UDC: 637.12.06:615.33
Original Scirntific Paper

ANALYTICAL SCREENING METHOD FOR DETECTING RESIDUES OF FLUOROQUINOLONE ANTIBIOTICS IN MILK

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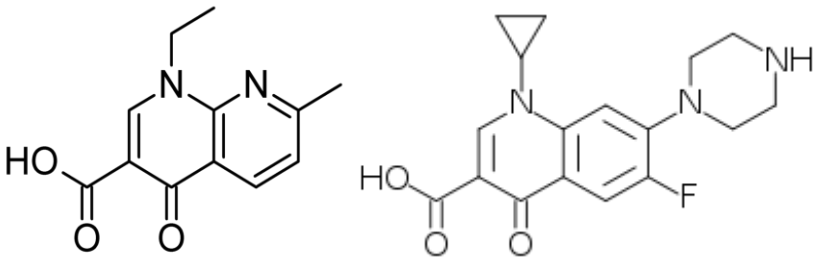
Abstract

Fluoroquinolones (FQs) are a group of broad-spectrum antibiotics that are widely used to prevent or treat bacterial infections and are a growth promoter to increase food efficiency. Their residues may cause bacterial resistance, allergic hypersensitivity, and toxic effects predicting a potential risk to human health. This study aimed to detect residues of fluoroquinolone antibiotics in milk using the ELISA screening method.
A total of 130 milk samples were examined for the presence of quinolone antibiotic residues and were collected in 2015 under a national residue monitoring plan. Samples were collected and delivered by authorized inspection personnel. Samples were analyzed for residues of quinolone antibiotics using an ELISA test kit (type AB685 from TECNA, Trieste, Italy). The methods were validated in accordance with the recommendations made by European Commission Decision 2002/657/EC.
The data obtained confirmed that the methods were appropriate for the detection of specific antibiotics, at the level of concentration of interest. The linear regression analysis revealed good correlation factors with R2=0.994. The validation procedure was carried out by the criteria laid down in Commission Decision 2002/657/EC. The detection limit (LOD) for fluoroquinolons was 6.17 (μg/kg) and the detection capability (CCβ) was 77.62 (μg/kg). Recoveries were in the range of 75.03%. The values obtained for the CCβ were below the MRL (maximum residue limits). None of the samples analyzed had a value higher than the minimum required level of performance of the screening method.
Validation of the successful method according to the requirements of the European Union and its sensitivity and specificity, simple and good, to arrive at the unambiguous identification of fluoroquinolone antibiotic residues in milk.

Keywords: fluoroquinolones, residues, milk, validation, ELISA.

1. Introduction

Quinolones are a group of antibiotics with a broad spectrum of activity; they are synthesized from a 3-quinolone carboxylic acid. Containing fluorine at position C-6 and piperazinyl at position C-7 are obtained from fluoroquinolone compounds, as shown in Figure 1. Antimicrobial agents are widely used to prevent or treat bacterial infections and growth promotion effects to increase dietary efficiency. (Daniel, et al., 2014, Huang, et al., 2010).



a) enrofloxacine b) ciprofloxacine

Figure1. Chemical structures of fluoroquinolones

They are used against all bacterial infections, particularly against urinary tract infections and acute respiratory diseases. These drugs have bactericidal or bacteristatic anti-microbial activity, namely, they behave through inhibition of DNA-gyrase bacteria in gram-negative and topoisomerase IV species in gram-positive species and prevent bacterial DNA from unfolding and duplicating as can be seen in Figure 2. (Pena, et al., 2010, Anne, et al., 2010, Iqbal, 2014).

