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Reverse ultrasound-assisted emulsification-microextraction of macrolides from chicken fat followed by electrophoretic determination

Anabela S. Lorenzetti, Adriana G. Lista[1], Claudia E. Domini*

INQUISUR, Departamento de Química, Universidad Nacional del Sur (UNS)-CONICET, Av. Alem 1253, 8000, Bahía Blanca, Argentina

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ABSTRACT

A new microextraction methodology, called reverse ultrasound-assisted emulsification-microextraction (R-USAEME) was developed to extract Tilmicosin (TILM) and Tylosin (TYL) from chicken fat samples, prior to their determination by capillary electrophoresis with UV-detection. The R-USAEME was based on the use of an aqueous ionic liquid ([Bmim]Cl) solution with sodium tartrate and sodium phosphate as extractant, applying an ultrasound probe (91 W; 7.5 min). A good linearity was obtained in a range from 35 to 200 μg kg^{-1} with relative standard deviations (RSDs) lower than 13% using matrix-matched calibration on five chicken fat samples. The quantification limits (LOQs), ranged from 17.4 to 55.0 μg kg^{-1} and from 22.1 to 47.0 μg kg^{-1} for TILM and TYL respectively. The obtained recoveries were between 73 and 117%. The analytical parameters clearly showed the applicability of the method for the extraction and quantification of macrolides in this complex biological sample.

1. Introduction

In the last decades, there has been a significant increase in the use of antibiotics in veterinary with therapeutic and prophylactic purposes or as growth promoters. The improper use of these drugs in different treatments for animals can leave residues in tissues or food products causing allergic reactions in some hypersensitive individuals and bacterial resistance (Lozano & Trujillo, 2012; McEvoy, 2002; J.; Wang, 2008). Macrolides are used against a wide variety of Gram-positive and Gram-negative bacteria (Tao et al., 2012) in the treatment of respiratory diseases, and to prevent microbial infections in cattle, sheep and poultry. Macrolides are lipophilic molecules, consisting of macrocyclic lactone rings with 14–16 carbons linked to carbohydrate molecules.

Two of the most prevalent macrolides used in veterinary are Tylosin (TYL) and Tilmicosin (TILM). TYL is produced by the microorganism *Streptomyces fradiae* while TILM is a semi-synthetic compound obtained from TYL (Katz & Baltz, 2016). The presence of these analyte residues in food products derived from animals has a significant impact on human health. Therefore, maximum residue limits (MRLs) for macrolides are established for each animal tissue. According to the Commission Regulation (EU), these limits for TYL and TILM in poultry are: 100 μg kg^{-1} and 75 μg kg^{-1} for skin and fat, 100 μg kg^{-1} and 1000 μg kg^{-1} for liver, respectively (The European Commission, 2010). For the Codex Alimentarius Commission, these MRLs are 100 μg kg^{-1} (fat and skin) and 100 μg kg^{-1} (liver) for TYL and for TILM are 250 μg kg^{-1}, 2400 μg kg^{-1} (FAO & WHO, 2015, p. 41).

Separation techniques, as liquid chromatography (LC) or capillary electrophoresis (CE) with ultraviolet detection, have been used to determine macrolides in different matrices (Blackwell et al., 2004; García-Mayor, Gallego-Picó, Garcinuño, Fernández-Hernando, & Durand-Alegría, 2012). Nowadays, the LC coupled to mass spectrometry in single or tandem mode (LC-MS, LC-MS/MS) is the most common technique used for macrolides determination in samples such as milk, muscle (Jank et al., 2015), honey (Jin et al., 2017), eggs (K. Wang, Lin, Huang, & Chen, 2017), kidney and liver (Rizzetti, de Souza, Prestes, Adaime, & Zanella, 2016).

The most difficult step in the analysis of these biological samples is the pretreatment, which involves the extraction/preconcentration of macrolides. Common procedures used for this are liquid-liquid extraction (LLE) (Patyra, Nebot, Gavilán, Cepeda, & Kwiatek, 2018), solid-phase extraction (SPE) (Feng et al., 2016) and dispersive solid-phase extraction (Boscher, Guignard, Pellet, Hoffmann, & Bohn, 2010), among others. However, these procedures are tedious, time-consuming and use a large volume of toxic organic solvents.

In this context, new microextraction methods, like liquid phase microextraction (LPME), have appeared as they are easier, faster and ecofriendlier sample pretreatment procedures. One of the most used

* Corresponding author.
E-mail addresses: claudia.domini@uns.edu.ar, cdomini@criba.edu.ar (C.E. Domini).
[1] In memoriam.

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ones is the dispersive liquid-liquid microextraction (DLLME) presented by Assadi and co-workers (Rezaee et al., 2006). Even though DLLME is a low-cost simple method which generally uses small amounts of organic solvents as extractant and/or dispersant, these solvents are still highly toxic. In order to solve this problem, the use of environmentally friendly extraction solvents, such as ionic liquids (ILs) and the replacement of the dispersive solvents by ultrasound energy, vortex, temperature, among others, are a good solution (Barfi, Rajabi, & Asghari, 2015).

