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Original research paper

Simultaneous determination of macrolides in water samples by solid-phase extraction and capillary electrophoresis

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ABSTRACT

Solid-phase extraction (SPE) coupled with capillary electrophoresis (CE) for the determination of macrolide antibiotics (azithromycin, clarithromycin, roxithromycin, tylosin) and tiamulin in water samples was described in this article. These compounds were extracted with different types of sorbents (Oasis HLB, C18, C8, SDB, and Strata-X) and different masses of sorbents (60 mg, 200 mg, and 500 mg) using different organic solvents (methanol, ethanol, and acetonitrile) and different pH values of water samples (pH 7.00, 8.00, and 9.00). It was found that the highest extraction efficiency of the studied compounds was obtained with 200 mg/3 mL C18 cartridges with methanol as eluent at pH 9.00 of the water sample. The developed SPE-CE method for macrolide antibiotics and tiamulin was validated for linearity, precision, repeatability, the limit of detection (*LOD*), the limit of quantification (*LOQ*), and recovery. Good linearity was obtained in the range of 0.3–30 mg L^{-1} depending on the drug, with correlation coefficients higher than 0.9958 in all cases except clarithromycin (0.9873). Expanded measurement uncertainties were calculated for each pharmaceutical, accounting for 20.31 % (azithromycin), 38.33 % (tiamulin), 28.95 % (clarithromycin), 26.99 % (roxithromycin), and 21.09 % (tiamulin). Uncertainties associated with precision and calibration curves contributed the most to the combined measurement uncertainty. The method was successfully applied to the analysis of production wastewater from the pharmaceutical industry.

Keywords: macrolides, tiamulin, solid-phase extraction, capillary electrophoresis, wastewater, measurement uncertainty

INTRODUCTION

Antibiotics represent a large group of natural and semisynthetic products that are used intensively for the treatment and prevention of diseases (1). There are several groups of antibiotics (penicillins, tetracyclines, macrolides, aminoglycosides, and amphenicols), with macrolides being the most commonly used after penicillins.

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The macrolide antibiotics comprise a family of antibacterial agents widely used in human and veterinary medicine. Consequently, vast usage in all aspects of medicine leads to their presence in the environment. There have been many research studies dealing with the occurrence of pharmaceuticals in the environment, among them macrolide antibiotics. The drugs are not being removed during conventional wastewater treatment plant (WWTP) and they are present not only in wastewater influent but also in effluent (2–5) and consequently in environmental waters (2, 6, 7). It can be expected that if the WWTP is not adapted to the expected compounds (*e.g.*, pharmaceuticals, which is usually the case, since new pharmaceuticals are constantly coming on the market), some of them will appear in the WWTP effluents, even in high concentrations, which is confirmed by numerous scientific papers (8–11). The highest concentration of pharmaceuticals is found in process wastewater, as these waters are generated by washing production equipment and mixing with wastewater effluent. Although the presence of pharmaceuticals is constantly monitored (both before or after wastewater treatment and in the environment), there is always a need to develop new analytical methods. Multiresidue analytical method for the simultaneous monitoring of pharmaceutical residues is a prerequisite for obtaining reliable data on the pharmaceutical's behavior in WWTPs. The information obtained from the analysis of WWTP influent and effluent samples can be used to optimize the treatment process and for a possible pretreatment step that would prevent the emission of undesirable pollutants into the receiving water.

Regardless of their definite presence in the environment, there are no legal regulations specifying their maximum discharge concentrations.

All macrolide antibiotics have a similar chemical structure which includes a macrocyclic lactone ring with usually 12 to 16 atoms and one or more neutral deoxy and/or aminosugars linked by glycosidic bonds (12). The first discovered macrolide antibiotic is erythromycin and its synthetic derivates such as clarithromycin, roxithromycin, and azithromycin are very important antibiotics for treating human infectious diseases.

The most common methods for the determination of pharmaceuticals in aquatic environmental matrices consist of solid-phase extraction as sample preparation followed by chromatographic analysis (gas chromatography or liquid chromatography). The main disadvantage of gas chromatography for pharmaceutical analysis is that this technique is limited to volatile and thermally stable compounds and that most pharmaceutical products, which are polar substances, must be derivatized beforehand. For this reason, liquid chromatography is much more widely used in pharmaceutical analysis (as well as in the analysis of macrolides), especially in combination with mass spectrometry (LC-MS) (13, 14). In addition to liquid chromatography coupled with a mass spectrometry detector, macrolides can also be analysed with other detectors such as UV, fluorescence, and electrochemical (15, 16, 17, 18). UV detection is often not a good choice and a good alternative is determination with a fluorescence detector, which, however, requires chemical derivatization (16, 17). Electrochemical detection, including coulometric and amperometric methods, is considered one of the most efficient and sensitive because most macrolides have a suitable electroactive group (18). However, when multiple macrolides need to be determined in one analysis and their identity confirmed, the aforementioned techniques are insufficient, and mass spectrometry (MS), for all its advantages, has a price disadvantage (19).

The literature contains little information on the determination of tiamulin. Liquid chromatography is usually used for the determination of medical formulations, although gas chromatography analysis is also possible after prior derivatization. However, considering

the physical and chemical properties of the compound, liquid chromatography with UV detection is preferred, with the current trend toward the use of LC-MS (20). After all that has been said before, it should be emphasized here that no method has been found in the literature for this group of macrolides (tylosin is always somehow separated from the others) and tiamulin, and we have undertaken the development of a new analytical method that is reliable but also economically acceptable for the given purpose. Capillary electrophoresis (CE) is a very promising analytical technique with a diverse application range (21–25). Originally considered primarily for the analysis of biological macromolecules it has proven to be efficient in separating many different compounds (amino acids, chiral drugs, vitamins, pesticides, inorganic ions…) (26–30). The extension of CE usage in different fields is due to its advantages when compared with more commonly used chromatographic techniques. These advantages are: high efficiency and selectivity, simpler method development, minimal sample volume requirements, low solvent consumption, and lack of organic waste which makes it a preferable green chemistry solution (31).

When speaking of analysis of environmental samples, the sample preparation step is unavoidable. The purpose of the sample preparation step is clean-up of the sample by removing potential interferences and achieving lower limits of detection and quantification. Another purpose is preconcentration which is beneficiary for the analysis with CE. The main drawback of CE is the lack of sensitivity especially for the most widely used ultraviolet absorbance detection (UV). This is because of the short optical path length and the low sample volume (32). Solid phase extraction (SPE) is one of the widely used analytical/pre-treatment techniques due to its many advantages. It is simple to use, affordable, and applicable to different analytes. It also yields higher recoveries, it is selective, specific, and reproducible and it uses low quantities of organic solvents (33).

