SIMULTANEOUS DETERMINATION OF ANTIBIOTIC RESIDUES (ENROFLOXACIN, CIPROFLOXACIN, DOXYCYCLINE AND CHLORAMPHENICOL) IN MILK OF JORDAN MARKET

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Abstract: A new multi-residue analytical method for simultaneous determination of four antibiotic residues (doxycycline (DOX), enrofloxacin (EN), ciprofloxacin (CP) chloramphenicol (CAM)))) in cow's milk has developed and validated by liquid chromatography–tandem mass spectrometry, was successfully applied to investigate commercially available cow's milk in Jordan. The analytes including etoricoxib as internal standard (IS) were extracted using liquid-liquid extraction and separated from their matrix chromatographically by using Fortis Universil Cyano column (50×2.1 mm, 5 µm), eluted by a mobile phase of 0.5 mM ammonium chloride

/methanol /formic acid (35:65:0.08%, v/v) and delivered isocratically at constant flow rate of 0.4 mL/min for total LC run time of 1 min. Twenty six cow's milk samples from different brands of dry powder milk, long shelf life milk and raw untreated milk were collected randomly from Jordanian market and analyzed in triplicate analysis. The calibration curve was linear within the dynamic range of 10–1000 ng/mL in spiked milk for each analyte and the correlation coefficients were greater than 0.9973 for all calibration curves during validation. The internal standard-normalized matrix effects extend from 0.901 to 1.11. The intra-assay and inter-assay precision normalized matrix effects extend from 0.901 to 1.11. The within-day and between day precision ranged from 2.60%-12.71% and 2.68%-12.66%, respectively, and the relative error of accuracy does not exceed 15%. The results obtained are less than the approved stated regulatory guidelines and all samples screened were found to be free of any of the antibiotics tested.

Keywords: Antibiotic residues, Milk. LC-MS/MS.

Abreviations: (MRL) Maximum Residue Limit, (EN) Enrofloxacin; (CI) Ciprofloxacin; (DOX) Doxycicline; (CAM) Chloramphenicol, (ADI) Acceptable Daily Intake, (LLOQ) Lower Limit of Quantitation, (IS) Internal Standard, (CV) Coefficient of variation, (LLOQ) Lower Limit of Detection Quntity, (BLOQ) Below Limit of Quantitation.

Introduction: Antibiotics have a widespread use in the veterinary and agriculture sector, besides their significant use to treat infected food-producing animals. Some classes of antibiotics are added to the feed or of animals for growth promotion or for prophylaxis (1). Most farmers in developing countries including Jordan, are misusing and or overusing antibiotics (2). Usually, farmers try to maximize their profits, using a lot of antibiotics for a long time to protect and maintain the health of birds and animals, to avoid them from diseases, without knowing that these antibiotics and their residues have negative and inappropriate effect on the health of society. Milk and other dairy

products are susceptible to contamination by antibiotic residue.

Inappropriate administration of various antibiotics including fluoroquinolones, tetracyclines, and amphenicols causes adverse health effects such as allergies (e.g. penicillin), and cancer induction (e.g., sulphamethazine) (3). Adding to that, non-prudent use of antibiotics is the reason for the development of antibiotic resistant strains that makes antibiotics ineffective as therapeutic agents (4). Moreover, the broad-spectrum antibiotics contribute to the disturbance of various gastrointestinal microbiota because they attack and kill a wide range of intestinal flora and benign bacteria (5).

International organizations such as the European Commission (EC), World Health Organization (WHO), Food and Agricultural Organization (FAO) and United States Food and Drug Administration (USFDA) authorize limits on the residue levels of antibiotic drugs that are permitted in animal derived food to protect consumers (6-7). Maximum residue levels (MRLs) and the acceptable daily intake (ADI) are established after performing toxicological analysis and pharmacokinetics studies. Table 1 shows some accepted MRLs values for residues of veterinary drugs in milk established by which international organization such as EC, WHO and FAO (8-9).

