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Validation of a method for the analysis of nine quinolones in eggs by pressurized liquid extraction and liquid chromatography with fluorescence detection

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ABSTRACT

A multiresidue method for the analysis, in egg matrices, of residues of nine quinolones used in veterinary medicine, has been developed and validated according to the provisions of Council Decision 2002/657. Compounds were extracted by a pressurized liquid extraction (PLE) technique using a 1:1 mixture of acetonitrile and a phosphoric acid buffer (pH 3.0) at 70 °C. The obtained extract was clear enough, so that no further clean-up was necessary. Analytes were determined by liquid chromatography (LC) with fluorescence detection (FL). Two chromatographic columns were compared: a high-purity silica Inertsil C_8 column and a newly developed Kinetex C_{18} core–shell technology column. Validation was carried out at four concentration levels, using both chromatographic columns. Precision in terms of reproducibility standard deviation was between 7% and 23%, and good recoveries were obtained. Decision limit (CCα) and detection capability (CCβ) values obtained with the Inertsil and Kinetex columns were in the 0.2–19.8 μg kg^{-1} and 0.4–33.5 μg kg^{-1} concentration ranges, respectively. The proposed method allows residues of quinolones banned for use with laying hens to be detected and quantified in eggs. About twenty-four samples per day can be processed.

1. Introduction

Quinolones are broad spectrum synthetic antimicrobial agents, derived from nalidixic acid, and are classified as first, second and third generation depending on their antibacterial spectrum, potency and pharmacology. The second and third generations include the fluoroquinolones, which contain a fluorine atom at the C-6 position and a piperazinyl group at the C-7 position and have better antimicrobial activity, as fluorine increases the activity against Gram-positive pathogens whereas the piperazino moiety improves the effectiveness against Gram-negative microorganisms. Quinolones inhibit the action of bacterial DNA gyrase enzymes, thus preventing DNA replication [1].

Quinolones are used in both human and veterinary medicine to treat an extensive range of diseases, including urinary, respiratory and gastrointestinal tract infections [2]. The widespread use of quinolones has led to a significant increase in antibacterial resistance, and this has had important consequences for public health [3]. In order to address these problems, and to increase food safety, the European Union (EU) has laid down a set of policies and measures, including the establishment of maximum residue limits (MRLs) for some antimicrobials, a network of reference laboratories and a system of alert notifications, among others [4]. A list of allowed substances with a MRL is given in the Annex I of Commission Regulation 37/2010 [5]. The quinolones included in this list are danofloxacin, difloxacin, enrofloxacin (expressed as the sum of enrofloxacin and ciprofloxacin), flumequine, marbofloxacin, oxolinic acid and sarafloxacin; MRL values depend on the tissue and the animal species. With the exception of marbofloxacin, all of them have been approved for the treatment of bacterial infections in poultry, but are banned for use in laying hens because there is evidence that residues of these antibiotics may reach the eggs and accumulate there [3,6–11].

As the use of quinolones as veterinary drugs for laying hens is banned in the EU, strict monitoring of the residues of these substances in eggs is crucial to enforce this ban and ensure food safety. In the last thirteen years, many studies have been published about the analysis of residues of quinolones in eggs; they are summarized in Table 1.

Preparation of the biological sample is one of the most crucial and difficult steps in residue analysis. Eggs in particular have a very complex matrix, with a high lipid and protein content; some analytes bind to lipoproteins, which hinders their extraction, while several organic solvents form emulsions and foams with the matrix [12]. In most cases, the analytes were extracted from the egg matrix by liquid extraction (LE), combined sometimes with a clean-up by either solid phase extraction (SPE) [13–21], microdialysis [10,22] or in tube solid phase microextraction (SPME) [7]. Acetonitrile (ACN) is considered to be the best extraction solvent, because it precipitates

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Table 1
Methods for the analysis of quinolones in eggs.

Analytes/compounds	Extraction	Clean-up	Analytical technique	Calibration	Limits ($ng\,g^{-1}$)	Recoveries	Remarks	Reference
ENR, CIP	• ACN	No	LC–UV	Standards in solvent	*LOD*: 19-156	36–85%	Analysis incurred eggs	[11]
CIP, DAN, decoquinate, enoxacin, ENR, FLU, lomefloxacin, MAR, nalidixic acid, NOR, ofloxacin, OXO, SAR	• ACN with acetic acid + sodium sulphate • ACN + sodium sulphate	SPE Bond Elut (cation exchange) SPE AGMP-1 resin(anion exchange)	LC –FL/UV	Standards in solvent	*LOQ*: 5–50	44–99%	Various food matrices and eggs	[13]
SAR	• ACN + NaCl	On-line microdialysis	LC–FL	Standards in solvent	*LOD*: 0.2 *LOQ*: 1	87–102%	Analysis incurred eggs	[10]
CIP, DIF, ENR, NOR, MAR	• HCl 1 M	SPE Sep-Pak C18	LC–UV	Standards in solvent	*LOD*: 1–4 ng injected	72–99%	Various tissues and eggs	[14]
NOR, CIP, SAR, ENR, DAN	• ACN with ammonium	On-line microdialysis	LC–FL	Standards in solvent	*LOD*: 0.3–3	65–110		[22]
CIP, ENR, SAR	• Phosphoric acid + ACN	No	LC–FL	Standards in solvent	*LOD*: 1–3	83–108%	Analysis incurred eggs	[9]
ENR, CIP	• Diphasic dialysis: citrate buffer pH 6.0	No	LC–MS	Standards in solvent	*LOQ*: 2–4	55–104%		[23]
CIP, NOR, DAN, ENR, orbifloxacin, SAR, DIF	• ACN with ammonium + hexane + NaCl	No	LC–FL LC–MS	Standards in solvent and matrix-matched standards	*LOQ*: 0.1–7 (egg white) 0.1–1.5 (egg yolk)	60–100%	Analysis incurred eggs	[8]
CIP, ENR, ofloxacin, NOR	• SFE: CO_2 + CO_2 with 20% MeOH	No	LC–FL	Standards in solvent	*LOD*: 10	76–97%		[24]
CIP, ENR	• MeOH with ammonium + hexane + ether + NaCl	No	LC–FL	Standards in solvent	$CC\alpha$: 2.8–3.1 $CC\beta$: 7.0–7.8	64–70%	D-optimal designs to optimization	[29]
CIP, DAN, DIF, ENR, FLU, MAR, nalidixic acid, NOR, OXO, SAR	• 5% trichloroacetic solution	No	LC–FL	Standards in solvent	$CC\alpha$: 44–235 $CC\beta$: 57–308	29–68%	Various food matrices and eggs	[30]
SAR, NOR, DIF, CIP, perfloxacin, ofloxacin, cinoxacin, DAN, ENR, MAR, lomefloxacin, enoxacin, FLU, OXO, nalidixic acid	• Phosphate-buffered saline pH 7.4/MeOH (1:1)	No	ELISA	Spiked blanks	$CC\alpha$: < 4 to < 200	–	Various food matrices and eggs	[33]
CIP, ENR, SAR, DIF Sulfonamides Tetracyclines B-lactans	• Aqueous sodium succinate buffer	SPE	LC–MS/MS	Standards in solvent, matrix-matched standards and spiked blanks compared	*LOD*: 10–20	45–55%	Screening purposes	[15]
Ofloxacin, NOR, CIP, ENR, SAR	• Phosphate buffer pH 4.1	In tube SPME	LC –FL/UV	Spiked blanks	*LOD*: 0.1–2.6 *LOQ*: 0.4–8.6	75–94%	Analysis incurred eggs	[7]
CIP, ENR, levofloxacin, NOR, ofloxacin, FLU, OXO, nalidixic acid	• Phosphate buffer/ACN (3:7)	SPE Bond Elute	LC–UV	Standards in solvent	*LOD*: 1–4	–	Poultry products and eggs	[16]
ENR, DIF, DAN, FLU, OXO	No	No	Microbial screening assay	Spiked blanks	$CC\beta$: ≤ 15 to ≤ 150	–	Poultry muscle and eggs	[32]
CIP, ENR, NOR, ofloxacin, pefloxacin, enoxacin, MAR, lomefloxacin, amifloxacin, DAN, OXO, FLU, SAR, DIF	• ACN + evaporation + phosphate-buffered saline + hexane	No	ELISA	Standards in solvent	*LOD*: 0.2 $ng\,mL^{-1}$ (NOR)	60–90%	Various food matrices and eggs	[34]
CIP, DAN, ENR, SAR	• ACN with acetic acid + NaCl + hexane	SPE Oasis HLB	LC–MS/MS	Matrix-matched standards	$CC\alpha$: 0.3–2.1 $CC\beta$: 0.6–4.23	86–110%		[17]

