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Analysis of Quinolones Residues in Milk using High Performance Liquid Chromatography

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A B S T R A C T

In the present study, High Performance Liquid Chromatography with Ultra-Voilet detector (HPLC-UV) technique was standardized and validated for the detection and quantitation of quinolones antimicrobial residues viz. enrofloxacin, norfloxacin and ciprofloxacin from milk. The standardization procedure showed that the values for the system precision (% RSD) for both the analytes was <11% for area and <0.9% for retention time), linearity (r^2>0.98), specificity and accuracy (70-110%) and precision (<10%) were within accepted range and demonstrated system suitability for analysis of milk samples. The standardized and validated method was applied for the detection of quinolones residues from 100 randomly milk samples collected from local market of Hisar (Haryana). Mean concentrations of norfloxacin and enrofloxacin antimicrobial residues in market milk samples were 3.54 and 2.02 µg/kg, respectively. A total of 8 samples were found to be containing quinolone antimicrobial residues. Comparison of antimicrobial concentration in each positive sample of milk with international MRLs showed that, none of the three antimicrobial was responsible for violations of set residue limits. It was concluded that milk is significant source of antimicrobial residues.

Introduction

Since the early 1960s, there has been two-fold increase in per capita milk consumption of developing countries. This increased demand of milk made it essential to adopt extensive animal husbandry practices. Use of veterinary drugs for taking cure of variety of ailments in farm animals is an integral component of such extensive animal husbandry practices. Antibiotics are the most widely used veterinary drugs for therapeutic and prophylactic purposes and also as growth promoter in dairy animals which may appear in milk as residues for a certain time period (Wassenaar, 2005). They are also be used at sub-therapeutic levels to increase feed efficiency, promote growth and prevent diseases (Ronquillo and Harnandez, 2016). According to one estimate, approximately 80% of the food-producing animals receive medication for part or most of their lives (Pavlov *et al.*, 2008). The use of antibiotics therapy to treat and prevent udder infections in cows is a key component of mastitis control in many countries. The extra-label use

treatment of human infections, insufficient withdrawal period and lack of records are the most common causes of these residues in milk. In addition the lack of good veterinary practice and illegal use of veterinary drugs by farmers will increase this problem (MacEven *et al.*, 1991).

Nowadays, beta-lactams (penicillin G, ampicillin, amoxicillin etc), aminoglycosides (streptomycin, neomycin etc) and tetracycline (tetracycline, oxytetracycline etc) antibiotics are the most frequently used antimicrobials for treatment of mastitis in dairy cows and consequently, the most commonly found residues in milk (Gustavsson *et al.*, 2004). Also several quinolones such as danofloxacin, difloxacin, enrofloxacin are specifically used in veterinary medicine (Reeves, 2012). Although use of antimicrobials is essential, its frequent use may result in occurrence of drug residues in food products viz. meat and milk obtained from exposed animals.

Fluoroquinolones are synthetic class broad spectrum antibacterials primarily active against Gram negative pathogens. These are effective for the therapy of serious infections, e.g. septicemia, gastroenteritis and respiratory diseases and also used for the treatment of infections of the urinary tract and soft tissues (Nizamlıoglu and Aydın, 2012). They are effective in the therapy of mycoplasma infections and infections caused by atypical bacteria (Navratilova *et al.*, 2011). In veterinary medicine, they are useful especially in the therapy for gastrointestinal and respiratory tract infections, enrofloxacin being the most widely used fluoroquinolone in veterinary medicine (Monica *et al.*, 2011). Fluoroquinolone preparations are also used for the prevention and treatment of mastitis in lactating cows and for dry cow therapy (Gruet *et al.*, 2001).

