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Validation of an HPLC-UV method according to the European Union Decision 2002/657/EC for the simultaneous determination of 10 quinolones in chicken muscle and egg yolk

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Abstract

Herein two different methods are proposed for the determination of 10 quinolones (enoxacin, ofloxacin, norfloxacin, ciprofloxacin, danofloxacin, enrofloxacin, sarafloxacin, oxolinic acid, nalidixic acid and flumequine) in chicken muscle and egg yolk. Two different HPLC systems were used comparatively and the respective methods were fully validated. The analytes were initially extracted from chicken muscle and egg yolk and purified by a solid phase extraction using LiChrolut RP-18 cartridges. Recoveries varied between 96.6 and 102.8% for chicken muscle and 96.4–102.8% for egg yolk. HPLC separation was performed at 25 °C using an ODS-3 PerfectSil® Target (250 mm × 4 mm) 5 μm analytical column (MZ-Analysentechnik, Germany). The mobile phase consisted of a mixture of 0.1% trifluoroacetic acid (TFA)–ACN–CH_3OH, delivered by a gradient program, different for each method. In both cases caffeine was used as internal standard at the concentration of 7.5 ng/μL. Column effluent was monitored using a photodiode array detector, set at 275 and 255 nm. The developed methods were validated according to the criteria of Commission Decision 2002/657/EC. The LODs for chicken muscle varied between 5.0 and 12.0 μg/kg and for egg yolk was 8.0 μg/kg for all examined analytes.

Keywords: Quinolones; Solid phase extraction; Chicken muscle; Egg yolk; HPLC; Commission decision 2002/657/EC

1. Introduction

Quinolones are synthetic antibiotics whose action is based on their anti-DNA activity. Nalidix acid was the first quinolone approved on 1963, by Food and Drug Administration (FDA) for the treatment of urinary tract infections. Quinolones are widely used till nowadays in human and in veterinary medicine, due to their safety with good tolerance and broad antibacterial spectrum. Fluoroquinolones belong to the second generation of quinolones and their characteristic is the greater effectiveness against both Gram-negative and Gram-positive pathogens that are resistant to other antibacterials [1].

Data collected from 25 European countries showed that fluoroquinolones represented more than 50% of the quinolones used. Ciprofloxacin is the most widely prescribed fluoroquinolone in the world, followed by ofloxacin [2].

The widespread use of quinolones in human and in veterinary medicine has led to a significant increase in antibacterial resistance, having therefore important consequences for public health. To minimize risks in human health by the consumption of quinolones' residues in foods, the European Union by the Council Regulation No. 2377/90 has established maximum residue limits (MRLs) of veterinary medicinal products in foodstuffs of animal origin and among them are some quinolones [3]. The MRLs according to this regulation in chicken muscle are 200 μg/kg for danofloxacin, 300 μg/kg for difloxacin, 100 μg/kg for enrofloxacin and 200 μg/kg for flumequine. Another provision according to this directive is that the use of quinolones is prohibited in animals from which eggs are produced for human consumption. Therefore, analytical methods as sensitive as possible are required in order to check food samples before their disposal to the markets for human consumption. Recently European Union has issued the decision 2002/657/EC which concerns the performance of analytical methods and the interpretation of results in the official control of residues in products of animal origin [4].

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The increasing number of published papers concentrating on the determination of quinolones' residues in food is illustrating the seriousness of this state. The last decade the majority of the articles propose a simultaneous analysis of more than five quinolones' residues in chicken tissue and in eggs. Huang and his team and Gigosos et al. have developed methods for the determination of five quinolones in eggs [5,6]. Hassouan et al. propose a method for the determination of seven quinolones in eggs [7] and Bailac et al. in two different papers develop methods for the determination of seven quinolones in chicken muscle [8,9]. Schneider and Donoghue have developed a method for the determination of eight quinolones in eggs and in chicken tissue [10,11]. Also York and Froc propose a method for the determination of eight quinolones in chicken tissue but in three groups [12]. Finally Zeng et al. have developed a method for the simultaneous determination of nine quinolones in eggs [13].

All published methods mentioned above involve the use of HPLC. Most of them are using a Fluorescence Detector single [7,8,12,13], or coupled with MS [10,11] or a photodiode array detector [5]. Gigosos et al. use a UV-diode array detector [6] and Baillac et al. use ESI-MS/MS detector [8].

A previous work of the authors deals with the simultaneous determination of five quinolones in chicken tissue by HPLC [14]. The innovation of the present work is the analysis of five more