ILs are organic salts consisting of a combination of organic or inorganic anions and organic cations. The ILs have many characteristic properties such as a wide liquid range, low volatility, good thermal stability and low toxicity (Pavlović, Babić, Horvat, & Kaštelan-Macan, 2007), making possible their use as extraction solvent for a wide array of analytes.

On the other hand, the use of ultrasound energy instead of dispersion solvents improves the performance of DLLME; being this energy an excellent tool to generate fine emulsions from two immiscible liquids with an increased analyte transfer between the two phases. This procedure is called ultrasound-assisted emulsification microextraction (USAEME) (Regueiro, Llompart, Garcia-Jares, Garcia-Monteagudo, & Cela, 2008). In the last ten years, the reverse phase extraction mode, which uses an aqueous solution as extractant, has emerged as an attractive alternative to the traditional extraction processes, mainly to avoid the use of organic solvents (Fernández, Vidal, & Canals, 2018; Hashemi, Raeisi, Ghiasvand, & Rahimi, 2010).

In this work, a new methodology based on reverse phase microextraction mode assisted by ultrasound energy was developed to determine TYL and TILM in chicken fat samples. Detection was performed by capillary electrophoresis system equipped with a diode array detector. In the ME method, a small volume of IL, used as extractant, and a hydrophobic sample were employed, giving way to a water-in-oil (W/O) emulsion. The new procedure was named reverse ultrasound-assisted emulsification-microextraction (R-USAEME). The extraction process was improved by adding salts (sodium tartrate and sodium phosphate) to an IL aqueous solution and using the ultrasound probe to accelerate the emulsion formation.

It is important to point out that, to the best of our knowledge, this is the first time that an extraction methodology has been developed to extract TYL and TILM from chicken fat samples.

2. Material and methods

2.1. Reagents and solutions

All reagents used were from analytical grade. TILM and TYL standards were acquired from Sigma–Aldrich (Buenos Aires, Argentina). Individual standard solutions (1000 mg L^{-1}) were prepared in methanol (Merck, Buenos Aires, Argentina) and kept in the dark at −18 °C maintaining their stability for at least one month. The standard working solutions were daily prepared by appropriate dilutions of stock solutions with methanol.

Both ILs, 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmim]BF_4) and 1-butyl-3-methylimidazolium chloride ([Bmim]Cl), as well as sodium monobasic phosphate, sodium tartrate and sodium hydroxide were purchased from Merck (Darmstadt, Germany).

The electrophoretic buffer solution was daily prepared dissolving the appropriate amount of sodium dibasic phosphate and phosphoric acid in ultrapure water (18 mΩ) provided by Milli-Q system (Millipore, Bedford, USA).

2.2. Instrumentation

Ultrasound-assisted extractions were carried out using a Sonics Vibra cell, VCX130 with a titanium probe tip (9.5 mm diameter, 130 W nominal power, 20 kHz frequency). A Rolco centrifuge was employed to separate the emulsified samples.

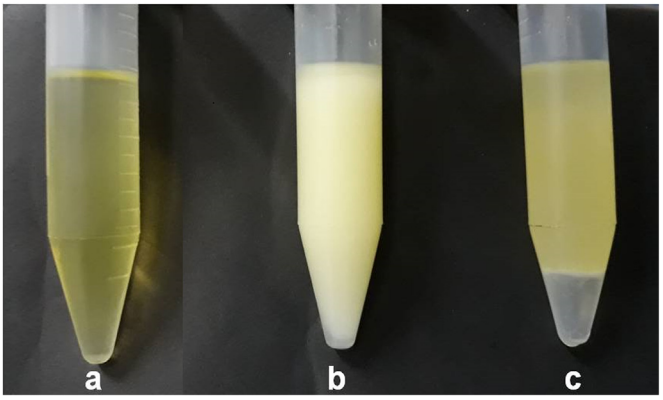

Fig. 1. Different extraction process steps applying the proposed method.
a) 5.0 g of liquid fat sample, heated at 75 ± 1 °C on a hot plate, before extraction.
b) Emulsified sample after adding 500 μL of extractant solvent (2.5 mmolL^{-1} [Bmim]Cl, 0.00625 mmolL^{-1} sodium tartrate and 0.00625 mmolL^{-1} sodium phosphate) and applying ultrasonic cycles of 40 s (on)/20 s (off) at 91 W of power for 7.5 min.
c) The two phases as the result of centrifugation at 2500 rpm for 5 min.

A Beckman Coulter CE instrument MDQ equipped with a diode array detector was used. The capillaries were also from Beckman Coulter. Control and data processing were carried out using a 32 Karat software.

2.3. Samples

With the aim of assessing the applicability of the proposed method, five chicken fat samples from different origins were analyzed. The first and second (A and B) were purchased in retail markets and the third (C) in a supermarket. In order to ensure the absence of antibiotics, the last two samples (D and E) came from ecologic farms of the zone of Bahía Blanca city, Buenos Aires province. All samples were from Argentina and they were acquired during 2017.

