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Simultaneous Determination of Five Coccidiostats in Veterinary Powders, Feed Premixes, and Baby Food by Micellar Electrokinetic Chromatography: Application to Chicken Tissues and Liver

Fathalla Belal[1] · Sawsan Abd El-Razeq[2] · Manal Fouad[3] · Sahar Zayed[4] · Fatma Fouad[2]

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Abstract

A new, simple, and reliable micellar electrokinetic chromatographic method was developed and validated for the simultaneous determination of amprolium, ethopabate, diaveridine, sulphadimidine, and sulphaquinoxaline. The separation was achieved using 50 mM tris buffer (pH 8.5) with 50 mM SDS and 15% acetonitrile (*v*/*v*) at 28 kV and the components were detected at 200 nm. Different factors affecting the electrophoretic mobility of the five investigated drugs were studied and optimized. Method validation showed that calibration plots were linear within the range from 0.5 to 100 μg/mL with a correlation coefficient > 0.998. Intraday and interday precision and accuracy evaluated by relative standard deviation were lower than 2%. The limits of detection were in the ranges of 0.02 to 0.07 μg/mL. The new method with simple sample pretreatment based on aqueous methanol extraction has been successfully applied for analysis of these drugs in powder preparations, feed premixes, baby food, chicken tissues, and liver samples with the recoveries of 97–101%. The present method is suitable and favorable for the analysis of the five coccidiostats drugs on account of its cost effectiveness, simplicity, rapidity, and sensitivity.

Keywords Micellar electrokinetic chromatography · Anticoccidial drugs · Powder preparations · Feed premixes · Baby food · Chicken tissues · Liver

Introduction

Coccidiostats are widely used in poultry farming as feed additives for the prevention and treatment of coccidiosis. This parasitic disease of the intestinal tract is caused by protozoa of the genera Eimeria and Isospora, causing bleeding and swelling of the intestines (Broekaert et al. 2011). This causes weight loss or poor weight gain and, thus, serious economical consequences in animal production. Among anticoccidial mainly used as feed additives for poultry are sulfonamides, diaveridine, amprolium, and ethopabate which are registered as veterinary drugs for the treatment of the clinical form of coccidiosis. Sulphadimidine (SDM) and sulphaquioxaline (SQL) are the most frequently used sulfonamides for both prevention and treatment of diseases and as feed additives to promote growth in animal feeding operations (Clarke et al. 2014). In the 1950s, amprolium (AMP) was developed, a coccidiostat still used today. It used to inhibit the growth of protozoan coccidian in chicken feed and for the treatment of the clinical form of coccidiosis (Song et al. 2007). Ethopabate (EPB) is usually used in combination with AMP. It has anticoccidial activity especially against intestinal forms (Clarke et al. 2014). Diaveridine (DVR) has remarkable activity against coccidia and used to treat intestinal infections. DVR is rarely used by itself in the clinic; it is used as a synergist with SDM (Wanga et al. 2014). The chemical structures of the studied drugs are shown in Fig. 1.

The continuous administration of coccidiostats often leads to the accumulation of veterinary drug residues in food products for human consumption, which may cause adverse toxic effects on consumers' health (Girardi and Odore 2008). Therefore, the control of veterinary drug residue is an important measure in ensuring consumer protection (Commission Recommendation 2005/

✉ Sahar Zayed
s1zayed@yahoo.com

[1] Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, University of Mansoura, Mansoura, Egypt

[2] Department of Analytical Chemistry, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt

[3] Department of Analytical Chemistry, Faculty of Pharmacy, Al-Azhar University, October University for Modern Sciences and Arts, Cairo, Egypt

[4] Unit of Drug Analysis, Faculty of Pharmacy, University of Mansoura, Mansoura, Egypt

