**Determination of quinolone residues in infant and young children powdered milk
combining solid-phase extraction and ultra-performance liquid chromatography-
tandem mass spectrometry**

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Abstract

The present work describes a method based on solid-phase extraction (SPE) and ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) for the simultaneous determination of three quinolones (pipemidic acid, oxolinic acid and flumequine) and twelve fluoroquinolones (marbofloxacin, fleroxacin, pefloxacin, levofloxacin, norfloxacin, ciprofloxacin, enrofloxacin, danofloxacin, lomefloxacin, difloxacin, sarafloxacin, and moxifloxacin) in different infant and young children powdered milks. After suitable deproteination of the reconstituted powdered samples, a SPE procedure was developed providing recovery values higher than 84% (RSDs lower than 13%) for all the analytes, with limits of detection between 0.04 and 0.52 µg/kg. UPLC-MS/MS analyses were carried out in less than 10 minutes. Sixteen infant and young children powdered milk samples of different origin, type and composition bought at Spanish markets were analyzed. Residues of the selected antibiotics were not detected in any of the analyzed samples.

Keywords: Quinolones, antibiotics, UPLC-MS/MS, SPE, powdered milk, infants

1. Introduction

During the last decade, special concern has arisen in the food safety field on infant foods, because infants' low body weight makes them more vulnerable to food contaminants or residues (pesticides, pharmaceutical compounds, etc.). This fact is of special importance in infants up to two years old since they have a relative food intake that can be three times higher than that of adults. The wide variety of food products nowadays available, as well as the high number of target analytes to be analyzed in food monitoring programs, demands the development of ultra-fast analytical techniques able to provide results in a short time with extremely low detection limits to fulfill the requirements established by the current legislation.

Infant foods are divided into infant formulae (usually for the first six months of life), follow-on formulae (when appropriate complementary feeding is introduced and indicated usually for babies between 6 and 12 months) and growth formulae according to the age of the children they are aimed at (usually babies older than one year). Their chemical composition is based on infants' nutritional requirements and in most cases they are partly manufactured from bovine milk resulting in a powder that should be carefully reconstituted in boiled water. Nowadays, there is a relatively high number of infant powdered milk products with a variable composition commercially available worldwide. As a result of their bovine origin, it is of high importance to ensure that such products are free of veterinary drug residues like, for example, antibiotics that can develop resistance at this initial stage of life. In this sense, and with the aim of protecting infants' health, the European Union (UE), has already established maximum residue limits (MRLs) for pesticides and mycotoxins in processed fruit, vegetables or cereal-based baby foods and infant formulae [1,2] because of the importance of both types of compounds. However, up to now, no legislation has been published regarding MRLs of pharmacologically active substances (including antibiotics) in such products, despite the fact that in the Commission Regulation 37/2010 [3] general MRLs are established for foodstuffs of animal origin. Even though, and concerning the analysis of antibiotics in infant and young children powdered milk, general MRLs established for bovine milk can be considered as a reference.

Quinolones constitute an important family of antibiotics with a broad spectrum activity against Gram-positive and Gram-negative bacteria which are widespread used worldwide for the treatment of respiratory diseases and bacterial infections in humans and some food producing animals. In this last case, it is well known that their residues

can appear in foods of animal origin like meat, milk or its derivatives. [4-6]. Concerning EU MRLs of quinolones, only seven of them have been included in Commission Regulation 37/2010 [3]: danofloxacin (DANO), sarafloxacin (SARA), enrofloxacin (ENRO), flumequine (FLUME), marbofloxacin (MARBO), oxolinic acid (OXO) and difloxacin (DIFLO) which is a metabolite of SARA. However, SARA's MRL has not been established in milk while DIFLO and OXO are forbidden in animals from which milk is produced for human consumption. MRLs for the other quinolones are 30 µg/kg for DANO, 100 µg/kg for ENRO expressed as sum of ENRO and ciprofloxacin (CIPRO, a metabolite of ENRO), 50 µg/kg for FLUME and 75 µg/kg for MARBO [3].

