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In-syringe automated extraction of selected antibiotics in surface waters exploring deep eutectic solvent coupled online to HPLC analysis

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ABSTRACT

Antibiotics are by design difficult to remove in the biological treatment of wastewater and are becoming ubiquitous in aquatic environments, which bears the risk for the formation of resistant bacterial strains. The new EU Urban Wastewater Treatment Directive demands fast, cheap, efficient, and environmentally-friendly methods for antibiotic determination. However, there is a general need for sample preparation and analyte preconcentration. For this purpose, liquid phase extractions remain reliable and often-used approaches, while they show drawbacks regarding procedural greenness. Herein, we applied dispersive liquid-liquid microextraction based on a hydrophobic natural deep eutectic solvent for the first time for the fully automated enrichment of the selected antibiotics tetracycline, oxytetracycline, doxycycline, chlortetracycline, sulfamethoxazole, and trimethoprim from surface waters. The methodology was based on the Lab-In-Syringe technique and was coupled online to high-performance liquid chromatography with spectrophotometric detection. Sample and solvent handling and extraction were carried out inside the void of a computer-controlled syringe with a multiposition head valve with an integrated magnetic stir bar for efficient solvent dispersion. The procedure required 200 µL solvent and was completed within 8 min, including all cleaning steps, and was operated in parallel to the chromatographic analysis of the previous extract. A greenness evaluation by the AGREE tool yielded a value of 0.8. Excellent linearity up to 1000 µg L^{-1}, detection limits ranging from 0.4 to 15 µg L^{-1}, and RSD values typically below 4.3% were achieved. The method was successfully applied to the determination of selected antibiotics in surface waters, demonstrating its practical application.

1. Introduction

Antibiotics are commonly used in veterinary medicine for controlling infectious diseases in animals, as well as humans [1]. Among these, tetracyclines, trimethoprim, and sulfamethoxazole are widely used antibiotics in human therapy. They are also used in subtherapeutic doses for prophylaxis or as growth promoters in animal feed since they are readily-absorbed, inexpensive agents that are effective against a broad spectrum of both Gram-positive and Gram-negative bacteria as well as some obligate anaerobes, protozoa, parasites, and fungi [2–6].

Antibiotics can be found in the aquatic environment at up to µg per litre concentrations, as most of the consumed antibiotics are excreted through urine and faeces in the form of the parent compounds or metabolites [7]. Studies have shown that wastewater treatment plants cannot remove these antibiotics completely, so they can be found in significant concentrations in sediments, surface water, and groundwater close to animal farms, hospitals, or pharmaceutical factories [8]. When used as growth promoters in aquaculture, excreta from farmed fish contribute to surface water pollution [9]. These contaminations assist in the development of antibiotic-resistant bacteria [10], and present major ecotoxicological threats given their low EC_{50} levels regarding plants and aquatic species [11]. In this context, there is a strong need for sensitive, reliable, and rapid methods for detecting antibiotics in aqueous samples.

Analysis of antibiotics in environmental waters is typically done by

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Table 1
List of synthesized NADES for preliminary tests.

Abbreviation	NADESs	Molar ratio
NADES1	[HA]/[Th]	1:1
NADES2	[OA]/[Th]	1:1
NADES3	[1,2-PD]/[Th]	1:1
NADES4	[1,3-PD]/[Th]	1:1
NADES5	[AA]/[Th]	1:17
NADES6	[2,3-BD]/[Th]	1:1
NADES7	[OA]/[TBA]	1:1
NADES8	[OA]/ [1,3-PD]/[Th]	1:1:2
NADES9	[1-HO]/[Th]	1:1
NADES10	[1,4-BD]/[Th]	1:1
NADES11	[1-HO]/[Mh]	1:1
NADES12	[OA]/ [1,3-PD]/[Th]	2:1:1
NADES13	[OA]/ [1,3-PD]/[Th]	1:2:1
NADES14	[OA]/ [1,3-PD]/[Th]	1:1:4
NADES15	[AA]/[1,3-PD]/[Th]	1:1:18
NADES16	[AA]/[1,3-PD]/[Th]	1:4:5
NADES17	[OA]/[Th]	1:2
NADES18	[OA]/[Th]	2:1
NADES19	[1,3-PD]/[Th]	1:2
NADES 20	[BA]/[Th]	1:1

Legend: [HA]/[Th]: hexanoic acid/thymol; [OA]/[Th]: octanoic acid/thymol; [1,2-PD]/[Th]: 1,2-propanodiol/thymol; [1,3-PD]/[Th]: 1,3-propanodiol/thymol; [AA]/[Th]: azelaic acid/thymol;[2,3-BD]/[Th]: 2,3-butanediol/thymol; [OA]/[TBA]: octanoic acid / tetrabutyl-ammonium bromide; [OA]/[1,3-PD]/[Th]: octanoic acid/1,3-propanodiol/thymol; [1-HO]/[Th]: 1-hexanol/thymol; [1,4-BD]/[Th]: 1,4-butanediol/thymol; [1-HO]/[Mh]: 1-hexanol/menthol; [OA]/ [1,3-PD]/[Th]: octanoic acid/1,3-propanodiol/thymol; [OA]/[1,3-PD]/[Th]: octanoic acid/1,3-propanodiol/thymol; [OA]/ [1,3-PD]/[Th]: octanoic acid/1,3-propanodiol/thymol; [AA]/[1,3-PD]/[Th]: azelaic acid/1,3-propanodiol/thymol; [AA]/[1,3-PD]/[Th]: azelaic acid/1,3-propanodiol/thymol; [OA]/[Th]: octanoic acid/thymol; [OA]/[Th]: octanoic acid/thymol; [1,3-PD]/[Th]: 1,3-propanodiol/thymol; [BA]/[Th]: benzyl alcohol/thymol.