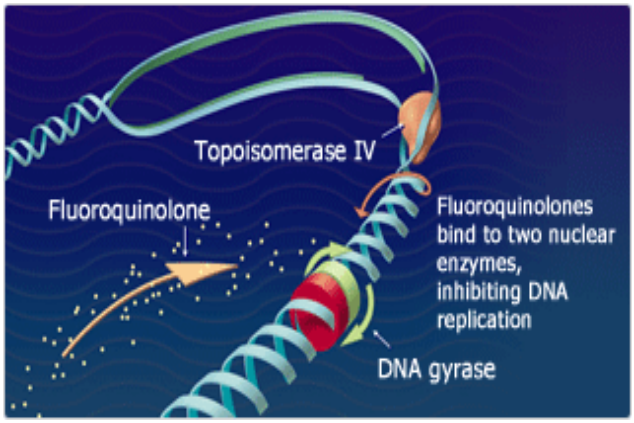


Figure 2. Mechanism of action

Fluoroquinolones are very effective drugs for the treatment of campylobacteriosis and salmonellosis. These residues may cause short-term health effects including allergic hypersensitivity and toxic effects, while long-term exposure could result in chronic toxic effects such as bacterial resistance development in humans. These resistant bacteria might then cause difficulty-treating human infections and disruption of normal human flora in the intestines. (Godfred, et al., 2017) The main concern for public health is related to possible transfer resistant zoon tic bacteria, especially Salmonella spp and Campylobacter spp., because of the emergence of resistance of Campylobacter to quinolones, the use of enrofloxacine for poultry was withdrawn by the U.S. Food and Drug Administration in 2005. Further research demonstrates that these antibiotics and their metabolites may directly cause oxidative damage to cell membranes. Some have also been suspected to affect the central nervous system. For example, in humans, they have been associated with some cases of severe disorders like headaches, dizziness, or convulsions (Chang, et al., 2010, Jinqing et al., 2010)

As a result of their side effects on public health, legislation on the control and monitoring of these residues is set out in EU Council Directive 9623EC (Council Directive 96/23/EC) and EU Council Regulation 37/2010/EU (Commission Regulation 37/2010). The EU has defined MRLs for several of these compounds in different food matrices of animal origin. According to Commission Regulation, quinolones range between 100-1900 μg/ kg in chicken liver and 30-100 μg/kg in milk. According to these requirements, the R of N. Macedonian legislation was fully aligned with the EU legislation (Official Gazette of the R.M. 80/2011). Monitoring fluoroquinolone residues in animal products is very important to ensure that human food is entirely free of potentially harmful residues. Lee et al. in 2007 in a paper described the LC-MS method with fluorescence detection developed for the determination of ofloxacin, norfloxacin, and ciprofloxacin in sewage (Lee, et al., 2007). In the case of Sun, et al., in 2010, they established an LC analysis with solid-phase extraction to simultaneously detect malachite green, enrofloxacin, and ciprofloxacin in fish farming water (Sun, et al., 2010). Jiang Jinqing, et al., 2010 reported the immunoassay method for the determination of fluoroquinolones residues in water samples (Jinqing, et al., 2010). Anne, et al., in 2006 developed simultaneous determination of (Fluoro)quinolone antibiotics in the kidney, marine products, eggs, and muscle by Enzyme-Linked Immunosorbent Assay (ELISA) (Anne, et al., 2010). José Barbosa, et al., in 2009 reported the determination and characterization of quinolones in foodstuffs of

animal origin by CE-UV, LC-UV, LC-FL, LC-MS AND LC-MS/MS (Barbosa, et al., 2009). For the determination of fluoroquinolones residues in marine products, other scholars have also developed different physicochemical methods (Schneider, et al., 2007, Dufresne, et al., 2007). However, they are all costly and need skilled personnel and lengthy sample preparation. The ideal assay for the ELISA screening method of fluoroquinolones should be fast, simple, and cost-effective, thanks to the very high specificity of the biospecific reagents used (antibodies) (Jinqing, et al., 2010). In many cases, liquid chromatography coupled with ultraviolet or fluorometric detection and with tandem mass spectrometry has been applied as a post-screening method for qualitative and quantitative analysis, the latter allowing increased sensitivity, selectivity, identification/confirmation of antibiotics residues in complex matrices (Barbosa, et al., 2009) All methods used for that purpose have to detect antibiotics at or below their permissible limits or MRLs and also have to be validated by the Council Directive 2002/657/EC (Council Directive 2002/657/EC).
This study aimed to detect residues of fluoroquinolone antibiotics in milk using the ELISA screening method.

2. Material and methods

Sample Collection

A total of 130 milk samples were examined for the presence of quinolone antibiotic residues and in 2015 were collected under a national residue monitoring plan. Samples were collected and delivered by authorized inspection personnel. Samples were stored at 4-8oC if analyzed within 24 hours after sampling or kept at less than -20oC for four weeks. The samples were kept frozen until use and the examinations were carried out according to the requirements of the European Community in five specialized diagnostic laboratories belonging to the Faculty of Veterinary Medicine, Food Institute in Skopje.

