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Determination of quinolones in chicken tissues by liquid chromatography with ultraviolet absorbance detection

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Abstract

This paper presents an analytical method for the determination of quinolones in chicken tissues. The procedure involves pre-treatment by solid-phase extraction (SPE) and subsequent liquid chromatography (LC) with UV absorbance detection. Different SPE disposable cartridges and extractants of the tissue samples were tested, and various columns were systematically tested. The mobile phase was composed of acetonitrile and citric buffer at pH 4.5, with an initial composition of acetonitrile–water (12:88, v/v) and using linear gradient elution. Recoveries were 66–91% in the concentration range 30–300 $\mu g\,kg^{-1}$. The detector response was linear in this range. The limits of detection were 16–30 $\mu g\,kg^{-1}$. These values were lower than the maximum residue limits established by the European Union.
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Keywords: Chicken tissues; Linear solvation energy relationships; Quinolones

1. Introduction

Quinolones, which act principally by inhibiting DNA-gyrase in bacterial cells, form a group with different chemical structures and spectra of activity [1]. These antimicrobial agents have demonstrated broad-spectrum activity against many pathogenic Gram-negative and Gram-positive bacteria. A significant increase in the use of quinolones in animal production was noted over the last decade. The use of antibiotics in food-producing animals has generated considerable interest and concern due to the growing problem of microbial resistance. In relation to this use, the European Union has established maximum residue limits (MRL) for quinolone residues in animal tissues [2]. Thus, the establishment of sensitive multi-residues screening methods is required in order to control these drugs. The MRL, for the quinolones studied in chicken muscle are given in Table 1.

Many papers have been published about the analysis of quinolone residues in animal products [3]. Liquid chromatography (LC) is generally used for separation [3,4], but gas chromatography (GC) [5,6], high-performance thin layer chromatography (HPTLC) [7,8], and capillary electrophoresis (CE) [9–12] have also been used. Fluorescence detection is extensively applied in LC because of its good selectivity and sensitivity [13,14]. For confirmatory methods mass spectrometry is the preferred technique [15,16]. However, UV absorbance detection is widely used in LC because it has a good combination of sensitivity and versatility. For example, five quinolones were determined in bovine kidney, muscle and eggs using a C_{18} Hypersil column [17]. Pecorelli et al. [18] proposed a multi-residue method for 13 quinolones in feeds using photodiode-array and fluorescence detection. The separation on a C_5 LUNA column took in less than 27 min.

We have published several papers on the behaviour of several quinolones as a function of the composition and pH of the mobile phase [19,20]. Here, we report a LC–UV separation on a C_8 Zorbax column for the simultaneous determination of the quinolones regulated in chicken muscle by European Union and sarafloxacin, which has not yet a MRL in chicken muscle but it is the main metabolite of difloxacin. Norfloxacin was the internal standard because is a quinolone forbidden in veterinary medicine. This column permits the separation of these eight quinolones with good resolution

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Table 1
MRL of the quinolones regulated for chicken muscle

	MRL ($\mu g\,kg^{-1}$)	Oasis HLB		Oasis MAX		SDB-RPS		Direct extraction	
		LOD ($\mu g\,kg^{-1}$)	R (%)	LOD ($\mu g\,kg^{-1}$)	R (%)	LOD ($\mu g\,kg^{-1}$)	R (%)	LOD ($\mu g\,kg^{-1}$)	R (%)
Ciprofloxacin	–[a]	5	87	10	23	5	70	30	66
Danofloxacin	200	10	61	10	68	10	66	20	69
Enrofloxacin	100[a]	5	84	5	84	5	91	25	80
Sarafloxacin	–	5	80	5	78	5	87	15	60
Difloxacin	300	10	81	10	79	10	87	10	66
Oxolinic acid	100	10	80	10	87	5	86	30	50
Flumequine	400	10	85	5	96	10	83	30	42

Limits of detection (LOD) and recoveries (R) obtained for the three types of SPE cartridges and the direct extraction method. Recoveries determined with spiked chicken muscle at $240\,\mu g\,kg^{-1}$.