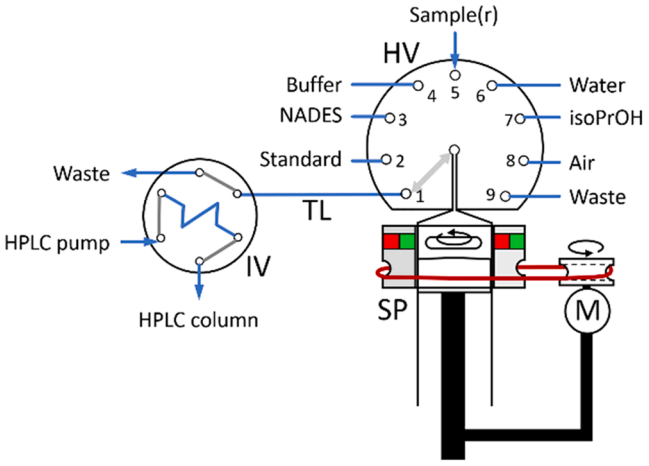

Fig. 1. Scheme of the used Lab-In-Syringe system. HV: multiposition head valve, M: motor, IV: injection valve, TL: transfer line.

liquid chromatography. Challenges are the low concentration levels and matrix complexity, which make sample preparation imperative. Classical methodologies, such as cartridge-based solid-phase extraction (SPE), e.g., using hydrophilic-lipophilic balanced sorbents, are effective for analyte preconcentration. However, they can be laborious and time-consuming, especially in combination with large sample volumes. Retention and recovery of analytes can be biased by the sample matrix, and automation of SPE protocols remains a tricky task [12]. More importantly, the retention of polar compounds such as the antibiotics discussed above for water samples may be insufficient and lead to poor extraction efficiencies. On the other side, liquid-liquid extraction (LLE) separates analytes based on their solubility between two immiscible liquids. It can readily be downscaled and automated, but it is less specific and typically not well-suited for polar analytes. Certain drawbacks, in particular the significant amount of hazardous solvents needed, have been minimized by what are termed Liquid-Phase Microextraction (LPME) procedures, which therefore are counted as greener approaches to LLE [13,14]. Among these, dispersive liquid-liquid microextraction (DLLME) stands out as a well-established approach, offering reduced solvent consumption, shorter extraction times, and higher enrichment factors [15]. Briefly, DLLME involves rapid analyte transfer within a turbid ternary system containing solvent droplets formed by rapidly injecting a mixture of disperser and extraction solvents into the donor phase, followed by phase separation [16].

One elegant way to tackle the difficulty of liquid-extracting polar compounds is the use of novel solvents capable of undergoing various types of interactions with the analytes. Deep eutectic solvents (DES) refer to a class of such solvent alternatives, all formed through hydrogen bonding between a hydrogen bond donor and a hydrogen bond acceptor [17]. By the choice of these components and molar ratios, their physicochemical properties can be tuned, including viscosity, density, polarity, or water solubility. This allows for purpose-designed DES and enhances extractions from aqueous matrices, even of analytes of high polarity [18]. Natural deep eutectic solvents (NADES) have gained special attention for being composed of two or more natural components, such as sugars, amino acids, organic acids, terpenes, or phenolic compounds, that can form an eutectic mixture [19]. NADES are gaining attention as a powerful, easily available, low-cost, and eco-friendly substitute for conventional organic solvents or ionic liquids in many applications [20], including analytical chemistry [21]. Herein, they have proven to be efficient extractants for polar or even charged solutes and to be compatible with liquid chromatography [22]. In this sense, NADES hold the potential and promise to develop greener, more sustainable analytical methods for environmental contaminants with a broad polarity range (logP from −1.37 to +0.91).

Efficient accomplishment and miniaturization of extraction methodologies, accompanied by high reproducibility, avoidance of handling errors, and improved user safety are typical benefits of procedural automation. Apart from versatile autosampler systems, this can be achieved by modern flow techniques [23]. Lab-In-Syringe (LIS) is a flow-batch technique, which has proven highly efficient in the automation of liquid phase microextraction, foremost DLLME [24,25]. Deriving from Sequential Injection Analysis [26], it is based on the use of an automatic syringe pump and a liquid selector, with the main difference that all operations, mixing, derivatization, or extraction, are carried out inside the syringe void and typically aided by in-syringe magnetic stirring. This enables a high operational versatility compared to tubing-based flow techniques, since neither gas content, nor solution ratio, viscosity, nor miscibility can affect solution mixing significantly. Moreover, pressure adaptation or further addition of needed solutions is straightforward [21,27]. Recent evaluations using the Blue Applicability Grade Index (BAGI) consistently give LIS systems high scores, reflecting their effectiveness for routine analytical tasks across diverse preparative procedures [28].

The aim of the present work was the development of a green, fully automated, and NADES-based DLLME method for the extraction of six commonly used veterinary antibiotics, namely trimethoprim, sulfamethoxazole, oxytetracycline, tetracycline, doxycycline, and chlortetracycline, in aqueous matrices. For analyte determination, the automated extraction system was online coupled to HPLC with spectrophotometric detection.

2. Materials and methods

2.1. Reagents and materials

Analytical standards (≥99%) for tetracycline (TC), chlortetracycline (CTC), oxytetracycline (OTC), doxycycline (DC), sulfamethoxazole

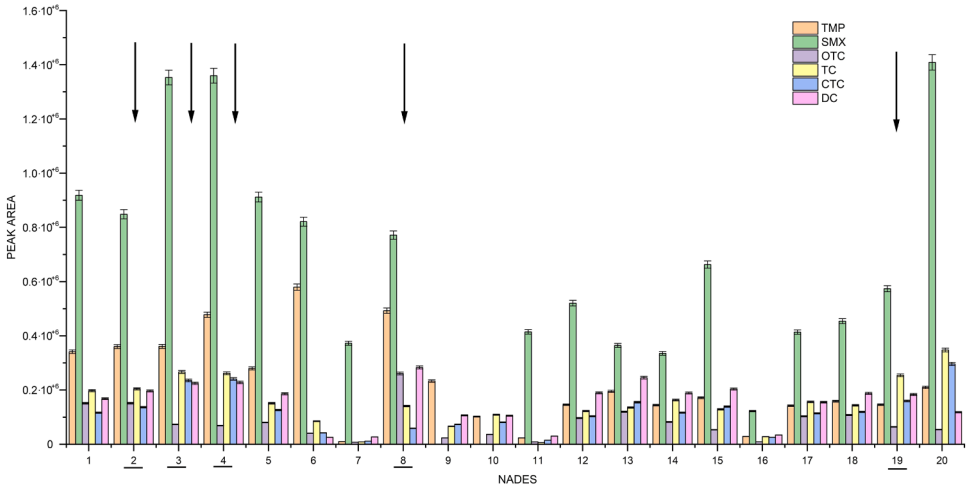

Fig. 2. Evaluation of the effect of the type of NADES on the extraction efficiency of selected antibiotics with errors bars representing the standard deviation ($n = 3$). Manual extraction with 1 mL sample in pH 6 and 200 µL NADES over 60 s and phase separation over 600 s.