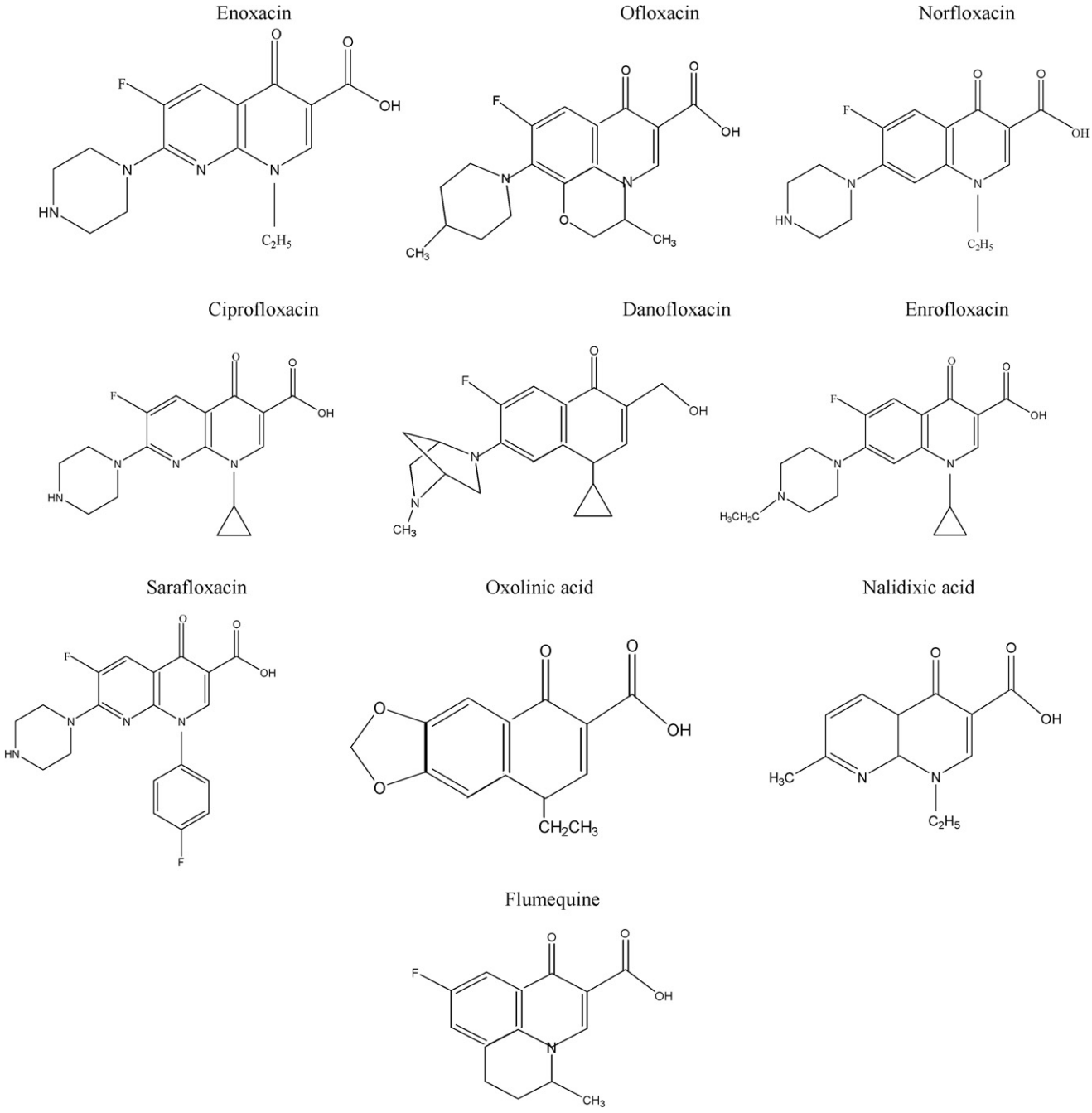

Fig. 1. Chemical structure of the examined quinolones.

quinolones, in total ten Quinolones: enoxacin (ENO), ofloxacin (OFL), norfloxacin (NOR), ciprofloxacin (CIP), danofloxacin (DAN), enrofloxacin (ENR), sarafloxacin (SAR), oxolinic acid (OXO), nalidixic acid (NAL) and flumequine (FLU), which are determined in chicken tissues and in eggs' yolk. Chemical structures of examined quinolones are illustrated in Fig. 1.

Two methods have been developed and validated using two different HPLC instruments. The comparison of the two methods proved that they are applicable for both matrices, with the same recoveries and the same limits of quantitation.

Solid phase extraction was selected for the sample preparation of both matrices as the fastest, easiest and most efficient technique.

2. Experimental

2.1. Reagents and materials

Enoxacin, ofloxacin, norfloxacin, nalidixic acid, flumequine, ciprofloxacin and internal standard caffeine, all of analytical grade were purchased from Sigma (Steinheim, Germany), enrofloxacin >98%, oxolinic acid 97% from Fluka (Steinheim, Germany), danofloxacin 98.4% and sarafloxacin 99.3% VETRANAL® from Riedel-de Haen (Buchs SG, Schweiz). HPLC grade methanol (99.8%), gradient grade acetonitrile (99.9%) were supplied by Carlo Erba (Milano, Italy) and analytical grade sodium hydroxide (NaOH, 1 mol/L) by Merck (Darmstadt, Germany). Trifluoroacetic acid 99% was obtained from Aldrich (Steinheim, Germany). Ultrapure water provided by a Milli-Q® purification system (Millipore, Bedford, MA, USA) was used throughout the study.

Merck LiChrolut RP-18 (200 mg/3 mL) SPE cartridges were used for the isolation of the analytes from any endogenous interference stemmed from chicken and egg yolk matrices.

2.2. Instrumentation

2.2.1. HPLC system 1

A Shimadzu (Kyoto, Japan) HPLC system was used for the analysis of the examined quinolones in chicken muscle. It was consisted of a mixer FCV-9AL for the mixing of the solvent lines, an LC-9A pump for delivering the mobile phase to the analytical column, a SIL-9A autosampler equipped with a 50 μL loop for sample injection a column oven for maintaining the temperature stable and an SPD-M6A Photodiode Array Detector. Degassing of the mobile phase was achieved by continuous helium sparking in the solvent reservoirs by a DGU-2A degassing unit. The whole system was complied by the software Class M-10A.

2.2.2. HPLC system 2

A Shimadzu (Kyoto, Japan) quaternary low-pressure gradient system was used for chromatographic determination of the examined quinolones in egg yolk. The solvent lines were mixed in an FCV-10AL$_{VP}$ mixer. An LC-10AD$_{VP}$ pump was used to deliver the mobile phase to the analytical column, equipped with a Shimadzu SCL-10AL$_{VP}$ System Controller, permitting fully automated operation, used to deliver the mobile phase to the analytical column. Sample injection was performed via a Rheodyne 7725i injection valve (Rheodyne, Cotati, California, USA) equipped with a 20 μL loop. Detection was achieved by an SPD-M10A$_{VP}$ Photodiode Array Detector, in compliance with data acquisition software LabSolutions-LCsolutions by Shimadzu. Functions of the whole system were controlled by an SCL-10A$_{VP}$ controller. Degassing of the mobile phase was achieved by continuous helium sparking in the solvents reservoirs by a DGU-10B degassing unit.

