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Determination of antibiotics by SPE-LC-MS/MS in wastewater and risk assessment

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Abstract. In this study, conditions of solid phase extraction (SPE) for determination of some antibiotics such as trimethoprim, oxytetracycline, erythromycin, clarithromycin, azythromycin, doxycycline, sulfamethazine, ciprofloxacin, chlortetracycline, sulfamethoxazole in wastewaters were optimized. After the optimum volume and pH of the sample were determined, the effect of the concentration of the compounds and matrix were investigated. The highest recovery rates for antibiotic compounds were determined between 82% and 105% in 200 mL sample volume and pH 2.5. Then, antibiotic compounds were investigated in influent and effluent samples taken from Konya Urban Wastewater Treatment Plant. The concentration of the antibiotics was detected range of 0.11-101 ng/L in influent waters and <dl-288 ng/L in effluent samples in wastewater treatment plant. Hazard quotients (HQs) of antibiotic compounds determined in WWTP effluents to evaluate the risk towards different aquatic organisms (algae, *Daphnia magna* and fish) were determined. Azythromycin for fish and erythromycin, sulfamethoxazole, ciprofloxacin, clarithromycin for algae posed a moderate risk while azythromycin, ciprofloxacin, clarithromycin, oxytetracycline posed a high risk for algae in the receiving environment.

Keywords: antibiotic; LC-MS/MS; risk assessment; SPE, wastewater

1. Introduction

Pharmaceuticals are groups of chemical substance having medical features. Wastewater treatment plant effluents, groundwater and surface water may involve 160 types of drugs. In fact, even pharmaceuticals in drinking water are encountered. Nowadays, pharmaceuticals have become indispensable and important element in areas such as medicine, veterinary, agriculture. In the 1970s, information on the presence of pharmaceuticals in nature has begun to emerge in recent years and their effects on behavior studies were started in the 1990s. The main source of pharmaceutical compounds is the metabolic wastes of patients going to the sewerage system. These contaminants are excreted by fecal and urinary because of not to be fully metabolized by humans and animals. In generally, it is assumed that the hospital wastewater has the same pollutant

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load as domestic wastewater and is discharged into the sewage system to be treated together with urban wastewater in our country as in many countries. The pharmaceuticals have stable, hydrophilic, and resistant of against hydrolysis and enzyme structure because of their removal is low in conventional treatment plants and so they are discharged to environment media such as surface water. Furthermore, farmland application of sewage sludge represents another input of pharmaceuticals into the environment. These contaminants access humans and other living creatures with water resources. Antibiotics that used mainly for treating bacterial infections are one of the pharmaceutical groups. They are among the most consumed pharmaceuticals worldwide. Some studies indicate that many of antibiotic residues have been found in different water samples at relatively low concentrations. For example; Brown *et al.* (2006) detected sulfamethoxazole at 400-2100 ng/L in hospital wastewater, 390-1000 ng/L in influent wastewater, 390 ng/L in effluent wastewater; trimethoprim at 2900-5000 ng/L in hospital wastewater, 590-1400 ng/L in influent wastewater, 180 ng/L in effluent wastewater. Hirsch *et al.* (1999) found 5 antibiotic compounds (clarithromycin, erythromycin, sulfamethazine, sulfamethoxazole, trimethoprim) in surface water at concentration 60-1700 ng/L. One of the major concerns about antibiotic residues in the environment is that some bacterial species acquire resistance to antibiotics. This causes the spread of antibiotic-resistant genes. So antibiotics may be in-effective in the treatment of some diseases. Also, their residues show adverse effect on microorganism even at the low concentrations. They can accumulate in aquatic organisms, especially fish (Huerta *et al.*, 2012; Ramirez *et al.*, 2009). For all these reasons, concentrations should be determined in environmental medium and potential risks must be identified. Their analysis is difficult; because of the fact that they were found low concentration in complex matrixes. So it is important to development of analytical methods for their analysis. In generally, a pre-concentration step by solid-phase extraction (SPE), followed by a liquid chromatographic (LC) and mass spectrometry (MS) or tandem mass spectrometry (MS/MS) is used determination of pharmaceuticals in wastewater. In this study, conditions of solid-phase extraction (SPE) were optimized and then some antibiotics were determined in wastewater treatment plant influent and effluent samples using determined method. Also, the potential ecotoxicological risk for aquatic organisms (fish, Daphnia magna and algae) in the receiving environment was evaluated.

