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Additives/Contaminants:	Veterinary drug residues - fluoroquinolones
Food Types:	Animal products – meat

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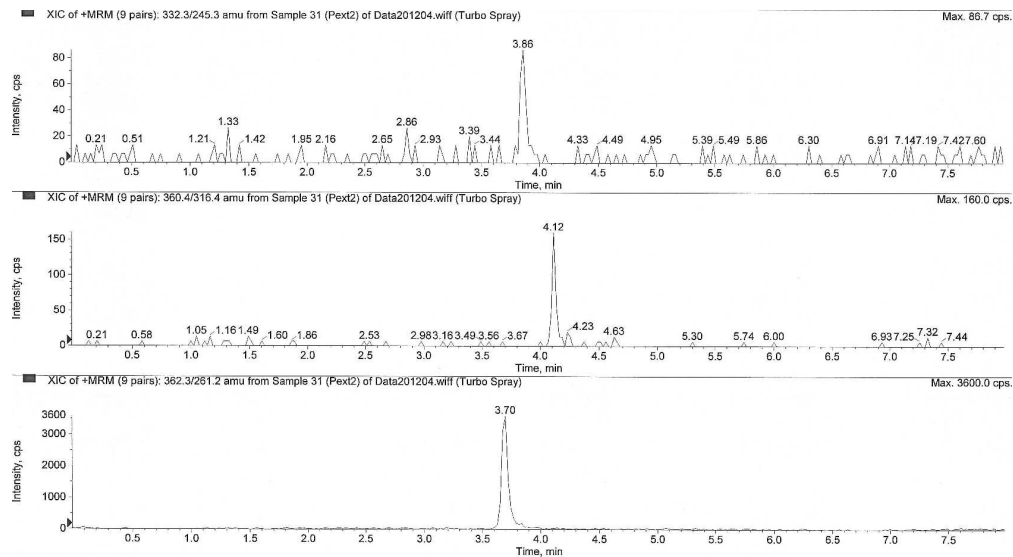


Figure1. Illustrative chromatogram of raw tissue spiked with 5 ngg-1 of ciprofloxacin (MRM: 332.3-245.3) and enrofloxacin (MRM: 360.4-316.4), using lomefloxacin (MRM: 362.3-261.2) as internal standard.

284x155mm (300 x 300 DPI)

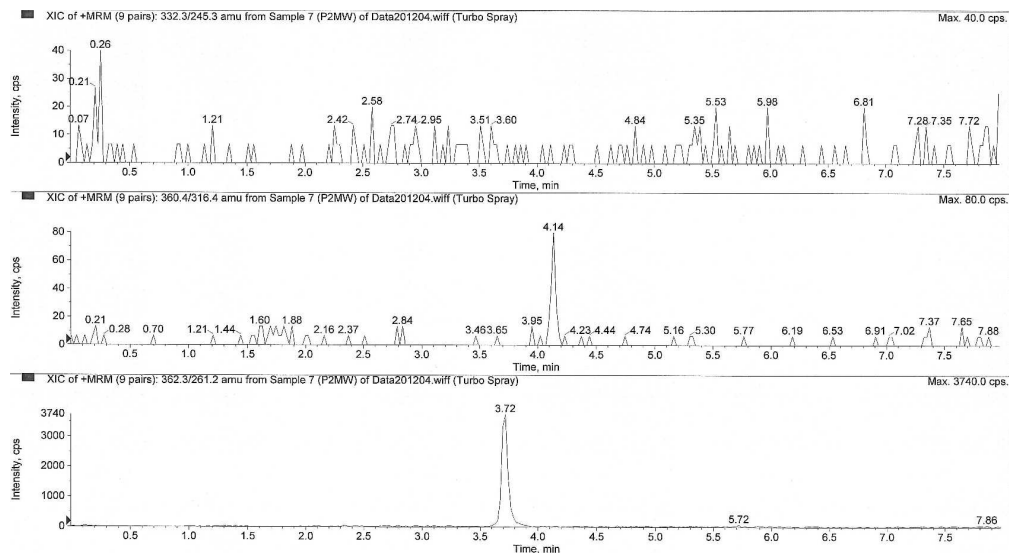


Figure2. Chromatogram of a breast portion cooking by microwaving from a chicken treated by injection, only peak corresponding enrofloxacin (MRM: 360.4-316.4) and internal standard lomefloxacin (MRM: 362.3-261.2) are presents.  
285x157mm (300 x 300 DPI)