Table 1: Maximum Residue Limits (MRLs) for Residues of Veterinary Drugs in Milk

Antibiotics	**MRL (μg/Kg body weight)**
Enrofloxacin	100
Ciprofloxacin	100
Doxycycline	3
Chloramphenicol	Should not be detected

Huge numbers of veterinary drugs containing fluoroquinolones had been introduced into the market by the end of the 1980s and beginning of the 1990s, due to their low toxicity and large antimicrobial spectrum. Fluoroquinolones cause cytostasis and cell death for bacteria or tumors by acting as DNA gyrase and DNA topoisomerase inhibitors (10). Enrofloxacin (EN) -a quinolinemono carboxylic acid that is 1,4-dihydroquinoline-3-carboxylic acid)- (Figure 1a), and

ciprofloxacin (CP) -1-cyclopropyl-6-fluoro-4-oxo-7-piperazin-1-ylquinoline-3-carboxylic acid; - (Figure 1b) are the most widely used fluoroquinolones for veterinary use. Doxycycline (DOX) - 4S, 4aR, 5S, 5aR, 6R, 12aS)-4-(Dimethylamino)-3,5,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a, 5,5a,6,11,12a-octahydrotetracene-2-carboxamide - (Figure 1c), that belongs to the tetracycline class and chloramphenicol (CAM) -2, 2-dichloro-N-[(1R, 2R)-1, 3-dihydroxy-1-(4-nitrophenyl) propan-2-yl]acetamide - (Figure 1d) are recognized as a broad-spectrum antibiotic which have high potency to prevent or cure infections caused by bacteria or some specific parasites for both humans and animals (11).

In order to determine the quantity of antibiotic residues in different forms of food including milk, several analytical methods have been developed utilizing liquid chromatography tandem mass spectrometry (LC-MS/MS). Most of the reported LC- MS/MS analysis methods ~~are suffering from~~ have a drawback of long analysis time (12) and are ~~so~~ too expensive (using solid phase extraction procedure) (13). Some studies using extraction methods including protein direct precipitation step by methanol (14,15). Other reported methods require acetonitrile usage, and this causes the MS detection system susceptible to contamination. (12,16, 17).

Although there are tremendous studies on analysis of antibiotic residues in milk, but none has yet described a fast and throughput method for the simultaneous determination of enrofloxacin, doxycycline, ciprofloxacin, and chloramphenicol in a single run. The present study describes the development and validation of an easy and fast analytical method for simultaneous determination of four antibiotics residues (enrofloxacin, doxycycline, ciprofloxacin, and chloramphenicol) in milk. The overall aim was to obtain a fast and simple LC-MS/MS method with an inexpensive sample preparation protocol.

