



A new approach for the extraction of tetracyclines from soil matrices: application of the microwave-extraction technique

Paulina Łukaszewicz¹ · Anna Białk-Bielińska¹ · Joanna Dołzonek¹ · Jolanta Kumirska¹ · Magda Caban¹ · Piotr Stepnowski¹

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Abstract

The widespread use of tetracyclines (TCs) in animal husbandry is associated with their constant penetration into the environment and the threat they might pose to living organisms. While the literature data on the analysis of these substances in such matrices as food, tissues, or wastewater are quite extensive, there are still only a few methods presented for the determination of these compounds in soil samples. Moreover, among the literature methods for the extraction of TCs from soil samples, microwave-assisted solvent extraction (MAE) was used only once and in combination with liquid chromatography with spectrophotometric detection (LC-UV). However, according to the EU Commission Decision 2002/657/EC, the use of LC-UV for the determination of compounds, including pharmaceuticals, in environmental samples is not sufficient. Therefore, the development and application of a sensitive and selective method using the MAE-SPE-LC-MS/MS(MRM) technique for the isolation and identification of a mixture of oxytetracycline (OTC), tetracycline (TC), and chlortetracycline (CTC) in soils is presented in our study. The credibility of this method has been confirmed with good parameters of validation. The optimal extraction conditions of three TCs using MAE techniques were to conduct double extraction for 10 min each, at 60 °C, using a mixture of ACN:McIlvaine buffer:0.1 M EDTA (2:1:1, v/v/v) and an additional cleaning of the extracts by SPE. The effectiveness of the extraction of these compounds was assessed based on two different ways (absolute recovery from 46 to 65.1% and relative recovery from 101.1 to 109.5%). Finally, the validated MAE-SPE-LC-MS/MS(MRM) method was used for the analysis of six samples from agricultural areas of northern Poland. OTC and TC, at concentrations of 11.7 and 14.5 µg kg⁻¹ were determined in two different samples. An assessment of risk quotients was also performed. The presented method was proven to be a useful tool in the analysis of residues of selected TCs in the soil ecosystem. Obtained data on the presence of these drugs in Polish soils is the first report for this country.

Keywords Veterinary antibiotics · Microwave-assisted extraction · Liquid chromatography-mass spectrometry · Environmental analysis

Introduction

The use of antimicrobial drugs in modern times is inevitable. However, in addition to a number of benefits arising from their

utilization-weight gain or a reduction in bacterial infections, there are also a number of negative consequences observed, including an increased antibiotic resistance of bacteria, allergies, and chronic toxicity [1].

Antibacterials today constitute the largest group of substances used in human and animal medicine. Their global production ranges from 100,000 to 200,000 t. Among the wide range of groups of medicines available in the market, tetracyclines (TCs) are in second place in terms of production and consumption in the world [2]. The broad spectrum of activity against Gram-positive bacteria (*Staphylococcus*, *Pneumococcus*, *Enterococcus*) and Gram-negative bacteria (*Neisseria gonorrhoea*, *Vibrio cholerae*, *Brucella*); efficacy against microorganisms of the genus *Rickettsia*, *Coxiella*,

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✉ Joanna Dołzonek
joanna.dolzonek@ug.edu.pl

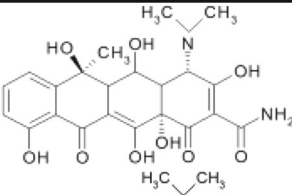
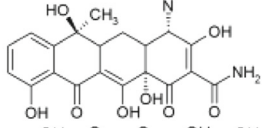
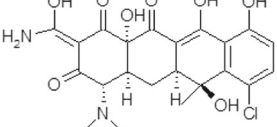
¹ Department of Environmental Analysis, Faculty of Chemistry, University of Gdańsk, ul. Wita Stwosza 63, 80-308 Gdańsk, Poland

and *Mycoplasma*, spirochetes and certain bacteria of the genus *Mycobacterium*; the relatively low cost and ease of production; and the lack of serious side effects have made them universally used since 1950, primarily in veterinary medicine [3, 4]. These antibiotics have been used for a long time in animal production as promoters of weight gain in livestock. From the first of January 2006, the EU banned the use of antibiotic growth promoters, but in Korea, the USA, and Canada, it is still allowed [4]. Seventy percent of applied TCs in their active parent forms is excreted to the environment with urine and feces [4]. The limited degree of removal of these compounds in sewage treatment plants (12–80%), as well as the use of sewage sludge or animal manure as fertilizer on fields, ultimately pollutes the soil with these substances [5]. TCs as new emerging pollutants are not covered by environmental monitoring programs and there are no regulations on permissible concentration levels of these compounds in natural ecosystems [6]. According to the literature, the residues of TCs are detected in the soil environment to a lesser extent than well-known environmental pollutants (e.g., pesticides), but their continuous introduction and long-term presence might pose a threat and should not be ignored [7]. Moreover, the effects of the presence of TCs in soils can be very extensive and include changes in structure and growth, and in resistance to various soil organisms, and can interfere with the functioning of the microsystem [8].

