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SIMULTANEOUS DETERMINATION OF RESIDUAL VETERINARY DRUGS IN BOVINE MILK USING LIQUID CHROMATOGRAPHY COUPLED WITH ELECTROSPRAY IONIZATION TANDEM MASS SPECTROMETRY

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ABSTRACT

A method based on Ultra High-Performance Liquid Chromatography coupled with Electrospray Ionization Tandem Mass Spectrometry (UHPLC-MS/MS) for the simultaneous identification and quantification of 9 veterinary drug residues in bovine milk was developed. The molecular separation was performed using C_{18} Synergi™, Fusion – RP, 80 Å, column (50 x 2 mm, 4.0 µm) at 40°C. The mobile phase was used in gradient mode with 0.1% pentafluoropropionic acid (PFPA) in water and acetonitrile (ACN), at a flow rate of 0.6 mL/min and an injection volume of 20 µL. The method was carried out on SCIEX QTRAP 6500+ system and set in the positive electrospray ionization mode (ESI+). The voltage was set at 5.5 kV and the Ion Drive Turbo V™ Ion source was kept at a temperature of 500°C. The mass spectrometer was set in Multiple Reaction Monitoring (MRM) mode. The calibration curves met the European Commission Regulation (EC) 2021/808 requirements and the correlation coefficient (r^2)were within the acceptable limits for each compound. The method validation disclosed an acceptable precision, repeatability ranging from 0.2% to 5.4%, and reproducibility from 0.00% to 2.6%. The recovery ranged from 98.9% to 104.0%, and the capacity of detection (CCβ) values were below the MRL or the target validation concentrations. The tolerance limits were included in acceptance limits for accuracy profiles and linearity profiles of the validated method. The risk profiles were below the limits which are 5% for molecules with MRLs and 1% for prohibited molecules.

Keywords: UHPLC/MS-MS, MRM mode, Veterinary drug residues, Bovine milk, method validation.

INTRODUCTION

Antibiotics are widely used in livestock to prevent diseases and promote growth. When used improperly, veterinary drugs in livestock operations can accumulate these drugs in animal tissues and other animal-derived foods such as milk[1]. The possible presence of veterinary drug residues and other contaminants in animal food products is one of the key issues for food safety which arouses great public concern. The presence of veterinary drug residues in animal food products especially milk implicates a negative impact on public health such as allergic reactions, carcinogenicity, and the promotion of bacterial resistance. To ensure food safety at national and international levels, several regulatory agencies have set Maximum Residue Limits for veterinary drugs in foods [2]. Therefore, analytical methods used to control veterinary drugs in foods are essential to monitor the consumer's exposure to the drugs and to regulate and facilitate the international trade of food. Analytical methods play a crucial role in ensuring the safety and quality of food products traded globally. The World Trade Organization (WTO) sets international standards for food safety and quality under the Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement). According to the SPS Agreement, analytical methods used to detect veterinary drug residues in foods must be scientifically valid and provide accurate results. These methods must also be sensitive, specific, and reliable, and the results should be consistent and reproducible[3]. To guarantee the safety of the world trade exchanges of foods, it is also important to have harmonized standards and regulations for veterinary drug residues in foods across different countries. The Codex Alimentarius Commission, an international food standards organization, develops guidelines and standards for food safety and quality, including veterinary drug residues. These standards provide a basis for establishing national regulations and ensuring the consistency and reliability of analytical methods used for detecting veterinary drug residues in foods. Harmonized standards and regulations across different countries can help ensure the safety and quality of food products traded globally [4]. Thus, the European Commission Regulation (EC) 2021/808 established requirements that should be fulfilled on the minimum performance criteria that must be met by analytical methods used for screening, confirmatory testing, and quantitative analysis of veterinary drug residues [5]. In order to ensure the fulfillment of these regulations regarding the control of antibiotic residues, it is necessary to employ sensitive, selective, and accurate analytical methods [6-11]. The European Union also provides guidance documents that describe the validation procedures for analytical methods used for the detection of veterinary drug residues. These documents provide detailed information on the validation requirements for different types of methods, including sample preparation, instrumental analysis, and data evaluation. Several methods were developed for the determination of veterinary drug residues in foods. Despite the availability of all the analytical techniques, currently, there is an increasing demand for fast, sensitive, and reliable multi-class multi-residue methods, which could reduce analysis times and cost[12-15]. Among the different mass analyzers usually applied for target analysis, triple quadrupole (QqQ) is the most widely used for routine screening and confirmation of residues of veterinary drugs in milk.

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Several methods have been published for the determination of veterinary drug residues using tandem mass spectrometry [16,18] due to its high sensitivity, rapid detection, and capacity to monitor multiple compounds in a variety of matrices simultaneously[19, 20]. The objective of this study was to report on a new validation of an ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) for analyzing veterinary drug residues in milk according to the current analytical performance method requirements of the European Commission Regulation (EC) 2021/808 [5].

EXPERIMENTAL PHASE

The method was chosen for the determination of 8 quinolone residues and one aminoglycoside residue in milk. This method was inspired by a multi-residue method that was developed for monitoring antibiotic residues in milk using liquid chromatography coupled to a tandem quadrupole mass spectrometer (LC/MS-MS) by the French Community Reference Laboratory for Antimicrobial Residues (Agence Française deSécurité Sanitaire des Aliments, Laboratoire d'Etude et de Recherche sur les MédicamentsVétérinaires et les Désinfectants)[21].

Principe of Tandem mass spectrometry (MS/MS)

Tandem mass spectrometry (MS/MS) as a powerful analytical technique was used for the identification and quantification of antibiotic residues in milk. It involves the use of two or more mass analyzers to selectively isolate and fragment ions of interest. The first stage of MS/MS involves the ionization of molecules in the sample, typically using ionization techniques such as electrospray ionization (ESI) or Atmospheric Pressure Chemical Ionization (APCI). The resulting ions are then introduced into the first mass analyzer, which separates them based on their mass-to-charge ratio (m/z). This produces a spectrum of ions, each with a specific m/z value [22]. Next, the ions of interest are selectively isolated from the ion population using a process called precursor ion selection. This is achieved by applying a voltage to a set of electrodes, known as a quadrupole, which only allows ions of a specific m/z value to pass through. These selected precursor ions are then subjected to a second stage of fragmentation, typically by collision-induced dissociation (CID), in which they have collided with neutral gas molecules[23]. The resulting fragments are then separated based on their m/z value in a second mass analyzer, known as a product ion analyzer. This produces a spectrum of fragment ions, which can be used to identify the structure of the molecule. The specific mass analyzer used in the second stage of MS/MS can vary depending on the application, with options including quadrupole, time-of-flight (TOF), and ion trap analyzers [24].

By selectively isolating and fragmenting ions of interest, tandem mass spectrometry provides increased sensitivity and specificity for the identification and quantification of molecules in complex samples. It is widely used in applications such as proteomics, metabolomics, and environmental analysis, among others[25].

Chemical and Reagents

The analytical grade reagent that was used as chemical extraction was Trichloroacetic acid (TCA)which was purchased from Carlo Erba (Val de Reuil, France). The sodium hydroxide was obtained from SCHARLAU (Sentmenat, Spain). The Acetonitrile (ACN) and the methanol used, were HPLC ultra gradient grade reagents and were procured from ROTH (Karlsruhe, Germany) while Pentafluoropropionic acid (PFPA) was supplied by SIGMA ALDRICH (St. Louis, MO, USA).Analytical standards of Nalidixic Acid (NAL), Oxolinic Acid (Oxo), Norfloxacin (Nor), Ciprofloxacin (Cip), Danofloxacin (Dan), Difloxacin (Dif), Enrofloxacin (Enr), Marbofloxacin (Mar) and Lincomycin (Lin), were ordered from SIGMA ALDRICH (St. Louis, MO, USA). Sulfaphenazole (Sul) was used as an internal standard and was purchased from SIGMA ALDRICH (St. Louis, MO, USA). Ultra-pure water (18.0 MΩ.cm-1) was obtained in-house using an ELGA PURELAB Prima water purification system (United Kingdom). A 5% TCA solution was obtained by dissolving 50 g of trichloroacetic acid in 1 liter of Ultra-pure-water (UPW) and 0.1% PFPA solution was also prepared by adding 1 mL of pentafluoropropionic acid in 900 mL Ultra-pure water. All quinolone standards were used for the preparation of individual stock standard solutions (concentration of 0.5 mg.mL-1) in methanol mixed with 4% of 1 M of sodium hydroxide. The lincomycin standard (concentration of 0.5 mg.mL-1) was stocked in 100 % of methanol. From these stock solutions, suitable concentrations of spiking solutions were prepared in ultra-pure water to be used during the blank milk supplementation process. An antibiotic mixture corresponding to the European Union MRL and level of concentration for the validation of the analytes containing 9 different antibiotics was used for the supplementation. The concentrations evaluated during the validation are given in Table 1.

