Steroids contents in waters of wastewater purification plants: determination with partial-filling micellar electrokinetic capillary chromatography and UV detection

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ABSTRACT
Partial-filling micellar electrokinetic capillary chromatography (PF-MEKC) with UV detection was applied for determination of human-based steroids in water samples of Finnish wastewater treatment plants. The samples were purified with solid-phase extraction (SPE) on octadecyl substituted polymer sorbents obtaining analyte enrichment of 20,000-fold. The steroids studied were androgens, estrogens, and progesterone. Three of the steroids could be quantified with the PF-MEKC method. The detection and quantification limits were 0.05–1.06 μg/mL and 0.15–3.2 μg/mL, meaning in the SPE concentrates as 2.5–53 pg/L and 7.5–160 pg/L, respectively. In the influent waters, the total amount of testosterone glucuronide, androstenedione, and progesterone was up to 350 ng/L. In effluent water samples the total steroid quantity was maximum at 320 ng/L. Remarkably high quantity of androstenedione was quantified in both influent and effluent water samples. The cleanest effluent waters were produced in Western Finland. Correspondingly, the highest quantities were located near the largest lake and river areas in South-Eastern Finland. The concentration variation in effluent waters was explained with differences in the purification materials and processes at the plants and with steroid adsorption on soil and organic material suspended into water.

1. Introduction
Existence of steroid hormones in environmental waters are a special point of interest because they are produced naturally in human body or chemically synthetised and primarily excreted as natural steroids or conjugates in body fluids. They are slightly water-soluble making their medicinal activity last and therefore allows treatment of infertility, cancer, menstrual and menopausal hormonal disorders, and birth control [1]. The drawback of their use reflects to their stability and hydrophobicity since their final destination as raw compounds and metabolites is the surface waters through wastewater treatment plants [2]. They belong to the target group in environmental monitoring and therefore they have concentration limits in water which is used for preparation of drinking water. The European Union Water Framework Directive [3,4] has a specific category for
endocrine disrupting compounds (EDCs) including steroid hormones because of their biological disadvantages [5,6]. Lately, also the list of the compounds was updated for containing estrogens which are frequently present in wastewater effluents. According to a recent study, certain other anabolic steroids and pharmaceutical products are also known to last longer in the environment as opposed to previously known. The researchers found that the synthetic steroid trenbolone acetate never completely degrades in the environment. In fact it can even partially regenerate itself [7].

The quality control of inland, surface, transitional, coastal, and ground waters should be obligatory to predict and prevent steroid load into environment. It is unclear whether their concentrations in surface water have effects on causing endocrine disruption or gender evolution [2]. The exact drifts of steroid hormones into the debits of water sources are rarely precisely known [8,9]. More research with the newest techniques is needed on that topic and steroid transformation products like their metabolites in respect of characterisation, occurrence, and fate in water types, especially, in effluent flows to groundwater area [10]. It is known that the purification techniques used at the plants for processing drinking water seldom remove all steroid hormones [11].

The purification methods for processes, present for both organic and biological wastes, like decomposing the organics in membrane bioreactors (MBRs), have attracted a significant amount of interest globally. There is evidence on their ability to clean wastewater and produce high-quality effluents which are suitable for water recycling [12]. Therefore, pretreatment plants have put efforts in processes for integration biological treatment of wastewaters. However, human-based sex hormones and their endogenic metabolites, such as estrogens may easily stay in water. They are excreted as inactive glucuronide and sulphate conjugates but are supposed then to be hydrolysed by enzymes into native form [2,13,14].

In addition, to be able to adsorb steroid hormones, environmental waters contain various kinds of inorganic and organic ions and other organic compounds, solid material, water-soluble particles and soluble chemicals, making the water profitable environment for the steroids exiting the purification systems [5,6,15–19].

Estrogens are known to cause feminisation in animal species, development of physical abnormalities, and birth defects [20,21]. Since estrogens migrate to environment, the waters in treatment plants may contain estrone (E1), 17β-estradiol (E2), estratriol (E3), and also progesterone (P4) from human waste sources [22–25]. Therefore, steroidal hormones are tracked in nearly all wastewater plant influent waters and even in the purified effluents [26–33]. As usual, the concentrations of estrogens in environmental water sources are low in river and water systems. It was documented that E2 concentrations reached only 27 ng/L in rivers in Japan, Germany, Italy, and the Netherlands [28–31]. In spite of the directives [3,4], there is no standardisation methodology nor official guidelines available for measuring individual steroids from environmental waters. Mostly they are studied as dissolved neutral compounds without sample hydrolysis needed for metabolites, although steroids are released as glucuronide and sulphate conjugates from body [32].

