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Analytical determination of oestrogenic endocrine disruptors: the method of choice for wastewater treatment plant effluents

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Environmental context. Endocrine disrupting compounds (EDCs) are among the most recently targeted micropollutants detected in wastewater treatment plant (WWTP) effluents and in aquatic environments. There is a need for the development of robust analytical methods for most relevant estrogenic EDCs. This study provides optimisation of analytical techniques and addresses several relevant aspects that are often overlooked in the literature. The method was finally successfully employed for the analysis of WWTP effluents.

Abstract. Two analytical approaches – liquid chromatography–tandem mass spectrometry (LC-MS/MS) and gas chromatography–tandem mass spectrometry (GC-MS/MS) methods – were compared for the simultaneous determination of the 19 most important oestrogenic endocrine disrupting chemicals (EDCs), such as 17β-oestradiol, oestrone, 17α-ethinyloestradiol, bisphenol A and triclosan in wastewater treatment plant effluents. To lower the instrument limits of detection (ILODs), a derivatisation step preceded detection in both methods. The stability, sensitivity and ease of use of dansylation (Dns) for LC-MS/MS and trimethylsilylation (TMS) for GC-MS/MS derivatives were evaluated before method validation. TMS derivatisation products were highly unstable over time. Parameters such as susceptibility to matrix effects and the stability of monodansylated and didansylated derivatisation products of phytohormones are discussed. Lower ILODs of highly potent EDCs (0.11 ng mL^{-1} for 17β-oestradiol, 0.01 ng mL^{-1} for 17α-ethinyloestradiol and 0.22 ng mL^{-1} for oestrone) and stability of derivatisation products within 7 days were achieved using LC–MS/MS; therefore, further validation of this method at environmentally relevant concentrations was conducted. The method limits of detection (MLODs) met the requirements of the European Union defined in Directive 2008/105/ES for 17α-ethinyloestradiol (0.035 ng L^{-1}) and 17β-oestradiol (0.4 ng L^{-1}). Twenty samples of wastewater treatment plant effluent from the Czech Republic were screened using LC-MS/MS. Fifteen of the EDCs were detected in at least one sample. The most abundant EDCs were bisphenol A, with a concentration up to 1107 ng L^{-1}, and triclosan, with a concentration up to 76 ng L^{-1}. No seasonal trend between late spring and autumn samples was observed in the frequency or quantity of analytes.

Keywords: BSTFA, dansyl chloride, oestrogens, phytoestrogens, WWTP effluent, liquid chromatography, gas chromatography, mass spectrometry.

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Introduction

There has been increasing interest in environmental contamination by endocrine-disrupting chemicals (EDCs) in recent decades. These substances have the potential to interfere with hormonal systems and cause adverse effects in exposed organisms. Moreover, even substances with a biologically plausible link to the endocrine-disrupting mode of action can be considered EDCs according to the European Chemicals Agency and European Food Safety Authority (ECHA et al. 2018).

Endocrine disruptors encompass endogenous hormones and a wide range of manmade chemicals that can affect various hormonal pathways. Wastewater treatment plant (WWTP) effluents are often referred to as secondary sources or as hot spots of EDC release into the environment owing to insufficient removal. Accordingly, effluents and downstream river water are among the most studied matrices (Grover et al. 2009; Gorga et al. 2013; Golovko et al. 2018; Kramer et al. 2018; Ronderos-Lara et al. 2018).

In the present European and US legislation, there are no specific regulations regarding target values for maximum levels of EDCs in WWTP effluents. To date, information about these limits is only in the form of recommendations or proposals (Drinking Water Candidate Contaminant List assembled by the US EPA, Watch List of Water Framework Directive of the European Union 2015/495). However, as a first step towards limiting EDCs, the European Commission included the three most potent and frequently occurring compounds, 17β-oestradiol (β-E2), oestrone (E1) and 17α-ethinyloestradiol (EE2), on a

watch list for aqueous matrices (Watch List referring to European Commission decision 2015/495 with regard to Directive 2008/105/ES). Methodological instructions for their determination were defined as maximum acceptable method limits of detection (MLODs; 0.035 ng L^{-1} for EE2 and 0.4 ng L^{-1} for E1 and β-E2).

Owing to increasing concerns about EDC pollution and the effects of their mixtures, reliable methods for screening a wider group of analytes and low MLODs are needed. Oestrogenic activity is the most described and usually the most frequently found hormonal activity in aqueous samples (Tousova et al. 2017). Oestrogenic active compounds occur at very low concentrations (ng L^{-1}); nevertheless, these concentrations are biologically relevant (Adams 1998; Routledge et al. 1998; Nash et al. 2004; Gross-Sorokin et al. 2006; Kidd et al. 2007). Therefore, method validation and subsequent analysis of environmental samples must be performed with the lowest possible concentration, at least at concentrations that are predicted to have no biological effect. This is challenging because these substances differ in physical and chemical properties. Furthermore, the complex matrix of environmental samples can lead to problems described as matrix effects (MEs), especially in the case of liquid chromatography (Antignac et al. 2005; Bienvenu et al. 2017).

Most of the current methods focus only on some known oestrogenic compounds represented by human endogenous hormones, the active substances in contraceptives, and industrial chemicals. With respect to the mixture of contaminants to which humans and wildlife populations are constantly exposed, it is highly necessary to monitor all substances in the groups (Preindl et al. 2019). In fact, derivatisation is often applied before separation methods to enhance ionisation and to reach lower LODs (higher sensitivity). The two most commonly used derivatisation reagents for hydroxyl groups are *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide with trimethylsilyl chloride (BSTFA:TMCS, 99:1, trimethylsilylation) and dansyl chloride (dansylation) for gas chromatography–tandem mass spectrometry (GC-MS/MS) and liquid chromatography–tandem mass spectrometry (LC-MS/MS), respectively (Tomsikova et al. 2012; Barreiros et al. 2016; Caballero-Casero et al. 2016; Kramer et al. 2018).

Compared with GC-MS, LC-MS systems are more susceptible to MEs, which decrease the signal-to-noise ratio; therefore, the ILODs increase (Matuszewski et al. 2003; Antignac et al. 2005; Grover et al. 2009; Bienvenu et al. 2017). Tandem mass spectrometry (MS/MS) is typically used for environmental samples to avoid noise. Diaz-Cruz et al. (2003) tested the detection of hormonally active compounds using two ionisation techniques for LC: electrospray ionisation (ESI) and atmospheric pressure chemical ionisation. ESI was considered the optimal ionisation source (Diaz-Cruz et al. 2003). The limitation of ESI for the detection of steroids is the selectivity of fragments (Vanderford et al. 2003; Glineur et al. 2018). Nevertheless, ESI is often used in such analyses, despite the low ionisation efficiency of the semipolar features of hormones (Glineur et al. 2018; Preindl et al. 2019).

The aim of the present study was to develop a reliable, sensitive and suitable method for the determination of the 19 most important oestrogenic active compounds, consisting of six endogenous compounds, three phytoestrogens, one mycoestrogen and nine xenoestrogens and progestins (Table 1). Various factors, such as the strength of the ligand binding to the oestrogenic receptor, environmental concentration, present consumption, worldwide increasing production of soybeans and pharmaceuticals, and legislative recommendations, were considered for inclusion in this method. Prior to method validation and application, the two most frequent analytical approaches for the measurement of EDCs were compared (LC-MS/MS and GC-MS/MS). The advantages and disadvantages of both methods are discussed in detail. During validation, special emphasis was given to environmentally relevant concentrations and to the stability of derivatisation products, which have rarely been studied. Finally, the optimised method was applied for screening 19 EDCs in 20 typical municipal WWTP effluents across the Czech Republic.

