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DETERMINATION OF ENROFLOXACIN RESIDUE IN CHICKEN EGGS USING LIQUID CHROMATOGRAPHY – MASS SPECTROMETRY METHOD

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ABSTRACT

The number of poultry farms in Kosovo is constantly increasing and the need to prevent infectious bacterial infections in poultry has led to the use of antibacterial preparations, which in addition to the beneficial effects of combating these diseases, also poses risk to public health. The purpose of this study was to confirm the presence or absence of enrofloxacin residues in 8 egg samples, after analyzing 180 eggs samples collected at commercial farms in Kosovo with the Elisa Test. The confirmation method used was the Liquid Chromatography Mass Spectrometry. 2 of 8 samples resulted with presence of enrofloxacin residues. The findings suggest that illegal use of enrofloxacine in chicken farms is present and highlight the fact that chicken farmers were using antibiotics as a promoter of egg production and body weight and to reduce the incidence of infectious diseases. Both the tests are performed at the Kosovo Food and Veterinary Laboratory. The testing was performed according to the Commission Decision 2002/657/EC

Keywords: Enrofloxacin, Eggs, Liquid Chromatography Mass Spectrometry .

1. INTRODUCTION

The discovery of antibiotics was one of the greatest achievements in the history of mankind. Antimicrobials are our most powerful allies in the fight against communicable diseases. Enrofloxacin is widely used in veterinary medicine because of the broad spectrum of activity and high efficacy against Gram-positive and Gram-negative bacteria (M G-Sikorska et al, 2013)

In the frame of food safety, the European Union (EU) has regulated the use of quinolones as veterinary drugs in food-producing animals and has established maximum residue limits (MRLs) of antibiotics in food. According to EU regulations, quinolones, such as ciprofloxacin, enrofloxacin, sarafloxacin and oxolinic acid are “not for use in animals from which eggs are produced for human consumption”, which means they are strictly forbidden in rearing laying hens. No MRLs have been stated for them, and eggs and industrially elaborated egg products

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containing quinolone residues at any level must be rejected (European Regulation (EU). No 37/2010, Regulation (EC). No 470/2009, J P Vicenteet al 2019).

To protect the consumer from exposure to residue levels that might constitute a health risk, the European Union has introduced legislation with regard to authorisation of veterinary medicine. The approval of veterinary medicinal products can only occur after an extensive safety and residue evaluation and subsequent registration in Annex I, II or III of Council Regulation 2377/90 (EC 1990) (Mariel G Pikkemaat et al 2007)

Council Directive 96/23 / EC (Council Directive 1996) requires Member States of the European Union to adopt and implement a national waste monitoring plan for specific groups of substances. However, data from the literature and Rapid Alert System for Food and Feed (RASFF) indicated that there were incidents of ENR illegal use in laying hens' therapy (RASF). Accordingly, their residues need to be controlled to verify the compliance of producers and importers with the regulation.

However, inappropriate or abusive use of antibiotics in poultry farms might provoke the transfer and accumulation of their residues in food products. This causes exposure to the consumer to these compounds in low concentrations which may produce toxic reactions and stimulate the emergence of quinolone-resistant pathogens (Pruden A et al 2006, Friedman, M et al 2015). It has been largely proven that eggs from poultry treated with pharmaceutical products contain drug residues, even those laid days to weeks after treatment cessation (J P Vicenteet al 2019)
Given the risk of large-scale use of antibiotics in Kosovo, the Ministry of Health of the Republic of Kosovo, is implementing the Strategic Plan for Microbial Resistance, supported by other relevant institutions including the Food and Veterinary Agency as the only institution dealing with the protection of animal health and public health respectively.
In the Report of EFSA 2018, on the results from the monitoring of veterinary medicinal product residues and other substances in live animals and animal product the examination of 4,476 samples of eggs for antimicrobial residues revealed positivity of 8 (0.18 %) samples for doxycycline, enrofloxacin, flumechin and sulfadiazine (EFSA 2018).
According to the Commission Decision 2002/657/EC (European Commission, 2002), the confirmation of suspect positive samples must be carried out by mass spectrometry (MS) coupled to adequate chromatographic separation (Shehu F, et al 2018).