Usually, compounds in waters are analysed by gas chromatography (GC) and liquid chromatography (LC) because of their sensitive detection and reproducible quantification [32–35]. Because GC separates volatile compounds, chemical approaches like derivatisation are used for decreasing volatility and increasing sensitivity of the steroids. The instruments cannot always provide simultaneous full structural identification of the
studied metabolites and the parent compound. Although derivatisation is not quantitative, the synthesis products help characterisation of the original sample. One of the newest techniques in this field is on-drop chemical derivatisation coupled with mass spectrometry analysis (DESI-MS) [36,37]. It uses desorption electrospray ionisation – MS for metabolite identification. The aqueous sample drop is prepared on a glass plate and dried following by derivatisation and analysis. Lately, also untargeted GC×GC-TOFMS technique with derivatisation and focusing on steroidal structure compound group was introduced [2]. The technique was demonstrated without model compounds to have potential in approaches to generate information on steroids in water matrices at wastewater purification plants. Then, calculations with statistical tools from data of concentrated water samples gave the lowest concentration of steroidal compounds at 5 ng/L. To enhance the amount of steroids in detection, LC methods are usually used. For multi-compounds with different polarities a single LC method with one type of column sorbent is not sufficient. However, a single injection analysis technique for samples with a large variety in polarity can be performed with ultra-high performance LC systems which can be constructed using sequentially hydrophilic interaction chromatography (HIC) and reversed phase liquid chromatography (RP-LC) techniques [38].

At present, determination of individual steroids might be more important than defining their total quantity in water. Hence, capillary electrophoresis (CE) with its high separation efficiency is a worthy alternative to chromatographic techniques in determination of steroids [39–42]. CE analyses are done in buffer solutions with electricity. CE allows very good separation for structurally similar compounds, such as steroids and their metabolites. However, the main reasons for preferring LC in the steroid analyses are larger injection volume (in LC 1–100 μL and in CE 1–10 nL) and the easiness to on-line couple LC system on-line with mass spectrometer [31]. The advantage of CE is that it also enables analyses even without enrichment of the analytes in multicomponent matrices because the compounds can be concentrated during the analysis.

Micellar electrokinetic capillary chromatography (MEKC) is a hybrid method that combines chromatographic and electrophoretic separation principles and extends the applicability of capillary electrophoretic methods to neutral analytes, such as steroids. MEKC is based on the addition of surfactant (e.g. sodium dodecyl sulphate, SDS) into the buffer solution. The surfactant acts as a micellar pseudostationary phase which interacts with neutral analytes according to partitioning mechanisms. One special case of MEKC is partial-filling micellar electrokinetic chromatography (PF-MEKC) [43], in which a small portion of the capillary is filled with a micellar solution for the separation of non-polar compounds. The separation is based on analyte interactions with the micelles. Instead of PF-MEKC field amplified sample injection (FASI) method or other on-line sample concentration methods may be used for fractioning the studied compounds into concentrated bands. Due to the very low amounts of the steroids, unfortunately those on-line stacking techniques do not give enough sensitivity for determination of steroids in environmental water by UV absorption. Therefore, the only way is to make offline concentration, extraction, and clean-up prior separation and detection. Micro-extraction techniques have been used in removing the biological matrices (e.g. human urine and serum) and cleaning the environmental samples (e.g. wastewaters, surface waters, tap waters, river waters, and sewage sludge, and marine and river sediments) for CE analyses.
Recently, the concentration of analytes was made with sorptive micro-extraction on molecularly imprinted polymers (MIPs) which showed to considerably improve the analyte recovery when compared to treatment of steroids with solid phase sorbents. MIP materials have specific capacity because of the molecule specific affinity but not yet at molecular level.

Even after purifying household wastewaters, androgens and progesterone flow to environment. Then, for example, testosterone (at 1–40 ng/mL in urine) is transferred to sewage and therefore androgen hormones exist in influent waters of water purification plants. According to literature, low ng/L levels of steroids in environmental samples can be measured. They can be determined after cleaning of water matrix and enrichment of the steroids by both solid-phase extraction (SPE) and liquid-liquid extraction (LLE). The SPE materials were polymeric C18 sorbents allowing the steroid recoveries from water to be around 90–120% with the method limit of detection at 0.01–0.24 ng/L. In the waters of wastewater treatment plants the concentrations of ECDs have been from below 10 ng/L till up to 1200 ng/L. The water samples are used in too small samples volumes (50 mL – 500 mL – 1 L) in cases where the steroid concentrations are ng/L level. The low concentrations show the importance of sample preparation, cleaning of the matrix before analysis, and detection of insignificant steroid concentrations.

The aim of the present study was to optimise a PF-MEKC for separation and determination of steroid hormones in influent and effluent waters of nine wastewater treatment plants in Finland. In order to detect the steroids, enrichment with SPE using non-polar polymer-based Strata-X sorbent, was needed. The target was also in the means of analytics to see the steroid removal quality in the water plants using different types of processes.

