Multi-residue Automated Turbulent Flow Online LC-MS/MS Method for the Determination of Antibiotics in Milk

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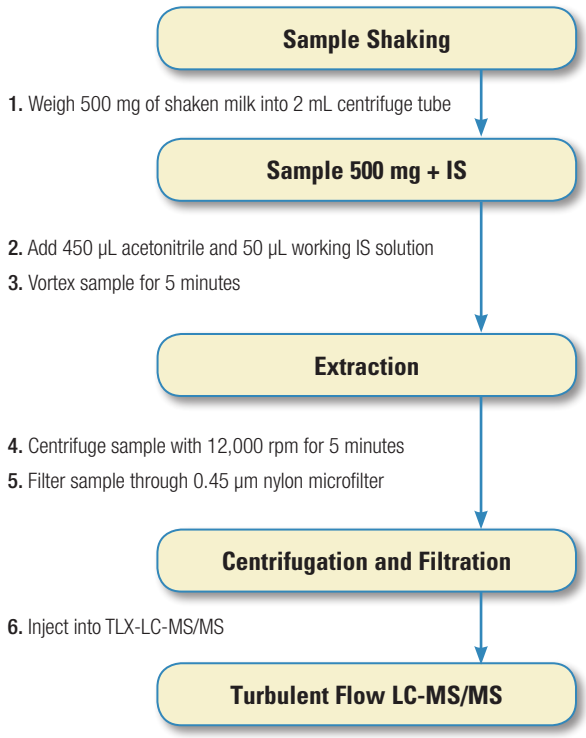
Method: 63551

Key Words
Transcend TLX, TSQ Quantum Access MAX, Antibiotics, Food Safety, Milk, TurboFlow Technology

1. Schematic of Method



2. Introduction

Antibiotics are a group of chemicals that are widely used in animal husbandry primarily for protection of animals from disease but also as growth promoters. The European Union (EU) has set maximum residue limits (MRL) for a variety of antibiotics in animal tissues, milk and eggs; suitable methods are required to be capable of detecting these residues at regulated levels. For the last decade laboratories have been using methods only for one class of antibiotics. However, an increasing number of multi-residue methods covering different classes of drugs are being developed as more efficient and cost-effective procedures.

For fast screening of antibiotics, microbiological or bioassay techniques are widely used. These techniques are not able to distinguish between the different types of antibiotics and provide only a semi-quantitative result for the total amount of drug residues. The big drawback is the incidence of false-negative or false-positive results because of low sensitivity and specificity. However, these screening assays are still very popular and widely used because of their cost-effectiveness and speed of analysis.

For quantitative analysis it is necessary to use instrumental techniques such as LC-MS/MS. This technique can also be used for screening, and provides much higher sensitivity and greater specificity. The use of LC-MS/MS for screening was described in a validated multi-residue screening method to monitor 58 antibiotics in milk[1].

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This note describes a multi-residue confirmatory method for the quantitative determination of antibiotics in milk using turbulent flow chromatography coupled to LC-MS/MS. This method fulfills the increasing need for a cost-effective and fast method by employing Thermo Scientific TurboFlow technology (via the Thermo Scientific Transcend TLX) for online sample cleanup. This approach has already been applied in the development of a screening method for antibiotics in milk[2]. The method in this note is different due to the increased number of antibiotics detected as well as the inclusion of quantitative results.

3. Scope and Application

This online TLX-LC-MS/MS method can be applied to detect and quantify the presence of 36 compounds from 7 different classes of antibiotics (aminoglycosides, sulfonamides, macrolides, quinolones, tetracyclines, lincosamides and trimethoprim) in milk. This multi-residue method fulfills legislative requirements described in the EU Commission Decision 2002/657/EC[3].

4. Principle

This method uses turbulent flow chromatography for online cleanup of the sample. Sample concentration, cleanup and analytical separation are carried out in a single run using a TurboFlow™ column connected to an analytical LC column. Macromolecules are removed from the sample extract with high efficiency, while target analytes are retained on the column based on different chemical interactions. After application of a wash step, the trapped compounds are transferred onto the analytical LC column and separated conventionally. Before applying the sample extract onto the TurboFlow column, the sample is thoroughly mixed to evenly distribute the fat and then fortified with an internal standard, extracted with acetonitrile and centrifuged. Cleanup using the TLX system was optimized for maximum recovery of targeted compounds and minimal injection of co-extractives into the mass spectrometer. Identification of antibiotics is based on retention time, ion-ratios using multiple reaction monitoring (MRM) of characteristic transition ions, and quantification using matrix matched standards of one of the selected MRM ions.

