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Quantitation of nine quinolones in chicken tissues by high-performance liquid chromatography with fluorescence detection

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Abstract

A simple reversed-phase high-performance liquid chromatographic method was developed and validated for simultaneous analysis of nine quinolones (ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, flumequine, marbofloxacin, nalidixic acid, oxolinic acid, sarafloxacin) in chicken tissue. The analytes were extracted from homogenized muscle using an acetonitrile basic solution. After centrifugation and partial evaporation, direct injection was possible. Three different HPLC conditions were applied to quantify the residual quinolones. Separation was achieved on a PLRP-S column and detection was performed with a monochromator fluorescence detector. The recovery, the limit of detection, the limit of quantification, the accuracy and the precision of the method were evaluated from spiked tissue samples at concentration levels ranging from 15 $\mu g\ kg^{-1}$ to 300 $\mu g\ kg^{-1}$ according to the maximum residue limit of each quinolone. This method is also suitable for porcine, bovine, ovine and fish muscle tissue.

Keywords: Food analysis; Validation; Quinolones; Antibiotics

1. Introduction

Quinolones are often used in livestock and fish farm industries in cases of pulmonary, urinary and digestive infections as they act by inhibiting bacterial DNA-gyrase. There is now a strict legislative framework controlling the use of such substances, with the aim of minimising the risk to human health associated with consumption of their residues. Therefore, to ensure human food safety the European Union (EU) has set tolerance levels for these compounds as maximum residue limits (MRLs) [1]. State laboratories of the EU have to put into practice methods for screening and confirming residues possibly present in the samples coming from slaughterhouses [2].

Recently MRLs have been fixed for several quinolones. The MRL in bovine, poultry and porcine muscle was fixed at 30 $\mu g\ kg^{-1}$ for the sum of enrofloxacin and ciprofloxacin [3]. The MRL in bovine and porcine muscle was fixed at 50 $\mu g\ kg^{-1}$ for difloxacin [4]. The MRL in chicken and turkey muscle was fixed at 300 $\mu g\ kg^{-1}$ for danofloxacin [5]. The MRL in bovine and porcine muscle was fixed at 150 $\mu g\ kg^{-1}$ for marbofloxacin [6]. The MRL was fixed at 50 $\mu g\ kg^{-1}$ for flumequine [7]. The MRL in fish was fixed at 30 $\mu g\ kg^{-1}$ for sarafloxacin [8]. Finally it was decided to add two other quinolones (nalidixic acid, oxolinic acid) with no MRL set but which are still currently used. Since a high-performance thin-layer chromatography (HPTLC) method for screening residual quinolones in muscle has been previously designed in our laboratory [9] it was proposed to develop a high-performance liquid chromatography (HPLC) method

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to confirm positive samples. Many HPLC methods with fluorescence detection have been published for the confirmation of quinolones, either as monoresidue or as multiresidue methods. For bovine, porcine or chicken tissue, quinolones are extracted with a mixture of methylenechloride methanol [10], with ethanol–acetic acid [11], with acetonitrile–ammonia [12], with metaphosphoric acid–acetonitrile [13] or with methanol–perchloric acid–phosphoric acid [14]. For fish tissue, quinolones are extracted with hexane–ethyl acetate [15], with water–acetonitrile [16] or with acetone [17].

However, these methods regard only one subclass of quinolones at a time. Among all the quinolone compounds used in veterinary medicine three different generations of drugs exist which could be classified into two different chemical groups. The first group includes the pyridonecarboxylic acid antibacterials (PCAs): oxonilic acid, nalidixic acid and the flumequine (Fig. 1), the second group includes the other quinolones having a piperazinyl moiety in the C-7 position (Fig. 2).

The aim of this study was to develop a multiclass method which can be transferred in the field laboratories. For that purpose it was decided to use HPLC with a fluorescence detector which is a commonly used apparatus.

2. Experimental

2.1. Reagents

Methanol, acetonitrile, tetrahydrofuran, hydrochloric acid fuming, hexane, sodium hydroxide and tris(hydroxymethyl)aminomethane were obtained from Merck (Darmstadt Germany). Ultrapure water was purified through an Alpha-Q system from Millipore (Molsheim, France). Orthophosphoric acid was obtained from Prolabo (Nogent sur Marne, France). Nitrogen C was purchased from Alphagaz (Air Liquide, St. Quentin en Yveline, France). Ciprofloxacin and enrofloxacin were supplied by Bayer

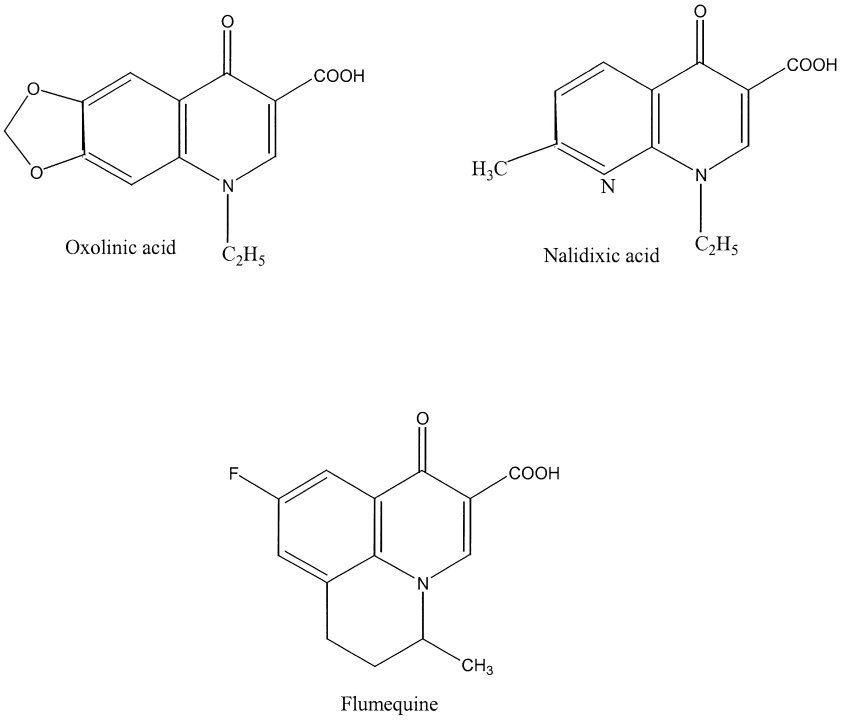

Fig. 1. Chemical structures of PCAs and flumequine.