Table 1 (*Continued*)

Analytes/compounds	Extraction	Clean-up	Analytical technique	Calibration	Limits ($ng\,g^{-1}$)	Recoveries	Remarks	Reference
CIP, ENR, DAN, DIF, FLU, OXO, SAR	• Ammonium + ACN + CH_2Cl_2	No	LC–FL	Spiked blanks	*LOD*: 4–12 *LOQ*: 12–41	70–98%		[31]
ENR, CIP, SAR	• PLE: Phosphate pH 3.0/ACN (1:1)	No	LC–FL	Matrix-matched standards	*CCα*: 17–24 *CCβ*: 30–41	66–89%		[25]
Enoxacin, ofloxacin, NOR, CIP, DAN, ENR, SAR, OXO, naxilidic acid and FLU	• NaOH in ACN	SPE Li-Chrolut	LC–UV	Spiked blanks	*CCα*: 25–29 *CCβ*: 26–31	96–103%	Chicken muscle and egg yolk	[18]
Ofloxacin, pefloxacin, NOR, CIP, ENR	• MIPs as selective MSPD		LC–FL	Spiked blanks	*LOD*: 0.05–0.09	86–105%	Chicken eggs and swine tissues	[26]
CIP, DAN, ENR, NOR	• Trichloro-acetic acid + Na_2SO_4 + ACN + hexane	No	LC–FL	Matrix-matched standards	*LOQ*: 0.6–9	74–87%	Incurred eggs. Firstly monitored by microbiological assay	[6]
Quinolone, naphthyridine pyridopyrimidine, crinoline Tetracyclines	• EDTA–McIlvaine buffer	SPE Oasis HLB	LC–MS/MS	Spiked blanks	*LOD*: 0.01–1.30 *LOQ*: 0.02–4.29	71–112%		[19]
MAR, enoaxacin, ofloxacin, pefloxacin, NOR, CIP, lomefloxacin, DAN, ENR, SAR, DIF, OXO, FLU	• Phosphate buffer pH 4.0 + hexane	SPE HLB 60 mg 3 mL^{-1}	LC–MS/MS	Matrix-matched standards	*LOD*: 0.05–0.2 *LOQ*: 0.5–1	67–93%		[20]
NOR, CIP, DAN, DIF, enoxacin, ENR, FLU, lomefloxacin, MAR, ofloxacin, OXO, pefloxacin, SAR	• ACN + evaporation + phosphate-buffered saline + hexane	No	Surface Plasmon resonance biosensor assay	Matrix-matched standards	*CCβ*: < 0.5 (NOR)	123%	Various food matrices and eggs	[35]
DIF, FLU, MAR, OXO	• Phosphoric acid in water/MeOH (60:40)	SPE Oasis HLB	ELISA	Standards in solvent	*CCβ*: < 10	–	Various food matrices and eggs. Two different kits	[21]
MAR, NOR, CIP, ENR, DAN, DIF, OXO, FLU	MSPD: hot water acidified with formic acid	No	LC–MS/MS	Spiked blanks	*CCα*: 0.4–2.6 *CCβ*: 0.6–3.7	89–104%		[27]

proteins and denatures enzymes that could degrade drug residues during sample treatment. Half of the papers reported in Table 1 used ACN or ACN-aqueous buffer mixtures in the extraction, but methanol was also used as organic solvent in some cases. Other sample preparation techniques used for the analysis of quinolones in eggs were diphasic dialysis [23], supercritical fluid extraction (SFE) [24], pressurized liquid extraction (PLE) [25] and matrix solid phase dispersion (MSPD) [26,27]; all of them were performed without the need of an extra clean-up step. PLE combined the benefits of high-throughput, automation and low solvent consumption [28].

Most of the reported methods in Table 1 used liquid chromatography (LC) with ultraviolet (UV) [7,11,13,14,16,18], fluorescence (FL) [6–10,13,22,24–26,29–31] or mass spectrometry (MS) detection [8,15,17,19,20,23,27]. Other reported techniques included microbiological screening assays [32], enzyme-linked immunosorbent assays (ELISA) [21,33,34] and biosensors [35], which were used in screening methods. LC combined with tandem mass spectrometry detection permits the quantification and confirmation of a great number of antibiotics at trace levels, but requires expensive instrumentation, which is not widely available in many laboratories.

