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Optimization and validation of the micellar electrokinetic capillary chromatographic method for simultaneous determination of sulfonamide and amphenicol-type drugs in poultry tissue

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ABSTRACT

The report describes a new approach enabling simple and rapid multi-residue screening of seven sulfonamides (SAs): sulfamethazine (SMZ), sulfamerazine (SMR), sulfathiazole (STZ), sulfachloropyridazine (SCP), sulfamethoxazole (SMX), sulfacarbamide (SC), and sulfaguanidine (SG); and three amphenicol-type antibiotics: chloramphenicol (CAP), thiamphenicol (TAP), and florfenicol (FF) in animal tissue by micellar electrokinetic chromatography (MEKC). The analytes were isolated from tissue samples through solid-phase extraction (with a C_{18} cartridges) following protein precipitation with acetonitrile. The evaluated LOD and LOQ values ranged from 1.3 to 7.8 and from 4.5 to 26.1 ng/g, respectively. These values are far lower than the maximum residue limits (MRLs) set by several control authorities. Intra- and inter-day precision data were less than 9.5% and 11.2% for SAs, and 8.4% and 14.9% for amphenicols. Moreover, the method was found accurate, with the recoveries ranging from 86.4% to 109.4%. The absolute recoveries of the analysed drugs were higher than 77.2%. The results obtained in the validation process demonstrate that the developed CE method is suitable for simultaneous determination of SA and amphenicol residues in poultry tissue, with the total run time less than 8 min.

1. Introduction

In the last decades, livestock production has notably increased, particularly thanks to intensive farming. Large quantities of veterinary drugs are extensively used in animal husbandry both for prophylactic and therapeutic purposes. Sub-therapeutic doses are, however, also used illegally for growth promotion. Improper use of chemotherapeutics in veterinary medicine and insufficient withdrawal time for treated animals may lead to the occurrence of drug residues in edible tissue, posing health hazard to the consumer. Such residues may have toxic effects on the humans or cause problems by introducing resistant strains of bacteria and inducing possible allergic hypersensitivity reactions.

Sulfonamides (SAs) (Fig. 1) as well as amphenicols (APhs) (Fig. 2) have played an important role in veterinary medicine practice because of their inexpensiveness and broad range of antimicrobial activity. Mixing the two chemotherapeutic groups is targeted at intensifying their efficiency achieved thanks to their synergic antimicrobial effects. Alone, SAs are bacteriostatic agents, but when combined with APhs a bactericidal effect occurs. However, because CAP can cause aplastic anemia, thrombo- and granulocytopenia in humans, the European Union (EU) has banned the use of this antibiotic for food-production. On the other hand, sulfamethazine is suspected to be carcinogenic and trigger thyroid tumors [1]. To safeguard human health, the EU has adopted the maximum residue level (MRL) legally permitted or accepted in food products of animal origin, and the minimum required performance limit (MPRL) for banned substances. The established MRL values are defined at 100 μg/kg for the sum of all SAs, and 50 μg/kg for TAP in all edible tissues. For FF and its major metabolite, FF amine (the sum of both), on the other hand, the values range between 100 μg/kg for muscle and 2500 μg/kg for liver [2]. MPRL value for CAP has been set at 0.3 μg/kg.

Numerous liquid chromatographic (LC) and capillary electrophoretic (CE) techniques with mass spectrometry (MS) or tandem MS detection have been developed [1,3–9] to detect and measure the residues of SAs or APhs in edible animal tissue. Unfortunately, the sensitive detection modes are not frequently applied in routine analyses because of the high instrumentation cost a common laboratory would have to incur. The UV detector coupled with CE equipment offers an alternative method for determination of SA [10–12] and APh compounds [13–15], carrying the benefit of simplicity, low cost, and sufficient sensitivity. In contrast to the earlier studies, the proposed method enables detecting both groups simultaneously under the micellar electrokinetic capillary chromatography (MEKC) technique, without any large volumes

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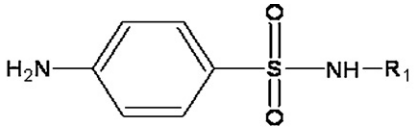


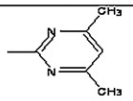
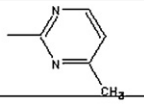
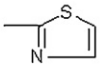
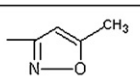
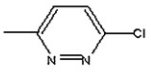
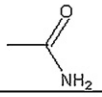
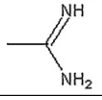
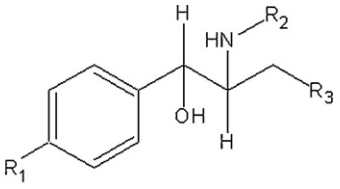
	R_1
Sulfamethazine (SMZ)	
Sulfamerazine (SMR)	
Sulfatiazole (STZ)	
Sulfamethoxazole (SMX)	
Sulfachloropyridazine (SCP)	
Sulfacarbamide (SC)	
Sulfaguanidine (SG)	

Fig. 1. Structures of sulfonamides.

of organic solvents, high number of extractions, or derivatizing treatment. The method has been fully validated against the EU guidelines. In the present study we provide a new strategy for solving the problem of sample treatment when different groups of chemotherapeutic drugs are simultaneously determined in animal tissue samples. In the same study we additionally investigated application of the MEKC method to screen real animal products for drug residues. To date, there has been no paper that would report simultaneous determination of both group of drugs (in a single run) in animal tissue. The obtained results confirm that the developed MEKC method is suitable for the EU statutory veterinary drug residue surveillance programme, particularly in the perspective of widespread application in routine laboratories.