5. Reagent List

		Part Number
5.1	Purified Water – Thermo Scientific Barnstead EASYpure II water system	D 13321
5.2	Methanol Fisher Scientific Optima, LC-MS grade	10767665
5.3	Water, LC-MS grade	10777404
5.4	Acetonitrile Optima® LC-MS grade	10001334
5.5	Isopropanol, HPLC grade	10674732
5.6	Acetone, HPLC grade	10131560
5.7	Formic acid, extra pure, >98%	10375990
5.8	Heptafluorobutyric acid, 99%	172800250
5.9	Ammonia, extra pure, 35%	10305170

6. Calibration Standards

6.1	Kanamycin	Sigma-Aldrich®
6.2	Amikacin	Sigma-Aldrich
6.3	Dihydrostreptomycin	Sigma-Aldrich
6.4	Streptomycin	Sigma-Aldrich
6.5	Lincomycin hydrochloride monohydrate	Sigma-Aldrich
6.6	Clindamycin hydrochloride	Sigma-Aldrich
6.7	Trimethoprim	Sigma-Aldrich
6.8	Josamycin	Sigma-Aldrich
6.9	Spiramycin	Sigma-Aldrich
6.10	Tilmicosin	Sigma-Aldrich
6.11	Tylosin tartrate	Sigma-Aldrich
6.12	Clarithromycin	Sigma-Aldrich
6.13	Erythromycin A dihydrate	Sigma-Aldrich
6.14	Oleandomycin phosphate dehydrate	Dr. Ehrenstorfer
6.15	Tylvalosin tartrate	FarmKemi®
6.16	Sulfadimethoxine	Sigma-Aldrich
6.17	Sulfadoxin	Sigma-Aldrich
6.18	Sulfaquinoxaline	Sigma-Aldrich
6.19	Sulfachlorpyridazine	Sigma-Aldrich
6.20	Sulfaclozine sodium	Dr. Ehrenstorfer
6.21	Oxytetracycline hydrochloride	Sigma-Aldrich
6.22	Doxycycline hyclate	Sigma-Aldrich
6.23	Marbofloxacin	Sigma-Aldrich
6.24	Ciprofloxacin	Sigma-Aldrich
6.25	Danofloxacin	Sigma-Aldrich
6.26	Enrofloxacin	Sigma-Aldrich
6.27	Difloxacin	Sigma-Aldrich
6.28	Oxolinic acid	Sigma-Aldrich
6.29	Flumequine	Sigma-Aldrich
6.30	Nalidixic acid	Sigma-Aldrich
6.31	Enoxacin	Sigma-Aldrich
6.32	Ofloxacin	Sigma-Aldrich
6.33	Lomefloxacin hydrochloride	Sigma-Aldrich
6.34	Norfloxacin	Sigma-Aldrich
6.35	Sarafloxacin hydrochloride trihydrate	Sigma-Aldrich
6.36	Cinoxacin	Sigma-Aldrich
Internal Standard		
6.37	Sulfaphenazole	Sigma-Aldrich

7. Standards Preparation

7.1 Stock Standard Solutions of Veterinary Drugs

Stock standard solutions (1000 µg/mL) are prepared individually by dissolving the analytes in methanol (lincosamides, macrolides, sulfonamides, tetracyclines and trimethoprim), in water (aminoglycosides) and in methanol with 2% 2M NH_4OH (quinolones). Solutions are stored at -20 °C.

7.2 Working Standard Solution

The working calibration standard solution containing 1000 µg/L is prepared by dilution of individual stock standard solutions with acetonitrile. Solution should be prepared fresh every time before using.

7.3 Stock Solution of Internal Standard

Stock standard solution of the internal standard (1000 µg/mL) is prepared by dilution of sulfaphenazole in methanol. Solution is stored at -20 °C.

7.4 Working Standard Solution of Internal Standard

The working standard solution of the internal standard (2000 µg/L) was prepared by dilution of stock standard solution (sulfaphenazole) with acetonitrile. Solution should be prepared fresh every time before using.

8. Aparatus

		Part Number
8.1	Turbulent flow chromatograph Transcend™ TLX-1 system	
8.2	Thermo Scientific TSQ Quantum Access MAX triple quadrupole mass spectrometer	
8.3	Fisher Science Education™ precision balance	02225102
8.4	Sartorius analytical balance	14557812
8.5	Barnstead™ EASYpure™ II water system	D 13321
8.6	Vortex shaker	14505141
8.7	Vortex universal cap	3205029
8.8	Accu-jet® pipettor	3140246
8.9	Thermo Scientific Heraeus Fresco 17 micro centrifuge	208590

