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Application of salting-out assisted liquid-liquid extraction for the determination of oxytetracycline, tetracycline, tilmicosin, and tylosin in cow milk by liquid chromatography with photodiode array detection

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SALLE

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ABSTRACT

This paper introduces a novel, sensitive, and rapid method for the quantification of oxytetracycline, tetracycline, tilmicosin, and tylosin residues in cow's milk. The method involves a two-step process of extraction and detection. The extraction process uses acetonitrile and salting-out assisted liquid-liquid extraction to isolate the antibiotics from the milk. The detection process employs Liquid Chromatography coupled with photo-diode array detector (PDA) to quantify the antibiotics. The method has been successfully applied to milk samples, demonstrating its effectiveness and potential for widespread use in residue analysis.

- The calibration curves for the antibiotics were found to be linear within the range of 0.06–3.0 μg/mL to 0.1–3.0 μg/mL.
- The limits of detection for oxytetracycline, tetracycline, tilmicosin, and tylosin were 0.03 μg/mL, 0.02 μg/mL, 0.04 μg/mL, and 0.02 μg/mL respectively.
- The method demonstrated an average recovery rate of over 90% from milk samples with peak values reaching up to 0.100–0.200 μg/mL.

Specifications table

Subject area:	Environmental Science
More specific subject area:	Sample preparation
Name of your method:	SALLE
Name and reference of original method:	SALLE
Resource availability:	It is not applicable

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Method details

Antibiotics are globally utilized to combat bacterial infections and promote the health of farm animals, thereby enhancing milk production. Despite their introduction into veterinary medicine over four decades ago, tetracyclines remain the most frequently used antibiotics in animal husbandry [1]. However, their presence in the milk supply is undesirable. These drugs exhibit high levels of activity against a broad spectrum of bacteria in food-producing animals. The US Food and Drug Administration's Center for Veterinary Medicine (CVM) has established tolerances for total residues of tetracyclines, including chlortetracycline (CTC), oxytetracycline (OTC), and tetracycline (TC), in bovine milk at 300 ng/g, while the European Union (EU) has set maximum residue limits (MRLs) at 100 ng/g for each drug, considering the sum of the parent drug and its 4-epimer [2,3]. To protect human health, it is crucial to develop analytical methods that can accurately determine tetracycline concentrations in animal tissue and evaluate its presence in edible animal products. Macrolide antibiotics, commonly used and consisting of a macrocyclic lactone ring of 14, 15, or 16 atoms to which sugars are attached through glycosidic linkages, include Tilmicosin and Tylosin. These are used almost exclusively in veterinary medicine. Tilmicosin is extensively used to treat respiratory diseases in cattle, pigs, and chickens, among others. Tylosin, a macrolide antibiotic produced by Streptomyces fradiae, is primarily active against gram-positive bacteria, anaerobic bacteria, and mycoplasma [4]. Tilmicosin is synthesized from tylosin and has a similar antibacterial spectrum. The EU has set MRLs in milk at 50 g/kg for tilmicosin and 100 g/kg for tylosin [5]. The structure of the selected antibiotics in this study is depicted in Fig. 1.

Numerous liquid chromatographic methods have been developed for the determination of the examined antibiotics [6–8]. Liquid-liquid extraction (LLE) using water-miscible organic solvents has been reported for the analysis of biological samples. Recently, there has been a growing interest in miniaturizing LLE to facilitate automation, expedite extractions, and reduce the consumption of organic solvents. An alternative to LLE extraction with acetonitrile is salting-out assisted liquid–liquid extraction (SALLE) [9,10].

