Analyze Quinolone Residues in Milk with an Agilent Poroshell 120, 4 µm Column

Application Note

Food Testing and Agriculture

Authors

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Abstract

A method for the simultaneous determination of quinolone residues in milk was developed and validated. The analytes were extracted and cleaned with Agilent Bond Elut Plexa solid phase extraction, separated on Agilent Poroshell 120 EC-C18, 4 µm and 2.7 µm columns, and quantified by liquid chromatography with fluorescence detection. The dynamic calibration range for danofloxacin was 30 to 150 µg/kg, and 50 to 250 µg/kg for flumequine. Dynamic calibration range for the other sulfonamides was 100 to 500 µg/kg. Overall recoveries ranged from 79.3 to 99.1%, with RSD values between 2.6 and 7.4%.

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Introduction

Quinolones are a family of synthetic broad-spectrum antibacterial drugs. The quinolone antibiotics (Figure 1) are synthetic antimicrobial agents widely used in human and veterinary medicine. Extensive use of these antibiotics in veterinary medicine and medicated feed plays a crucial role in intensive production of animals bred for food. This leads to a significant increase in antibiotic resistance and allergic reactions, having therefore important consequences for public health. To safeguard human health, the European Union set maximum residue limits (MRLs) of 30 µg/kg for the sum of enrofloxacin and its metabolite, ciprofloxacin, in muscle, kidney, and liver [1]. Because of the great variety of quinolones, and the possibility of trace residues in edible tissue, it is necessary to develop sensitive multiresidue screening methods for their determination.

The China regulation of GB 29692-2013 set MRLs of 11 quinolines for the concentrations of antibiotic residues in milk. Residue limits vary from 15 to 50 µg/kg [2]. The objective of this work was to develop a multiresidue method that would be simple and fast for routine regulatory analysis of quinolone antibiotic residues in milk. The method relies on a simple SPE step using a polymer sorbent (Agilent Bond Elut Plexa) and an HPLC column separation using a Poroshell 120, 4 µm column. Figure 1 shows details of the quinolones.

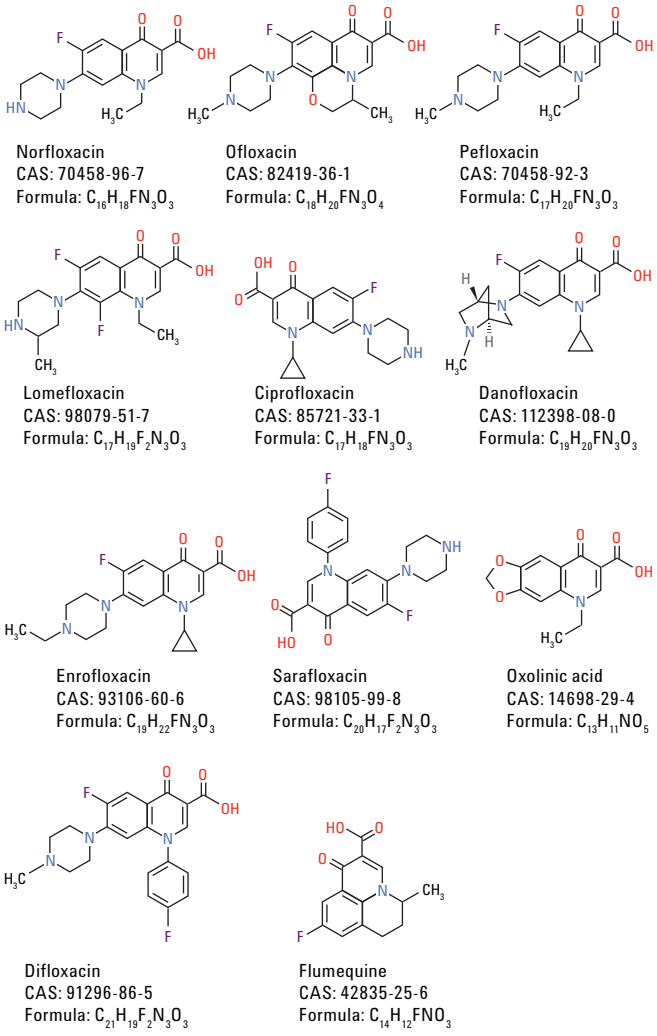

Figure 1. Quinolone compounds used in this study.