9. Consumables

		Part Number
9.1	Thermo Scientific TurboFlow Cyclone P (50 × 0.5 mm) column	CH-953289
9.2	Thermo Scientific BetaSil phenyl-hexyl (50 × 2.1 mm, 3 µm) column	73003-052130
9.3	LC vials	3205111
9.4	LC caps	3151266
9.5	Thermo Scientific Pipette Finnpipette 100–1000 µL	3214535
9.6	Pipette Finnpipette™ 20–200 µL	3214534
9.7	Pipette Finnpipette 10–100 µL	3166472
9.8	Pipette Finnpipette 500–5000 µL	3166473
9.9	Pipette Finnpipette 1000–10,000 µL	3214536
9.10	Pipette holder	3651211
9.11	Pipette tips 0.5–250 µL, 500/box	3270399
9.12	Pipette tips 1–5 mL, 75/box	3270420
9.13	Pipette tips 100–1000 µL, 200/box	3270410
9.14	Pipette tips 20,000–10,000 µL, 40/box	3270425
9.15	Pipette Pasteur soda lime glass, 150 mm	FB50251
9.16	Pipette suction device	3120891
9.17	Spatula, 18/10 steel	3458179
9.18	Spatula, nylon	3047217
9.19	1 mL Single-use syringes	1066-4161
9.20	17 mm nylon syringe filter, 0.45 µm	F2513-1
9.21	Vial rack (2 mL)	12211001
9.22	Centrifuge plastic tube (2 mL)	3150968
9.23	Rack for 50, 15, 2 and 0.5 mL tubes	10321031
9.24	Pipette tips 20,000–10,000 µL, 40/box	3270425
9.25	Pipette Pasteur soda lime glass, 150 mm	FB50251

Glassware

		Part Number
9.26	Beaker, 50 mL	10527211
9.27	Beaker, 100 mL	10769541
9.28	Beaker, 25 mL	10683771
9.29	Volumetric flask, 25 mL	10107901
9.30	Volumetric flask, 10 mL	10406681
9.31	Volumetric flask, 5 mL	10770803
9.32	Volumetric flask, 100 mL	10675731
9.33	5 mL glass pipette	10179522

10. Procedure

10.1 Sample Preparation

The sample of milk is shaken vigorously by hand. The sample (500 mg) is then weighed into a 2 mL polypropylene tube. Working internal standard solution (50 µL) and acetonitrile (450 µL) are added to the sample. The sample is shaken for 5 min on the vortex and then centrifuged at 12000 rpm for 5 min for removal of protein. The supernatant is filtered through a nylon micro filter (0.45 µm pore size) directly into the LC vial and the sample is analyzed by TLX-LC-MS/MS.

10.2 The LC Conditions

LC analysis is performed on a Transcend TLX-1 System.

TurboFlow column: TurboFlow Cyclone P (50 × 0.5 mm)
Analytical column: BetaSil™ phenyl-hexyl (50 × 2.1 mm, 3 µm)
Total run time: 19 min
Mobile phases: A = 1mM heptafluorobutyric acid and 0.5% formic acid in water
B = 0.5% formic acid in acetonitrile/methanol (1/1)
C = 2% methanol in water
D = acetone/acetonitrile/isopropanol (20/40/40)

10.2.1 Injector Settings

Injector: Thermo Scientific Pal injector with 100 µL volume injection syringe
Tray temperature: 10 °C
Cleaning solvents for the autosampler:
Solvent 1: acetonitrile/water (20/80)
Solvent 2: acetone/acetonitrile/isopropanol – 20/40/40

- Pre clean with solvent 1 [steps]: 3
- Pre clean with solvent 2 [steps]: 3
- Pre clean with sample [steps]: 1
- Filling speed [µL/s]: 50
- Filling strokes [steps]: 1
- Injection port: LC Vlv1 (TX channel)
- Injection speed [µL/s]: 100
- Pre inject delay [ms]: 500
- Post inject delay [ms]: 500
- Post clean with solvent 1 [steps]: 5
- Post clean with solvent 2 [steps]: 5
- Valve clean with solvent 1 [steps]: 5
- Valve clean with solvent 2 [steps]: 5
- Injection volume: 25 µL

Sample concentration, cleanup and analytical separation are carried out in a single run using an automated online sample preparation system, which includes the Transcend TLX system and Thermo Scientific Aria operating software. First the sample is applied during the loading step by the loading pump onto the TurboFlow column. During the same step the macromolecules are removed, while the target analytes are retained on the TurboFlow column based on their different chemical interactions. In the next step, the trapped analytes are transferred with the help of an eluting pump, and an adequately strong solvent (eluent) in the loop onto the analytical LC column where the analytes are separated conventionally. While the separation on the analytical column is running, the loop is filled with the eluent, and the TurboFlow column is washed and conditioned to be ready for the injection of the next sample. The TLX and LC conditions are presented in Table 1.

The analytical column is conditioned during loading of the sample onto the TurboFlow column. The separation of the analytes on the analytical column is done by gradient (Table 1). To prevent the possibility of carry-over and cross contamination, the injection syringe as well as the injection valve are washed five times with cleaning solvent 1 (acetonitrile/water – 20/80) and cleaning solvent 2 (acetone/acetonitrile/isopropanol – 20/40/40) before and five times after injection.