Up to now, very few articles have been published dealing with the analysis of antibiotics in baby foods [7-11], most of them [7-10] focusing on the analysis of some quinolones and fluoroquinolones (quinolones with a fluor atom in C-6 and a piperazinyl moiety in C-7). Such low number of works clearly indicates the need to develop suitable analytical approaches for this task at very low levels. As an example, Díaz-Álvarez et al. [8] analyzed nine quinolones (cinoxacin, nalidixic acid, enoxacin, OXO, FLUME, norfloxacin (NORFLO), ENRO, CIPRO and DANO) in baby foods based on chicken meat, vegetables, potatoes puree and olive oil. Rodríguez et al. [7] extracted five fluoroquinolones (CIPRO, ENRO, DANO, SARA and lomefloxacin (LOME) as internal standard) from infant milks and later extended this work to the determination of six fluoroquinolones (NORFLO, CIPRO, ENRO, DANO, SARA and LOME) in powdered milk-based infant formulae and poultry-based baby foods [9]. In all these cases, high-performance liquid chromatography (HPLC) was used either in combination with UV [8] or fluorescence detection [7, 9, 10] with MS/MS confirmation [7, 9]. Ultra-performance liquid chromatography (UPLC) usually provides lower analysis time and higher efficiency and sensitivity compared to HPLC. UPLC has been previously used with success for the analysis of several small groups of quinolone antibiotics in milk [12-16] although no infant powdered milk products have been investigated so far applying a multiresidue method for the simultaneous determination of quinolones and fluoroquinolones.

In this work, different quinolones, namely pipemidic acid (PIP), MARBO, fleroxacin (FLERO), levofloxacin (LEVO), pefloxacin (PEFLO), NORFLO, CIPRO, ENRO, DANO, LOME, DIFLO, SARA, moxifloxacin (MOXI), OXO and FLUME (Figure 1) are studied, including those for which the EU has established MRLs in milk. Thus, the aim of this contribution was to develop and apply a multiresidue method

based on the use of UPLC-MS/MS to simultaneously determine these antibiotics in different types of infant powdered milk products (infant, follow-on and growth formulae).

2. Experimental

2.1. Chemicals and samples

All chemicals used were of analytical reagent grade. LOME hydrochloride, MARBO, CIPRO, DANO, ENRO, SARA hydrochloride, DIFLO hydrochloride, PIP, PEFLO mesylate dehydrate, FLERO, FLUME, and NORFLO were provided by Sigma Aldrich (Madrid, Spain). LEVO and OXO were obtained from Fluka (Madrid, Spain). MOXI was kindly supplied by Bayer HealthCare (Wuppertal, Germany). Quinolones were used without further purification (purity $\geq 98\%$). Individual standard solutions (100 mg/L) were prepared in MeOH and kept in the dark under refrigeration at 4°C. Mixtures of quinolones were prepared by appropriate combination and dilution with MeOH.

Acetonitrile (ACN) and methanol (MeOH) were HPLC-grade from Lab-Scan (Gliwice, Sowinskiego, Poland) and distilled water was deionized by using a Milli-Q system (Millipore, Bedford, MA, USA). Acetic acid was purchased from Scharlau (Sentmenat, Spain), trichloroacetic acid (TCA) was from Merck (Darmstadt, Germany) and dehydrated ethylenediaminetetraacetic acid disodium salt (EDTA) and formic acid were from Sigma and Fluka, respectively.

Infant formulae (for the first six months of life), follow-on formulae (indicated for babies between 6 and 12 months old) and growth formulae (for children above one year old) were bought in local supermarkets and chemists (Madrid, Spain). Samples were spiked with the selected antibiotics at several concentrations (see Results and Discussion section) in order to identify and quantify the analytes in the real samples.

2.2. UPLC-MS/MS

UPLC-MS/MS analyses were carried out using an Accela liquid chromatograph (Thermo Scientific, San Jose, CA) also equipped with a diode array detector (DAD) and an autosampler. The chromatograph was coupled to a TSQ Quantum (Thermo Scientific) triple quadrupole analyzer via an electrospray (ESI) interface. For data storage and evaluation the Xcalibur software (Thermo Scientific) was used. Analytical conditions employed consisted of the use of a Hypersil Gold C₁₈ column (50 mm $\times$ 2.1 mm and 1.9 μ m of particle diameter) (Thermo Scientific) using 0.1% (v/v) formic acid

in Milli-Q water as mobile phase A and MeOH as mobile phase B. Elution was carried out according to the following gradient: 0 min, 10% B; 6 min, 40% B; 9 min, 50% B; 9.1 min, 10% B; 13 min, 10% B. The optimum flow rate was 0.3 mL/min whereas the injection volume was 10 μ L. The DAD recorded the spectra from 200 to 450 nm. Column and autosampler compartments were thermostated at 25°C.

To quantify the quinolones, the mass spectrometer was operated in the positive ESI mode. Spray voltage and capillary temperature were set at 3500 V and 250°C, respectively. Nitrogen was used as sheath and auxiliary gas at pressures of 40 and 20 arbitrary units, respectively. Ion sweep gas pressure was 2 units and collision gas (Ar) pressure, 1.5 mTorr. Scan width and the scan time were fixed at 0.020 (m/z) and 0.100 s and system was operated in selected reaction monitoring (SRM). SRM parameters were optimized by direct infusion of standards. Two transition ions were monitored for identification but only the most intense one for each precursor ion was used for quantification. The values corresponding to the tube lens offset voltage and collision energy for each selected ion transitions are indicated in Table 1. The StatGraphics Plus Software (Statistical Graphics, Rockville, USA) was used for data processing.