2.MATERIALS AND METHODS

2.1 The regions included in the study

180 egg samples from commercial farms were collected in 7 regions of Kosovo, respectively: Pristina, Prizren, Ferizaj, Peja, Gjilan, Gjakova and Mitrovica. 1 sample is composed of 12 eggs. The number of farms and poultry eggs by regions under study are in Table 1.

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Table 1. The number of farm and poultry eggs by region under study

Region	No / heads	No of poultry farms	Samples under the study
Pristina	125648	25	1 x 12
Prizren	125430	28	2 x 12
Ferizaj	95421	15	1 x 12
Peja	125478	18	1 x 12
Gjilan	62541	12	1 x 12
Gjakova	365214	16	1 x 12
Mitrovica	75621	13	1 x 12
	975353	127	8 x 12

The purpose of this method is to identify and quantify enrofloxacin in the egg. Testing is performed using the analytical method of liquid chromatography with mass spectrometer detector (LC-MS).

The observed limits of detection are in accordance with the various MRLs for the four classes of veterinary drugs, and the average recoveries exceed 50%, thus meeting the requirement for routine analysis (Jin-Lan Sun et al 2012)

2.2 Reagents and standards used

Enrofloxacin, True acetonitrile for waste testing, Methanol suitable for byte testing, Formic acid, Phosphoric acid, Dinateriumhydrogen phosfate dihidrat and Citric acid

2.3 Instrument Parameters

Optimization of enrofloxacine for the LCMS method was performed for its determination in suspected samples.

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Table 2 LCMS - Operating conditions for egg enrofloxacin testing

Column	Phenomenex Gemini 150x3.0mm
Eluenti A	Methanol me 0.12% acid formik
Eluenti B	0.1% Acid formik
Time (min) 0	Eluent B (%)
0	90
1	90
10	20
20	20
21	90
30	90
Injection Volume	10.0 µL
Flow	0.4 ml /min
MS Parameters:	
Detection	ESI+MS/MS
Polarity	Positive
Scan Type	Schedueld MRM
Jon suorce	Turbo spray
Rezulution	Q1,Q2:unit
ISO voltage	5500V
Temperature	150°C
Gas 1 (N2)	45 psi
Gas 2 (N2)	55 psi
CAD gas	High
Curtain gas:	30psi

2.4 Sample homogenization (Preparation of egg samples)

The 100 g eggs samples were homogenized. then stored at -20 °C.

2.5 Extraction

In 2 g of homogenized sample, 100 µL of internal standard solution and 10 mL of EMI Buffer were transferred, stirred in vortex for 1 min, and then placed for 10 min in Shaker at maximum shaking. The mixture was centrifuged for 5 min at 4000 U / min. The upper aqueous phase (supernatant) was filtered through a filter in a 50 mL PE-centrifuge tube. The residue was

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extracted with 6 mL EMI Buffer. The collected filtrate was used for purification in SPE. Cleaning is done with methanol and Evaporates with nitrogen. After this, concentrations in the mixture of acetonitrile and water, mixed / centrifuge and put in vials and put into to autosampler.

2.6 Purification of sample extract with SPE-colon

The filtrate collected in PE-centrifuge tubes was washed with 6 mL Wash solution and dried for 10 min under vacuum. It was diluted with 6 mL of methanol and the collected filtrate was placed in PE-centrifuge tubes and then subjected to drying in a nitrogen evaporator at 70 ° C.

2.7 Liquid Chromatography-Mass Spectrometry

After processing the samples we started the analysis in LC immediately. If the analysis was not possible on the same day, then the samples were stored at -20ºC. Before the instrument started, we made sure that all solvents, wash lines, columns, and detectors were about 5 min in advance.

HPLC parameters were set: Eluent A: formic acid in water LC-MS 0.2% and Eluent B: formic acid in acetonitrile LC-MS 0.2%. Flow: 0.35 mL / min. Column temperature: 25 ° C. Injection volume: 10µl. Injection temperature 10 ° C and MS / MS parameters set.