Step			TurboFlow Column[a]					Cut-in Loop		Analytical LC Column[b]			
Description	**Start [min]**	**Time [s]**	**Flow [mL/min]**	**A%**	**B%**	**C%**	**D%**	**Tee**	**Loop**	**Flow [mL/min]**	**Step**	**A%**	**B%**
1. loading	0	60	1.5	–	–	100	–	–	out	0.3	Step	100	–
2. transferring	1	60	0.2	100	–	–	–	T	in	0.6	Step	100	–
3. washing	2	60	1.5	–	–	50	50	–	in	0.3	Step	100	–
4. washing	3	720	1.5	–	–	–	100	–	in	0.3	Ramp	5	95
5. filling loop	15	120	1.5	50	50	–	–	–	in	0.3	Step	5	95
6. equilibrating	17	120	1.5			100	–	–	out	0.3	Step	100	–

[a]mobile phases for the TurboFlow method:
A: 1 mM heptafluorobutyric acid + 0.5% formic acid in water,
B: 0.5% formic acid in acetonitrile / methanol – 1/1,
C: 2% methanol in water and
D: acetone/acetonitrile/isopropanol – 20/40/40

[b]mobile phases for the analytical method:
A: 1 mM heptafluorobutyric acid + 0.5% formic acid in water and
B: 0.5% formic acid in acetonitrile/methanol – 1/1

Table 1: Gradient program table for TurboFlow system coupled with an analytical column

10.3 Mass Spectrometric Conditions

Mass spectrometric analysis is carried out using a TSQ Quantum Access MAX™ triple quadrupole system. Data acquisition for quantification and confirmation are performed in MRM mode. All selected reaction monitoring (SRM) traces (parent, qualifier and quantifier ion) are individually tuned for each target analyte by direct injection of the individual working standard solution (10 mg/mL). Data acquisition and processing is performed using Thermo Scientific Xcalibur 2.1 software.

- Ionization mode: Electrospray (ESI)
- Scan type: SRM
- Polarity: positive ion mode
- Spray voltage [V]: 3500
- Ion sweep gas pressure [arb]: 0
- Vaporizer Temperature [°C]: 400
- Sheath gas pressure [arb]: 50
- Aux gas pressure [arb]: 10
- Capillary temperature [°C]: 370
- Collision gas pressure [mTorr]: 0
- Cycle time [s]: 0.6

Peak width: Q1/Q3 the full width of a peak at half its maximum height (FWHM) of 0.70 Da

The parameters for SRM analysis for targeted compounds and internal standards are displayed in the Table 2.

11. Calculations

11.1 Identification

Identification of the antibiotics is confirmed by the presence of transition ions (quantifier and qualifier) at retention times (± 2.5%) to the corresponding standards. In MRM mode, the measured peak area ratios for qualifier to quantifier ions should be in close agreement (according to EU Commission Decision 2002/657/EC) with the ratios of the standards, as shown in Table 3. The quantifier and qualifier ions were selected among the product ions produced by the fragmentation of the selected parent ion on the basis of the intensity. Representative chromatogram is shown in Figure 1.

11.2 Quantification

For quantification, internal standardization is used measuring peak area ratios for standards in matched matrixes. Sulfaphenazole is used as the internal standard for all target antibiotics. Calibration curves are plotted as the relative peak areas (analyte versus the corresponding standard) as a function of the compound concentration. The antibiotic concentration in the samples is determined from the equation:

$$c_a = \left(\frac{A_a}{A_{IS}} - b\right)/a$$

c_a – antibiotic concentration in µg/kg

A_a – peak area of the antibiotic

A_{IS} – peak area of internal standard

b – y-intercept

a – slope of the calibration curve.

12. Method Performance

The method was validated in-house according to the criteria specified in EU Commission Decision 2002/657/EC for a quantitative method. The validation parameters were determined by spiking blank milk at levels of 0.5, 1 and 1.5 times the MRL. For compounds without MRL, samples were spiked at 10, 50 and 100 µg/kg for clindamycin, macrolides and quinolones; at 100, 200 and 300 µg/kg for aminoglycosides; and 50, 100 and 150 µg/kg for doxycycline. The measured parameters were specificity, linear range, repeatability, accuracy, limit of detection (LOD), and limit of quantification (LOQ), decision limit (CCα), and detection capability (CCβ).

12.1 Samples and Quality Control Materials

For preparation of matrix-matched calibration standards and spiked samples for validation, skim milk with a fat content of 0.3% obtained from a local market was used. Before use, the milk was checked by repeated measurements to confirm that it was free of antibiotics. For determination of accuracy, a certified reference material ERM® – BB492 of partially skim milk containing a certified amount of oxytetracycline was used, obtained from the Institute for Reference Materials and Measurements (Geel, Belgium). The skim milk powder was reconstituted according to instructions.

12.2 Specificity

Using SRM, the specificity is confirmed based on the presence of the transition ions (quantifier and qualifier) at the correct retention times corresponding to those of the respective antibiotics. The measured peak area ratios of qualifier/quantifier are within the range defined in EU Commission Decision 2002/657/EC when compared to the standards (Table 3).

