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# Method Optimization and Validation of Antibiotics Residues in Milk Sample Using LC-MS/MS

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**Abstract**

Veterinary drugs, that are utilized to treat diseases in animals, are broad-spectrum antibacterial antibiotics. Numerous nations have previously prohibited the utilization of veterinary medications due to the formation of residues for example nitrofurans, chloramphenicol etc. Veterinary drugs have already been supplied to animals to enhance milk production for increased profit and economic incentives. The rising utilization of illegal medicines in animal milk production is largely ineffective for consumers. It affects food products derived from animals when consumers purposefully consume the medication since it leaves behind many antimicrobial residues. It causes serious health problems in milk consumers, including allergic disorders as well as cancer. This necessitates a limitation on illegal consumption of drugs for veterinary purposes; the Indian government needs to put restrictions on this drug utilization in animals. It is possible to find and confirm the presence of different veterinary medication residues in milk; dairy products can be examined with liquid chromatography and mass spectrometry, among other analytical methods. These are all advanced devices, and the requisite equipment is now readily accessible. According to the European Commission, 2021/808/EC should govern the validation of analytical methods. The application of veterinary drugs consistently presents significant challenges regarding efficiency, prolonged usage, and authorization. Various analytical procedures can be efficiently employed to protect consumer health in a short time; multiple compound groups, including tetracyclines, macrolides, sulphonamides etc. are analyzed utilizing a single multi-residual technique. Each of these techniques is employed within predetermined validation parameters, which consist of precision, quantification, accuracy, detection limit, along with calibration curve. This current research aims to develop a multi-residual fast test for milk samples in a reduced timeframe.

**Keywords**: Veterinary Drugs Residues; LC-MS/MS; Method Validation; Method development; Antibacterial.

## Introduction

Numerous antimicrobial agents are extensively regarded in the management of dairy cattle. Disease treatment is the primary focus of cattle management, particularly in the application of antimicrobial agents [1]. Improper management by farmers and veterinarians, particularly in failing to monitor the welfare of treated animals and adhere to prescribed withdrawal periods, can lead to significant consequences. This includes the existence of antimicrobial residues in milk along with processed foods, which support the spread of bacterial resistance and microbial effects on medicine resistance, leading to serious health issues [2]. Veterinary drugs have been mostly utilized to suppress proliferation of bacteria within the animal body and are extensively employed to treat ailments in animals[23]. Research on drug residues and antimicrobials in animal-derived food items commenced in the late 1960s as well as early 1970s, typically in European countries for example Belgium, Luxembourg, along with Netherlands [24]. The causes include the potential for drugs and antimicrobials to leave residues in dairy products, unlawful use of unlicensed drugs and antibiotics, excessive dosages beyond label recommendations, inadequate monitoring during withdrawal periods, significant contamination of animal feed, and treatment of animals with antimicrobials. Meat and milk from animals raised for food are also impacted by antibiotic residues from prohibited sources, which is an unlawful activity [3]. Currently, dairy products, particularly goat milk, exhibit distinctive features,

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notably in their amino acid and milk protein ratios, as well as their digestibility. The detrimental medicinal effects of veterinarians can adversely impact human health. Understanding the role of surveillance in the control of antibiotic residues is essential [4]. The EU has already implemented monitoring of veterinary drug usage and the management of residues at the borders. This is accomplished by following the guidelines in "council Directive 96/23/EC." The "97/747/EC" commission ruling also contributed to that necessity. Since 2000, Croatia has implemented a residue monitoring program with a specific focus on animal products along with living animals. Investigations are conducted on the distribution of the sample among all monitoring groups that are below observation for the elements under analysis [5]. The Council directive "96/23/EC" requires the testing as well as analysis of antimicrobial compounds in the samples of milk obtained from various species of animals. According to the comprehensive surveillance along with annual monitoring plans, State Veterinary Inspection groups carry the primary responsibility, must perform annual administrative controls for the examination of animal products and the veterinary drug application procedure [6].

The HPLC approach is occasionally employed to quantify drug residues; however, its efficiency is limited [15]. This quantification methodology possesses certain limitations [8]. The method of collecting instruments and various compound groups simultaneously is time-consuming and not very straightforward. It is essential to employ distinct quantification methods for various groups, and the more advanced LC-MS/MS conditions are presently regarded as optimal [7].

This procedure is less time-consuming, and quantifying specific molecules is significantly easier than employing HPLC. The examination of veterinary medication residues including many antibiotic groups in a singular multi-residue procedure [9]. The primary benefit is the capacity to do both quantitative as well as confirmatory analysis at extremely low levels, and this is done under LC-MS/MS settings [10]. One of the most important parts of the fight against antimicrobial resistance is the detection and tracking of antibiotic use. The detection range of antibiotic residue testing using current LC-MS/MS techniques is constrained.[24].The general perception that these medications have been abused or misused in animals and that this misuse has resulted in residues in derived foods, like milk and dairy products, has raised concerns in recent decades.[25].

Even though milk and other dairy goods are nutritious and healthful, they may contain potentially dangerous drug

residues. Milk is consumed all over the world and is important both economically and nutritionally. Polluted feed

and water, inappropriate veterinary medication use, careless milk extraction, or faulty milk collection and processing are some of the ways that residues get into the milk. [26]. They significantly boost allergic reactions and antimicrobial resistance against several dangerous pathogens. [27].