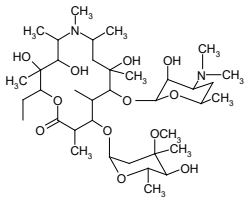
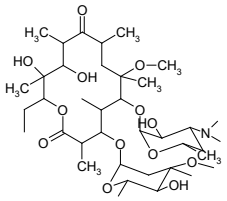
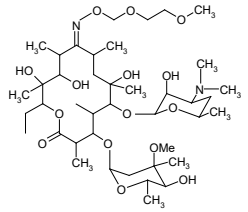
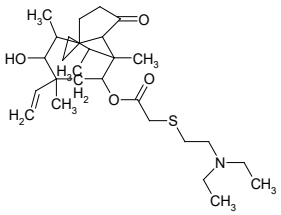
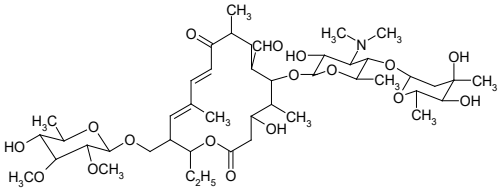
The aim of this work was to optimize and validate a fast, reliable, and affordable analytical method for the determination of chosen macrolide antibiotics (azithromycin, clarithromycin roxithromycin, tylosin, and tiamulin) in wastewaters. Solid phase extraction was used for sample preparation because of its wide usage for the preparation of water samples and its effectiveness. The samples were analysed by CE and good separations were achieved. CE was the method of choice because it has been proven to be a highly efficient and rapid analytical technique for the analysis of diverse types of analytes. The method was successfully applied for the analysis of real wastewater samples. Using straightforward techniques such as SPE and CE satisfactory results were obtained in the analysis of macrolide antibiotics in water samples which indicates that this method can be used for routine analysis of macrolide antibiotics in water samples. The need for monitoring macrolide antibiotics in water samples is becoming even more significant considering their potential negative influence on the environment.

EXPERIMENTAL

Chemicals and materials

The pharmaceuticals used in the study are: azithromycin (AZI) was obtained from Pliva, Croatia; clarithromycin (CLARY) and roxithromycin (ROXY) were purchased from Sigma-Aldrich, Germany; tylosin (TYL) and tiamulin (TIA) were obtained from Genera d.o.o., Croatia. All pharmaceutical standards were high-purity grade (> 99 %). The studied pharmaceuticals and their physiochemical properties are shown in Table I.

Table I. Chemical structures and physicochemical properties of studied pharmaceuticals

	Empirical formula	CAS no.	pK_a (34)	log K_{ow} (35)
AZI		083905-01-5	8.96 ± 0.04	3.24
CLARY		081103-11-9	8.79 ± 0.06	3.18
ROXY		80214-83-1	8.82 ± 0.04	2.75
TIA		055297-95-5	8.90 ± 0.09	4.75
TYL		001401-69-0	7.71 ± 0.03	1.05

AZI – azithromycin, TIA – tiamulin, CLARY – clarithromycin, ROXY – roxithromycin, TYL – tylosin

Table II. Composition of buffers (50 mmol L^{-1} ionic strength)

pH	Buffer composition
4.89	5.2 mL 1 mol L^{-1} HCOOH + 5 mL 1 mol L^{-1} NaOH in 100 mL water
5.51	5.6 mL 1 mol L^{-1} CH_3COOH + 5 mL 1 mol L^{-1} NaOH in 100 mL water
6.02	19 mL 0.5 mol L^{-1} NaH_2PO_4 + 2 mL 0.5 mol L^{-1} Na_2HPO_4 in 250 mL water
6.50	5.3 mL 0.5 mol L^{-1} NaH_2PO_4 + 1.5 mL 0.5 mol L^{-1} Na_2HPO_4 in 250 mL water
7.10	2 mL 0.5 mol L^{-1} NaH_2PO_4 + 2.5 mL 0.5 mol L^{-1} Na_2HPO_4 in 100 mL water
7.88	1 mL 0.5 mol L^{-1} NaH_2PO_4 + 8 mL 0.5 mol L^{-1} Na_2HPO_4 in 250 mL water
9.19	4.2 mL 1 mol L^{-1} $NaHCO_3$ + 0.3 mL 1 mol L^{-1} NaOH in 100 mL water

A stock solution of the pharmaceutical mixture was prepared by dissolving accurate quantities of the powdered standards in 50 % methanol. The mass concentrations of each pharmaceutical in the mixture were 1.0 mg mL^{-1}. Calibration standards were made by serial dilution of a stock standard solution in the working range from 0.06 to 3.0 mg mL^{-1} for AZI, and from 0.02 to 1.0 mg mL^{-1} for CLARY, ROXY, TYL, and TIA. All standard solutions of pharmaceuticals were stored protected from light at 4 °C and were stable for four weeks. The stability of the solution is confirmed by periodically checking the solution, paying attention to the reactions of the components in the standard solution, good separation, the unaffectedness of the measured signal by other substances, and the absence of additional reactions that could be a consequence of decomposition.

The following chemicals were used to prepare the buffers: sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium hydrogen carbonate, formic acid, acetic acid, boric acid, sodium chloride, and sodium hydroxide. The manufacturer of all chemicals listed except disodium hydrogen phosphate was Kemika (Croatia), while disodium hydrogen phosphate was purchased from Merck (Germany). All chemicals were *p.a.* grade. Deionized water (Milli-Q deionizer, Millipore, USA) was used to prepare the solvent mixture. Stock solutions of 1 mol L^{-1} formic acid, 1 mol L^{-1} sodium hydroxide, 0.5 mol L^{-1} sodium dihydrogen phosphate, 1 mol L^{-1} sodium hydrogen carbonate, and 0.5 mol L^{-1} disodium hydrogen phosphate were used to prepare buffer solutions for the pH range of 4.89 to 9.19. Buffer solutions with a constant ionic strength of 0.05 mol L^{-1} were prepared by mixing the appropriate amounts of the solutions shown in Table II.

Methanol (20, 50, and 80 %) was used as an electroosmotic flow (EOF) marker. All solutions were filtered through a 0.45 μm pore size hydrophilic polypropylene membrane filter (PALL Life Science, USA) before analysis.

