1 **A rapid and reliable assay to determine flumequine, marbofloxacin, difloxacin, and**

2 **sarafloxacin in commonly consumed meat by micellar liquid chromatography**

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7 Juan Peris-Vicente[a]*, Jesús Javier Iborra-Millet[b], Jaume Albiol-Chiva[a], Samuel Carda-

8 Broch[a], and Josep Esteve-Romero[a]

9

10 *a Química Bioanalítica,Department of Physical and Analytical Chemistry, ESTCE, Universitat Jaume I,*

11 *Castelló, Spain*

12 *b Laboratori d'Anàlisi Clínics, Hospital Universitari General de Castelló, Castelló, Spain*

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14 **Corresponding author. Phone: +34964728099 e-mail: vicentej@uji.es*

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Abstract

BACKGROUND: Micellar liquid chromatography – fluorescence detection was used to determine the antibiotics flumequine, marbofloxacin, difloxacin, and sarafloxacin in porcine, bovine, poultry, ovine, caprine, rabbit, and equine meat, to verify compliance with EU Regulation 37/2010 with regard to the occurrence of veterinary drugs in food.

RESULTS: The analytes were isolated from the matrix by ultrasonication-assisted leaching in a micellar solution, and the supernatant was filtered and directly injected. The fluoroquinolones were resolved in < 19 min using a C18 column, with an isocratic mobile phase of 0.05 mol L^{-1} sodium dodecyl sulfate - 8% 1-butanol – 0.5% triethylamine buffered at pH 3. The limits of quantification (0.01–0.05mg kg^{-1}) were below the maximum residue limits (0.15–0.4mg kg^{-1}). The method was validated by EU Commission Decision 2002/657/EC guidelines.

CONCLUSION: The method shows practical advantages such as simplicity, lowcost, eco-friendliness, safety, and applicability for routine analysis, and is useful for surveillance programs.

Keywords: Animal; Fluoroquinolone; Food safety; Micellar; Muscle; Validation

This paper has been published (DOI 10.1002/jsfa.9314). The authors acknowledge the publication of the paper.

1. Introduction

Meat is a highly-appreciated foodstuff due to their taste and elevated content of proteins, fats, vitamins, minerals and micronutrients, which must be included in a balanced diet and are essential for growth. In the last years, the consumption of meat has increased worldwide, because of the augment of the population, urbanization and income, although it has remained stable at a high level in developed countries [1,2]. The production of meat is an important economic activity in the EU, because of its high production, consumption and trading. The production of pork (22.6 million tons), beef (7.7 million tons) and poultry (12.6 million tons) meat is directed to the inner market (110 %, slightly above 100% and 104% of self-sufficiency rate, respectively) and the exportation, mainly to Russia and East Asia [3]. Although 0.92 tons of sheepmeat and goatmeat are annually produced, the EU is a net importer (88 % of self-sufficiency rate), mainly from New Zealand and Australia [4]. The production of other kind of meats, such as rabbit (0.6 million tons) and horse (62.8 million tons) has also reached a high economic relevance [5,6]. Most of these animals are reared in farms at higher stocking densities and fed with a manufactured feed to reduce the high production costs, and maintain an affordable retail price. However, this practice stimulates the incidence and propagation of infectious diseases among cattle and swine, thus increasing their morbidity and mortality and affecting the productivity of the farm [7].

76 Fluoroquinolones are synthetic broad-spectrum antimicrobials and have a significant
77 post-antibiotic effects against gram positive and negative bacteria. Among them, flumequine
78 (FLU), marbofloxacin (MARBO), difloxacin (DIF) and its main metabolite sarafloxacin
79 (SAR) are widely prescribed in medical and veterinary practice against a wide range of
80 diseases originated by bacterial infections [8]. Their structure and properties can be seen in
81 Figure 1 and Table 1 (respectively) [9,10]. In farms, antimicrobial drugs are administered,
82 either orally or in injected, to the food-producing animals as prophylactic and curative agents,
83 to safeguard their welfare, as well as to promote growing [11]. However, their indiscriminate
84 use has resulted in the occurrence of antibiotic residues in edible tissues. The unnoticed
85 exposure to sub-therapeutic amounts has been associated with severe long-term health
86 problems for consumers, such as hazardous effects, allergies and the emergence of
87 fluoroquinolone-resistant human pathogens [12,13]. This stimulates the boost of infectious
88 epidemics, that cannot be treated by the current antibacterial arsenal, and may provoke
89 serious consequences for individual patients and increase the costs for medical care [11].
90 Nowadays, there is a worldwide concern among population and international agencies
91 about the potential risks originated by the abusive use of floroquinolones [11]. Therefore,
92 several governments have established regulations and actions to avoid the misuse of
93 antibiotics in animal farming [14]. Within the frame of its policy to protect human health and
94 keep the image of European meat as healthy and high-quality, the EU has set maximum
95 residue limits (MRLs) for FLU, MARBO and DIF in muscle tissue of several animals,
96 produced and distributed in its area (EU Regulation 37/2010) [15]. No MRL has been
97 established for SAR, but its residue would not be higher than that of DIF (Table 1). Their
98 monitoring is necessary to verify the compliance with the regulation and ensure food safety.
99 Several multiresidue methods have been developed for the determination of
100 fluoroquinolones in animal muscle tissues using microbiological tests [16], immunoassay