Glass vacuum-filtration apparatus obtained from Alltech Associates (Deerflied, IL, USA) was used for the filtration of buffer solutions through Whatman Cellulose Nitrate 0.2 μm-WCN Type (47 mm DIA) (Whatman Laboratory Division, Maidstone, England) membrane filters. A Glasscol (Terre Haute, IN 47802, USA) small vortexer, a Hermle centrifuge, model Z-230 (B. Hermle, Gosheim, Germany) and an Ultrasonic bath Transonic460/H (Elma, Germany) were employed for sample pre-treatment. All evaporations were performed with a Supelco 6-port Mini-Vap concentrator/evaporator (Bellefonte, PA, USA). SPE was carried out on a 12-port vacuum manifold from Supelco.

2.3. Chromatographic conditions

A PerfectSil®Target ODS-3 analytical column (250 mm × 4 mm), 5 μm, purchased from MZ-Analysentechnik (Mainz, Germany) was used for the separation of the studied quinolones, operated at 25 °C. The mobile phase consisted of A: 0.1% TFA, B: ACN and C: CH_3OH. Two different gradient programs were used for the separation of the 10 quinolones, which are described in Table 1. Column effluent was monitored

Table 1
Gradient timetable for the two HPLC systems

HPLC system 1				HPLC system 2			
t (min)	TFA (0.1%)	ACN	CH_3OH	*t* (min)	TFA (0.1%)	ACN	CH_3OH
0	80	10	10	0	80	4	16
10	80	10	10	17	80	4	16
20	80	20	0	50	30	70	0
30	45	55	0				
36	45	55	0				
36.1	80	10	10				

at 275 nm for all analytes except OXO, NAL and FLU, which were monitored at 255 nm. The flow rate was 1.2 mL/min for both systems but the inlet pressure ranged from 290 to 310 kg/cm^2 for HPLC system 1 and from 230 to 240 kg/cm^2 for HPLC system 2. Caffeine (CAF) was used as internal standard at a concentration of 7.5 ng/μL. Injected sample volume was 50 μL for HPLC system 1 and 20 μL for HPLC system 2.

2.4. Preparation of standard solutions

Stock solutions at a concentration of 100 ng/μL for ENO, OFL, NOR, CIP, DAN, ENR and SAR were prepared every 2 months, while those for OXO, NAL and FLU, proved to be less stable and were prepared every 2 weeks. All stock solutions were prepared in water by dissolving the appropriate amount of quinolone and by adding an aliquot of 100 μL of NaOH 0.1 mol/L per 10 mL to enhance solubility of the compounds. Stock solutions were stored at 4 °C. All working standards were prepared every day by appropriate dilutions of the concentrated stock standard solutions. Aqueous stock solution of CAF was prepared at the concentration of 100 ng/μL.

2.5. Sample preparation

2.5.1. Sample preparation of chicken muscle

Chicken tissues were chopped, homogenised and stored at −20 °C at packages of 1 ± 0.0001 g. According to the protocol about 1 g of tissue was either spiked with 200 μL of the mixture of quinolones (including the internal standard at the concentration of 7.5 ng/μL) or not (in case of blank sample). A volume of 4 mL of 0.1% TFA in CH_3OH (extraction solvent) was added and the mixture was vortexed and left to settle in dark for 10 min. The solution was then sonicated for 15 min and directly centrifuged at 800 × *g* for 10 min. The supernatant was collected in a test tube and was evaporated under stream of N_2. The sample was re-extracted a second time with 4 mL of the same extraction solvent. The solution was vortexed, left to settle in dark, sonicated for 15 min and centrifuged. The supernatant was added to the same test tube and was also evaporated to dryness. The residue was dissolved then in 2 mL of an aqueous solution of 0.1% TFA. The solution was applied to the SPE cartridge, which was previously conditioned with 2 mL CH_3OH and 2 mL water. The elution was performed with 1.5 mL solution of 0.1% TFA in ACN and 0.5 mL ACN. The eluent was evaporated to dryness at 45 °C under a gentle stream of N_2. Finally, the dry residue of the quinolones in case of spiked sample or of the blank sample was dissolved in 200 μL of an aqueous solution of TFA 0.1% and 50 μL was injected into the HPLC system 1.

Table 2
Resolution factors of the analytes in the different methods

	HPLC system 1	HPLC system 2
CAF(IS)-ENO	15.0	4.9
ENO-OFL	1.3	1.2
OFL-NOR	1.6	1.1
NOR-CIP	1.8	1.3
CIP-DAN	3.2	2.3
DAN-ENR	1.7	2.0
ENR-SAR	3.7	4.4
SAR-OXO	4.0	6.5
OXO-NAL	6.0	12.6
NAL-FLU	1.6	4.25
Total analysis time	33 min	27 min

2.5.2. Sample preparation of egg yolk

1 ± 0.0001 g of egg yolk sample was either spiked with 200 μL of the mixture of quinolones (including the internal standard at the concentration of 7.5 ng/μL) or not (in case of blank sample). Two milliliters of NaOH 0.75 M in ACN (extraction solvent) were added and the mixture was vortexed and left to settle in dark for 10 min. The solution was then sonicated for 15 min and directly centrifuged at 800 × *g* for 10 min. The supernatant was collected in a test tube and was evaporated under stream of N_2. The sample was re-extracted a second time with 2 mL of the same extraction solvent. Again the solution was vortexed, left to settle in dark, sonicated for 15 min and centrifuged. The supernatant was added to the same test tube and was also evaporated to dryness. The extracted was dissolved then in 2 mL of an aqueous solution of 0.1% TFA. The solution was applied to the SPE cartridge, which was previously conditioned with 2 mL CH_3OH