2.4. Sample preparation and microextraction procedure

The solid chicken fat was heated at 75 ± 1 °C on a hot plate. The liquid fat was filtered under vacuum through a 22 μm paper filter (Fig. 1 a). 5 g of the filtered sample were introduced in a centrifuge tube and 500 μL of extraction solvent ([Bmim]Cl, sodium tartrate and sodium phosphate) were added. The ultrasound probe was immersed in the tube containing the mixture and then it was placed in an ice bath. The microextractions were performed at 91 W for 7.5 min applying ultrasonic cycles of 40 s (on)/20 s (off). As a result, water-in-oil (W/O) emulsion was formed (Fig. 1 b). Then, by centrifugation at 2500 rpm for 5 min, the emulsion was disrupted and the aqueous phase was sedimented at the bottom of the conical tube (Fig. 1 c). After the oil phase was discarded, the aqueous phase was cleaned through a nylon syringe filter (Gamafil, Buenos Aires, Argentina) and collected in a CE vial for the subsequent detection step. All analytical process is illustrated in Fig. 2.

2.5. CE analysis

The separation was carried out in a fused-silica capillary (62 cm effective length, 50 μm id) with a separation voltage of 22.5 kV at 25 °C. All solutions were filtered through a 0.22 μm filter (Gamafil, Buenos Aires, Argentina) before being introduced into the electrophoretic system. Then, a mixture of 50 mmolL^{-1} sodium dibasic phosphate and phosphoric acid at pH 4.50 was used as background electrolyte. The

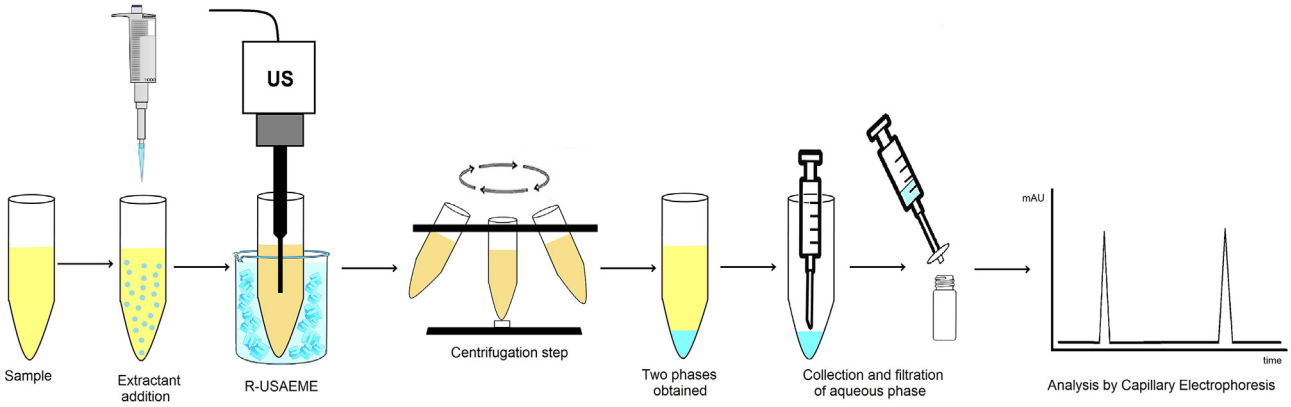

Fig. 2. The analytical process to determine TILM and TYL, including extraction, preconcentration and detection steps.

Table 1
Optimization study: assessed ranges and their optimal values.

Variable	Studied range	Optimum value
Ionic liquid [mmolL^{-1}]	0–10	2.5
Salts [molL^{-1}]	0–0.05	0.00625
Extractant volume [μL]	200–600	500
Cycles [s]		
- on	20–60	40
- off	5–40	20
Time (on + off) [min]	1–10	7.5
Power [W]	52–117	91

capillary was conditioned by flushing 0.1 mol L^{-1} HCl for 5 min, ultrapure water for 3 min, and finally by buffer solution for 5 min between runs. This sequence improved the reproducibility of separation when fat samples were analyzed. A hydrodynamic injection mode was used applying 0.8 psi for 10 s. The TILM and TYL electropherograms were recorded at 290 nm.

2.6. Statistical data analysis

Every parameter of the calibration curves has been calculated with ULC 2.0 (Univariate Linear Calibration) computer software. Additional statistical calculations were performed using Microsoft Office Excel ® 2010 (Microsoft, Redmond, WA, USA).

3. Results and discussion

3.1. Optimization of R-USAEME variables

The variables of the R-USAEME procedure were optimized taking into account the CE separation and using a univariate method. This study was carried out at 290 nm with spiked samples at MRL for TILM (75 μg kg^{-1}) and TYL (100 μg kg^{-1}) allowing for a fast, simple and environmentally friendly methodology. The fortification was performed adding the suitable amount of each analyte to the filtered sample. This mixture was then homogenized for 1 min using a vortex, kept at room temperature and applied to real chicken samples in order to demonstrate the applicability of the new method.