*Aim and objectives*

This analysis was examined at a Mass Spectrometry Chromatography. The objective is to employ liquid chromatography Mass Spectrometry to quickly test, optimize, as well as validate the method for identifying veterinary medicine residues in milk products. These are the objectives of this study:

- Optimization "of extractions procedure of antibiotic residues in milk.
- Optimization of Compound and source dependent parameters.
- Method development and validation of antibiotic residues in milk using liquid chromatography mass-spectrometry as per regulatory 2021/808/EC.

The technique entails a simple extraction" procedure as well as standard laboratory evaluations for analysing a substantial volume of samples regularly, accommodating various compound categories.

## Methods

*Requirement, Chemical Reagents*

The commercial guidelines for the antibiotics remain upheld, including Acetic Acid Merck, Acetonitrile at Rankem/JT Baker Milli-Q water at Merck/JT Baker, Methanol JT Baker, Magnesium Sulphate Labroratory grade, LC-MS Grade Ammonium Formate, Formic Acid at MS Grade, and C18, along with Sodium Chloride Laboratory Grade [11]. Additional Antitiotics analyte names that are proposed for the groups for veterinary drugs as well as testing range encompass Meloxicam (10 µg/kg, 1.0- 20), Flunixin (10µg/kg, testing range: 1-20), Lincomycin (150µg/kg, testing range: 15-300), Monensin (1µg/kg, testing range: 0.25-4), Trimethoprim (10µg/kg, 1.0-20), Tylosin (100µg/kg, 10 to 200 µg/kg), Virginiamycin (10µg/kg, 1.0-20 ), as well as Diminazene (150 µg/kg, 15-300). All of these are HPC standards-based certified reference products; the only exceptions are Virginomycin from

Sigma-Aldrich and Trimethoprim and Tylosin from Pherma A2S [12].

Standard stock solutions have also been utilized for particular substances at concentration levels ranging from 200 to 300mg/L. These were created by precisely weighing 100ml of methanol as well as the powder. In 100ml of HPLC-grade methanol, the powder was solubilized, primarily from Sigma [12]. The individual component was utilized by preparing appropriately diluted stock solutions maintained in methanol within screw-capped glass tubes at -20℃. Through Milli-Q gradient water method, the ultrapure water was obtained [16].

*Mobile Phase Preparation & cleaup details:*

Within the secure lock reaction vessels, a DSPE cleanup procedure weighing approximately 50mg and 150mg of magnesium sulfate has been executed. Subsequently, 1 percent acetic acid was prepared in an acetonitrile (v/v) phase by adding 1ml of glacial acetic acid (concentrated acetic acid) to a 100ml volumetric flask. A fill line was marked with a correctly mixed acetonitrile solution. The mobile phase A consists of 5mM ammonium formats as well as 0.1 percent formic acid in the water, produced by dissolving 0.3153g of ammonium formats and added 1ml of Formic Acid in 100% HPLC Grade Water. This was sonicated also well blended. The subsequent component had been mobile phase B, containing 0.1percent formic acid in the acetonitrile. In 1000ml of acetonitrile mixture, 1ml of formic acid was dissolved followed by thoroughly sonicated.

*Referances Stock Solution Preparation*

Initially, a standard stock solution approach was employed, which included standard procurement at specified temperatures and from reliable, certified sources. ~10mg of standard is weighed, then into a 10ml volumetric flask that was transferred. A 10ml suitable solvent was utilized to dissolve the standard. And compute the concentration utilizing the formula "below.

$$\text{Stock solution conc. (mg/L)} = \frac{\text{Standard weight in (mg)} \times \text{Purity} \times 1000}{\text{Make up volume (ml)} \times 100}$$

Subsequently", a 10mL volumetric flask was filled with 100µL of individual 1000µg/ml stock standard (the volumes might differ depending on the stock standards' concentrations). Acetonitrile was then added to the flask, and the mixture was labelled as a 10µg/ml working standard solution. Subsequently, it was shifted into a 10mL volumetric flask together with 1mL of a standard containing 10µg/ml and labelled as 1000ng/ml [13]. The production phase for the calibration curve standard then started, and tables with dilutions (Table 1) utilizing a solvent with a working solution mixture of 1000ng/ml as well as 100ng/ml solutions were included. Finally, employing 2.0g matrix blanks, the matrix preparation complied with the calibration curve standards.

The sample extraction technique described above was executed by the sample processing.