[a] $100\,\mu g\,kg^{-1}$ is the MRL corresponding to the sum of enrofloxacin and ciprofloxacin.

in less time. A systematic study on the optimisation of the mobile phase, and peak resolution was optimised by using the linear solvation energy relationship (LSER) formalism [21]. Secondly, different SPE cartridges were tested and the results compared with a direct extraction method. Finally, the analytical performance of the optimised method was assessed.

2. Experimental

2.1. Reagents

The quinolones were obtained from different pharmaceutical firms: norfloxacin (Liade, Boral Química), ciprofloxacin (Lasa), sarafloxacin and difloxacin (Abbott), enrofloxacin (Cenavisa), danofloxacin (Pfizer), oxolinic acid and flumequine (Sigma).

All reagents were of analytical grade. Merck supplied phosphoric acid, sodium hydroxide, dichloromethane, trifluoroacetic acid, potassium hydrogenphosphate, potassium bromide, ammonium acetate, oxalic acid, acetonitrile and methanol; citric acid was supplied by Flucka and acetic acid was obtained from Carlo-Erba. Triethylamine was obtained from Baker. The SPE cartridges were HLB ($3\,cm^3$/60 mg) and MAX ($3\,cm^3$/60 mg) obtained from Oasis, and SDB-RPS ($1\,cm^3$/4 mg) from 3 M Empore (Supelco).

2.2. Preparation of standard and stock solutions

Individual standard solutions of ciprofloxacin, danofloxacin, enrofloxacin, sarafloxacin and difloxacin were prepared in a concentration of $100\,\mu g\,ml^{-1}$, by dissolving the quantity of each compound exactly weighted in 50 mM acetic acid aqueous solution. Standard solutions of oxolinic acid and flumequine were prepared by dissolving the proper quantity of each compound in acetonitrile at concentration of $100\,\mu g\,ml^{-1}$. Norfloxacin, used as internal standard, was prepared by dissolving it in a 50 mM acetic acid–acetonitrile mixture (80:20, v/v) at a concentration of $100\,\mu g\,ml^{-1}$. The stock solution, used to spike the chicken samples, was prepared by mixing the individual standard solutions and diluting it to a concentration of 10 and $5\,\mu g\,ml^{-1}$ with acetonitrile. The quinolone mixture was prepared by diluting a suitable standard with acetonitrile–water (12:80, v/v). The samples were filtered through a 0.45 μm nylon filter membrane (MSI) before injection.

2.3. Instrumentation

The LC equipment consisted of an HP 1100 series with an injection valve with a 20 μl sample loop. Detection was performed by a diode array detector (DAD), using a detection wavelength of 250 nm for oxolinic acid and flumequine and a wavelength of 280 nm for the other quinolones. The LC columns were a 4.6 mm i.d. × 250 mm length Kromasil C_8 column (Aplicaciones Analíticas), a 4.6 mm i.d. × 250 mm Inertsil C_8 (Alltech, Deerfield, IL, USA), a 4.6 mm i.d. × 150 mm Zorbax Eclipse XDB-C_8 (Agilent Technologies), a 4.6 mm i.d. × 250 mm Lichrospher C_{18} (Merck) and a 4.6 mm i.d. × 250 mm Nucleosil C_{18}.

The pH of the mobile phase was measured with a CRISON 2002 potentiometer (±0.1 mV) using an Orion 8102 ROSS combination pH electrode. A centrifuge Macrotronic SELECTA was used in order to perform the extractions. SPE was carried out on a Supelco vacuum manifold for 12 columns connected to a SUPELCO vacuum tank.

2.4. Procedure

2.4.1. Mobile phase

The solution used for the optimisation of the mobile phase composition was a $0.010\,mol\,l^{-1}$ citrate solution adjusted at pH 4.5 with ammonia, with up to 40% (v/v) of acetonitrile percentages. The flow rate was $1.5\,ml\,min^{-1}$. The hold-up time was measured by the injection of $0.05\,mol\,l^{-1}$ potassium bromide.