Fresh pasteurized free of antibiotic residues was used as blank milk in this study. The samples were storedat -20°Cuntil analysis.

Table 1: European Union MRL and level of concentration for the validation of the analytes extracted.

Analyte	MRL (µg/kg)	Validation concentration
Oxolinic Acid (Oxo)	Forbidden(1)	100
Difloxacin (Dif)	Forbidden(1)	300
Marbofloxacin (Mar)	75	75
Ciprofloxacin (Cip)	100(2)	100
Danofloxacin (Dan)	30	30
Enrofloxacin (Enr)	100(2)	100
Nalidixic Acid (Nal)	-/(3)	100
Norfloxacin (Nor)	-/(3)	100
Lincomycin (Lin)	150	150

[1]Forbidden for use in milk-producing animals; [2]MRL established for the parent drug plus the metabolites; [3]No authorization in veterinary medicine

Sample preparation

Blank milk samples were first thawed and then homogenized to thoroughly mix the cream with the milk using an ultrasonic bath. For blank samples, a portion of 2 ± 0.04 g of fresh milk was weighed to which 200 µL of the internal standard at a concentration of 5 µg/mL was added with 800 µL of water. For the supplementation, the antibiotic mixture was directly spiked into 2g of blank milk by adding the aqueous working mixed standard into a centrifuge tube to which 200 µL of internal standard was added with a volume of water according to the calculated proportions and then mixing by vortex in order to get the concentration levels corresponding to 0.1 MRL, 0.5 MRL, 1.0 MRL, 1.5 MRL, and 2.0 MRL. Then all the samples were allowed to stand in the dark for 15 min to permit the antibiotic mixture to be absorbed into the liquid samples. After, chemical extraction was carried out by adding a volume of 8 mL of 5% of TCA to each sample. Then the samples were shaken for 10 min on the rotary shaker at 100 rpm before they were centrifuged at 14 000 g for 5 min at 4°C. About 1 mL of the supernatant was filtered through a 0.45 µm ROTH Rotilabo®-Spritzenfilter, PVDF, sterile filter, and a volume of 20 µL was injected in UHPLC-ESI-MS/MS.

UHPLC-MS/MS

Ultra High-Performance Liquid Chromatography (UHPLC) analysis was performed using a SCIEX ExionLC AD system. A volume of 20 µL was injected onto a C_{18} Synergi™, Fusion – RP, 80 Å, column (50 x 2 mm, 4.0 µm) at 40°C. The flow rate was set to 0.6 mL.min−1. The mobile phase in a gradient mode (eluent A: 0.1% of pentafluoropropionic acid, eluent B: acetonitrile) was set up as follows: 0 min, 10% B; 0.5 min, 50% B over 4 min and decreased to 10% at 4.5 min and held up to 6 min to equilibrate the system before the next injection. The Electrospray interface ionization was carried out on SCIEX QTRAP 6500+ system and set in the positive ionization mode (ESI+) for all antibiotic residues. The voltage was set at 5.5 kV and the Ion Drive Turbo V™ source was kept at a temperature of 700°C, gas1 and gas2 were set at 45 psi and 50 psi respectively, Collision Gas (CAD) in medium and Curtain Gas (CUR) was set at 25 psi. MRM conditions, Entrance Potential (EP), Declustering Potential (DP), Collision Energy (CE), and Collision Cell Exit Potential (CXP)were first optimized for each antibiotic by infusing solutions of the antibiotic standards prepared in the mobile phase. The mass spectrometer was set in Multiple Reaction Monitoring (MRM) mode, with specific transition parameters as reported in Table 2.

Table 2. MRM conditions

Analytes	Precursor ion (m/z)	Product ion (m/z)	DP (V)	CE (eV)	EP (V)	CXP (V)
Sulfaphenazole (Sul)	315	156	50	30	10	12
Nalidixic Acid (Nal)	233.2	221	42	22	10	12
		187	42	55	10	12
Oxolinic Acid (Oxo)	262.2	244,1	53	25	10	12
		216	53	40	10	12
Danofloxacin (Dan)	358.3	340	60	33	10	12
		255	60	50	10	12
Enrofloxacin (Enro)	360.1	342	72	30	10	12
		286	72	50	10	12
Marbofloxacin (Mar)	363	345	70	30	10	12
		320	70	22	10	12
Ciprofloxacin (Cip)	332.2	314	61	30	10	12
		231	61	47	10	12
Difloxacin (Dif)	400	382	80	30	10	12
		356	80	26	10	12
Norfloxacin (Nor)	320	302	60	33	10	12
		231	60	50	10	12
Lincomycin (Lin)	407.5	126	60	40	10	12
		359	60	26	10	12

Method Validation

The quantities measured for an analyte during a measurement by tandem mass spectrometry chromatography were the relative retention time which might be less than 2.5%, the relative intensity of the ionic signals of the target molecules (height of the signal) compared to the signal of the internal standard, the relative intensity between ionic signals characteristic of the analyte (ion or transition ratios) which might be less than 20%. With the specific techniques and combinations of techniques used, a number of 5 identifications points should be fulfilled according to European Commission Regulation (EC) 2021/808[5].

The validation parameters were the precision, recovery, linearity, limit of detection (LOD), limit of quantification (LOQ), specificity, sensitivity, decision limit (CCα,) and detection capability (CCβ). To estimate them, it was necessary to calculate the “Threshold value T” and the “Fm cut-off value” parameters first.

Threshold value T and Fm cut-off value

The T-value is a threshold value corresponding to the minimum analytical response above which the sample will be truly considered positive[26]. For each antibiotic residue, 20 blank milk samples from different origins were analyzed to determine the threshold value "T". The Threshold value Tor technical threshold was estimated as follows: T = B+1.64×SDb. Where B is the mean response and “SDb” is the standard deviation of blank samples. Meanwhile, the supplemented samples were fortified at 0.1 MRL, 1.0 MRL, 1.5 MRL, and then analyzed. Each level of spiked sampleswas repeated 7 times over 3 non-consecutive days (63 results at the level of interest per analyte) to determine the Fm cut-off value. The cut-off Level or Fm cut-off valueis the response or signal from a screening test that indicates that a sample contains an analyte at or above the screening target concentration. If the cut-off Level is exceeded a subsequent confirmatory test is carried out. During the initial validation process, the cut-off Level may be established through the analysis of matrix blank samples and replicates of those same samples spiked (fortified) at the screening target concentration[27]. The cut-off factor Fm was estimated as follows: Fm = M−1.64×SD, where M was the mean response and

"SD" the standard deviation of spiked samples. Then the detection limits (LODs), sensitivity, specificity, and CCβ were estimated for each analyte.

Detection capability (CCβ)

Detection capability for screening (CCβ) means the smallest content of the analyte that may be detected or quantified in a sample with an error probability of β. In the case of substances for which no permitted limit has been established, the detection capability is the lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of 1–β. In the case of substances with an established permitted limit, this means that the detection capability is the concentration at which the method is able to detect permitted limit concentrations with a statistical certainty of 1–β [28].

In our case, the system is a diagnostic ion chromatogram of the target analyte, and the ground state corresponds to this ion chromatogram for a blank sample (prohibited substances) or for a sample containing the analyte at a concentration equal to the MRL (substances subject to MRLs).According to Decision 2004/25/EC [29], CCβ for screening methods is acceptable (CCβ ≤ MRL) when the false negative rate is less than 5% at the level of interest, i.e. Fm > T. An intensity/concentration relationship was given by a calibration curve produced from a mixture of different blank samples (n=20), supplemented with at least six concentration levels including 0 x.MRL; 0.1 MRL; 0.5 MRL; 1.0 MRL; 1.5 MRL and 2.0 MRL where x and y were set according to needs.