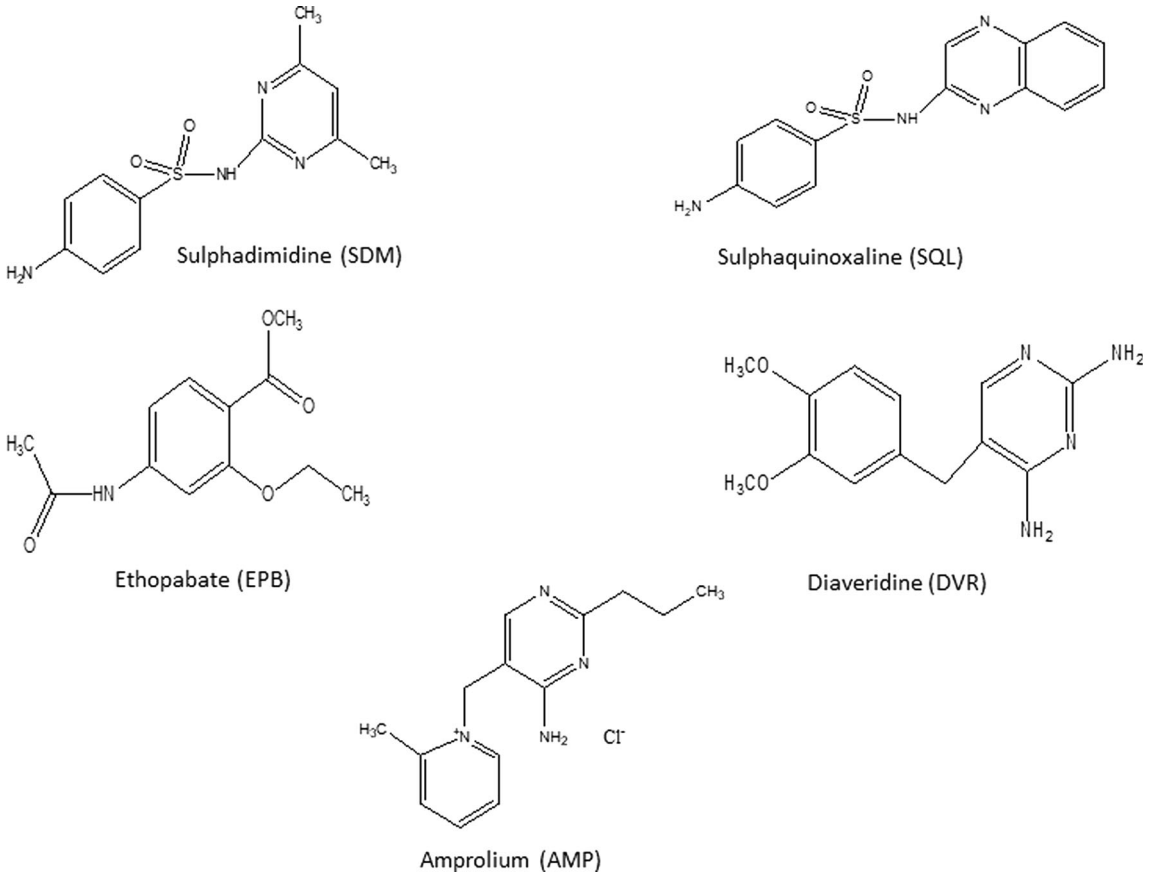

Fig. 1 Structural formulae of the studied drugs

925/EC 2005). So, an urgent need arises for the development of analytical methods for their determination. To protect consumers' health, American and European institutions have established maximum residue levels (MRLs) of 100 μg/kg for sulfonamides, EPB, and DVR, and 200 μg/kg for AMP in foodstuffs of animal origin (European Union 2010). However, with the improper use of these drugs, it would increase the risk of their residue in animal tissue beyond the MRL range (Clarke et al. 2014; Vinay et al. 2013). In addition, the feed premix samples could be contaminated with high levels of coccidiostats during the manufacture process (Mcevoy 2002).

Literature survey revealed that these selected anticoccidial drugs were analyzed by spectrophotometric (Hussein et al. 2015; Abd-El-Sattar 2002; Alomary 2004; Nour El-Dien et al. 2010; Rizk et al. 2013), spectrofluorimetric (El-Kosasy et al. 2015; Nasr and Shalan 2014) in addition to chromatographic methods (Smallidge Jr. 1978; Salama et al. 2012; Martinez-Villalba et al. 2010; Kaklamanos et al. 2013; Ghanem and Abu-Lafi 2013; Kim et al. 2012; Furusawa 2002; Kao et al. 2001; Ortelli et al. 2009; Van Rhijna et al. 2002; Petersa et al. 2009; Aguilera-Luiz et al. 2012; Hartig et al. 1999; Liu et al. 2011). Capillary electrophoresis (CE) has been used for the determination of some anticoccidial drugs (Martínez-Villalba et al. 2013; Křivánková and Boček 1985; Hows et al. 1997; Font et al. 2007; Ching-Erh 1997). However, a literature survey revealed that no capillary electrophoretic method was applied for simultaneous determination of AMP, EPB, DVD, SDM, and SQL. Therefore, the development of a general and selective method for the simultaneous analysis of these drugs in different matrices is highly significant.

Micellar electrokinetic chromatography (MEKC) is a hybrid of electrophoresis and chromatography introduced by Terabe in 1984 (Heiger 1992). MEKC is one of the most widely used CE modes. It is used for the separation of neutral solutes as well as charged ones. The separation of neutral species is accomplished by the use of surfactant in the running buffer in concentration above its critical micelle concentrations. The neutral solutes arrange themselves in and out of the micelles and move according to the micelles velocity not by their electrophoretic mobility resulting in good separation of the mixture (Riekkola et al. 2004). MEKC has been proven to be a useful technique for various applications (El-Awady et al. 2013; Belal et al. 2016; Springer and Lista 2012).

This paper reports for the first time, a simple, economic, and fast MEKC method based on aqueous methanol extraction (AME) for the separation and quantification of five anticoccidial drugs in dosage forms, feed premixes, and baby food. The procedure was also extended to the analysis of these drugs in chicken tissues and liver. The proposed method was optimized for different experimental parameters and validated according to ICH guidelines (International Conference on Harmonization (ICH) 2005).

Experimental

Chemicals and Reagents

Pure AMP HCl and DVR were supplied by Arab Company for Medical Products Cairo, Egypt. EPB was supplied by the Egyptian Company for Chemicals and Pharmaceuticals, Cairo, Egypt. SDM and SQL were supplied by the Universal Pharmaceutical Industries Company. Pharmaceutical powder samples assayed in the study included Amprobate®, each 100 g contains AMP HCl 25 g and EPB 1.6 g, a product of Memphis for Pharmaceuticals and Chemical Industries, Cairo, Egypt. Medacox®, each 100 g contains SDM sodium 86 g (equivalent to SDM base 80 g) and DVR 8 g, a product of Arab Company for Medical Products, Cairo, Egypt. Unisulphaquin® 20% (each 100 g contains 20 g SQL) and Sulphadin® 33% (each 100 g contains 33 g SDM), products of Universal Pharmaceutical Industries Company were obtained from commercial sources. Analytical grade sodium dihydrogen phosphate, disodium tetraborate decahydrate, sodium dodecyl sulfate (SDS), hydrochloric acid, and sodium hydroxide were from Fisher Scientific, UK. Tris, acetonitrile, methanol, n-propanol were purchased from Sigma-Aldrich (Germany). Chicken tissues and liver samples were purchased from the local market. Chicken noodles baby food precooked, was from Gerber® (USA). Poultry feed premixes were purchased from a commercial veterinary retailer. Membrane filters (0.45 μm) purchased from Phenomenex (USA) were used for filtration of samples and background electrolyte (BGE). All buffers were prepared in ultrapure deionized (DI) water from an Easypure RODI water system (Barnstead Int., USA).

Equipment

All measurements were done with an Agilent 7100 Capillary Electrophoresis system (Agilent Technologies, Waldbronn, Germany) equipped with a diode-array detector. Data were recorded with Agilent Open LAB CDS software. Uncoated fused-silica capillaries 75 μm i.d × 55.2 cm (46.7 cm effective length) from Polymicro Technologies (Phoenix, AZ, USA) was used. A Consort NV P-901 pH–Meter (Belgium) was used for pH measurements. Ultrasonic bath used was BHA-180T (Abbotta, USA). Centrifugation was carried out using a TDL-60 B Centrifuge (Anke, Taiwan). Tissue Master-125 with 7-mm stainless steel generator probe (Omni International, GA, USA) was used for tissue homogenization.

Operating Conditions

Before the first use, the capillary was conditioned by flushing with 0.2 M NaOH for 60 min, then with water for 30 min and BGE for 15 min. At the beginning of each working day, the capillary was rinsed with 0.5 M NaOH for 5 min, water for 5 min and then with BGE for 10 min. Before each injection, the capillary was preconditioned with 0.5 M NaOH (2 min), water (1 min), and BGE (3 min) to maintain proper repeatability of run-to-run injections. The BGE was 50 mM tris buffer of pH 8.50 (adjusted with 1 M HCL) containing 50 mM SDS and 15% acetonitrile. Sample introduction was carried out under hydrodynamic pressure at 50 mbar for 20 s. Photodiode array (PDA) detector was set at 200 nm. Separations were performed at 25 °C and a voltage of 28 kV was applied.

