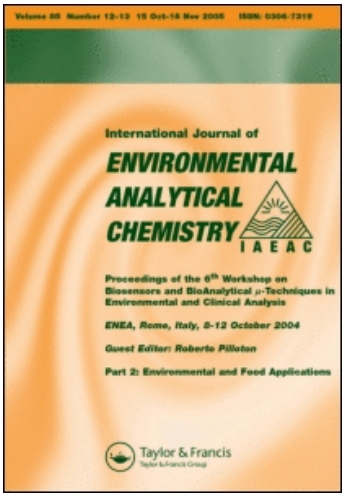

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SPE-HPLC/DAD determination of trimethoprim, oxytetracycline and enrofloxacin in water samples

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In this paper high-performance liquid chromatography with diode array UV detection (HPLC/DAD) after SPE sample pretreatment for simultaneous analysis of pharmaceuticals from three different classes: enrofloxacin, oxytetracycline and trimethoprim, has been developed, optimised and validated. The chromatographic separation was developed on spiked wellspring water samples and checked on model wastewater samples of veterinary pharmaceuticals before and after RO/NF membrane treatment (feed and permeate streams) and on process wastewaters of industrial origin. Chromatographic separation was performed on Varian ProStar HPLC/DAD with C-18 column (Microsorb-MV 100 C18, 150 × 4.6 mm; 5 μm, Varian, USA). Detection and quantification was performed at 254 nm. The best separation was achieved with mobile phase 0.5% formic acid and 1% trifluoroacetic acid in 0.05 M ammonium acetate-methanol, 70 + 30, (*v*/*v*) after extraction procedure on polystyrenedivinylbenzene Varian Empore extraction disks. The extraction efficiency was checked by recovery experiments.

Keywords: veterinary antibiotics; trimethoprim; oxytetracycline; enrofloxacin; water; solid-phase extraction (SPE); high-performance liquid chromatography (HPLC); membrane treatment

1. Introduction

In the last decade it is documented with increasing frequency that many chemical constituents that have not historically been considered as contaminants are present in the environment on a global scale. Among them are veterinary and human antibiotics. These 'emerging contaminants' are commonly derived from municipal, agricultural (livestock, poultry production and fish farming), and industrial wastewater (process wastewater) sources and pathways. The amount of pharmaceuticals and their bioactive metabolites being introduced into the environment is likely to be low. However, their continuous environmental input may lead to a high long-term concentration and promote continual, but unnoticed adverse effects on aquatic and terrestrial organisms. Many believe that of all the emerging contaminants, antibiotics are ones the biggest concern

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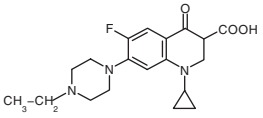
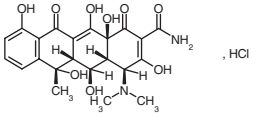
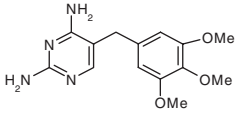
Table 1. Physicochemical properties of enrofloxacin, oxytetracycline and trimethoprim.

Compound	Antibiotic class	CAS no.	M_w/ g mol^{-1}	pK_a	Log K_{ow}
Enrofloxacin	Fluoroquinolones	93106-60-6	359.4	6.27; 8.3	1.1
					
Oxytetracycline	Tetracyclines	79-52-2	496.5	3.27; 7.32; 9.11	-1.22
					
Trimethoprim	Diaminopyrimidines	738-70-5	290.3	3.23; 6.76	0.73
					

because of the potential risk for antibiotic resistance. The increasing use of these drugs during the last five decades has caused a genetic selection of more harmful bacteria, which is a matter of great concern [1–3].

Antibiotics are defined as chemical compounds that are synthesised through the secondary metabolism of living organisms, with exceptions for semi- or completely synthetic substances. In Europe, two thirds of all antibiotics are used in human medicine and one third for veterinary purposes [3,4]. These newly recognised contaminants represent a shift in traditional thinking as many are produced industrially yet are dispersed to the environment from domestic, commercial and industrial uses.

