Master's Thesis

Simultaneous determination of five antibiotics in urban wastewater using SPE–HPLC–MS/MS

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ABSTRACT

The detection of antibiotics in wastewater is a challenge due low concentration and complex matrix. In this work the method with solid phase extraction, liquid chromatography and tandem mass spectrometry was developed to analyze five antibiotics simultaneously in wastewaters. The method was applied in practice by measuring concentration of antibiotics in three Finnish medium size WWTPs.

Complex matrix can suppress the signal of antibiotics in mass spectrometry and this was taken into account by using matrix matched external standard. The developed method was simple, fast and repeatable and the limit of detections range from <10–150 ng/L. Three of five antibiotics (trimethoprim, sulfamethoxazole and ciprofloxacin) were detected in all influents. Doxycycline and norfloxacin were not detected neither influents nor effluents. In influent ciprofloxacin had the highest concentration: 300 ng/L. The concentrations were similar to published studies. Trimethoprim was the only one detected in effluents, and the concentrations were higher in all effluents than in influents. This may be because slightly transformed metabolites can be transformed back to parent compounds during the treatment process. As there are large amount of different antimicrobials in wastewaters and all of them cannot be detected at the same time, further investigate is needed to find the indicate compounds that can be detected simultaneously and assess the risk of antimicrobial resistance.

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Kosunen Päivi:	Viiden antibiootin yhtäaikainen määrittäminen urbaanista jätevedestä SPE–HPLC–MS/MS -menetelmää käyttäen
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TIIVISTELMÄ

Antibioottien havaitseminen jätevesistä on haastavaa johtuen matalista pitoisuuksista ja monimutkaisesta matriisista. Tässä työssä kehiteltiin kiinteäfaasiuuttoa, nestekromatografia ja tandem massaspektrometriä hyväksi käyttävä menetelmä, jolla voitiin analysoida viittä antibioottia yhtäaikaisesti matalina pitoisuuksina jätevesistä, ja sitä testattiin käytännössä kolmen keskisuuren suomalaisen jätevedenpuhdistamon jätevesiin.

Monimutkainen matriisi heikentää usein antibioottien signaalia massaspektrometrissä ja tässä työssä käytettiin matriisi-korjattua ulkoista standardia huomioimaan efektiä. Menetelmä oli yksinkertainen, nopea ja toistettava. Määritysrajat vaihtelivat <10–150 ng/l välillä. Viidestä tutkitusta antibiootista kolmea (trimetopriimi, sulfametoksatsoli ja siprofloksasiini) havaittiin kaikissa tutkituissa puhdistamattomissa jätevesissä. Doksisykliiniä ja norfloksasiinia ei havaittu jätevesissä lainkaan. Korkein havaittu pitoisuus oli siprofloksasiinilla, 300 ng/L. Pitoisuudet olivat samaa luokkaa kuin mitä aiemmin tutkimuksissa on havaittu. Ainoastaan trimetopriimiä havaittiin puhdistetuissa jätevesissä, ja sen pitoisuus oli korkeampi jokaisessa tutkitussa puhdistetussa jätevedessä kuin puhdistamattomassa. Tämä johtunee siitä, että vain vähän muuntuneet aineenvaihduntatuotteet voivat muuntua takaisin alkuperäisiksi lääkeaineiksi jäteveden puhdistusprosessin aikana. Mikrobilääkkeitä on suuri määrä eikä niitä kaikkia voi tutkia yhtä aikaisesti jätevesistä. Jatkotutkimuksia tarvitaankin, jotta löydetään indikaattoriyhdisteet, joita voidaan analysoida yhtäaikaisesti ja joiden avulla voidaan määritellä mikrobilääkkeiden aiheuttama resistenttien kantojen riski.

TABLE OF CONTENT

ABBREVIATIONS

ACN	Acetonitrile
CIP	Ciprofloxacin
DDD	Defined daily dose
DOX	Doxycycline
ESI	Electrospray ionization
FA	Formic acid
HAc	Acetic acid
HPLC	High performance liquid chromatography
Koc	Soil organic carbon partitioning coefficient
LOD	Limit of detection
LOQ	Limit ofication
MS/MS	Tandem mass spectrometry
MeOH	Methanol
NOR	Norfloxacin
S/N	Signal to noise -ratio
SPE	Solid phase extraction
SMX	Sulfamethoxazole
STP	Sewage treatment plant
TRI	Trimethoprim
WWTP	wastewater treatment plant

1 INTRODUCTION

1.1 Pharmaceuticals as emerging micropollutants in the environment

Pharmaceuticals in the aquatic environment have been recognized as emerging issue at 1990's (Daughton & Ternes 1999, Heberer 2002) although the very first prescription pharmaceutical in aquatic environment has been reported at 1970's (Daughton & Ternes 1999). The main sources of pharmaceutically active compounds are the effluents of the sewage treatment plants (STP): after consuming pharmaceuticals are excreted in urine and faeces as parent compounds, only slightly transformed metabolites or conjugated with inactivating compounds that may cleave back to the parent compound during wastewater treatment (Heberer 2002). Recently, a lot of researches have been carried out to study the fate of hydrophobic pharmaceuticals. Besides of biodegradation, the common removal mechanism is adsorption into the sludge. Some pharmaceuticals are highly polar compounds and they have low tendency to adsorb during the wastewater treatment processes. Typically STP's are not designed to remove pharmaceuticals and therefore these compounds are discharged into receiving waters (Ternes & Joss 2006). Pharmaceuticals are removed by the STPs only partly and there is low, but continuous flow of pharmaceuticals to the aquatic environment. The other sources of pharmaceuticals are e.g. landfills, disposal of unused medicine via drain and pharmaceuticals used in agriculture (Heberer 2002).

There are several concerns with connection to pharmaceuticals in the environment. Although these pollutants are usually found at low ng/L-concentration in the aquatic environment there has been reported both acute and chronic effect in aquatic organisms. Mixture of thousands compounds can also cause unpredictable effects (Kümmerer 2009a). Antibiotics are one of the most frequently used group of pharmaceuticals and they have been detected in the aquatic environment worldwide including wastewaters, surface water and groundwater, but also in drinking water (Seifrtová *et al.* 2009). Vieno *et al.* (2006) reported occurrence of antibiotics up to 650 ng/L in influents. They were also found in effluents up to 40 ng/L and in river waters at low ng/L-concentrations. Also Äystö *et al.* (2014) reported occurrence of antibiotics in effluents and in surface waters, usual at low ng/L-concentrations. Despite of the low concentrations, the continuous input from the wastewater treatment plants to the surface water makes them pseudo-persistent micropollutants in the environment. When antimicrobial pharmaceuticals (including antibiotics and antiviral pharmaceuticals) are present in the environment, one of the main concerns is the risk of the development and maintenance of antimicrobial resistance (Kümmerer 2009b, Jain *et al.* 2013).

1.2 Analysis of pharmaceuticals

Analysis of pharmaceuticals in liquid matrix, such as wastewater influent or effluent, may be challenging: they exist in complex matrix and usual at low concentration (ng/L–level) (Seifrtová *et al.* 2009). Most pharmaceuticals are also rather small and usually polar molecules (McArdell *et al.* 2006). Simultaneous analysis of the pharmaceuticals is a challenge since there is large variety of compounds with dissimilar properties such as polarity, solubility and partition coefficients. There occur also not only primary compounds in the environment but also transformed products, and specific standard substances are not necessarily available (Jakimska *et al.* 2014). Suitable analysis method has to be both specific and sensitive enough for the detection of the target compounds among large amount of impurities. These high demands can be fulfilled by sophisticated analytical techniques such as high performance liquid chromatography (HPLC) or gas chromatography (GC) followed by tandem mass spectrometry (MS/MS) (McArdell *et al.* 2006).

1.3 Objective

The aim of this study was to develop the fast, simple and reliable, simultaneous SPE–HPLC–MS/MS method for analysis of five (ciprofloxacin, doxycycline, norfloxacin, sulfamethoxazole and trimethoprim) antibiotics that belong in several groups. This included optimization of the solid phase extraction (SPE) method: optimum pH adjustment, optimum composition and volume of the eluent to get target compounds from SPE cartridge, and choosing the suitable filters. The optimum conditions were found by comparing the recoveries of compounds between each treatment. Also optimum MS/MS parameters: cone voltage and collision energies, were determined. The objective was to validate the method with regard to calibration, recovery, matrix effect and repeatability. The limit of detection and limit of quantification was desired to be low enough to investigate target compounds in Finnish wastewaters.

The other aim was to employ the method and analyse wastewaters to discover the concentrations of selected pharmaceuticals if they exist in the influents or effluents of medium size municipal wastewater treatment plants in Finland. The aim was to use matrix matched external standard, prepared in lake water, to define the concentrations of target compounds in wastewaters. Also the theoretical concentrations of pharmaceuticals were calculated based on their consumption in Finland and excretion portion as parent compounds. These values were compared to each other.

2 BACKGROUND

At 1990's started the discussion of pharmaceuticals in the aquatic environment and first methods for analysis of pharmaceuticals were developed. Ternes (2001) reported the methods that were used to detect many groups of pharmaceuticals, including antibiotics, in ng/L-level. These methods were used to investigate pharmaceuticals in German sewage treatment plants (STPs) in 1996–98.

The analytical procedure includes usually five steps starting from sampling and sample preparation followed by chromatographic separation, detection and data analysis (Seifrtová *et al.* 2009). Sampling and sample preparation can take 80% of time of the whole analysis process (Kataoka 2003). According to McArdell *et al.* (2006) the development and validation of the analysis method for pharmaceuticals can take months.

The sampling is a critical part of the analysis: the errors made during the sampling cannot be corrected later. Occurrence of pharmaceuticals has seasonal variations: certain pharmaceuticals are used in different season (winter–summer), also the removal rates may vary between cold winter and warm summer (McArdell *et al.* 2006). The flow is also not constant around the clock: people use toilets more at daytime than at nights. Some pharmaceuticals are originated in only a few persons. Variation of the occurrence may be difficult to predict. Integrated samples over the time, such as 24-hours composite samples, are required, when the aim is to evaluate the mass flux or loads and the series of grab samples are suitable for assessing the peak concentrations (McArdell *et al.* 2006).

2.1 Sample preparation

The samples should be injected in HPLC without any pre-treatment if it is possible, but almost always at least some preparation is required. Developing the sample preparation method can be more laborious than developing the HPLC method itself (Snyder *et al.* 1997).

Sampling and storing the samples should be arranged such way the target compounds are not transformed before analysis (McArdell *et al.* 2006). In the optimum case, the samples are prepared and analysed immediately after sampling. Aqueous samples require filtration at first as the particles may impede the later steps of the sample praparation. Also pH needs to be adjusted according to the properties of the target compounds, and it is usual essential to reach as good as possible recovery of the compound. Microbial processes (which can transform some compounds) may be inhibited by adding disinfectants or lower the pH below 2. Samples can be stored in amber glass bottles in a freezer, if they are not analysed immediately. Storing samples in amber glass bottles prevent the degradation by UV-radiation. The samples with volatile compounds or compounds, which are easily oxidized by oxygen, should be stored completely filled bottles. Sorption to the surface of the container should also be considered (McArdell *et al.* 2006).

Sampling and sample preparation together is usually the most time-consuming step in environmental analysis. The sample preparation is needed to extract, isolate and concentrate the target compounds from matrix as it can disrupt the operation of analytical device (Kataoka 2003). The selected factors for sample preparation depend on physical and chemical properties of the target compounds (Seifrtová *et al.* 2009).

According to Kataoka (2003) good sample preparation has to meet five aims: minimal loss of the sample, efficient removal of coexisting components, quick to conduct, economical and not cause any problems in the chromatography process. For routine analysis it is good to have easily automated method and to use the solvent as less as possible to save resources (Samanidou & Karageorgou 2010).