101 [17], electrophoresis [18] and reverse phase high performance liquid chromatography (RP-
102 HPLC) [19]. This last one is the technique-of-choice by its higher versatility and selectivity.
103 Several HPLC methods have been developed for the analysis of FLU, MARBO, DIF and
104 SAR in porcine, bovine, ovine and poultry meat. In general, they require a careful multistep
105 sample preparation [19]. Firstly, the antimicrobials must be extracted with a solvent (aqueous
106 [20-22] or hydroorganic [12,23-28]), by simple mixing [20], vortexing [12,21,22], shaking
107 [23-27,29], ultrasonication [23,26], microwave assisted-[27] followed by centrifugation
108 [12,20-27,29]. Sometimes, several successive extraction steps are even required. Afterwards,
109 the supernatant is often purified before injection to avoid the introduction of particles,
110 proteins, macromolecules, or other small endogenous compounds, which may be harmful for
111 the column and/or overlap with the analytes, by solid phase extraction using a C18 [20],
112 hydrophilic-lipophilic [21,23,29] or hydroxylated polystyrene-divinylbenzene [24,27],
113 immunoaffinity [22] or metalchelate affinity [28] coating, liquid/liquid extraction [12,26] or
114 QuEChERS [25] extraction. These procedures enlarge the time, effort, economic and
115 laboratory resources, and amount of toxic chemicals required for the analysis. Besides, they
116 provide variable recoveries and increase the sources of variance of the method. Finally, the
117 drugs are separated in a polystyrene-divinylbenzene [26], C8 [24] or C18
118 [12,20,21,23,25,27,29] columns, a mobile phase with a high concentration of organic solvent
119 (up to 100 %), usually programmed as a gradient [12,20-27,29], and detected by mass
120 spectrometry [20,23-25], UV-Visible absorbance [24,27] or fluorescence [12,21,22,26,28,29].
121 This last one is preferred because of its higher analytical performance-per-cost ratio.
122 However, at our knowledge, no HPLC method has been published about the analysis of these
123 antibiotics in caprine, rabbit or horse meat. Liquid chromatography with acidic hybrid
124 mobile phases, using sodium dodecyl sulphate (SDS) as surfactant and triethylamine (TEA)
125 as sacrificial base, has been proven as an interesting alternative to the determination of

126 quinolones in food [30-32]. Micellar solutions are able to solubilize compounds within a
127 large range of molecular mass, hydrophobicity and charge. Therefore, proteins and other non-
128 water soluble compounds are harmless eluted at the front of the chromatogram, and does not
129 interfere with less retained analytes. This avoids the injection of aqueous suspensions without
130 cleanup after a simple filtration, thus simplifying the sample pretreatment [33]. Besides, the
131 negative layer on the stationary phase and the presence of the micellar pseudophase increase
132 the versatility and the reproducibility of the retention mechanism, and allows the resolution of
133 a mixture of cationic and neutral drugs with different hydrophobicities in the same run using
134 a mobile phase containing <12.5% of organic solvents working under isocratic mode. In
135 addition, the fluorescence is enhanced in organized environments [34]. The use of acidic pure
136 micellar solutions followed by ultrasonication has been also used to extract fluoroquinolones
137 from flesh with a high yielding [30]. The aim of the work was the development of an
138 analytical method for the screening of flumequine, marbofloxacin, difoloxacin and
139 sarafloxacin in edible muscle from several animals (pork, beef, chicken, turkey, duck, sheep,
140 goat, rabbit and horse) using micellar liquid chromatography - fluorescence detection. It must
141 be appropriate for quality control to verify the compliance of commercial samples with the
142 EU Regulation 37/2010 [15]. Therefore, it should be practical, easy-to-handle, safe,
143 environmentally friendly, inexpensive and sensitive enough to provide consistent values close
144 to the maximum residue limits for each fluoroquinolone. The analytical performances of the
145 method were verified by validation through the guidelines of EU Commission Decision
146 2002/657/EC [35]. The suitability of the method for routine analysis would be demonstrated
147 by the analysis of incurred samples from retail stores.

