Screening 36 Veterinary Drugs in Animal Origin Food by LC/MS/MS Combined with Modified QuEChERS Method

Application Note

Food Testing and Agriculture

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Abstract

This application note introduces a modified QuEChERS method that screens food for four classes of veterinary drugs-sulfanilamides, macrocyclic lactones, quinolones, and clopidols. The modified QuEChERS consists of an extraction kit (4 g Na_2SO_4+ 1 g NaCl) and a dispersive-SPE kit (50 mg PSA, 150 mg, C18EC, 900 mg Na_2SO_4); the extraction solvent is 1% acetic acid in acetonitrile. Satisfactory recoveries were achieved by this method for all four classes of veterinary drugs. The veterinary drugs were quantified by LC/ESI/MS/MS using Dynamic Multiple Reaction Monitoring (DMRM). The observed limits of detection are in accordance with the various MRLs for the four classes of veterinary drugs, and the average recoveries exceed 50%, thus meeting the requirement for routine analysis.

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Introduction

The QuEChERS method was first introduced for the extraction of pesticides from fruits and vegetables [1]. The QuEChERS methodology can be divided into two steps, extraction/partitioning and dispersive SPE (d-SPE). In the first step, acetonitrile is used as the extraction solvent; magnesium sulfate together with salts facilitate partitioning/extraction. The second step, d-SPE removes matrix interferences from the extract. Common d-SPE materials are primary secondary amine (PSA), C18 end-capped (C18EC), and graphite carbon black (GCB).

Since its validation, the QuEChERS method has been used for many types of sample matrices. When compared to fruit and vegetables, animal origin food samples presented in this application require the use of PSA and C18EC in the d-SPE to remove additional interferences from protein and lipids found in these types of samples. The veterinary drugs analyzed in this application did not require the use of the buffered QuEChERS salts, namely AOAC or EN versions employed in the extraction of pH-labile pesticides. This highly selective and sensitive methodology has proven remarkably rugged and rapid for analyzing targeted pesticides at trace levels in complex edible food matrices.

Compared to solid phase extraction (SPE), the QuEChERS method will result in more matrix interferences because it is a "just enough" sample preparation technique. Therefore, highly selective instrumentation, like LC/MS/MS with DMRM, is required for the analysis of veterinary drugs with ease and accuracy.

With the LC/MS/MS method described in this application note, 36 veterinary drugs in animal origin food can be effectively separated in less than 9 minutes. Combined with a rapid QuEChERS extraction, this method saves a significant amount of analysis and analyst time, providing a reliable approach to screen veterinary drugs in routine work

Experimental

Reagents and standards

All reagents and solvents were HPLC or analytical grade. Methanol (MeOH) and Acetonitrile (ACN) were from Honeywell (Muskegon, MI, USA). Formic acid (FA) and acetic acid were from Sigma-Aldrich (St Louis, MO, USA)

Solutions and standards

A 1% formic acid solution in ACN was made fresh daily by adding 1 mL of formic acid to 100 mL of ACN, then mixing well. A 1% acetic acid solution in ACN was made fresh daily by adding 1 mL of acetic acid to 100 mL of ACN, then mixing well.

Standard solutions were made at the concentration of 10 µg/mL (sulfanilamides and macrocyclic lactones), 5 µg/mL (clopidols), and 1 µg/mL (quinolones).

Equipment and material

Agilent 1260 HPLC with Diode Array Detector (Agilent Technologies, Inc., CA, USA).

Agilent 6460 Triple Quadrupole LC/MS system with AJST Electrospray Ionization source (Agilent Technologies, Inc., CA, USA).

Agilent Bond Elut QuEChERS Magnesium Sulfate (p/n 5982-8082)

Agilent Bond Elut Sodium Chloride (p/n 5982-5750)

Agilent Bond Elut PSA (p/n 5982-8382 or 5982-5753)

Agilent Bond Elut C18EC (p/n 5982-1382 or 5982-5752)

Agilent Bond Elut SAX (p/n 12213042)

Agilent Bond Elut NH2 (p/n 12213021)

Agilent Bond Elut d-SPE for drug residues (p/n 5982-4956)

Agilent Bond Elut Non-buffered QuEChERS extraction kit (p/n 5982-5550)

Agilent ZORBAX Solvent Saver HD Eclipse Plus C18 3.0 × 100 mm, 1.8 µm column (p/n 959757-302)

