Steroid hormone determination in water using an environmentally friendly membrane based extraction technique

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A B S T R A C T
In this study, a method was developed for determination of steroid hormones (17β-estradiol, estrone, 17α-ethynylestradiol) in tap and sewage water samples from Sweden. Sample preparation and analysis were performed by a hollow-fibre microporous membrane liquid–liquid extraction (HF-MMLE) set-up combined with gas chromatography–mass spectrometry (GC–MS). In this approach, only the organic liquid in the lumen (10 μL) of the hollow-fibre membrane was utilised for depleting extraction. Several parameters were studied, including: type of organic solvent, sample pH, salt and humic acid content. The optimised method allowed the determination of the analyte at the low ng L−1 level in tap and sewage water. A linear plot gave correlation coefficients better than 0.995 and resulted in a method limit of detection of 1.6, 3 and 10 ng L−1 for 17β-estradiol, estrone, and 17α-ethynylestradiol, respectively, in sewage water. Enrichment factors were over 1400 after derivatisation. The repeatabilities at 50 and 600 ng L−1 were better than 10% and 6%, respectively.

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1. Introduction

The earliest report on human hormones in water was published in 1965, indicating that steroids were not completely eliminated during wastewater treatment [1]. However, the presence of hormones in the aquatic environment was not given much attention until the 1990s when a link was established between a synthetic oestrogenic compound contamination. Estrogenic substances have been identified in a wide variety of municipal effluents at mean concentrations of 1.8 and 17 ng L−1 for 17β-estradiol and estrone, respectively [7], as well as in urban and agricultural runoff in Canada [8]. In Kolpin et al. [9] performed an extensive mapping of US surface waters in which 139 streams were sampled across 30 states and reproductive hormones were found in approximately 40% of the streams sampled.

Current analytical methods for water-associated estrogens include gas chromatography–mass spectrometry (GC–MS) where method detection limits (MDLs) have been reported to range from 0.1 to 1.5 ng L−1 [10–12], and 0.1 to 2.4 ng L−1 [10,11,13,14] for tandem GC–MS/MS. However, high performance liquid chromatography–mass spectrometry (LC–MS) methods have the advantage of direct analysis of the samples without a need for a derivatisation step. Reported MDLs or LC–MS and LC–MS/MS analyses of different types of waters vary between 1 and 20 ng L−1 [15,16] and 0.05 and 3 ng L−1 [16–22], respectively. However, LC–MS methods often require extensive clean-up steps to remove interferences arising from the matrix that result in ion suppression of the analytes. To less extent, ELISA [23] has been the preferred method for final analysis. However, this more cost-effective and sensitive method needs to be improved in terms of accuracy for very complex waters. The determination of estrogens in wastewater is a difficult task because of the low limit of detection required and the complex sample matrix. This often leads to complicated, time-consuming extraction and clean-up processes. Most extraction procedures for sex hormones from wastewater have been predominantly per-
formed by off-line solid-phase extraction (SPE) with either disks or cartridges [24,25]. Octadecyl (C18)-bonded silica has been the adsorbent most widely used, but polymeric sorbents have also been employed.

Alternative methods to SPE have been developed to quantify hormones in aquatic environment. Conventional extraction techniques such as liquid–liquid extraction are still in use. An on-line continuous liquid–liquid extraction with dichloromethane coupled to a GC–MS was presented in 2004 [26]. The method had the advantages of rapidity, simplicity and there was no need of derivatisation. However, the use of dichloromethane as extracting solvent, 40–60 mL for each sample, makes the method questionable from an environmental point of view. In 2006 a chemiluminescent recombinant yeast assay based method was presented [27] with the advantages of simplicity and high analytical throughput. However, sample matrix effects were in some occasions considered to strongly affect the yeast cells response. Automated solid-phase microextraction (SPME) has as well been tested for steroid hormone analysis in environmental aqueous samples with simultaneous derivatisation and GC–MS analysis [28]. The method limit of detection in pure water was 7 and 14 ng L−1 for estradiol and estrone, respectively. Two studies using stir bar sorptive extraction (SBSE) have been presented using HPLC–DAD [29] or GC–MS [30] in the final analysis. The recovery was poor, below 50% for most of hormones, when liquid desorption was used [29]. A dual derivatisation method and thermal desorption in GC–MS resulted in better results for 17β-estradiol reaching a method limit of detection of 0.5 ng L−1 in river water [30]. An alternative extraction method using an automated two-phase hollow-fibre membrane set-up combined with GC–MS for determination of pharmaceutical and endocrine disrupting compounds (17α-ethynylestradiol) in distilled water samples gave a MDL of 20 ng L−1 [31].