Materials and methods

Chemicals

Bisphenol A (BPA; 99+ %), bisphenol F (BPF; ≥98 %), bisphenol S (BPS; ≥98 %), equilin (EQN; ≥98 %), estriol (E3; ≥ 97 %), E1 (≥99 %), triclosan (TCS; ≥97 %), hexachlorobenzene (HCB; 99 %), mestranol (MES; ≥99 %), 19-norethindrone (NORE; ≥98 %), EE2 (99.4 %) and β-E2 (≥98 %) were purchased from Sigma–Aldrich (Germany). Standards of (*R*,*S*)-equol (EQ; 98 %), daidzein (DAID; 98 %), α-zearalenol (ZEA; 96.92 %), 17α-oestradiol (α-E2; 98 %) and all labelled standards, except [$^2H_4$]-mestranol, were obtained from Toronto Research Chemicals, Inc. (Canada). [$^2H_4$]-Mestranol was purchased from Alsachim (France). Genistein (GEN; 99+ %) and dansyl chloride (≥96 %) were obtained from Alfa Aesar (Germany). Equilenin (LEN) solution in acetonitrile (≥98 %), D(–)-norgestrel (NRG; ≥99 %) and derivatisation reagent BSTFA + TMCS (99:1) were acquired from Supelco (Sigma–Aldrich, Germany). 4-Nonylphenol (NP; 99.9 %) was purchased from Fluka (Germany). Analytes and some of their physicochemical properties are listed in Table 1.

Ethyl acetate (EtAc, ≥99.8 %), methanol (MeOH, ≥99.9 %) and LC-MS-grade methanol (MS-MeOH, ≥99.9 %) were purchased from VWR (Czech Republic). Pyridine (PYR, >99.9 %) and dimethylformamide (DMF, >99.9 %) were acquired from Sigma–Aldrich. Formic acid (FA; LC-MS quality) was obtained from Labicom (Czech Republic). Ultrapure water (MQ; 18.2 MΩ cm) was prepared using a Milli-Q purification system (Millipore) or a Barnstead™ Smart2Pure™ system (Thermo Fisher).

Sample collection and preparation

Sample collection

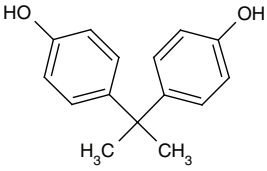
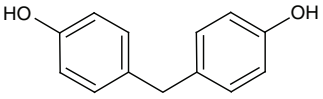
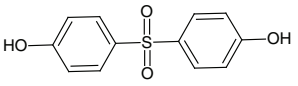
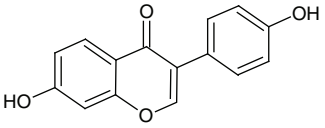
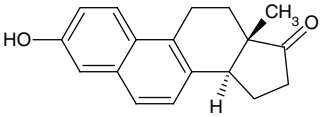
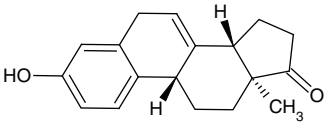
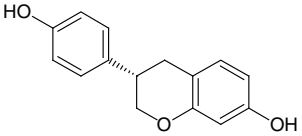
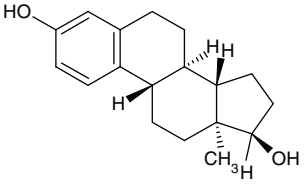
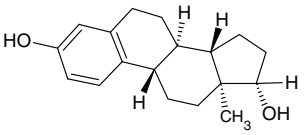
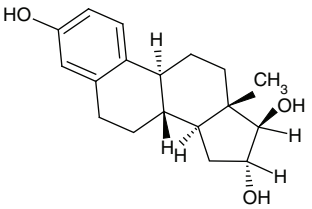
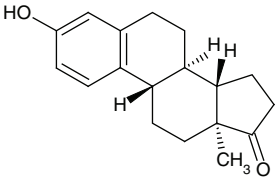
Twenty municipal WWTP effluents were sampled twice in two seasons (autumn 2018 and late spring 2019) in the Czech Republic. The locations are not named to protect the privacy of the operators. The WWTPs receive mainly household wastewater and their population equivalent ranged between hundreds and hundreds of thousands. Mechanical removal, activated sludge treatment and phosphorus precipitation were the main processes involved. The total organic carbon (TOC) content was measured to characterise the effluents using a FORMACS^{HT} TOC/TN analyser (Skalar).

Samples were collected in clean amber glass bottles with Teflon-lined caps, transported to the laboratory on the same day and stored at 4 °C until extraction was performed within 36 h.

Solid-phase extraction

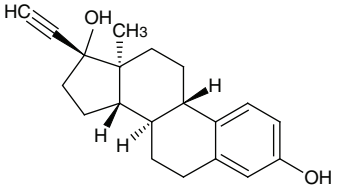
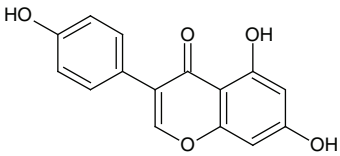
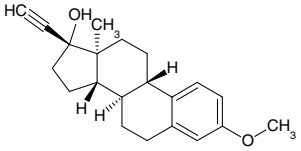
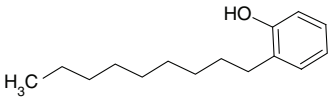
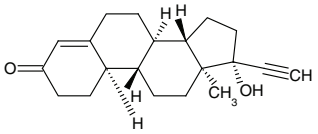
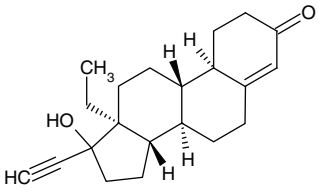
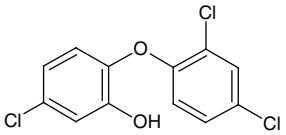
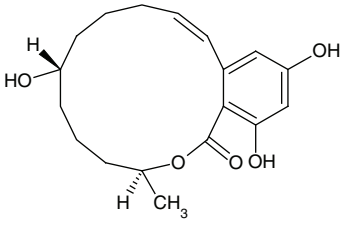
The grab samples were divided into three equal samples with volumes of 0.85–1 L. The sample pH was adjusted to 2.5 with hydrochloric acid (35 %). Then, the sample was filtered through paper filters (0.5 μm, Macherey–Nagel) and cellulose nitrate membrane filters (0.45 μm, Whatman) purchased from P-LAB (Czech Republic). Solid-phase extraction was performed using Chromabond® C18 ec cartridges (6 mL, 500 mg, BDL, Czech Republic) according to previously published methods (Samaras et al. 2011; Kresinova et al. 2018). Each column was

Table 1. List of the studied oestrogenic endocrine disruptors and their physicochemical properties