3.1.1. Extraction solvent

In order to develop an efficient extraction, the selection of the extractant is one of the most important parameters. Factors such as: low solubility in the oily phase, high affinity for the target analytes, easy dispersion in oil during sonication process and, in this case, compatibility with CE were considered.

In other extraction procedures of macrolides from biological samples, solvents such as methanol, methanol/water, McIlvaine buffer solution, and acetonitrile/water were successfully used (Carmona, Andreu, & Picó, 2017; Jank et al., 2015; Jin et al., 2017). Preliminary tests were performed to assess the above mentioned solvents without achieving good results.

Due to the fact that ILs have a high ability to extract lipophilic molecules (Flieger, Czajkowska-zelazko, Rzadkowska, Szacoń, & Matosiuk, 2012), [Bmim]BF_4 and [Bmim]Cl were tested. Since the obtained recoveries (between 49.2 and 74.8%) were not satisfactory, an extraction process using both ILs assisted by an ultrasound probe was carried out. However, low recovery values were obtained again.

According to the literature, TYL and TILM increase their water solubility in salt form and, being tartrates and phosphates their most soluble ones (Chen et al., 2014; Hamscher, Limsuwan, Tansakul, & Kietzmann, 2006), the addition of sodium tartrate and sodium phosphate to both IL solutions was tested. The recovery values showed notorious improvement, especially when [Bmim]Cl was used.

An IL aqueous solution with sodium tartrate and sodium phosphate assisted with ultrasound energy, was finally selected as the most appropriate extraction method. This can be explained because a new interphase is created when the cation of the IL undergo adsorption on the hydrophobic surface as a lipophilic specie (Flieger et al., 2012). Besides, the major solubility of TYL and TILM salts favors their extraction from fat samples, and as [Bmim]Cl is less chaotropic (water-destructuring/more hydrated) (Wu, Zhang, & Wang, 2008) than BF_4^-, its contact with the oily phase enhances the extraction process.

The IL and both salt concentrations were optimized and the studied ranges and their optimal values are shown in Table 1. Also, the effect of the extraction solvent volume on macrolide recoveries was evaluated. For that, different volumes of the mixture of IL and salts were tested. The results showed that by increasing the volume better recoveries were obtained, however, higher volumes than 500 μL produced a dilution effect (see Table 1).

3.1.2. Ultrasound

On the other hand, the ultrasonic probe variables were also tested. It was observed that working at high power and/or continuous sonication, the temperature in the sample exceeded the allowed one by the ultrasound manufacturer, causing an interruption in the sonication process. Therefore, different ultrasonic cycles were evaluated, taking into account that an ultrasonic cycle is determined as the sonication time (on) and the intermittent time (off). Because the temperature was still high, an ice bath was used to keep the temperature constant. By working with low power and/or short time, the emulsification was incomplete because the contact between both immiscible liquids was not reached. This can be due to the fact that oil viscosity hinders the process of dispersion (López-García, Vicente-Martínez, & Hernández-Córdoba, 2014). In conclusion, to obtain the best emulsion formation, the studied

Table 2
Results of analyzed fat samples by the proposed R-USAEME method, using capillary electrophoresis with UV detection. Recovery and RSD (%) of TILM and TYL spiked at three concentration levels (0.5 MRL, MRL and 1.5 MRL).

Sample	Tilmicosin[a]						Tylosin[b]					
	Linear range (μg kg^{-1})	0.5 MRL		MRL		1.5 MRL	Linear range (μg kg^{-1})	0.5 MRL		MRL		1.5 MRL	
		Recovery* (%)	RSD (%)	Recovery* (%)	RSD (%)	Recovery* (%)	RSD (%)		Recovery* (%)	RSD (%)	Recovery* (%)	RSD (%)	Recovery* (%)	RSD (%)
A	35–200	107.0	1.7	73.0	11.1	87.0	9.1	35–200	117.0	0.5	73.0	6.2	89.4	7.6
B		93.4	0.7	98.0	4.4	101.3	12.4		99.0	3.4	86.4	4.9	108.0	5.9
C		80.3	4.0	96.5	6.7	94.5	4.1		109.0	0.9	100.9	2.4	90.9	5.5
D	50–200	88.6	7.6	75.5	11.1	94.5	6.2		83.4	2.9	92.3	6.4	96.5	2.5
E	–	–	–	–	–	–	–		87.7	7.8	86.5	11.7	83.0	10.1

*average of three replicates.
[a] TILM MRL: 75 μg kg^{-1}
[b] TYL MRL: 100 μg kg^{-1}

ranges and optimal values for ultrasonic probe variables are shown in Table 1.

3.2. Optimization of capillary electrophoresis analysis

The electrophoretic analysis was done using the CZE mode. The pH buffer solution and its concentration were tested taking into account the extraction step. Since the pH of the extractant was around 5.00, a pH background electrolyte solution was tested at 2.50, 4.50 and 7.50 using sodium dibasic phosphate and phosphoric acid mixture. At pH 2.50, the peak areas were higher but the time of analysis was too long. When pH 7.50 was used, the peaks of analytes were overlapped with the electroosmotic flow signal. Even though, the peak areas of the analytes were slightly lower at pH 4.50, the time of analysis was reduced from 22 min to 14 min, so this pH value was selected. Then, other buffer solutions at this pH were tested (citric acid-citrate, acetic acid-acetate) and taking into account the reproducibility of the signals, the best results were obtained using phosphoric acid-phosphate buffer solution.

