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Application Note

No. LC-14-ADI-035

LCMS-8040

Quantitative analysis of multi-class antibiotic residues in milk using LC/MS/MS

❑ Introduction

Antimicrobial drugs are widely used for treatment and prevention of diseases in dairy cattle. Residues of these drugs may, therefore, be present in milk and can be a health hazard to consumers. They may cause allergic reactions in sensitive persons and can increase risk of developing antibiotic resistant pathogenic bacteria. Hence, monitoring of antimicrobial residues in commercial milk is essential for ensuring the safety and adequacy of food.
Antibiotics belonging to different classes such as β-lactams, Sulfonamides, Macrolides etc. are generally administered to cattle to treat various bacterial infections. It is, therefore, desirable to develop a single analytical method for simultaneous determination of multi-class antibiotics. High specificity and sensitivity offered by LC/MS/MS technique makes it a preferred method of choice for multi-component analysis from complex matrices[1].
Multi-class antibiotic analysis using LC/MS/MS requires a system capable of acquiring data in both positive and negative polarities simultaneously due to different ionization tendencies of analytes. Multiple antibiotics belonging to the same class may fragment to give same product ions during MRM analysis. Hence, it is essential to have minimum crosstalk. LCMS-8040 with ultrafast polarity switching (15 msec), UFsweeper^{TM} II technology (ensuring minimum cross talk), lowest dwell time and pause time (0.8 and 1 msec respectively) is, therefore, well suited for multi-class antibiotic analysis.

❑ Experimental

Preparation of matrix matched standards

2 mL of raw milk sample was mixed with 8 mL of acetonitrile using ultra sonicator for 5 mins. Mixture was centrifuged and 8 mL of the supernatant was collected. This supernatant was evaporated to dryness using low volume nitrogen evaporator. Dried residue was then reconstituted in 8 mL of water. The reconstituted solution was filtered through 0.22 µ syringe filter. This filtered solution was then used as a diluent to prepare antibiotics matrix matched mix standards at concentration levels of 0.5 ppb, 1 ppb, 2 ppb, 4 ppb, 5 ppb, 8 ppb, 10 ppb and 20 ppb.
Note: Matrix effect is a phenomenon seen in Electro Spray Ionization (ESI) LC/MS/MS analysis that impacts the data quality, especially when matrix is complex like food items. Milk is one such matrix that can exhibit matrix effect (either ion suppression or enhancement). A calibration curve based on matrix matched standards can demonstrate true sensitivity of the analyte in presence of matrix. Therefore, this approach was used to obtain more reliable and accurate data as compared to quantitation against neat (solvent) standards[2].

Analytical conditions

Matrix matched antibiotics were analyzed using Ultra High Performance Liquid Chromatography (UHPLC) Nexera coupled with LCMS-8040 triple quadrupole system (Shimadzu Corporation, Japan). The details of analytical conditions are given below:

Nexera parameters	
Column	Shim-pack XR-ODS (50 mm L x 3 mm I.D.; 2.2 µ)
Mobile phase	A: water B: 0.1 % formic acid in acetonitrile
Flow rate	0.3 mL / min
Oven temp.	40 ˚C
Injection volume	15 µl

Gradient time program		
Time (min)	A conc. (%)	B conc. (%)
0.50	90	10
2.00	60	40
2.70	0	100
3.50	0	100
3.51	90	10
6.00	Stop	

LCMS-8040 parameters	
Interface	ESI
Polarity	positive and negative
Nebulizing gas flow	2 L / min
Drying gas flow	10 L / min
Desolvation line temp.	250 ˚C
Heat block temp.	350 ˚C

❑ Results and discussion

LC/MS/MS method was developed for analysis of ten antibiotics belonging to different classes like β-lactams, Sulfonamides, Tetracyclines, Macrolides etc. in a single run. LOQ was determined for each antibiotic based on following criteria – **A**. % RSD for area < 16% (n=6), **B**. % accuracy between 80-120 % and **C**. Signal to noise ratio (S/N) > 10. Linearity and LOQ results for each antibiotic has been summarized in Table 1. Representative MRM chromatograms of blank matrix and matrix matched antibiotics at 1 ppb level are shown in Figures 1 and 2 respectively. MRM chromatograms of all antibiotics at LOQ levels and calibration graphs have been shown in Figure 3.

