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Screening for Enrofloxacin and Ciprofloxacin Residues in Chicken Liver by Liquid Chromatography Tandem Mass Spectrometry Accompanied by Optimal Liquid-liquid Extraction with Phosphoric Acid

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ABSTRACT

Background: Enrofloxacin used considerably on large scale among poultry birds. Detection and monitoring of its residues is vital in the domestic and export market. A simple and rapid LCMS/MS method for screening of Enrofloxacin and its metabolite residues in poultry liver samples was described.

Methods: Target analytes were extracted with a solvent combination of 5% phosphoric acid in 50% acetonitrile in water. It was followed by deep freezing for efficient separation of the liquid phase. Later extractants were subjected to centrifugation at 4°C, 10,000 rpm for 20 minutes and supernatant was filtered and injected into LCMS/MS. 0.1% formic acid is used as mobile phase additive for both aqueous and organic mobile phase acetonitrile.

Result: Accuracy and precision values of the method are within the acceptable limit of (<5% RSD) accorded by the European commission. Standardized method has been applied to determine the Enrofloxacin and Ciprofloxacin residues in liver samples collected in local markets and found within maximum residue limits.

Key words: Chicken liver, Ciprofloxacin, Enrofloxacin, LC-MS/MS, Low temperature partitioning, Residues.

INTRODUCTION

The residues of antibiotics in foods of animal were as a result of improper usage of drugs weather it is extended or off label in the animals or birds. Use of antibiotics in food animals became highly significant in community health recently. It was reported that exposure to antimicrobial residues in animal products could lead to transfer of resistant strains of microorganisms to humans thereby posing risk to the consumer's health (Nonga *et al.,* 2009; Hussein and Khalil, 2013).

Enrofloxacin is a fluoroquinolone drug which is used in considerably large scale in poultry including India (Lolo *et al.,* 2006; Ramakant *et al.,* 2014) as it is highly effective against important poultry pathogens. Whereas its primary metabolite ciprofloxacin was de-ethylated compound is used both in human and poultry medicine (Chang *et al.,* 2008). FDA has completely prohibited the use of enrofloxacin in poultry because of the emergence of Campylobacter, *E.coli* quinolone resistant strains (FDA, 2005).

Currently, regulatory agencies like European Union (EC, 2002) established MRLs (Maximum residue limits) *i.e.* legally permitted level for Enrofloxacin and its metabolite together as a marker residue in different matrices such as meat and liver as 100 µg/kg and 200 µg/kg. Export inspection council of India (EIC, Residue monitoring plan 2017-18) is also monitoring at 100 µg/kg and whereas 20 µg/kg in edible tissues as prescribed by Japan. At the same time, drug withdrawal periods were also set by regulatory agencies for intended use in animals or poultry to be at the safe levels finally in meat or milk (Lolo *et al.,* 2006).

Current methods of analysis of quinolone drug residues in foods of animal origin were based on High performance liquid chromatography (HPLC) with UV (Chang *et al.*, 2008; Ramakant *et al.*, 2014) or advanced techniques like LC-MS/MS (Marni *et al.,* 2011; Junza *et al.*, 2011; Sunil Chandra

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et al., 2015). Many different conditions of extraction of target analytes from meat and liver matrices were reported by employing simple extraction methods using 5% trichloroacetic acid (Verdon *et al.,* 2005), a mixture of acetonitrile and 0.1 M citrate, 150 mM $MgCl_2$ (Schneider and Donoghue, 2003), phosphoric acid in acetonitrile (Hermo *et al*., 2006; Chang *et al*., 2008), 25% ammonia solution (vol/vol) with acetonitrile (San martin, 2007), Hydrochloric acid (Pena *et al*., 2010; Marni *et al.,* 2011), combined with solid phase extraction (Chung *et al*., 2018) and with different solvents like 1% formic acid and 0.01M EDTA (Acaröz and Sözbilir, 2020). Phosphoric acid was used as mobile phase for extraction of Fluroquinolone Norfloxacin by Meena *et al.* (2020).