	R_1	R_2	R_3
Chloramphenicol (CAP)	$-NO_2$	$-COCHCl_2$	-OH
Thiamphenicol (TAP)	H_3C-SO_2-	-H	-OH
Florfenicol (FF)	H_3C-SO_2-	$-COCHCl_2$	-F

Fig. 2. Structures of amphenicols.

2. Experimental

2.1. Reagents and standard solutions

SMZ, SMR, STZ, SMX, SCP, SC and SG were supplied by Sigma–Aldrich (St. Louis, MO, USA). FF, TAP and flumequine used as internal standard (I.S.) were obtained from Vetos-Farma (Bielawa, Poland), while CAP was purchased from Pliva (Kraków, Poland). Sodium tetraborate dehydrate was purchased from POCH (Gliwice, Poland). Sodium dodecylsulfate (SDS), acetonitrile and methanol were supplied by Merck (Darmstadt, Germany). All chemicals were of analytical grade and applied without further purification. The water used in all experiments was purified through triple distillation. LiChrolut C-18 SPE cartridges (100 mg, 1 ml) were purchased from Merck (Darmstadt, Germany).

Stock standard solutions of individual SAs were prepared by dissolving each of the substances in 2 ml of methanol and diluting them with the sodium tetraborate decahydrate buffer (pH 9.3, 15 mM) to the final concentration of 1 mg/ml. Stock standard solutions (1 mg/ml) of individual APh compounds were prepared in the sodium tetraborate decahydrate buffer (pH 9.3; 15 mM). The optimized phosphate-borate solution used for SPE was: 15 mM sodium tetraborate decahydrate adjusted to pH 6.5 with 20 mM monosodium phosphate solution. All stock standard solutions were stored in the dark at the temperature 4 °C until use to avoid possible decomposition. They proved stable for at least 2 months. Mixed working solutions of SAs, and individual working solutions of CAP, TAP, and FF at the concentrations of 1.0 and 10.0 μg/ml, were prepared daily by diluting the stock solutions as appropriate with triple distilled water. These were stored in a refrigerator until use.

2.2. Experimental design

All procedures involving poultry complied with the local regulations of animal ethics and were conducted under the guidance of an experienced veterinarian. In the small-animal study, 8 turkeys and 8 hens were collected from a poultry farm – PFO "Vetos-Farma" (Bielawa, Poland). Before the experiment, the animals were physically examined and determined to be healthy, marked with numbers, and weighed to establish the individual weight of each. The average weight of the birds was: 2.84 ± 0.52 kg for hens, and 7.85 ± 1.32 kg for turkeys. The birds were divided into two experimental groups: one control group and one treatment group. The veterinary drugs were added to food, and each bird of the treatment group was medicated with TAP or FF. The therapeutic single dose of 50 mg/kg for TAP and 20 mg/kg of body weight (b.w.) per day for FF was administered for five consecutive days. Each group of animals was treated with only one of the compounds. The birds of the control group (2 turkeys and 2 hens) were fed a non-drugged feed. Several samples of the turkey and hen meat were prepared upon slaughtering the birds 10, 18, and 28 days after the end of the medication period. The samples of edible tissues (muscles, liver, and fat with skin) were minced and stored at −20 °C until the assay.

Finally, twenty commercial poultry samples (muscle, liver and skin with fat) were purchased from local supermarkets and butcher's shops (Gdańsk, Poland) and treated as unknown samples. All were stored at −20 °C until processing.

2.3. Instrumental parameters and conditions

All capillary electrophoretic experiments were performed using the Beckman P/ACE 5000 (Fullerton, CA, USA) system, equipped with an autosampler, fluid-cooled capillary cartridge, and fixed-wavelength UV detector with a 200 nm filter. Separations were carried out using fused-silica capillary, 75 μm I.D., 57 cm (51 cm effective length). All experiments were performed at 22.0 (±0.1) °C,

and the voltage was 22 kV. Analytes were loaded into the capillary at the anode through 2 s long argon pressure injection at 3.45 kPa, whereas the detector was set on the cathode end of the capillary. The best separation of the analytes was obtained when using the buffer solution composed of methanol and a mixture of sodium tetraborate decahydrate (pH 9.3, 15 mM) and sodium dodecylsulfate (25 mM) (20:80, v/v). At the beginning of each day, the capillary was conditioned with 0.1 M sodium hydroxide for 5 min, then rinsed with triple distilled water (5 min), and finally with the running buffer (5 min). Additionally, in order to improve peak repeatability, the capillary was flushed in between the runs with 0.1 M hydrochloric acid (0.5 min), 0.1 M sodium hydroxide (1 min), and triple distilled water (1 min). Before injection, the capillary was rinsed with the running buffer for 1 min.