Materials and Methods

Reagents and chemicals

All reagents were HPLC or analytical grade. Acetonitrile was from J&K Scientific Ltd, Beijing. The standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Milk samples were produced in China and purchased from a local supermarket. Standard solutions (1.0 mg/mL) were made in methanol individually and stored in a freezer at –20 °C. A combined working solution was made in water and also stored at –20 °C. The spiked solutions were then made daily by appropriately diluting the combined working solution in water.

Sample preparation

The milk sample (1 g) was weighed into a polypropylene centrifuge tube and 15 mL of 10% trichloroacetic acid in acetonitrile added. The mixture was vortexed thoroughly for 1 minute and then ultrasonicated for 5 minutes. The solution was then centrifuged at 5,000 rpm at 4 °C for 10 minutes. The upper level of clean liquid was collected and then ready for the SPE procedure.

Solid phase extraction

The SPE procedure is shown in Figure 2. SPE cartridges (Agilent Bond Elut Plexa, 60 mg, 3 mL, p/n 12109603) were preconditioned with 3 mL of methanol, and then equilibrated with 3 mL of water. The sample solution was then loaded onto a cartridge and passed through under gravity (about 1 mL/min). The cartridges were washed with 3 mL 10% methanol in water. A full vacuum (Agilent Vac Elut 20 manifold, p/n 12234101) was applied to the cartridge for 5 minutes to completely dry the resin. The compounds were eluted with 3 mL methanol at a rate of 1 mL/min, and the eluent dried under nitrogen at 40 °C. The residue was reconstituted in 0.5 mL of initial mobile phase. The sample was then vortex mixed and ultrasonicated to completely dissolve the residue, and centrifuged at 5,000 rpm using an Eppendorf MiniSpin Plus. The liquid was finally transferred to a 2 mL chromatography vial for HPLC analysis.

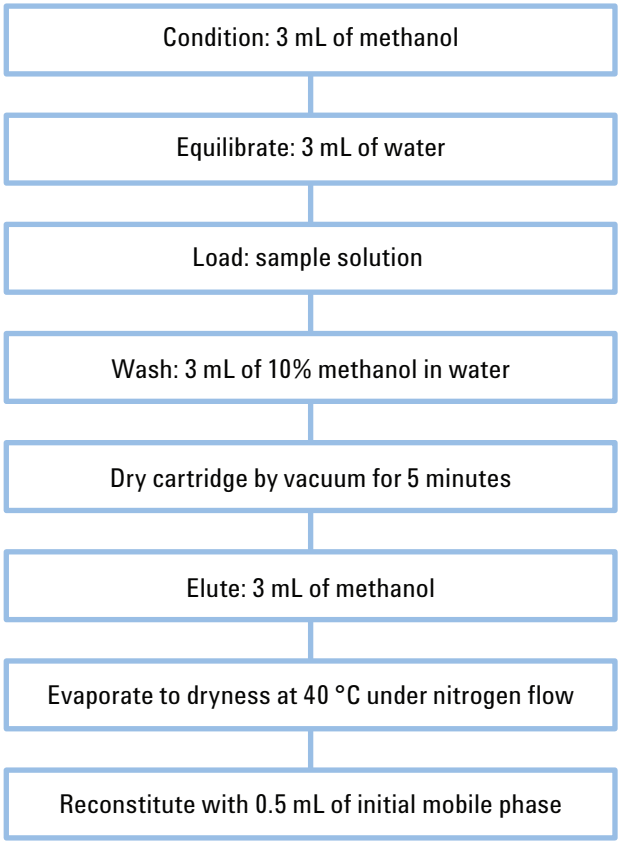

Figure 2. Milk cleanup and enrichment using Agilent Bond Elut Plexa SPE.