Reagents and standard solutions

TECNA quinolones test kit (type AB685 from, Trieste, Italy) and its reagents were used to determine the presence and the fluoroquinolones levels in milk.
The fluoroquinolone standards, ENR (99.8%) and CIP (99.5%) were obtained from Fluka-Vetranal. Methanol, dichloromethane used were of analytical grade, 3 N hydrochloric acid, extraction buffer 10x (dilute the concentrated buffer 1:10 with distilled water).
Standard stock solutions with concentration of 1 mg/mL were prepared on monthly basis by dissolving the analytes in methanol.
Quinolone solutions were prepared in sample dilution buffers, provided in the ELISA kits. These solutions were used for spiking blank milk samples at different levels. Following fortification, samples were allowed to equilibrate for 15 minutes before extraction. The methods were validated according to the recommendations laid down by European Commission Decision 2002/657/EC.

Sample preparation for the screening methods

Sample preparation, as well as preparation of all reagents for quinolones, was according to the manufacturer's instructions. The milk samples for analysis of quinolones sample were homogenized and 4 mL of the homogenized sample was transferred to centrifuge tubes were centrifuged at 4ºC (3000rpm for 10 minutes). Add to a glass tube, 2 mL of sample and 4 mL of dichloromethane and mix for 15 minutes. Centrifuged at 6000 rpm for 10 minutes. Colleceted 0.5 mL of the clear lower phase and transferred it to a

glass tube. Evaporate at 50 ºC to dry under a nitrogen stream. Resuspended with 2.5 mL extraction buffer 1x, containing 8% (v/v) of methanol.

Quinolones analysis by ELISA

ELISA test kit for detection of quinolones provided by TECNA (Trieste, Italy). Each kit contained a microtiter plate with 96 determinations including standards (1.3 mL each) 0, 0.3, 1.1, 3.3, 10, 30 ng/mL, Quinolones spiking solution 100 ng/L, enzyme conjugate, extraction buffer, washing buffer, developing solution, stop solution. The extraction and clean-up procedures were those described by the ELISA kit manufacturer (TECNA, Trieste, Italy).

Assay procedures

All the necessary reagents were brought to normal room temperature (20-25°C) by lifting them for about 30 minutes. Predisposed an assay layout, recording the standard and sample positions, considering that all must be run in duplicate. 100 µl of diluted antibody was added to each well, added 50 µL of enzyme conjugate in each well was mixed gently by shaking the plate manually and incubated for 30 min at room temperature (20-25°C).
All wells were filled with washing buffer. Washing was repeated three (3) more times. 100 µL of developing solution was added to each well, mixed gently by shaking the plate manually, and incubated for 20 min at room temperature (20-25°C). 50 µl of stop solution (2M H2SO4) was added and mixed gently by shaking the plate manually.
After mixing, the absorbance was read at 450 nm by using a spectrophotometer, presented in Figure 3 (BIO-RAD model 680).

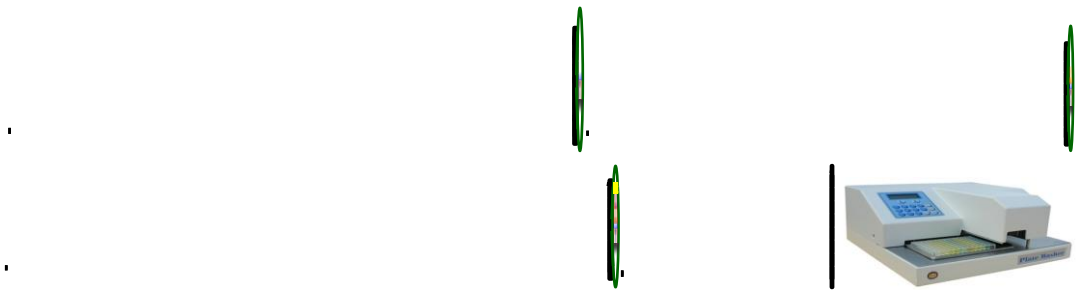


Figure 3. Assay procedures

Validation of the screening methods

Performance characteristics of ELISA methods were determined as prescribed for qualitative screening methods in Commission Decision 2002/657/EC. Also, the limit of detection (LOD) was obtained by adding 3 and 10 times the standard deviation of 20 blank samples to the mean blank value. Recovery was assessed by performing the experiments where fortified milk samples were analyzed in ten replicates, at the respective maximum residue level (MRL) values for the substances being analyzed. From the recovery experiments, the method's precision was obtained, as well. The detection capability (CCβ) for quinolones was determined by spiking 20 blank samples at the one-half of the MRL value set at 100 µg/kg. The calculations were performed by the formula provided in the EU Commission Decision.

