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Determination of Quinolononones in Food of Animal Origin by Liquid Chromatography Coupled with Fluorescence and Mass Spectrometric Detection

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Summary. High-performance liquid chromatography coupled with fluorescence (HPLC–FD) and tandem mass spectrometric detection (LC–MS/MS) was studied as a versatile tool for fast and reliable determination of nine regulated quinolones in food of animal origin (Council Regulation 2377/90/ECC). The sample pre-treatment protocol includes double step extraction with acetonitrile followed by solid phase extraction (SPE) cleanup on hydrophobic–lipophilic balance (HLB) cartridge. The separation of quinolones in HPLC–FD determination was performed on C18 Zorbax column with a gradient mixture of aqueous formic acid, methanol, and acetonitrile. A multi-wavelength excitation/emission program was used for sensitive quinolones detection. The separation efficiency of newly available chromatographic columns: Gemini C18 and Synergi Polar RP (fully porous particles), as well as Kinetex PFP and Poroshell 120 EC-C18 (core–shell particles), was studied in liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS) analysis. Appropriate gradient elution program was designed for each column. Multiple reaction monitoring was used for selective determination of each quinolone. LC–MS/MS allowed quinolones determination in less than 5 min. Both methods showed detection limits below maximum residue limits for quinolones residues in food commodities.

Key Words: quinolones, food analysis, fluorescence detection, HPLC, LC–MS/MS

Introduction

Quinolones are broad-spectrum synthetic antimicrobial agents used for treatment and prevention of diseases or for mass-growth promotion of livestock and aquacultures. The quinolone-based antibiotics are derivates of nalidixic acid containing different alkyl or aryl groups at position 1 and fluoro and piperazinyl substitutions at positions 6 and 7, respectively. The derivates are classified as first, second, and third generation depending on their biological and pharmacological activities [1, 2]. The structure and chemical properties of quinolones are well studied and described in a number of papers [1–5].

Widespread administration of quinolones to animals leads to occurrence of quinolone residues in food products of animal origin. The residues provoke antibiotics-resistance of foodborne pathogens. The European Human Health authorities have imposed severe criteria for food quality and have recently published maximum residue limits (MRLs) for residues of veterinary drugs in food: Regulation (EC) 470/2009 and 37/2010 [6]. Thorough reviews on the recent advances in quinolones determination in food and environmental samples have been published [1, 2, 5, 7]. The mostly reported methods are based on liquid chromatography coupled with fluorescence detection (FD) [4, 5, 8–12] or different mass spectrometric techniques (MS) [3, 13–18]. Almost all of them are multiresidue methods aiming to increase the rapidity and simplicity of the existing ones. Detection limits of methods based on fluorescence or MS detection range between tenths ng kg^{-1} to a few ng kg^{-1}. All the methods are sensitive enough to detect quinolones far below the MRL. The need for monitoring of a number of drug residues in a broad spectrum of matrices provokes intense research on developing and validation of fast and cost-effective analytical methods for residue determination at trace levels in ecologically and user-friendly manner.

New trends in fast liquid chromatography and online sample preparation techniques applied in food and environmental analysis have been recently published [19, 20]. They include a selection of the most relevant papers recently published regarding instrumental and column technology and the use of new stationary phases focusing on environmental and food applications, particularly monolith columns, high temperature separations, ultra high-performance liquid chromatography (UHPLC) methods with sub-2 μm columns, and novel porous shell particle packed columns. The application of new generation chromatographic separation techniques based on commercially available columns in quinolones determination in food samples is very scarce. Although the sub-2 μm chromatographic columns allow fast separations with improved efficiencies [21], only two of their applications for determination of quinolones in food were mentioned in the reviews [22, 23]. We can also add the newly published works on the development of multiclass ultra-high pressure liquid chromatography tandem mass spectrometry (UHPLC–MS/MS) methods for antibiotics residue determination in chicken, fish, bee products, baby food, and in milk [14, 24–28], or UHPLC-Orbitrap MS for veterinary drugs determination in honey [29]. A multiclass determination of 41 antibiotics in one chromatographic run of 13 min [29] and determination of 23 quinolones in 10 min [14] was achieved. Another trend in chromatographic separation described by Nunez et al. [19] and Farré et al. [20] is the use of monolith columns, which provide higher separation efficiency than in the case of fully porous columns. A liquid

chromatography–ultraviolet (LC–UV) method for quinolones determination after separation on monolith C18 column was proposed for analysis of environmental samples achieving analysis time of 14 min [30]. A hydrophilic interaction liquid chromatography with MS detection (HILIC–MS) for determination of quinolones in food samples was proven to allow determination of antibiotics from different classes in 8 min [31]. Although core–shell (fused-core) particles make possible to achieve a fast chromatographic separation using conventional HPLC systems, to the best of our knowledge, they are used only in a few methods for quinolone determination in food analysis [32, 33]. The work of Jimenez et al. [32] presents a comparative study of HPLC–FD after separation on C8 fully porous and Kinetex core–shell C18 columns. Nine quinolones were separated on the core shell particles packed column in 27 min with LOD between 1 μg kg^{-1} and 75 μg kg^{-1} in eggs extracts.