The objective of the present work was to develop a multiresidue method for the analysis in eggs of residues of quinolones that are currently used in veterinary medicine but are banned for use in laying hens. The analytes were extracted by PLE. Advantage was taken of the natural fluorescence of quinolones to develop a LC method with FL detection, allowing a sensitive and selective determination of these substances for routine residue analysis without any derivatization step. Two chromatographic columns were compared for the separation of the quinolones, a high-purity silica Inertsil C_8 column and a newly developed Kinetex core–shell technology column. The method was validated according to the provisions of Council Decision 2002/657 [36]. The names and structures of the nine quinolones studied are shown in Fig. 1.

2. Experimental

2.1. Chemicals and reagents

Marbofloxacin vetranal (MAR), norfloxacin vetranal (NOR), ciprofloxacin vetranal (CIP), danofloxacin vetranal (DAN), enrofloxacin vetranal (ENR), sarafloxacin hydrochloride trihydrate vetranal (SAR) and difloxacin hydrochloride vetranal (DIF) were purchased from Fluka (Buchs, Switzerland). Oxolinic acid (OXO) and flumequine (FLU) were obtained from Sigma (St Louis, MO, USA)

Acetonitrile was HPLC gradient grade and purchased from Panreac (Castellar del Vallès, Spain). Phosphoric acid 85% was also from Panreac. The solid reagents used were all analytical grade; oxalic acid dihydrate was purchased from Fluka and sodium hydroxide from Merck (Darmstadt, Germany). Diatomaceous earth was purchased from Dionex (Sunyvale, CA, USA). Extran AP 13 containing detergents and sodium hydroxide was from Merck. Double-deionized water (Milli-Q, Millipore, Molsheim, France) of 18.2 $M\Omega\,cm^{-1}$ resistivity was used.

Stock standard solutions (200 $mg\,L^{-1}$) of all quinolones were prepared by dissolving the compounds in appropriate solvents, which were 0.02 $mol\,L^{-1}$ phosphoric acid for MAR, NOR, CIP, DAN, ENR, SAR and DIF, and 0.1 $mol\,L^{-1}$ sodium hydroxide for OXO and FLU. These solutions were kept at 4 °C in dark glass bottles for up to six months.

Working solutions containing all quinolones with variable concentrations were freshly prepared by dilution of the stock solutions with 0.01 $mol\,L^{-1}$ oxalic acid.

The pH 3.0 phosphoric acid buffer was prepared by diluting 3.4 mL of the concentrated acid in water and making up to 1 L with more water. The pH 4.0 oxalic acid buffer was prepared by dissolving 1.26 g oxalic acid dihydrate in water and making up to 1 L with more water. The final pH of both solutions was adjusted with a 3 $mol\,L^{-1}$ sodium hydroxide solution. These solutions were filtered through a 0.22 μm nylon filter (Lida, Kenosha, WI, USA).

2.2. Instrumentation

Chromatographic analysis was carried out in a Shimadzu system (Kyoto, Japan) consisting of an LC-10AD VP quaternary pump, a SIL-10AD VP automatic injector and a RF-10A XL fluorescence detector with a 150 W xenon lamp. The columns were an Inertsil C_8 (250 mm × 4.6 mm, 5 μm particle size) from GL Sciences Inc. (Tokyo, Japan) equipped with a similar pre-column, and a Kinetex C_{18} (150 mm × 4.6 mm, 2.6 μm particle size) from Phenomenex (Torrance, CA, USA) equipped with a SecurityGuard guard cartridge from Phenomenex.

Pressurized liquid extraction of the analytes from the egg matrix was performed with an ASE 350 system from Dionex (Sunyvale, CA, USA), equipped with 5 mL stainless steel cells with cellulose filters from Dionex. A turbo Vap LV evaporation system from Caliper (Hopkinton, MA, USA) was used for the evaporation of the extracts. A vortex mixer SA8 from Stuart (Stone, Staffordshire, UK) and a Heraeus Christ Labofuge 400 centrifuge (Osterode am Harz, Germany), with a 600–6000 rpm speed range, were also used in the treatment of the samples. The pH was measured with a Crison GLP 21 pH meter (Alella, Spain) equipped with a Crison 52–02 Ag/AgCl combined glass electrode.

2.3. Samples

Egg samples were purchased from retail markets in Catalonia (Spain). Preliminary analyses were performed to check that they were free from analytes. Samples were stored at 4 °C until analysis.

Spiked samples were prepared by adding the proper amount (range 50–100 μL) of a solution containing each of the quinolones at suitable concentrations to each portion of the weighed samples, which were gently homogenized before analysis.

2.4. Procedures

2.4.1. Extraction

Samples weighing 1 g of the homogenized whole eggs (spiked with quinolones, if required) were mixed in a ceramic mortar with 1.5 g of diatomaceous earth. The mixture was then placed in the 5 mL extraction cells. The extraction buffer consisted of a 1:1 (v/v) mixture of ACN and 0.05 $mol\,L^{-1}$ phosphoric acid buffer pH 3.0. The PLE program was as follows: the cell was first heated with approximately 10 mL of extraction buffer for 5 min at 70 °C and 1500 psi, and this was followed by two static extractions of 3 min at the same temperature and pressure, rinsing the cell with an extra 3 mL of extraction buffer in two steps (one after each static cycle); the cell was then purged for 1 min with nitrogen and all the extract was collected into the same vial (approximately 13 mL). Extraction cells were cleaned between each run by sonication for around 15 min in an Extran AP 13 solution, then 15 min in water and, finally, 15 min in acetone.

After the extraction, all extracts were made up to a final volume of 25 mL, placed in centrifuge tubes and centrifuged for 10 min at 3500 rpm. Next, 4 mL aliquots of the clear extracts were evaporated to near dryness at 50 °C in a nitrogen evaporator and, subsequently, re-dissolved by vortex mixing in 1 mL of pH 4.0 oxalic acid. Finally, the extracts were filtered through 0.45 μm nylon membrane filters, and 50 μL of each one was injected into the chromatographic system.