CCβ was estimated as follow: $CC\beta = (\mu_{LMR}-\mu_B+1.64.\sigma_{LMR}+1.64.\mu_B.CV_C) / a\ (1-1.64.CV_C)$.

Where:

- μ_{LMR} and σ_{LMR} represented respectively the mean and the standard deviation (SD) of the amplitude of the signal of the analyte at the concentration equal to the MRL, calculated on different supplemented samples (n=20).
- μ_B, CV_C and a represented respectively the mean relative response of the blank samples, the Relative Standard Deviation (RSD) of the relative amplitude response of the analyte at the concentration equal to the MRL, calculated on different supplemented samples (n=20), and the slope of the calibration curve.

Decision limit for confirmation (CCα)

The decision limit for confirmation (CCα) means the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant and the value 1–α means statistical certainty in percentage that the permitted limit has been exceeded. It was determined as follows: $CC\alpha = (\mu_{LMR}-\mu_B+1.64.\sigma_{LMR}) / a$, where μ_{LMR}, σ_{LMR}, and μ_B were defined as above.

Specificity/Sensitivity

The specificity is the probability that the method classifies a sample as negative knowing that this sample is a true negative. We will then speak of a negative agreement (NA). The specificity of the method was checked by observing the ion chromatograms of a significant number of different blank samples (n=20) and verifying the absence of co-elution which could disturb the interpretation. It was estimated as follows:

Specificity = $(NA/N^-).100\%$, where N^- was the total number of true negatives.

Sensitivity is defined as the positive agreement (PA), which means that a truly positive sample (containing the analyte in a concentration above the MRL) is detected positive by the method to be validated. Sensitivity is then, the probability that the method classifies a sample as positive knowing that this sample is a true positive. We will then speak of positive agreement (PA). It was estimated in a significant number of fortified samples (n=63) as follows:

Sensitivity = $(PA/N^+).100\%$, where N^+ was the total number of true positives.

Precision/Trueness/Tolerance intervals

To check the fitness for the purpose of the analytical method, the accuracy profile procedure was combined with the European Union analytical performances' procedure to evaluate the capability of the method to quantify samples with a known accuracy and a fixed risk. The value of the acceptability limit noted λ of each compound was defined at 10% around the target value. Precision, trueness, and tolerance intervals of the method were determined using fortified samples at low (0.1 MRL); medium (1.0 MRL);and high (1.5 MRL) concentrations relative to the calibration range of target concentration values of each analyte. Each fortified sample was analyzed in 7 replicates on 3 non-consecutive days. Intraday (sr), interday (sL), and intermediate (sFI) precision standard deviation precision values were calculated using analysis of variance and expressed as relative standard deviation (%RSD). From the data obtained, the concentrations of the fortified samples were back-calculated to determine the mean relative bias, the standard deviation for intermediate precision, and finally the upper and lower β-expectation tolerance limits at 95% level. The %RSD for the repeated analysis of the fortified materials, under within-laboratory reproducibility conditions, might not exceed the level calculated by the Horwitz Equation [30].

Risk profile

The risk profile expressed by level of concentration, evaluates the probability that the result of a measurement carried out in routine analysis may fall outside the acceptance limits [26]. It is established graphically by considering the absolute value of the biases obtained in the validation phase. The probability of risk was set at 5% for the compounds with established MRLs and at 1% for the forbidden compounds for use in milk-producing animals and for non-authorized compounds in veterinary medicine.

Linearity

The linearity of method validation is an important aspect of analytical that involves testing the relationship between the concentration of an analyte and the corresponding response of the analytical method. Five-point (0.1MRL, 0.5 MRL, 1.0 MRL, 1.5 MRL and 2.0 MRL) calibration curves were constructed by plotting peak height against nominal concentrations of the calibration standards. The curves were fitted using weighted least squares linear regression with a weighting factor of $1/x^2$. The correlation coefficient(r^2) might be greater than 0.98(r^2>0.98) for all the analytes.

The other approach was based on absolute tolerance limits. The linearity of the method was also graphically checked according to the rules of proportionality existing between the introduced concentrations and the found concentrations. The linearity of the method was established on 3-point levels of the studied range with the data used for the determination of precision. A regression line is only linear if the absolute tolerance limits are within the predefined acceptance limits.

Limit of detection LOD / Limit of quantification LOQ

The limit of detection (LOD) is the lowest concentration of an analyte that can be reliably detected by an analytical method. It is defined as the concentration that gives at signal-to-noise ratios(S/N) > 3[27]. The height of the background noise was determined graphically on each chromatogram over an area corresponding to the 10% retention time. The limit of detection (LOD) was estimated as follows:

$LOD = [C]_{0.1\ LMR} \times H_{3xB} / H_{sup\ 0.1\ LMR}$

Where:

- LOD = limit of detection (µg/kg);
- $[C]_{0.1\ LMR}$ = Concentration corresponding to the 0.1 MRL (µg/kg);
- H_{3xB} = response (peak height) corresponding to 3 times the average background noise;
- $H_{sup0.1\ LMR}$ = response (peak height) of supplemented 0.1 MRL.

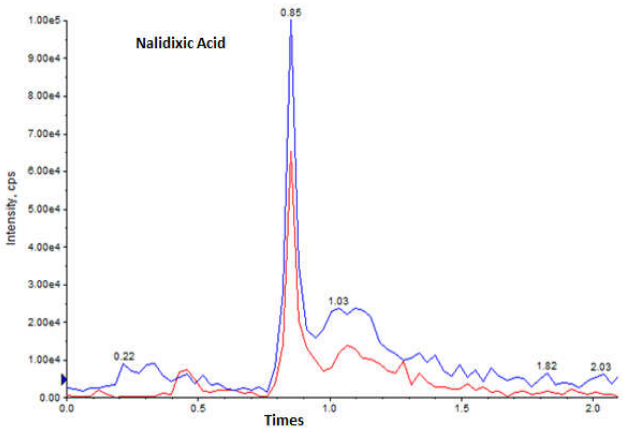
The limit of quantification (LOQ) is the lowest concentration of an analyte that can be reliably quantified by an analytical method. It is defined as the concentration that gives a signal-to-noise ratio(S/N)> 10[28]. The limit of Quantification (LOQ) was estimated as follows:
$LOQ = [C]_{0.1\ LMR} \times H_{10xB} / H_{sup\ 0.1\ LMR}$

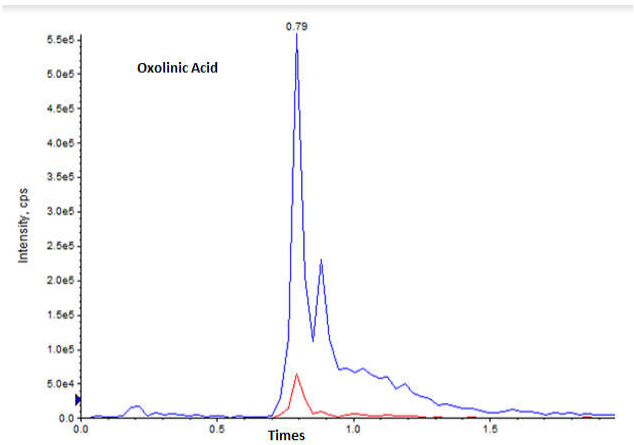
Where:

- LOQ = limit of quantification(µg/kg);
- $[C]_{0.1\ LMR}$ = Concentration corresponding to the 0.1 MRL (µg/kg);
- H_{10xB} = response (peak height) corresponding to 10 times the average background noise;
- $H_{sup\ 0.1\ LMR}$ = response (peak height) of supplemented 0.1 MRL.

RESULTS AND DISCUSSION

All analytes were identified. The results of identification based on retention time matching and MRM ratio comparison, were found satisfactory. All 9 vet drug residues were confidentially identified in all 7 spiked samples. The chromatograms obtained are summarized in Figure 1.





