
PEFLO	333.9	$[M+H]^+$	289.9	$[M+H-CO_2]^+$	0.05	30	15	1.5	1.62
			316.3	$[M+H-H_2O]^+$	0.05	30	20		
OFLO	362.0	$[M+H]^+$	261.2	$[M+H-H_2O]^+$	0.05	30	25	2.6	1.71
			318.0	$[M+H-CO_2]^+$	0.05	30	20		
ENRO	359.9	$[M+H]^+$	316.1	$[M+H-CO_2]^+$	0.05	30	20	3.5	2.00
			341.9	$[M+H-H_2O]^+$	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-

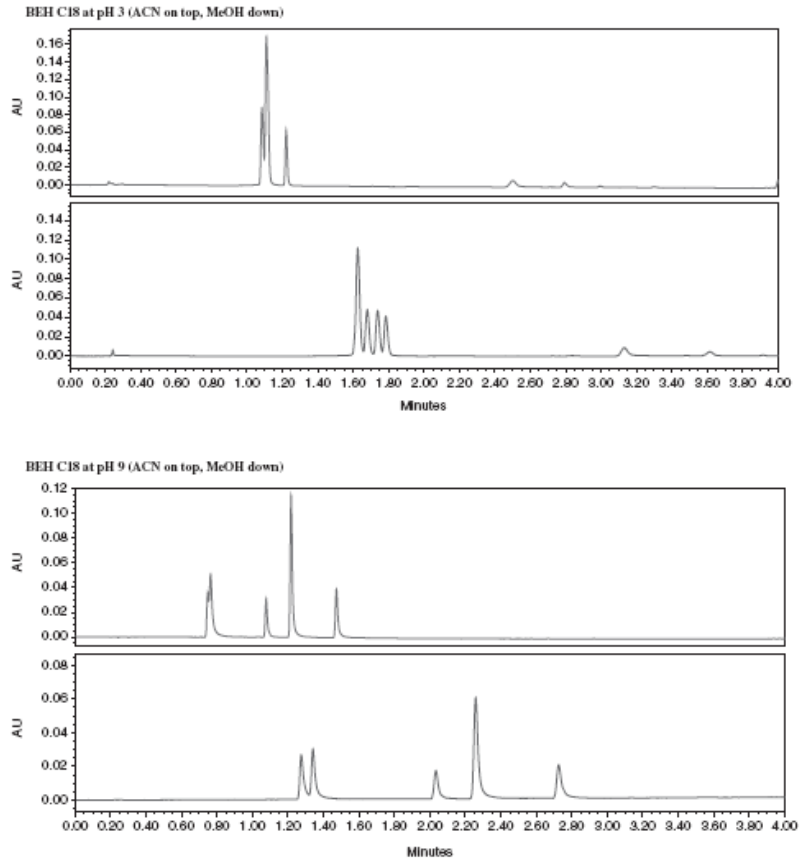

Figure 3. The influence of organic modifier and pH on separation of FQs on C18 analytical columns, conventionally used in FQs analysis.

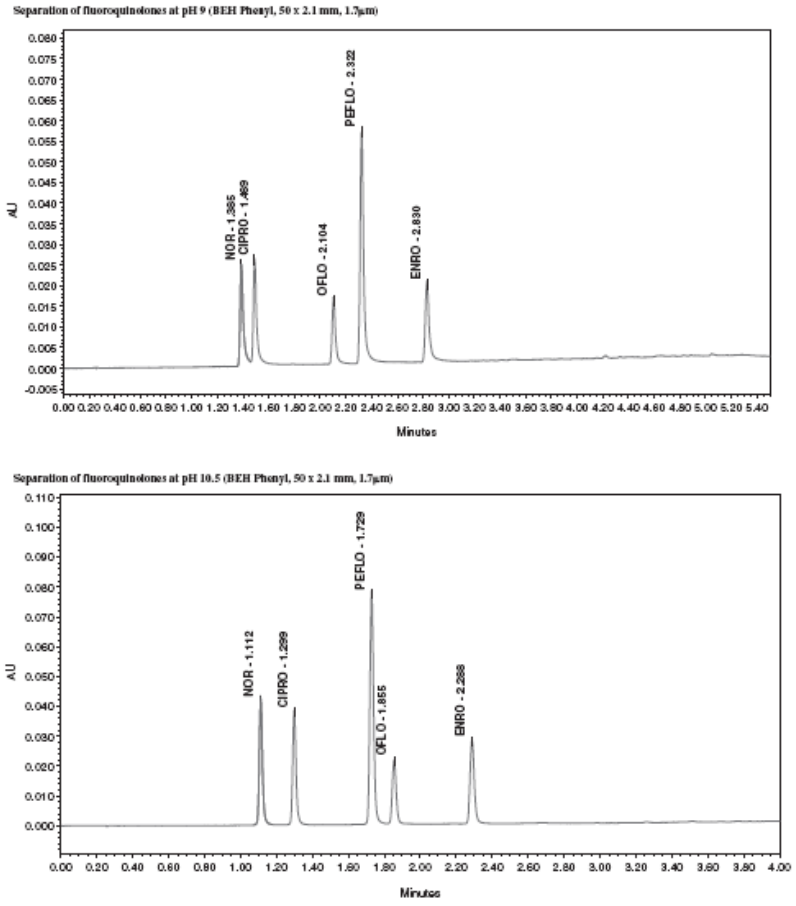

Figure 4. Chromatogram of standard solution at pH 9 (upper) and at pH 10.5 (lower) obtained on UHPLC system. The peak order switch is demonstrated in dependence on relatively small change of pH.

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

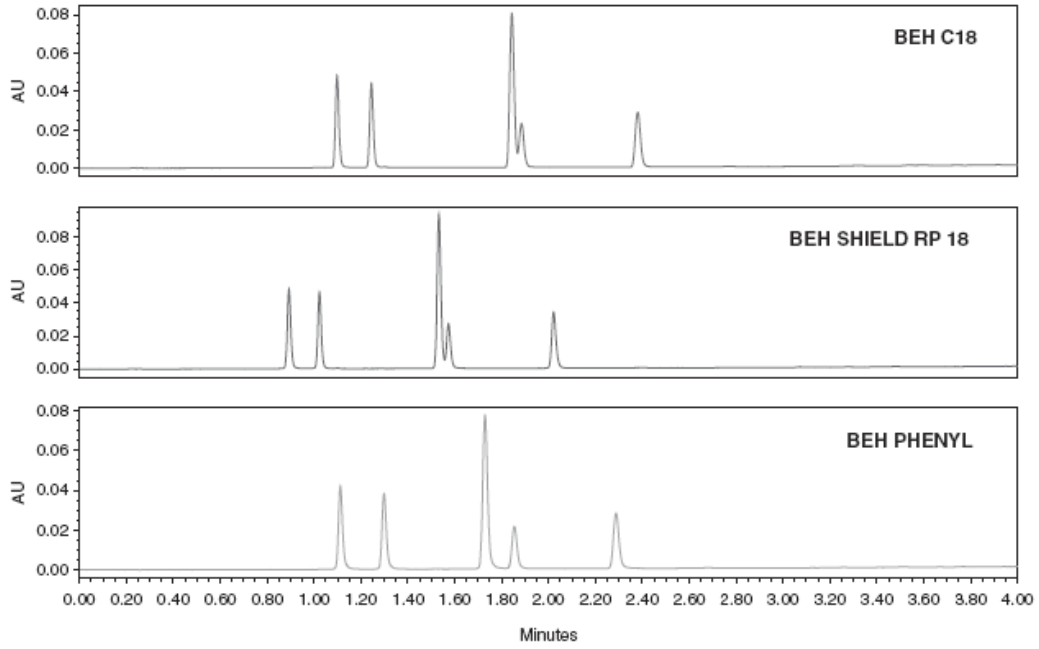

Figure 5. Chromatograms obtained during systematic method development on different stationary phases. Mobile phase composition: MeOH and AmAc pH 10.5.

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 × 2.1 mm, 1.7 μm) analytical column. Recently, there was a published study dealing with the stability of analytical columns [24]. They reported the unstability of XBridge phenyl analytical column (Waters) over pH 7. However, in this study, we have not observed any changes of peak shapes of 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 λ_{exc} = 310 nm and λ_{em} = 447 nm, whereas at basic pH the optimal λ_{exc} and λ_{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 λ_{exc} and λ_{em} at acidic pH were 311 and 447 nm, whereas at basic pH these optimal λ_{exc} and λ_{em} were 308 and 415 nm, respectively. OFLO showed completely different spectra from the other FQs. At acidic, λ_{exc} = 298 nm and λ_{em} = 490 nm, whereas at basic pH the optimal λ_{exc} and λ_{em} wavelengths were 295 and 464 nm, respectively. Consequently, further assay comparing the sensitivity of FD of FQs at commonly used λ_{exc} = 278 nm and λ_{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[a)]

	UHPLC-FD at basic pH					UHPLC-MS/MS				
SST	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					
t_R	2.49	3.07	3.89	4.31	5.23	1.25	1.37	1.62	1.71	2.00
Repeatability t_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
Validation										
Linearity (r^2)	0.9998	0.9985	0.9980	0.9991	0.9999	0.9990	0.9989	0.9986	0.9994	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	1.00–500.00	1.00–500.00	5.00–500.00	0.50–500.00	0.50–500.00
IDL (pg injected into column)						1.00	1.00	2.00	0.20	0.20
LOD (ng/mL)	50.00	25.00	25.00	10.00	25.00	0.50	0.50	1.00	0.10	0.10
LOQ (ng/mL)	150.00	100.00	50.00	50.00	100.00	1.00	1.00	5.00	0.50	0.50
Tested levels (L1, L2 and L3) (ng/mL)	250, 1000, 2500	250, 1000, 2500	250, 1000, 2500	250, 1000, 2500	250, 1000, 2500	50, 100, 250	50, 100, 250	50, 100, 250	50, 100, 250	10, 50, 100
Recovery L1 (%)	128.95	606.57	79.11	88.97	88.99	100	102	92	100	102
Recovery L2 (%)	108.65	292.00	63.56	75.21	74.66	99	100	119	101	102
Recovery L3 (%)	80.63	37.76	83.39	92.37	96.18	98	90	85	90	100
Intra-day precision L1 (%)	0.22	1.54	1.71	1.22	1.92	5.00	3.17	4.02	2.10	4.29
Intra-day precision L2 (%)	4.21	9.95	2.08	1.47	0.42	3.00	2.58	0.96	1.92	2.27
Intra-day precision L3 (%)	4.99	9.45	1.59	1.13	1.32	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

	UHPLC-FD at acidic pH				
Linearity (r^2)	0.9998	0.9986	0.9993	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.

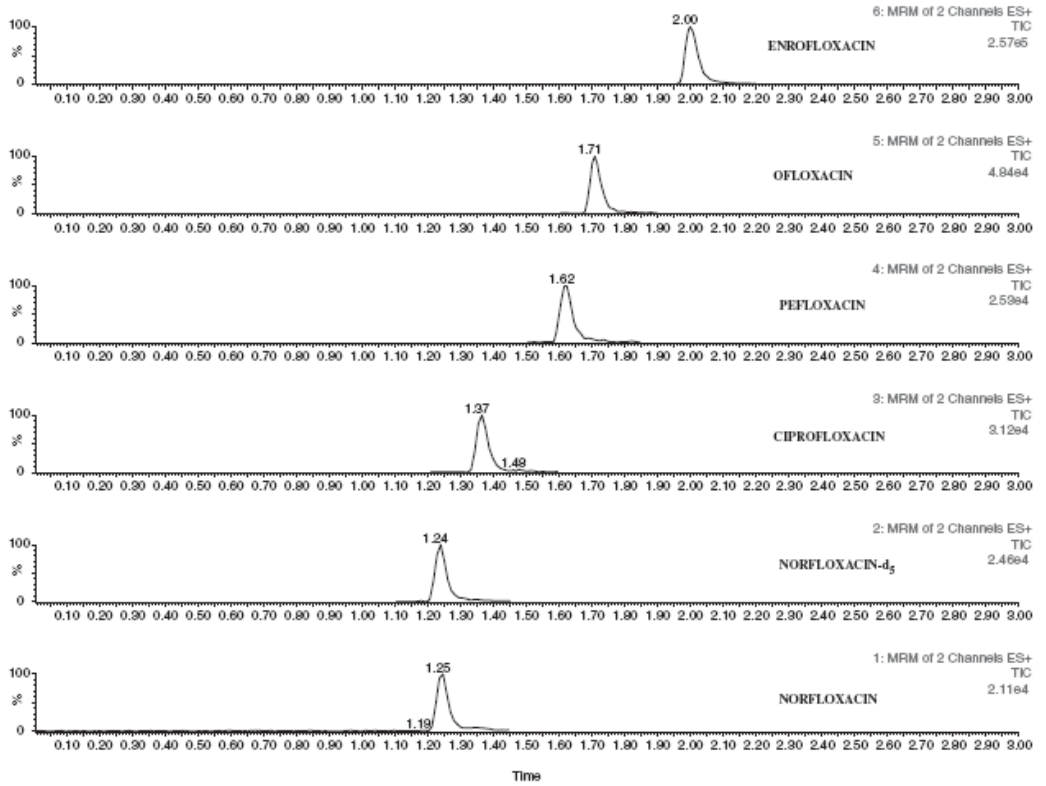

Figure 6. Chromatogram of standard mixture of FQs.

[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_5 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_5 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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Supplement II

Jana Aufartová, Cristina Mahugo-Santana, Zoraida Sosa-Ferrera, José Juan Santana-Rodríguez, Lucie Nováková, Petr Solich

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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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.

Jana Aufartová received her Master Degree in Pharmacy in 2008 at Charles University in Prague (Czech Republic). Since that year, she is working as a Ph.D. student under the guidance of Professor Solich and currently under the guidance of Professors Santana Rodríguez and Sosa-Ferrera at University of Las Palmas de Gran Canaria (ULPGC) (Spain). Her research is focused on development of new methodologies for the extraction, preconcentration and clean-up in the determination of pharmaceuticals (mainly fluoroquinolones and steroids) in environmental samples.