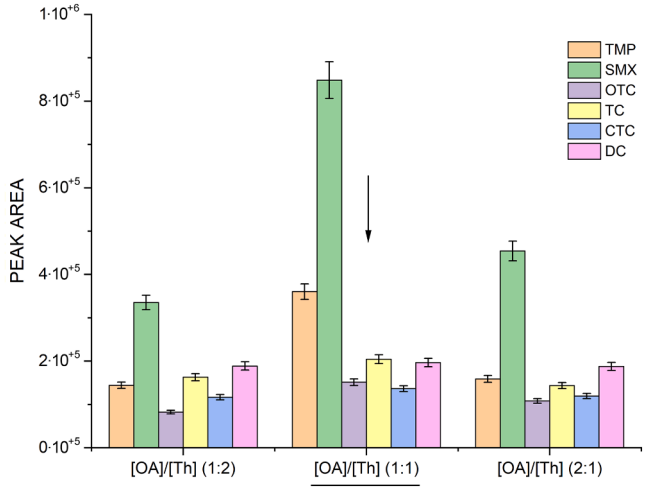

Fig. 3. Evaluation of the molar ratios of selected NADES on the extraction efficiency of selected antibiotics with errors bars representing the standard deviation ($n = 3$). Conditions as in Fig. 2.

(SMX), and trimethoprim (TMP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile (ACN, 99.9%) and formic acid (FA, >96%) were used as mobile phase components, isopropanol (99.9%) served as a cleaning solvent. Components for buffer preparation, including citric acid and sodium dihydrogen phosphate dihydrate (99%). Components for NADES synthesis, including thymol (Th, 99.5%), octanoic acid (OA, >98%), hexanoic acid (HA, > 99%), azelaic acid (AA, 98%), 2,3-butanediol (2,3-BD, 98%), 1-hexanol (1-HO, 98%), 1,4-butanediol (1,4-BD, ≥99%), menthol (Mh, code M2772, 99%), benzyl alcohol (BA, purris.) were also purchased from Sigma-Aldrich, 1,3-propanodiol (1,3-PD, 98%), 1,2-propanodiol (1,2-PD, 99.8%) were purchased from POL-AURA (Poland), tetrabutylammonium bromide (TBA, 99%) was purchased from FLUKA. Purified water (18.2 MΩ/cm) used for all work was generated using a Merck Millipore purification system (Burlington, MA).

Individual stock solutions for all antibiotics were prepared at 1 mg mL^{-1} in ACN (for CTC, MeOH was used). The dilutions were freshly prepared daily by diluting the standard solutions with the appropriate volume of ACN.

Five surface water samples were collected around the city of Hradec Králové, from the Labe River, an old arm of the Labe River, the Orlice River and a wastewater treatment plant (WWTP) effluent. Samples were stored at +4 °C in the dark until used. Samples were filtered directly before use through KA 1 filter paper (Fisher Scientific).

The pH of buffer or NADES solutions was measured with an Accumet XL200 pH meter (Fisher Scientific).

2.2. Preparation of NADES

A total of 20 NADES, consisting of either two or three components, were synthesized by stirring the components at 80 °C for 20 min until a colourless liquid was formed. The molar ratios were selected based on previous articles [27,29]. A selected number of NADES were then synthesised in different molar ratios (1:3, 1:2, 1:1, 2:1, 3:1) to study the influence of this ratio on the extraction efficiency. The list of all synthesized NADES for the preliminary tests is given in Table 1. The applied NADES are well documented in the literature [27,30] with confirmed structural characteristics and established physicochemical properties. Therefore, no additional structural characterization (e.g., FT-IR analysis) was performed in this study. To ensure complete formation and homogeneity of the eutectic system, the mixing time was extended from 15 to 20 min compared to the standard preparation protocol, guaranteeing proper synthesis and reproducibility of the NADES between batches.

2.3. HPLC instrumentation and method

Chromatographic analysis was performed using a Nexera series HPLC system (Shimadzu, Japan) and SPD-M40 diode array detector (DAD), CTO-40 S column oven, and SDP-M40 photodiode array detector (PDA). The analytical column was an Ascentis® Express C18 HPLC Column (15 cm x 4.6 mm, 2.7 µm, Supelco™ Analytical, Sigma Aldrich), which was used with an opti-guard® 1 mm C18 guard column. The separation was operated at 30 °C with gradient elution. The flow rate was 1 mL min^{-1}. Gradient elution was carried out using 0.1%(v/v) formic acid in water (mobile phase A) and ACN (mobile phase B). Separation was performed with the following gradient conditions: 15% to 60% B from 0 to 7.5 min, 60% to 100% B from 7.5 to 8 min, holding 100% B until 11.5 min, and column equilibration at 15% B from 12 to 14 min. Absorbance signals were measured at a wavelength of 270 nm for TMP, SMX, and 360 nm for TC, CTC, OTC, and DC, where the analytes showed their absorbance maximum. For data collection and system control, the software LabSolutions (Shimadzu, Kyoto, Japan) was used.

During the study of NADES solvents and initial experiments, extracts were collected and analysed offline using the autosampler of the HPLC