The target compounds in aqueous samples have to be concentrated and extracted, and there are some techniques available such as liquid/liquid extraction and solid phase extraction (SPE). The latter is nowadays the most widely used sample preparation technique to analyse pharmaceuticals in waters (Jakimska *et al.* 2014). It is usable for both enriching the trace amount of the target compound and simplifying the matrix (Pavlović *et al.* 2010). If the analysis will be performed later, the SPE cartridges can also be stored when they are dried first. The final extracts should be stored frozen or at least cooled in amber glass or plastic vials if they are not used immediately (McArdell *et al.* 2006).

When using HPLC to separate the compounds, the sample pre-treatment is necessary because of removal of impurities (Samanidou & Karageorgou 2010). This helps to prolong the lifespan of the apparatus and especially the column. Pre-treatment also improves detection, because when samples are concentrated in smaller volume (e.g. from 500 mL to 1 mL) the values of the limit of detection (LOD) and the limit of quantification (LOQ) are lower. The solvent is also changed to suitable one for HPLC analysis during sample preparation (Samanidou & Karageorgou 2010).

A review by Petrović *et al.* (2005) reported the SPE is the most common extraction method for pharmaceuticals including antibiotics, non-steroidal anti-inflammatory drugs, β-blockers, lipid regulating agents and psychiatric drugs. Method is based on different interactions between stationary phase (sorbent) and mobile phase (sample). The extraction takes place in cartridge containing SPE sorbent material (McArdell *et al*. 2006). There are wide range of SPE sorbents available, and the separation is based on different interactions between compound and phases (Kataoka 2003). SPE has four steps (Figure 1): sorbent conditioning, sample loading, washing and elution (Samanidou & Karageorgou 2010). Sorbent conditioning prepares the sorbent in the cartridge to be ready to receive the sample. The sorbent must not dry between conditioning and sample loading. Sample is forced through the cartridge for example by vacuum manifold. Washing with suitable eluent removes unwanted matrix compounds. Elution with suitable solvent collects the target compound. The eluent can be either injected directly into HPLC or evaporated and reconstituted in suitable solvent (mobile phase). Each step needs to be optimized for the best possible recovery (Samanidou & Karageorgou 2010).

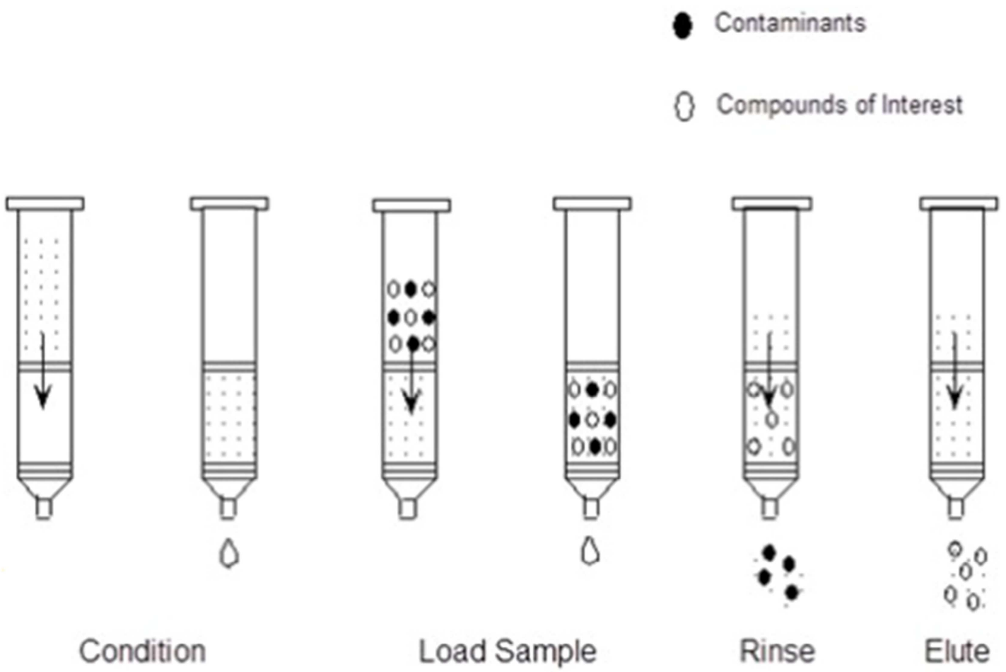

Figure 1: The steps of solid phase extraction. (Source: University of Florida. Available at https://ufl.instructure.com/courses/313181/pages/partitioning-extractions-solid-phase-extraction-sequence).

Pavlović *et al.* (2010) investigated different types of SPE sorbents for eight antibiotics including norfloxacin, sulfamethoxazole and trimethoprim. The efficiency of extraction was determined by HPLC followed by diode array detection (DAD). They used both spring and wastewater in their research. Their preliminary studies showed very poor recovery (11%) for some pharmaceuticals with Oasis HLB -cartridge, which is one of the most widely used SPE-cartridges. They tested five SPE cartridges and the results varied from not detected to 107%. They discovered the recoveries vary even when the sorbent materials were similar.

The filtration is an important step in the sampling process when treating wastewater. The particles in the water sample may cause clogs in the SPE-cartridges and slow down or even prevent the water flow. Most generally used filters are glass-fibre filters with pore size of 0.2 or 0.45 µm. Filtration may lead the loss of target compounds if they are hydrophobic and absorb in the particles of the water (Seifrtová *et al.* 2009).

Hebig *et al.* (2014) investigated impact of the material of syringe filters on the recovery of the organic micropollutants. Syringe filters are used for filtration right prior injecting the sample into HPLC. They tested seven different filters with 43 acidic, basic and neutral organic micropollutants including antibiotics and other pharmaceuticals. Sulfamethoxazole and trimethoprim were also included. Only four out of 43 compounds showed mass loss over 20% with one or more filter. The largest losses were over 80%. There were no systematic correlations between mass loss and chemical or physical propertied of compounds (molar mass, ionic character or partitioning behaviour), so the behaviour of compound during filtration can be unpredicted (Hebig *et al.* 2014).

2.2 HPLC

High performance liquid chromatography (HPLC) is frequently used analytical technique to separate and analyse compounds in aqueous samples. Earlier the most used analytical method for pharmaceutical residues along literature was gas chromatography–mass spectrometry (GC–MS), but liquid chromatography-mass spectrometry (LC–MS) and LC–MS/MS (also known tandem–MS) have replaced GC (Petrović *et al*. 2005). The GC method requires often derivatization of the compounds to make them volatile: for polar pharmaceuticals the HPLC is a better choice as the derivatization is not required (McArdell *et al.* 2006).

The compounds of interest are dissolved in a suitable solvent and then forced to flow with the mobile phase through the column (stationary phase) under high pressure (Harris 2007). The main parts of HPLC device are a pump, an injector, a separation column and a detector. The sample is loaded in the sample loop and then injected to the mobile phase flow. The separation in a column is based on different partitioning behaviour between mobile phase and stationary phase (Harris 2007).

The most important separation goals in HPLC method development are linked to resolution and separation time (Snyder *et al.* 1997). Resolution is the difference in retention times between two elution peaks divided by sum of width of the peaks. According to Snyder *et al.* (1997) the resolution should be at least 1.5 in precise and rugged quantitative analysis with only a few compounds (<6) in which case compounds can be easily obtained. The resolution 1.0–1.5 is good enough for samples containing more than 10 compounds. The length of HPLC run time (≈ retention time of the slowest compound) should be as short as possible for practical reason, especially when there are large amount samples to analysis. Run time of less than 10 min is preferred (Snyder *et al.* 1997).

2.3 MS/MS detection

Mass spectrometry (MS) is the most used detector for analysing the pharmaceuticals because of the highly selectivity and sensitivity of technique (McArdell *et al.* 2006). It provides structural information of the target compounds. The aim of MS is to separate ionized molecules (or their fragments) according their mass-to-charge ratio (*m/z*). The separation occurs in electric and/or magnetic fields. The main parts of MS are an ion source, a mass separator and an ion detector. Precursor ions (also known parent ions) and their product ions (also known fragment or daughter ions) are used for quantification of the compounds in MS/MS (Petrović *et al*. 2005).

Electrospray ionisation (ESI) is widely used for the ionisation of the compounds separated in LC and analysed in MS/MS. It is so called soft-ionisation technique which means that only small amount of energy is used for ionisation. ESI+ produces positive ions (McArdell *et al*. 2006).

2.4 Matrix effect

Although HPLC–MS/MS is efficient technique for analysis of pharmaceuticals due high sensitivity and selectivity, it is susceptible to matrix effect and this needs to be taken account when analysing compounds in complex matrix such as wastewater (Hao *et al.* 2007). All of the compounds other than the compound of interest in the environmental LC–MS analysis are termed to matrix (Seifrtová *et al.* 2009). Chambers *et al.* (2007) described matrix effect as a difference between the mass spectrometric response values for the compound in standard solution and in sample matrix (such as plasma). Like plasma, the wastewater as well, is a complex solution with numerous different compounds. Co-eluted metabolites, impurities and degradation products may cause matrix effect because they have influence on the ionization of the target compound (Chambers *et al.* 2007). The matrix effect is difficult to prevent and it is hard to predict beforehand as it fluctuates between compounds and matrixes (Seifrtová *et al.* 2009). The response may be increased or decreased due to matrix effect and this leads to inaccuracy and imprecision of analysis (Chambers *et al.* 2007). The signal intensity of antibiotics in MS/MS may be significantly suppressed in wastewater matrixes (Seifrtová *et al.* 2009). The suppression level may differ between an analyte and an internal standard in the same matrix which affects the accuracy of the method (Matuszewski *et al.* 2003). One of frequently used method for ionization of molecules in MS is ESI, and it is vulnerable to other compounds in the matrix and this may decrease the results or sometimes, rarely, increase the results, although the matrix effect is not thoroughly understood (Stüber & Reemtsma 2004).

According to Chamber *et al.* (2007) the post-column infusion method and the post-extraction spike method are the two most widely used methods to assess matrix effect. The first mentioned method identifies the region of HPLC–MS/MS system where matrix effect most likely has some influence: practically at first the extract of the sample is injected in system and then constant flow of analyte is infused. The variation of response is monitored. This method is time-consuming and do not provide quantitative understanding of the effect. Whereas the post-extraction spike method assesses matrix effect comparing between response of the target compound in pure solution (e.g. mobile phase) and response of the target compound spiked in the blank matrix after sample preparation. This method provides quantitatively assessment of matrix effect (Chambers *et al.* 2007).

Chamber *et al.* (2007) discovered the mixed-mode SPE, appropriate mobile phase and ultra performance liquid chromatography were the most suitable combination of techniques is used to reduce matrix effect in analysis of pharmaceuticals in plasma.

2.5 Quantification and method validation

To assure the quality of the analysis method for pharmaceuticals the used method needs to be described completely. This includes not only information of chemicals, devices, sampling, filtration, pH adjustment but also use of surrogate and instrumental standards, method of quantification, determination of recoveries, limit of detection (LOD) and quantification (LOQ), limitation caused by matrix effects and employment of instrumental blanks (McArdell *et al.* 2006).

Surrogate standard is used to determine the systematic loss during the sample preparation and detection (McArdell *et al.* 2006). A compound with similar physical and chemical properties may be used as surrogate standard, the typical ones are the isotope-labelled compounds. The surrogate standard is spiked into the samples at very beginning of the sample preparation to figure out all the losses of the compound during the preparation. The disadvantage of this method is availability of isotope-labelled substance or other surrogate (McArdell *et al.* 2006).

Compound with similar properties as the target compound may be used as instrumental standard. It is spiked into reconstitute sample right before injecting to LC–system and it can be used to determine the absolute recovery (McArdell *et al.* 2006).

