

Technical Report

The Selective Extraction of Fluoroquinolones in Veterinary Samples using Molecularly Imprinted Polymer SPE

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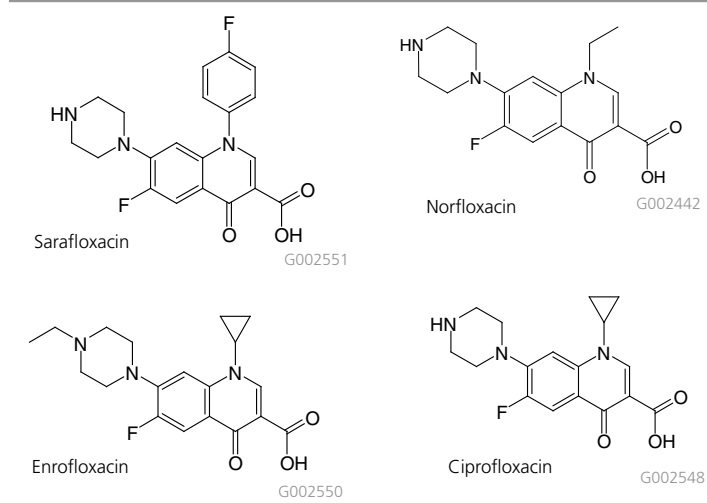
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Introduction

Fluoroquinolones (FQLs) are a class of broad-spectrum antibiotics heavily used in veterinary and human medicine. Widespread usage of this class of antibiotics has resulted in the emergence of resistant bacterial strains (1), the presence of this antibiotic class in the environment at sub-therapeutic levels can lead to multiple FQL-resistant bacterial strains. When used to treat animals, FQLs may end up in the human food chain causing allergic reactions while at the same time encouraging development of resistant bacteria. The surveillance of FQLs is mandated by law. The EU has set strict Maximum Residue Limits (MRLs), the values of which depend on the particular compound and matrix. For example, the MRL for enrofloxacin in bovine kidney is set at 200 µg/kg. The US, Canada and Japan have also set MRLs but for a more limited range of fluoroquinolones.

Figure 1. Structures of Fluoroquinolones



In this report, we discuss the extraction of FQLs from bovine kidney and honey using molecularly imprinted polymer SPE technology (SupelMIP™ SPE – Fluoroquinolones). For bovine kidney, the SupelMIP approach was compared against a tandem polymer mixed-mode SPE method. The specific FQLs examined in this report include: sarafloxacin, norfloxacin, enrofloxacin, and ciprofloxacin (Figure 1).

SupelMIP SPE - Molecularly Imprinted Polymers

SupelMIPs are based on molecularly imprinted polymers (MIPs). MIPs are a class of highly cross-linked polymer-based molecular recognition elements engineered to bind one target compound or a class of structurally related compounds with high selectivity. Selectivity is introduced during MIP synthesis in which a template molecule, designed to mimic the analyte, guides the formation of specific cavities that are sterically and chemically complementary to the target analyte(s). As a result, multiple interactions (e.g., hydrogen bonding, ionic, Van der Waals, hydrophobic) can take place between the MIP cavity and analyte functional groups. The strong retention offered between a MIP phase and its target analyte(s) allows for the use of exhaustive wash procedures during solid phase extraction that results in superior sample cleanup prior to analysis.

Extraction of FQLs from Bovine Kidney

Sarafloxacin, norfloxacin, enrofloxacin, and ciprofloxacin were spiked into bovine kidney at the levels of 0-75 µg/kg and I.S. d₅ – norfloxacin (75 µg/kg). 2 g of spiked kidney sample was homogenized with 30 mL 50 mM NaH₂PO₄, pH 7.4 and centrifuged for 10 min. at 5000 rpm. The resulting supernatant was filtered using a 0.45 µm filter and processed using the SupelMIP procedure described in Table 1 and the tandem polymer MAX and MCX procedure described in Table 2. LC-MS/MS analysis was conducted using the method described in Table 3.