	Monoisotopic mass (g mol^{-1})	Log P^{A}	pK_a	Usage or origin	Structure
Bisphenol A (BPA)	228.1	3.30	9.78	Industry	
2,2-Bisphenol F (BPF)	200.1	2.90	9.84	Industry	
Bisphenol S (BPS)	250.0	1.90	7.42	Industry	
Daidzein (DAID)	254.1	2.50	6.48	Phytoestrogen	
Equilenin (LEN)	266.1	3.50	9.78	Horse hormone	
Equilin (EQN)	268.1	2.90	9.41	Horse hormone	
R,*S*-Equol (EQ)	242.1	3.00	9.63	Phytoestrogen	
17α-Oestradiol (α-E2)	272.2	4.00	10.33	Human hormone	
17β-Oestradiol (β-E2)	272.2	4.00	10.33	Human hormone	
Estriol (E3)	288.2	2.50	10.33	Human hormone	
Estrone (E1)	270.2	3.10	10.33	Human hormone	

(*Continued*)

Table 1. (Continued)

	Monoisotopic mass (g mol^{-1})	Log P[A]	pK_a	Usage or origin	Structure
17α-Ethinyloestradiol (EE2)	296.2	3.70	10.33	Synthetic hormone	
Genistein (GEN)	270.1	2.70	6.55	Phytoestrogen	
Mestranol (MES)	310.2	4.00	17.59	Synthetic hormone	
4-Nonylphenol (NP)	220.2	5.60	10.41	Industry	
19-Norethindrone (NORE)	298.2	3.00	17.59	Synthetic hormone	
D(–)-Norgestrel (NRG)	312.2	3.30	17.91	Synthetic hormone	
Triclosan (TCS)	288.0	5.00	7.68	Personal care product	
α-Zearalenol (ZEA)	320.2	4.00	8.54	Mycohormone	

[A]Values of the partition coefficients were calculated according to the atom-additive method and taken from PubChem.

conditioned with 6 mL EtAc, 2 × 3 mL MeOH, 2 × 3 mL MQ water and 4 mL MQ water (pH 2.5). After loading the whole water sample into the system, the sorbent was washed with 6 mL MQ water (pH 2.5) and dried under vacuum. The retained analytes were eluted with 6 mL EtAc, and the final volume was reduced to ~2 mL. The exact volume was determined by weighing, and the typical concentration factor was at least 400. After derivatisation with dansyl chloride, the final measurement was performed using LC-MS/MS.

MQ water from the two purification systems served as method blanks for an evaluation of potential background contamination.

Derivatisation

The derivatisation reaction for LC-MS/MS was adopted from the literature (Anari et al. 2002; Backe 2015) and slightly modified (incubation time and reagent volume were optimised): 400 µL of standard in MeOH or sample solution was dried under a gentle stream of nitrogen (N_2). After that, 200 µL 100 mM sodium bicarbonate buffer and 200 µL dansyl chloride in acetone (1 mg mL^{-1}) were added to the dry residues. The samples were vortexed, incubated (60 °C, 5 min), cooled to ambient temperature, and injected into the LC-MS/MS system. Four incubation intervals (3, 5, 8 and 20 min) and two volumes

of derivatisation reagents (40 or 100 % of final sample volume) were tested.

The stock solution of dansyl chloride was prepared by adding exact aliquots of acetone to weighed dansyl chloride. The vial was sonicated between each addition of solvent and filtered through a PTFE membrane syringe filter (0.2 μm, Rotilabo®, P-LAB, Czech Republic). Sodium bicarbonate buffer was prepared by dissolving 0.42 g sodium bicarbonate in 50 mL MQ water. The buffer pH was adjusted to 10.5 with 1 M sodium hydroxide (Sosvorova et al. 2017).

Two derivatisation treatments were investigated for GC-MS/MS using PYR or DMF addition as recommended in Shareef et al. (2006) and Zhang et al. (2006). For the first approach, 1 mL of sample was dried under a stream of N_2, and 100 μL PYR was added. For the second approach, 100 μL DMF was added to 1 mL of the sample, and the mixture was evaporated to 100 μL. In both cases, the solution was mixed with 200 μL of derivatisation reagent BSTFA + TMCS (99:1) and incubated (70 °C, 30 min). After cooling to room temperature, the BSTFA + TMCS was evaporated to dryness (PYR treatment) or to 100 μL (DMF treatment) under a gentle stream of N_2. Finally, 100 μL internal standard (IS; HCB in EtAc, 1 mg mL^{-1}) was added, and the sample was reconstituted with EtAc (final volume was ~1 mL). The MS signal of each sample was recalculated according to the response of the IS to correct the final volume. The influence of incubation time has already been tested by Shareef et al. (2006); in addition, a 30-min interval was recommended in the manufacturer's instructions.

Derivatisation stability

The short- and long-term stability of dansylated (Dns) and trimethylsilylated (TMS) products was tested using a standard stock solution at three concentration levels covering a linear range; these concentrations were designated low, middle and high (see Tables 2 and 3 for the specific concentration range of each analyte with respect to the instrument used). Short-term stability was assessed by repeated injections of triplicate solutions within 20 h (samples at point 0 were injected 2 h after derivatisation). The long-term stability of the samples was measured with triplicate solutions after 0, 1, 3, and 7 days of storage at 4 °C. The long-term stability test was repeated twice to confirm the trends.

Statistical analysis using one-way ANOVA was applied to the data from the derivatisation stability tests. The dataset for ANOVA included all three concentrations, and the significance of time was tested.

Instrumentation

LC-MS/MS conditions

A Shimadzu Nexera X2 LC system coupled to a triple quadrupole Sciex 4500 mass spectrometer (Sciex) was used to separate and detect target analytes. A Kinetex Phenyl-hexyl 2.6 μm analytical column (100 × 2.1 mm, Phenomenex) preceded by a same-phase security guard column (ULTRA, 2.1 mm) was used for chromatographic separation.

The mobile phase consisted of MQ water and MS-MeOH, both with the addition of 0.1 % FA. All 19 target compounds were separated and eluted within 40 min in gradient mode as follows: the initial conditions were set to a total flow of 0.3 mL min^{-1} of 50 % MS-MeOH, followed by a gradient change to 80:20 MS-MeOH:MQ water in 7 min. The mobile phase was isocratically held till 13 min; then, the MS-MeOH content was increased linearly to 100 % by 20 min; 100 % MS-MeOH solution was held to 30 min, and a final gradient returned the solution to the initial conditions. The injected sample volume was 5 μL, and the column temperature was 30 °C. The separation gradient was a result of a previous optimisation.