There are few methods to quantify the amount of compound separated in HPLC–MS/MS. Surrogate standard is highly recommended method to quantify the analytes by McArdell *et al.* (2006) if the suitable compound is available. Also the external or internal calibrations are suitable. Standard addition method can be used in environmental analysis even though it is time-consuming and require lot of resources and an extra step of calculations (Stüber & Reemtsma 2004).

Accuracy, precision and linearity are the demands of the good HPLC method. The method is accurate when measured values are close to the true values and precise when measurements are reproducible. The method is linear when calibration plot between the concentration and response is straight line (Snyder *et al.* 1997). Limit of detection (LOD) is the minimum amount of analyte that can be detected by method, and it is usually defined as peak signal-to-noise-ratio (S/N) 3. Limit of quantification (LOQ) is defined similarly: minimum amount of analyte that can be quantified by method and in that case the S/N is 10 (Snyder *et al.* 1997).

2.6 Analysis methods for antibiotics

There are numerous of studies available to determined antibiotics simultaneously in waters, also studies of ciprofloxacin, doxycycline, norfloxacin, sulfamethoxazole and trimethoprim. At least Karthikeyan & Meyer (2006), Lindberg *et al.* (2004) and Ye *et al.* (2007) have studied all of these antibiotics simultaneously in wastewaters by SPE–HPLC.

In all of these studies pH was adjusted to 3. Ye *et al.* (2007) used Oasis HLB SPE-cartridges, Lindberg et al. (2004) used layered C2/ENV+ SPE columns and Karthikeyan & Meyer (2006) used Oasis HLB followed by Oasis MCX to extract the compounds. The elution solvent in all three studies was acidified methanol. Recoveries for all studied antibiotics varied from 55–161%.

2.7 Selected antibiotics

Antibiotics are used to treat bacterial infection and the selected ones are commonly used worldwide, also in Finland. Some of them were selected based on earlier experiments by supervisor as well as the wide consumption in Finland but above all by availability of pure substance. The selected antibiotics were ciprofloxacin (CIP), doxycycline (DOX), norfloxacin (NOR), sulfamethoxazole (SMX) and trimethoprim (TRI). The chemical and physical properties of the selected pharmaceuticals are listed in Table 1.

Table 1: The physical and chemical properties of studied antibiotics

Compound	CAS	Molecular weight	pKa	log K_{ow}	Water solubility (mg/mL)
Ciprofloxacin	85721-33-1	331.34	6.09	0.28	30.0
Doxycycline	564-25-0	462.46	7.75	-0.72	0.63
Norfloxacin	70458-96-7	319.33	6.34; 8.75	-1.03	1.01
Sulfamethoxazole	723-46-6	253.28	5.77	0.89	0.459
Trimetoprim	738-70-5	290.32	7.12	0.91	0.615

Fimea (Finnish Medicines Agency) publish the statistics of pharmaceutical consumption annually. The consumption of pharmaceuticals has apprised in DDD (defined daily dose) /1 000 inhabitants/day which shows the portion of the population (per mil) who has consume DDD of pharmaceutical daily (Table 2). World Health Organization (WHO 2015) has calculated the DDD for each pharmaceutical based on their actual consumption. DDD fluctuate between 100 to 2000 mg among these five pharmaceuticals. Thus ciprofloxacin is most used antibiotic in Finland (226 mg per year per capita) among these five pharmaceuticals when calculate in grams. According to European Centre for Disease Prevention and Control (ECDC) (2015) the consumption of antibiotics is usually about 25% higher in winters than in summers.

Table 2: Consumption of studied antibiotics in Finland in 2013 (Fimea), defined daily dose (WHO) and portion of excretion as parent compound (Drugbank), except sulfamethoxazole (Vree 1995)

Compound	DDD/1000 inhabitants/d	DDD (mg)	Usage per capita per year (g)	Excretion as parent compound (%)
Ciprofloxacin	0.62	1 000	0.226	50
Doxycycline	2.81	100	0.103	40
Norfloxacin	0.07	800	0.020	40
Sulfamethoxazole	0.08	2 000	0.058	11
Trimethoprim	1.01	400	0.147	90

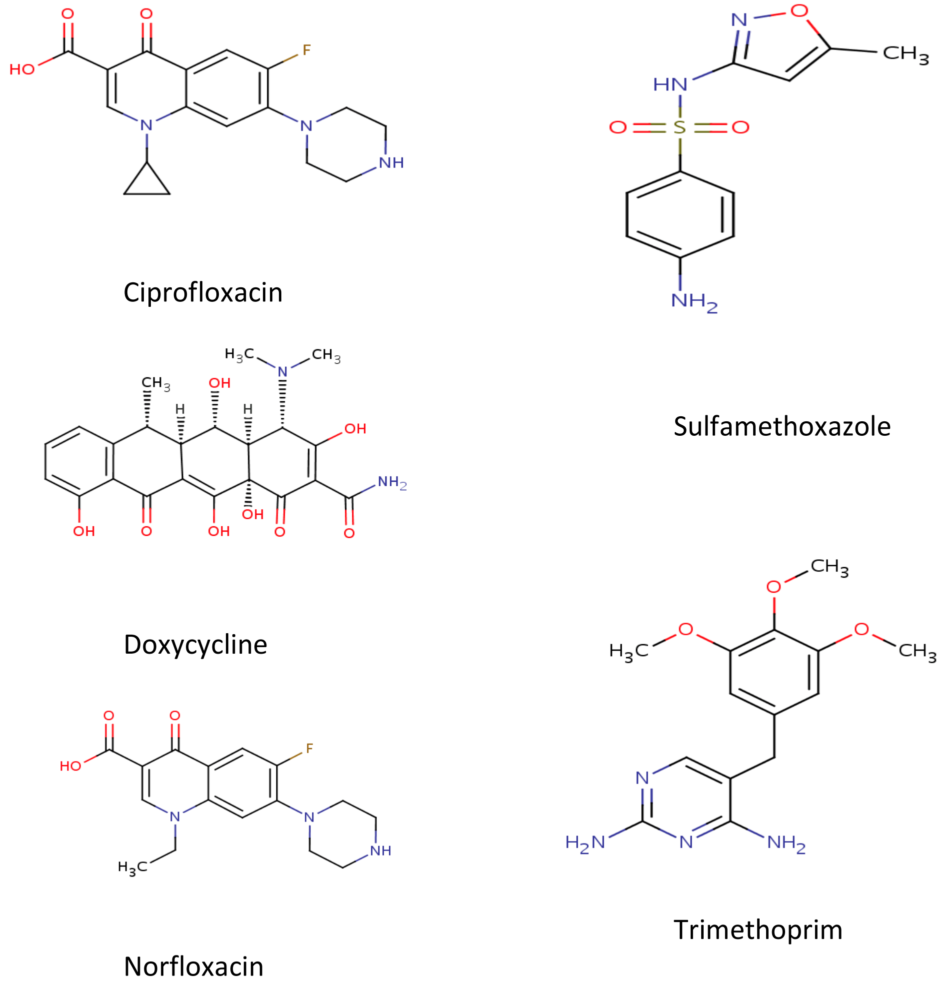

Figure 2: Molecular structures of studied antibiotics.

2.7.1 Ciprofloxacin

Ciprofloxacin (CIP) (Figure 2) belong to the group of the fluoroquinolone antibiotics (Quinoline carboxylic acids). Ciprofloxacin is soluble in water (30 000 mg/L at 20°C) and practically insoluble in ethanol. Estimated vapour pressure is 2.85×10-13 mm Hg (at 25°C) (Toxnet 2015). Ciprofloxacin is metabolized to at least four metabolites (Toxnet 2015). From 40 to 50% of pharmaceutical is excreted in the urine as unchanged, parent drug (Drugbank 2015).

If ciprofloxacin is released to air it is expected to exist only in the particulate phase and will be removed by wet or dry deposition. In aquatic environment ciprofloxacin is photo-degradable. In soil compound is expected to be immobile based on soil organic carbon partitioning coefficient K_{oc} of 61 000. Volatilization is not important fate process. Ciprofloxacin is not easily biodegradable. Bioconcentration factor (BCF) is 3, which indicates the potential for bioconcentration in aquatic organisms is low (Toxnet 2015).

2.7.2 Doxycycline

Doxycycline (DOX) (Figure 2) is an antibiotic and belongs to the class of tetracyclines. Doxycycline is very slightly soluble in water and sparingly soluble in alcohol (Toxnet 2015). The estimated vapour pressure is 0.0 ± 2.2 mm Hg (at 25°C) and soil organic carbon partitioning coefficient Koc is estimated to be 155. It has low potential for bioaccumulation (Chemspider 2015). Approximately 40% of doxycycline is excreted in urine (Drugs.com 2015).

2.7.3 Norfloxacin

Norfloxacin (NOR) belongs to same group, fluoroquinolones, with ciprofloxacin (Figure 2). It is soluble in water (280 mg/L at 25°C), solubility is pH depend: it will increase sharply when pH is lower than 5 or higher than 10. It is also soluble in methanol or ethanol (Toxnet 2015).

Norfloxacin is metabolized to six metabolites. From 25 to 40% of compound is excreted in urine as unchanged, parent drug. It is also excreted in faeces (10–50%) (Toxnet 2015).

If norfloxacin is released to air it is expected to exist only in in the particulate phase and will be removed by wet or dry deposition. It is photodegradable in aquatic environment and immobile in the soil like ciprofloxacin. Norfloxacin is not expected to be volatile. It has low potential for bioconcentration (BCF of 3) (Toxnet 2015).

2.7.4 Sulfamethoxazole

Sulfamethoxazole (SMX) (Figure 2) is an antibiotic and belongs to the class of sulphonamides (benzenesulphonamides). It is very slightly soluble in water. Most sulphonamides are metabolized by N4–acetylation up to 40% (Toxnet 2015). Also some glucuronide conjugate has been identified but data for elimination route is not available (Drugbank 2015).

An estimated vapour pressure is 6.9×10^{-8} mm Hg (at 25°C) and this indicates sulfamethoxazole would exist in both vapour and particles if released to the air. In the atmosphere it will be degraded photochemically. Sulfamethoxazole is expected to have high mobility (Koc of 72) if released in soil, but it is not volatile (upon low Henry's law constant) from soil or from surface of water. It is not readily biodegradable and may be persist in soil. In water sulfamethoxazole is not expected to adsorb solids or sediments (Toxnet 2015).

2.7.5 Trimethoprim

Trimethoprim (TRI) (Figure 2) is slightly soluble in water (Toxnet 2015). Trimethoprim is mainly (80–90%) excreted unchanged in the urine (Drugbank 2015).

If trimethoprim is released to air, it will exist in particulate phase (vapour pressure of 9.9×10^{-9}) and it will be removed from atmosphere by wet or dry deposition. High mobility is expected upon an estimated K_{oc} 75. Trimethoprim is not volatile compound. In water, the neutral form of trimethoprim is not expected to adsorb in solids or sediments, but protonated form is. Estimated BCF is 3 (expected bioconcentration in aquatic organisms is low) (Toxnet 2015).

3 MATERIALS AND METHODS

3.1 Chemicals

All of the pure substances of pharmaceuticals (purity ≥ 95%) were received from Universal Corporation Ltd., Kenya. Acetonitrile (ACN), methanol (MeOH) and acetone were purchased from Merck (Darmstadt, Germany) and they were HPLC–grade. Formic acid (FA) (98%) was from Fluka (Darmstadt, Germany) and acetic acid (anhydrous) (HAc) from Merck (Darmstadt, Germany). Acidified Milli-Q water was filtered before using in LC. Hydrocloric acid (HCl) and sodium hydroxide (NaOH) were used for pH adjustment.

