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Determination of steroids in the dissolved and in the suspended phases of wastewater and Danube River samples by gas chromatography, tandem mass spectrometry

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ABSTRACT

In this paper, a new working approach is described for the analysis of steroids as environmental water pollutants. As novelty to the field, steroids were identified and quantified both in the dissolved and in the suspended phases, as their trimethylsilyl-(oxime)-ether derivatives, applying a recently developed tandem gas chromatographic mass spectrometric (GC–MS/MS) method, applying multiple reaction monitoring (MRM) acquisition, suitable for their quantitation in the low ng/L level, in wastewater and in Danube River samples.

In addition to the analysis of filtrates obtained by the common solid phase extraction (SPE) enrichment, even the insoluble, isolated by filtration prior to the SPE, and usually discarded part of steroids were identified and quantified, simultaneously, for the first time. For this purpose a new, time, labor, cost efficient and quantitative, ultrasound assisted extraction process was developed.

Reproducibility, reliability and practical utility of the ultrasound assisted extraction process were proved by the proportionality of the extracted suspended steroids obtained from different sample volumes: prepared from 0.5 L and 1.0 L influent wastewater, as well as from 3 L, 5 L and 10 L Danube River water samples. Steroids' concentrations, identified and quantified in suspended conditions, showed proportionality, characterized with the relative standard deviation percentages (RSD%) of analyses: varying in case of Danube River water in the range of 0.92–6.0%, with an average of 4.10% RSD, while in the case of influent wastewater in the range of 1.59–5.8%, with an average of 4.03% RSD.

Partition of steroids, between the dissolved and suspended phases of influent and effluent wastewaters and river water samples, meaning, the total amounts of steroids that the ecosystem is liable to, were defined in river water samples for the first time.

Distribution of found steroids revealed that their considerable and/or overwhelming part (relating to their total amounts), are present in suspended phases: in average, 71% from wastewater and 64% from Danube River samples.

1. Introduction

The presence of steroids in environmental waters, their hazardous effect on aquatic ecosystems is widely known; summed up also in our recent papers [1,2] related to their identification and quantification, as their trimethylsilyl (oxime) ethers, by GC–MS/MS. In the knowledge of steroids' limited solubility we extended our attention from 2010 on to the analysis of the insoluble (suspended) parts of water samples, isolated by filtration and usually, discarded, except to a very recent, excellent two dimensional gas chromatography–time-of-flight mass spectrometry based analysis of wastewater samples, only [3]. Our decision was also inspired by the European Water Framework Directive (EWFD) [4] addressed to the Member States of the European Union, i.e., "As sediments appropriate for monitoring contaminants may not always be available…" consequently, "…suspended particulate matter as an alternative to sediments …" should be monitored [5].

Steroids' analysis in the suspended phases of environmental water samples is of primary importance since even under suspended conditions they are biologically available, as proven by bioassays [6,7], by in vitro [8] and by in vivo tests [9]. Suspended steroids contribute to the total estrogenic activities of waters [6], consequently, they should be considered as an integral part of environmental samples.

Based on the overview of recent, relevant papers it turned out that distribution studies of steroids, performed simultaneously, between the dissolved and suspended phases, considering influent and effluent wastewater and river water samples are not available.

Extraction approaches proposed for the analysis of the suspended phases proved to be labor-intensive and time-consuming.

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They involved the costly, pressurized liquid extraction (PLE) for drugs of abuse [10,11] for selected pharmaceuticals [12] and for the analysis of steroid hormone runoff from agricultural tests obtained from municipal biosolids [13]. Enrichment of antidepressant and non-steroidal anti-inflammatory drugs was performed via ultrasonic extraction, freeze-drying of samples followed by SPE cleanup [14]. Polyfluoro alkyl compounds were filtered on syringe nylon membrane polypropylene filters [15], while alkylphenol derivatives, ß-estradiol and ethinylestradiol were isolated on membrane filters [16], followed membrane filters' immersion into ultrasonic bath. Steroidal hormone profile of particular matter from Jalle d'Eysines River and from wastewater effluent was isolated applying three consecutive steps [17] (1: 0.7 μm pore size filter, 2: filters' focused microwave extraction, and 3: residue's purification with HLB and NH_2 cartridges). Estrogens of suspended materials from Scheldt estuary (Belgium – The Netherlands) were collected using flow through centrifuge followed residue's treating with accelerated solvent extraction (ASE) [18]. Xenobiotics of suspended materials – from maximum 1 L surface water – were collected on SPE disks: preventing plugging of SPE cartridges and permitting the analysis of suspended residues, dissolving them from the disks, separately [19]. The analysis of fecal sterols, one by one, from catchment waters' suspended parts consisted of a 2 h long Soxhlet extraction followed by saponification, continued by ethanol extraction and freeze-drying [20].