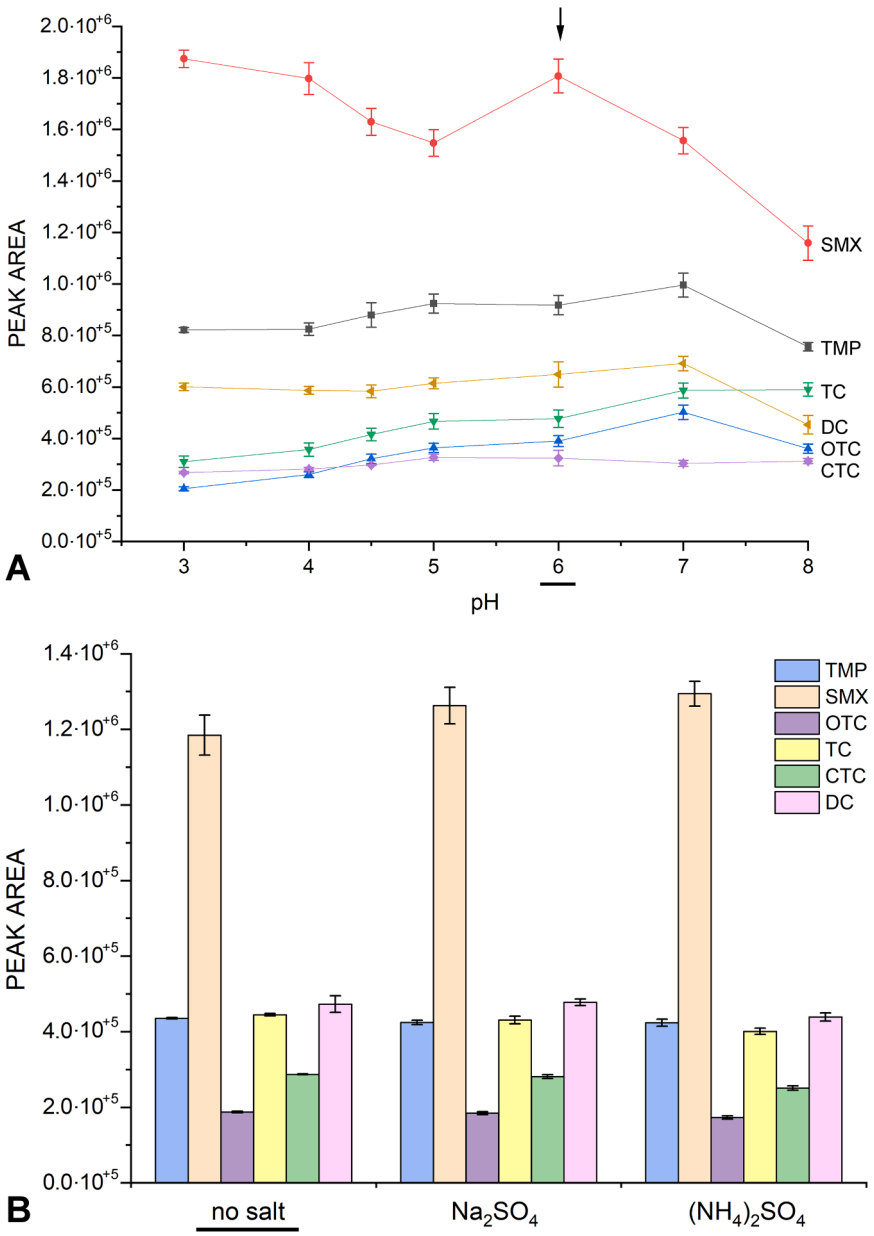

Fig. 4. Evaluation of the effect of A) pH, B) salting-out on the extraction efficiency on the extraction efficiency of selected antibiotics with errors bars representing the standard deviation ($n = 3$). Automated extraction with 1 mL sample and 200 µL NADES 2 over 60 s and phase separation over 240 s.

system for injections of 10 µL. During online coupling of the LIS system to the HPLC, an external 6-port high-pressure injection valve (type FCV-S) from Shimadzu, equipped with a 10 µL injection loop (0.25 mm id, 20 cm length), was placed between the pressure pump and column, and the autosampler was removed from the configuration.

2.4. Lab-In-Syringe instrumentation

The LIS system used for automated DLLME is shown in Fig. 1 and was based on a Cavro XC9+ automatic syringe pump (Tecan, City, Switzerland) used in an upright orientation, given that hydrophobic NADES a typically of lower density than water (floating) [24] and as pushing out the extract before emptying the syringe from the remaining sample ensures selective sampling of the separated upper layer and minimizing any disturbance of the phase boundary.

It featured a 9-port ceramic distribution head valve (HV) and was equipped with a 2.5 mL glass syringe of 3 cm piston stroke. The HV ports were used for the aspiration of standard (port 2), NADES (3), buffer (4), and sample (5), as well as water (6) and isopropanol (7) for system cleaning from individual reservoirs through FEP tubing (0.8 mm id, ca 40 cm). Air (8) was used to promote vortex formation inside the syringe. Discharge of the syringe content to waste was possible via HV port 9. On port 1, a transfer line of 45 cm (0.5 mm i.d., ca. 90 µL) connected the LIS system to the injection valve during online coupling.

During method validation and sample measurement, the LIS system was connected via HV port 5 to an AIM 3200 autosampler (Aim Lab Automation Technologies, Virginia, Australia) housing two racks of up to 60 Falcon tubes (15 mL) for standards and sample solutions, so that manual intervention was not necessary. The extracts were then transferred from the LIS to the online coupled HPLC system so that the HPLC autosampler was not used.

A magnetic stir bar (6 mm in length and 2 mm in diameter) was placed inside the syringe void, allowing in-syringe mixing of the aspirated solutions at computer-controlled stir rates. Driving the stir bar was done as formerly described [27] via a driver ring placed onto the syringe barrel, and holding two small neodymium magnets. It was driven via a

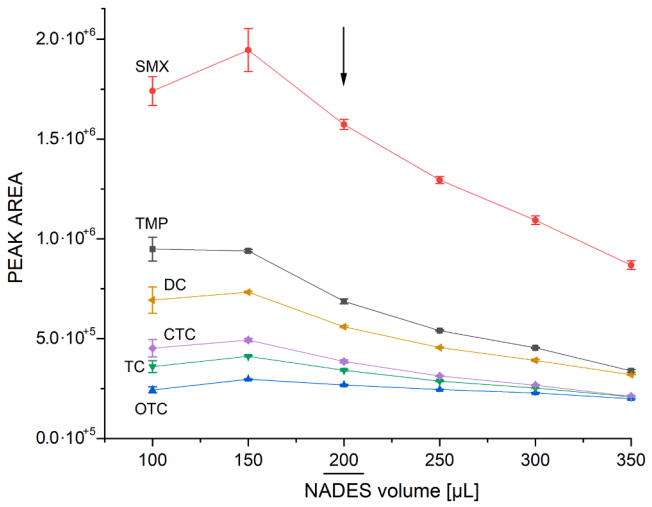

Fig. 5. Evaluation of the effect of NADES volume on the extraction efficiency of selected antibiotics, with error bars representing the standard deviation ($n = 3$). Automated extraction with 1 mL sample in pH 6 and NADES 2 over 60 s and phase separation over 240 s.

Table 2
Analytical figures of merit for investigated antibiotics with aqueous standards.

