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Short communication

Multiresidue determination of quinolone antibacterials in eggs of laying hens by liquid chromatography with fluorescence detection

M.K. Hassouan [a], O. Ballesteros [a], J. Taoufiki [a], J.L. Vílchez [a], M. Cabrera-Aguilera [b], A. Navalón [a,*]

[a] *Research Group of Analytical Chemistry and Life Sciences, Department of Analytical Chemistry, University of Granada, Avda, Fuentenueva s/n, E-18071 Granada, Spain*
[b] *Servicio Andaluz de Salud (SAS), E-18071 Granada, Spain*

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Abstract

An analytical method for the simultaneous determination of seven quinolones (ciprofloxacin, enrofloxacin, danofloxacin, difloxacin, flumequine, oxolinic acid and sarafloxacin) in egg samples of laying hens was developed. Their use is totally prohibited in animals from which eggs are produced for human consumption. Protein precipitation was achieved by addition of acetonitrile and ammonia, removal of acetonitrile with dichloromethane, the quinolones remaining in the basic aqueous extract. The aqueous extract was analysed by liquid chromatography with fluorescence detection (LC–FD). The mobile phase was composed of acetonitrile and 10 mM citrate buffer solution of pH 4.5, with an initial composition of acetonitrile–water (12:88, v/v) and using linear gradient elution. Norfloxacin was used as an internal standard. The limits of detection found were 4–12 ng g^{-1}. These values were lower than the maximum residue limits (MRLs) established by the European Union for these compounds in different tissues of eggs-producing animals.

Keywords: Quinolones; Eggs analysis; Liquid chromatography–fluorescence detection (LC–FD)

1. Introduction

Antibiotics are widely used in animal husbandry for treatment and prevention of diseases and as feed additives to increase the animal mass. Their misuse can leave residues in edible animal tissues, giving rise to public health concern (toxic effects, development of resistant strains of bacteria, allergic hypersensitivity reactions, etc.) as well as environmental and industrial (cheese or yoghurt production, etc.) problems [1,2].

Quinolones, which act principally by inhibiting DNA-gyrase in bacterial cells, form an important group of synthetic antimicrobial agents with different chemical structures and spectra of activity [3]. A significant increase in the use of quinolones in animal production was noted over the last decade. The European Union has established maximum residue limits (MRLs) for quinolone residues in animal tissues [4]. Thus, the establishment of sensitive multiresidues screening methods is required in order to control these drugs.

Multiresidue analysis of quinolones in biological samples and animal tissues [5–8] involves liquid chromatography with ultraviolet (LC–UV) [9–12], fluorescence (LC–FD) [13–18] or mass spectrometric detection (LC–MS) [19–22]. Only a few methods [9,23–29] have focused however on the determination of quinolone residues in eggs. Gorla et al. [23] proposed a LC–UV method for the determination of enrofloxacin and its metabolite, ciprofloxacin, in egg yolk or egg white, but the extraction recoveries obtained were low (36–50% for ciprofloxacin and 49–85% for enrofloxacin).

Maxwell et al. [24] developed a method for the determination of sarafloxacin in whole eggs using an automated sequential trace enrichment of dialysates (ASTED) system and LC–FD; the recovery of the method was 87–102% and the limit of quantification found was 1 ng g^{-1}. Also, using an ASTED system, Schneider and Donoghue [25] proposed a LC–FD method for the determination of six fluoroquinolones in whole eggs, achieving good sensitivity and satisfactory recovery for the six compounds

* Corresponding author. Tel.: +34 958 243326; fax: +34 958 243328.
E-mail address: anavalon@ugr.es (A. Navalón).

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studied (65–110%). But the ASTED system restricts the methods to a few laboratories.

Using solid-phase extraction, Gigosos et al. [9] developed a LC–UV method for simultaneous determination of five fluoroquinolones in animal tissues (bovine kidney and muscle) and eggs; the recoveries obtained and the detection limits found were satisfactory. The multiresidue method described by Rose et al. [26] was found suitable for the determination of nine fluoroquinolone residues and three acidic quinolone residues, but required multiple LC runs and some modification for acidic quinolones; several extraction procedures were used and only four compounds were simultaneously determined in one run.

