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Kopperi, Matias

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# Non-targeted evaluation of the fate of steroids during wastewater treatment by comprehensive two-dimensional gas chromatography – time-of-flight mass spectrometry

Matias Kopperi, Jevgeni Parshintsev, José Ruiz-Jiménez, Marja–Liisa Riekkola*

Laboratory of Analytical Chemistry, Department of Chemistry, P.O. Box 55, FI-00014 University of Helsinki, Finland

*Corresponding author. Tel.: + 358 2941 50268; E-mail: marja-liisa.riekkola@helsinki.fi

**ABSTRACT**

Emerging organic contaminants in wastewater are usually analyzed by targeted approaches, and especially estrogens have been the focus of environmental research due to their high hormonal activity. The selection of specific target compounds means, however, that most of the sample components, including transformation products and potential new contaminants, are neglected. In this study, the fate of steroidal compounds in wastewater treatment processes was evaluated by a non-targeted approach based on comprehensive two-dimensional gas chromatography – time-of-flight mass spectrometry. The potential of the non-targeted approach to generate comprehensive information about sample constituents was demonstrated with use of statistical tools. Transformation pathways of the tentatively identified compounds with steroidal four-ring structure were proposed. The purification efficiency of the wastewater treatment plants was studied, and the distribution of the compounds of interest in the suspended solids, effluent water, and sludge was measured. The results showed that, owing to strong adsorption of hydrophobic compounds onto

the solid matter, the steroids were mostly bound to the suspended solids of the effluent water and the sewage sludge at the end of the treatment process. The most abundant steroid class was androstanes in the aqueous phase and cholestanes in the solid phase. Estradiol was the most abundant estrogen in the aqueous phase but it was only detected in the influent samples indicating efficient removal during the treatment process. In the sludge samples, however, high concentrations of an oxidation product of estradiol, estrone, were measured.

**KEYWORDS**

Chemometrics, GC×GC-TOFMS, Non-target, Steroid, Wastewater, Transformation product

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## INTRODUCTION

A current environmental concern is the widening range of chemicals used by society and the flux of these contaminants into surface waters through wastewater treatment plants (WWTP). The European Commission upholds the Water Framework Directive, which sets environmental quality standards (EQS) for selected priority substances in surface waters. However, an unknown number of emerging organic contaminants (EOC) are missing from the list because of insufficient ecotoxicological data and inadequate knowledge of their concentrations or long-term effects in the environment. The list of priority substances can be found in the amended Annex X of the Water

Framework Directive (Directive 2013/39/EU). Some of the most recent compounds added to the Annex X are estradiol and the synthetic ethynylestradiol. They have been included due to their hormonal activity and frequent presence in wastewater effluents, which motivates further research on the occurrence of similar steroidal compounds.

Emerging organic contaminants have been widely studied (Richardson and Ternes 2011; Loos et al. 2013; Vieno 2014). Unfortunately, the majority of the studies in the field have focused only on the aqueous phase and ignored the compounds adsorbed onto sewage sludge and solid particles suspended in the effluent, which is essential for the evaluation of the fate of EOCs. Moreover, most studies have targeted only substances with known hazardous properties. However, the bacteria present in wastewater and municipal treatment processes are able to induce functional changes in the structures of the EOCs (by hydrogenation, hydroxylation, deconjugation) (Lishman et al. 2006; Racz and Goel 2010; Vieno 2014), rendering them invisible to targeted analysis. These transformation products of the parent compounds can also be formed by anthropogenic metabolism before excretion (conjugation) and by the specific chemical conditions during wastewater treatment (oxidation, reduction) (Haddad et al. 2015). The biotransformation of progesterone, for example, can be accomplished by the microalgae in aquatic environment (Peng et al. 2014). Non-targeted analysis can reveal these transformation products along with their parent compounds, although, the limitations of sample preparation and analytical techniques often require focus on a specific class of compounds.

Some studies have reported non-targeted screening for EOCs by gas chromatography (Slobodnik et al. 2012) and liquid chromatography (Hernández et al. 2014). Comprehensive two-dimensional gas chromatography – time-of-flight mass spectrometry (GC×GC–TOFMS) has also been recently

utilized to enable automated comparison of environmental samples and blanks to identify EOCs as outliers of the normal sample matrix (Prebihalo et al. 2015). Non-targeted analysis has previously been carried out in Finland by Nurmi et al. 2012 and Jernberg et al. (2013a, 2013b). Strict criteria are required to verify tentative identifications and reduce the possibility of false positives and negatives when certified reference materials are not available. Besides the uncertainties in identification, also quantification is problematic in non-targeted approaches. For this, a surrogate approach is often exploited if calibration standards are not available for all identified compounds. The high prediction error for concentrations calculated with the surrogate approach can be reduced by implementing multiple surrogates and their mass spectral similarities in a chemometric model. This approach was employed in a previous study, achieving good prediction accuracy for the concentrations of unknown steroidal compounds (Kopperi et al. 2013).

The aim of the present study was to apply non-targeted analysis of compounds with steroid structure by GC×GC–TOFMS to determine their fate during wastewater treatment as well as to identify possible transformation products of the parent compounds. The application of statistical methods to the non-targeted screening data enabled an inclusive comparison of results, not possible with traditional targeted approaches. The purification efficiency as well as the distribution of the studied compounds into sludge, suspended solids, and effluent water was determined for 10 Finnish WWTPs. Possible accumulation of EOCs into environment through the reuse of sewage sludge for landscaping and agricultural purposes was also evaluated. Dry sludge samples were analyzed from Viikinmäki WWTP and the results compared with those from commercial soil products generated from this sludge after composting.