Analyte	RT [min]	LDR [µg L^{-1}]	R^2	LOD [µg L^{-1}]	LOQ [µg L^{-1}]	Extraction efficiency [%]
TMP	2.8 ± 0.1	50.0–1000	0.9995	15.0	50.0	110.2 ± 4.1
OTC	3.2 ± 0.1	6.3–1000	0.9995	1.9	6.3	38.7 ± 1.0
TC	3.5 ± 0.1	5.0–1000	0.9996	1.5	5.0	52.1 ± 1.1
CTC	4.4 ± 0.1	10.0–1000	0.9996	3.0	10.0	71.9 ± 2.0
DC	4.7 ± 0.1	4.8–1000	0.9998	1.4	4.8	96.9 ± 3.0
SMX	5.7 ± 0.1	1.4–1000	0.9990	0.4	1.4	87.0 ± 3.3

Legend: CTC: chlortetracycline; DC: doxycycline; LDR: linear dynamic range; LOD: limit of detection; LOQ: limit of quantification; OTC: oxytetracycline; Rec, recovery ± RSD, relative standard deviation, $n = 6$); RT: retention time; SMX: sulfamethoxazole; TC: tetracycline; TMP: trimethoprim.

rubber ring by a DC motor prepared from a pulse width modulated (PWM) computer fan. The assembly is shown as photography in the Electronic Supplementary Material S-1. Required devices for in-syringe stirring were fabricated by 3D printing on a DeltaQ FDM printer (Trilab company, Hradec Králové, Czech Republic) from polylactic acid (PLA) filament [31].

The entire system was controlled by the software Cocosoft 6.1, written in Python [32]. A Trinket M0 microcontroller (Adafruit, New York, USA), connected to the controlling PC via a USB-serial converter (COM 23) served to activate the stirring motor as well as speed control, triggering of the HPLC run via relay (contact closure), and analogue control of the injection valve position via an SPDT relay.

It should be pointed out that all wetted materials used, both of the LIS and the HPLC instrument were chemically inert also regarding the use of various deep eutectic solvents.

2.5. Operation protocol for automated dispersive liquid-liquid microextraction procedure

The operational method explained in the following is given in tabular form in the supplementary materials (Table S-1) and is visualized in the graphical abstract. Specific parts of the method, such as syringe cleaning, were coded as routines, while for method or routine parameters, e.g., positions of head valve or sample location on the autosampler, volumes, number of repetitions, variables were used to achieve high operational flexibility and allow straightforward adaptation of these parameters, e.g., during optimization studies.

The method started by cleaning both the syringe and transfer line once with isopropanol. For this, small volumes of the solvent (500 µL and 200 µL, respectively) were aspirated with activated fast stirring, and then dispensed to either waste or the transfer line. These procedures were then repeated with water (1000 µL and 500 µL, respectively). Finally, the syringe was cleaned with the sample aspirated via head valve port 5 from the autosampler.

The actual extraction method, given with the final chosen parameters, started by the aspiration of 1700 µL sample, followed by 200 µL McIlvaine buffer, and 25 µL air to drive all liquid into the head valve and magnetic stirring at ca. 1000 rpm. Afterwards, 200 µL of NADES was aspirated, followed by another 50 µL of air. This also served vortex formation at the beginning of solvent dispersion, for which the stirring rate was increased to 1400 rpm for 180 s extraction time. Then, the stirring rate was reduced to the possible lowest rate, which facilitated droplet coalescence, and was then deactivated for phase separation over 240 s.

Afterwards, the injection valve was turned to position LOAD, and 240 µL were expulsed from the syringe through the transfer line, which was the volume adjusted for effective loading of the floating NADES extract. Then, the HPLC method was triggered by contact closure, and the injection valve was switched to position INJECT. Finally, the syringe was emptied into waste (port 9).

The total time for the LIS-automated DLLME, including syringe and autosampler cleaning, required just 8 min. To synchronize the LIS-automated DLLME procedure with the longer-lasting chromatographic separation, the LIS method included a final wait of 6.5 min. After this predetermined waiting time, the LIS procedure started from the beginning with syringe cleaning and the extraction procedure using the following sample. This way, the extract was ready to load to HPLC just when the chromatographic separation of the previous injection was completed.

3. Results and discussion

3.1. Chromatographic method

TMP, OTC, TC, CTC, DC, and SMX were selected as model analytes, as one of the most commonly used veterinary antibiotics [33]. During method development, five different separation columns were tested, namely Ascentis® Express C18 HPLC Column (15 cm x 4.6 mm, 2.7 µm), YMC-Triart C18 (100×4.6 mm, 5 µm), Kinetex® Polar C18 (100×3 mm, 2.6 µm), ACE C18 (150×4.6 mm, 5 µm), and YMC-Triart C18 ExRS (150×4.6 mm, 3 µm). Moreover, the effect of the mobile phase compositions studied, in particular the elution gradients using acetonitrile as mobile phase B, and the effect of mobile phase composition.

Using formic acid (0.1%v/v) and acetonitrile as mobile phases A and B, the Ascentis® Express C18 HPLC column was found to provide the best analyte separation in terms of peak shape and resolution (see Electronic Supplementary Material, Figure S-1) with gradient conditions as given in Section 2.3. The tested columns differed in particle morphology, including core-shell particles (Ascentis® Express C18 and Kinetex® Polar C18) and fully porous particles (YMC-Triart C18, ACE C18, YMC-Triart C18 ExRS). Core-shell technology is generally associated with improved chromatographic performance due to reduced mass transfer resistance and shorter diffusion paths, which can result in narrower peaks and higher theoretical plate numbers compared to fully porous particles. The Ascentis® Express C18 and Kinetex® Polar C18 column used are both based on core-shell particles but differed in column dimensions and surface chemistry. The Ascentis® Express C18

Table 3
Precision and accuracy for antibiotics in river waters and WWTP effluent samples. ($n = 3$).