148

149 **2. Experimental**

150

151 *2.1 Standards and chemicals*

152

153 Solid standards of FLU (purity>98%), MARBO (>98%), DIF (>99.8%) and SAR
154 (>97.2 %) were obtained from Sigma (St-Louis, MO, USA). SDS (>99.0%) was supplied as a
155 powder by Merck (Darmstadt, Germany). Sodium dihydrogen phosphate monohydrate
156 (>99.0%), 1-propanol, 1-butanol and 1-pentanol (HPLC grade) were bought from Scharlab
157 (Barcelona, Spain). Hydrochloric acid (37.0 %), ethanol (HPLC grade) and trimethylamine
158 (>99.5 %) were purchased from J.T. Baker (Deventer, The Netherlands). Ultrapure water was
159 in-lab produced from deionized water (supplied by the University as tap water) using an
160 ultrapure generator device Simplicity UV (Millipore S.A.S., Molsheim, France).

161

162 *2.2 Preparation of solutions*

163

164 Micellar solutions were prepared by weighting the appropriate amount of SDS and
165 $NaH_2PO_4.H_2O$, and solving them in ultrapure water using a magnetic stirrer. The adequate
166 amount of trimethylamine was added, and then the pH was set to 3 by adding drops of HCl
167 solutions. Furthermore, the organic solvent was added to reach the selected proportion, and
168 the flask was filled-up with ultrapure water. Finally, the solution was ultrasonicated for 5 min
169 to achieve solubilization and filtered through a 0.45 µm membrane filter (Micron
170 Separations, Westboro, MA, USA) placed on a Büchner funnel, with the aid of a vacuum
171 pump.

172 Individual solutions of each fluoroquinolone (100 mg L^{-1}) were prepared by solving the
173 adequate amount of the powdered standard and solving it in 5 % of ethanol in a volumetric
174 flask, and then a solution of 0.05 M SDS buffered with phosphate salt 0.01 M at pH 3 was
175 added up to the mark. These solutions were ultrasonicated for 5 min to assure the complete

176 solubilization. Working solutions were prepared by successive dilutions of the stock solutions
177 in the same micellar solution. All the standard solutions were kept at +4ºC a maximum of two
178 months.
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182 *2.3 Chromatographic instrumentation and conditions*
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184 The chromatograph was an HP1100 (Agilent Technologies, Palo Alto, CA, USA),
185 equipped with an isocratic pump, a degasser, a 20-µL loop, an autosampler and a
186 fluorescence detector. The control of the instrumentation and the registration of the signal
187 was performed using the Chemstation Rev.A.10.01 (Agilent Technologies) software. The
188 efficiency (N) was calculated as indicated in [36], using the half-peak width obtained by the
189 software. The dead time (t_0) and retention time (t_R) were directly taken from the
190 chromatogram. The asymmetry was evaluated by visual appreciation.
191 The stationary phase was in a C18 Kromasil column (Scharlab) with the following
192 characteristics: length, 150 mm; internal diameter, 4.6 mm; particle size, 5 µm; pore size, 10
193 nm). The mobile phase was an aqueous solution of 0.05 M SDS – 8 % 1-butanol – 0.5 %
194 trimethylamine, buffered at pH 3 with 0.01 M phosphate salt, running at 1 mL min^{-1} under
195 isocratic mode. The detection was performed by fluorescence, and the excitation/emission
196 wavelengths (nm) were programmed in-time as follows: 0.0-8.5 min, 240/370; 8.5-11.5,
197 300/488; 11.5-20, 280/455. The solutions were filtered through a 0.45-µm Nylon membrane
198 filter before introduction into the vials. The special care required with the chromatographic
199 instrumentation when dealing with micellar mobile phases (change of mobile phase, cleaning
200 before switching off, *etc.*) is detailed in [33].

2.4 Sample processing

Samples of pork, beef, chicken, turkey, duck, sheep, goat, rabbit and horse meat were bought from a local supermarket, finely minced and stored at -20ºC in a freezer for a maximum of two months. Before processing, sample meat was thawed for 30 min at room temperature.