Instrument conditions

HPLC conditions

Column:	Agilent ZORBAX Solvent Saver HD Eclipse Plus C18, 3.0 × 100 mm, 1.8 µm		
Flow rate:	0.5 mL/min		
Column temperature:	30 °C		
Injection volume:	5 µL		
Mobile phase:	A: H_2O 0.1% formic acid B: ACN		
Gradient:	Time	%A	%B
	0.0	90	10
	0.5	90	10
	1.0	80	20
	4.0	75	25
	8.0	40	60
	9.0	5	95
	12.0	5	95
	12.1	90	10
	15.0	90	10

MS conditions

Polarity:	Positive
Gas temperature:	300 °C
Gas flow:	7 L/min
Nebulizer:	50 psi
Capillary:	3,000 V
Sheath gas temperature:	350 °C
Sheath gas flow:	10 L/min
Scan mode:	DMRM

Sample Preparation

Sample homogenization

Blank pork, milk, honey, and eggs were purchased from a local grocery store. The samples were washed and chopped into small pieces (if necessary), then stored at -20 °C.

Extraction

Two grams (±0.05 g) of each sample (homogenized if required) were placed into 50 mL centrifuge tubes. Samples were then fortified with 200 µL of veterinary drugs standard to make the concentration at 10 ng/g (sulfanilamides and macrocyclic lactones), 5 ng/g (clopidols), and 1ng/g (quinolones). Four mL of water were added, and samples were vortexed for 1 minute. A 10 mL solution of 1% acetic acid in ACN were added to each tube. Tubes were capped and vortexed for 1 minute. The extraction salts (4 g Na_2SO_4, 1 g NaCl) were added to each tube. Sample tubes were capped tightly and vigorously shaken for 1 minute. Tubes were centrifuged at 5,000 rpm for 5 minutes at 4 °C, then allowed to stand for 30 minutes.

Dispersive-SPE

A 6 mL aliquot of the upper ACN layer was transferred into a 15 mL tube, which contained 50 mg of PSA, 150 mg of C18EC and 900 mg of anhydrous Na_2SO_4. The tubes were tightly capped and vortexed for 1 minute and then centrifuged at 5,000 rpm for 5 minutes. A 4 mL aliquot of the upper ACN layer was transferred into another tube and dried by N_2 flow at 40 °C. Samples were reconstituted into 1 mL of 2:8 ACN/H_2O, and then centrifuged at 10,000 rpm for 10 minutes. The upper layer was transferred to an autosampler vial.

Results and Discussion

Optimize chromatographic conditions

The Agilent 1260 Infinity Binary LC system delivers fast results with significantly high data quality at maximum pressure of 600 bar. Combined with the Eclipse Plus C18 sub-2 µm particle column, the system can improve the resolution, shorten the analysis time, and improve sensitivity, which is extremely important to screen veterinary drugs. The Agilent 6460 MS/MS delivers sensitivity, advanced hexapole collision cell eliminates background noise and crosstalk, and the innovative dynamic multiple reaction monitoring (DMRM) method builds ion transition lists during the LC separation based on a retention time window for each analyte.

Separation of 36 veterinary drugs (sulfanilamides and macrocyclic lactones 10 ng/g, clopidols 5 ng/g, and quinolones 1 ng/g) in a matrix standard solution by LC/MS/MS is shown in Figure 1. The MS conditions for each compound are given in Table 1.