Table 1. SupelMIP SPE – Fluoroquinolones SPE Procedure

SPE Cartridge:	SupelMIP SPE – Fluoroquinolones, 25 mg/3 mL (Cat. No. 53269-U)
1. Condition / Equilibrate:	1 mL methanol followed by 1.5 mL DI water
2. Load:	1 mL of sample extract
3. Wash in the described order*:	3 mL DI Water Apply strong vacuum through the cartridge for 2 min. 1 mL acetonitrile 1 mL 0.5% acetic acid in acetonitrile Apply a strong vacuum through the cartridge for 2 min. 1 mL 0.1% ammonia in DI water Apply a strong vacuum through the cartridge for 2 min.
4. Elute:	Elute FQLs with 1 mL 2% ammonia in methanol
5. Evaporate / Reconstitute:	The SPE eluent was evaporated gently under nitrogen at 35 °C and reconstituted 150 µL 50% acetonitrile in 0.1% formic acid prior to analysis.

*Note that for honey samples, a strong vacuum was applied to the cartridge for 2 min. between each wash step.

Table 2. Tandem Polymer MAX / MCX SPE Procedure (2)

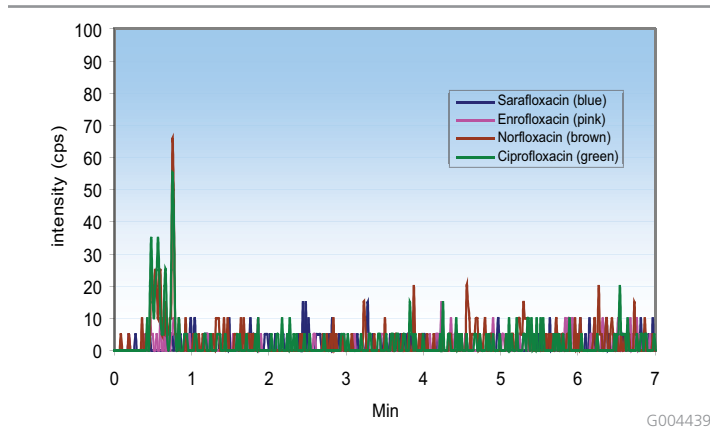
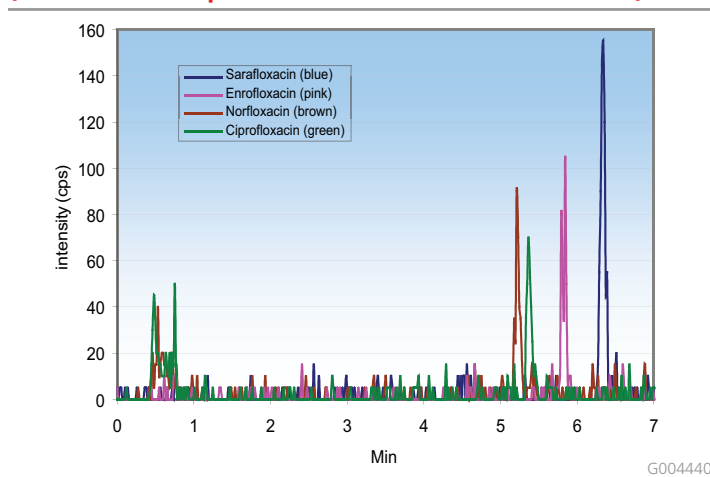
SPE Cartridge 1:	Polymer Mixed-Mode Anion Exchange (MAX) SPE, 150 mg/6 mL
1. Condition / Equilibrate:	1 mL methanol followed by 1 mL 5N NaOH and 1 mL DI water
2. Load:	5 mL of bovine kidney sample extract
3. Wash in the described order:	1 mL 5% ammonia in DI water 1 mL methanol
4. Elute:	Elute FQLs with 2 mL 0.2 N HCl in methanol
SPE Cartridge 2:	Polymer Mixed-Mode Cation Exchange (MCX) SPE, 30 mg/1 mL
5. Condition / Equilibrate:	1 mL methanol
6. Load:	2 mL MAX eluent obtained from step 4
7. Wash in the described order:	2 mL methanol
8. Elute:	0.5 mL 10% ammonium hydroxide in methanol into a 1 mL volumetric flask
9. Neutralize/Volume adjustment:	Neutralize with formic acid and bring to 1 mL volume with methanol