## MATERIALS AND METHODS

**Reagents and solutions**

Methanol, acetonitrile, dichloromethane, acetone and hexane were from VWR International (Radnor, PA, USA). Pyridine was purchased from J.T.Baker (Deventer, The Netherlands). All solvents were HPLC grade, and pyridine was purified by distillation in the laboratory every two months. Distilled water was purified with a Millipore Direct-Q 3 UV system (0.05 μS $cm^{-1}$ conductivity; Billerica, MA, USA). Estriol (≥ 97%), estrone (≥ 99%), β-estradiol (≥ 98%), 17α-ethynylestradiol (≥ 98%), and *trans*-androsterone (≥ 98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Testosterone (≥ 99%) and androstenedione (≥ 99%) were purchased from Fluka Chemie GmbH (Buchs, Switzerland). Progesterone (≥ 97%) was from Merck Group (Darmstadt, Germany). Stock solutions (1 mg $mL^{-1}$) of the steroid standards were prepared in methanol and stored at 4 °C. Internal standard 1,1′-binaphthalene (≥ 97%) was purchased from Acros Organics (Geel, Belgium) and a stock solution (1 ng $mL^{-1}$) was prepared in dichloromethane. Silylation of analytes was done with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS). The derivatization reagent was purchased from Sigma-Aldrich (St. Louis, MO, USA) and stored at 4 °C.

**Sampling**

Twenty-four-hour flow-proportional composite samples were taken from ten wastewater treatment plants around Finland (Table 1). Five-liter samples were collected at the beginning (influent) and end (effluent) of the purification process. Some of the WWTPs included a biological filtration unit as secondary or tertiary treatment, and additional samples were taken immediately before this.

Samples were stored in plastic (high-density polyethylene) containers at 4 °C and extracted within 48 h after sampling. Three parallel one-liter samples were filtered through a glass microfiber filter

(Whatman GF/C) from GE Healthcare Life Sciences (Buckinghamshire, England) and then through a 0.45 µm membrane filter from Millipore (Billerica, MA, USA). The resulting solid and aqueous phases were analyzed separately. In addition, sewage sludge samples from Viikinmäki, Helsinki were studied. The processed sludge is relocated to a composting field where it is combined with peat and, after a certain composting period, applied as soil in private gardens and public parks. Samples of dry sludge and final soil products were received from the WWTP to determine the elimination of compounds during composting and to evaluate the safety of applying wastewater sludge in agriculture and landscaping. The samples were stored in ambient temperature before extraction.

**Sample Preparation**

Samples were prepared by a methodology adapted from previous study (Kopperi et al. 2013). The scheme is illustrated in Online Resource 1, including the workflow for data processing. Compounds in the filtered aqueous phase were extracted by pumping the samples through Strata-X cartridges in reverse direction. A peristaltic pump was connected to the tip of the cartridge, and methanol and Direct-Q water were pumped through to activate the sorbent. The tubing of the pump was then transferred to the sample containers and wastewater was loaded into the sorbent at a flow rate of 8 mL $min^{-1}$. Three parallel samples were extracted simultaneously. After the one-liter samples had been loaded, the cartridges were vacuum dried for 20 min in a VacMaster-10 manifold. Finally, the samples were eluted from the cartridges, in normal direction, with 6 mL of methanol, of which 2 mL aliquots were sampled for analysis.

Sludge and soil samples (50 mg) as well as solid particles collected from wastewater samples during filtering were dried (48 h, 45 °C) before extraction. Sludge and soil samples were

homogenized by grinding after drying and then extracted by methodology adapted from previous study (Kopperi et al. 2013), which was also utilized to samples collected during filtration. Three parallel samples were simultaneously extracted. The test tubes containing the filters and solid material were filled with acetonitrile and placed in the ultrasound bath for 60 min. The extract was removed and the procedure repeated once. Collected extracts were combined, evaporated, and reconstituted in 6 mL of acetonitrile, of which 2 mL aliquots were sampled for analysis.

The samples were analyzed by GC×GC–TOFMS. Before the analysis, lipid content was removed from the extracts in a Florisil column by a method adapted from the previous study (Kopperi et al. 2013). Aliquots of 2 mL were evaporated and reconstituted in hexane:$CH_2Cl_2$ (3:1, v:v). Samples were loaded into Florisil cartridges that had been conditioned with hexane, and analytes were eluted from the cartridges with 2 mL 10% acetone in dichloromethane. The extracts were evaporated under nitrogen flow and silylation of the hydroxyl-containing sample constituents was performed by adding 10 µL BSTFA (1% TMCS) and 2 µL of pyridine, then heating the mixture at 60 °C for 30 min. After the derivatization, the samples were diluted with $CH_2Cl_2$ to 50 µL, and 1,1´-binaphthalene (0.75 ng $\mu L^{-1}$) was added as internal standard for the injection.

**Instrumentation**

A Bransonic 5510 ultrasound bath from Branson Ultrasonics Corporation (Danbury, T, USA) was used for the ultrasound-assisted solvent extraction of solid particles. An 8-line 205S peristaltic pump from Watson Marlow (Wilmington, MA, USA) and a VacMaster-10 manifold from Biotage (Uppsala, Sweden) were used for the solid-phase extraction of liquid samples. Solid-phase extraction cartridges Strata-X (500 mg / 6 mL) were purchased from Phenomenex (Torrance, CA, USA) and Florisil (100 mg / 1 mL) from Agilent Technologies (Santa Clara, CA, USA).