2.4.2. SPE

Five grams of chicken muscle tissue were placed in 50 ml centrifuge tube. Samples were spiked by adding the appro-

priate stock solutions. Then water was added to obtain a final spiking volume of 1 ml, and the samples were allowed to stand for 20 min in the dark before extraction.

Two volumes of dichloromethane (10 and 20 ml) were added to the sample in order to extract the quinolones. After shaking for 5 min, the mixture was centrifuged at 366.5 rad·s^{-1} (5 min). The organic phases were transferred into a 40 ml glass tube and were extracted with two portions of 5 ml of 1 M NaOH, and centrifuged at 209.4 rad·s^{-1} (5 min). The aqueous extracts were adjusted to pH 3 with phosphoric acid. The SDB-RPS cartridges were activated with 2 ml of methanol and 2 ml of water. After the samples were passed, the cartridges were cleaned with 2 ml of water. The quinolones were eluted with 2 ml of 2% trifluoroacetic acid in water and acetonitrile (25:75), followed by 1 ml of acetonitrile. The Oasis HLB cartridge were activated with 2 ml of methanol, 2 ml of water and 2 ml 50 mM KH_2PO_4 to pH 3. The cartridges were cleaned with 2 ml of water and the analytes were eluted with 4 ml of 2% trifluoroacetic acid in water and acetonitrile (25:75), followed by 1 ml of acetonitrile. The activation of Oasis MAX was made with 2 ml of methanol and 2 ml of water. The cartridges were cleaned with 2 ml of water and quinolones were eluted with 2 ml of 2% trifluoroacetic acid in water and acetonitrile (25:75), followed by 1 ml of acetonitrile. In all cases, the samples were evaporated to dryness at 50 °C under a stream of nitrogen. Two hundred microlitres of mobile phase was added in order to resuspend the residue. The resulting solution was injected into the LC system.

2.4.3. Calibration and validation

Validation was performed according to the FDA guideline for bioanalytical assay validation [22]. For the calibration, spiked standard samples at six concentration levels (30–300 μg kg^{-1}) were extracted following the extraction procedure method previously explained (each level was prepared by duplicate, and each calibration sample was analysed twice). Calibration curves were constructed using analyte/internal standard peak area ratio versus concentration of analyte. The LODs have been considered as the signal that represents the blank signal plus 3 times the blank standard deviation and in the LOQs we considered instead of 3, 10 times the blank standard deviation. The methodology applied to calculate the LODs and LOQs are based on the calibration curve.

To assess accuracy and precision of the assay, three spiked samples at three concentration levels each (50, 100 and 200 μg kg^{-1}) were extracted and analysed by duplicate. The procedure was repeated on three consecutive days to determine inter-day variability. Each day, separately weighted stock solutions of the analytes were prepared.

Recovery experiments were performed by comparing the analytical results for extracted standard samples of chicken muscle at the concentration of 30, 50, 75, 150 and 300 μg kg^{-1}, with unextracted standards prepared at the same concentrations in blank extract representing 100% recovery. The recoveries were obtained from the slope of the plot of amount of quinolone spiked versus the quinolone recovered.

3. Results and discussion

Usually, the LC separation of quinolones is performed with reversed-phase C_{18} or C_8 silica-based columns and water–acetonitrile mixtures as mobile phases. In preceding papers, other separation methods have been developed. A separation of a mixture of standards of four and six quinolones, respectively, was developed on a Lichrospher C_{18} and the mobile phase was a 0.025 mol l^{-1} phosphoric acid solution in MeCN–H_2O (7:93, v/v) [23,24]. Several quinolones were separated with a Kromasil C_8 column, and the mobile phase was a 0.020 mol l^{-1} ammonium acetate solution [20]. Using a Lichrospher C_{18} column, a peak broadening was observed that made difficult the separation of the series of eight quinolones studied. Using C_8 Kromasil, a coeluation of sarafloxacin and difloxacin was presented. For this reason, the development of a new separation method of these eight quinolones was necessary. Considering these papers and other works found in bibliography [25,26], we have tested the chromatographic behaviour of the seven quinolones regulated in chicken tissues and sarafloxacin with different stationary phases based on C_8 (Zorbax, Inertsil and Kromasil) and other columns based in C_{18} (Lichrospher, Nucleosil) in order to improve the separation, to obtain shorter retention times with a good resolution between peaks.