2.4. Sample preparation

The calibration standard, quality control (QC), control blank tissue samples, as well as experimental samples were analyzed in the same manner. The thawed muscle sample (weighing accurately 3 g) was homogenized at high speed for 3 min using Polytron TM (Germany). After fortification with analysed compounds and I.S., the sample was deproteinized using 6 ml of acetonitrile and ultrasonic bath (10 min). The homogenate was centrifuged at 8000 rpm for 5 min, decanted, and evaporated to dryness in water bath at 40–45 °C. After reconstitution in 3 ml of water and centrifugation (8000 rpm, for 5 min), the extract was passed through an SPE cartridge (C-18) preconditioned with 2 ml of methanol and 2 ml of water. Next, the cartridge was washed with 2 ml of triple distilled water and the analytes were eluted with 300 μl of methanol. Finally, after evaporation to dryness, the residue was reconstituted in 100 μl of 2 mM sodium tetraborate decahydrate, centrifuged again in the same conditions as before, and stored at −20 °C until the time of the analysis.

2.5. Validation of the analytical method

The validation of the method was performed in accordance with the EU guidelines. Calibration was performed by adding known amounts of analyzed drugs to blank edible animal tissue to yield concentrations over the range of 0.025–1 μg/g for each SA and 0.005–1, 0.01–1, 0.025–5 μg/g for CAP, TAP and FF, respectively, and the fixed concentration of flumequine (I.S.) (0.5 μg/g). These standards were maintained over the whole procedure described above. Calibration curves were constructed based on the peak area ratio of the analyte to I.S. versus the known concentrations of the analyte, and calculated by in the weighted ($1/x$) linear regression analysis. The limits of detection (LOD) and quantification (LOQ), as well as the decision limit (CCα) and detection capability (CCβ) of the analyzed drugs were determined based on the standard deviation (SD) of the intercepts (b) of the regression line and the slope (S) evaluated from the calibration curves of each of the drugs ($n = 36$): LOD = 3SD/S; LOQ = 10SD/S; CCα = 2.33 × SD of the y intercept; CCβ = CCα + (1.64 × SD). The CCα was calculated at the statistical certainty of $1 - \alpha$ ($\alpha = 0.05$) and CCβ was $1 - \beta$ ($\beta = 0.05$) to detect the concentration at the MRL level. The selectivity of the method was determined on the basis of an analysis of different drug-free poultry muscle samples and extracts of muscle spiked with selected compounds and internal standard ($n = 6$). Intra-and inter-day precision and accuracy of the assay were evaluated in a replicate analysis of samples containing known amounts of each drug at 0.5× MRL, 1× MRL and 2× MRL values for poultry muscle, corresponding with the QC samples. These QCs at the concentration of 0.05, 0.1 and 0.2 μg/g for each SA and 0.01, 0.05 and 0.25 μg/g for CAP, 0.025, 0.05 and 0.1 μg/g for TAP and 0.05, 0.1 and 0.2 μg/g for FF were prepared and analyzed on the same day (intra-day) and on ten different days (inter-day variability). The precision of the method was expressed as the relative standard deviation (RSD%), while the accuracy was defined as correspondence of the obtained assay result with the nominal concentration value. The absolute recovery was calculated by comparing peak areas of the extracted animal muscle samples versus the unextracted standard solutions at three QC levels ($n = 6$). As part of the validation process, a test of freeze–thaw stability of all drugs in poultry tissue was performed by measuring three replicates of the samples at three QC levels over three cycles of freezing (−20 °C) and thawing (room temperature) over two months. The evaluation was based on the back-calculated concentrations.

3. Results and discussion

Although SAs and APhs have been studied extensively, the present work is the first example of a validation procedure for simultaneous quantification of both groups by MEKC in tissue samples of animal origin. One of the main difficulties in the development of an analytical method for the biological matrix lies in sample treatment, which become more complicated when compounds of different chemical properties (i.e., polarities, solubilities and pK_a values) are attempted. An animal tissue sample is a highly complex mixture consisting of proteins, fats, carbohydrates, and other substances. Because veterinary drugs generally appear in the biological samples at trace levels, the SPE procedures were adopted to extract and pre-concentrate them. The main purpose of the discussed study was to develop a relatively simple, quick method, involving minimum volumes of organic solvents. The pH value of the solution was anticipated to influence extraction of the compounds significantly due to the presence of basic or acidic functional groups in their molecular structures. Both SAs and APhs are amphoteric compounds and are easily extracted by organic solvents when present in the molecular form. Because the analytes have different pK_a values, it was important to select the appropriate pH conditions for both extraction, and electrophoretic separation.