In this work different SPE columns were used: 60 mg/3 mL Oasis HLB was purchased from Waters, Labtim, Croatia; 200 mg/3 mL Strata X, Strata SDB-L, Strata C18, and Strata C8 were purchased from Phenomenex, Vita Lab Nova d.o.o, Croatia.

The pharmaceuticals free water sample was collected from the wellspring Borčec (45°49′ N, 15°52′ E) in the surrounding of Zagreb, Croatia.

Water sample preparation

In the development and optimization of the method, real water without pharmaceutical additives was used. The water was taken from the Borčec Spring near Zagreb, Croatia. Immediately before analysis, water samples were filtered through 0.45 μm nylon filters (Pall Corporation, USA) to remove particles.

The optimized SPE-CE method was applied to real wastewater samples analysis. The wastewater samples were collected from a pharmaceutical industry wastewater treatment plant. The wastewater samples also had to be filtered immediately before analysis, and since they were much more contaminated than source water, a Büchner funnel (black Whatman) was used first, followed by filtration with a 0.45 μm nylon membrane.

Amber glass bottles pre-rinsed with ultrapure water were used for sample collection. All water samples were stored at 4 °C until SPE extraction, which was performed within 24 h.

Solid-phase extraction

The solid-phase extraction procedure was developed by using 100 mL of spring water to which 1 mL of stock standard solution of the mixture of the tested pharmaceuticals was added, resulting in a concentration of 10 mg L^{-1} of the tested pharmaceuticals in the water. The tested pharmaceuticals from the samples thus prepared were extracted using an SPE device (Supelco, USA). Before extraction of each sample, the SPE cartridges were preconditioned with 5 mL of methanol, followed by 5 mL of deionized water. After passing the water sample through the SPE column, the column had to be dried under vacuum, after which the retained pharmaceuticals were eluted with 5 × 2 mL of methanol. The extracts were evaporated on a rotary evaporator (140 rpm) at 40 °C to form a dry residue, and the dry residue was dissolved in 1 mL of 50 % methanol.

In parallel with the extraction of the water samples prepared with a pharmaceutical mixture, an extraction experiment with pure water (without added pharmaceuticals) was performed in all experiments, which allowed the possible contribution of the water matrix to the pharmaceutical signals of the enriched samples to be identified.

The extraction efficiencies of SPE cartridges were determined by capillary electrophoresis. In the first set of experiments, extraction efficiency was tested using different SPE cartridges (C18, C8, SDB, Strata-X, and Oasis HLB) with methanol as the elution solvent. pH values of the water sample were set to 9.00. In this set of experiments, the optimal SPE cartridge was selected.

In the second set of experiments, the optimal elution solvent was tested. For this purpose, methanol, ethanol, and acetonitrile were used. The pH of the water sample was the same as in the previous series of experiments.

In the third set of experiments, based on the previous results, the optimal pH value of the water sample was investigated. For this reason, the pH was adjusted to 7.00, 8.00, and 9.00.

In the final set of experiments, the optimal sorbent, the optimal elution solvent, and the optimum pH of the sample were applied in order to determine the optimal mass of the SPE column. For this purpose, 500 mg/3 mL C18 SPE column was used.

Capillary electrophoresis

CE experiments were carried out with an HP3DCE system (Hewlett-Packard, Germany) and Agilent High-Performance Capillary Electrophoresis (7100) system (Agilent Technologies, USA) equipped with an autosampler, automatic injector, photodiode array detector, and an air-cooling unit for the capillary. For controlling the CE system, data acquisition, and data analysis the ^{3D}CE/MSD ChemStation and OpenLab CDS, ChemStation Edition (Agilent Technologies, Rev. C.01.10 (201)) software were used.

Uncoated fused-silica capillaries (Agilent Technologies, Germany) with an inner diameter of 50 μm, an outer diameter of 363 μm, a total length of 56.0 cm, and an effective length (to the detector) of 48.0 cm were used for the analysis. The temperature of the capillary cassette was maintained at 25 °C with air cooling. Samples were hydrodynamically injected at a pressure of 50 mbar for 4.0 s. The operating voltage was 20 kV positive polarity, and the resulting electric current was 25 μA. The operating voltage was chosen based on a preliminary study in which operating voltages between 10 and 25 kV were tested. The optimized running buffer was 0.05 mol L^{-1} phosphate buffer pH 6.02 with 20 % methanol.

At the beginning of each working day, the fused-silica capillary was conditioned at 25 °C as follows: 15 min with 0.1 mol L^{-1} NaOH, 15 min with water, and finally 30 min with buffer pH 6.00. All solutions were filtered with 0.45 mm hydrophilic polypropylene membrane (Pall Life Sciences, USA) before being introduced into the instrument. UV detection was performed at 254 nm for 50 % methanol as an EOF marker. Analytes were detected at 214 nm (AZI, CLARY, ROXY, TIA, and TYL). After each working session, the capillary was flushed for 5 min with 0.1 mol L^{-1} NaOH, 5 min with deionized water, and dried for 10 min with air. The capillary was stored dry. Running buffers were replenished every five runs.

Validation

After the development of the SPE-CE method, validation was started, *i.e.*, the determination of method performance characteristics such as linearity, precision, detection, and quantification limit. Linearity was determined using five standard solutions of the pharmaceutical mixture with concentrations in the working range of 3.0 to 30.0 mg L^{-1} for AZI and seven concentrations in the range of 0.3 to 10.0 mg L^{-1} for CLARY, ROXY, TYL, and TIA in 50 % of methanol. Blank samples were prepared in parallel with the aforementioned samples but were not included in the regression analysis. The results were evaluated by the linear regression method.

The precision of the method was determined by repeating the analyses of the same extracts for one day (intra-day precision), but also on three consecutive days (inter-day precision), using the same equipment and the same analytical procedure. Limit of detection (*LOD*) and limits of quantification (*LOQ*) are determined by the signal-to-noise ratio. The limit of detection was determined as the signal-to-noise ratio of 3 and 10 for the limit of quantification.

Measurement uncertainty

The measurand, the mass concentration of each pharmaceutical, was calculated from the calibration Equation (*1*):

$$\gamma_0 = \frac{A - a}{b} \tag{1}$$

where γ_0 is an analyte concentration (mg L^{-1}), A is a peak area (number of pixels), a is the intercept and b is the slope of the calibration curve. The calibration curve already considers the pre-concentration of the sample by SPE.