The determination of antibiotics residue in food commodities is always challenging due to the complexity and diversity of sample matrices. Matrix-dependent signal suppression or enhancement represents a major drawback in quantitative analysis of complex samples even when MS/MS detection is used [13]. Because the matrix effects might exert a negative impact on the analytical method characteristics, sample pre-treatments, involving isolation of analytes, purification of extracts and pre-concentration, are required. These steps often constitute the most time- and labor-consuming parts of the analytical process, requiring the optimization in each of the studied matrices. Recently, we have proposed a new sample pre-treatment protocol and a HPLC–FD method for quinolones determination in food of animal origin [34]. The protocol consists of a two-step acetonitrile-based liquid extraction and a solid phase extraction (SPE) cleanup on hydrophobic–lipophilic balance (HLB) cartridge. The procedure was efficient enough to be applied for nine quinolones separation from six matrices without additional modification. The HPLC–FD determination of nine quinolones took 22 min, resulting in a reduction of solvent consumption and analysis time. The method was validated in eggs, milk, fish, ovine muscle, chicken muscle, and porcine kidney according to Commission Decision 2002/657 EC [35].

Here we present the analytical performance of HPLC–FD method at lowest detectable concentration levels of nine regulated quinolones. In an attempt to improve the analysis time and sensitivity, we studied the efficiency of four types of new chromatographic columns (fully porous and core–shell particles based on different phases) for separation of nine quinolones followed by tandem mass spectrometric detection. Quinolones were determined using previously described protocol for sample pre-treatment [34].

Experimental

Reagents, Solutions, and Samples

Norfloxacin (Nor), ciprofloxacin (Cip), enrofloxacin (Enro), difloxacin hydrochloride (Dif), and nalidic acid (Nal) were purchased from Sigma Aldrich (Steinheim, Germany); flumequine (Flu), from Fluka (Buchs, Switzerland); danofloxacin (Dan), from Riedel de Haen (Seelze, Germany); and sarafloxacin hydrochloride trihydrate (Sar) and oxolinic acid (Oxo), from Dr. Ehrenstorfer (Augsburg, Germany). All reagents were of Vetranal grade. Acetonitrile (ACN) and methanol from Labscan (Dublin, Ireland) used in sample pre-treatment or HPLC experiments were of p.a. and HPLC grade, respectively. Formic acid and NaOH (p.a. grades) were supplied from Merck (Darmstadt, Germany). All working solutions were prepared with deionized water (18.2 MΩ cm resistivity) generated by ELGA system (Marlow, United Kingdom).

Stock standard solutions of quinolones (0.1 g L^{-1}) were prepared by dissolving 10 mg of quinolone in 50 μL of 3 mol L^{-1} NaOH and diluting up to 100 mL with methanol. The stock solutions were kept at 4°C in polypropylene volumetric flasks up to 3 months. Working standard solutions (2.5 mg L^{-1}) were prepared by appropriate dilution of stock solutions with methanol. Working solutions for HPLC analysis were freshly prepared by appropriate dilution with mobile phase: 0.1% aqueous HCOOH and acetonitrile (90:10; *v*/*v*). Spiking solutions were prepared by mixing appropriate volumes of individual quinolones stock solutions and diluting to the final concentrations. The ammonium acetate buffers (pH 9 and 4) were prepared by dissolving 0.15 g ammonium acetate in 100 mL deionized water and adding appropriate quantity of acetic acid or ammonium hydroxide solution. The final pH of buffer solutions was potentiometrically controlled with an Inolab Level 1 pH meter WTW 282 (Wissenschaftlich-Technische Werkstätten, Weilheim, Germany) equipped with a combined glass electrode. The total concentration of the buffers was 20 mM.

Chicken muscle, ovine muscle, porcine kidney and muscle, milk, fish, and eggs samples were purchased from the Bulgarian market. To ensure that they are blank tissues, regarding antimicrobial/quinolone residues with absence of bacterial inhibition, the method of five-plate test was conducted. The samples were minced and stored at −18°C. They were thawed before analysis. Spiked samples were prepared by adding appropriated volumes of spiking solution of quinolones to each portion of homogenized samples.