2. Experimental

2.1. Chemicals

The steroid chemicals used in the PF-MEKC method validation and determination of the water samples are listed in Table 1. In addition, estrone (E1), 17β-estradiol (E2), 1,3,5(10)-estratrien-3,17-β-diol 3-glucosiduronate (estradiol-gluc, E2-gluc), 3-hydroxyestr-1,3,5(10)-tri-en-17-one glucuronide (estrone-gluc, E1-gluc), and 1,3,5(10)-estratrien-3,16α,17β-triol glucuronide (estriol-gluc, E3-gluc) were used. The steroids were purchased from Steraloids Inc. (Newport, RI, U.S.A.), Riedel-de Haën (Seelze, Germany), and Sigma-Aldrich Co. (Darmstadt, Germany). They were used as received and stored in a dark and cold cupboard (+4°C). The other chemicals and solutions used were ammonium acetate (Sigma-Aldrich Co, Germany) and ammonia (25%, VWR International S.A.S. France). Buffer solutions at pH 4 (phthalate, pH 3.98 at 20°C), pH 7 (phosphate, pH 7.02 at 20°C) and pH 10 (borate, pH 9.99 at 20°C) (Fisher Scientific UK), methanol (HPLC grade, Fisher Scientific UK, used as the marker of electro-osmosis), sodium dodecyl sulfate (approx. 99%, Sigma-Aldrich Co., Germany), sodium hydroxide (1.0 mol/L, Oy FF-Chemicals Ab, Finland), and sodium salt of taurocholic acid as the monohydrate (BioXtra, ≥ 95% (TLC), Sigma-Aldrich Co., Germany). The ICP-AES standards (Ag, As, Ba, Ca, Cd, Co, Cr, Cu, Fe, Li, Mg, Mn, Na, Ni, P, Pb, S, Sn, Ti, V, Zn, and K) were commercial products. Calcium and silver solutions were from ROMIL (ROMIL Ltd, The Source,
Titanium and other elements were from AccuStandard (AccuStandard Europe, Niederbipp, Switzerland). All chemicals were analytical grade and they were used as received.

### 2.2. Instruments

The capillary electrophoresis separations were performed with a Hewlett-Packard 3D CE system (Agilent, Waldbronn, Germany) equipped with a diode array detector (λ 190–600 nm). Bare fused silica capillaries (i.d. 50 μm, o.d. 375 μm) were purchased from Polymicro Technologies (Phoenix, AZ, USA). Their total and effective lengths were 80 cm and 71.5 cm, respectively. Before use they were conditioned by sequentially flushing with 0.1 M NaOH, milli-Q water, and the electrolyte solution for 20 min each.
The CE instrument was applied with ChemStation programmes (Agilent) for instrument running and data handling. The temperature during the analyses was +25°C. Positive polarity and the voltage of +25 kV was set as constant value. The current was detected during all analyses. It was +17 µA in all analyses. The simultaneous peak detection was made at 214, 220, 240, 247, and 260 nm, but 247 nm was used as the selective wavelength for all native steroids.

For separation of the steroid hormones, the micellar solution was introduced at 0.50 p.s.i (34.5 mbar) for 75 s against the electrolyte solution in the capillary. The volume of the hydrodynamic injection was .56 nL (calculated by CE Expert Lite program, SCIEX). After the micellar plug, the sample was introduced at 0.725 p.s.i (50 mbar) for 6 s (volume in hydrodynamic injection was 6.46 nL) from inlet of the capillary towards the detector.

Before each analysis, the capillary was flushed with 0.1 M NaOH in water and with the electrolyte solution for 2 min and 5 min, respectively. After every eight runs, the capillary was washed by flushing with 0.1 M NaOH in water, milli-Q water, and the electrolyte solution for 7, 5, and 10 min, respectively.

Inorganic cations (Table S1) in the water samples were determined using an ICP-AES (Iris Intrepid II XDL, Thermo Electron Corporation, UK). The flow gas was argon (99.998%). The data collecting and handling was made with Teva kp software. Gases used were technical air containing N₂ and 20.9% O₂ that was made of natural compressed air, acetylene (purity 99.5%) and nitrous oxide gas (purity 99.0%). The temperature of the flame was 3000°C and the flows of C₂H₂ gases were 4.3 L/min. All gases were from Aga Oyj (Espoo, Finland).

In determination of inorganic anions (S₂O₃²⁻, SO₄²⁻, F⁻, Cl⁻, NO₃⁻, NO₂⁻, PO₄³⁻, B₄O₇²⁻) the ion chromatograph used was a Dionex DX-120 (Sunnyvale, California, USA) apparatus. The column used was Dionex On Guard® II 1cc column that removes Be, Mg, Ca, Sr, Ba, Ra, Mn, Fe, Ni, Cu, Zn, and Al from the samples. Detection was made with a suppressed conductivity detector CD 25. The suppressor was self-cleaning ASRS® ULTRA II 4 mm Autosuppression® in recycle mode with current of 31 mA. The injection volume was 10 µl. Flow rate of the eluent was 1.07 mL/min. Temperature during the measurements was kept at 20°C.