3. Results

The data obtained confirmed that the method was suitable for the detection of specific antibiotics at the level of interest. The data have been analyzed with special software RIDAWIN ELISA (R-Biopharm, Darmstadt, Germany). The mean absorbance values obtained for the standards and the samples divided by the absorbance value of the first standard (zero standards) and multiplied by 100 were the % absorbance. The zero standards were thus established at 100 and the absorbance values were given in percentages. But because of the different samples, the optimal curve fitting models of the standard curve have certain differences. The linear regression equation can be used to meet the needs of the experiment for ELISA detection and reduce computational difficulty. So, in this experiment, the fitting form of linear regression is selected.

The absorption is inversely proportional to the concentration of fluoroquinolones. As can be seen in Figure 4, the fluoroquinolones calibration curve was found to be virtually linear between 0.0-100 ppb. In Figure 5 the correlation between the absorbance ratio and fluoroquinolones concentration was evaluated over the range of 0.00- 100 ppb.

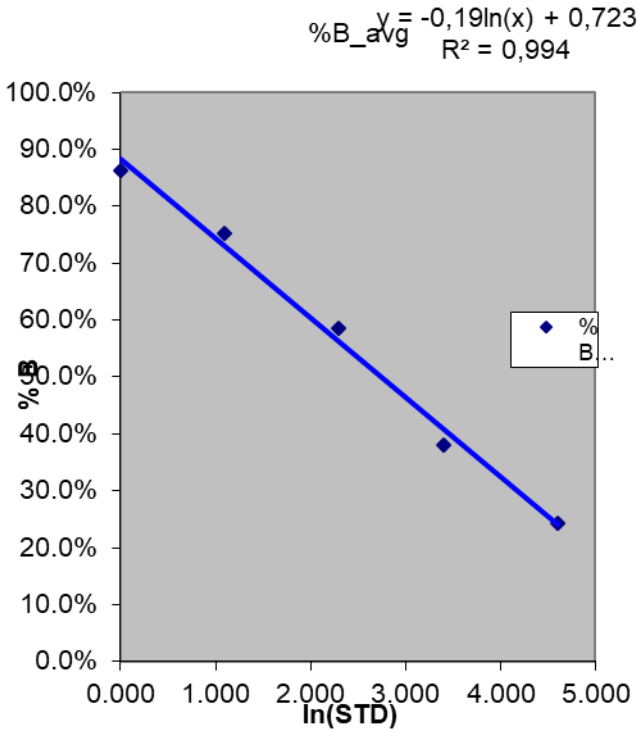


Figure 4. Linearity of Calibration curve for Fluoroquinolones standards (0- 100 ppb)

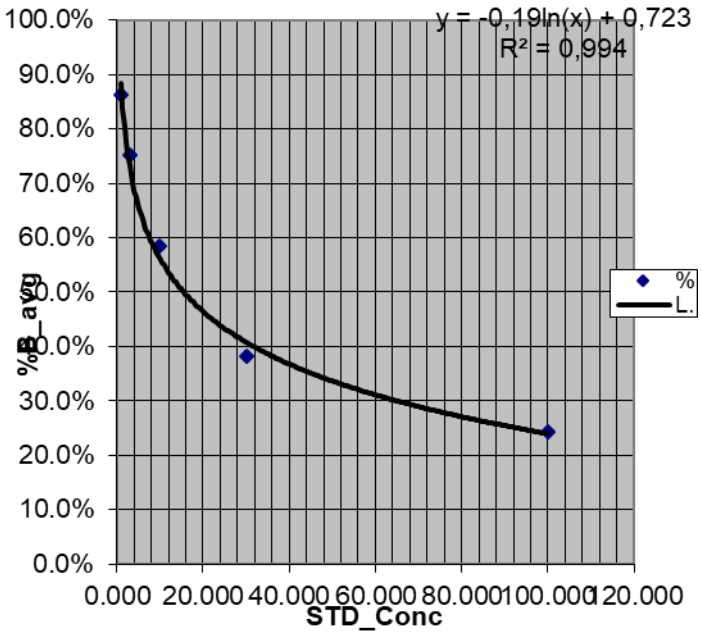


Figure 5. Calibration curve for Fluoroquinolones standards (0- 100 ppb)

The linear regression analysis revealed good correlation factors with R2=0.994, (y = - 0.1453 x + 1.4057), where y was relative absorbance (%) and x was quinolones concentration in ppt (Table 1).