Table 2. Parameters of the LC-MS/MS method with instrumental limits of quantification and detection (ILOQ and ILOD)

'Mono' and 'di' indicate monodansylated and didansylated derivatisation products used for final validation. The linear range was individually optimised for each analyte according to the sensitivity of the instrument. At least two multiple reaction monitoring (MRM; with collision energy (V) in brackets) transitions were used for analyte identification. The matrix effect (ME) was tested in two matrices with low (7.48 mg L^{-1}) and high (12.46 mg L^{-1}) contents of total organic carbon (TOC). MEs corrected to the respective labelled standards and without any correction are shown. n.d., not determined

	Dansylation level	ILOQ (ng mL^{-1}) ($n = 6$)	ILOD (ng mL^{-1}) ($n = 6$)	Linear range (ng mL^{-1})	R^2 ($n = 6$)	Retention time (min)	MRM transitions (collision energy)	ME (%) (low TOC; high TOC)	ME (%) corrected (low TOC; high TOC)
BPA	di	13.39	4.02	10–500	0.9994	21.0	694.6 >155.8 (103), 169.8 (51)	85; 85	113; 94
BPF	di	19.57	5.87	10–500	0.9998	20.3	666.6 >170.9 (47), 169.9 (47)	88; 87	119; 98
BPS	di	19.44	5.83	20–1000	0.9989	20.0	716.6 >155.8 (99), 170.9 (49)	97; 92	96; 87
DAID	mono	0.53	0.16	0.2–10	0.9994	10.1	488.1 >170.9 (39), 169.8 (39)	86; 54	12; 15
LEN	mono	6.10	1.83	10–500	0.9980	17.2	499.7 >155.8 (75), 170.8 (39)	94; 96	n.d.
EQN	mono	0.007	0.002	0.01–0.5	0.9993	17.3	502 >155.8 (73), 170.9 (41)	93; 104	78; 81
EQ	di	21.98	6.59	1–50	0.9987	20.7	709.0 >170.0 (49), 156.0 (103)	74; 84	69; 84
α-E2	mono	0.29	0.09	0.2–10	0.9990	16.4	506.4 >155.8 (75), 170.8 (43)	111; 100	94; 101
β-E2	mono	0.35	0.11	0.2–10	0.9992	16.5	505.9 >155.8 (77), 170.8 (43)	98; 119	95; 109
E3	mono	0.96	0.29	1–50	0.9997	11.3	521.8 >170.8 (35), 155.8 (73)	97; 105	90; 87
E1	mono	0.73	0.22	0.1–5	0.9995	17.9	504.0 >169.8 (39), 170.8 (39)	102; 115	96; 96
EE2	mono	0.05	0.01	0.1–5	0.9999	16.5	529.9 >114.9 (113), 170.8 (45)	79; 109	81; 104
GEN	mono	3.59	1.08	10–500	0.9997	9.9	504.0 >169.8 (39), 170.8 (39)	103; 129	113; 118
MES		2.42	0.73	5–250	0.9986	8.9	311.1 >120.9 (25), 158.9 (19)	84; 93	104; 86
NP	mono	0.28	0.08	1–50	0.9994	19.8	454 >170.8 (35), 155.9 (35)	97; 100	105; 102
NORE		8.89	2.67	10–500	0.9994	6.6	298.7 >91.00 (63), 108.9 (31)	94; 106	105; 104
NRG		7.31	2.19	10–500	0.9994	7.4	312.6 >91.0 (61), 108.9 (29)	88; 107	103; 100
TCS	mono	0.10	0.03	0.1–5	0.9997	18.4	527.8 >155.8 (73), 170.8 (35)	89; 109	87; 96
ZEA	di	4.35	1.30	10–500	0.9993	19.4	787.9 >169.8 (67), 170.8 (67)	103; 114	92; 96

Table 3. Parameters of the GC-MS/MS method with instrumental limits of quantification and detection (ILOQ and ILOD)
The linear range was optimised individually for each analyte according to the sensitivity of the instrument. Hexachlorobenzene (HCB) was used as an internal standard for the volume correction. At least two multiple reaction monitoring (MRM) transitions were used for analyte identification. The collision energy of 30 V was the same for all target compounds except 10 V for HCB

	Trimethylsilylation level	ILOQ (ng mL^{-1}) ($n = 6$)	ILOD (ng mL^{-1}) ($n = 6$)	Linear range (ng mL^{-1})	R^2 ($n = 6$)	Retention time (min)	MRM transitions
BPA	di	0.62	0.19	0.5–100	0.9992	17.5	357.0 >357.0, 372.0 >357.0
BPF	di	0.33	0.10	0.5–100	0.9994	16.4	344.0 >344.0, 344.0 >179.0
BPS	di	8.78	2.63	10–1000	0.9999	26.1	394.0 >379.0, 379.0 >379.0
DAID	di	7.82	2.35	5–1000	0.9993	30.9	398.0 >383.0, 383.0 >383.0
EQN	mono	5.64	1.69	0.005–0.5	0.9995	25.7	216.0 >216.0, 283.0 >283.0
EQ	di	5.48	1.64	5–1000	0.9998	24.3	386.0 >191.0, 191.0 >191.0
HCB						9.6	284.0 >284.0
α-E2	di	3.50	1.05	5–1000	0.9988	25.5	416.0 >285.0, 326.0 >326.0, 285.0 >285.0
β-E2	di	3.3	0.99	5–1000	0.9970	26.4	416.0 >285.0, 326.0 >326.0, 285.0 >285.0
E3	tri	4.48	1.34	5–1000	0.9951	30.4	504.0 >311.0, 504.0 >297.0, 311.0 >311.0
E1	mono	6.75	2.02	5–1000	0.9991	25.4	342.0 >342.0, 257.0 >257.0
EE2	di	5.64	1.69	5–1000	0.9996	29.2	425.0 >425.0, 285.0 >285.0
MES	mono	70.86	21.26	50–10000	0.9960	26.7	382.0 >367.0, 367.0 >367.0
NP	mono	0.9	0.27	5–1000	0.9960	12.4	179.0 >179.0, 292.0 >179.0
NORE	mono	28.97	8.69	50–10000	0.9990	27.9	370.0 >355.0, 355.0 >355.0
NRG	di	45.85	13.75	50–10000	0.9978	29.9	355.0 >355.0, 317.0 >317.0
TCS	mono	2.06	0.62	0.5–100	0.9993	15.9	345.0 >345.0, 345.0 >200.0, 200.0 >200.0
ZEA	tri	66.89	20.07	50–10000	0.9996	30.6	536.0 >521.0, 446.0 >446.0, 536.0 >446.0

The mass spectrometer was operated in positive ESI mode. Multiple reaction monitoring (MRM) mode transitions were recorded in schedule mode. The parameters for ESI were set as follows: curtain gas, 20 psi (1 psi = 6.89 kPa); ion spray voltage, 5.5 kV; vaporiser temperature, 650 °C; ion source gas 1, 50 psi; and ion source gas 2, 50 psi. The MS/MS conditions were optimised for each compound (specific transitions and collision energies are listed in Table 2). Mono-Dns and di-Dns analytes were evaluated for compounds with multiple hydroxyl groups. Only one of the derivatisation products (the most abundant) was finally chosen for the analysis and quantification of the respective analyte (see *Derivatisation: Dns versus BSTFA*). *Analyst Software 1.6.3* was used for data acquisition and handling.

GC-MS/MS conditions

A Scion 436-GC instrument coupled to a triple quadrupole Evoq TQ (Bruker) with an electron-impact ionisation (EI) interface (70 eV) was used for separation and detection of target compounds. The instrument was equipped with a DB-5ms column (0.25 µm, 30 m × 0.25 µm, Agilent Technologies). Helium served as a carrier gas with a constant flow rate of 1.2 mL min^{-1} and argon as a collision gas. The collision energy was 30 V for all compounds except the internal standard (HCB), which had a collision energy of 10 V. The injection on the column was performed in split/splitless mode at a ratio of 1:50. Injected volume of the sample was 1 µL. The injector temperature was set to 250 °C.