2.3. Sample deproteination and SPE procedure

Powdered milk samples were prepared according to manufacturers' indications. SPE procedure was adapted from a method previously developed by our group to extract seven fluoroquinolones from bovine, ovine and caprine milk [17]. Briefly, reconstituted milk samples (2 mL) were spiked with the selected quinolones and deproteinated by mixing with 6 mL of ACN containing 20% w/v TCA and sonicated for 30 min. Then, the mixture was kept in dark for 15 min and centrifuged for 15 min at 4500 rpm. After suitable filtration of the supernatant with Chromafil Xtra PET-45/25 filters of 0.45 μ m pore size (Macherey-Nagel GmbH & Co. KG, Düren, Germany) the organic solvent was evaporated using a rotary evaporator R-200 equipped with a V-800 vacuum controller and a V-500 vacuum pump (Büchi Labortechnik, Flawil, Switzerland). The residue was dissolved in water containing 11.24 g/L of EDTA. pH was adjusted to 4.0 with NaOH (1 M) and the sample was centrifuged again for 15 min at 4500 rpm. Then, the supernatant was subjected to a SPE procedure in a Vac-Master manifold (IST, Hengoed, South Wales, UK) using Oasis HLB cartridges (6 mL, 500 mg) from Waters, previously activated with 5 mL of MeOH and 5 mL of Milli-Q water. When the retention step was completed, the cartridge was dried under vacuum of -10

mmHg (1 mmHg=133.322 Pa) for 20 min and elution of the retained antibiotics was carried out with 10 mL MeOH containing 1.5% (w/v) acetic acid. The eluate was then evaporated to dryness at 40°C and 200 mbar. The dry residue was redissolved in 1 mL of MeOH and directly injected into the UPLC instrument.

Recovery values were assessed by comparing the peak areas obtained for standards spiked in milk samples before the extraction procedure with those obtained for standards spiked after the extraction step. In both cases, the samples did not contain the target analytes.

3. Results and discussion

3.1. UPLC-MS optimization

The first step in the optimization of the separation of the selected antibiotics was the determination of the optimum UPLC separation parameters. To do that, a short C₁₈ chromatographic column was employed containing sub 2-µm particles (50 x 2.1 mm, 1.9 µm particle diameter). The use of this kind of columns allows the attaining of fast separations with improved efficiencies compared to conventional columns [18]. According to the nature of the fifteen selected quinolones, different mobile phases were tested. Namely, water was employed containing 0.1-0.2 % v/v formic acid as mobile phase A whereas 100% methanol and 100% acetonitrile (both with and without 0.1% v/v formic acid) were tested as mobile phase B. The best results in terms of peak shapes and best signal-to-noise ratios in the positive ESI mode were provided by the combination of acidified water (0.1% v/v formic acid) and 100% methanol as mobile phases. Once these mobile phases were selected, the gradient of elution was adapted to maximize the separation among the interesting compounds while reducing the elution time. Temperature effect in the column compartment was studied between 25 and 40°C to improve separation. However, although high temperature values could speed up the analyte elution, a decrease in the separation efficiency was observed at 40°C. Thus, 25°C was selected for subsequent experiments. In section 2.2 the optimum separation parameters finally selected are described. Using these conditions, the separation of the 15 compounds investigated was performed in less than 10 min.

Once the UPLC separation was optimized, the different MS/MS detection parameters were studied. In this sense, the UPLC-MS/MS analysis of contaminants must fulfill the number of identification points required by the current EU legislation for the analysis of these components in foods [19]. In this work, two product ions for each

determined compound have been selected (from their corresponding precursor ions) which complies with the requirements proposed in this legislation. In this way, each compound is precisely characterized by its retention time as well as by two precursor-product ion transitions. The most intense product ion was employed for quantification whereas the other one was used for peak identification. Although the selective detection of the antibiotics through the selection of appropriate ion transitions was optimized infusing directly those compounds into the MS instrument (see Table 1), the sensitivity of the analytical method might be significantly increased fine tuning the rest of ionization conditions. In this sense, the different parameters involved in the positive ESI of the antibiotics were studied, namely, capillary temperature (250-350°C), spray voltage (3000-4500 V), sheath gas pressure (30-50 a.u.) and auxiliary gas pressure (0-5 a.u.). The different parameters were optimized using a univariate method. The values which provided best total ion intensities were selected. The final detection and ionization conditions included the use of a capillary temperature of 250°C, 3500 V in the spray voltage, and N₂ pressures of 40 and 20 a.u. for the sheath and auxiliary gas, respectively, in the positive ESI mode.