3.2 Used chromatographic system

The apparatus used in chromatographic separation and detection was HPLC system (Waters Alliance 2795) and tandem-MS (Quatro Micro triple-quadrupole). In the HPLC the used column was a reversed phase C18 column (Waters XBridge 3.5 μm, 2.1x100 mm) with guard column (2.1x10 mm) with same material as the main column. Temperature of the column oven was set to 30 °C and temperature of autosampler to 20 °C. Positive electrospray ionization (ESI+) technique was used for ionization the compounds. In MS/MS nitrogen was used as desolvation gas (500 L/h) and as cone gas (50 L/h). Argon was used as collision gas.

MassLynx V4.1 software (Waters) was used for instrument control and data analysis.

3.3 Mobile phase

Composition of the mobile phase was selected by supervisor beforehand: initial gradient conditions were 20% acetonitrile (ACN) and 80% acidified Milli-Q water. Acid enhanced the ionisation. One mL of FA was added in 1 L Milli-Q water to make it acidic and the solution was filtered with Whatman hydrophilic membrane filter (poresize 0.2 μm). The injection volume was 10 μL and the flow rate was 0.25 μL/min. Total run time was 12 minutes.

3.4 Standards

The individual stock standard solutions of pharmaceuticals were prepared in methanol except ciprofloxacin, which was prepared in Milli-Q water. The concentrations of stock standards were 1000 mg/L except norfloxacin, which is soluble in methanol only slightly. The concentration of the stock standard of norfloxacin was 500 mg/L. 0.1000 g of each pharmaceutical (0.0500 g of norfloxacin) was weighted and dissolved to 100 mL of solvent. The standard of norfloxacin was stirred with magnetic stirrer for three hours until it was dissolved. Stock standards were stored in amber glass bottles at cool temperature (+4°C).

Two mixtures of all standards (50 mg/L and 10 mg/L) were prepared to act as working solution. Those standards contained all of the five pharmaceuticals with 50:50 (v/v) Milli-Q water/methanol. Both working solutions were also stored in amber glass bottles in fridge. The working standards used in analysis of each experiment were prepared by diluting the 10 mg/L standards with mobile phase right prior the analysis.

3.5 Optimization of MS/MS

The used precursor and product (fragment) ions were sought for in the literature. All of the precursor ions are positive charged molecule ions [M+H] and the selected product ions are the most abundance ones. The structures of the product ions are listed at Table 3.

Table 3: Mass-to-charge -ratio and structure of product ions

Compound	Product ion (*m/z*)	Structure
Ciprofloxacin	288	$[M–H_2O–CO_2+H]^+$
Doxycycline	428	$[M–NH_3+H]^+$
Norfloxacin	233	$[M+H–CO_2–C_2H_5N]^+$
Sulfamethoxazole	156	$[H_2NPhO_2]$
Trimethoprim	123	[M–trimeoxyphenyl]+

The best parameters were defined for each compound one at a time by infusing the individual stock solution in MS/MS via syringe pump. To reach the best possible signal, the cone voltage and collision energies were determined for each individual pharmaceutical. The best collision energy gives the most abundant fragmentation. The ion current was monitored for each compound individually and the values with maximum response were determined. The objective was to analyse all of the selected pharmaceuticals simultaneously, thus the compromised method was developed.

3.6 Sample preparation

Sample preparation and analysis steps in this work are present at Figure 3. Pre-treatment started by filtration as the particles in un-filtered sample may clog the SPE cartridge. The filtration took place in a Büchner funnel which was fixed in Büchner flask with rubber bung. The Büchner flask was fixed in water tap with rubber tubing to create partial vacuum. Whatman glass-microfibre filters GF/F (47 mm), pore size 0.7 µm, were used for sample filtration. The samples of untreated waste water were filtered first with Whatman glass-microfibre filters (GF/A) with pore size 1.6 µm because the smaller pore size filter got clogged easily. After filtration the pH was adjusted with 0.1 M HCl or 0.1/1.0 NaOH depending the target pH.

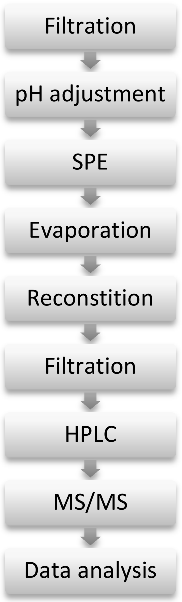

Figure 3: Sample preparation and analysis steps.

3.6.1 Solid phase extraction

The used cartridges in this study were Oasis HBL (Waters). The sorbent is strongly hydrophilic and manufacturer recommends the cartridge for all purposes, acidic, basic and neutral compounds. The cartridges used for the wastewater samples were 6 mL by volume. To save resources while developing method the used cartridges volume were 3 mL, but in eluent experiments the used ones were 1 mL. First the SPE cartridges were conditioned with 4.0 mL MeOH followed by 4.0 mL Milli-Q water. The cartridges were not let run dry before the samples were introduced. The vacuum manifold was used to achieve liquid flow in SPE cartridges. The flow rate was approx. 5–7 mL/min. The pressure was under 20 mmHg all the time, and the easiest way to control flow rate was adjust the stop-cock valves. Before elution the cartridges were let to run dry at least five minutes. The samples were not washed with any eluent before elution. Compounds in SPE cartridges were eluted to Kimax tubes. Different solvents and optimum volume of solvent were determined.

3.6.2 Elution and filtration

As the SPE eluent was not the solvent used in HPLC, the next step after elution was evaporation of samples. Evaporation took place with gentle nitrogen stream in warm water bath until dry. The samples were reconstituted with solution of ACN and acidified Milli-Q water 20:80 (V:V). Samples were stirred with vortex for 30 seconds when analysing spiked Milli-Q water and tap water and 1 min when analysing lake water or wastewater samples.

The last step before chromatographic separation is the filtration of the sample. The polyethersulfone syringe filters (PES, manufactured by VWR) were used during developing the method until the influence of the different materials were tested.

Five different syringe filters were tested with post-spiked Milli-Q water: Millipore PTFE (biopore hydrophilic polytetrafluoroethene membrane, PVC housing, pore size 0.2 μm), Millex GP PES (polyethersulfone membrane with modified acrylic housing, pore size 0.22 μm), Whatman PES (polyethersulfone membrane, polyethene housing, pore size 0.2 μm), VWR international PES (polyethersulfone membrane with acrylic housing, pore size 0.2 μm) and Whatman acetate (cellulose acetate membrane, polypropylene housing, pore size 0.2 μm).

3.7. Method validation

When comparing results in experiments the recovery of each compound were calculated using equation 1.

$$Recovery = \frac{c_{Measured}}{c_{Theoretical}} \times 100\% \qquad (1)$$

The developed SPE-HPLC-MS/MS method was evaluated by calibration, matrix effect and repeatability. Calibration curves with five points were prepared in lake water by spiking the target compounds. Repeatability was tested with standard solutions in three different days. Limit of detection (LOD) and limit of quantification (LOQ) were determined for each compound individually in lake water matrix. For LOD was used signal to noise -ratio, S/N = 3 and for LOQ was used S/N = 10. Matrix effect was calculated by comparing post-spiked lake water with matrix matched external standards (equation 2). Post-spiked method means that the known amount of the standard solution is added into sample right before injection to the HPLC.

$$ME = \frac{P}{E} \times 100 \qquad (2)$$

Where *ME* is matrix effect (%), *P* is post spiked response and *E* is external standard response.

3.8 Samples

Wastewater samples were collected from three municipal wastewater treatment plants (WWTP): Nenäinniemi in Jyväskylä, Viinikanlahti in Tampere and Lehtoniemi in Kuopio where Tampere WWTP serviced the largest population and Kuopio the smallest. The samples were collected by the employee of the WWTP's. Both influent and effluent samples were collected. There was one sampling per WWTP.

The samples from Jyväskylä wastewater treatment plant were collected on in February 2015. They were 24-hours composite samples. Nenäinniemi WWTP services 155 000 inhabitants and the average flow of wastewater is 35 591 m^3/d (in 2014). The water flow was 33 190 m^3 during the sampling.

The samples from Tampere wastewater treatment plant were collected on in March 2015. They also were 24-hours composite samples. Viinikanlahti WWTP services 247 000 inhabitants and on that day the flow rate was 62 949 m^3 during the sampling

The samples from Kuopio wastewater treatment plant were collected in May and they were 24-hours composite samples. Lehtoniemi WWTP service 80 000 consumers and the flow rate on that day was 26 735 m^3 during the sampling.

The wastewater in Jyväskylä was collected in transparent glass bottles and in Tampere and in Kuopio in low-density polyethylene (LDPE) plastic bottles. All of the samples were stored at cool temperature (+4°C) until sample preparation and analysis. The samples from Jyväskylä were prepared and analysed at the same day. The samples from Tampere were stored at cool temperature for four days until preparation. The last samples from Kuopio were filtered and extracted following day and evaporated and analysed day after that. The analysis was performed with 500 mL samples and duplicates were used.

In order to measure pH-level of the samples, PHM220 (MeterLab) was used except for samples from Kuopio when Mettler Toledo (SevenEasy) was used.

3.9 Calculated concentration

The theoretical concentrations of pharmaceuticals in the influent can be calculated using information of consumption and degree of metabolism of pharmaceutical. The flow rate of wastewater is also needed (equation 3) (Vieno 2007).

$$C_{calc} = \frac{A \times P \times e \times 10}{365 \times Q} \qquad (3)$$

where C_{calc} is the theoretical concentration of pharmaceutical (µg/L), A is the amount of pharmaceutical used per year per capita (in grams per inhabitant per year), P is number of inhabitant serviced by WWTP, e is the degree of pharmaceutical excreted as parent compound (in %) and Q is the flow rate of the wastewater (m3/d).

4 RESULTS AND DISCUSSION

Optimization of the SPE method required several separate experiments to optimise the composition and the volume of the elution solvent, pH and the filtration material.

4.1 Optimum elution

The composition and the volume of the elution solvent to remove compounds from cartridge after extraction were studied only for CIP, TRI and SMX. The spiked Milli-Q water (10 µg/L of each pharmaceutical) was used in experiment and the volume of sample was 20 mL. The used cartridges were 1 mL and the volume of eluent was 1 mL. The samples were not filtered or pH adjusted before SPE. The equation 1 was used to calculate the absolute recoveries. These experiments cannot explain in which preparation step the target compounds were lost during the preparation and analysis. The difference was in some case insignificant but the eluent conditions that produced somehow better recovery than the others has been chosen.

At first experiment four different eluents were studied: methanol (MeOH), acetonitrile (ACN), acetone and the mixture of ACN, MeOH and acetic acid (HAc) (50:50:2 by volume). Elution with MeOH gave no recovery for any of the studied compounds (Figure 4). Ye *et al.* (2007) and Lindberg *et al*. (2004) used acidified MeOH to elute the extract but the acidified MeOH was not tested in this work. Elution with ACN gave the second lowest recoveries. Acetone and the studied mixture gave similar recoveries: the mixture gave the highest recovery for TRI and CIP and pure acetone gave slightly higher recovery for SMX.

