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Electromembrane extraction of tetracyclines in foods and human plasma prior to LC-MS/MS analysis: Impact of sample matrix on extraction performance

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ABSTRACT

Tetracyclines are widely used antibiotics in animal farming, raising concerns over their residues in food and associated health risks. Current methods for residue determination are complex, time-consuming, and produce substantial solvent waste. Electromembrane extraction (EME) offers high clean-up efficiency, minimal solvent use, and direct LC-MS/MS compatibility. This study evaluates EME for extraction of four tetracyclines (oxytetracycline, tetracycline, chlorotetracycline, doxycycline) from complex matrices including milk, egg white, honey, and human plasma. Optimal parameters varied across matrices, underscoring the need for matrix-specific optimization. Optimized methods achieved recoveries of 66–101 %, excellent linearity (R^2 = 0.9929–0.9998), and minimal matrix effects (-14 % to 1 %). LLOQs ranged from 0.5 to 2.1 ng/g, with calibration curves extending to 1000 ng/g, covering concentrations relevant for residue monitoring. These results demonstrate EME's potential for reliable analysis of challenging polar and zwitterionic compounds in food and biological samples. Finally, the greenness was evaluated by the AGREEprep metric tool, gaining a total score of 0.64.

1. Introduction

Tetracyclines are broad-spectrum antibiotics used to treat bacterial infections in humans and animals. They act by inhibiting protein synthesis, preventing bacterial growth and replication [1,2]. In humans, doxycycline (DC) is commonly prescribed, whereas oxytetracycline (OTC), tetracycline (TC), and chlorotetracycline (CTC) are primarily used in livestock due to their low cost, low toxicity, and high availability [1,3]. These antibiotics are used both to treat infections and as feed additives to promote growth and weight gain. They may also be administered before or after slaughter to prolong meat shelf life [2,4]. In the US, they accounted for 65 % of domestic sales of medically important antimicrobial drugs approved for use in food-producing animals in 2022 [5]. The same year, they also represented the second highest selling class of antibiotics in Europe [6]. Frequent use can lead to tetracycline resistance in livestock, and their strong chelation with metal cations (e.g., Ca^{2+}, Mg^{2+}) prolongs elimination and promotes accumulation in animal tissues. Consequently, residues can enter the human food chain via milk, eggs, and meat. This poses a public health concern because tetracycline residues may contribute to resistant bacteria, dysbacteriosis, and allergic reactions [2]. Regulatory authorities have therefore established maximum residue limits (MRLs) to mitigate this risk. In the European Union, the limits are 0.2 μg/g for eggs and 0.1 μg/g for milk [4]. Sensitive and reliable analytical methods are essential for detecting tetracycline residues in livestock products and ensuring compliance with MRLs.

Interest in studying tetracycline residues in food samples has grown in recent years (Scopus). Milk, eggs, and honey are the most studied matrices, and numerous approaches have been developed to quantify tetracyclines in these samples [4]. For the sample preparation, methods such as solid-phase extraction (SPE) [7], protein precipitation (PP) [8], liquid-liquid extraction (LLE) [9], ultrasonic-assisted extraction (UAE) [10], and pressurized liquid extraction (PLE) [11], or various combinations of these, are some of the most commonly used. Methods such as SPE, PP, and LLE are well studied and widely used, making them reliable and cost-effective for tetracycline quantification. However, these methods involve multiple preparation steps and generate large amounts of organic waste, making them cumbersome and environmentally unfriendly. Additionally, some analytical methods lack the sensitivity to detect tetracycline residues at MRLs, requiring time-consuming and costly preconcentration steps [4]. Molecularly imprinted polymers (MIPs) have also been used for tetracycline extraction and

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preconcentration in food matrices, offering excellent selectivity [12]. However, this technique is limited by high cost and methodological complexity. Moreover, a preliminary cleanup step such as PP is commonly needed when dealing with protein-rich biological samples.

Electromembrane extraction (EME) is a miniaturized form of LLE, first developed in 2006 by Pedersen-Bjergaard and Rasmussen [13]. EME employs a three-phase system comprising two aqueous phases - the sample and acceptor solutions - separated by a water-immiscible organic liquid membrane (SLM). Successful extraction requires analytes to be charged, typically achieved by adjusting the pH of both sample and acceptor solutions. For basic compounds, both solutions are acidified to promote protonation; for acidic compounds, the reverse applies. An external electric field drives analyte transport across the SLM. For basic compounds, the positively charged electrode (anode) is placed in the sample solution, and the negatively charged electrode (cathode) in the acceptor solution. When voltage is applied, positively charged analytes migrate through the SLM into the acceptor solution.

EME offers high efficiency, strong clean-up capability, environmental benefits, and potential for automation. EME is a one-step preparation method, and its aqueous acceptor solution is directly compatible with LC-MS/MS. The electric field accelerates analyte transfer, improving speed compared to traditional LLE. These aspects contribute to reducing valuable laboratory time. EME's selectivity ensures effective clean-up, as neutral and oppositely charged analytes remain in the sample. The SLM excludes salts, proteins, lipids, phospholipids, and many endogenous metabolites, reducing risk of column degradation and ion suppression during LC-MS/MS. This also eliminates the need for a preliminary protein removal step. Moreover, EME uses only microliters of organic solvent, aligning with green chemistry principles. EME also shows strong potential for automation, as demonstrated in recent studies [14,15]. EME can be implemented in 96-well formats to increase throughput [16,17].

A major challenge in EME, as in many extraction techniques, is recovering polar, zwitterionic compounds - such as tetracyclines - due to their complex ionization and poor partitioning into conventional EME solvents [18,19]. This study reports the first successful application of EME for extracting tetracyclines (OTC, TC, CTC, DC) from diverse matrices including milk, egg white, honey, and human plasma. These compounds were extracted with recoveries ranging from 66 to 101 %, demonstrating robust performance across matrices with markedly different compositions in terms of protein content, viscosity, and sugar levels. Beyond method development, this work provides fundamental insights into EME behavior of polar, zwitterionic analytes, including transport mechanisms and solvent compatibility. By systematically assessing matrix complexity, this study clarifies how matrix constituents affect extraction efficiency and system stability - an aspect largely overlooked in previous EME research [20–25]. These findings expand the scope of EME, positioning it as a versatile tool for analyzing challenging analytes in complex biological and food samples.