After measurement definition, the next step is to identify uncertainty sources. For the developed SPE-CE method main uncertainty sources are: 1. uncertainty of sample volume measurement; 2. uncertainty due to the sample preparation, 3. uncertainty associated with the predicted concentration using the calibration curve, and 4. uncertainty due to the precision.

Uncertainty of sample volume measurement (100 mL ± 0.080 mL volumetric pipette) was calculated from the manufacturer's tolerance assuming triangular distribution:

$$u(V) = 0.080/\sqrt{3} = 0.0462 \text{ mL}.$$

The recovery was estimated from a triplicate analysis of spiked water samples at six concentration levels within a linear range. The exception was AZI, for which recovery was estimated at five concentration levels. According to the EURACHEM guide (36), the standard uncertainty for each pharmaceutical was calculated as the standard deviation of the mean (Equation *2*):

$$u(\bar{R}) = \frac{s}{\sqrt{n}} \tag{2}$$

The standard uncertainty for γ_0 due to the uncertainty of the prediction using calibration equation was calculated according to Equation *3* (36, 37):

$$u(\gamma_0) = \frac{s_{residual}}{b}\sqrt{\frac{1}{p} + \frac{1}{n} + \frac{(\gamma_0 - \bar{\gamma})^2}{\sum_{i=1}^{n}(\gamma_i - \bar{\gamma})^2}} \tag{3}$$

where $s_{residual}$ is the residual standard deviation of the calibration curve (Equation *4*):

$$s_{residual} = \sqrt{\frac{\sum_{i=1}^{n}\left[A_i - (b\gamma_i + a)\right]^2}{n-2}} \tag{4}$$

Uncertainty associated with the method precision was estimated for each pharmaceutical from the standard deviation obtained from intra-day precision experiments performed under method validation: u(precision) = s.

After the quantification of all identified sources of uncertainty for each analyte, the combined uncertainty $u_C(\gamma_0)$ was calculated using the Equation *5*:

$$u_c(\gamma) = \gamma \cdot \sqrt{\frac{u(V)}{V} + \frac{u(\bar{R})}{\bar{R}} + \frac{u(\gamma_0)}{\gamma_0} + u(precision)} \tag{5}$$

The final step in the evaluation of measurement uncertainty is to calculate the expanded uncertainty by multiplying the combined uncertainty by the coverage factor (k).

The expanded uncertainty U at a 95 % confidence level is obtained using k = 2. The expanded measurement uncertainty provides an interval ($\gamma \pm U$) within which the value of the measurand is believed to lie with a higher level of confidence.

RESULTS AND DISCUSSION

Optimization of capillary electrophoresis

The aim of the capillary electrophoretic separation of the five analytes of interest is to ensure good resolution, peak shape, and symmetry in a satisfactory time of analysis. The first step during CE method development was choosing the optimal buffer type and pH. This is the most important parameter since the pH value affects both the ionization of the analytes as well as has a direct impact on the EOF, the driving force in CE. During method development, seven different pH values in the range of 4.89–9.19 were tested as reported in Table II. pH values above 9.19 were discarded since tylosin was not charged (34) and hence would require using micellar electrokinetic chromatography for the analysis and would prolong the analysis time (Fig. 1). Three pH values (4.89, 5.51, and 6.02) were chosen for further experiments based on peak shape and symmetry, separation efficiency, and analysis time. For these buffer systems, different separation voltages were tested. Based on Joule heating, by comparing the electric current to the applied electric field, and the obtained Ohm's law plots, the applied voltage was kept in range from 10 to 25 kV. The lowest pH value 4.89 gave similar results to pH 5.51 in terms of migration time, separation, and peak shape, while higher pH values, due to stronger EOF, gave faster analysis times. Therefore, in further experiments, only pH values 5.51 and 6.02 were evaluated. Three important parameters were investigated at these buffer pH values, the separation voltage, and methanol content in both the sample solution and in the buffer solution. At all investigated parameters, pH 6.02 provided significantly better peak shape and height, and shorter migration times, and therefore was chosen as optimal. Applying higher separation voltage resulted in shorter analysis time, and slightly smaller peak areas (Fig. 2). The addition of organic solvent to the sample solution was necessary to ensure good solubility of the analytes. The influence of the methanol volume ratio in the sample solution was investigated from 20 % to 100 %. The peak height increased with higher methanol volume proportion, but so did the peak width, leading to a loss of resolution and unsatisfactory peak symmetry. The lower methanol volume proportion resulted in a poor baseline, while a very high proportion of methanol in the sample solution led to the mentioned poor peak shape and loss of resolution. The best peak shape, low capillary current, and satisfactory analysis times were achieved using 50 % methanol in sample solutions. The final step during method optimization was the addition of methanol as the organic solvent in the buffer solution, at 0 %, 20 %, and 40 % volume proportion. As expected, the addition of the organic modifier to the buffer solution directly influenced both the electroosmotic and electrophoretic mobilities. No change in the elution order was observed, only the decrease in the electroosmotic flow, resulting in a significantly longer analysis time. The addition of 20 % methanol resulted in better peak shapes, providing higher and narrower peaks, and enhancing method selectivity and sensitivity. A further increase to 40 % methanol increased migration times extensively (t_m > 17 min) while no improvement of peak shape and resolution was observed. Hence, 20 % methanol was chosen as the optimal organic modifier in the buffer solution.

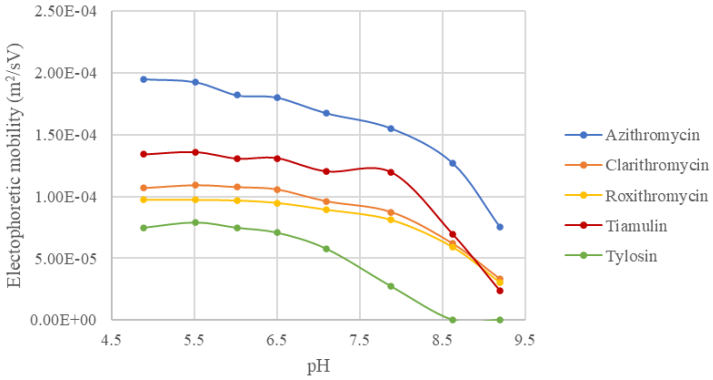


Fig. 1. Dependence of the electrophoretic mobilities on pH value.