3.2. Intraday/interday precision and calibration studies.

Once optimum separation conditions and MS/MS detection parameters were selected, instrumental intraday and interday precision was assessed. For this purpose, mixtures of the selected quinolones at two levels of concentration (between 20 µg/L and 800 µg/L) were consecutively injected five times in the same day (n=5) and also in three different days (n=15). RSD values obtained in the same day were in the range between 0.3% and 8.1% for peak areas and between 0.1% and 0.6% for retention times for the highest concentration level. Day-to-day RSD values were slightly higher in the range between 5.1% and 10.2% for peak areas and between 0.2% and 1.3% for retention times. Results obtained for the lowest concentration level were similar to the ones described above: RSD values obtained in the same day were in the range 2.4-10.1% for peak areas and 0.2-0.8% for retention times, while day-to-day RSD values were in the range 6.5-10.8% for peak areas and 0.4-1.6% for retention times.

Besides, calibration curves were obtained by the triplicate injection of eight different concentration levels (n=8) (data not shown). Determination coefficients (R²) higher than 0.99 were obtained in all cases. Instrumental LODs values, which were calculated as three times the signal-to-noise (S/N) ratio, were lower than 1.06 µg/L

while instrumental LOQs values (calculated as ten times the S/N) were lower than 3.52 µg/L. The obtained data demonstrated the suitability of the optimized UPLC-MS/MS for the fast and sensitive detection of the 15 studied quinolones in a single run.

3.3. Optimization of the deproteinization procedure.

Samples investigated in this work have a protein and fat content in the range 10-15% and 14-30%, respectively, and therefore, suitable protein precipitation and defatting of the samples is necessary. Initial experiments were carried out considering 2 mL of a reconstituted infant formulae (as indicated by each manufacturer) and deproteinization with 6 mL of 20% (w/v) TCA in MeOH. After suitable sonication, centrifugation and filtration, the supernatant was evaporated to dryness and redissolved in 50 mL of water containing 0.562 g of EDTA and adjusted to pH 4.0. Afterwards, the sample was passed through a previously activated Oasis HLB SPE cartridge of 500 mg. Addition of EDTA is necessary since the formation of complexes between these quinolones and EDTA clearly improves their SPE extraction with Oasis HLB cartridges [20]. After elution with 10 mL of MeOH containing 1.5% (w/v) of acetic acid, the evaporated sample was reconstituted in 1 mL MeOH and injected in the UPLC system. As indicated in the experimental section, recovery values were assessed by comparing the peak areas obtained for standards dissolved in the final sample matrix (free of the target quinolones) with those obtained for spiked samples. Under these conditions, mean recovery values of the selected quinolones were in the range 57-85%, which are clearly lower than our previous results obtained for the extraction of a reduced number of quinolones from raw milk which were between 73 and 113% (RSDs<16%) [17].

Therefore, the extraction protocol had to be modified to improve the obtained results. To do that, several modifications of the deproteinization conditions were carried out. For this purpose, higher amounts of MeOH and TCA percentages were used, as well as the use of ACN instead of MeOH, which has also shown to provide good results [13]. Besides, EDTA amount was also increased since our previous experience with the method clearly showed that depending of the calcium or magnesium content of the sample, competition of both cations for the EDTA may take place and therefore, higher amounts of the complexing agent might be necessary [20]. For comparison purposes, blank samples, which were found to be free of the selected analytes, were also analyzed. Figure 2 shows the recovery values obtained for each quinolone when some of the deproteinization parameters as well as EDTA amount were changed. As it can be seen in

that Figure, a clear and comparable improvement in the recovery values were obtained (77-107 %) for two of the tested conditions, namely when 9 mL of MeOH containing 20% (w/v) of TCA or 6 mL ACN containing 20% (w/v) TCA were used for the deproteination. These recoveries were also comparable to those attained in our previous work for raw milk [17]. As a result, and in order to minimize the volumes of organic solvent consumed, 6 mL of ACN containing 20% (w/v) TCA were used for subsequent experiments. The increase in the amount of EDTA did not improve the recovery values for any of the analyzed quinolones, being the initial amount (0.562g) enough to ensure a quantitative complexation for all analytes and, therefore, a suitable retention of the complexed analytes onto the SPE stationary phase.