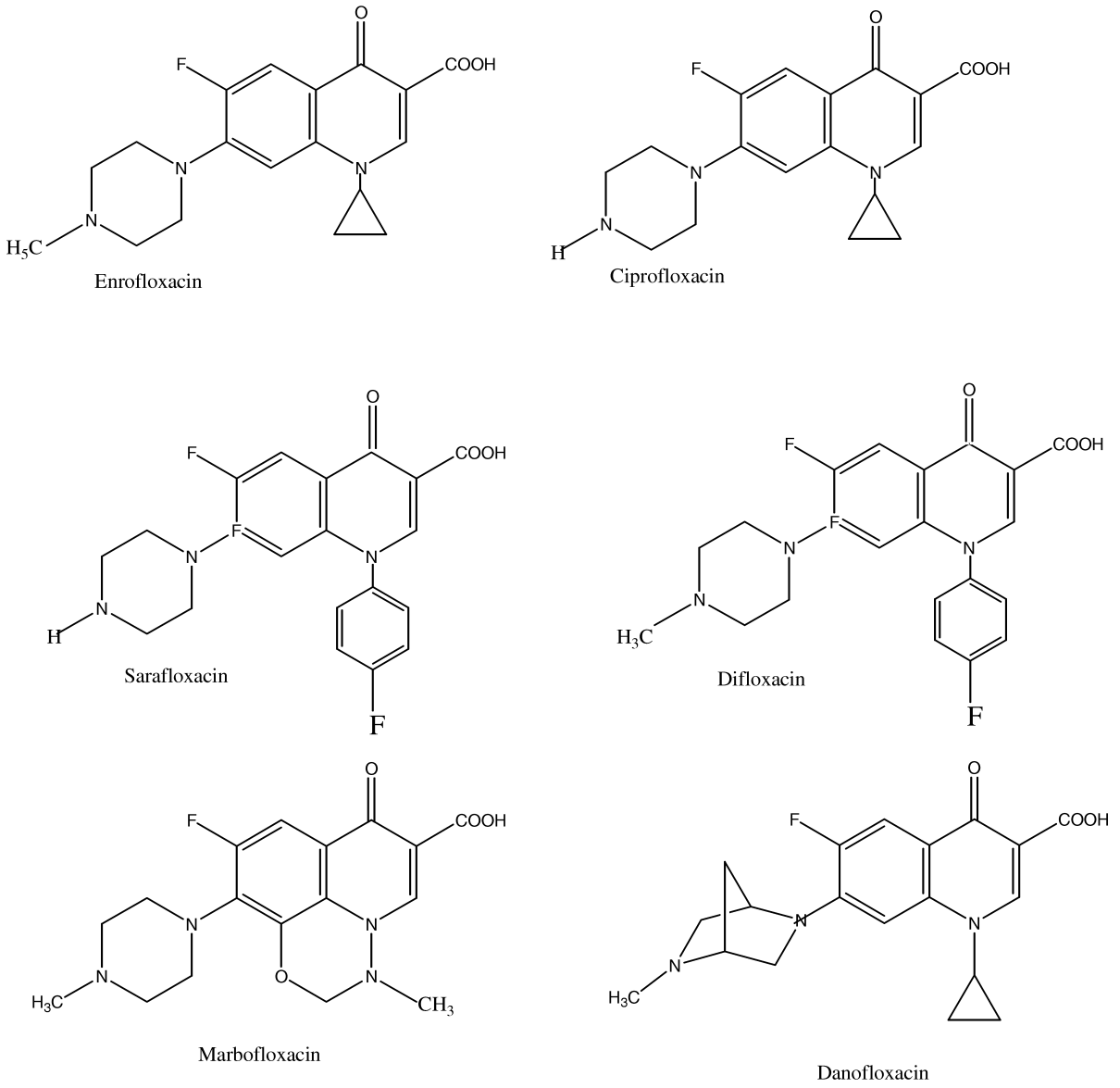

Fig. 2. Chemical structures of quinolones with a piperazinyl moiety.

(Puteaux, France). Danofloxacin was supplied by Pfizer (Amboise, France). Marbofloxacin was supplied by Vétoquinol (Lure, France). Sarafloxacin and difloxacin were supplied by Solvay Duphar (Weesp, The Netherlands). Flumequine was supplied by Sanofi Santé Nutrition Animale (Libourne, France). Oxolinic acid and nalidixic acid were purchased from Sigma (Saint Quentin Fallavier, France).

2.2. Sample preparation equipment

A refrigerated centrifuge type MR 1822 was purchased from Jouan (St. Herblain, France) and a stirrer type Vortex was from Bioblock (Illkirch, France). An ultrasonic probe type sonopuls hd 60 Bandelin was purchased from Labo Moderne (Paris, France).

Table 1
Concentration of the working solutions

Solution	Concentration ($\mu g\ kg^{-1}$)				
	Dilution 1/40	Dilution 1.5/40	Dilution 1/20	Dilution 1.5/20	Dilution 1/10
A					
Ciprofloxacin	37.5	56.25	75	112.5	150
Enrofloxacin	37.5	56.25	75	112.5	150
Sarafloxacin	125	187.5	250	375	500
Difloxacin	125	187.5	250	375	500
B					
Marbofloxacin	375	562.5	750	1125	1500
Danofloxacin	750	1125	1500	2250	3000
C					
Oxololinic acid	125	187.5	250	375	500
Nalidixic acid	125	187.5	250	375	500
Flumequine	125	187.5	250	375	500

2.3. Chromatography equipment

A polymeric column, PLRP-S (150×4.6 mm I.D., 5 μm, 100 Å) packed by Polymer Labs. obtained from Touzart & Matignon (Courtaboeuf, France) and a guard column packed with RP18-E, 4×4 mm I.D. (Merck) were used at flow-rate of 0.8 ml min^{-1} provided by a pump–autosampler device, Model Alliance 2690 from Waters (St. Quentin en Yvelines, France). The column was heated at 50°C using an external oven, Croco-cil, from Cil Cluzeau (Bordeaux, France). A fluorescent detector, Model FP1520 Jasco, obtained from Prolabo (Nogent sur Marne, France) was utilised. Data were acquired with a Millennium 32 computer data system through a Waters sat/in module.

2.4. Standard and buffer solutions

A buffer solution, pH 9.1 was prepared by dissolving 6.05 g tris(hydroxymethyl)aminomethane and 0.475 ml of hydrochloric acid fuming in 500 ml water and completing it to 1 l. This solution was filtered through a 0.45-μm filter from Millipore (St. Quentin en Yvelines, France).

Individual stock solutions at 0.1 g l^{-1} were prepared by dissolving each standard with a solution of 1 ml of 1 *M* sodium hydroxide solution in 1 l of methanol in ambered volumetric flasks. The stock solutions were stable for at least 3 months when stored in a refrigerator. Three quinolone combined stock solutions were prepared by diluting individual stock solutions with the buffer, pH 9.1 in order to obtain multi-component and multi-concentration solutions as follows: solution A: ciprofloxacin and enrofloxacin at 1.5 mg l^{-1}, sarafloxacin and difloxacin at 5 mg l^{-1}; solution B: marbofloxacin at 15 mg l^{-1} and danofloxacin at 30 mg l^{-1}; solution C: nalidixic acid, oxolinic acid and flumequine at 5 mg l^{-1}. Working solutions were prepared by diluting with the buffer solution, pH 9.1 each of the three

Table 2
Composition of the mobile phase

Quinolones analysed	Flow (ml min^{-1})	0.02 *M* H_3PO_4 (%)	Acetonitrile (%)	Tetrahydrofuran (%)
Ciprofloxacin, enrofloxacin, sarafloxacin, difloxacin	0.8	85	15	0
Marbofloxacin, difloxacin	0.8	92.5	6	1.5
Oxolinic acid, nalidixic acid, flumequine	0.8	72	16	12

Table 3
Conditions for the fluorescent detection of the nine quinolones

Quinolones analysed	Excitation wavelength (nm)	Emission wavelength (nm)
Ciprofloxacin, enrofloxacin, sarafloxacin, difloxacin	280	450
Marbofloxacin, danofloxacin	294	514
Oxolinic acid, nalidixic acid, flumequine	312	366

solutions A, B, C, concentrations of these solutions are given in Table 1. The 15 working solutions obtained were stable for at least 2 weeks when stored in a refrigerator.