In this work we present for the first time a validated microextraction method for ultra-trace analysis of three steroid hormones in complex samples as sewage water. The method is based on one-step hollow-fibre microporous membrane liquid–liquid extraction (HF-MMLLE). This is an environmentally friendly, simple, cheap, precise and high sample throughput extraction technique, which provides as well a high selective enrichment. This leads to detection limits in the low ng L−1 range, when GC–MS is used for final analysis after a simple derivatisation step.

2. Experimental

2.1. Reagents and standards

The analytes considered were 17β-estradiol (E2, >97%) purchased from Fluka (Buchs, Germany), estrone (E1, >99%) and 17α-ethynylestradiol (EE2, >98%) purchased from Sigma (Stenheim, Germany). Surrogate internal standards (IS) were estrone-d4 (E1-d4) and 17β-estradiol-d3 (E2-d3) from Aldrich and 17α-ethynylestradiol-d4 (EE2-d4) from CDN isotopes (Point-Claire, Quebec, Canada). Properties of these compounds are given in Table 1. Isooctane was from Riedel-de Haen (Seelze, Germany) while acetonitrile, methanol and acetone were from Sigma. Isooctane and methanol were ACS grade, while acetonitrile and acetone were LC grade. Sodium chloride, sodium bicarbonate and di-n-hexylether (DHE) were bought from Fluka. N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA, derivatisation grade), tri-n-octylphosphine oxide (TOPO) and undecane were obtained from Aldrich. Hydrochloric acid, di-sodiumhydrogen phosphate dihydrate, sodium-dihydrogenorthophosphate 1-hydrate were purchased from Merck (Darmstadt, Germany). Humic acids-sodium

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Structure</th>
<th>tR (min)</th>
<th>Target ion (m/z)</th>
<th>Confirmation ions (m/z)</th>
<th>pKa</th>
<th>log Kow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrone (E1)</td>
<td></td>
<td>14.2</td>
<td>342</td>
<td>257, 244</td>
<td>10.25</td>
<td>3.69</td>
</tr>
<tr>
<td>Estradiol (E2)</td>
<td></td>
<td>15.35</td>
<td>416</td>
<td>326, 285</td>
<td>10.27</td>
<td>4.13</td>
</tr>
<tr>
<td>Ethynylestradiol (EE2)</td>
<td></td>
<td>18.1</td>
<td>425</td>
<td>440, 193</td>
<td>10.24</td>
<td>4.52</td>
</tr>
</tbody>
</table>

Values calculated using a computer program, ACD/Labs for Chemistry, Advanced Chemistry Development Inc., Canada.
salt (tech) with 35% TOC was obtained from Sigma–Aldrich. Ultra-
pure reagent water purified by a Milli-Q gradient system (Millipore,
Bedford, MA, USA) was used throughout this work. Q3/2 Accurel
PP polypropylene hollow-fibre membranes (HF) (200 μm wall-
thickness, 600 μm inner diameter, 0.2 μm pore size) were obtained
from Membrana (Wuppertal, Germany).

Standard stock solutions of E1, E2 and EE2 at 1 mg mL\(^{-1}\) for
each hormone were prepared in acetonitrile and further diluted to
appropriate concentration in isooctane (GC–MS standards) and
in methanol:water (1:1, v/v) when used for spiking. Standard stock
solutions of surrogate internal standards (IS) were prepared in ace-
onitrile at concentrations of 1 mg mL\(^{-1}\) for each E1-d4 and E2-d3,
and at concentration of 0.4 mg L\(^{-1}\) for EE2-d4. These solutions were
then further diluted in acetonitrile or water–methanol to appro-
iate working concentrations. Standards dissolved in isooctane were
stored at \(-20^\circ\text{C}\) and the other solutions at 4 \(^\circ\text{C}\).