The objective of this work was to develop and validate a simple, sensitive, accurate and rapid method for determination of two commonly used antibiotics Enrofloxacin and Ciprofloxacin in poultry liver samples. Consequently, this method was applied to assess the occurrence of Enrofloxacin and Ciprofloxacin residues in chicken liver samples collected in retail markets of Chennai.

MATERIALS AND METHODS

Chemicals and materials

Enrofloxacin (VETRANAL™) ($C_{19}H_{22}FN_3O_3$) of molecular weight 359.39 g/mol, Ciprofloxacin (VETRANAL™) ($C_{17}H_{18}FN_3O_3$) of molecular weight 331.34 g/mol, with purity of ≥ 99 per cent, Methanol, Acetonitrile, Formic acid of LC-MS grade were obtained from Sigma Aldrich (St. Louis, USA). Meta-phosphoric acid of HPLC grade was obtained from Thermofischer scientific (Germany). Deionized Millipore water was used for mobile phases and reagents preparation throughout the study. HPLC column of 4.6 × 50 mm of 2.1 µ C18 particle size used in the study was obtained from Agilent.

Preparation of stock standard solutions

The standard stock solutions were prepared by accurately weighing 10 mg of both the standards transferred each into 10 mL to get 1000 ppm in a volumetric flask and made up to the mark with methanol and stored at 2-8°C until further use. Intermediate standards (10 ppm) were also prepared by proper dilution and further diluted for the preparation of working calibration standards in methanol.

Equipment

The Agilent LC/ESI/MS/MS system was equipped with 1260 model HPLC and triple Quadrupole mass spectrometer (Agilent, 6460C) consists of an electrospray ionization source with configuration of Agilent Jet Stream Technology. All the acquisition performances were controlled with MassHunter Workstation Software (Agilent®).

Liver samples were homogenized with laboratory mixer emulsifier (Silverson). Digital ultrasonic bath (Labman scientific equipments) with ultrasonic wattage 50 W was used for sample processing and cleaning activities. The extracts were centrifuged using a high speed refrigerated centrifuge (Thermo scientific). LCMS/MS instrument was supplied with nitrogen gas of purity 99.995% which acts as a sheath gas and drying gas continuously at a pressure of 8 bar or slightly greater than or equal to 100 psi with the help of nitrogen generator (Cherry precision products, Nitron 70®). Nitrogen gas of purity >99.999 at a pressure of 30-45 psi was used as collision gas. Finally, data was analyzed by Agilent quantitative and qualitative analysis software.

Sample preparation

Liver samples (n=45) were collected from different poultry local retails outlets of Chennai in the year 2018-19. Target analytes were extracted from liver samples by liquid-liquid extraction as per the methodology described by Hermo *et al.* (2006) and Chang *et al.* (2008) with modifications in composition of solvent with additional ultrasonication step, low temperature partition via liquid-liquid extraction. Homogenized chicken liver tissue sample 2±0.05 gram was weighed and suspended in 10 ml of 50:50 acetonitrile: water with 5% phosphoric acid in a 50 ml centrifuge tube with ceramic in the tube for better homogenization. The resultant mixture was homogenized in meat homogenizer for 5-10 minutes. Subsequently, sample was vortexed for 2-3 minutes. Then mixture was ultrasonicated for 10 minutes and kept in a freezer at -20°C for 1 hour. Then the total mixture was subjected to centrifugation at 4°C, 10000 rpm for 20 minutes for efficient separation of supernatant. The supernatant was carefully collected into a 5ml syringe and filtered with PVDF 0.22 µ syringe filter (13 mm) into autosampler vial with tube inserted loaded to autosampler tray of liquid chromatography unit.