		Labe river		Old Labe river		Orlice river		WWTP effluent	
Analyte	Spiked [μg L^{--1}]	Detected [μg L^{-1}]	Recovery [%]	Detected [μg L^{-1}]	Recovery [%]	Detected [μg L^{-1}]	Recovery [%]	Detected [μg L^{-1}]	Recovery [%]
TMP	0	< LOD		< LOD		< LOD		< LOD	
	250	217.7	87.1 ± 2.1	231.2	92.5 ± 3.0	275.1	110.0 ± 2.1	268.8	107.5 ± 3.4
	500	406.5	81.3 ± 3.5	415.1	83.0 ± 2.7	520.2	104.0 ± 3.6	452.5	86.5 ± 2.1
OTC	0	< LOD		< LOD		< LOD		< LOD	
	250	188.2	70.5 ± 2.9	186.0	74.4 ± 7.8	225.8	90.3 ± 2.1	149.2	61.8 ± 4.8
	500	472.9	100.8 ± 2.6	399.8	80.0 ± 5.6	512.0	102.4 ± 3.3	381.1	72±5.1
TC	0	< LOD		< LOD		< LOD		< LOD	
	250	220.6	88.2 ± 0.8	189.3	75.7 ± 2.3	240.7	96.3 ± 2.0	194.5	77.8 ± 1.2
	500	524.8	105.0 ± 1.1	478.3	95.7 ± 2.3	584.7	116.9 ± 2.1	470.6	94.1 ± 1.4
CTC	0	< LOD		< LOD		< LOD		< LOD	
	250	155.4	62.1 ± 5.1	122.2	48.9 ± 5.4	132.4	52.9 ± 2.9	111.8	44.7 ± 2.2
	500	317.5	63.5 ± 5.7	283.3	56.7 ± 1.7	302.4	60.5 ± 2.5	247.0	49.4 ± 3.0
DC	0	< LOD		< LOD		< LOD		< LOD	
	250	196.4	78.6 ± 3.6	168.3	67.3 ± 1.5	172.6	69.0 ± 4.3	144.7	57.9 ± 3.6
	500	411.9	82.4 ± 2.3	392.9	78.6 ± 3.3	391.5	78.3 ± 2.1	346.2	69.2 ± 0.8
SMX	0	< LOD		< LOD		< LOD		< LOD	
	250	256.9	102.8 ± 1.6	258.6	103.5 ± 5.4	273.2	109.3 ± 2.4	261.3	104.5 ± 5.2
	500	498.2	99.6 ± 2.9	510.7	102.1 ± 2.4	526.4	105.3 ± 5.3	512.1	102.4 ± 3.3

Abbreviations: LOD: limit of detection.

Table 4
Comparison of the optimised extraction procedure with other studies.

Extraction procedure	Analytes	Sample volume [mL]	Extraction solvents/ extractant phase	Extraction procedure time [min]	Separation time [min]	RSD [%]	LOQ [μg L^{-1}]	Recovery [%]	AGREE Score [46]	CF	Ref
DSPE assisted CPE	OTC, TC, CTC, DC	1	iron-tannic nano solids	31	20	≤ 10	2.63–4.01	79.3–107.1	0.51	100	[40]
SPE	OTC, TC, DOX	20	50 mg polythiophene	8	8	≤ 7.3	1.2–2.7	69.5–114.1	0.35	20	[41]
DSPE	OTC, TC, DC	0.5	basil seed mucilage	21	18	≤ 13	15.0	83.1–109.9	0.43	100	[42]
VA-ELLME	OTC, TC, DC	5	0.4 mL DES	6.5	16	≤ 9.0	3–9.32 (μg kg^{-1})	69–102	0.54	12.5	[43]
DSPE–DLLME	OTC, TC, CTC	10	bio-MOF, MDES	12	20	≤ 7.5	0.83–0.97	89–104	0.47	182	[44]
DLLME	OTC, DC, TC	10	0.175 mL DES	11	17	≤4.7	4.53–14.60	74–114	0.59	57	[30]
LIS-automated LLME	TMP, SMC, TC, OTC, CTC, DC	1.7	0.2 mL NADES	8	14	≤ 4.3	1.4–50 For TCs: 4.8–10	55–104	0.80	8.5	This work

Abbreviations: CF: concentration factor; CPE: Cloud point extraction; DLLME: Dispersive liquid–liquid microextraction; DSPE: Dispersive solid phase extraction; MDES: Magnetic deep eutectic solvent; MOF: Metal-organic framework; LOQ: limit of quantification; SPE: Solid phase extraction; VA-ELLME: Vortex-assisted emulsification liquid–liquid microextraction.

column (150 mm length) provided better peak resolution compared to the shorter Kinetex® Polar C18 column (100 mm) under the tested gradient conditions, which was deduced to the embedded polar groups of the second column, adding to additional retention mechanism for amphoteric analytes such as the tetracyclines as our analytes of interest.

The addition of 0.01 M oxalic acid at both pH 2 and 4 was tested instead of formic acid. However, with the column selected for analysis, the addition of oxalic acid (both pH 2 and 4) showed to prolong the elution of the analytes by about 2 to 4 min min as well as to lead to wider peaks. In addition, the retention time of the last peak (SMX) was 9.5 for oxalic acid and 7.0 for formic acid (see Electronic Supplementary Material, Figure S-2).

Methanol was tested as an alternative to ACN with the possibility of a faster elution of NADES residues (protic solvent), but due to the significantly higher pressure and no positive effect on peak shape or resolution was found, so that due to an increased backpressure above 30 MPa, further tests were discontinued.

It should be highlighted that the injection of the hydrophobic NADES taken into account as selection from manual screening, did not course any baseline or signal disturbance compared to aqueous standards using either 5 μL or 10 μL injection volumes, for which an injection volume of 10 μL NADES was selected for further work.

3.2. Optimization of extraction conditions

3.2.1. Effect of different nades and their molar ratios

For this research, we studied a total of 20 NADES for the extraction of the given analytes. In the selection of NADES, water solubility, straightforward achievement of phase separation, possible peak overlay with the analytes of interest, and a high extraction capacity were of the highest interest [34,35]. Screening tests with the 20 synthesized NADES were carried out manually in 1.5 mL polypropylene vials, with results shown in Fig. 2. For this, 1 mL aqueous standard (1 mg/L) and 200 μL NADES were vortexed for 1 min. After allowing phase separation for 10 min, 10 μL of the NADES supernatant was injected into the HPLC for analysis. All extractions were done in triplicate. The relatively high analyte concentration was chosen during optimization to allow evaluation of reliable signals and consequently extraction efficiency and comparison of experiment outcomes during optimization of the method parameters.