The broadening of peaks was observed using columns based on C_{18} while with C_8 columns better peaks were obtained. With Inertsil and Zorbax C_8 columns, a baseline separation of sarafloxacin and enrofloxacin was also achieved.

Different electrolytes were tested as aqueous fraction of the mobile phase: ammonium acetate, oxalic acid, phosphoric acid, trifluoroacetic acid, triethylamine, acetic acid and citric acid. The C_8 Zorbax column and the citric acid solution were chosen because shorter retention times were obtained with narrow and good resolved peaks.

To optimise the separation of the quinolones of interest in this new column, the linear solvation energy relationship methodology has been applied in the same way that has been described in other manuscripts written by our research [20]. Resolution between two adjacent peaks was calculated at 10 acetonitrile percentages (from 10 to 40) to predict the optimum percentage of organic phase. In Fig. 1, solid lines indicate theoretical resolution and symbols indicate experimental values of resolution, obtaining a good relationship between them.

In relation with the pH optimisation of mobile phase, the retention factors for the quinolones at different pH (from 3 to 5) were determined from three different injections at every pH considered, and the resolution between adjacent peaks was calculated. Fig. 2 shows the experimental (symbols) and theoretical values (solid lines) of resolution (R_s) for the

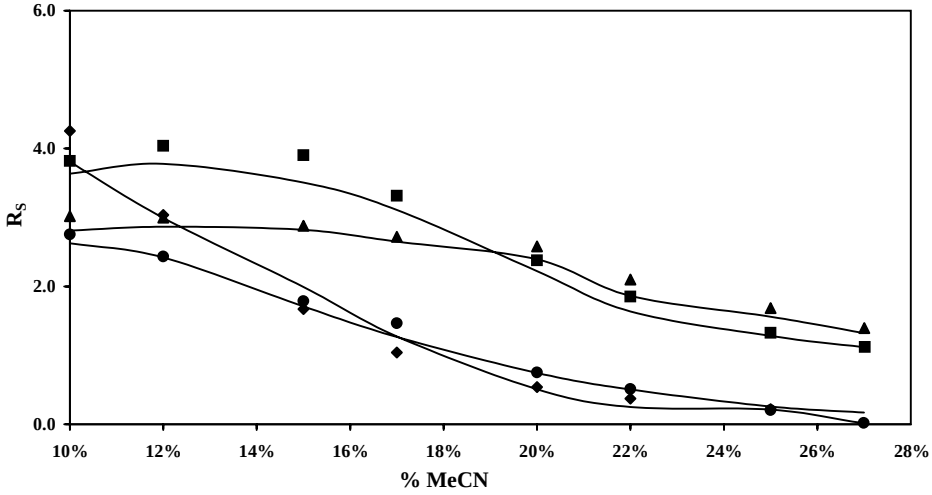

Fig. 1. Plot of the resolution (R_s) between adjacent peaks at different percentages of acetonitrile: (●) norfloxacin/ciprofloxacin; (◆) ciprofloxacin/danofloxacin; (■) danofloxacin/enrofloxacin; (▲) sarafloxacin/difloxacin.

solute pairs studied, with the pH of the mobile phase at 12% of acetonitrile, calculated considering the theoretical retention factors obtained from Eq. (1):

$$k = x_{HB^+} k_{HB^+} + x_B k_B \quad (1)$$

Figs. 1 and 2 indicate that good separation can be obtained at 12% of acetonitrile and a pH of 4.5, but the separation of the eight compounds studied need more than 60 min since flumequine is highly retained. Thus, was necessary a gradient elution. The optimised stepwise linear gradient elution used is following: from 0 to 11 min, the initial mobile phase contains a 12% acetonitrile; from 11 to 15 min, the percentage of acetonitrile linearly increases to 30%; from 15 to 22 min, this percentage is maintained; from 22 to 27 min, the acetonitrile decreases to 12%. The chromatogram obtained with the stepwise linear gradient optimised is shown in Fig. 3. The separation of these eight quinolones was achieved in less than 25 min.