3.4. Matrix-matched calibration

With the aim of verifying the influence of the sample matrix in the MS response, matrix effect of each sample type was studied by obtaining the calibration curves of the standards dissolved in methanol and in each of the three different sample matrices (using also methanol as solvent) at eight concentration levels. Each level was injected in triplicate in the UPLC-MS system. Table 2 shows matrix-matched calibration parameters including the regression equation (based on peak areas), the determination coefficient (R^2) and standard deviation of the residuals ($S_{y/x}$) for the three types of matrices studied. Determination coefficients (R^2) higher than 0.994 were obtained in all cases. Statistical comparison (calculation of F and p values) of matrix-matched calibration curves with the ones obtained in pure solvent was developed in order to evaluate if there is a change in the values of the intercepts or the slopes of the regression curves for each quinolone. If this happens, then there is a matrix effect for the compound under study and it would be necessary to develop a calibration in the sample matrix to get truthful results. In this sense, it could be clearly seen that a matrix effect occurred for almost all the quinolones. Besides, statistical comparison between matrix-matched calibration curves revealed that there were also clear differences between them. Consequently, for quantification purposes matrix-matched calibration should be developed and applied for each sample type.

3.5 Extraction recovery study.

Once optimum SPE conditions were achieved, a recovery study was carried out at two levels of concentration for the three types of powdered milk samples (infant,

follow-on and growth formulae), with the aim of demonstrating the applicability of the method. Five replicates were carried out at each level. A previous extraction was developed for each sample in order to verify the absence of the selected quinolones. Results of the recovery study as well as the spiking levels are shown in Table 3. As it can be appreciated, recovery values were between 84 and 108% with RSDs values lower than 17% for infant formulae, between 98 and 117% with RSDs values lower than 11% for follow-on formulae and between 88 and 106% with RSDs lower than 9% for growth formulae. These results demonstrate the applicability of the developed method to extract quinolone residues from different types of powdered milk samples devoted to infants' consumption, independently on their fat, protein and carbohydrate contents. LODs and LOQs of the method obtained for the reconstituted samples were in the range 0.04-0.54 $\mu\text{g/L}$ and 0.14-1.80 $\mu\text{g/L}$, respectively. Considering the sample weight, LOD values were between 0.04 and 0.52 $\mu\text{g/kg}$, while LOQ values were between 0.13 and 1.73 $\mu\text{g/kg}$. They were calculated as the lowest analyte concentration in the real sample that provided a signal-to-noise ratio of 3 and 10, respectively. Such quantification limits are well below EU MRLs already established for some of these compounds (30–100 $\mu\text{g/kg}$) in bovine raw milk [3], which can be taken as a reference, although, as previously indicated, there is no current legislation regarding MRLs for quinolones in this type of samples. Figure 3 shows the UPLC-MS/MS chromatograms of a spiked powdered milk (growth formula) after the deproteination and SPE procedure. As it can be deduced from the figure, the selected quinolones can be perfectly identified and quantified in such complex samples, since clean extracts can be observed without interferences. Furthermore, it can be noted that, although complete resolution is not obtained for some of the quinolones, MS/MS allows the selective determination of these compounds since unique transitions are monitored. Similar chromatograms were obtained for the other two types of samples.

Concerning previous works dealing with the analysis of some of these quinolones in baby foods, LOQs reached in our work are lower than those previously obtained for the analysis of CIPRO, ENRO, DANO, SARA and LOME (as internal standard) by SPE-HPLC-FD (LOQs of 6-80 $\mu\text{g/kg}$) [7] as well as lower than those attained after the pressurized liquid extraction and HPLC-FD analysis of NORFLO, CIPRO, ENRO, DANO, SARA and LOME (LOQs of 12-22 $\mu\text{g/kg}$) in powdered infant formulae [9].

As it has been already stated, this is the first time that UPLC-MS/MS is employed for the analysis of quinolone residues in infant powdered milk. Nevertheless, the obtained LODs are comparable to those attained in previous UPLC-MS/MS-based applications for the analysis of quinolones in raw milk samples [14] and lower than the ones obtained by QuEChERS-UPLC-QqQ-MS [12, 16] and by ultrafiltration-UPLC-TOF-MS [13].

3.6. Powdered milk samples analysis

In order to fully demonstrate the applicability of the proposed method, 16 samples of different type, origin and composition commercialized in Spain were analyzed. Samples included five infant formulae, eight follow-on formulae and three growth formulae (one of them with cereals). None of the analyzed samples presented any detectable residues of the selected quinolones in good agreement with previous works in which a more reduced number of quinolones were analyzed in similar samples [7, 9].

4. Conclusions

In this work, the combined use of SPE and UPLC-MS/MS is proposed for the simultaneous and rapid analysis of 15 quinolones in infant powdered milk of different type, origin and composition. The method, which involves a previous deproteination of the samples with ACN containing 20% (w/v) of TCA, is highly sensitive (LODs below 0.52 µg/kg for the reconstituted samples), repeatable and selective, with high recovery values (>84%). The analysis of 16 commercialized powdered milk samples (infant, follow-on and growth formulae) revealed the absence of these compounds in all the samples.

