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Applicability of comprehensive two-dimensional gas chromatography – time-of-flight mass spectrometry to environmental non-target screening: Special emphasis on wastewater

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ACADEMIC DISSERTATION

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Preface

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Abstract

The flux of emerging organic contaminants into environment is a global threat, which is widely studied and monitored. However, current regulation is not able to keep up with the increasing variety of new compounds released to the environment. More efficient and comprehensive analytical methodologies are required to enable sufficient monitoring of these compounds for legislative purposes. Non-targeted analytical approaches are able to identify previously unknown contaminants, which is not possible with conventional targeted methods. Therefore, the development of novel non-target methodologies is important.

The goal of the thesis was to look for new ways to utilize non-targeted data for environmental applications with a special emphasis on wastewater analysis. The developed methodologies focused on chemometric quantification of non-target compounds, identification of steroidal transformation products, statistical cross-sample analysis of wastewater and atmospheric particles as well as non-targeted approaches to quantify selectivity of adsorbents employed in sample preparation.

The samples were analyzed by comprehensive two-dimensional gas chromatography – time-of-flight mass spectrometry utilizing mass spectral libraries and retention indices for compound identification. Different solid-phase extraction procedures were applied to aqueous samples, and ultra-sound assisted extraction to solid samples. The study included also the synthesis of novel polymeric adsorbents with increased selectivity towards steroidal compounds. Modern statistical software was utilized for data handling and chemometrics.

The multidimensional system enabled the analysis of complex wastewater samples, and several steroids and their transformation products were identified from the samples. It was concluded that hydrophobic steroids were efficiently removed from wastewater by adsorption to sewage sludge. However, elimination from sludge was less efficient and steroids were also found in the processed sludge destined for agricultural purposes. The chemometric model for the prediction of concentrations of non-target compounds with steroidal structure demonstrated good accuracy. Non-targeted approaches allowed the arithmetic comparison of adsorbent selectivity, when previously only relative methods have been used. Fast comparison of different wastewater and aerosol samples was possible through cross-sample analysis with non-targeted data.

Non-targeted approaches presented in this thesis can also be applied to other groups of contaminants and thus promote the available knowledge about environmental pollution. New ways to utilize non-targeted methodologies and cross-sample analyses demonstrated their value in this thesis and hopefully inspire future studies in the field.

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Appendices: Papers I–IV

List of original publications

This doctoral thesis is based on the following papers, hereafter referred to by their Roman numerals [I–IV]:

I **Kopperi, M**., Ruiz-Jiménez, J., Hukkinen, J.I., Riekkola, M.-L., New way to quantify multiple steroidal compounds in wastewater by comprehensive two-dimensional gas chromatography – time-of-flight mass spectrometry, *Analytica Chimica Acta*, 2013, 761, 217–226. DOI: 10.1016/j.aca.2012.11.059.

II **Kopperi, M**., Parshintsev, J., Ruiz-Jiménez, J., Riekkola, M.-L., Nontargeted evaluation of the fate of steroids during wastewater treatment by comprehensive two-dimensional gas chromatography – time-of-flight mass spectrometry, *Environmental Science and Pollution Research*, 2016, in press. DOI: 10.1007/s11356-016-6800-4.

III **Kopperi, M**., Riekkola, M.-L., Non-targeted evaluation of selectivity of water-compatible class selective adsorbents for the analysis of steroids in wastewater, *Analytica Chimica Acta*, 2016, 920, 47–53. DOI: 10.1016/j.aca.2016.03.036.

IV Booyens, W., Van Zyl, P.G., Beukes, J.P., Ruiz-Jiménez, J., **Kopperi, M.**, Riekkola, M.-L., Josipovic, M., Venter, A.D., Jaars, K., Laakso, L., Vakkari, V., Kulmala, M. and Pienaar, J.J., Size-resolved characterisation of organic compounds in atmospheric aerosols collected at Welgegund, South Africa, *Journal of Atmospheric Chemistry*, 2015, 72, 43–64. DOI: 10.1007/s10874-015-9304-6.

The contribution of the author:
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Abbreviations and Symbols

ANOVA	Analysis of Variance
BCD	β-Cyclodextrin
CRM	Certified Reference Material
ECD	Entrapped β-cyclodextrin–epichlorohydrin
EOC	Emerging Organic Contaminant
EPE	External Compounds Prediction Error
EPI	Epichlorohydrin
ESI	Electrospray Ionization
FID	Flame Ionization Detector
GC×GC	Comprehensive Two-dimensional Gas Chromatography
GC–GC	Heart-cutting Two-dimensional Gas Chromatography
GC–MS	Gas Chromatography – Mass Spectrometry
HCl	Hydrochloric Acid
HLB	Hydrophilic-lipophilic Balance
HRMS	High Resolution Mass Spectrometry
HRT	Hydraulic Retention Time
I.D.	Internal Diameter
LC–MS	Liquid Chromatography – Mass Spectrometry
MDGC	Multidimensional Gas Chromatography
MIP	Molecularly Imprinted Polymer
MS/MS	Tandem Mass Spectrometry
NaOH	Sodium Hydroxide
NIP	Non-imprinted Polymer
NRF	Normalized Response Factor
ODS	Octadecyl-silica
PCA	Principal Component Analysis
RF	Response Factor
SIM	Selected Ion Monitoring
SPE	Solid-phase Extraction
SRT	Solids Retention Time
TIC	Total Ion Chromatogram
TOFMS	Time-of-flight Mass Spectrometry
WWTP	Wastewater Treatment Plant
XIC	Extracted Ion Chromatogram

1. Introduction

The amount of chemicals consumed by the society is increasing steadily and part of this chemical load ends up in the environment. The potential ecological risk imposed by the variety of anthropogenic compounds has motivated environmental research for decades. However, regulative legislation is slow and improved analytical tools are required to identify hazardous compounds as soon as they emerge in the environment.

Non-targeted approaches are essential to regain information about new contaminants as well as to identify the possibly hazardous transformation products of the known contaminants. It has been estimated that estrogens are one of the most hormonally active compounds in aquatic environment. Therefore, the focus of this thesis was on the analysis of all compounds with similar steroid structures. The main objective was the development and application of novel non-targeted approaches utilizing comprehensive two-dimensional gas chromatography – time-of-flight mass spectrometry (GC×GC-TOFMS) in the field of environmental chemistry.

The focus of the first study (Paper I) was to develop a reliable method for the analysis of all steroidal compounds present in the wastewater. A new analytical method was developed for separate analysis of steroids in aqueous and solid phase of wastewater by GC×GC-TOFMS. A novel chemometric model was also developed to predict analyte concentrations without the need for commercial reference materials for quantification.

In the second study (Paper II), the previously developed method was applied to several wastewater samples from different treatment plants around Finland. The concentrations of steroidal compounds in wastewater, suspended solid material and sewage sludge were compared in order to evaluate their fate during the treatment process, including also the identification of possible transformation products.

To further improve the selectivity of the sample preparation towards compounds with steroidal structure, a study was made to compare synthetic and commercial adsorbents (Paper III). Non-targeted approaches were utilized to evaluate qualitative and quantitative selectivity of the studied extraction materials.

In the final part of the thesis (Paper IV), another approach for non-target screening was considered when GC×GC-TOFMS was applied to atmospheric aerosol particles. Instead of measuring individual compounds, the composition of aerosol particles was determined based on different chemical functional groups of the compounds. With this approach, useful cross-sample analysis was possible without the need for reference materials.

The specific aims of the study were the following:

- To develop sample preparation methodologies for steroidal compounds in aqueous wastewater as well as in suspended solid particles and sewage sludge.
  (Papers I and II)

- To develop quantification methodology for non-targeted steroidal compounds without reference materials utilizing statistical methods and chemometrics.
  (Paper I)

- To evaluate the fate of steroidal compounds in wastewater and to identify possible transformation products by a non-targeted approach.
  (Paper II)

- To utilize non-targeted cross-sample analysis to compare the purification efficiency of several wastewater treatment plants in Finland.
  (Paper II)

- To synthetize new adsorbents with improved selectivity towards steroidal compounds in wastewater.
  (Paper III)

- To evaluate the selectivity of synthetic and commercial adsorbents towards steroidal compounds by non-targeted approaches.
  (Paper III)

- To utilize non-targeted cross-sample analysis to compare the organic composition of different size fractions of atmospheric aerosols.
  (Paper IV)

2. Background to the work

2.1. Environmental analysis of emerging organic contaminants

The major research in environmental analysis during the last decades has focused on the evaluation of anthropogenic chemicalization. Especially, the impact of ecotoxicological chemicals on aquatic ecosystems has been of concern, which has led to improved regulating legislation, including the Water Framework Directive of the European Commission adapted on 23 October 2000. However, the update of the current list of monitored priority substances in Annex X (Directive 2013/39/EU) is slow because the occurrence of many trace pollutants and emerging organic contaminants (EOC) in the environment is not well documented or their toxicity is not established. Most of the currently studied contaminants can be classified into three categories (Murray et al. 2010):

1. *Industrials*: antioxidants, perfluorates, phenols, phthalates, polybrominated diphenyl ethers, triazoles
2. *Pesticides*: carbamates, chloroacetanilides, chlorophenoxy acids, organochlorines, organophosphates, pyrethroids, triazines
3. *Pharmaceuticals and Personal Care Products*: analgesics, anti-epileptic drugs, antihyperlipidemics, antimicrobials, polycyclic musks, non-steroidal anti-inflammatory drugs, synthetic hormones

Due to the development of more sensitive analytical instrumentation, more and more information is available of the occurrence of EOCs in the environment, as demonstrated in several reviews on their observed concentrations and fate (Lapworth et al. 2012, Li 2014, Pal et al. 2010) as well as on the utilized analytical methodologies (Richardson 2009, Wille et al. 2012). It has been concluded that estrogens are the most potent group of endocrine-disrupting compounds found in the aquatic environment and they were therefore also the focus of this thesis along with androgens and other compounds with similar steroidal structures.

Wastewater treatment plants (WWTP) are the most important point source of EOCs in surface and groundwater, and also one of the diffuse sources might be the runoff from agriculture after the application of treated sewage sludge as a fertilizer (Lapworth et al. 2012). The knowledge about the fate of EOCs during the wastewater treatment process is therefore of vital importance. The fate of estrogens has been exhaustively reviewed (Cirja et al. 2008, Clarke and Smith 2011, Khanal et al. 2006, Koh et al. 2008) and the most important parameters for their elimination in WWTPs are as follows (Cirja et al. 2008):

1. *Hydrophobicity*: Hydrophilic compounds (log K_{OW} < 2.5) remain mostly in the aqueous phase and hydrophobic compounds are collected in the sludge.
2. *Chemical structure*: Compounds with complex structures are more resistant to biodegradation.
3. *Hydraulic and Solids Retention Time (HRT and SRT)*: Especially SRT should be long enough (> 10 days) for efficient removal from aqueous phase into sludge.

4. *pH*: The adsorption of estrogens is highest in low pH but they can be desorbed back to aqueous phase from suspended solids if pH is too high (> 9)
5. *Temperature*: It has been observed that the activity of the biomass is reduced in low temperatures and the elimination by biodegradation is therefore slower during winter.

It has been established that estrogens are efficiently eliminated from wastewater but more research is required on their transformation products as well as on the fate of other steroidal compounds during purification processes in WWTPs. Also, the development of more sensitive instruments can unveil trace amounts of estrogens previously hidden in wastewater effluents.

Water samples consist of a high number of different substances in a large variety of concentrations. Because EOCs are most often found in very low concentrations (~ng L^{-1}), a preconcentration step is required to remove matrix components and to decrease method detection limits. To accomplish this, solid-phase extraction (SPE) is most often utilized with Oasis HLB cartridges, because the hydrophilic-lipophilic adsorbent is capable of retaining a large variety of different contaminants (Wille et al. 2012). Liquid chromatography coupled with mass spectrometry (LC–MS) is most often utilized due to its applicability to polar compounds. Gas chromatography is more suitable for hydrophobic compounds like steroids, which can also be difficult to ionize by electrospray most often utilized in the coupling of LC–MS. Different approaches for targeted and non-targeted analysis of EOCs are described in the next chapter.

2.2. Targeted and non-targeted screening approaches

Conventional analytical methodology involves the identification of target analytes by comparison of retention time and mass spectrum to those of certified reference materials (CRM). European Commission has implemented identification points with a minimum of four points as the criterion for unequivocal identification (Decision 2002/657/EC). Points are calculated as the number of matching ions in the mass spectrum so that each low resolution ion gives one point, each transition ion gives 1.5 points and the use of high resolution mass spectra (HRMS) awards one additional point per ion. Therefore, unequivocal identification by LC–MS/MS or GC–MS/MS requires one matching precursor ion and two daughter ions, and if HRMS is utilized only one precursor and daughter ion is required. The main problem with targeted approaches is the requirement for CRMs and prior knowledge of the studied analytes. In multiresidue analysis, for example, hundreds of CRMs can be purchased and still only the previously selected compounds can be detected. Therefore possible transformation products and new contaminants remain hidden and non-targeted approaches are required to unveil them.

The screening approaches for unknown compounds can be divided into suspect screening and non-target screening. Suspect or post-target screening is accomplished by comparison of accurate mass data of possible environmental pollutants to experimental extracted ion chromatograms (XIC) with HRMS full scan spectra. In non-targeted screening, no prior

information is available and the whole chromatogram is searched for peaks and the resulting peak table is compared to libraries for compound identification based on their spectral fragmentation patterns, accurate mass information and estimated retention times.