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Petr Solich is currently Professor and Head of the Analytical Chemistry Department at the Faculty of Pharmacy, Charles University in Hradec Králové, Czech Republic. His research interests are automation of analytical procedures; flow methods (flow injection analysis, sequential injection analysis and sequential injection chromatography); chromatographic methods (mainly UHPLC with sub-2-micron columns or use of monolithic columns) applied to environmental analysis of low concentrations of pharmaceuticals or bioanalytical analysis for determination of biomarkers and modern sample-preparation methods. He has published more than 140 research papers and has been responsible for more than 20 research grants from different disciplines (pharmaceutical, environmental, and bioanalytical).

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 estriol (E3), are C_{18} steroids that differ in the oxidation state of their rings. These C_{18} 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_{21}-steroid pregnane such as progesterone (P). Androgens are C_{19} 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 $ng\,L^{-1}$, but values of 51 $ng\,L^{-1}$ 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]. Influents 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.

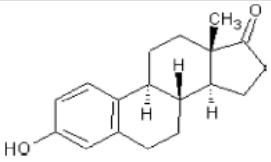
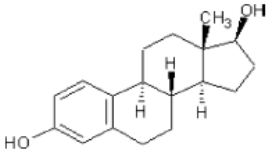
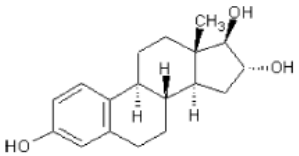
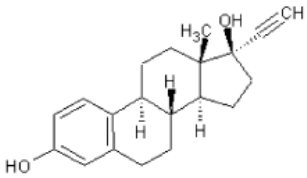
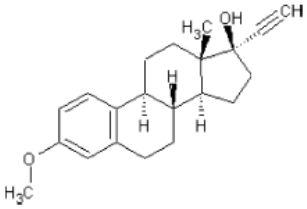
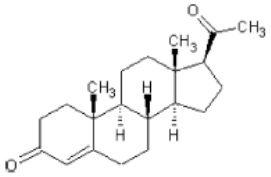
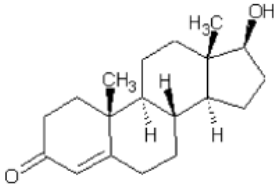
Name (Abbr.)	Systematic name	Synthetic/ natural (S)/(N)	Group	pK$_a$ log Kow	Summary structure	Structure
Estrone (E1)	Estra-1,3,5(10)-trien-17-one	N	C_{18}	10.77 3.4	$C_{18}H_{22}O_2$	
Estradiol (E2)	Estra-1,3,5(10)-triene-3,17diol	N	C_{18}	10.08 4.0	$C_{18}H_{24}O_2$	
Estriol (E3)	(16-alpha,17-beta)-estra-1,3,5(10)-triene-3,16,17-triol	N	C_{18}	10.4 2.4	$C_{18}H_{24}O_3$	
Ethynylestradiol (EE2)	19-norpregna-1,3,5(10)-triene-20-yn-3,17-diol	S	C_{18}	10.4 3.7	$C_{20}H_{24}O_2$	
Mestranol (MES)	19-norpregna-1,3,5(10)-triene-20-yn-17-ol	S	C_{21}	13.1 4.7	$C_{21}H_{26}O_2$	
Progesterone (P)	Pregn-4-ene-3,20-dione	N	C_{21}	n.a. 3.9	$C_{21}H_{30}O_2$	
Testosterone (T)	Androst-4-en-3-one	N	C_{19}	9.74 3.3	$C_{19}H_{28}O_2$	

Table 1 (*Continued*)

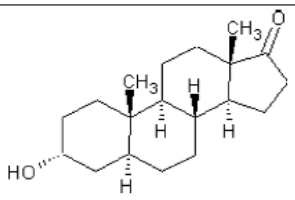
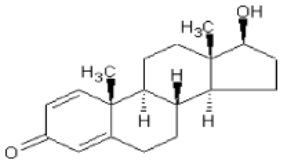
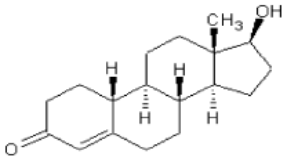
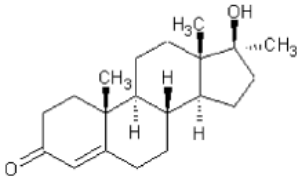
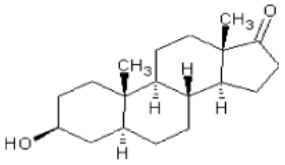
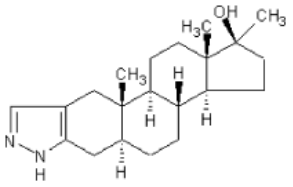
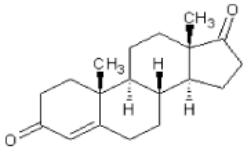
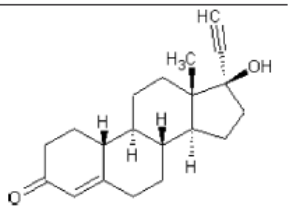
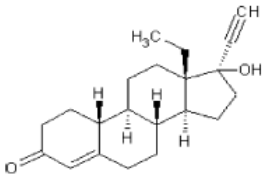
Name (Abbr.)	Systematic name	Synthetic/ natural (S)/(N)	Group	pK_a log Kow	Summary structure	Structure
Androsterone (ADR)	Androstan-17-one	S	C_{19}	15,14 3,7	$C_{19}H_{30}O_2$	
Boldenone (BOLD)	Androsta-1,4-dien-3-one	S	C_{19}	15,05 3,1	$C_{19}H_{26}O_2$	
Nandrolone (NDL)	Estr-4-en-3-one	S	C_{18}	15,06 2,6	$C_{18}H_{26}O_2$	
Methyltestosterone (MeT)	Androst-4-en-3-one	S	C_{19}	15,13 3,4	$C_{20}H_{30}O_2$	
Epiandrosterone (EADR)	Androst-2-[3,2-c]pyrazol-17-ol	N	C_{19}	15,14 3,8	$C_{19}H_{30}O_2$	
Stanozol (SZL)	Androst-2-eno[3,2-c]pyrazol	S	C_{21}	3,84; 15,15 5,5	$C_{21}H_{32}N_2O$	
Androstenedione (ADD)	Androst-4-ene-3,17-dione	N	C_{19}	n.a. 2,8	$C_{19}H_{26}O_2$	

Table 1 (*Continued*)

Name (Abbr.)	Systematic name	Synthetic/ natural (S)/(N)	Group	pK$_a$ log Kow	Summary structure	Structure
19-Norethisterone (19-No)	19-Norpregn-4-en-20-yn-3-one	S	C_{21}	13.9 3.0	$C_{20}H_{26}O_2$	
Norgestrel (Ng)	13-ethyl-17-ethynyl-19-nortestosterone	S	C_{21}	13.09 3.5	$C_{21}H_{28}O_2$	

[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 pg g^{-1} to a few 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 sub-ng g^{-1} to 11.9 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 (CCα) and detection limit (CCβ).

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^{-1} or sub-ng g^{-1} 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 desire 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, trimethylolpropanetrimethacrylate (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

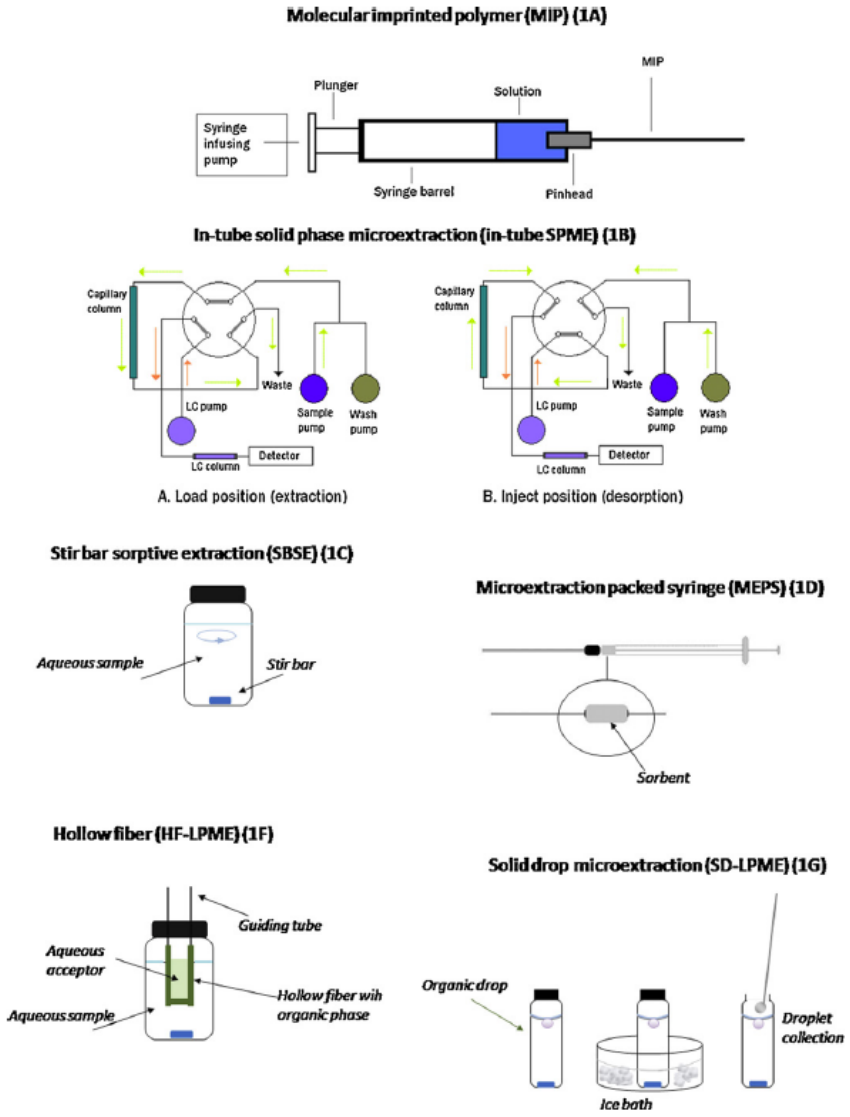


Fig. 1. Microextraction approaches used in the determination of hormones in environmental samples.

the determination of androsterone (ADR), stanolone (STAN), androstenedione and methyltestosterone in spiked human urine. The obtained LODs were in the range of 0.2–0.8 μg 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 17β-ethynylestradiol, in fishery samples [57]. An MIP-coated SPME fiber with 17β-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 0.98–2.39 μg 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 mg L^{-1} were obtained by Jiang et al. [58] for the determination of estrogenic compounds (17β-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 17β-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

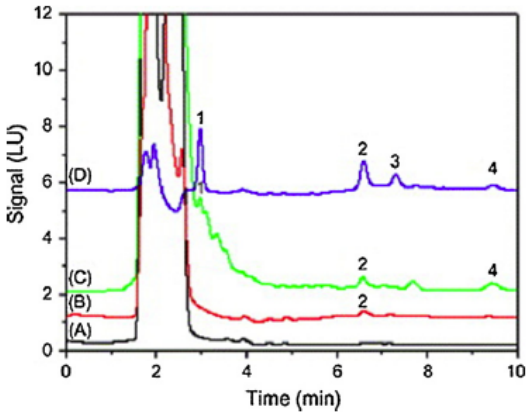


Fig. 2. Analysis of endocrine-disruptor compounds using in-tube SPME–HPLC. Tap water sample (A), lake water sample (B), sewage water sample (C) and standard sample at 2 ng mL^{-1} level (D). The PEEK tube extraction column was applied to the microextraction device. Peaks: 1: estriol, 2: bisphenol A, 3: 17β-estradiol, and 4: 17α-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 absorbents. 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 (17β-estradiol, estriol, bisphenol A and 17α-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 μg mL^{-1} and 0.02–0.35 μg 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, ethynylestradiol, 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 μL 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 μg 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 stanozol from human urine samples [73]. The optimum in-tube SPME conditions were also 20 draw/eject cycles with a sample size of 40 μL. However, the steroid LODs were higher, in the range of 0.009–0.182 μg 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 automatisation 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 twisters® (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 μg L^{-1} sample level in ion extraction mode and extended to the ng L^{-1} and sub-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, 17β-estradiol and 17α-ethynylestradiol, in river water samples by derivatisation with acetic acid anhydride. The detection limits were also in the ng L^{-1} range. Later, the same authors [78] developed a "dual derivatisation method" to determine 17β-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). 17β-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 17β-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 μg 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, 17α-estradiol, 17β-estradiol, 17α-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 μL of PDMS were used, yielding recoveries ranging from 11.1% (17β-estradiol) to 100.2% (mestranol) and limits of detection between 0.3 and 1.0 $\mu g\,L^{-1}$.

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 β-cyclodextrin (PDMS/β-CD) prepared by a sol–gel technique. They used this SBSE and LC-FD to determine estrogens (estriol, estrone, 17β-estradiol and 17α-ethynylestradiol) in environmental water samples. This technique allowed LODs within the range of 0.04–0.11 $\mu g\,L^{-1}$ 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 $\mu g\,L^{-1}$, 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 determined estrone, 17β-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 $ng\,L^{-1}$ for water samples and 0.02 $ng\,g^{-1}$ for solid samples, whereas the LOQs were 5.0 $ng\,L^{-1}$ for water samples and 0.06 $ng\,g^{-1}$ 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 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 automatised 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.