12.3 Linearity and Calibration Curve

The linearity of calibration curves is assessed over the range from 0-500 µg/kg for all target compounds. In all cases, the correlation coefficients of linear functions have to be >0.99. The calibration curves are created from 8 matrix-matched calibration standards that are injected in each batch in duplicate.

12.4 Precision

Precision (repeatability) of the method was determined using independently spiked blank samples at three different levels. In one day, the set of samples at three levels was measured with six repetitions. To determine between-day precision, two other sets at one level were measured with six repetitions over the next two days. The results for repeatability ranged from 4% to 28% (Table 4).

12.5 Accuracy

Method accuracy was determined using independently spiked blank samples at three different levels. Accuracy was evaluated by comparing found values with standard additions in spikes. Recovery values ranged between 78–120% (Table 5). Additionally, accuracy was established for oxytetracycline by analyzing the certified reference material ERM – BB492 which was partially skim milk powder. All measured concentrations of oxytetracycline were within the acceptable range (Table 6).

12.6 LOD and LOQ

LOD and LOQ are estimated following the IUPAC approach which consists of first analyzing the blank sample to establish noise levels and then estimating LODs and LOQs for signal/noise, 3 and 10 respectively. The values for milk are shown in Table 7, and in all cases, they are under the level of MRL for all analytes that have an assigned MRL.

12.7 Limit of Decision (CCα) and Limit of Capability (CCβ)

Both CCα and CCβ were established by the calibration curve procedure according to ISO 11843[4]. The blank material fortified at and below the MRL (for analytes with MRL) or at and above the lowest possible level (for analytes without MRL) in equidistant steps was used. The calculated values are shown in Table 7.

13. Conclusion

This in-house validated method enables quantification of 36 residues from seven different classes of antibiotics in milk. For all 36 compounds, only one extraction procedure was used although they come from different groups with widely varying polarities and solubilities. The use of turbulent flow chromatography combined with LC-MS/MS detection for analytical separation saves a significant amount of time in sample preparation and increases the sample throughput. The in-house validation results, according to IUPAC/AOAC harmonized protocol, reflected that this method is suitable for regulatory purposes. This method can be strongly recommended for use because it significantly speeds up sample analysis compared to traditional methods, is applicable for a large number of antibiotic residues and is convenient for regulatory purposes.

14. References

1. Gaugain-Juhel M, Delepine B, Gautier S, Fourmond MP, Gaudin V, Hurtaud-Pessel D, Verdon E, Sanders P Validation of a liquid chromatography-tandem mass spectrometry screening method to monitor 58 antibiotics in milk: a qualitative approach. *Food Addit. and Contam.* **2009**, 26, 1459-1471
2. Stolker AAM, Ruud JBP, Zuiderent R, DiBussolo JM, Martins CPB Fully automated screening of veterinary drugs in milk by turbulent flow chromatography and tandem mass spectrometry. *Anal Bioanal Chem.* **2010**, 397, 2841-2849
3. EU Commission Decision 2002/657/EC. *Off. J. Eur. Commun.* **2002**, L221/8
4. ISO 11843: Capability of detection (1997)

Thermo Scientific Transcend TLX system coupled with the TSQ Quantum Access MAX triple quadrupole mass spectrometer