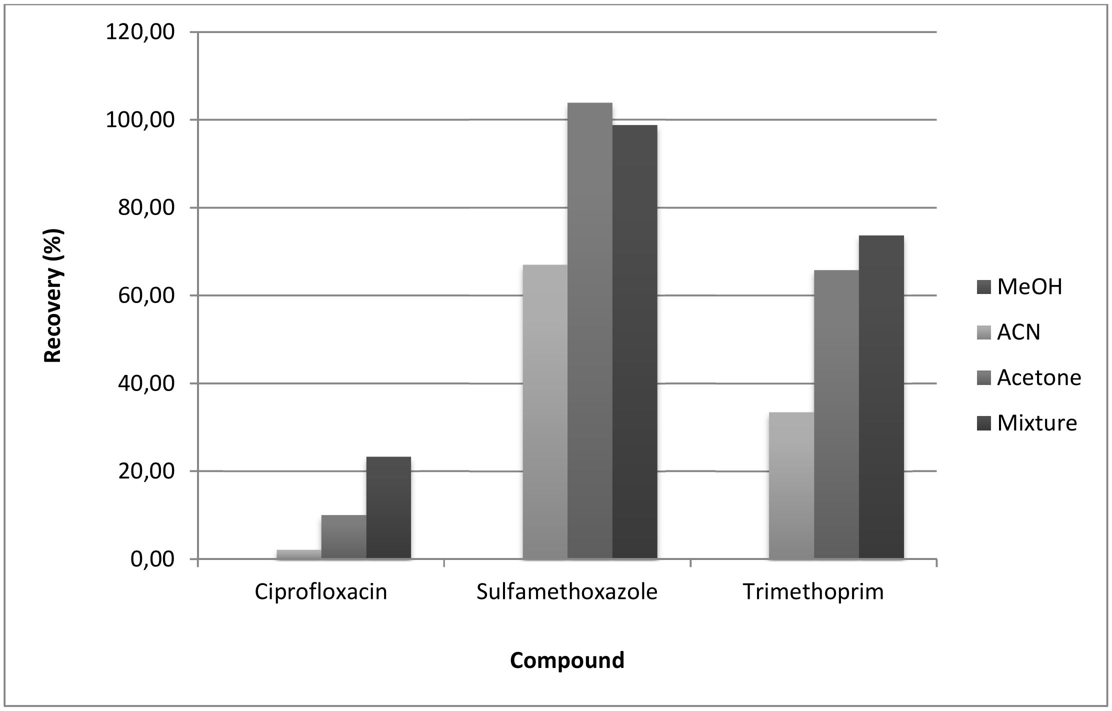

Figure 4: Recoveries of four eluents: methanol (MeOH), acetonitrile (ACN), acetone and mixture of ACN:MeOH:HAc (acetic acid), 50:50:2 by volume. Methanol gave no recovery for any of these three antibiotics.

Also two mixture eluents were tested. Mixture 1 was same as previous experiment (ACN:MeOH:HAc, 50:50:2 by volume) and the mixture 2 was ACN:MeOH:acetone:HAc, 50:30:20:2 by volume. The elution volume was 3 mL and the duplicates were used. Recoveries varied from 33–65%. Recoveries with mixture 1 for SMX were 15% and 59%, so the standard deviation is high (Figure 5), so there may be an error. The mixture 1, the one without acetone, gave better recovery for CIP and TRI (Figure 5).

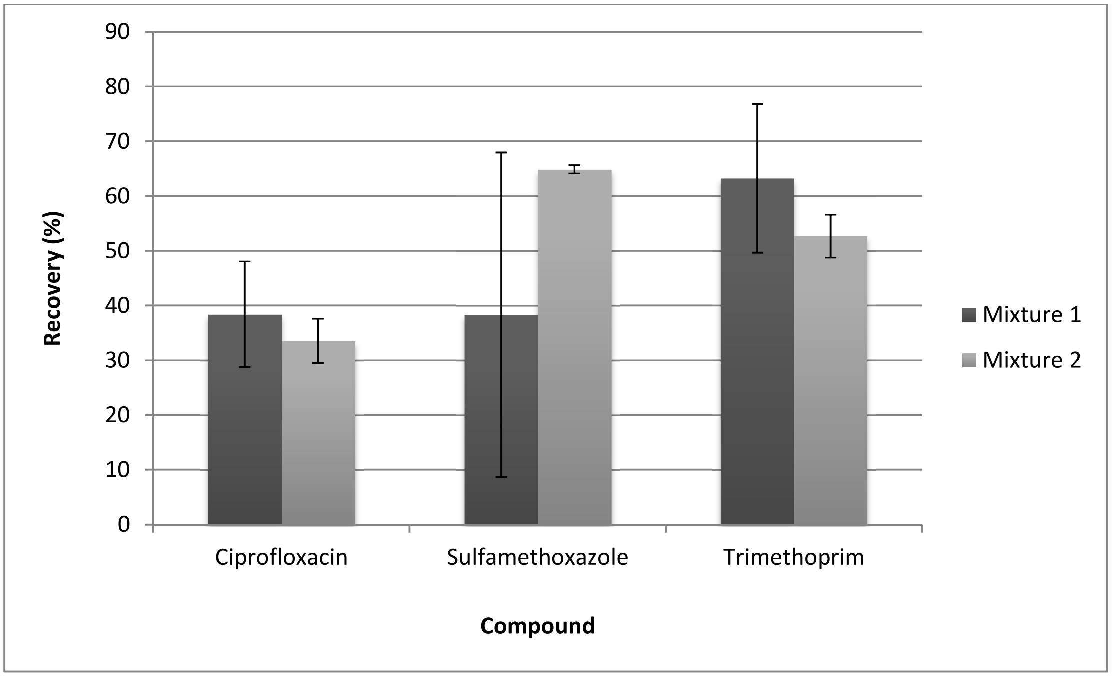

Figure 5: Two mixture eluents were studied. Mixture 1 contain ACN:MeOH:HAc, 50:50:2 by volume and mixture 2 ACN:MeOH:acetone:HAc, 50:30:20:2 by volume. Average and standard deviation, *n=2*.

Also acidic and basic eluents were compared. At first pH of the samples were adjusted at 2, 4 and 6 by 0.1M or 1.0M HCl and at pH 8 and 10 by 0.1M NaOH. Two eluent solution were used: basic mixture A: ACN:MeOH:NH_4OH (ammonium hydroxide), 50:50:2 by volume and acidic mixture B: ACN:MeOH:HAc, 50:50:2 by volume. The elution of acidified samples (at pH 2,4 and 6) was done by basic mixture A and the elution of basic samples (pH 8 and 10) by acidic mixture B. Samples were prepared by spiking antibiotics in milliQ-water and the volume of the samples were 20 mL. The elution volume was 3 mL.

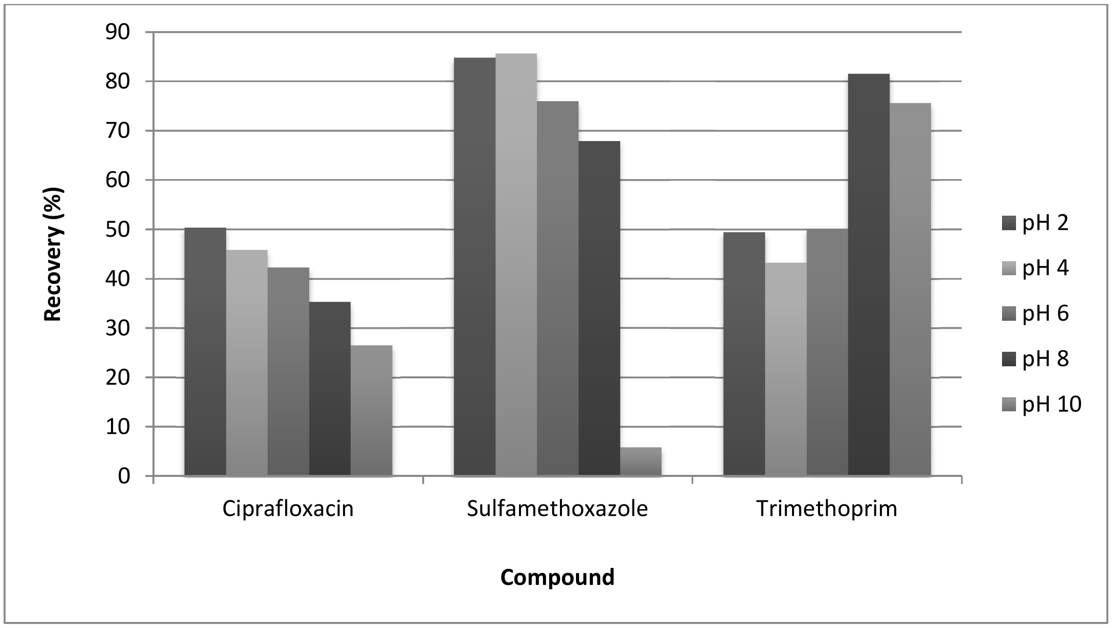

Figure 6: Comparison of the acidic and basic eluent. The acidified samples (pH 2, 4 and 6) were eluted by mixture A and samples with pH 8 and 10 by mixture B. Mixture A: ACN:MeOH:NH_4OH (50:50:2 by volyme) and mixture B: ACN:MeOH:HAc (50:50:2 by volume).

The recoveries varied from 6–86%. The trend of recoveries was decreasing from acidic to basic pH for both CIP and SMX. In pH 10 SMX gave poor recovery. As average, the pH 8 showed the highest recovery for these three antibiotics. In earlier studies (Karthikeyan &Meyer 2006, Ye *et al.* 2007) the acidified eluent was used with low pH (pH 3) and that should have been also tested.

The optimum volume of the eluent was also studied. The used eluent was mixture of ACN:MeOH:HAc, 50:50:2 by volume and the studied volumes were 1, 2, 3 and 4 mL. It was presumed the recovery of the compounds will increase when the volume of elution increases then remain that level with greater volume. This trend was not observed in recovery (Figure 7), because the largest volume of the eluent showed the lowest recovery for SMX and TRI. There is no sensible explanation for this result. One reason may be difference between flow speeds of elution solvents. However elution volume 3 mL was chosen as it showed the largest average recovery for these three antibiotics.

These experiments were performed only three out of five target antibiotics. The results may be different if conditions were optimized for all five antibiotics.

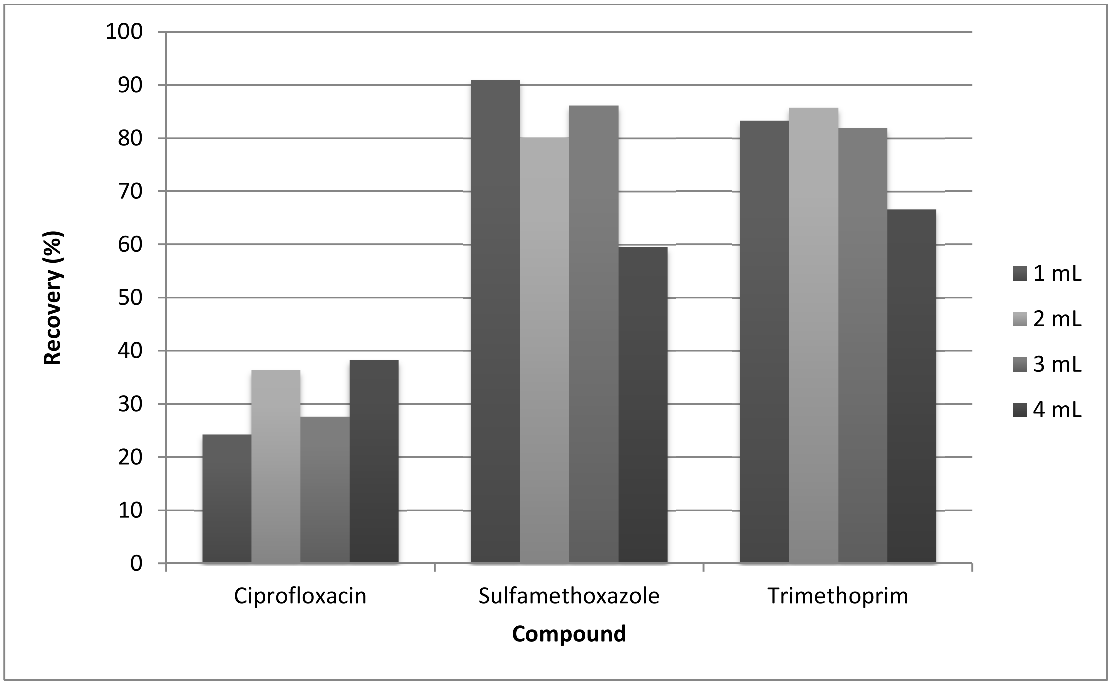

Figure 7: Volume of eluent (ACN:MeOH:HAc).