Analytes were separated in gradient mode within 40 min. The column temperature began at 60 °C, which was held for 2 min. The temperature was increased to 180 °C at a rate of 30 °C min^{-1}. Then, it increased to 250 °C (rate 4 °C min^{-1}) and was held for 5 min. Finally, the temperature increased to 280 °C at a rate of 25 °C min^{-1} and was held for 10 min.

Derivatised analytes were detected in the positive MRM mode. The MS/MS conditions were optimised for each compound (specific transitions are listed in Table 3). The compounds were quantified using one derivatisation product because only one product was usually recognised. An MS Workstation 8 was used for data acquisition and handling.

LC-MS/MS and GC-MS/MS method performance and LC-MS/MS method validation

The repeatability of retention times and peak heights, linearity, ILODs and instrumental limits of quantification (ILOQs) were assessed for both methods using standard mixtures in the respective solvents. The optimised derivatisation was applied before measurements – for LC (100 % reagents in total sample volume, 60 °C and 5 min) and for GC (20 % reagent in total sample volume, 70 °C and 30 min). Trueness, precision, ME and solid-phase extraction (SPE) method recovery were subsequently evaluated only for the LC-MS/MS method since it better met the requirement for the determination of targeted analytes in environmental samples.

Stock solutions of individual standards and IS were gravimetrically prepared in MS-MeOH (LC-MS/MS) and EtAc (GC-MS/MS) and stored at –20 °C. The calibration solutions were prepared by diluting stock solutions in MS-MeOH and EtAc over the specific calibration range. At least six concentration levels were used for the calibration curve. Dynamic linear ranges for each substance were adjusted according to the sensitivity of the respective instrument (Tables 2 and 3). The final concentrations of labelled ISs for the LC-MS/MS method were 25 or 50 ng mL^{-1}.

The ILOD and ILOQ were calculated using the following equations: $\mathrm{ILOD} = 3\frac{\sigma}{m}$ and $\mathrm{ILOQ} = 10\frac{\sigma}{m}$, where σ is the standard deviation of the peak heights and m is the slope of the calibration curve (Vidova and Spacil 2017). The lowest calibration level with a precision <20 % ($n = 6$) was used for ILOD and ILOQ calculations (FDA 2018). The MLODs were assessed from the ILODs and concentration factor resulting from the preparation procedure (see *Solid-phase extraction*).

The following parameters were determined only for LC-MS/MS. The method trueness (also called matrix recovery) and

precision were assessed in matrix-matched standards at three concentrations and are expressed as concentration accuracy and relative standard deviation (RSD), respectively. The ME was estimated by comparing the slopes of calibration curves measured in the matrix and in the neat solvent. The result of the ME analysis for each analyte is expressed as a percentage of the slopes obtained. Both tests were performed for two extracts of effluents with low and high amounts of TOC. In addition, the ME was checked using the standard addition of labelled IS.

To determine the SPE method recovery, the MQ water was spiked with the standard solution at three concentration levels (specific concentration levels for individual analytes are defined in Table 4). The method recovery and ME can be assessed separately for each sample and then used for correction of a sample concentration (Valitalo et al. 2016). In our case, the SPE method recovery and ME were not used to correct the sample concentration because the validation was not performed separately for each sample.

Results and discussion

In line with the high biological potency of EDCs at low concentrations, there is a demand for highly sensitive analytical methods. The two most commonly used methods (LC-MS/MS and GC-MS/MS) for the determination of the chosen 19 oestrogenic active compounds were developed and compared in this study. The derivatisation of hydroxyl groups and the same type of detector were used for a full comparison. The more sensitive and reliable method was applied to real environmental samples of WWTP effluents. Most of the validation was performed at three concentration levels covering the linear range specific for each analyte and instrument (Tables 2 and 3).

Derivatisation: Dns versus BSTFA

Derivatisation with dansyl chloride was not applicable to MES, NRG or NORE for LC-MS/MS systems (Table 2). Ding and Chiang (2003) and Diaz-Cruz et al. (2003) published that derivatisation of MES with BSTFA, N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) and N-methyl-N-(t-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) resulted in a negligible amount of TMS product. The hydroxyl group is probably sterically hindered by the ethinyl group at the same position. The common structural motif is also present in NORE and NRG. Nevertheless, only TMS products for these analytes were present in the GC-MS/MS spectra in this study (trimethylsilylation levels are summarised in Table 3).

Analytes with more hydroxyl groups could be detected as mono-Dns and di-Dns products by LC-MS/MS. This fact was considered during the development and validation of the method for phytoestrogens and mycoestrogen in which both derivatisation products occurred. For example, di-Dns DAID and di-Dns GEN showed lower MEs but were not stable at all for 20 h, unlike the mono-Dns compounds. Therefore, mono-Dns products of GEN and DAID were used for quantification. The labelled standards were recommended for correction of MEs in the case of DAID and GEN (as is further discussed in the section *LC-MS/MS and GC-MS/MS method performance and LC-MS/MS method validation results*). Mono-Dns EQ exhibited a high ILOD (29 ng mL^{-1}) and showed unacceptable MEs (more than 150 %). Moreover, the di-Dns product was more stable than mono-Dns for 20 h. Fig. 1 shows the influence of changing derivatisation conditions on the product abundance of ZEA. While the derivatisation time (ranging from 3 to 20 min) did not influence product abundance, the volume of derivatisation reagent was the driving factor. A higher volume of derivatisation reagent (100 % of the total volume) increased the formation of didansylated compound.

Addition of the two most commonly used catalysts for derivatisation with BSTFA for GC-MS/MS, PYR and DMF, was tested. Shareef et al. (2006) suggested PYR and DMF use owing to the formation of a single TMS-EE2 product as an alternative to derivatisation in organic solvents alone (acetonitrile, dichloromethane and EtAc). In our study, compared with the process

Table 4. Recovery ± relative s.d. (%) for the whole method determined in triplicate at three concentration levels (low, middle and highest)
The tested concentrations (ng L^{-1}; in brackets) were determined using the sensitivity of the instrument and covered the calibration curve range

	Low concentration		Middle concentration		Highest concentration	
BPA	134 ± 33	(33)	88 ± 6	(267)	88 ± 13	(1333)
BPF	113 ± 32	(33)	108 ± 34	(267)	78 ± 31	(1333)
BPS	86 ± 12	(67)	94 ± 1	(533)	82 ± 18	(2667)
DAID	77 ± 10	(1)	74 ± 22	(5)	107 ± 11	(27)
LEN	76 ± 18	(3)	61 ± 19	(27)	53 ± 9	(133)
EQN	52 ± 14	(0.03)	73 ± 20	(0.3)	65 ± 30	(1.3)
EQ di	84 ± 5	(3)	82 ± 9	(27)	72 ± 24	(133)
α–E2	55 ± 19	(1)	86 ± 16	(5)	84 ± 15	(27)
β–E2	71 ± 29	(1)	73 ± 8	(5)	90 ± 17	(27)
E3	75 ± 10	(3)	89 ± 5	(27)	112 ± 14	(133)
E1	70 ± 8	(0.3)	80 ± 2	(3)	84 ± 12	(13)
EE2	93 ± 6	(0.3)	81 ± 12	(3)	86 ± 14	(13)
GEN	99 ± 4	(33)	77 ± 16	(267)	96 ± 13	(1333)
MES	105 ± 10	(17)	88 ± 3	(133)	89 ± 7	(667)
NP	28 ± 13	(3)	20 ± 6	(27)	26 ± 2	(133)
NORE	85 ± 8	(33)	104 ± 3	(267)	91 ± 12	(1333)
NRG	71 ± 8	(33)	97 ± 4	(267)	88 ± 8	(1333)
TCS	74 ± 1	(0.3)	84 ± 7	(3)	80 ± 7	(13)
ZEA	66 ± 4	(33)	79 ± 14	(267)	71 ± 12	(1333)