2. Material and methods

2.1 Chemicals and reagents

All reagents used in this study were of analytical grade. Azithromycin (AZI), sulfamethoxazole (SMX), trimethoprim (TMP), chlortetracycline hydrochloride (CTC), ciprofloxacin (CIPRO), clarithromycin (CLAR), oxytetracycline (OXY), doxycycline (DOXY) were purchased from Fluka (Munich, Germany), while erythromycin (ERY), and sulfamethazine (SMZ) from Sigma (Munich, Germany).Codeine (CO) was purchased from Cerilliant (Munich, Germany). HPLC-grade methanol, acetonitrile, hydrochloric acid (37%), formic acid (98%), Na_2EDTA (ethylene diamine tetra acetic acid disodium salt solution) were purchased from Merck Co (Darmstadt, Germany). Glass fiber filters (1.2 µm pore size) were obtained from Whatman, while nylon membrane filters (0.45 µm pore size) were taken from Sartorius (Göttingen, Germany). Oasis HLB cartridge (60 mg, 3 mL) for SPE were obtained from Waters Corporation. High-purity nitrogen gas was obtained from the nitrogen generator (Peak Scientific). De-ionized water was purified with Millipore milli-Q Plus water purification system (Darmstadt, Germany). CIPRO stock solution

was prepared in methanol/acetonitrile (17.5/7.5, v/v) containing 0.2% HCl. While TMP and CTC stock solutions were prepared in methanol/acetonitrile (1/1, v/v), the other compounds were prepared in methanol. Stock solutions were stored in amber vials in dark at −20°C and were prepared every 3 months.

2.2 SPE Procedure

Analytical procedures based on EPA Method 1694 (US EPA, 2007) has been developed with some modifications to be used in the wastewater sample. The lipophilic/hydrophilic balanced Oasis HLB cartridge was used to optimize the conditions of the extraction method. Oasis HLB cartridge was pre-conditioned with 2×2.5 mL of methanol and 2×2.5 mL of de-ionized water (pH 2.5). After water sample was applied to the SPE column (1 mL/min flow rate), 2×2.5 mL of de-ionized water passed through the column (2 mL/min flow rate). Then the air was passed through the cartridge for 5 minutes. The extracts was eluted with 4×2.5 mL methanol and were evaporated to dryness and re-constituted with 1 mL of methanol/water (50/50, v/v). Antibiotic compounds were analyzed by LC/MS-MS system. After the effect of sample volume, pH of the sample, the concentration of antibiotic compounds was determined, the effect of filtering and matrix on SPE efficiency was investigated. The developed method was used to determine of antibiotics in wastewater samples.

2.3 LC/MS-MS analysis

The separation of compounds was performed using Agilent 1260 HPLC equipped with Agilent Poroshell 120 EC-C18 (3.0x100 mm, 2.7 µm) columns. HPLC-MS/MS system was operated positive and negative ion mode electrospray ionization (ESI). The binary mobile phase was composed of eluent A (0.1% formic acid in ultrapure water), and eluent B (methanol). The gradient program began with a hold for 1 min at 90% of A and 10% B, followed by a 3 min gradient to 30% of, then a 8 min gradient to 70% and a further 2 min gradient to 95% of eluent B and it was held constant at 95% for 2 min. Column temperature was 35°C and the injection volume was 2 µL. Flow rate of mobile phase was 0.6 mL/min was used.

2.4 Wastewater samples

Wastewater of Konya is collected in a combined sewerage system from residential and industrial areas, and is discharged to Konya Main Drainage Channel. Water of the channel is finally discharged to the Salt Lake. Konya basin is a closed basin. Salt Lake is located in the basin, is the second largest lake in the country, and is the third saltiest lake in the world. The drainage channel is open and about 150 km long. It was built for floods controls and for the drainage fields. Wastewater treatment plant serving 1300000 population and consists of screens, aerated grit and grease tanks, primary clarifiers, aeration tanks, final sedimentation tanks, sludge thickeners, anaerobic digesters, ultraviolet disinfection units. 24-hour composite wastewater samples were taken from the treatment plant inlet and outlet and they kept refrigerated at 4 °C without preservatives until they were processed. The influent and effluent samples were filtered through 1.2 µm glass fiber filters followed by 0.45 µm nylon membrane filters. Na_2EDTA reduces pharmaceutical compounds binding to cations in water (Lopez-Serna *et al.*, 2011). Na_2EDTA solution was added to filtered wastewaters to achieve a final concentration of 0.1% (g solute/g solution).