The total amount of organic compounds (TOC) were measured as TC and TOC using a Shimadzu TOC analyzer (TOC-L, Vantaa, Finland). The instrument provides a concentration range of 4 µg/L–30,000 mg/L. TOC analyzer uses a 680°C temperature and the combustion with ozone catalytic oxidation.

The pH value of the electrolyte solution was adjusted using an InoLab pH7110 (WTW) instrument, which was calibrated with 3-point-calibration buffers of pH 4, 7, and 10 (Fisher Scientific, Loughborough, UK). The SPE device VacMaster was used for sample concentration. The Reacti-Vap Evaporation unit (Thermo Scientific, Vantaa Finland) was used for evaporation of the extracts under N₂ gas. All waters used were purified with a Direct-Q UV Millipore water purification system (Millipore S.A., Molsheim, France).

### 2.3. Filters and sorbents in sample preparation

In laboratory, the pretreatment of the waters was made with filtration of the water samples with glass microfibre and membrane filters (GE Healthcare Life Sciences,
Whatman™, Glass Microfiber Filters GF/C™, Diameter 90 mm and Millipore, Durapore® Membrane Filters, 0.45 μm HV), respectively. Next, the water samples were purified and steroids were concentrated with Strata-X 33u polymer-based hydrophobic sorbents (500 mg/6 mL) which were obtained from Phenomenex (Copenhagen, Denmark).

2.4. Preparation of standard solutions

2.4.1. Stock solutions of steroids

The stock solutions of steroid hormones at 1000 μg/mL were prepared in methanol and stored at +4°C in 2 mL glass vessels until used. The working solutions were prepared from the stocks by diluting a specific concentration into methanol. Before the stock solutions were used, they were let to warm up to room temperature and mixed with a vortex mixer. Optimisation and calibration was made with 4-androsten-17β-ol-3-one glucosiduronate (testosterone glucuronide). The working solutions were 4, 8, 12, 16, 20, and 24 μg/mL. Progesterone was made as 1, 2, 4, 8, 12, and 16 μg/mL solutions, while the rest steroids were as 2, 4, 8, 12, 16, and 20 μg/mL solutions. The solvent used was either the electrolyte solution, milli-Q water, or methanol for the desired concentrations. No differences were noticed in the solvents.

2.4.2. Electrolyte and micelle solutions

The electrolyte solution was 20 mM ammonium acetate. Its pH was adjusted exactly to pH 9.68 with 25% ammonia, because ammonium acetate in water did not give the desired alkalinity. A 100 mM SDS stock solution was used as one part of the micelle solution. It was made into the electrolyte solution (pH 9.68). The other part of the micelle solution was 100 mM sodium taurocholate which was prepared into milli-Q water. Its pH was not adjusted. The both micelle solutions were stored at room temperature in glass vessels. Neither electrolyte nor the micelle solutions were filtered before use. If not used, taurocholic acid, SDS, and the electrolyte solutions needed to be stored always at room temperature (+20°C to +23°C). Before PF-MEKC analyses, the electrolyte solution was ultrasonicated in a water bath at room temperature for 15 min. To prevent adsorption, the stock steroid solutions were kept in glass vials.

The final micelle mixture for the analysis was made from the two micelle solutions for adding 1000 μL of 20 mM ammonium acetate solution (pH 9.68), 440 μL of 100 mM SDS in 20 mM ammonium acetate solution (pH 9.68), and 50 μL of 100 mM sodium taurocholate solution together. The specific order was important. The micelle and the electrolyte solutions were sequentially introduced into the capillary [39,43]. In addition, the micelle mixture and the electrolyte solution needed to be replaced with a fresh solution between each analysis sequence.

2.5. Sampling and sample preparation

Twenty-four-hour flow-proportional composite samples were taken from nine wastewater treatment plants at distance of max 600 km from each other (Table 2). The water sampling was done in 2014 on March and on April from plants in Eastern, Southern, and Western Finland. They were located in Kajaani (Peuraniemi, March 24–25), Joensuu (Kuhasalo, March 25–26), Mikkeli (Kenkäveronniemi, March 25–26), Kouvola (Mäkikylä,
March 25–26), and in Espoo (Suomenoja, April 10–11), Porvoo (Hermanninsaari, March 18–19), and in Pori (Luotsinmäki, March 19–20), Uusikaupunki (Häpönniemi, March 26–27), and Turku (Kakolanmäki, March 24–25). Two of the plant processes included a biological purification step as the secondary and tertiary treatments.