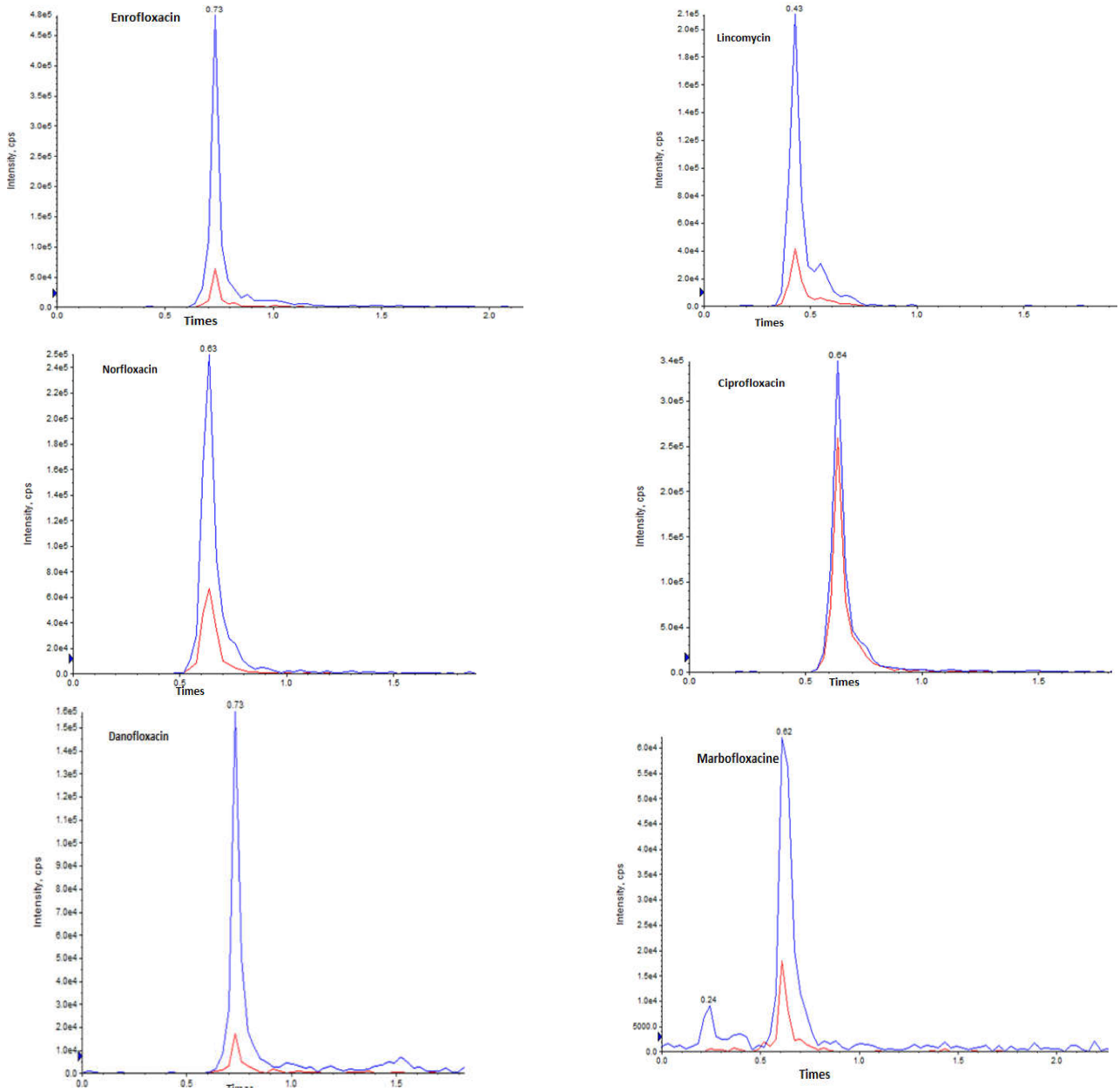


Figure 1. MRM chromatograms of vet drug residues in milk

T value, cut-off (Fm), CCα, CCβ, LOD, and LOQ

The results obtained from the Threshold value-T, the Fm cut-off value, the limit of detection (LOD), the limit of quantification (LOQ), the specificity/sensitivity, the decision limit (CCα), and the detection capability (CCβ) are presented in Table 3.

Table 3. T-values, Fmcut-off values, CCβ, CCα, and Specificity/Sensitivity results

	Transition 1						Transition2					
Analytes	T/Fm	CCβ	CCβ /MRL	CCα	Specificity/ Sensitivity	LOQ (µg/kg)	T/Fm	CCβ	CCβ/MRL	CCα	Specificity/ Sensitivity	LOD (µg/kg)
Nalidixicacid (Nal)	T<Fm	42.6	CCβ<MRL	38.3	100%/100%	14,9	T<Fm	43.8	CCβ<MRL	36.3	100%/100%	4,92
Oxolinic Acid (Oxo)	T<Fm	41.3	CCβ<MRL	36.3	100%/100%	8,92	T<Fm	91.6	CCβ<MRL	75.6	100%/100%	5,2
Ciprofloxacin (Cip)	T<Fm	41.1	CCβ<MRL	36.2	100%/100%	2.7	T<Fm	51.1	CCβ<MRL	42.8	100%/100%	0.82
Danofloxacin (Dan)	T<Fm	12.8	CCβ<MRL	11.3	100%/100%	4,5	T<Fm	15.2	CCβ<MRL	12.6	100%/100%	0,59
Difloxacin (Dif)	T<Fm	135.24	CCβ<MRL	118.07	100%/100%	8,3	T<Fm	137.55	CCβ<MRL	107.53	100%/100%	3,6
Enrofloxacin (Enr)	T<Fm	43.54	CCβ<MRL	38.65	100%/100%	6,12	T<Fm	40.49	CCβ<MRL	33.95	100%/100%	5,50
Marbofloxacin (Mar)	T<Fm	32.03	CCβ<MRL	28.21	100%/100%	3,32	T<Fm	38.23	CCβ<MRL	30.10	100%/100%	2,5
Norfloxacin (Nor)	T<Fm	44.94	CCβ<MRL	39.56	100%/100%	8,7	T<Fm	52.51	CCβ<MRL	43.45	100%/100%	6,7
Lincomycin (Lin)	T<Fm	69.2	CCβ<MRL	60.62	100%/100%	4,8	T<Fm	83.92	CCβ<MRL	62.98	100%/100%	3,0

The results showed that the Fm-values were found above the T-values for all the analytes. The LODs were ranging from 0.59 µg/kg to 6.7 µg/kg and the LOQs were ranging from 2.7 µg/kg to 14.9 µg/kg. The estimated CCβ were below than the screening target concentrations or the MRLs for all the antibiotics. The results highlighted that the method was estimated 100% specific and 100% sensitive. No endogenous of co-elutions

were observed in extracts from the fortified samples that could disturb the interpretation. The number of false negatives was then truly below 5%. It can then be deduced that CCβ values for this screening method were definitely acceptable (CCβ ≤ MRL).

Accuracy profiles

The trueness is represented by the relative bias (%) and the precision is symbolized by the lower tolerance limit (LTL) and the upper tolerance limit (UTL) calculated with intermediate precision relative standard deviation (RSD). The results obtained are summarized in Table 4.