2. Materials and methods

2.1. Chemicals and reagents

Tetracycline hydrochloride (TC, ≥95 %), chlorotetracycline hydrochloride (CTC, ≥90 %), oxytetracycline hydrochloride (OTC, ≥90 %), and doxycycline (DC, ≥95 %) were purchased from Merck (Darmstadt, Germany). Structures, molecular masses, isoelectric points, and Log P values are shown in Table S1; isoelectric plots are in Fig. S1.

Furthermore, 6-methylcoumarin (6-MC, ≥ 99 %), thymol (Thy, 99–101 %), formic acid (≥ 99 %), hydrochloric acid (37 %), 1-octanol (≥ 99 %), 2-undecanone (99 %), 2-nitrophenyl octyl ether (NPOE, ≥ 99 %), di(2-ethylhexyl) phosphite (DEHPi, 96 %), di(2-ethylhexyl) phosphate (DEHP, 97 %), Aliquat®336, and LC-MS grade acetonitrile, were all purchased from Merck (Darmstadt, Germany). Milli-Q grade water was produced by a Millipak® (0.22 μm filter) purification system.

Milk (1 % fat, homogenized and pasteurized), chicken eggs, and acacia honey was obtained from the local grocery store. Human drug-free citrate plasma was purchased from Oslo University Hospital (Oslo, Norway) and stored at −28 °C.

2.2. Preparation of stock and standard solutions

Individual stock solutions of TC, OTC, CTC, and DC (≈1 mg/mL in MeOH) were stored at −28 °C. Working solutions (10 μg/mL) were prepared by dilution with water and aliquoted into 1.5 mL Eppendorf tubes. For optimization and performance evaluation, samples were spiked to 0.1 μg/mL. The preparations of the sample solutions are described below.

2.3. Preparation of sample solutions

Milk samples were prepared by mixing equal volumes of milk and water. To 1800 μL of this mixture, 48, 33, 17, or 0 μL of 3 M HCl was added to adjust pH to 1.5, 2.4, 4.0, or 7.0 for optimization experiments.

To prepare the egg samples, the egg white was separated from the yolk. For the egg yolk extractions, 1 part of egg yolk was mixed with 2 parts of water (w/w) and vortexed. To 1000 mg of this solution, 26 μL of 3 M HCl was added to reach pH 2.4 for initial experiments. For the egg white extractions, equal weight of egg white and water were mixed and centrifuged to ensure a homogenous solution. To 1000 mg of this solution, 33, 26, 15, and 5 μL of 3 M HCl was added to reach pH values of 1.5, 2.4, 4.0, and 7.0, respectively, for optimization experiments.

Thawed human plasma was diluted with an equal volume of 100 mM formic acid and vortexed. To 1000 μL of this solution, 35, 25, and 0 μL of 3 M HCl was added to adjust the pH to 1.5, 2.4, and 4.0, respectively. To prepare pH 7.0, equal volumes of plasma and 50 mM citric acid/Na_2HPO_4 buffer pH 7.0 were mixed and vortexed.

Honey was initially diluted 3-fold with water (w/w). To 2000 mg of this solution, 8.0, 4.6, and 0.0 μL of 3 M HCl was added to obtain pH values of 1.5, 2.4, and 4.0, respectively. At a later stage, a 2-fold dilution was found to be sufficient, and the final composition of the honey samples was adjusted to a honey-to-water ratio of 1:1 (w/w) with a pH of 4.0.

2.4. Preparation of the supported liquid membrane (SLM)

Several liquid membrane compositions were tested in this study. The SLM providing the best extraction and used in all subsequent optimization experiments was a 1:2 (w/w) mixture of 6-methylcoumarin and thymol. The components were weighed into a 5 mL Eppendorf tube, heated at 80 °C for 15 min, and vortexed for 10 s to obtain a homogeneous liquid.

2.5. EME setup and procedure

Electromembrane extraction was performed in a 10-position prototype device developed by Extraction Technologies Norway (ETN, Ski, Norway) (Fig. 1). The vials housing the sample and acceptor solutions were made of a conductive polymer and had total volumes of 200 μL. For each extraction, working volumes of 120 μL sample and acceptor solution were pipetted into their respective vials. A porous polypropylene membrane (Accurel PP2E, Membrana GmbH, Wuppertal, Germany) was placed inside a leak-tight union using the bottom of a pipette tip. The union was attached to the acceptor vial. Then, 9 μL of SLM solvent was pipetted onto the polypropylene membrane and allowed to distribute evenly for approximately 1 min. The sample vial was secured to the other side of the union, forming the complete EME-unit. The assembled unit was placed in a shaking device. A top cover filled with electrodes, connected to an external power supply (model ES 0300-0.45, Delta Elektronika BV, Zierikzee, Netherlands) was positioned over the vials. When the negatively charged cathodes were in contact with the acceptor