Sampling of the influent and effluent waters were made by the personnel of the water treatment plants. The samples were collected at the beginning (influent) and end (effluent) of the purification processes. The waters were taken into 5 L-volume canisters from which they were divided to four 1 L water portions. These portions were then used as the main samples, influent separately from the effluent. In Kajaani and Uusikaupunki the samples were taken after the biological filtration (biofilter) at the end of the whole purification process. Other details about the sampling and sample storage are explained elsewhere [2].

In the laboratory, the influent and effluent waters were filtrated through fibreglass and then through membrane (0.45 μm) filters. Then, the liquid fractions were treated with SPE to adsorb the steroids onto polymer sorbents. Before use, the column materials were conditioned with methanol and water. Then the samples were introduced onto the materials by pumping at 8 mL/min flow rate. The eluent passed through the column and the SPE materials with the steroids were dried under vacuum for 30 min. The steroids adsorbed on the material were eluted with 6 mL of methanol. Then, the eluent was evaporated under N₂ flow at 40°C. The precipitate was dissolved into 250 μL of methanol. The studies were performed with 1–3 replicates and with three sequential analyses.

### 2.6. Optimisation of the separation parameters

Our earlier developed PF-MEKC-UV method for corticosteroids [47] was used as the starting procedure for optimising the method for steroid hormones and metabolites in the water samples of water purification plants. Since Beckman-Coulter CE instrument was earlier used, the Agilent CE method required additional optimisation and validation. First, testosterone at 0.5–20 μg/mL concentration level was used with 12 different method set-ups including chemical changes (pH and concentration of the electrolyte, the micelle, and sample solvent) and instrumental modifications (injection volume, electric field, detection, and length of the micelle plug). The length of the capillary was fixed due to the possibility to couple the system with a mass spectrometer.

| City (location of the water treatment plant) | Influent (water to the plant) | Biological filtration used in the plant (water in the plant process) | Effluent (water from the plant) |
|---------------------------------------------|------------------------------|-------------------------------------------------------------------|---------------------------------|
| Espoo                                       | x                            |                                                                   | x                               |
| Joensuu                                     | x                            |                                                                   | x                               |
| Kajaani                                     | x (aerobic)                  |                                                                   | x                               |
| Kouvolá                                     | x                            |                                                                   | x                               |
| Mikkeli                                     | x                            |                                                                   | x                               |
| Uusikaupunki                                | x (aerobic)                  |                                                                   | x                               |
| Pori                                        | x                            |                                                                   | x                               |
| Porvoo                                      | x                            |                                                                   | x                               |
| Turku                                       | x                            |                                                                   | x                               |

*Sampling after the biological process.

*Sand filtration.*
2.7. Identification of steroids

First, all steroid compounds were analysed individually to monitor their migration order and detection sensitivity. Then, the optimisation was made by adding one steroid at a time into various mixture compositions prepared in methanol. The identification was made with five selective UV wavelengths to verify the existence of the steroid (andro-gen, progesterone) by absorption relations because the CE instrument could not be on-line coupled with a mass spectrometer. In addition, sequential steroid spiking to a standard mixture was done with individual steroid at 2 μg/mL into the water sample concentrate.

2.8. Calibration and optimisation

Concentration calibration was made from either 500 μg/mL or 100 μg/mL steroid solutions in methanol. From these solutions, five to six diluted mixture solutions at different concentrations were prepared into methanol. The method linearity and the compound sensitivity were determined by plotting the average steroid peak areas calculated from the electropherograms against their corresponding concentrations at 0.5–10 μg/mL level. The LOD value correlating the signal-to-noise ratio of 3 was measured from the concentrations of the steroids when they were as a mixture. The LOQ value for each analyte was then calculated with signal-to-noise ratio of 3 × LOD (9 × S/N).

Intraday analyses were measured with a mixture containing the steroids at 2 μg/mL. It was injected for five times with five repetitions on the same day. In addition, the inter-day repeatability was calculated from the data measured with the steroid mixture for five times during 7 days in 3 months. The intraday reproducibility was measured during 24 h from migration times of EOF and progesterone with 30 values of each (totally 60 values). By now, the method has been used successfully for 15 months. The day-to-day reproducibility was calculated as above with 65 values of each (totally 130 values).

3. Results

3.1. Method optimisation

There are a few reasons which are to be considered when capillary electrophoresis with UV detection is used as the analysis method for steroids in water samples. First, the method development needs extensive optimisation due to the low detection sensitivity when dilute samples are studied. Second, the quantities of the steroids in multicomponent water need matrix construction. In this project, therefore to enhance the sensitivity the steroid hormones were studied with the PF-MEKC using selectively optimised UV absorption. In addition, for determination of androgens, estrogens, and progesterone in water samples from nine communal water treatment plants, steroids needed to be isolated from the matrix by sample preparation process. In the study, the steroids were endogenous steroids and crucial metabolic intermediates in the production of other endogenous steroids except synthetic fluoxymesterone which is used as an anabolic steroid in sports and here tested as an internal standard in PF-MEKC separation. The analysis of testosterone and its urine metabolites, progesterone and its metabolite, and androstenedione (precursor of testosterone and estrogens in the metabolic
pathway) are important contaminants from households since the wastewater is a mixer containing crap and urine with sex hormones.