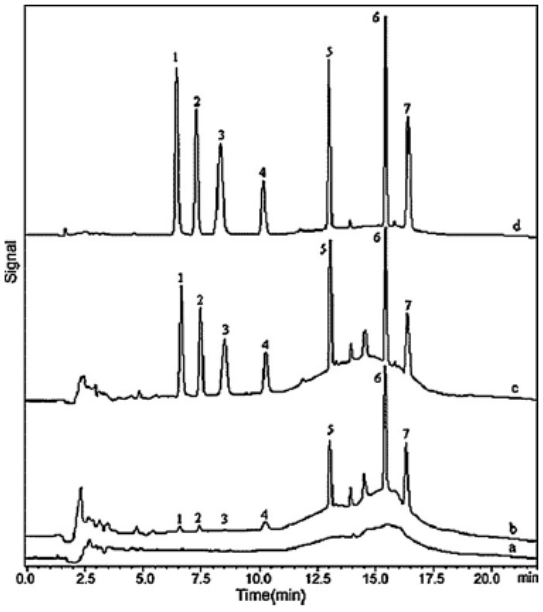


Fig. 3. Chromatograms of seven steroid hormones (SSHs). (A) Direct injection of spiked Milli-Q-purified water sample with each SSH at 50 $ng\,mL^{-1}$. (B) Spiked Milli-Q-purified water sample with each SSH at 50 $ng\,mL^{-1}$ and treated with stir bar based on polydimethylsiloxane material (SBSEC). (C) Spiked Milli-Q-purified water sample with each SSH at 50 $ng\,mL^{-1}$ and treated with stir bar based on monolithic material (SBSEM). (D) Standard sample with each SSH at 5,0 $\mu g\,mL^{-1}$. Peaks: 1: nortestosterone, 2: testosterone, 3: diethylstilbestrol, 4: methyltestosterone, 5: progesterone, 6: testosterone propionate and 7: nandrolone phenylpropionate. Chromatogram is taken from [84].

2.1.4. 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 μL) 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 μL, 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_{18}, C_8, and C_2), 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 17α-ethynylestradiol, in water samples. The microextraction was carried out with a MEPS device made with a 100 μL 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_{18}. 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 μL of sample were between 0.2 and 266 ng L^{-1} 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 (16α-hydroxyandrosterone, 2α-hydroxytestosterone, 11-keto,5β-androstanedione, 6α-hydroxyestradiol and 7β-hydroxypregnenolone) and then applied to urine samples collected from control versus androstenedione-treated bovines. They used the BIN assembly with a C_{18} 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 μg L^{-1} 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, 17β-estradiol and 17α-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^{-1} 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 (17β-estradiol, estrone and 17α-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 μL) of the hollow fiber membrane was utilised for depleting extraction. LODs of 1.6–10 ng L^{-1} 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 μg L^{-1}.

Another steroid hormone, progesterone, was determined in human serum sample using this microextraction technique coupled to GC–MS. The 10 μL microsyringe used for this miniaturised HF-LPME procedure allowed an LOD of 0.5 μg L^{-1} 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, α-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 μg L^{-1} 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 17β-estradiol, 17β-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

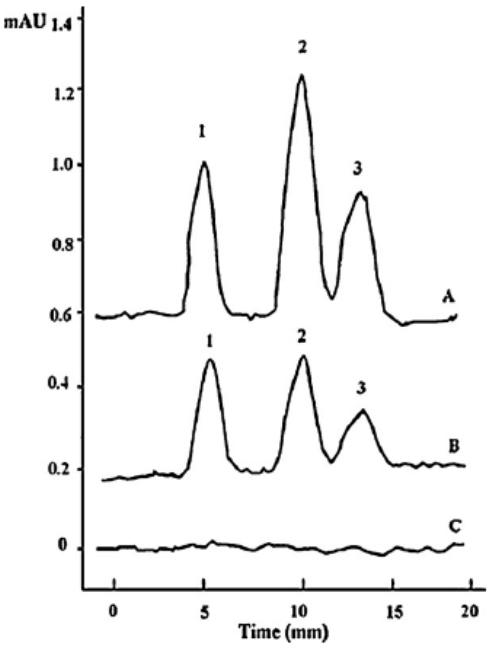


Fig. 4. Chromatograms of 100 μg L^{-1} estrogen spiked solutions of milk samples and determination of three estrogens in spiked sample solutions with molecularly imprinted polymer-coated polypropylene hollow fiber tube (MIP-HFT) and the non-imprinted polymer hollow fiber tube (NIP-HFT). (A) Spiked sample 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 μg L^{-1} for spiked river water and from 1.4 to 3.1 μg L^{-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 Na_2SO_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 μg L^{-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^{-1}	[50]
E3, E1, 17β-EE2, 17β-E2	Fish and shrimp tissue	SPME MIP	LC-UV	80,0–94,0	0,98–2,3 μg L^{-1}	[51]
17β-E2, E3, DES	Fish and prawn tissue	MISPE	LC-UV/FD	78,3–84,5	0,023–0,76 mg L^{-1}	[52]
17β-E2, E3,BPA, 17α-EE2	Surface waters	In-tube SPME	LC-FD	86–116	0,006–0,1 ng mL^{-1}	[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^{-1}	[55]
BOLD, NDL, T, MeT, EADR, ADR, STAN	Urine	In-tube SPME	LC–MS	85,7–117,3	9–182 pg mL^{-1}	[56]
E1, 3α-H-5β-A-16, 3β-H-5,16-ADD, 3α-H-5α-A-16, 5α-A-16-e-3, δ,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^{-1}	[59]
E1, 17β-E2, 17β-EE2	Water	SBSE-TD	GC–MS	90,3–105,7	0,2–1 pg mL^{-1}	[60]
17β-E2	River water	SBSE-TD	GC–MS	93,1–98,4	0,5–100 pg mL^{-1}	[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^{-1}	[63]
E3, 17β-E2, 17α-EE2, E1, BPA	Water	SBSE	LC-FD LC-UV	84,6–124,1	0,04–0,11 μg L^{-1} 8 ng L^{-1}	[64]
T, P, MeT, DES, NDL phenylpropionate, T propionate	Urine	SBSE	LC-DAD	21,2–81,8	0,062–0,38 ng mL^{-1}	[65]
NDL, T, DES, MeT, P, T propionate, NDL phenylpropionate	Water	SBSEM	LC–MS GC–MS	n,a.	0,036–0,068 ng mL^{-1}	[66]
NT, P, DES, T, MeT, T propionate, NDL phenylpropionate	Wastewater	SBSEM	LC-DAD	48,2–110	0,14–0,26 ng mL^{-1}	[67]
ADR, E1, 17β-E2, and their acryl derivates	Water, sludge	SBSE	GC–MS	73,8–100	0,2; 0,02 ng g^{-1}	[68]
MES,17α-EE2, BPA	Water	MEPS	LVI-GC–MS	97–113	48,5–292,6 ng L^{-1}	[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^{-1}	[78]
DES, E1, 17β-E2, 17α-EE2	Water	PC-HFME	GC–MS	86–110	0,03–0,8 ng L^{-1}	[88]
17β-E2, E1,17α-EE2	Sewage water	HF-MMLLE	GC–MS	45–98	1,6–10 ng L^{-1}	[89]
DES, dienestrol, hexestrol	Wastewater	HF-LPME	LC-UV	86,0–95,5	0,25–0,5 ng L^{-1}	[90]
DES, dienestrol, hexestrol	Milk	MIP-HFT	LC-UV	83,7–90,6	2,5–3,3 μg L^{-1}	[91]
E1, E2, EE2, E3	River and tap water	DLLME-SFO	UPLC-PDA	87–116	0,8–2,7 μg L^{-1}	[94]
E3, E2, E1, P	Water	CPE	LC-UV	81,2–99,5	0,23–5,0 ng mL^{-1}	[98]

Estrone (E1), estradiol (E2), estriol (E3), ethynylestradiol (EE2), diethylstilbestrol (DES), androsterone (ADR), epiandrosterone (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), stanazol (STAN), nortestosterone (NT), 16α-hydroxyandrosterone (16α-hydroxy-ADR), 2α-hydroxytestosterone (2α-hydroxy-T), 11-keto,5β-androstenedione (11-keto,5β-ADD), 6α-hydroxyestradiol (6α-hydroxy-E2), 7β-hydroxypregnolone (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), δ,4,16-androstadien-3-one (δ,4,16-ADD-3), 5α-androst-3,17-dione (5α-A-3,17-DD), etiocholanolone (EC), iso-androsterone (iso-A), 3α-hydroxy-5β-pregnan-20-enoe (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 (DLLME-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 determined 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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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.

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

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

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

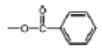
1. Introduction

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

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

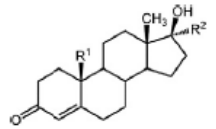
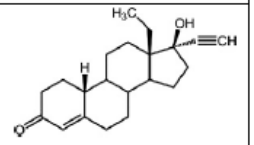
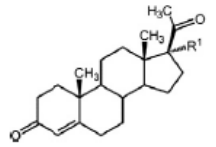
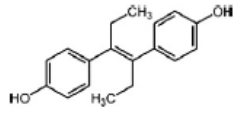
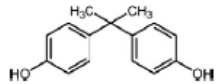
Another serious problem caused by steroid therapy involves pollution of the global environment. The occurrence of estrogenic substances in aquatic systems has already been described [9]. Estrogens are usually not entirely metabolized and they reach the aquatic environment mainly via effluents from wastewater-treatment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

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

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

(*continued on next page*)

Table 1. (*continued*)

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

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

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

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

2. Sample preparation

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

2.1. Aqueous samples

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

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

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

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

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

Table 2. Sample preparation in analysis of estrogens and progestogens

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

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

(continued on next page)

Table 2. (*continued*)

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

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

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

Elution was typically accomplished by methanol (MeOH) [4,5,13,15,21,26] and ethyl acetate (EtAc) (very often with connection to derivatization) [10,27,28] or in combination (Table 2). For the elution of estrogen-conjugates triethylamine (TEA) [10,29,30] as an ion-pair reagent and 2% ammonium hydroxide (AmOH) [21,31] were added into MeOH, while free estrogens were eluted with the help of EtAc or MeOH [13,29–31]. Moreover, the addition of 5% acetonitrile (ACN) into MeOH improved the recoveries of real samples [16]. In general, more hydrophobic EDCs (with a higher 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^2) [23,39]. This technique was successfully used for estrogen determination in influent/effluent WWTPs with the combination of derivatization with dansyl chloride [25]. Matrix effects, less flexibility, limited portability, expensive equipment and absence of extracts for further analysis or verification are among the disadvantages of on-line SPE [23].

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

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

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

2.2. Solid samples

Steroids and phenolic compounds tend to accumulate a great deal in solid matrices due to their lipophilicity expressed by their higher 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^2>MS>FD>UV (ordered by decreasing sensitivity) [10,12,23,25,37,41,45,47]. It is difficult to achieve trace analysis with conventional detectors (UV and FD). Table 4 confirms this premise. Considering the low LODs (of the order of ng/L or ng/g), GC-MS/GC-MS^2 and especially LC-MS/LC-MS^2 are the methods of choice in the steroid-hormone analysis (Tables 3 and 5).

3.1. Liquid chromatography coupled to mass spectrometry

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

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

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

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

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

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

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

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

(continued on next page)

Table 3. (*continued*)

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

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

(*continued on next page*)

Table 3. (*continued*)

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

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

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

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

3.1.2. Mass-spectrometry detection

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

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

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

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

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

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

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

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

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

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

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

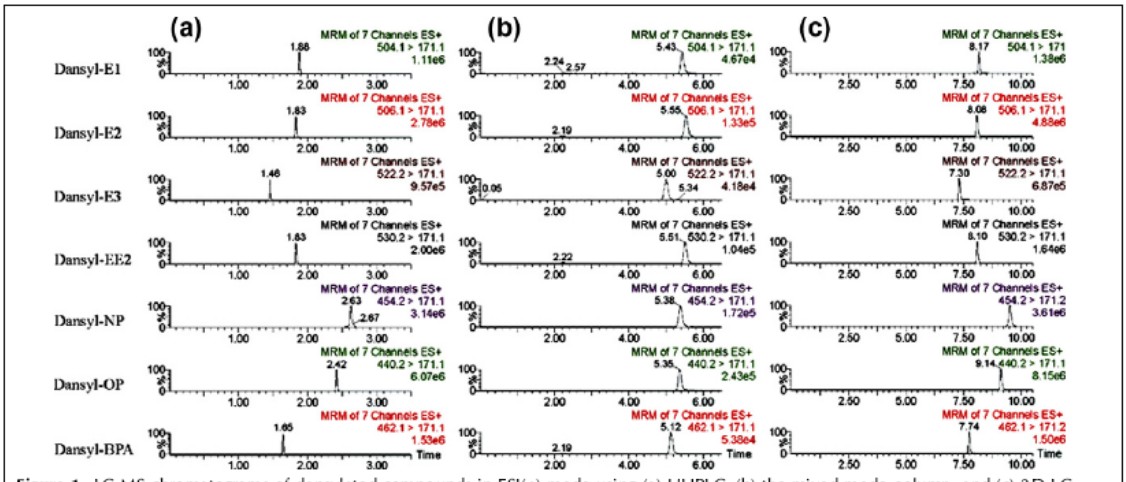


Figure 1. LC-MS chromatograms of dansylated compounds in ESI(+) mode using (a) UHPLC, (b) the mixed-mode column, and (c) 2D-LC (Reprinted from [58] with permission).

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

Labadie and Hill [9] compared the suitability and the performances of LC-TOF-MS and LC-MS^2 (QqQ) for determination of estrogens in river sediment. LC-MS^2 achieved 1 3 times lower LODs than LC-TOF-MS, and also showed better selectivity [9]. Farré et al. [54] also compared UHPLC-Q-TOF-MS with HPLC-MS^2 (QqQ). The sensitivity of HPLC-MS^2 (selective reaction monitoring mode – SRM) was up to one order of magnitude better than using the UHPLC-Q-TOF-MS method (scan mode). In terms of selectivity, both HPLC-MS^2 (QqQ) and UHPLC-Q-TOF-MS showed excellent performance, but UHPLC achieved shorter analysis times [54]. The use of QqQ together with the SRM (Fig. 2) will enhance the sensitivity, and reach low LODs and limits of quantification (LOQs) as well as low background noise [4,13].