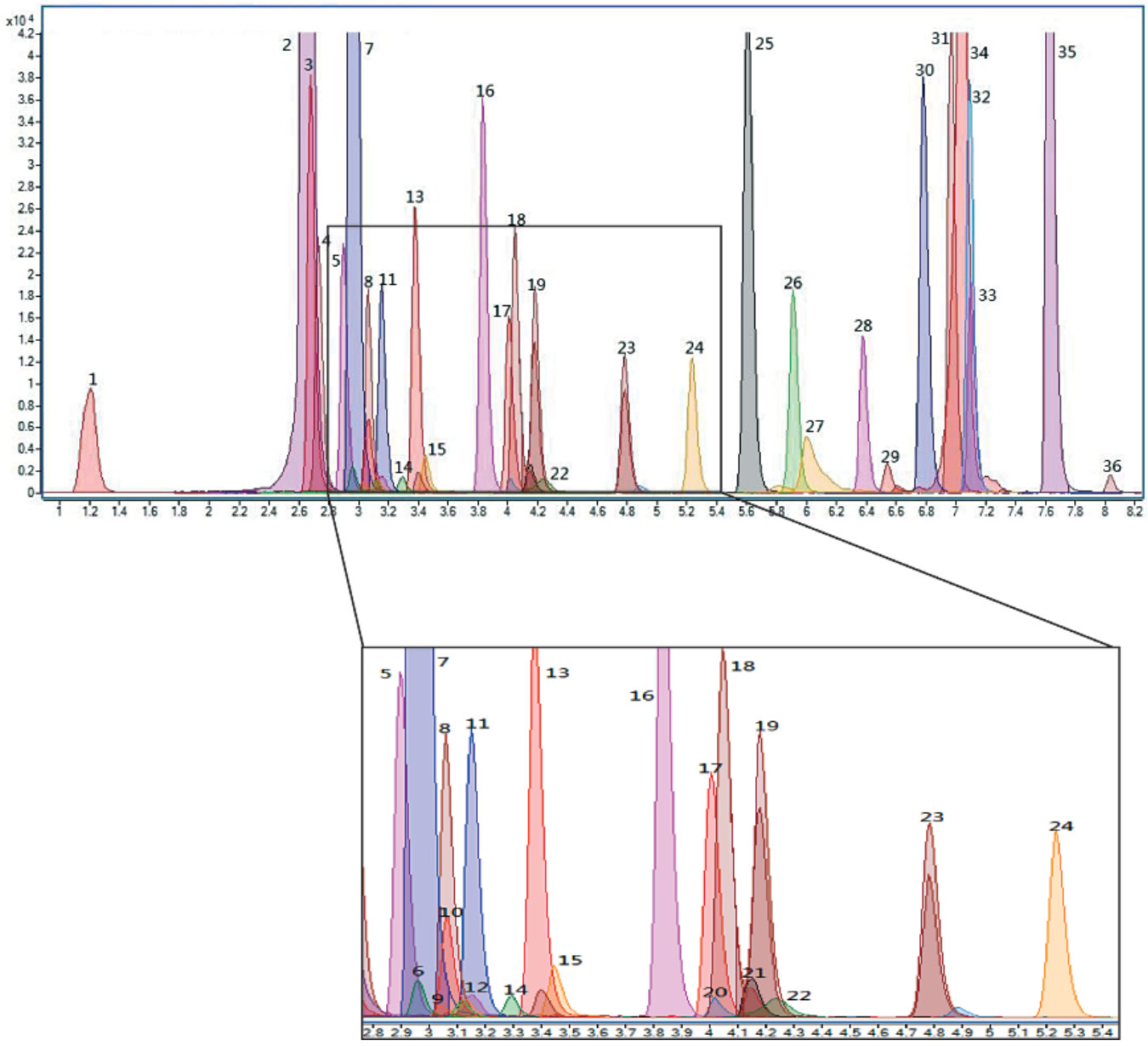

Figure 1. MRM extracted chromatogram for veterinary drugs.

Table 1. MRM transitions and MS operating parameters.