The antibiotic compound classes primarily administered in veterinary medicine are tetracyclines, sulfonamides + diaminopyrimidines, aminoglycosides, beta-lactams and macrolides. In Croatia the most prescribed and manufactured antimicrobial agents are oxytetracycline (OTC), enrofloxacin (ENRO) and trimethoprim (TMP) (Table 1).

OTC is from tetracycline (TCs) compound class, used to treat infections of the respiratory and urinary tracts, skin, ear, eye and Gonorrhoea. The drug is particularly useful when penicillins and/or macrolides cannot be used due to allergy [4]. ENRO is fluoroquinolone (FQs), used for treatment of individual pets and domestic animals, and also in water to treat flocks of poultry [4]. TMP is of diaminopyrimidine compound class. It is a dihydrofolate reductase inhibitor, commonly prescribed with sulfonamides antimicrobial agents as synergist and mainly used in the prophylaxis and treatment of urinary tract infections [5].

Many analytical methodologies are available for determination of emerging contaminants, and also for antibiotics, mainly based on LC-MS or GC-MS. The use of expensive instrumentation, such as LC-MS(-MS) and GC-MS(-MS), with more sensitive and selective detection means that a less rigorous sample clean up is required [6,7].

Even though current analytical methodologies developed for the determination of antibiotics in environmental samples are based on LC-MS, HPLC-DAD has been the common method widely used in the determination of antibiotics. High-performance liquid chromatography with diode array detector (HPLC/DAD) after solid phase extraction is one of the most popular methods to resolve this problem, because if its simplicity and robustness. Our research activities were focused to develop an analytical method to measure and monitor these pharmaceutically active substances in a variety of matrices and to check the method for their removal. These three substances were chosen due to their high production in the plant of veterinary pharmaceutical industry.

To unburden surface recipients from pharmaceutical active compounds, their efficient removal is recommended. As conventional water treatment processes are often ineffective in the removal of emerging contaminants, methods such as carbon adsorption, advanced oxidation processes (AOP) and modern pressure driven membrane processes reverse osmosis and nanofiltration (NF/RO) are used for this purpose [8]. The second aim of this study was to investigate the antibiotics removal efficiency RO/NF pilot plant to find the optimal membrane type and operating conditions. The efficiency of several RO/NF membranes tested in a laboratory set-up, shown and described previously [9], was determined by relating the solute rejections to membranes' porosity. Membranes chosen for examinations were: the reverse osmosis membranes: XLE and HR95PP (Dow/FilmTec, Midland MI), TFC-S (Koch Membrane Systems, Wilmington, MA), the nanofiltration membranes: NF90 (Dow/FilmTec) and HL (Desal, Osmonics, GE Infrastructure Water Process Techn., Vista, CA). The NF/RO experiments were carried out with the artificial model wastewaters.

2. Experimental

2.1 *Antibiotic standards and reagents*

The antibiotics studied were: trimethoprim (TMP), oxytetracycline (OTC) and enrofloxacine (ENRO). All antibiotic standards (Veterina, Animal Health Ltd., Kalinovica, Croatia) were of analytical grade (>98%). The chemical structures and physicochemical properties of the studied antibiotics are shown in Table 1.

Stock solution of antibiotic mixture was prepared by dissolution of accurate quantities of powdered samples in 100 μL of 0.1 M sodium hydroxide and adjusting to 100 mL with methanol. Mass concentration of each antibiotic in the mixture was 100 mg L^{-1}. The solution was stored at 4°C in the dark. Calibration standards were made by serial dilution of the stock solution with methanol in the range of 0.1–70 mg L^{-1}.

Solvents used for solid-phase extraction (SPE) were p.a. grade (Kemika, Zagreb, Croatia) and for high-performance liquid chromatography were HPLC grade (Merck, Darmstadt, Germany).

2.2 *Sample preparation*

Water samples free of antibiotics were taken from the wellspring Borčec, Zagreb, Croatia. Physical and chemical tests were done on Borčec water, whose characterisation was presented elsewhere [5].

Production wastewater was collected from a wastewater treatment plant which consists of a primary settlement and receives only industrial wastewater. The chemical synthesis

Table 2. Compositions of artificial model wastewaters (containing ~130 mg L^{-1} Ca^{2+}).