4.2 Optimum pH, filtration and matrix effect

The optimum pH was determined with all five antibiotics. At the first experiment the used water was spiked Milli-Q water and sample volume was 100 mL.

The optimum pH fluctuated with different compounds (Figure 8). The recoveries varied from 2–78%. The recovery of CIP and DOX were poor (<12%) with all pH. Only for DOX the optimum pH was the lowest one: 4. For NOR and TRI the optimum pH was 6 and for SMX there were no difference between pH 4 and 6.

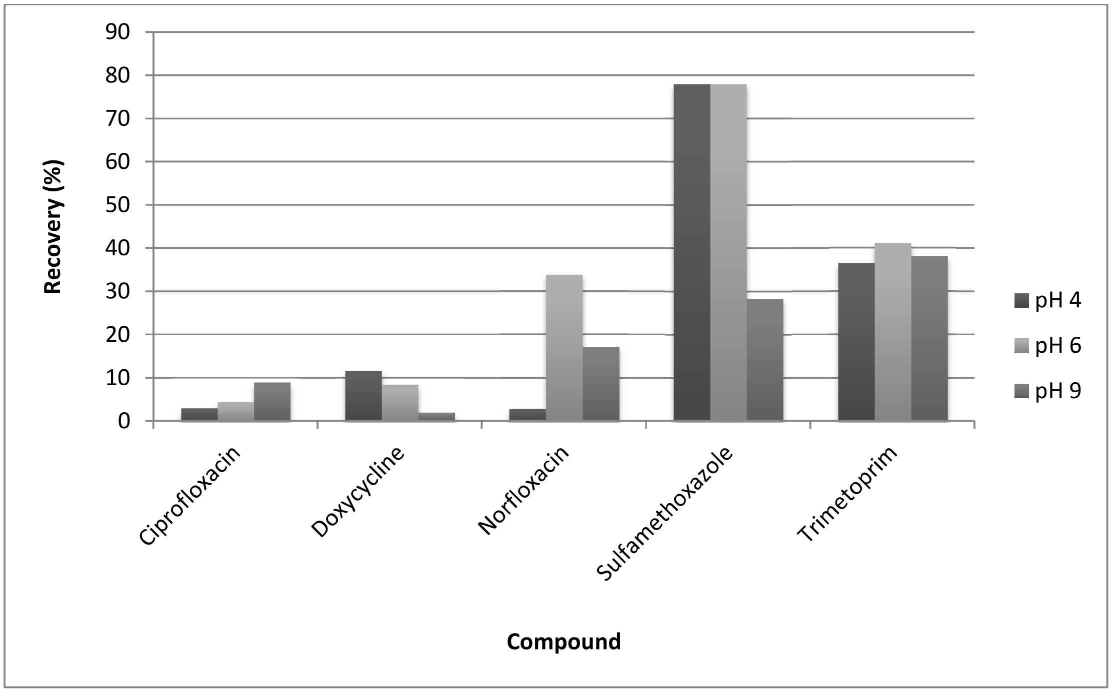

Figure 8: Recoveries for pharmaceuticals in different pH.

In a new experiment pH 8 and 9 were tested. Known amount of pharmaceuticals were spiked in lake water and then samples were prepared as usual and the duplicates were used. Recoveries varied from 10–91%. The recovery for DOX was poor (≈10%) with both pH. The recoveries for TRI and NOR were quite similar with both pH 8 and 9. pH 8 was chosen because it showed higher recovery of SMX. Also the average recovery at pH 8 was slightly higher than pH 9 (45% and 49%, respectively).

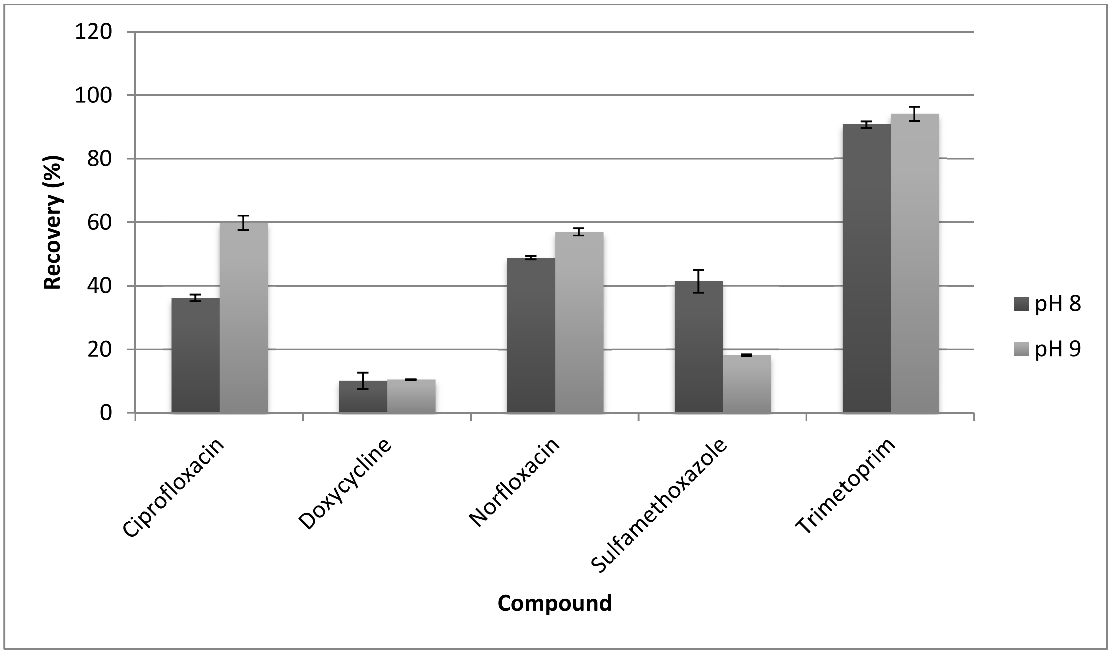

Figure 9: Recoveries of pharmaceuticals at pH 8 and 9. Average with standard deviation, *n=2*

Filtration of reconstituted sample is the last step in the sampling preparation where compounds may get lost. This was realized during the experiments. The used syringe filters at the eluent experiments and the first pH experiment were VWR international polyethersulfone (PES). The last pH experiment (comparing pH 8 and pH 9) was carried out with Whatman acetate syringe filters that gave better recovery for all of studied antibiotics. The recovery for DOX was very poor (<5%) with both syringe filters at all pH.

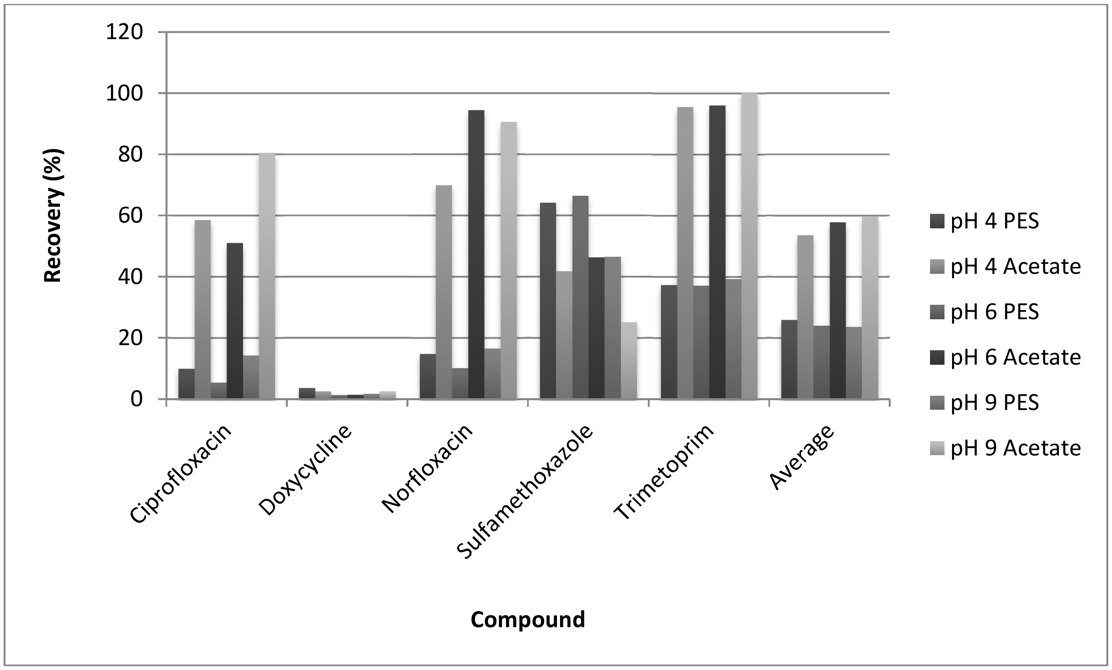

Figure 10: Two different syringe filters were tested at three pH. The studied filters were VWR international polyethersulfone (PES) and Whatman cellulose acetate filter.

The syringe filters were studied also with milliQ-water at pH 8. The samples were post-spiked after reconstitution. The studied filters were Millipore PTFE (biopore hydrophilic polytetrafluoroethene membrane, PVC housing, pore size 0.2 µm), Millex GP PES (polyethersulfone membrane with modified acrylic housing, pore size 0.22 µm), Whatman PES (polyethersulfone membrane, polyethene housing, pore size 0.2 µm), VWR international PES (polyethersulfone membrane with acrylic housing, pore size 0.2 µm) and Whatman acetate (cellulose acetate membrane, polypropylene housing, pore size 0.2 µm).

The recoveries varied from 2–122%. DOX showed very poor recovery for all five filters (<4%) so further studies are needed to test different filter materials, also in lower pH. Filtration with Whatman cellulose acetate filter showed better recoveries for CIP, NOR and TRI, and the rest of experiments and analysis were performed with acetate filter. The analysis without filtration of the reconstituted sample was not tested.

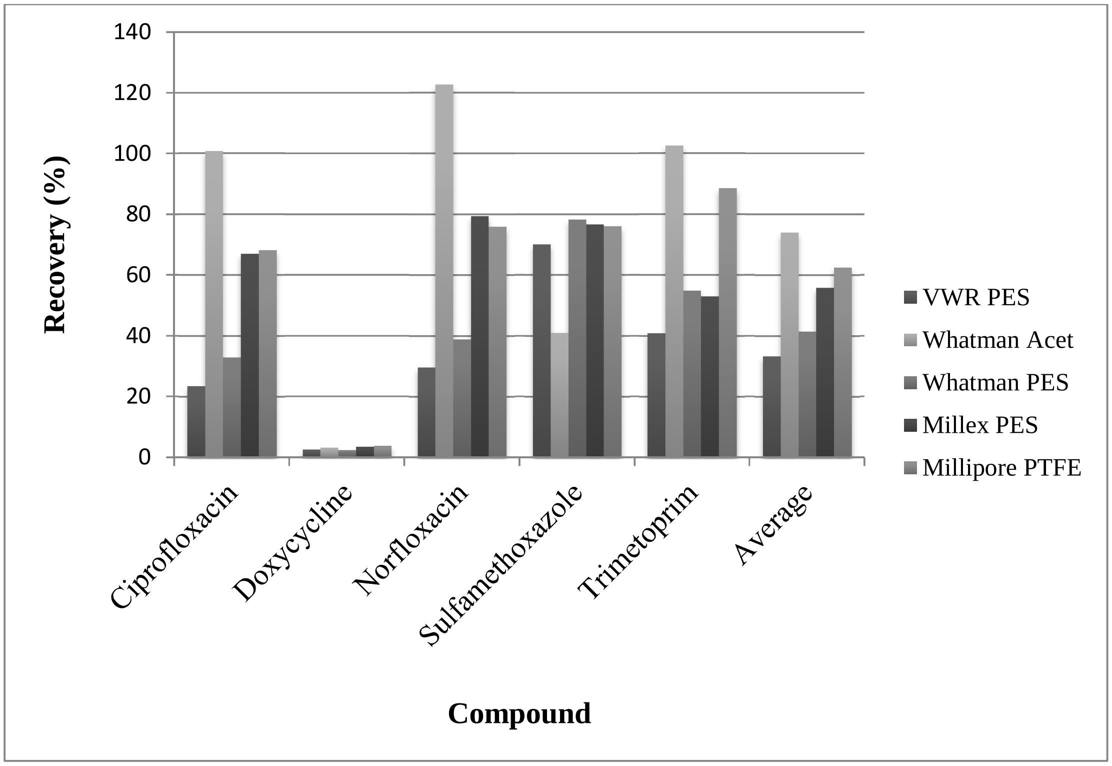

Figure 11: Recoveries of pharmaceuticals filtered with five different syringe filters. The samples were post-spiked after reconstituting and then filtered right prior injecting into HPLC.