Table 4. Accuracy and Precision Results

Analytes	Spiked level ($\mu g.kg^{-1}$)	Recoveries (%)	%RSD of intraday precision	%RSD of interdayprecision	%RSD of intermediate precision	Absolute Bias (%)
Nalidixic Acid (Nal)	10	102 .10	5.4	2.00	1.04	2.09
	100	100.70	1.6	0.00	1.02	0.76
	150	100.20	1.5	0.00	1.02	0.19
Oxolinic Acid (Oxo)	10	99.53	1.0	0.00	1.02	-0.47
	100	100.15	1.8	0.00	1.02	0.15
	150	99.94	0.6	0.00	1.02	-0.06
Ciprofloxacin (Cip)	10	102.53	2.3	2.60	1.19	2.53
	100	99.27	1.3	0.60	0.20	-0,73
	150	100.31	1.3	0.00	0.00	0.31
Danofloxacin (Dan)	3	101.90	1.3	1.30	1.09	1.90
	30	99.44	3.1	0.00	1.02	-0.56
	45	100.24	2.7	0.00	1.02	0.24
Difloxacin (Dif)	30	101.55	0.9	0.20	1.03	1.55
	300	99.52	0.8	0.00	1.02	-0.48
	450	100.21	0.2	0.00	1.02	0.21
Enrofloxacin (Enr)	10	100.75	1.9	0.00	1.02	0.75
	100	99.81	2.4	0.00	1.02	-0.19
	150	100.08	2.4	0.00	1.02	0.08
Marbofloxacin	7.5	98.99	2.5	0.14	1.07	-1.01
(Mar)	75	100.27	2.5	0.00	1.02	0.27
	112.5	100.10	1.6	0.00	1.02	-0.12
Norfloxacin (Nor)	10	100.80	0.9	1.10	1.11	0.77
	100	99.80	1.0	0.00	1,02	-0.21
	150	100.10	1.2	0.00	1.02	0.09
Lincomycin (Lin)	15	104.00	4.9	0.00	1.02	4.01
	150	98.90	1.8	0.00	1.02	-1.1
	225	100.50	1.1	0.00	1.02	0.47

The results showed that the accuracy in terms of average recoveries ranged from 98.9 to 102.53%. For all spiking levels studied in milk, the RSD of the repeatability (intraday precision) ranged from 0.2 to 5.4% and the RSD of the reproducibility (interday precision) ranged from 0.0 to 2.6%. These results showed that the % RSD for the repeated analysis of fortified materials, under within-laboratory reproducibility conditions, was found to be within the European Commission specifications on the performance of analytical methods for residues of pharmacologically active substances used in food-producing animals[30].

Table 5 shows acceptability and tolerances results.

Table 5. Acceptability and tolerance limits results

Analyses	Levels	Low tolerance limit (%)	High tolerance limit (%)	Low limit of acceptability (%)	High limit of acceptability (%)	Absolute tolerance low limit	Absolute tolerance high limit	Low limit of acceptability	High limit of acceptability	Fixed level of risk in %
Nalidixic Acid	10	89.2	115.0	90.0	110.0	8.9	11.5	9	11	5
(Nal)	100	97.3	104.2	90.0	110.0	97.33	104.2	90	110	5
	150	97.0	103.3	90.0	110.0	145.6	155.0	135	165	5
Oxolinic Acid	10	97.45	101.62	90.0	110.0	9.74	10.1	9	11	1
(Oxo)	100	96.38	103.92	90.0	110.0	96.4	103.9	90	110	1
	150	98.64	101.24	90.0	110.0	147.9	151.8	135	165	1
Ciprofloxacin	10	92.6	112.5	90.0	110.0	9,3	11.2	9	11	5
(Cip)	100	96.2	102.4	90.0	110.0	96.2	102.4	90	110	5
	150	97.5	103.1	90.0	110.0	146.3	154.7	135	165	5
Danofloxacin	3	96.9	106.9	90.0	110.0	2.91	3.21	9	11	5
(Dan)	30	92.8	106.1	90.0	110.0	27.8	31.8	90	110	5
	45	94.3	106.1	90.0	110.0	42.4	47.76	135	165	5
Difloxacin (Dif)	30	99.6	103.5	90.0	110.0	29.9	31.0	9	11	5
	300	97.7	101.3	90.0	110.0	293.1	304.0	90	110	5
	450	99.8	100.6	90.0	110.0	449.1	452.8	135	165	5
Enrofloxacin	10	96.7	104.8	90.0	110.0	9.7	10.5	9	11	5
(Enr)	100	96.7	102.9	90.0	110.0	96.7	102.9	90	110	5
	150	94.8	105.3	90.0	110.0	142,3	158.0	135	165	5
Lincomycin	15	93.0	115.0	90.0	110.0	14.0	17.2	9	11	1
(Lin)	150	95.1	102.7	90.0	110.0	142.7	154.0	90	110	1
	225	98.0	102.9	90.0	110.0	220.5	231.6	135	165	1
Marbofloxacin	7.5	91.5	106.5	90.0	110.0	6.9	8.0	6,75	8.25	5
(Mar)	75	94.8	105.7	90.0	110.0	71.1	79.3	67,5	82.5	5
	112.5	96.5	103.3	90.0	110.0	108,6	116.2	101,2	123.7	5
Norfloxacin	10	96.6	104.9	90.0	110.0	9.7	10.5	9	11	1
(Nor)	100	97.7	101.9	90.0	110.0	97.7	101.9	90	110	1
	150	97.5	102.7	90.0	110.0	146.2	154.0	135	165	1

The accuracy profiles for the studied antibiotic residues are illustrated in Figures. 2a,2b and 2c.