Antimicrobials causes broad range of health effects, to summarize they can cause development anomalies e.g. bone marrow aplasia and can alter the normal gastrointestinal microflora resulting in GI disturbances and development of resistant strains of bacteria. Therefore, the use of antimicrobials may result in emergence of antibiotic resistant strains of pathogens, complicating the treatment for both human and animal diseases (Dewdney *et al.*, 1991; Goffova *et al.*, 2012). In addition some of the antibacterial may act as carcinogens and pro-carcinogens.

Widespread use of antimicrobials has created potential residue problems in milk and milk products making it an important public health hazard. In India especially Haryana, there is a paucity of reports related to occurrence of antimicrobial residues in milk. Therefore, the present investigation was planned with the objective to standardize the high performance liquid chromatography (HPLC) technique for detection and quantification of quinolones antimicrobial residues.

Materials and Methods

Collection of samples

The present work was carried out in the Department of Veterinary Public Health and Epidemiology, LUVAS, Hisar. For this, 100 milk samples were randomly collected from local market of Hisar, among which, 80 samples of raw milk and 20 samples of pasteurized milk of various brands were included. Samples were collected in sterile plastic bottles and stored at -20°C till analysis.

Chemicals and Reagents

The analytical standards of antimicrobials viz. norfloxacin, ciprofloxacin, enrofloxacin having purity more than 98% were procured from Sigma-Aldrich. Supelclean™ LC-18 SPE

Tube having bed wt. 500 mg and volume 3 mL were also procured from Sigma-Aldrich. HPLC grade solvents namely methanol and acetonitrile were procured from Fisher Scientific whereas anhydrous sodium sulphate was procured from Qualigens. HPLC grade water was prepared in the laboratory using Millipore (Bedford, MA, USA) Milli-Q system to give a resistivity of at least 18.2 M Ω cm.

Preparation of standards

The primary standard solution of each antimicrobial was prepared by dissolving neat standards of quinolones in methanol by using class A glassware (Final volume 25 ml) so that effective concentration remained more than 100 µg/mL. Secondary standard solutions, the maximum residue limits (MRLs) prescribed by European Union (EU, 2010) for all antibiotics were considered. Based on these MRL values, a linearity range was selected (50, 100, 150, 200, 250 ng/ml) for quinolones, Then appropriate dilutions of secondary standard solution in same solvent were made to produce a required dilution of working solution. Mobile phase used for the instrumental analysis of quinolones was composed of solvent A (water: formic acid at 1000:1 v/v) and solvent B (water: acetonitrile: formic acid (at 100:900:1 v/v/v). In the present study, HPLC-UV method was standardized and validated for the determination of quinolones i.e. enrofloxacin, norfloxacin and ciprofloxacin based on the method reported by Stolker *et al.,* (2008) with slight modifications.

Sample extraction and cleanup

Laboratory method for detection of quinolone residues in milk was standardized as per the protocol proposed by Stolker *et al.,* (2008) with slight modifications. 10 ml of spiked milk sample was taken in centrifuge tube and mixed with 25-30 g sodium sulphate until slurry was formed. Twenty millilitre acetonitrile was added to it and centrifuged at 7000 rpm for 15 minutes. 15 mL of the supernatant was taken out in a beaker and 10 mL of acetonitrile was again added to the centrifuge tube and re-centrifuged (7000 rpm/15 minutes). Supernatant was collected in a 50 mL beaker. This procedure was repeated again and supernatant was added to previously collected extract in measuring cylinder.

For sample cleanup, solid phase C_{18} cartridge was attached to vacuum manifold and activated with 6 ml methanol followed by 6 ml water using vacuum manifold. Sample extract was loaded on the activated cartridge. Then cartridge was eluted using 15 mL methanol. The cleaned up extract as well as eluent was collected in pear shaped evaporating flask and evaporated to dryness at 55ºC using a rotary evaporator. Residue in flask were redissolved in 2 mL methanol and subjected for chromatographic analysis for quinolones.