	Water no. 1	Water no. 2
NaCl	1000 mg L^{-1}	1000 mg L^{-1}
Na_2HPO_4	230 mg L^{-1} (50 mg L^{-1} P)	–
$MgCl_2$	–	200 mg L^{-1}
pH	7.4–7.5	7.4–7.5
Citric acid	50 mg L^{-1}	50 mg L^{-1}
Ascorbic acid	30 mg L^{-1}	–
Sucrose	100 mg L^{-1}	–
Ca gluconate	–	100 mg L^{-1}

processes of the veterinary pharmaceuticals industry produce wastewaters which are variable in character depending on the production. Wastewater was slightly opalescent to turbid, colourless to grey or light yellow. Before analysis, wastewater samples were filtered through a Büchner, black Whatman and 0.45 μm nylon filters.

Two artificial model wastewaters similar to those from equalising basins of the veterinary pharmaceuticals works were prepared. The compositions (relevant values) are given in Table 2.

Wellspring water samples and wastewater samples were collected in amber glass bottles. Prior to extraction, all water samples used in this study were filtered with Whatman filter paper to eliminate the suspended matter and then filtered with the 0.45 μm nylon membrane filter. The samples were stored at 4°C until SPE extraction, which was performed within 24 h in order to avoid any degradation.

The spiked wellspring water samples were prepared from pre-filtered water by addition of 1 mL of stock standard solutions of antibiotics mixture to 500 mL of water. The artificial model wastewaters were spiked with the mixture of antibiotics so the final concentration of each antibiotic was 10 mg L^{-1}.

2.3 *Solid-phase extraction*

Solid-phase extraction experiments were performed using 47 mm Empore extraction disks (polystyrene divinylbenzene (SDB) and C18) and a Vac Elute extraction manifold (Varian, Harbor City, USA). Before sample application, the extraction disks were washed with 20 mL of eluent and conditioned by passing 20 mL of methanol under vacuum. Prior to extraction, sample pH was adjusted to pH = 3 (HCl conc.) and afterwards, 500 mL of water sample were pre-concentrated onto the extraction disks. The adsorbent (disc) was never allowed to dry during the conditioning and sample-loading procedure. After the passage of the sample, the disks were air dried for 10 min. The analytes retained were eluted with 2 × 10 mL of ethanol. Following elution, the eluates were evaporated on a rotary evaporator (R-114/A; Büchi, Switzerland) at 40°C to dryness and the residues were dissolved in 1 mL of methanol.

2.4 *High-performance liquid chromatography*

The analyses were carried out on a Varian ProStar 500 (Walnut Creek, California, USA) separations module equipped with a ProStar 230 tertiary solvent delivery module and

a manual injection system with 20 μL loop, coupled with a ProStar 330 photodiode array detector. Chromatographic separation of standard solutions and sample extracts were obtained on a commercial Microsorb-MV 100 C18 (150 × 4.6 mm; 5 μm) (Varian, USA) column at 30°C. Isocratic elution was carried out with aqueous 0.5% formic acid and 1% trifluoroacetic acid in 0.05 M CH_3COONH_4 : MeOH (70 : 30 v/v) at a flow rate of 1 mL min^{-1}. The acquisition wavelengths were 254 nm for all tree antibiotics. Chromatographic system and data collection were controlled with a Star Chromatography Workstation System Control Version 5.52 chromatographic software interfaced to a personal computer.

3. Results and discussion

3.1 *HPLC-DAD*

Chromatographic separation was achieved on C18 chromatographic column. Improved resolution of different components was achieved by manipulating the solvent and additive composition (formic acid, trifluoroacetic acid, acetonitrile and methanol were used), the volume proportion of solvents in mobile phase and the concentration of formic acid. The first experiments for separation of studied analytes were made using the mobile phase from the literature. Based on this, 0.1% formic acid in water and 0.1% formic acid in 0.5 M NH_4CH_3COOH were tested as aqueous phase and acetonitrile as organic phase. Both mobile phases were applied in different ratios. With these different mobile phases separation was not good, and analytes were not retained. Retention time for all studied analytes was maximum three minutes and chromatographic peaks were overlapped. For this reason, acetonitrile was replaced by methanol and trifluoroacetic acid was added in the aqueous phase in order to narrow chromatographic peaks. Ratios of organic and aqueous phase were chosen and tested using experimental design. Therefore, the best resolution was achieved with mobile phase composed of 0.5% formic acid and 1% trifluoroacetic acid in 0.05 M ammonium acetate-methanol, 70 : 30 (v/v).