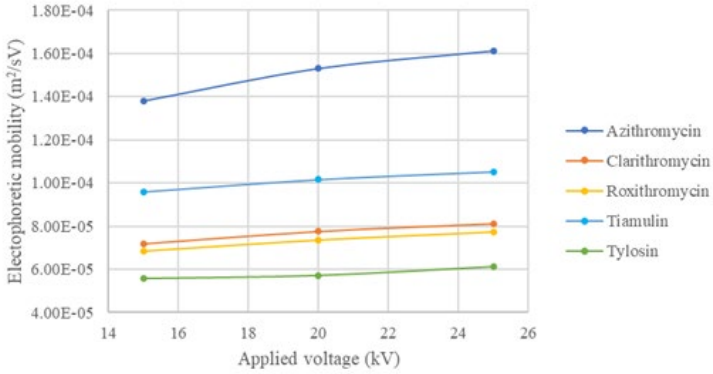


Fig. 2. Dependence of the electrophoretic mobilities on applied separation voltage.

The influence of the methanol volume proportion in the standard solution on the peak area and resolution is shown in Fig. 3. In all these experiments, the proportion of methanol in the buffer was 20 %. In Fig. 3, it can be seen that increasing the methanol content in the standard solution increases the peak areas (width and height) and consequently decreases the electrophoretic resolution of the peaks in the standard solution as well as the height of the peak, resulting in the inability to separate the curves of CLARY and ROXY. Fig. 3 shows that the best option is the presence of 50 % methanol in the standard solution, which was previously mentioned.

Optimization of solid-phase extraction

In order to isolate and analyze the target analytes from a complex sample by solid--phase extraction, a suitable sorbent must be chosen. This choice depends strongly on the physicochemical properties of analyzed analytes, but also on the interactions of the chosen sorbent with the functional groups of the analytes. Moreover, we should not forget

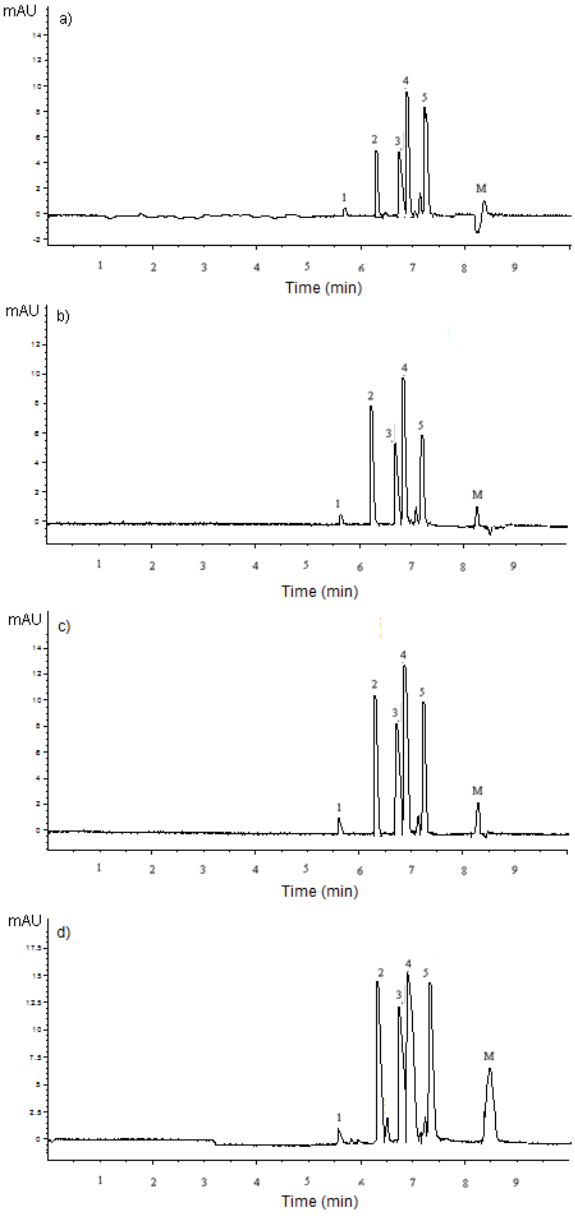


Fig. 3. Influence of methanol content in standard solution on the appearance of electroferograms obtained from pharmaceutical mixtures: a) standard solution (1 mg mL^{-1} in 20 % methanol; 20 % methanol in buffer); b) standard solution (1 mg mL^{-1} in 50 % methanol; 20 % methanol in buffer); c) standard solution (1 mg mL^{-1} in 80 % methanol; 20 % methanol in buffer); d) standard solution (1 mg mL^{-1} in 100 % methanol; 20 % methanol in buffer). 1 – azithromycin, 2 – tiamulin, 3 – clarithromycin, 4 – roxithromycin, 5 – tylosin, M – EOF marker.

the nature of the sample matrix and the interactions occurring between it and the sorbent and between it and the analyte (26). Among the physicochemical properties of the analytes tested, we should mention the hydrophobicity expressed by the octanol/water partition coefficient, K_{ow}. Based on known log K_{ow} values of tested pharmaceuticals (AZI, CLARY, ROXY, TIA, and TYL) which range from 1.05 (for TYL) to 4.75 (for TIA), can be concluded that tested pharmaceuticals are mainly hydrophobic components (tylosin is only moderately hydrophobic). For this reason, and based on the available literature, five types of sorbents were selected for the preliminary investigation; Oasis HLB, Strata-X, and SDB as polymer sorbents and C8 and C18 as silica gel sorbents. The predominant retention mechanism (or one of the predominant) in all SPE cartridges used is the reversed-phase. The reversed phase was an obvious choice as it implies the transition of the pharmaceuticals from water as a polar medium to a non-polar phase represented by the selected sorbents. In addition to the selection of appropriate sorbents, the pH of the water sample is also one of the most critical parameters. Pharmaceutical ionization depends on the acid dissociation constants (pK_a values), and the pH of the solution can be controlled. Therefore, based on the known pK_a values of tested pharmaceuticals (Table I), the pH value of the water samples was set at 9.0 for preliminary experiments. The results are presented in Table III.

From Table III it can be seen that the best extraction sorbent for the mixture of all five pharmaceuticals is C18. Such a result is in line with our expectations since C18 is the most hydrophobic sorbent. Nevertheless, the highest extraction efficacy of clarithromycin is achieved by using an SDB column. From the presented results it is also noted that the worst extraction efficiency of the test mixture was achieved by the use of the Strata-X column, not the Oasis HLB column. This finding is quite surprising given that the weight of the Oasis HLB column used was the lowest (60 mg) compared to other columns.