Sample Preparation

Extraction Procedure

One gram of minced sample was placed into polypropylene tube, 10 mL ACN was added, and the sample was homogenized for 10 min at room temperature. Then the sample was centrifuged for 10 min at 0°C, 7500 rpm using a centrifuge Sigma 3K15 (Osterode am Harz, Germany). The liquid phase was separated, and the extraction was repeated with addition of 10 mL ACN to the precipitate. Both liquid phases were combined and evaporated under N_2 stream at 60°C maintained by water bath using a vacuum evaporation system (Buechi Labortechnik, Flawil, Switzerland). The dry extract was dissolved in 5 mL 20 mM ammonium acetate buffer (pH 9) by a vortex mixer (Heidolph Instruments, Schwabach, Germany) and centrifuged for 5 min (0°C, 7500 rpm).

Clean-up Procedure

The SPE was carried out on solid-phase extraction manifold SUPELCO with OASIS hydrophobic–lipophilic balance (HLB) cartridge (200 mg, 3 mL) with 3-µm particle size from Waters Corporation (MD, USA). The HLB cartridge was activated with MeOH (1 mL) and equilibrated using ammonium acetate buffer (pH 3; 1 mL). After the samples loading, the cartridge was cleaned with 1 mL deionized water. The analytes were eluted with 10 mL 0.2% HCOOH in ACN. The obtained eluate was evaporated in a nitrogen evaporator at 70°C (water bath) and the dry residue was dissolved in 1 mL mixture of 0.1% aqueous HCOOH and ACN (90:10; v/v). The sample was filtered through a 0.45-µm nylon filter into an LC vial.

HPLC–FD Analysis

The separation of the analytes was carried out using LC System Agilent 1100 (Agilent Technologies, Morges, Switzerland) equipped with an analytical column and a pre-column, both Zorbax Eclipse XDB C18 (150 × 3 mm, 5 µm particle size). The mobile phase consisted of 0.1% (v/v) formic acid in water (eluent A), acetonitrile (eluent B) and MeOH (eluent C) delivered under gradient program. The analysis started with 83% of eluent A, 15% eluent B and 2% eluent C. After 9 min, the composition was 78% of eluent A and 20% of eluent B. The percentage of eluent C was kept constant (2%) during the whole elution. After 8 min, the percentage composition of mobile phase was changed to: 63% of eluent A and 35% of eluent B and kept for 5 min. The next change of mobile phase composition was performed at

23 min after the beginning of the experiment: 58% of eluent A and 40% of eluent B. At 26 min, from the beginning of the experiment, the composition of the mobile phase was 83% of eluent A and 15% of eluent B. Total running time was 27 min. The flow rate was 0.5 mL min^{-1}, and the column temperature was set at 50°C. Aliquots of 25 μL of the sample extract were injected into the chromatographic system.

The HPLC system was coupled to a fluorescence detector with a 150-W xenon lamp. The fluorescence detection was performed under a multi-step detection mode [33]: from 0 to 12 min: λ_{ex} = 280 nm and λ_{em} = 450 nm; from 12 to 23 min: λ_{ex} = 321 nm and λ_{em} = 360 nm. Instrumental control and data analysis were performed by Chemstation application software from Agilent Technologies.

LC–MS/MS Analysis

The quinolones determination was carried out in a LC–MS/MS System TSQ Quantum Discovery MAX (Thermo Electron Corporation). The evaluated analytical chromatographic columns were Gemini C18 (150 mm × 2.00 mm, 5 μm, 110 Å); Kinetex PFP (50 mm × 2.10 mm, 2.6 μm, 100 Å) and Synergi Polar RP (50 mm × 2.00 mm; 2.5 μm) from Phenomenex Inc. (Torrance, USA), as well as Poroshell 120 EC-C18 (3.0 mm × 100 mm, 2.7 μm) from Agilent Technologies (Morges, Switzerland) equipped with the same pre-columns. Different gradient programs were followed using a mixture of 0.1% (*v*/*v*) formic acid in acetonitrile and 0.1% (*v*/*v*) formic acid in deionized water as shown in *Table I*. The flow rate was 350 μL min^{-1} (Gemini) and 500 μL min^{-1} (others). The column temperature was set at 25°C.