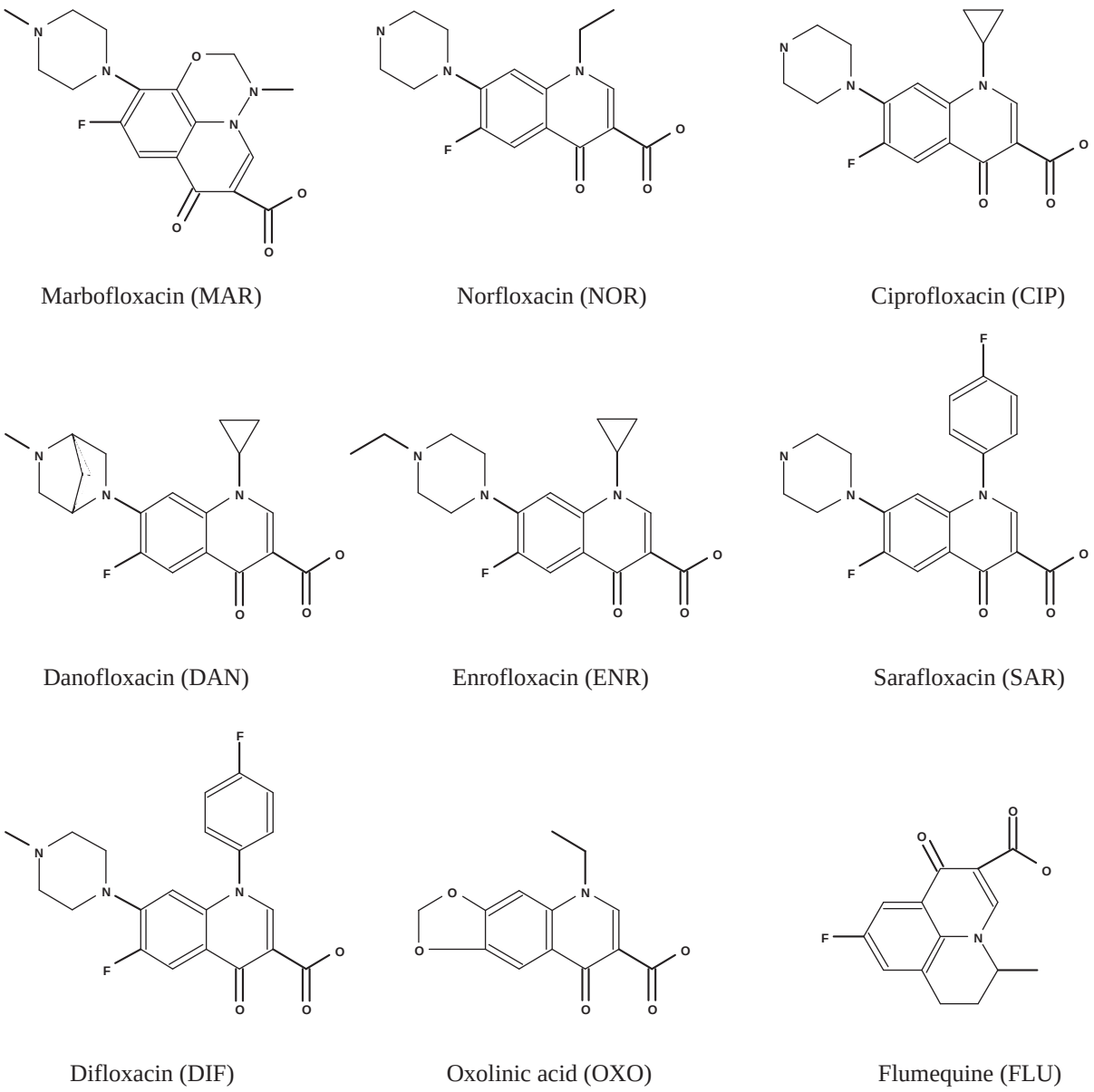

Fig. 1. Structural formulae of the quinolones under study.

2.4.2. Determination by liquid chromatography with fluorescence detection

A binary mobile phase with two gradient elution programs was used for the chromatographic separation of the nine quinolones. Mobile phase A was an aqueous solution of 0.01 mol L^{-1} oxalic acid, while mobile phase B was ACN. The separation was performed at 25 °C and the following gradient programs were applied, depending on the column used:

- Inertsil column: 0–12 min, 88% A; 12–22 min, linear decrease to 74% A; 22–29 min, linear decrease to 55% A; 29–32 min, linear increase to 88% A; and finally, 32–38 min, 88% A. The mobile phase flow rate was 1.5 mL min^{-1} and the injection volume was 50 μL.
- Kinetex column: 0–8 min, 88% A; 8–15 min, linear decrease to 70% A; 15–18 min, linear decrease to 50% A; 18–23 min, linear increase to 88% A; and finally, 23–27 min, 88% A. The mobile phase flow rate was 1.2 mL min^{-1} and the injection volume was 50 μL.

The fluorescence detector was set in a multistep detection mode (Table 2).

Calibration curves were produced by preparing standards in pH 4 oxalic acid buffer. For OXO and FLU quantification at the levels close to limit of quantification (LOQ), calibration curves were produced from six matrix-matched standards prepared by spiking the blank egg extract just before injection.

Table 2
Fluorescence detector multistep detection mode.

Detected compounds	Time (min)		λ_{Exc} (nm)[a]	λ_{Em} (nm)[a]
	Inertsil column	Kinetex column		
MAR	0–10	0–6.5	297	507
NOR, CIP, DAN, ENR, SAR, DIF	10–26	6.5–18	280	458
OXO	26–30	18–21	263	380
FLU	30–38	21–27	248	361

[a] λ_{Exc}: Excitation wavelength and λ_{Em}: emission wavelength.