3.2. Gas chromatography coupled to mass spectrometry

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

4. Derivatization in chromatographic methods

4.1. Liquid chromatography coupled to mass spectrometry

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

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

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

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

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

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

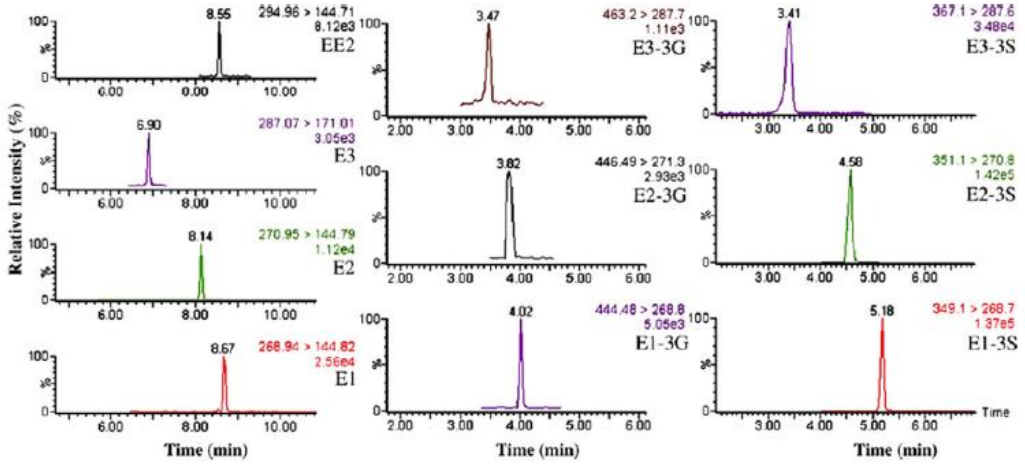


Figure 2. The UHPLC-MS^2 chromatograms (SRM) of four free estrogens and their six conjugates with relative intensity (%). Mass spectrometry was performed on Micromass Quattro Premier tandem MS in ESI(−) mode (Reprinted from [13] with permission).

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

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

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

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

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

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

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

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

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

4.3. Gas chromatography coupled to mass spectrometry

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

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

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

Table 7. Derivatization in estrogen and progestogen analysis

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

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

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

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

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

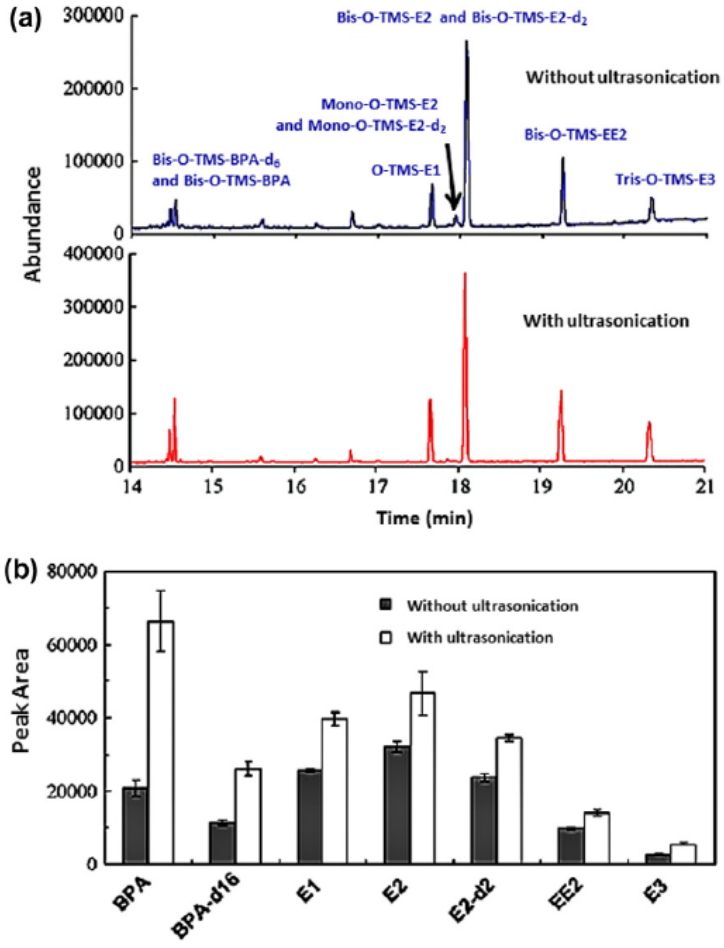


Figure 3. GC-MS chromatograms demonstrated the effect of ultrasonication on derivatization of studied EDCs and IS. (a) TIC of the derivatives; and (b) Selective ion monitoring (SIM) peak areas of the derivatives (n = 3). A derivatizing agent was BSTFA with 1% TMCS; electron ionization (EI) was used.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

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

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

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

5. Conclusions

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

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

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

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Supplement IV

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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

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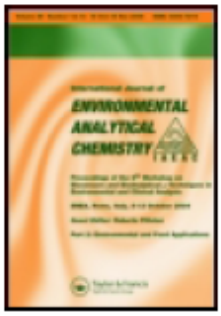

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Optimisation of an in-tube solid phase microextraction method coupled with HPLC for determination of some oestrogens in environmental liquid samples using different capillary columns

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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™ and Carboxen™ 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^{-1} and 0.12–1.9 ng mL^{-1}, 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^{-1} and LOQs of 0.015–0.08 ng mL^{-1} using Supel-Q and LODs of 0.01–0.015 ng mL^{-1} and LOQs of 0.025–0.04 ng mL^{-1} 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 Pawliszyn published the first approach to develop an automated in-tube SPME coupled to HPLC [18]. In 1999, Kataoka, Lord and Pawlizyn 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 17α-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 *Instumentation*

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*

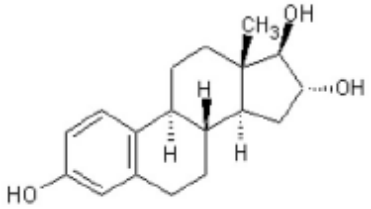
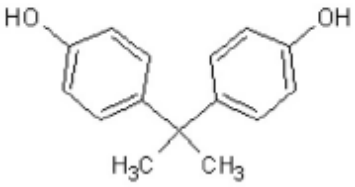
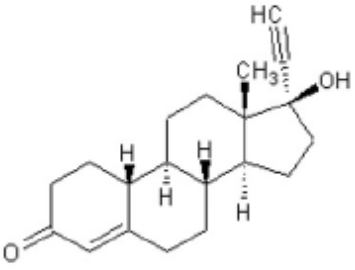
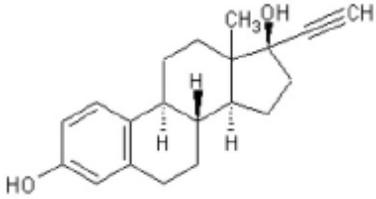
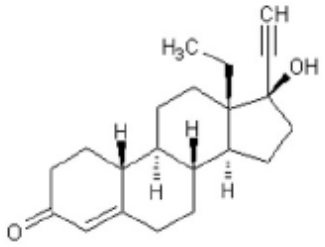
The EDCs selected in the present study, which includes: oestriol (E3), bisphenol A (BPA), 19-norethisterone (NORE), 17α-ethynylestradiol (EE2) and D–norgestrel (NORG) were purchased from Sigma–Aldrich (Steinheim, Germany). All pharmaceutical standards were 98–99% pure. Individual standard solution of these compounds were prepared in methanol at a concentration of 100 μg 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 μg 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_{18} (3.9 mm × 150 mm, 4 μm 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 ± 0.2°C.

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

N°	Structure	Analyte	Abbreviation	t_R (min)	Log K_{ow}	pK_a	λ (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

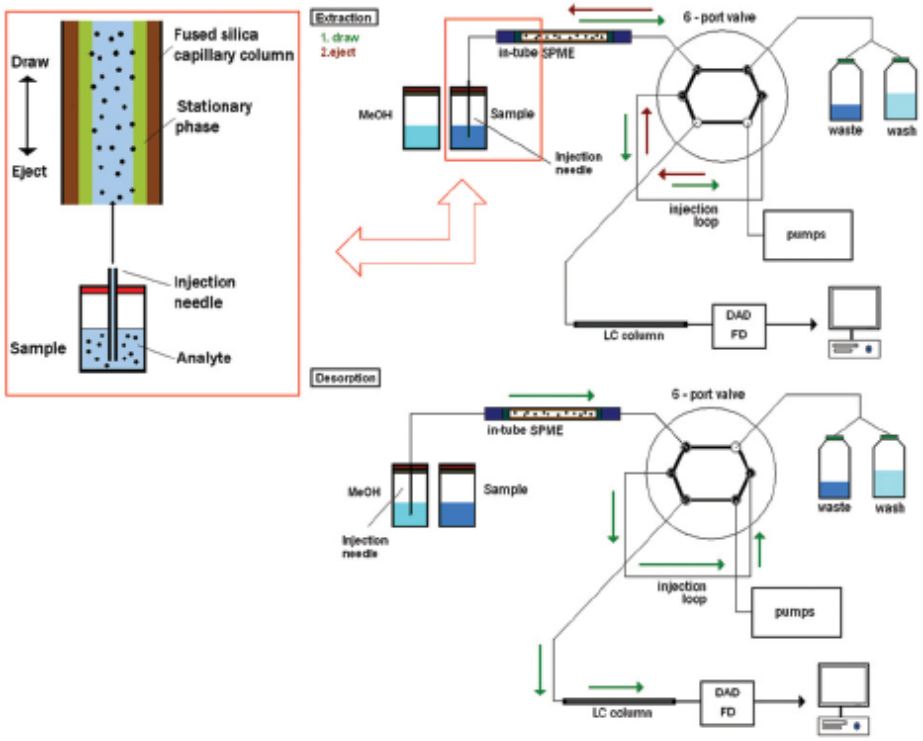


Figure 1. Schematic diagram of the automated in-tube SPME-HPLC system.

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^{-1}; 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^{-1} 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

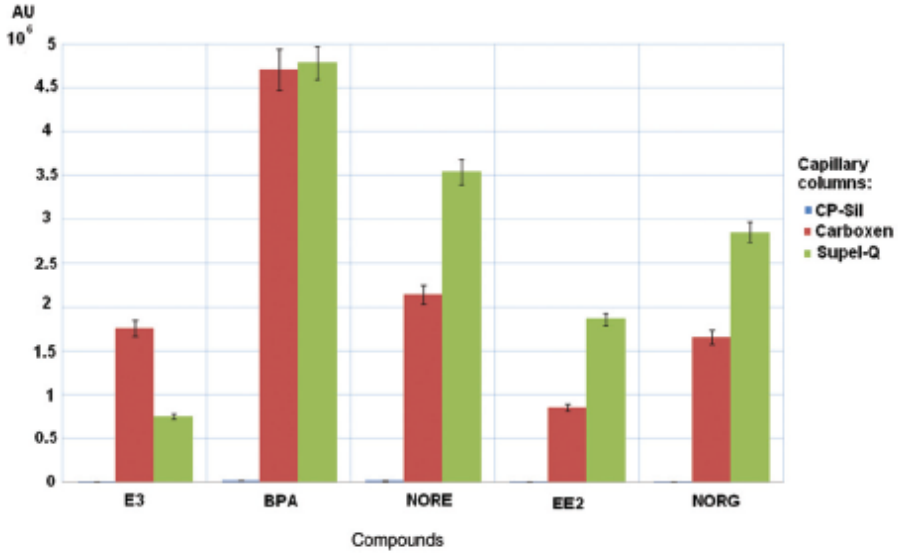


Figure 2. Influence of different capillary coatings in the extraction efficiency of the selected EDCs. Each compound was initially extracted by 14 draw/eject cycles of 90 µL of standard solution (1µg mL^{-1} of each) at a flow of 0.31 mL min^{-1} and desorbed with 50 µL of methanol.

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 µL per cycle, 14 draw/eject cycles and 50 µL of methanol as desorbent. The draw/eject cycle flow rates was 0.31 mL/min. Using these conditions, three different capillary columns (Supel-Q™ PLOT, Carboxen™ 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 pK_as 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 µL. 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 µL of sample volume for Supel-Q and 40 draw/eject cycles and 100 µL 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 µL of sample volume for Supel-Q and 20 draw/eject cycles and 75 µL of sample volume for Carboxen. Best results for E3 were obtained with 20 draw/eject cycles and 100 µL of sample volume for Supel-Q and 40 draw/eject cycles and 50 µL of sample volume for Carboxen.

The final optimal conditions for the selected EDCs were 20 draw/eject cycles with 75 µL of sample volume for Supel-Q column and 40 draw/eject cycles with 100 µL 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 µL, the

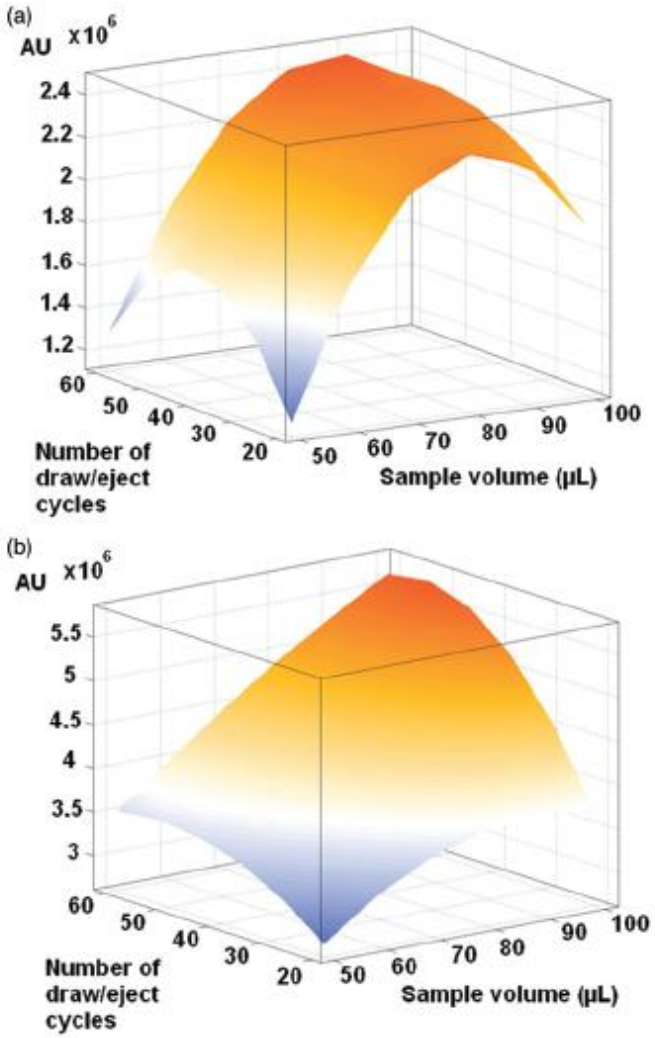


Figure 3. Response surface for optimal conditions of EE2: (a) Supel-Q column and (b) Carboxen column. Standard solution contained 1 µg mL^{-1} of each oestrogen at a flow of 0.3 mL min^{-1} and desorbed with 50 µL of methanol.