The presence of TCs and their mobility in the environment depends on many factors—their physico-chemical properties, the properties of the components of the environment, or the prevailing climatic conditions [9]. TCs are polar substances of relatively high molecular weight (Table 1). The basis of their

chemical structure are four benzene rings. They are amphoteric compounds with three functional groups which may be present in cationic, anionic, or zwitterionic forms depending on the pH of the medium (Table 1) [8]. From the point of view of their presence in the soil environment, it is important because in a typical soil of pH 6–8, these drugs have the ability to interact with both cationic and anionic ions present in this matrix. An unusual feature of TCs is the presence of keto groups in their molecules, so that they are capable of chelating divalent cations [8]. The already proved high sorption potential of TCs to soils ($K_d > 300 \text{ L kg}^{-1}$) demonstrates their strong retention in soil matrices [10]. Due to the widespread use of TCs in the farming industry, the literature on the analysis of these compounds is quite extensive and includes methods for the determination of these drugs in such matrices as food and animal tissue [11, 12], plasma [11], urine, surface water, and wastewater [11, 13]. On the other hand, there is still little information on the determination of these pharmaceuticals in soil samples, which may be due to problems associated with the extraction step of the determination of TCs [14]. The very complex matrix of multivalent metals in soils, with which TCs form strong complexes, significantly hinders the extraction efficiency of these drugs [15]. Most of the methods for the extraction of TCs from solid matrices presented in the literature are based on the use of ultrasound-assisted extraction (UAE) [8], extraction in a Soxhlet apparatus, and shaking/vortexing samples with polar solvents [14]. Currently, methods are aimed at being cheap, fast, and effective, and the amount of organic solvents, often toxic and carcinogenic, are as small as possible. Recent studies in the direction of sample preparation are focused on the use of more efficient,

Table 1 Physico-chemical parameters of the tested pharmaceuticals

Substance, Abbrev.	Chemical structure	Selected physico-chemical properties
Oxytetracycline, OTC		$M^a = 460.5 \text{ g mol}^{-1}$; $\text{Log } K_{ow}^a = -1.22$; $S^b = 0.062 \text{ mol L}^{-1}$; $\text{pKa}^a: 3.22, 7.46, 8.94$
Tetracycline, TC		$M^a = 444.4 \text{ g mol}^{-1}$; $\text{Log } K_{ow}^a = -1.19$; $S^b = 0.041 \text{ mol L}^{-1}$; $\text{pKa}^a: 3.32, 7.78, 9.58$
Chlortetracycline, CTC		$M^a = 478.8 \text{ g mol}^{-1}$; $\text{Log } K_{ow}^a = -0.41$; $S^b = 0.008 \text{ mol L}^{-1}$; $\text{pKa}^a: 3.33, 7.55, 9.33$

M molecular weight, S solubility in water

^a(8)

^b(4)

economical, and environmentally friendly methods, which include pressurized liquid extraction (PLE/ASE), supercritical fluid extraction (SFE), solid-phase microextraction (SPME), stir bar sorptive extraction (SBSE), or microwave-assisted extraction (MAE) [7, 14]. While there are reports in the literature demonstrating the use of the PLE technique [7, 16] for extracting these compounds from soil samples, so far, according to our best knowledge, MAE was applied for the isolation of TCs from this matrix only once [17]. MAE is an innovative technique that uses microwave radiation to heat samples above the boiling point of the solvent used in extraction [8], thereby increasing the solubility of the analytes in a solvent mixture, the contact of this mixture with the surface of the matrix, and the extraction kinetics, what may increase extraction efficiency especially in the case of TCs characterized by strong sorption potential to soil. Moreover, less solvent consumption, shorter times and automation, significantly reduce the costs of the entire process [8]. Although this method is not as widely used as UAE, it has already been used successfully for the extraction of antibiotics from different matrices [18].

The use of an appropriate solvent mixture for the extraction of antibiotics from environmental samples is crucial and highly dependent on the physico-chemical properties of analytes [14]. According to the data presented in the literature, the best choice of extractants for isolating TCs are McIlvaine buffer (pH 4.0), 0.1 M EDTA, methanol, acetonitrile, citrate buffer at pH 4.7, or mixtures of these solvents [8].

However, it must also be highlighted that the development of methods for the determination of contaminants in solid samples requires an additional step, which is cleaning the extracts to remove undesirable components from samples [1]. The most commonly used technique for this purpose is solid-phase extraction (SPE) [19]. The obtained extracts are dissolved in water to reduce the content of the organic reagent, and applied to a pre-prepared cartridge [14].

However, one of the major problems in the isolation of antibiotics from soil matrices is the repeatability of the proposed method. This is largely due to the different physico-chemical properties of the soils selected for testing, differing in the content of clay or of organic matter, as well as different contact times of the analyte with the sample. Moreover, there is no recommended method for the determination of extraction effectiveness. In the literature, there are various methods for determining the effectiveness of this process, i.e., by presenting the values of process efficiency (PE), absolute recovery (AR), or relative recovery (RR), which makes it difficult or impossible to compare the obtained data [20]. Moreover, it must be also highlighted that RR is even determined in two different ways—in relation to the control sample (containing the known amount of analytes but without the matrix that is subjected to the whole extraction procedure) or to the response of the detector to a surrogate internal standard added to the sample before extraction. For these reasons, RR values are

generally high, especially if the standard is an isotope-labeled counterpart of the determined analyte, as the loss of both compounds are similar [20]. In contrast, the AR parameter is determined in reference to a sample in which the analytes are dissolved in pure solvent, or added to an extract of blank soil after the extraction process. AR shows the influence of the sample matrix on the whole method of determination (including the loss of analytes during the extraction step), as the obtained values are often lower in comparison to RR [20].

Considering all the above information, the main objective of this study was to develop and validate a sensitive and selective method for the determination of the mixture of three TCs in soil samples. For this purpose, the application of the MAE technique combined with LC-MS/MS(MRM) was investigated for the first time. The extraction effectiveness was assessed by determining both the AR and RR values. The matrix effects were also investigated. Finally, the newly developed method was applied for the analysis of soils samples from agricultural areas collected from regions of northern Poland. Information on the presence of TCs in agricultural soils of the Pomeranian Voivodeship is of pioneering nature in the field of preliminary assessment of soil contamination in northern Poland by these drugs.