In order to recover the analytes, 5 g of meat were mixed with 50 mL of a 0.05-M SDS solution buffered at pH 3. The obtained solutions were placed in an Erlenmeyer flask, shaken using a magnetic stirrer for 1 h, and ultrasonicated for 15 min. Finally, the supernatant was taken by decantation and filtered through a 0.45-μm Nylon membrane filter using a Büchner funnel, with the aid of a vacuum pump. This supernatant was immediately injected or kept at +4ºC in the fridge a maximum of two months, until analysis.

For spiked samples, the appropriate volume of the standard solution was injected in the minced meat. Furthermore, the sample was kept overnight at room temperature to provoke the slow vaporization of the solvent and the incorporation of the antibiotic to the matrix. Therefore, these fortified samples adequately imitate those biologically contaminated [37]. Afterwards, the analytes were extracted as indicated above.

Before the analysis, the stored solutions (standard or supernatant) were warmed at room temperature for 30 min to dissolve the crystals of SDS formed overnight.

3. Results and discussion

3.1 Optimization of the chromatographic conditions

226 The main separation conditions were taken from other methods devoted to the
227 determination of fluoroquinolones in honey [31,32] and fish flesh [30], which have provided
228 adequate results: stationary phase, C18; flow rate, 1 mL min^{-1} under isocratic mode;
229 surfactant, SDS; required organic solvent, 1-propanol or 1-butanol; pH, 3 and 0.5 %
230 triethylamine. In this work, we optimize the composition of the hybrid micellar mobile phase
231 (concentration of SDS, and the nature and concentration of the organic solvent) and the
232 detection conditions, in order to resolve a mixture of FLU, MARBO, DIF and SAR with a
233 good peak shape, at the minimum analysis time. The studies were performed using a standard
234 solution containing 0.02 mg L^{-1} of each fluoroquinolone.

235 According to the previous studies, these antimicrobials show a binding behaviour with
236 the micelles, and then the retention times and the efficiency decrease at higher concentrations
237 of SDS. Indeed, depending on their hydrophobicity and charge, they have the possibility to
238 interact with the polar, anionic and hydrophobic sites of the micelles [34]. In order to
239 maximize the efficiency, the concentration was set to the minimal value recommended for
240 MLC: 0.05 M.

241 The pure micellar mobile phase provided too long analysis times and broad peaks. In
242 order to avoid it, the addition of 1-propanol (2.5 to 12.5 %) or 1-butanol (1 to 10 %) [34] was
243 tested. In both cases, lower retention times and higher efficiencies were obtained. This effect
244 was higher for 1-butanol than for 1-propanol, and augmented at increasing concentrations of
245 alcohol. Sarafloxacin was too retained using 1-propanol, even at larger proportions, and then
246 it was discarded. Using 1-butanol, a proportion of 8 % provided the maximal resolution at the
247 minimal analysis time. The less retained peak was flumequine ($t_R \approx 7.3$ min), enough far from
248 the front of the chromatogram. Adequate efficiencies and low tailings were obtained for the
249 four fluoroquinolones.

250 A standard solution of the four quinolones was analyzed using the optimized mobile
251 phase: 0.05 M SDS - 8 % v/v 1-butanol - 0.5 % v/v triethylamine, buffered at pH 3 with 0.01
252 M phosphate salt. The obtained values of (t_R; N) were: flumequine, (7.3 min; 3842);
253 marbofloxacin, (10.2; 2985), difloxacin (13.6; 4580) and sarafloxacin (16.9; 3214). The
254 analytes were adequately resolved. According to the retention time of the first eluting
255 fluoroquinolone, no overlapping with the front of the chromatogram or the less retained
256 compounds of the matrix is expected.

257 The analytes were resolved using a mobile phase containing a less proportion of toxic,
258 volatile and flammable solvent (<8.5 %), than usually required in hydroorganic HPLC (up to
259 100 %). Besides, the interaction with SDS even reduced its volatility. The mobile phase
260 works under isocratic mode, which improves the baseline stability, the reproducibility of the
261 results and enlarges the column lifespan. Besides, a reequilibration time is not needed
262 between two successive injections, thus reducing the analysis time per sample [38].

263

264 *3.2 Detection conditions*

265

266 Fluorescence was selected as a detection technique due to its higher selectivity and
267 sensitivity than absorbance, and lower cost than mass spectrometry. A derivatization was not
268 required, because the studied fluoroquinolones show natural fluorescence. As the
269 spectrophotometric properties of the fluorophore depends on the chemical environment, the
270 excitation/emission wavelengths (nm) of maximal emitted intensity were chosen from several
271 methods about the analysis of these antimicrobials using similar mobile phases: FLU,
272 240/370; MARBO, 300/488 [32]; DIF and SAR, 280/455 [31].