Concerning chromatographic methods applied for the analysis of suspended steroids liquid chromatography tandem mass spectrometry (LC–MS/MS) [10–12,14–16], GC–MS [17,20] GC–MS/MS [13,18,21] and GC × GC–TOFMS [3] were performed). (Note: due to the sample preparation of suspended steroids, performed usually with organic solvents it is obvious that this is one of the reasons why GC–MS/MS proved to be the preferable chromatographic technique compared to LC–MS/MS).

Out of the above detailed approaches, identification and quantification of suspended steroids were planned in six cases [13,16–19,21] and found effectively in four cases, only [3,13,16,18]. The amounts of steroids in suspended phases, with two exceptions [3,13], were defined as μg/g dried suspended material, without any correlation with the volume of water samples they have been prepared from.

Based on our recent experiences [1,2,22], optimum conditions for the derivatization, mass fragmentation, gradient elution and tandem mass spectrometric acquisitions for steroids, together with cholic acids, were available [23].

This work was undertaken in order

(1) to develop a simple, time, cost, labor efficient and quantitative method for the analysis of steroids existing in the suspended phases of target waters;
(2) to identify and quantify steroids in their counterpart phases of water samples at the same time (omitting storage steps), under the same analytical conditions, simultaneously;
(3) to define their distribution between the dissolved and suspended phases of water samples, expressed in ng/L or μg/L values to be comparable with their corresponding parts in dissolved condition;
(4) to convince analytical chemists that pollutants' analysis of low solubility, like steroids, is obligatory, both in suspended and dissolved phases, as they are available for living organisms, as a whole, and
(5) to prove the analytical performance and the practical utility of the optimized technique by the analysis of suspended steroids isolated from the influent and effluent samples of wastewater treatment plants (WWTPs) and from the Danube River water.

2. Experimental

2.1. Materials and reagents

All reagents were of analytical reagent grade. Pyridine, hydroxylamine · HCl and concentrated hydrochloric acid were purchased from Reanal (Budapest, Hungary). Hexane, methanol, ethyl acetate, hexamethyldisilazane (HMDS), trifluoroacetic acid (TFA) and model compounds like, steroids were the same described in our previous papers [1,2]. Glass micro-fiber filters (GF/A 125 mm, ∅, Cat no. 1820-125) were from Whatman (Maidstone, UK). Cartridges (Oasis, HLB 6cc), for solid phase extraction (SPE), were from Waters (Milford, MA, USA). SPE extractions were performed on the Visiprep DL Vacuum Manifold for 12 samples (Cat no. 57044) from Supelco (Bellefonte, PA, USA). Ultrasonic extraction were performed on the Bandelin Sonorex (RK 52 H) apparatus (Bandelin electronic), Berlin, Germany.

2.2. Sample preparation

To separate steroids present in the suspended and in the dissolved phases of environmental waters, samples were collected in amber bottles with glass stopcocks, stored in the dark at 4 °C for maximum 18 h, before being analyzed. Appropriate aliquots (0.5 L or 1.0 L wastewater, 3 L, 5 L or 10 L of Danube River water), were taken from the (by shaking) homogenized samples and filtered on glass microfiber filter papers previously weighed with analytical precision.

2.2.1. SPE extraction of dissolved phase

Cartridges, prior to extraction were conditioned with 5 mL hexane, 5 mL ethyl acetate, 10 mL methanol and 10 mL distilled water. Filtered aliquots were adjusted to pH 4 with hydrochloric acid. Extractions were followed with a flow rate of 4–5 mL/min. Cartridges have been dried by vacuum and elutions were performed, in order of listing with 5 mL hexane, 5 mL ethyl acetate and 10 mL methanol.

2.2.2. Ultrasound assisted solvent extraction of suspended solid phase

Glass microfiber filter papers were dried overnight at ambient temperature (prior to extraction, until constant weight) then cut to 5 × 5 mm pieces and put in glass beakers (150 mL). Extractions were made with a solvent mixture of hexane/ethyl acetate/methanol 1/1/2 (v/v/v%) (applying the same solvent ratios as used for the SPE process). At first, 40 mL of solvent mixture was added to the glass beakers and sonicated for 20 min. This step was repeated two times with 20 mL solvent mixture. Solvent portions were filtered on glass micro-fibre paper, unified and treated the same way as the eluents of the SPE process.