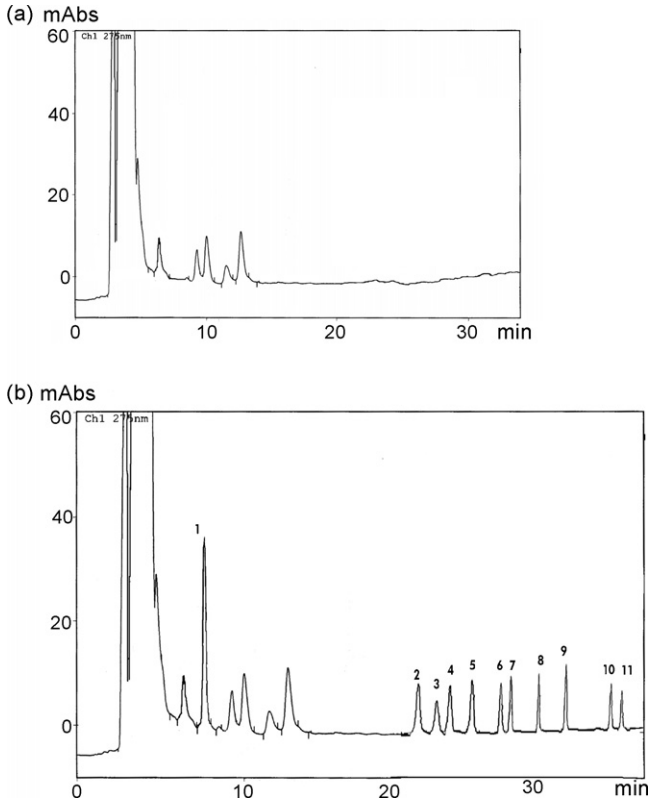

Fig. 2. (a) Chromatogram of blank chicken muscle monitored at 275 nm. (b) Chromatogram of chicken muscle spiked with a mixture of the 10 quinolones at near MRL level monitored at 275 nm: (1) Caffeine: 7.6 min (7.5 ng/μL), (2) ENO: 20.2 min (3.0 ng/μL), (3) OFL: 21.3 min (3.0 ng/μL), (4) NOR: 22.1 min (3.0 ng/μL), (5) CIP: 23.5 min (3.0 ng/μL), (6) DAN: 25.3 min (4.0 ng/μL, MRL), (7) ENR: 26.0 min (2.0 ng/μL, MRL), (8) SAR: 27.7 min (3.0 ng/μL), (9) OXO: 29.3 min (3.0 ng/μL), (10) NAL: 32.0 min (3.0 ng/μL), and (11) FLU: 32.6 min (3.0 ng/μL).

and 2 mL water. The elution was performed with 1.5 mL solution of 0.1% TFA in ACN and 0.5 mL ACN. The eluent was evaporated to dryness at 45 °C under a gentle stream of N_2. Finally, the dry residue of the quinolones in case of spiked sample or of the blank sample was dissolved in 200 μL of an aqueous solution of 0.1% TFA and 20 μL was injected into the HPLC system 2.

2.6. Method validation

Both methods were validated in order to accomplish the criteria specified by the European's Commission Decision 2002/657/EC.

Both methods were checked for the linearity and the sensitivity. Linearity was studied by injecting a series of mixture of the analytes at different concentration levels, in order to cover the whole working range. Calibration curves of spiked samples for every quinolone, with the respective correlation coefficient, were calculated by least-squares linear regression analysis of the peak area ratio of each analyte to IS of the respective results versus analyte concentration. The calculations for the limits of detection (LODs) were based on the standard deviation of *y*-intercepts of regression analysis (σ) and the slope (S), using the following equation $LOD = 3.3\sigma/S$ [15]. In turn, the limits of quantitation (LOQs) were calculated by the equation $LOQ = 10\sigma/S$ [15].

Selectivity of the methods was assessed by studying the absence of any interference in same chromatographic run as the examined quinolones with the respective method for chicken tissue and egg yolk samples.

The methods were also validated with respect to accuracy, intra-day ($n = 6$) and inter-day ($n = 6$) precision. Accuracy was studied by analyzing six times three concentration levels.

Decision limits (CC_α) and detection capability (CC_β), the new criteria according to European Decision 2002/657/EC were also calculated. For the measurement of CC_α samples were spiked at the respective LOQ level of each method as well as at the concentration of MRL for those quinolones with specified permitted limits. The decision limits (CC_α) were calculated as the mean values of the found concentrations plus 1.64 times the corresponding standard deviations. The detection capability (CC_β) values were obtained after spiking the samples at the CC_α levels by adding 1.64 times the corresponding standard deviation. Statistical analysis for CC_α and CC_β was performed at the 95% confidential level and the number of replicate analyses was 20.