2.5. Spiked control sample

Fortified muscle samples (A, B, C) were prepared by spiking 0.50±0.01 g of minced blank muscle

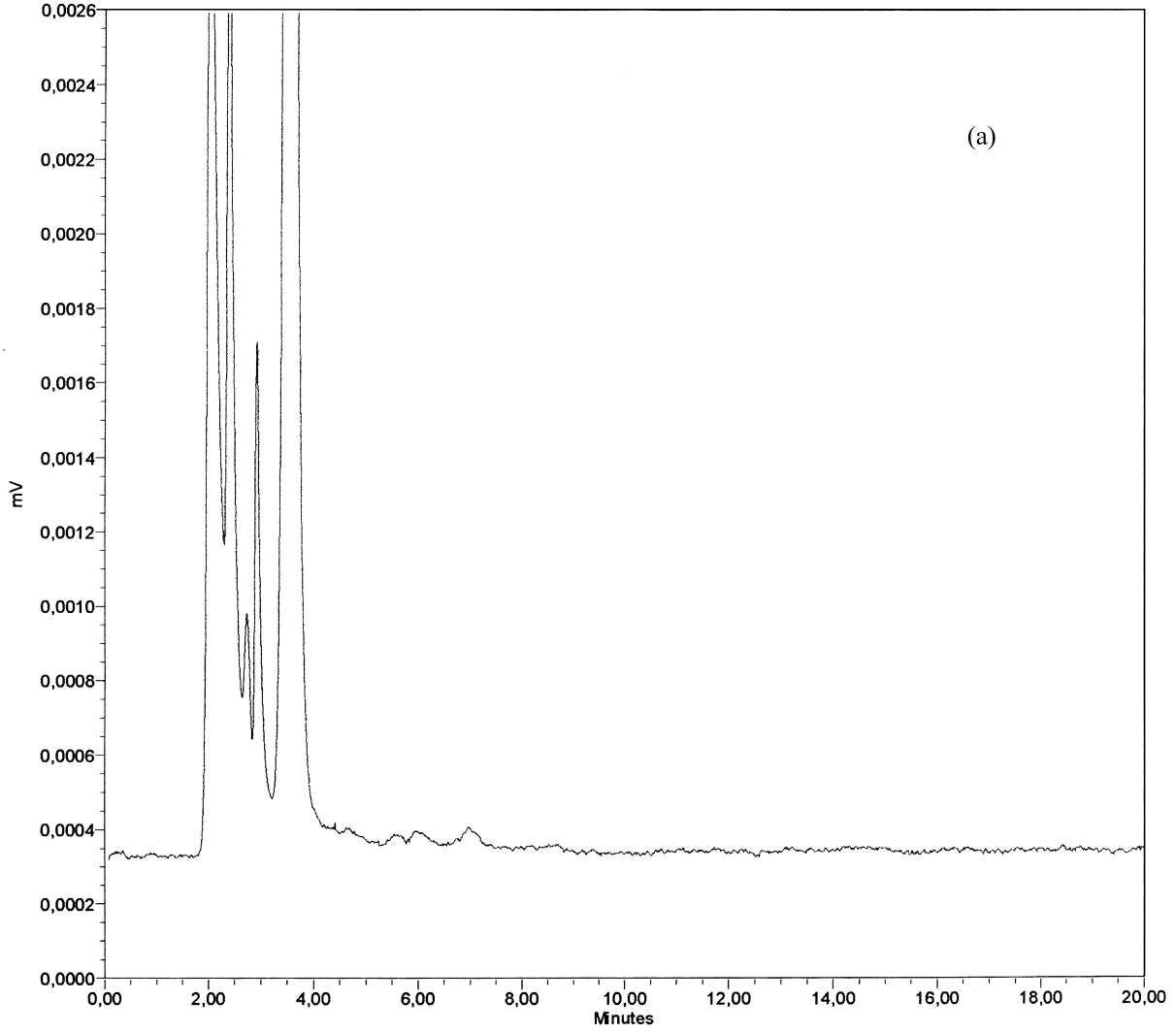

Fig. 3. Chromatograms of the first group of quinolones obtained with (a) blank chicken muscle tissue, (b) extract from chicken muscle tissue fortified with ciprofloxacin and enrofloxacin at 15 μg kg^{-1} and sarafloxacin and difloxacin at 50 μg kg^{-1}, (c) standard solution containing ciprofloxacin and enrofloxacin at 15 μg l^{-1} and sarafloxacin and difloxacin at 50 μg l^{-1}.

tissue in a 2-ml microcentrifuge tube, with 100 μl of the working solutions (A, B, C)/(dilution 1/20). They were frozen until analysis and stored for less than 3 months in the freezer (−20°C).

2.6. Extraction procedure

The sample was thawed and minced, then 0.50 g of muscle was weighed accurately into a 2-ml microcentrifuge tube and 300 μl of buffer solution, pH 9.1 (200 μl in case of spiked control muscle) was added. The sample was stirred for 1 min, let in contact for 15 min, then 200 μl of acetonitrile was added and the tissue was pulverised during 20 s with an ultrasonic probe (power 50% and emission 90%). The probe was rinsed with 800 μl of acetonitrile and the solvent was collected in the microcentrifuge tube. The probe was cleaned by immersing it in a beaker containing methanol and by switching on the power for a few seconds. This operation was repeated with a beaker containing water. The homogenate was stirred by a vortex mixer for 1 min to perform the extraction, then centrifuged during 3 min at 17 000 *g* with the temperature set at +5°C. The supernatant was poured into a graduated 2-ml microcentrifuge tube and evaporated at 50°C under a nitrogen stream