No.	RT	Compound name	Precursor ion	Fragmentor (V)	Quantifier ion	Quantifier CE(V)	Qualifier ion	Qualifier CE(V)
1	1.24	Sulfaguanidine	215.0	80	108.0	20	156.0	9
2	2.75	Lincomycin	407.2	150	126.0	30	359.0	15
3	2.68	Clopidol	192.1	110	101	25	87	30
4	2.78	Sulphacetamide	215.0	70	92.0	19	156.0	3
5	2.97	Sulfadiazine	251.1	100	108.0	22	156.0	10
6	3.1	Marbofloxacin	363.0	120	320.1	9	345.1	17
7	3.11	Trimethoprim	291.2	150	123.0	22	230.1	22
8	3.15	Sulfathiazole	256.0	100	108.0	21	156.0	9
9	3.23	Norfloxacin	320.0	140	276.1	13	302.1	17
10	3.26	Ofloxacin	362.0	140	261.1	26	318.1	14
11	3.27	Sulfapyridine	250.1	100	156.0	10	184.0	14
12	3.36	Ciprofloxacin	332.1	130	231.0	42	314.1	18
13	3.52	Sulfamerazine	265.1	120	92.0	30	172.0	13
14	3.57	Danofloxacin	358.2	140	255.0	46	340.1	22
15	3.76	Enrofloxacin	360.0	130	316.2	18	342.1	18
16	4.05	Sulfamethazine	279.1	120	124.0	18	186.0	14
17	4.2	Sulfamethizole	271.0	100	108.0	22	156.0	10
18	4.27	Sulfamethoxypyridazine	281.1	125	108.0	22	156.0	14
19	4.38	Sulfameter	281.1	120	108.0	26	156.0	14
20	4.43	Sarafloxacin	386.1	140	342.1	14	368.1	18
21	4.57	Difloxacin	400.0	140	356.1	18	382.1	18
22	4.91	Spiramycin	843.5	200	101.0	46	174.0	42
23	5.07	Sulfamonomethoxine	281.1	120	108.0	26	156.0	14
24	5.54	Sulfachloropyridazine	285.0	100	108.0	22	156.0	10
25	6	Sulfadoxine	311.1	120	92.0	30	156.0	14
26	6.21	Sulfamethoxazole	254.1	100	92.0	26	156.0	10
27	6.45	Tilmicosin	869.6	250	174.0	50	696.4	45
28	6.65	Sulfisoxazole	268.1	100	113.0	10	156.0	10
29	6.83	Oxolinic acid	262.1	100	216.0	30	244.0	13
30	7.12	Erythromycin	734.5	170	158.1	30	576.3	14
31	7.21	Sulfabenzamide	277.1	80	108.0	22	156.0	6
32	7.34	Sulfadimethoxine	311.1	125	108.0	26	156.0	17
33	7.36	Sulfaquinoxaline	301.1	110	92.0	29	156.0	11
34	7.37	Tylosin	916.5	240	101.0	54	174.0	42
35	7.96	Roxithromycin	837.5	170	158.0	38	679.4	18
36	8.28	Flumequine	262.0	150	216.0	30	244.0	13

Modification of Extraction and Dispersive-SPE Parameters

The Agilent Bond Elut non-buffered salts and the Agilent Bond Elut QuEChERS d-SPE kits for drug residues were used in our initial evaluation. During development of the method, we found that modifications of the procedure were necessary. These modifications resulted in recoveries meeting the requirements for routine analysis and are described below. The procedure was applied successfully to the following food matrices: meat, honey, egg, and milk.

Optimization of the extraction step

At first, the d-SPE for drug residues (150 mg C18, 900 mg $MgSO_4$) was used in methods 1, 2, 3, and 4 (Table 2) to evaluate modifications within the extraction step.

Extraction salt

The QuEChERS method uses $MgSO_4$ to remove water within the sample. Experimental evaluation showed that $MgSO_4$ negatively influenced the recovery of many compounds, especially the sulfanilamides and macrocyclic lactones. Na_2SO_4 was substituted for $MgSO_4$, and samples were allowed to stand after centrifugation for 30 minutes to efficiently absorb the water [2]. The results show that replacement of $MgSO_4$ with Na_2SO_4 increased the recovery of sulfanilamides and macrocyclic lactones (Method 1 and Method 2 in Table 2).

Extraction solvent

The ratio of water to acetonitrile was also evaluated in the extraction step. The results show that the recovery is better with the water /acetonitrile ratio of 1:2 versus 1:1 in the extraction step (Method 2 and Method 3 in Table 2).

Many sample preparation techniques for meat matrices use acid to disrupt compound-protein binding which directly affects recovery. Common acids used for this purpose are formic and acetic acids [3]. Comparison showed that the recovery with 1% acetic acid acetonitrile is greater than with 1% formic acid acetonitrile treatment. (Method 3 and Method 4 in Table 2).

The recovery of lincomycin is very low in all 4 methods evaluated. It is proposed that the polarity of lincomycin (log P = 0.56) is limiting its extractability into the acetonitrile.

Table 2. Results of various optimization of extraction step.