The unified eluents of both the SPE and the ultrasonic extraction process were reduced in volume, evaporated to dryness by means of a rotary evaporator {Büchi Rotavapor R-200 and Büchi Vacuum pump V-700, both from Büchi (Flawil, Switzerland)} at 30–40 °C (further on: extract). Blank tests (reagent blanks, SPE blanks and glass filter paper blanks) were carried out with each series.

2.3. Preparation of the TMS and TMS (oxime) ether/ester derivatives

Model compounds (20–25 mg/100 mL), weighed with analytical precision were dissolved in ethanol, or in water/ethanol = 1/1 (v/v) solution and further diluted for 10 ×, 100 ×, and 1000 ×. Model solutions and the extracts were rotary evaporated to dryness at 30–40 °C. The residues were treated with 125 μL

hydroxylamine · HCl containing pyridine (2.5 g hydroxylamine · HCl/100 mL) heated in oven at 70 °C for 30 min. Thereafter silylation was continued with 225 μL HMDS+25 μL TFA and heated at 70 °C for 90 min. Samples were taken for the analysis after dilutions with HMDS, 1 μL of the diluted solutions was injected into the GC–MS system. Optimized conditions, including derivatization [1,22] and acquisition validations for steroids and cholic acids [2] were given in details.

2.4. Instrumentation

The apparatus consisted of a Varian 240 GC–MS/MS system (Varian, Walnut Creek, CA, USA), equipped with a Varian CP-8400 Autosampler, and with the Septum-equipped Programmable Injector (SPI). The column used was a product of SGE (Victoria, Australia); SGE forte capillary: 30 m × 0.25 mm; df = 0.25 μm. The temperature of the transfer line, ion trap and manifold were, in order of listing 300 °C, 210 °C and 80 °C, respectively.

Under gradient conditions, the optimized temperature programs, different for both the column and the septum equipped programmable injector (SPI), were as follows: injections were made at 100 °C, and held at 100 °C for 0.5 min, then heated to 300 °C (200 °C/min), with a 3 min hold at 300 °C, column temperature starts at 100 °C, held for 1 min, then heated up to 260 °C for 10 °C/min, with 6 min hold and finally heated up to 300 °C for 10 °C/min with a 7.5 min hold at 300 °C (total elution time 34.5 min).

2.5. MRM parameters

The selective ions and transitions used for quantitation of steroids during the multiple reaction monitoring (MRM) detection method were published in our previous paper [2].

The general MS/MS parameters were: Fil/Mul delay: 20.00 min; mass defect: 0 mmu/100 μ; filament current: 40 μA; target TIC: 5000 counts; prescan ion time: 1500 μs; scan mode: fast; scan time: 0.17 s/scan; multiplier offset: autotune+300 V and electron energy: 70 eV. Ion preparation method (IPM) parameters in each segment were: isolation window: 3.0 m/z; ionization storage level: 35 m/z; high mass ejection: 35 V; excitation time: 20 ms; modulate RF: yes; frequency number: 1 and CID frequency offset: 0.0 kHz.

3. Results and discussion

3.1. Reliability and reproducibility of the ultrasonic extraction process of suspended steroids

Regarding literature premises addressed to the analysis of suspended solids' reliability/reproducibility studies were either neglected [12,14], or carried out by spiking the isolated suspended solids [3,11] with unknown [3], or with enormously high amounts of standards [11].

In our understanding, the interaction between the spiked steroids and the dried solid particles is considerably different compared to that of the real samples: due to the limited/shorter time of possible interactions and in shortage of all intrinsic characteristics associated with real samples (adsorption, co-precipitation, solubility influenced by other constituents, living organisms, etc.). We applied a more realistic and convincing approach. The efficiency of the ultrasound assisted extraction process was consciously verified (Table 1) and its utility exhaustively confirmed (Tables 2, 3).