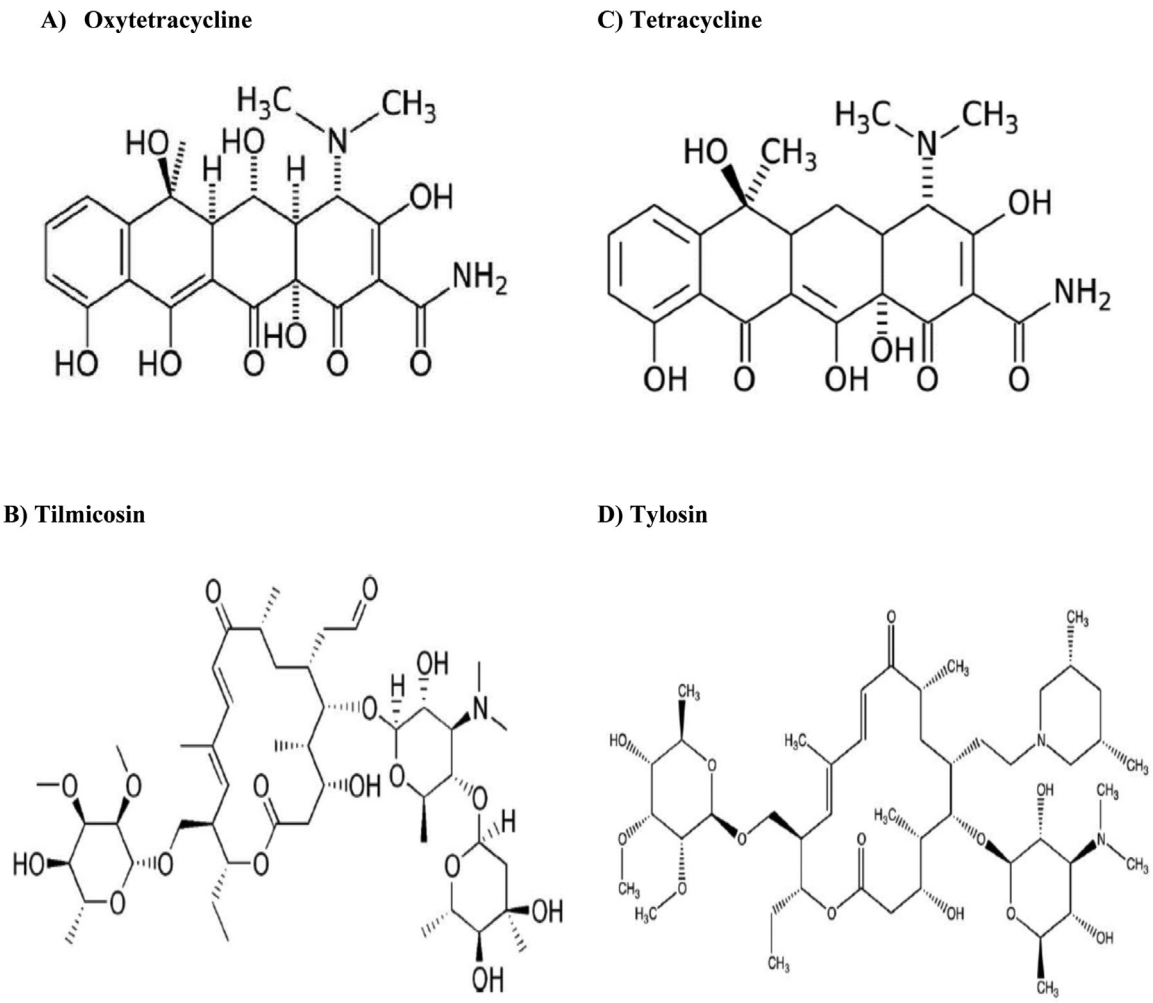

Fig. 1. The structure of the antibiotics.

A high throughput, rapid, salting-assisted liquid/liquid extraction (SALLE) technique has been recently developed. SALLE is performed by adding a concentrated inorganic or organic saline solution to a mixture of drug-containing biological samples and a water-miscible solvent such as acetonitrile, methanol, or acetone. The mixture of a biological sample and a water-miscible organic solvent can be forced to phase separate from solvated salts by increasing the ionic strength. Deproteinization is a common method for the extraction/purification step of antibiotic sample preparation in biomatrices. It is used where there is a need to eliminate interference while maintaining good recovery of the analytes of interest. An advantage of protein precipitation is that it is a relatively simple and inexpensive off-line process. In this method, the analyte was determined using acetonitrile as the deproteinization solution. Advantages of salting out with acetonitrile have been demonstrated, including ease of purification of biological samples and analyte enrichment [11,12].