*Instrument Details*

The UPLC system was the apparatus that had been employed for this chromatographic analysis. Waters, Milford, USA, along with MA are all included in that system. This is Nexon HPLC with ODS column ((“100mm*2.1mm, 1.7ɥ particular size”). The primary apparatus includes analytical balance (Simdazu AUR, range 0.00001 to 220g), micropipettes (MICROLIT, 10-100µL, 10-200µL, 100-1000µL), LC-MS/MS (AB SCIEX-4500), and centrifuge (NEYA 16 R (2000 to 10000RPM)). Chromatography has been conducted in accordance with Table 2:

*LC-MS/MS conditions*

Exion LC standard for HPLC, 40 ºC column oven temperature, 15 ºC autosampler temperature, and mass spectrometry utilizing the AB SCIEX TRIPLE QUAD-4500 have all been considered when optimizing parameters in LC-MS/MS conditions. Both positive and negative ion modes have been assessed at ESI for ion sources. The mobile phase comprised Eluent A– 5mM ammonium formate in water with 0.1% formic acid, and Eluent B– 0.1 % Formic Acid in ACN. Utilizing a diluent composed of 0.1 % of Formic Acid in Methanol: water (80:20), the rate of the flow had been set at 0.500mL/min. A 4µL injection volume, MRM scan type, and a 14-minute run period were employed. With the exception of ivermectin and abamectin, ions are continuously plentiful in all cases; also, these ions have been important forerunners in understanding among the greatest sensitive transitions for both confirmation as well as quantification purposes. In several published works, the application of the APCI use probe in negative mode for anthelmintics level determination is also discussed. In MS/MS circumstances, positive ESI mode can also yield good sensitivity.

*HPLC Gradient Details*

In this work, chromatographic separation is critical to achieving optimal analyte retention and separation. First, a number of mobile preparation tests using methanol were conducted at varying concentrations in acetic acid.

Chromatographic separation has been developed utilizing the following HPLC programming:

*Optimization of Sample Extraction Procedures*

The test milk sample weighs 5.0±0g after it has been thoroughly homogenized, and it is put into a Poly Propaline Tarson Tube (50mL). Subsequently, added 5mL of 0.5 % Glacial Acetic Acid in ACN

was poured. The tarson tube was closed as well as vortexed for five minutes. To a multitube vortex travelling, it was attributed at a high speed. The tube was then sealed after 0.5g of MgSO4 and 0.5g of NaCl were added. After that, it was quickly given a vigorous one-minute shake and centrifuged for five minutes at 4200 rpm. Magnesium sulfate tends to cluster together when wet and can solidify quickly. If the salt mixture is added and then agitated violently right away, this can be prevented.

A dSPE cleanup salt mixture was placed in a ria vial with an aliquot of 4ml. A cap was placed on the tube, and it was vigorously shaken for 30seconds before being centrifuged for 5minutes at 10,000rpm. Subsequently, 1mL of supernatant was placed into a ria vial. An evaporative procedure was required for drying under nitrogen at 40ºC. Subsequently, reconstitute with 0.5 mL of a 8:2 (Water: MeoH) solution containing 0.1 percent formic acid, then the mixture was sonicated, vortex, centrifuge, as well as passed through a 0.45µm syringe filter into an 2mL HPLC vial for injection into LC-MS/MS.

## Result and Discussion

This effort aimed to quickly test, optimize, as well as validate the liquid chromatography-mass spectrometry method for detection of antibiotic residues in various products of milk. The primary aim is focused on optimizing the extraction procedures for veterinary drug residues in milk. The parameters reliant on the source, development of the method, and validation of samples from milk liquid chromatography are all included in the validation research, which has been completed in accordance with the mass spectrometry regulations of 2021/808/EC.

### *Instrumental Parameters Optimization*

The formulation of the sample has consistently been a crucial phase in the current investigation because of the complexity of this multiresidue antibiotic methodological rapid test. The selected compounds undergo simultaneous extraction of diverse characteristics. Moreover, the extraction of antibiotics from milk was previously performed utilizing a conventional protein precipitation method involving a mixture of strong acids or an organic solvent, for example, trichloroacetic acid, succeeded by cleanup via solide phase extration system as well as sample enrichment. The buffered QuEChERS technique has streamlined this procedure.

### *Matrix Effect*

The process of ionization in mass spectrometry utilized ESI. The primary problem stemmed from the analytes' enhancements, specifically the signal suppression caused by the matrix effects of the other elements. For this validation investigation, three varied samples of milk (full-cream, semi-skimmed, along with skimmed) were considered in order to assess these matric effects. In pure solvents, various concentration standards were examined within the matrices..

### *Validation*

Numerous stages were taken in the development and validation of the complete process. These procedures include the linearity factor, sensitivity, and certain intraday points. The method's linearity factor was measured utilizing all matrix-matched calibration spiked milk samples and the calibration curve performance (the antibiotics' specified ranges are 5-200µg/kg); When it comes to coefficient determination, in the assayed range a linear response was observed. The coefficient of determination exceeded 0.99 in all instances analyzed. A recovery investigation was performed at 2 specific concentrations (10 & 50µg/kg), utilizing six blank samples of milk enhanced at every level of antibiotic. The results obtained are reported below in Tables 4, 5, and 6. The primary validation processes in this work encompass system precision, selectivity, specificity, precision (repeatability study, RSDr), recovery study, reproducibility, limit of quantification, limit of detection, ruggedness, as well as measurement uncertainty.