(1) The suspended steroids' content of an influent wastewater sample (Dél-Pest, June 2010, 0.5 L; Table 1), were quantified in the consecutive ultrasound assisted solvent portions: the study was performed in three parallel tests, injected two times of each. The reproducibilities of parallel tests and injections, for the fractions one by one, were shown in round brackets, varying between 1.13% RSD and 7.5% RDS, with an average of 3.83% RSD.
Distribution of steroids in solvent fractions is presented in square braces. The first portion of 40 mL solvent mixture contained 91–100%, the second of 20 mL 6.5–7.7% and the final of 20 mL 0.094–1.56%, expressed in the total amount of each steroid found. The proportionality study of suspended steroids obtained from different volumes of target waters were carried out simultaneously with their dissolved phases (Tables 2, 3).
The total amounts of fractions reflect a convincing agreement, varying within the found steroids between 1.00% and 3.69% RSD.

(2) Steroids' amounts determined in the suspended phases, applying the ultrasound assisted solvent extraction from different sample volumes (Table 2: 0.5 L, 1.0 L of influent wastewaters; Table 3: 3 L– 10 L of Danube River samples) showed excellent proportionalities (Bold printed average values in columns Susp. Av.**). Amounts of suspended steroids extracted from 0.5 L and 1.0 L influent wastewater (Table 2, Telki, July 2010) altered between 1.59% and 5.8% RSD, while, from 3 L and 5 L, as well as, from 5 L and 10 L Danube River (Table 3, August 2011, as well as January 2012) varied between 1.00% and 6.0%, as well as, between 0.92% and 4.65% RSD percentages, respectively).

(3) Reproducibilities of suspended steroids, one by one, from parallel samples, carried out in three tests and two injections of each, varied in order of listing, in cases of influent, effluent wastewaters (Table 2) and in the case of Danube River (Table 3), in the ranges of 0.43–12% RSD, 0.83–11%, and 0.87–18% RSD with the averages of 3.64%, 4.58% and 6.7% RSD, respectively.

Table 1
Steroids of the suspended solids obtained from 0.5 L influent wastewater samples' (Dél-Pest, June 2010), via ultrasound assisted solvent extraction, in three consecutive solvent mixture fractions determined as TMS ether derivatives by GC–MS/MS (MRM).

↓ Compounds Solvent mixture* fractions 1.–3. (mL)→	Steroids obtained from consecutive solvent portions of suspended solid phase (μg/L)			
	1. 40 mL	2. 20 mL	3. 20 mL	Total**
β-Estradiol	0.032 (1.13) {100}	< LOQ	< LOQ	**0.322 (1.13)**
Coprostanol	454 (1.33) {93}	31.2 (0.42) {6.5}	0.46 (3.15) {0.094}	**488 (1.30)**
Cholesterol	137 (3.13) {92}	9.1 (2.40) {6.9}	1.32 (5.9) {0.898}	**147 (1.06)**
Stigmasterol	44.1 (2.99) {91}	3.73 (7.3) {7.7}	0.72 (7.3) {1.48}	**48.5 (3.69)**
β-Sitosterol	69.2 (5.2) {91}	5.78 (7.5) {7.6}	1.19 (2.07) {1.56}	**76.0 (1.00)**

Indications: ()=in round brackets relative standard deviation percentages, obtained from three separate tests and two injections of each; *=solvent mixture: hexane/ethyl acetate/methanol=1/1/2, the same ratio as used during the SPE process; {}=percentages based on the total amount quantified; total**=these bold printed values, the unified ones of fractions, are the same listed in Table 2.

Table 2
Steroids and cholic acids determined in the dissolved (Diss.) and suspended (Susp.) phases of influent (Infl.) and effluent (Effl.) wastewater samples (0.5 L, except Infl. Telki July 2010: 05 l and 1.0 L), determined as their trimethylsilyl (oxime) ether derivatives by GC–MS/MS (MRM).