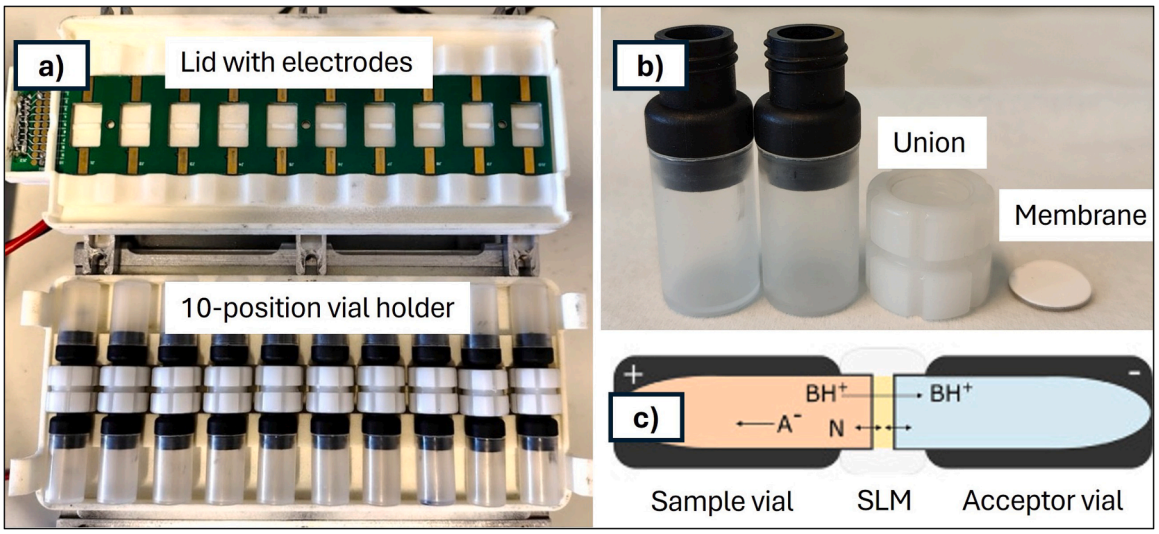


Fig. 1. (a) Photo of the prototype EME device. (b) Photo of sample and acceptor vial in transparent plastic holders to mimic the size of a HPLC vial, a leak-tight union, and a polypropylene membrane. (c) Illustration of the EME principle of the extraction of a protonated basic compound. The figure was adapted from Skaalvik et al. [26].

vials, and the positively charged anodes were in contact with the sample vials, an electrical circuit was established. During the extraction, the agitation rate was 900 RPM. The current was monitored using a Fluke287 multi-meter (Everett, WA, USA). After extraction, the acceptor solutions were collected and transferred to HPLC-vials with inserts, and analyzed by UHPLC-MS/MS. The polypropylene membranes were single-use. For the preparation of post-spiked acceptor solutions used to calculate recovery and matrix effects, 90 μL blank, extracted matrix was mixed with 10 μL standard solution prepared in the same solvent as the acceptor solution. The 10 % dilution of the extracted matrix was considered to have a minimal influence on the calculation of matrix effects.

2.6. UHPLC-MS/MS determination

Chromatographic separations were performed using an Agilent 1290 Infinity II UHPLC system (Agilent Technologies, Santa Clara, CA, USA). The column used was a C18 Acquity UPLC® HSS T3 (2.1 × 50 mm, 1.8 μm, Waters, Wexford, Ireland), kept at a temperature of 40 °C. Mobile phases A and B were composed of deionized water and acetonitrile in 95:5 and 5:95 ratios (v/v), respectively, and acidified with 0.1 % formic acid. The injection volume was 1 μL. Elution was performed with the following gradient: Mobile phase B increased from 10 % to 40 % over 3 min, held at 100 % until 4 min, then returned to 10 % for re-equilibration until 5.5 min. The flow rate was 0.4 mL/min. A chromatogram showing the separation and retention times of the analytes is provided in supplementary information (Fig. S2).

Detection was performed with an Agilent 6495 LC/TQ mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) operated with positive electrospray ionization (ESI) at 3.5 kV. The gas temperature was 160 °C, and the gas flow was 13 L/min. Each analyte was measured by two MRM-transitions: *m/z* (quant) 461.2 → 426.2 (CE = 19 V) and *m/z* 461.2 → 443.2 (CE = 11 V) for OTC, *m/z* (quant) 445.2 → 410.2 (CE = 19 V) and *m/z* 445.2 → 154.1 (CE = 27 V) for TC, *m/z* (quant) 479.1 → 444.1 (CE = 19 V) and *m/z* 479.1 → 462.2 (CE = 15 V) for CTC and *m/z* (quant) 445.2 → 428.2 (CE = 15 V) and *m/z* 445.2 → 154.1 (CE = 30 V) for DC. Sheath gas temperature and flow was 200 °C and 12 L/min, respectively. Nebulizer was 50 psi, and the nozzle voltage was 0 V.

2.7. Calculations

Extraction recovery (R) was calculated as the average of replicate extractions using the Matuszewski method [27], which corrects for matrix effects, as shown in Eq. (1):

$$R = \frac{AUC\ acceptor,\ pre\ spiked\ matrix}{AUC\ acceptor,\ post\ spiked\ matrix} x100\% \quad (1)$$

Where "AUC acceptor, pre spiked matrix" is the peak area for the extracted sample, and "AUC post spiked matrix" is the peak area for a blank, extracted sample, spiked after extraction.

Matuszewski's method was also used to calculate the matrix effects (ME):

$$ME = \left(1 - \frac{AUC\ acceptor,\ post\ spiked\ matrix}{AUC\ neat\ standard}\right) x100\% \quad (2)$$

Where "AUC neat standard" is the peak area for the analyte dissolved in 500 mM formic acid with identical concentration as the acceptor, post spiked matrix. In the case of ME = 0 %, there is no change in signal (ion suppression/enhancement) due to influence from matrix components in the sample.

2.8. Evaluation of analytical merits

The linearity, precision and accuracy of four tetracyclines in milk, egg white, honey and human plasma samples were evaluated using spiked blank samples, and the results are given in Table 1. For the determination of the lower level of quantification (LLOQ), following equation was used:

$$LLOQ = (3.3\ x\ S_y)/S \quad (3)$$

Where S_y represents the standard deviation of the y-intercept, and S represents the slope of the calibration curve. The upper limit of quantification (ULOQ) was determined experimentally and represents the highest point on the calibration curve.

The accuracy was calculated based on Eq. (4):

$$Accuracy = C/C_0\ x\ 100\% \quad (4)$$

Where C is the estimated concentration and C_0 is the true concentration.

Table 1
Analytical merits for the determination of tetracyclines in milk, egg white, honey and human plasma samples. Samples were extracted with four replicates per concentration level. Concentrations are given in ng/g for milk, egg white and honey, and ng/ml for human plasma and 100 mM formic acid. Calibration curves were weighted by 1/x. LLOQ=lower limit of quantification, ULOQ=upper limit of quantification.