### *Conclusion*

Each technique employed in this study holds unique significance according to its application process. A systematic technique has been employed sequentially to analyze antibiotic residues in the milk samples. Alternative approaches, like HPLC, LCMS, as well as ELISA, are indeed valuable procedures for the objective. To investigate antigen-antibody interactions, ELISA is employed; this method is predominantly knit-based as well as semi-quantitative in nature. HPLC has emerged as an important tool for the quantitative assessment of microbial antibiotic presence. This approach has gained use due to its effectiveness with a broader range of unknown samples. The sample and interpretation have been well aligned; additionally, a consistent wavelength standard and pharmaceutical conditions with identical retention time have been adhered to. All of these are applicable in the food industry and various research domains. Effective validation parameters have been employed for recovery, linearity, precision, etc. There have been fifteen validation samples examined in all, and the extraction process was completed in under 2 hours. The ESI technique is widely recognized and appropriate for mid-polar and polar chemicals, including residues of antibiotics and pesticides. For weekly polar as well as non-polar molecules, the APPI is beneficial in terms of the gentle ionization process. The suggested approach, in conjunction with MS/MS, provides enhanced sensitivity and resolution. It can extensively identify unidentified

remnants of veterinary pharmaceuticals. The analyte method has been conducted on three types of samples of milk: skimmed, semi-skimmed, as well as full-cream. The components in this investigation demonstrate a diverse range of physicochemical properties, demonstrating the potential of QuEChERS for the multi-residual extraction of veterinary antibiotics in the milk.

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This study didn't receive any funding support

Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

**TABLE 1. Repeatability of Flunixin-01 (297.0 / 279.0) at MRL Level.**

| Test Details | Details of Sample particular | Time and date of acquisition | Area of std | RTmin. | ug/kg | Observe concentration | Accuracy % | Ion Ratio |
|---|---|---|---|---|---|---|---|---|
| Blank-1 | Matrix | 1/14/2023 6:22:23 PM | NA | NA | NA | NA | NA | NA |
| CC_01 | STD | 1/14/2023 6:38:19 PM | 306527 | 7.38 | 0.50 | 0.500 | 99.94 | 0.023 |
| CC_02 | STD | 1/14/2023 6:54:11 PM | 580288 | 7.38 | 1.00 | 0.930 | 93.42 | 0.030 |
| CC_03 | STD | 1/14/2023 7:10:06 PM | 1390269 | 7.38 | 2.00 | 2.220 | 110.98 | 0.030 |
| CC_04 | STD | 1/14/2023 7:26:10 PM | 3020431 | 7.37 | 5.00 | 4.810 | 96.13 | 0.027 |
| CC_05 | STD | 1/14/2023 7:42:03 PM | 6221420 | 7.38 | 10.00 | 9.890 | 98.86 | 0.033 |
| CC_06 | STD | 1/14/2023 7:57:54 PM | 9406043 | 7.38 | 15.00 | 14.940 | 99.60 | 0.029 |
| CC_07 | STD | 1/14/2023 8:13:48 PM | 12728770 | 7.37 | 20.00 | 20.210 | 101.07 | 0.030 |
| Reagent Blank-1 | Unknown | 1/14/2023 8:29:40 PM | 9019 | 7.33 | NA | 0.030 | NA | NA |
| Reapeatability at 0.1 x MRL-01 | QC-01 | 1/14/2023 9:01:26 PM | 620106 | 7.36 | 1.00 | 1.000 | 99.73 | 0.028 |
| Reapeatability at 0.1 x MRL-02 | QC-02 | 1/14/2023 9:17:22 PM | 655564 | 7.37 | 1.00 | 1.050 | 105.36 | 0.029 |
| Reapeatability at 0.1 x MRL-03 | QC-03 | 1/14/2023 9:33:15 PM | 594733 | 7.37 | 1.00 | 0.960 | 95.71 | 0.029 |
| Reapeatability at 0.1 x MRL-04 | QC-04 | 1/14/2023 9:49:10 PM | 667035 | 7.36 | 1.00 | 1.070 | 107.18 | 0.027 |
| Reapeatability at 0.1 x MRL-05 | QC-05 | 1/14/2023 10:05:03 PM | 548467 | 7.37 | 1.00 | 0.880 | 88.37 | 0.033 |
| Reapeatability at 0.1 x MRL-06 | QC-06 | 1/15/2023 2:19:25 AM | 687261 | 7.37 | 1.00 | 1.100 | 110.39 | 0.032 |
| Matrix Blank-2 | Unknown | 1/14/2023 10:20:58 PM | N/A | N/A | N/A | N/A | N/A | N/A |