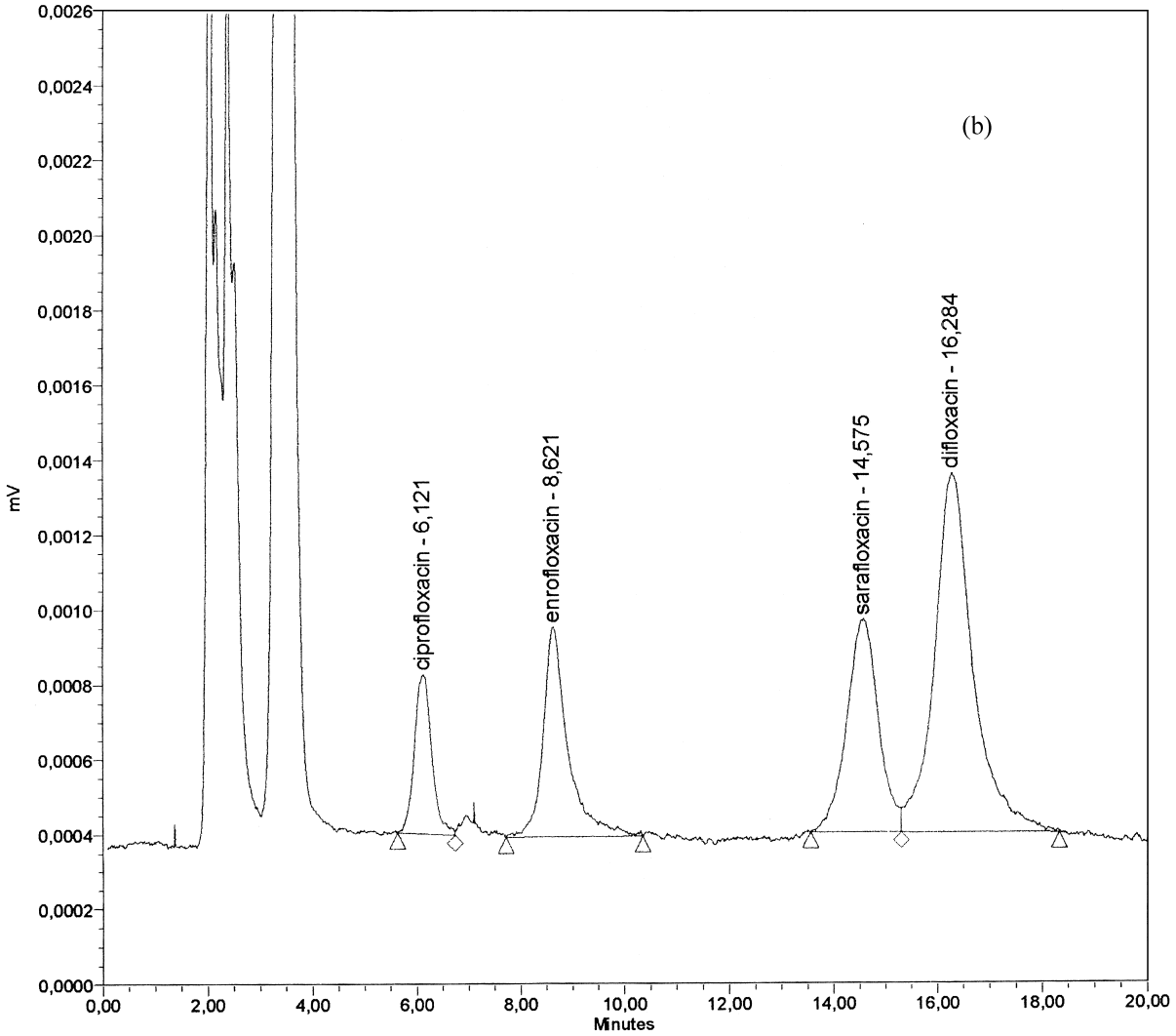

Fig. 3. (*continued*).

until the volume of the extract was less than 500 μl. The sample was reconstituted with the buffer solution, pH 9.1 up to 500 μl. A 300-μl portion of hexane was added before vortex mixing for 10 s. The extract was centrifuged during 3 min at 17 000 *g* (+5°C) then 400 μl of the aqueous phase was transferred to the HPLC vial.

2.7. HPLC

Separation of quinolones from matrix interferences was achieved on a PLRP-S analytical column heated at 50°C by using one of the three sets of analytical conditions given in Table 2. Detection was performed with a fluorescent detector set at different excitation–emission wavelengths as given in Table 3.

2.8. Calculation

Injections of 20 μl of the working standard solutions allowed one to plot the five concentration levels of each calibration curve. Volumes of 100 μl of extracts (samples or spiked control samples) were injected. Quantification was performed by using a five-level external standard calibration curve. A

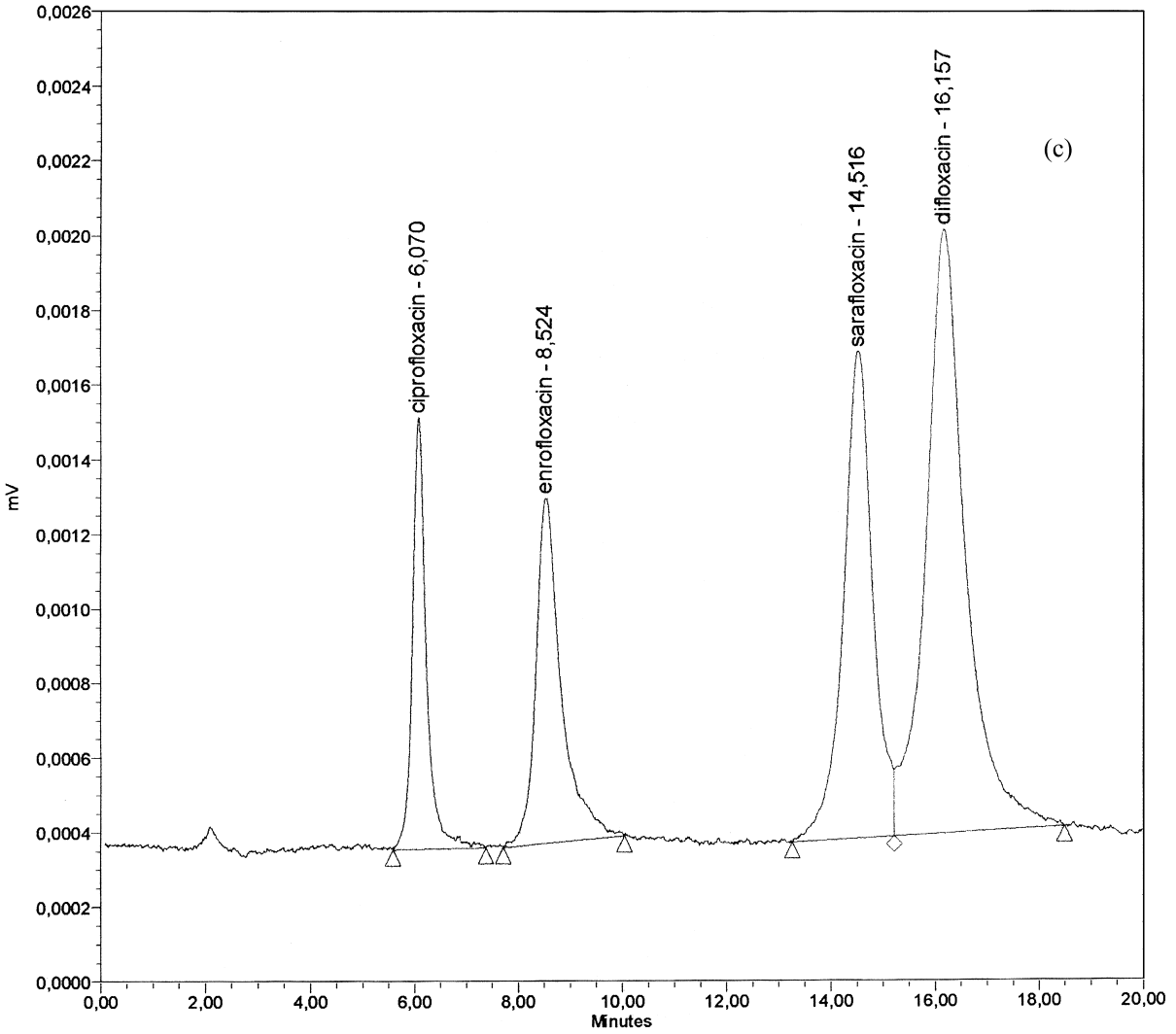

Fig. 3. (*continued*).

correction for recovery was applied taking into account the spiked control samples.

2.9. Validation procedure

The method was validated according to recommendations for analytical methods developed for controlling veterinary drug residues [18]. Raw data for the standard solutions and for the spiked samples were analysed with a laboratory-made computer program (Logval). Linearity and goodness of fit were tested by analysis of the variance as recommended by Schwartz [19]. The limit of quantification, the limit of detection and the accuracy were evaluated. Recovery and precision in term of intra- and inter-day repeatability was also checked. Selectivities of the three HPLC methods were evaluated. A stability study on incurred muscle is under development.