The gas chromatographic analysis was performed with a LECO Pegasus 4D GC×GC–TOFMS system with a gas chromatograph (7890A) and an autosampler (7683B) from Agilent Technologies (Santa Clara, CA, USA). A nonpolar first column (BGB-5MS, 30 m × 0.25 mm i.d., film thickness 0.25 µm; BGB Analytik AG Boeckten, Switzerland) was connected to a semi-polar second column (DB-17, 1 m × 0.1 mm i.d., film thickness 0.10 µm; Agilent Technologies, Santa Clara, CA, USA). The first column was connected to a 2.5 m × 0.53 mm i.d. deactivated retention gap (Agilent Technologies, Santa Clara, CA, USA). The temperature program was as follows: 30 °C (1 min) — 10 °C $min^{-1}$ — 250 °C — 5 °C $min^{-1}$ — 285 °C (6 min). Temperature of the secondary oven was always five degrees higher, with a final temperature of 290 °C. 1 µL splitless injection (injector 280 °C) was employed. Helium was used as carrier gas at a flow rate of 1.3 mL $min^{-1}$. Modulator temperature offset was 15 °C and the second-dimension separation time was 4 s. Transfer line and ion source temperatures were 290 °C and 200 °C, respectively. Acquisition delay was 7 min, mass range 50–700 m/z, and ionization energy 70 eV. The analysis time was 36 min.

**Data Handling**

The GC×GC–TOFMS data was first processed with LECO ChromaTOF software for automated identification of peaks by comparison of their mass spectra to NIST2005 mass spectral database. Retention indices were automatically assigned based on the retention times of previously analyzed linear hydrocarbons. The data from parallel extracts and successive injections was then aligned with Guineu metabolomics data analysis software (Castillo et al. 2011). The aligned data was manually processed by subtraction of zero samples (pure water samples, which underwent the same sample preparation methodology) and removal of compounds not found in all parallel samples and

successive injections (n=9). The reliability of tentative identification was increased for steroidal compounds by manual comparison of retention indices and mass spectra with data in the GOLM metabolome database (Hummel et al. 2010). The final criteria for tentative identification of steroidal compounds was decided by evaluating the identification requirements of the analyzed steroid standards: spectral match >700 and maximum difference between experimental and library Kováts indexes ± 200 (NIST); 1-dot-product distance < 0.2; Euclidean distance < 0.05; Jaccard distance < 0.6; 12GowLeg distance < 0.6 (GOLM).

Quantification of the tentatively identified compounds with the four-ring steroid structure was carried out with use of previously described chemometric model (Kopperi et al. 2013). Briefly, eight steroid standards (estriol, estrone, β-estradiol, 17α-ethynylestradiol, androsterone, testosterone, androstenedione, progesterone) were directly analyzed from stock solution dilutions in eight different concentrations (0.1–50 ng $\mu L^{-1}$) relevant to environmental samples after extraction and pre-concentration. Response factors were calculated by dividing peak areas by peak area of the internal standard for injection (1,1´-binaphthalene). Response factors derived from total ion chromatograms were then converted to ion intensities on the basis of relative abundances of individual ions in the mass spectra. The data collected from the standards was used to estimate the concentrations of identified steroidal compounds on the basis of partial least squares regression of the ion intensities.

**RESULTS**

In the next sections, the performance of the optimized method is first briefly evaluated. Then, the findings of the non-targeted screening study are presented, including novel statistical approaches to evaluate sample composition and to compare the differences between the ten WWTPs. Special

attention is put on the structure of the tentatively identified steroidal compounds, and pathways for the identified transformation products are proposed. Finally, the fate of steroidal compounds is evaluated by calculating daily loads of the tentatively identified compounds into receiving waters and their concentrations in the collected sewage sludge and suspended particle phases.

During optimization of the methodology, available steroid standard compounds were utilized to estimate the applicability of the methodology for non-targeted analysis of all compounds with steroidal four-ring structure. Therefore, the values of recovery and limits of detection of the non-targeted methodology are estimated averages for the whole steroid class. The methodology was developed earlier (Kopperi et al. 2013) but to manage the high sampling rate of the current study, some changes were implemented. Dynamic ultrasound-assisted extraction with sonifier tip was replaced by static extraction in a sonication bath. To confirm the comparability of the methods, a sewage sludge sample was repeatedly analyzed and the recovered amounts of some target steroids (estrone, progesterone, androstenedione, and androsterone) present in the sample were compared. It was observed that the efficiency of extraction with the sonifier tip depended greatly on the distance between the tip and the sample. This was a major drawback, which reduced the robustness of the method when multiple samples were placed in the extraction vessel simultaneously. It was also observed that the recovery of steroids during multiple sample extraction with the sonication bath was twice as high as with the sonifier tip, although the measured relative amount of steroids in the samples was comparable between the techniques. To analyze the aqueous phase more efficiently, the previous method was automated so that the samples were pumped through the cartridges in reversed direction. The recoveries of the target steroids were acceptable (> 90%). Instrumental detection limits for non-targeted steroidal compounds were estimated from the lowest

measured concentration of the target steroids during method development, and the resulting method detection limits (MDL) were < 15 ng $L^{-1}$.

The main advantages of GC×GC–TOFMS in non-targeted screening are the increased peak capacity and the generation of trilinear data (mass spectrum + retention time coordinates) to help with the identification and classification of unknown compounds. The technique can also be used to compare the analytical profiles of several samples through statistical analysis of the ´raw´ data. The number of tentatively identified compounds, for example, can be utilized to screen for variation between samples without the time-consuming process of confirming the identification of individual compounds and quantifying them. Fig. 1 illustrates differences between the various treatment plants by comparing the number of all tentatively identified peaks in the samples (library match > 600).