To maximise the sensitivity of the analytes for evaluating the migration order of the steroids total method optimisation was made. The result was that the steroid hormones migrated in the order: testosterone-gluc, fluoxymesterone, androstenedione, testosterone, 17α-hydroxyprogesterone, 17α-methyltestosterone, and progesterone. Some glucuronide metabolites of estrogens (Figure 1) were also studied, such as estradiol-glucuronide (E2-gluc), estrone-glucuronide (E1-gluc), and estriol-glucuronide (E3-gluc). The estrogens were detected in the standard solutions but could not be measured reliable enough in the actual water samples due to the very low concentrations and the moderately high background in the effective separation profile of the PF-MEKC-UV method.

The PF-MEKC-UV method enabled movement of the non-polar and nonionic steroid compounds in the capillary because of the micelles which interacted better with the polar steroids better than with the conjugated ones. Repeatability (RSD) of the method was very good, as it is noticed from the absolute migration times, electrophoretic mobilities, and electro-osmosis values of 2.9–8.2%, 0.6–5.0%, and 1.5%-3.8%, respectively (Table S2). The results showed that also the inter-day precision of the migration was excellent and accurate (RSD 4–8%) during the method development and analysis period.

The sensitivities of the testosterone glucuronide and progesterone were much higher when compared with androstenedienone, most probably because they are moderately soluble in water. The parent compounds of the sensitive steroids are the strongest acids with pKₐ around 19 (Table 1).

Figure 1. Electropherograms of PF-MEKC-UV separation of the studied androgens, estrogens, and progesterone. Steroid mixture (2 μg/mL). Peaks: (1) testosterone-glucuronide, (2) fluoxymesterone, (3) androstenedione, (4) testosterone, (5) 17α-hydroxyprogesterone, (6) 17α-methyltestosterone, (7) progesterone, (8, 1st peak) estrone-glucuronide (E1-gluc), (8, 2nd peak) estradiol-glucuronide (E2-gluc), (9) estrane (E1), and (10) estradiol (E2). Migration of electro-osmosis 5.2 min (with methanol); electric field: voltage (+25 kV) and current (+17 μA). Detection of compounds 1–7 at λ 247 nm and compounds 8–10 at λ 214 nm. Electrolyte preparation with other details are informed in Experimental.
3.2. Concentration linearity and sensitivity

The linearity of the method was measured at concentration range of 0.5–10 μg/mL using steroid mixtures. The result was a linear correlation between concentration and the peak area of the analyte. The intraday and the day-to-day repeatability (RSD %) were 9.7% and 19%, respectively.

| Steroids                  | Linear equation    | $R^2$ value | Method concentration range [μg/mL] | LOD* [μg/mL] | LOQ** [μg/mL] |
|---------------------------|--------------------|-------------|-----------------------------------|--------------|---------------|
| Testosterone-gluc         | $y = 1.2702x + 0.005$ | 0.913       | 0.5–8                             | 0.05         | 0.15          |
| Androstenedione           | $y = 0.632x + 0.029$ | 0.940       | 0.5–8                             | 0.06         | 0.19          |
| Testosterone              | $y = 0.779x + 0.214$ | 0.962       | 0.5–8                             | 0.94         | 2.82          |
| 17α-hydroxyprogesterone   | $y = 1.1506x − 0.354$ | 0.947       | 0.5–6                             | 0.38         | 1.15          |
| 17α-methyltestosterone    | $y = 2.9447x − 3.040$ | 0.969       | 0.5–10                            | 1.06         | 3.19          |
| Progesterone              | $y = 4.3152x − 4.077$ | 0.967       | 0.5–10                            | 0.97         | 2.90          |

*LOD was measured from the electropherogram peak area of know steroid concentration (S, signal) divided with the average noise peak area (N, noise) with S/N = 3; **LOQ was measured from the corresponding LOD of the steroid by multiplying with 3 (LOQ = 9 × S/N). It was verified with the SPE extract containing the remaining matrix.

3.3. Determination of steroid hormones and metabolites in water

The present study was focused in measuring human-based androgens, estrogens, and synthetic progesterone (Figure 2). Urinary androstenedione, testosterone, 17α-methyltestosterone, and progesterone are usually determined in environmental water [19,40–43,48]. In spite of that, estrogenic steroids are studied the most intensively. In the purified effluent water samples of sewage water treatment plants the measurable estrogen quantities have noticed to be even higher such as 70 ng/L (E1), 64 ng/L (E2), 18 ng/L (E3), and 42 ng/L (EE2) [21,49]. It is not surprising, since almost 80% of effluent water samples from the plants have noticed to contain female hormones after clean-up of the water [46]. Since the concentrations are very low, steroids in the water sample need to be enriched and extracted from the main matrix. Therefore in the present study the sampled water volume was increased from the traditional volumes (500 mL–1 L) to 2 L in order to enrich the steroid concentrations for maximal UV detection in PF-MEKC analyses and to validate the system capability for long-terminal steroid monitoring.