Table I. Gradient elution programs for LC–MS/MS using different chromatographic columns

Column	Poroshell 120 EC-C18 Kinetex PFP		Synergi Polar RP		Gemini C18		
Time, min	ACN[a], %	H_2O[a], %	ACN[a], %	H_2O[a], %	Time, min	ACN[a], %	H_2O[a], %
0	80	20	85	15	0	95	5
0.5	80	20	85	15	6.0	70	30
4.0	45	55	45	55	10.0	30	70
4.2	20	60	20	60	12.0	30	70
5.0	80	20	85	15	12.5	95	5
8.0	80	20	85	15	15.0	95	5

[a]All solvents contained 0.1% (*v*/*v*) HCOOH.

The electrospray ionization–tandem mass spectrometry (ESI–MS/MS) detection of the quinolones was achieved using a triple stage quadrupole instrument. The positive ionization mode was used, and the ions were monitored in the multiple-reaction monitoring (MRM) mode. The ESI–MS/MS conditions were the following: spray voltage 3 kV; sheath gas (N_2, >95%) 23 (arbitrary units); auxiliary gas (N_2, >95%) 0 (arbitrary units); capillary offset/voltage 35 V, capillary temperature 300°C. The cone voltage and collision energy for MRM acquisitions are presented in *Table II*. The dwell time was 100 ms/transition. Two transitions were followed for identification but only one was used for quantitation (in bold in *Table II*). Instrumental control and data analysis were performed by Qualbrauser application software from Thermo Electron Corporation.

Table II. MS/MS conditions selected in multiple reactions mode for quinolones identification and quantification (fragment ions in bold were used for quantification)

Compound	Parent ion (m/z)	Fragment ion (m/z)	Collision energy (eV)
Norfloxacin	320.1	**276**, 302	14
Ciprofloxacin	332.2	**231**, 288	14
Danofloxacin	358.1	283, **340**	14
Enrofloxacin	360.3	**245**, 316	12
Difloxacin	400	**299**, 356	12
Sarafloxacin	386.2	**299**, 342	12
Oxolinic acid	262.1	**216**, 244	14
Nalidixic acid	233.2	187, **215**	12
Flumequine	262.1	**202**, 244	14

Analytical Performance

The evaluated characteristics were: specificity, linear range, recovery, and precision (repeatability and intra-laboratory reproducibility). For analytes with established maximum residue limit (MRL), analytical characteristics were determined at concentrations equal to 0.5 MRL, 1 MRL, 1.5 MRL, 2 MRL, and 2.5 MRL. For quinolones without established MRL or maximum required performance limit, recovery and precision were estimated at concentration levels corresponding to the lowest linear calibration point, here labelled as LCP: 1, 1.5, 2, and 2.5 times LCP. Six samples at each concentration level were prepared by spiking with a mixture of quinolones. All spiked samples were submitted to liquid extraction, SPE, and HPLC proce-

dure in triplicate. The calibration range of each quinolone was (0.5–2.5) × MRL or (1–2.5) × LCP. The method specificity was estimated by comparing the chromatograms of a mixture of nine studied quinolones obtained in a standard solution, spiked matrix, and blank matrix samples. Accuracy and precision of the method were evaluated by estimating recoveries and reproducibility for all analytes in each studied matrix. Six blank samples of each matrix (1 g) were spiked by standard mixture of nine quinolones at four concentration levels from 0.5 to 2.5 MRL/LCP. Spiked samples were pre-treated and analyzed in triplicate. The recoveries were calculated as the ratio of obtained mean over nominal concentration (in per cent). Matrix-matched calibration curves were used.

Results and Discussion

HPLC with Fluorescence Detection (HPLC/FD)

The optimization of the liquid extraction and SPE cleanup procedures in terms of number of the extractions, type and volume of the extraction solvent, solvent evaporation technique, SPE sorbent, and elution solvents were described in our previous study [34]. The nine studied quinolones were separated by liquid chromatography on C18 Zorbax column. Aqueous formic acid, methanol, and acetonitrile were used as a mobile phase with gradient elution program and a two-phase multi-wavelength excitation/emission program for sensitive fluorescence detection of quinolones. Good separation of all of nine quinolones was achieved in run of 22 min *(Fig. 1a)*. The chromatograms of blank and spiked with nine quinolones porcine muscles are presented in *Fig. 1*. Matrix effect was evaluated by comparing the calibration parameters obtained from standards in solvent and from spiked blank matrix. The lowest and the highest slope ratios are presented in *Fig. 2*. As can be seen from the figure, eggs and porcine kidney influenced negatively the slope of the calibration plots of all studied quinolones. A calibration slope above 100% was observed in limited number of cases: Enro in eggs, Oxo and Flu in ovine muscle, and Nor in chicken muscle. Therefore, matrix-matched calibration was recommended for quinolones quantification. Linearity was evaluated in the range (0.5–2.5) × MRL or (1–2.5) × LCP (LCP is the lowest linear calibration point), and the calibration results are presented in *Table III*. Trueness was estimated in terms of recovery. As recovery is affected by the matrix and might vary as a function of the concentration [35], recovery was calculated at each concentration level corresponding to 0.5, 1, 1.5, 2, and 2.5 MRL/LCP by spiking the blank sample extracts obtained from every of the studied matrices. The recoveries at lowest evaluated concentrations are presented in *Table IV*. Precision of the method was