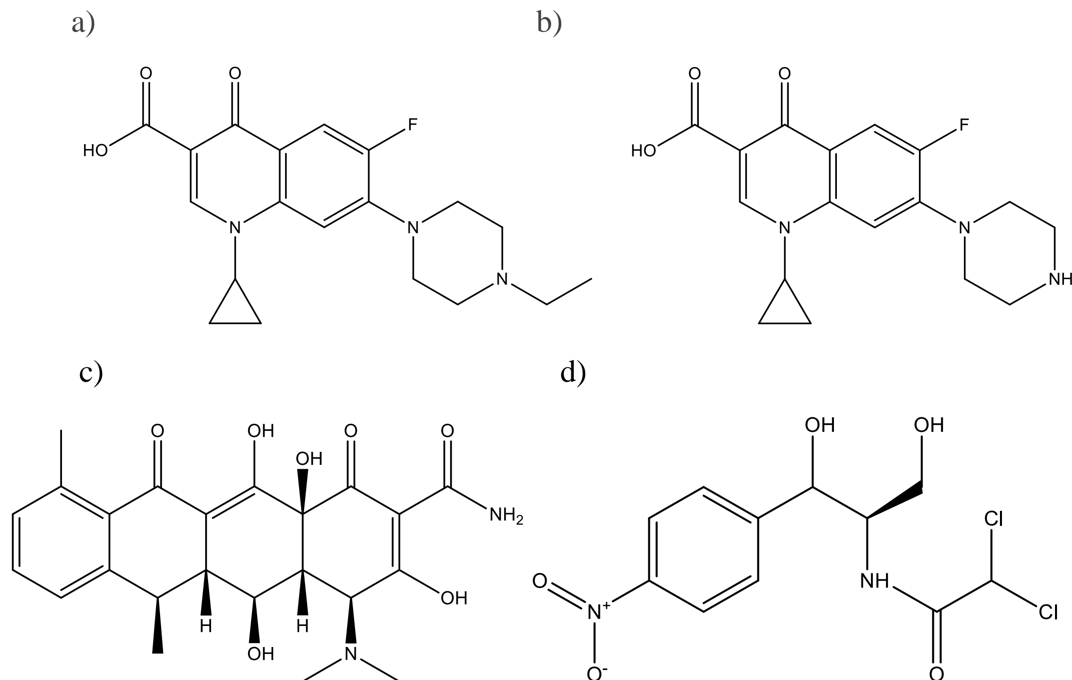

Figure 1: Chemical structure of (a) Enrofloxacin (b) Ciprofloxacin (c) Doxycycline (d) Chloramphenicol

EXPERIMENTAL

Materials

Chloramphenicol palmitate (purity 99.6 %, working standard) was supplied by Mehta Api Private Limited (Mumbai, India). Ciprofloxacin hydrochloride (purity >98%, working standard) was purchased from Shangyu Jingxin (Shangyu, China). Enrofloxacin base (purity 99%, working standard) was provided by Zhejiang Guobang Pharmaceutical (Zhejiang, China). Doxycycline hyclate was purchased from Wuhan Lipharma Chemicals (Wuhan, China) and etoricoxib was supplied by Virdev Intermediates Pvt (Palsana, India). Methanol (chromatographic grade), tert-butyl methyl ether (TBME; chromatographic grade), acetic acid (Optima LC/MS grade), and formic acid (99.0%; LCMS grade) were obtained from Fischer Scientific (Loughborough, USA).

Blank milk for spiking of calibration and quality control samples was obtained from a green cow's farm in a rural region close to Ajloun city in Jordan.

Instrumentation

LC –MS/MS was used consisting of a liquid chromatograph (LC) Agilent 1200 series (USA) and an API 4000 mass spectrometer (AB Sciex, USA). Separation was performed on a 50 mm × 2.1 mm column packed with 5 µm particles (Fortis Universil Cyano; England). A mobile phase consisted of 35% of aqueous 0.5 mM ammonium chloride, 0.08% formic acid and 65% methanol (v/v). The flow rate was 0.4 mL/min and total LC run time was 1 min. The injected volume and injector temperature were 5 µL and 10 ºC, respectively. All analytes and the internal standard were detected on mass spectrometer equipped with a Turbo ion spray ™ interface at 500 ºC evaporation temperature. The operated ionization mode and detection mode were positive Electro-spray (ESI+) and multiple reaction monitoring (MRM), respectively. The optimized precursor ions pairs were m/z 445.1 → 427.5 for DOX, 360.4 → 340.9 for EN, 332.2 → 287.9 for CP, 322.7 → 274.9 for CAM, and 359.0 → 280.0 for etoricoxib. The MS parameters were set as the following: Air (zero grade) as nebulizer and auxiliary gas, nitrogen (ultra-pure) as a curtain and collision gas, curtain gas pressure was 10 psi, collision gas pressure was 10 psi, ion spray voltage was 5500 V, ion source gas one and ion source gas two were 25 and 45 psi, respectively and ion spray temperature was 500 °C. The computational analysis was done utilizing Analyst Software version 1.6.3.

Sample Collection

Three forms of whole fat milk samples, including nine brands of powdered milk, fourteen brands of long – term milk and three brands of untread raw milk, were collected from the Jordanian market and analysed for the presence of antibiotics residues (CAM, EN, CP and DOX).

The collected blank milk samples for spiking of calibrators and quality control samples were obtained from a green cow's farm in a rural region close to Ajloun city in Jordan. Furthermore, an antimicrobial activity screening test was conducted for a long life full fat milk using seeded media with St. aureus gem + be bacteria.

Preparation of Stock and standard working solution

Standard stock solutions of DOX, CAM, EN, and CP were prepared by weighing 10 mg of each individual standard in a10 mL volumetric flask and dissolving it in water: methanol mixture (3:7, v/v) to obtain a final concentration of 1000 µg/mL. A working mixed standard solution with a concentration of 25.0 µg/mL of DOX, CAM, EN, and CP was freshly prepared by transferring 250 µL of the aliquots of the stock solution of each analyte into a 10 mL volumetric flask and making up to volume using a water-methanol mixture (1:1, v/v).