3. Results and discussion

3.1 Optimization of LC-MS/MS parameters

Mix standard of antibiotic was used for the best separation and peak formation by varying mobile phase type, flow rate. Eluent A (0.1% formic acid in ultrapure water), and eluent B (methanol) as binary mobile phase that was chosen within from tested different mobile. Flow rate was tested in the range of 0.3-0.6 mL/min and optimal flow rate was found 0.6 mL/min. LOD (limit of detection), LOQ (limit of quantification), R^2 and m/z values for investigated compounds are given in Table 1.

3.2 Optimization of SPE procedure

Sample volume is important because it can lead to elution of the compounds and increases matrix effect. Therefore, the effect of sample volume on extraction efficiency was investigated.

Table 1 LOD (limit of detection), LOQ (limit of quantification), R^2 and m/z values of investigated compounds in HPLC-MS/MS system

Antibiotics	m/z value	LOD (pg/L)	LOQ (pg/L)	R^2
AZI	749.5, 158.1 $[M+H]^+$	0.015	0.005	0.9928
CTC	479.1, 444.1 $[M+H]^+$	0.096	0.321	0.9984
CIPRO	332.1, 314.1 $[M+H]^+$	0.014	0.048	0.9989
CLAR	748.5, 590.4 $[M+H]^+$	0.004	0.013	0.9939
DOXY	445.2, 428.1 $[M+H]^+$	0.225	0.749	0.9989
ERY	734.5, 576.4 $[M+H]^+$	0.005	0.017	0.9992
OXY	461.2, 443.1 $[M+H]^+$	0.867	2.890	0.9995
SMZ	279.1, 186 $[M+H]^+$	0.060	0.201	0.9974
SMX	254.1, 156 $[M+H]^+$	0.302	1.006	0.9998
TMP	291.1, 261.1 $[M+H]^+$	0.022	0.073	0.9967