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Figure captions

Figure 1.- Structures, acronyms and monoisotopic molecular weight of the selected quinolones.

Figure 2.- Effect of deproteination conditions on the recovery values of the selected quinolones: (1) 6 mL MeOH 20% TCA, (2) 6 mL MeOH 30% TCA, (3) 9 mL MeOH 20% TCA, (4) 6 mL ACN 20% TCA; and (5) effect of the addition of a higher amount of EDTA (0.750 g) on the recoveries (in this case 6 mL MeOH 20% TCA were used for deproteination of the sample). See experimental section for details.

Figure 3.- UPLC-MS/MS chromatogram of a growth powdered milk spiked at 400 µg/L with the studied quinolones.

444 **Table 1.-** Retention times and MS/MS detection parameters for the selected quinolones.

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Peak	Antibiotic	t _R (min)	Precursor ion (m/z) [M+H] ⁺	SRM transitions		Tube lens offset (V)
				Quantifier ion (m/z) (Collision energy, V)	Qualifier ion (m/z) (Collision energy, V)	
1	PIP	3.36	304.1	217.1 (21)	189.0 (31)	87
2	MARBO	3.69	363.1	320.0 (12)	276.2 (13)	94
3	FLERO	3.83	370.1	326.1 (17)	269.1 (27)	93
4	LEVO	4.21	362.1	318.1 (19)	261.1 (26)	92
5	PEFLO	4.24	334.1	290.2 (17)	233.1 (25)	86
6	NORFLO	4.42	320.1	302.1 (20)	276.1 (15)	88
7	CIPRO	4.63	332.1	314.2 (21)	288.1 (15)	85
8	ENRO	4.77	360.2	316.1 (18)	245.1 (25)	93
9	DANO	4.85	358.1	340.2 (23)	314.1 (19)	91
10	LOME	4.90	352.1	265.1 (22)	308.1 (22)	90
11	DIFLO	5.20	400.2	356.1 (20)	299.0 (28)	94
12	SARA	5.45	386.1	342.1 (16)	299.0 (21)	97
13	MOXI	6.56	402.2	384.1 (23)	261.0 (22)	97
14	OXO	6.89	262.1	244.0 (20)	130.2 (35)	84
15	FLUME	9.07	262.1	244.1 (16)	202.0 (32)	85

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Table 2.-Matrix matched calibration data of the selected quinolones.