Experimental

Chemicals and materials

Pure standards (> 98%) of oxytetracycline hydrochloride (OTC) [CAS No. 2058-46-0], tetracycline hydrochloride (TC) [CAS no. 64-75-5], and chlortetracycline hydrochloride (CTC) [CAS 64-72-2] were purchased from Sigma-Aldrich (Steinheim, Germany). The organic solvents: methanol (MeOH) and acetonitrile (ACN), were purchased from Avantor (Gliwice, Poland). The acetonitrile (LC-MS Chromasolv®) used for the LC-MS/MS analysis was purchased from Sigma-Aldrich (Steinheim, Germany). The reagents used for the extraction or preparation of the mobile phase—all analytical grade (acetic acid 100%, oxalic acid, formic acid 80%, ammonium hydroxide 25%, disodium phosphate, citric acid, ethylenediaminetetraacetic acid (EDTA), ammonium acetate, orthophosphoric acid 85%, *n*-hexane, and methylene chloride)—were supplied by Avantor (Gliwice, Poland). Deionized water was produced by the HYDROLAB System (Gdańsk, Poland). Oasis HLB (Waters) cartridges with 200 mg packing material and a 6 mL reservoir were used for the purification of extracts. Syringe filters, PET (ø 15 mm), were bought from Witko, Poland. Standard stock solutions of the target compounds (100 mg L⁻¹) were prepared in MeOH. All the stock solutions were stored at -18 °C prior to use (for less than one month).

Working calibration standard solutions were prepared by diluting the standard stock solutions in MeOH, and stored at $-18\text{ }^{\circ}\text{C}$.

Soils selected to determine the extraction conditions of TCs

The experimental materials which were selected to determine the optimal conditions for the procedure of the extraction methods were collected from the surface layer of soils from agricultural areas of Pomeranian Voivodeship into amber glass bottles and then dried at room temperature, homogenized in porcelain mortar, and passed through a 2 mm sieve. Next, their main physico-chemical properties were determined and presented in Table S1 in the Electronic Supplementary Material (ESM).

Preparation of spiked soil samples

The soils ($1 \pm 0.01\text{ g}$) were weighed in 30 mL screw-top plastic centrifuge tubes, mixed with MeOH containing an appropriate amount of the mixture of TCs. Samples with analytes at a concentration of $1000\text{ }\mu\text{g kg}^{-1}$ were prepared in order to optimize the conditions of their MAE-SPE extraction using HPLC-UV for the determination of analytes. Samples with analytes at a concentration of 1000 and $100\text{ }\mu\text{g kg}^{-1}$ were prepared in order to test the repeatability of the procedure for extracting TCs from soil using the MAE-SPE-LC-MS/MS method. The validation of the developed MAE-SPE-LC-MS/MS method was performed using soil samples spiked with seven different concentrations of TCs (in a range from 2.5 to $1000\text{ }\mu\text{g kg}^{-1}$). Each concentration was prepared in triplicate. All samples after spiking were left to dry in the dark. Blank (non-spiked) samples were also prepared in order to correctly assess recoveries. Moreover, control samples (samples containing the known amount of analytes but without the matrix (soil)) were also prepared in order to assess the RR value. The control samples were also subjected to the whole extraction procedure.

Determination of the mixture of TCs by LC-MS/MS technique

The LC-MS/MS system consisted of an Agilent 1200 Series LC system (Agilent Technologies Inc., Santa Clara USA) and an HCT Ultra ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization source. The mobile phases were ACN (component A) and 0.4% formic acid (pH 2.3) as component B. The separation was performed on a C18 Hypersil GOLD aQ end-capped column ($150 \times 4.6\text{ mm}$, $5\text{ }\mu\text{m}$, Thermo Scientific, USA). A gradient elution mode was also used in the chromatographic analysis (from 18% A in 15 min to 60% A, 60% A for 1 min, 2 min to 80% A, 80% A in 4 min to 18% A, 18% A in 2 min).

The mobile phase flow rate was 0.6 mL min^{-1} . The injection volume was $50\text{ }\mu\text{L}$. The MS/MS parameters such as nebulizer gas pressure (30–50 psi), dry gas flow rate ($8\text{--}10\text{ L min}^{-1}$), dry temperature ($350\text{--}365\text{ }^{\circ}\text{C}$), and positive ion mode were optimized, separately for each compound. The capillary voltage was -4 kV . The transitions in multiple reaction monitoring mode (MRM) for the qualitative and quantitative analysis of TCs were selected, and are presented in Table 2. For the quantitative analysis, MRM transitions of the highest intensity were selected. The compounds were identified based on retention time and selected characteristic MRM transitions.

Choice of conditions for the isolation of TCs from soil

The conceptual approach of this set of experiments is presented in Fig. 1. The MAE technique was used for the isolation of a mixture of TCs from two selected types of soil (Table S1 in ESM). MAE was performed by a MarsXpress microwave system supplied by CEM (CEM Corporation, UK) equipped with an 8 PTFE vessel carousel and internal temperature control. At the beginning, 1 g of spiked soils (soil A and soil B) were transferred to the PTFE vessels. Ten milliliters of each of the five different solvent mixtures (ES), i.e., A-McIlvaine buffer (pH 4.0); B-0.1 M EDTA; C-ACN:0.1 M EDTA:McIlvaine buffer (pH 4.0), (2:1:1, v/v/v); D-MeOH:0.1 M EDTA:McIlvaine buffer (pH 4.0) (2:1:1, v/v/v); E-MeOH:ACN:0.1 M EDTA:McIlvaine buffer (pH 4.0) (1:1:1:1, v/v/v/v) was added to different vessels and agitated for 30 s using a vortex system. Next, each sample was subjected to triple extraction with the use of fresh portion of respective ES at $60\text{ }^{\circ}\text{C}$ (irradiation time-10 min, microwave power 400 W, 8 min temperature rise to final temperature). After each extraction, the vessels were left in the extraction system to cool down to room temperature and next the decanted extract was transferred to a 30 mL screw-top plastic tube, centrifuged for 10 min at 4000 rpm, filtered using syringe filters into a 500 mL glass flask. Next, the combined extract was diluted in demineralized water and cleaned up by SPE according to SPE_3 procedure (see Table S3, ESM). Further experiments were focused on the evaluation of different temperatures (40, 60, and $80\text{ }^{\circ}\text{C}$), extraction cycles (triple, double, and single), and irradiation times (10, 15, and 20 min) on the extraction effectiveness. Each extraction was prepared in triplicate, and the obtained samples were analyzed twice using the HPLC-UV technique. Information on the instrumental method for the determination of analytes with HPLC-UV technique (Section S2) and its validation parameters (Table S2) are presented in the ESM.