Chromatographic analysis

A Shimadzu prominence UFLC system equipped with DGU-20A5R degasser, SIL-20A HT autosampler and LC-20AD pump connected to C_8 column (Enable 4.6 mm x 250 mm porosity 5 µm) housed in CTO-10AS column oven with SPD-20A UV-VIS detector was used. Operating conditions of the instrumental methods were as detailed Table 1.

Results and Discussion

Standardization and validation studies

System precision

The system precision was evaluated by studying the reproducibility of the

instrumental response with respect to retention time and area of an analyte Retention time of the analytes were 4.293 ± 0.035, 4.604 ± 0.009 and 5.426 ± 0.007, for norfloxacin, ciprofloxacin, enrofloxacin, respectively. Relative standard deviation (RSD) of retention time was in range of 0.13 - 0.82 % for quinolones. Relative standard deviation (RSD) of the area under curve was in the range of 2.98–10.65% % for quinolones. Chromatograms of analytical standard mix solution demonstrating separation efficiency in comparison with solvent blank are shown for quinolones (Figure 1).

Specificity

It was evaluated by visual observation of chromatograms of blank sample matrix and sample matrix spiked with standard mixture. For milk, chromatogramic signals at the retention times of quinolones viz. enrofloxacin, norfloxacin and ciprofloxacin were absent in blank sample matrix. The zoomed portion of chromatogram covering the time scale of retention time of each of analytes is depicted in Figure 2 (A to C).

Linearity

The standard calibration curves of the analyzed quinolones standards presented a good regression line (r2>0.98) in the range of explored concentrations i.e. 50 to 250 µg/kg for all three analytes.

Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ were determined by measuring the magnitude of the background response was analyzed by 10 blank samples and calculated by standard deviation of this response. Table 2 summaries the LOD and LOQ obtained for each analytes of quinolones group.

Accuracy

Accuracy was estimated on the basis of ability of the method to recover the known spiked quantity of quinolones antimicrobials in milk. It is expressed as percent average recovery and evaluated for each analyte of quinolones group at five different fortification levels i.e. 50 to 250 µg/kg for all three analytes i.e. enrofloxacin, norfloxacin and ciprofloxacin. Table 3 shows the accuracy of method for detection of quinolones.

Precision

The precision expressed as relative standard deviation and was assessed at five concentration levels i.e. 50 to 250 µg/kg for all quinolones. Repeatability and intermediate precision values, (CV percent) were found less than 9 for all analytes of quinolones (Table 4).

Overall the method followed for multiresidue detection and quantification of quinolones antibiotic residues in milk was subjected to rigorous validation parameters. The system precision values indicated a good consistency in response by the HPLC instrument used during present study. A good linearity was noted for standards and spiked milk samples. Absence of interfering peaks in blank samples was indicating good specificity of extraction and cleans up method. In comparison with international guidelines the, accuracy and precision of the method were found to be in accepted range. These results of validation studies were evident that the present method is suited for routine analysis of quinolones in milk.

Determination of residues of quinolones in milk

After successful standardization and validation, the technique for detection of

quinolones residues was implemented for on extraction, detection and quantification of 100 milk samples randomly collected from the local market of which 40 samples were obtained from vendors, 40 samples from mini dairies (private milk collection and selling counters), whereas, 20 samples of pasteurized milk were obtained from retail shops of Hisar city. The occurrence of quinolones residues with their mean concentration in milk samples is presented in Table 5. The results revealed that absolute mean concentration of quinolones was 5.56 µg/kg in which the residual concentrations of norfloxacin and enrofloxacin were 3.54 and 2.02 µg/kg respectively.

In the present study, out of 100 samples analysed for antimicrobial residues in the present study, 8 (8%) samples were found positive for quinolone antimicrobials with highest occurrence of norfloxacin residues followed by enrofloxacin residues. In the present study, none of the milk sample was found positive for ciprofloxacin residues. However, Gaurav *et al.,* (2014) reported the presence of ciprofloxacin 9.2 % milk sample collected from various districts of Punjab.