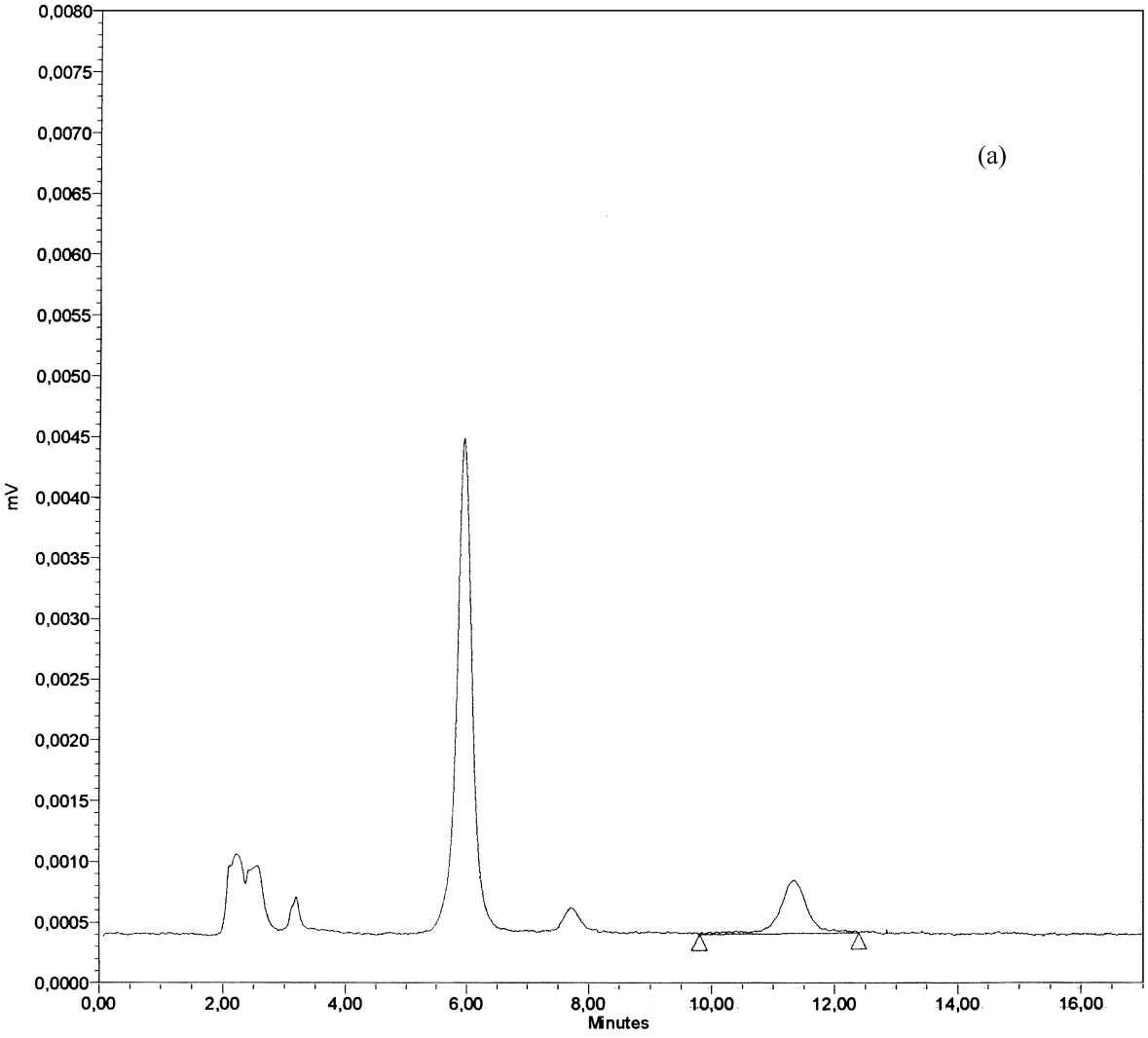

Fig. 4. Chromatograms of the second group of quinolones obtained with (a) blank chicken muscle tissue, (b) extract from chicken muscle tissue fortified with marbofloxacin at 150 μg kg^{-1} and danofloxacin at 300 μg kg^{-1}, (c) standard solution containing marbofloxacin at 150 μg l^{-1} and danofloxacin at 300 μg l^{-1}.

3. Results and discussion

The extraction step was performed by testing different solvents. Organic solvents (ethyl acetate, methylenechloride) were first tried because of the easiness of their evaporation. Unfortunately the recoveries were very low for the six quinolones with a piperazinil moiety (<40%). More polar solvents like acetonitrile and phosphate buffer were then evaluated. The extract obtained with the phosphate buffer needed a further clean-up step with a solid-phase extraction column which was time consuming and costly. The extraction with the acetonitrile gave the best results and allowed, after a centrifugation step and a partial evaporation, a direct injection into the HPLC system.

The influence of the pH on the recovery of the extracted quinolones with a piperazinil moiety was checked. Three different pH values (4.65; 7; 9.18) were applied. Good recoveries were obtained at pH 4.65 and 9.18 (from 70% to 80%). Lower recoveries were obtained at pH 7 (from 40% to 50%). These results are in accordance with the theory, quinolones with a piperazinil moiety have two pK_a values:

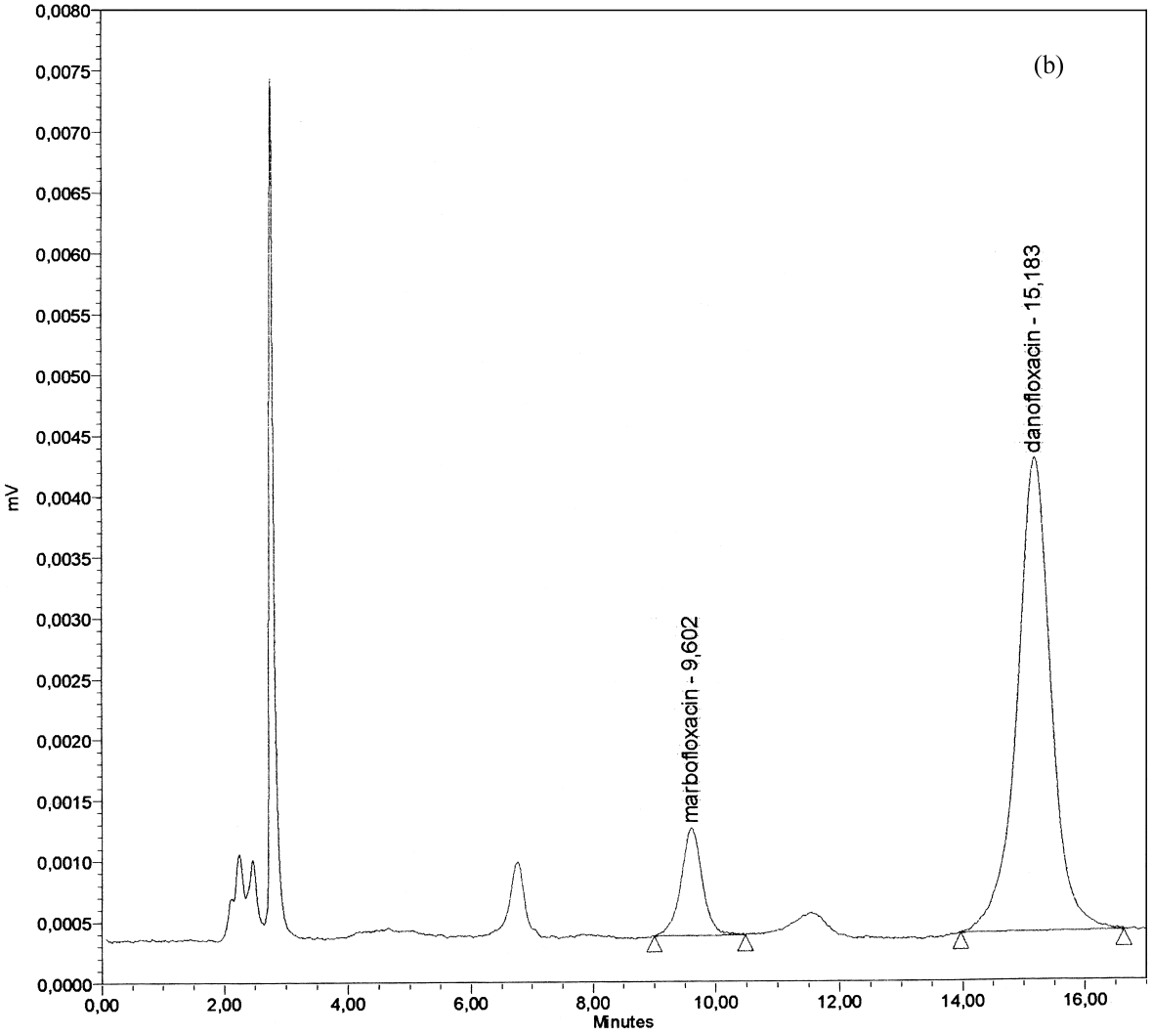

Fig. 4. (*continued*).