The objective of this study is to develop a method based on SALLE for determining residues of tetracycline, oxytetracycline, tylosin, and tilmicosin in cow milk using liquid chromatography with photo-diode array detector (LC/UV/DAD).

Materials and methods

Reagents

All reagents used were of recognized analytical quality. Extreme care was taken to prevent any possible contamination of water, solvents, and inorganic salts. The following reagents were used:

1. **Solvents**: HPLC grade methanol and acetonitrile (Merck, Darmstadt, Germany).
2. **Water**: Deionized (Millipore, Molsheim, France).
3. **Inorganic Salts**: Oxalic acid, ammonium acetate, sodium hydroxide, sodium chloride (Merck Darmstadt, Germany).
4. **Trifluoroacetic Acid**: UV grade (Merck).
5. **0.1% NaOH Solution**: Prepared by dissolving 0.1 gram of sodium hydroxide in 100.0 mL of water.

Standards

The following standards were used:

1. **Reference Standards**: Tetracycline (98.9% w/w) and oxytetracycline (98% w/w) were obtained from Merck (Darmstadt, Germany), while tilmicosin (85%) and tylosin tartrate (88%) were procured from Sigma-Aldrich (USA).
2. **Individual Stock Solutions**: Stock solutions of tetracycline and oxytetracycline (1000.0 μg/mL) were prepared by dissolving the respective compounds in 100.0 mL of reagent water at appropriate concentrations. Similarly, stock solutions of tilmicosin and tylosin tartrate (1000.0 μg/mL) were prepared by dissolving tilmicosin in acetonitrile and tylosin tartrate in water.
3. **Intermediate Standard Solution**: Two intermediate solutions were prepared by diluting 10.0 mL of the tetracycline and oxytetracycline stock solutions in 100.0 mL of water, and by diluting 10.0 mL of the tilmicosin and tylosin stock solutions in 100.0 mL of water, respectively.
4. **Calibration Standards**: Varying volumes of the intermediate solution were diluted in 50.0 ml blank milk to prepare calibration standards with concentrations ranging from 0.05 to 3.00 μg/mL for tetracycline, oxytetracycline, tilmicosin, or tylosin.
5. **Spiking Solution**: Varying volumes of the intermediate solution were diluted in 50.0 ml blank milk to prepare spiking solutions with concentrations ranging from 0.05 to 0.200 μg/mL for tetracycline, oxytetracycline, tilmicosin, and tylosin. All solutions were stored at a temperature of 4 °C.

Apparatus

1. **Filters**: Millex HV13 filters (0.45 μm, 13 mm id) were used (Millipore, Saint Quentin Yvelines, France).
2. **Analytical Column**: A Nova Pak C18 column (3.9 i.d. × 150 mm) containing C18 particles of size 5.0 μm was used (Waters Ireland).
3. **Liquid Chromatograph**: The chromatography system used was a Model 2695 Waters liquid chromatography system, which included an autosampler, an in-line degasser, a 600E pump, a PDA detector (2996), and a Millennium 32 workstation (Waters, Milford, MA, USA).

Chromatographic determination

Tetracycline LC Mobile Phase was 10 mM oxalic acid in water–methanol (80:20, V/V) in an isocratic mode at a flow rate of 0.5 mL/min and they were detected at a wavelength of 355 nm. Tilmicosin and Tylosin LC Mobile Phase was 10 mM ammonium acetate and 1% trifluoroacetic acid in water–methanol (70:30, V/V) with a flow rate set at 1.0 mL/min and they were detected at 285 nm. The injection volume for each standard and sample solution was set at 20 μL.