Statistical analysis

Statistical analysis was performed for six replicates using PQStat Software (Poland). Homogeneity of variance was

Table 2 Validation parameters of the developed MAE-SPE-LC-MS/MS method and chosen MRM transitions; ($n = 5$)

MAE-SPE-LC-MS/MS(MRM)									
Analyte	Identification parameters	Linearity range ($\mu\text{g kg}^{-1}$)	R^2	Accuracy (%)	Precision (%)	MQL ($\mu\text{g kg}^{-1}$)	MDL ($\mu\text{g kg}^{-1}$)	AR ^a (%); RR ^b (%)	ME ^b (%)
Retention time (min) Precursor and product ions									
OTC	461 [M + H] ⁺ 443; 426	5.0–500.0	0.9997	88.3–106.1	2.7–8.9	5.0	1.7	65.1 (6.5); 103.8 (7.4)	36.0
TC	445 [M + H] ⁺ 427; 410	7.5–500.0	0.9965	79.1–124.5	1.5–9.2	7.5	2.5	60.4 (9.9); 101.1 (15.6)	33.1
CTC	479 [M + H] ⁺ 462; 444	7.5–500.0	0.9997	73.3–128.3	1.8–9.3	7.5	2.5	46.0 (7.5); 109.5 (20.6)	27.3

Selected transitions for quantitative analysis are written in italic; isolation width was ± 2 ; optimal fragmentation amplitude for the transitions selected for quantitative analysis was 0.75 V, SD is presented in the brackets

^a Averaged value for the entire range of linearity

^b Averaged value for the four concentrations of TCs in soils from the linearity range

checked using the Levene's test, while normality using the Shapiro-Wilk test. One-way ANOVA followed by post-hoc Tukey HSD or Welch's ANOVA followed by post-hoc Tamphane's T2 (significance level 0.05) was performed to verify the significance of the effect of the investigated factors on the extraction efficiency.

Assessment of the effectiveness and repeatability of procedures

In order to check the effectiveness of the tested procedures, absolute recovery (AR) and relative recovery (RR, only for LC-MS/MS analysis) were determined according to the formulas presented below.

$$\text{AR} = \frac{P_{\text{pre-ext}} - P_{\text{non-spiked}}}{P_{\text{standard}}} \times 100\% \quad (1)$$

$$\text{RR} = \frac{P_{\text{pre-ext}} - P_{\text{non-spiked}}}{P_{\text{control-sample}}} \times 100\% \quad (2)$$

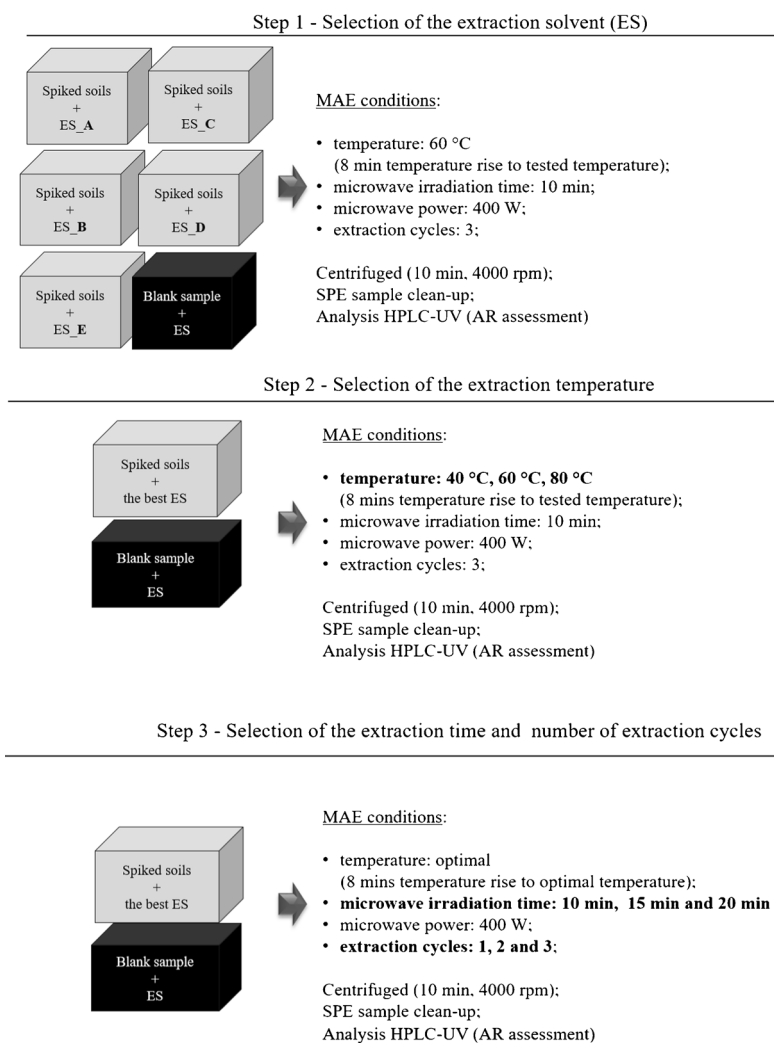
Where $P_{\text{pre-ext}}$ is the peak area of the analyte recorded for the sample, spiked with the target compound prior to extraction, $P_{\text{non-spiked}}$ is the peak area of the analyte recorded for the non-spiked sample, P_{standard} is the peak area of the analyte recorded for the standard solution, $P_{\text{control-sample}}$ is the peak area of the analyte recorded for the sample spiked with the target compound before extraction, but without soil.