pK_{a1}≈6 and pK_{a2}≈9 and are thus always charged. They exist mostly in cationic form at acidic pH, anionic form at basic pH and as zwitterionic form at neutral pH. The zwitterionic form can be considered as a neutral species and consequently is less solvable with polar solvents. Moreover the pyridonecarboxylic acid antibacterials (PCAs) have one pK_a value (pK_a≈6) and therefore exist as neutral compounds at acidic pH and as anionic form at neutral and basic pH. Finally pH 9.1 was chosen because all quinolones behave as anions. The volume of solvent for the extraction step was fixed at 1.3 ml and contained 1 ml of acetonitrile and 0.3 ml of buffer, pH 9.1 allowing microcentrifugation. The composition of the solvent extraction allowed a partial evaporation of the extract (complete elimination of the acetonitrile phase) in 25 min. A defatting step with hexane prior the HPLC run has been added when the method was applied to species like fish or pork. This extraction improved the life time of the guard column and therefore has been added to the initial procedure.

Separation of quinolones from endogenous compounds was performed by using a PLRP-S column.

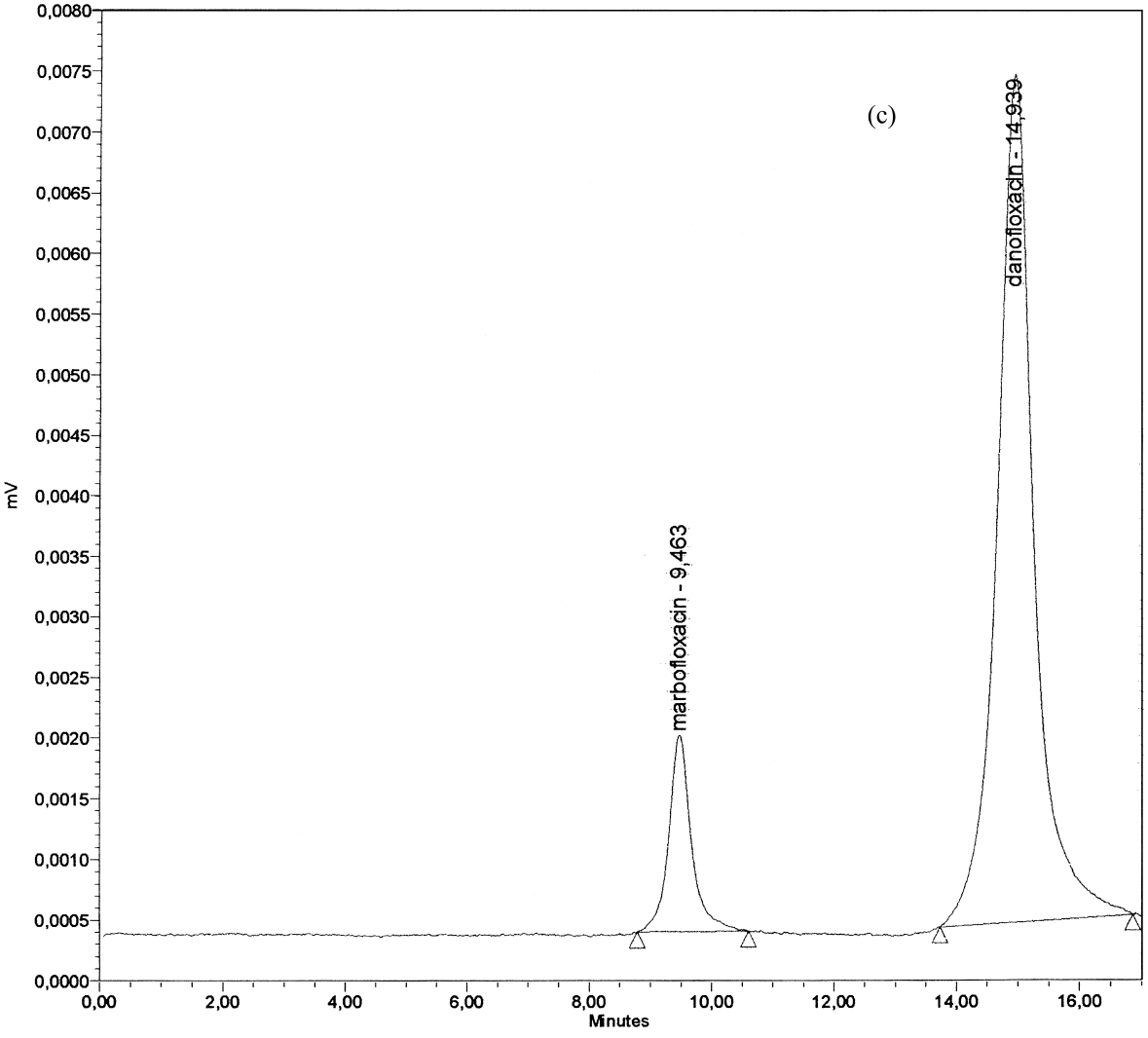

Fig. 4. (*continued*).

Quinolones have been gathered in three different HPLC runs. Ciprofloxacin, enrofloxacin, sarafloxacin and difloxacin were in the first group, marbofloxacin and danofloxacin in the second group, oxolinic acid, nalidixic acid and flumequine in the third group. The conditions of elution and of detection are reported in Tables 2 and 3, respectively. The composition of the mobile phases were found in the literature, for the first group [11] and for the third group [17]. They were applied with slight modifications for the first group: triethylamine was suppressed and the quantity of acid was doubled. Mobile phase for the second group was developed in the laboratory. The chromatograms presented in Figs. 3–5 correspond to analyses performed on blank chicken tissue, on spiked chicken tissue and on standard solutions for each of the three analytical conditions.