The results are summarized in Table 6. Positive samples were equally associated with vendor milk and dairy milk and not with pasteurized milk. Studies reported by other scientists also showed presence of quinolones in milk from different countries. Chung *et al.,* (2009) recorded a minor prevalence (0.3 %) of quinolones in milk samples obtained from Korean market. In an another study conducted by Junza *et al.,* (2010) in Spain for detection of quinolones and β-lactams in milk using LC, 3% samples were found to be positive for quinolones out of 49 samples analysed. A very high prevalence of 87.3% of flouroquinolones was reported by Navratilova *et al.,* (2011) in bulk samples of raw cow's milk from Czech Republic. Similarily, Zhang *et al.,* (2014) analyzed 120 samples in China for the detection of quinolones residues in milk and found 86 % samples with detectable levels of residues. In India, Moharana *et al.,* (2015) reported the presence of enrofloxacin residues in 21% milk samples out of 120 samples analysed.

The concentration of each of the antimicrobial under study in each of the milk samples (if detected) was compared with available MRLs set forth by the EU. Amongst the antimicrobials included in the present study, EU MRLs are available only for enrofloxacin (100 µg/kg) in milk. No sample was found to have antimicrobial residue above the set residue limits.

Table.1 Specific HPLC conditions for each antibiotic

Parameters	Enrofloxacin	Norfloxacin	Ciprofloxacin
Mobile-phase A:B	75: 25	75: 25	75: 25
Detection wavelength	280 nm	280 nm	280 nm
Flow rate	1 ml/min.	1 ml/min.	1 ml/min.
Oven temperature	30 ºC	30 ºC	30 ºC
Injection volume	40 µl.	40 µl.	40 µl.
Runtime	20 min.	20 min.	15 min.

Table.2 Limit of detection (LOD) and limit of quantitation (LOQ) for quinolones antimicrobials

Group of antimicrobials	Analyte	LOD(µg/kg)	LOQ(µg/kg)
Quinolones	Norfloxacin	146.96	270.83
	Ciprofloxacin	38.55	98.49
	Enrofloxacin	24.56	47.91

Table.3 Accuracy of quinolones antimicrobials spiked in milk

Analyte	Accuracy (%Average recovery ± SD)				
	50	**100**	**150**	**200**	**250**
Norfloxacin	108.15±5.92	104.08±3.96	112.70±3.18	109.46±3.85	107.09±2.90
Ciprofloxacin	104.35±4.02	103.47±6.77	109.38±4.18	102.16±2.60	101.77±2.14
Enrofloxacin	109.03±9.06	107.13±5.43	107.51±3.48	104.14±3.38	101.51±1.60

SD= Standard deviation, RSD = Relative Standard Deviation

Table.4 Precision of quinolones antimicrobials spiked in milk

Group of antimicrobials	Analyte	Precision (% RSD)				
		50	**100**	**150**	**200**	**250**
Quinolones	Norfloxacin	5.47	3.81	2.82	3.52	2.71
	Ciprofloxacin	3.85	6.55	3.82	2.54	2.10
	Enrofloxacin	8.31	5.07	3.24	3.71	1.57

Table.5 Mean concentrations of quinolones in milk samples

Group of antimicrobials	Analyte	Mean concentration (µg/kg)			
		Raw milk-Vendor (n=40)	**Raw milk-Dairy (n=40)**	**Pasteurized milk (n=20)**	**Total (n=100)**
Quinolones	Norfloxacin	8.89	BDL	BDL	3.54
	Ciprofloxacin	BDL	BDL	BDL	-
	Enrofloxacin	5.05	BDL	BDL	2.02

BDL- Below detection limit

Table.6 Distribution of positive samples for each analyte

Group of antimicrobials	Analyte	Raw milk samples		Pasteurized milk samples (n=20)
		Vendor milk (n=40)(% positive)	Mini dairies milk(n=40)	
Quinolones	Norfloxacin	3 (7.5%)	4(10%)	0(0%)
	Ciprofloxacin	0 (0%)	0 (0%)	0(0%)
	Enrofloxacin	1(2.5%)	0 (0%)	0(0%)