2.2. Sample collection

Tap water was sampled from the water supply network of Lund,
Sweden. Sewage water was collected from the sewage system of
the city of Kristianstad, Sweden. This plant serves a population of
\(\sim 53,000\). The four sampling points were an area with dwelling
houses, near a hospital and in the inlet and outlet of the STP.
The sample was kept in the dark at 4 \(^\circ\text{C}\) until analysis, which was
performed within a week. Sewage water used for validation was fil-
tered through a 1 μm glass fibre filter (Whatman, Mainstone, UK)
 prior to extraction.

2.3. Hollow-fibre microporous membrane liquid–liquid
extration (HF-MMLLE)

The extraction procedure was based on a previous work [32].
Shortly, prior to extraction, hollow-fibre (HF) pieces of ca. 3.7 cm
length were heat-sealed at the two ends using a hot surface, and
then cleaned with acetone and dried. After this preparation, the
sampling HF had an effective length of ca. 3.5 cm with sampling
phase volume of ca. 10 μL in the lumen and ca. 11 μL in the pores
of the wall (taking into account that the porosity in the HF wall is ca.
66%). Then, fibres were filled by sonication for about 1 h with the
organic solvent (DHE containing 10% (w/v) TOPO). Before starting
the extraction procedure 10% (w/v) sodium chloride was added to
each sample and the sample was spiked with IS to a final concen-
tration of 400 ng L\(^{-1}\). The filled HFs were taken from the organic
solvent, immersed and briefly shaken in reagent water one by one
to wash away any excess of organic solvent from the surface. Each
HF was placed in the aqueous sample (100 mL) for extraction.
During the extraction, the solution was stirred at maximal speed of
1100 rpm in a Multi 6 Velp Scientifica (Usmate, Italy) stirrer. Up to
six samples could be simultaneously extracted. After the extraction,
each HF was taken out and the solvent in the lumen was collected
by just puncturing one end of the HF with a 25 μL 702 N Hamilton
(Bonaduz, Switzerland) GC syringe. Normally, 10 μL of acceptor sol-
vent was obtained. To this solvent volume 20 μL MSTFA was added
for hormone derivatisation at 65 \(^\circ\text{C}\) in an oven for 1 h. Afterwards,
within a few hours, 2 μL of the derivatised extract was injected in
the GC for final analysis.

2.4. GC–MS analysis

All analyses were performed using a 6890 series gas chromato-
graph equipped with a split/splitless injector, autosampler and a
5973-N mass spectrometric detector (Agilent Technologies, Palo
Alto, CA, USA). Analytes were separated using a VF-1 ms (Var-
ian, Darmstadt, Germany), 30 m x 0.32 mm fused silica capillary
column coated with a stationary phase of 100% dimethylpolysilox-
ane with a phase thickness of 0.25 μm. The temperature program
was: 70 \(^\circ\text{C}\), hold 1 min, rate 30 \(^\circ\text{C}\) min\(^{-1}\) to 220 \(^\circ\text{C}\), rate 1 \(^\circ\text{C}\) min\(^{-1}\) to
230 \(^\circ\text{C}\), rate 2 \(^\circ\text{C}\) min\(^{-1}\) to 235 \(^\circ\text{C}\), rate 20 \(^\circ\text{C}\) min\(^{-1}\) to a final tempera-
ture of 300 \(^\circ\text{C}\) and then hold for 2 min. Helium (Purity 99.9999%,
Strandmøllen, Klampenborg, Denmark) was used as carrier gas at
a flow rate of 1.2 mL min\(^{-1}\). Injection volume was 2 μL. The injector
temperature was set at 250 \(^\circ\text{C}\) with a splitless time of 2 min. The MS
was operated in electron impact ionisation (EI) mode (70 eV). The
transfer line, quadrupole and ion source temperatures were 280,
150 and 230 \(^\circ\text{C}\), respectively. Samples were analysed in selected
ion monitoring (SIM) mode. Scan runs were made with m/z range
from 50 to 500. Specific ions were selected for each hormone con-
gener and the most abundant ion was selected as a quantitative ion,
while two other ions were used as qualifiers (Table 1). Quantification
was based on three deuterated surrogate internal standards
E1-d4, E2-d3 and EE2-d4.