Analyte	Retention Time (min)	Molecular Weight	Precursor Ion	Quantifier Ion	Qualifier Ion	CE for Quantifier Ion (V)	CE for Qualifier Ion (V)	Tube Lens
Kanamycin	1.74	484.5	485.28	163.1	324.2	25	15	90
Amikacin	1.73	585.6	586.29	163.1	425.2	33	21	102
Dihydrostreptomycin	1.67	583.6	584.29	263.1	246.0	29	33	109
Streptomycin	1.66	581.6	582.26	263.1	221.1	30	40	141
Lincomycin	8.24	406.5	407.14	126.2	359.2	28	17	97
Clindamycin	10.75	425.0	425.14	126.2	377.2	28	18	86
Trimethoprim	8.84	290.3	291.1	230.1	261.1	23	24	93
Josamycin	12.55	828.0	828.43	174.0	109.1	30	34	18
Spiramycin	10.50	843.1	843.31	174.0	142.0	32	32	146
Tilmicosin	11.21	869.1	869.62	696.4	174.0	40	41	132
Tylosin	11.65	916.1	916.51	174.0	772.4	35	26	141
Clarithromycin	12.05	748.0	748.51	158.2	590.4	28	17	108
Erythromycin	11.40	733.9	734.46	576.4	158.0	17	28	103
Oleandomycin	11.13	687.8	688.44	544.4	158.1	14	25	106
Tylvalosin	13.25	1042.3	1042.64	109.1	174.0	41	37	133
Sulfadimethoxine	10.20	310.3	311.03	156.1	108.1	21	27	88
Sulfadoxin	9.17	310.3	311.04	156.0	108.1	18	26	88
Sulfaquinoxaline	10.40	300.3	301.04	156.0	92.2	17	28	92
Sulfachlorpyridazine	8.83	284.7	284.97	156.0	92.2	15	26	90
Sulfaclozine	10.14	284.7	284.96	92.2	108.1	29	26	87
Sulphafenazole	10.39	314.4	315.06	158.1	160.1	28	22	94
Oxytetracycline	8.94	460.4	461.11	426.1	337.0	18	29	93
Doxycycline	10.60	444.4	445.14	428.2	321.1	18	31	82
Marbofloxacin	8.90	362.4	363.11	72.3	320.1	22	14	97
Ciprofloxacin	9.17	331.3	332.08	288.1	314.1	18	22	89
Danofloxacin	9.26	357.4	358.11	340.1	314.2	24	16	99
Enrofloxacin	9.41	359.4	360.1	316.1	345.1	19	25	96
Difloxacin	9.90	399.4	400.1	356.1	299.0	19	28	98
Oxolinic acid	9.95	261.2	262.01	244.0	216.0	18	29	84
Flumequine	11.06	261.3	262.02	244.1	202.0	19	33	84
Nalidixic acid	10.77	232.2	233.04	215.1	187.1	15	25	77
Enoxacin	8.85	320.3	321.09	303.1	257.1	19	17	93
Ofloxacin	9.10	361.4	362.12	318.1	261.1	18	27	91
Lomefloxacin	9.34	351.3	352.1	265.1	308.1	23	15	100
Norfloxacin	9.07	319.3	320.07	276.1	302.1	16	22	94
Sarafloxacin	9.90	385.4	386.08	342.1	299.1	18	27	94
Cinoxacin	9.41	262.2	263.02	245.0	189.0	16	27	90

Table 2: LC-MS/MS parameters for selected reaction monitoring of analytes

Analyte	Ion Ratio (Std Mix)	Ion Ratio (Milk)
Kanamycin	0.74	0.64
Amikacin	0.65	0.61
Dihydrostreptomycin	0.26	0.26
Streptomycin	0.46	0.49
Lincomycin	0.24	0.30
Clindamycin	0.09	0.07
Trimethoprim	0.90	0.83
Josamycin	0.85	0.93
Spiramycin	0.26	0.23
Tilmicosin	1.19	0.72
Tylosin	0.20	0.22
Clarithromycin	0.48	0.57
Erythromycin	0.54	0.53
Oleandomycin	1.22	0.95
Tylvalosin	0.63	0.76
Sulfadimethoxine	0.11	0.14
Sulfadoxin	0.31	0.31
Sulfaquinoxaline	0.28	0.30
Sulfachlorpyridazine	0.27	0.29
Sulfaclozine	0.44	0.55
Sulphafenazole	0.89	0.88
Oxytetracycline	0.13	0.13
Doxycycline	0.04	0.05
Marbofloxacin	0.73	0.68
Ciprofloxacin	0.15	0.14
Danofloxacin	0.03	0.03
Enrofloxacin	0.53	0.48
Difloxacin	0.74	0.71
Oxolinic acid	0.05	0.06
Flumequine	0.22	0.28
Nalidixic acid	0.25	0.29
Enoxacin	0.02	0.01
Ofloxacin	0.73	0.79
Lomefloxacin	0.60	0.66
Norfloxacin	0.09	0.11
Sarafloxacin	0.28	0.34
Cinoxacin	0.30	0.35

Table 3: Ion ratios (Qual/Quant) in matrix and in standard mixture (the agreement between ion ratios should be within the permitted tolerance, which is defined in EU Commission Decision 2002/657/EC)

	RSD (%) – spiking level 2			Milk – RSD (%)		
Analyte	Day 1	Day 2	Day 3	Level 1 (µg/kg)	Level 2 (µg/kg)	Level 3 (µg/kg)
Kanamycin	17	12	21	24	17	10
Amikacin	13	11	23	23	13	16
Dihydrostreptomycin	14	21	22	10	14	6
Streptomycin	6	26	15	17	6	12
Lincomycin	16	19	15	17	16	9
Clindamycin	9	8	7	13	9	12
Trimethoprim	10	9	11	19	10	8
Josamycin	7	11	12	12	7	13
Spiramycin	15	6	7	21	15	6
Tilmicosin	4	6	6	10	4	12
Tylosin	4	9	7	12	4	9
Clarithromycin	8	12	8	28	8	12
Erythromycin	4	6	6	13	4	9
Oleandomycin	13	7	10	21	13	9
Tylvalosin	6	11	6	18	6	6
Sulfadimethoxine	14	9	4	9	14	6
Sulfadoxin	9	9	8	5	9	5
Sulfaquinoxaline	12	9	9	12	12	16
Sulfachlorpyridazine	11	14	16	24	11	5
Sulfaclozine	17	18	16	11	17	13
Oxytetracycline	26	27	17	28	26	15
Doxycycline	8	9	9	12	8	8
Marbofloxacin	9	17	15	15	9	11
Ciprofloxacin	6	9	10	8	6	10
Danofloxacin	11	18	23	18	11	11
Enrofloxacin	9	6	6	14	9	10
Difloxacin	11	10	18	16	11	5
Oxolinic acid	20	12	13	21	20	14
Flumequine	4	6	4	9	4	9
Nalidixic acid	10	12	6	10	10	10
Enoxacin	14	14	16	26	14	14
Ofloxacin	14	8	5	12	14	14
Lomefloxacin	19	14	22	8	19	11
Norfloxacin	10	15	11	13	10	14
Sarafloxacin	11	9	11	14	11	16
Cinoxacin	15	15	14	24	15	11

