Liquid Chromatography/
Mass Spectrometry

Authors:

Avinash Dalmia

PerkinElmer, Inc.
Shelton, CT

Determination, Confirmation and Quantitation of Multi-Class Antibiotic Residues in Milk by UHPLC MS/MS

Introduction

Antibiotics are widely used in veterinary medicine for treatment and prevention of disease and as growth promoters. Excessive usage of antibiotics might result in residue violations in animal originated food such as milk, meat and others, and this can pose a risk to health of humans. These antibiotic residues in food can cause toxicity and side effects such as allergic reactions, rash and nausea, etc. In addition, the low levels of antibiotics in food products, consumed for long time, can lead to the spread of drug-resistant bacteria. Therefore, the regulation of antibiotics for use in animals is enforced by every country in the world. For example, the European Union and Canada set maximum residue levels (MRLs) of drug residues in food and in the USA, these are called tolerance levels. The antibiotic residue classes of regulatory interest in milk include sulfonamides, β-lactams, fluoroquinolones, macrolides and tetracyclines. The tolerance limits of different classes of antibiotics, analyzed in this study, in milk are given in Table 1.

PerkinElmer
For the Better

In this work, we present a fast and sensitive LC/MS/MS method for the quantitative and confirmatory analysis of multi-class antibiotics in milk. The analyzed antibiotics in the study included ciprofloxacin, sarafloxacin, ofloxacin, penicillin-G, cloxacillin, tilmicosin, tylosin, erythromycin, sulfamethazine, sulfamethoxazole, sulfadimethoxine, tetracycline and chlortetracycline. The two internal standards in the method were ciprofloxacin-d_8 and flunixin-d_3. A modified and simple QuEChERS method was used for extraction of antibiotics from milk. The developed method showed good sensitivity, linearity, recovery, precision and selectivity required for analysis of antibiotics in milk at low tolerance levels set by different regulatory bodies in the world.

Table 1. List of antibiotics from different classes and their tolerance limit in milk.

Compound	Antibiotic Class	Tolerance Level[a]
Sulfamethazine	Sulfonamide	10 ng/ml[b]
Sulfamethoxazole	Sulfonamide	10 ng/ml[b]
Sulfadimethoxine[f]	Sulfonamide	10 ng/ml[b]
Tilmicosin	Macrolide	100 ng/ml[c]
Tylosin	Macrolide	50 ng/ml
Erythromycin	Macrolide	50 ng/ml
Penicillin-G	β-lactam	5 ng/ml
Cloxacillin	β-lactam	10 ng/ml
Ofloxacin	Fluoroquinolone	5 ng/ml[d]
Ciprofloxacin	Fluoroquinolone	5 ng/ml[d]
Sarafloxacin	Fluoroquinolone	5 ng/ml[d]
Tetracycline	Tetracycline	300 ng/ml[e]
Chlortetracycline	Tetracycline	300 ng/ml[e]

[a] Tolerance or safe levels in milk from 9/27/05 FDA/CFSAN Milk Safety Branch memo[1]

[b] Amounts listed are "safe level" not a tolerance

[c] No tolerance set for milk ; method target level set at 100 ng/ml (tolerance in muscle)

[d] No tolerance or safe levels have been established; method target levels set at 5 ng/ml

[e] Tolerance includes sum and individual residues; method target levels set at 100 ng/ml each

[f] Extra-label use of sulfonamide drugs in lactating dairy cattle (except sulfadimethoxine) is prohibited[1]

Hardware/Software

Samples and Sample Preparation
For the chromatographic separations, a PerkinElmer Altus™ UPLC® System was used, including the Altus A-30 Solvent/Sample Module, integrated vacuum degasser and column heater. For detection, a QSight™ 210 Triple Quadrupole MS/MS detector was used. All instrument control, analysis and data processing was performed using the Simplicity 3Q™ software platform.