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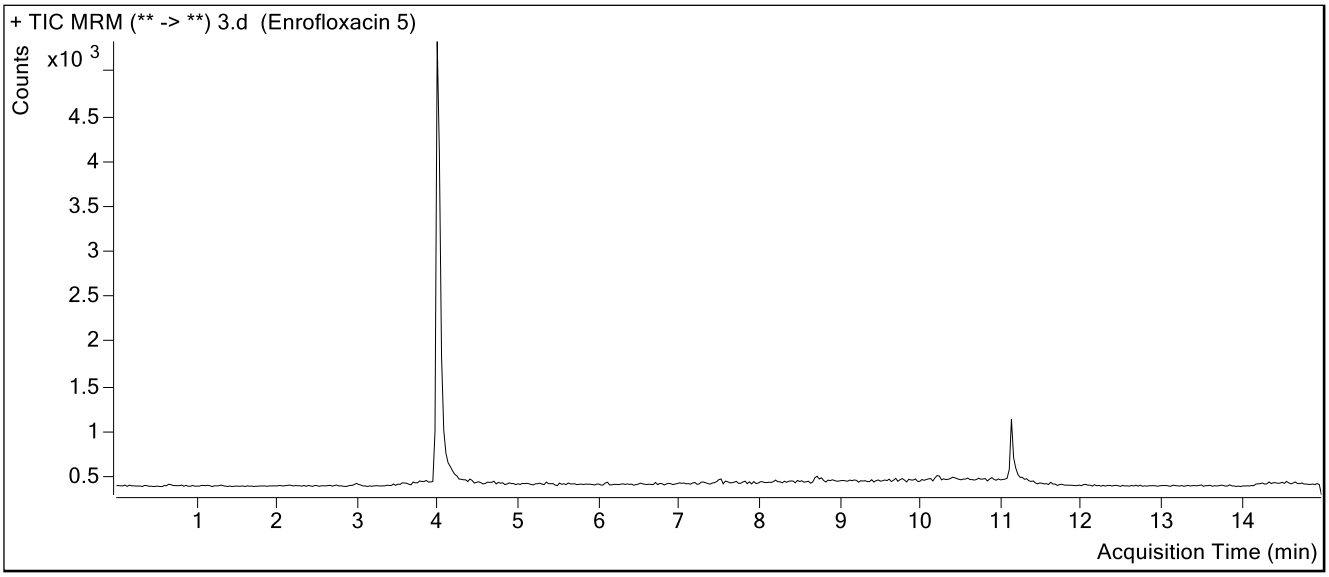