Table 1. Linearity of the method

Analyte	**Equation of the calibration curve**	**R^2**
Fluoroquinolones (Enrofloxacine, Ciprofloxacin)	y=-0.19ln(x) + 0.723	0.994

The validation process was performed by the criteria set out in Commission Decision 2002/657/EC criteria. The detection limit (LOD) for fluoroquinolons was 6.17 (µg/kg) and the detection capability (CCβ) was 77.62 (µg/kg). The values obtained for the CCβ were below the MRL (maximum residue limits). None of the samples analyzed had a value higher than the minimum required level of performance of the screening method (Table 2).

Table 2. Detection capability (CCβ) and Maximum Residue Limits (MRL)

Analyte	**CCβ (µg/kg)**	**MRL µg/kg**
Fluoroquinolones (Enrofloxacine, Ciprofloxacin)	77.62	100

Recoveries were in the range of 75.03% and reproducibility was evaluated by CV, % (coefficients of variation, %), and ranged from 15.23 to 20.295%. The obtained values were satisfactory which indicated the good performance of the proposed analytical method (Table 3).

Table 3. Accuracy, precision of the method

Analyte	**Recovery (%)**	**CV(%)**

Fluoroquinolones (Enrofloxacine, Ciprofloxacin)	75.03%.	20.295%

Validation data corresponding to the screening method performance (LOD, CCβ, recovery, and precision) and their results were calculated using the Excel program, which is presented in Table 2 and Table 3. Validation of the successful method according to the requirements of the European Union and its sensitivity and specificity to arrive at the unambiguous identification of fluoroquinolone antibiotic residues in milk.

4. Discussion and conclusion

Raw milk and dairy products, which are important for human consumption, should be safe and should not contain factors or substances harmful to human health. Residues of antimicrobial agents pose a potential danger to the consumer and may cause allergic reactions, interferences in the intestinal flora, and toxic effects.

The linearity of the method with the high coefficient determination showed reliable results.

This study has resulted in better precision, as well as better recovery (>73%) compared with other the study. The values obtained for the CCβ were below the MRL (maximum residue limits). None of the samples analyzed had a value higher than the minimum required level of performance of the screening method MRLs (Maximum Residue Limits). In this study, ELISA has been used to unambiguously identify residues of quinolone antibiotics in milk samples. This method has been validated by the criteria laid down in Commission Decision 2002/657/EC and is used in our laboratory's routine analyses. Due to the simplicity, speed, good sensitivity, specificity, and cost-effectiveness of the method and its good recovery and accuracy, it can be applied in official control laboratories as a screening method.

Dimitrieska-Stojkovic, et al., in 2011 in a previous study reported residues of antimicrobic drugs were detected in 4.3 % of milk samples collected in Macedonia during 2010, using a screening diffusion test (Delvo test, DSM Food Specialities, Delft, and The Netherlands).

Shu-chu Su, et al., (2003) in Taiwan using high performance liquid chromatographic (HPLC method to detect quinolones in 63 samples of chicken, pork, and fish purchased from markets, enrofloxacin residue was found in 9 chicken muscle samples and 4 chicken liver samples.

Hamdi Aliu reported in Kosovo that the analytical control carried out for three fluoroquinolones in 2014 confirmed the incidence of 12.7%.

Pena, et al., (2010) In Portugal reported that comparison with fluoroquinolones occurrence data at European and international levels is difficult because few studies are available in the scientific literature, that only one sample of 61 chicken muscle samples was contaminated with enrofloxacine at levels higher than the MRLs.

As a result of the study performed by Iqbal A. Sultan, (2014) in Iraq Detection of Enrofloxacin Residue in Livers of Livestock Animals results show that 56.66% of poultry liver samples were positive for enrofloxacin. Moreover, it was reported that mycotoxin contamination of broiler feed may increase the residual values of enrofloxacin in poultry carcasses.

In a study undertaken by Ferhan Nizamlıoğlu, (2012) in Turkey fifty samples of raw milk and 50 of chicken liver were analyzed using ELISA method for the presence of quinolone antibiotics. All of the analyzed samples except one showed the presence of quinolone residues below the MRLs established by EU and Turkish Legislation.

However, veterinarians need to be knowledgeable about the importance of drug residues in food intended for human consumption and they should inform livestock producers of good agricultural practices and the responsible use of antibiotics, and the risks they pose to the public.

Surveillance of antibiotic residues is required to ensure food safety and prevent consumer exposure. Successful validation methodology by EU requirements (2002/657/EC). The ELISA method is simple,

sensitive, and specific to obtain unambiguous identification of quinolone antibiotic residues in milk. This method can be used in routine sample analysis. Milk in Macedonia on average has low levels of antibiotic residues and may be considered safe for human consumption.

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