Etoricoxib 2 µg/mL was used as internal standard was prepared using water-methanol mixture (1:1 v/v) as the diluent solvent.

Calibration Curves and Quality Control Samples

Blank milk samples were spiked with 50 µL of IS (2.0 µg/mL etoricoxib). After that, the appropriate volume of working mixed standard solution or standard stock solutions of DOX, CAM, EN, and CP was added to the blank milk samples to get standard solutions. Standards solutions having concentrations equivalent to 10, 20. 50, 100, 300, 600, and 1000 ng/mL DOX, CAM, EN and CP were obtained and used to plot calibration curves. Calibration curves were established by identifying the best fit of peak area ratios (peak - area analytes/ peak area internal standard) versus concentration and fitted to the equation $y = mx + b$ by weighted least-squares regression (1/x).

Quality control (QC) samples were prepared at three concentration levels (low QC= 60 ng/mL, medium QC= 400 ng/mL and high QC= 800 ng/mL).

Extraction

A 5 mL of solvent (dichloromethane- TBME, 15:85, v/v) was added to the 0.5 mL of milk spiked with 2.0 µg/mL of IS. Then, the milk was vortexed for 5 min, centrifuged at 4400 rpm at 5 ºC for 5 min and the organic layer was separated. Then, the organic layers were collected together and evaporated to dryness under dried ultra- pure compressed air at 40°C. The residue was reconstituted with 250 µL of methanol/ water/ acetic acid (70:30: 0.1; v/v/v), vortexed for a 1 minute, and kept in HPLC vials until analysis time. All milk samples were extracted and analysed in triplicate.

Bioanalytical method validations

The method was validated for analyzing antibiotic residues in cow milk in concordance with the European guideline (18), taking into consideration the United States FDA guideline requirements (19). Both guidelines were considered as protocols for all validation sections. The validation was performed in order to evaluate the method in terms of specificity, sensitivity, calibration curve (linearity of response), accuracy, precision, matrix effect and recovery.

Specificity and Selectivity

The presence of endogenous interfering peaks was investigated the by analyzing the extracted blank milk samples from eight different sources and compared to samples spiked with DOX, CAM, EN, and CP at the lower limit of quantitation (LLOQ; 10 ng/mL for DOX, CAM, EN, and (CP). The interfering peak area should not exceed 20% of the peak area of the analyte and 5% of the peak area of the IS at the LLOQ (18,19).

Matrix Effect

The matrix effect for DOX, CAM, EN, and CP was investigated to assess the ion enhancement or suppression of each analyte.. The mean peak area for quality control samples (at low and high QCs) was prepared in 50% methanol and compared to the corresponding samples included extracted blank. The matrix effect was expressed as a matrix factor (MF) and internal standard normalized matrix factor. The MF and internal standard normalized factors were calculated by the following equations:

$$\text{Internal standard normalized factor} = \frac{\text{Matrix factor of analyte}}{\text{Matrix factor of internal standard}}$$

Coefficient of variation (CV) of the IS-normalized MF should not exceed 15%.

Accuracy and Precision

Intra-day precision and accuracy were evaluated by analyzing six replicates of each QC sample on the same day. The selected concentrations of QC samples were 10 ng/mL for DOX, CAM, and EN and CP (LLQC), 60 ng/mL (Low QC), 600 ng/mL (medium QC), and 800 ng/mL (high QC). To evaluate the inter-day precision, the same quality control samples (freshly prepared) were analyzed together with one independent calibration reference curve in three different days. Intra-day precision and inter-day precision were expressed as CV. The acceptance criteria for intra-day and inter-day precision are up to 15% for low OQ, medium OQ, and high OQ samples and up to 20% for LLOQ samples.