Sample preparation

The sample preparation involved the following steps:

1. To a spiked cow's milk sample (1.5 mL), NaOH (0.1%, 0.1 mL) and acetonitrile (3.0 mL) were added in a polypropylene centrifuge tube.
2. The sample mixture was gently shaken for 1 min and then centrifuged at 3000 rpm for 5 min at room temperature.
3. The upper phase was transferred to a 1.5 mL centrifuge bottle and one gram of NaCl was added.
4. After gentle shaking and phase separation, the acetonitrile (extraction solvent) separated at the top of the measuring tube.
5. The acetonitrile was removed with a Hamilton syringe (0.30 mL) and transferred to a conical vial.
6. A volume of 20 µL from this vial was then injected into the LC/UV for quantification.

Method validation

The theory underlying salting-assisted liquid/liquid extraction (SALLE) is akin to that of Liquid-Liquid Extraction (LLE). The final concentration of the analyte in the milk sample solution can be expressed as follows:

$$C_f(\mu g\,mL^{-1}) = \frac{C_{eq}^{ex} V_{ex}}{V_{sam} E_r}$$

where, $C_{eq}^{ex}(\mu g\,mL^{-1})$ is the final concentration of analyte in the extracting phase, and V_{ex}(mL) is the final (separated) volume of the extracting phase, E_r is the extraction recovery and V_{sam} (mL) is the volume of the sample, respectively. Preliminary experiments indicate that the antibiotics under study are extracted more efficiently in an alkaline medium than in an acidic one. Consequently, we employed Sodium Hydroxide (NaOH) for the extraction of antibiotics. Acetonitrile was added to milk to extract drug residues and precipitate milk proteins. To validate the developed SALLE method, we tested linearity, correlation coefficient, detection limits, and repeatability using spiked samples. We prepared a matrix-matched calibration curve of oxytetracycline, tetracycline, tylosin, and tilmicosin in blank milk at concentrations of 0.05, 0.10, 0.50, 1.00, 2.00, and 3.00 µg/mL using external standard methods (average of three sets of data). We individually injected 20.0 µL of each sample and standard solution into the LC system. Peak identification was achieved by comparing the retention times and spectra of samples with those of standards, yielding correlation coefficients (R2) of > 0.99. The limits of detection (LOD), based on a signal-to-noise ratio of 3, were determined to be 0.03 µg/mL for OTC, 0.02 µg/mL for TC, 0.04 µg/mL for tilmicosin, and 0.02 µg/mL for tylosin. The limits of quantification (LOQ), defined as the lowest concentration of an analyte that can be determined with acceptable precision and accuracy under stated test conditions, are presented in Table 1.

We obtained inter-day relative errors, inter-day relative standard deviations, and recoveries by spiking blank samples at three different levels (50.0, 100.0, 150.0 and 200.0 ng/mL), plus a zero level. Recoveries for fortified samples are reported in Table 2.

We applied this method to real samples collected from local markets in Tehran. Figs. 2 and 3 present typical chromatograms of a spiked sample with OTC, TC, tylosin, and tilmicosin.

The salting-assisted liquid/liquid extraction (SALLE) method, as demonstrated in this study, is a straightforward and efficient technique for the extraction and quantification of four antibiotics (oxytetracycline, tetracycline, tylosin, and tilmicosin) in milk

Table 1
Table of merit values for oxytetracycline, tetracycline, tilmicosin, and tylosin extracted from fortified cow milk samples.

Analyte	Regression equation	Correlation coefficient	(µg/mL)			
			LOD	LOQ	DLR	MRL
Oxytetracycline	Area = 3273 C_f −57	0.994	0.03	0.09	0.09–3.0	0.05
Tetracycline	Area = 6615 C_f −358	0.997	0.02	0.06	0.06–3.0	0.05
Tilmicosin	Area =15,690 C_f + 330	0.999	0.04	0.1	0.1–3.0	0.05
Tylosin	Area = 18,173 C_f + 651	0.999	0.02	0.06	0.06–3.0	0.1

LOD: Limit of detection, LOQ: Limit of quantification, DLR: Dynamic linear range,.
MRL: Maximum residue level.