The repeatability of the MAE-SPE-LC-MS/MS method was investigated. For this purpose, samples were spiked with analytes at two concentration levels 100 and 1000 $\mu\text{g kg}^{-1}$. The spiked soil samples were prepared according to the description given in the "Preparation of spiked soil samples" section. The evaluation of its effectiveness and repeatability was based on AR and RR values, as well as standard deviation (SD).

Validation of the MAE-SPE-LC/MS/MS method

The validation of the analytical method (MAE-SPE-LC/MS/MS) was carried out using the matrix-matched calibration solutions and working calibration standard solutions. The matrix-matched calibration solutions were prepared by spiking soil samples with seven different concentrations of TCs in a range from 2.5 to 1000 $\mu\text{g kg}^{-1}$. Linearity, correlation coefficient (R^2), precision (expressed by RSD), and accuracy were calculated according to the procedures described in the paper of Migowska et al. [21]. The limits of detection (MDLs) and quantification (MQLs) were determined according to Kumirska et al. [22] but permissible precision and accuracy limits were adopted, based on the guidelines described in US EPA [23]. AR was determined for each concentration of TCs in soil samples, while RR for four concentrations from the range of linearity of the method. Moreover, because of the fact

Fig. 1 Conceptual approach for the establishment of optimal extraction conditions of TCs from soil samples using the MAE technique. A-McIlvaine buffer (pH 4.0); B-0.1 M EDTA; C-ACN:0.1 M EDTA:McIlvaine buffer (pH 4.0), (2:1:1, v/v/v); D-MeOH:0.1 M EDTA:McIlvaine buffer (pH 4.0) (2:1:1, v/v/v); E-MeOH:ACN:0.1 M EDTA:McIlvaine buffer (pH 4.0) (1:1:1:1, v/v/v/v); (ES—extraction solvent)



that matrix effects (ME) are common during the application of the LC-MS/MS technique, this parameter was also calculated, based on a formula presented by Caban et al. [20].

Testing the applicability of the MAE-SPE-LC-MS/MS method

The developed method was used for the analysis of target compounds in six soils sampled from the agricultural region of northern Poland. Samples were collected in March 2015 from the surface layer of soil (up to 20 cm) into amber glass bottles, dried at room temperature, homogenized in porcelain mortar, and passed through a 2 mm sieve. Each sample was prepared in three parallel replicates. The analytes were identified based on the retention times and characteristic MRM transitions (Table 2). Quantitative analysis was done using solvent standard calibration curve. During the

determination of the pharmaceutical concentrations in the environmental samples, the AR values of the analytes were taken into account.

Preliminary assessment of risk quotients

Environmental risk assessment was conducted based on environmental risk quotients (RQ), according to the European Technical Guidance Document (TGD) [24]. RQ values were determined according to the formula presented below.

$$RQ = \frac{MEC}{PNEC_{soil}} \quad (3)$$

Where MEC is the measured environmental concentration, $PNEC_{soil}$ is the predicted no-effect concentration, and PNEC soil values were taken from literature [25].

Results and discussion

Development of the MAE procedure

Selection of the extraction solvent mixture

Based on the observations made by Zheng et al., who demonstrated that the effectiveness of the isolation of TCs using a McIlvaine buffer with pH 4.0 is higher compared to a buffer pH 8.0 [15], in conducted experiments a buffer solution with a pH of 4.0 and different solvent mixtures containing this buffer (extraction solvents, ESs A-E) were used. The obtained AR values using MAE are presented in Fig. 2. Although Zheng et al. achieved a recovery from 23.3 to 155.2% using a this buffer for the isolation of TC, CTC, and OTC from agricultural

soil [15], the use of a pure McIlvaine buffer (ES_A) or 0.1 M EDTA (ES_B) was not sufficient to isolate a mixture of TCs from soil. The value of AR achieved for TCs extracted using a McIlvaine buffer did not exceed 9.8% for soil A and 5.5% for soil B. The application of 0.1 M EDTA only marginally improved the effectiveness of the isolation of analytes-AR from 9.7 to 23.6%, depending on the soil type.

Therefore, in the next step, the addition of an organic solvent to a mixture of McIlvaine buffer and 0.1 M EDTA, what could improve the extraction effectiveness due to the increased solubility of TCs, was tested (Fig. 2a, b). For example, the use of the ES_D variant, where the organic additive was MeOH alone, provided the extraction effectiveness of TCs at a level of 28.1 to 46.2% AR, for both soil types. The introduction of ACN to a mixture consisting of a buffer, 0.1 M EDTA, and MeOH (ES_E) caused