In Kajaani and Uusikaupunki, a biological filter replaces the traditional biological purification process (secondary treatment), while in Helsinki a biofilter process is additional to chemical and biological purification (tertiary treatment). Thus, in Helsinki, the water is cleaner before entering the biofilter. Furthermore, several tentatively identified steroids were found in the samples taken before the biofiltration in Kajaani and Uusikaupunki, whereas in Helsinki the steroids were mostly eliminated already at this stage. The larger number of compounds found in Helsinki after the biofiltration suggests that some new compounds may have formed or been released during the biofilm process. These compounds may have been released during the biofiltration process in Kajaani and Uusikaupunki as well, since the number of compounds in the effluent water is similar in the three WWTPs. Probable sources for these compounds are plastic carrier material or dead bacterial cell matter released from the biofilter.

The purification profiles of the other cities were very similar with a few small exceptions in the aqueous samples. The number of compounds in effluent samples of Turku was slightly higher than average, although the WWTP has sand filtration as a tertiary treatment. This can be explained by the high number of population serviced and the resulting high number of compounds detected also in influent samples. Another exception was the low removal efficiency of the Porvoo WWTP for compounds in aqueous samples, which cannot be explained with the operational parameters. Elimination rate for an average WWTP can be estimated by comparing the number of compounds found in the influent and effluent samples. The average elimination rate was ~50% for the compounds found in aqueous phase (Fig. 1a) and ~90% for those bound to suspended particles (Fig. 1b), which sums up to a total elimination rate of ~70% in the whole-water (Fig. 1c). While most of the compounds bound to the influent particles were collected into the sewage sludge, 10% were still present in the suspended solid material of the effluent. These suspended solids end up in the environment and require further research.

Conventional targeted analysis of EOCs typically concentrates on a few specific compounds of high environmental concern. With non-targeted analysis, it is possible to find transformation products as well, and to classify these according to their structural similarities. The structures of the tentatively identified compounds with steroidal structure have been illustrated in Fig. 2 and Fig. 3 along with proposed reaction pathways for transformation products. The pathways have been proposed according to the simplest route based on the most common transformation reactions found in the literature (Lishman et al. 2006; Racz and Goel 2010; Peng et al. 2014; Vieno 2014; Haddad et al. 2015). The authors would like to point out that due to similarities in the structure of

steroids and the variety of possible reactions during wastewater treatment, other pathways are also possible originating from different parent compounds.

The benefit of using two mass spectral libraries is the added accuracy of tentative identification, although the final confirmation of compound structure would require the use of reference materials. Comparison of mass spectral libraries also gives indication about the reliability of identification at different levels of structural detail. Experimentally observed reliability of identification for the studied compounds, for example, can be listed in decreasing order as follows: presence of a four-ring skeleton > identity of substituents at key locations (Fig. 2: C3 and C17–C20) > identity of substituents at other locations > number and location of double bonds > stereoisomerism. Therefore, after the concentrations of the tentatively identified steroidal compounds had been calculated with the chemometric model, they were classified into groups on the basis of their skeletal structure and key substituents (Table 2). The classification was done to reduce the uncertainty arising from the identification of a more detailed compound structure. The benefit of using the chemometric model is that concentrations can be predicted on the basis of mass spectrum and detector response without prior knowledge of compound identity.

As can be seen, the major class of steroids in the aqueous phase was the androstanes, and estranes were present only in this phase. Cholestanes were more abundant in the suspended particles than in the aqueous phase, which is not surprising considering their hydrophobicity. Most of the steroids were removed from the aqueous phase during the treatment. Only a few compounds were detected in the effluents of Espoo WWTP. This WWTP services the second largest population after Helsinki. Because high steroid concentrations were detected also in the influent samples of Espoo, additional tertiary treatment might be recommended. In order to evaluate the daily load of steroids

flowing through WWTPs in Finland, their concentrations in whole water (aqueous phase + suspended solids) were normalized by the flow volume of the WWTPs during sampling and the population of the city in question. In an average WWTP, the daily loads (mg / 1000 inhabitants) were reduced during treatment as follows: androstanes (4000→180); cholestanes (670→130); estranes (160→30); pregnanes (800→0). Elimination of steroids from the wastewater during treatment was efficient.

Numerous pharmaceutical compounds and consumption habit markers were also tentatively identified in the aqueous samples (Table 3) with ibuprofen, carbamazepine, caffeine and cotinine being the most abundant. Their frequent presence in the effluent indicated that they were removed from the wastewater less efficiently than were the steroidal compounds. This was confirmed also when only few of these compounds were found in the suspended solids and sludge samples, which indicates their low adsorption to solids and therefore poor elimination through activated sludge process in WWTPs.

In the final part of the study, concentrations of the EOCs were measured in dried sewage sludge from Viikinmäki WWTP and in composted soil products processed from the sludge. Several steroidal compounds were tentatively identified in the samples. Most of these were the same compounds also found in the aqueous samples including several androstanes (n = 7) and pregnanes (n = 7) as well as high concentrations of estrone (10 mg $kg^{-1}$ in sludge). The presence of estrone in the sludge samples can be the result of oxidation of estradiol frequently detected in influent samples. The main difference in the composition of sludge and soils samples compared to aqueous samples was the high number of different cholestane compounds (n = 15), which were mainly based on the structure of cholesterol with variation in the number and location of double bonds and

hydroxyl groups. The concentration of steroidal compounds were reduced by 70–100% during the composting process, but still concentration levels of mg $kg^{-1}$ were found in the soil. Considering that the sludge is used for agricultural purposes, these values are of concern. The no-effect concentration (PNEC) for estrogens in soil, for example, has been estimated to be in the range of 1-700 μg $kg^{-1}$ (Martin et al. 2012). No pharmaceutical compounds were found in the sludge and soil samples. Many studies conclude that the adsorption of EOCs into solids is driven by their logP values so that compounds with logP < 2 remain in the aqueous phase and those with logP > 3 may be adsorbed, especially during activated sludge processes (Hamid and Eskicioglu 2012; Evgenidou et al. 2015). The logP values for steroidal compounds often lie in the range of 3–4, and values as high as 6–7 can be expected for cholestanes (Law et al. 2014). It has also been documented that the complete removal of steroids from solids is challenging (Silva et al. 2012), which therefore presents an environmental risk where treated sewage sludge is applied for agricultural purposes.