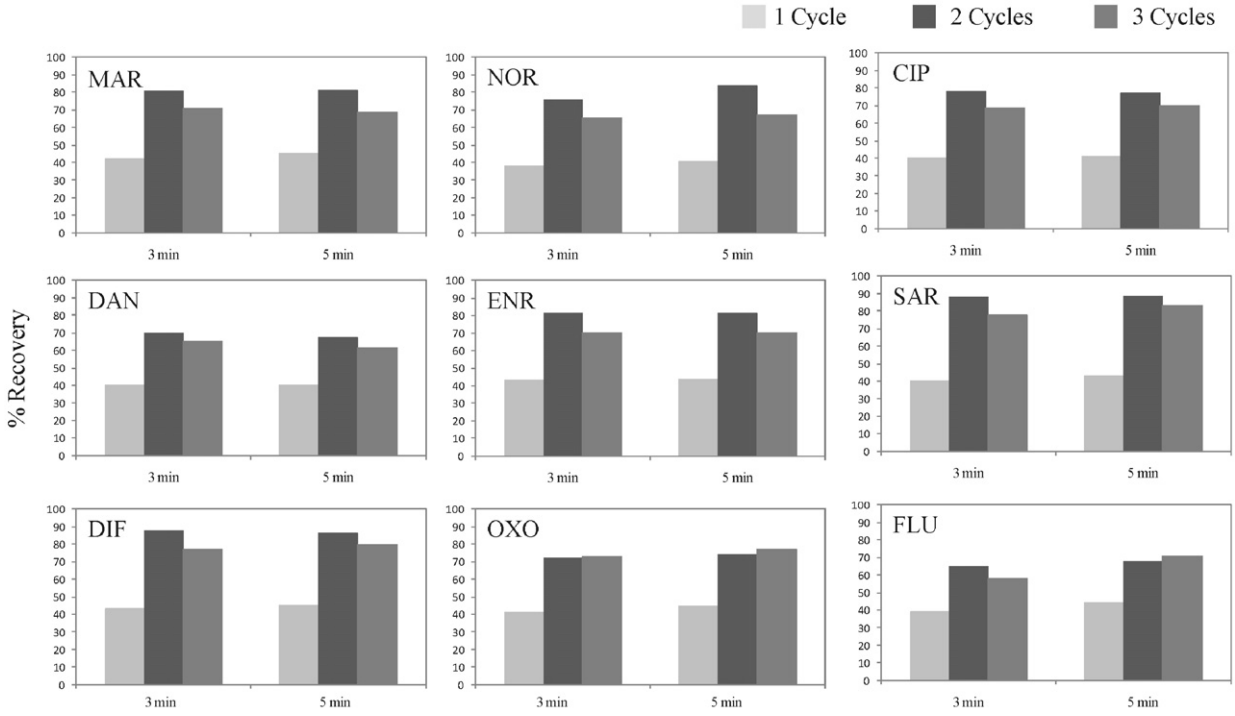

Fig. 2. Influence of number of cycles and static time on the extraction of analytes.

3. Results and discussion

3.1. Extraction procedure

PLE was used as extraction technique in the present work; it was already used in the extraction of three quinolones from egg matrix [25], where a 0.05 mol L^{-1} aqueous phosphoric acid pH 3.0/ACN (1:1, v/v) mixture was used as the extraction solvent. In the present work, besides a 0.05 mol L^{-1} aqueous phosphoric acid pH 3.0/ACN (1:1, v/v) mixture, a simpler water/ACN (1:1, v/v) mixture was tested. Triplicate extractions of egg samples spiked with quinolones at different concentration levels (200 μg kg^{-1} for DAN, 400 μg kg^{-1} for DIF and OXO, 1000 μg kg^{-1} for MAR, NOR, CIP, ENR, SAR and FLU) were performed at 70 °C, with one cycle of 5 min and the two extraction mixtures tested. Recoveries obtained with the 0.05 mol L^{-1} aqueous phosphoric acid pH 3.0/ACN (1:1, v/v) mixture were in all cases higher than those obtained with the water/ACN (1:1, v/v) for all quinolones. When acidic conditions were used in the extraction, the aqueous solubility of fluoroquinolones generally increased, as they were in the cationic form because of their piperazinyl group at the 7 position, the only exceptions being OXO and FLU, which only have a carboxylic group at C-3 [37] and, therefore, are acidic compounds.

To study the effect of the number of cycles and the time of each static cycle on the recoveries of the quinolones, triplicate extractions were performed with static times of three and five minutes and applying 1, 2 and 3 cycles, at 70 °C, using a 0.05 mol L^{-1} aqueous phosphoric acid pH 3.0/ACN (1:1, v/v) mixture as extraction solvent. Fig. 2 shows that for all quinolones the extractions with 2 cycles were more effective than those with 1 cycle and similar to those with 3 cycles, and that there were no differences between 3 and 5 min of static time. Therefore, 2 cycles of 3 min were finally selected.

Extraction temperature is a very important parameter in PLE, because analytes are better extracted at higher temperatures, provided that they are stable. This effect was studied performing triplicate extractions of spiked eggs at three temperatures, 50, 70 and 90 °C. Higher recoveries were obtained at 70 °C than at 50 °C, while recoveries obtained at 90 °C were only slightly better than at 70 °C, and only for some quinolones (Fig. 3). As the extracts obtained at 70 °C were cleaner, this was selected as the optimal temperature for this method.

All extracts were made up to a final volume of 25 mL, because the obtained volumes (about 13 mL) were not totally reproducible, and evaporation and reconstitution of the extracts would unnecessarily lengthen the method. After centrifugation of the diluted extracts, 4 mL aliquots of the clear solutions were evaporated to near dryness at 50 °C in a nitrogen evaporator. This evaporation was necessary because previous experiments demonstrated that higher and better shaped peaks in the chromatogram were obtained when the injection solvent was oxalic acid at pH 4.0. Furthermore, this evaporation step has a pre-concentration effect of the analytes in the injected extract.

Contamination of the extraction cells with the analytes was observed after performing the first extractions of this study. Two

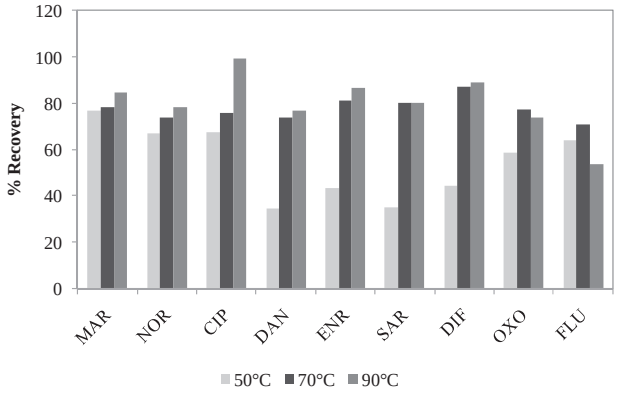

Fig. 3. Influence of temperature on the extraction of analytes.

Table 3
LOD, LOQ, CCα and CCβ values obtained with Inertsil and Kinetex columns.

Compound	Inertsil column				Kinetex column			
	LOD ($\mu g\,kg^{-1}$)	LOQ ($\mu g\,kg^{-1}$)	CCα ($\mu g\,kg^{-1}$)	CCβ ($\mu g\,kg^{-1}$)	LOD ($\mu g\,kg^{-1}$)	LOQ ($\mu g\,kg^{-1}$)	CCα ($\mu g\,kg^{-1}$)	CCβ ($\mu g\,kg^{-1}$)
MAR	15	30	6.7	11.8	5	8	3.8	9.1
NOR	5	8	5.5	14.1	30	40	14.9	21.1
CIP	30	50	19.8	33.5	5	8	6.4	14.3
DAN	1	1.5	0.2	0.4	1	1.5	0.2	0.4
ENR	5	8	1.0	3.0	5	8	1.2	3.3
SAR	10	15	2.2	4.1	5	8	2.1	4.3
DIF	1.5	3	0.5	1.5	1	1.5	0.4	1.2
OXO	10	15	1.4	2.8	10	15	1.8	4.7
FLU	20	40	9.7	22.4	75	100	15.3	30.7

different cell cleaning procedures were tested. The first involved sonication of cells for 15 min in a 0.05 mol L^{-1} phosphoric acid solution followed by sonication in water for 15 min, while the second consisted of sonication for around 30 min in methanol. However, none of these procedures fully removed the contamination. Finally, a cleaning approach consisting of sonication for around 15 min in an alkaline solution of Extran AP 13, followed by sonication in water for 15 min and a final sonication in acetone for 15 min gave successful results, avoiding any carry-over contamination.