2.5. Definitions

The enrichment factor, \(E_r\), is defined as the ratio of the concen-
tration of analyte in the acceptor after the extraction to that in the
sample before extraction. Extraction efficiency, \(E\), is calculated as
the ratio of moles of analyte in the acceptor solvent and in the sample.

3. Results and discussion

3.1. Method optimisation

Since the extraction in MMLLE is based on an equilibrium dis-
tribution process, the amount of analyte extracted at a certain time
depends on the mass transfer of the analyte from the aqueous sam-
ple to the organic solvent in the HF. There are several parameters
such as type of organic solvent, extraction time, addition of salt and
sample pH that can enhance this distribution process. Thus, we first
optimised all of the above-mentioned factors affecting the extrac-
tion efficiency. To evaluate the significance of these factors, a series
of 100 mL aqueous samples spiked at 1000 or 2000 ng L\(^{-1}\) of
each hormone were extracted in triplicate. Stirring speed is a funda-
mental parameter in the extraction and higher stirring speed has been
previously demonstrated to give faster mass transfer up to the high-
est speed investigated, which was 1200 rpm [32]. Thus, 1100 rpm
(highest speed attainable in the used instrument) was chosen.

3.1.1. Selection of organic solvent

The selection of organic solvent was based on the capability
of the solvent to selectively extract the analytes of interest and
low solvent evaporation during the extraction process. For this
purpose isooctane, DHE and \(n\)-undecane were examined. We first
attempted to use different mixtures with all three solvents to cus-
tomise selectivity of the solvent towards the analytes. However,
mixtures containing isooctane (b.p. 95 \(^\circ\text{C}\)) gave partial or total evap-
oration during the extraction or afterwards upon manual handling
of the hollow-fibres. Therefore, irreproducible volumes and results
were obtained. Consequently, only the two solvents undecane (b.p.
196 \(^\circ\text{C}\)) and DHE (b.p. 220 \(^\circ\text{C}\)) with high boiling points were tested
in further experiments. Pure solvents or different mixtures of these
two solvents were studied. Results are shown in Fig. 1.

Pure DHE gave the highest enrichment with good selectivity for
extraction of medium hydrophobic compounds such as hormones
(log \(K_{ow}\) values in Table 1). Furthermore, DHE showed the best sta-
bility during and after the extraction resulting in lower RSD values.
One more advantage of DHE over undecane is that polar aprotic
solvents are recommended for silylation reactions [33]. Therefore,
DHE was chosen as extraction solvent.
Fig. 1. The influence of the organic solvent composition on enrichment in hollow-fibre extraction of hormones at a concentration of 2 μg L⁻¹ (n = 3). Error bars correspond to standard deviation.

3.1.2. Salt addition and sample pH

NaCl is often added to the sample in order to increase the ionic strength and enhance the analyte extraction by increasing salting-out power. With the change of the ionic strength, the viscosity will change accordingly and these two factors will alter the partition coefficient between the donor phase and the acceptor phase. However, increased viscosity and density of the aqueous phase can negatively affect the kinetics of the process [34] and might also lead to analyte losses due to surface (glass or polymer) adsorption [32,35] and consequently decreased extraction efficiency.