Table 3. LC-MS/MS Conditions for Fluoroquinolones Analysis

column:	Ascentis® C18, 5 cm x 3 mm I.D., 3 µm particles (581307-U) w/ guard column	
instrument:	LC-MS/MS Triple Quadrupole	
mobile phase A:	0.1% formic acid	
mobile phase B:	acetonitrile	
temp.:	ambient	
flow rate:	0.5 mL/min.	
gradient:	Min	%A %B
	0.0	95 5
	7.0	85 15
	7.2	20 80
	8.2	5 5
	11.0	95 5
det.:	MS/MS, MRM transitions sarafloxacin (386.1/299.1), norfloxacin (320.6/276.2), enrofloxacin (360.2/245.2), ciprofloxacin (332.4/288.2), d ₅ -norfloxacin I.S. (325.3/288.1)	
polarity:	Positive	
ion source:	TurboSpray	
ion spray voltage:	4500 V	
decluster potential:	sarafloxacin – 46 V, norfloxacin – 41 V, enrofloxacin – 49 V, ciprofloxacin – 45 V, d ₅ - norfloxacin – 46 V	
entrance potential:	sarafloxacin – 5 V, norfloxacin – 3 V, enrofloxacin – 4 V, ciprofloxacin – 4 V, d ₅ - norfloxacin – 4 V	
source temp:	500 °C	
collision gas:	5 psi	
curtain:	15 psi	
lon-source gas 1:	50 psi	
lon-source gas 2:	60 psi	
dwell time:	200 msec.	
run time:	10 min.	
inj.:	3 µL	

Evaluation of FQL SPE Procedures for Bovine Kidney

Spiked bovine kidney was spiked with FQLs and extracted and analyzed using the procedures described in Tables 1-3. LC-MS/MS chromatograms of both blank and spiked (3 µg/kg) kidney using the SupelMIP approach are described in Figures 2 and 3, respectively. The SupelMIP approach provided low background and good analyte response at the mass transitions monitored.

Figure 2. LC-MS/MS chromatogram of Blank Kidney Extracted with SupelMIP SPE (colored lines represent m/z transitions monitored)**Figure 3. LC-MS/MS chromatogram of Fluoroquinolone spiked Kidney (3 µg/kg) Extracted with SupelMIP SPE (colored lines represent m/z transitions monitored)**

Absolute and relative recovery values for both the SupelMIP and tandem polymer mixed-mode SPE procedures across a range of spike levels tested were comparable. Absolute and relative recovery values for the SupelMIP approach are described in Table 4. Recovery values for polymer MAX / MCX SPE methods are not shown. Note that although the recovery values were comparable, the SupelMIP procedure only required one SPE cartridge. In contrast, the tandem polymer MAX / MCX SPE approach required two separate SPE procedures for sample cleanup.

Table 4. Absolute & Relative Recovery of FQLs in Bovine Kidney using SupelMIP SPE

Recovery (%) Spike Level	Sarafloxacin		Norfloxacin		Enrofloxacin		Ciprofloxacin	
	Absolute	Relative	Absolute	Relative	Absolute	Relative	Absolute	Relative
3 µg/kg	78	113	95	165	48	85	60	132
7.5 µg/kg	70	79	105	123	56	68	90	111
15 µg/kg	65	84	83	112	47	63	75	104
45 µg/kg	72	84	93	106	54	63	94	109
75 µg/kg	77	78	106	106	60	60	103	103

The decision and detection limits for both the SupelMIP and tandem polymer MAX / MCX approach were calculated. The CC α (decision limit where alpha error is 1%) and CC β (detection capability where beta error is 5%) were calculated (Table 5) (3). Note that both decision and detection limits CC α and CC β values were lower than the Polymer MAX / MCX SPE approach.

Table 5. Detection Limits for FQLs in Bovine Kidney using SupelMIP and Tandem Polymer MAX / MCX SPE

Detection Limit (µg/kg)	Sarafloxacin	Norfloxacin	Enrofloxacin	Ciprofloxacin
SupelMIP CC α (Decision Limit)	3.9	3.5	6.1	4.5
SupelMIP CC β (Detection Limit)	7.4	6.0	10.8	7.9
Polymer MAX / MCX CC α (Decision Limit)	7.4	4.1	11.3	3.7
Polymer MAX/MCX CC β (Detection Limit)	12.3	6.5	19.1	5.8

Extraction of FQLs from Honey using SupelMIP SPE

In this application, honey samples were spiked with FQLs at the level of 1 ng/g and 2 ng/g, respectively. I.S. d₅ – norfloxacin was also added at 2 ng/g level.