Method Parameters
The LC method and MS source parameters are shown in Table 2.

Solvents, Standards and Sample Preparation
All solvents, reagents and diluents used were LC/MS grade. All antibiotic standards were obtained from Sigma-Aldrich® Inc., Saint-Louis, MO and stored at 4 °C in refrigerator to prevent their degradation. Stock and mixed drug solutions for spiking and calibration were prepared in methanol for all antibiotics except β-lactams for which water was used as a solvent. β-lactam

Table 2. LC Method and MS Source Parameters.

LC Conditions	
Column:	PerkinElmer Brownlee™ SPP C18, 2.7 µm, 2.1 x 100 mm (Part # N9308404)

Mobile Phase: Solvent A: 5mM ammonium formate, 0.1 % formic acid in water

Solvent B: 95 % Acetonitrile (ACN)/ 5 % Methanol with 0.1 % formic acid

	Time (min)	%A	%B	Curve
1	Initial	95.0	5.0	
2	0.50	95.0	5.0	6
3	7.50	40.0	60.0	6
4	10.00	0.0	100.0	6
5	15.00	0.0	100.0	6
6	15.30	95	5.0	6

Analysis Time:	12 min; Column wash time with 100% B: 3 min; re-equilibration time: 4.2 min
Flow Rate:	0.4 mL/min **Pressure:** 4200 psi/285 bar (maximum)
Oven Temp.:	30 °C
Injection Volume:	10 µL
Sample Temp:	7 °C

MS Conditions	
Ionization Mode:	ESI - positive
Drying Gas (Nitrogen) Setting:	75 arbitrary units
HSID™ Temp:	320 °C
Electrospray Voltage:	5500 V
Source Temp:	425 °C
Nebulizer Gas (Nitrogen) Setting):	175 arbitrary units
Detection Mode:	MRM Mode

standards need to be stored in plastic vials; whereas other antibiotic standards can be stored either in plastic or glass containers. The sorbent end capped C18 was obtained from Supelco. The vials were maintained at 7 °C in autosampler tray to prevent degradation of analytes. To prevent degradation of standards, all stock and working standards were stored under refrigeration until used, and only amber 2-mL LC vials were used. For calibration and quantitation purposes, two internal standards were used: ciprofloxacin-d_8 for fluoroquinolones, sulfonamides and tetracyclines and flunixin-d_3 for macrolides and β-lactams.

We used organic whole milk samples from a local market as a controlled blank matrix. Also five different varieties of milk samples were analyzed with different fat content to check for presence of antibiotics. We followed a simple and modified QuEChERS method for sample preparation[2,3]. The sample preparation method included the following steps: (1) add 20 ml of organic solvent(methanol or acetonitrile) to 5 ml of either blank organic milk or milk fortified with different levels of antibiotics and internal standards at 15 ng/ml; (2) pulsed-vortexing for 1 min. and centrifuge at 7800 rpm for 10 min; (3) take supernatant out and add 1.2 gm of C18 for dispersive-SPE for fat removal; (4) pulsed-vortexing for 5 minutes and centrifuge at 7800 rpm for 5 min; (5) take 12.5 ml of supernatant out and dry down to 1.5 to 2 ml with dry nitrogen

for about 1 hr at 40 °C; (6) reconstitute with 15 % methanol in water and bring the total volume up to 2.5 ml of extract; (7) filter the extract through PVDF filter (0.22 μm). For matrix matched calibration, the spiking and internal standard solutions were added after step 7 to give equivalent analyte concentrations in the samples ranging from 0.1 ng/ml to 1000 ng/ml. The number of replicates at each calibration level were five.

