Quantification of multiple antibiotics in milk using the SCIEX Triple Quad™ 3500 LC-MS/MS System

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The nutritional well-being of the livestock is often maintained by the administration of antibiotics. However, the usage of these antibiotics is a major threat to human health. Over-usage creates the risk of creating resistant microorganisms. Individuals can be allergic to certain antibiotics. Additionally, the presence of these antibiotics in milk can affect the manufacturing of products like cheese, yogurt, etc. by inhibiting the starter culture.[1]

Each country had set a permissible limit of antibiotic that can be present in their food products based on human studies to determine tolerance limits. The permissible limit set for an antibiotic is typically in microgram per kg, which is difficult to quantify accurately using traditional methods like HPLC or microbial techniques. For the dairy industry, a simple assay that provides the accurate determination of various antibiotics at low levels in milk plays a vital role in helping them ensure the safety of their product and the health of their consumers.[2,3]

Here, a method was developed using SCIEX Triple Quad 3500 System, to determine residual antibiotics in milk. Local market milk samples were analyzed to check the presence of albendazole, fenbendazole, tylosin and tilmicosin and some metabolites. Sufficient sensitivity was provided with this assay to meet the MRL levels for these analytes and good assay performance was observed.

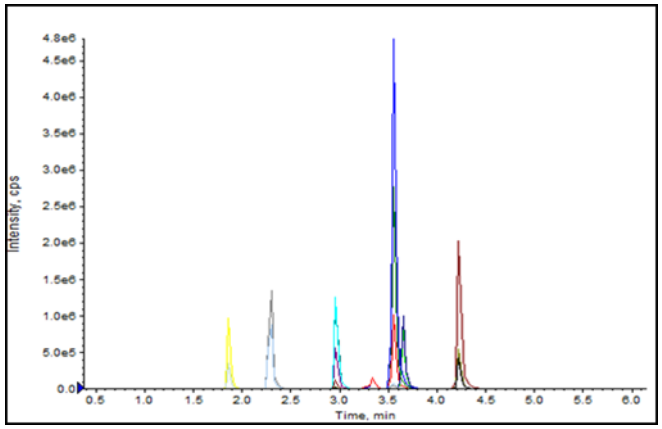


Figure 1: Chromatographic separation of antibiotics. LC separation is shown for albendazole, fenbendazole (and its metabolites), tilmicosin, tylosin at 100 ppb.

Key features for the multi-antibiotic assay in milk

- A quantitative method for the analysis of multiple veterinary drugs in milk was developed, namely albendazole, fenbendazole and its metabolites (albendazole sulfone, albendazole sulfoxide, albendazole-2-amino sulfone, fenbendazole sulfone) tylosin and tilmicosin
- Using the SCIEX Triple Quad 3500 System, sufficient sensitivity was provided to meet the regulatory requirements
- A linear dynamic range of 2.5-300 ppb was analyzed and excellent quantitative performance was achieved
- The robustness of the Turbo V™ Ion Source with the Curtain Gas™ Interface ensures high uptime and maximum productivity
- The method development was performed and partially validated per the regulatory guidelines described in 2002/657/EC directive recommendations

Methods

Chemicals: Standard albendazole, fenbendazole and their metabolites (albendazole sulfone, albendazole sulfoxide, albendazole-2-amino sulfone, and fenbendazole sulfone), tylosin and tilmicosin were obtained from collaborator. All other chemicals used were of LC-MS grade and commercially available. Milk samples were purchased from the local market of Delhi, and Gurgaon and stored in refrigerator at 2 to 8 ºC until the analysis was completed.

Sample preparation: A generalized extraction procedure was performed in which 1 mL of milk was mixed with 5 mL of water and vortexed. 10 mL of acetonitrile with 0.1% formic acid was added and vortexed for 10 min. Then, 2 g of NaCl was added to the solution, the solution was mixed well and centrifuged at 2598 g (4000 rpm) for 5 min. After centrifugation, the supernatant was collected and evaporated to dryness. The sample were reconstituted with 1 mL of acetonitrile/water (20:80, V/V) with 0.1% formic acid.

LC conditions: LC separation was performed on an ExionLC™ System using a Phenomenex Luna C18(2) (4.6×150 mm, 5.0 µm) column. The injection volume of the method was 10 µL. See Table 1 for gradient information.

Table 1. Gradient profile and mobile phase composition.