In all these studies, the determination of the quinolones by LC–FL was performed with the Inertsil C_8 (250 mm × 4.6 mm, 5 μm particle size) column.

3.2. Determination by liquid chromatography with fluorescence detection

The chromatographic analyses were based on a previously reported LC–FL methodology for the determination of quinolones in water [38]. In the aforementioned method, a C_8 silica-based reversed-phase column and a mobile phase consisting of ACN–water mixture containing oxalic acid as a buffer were used. Since OXO and FLU are more hydrophobic than fluoroquinolones, the chromatographic conditions for the separation of these two were different from those for the separation of the other quinolones. ACN content ranged from 12% to 26% for fluoroquinolones and was about 45% for OXO and FLU, when an Inertsil C_8 column was used. The use of this column with a suitable elution gradient program led to a good separation of the nine quinolones studied in a chromatographic run of 38 min. To obtain the optimum sensitivity, detection was performed by wavelength programming, so that each analyte was detected at its optimal wavelengths. Fig. 4A shows the chromatogram obtained with an egg sample spiked at 2 times the LOQ of MAR, CIP, DAN, ENR, SAR, DIF and OXO, 5 times the LOQ of FLU and 10 times the LOQ of NOR, compared with the chromatogram of a blank egg extract. As can be observed, no important matrix-interferences existed, except in the cases of CIP and OXO, where small matrix peaks co-eluting closely to those of these analytes resulted in an increase in the limit of detection (LOD) and LOQ values for these compounds (Table 3). In the case of FLU, distortions in the baseline, provoked by the sharp change in the mobile phase composition between the minutes 22 and 29, were more intense in the presence of egg matrix; FLU could still be detected and integrated, but higher LOD and LOQ were obtained.

In order to improve the chromatographic analysis, a new generation column was also used and results were compared with those obtained with the Inertsil C_8 column. The columns with sub-2.0 μm particles are operated at very high backpressures (~1000 bar) to reach their expected efficiency, which requires costly ultra-high-pressure liquid chromatography (UHPLC) instruments. Columns packed with a new brand of fine shell particles [39–42], such as the Halo (Advanced Material Technology) and Kinetex (Phenomenex) columns, can also be operated with conventional high-pressure liquid chromatography (HPLC) systems (which can operate at a maximum inlet pressure of 400 bar) and still give results similar to those from columns packed with sub-2 μm particles. In this work, a Kinetex C_{18} (150 mm × 4.6 mm, 2.6 μm particle size) was used for the separation of the nine studied quinolones. The mobile phase was the same as with the Inertsil column, but at a lower flow rate (1.2 mL min^{-1}), and with a slightly modified elution gradient program. A good separation of the nine quinolones was obtained in a chromatographic run of 27 min, resulting in a considerable reduction in solvent consumption and time, 25 mL and 11 min per sample, respectively. Fig. 4B shows the chromatogram obtained with an egg sample spiked at the 2 LOQ level for NOR, DAN, ENR, OXO and FLU, 4 LOQ for SAR and DIF, 7 LOQ for MAR and 12 LOQ for CIP, compared with the chromatogram of a blank egg extract. The matrix component that interfered with CIP in the Inertsil column, now interfered with NOR, producing an increment in its LOD and LOQ values (Table 3). This interference was not subtracted in the quantification of NOR because, as can be seen in Table 4, it only affected NOR recoveries at the LOQ level (103%), and from 1.5 LOQ recoveries were not affected by this interference. The same happened with CIP when the Inertsil column was used: the interference only affected CIP recoveries at the LOQ (122%), but from 1.5 LOQ its influence was negligible. In the case of FLU, distortions in the baseline were more intense than in the chromatogram obtained with the Inertsil column, leading to higher LOD and LOQ values.

3.3. Validation procedure

The method was validated in egg matrix according to Council Decision 2002/657. This Decision establishes criteria and procedures for the validation of analytical methods to ensure the quality and comparability of analytical results obtained by laboratories.

The validation parameters measured to evaluate the method were specificity, linear range, recovery, precision (repeatability and intra-laboratory reproducibility), decision limit (CCα) and detection capability (CCβ). Although Decision 2002/657 does not require the determination of LOD and LOQ, they were evaluated in the present work, because these performance parameters are very used in analytical methodologies and, moreover, a low concentration limit was necessary for the validation, as quinolones are banned substances in eggs. The validation was carried out at three low concentration levels and at a higher concentration level. The low levels roughly corresponded, in most cases, to LOQ, 1.5 LOQ and 2 LOQ; if the LOQ of the two columns did not coincide, the higher value was used. The higher level was 200 $\mu g\,kg^{-1}$ of DAN, 400 $\mu g\,kg^{-1}$ of DIF and OXO, 1000 $\mu g\,kg^{-1}$ of MAR, NOR, CIP, ENR, SAR and FLU

LOD and LOQ were calculated on the basis of three and ten times, respectively, the baseline standard deviation of the chromatogram obtained with a blank egg extract, using low-concentration standard solutions. They were checked to be correct by injection of egg samples spiked at low concentration levels. All the LOD and LOQ values obtained for both columns are shown in Table 3. With the

Table 4
Precision and recovery in different validation levels, obtained with the Inertsil and the Kinetex columns.