| Test Details | Details of Sample particular | Time and date of acquisition | Area of std | RTmin. | ug/kg | Observe concentration | Accuracy % | Ion Ratio |
|---|---|---|---|---|---|---|---|---|
| Reapeatability at 1.0 x MRL-01 | QC-01 | 1/14/2023 10:36:51 PM | 5535708 | 7.37 | 10.00 | 8.800 | 87.98 | 0.031 |
| Reapeatability at 1.0 x MRL-02 | QC-02 | 1/14/2023 10:52:44 PM | 5641190 | 7.37 | 10.00 | 8.970 | 89.66 | 0.030 |
| Reapeatability at 1.0 x MRL-03 | QC-03 | 1/14/2023 11:08:36 PM | 6652352 | 7.36 | 10.00 | 10.570 | 105.70 | 0.024 |
| Reapeatability at 1.0 x MRL-04 | QC-04 | 1/14/2023 11:40:22 PM | 6305267 | 7.38 | 10.00 | 10.020 | 100.19 | 0.028 |
| Reapeatability at 1.0 x MRL-05 | QC-05 | 1/14/2023 11:56:16 PM | 5863731 | 7.37 | 10.00 | 9.320 | 93.19 | 0.033 |
| Reapeatability at 1.0 x MRL-06 | QC-06 | 1/15/2023 2:51:14 AM | 6577694 | 7.37 | 10.00 | 10.450 | 104.52 | 0.029 |
| Matrix Blank-3 | Unknown | 1/15/2023 12:12:09 AM | N/A | N/A | N/A | N/A | N/A | N/A |
| Reapeatability at 1.5 x MRL-01 | QC-01 | 1/15/2023 12:28:03 AM | 11158991 | 7.37 | 15.00 | 17.720 | 118.15 | 0.026 |
| Reapeatability at 1.5 x MRL-02 | QC-02 | 1/15/2023 12:43:56 AM | 10586359 | 7.37 | 15.00 | 16.810 | 112.09 | 0.033 |
| Reapeatability at 1.5 x MRL-03 | QC-03 | 1/15/2023 12:59:51 AM | 10182160 | 7.37 | 15.00 | 16.170 | 107.81 | 0.028 |
| Reapeatability at 1.5 x MRL-04 | QC-04 | 1/15/2023 1:15:46 AM | 10358043 | 7.37 | 15.00 | 16.450 | 109.67 | 0.030 |
| Reapeatability at 1.5 x MRL-05 | QC-05 | 1/15/2023 1:31:40 AM | 8885250 | 7.38 | 15.00 | 14.110 | 94.09 | 0.035 |
| Reapeatability at 1.5 x MRL-06 | QC-06 | 1/15/2023 3:38:55 AM | 10481514 | 7.37 | 15.00 | 16.650 | 110.98 | 0.028 |
| Matrix Blank-4 | Unknown | 1/15/2023 2:03:29 AM | 8326 | 7.37 | N/A | 0.030 | N/A | N/A |

Regression Equation: y = 24081.97625 x + 10782.26647 (r = 0.99912, $r^2$ = 0.99824) (weighting: 1 / x)

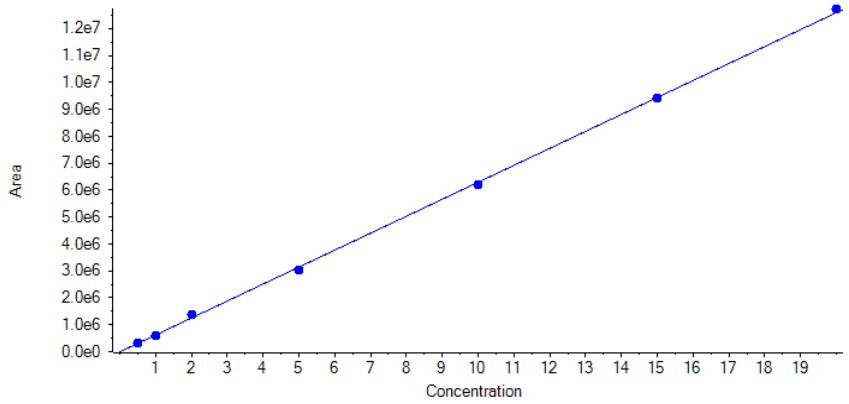


**Fig. 1. Instrumental graphs of calibration curve with Area v/s different concentration of parameter for Flunixin in method validation on LC-MS/MS**