Constituents	Steroids and cholic acids obtained from Hungarian wastewaters treatment plants (WWTPs): μg/L													
	Dél-Pest, June 2010				Telki, July 2010						Telki, October 2010			
	Infl.		Effl.		Infl.				Effl.		Infl.		Effl.	
	Diss.	Susp.	Diss.	Susp.	Diss.	Susp. 0.5 L*	Susp.1.0 L*	Susp. Av.**	Diss.	Susp.	Diss.	Susp.	Diss.	Susp.
Suspended, total (mg/L)	91 (1.56)		2.98 (6.5)		229 {3.70}				2.15 (1.87)		242 (4.75)		9.6 (7.4)	
Androsterone	< LOQ	< LOQ	< LOQ	< LOQ	2.32 (1.99)	< LOQ			< LOQ	< LOQ	7.2 (4.87)	< LOQ	< LOQ	< LOQ
***β*-Estradiol**	0.027 (1.65)	0.032 (1.13)	< LOQ	< LOQ	0.016 (13)	0.0089 (6.2)	0.0081 (3.16)	**0.0085 {4.71}**	0.0075 (21)	0.020 (1.05)	0.017 (13)	0.0066 (6.5)	0.0062 (11)	0.0049 (3.21)
Transandrosterone	< LOQ	< LOQ	< LOQ	< LOQ	4.00 (7.8)	< LOQ			< LOQ	< LOQ	5.81 (4.63)	< LOQ	< LOQ	< LOQ
Estriol	0.085 (1.01)	< LOQ	< LOQ	< LOQ	0.39 (0.26)	< LOQ			< LOQ	< LOQ	0.275 (13)	< LOQ	< LOQ	< LOQ
Coprostanol	39.1 (0.68)	488 (1.30)	4.69 (5.4)	9.3 (3.36)	36.6 (2.42)	22.4 (0.43)	21.7 (1.58)	**22.1 {1.59}**	0.653 (6.8)	36.2 (1.73)	46.3 (1.06)	28.3 (4.49)	4.01 (0.63)	18.6 (2.96)
Cholesterol	7.6 (3.06)	147 (1.06)	0.36 (3.84)	0.79 (3.54)	43.0 (1.58)	412 (2.98)	442 (4.62)	**427 {3.51}**	0.68 (3.98)	22.4 (4.72)	27.5 (0.95)	95 (12)	2.65 (1.59)	10.3 (1.93)
Lithocholic acid	28.9 (4.61)	< LOQ	< LOQ	< LOQ	113 (2.72)	< LOQ			< LOQ	< LOQ	24.9 (4.30)	< LOQ	< LOQ	< LOQ
Stigmasterol	7.50 (3.38)	48.5 (3.69)	1.10 (5.5)	0.17 (11)	< LOQ	11.5 (5.5)	12.9 (3.27)	**12.2 {5.8}**	< LOQ	8.80 (1.54)	7.00 (3.71)	39.5 (5.6)	< LOQ	2.62 (0.83)
Cholic acid	59.1 (5.2)	< LOQ	7.8 (5.2)	< LOQ	64.4 (4.05)	< LOQ			0.70 (2.19)	< LOQ	42.7 (1.96)	LOQ	< LOQ	< LOQ
Chenodeoxycholic acid	31.8 (0.50)	< LOQ	8.4 (8.8)	< LOQ	40.8 (2.16)	< LOQ			0.42 (4.86)	< LOQ	13.8 (11)	LOQ	< LOQ	< LOQ
***β*-Sitosterol**	7.0 (1.17)	76.0 (1.00)	0.57 (7.4)	0.42 (3.63)	LOQ	< LOQ			LOQ	< LOQ	41.9 (4.34)	130 (1.14)	4.62 (0.41)	9.2 (3.59)
Ursodeoxycholic acid	39.1 (11)	< LOQ	< LOQ	< LOQ	45.7 (11)	< LOQ			0.32 (1.83)	< LOQ	28.5 (2.70)	< LOQ	< LOQ	< LOQ
3-OH-7-ketocholanic acid	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ			< LOQ	< LOQ	16.6 (5.2)	< LOQ	< LOQ	< LOQ

Indications: as in Table 1, and/or: {}=in braces relative standard deviation of suspended phases isolated from 0.5 L∗ and 1.0 L∗ sample volumes; Av.**=bold printed values obtained with the ultrasound assisted solvent extraction of the suspended phases of 0.5L and 1.0 L volumes of the same sample, calculated from three parallel tests and two injections of each. *Note*: identifications were performed from twofold up to tenfold diluted, 375 μL stock solutions of derivatives; < LOQ values were taken from paper [2].

Table 3
Steroids and cholic acids determined in the dissolved (Diss.) and suspended (Susp.) phases of Danube River samples, determined from Susp. phases of 3.0 L, 5.0 L and 10 L volumes as their trimethylsilyl (oxime) ether derivatives by GC–MS/MS (MRM).