Compound	Matrix	LLOQ	ULOQ	R^2	RSD (%)		Accuracy (%)	
					Lowest calibration level	ULOQ	Lowest calibration level	ULOQ
OTC	Milk	1.6	1000	0.9929	3	5	86	103
	Egg white	0.7	1000	0.9988	6	3	95	99
	Honey	0.8	1000	0.9995	11	2	106	101
	Human plasma	2.1	1000	0.9936	15	8	77	100
	100 mM formic acid	2.5	250	0.9956	11	5	80	99
TC	Milk	1.0	1000	0.9975	1	3	75	101
	Egg white	0.5	1000	0.9994	3	1	91	99
	Honey	0.5	1000	0.9998	11	2	98	100
	Human plasma	1.1	1000	0.9983	5	3	74	99
	100 mM formic acid	1.7	250	0.9958	5	3	87	101
CTC	Milk	0.9	1000	0.9977	7	4	86	101
	Egg white	0.7	1000	0.9986	8	2	102	100
	Honey	0.7	1000	0.9997	16	4	103	100
	Human plasma	0.9	1000	0.9989	7	2	78	99
	100 mM formic acid	0.6	250	0.9954	9	3	97	102
DC	Milk	1.2	1000	0.9960	11	3	94	101
	Egg white	0.7	1000	0.9988	12	1	108	100
	Honey	0.7	1000	0.9990	17	1	107	101
	Human plasma	1.0	1000	0.9986	5	2	74	99
	100 mM formic acid	1.3	250	0.9950	11	3	86	101

3. Results and discussion

3.1. Selection of ionization mode and SLM

Tetracyclines are zwitterionic compounds, capable of carrying either a positive or negative charge depending on sample pH. Their isoelectric points (4.8–5.2; Table S1) indicate that they are negatively charged above pH 5.2 and positively charged below pH 4.8. Charge variation with pH is shown in Fig. S1.

Extraction was first attempted with tetracyclines in their negatively charged form for two reasons: minimal pH adjustment was needed (native matrix pH ≈7), and tetracyclines remain negatively charged over a broader pH range, making small pH shifts in the acceptor less impactful. The negatively charged tetracyclines were extracted from a neat standard solution using four different SLMs suitable for acidic compounds: 1-octanol, 1-octanol + 1 % Aliquat 336, a deep eutectic solvent consisting of 6-methylcoumarin and thymol in a 1:2 ratio (w/w) (6-MC:Thy, 1:2), and a generic membrane developed for moderately polar acids termed A2 [28], which is 6-MC:Thy (1:2) diluted with 2-nitrophenyl octyl ether (NPOE) in a 2:1 ratio (w/w). 1-octanol was chosen as it is one of the most predominantly used SLMs in the literature for the extraction of acidic compounds, due to its hydrogen bond donor properties [29]. Aliquat 336 was added to 1-octanol to facilitate ionic interactions between the SLM and the polar tetracyclines to enhance extraction efficiency. The deep eutectic solvent was tested because it has proved successful for the extraction of zwitterionic peptides and acidic drugs [30,31]. The generic membrane A2 [28], a deep eutectic solvent with NPOE included for stability purposes, was also tested in this study.

As anions, the tetracyclines were initially extracted from 50 mM citric acid/Na_2HPO_4 buffer pH 7.4, 50 mM citric acid/Na_2HPO_4 buffer pH 9.0, and 50-mM phosphate buffer pH 10.0 to evaluate the influence of varying degrees of negative charge. Recoveries were however poor (Fig. S3), confirming previous reports that extracting polar acids with EME is challenging due to limited suitable solvents and the aqueous nature of sample matrices [29]. Additionally, since sample matrices in EME are aqueous, the selective extraction of polar analytes becomes more difficult. Another limitation is CTC instability at alkaline pH, which causes degradation [32]. Consequently, only three analytes were tested under these conditions.

In contrast, all tetracyclines were stable under acidic conditions (pH 2.4), and EME of positively charged tetracyclines from acidified buffer was considerably more effective. Five different organic solvents were tested as SLMs: NPOE, 6-MC:Thy (1:2), DEHPi + 10 % DEHP, NPOE + 10 % DEHP, and a generic membrane for polar bases termed B3 [33], consisting of 6-MC:Thy (1:2) diluted 1:1 with 2-undecanone and containing 1 % DEHP (Fig. 2). These SLMs were selected based on previously demonstrated suitability for extracting basic analytes [29,33,34]. In this study, using NPOE as SLM, none of the tetracyclines were extracted as they were too polar to partition into the hydrophobic liquid membrane. The remaining SLMs, less hydrophobic in nature, were all able to extract the polar tetracyclines to various extents, with 6-MC:Thy (1:2) providing the best performance. This SLM could extract all tetracyclines with recoveries exceeding 80 % and showed excellent operational stability, with no increase of electrical current throughout the extraction period. Consequently, EME of positively charged tetracyclines using 6-MC:Thy (1:2) as the SLM was selected for further optimization experiments.

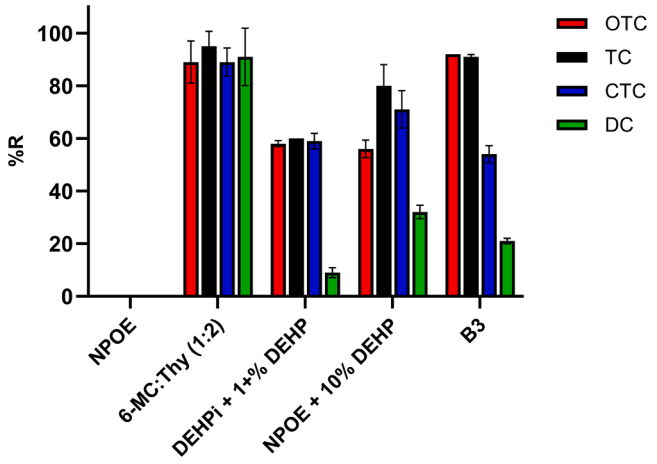


Fig. 2. %R of 4 tetracyclines (0.1 μg/mL) extracted from 100 mM formic acid (pH 2.4), through five different SLMs. All samples were extracted at 30 V, except when extracting through DEHPi + 10 % DEHP (5 V). Extraction times were 20 min. Error bars represent the standard deviation (n = 3).