**Chromatogram**

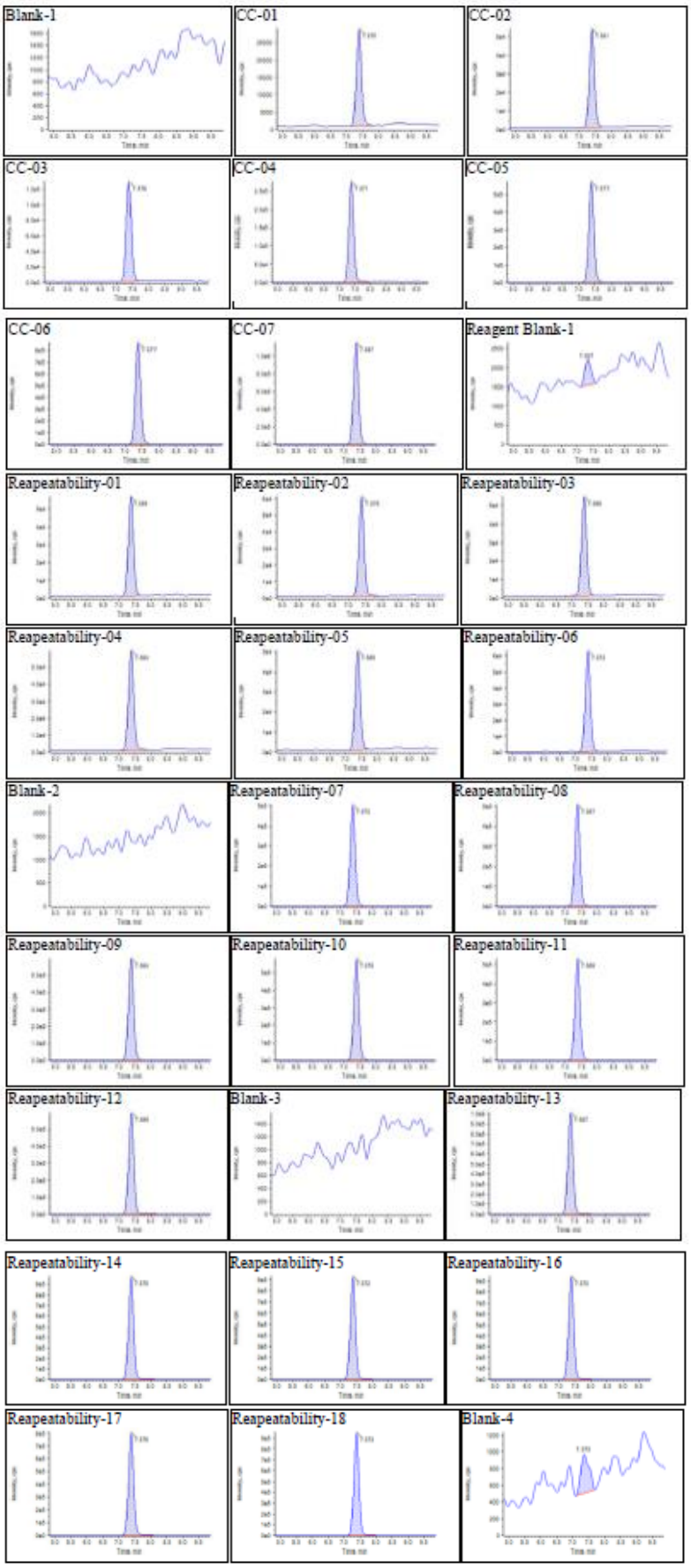


**Fig. 2. Instrumental Chromatograms of Repeatabiity parameter for Flunixin in method validation on LC-MS/MS with different maximum residual limit (MRL)**

**TABLE 2. Repeatability Test of Meloxicam at 0.1 * MRL Level method validation data in milk samples.**

| **Name of the Parameter** | | **Repeatability Test** | | |
|---|---|---|---|---|
| **Validation Name** | | **Antibiotics in Milk** | | |
| **Acceptance criteria** | | **Recovery- 50 to 120%, % RSD-20%** | | |
| | **Concentration** | **At 0.1 x MRL** | | |
| **S. No.** | **Analyte** | **Actual Conc at 0.1*MRL (ppb)** | **Calculated Concentration** | **% Recovery** |
| 1 | Meloxicam | 1 | 1.02 | 102 |
| 2 | Meloxicam | 1 | 0.77 | 77 |
| 3 | Meloxicam | 1 | 0.79 | 79 |
| 4 | Meloxicam | 1 | 0.86 | 86 |
| 5 | Meloxicam | 1 | 0.86 | 86 |
| 6 | Meloxicam | 1 | 1.03 | 103 |
| | | **Average** | **0.89** | |
| | | **SD** | **0.112** | |
| | | **% RSD** | **12.60** | |

## Milk Sample Analysis Details

The developed method has been applied for the antibiotic residue determination in terms of all the 20 milk samples. The sample analysis table has given below:

**TABLE 3. Specificity of Flunixin-01 (297.0 / 279.0) in milk samples.**

| Test Details | Details of Sample particular | Time and date of acquisition | Area (cps) |
|---|---|---|---|
| Specificity-1 | Milk-01 | 12/25/2023 7:42:46 PM | Not Applicable |
| Specificity-2 | Milk-02 | 12/25/2023 7:58:39 PM | Not Applicable |
| Specificity-3 | Milk-03 | 12/25/2023 8:14:35 PM | Not Applicable |
| Specificity-4 | Milk-04 | 12/25/2023 8:30:27 PM | Not Applicable |
| Specificity-5 | Milk-05 | 12/25/2023 8:46:23 PM | Not Applicable |
| Specificity-6 | Milk-06 | 12/25/2023 9:02:17 PM | Not Applicable |
| Specificity-7 | Milk-07 | 12/25/2023 9:18:12 PM | Not Applicable |
| Specificity-8 | Milk-08 | 12/25/2023 9:34:16 PM | Not Applicable |
| Specificity-9 | Milk-09 | 12/25/2023 9:50:12 PM | Not Applicable |
| Specificity-10 | Milk-10 | 12/25/2023 10:06:07 PM | Not Applicable |
| Specificity-11 | Milk-11 | 12/25/2023 10:22:01 PM | Not Applicable |
| Specificity-12 | Milk-12 | 12/25/2023 10:37:56 PM | Not Applicable |
| Specificity-13 | Milk-13 | 12/25/2023 10:53:51 PM | Not Applicable |
| Specificity-14 | Milk-14 | 12/25/2023 11:09:46 PM | Not Applicable |
| Specificity-15 | Milk-15 | 12/25/2023 11:25:41 PM | Not Applicable |
| Specificity-16 | Milk-16 | 12/25/2023 11:41:38 PM | Not Applicable |
| Specificity-17 | Milk-17 | 12/25/2023 11:57:34 PM | Not Applicable |
| Specificity-18 | Milk-18 | 12/26/2023 12:13:28 AM | Not Applicable |
| Specificity-19 | Milk-19 | 12/26/2023 12:29:22 AM | Not Applicable |
| Specificity-20 | Milk-20 | 12/26/2023 12:45:16 AM | Not Applicable |