Conditions

Columns: Agilent Poroshell 120 EC-C18, 4.6 × 100 mm, 4 µm (p/n 695970-902),
Agilent Poroshell 120 EC-C18, 4.6 × 150 mm, 4 µm (p/n 693970-902),
Agilent Poroshell 120 EC-C18, 4.6 × 100 mm, 2.7 µm (p/n 695975-902),
Agilent ZORBAX Eclipse Plus C18, 4.6 × 150 mm, 5 µm (p/n 959993-902)

Mobile phase: A) Dissolve 11 g citric acid and 7.87 g ammonium acetate in water, adjust to pH 4.0 with triethylamine and add water to 1,000 mL
B) Acetonitrile

Gradient (for 100 mm column):

time (min)	B (%)
0	8
7	12
9	13
15	35
15.5	90
20	90
20.5	8
25	8

Gradient (for 150 mm column):

time (min)	B (%)
0	8
10.5	12
13.5	13
22.5	35
23.0	90
30	90
30.5	8
37.5	8

Column temperature: 50 °C

Injection volume: 10 µL

Flow rate: 2.0 mL/min

FLD wavelength (for 100 mm column):

time (min)	Ex (nm)	Em (nm)
0	278	465
9.5	312	366
15	278	366

FLD wavelength (for 150 mm column):

time (min)	Ex (nm)	Em (nm)
0	278	465
15.7	312	366
22	278	366

Instrument: Agilent 1290 Infinity LC with Agilent 1260 Infinity Fluorescence Detector

Results and Discussion

Separation

An optimized gradient method separated the 11 quinolone compounds very well on the Poroshell 120 EC-C18, 4 µm column, shown in the upper chromatogram of Figure 3. The separation was completed in 14 minutes. The blank milk sample (mid chromatogram) and the sample spiked with standards mixture (lower chromatogram) were tested on the same column. It is clear that there were no interferential substances in the sample matrix for the target compounds. A wash step was followed after the elution to reduce column contamination.

The method was run on 4 µm and 2.7 µm columns with the same dimensions of 4.6 × 100 mm. As predicted, the 2.7 µm column gave better resolution and efficiency than the 4 µm column. The peak width in half height ($W_{1/2}$) with the 4 µm column increased by 30% compared to the 2.7 µm column. However, pressure on the 4 µm column decreased by 45%, and still yielded adequate resolution for the separation. Therefore, it is better suited for use on a 400-bar HPLC, whereas the 2.7 µm column is suitable for 600-bar HPLC.

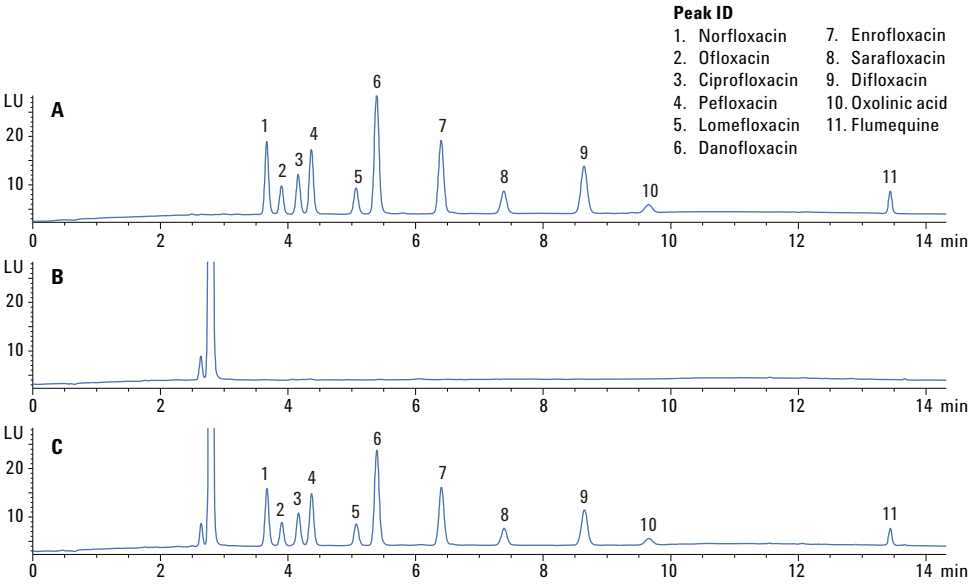

Figure 3. Chromatograms using an Agilent Poroshell 120 EC-C18, 4.6 mm × 100 mm, 4 µm column. A) quinolone standards mixture (0.2 µg/mL); B) blank milk sample; C) sample spiked with standards mixture (100 µg/kg).