Extraction Recovery

Extraction recovery was performed to evaluate the loss of analytes and/or internal standards during sample extraction. Extraction recovery was observed in triplicate at three QC concentration levels

(low QC, medium QC and high QC). Both the extract of spiked samples of blank milk and references spiked to analyte free milk extract (post extract) were analyzed and the extraction recoveries were calculated based on the following equation:

$$\text{Recovery } \% = \frac{\text{Peak area of extracted milk sample}}{\text{Area of blanks spiked with the analyte post extraction}} * 100\%$$

RESULTS AND DISCUSSION

Mass Spectrometric analysis

The optimized MS parameters including precursor ion (Q1), product ion (Q3), declustering potential (DP), entrance potential (EP), collision energy (CE), and cell exit potential (CP) for all analytes and internal standard are listed in Table 2. Intense and stable precursor and product ions were obtained for all analytes and internal standard (IS). In this study a single parent ion and single product ion were detected for each analyte. The parent ions (Q1 [m/z] = 445.1, 360.4, 332.2, and 359.0 correspond with the protonated molecular ions ($[M + H]^+$) of DOX, EN, CP and etoricoxib (IS) respectively, the product ions (Q3 [m/z] = 427.5) corresponds with loss of ammonia from the protonated molecular ions $[M + H\text{-}17]^+$) of DOX. The product ion (Q3 [m/z] = 245.0) is associated with the loss of 1-Ethylpiperazine ($C_6H_{14}N_2$) ring from protonated molecular ion of EN. The product ions (Q3 [m/z] = 287.9 and 280.0) correspond with the loss of CO_2 from the protonated molecular ions $[M + H\text{-} 44]^+$) of CP and the methyl sulfone group from protonated molecular ions ($[M{+}H\text{-} CH_3SO_2]^+$) of etoricoxib, respectively. Analyzing CAM by applying positive ionization mode led to formation of the molecular ion at m/z 322.7 and a fragment ion at m/z 274.9. Comparing fragments obtained from positive ionization of CAM with that reported from negative

ionization, fragments were observed at m/z 321, 152, 194 and 257 when CAM analyzed in the negative ionization mode.

Table 2: Optimized MS/MS Parameters Used in Validation of DOX, CAM, EN, and CP

Analyte	Q1 (m/z)	Q3 (m/z)	DP (V)	EP (V)	CXP (V)
DOX	445.074	427.500	50	7	25
CAM	322.705	274.900	30	10	12
EN	360.411	245.00	75	9	15
CP	332.187	287.900	75	15	15
Etoricoxib (IS)	359.000	280.000	70	10	12

Figure 2A represents MRM chromatograms for extracted drug-free milk sample with IS only. In this chromatogram, the interfering peaks are almost absent at the retention times of DOX, CAM, EN, and CP. Figures 2B, 2C, 2D, and 2E are representative chromatograms for CAM, EN, and CP, respectively, at the LLOQ level (10 ng/mL). The peak areas observed at the retention time of all analytes in Figure 2a were less than 20% of the LLOQ peak areas of EN, DOX, CAM, and CP in the Figures from 2b to 2e.