Chromatographic conditions

Chromatographic conditions were applied as per the conditions mentioned by Marni *et al.* (2011) with modifications in the column used. The separation of Enrofloxacin and Ciprofloxacin was accomplished using reversed phase chromatography with Agilent poroshell EC C18, 4.6×50 mm 2.7 micron size with maximum of 600 bar pressure. Mobile phases used were 0.1% formic acid aqueous (A) and organic solvent acidified acetonitrile with 0.1% formic acid (B). 0 to 5.00 minutes A-90%,B-10%, 6.00 to 7.00 minutes 35% A and 65% B and from 7.00 minutes to 9.00 minutes 5% A and 95% B. Column temperature was maintained at 35°C. The sequence given initially with working calibration standards followed by samples and the injection volume was 5 µl. Total run time was 13 minutes including post run time of four minutes.

Mass spectrometric conditions

Mass spectrometric conditions maintained for acquisition were presented in Table 1 and acquisition scan segments (MRM parameters) for Enrofloxacin and Ciprofloxacin were presented in Table 2.

Analytical validation studies

Method validation was performed according to the guidelines prescribed by European commission (EC, 2002) and Codex

alimentarius commission (CAC, 2009). As part of method validation, linearity calibration curve, precision, selectivity and specificity, limit of quantification (LOQ) and limit of detection (LOD), Accuracy and Precision were performed.

Linearity of calibration curves for both standards over a linear calibration range of 0 ng/g to 200 ng/g. The calibration standards prepared were injected before each series of analysis. The processing of chromatograms, the regression parameters of slope, intercept and correlation coefficient were calculated automatically by quantitative analysis software. The selectivity of the method was performed by analyzing blank tissue samples (n=15) to determine whether substances can interfere with the retention time of the Enrofloxacin and Ciprofloxacin. Specificity of the method was tested based on the ratios of quantifier and qualifier ions of three spiked levels whether they are within the prescribed range specified by European commission decision compared to working standards. The limit of detection of the quantitative analysis indicated the lowest level of the analyte that can be measured with stastical certainty in a sample, which gave a signal to noise ratio of 3:1 (The ratio between the peak intensity and the noise intensity was used). The LOQ was calculated from the concentration of the analytes that provided a signal to noise (S/N) ratio of 10:1 on analysis as recommended in decision. The accuracy of the method was expressed as the mean recoveries of spiked analytes in chicken muscle at three concentration levels. Recovery obtained at each concentration was calculated using the formula:

$$R = \frac{C_1}{C_2} \times 100$$

Which considers measured content (C_1) and the fortification level (C_2). The precision of the method was tested repeatedly by analyzing the spiked chicken samples. The intra-day precision of the method was determined in chicken muscle using six determinations (n=6) at three concentration levels (5 ng/g, 10 ng/g, 15 ng/g). The intra-day precision analysis was done on the same day. The inter day precision was determined by repeating the study for two consecutive days. The precision of the method was expressed by the relative standard deviation (RSD%) of multiple analyses at different concentration levels.

Table 1: Mass spectrometric conditions maintained for acquisition of Enrofloxacin and Ciprofloxacin.

Parameter/s	Conditions
Gas temperature	350°C
Gas flow	10 l/minute
Nebulizer	45 psi
Sheath gas temperature	350°C
Sheath gas flow	10 L/minute
Capillary voltage	Positive: 4500V, Negative: 3500 V, 32nA
MS1 Heater and MS2 Heater	100°C
Turbo 1 and turbo 2 speed	100%
Nozzle voltage	Positive: 500 V, Negative: 500 V
Chamber current	0.25 µA

RESULTS AND DISCUSSION

Sample preparation, chromatographic and mass spectrometric conditions

Acetonitrile as an extraction solvent is known for precipitation of proteins and other enzymes which will help in good recovery of the analyte (Stolker and Danehar, 2012). A combination of different disruption techniques like homogenization, centrifugation and ultrasonication were also utilized and compared to obtain good extractability of target analytes into the extraction solvent from the meat matrix. Precipitation with 1% phosphoric acid in 50:50 acetonitrile was used for meat matrix in this study which has the best capability of extracting and removing protein interference. Similar extraction capability with phosphoric acid and acetonitrile for extraction of quinolone group of compounds was achieved by several other researchers (Su *et al.,* 2003; Chang *et al.,* 2008).