Experimental calibration curves were prepared for each compound by plotting the peak area versus the analyte concentration. The results were analysed by linear regression method. Coefficients of correlation were higher than 0.997 thus confirming the linearity of the method (Table 3).

Limits of detection (LOD) and quantification (LOQ) were determined from the signal-to-noise ratio. Detection limit was determined as a signal-to-noise ratio of 3 and limit of quantification as a signal-to-noise ratio of 10. The limits of detection and quantification are reported in Table 3.

Repeatability, expressed as relative standard deviation (RSD), was determined by repeated analysis of the antibiotic mixture using the same equipment and the same analytical procedure. Repeatability was evaluated in the middle of the linearity range. Results are listed in Table 3. Satisfactory results were achieved for all antibiotics with RSDs lower than 0.02% for injection of samples five times, indicating high measurement repeatability of the HPLC-DAD method.

3.2 *SPE*

When an analyte is present at low concentration in complex environmental samples, such as wastewaters, extraction and pre-concentration must precede HPLC analysis.

Table 3. Validation parameters of antibiotics by HPLC-DAD.

Antibiotic	Linearity range ($mg\,L^{-1}$)	R^2	LOD ($mg\,L^{-1}$)	LOQ ($mg\,L^{-1}$)	RSDinj (%) ($n=5$)
Trimethoprim	1.4–70	0,9998	0.5	1.4	0.06
Oxytetracycline	1.4–70	0.9974	0.5	1.4	0.02
Enrofloxacin	1.0–100	0.9983	0.1	1.0	0.03

Table 4. Mean recoveries (%) and RSDs (%) of antibiotics investigated obtained with C18 and SDB Empore extraction disks.

Empore disks	pH of water samples	Solvent	Sample recovery, % ($n=3$)		
			Trimethoprim	Oxytetracycline	Enrofloxacin
SDB	pH = 3.0	Methanol	74.0 ± 4.7	76.4 ± 3.0	90.7 ± 8.7
		Ethanol	69.8 ± 7.7	77.3 ± 9.1	96.1 ± 6.2
	pH = 7.8	Methanol	118.2 ± 5.4	55.0 ± 12.2	76.7 ± 31.2
		Ethanol	115.8 ± 5.1	42.1 ± 13.4	74.8 ± 6.5
C18	pH = 3.0	Methanol	10.0 ± 26.4	12.0 ± 10.4	87.8 ± 1.9
		Ethanol	9.3 ± 58.4	14.5 ± 3.6	72.9 ± 8.6

In this work, solid-phase extraction with SDB and C18 Empore extraction disks was used for sample preparation. The extraction recoveries of the analytes were estimated using spiked wellspring water samples. Antibiotics were extracted from water adjusted at pH 3.0 and/or 7.8 they were eluted from the disks with methanol or ethanol. The spiked samples were extracted in triplicate and analysed by HPLC-DAD. The recovered amount was calculated as peak area ratio of extract and standard solution. Extraction recoveries for all investigated antibiotics are given in Table 4.

C18 Empore disks showed very low recoveries, especially for OTC and TMP. These results were expected, because silica based sorbents have several disadvantages, like low recovery of polar compounds. Moreover, the residual silanol group affect on sorption of tetracyclines. For instance, oxytetracycline irreversibly binds silanol groups through metal ions present in water. This situation could be avoid by adding a chelating agent, such as EDTA (ethylenediaminetetraacetic acid), but that would imply one more step in sample preparation. Besides this, C18 is the most hydrophobic sorbent but all investigated compounds are polar (see $\log K_{OW}$ value in Table 1). Applying the C18 Empore discs, the highest average recovery was obtained for ENRO, because it is the most hydrophobic compound among the three studied. In contrast with the poor retention of antibiotics investigated observed when using C18 discs, SDB proved to be a more efficient choice for all of them.