273 In order to maximize the sensitivity, the detector was programmed to detect each
274 fluoroquinolone at its optimal excitation/emission wavelengths. At the beginning of the

275 chromatography run, the signal was monitored at 240/370. Once flumequine has been eluted
276 (8.5 min), the detection wavelengths turned into 300/488, until the complete elution of
277 marbofloxacin (11.5 min). From this point to the end of the chromatograms, the signal was
278 registered at 280/455. The baseline noise was similar for the three sets of wavelengths, and
279 no sudden oscillation of the baseline was observed at the wavelength changes.

280

281 *3.3 Sample preparation*

282

283 The sample preparation was based on that described in [30]: extraction of the
284 fluoroquinolones from the flesh to a solvent (1/10, w/v) by shaking, followed by filtration of
285 the supernatant and direct injection. Several solvents (methanol and 0.05 M SDS at pH 3)
286 were tested and the duration of the stirring were optimized. The studies were performed using
287 a sample of porcine meat spiked at 0.2 mg kg^{-1} of each antibiotic. The recoveries were
288 compared considering the area of the corresponding chromatographic peaks.

289 A at glance, it can be observed that, the micellar solutions contain a larger particles,
290 and then it must be ultrasonicated for 15 min to reduce their size to favour the filtration. The
291 chromatographic peaks were sharper using the micellar solution, although the recoveries were
292 similar with both solvents. The use of methanol was discarded, because the volume of
293 organic solvent handled and wasted would be too high, and it can partially vaporize during
294 the processing, thus providing variable and falsely enhanced recoveries.

295 Several stirring times, from 10 min to 3 h were tested. The recovery strongly
296 increased from 0 min to 30 min, augments at a low rate to 60 min, and does not show
297 significant variations beyond this value. Therefore, the stirring time was fixed at 60 min.

298 The sample preparation was easy-to-handle, as it only includes a simple solid/liquid
299 extraction and the direct injection of the supernatant. Time-consuming and cumbersome

cleanup steps are not needed and no reactions are involved. The used reagents are available, stable, innocuous and biodegradable, and no toxic organic solvent was required. Therefore, the loss of analyte, either by incomplete recuperation or by chemical change, and the risk of contamination of the sample are reduced, thus enhancing the reliability of the procedure. Besides, several samples can be simultaneously processed by the same operator, which is an interesting practical feature.

3.4 Method validation

The procedure was *in-lab* validated following the guidelines of the European Commission Decision 2002/657/EC in terms of selectivity, calibration range, linearity, trueness, precision, sensitivity, decision limit (CCα), detection capability (CCβ), ruggedness and stability [35].

3.4.1 Selectivity

Free-fluoroquinolone samples of each studied meat were analyzed by the developed method. The front of the chromatogram cover from the dead time to 2.5 min, and other small peaks were observed, but far from the window time ± 2.0 min of the studied antibiotics. The chromatograms obtained from all of them were similar.

The same samples were fortified to 0.2 mg kg^{-1} FLU, MARBO, DIF and SAR, and analyzed. The chromatogram obtained from the spiked porcine meat sample can be seen in Figure 1. In all cases, peaks corresponding to the four antibiotics appeared at similar retention times (<2 %) and peak areas (<4 %) to those obtained by the analysis of a standard solution. The excitation and emission wavelength were taken, and the wavelengths of maximal emitted

325 fluorescence were the same as those indicated in Section 3.2. These results prove the absence
326 of matrix effect. Besides, no overlapping with meat compounds was observed.
327 The high selectivity of the method was reached because of the low retention of the
328 proteins, fats and other macromolecules, because their strong interaction of the micelles; and
329 the specificity of fluorescence, which reduces the number of potential interfering compounds.
330
331 *3.4.2 Calibration range and linearity*
332
333 Standard solutions containing increasing concentrations (up to 0.8 mg L^{-1}) of the
334 studied fluoroquinolones were 1/10 diluted, to include the dilution caused by the transfer of
335 the analytes from the meat to the supernatant, and analyzed by triplicate. Therefore, the
336 quantitative values refer to concentrations in meat, not in the injected solution. The average
337 peak area was related to the corresponding concentration by a first-grade equation by least-
338 square linear regression [39]. The slope, y-intercept and determination coefficients can be
339 seen in the Table 2.
340 The limits of detection (LOD) and quantification (LOQ) were calculated as 3 and 10
341 times the standard deviation of the blank divided by the sensitivity [39]. The calibration range
342 was from LOQ to 0.8 mg kg^{-1}. The results can be seen in the Table 2. The chromatogram
343 obtained from the analysis of a porcine meat sample spiked with the studied antibiotics at
344 their corresponding LOQ can be seen in Fig. 2.
345 A satisfactory linearity was reached, according to the high goodness of fit of the
346 regression ($r^2 > 0.9994$). For each fluoroquinolone, the calibration ranges cover the maximum
347 residue limits in porcine and bovine muscle, mainly thanks to the high sensitivity of
348 fluorescence detection.
349