MS/MS Parameters

Electrospray ionization in positive mode was used for analysis of all residues. Source parameters including gas flows, source temperature and position settings were optimized by infusing a 0.5 μg/ml solution of sulfamethazine at 10 μl/min into a stream of 90:10 water:acetonitrile with 0.1 % formic acid and 5 mM ammonium formate at a flow rate of 0.5 ml/min. The solutions for each residue were infused at 0.5 μg/ml to determine optimal collision energies for each MRM transition. The quadrupole peak widths (Q1 and Q3) were set at 0.7 amu. The specific parameters for each MRM transitions are listed in Table 3. Three MRM transitions were monitored for each antibiotic residue to reduce number of false positive and negative in the method[4].

Results and Discussion

The LOQ and regression values for the calibration curves for each of the antibiotics are summarized in Table 4. The LOQ for each analyzed antibiotic was lower than their allowed tolerance level in milk by a factor of 5 to 100. This demonstrates that the method is more than adequately sensitive for antibiotic analysis in milk at tolerance level. Figure 1 shows 3 MRM transitions for one of the antibiotics, sulfamethazine, spiked at 5 ng/ml level in milk matrix, with good signal to noise. The retention time for each analyte was reproducible and was within ± 0.1 minute over 24 hour period. A matrix matched calibration curve was generated by spiking blank milk samples with varying concentrations of antibiotics (0.1-1000 ng/mL) at nine different concentration levels along with internal standards. Figure 2 shows an example of calibration curve for

sulfamethazine over four orders of magnitude. The calibration curves were linear with calibration fit of R^2 greater than 0.9958 for all the analytes. Table 5 shows excellent precision of response for each analyte for five replicates at different concentration levels. The data showed that the RSD of the response was lower than 5% at concentration level of half of the tolerance level or higher for each analyte in milk extract.

Table 6 summarizes the recovery of all 13 antibiotics at concentration levels close to their tolerance limit in milk. The absolute recoveries of antibiotics of the macrolides, sulfonamides and β-lactam classes were in range of 70-120% with RSD% <20% for replicate samples (n=5). The absolute recoveries for fluoroquinolones were in the range of 60-70%; whereas the absolute recoveries for tetracycline were less than 30%. The recovery for internal standards-: ciprofloxacin-d_8 and flunixin-d_3 was about 70% and 90%, respectively. Since both fluoroquinolones and deuterated fluoroquinolones are expected to undergo similar losses during extraction process, it is reasonable to compensate for lower absolute recoveries for fluoroquinolones using an internal standard. The recoveries for fluoroquinolones and tetracyclines listed in Table 1 were corrected for losses during the extraction process using ciprofloxacin-d_8 as an internal standard. After corrections of recovery with deuterated fluoroquinolone as an internal standard, the recoveries of fluoroquinolones were in the range of 80-110 %, where as it was about 30% for tetracyclines. In future, the use of a deuterated tetracycline can be used to compensate for losses of tetracyclines with this extraction process. Table 6 also lists the effect of using different organic solvent during protein precipitation on the recovery of analytes and ion suppression from matrix. The recovery of all analytes except for erythromycin was similar with both solvents. The recovery of erythromycin with acetonitrile and methanol was about 27% and 98%, respectively. The matrix suppression/ enhancement was calculated by taking a ratio of response of analyte in milk matrix to a solvent standard and was found to be

Table 3. MS/MS parameters of LC/MS/MS method.