From results shown in Table 4 it is evident that better antibiotic recoveries were obtained by SDB at pH 3.0. Reason for that probably lies in physico-chemical properties (pK_a values) of investigated antibiotics shown in Table 1. Oxytetracycline is an amphoteric molecule more stable in acids, as opposed enrofloxacin which is very stable compounds in all media. Trimethoprim shown good recoveries in both media although high recovery (>100%) of TMP at pH 7.8 is a probably consequence of some different mechanism.

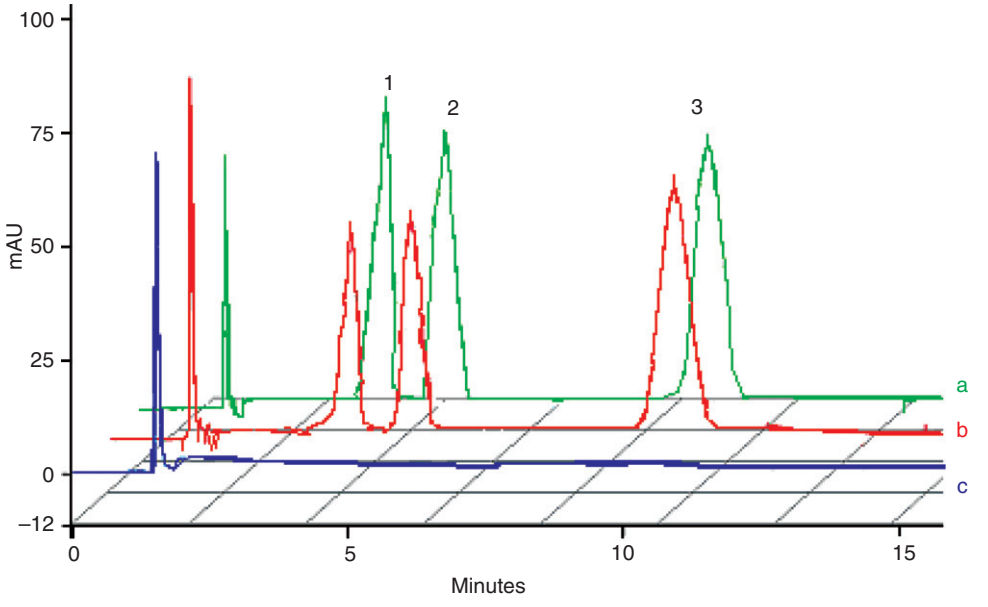


Figure 1. Chromatogram of antibiotic mixture: (a) standard solution (10 mg L^{-1}), (b), antibiotic mixture extracted from spiked wellspring water sample (SDB disc, 20 mL of ethanol, pH = 3.0), (c) blank extract; 1 – trimethoprim; 2 – oxytetracycline; 3 – enrofloxacin.

Recoveries obtained with methanol and ethanol are very similar at same pH value, so we suggest the use of ethanol because of its lower toxicity for the environment. Apparent recoveries were 96.1 ± 6.2 for enrofloxacin, 77.3 ± 9.1 for oxytetracycline, and 69.8 ± 7.7 for trimethoprim.

Blanks (non-spiked water samples) were also analysed in all experiments, using the same procedure, in order to detect any possible contribution of water matrix to antibiotic signals in the spiked samples. Signals obtained from spiked wellspring water samples were compared with signals from wellspring water without standard addition and with those obtained from standard solutions. Since no interference peaks were detected in extracted samples, they could be chromatographed without additional clean-up steps.

Chromatograms of standard antibiotic mixture, extracted samples and blank extract are shown in Figure 1.

3.3 *Applications*

The described method was applied to the determination of TMP, OTC and ENRO in two wastewater samples from pharmaceutical industry. Before analysis, wastewater samples were filtered and acidified to pH 3.0. Five hundred millilitres (500 mL) of prepared wastewater samples were applied to SDB disk and ethanol eluate were evaporated to dryness, residues were dissolved in 1 mL of methanol and injected to HPLC system. The factors used to confirm the presence of the target compounds in these samples were: retention time and UV spectrum. Chromatograms of wastewater samples obtained with diode array detector are shown in Figure 2.