Method	**1**	**2**	**3**	4
Extract salt	$MgSO_4$+NaCl	Na_2SO_4+NaCl	Na_2SO_4+NaCl	**Na_2SO_4+NaCl**
Dispersive-SPE mix	C18EC+$MgSO_4$	C18EC+Na_2SO_4	C18EC+Na_2SO_4	**C18EC+Na_2SO_4**
Extract solvent	1% formic acid acetonitrile	1% formic acid acetonitrile	1% formic acid acetonitrile	**1% acetic acid acetonitrile**
Water	8 mL	8 mL	4 mL	**4 mL**
Average recovery of macrocyclic lactones	22.95%	43.66%	45.12%	**70.46%**
Average recovery of sulfanilamides	10.86%	25.96%	33.25%	**54.35%**
Average recovery of quinolones	86.79%	47.69%	62.69%	**64.44%**
Average recovery of clopidols	55.12%	38.02%	49.89%	**65.37%**

Optimization of d-SPE

When investigating modifications in the d-SPE to enhance matrix interference removal, all salts included C18EC sorbent to absorb proteins and lipids from the matrix [4] and additional anhydrous Na_2SO_4 to remove water. Modifications included PSA (Methods 1 and 2), SAX (Method 3), and NH_2 (Method 4). In the QuEChERS method PSA, NH_2, and SAX have been used as d-SPE material because of their anionic exchange properties. They can strongly interact with acidic interferences in the matrix, such as polar organic acids, sugars, and fatty acids.

Table 3. Results of dispersive SPE parameters optimization.

Method	1	2	3	4	5
Extract salt	**Na_2SO_4+NaCl**	Na_2SO_4+NaCl	Na_2SO_4+NaCl	Na_2SO_4+NaCl	**Na_2SO_4+NaCl**
Dispersive-SPE mix	**50 mg PSA+ 150 mg C18EC+ 900 mg Na_2SO_4**	100 mg PSA+ 150 mg C18EC+ 900 mg Na_2SO_4	50 mg SAX+ 150 mg C18EC+ 900 mg Na_2SO_4	100 mg NH_2+ 150 mg C18EC+ 900 mg Na_2SO_4	**300 mg C18EC+ 900 mg Na_2SO_4**
Extract solvent	**1% acetic acid acetonitrile**	1% acetic acid acetonitrile	1% acetic acid acetonitrile	1% acetic acid acetonitrile	**1% acetic acid acetonitrile**
Water	**4 mL**	4 mL	4 mL	4 mL	**4 mL**
Average recovery of macrocyclic lactone	**54.57%**	42.23%	59.87%	33.70%	**66.10%**
Average recovery of sulfanilamide	**64.37 %**	63.27 %	77.35%	51.71%	**71.80%**
Average recovery of quinolone	**73.88%**	88.34 %	76.82%	97.03%	**84.66%**
Average recovery of clopidol	**85.12%**	100.11%	71.57%	70.27%	**91.17%**

The results in Table 3 show that the best overall recoveries were achieved with Methods 1 and 5, which incorporated C18EC with or without PSA. Our goal was to define a d-SPE that could be used with all our sample matrices. Therefore, it is important to include PSA in the d-SPE because of its capabilities of removing organic acids and sugars prevalent in the honey matrix. The d-SPE of choice is 50 mg PSA, 150 mg C18EC, and 900 mg Na_2SO_4.

Other Sample Matrices

Other than the meat matrix, this method was successfully used on egg, milk, and honey matrices. The recoveries for these matrices were also acceptable and met the requirements for routine determination of veterinary drugs (Appendix I).

Conclusion

A modified QuEChERS method, combined with LC/MS/MS, provides successful and time-efficient screening of sulfanilamides, macrocyclic lactones, quinolones, and clopidols in an animal origin matrix. The optimum QuEChERS composition defined in this application is a combination of 4 g Na_2SO_4, 1 g NaCl as extraction salt with acetonitrile (1% acetic acid) as extraction solvent, 50 mg PSA,150 mg C18EC, and 900 mg Na_2SO_4 as d-SPE. The recovery obtained by the modified QuEChERS method met the requirement for routine veterinary drugs screening.

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Ordering Information

Description	Quantity per pack/size	Contents	Part no.
QuEChERS Extraction Tubes	50 packets and tubes	4 g Na_2SO_4, 1 g NaCl	5982-0032
Dispersive-SPE	50-15 mL tubes	50 mg PSA, 150 mg C18EC, 900 mg Na_2SO_4	5982-4950

Appendix 1. The recovery and LOQ of veterinary drugs in four matrices using the modified QuEChERS method.