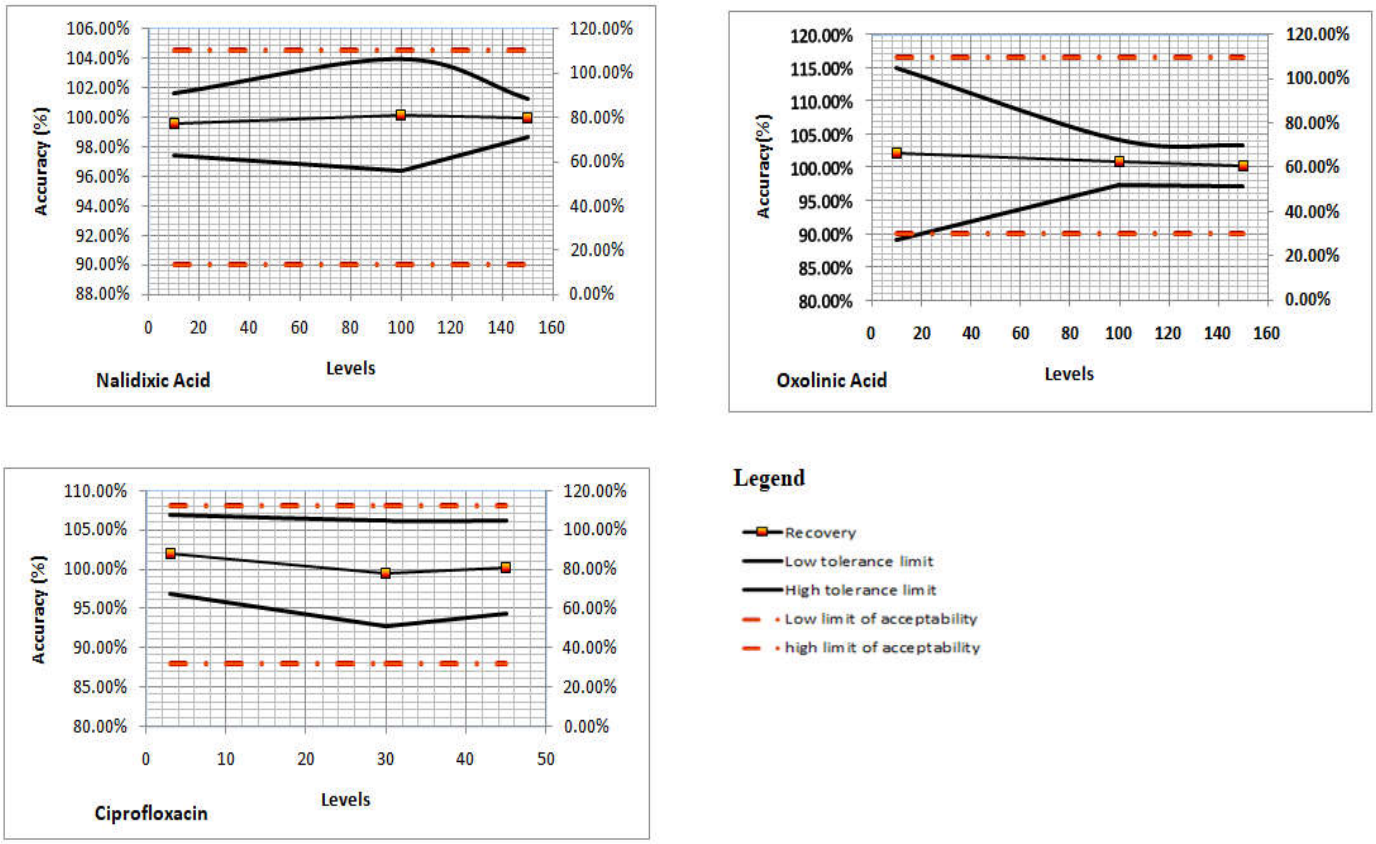


Figure 2a. Nalidixic acid, oxolinicacid, and ciprofloxacinaccuracy profiles

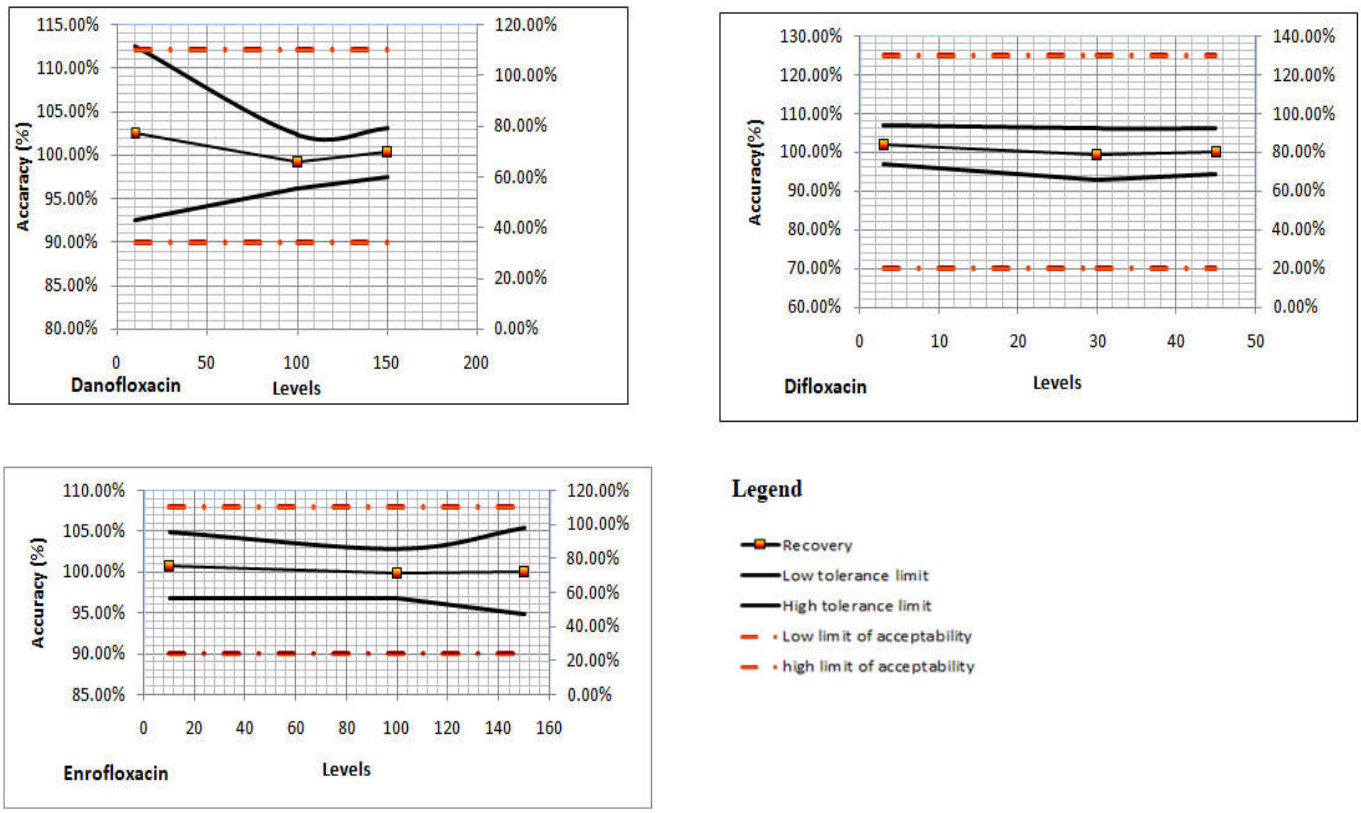


Figure 2b. Danofloxacin, Difloxacin, and Enrofloxacin accuracy profiles

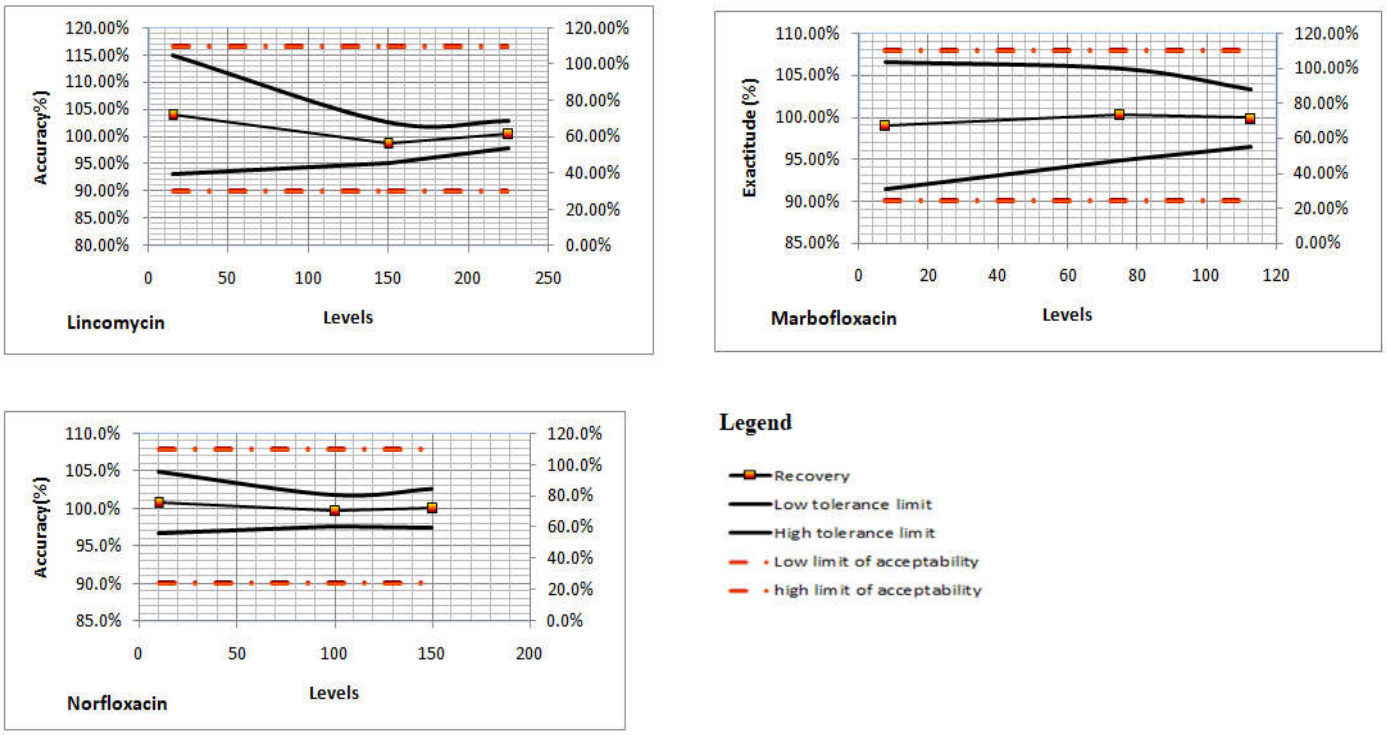
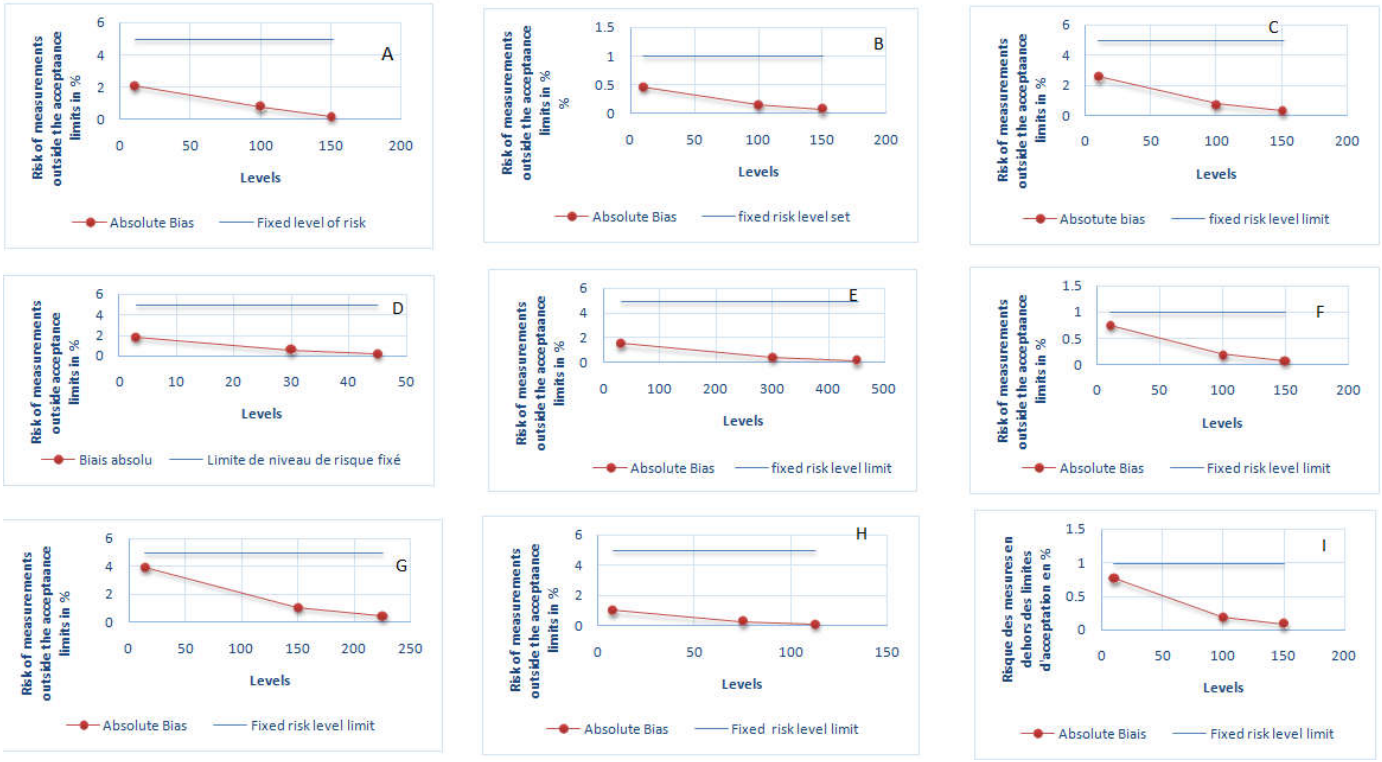