The buffer ionic strength was also evaluated changing the concentration of the sodium dibasic phosphate solution from 20 to 100 mmolL^{-1}. The best results in terms of selectivity and sensitivity were obtained working with 50 mmolL^{-1}.

The optimization of instrumental variables was performed varying the applied voltage (range: 15–25 kV) and injection time (5, 10 and 15 s) at 0.8 psi to evaluate their effects on time and resolution of the analyte peaks. As is well known, the resolution was better with lower voltages and the time of migration were lower with higher voltage. When 22.5 kV were applied, a better compromise between both parameters was obtained. Regarding the injection time, it was noted that after 10 s, the peaks in the electropherograms were flattened and the migration time were longer. Therefore, this value was selected as optimum, in hydrodynamic mode. The migration time for TILM and TYL were 8.5 min and 14.1 min respectively, taking into account the optimal values of each variable.

3.3. Analytical parameters and analysis of real samples

As it can be seen in Section 2.3, five different chicken fat samples were analyzed. After applying the whole proposed method no analytes were found.

Since the samples presented a complex matrix, it was necessary to evaluate its effect. For this purpose, a comparison between the slopes of the calibration curves, obtained using standard solutions prepared in extraction solvent and the ones obtained with matrix–matched standards, was carried out working at the same linear ranges. The residual variances of both linear regressions were statistically comparable (comparison performed by means of an F test), so a *t*-test was carried out by using the following equation:

$$t_{cal} = \frac{b_1 - b'_1}{\sqrt{S^2\left(\frac{1}{\sum(x_{i1} - \bar{x}_1)^2 + \sum(x_{i,1} - \bar{x}'_1)^2}\right)}}$$

where, b_1 and b'_1 are the slopes of the two regression lines, S^2 is the pooled estimated variance, x and x^- correspond to the concentration of the standards in the calibration curves (Massart, D. L., Vandeginste, B. G., Buydens, L. M. C., De Jong, S., Lewi, P. J., Smeyers-Verbeke, J., & Mann, 1998).

The results indicated that both slopes were significantly different which came to show there was matrix effect. As an example, the $t_{calculated}$ value for TILM in sample C was 12,454 which was much greater than $t_{tabulated\ (0.025;\ 6)}$ value (2.44). Therefore, the quantification of the analytes was performed by using matrix-matched standard solutions.

In addition, the homoscedasticity was tested for the matrix-matched calibration curves applying the Hartley test (Andrade et al., 2019;

Table 3
Previously described analytical methods for antibiotic extraction from food-producing animals from 1998 to 2018.