Constituents	Steroids obtained from Danube River samples (ng/L)											
	February 2011		March 2011		August 2011				January 2012			
	Diss. 3 L	Susp 3 L	Diss. 3 L	Susp. 3 L	Diss. 3 L	Susp. 3 L*	Susp. 5 L*	Susp. Av.*	Diss. 3 L	Susp. 5 L∗	Susp. 10 L*	Susp. Av.**
Suspended, total (mg/L)	5.7 (1.63)		8.6 (0.65)		10.6 {5.7}				25.6 {2.15}			
ß-Estradiol	0.449 (7.3)	< LOQ	< LOQ	< LOQ	0.728 (5.2)	< LOQ			0.33 (6.4)	< LOQ		
Ethinylestradiol	1.16 (12)	0.35 (2.08)	< LOQ	0.46 (5.3)	< LOQ	< LOQ			1.14 (6.3)	0. 39 (6.5)	0.41 (9.6)	**0.40 {2.28}**
Coprostanol	42.1 (8.4)	218 (18)	28.8 (13)	254 (6.5)	36.1 (4.91)	101 (4.70)	99 (7.15)	**100 {1.00}**	18.6 (9.4)	260 (5.3)	266 (5.3)	**263 {1.10}**
Cholesterol	96 (9.2)	106 (0.87)	105 (9.0)	48.2 (11)	170 (4.50)	15.6 (7.5)	13.9 (7.3)	**14.5 {6.0}**	88 (8.7)	516 (5.3)	534 (4.70)	**525 {1.72}**
Stigmasterol	< LOQ	18.3 (9.5)	< LOQ	45.1 (8.2)	< LOQ	< LOQ			23.3 (7.1)	171 (2.32)	188 (1.69)	**179 {4.65}**
Cholic acid	19.2 (16)	< LOQ	128 (14)	< LOQ	< LOQ	< LOQ			8.1 (8.5)	< LOQ		
***β*-Sitosterol**	317 (10)	255 (3.75)	379 (4.22)	463 (13)	128 (9.0)	23.1 (6.23)	21.5 (7.3)	**22.3 {3.59}**	260 (2.00)	1796 (12)	1764 (5.3)	**1780 {0.92}**

Indications: as in Tables 1, 2 and/or: {}=in braces relative standard deviation percentages of the suspended phases isolated from 3 L∗, 5 L∗ and 10 L* sample volumes; Av.**: bold printed values obtained with the ultrasound assisted solvent extraction of the suspended phases filtrated from 3 L, 5 L and 10 L volumes of the same sample.

(4) Inter-day repeatability was evaluated for the January 2012 Danube River sample immediately after derivatization (Table 3.) and a week later (detailed data not shown). *β*-estradiol, ethinylestradiol, coprostanol, cholesterol, stigmasterol and *β*-sitosterol determined in the 3 L dissolved phase provided, in order of listing, 4.61%, 2.31%, 7.8%, 0.621%, 13%, 19% and 3.60% RSD, while, ethinylestradiol, coprostanol, cholesterol, stigmasterol and *β*-sitosterol determined in the 5 L suspended phase, in order of listing, 11.0%, 2.25%, 7.9%, 4.47% and 2.21% RSD.