Review Only

## The effect of cooking on enrofloxacin residues in chicken tissues

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### Abstract

The aim of this study was to determine the effect of different cooking processes (microwaving, roasting, boiling grilling and frying) on enrofloxacin residues in naturally incurred chicken muscle. Enrofloxacin and its metabolite ciprofloxacin were analysed using a validated LC-MS method with limits of detection (LOD) and quantification (LOQ) of respectively 2 and 5 ng g<sup>-1</sup> of quinolones in muscle samples. The method was demonstrated to be linear over the range of 5-500 ng g<sup>-1</sup>. The mean intra-day RSD at a concentration of 50 ng g<sup>-1</sup> (n=6) was 6%; inter-day RSD was 12%. A recovery study demonstrated that 65-101%, of the drug and metabolite could be recovered from the tissue. The RSD with naturally incurred roasted chicken breast was 9.18% at a concentration of 11 ±1,01 ng g<sup>-1</sup> (n=6). In water, enrofloxacin remained stable for 3 h when heated at 100 °C. It was concluded that residue data from raw tissues are valid for the estimation of the exposure of the consumers to this drug as well as the ADI calculations because cooking procedures did not affect the enrofloxacin residues which remained stable during heating. However, there was an apparent decrease in quinolone concentration in the tissues because some was lost by exudation or into the liquid used for cooking. Conversely, for a cooking procedure with water loss, there was an apparent increase in residue concentration.

**Keywords:** Enrofloxacin, veterinary residues, chicken, cooking.

## 1 Introduction

2  
3 Enrofloxacin belongs to the group of potent antibiotics known as fluoroquinolones  
4 that are extensively used in human and veterinary medicine. These antibiotics have a  
5 wide spectrum of action and high efficacy against infectious disease. They are highly  
6 effective in treating against Gram positive, Gram negative and *Mycoplasma* in  
7 infection processes (Hooper et al., 2001a). The high level of use in animals and  
8 humans, and to some degree of misuse in the sense of unnecessary use or use of  
9 quinolones with poor activity in some developing countries, has been blamed for the  
10 rapid development of bacterial resistance to these agents (Hooper, 2001b) which  
11 means a hazard to human health.  
12

13 Currently the levels of drug residues in raw food (meat and animals products) are  
14 regulated. In this regard, the maximum residue limits (MRL) for the fluoroquinolone  
15 enrofloxacin and its metabolite ciprofloxacin legally permitted in food under the laws  
16 of the European Union (EEC, 1990) have been established for muscle, fat, liver, and  
17 milk from several animal species, including chicken.  
18

19 Since most of this type of food is cooked before consumption, more information  
20 about the effect of cooking on residues is required to give a more accurate estimate  
21 of consumer exposure to these chemicals and any breakdown products. However,  
22 few studies have been carried out on the occurrence of the veterinary drug residues  
23 in cooked foods, nor on the stability of these compounds following heat treatment  
24 (Rose et al, 1995 a,b,c; Rose et al., 1996; Rose et al, 1997a,b,c; Rose et al., 1998;  
25 Rose et al., 1999; Moats, 1999). Furthermore, none of these studies is related to the  
26 widely used enrofloxacin or other fluoroquinolones.  
27

## 28 Experimental

### 29 *Chemicals*

30 Ciprofloxacin and enrofloxacin were supplied by Bayer AG (Wuppertal, Germany).  
31 Ofloxacin was supplied by Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile, formic  
32 acid and sodium hydroxide were supplied by Merck (Darmstadt, Germany). Milli-Q  
33 organic free water from Millipore (Bedford, MA, USA) was used. All reagents were of  
34 analytical grade. All chemicals used were analytical grade.

## 1 Apparatus

2 Ultra-Turrax® T25 homogenizer (Staufen, Germany). Ultrasounds bath UCI-200  
3 Raypa (Barcelona, Spain). Centrifuge Eppendorf 5415D (Hamburg, Germany).