10 g of spiked honey was dissolved with equal amounts of 10 mM ammonium acetate, pH 7. The sample was heated at 40 °C and shaken until complete dissolution was achieved. The final pH of the sample was adjusted to 7 using ammonium hydroxide and acetic acid. The sample was then centrifuged and 1.6 mL of the resulting supernatant (equivalent to 1 g of honey) was processed using the SupelMIP procedure described in Table 1.

To study the ion-suppression / ion-enhancement effects after SupelMIP SPE cleanup, blank samples of honey were extracted as described above. The resulting blank SPE eluent from each matrix was spiked with fluoroquinolones and I.S. at 10 ng/g.

The samples were analyzed using LC-MS/MS as described in Table 3. Note that for the honey study, fluoroquinolones ofloxacin (m/z 362.2/261.1) and lomefloxacin (m/z 352.2/265.1) were also included with the test compounds listed in Figure 1.

Evaluation of SupelMIP SPE for FQLs in Honey

The SupelMIP SPE procedure offered good recovery levels for FQLs spiked in honey at the level of 1 ng/g. Relative recovery averaged at 102% for the FQLs tested, and absolute recovery averaged at 79%. Precision (%RSD) was less than 13% for each of the FQLs tested and the detection limit was estimated to be between 0.12-0.40 ng/g. Post-SPE spike recovery ranged from 81-140% indicating some levels of ion-suppression and ion-enhancement; however, the effects were marginal. The data is compiled in Table 6.

Table 6. Performance Data for FQLs (1 ng/g spike) in Honey using SupelMIP SPE

	Ciprofloxacin	Enrofloxacin	Lomefloxacin	Norfloxacin	Ofloxacin	Sarafloxacin
Relative Recovery (n=5)	76%	123%	114%	83%	134%	77%
Absolute Recovery (n=5)	53%	90%	81%	60%	100%	55%
Precision (RSD) (n=5)	13%	9%	5%	12%	10%	11%
Recovery – Post SPE Spike	101%	117%	112%	88%	140%	81%
Est. Detection Limits** (ng/g)	0.40	0.13	0.12	0.24	0.19	0.21

** Detection limits estimated S/N value of 3 to corresponding analyte responses at 1 ng/g spike level.

Conclusion

In this report, we discussed the use of a molecularly imprinted polymer SPE procedure engineered and optimized for the selective extraction of fluoroquinolones in difficult sample matrices such as bovine kidney and honey. The bovine kidney was compared against a tandem polymer MAX / MCX SPE procedure. For the comparison study, recovery values were comparable across the two techniques; however, the SupelMIP approach only required one SPE method whereas the tandem polymer MAX / MCX approach required two SPE methods thereby significantly reducing overall assay time. In addition, decision ($CC\alpha$) and detection limits ($CC\beta$) were lower on the SupelMIP method relative to the tandem polymer MAX / MCX procedure. Honey samples were also evaluated using SupelMIP SPE, resulting in high recoveries, good precision, and low detection limits.

Ordering Information

Description	Cat. No.
SupelMIP SPE - Fluoroquinolones, 25 mg/3 mL, pk. 50	53269-U
Ascentis C18, 5 cm x 3 mm I.D., 3 μ m particles	581307-U

For more information on SupelMIP SPE, please visit sigma-aldrich.com/supelmip

References

1. Fluoroquinolone resistance – Overuse of fluoroquinolones in human and veterinary medicine can breed resistance, Piddock L, BMJ 1998; 317:1029-1030.
2. Fluoroquinolone Antibiotics in Beef Kidney – Tandem Oasis MAX-MCX Method (Excerpt from Oasis Applications Notebook) – Available at http://www.waters.com/waters/library.htm?locale=en_US&lid=1534786.
3. European Commission Council Directive 2002/657/EC on implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results.

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