***Chromatograms***

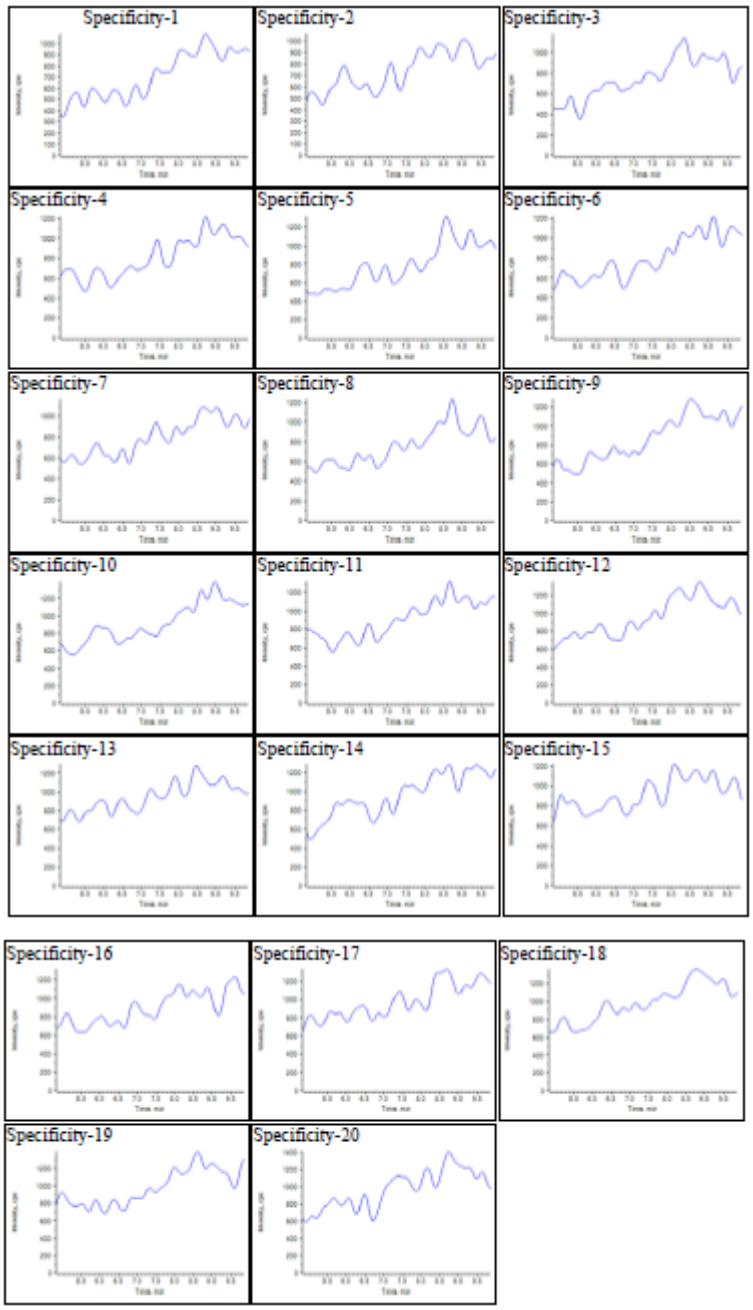


**Fig.3. Instrumental Chromatograms of Control milk samples (Specificity) using for method validation on LC-MS/MS.**

**TABLE 4. Linearity of milk based with concentration of 0.25ug/ml to 20ug/ml prepared in matrix blank with milk sample of stock solutions.**

| S. No | Linearity Details | Linearity (ppb) | Weight of Milk (ml/gm) | Stock Concentration (ug/kg/ppb) | Added vol' (uL) |
|---|---|---|---|---|---|
| 1 | Reference Standard-1 | 0.25 | 2 | 10 | 50 |
| 2 | Reference Standard-2 | 0.50 | 2 | 10 | 100 |
| 3 | Reference Standard-3 | 0.75 | 2 | 10 | 150 |
| 4 | Reference Standard-4 | 1.50 | 2 | 20 | 150 |
| 5 | Reference Standard-5 | 3.0 | 2 | 100 | 60 |
| 6 | Reference Standard-6 | 10.0 | 2 | 100 | 200 |
| 7 | Reference Standard-7 | 20.0 | 2 | 1000 | 40 |

**TABLE 5. UPLC Mobile phase details of method validation with pump A and Pump B and flow rate 600 µl/min, Total Run Time 14 minute.**

| Programme details | Minute | Flow (uL/minute) | A (%) | B (%) |
|---|---|---|---|---|
| I | 0.01 | 500 | 90 | 10 |
| II | 1.20 | 500 | 90 | 10 |
| III | 5.00 | 500 | 5 | 95 |
| IV | 7.50 | 500 | 5 | 95 |
| V | 12.00 | 500 | 90 | 10 |
| VI | 14.00 | 500 | 90 | 10 |

To achieve a quick and accurate chromatographic separation, a number of flow rates, gradient profiles, injection volume, column temperature, total time, etc. were investigated.