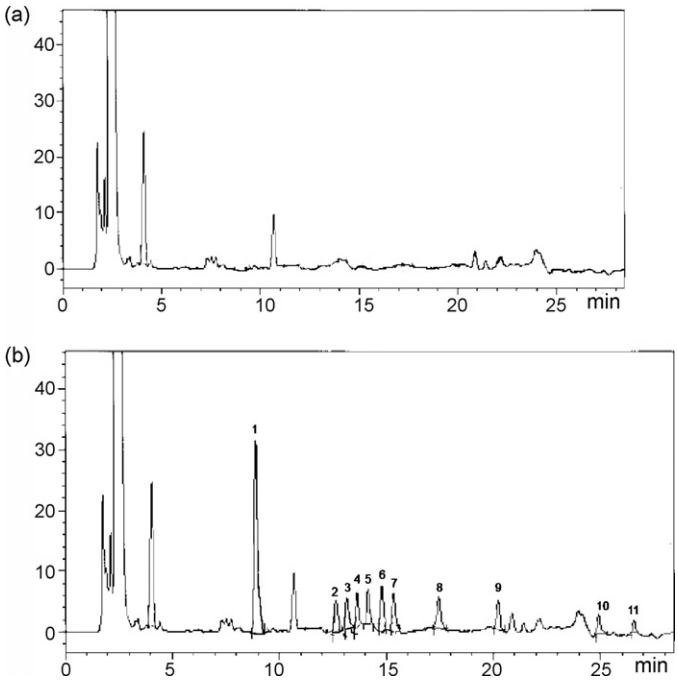

Fig. 3. (a) Chromatogram of blank egg yolk sample monitored at 275 nm. (b) Chromatogram of egg yolk spiked with a mixture of the 10 quinolones near LOQ level (0.5 ng/μL) monitored at 275 nm. (1) Caffeine: 8.9 min (7.5 ng/μL), (2) ENO: 13.0 min, (3) OFL: 13.3 min, (4) NOR: 13.6 min, (5) CIP: 14.2 min, (6) DAN: 14.8 min, (7) ENR: 15.2 min, (8) SAR: 17.5 min, (9) OXO: 20.2 min, (10) NAL: 25.0 min, and (11) FLU: 26.6 min.

3. Results and discussion

3.1. Chromatography

The mobile phase for both methods consisted of a mixture of 0.1% TFA–CH_3OH–ACN delivered to the analytical column according to the corresponding gradient program described in Table 1. In HPLC system 1 the separation of 10 quinolones was achieved in 33 min while using HPLC system 2 all analytes were separated in 27 min. This difference is expected if we take into

Table 3
Optimization of egg yolk pre-treatment

Protocol	Extraction solution	Recoveries (%)									
		ENO	OFL	NOR	CIP	DAN	ENR	SAR	OXO	NAL	FLU
1	0.5 M NaOH	45.8	48.3	42.5	53.5	50.7	55.1	46.6	37.1	38.2	41.3
2	0.75 M NaOH	51.3	53.4	50.6	55.3	57.4	60.3	52.3	38.5	37.9	42.5
3	1 M NaOH	57.3	60.1	58.2	57.4	60.7	62.3	55.4	40.3	39.1	42.8
4	0.5 M NaOH in CH_3OH	52.3	55.3	50.8	65.3	56.3	59.1	48.5	51.6	53.1	49.8
5	0.75 M NaOH in CH_3OH	63.0	60.8	63.2	70.6	65.8	65.4	63.4	54.9	57.4	51.3
6	1 M NaOH in CH_3OH	52.8	56.7	51.3	66.2	55.6	57.7	50.6	52.7	55.0	50.8
7	0.5 M NaOH in ACN	72.8	68.3	65.4	73.2	74.7	71.3	72.0	63.3	71.9	64.4
8	0.75 M NaOH in ACN	83.4	87.9	85.7	83.7	84.2	80.2	81.6	75.4	80.6	75.6
9	1 M NaOH in ACN	65.5	68.3	64.4	65.2	67.3	71.0	72.3	65.5	60.9	69.3

Table 4
Calibration curves and sensitivity data of both the developed methods for the determination of the ten examined quinolones using as IS CAF 7.5 ng/μL as internal standard

Quinolones	Chicken muscle HPLC system 1					Egg yolk HPLC system 2				
	Slope $(\mu g/kg)^{-1}$	Intercept	R	Range (μg/kg)	LOD (μg/kg)	Slope $(\mu g/kg)^{-1}$	Intercept	R	Range (μg/kg)	LOD (μg/kg)
ENO	0.0053 ± 0.0002	−0.0320 ± 0.0080	0.9918	15–500	5	0.0024 ± 0.0001	0.0702 ± 0.0060	0.9954	25–500	8
OFL	0.0016 ± 0.0001	−0.0283 ± 0.0040	0.9958	25–600	8	0.0084 ± 0.0001	0.0126 ± 0.0210	0.9973	25–500	8
NOR	0.0043 ± 0.0001	−0.0210 ± 0.0064	0.9973	15–600	5	0.0016 ± 0.0001	0.0763 ± 0.0040	0.9987	25–500	8
CIP	0.0038 ± 0.0001	0.0867 ± 0.0095	0.9982	25–500	8	0.0024 ± 0.0001	0.0471 ± 0.0060	0.9987	25–500	8
DAN	0.0037 ± 0.0003	−0.0087 ± 0.0137	0.9903	37–500	12	0.0022 ± 0.0001	0.0532 ± 0.0055	0.9984	25–500	8
ENR	0.0048 ± 0.0002	0.1192 ± 0.0178	0.9983	37–500	12	0.0028 ± 0.0001	0.0131 ± 0.0070	0.9966	25–500	8
SAR	0.0042 ± 0.0001	0.0895 ± 0.0063	0.9976	15–500	5	0.0031 ± 0.0001	0.0958 ± 0.0078	0.9991	25–500	8
OXO	0.0078 ± 0.0003	−0.1403 ± 0.0117	0.9925	15–600	5	0.0055 ± 0.0002	0.0858 ± 0.0138	0.9971	25–500	8
NAL	0.0014 ± 0.0001	−0.0260 ± 0.0035	0.9949	25–600	8	0.0028 ± 0.0001	0.0142 ± 0.0070	0.9974	25–500	8
FLU	0.0027 ± 0.0001	−0.0083 ± 0.0068	0.9924	25–600	8	0.0021 ± 0.0001	0.0259 ± 0.0052	0.9992	25–500	8