Figure 2c. Lincomycin, Marbofloxacin, and Norfloxacin accuracy profiles

When considering these profiles, it can be concluded that the method is valid for the quantification of allantibiotic residues because all tolerance intervals are included within the acceptance limits of ±10%.Therefore, the analyst can guarantee that the method gives "acceptable" results, for any milk sample, in each analyte range of concentrations. The method is valid from 10.0 to 150.0 µg/kg for nalidixic acid (Nal), oxolinic acid (Oxo), ciprofloxacin (Cip), norfloxacin (Nor) and enrofloxacin (En). It is also valid from 3.0 to 45.0 µg/kg for danofloxacin (Dan), from 30.0 to 450.0 µg/kg for difloxacin (Dif), from 7.5 to 112.5µg/kg for marbofloxacin (Mar) and from 15.0 to 225.0 µg/kg for lincomycin (Lin).

Risk profile

It shows that the maximum risk of having future measurements outside the acceptance limits is set at 5% for the compounds with established MRLs and at 1% for the forbidden compounds for use in milk-producing animals and for non-authorized compounds in veterinary medicine. The risk profile obtained with these data is illustrated in Figure 3.



A: Nalidixicacid, B: Oxolinic acid, C:Difloxacin, D:Ciprofloxacin, E: Danofloxacin, F:Enrofloxacin, G:Marbofloxacin, H:Lincomycin and I:Norfloxacin.

Figure 3. The risk profile of all target analytes

In any case, the results demonstrated that the probability of obtaining future results outside the acceptance limits, within the range of validity, is less than the corresponding limit for each compound (5% or 1%). The results showed that the method is reliable enough to measure the milk samples with a fixed risk level.

Linearity

The relationship between the concentration of an analyte and the corresponding response of the analytical method of the 5-point spiked samples for each analyte leads to linear models with slopes close to the unit and y-intercept. The results are described in Table 6. The correlation coefficients found for all analytes were higher than 0.98.We could say from there that the method is linear. However, VAN LOCO [32] and HIBBERT [33] demonstrated that the value of the coefficient of correlation close to 1 is insufficient to determine the quality of the linearity. According to these authors, even a curvilinear regression can present an r^2 close to unity. This is why we used the approach based on absolute tolerance limits. Since the tolerance limits are within the acceptance limits, the method of analysis is therefore linear. In order to assess the linearity with the second approach, the absolute β-expectation tolerance intervals were used. Linearity for antibiotic residues was also demonstrated since the β-expectation tolerance limits were included in the absolute acceptance limits for the previously defined concentration range of each analyte (Table. 6).

Table 6. Five points of linearity results

Analytes	Nalidixic Acid (Nal)	Oxolinic Acid (Oxo)	Ciprofloxacin (Cip)	Danofloxacin (Dan)	Difloxacin (Dif)	Norfloxacin (Nor)	Enrofloxacin (Enr)	Lyncomycin (Lyn)	Marbofloxacin (Mar)
Slopes	767.2	858.7	896.6	1129.2	669.89	858.7	718.06	961.1	475.5
Intercept	5945	3995.28	-44.5	833.78	-3501.7	3995.2	-2681	1414.8	641.04
r^2	0.999	0.9988	0.9978	0.9987	0.9996	0.9978	0.9985	0.9982	0.9982

The curves are shown in Figures4a, 4b, and 4c.