3.2. Effect of sample matrix

The applicability of 6-MC:Thy as the SLM was tested using several relevant food and biological matrices: cow milk, egg yolk, honey and human plasma. Cow milk and egg yolk were selected as matrices because tetracyclines are widely used in farming and are readily transferred from animal tissue to their food products after administration. Regarding eggs, the yolk residue concentration is generally higher than in the egg white [35]. Therefore, yolk was initially tested to enhance the sensitivity of the method. Honey was included because tetracyclines are frequently used by beekeepers to prevent bacterial infections such as foulbrood [36]. Human plasma was chosen because DC is a commonly prescribed antibiotic in clinical settings. Although DC is not typically relevant for therapeutic drug monitoring, the experiments in the present study provide valuable insights into the extraction of polar, zwitterionic compounds from human plasma.

All matrices yielded stable extractions with acceptable recoveries (data not shown), except egg yolk. Its high fat and protein content likely caused partial SLM dissolution, preventing stability, and the extraction current increased markedly during the experiment, even after 5-fold dilution. To address stability issues, the second-best SLM from Fig. 2 (NPOE + 10 % DEHP) was tested as an alternative. Its higher hydrophobicity improved resistance to fat and protein interference, enhancing stability. However, this also reduced recovery, likely due to limited analyte partitioning and possible protein interactions with DEHP (Fig. S4).

Due to poor yolk recoveries, a 2-fold diluted egg white matrix was tested as an alternative. Using 6-MC:Thy (1:2) as the SLM, stable extractions with acceptable recoveries were achieved (data not shown). Although tetracyclines generally partition less into egg white than into yolk, reported egg white/yolk concentration ratios vary considerably: 0.25 to 1.0 for CTC, 0.1 to 1.2 for OTC, and 0.19-0.22 for TC [35]. Importantly, even under the lowest reported ratios, the LC-MS/MS method developed in this study provided sufficient sensitivity to detect tetracyclines in egg white at relevant levels. Accordingly, 2-fold diluted egg white was selected as egg matrix for further optimization.

To assess the general compatibility of egg yolk with EME, independent of tetracyclines, a mixture of 91 basic compounds with a wide range of hydrophobicity ($-2 < \text{Log } P < 8$) was extracted from 3-fold diluted egg yolk using NPOE + 10 % DEHP as SLM. For a comprehensive list of compounds, their log P values and the LC-MS method used for quantification we refer to Chen et al. [37]. The results, presented in Fig. S5, show that a substantial number of compounds can indeed be successfully extracted from egg yolk. Compounds with Log P values between 1 and 2 were more readily extracted compared to those that were either more hydrophilic or more hydrophobic. This highlights the critical role of the SLM in achieving efficient and selective extraction for different kinds of matrices.

3.3. Optimization of extraction parameters

In the next set of experiments, the sample pH, acceptor pH, applied voltage and extraction time were optimized for each matrix. This approach was taken to assess potential variations in the optimal extraction parameters across different matrices. All extractions were performed using 6-MC:Thy (1:2) as the SLM, at 900 RPM for 20 min unless otherwise specified. All matrices were initially 2-fold diluted, except for honey, which was 3-fold diluted during the preliminary experiments. From Section 3.3.3 onward, honey was also 2-fold diluted.

3.3.1. Selection of sample pH

The sample pH is a critical parameter, as it determines the ionization state of the analytes. The analytes must be in their charged state to be influenced by the electrical field and extracted. Typically, the sample pH for extraction of protonated compounds is about 2 pH levels below the pKa-value of the analytes [38]. For each matrix, pH levels of 1.5, 2.4, 4.0, and 7.0 were tested to identify optimal extraction efficiency (Fig. 3). In the case of honey, pH 7 was excluded to avoid adjusting the pH upwards, as the natural pH of a 1:2 (w/w) honey-to-water mixture was already around 4.0. For the milk samples, coagulation was observed at pH 4.0, likely due to the behavior of casein, a major milk protein. Casein has an isoelectric point around 4.0, causing it to become electrically neutral, leading to aggregation and precipitation. Nevertheless, extraction was performed, and no detrimental effects on system stability were observed, highlighting the suitability of EME also for non-homogenous samples.

Across all matrices, recoveries dropped significantly at pH 7, due to loss of positive charge and reduced mass transfer. Extractions at pH 7.0 also showed higher electrical currents, likely from increased ion mobility at neutral pH. The optimal pH varied slightly between matrices: for human plasma, the highest average %R was obtained at pH 1.5; for egg white and milk, pH 2.4 yielded the best results. For honey, the optimal pH was 4.0, which was advantageous because it eliminated the need for pH adjustment with strong acid. Honey's high extraction efficiency may result from its sugar content, which induces a "sugaring-out effect" that reduces matrix competition and improves mass transfer [39].

While recoveries in all matrices remained high up to pH 4.0, the data also confirm the importance of individual optimization of pH in different matrices when developing new EME methods.

3.3.2. Selection of acceptor pH

Like the sample pH, a sufficiently low acceptor pH is critical to prevent loss of analyte charge and subsequent back-extraction into the SLM. Also, it is important to overcome the local pH effects in the SLM/acceptor interface, which previously has been demonstrated to be elevated relative to the bulk solution [40]. For the selection of acceptor pH, human plasma was used as the representative matrix, based on the assumption that analyte transport from the SLM into the acceptor is largely independent of the sample matrix. Three different acceptor solutions were tested: 20 mM formic acid (pH 2.7), 100 mM formic acid (pH 2.4) and 500 mM formic acid (pH 2.0). Lower pH levels were not tested to avoid incompatibility with the analytical column. The average recovery of the four tetracyclines were 65 %, 82 % and 84 %, for 20-, 100- and 500-mM formic acid, respectively. Based on the results, 500 mM formic acid (pH 2.0) was selected as the optimal acceptor solution.