Preparation of Standard Stock Solutions

Stock solutions 1.0 mg/mL of each of AMP, EPB, DVR, SDM, and SQL were prepared by dissolving 50.0 mg of each separately in 50 mL 50% aqueous methanol. Working solutions were prepared by further dilution of the stock solutions with the same solvent then with deionized water as appropriate to give a final desired concentration of 5.0–100.0 μg/mL for AMP, SDM, and SQL, and 1.0–20.0 μg/mL for EPB and 0.5–25.0 μg/mL for DVR. The stock solutions were stable for at least 2 weeks when kept in the refrigerator at 4 °C.

Analysis of Dosage Forms

An accurately weighed amount of Sulphadin® or Unisulphaquin® powders equivalent to 0.076 and 0.125 g, respectively were transferred to 25-mL flasks and dissolved in 50% aqueous methanol, then diluted to the final volume with the same diluent to obtain a solution claimed to contain 1.0 mg/mL.

For the determination of Amprobate® or Medacox® powders, an accurately weighed amount of the powder equivalent to 25.0 mg were transferred and dissolved each separately into 25 mL of 50% aqueous methanol to obtain a solution claimed to contain 250 μg/mL AMP and 16 μg/mL EPB for amprobate® or 860 μg/ mL SDM and 80 μg/mL DVR for medacox®. The prepared solution was diluted quantitatively with the same

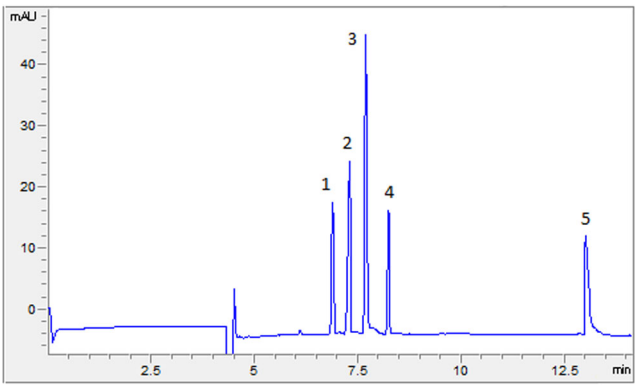

Fig. 2 Electropherogram for the studied analytes using the optimized experimental conditions (analyte concentration: 10.0 μg/mL for each analyte; (1) SDM, (2) SQL, (3) EPB, (4) DVR, and (5) AMP).

Table 1 Parameters of the linear regression, limits of detection (LOD), limits of quantification (LOQ) for the five coccidiostats

Drug	Linearity range (μg/mL)	Regression equation	Correlation coefficient (r)	LOD (μg/mL)	LOQ (μg/mL)
SDM	5–100	$y = 0.348\times + 0.059$	0.9993	0.161	0.463
SQL	5–100	$y = 0.429\times + 1.01$	0.9997	0.124	0.447
EPB	1–20	$y = 0.648\times + 0.024$	0.9999	0.065	0.183
DVR	0.5–25	$y = 0.277\times - 0.023$	0.9999	0.159	0.485
AMP	5–100	$y = 0.301\times - 0.006$	0.9999	0.172	0.493

solvent and then with deionized water as appropriate to obtain the required concentration for the assay.

Sample Preparation

The samples of feed premixes, baby food, chicken tissues, and liver (5 g each) were accurately weighed and spiked with aliquots of the studied drugs. The spiked samples were mixed with 20 mL of extracting solvent (50% aqueous methanol) and homogenized at 5000 rpm for 10 min. Then the homogenate was sonicated in an ultrasonic bath for 15 min, stirred for 15 min at room temperature and then centrifuged at 3000 rpm for 5 min. The supernatant of all samples was filtered through 0.45-μm syringe filters. The filtrate was transferred to 50-mL volumetric flasks and diluted to the mark with the same solvent. Different known aliquots were transferred into 10-mL calibrated flasks and completed to the volume with deionized water to obtain final concentrations in the range 1.0 to 10.0 μg/mL for the studied drugs in feed premixes and 0.5 to 5.0 μg/mL for the studied drugs in baby food, chicken tissues, and liver.

Results and Discussion

Optimization of Separation Conditions

Effect of Running Buffer Composition and pH

Several running buffers were investigated, including phosphate, borate, mixture of borate and phosphate, and tris buffer. The results showed that a better resolved peaks with low background current was obtained in tris buffer. The effect of pH on the resolution and migration time was investigated over the pH range 8.0–10.0, using 50 mM buffer solutions prepared at different pH.

Table 2 Intraday and interday precision of the developed MEKC method

Analyte	Conc. taken (μg/mL)	Intraday[a]			Interday[b]		
		Conc. found ± S.D. (μg/mL)	% RSD	% Error [c]	Conc. found ± S.D. (μg/mL)	% RSD	% Error[c]
SDM	10	9.83 ± 0.16	0.17	0.11	10.09 ± 1.23	1.21	0.69
	25	24.54 ± 1.18	1.19	0.69	24.29 ± 0.56	0.54	0.31
	50	49.25 ± 0.49	0.50	0.29	49.43 ± 1.27	1.25	0.72
SQL	10	10.19 ± 1.24	1.25	0.72	10.99 ± 0.94	0.88	0.51
	25	25.22 ± 0.15	0.16	0.09	25.14 ± 1.75	1.77	1.02
	50	50.52 ± 0.35	0.36	0.21	50.07 ± 0.74	0.71	0.41
EPB	2.5	2.55 ± 0.69	0.72	0.42	2.52 ± 1.06	1.04	0.60
	5	5.12 ± 0.37	0.32	0.18	5.01 ± 0.67	0.62	0.36
	10	9.96 ± 1.51	1.52	0.88	10.11 ± 1.25	1.22	0.71
DVR	2.5	2.52 ± 0.58	0.57	0.33	2.58 ± 1.03	1.09	0.63
	5	4.98 ± 0.92	0.93	0.54	4.99 ± 0.78	0.81	0.47
	10	9.95 ± 1.18	1.29	0.75	10.09 ± 1.03	1.08	0.62
AMP	10	10.08 ± 1.19	1.21	0.70	10.26 ± 1.61	1.64	0.95
	25	24.81 ± 0.38	0.39	0.23	24.33 ± 0.53	0.57	0.33
	50	49.46 ± 0.42	0.44	0.25	50.14 ± 1.14	1.12	0.65

Each result is the average of three separate determinations

[a] Within the day

[b] Three consecutive days

[c] % Error = % RSD/$\sqrt{n}$

The migration times were shown to increase as the pH increased with accompanying peak broadening, however, lower pH values resulted in decrease in the migration time and peak width, with excellent peak symmetry. The optimum pH value was taken as pH 8.5 as it provided the highest efficiency, best peak shape, and sensitivity. Different concentration (20–60 mM) of tris buffer was examined with 50 mM SDS using a temperature of 25 °C, a voltage of 25 kV and a pH value of 8.5. BGE with buffer concentration more than 40 mM resulted in an increase in the symmetry and sharpness of all the analyte peaks. A concentration of 50 mM tris buffer was chosen in order to maintain good peak shape within a reasonable migration time.