## CONCLUSIONS

The results presented allow us to draw several conclusions about the fate of the studied EOCs during wastewater treatment. Non-targeted analysis confirmed that a wide variety of steroidal compounds are present in high concentrations in influent wastewater, and that the concentrations in effluent water are significantly reduced. Strong tendency to bind to solid matter correlates well with their logP values and results in high concentrations in the residual sludge but some steroids were also present in the suspended solids in the effluent water. The adsorbed steroids are relatively persistent and can be detected in the dry sludge even after a long period of composting. The results indicate a need for further studies on the impact of steroidal compounds where composted sludge is applied in agriculture and landscaping. Many transformation products were tentatively identified and their analysis should be considered in studies where the fate of steroids is evaluated.

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**Table 1** Description of the wastewater treatment plants and sampling.

| WWTP | Population serviced | Indrustrial wastewater (%) | Recipient | Sampling date | Flow during sampling ($m^3$ $d^{-1}$) | Tertiary treatment |
|---|---|---|---|---|---|---|
| Kajaani, Peuraniemi | 33 000 | 0 | River Kajaani | 24.3.2014 | 9 600 | Biological filtration |
| Uusikaupunki, Häpönniemi | 25 000 | 15 | Baltic Sea (Gulf of Bothnia) | 26.3.2014 | 7 600 | Biological filtration |
| Helsinki, Viikinmäki | 800 000 | 8 | Baltic Sea (Gulf of Finland) | 1.4.2014 | 286 000 | Biological filtration |
| Espoo, Suomenoja | 320 000 | 8 | Baltic Sea (Gulf of Finland) | 10.4.2014 | 92 000 | - |
| Joensuu, Kuhasalo | 75 000 | 15 | River Pielisjoki | 25.3.2014 | 19 800 | - |
| Kouvola, Mäkikylä | 70 000 | 8 | River Kymijoki | 25.3.2014 | 18 000 | - |
| Mikkeli, Kenkäveronniemi | 43 000 | 5 | Lake Saimaa | 25.3.2014 | 11 300 | - |
| Porvoo, Hermanninsaari | 50 000 | 3 | Baltic Sea (Gulf of Finland) | 18.3.2014 | 12 300 | - |
| Pori, Luotsinmäki | 115 000 | 8 | River Kokemäenjoki | 19.3.2014 | 33 700 | - |
| Turku, Kakolanmäki | 275 000 | 7 | Baltic Sea (Gulf of Finland) | 24.3.2014 | 102 000 | Sand filtration |

**Table 2** Average (n = 9) concentrations of the identified steroid species measured in the samples (ng $L^{-1}$) from ten WWTPs by GCxGC–TOFMS (I = influent samples, E = effluent samples, B = samples before biological filtration).

**Aqueous Phase**

| **Androstanes** | | **Kajaani** | | | **Uusikaupunki** | | | **Helsinki** | | | **Espoo** | | **Joensuu** | | **Mikkeli** | | **Pori** | | **Kouvola** | | **Turku** | | **Porvoo** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| substituent at C3 | substituent at C17 | I | B | E | I | B | E | I | B | E | I | E | I | E | I | E | I | E | I | E | I | E | I | E |
| – OH | – OH | 2770 | 960 | - | 570 | 100 | - | 1610 | - | - | 6640 | - | 7030 | - | 6500 | - | 600 | - | 6620 | - | 4550 | - | 4040 | - |
| – OH | = O | 4500 | 2670 | - | 560 | 770 | - | 3850 | - | - | 9740 | 520 | 5310 | - | 5870 | - | 3360 | - | 2540 | - | 6570 | - | 17400 | - |
| = O | – OH | 1200 | - | - | 1370 | - | - | - | - | - | 5650 | - | 5710 | - | 6910 | - | 820 | - | 5190 | - | 1820 | - | - | - |
| = O | = O | 340 | - | - | 230 | 240 | - | 320 | 70 | - | 520 | 100 | - | - | - | - | 180 | - | - | - | 90 | - | - | - |
| | = O | - | - | - | - | - | - | - | - | - | 900 | - | - | - | - | - | - | - | - | - | - | - | 9040 | - |
| **Estranes** | | | | | | | | | | | | | | | | | | | | | | | | |
| substituent at C3 | substituent at C17 | | | | | | | | | | | | | | | | | | | | | | | |
| – OH | = O | - | - | - | - | - | - | - | - | - | - | 110 | - | - | - | - | - | - | - | - | 40 | - | - | - |
| – OH | – OH | - | 150 | - | 370 | - | - | 620 | - | - | 480 | - | - | - | 690 | - | 380 | - | - | - | 500 | - | - | - |
| **Pregnanes** | | | | | | | | | | | | | | | | | | | | | | | | |
| substituent at C3 | substituent at C20 | | | | | | | | | | | | | | | | | | | | | | | |
| – OH | – OH | 1560 | 420 | - | 1130 | 630 | - | 4000 | - | - | 2230 | - | 2770 | - | 3260 | - | 60 | - | 2990 | - | 4180 | - | - | - |
| – OH | = O | - | 270 | - | - | 170 | - | - | - | - | 780 | - | - | - | 450 | - | 280 | - | 550 | - | 370 | - | - | - |
| **Cholestanes** | | - | - | - | - | - | - | 370 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 1840 | - |