* Values in parenthesis indicate percentage

Fig.1 Chromatogram of solvent blank and standard mix of quinolones

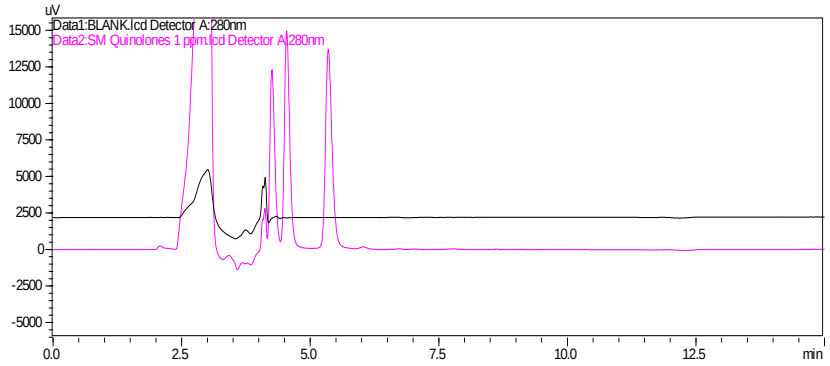
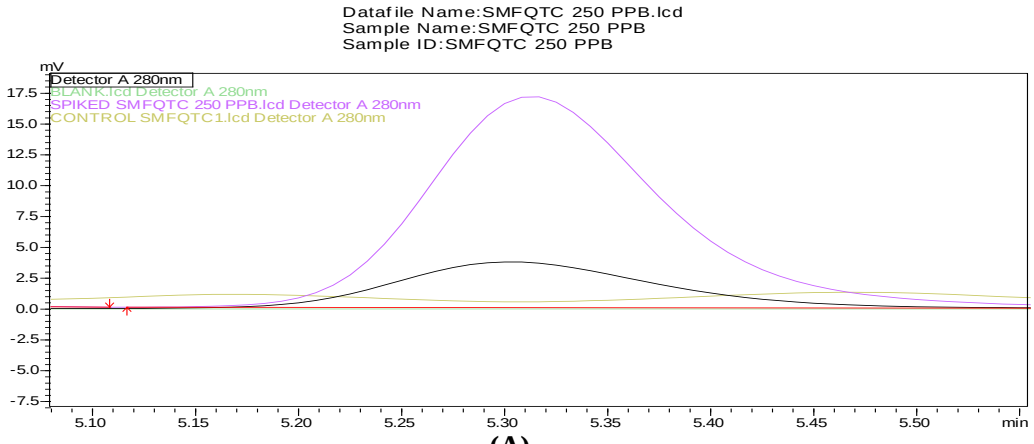
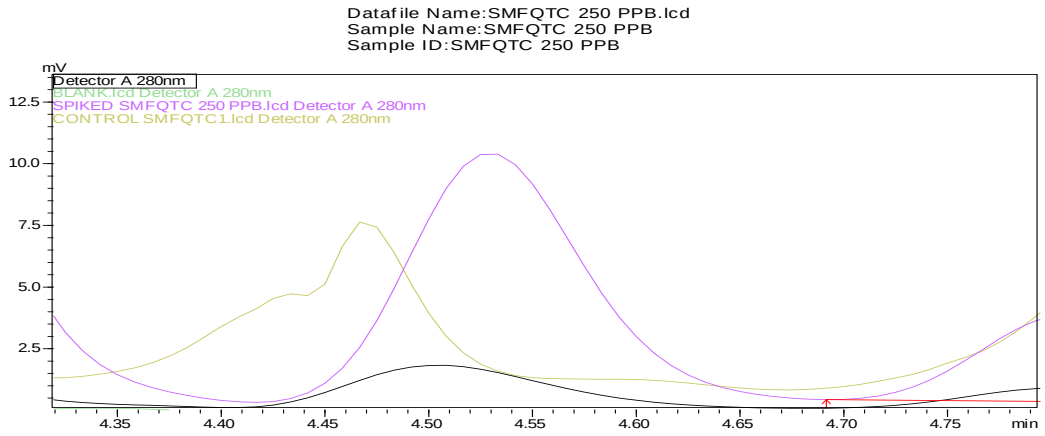