Currently, the most popular instrumental setup for non-targeted screening of EOCs in aquatic environment is LC–HRMS by Orbitrap or quadrupole–time-of-flight (QTOF) mass spectrometry (Aceña et al. 2015, Leendert et al. 2015). The development of user-friendly HRMS-instruments and their coupling to chromatographic systems with high separation capacity has multiplied the number of non-targeted studies in the field. The major benefit of HRMS is their increased selectivity due to very narrow mass windows, which result in accurate mass XICs that are characterized by the absence of baseline noise, which reduces the requirements for sample preparation. When the mass precision is increased, the number of possible molecular formulas is decreased significantly and the identification of compounds can be accomplished by comparing experimental accurate masses to calculated monoisotopic exact masses. The minimum criteria of identification procedures can be optimized by treating known target compounds as ´unknowns´ while identifying them with the non-targeted approach (Gago-Ferrero et al. 2015). The identification protocols usually contain the following procedures (Leendert et al. 2015):

1. Application of exact mass filters, often with mass error < 2 mDa.
2. Peak-noise filters (blank subtraction)
3. Isotopic pattern recognition
4. Retention time prediction with log K_{OW} or linear solvation energy relationship
5. Fragmentation patterns

As mentioned previously, transformation products of EOCs can be hidden from targeted approaches, although their toxicity might still be as potent as that of the parent compounds. Transformation products can be classified into biotic (human, animal or microbial metabolites) and abiotic (products of chemical processing during treatment) ones, which are mostly formed through hydroxylation, oxidation and reduction, dealkylation, conjugation and deconjugation (Bletsou et al. 2015, Evgenidou et al. 2015). In order to study the transformation pathways of these compounds, additional non-targeted identification procedures are required. In kinetic batch reactor studies, for example, spiked and non-spiked samples are analyzed at certain intervals and possible transformation products are identified by automated comparison of generated peak tables. Extra peaks found in the spiked samples after filtering of the target compounds and matrix constituents are candidates for further study (Boix et al. 2016).

Although, LC–MS has been frequently used for the analysis of EOCs in aquatic environment, there are two major benefits for non-targeted GC–MS approaches. The reproducibility of electron ionization enables the automated comparison of mass spectra to mass spectral libraries for compound identification, whereas LC–MS with electrospray ionization has to rely on HRMS and accurate mass for the construction of chemical structure (Hernández et al. 2009). Another benefit is the higher separation power, which results in narrow chromatographic peaks and high peak capacity. With narrow peaks, more precise

retention time locking can be utilized to aid in identification (Gómez et al. 2009). Furthermore, the reliability of identification via mass spectral matching is dependent on the purity of the mass spectrum and increased peak capacity reduces the possibility of overlapping peaks. The highest peak capacity can be generated by multidimensional gas chromatography (MDGC), which also includes novel non-targeted cross-sample analysis potential (Gómez et al. 2011). The theory and application of MDGC will be discussed in detail in chapter 3.

Most non-targeted application are focused only on the identification of unknown compounds and the quantification of the found compounds are then done after corresponding CRMs have been purchased. The literature on purely non-targeted quantification is scarce. Semi-quantification of the analytes can be accomplished by comparison of normalized peak areas or estimation of concentrations with a surrogate approach, where one or few compounds are used to predict the concentration of similar compounds. This is sufficient for cross-sample analysis but in order to monitor the concentrations of EOCs with non-targeted approaches, more research is required on non-targeted quantification.

3. Comprehensive two-dimensional gas chromatography – time-of-flight mass spectrometry

3.1. Main principles

The main motivation behind multidimensional separation techniques is the effort to increase the resolving power of chromatographic systems to enable the analysis of complex samples. A practical measure of the chromatographic resolving power is the peak capacity, which equals the number of peaks that can fit in a chromatogram with a selected resolution. However, to successfully separate all sample constituents, theoretical peak capacity must greatly exceed the number of compounds in sample due to uneven distribution of peaks in real applications. Fortunately, only the separation of analytes from matrix components is usually required and even unresolved peaks can be separated by mass spectrometry. Regardless, the peak capacity of conventional gas chromatography is not sufficient for complex samples.

The most efficient way to increase peak capacity in gas chromatographic analysis is to combine two analytical capillary columns with orthogonal separation mechanisms. Orthogonality in MDGC is most often achieved by combining nonpolar capillary in the first dimension (volatility based separation) and semi-polar capillary in the second dimension (polarity based separation) (Seeley and Seeley 2013). However, theoretical orthogonality cannot be achieved in practice because volatility and polarity are interconnected; increasing polarity often decreases the volatility of a compound (Blumberg 2011). Column variations in MDGC will be described in more detail in chapter 3.2.

Two main separation modes exist in multidimensional separations. If only part of the first dimension flow is directed to the second dimension, the technique is called heart-cutting two-dimensional gas chromatography (GC–GC) introduced already in 1968 (Deans 1968). The peak capacity in such a system is estimated from the sum of the capacities of first dimension separation and second dimension heart-cuts. When the entire flow from the first dimension is divided into fractions and directed to the second dimension, heart-cutting approach becomes comprehensive two-dimensional gas chromatography (GC×GC) introduced in 1991 (Liu and Phillips 1991). Then the peak capacity is estimated from the multiplication of the first dimension and second dimension capacities. The focus of this study will only be on comprehensive gas chromatography. Instrumental setup of two-dimensional gas chromatography and the two separation modes are illustrated in Figure 1, which also clarifies how two-dimensional contour plots are formed from the aligned second-dimension chromatograms in GC×GC.

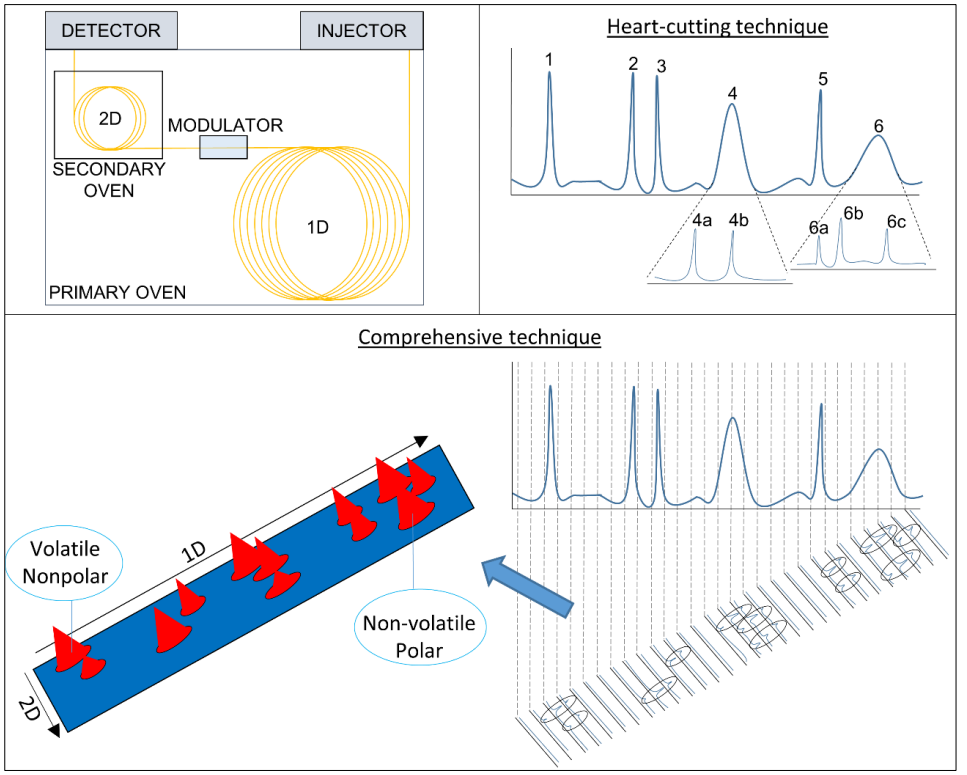


Figure 1 Instrumental setup of two-dimensional gas chromatography with heart-cutting and comprehensive separation modes.

The most important component of MDGC is the modulator and its main responsibility is to maintain the resolution achieved in the first dimension. Without modulation between the capillaries, resolved peaks of the first dimension could overlap during the second dimension separation. Modulator is responsible for the collection of first dimension flow into focused narrow fractions and their injection into the second dimension. The next fraction will be collected during the separation of the previous fraction in the second dimension column. Therefore it follows, that the modulation period should be equally long or longer than the separation time in the second dimension. Otherwise ´wrap-around´ of the compounds can occur, where the slow-eluting compounds of the previous fraction emerge in the beginning of the following fraction. In some cases, however, ´wrap-around´ can be beneficial by randomizing the distribution of analytes in the two-dimensional chromatogram and thus increasing the experimental peak capacity (Mondello et al. 2008). If the modulation period is too long, peaks already separated in the first dimension will be collected in the same fraction during modulation. To maintain the separation achieved in the first dimension and to generate the maximum peak capacity, first dimension peaks should theoretically be sampled into three fractions (Murphy et al. 1998). Therefore, temperature programs in GC×GC are usually slow (1–3 °C min^{-1}) in order to broaden the first dimension peaks, which enables their proper modulation with increased separation times in the second dimension (Mondello et al. 2008). The temperature program during a single second dimension fraction

is always isothermal due to the short separation time. Different modulation technologies will be discussed in more detail in chapter 3.3.

The refocusing of analytes in the modulator results in very narrow peaks in the second dimension, where peak widths are usually in the range 0.1–0.5 s (Mostafa et al. 2012). Quantification of a chromatographic peak requires enough data points (usually > 10) to correctly determine the peak shape, which means that very fast scan rates are demanded from the detector coupled to the GC×GC. Therefore flame ionization detection (FID) or time-of-flight mass spectrometry (TOFMS) are often utilized with possible scan rates up to 500 Hz (Seeley and Seeley 2013). If quadrupole-MS, for example, would be utilized, narrow scan range or selected-ion-monitoring (SIM) are required to compensate for the slow scan rate caused by the physical restrictions of the quadrupole analyzer (Mostafa et al. 2012). These approaches, however, would only be suitable for targeted analyses. Due to increased size of the data files with high acquisition rates, 50 Hz scan rate is usually applied in GC×GC–TOFMS. For identification purposes, high resolution instruments (HR–TOFMS) are a great tool to increase the reliability of identification (Tranchida et al. 2014a). However, due to slower scan rates (~25 Hz) they are not so suitable for quantification purposes (Mostafa et al. 2012). The large quantity of data generated with GC×GC–TOFMS requires advanced software for efficient data handling. A short review of data processing methods will be described in chapter 3.4.

3.2. Column variations

The separation in the second dimension takes usually only few seconds in order to enable sufficient sampling of first dimension peaks. Therefore the 2D capillary length is usually only 0.5–1.5 m whereas the length of the 1D capillary is 15–30 m. Consequently, the 2D capillary (~0.1 μm I.D.) has smaller internal diameter than the 1D capillary (~0.25 μm I.D.) in order to increase its efficiency (Mostafa et al. 2012). Due to different volumes of the two capillaries, the linear flowrate of the carrier gas is different and usually only optimized for the 1D capillary. Therefore, the flow rate in 2D capillary is higher than the optimal value derived from the van Deemter equation, which decreases the achieved peak capacity compared to the theoretical maximum value. Wider bore capillaries can be used in the second dimension but the efficiency is then decreased (Mondello et al. 2008). One potential approach to reduce the flow rate in the second dimension would be the application of a split-flow valve between the capillaries, which was actually already proposed by Liu & Phillips in 1991.

The stationary phase in 1D capillary is usually non-polar so the compounds elute according to decreasing volatility. The stationary phase in the 2D capillary is semi-polar so the compounds elute according to increasing polarity. This column configuration is the most popular one and it was also applied in this thesis. However, increasing the orthogonality by polarity difference is not always the best option for column selection and the different selectivity of stationary phases with analytes and matrix components should be considered instead (Seeley and Seeley 2013). Also, completely orthogonal setup with apolar-polar columns can never be achieved because the effect of volatility is always present in gas

chromatography. Therefore, most GC×GC separations are characterized by a diagonal fan-shaped formation of peaks in the contour plot, where the areas in the upper left corner (volatile and polar compounds) and lower right corner (non-volatile and non-polar compounds) are devoid of analyte peaks (Mondello et al. 2008).

3.3. Modulation technologies

There are three major requirements for a modulator. First of all, its performance must be repeatable and precise. Modulation must happen in the same way during the whole analysis without breakthrough of analytes into second dimension during sampling of the first dimension flow. Secondly, it must maintain the resolution gained in the first dimension. Finally, the sampling of first dimension flow into the second dimension must be representative so no information is lost during modulation. Modulators can be categorized into two classes: thermal modulators and flow modulators (also known as pneumatic modulators or valve-based modulators). The development of modulators since 1991 until 2011 have been exhaustively reviewed (Edwards et al. 2011, Seeley 2012, Tranchida et al. 2011). Majority of current applications are utilizing cryogenic modulation but the development of new modulators is mainly focused on flow-modulation, which might increase their popularity in the future (Tranchida et al. 2014b,c, Duhamel et al. 2015, Tranchida et al. 2016).

3.3.1. Thermal modulators

The first modulators, beginning with the innovation of Liu and Phillips (1991), were heater based and the trapping of analytes was accomplished with a segment of capillary with thicker stationary phase. The release of analytes into the second dimension was accomplished by a fast heating of the modulator.