3.1. Optimization of analyte isolation from animal tissue

To determine the best deproteinizing agent, different organic solvents like acetonitrile, trichloroacetic acid (TCA) in acetonitrile, methanol and its mixtures with buffer solutions (in the range pH 3–9) were tested. The impact of the selected deproteinizing agents and the matrix nature of the recovery of the compound of interest are illustrated in Figs. 3 and 4. By conducting series of experiments and comparing the corresponding electropherograms it was found that acetonitrile was most efficient in removing interferences. The recoveries of all analytes went up by about 12% when the volume of acetonitrile was increased from 5 to 6 ml. Above the ceiling the values did not change irrespective of any further increase in the volume of the organic solvent. During the extraction optimization process the effect of the ultrasonic bath duration (ranging from 5 to 20 min) on the recoveries of the analytes was investigated. It was observed, that mean recoveries of SA and APh drugs in ultrasonic bath increased from 67% to 84% when the duration of the bath was extended from 5 to 10 min. No further extension of the ultrasonic bath time (to 20 min) resulted in any increase of the recovery values.

3.2. SPE optimization

Since the concentration of antibacterial drugs commonly found in food samples is extremely low, pretreatment such as the SPE is necessary for their sensitive detection and quantification. In order to maximize the recovery of the analytes, sample extract solutions of different pH values (ranging 2.5–9) were, upon evaporation of

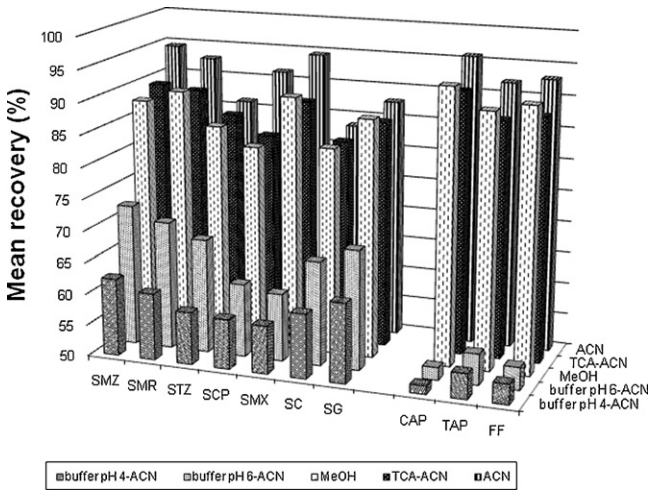


Fig. 3. The influence of selected deproteinizing agents on the extraction efficiency of compound of interest. ACN, acetonitrile, TCA, 3% trichloroacetic acid in acetonitrile, MeOH–methanol, buffer pH 6-ACN-mixture of acetonitrile and phosphate buffer at pH 6 (1:1, v/v), and buffer pH 4-ACN-mixture of acetonitrile and phosphate buffer at pH 4 (1:1, v/v).

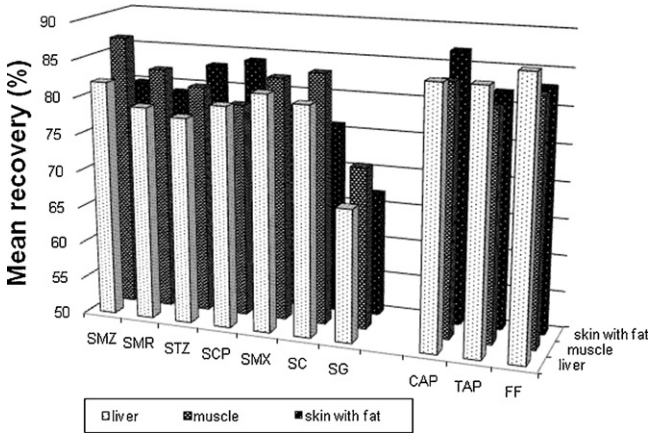


Fig. 4. The influence of matrix nature on the extraction efficiency of analytes.