A

Reagents	Penalty points
Water	0
Aacetonitrile	4
Formic acid	4
Standard solution	2
Isopropanol	4
Octanoic acid	2
Thymol	4
	Σ 20
Instruments	**Penalty points**
Transport	1
HPLC	1
Occupational hazard	0
Waste 1-10 mL	3
Waste no treatment	3
	Σ 8
Total penalty points:	28
Analytical Eco scale total score:	72

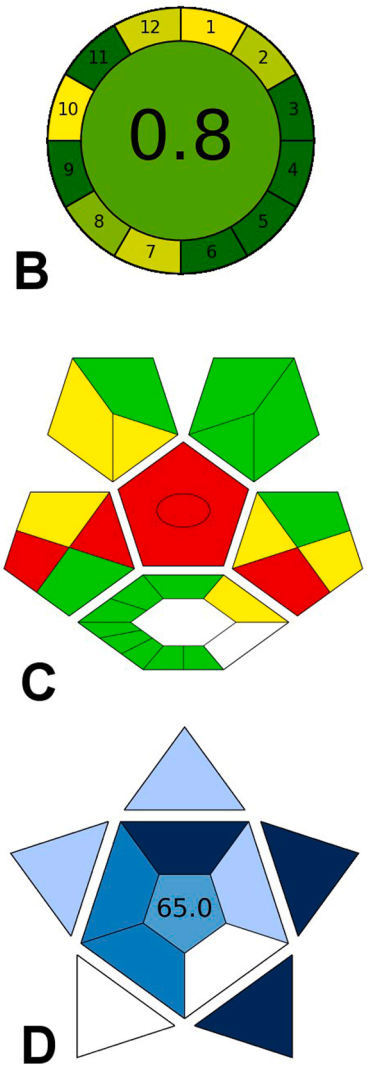

Fig. 6. Assessment of greenness and practicality of developed method: a) calculated penalty points based on the Analytical Eco-Scale proposed by [46], b) AGREE pictogram [45], c) GAPI pictogram [47], and d) BAGI index pictogram [48].

NADES based on octanoic acid generally showed better extraction efficiency and faster phase separation after extraction. In contrast, DES based on propanediols needed significant time for phase separation, limiting their use in the LIS system. NADES prepared with hexanoic acid and octanoic acid showed similar extraction efficiencies. However, hexanoic acid is more volatile, water soluble, and toxic than octanoic acid, and was very unpleasant to work with (smell), so this component and related NADES were finally rejected.

The studied antibiotics are compounds over a broad polarity range (logP values: TMP 0.91; OTC −0.9; TC −1.37; CTC −0.62; DC −0.02; SMX 0.89) [6,36,37], so unsurprisingly NADES composed of Th with 1, 2-PD, 1,3-PD, and OA as the second component showed the highest extraction recoveries since they are the most polar ones. Furthermore, different molar ratios for these components were tested (Table 1, Fig. 3).

During LIS experiments, the best NADES for all selected antibiotics was found to be NADES 2. Using NADES with 1,2-propanediol and 1,3-propanediol, OTC extraction was very low (about 10%, see Fig. 2). This may be due to the partial miscibility of the used NADES with water and the high polarity of OTC. NADES 2 shows a lower polarity and water solubility, and yielded increased extraction efficiency. This was also attributed to dipole-dipole and hydrogen interactions between the NADES components and the analytes [34].

Based on the observations and using the geometric mean of the extraction efficiency of all analytes as desirability, ranging from 54–65% for all antibiotics and 46–57% solely for the tetracyclines, five NADES were eventually selected for further studies of the automated DLLME LIS corresponding to the numbers 2, 3, 4, 8, and 19.

All hydrophobic NADES showed density lower than water but a significantly high viscosity so that reduced flow rates were used for reliable solvent aspiration or dispense. Taking this into account as well as their genuine ability to form hydrogen bridges leading to a higher water affinity than many classical immiscible solvents, coalescence of solvent droplets also requires a longer time reflected in the phase separation time used of 240 s.

3.3.2. *Effect of sample pH*

The pH value for the extraction of the analytes was tested in the pH range from 3 to 8. McIlvaine buffer in various proportions was used to prepare the appropriate pH, and the pH was controlled with a pH meter. The choice of pH is important because of the different pKa values of the selected analytes. Extreme pH values were not examined due to the chemical transformation of tetracyclines (at pH values ⟨ 3.1 they change into anhydrotetracyclines, while at values ⟩ 9.0 they form iso-tetracyclines) [38,39]. The extraction efficiency increased up to pH 7

and then decreased significantly from 3% (CTC) to even 30% (SMX, DC). This was presumably due to the dissociation of octanoic acid (see Fig. 4A), finding also that starting at pH 7, NADES 2 demonstrated surfactant properties due to the dissociation of the octanoic acid, which inhibited phase separation. Therefore, a pH value of 6 was selected for further experiments. Under these conditions, the effects of buffer addition, EDTA, and sample container material (plastic or glass) were assessed and found to have no significant impact on recovery, except for a slight negative effect observed for EDTA.

3.3.3. Effect of the ionic strength

The extraction efficiency can often be enhanced by the addition of salts due to a decreased analyte and solvent solubility in the aqueous phase (salting-out effect) and by assisting phase separation due to an increase in density of the aqueous phase. Therefore, the effect of salt addition was studied for both Na_2SO_4 and $(NH_4)_2SO_4$ at 1.6 and 4.5 mol/L (80% saturated), respectively. However, salt addition showed no significant effect on extraction efficiency over the studied range (see Fig. 4B) and it was therefore omitted in the following.

3.3.4. Effect of stirring speed

The speed of in-syringe magnetic stirring was studied in the range of 1400 to 2300 rpm. The recovery values indicate that the extraction efficiency did decrease only slightly over this range. In particular, for reasons of phase separation speed and solvent recovery, also related to yielding the lowest overall RSD (relative standard deviation) values, a stirring rate of 1400 rpm was selected (see Electronic Supplementary Material, Figure S-3).

3.3.5. Effect of nades volume

In the next step, the effect of the NADES volume on the extraction efficiency was studied in the range of 100 to 300 µL, with results and experimental conditions given in Fig. 5. For lower volumes of NADES (100 and 150 µL), RSDs hinder reliable NADES transport and loading into the HPLC injection loop, which we consider to be due to unreproducible solvent recovery. For larger volumes, we found a dilution effect of the extracts, so as a compromise between sensitivity and method reliability, a DES volume of 200 µL was selected for all further work.

3.3.6. Effect of extraction and separation time

The extraction time is a crucial parameter influencing extraction efficiency and was therefore tested in 30 s intervals ranging from 30 to 210 s. The extraction efficiency stopped increasing after 180 s (see Electronic Supplementary Material, Figure S-4). Therefore, an extraction time of 180 s was adopted for further experiments.

Moreover, the time needed for complete phase separation was examined, which is crucial considering that the organic phase should be homogeneous and representative for the extraction before injection to HPLC. Moreover, too short a time can lead to the erroneous injection of droplets of water into the instrument. The separation time was studied by visually assessing phase separation from 0 to 240 s. Due to the small difference in phase densities, the longest time was selected for further experiments.