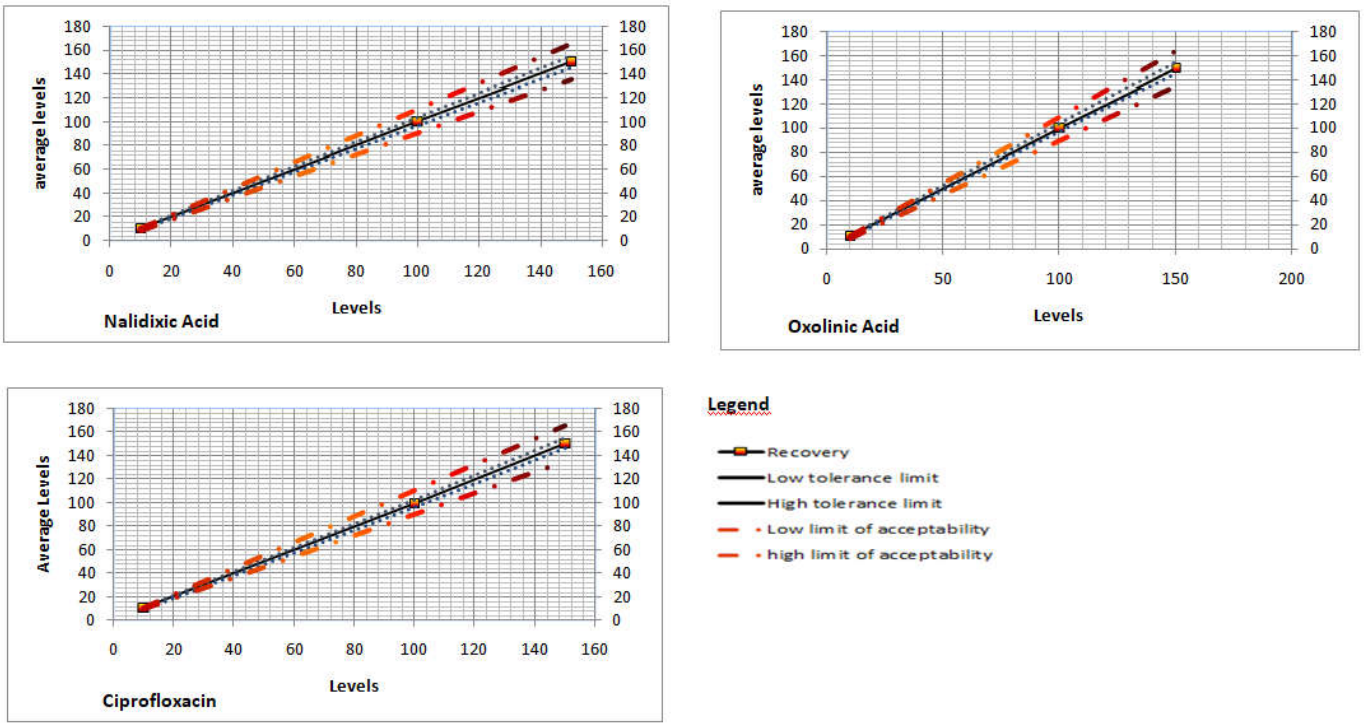


Figure 4a. Nalidixic acid, oxolinicacid, and ciprofloxacin linearity profiles

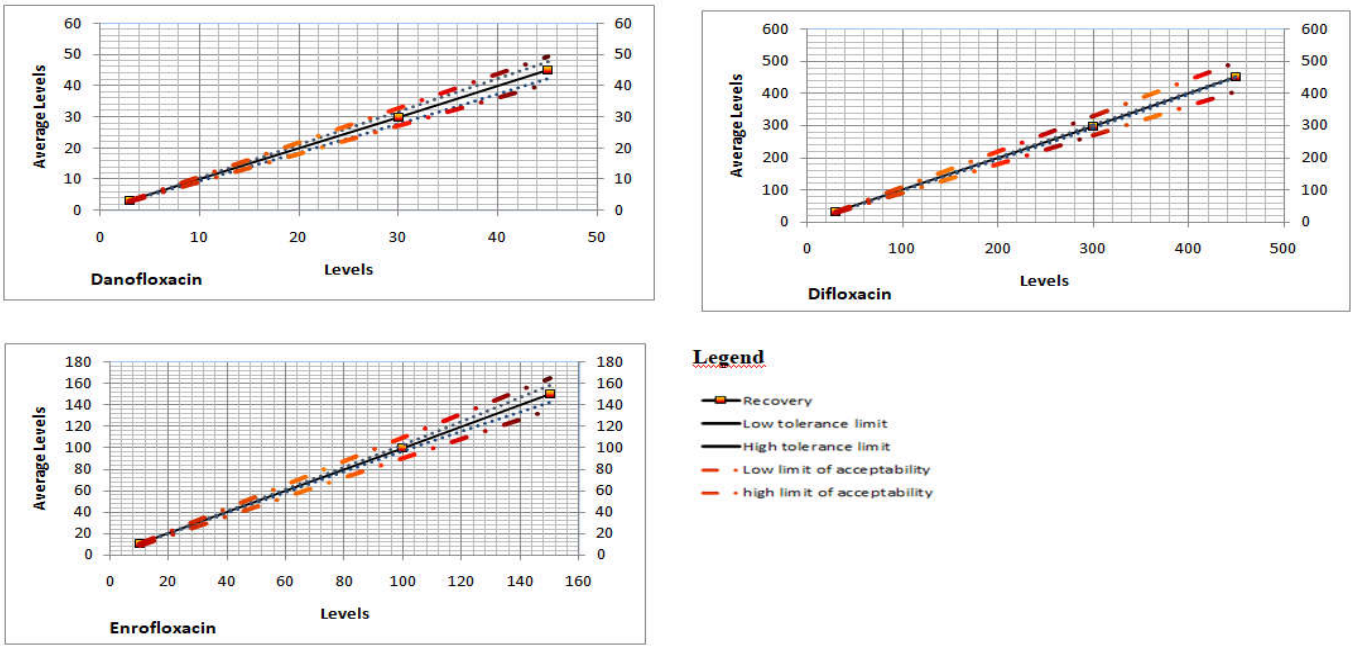


Figure 4b. Danofloxacin, Difloxacin, and Enrofloxacin linearity profiles

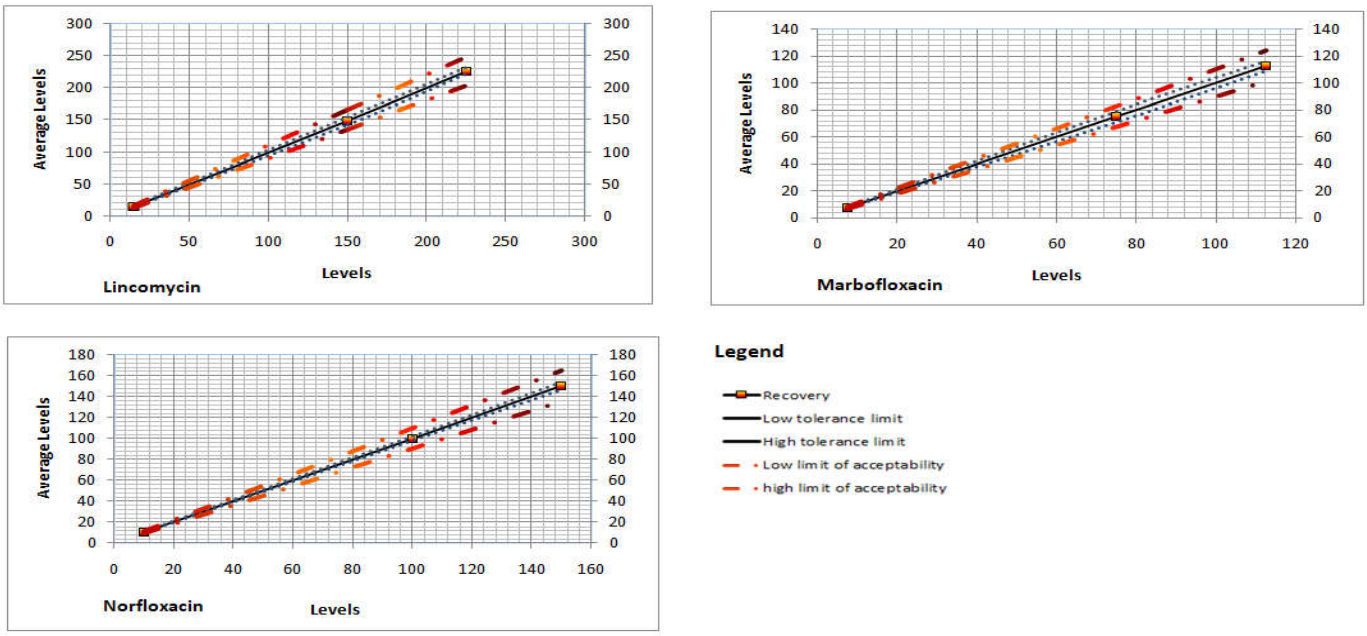


Figure 4c. Lincomycin, Marbofloxacin, and Norfloxacin linearity profiles

The data obtained confirms the linear relationship between the concentrations of analytes and the corresponding analytical responses of the method because the β-expectation tolerance limits were included in the absolute acceptance limits. Therefore, the data demonstrated the linearity of the method.

CONCLUSION

The proposed method is useful for the simultaneous identification and quantification of 9 veterinary drug residues (8 quinolones and 1 aminoglycoside) in bovine fresh milk based on Ultra High-performance Liquid Chromatography coupled with Electrospray Ionization Tandem Mass Spectrometry (UHPLC-MS/MS). The sensitivity and the specificity of the method were suitable to help meet the MRLs or the target concentration values of prohibited compounds in milk. The performances characteristics for the studied vet drug residues were acceptable according the European guideline for the method validation. Good calibration curves linearity and acceptable average recoveries with low relative standard deviations results were found for all analytes in the milk matrix. This method can be recommended for the routine analysis in dairy industry and in monitoring studies on vet drug residues in the local market's food.

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