Based on the moderate log Kow of these compounds a “salting-out effect” can be expected when adding sodium chloride. The possible salting-out effect was evaluated by adding different amounts of NaCl (0–20%, w/v) to the aqueous solution spiked at analyte concentration of 2000 ng L⁻¹. Fig. 2 depicts that the optimal extraction was achieved when the highest investigated concentration (20% NaCl) was considered for E1 and E2 and at 10% for EE2. The reason why the extraction of EE2 decreased above 10% NaCl may depend on several reasons. In principle the higher ionic strength in the sample the lower the solubility of these compounds would be, leading to a higher partitioning to the organic solvent in the fibre. This process is attributed to the decrease of the affinity of organic compounds to water layer. However, at high ionic strength other processes can become relevant such us adsorption to glassware being very important at trace levels. This could be the cause for the decrease in enrichment of EE2 (having largest Kow of the considered hormones) at NaCl concentrations above 10%. A second possible reason could be that the high salt content in water affects hydration causing increased solubility in the aqueous phase [36]. Since the enrichment was lowest for EE2, 10% NaCl was used in the following experiments to prioritise and enhance the enrichment of this compound. These results are in agreement with another work [28] where 10% was selected as the optimum value. Studies using other microextraction techniques, such as SPME and polymer-coated hollow-fibre microextraction (PC-HFME), have shown optimal values at concentration as high as 30% (w/v) NaCl [37,38].

In membrane extraction pH can be an important parameter when working with ionisable compounds. Generally it is recommended that for acidic analytes the pH in the sample should preferably be 2 units below the pKa value of the analytes. In this way most of the analyte species are uncharged in the sample solution. This is a requirement for an optimal partitioning and therefore enrichment in the organic phase. Since the pKa value of steroid hormones is around 10.2 it was considered necessary to check pH influence under likely sewage and environmental water pH. The effect of pH on the enrichment was accordingly investigated in the pH range from 6 to 9. As expected considering the pKa values of hormones (Table 1), no significant effect of sample pH was observed, which means that pH adjustment of environmental samples generally is not needed.

3.1.3. TOPO content

The addition of TOPO to the organic phase is often used in SLM to enhance the enrichment of compounds containing acidic or alcohols groups [39,40]. The mechanism by which TOPO increases the mass transfer into the organic phase is via hydrogen bonding to the analytes. The effect of the addition of 2 to 10% (w/v) of TOPO to the DHE was studied and the results are shown in Fig. 3. Fig. 3 shows that generally when the higher amount of TOPO is added the higher enrichment is obtained. Ten percent TOPO was found to be the optimum value for E1 and E2. The largest improvement is seen by going from 0% to 2% of TOPO, which leads to an increase in peak area, in Fig. 3, of at least a 200%. Since E1 and E2 were not negatively affected by a TOPO increase to 10% and the
Table 2
The analytical performance of the methodology based on HF-MMMLLE–GC–MS for determination of the hormones E1, E2 and EE2 in tap and sewage water samples

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Linearity</th>
<th>Limits</th>
<th>Accuracy (200 ng L(^{-1}))</th>
<th>Precision at 50 ng L(^{-1}) (%RSD)</th>
<th>Precision at 600 ng L(^{-1}) (%RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range (ng L(^{-1}))</td>
<td>(r^2)</td>
<td>MDL (ng L(^{-1}))</td>
<td>MQL (ng L(^{-1}))</td>
<td>Relative error (%)</td>
</tr>
<tr>
<td>Tap water</td>
<td>E1 10–800</td>
<td>0.9995</td>
<td>3</td>
<td>12</td>
<td>+0.5</td>
</tr>
<tr>
<td></td>
<td>E2 10–800</td>
<td>0.9994</td>
<td>1.6</td>
<td>5.5</td>
<td>+11.9</td>
</tr>
<tr>
<td></td>
<td>EE2 10–800</td>
<td>0.9994</td>
<td>9</td>
<td>30</td>
<td>−0.8</td>
</tr>
<tr>
<td>Sewage water</td>
<td>E1 10–800</td>
<td>0.996</td>
<td>3</td>
<td>12</td>
<td>+6.6</td>
</tr>
<tr>
<td></td>
<td>E2 10–800</td>
<td>0.998</td>
<td>1.6</td>
<td>5.5</td>
<td>+7.2</td>
</tr>
<tr>
<td></td>
<td>EE2 10–800</td>
<td>0.995</td>
<td>10</td>
<td>30</td>
<td>+1.4</td>
</tr>
</tbody>
</table>

* Signal-to-noise ratio of 10.

response of E2 at this concentration was nearly doubled. 10% TOPO was used in further experiments. Higher concentrations of TOPO than 10% were not tested because of the limited solubility of TOPO in DHE. It is interesting to note that E2, compared to E1, reaches higher enrichment at higher TOPO content probably because of the presence of two alcohol groups instead of one. EE2 has two alcohol groups as well. However, one of them is hindered by the ethynyl group making the interaction with the alcohol group more difficult.