consideration that the two instruments differ by 8 min in their dwell volume. In Table 2 resolution factors (R_s) of the 10 analytes and the internal standard are calculated according to the formula: $R_s = 2(t_2 - t_1)/(t_{w1} + t_{w2})$, where t_1 and t_2 are the retention times and t_{w1} and t_{w2} the baseline peak widths of successive peaks. The separation of the analytes in both systems is quite satisfactory, as it is proved from the resolution factors. Retention times of the examined analytes in HPLC system 1 were 7.602 ± 0.032 min for CAF, 20.215 ± 0.017 min for ENO, 21.311 ± 0.026 min for OFL, 22.112 ± 0.013 min for NOR, 23.522 ± 0.021 min for CIP, 25.331 ± 0.031 min for DAN, 26.015 ± 0.025 min for ENR, 27.731 ± 0.018 min for SAR, 29.341 ± 0.011 min for OXO, 32.023 ± 0.024 min for NAL and 32.603 ± 0.022 min for FLU. Column effluent was monitored using a photodiode array detector, set at 275 and 255 nm. Typical chromatograms of blank and spiked chicken muscle at near MRL level monitored at 275 nm are shown in Fig. 2(a and b). Retention times of the examined analytes in HPLC system 2 were 8.905 ± 0.012 min for CAF, 12.961 ± 0.024 min for ENO, 13.331 ± 0.022 min for OFL, 13.650 ± 0.033 min for NOR, 14.187 ± 0.023 min for CIP, 14.807 ± 0.030 min for DAN, 15.178 ± 0.013 min for ENR, 17.457 ± 0.022 min for SAR, 20.193 ± 0.024 min for OXO, 24.960 ± 0.009 min for NAL and 26.549 ± 0.018 min for FLU. Typical chromatograms of egg yolk blank and spiked at near LOQ level monitored at 275 are shown in Fig. 3(a and b). OXO, NAL and FLU were monitored at 255 nm, since they present higher sensitivity.

3.2. Optimization of sample preparation

Optimization of sample preparation was focused on the extraction of the 10 quinolones from egg yolk. The extraction solvent used for chicken muscle (TFA 0.1% in CH_3OH) did not give satisfactory recoveries. Also other acidic extraction solvents were used such as 1% TFA in ACN, 2% CH_3COOH in ACN and HCl 1 M but none of them gave recoveries higher than 55%. Various concentrations of NaOH in water and in organic solvents were tried for the extraction of the 10 analytes from egg yolk. Results are recorded in Table 3. It is obvious that *protocol 8* gave the highest recoveries. This protocol provides higher recovery and cleaner sample than the one previously described by the authors [14]. All trials were performed with 4 mL extraction solvent and the extraction was repeated twice, followed by the SPE for a further clean-up.

3.3. Method validation

3.3.1. Linearity and sensitivity

Calibration curves were obtained by least-squares linear regression analysis of the peak area ratio of analyte to internal standard versus analyte concentration. The two methods were linear up to 500 μg/kg for all analytes except OFL, NOR, OXO, NAL and FLU in chicken muscle which were linear up to 600 μg/kg. Regression analysis revealed correlation coefficients between 0.9903 and 0.9983 for chicken muscle and 0.9954–0.9992 for egg yolk. The LODs for chicken muscle varied between 5.0 and 12.0 μg/kg and for egg yolk were 8.0 μg/kg

for all examined analytes. All calibration data are presented in Table 4.

3.3.2. Selectivity

Selectivity of both methods was assessed by the absence of any interference at the elution times of the studied analytes in the same chromatographic run as shown in blank chromatograms. To check the selectivity of the methods ten different samples of chicken muscle and ten different samples of egg yolk were analyzed with the respective method, after being pre-treated as described above, without any spiking.

3.3.3. Precision and accuracy

To check the repeatability of the each method, spiked sample of chicken muscle and of egg yolk, respectively, were measured at three different concentrations. For chicken muscle the spiking levels were the MRLs/2, the MRLs and the MRLs × 1.5 for quinolones with limits defined by the Council Regulation EEC 2377/90 and for the other quinolones the spiking levels were the LOQ of the method and two more concentrations. According to the same regulation the use of quinolones is prohibited in chickens from which eggs are produced for human consumption. So the LOQ of the respective method and moreover two more concentrations were chosen for the within-day repeatability and between-day precision assay. At each spiking level six different samples were prepared.

To study the reproducibility of the method between six consecutive days the same experimental procedure was followed with spiked samples at the same concentration level as mentioned above (measurements for three samples per day, analyzed in triplicate). Precision and accuracy results are summarized in Table 5 for chicken muscle and in Table 6 for egg yolk.

The accuracy of the methods was tested by studying the obtained average recoveries ranging between 96.6 and 102.8%

Table 5
Within-day ($n = 6$) and between-day (over a period of six consecutive days) precision and accuracy data for the determination of quinolones in chicken muscle