Since the beginning of the 21st century, heater-based modulators have been replaced by cooling-based modulators where the trapping of analytes is accomplished by fast cooling of the capillary most often utilized by cryogenic fluids. The subsequent release of analytes into the second dimension is accomplished by heating usually with a hot pulse of air onto the capillary. The principle of the cryogenic modulator utilized in this work is illustrated in Figure 2. The gaseous nitrogen was cooled down by passage through a Dewar bottle filled with liquid nitrogen. Then the cold cryogen (N_2) was sprayed from the cryojets of the modulator onto the surface of the second dimension capillary to enable trapping of the compounds.

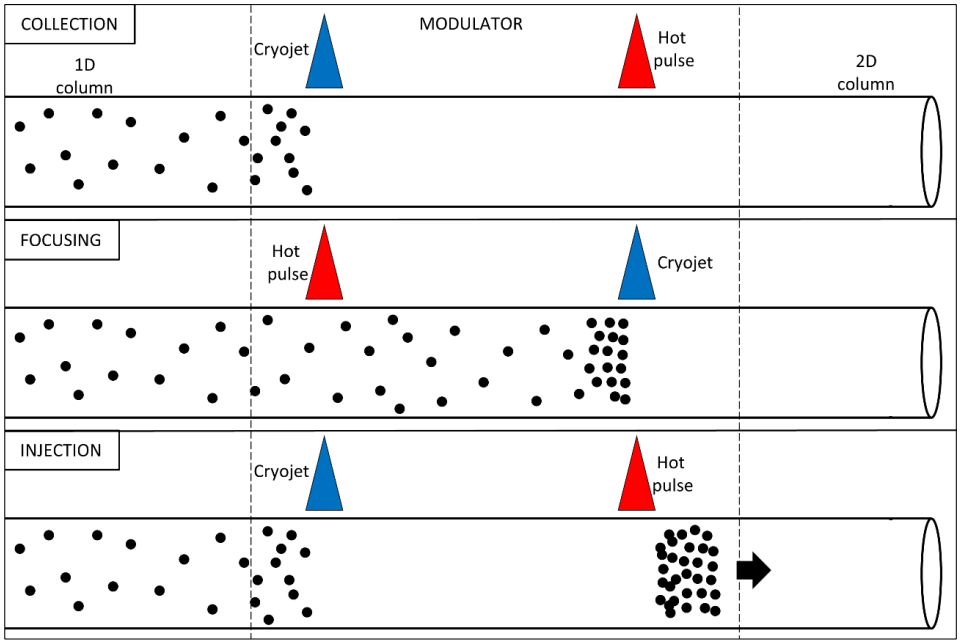


Figure 2 Principle of the cryogenic modulator in the LECO Pegasus 4D instrument.

3.3.2. Flow modulators

The first flow modulator was introduced in 1998 (Bruckner et al. 1998) and their development has been extensive ever since. The motivation for the replacement of thermal modulators is their high price and the consumption of expensive cryogenic fluids. Flow modulators are cheap and not dependent on the availability of the cryogen. However, the main drawback of flow modulators is the broadness of the second dimension pulses due to the lack of a focusing step during modulation.

Flow modulators can be divided into low duty cycle instruments, where only a small portion of the first dimension flow is diverted to second dimension during a modulation period, and high duty cycle instruments where most of the flow is sampled to the second capillary. Most low duty cycle modulators utilize diaphragm valves fitted with sample loops (Seeley 2012). During collection the flow from first dimension goes through the sampling loop, which is then purged into the second dimension with auxiliary gas flow by briefly turning the diaphragm valve (Figure 3a). The benefit of low duty cycle modulators is the generation of very sharp pulses into the second dimension and the increased resolution. Additionally, the flow rates of the auxiliary gas in the second dimension can be reduced with low duty cycle instruments, which makes them applicable for mass spectrometry. However, sensitivity is decreased and representative sampling of the first dimension flow can be compromised. Therefore, the development of high duty cycle modulators has been more popular and they are usually based on fluidic modulators that employ differential flow conditions (Seeley 2012), where the higher flow rate of the auxiliary gas momentarily blocks the flow from the first dimension consequently at the end or at the beginning of the sampling loop (Figure 3b).

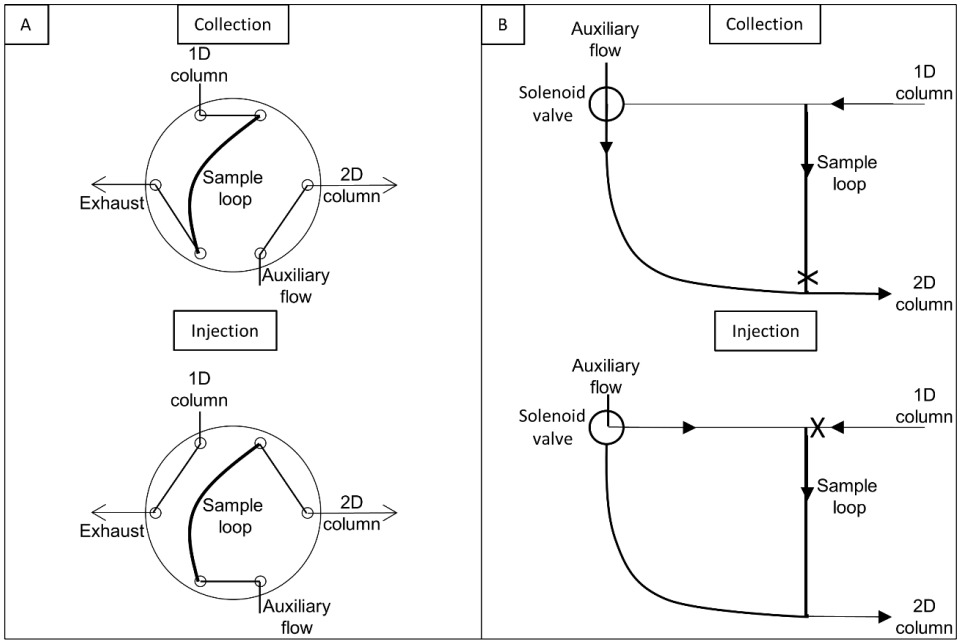


Figure 3 Schematics of flow modulators based on a) diaphragm valve or b) fluidic device, reproduced from (Seeley 2011).

3.4. Data processing

3.4.1. Preprocessing

The processing of two-dimensional data begins usually with automated preprocessing of the chromatograms, which can include, for example, baseline corrections and noise reduction (Pierce et al. 2012). The most important thing, however, is to correctly combine the modulated sub-peaks of the corresponding primary peak as well as to separate overlapping peaks by deconvolution and to manage possible retention time shifts between samples (Zeng et al. 2014). There are many groups, who are further developing chemometric approaches for these issues but one of the most sophisticated commercial tool for preprocessing of GC×GC–TOFMS data is the ChromaTOF-software from LECO Corporation (Amador-Muñoz and Marriott 2008), which was also utilized in this thesis.

3.4.2. Identification

After the data has been preprocessed, non-targeted analysis can be accomplished by automated comparison of mass spectra to spectral libraries or by the calculation of molecular structures from the accurate monoisotopic mass of the analytes. The reliability of tentative identification can then be increased by comparison of experimental retention indices to estimated ones. Several ways to assign retention indices are available depending on the stationary phase chemistry and analytes of interest, but the most common approach is to use linear *n*-alkanes and Kovats indexing (von Mühlen and Marriott 2011).

A third level of identification can be provided by structured patterns in the two-dimensional chromatograms. Homologous series of compounds with same functionalities can be aligned in a specific area of the separation space if the columns and other chromatographic conditions have been optimized accordingly. All compounds of such a structure can be tentatively identified based on the identification of a single compound in the series. Structured patterns are common in samples that contain a large number of isomers and homologs, analyzed with orthogonal column configuration (apolar – polar), although these structures can also be formed with the reverse configuration (polar – apolar) (Murray 2012). Structured chromatograms can be a great benefit for non-target identification, especially in the analysis of petroleum products by GC×GC–FID. However, the separation of analytes from matrix components is often a more important aim, especially when mass spectrometry can be utilized. The ´wrap-around´ of analytes, for example, can be beneficial in order to exploit the whole separation space for increased peak capacity and generation of single-component mass spectra, but this might destroy the structural patterns.

3.4.3. Quantification

Quantification of the target compounds can be problematic in GC×GC due to difficulties in correctly combining modulated sub-peaks of the first-dimension peak (Amador-Muñoz and Marriott 2008). In order to cope with sample-to-sample variation of the injection volume and detector response, normalization of the data is often utilized by the addition of a suitable internal standard (Pierce et al. 2012) and the calculation of relative response factors. Non-targeted approaches can then be utilized to characterize samples by comparing the summed response factors of different species tentatively identified, for example, by structured patterns in the chromatogram (Murray 2012). A novel chemometric approach for the quantification of non-target compounds with steroidal structure has been presented in this thesis.

3.4.4. Cross-sample analysis

The potential of GC×GC–TOFMS over 1D GC becomes evident in cross-sample analysis, where hundreds or thousands of samples are compared semi-automatically with non-targeted methodologies utilizing modern chemometrics with principal component analysis (PCA) or analysis of variance (ANOVA). The aims for cross-sample analysis are, for example, to determine the origin of a sample based on chromatographic features (fingerprinting) or to find biomarkers for cancer diagnosis based on the differences between the chromatographic features in samples received from healthy and sick patients. Cross-sample analysis can also simplify the identification of new EOCs in wastewater. Automated comparison between fresh wastewater samples and previously characterized samples or method blanks can be utilized to reveal possible EOCs as outliers in the sample data (Prebihalo et al. 2015).

The most important part of cross-sample analysis is the generation of features from the preprocessed chromatograms and their alignment between samples. The five features most often utilized in non-targeted cross-sample analysis have been recently reviewed (Reichenbach et al. 2012):

1. *Visual images*: The comparison of samples based on visual differences in their chromatograms. Although, modern imaging techniques have been used, this approach is usually not quantitative due to insufficient resolution of the images.
2. *Data points*: The comparison of samples based on the intensity (detector response) at each data point (pixel) in the chromatograms. This feature is often too selective because even small misalignment of data points from sample to sample can affect the results.
3. *Peaks*: The problematic selectivity of data points can be decreased by utilizing multiple data points as peak features, which was also applied during this thesis by Guineu-software developed originally for metabolomic cross-sample studies (Castillo et al. 2011). The approach should be carefully optimized to correctly match peaks in order to avoid problems arising from random trace peaks and co-eluting compounds.
4. *Regions*: Instead of a single peak, a region where the peak is found can be utilized to decrease sensitivity to misalignment even more. This approach becomes problematic when a region encompasses multiple analytes or a single analyte is spread across multiple regions.
5. *Peak-regions*: The fifth approach attempts to define regions so that only one analyte lies within a single region.

Most of the problems with feature generation are related to optimization during the preprocessing of the data, as was also the case with quantification. The most important thing is the correct merging of modulated sub-peaks and deconvolution of overlapping peaks.

3.5. Benefits and drawbacks compared to conventional gas chromatography

The benefits and drawbacks of GC×GC are summarized in Table 1. Due to the high price of the instrument, especially with cryogenic modulation, MDGC should be considered only if some of its benefits are required for the application in question. The non-targeted screening of a large and complex sample set, for example, is only possible with GC×GC supported by automated data processing and statistical analysis of the results.

Table 1 Benefits and drawbacks of GC×GC over 1D GC.

Benefits	Drawbacks
Optimal for non-target screening	Fast detector required
High peak capacity = high quality mass spectra	Large data files
Increased sensitivity through refocusing	More complex optimization
Structural patterns for group identification	Expensive
Possibility to sample ´fingerprinting´	
Reduced requirements for sample preparation	

A drawback of sorts, is also the unrealized potential of the theoretical peak capacity of GC×GC. The main reasons for this have been described in previous chapters and are summarized as follows:

- lack of orthogonality in the column selection
- sub-optimal flow rate of carrier gas in the second dimension
- slow reinjection from the modulator, which generates broad analyte bands in the second dimension

3.6. Applications of multidimensional gas chromatography

The applications of MDGC since 1991 have been exhaustively covered by multiple reviews. The complete overview of the published literature is beyond the scope of this thesis but some of the most influential reviews are listed in Table 2.

Table 2 Application focused reviews of multidimensional gas chromatography.

Coverage	Title	Ref.	Citation
1991–2002	Comprehensive two-dimensional gas chromatography: a powerful and versatile analytical tool	109	(Dallüge et al. 2003)
2003–2005	Recent developments in comprehensive two-dimensional gas chromatography (GC×GC)		
	I. Introduction and instrumental set-up	280	(Adahchour et al. 2006a)
	II. Modulation and detection		(Adahchour et al. 2006b)
	III. Applications for petrochemicals and organohalogens		(Adahchour et al. 2006c)
	IV. Further applications, conclusions and perspectives		(Adahchour et al. 2006d)
2004–2007	Recent developments in the application of comprehensive two-dimensional gas chromatography	253	(Adahchour et al. 2008)
2007–2008	Comprehensive two dimensional gas chromatography	141	(Cortes et al. 2009)
2005–2011	Multidimensional gas chromatography	201	(Marriott et al. 2012)
2011–2012	Multidimensional gas chromatography: Fundamental advances and new applications	171	(Seeley and Seeley 2013)

The main application of MDGC has always been in the field of petroleum product characterization because the samples contain usually over 1000 compounds, which also form group-type patterns in the two-dimensional chromatograms due to structural similarities of homologue series. Another increasing field is the screening of environmental samples for targeted and non-targeted organic analytes (Hamilton 2010, Panić and Górecki 2006). In a more recent review by Seeley and Seeley (2013), over 100 applications were considered, which included the analysis of petroleum products (31), environmental samples (33), food, flavor and fragrances (20) and biological studies (23).