3.1.4. Humic acid influence

Humic acids are frequently found at different concentrations in natural water, but often at concentrations below 5 ppm [41]. Consequently, the influence of these compounds on the extraction was studied. Four different concentrations, 0, 5, 10 and 20 ppm, of HAs were added to 100-mL reagent water samples spiked to a final concentration of 1 \(\mu\)g L\(^{-1}\) of hormones. None of the hormones were significantly affected by the presence of HAs at 95% confidence level. There was no significant change in peak areas of any of the three considered hormones in the range of 5–20 ppm HAs. For E1, E2 and EE2 the changes in peak area were 0.3%, 6.1% and 14.0%, respectively. This was not significant at the 95% level taking into consideration that triple determination at 0 and 20 ppm HAs addition gave RSD values of the peak area of 4.5% and 9.4% for E1, 6.0% and 9.0% for E2 and 6.5% and 12.6% for EE2. This suggests that there are no important losses due to the presence of a more complex matrix such as sewage water.

3.1.5. Extraction time

To determine the influence of the extraction time, aqueous standard solutions were extracted for different extraction times ranging from 30 min to 5 h at a stirring rate of 1100 rpm. Fig. 4 shows the extraction time profile for all the analytes.

The enrichment factor, \(E_e\), for the extraction increased with the extraction time up to 90–120 min, where after no increase was observed indicating that equilibrium was attained. During prolonged extraction time (8 h, data not shown) a slight decrease could be observed, most probably due to solvent losses. On the basis of these findings and also considering the high \(E_e\) for all analytes after 2 h, we selected this time as optimum extraction time for the subsequent experiments.

3.2. Enrichment factor and extraction efficiency

After evaluating the different parameters that might affect the extraction, the following optimised conditions were selected for all further experiments: 3.5 cm HF filled (lumen volume ca. 10 \(\mu\)L) and impregnated (membrane pore volume ca. 11 \(\mu\)L) with DHE solution containing 10% TOPO, and placed in 100 mL aqueous sample (with 10% salt addition and no pH adjustment) stirred at 1100 rpm for 2 h.

IS was added to the sample to a final concentration of 400 ng L\(^{-1}\). Fig. 5 depicts \(E_e\) values for all studied compounds under the above-optimised conditions in reagent water.

Extraction efficiency (\(E_e\) in lumen, was 45%, 85% and 98% for EE2, E1 and E2, respectively. The lower values found for EE2 and E1 may...
depend on analyte remaining in membrane pore liquid but, as confirmed in the validation exercise (Section 3.3), this will not influence quantification since sample and standard are treated in the same way. Before analysis the collected 10 μL of HF lumen is diluted with 20 μL of derivatisation reagent and final enrichment, Ee, in Fig. 5 range from 1500 ± 8% times for EE2 to 3400 ± 11% times for E2. Less derivatisation reagent would mean higher final enrichment but, after testing from 10 to 50 μL of MSTFA, the best-normalised response was found using 20 μL.

In a previous study dealing with membrane technology endocrine disrupting chemicals and pharmaceuticals where extracted using a similar two-phase extraction system and Ee values between 13 and 415 were reported [31]. In our study Ee values were at least one order of magnitude higher, but sample volume (100 mL) was 20 times higher. In contrast to the referred work, where concentrations at 10 μg L−1 were used, the Ee values in the present study were obtained by spiking samples at the levels likely to be found in sewage waters (0.01–1 μg L−1). Another attractive feature of the extraction method presented here is that it is capable of extracting E1 and E2 with high extraction efficiency (E). Highest extraction efficiency (ca. 60%) for EE2 was found using 2% TOPO. On the contrary, the other two analytes of interest had a negative effect under this concentration. Higher efficiency than obtained would be expected for EE2 considering its comparable log Kow to the other two hormones. The lower extraction efficiency for EE2 could also be related to an inferior yield in the derivatisation due to the fact that one of the two alcohol groups is hindered. Another less probable explanation is adsorption to the HF polymer or glassware.