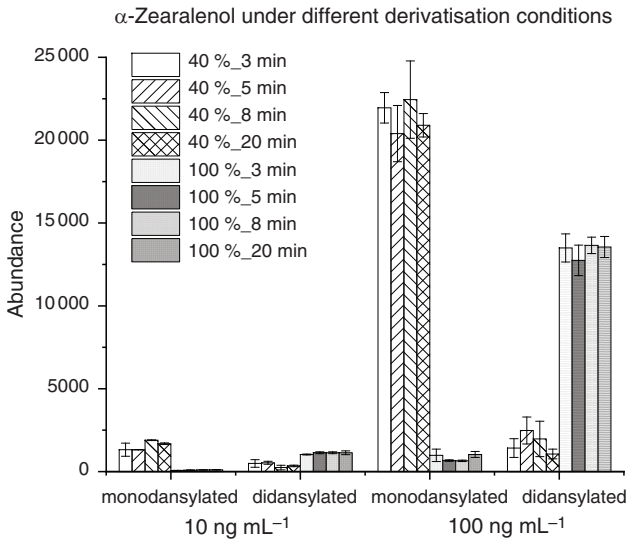


Fig. 1. Various derivatisation conditions and LC-MS/MS determined abundances of monodansylated and didansylated products of mycoestrogen α-zearalenol. Data are shown for two concentrations of the compound (10 and 100 ng mL^{-1}). Four incubation intervals (3, 5, 8, 20 min) and two volumes of derivatisation reagents (40 and 100 % of the total volume) were tested.

with DMF treatment, the presence of PYR resulted in a lower amounts of derivatisation products except for E3, α-E2 and β-E2 at the highest tested concentration (data not shown). Moreover, EQN (1 μg mL^{-1}) was not even observed using PYR treatment. Therefore, DMF was selected for the whole method validation.

Derivatisation stability

The short-term stability of derivatisation products was tested at three concentrations (covering the linear range of the calibration curves) within 20 h. None of the products exhibited significant changes within the monitored period (single factor ANOVA: *P* value for LC-MS/MS = 0.90–0.999; *P* value for GC-MS/MS = 0.34–0.90). However, a decrease in response at the highest tested concentration was observed for GC-MS/MS (Fig. 2d).

Regarding long-term stability, the Dns products differed slightly in time, but no significant trend was observed for the derivatised analytes within 7 days of storage at 4 °C (Fig. 2a). Backe (2015) showed (±20 %) of Dns products of E1, β-E2, E3, EE2 and EQN to be stable over 28 days in frozen samples.

Long-term stability results showed substantial instability of the TMS products monitored over 7 days (Fig. 2b). An increase in the responses of the derivatisation products was observed for all the analysed substances except for BPS, DAID and EQN (the highest being up to 245 % for ZEA). The ongoing derivatisation of EE2 at 4 °C was demonstrated by Zhang et al. (2006), while the stability of three endogenous oestrogens E1, β-E2 and E3 within 120 h was documented in their work. As in our case, an increase in the EE2 response was observed after 48 h. However, Shareef et al. (2006) outlined the susceptibility of the EE2 derivatisation product to hydrolysis when derivatisation was performed in the presence of DMF.

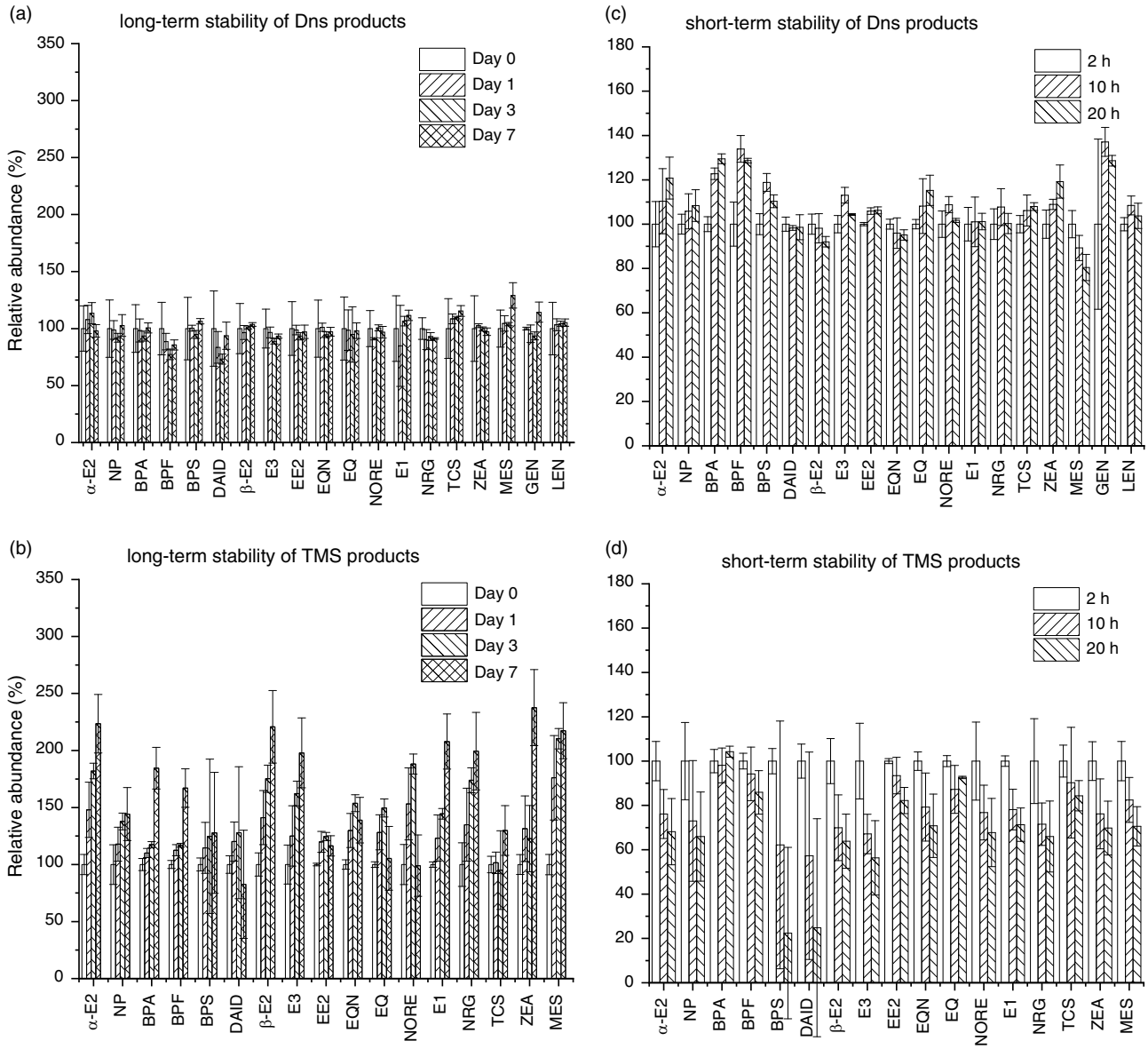


Fig. 2. Comparison of long-term (a, b), and short-term (c, d) stability using derivatisation with BSTFA (TMS, trimethylsilylation) and dansyl chloride (Dns, dansylation). Data are shown for the concentration representing the highest point of the linear calibration curve for each individual analyte (summarised in Tables 2 and 3). The abundances are compared with Day 0 or 2 h, which represented 100 %. The upper graphs show Dns analytes determined by LC-MS/MS, and the lower graphs show TMS compounds in the presence of dimethylformamide used for GC-MS/MS analysis.