Using supercritical fluid extraction, Shim et al. [27] reported a LC–FD method for the determination of four fluoroquinolones in eggs; good recoveries (83–96%) were obtained.

Schneider and Donoghue [28] developed a novel LC–FD–MS^n method for the determination of eight fluoroquinolones in mixed eggs, egg white and egg yolk. Their acetonitrile extraction followed by hexane defatting gave good recoveries for seven of the eight compounds, but the recovery obtained for desethylene ciprofloxacin (46.0–65.4%) and norfloxacin (55.6–75.9%) in fortified egg yolk samples was low.

Zeng et al. [29] proposed a LC–FD multiresidue method for the determination of nine fluoroquinolones in eggs (egg white and egg yolk). Egg white samples were deproteinised followed by defatting with hexane once (white) or twice (yolk), and extracting the compounds into acetonitrile. After acetonitrile was evaporated, the residue was dissolved in mobile phase. Good recoveries were obtained (74.7–91.2%) for nine fluoroquinolones and the quantification limits found were 5–20 ng g^{-1}.

This paper reports the development of a simple, selective and sensitive LC–FD method for the simultaneous determination in whole eggs of seven fluoroquinolones: ciprofloxacin (CIPRO) (major active metabolite of ENRO), danofloxacin (DANO), difloxacin (DI), enrofloxacin (ENRO), flumequine (FLU), oxolinic acid (OXO) and sarafloxacin (SARA) (major active metabolite of DI). Although their use in poultry for meat is regulated, they are totally prohibited in poultry laying eggs birds.

A simple sample pretreatment, based on common extraction of the quinolones from the samples, was used.

Here, an advantageous single chromatographic run allowing the determination of five zwitterionic quinolones (CIPRO, DANO, DI, ENRO and SARA) and two acidic quinolones (FLU and OXO) is presented. The limits of detection found were between 4 and 12 ng g^{-1}. The proposed method was applied to the determination of these compounds in commercial egg samples.

2. Experimental

2.1. Chemicals and reagents

All reagents were analytical grade, unless stated otherwise. Water (18 MΩ cm^{-1}) was purified by means of a Milli-Q plus system (Millipore, Bedford, MA, USA).

Quinolones were obtained from different pharmaceutical firms: ciprofloxacin (Ipsen Pharma, Barcelona, Spain), danofloxacin (Pfizer, Karlsruhe, Germany), difloxacin (Abbott, Madrid, Spain), enrofloxacin (Cenavisa, Tarragona, Spain) flumequine (Sigma–Aldrich, Madrid, Spain), norfloxacin (Sigma–Aldrich), oxolinic acid (Sigma–Aldrich) and sarafloxacin (Abbott).

Individual stock solutions of CIPRO, ENRO, DANO, DI, NOR and SARA were prepared in ethanol (99.9%, v/v) at a concentration of 100 μg ml^{-1}. Individual stock solutions of FLU and OXO were prepared in acetonitrile at a concentration of 100 μg ml^{-1}. These solutions were stored at 4 °C in the dark for not longer than 2 months. Individual working solutions were prepared by diluting suitably with an acetonitrile–water mixture (12:88, v/v). The working solutions, used to spike the egg samples, were prepared by mixing the individual stock solutions and diluting suitably with an acetonitrile–water mixture (12:88, v/v).

Acetonitrile (HPLC-gradient grade) and citric acid were obtained from Panreac (Barcelona, Spain). Ammonia (25%), dichloromethane (HPLC-grade) and ethanol (99.9%, v/v) were supplied by Merck (Darmstadt, Germany). The 10 mM citrate buffer solution of pH 4.5 was prepared from citric acid and ammonia.

All solutions prepared for LC were filtered through 0.22 μm nylon filter membranes (Sharlab, Barcelona, Spain) before use.

2.2. Apparatus and software

The chromatographic system consisted of an Agilent 1100 series high performance liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump, an on-line degasser, an autosampler, an automatic injector, a thermostated column compartment and a fluorescence detector (G1321A) connected on-line. ChemStation for LC 3D (Agilent) software package was used to control the instrument, data acquisition and data analysis.

All pH measurements were made with a Crison (Crison Instruments SA, Barcelona, Spain) combined glass-saturated calomel electrode using an earlier calibrated Crison 2000 digital pH-meter.