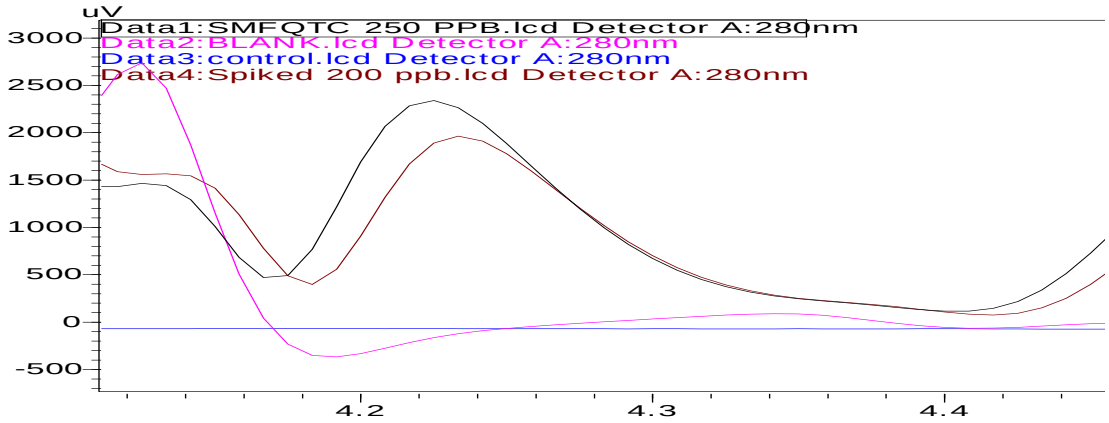
Fig.2 Comparison of chromatograms of blank and spiked milk samples demonstrating specificity Enrofloxacin (A), Ciprofloxacin (B), Norfloxacin (C)



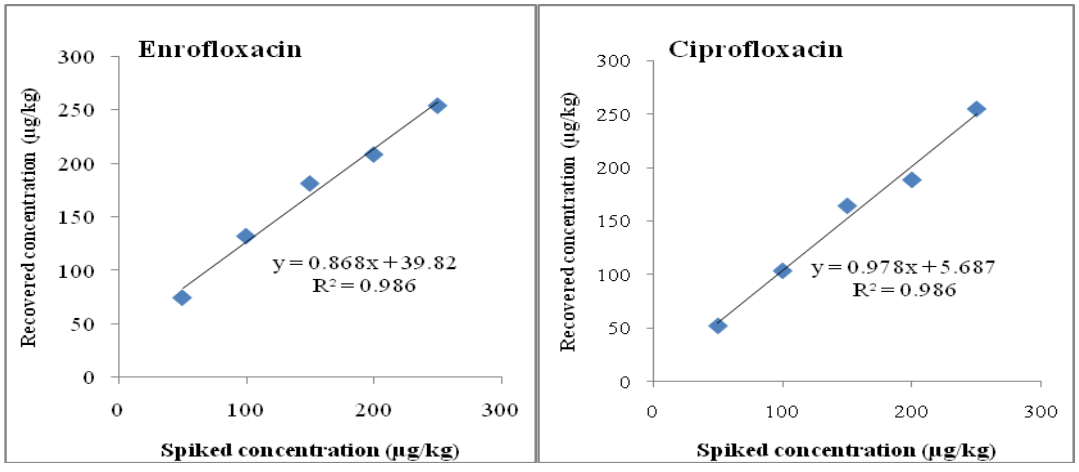
(A)