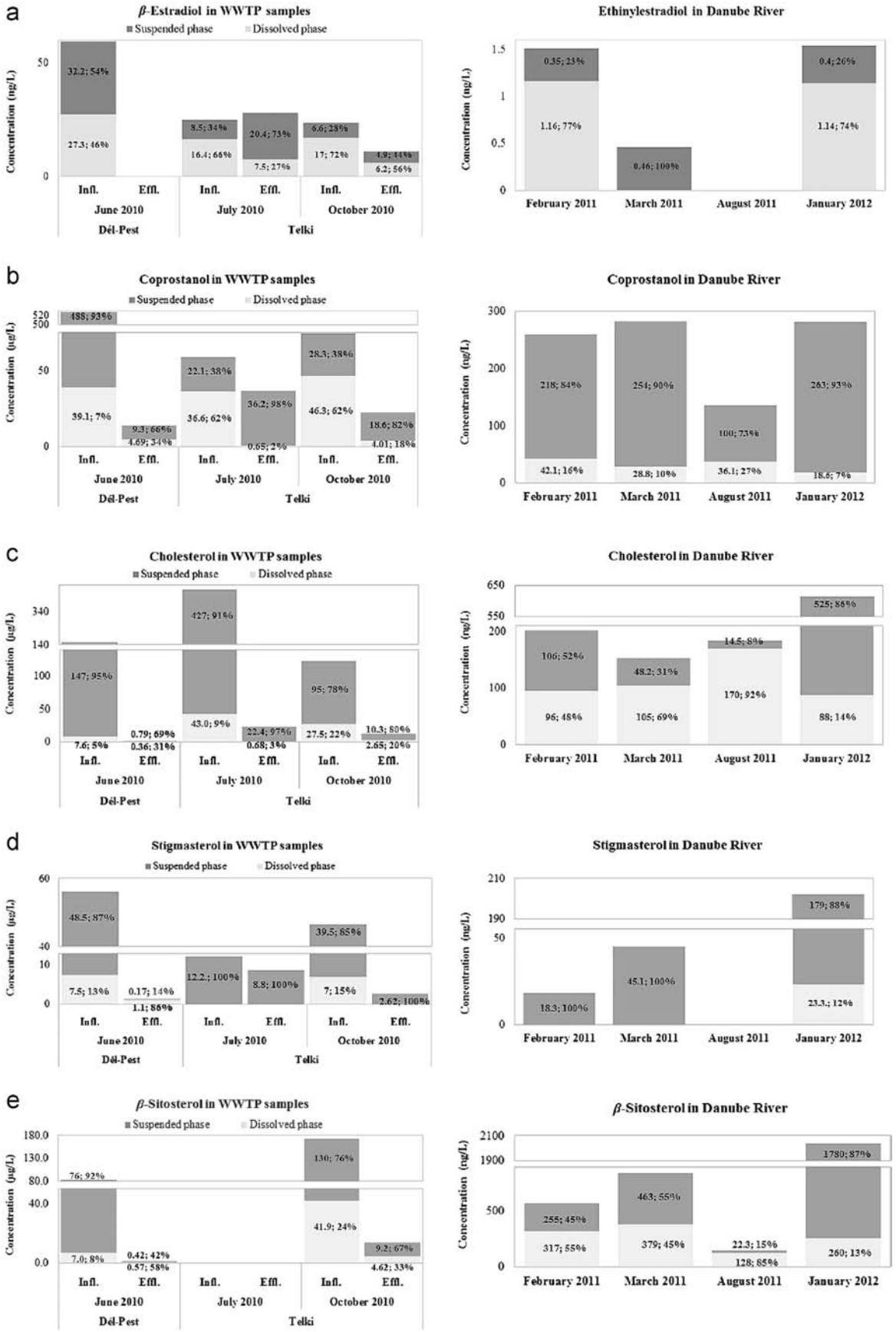

Fig. 1. (a–e) Distribution of natural and synthetic steroids in the dissolved and suspended phases of wastewater and Danube River samples: concentrations are expressed in μg/L and/or in ng/L (detailed analyses in Tables 2 and 3); percentages show the distribution of the total amount of steroids identified and quantified in the dissolved and suspended phases, separately.

3.2. Steroids and cholic acids determined in the dissolved and suspended phases of samples from two Hungarian WWTPs

During a five months period of time, three samples were analyzed.

As to the total of isolated suspended solids, their amounts decreased drastically during the wastewater treatment process (Table 2, difference values in the first horizontal line: by 96.7% (Dél-Pest, June 2010: calculated as example: 91.0–2.98=88.0; $88 \times 100/91 = 96.7$), by 99% (Telki, July 2010) and by 96% (Telki, October 2010).

The natural steroid hormones, like androsterone, transandrosterone and estriol were determined only in the dissolved phases of these samples, despite the high amount of androsterones found in the influent samples (2.32 and 7.2 μg/L for androsterone, 4.00 and 5.81 μg/L for transandrosterone). This can be attributed to their good water solubility among the steroid group.

The cholic acids (litocholic-, cholic-, chenodeoxycholic-, ursodeoxycholic-, and 3-hydroxy-7-ketocholanic acids), were present in the dissolved phase, only. This can be explained by their acidic properties: the pH of the water samples was neutral–slightly basic (pH 7–8), while pKa of these cholic acids is below pKa 6 [22]. Their dissociation to ionic form increases their solubility.

Steroids quantified in the suspended phases confirmed our assumptions (Table 2). Steroids' concentrations, found in these phases, expressed in μg/L values, proved to be higher in comparison to the amounts determined in their corresponding dissolved ones. Even effluent wastewaters' suspended phases contained steroids in wide concentration ranges. The natural estrogen β-estradiol showed up from 0.0049 to 0.032 μg/L, while fecal sterols (coprostanol, cholesterol) and phytosterols, (stigmasterol and β-sitosterol) manifested considerable higher amounts: coprostanol varied from 9.3 μg/L to 488 μg/L, cholesterol from 0.79 to 427 μg/L, stigmasterol from 0.17 μg/L to 48.5 μg/L and β-sitosterol from 0.42 μg/L to 130 μg/L.