A vortex-mixer IKA MS2 (Staufen, Germany) was used to mix and homogenize egg samples during pretreatment and a centrifuge Hettich Zentrifugen Universal 32 (Tuttligen, Germany) was used in order to perform the extractions.

Statgraphics [30] software package was used for statistical analysis of the data and for regression analysis (linear model).

2.3. Chromatographic conditions

Chromatographic separation of the quinolones was performed on a Zorbax Eclipse XDB-C_8 column (150 mm × 4.6 mm i.d., 5 μm particle) from Agilent. The column was protected with an Eclipse XDB-C_8 (Agilent) pre-column (12.5 mm × 4.6 mm i.d., 5 μm particle).

A gradient program was used with the mobile phase, combining solvent A (10 mM citrate buffer solution of pH 4.5)

and solvent B (acetonitrile) as follows: 12% B (10 min), 12–30% B (5 min), 30% B (4 min), 30–12% B (1 min), 12% B (6 min).

The flow-rate was 1.5 ml min^{-1}, the injection volume 20 μl and the column temperature was maintained at 35 °C.

The excitation and emission wavelengths selected for the detection of CIPRO, ENRO, DANO, DI, NOR and SARA were 280 and 450 nm, respectively, and for the detection of FLU and OXO 325 and 365 nm, respectively.

2.4. Extraction procedure

An aliquot (1 g) of previously homogenized whole egg containing the seven quinolones was placed in a 10 ml glass centrifuge tube containing 200 μl of 1 μg ml^{-1} solution of norfloxacin (internal standard) and then 250 μl of concentrated ammonia were added to the mixture. After shaking the mixture briefly (about 5 s) on a vortex-mixer, 2 ml of acetonitrile were added. The mixture was vortexed for about 10 s at high speed and then centrifuged for 5 min at 4000 rpm (2630 × *g*). The supernatant was decanted into another 10 ml glass centrifuge tube, and 4 ml of dichloromethane were added. The mixture was vortexed for about 10 s at high speed and then centrifuged for 5 min at 4000 rpm (2630 × *g*). The upper, aqueous layer was transferred into an autosampler vial using a Pasteur pipette. The aqueous egg extracts were then analysed by LC–FD using the conditions above described.

Four different commercial egg samples were purchased in several markets in the area of Granada and were treated with the same procedure optimised for spiked samples.

3. Results and discussion

3.1. Optimisation of the LC conditions

As in other studies made by the authors [11], a Zorbax Eclipse XDB-C_8 column and a gradient elution were used for quinolones separation. To optimise the separation of the compounds of interest in this column the linear solvation energy relationship (LSER) methodology was applied in the same way that has been previously described [11,31]. Resolution between adjacent peaks (R_S) [32] was calculated at 10 different acetonitrile percentages (from 10 to 40%) to predict the optimum percentage of organic phase.

In relation with the pH optimisation, the retention factors [32] for the quinolones at different pH values of buffer solution (between 3.0 and 5.0) were determined from three different injections at every pH considered, and the R_S was calculated. The optimised LC conditions are described in Section 2.3. R_S values found under these conditions for the eight quinolones were in all cases higher than 2.0. Norfloxacin (used in human medicine) was selected as internal standard for LC quantification, because this quinolone was efficiently extracted from egg (88 ± 2%) and did not coelute with any of the evaluated quinolones. A typi-