4. Experimental

Experimental procedures of the thesis are explained in the following chapters, including lists of reagents and materials, sampling and sample preparation methodologies, instrumental conditions and data processing approaches. More detailed information is available in Papers I–IV.

4.1. Materials

The equipment and reagents used in this work are listed in Tables 3 and 4.

Table 3 List of equipment and instruments used in the studies.

Equipment / Instrument	Manufacturer / Model	Paper
Aerosol sampler	Dekati, PM10 cascade impactor	IV
BGB-5MS GC-capillary (0.25 mm I.D.)	BGB Analytik	I–III
DB-17 GC-capillary (0.1 mm I.D.)	Agilent Technologies	I–IV
Deactivated retention cap for GC (0.53 mm I.D.)	Agilent Technologies	I–IV
GC×GC–TOFMS	LECO, Pegasus 4D	I–IV
Glass microfiber filter GF/C	Whatman	I–III
HP-1 GC-capillary (0.25 mm I.D.)	Agilent Technologies	IV
Ion trap mass spectrometer	Bruker Daltonics, Esquire 3000+	III
LC-column, Luna C18	Phenomenex	III
Liquid chromatograph	Agilent Technologies, HP 1100	III
Membrane filter 0.45 µm	Millipore	I–III
Nitrogen evaporator	Thermo Fisher	I–IV
Peristaltic pump	Watson Marlow, 8-line 205S	II
Pump	Jasco, PU-980	I, IV
Quartz filter 25 mm	Whatman	IV
Quartz filter 47 mm	Whatman	IV
Sieves 53 µm and 106 µm	Retsch GmbH	III
Sonication bath	Branson, Bransonic 5510	II, IV
Sonifier	Branson, sonifier 250	I
SPE-cartridge: AffiniMIP Estrogens 100 mg/1mL	Polyintell, Affinisep	III
SPE-cartridge: C18 500 mg/3mL	Agilent Technologies	I
SPE-cartridge: Florisil 100 mg/1mL	Agilent Technologies	I, II
SPE-cartridge: Strata-X 500 mg/6mL	Phenomenex	II, III
SPE-manifold	Biotage, VacMaster-10	I–III
Water purification system	Millipore, Direct-Q 3 UV	I–III
Vortexer	Scientific Industries	III

Table 4 List of chemicals used in the studies.

Chemical	Manufacturer	Paper
1´,1-Binaphthalene	Acros Organics	I–IV
17α-Ethynylestradiol	Sigma-Aldrich	I–III
17β-Estradiol	Sigma-Aldrich	I–III
2-Hydroxyethyl methacrylate	Sigma-Aldrich	III
Acetic acid	Sigma-Aldrich / Merck	III
Acetone	VWR / J.T. Baker	I–IV
Acetonitrile	VWR	II
Acrylamide	Sigma-Aldrich	III
Androstenedione	Fluka	II
Chloroform	VWR	III
Dichloromethane	VWR	I–IV
Divinylbenzene	Sigma-Aldrich	III
Epichlorohydrin	Fluka	III
Estriol	Sigma-Aldrich	I–III
Estrone	Sigma-Aldrich	I–III
Ethyl acetate	VWR	I
Ethylene glycol dimethacrylate	Sigma-Aldrich	III
Hexane	VWR	I, II
Hydrochloric acid	VWR / J.T. Baker	I
Methacrylic acid	Sigma-Aldrich	III
Methanol	VWR	I–IV
N,O-Bis(trimethylsilyl)trifluoroacetamide	Sigma-Aldrich	I–IV
Progesterone	Merck	II, III
Pyridine	J.T. Baker	I–IV
Testosterone	Fluka	I–III
Toluene	VWR	III, IV
trans-Androsterone	Sigma-Aldrich	I–III
Trimethylpropane trimethacrylate	Sigma-Aldrich	III
α,α′-Azoisobutyronitrile	Sigma-Aldrich	III
β-Cyclodextrin	Sigma-Aldrich	III
β-Estradiol 17-(β-D-glucuronide) sodium salt	Sigma-Aldrich	I

4.2. Sampling

4.2.1. Wastewater samples

Wastewater samples were mainly collected from the WWTP in Viikinmäki, Helsinki, but also from other cities during the survey study in Paper II (Table 5). Twenty-four-hour flow-proportional composite sampling was utilized for the actual samples but grab sampling was applied when only the wastewater matrix was required for method development purposes. Samples were collected mainly in high-density polyethylene containers but glass amber bottles were used in Paper I.

Table 5 Wastewater sampling sites during the survey study (Paper II)

WWTP	Population served	Indrustrial wastewater (%)	Recipient	Sampling date	Flow during sampling (m^3 d^{-1})
Kajaani	33 000	0	River Kajaani	24.3.2014	9 600
Uusikaupunki	25 000	15	Baltic Sea (Gulf of Bothnia)	26.3.2014	7 600
Helsinki	800 000	8	Baltic Sea (Gulf of Finland)	1.4.2014	286 000
Espoo	320 000	8	Baltic Sea (Gulf of Finland)	10.4.2014	92 000
Joensuu	75 000	15	River Pielisjoki	25.3.2014	19 800
Kouvola	70 000	8	River Kymijoki	25.3.2014	18 000
Mikkeli	43 000	5	Lake Saimaa	25.3.2014	11 300
Porvoo	50 000	3	Baltic Sea (Gulf of Finland)	18.3.2014	12 300
Pori	115 000	8	River Kokemäenjoki	19.3.2014	33 700
Turku	275 000	7	Baltic Sea (Gulf of Finland)	24.3.2014	102 000

Samples were taken from the wastewater coming to the WWTP (influent), from the wastewater after the purification (effluent), and some samples were taken before the biological filter (biofilter) in order to evaluate its effect on the purification process. These sampling sites are illustrated in Figure 4 for the WWTP in Helsinki.

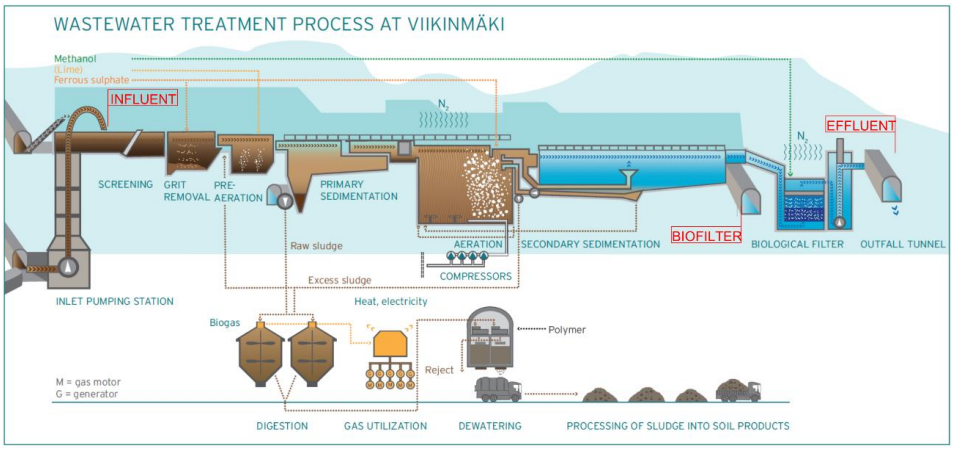


Figure 4 Sampling sites in the Viikinmäki WWTP. (Adapted from www.hsy.fi/ en/experts/water-services/wastewater-treatment- plants/viikinmaki/Pages/ default.aspx)

Wastewater was filtered directly after sampling with a Whatman glass microfiber filter (1.2 µm) and finally with a Millipore membrane filter (0.45 µm). Filtered liquid phase was stored in the fridge (+4 °C) and extracted within 48 h. Solid material collected on the filters as well as some sludge samples collected from Viikinmäki (Paper II) were oven dried (+45 °C) and weighed before extraction.

4.2.2. Aerosol samples

Aerosol samples for Paper IV were collected in Welgegund measuring station, South Africa. Twenty-four-hour sampling was done once a week from April 2011 to April 2012 with a Dekati PM_{10} cascade impactor at a flow rate of 30 L min^{-1}. Size fractions 2.5–10 µm (PM_{10}) and 1.0–2.5 µm ($PM_{2.5}$) were collected on a 25 mm quartz filter and the particles under 1µm (PM_1) were collected on a 47 mm back filter. After sampling, the filters were placed in petri dishes, covered with parafilm and stored in freezer until extraction.

4.3. Sample preparation

4.3.1. Wastewater samples: solid phase

For the extraction of analytes from suspended wastewater particles and sewage sludge ultrasound-assisted extraction was used in both dynamic (Paper I) and static (Paper II) operating modes. Sonifier tip was used for the extraction in Paper I but the sonication bath was favored during Paper II because it allowed for the simultaneous extraction of multiple samples.

In the static extraction, 50 mg of homogenized sludge or the dried filter papers were placed in test tubes with acetonitrile. The test tubes were placed in the sonication bath and extracted for 60 min. The test tubes were then centrifuged, supernatant removed and the extraction procedure repeated. Supernatants were finally combined and their volume adjusted to 6 mL.

In the dynamic extraction, the dried filter papers were placed in an extraction chamber (PEEK cylinder with 5 cm length and 7.5 mm i.d) and methanol was pumped through for 20 min at a flow rate of 0.5 mL min^{-1}. During extraction, the chamber was immersed in a

water bath and a sonifier tip (15 mm diameter) was placed just above the chamber. The final extract was evaporated to 0.5 mL and diluted with water to 100 mL before it was subjected to the SPE procedure for the isolation of free and conjugated steroids (Paper I).

4.3.2. Wastewater samples: liquid phase

Three different modes of SPE was utilized during the work. Single samples were extracted with vacuum driven SPE (Paper I), multiple parallel samples were extracted with pump driven SPE (Paper II) and dispersive SPE was utilized to evaluate the performance of different adsorbents (Paper III).

In Paper I, a sequential elution was used to isolate free and conjugated steroid fractions in wastewater samples. After conditioning the ODS-adsorbent with methanol and water, 1 L wastewater samples and diluted extracts of the suspended solids were loaded at a flow rate of 10 mL min^{-1} with vacuum. Free steroids were then eluted with 3 mL ethyl acetate followed by the elution of conjugated steroids with 3 mL methanol.

A more comprehensive set of samples was studied in Paper II, which required more automated extraction methodologies. Peristaltic pump was utilized to extract three parallel samples simultaneously. SPE cartridges were attached to the pump tubing in such a way that the sample flow through the cartridge was reversed. In Paper II, ODS-adsorbent was replaced with the more generic Strata-X-adsorbent in order to retain both steroids and polar pharmaceuticals. After conditioning the Strata-X adsorbent with methanol and water, 1 L samples were pumped through the cartridges at a flow rate of 8 mL min^{-1}. After sample loading, the tubing was removed and the cartridges were vacuum dried in the SPE-manifold before elution with 6 mL methanol.

In Paper III, the performance of several synthetic and commercial adsorbents was evaluated and compared. In order to avoid problems arising from the unrepeatable packing of adsorbents into cartridges, a simpler approach was utilized. In dispersive SPE, 500 mL of filtered effluent was spiked with 50 ng of target compounds and then magnetically stirred for 60 min in the presence of 100 mg adsorbent. After extraction, the adsorbent was filtered out, dried, and finally the compounds were eluted with 5 mL methanol via vacuum. These extracts were then evaporated, derivatized and injected to GC×GC–TOFMS. A reversed version of dispersive SPE was used to optimize the synthesis of the sorbents. 10 mg of adsorbent was measured in a sample vial and 1 mL of pure water was added spiked with various concentrations of target steroids. Vials were vortexed for 60 min and then centrifuged. An aliquot of the supernatant was injected to LC–MS and the decrease of analyte concentration was measured in order to evaluate the affinity of steroids towards the adsorbent.

Hydrolysis of the conjugated steroids was studied in Paper I. The isolated steroid conjugate fraction was first evaporated and then reconstituted in 2 mL of 2 mol L^{-1} hydrochloric acid. The solution was refluxed for 30 min and then neutralized with 1 mol L^{-1} sodium hydroxide and diluted to 50 mL before performing a solvent exchange to ethyl acetate with SPE.

4.3.3. Florisil clean-up of the wastewater extracts

In Paper I, the extracts were first evaporated to dryness and reconstituted in hexane:dichloromethane (3:1, v:v). They were then loaded into Florisil cartridges that had

been conditioned with hexane, and the analytes were finally eluted with 5 mL dichloromethane (5% acetone). In Paper II, the volume of acetone in the elution solvent was increased to 10%, and therefore only 2 mL of solvent was required for sufficient elution recovery.

4.3.4. Aerosol samples

Dynamic ultrasound-assisted extraction was utilized also for the aerosol samples (Paper IV). The filter papers were fitted in the extraction chamber in sonication bath. Methanol:acetone (1:1, v:v) mixture was pumped through for 40 min at a flow rate of 1 mL min^{-1}. The extracts were then evaporated with nitrogen flow and finally reconstituted in 5 mL methanol. Few drops of toluene was added to the extracts before evaporation in order to prevent loss of the more volatile compounds.