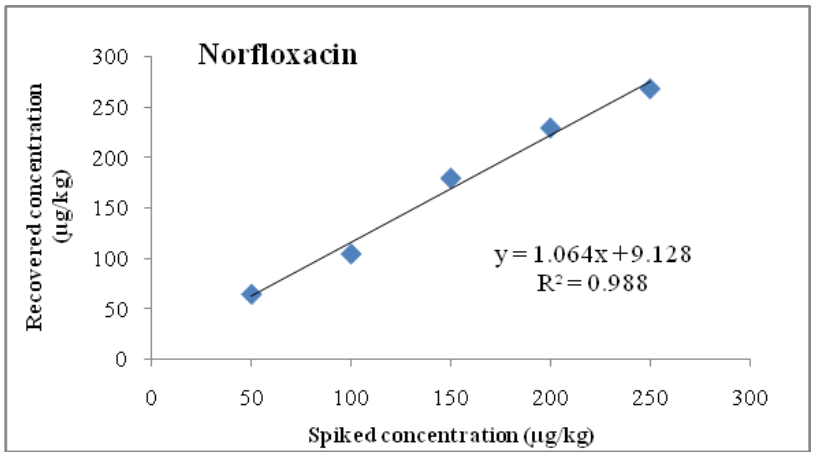

Datafile Name:SMFQTC 250 PPB.lcd
Sample Name:SMFQTC 250 PPB
Sample ID:SMFQTC 250 PPB
mV
Detector A 280nm
BLANK.lcd Detector A 280nm
SPIKED SMFQTC 250 PPB.lcd Detector A 280nm
CONTROL SMFQTC1.lcd Detector A 280nm
12.5
10.0
7.5
5.0
2.5
4.35
4.40
4.45
4.50
4.55
4.60
4.65
4.70
4.75
min

(B)


uV
Data1:SMFQTC 250 PPB.lcd Detector A:280nm
Data2:BLANK.lcd Detector A:280nm
Data3:control.lcd Detector A:280nm
Data4:Spiked 200 ppb.lcd Detector A:280nm
3000
2500
2000
1500
1000
500
0
-500
4.2
4.3
4.4

(C)


Enrofloxacin
Recovered concentration (µg/kg)
Spiked concentration (µg/kg)
y = 0.868x + 39.82
R² = 0.986
Ciprofloxacin
Recovered concentration (µg/kg)
Spiked concentration (µg/kg)
y = 0.978x + 5.687
R² = 0.986



Based on the frequency of detection and concentration of analytes, the milk samples were found to be contaminated with antimicrobial residues of quinolones group. On the basis of findings of the present study it can be concluded that, the antibiotic residues in milk is more it may be because of lack of awareness of farmers about the withdrawal period of milk during the treatment period. However, further monitoring studies are required to produce residue free milk for consumers.

In conclusion, the present work was envisaged to standardize and validate the liquid chromatographic methods for detection of quinolones antimicrobials (enrofloxacin, norfloxacin and ciprofloxacin) in milk. Total 8% samples were found positive for quinolones residue with high prevalence of residues in raw milk samples. Out of the all raw milk samples, vendor milk samples were found highly contaminated with quinolones residues followed by mini dairy samples. None of the pasteurized milk sample was having any residues.

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References

Chung, H.H., Lee, J.B., Chung, Y.H. and Lee, K.G. (2009). Analysis of sulfonamide and quinolone antibiotic residues in Korean milk using microbial assays and high performance liquid chromatography. Food Chem., 113(1): 297-301.

Dewdney, J.M., Maes, L., Raynaud, J.P., Blanc, F., Scheid, J.P., Jackson, T. and Verschueren, C. (1991). Risk assessment of antibiotic residues of β-lactams and macrolides in food products with regard to their immuno-allergic potential. Food Chem. Toxicol. 29(7): 477-483.