3.3.3. Voltage and time optimization

Voltage is the driving force that stimulates analyte transport across the SLM. Higher voltages generally increase recovery until instability occurs, causing excessive current and electrolytic reactions that reduce recovery and precision [38]. To optimize voltage, all matrices were extracted at 0, 5, 10, 20, 30, and 50 V (Fig. 4). Recoveries increased sharply between 0 and 5 V for all tetracyclines, but plateaued beyond 10 V. Considering recovery and system stability, 10 V was selected as optimal for all matrices, indicating that voltage-assisted partitioning was largely matrix-independent.

Extraction time was optimized for egg white, honey, plasma, and milk. As with voltage, recoveries increased with time until reaching equilibrium [41]. Samples were extracted at 10 V for 5, 10, 15, 20, and 30 min (Fig. 5). Equilibrium was reached after 20 min for plasma and milk, but required 30 min for egg white and honey. The longer time for honey was attributed to high viscosity reducing mass transfer, while in egg white, high protein content likely caused SLM surface fouling and therefore longer time was required. These findings also underscore the need for matrix-specific optimization.

The optimal extraction parameters for each sample matrix are outlined in Table S2, and their extraction current profiles are shown in Fig. S6. All matrices provided low current in the range 2–14 μA throughout the extraction time, which indicates excellent system stability.

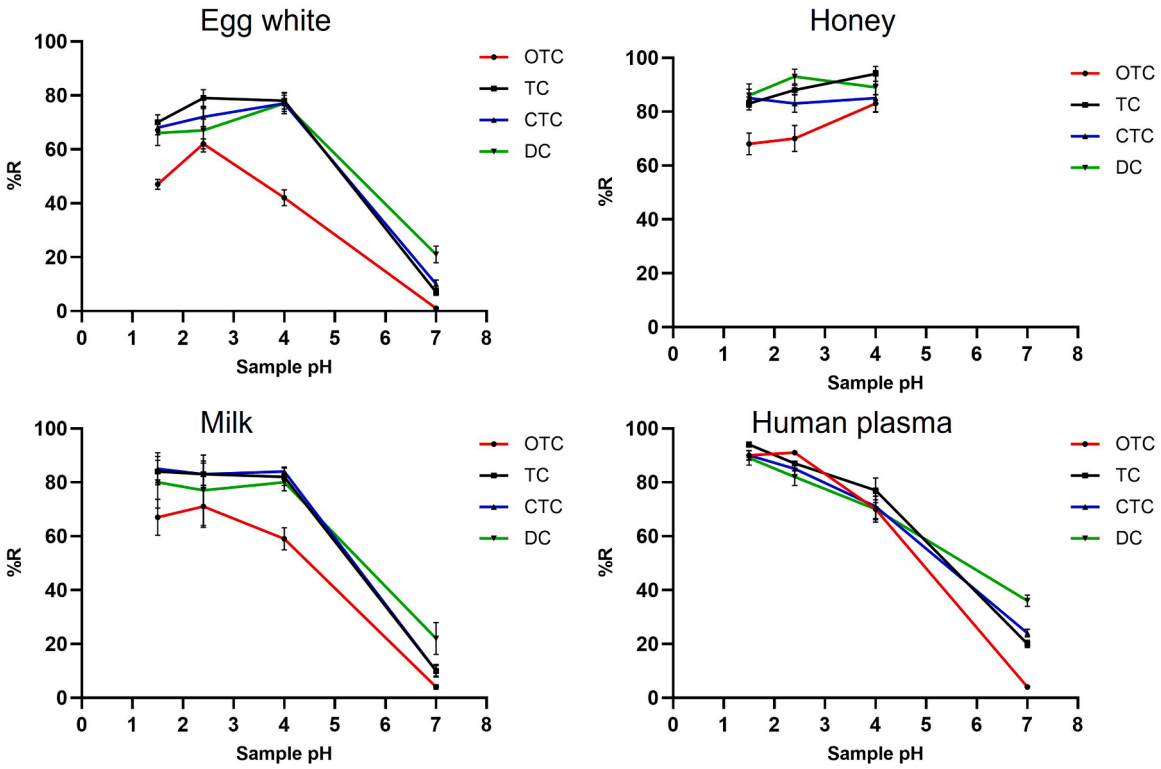


Fig. 3. %R of 4 tetracyclines extracted from egg white, honey, milk and human plasma at pH 1.5, 2.4, 4 and 7. All samples were extracted at 10 V. The concentrations of tetracyclines are 0.1 μg/g for egg white, honey and milk, and 0.1 μg/ml for human plasma. Error bars represent the standard deviation ($n = 3$).