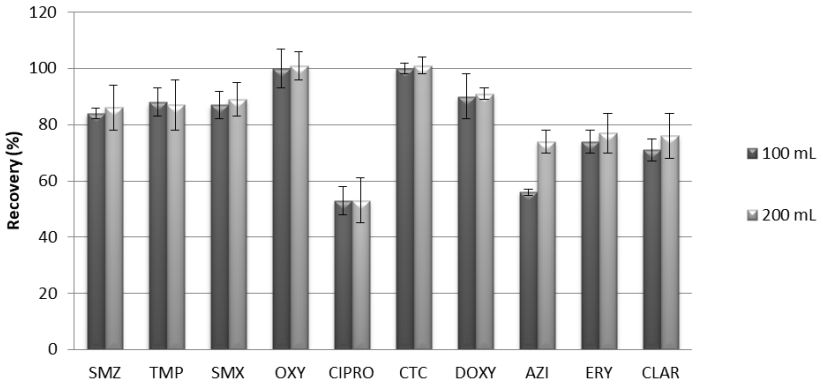

Fig. 1 Recovery values of antibiotic compounds for different sample volume

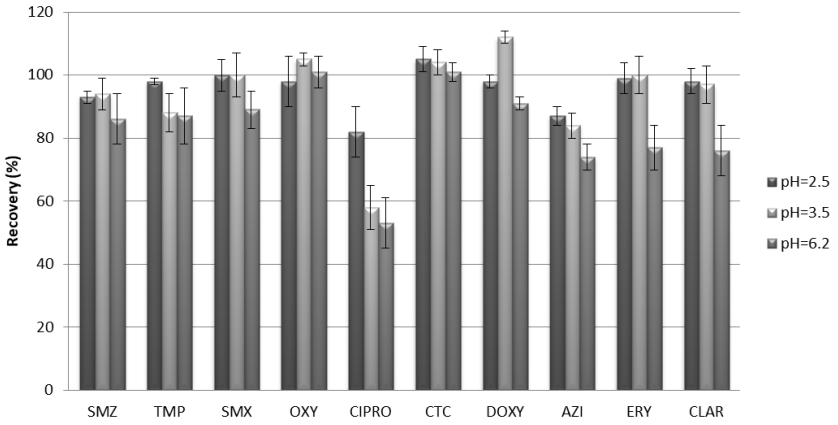

Fig. 2 Recovery values of antibiotic compounds for different pH values

Antibiotic compounds were spiked 5000 ng/L in 100 and 200 mL de-ionized water. Oasis HLB cartridge was used for extraction of antibiotic compounds. The cartridge was pre-conditioned with 2×2.5 mL methanol, 2×2.5 mL ultrapure water. After water sample was applied to the SPE column with 1 mL/min flow rate, 2×2.5 mL ultrapure water was passed through the cartridge. Then the cartridge was dried with air under vacuum during 5 min. SPE column was eluted with 4×2.5 mL methanol and extracts were evaporated to dryness and reconstituted with 1 mL of methanol/water (50/50, v/v). The recovery values of antibiotics are given in Fig. 1.

The results have shown that recoveries of antibiotic compounds are ranges from 53% to 100% for 100 mL sample volume and ranges from 53% to 101% for 200 mL sample volume. As shown in Fig. 1, while recovery efficiency for AZI compounds in 100 mL sample volume was 56%, it was 75% in a sample volume of 200 mL. 200 mL sample volume was better for the extraction of the AZI compound. Therefore, further experiments were carried out with 200 mL sample volume.

Antibiotics are generally acidic compounds. pH effect on recovery rates of these compounds in SPE are changeable according to their different physical-chemical properties. Sample pH has an important role for extraction of antibiotics and pH value usually adjusts acidic values. The highest recoveries were obtained at pH 3 values in SPE procedure in many studies for fluoroquinolones and some tetracyclines (Tong *et al.* 2016). Researchers suggest that pH of sample must be lower than pKa values of antibiotics because antibiotics are in soluble form with a pH value lower than the pKa. Determination of pH effect on method optimization, antibiotic compounds was spiked 5000 ng/L in 200 mL ultrapure water. pH of water samples were adjusted 2.5, 3.5 and 6.2 with HCI. Then SPE procedure was applied. the recoveries of antibiotics are given in Fig. 2.

The recoveries of antibiotic compounds were ranges from 72% to 105% at pH 2.5, ranges from 58% to 112% at pH 3.5 and ranges from 53% to 101% at pH 6.2. The recoveries of antibiotic compounds other than CIPRO were determined close to each other at different pH values. But recoveries of CIPRO were determined 82% at pH 2.5, 58% at pH 3.5, 53% at pH 6.2. Since the highest recovery for CIPRO was pH 2.5, it was selected as optimum pH value. Also, in statistical evaluation, a significant difference was found between performed extraction studies with pH 2.5 and pH 6.2 and between performed extraction studies with pH 3.5 and pH 6.2 (Table 2). Extraction studies were continued with pH 2.5.

In generally, all antibiotic compounds have high recovery values at low pH. Gros *et al.* (2013), investigated antibiotics in hospital, urban wastewater and river water by ultra-high-performance

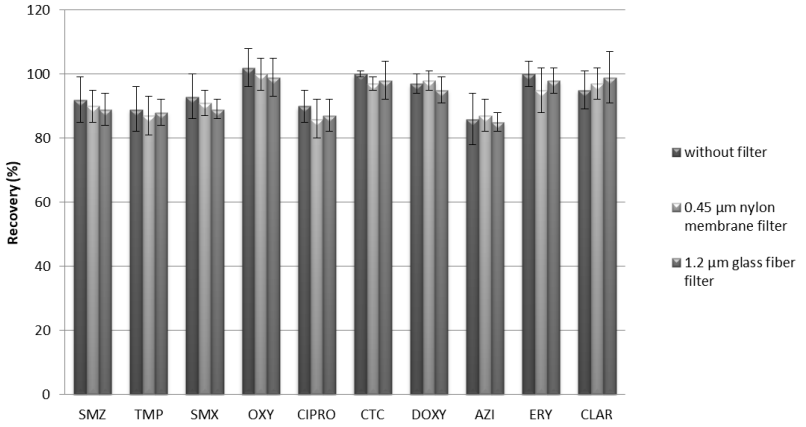

Fig. 3 The effect of filtration process on recovery efficiency of antibiotic compounds