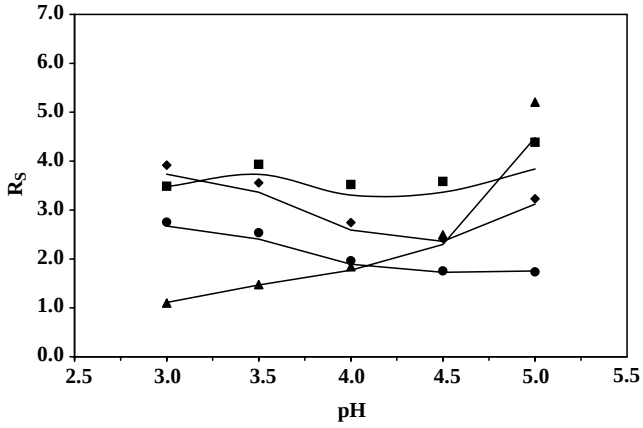

Fig. 2. Plot of the resolution (R_s) between adjacent peaks at different pH studied: (●) norfloxacin/ciprofloxacin; (◆) ciprofloxacin/danofloxacin; (■) danofloxacin/enrofloxacin; (▲) sarafloxacin/difloxacin.

The method previously optimised [9,12] has been proved that is valid to extract the seven quinolones regulated by European Community in chicken muscle. In a previous work developed by capillary electrophoresis [27], a comparative study of different silica and polymeric based sorbents has been done to achieve maximum recoveries and optimal clean-up efficiency of a series of quinolones. In this preliminary study with standards, better results were obtained with polymeric and anionic exchanged cartridges. For this reason Oasis HLB, Oasis MAX and SDB-RPS sorbents have been chosen for the study of quinolones regulated in chicken sample by LC. Oasis HLB cartridges are formed by a polymeric macroporous poly(divinylbenzene-co-*N*-vinylpyrrolidone), Oasis MAX cartridges, formed by a mixed-mode polymeric sorbent with strong anion-exchange quaternary amine groups on the surface of the copolymer poly (divinylbenzene-co-*N*-vinylpyrrolidone), and SDB-RPS Empore cartridges are formed by a poly(styrenedivinylbenzene) copolymer sorbent that displays lightly cation-exchange interactions due to sulphonic groups.

In order to evaluate the cartridges that supplied best recoveries, the peak areas obtained with a spiked tissue sample subjected to the whole process of extraction and a chicken sample with quinolones added just before the injection, which supposes the 100% of recovery, were compared. Table 2 shows the recoveries obtained for all quinolones in spiked muscle samples at a concentration level of 240 $\mu g\,kg^{-1}$. These values were high for all quinolones except for ciprofloxacin when Oasis MAX cartridges were used, to which a recovery to less than 25% was obtained.

Calibration curves of each quinolone were obtained for each cartridge in the concentration range 25–500 $\mu g\,kg^{-1}$ and the limits of detection obtained are shown in Table 1. The values are similar for all the quinolones and between the three types of cartridges, and are in a concentration below