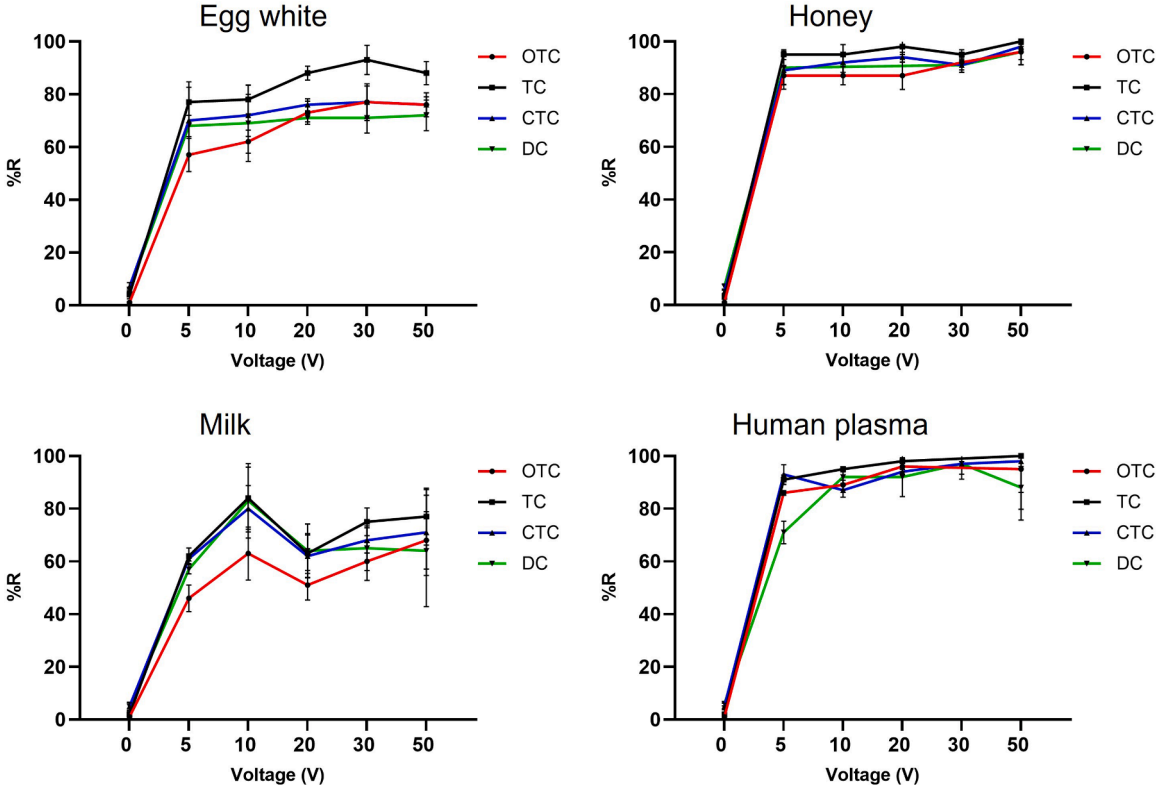


Fig. 4. Optimization of voltage. %R for four tetracyclines in egg white, honey, milk, and human plasma, at 0, 10, 20, 30, and 50 V. Extraction time was 20 min. The concentrations of tetracyclines are 0.1 μg/g for egg white, honey and milk, and 0.1 μg/ml for human plasma. Error bars represent the standard deviation ($n = 3$).

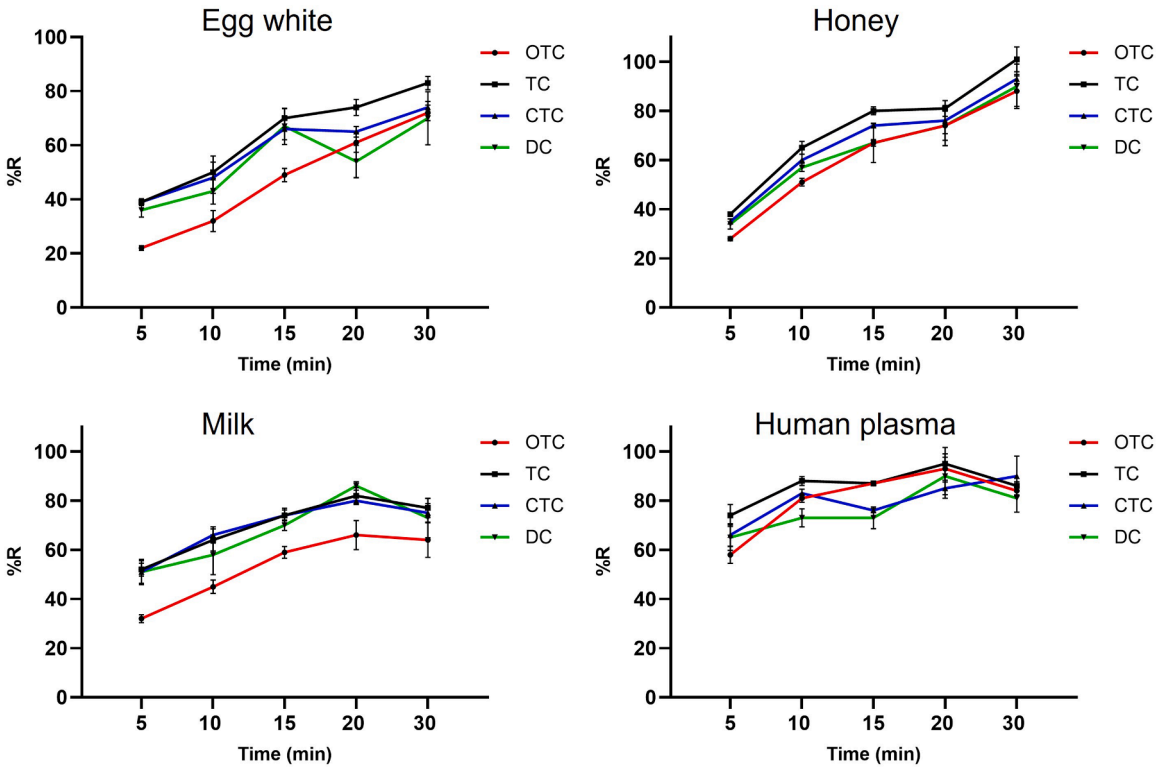


Fig. 5. Optimization of time. %R of four tetracyclines in egg white, honey, milk and human plasma, at 5, 10, 15, 20, and 30 min. Extraction voltage was 10 V. The concentrations of tetracyclines are 0.1 μg/g for egg white, honey and milk, and 0.1 μg/ml for human plasma. Error bars represent the standard deviation ($n = 3$).