Although the pH of the water sample in the first preliminary experiments was adjusted to pH 9.0 based on ionization constants (the pK_a values of the tested pharmaceuticals were in the range of 7.71–8.96 with the exception of tylosin), the selected pH value in preliminary experiments might not be the best option for all five pharmaceuticals. For this reason, the pH of the water sample was adjusted further to 7.0 and 8.0. The results of this series of experiments are shown in Table IV.

Table III. Comparison of extraction recoveries, obtained from the analysis of the 100 mL spiked water samples on different sorbents (elution solvent was methanol; all cartridges used were 200 mg/3 mL except 60 mg/3 mL Oasis HLB)

	Recovery ± RSD (%)[a]				
	C8	C18	SDB	Strata-X	Oasis HLB
AZI	64.2 ± 10.2	68.3 ± 13.1	59.9 ± 10.7	57.5 ± 11.1	54.6 ± 17.4
TIA	78.4 ± 11.8	80.0 ± 5.6	73.4 ± 12.4	59.9 ± 12.6	67.2 ± 7.4
CLARY	71.0 ± 9.4	76.2 ± 5.0	88.7 ± 9.2	69.6 ± 10.4	82.5 ± 12.6
ROXY	74.4 ± 7.3	80.4 ± 7.4	55.7 ± 6.6	47.0 ± 11.1	51.0 ± 10.4
TYL	78.5 ± 7.5	80.2 ± 7.0	73.4 ± 12.3	60.1 ± 9.3	67.0 ± 10.7

[a] n = 3; AZI – azithromycin, TIA – tiamulin, CLARY – clarithromycin, ROXY – roxithromycin, TYL – tylosin

Table IV. Comparison of extraction recoveries, obtained from the analysis of the 100 mL spiked water samples using different pH values of water samples (elution solvent was methanol; 200 mg/3 mL C18)

	Recovery ± RSD (%)		
	pH = 7.0	pH = 8.0	pH = 9.0
AZI	23.4 ± 10.4	39.9 ± 14.0	68.3 ± 13.1
TIA	40.1 ± 12.5	40.3 ± 14.9	80.0 ± 5.6
CLARY	54.8 ± 13.9	62.8 ± 11.2	76.2 ± 5.0
ROXY	42.6 ± 12.1	49.5 ± 12.5	80.4 ± 7.4
TYL	43.7 ± 7.3	50.2 ± 11.2	80.2 ± 7.0

[a] $n = 3$; AZI – azithromycin, TIA – tiamulin, CLARY – clarithromycin, ROXY – roxithromycin, TYL – tylosin

The results of the above experiment, confirm that for all individual pharmaceuticals as well as for the tested pharmaceutical composition, the best pH value of the water sample is 9.0.

After selecting the optimal sorbent and optimal pH value of the water sample, the determination of the optimal elution solvent for the tested pharmaceutical mixture was started (Table V). This was achieved by repeating the experiments on the selected Strata C18 SPE cartridges using different elution solvents (acetonitrile, ethanol, and methanol).

From the table above, it was evident that methanol provided the best recoveries (68.3–80.4 %) for all pharmaceuticals. Extraction efficiencies obtained with the other two elution solvents were not so good, especially in the case of acetonitrile.

Once the best sorbent was determined, it was necessary to select the appropriate, optimal sorbent mass. This is extremely important because the optimal sorbent mass must have a capacity sufficient to hold the desired analytes as well as all other interfering constituents that may also be retained on the sorbent. If the capacity of the selected sorbent mass is insufficient, a breakthrough may occur resulting in low or non-reproducible yields. On the other hand, if sorbent mass exceeds the required amount for retention of all compounds from the samples (analytes and interferences), the certainty nothing will be lost

Table V. Comparison of extraction recoveries, obtained from analysis of 100 mL spiked water samples using different elution solvents (pH=9.0; 200 mg/3 mL C18)

	Recovery ± RSD (%)[a]		
	acetonitrile	ethanol	methanol
AZI	35.4 ± 4.4	68.5 ± 15.8	68.3 ± 13.1
TIA	55.5 ± 5.5	60.4 ± 7.5	80.0 ± 5.6
CLARY	66.2 ± 2.9	69.5 ± 6.0	76.2 ± 5.0
ROXY	55.1 ± 4.6	59.5 ± 9.7	80.4 ± 7.4
TYL	44.6 ± 4.8	50.6 ± 4.1	80.2 ± 7.0

[a] $n = 3$; AZI – azithromycin, TIA – tiamulin, CLARY – clarithromycin, ROXY – roxithromycin, TYL – tylosin

Table VI. Comparison of extraction recoveries, obtained from the analysis of the 100 mL spiked water samples using different sorbent mass (sorbent C18; pH = 9.00; elution solvent was methanol)[a]

	Recovery ± RSD (%)	
	200 mg	500 mg
AZI	68.3 ± 13.1	70.7 ± 15.6
TIA	80.0 ± 5.6	79.5 ± 15.0
CLARY	76.2 ± 5.0	80.7 ± 14.1
ROXY	80.4 ± 7.4	57.0 ± 10.6
TYL	80.2 ± 7.0	69.3 ± 11.8

[a] $n = 3$; AZI – azithromycin, TIA – tiamulin, CLARY – clarithromycin, ROXY – roxithromycin, TYL – tylosin

during the extraction procedure usually remains unchanged. However, a higher sorbent mass requires larger amounts of elution solvents which leads to an increase in the cost of the sample preparation process, and the resulting extract may be less pure. So, the next series of experiments was conducted with the goal of determining the optimal sorbent mass. For that purpose, 200 mg/3 mL and 500 mg/3 mL Strata C18 sorbent were used and the results are shown in Table VI.

As can be seen from the results presented, the extraction efficiency of the tested pharmaceuticals is not significantly altered by the application of a 500 mg sorbent mass. The only exceptions obtained are for roxithromycin and tylosin, for which the extraction efficiencies are even reduced. However, such a result also confirms our assumptions that the increase in the mass of applied sorbent also increases the need for a larger volume of elution solvent. Since increasing the volume of the elution solvent was not the goal, it can be concluded that the optimal sorbent mass is 200 mg/3 mL.

Electropherograms obtained from the standard mixture of pharmaceutical compounds, from extracts of spiked samples, and from extracts of the blank samples (200-mg Strata C18 cartridges, pH = 9.0, methanol as elution solvent) are shown in Fig. 4. No interference peaks were detected.