3.3. Method validation and comparison with other microextraction techniques

To validate the analytical methodology, tap and sewage water samples were spiked at low ng L−1 levels. Linearity of the method, instrumental (IDL) and method detection limits and method precision and accuracy were obtained for both sample matrices. Performance parameters for tap and sewage water spiked with the three hormones at concentrations ranging from 10 to 800 ng L−1 are tabulated in Table 2. A total ion chromatogram of spiked tap and sewage sample is shown in Fig. 6.

Each level of concentration was analysed in triplicate. All analytes in Table 2 exhibit good linearity in spite of a very complex matrix with squared regression coefficient (r²) better than 0.995. Thus, the method has good precision even at extremely low concentrations. The repeatability and reproducibility (two consecutive days with triplicate analyses every day) were tested with water samples spiked at 50 and 600 ng L−1. The precision of the experimental procedure, expressed as relative standard deviations (%RSD), was 1.4–9.3% for repeatability, and 3.5–10.3% for reproducibility. Thus the methodology gives good precision even at extremely low concentration. To reach such good precision it is necessary to use deuterated surrogate standards for all considered hormones, e.g. the use of deuterated EE2 is improved the reproducibility from 31% to 9% in sewage samples.

The MDL, determined as three times the noise of the baseline near the analyte are in low ng L−1 range. Considering the high enrichment obtained the detection limits are somewhat high because of the relative high IDL (10, 5 and 13 ng mL−1, for E1, E2 and EE2, respectively). Other works have also reported on inferior IDL leading to lower MDL [10,12]. Instrumentation was a limiting factor and detection limits could probably be improved by the use of GC–MS/MS. Using high resolution mass spectroscopy (HR-MS) it is expected that the MDL for the HF-MMLLE method would be lower in accordance with works presented based on GC–MS/MS.

### Table 3

<table>
<thead>
<tr>
<th>Extractiona</th>
<th>Determination</th>
<th>Sample typeb</th>
<th>Sample volume (mL)</th>
<th>Processing time (min)</th>
<th>Derivatisation</th>
<th>Organic solvent (μL)</th>
<th>LOD/LOQ (ng L−1)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPME GC–MS</td>
<td>GC–MS</td>
<td>River</td>
<td>18</td>
<td>135</td>
<td>0</td>
<td>MSTFA</td>
<td>0</td>
<td>0.7–3.5</td>
</tr>
<tr>
<td>SPME GC–MS</td>
<td>GC–MS</td>
<td>River</td>
<td>10</td>
<td>125</td>
<td>0</td>
<td>BSTFA</td>
<td>0</td>
<td>0.0-0.25</td>
</tr>
<tr>
<td>SRSE GC–MS</td>
<td>GC–MS</td>
<td>Ultrapure</td>
<td>20</td>
<td>100</td>
<td>0</td>
<td>MSTFA</td>
<td>0</td>
<td>1.5–25</td>
</tr>
<tr>
<td>PC-HFME GC–MS</td>
<td>GC–MS</td>
<td>Ultrapure</td>
<td>20</td>
<td>100</td>
<td>0</td>
<td>MSTFA</td>
<td>0</td>
<td>2.0–15</td>
</tr>
<tr>
<td>HF-MMLLE (LPME) GC–MS</td>
<td>GC–MS</td>
<td>Sewage</td>
<td>100</td>
<td>180</td>
<td>0</td>
<td>MSTFA</td>
<td>0</td>
<td>2.0/10–5</td>
</tr>
</tbody>
</table>

a SPME, solid-phase microextraction; SBSE, stir bar sorptive extraction; PC-HFME, polymer-coated hollow-fibre microextraction; 2P-HF-LPME, 2 phase hollow-fibre liquid phase microextraction; HF-MMLLE, hollow-fibre microporous membrane liquid–liquid extraction. 
b Sample type used for validation.
The MDLs were the same as in tap water since the extraction was not highly influenced by the matrix. This was confirmed by comparing the regression lines obtained for the hormones in spiked tap and sewage water. For all hormones the intercepts in tap water as well as in sewage water were close to the origin. A comparison of the slopes for E1, E2 and EE2 showed deviations of 3.8%, 3.5% and 3.3%, respectively. A two-tailed test gave no significant deviation between the slopes at 95% confidence limit for any of the hormones, which shows that the sewage matrix has small influence on the results.