| Analyte	Retention Time	Dwell Time	Precursor Ion	Quant Ion	CE	Qual Ion 1	CE	Qual Ion 2	CE
Sulfamethazine	3.37 min	10 ms	279.1	186	20 V	156	25 V	107.9	40 V
Sulfamthoxazole	4.38 min	10 ms	254.1	107.9	40 V	91.7	55 V	156	30 V
Sulfadimethoxine	5.15 min	10 ms	311.1	156	25 V	91.7	40 V	107.9	50 V
Tilmicosin	4.93 min	30 ms	435.3	695.4	20 V	174	30 V	87.9	55 V
Tylosin	5.70 min	30 ms	916.5	174	46 V	100.9	64 V	144.9	48 V
Erythromycin	5.41 min	20 ms	734.4	158	40 V	576.2	22 V	115.9	66 V
Penicillin-G	5.38 min	30 ms	334.9	160	22 V	176	22 V	113.9	48 V
Cloxacillin	6.6 min	30 ms	436.2	277.1	18 V	113.9	62 V	178	50 V
Ofloxacin	3.30 min	10 ms	361.9	317.9	25 V	343.9	25 V	260.9	35 V
Ciprofloxacin	3.38 min	10 ms	331.9	313.9	24 V	230.9	51 V	187.9	75 V
Sarafloxacin	3.93 min	10 ms	385.9	367.9	28 V	341.9	24 V	298.9	36 V
Tetracycline	3.52 min	30 ms	445.1	410.1	16 V	154	24 V	97.9	36 V
Chlortetracycline	4.33 min	30 ms	479	444	26 V	462	22 V	154	36 V
Ciprofloxacin-d_8	3.36 min	10 ms	340.1	235	48 V	-	-	-	-
Flunixin-d_3	7.20 min	10 ms	300	282	30 V	-	-	-	-

negligible for sulfonamides, macrolides and β-lactams, when sample preparation used either methanol or acetonitrile. Both fluoroquinolones and tetracyclines showed ion enhancement from the matrix. On average, the use of methanol compared to acetonitrile resulted in higher ion enhancement effects due to the matrix for fluoroquinolones and tetracyclines. Five milk samples, with different fat content (1% to 5%), fortified with internal standards, were screened for antibiotics with the method developed in this work. Based on retention time and quantifier ion response, none of target antibiotic analytes were detected even at a level of three times lower than method LOQ (0.1 to 1 ng/ml) for each analyte. The RSD of recovery of two internal standards from milk samples with different fat content was less than 15%. This suggests that fat content in milk does not appear to affect recovery of these analytes with the sample preparation method used in this work. Three ion transitions instead of two transitions were monitored for identification and confirmation purposes. This results in two product ion ratios instead of just one ratio and provides higher selectivity and specificity in identification. All ion ratios were computed by calculating peak area for less intense ion to peak area for more intense ion to generate ion ratios less than 100%. Table 7 shows the average ion ratio for all target analytes at concentration levels of half of the tolerance level to 10 times the tolerance level in milk extract. The relative ion ratios at different concentration levels were within ± 11 % of an average ion ratio, which is lower than the permitted relative tolerance level limit of ± 30 % set by SANCO document for analysis of chemical residues in food[5].