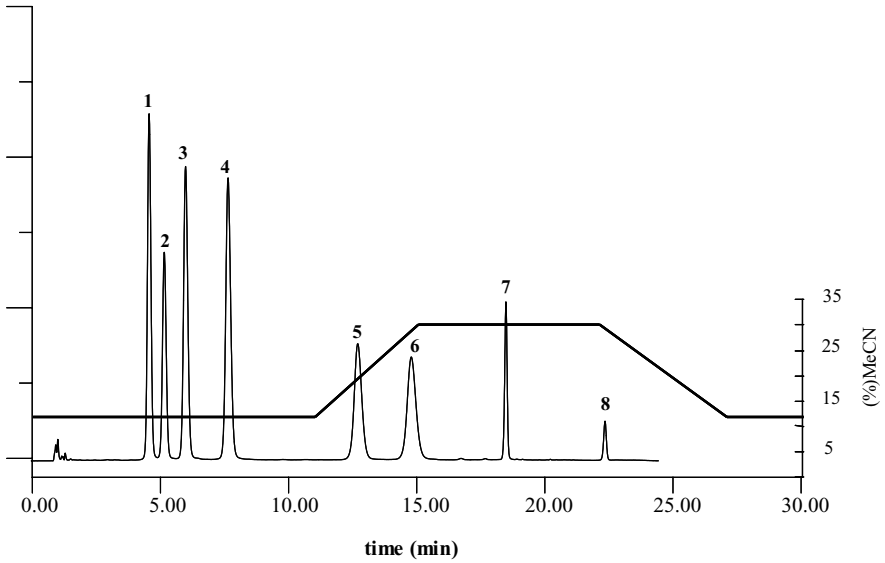

Fig. 3. Chromatograms of a standard solution of quinolones injected in the optimised mobile phase, 0.010 mol l^{-1} citrate buffer at pH 4.5 in 12% MeCN, with the gradient optimised: (1) norfloxacin (IS); (2) ciprofloxacin; (3) danofloxacin; (4) enrofloxacin; (5) sarafloxacin; (6) difloxacin; (7) oxolinic acid; (8) flumequine.

Table 2
Quality parameters for quinolones studied using SDB-RPS cartridges

Parameter	CIP	DAN	ENR	SAR	DIF	OXO	FLU
Calibration curves ($n = 24$)							
Intercept	7.80×10^{-3}	2.20×10^{-2}	2.26×10^{-2}	5.92×10^{-3}	7.82×10^{-3}	2.20×10^{-2}	4.10×10^{-2}
Slope	1.20×10^{-3}	1.19×10^{-3}	2.03×10^{-3}	1.16×10^{-3}	1.36×10^{-3}	1.96×10^{-3}	1.43×10^{-3}
Correlation coefficient	0.9993	0.9988	0.9996	0.9988	0.9990	0.9996	0.9994
Recovery (%)	65	69	89	90	116	119	100

the MRL established by European Community. According to these results, the three cartridges could be used to extract quinolones from chicken tissues. Fig. 4 shows the chromatograms of spiked chicken samples at a concentration of 200 $\mu g\,kg^{-1}$ passed through the different SPE cartridges. In the chromatogram obtained using a HLB cartridge, was observed an impurity coeluating with danofloxacin that impede the determination of its area. Because of this fact and the low recovery obtained for ciprofloxacin using MAX cartridges, the SDB-RPS cartridges have been chosen.

A direct extraction method of the chicken muscle with different acids solutions [3,17,28] has been tested in order to develop a method faster than the sample treatment established. Thus, formic, phosphoric, acetic and trichloroacetic acids, at a concentration of 1 M with different percentage of acetonitrile were tested. The best results were obtained with a formic acid solution. Also, a calibration curve was established. The values of recoveries and LOD are summarised in Table 1. As expected, although this direct extraction method was shorter than the SPE method, recoveries obtained are lower and the LOD values were higher compared with those obtained by SPE method.

The LC with UV detection method developed, using SDB-RPS cartridges, was validated for the seven quinolones regulated in chicken muscle, using norfloxacin as the internal standard. The calibration curves with the correlation coefficients and the recovery obtained are shown in Table 2. Values of the recoveries of quinolones differ of the values shown in Table 1 because of the different way to obtain them. The values presented in this table are based on the slope of the calibration curve, following the FDA guideline.