**Suspended solid particles**

| **Androstanes** | | **Kajaani** | | | **Uusikaupunki** | | | **Helsinki** | | | **Espoo** | | **Joensuu** | | **Mikkeli** | | **Pori** | | **Kouvola** | | **Turku** | | **Porvoo** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| substituent at C3 | substituent at C17 | I | B | E | I | B | E | I | B | E | I | E | I | E | I | E | I | E | I | E | I | E | I | E |
| | | 149* | 35 | 10 | 242 | 52 | 7 | 226 | 20 | 16 | 169 | 9 | 133 | 6 | 163 | 16 | 236 | 5 | 135 | 13 | 180 | 5 | 135 | 5 |
| – OH | = O | 60 | - | - | - | - | - | 40 | - | - | - | - | 20 | - | 70 | - | 140 | - | - | - | 100 | - | 100 | - |
| = O | = O | - | - | - | 20 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 20 | - | 200 | - |
| **Pregnanes** | | | | | | | | | | | | | | | | | | | | | | | | |
| substituent at C3 | substituent at C20 | | | | | | | | | | | | | | | | | | | | | | | |
| – OH | – OH | 190 | - | - | - | - | - | - | - | - | 70 | - | 50 | - | 110 | - | 490 | - | 60 | - | 300 | - | 200 | - |
| – OH | = O | 20 | - | - | - | - | - | - | - | - | - | - | - | - | 20 | - | 170 | - | - | - | 120 | - | - | - |
| = O | = O | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 20 | - |
| **Cholestanes** | | - | - | 620 | 1630 | 4630 | 190 | 170 | - | 620 | 4950 | 470 | - | - | - | 130 | - | - | 100 | - | 2800 | - | 1540 | - |

*Average mass of solid material (mg $L^{-1}$) in the parallel samples.

**Table 3** Tentatively identified EOCs in liquid samples by GC×GC–TOFMS. Average (n = 9) concentrations are listed as response factors (I = influent samples, E = effluent samples).

| | **Kajaani** | | **Uusikaupunki** | | **Helsinki** | | **Espoo** | | **Joensuu** | | **Mikkeli** | | **Pori** | | **Kouvola** | | **Turku** | | **Porvoo** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | I | E | I | E | I | E | I | E | I | E | I | E | I | E | I | E | I | E | I | E |
| 1,7-Dimethylxanthine | 2.0 | - | 1.2 | - | 1.4 | - | 2.0 | - | - | - | 1.7 | - | 0.2 | - | 0.9 | - | 2.5 | - | - | - |
| 3-Hydroxycotinine | 3.6 | - | - | - | - | - | 6.5 | - | 6.3 | - | 8.3 | - | - | - | - | - | - | - | 34 | - |
| Amitriptyline | - | 0.3 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 2.9 | 2.4 | 0.6 |
| Caffeine | - | 8.6 | 170 | 240 | - | - | - | 0.1 | - | - | 550 | - | 860 | - | 490 | - | 190 | - | 520 | - |
| Carbamazepine | 0.5 | 0.6 | - | 0.1 | 0.3 | 0.5 | 0.4 | 0.6 | - | 1.0 | 0.4 | 0.8 | 0.3 | 0.4 | - | 0.6 | 0.2 | 0.3 | - | 0.6 |
| Clomethiazole | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 7.9 | 5.9 | - | - |
| Clozapine | 0.1 | - | - | - | - | - | - | - | - | - | 0.2 | - | - | - | - | - | - | - | - | - |
| Codeine | - | 2.1 | - | 1.5 | - | - | 1.8 | - | 2.3 | 0.8 | - | - | - | - | - | - | - | 2.2 | 6.5 | 9.4 |
| Cotinine | - | - | - | - | - | - | - | - | 8.7 | - | - | - | - | - | 3.2 | - | 8.9 | - | 32 | - |
| Dihydromorphine | - | - | - | - | 120 | - | - | - | 50 | - | - | - | - | - | - | - | - | - | - | - |
| Ibuprofen | - | - | - | - | - | - | 54 | - | - | - | - | - | 150 | - | 60 | - | 55 | - | 540 | - |
| Leveorphanol | - | - | - | - | 10 | - | 8.4 | - | - | - | 6.8 | - | - | - | - | - | - | - | - | - |
| Lidocaine | 0.8 | - | 11.1 | - | - | 3.7 | - | 6.5 | 5.4 | 4.5 | 1.5 | 2.3 | - | 7.1 | - | - | - | - | - | - |
| Mirtazapine | - | 0.1 | - | - | - | - | - | - | 0.2 | - | - | - | 0.1 | - | - | - | - | 0.1 | - | 0.6 |
| Moclobemide | - | - | - | - | 0.2 | 0.2 | 0.2 | - | - | - | - | - | - | - | - | - | - | - | - | 0.5 |
| Temazepam | - | 1.1 | - | - | - | 1.9 | - | 2.2 | - | 1.1 | - | 1.7 | - | 2.3 | - | - | - | 2.8 | - | 2.0 |
| Timolol | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 100 | 13 | - | - |
| Tramadol | - | - | - | 3.3 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Venlafaxine | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 11 | - |