4.3.5. Synthesis of molecularly imprinted polymers

Several reagents were tested in order to synthetize a water-compatible polymer, whose affinity towards steroids could be improved by imprinting with a suitable template molecule (Paper III). In the optimized method, testosterone (template, 0.5 mmol) was first dissolved in methanol (porogen, 6 mL) and mixed with acrylamide (functional monomer, 4.0 mmol). Ethylene glycol methacrylate (cross-linker, 12.5 mmol) was then added with α,α′-azoisobutyronitrile (initiator, 0.3 mmol). The mixture was purged with nitrogen gas for 5 min in a test tube, which was then sealed. Polymerization was carried out in a heating oven (60 °C) for 24 hours. After polymerization was completed, the test tube was crushed and the polymer was ground to powder and wet-sieved to particle size 50–100 μm. Finally, the template was extracted from the molecularly imprinted polymer (MIP) by soxhlet-extraction (24h) first with methanol:acetic acid (9:1, v:v) and then with methanol.

4.3.6. Synthesis of β-cyclodextrin–epichlorohydrin polymers

Another synthetic adsorbent studied in Paper III was an entrapped β-cyclodextrin–epichlorohydrin polymer. The optimized procedure started by dissolving 2.6 g of sodium hydroxide in 7.5 mL Direct-Q water. 2.5g of β-cyclodextrin was added and dissolved with vigorous stirring in 50 °C. When the solution was clear, 7.0 mL of epichlorohydrin was slowly added resulting in molar ratios of 1:30:40 (βCD:NaOH:EPI). The stirring and heating was continued for 5 hours. After polymerization, 20 mL of acetone was added and the solution was cooled down. The mixture was poured into a large quantity of Direct-Q water and vacuum filtered. The resulting gel/crystals was purified by soxhlet extraction with acetone for 18 hours and dried in a heating oven for 2 hours in 45 °C. The resulting white powder was grinded and further purified by soxhlet with Direct-Q water (5 h) to remove residual NaOH followed by soxhlet with acetone (18 h). The final product was then dried, grinded and dry-sieved to particle size 50–100 μm.

4.3.7. Derivatization for gas chromatographic analysis

Derivatization of the analytes was required before subjecting samples to gas chromatographic analysis. In Paper I, silylation was performed by adding 5 μL N,O-bis(trimethylsilyl)-trifluoroacetamide containing 1% trimethylchlorosilane and 1 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 μL^{-1}) was added as internal standard for the injection. In Papers II and III the amounts of the silylation reagent and pyridine were doubled in order to increase repeatability of derivatization. In Paper IV,

silylation was done in 35 °C in sonication bath 40 min. Aerosol samples were analyzed also without pyridine and underivatized.

4.4. Instrumentation

The instrumental conditions used in the study are listed in Tables 6 and 7.

Table 6 Experimental parameters for GC×GC–TOFMS.

Parameter	Details	Paper
Capillary 1	2.5 m × 0.53 mm retention gap	I–IV
Capillary 2	30 m × 0.25 mm × 0.25 μm (BGB-5MS)	I–III
	30 m × 0.25 mm × 0.25 μm (HP-1)	IV
Capillary 3	1.0 m × 0.10 mm × 0.10 μm (DB-17)	I–IV
Temperature gradient of 1st oven	150 °C (2 min) –$^{4.2\ min}$→ 255 °C –$^{10\ min}$→ 275 °C –$^{2\ min}$→ 285 °C (5 min)	I
	30 °C (1 min) –$^{22\ min}$→ 250 °C –$^{7\ min}$→ 285 °C (6 min)	II, III
	70 °C (2 min) –$^{42\ min}$→ 280 °C (5 min)	IV
2nd oven offset	+5 °C	I–IV
Modulation time	5 s	I, IV
	4 s	II, III
Modulation offset	+15 °C	I–IV
Injection volume	1 μL	I–IV
Carrier gas	Helium, 1.3 mL/min	I–IV
Injector	280 °C	I–IV
Transfer line	290 °C	I–IV
Ion source	200 °C	I–IV
Ionization	Electron ionization, 70 eV	I–IV
Mass range	50–600 amu	I
	50–700 amu	II, III
	50–450 amu	IV

Table 7 Experimental parameters for LC–MS (Paper III).

Parameter	Details	
Column	2.0 mm × 150 mm × 3 µm C18 (Luna)	
Eluents	A: direct-Q water ; B: methanol	
Gradient of B	5% (1 min) $-^{2\ min}\rightarrow$ 80% (5 min) → 95% (7 min) → 5% (8 min)	
Injection volume	5 µL (ESI-); 1 µL (ESI+)	
Flow rate	0.25 mL/min	
Capillary voltage	4500 V	
End-plate offset	500 V	
Nebulizer pressure	50 psi	
Drying gas	Nitrogen, 9 L/min, 350 °C	
Detection mode	SIM	
Ionization and target ions	Androstenedione	287 (ESI+)
	Progesterone	315 (ESI+)
	Testosterone	289 (ESI+)
	Estrone	269 (ESI–)
	17β-Estradiol	271 (ESI–)
	Estriol	287 (ESI–)
	17α-Ethynylestradiol	295 (ESI–)

4.5. Processing of GC×GC–TOFMS data

4.5.1. Automated peak defining

The first step in data processing was the automated peak defining from two-dimensional chromatograms by LECO ChromaTOF -software. The mass spectra of the peaks (S/N > 10) were matched with NIST2005 -mass spectral database and their retention times converted to Kováts retention indices based on the experimental retention times of linear hydrocarbons (C8–C30). Polycyclic aromatic hydrocarbons, silylated polyols and carboxylic acids were also utilized to calculate retention indices in Paper IV.

Guineu –software (Castillo et al. 2011) was then used for the alignment of peaks from separate and parallel samples and their automated comparison to GOLM -mass spectral database. Guineu was also utilized to compare retention indices to theoretical indices in GOLM and NIST databases.

4.5.2. Application of statistical criteria for tentative identification

The automated peak defining generates tables with over 10 000 peaks per sample. Therefore strict selection criteria must be utilized in order to tentatively identify actual compounds with reasonable reliability. These methods included the subtraction of blank samples, removal of duplicates and removal of compounds not found in all successive injections of a sample. Additional criteria for identification were sufficient similarity to library spectra (> 700 in Papers I, II, IV; > 600 in Paper III) and low deviation of measured retention indices

from theoretical ones (< 150 in Paper IV; ±200 in Papers I, II). Several statistical criteria were also applied for the identification of compounds with GOLM-library.

4.5.3. Non-targeted quantification during the studies

The quantification of non-targeted steroid compounds was accomplished with a chemometric model derived from the standards of several targeted steroids (Paper I: 6 compounds, 7 concentrations in a range 0.1–10 ng L^{-1}; Paper II: 8 compounds, 8 concentrations in a range 0.1–50 ng L^{-1}). Response factors (RF) were first calculated by dividing peak areas of the standards by the peak area of the internal standard. Response factors derived from total ion chromatograms (TIC) were then converted to ion intensities on the basis of relative abundances of individual ions in the mass spectrum according to equation 1.

$$RF_i(m/z) = RF_i \times \left(\frac{I_i m/z_{50-600}}{\sum I_i m/z_{50-600}}\right) \quad (1)$$

, where RF_i is the response factor of a compound in a certain concentration derived from TIC; $I_i m/z_{50–600}$ is the normalized detector signal intensity of a single ion in the mass spectrum (mass range 50–600 amu).

Finally, the data collected from the standard runs was built into a chemometric model on the basis of partial least squares regression equations, where the response factors (RF_i (m/z)) calculated as a function of the mass spectra were used as explanatory variables, and the concentrations of the standards were used as response variables.

Due to the large variance of compound structures in aerosol samples, semi-quantification was utilized in Paper IV. Response factors were calculated for all the tentatively identified compounds as before but the data was then normalized with the volume of the air samples (NRF). Samples were compared by calculating the summed NRF-values and the total number of compounds belonging to a specific chemical group, for example, halogenated organic compounds.

In Paper III, the selectivity of the studied adsorbents was quantified with non-targeted approaches utilizing the number of identified compounds as well as their summed response factors. First, the summed response factors of the target steroids were divided by the summed response factors of all tentatively identified compounds in the raw data (ChromaTOF processing only). After application of statistical criteria, the number of tentatively identified four-ring steroid compounds was divided by the total number of tentatively identified compounds in the samples.

5. Results and Discussion

5.1. Methodological developments

The analytical methodologies for wastewater analysis (Papers I and II) are illustrated in Figure 5. The main differences between the methods and their optimization are described in this chapter along with additional comments regarding Papers III and IV. Although, the methods were optimized with selected reference compounds, when applied to non-targeted analysis, the values of recoveries, for example, are only estimates for the whole class of analytes in question.

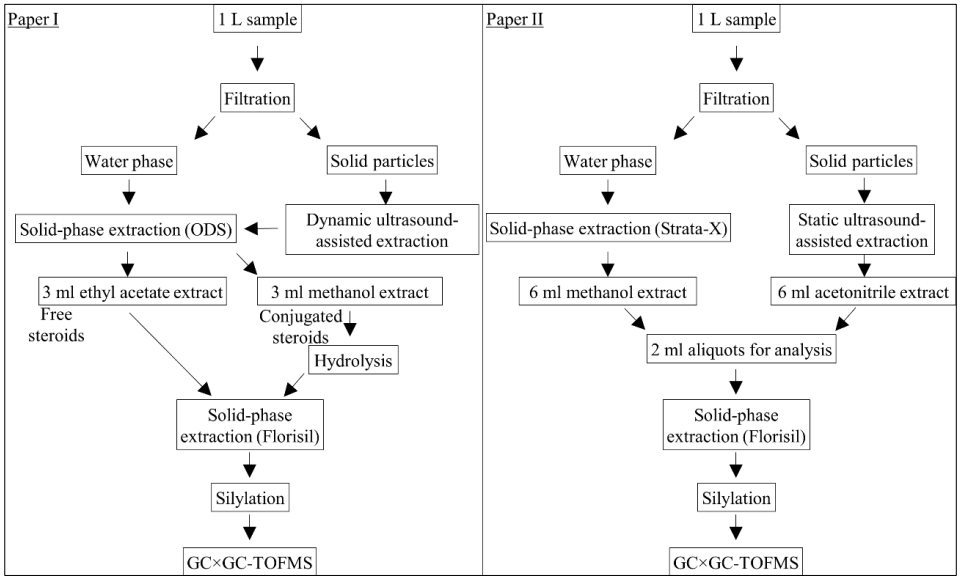


Figure 5 Developed methodologies for wastewater analysis.

5.1.1. Extraction methods for solid particles

It was noticed in the beginning of the work, that in order to avoid clogging problems during SPE, filtration was required even for the treated effluent water. Therefore it was concluded that also the filtered solid matter has to be analyzed to gain knowledge about the fate of EOCs in the municipal wastewater treatment process. Interestingly, the analysis of suspended particles of the wastewater has been somewhat neglected in the majority of previous studies (Andrási et al. 2013).

The problem with the optimization of extraction methodologies for solid matter is the difficulty of matching the analyte-matrix interactions in spiked reference material and actual environmental sample (Clement and Hao 2010). Simple spiking procedures often result in the adsorption of the analyte only onto the surface of the matrix. Therefore, the release of analytes from the matrix during extraction is not comparable between the spiked material and environmental sample where analytes are equally distributed also inside the pores of

the sample. In Paper I, spiking of the solid material was done by magnetically stirring the analytes and the grinded sewage sludge in acetone until all of the solvent had evaporated. The material was then dried, grinded and used during the optimization. After 20 min of dynamic ultrasound-assisted extraction, 90% of the spiked steroids were recovered from the matrix. Similar methodology for the aerosol samples in Paper IV was previously developed (Ruiz-Jiménez et al. 2011) by relative comparison of results from actual samples in order to find optimal parameters for extraction.

In Paper II, the number of samples and the sampling frequency was very high, so multiple samples had to be extracted simultaneously. Therefore, dynamic extraction was changed to a static version. Our earlier studies indicated that recoveries should be comparable, especially when extraction time was increased and the extraction repeated twice as done in Paper II. Another change required to cope with multiple samples was to replace the sonifier tip with a sonication bath. The comparison of these two methods was done in another study (Hopland 2015) by analyzing the amount of targeted steroids extracted in an actual sewage sludge sample. The results confirmed that the methods were comparable but also that the sonication bath was more robust. The extraction results of the sonifier tip depend heavily on the distance of the tip from the sample. With multiple samples, the variance of this distance would make the results incomparable. Methanol was also changed to acetonitrile to increase the extraction efficiency due to lower viscosity.

5.1.2. Extraction methods for liquid samples

The aim of the SPE in Paper I was to isolate free and conjugated steroid fractions. This procedure was applied also to the extracts recovered from solid particles. The methodology was adapted from literature (Isobe et al. 2003) and its performance was evaluated with selected free steroid standards and β-estradiol 17-β-D-glucuronide standard. The recovery of free steroids into the ethyl acetate fraction was very good (100 ± 5%) and the recovery of the conjugate into methanol fraction was acceptable (> 70%). Traces of the conjugate (< 5%) were also present in the ethyl acetate fraction.

The aim of Paper II was to collect several wastewater samples from multiple WWTPs in a short three weeks' time period for the non-targeted analysis of steroid compounds. Therefore, the methodology from Paper I was adapted for a higher throughput and more generic selectivity. The traditional vacuum driven SPE procedure was changed to a pump driven method, which allowed the simultaneous extraction of multiple samples. The ODS-adsorbent was changed to a hydrophilic-lipophilic balanced Strata-X material. 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 were < 15 ng L^{-1}. .