The recovery of each compound was defined both in spiked lake water and post-spiked lake water. Recovery of spiked sample describes amount of analyte both lost during sample preparation and also enhanced or suppressed in analysis. Recovery of post-spiked samples describes only the latter. The relative difference between them is called matrix effect (equation 3) and in this study it varied from 25–93% (Figure 12). This recovery should not be affected the final quantification as the calibration standards have gone through the same preparation steps as the samples. Indeed the recovery of DOX is so low and fluctuate in experiments there may be possibility to loose almost all of that pharmaceutical during the process and get no results even it was present in the sample.

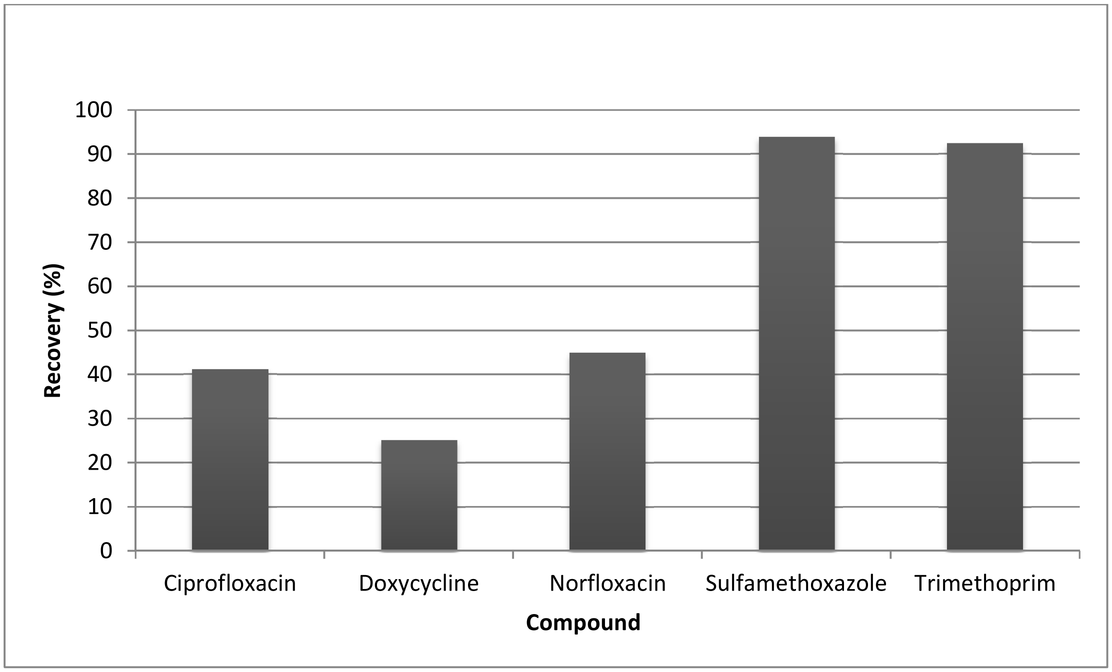

Figure 12: Matrix effect of each compound.

The chosen conditions for sample preparation were the following: pH of the samples were adjusted at 8, the used eluent was mixture of ACN:MeOH:HAc (50:50:2 by volume) and elution volume was 3 mL. The filtration right prior injection in LC was performed with Whatman acetate filter.

4.3 MS/MS optimization

The MS/MS detection was optimized first for each compound individually by infusing stock standard in MS/MS. The optimization was based on the precursor and product ions that are listed at Table 4. The optimum cone voltages and collision energies for each compound are listed at Table 5.

Table 4: Precursor and product ions and retention times for each compound. Source of the ions: Petrovic *et al.* (2005), except trimethoprim and norfloxacin (Rao *et al.* 2008)

Compound	Precursor ion *(m/z)*	Product ion *(m/z)*	Retention time (min)
Ciprofloxacin	332.2	288	2.05
Doxycycline	445	428	5.80
Norfloxacin	320.2	233	1.98
Sulfamethoxazole	254	156	5.10
Trimethoprim	291.1	123	2.02

Table 5: Optimum parameters for studied antibiotics

Compound	Cone voltage (V)	Collision energy (eV)
Ciprofloxacin	34	19
Doxycycline	30	19
Norfloxacin	34	25
Sulfamethoxazole	28	18
Trimetoprim	34	19

4.4 Method validation

The repeatability of the developed HPLC–MS/MS method was tested by running standard solutions of three concentrations (100, 500 and 1000 µg/L of each individual pharmaceutical) into system three times in different days. The mean values, standard deviations and relative standard deviations (RSD) of responses were calculated. The RSD values were 3–19% (Table 6) and since they were <20%, SPE–HPLC–MS/MS may considered to be repeatable.

Table 6: Repeatability (in relative standard deviation, RSD) of the method at three standard concentrations, *n=3*

	Repeatablity, RSD (%)		
	100 µg/L	500 µg/L	1000 µg/L
Ciprofloxacin	9	3	3
Doxycycline	13	10	7
Norfloxacin	8	2	3
Sulfamethoxazole	19	17	17
Trimethoprim	13	12	13

The SPE–HPLC–MS/MS method was validated for lake water. The recoveries were determined from five spiked lake water samples except for CIP and NOR where the lowest concentrations gave negative results and one and two lowest point, respectively, have been omitted (Table 7). Trimethoprim showed the best recovery (72%) and also sulfamethoxazole showed tolerable recovery (42%), but the rest three showed recoveries below 20%.

Table 7: Recoveries of studied pharmaceuticals in spiked lake water. Average with standard deviation, *n* = 5, except for ciprofloxacin *n=4* and norfloxacin *n=3*

Compound	Recovery (%)
Ciprofloxacin	14±8
Doxycycline	6±3
Norfloxacin	14±4
Sulfamethoxazole	42±6
Trimethoprim	72±9

The limit of detection (LOD) and limit of quantification (LOQ) of method for lake water were determined from signal-to-noise –ratio (S/N). The highest LOD value was 50 ng/L for SMX and the rest of them were <10 ng/L. The LOQ values varied from below 10 ng/L up to rather high 150 ng/L for SMX. The used sample volume was rather high: 500 mL.

Table 8: Limit of detection (LOD) and limit of quantification (LOQ) for studied compounds in given method (sample volume 500 mL)

	LOD (ng/L)	LOQ (ng/L)
Ciprofloxacin	<10	<10
Doxycycline	<10	30
Norfloxacin	<10	20
Sulfamethoxazole	50	150
Trimethoprim	<10	<10

Although recoveries were poor for some compounds the method can be described as reliable because of using the matrix matched external standards that has prepared as samples: the losses during sample preparation and analysis were similar between samples and standards. More precise results can be obtained by using standard addition method, but because it is time-consuming and requires large amount of replicates prepared at same time, it is not practical and therefore rarely used. Zhou & Kang (2013) studied how precise results matrix matched external calibration method give. According to them for e.g. tetracycline (belong the same group with DOX) matrix matched external calibration is suitable method but not for all antibiotics. The common convention is to use suitable internal standard (prepared in pure solvent) and Zhou & Kang (2013) discovered the combined use of matrix matched external calibration and internal standard showed the best results. There are still questions how similar matrix lake water and wastewater are; the further investigations are needed to compare them. The better option would be to use the wastewater without pharmaceuticals as matrix matched external standard.

The developed method was simple and fast, and the LOD and LOQ were rather low except for SMX. DOX would show better recovery if the sample preparation steps, also pH adjustment, were also optimized for it. Sample preparation with acid pH should be tested like Karthikeyan & Meyer (2005), Lindberg *et al.* (2004) and Ye & Weinberg (2007) did. More syringe filter materials should be tested for DOX.

4.5 Measured concentration

The matrix matched external standard was used for analysing wastewater samples. The lake water was spiked with five different amount of standard solution thus the concentration of the external standards were from 200–2 000 ng/L. The standards were prepared as to figure out the losses of pharmaceutical during the process. The standards were prepared in lake water for observing also the matrix effect of the water.

Using the concentration and the corresponding peak areas the external calibration graph equations were determined and they can be used to calculate the concentration of unknown samples. The peak area corresponding concentration of 1 000 ng/L was clearly outlier especially for DOX and this concentration has left aside in the formation of calibration graph. Detection of TRI is the most sensitive whereas detection of NOR is the least sensitive (Figure 13).

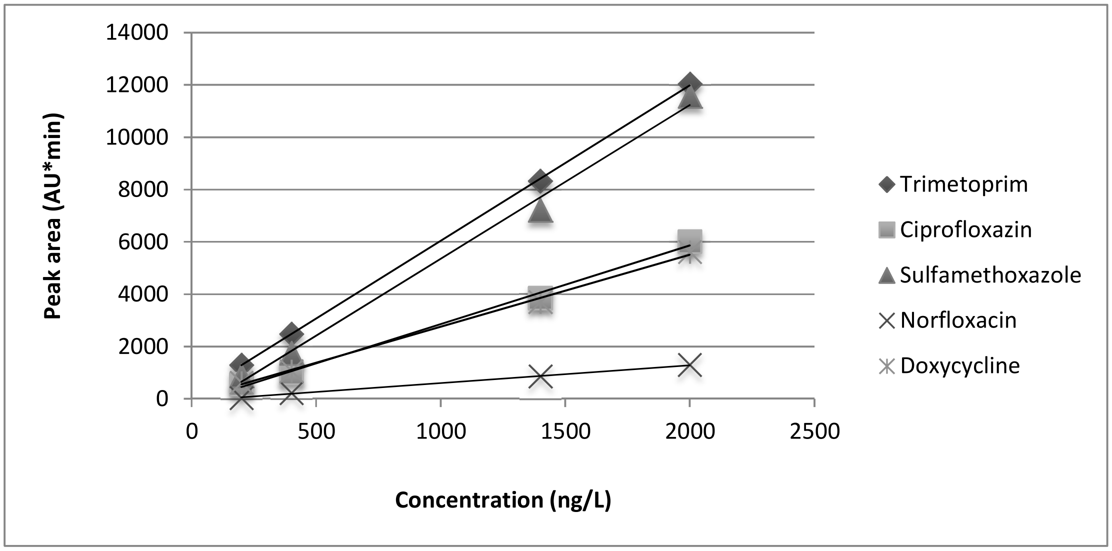

Figure 13: Graphs of peak areas versus concentrations of pharmaceuticals.

The regression coefficients (R^2) for all compounds are greater than 0.99 (Table 9). The regression coefficients of sulfamethoxazole and ciprofloxacin are less than 0.996 (one of the definition for highly linear).