The method was also run on Poroshell 120 EC-C18, 4 µm and ZORBAX Eclipse Plus C18, 5 µm columns with the same dimensions of 4.6 × 150 mm (Figure 5), to demonstrate the significant improvements of using the 4 µm column as a drop-in replacement for the 5 µm column. Separation on the 5 µm column did not resolve all the compounds.

Peaks 3 and 4 were not baseline separated with a resolution value of 1.25. In contrast, the 4 µm column totally separated these two peaks with a resolution value of 1.96. The peak width at half height ($W_{1/2}$) with the 5 µm column increased by 40% compared to that of the 4 µm column, which leads to a higher signal-to-noise (S/N) ratio.

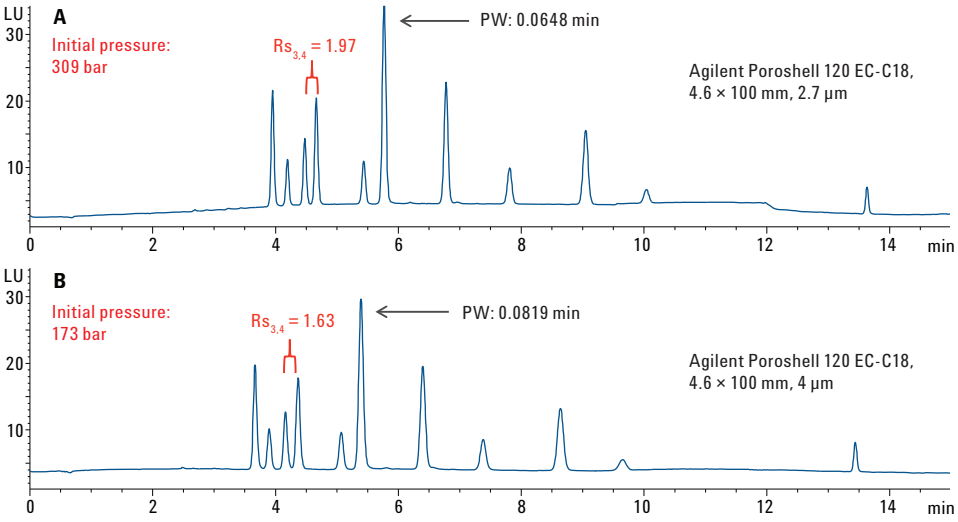

Figure 4. Comparison between Agilent Poroshell 120 EC-C18, 4.6 mm × 100 mm, 4 µm and 2.7 µm columns.

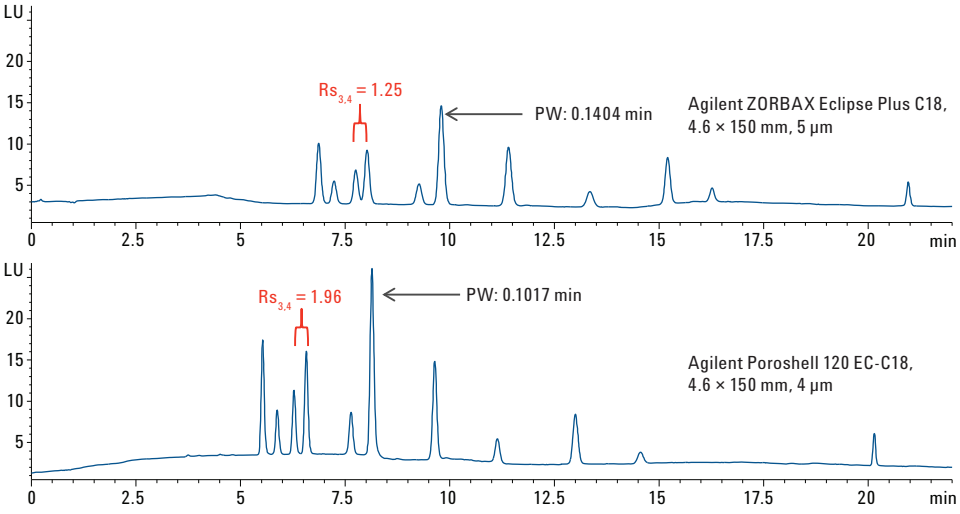

Figure 5. Comparison between Agilent Poroshell 120 EC-C18, 4.6 mm × 150 mm, 4 µm and Agilent ZORBAX Eclipse Plus C18, 4.6 × 150 mm, 5 µm columns.