Evaluation of accuracy is difficult for the analysis of estrogens in environmental matrices, as no certified reference material is currently available. To get a value of the accuracy the methodology proposed in [38,42] was used. The experimentally determined average difference of four standard additions was compared with the theoretical concentration (200 ng L\(^{-1}\)). Found values ranged from 99% to 112% of the spiked concentrations. These results suggest a reasonable good accuracy of the method developed for sewage samples.

Most significant applications of microextraction methods [28,30,31,37,38] for the determination of steroid hormones in water samples and analysed by GC–MS/(MS) are summarised in Table 3. All the different microextraction techniques require none or extremely low amounts of organic solvent and small sample volume compared to SPE. The time and sample volume for all these microextraction techniques are below 180 min and 100 mL, respectively. Table 3 reveals that sample volume and processing time of this work is in the same range or slightly above. However, the presented methodology is the only method that was validated using highly complex matrix such as sewage water. Thus, the described HF-MMLLE–GC–MS methodology used has a great potential for analysing trace levels of hormones in environmental water samples.

### Table 4

<table>
<thead>
<tr>
<th>Sewage water type</th>
<th>E1 95% confidence limit (ng L(^{-1}))</th>
<th>E2 95% confidence limit (ng L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw hospital sewage (H)</td>
<td>151.0 ± 15.7</td>
<td>17.1 ± 5.1</td>
</tr>
<tr>
<td>Raw household sewage (O)</td>
<td>32.2 ± 6.1</td>
<td>26.3 ± 5.6</td>
</tr>
<tr>
<td>Influent (STP1)</td>
<td>14.5 ± 4.85</td>
<td>3.4 ± 4.1*</td>
</tr>
<tr>
<td>Effluent (STP2)</td>
<td>3.0 ± 4.1*</td>
<td>&lt;LOD</td>
</tr>
</tbody>
</table>

Standard addition method was used to measure the concentrations in sewage water (n = 3).

* Below method quantification limit.

### 3.4. Analysis of real samples

The developed procedure was applied to the analysis of estrogens at different points in a sewage system including at the influent and effluent from a municipal STP. This STP includes mechanical, biological and chemical cleaning followed by membrane filtration process. All samples except raw household sewage were collected as time integrated samples over 24 h. The detection of these samples was performed by standard addition method. Table 4 shows the results of these measurements.

Ethynylestradiol was not detected in any of the samples. However, measured concentrations of the natural estrogens, E1 and E2, were in the low ng L\(^{-1}\) level range as shown in Table 4. Similar concentrations of E1 and E2 have previously been found in a Canadian study [7]. The concentration of E1 in raw hospital sewage water was relatively high, but at the outlet of the STP the concentration was around the LOD. E2 was not detected at the outlet and altogether the results in Table 4 suggest that E2 is largely or completely removed in the STP, while removal efficiency for E1 is about 80%. Previous studies showed that to a great extend these two hormones are elim-
inated in a STP [7]. The removal of naturally occurring estrogens, estradiol and estrene, in different Canadian wastewater treatment plants (WWTPs) varied between 46% and 98%.

4. Conclusions

A novel method has been developed for determination of steroid hormones in aqueous samples, such as tap and sewage water. This method is based on HF-MMLLE for sample pre-treatment and GC–MS for final analysis. The employed HF-MMLLE procedure can be considered environmentally friendly since it minimises the consumption of organic solvent (20 μL). A conventional hormone extraction method using GC–MS (MS) would use at least 150 times more organic solvent per extraction and in some cases hazardous hexane or dichloromethane. The presented methodology has unsurpassed simplicity avoiding an extra clean-up step, and GC–MS for final analysis. The employed HF-MMLLE technique even lower concentrations can be quantified.

This method is based on HF-MMLLE for sample pre-treatment and Spatial planning (FORMAS).

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References