Dispersive SPE was utilized in Paper III for the comparison of synthetic and commercial adsorbents in spiked effluent sample. The proof of principle for this methodology was done by comparing the recoveries of target steroids in pure water with Strata-X adsorbent and different extraction times (30–120 min). The recoveries were low (> 30%) after 30 min

extraction but they were acceptable (> 80%) after 60 min and stayed constant when extraction time was increased further. Therefore, the mixing speed was not optimized but kept constant between magnetic stirrers to achieve good repeatability.

5.1.3. Hydrolysis of conjugated analytes

The hydrolysis of the conjugated steroids was undertaken because there are studies describing the deconjugation of the EOCs into their free forms during the wastewater treatment process or in the sewage system before the WWTP (Lishman et al. 2006, Racz and Goel 2010). If the concentration of free species can increase through deconjugation, their measurement does not give accurate estimate of the actual impact of wastewater effluents on the environment. Therefore, also conjugated species must be analyzed, or hydrolyzed to free species before analysis.

Hydrolysis of glucuronides and sulfates is often accomplished either by acidic or enzymatic hydrolysis (Gomez et al. 2014). Enzymatic treatment is considered a softer technique where acidic hydrolysis breaks most of the bonds in the sample. Due to a high price of the enzymes, acidic hydrolysis was preferred in this work. Acidic hydrolysis of the conjugated steroids in 2 M HCl solution was optimized in Paper I by monitoring the decreasing peak area of β-estradiol 17-β-D-glucuronide and the increasing peak area of the hydrolyzed free estradiol. After initial tests in sonication bath failed, different reflux times (30–120 min) were tested. The highest peak area for the free species was observed after 30 min refluxing with a yield of 80%.

Some conjugated compounds were found in Paper I, but there was a possibility that the SPE isolation was not complete and some free steroids ended up in the conjugate fraction. To test the hypothesis of hydrolysis during the wastewater treatment, possible deconjugation of compounds in influent and effluent samples was clarified in Paper II. The aim was to measure if the concentration of free compounds was increased after the hydrolysis of influent water, and also to measure if the concentrations stay constant in effluent where compounds should already be hydrolyzed after the treatment. The results did not support the hypothesis that metabolites are deconjugated during the treatment process and therefore hydrolysis was omitted from the sample preparation methodology in Paper II.

5.1.4. Clean-up and derivatization procedures

The application of ODS-adsorbent (Paper I) or Strata-X-adsorbents (Paper II) for wastewater resulted in dirty extracts, which required further clean-up, especially to remove lipophilic substances, which might interfere with the analysis. The Florisil methodology was adapted from literature (Isobe et al. 2003) and optimized further in our laboratory (Hopland 2015). It was noticed that the average recovery of steroids from the sorbent was increased from 75% to 100% by increasing the acetone percentage from 5% to 10%. The application of the Florisil purification provided a significant improvement in the chromatograms and thus cleaner spectra was available for more accurate identification of compounds.

Previously developed methodology for the aerosol samples (Ruiz-Jiménez et al. 2011) was utilized in Paper IV with sonication. The silylation procedure of steroids was developed for the study in Paper I. The volumes and ratios of the silylation reagent and pyridine were optimized so that their consumption was as low as possible in order to protect the analytical column in GC. The amount of pyridine in the optimized method was 15%. The silylation of the steroids was efficient already in room temperature but the completion of the reaction required 30 min at 60 °C. Sonication was not required for the silylation of steroids.

5.1.5. Development of instrumental methodologies

Temperature program of the GC×GC was optimized so that the resolution was highest within the retention time range of the steroid class. Because the aim of the study in Paper I was to concentrate only on the steroids, the program was started at high temperature (150 °C) and was increased quickly to 250 °C where steroids began to elute. The gradient was then slowed down to increase the separation of the steroids. In Papers II and III, a more generic gradient was applied (starting at 30 °C) to screen a wide variety of compounds. In Paper IV, a generic program was used without focusing on steroids at 250 °C.

Several stationary phases were evaluated for the separation in the second dimension and the best resolution was achieved with a mid-polar (50%-phenyl)-methylpolysiloxane column (DB-17). The separation in the first dimension is mainly based on volatility so a non-polar capillary was utilized: (5%-phenyl)-methylpolysiloxane (BGB-5MS, Papers I–III) and 100% dimethylpolysiloxane (HP-1, Paper IV). Modulation was kept as fast as possible to obtain multiple segments for each first dimension peak but making sure that all the compounds of a segment were eluted from the second dimension capillary during a modulation period.

The LC–MS method employed in Paper III was based on our previous study (Lizcano 2013) about the ionization methods for steroidal compounds, which concluded that electrospray ionization was the most suitable option for steroids. Very high ion suppression was observed, which motivated further research on the synthesis of selective adsorbents (Paper III). The chromatographic separation of estrogens was very difficult but different m/z-values enabled their analysis when using SIM-mode. Tandem MS was not required because the LC-MS method was only used for the comparison of the affinities of different polymers in pure water without matrix interferences.

5.1.6. Data processing

Careful optimization was required for the data processing methods (Papers I–IV). The most important step of the data processing method in ChromaTOF –software was the selection of optimal parameters for the reconstruction of the modulated first dimension peaks based on modulation time, compound mass spectrum and estimated peak width in the first dimension. Theoretically, in order to retain the resolution obtained in the first dimension, each peak should be modulated at least into 3 segments (Murphy et al. 1998). The data processing must then correctly combine the second dimension chromatograms in order to match the modulated first dimension peak. Target steroids were run multiple times and software parameters adjusted until correct reconstruction was observed.

Similar optimization was required for the Guineu –software, which was used to align multiple samples into one file. Alignment was based on matching mass spectra and retention times in first and second dimensions. Therefore, the parameters for the calculation of the match score required careful optimization utilizing standard compounds and adjustment of penalties given for retention time variation in the alignment macro.

The selected identification criteria were based on the minimum requirements for the identification of the standards. The optimized identification criteria were retention index range, similarity to mass spectrum libraries (NIST and GOLM) as well as additional statistical parameters utilized in the GOLM-library. Experimental Kováts indices of the steroids were calculated by comparison of retention times to those of the linear hydrocarbons (Papers I–III). Various other compound groups were also tested for the retention index method, but their results were not comparable to the estimated library indices. With linear hydrocarbons, experimental values of all steroids were, on average, 400±200 units higher than the estimated library indices, which was selected as the identification criteria for unknown steroids. Experimental indices for the compounds in the aerosol samples were calculated based on linear hydrocarbons and polycyclic aromatic hydrocarbons (for semi-volatile compounds) as well as silylated polyols and carboxylic acids (for silylated low-volatility compounds) and the identification criteria of < 150 units compared to estimated library values was chosen (Paper IV).

5.1.7. Chemometric model for non-target quantification

The performance of the chemometric quantification model for steroids was evaluated by cross validation and comparison of external compounds prediction errors (EPE) with other suitable approaches (Table 8). During cross validation, one of the steroid standards was left out of the model and its concentration was predicted with the modified model. The predicted concentration was then compared to the actual value for the calculation of EPE (%). This was done to all the model standards within the studied concentration range and the calculated average EPE was used to describe the error of the chemometric model.

The performance of the chemometric model was compared to those of the surrogate approach, where the calibration curve (TIC) of a single compound was used to evaluate the concentration of similar compounds. The third approach in the comparison was the surrogate approach utilizing only specific m/z-ratios that were common for all the steroid standards.

Table 8 External compounds prediction errors (%) with different approaches.

	Calibration curve (TIC)	Calibration curves (m/z: 53; 55; 77; 93)	Chemometric model (m/z: 50-600)
trans-Androsterone	40	50	17
Estrone	188	47	16
17β-Estradiol	123	67	14
Testosterone	60	51	15
17α-Ethynylestradiol	107	51	15
Estriol	53	70	17
Average	**95**	**56**	**16**

Table 8 illustrates the improved quantification of unknown compounds with the chemometric model. The values in the first column (TIC) represent the estimation error when the corresponding compound was used to predict the concentration of other standard steroids. The average EPE with this method was 95%. In the second column, calibration curves of specific m/z-ratios of the corresponding compound was used instead of TIC, and the average EPE was slightly improved. The values in the third column represent the EPE of the modified model when calculating the concentration of the corresponding standard. The average EPE of all the standards with the chemometric model was only 16%.

The utilization of comprehensive calibration data with statistical methods seems to improve the accuracy of the quantification of non-targeted compounds compared to other viable options. Similar methodologies could be applied to other compound groups after detailed research on the dependency of prediction accuracy on structural variation. Large structural differences between the unknowns and the compounds used in the model can affect the prediction accuracy due to variation in fragmentation and detector response in mass spectrometry.

5.2. Comparison of synthetic and commercial adsorbents (Paper III)

5.2.1 Synthesis of molecularly imprinted polymers

The problem with MIPs is often the hydrophobic nature of the material, which increases nonspecific adsorption of nonpolar compounds in aqueous samples due to hydrophobic effects (Horemans et al. 2012). These compounds can then interfere with the analysis of the selectively bound target analytes. To reduce this unwanted effect, the study focused first on the synthesis of non-imprinted polymeric adsorbents (NIP) with low affinity towards steroids (Table 9).

Table 9 Composition of the non-imprinted polymers and their adsorption capacity for the studied steroids in pure water at concentration range 1.0–9.0 ng μL^{-1} (Paper III).

NIP	Monomer 4 mmol	Cross-linker 12.5 mmol	Initiator 0.3 mmol	Porogen 6 mL	Average adsorption %
1	MAA	EGDMA	AIBN	Acetone	97
2	MAA	EGDMA	AIBN	Methanol	88
3	MAA	EGDMA	AIBN	Chloroform	99
4	MAA	EGDMA	AIBN	Toluene	95
5	HEMA	EGDMA	AIBN	Acetone	97
6	HEMA	EGDMA	AIBN	Methanol	97
7	HEMA	EGDMA	AIBN	Chloroform	97
8	HEMA	EGDMA	AIBN	Toluene	95
9	MAA / HEMA 50%	EGDMA	AIBN	Acetone	97
10	MAA / HEMA 50%	EGDMA	AIBN	Methanol	86
11	MAA / HEMA 50%	EGDMA	AIBN	Chloroform	99
12	MAA / HEMA 50%	EGDMA	AIBN	Toluene	96
13	Acrylamide	EGDMA	AIBN	Acetone	97
14	Acrylamide	EGDMA	AIBN	Methanol	**65**
15	Acrylamide	EGDMA	AIBN	Chloroform	98
16	MAA	TMPTMA	AIBN	Methanol	99
17	MAA	TMPTMA	AIBN	Chloroform	100
18	Acrylamide	TMPTMA	AIBN	Methanol	99
19	MAA	DVB	AIBN	Methanol	99
20	Acrylamide	DVB	AIBN	Methanol	97

The optimal adsorbent (NIP14) was then imprinted with a suitable template molecule. Due to solubility issues in methanol, only testosterone and estradiol were suitable candidates. The affinity of the MIP towards these compounds was then measured and compared with that of the NIP. The average adsorption of the studied steroids was increased from 57% (NIP) to 76% (MIP: estradiol) and 90% (MIP: testosterone) due to the selective binding at the imprinted active sites in the polymer cavities. The adsorption for estrogenic compounds is illustrated in Figure 6.

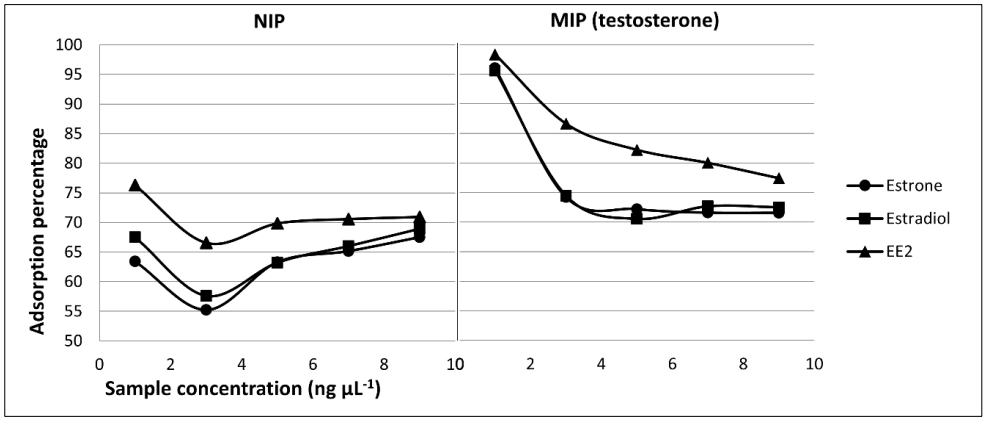


Figure 6 Comparison of adsorption percentages onto NIP and MIP materials for environmentally relevant estrogenic compounds (Paper III).

It can be seen from Figure 6 that the adsorption percentage of estrogens onto the MIP decreases as the concentration increases due to the limited capacity of the specific binding sites imprinted in the polymer. As the cavities are filled, the affinity of the MIP approaches that of the NIP.