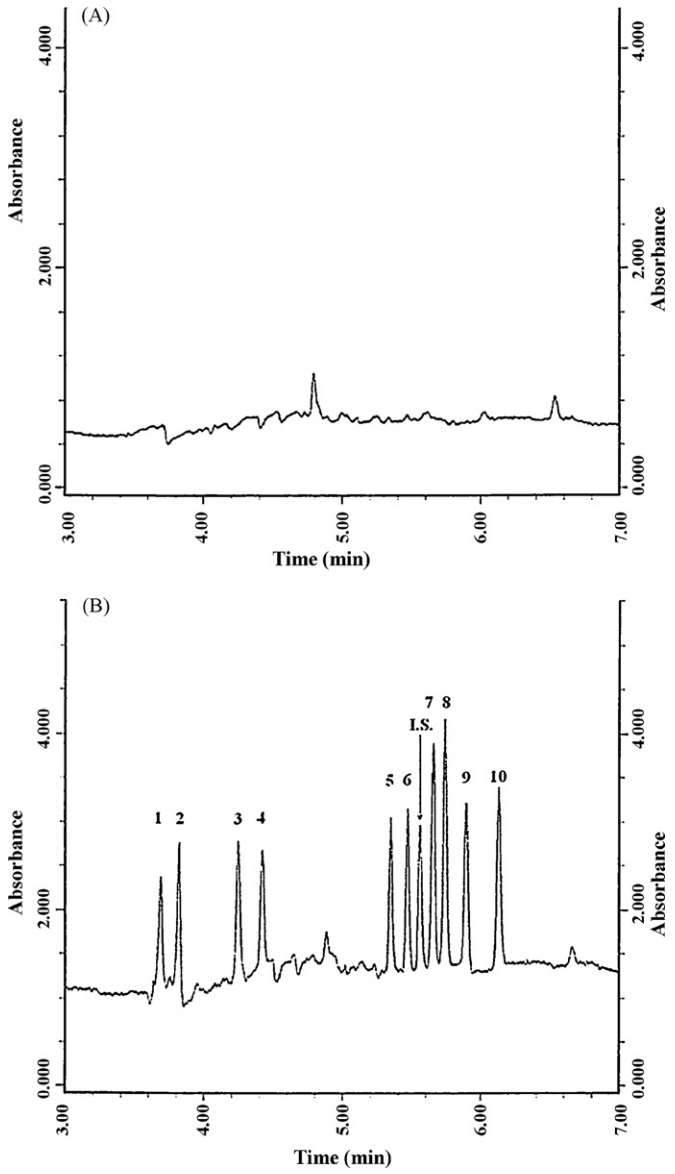


Fig. 5. Typical electropherogram of (A) drug-free turkey muscle and (B) blank turkey muscle spiked with SG (1), FF (2), TAP (3), CAP (4), SMZ (5), SMR (6), SCP (7), SMX (8), STZ (9), SC (10) and flumequine (I.S.). Concentration for APhs-100 ng/g, SAs-200 ng/g, I.S.-500 ng/g of sample. *Conditions*: UV detection at 200 nm, unmodified silica capillary (57 cm × 50 μm I.D.), temp. 22 °C, running buffer composed of (20:80, v/v) methanol and mixture of 15 mM $Na_2B_4O_7$ and 25 mM SDS.

the organic solvent, let drift in the SPE columns. It was observed that the recoveries of the analytes diminished notably when the pH of the sample went down below 3. Because the constants (pK_a) of the analyzed SAs in first ionization range between 1.8 and 2.8, the compounds of strong acidic pH values are transformed to cations, hence show no tendency to penetrate the C18 cartridge. The second pK_a values of the SAs range from 5 to 8 [16], and the pK_a values for APhs fall in the range of 5.5–7.2. Thus, in alkaline solutions both groups of interest are converted to anions. Ultimately, the pH 6.5 was selected because most compounds of interest were expected to be uncharged at that pH value. The results confirmed that the highest recoveries (more than 77%) for all tissues spiked with analytes were obtained when the phosphate-borate buffer (pH 6.5) was used to dissolve drug residues.

Table 1
Summary of validation data for SAs obtained with MEKC calibrations.

	SMZ	SMR	STZ	SMX	SCP	SC	SG
Linearity range (μg/g)	0.025–1						
Slope	1.715 ± 0.009	1.869 ± 0.009	2.151 ± 0.011	2.735 ± 0.012	2.433 ± 0.012	2.221 ± 0.012	1.339 ± 0.007
Intercept	0.007 ± 0.004	0.002 ± 0.004	0.012 ± 0.005	-0.003 ± 0.006	0.001 ± 0.006	0.010 ± 0.005	0.006 ± 0.003
Standard error	0.007	0.007	0.010	0.010	0.010	0.010	0.006
LOD (ng/g)	7.2	6.4	7.4	6.2	7.3	7.4	7.8
LOQ (ng/g)	23.9	21.4	24.6	20.8	24.2	24.8	26.1
CCα (ng/g)	16.3	11.5	24.3	9.9	14.8	22.8	13.8
CCβ (ng/g)	23.1	18.1	33.0	19.2	24.5	31.8	19.6

Moreover, different modes of the SPE cartridges were tested to separate the analytes from impurities in the tissue sample. In order to eliminate the influence from the matrix in an effective clean-up procedure, several kinds of sorbents (C8, C18, Alumina N, Lichrolut EN) were tested as the candidate packing materials of the SPE cartridge. The C18 cartridge allowed to obtain recoveries of the highest values. To choose the most effective eluent for the C18 cartridges we tested several organic solvents (acetonitrile, methanol, and ethyl acetate). Due to strong polarity pure methanol turned out to enable good recoveries of the extracted chemotherapeutics. Moreover, the methanol extracts were free from any interfering compounds. The whole clean-up procedure was applied to all matrices, including muscle, liver, and skin with fat.

3.3. MEKC method development

The main objective of developing analytical methodologies, especially when a number of compounds are considered simultaneously, is to obtain adequate separation of each analyte. Because of the diversity of the chemotherapeutics, especially their pK_a values, we tested a variety of electrophoretic conditions including the buffer composition, pH values and the molarity in order to achieve full separation of all peaks. We evaluated the effect of the pH value of the running buffer solutions, ranging between 4 and 10, on the analyte separation. APhs and most SAs are positively charged in acidic, and negatively charged in alkaline conditions. Therefore, an alkaline buffer solution seems to be an adequate electrolyte carrier as it causes different net charges of the chemotherapeutics. Mamani et al. [17] have proven that simultaneous separation of the antimicrobial agents can be performed in the pH range of 7.5–9, when all analytes have a negative charge.