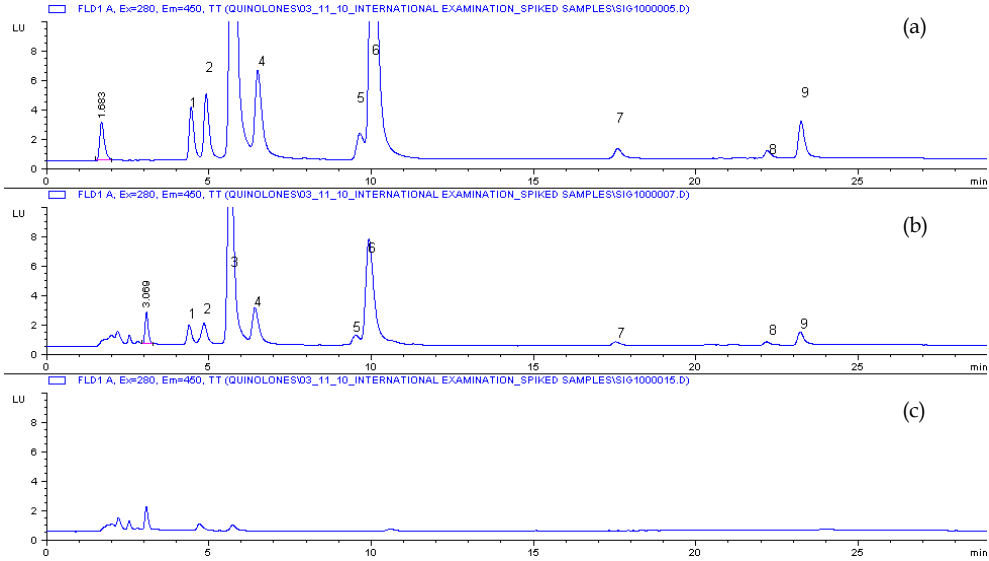

Fig. 1. HPLC–FD chromatograms of (a) quinolone standards, (b) spiked porcine muscle, and (c) blank porcine muscle obtained using C18 Zorbax column with ACN–MeOH–HCOOH mobile phase and gradient elution program: (1) norfloxacin 25 μg kg^{-1}; (2) ciprofloxacin 100 μg kg^{-1}; (3) danofloxacin 200 μg kg^{-1}; (4) enrofloxacin 100 μg kg^{-1}; (5) sarafloxacin 10 μg kg^{-1}; (6) difloxacin 300 μg kg^{-1}; (7) oxolinic acid 100 μg kg^{-1}; (8) nalidixic acid 25 μg kg^{-1}; (9) flumequine 400 μg kg^{-1}

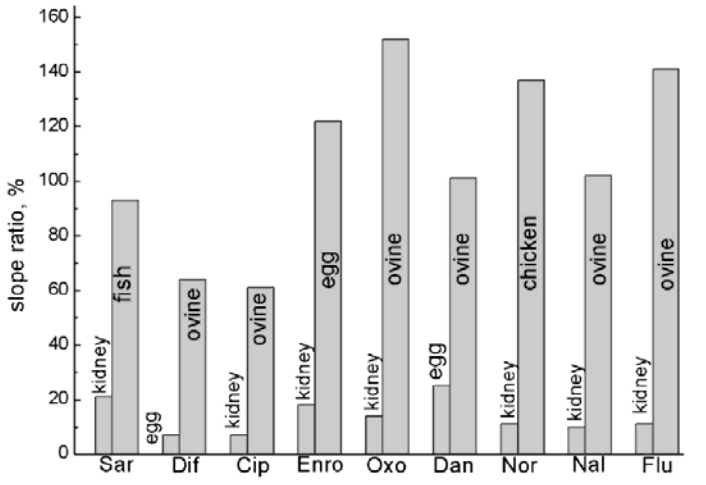

Fig. 2. Evaluation of the matrix effect on quinolones determination by HPLC–FD. Slope ratio is determined as a ratio between the slope of the calibration curve obtained from standard solutions and the slope of the calibration curve obtained from spiked blank tissue extracts

Table III. Calibration parameters in different matrices obtained by HPLC/FD method