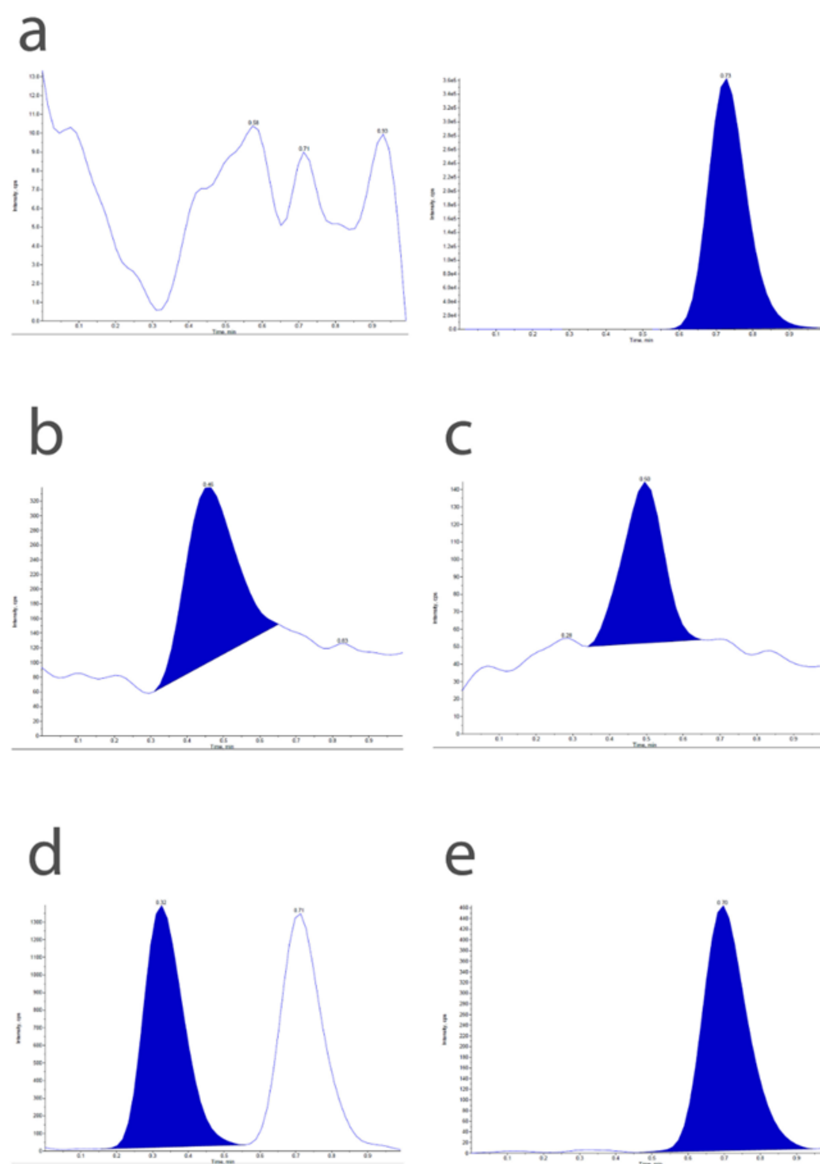

Figure 2: MRM chromatograms of (a) spiked blank milk sample with IS only (b) DOX at LLOQ concentration, 10 ng/ml, (c) at LLOQ concentration, 10 ng/mL (d) EN at LLOQ concentration,, 10 ng/mL and € CP at LLOQ concentration 10 ng/mL

Linearity

The analytical performance of this method is summarized in Table 3. The mean linear equations for calibration curves were y = 0.00016 x -0.00083 for DOX, y = 0.00018 x + 0.00040 for CAM, y = 0.00049 x -0.00173 for EN and y = 0.00010 x + 0.00009 for CP. The studied concentration

ranges were 10.0-1000 ng/mL for DOX, CAM, EN, and CP. The correlation coefficients (R^2) were equal to or greater than 0.9972 for all analytes in the studied concentration ranges, and this confirms good linearity relationships by the internal standard calibration curve. LLOQ values were in the range of 10 ng/mL for EN, DOX, CAM and CP. All values of LLOQ were below the regulatory limits (MRLs) which set by the European Commission.

Table 3: Retention Times (t_{RS}), Calibration Curve Equilibrations, (R^2), and LLOQs, for DOX, CM, EN, and CP.

Analyte	t_R (min)	Linear equation	Linear range (ng/L)	R^2	LLOQ (ng/L)
DOX	0.52	Y = 0.00016 X -0.00083	10.000 – 1000.0	0.9977	10
CAM	0.43	Y = 0.00018 X + 0.00040	10.000 – 1000.0	0.9987	10
EN	0.52	Y = 0.00049 X -0.00173	10.000 – 1000.0	0.9987	10
CP	0.52	Y = 0.00010 X + 0.00009	10.000 – 1000.0	0.9973	10
Etoricoxib (IS)	0. 71	2.0 µg/mL	-	-	-

Matrex Effect

The matrix effect for DOX. CAM, EN, and CP in milk were studied by calculating the matrix factor (MF) and the internal standard - normalized matrix factor (IS-normalized MF). The results of the matrix effect are summarized in Table 4. No apparent matrix effect was observed for CAM, and CP (MF is higher than 0.85), while a slight ion suppression was observed for DOX in high QC samples (MF = 0.84), and for EN in high and low QC samples (MF= 0.83 and 0.81) respectively. The internal standard-normalized matrix factor was in the range of 0.90 -1.11; this confirms that the internal standard can correct the change in any potential matrix effect for DOX, CAM, EN and CP. Maximum observed CV referred to a low level of QC samples of CP and was equivalent to 5.7%. This data meets the acceptance criteria set by the European Medicines Agency

(18, 19). Although milk is considered a complex matrix and contains a high content of protein and fat, our sample pretreatments is effective in reducing the matrix effect.