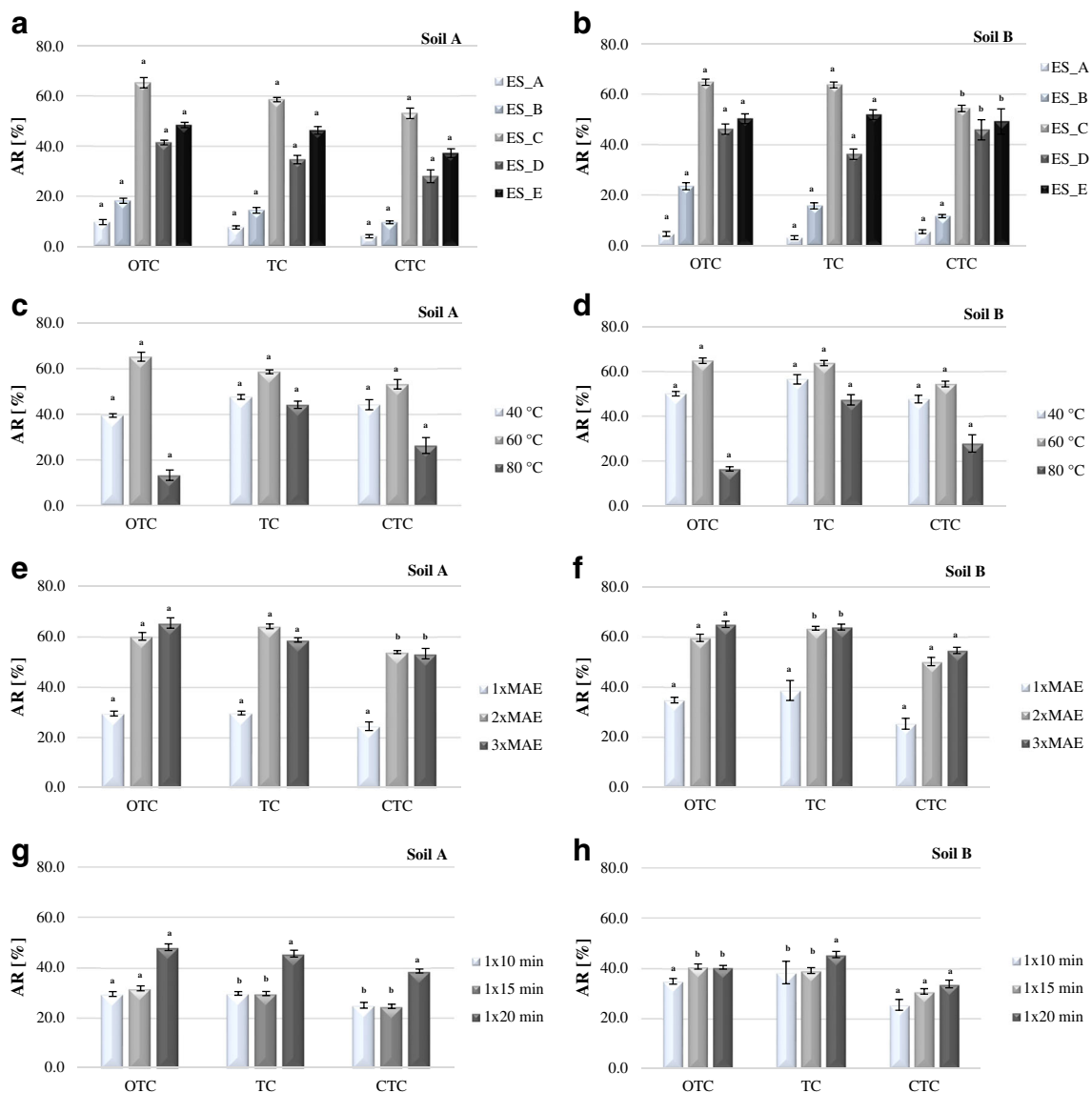


Fig. 2 AR values of TCs depending on the tested conditions: extraction mixture (a, b), extraction temperature (c, d), extraction multiplicity (e, f), and time (g, h) using MAE-SPE-HPLC-UV techniques; ^a-significant

differences in post-hoc test ($p < 0.05$), ^b-no significant difference in post-hoc test ($p > 0.05$)

an increase in the AR values of target TCs. The values obtained were within the ranges of AR 37.3–52.0%.

The highest AR of the tested TCs (53.2–65.4%) was achieved using the ES_C (mixture of ACN:McIlvaine buffer:0.1 M EDTA, 2:1:1, v/v/v), therefore it was chosen for further experiments. According to our best knowledge, this mixture was used for the extraction of these antibiotics from soil samples for the first time.

One-way ANOVA tests showed that in at least one case, the type of ES used significantly influenced the AR value of the test compounds ($p < 0.05$). In order to determine where the differences occur, post-hoc tests were performed. These tests showed significant differences in extraction efficiency of TCs from soil A and OTC and TC from soil B. For CTC (soil B), the isolation efficiency of this compound using ES_C and ES_E ($p = 0.42$) and ES_D and ES_E ($p = 0.93$) does not differ significantly (see Fig. 2).

Selection of the MAE temperature

During the study, three MAE extraction temperatures (40, 60, and 80 °C) were tested, and the ES_C was selected in the previously described optimization step. The obtained results are presented in Fig. 2c, d. In contrary to Hu et al., who obtained comparable recoveries of TC and CTC at 40 and 60 °C during the extraction of these compounds from manure [26], it was observed that reducing the extraction temperature from 60 to 40 °C decreased the extraction effectiveness for all TCs. In the case of OTC, the AR value was reduced by about 25% points for soil A, and about 15% points for soil B. For TC and CTC, the differences in recoveries were about 10% points (see Fig. 2c, d). The use of 80 °C proved to be ineffective and deteriorated the recoveries (by about 50, 15, and 27% points for OTC, TC, and CTC respectively) in comparison to 60 °C. Similar observations were reported by Jiao et al. during extraction of TCs from soil samples using MAE- μ -SPE technique. The extracted amount of TCs increased from 40 to 70 °C and decreased with increasing temperature [17]. Less efficient extraction could be attributed to the simultaneous extraction of undesired matrix components that can interfere with the target compounds or the degradation of these compounds. TCs, when used at high temperatures, tend to be converted to their epi- or anhydroforms [27, 28], therefore the temperature ranging from room temperature to 100 °C is usually used to isolate these compounds [29]. Statistical analysis confirmed the significant effect of temperature on the extraction efficiency of TCs from soil samples ($p < 0.05$). Finally, the temperature of 60 °C was chosen as optimal for the isolation of the mixture of TCs from soil by MAE.