Long term stability of the system was studied over six days by injecting milk extract spiked with 10 ng/ml of sulfonamides, fluoroquinolones, β-lactams and 50 ng/ml of macrolides and tetracyclines. Figure 3 shows long term normalized response of 10 ng/ml of sulfamethazine in milk extract with respect to internal standard. The response for sulfamethazine did not decrease after six days of injections and this demonstrated the excellent stability
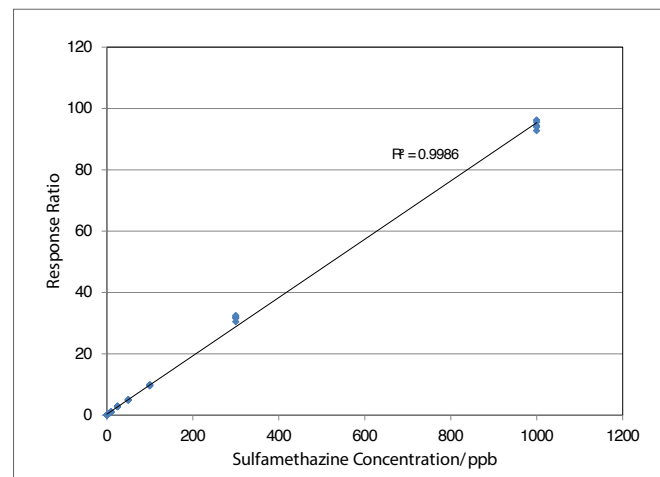
of a triple quad MS/MS system used in the study. The response for 10 out of 13 antibiotic residues in milk extract did not degrade after six days of injections. The response for three (erythromycin, tylosin and ofloxacin) out of 13 antibiotics residues decreased only by 30% over six days, which could be explained by decomposition of these antibiotics with time.
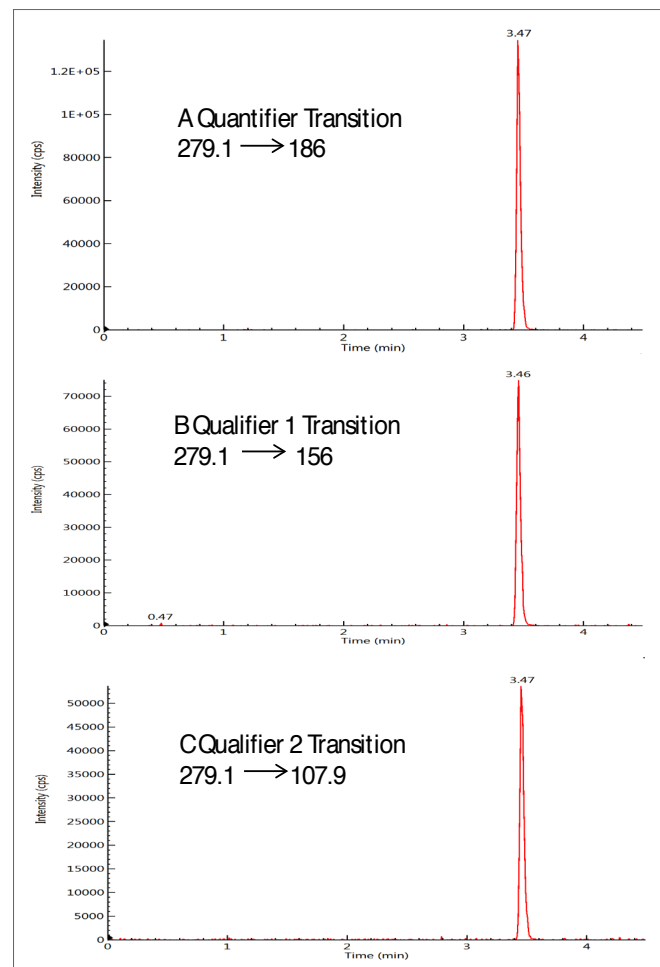
Figure 1. Chromatograms of three MRM transitions(one quantifier-A and two qualifiers-B,C) of sulfamethazine spiked at 5ng/ml in milk extract.

Table 4. LOQ and Linearity Correlation coefficients for different antibiotic residues in milk extract.

Compound	LOQ in Milk	Linear Calibration Curve Concentration Range	Correlation Coefficient R²
Sulfamethazine	0.1 ng/ml	0.1-1000 ng/ml	0.9986
Sulfamethoxazole	0.1 ng/ml	0.1-1000 ng/ml	0.9996
Sulfadimethoxine	0.1 ng/ml	0.1-1000 ng/ml	0.9991
Tilmicosin	0.1 ng/ml	0.1-1000 ng/ml	0.9990
Tylosin	1 ng/ml	1-1000 ng/ml	0.9980
Erythromycin	0.3 ng/ml	0.3-1000 ng/ml	0.9958
Penicillin-G	1 ng/ml	1-1000 ng/ml	0.9981
Cloxacillin	1 ng/ml	1-1000 ng/ml	0.9985
Ofloxacin	0.1 ng/ml	0.1-1000 ng/ml	0.9982
Ciprofloxacin	0.1 ng/ml	0.1-1000 ng/ml	0.9978
Sarafloxacin	0.1 ng/ml	0.1-1000 ng/ml	0.9968
Tetracycline	0.3 ng/ml	0.3-1000 ng/ml	0.9956
Chlortetracycline	0.3 ng/ml	0.3-1000 ng/ml	0.9984

Figure 2. Calibration curve for sulfamethazine over concentration range from 0.1 to 1000 ng/ml for n=5 at each level in milk extract.