Typical RPLC (Reverse phase liquid chromatography) principles as outlined by Wang and Turnipseed, (2012) were utilized for optimization of chromatographic conditions like usage of mobile phases with acetonitrile and aqueous mobile phases with additives, RPLC gradient profile starting with > 90% of aqueous mobile phase, followed by a gradient with organic solvent as elution solvent. The chromatographic run time was kept as short as possible by adjusting chromatographic conditions to achieve proper separation of compounds. Co-eluting the compounds that are originating from the matrix can enhance or suppress signals by affecting the ionization efficiency of the analyte. The improvement in the chromatographic separation was important in the method development; otherwise, it could result in increased amounts of matrix compounds by co-eluting the analyte (Junza *et al.,* 2011). For this an additional step of low temperature partition was done which is the step efficient in freezing of the matrix interference compounds such as fat which is rich component in meat and liver matrices in common. This was observed in tetracycline extraction by previous research works (Desmarchelier *et al.*, 2018) and multianalyte extraction by Lopes *et al.* (2012). Immediate centrifugation of the contents definitely helped

Table 2: Acquisition scan segments (MRM parameters) for Enrofloxacin and Ciprofloxacin.

Compound name	Precursor ion	Production	Collision energy	Fragmentor voltage	Polarity
Enrofloxacin	360.2	245.1	32	100	Positive
Enrofloxacin	360.2	316	20	100	Positive
Ciprofloxacin	332	314	20	100	Positive
Ciprofloxacin	332	231.1	40	100	Positive

in efficient extraction of target analytes into the solvent which was evident obtained higher recovery values for both the analytes in the study.

Analytical validation study

The limit of detection and limit of quantification achieved in the developed method for Enrofloxacin was 0.22 ng/g and 0.85 ng/g and for Ciprofloxacin 0.47 ng/g and 1.44 ng/g respectively and were found to be far below the fixed minimum performance level of 10 ng/g for both the fluoroquinolone residues in this study. The sensitivity achieved in this method was sufficient enough to determine the analytes at the concentration of very low levels of interest. Similar LODs and LOQs for Enrofloxacin as 1 ng/g and 3 ng/g respectively in different matrices were reported by Panzenhagen *et al.* (2016).

Specificity as depicted by ion ratios was determined for both the analytes from replicate which are samples spiked with all the analytes at each of calibration ranges (0-200 ng/g). The two most abundant product ions were chosen for LC-MS/MS analyses. The correlation coefficient was >0.999 for both Enrofloxacin and Ciprofloxacin (Fig 1 and Fig 2). Product ion combination of m/z 360/316, 360/245 for Enrofloxacin and m/z 332/314, 332/231 for Ciprofloxacin were used successfully according to selected reaction monitoring mode (SRM) in this study. Ion ratios acquired and analyzed by quantitative analysis of the study were all within the tolerance limit of 58.9 to 89.3 according to European Union guidelines (EC, 2002).

Enrofloxacin and Ciprofloxacin accuracy and precision values were presented in the Table 3 and chromatograms are shown in Fig 3. Good recoveries were obtained for both the compounds at three spiked levels of 5, 10, 15 ppb levels. The mean recoveries obtained in this study are in similar line with recoveries obtained by Chang *et al.* (2008). Accuracy and precision studies done in the present study were 10 times below the level of MRL *i.e* 10 ppb. The recoveries observed in this study for both the compounds at all three spiked levels were within the acceptable range as prescribed by EU Commission (EC, 2002) and Codex