Sample (amount) –Analytes- Detection technique	Extraction metodology	Extraction solvent Volume of extraction solvent	Recovery	Analytical parameters	Ref.
Muscle (pig, cattle, sheep, horse, deer, reindeer) Kidney (pig, cattle, sheep and horse) (3 ± 0.03 g) Tetracyclines, sulfonamides, quinolones, β-lactams and macrolides **LC–MS/MS**	-Solvent extraction (shaking)	− 0.1 M EDTA − 70% methanol − 200 μL − 15 mL	Macrolides - Pig muscle: 80–86% - Other muscles: 77–104% - Kidney: 44–68%	Pig muscle LOD 3 μg kg^{-1} The worst results were obtained for the macrolides which had S/N > 8 at MRL.	Granelli and Branzell (2007)
Liver and kidney (beef, chicken, lamb, pig and rabbit) (5.00 ± 0.02 g) Erythromycin, TYL, spiramycin, roxithromycin, troleandomycin, TILM and josamycin **LC–DAD**	- Homogenizing the tissue with extractant solvent and extracted with an Oasis HLB cartridge	-EDTA–McIlvaine's buffer − 50 mL	Intra-day recovery TILM Spiking level −100 μg kg^{-1}: 70% −200 μg kg^{-1}: 74% −300 μg kg^{-1}: 93% TYL Spiking level −100 μg kg^{-1}: 65% −200 μg kg^{-1}: 70% −300 μg kg^{-1}: 72%	CCα TILM 1005 μg kg^{-1} TYL 116 μg kg^{-1} CCβ TILM: 1010 μg kg^{-1} TYL: 132 μg kg^{-1}	Berrada, Borrull, Font, Moltó, and Marcé (2007)
Meat (pork, beef, mutton, chicken, pork liver, lambs' liver, and chicken liver) (2.50 ± 0.01 g) β-agonists, sulfanilamides, quinolones, macrolides, tetracyclines, β-lactams, nitroimidazoles, glucocorticoids, sex hormones, chloromycetins, sedatives, and olaquindox metabolite **UPLC-MS/MS**	- Vortex - Sonication - Waters Oasis PRiME HLB cartridge	- Acetonitrile/water (80/20, v/v) −10 mL	Mean recoveries for all analytes ranged from 80 to 116%	LOQ were in the range 0.05–3.0 μg kg^{-1} and limits of detection were in the range 0.1–10 μg kg^{-1}	(Zhang, Li, et al., 2018b)
Chicken muscle (2 g) Fluoroquinolones, sulfonamides, and macrolides (TILM, TYL) **LC–MS/MS**	- Vortex	− 1% acetic acid in ACN - dilution with 0.1% formic acid in 20% methanol aqueous solution. − 10 mL - diluted to 20 mL	- TILM 71–106% - TYL 63–119%	LOQ - TILM: 5 ng g^{-1} - TYL: 5 ng g^{-1}	(Zhang, Li, et al., 2018a)
Deep-fried chicken and non-fried (5.0 g) 4 antifolics, 4 benzimidazoles, 5 macrolides, 7 polyethers, 2 quinolones, 7 sulfonamides, and 8 other clases **LC–MS/MS**	- InertSep® K-solute cartridge	- Ethyl acetate - Distilled water - ACN −10 mL −5 mL −30 mL	TILM Spiking level: 10 μg kg^{-1} - deep-fried chicken: 79% - non-fried chicken cutlet: 71% - muscle: 73% Spiking level: 50 μg kg^{-1} - deep-fried chicken: 78% - non-fried chicken cutlet: 72% - muscle: 76%	TILM LOQ - deep-fried chicken: 1 μg kg^{-1} - non-fried chicken cutlet: 0.4 μg kg^{-1} - muscle: 0.4 μg kg^{-1}	Yoshikawa et al. (2017)
Chicken Eggs (2.0 g) Sulfonamides, quinolones, tetracyclines, macrolides, lincosamide, nitrofurans, β-lactams, nitromidazoles, and cloramphenicols **LC-MS/MS**	-Homogenized by a high-speed dispersion rotor, ultrasonically oscillated, and centrifuged.	- ACN − H_2O (90:10, v/v) - 0.1 mol L^{-1} Na_2EDTA − 7.5 mL − 0.5 mL	Spiking level 5 μg kg^{-1} TYL 107.3% TILM 93% Spiking level 20 μg kg^{-1} TYL 103.3% TILM 84.8%	LOD - TYL: 0.07 μg kg^{-1} - TILM: 0.75 μg kg^{-1} LOQ - TYL: 0.24 μg kg^{-1} - TILM: 2.50 μg kg^{-1}	(K. Wang et al., 2017)

(*continued on next page*)

Table 3 (*continued*)