Table 4: Matrix Factor and Internal Standard normalized Matrix Factor

Analyte	Concentration ng/mL	MF	IS-Normalized MF	CV (%)
DOX	low	0.869	0.912	3.31
	high	0.839	0.902	2.24
CAM	low	0.902	0.912	3.86
	high	0.890	1.110	3.93
EN	low	0.830	0.910	4.69
	high	0.810	0.900	4.20
CP	low	0.942	1.098	5.74
	high	0.958	0.901	4.08

Specificity and Selectivity

The presence of endogenous interfering peaks was investigated the by analyzing the extracted blank milk samples from eight different sources and compared to samples spiked with DOX, CAM, EN, and CP at the lower limit of quantitation (LLOQ; 10 ng/mL for DOX, CAM, EN, and CP). The interfering peak area should not exceed 20% of the peak area of the analyte and 5% of the peak area of the IS at the LLOQ (18,19). Furthermore, to confirm the validity of used blank, an antimicrobial activity screening test was conducted for a long life full fat milk using seeded media with St. aureus gem + be bacteris, the seeded media inculated with the milk as sample and purified water as blank (- be control). After incubation overnight good growth of bacterial appears with no sign of inhibition zone of growth around the milk sample or the -be control.

Accuracy and Precision

The accuracy and precision (within-day and between day) were estimated by analyzing the spiked blank samples of milk at four concentration levels, LLOQ (10 ng/mL for CAM, DOX, EN and CP), low QC (60 ng/mL), medium QC (400 ng/mL), and High QC (800 ng/mL). In accordance with the Commission Decision 2002/657/CE of the EU, accepted accuracies are extended from 85% -110%. The maximum acceptable variance coefficient (CV) should not be more than 15% for analyte concentration at low QC, medium QC and high QC or 20 % for analyte concentration at LLOQ. As shown in Table 5, data showed accuracies were ranged from 92.48% to 110.05% and the relative error of accuracy did not exceed 20%. The CV values of both inter-day and intra-day were below 12.66% and 11.76%, respectively. These results revealed that the validated method has good accuracy, precision, and convenient for daily routine analysis.

Table 5: Accuracy (%) with Relative Standard Deviation, within- and between-Day Precision Values for Quality Control Samples

Within Run Accuracy (%) and R.S.D				
Concentration				
Analyte	**10 ng/mL**	**60 ng/mL**	**400 ng/mL**	**800 ng/mL**
DOX	104.57 ± 13.41	100.21 ± 11.33	96.59 ± 9.80	109.36 ± 7.93
CAM	94.91 ± 9.43	100.11 ± 4.89	98.26 ± 2.94	99.32 ±11.47
EN	110.05± 4.05	105.12 ± 8.72	98.65 ± 8.46	108.56 ± 8.36
CP	92.48 ± 19.48	100.19 ± 4.50	101.62 ± 6.19	98.80 ± 2.58
Within-day precision (CV %)				
DOX	9.68	2.60	5.43	9.68
CAM	10.09	11.76	12.71	10.09
EN	10.09	11.76	12.71	10.09
CP	19.67	3.03	12.28	4.78
Between-day precision (CV %)				
DOX	12.09	9.68	2.68	5.44
CAM	11.89	10.08	11.78	11.52
EN	12.60	8.52	12.66	5.11
CP	10.09	3.63	2.81	7.75

Extraction recovery

Extraction recoveries are listed in Table 6. The mean recoveries were 88.70% for CAM and 61.67% for EN. This data confirms that the extraction method has been used is stable and efficient for CAM and EN. On the other hand, the extraction recoveries were reported as 4.50 % for CP and 10.02% for DOX. The extraction recovery of IS at 2.0 µg /mL was estimated to be 61.57% ±7.72. In spite of low recovery for CP, it was accepted based on the guideline's acceptance criteria.