Compounds	RT (min)	Recovery of meat (pork) (%)	LOQ of meat (pork) (ng/g)	Recovery of egg (%)	LOQ of egg (ng/g)	Recovery of milk (%)	LOQ of milk (ng/g)	Recovery of honey (%)	LOQ of honey (ng/g)
Lincomycin	2.7	12.61	0.012	10.77	0.013	12.74	0.018	7.18	0.025
Spiramycin	4.9	75.31	0.813	45.57	0.293	74.46	0.476	54.72	0.431
Tilmicosin	6.5	99.43	0.085	106.67	0.130	161.03	0.144	66.40	0.184
Erythromycin	7.1	26.10	0.027	39.76	0.017	36.67	0.013	25.60	0.030
Tylosin	7.4	68.29	0.083	47.08	0.126	69.18	0.524	63.89	0.145
Roxithromycin	8.0	86.83	0.015	95.17	0.008	96.42	0.007	71.19	0.017
Sulfaguanidine	2.7	23.96	0.678	43.95	0.719	42.16	0.339	46.59	0.127
Sulphacetamide	2.7	50.15	0.500	75.50	0.293	72.04	0.289	70.66	0.457
Sulfadiazine	2.9	50.78	0.420	75.14	0.025	65.35	0.043	66.62	0.060
Trimethoprim	3.0	83.71	0.026	83.22	0.009	83.53	0.013	82.72	0.014
Sulfathiazole	3.1	37.41	0.133	59.15	0.094	57.37	0.116	53.09	0.113
Sulfapyridine	3.2	46.22	0.037	70.50	0.029	63.07	0.035	64.50	0.024
Sulfamerazine	3.4	54.98	0.373	68.24	0.106	63.83	0.052	66.89	0.060
Sulfamethazine	3.9	45.70	0.206	69.76	0.044	61.04	0.024	69.42	0.035
Sulfamethizole	4.1	33.43	0.460	64.39	0.136	58.59	0.081	54.69	0.228
Sulfameter	4.1	41.96	0.025	71.82	0.010	59.34	0.022	60.48	0.022
Sulfamethoxypyridazine	4.2	40.36	0.039	75.35	0.066	70.40	0.123	68.04	0.097
Sulfamonomethoxine	4.9	50.13	0.113	74.10	0.077	64.10	0.089	71.37	0.101
Sulfachloropyridazine	5.3	48.26	0.124	71.89	0.107	63.97	0.108	66.32	0.042
Sulfadimethoxine	5.8	58.76	0.029	76.94	0.013	48.33	0.015	69.93	0.020
Sulfadoxine	5.8	50.91	0.032	74.45	0.074	64.35	0.060	69.93	0.032
Sulfamethoxazole	6.0	45.82	0.135	76.80	0.072	69.80	0.050	71.15	0.077
Sulfisoxazole	6.5	51.23	0.154	72.43	0.056	66.84	0.051	68.77	0.160
Sulfabenzamide	7.1	55.37	0.035	73.00	0.038	47.34	0.020	62.96	0.040
Sulfaquinoxaline	7.3	51.06	0.073	73.89	0.035	51.43	0.030	69.25	0.093
Clopidol	2.7	75.69	0.056	78.79	0.039	78.26	0.037	81.13	0.020
Norfloxacin	3.2	66.37	1.587	72.83	3.846	55.25	2.703	71.68	0.469
Ofloxacin	3.2	57.10	0.102	46.76	0.114	49.68	0.074	67.19	0.079
Ciprofloxacin	3.3	107.17	1.370	38.30	0.082	48.53	0.007	59.34	0.110
Danofloxacin	3.6	56.26	0.053	55.68	0.031	30.23	0.641	79.00	0.526
Enrofloxacin	3.7	60.82	0.179	54.61	0.071	49.82	0.143	76.53	0.102
Sarafloxacin	4.4	56.04	1.087	93.79	0.588	57.33	0.227	72.75	0.667
Difloxacin	4.5	69.74	0.340	60.40	0.065	62.27	0.157	85.53	0.222
Flumequine	6.8	79.34	1.299	68.91	0.244	47.87	1.389	84.74	0.121
Oxolinic acid	8.2	72.96	2.000	76.20	1.333	56.73	0.518	86.55	0.382
Marbofloxacin	7.3	56.81	0.546	39.71	0.455	53.80	0.333	106.56	10.000

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