The determination of the APhs in the conventional capillary zone electrophoresis (CZE) is difficult because FF contains no functional groups which could be ionized in the pH range between 2 and 12 [14]. Differently, TAP and CAP become charged in the buffer electrolyte because they contain vicinal hydroxyl groups and can form complexes with borate ions thus becoming charged. Our earlier work [13] revealed that the buffer solution composed of 25 mM SDS and 10 mM sodium tetraborate decahydrate can be successfully used only for electrophoretic determination of APh antibiotics. However, in order to increase sensitivity and quality of separation for simultaneous determination of SAs and APhs, acetonitrile and methanol (in different volume proportions) were added to the running buffer solution. Experimental results indicated that effective separation of the peaks of interest could be observed when 20% of methanol (v/v) was added to the buffer solution. Therefore, the mixture of sodium tetraborate decahydrate (pH 9.3, 15 mM), sodium dodecylsulfate (25 mM) and methanol (80:20, v/v) was selected to separate all analytes at the highest resolution without disturbances.

3.4. Assay validation

The developed method was validated for linearity, precision, accuracy, selectivity, recovery and stability. Details of the calibration curves are shown in Table 1 (SAs) and Table 2 (APhs). LODs and LOQs for selected SAs were proved to range between 6.2–7.8 and 20.8–26.1 ng/g, whereas the same values for APhs ranged from 1.3 to 5.7 and from 4.5 to 18.9 ng/g, respectively. We also established the CCα and CCβ values for all analytes finding them fall in the range of 9.9–24.3 and 18.1–33.0 ng/g for SAs, and below 15.9 and 21.1 ng/g for APhs, respectively. The results of intra- and inter-day assay precision and accuracy tests are presented in Tables 3 and 4. The obtained values were lower than the limits required for biological samples and confirmed high precision and accuracy of the developed method. The mean absolute recoveries for all analysed drugs and the internal standard in poultry muscle samples were above 77.2%, which means were comparable to those of the published methods of SAs detection in tissue samples [11].

Table 2
Summary of validation data for APhs obtained with MEKC calibrations.

	CAP	TAP	FF
Linearity range (μg/g)	0.005–1	0.01–1	0.025–5
Slope	2.439 ± 0.003	2.040 ± 0.004	1.690 ± 0.002
Intercept	0.001 ± 0.001	0.0004 ± 0.002	0.008 ± 0.003
Standard error	0.002	0.004	0.007
LOD (ng/g)	1.3	2.6	5.7
LOQ (ng/g)	4.5	8.8	18.9
CCα (ng/g)	3.7	4.6	15.9
CCβ (ng/g)	5.5	7.5	21.1

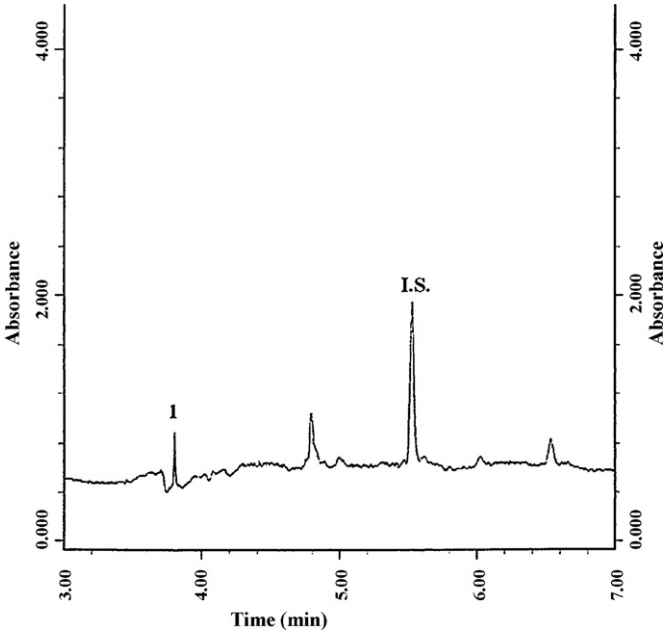


Fig. 6. Electropherogram of the muscle extract of turkey, which was treated in medicated feed with FF (1) at daily dose of 20 mg/kg b.w., for five consecutives days. Concentration for FF-38 ng/g, I.S.-500 ng/g of sample.

The typical electropherograms of the extracts of blank poultry tissue samples subject to extraction, and extracts of poultry tissue samples spiked with drugs are illustrated in Fig. 5A and B. No signal at the same migration time as that of the selected drugs indicates that there were no matrix interferences.

In the course of the validation process the freeze–thaw stability of the analytes in animal tissue was tested by analysing three replicates of each of the three QC levels after three cycles of freezing (−20 °C) and thawing (at room temperature) over two months. It was found, that the recovery values after one week, one month and two months were 98.3%, 96.9%, and 95.4% for SAs, and 100.2%, 97.3%, and 96.8% for APhs, respectively. The test of the freeze–thaw stability of the selected drugs confirmed that no significant degradation of compounds occurred in the poultry tissue samples.