Inertsil column

Compound	LOQ				2 LOQ				Higher concentration level[a]			
	RSD_r (%)[b]	RSD_R (%)[b]	Recov.sc (%)[c]	Recov.mm (%)[c]	RSD_r (%)[b]	RSD_R (%)[b]	Recov.sc (%)[c]	Recov.mm (%)[c]	RSD_r (%)[b]	RSD_R (%)[b]	Recov.sc (%)[c]	Recov.mm (%)[c]
MAR	12	15	92	79	8	15	83	70	4	10	78	75
NOR[d]	6	10	73	77	5	9	68	71	5	15	74	78
CIP	7	17	122	–	6	13	79	–	5	14	76	77
DAN	11	14	80	84	7	12	85	90	5	15	74	78
ENR	6	20	82	84	4	14	81	83	4	13	81	83
SAR	7	12	75	68	7	8	68	65	5	15	80	72
DIF	13	19	91	96	6	9	73	77	5	12	87	92
OXO	10	12	48	69	8	13	49	70	8	16	77	94
FLU[f]	9	23	40	69	9	12	44	74	7	16	71	91

Kinetex column

Compound	LOQ				2 LOQ				Higher concentration level[a]			
	RSD_r (%)[b]	RSD_R (%)[b]	Recov.sc (%)[c]	Recov.mm (%)[c]	RSD_r (%)[b]	RSD_R (%)[b]	Recov.sc (%)[c]	Recov.mm (%)[c]	RSD_r (%)[b]	RSD_R (%)[b]	Recov.sc (%)[c]	Recov.mm (%)[c]
MAR[d]	8	19	67	64	4	10	66	63	5	11	68	65
NOR	8	15	103	–	6	8	74	–	5	7	74	78
CIP[e]	8	16	74	75	7	13	68	69	5	9	72	73
DAN	9	20	74	78	8	15	80	84	7	12	75	79
ENR	10	10	85	87	6	8	71	73	6	9	76	78
SAR[f]	10	12	74	82	6	16	65	79	6	14	70	83
DIF[f]	11	22	71	75	8	11	61	64	9	12	75	79
OXO	8	16	48	69	5	12	39	58	7	13	70	96
FLU	10	12	33	64	5	14	29	62	8	15	69	94

[a] Higher concentration level: 200 μg kg^{-1} for DAN, 400 μg kg^{-1} for DIF and OXO, 1000 μg kg^{-1} for MAR, NOR, CIP, ENR, SAR and FLU.
[b] RSD_r (%): Repeatability and RSD_R (%): Intra-laboratory reproducibility.
[c] Recov.sc (%): % recoveries calculated by calibration standards prepared in solvent, and Recov.mm (%): recoveries calculated by matrix-matched calibration standards.
[d] Lower concentration levels: 2 LOQ and 6 LOQ.
[e] Lower concentration levels: 3 LOQ and 9 LOQ.
[f] Lower concentration levels: 1 LOQ and 3 LOQ.

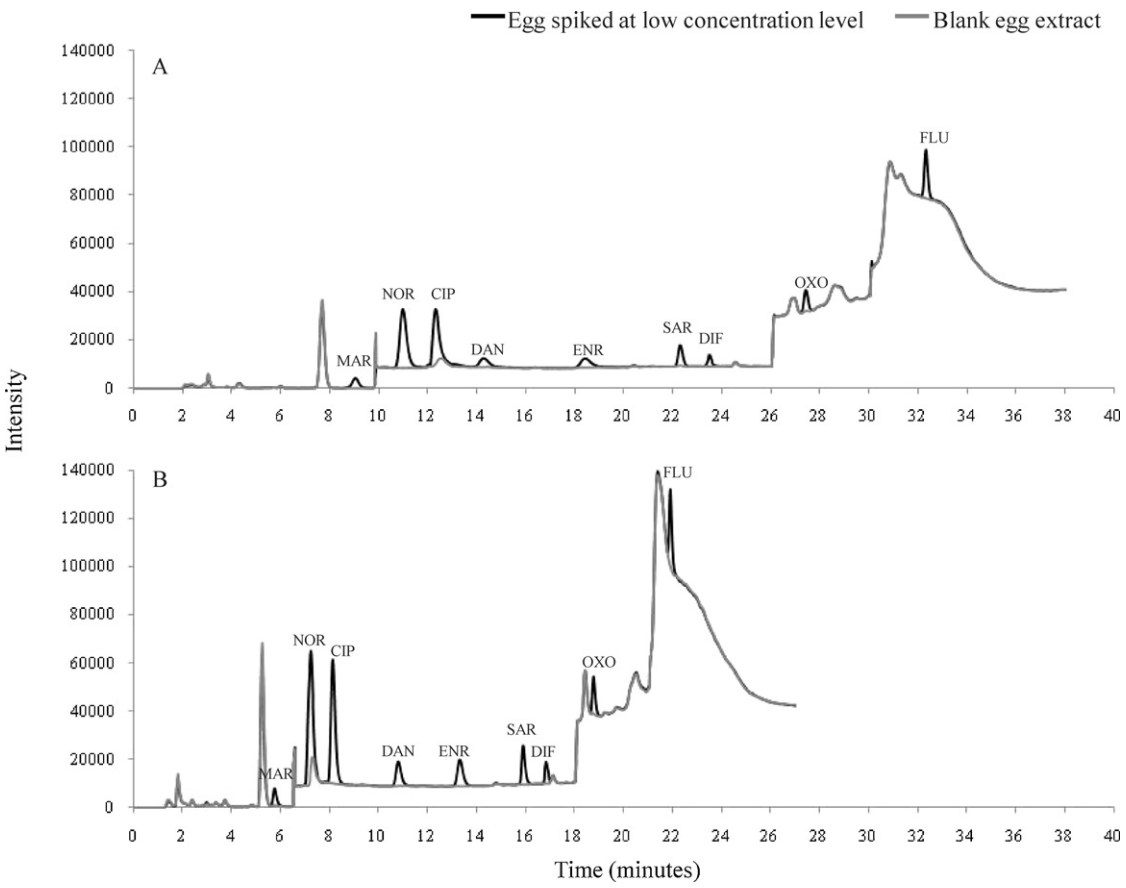

Fig. 4. LC–FL chromatograms of a sample of egg spiked with quinolones at low concentrations (3 μg kg^{-1} of DAN, 6 μg kg^{-1} of DIF, 16 μg kg^{-1} of ENR, 30 μg kg^{-1} of SAR and OXO, 60 μg kg^{-1} of MAR, 80 μg kg^{-1} of NOR, 100 μg kg^{-1} of CIP and 200 μg kg^{-1} of FLU) and of a blank egg extract, obtained with the Inertsil column (A), as well as with the Kinetex column (B).

Kinetex column, better LOD and LOQ values were obtained for MAR, CIP, SAR and DIF, and they were worse for NOR and FLU, which were affected by matrix interferences. LOD and LOQ for DAN, ENR and OXO were identical with both columns.

Specificity was assayed by analyzing six blank egg samples of different origin and checking the absence of matrix interference peaks higher than those corresponding to the LOD of the studied quinolones.