Table 6: Extraction Recovery with Relative Standard Deviation (RSD) for DOX, CAM, EN and CP

Analyte	60 ng/mL	400 ng/mL	800 ng/mL	Mean recovery (%)	CV (%)
DOX	11.35	9.14	9.56	10.02	11.72
CAM	71.89	86.38	107.83	88.70	20.39
EN	59.48	55.28	70.25	61.67	12.52
CP	4.15	4.80	4.54	4.50	7.27

Application of the Method to Quantify DOX, CAM, EN and CP in Milk Samples

The proposed method was applied to determine DOX, CAM, EN, and CP in 26 milk samples (14 samples of long-term milk, 9 samples of powdered milk, and 3 samples of untreated raw milk samples). First, non-targeted screening was performed from the obtained samples of green cow's farm (which already confirmed by antibacterial activity test) to find samples that may contain one or more of the analytes (DOX, CAM, EN and CP) in the absence of the IS. Then, IS was applied to validate and quantify the analytes. In this study, out of the total 26 samples of milk, ciprofloxacin was detected in only two samples of untreated raw milk (2.5%). Ciprofloxacin was detected at a trace level (below LLOQ (BLLOQ), 8.75 and 9.77 ng/L). Although the extraction recovery of CP suffers from limitation, an adequate response to CP was observed. Concentrations of the detected antibiotic residues summarized in Table 7.

Table7: Concentrations in ng/mL found in Milk Samples

Sample tested (Milk Code number)	Milk sample form	DOX	CAM	EN	CP	Comment
01	Long term liquid	negative	negative	negative	negative	BLLOQ
02	Long term liquid	negative	negative	negative	negative	BLLOQ
03	Long term liquid	negative	negative	negative	negative	BLLOQ
04	Long term liquid	negative	negative	negative	negative	BLLOQ
05	Long term liquid	negative	negative	negative	negative	BLLOQ
06	Long term liquid	negative	negative	negative	negative	BLLOQ
07	Long term liquid	negative	negative	negative	negative	BLLOQ
08	Long term liquid	negative	negative	negative	negative	BLLOQ
09	Long term liquid	negative	negative	negative	negative	BLLOQ
10	Long term liquid	negative	negative	negative	negative	BLLOQ
11	Long term liquid	negative	negative	negative	negative	BLLOQ
12	Long term liquid	negative	negative	negative	negative	BLLOQ
13	Long term liquid	negative	negative	negative	negative	BLLOQ
14	Long term liquid	negative	negative	negative	negative	BLLOQ
15	Powdered	negative	negative	negative	negative	BLLOQ
16	Powdered	negative	negative	negative	negative	BLLOQ
17	Powdered	negative	negative	negative	negative	BLLOQ
18	Powdered	negative	negative	negative	negative	BLLOQ
19	Powdered	negative	negative	negative	negative	BLLOQ
20	Powdered	negative	negative	negative	negative	BLLOQ
21	Powdered	negative	negative	negative	negative	BLLOQ
22	Powdered	negative	negative	negative	negative	BLLOQ
23	Powdered	negative	negative	negative	negative	BLLOQ
24	Raw untreated	negative	negative	negative	Negative	8.75
25	Raw untreated	negative	negative	negative	negative	9.78
26	Raw untreated	negative	negative	negative	negative	BLLOQ

All selected cow's milk samples including their natural fat contents, to avoid pre-extraction for targeted analyts. Such current findings are in agreement with the previous studies of simlar objective that found a small percentage of antibiotic residiues with many of cow's milk samples of different brands (12, 20).

CONCLUSION

This research is establising a suitable method of analysis which can be ussed as a simple method for detection some antibiotic residues, especially these antibiotics widely used for the prevention and treatment of disease in the milk producing animals. This validated LC-MS /MS method proposes a fast and high throughput method for simultaneous quantification of four antibiotic (DOX, CAM, EN, and CP) in milk samples has been obtained from the Jordanian market. The developed extraction method and sample pretreatment were simple, low cost and easy to use. The LLOQ was also much lower than the maximum residue limit. Morever this method shows good linearity, high accuracy and high precision which can be used for QC analysis of the residues of the above mentioned products.

Development of the method of analysis using LC/MS for some antibiotics residues in milk in the Jordan market is a very important for safety of consumer health and at the same time to remove or reject the contaminated milk product from the market place.

Conflict of interest: The authors declare no conflict of interest.

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