Total Time (min)	Flow Rate (µL/min)	A%	B%
0.0	*600*	*90*	*10*
7.0	*600*	*10*	*90*
8.5	*600*	*10*	*90*
8.6	*600*	*90*	*10*
10.0	*600*	*90*	*10*

Mobile phase A: water + 0.1% formic acid
Mobile phase B: acetonitrile + 0.1% formic acid

MS/MS conditions: The SCIEX Triple Quad 3500 LC-MS/MS System was operated in positive ion mode, using the Turbo V™ Ion Source with an electrospray ionization (ESI) probe. Data were collected using multiple reaction monitoring (MRM) with two transitions per analyte using Analyst® Software 1.6.

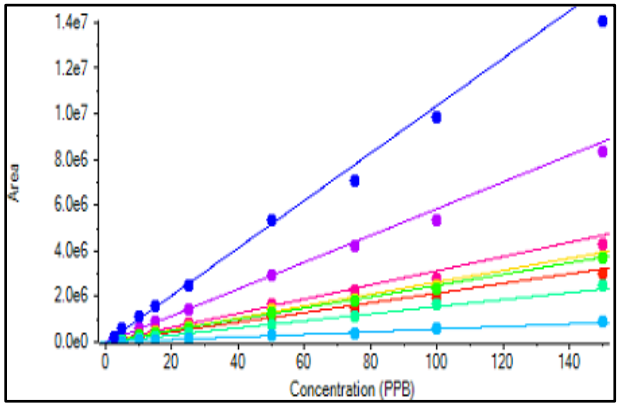
Data processing: LC-MS/MS data were processed using the Multiquant™ Software 3.0.2.

Results

The SCIEX Triple Quad 3500 System showed very good sensitivity for multi-residue antibiotic analysis in milk. The experimental data were acquired and partially validated in accordance with 2002/657/EC directive recommendations. The antibiotic mix was prepared and spiked at 5% in the milk matrix to determine the accuracy, precision, and reproducibility. The linearity in matrix was assessed from the range of 2.50 to 300 ppb for albendazole, fenbendazole and its metabolites (albendazole sulfone, albendazole sulfoxide, albendazole-2-amino sulfone, fenbendazole sulfone) tylosin, tilmicosin, trimethoprim, and tilmicosin (Figures 2-4). For all the analytes a regression coefficient was found to be above 0.99, where the weighing factor used was $1/x^2$.

Table 2. MRM transitions of antibiotics.

Compound	Precursor Ion	Product Ion (Quantifier)	Product Ion (Qualifier)
Albendazole	*266.0*	*234.3*	*191.1*
Albendazole Sulfone	*298.0*	*266.0*	*159.1*
Albendazole Sulfoxide	*282.0*	*240.0*	*208.1*
Albendazole-2-Amino Sulfone	*240.0*	*133.2*	*197.9*
Fenbendazole	*300.0*	*268.0*	*158.7*
Fenbendazole Sulfone	*332.0*	*159.0*	*300.1*
Tilmicosin	*869.8*	*174.2*	*696.7*
Tylosin	*916.7*	*174.1*	*772.4*



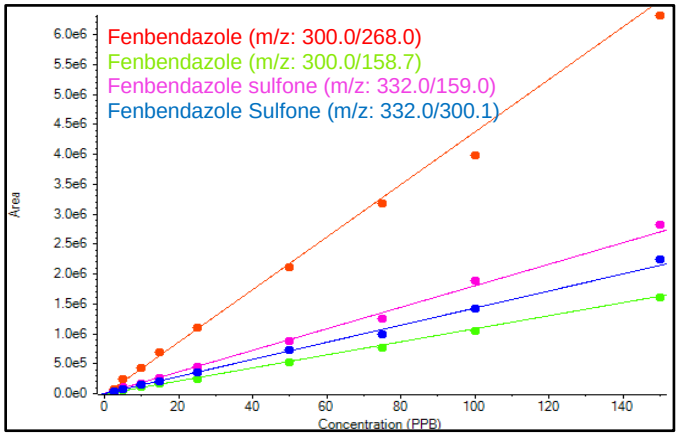
Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV
ALBENDAZOLE_01	0.05	6 of 6	1.699e4	1.308e3	7.70

Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV
ALBENDAZOLE SULFONE_01	0.10	6 of 6	3.900e3	2.544e2	6.52

Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV
ALBENDAZOLE SULFOXIDE_01	0.30	6 of 6	7.652e3	6.072e2	7.93

Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV
ALBENDAZOLE 2 AMINO SULFONE_01	1.00	6 of 6	1.025e4	7.313e2	7.13