Compound	Sara	Dif	Cip	Enro	Dan	Oxo	Nal	Nor	Flu
Eggs linear range, μg kg^{-1}	25–125	15–90	15–125	25–125	20–100	25–125	25–125	25–125	40–200
0	5.15	0.17	0.17	2.07	4.23	4.78	0.30	0.05	0.30
Intercept	5.17	0.69	0.28	4.42	11.1	0.37	0.14	0.38	0.26
r	0.987	0.993	0.994	0.992	0.982	0.979	0.970	0.996	0.982
Fish linear range, μg kg^{-1}	15–90	150–900	50–300	50–300	50–300	50–250	15–90	15–90	300–1800
Slope	7.84	0.02	0.26	0.88	4.23	0.09	0.50	0.15	0.11
Intercept	30.7	0.92	9.02	20.9	104.4	2.41	0.30	1.09	16.5
r	0.973	0.929	0.971	0.984	0.982	0.988	0.986	0.975	0.989
Milk linear range, μg kg^{-1}	15–75	15–75	25–125	25–125	15–75	15–75	25–125	25–125	25–125
Slope	1.53	0.50	0.722	1.83	10.3	0.96	0.71	0.74	0.27
Intercept	1.06	6.48	4.13	13.1	27.1	1.37	0.05	3.98	0.83
r	0.991	0.970	0.972	0.951	0.922	0.984	0.993	0.952	0.998
Chicken muscle linear range, μg kg^{-1}	5–25	150–750	25–125	25–125	100–500	50–250	25–125	25–125	200–2000
Slope	5.73	0.24	0.05	0.83	6.07	0.02	0.05	0.21	0.10
Intercept	2.43	0.21	1.40	2.39	19.3	0.92	0.03	0.87	1.00
r	0.991	0.953	0.993	0.989	0.996	0.951	0.992	0.988	0.992
Ovine muscle linear range, μg kg^{-1}	50–250	200–1000	25–125	25–125	100–500	50–250	50–250	50–250	100–500
Slope	2.41	0.04	0.20	0.64	3.03	0.11	0.04	0.25	0.11
Intercept	11.6	0.36	1.15	3.32	48.1	0.81	0.07	1.49	4.69
r	0.975	0.971	0.956	0.973	0.968	0.953	0.962	0.983	0.955
Porcine kidney linear range, μg kg^{-1}	400–2000	400–2000	100–500	100–500	200–1000	75–375	75–375	75–375	750–3750
Slope	0.33	0.11	0.24	0.52	1.27	0.04	0.02	0.32	0.04
Intercept	219.1	71.3	18.9	53.4	102.5	4.67	3.87	39.6	122.4
r	0.998	0.989	0.981	0.981	0.971	0.991	0.988	0.989	0.960

evaluated by estimating repeatability (standard deviation (SD)) and intra-laboratory reproducibility (relative standard deviation (RSD), %) at each concentration level. The reproducibility at MRL or LCP levels was between 1% and 34%.

Table IV. Recovery in different matrices at different concentration levels, obtained by LC–FD method

Compound	Sara	Dif	Cip	Enro	Dan	Oxo	Nal	Nor	Flu
Eggs MRL, μg kg^{-1}	No								
Evaluated level, μg kg^{-1}	5	15	25	25	20	25	25	25	40
Recovery, %	65	106	92	55	95	50	124	77	107
Fish MRL, μg kg^{-1}	30	300	–	–	100	100	–	–	600
Evaluated level, μg kg^{-1}	15	150	50	50	50	50	15	15	300
Recovery, %	120	128	118	120	122	116	119	111	116
Ovine muscle MRL, μg kg^{-1}	–	400	100		200	100	–	–	200
Evaluated level, μg kg^{-1}	50	200	50	50	100	50	50	50	100
Recovery, %	84	64	96	81	80	81	86	78	119
Milk MRL, μg kg^{-1}	–	–	100		30	–	–	–	50
Evaluated level, μg kg^{-1}	15	15	50	50	15	15	25	25	25
Recovery, %	91	75	100	111	102	61	108	117	97
Chicken muscle MRL, μg kg^{-1}	10	300	100		200	100	–	–	400
Evaluated level, μg kg^{-1}	5	150	50	50	100	50	25	25	200
Recovery, %	80	57	75	90	100	124	113	95	99
Porcine kidney MRL, μg kg^{-1}	–	800	200		400	150	–	–	1500
Evaluated level, μg kg^{-1}	400	400	100	100	200	75	75	75	750
Recovery, %	100	120	90	67	125	56	40	60	42

High-Performance Liquid Chromatography–Tandem Mass Spectrometry (HPLC–MS/MS)