Table 9: Calibration graph equation for each compound and their regression coefficients (R^2)

Compound	Calibration graph equation	R^2
Ciprofloxacin	y=3,01x-150,87	0,9955
Doxycycline	y=2,75x-0,32	0,9972
Norfloxacin	y=0,68x-70,34	0,9984
Sulfamethoxazole	y=5,88x-522,29	0,9944
Trimethoprim	y=5,95x+86,95	0,9998

TRI, CIP and SMX were those pharmaceuticals that were detected in the influent samples (Figure 14). Only TRI was detected all influent and effluent samples. Concentrations of TRI in the influent samples were 171, 148 and 136 ng/L in Jyväskylä, Kuopio and Tampere, respectively. The concentrations of TRI in the effluent samples were slightly higher than in influent in all WWTPs (230, 233 and 175 ng/L). The highest measured concentration was 301 ng/L for CIP in the influent sample in Jyväskylä.

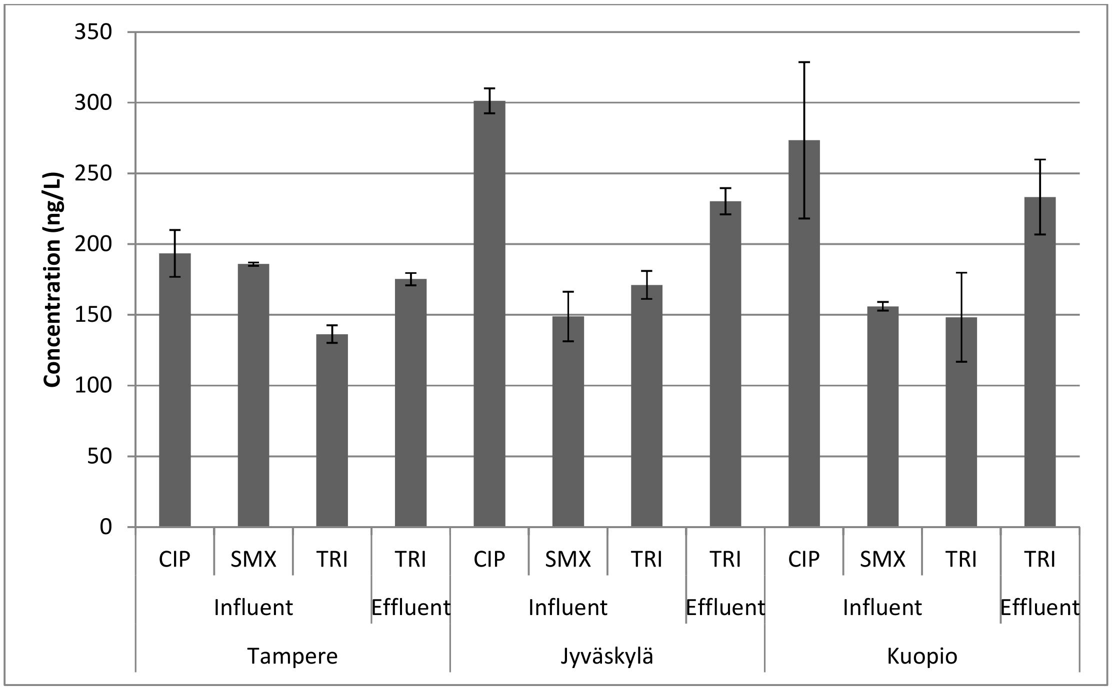

Figure 14: Concentration of the influent and effluent samples.

TRI was detected both from influent and effluent in all three WWTP’s and the concentrations were higher in every effluent than in influents (Figure 14). The higher concentration in effluent than in influent may be due biological activity in wastewater treatment process: metabolites that are conjugated with inactivating compounds may cleave back to the parent compound (Heberer 2002). Although TRI is excreted mainly as parent compounds it has also some metabolites (Drugs.com).

Dai *et al.* (2014) studied 15 pharmaceuticals and personal care products, including TRI, in wastewaters in Xiaohongmen, China. The concentration of TRI was 390 ng/L in influent and slightly higher in effluent. On the other hand Birosova et al. (2014) reported some removal of TRI during wastewater treatment processes in their study: 12–14% in one WWTP and 44–52% in other WWTP. Also Verlicchi *et al.* (2014) found difference of TRI concentration between influent and effluent samples, 59 and 40 ng/L respectively.

Detected concentrations were similar than in literature. Äystö et al. (2014) investigated occurrence of pharmaceuticals in Finnish WWTP's effluents and in receiving waters. Effluent samples were collected from four WWTP: Turku, Tampere, Riihimäki and Mäntsälä in autumn 2013. The WWTP in Tampere (Viinikanlahti) was the same plant as in this thesis. They studied 27 pharmaceuticals including antibiotics DOX, SMX and TRI. DOX was detected neither in effluent nor surface water, but both SMX and TRI were detected in all four effluents. TRI was detected in all effluents with higher concentration than sulfamethoxazole. The concentration of TRI varied from 100–460 ng/L. The highest concentration was in Tampere. The concentration of SMX was 10 ng/L in all four effluents.

Occurrence of some pharmaceuticals, including CIP and NOR, in influents and effluents has reported in research by Vieno *et al.* (2006). They studied 21 samples in 12 WWTPs. In the influents the highest concentration of CIP was 4230 ng/L and median 390 ng/L (n=20). The highest concentration of NOR was 960 ng/L and median 80 ng/L (n=13). In effluents NOR was detected once (110 ng/L) and CIP 18 times with maximum concentration 130 ng/L (median 70 ng/L).

DOX was detected neither in influent nor effluents in this study. Also Äystö *et al.* (2014) reported they could not detected DOX in effluents and their LOD was 10 ng/L.

4.6 Calculated concentration

Theoretical concentration of each antibiotic has been calculated using equation 2. These calculated concentrations were the highest in Jyväskylä and lowest in Kuopio ranging from 50 ng/L to 1.7 µg/L (Table 10).

Table 10: Theoretical concentration in wastewater influent at Jyväskylä, Tampere and Kuopio

	Calculated concentration (µg/L)		
Compound	Jyväskylä	Tampere	Kuopio
Ciprofloxacin	1.45	1.22	0.93
Doxycycline	0.52	0.44	0.34
Norfloxacin	0.10	0.09	0.07
Sulfamethoxazole	0.08	0.07	0.05
Trimetoprim	1.70	1.43	1.09

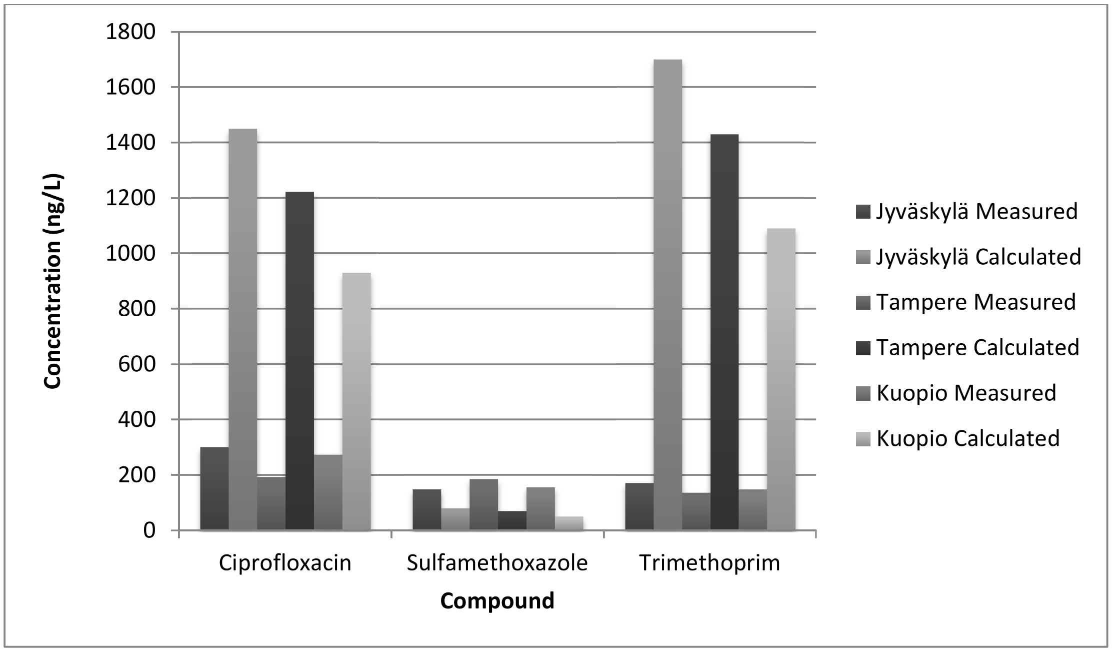

Figure 15: Comparation of measured and calculated concentrations of three antibiotics.

The measured concentrations in influents were lower in every WWTP than theoretical concentrations for CIP and TRI. The measured concentrations for these two compounds range from 136–301 ng/L while the calculated concentrations were from 930–1 700 ng/L. The seasonal variation does not explain the differences between measured and calculated concentrations as the consumption of antibiotics is higher in winters than in summers (ECDC 2012). Sampling in Jyväskylä was made in winter (February) and in Tampere and Kuopio during the spring. For SMX the measured concentrations (149–186 ng/L) were slightly higher than calculated concentrations (50–80 ng/L). NOR and DOX were not detected at all.

SMX is excreted as metabolites and according to literature only 10% is excreted as parent compounds and calculation in this work has performed with this 10%. If calculation is performed with the worst case scenario (the excretion portion as parent compound 100%), the theoretical concentration will be 480–750 ng/L instead of 50–80 ng/L. It is possible at least some of the metabolites can transform back to the parent compound even before wastewater enter in WWTP.

DOX and NOR were not detected at all and the reason could also be the fact, some of compounds may excreted in faeces (DOX) or be adsorbed in the sludge as only the liquid phase of wastewater was studied. Vieno (2006) discovered in her studies the calculated and measured concentrations were similar for several pharmaceuticals (e.g. CIP and NOR), but for ofloxacin the measured ones were five times lower than calculated values. For some pharmaceuticals (but not antibiotics) the measured values were higher than calculated ones.

5 CONCLUSIONS

Simultaneous SPE–HPLC–MS/MS method for five antibiotics (ciprofloxacin, doxycycline, norfloxacin, sulfamethoxazole and trimethoprim) was developed. The optimum conditions for SPE and MS/MS were determined. Despite of relatively poor recoveries for doxycycline, ciprofloxacin and norfloxacin, the method was rather reliable since the analysis was carried out with the matrix matched external standard prepared in lake water. Matrix matched external standard method was chosen because suitable internal standards were not available and standard addition method is too time- and resource-consuming. The developed method is simple and fast. Limits of detection and quantification were low enough to study these antibiotics in wastewaters, only sulfamethoxazole had a bit higher LOD and LOQ, 50 ng/L and 150 ng/L, respectively.

Method was employed to determine occurrence of these pharmaceuticals in wastewater influent and effluent in three Finnish WWTPs. Ciprofloxacin, sulfamethoxazole and trimethoprim were detected in all influents and the latter also in all effluents. Ciprofloxacin was detected with the highest concentration: 301 ng/L. The concentrations of trimethoprim were higher in effluents than in influents. This may be due the cleavage of glucuronide conjugates from slightly transformed metabolites during wastewater treatment process, but usual some removal of trimethoprim is reported.

Norfloxacin and doxycycline were not detected in this study. Doxycycline was almost vanished during filtration and more investigation is needed to find the best laboratory materials for it.

As there are over 3 000 pharmaceutically active compounds and dozens of different antibiotics from several groups in the market, so it is not practical to try to detect them all. Further studies are needed to define few indicate compounds, that can be analyzed simultaneously, to show the level of contamination of wastewater and assess the risk antimicrobial resistance.

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