5.2.2 Synthesis of entrapped β-cyclodextrin–epichlorohydrin polymers

The recipe for the synthesis of entrapped β-cyclodextrin–epichlorohydrin (ECD) polymers was adapted from the literature (Crini et al. 1998, Jiang et al. 2012, Moon et al. 2008) and optimized further. It was first observed that moderate heating (50 °C) was required to produce water-insoluble polymers. The volume of solvent (7.5 mL Direct-Q water) was also critical and larger volumes (> 10 mL) prevented the polymerization. Molar ratio of the constituents was also optimized to 1:30:40 (βCD:NaOH:EPI), which resulted in hard gel with large crystals after drying. Smaller quantities of EPI (1:30:30) resulted in a brittle gel, which was mostly soluble in water. The final parameter to optimize was synthesis time and it was observed that even 5 h was long enough for completion. The performance of the different batches were evaluated by measuring their affinity towards steroids in pure water with dispersive SPE. The affinity of the final polymer was equal to the commercial Strata-X adsorbent. Regarding future applications, one major problem with the polymer was that after swelling in aqueous sample, it did not retain its particulate structure during drying. The resulting clay-like monolith had to be grinded and sieved before reuse.

5.2.3. Comparison of adsorbent performance

In addition to the development of synthetic methodologies, another aim was to explore the suitability of non-targeted statistical methods for the quantification of analytical selectivity allowing the comparison of synthetic and commercial adsorbents. Definitions of semi-quantitative and semi-qualitative selectivity were proposed. Summed relative peak areas of the target steroids were divided by those of all compounds (S/N > 10) in the chromatogram. This value represented the semi-quantitative selectivity of an adsorbent, which describes the intensity of target compounds compared to other constituents in the chromatogram. After

applying several identification criteria, the number of tentatively identified steroidal compounds was divided by the number of all tentatively identified compounds in a sample. This value represented the semi-qualitative selectivity, which describes how many compounds were extracted alongside the steroid compound class.

Other measures of selectivity in Paper III were the matrix removal potential and the recovery of target compounds from the adsorbents. Matrix removal potential was evaluated by measuring the ion suppression (ESI–LC–MS) of target compounds spiked in effluent samples after extraction with the studied adsorbents. Recovery was calculated from the effluent samples spiked before extraction (GC×GC–TOFMS). These results are listed in Table 10.

Table 10 Properties of the adsorbents in wastewater effluent samples (Paper III).

	Strata-X	**AffiniMIP**	**NIP14**	**MIP**	**ECD**
[a]Semi-quantitative selectivity	14.7	13.7	7.1	9.1	3.1
[b]Semi-qualitative selectivity	1.5	2.9	3.9	3.7	4.4
[c]Recovery %	300	5	18	67	40
[d]Matrix removal potential (Ionization suppression %)	-80	-45	-10	-15	-29

a) The summed response factors of the spiked target steroids divided by the summed response factors of all tentatively identified compounds in the raw data ($\times 10^5$).

b) The number of tentatively identified four-ring steroid compounds divided by the total number of tentatively identified compounds in the processed data (%).

c) Average recovery of the spiked steroids (100 ng L^{-1}) in effluent samples (500 mL) with different sorbents (100 mg).

d) The suppression factors of the studied steroids (0.5 ng μL^{-1}) spiked in effluent samples after extraction (500 mL to 0.5 mL) with different sorbents (100 mg).

The recovery of spiked steroids with Strata-X was very high indicating positive ion suppression, which also explains the measured high semi-quantitative selectivity. On the other hand, high semi-quantitative selectivity was measured for the AffiniMIP -material because the adsorption of both steroids and other compounds was very low. In this way, semi-quantitative selectivity was closely related to recovery and cannot be utilized for comparison of adsorbents if large variation in affinities is expected

Semi-qualitative selectivity seems to be a more reasonable parameter for the comparison of adsorbent selectivity. The synthetized polymers (ECD and MIP) had relatively high affinity towards steroids and good semi-qualitative selectivity. Strata-X, on the other hand, had the lowest semi-qualitative selectivity due to low identification certainty caused by the complexity of the extract (Figure 7). The generic nature of Strata-X was also evident in the high matrix induced ion suppression measured in the spiked extract. AffiniMIP -adsorbent was advertised to be selective only for estrogens but it had similar affinity also towards androgens. All of the synthetized polymers performed better than this commercial MIP but the total affinity was lower in comparison with Strata-X.

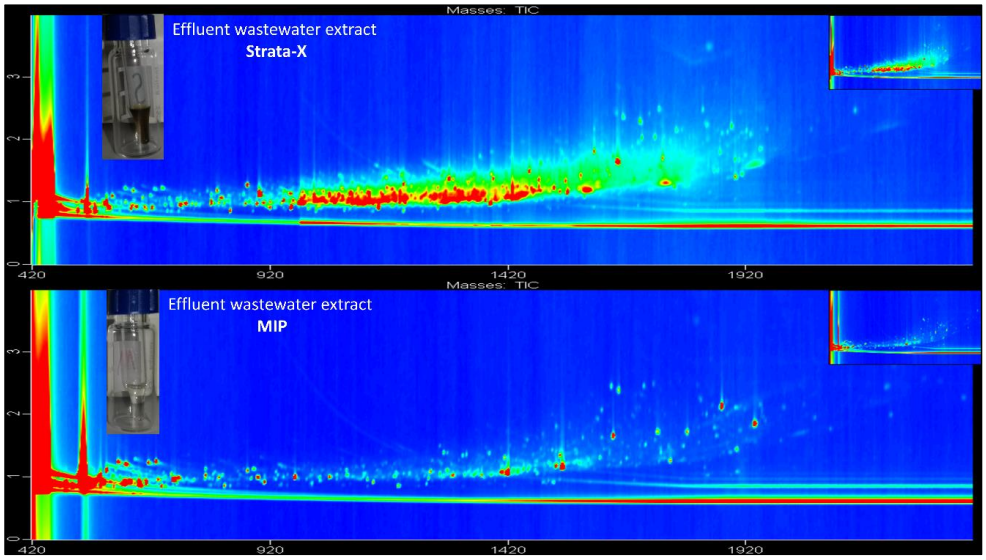


Figure 7 GC×GC–TOFMS contour plots of the spiked effluent samples extracted with the MIP and Strata-X adsorbents.

5.3. Comparison of environmental concentrations of the studied compounds

In Paper II, non-targeted approach was utilized to compare the purification efficiency of several WWTPs as well as to evaluate the fate of steroidal compounds during the treatment process. Raw data was first lightly processed (chapter 4.5.2.) and utilized to compare the number of tentatively identified compounds in different samples. These results are illustrated in Figure 8.

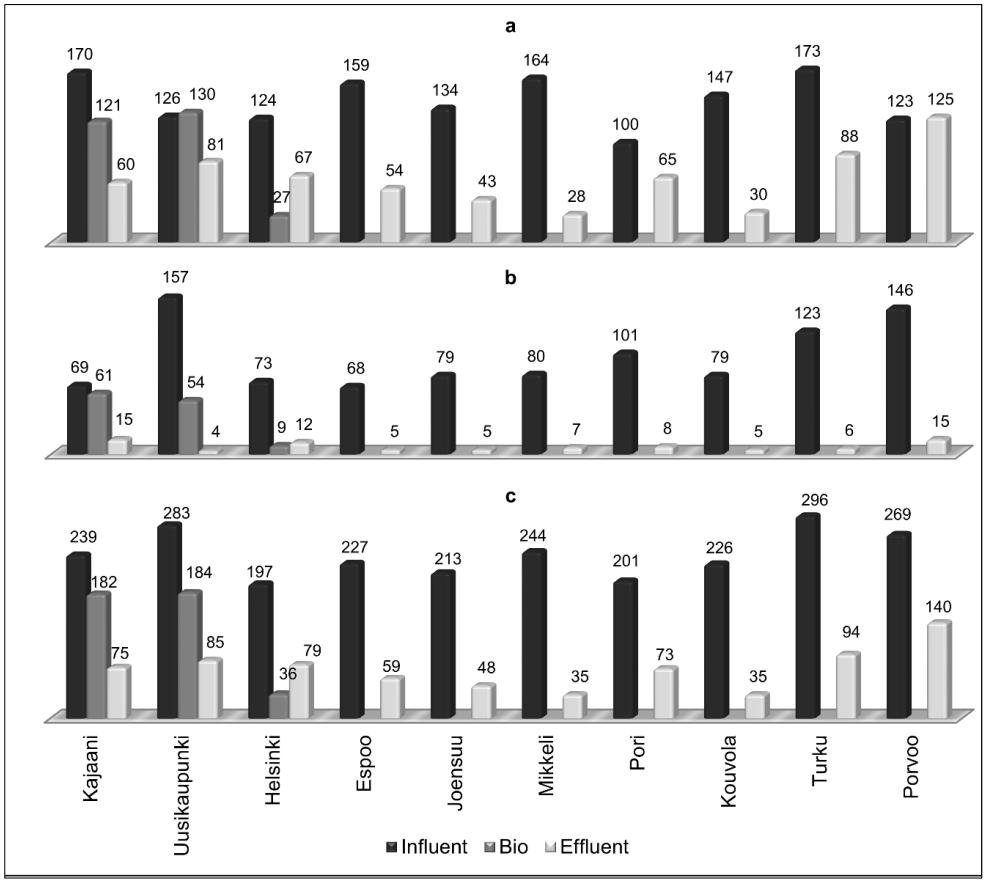


Figure 8 Average number (n = 9) of tentatively identified peaks in influent water, water before biological filtration, and effluent water of the WWTPs (library match > 600) analyzed separately from aqueous phase (a) and suspended solids (b). The summation of these concentrations (c) represents the total number of compounds in wastewater. (Paper II).

Several conclusions can be drawn from this sort of profiling data. Surprisingly small variation was observed in the number of compounds found in the suspended solids. However, when taking into account the combined results of liquid and solid samples, some differences in the purification percentage can be seen. In Kajaani and Uusikaupunki, a biological filter replaces the traditional biological purification process, while in Helsinki a biofilter process is additional to chemical and biological purification. Thus, in Helsinki, the water is cleaner before entering the biofilter. The increased number of compounds found in Helsinki after the biofilter stage suggests that some new compounds may have formed or been released during the biofilm process. The same compounds may have been released in Kajaani and Uusikaupunki as well, since the number of compounds in the effluent water is similar in the three WWTPs with a biological filtration system.

5.3.1. Fate of steroids during wastewater treatment

The collected data was processed further to increase the reliability of tentative identification for steroidal compounds and to propose possible pathways for the found transformation products. Structures of the tentatively identified cholestanes, pregnanes and estranes are illustrated in Figure 9 and the transformation products of androstanes in Figure 10. However, 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.

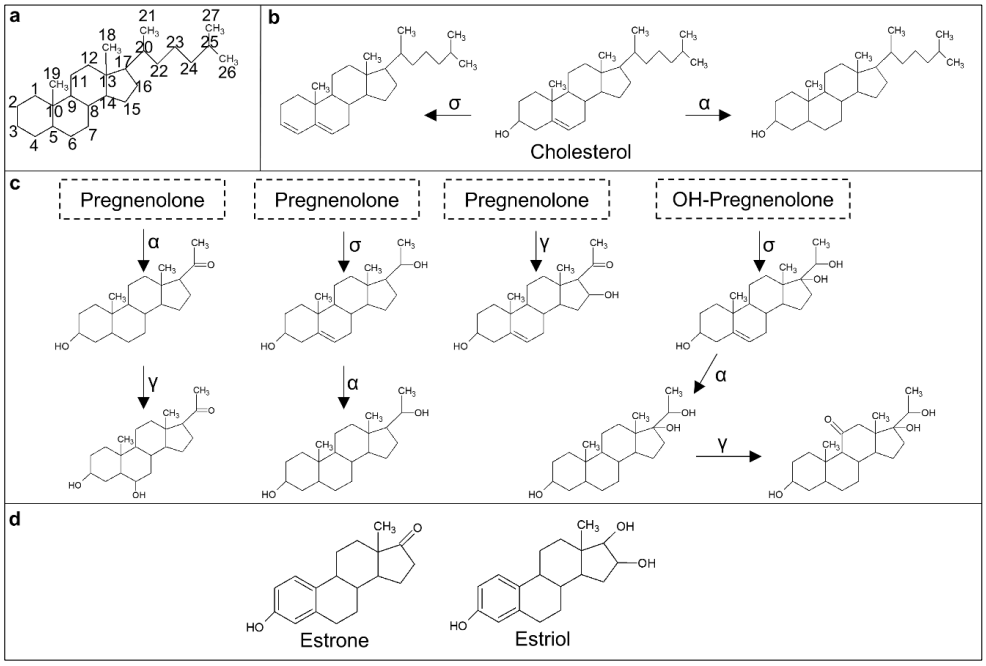


Figure 9 The numbering of the steroid skeleton carbon atoms (a) and the structures of the tentatively identified cholestanes (b), pregnanes (c) and estranes (d). α = hydrogenation; γ = oxidation; σ = reduction. (Paper II)