In order to shorten the separation time, reversed phase HPLC separation on a new generation chromatographic columns was studied: Kinetex PFP (core–shell particles), Poroshell 120 EC C18 (core–shell particles), Synergi Polar RP (fully porous particles), and Gemini C18 (fully porous particles). The core–shell particles are specially designed to reduce diffusion and to work at low back pressure generating at the same time high plate counts, less band broadening, and high efficiency of the separation process. The phenyl bonded phase (Synergi and Kinetex columns) provides π–π interactions with conjugated compounds, increasing the retention of polar compounds. The exhausted polar or non-polar endcapping (Synergi and Poroshell columns) reduces the interactions between the free silanol groups and the nitrogen moiety of the quinolones reducing peak tailing. The composition of the mobile phase and the gradient elution programs was optimized to be effective in different columns in an attempt to ensure easy

transfer across HPLC systems. The mobile phase consisted of a mixture of 0.1% formic acid in acetonitrile and 0.1% formic acid in water. Several gradient programs were tested to ensure optimum quinolones separation on each of the studied chromatographic columns taking into account that two of the studied quinolones have the same molecular mass and may interfere in the case of co-elution [37, 38]. The results showed that all of the studied quinolones can be separated using the same gradient program on the two studied core–shell particles based columns: Kinetex PFP and Poroshell 120 EC-C18 *(Table I)*. Good separation on Synergy Polar RP column was obtained by increasing the organic phase up to 85% following the same time program as in the case of Kinetex and Poroshell columns *(Table I)*. Higher content of organic phase (95–30%) and a different elution program were used to achieve good separation on Gemini column *(Table I)*. The achieved analysis time for separation of all of the nine studied quinolones was 5 min on Poroshell column, 8 min on Kinetex and Synergy columns, and 15 min on Gemini column. Although the separation time on Gemini column was higher than other studied columns, it is short enough to ensure good separation at low reagent and analysis time consumption.

The MS/MS detection parameters were also optimized starting with the parameters described in [37, 39]. In our work, two product ions for each determined compound have been selected (from their corresponding precursor ions), which complies with the requirements proposed in current EU legislation for a minimum total score of 3 identification points for confirmatory MS method [35]. In this way, each compound is precisely characterized by its retention time as well as two precursor–product ion transitions. The most intense product ion was employed for quantification whereas the other ions were used for conformation *(Table II)*. The MS spectra obtained at 10 μg kg^{-1} spiking level of each of the studied quinolones after separation on four tested chromatographic columns are presented in *Fig. 3*. As can be seen from the figure, the peaks are well shaped and separated, allowing selective and sensitive quinolones determination. Because the molecular weights of Oxo and Flu are identical, their identification was also based on the retention time. The most pronounced difference was observed on Kinetex PFP and Poroshell 120 EC-C18 columns (1.13 and 1.07 min, respectively). Despite of the fact that the separation of Oxo and Flu on Gemini and Synergy columns was 0.7 min, it is enough for the identification of two compounds.