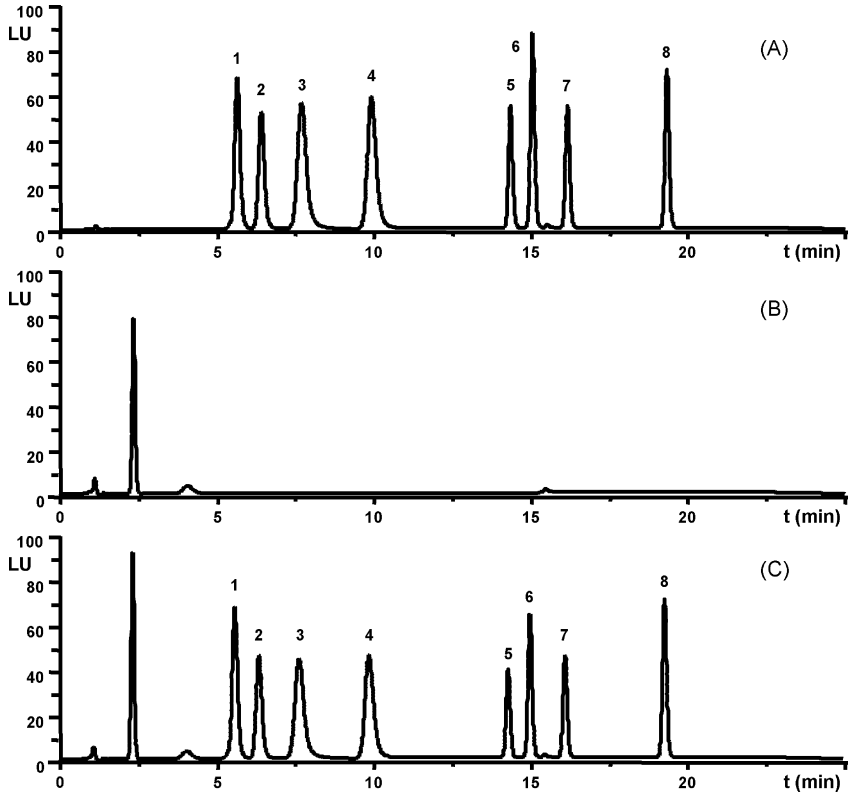

Fig. 1. Chromatograms of: (A) a standard mixture of the selected quinolones; (B) an unspiked whole egg sample; (C) a spiked whole egg sample. Chromatographic conditions are described in Section 2.3. Peaks identifications: (1) norfloxacin, (IS), 100 ng g^{-1}; (2) ciprofloxacin, 100 ng g^{-1}; (3) danofloxacin, 25 ng g^{-1}; (4) enrofloxacin, 100 ng g^{-1}; (5) sarafloxacin, 100 ng g^{-1}; (6) difloxacin, 100 ng g^{-1}; (7) oxolinic acid, 200 ng g^{-1}; (8) flumequine, 200 ng g^{-1}.

Table 1
Analytical and statistical parameters

Parameter	CIPRO	DANO	DI	ENRO	FLU	OXO	SARA
Intercept (a)	−0.0120	−0.0013	−0.0071	−0.0039	−0.0011	−0.0011	0.0063
Intercept standard deviation (S_a)	0.0070	0.0122	0.0051	0.0050	0.0012	0.0011	0.0034
Slope (b) (g ng^{-1})	0.0040	0.0170	0.0030	0.0040	0.0009	0.0004	0.0010
Slope standard deviation (S_b)	0.0001	0.0003	0.0001	0.0001	0.0001	0.0001	0.0001
Correlation coefficient (r^2)	0.9983	0.9989	0.9979	0.9989	0.9988	0.9967	0.9968
Regression standard deviation ($S_{y/x}$)	0.0132	0.0231	0.0096	0.0095	0.0023	0.0021	0.0065
Lack-of-fit test (P-value)	0.32	0.62	0.39	0.97	0.63	0.80	0.95
Linear dynamic range (ng g^{-1})	30–300	15–150	35–300	25–250	25–250	45–350	40–350
Detection limit (ng g^{-1})	9	4	10	7	7	12	12
Quantification limit (ng g^{-1})	29	12	32	23	24	41	40

cal chromatogram corresponding to a standard mixture of the selected antibiotics is shown in Fig. 1(A). The separation of these eight quinolones was achieved in less than 20 min.

3.2. Selection of the extraction procedure

Eggs are a difficult food matrix for residue analysis because of significant binding between the lipoprotein matrices of eggs and drugs, resulting in poor extraction and isolation of quinolones [27]. The extraction procedure, described in Section 2.4, was adopted after an extensive investigation of methods for the extraction of several quinolones from biological matrices. It was based on the researches by Idowu and Peggins [33] for the extraction of ENRO and CIPRO from milk being simple and fast. There was an initial precipitation of proteins with a combination of acetonitrile and *o*-phosphoric acid and the removal of acetonitrile and fat by extraction with dichloromethane led to the extraction of quinolones from whole egg samples but low recoveries were achieved for both flumequine and oxolinic acid.