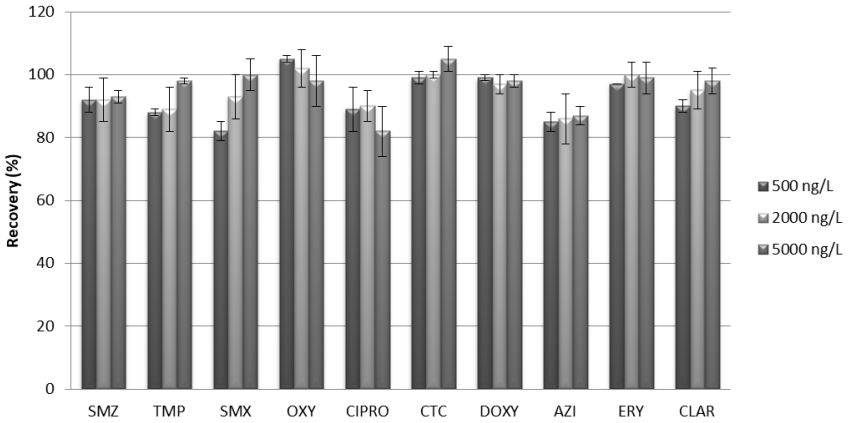

Fig. 4 Recoveries values of antibiotic compounds obtained for different analyte concentrations

liquid chromatography. They developed an analytical method working for determination of 53 antibiotic compounds. They determined high recoveries for antibiotics at low pH values with Oasis HLB cartridge in SPE procedure. Dorival García *et al.* (2013) determined 13 antibiotics in wastewater using SPE-UPLC-MS/MS. They optimized SPE conditions for using different cartridges. They determined the highest recovery rates with Oasis HLB cartridges at pH 3 value.

Filtration is one of the important steps in SPE procedure for analysis of antibiotics. Filtration effect must be investigated because adsorbed compounds in filter may be reduced recovery values. Antibiotic compounds were spiked 2000 ng/L to 200 mL ultrapure water to determine effect of filtration as a sample pre-treatment on method. pH of water samples were adjusted 2.5 with HCl and filtered through 1.2 µm glass fiber filters followed by 0.45 µm nylon membrane filters. Then SPE procedure was applied. The recovery values of antibiotics are given in Fig. 3.The recoveries without filtration was ranges from 86% to 102% for antibiotics. The recoveries filtrated nylon membrane filters with a 0.45 µm pore diameter were ranges from 87% to 100% for antibiotics, filtrated glass fiber filters with a 1.2 µm pore diameter ranges from 87% to 99% for antibiotics. No significant difference was found between the obtained recoveries (Table 2). The filtration process has proven to have no adverse effect on the extraction process.

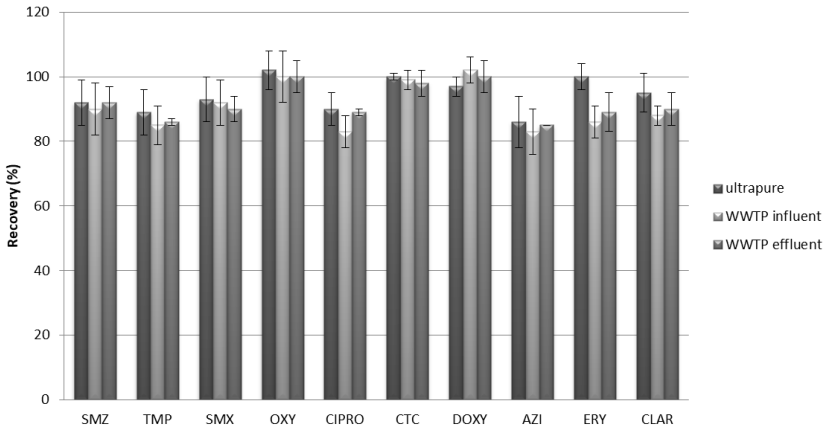

Fig. 5 Effect of matrix on the recovery of antibiotics

Table 2 Statistical evaluation of the impact of factors affecting the recovery efficiency of the antibiotic compound (significance level: 5%)