**TABLE 6. Antibiotics residues with Precursor Ion , Dauther ion and Mass spectrometry parameters for method validation for mass parameter optimization.**

| Precursor Ion | Daughter Ion | Veterinary Drug | Declustering Potential | Entrance Potential | Collision Energy | Collision Cell Exit Potential |
|---|---|---|---|---|---|---|
| 352.06 | 114.99 | Meloxicam-01 | 70 | 10 | 18 | 10 |
| 352.06 | 141.01 | Meloxicam-02 | 70 | 10 | 20 | 10 |
| 297 | 279.0 | Flunixin-01 | 57 | 10 | 32 | 10 |
| 297 | 264.0 | Flunixin-02 | 57 | 10 | 30 | 10 |
| 297 | 235.9 | Flunixin-03 | 57 | 10 | 62 | 11 |
| 407.3 | 126.1 | Lincomycin-01 | 30 | 10 | 32 | 10 |
| 407.3 | 359.2 | Lincomycin-02 | 30 | 10 | 27 | 10 |
| 693.2 | 461.3 | Monensin-01 | 70 | 10 | 71 | 10 |
| 693.2 | 479.3 | Monensin-02 | 70 | 10 | 74 | 10 |
| 291.4 | 230.1 | Trimethoprim-01 | 152 | 10 | 34 | 14 |
| 291.4 | 123.0 | Trimethoprim-02 | 152 | 10 | 34 | 14 |
| 291.4 | 110.0 | Trimethoprim-03 | 152 | 10 | 34 | 14 |
| 916.4 | 174.2 | Tylosin- 01 | 91 | 12 | 49 | 6 |
| 916.4 | 772.0 | Tylosin -02 | 91 | 12 | 49 | 6 |
| 526.1 | 508.3 | Virginiamycin -01 | 68 | 10 | 21 | 10 |
| 526.1 | 355.2 | Virginiamycin -02 | 68 | 10 | 29 | 10 |
| 282.2 | 254.1 | Diminazene-01 | 60 | 10 | 13 | 10 |

**TABLE 7. System Precision of Meloxicam in milk sample in method validation parameter**

| Test Details | Types | Time and date of acquisition | Standard Area | Retention Time | Nominal Con'c (ppb) | Ion Ratio |
|---|---|---|---|---|---|---|
| *System Precision_01* | STD | 12/26/2023 1:01:11 AM | 1538186 | 6.96 | 10 | 0.350 |
| *System Precision_02* | STD | 12/26/2023 1:17:04 AM | 1472536 | 6.96 | 10 | 0.409 |
| *System Precision_03* | STD | 12/26/2023 1:32:58 AM | 1488891 | 6.97 | 10 | 0.389 |
| *System Precision_04* | STD | 12/26/2023 1:48:53 AM | 1503203 | 6.96 | 10 | 0.388 |
| *System Precision_05* | STD | 12/26/2023 2:04:48 AM | 1332176 | 6.96 | 10 | 0.444 |
| *System Precision_06* | STD | 12/26/2023 2:20:42 AM | 1427403 | 6.97 | 10 | 0.420 |

**Chromatogram:**

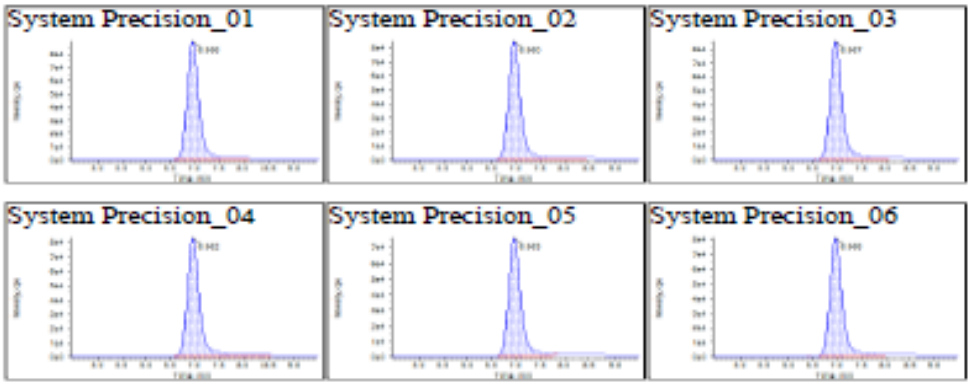


**Fig.4. Instrumental Chromatograms of System Precision parameter for Meloxicam of method validation on LC-MS/MS.**

**TABLE 8. System Precision Test of Meloxicam method validation data in milk samples.**

| **Name of the Parameter** | | **System Suitability** | |
|---|---|---|---|
| **Validation Name** | | **Antibiotics in Milk** | |
| **Acceptance criteria** | | **% RSD of Area is < 20% & RT is < 2%** | |
| **S. No.** | **Analyte** | **Standard Area** | **Retention Time** |
| 1 | Meloxicam | 1538186 | 6.96 |
| 2 | Meloxicam | 1472536 | 6.96 |
| 3 | Meloxicam | 1488891 | 6.97 |
| 4 | Meloxicam | 1503203 | 6.96 |
| 5 | Meloxicam | 1332176 | 6.96 |
| 6 | Meloxicam | 1427403 | 6.97 |
| | Mean | 1460399 | 6.96 |
| | STDEV | 72625 | 0.01 |
| | % RSD | 4.973 | 0.074 |

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