Sample (amount) –Analytes- Detection technique	Extraction metodology	Extraction solvent Volume of extraction solvent		Recovery	Analytical parameters	Ref.
				Spiking level 50 μg kg^{-1} TYL 88.5% TILM 80.9%		
Swine, cattle and chicken muscles samples (2 g) Ten macrolide drugs **LC–MS/MS**	- Molecularly imprinted solid-phase extraction	- Sodium borate buffer solution (pH = 10) - Ethyl acetate	-5 mL -10 mL	TILM (Chicken) Intra-day recovery: Spiking level −1 μg kg^{-1}: 67.6% −5 μg kg^{-1}: 63.0% −20 μg kg^{-1}: 85.0%	TILM (Chicken): LOD: 0.2 μg kg^{-1} LOQ: 0.6 μg kg^{-1}	Song et al. (2016)
Chicken muscle tissue (2.0 g) Benzimidazoles, quinolones, nitromidazoles, β-lactams, macrolides, triphenylmethane dyes, sulphonamides and tetracyclines **LC-MS/MS**	-Homogenisation system FASTH21	- Ethylenediamine tetraacetic acid-succinate buffer - Acetonitrile	− 5 mL − 10 mL	TYL 72% TILM 58%	LOD - TYL: 9 ng g^{-1} - TILM: 8 ng g^{-1} LOQ - TYL: 28 ng g^{-1} - TILM: 24 ng g^{-1}	Biselli, Schwalb, Meyer, and Hartig (2013)
Muscle chicken (5.0 g) Quinolones, sulfonamides, macrolides, anthelmintics, avermectins and diamino derivatives, and benzathine, used as a marker of the presence of penicillin, **UHPLC–MS/MS**	-QuEChERS methodology	-1% of acetic acid in a solution of acetonitrile:water (80:20, v/v).	− 10.0 mL	TILM Spicking level: 20 μg kg^{-1}: 75.5% − 50 μg kg^{-1}: 81.4% − 100 μg kg^{-1}: 90.1% TYL Spicking level: 20 μg kg^{-1}: 83.2% − 50 μg kg^{-1}: 91.6% − 100 μg kg^{-1}: 75.4%	Limits of detection (LODs) and quantification (LOQs) ranged from 3.0 to 6.0 μg kg − 1 and 10.0–20.0 μg kg − 1, respectively, except for TYL that showed a LOD and LOQ of 9.0 and 30.0 μg kg − 1.	Lopes, Reyes, Romero-González, Frenich, and Vidal (2012)
Poultry meat (2 g) Dihydrostreptomycin, spectinomycin, spiramycin, streptomycin, TILM, and TYL **LC-ESI-MS**	- Pressurized liquid extraction	- Methanol	− 22 mL	Within-day TILM - MRL/2: 82% - MRL: 79% − 1.5 MRL: 83% TYL - MRL/2: 91% - MRL: 92% − 1.5 MRL: 94%	LOD: 1–6 μg kg^{-1} LOQ: TILM: 10 μg kg^{-1} -TYL: 5 μg kg^{-1}	Berrada, Moltó, Mañes, and Font (2010)
Pultry meat and fish (5 g) Erythromycin, josamycin, roxithromycin, spiramycin, TILM, troleandomycin and TYL. **LC–(ESI)MS**	-Pressurized liquid extraction	-Methanol	-40 mL	Intra-day recovery TILM Spiking level −50 μg kg^{-1}: 73% − 100 μg kg^{-1}:78% − 200 μg kg^{-1}: 82% TYL Spiking level −50 μg kg^{-1}: 72% − 100 μg kg^{-1}: 83% − 200 μg kg^{-1}: 90%	LOD TILM: 23 μg kg^{-1} TYL: 18 μg kg^{-1} LOQ TILM: 25 μg kg^{-1} TYL: 25 μg kg^{-1}	Berrada, Borrull, Font, and Marcé (2008)
Poultry muscle (2.5 g) Spiramycin, TYL tartrate, oleandomycin phosphate, roxithromycin and erythromycin **LC–MS**	-Solvent extraction and cation-exchange cartridge	-0.3% metaphosphoric acid–methanol (7:3, v/v)	-17 mL	TYL Spiked level: 50–200 μg kg^{-1} Recovery: 56% TILM Spiked level: 40–150 Recovery: 93%	LOD TYL: 1 μg L^{-1} TILM: 8 μg L^{-1}	Codony, Compañó, Granados, Garcí;a-Regueiro, & Prat (2002)
Food producing animals (2.5 g) Spiramycin, TILM, TYL, kitasamicin	- Manually shaken with extraction solvent	-0.3% metaphosphoric acid–methanol (7:3, v/v)	-17 mL	TILM: 65% TYL: 60%	LOD - TILM: 10 μg kg^{-1}	Leal, Codony, Compañó,

(*continued on next page*)

Table 3 (*continued*)

Sample (amount) –Analytes- Detection technique	Extraction metodology	Extraction solvent Volume of extraction solvent		Recovery	Analytical parameters	Ref.
josamicin erythromycin and oleandomycin. **LC–UV**					- TYL: 30 μg kg^{-1} LOQ - TILM: 30 μg kg^{-1} - TYL: 80 μg kg^{-1}	Granados, and Prat (2001)
Chicken, Cattle, Swine, and Sheep Tissues (4.50–5.50 g) TILM **LC-UV**	-Homogeinization with ultrasonic probe and C18 cartridge for clean up	- Methanol – 100mM pH 2.5 phosphate buffer	– 20 ± 1 mL – 5 ± 1 mL	Abdominal fat, TILM Fortified conc.-0.025 μg/g: 89% – 0.05 μg/g: 80% – 1.00 μg/g: 96%	LOD - chicken kidney and liver: 0.013 μg/g - chicken abdominal fat, skin, and muscle 0.0053 μg/g - swine tissue 0.0066 μg/g - cattle tissue 0.0075 μg/g - sheep tissue 0.011 μg/g LOQ - for all tissue except chicken kidney and liver: 0.025 μg/g - chicken kidney and liver: 0.06 μg/g	Stobba-Wiley et al. (2000)
Pork Muscle (5 g) TILM, TYL, spiramycin, and neospiramycin, **LC-UV**	-Liquid–liquid extraction and cleaned on Bond Elut C18 cartridges	-ACN -Isooctane	-10 mL -10 mL	TILM −1/2 MRL: 61.5% -MRL: 59.9% −2 MRL: 58.2% −4 MRL: 44.1% TYL −1/2 MRL: 64.6% -MRL: 64.2% −2 MRL: 63.0% −4 MRL: 52.2%	LODs 15 μg kg^{-1} for TILM and TYL	Juhel-Gaugain, Anger, and Laurentie (1999)
Chicken muscle and liver, swine muscle, liver and kidney, and cattle muscle and liver (5 g) Josamycin, kitasamycin, mirosamicin, spiramycin and TYL **HPLC**	- Liq-liq extraction and clean up on a Bond Elut SCX cartridge	– 0.3% metaphosphoric acid–methanol (7:3, v/v)	– 100 mL	TYL Chicken muscle: 71.7% Chicken liver: 70.8%	LOD: 0.05 μg/g	Horie et al. (1998)
Chicken Fat (5 g) TILM and TYL **CE-DAD**	- Ultrasound-assisted emulsification microextraction (R-USAEME)	[Bmim]Cl, sodium tartrate and sodium phosphate)	– 500 μL	TILM: 73–107% TYL: 73–117%	LOD - TILM: 5.2–18.9 μg kg^{-1} - TYL: 6.6–12.8 μg kg^{-1} LOQ - TILM:17.4–55.0 μg kg^{-1} - TYL:22.1–47.0 μg kg^{-1}	This work