Peak	Antibiotic	Matrix	Range of concentration tested (µg/L)	Slope (n=8)	Intercept (n=8)	R ²	S _{y/x}
1	PIP	Infant powdered milk	1.0-1200	(1.00±0.05) 10 ⁴	(6.19±28.1) 10 ⁴	0.998	2.08·10 ⁵
		Follow-on powdered milk		(9.98±0.50) 10 ³	(5.79±3.08) 10 ⁵	0.999	1.99·10 ⁵
		Growth powdered milk		(1.29±0.06) 10 ⁴	(3.65±31.6) 10 ⁴	0.999	2.53·10 ⁵
2	MARBO	Infant powdered milk	1.5-1200	(3.74±0.41) 10 ³	(2.03±26.2) 10 ⁴	0.999	8.94·10 ⁴
		Follow-on powdered milk		(4.04±0.28) 10 ³	(1.58±1.72) 10 ⁵	0.998	1.10·10 ⁵
		Growth powdered milk		(5.27±0.21) 10 ³	(-7.91±119) 10 ³	0.999	9.44·10 ⁴
3	FLERO	Infant powdered milk	1.5-1200	(2.59±0.06) 10 ⁴	(-1.72±28.6) 10 ⁴	1.000	2.09·10 ⁵
		Follow-on powdered milk		(2.39±0.12) 10 ⁴	(1.01±0.71) 10 ⁶	0.999	4.56·10 ⁵
		Growth powdered milk		(3.06±0.09) 10 ⁴	(1.35±5.14) 10 ⁵	0.999	4.07·10 ⁵
4	LEVO	Infant powdered milk	1.0-1200	(2.33±0.10) 10 ⁴	(2.19±5.18) 10 ⁵	0.999	3.83·10 ⁵
		Follow-on powdered milk		(2.53±0.21) 10 ⁴	(1.02±1.29) 10 ⁶	0.996	8.38·10 ⁵
		Growth powdered milk		(3.28±0.10) 10 ⁴	(1.20±5.45) 10 ⁵	0.999	4.37·10 ⁵
5	PEFLO	Infant powdered milk	2.0-1200	(1.12±0.14) 10 ⁴	(2.15±7.77) 10 ⁵	0.996	4.26·10 ⁵
		Follow-on powdered milk		(1.28±0.06) 10 ⁴	(4.30±3.42) 10 ⁵	0.999	2.15·10 ⁵
		Growth powdered milk		(1.49±0.04) 10 ⁴	(1.36±2.38) 10 ⁵	0.999	1.86·10 ⁵
6	NORFLO	Infant powdered milk	5.0-1200	(2.57±0.08) 10 ⁴	(3.21±45.9) 10 ⁴	1.000	2.59·10 ⁵
		Follow-on powdered milk		(2.84±0.17) 10 ⁴	(1.56±1.09) 10 ⁶	0.998	6.26·10 ⁵
		Growth powdered milk		(3.50±0.16) 10 ⁴	(-1.77±9.51) 10 ⁵	0.998	6.94·10 ⁵
7	CIPRO	Infant powdered milk	2.0-1200	(2.99±0.18) 10 ⁴	(-1.84±9.32) 10 ⁵	0.998	6.74·10 ⁵
		Follow-on powdered milk		(3.19±0.20) 10 ⁴	(9.26±12.2) 10 ⁵	0.998	7.70·10 ⁵
		Growth powdered milk		(3.79±0.13) 10 ⁴	(-9.43±71.5) 10 ⁴	0.999	5.59·10 ⁵
8	ENRO	Infant powdered milk	2.0-1200	(2.58±0.15) 10 ⁴	(1.85±7.96) 10 ⁵	0.998	5.76·10 ⁵
		Follow-on powdered milk		(3.04±0.17) 10 ⁴	(8.49±10.3) 10 ⁵	0.998	6.49·10 ⁵
		Growth powdered milk		(3.58±0.14) 10 ⁴	(5.54±78.5) 10 ⁴	0.999	6.14·10 ⁵
9	DANO	Infant powdered milk	2.0-1200	(2.20±0.12) 10 ⁴	(1.77±6.36) 10 ⁵	0.998	4.60·10 ⁵
		Follow-on powdered milk		(2.43±0.16) 10 ⁴	(6.52±9.57) 10 ⁵	0.998	6.02·10 ⁵
		Growth powdered milk		(2.98±0.16) 10 ⁴	(-2.92±8.86) 10 ⁵	0.998	6.93·10 ⁵
10	LOME	Infant powdered milk	1.5-1200	(2.18±0.09) 10 ⁴	(1.73±4.54) 10 ⁵	0.999	3.32·10 ⁵
		Follow-on powdered milk		(2.95±0.25) 10 ⁴	(1.48±1.51) 10 ⁶	0.996	9.65·10 ⁵
		Growth powdered milk		(3.47±0.09) 10 ⁴	(4.72±4.92) 10 ⁵	1.000	3.89·10 ⁵
11	DIFLO	Infant powdered milk	3.0-1200	(1.74±0.05) 10 ⁴	(5.56±30.8) 10 ⁴	1.000	1.73·10 ⁵
		Follow-on powdered milk		(2.24±0.09) 10 ⁴	(6.81±5.37) 10 ⁵	0.999	3.28·10 ⁵
		Growth powdered milk		(2.75±0.16) 10 ⁴	(-7.70±89.4) 10 ⁴	0.998	6.83·10 ⁵
12	SARA	Infant powdered milk	5.0-1200	(9.82±0.84) 10 ³	(-1.14±4.48) 10 ⁵	0.996	3.03·10 ⁵
		Follow-on powdered milk		(1.02±0.05) 10 ⁴	(5.80±3.27) 10 ⁵	0.999	1.88·10 ⁵
		Growth powdered milk		(1.22±0.02) 10 ⁴	(6.71±11.7) 10 ⁴	1.000	8.56·10 ⁴
13	MOXI	Infant powdered milk	3.0-1200	(1.82±0.15) 10 ⁴	(-4.11±9.01) 10 ⁵	0.997	5.62·10 ⁵
		Follow-on powdered milk		(1.15±0.27) 10 ⁴	(5.33±5.90) 10 ⁵	0.994	1.80·10 ⁵
		Growth powdered milk		(2.41±0.10) 10 ⁴	(-4.30±5.67) 10 ⁵	0.999	4.33·10 ⁵
14	OXO	Infant powdered milk	6.0-1200	(6.52±0.85) 10 ⁴	(3.42±4.84) 10 ⁶	0.995	2.65·10 ⁶
		Follow-on powdered milk		(7.600±0.72) 10 ⁴	(7.16±4.87) 10 ⁶	0.997	2.19·10 ⁶
		Growth powdered milk		(6.84±0.68) 10 ⁴	(2.57±4.53) 10 ⁶	0.997	2.15·10 ⁶
15	FLUME	Infant powdered milk	2.0-1200	(3.43±0.11) 10 ⁵	(1.46±5.65) 10 ⁶	0.999	4.09·10 ⁶
		Follow-on powdered milk		(3.17±0.05) 10 ⁵	(-1.71±2.98) 10 ⁶	1.000	1.87·10 ⁶
		Growth powdered milk		(2.76±0.04) 10 ⁵	(3.99±21.5) 10 ⁵	1.000	1.68·10 ⁶