Calibration curves at two concentration ranges, both with and without matrix matched standards, were obtained. The linear regression analyses were carried out by plotting analyte peak areas versus analyte concentrations. The calibration parameters obtained with standards in pH 4.0 oxalic acid buffer are summarized in Table 5. They do not differ significantly from the values obtained with matrix matched standards and because calibration with standards in buffer is simpler, this is the approach proposed except in the case of FLU and OXO at concentrations close to the LOQ, which requires matrix matched standards. Slopes of the equations obtained with the Kinetex column were in all cases higher than those obtained with the Inertsil column, indicating higher sensitivity. Correlation coefficients were, in all cases, equal to or higher than 0.998, and residuals were lower than 6% at all points of the calibration curves for all analytes with the two columns, which is an indication of good linearity.

Accuracy of the method was evaluated by estimating recoveries and precisions for all analytes. Six 1 g fractions of blank samples were fortified at each validation level using variable volumes (50, 75 and 100 μL) of spike solutions containing all analytes at suitable concentrations. Sample extracts were injected in both assayed chromatographic columns. This procedure was also repeated on two additional days. Recoveries were calculated at each validation level as the ratio of obtained mean over nominal concentration (%). Results at the LOQ, 2 LOQ and the higher concentration level are shown in Table 4. Recoveries at the lower concentration levels were satisfactory (>60%) for most analytes and similar to those obtained at the higher concentration level, except for OXO and FLU, whose recoveries (25–50%) were lower than those obtained at the higher concentration (69–77%). Baseline distortions provoked by the egg matrix disguised part of the OXO and FLU peaks, and lower recoveries were obtained at the lower concentration levels when quantification was performed with a calibration curve in solvent. When samples at these lower concentrations were quantified by means of a matrix-matched calibration curve, recoveries for OXO and FLU were between 60% and 80%. Values of repeatability (RSD_r) and intra-laboratory reproducibility (RSD_R) were calculated by applying a one-way (day) ANOVA for a 95% confidence level to obtain inter-session (s_L), intra-session (s_r) and total intra-laboratory standard deviation (s_R). According to Decision 2002/657, the calculated RSD_R must not exceed the level calculated by the Horwitz equation. RSD_R values were equal to or lower than 23% at the lower concentrations (below 100 μg kg^{-1}, except for FLU), significantly better than the limits calculated by the Horwitz Equation, which is desirable because these limits are rather permissive at low concentrations. RSD_R at higher concentrations were, in general, lower than 16%, also better than the values calculated by Horwitz equation. RSD_r were always equal to or better than 13%.

Finally, CCα and CCβ values were determined (Table 3). The CCα, defined as the concentration above which it can be concluded that a sample is non compliant with an error probability α, was calculated using the approach of the calibration curve procedure. CCα

Table 5
Calibration parameters obtained injecting standards in pH 4.0 oxalic acid buffer in the Inertsil and the Kinetex columns.

Inertsil column								
Compound	Low concentration range				High concentration range			
	Range ($\mu g\,L^{-1}$)	Slope	Intercept	R	Range ($\mu g\,L^{-1}$)	Slope	Intercept	R
MAR	5.0–33.5	4362	−533	0.997	5–500	5886	−4972	1.000
NOR	2.0–13.4	37,225	−85	0.999	5–500	35,120	−93,859	1.000
CIP	8.0–53.6	29,708	−2317	0.999	50–500	25,296	−58,948	1.000
DAN	0.24–1.6	191,420	−2547	0.999	1–100	192,706	−72,949	1.000
ENR	1.3–8.7	41,548	−7533	0.999	5–500	49,724	−118,068	1.000
SAR	2.4–16.1	23,821	1729	1.000	5–500	23,460	−45,818	1.000
DIF	0.5–3.4	37,317	−353	0.999	2–200	41,434	−5035	1.000
OXO*	2.4–16.1	21,835	2320	0.998	2–200	32,731	−1627	1.000
FLU*	6.4–42.9	6476	5741	0.999	50–500	9421	−12,197	1.000
Kinetex column								
Compound	Low concentration range				High concentration range			
	Range ($\mu g\,L^{-1}$)	Slope	Intercept	R	Range ($\mu g\,L^{-1}$)	Slope	Intercept	R
MAR	1.5–33.5	6340	1076	1.000	5–500	7924	−35,634	0.999
NOR	6.4–13.4	49,810	12,263	1.000	50–500	40,943	85,139	1.000
CIP	2.5–53.6	39,815	9088	1.000	5–500	33,051	−148,691	0.999
DAN	0.24–1.6	294,185	237	0.999	1–100	257,670	26,411	1.000
ENR	1.3–8.7	62,231	−802	0.999	5–500	59,418	17,114	1.000
SAR	1.3–16.1	32,989	1589	0.999	5–500	29,204	−58,293	0.999
DIF	0.28–3.4	52,181	3.6	0.998	2–200	53,344	−93,358	0.999
OXO*	2.4–16.1	26,598	−2180	0.998	2–200	40,211	−54,525	1.000
FLU*	16–42.9	7478	−1682	1.000	50–500	11,738	−47,609	1.000

* Matrix-matched standards at the low concentration range.

was established as the concentration at the *y*-intercept plus 2.33 its standard deviation at the lowest level using data from the intra-laboratory reproducibility study at low concentrations. For banned substances, CCβ is the minimum concentration level at which the method can detect contaminated samples with an error probability β. It was calculated as the CCα plus 1.64 times the corresponding standard deviation when analyzing at least 18 egg samples spiked at the CCα level. These deviations were considered to be the same as those obtained at the lowest level studied (LOQ).

4. Conclusions

As stated earlier, many methods have been proposed in the last decade for the analysis of quinolones in egg samples (Table 1). The method reported in the present paper complements that of Herranz et al. [25], increasing from three to nine the number of determined quinolones and optimizing the PLE procedure in terms of number of cycles, static time and extraction temperature. The use of a PLE technique contributes to increase the sample throughput, as there is no need of further clean-up steps and it lends itself to a high degree of automation, and moreover, good recoveries were obtained. The proposed method allows nine quinolones to be determined at trace levels in egg samples without the need of costly MS instrumentation, which is not widely available in many laboratories. If the data reported for ENR, CIP and SAR in the aforementioned paper [25] are compared with those of the present work, the conclusion is that recoveries are similar, but CCα and CCβ have been improved.

Separation with the newly developed Kinetex core–shell technology column resulted in a significant reduction in solvent consumption and in analysis time, combined with good resolution for all analytes and better sensibility for MAR, CIP, SAR and DIF. Validation of the method according to Council Decision 2002/657 was performed using both columns, and the obtained parameters were satisfactory, showing that the method can be useful to control the illegal use of these nine quinolones in laying hens.

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