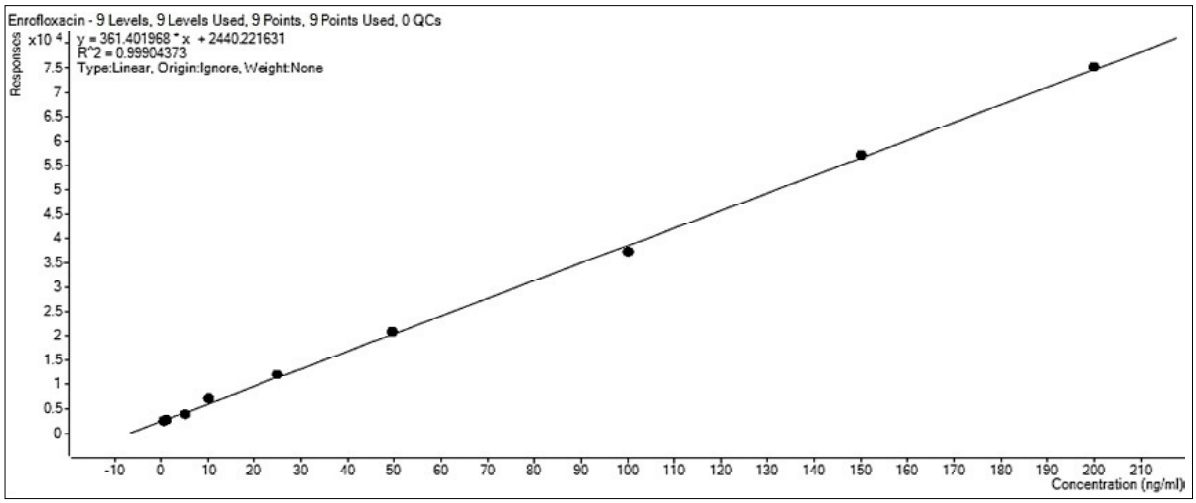


Fig 1: Calibration curve of enrofloxacin abundance (response) and concentration (ng/ml) of ions.

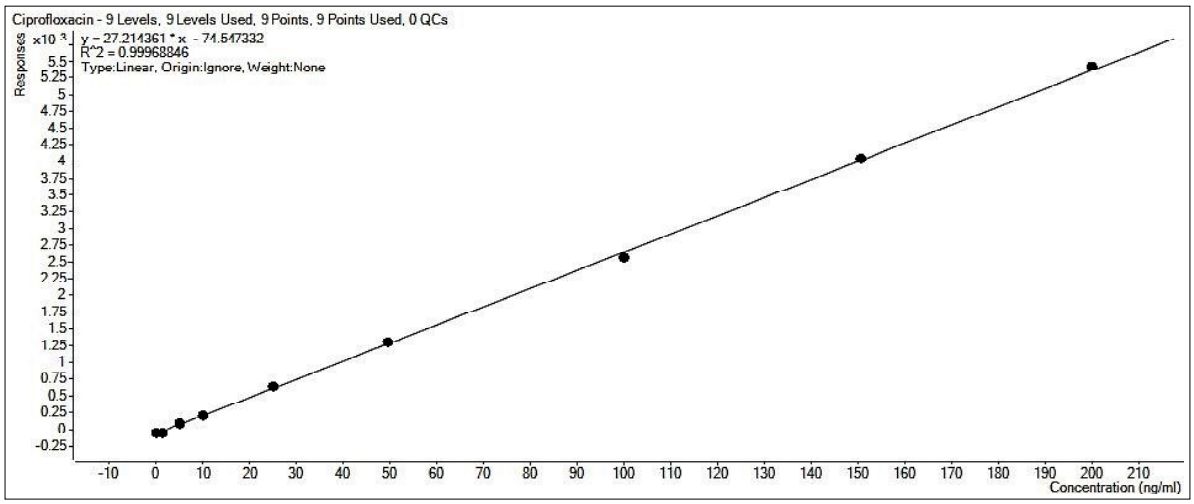


Fig 2: Calibration curve of ciprofloxacin abundance (response) and concentration (ppb or ng/ml) of ions.

Table 3: Calibration parameters, LOD, LOQ, Accuracy and Precision values for Enrofloxacin and Ciprofloxacin.