Table 5. Repeatability of response for antibiotic residues at different levels in milk extract.

Compound	2.5 ng/ml RSD (n=5)	5 ng/ml RSD (n=5)	10 ng/ml RSD (n=5)	25 ng/ml RSD (n=5)	50 ng/ml RSD (n=5)	100 ng/ml RSD (n=5)
Sulfamethazine	2.4 %	2.4 %	1.1 %	1.4 %	0.8 %	0.7 %
Sulfamethoxazole	3.5 %	4.7 %	2.8 %	1.7 %	2.9 %	1.4 %
Sulfadimethoxine	3.0 %	0.8 %	2.0 %	1.6 %	1.2 %	1.7 %
Tilmicosin	0.7 %	1.5 %	1.8 %	0.7 %	0.8 %	1.1 %
Tylosin	7.8 %	4.3 %	3.9 %	2.1 %	2.2 %	1.4 %
Erythromycin	4.7 %	3.8 %	4.0 %	1.7 %	2.3 %	0.8 %
Penicillin-G	3.0 %	4.4 %	4.2 %	1.4 %	2.6 %	1.5 %
Cloxacillin	4.5 %	4.4 %	3.7 %	2.5 %	1.8 %	1.4 %
Ofloxacin	2.9 %	1.9 %	2.6 %	1.5 %	1.7 %	1.8 %
Ciprofloxacin	2.2 %	2.3 %	0.9 %	3.1 %	1.0 %	1.1 %
Sarafloxacin	2.4 %	2.8 %	2.0 %	1.5 %	2.0 %	0.7 %
Tetracycline	6.1 %	5.9 %	6.1 %	4.7 %	2.8 %	1.3 %
Chlortetracycline	5.6 %	5.0 %	5.7 %	3.6 %	4.1 %	2.1 %

Table 6. Average and RSD of recovery and matrix effect for different antibiotics from milk extract with different extraction solvents (acetonitrile and methanol) during protein precipitation step of extraction process.

Compound	Fortified Level (ng/ml)	Recovery with Methanol	Recovery RSD with Methanol N=5	Matrix Effect with Methanol	Recovery with Acetonitrile	Recovery RSD with Acetonitrile N=5	Matrix Effect with Acetonitrile
Sulfamethazine	10	102 %	8 %	110 %	101 %	9 %	102 %
Sulfamethoxazole	10	103 %	10 %	93 %	105 %	13 %	97 %
Sulfadimethoxine	10	96 %	7 %	111 %	104 %	7 %	106 %
Tilmicosin	50	89 %	10 %	127 %	82 %	15 %	103 %
Tylosin	50	97 %	14 %	83 %	92 %	17 %	97 %
Erythromycin	50	98 %	11 %	80 %	27 %	17 %	91 %
Penicillin-G	10	103 %	10 %	94 %	84 %	10 %	96 %
Cloxacillin	10	94 %	13 %	97 %	96 %	13 %	102 %
Ofloxacin	10	101 %	17 %	163 %	97 %	1.7 %	181 %
Ciprofloxacin	10	102 %	16 %	174 %	87 %	1.0 %	163 %
Sarafloxacin	10	104 %	11 %	162 %	96 %	9 %	142 %
Tetracycline	50	32 %	5 %	152 %	24 %	5 %	120 %
Chlortetracycline	50	25 %	8 %	177 %	21 %	10 %	130 %

Table 7. Average of 2 ion ratios and their ranges for antibiotic residues at different concentration levels in milk extract.