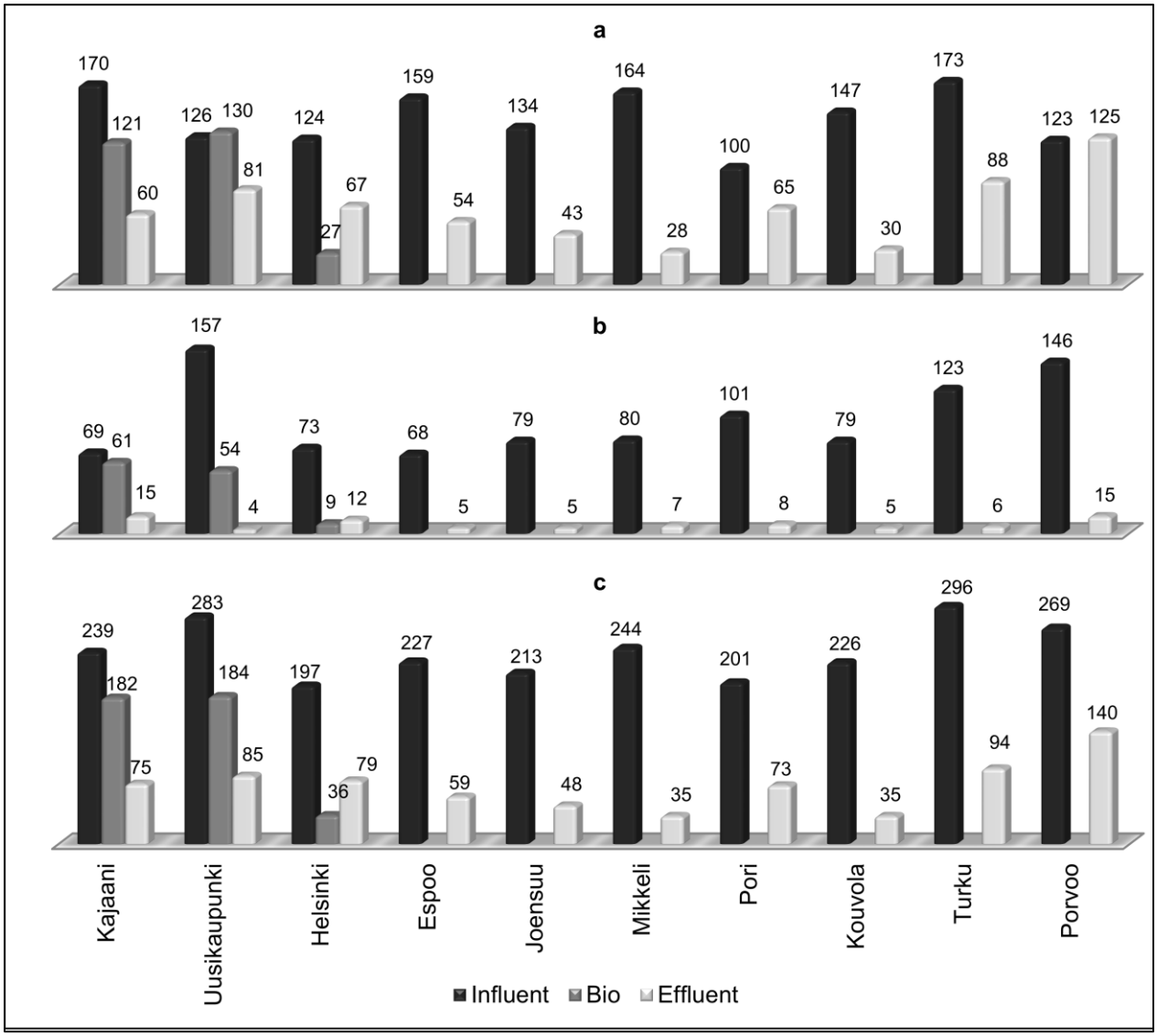


**Fig. 1** Average number (n = 9) of tentatively identified peaks in influent water, water before biological filtration, and effluent water of the WWTPs (library match > 600) analyzed separately from aqueous phase (a), suspended solids (b) and whole-water (c)