Ongoing derivatisation at 4 °C suggested that the incubation during the derivatisation process was insufficient and unreacted residuals of the reagents were present after the 30-min incubation. Shareef et al. (2006) tested three incubation intervals of 30, 60 and 120 min, and no influence on the yield of derivatisation products was observed. Incubation for 30 min was recommended in the manufacturer's instructions. Notably, the unreacted BSTFA was substantially removed after the derivatisation procedure when the samples were evaporated to dryness except for less-volatile DMF.

Chromatographic separation: LC versus GC

One of the challenging parts of this study was to separate all the target analytes. In particular, α- and β-E2 isomers had to be separated owing to exhibiting the same MRM transitions. The subtype α-E2 has lower oestrogenic potential (Blair et al. 2000); therefore, combined quantification of both isomers is not biologically and environmentally relevant. The oestradiols were not baseline-separated in LC (peak resolution determined at half-height $R_s = 0.34$, half-height method), and their quantification had to be carried out by measuring the peak height instead of the more usual area integration (retention times are summarised in Tables 2 and 3). In contrast, GC enabled their complete separation ($R_s = 8.61$). GC also enables separation of nonylphenol isomers in technical mixtures (Ieda et al. 2005). The separation of α- and β-E2 by LC in a mixture of the top six analytes in serum, river water, and WWTP effluents and influents has already been published (e.g. Miege et al. 2009 and Szarka et al. 2013). Our proposed LC method is a compromise for the determination of 19 oestrogenic compounds in an environmental matrix where separation of two oestradiol isomers is feasible.

LC-MS/MS and GC-MS/MS method performance and LC-MS/MS method validation results

The final derivatisation conditions – LC-MS/MS: 100 % reagents in total sample volume, 60 °C and 5 min; and GC-MS/MS: 100 μL DMF + 20 % reagent in total sample volume, 70 °C and 30 min – were used for determination of method performance and method validation. The repeatability of peak heights did not exceed RSD = 5 % for six repeated injections in LC-MS/MS and GC-MS/MS. Retention time repeatability reached RSD = 0.18 % for both methods. The methods were linear for all analytes in the ranges indicated in Table 2 (LC-MS/MS) and Table 3 (GC-MS/MS) with $R^2 > 0.995$. The lowest calibration level with precision <20 % ($n = 6$) was used for ILOQ calculation, and the values are summarised in Tables 2 and 3. A few studies have considered a precision of <30 % acceptable for the calculation of ILOQ (Golovko et al. 2018). Generally, more approaches for ILOD and ILOQ determination have been reported (Shrivastava and Gupta 2011). In general, LC-MS/MS was more sensitive for detecting the analysed compounds, except for the bisphenols and EQ, as the ILOQs were 2–60 times lower for GC-MS/MS. However, the LC-MS/MS method met the requirements of the European Commission (Decision 2015/495/EU) for the maximum acceptable MLODs for the two most potent oestrogenic compounds, EE2 (0.035 ng L^{-1}) and β-E2 (0.4 ng L^{-1}). However, the method did not meet the requirements for the most frequently detected oestrogen E1, as the LC-MS/MS limit was slightly higher than the requirement (0.53 instead of 0.4 ng L^{-1}). Similar levels of MLODs for human natural oestrogens in river and wastewater can also be reached without any derivatisation (Celic et al. 2017). The analytes GEN and LEN were not detectable at all using GC-MS/MS.

When evaluating method selectivity, no analyte was detected in the method blank (MQ water extract) except TCS. Two sources of MQ water and four drinking water samples were analysed to examine background contamination by TCS. All of the samples contained measurable concentrations of TCS <0.8 ng L^{-1}. The individual steps of sample preparation were separately tested for the presence of TCS (filtration, SPE procedure, derivatisation, and mobile phase). An omission of filtration did not influence the TCS concentration. Since the instrumental blanks, extraction solvents and other steps did not show any TCS contamination, background residues of TCS in environmental matrices were considered. Vanderford et al. (2003) observed similar behaviour for progesterone. TCS was also detected close to the LOD (20 ng L^{-1}) in drinking water in the US (Shelver et al. 2007). It was also detected in indoor environments (Laborie et al. 2016). However, no TCS was detected in drinking water samples in Spain; nevertheless, the respective LODs were not shown (Rodil et al. 2012).

The method trueness ranged between 80 and 123 % with a precision <14.82 %, reflecting injection errors, MEs and derivatisation efficiency. The LC-MS/MS ME was reduced for phytohormones owing to optimisation of the derivatisation step (Fig. 1). The ME was evaluated in two effluents with low and high TOC levels (7.48 and 12.46 mg L^{-1}); generally, the WWTP effluents from our study contained 7.48–12.81 mg TOC L^{-1}. The final values of the MEs ranged from 74 to 129 %, except for an ME of 54 % for DAID (Table 2). According to the Decision of the European Commission, the maximum acceptable ME value is 30 % (2002/657/EC). The correction of MEs using isotopically labelled ISs did not substantially improve the results (Table 2). The use of *d*6-daidzein made the ME of DAID even worse (12 and 15 % for low and high TOCs, respectively). The ME can never be completely eliminated using labelled standards (Glineur et al. 2018). Wang et al. (2007) demonstrated that small differences between the retention time of standards and their respective deuterated analogues may result in peak ratio changes in the presence of matrix. This observation could explain our findings regarding the ME of DAID.

Method recovery

The results obtained from the method recovery experiment with the corresponding RSD and concentration used for the experiment are summarised in Table 4. The values were satisfactory for most of the analytes, ranging between 70 and 130 % (except for 52 % for EQN at 0.03 ng L^{-1}, 53 % for LEN at 133 ng L^{-1}, 55 % for α–E2 at 1 ng L^{-1}, 61 % for LEN at 27 ng L^{-1}, 65 % for EQN at 1.3 ng L^{-1}, 66 % for ZEA at 33 ng L^{-1}, 134 % for BFA at 33 ng L^{-1}, and NP at all concentration levels). These values are comparable with results published by other authors studying a wide group of hormones and other EDCs (Locatelli et al. 2016; Valitalo et al. 2016; Andaluri et al. 2017; Golovko et al. 2018; Preindl et al. 2019). Low recovery was observed only for NP (max 28 %), which has the highest partition coefficient between *n*-octanol and water (log P) compared with other target analytes. This result indicates that the feasibility of using a C18 sorbent for NP is limited. Recoveries of 101–106 % were achieved through recalculation with $^{13}C_6$-4-nonylphenol. In the study of Samaras et al. (2011), the average recovery of a nonylphenol technical mixture was 76 ± 3 % without the labelled standard. The SPE conditions were selected as a compromise with respect to all analytes.