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 1–1000 ng mL^{-1} (HPLC-DAD) and 0.1–100 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.

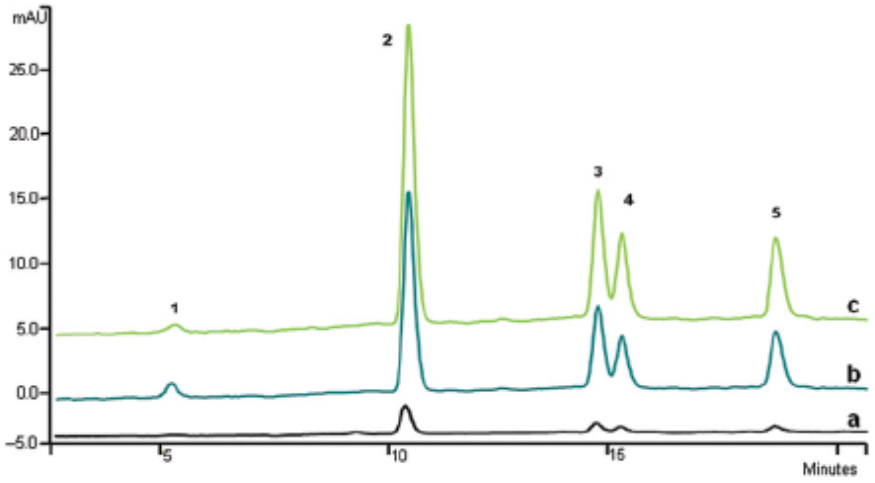


Figure 4. Chromatograms of the selected EDCs obtained by in-tube SPME-HPLC-DAD with direct injection (a) using Carboxen column (b) and using Supel-Q column (c), in optimal conditions. Standard solution contained 500 ng mL^{-1} of each target analyte in Milli-Q water. The numbering refers to Table 1.

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 0.04–0.63 ng mL^{-1} and 0.12–1.9 ng mL^{-1} using Supel-Q and 0.04–0.46 ng mL^{-1} and 0.12–1.4 ng mL^{-1} using Carboxen. Fluorescence detection improved these parameters for E3, BPA and EE2: LODs of 0.005–0.03 ng mL^{-1} and LOQs of 0.015–0.08 ng·mL^{-1} were obtained using the Supel-Q; LODs of 0.01 ng mL^{-1} for BPA and EE2, LODs of 0.015 ng mL^{-1} for E3 and LOQs of 0.025–0.04 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.

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

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

[a]Detection limits are calculated as signal to noise ratio of 3 (in $ng\,mL^{-1}$); [b]Quantification limits are calculated as signal to noise ratio of 10 (in $ng\,mL^{-1}$); [c]In-day precision, %RSD ($n=6$; 200 $ng\,mL^{-1}$ for E3 and 100 $ng\,mL^{-1}$ for the rest); [d]Intra-day precision, %RSD ($n=6$; 200 $ng\,mL^{-1}$ for E3 and 100 $ng\,mL^{-1}$ for the rest); [nd]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^{-1} of target analytes.

	% Recovery*		
	In-tube SPME		
Analyte	Carboxen	Supel-Q	SPE [32]
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™ PLOT, Carboxen™ 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), 17α-ethynylestradiol (EE2) and D–norgestrel (NORG) in aqueous samples using in-tube solid phase microextraction

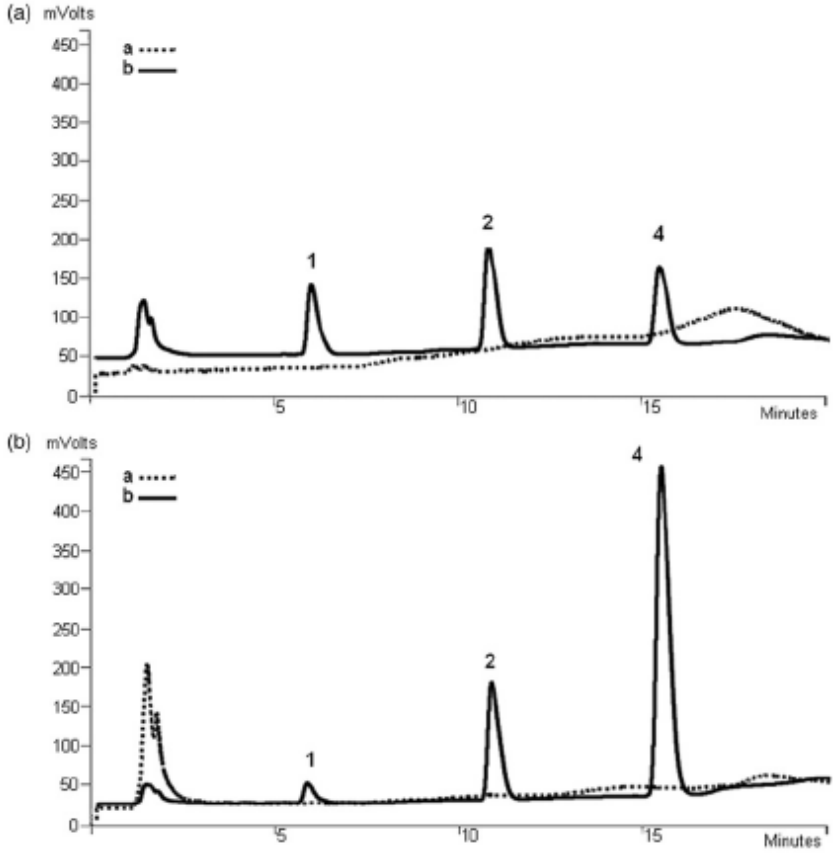

Figure 5. Chromatograms of the selected EDCs obtained by in-tube SPME-HPLC-FLD with (a) Carboxen column (b) Supel-Q column for (a) non-spiked and (b) spiked with 50 ng mL^{-1} of each target analyte real WWTPs sample. The numbering refers to Table 1.

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

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

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

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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

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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

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Abstract

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, 17α-ethynylestradiol and D-norgestrel) in environmental samples. For the first time, in the present study, a micellar desorption was used with a GC Supel-Q^{TM} 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^{-1} and 0.1-6 ng mL^{-1} respectively. In contrast, using a FD detector, the LODs and LOQs were 0.02-0.14 ng mL^{-1}and 0.06-0.4 ng mL^{-1} for estriol, bisphenol A and 17α-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.

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

1. Introduction

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

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

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

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

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

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

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

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

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

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

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

2. Experimental

2.1. Instrumentation

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

2.2. Reagents and solutions

Endocrine disruptor compounds evaluated in the present study, including estriol (E3), bisphenol A (BPA), norethisterone (NORE), 17α-ethynylestradiol (EE2) and D-norgestrel (NORG), were purchased from Sigma–Aldrich (Steinheim, Germany). All pharmaceutical standards were 98–99% pure. Individual standard solutions of the aforementioned compounds were prepared in methanol at a concentration of 100 $\mu g \cdot mL^{-1}$ and were stored in the dark at 4 °C prior to use. Working solutions were prepared by diluting the stock solutions with water, and a concentration of 1 or 0.5 $\mu g \cdot mL^{-1}$ was used for the experiments. Non-ionic surfactants, including polyoxyethylene 10 lauryl ether (POLE) and oligoethylene glycol monoalkyl ether (Genapol), were obtained from Sigma-Aldrich (Madrid, Spain) and were prepared in ultra-high-quality water. HPLC-grade methanol was obtained from Panreac Quimica S.A. (Barcelona, Spain). Ultra-high-quality water obtained from a Milli-Q water purification system (Millipore, USA) was used throughout the study.

2.3. Column, mobile phase and chromatographic conditions

A NovaPack C_{18} (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 $mL \cdot min^{-1}$. 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™ 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^{-1} of the standard in

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

2.5. Experimental design and Statistical analysis

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

3. Results and Discussion

3.1. Optimization of in-tube SPME conditions

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

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). Kataoka et al. [28] and Mitani et al. [25, 29, 37] found that a Supel-Q-PLOT capillary was more efficient for the analysis of steroid compounds. Therefore, we chose initially a Supel-Q capillary with a length of 60 cm and used methanol as the elution reagent in a single cycle for the initial optimization.

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

3.1.1. Experiment design – screening

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

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

The first stage of the in-tube SPME parameter optimization study consisted of a 2^4 factorial design (two levels and four variables), 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 μL) and desorption solution volume (25-50 μL). By applying a factorial design, the influence of each variable on the analyte recovery rate could be determined. Partial and bivariate correlations between dependent and independent variables were investigated and are shown in Table 2. For all of the compounds, the strongest correlations were obtained for the sample volume. The E3 and EE2 desorption volume negatively affected the extraction; however, we believe that this result was caused in part by the shape of the peak because the corresponding recoveries were on the same level. For the other variables (extraction and desorption time), the results showed only a slight dependence. According to the aforementioned results, an extraction time of 10 minutes, a desorption time of 10 minutes, a sample volume of 50 μL and a desorption volume of 25 μL were used in subsequent studies.

3.1.2. Selection of the capillary column length

The optimal length of a capillary column ranges from 20 to 100 cm and the internal diameter of a capillary used in combination with a LC varies between 0.25 and 0.32 mm [28]. Under the conditions outlined in section 3.1.1. (extraction time of 10 min, desorption time of 10 min, standard volume of 50 μL, 25 μL of methanol as the desorbent), we studied capillary lengths of 40, 60 and 80 cm. The results showed that a capillary length of 40 cm presented technical problems, like the 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. Therefore, a capillary column with a length of 80 cm was selected.

3.1.3. Optimization of extraction time and sample volume

After optimizing the capillary length, a 3^2 experimental design (two variables and three levels) was performed, and the extraction time (2, 6, and 10 min) and sample/standard solution volume (35, 50 and 65 μL) were evaluated using surface response methodology. The experiments were performed at a desorption time of 10 min,

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

Then, the desorption time (1, 2, and 10 min) was studied under the optimal extraction conditions. The results obtained at a desorption time of 1 min were not significantly different from those obtained at a desorption time of 2 and 10 min. 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 µL and desorption time of 1 min.

3.1.4. Optimization of cycle number

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.

Under the optimal conditions, experiments were performed in 1, 2, 4, 8, 12 and 15 cycles, and 65 µL of sample was used in each cycle. As shown in Figure 3, as 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.

3.1.5. Selection of micellar media as the desorbent agent

The selection of an appropriate desorption solvent is a very important for in tube SPME. Micellar media can be used as an alternative to organic solvents for desorption and have been used as desorbents in previous studies [31-33]. Under the optimal conditions for the proposed conventional in-tube SPME method, we replaced methanol with more environmentally friendly eluents in the desorption step. In this work, two non-ionic surfactants were selected instead of organic solvent, including POLE (polyoxyethylene 10 lauryl ether) and Genapol (oligoethylene glycol monoalkyl ether). The experiments were conducted under the following conditions: capillary column length of 80 cm, 12 cycles, standard solution volume of 65 µL, extraction time of 2 min, desorbent agent volume of 25 µL and a desorption time of 1 min. 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 further research.

3.1.6. Optimization of micellar desorption

Variables that affect the desorption step were studied using 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 μL) 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 μL were employed. The surface response of NORE and EE2 is shown in Figure 4.

In agreement to the obtained results, the optimal conditions for in-tube SPME using micellar desorption with Genapol were as follows: capillary column length of 80 cm, 12 cycles, sample standard solution volume of 65 μL for each cycle, extraction time of 2 min, desorption time of 1 min, 20 μL of 0.1% (v/v) of Genapol as the desorption reagent.

Figure 5 shows the chromatograms obtained from the direct injection of target analytes in a solution of methanol and injection with in-tube SPME using methanol and Genapol (0.1%, v/v) as desorption eluents. As shown in the figure, in-tube SPME with methanol and micellar desorption enabled the satisfactory separation and identification of the target compounds.

3.2. Analytical performance characteristics

Under the optimum extraction conditions, calibration curves were constructed using concentrations ranging from 1 ng·mL^{-1} to 1000 ng mL^{-1} (LC-DAD) and 0.1 ng·mL^{-1} to 1000 ng mL^{-1} (LC-FD). In all cases, the correlation coefficients (R^2) were greater than 0.99. Each level of concentration was prepared and injected three times.

The reproducibility of the method was evaluated by determining the inter-day and intra-day precision. As shown in Table 3, excellent reproducibility was obtained for both desorption reagents. Namely, intra-day % RSDs of less than 6.9 and 7.1 and inter-day % RSDs of less than 6.5 and 7.6 were obtained for methanol and Genapol in LC-DAD, respectively.