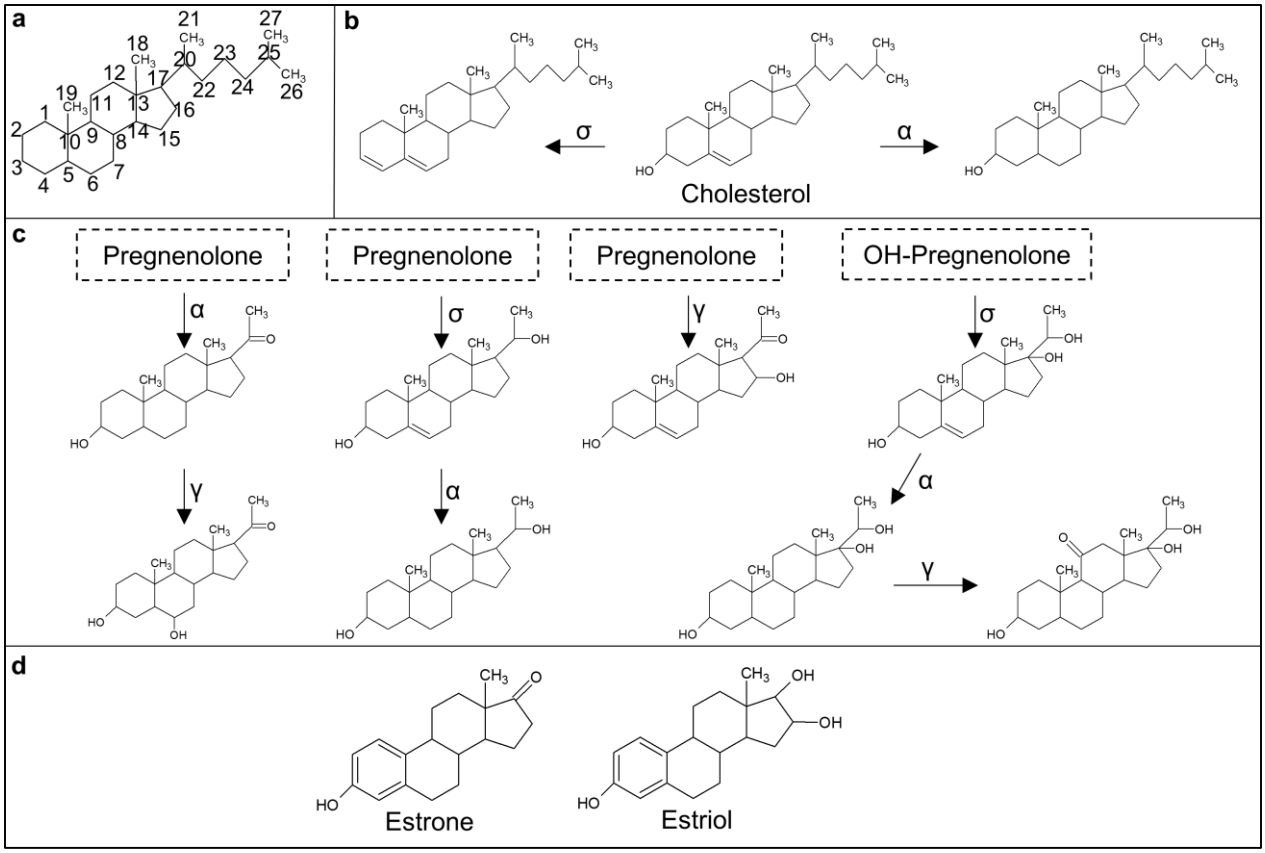


**Fig. 2** The numbering of the steroid skeleton carbon atoms (a) and the structures of the tentatively identified cholestanes (b), pregnanes (c) and estranes (d). (α = hydrogenation; γ = oxidation; σ = reduction)

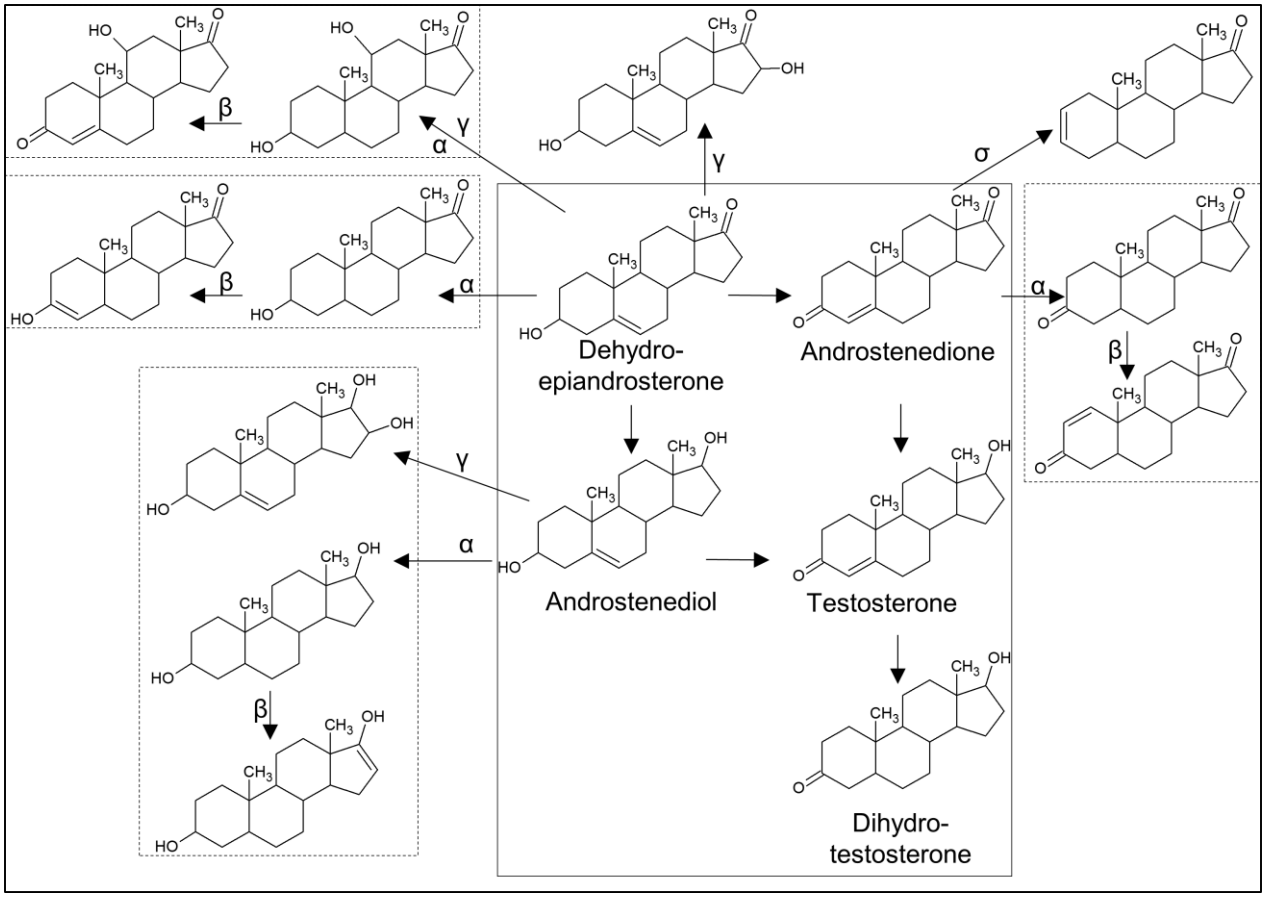


**Fig. 3** The structures of the tentatively identified androgens, including anthropogenic steroids (in the middle) and their proposed transformation products. (α = hydrogenation; β = dehydrogenation; γ = oxidation; σ = reduction.)