Effect of SDS Concentration

The SDS concentration was the key parameter regarding separation quality. The influence of the SDS was investigated in the concentration range from 30 to 60 mM at constant 50 mM tris buffer concentration at pH 8.5. The study revealed that, the increase of SDS concentration resulted in an improvement of separation between SDM and SQL with an increase in the migration time and the current. Better efficiency combined with short analysis time was achieved with 50 mM SDS.

Choice of Organic Modifier and Its Concentration

The addition of organic additives in the buffer could influence both resolution and theoretical plate number because they could cause a difference in affinity between micelles and analytes. In our experiments, methanol, n-propanol, and acetonitrile were added at a concentration of 10 *v/v* to the running background electrolyte of 50 mM tris buffer, pH 8.50 containing 50 mM SDS. The addition of 10% methanol resulted in good separation of the five studied drugs but the migration time of AMP increased significantly. Reasonable migration times were obtained upon the addition of 10% n-propanol but with change in selectivity and bad resolution

Table 3 Accuracy of the developed MEKC method using standard addition technique

Pharmaceutical preparation	Concentration taken	Concentration added (μg/mL)		% Recovery[a]	
Amprobate® powder	25, 1.6 μg/mL	AMP	EPB	AMP	EPB
		15	3	97.71	98.79
		25	8	98.37	99.42
		50	15	99.02	99.12
Mean ± SD				98.37 ± 0.66	99.11 ± 0.32
% RSD				0.67	0.32
% Error				0.39	0.18
Medacox® powder	21.5, 2 μg/mL	SDM	DVR	SDM	DVR
		20	4.0	102.69	97.37
		40	8.0	103.20	98.27
		60	15.0	101.09	98.70
Mean ± SD				102.33 ± 1.10	98.11 ± 0.69
% RSD				1.07	0.70
% Error				0.62	0.40
Sulphadin® powder	20 μg/mL	SDM		SDM	
		20		103.02	
		40		100.29	
		60		100.33	
Mean ± SD				101.21 ± 1.56	
% RSD				1.54	
% Error				0.89	
Unisulphaquin® powder	20 μg/mL	SQL		SQL	
		20		99.01	
		40		99.12	
		60		99.04	
Mean ± SD				99.06 ± 0.06	
% RSD				0.07	
% Error				0.04	

[a] Each result is the average of three separate determinations

Table 4 Robustness evaluation of the proposed MEKC method

Variation	Resolution			Theoretical plates					Run time
	SDM/SQL	SQL/EPB	EPB/DVR	SDM	SQL	EPB	DVR	AMP	
14% ACN	3.31	5.04	4.95	58,918	61,397	103,123	106,498	76,897	13.54
16% ACN	3.59	4.21	4.54	59,756	59,627	104,234	106,629	79,517	12.24
45 mM SDS	3.41	4.57	4.79	59,245	61,592	103,786	106,672	77,564	12.64
55 mM SDS	3.48	4.68	4.74	59,497	61,869	103,576	106,298	78,632	13.24
pH 8.3	3.42	4.65	4.81	59,456	61,656	103,578	106,587	78,234	12.85
pH 8.7	3.47	4.59	4.72	59,296	61,612	103,754	106,386	77,978	12.92
45 mM Tris buffer	3.37	4.56	4.96	58,167	60,170	103,317	107,853	77,897	12.83
55 mM Tris buffer	3.52	4.71	4.56	60,874	62,854	103,879	105,132	78,554	12.93
Injection time (19 s)	3.54	4.59	4.79	61,218	63,850	108,902	111,916	79,817	12.93
Injection time (21 s)	3.36	4.67	4.74	57,464	59,744	103,802	101,861	76,433	12.85
Capillary temp. 23 °C	3.48	4.65	4.76	59,573	61,892	103,867	106,579	78,465	12.91
Capillary temp. 27 °C	3.42	4.61	4.64	59,167	61,456	103,572	106,383	77,876	12.87
Applied voltage (27 kV)	3.49	4.66	4.81	59,345	61,567	103,613	106,398	77,976	13.76
Applied voltage (29 kV)	3.38	4.59	4.75	59,411	61,786	103,768	106,589	78,345	12.51
Without variation	3.45	4.63	4.78	59,382	61,662	103,698	106,492	78,025	12.89

between EPB and SQL. The addition of 10% acetonitrile resulted in a better resolution between SDM and SQL. The effect of acetonitrile concentration was studied in the range 10–20% *v/v*. As the concentration increased, the separation efficiency of the analytes improved. A concentration of 15% provided adequate resolution with short analysis time.

Effect of Separation Voltage

The effect of the applied voltage on the migration time and resolution was investigated in the range from 20 to 30 kV. As expected, increasing the applied voltage increases the EOF, leading to shorter analysis time and higher efficiencies. A lower voltage