The limits of detection (LODs) and quantification (LOQs) were determined using six independent sample blanks and were calculated as a signal-to-noise ratio of 3 and 10, respectively. The data are shown in Table 3. With a DAD detection, the

LODs and LOQs of the proposed method ranged from 0.03 to 1.04 ng·mL^{-1} and 0.1 to 3.13 ng mL^{-1} when methanol was used and 0.03-1.95 ng·mL^{-1} and 0.10-5.95 ng mL^{-1} when Genapol (0.1%, v/v) was employed. Fluorescence detection improved these parameters for E3, BPA and EE2. Specifically, LODs of 0.02 - 0.15 ng mL^{-1} and LOQs of 0.05-0.47 ng mL^{-1} were obtained with methanol, and LODs of 0.02-0.14 ng·mL^{-1} and LOQs of 0.06-0.42 ng·mL^{-1} were obtained using micellar desorption.

Previous works [15, 17] reported determination of steroid sex hormones in water and urine matrices with DAD detection with similar results to the obtained in this work.

The obtained LODs for the combination of in-tube SPME- MD-LC-DAD-FD approach could be improved using MS detection. However, it is demonstrated that the proposed methodology can be an alternative to the use of organic solvents which makes possible to use it like an easier and cheaper approach to analyse EDCs in another type of liquid matrices like facultative pond wetland systems.

3.3. Application to the analysis of environmental waters

To study 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. Known amounts of the five EDCs were spiked into each water sample, and their recoveries were calculated. All samples were analysed in triplicate. Non-spiked real samples were analysed using in-tube SPME-LC-DAD-FD with methanol and Genapol, as described above. EDCs or interference were not detected at the retention times of the selected compounds. This fact was verified using a SPE procedure.

Chromatograms of seawater and WWTP samples are shown in Figure 6. Good peak shapes and resolution were achieved for all compounds with no interference from water matrix. The results are outlined in Table 4. As shown in the table, when methanol was used with a DAD, 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 detection. These results indicate that no appreciable difference is found when the extraction is carried out in different

matrices and therefore the applicability in environmental water samples is demonstrated.

4. Conclusions

A novel in-tube SPME-MD methodology was developed for the determination of EDCs in environmental liquid samples. An innovative feature of the present work was the use of micellar media instead of organic solvent and the use of experimental design methodology to optimize the proposed method. The extraction efficiency obtained using Genapol (0.1%, v/v) was comparable to that of methanol, which is a conventional solvent. The manually handled in-tube SPME was connected to LC-DAD and LC-FD systems. The proposed extraction method displayed good reproducibility, and recoveries greater than 86% were obtained. Finally, the optimal procedure was successfully applied to the analysis of EDCs in two different environmental water matrices. The developed method is simple, efficient and precise, and conventional equipment can be employed. Moreover, the proposed method is environmentally friendly and inexpensive, due to its low solvent volumes. After a careful selection of the extraction and desorption conditions, it was demonstrated that the proposed method could be useful for the screening and determination of EDCs in some environmental matrices. The obtained results from the application of this methodology in environmental water samples suggest that this process can be a promising alternative to other conventional procedure like SPE.

Acknowledgments

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Table 1: Abbreviations, structure, CAS number, log K_{ow} and detection wavelength (λ) used for monitoring the target analytes.

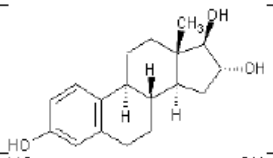
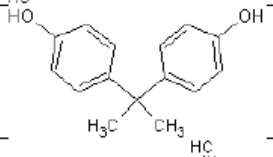
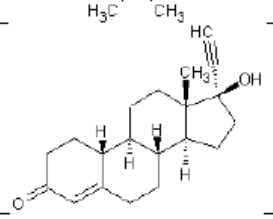
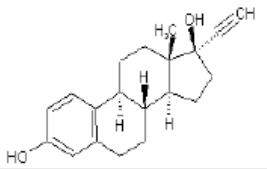
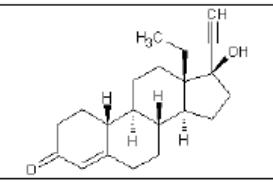
	Name	Abbreviation	Structure	CAS number	log K_{ow}	DAD λ (nm)	FD λexc/λem (nm)
1	Estriol	E3		50-27-1	2.5	224	228/315
2	Bisphenol A	BPA		80-05-7	3.3	224	228/315
3	Norethisterone	NORE		68-22-4	3.3	244	-
4	Ethynyl estradiol	EE2		57-63-6	3.7	224	228/315
	Norgestrel	NORG		83150-76-9	3.5	244	-

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Table 2. Partial and bivariate correlations between selected variables (maxima correlation are +1 and -1).

Experimental design – screening	E3	BPA	NORE	EE2	NORG
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

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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			
			precision				precision				precision				precision	
	LOD[a]	LOQ[b]	Inter-day[c]	intra-day[d]	LOD[a]	LOQ[b]	inter-day[c]	intra-day[d]	LOD[a]	LOQ[b]	inter-day[c]	intra-day[d]	LOD[a]	LOQ[b]	inter-day[c]	intra-day[d]
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.[e]	n.d.[e]	n.d.[e]	n.d.[e]	0.10	0.33	5.0	5.4	n.d.[e]	n.d.[e]	n.d.[e]	n.d.[e]
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.[e]	n.d.[e]	n.d.[e]	n.d.[e]	0.07	0.19	6.7	7.1	n.d.[e]	n.d.[e]	n.d.[e]	n.d.[e]

[a] **Limits of detection are calculated as signal to noise ratio of 3 (in ng·mL^{-1})**
[b] **Limits of quantification are calculated as signal to noise ratio of 10 (in ng·m^{-1})**
[c] **Inter-day precision, % RSD (n=6, 100 ng·mL^{-1} for all compounds)**
[d] **Intra-day precision, % RSD (n=6, 100 ng·mL^{-1} for all compounds)**
[e] **n.d.: not detected**

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Table 4.: Recovery percentages (n=3) obtained for sea water and WWTP effluent samples spiked with 100 ng mL^{-1} of selected compounds using methanol and Genapol (0.1%, v/v) as desorption agents.

MeOH	DAD		FLUORESCENCE	
Analyte	Sea water	Effluent of WWTP	Sea water	Effluent of WWTP
E3	103.4±2.6	95.5±6.9	100.7±4.7	94.4±2.6
BPA	98.3±9.1	98.7±3.3	98.5±9.1	98.8±7.0
NORE	96.1±3.5	101.2±3.6	n.d.[a]	n.d.[a]
EE2	95.5±4.6	94.7±2.2	86.8±6.9	94.1±7.2
NORG	93.0±5.2	93.0±3.8	n.d.[a]	n.d.[a]
Genapol	DAD		FLUORESCENCE	
Analyte	Sea water	Effluent of WWTP	Sea water	Effluent of WWTP
E3	94.2±3.1	91.0±2.7	100.1±1.7	90.9±2.5
BPA	99.2±5.4	96.3±6.8	92.5±4.7	94.2±3.1
NORE	93.7±3.3	106.9±2.7	n.d.[a]	n.d.[a]
EE2	104.4.2±2.8	97.7±1.5	103.2±3.0	93.3±3.1
NORG	97.7±5.9	90.5±6.1	n.d.[a]	n.d.[a]

[a)] **n.d.: not detected**

Figure 1. Schematic diagram of the in-tube SPME connection (A) Load position (B) Inject position.

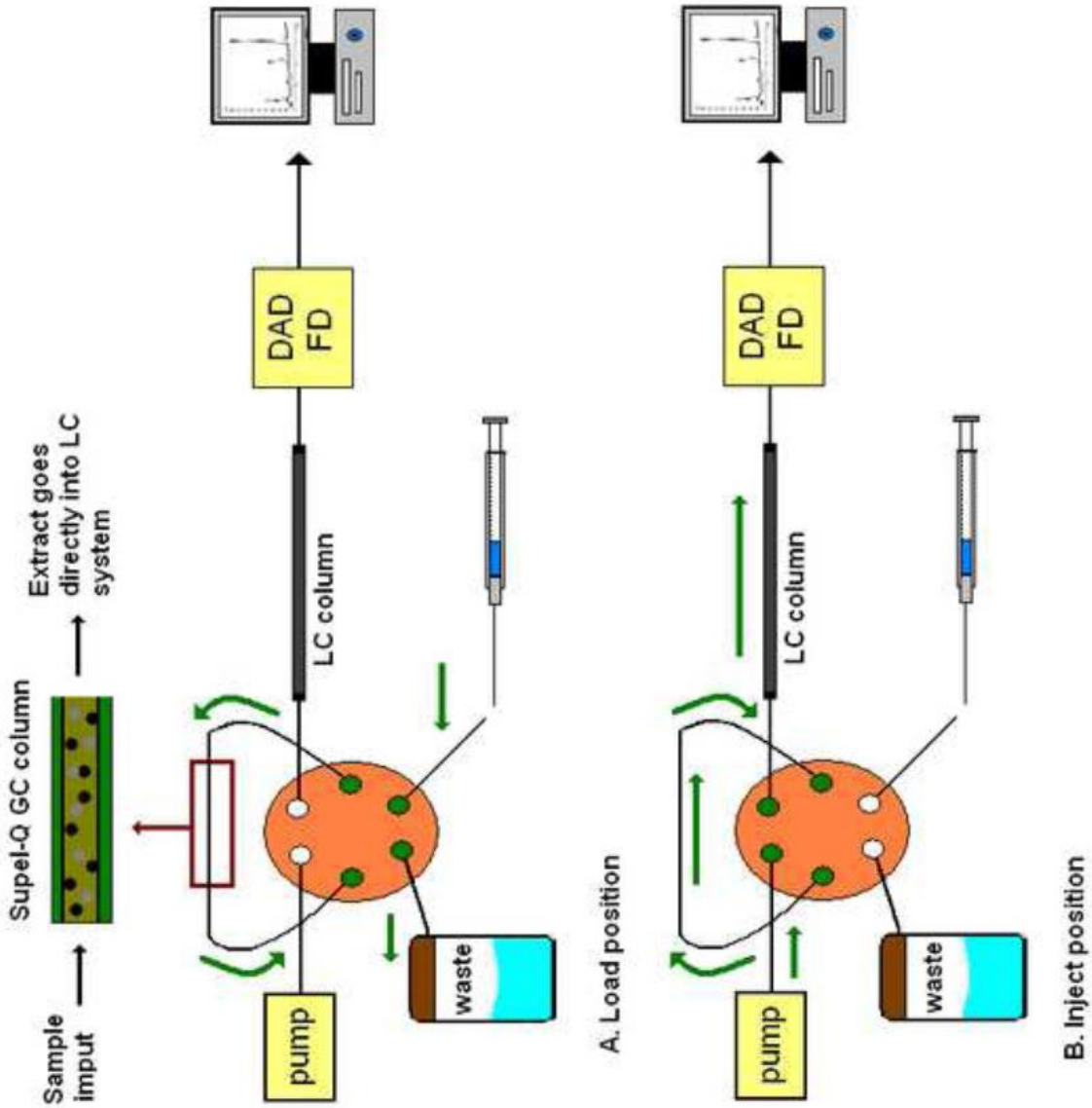
Figure 2. Surface response of sample volume and extraction time of NORE (A) and EE2 (B). Each compound was initially extracted with one cycle of 65µL of standard solution (1µg·mL^{-1} of each compound). Desorption was provided during 10 min by 25 µL of methanol.

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 65µL of standard solution (1µg·mL^{-1} of each compound). Desorption was provided during 10 min by 25 µL of methanol.

Figure 4. Surface response in the optimization of micellar desorption using Genapol of (A) NORE and (B) EE2. Standard solution (1µg·mL^{-1} of each compound) was extracted with 12 cycles of 65µL 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 µg·mL^{-1} 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·mL^{-1} of each compound. The numbering refers to Table 1.



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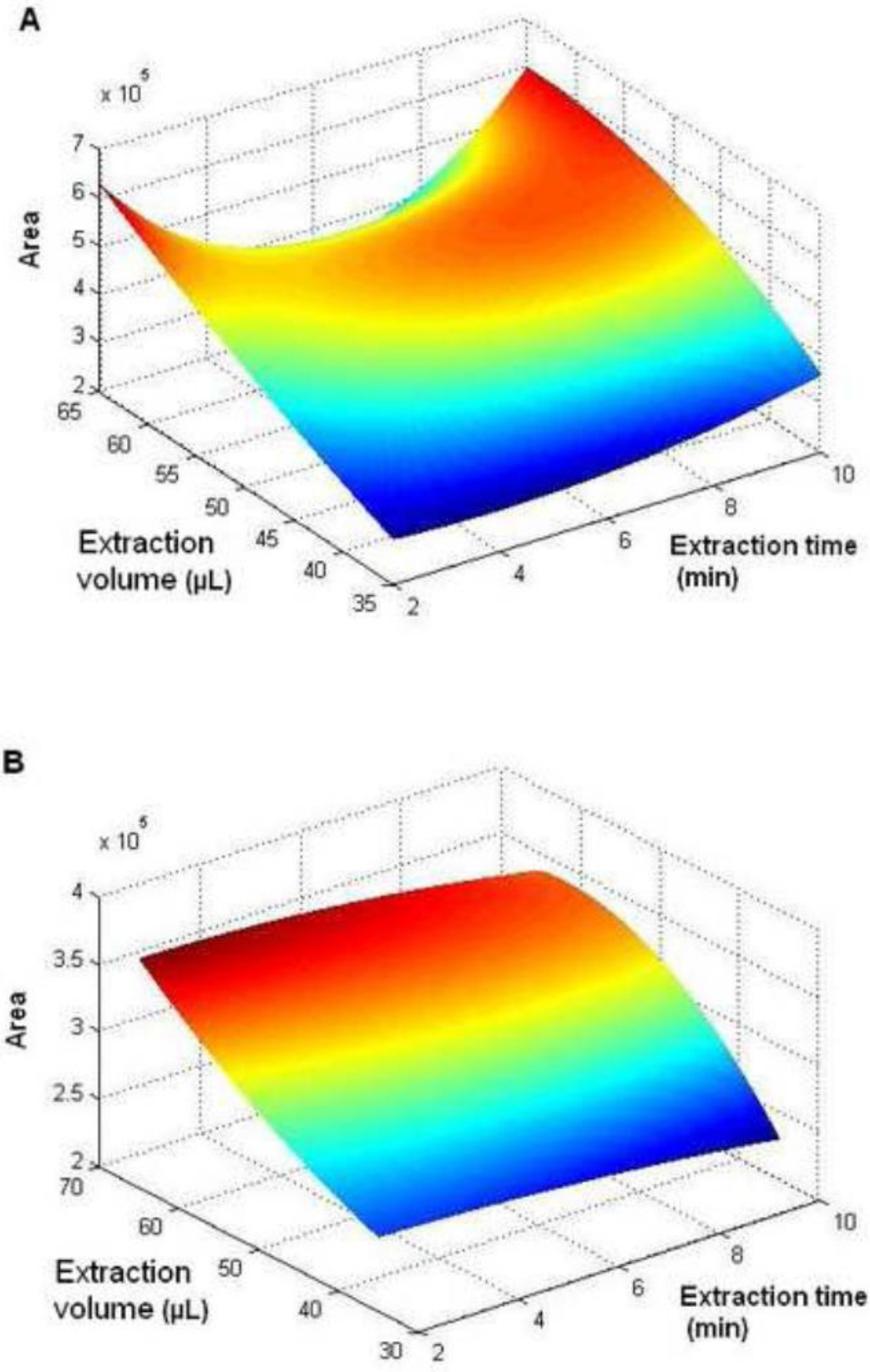