Analytes	Added (μg/kg)	Within-day			Between-day		
		Found ± S.D. (μg/kg)	R.S.D.	Recovery (%)	Found ± S.D. (μg/kg)	R.S.D.	Recovery (%)
Chicken muscle (HPLC system 1)							
ENO	15	15.3 ± 0.6	1.6	102.0	14.9 ± 0.3	1.1	99.3
	50	48.8 ± 0.7	1.5	97.6	50.3 ± 1.8	2.2	100.6
	100	100.7 ± 1.0	0.4	100.7	98.6 ± 2.8	1.7	98.6
OFL	25	24.2 ± 0.8	2.5	96.8	25.4 ± 1.1	3.2	101.6
	50	48.3 ± 0.7	1.7	96.6	48.3 ± 2.4	2.5	96.6
	100	100.7 ± 0.7	2.4	100.7	99.7 ± 0.7	1.2	99.7
NOR	15	14.5 ± 0.9	0.3	96.7	15.7 ± 0.6	3.3	104.7
	50	48.5 ± 1.7	0.3	97.0	50.6 ± 0.8	1.5	101.2
	100	100.6 ± 0.6	2.6	100.6	99.7 ± 1.2	1.6	99.7
CIP	25	24.8 ± 0.1	3.1	99.2	24.7 ± 2.5	4.1	98.8
	50	49.2 ± 0.8	1.9	98.4	50.2 ± 1.1	1.2	100.4
	100	100.3 ± 0.3	2.2	100.3	100.8 ± 1.9	2.0	100.8
DAN	37	37.2 ± 0.7	1.6	100.5	36.0 ± 0.5	1.4	97.3
	100	99.8 ± 1.3	0.8	99.8	99.9 ± 1.8	3.6	99.9
	200[a]	200.8 ± 0.2	4.2	100.4	200.2 ± 0.4	1.7	100.1
	300	300.1 ± 0.6	1.3	100.0	298.8 ± 0.9	2.3	99.6
ENR	37	36.9 ± 1.1	1.2	99.7	36.1 ± 0.4	1.5	97.6
	50	48.6 ± 1.2	2.1	97.2	49.6 ± 1.3	2.8	99.2
	100[a]	99.8 ± 1.1	2.1	99.8	99.2 ± 1.5	0.7	99.2
	150	148.7 ± 3.2	2.7	99.1	148.7 ± 2.3	1.7	99.1
SAR	15	15.1 ± 0.7	3.2	100.7	14.5 ± 1.4	2.6	96.7
	50	51.4 ± 0.6	1.2	102.8	50.3 ± 2.1	1.6	100.6
	100	98.4 ± 0.4	3.7	98.4	100.2 ± 1.1	0.8	100.2
OXO	15	14.9 ± 0.5	1.1	99.3	15.3 ± 1.3	2.7	102.0
	50	50.1 ± 1.2	1.6	100.2	50.6 ± 1.3	1.3	101.2
	100	100.2 ± 1.4	0.8	100.2	99.4 ± 1.2	0.8	99.4
NAL	25	24.8 ± 0.4	2.8	99.2	25.2 ± 1.8	1.3	100.8
	50	49.6 ± 0.6	3.2	99.2	49.6 ± 1.2	2.1	99.2
	100	99.7 ± 1.3	3.7	99.7	99.6 ± 2.5	1.7	99.6
FLU	25	25.2 ± 0.4	1.7	100.8	24.9 ± 0.7	1.6	99.6
	200	199.7 ± 0.4	1.2	99.8	199.8 ± 1.0	2.0	99.9
	400[a]	399.0 ± 3.2	0.6	99.8	399.8 ± 1.5	1.8	100.0
	600	597.1 ± 2.3	3.1	99.5	598.1 ± 1.6	1.2	99.7

[a] Maximum residue level according to Council Regulation (EEC) No. 2377/90.

Table 6
Within-day ($n = 6$) and between-day (over a period of six consecutive days) precision and accuracy data for the determination of quinolones in egg yolk

Analytes	Added (μg/kg)	Within-day			Between-day		
		Found ± S.D. (μg/kg)	R.S.D.	Recovery (%)	Found ± S.D. (μg/kg)	R.S.D.	Recovery (%)
Egg yolk (HPLC system 2)							
ENO	25	25.7 ± 0.1	1.3	102.8	24.3 ± 3.1	1.7	97.2
	50	48.2 ± 1.5	0.7	96.4	50.1 ± 0.9	2.8	100.2
	100	99.4 ± 1.3	0.5	99.4	99.3 ± 1.2	3.0	99.3
OFL	25	25.7 ± 0.4	2.4	102.8	24.6 ± 1.2	3.2	98.4
	50	49.6 ± 0.3	3.0	99.2	49.3 ± 1.1	2.8	98.6
	100	99.8 ± 1.2	1.4	99.8	97.5 ± 2.7	1.4	97.5
NOR	25	24.6 ± 2.3	2.1	98.4	25.6 ± 1.3	1.8	102.4
	50	49.7 ± 1.3	1.8	99.4	49.7 ± 1.2	2.8	99.4
	100	101.2 ± 1.5	1.3	101.2	102.1 ± 0.4	1.7	102.1
CIP	25	25.4 ± 1.1	1.9	101.6	25.6 ± 1.3	3.2	102.4
	50	50.4 ± 1.3	0.4	100.8	50.2 ± 2.2	4.1	100.4
	100	99.3 ± 0.8	2.5	99.3	98.8 ± 1.2	1.3	98.8
DAN	25	24.1 ± 1.2	2.1	96.4	24.6 ± 1.3	2.7	98.4
	50	49.4 ± 0.2	1.1	98.8	50.6 ± 1.1	1.8	101.2
	100	98.7 ± 1.4	1.2	98.7	97.8 ± 2.4	4.0	97.8
ENR	25	24.4 ± 1.4	3.2	97.6	25.0 ± 2.3	1.8	100.0
	50	48.3 ± 0.7	1.3	96.6	48.3 ± 3.2	1.3	96.6
	100	99.2 ± 0.8	2.3	99.2	98.7 ± 1.6	2.4	98.7
SAR	25	25.6 ± 0.4	3.1	102.4	24.5 ± 1.4	3.1	98.0
	50	51.2 ± 1.1	1.6	102.4	48.3 ± 2.1	2.7	96.6
	100	96.4 ± 2.4	3.2	96.4	99.2 ± 1.8	0.7	99.2
OXO	25	25.2 ± 1.4	3.4	100.8	24.9 ± 1.3	3.1	99.6
	50	50.1 ± 0.7	4.0	100.2	50.4 ± 1.3	2.7	100.8
	100	101.3 ± 0.6	1.8	101.3	98.4 ± 2.3	2.9	98.4
NAL	25	25.6 ± 0.3	0.8	102.4	25.4 ± 1.7	3.7	101.6
	50	49.3 ± 1.2	1.2	98.6	50.4 ± 2.1	3.2	100.8
	100	99.7 ± 2.0	4.0	99.7	99.6 ± 1.5	1.3	99.6
FLU	25	24.7 ± 1.4	2.6	98.8	24.9 ± 0.4	2.3	99.6
	50	50.2 ± 0.4	2.2	100.4	48.8 ± 1.6	2.8	97.6
	100	98.1 ± 2.3	3.1	98.1	99.1 ± 2.6	3.3	99.1

Table 7
Calculations of error α and β, as well as decision limits (CC_α) and detection capabilities (CC_β) at the LOQ levels of the method and at the MRLs for the quinolones which are specified for chicken tissues (μg/Kg).