Factor	p-value	F-value	Effect
Sample volume: 100 mL-200 mL	0.096	3.44	Not significant
Sample pH: 2.5-3.5	0.626	0.25	Not significant
Sample pH: 2.5-6.2	0.002	16.4	Significant
Sample pH: 3.5-6.2	0.002	17.1	Significant
Effect of filtration: Without filter-0.45 µm nylon membrane filter	0.052	4.97	Not significant
Effect of filtration: Without filter-1.2 µm nylon membrane filter	0.061	5.70	Not significant
Effect of different concentration: 500 ng/L-2000 ng/L	0.182	2.09	Not significant
Effect of different concentration: 500 ng/L-5000 ng/L	0.219	1.74	Not significant
Effect of different concentration: 2000 ng/L-5000 ng/L	0.402	0.77	Not significant
Matrix effect: WWTP influent water	0.061	3.93	Not significant
Matrix effect: WWTP effluent water	0.059	4.67	Not significant

For the effect of analyte concentration, antibiotic compounds were spiked 500, 2000, 5000 ng/L in 200 mL ultrapure water. After pH was adjusted to 2.5 and then SPE procedure was applied. Recoveries are given in Fig. 4. Recoveries of antibiotic compounds were obtained 82-105% for 500 ng/L spike concentration, 86-102% for 2000 ng/L spike concentration, 82-105% for 5000 ng/L spike concentration. There was no significant difference in the recovery values obtained at different concentrations.

Matrix effect is an also important parameter in method optimization study. It has proven that ionization efficiency of the some antibiotic groups may be reduced because of the matrix effect in literature works (Dorival García *et al.* 2013). Many studies declared that matrix effect of samples were removed by pre-treatment of samples. To determine matrix effect on method, pH of wastewater treatment plant inlet and outlet water samples was adjusted to 2.5. Then samples filtered through 1.2 µm glass fiber filters followed by 0.45 µm nylon membrane filters and 0.1 M Na_2EDTA was added in samples. Matrix effects on antibiotics are given in Fig. 5. For antibiotic compounds, recoveries were obtained for spiked to pure water was changeable between 86-102%, for spiked to influent water 83-102% and effluent water 85-100%. No significant difference was observed between studies with different matrixes. It was observed that there was no negative effect of working with complex matrices.

The effects of each factor on the extraction efficiency were evaluated by means of the analysis of variance (ANOVA) using Tool Pak in Microsoft Excel. The strength of the effect of a factor is determined by the p-values at 5% significant level. p and F values are given in Table 2. Only effect of sample pH was determined as significant, other parameters were determined as not significant.