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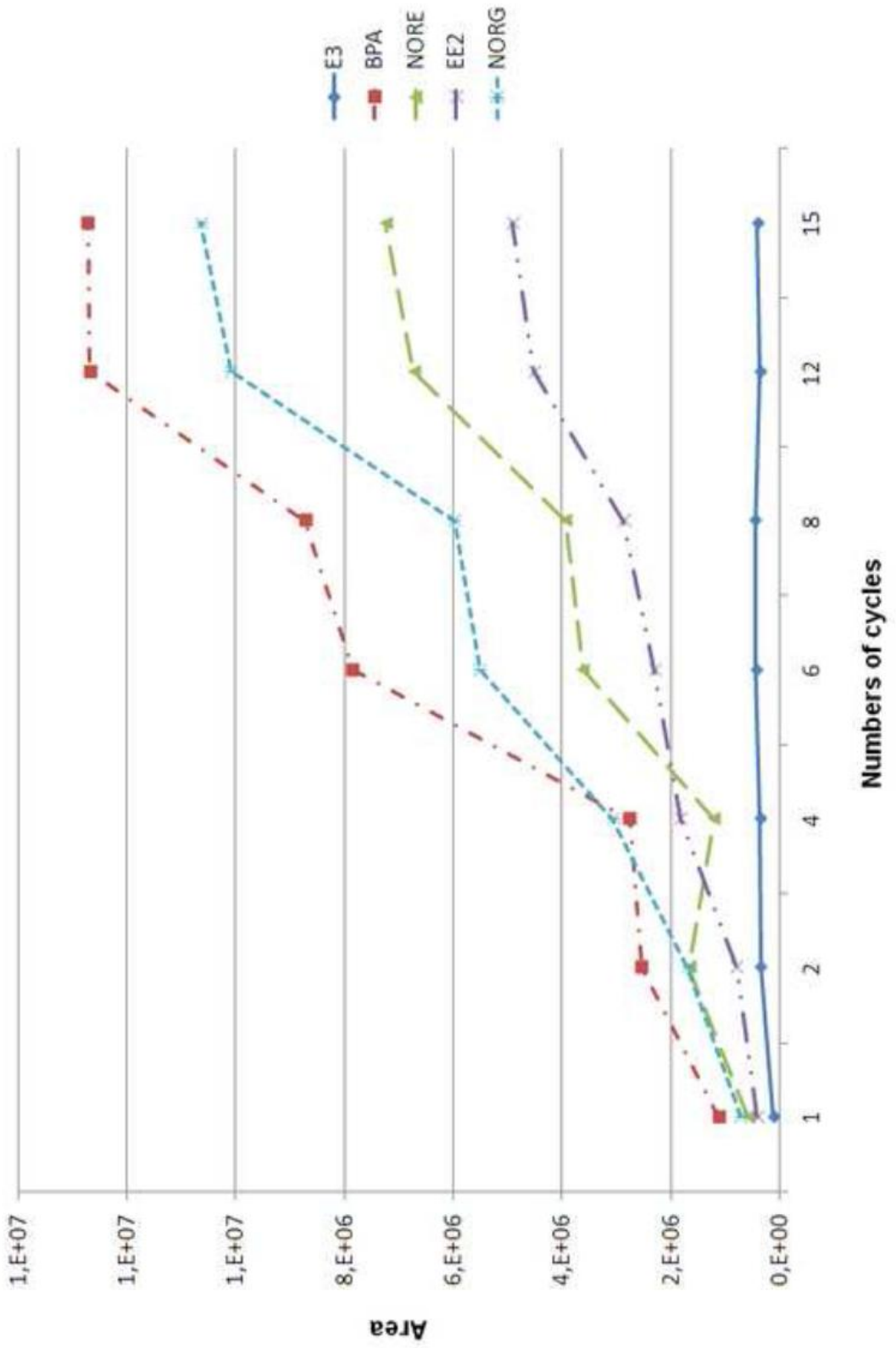

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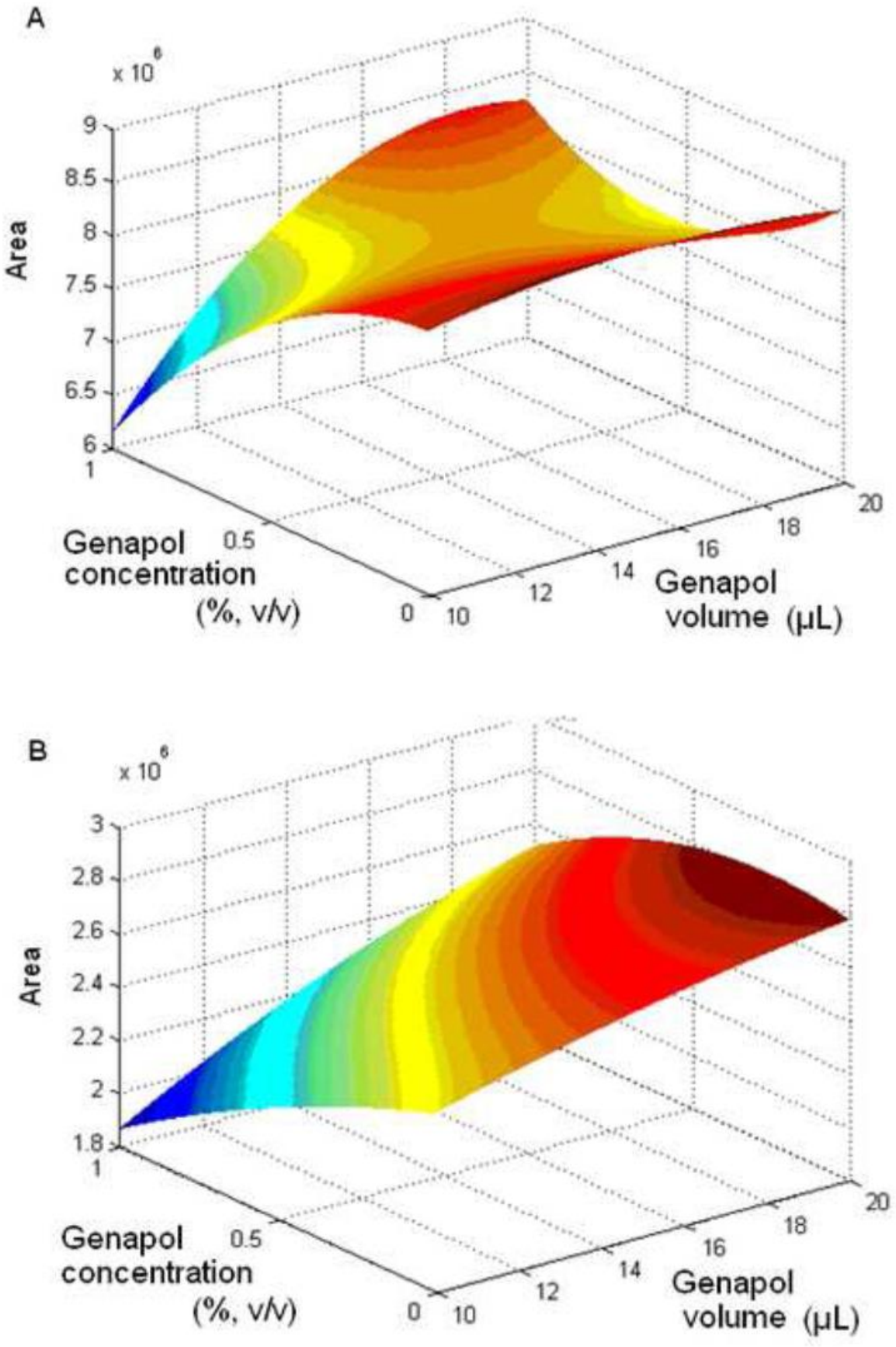

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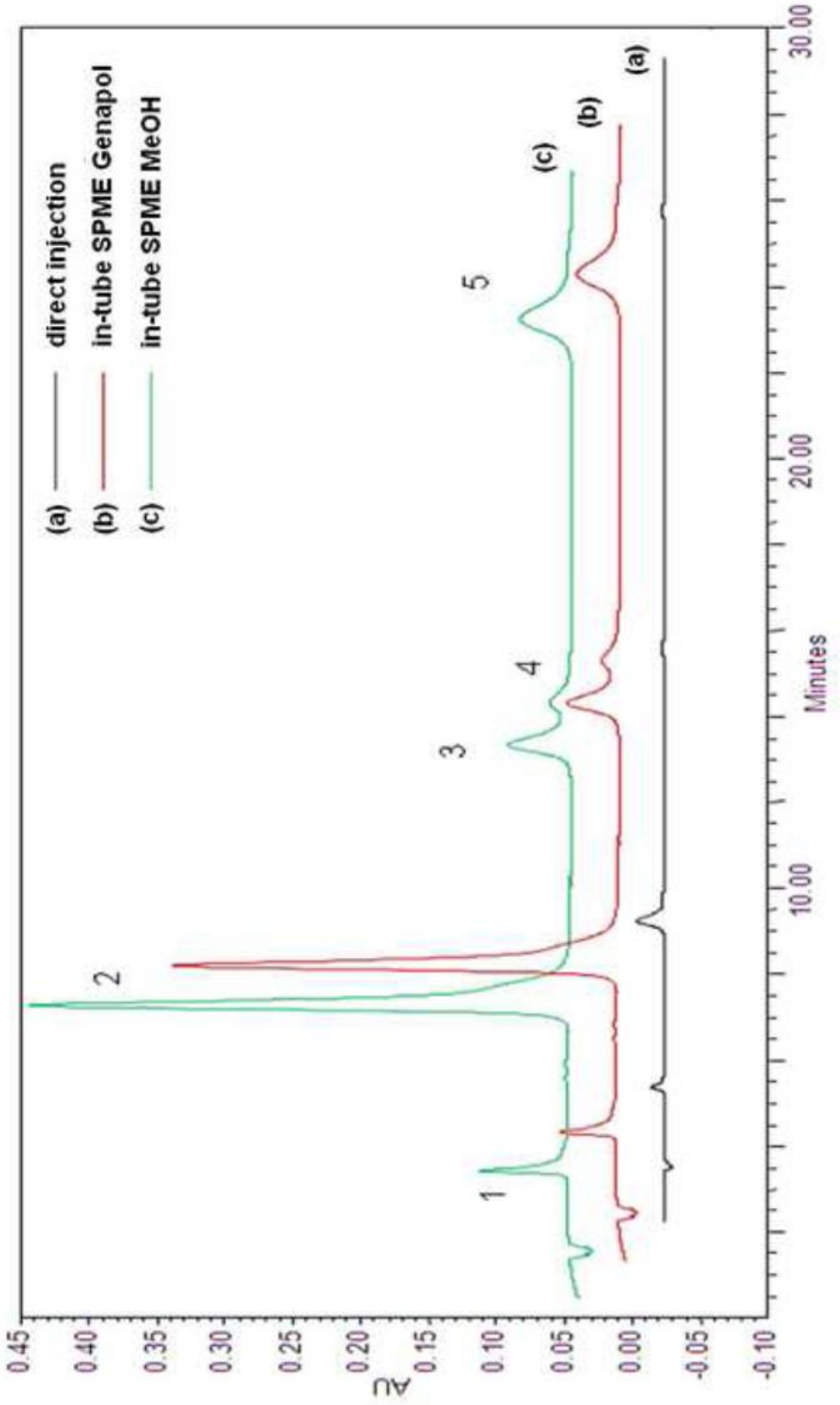
(a) direct injection
(b) in-tube SPME Genapol
(c) in-tube SPME MeOH
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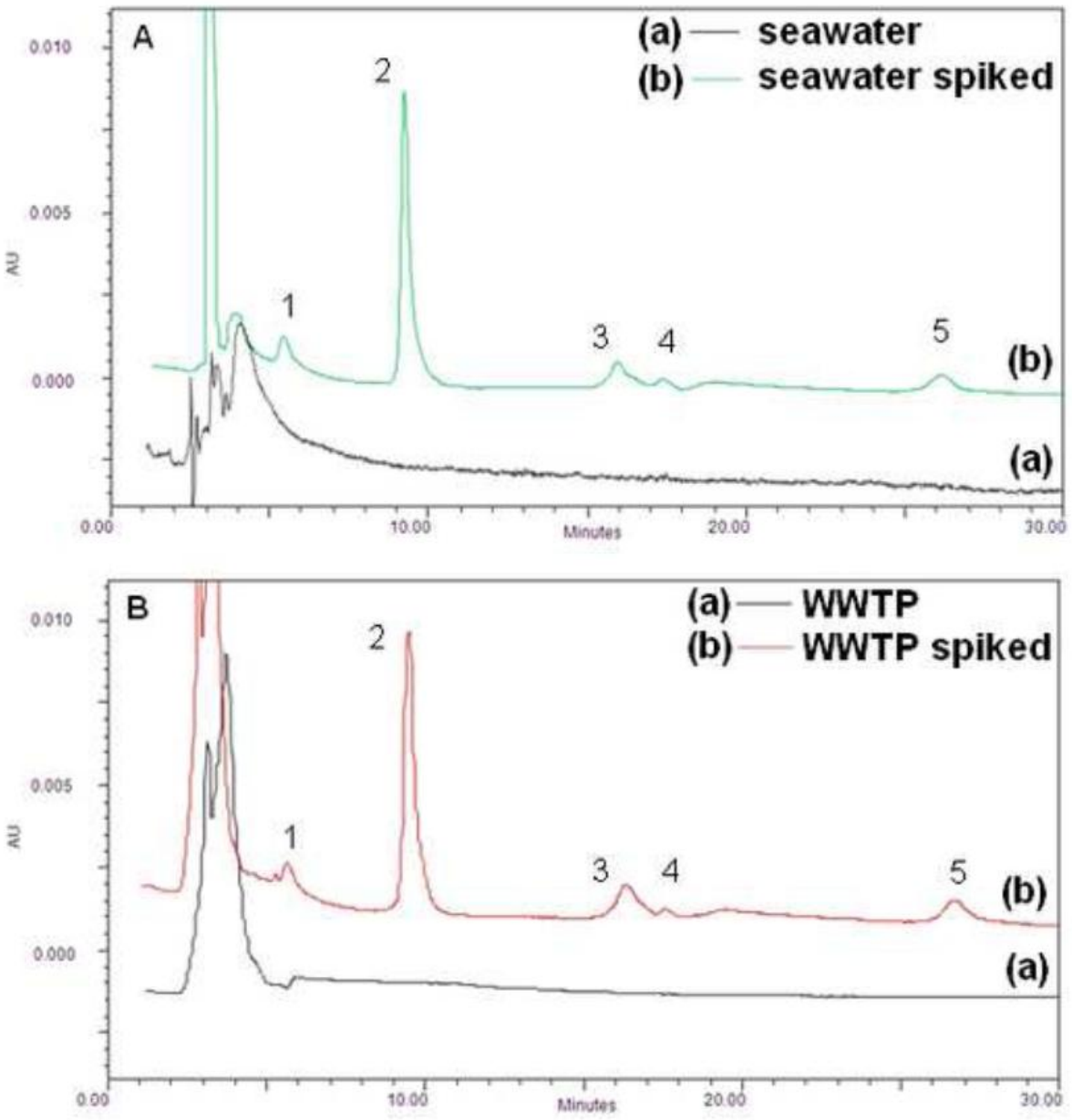