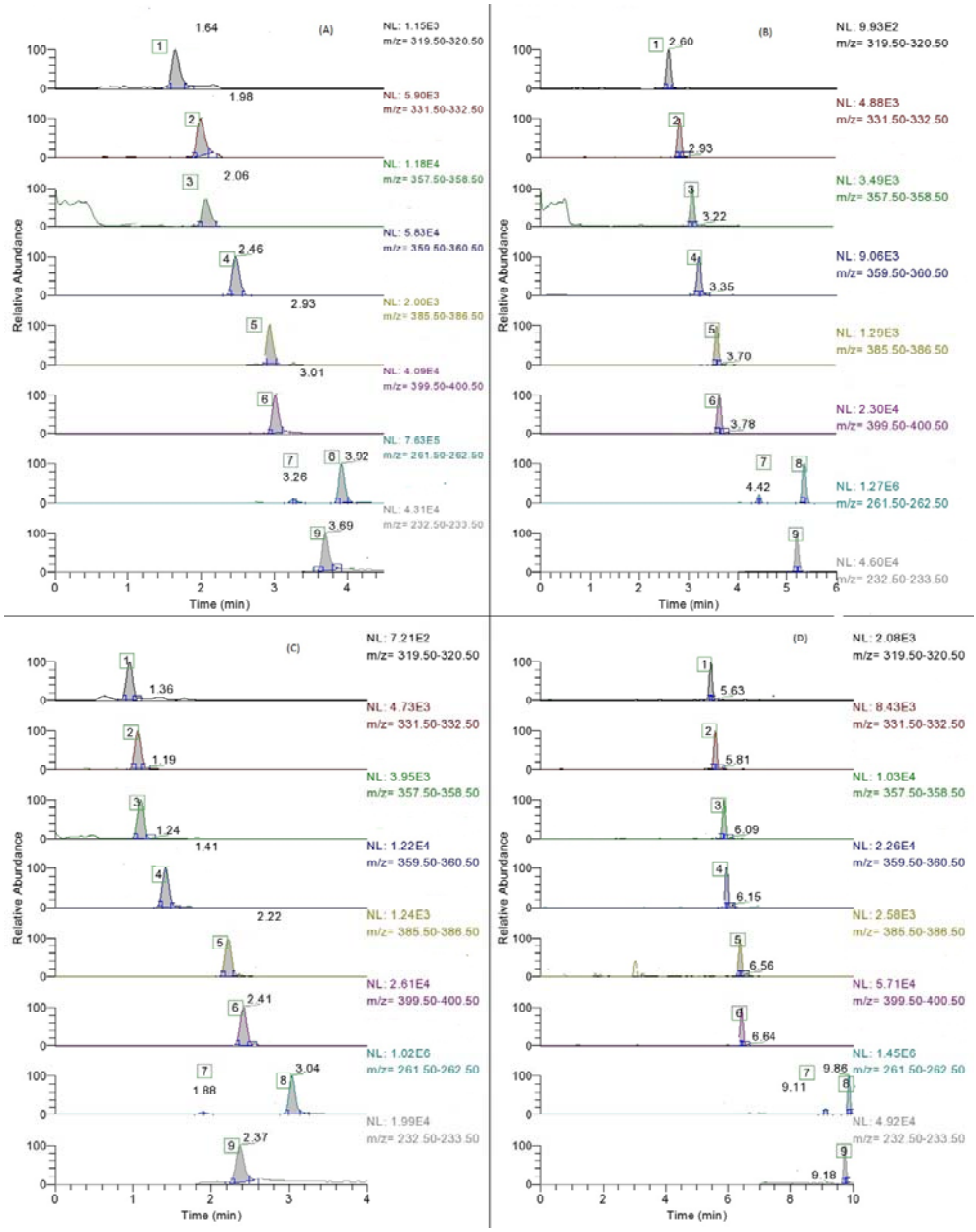

Fig. 3. LC–MS/MS chromatograms of quinolones in MRM mode at 10 μg kg^{-1} each: (1) norfloxacin, (2) ciprofloxacin, (3) danofloxacin, (4) enrofloxacin, (5) sarafloxacin, (6) difloxacin, (7) oxolinic acid, (8) nalidixic acid, (9) flumequine, obtained by separation on: (A) Synergi Polar RP, (B) Poroshell 120 EC C18, (C) Kinetex PFP, and (D) Gemini C18 columns

The quality parameters of LC–MS/MS method, linear range, matrix effect, recovery, and precision, were studied in fish tissue. The previously reported sample pretreatment protocol based on double step acetonitrile extraction and SPE cleanup on HLB column [34] was applied for LC–MS/MS determination of five regulated quinolones in fish: Sar, Dif, Dan, Oxo, and Flu [40]. When using electrospray ionization, the matrix components may affect the ionization of the target analytes by either reducing or increasing analyte signal. The matrix effect was estimated by comparing the slope and intercept of two sets of calibration curves: one obtained from standard solutions of quinolones and the other obtained from spiked fish extracts. Relative MS responses ranged from 40% (for Flu) to 100% (Dif), indicating some extent of signal suppression in the case of Sara, Dan, Oxo, and Flu and no matrix effect in the case of Dif. Hence, matrix-matched calibration curves, obtained by spiking with quinolones fish tissues extracts, were further used. The linearity was examined ranging from 0.5 MRL to 2 MRL for each of regulated quinolones, and correlation coefficients >0.96 were obtained. The recovery was used to assess the method accuracy, and 94% for Sar, 79% for Dif, 56% for Dan, 83% for Oxo, and 73% for Flu were obtained at MRL level with RSD below 30%.

Conclusion

The HPLC–fluorescence detection and LC–tandem mass spectrometry methods for nine quinolone determination in food of animal origin after liquid extraction and SPE cleanup presented here showed detection limits below MRL for quinolones residues in food commodities. The sample pretreatment protocol was applicable to both methods. The LC–MS/MS method offers the advantage of high sensitivity and selectivity as well as the possibility of identification and elucidating structures. LC–MS/MS coupled with newly available columns allows quinolones determination in less than 5 min. The HPLC–FD method offers ease of using and less expensive equipment. The selection of the detection technique would be according to the needs of the analysis.

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