449 **Table 3.-** Mean recovery percentages (n=5), RSDs (between parenthesis in italics), spiked concentration (between parenthesis in bold), LOD and
450 LOQ values of method.
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Peak	Antibiotic	Infant powdered milk				Follow-on powdered milk				Growth powdered milk			
		Level 1, recovery ^a	Level 2, recovery ^a	LOD method	LOQ method	Level 1, recovery ^a	Level 2, recovery ^a	LOD method	LOQ method	Level 1, recovery ^a	Level 2, recovery ^a	LOD method	LOQ method
		(%RSD) (added µg/L)	(%RSD) (added µg/L)	(µg/kg)	(µg/kg)	(%RSD) (added µg/L)	(%RSD) (added µg/L)	(µg/kg)	(µg/kg)	(%RSD) (added µg/L)	(%RSD) (added µg/L)	(µg/kg)	(µg/kg)
1	PIP	95 (17) (10)	92 (6) (400)	0.05	0.16	109 (4) (10)	110 (4) (400)	0.04	0.13	88 (9) (10)	99 (6) (400)	0.05	0.15
2	MARBO	95 (15) (15)	106 (5) (400)	0.09	0.31	106 (9) (15)	111 (8) (400)	0.09	0.29	98 (9) (15)	98 (7) (400)	0.10	0.32
3	FLERO	88 (15) (15)	93 (8) (400)	0.11	0.36	104 (9) (15)	117 (6) (400)	0.09	0.29	94 (5) (15)	94 (6) (400)	0.10	0.35
4	LEVO	89 (13) (10)	86 (7) (400)	0.06	0.22	109 (4) (10)	114 (7) (400)	0.05	0.17	95 (5) (10)	102 (8) (400)	0.06	0.19
5	PEFLO	91 (11) (20)	95 (7) (400)	0.19	0.64	110 (8) (20)	114 (9) (400)	0.16	0.53	94 (5) (20)	106 (7) (400)	0.18	0.59
6	NORFLO	97 (12) (50)	97 (13) (400)	0.32	1.07	110 (6) (50)	114 (6) (400)	0.28	0.93	89 (6) (50)	103 (5) (400)	0.33	1.08
7	CIPRO	98 (14) (20)	90 (9) (400)	0.16	0.53	111 (5) (20)	110 (7) (400)	0.14	0.45	90 (5) (20)	104 (5) (400)	0.15	0.51
8	ENRO	94 (9) (20)	91 (6) (400)	0.19	0.64	109 (4) (20)	105 (7) (400)	0.17	0.56	97 (7) (20)	95 (6) (400)	0.19	0.62
9	DANO	87 (10) (20)	89 (3) (400)	0.17	0.56	111 (3) (20)	118 (7) (400)	0.13	0.44	95 (8) (20)	102 (7) (400)	0.15	0.50
10	LOME	92 (15) (15)	87 (10) (400)	0.12	0.38	112 (6) (15)	117 (6) (400)	0.09	0.30	101 (5) (15)	102 (8) (400)	0.10	0.34
11	DIFLO	89 (5) (30)	89 (7) (400)	0.24	0.79	117 (4) (30)	114 (6) (400)	0.18	0.61	101 (7) (30)	103 (3) (400)	0.21	0.69
12	SARA	97 (11) (50)	91 (3) (400)	0.31	1.02	116 (4) (50)	117 (8) (400)	0.25	0.82	99 (8) (50)	97 (6) (400)	0.29	0.97
13	MOXI	94 (14) (30)	84 (5) (400)	0.23	0.78	108 (7) (30)	100 (8) (400)	0.20	0.67	98 (9) (30)	101 (4) (400)	0.21	0.70
14	O XO	94 (8) (60)	108 (8) (400)	0.51	1.69	98 (3) (60)	109 (11) (400)	0.49	1.65	99 (3) (60)	97 (6) (400)	0.52	1.74
15	FLUME	94 (3) (20)	96 (1) (400)	0.15	0.51	98 (6) (20)	98 (4) (400)	0.15	0.49	101 (2) (20)	100 (1) (400)	0.14	0.48

452 ^a Mean of five extractions (n=5).
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