Parameters	Enrofloxacin	Ciprofloxacin
Linear calibration range	0 ppb-200 ppb	0 ppb-200 ppb
Linear regression equation	y=361.401968*x+2440.221631	y=27.21436*x-74.547332
Correlation coefficient	0.99904373	0.99968846
Retention time	5.03±0.03	4.803±0.03
LOD (ng/g)	0.22	0.47
LOQ (ng/g)	0.85	1.44
Ion ratios	63.6	103.2
Accuracy	5 ng/g- 90.4-102.2 (4.10)	5 ng/g- 86.4-98.6 (5.65)
(Recovery range%)	10 ng/g- 92.5-103.22 (4.59)	10 ng/g- 93.25-101.94 (4.24)
(% RSD)	15 ng/g- 90.7-102.3 (4.67)	15 ng/g- 88.8-98.8 (3.54)
Intraday precision	5 ng/g-6.20	5 ng/g-7.01
(Repeatability)	10 ng/g-5.58	10 ng/g-6.53
(% RSD)	15 ng/g-3.30	15 ng/g-6.65
Interday precision	5 ng/g-5.25	5 ng/g-5.55
(Reproducibility)	10 ng/g-4.17	10 ng/g-5.23
(% RSD)	15 ng/g-4.20	15 ng/g-5.23

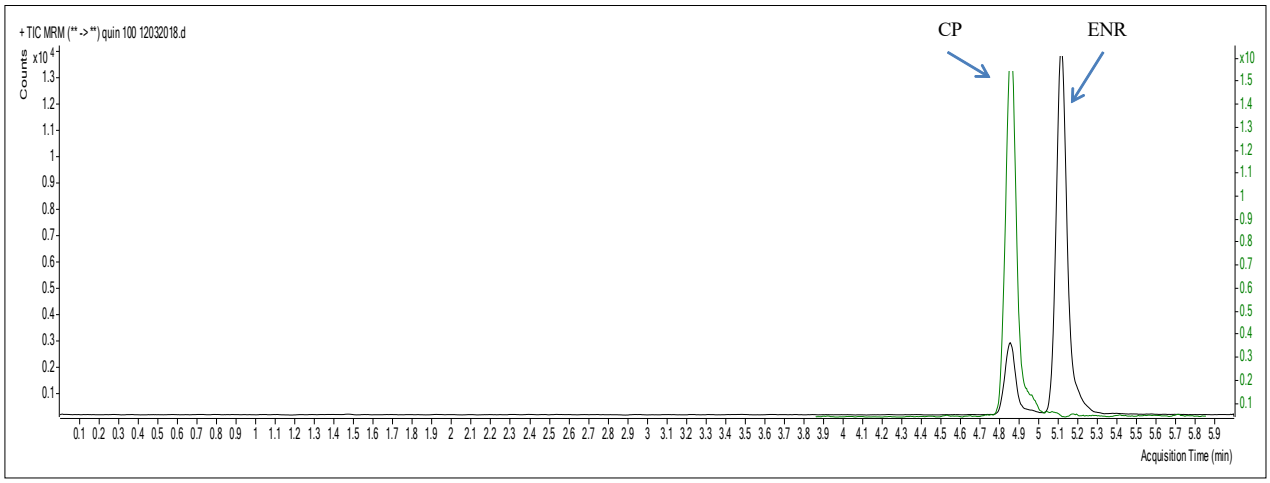


Fig 3: Typical chromatogram of ciprofloxacin (CP) and enrofloxacin (ENR) (100 ppb) with their retention times abundance (response) and acquisition time (minutes).

alimentarius commission regulations (CAC, 2009) who reported, < 1 ppb, 1-10 ppb and >10 ppb spiking levels to be as 50 to 120%, 70 to 110% and 80 to 110% respectively. The percent relative standard deviation values obtained in this study for all three spiked levels at each day for both the analytes was less than 7.01 demonstrating very good method precision and they were below the maximum acceptable limit of 15% for the performance of analytical methods as prescribed by EC, (2002).