In the study, the effluent water in the plant process was made without and with biological clean-up. However, in the latter case water sampling was done after the bioprocess treatment at the end of the whole process. During cleaning and enrichment with SPE the steroids were eluted in 20,000 times more concentrated than the original
plant sample (Figure 2). Then, based on the PF-MEKC analyses the influents and effluents contained glycosylic form of testosterone, but also androstenedione and progesterone without glucose conjugation. The other androgens used in the standard mixture could not be detected at quantification level. Interestingly, the steroids detected happened to be the strongest bases among the studied compounds (Table 1). As the reference, steroids determined with GC×GC-TOFMS the steroids had the highest log $P$ values [2].

The results also showed that in general the steroid quantities were significant and varied from 0.1 ng/L to tens of ng/L in the original water sample from the plant at detection wavelength of 247 nm. Glucuronide conjugates of estrogens could be seen at 214 nm, but their concentrations at water samples were very low at S/N < 3 (Figures 1 and 3).
recovery of the SPE treatment was 80–90% for testosterone, which is in correlation with the literature values.

According to literature, in wastewater treatment plant the steroid hormones are supposed to become deconjugated and deactivated by enzymes for degradation of biological material [33,39–42,48,50–52]. Our results correlate with the published results, since in our case some steroid amounts seemed to increase in the biochemical treatment. The SPE enrichment followed by PF-MEKC-UV analysis showed testosterone glucuronide and androstenedione, both being metabolites of both testosterone [32], and progesterone. However, the other androgenic steroids were not observed. The results showed that the enzymatic procedures used in the plants decreased remarkably the amount of progesterone (Table 4). The influent waters contained 1–360 ng/L of testosterone glucuronide, androstenedione, and progesterone. Naturally, the quantities in the effluents were lower than in the influents, being at 0–320 ng/L level. When the analyte in a sample concentrate was clearly identified (based on specific wavelengths, absolute migration time, and relative migration time) in the electropherogram, the sample was spiked with a 2 μg/mL standard and quantified using the standard addition method.

Our results showed that the differences between the steroid concentrations were influenced by the sampling places in the water plant process. The biological filtration units showed the increase in concentration of especially androstenedione, even up to 85.3 ng/L. The reasons may be that there are mechanisms in which progesterone can produce androstenedione, which is the precursor in metabolism of testosterone (andro-gen) and estrogens such as estradiol [53]. Earlier, it has also been reported that biological cleaning processes are not the most efficient ones compared to nitrifying activated sludge and to removing estrogens from the final effluents [51]. According to literature [54] estradiol (E2) is noticed in the waters process before the biological filtration unit. The average of total estrane (E1) concentration of surface waters is reported to be 0.005 ng/L [55]. In addition, in Germany their concentrations are noticed to be around 0.5 ng/L. Our study showed that the total amount of estrogens was 8–60 ng/L. Most probably, the reasons for the differences are the origin of the wastewater, water volume in the analyses, analysis method and calibration, detection sensitivity, and the assumed benefit of non-quantitative silyl derivatisation.

The differences between the biological cleaning processes and the process without it can be noticed (Table 4, Figure 3). In Uusikaupunki plant the process uses two steps for movement of water to the nitrification filter. In the processes, methanol is added to keep carbon source in the water and to stabilise the bacteria involved in denitrification. It may be the reason why Uusikaupunki in the Eastern and Pori in the Western Finland process produce the cleanest water. Based on results the most efficient clean-up procedures of the studied steroids were found in Pori and Espoo. On the contrary, the highest steroid amounts were in the waters of the Eastern cities Kajaani and Mikkeli and also in Porvoo. As to the cleanest water, the top three plants were in Uusikaupunki, Kouvola, and Espoo.

The purification process of the influent to effluent samples at the plant was extremely good (100%) in Pori and Uusikaupunki and good (80%) in Kouvola. However, normally the steroid loss in purification was only between 15% and 37%. The steroids contamination left in effluent was at 72% (Kajaani), 87% (Joensuu), 81% (Porvoo), and 67% (Turku). In Espoo the amount of only androstenedione was for some reason enriched with 100%.
As to take a reference, the reported efficiency of wastewater purification ranged from 10% to 90% depending on the nature of the analytes [28]. However, the purification materials and processes at the plants have the most important role in clean water
Figure 3. Steroids concentrations in influents (left) and effluents (right) of water pretreatment plants in Southern, Western, and Eastern Finland. Upper figures: Concentrations measured using the SPE concentrates and the concentration calibration of the PF-MEKC-UV method. Figures below: Concentrations calculated from the measured value by using the 1:20,000 dilution factor to get the quantity in the plant water samples. Compounds are marked on the top of the figure (right): (black) 4-testosterone glucoside (T-gluc); (grey) androstenedione (Andr); (light grey) progesterone (Prog). Details of the sample concentration and clean-up are explained in Experimental.
production. The removal of polar compounds was noticed to vary from 12% to 90% depending on the purification process. The highest efficiencies were obtained in activated sludge processes which even showed to decrease the contaminant level more than biofilms used as the reference [31].