350 *3.4.3 Trueness and precision*

351

352 These parameters were determined under repeatability and within laboratory
353 reproducibility conditions. Each level, fluoroquinolone and kind of meat were separately
354 investigated.

355 For the repeatability measurements, samples of porcine and bovine meat were fortified
356 with each fluoroquinolone at 0.5x; 1x and 1.5x the corresponding MRL (the lowest
357 concentration evaluated for MARBO was 0.1 mg kg^{-1}, as the 0.5xMRL falls under LOQ).
358 The processed samples were analyzed by six successive injections. The trueness was
359 calculated as the average of the concentrations provided by the calibration curve minus the
360 true value, divided by the true value, while the precision was the relative standard deviation
361 of the six peak areas. For the within laboratory reproducibility studies, the same protocol was
362 performed five separate days over a three-month period, by renewing the fortified samples.
363 The trueness was the average of the five average found concentrations measured each day
364 minus the true value, divided by the fortified concentration, whereas the precision was the
365 relative standard deviation (RSD) of the five average values of the peak areas obtained each
366 day. The results are shown in Table 3 (for flumequine and marbofloxacin) and in Table 4 (for
367 difloxacin and sarafloxacin).

368 The values of bias (from -16.1 to +7.8 %) and variability (RSD <9.4%) provided by the
369 procedure were adequate for the studied levels, analytes, and matrices, and fulfil the
370 requirements stated by the validation guideline (from -20 to +10 % and <12 %, respectively)
371 by the EU guidelines. This demonstrated the high and stable yielding of the extraction step,
372 and the advantages of the direct injection of the supernatant.

373

374 *3.4.4 Decision limit and detection capability*

375

376 These parameters have been proposed by the EU Decision Commission 2002/657/EC, 377 in order to consider the disturbance in the recognition of compliant and non-compliant 378 samples, because of the uncertainty of the quantitative measurements. A more detailed 379 description of these parameters can be seen in [35]. In brief, the decision limit is the minimal 380 found concentration resulting in a rejection, with a reduced probability (< 5%) of making a 381 wrong decision. However, this increases the probability to accept a contaminated sample. The 382 CCβ is the minimal concentration in a sample that the method is able to classify as non-383 compliant with a certainty of >95%.

384 CCα and CCβ were separately measured for each kind of meat and fluoroquinolone. 385 The decision limit was the MRL plus 1.64 times the standard deviation obtained by the 386 analysis of a muscle piece spiked at the MRL (n=20). The detection capability was the CCα 387 plus 1.64 times the standard deviation obtained by the analysis of a sample fortified at the 388 CCα [35]. The results can be seen in Table 5.

389 For both kinds of meat and antimicrobial, the decision limits (<13% over MRL) and 390 the detection capabilities (<27% over MRL) were close to the MRL. Therefore, the 391 probability to obtain a result, leading to the acceptance of a potential non-compliant sample is 392 relatively low. Besides, the concentration range at which the method is unable to correctly 393 classify a contaminated meat sample is quite narrow. Therefore, random errors would 394 provoke a false decision only in a few situations.

395

396 *3.4.5 Ruggedness*

397

398 The changes in the retention and sensitivity caused by small variations of the 399 experimental conditions was examined, in the range that can occur in the normal laboratory

400 practice, using a Youden approach [35]. The ruggedness was separately studied for each
401 fluoroquinolone, and instrumental response (retention time and peak area), using a standard
402 solution of 0.02 mg L^{-1} of FLU, MARBO, DIF and SAR.

403 The considered factors and their intervals were: SDS, 0.045-0.055 M (A); 1-butanol
404 proportion, 7.8-8.2 % (B); pH, 2.8-3.2 (C); TEA, 0.45-0.55 % (D); flow-rate, 0.98-1.02 mL
405 min^{-1} (E); excitation wavelength; optimal value ± 5 nm (F) and emission wavelength: optimal
406 value ± 5 nm (G). The standard deviation of the method was determined under within-
407 laboratory reproducibility using the optimal instrumental conditions, as indicated in Section
408 3.4.2, but using the standard solution.