3.3. Steroid content of the dissolved and suspended phases of Danube River samples

During a twelve months period of time, four samples were taken (Table 3.).

The total amounts of the suspended solid particles in the Danube River samples varied between 5.7 and 25.6 mg/L.

In the dissolved phases, coprostanol, cholesterol and β-sitosterol were detected in all cases, while β-estradiol and cholic acid in three, ethinylestradiol in two, stigmasterol in only one sample. Their concentration ranged 0.33–0.45 ng/L (β-estradiol), 1.14–1.16 ng/L (ethinylestradiol), 18.6–42.1 ng/L (coprostanol), 88–170 ng/L (cholesterol) 23.3 ng/L (stigmasterol), 8.1–128 ng/L (cholic acid) and 128–379 ng/L (β-sitosterol).

Suspended phases all contained coprostanol, cholesterol and β-sitosterol while ethinylestradiol and stigmasterol in three cases out of four were found. The concentration of these steroids ranged 0.35–0.46 ng/L (ethinylestradiol), 218–263 ng/L (coprostanol), 14.5–525 ng/L (cholesterol), 18.3–179 ng/L (stigmasterol) and 22.3–1780 ng/L (β-sitosterol).

3.4. Distribution of steroids between the dissolved and suspended phases of wastewater and Danube River samples

Steroid contents' comparisons in the dissolved and in the suspended phases of each sample, expressed in μg/L (β-estradiol and ethinylestradiol in ng/L), were completed with their percentages, based on their total amounts determined (Fig. 1a–e)

Steroids' ratios found in the dissolved and suspended phases revealed considerable differences; special distinction between sample types (influent and effluent wastewater, Danube River water) could not be confirmed.

The percentages of the amount of steroids in the suspended phases, with few exceptions, proved to be higher compared to their corresponding dissolved ones.

Steroids' distributions, in order of listing, proved to be in WWTP and Danube River samples in the range of 28–54% (β-estradiol, WWTPs only, Fig. 1a), 23–100% (ethinylestradiol, Danube River only, Fig. 1a), 38–98% and 73–93% (coprostanol, Fig. 1b), 69–97%, 8–86% (cholesterol, Fig. 1c), 14–100%, 88–100% (stigmasterol, Fig. 1d), 42–92% and 15–87% (β-sitosterol, Fig. 1e).

The overwhelming part of steroids, (expressed in their total amounts), were found in samples' suspended phases: in averages 71% from WWTPs and 64% from Danube River samples.

The variability of steroids' distribution can be explained by the random differences in the size and compositions of the suspended solid particles, and by the actual characteristics of waters they are isolated from (sorption behavior of other constituents) [24].

4. Conclusions

The identification and quantification of steroid pollutants, and their distribution between the dissolved and suspended phases of environmental waters, in comparable manner, were confirmed for the first time, as their trimethylsilyl-(oxime)-ether derivatives, applying the recently developed GC–MS/MS (MRM) method.

A simple, time, cost, labor efficient and quantitative ultrasound assisted extraction method was presented. The reliability, reproducibility, proportionality and practical utility of suggested proposal was confirmed by the analysis of suspended steroids' contents extracted from different volumes of influent wastewater and Danube River samples. Analytical performances of results were characterized with the relative standard deviation percentages of values. This means, that steroids' partition of three samples from two wastewater treatment plants (WWTPs), collected during a five months and from four Danube River samples of 12 months periods of time have been compared. Suspended steroid contents obtained from 0.5 and 1.0 L wastewater and from 3 L, 5 L and 10 L Danube River samples confirmed excellent proportionality, characterized with the relative standard deviation percentages: ranging from 1.59 RSD% to 5.8 RSD% (WWTP) and from 0.92 RSD% to 6.0 RSD% (Danube River).

Results confirmed that the steroid contents of dissolved and suspended phases of environmental water samples need to be considered simultaneously: they are integral parts of each others. Meaning, that the steroid contents of the suspended phases cannot be omitted from the analysis. This statement is well documented by the fact that the overwhelming part of steroids, expressed in their total amounts, were found in their suspended phases (71% from WWTPs, 64% from Danube River samples).

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