## 4 LC-MSMS System

5 The LC-MS-MS system consisted of an Agilent Technologies 1100 HPLC (Agilent  
6 Technologies, Minnesota, USA) and a Q-Trap mass spectrometer with turbo ion  
7 spray interface Applied Biosystems MSD Sciex (Toronto, Canada). The HPLC  
8 column used was a Synergi Fusion-RP (50×2 mm) 4 μm, Phenomenex (Torrance,  
9 CA, USA). The mobile phase was aqueous formic acid (0.1 %) mixed in gradient  
10 mode (95 % to 65 % in 5 min) with acetonitrile. Flow-rate was 500 μL min<sup>-1</sup>.

11  
12 MS/MS detection was performed in mode MRM from the protonated molecular ion  
13 [M+H]<sup>+</sup> of analyte to its product ion in positive ion mode. The product ion efficiency of  
14 the [M+H]<sup>+</sup> was optimized by varying the spray, orifice and voltages. The collision  
15 activated dissociation (CAD) of the [M+H]<sup>+</sup> of analytes was performed at quadrupole  
16 using N<sub>2</sub> gas atmosphere. These CAD parameters were optimized by varying the  
17 collision gas pressure and collision energy using a programme to obtain the  
18 maximum intensity and reproducibility. The parameters are showed in the table 1.

19  
20 “[insert Table 1 about here]”

## 21 Animal treatment

22 Five chickens housed in individual cages were used. One of them were considered  
23 as blank. The other four were dosed over 5 consecutive days with 15 mg/day of  
24 enrofloxacin through the intramuscular route. The pharmacological speciality  
25 Ganadexil® (Enrofloxacin 5%), obtained from Industrial Veterinaria, S.A. (Esplugues  
26 de Llobregat, Barcelona, Spain) was employed.

## 27 Cooking Procedure

28  
29 Three samples (breast, whole leg and liver) from each chicken were subjected to five  
30 different cooking treatments. Breast and whole leg were cooking using methods that  
31 reflect the wide variation in domestic practices: microwaving, roasting, grilling, boiling  
32 and frying. The liver was fried and roasted in an oven. All the pieces of tissue were  
33

1 analysed three times in the raw state to ensure the homogeneity of residue  
2 distribution. In addition, the water used -or the exudates released- for the boiling or  
3 microwave cooking were also analysed.  
4

5 *Cooking by frying.*

6 A 5 g portion of chicken tissue (breast, whole leg and liver) was fried for 10 min with  
7 5 g of sunflower oil in a pan using an electric hob, turning occasionally. The cooked  
8 muscle had a “well done” appearance on the outside.  
9

10 *Cooking by boiling.*

11 A 5 g portion of chicken (breast and whole leg) was placed with 10 ml of water in a  
12 vessel and boiled for 10 min, using an electric hob.  
13

14 *Cooking by microwaving.*

15 A 5 g portion of chicken (breast and whole leg) with 1 g of sunflower oil was cooked  
16 for 3.5 min at 800 W.  
17

18 *Cooking by roasting in an oven.*

19 A 5 g portion of chicken (breast, whole leg and liver) with 1 g of sunflower oil was  
20 cooked in a electric oven at 200 °C for 10 min.  
21

22 *Cooking by grilling.*

23 A 5 g portion of chicken (breast and whole leg) was grilled for 10 min.  
24

25 *Sample preparation*

26 After the cooking treatments, the chicken muscle was homogenized using an Ultra-  
27 Turrax® blender. An accurately weighed sample ( $0.5 \pm 0.05$  g) was placed in an  
28 Eppendorf tube and then 500  $\mu$ L of a sodium hydroxide aqueous (10 mM) –  
29 acetonitrile (1:1) solution was added. The sample was sonicated for 10 min and  
30 centrifuged at 13000 rpm for 20 min. Finally, 250  $\mu$ L of the upper layer was placed in  
31 an injection vial and 25  $\mu$ L of internal standard (Ofloxacin,  $1 \mu\text{g mL}^{-1}$ ) was added and  
32 5  $\mu$ L of this mixture were injected into the LC/MS/MS system.  
33



### 1 *Stability of enrofloxacin in heated water*

2 Solutions containing 25 ngmL<sup>-1</sup> enrofloxacin were prepared in water and dispensed  
3 into a number of sealed vials. The vial solutions were placed in a thermostatic oven  
4 and removed at intervals of 30 min during 3h. A 0.5 mL aliquot was used to analyse  
5 enrofloxacin in the same way than tissue samples. The experiment was carried out at  
6 100 °C.