Three different HPLC runs have been developed because it was difficult to get a single chromatogram of the nine quinolones in less than 45 min in gradient

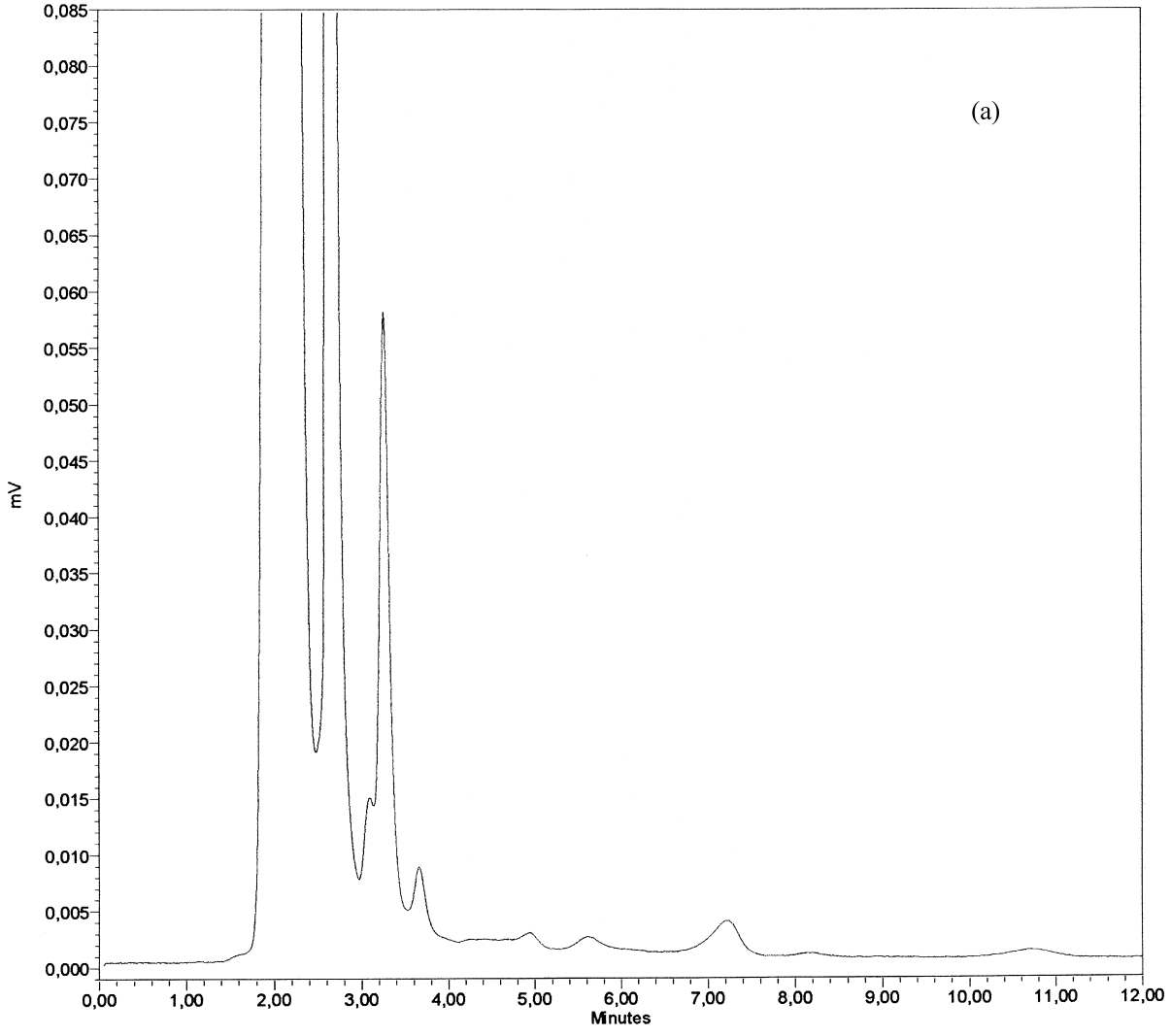

Fig. 5. Chromatograms of the third group of quinolones obtained with (a) blank chicken muscle tissue, (b) extract from chicken muscle tissue fortified with oxolinic acid, nalidixic acid and flumequine at 50 μg kg^{-1}, (c) standard solution containing oxolinic acid, nalidixic acid and flumequine at 50 μg l^{-1}.

mode. Moreover quinolones have different MRLs and do not present the same fluorescent maximum for excitation as well as for emission. It would have been necessary to carry out a simultaneous gradient elution and time programming detection mode in order to perform a single HPLC run of the nine quinolones which is not the most reliable and rugged.

The main data obtained during the validation are given in Tables 4 and 5. The selectivity for the nine quinolones quantified with the three HPLC methods was achieved. Other quinolones were also tested, among these, 7-hydroxyflumequine was partially resolved from oxolinic acid, likewise norfloxacine was partially resolved from marbofloxacin. The precision (RSD≤15%) for all the quinolones was better than those recommended (RSD≤23%). The accuracy varied from −4.8% to +7% for the quantification of quinolones and was in agreement with the recommendation [18]. The limits of detection (from

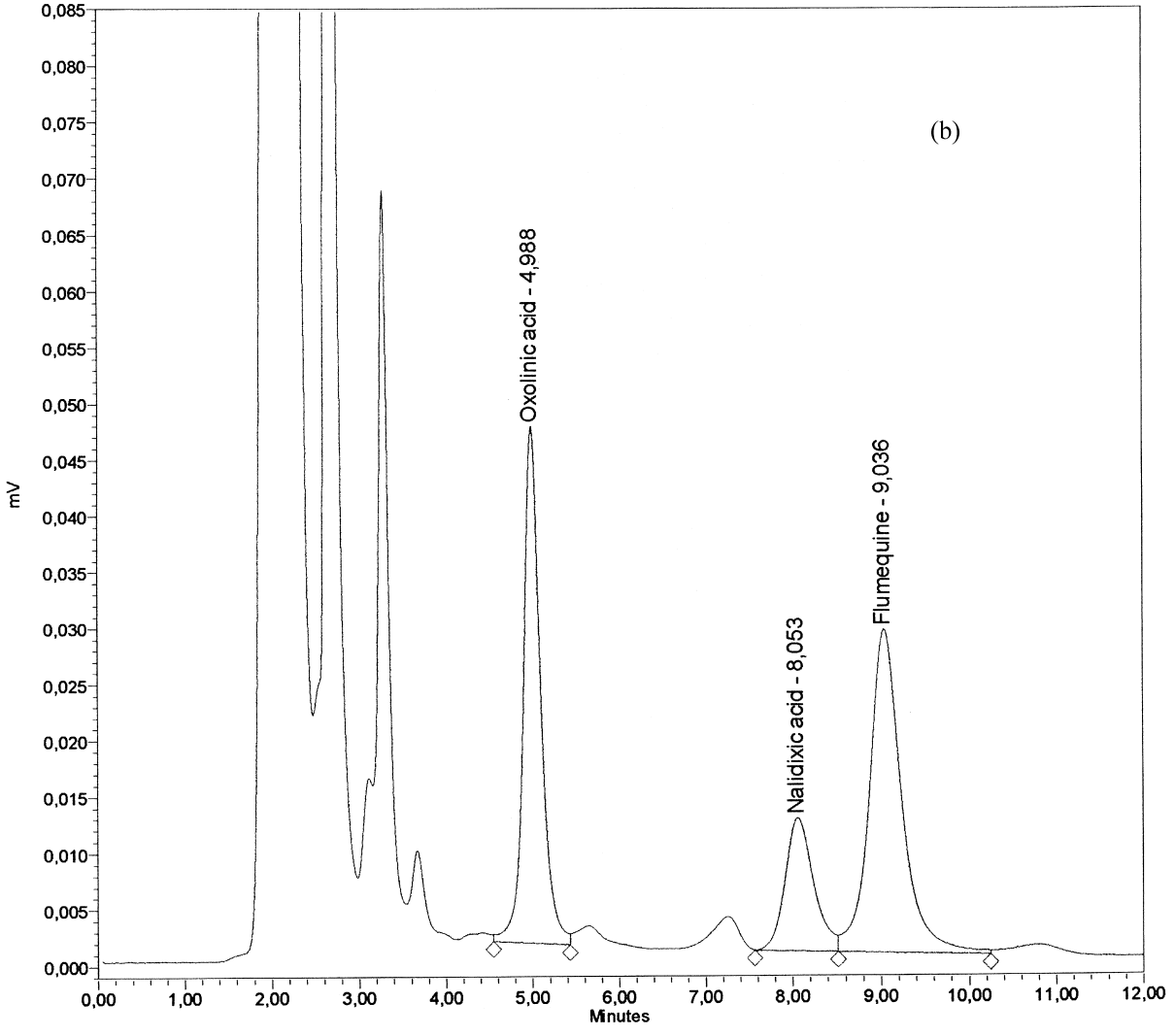

Fig. 5. (*continued*).