3.4. Method validation and performance

The analytical performance of the method combining LIS-automated DLLME with online coupled HPLC is summarized in Table 2. Calculations were performed from calibrations (peak area versus analyte concentration, six concentration points ranging from 0 to 1000 µg L^{-1}, n = 3). Method validation was carried out using river waters as the sample matrix. The extraction recovery (ER%) was calculated by comparing the measured concentration after extraction with the spiked concentration in the sample: ER (%) = (C_found / C_spiked) × 100%, where C_found is the concentration determined after extraction and C_spiked is the spiked concentration. The recoveries for each substance are reported in Table 2. It should be highlighted that values for TC and OTC are usually lower due to their higher polarity and water solubility.

The procedure's repeatability was evaluated for all analytes at a concentration of 250 µg L^{-1} (n = 6), and the RSD were below 4% for all analytes. Intra-day and inter-day accuracy were evaluated as the relative difference between the measured concentration and the nominal spiked concentration. The intra-day accuracy (n = 9, 1 day) of the developed method ranged from 86.8% to 96.4%. The inter-day precision (n = 9, 3 days) resulted in RSD values below 4.3% for all analytes.

Method linearity was proven within the concentration range of 1.4–1000 µg L^{-1}, with r^2 values exceeding 0.999. The limits of detection (LOD) and limits of quantification (LOQ) were calculated based on signal-to-noise ratios of 3 and 10, respectively, yielding values ranging from 0.4 to 15 µg L^{-1} for LOD and 1.4 to 50 µg L^{-1} for LOQ. The preconcentration factor (PF) was calculated as the ratio between the initial sample volume and the volume of the selected NADES. Based on this calculation, a preconcentration factor of 8.5 was achieved. The selectivity of the method was also verified, as no impurities were observed at the retention times of the target analytes during experiments on real samples.

It should be highlighted that LIS-automated sample preparation run in parallel to the chromatographic separation of the previously injected sample, so that a sample throughput of 4 per hour was achieved during calibration and sample analysis. A further increase could be achieved by speeding up the chromatographic separation (e.g., use of shorter column with smaller particles). Our results highlight the potential of NADES techniques and automation in improving analytical methods, especially for pharmaceuticals in complex matrices.

No carry-over effects nor progressive system contamination were observed, e.g. verified by blank injections, which could have been related to improper cleaning of the syringe, transfer line, or injection valve nor due to NADES remains on the separation column. performed prior to each analytical sequence; no analyte peaks were detected.

3.4. Analysis of real samples

The method was successfully applied for the determination of selected antibiotics in real water matrices, demonstrating its practical application. Samples collected from three local rivers, a WWTP effluent, and treated as given in Section 2.1. The tested water samples were analysed both with analyte spiking on two concentration levels (250 and 500 µg L^{-1}) and without spiking (genuine). The results are summarized in Table 3. In non-spiked samples, concentrations of all analytes were below their respective LOD values. Variability in recovery for tetracyclines was observed in the analysis of surface water samples, which could be compensated by an appropriate internal standard. It should be highlighted that RSD values were below 5.4% for sample analyses.

3.5. Comparison of NADES-LIS-HPLC-UV method with other extraction methods

The developed method has key benefits being foremost fully automated sample preparation and extract analysis, a small sample volume needed (1.7 mL), and utilizing a small volume of NADES as a green extraction solvent, which enabled efficient extraction while enhancing method greenness. The optimized NADES-LIS-HPLC-UV method's analytical parameters were compared with previously developed techniques for tetracycline determination in aqueous matrices (Table 4) [30, 40–44]. It should be pointed out that our method was the only one that can be classified as fully automated, while the others were entirely manual. Compared to the other methods, our methodology achieved faster extraction, i.e. within 3 min, and chromatographic separation of the analytes within 14 min. Running the separation in parallel, this allowed a higher throughput than in most of the previously reported methods. Moreover, procedural automation yielded significantly

improved reproducibility compared with the reported manual method yet with a lower preconcentration factor of 8.5. Furthermore, the method distinguishes itself through its high extraction efficiency and recoveries from 45 to 110%. It is characterized by its greenness and high efficiency, with minimal processing time and low sample volume requirements. However, extraction of oxytetracycline was rather unsatisfactory and higher extraction efficiencies have been achieved by formerly reported solid phase extraction methodologies, so that further studies will be required to improve this protocol. The automation method is applicable also to other analytes yet a critical study in particular of the extraction solvent would be required. Moreover, further decrease of the solvent volume used and fastening the chromatographic separation, e.g. by a shorter column, is desirable to further enhance method performance.

3.6. Greenness and practical assessment of NADES-LIS-HPLC-UV method

The greenness of the developed method was evaluated according to three assessment tools with results summarized in Fig. 6: the Analytical Eco-Scale [45], the Analytical GREEnness Metric Approach (AGREE) [46], and the complementary green analytical procedure index (GAPI tool) [47]. The results according to the eco-scale and AGREE calculator assessment are 72 and 0.8, respectively, confirming the greenness of the methodology. Among the assessed methodologies (Table 4), the presented approach achieved the highest AGREE Score, indicating it is the most environmentally sustainable. The HPLC method was also evaluated in terms of practicality using the BAGI calculator, with a score of 65.0, while scores above 60.0 are recommended in terms of practicality of the method [48].

4. Conclusion

In this research, an automated LLME online to HPLC method based on NADES was validated for the quantification of six antibiotics (tetracycline, chlortetracycline, oxytetracycline, doxycycline, sulfamethoxazole, and trimethoprim) in aqueous samples with adequate analytical performance for routine analysis. The developed methodology is characterized by a fast preparation process of 8 min in parallel with the running analyte separation and low need of a separation friendly extractant (200 μL solvent). The validated methodology has high reproducibility and avoids potential errors of the analyst during extraction. The method showed excellent linearity, detection limits ranging from 0.4 to 15.0 μg L^{-1}, high precision with RSD values typically below 4.3 % and analyte recoveries similar to previously described methodologies. On the other hand, the method did not overcome the often-found critical recovery of oxytetracycline due to the basic functional group present to which end, ion pairing could aid.

Data availability

Experimental data are shared via Zenodo database under the reference DOI: 10.5281/zenodo.19367053.

CRediT authorship contribution statement

Joanna Antos: Writing – original draft, Validation, Investigation, Formal analysis, Data curation. **Derya Demir:** Writing – original draft, Investigation. **Dobrochna Ginter-Kramarczyk:** Writing – review & editing. **Joanna Zembrzuska:** Writing – review & editing. **Petr Solich:** Writing – review & editing, Funding acquisition. **Burkhard Horstkotte:** Writing – review & editing, Visualization, Supervision, Software, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no competing financial interests nor personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sampre.2026.100233.

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