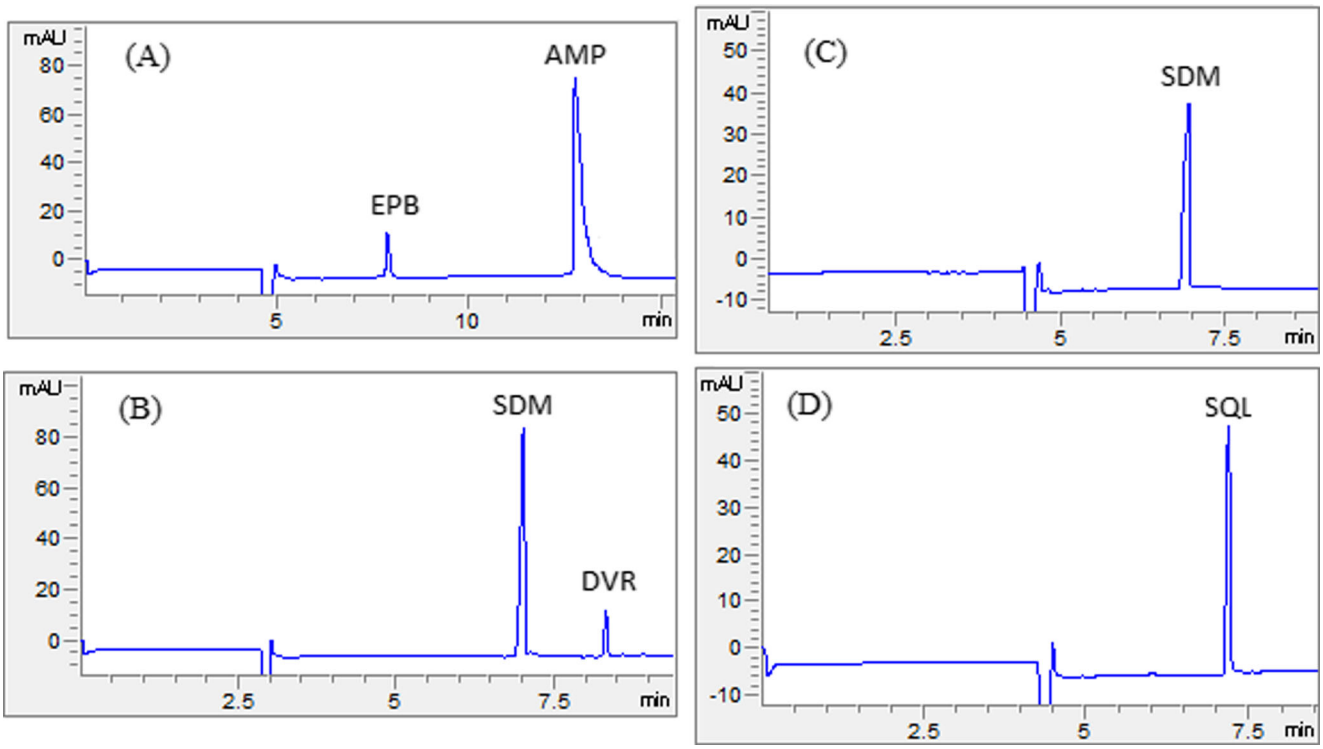

Fig. 3 Electropherograms obtained from the application of the proposed method to the analysis of **a** AMP (50 μg/mL) and EPB (3.2 μg/mL) in Amprobate® powder. **b** SDM (43 μg/mL) and DVR (4.0 μg/mL) in Medacox® powder. **c** SMD (20.0 μg/mL) in Sulphadin® powder and **d** SQL (20.0 μg/mL) in Unisulphaquin® powder

Table 5 Assay results of the five coccidiostats in commercial powder preparations

Formulation	Label claim (g/100 g)		Mean found	% Recovery[a]	% RSD	% Error
Amprobate®	AMP	50	50.04	100.08	0.17	0.10
	EPB	3.2	3.23	100.94	1.05	0.61
Medacox®	SDM	86	86.27	100.33	0.77	0.45
	DVR	8	8.02	100.25	0.91	0.53
Sulphadin®	SDM	33%	32.91	99.73	1.11	0.64
Unisulphaquin®	SQL	20%	20.07	100.35	0.93	0.54

[a] Each result is the average of three separate determinations

would increase the migration time and cause peak broadening. The voltage of 28 kV was chosen because there was adequate resolution of the five drugs and within a reasonable time and acceptable current.

Effect of Injection Time and Capillary Temperature

In order to reduce the detection limits, the injection time was varied from 5 to 30 s. Using hydrostatic injection at 50 mbar, a 20-s injection time was selected as the optimal value. The capillary temperatures ranging from 20 to 30 °C were investigated. In this study, the best operating CE conditions were obtained at 25 °C.

Selection of Detection Wavelength

The optimal wavelength was established experimentally with the aid of the photodiode array detector. Several electropherograms were run at different detection wavelengths, 200, 230, and 270 nm. The best electropherograms were obtained at 200 nm, so this wavelength was used for detection to maximize the sensitivity of the studied drugs.

Effect of Sample Solvent

To study the influence of sample solvent on peak shape, standard solutions were prepared using BGE, water and tenfolds diluted BGE from each stock solution. The most favorable results were obtained when standard solution was prepared by dilution of stock solutions with water. The samples prepared in BGE showed less enrichment and a larger peak width compared with those prepared in water. Therefore, water was selected as the sample matrix.

The optimized experimental conditions were the following: BGE composed of 50 mM tris buffer (pH 8.5) containing 50 mM SDS and 15% acetonitrile, 28 kV applied voltage at 25 °C with detection at 200 nm. Under these conditions, all

Table 6 Linearity equations for the five coccidiostats in different matrices

Matrix	Analyte	Regression equation	Correlation coefficient (r)
Feed premix	SDM	$y = 0.323\times + 0.044$	0.9995
	SQL	$y = 0.377\times - 0.046$	0.9991
	EPB	$y = 0.334\times - 0.165$	0.9993
	DVR	$y = 0.261\times + 0.099$	0.9999
	AMP	$y = 0.263\times + 0.032$	0.9995
Baby food	SDM	$y = 0.246\times + 0.068$	0.9992
	SQL	$y = 0.645\times - 0.156$	0.9987
	EPB	$y = 0.67\times - 0.033$	0.9996
	DVR	$y = 0.241\times + 0.001$	0.9996
	AMP	$y = 0.309\times - 0.009$	0.9991
Chicken tissue	SDM	$y = 0.371\times + 0.012$	0.9991
	SQL	$y = 0.647\times - 0.124$	0.9990
	EPB	$y = 0.631\times + 0.005$	0.9998
	DVR	$y = 0.229\times + 0.019$	0.9986
	AMP	$y = 0.302\times - 0.01$	0.9999
Chicken liver	SDM	$y = 0.331\times + 0.003$	0.9983
	SQL	$y = 0.64\times - 0.144$	0.9993
	EPB	$y = 0.64\times - 0.003$	0.9999
	DVR	$y = 0.237\times + 0.035$	0.9995
	AMP	$y = 0.308\times - 0.009$	0.9995

analytes are baseline-separated in less than 15 min. A typical electropherogram of the five drugs is given in Fig. 2.

Method Validation

The method was validated according to the International Conference on Harmonization (ICH) (International Conference on Harmonization (ICH) 2005) guidelines for validation of analytical procedures.