Table 1: Results of multi-class antibiotics analysis

Compound name	**Antibiotic class**	**MRM transition**	**Retention time (min)**	**Calibration range (ppb)**	**Correlation coefficient (r^2)**
Amoxicillin	β-lactam	366.10>134.20	1.17	1 - 20	0.9982
Trimethoprim	Dihydrofolate reductase inhibitor	290.50>261.00	2.09	0.5 - 20	0.9930
Ampicillin	β-lactam	350.10>106.10	2.20	1 - 20	0.9970
Oxytetracycline	Tetracycline	461.00>426.10	2.50	1 - 20	0.9993
Tetracycline	Tetracycline	445.00>154.10	2.55	1 - 20	0.9964
Sulfadiazine	Sulfonamide	250.60>156.00	2.58	0.5 - 20	0.9934
Chlortetracycline	Tetracycline	479.20>444.20	2.86	1 - 10	0.9990
Tylosin	Macrolide	916.20>174.20	3.14	1 - 20	0.9926
Sulfadoxine	Sulfonamide	310.80>156.00	3.35	0.5 - 20	0.9984
Chloramphenicol	Amphenicol	321.20>152.20	3.38	1 - 20	0.9973

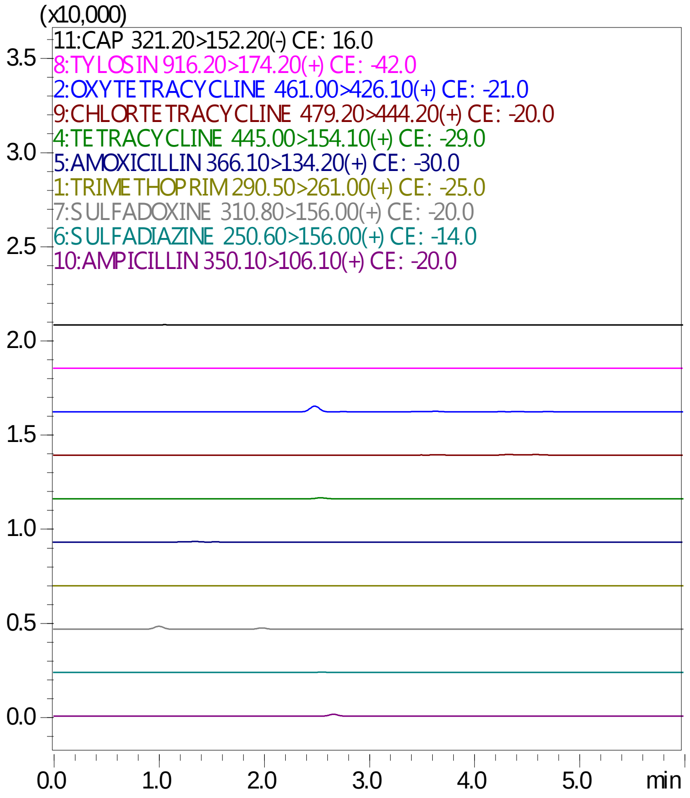

Figure 1: MRM chromatogram of blank matrix

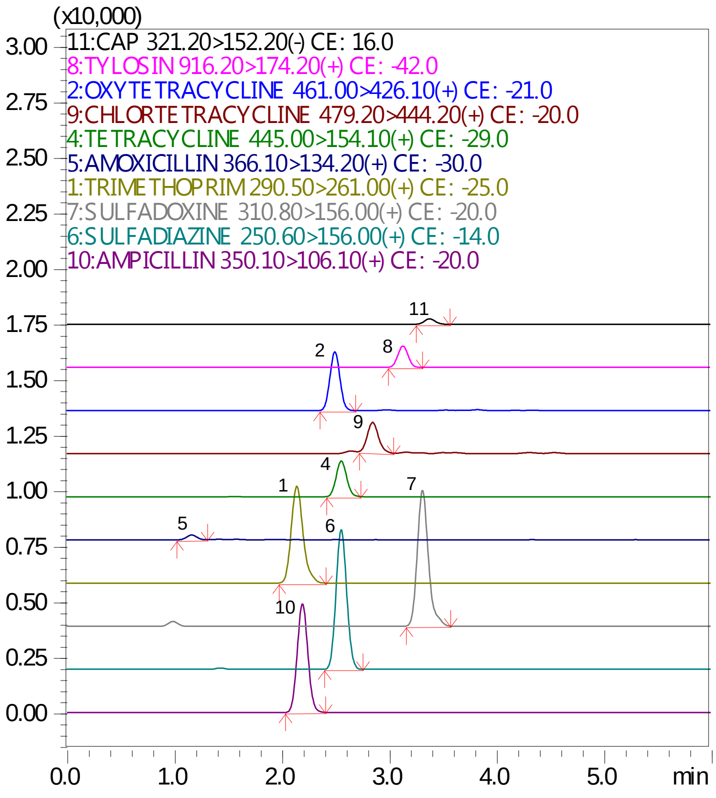

Figure 2: MRM chromatogram of antibiotics at 1 ppb level

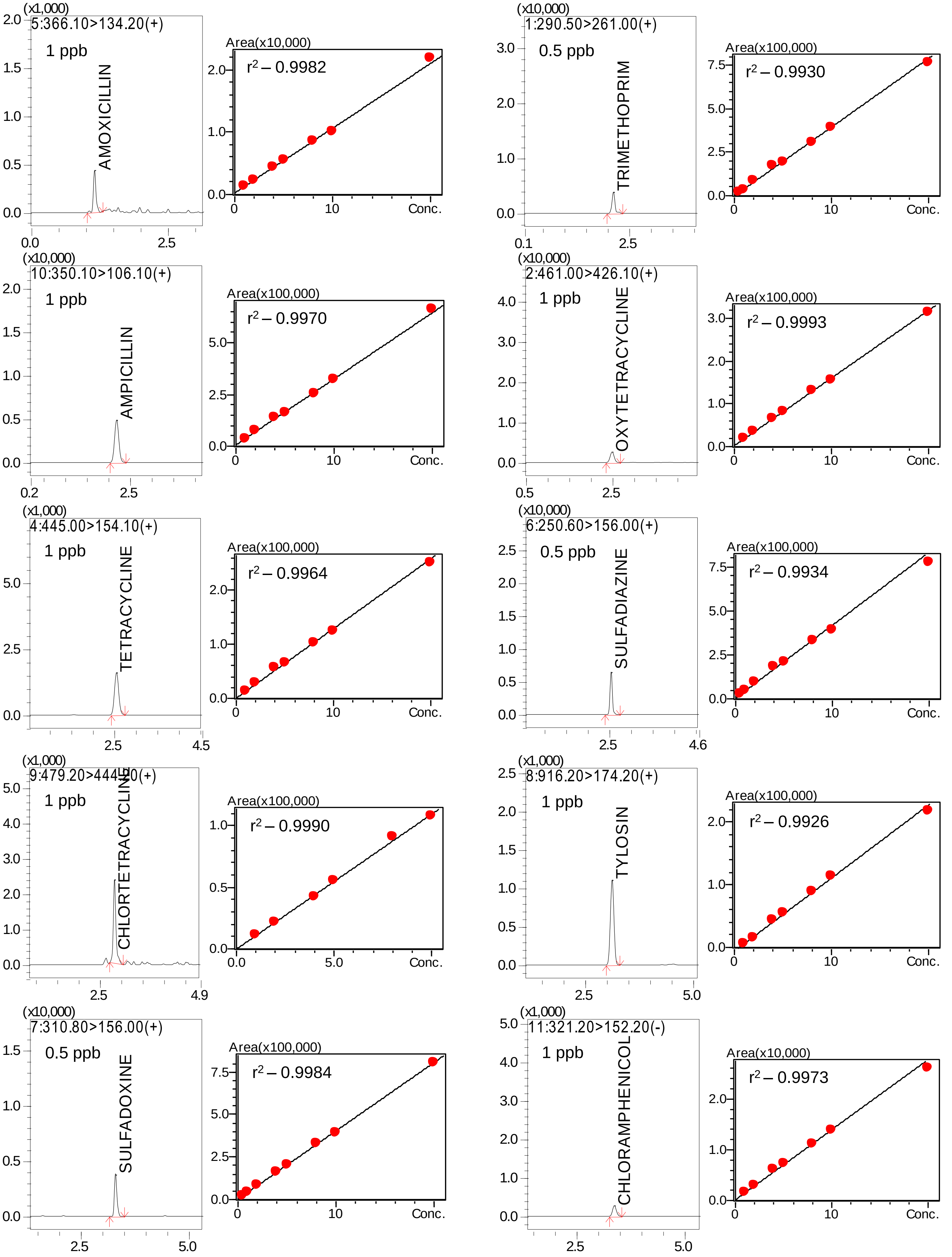

Figure 3: MRM chromatograms at LOQ levels and calibration graphs of antibiotics

❑ Conclusion

Quality checking of the milk sample becomes tedious when antibiotics belonging to different classes have to be analyzed. This often requires multiple LC/MS/MS methods. This task has been simplified here, by developing a single method for simultaneous analysis of ten antibiotics belonging to six different classes.

Ultra-high sensitivity, ultra fast polarity switching (UFswitching), low pause time and dwell time along with UFsweeper^{TM} II technology of LCMS-8040 system has enabled sensitive and selective multi-class antibiotic analysis from matrix like milk.

❑ References

1. Helio A. Martins-Junior, Tereza A. Kussumi et al., A rapid method to determine antibiotic residues in milk using liquid chromatography coupled to electrospray tandem mass spectrometry, J.Braz.Chem.Soc., Volume 18, No. 2 (2007), 1781–1788.
2. Kwon H, Lehotay SJ, Geis-Asteggiante L., Variability of matrix effects in liquid and gas chromatography-mass spectrometry analysis of pesticide residues after QuEChERS sample preparation of different food crops, J. Chromatogr A, Volume 1270, (2012), 235–245.

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