0.5 to 35 μg kg^{-1}) were also far below the MRLs. The linearity of the response for the standards as for the spiked samples was good enough to quantify the extracts with the response factor obtained from diluted standards after correction of the recovery obtained from quality control samples.

This procedure was tested with success to spiked muscle tissues from porcine, bovine, ovine and fish species. Moreover this method was applied in collaborative study for the determination of oxolonic acid in incurred fish.

4. Conclusion

This paper describes a new HPLC method for the quantification of nine quinolones (ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, flumequine, marbofloxacin, nalidixic acid, oxolinic acid, sarafloxacin) in chicken, porcine, bovine, ovine and fish muscle. This assay which has been designed to achieve a high throughput samples with a short time for the preparation step could be used to confirm positive samples coming from screening process.

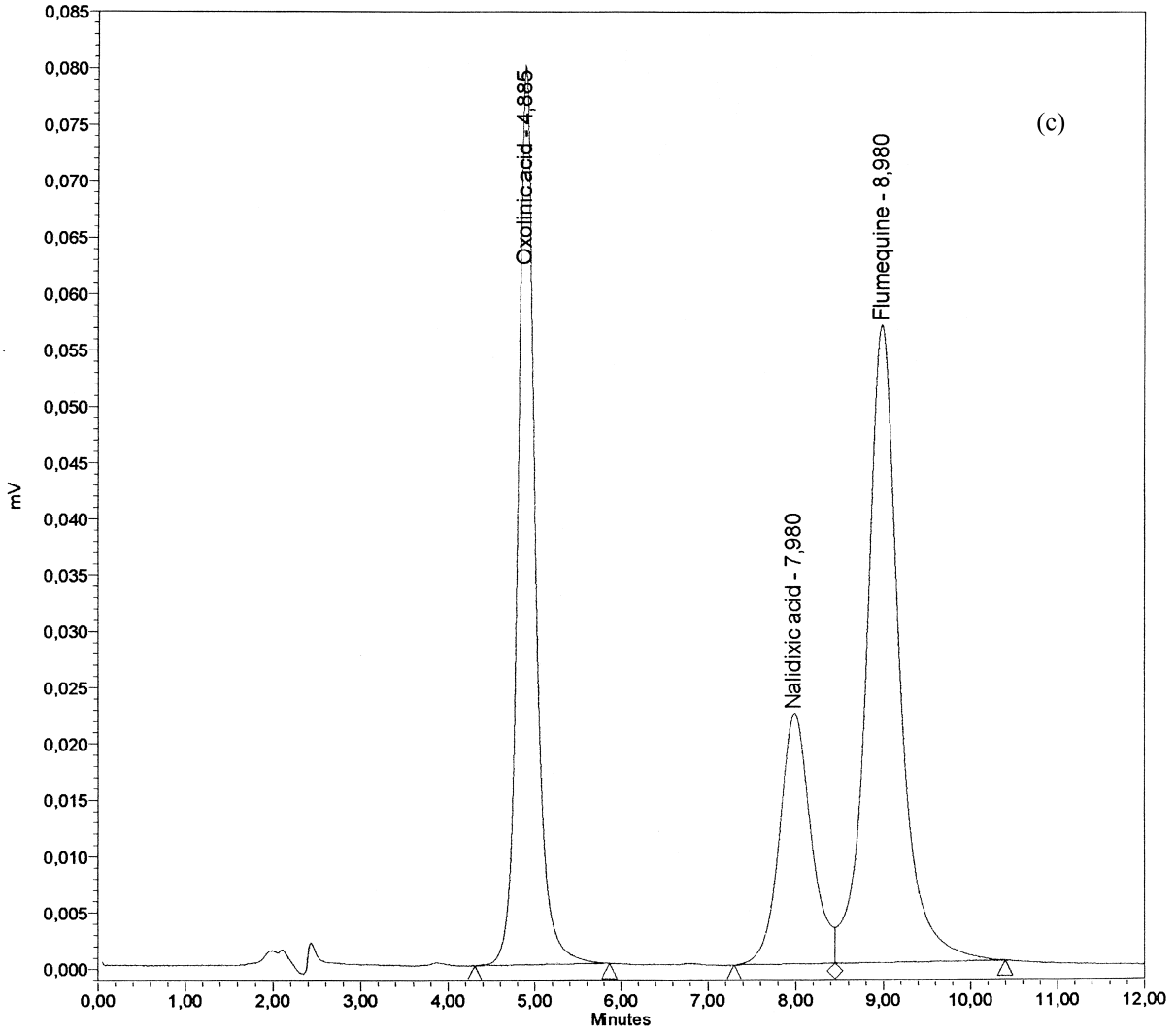

Fig. 5. (*continued*).

Table 4
Limits of detection, limits of quantification and accuracy of the method verified for the nine quinolones analysed in chicken muscle tissue

Compound	LOD[a] (μg kg^{-1})	LLOQ[b] (μg kg^{-1})	ULOQ[c] (μg kg^{-1})	Accuracy[d] (%)
Ciprofloxacin	2	7.5	30	−4.8
Enrofloxacin	0.5	7.5	30	−1.8
Sarafloxacin	1	25	100	−3.1
Difloxacin	0.5	25	100	−2.4
Marbofloxacin	35	75	300	−0.7
Danofloxacin	7.5	150	600	−1.0
Oxolinic acid	12	25	100	+4.0
Nalidixic acid	7.5	25	100	+7.2
Flumequine	3.0	25	100	+3.6

[a] LOD: Limit of detection calculated with 20 different samples from blank chicken muscle tissue as LOD=$R(h+3\times SD)$, R=response factor and h=average height of the blank samples.
[b] LLOQ: Lower validated concentration.
[c] ULOQ: Upper validated concentration.
[d] Accuracy: Calculated for the middle level of the range of concentration (2×LLOQ).

Table 5
Recovery and precision of the method for the nine quinolones analysed in chicken muscle tissue

Compound	Level[a]	Replicate[b]	Day[c]	N[d]	Recovery[e]±SD (%)	RSD_r[f] (%)	RSD_R[g] (%)
Ciprofloxacin	5	3	4	60	67±10.5	8.4	12.4
Enrofloxacin	5	3	4	60	77±8.5	10.7	12.7
Sarafloxacin	5	3	4	60	71±7.5	5.4	9.8
Difloxacin	5	3	4	60	75±6.75	5.4	8.5
Marbofloxacin	5	2	3	30	64±7.5	4.2	15.5
Danofloxacin	5	2	3	30	59±5.25	4.7	9.9
Oxolinic acid	5	3	3	45	73±6.5	7.3	7.3
Nalidixic acid	5	3	3	45	71±7	7.2	7.5
Flumequine	5	3	3	45	70±5.5	7.7	7.7

[a] Range of concentrations (1×LLOQ, 1.5×LLOQ, 2×LLOQ, 3×LLOQ, 4×LLOQ).
[b] Number of spiked samples at each concentration.
[c] Number of days for the experiment.
[d] Number of spiked samples (level×replicate×day).
[e] Average recovery calculated with N samples.
[f] Relative standard deviation of the recovery calculated with the intra-day data.
[g] Relative standard deviation of the recovery calculated with the inter-day data.

Acknowledgements

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