409 For both peak area and retention time, the differences obtained for each factor were
410 similar. Besides, these differences and the standard deviation of the differences were slightly
411 over the standard deviation obtained under optimal conditions. Therefore, the method is
412 enough robust to be unaffected by the modifications of the instrumental conditions in the
413 considered ranges, mainly because of the reproducibility of MLC.

414

415 *3.4.6 Stability*

416

417 The degradation of the fluoroquinolones in the standard solutions and in the studied
418 muscle tissues was investigated at their common storage conditions (as indicated in Section
419 2), in order to corroborate the adequacy of the selected storage time.

420 A standard solution of MRL/10 mg L^{-1} of each fluoroquinolone was stored in a fridge
421 and analyzed each day. The peak areas remained nearly constant for two months, and no
422 other peaks appeared in the chromatogram.

423 Samples of each studied meat were fortified at their respective MRLs of the studied
424 antimicrobials and kept in a freezer. On the day 0 and each week, a sample was analyzed. The

425 concentration of the antibiotics does not undergo a significant declining after two months,
426 and no degradation products were observed.
427 The fluoroquinolones remain stable in both micellar standard solution at +4ºC and in
428 meat at -20ºC, in the darkness, for at least two months. The standard solutions were discarded
429 after two months, and samples meats can be stored during this period until analysis.
430
431 *3.5 Analysis of real samples*
432
433 The developed method was used to determine the quantity of FLU, MARBO, DIF and
434 SAR in incurred samples from pig, beef, chicken, turkey, duck, sheep, goat, rabbit and horse
435 meat (five samples each one) purchased from a local supermarket, in order to evaluate its
436 applicability for routine analysis. Fluoroquinolone residues were not detected in any sample,
437 and then they can be sold without risk for the population.
438 A single operator was able to analyzed the whole set of samples in one day. Indeed, the
439 meat pieces were simultaneously processed in < 2 h, and the total chromatographic sequence
440 takes nearly 14.5 h. The participation of the operator was restrained to the preparation of the
441 solutions, mixtures, filtration, control of the instrumentation and apparatus, as well as the
442 supervision of the whole process, as the other tasks (stirring, ultrasonication, injection and
443 chromatographic separation) were fully automated.
444 The procedure is able to study a large number of samples per day, using basic
445 laboratory instrumentation and material, and a low amount of chemicals. Besides, the method
446 does not suppose a risk for the health of the operator or the environment, because of the
447 limited toxicity of the prepared solutions. In addition, this allows the reduction of the costs
448 for waste segregation and treatment. Therefore, the analyses were performed at a reasonable
449 price. These practical features make the developed method useful for routine analysis.

4. Conclusions

The determination of residues of FLU, MARBO, DIF and SAR in the most consumed meats can be reliably performed by micellar liquid chromatography - fluorescence detection. The designed procedure reached a high sample throughput with an easy-to-handle pretreatment and a minimal participation of the operator, in spite of the complexity of the matrix. Besides, it was eco-friendly, safe for the laboratory staff, relatively inexpensive and useful for routine analysis. These can be considered the main advantages of the procedure. The analytical quality (selectivity, calibration range, linearity, trueness, precision, decision limit, detection capability, robustness and stability) was thoroughly evaluated following the guidelines of the EU Commission Decision 2002/657/EC, with satisfactory results. It was observed that the method provides consistent quantitative values around the maximum residue limits (0.15 - 0.4 mg kg^{-1}). The remarkable analytical and practical performances were reached mainly by the specific properties of micellar solutions. Therefore, this analytical method is a suitable alternative for quality-control laboratories to evaluate the compliance of commercial edible animal muscle samples with the EU regulation 37/2010, regarding to the occurrence of the antimicrobials flumequine, marbofloxacin, difloxacin and sarafloxacin.

5. Future perspective

The current trend in Analytical Chemistry is the development of inexpensive, simple, automated and ecofriendly analytical procedures. Therefore, the implementation of these kinds of methods will increase in the future in routine analysis and official quality control. An

475 interesting approach is the substitution of current analytical methods by other ones, based on
476 direct injection, and using a lower quantity of toxic chemicals. Micellar liquid
477 chromatography can play a major role in this process.

478 The application of the here-described method may be enlarged to determine other
479 veterinary drugs used in farming, by a small variation in the separation conditions. Besides, it
480 can also be applied to other edible meats, and further to other solid foodstuff. In this case, a
481 modification of the extraction conditions would be necessary. The chromatographic
482 conditions will be similar, as the micellar environment prevents the matrix effect.