However, when the *o*-phosphoric acid replaced by ammonia good recoveries were achieved for the eight quinolones we assayed. Thereafter, the amounts of acetonitrile, concentrated ammonia and dichloromethane used were optimised. Two millilitres of acetonitrile, 250 μl of concentrated ammonia and 4 ml of dichloromethane were selected as optimum values for the extraction of the seven quinolones here studied as well as the internal standard (norfloxacin) from the egg samples.

3.3. Calibration and method performance

For the calibration, spiked standard samples at six concentration levels were extracted following the extraction procedure previously explained (each level was prepared by duplicate, and each calibration sample was analysed twice). Calibration curves were constructed using analyte/internal standard peak area ratio versus concentration of analyte. The lack-of-fit test [34] was used to check the linearity of the calibration graphs. The limits of detection and quantification were calculated according with the IUPAC criterion [35].

The analytical and statistical parameters for each quinolone studied are summarised in Table 1.

In order to determine the intra- and inter-day repeatability, blank egg samples were spiked at three concentration levels (50, 100 and 150 ng g^{-1} for CIPRO, ENRO, DI, FLU, OXO and SARA and 25, 50 and 75 ng g^{-1} for DANO) and six analyses were performed on 3 days. Recoveries were achieved by comparing the analytical results for extracted standard samples of egg at aforementioned concentrations with unextracted standards prepared at the same concentrations in blank extract representing 100% recovery. The results obtained, summarised in Table 2, fulfil the requirements defined by the European Union legislation [36].

Table 2
Intra- and inter-day recovery (%) and precision (R.S.D., %) data obtained for the determination of studied quinolones in egg samples ($n = 6$)

Compound	Concentration level (ng g^{-1})[a]		
	50	100	150
CIPRO			
Day 1	85 (5)	87 (3)	92 (2)
Day 2	85 (4)	88 (3)	89 (3)
Day 3	86 (4)	88 (2)	88 (3)
DANO[a]			
Day 1	80 (4)	79 (5)	79 (3)
Day 2	81 (5)	79 (5)	80 (3)
Day 3	79 (4)	78 (5)	79 (4)
DI			
Day 1	70 (5)	73 (3)	72 (4)
Day 2	71 (4)	72 (1)	73 (3)
Day 3	71 (4)	71 (2)	74 (3)
ENRO			
Day 1	79 (4)	77 (5)	78 (4)
Day 2	78 (3)	76 (5)	77 (4)
Day 3	78 (2)	78 (5)	78 (5)
SARA			
Day 1	77 (5)	78 (4)	79 (3)
Day 2	79 (5)	78 (4)	79 (3)
Day 3	78 (5)	80 (4)	79 (2)
OXO			
Day 1	95 (5)	93 (4)	96 (4)
Day 2	96 (3)	94 (3)	97 (3)
Day 3	95 (4)	95 (4)	98 (4)
FLU			
Day 1	87 (5)	84 (4)	84 (3)
Day 2	85 (3)	83 (4)	82 (3)
Day 3	84 (3)	83 (5)	84 (2)

[a] For DANO, the concentration levels were 25, 50 and 75 ng g^{-1}.

Table 3
Results of recovery assays in egg samples to check the accuracy of the proposed method