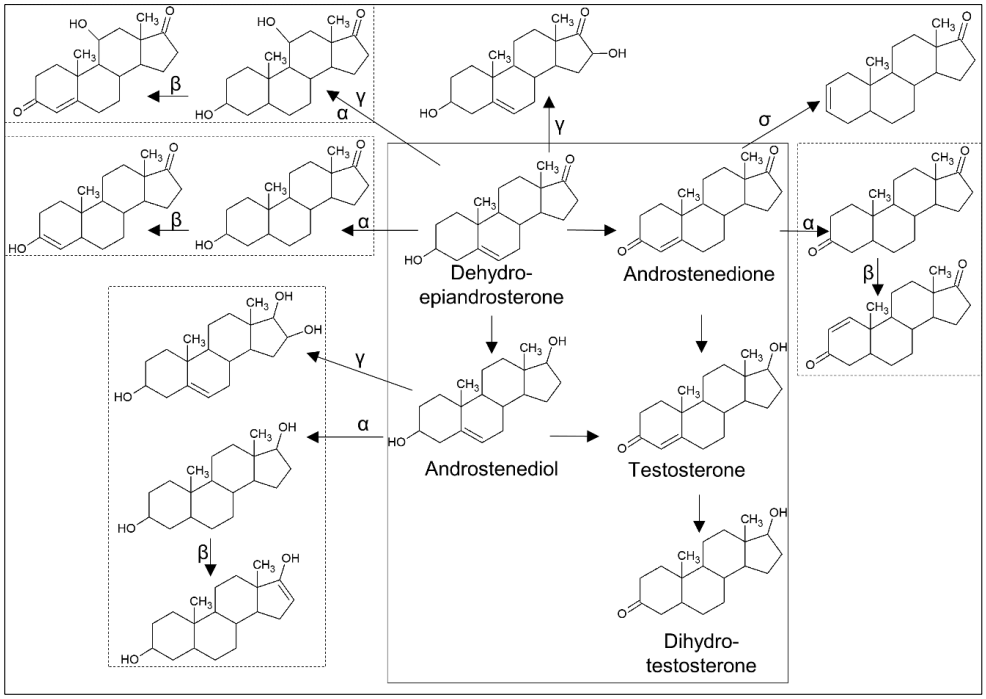
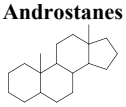
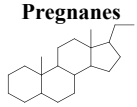
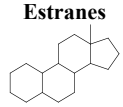
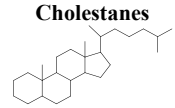


Figure 10 The structures of the tentatively identified androgens, including anthropogenic steroids (in the middle) and their proposed transformation products. α = hydrogenation; β = dehydrogenation; γ = oxidation; σ = reduction. (Paper II)

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 CRMs. 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 (C3 and C17–C20) > identity of substituents at other locations > number and location of double bonds > stereoisomerism. The concentrations of steroidal compounds was calculated with the developed chemometric model and then classified according to their steroid skeleton. The results from Papers I and II are collected in Table 11. 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.

Table 11 Total concentrations (ng L^{-1}) of different steroid classes in the filtered water (aq.) and suspended solids (s.) of the influent and effluent samples (Papers I and II).

	Androstanes 				**Pregnanes** 				**Estranes** 				**Cholestanes** 			
	Influent		**Effluent**		**Influent**		**Effluent**		**Influent**		**Effluent**		**Influent**		**Effluent**	
	aq.	**s.**	**aq.**	**s.**	**aq.**	**s.**	**aq.**	**s.**	**aq.**	**s.**	**aq.**	**s.**	**aq.**	**s.**	**aq.**	**s.**
Helsinki 11.01.2012	1080	250	30	5	500	95	-	15	55	45	-	25	110	1190	110	150
Helsinki 31.01.2012	1940	190	155	55	345	85	10	5	85	60	10	-	55	1215	40	105
Helsinki 01.04.2014	5785	36	-	-	3995	-	-	-	625	-	-	-	370	170	-	620
Kajaani 24.03.2014	8800	60	-	-	1560	210	-	-	-	-	-	-	-	-	-	615
Uusikaupunki 26.03.2014	2730	25	-	-	1125	-	-	-	370	-	-	-	-	1630	-	190
Espoo 10.04.2014	23430	-	620	-	3015	70	-	-	480	-	110	-	-	4950	-	475
Joensuu 25.03.2014	18040	20	-	-	2765	55	-	-	-	-	-	-	-	-	-	-
Mikkeli 25.03.2014	19280	70	-	-	3710	130	-	-	685	-	-	-	-	-	-	135
Pori 19.03.2014	4970	145	-	-	335	665	-	-	380	-	-	-	-	-	-	-
Kouvola 25.03.2014	14350	-	-	-	3540	60	-	-	-	-	-	-	-	100	-	-
Turku 24.03.2014	13040	100	-	-	4550	415	-	-	540	-	-	-	-	2800	-	-
Porvoo 18.03.2014	30470	100	-	-	-	225	-	-	-	-	-	-	1840	1535	-	-

The most abundant class of steroids in the aqueous phase were androstanes followed by pregnanes. Cholestanes were mainly found in suspended solids due to their high hydrophobicity. The lowest concentrations were found for estrogens. However, because they are the most hormonally active of all the steroids (Kozlowska-Tylingo et al. 2010), even small concentrations can have an impact on aquatic ecosystems. Relatively high concentrations of steroids were occasionally found in the suspended particles of the effluent samples. The average mass of solids was reduced from 180 mg L^{-1} in influent to 10 mg L^{-1} in effluent but the concentration of steroids was not decreased in the same proportion. This enrichment on solid particles can be explained by the hydrophobic nature of steroids and

their adsorption to solid particles during the treatment process depending on the solids retention time of a WWTP. Another explanation is the different average particle size of suspended solids in influent and effluent water. Because of the smaller particle size, a mass of solid material in the effluent has larger surface area for adsorption than the same mass in the influent when comparable pore size is assumed. To quantify the differences in particle size, we carried out studies by field-flow fractionation (FFF). The results confirmed that the average diameter of the effluent particles (25 nm) was smaller than the average diameter of the influent particles (145 nm).

The removal percentage of steroids during the treatment process was very high due to their adsorption onto solid material during the activated sludge process. 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). These results are in good agreement with the recent literature on steroid removal in WWTPs. Many studies conclude that the adsorption of EOCs onto 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 (Evgenidou et al. 2015, Hamid and Eskicioglu 2012). Collection into sludge was confirmed with the results received from the analysis of sludge samples taken from Helsinki WWTP as well as from composted soil products processed from the sludge. Several steroidal compounds were tentatively identified in the samples. Most of these had the cholestane structure, but also several androstanes and pregnanes (n = 7) were identified. The concentration of steroidal compounds were reduced by 70 to 100% during the composting of the dried sludge, but still concentration levels of mg kg^{-1} were found in the soil products after processing. Considering that the composted sludge is reused for agricultural purposes, these values are of concern. The predicted no-effect concentration for estrogens in soil, for example, has been estimated to be in the range of 1-700 μg kg^{-1} (Martín et al. 2012). It has been previously 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.

5.3.3. Organic composition of aerosol particles (Paper IV)

The organic composition of aerosol particles was studied in Paper IV. Over 1000 compounds were tentatively identified and semi-quantified in three different size fractions of aerosols (PM_1, $PM_{2.5}$ and PM_{10}) collected on quartz filters. The number of compounds as well as their division into chemical groups was relatively constant between the aerosol size fractions. The average composition of an aerosol sample is illustrated in Figure 11.

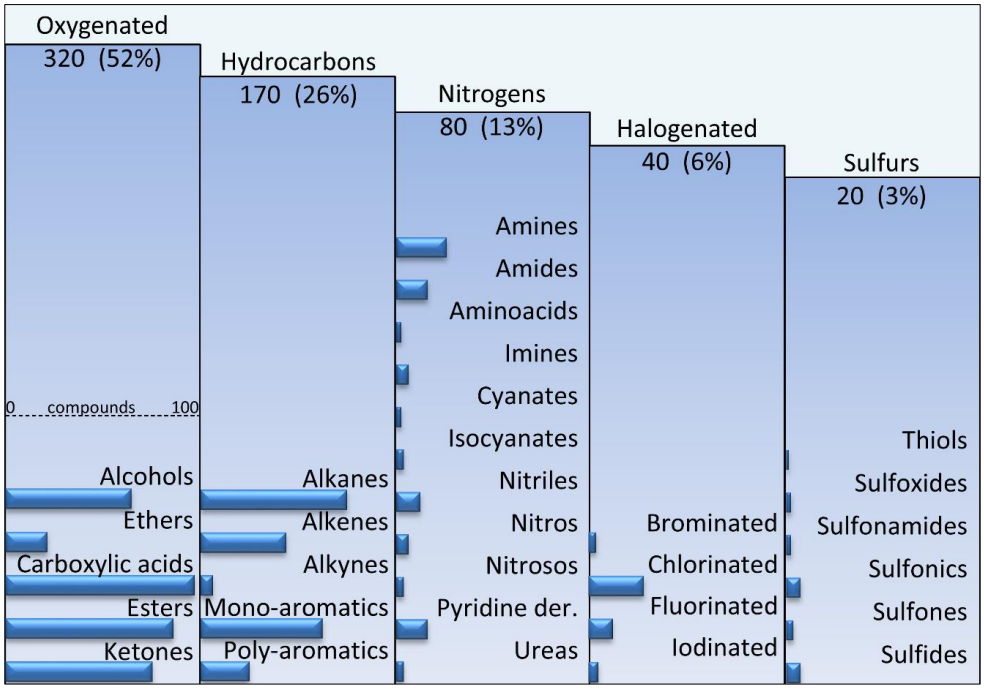


Figure 11 Average number of tentatively identified compounds in the aerosol samples with classification based on chemical functionalities (Paper IV).

Oxygenated compounds and hydrocarbons were the main components of the aerosols with 320 and 170 tentatively identified compounds, respectively. Most of the oxygenated species were carboxylic acids and esters, and the main components of the hydrocarbon group were alkanes and mono-aromatic compounds. Several nitrogen and sulfur containing compounds were identified as well as halogenated compounds with a majority of chlorinated compounds.

The concentration of compounds was semi-quantified by normalizing the data with the internal standard. When the normalized response factors (∑NRF) of all tentatively identified compounds were summed, some variation was observed between the size fractions of the aerosols (Figure 12).

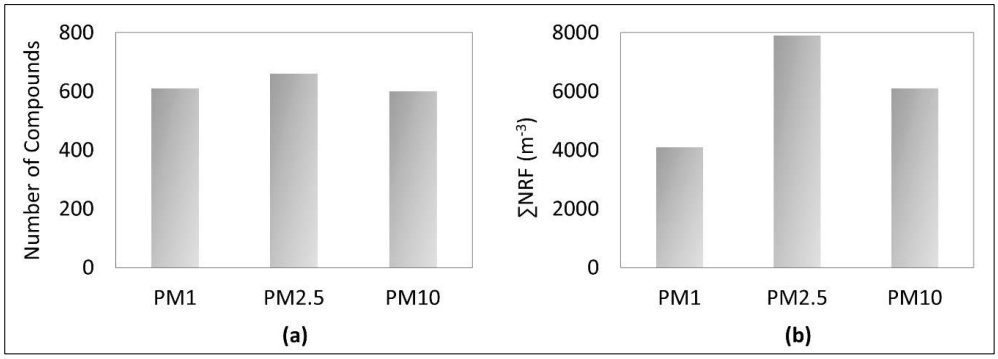


Figure 12 Total number of tentatively identified compounds (a) and the summation of the normalized response factors ∑NRF (b) in each size fraction of the aerosol samples.

Differences were also observed in the ratio of different functionalities when semi-quantitative data was compared to qualitative data (Figure 11) collected from all the samples. Oxygenated compounds were still the largest fraction (55%) followed by hydrocarbons (16%) and halogenated compounds (16%), nitrogen containing compounds (12%) and compounds with sulfur (1%). The ratio of halogenated compounds was increased and the ratio of hydrocarbons was decreased in the ∑NRF data. This means that the mass of halogenated compounds is divided between a few different compounds whereas the hydrocarbon fraction is more diverse. The majority of oxygenated species can be explained by the location of the sampling site in Welgegund, which is affected by aged air masses from different anthropogenic source regions.

6. Conclusions

The main goal of this thesis was to employ novel methods for the utilization of non-targeted approaches. The developed chemometric model for the quantification of tentatively identified compounds with steroidal structure demonstrated improved accuracy (prediction error < 16%) compared to other approaches like quantification with a surrogate compound. The model could be adapted also for other compound classes after consideration of required structural similarity within the studied class and the standards utilized in the model development; the accuracy of the model is dependent on similarities in mass spectral fragmentation and detector response.

The fate of steroidal species in wastewater was evaluated with non-targeted methodologies. Efficient elimination (~100%) was observed during municipal treatment process through hydrophobic adsorption into sludge and degradation into transformation products. However, some of the suspended solid particles remained in the effluent water, which increases the possibility of steroid flux through the treatment plants via these solid carriers. The elimination of steroids from sludge was less efficient and the reuse of composted sludge in agriculture proposes a risk of their environmental accumulation. Furthermore, due to increased method detection limits of non-targeted methods, trace amounts of steroids can possibly be detected from effluent samples with targeted analysis.

Novel non-targeted approach to quantify adsorbent selectivity was proposed. The comparison of the number and relative amounts of steroidal compounds and other tentatively identified sample components enabled arithmetic evaluation of selectivity. Synthetized water-compatible molecularly imprinted polymers demonstrated higher selectivity towards steroids but further improvement of overall affinity is required for their efficient application into wastewater analysis.

The utilization of non-targeted cross-sample analysis with peak features enabled fast comparison of the purification efficiency of several wastewater treatment plants. Similar methodology was also utilized for the evaluation of organic compound composition of atmospheric aerosols in different size fractions.

The proposed non-targeted approaches can be utilized for fast evaluation of environmental samples by allowing the identification of possible emerging organic contaminants more comprehensively than is possible with conventional targeted methods. The reliability of tentative identification can be further improved by utilizing modern high resolution mass analyzers and development of more efficient statistical tools for cross-sample analysis.

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