Application to real samples

Out of 45 liver samples about 14 samples and 13 samples of liver samples tested positive with possible detection levels of Enrofloxacin and Ciprofloxacin respectively. Enrofloxacin and Ciprofloxacin drug residue concentration ranged from 4.6 ppb to 133.5 ppb, 7.72 ppb to 33.47 ppb respectively. None of the liver samples exceeded the maximum acceptable levels of residual sum of Enrofloxacin and Ciprofloxacin of 200 ppb as ordained by EC, (2002) and Export Inspection Council of India (EIC, 2017).

A higher concentration of Enrofloxacin was recorded in Liver sample in this study (Fig 4). This fact was supported by Petrovic, (2006) reported that Enrofloxacin concentrations normally will be 3.78 times higher in the liver than in muscle 24 hours after the beginning of the treatment. A similar higher concentration of Enrofloxacin and Ciprofloxacin in liver samples than breast muscle samples was reported by Attari *et al.* (2014) and Panzenhagen *et al.* (2016).

Although the detectable levels of both Enrofloxacin and Ciprofloxacin were less than the MRL obtained in this study, there is plausible threat *via* development of resistant microorganisms and their transfer in the food chain. FDA in United States imposed a zero tolerance policy for residues of Enrofloxacin and Ciprofloxacin in broilers, with the target tissue monitoring these residues being muscle, this is the tissue with the greatest tissue antibiotic concentration and

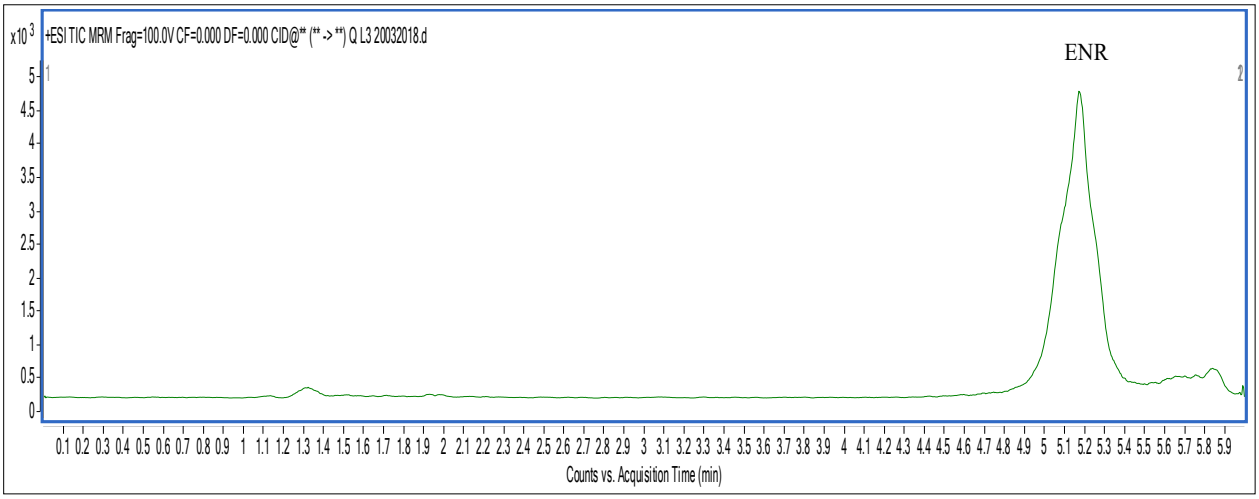


Fig 4: Typical chromatogram of enrofloxacin (ENR) in liver samples.

persistence, with the highest concentration of residues found in the breast muscle (De Assis *et al.,* 2016).

CONCLUSION

A simple and rapid extraction procedure coupled with Electron spray ionization liquid chromatography and mass spectrometry in positive mode was established for the analysis of Enrofloxacin and its primary metabolite Ciprofloxacin in liver samples. The method was applied to analysis of the target analytes in the real samples. It was demonstrated that method has the potential to execute as a good technique for analysis of these two residues. None of the liver samples had shown residue levels above MRL prescribed by European Union. Continuous monitoring, surveillance and farm traceability are to be strictly implemented to curb improper usage of drugs.

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Conflict of interest: None.

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