3.5. Real samples

The effectiveness of MEKC method was checked by analyzing tissue samples from the turkeys and hens, which were medicated with TAP at the dose of 50 mg/kg, or with FF at the dose of 20 mg/kg of body weight per day. Both drugs were administered for five consecutive days. In the small-animal study we used 8 turkeys and 8 hens to experiment. Electropherogram of the muscle extract of turkey, which was treated in medicated feed with FF at daily dose of 20 mg/kg b.w., for five consecutives days is given in Fig. 6.

Table 3
Assay validation results obtained from intra-day and inter-day experiments analysed for SAs.

Nominal concentration (μg/g)	Intra-day			Inter-day			Mean recovery ($n=6$) (%)	Overall RSD (%)
	Concentration found ($n=6$) (μg/g)	Precision RSD (%)	Accuracy (%)	Concentration found ($n=6$) (μg/g)	Precision RSD (%)	Accuracy (%)		
SMZ								
0.05	0.0471	8.8	94.1	0.0453	9.7	90.6	89.4 ± 1.6	1.8
0.1	0.0971	7.3	104.0	0.0938	9.1	93.8		
0.2	0.2050	5.5	99.0	0.2060	7.2	103.0		
SMR								
0.05	0.0508	9.5	101.6	0.0523	10.4	104.6	89.4 ± 1.6	1.8
0.1	0.0983	6.8	98.2	0.0928	9.2	92.8		
0.2	0.2048	5.7	102.4	0.2084	7.5	104.2		
STZ								
0.05	0.0492	9.0	98.4	0.0474	9.8	94.8	82.5 ± 2.7	3.3
0.1	0.0972	6.8	97.2	0.0895	8.4	89.5		
0.2	0.2002	5.3	100.1	0.1948	5.9	97.4		
SMX								
0.05	0.0502	7.5	93.8	0.0468	9.2	93.6	92.4 ± 1.1	1.2
0.1	0.0990	5.4	91.4	0.0898	8.6	89.8		
0.2	0.1957	4.7	91.3	0.1824	5.2	91.2		
SCP								
0.05	0.0542	8.2	108.4	0.0547	8.9	109.4	86.3 ± 1.9	2.2
0.1	0.0989	7.5	98.9	0.0938	9.1	93.8		
0.2	0.1931	4.8	96.5	0.1935	5.7	96.8		
SC								
0.05	0.0483	7.7	96.6	0.0465	9.0	93.0	77.2 ± 0.9	1.2
0.1	0.0980	6.3	98.0	0.0875	9.5	87.5		
0.2	0.2025	5.6	101.3	0.1908	7.1	95.4		
SG								
0.05	0.0537	9.0	107.5	0.0538	10.2	107.6	80.2 ± 1.5	1.9
0.1	0.0942	8.6	94.2	0.0865	11.2	86.5		
0.2	0.2054	6.6	102.7	0.2074	6.8	103.7		

The mean concentrations of the drugs in the tissue (muscle, liver, and skin with fat) following administration in medicated feed are illustrated in Fig. 7 and summarised in Table 5. The present study demonstrated that both TAP and FF tend to accumulate in poultry edible tissue after administration in medicated feed. This may be attributed to the ability of the APh drugs to bind with the tissue proteins, as well as to re-absorption of the free fraction from the renal tubules. In present study, the analytes continued to be detectable in all tested tissues on the 10 and 18 day after stopping drug administration. The content of TAP in poultry samples recorded on the 10 and 18 day was above the MRL value in all tissues. The same was true for FF in muscle and skin with fat on the 10 day. It was only on 28 day after last drug administration that their concentration in the liver was under the MRL and no drug residues were found in the muscle and skin with fat samples. Administration of FF and TAP resulted in higher concentration of drug residues in the tissue than the values recorded in other studies. The literature lacks specific reports on the APh residues in the edible tissues of animals treated with the drug and slaughtered at different times up to 28 days after cessation of administration. Unfortunately, the residue depletion studies for turkeys and hens stopped at 7 or 8 days after cessation of treatment [18,19]. Villa and Brightwell [20] found that

Table 4
Assay validation results obtained from intra-day and inter-day experiments for APhs.

Nominal concentration (μg/g)	Intra-day			Inter-day			Mean recovery ($n=6$) (%)	Overall RSD (%)
	Concentration found ($n=6$) (μg/g)	Precision RSD (%)	Accuracy (%)	Concentration found ($n=6$) (μg/g)	Precision RSD (%)	Accuracy (%)		
CAP								
0.01	0.0094	8.2	94.0	0.0104	14.9	104.0	86.3 ± 6.4	7.4
0.05	0.0501	6.7	100.2	0.0490	6.8	98.0		
0.25	0.2490	4.8	99.6	0.2482	4.3	99.3		
TAP								
0.025	0.0229	8.0	91.7	0.0246	12.2	98.4	81.6 ± 5.2	6.3
0.05	0.0514	5.5	102.7	0.0503	7.7	100.6		
0.1	0.0103	3.1	103.0	0.0995	4.3	99.5		
FF								
0.05	0.0461	8.4	92.2	0.0498	10.6	99.7	85.6 ± 4.2	4.9
0.1	0.1019	7.5	101.9	0.1033	6.2	103.3		
0.2	0.2071	5.1	103.5	0.2014	3.7	100.7		