Linearity and Detection Limits

A linear relationship was established by plotting the corrected peak area against the drug concentration in μg/mL. The results of the statistical analysis of the data are summarized in Table 1 showing the range of the developed method for each analyte. A high value of the correlation coefficient r of the regression line, of intercept S_a, and of slope S_b indicate the linearity of the calibration graphs. The limit of quantitation (LOQ) was determined by establishing the lowest concentration of the analyte that can be measured according to ICH Q2B recommendations (International Conference on Harmonization (ICH) 2005) and below which the calibration graph is non-linear. The limit of detection (LOD) was determined by establishing the minimum level at which the analyte can be reliably detected; LODs and LOQs are shown in Table 1 for each compound.

Precision and Accuracy

The precision of the method was calculated by repeatability (intraday) and intermediate precision (interday). Intraday and interday precisions were confirmed using three concentrations and three replicates of each concentration. For each set of results, the RSD was calculated for the percentage recovery. An acceptable precision was obtained in all cases with intraday RSD values below 1.29% and interday RSD values within 1.57%. The results are illustrated in Table 2. The accuracy of the method was tested by the standard addition method on the dosage form to which known amounts of standard pure drugs have been added at different concentrations. The response was measured for each solution and the results are listed in Table 3. The calculated recoveries

Table 7 Recovery percentage of the five coccidiostats in different matrices

	Concentration taken (μg/mL)	% Recovery[a]				
Matrix		SDM	SQL	EPB	DVR	AMP
Feed premix	1	100.43	98.76	97.84	99.88	102.26
	2	97.46	99.31	101.43	100.77	99.16
	4	98.56	101.12	100.15	102.86	98.78
Mean ± SD		98.82 ± 1.5	99.73 ± 1.23	99.81 ± 1.82	101.17 ± 1.53	100.07 ± 1.91
% RSD		1.52	1.24	1.82	1.52	1.91
% Error		0.88	0.72	1.05	0.88	1.10
Baby food	0.5	96.87	100.25	98.19	98.67	97.76
	1	100.05	97.35	100.04	99.45	99.56
	2	99.25	101.13	97.96	99.43	99.13
Mean ± SD		98.72 ± 1.65	99.58 ± 1.98	98.73 ± 1.14	99.18 ± 0.44	98.82 ± 0.94
% RSD		1.68	1.99	1.15	0.45	0.95
% Error		0.97	1.15	0.66	0.26	0.55
Chicken tissue	0.5	99.12	97.81	98.27	96.83	98.47
	1	98.35	99.32	97.05	97.45	99.35
	2	100.81	99.13	99.57	99.13	100.43
Mean ± SD		99.43 ± 1.26	98.75 ± 0.82	98.31 ± 1.26	97.81 ± 1.19	99.42 ± 0.98
% RSD		1.27	0.83	1.28	1.22	0.99
% Error						
Chicken liver	0.5	98.05	95.29	97.62	97.65	97.26
	1	97.35	97.35	98.45	99.44	96.01
	2	96.15	98.43	95.17	99.17	98.11
Mean ± SD		97.18 ± 0.96	97.02 ± 1.59	97.08 ± 1.71	98.75 ± 0.97	97.13 ± 0.88
% RSD		0.99	1.64	1.76	0.98	1.06
% Error		0.57	0.95	1.02	0.57	0.61

[a] Each result is the average of three separate determinations

percentage (98.37–102.33%) demonstrate that the proposed method has excellent accuracy.

Robustness

The reliability of the established MEKC method was examined by testing the robustness. The experimental conditions, such as tris buffer pH 8.5 ± 0.2, tris buffer concentration 50 ± 5 mM, SDS concentration ± 5 mM, acetonitrile percentage ± 1% and capillary cartridge temperature 25 ± 2 °C) were altered purposely. These changes did not greatly affect the resolution, no. of theoretical plates, and analysis time which prove the robustness of the developed method. The obtained results are summarized in Table 4. The most critical parameter was found to be the percentage of acetonitrile.

Specificity

The specificity of the proposed MEKC method was established by its ability to determine the studied drugs in pharmaceutical powder preparations without interference from any additives. Furthermore, to evaluate the specificity of the method to