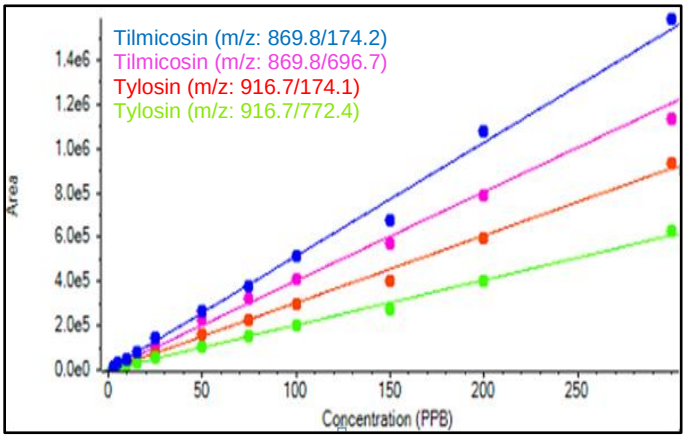
Figure 2: Calibration curve of albendazole and its metabolites. Good linearity was observed for these analytes from 2.5-150 ppb. Albendazole (m/z: 266.0/234.3), Albendazole (m/z: 266.0/191.1), Albendazole sulfone (m/z: 298.0/266.0), Albendazole sulfone (m/z: 298.0/159.1.0), Albendazole Sulfoxide (m/z 282.0/240.0), Albendazole Sulfoxide (m/z 282.0/208.1), Albendazole-2-amilno sulfone (m/z:240.0/133.2),Albendazole-2-amilno sulfone (m/z:240.0/197.9). Good reproducibility was observed at the lowest concentration of each for 6 replicate injections.



Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV
FENBENDAZOLE_01	0.05	6 of 6	1.030e4	9.120e2	8.85

Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV
FENBENDAZOLE SULFONE_01	0.10	6 of 6	2.019e3	1.463e2	7.25

Figure 3: Calibration curve of fenbendazole and its metabolites. Good linearity was observed for these analytes from 2.5-300 ppb. Good reproducibility was observed at the lowest concentration of each for 6 replicate injections.



Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV
TILMICOSIN_01	0.50	6 of 6	1.461e3	1.182e2	8.09

Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV
TYLOSIN_01	0.50	6 of 6	1.903e3	1.667e2	8.76

Figure 4: Calibration curve of tilmicosin and tylosin. Good linearity was observed for these analytes from 2.5-300 ppb. Good reproducibility was observed at the lowest concentration of each for 6 replicate injections.

The accuracy for each analyte across the concentration curve was 90-110% , which is in compliance with EU guidelines and the repeatability %CV were found to be less than 10% (n=6), at the respective MRL level of each analyte. Two MRM transitions were used for each analyte as quantifier and qualifier ions, to allow the determination of the ion ratio for analyte. MultiQuant Software was used for the data processing, for automatic calculation of statistics of the calibrations curves as well as the MRM ratios for the analytes.

Table 3. %CV at LOD level in aqueous standard (AQS) and MRL level in extracted samples.

Compound	LOD in AQS (ppb)	%CV at LOD (n=6)	MRL Value (ppb)	%CV at MRL (n=6)
Albendazole	*0.05*	*7.70*	*100*	*3.38*
Albendazole Sulfone	*0.10*	*6.52*	*100*	*5.37*
Albendazole Sulfoxide	*0.30*	*7.93*	*100*	*5.69*
Albendazole-2-Amino Sulfone	*1.00*	*7.13*	*100*	*5.94*
Fenbendazole	*0.05*	*8.85*	*10*	*4.94*
Fenbendazole Sulfone	*0.10*	*7.25*	*10*	*4.73*
Tilmicosin	*0.50*	*8.09*	*50*	*5.75*
Tylosin	*0.50*	*8.76*	*25*	*4.49*

Analysis of milk samples

Milk samples collected from local markets of Delhi and Gurgaon, India, were tested for the presence of multi-residue analyte. Albendazole, fenbendazole and its metabolites, tilmicosin and tylosin were absent in all the tested samples.

Conclusions

The method presented here on the SCIEX Triple Quad 3500 System shows a fast and accurate solution for the quantification of 8 antibiotics in milk samples using simple sample preparation combined with LC-MS/MS. The method developed as per 2002/657/EC directive recommendations showed acceptable accuracies (80%-120%) for analysis in matrix samples, linearity with r ≥ 0.99 for both the MRM transitions, and %CV for repeatability was ≤10% at the LLOD levels. No significant matrix interferences were observed. Automatic MRM ratio calculation provides quick confirmation of each analyte for increased assay confidence.

References

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