3.4. Analytical performance

All four tetracyclines demonstrated acceptable linearity, precision, and accuracy across all matrices (Table 1). In human plasma, accuracy at the lowest concentration level ranged from 74 % to 78 %, but this deviation is unlikely to be clinically significant since doxycycline plasma levels typically exceed 1 μg/mL [42]. For food matrices, calibration curves covered all relevant concentrations and MRLs. For example, the MRL for tetracyclines in eggs is 100 ng/g. In this study, LLOQs ranged from 0.5 to 2.1 ng/g, and calibration curves showed excellent linearity $0.9986 < R^2 < 0.9994$) up to 1000 ng/mL or ng/g. Recoveries were high across all analytes and matrices, ranging from 66 % to 101 % (Table 2). Matrix effects were acceptable (−14 % to 1 %), indicating that EME effectively retains components such as proteins and lipids that typically cause ion suppression in LC-MS/MS. Tetracyclines were also extracted from 100 mM formic acid (pH 2.4) for comparison with matrix-based extractions, under the same conditions as milk, with no additional optimization needed since recoveries were ≥92 %. Tables 1 and 2 show that LLOQ, ULOQ, R^2, RSD, accuracy, and ME values were comparable to matrix-based extractions, while slightly higher recoveries were achieved - likely due to the absence of protein binding, matrix viscosity, and membrane fouling. It may be noted that the ULOQ of neat 100 mM formic acids was lower than for the matrices, which was caused by higher recoveries bringing the response at 1000 ng/mL slightly outside the linear range.

Potential interferences were evaluated by extracting blank, non-spiked matrices using their respective optimized methods and analyzing the resulting extracts by LC-MS/MS. The chromatograms provided in Fig. S7 show that no interfering peaks were observed from the matrices relative to the neat 100 mM formic acid.

Table 2
% Recovery (R), % RSD and % matrix effects (ME) of tetracyclines for the optimized methods. The concentrations extracted are 0.1 μg/g for milk, egg white and honey, and 0.1 μg/ml for human plasma and 100 mM formic acid.

Compound	Matrix	R(%)	RSD (%), $n = 3$	ME (%)
OTC	Milk	66	9	−5
	Egg white	72	4	1
	Honey	88	7	−6
	Human plasma	93	5	1
	100 mM formic acid	101	5	−7
TC	Milk	82	4	−1
	Egg white	83	3	0
	Honey	101	5	−12
	Human plasma	95	7	1
	100 mM formic acid	97	4	−7
CTC	Milk	80	2	−3
	Egg white	74	3	−4
	Honey	93	2	−14
	Human plasma	85	3	−1
	100 mM formic acid	96	6	−9
DC	Milk	86	2	−4
	Egg white	70	14	0
	Honey	90	10	−2
	Human plasma	90	10	−6
	100 mM formic acid	92	3	−10

3.5. Evaluation of greenness

Greenness was evaluated using the Analytical Greenness Metric for Sample Preparation (AGREEprep) tool [43], which assigns a score from 0 to 1, with 1 representing the most environmentally friendly system. The score reflects compliance with the ten principles of Green Sample Preparation, considering factors such as solvent and reagent toxicity, waste generation, sample throughput, and energy consumption [44]. Results are shown in Fig. 6: all matrices achieved an overall score of 0.64. The methods scored highest in categories #2 (safe solvents and reagents), #4 (waste minimization), #5 (minimal sample, chemical, and material use), and #8 (energy efficiency). The lowest scores were in categories #1 and #10. Category #1 favors in situ sample preparation, which was not feasible due to laboratory-based requirements. Category

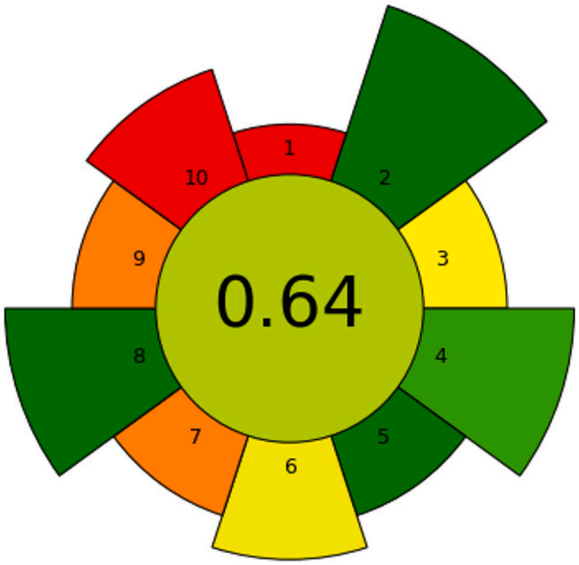


Fig. 6. AGREEprep pictogram for all four methods for extracting tetracyclines from different matrices. The number in the center of the pictogram represents the greenness score. The color and size of the ten surrounding categories represent their score and weight, respectively. The pictogram was generated using the web app https://agreeprep.anvil.app/.

#10 evaluates operator safety based on hazard pictograms; despite the very small amounts of 6-methylcoumarin, thymol, formic acid, and hydrochloric acid used, their presence resulted in more than four hazard pictograms, lowering the score. A detailed breakdown of all ten categories and input data is provided in Table S3.

4. Conclusion

This study demonstrates that EME is an effective alternative for extracting four commonly used tetracyclines from food matrices such as egg, milk, and honey, as well as from human plasma. Only minor differences in optimal parameters were observed among matrices, suggesting that EME is suitable for a wide range of complex samples. Compared to traditional methods, EME offers a greener approach by significantly reducing solvent consumption and waste, while providing a highly efficient procedure with minimal steps and limited sample dilution. A current limitation is its relatively low throughput, as no more than 10 samples can be processed simultaneously; however, the method is readily adaptable to a 96-well format, which would substantially increase throughput.

Tetracyclines were extracted with high recoveries (66–101 %) across relevant concentration ranges, low RSD values (1–17 %), negligible matrix effects (−14 % to 1 %), and acceptable accuracies (74–108 %). Greenness was assessed using the AGREEprep metric, yielding a score of 0.64. Consistent with previous findings, this study confirms that zwitterions are more efficiently extracted as positively charged cations than as negatively charged anions [45]. Furthermore, we observed that optimal parameters such as sample pH and extraction time vary by matrix, whereas voltage appears to be matrix-independent. These results demonstrate that EME is compatible with complex sample matrices, including non-homogeneous milk samples containing precipitated proteins, and is well suited for extracting challenging polar and zwitterionic compounds with high analytical performance across diverse matrices.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT-5 (OpenAI) for language editing. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

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CRediT authorship contribution statement

Anne Oldeide Hay: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Frederik André Hansen:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sampre.2025.100227.

Data availability

Data will be made available on request.

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