Massart, D. L., Vandeginste, B. G., Buydens, L. M. C., De Jong, S., Lewi, P. J., Smeyers-Verbeke, J., & Mann, 1998) and preparing each concentration level by triplicate. In all cases homoscedasticity was not fulfilled. As an example, the values obtained for TILM in sample C were $F_{calculated} = S^2_{major}/S^2_{minor} = 40.32$ and $F_{critic(0.05;2;2)} = 39.00$. Since the calculated F value was much greater than F_{critic}, the variances presented statistically significant differences. Thus, a weighted least-squares regression was used for quantification purpose instead of ordinary least-squares applying the following equation:

$$b = \frac{\sum (w_i(x_i - \bar{x}_w).(y_i - \bar{y}_w))}{\sum (x_i - \bar{x}_w)^2}$$

$$a = \bar{y}_w - b\bar{x}_w$$

where $Wi = {}^{1}/_{S_y^2}$, $\bar{x}_w = {}^{\sum w_i x_i}/_{\sum w_i}$, $\bar{y}_w = {}^{\sum w_i y_i}/_{\sum w_i}$, x_i and y_i represent the components of each point in the calibration curve.

In addition to the selectivity assessment (in terms of matrix effects), the whole validation method was performed by evaluating the following analytical parameters: linearity, LOD, LOQ, trueness and precision in terms of repeatability.

Linearity was investigated with replicates of matrix-matched standard solutions (n = 3), in the range from 35 to 200 $\mu g\,kg^{-1}$ for both analytes ($p < 0.05$ for linearity test).

The detection and quantification limits (LODs and LOQs) were both calculated from weighted least-squares regression data (Miller & Miller, 1993). The obtained LOD values were ranged from 5.2 to 18.9 $\mu g\,kg^{-1}$ and from 6.6 to 12.8 $\mu g\,kg^{-1}$ for TILM and TYL respectively. Regarding the LOQ values, they were ranged from 17.4 to 55.0 $\mu g\,kg^{-1}$ and from 22.1 to 47.0 $\mu g\,kg^{-1}$ for TILM and TYL respectively. It is important to point out that all LOQ values were much lower than the MRL ones.

Trueness was assessed from recovery studies. Thus, the fortified samples were prepared taking into account the MRL values for TILM (75 $\mu g\,kg^{-1}$) and TYL (100 $\mu g\,kg^{-1}$). The added concentrations for this study were 0.5 MRL, MRL and 1.5 MRL of each analyte. The obtained values are reported in Table 2, for the five analyzed samples and the two analytes. As it can be seen, satisfactory recoveries between 73 and 117% (calculated as $(Value_{found}*100\%)/Value_{added}$) were obtained, considering the recommendation criterion (European Commission, 2000), except for the sample E in the TILM determination. In this case, the recovery values were too high at the three concentration levels, probably due to the interaction of TILM with some components of the sample which were co-extracted. Thus, TILM was not determined in this sample.

The repeatability of the method was also evaluated by analyzing 3 replicates of the matrix-matched calibration solutions at the three concentration levels for both analytes. Table 2 shows that the obtained values were lower than 20% (European Commission, 2000). The good results obtained demonstrated the applicability of the method in the determination of TYL and TILM in chicken fat samples.

Table 3 includes different characteristics of previously described analytical methods, highlighting the extraction step in the determination of macrolide antibiotics. It is of utter importance to mention that most of them use large volumes of organic solvents in comparison with the proposed method that utilizes the lowest solvent volume. This can be explained because it takes advantage not only of the ability of water-soluble tartrates and phosphates, but also it is the only one that uses IL and ultrasound energy as strategy for extraction.

4. Conclusion

A new analytical method for the determination of TILM and TYL in chicken fat samples was developed. The extraction as well as the preconcentration of the analytes using an IL aqueous solution as extractant and an oily sample, was achieved with a new methodology called reverse ultrasound-assisted emulsification-microextraction (R-USAEME). This procedure takes just 12.5 min in the extraction process and without the use any toxic organic solvent. The extractant is directly injected into the CE equipment, taking just 15 min to complete the analysis. The obtained RSD% values were satisfactory (lower than 12.4%) and the LOQs were lower than MRLs established by European Legislation and Codex Alimentarius Commission.

Therefore, we have achieved a simple, fast, low cost and environmentally friendly methodology to detect TILM and TYL in chicken fat samples.

This new procedure is a promising approach which opens the doors to new protocols that may include other lipophilic antibiotics as well as fat from other sources. In this way, it contributes to the monitoring of products of animal origin with the premise of improving their quality as food for human consumption.

Conflicts of interest

The authors declare that the research was carried out in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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