### 7 *Validation*

8 The analytical method was characterized for raw tissue with the repeated generation  
9 of calibration graphs of raw tissue spiked prior to extraction with different amounts of  
10 enrofloxacin and ciprofloxacin, using ofloxacin as the internal standard. Three  
11 repetitions for each level were made each day over three days. Two analysts, using  
12 different solutions each day, generated the data. The calibration graph was described  
13 by the equation  $Y = mX + b$ , where Y represents the response value of the analyte in  
14 the solution (peak area), and X the number of nanograms of each quinolone on-  
15 column. To check the extraction methodology, we added 500 µL of a known  
16 concentration mixture of enrofloxacin and ciprofloxacin in a sodium hydroxide  
17 aqueous (10 mM) –acetonitrile (1:1) solution to 0.5 ± 0.05 g of muscle sample. The  
18 spiked muscle (5, 50, 250 ng g<sup>-1</sup>) was then extracted as above. This was carried out  
19 on three consecutive days three times each day. The intra-day assay was carried out  
20 at a level of 50 ng g<sup>-1</sup> repeated six times. To evaluate the method for cooked tissue,  
21 the same breast piece naturally incurred and roasted in an oven at 200°C was  
22 analysed six times.

## 23 **Results and Discussion**

### 24 *Method validation*

25 Figure 1 shows a chromatogram of an extract of raw tissue spiked with 5 ng g<sup>-1</sup> of  
26 ciprofloxacin (m/z 332.3 → 245.3), enrofloxacin (m/z 360.4 → 316.4), using  
27 lomefloxacin (m/z 362.3 → 261.2) as internal standard. Figure 2 show a  
28 chromatogram of a breast portion cooked by microwaving from a chicken treated by  
29 injection. Only peak corresponding enrofloxacin and lomefloxacin are present.

30 “[insert Table 2 about here]”  
31  
32  
33  
34

1 The specificity of the method was assessed by testing a number of representative  
2 blank muscle samples (n=10) to verify the absence of potential interfering  
3 compounds. The LODs and LOQs, defined as the blank response (n= 10) plus three  
4 or ten times the standard error of the blank response, was 10 pg and 25 pg  
5 respectively, both injected on-column. These values correspond to 2 and 5 ng·g<sup>-1</sup> of  
6 quinolones in muscle samples. The chromatographic method was demonstrated to  
7 be linear for the range studied (5-500 ng g<sup>-1</sup>) with P-value for lack-of-fit in the ANOVA  
8 table greater or equal to 0.10 (calibration coefficient 0,9998 and 0,9996, for  
9 ciprofloxacin and enrofloxacin, respectively). The mean intra-day RSD (n=6, c=50 ng  
10 g<sup>-1</sup>) was 6%; inter-day assay gave a RSD of 12%. The extraction percentages were  
11 within the established range 65-101%, for the drug and their metabolite. Appropriate  
12 relative standard deviation was obtained with naturally incurred roasted breast of  
13 9.18% (n=6, 11 ng g<sup>-1</sup>±1,01). Ciprofloxacin and enrofloxacin concentrations in the  
14 samples were interpolated from the five-point calibration curves made with 0.5 g of  
15 tissue as described above. All results quoted were corrected for analytical method  
16 recovery daily obtained with a blank sample fortified at the LOQ (5 ng g<sup>-1</sup>).