Linearity and limits of detection

Solutions used to create external calibration curves were prepared by using a combined working solution to spike matrix blanks (30, 60, 90, 120, and 150 µg/kg for danofloxacin, 50, 100, 150, 200, and 250 µg/kg for flumequine, and 100, 200, 300, 400, and 500 µg/kg for the other quinolones). Matrix blanks were created by taking milk through the entire procedure, including pretreatment and SPE procedures. The limits of detection (LODs) were chosen as the concentration of each compound that gave a S/N ratio greater than 3:1. All of the LODs for the compounds were below 5 µg/kg. The results for the calibration curves are shown in Table 1.

Table 1. Linearity of quinolones in milk.

Quinolone	Regression equation	Correlation coefficient (R^2)
Norfloxacin	y = 270.9x + 0.192	0.9996
Ofloxacin	y = 100.6x – 0.078	0.9998
Ciprofloxacin	y = 161.2x – 0.015	0.9998
Pefloxacin	y = 267.8x + 0.075	0.9995
Lomefloxacin	y = 107.9x – 0.214	0.9996
Danofloxacin	y = 527.1x + 0.404	0.9993
Enrofloxacin	y = 358.7x + 0.337	0.9998
Sarafloxacin	y = 118.3x – 0.114	0.9997
Difloxacin	y = 275.1x + 0.028	0.9995
Oxolinic acid	y = 51.40x + 0.004	0.9992
Flumequine	y = 89.58x – 1.778	0.9998

Recovery and reproducibility

The recovery and repeatability for the method were determined at three levels; milk spiked to three different concentrations is shown in Table 2. The analysis was performed with six replicates at each level. The chromatograms of spiked milk extracts (100 µg/kg) are shown in Figure 3.

Table 2. Recoveries and reproducibility of quinolones in milk.

Compounds	Spiked level (µg/kg)	Recovery (%)	RSD (n = 6) (%)
Norfloxacin	50	92.37	3.11
	100	95.66	3.72
	200	91.23	2.64
Ofloxacin	50	89.77	3.12
	100	91.97	3.41
	200	93.58	3.96
Ciprofloxacin	50	92.46	4.12
	100	94.54	4.43
	200	91.75	3.21
Pefloxacin	50	89.21	3.87
	100	92.61	2.95
	200	90.42	3.01
Lomefloxacin	50	88.49	4.73
	100	91.85	3.44
	200	90.64	3.09
Danofloxacin	15	83.76	2.91
	30	87.75	3.50
	60	91.08	3.24
Enrofloxacin	50	84.09	5.13
	100	88.69	3.68
	200	87.63	6.29
Sarafloxacin	50	89.18	3.74
	100	84.21	3.67
	200	81.63	4.94
Difloxacin	50	80.67	3.86
	100	82.82	3.95
	200	79.28	7.42
Oxolinic acid	50	80.17	3.64
	100	84.56	3.90
	200	86.35	3.27
Flumequine	25	98.04	4.73
	50	99.14	2.98
	100	93.20	3.17

Conclusions

The HPLC method developed with the Agilent Poroshell 120, 4 µm column is a reliable technique for the simultaneous quantification of quinolones in milk. Both the Poroshell 120, 4 µm and Poroshell 120, 2.7 µm columns are effective for the separation for multiple quinolones, and offer a flexible, scalable family for Fast LC method development on superficially porous particles Moreover, the results of this study show that Agilent Bond Elut Plexa can be used as an effective method for purification and enrichment of multiple quinolones in a complex milk matrix. The recovery and reproducibility results based on matrix spiked standards are acceptable for quinolone residue determination in milk subject to regulation. The impurities and matrix effects are minimal, and do not interfere with the quantification of any target compound.

References

1. Anon. Commission of the European Communities, Diario Oficial de las Comunidades Europeas (DOCE) 2377/90 L224, 991, 2608, August 18, **1990**.
2. Anon. GB 29692-2013, Determination of quinolones residues in milk. China Standard. General Administration of Quality Supervision, Inspection and Quarantine, Beijing, China, **2013**.

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