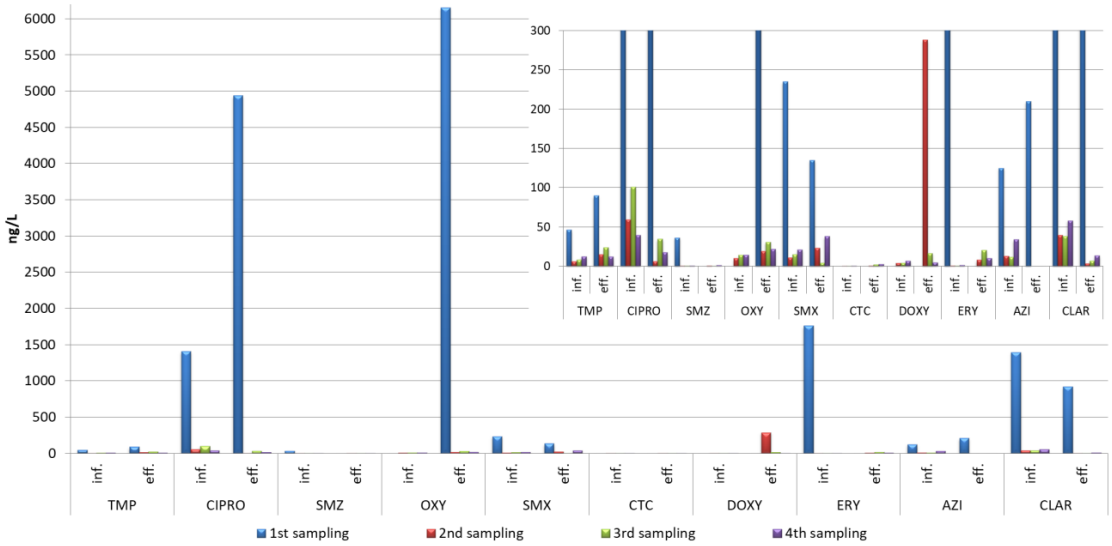

Fig. 6 Concentrations of target antibiotics in WWTP influent and effluent

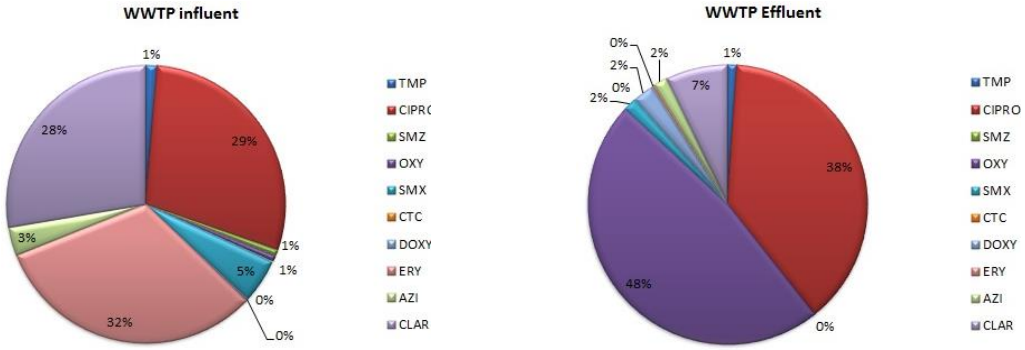

Fig. 7 Distribution of determined antibiotics in influent and effluent waters

Table 3 Antibiotic concentrations determined wastewater treatment plant in literature

Compound	Influent water (ng/L)	Effluent water (ng/L)	References
ERY	<100-250	<100-280	Petrović *et al.* (2006)
	14-600	27-270	Guerra *et al.* (2014)
	10-72	10-33	Verlicchi *et al.* (2012)
CTC	<dl	<dl	Gros *et al.* (2013)
	<26	<12	Guerra *et al.* (2014)
	<dl	<dl	Verlicchi *et al.* (2012)
TMP	590-1400	180	Brown *et al.* (2006)
	40-650	<5-230	Petrović *et al.* (2006)
	178	108	Gros *et al.* (2013)
	79-810	18-580	Guerra *et al.* (2014)
	39-72	36-51	Verlicchi *et al.* (2012)
SMZ	<dl	<dl	Brown *et al.* (2006)
	17-45	<7.3	Guerra *et al.* (2014)
	10-33	10-15	Verlicchi *et al.* (2012)
SMX	390-1000	310	Brown *et al.* (2006)
	<150-960	<150-800	Petrović *et al.* (2006)
	2020±368		Chang *et al.* (2010)
	528	198	Gros *et al.* (2013)
	59-3100	33-1800	Guerra *et al.* (2014)
CIPRO	17-2500	22-620	Guerra *et al.* (2014)
	1100-3700	290-1100	Verlicchi *et al.* (2012)
	200-1000		Brown *et al.* (2006)
DOXY	<dl	<dl	Gros *et al.* (2013)
	24-78	19-53	Guerra *et al.* (2014)
	<dl	<dl	Verlicchi *et al.* (2012)
CLAR	632	172	Gros *et al.* (2013)
	48-8000	130-7000	Guerra *et al.* (2014)
	110-780	260-310	Verlicchi *et al.* (2012)
OXY	41±15		Chang *et al.* (2010)
	<26	<12	Guerra *et al.* (2014)
AZI	<70-450	<70-300	Petrović *et al.* (2006)
	437	403	Gros *et al.* (2013)
	61-2500	57-1300	Guerra *et al.* (2014)
	10-330	70-180	Verlicchi *et al.* (2012)

<dl: below of detection limit

The recovery values determined for different methods in literature and our work were

compared. Gros *et al.* (2013), determined 50-137% recovery rates for investigated antibiotics in hospital wastewater samples, 74-180% in influent samples, 63-150% in effluent samples, 55-108% in river water samples. Khan *et al.* (2012) obtained recovery rates as 40-152% for antibiotics in surface water samples and 62-157% for effluent water samples in their method works. In this work recovery values changed between 86-102% for ultrapure water samples, 83-102% for influent samples and 85-100% in effluent samples. Our results are comparable with literature works.