17  
18 There are no references in the literature to the effect of cooking on enrofloxacin  
19 residues or related drugs in foods. Ciprofloxacin has been described as the main  
20 metabolite of enrofloxacin, with extensive metabolism in chickens (Delaporte et al.,  
21 1994). In light of this, we analysed for both substances but, as only small amounts, or  
22 undetectable levels of ciprofloxacin found were in all samples, only the enrofloxacin  
23 residue levels are given in this paper. The stability of enrofloxacin in water at 100 °C  
24 was demonstrated over a period of 3 h (n=6, c=25 ng mL<sup>-1</sup> RSD=14.3%). The  
25 concentrations of enrofloxacin in each piece from each chicken used in the  
26 experimental work are given in Table 2. The homogeneity of enrofloxacin residues  
27 was demonstrated for the same piece of each chicken (see results from raw pieces).  
28 Nevertheless, although the birds had been fed and treated in a similar way,  
29 significant variations due to metabolic or other differences between individuals were  
30 noted.

31  
32 “[insert Table 2 about here]”  
33

1  
2  
3 1 As it can be observed in table 3, the change in enrofloxacin levels in the tissue is  
4 2 dependant on the type of cooking. We noted a very different concentration of  
5 3 quinolone in the tissues from different birds and, for that reason, we have presented  
6 4 the data for the four chickens separately. The change in the concentration of  
7 5 enrofloxacin residue induced by the different cooking procedures were presented as  
8 6 a percentage increase or decrease. As shown in the table, extraction of residues took  
9 7 place when we boiled or microwaved the chicken pieces. There was always a  
10 8 reduction in the concentration, and the lost amount of analyte in the tissue was found  
11 9 in the water or in the exudates. On the other hand, the amount of residue increased  
12 10 in the case of roasting and grilling and this can be explained because the minor  
13 11 moisture content of the treated piece caused the apparent concentration of the  
14 12 quinolone residue. This takes to us to conclude that the cooking procedure did not  
15 13 therefore affect the levels of quinolone. Thus we concluded that residue data from  
16 14 raw tissues are valid for estimating consumer exposure and for making the ADI  
17 15 (Acceptable Daily Intake) calculation because the cooking procedure did not affect  
18 16 the enrofloxacin residues as they remained stable during the heating.

17  
18 18 The results reported here were consistent with these previously reported by other  
19 19 authors that studied the fate of drug residues. As an example, streptomycin in eggs  
20 20 subjected to boiling or frying did not change residue concentrations (Inglis and Katz,  
21 21 1978; O'Brien *et al.*, 1980), neomycin residues were not affected by cooking (Inglis  
22 22 and Katz, 1978; Katz and Levine, 1978), whereas oxytetracycline levels were  
23 23 reduced to a monor extent by frying and roasting procedures (O'Brien *et al.*, 1980).  
24 24 Other veterinary drugs such as clenbuterol (Rose *et al.*, 1995a), levamisole (Rose *et*  
25 25 *al.*, 1995b), sulphametazine (Rose *et al.*, 1995c), oxytetracycline (Rose *et al.*, 1996),  
26 26 oxfendazole (Rose *et al.*, 1997a), lasalocid (Rose *et al.*, 1997b) or ivermectin (Rose *et*  
27 27 *al.*, 1998), remained quite stable during cooking procedures.

### 28 29 **Acknowledgements**

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Table 1. Mass spectrometer parameters and MRM transition ions.

Parameter	Value
Source Temperature	450.0 °C
Courtain gas	20
CAD:	High
IS:	5500
Gas 1:	40
Gas 2:	60

MRM	
Ciprofloxacin	Enrofloxacin
332.30 → 314.30	360.40 → 342.30
332.30 → 288.40	360.40 → 316.40
332.30 → 245.30	360.40 → 245.30

Table 2: Homogeneity data. Enrofloxacin residue levels ( $\text{ng g}^{-1}$ ) in raw tissues from the same piece ( $n=3$ ) in different animals.

Animal		Whole leg	Breast	Liver
1	Mean	29,00	28,30	10,03
	RSD%	9,12	7,30	9,98
2	Mean	10,45	5,25	29,63
	RSD%	11,85	13,33	10,11
3	Mean	50,24	17,86	32,61
	RSD%	5,28	6,35	6,87
4	Mean	25,52	15,05	65,71
	RSD%	10,79	13,46	3,87
Blank	Mean	<LOQ	<LOQ	<LOQ
	RSD%	<LOQ	<LOQ	<LOQ



Table 3: Effect of cooking on enrofloxacin residues (ng g<sup>-1</sup>) in chicken tissues.