European Commission (EC). (2010). Council Regulation No. 37/2010. On pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. O.J.EU. 15: 1-72.

Gaurav, A., Gill, J.P.S., Aulakh, R.S. and Bedi, J.S. (2014). ELISA based monitoring and analysis of tetracycline residues in cattle milk in various districts of Punjab. Vet. World. 7(1): 26-29.

Goffova, Z.S., Kozarova, I., Mate, D., Marcincak, S., Gondova, Z. and Sopkova, D. (2012). Comparison of detection sensitivity of five microbial

inhibition tests for the screening of aminoglycoside residues in fortified milk. Czech J. Food Sci. 30(4): 314-320.

Gruet, P., Maincent, P., Berthelot, X. and Kaltsatos, V. (2001). Bovine mastitis and intramammary drug delivery: review and perspectives. Advanced Drug Delivery Reviews: 50, 245-259.

Gustavsson, E., Degelaen, J., Bjurling, P. and Sternesjo, A. (2004). Determination of beta-lactams in milk using a surface plasmon resonance based biosensor. J. Agric. Food Chem. 19: 2791-2796.

Junza Martinez, A., Amatya, R., Perez Burgos, R., Gokce, G., Grzelak, E., Barron Bueno, D. and Barbosa Torralbo, J. (2010). Residues of b-lactams and quinolones in tissues and milk samples. Confirmatory analysis by liquid chromatography mass spectrometry. Ovidius Univ. Annal. Chem. 21(2): 109-122.

McEwen, S.A., Black, W.D. and Meek, A.H. (1991). Antibiotic residue prevention methods, farm management, and occurrence of antibiotic residues in milk. Journal of Dairy Science. 74: 2128- 2137.

Moharana, B., Karthick Venkatesh, P., Preetha, S.P. and Selvasubramanian, S. (2015). Quantification of enrofloxacin residues in milk samples using RPHPLC. World J. Pharm. Pharm. Sci. 4(10): 1443-1450.

Monica, B., Patra, P.H., Khargaria, S., Manna, S., Chakraborty, A.K. and Mandal, T.K. (2011). Immunological and haematobiochemical studies of enrofloxacin with special reference to residue level following oral administration in goats, Indian Journal of Animal Sciences. 81: 904-907.

Navratilova, P., Borkovcova, I., Vyhnalkova, J. and Vorlova, L. (2011). Fluoroquinolone residues in raw cow's milk. Czech J. Food Sci. 29(6): 641-646.

Nizamlioglu, F. and Aydin, H. (2012). Quinolone antibiotic residues in raw milk and chicken liver in Konya. Eurasian J. Vet. Sci. 28: 154-158.

Reeves, P.T. (2012). Antibiotic groups and properties. In: Chemical analysis of antibiotic residues in food. New Jersey (USA): Wiley Publishing, pp.30-31.

Ronquillo, M.J. and Hernandez, J.C.A. (2016). Antibiotic and synthetic growth promoters in animal diets: review of impact and analytical methods. Food Control. 30: 1-13.

Stolker, A.A., Rutgers, P., Oosterink, E., Lasaroms, J.J., Peters, R.J., Van Rhijn, J. A. and Nielen, M. W. (2008). Comprehensive screening and quantification of veterinary drugs in milk using UPLC–ToF-MS. Anal. Bioanal. Chem., 391(6): 2309-2322.

Wassenaar, T.M., (2005). Use of antimicrobial agents in veterinary medicine and implications for human health. World Applied Sciences Journal. 31: 155-169.

Zhang, Y.D., Zheng, N., Han, R.W., Zheng, B.Q., Yu, Z.N., Li, S.L. and Wang, J.Q. (2014). Occurrence of tetracyclines, sulfonamides, sulfamethazine and quinolones in pasteurized milk and UHT milk in China's market. Food Control., 36(1): 238-242.

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