Table 4: Method intermediate precision as RSD (%) – 1 level – 3 sets with 6 replicates in 3 days and method repeatability expressed as RSD (%) and measured at 3 levels every time with 6 replicates

	Spiking levels			Milk – REC (%)		
Analyte	**Level 1 (µg/kg)**	**Level 2 (µg/kg)**	**Level 3 (µg/kg)**	**Level 1**	**Level 2**	**Level 3**
Kanamycin	75	150	225	104	78	102
Amikacin	100	200	300	90	101	111
Dihydrostreptomycin	100	200	300	111	104	111
Streptomycin	100	200	300	106	101	107
Lincomycin	75	150	225	74	81	96
Clindamycin	10	50	100	95	96	104
Trimethoprim	10	50	100	90	84	101
Josamycin	10	50	100	95	99	106
Spiramycin	100	200	300	84	102	94
Tilmicosin	25	50	75	98	95	101
Tylosin	25	50	75	103	95	100
Clarithromycin	10	50	100	98	108	94
Erythromycin	20	40	60	86	80	95
Oleandomycin	10	50	100	109	95	97
Tylvalosin	10	50	100	109	104	104
Sulfadimethoxine	50	100	150	93	97	104
Sulfadoxin	50	100	150	100	98	108
Sulfaquinoxaline	50	100	150	99	97	107
Sulfachlorpyridazine	50	100	150	96	99	109
Sulfaclozine	50	100	150	88	92	116
Oxytetracycline	50	100	150	108	98	105
Doxycycline	50	100	150	96	85	100
Marbofloxacin	37.5	75	112.5	102	98	120
Ciprofloxacin	50	100	150	94	85	106
Danofloxacin	15	30	45	94	80	95
Enrofloxacin	50	100	150	90	86	104
Difloxacin	10	50	100	102	99	99
Oxolinic acid	10	50	100	120	91	89
Flumequine	25	50	75	103	95	102
Nalidixic acid	10	50	100	100	90	98
Enoxacin	10	50	100	97	87	96
Ofloxacin	10	50	100	86	89	107
Lomefloxacin	10	50	100	87	81	99
Norfloxacin	10	50	100	95	90	103
Sarafloxacin	10	50	100	97	90	100
Cinoxacin	10	50	100	92	84	98

Table 5: Recoveries (%) for spiked samples of milk at 3 different spike levels (6 replicates)

Sample	Concentration [found] (µg/kg)
CRM 1	105
CRM 2	95
CRM 3	112
CRM 4	97

Table 6: Results of certified reference material – milk ERM-BB492 – oxytetracycline – c = 101 ± 11 µg/kg

Analyte	LOD (µg/kg)	LOQ (µg/kg)	MRL (µg/kg)	CCα (µg/kg)	CCβ (µg/kg)
Kanamycin	15.0	45.0	150	168	186
Amikacin	25.0	75.0	–	54	108
Dihydrostreptomycin	25.0	75.0	200	242	285
Streptomycin	25.0	75.0	200	231	261
Lincomycin	3.0	10.0	150	175	199
Clindamycin	0.3	1.0	–	8	16
Trimethoprim	1.5	5.0	–	6	12
Josamycin	0.3	1.0	–	11	23
Spiramycin	0.3	1.0	200	232	264
Tilmicosin	0.3	1.0	50	62	74
Tylosin	1.0	3.0	50	62	73
Clarithromycin	0.3	1.0	–	4	7
Erythromycin	0.3	1.0	40	56	72
Oleandomycin	0.3	1.0	–	7	13
Tylvalosin	0.3	1.0	–	8	17
Sulfadimethoxine	0.3	1.0	100[a]	116	131
Sulfadoxin	0.3	1.0	100[a]	117	133
Sulfaquinoxaline	1.5	5.0	100[a]	113	126
Sulfachlorpyridazine	15.0	45.0	100[a]	121	141
Sulfaclozine	15.0	45.0	100[a]	118	137
Oxytetracycline	15.0	45.0	100	117	134
Doxycycline	3.0	10.0	–	10	20
Marbofloxacin	1.5	5.0	75	85	96
Ciprofloxacin	0.3	1.0	100	115	129
Danofloxacin	0.3	1.0	30	36	42
Enrofloxacin	0.3	1.0	100	111	123
Difloxacin	0.3	1.0	–[b]	4	8
Oxolinic acid	0.3	1.0	–[b]	4	9
Flumequine	0.3	1.0	50	55	61
Nalidixic acid	0.3	1.0	–[c]	5	11
Enoxacin	0.3	1.0	–	5	11
Ofloxacin	0.3	1.0	–	6	12
Lomefloxacin	0.3	1.0	–	8	16
Norfloxacin	0.3	1.0	–	7	13
Sarafloxacin	0.3	1.0	–	5	11
Cinoxacin	1.5	5.0	–	13	27