Validation

A developed method SPE-CE was validated for quantitative purposes. From a qualitative point of view, the method was evaluated based on the precision of the retention factor (t_R-value) and selectivity. Fig. 4 shows a comparison of the electropherograms of the extracts with those of the standard solution confirming the agreement of the t_R-values. There are no false positives in any of the blanks, confirming the good separation of all tested components without the influence of excipients on the measured signal. Therefore, the selectivity was considered acceptable.

Using seven standard solutions for each pharmaceutical tested in water samples, linearity was determined in the concentration range of 0.3–30 mg L^{-1}, depending on the

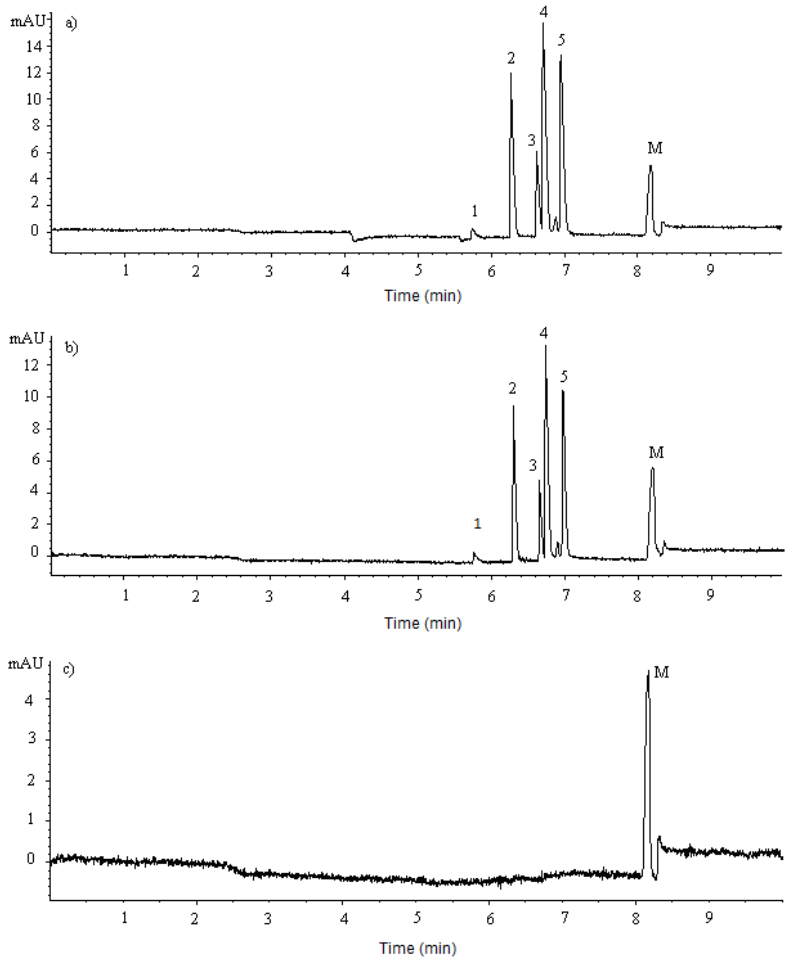


Fig. 4. Electropherograms obtained from pharmaceutical mixtures: a) standard solution (1 mg mL^{-1} in 50 % methanol); b) sample extract from spiked water at optimal solid-phase extraction parameters; c) blank extract. 1 – azithromycin, 2 – tiamulin, 3 – clarithromycin, 4 – roxithromycin, 5 – tylosin, M – EOF marker.

pharmaceutical. The exception was AZI, for which five standard solutions were used. Each calibration solution was measured in triplicate. These results were analysed by the linear regression method. The $R^2 > 0.9871$ was found for all compounds, confirming the linearity of the method (Table VII).

The limits of detection and quantification determined are listed in Table VII along with all other validation parameters. The *LOD* ranges from 0.2 to 1.2 mg L^{-1} and the *LOQ* ranges from 0.3 to 3.0 mg L^{-1}. The precision of the method studied was determined by analysing water samples in three replicates with the addition of a concentration from the middle of a specified linearity range. The RSD values obtained ranged from 3.1 to 15.8 %

Table VII. Results from quantitative determination of pharmaceuticals in water by SPE–CE

	Linearity range (mg L^{-1})	Calibration equation	R^2	*LOD* (mg L^{-1})	*LOQ* (mg L^{-1})
AZI	3–30	$A = 0.443\gamma - 0.2253$	0.9958	1.2	3
TIA	0.3–10	$A = 4.5804\gamma - 0.1663$	0.9973	0.2	0.3
CLARY	0.3–10	$A = 2.7699\gamma + 0.6118$	0.9873	0.2	0.3
ROXY	0.3–10	$A = 8.1457\gamma - 0.8031$	0.9982	0.2	0.3
TYL	0.3–10	$A = 6.4438\gamma + 0.3529$	0.9983	0.2	0.3

AZI – azithromycin, TIA – tiamulin, CLARY – clarithromycin, ROXY – roxithromycin, TYL – tylosin

for intraday precision and from 3.4 to 21.1 % for interday precision, indicating high repeatability of the developed method.

In testing all validation parameters, especially in the precision tests, the standard solution was always prepared fresh.

Measurement uncertainty

Measurement uncertainty is considered an essential parameter for method evaluation (36), it is necessary for the measurement results comparison. Correct identification of all possible sources of uncertainty is a prerequisite for a good estimation of measurement uncertainty.

In this work, sample volume, sample preparation, precision, and recovery are considered as the main components of the uncertainty budget. Uncertainty of calibration solution, comprising of uncertainty due to the purity of the standard, the weight of the standard, and preparation of calibration solutions, are included in the uncertainty associated with the linear least square fitting procedure ($u(\gamma_0)$). The results of the measurement uncertainty evaluation are shown in Table VIII.

As can be seen from the obtained results (Table VIII), uncertainty associated with sample volume has the smallest, negligible influence on the combined uncertainty for all

Table VIII. Relative combined and expanded measurement uncertainties

Compound	$\frac{u(V)}{V}$	$\frac{u(\bar{R})}{\bar{R}}$	$\frac{u(\gamma_0)}{\gamma_0}$	u(precision)	u_C (mg L^{-1})	U (%)
AZI	4.62×10^{-3}	0.0284	0.0974	0.0027	0.6093	20.31
TIA	4.62×10^{-3}	0.0236	0.0269	0.1883	0.9583	38.33
CLARY	4.62×10^{-3}	0.0400	0.0377	0.1339	0.7237	28.95
ROXY	4.62×10^{-3}	0.0399	0.0845	0.0974	0.6748	26.99
TYL	4.62×10^{-3}	0.0399	0.0637	0.0739	0.5271	21.09

AZI – azithromycin, TIA – tiamulin, CLARY – clarithromycin, ROXY – roxithromycin, TYL – tylosin

pharmaceuticals, which is expected. For AZI, the main contribution is uncertainty associated with the prediction of its concentration from the calibration curve. For all other pharmaceuticals, the contribution of uncertainty associated with the precision is the main contribution to the combined uncertainty of measurement results, with the significant contribution of uncertainty associated with the calibration curve for ROXY and TYL. In real sample analysis, it is quite common that the uncertainties associated with the precision and calibration are the main contribution to the uncertainty budget.