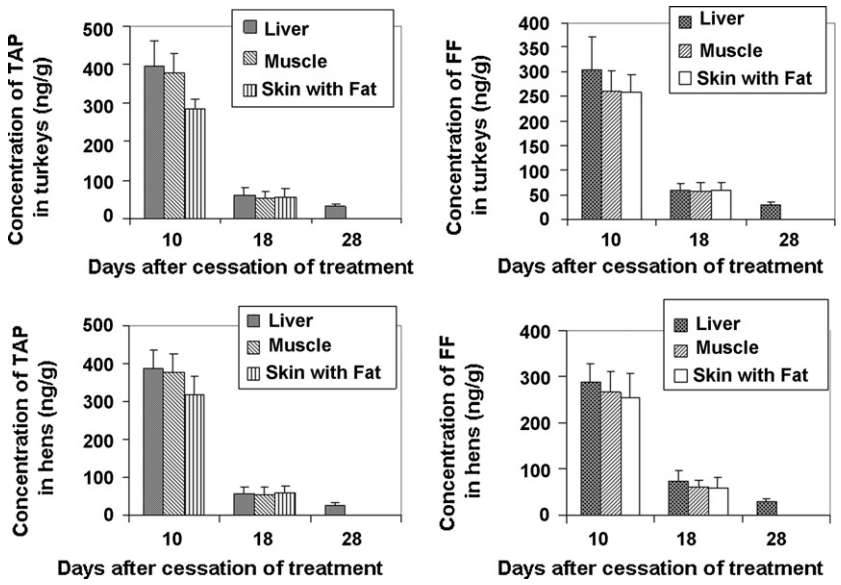


Fig. 7. Concentrations (mean ± SD; $n = 6$) of FF and TAP in tissues of turkeys and hens after administration in medicated feed at daily dose of 50 mg/kg b.w. for TAP and 20 mg/kg b.w. for FF, for five consecutives days.

Table 5
Range and mean (±SD) values of poultry tissue concentration of TAP and FF determined after administration in medicated feed at daily dose of 50 mg/kg b.w. for TAP and 20 mg/kg b.w. for FF, for five consecutives days.

Drugs	Animals	Tissues	Drug concentration (ng/g)					
			10 days		18 days		28 days	
			After the cessation of medication period ($n = 6$)					
			Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD
TAP	Turkeys	Liver	304–495	396 ± 66	36–93	62 ± 20	28–36	31 ± 3
		Muscle	312–449	378 ± 53	31–80	53 ± 17	ND	
		Skin with fat	267–303	285 ± 25	38–72	55 ± 24	ND	
	Hens	Liver	310–464	386 ± 49	38–87	58 ± 17	19–38	25 ± 8
		Muscle	302–446	376 ± 51	32–87	55 ± 19	ND	
		Skin with fat	283–353	318 ± 49	46–71	59 ± 18	ND	
FF	Turkeys	Liver	210–405	305 ± 65	51–88	73 ± 14	25–39	30 ± 6
		Muscle	199–321	261 ± 41	41–86	67 ± 19	ND	
		Skin with fat	232–285	259 ± 37	82–103	63 ± 15	ND	ND
	Hens	Liver	216–340	288 ± 40	40–106	72 ± 24	22–38	29 ± 5
		Muscle	195–303	265 ± 45	45–79	60 ± 16	ND	
		Skin with fat	216–292	254 ± 54	72–103	58 ± 22	ND	ND

ND, not detected.

when TAP was administered to pigs at the dose of 30 mg/kg, low levels of TAP were detected (27 ng/g in the liver and 94 ng/g in the skin). In comparisons to the studies where APhs were administered to other species, the present study indicates that depletion in poultry is slower than in fish [21,22], and quicker in turkeys and hens than in sheep [23].

As part of the study, the presented MEKC method was successfully applied in determination of the selected SAs and APhs residues in poultry tissue samples (muscle, liver, and skin with fat) ($n = 30$) purchased from different local markets. The extraction procedure was applied to all matrices. None of the commercial samples analysed yielded a positive result for the analysed compounds.

4. Conclusion

In this study, a rapid clean-up procedure and a reliable MEKC method for simultaneous determination of seven SA and three APh antibiotics in poultry tissue has been developed, optimized and validated. By applying the SPE during the sample treatment procedure and by using low wavelength detection (200 nm) it was possible to obtain satisfactory LOD for the MEKC-UV method. The method has significant advantages such as: short analysis time, low consumption of organic solvents, very good linearity, satisfactory precision and accuracy. Moreover, MEKC method gives quantitative results for the assayed chemotherapeutics providing selective determination of the analytes without interferences of other compounds during a single run. The method can be successfully adopted for routine screening of foodstuffs, which has been confirmed in research on real biological samples. In comparison with the LC–MS methods, the standard CE system equipped with a UV detector produces a slightly higher [7] or similar [1,8,24] values of LOD, but the achieved sensitivity of the method is sufficient to identify residues in edible tissues of animals at levels lower than the established EU MRL values.

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