The results of accuracy and precision are summarised in Table 3. The accuracy ranged between 85 and 115% for the most quinolones indicating that the assay fulfilled the requirements of FDA. In terms of precision, the values obtained were lower than the 15% considered by the FDA in the analysis of biological samples.

Considering that the MRL established for quinolones in muscle chicken tissue are comprised between 100 $\mu g\,kg^{-1}$ for enrofloxacin plus ciprofloxacin and 400 $\mu g\,kg^{-1}$ for flumequine, the proposed method is enough sensible for the analysis of these quinolones in chicken tissues, because the values of LOD and LOQ obtained were below the MRL established for these drugs in the Council Regulation 2377/90 of European Union.

Table 3
Accuracy and precision data of quinolones using SDB-RPS cartridges

Parameter	CIP	DAN	ENR	SAR	DIF	OXO	FLU
Accuracy (arithmetic mean value, %)							
Intra-day ($n = 9$)	58–62	72–78	98–105	97–102	98–100	102–110	102–105
Inter-day ($n = 27$)	61	74	101	99	99	107	103
Precision (relative standard deviation, %)							
Intra-day ($n = 9$)	4–5	6–8	5–9	4–5	8–10	12–15	4–5
Inter-day ($n = 27$)	5	8	9	5	9	13	5

Averages of results obtained at 50, 100 and 200 $\mu g\,kg^{-1}$; measurements on days 1–3 ($n = 9$ for each day).

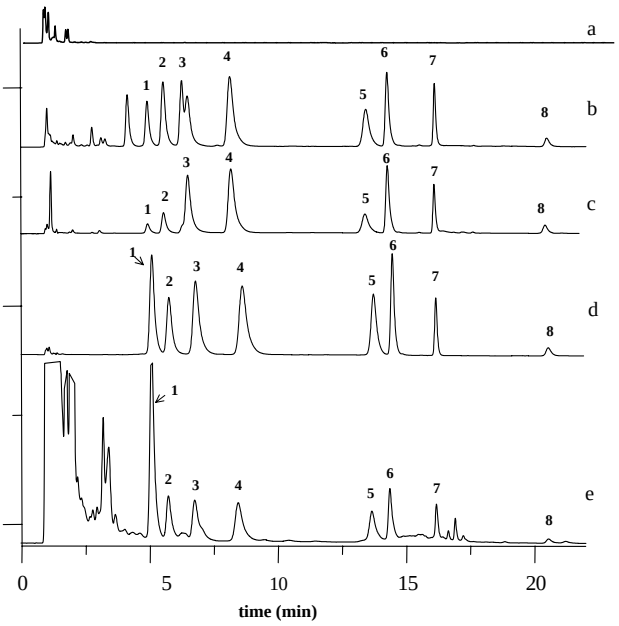

Fig. 4. Chromatogram of a blank chicken sample extracted using SDB-RPS cartridges (a), and chromatograms of extracted chicken samples spiked at 200 $\mu g\,kg^{-1}$ with the three types of cartridges (Oasis HLB (b), Oasis MAX (c), SDB-RPS (d)), and direct method of extraction (e). In chromatograms (d) and (e), the standard internal, corresponding to peak 1, was added at a concentration of 1000 $\mu g\,kg^{-1}$. (1) Norfloxacin (IS); (2) ciprofloxacin; (3) danofloxacin; (4) enrofloxacin; (5) sarafloxacin; (6) difloxacin; (7) oxolinic acid; (8) flumequine.

4. Conclusions

The determination and quantification of a series of quinolones by LC with UV detection in chicken tissues was successfully performed on a C_8 Zorbax column, with a linear gradient composed of acetonitrile and citrate buffer (pH 4.5). SDB-RPS polymeric cartridges were found best for sample handling. The analytical performance of the final procedure was validated by using the high quality FDA guidelines. The detection and quantification limits were found to be low enough to determine quinolone residues in animal tissues below the permissible MRLs established by the European Community.

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