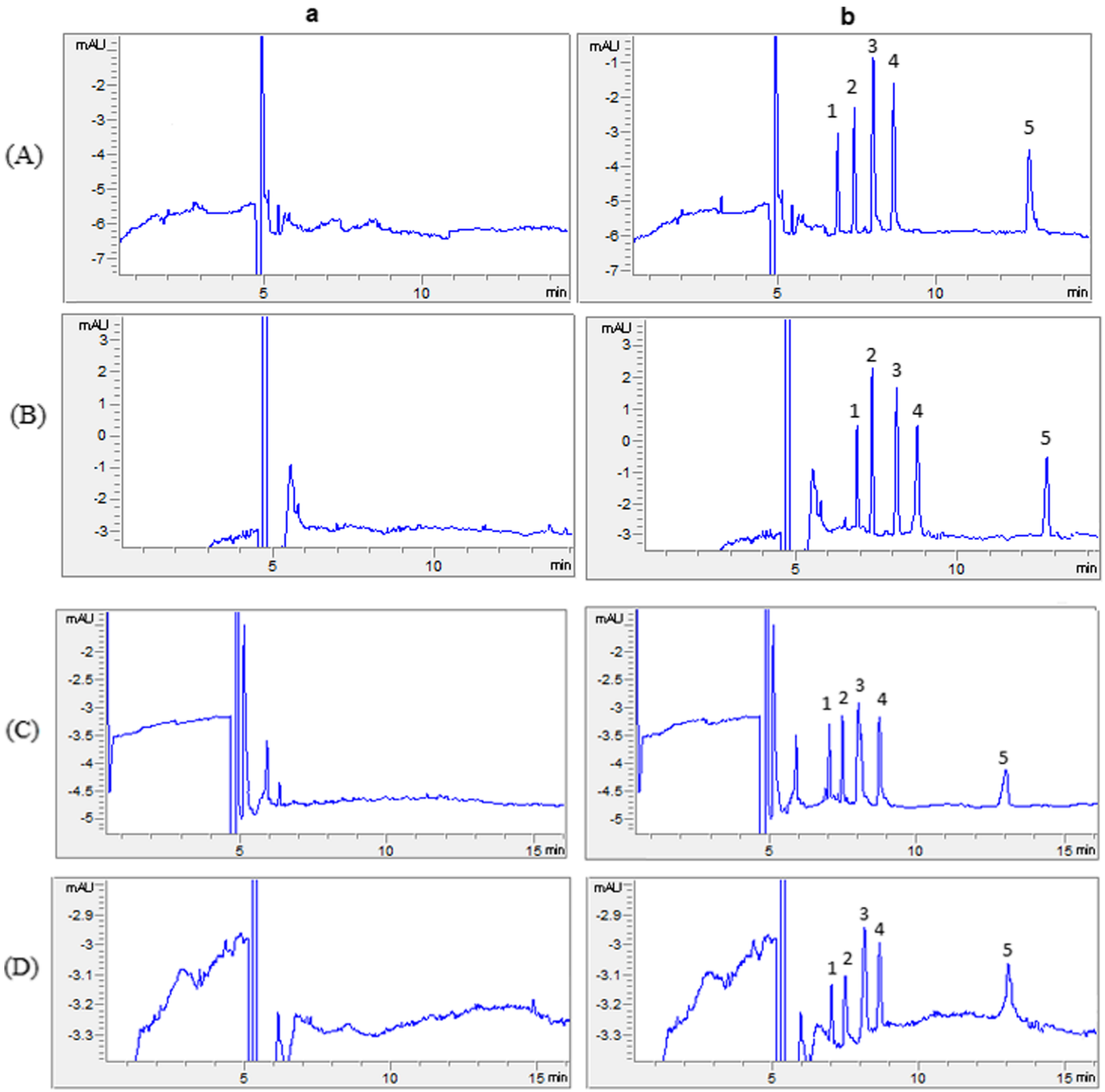

Fig. 4 Electropherograms obtained from the application of the proposed method to the analysis of the studied drugs in (A) feed premix (2.0 μg/mL), in (B) baby food (2.0 μg/mL), in (C) chicken tissues (1.0 μg/mL) and in (D) chicken liver (0.5 μg/mL); **a** blank sample; **b** spiked sample

determine the cited drug in feed premixes, baby food, chicken tissue, and liver, blank samples was prepared and analyzed under the recommended conditions. No interfering peaks were observed at the retention time of the studied drugs, which proved the homogeneity and purity of the peak.

Method Applications

Determination of the Studied Drugs in Their Single and Combined Pharmaceutical Preparations

The developed method was applied successfully for the assay of the studied drugs in single and combined pharmaceutical powder preparations. Simultaneous analysis of AMP and EPB in Amprobate® powder (Fig. 3a), SDM, and DVR in Medacox® powder (Fig. 3b), SQL in Unisulphaquin® powder (Fig. 3c), and SDM in Sulphadin® powder (Fig. 3d), were performed successfully with high percentage recovery (99.73–100.63) and good percentage RSD < 2 as shown in Table 5. This acceptable value indicated the applicability of the method for the routine quality control of the studied drugs without interference from the excipients.

Determination of the Studied Drugs in Spiked Feed Premixes, Baby Food, Chicken Tissues, and Liver

The applicability of the procedure developed to determine the studied drugs was tested by analyzing the drugs in spiked feed premixes, baby food, chicken tissues, and liver. Under the previously described experimental conditions, a linear relationship was established by plotting the average corrected peak areas versus drug concentrations (μg/mL). The calibration curves were linear as shown in Table 6. The results of three replicate experiments of each sample are listed in Table 7. Due to the limited optical path length of UV detector and low volume of injected sample, the proposed method could not determine concentrations below the permissible MRL in real samples. However, the possibility to clearly distinguish all analytes from matrix interferences and the satisfactory recoveries for each drug in all samples (Table 7) is sufficient for the determination of the studied drugs in real samples in case of their improper use. Figure 4 shows typical electropherograms obtained from drug-free and spiked samples of the studied drugs analyzed with the optimum operating conditions.

Conclusion

A novel and simple MEKC method for analysis of coccidiostats drugs (AMP, EPB, DVR, SMD, and SQL) was developed and validated. The proposed method was successfully applied to the analysis of studied drugs in veterinary powder preparations. In addition, it is a reliable analytical tool for the simultaneous analysis of the studied drugs in feed premixes, baby food, chicken tissues, and liver. Moreover, it has the advantage of simple sample extraction procedure with adequate recovery results and short analysis time, thus reducing the overall turnaround time. The good validation criteria of the proposed method allowed its utilization for quality control laboratories. It is possible to increase the method sensitivity using different online or/and offline concentration methods, and/or using different injection conditions.

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Compliance with Ethical Standards

Conflict of Interest Fathalla Belal declares that he has no conflict of interest. Sawsan Abd El-Razeq declares that she has no conflict of interest. Manal Fouad declares that she has no conflict of interest. Sahar Zayed declares that she has no conflict of interest. Fatma Fouad declares that she has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Informed consent is not applicable in this study.

References

Abd-El-Sattar OA (2002) Simultaneous determination of sulphaquinoxaline and amprolium in veterinary mixture by HPLC and first derivative ultraviolet spectrophotometry. Saudi Pharm J 10(4):190–195

Aguilera-Luiz MM, Martenez Vidal JI, Romero-Gonzllez R, Garrido Frenich A (2012) Multiclass method for fast determination of veterinary drug residues in baby food by ultra-high-performance liquid chromatography–tandem mass spectrometry. Food Chem 132:2171–2180

Alomary A (2004) Simultaneous quantification of amprolium and ethopabate in pharmaceutical preparation by derivative ultraviolt spectroscopy. Abhath Al-Yarmouk Pure Science and Engineering Series 13(1):59–70

Belal F, Sharaf El-Din MK, Tolba M, El-Awady M, El-Mansi H (2016) Analysis of four antimigraine drugs in two ternary mixtures by sweeping-micellar electrokinetic chromatography with retention factor gradient effect and dynamic pH junction. Microchem J 127:11–21

Broekaert N, Van Peteghem C, Daeseleire E, Sticker D, Van Poucke C (2011) Development and validation of an UPLC-MS/MS method for the determination of ionophoric and synthetic coccidiostats in vegetables. Anal Bioanal Chem 401:3335–3344

Ching-Erh L (1997) Migration behavior and separation of sulfonamides in capillary zone electrophoresis III. Citrate buffer as a background electrolyte. J Chromatogr A 768(1):105–112

Clarke L, Fodey TL, Crooks SRH, Moloney M, O'Mahony J, Delahaut P, O'Kennedy R, Danaher M (2014) A review of coccidiostats and the analysis of their residues in meat and other food. Meat Sci 97:358–374

Commission Recommendation 2005/925/EC (2005) Off J Eur Commun L337/51

El-Awady M, Belal F, Pyell U (2013) Robust method for the analysis of hydrophobic base analytes loratadine and desloratadinein pharmaceutical preparations and biological fluids by sweeping-cyclodextrin modified micellar electrokinetic chromatography. J Chromatogr A 1309:64–75