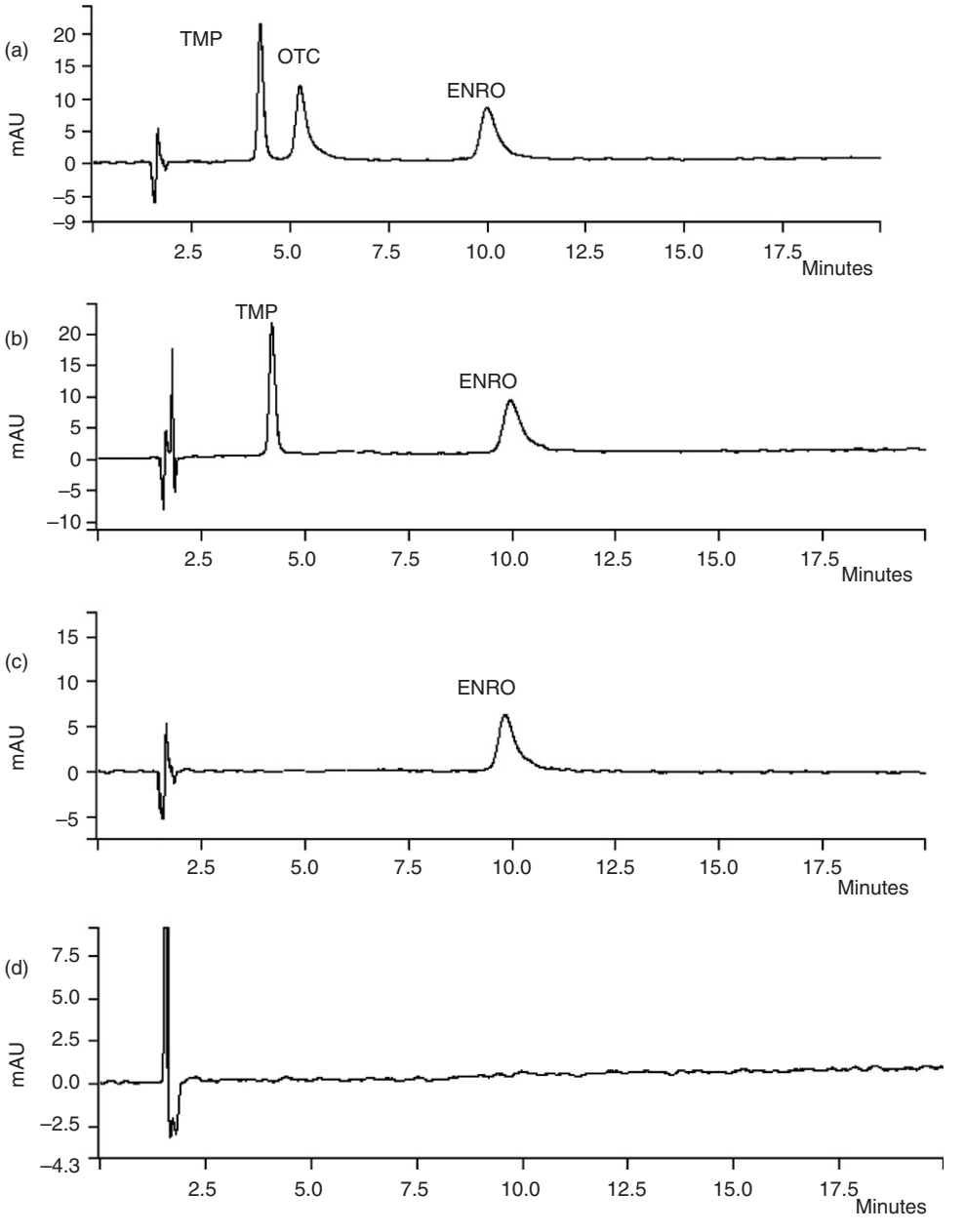


Figure 2. HPLC-DAD chromatograms of: (a) standard antibiotic mixture ($10\,mg\,L^{-1}$), (b) wastewater sample 1 (WWS1), (c) wastewater sample 2 (WWS2) and (d) blank extract.