Compound	Spiked (ng g^{-1})	Found[a] (ng g^{-1})/recovery (%)			
		Sample 1	Sample 2	Sample 3	Sample 4
CIPRO	50	50.8 ± 0.4/100	50.0 ± 0.8/100	49.8 ± 0.6/100	49.8 ± 0.7/100
	100	99.8 ± 1.2/100	100.1 ± 1.1/100	99.5 ± 1.1/99	99.7 ± 0.6/100
	150	151.8 ± 2.4/101	150.6 ± 2.3/100	151.0 ± 1.7/100	149.2 ± 1.5/100
	200	201.0 ± 2.1/100	200.9 ± 2.4/100	198.3 ± 2.5/99	201.3 ± 2.6/101
DANO	20	20.1 ± 0.1/100	20.0 ± 0.3/100	19.9 ± 0.2/100	20.2 ± 0.6/101
	40	40.1 ± 0.2/100	40.4 ± 0.8/101	40.3 ± 0.7/100	39.8 ± 0.5/99
	80	80.4 ± 0.5/100	79.4 ± 1.4/99	80.1 ± 0.6/99	79.4 ± 1.2/99
	150	149.2 ± 1.7/99	150.7 ± 0.9/100	151.3 ± 2.3/101	149.6 ± 1.2/100
DI	50	49.8 ± 0.6/100	49.7 ± 0.5/99	50.1 ± 0.7/100	49.9 ± 0.6/100
	100	100.3 ± 0.9/100	99.8 ± 0.8/100	99.7 ± 0.9/100	99.6 ± 0.8/100
	150	149.7 ± 0.9/100	150.6 ± 1.0/100	149.5 ± 1.3/100	149.4 ± 1.0/100
	200	200.9 ± 2.4/100	199.4 ± 2.3/100	200.9 ± 2.9/100	201.4 ± 2.4/101
ENRO	50	50.2 ± 0.4/100	49.6 ± 0.7/99	50.1 ± 0.41/100	49.7 ± 0.6/99
	100	99.2 ± 1.2/99	100.1 ± 1.0/100	100.8 ± 1.3/101	99.9 ± 0.9/100
	150	150.7 ± 1.4/100	149.6 ± 1.8/100	150.7 ± 1.6/101	150.5 ± 1.4/100
	200	199.5 ± 2.6/100	201.0 ± 2.1/101	201.1 ± 2.4/101	201.4 ± 2.4/101
SARA	50	49.7 ± 0.5/99	49.8 ± 0.7/100	50.3 ± 0.7/101	49.6 ± 0.7/99
	100	99.4 ± 0.8/99	99.8 ± 1.2/100	99.6 ± 0.9/100	100.6 ± 1.2/101
	150	149.3 ± 1.6/100	150.6 ± 1.5/100	149.4 ± 1.3/100	149.2 ± 1.2/100
	200	200.6 ± 1.5/100	201.1 ± 2.3/101	200.5 ± 1.9/100	198.8 ± 1.9/99
OXO	50	49.8 ± 0.5/100	50.2 ± 0.5/100	49.8 ± 0.4/100	50.1 ± 0.4/100
	100	100.6 ± 1.1/101	99.7 ± 0.6/100	100.5 ± 0.8/101	99.7 ± 1.2/100
	150	150.4 ± 1.6/100	150.9 ± 1.5/100	150.3 ± 0.9/100	149.8 ± 1.0/100
	200	199.4 ± 2.3/100	199.6 ± 2.2/100	200.7 ± 2.3/100	200.3 ± 1.9/100
FLU	50	50.2 ± 0.6/100	50.0 ± 0.4/100	49.9 ± 0.6/100	49.9 ± 0.6/100
	100	100.8 ± 1.2/100	99.5 ± 0.8/99	99.8 ± 1.2/100	100.1 ± 1.1/100
	150	149.3 ± 1.4/99	150.3 ± 1.8/100	149.6 ± 1.3/100	150.6 ± 1.3/100
	200	199.5 ± 1.9/100	199.3 ± 1.6/100	200.9 ± 1.5/101	200.8 ± 1.9/100

[a] Average value ± standard deviation of six determinations.

3.4. Application and validation of the method

The proposed method was applied to the determination of possible quinolones in four different commercial egg samples. None of the samples analysed gave a positive result for these compounds.

In Fig. 1(B and C), representative chromatograms of unspiked and spiked whole egg samples are shown.

Egg samples were spiked at different levels: 50, 100, 150 and 200 ng g^{-1} for CIPRO, ENRO, DI, FLU, OXO and SARA and 20, 40, 80 and 150 ng g^{-1} for DANO. The validation of the proposed method for these samples was tested by using a recovery test (Student *t*-test) [37]. As the *P*-values calculated in all cases are greater than 0.05, the null hypothesis appears to be valid, i.e., recoveries are close to 100%. The results obtained are shown in Table 3.

4. Conclusions

The determination and quantification of a series of quinolones by liquid chromatography with fluorescence detection in poultry eggs was successfully performed on a Zorbax Eclipse XDB-C_8 column, with a linear gradient composed of acetonitrile and 10 mM citrate buffer of pH 4.5. A rapid and simple treatment was used in order to extract quinolones from spiked egg samples. The method was validated by a recovery assay with spiked samples.

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