Environmental samples

Based on the results from the method development, the environmental samples of effluents from WWTPs were analysed using the LC-MS/MS method. Although GC-MS/MS was more sensitive for bisphenols and EQ, derivatisation for LC-MS/MS was faster, more stable and reproducible. The advantages and disadvantages of both methods are summarised in Table 5.

The frequency of appearance of the analytes in the effluents is shown in Fig. 3. The detected concentrations of synthetic and natural hormones are in units of nanograms per litre; however, phytohormones and bisphenols were detected at concentrations one order of magnitude higher. The highest concentration was observed for BPA (1107 ng L^{-1}). No trend was observed between spring and autumn in the sum of concentrations or frequency of detection, as is often described in the literature (Nie et al. 2012; Lindholm-Lehto et al. 2016).

Two analytes, BPA and TCS, were detected in all samples. The occurrence of TCS is discussed in the previous section, and no BPA interference from the method blank was observed. The same results on the ubiquity of BPA were reported from Spain in various aquatic matrices (Rodriguez-Mozaz et al. 2004). BPA was detected in 89 % of surface water samples in four European countries (Tousova et al. 2017). While BPA occurred in all samples in the present study, its analogues BPF and PBS were detected less often. Another target industrial compound – NP – is a well-known pollutant that is often detected in water bodies (Fairbairn et al. 2016; Vystavna et al. 2018; Vargas-Berrones et al. 2020). In the present study, NP was detected in four samples. Its monitoring can be difficult because of the common presence of the technical mixture of nonylphenols in real samples.

E1 is the most common endogenous hormone in all water bodies (Rodriguez-Mozaz et al. 2004; Konemann et al. 2018), as observed in our study. Natural hormones E1, E2, E3 and the synthetic hormone EE2 are broadly considered the main oestrogenic pollutants in the effluents from WWTPs (Desbrow et al. 1998; Salste et al. 2007; Ting et al. 2017). Along with these hormones, horse-specific oestrogens EQN and LEN are listed in the US EPA Drinking Water Contaminant Candidate List. Therefore, all of these compounds were investigated in this study and were detected in at least one sample. Both equine oestrogens were compared with other natural oestrogens rarely screened for and detected in waterbodies (Chimchirian et al. 2007; Tyler et al. 2009; Jaukovic et al. 2017). Matejicek et al. (2013) analysed all mentioned oestrogens except α-E2 in 20 aqueous and sediment samples, and only E1 was detected. The LOD of EQN and LEN was 0.8 ng L^{-1} in their study. The hormones β-E2, EE2 and E1 were also analysed (LOD 10 ng L^{-1}) in a Europe-wide survey of WWTP effluents, and none of these compounds were observed (Loos et al. 2012). The survey also included data from the Czech Republic presented in Jarosova et al. (2014). While oestrogenic activity was found in five out of seven Czech effluent samples, no target oestrogens were detected (LOD 10 ng L^{-1}). In our study, the LODs were substantially lower, and the analytes were detected across the tested environmental samples; therefore, the need for a highly sensitive method is justified.

Although free oestrogens were not detected in river water in the state of Rio de Janeiro, the isoflavonoid phytohormones GEN and DAID were detected up to concentrations of 366 and 276 ng L^{-1}, respectively (Kuster et al. 2009). GEN, DAID and EQ were not detected in any of 20 effluent samples in Ireland (Cahill et al. 2015), whereas we detected all of these compounds several times. The LOQs of GEN and DAID were higher in this previously published method than in our method. However, the LOQ for EQ – a DAID metabolite – was five times lower in the study by Cahill et al. (2015). Mycoestrogen α-zearalenol was found in only one of our samples. Kolpin et al. (2014) reported the detection of this compound in 10 % of more than 100 samples of effluents and streams around the USA, with an average concentration of ~10 ng L^{-1}. A parent compound of α-zearalenol, i.e. zearalenone, and its other metabolite β-zearalenol, also showed a similar concentration. These compounds were detected at least twice as often, in 26 and 20 % of samples for zearalenone and β-zearalenol, respectively. However, these compounds are not as important from a biological point of view because their oestrogenic potential is more than 70 times lower than that of α-zearalenol (Frizzell et al. 2011).

The analytes NORE, NRG, α-E2 and MES were not detected in any samples. The first two compounds listed have already been surveyed in the Czech Republic, and only NORE was detected in one sample (0.85 ng L^{-1}) (Golovko et al. 2018).

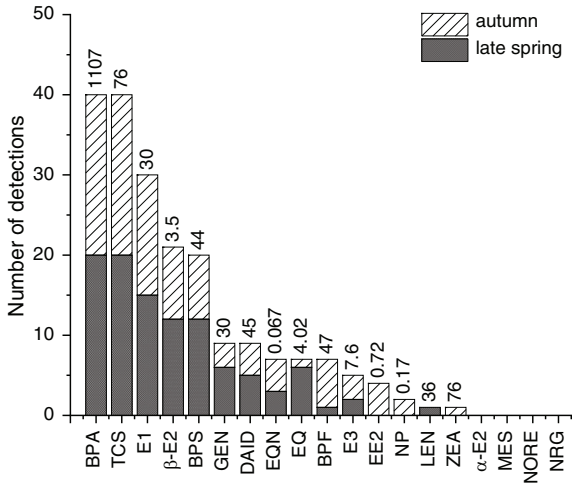


Fig. 3. Frequency of analyte appearance in WWTPs effluents. The respective maximum concentration is displayed above the columns (ng L^{-1}).

Table 5. Advantages and disadvantages of the methods presented (LC-MS/MS and GC-MS/MS)

	LC-MS/MS	GC-MS/MS
Number of detectable analytes	19	17
Stability of derivatisation products	More stable	Less stable
Derivatisation procedure	Fast	Time-consuming
Chromatographic separation	Limited for oestradiol isomers	Complete
Limits of detection	Lower for steroids	Lower for bisphenols

Conclusions

The feasibility of detecting and quantifying the 19 most relevant oestrogenic EDCs in the effluents of municipal WWTPs in the Czech Republic by means of LC-MS/MS and GC-MS/MS was studied, the two methods were compared, and the drawbacks of both analytical methods are critically described. Derivatisation with the two most frequently used derivatising reagents, dansyl chloride (LC-MS/MS) and BSTFA (GC-MS/MS), was comprehensively studied. Particular emphasis was given to determining the stability of dansylated and trimethylsilylated products. The TMS products were recognised to be highly unstable and thus not suitable for reliable determination of all EDCs. The LC-MS/MS method was considered superior because the derivatisation products were more stable, and the ILODs were generally lower. The results of the LC-MS/MS method validation confirmed the applicability for the determination of all 19 EDCs in effluent samples. The MLOD meets the criteria of the watch list published by the European Commission (Directive 2008/105/ES). Monitoring of 20 real WWTP effluents revealed the presence of each target pollutant except α-E2, MES, NRG and NORE in at least one water sample. No seasonal variation between late spring and autumn samples was observed. The results emphasise the need for efficient analytical methods for the detection of low concentrations of oestrogenic active substances, which are not the main targets of WWTPs and can further affect organisms in the receiving aquifers.

Conflicts of interest

The authors declare no conflicts of interest.

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