ENRO was determined in both samples ($\gamma(ENRO)_{WWS1} = 18.8\,\mu g\,L^{-1}$ and $\gamma(ENRO)_{WWS21} = 10.0\,\mu g\,L^{-1}$), but TMP was determined only in WWS1 at concentration $28.0\,\mu g\,L^{-1}$. OTC was not detected in analysed wastewater samples.

The developed method was also applied to optimisation of laboratory-scale membrane filtration unit for the removal of selected antibiotics. Different membranes were chosen for

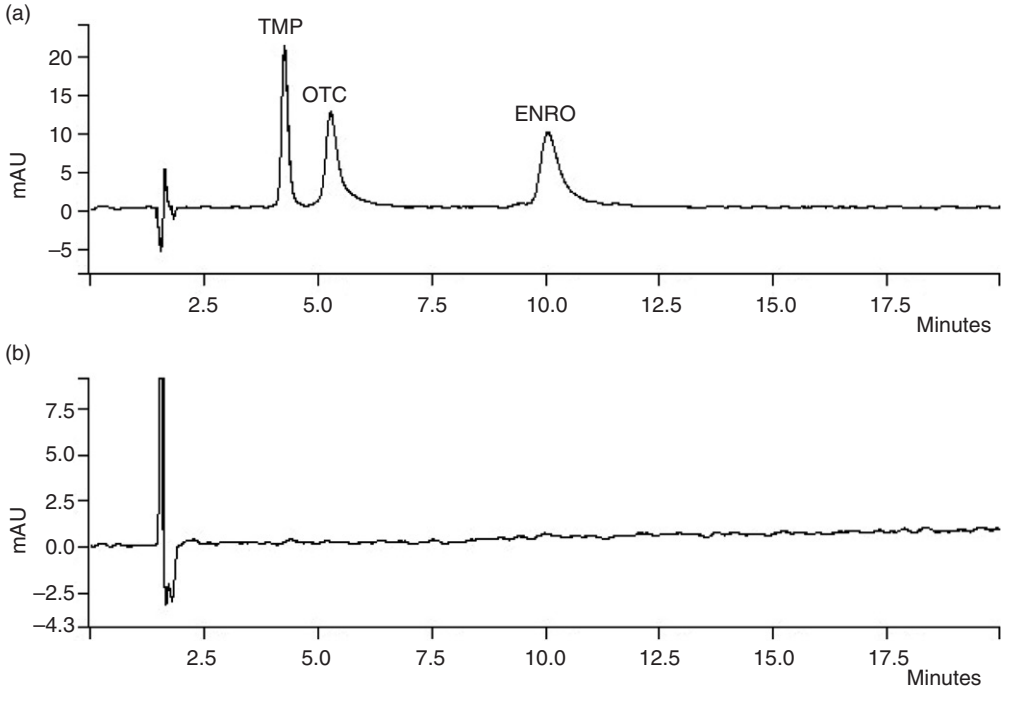


Figure 3. Chromatograms of artificial model wastewater 1 (AMWW1) before (a) and after (b) RO membrane treatment (HR95).

examinations: the reverse osmosis membranes: XLE and HR95PP (Dow/FilmTec, Midland MI), TFC-S (Koch Membrane Systems, Wilmington, MA), the nanofiltration membranes: NF90 (Dow/FilmTec) and HL (Desal, Osmonics, GE Infrastructure Water Process Techn., Vista, CA). The surface area of the investigated membranes was 10.8 cm^2. The membranes were pressurised at 25 bars for 8 h before the pure water flux measurements. The NF/RO experiments with the binary solutions of sodium and calcium chloride, the organic markers and the antibiotics were of the short-run type, each lasting for about three hours. The experiments were carried out at laboratory temperature and various pressures. The NF/RO experiments with the artificial model waters were carried out until the sufficient quantity of the permeate for the analysis was collected.

The performed experiments showed the complete rejection of all three antibiotics by the RO membranes (HR95, XLE, TFC-S) and by the nanofiltration membranes (NF90, HL), respectively, all concentration of antibiotics in permeates after RO/NF membrane processes are less than the limit of quantification (LOD, $\mu g\,L^{-1}$). Chromatograms of modal wastewaters before and after membrane treatment are shown in Figures 3 and 4.