3.4. Metals of the environmental waters in Eastern Finland

The reasons for elevated steroid concentrations of the incoming wastewater and the effluents of the plants of Eastern Finland compared with the other districts were evaluated by studying concentrations of metals and organic materials as the absorbents of steroids. Because the environmental water is flown from lakes, rivers, and swamp districts and the recipient of the wastewater are the river (Joensuu and Kouvola) and the lake (Mikkeli), the sources of the influent waters may have high contamination levels from organics and metals. Since the plant waters of Joensuu, Kouvola, and Mikkeli contained industrial wastewater (%) 15, 8, and 5, respectively [2], and the steroids are known to form complexes with metals, it was needed to know the correlation between steroid-load and the contamination.

The analytical samples were taken from 18 lake and river systems, ground waters, and waters of swamp deposits in Saimaa Lake area (Mikkeli – Joensuu district). We thought that high metal concentration, pH, and organic material would be partly the reason since they support the adsorption and solubility of steroids. Thus, the study was focused to alkali and earth alkali metals, heavy metals, inorganic and organic compounds for measuring potassium, calcium, magnesium, barium, copper, zinc, nickel, iron, manganese, lead, fluoride, chloride, bromide, nitrite, nitrate, and phosphate, but also pH, TC, TOC, and IC (Table S3, Table S4).

Based on the earlier results, the waste and toilet waters of households and wastewater of industry were known to be mixtures which should be purified to clean enough water suitable to be pumped to environment. The influent waters in Joensuu, Kouvola, and Mikkeli plants were mixtures of 5–15 industrial waters and household waters [2]. Our results showed that the metals in the waters were in balance with the regulatory limits set for barium, copper, iron, zinc, and nickel. However, the levels of manganese and lead were abnormal, being 221 and 34.4 µg/L, respectively. They also exceeded the limit values of the Finnish degree of drinking water standard [56] by 4.4 and 3.4 times, respectively. Manganese has high concentration since it is an essential element in both humans and animals. It is required for the functioning of many cellular enzymes (e.g. manganese superoxide dismutase, pyruvate carboxylase) and it can activate many other enzymes as well (e.g. kinases, decarboxylases, transferases, and hydrolases) [57].

It could be noticed a few trends from the measurements: When the pH was high, TOC was high and when the water was rich in sulphate, it indicated possible steroid complexation. The pH of the samples ranged from 3.35 to 8.19. The waters especially from swamp, lake, and river sources were contaminated with organics. The concentration of inorganic carbon was low in lake, river, ground, swamp deposit, and wastewater waters. On the contrary, organic carbon was quite high (2.42–47.56 mg/L) which correlated with the low pH of the water. Some swamp deposits contained also high iron (5054 µg/L) and manganese (381 µg/L) levels. The study shows correlation with the analytical quantities...
of organic material and metals. The amount of chloride was low, excluding two samples where it was as much as 2172 and 1652 mg/L. It can then be assumed that manganese and lead were correlating with phosphate. When hardness of water was high, copper and manganese were high in swamp waters. The organic soluble material is an excellent adsorbent for steroids.

According to our results, the clean-up techniques used in the water plants most likely do not take total hold in the steroid hormones in aqueous media. As supposed earlier, the environmental waters contain organics and metals which may complex the steroids from external sources. They are formulated during the pretreatment process, by deconjugation and reactivation in the process or by enzymes used in water treatment. Therefore, efficient sample preparation methods are needed to cover selectively a wide range of steroid compounds at insignificant low concentrations. In addition, hydrolysis is needed when the conjugates are not included in the main analyses.

4. Conclusions

The PF-MEKC method for the simultaneous determination of the androgens, estrogens, and progesterone has been evaluated in this study. In addition, the optimum conditions of a SPE technique to produce water concentrates has been obtained. The established SPE – PF-MEKC-UV method could be applied to determine concentrations of three steroids which has been identified in influents and effluents of wastewater pretreatment plants. The present method has been assumed to be an alternative for chromatographic methods. In the method validation it has been demonstrated to be both practical and viable technique, but also sensitive enough for concentrated water samples. Adequate repeatability and reproducibility showed that it was reliable and accurate for quantitative analysis of the steroids in real water samples.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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