		CHICKEN1	CHICKEN 2	CHICKEN 3	CHICKEN 4	<sup>a</sup> %
WHOLE LEG	Raw	29.00	10.45	50.24	25.52	
	Boiled	16.00	<LQ	26.07	14.40	-48.11 to -43.57
	Fried	14.00	9.00	25.62	8.60	-66.30 to -13.88
	Oven	41.80	22.78	102.91	64.19	44.14 to 151.53
	Microwave	17.00	<LQ	22.50	5.86	-77.04 to -41.38
	Grilled	42.13	14.80	75.00	51.50	41.63 to 101.80
	Water-Boiled	10.00	<LQ	53.60	22.46	
	Water Microwave	14.54	<LQ	24.00	24.36	
BREAST	Raw	28.30	5.25	17.86	15.05	
	Boiled	12.30	<LQ	7.94	5.60	-62.79 to -55.54
	Fried	16.00	<LQ	12.22	5.28	-64.92 to -31.58
	Oven	45.01	12.46	32.06	61.69	59.05 to 309.90
	Microwave	10.74	<LQ	9.43	5.20	-65.45 to -47.20
	Grilled	45.20	8.50	28.33	24.20	58.62 to 61.90
	Water-Boiled	38.00	<LQ	27.60	20.66	
	Water Microwave	8.16	<LQ	5.20	14.38	
LIVER	Raw	10.03	29.64	32.61	65.71	
	Fried	8.00	19.00	29.25	25.30	-61.50 to -10.30
	Oven	13.36	30.01	41.96	91.71	28.67 to 39.57

<sup>a</sup>%. Percentage of increase or decrease in enrofloxacin residue concentration. LQ: Limit of quantification.

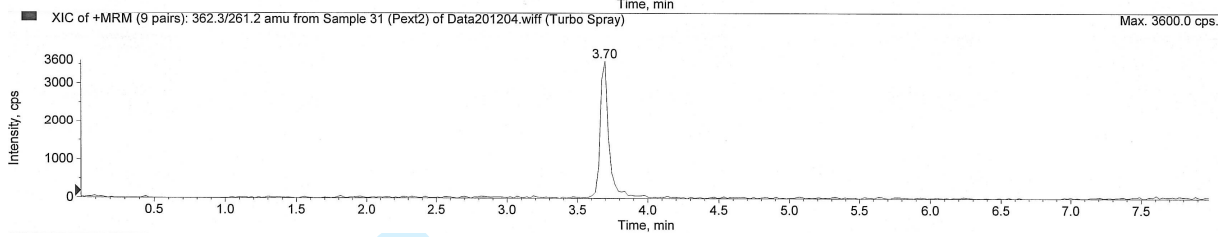
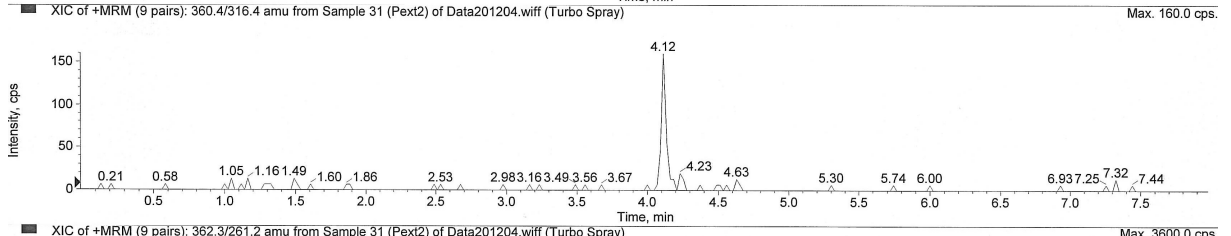
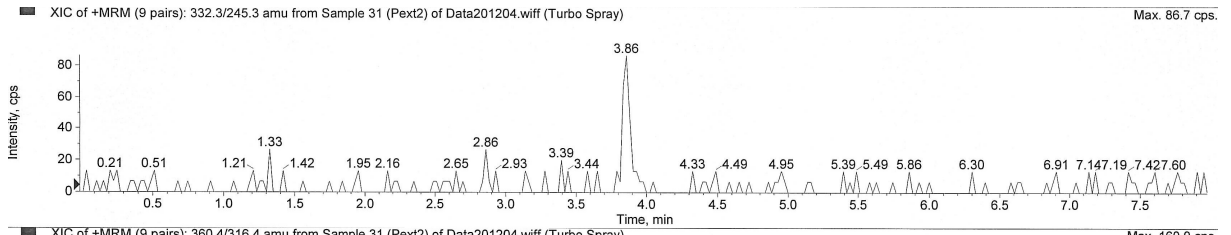
## FIGURE CAPTIONS