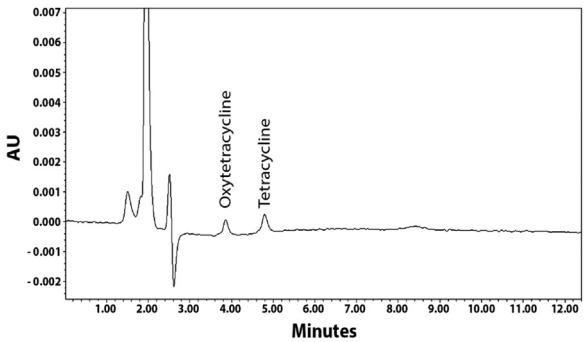

Fig. 2. Chromatogram obtained for spiked milk at a concentration of 0.100 µg/mL after extraction oxytetracycline and tetracycline.

Table 2
Mean recoveries of oxytetracycline, tetracycline, tilmicosin and tylosin from cow milk samples fortified at different levels ($n = 3$ replicates).

Sample	Added (ng/mL)	Intra-day recovery ± RSD (%)	Inter-day recovery ± RSD (%)
Oxytetracycline	50.0	75 ± 21	64 ± 17
	100.0	109 ± 6	107 ± 7
	150.0	111 ± 8	99 ± 3
	200.0	112 ± 7	101 ± 5
Tetracycline	50.0	81 ± 15	72 ± 14
	100.0	98 ± 7	90 ± 10
	150.0	103 ± 9	97 ± 4
	200.0	106 ± 5	102 ± 6
Tilmicosin	50.0	20 ± 70	55 ± 81
	100.0	119 ± 8	117 ± 9
	150.0	116 ± 6	116 ± 5
	200.0	107 ± 2	111 ± 8
Tylosin	50.0	35 ± 48	64 ± 29
	100.0	93 ± 4	93 ± 3
	150.0	95 ± 5	95 ± 5
	200.0	94 ± 10	98 ± 7

RSD: Relative standard deviation.

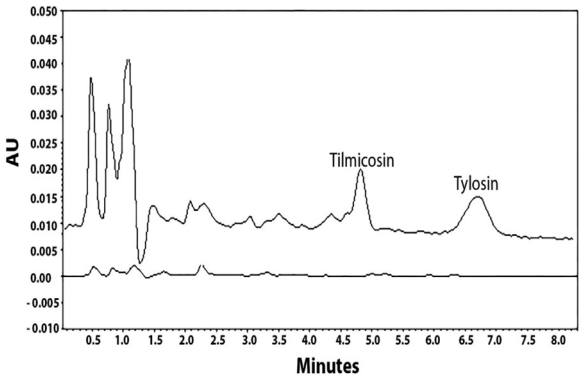

Fig. 3. Chromatogram obtained for spiked milk at a concentration of 0.100 μg/mL after extraction of tilmicosin and tylosin.

samples. This method, grounded in the principles of Liquid-Liquid Extraction (LLE), employs Sodium Hydroxide (NaOH) as the extraction agent and acetonitrile as the protein precipitant. The validation of this method was achieved by assessing its linearity, correlation coefficient, detection limits, and repeatability using spiked samples. The results confirm the accuracy and precision, with correlation coefficients exceeding 0.99, Limits of Detection (LODs) ranging from 0.02 to 0.04 μg/mL, and recoveries between 80% and 110%.

The successful application of this method to real samples sourced from local markets in Tehran further underscores its efficacy. The resulting chromatograms exhibited distinct peaks for the analytes. The SALLE method presents a novel and convenient approach for analyzing antibiotic residues in milk, making it a valuable tool for quality control and food safety applications. Notably, this method is more environmentally friendly than conventional methods due to its minimal consumption of organic solvents. SALLE's ability to effectively clean milk samples without any carryover effects are another significant advantage, given that the extraction units are single-use devices. In conclusion, this study has developed a simple, cost-effective, rapid, and reproducible method for determining tetracycline, oxytetracycline, tylosin, and tilmicosin in cow's milk using the SALLE technique.