Analytes	Added (μg/kg)	Measured ± S.D. (μg/kg)	Error α (1.64 × S.D.)	CC_α (μg/kg)	Added (μg/kg)	Measured ± S.D. (μg/kg)	Error β(1.64 × S.D.)	CC_β (μg/kg)
ENO	15	16.68 ± 1.75	2.87	17.87	18	18.43 ± 0.18	0.30	18.30
OFL	25	24.21 ± 0.38	0.62	25.62	26	25.75 ± 0.12	0.20	26.20
NOR	15	12.53 ± 0.39	0.64	15.64	16	16.42 ± 0.16	0.26	16.26
CIP	25	24.69 ± 0.04	0.06	25.06	25	24.97 ± 0.14	0.23	25.23
DAN	37	35.63 + 0.40	0.66	37.66	38	37.70 + 0.31	0.51	38.51
	200[a]	197.19 ± 3.20	5.25	205.25	205[a]	206.10 ± 0.23	0.38	205.38
ENR	37	35.89 ± 0.543	0.88	37.88	38	38.32 ± 1.32	2.16	40.16
	100[a]	96.52 ± 4.38	7.18	107.18	107[a]	107.60 ± 1.32	2.16	109.16
SAR	15	14.30 ± 0.98	1.61	16.61	17	17.10 ± 0.59	0.97	17.97
OXO	15	16.99 ± 0.01	0.02	15.02	15	16.99 ± 0.01	0.02	15.02
NAL	25	25.63 ± 0.17	1.08	25.08	25	25.00 ± 0.07	0.11	25.11
FLU	25	25.54 ± 1.05	1.72	26.72	27	28.55 ± 0.81	1.33	28.33
	400[a]	399.93 ± 2.99	4.90	404.90	405[a]	401.85 ± 1.25	2.05	407.05

[a] Maximum residue level according to Council Regulation (EEC) No. 2377/90.

Table 8
Calculations of error α and β, as well as decision limits (CC_α) and detection capabilities (CC_β) at the LOQ levels of the method and at the MRLs for the quinolones which are specified for egg yolk (μg/kg)

Analytes	Added (μg/kg)	Measured ± S.D. (μg/kg)	Error α (1.64 × S.D.)	CC_α (μg/kg)	Added (μg/kg)	Measured ± S.D. (μg/kg)	Error β (1.64 × S.D.)	CC_β (μg/kg)
ENO	25	24.53 ± 1.63	2.67	27.67	28	29.14 ± 1.83	3.00	31.00
OFL	25	25.20 ± 0.52	0.85	25.85	26	25.37 ± 0.24	0.39	26.39
NOR	25	25.17 ± 1.76	2.89	27.89	28	28.33 ± 1.76	2.89	30.89
CIP	25	24.81 ± 2.17	3.56	28.56	28	29.02 ± 1.46	2.39	30.39
DAN	25	24.48 ± 1.18	1.94	26.94	27	26.00 ± 0.63	1.03	28.03
ENR	25	25.10 ± 0.37	0.61	25.61	26	25.98 ± 1.91	3.13	29.13
SAR	25	24.27 ± 0.40	0.66	25.66	26	25.77 ± 1.79	2.94	28.94
OXO	25	24.58 ± 1.18	1.94	26.94	27	26.02 ± 1.46	2.39	29.39
NAL	25	26.00 ± 0.22	0.36	25.36	25	25.27 ± 1.61	2.64	27.64
FLU	25	24.68 ± 0.47	0.77	25.77	26	25.90 ± 0.64	1.05	27.05

for chicken muscle and between 96.4 and 102.8% for egg yolk. All R.S.D. values were lower than 4.2% for chicken muscle and lower than 4.0% for egg yolk.

3.3.4. Decision limit and detection capability

According to the 2002/657/EC decision the two novel criteria CC_α (limit of decision) and CC_β (capability of detection) were calculated for both methods in order to complete the validation procedure. The decision limits (CC_α) were calculated as the mean values of the found concentrations plus 1.64 times the corresponding standard deviations. The detection capability (CC_β) values were obtained after spiking the samples at the CC_α levels by adding 1.64 times the corresponding standard deviation. Table 7 summarises the obtained CC_α and CC_β for chicken tissues at the LOQ level of the method for each quinolone and at the MRL for DAN, ENR and FLU. For the measurements of CC_α and CC_β 20 blank milk samples were spiked, respectively. The same procedure was followed for the method of spiked egg yolk samples. Table 8 summarises the obtained CC_α and CC_β for egg yolk at the LOQ level of the method for each quinolone.

4. Concluding remarks

In the present work two different methods were developed for the simultaneous determination of ten quinolones in chicken muscle and in egg yolk, respectively. Both methods were validated according to 2002/657/EC European decision and the results of validation process demonstrate that the method is suitable for any surveillance programme for veterinary drug residue in European Union.

Following these two methods 10 samples of chicken tissues and 10 of egg yolk were analyzed all from different sources. No residues of quinolones were detected.

The methods proved to be quite flexible. *HPLC method 1* developed for chicken muscle proved to be suitable for egg yolk and vice versa for *HPLC method 2*. The major difference between the two analytical methods is the instrumentation, but both methods are applicable for both matrices.

The accuracy of the methods was tested by obtaining average recoveries ranging between 96.6 and 102.8% for chicken muscle and between 96.4 and 102.8% for egg yolk. All R.S.D. values were lower than 4.2% for chicken muscle and lower than 4.0% for egg yolk.

To conclude both methods developed herein are quite easy to be applied considering also the section of sample preparation which is also easy to implement for both methods with quite good recoveries.

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