Application of the validated method

The newly developed and validated SPE-CE method has been applied for the determination of azithromycin, clarithromycin, roxithromycin, tiamulin, and tylosin in two wastewater samples from the pharmaceutical industry. The aim was to determine the type and amount of macrolide antibiotics in wastewater that have not been removed during conventional wastewater treatment. The two samples were taken one month apart. The samples were slightly opalescent, colourless to light gray. Before the analysis samples were filtered through a nylon 0.45 μm-filter to remove particulate matter. The samples were alkalified to pH 9. Samples were then treated using an optimised SPE procedure and analysed by the validated CE method. Obtained electropherograms are shown in Fig. 5.

Identification was based on the migration time of the compound and its corresponding UV spectrum (Table S1 in Supplementary material). Two peaks were observed in

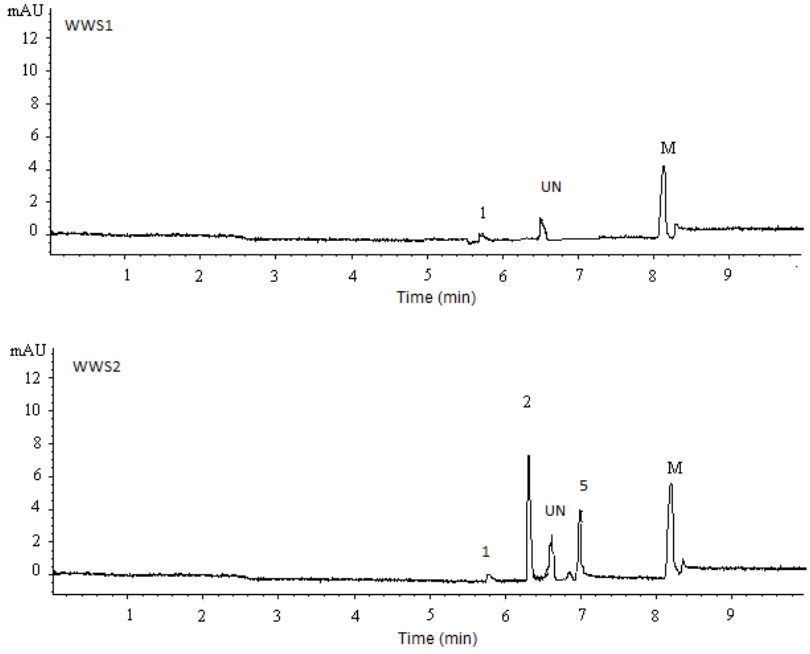


Fig. 5. Electropherograms of two wastewater samples using optimized electrophoretic parameters. UN – unknown, M – EOF marker.

Table IX. Results of wastewater samples analysis

Pharmaceutical	Standards	WWS1		WWS2	
	t_M, min	t_M, min	$\gamma \pm U$ (mg L^{-1})	t_M (min)	$\gamma \pm U$ (mg L^{-1})
AZI	5.803	5.810	5.6 ± 1.1	5.795	6.4 ± 1.3
TIA	6.319	–	n.a.	6.329	9.2 ± 3.5
CLARY	6.625	–	n.a.	–	–
ROXY	6.725	–	n.a.	–	–
TYL	6.952	–	n.a.	6.958	3.5 ± 0.7

AZI – azithromycin, TIA – tiamulin, CLARY – clarithromycin, ROXY – roxithromycin, TYL – tylosin

WWS1. The first corresponded to azithromycin with its similar migration time and UV spectrum. The migration time of the second peak does not correspond to any of the studied pharmaceuticals, and the identified UV spectrum is completely different from the spectra known so far. In addition, only azithromycin was expected, as information was obtained from the wastewater sampling, that only azithromycin was produced in the past. The second sample, WWS2, showed four different peaks. Three of these corresponded to azithromycin, tiamulin, and tylosin based on matching migration times and UV spectra. The remaining peak showed a similar migration time to the unknown compound from WWS1, but also an identical UV spectrum. Since only the produced components were identified in the process wastewater samples (only azithromycin in the first cycle (WWS1), while in the second cycle (WWS2) the production of azithromycin continued with two other smaller production units producing tiamulin and tylosin), it can be assumed that the unknown peaks correspond to the matrix component, a degradation product, or an agent used to flush the production unit. Clarithromycin and roxithromycin were not detected in any of the samples tested. The quantitative analysis was performed using the obtained linear regression equations. The determined amounts of the identified compounds are listed in Table IX.

CONCLUSIONS

SPE followed by CE-DAD determination was proposed for the simultaneous analysis of five pharmaceutical compounds: AZI, CLARY, ROXY TIA, and TYL. Identification of pharmaceuticals was possible by correlation of migration times and UV spectra. The optimized SPE method yielded acceptable recoveries, all above 80 %, except for azithromycin at 68.3 %. SPE-CE-DAD yielded detection limits from 0.3 to 3 mg L^{-1}, allowing the determination of pharmaceuticals in heavily polluted wastewater. The developed method is ideal for monitoring the concentrations of macrolides and tiamulin in process wastewater and evaluating the effect of conventional WWTPs. In the same way, the effect of washing process equipment before switching to the production of another pharmaceutical preparation could be monitored. The major advantage of the developed method is that a relatively inexpensive analytical technique compared to much more expensive techniques such as HPLC coupled with mass spectrometry. This simple method can become part of the rou-

tine analysis of wastewater after treatment in WWTPs to evaluate the performance of conventional WWTPs in eliminating pharmaceutical compounds.

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Authors contributions. – Conceptualization, D.M.P.; methodology, D.M.P. and S.B.; analysis, T.P.; investigation, T.P.; writing, original draft preparation, D.M.P., S.B., M.S. and M.Č.; writing, review and editing, D.M.P., S.B. and M.S. All authors have read and agreed to the published version of the manuscript.

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