| Compound	Ion Ratio 1	Relative Ion Ratio 1 Difference	Ion Ratio 2	Relative Ion Ratio 2 Difference
Sulfamethazine	57 %	± 5 %	43 %	± 6 %
Sulfamethoxazole	99 %	± 5 %	90 %	± 6 %
Sulfadimethoxine	38 %	± 6 %	29 %	± 5 %
Tilmicosin	76 %	± 5 %	64 %	± 5 %
Tylosin	44 %	± 6 %	26 %	± 11 %
Erythromycin	46 %	± 5 %	24 %	± 6 %
Penicillin-G	78 %	± 5 %	50 %	± 10 %
Cloxacillin	43 %	± 5 %	38 %	± 6 %
Ofloxacin	69 %	± 9 %	62 %	± 7 %
Ciprofloxacin	43 %	± 5 %	2.5 %	± 12 %
Sarafloxacin	13 %	± 7 %	8.1 %	± 7 %
Tetracycline	23 %	± 6 %	21 %	± 10 %
Chlortetracycline	68 %	± 6 %	37 %	± 6 %
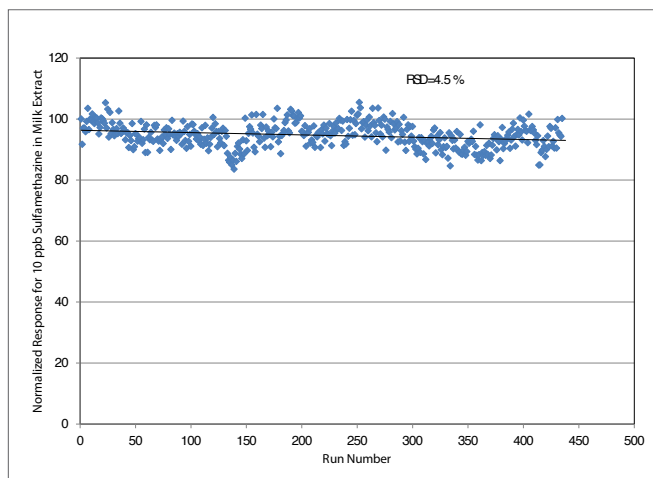
Figure 3. Long term stability data over six days for injections of milk spiked with 10 ng/ml of sulfamethazine.

Conclusions

This study demonstrates rapid, rugged and reliable LC/MS/MS method with sufficient sensitivity and selectivity for analysis of different antibiotic classes in milk using a modified QuEChERS sample preparation method. The sample preparation method was simple and showed good recoveries for most of the antibiotic classes. The method allowed identification and quantification of target compounds in low ppb range (0.1 to 1 ppb) in milk with good precision and retention time stability. Long term stability data demonstrated that a LC/MS/MS system can be used for analysis without any maintenance downtime for running samples with dirty matrices.

References

1. US FDA/CFSAN Tolerance and /or Safe Levels of Animal Drug Residues in Milk; memo dated Sep 27, 2005.

2. S. B. Clark, J. M. Storey, S. B. Turnipseed, FDA Laboratory Information Bulletin, Lib # 4443, http://www.fda.gov/downloads/ScienceResearch/FieldScience/UCM239311.pdf.

3. L. Geis-Asteggiante, S. J. Lehotay, A. R.Lightfield, T. Dutko, C. Ng, L. Bluhm, J. Chrom. A, 2012, 1258, 43.

4. T. Yamaguchi, M. Okihashi, K. Harada, K. Uchida, Y. Konishi, K. Kajimura, K. Hirata, Y. Yamamato, J. Agri. Food Chem., 2015, 63, 5133.

5. European Commission Guidance Document on Analytical Quality Control and Validation Procedures for Residues Analysis in food and feed, SANCO12571(2013), http://ec.europa.eu/food/plant/pesticides/guidance_documents/docs/qualcontrol_en.pdf

For a complete listing of our global offices, visit www.perkinelmer.com/ContactUs