Table 7: LOD and LOQ, MRL, CCα and CCβ for antibiotics in milk

[a] – *Expressed in form of sum-MRLs of all sulfonamides.*

[b] – *Banned for use in milk-producing animals.*

[c] – *No authorization in veterinary medicine.*

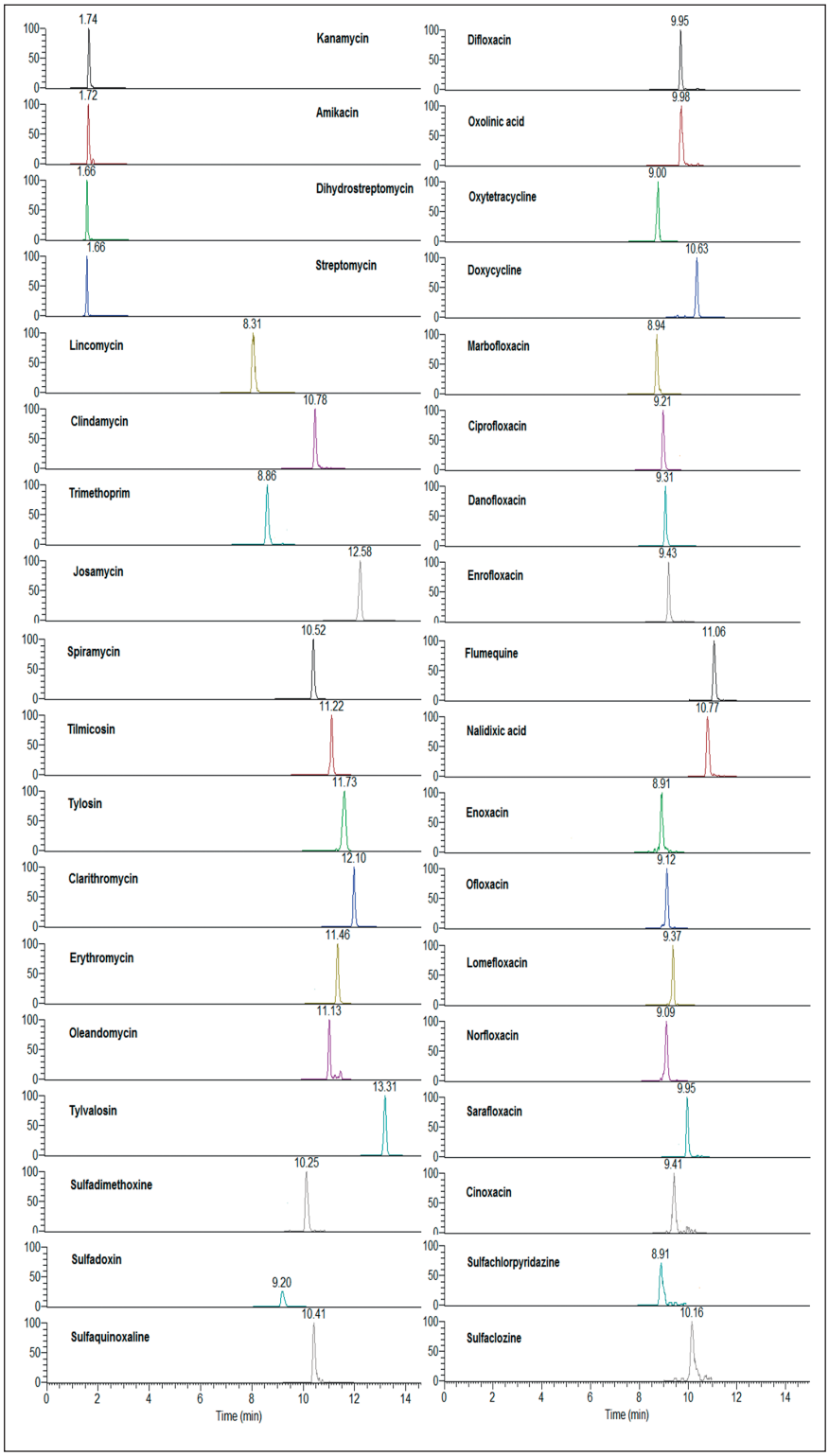

Figure 1: MRM chromatogram of all 36 antibiotics in spiked milk

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