El-Kosasy AM, Hussein LA, Magdy N, Abbas MM (2015) Sensitive spectrofluorimetric methods for determination of ethopabate and amprolium hydrochloride in chicken plasma and their residues in food samples. Spectrochim Acta A Mol Biomol Spectrosc 150:430–439

European Union (2010) Commission Regulation No 37/2010 of 22nd December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Off J Eur Communities L15:–1, 72

Font G, Juan-García A, Picó Y (2007) Pressurized liquid extraction combined with capillary electrophoresis–mass spectrometry as an improved methodology for the determination of sulfonamide residues in meat. J Chromatogr A 1159(1–2):233–241

Furusawa N (2002) Simplified high-performance liquid chromatographic determination of residual amprolium in edible chicken tissues. J Chromatogr Sci 40:355–358

Ghanem M, Abu-Lafi S (2013) Validation of a stability-indicating assay of amprolium hydrochloride in water soluble powder formulation using hydrophilic interaction liquid chromatography. J Appl Pharm Sci 3(10):51–58

Girardi C, Odore R (2008) Pharmacological treatments and risks for the food chain. Vet Res Commun 32:S11

Hartig C, Storm T, Jekel M (1999) Detection and identification of sulphonamide drugs in municipal waste water by liquid chromatography coupled with electrospray ionisation tandem mass spectrometry. J Chromatogr A 854:163–173

Heiger DN (1992) An introduction high performance capillary electrophoresis, 2nd edn. Hewlett-Packard Company, France

Hows MEP, Perrett D, Kay J (1997) Optimization of a simultaneous separation of sulphonamides, dihydrofolate reductase inhibitors and β-lactam antibiotics by capillary electrophoresis. J Chromatogr A 768:97–104

Hussein LA, Magdy N, Abbas MM (2015) Five different spectrophotometric methods for determination of amprolium hydrochloride and ethopabate binary mixture. Spectrochim Acta A Mol Biomol Spectrosc 138:395–405

International Conference on Harmonization (ICH) (2005) Technical requirements for the registration of pharmaceutical for human use, validation of analytical procedures; text and methodology Q2 (R1). Geneva, pp 1–13

Kaklamanos G, Vincent U, Von Holst C (2013) Analysis of antimicrobial agents in pig feed by liquid chromatography coupled to orbitrap mass spectrometry. J Chromatogr A 1293:60–74

Kao Y, Chang M, Cheng C, Chou S (2001) Multiresidue determination of veterinary drugs in chicken and swine muscles by high performance liquid chromatography. J Food Drug Anal 9(2):84–95

Kim B, Ham H, Lee J, Cheong N, Myung S (2012) Determination of coccidiostats (amprolium and decoquinate) in cattle and chicken's muscle using high performance liquid chromatography. Bull Kor Chem Soc 33(2):559–563

Křivánková L, Boček P (1985) Determination of amprolium in feed stuff by capiliary isotachophoresis. Electrophoresis 6(3):143–144

Liu R, He P, Li Z, Li R (2011) Simultaneous determination of 16 sulfonamides in animal feeds by UHPLC–MS–MS. J Chromatogr Sci 49(8):640–646

Martinez-Villalba A, Moyano E, Teresa Galceran M (2010) Analysis of amprolium by hydrophilic interaction liquid chromatography–tandem mass spectrometry. J Chromatogr A 1217:5802–5807

Martínez-Villalba A, Nunez O, Moyano E, Teresa Galceran M (2013) Field amplified sample injection-capillary zone electrophoresis for the analysis of amprolium in eggs. Electrophoresis 34:870–876

Mcevoy JDG (2002) Contamination of animal feedingstuffs as a cause of residues in food: a review of regulatory aspects, incidence and control. Anal Chim Acta 473:3–26, 2002

Nasr JJ, Shalan S (2014) Spectrofluorimetric analysis of ethopabate in veterinary formulations with application to residue determination in chicken muscles and liver. Luminescence 29(8):1188–1193

Nour El-Dien FA, Mohamed GG, Khaled E, Frag EYZ (2010) Extractive spectrophotometric determination of sulphonamide drugs in pure and pharmaceutical preparations through ion-pair formation with molybdenum(V) thiocyanate in acidic medium. J Adv Res 1:215–220

Ortelli D, Cognard E, Jan P, Edder P (2009) Comprehensive fast multiresidue screening of 150 veterinary drugs in milk by ultra-performance liquid chromatography coupled to time of flight mass spectrometry. J Chromatogr B 877:2363–2374

Petersa RJB, Bolcka YJC, Rutgersa P, Stolkera AAM, Nielena MWF (2009) Multi-residue screening of veterinary drugs in egg, fish and meat using high-resolution liquid chromatography accurate mass time-of-flight mass spectrometry. J Chromatogr A 1216:8206–8216

Riekkola ML, Joensson JA, Smith RM (2004) Terminology for analytical capillary electromigration techniques (IUPAC Recommendations 2003). Pure Appl Chem 76(2):443–451

Rizk MS, Zaky Frag EY, Mohamed GG, Tamam AA (2013) Spectrophotometric determination of distigmine bromide, cyclopentolate HCL, diaveridine HCL and tetrahydrozoline HCL via charge-transfer complex formation DDQ reagent. Iran J Pharm Res 3(2):168–183

Salama NN, Fouad MM, Rashed NM (2012) Validated chromatographic methods for simultaneous determinations of amprolium hydrochloride and ethopabate in veterinary preparations. IJPBR 3(4):185–190

Smallidge RL Jr (1978) Gas-liquid chromatographic determination of ethopabate in feed premixes. J Assoc Off Anal Chem 61(3):561–563

Song W, Huang M, Rumbeiha W, Li H (2007) Determination of amprolium, carbadox, monensin, and tylosin in surface water by liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom 21:1944–1950

Springer VH, Lista AG (2012) Micellar nanotubes dispersed electrokinetic chromatography for the simultaneous determination of antibiotics in bovine milk. Electrophoresis 33(13):2049–2055

Van Rhijna JA, Lasaromsa JJP, Berendsena BJA, Brinkmanb UAT (2002) Liquid chromatographic–tandem mass spectrometric determination of selected sulphonamides in milk. J Chromatogr A 960:121–133

Vinay K, Pardeep S, Pawan K, Mehlabs S, Anu G, Vijayta G (2013) Anticoccidial drugs used in the poultry: an overview. Sci Int 7:261–265

Wanga H, Yuana B, Zenga Z, Hea L, Dinga H, Guoa C, Konga X, Wanga W, Huang X (2014) Identification and elucidation of the structure of in vivo metabolites of diaveridine in chicken. J Chromatogr B 965: 91–99