3.3 Antibiotic concentrations in wastewaters

The concentrations of antibiotics in wastewater treatment plant influent and effluent samples are given in Fig. 6. In wastewater treatment plant influent samples, ERY (up to 1760 ng/L), CIPRO (up to 1405 ng/L) and CLAR (up to 1395 ng/L) were determined at higher concentrations. The detected mean concentrations for the remaining compounds in influent samples were 70 ng/L for SMX, 46 ng/L for AZI, 19 ng/L for TMP, 9.9 ng/L for SMZ and OXY, 4 ng/L for DOXY, 0.4 ng/L for CTC. The highest mean concentration was obtained for OXY compound as 1555 ng/L (19-6150 ng/L) in effluent samples. CIPRO compound also determined at high level as approximately 1250 ng/L (6.5-4940 ng/L). the mean antibiotic concentrations for other compounds in effluent samples were determined as 236 ng/L for CLAR, 77 ng/L for DOXY, 53 ng/L for AZI, 50 ng/L for AZI, 35 ng/L for TMP, 10 ng/L for ERY, 1.6 ng/L for CTC, 0.7 ng/L for SMZ.

Percentage distributions of detected antibiotics in influent and effluent water are given in Fig. 7. While in influent samples ERY (32%), CIPRO (29%) and CLAR (28%) compounds were determined at large proportion, TMP, SMZ, OXY and CTC compounds were determined at lower rates. OXY (48%) and CIPRO (38%) compounds were detected the biggest rate in effluent water in average of four sampling results. Other investigated antibiotics were detected at lower rate (<7%) in effluent water.

The antibiotic concentrations determined at wastewater treatment plant influent and effluent waters in different literature works are given in Table 3. Our results were compared to literature results. The concentrations of TMP, SMX, CIPRO, AZI compounds are lower than the literature. Concentrations of CTC, DOXY, OXY compounds are similar to literature. ERY, SMZ, CLAR are found to be higher than in the literature at the 1st sample.

Table 4 Calculated MEC/PNEC values

Compound	Fish	*Dapnia magna*	Algae
AZI	0-4.2	0-0.002	0-10.5
ERY	0	0-0.022	0-1.02
SMX	0	0-0.002	0-1.2
TMP	0-0.001	0.002-0.011	n.d.
CTC	0	0	0-0.14
CIPRO	0.001-0.092	0.001-0.072	0.653-92
CLAR	0-009	0-0.049	0.351-92
OXY	0-0.098	0.001-0.33	0.11-36
SMZ	0	0	0
DOXY	n.d.	n.d.	0-0.96

n.d: not determined

3.4 Risk assessment

Risk assessment is made based on measured environmental concentration (MEC)/Predicted No Effect Concentration (PNEC) ratios for fish, *Daphnia magna* and algae. MEC/PNEC ratios are between 0.1 and 1, the risk is low. MEC/PNEC ratios are between 1 and 10, the risk is moderate and MEC/PNEC ratios are above 10, the risk is high. Table 4 shows MEC/PNEC ratios. AZI for fish, ERY, SMX, CIPRO and CLAR for algae exhibited moderate risk. AZI, CIPRO, CLAR, OXY for algae exhibited high risk.

4. Conclusions

An analytical method has been developed for the simultaneous analysis of ten antibiotics using SPE-LC-MS/MS. Oasis HLB cartridge has provided high recovery rates for antibiotics in SPE. The results show that 200 mL sample at pH 2.5 volume is most suitable for extraction of antibiotics. Many researchers reported that the acidic pH values are most effective for antibiotic compounds in SPE procedure. Adverse effects of different pharmaceuticals concentrations, filtering and matrix on SPE were not detected. Optimized method was applied to real domestic treated and un-treated wastewaters. Antibiotics were determined up to 1760 ng/L (ERY) in influent water. In effluent water, the highest antibiotic concentration determined as 6150 ng/L (OXY).

This work showed that antibiotic compounds cannot be removed with conventional treatment processes because of low biodegradability. Effluent waters include antibiotics at different concentrations. Advanced treatment methods such as advanced oxidation processes are required for treatment of pharmaceuticals. Pharmaceuticals may be determined lower in effluent water than influent water. Pharmaceuticals may be adsorbed at sewage sludge and are determined at lower concentrations in effluent water. So, sewage sludge must be analyzed too. Risk assessment was also evaluated at this work. AZI for fish and ERY, SMX, CIPRO, CLAR for algae exhibited moderate risk while AZI, CIPRO, CLAR, OXY for algae exhibited high risk.

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