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.

CRediT authorship contribution statement

Jalal Hassan: Methodology, Writing – review & editing. **Gholam-Reza Shams:** Funding acquisition. **Mostafa Pourrastegar:** Funding acquisition, Writing – review & editing. **Ali Pourshaban-Shahrestani:** Funding acquisition, Writing – review & editing.

Data availability

Data will be made available on request.

Acknowledgment

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References

[1] V. Economou, P. Gousia, Agriculture and food animals as a source of antimicrobial-resistant bacteria, Infect. Drug Resist. (2015) 49–61.
[2] S.F. Zhong, B. Yang, Q. Xiong, W.W. Cai, Z.G. Lan, G.G Ying, Hydrolytic transformation mechanism of tetracycline antibiotics: reaction kinetics, products identification and determination in WWTPs, Ecotoxicol. Environ. Saf. 229 (2022) 113063.
[3] Y. Zheng, Y. Liu, H. Guo, L. He, B. Fang, Z. Zeng, Molecularly imprinted solid-phase extraction for determination of tilmicosin in feed using high performance liquid chromatography, Anal. Chim. Acta 690 (2) (2011) 269–274.
[4] C. Prats, R. Francesch, M. Arboix, B Perez, Determination of tylosin residues in different animal tissues by high performance liquid chromatography, J. Chromatogr. B 766 (1) (2002) 57–65.
[5] M. Herrera, H. Ding, R. McClanahan, J.G. Owens, R.P. Hunter, Quantitative determination of tilmicosin in canine serum by high performance liquid chromatography–tandem mass spectrometry, J. Chromatogr. B 857 (1) (2007) 9–14.
[6] E. Cristofani, C. Antonini, G. Tovo, L. Fioroni, A. Piersanti, R Galarini, A confirmatory method for the determination of tetracyclines in muscle using high-performance liquid chromatography with diode-array detection, Anal. Chim. Acta 637 (1–2) (2009) 40–46.
[7] A. Pena, N. Pelantova, C. Lino, M. Silveira, Solich P. Validation of an analytical methodology for determination of oxytetracycline and tetracycline residues in honey by HPLC with fluorescence detection, J. Agric. Food Chem. 53 (10) (2005) 3784–3788.
[8] A. Pena, C.M. Lino, R. Alonso, D Barceló, Determination of tetracycline antibiotic residues in edible swine tissues by liquid chromatography with spectrofluorometric detection and confirmation by mass spectrometry, J. Agric. Food Chem. 55 (13) (2007) 4973–4979.
[9] J. Zhang, H. Wu, E. Kim, T.A. El-Shourbagy, Salting-out assisted liquid/liquid extraction with acetonitrile: a new high throughput sample preparation technique for good laboratory practice bioanalysis using liquid chromatography–mass spectrometry, Biomed. chromatogr. 23 (4) (2009) 419–425.
[10] F. Myasein, E. Kim, J. Zhang, H. Wu, T.A. El-Shourbagy, Rapid, simultaneous determination of lopinavir and ritonavir in human plasma by stacking protein precipitations and salting-out assisted liquid/liquid extraction, and ultrafast LC–MS/MS, Anal. Chim. Acta 651 (1) (2009) 112–116.
[11] S. Song, E.N. Ediage, A. Wu, S. De Saeger, Development and application of salting-out assisted liquid/liquid extraction for multi-mycotoxin biomarkers analysis in pig urine with high performance liquid chromatography/tandem mass spectrometry, J. Chromatogr. A 1292 (2013) 111–120.
[12] J. Hassan, A. Farahani, GC–MS determination of PAHs in fish samples following salting-out-assisted solvent extraction-gel permeation chromatography, Chromatographia 74 (5–6) (2011) 477–482.