Figure 1. Chromatogram of enrofloxacine in spike sample 5 ug / kg

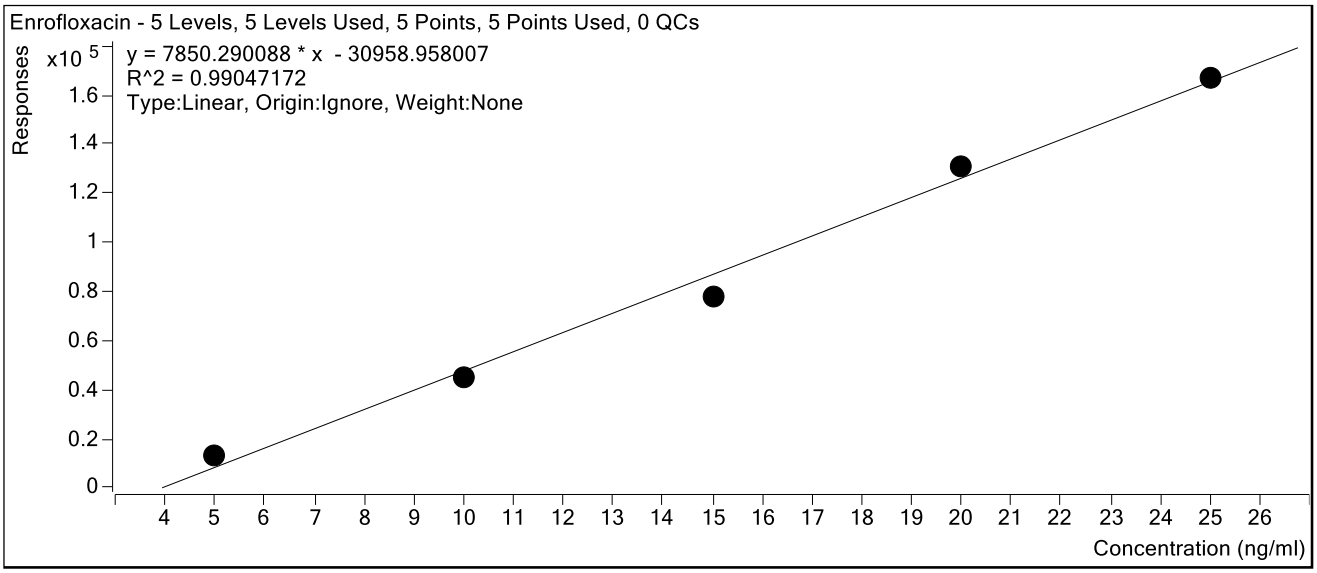

Figure 2. Calibration curve with spiking samples (5,10,15,20.25 ug / kg)

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3. RESULTS AND DISCUSSIONS

The basis of this study was the determination of enrofloxacin residues in eggs at commercial farms in Kosovo. All samples were taken as part of the annual plan for control and monitoring of antibiotics in chicken eggs, compiled by the competent authority - the Kosovo Food and Veterinary Agency.

The high performance liquid chromatographic method has been used to determine the maximum residual limit of enrofloxacin in the egg as an accurate and reliable method. The 8 from 180 egg samples were resulted suspected using Elisa test, and these 8 egg samples were analysed for confirmation. From these 8 samples we found that in 2 of them are present the enrfloxacin residues (Fig. 3).

The analytical quality was evaluated by the guidelines of the EU Commission Decision 2002/657/EC (specificity, calibration range, linearity, trueness, precision, decision limit, detection capability, robustness, and stability).

According to this study, it turns out that chicken farmers were using antibiotics as a promoter of egg production and body weight gain and in some cases to reduce the incidence of infectious diseases.

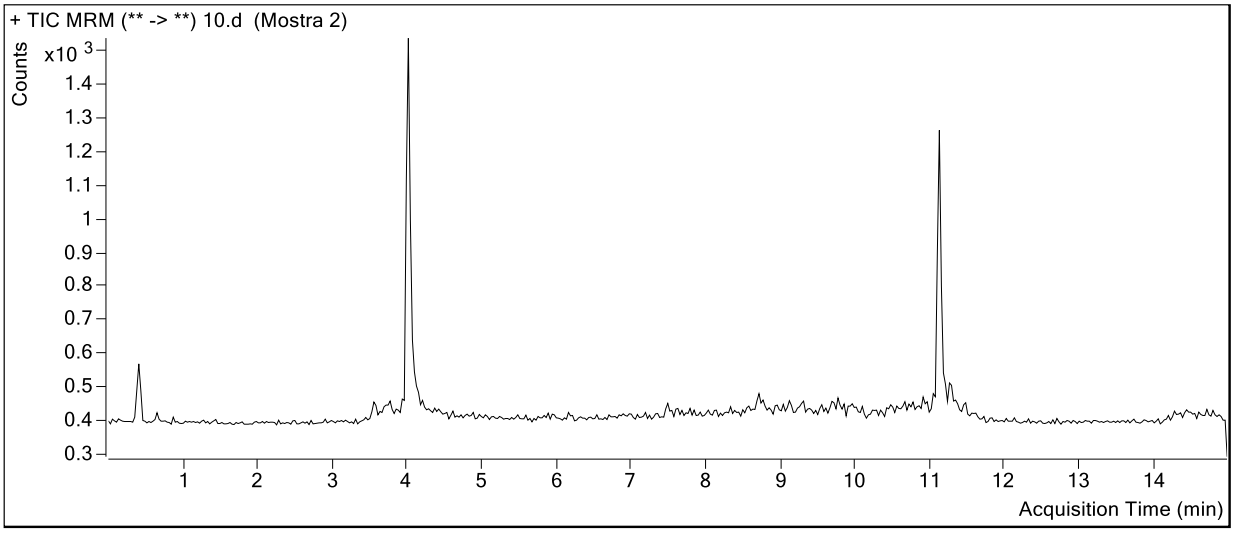

Figure 3. Sample chromatogram with MRL concentration

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