Selection of the number of cycles and the extraction time

The obtained results are presented in Fig. 2e, f. A single extraction cycle resulted in approximately 30% points lower ARs compared with the AR values reached during triple

extraction. During double extraction, AR recoveries were similar to those obtained with the triple cycle. Slightly different results were obtained by post-hoc tests, where only TC extracted from soil B ($p = 0.77$) and CTC extracted from soil A ($p = 0.88$) showed no significant differences between the two and three extraction cycles ($p > 0.05$). To determine whether an extended time of a single cycle will sufficiently improve the recoveries, the experiment was performed for 15 and 20 min for a single cycle. It was observed (Fig. 2g, h) that along with the increase in the extraction time, the AR values slightly increased. One-way ANOVA analysis indicated that at least one significant difference in AR was observed in relation to extraction time ($p < 0.05$) (Fig. 2). The post-hoc tests showed no significant differences in isolation efficiencies between 15 and 20 min for OTC (soil B), and between 10 and 15 min for CTC, TC in case soil A and TC in case soil B ($p > 0.05$) (Fig. 2). However, the AR values obtained with extended extraction time ($< 48.0\%$) were lower than the values obtained during the double extraction (10 min each). Although post-hoc tests show differences in extraction efficiency depending on the number of cycles (except TC (soil B) and CTC (soil A)), the maximum differences in AR values are 6%. Therefore, finally it was decided that the optimal MAE conditions for the extraction of three TCs from soil will be carried out with two extraction cycles, 10 min each at 60 °C. Moreover, the proposed conditions were also suitable for the extraction of TCs from the two types of soils (comparable recoveries), independently of their different characteristic (Table S1, ESM); thereby, in further experiments only soil A was taken.

Repeatability of the proposed MAE-SPE-LC-MS/MS conditions

All the results already presented refer to the MAE-SPE-HPLC-UV method. In the subsequent step, the obtained conditions were transferred to MAE-SPE-LC-MS/MS, and its repeatability was tested at two concentration levels of TCs (see the “Assessment of the effectiveness and repeatability of procedures” section). The obtained recoveries (AR and RR) are presented in Table 3.

Table 3 The recovery values obtained during the repeatability of the proposed MAE-SPE-LC-MS/MS conditions

Analyte	Concentration ($\mu\text{g kg}^{-1}$)	AR (%)	RR (%)
OTC	1000	62.6 \pm 6.3	116.6 \pm 3.1
	100	66.1 \pm 5.9	117.2 \pm 6.0
TC	1000	51.8 \pm 1.5	114.1 \pm 6.5
	100	58.5 \pm 5.0	102.8 \pm 15.3
CTC	1000	50.7 \pm 3.2	120.2 \pm 9.7
	100	50.9 \pm 2.7	125.7 \pm 3.0

The results indicate that the MAE-SPE-LC-MS/MS method is characterized by good reproducibility, with a satisfactory SD < 15.3% for each compound [23]. High differences were observed for AR and RR values, while the second one was twice as high for each analyte. As was mentioned in the “Introduction” section, the AR values include not only the loss of analytes during the extraction step but also the influence of the matrix components onto the whole procedure. Due to the comparable loss of analytes in the spiked sample and in the control sample during the extraction process (Eq. 2), the obtained values of RR exceed 100% in some cases, especially if the matrix effect was positive.

Nevertheless, in our opinion, the AR parameters are more informative and should be presented during the optimization of the extraction procedures from complex matrices.

Validation of method

The validation of the MAE-SPE-LC-MS/MS(MRM) method was performed using soil extracts containing seven concentrations of TCs. Each concentration level was analyzed five times. The obtained validation parameters are presented in Table 2. The sensitivity (expressed as MDL) and the reliability (represented as accuracy) of the developed methods were proven. Method linearity was established on the basis of at least five different concentrations. The values of correlation coefficients (R^2) confirmed a good linearity of the proposed method for each compound (> 0.9965). The accuracy range 73.3–128.3% and precision 1.5–9.3% meet the accepted criteria [23, 30]. The obtained values of RSD were lower than those presented by Jiao et al. indicating better precision of our method [17]. An analysis of the blank sample of soil, in which none of the analyzed compounds were detected, showed that the optimized method also exhibits good selectivity. The MDL and MQL for the whole procedure were 1.7–2.5 and 5.0–7.5 $\mu\text{g kg}^{-1}$, which proves the usefulness of this method for the analysis of environmental samples. These values are comparable [31] or even lower than those presented in the literature [16, 32, 33]. For

example, Li et al. presented MQL values of 14.1 and 47.4 $\mu\text{g kg}^{-1}$ for OTC and CTC respectively [33], whereas O'Connor et al. obtained the values of MQL within the range of 14–19 $\mu\text{g kg}^{-1}$ [16]. Nevertheless, the authors of these papers used different methods to determine these values, thus direct comparison is difficult. The obtained AR and RR values were within 46 to 65.1% (AR) and from 101.1 to 109.5% (RR) (Table 2). For comparison, the efficiency of extraction of TCs from soils obtained by Salvia et al. was between 25.4 and 41.7% when using the PLE technique [34]. In turn, in the work of Jiao et al., where the MAE- μ -SPE technique was used to extract TCs from soils, the recovered values were 70.6–110.5% [17], but the authors did not provide information on the type and method of their determination. Therefore, interpretation of those results is difficult and comparing them with the efficiency of extraction of method developed by us was impossible. The obtained ME (27.3–36.0%, see Table 2) indicate an enhanced signal in the sample matrix. Not only are these values at a satisfactory level, but also comply with the criteria-50% < ME < 50% [35]. The chromatograms of the mixture of TCs obtained using the developed methods are presented in Figs. S3 and S4 (see ESM).