Figure1. Illustrative chromatogram of raw tissue spiked with  $5 \text{ ngg}^{-1}$  of ciprofloxacin (MRM: 332.3-245.3) and enrofloxacin (MRM: 360.4-316.4), using lomefloxacin (MRM: 362.3-261.2) as internal standard.

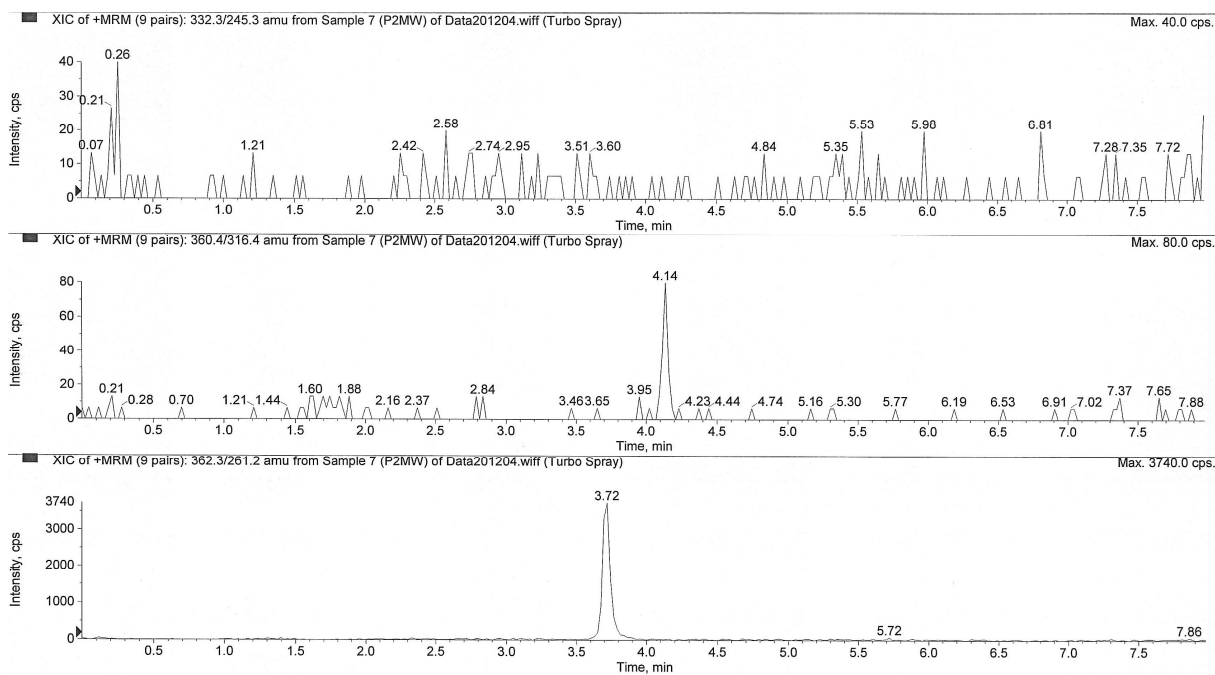
Figure2. Chromatogram of a breast portion cooking by microwaving from a chicken treated by injection, only peak corresponding enrofloxacin (MRM: 360.4-316.4) and internal standard lomefloxacin (MRM: 362.3-261.2) are presents.

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