The rejection factor R, is defined as

$$R = 1 - \frac{c_p}{c_f} \tag{1}$$

where c_p and c_f are antibiotic concentration ($mg\,L^{-1}$) in permeate and feed, respectively.

In order to determine the most suitable membranes for the removal of antibiotics from the plant wastewaters by the planned pilot experiments, the porosity of five membranes

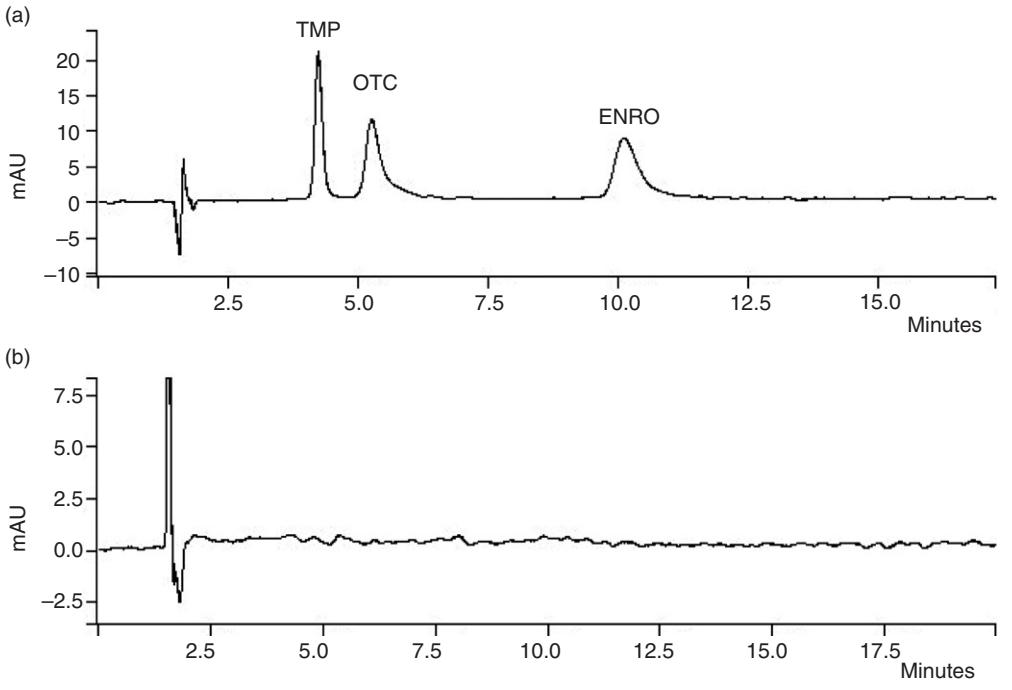


Figure 4. Chromatograms of artificial model wastewater 2 (AMWW2) before (a) and after (b) NF membrane treatment (HL).

was previously determined by the modified examination method based on the specific solutes (markers) transport [9–13]. The results are presented in literature [8]. Pore size distribution (PSD) of the examined membranes' active layer obtained at 8 bar showed differences between the examined membranes. The corresponding calculated 'effective numbers of pores' altogether with the water permeation show that rejection of solutes is governed by size exclusion and strongly depend on the distribution of the membrane pore sizes. The higher membrane flux of the NF90 membrane type would assure the lower energy consumption and lower operating costs for the antibiotics removal, and its on average wider pores would be less susceptible to pore fouling in the real wastewater cleaning process [14–18].

4. Conclusion

Enrofloxacin, oxytetracycline and trimethoprim, which have very different physico-chemical properties, were extracted and pre-concentrated from spiked water samples by solid-phase extraction (SPE) and quantitatively determined by high-performance liquid chromatography with diode array detector (HPLC). This procedure resulted in good recoveries for all the antibiotics used in this study. Because the extracts of SPE can be chromatographed without further pretreatment, the analysis time is substantially reduced.

The developed method was successfully applied for wastewater analysis and for testing the RO/NF membrane pilot facility.

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