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Supplement VI

Maria Esther Torres-Padrón, Jana Aufartová, Zoraida Sosa-Ferrera, José Juan Santana-Rodríguez

Benzimidazole Fungicides in Environmental Samples: Extraction and Determination 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

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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~5-6) or deprotonated (pK_a~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 lymphoadenopathy.

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 $\cdot kg^{-1}$, 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 $\cdot L^{-1}$ for most benzimidazole compounds present in natural waters, and a total concentration of all pesticides of 0.5 µg $\cdot L^{-1}$. 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.

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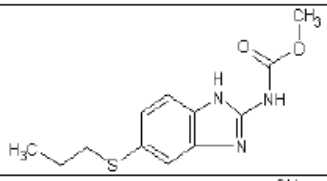
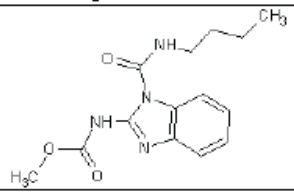
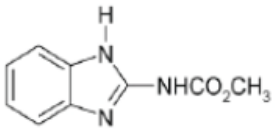
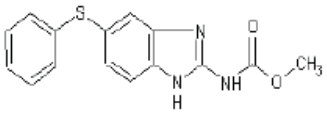
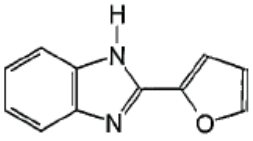
In this chapter some methodologies for the determination of BFs in environmental samples are presented. Its scope is the coverage of the main aspects which are involved in their determination in environmental matrices: sample handling, extraction/preconcentration and determination. We will conclude with 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_{oc}	pK_{a1}	pK_{a2}
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	

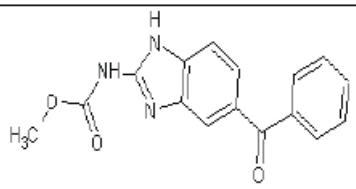
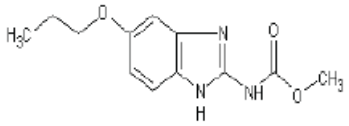
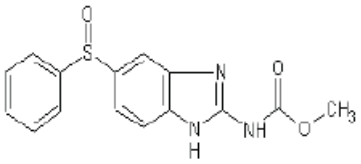
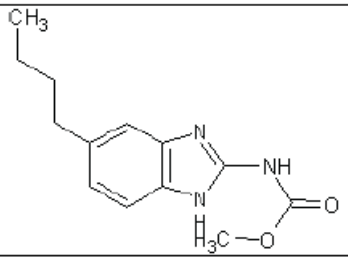
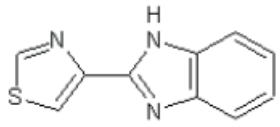
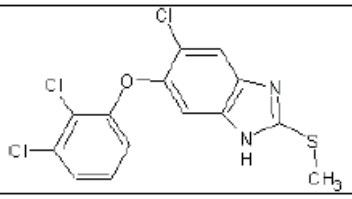
Name	Chemical structure	K_{oc}	pK_{a1}	pK_{a2}
Mebendazole (MBZ)		2.44-2.52	4.13	11.79
Oxibendazole (OXI)		1.86-2.63	6.26	13.78
Oxfendazole (OFZ)		1.88-2.13	4.13	11.79
Parbendazole (PAR)		1.86-2.63	5.99	13.53
Thiabendazole (TBZ)		1.58-1.76	5.82	12.79
Triclabendazole (TCB)		4.90-6.66	5.31	12.91

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.

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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), polystyrenedivinylbenzene (Guenu et al., 1996), ethylvinylbenzene-divinylbenzene (JunkerBuchheit, 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 $\mu g \cdot L^{-1}$ of each pesticide Limits of detections (LODs) were at the 0.05-0.3 $\mu g \cdot L^{-1}$ 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 $\mu g \cdot L^{-1}$. 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 (>200mL) and organic solvent (12–50 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 2.3–5.7 $ng \cdot L^{-1}$ 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

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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^{-1} for the different analytes. A chromatogram of this process is shown in Figure 1.

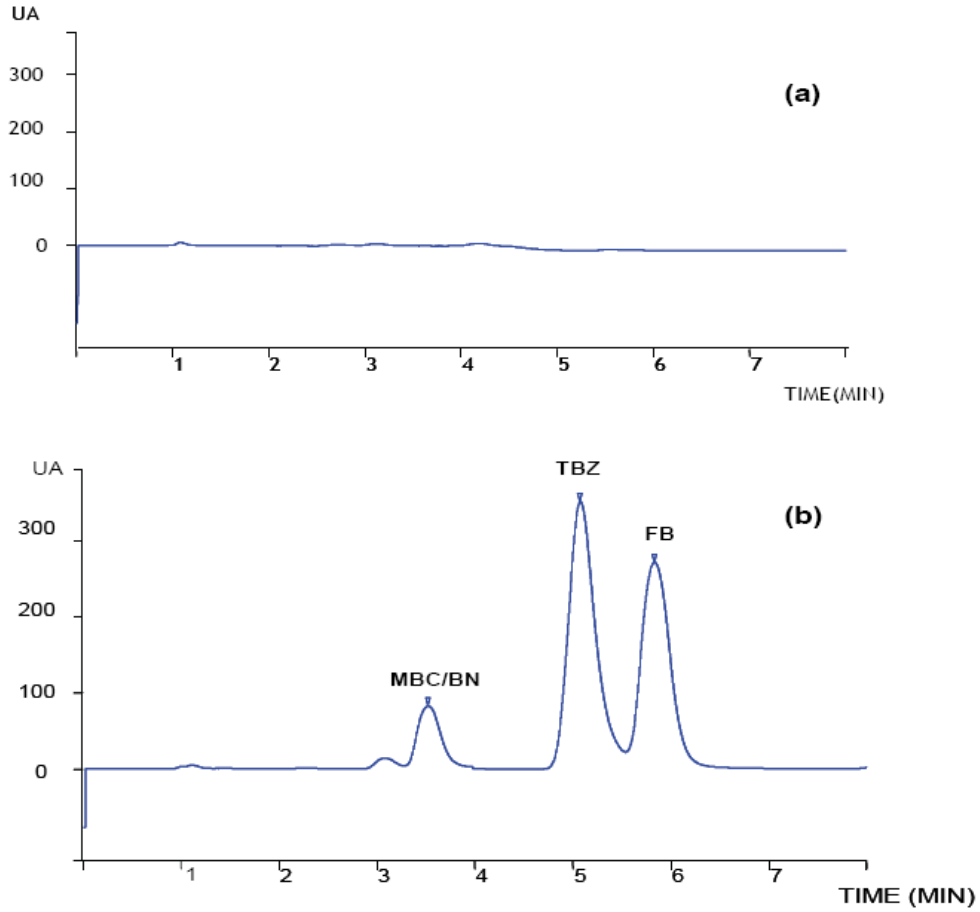

Fig. 1. Obtained chromatogram for a blank sample (a) and for the extract of benzimidazole fungicides from a spiked seawater sample (b) under optimum SPME extraction (López-Monzón et al., 2007).

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^{-1} by SLME and 2.7 times min^{-1} 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).

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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 ng mL^{-1} for carbendazim, ng mL^{-1} benomyl, 0.15 ng mL^{-1} for thiabendazole and 0.01 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 µL of $CHCl_3$ as extraction solvent (Wu et al., 2009). Obtained limits of detection for MBC and TBZ were 0.5 and 1.0 ng·mL^{-1} for liquid samples and 1.0 and 1.6 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

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 permited the favourable partition of analytes from environmental water using a quite low volume of supramolecular solvent (100 µL). 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 ng·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 pg·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 $\mu g \cdot g^{-1}$ for FDZ and TBZ and 25-30 $\mu g \cdot g^{-1}$ 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 (H_2O_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 mg mL^{-1} and a limit of detection of 7.24 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 Cu^{+2} and H_2O_2 was carried out in an alkaline Na_2CO_3 solution without CL reagent. On the addition of a trace amount of BFs to a mixture of Cu^{+2}–H_2O_2–Na_2CO_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 been 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 $\mu g \cdot kg^{-1}$ 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 $ng \cdot mL^{-1}$ 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 $ng \cdot L^{-1}$ 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 ng mL^{-1} for CBZ, 0.004 ng mL^{-1} for TBZ and 0.0001 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 $ng \cdot mL^{-1}$ for MBC and 1.0 $ng \cdot mL^{-1}$ for TBZ. In the case of the soil samples, LODs were 1.0 $ng \cdot g^{-1}$ for MBC and 1.6 $ng \cdot g^{-1}$ for TBZ.

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).

Balizs (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. Balizs (1999) obtained detection limits between 3 and 20 $\mu g \cdot kg^{-1}$. Gou et al. (2001) improved these

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^{-1}.

Compound	Q_1	Q_3
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^{-1}.

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

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 ng mL^{-1} for HPLC-MS and 0.075 ng mL^{-1} for HPLC-DAD) than ELISA method (0.10 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 tecnique	Analytical Parameters	Ref.
CBZ	soil	-	HPLC-UV/Vis	RSDs: 4.5% LODs: 0.3 µg mL^{-1}	Huebra et al. (2000)
CBZ	water	SLM, MMLLE	HPLC-UV	RSDs: < 5% LODs: 0.1 – 0.5 µgL^{-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 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 µg $\cdot L^{-1}$	Garrido et al. (2002)
CBZ, FDZ, TBZ	water	SPE	luminescence spectrometer	Recovery: 97-109% RSDs: 3.3-6.4% LODs: 0.001-0.13 µg $\cdot L^{-1}$	Zamora et al. (2002)
CBZ, BNM, FDZ, TBZ	water	CPE	HPLC-Fluorescence	Recovery: 70-96 % LODs: 0.01-6 ng mL^{-1}	Halko et al. (2004)

Analytes	Samples	Extraction	Determination tecnique	Analytical Parameters	Ref.
FDZ	water	-	fluorescence spectrophosphorimeter	RSDs: 3.1% LODs: 95 ng·L^{-1}	Salinas et al. (2005)
CBZ, BNM, FDZ, TBZ	soil	MAME	HPLC-Fluorescence	Recovery: 70-105 % LODs: 0.02-0.06 µg·g^{-1} 25-30 µg·g^{-1} (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^{-1}	Lesueur et al. (2007)
CBZ, BNM, FDZ, TBZ	water	SPME	HPLC-Fluorescence	RSDs: <9.0 % LODs: 0.03-1.3 ng·mL^{-1}	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^{-1}	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^{-1}; 1.0–1.6 ng·g^{-1}	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^{-1}	Moral et al. (2009)
BNM, CBZ, TBZ	water	MISPE online	HPLC-DAD	Recovery: 87-95% RSDs: <5% LODs: 2.3–5.7 ng·L^{-1}	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.

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 fluoresence 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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