The presence of TCs in soil samples

The validated method was used for the determination of TCs in six agricultural soil samples (Table 4). CTC was not detected in any of the studied samples. Comparable levels of TC and OTC were determined in the soil coming from Karłowo and Saduny, 11.7 $\mu\text{g kg}^{-1}$ (OTC) and 14.5 $\mu\text{g kg}^{-1}$ (TC) respectively. This may be related to the fact that the real samples were collected after season when soil is usually fertilized with manure, which is considered to be the main source of veterinary antibiotics. In comparison to other countries, TCs concentrations in agricultural soils in northern Poland are generally much lower. For example, in the German soils, the detected TC and OTC levels are between 450 and 900 $\mu\text{g kg}^{-1}$. Most of the soil samples tested in Italy contained OTC concentrations above 100 $\mu\text{g kg}^{-1}$ [9]. In Turkey, the OTC was

Table 4 Results of the determination of TCs using the developed MAE-SPE-LC-MS/MS method in agricultural soils collected in Poland (nd not detected) and established RQ values

Place	Geographic coordinates	Analyte concentrations ($\mu\text{g kg}^{-1}$); ($n = 3$); (SD)			PNEC _{soil} ($\mu\text{g kg}^{-1}$) ^a		RQ	
		OTC	TC	CTC	OTC	TC	OTC	TC
Tczew	54° 05' 31.89" N 18° 46' 38.39" E	<MQL	nd	nd	85.2	631.0	0.14	0.02
Kamień	54° 29' 12" N 18° 13' 32" E	nd	nd	nd				
Malbork	54° 02' 06" N 19° 01' 41" E	nd	nd	nd				
Saduny	54° 09' 57" N 21° 15' 43" E	nd	14.5 (0.8)	nd				
Pruszcz Gd	54° 15' 43" N 18° 38' 11" E	nd	nd	nd				
Karłowo	54° 18' 14" N 17° 51' 01" E	11.7 (2.0)	nd	nd				

^a[25]

determined in four independent soil samples at a concentration of 19–144 $\mu\text{g kg}^{-1}$ [36]. In turn, Xiang et al. reported the presence of TCs in each of investigated Chinese soils in quantities up to 184.8 $\mu\text{g kg}^{-1}$ [25]. The OTC and TC concentrations determined by us are comparable to the concentrations reported by Zheng et al. (9.9 $\mu\text{g kg}^{-1}$ for OTC and 13.2 $\mu\text{g kg}^{-1}$ for TC [15]), Huang et al. (19.3 $\mu\text{g kg}^{-1}$ for OTC [37]), and Salvia et al. (6.9 $\mu\text{g kg}^{-1}$ for TC [34]).

Preliminary environmental risk assessment was carried out in accordance with the TGD document [24]. PNEC_{soil} values estimated on the basis of PNEC_{water} values are presented in Table 4. RQ values are divided into three categories of risk levels, i.e., low (RQ 0.01–0.1), medium (RQ 0.1–1.0), and high (RQ > 1.0) [25]. On the basis of the designated RQ values (successively 0.14 and 0.02 for OTC and TC), it can be concluded that there is a low risk associated with the presence of TC in soil, in turn in the case of OTC medium. However, the presence of these drugs in the natural environment should not be ignored, as these compounds are continuously introduced into soil.

As it is well known, the intensification of agriculture and the development of the pharmaceutical industry make it impossible to avoid the contamination of natural ecosystems with antibacterial drugs. However, the continuous application of these substances and their presence in differing environmental components indicate that it is necessary to establish permanent monitoring of pollution of environmental matrices with residues of pharmaceuticals. This would probably reduce or avoid potential adverse effects that may result from the presence of these drugs in the environment. The presented and developed method is fully capable of performing further analyses for the presence of tetracycline antibiotics in soils.

Conclusion

An innovative, sensitive, and selective MAE-SPE-LC-MS/MS(MRM) method for the extraction of three TCs from soils was developed for the first time. A number of experiments proved that MAE is an effective tool for the isolation of these contaminants from such a complex matrix. Due to discrepancy and the lack of an embodied way of assessing the effectiveness of the extraction, in the presented studies, we evaluated the effectiveness of the isolation of TCs on the basis of two types of recoveries, i.e., relative (RR) and absolute (AR). The obtained RR and AR values were significantly different from each other, which confirms the need to standardize the method for determining the extraction effectiveness in environmental samples, which would make the comparison of the developed conditions easier, and the whole determination more reliable. The RR values of the target TCs were generally higher due to the comparable loss of analytes in the spiked soil sample with TCs and the control sample during the extraction

process. Smaller AR values indicate a total loss of analytes during the sample preparation step and also the influence of the matrix components onto the final determination.

The developed procedure proved to be effective for the two types of soil. The organic matter content did not significantly affect the value of recoveries. It was also shown that the choice of the appropriate MAE extraction temperature for the isolation of TCs is important and affects the effectiveness of the extraction of these compounds. Finally, the good validation parameters of the developed MAE-SPE-LC-MS/MS method proved its suitability for the analysis of these compounds in soil samples. Hence, it was applied to the analysis of these compounds in real soil samples. TC and OTC were detected in two different environmental samples at levels of successively 11.7 and 14.5 $\mu\text{g kg}^{-1}$, which supports the statement that the occurrence of antimicrobials in agricultural soils in northern Poland is of high concern. However, to determine the scale of the pollution of the ecosystem with residues of TCs, it is necessary to increase the scope of research. The developed method can be successfully used for this purpose in the future. The obtained results are the first ones concerning the presence of TCs in soils of Poland.

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Compliance with ethical standards The authors state that the research did not include human and/or animal participants.

Conflict of interest The authors declare that they have no conflict of interest.

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