483 A modification of micellar liquid chromatography, based on the use of pure mixed
484 micellar mobile phases (using a biodegradable and safe nonionic surfactant), instead of
485 hybrid ones (using toxic, flammable and volatile organic solvent), has been recently proposed
486 and has attracted a huge interest. This new technique can be applied to this method, in order
487 to totally remove the use of hazardous chemicals and then totally fulfil the requirements of
488 "green" chemistry.

489

490 **6. Executive summary**

491

492 *Background*:

493 - Antimicrobial drugs may occur in edible muscle of several food-producing animals due to
494 their abusive use in intensive farming. This represents a worldwide threat to the health for the
495 population, as it can stimulate the emergence of drug-resistant pathogens.

496 - The European Commission has established maximum residue limits about the presence of
497 flumequine, marbofloxacin, difloxacin and sarafloxacin in meat (EU regulation 37/2010), in
498 order to reduce the abusive administration of these medications and prevent hazardous effects
499 to the consumer.

500 - Analytical methods must be developed to determine these antibiotics in meat, in order to
501 evaluate the compliance of producers with the regulation.

502 *Experimental*:

503 - The analytes were extracted from the matrix by mixing with a pure micellar solution,
504 stirring and ultrasonication. The supernatant was filtered and directly injected.

505 - The four quinolones and the extracted matrix were resolved by HPLC using a hybrid
506 micellar mobile phases made of: sodium dodecyl sulfate as surfactant, 1-butanol as organic
507 solvent, triethylamine as sacrificial base and phosphate salt as a pH buffer.

508 - The analytes were detected by fluorescence.

509 *Results and discussion*:

510 - The composition of the mobile phase, the detection conditions and the sample preparation
511 were optimized.

512 - The fluoroquinolones showed a binding behavior to the micelles, and then the retention time
513 and the efficiency diminished at increasing concentrations of sodium dodecyl sulphate.

514 - The retention time and the broadness of the peaks decrease at higher proportions of organic
515 solvent.

516 - The selected mobile phase contains only < 9 % of organic solvent.

517 - The excitation/emission wavelengths were specifically optimized for a micellar
518 environment. These values were changed throughout the chromatographic run to maximize
519 the signal to noise ratio.

520 - The use of a 100 % aqueous micellar solution provides a good recovery, and interesting
521 practical advantages.

522 - The stirring time was optimized.

523 - The method was validated by the guidelines of the European Commission Decision
524 657/2002/EC, in terms of selectivity, sensitivity, trueness, precision, decision limit, detection

capability, ruggedness and stability. The values of the validation parameters were under the requirements of the guideline.

- The analytical procedure was successfully applied to commercial samples of the studied meats.

Conclusions:

- The method is suitable to monitor the selected antibiotics in meats available to the consumer.
- The procedure provides reliable results and is able to distinguish between compliant and non-compliant samples.
- It has interesting practical advantages, such as simple, easy-to-handle, short, environmentally friendly, safe, inexpensive, able to process of a large number of samples per day and useful for routine analysis.
- It can be implemented for official quality control to evaluate the compliance of meats with the regulation.

7. Conflict of interest

The authors state that there is no financial/commercial conflict of interest.

8. Acknowledgment

This work was supported by Project P1.1B2012-36 del Pla de Promoció de la Investigació de la Universitat Jaume I.

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684

685

686 **FIGURE CAPTIONS**

687

688 **Figure 1**. Chromatograms obtained by the analysis of a sample of porcine meat spiked at 0.2
689 mg kg^{-1} of each quinolone. The structure of each antimicrobial is also shown.

690 **Figure 2**. Chromatogram obtained from a sample of porcine meat spiked at their
691 corresponding LOQ.

TABLES

Table 1. Characteristics and MRL (mg kg^{-1}) of the studied fluoroquinolones [9,10,15].

Antibiotic	Flumequine	Marbofloxacin	Difloxacin	Sarafloxacin
pKa COOH group (acidic)	6.4	5.7	5.7	5.6
pKa N-piperazynil moiety (basic)	---------	8.0	7.2	8.2
Log Po/w	2.3	-2.9	1.3	1.1
MRL in porcine and bovine meat	0.2	0.15	0.4	0.4[a]
MRL in poultry meat	0.4	0.15[b]	0.3	0.3[a]
MRL in ovine meat	0.2	0.15[b]	0.3	0.3[a]
MRL in caprine meat	0.2	0.15[b]	0.4	0.4[a]
MRL in rabbit and horse meat	0.1	0.15[b]	0.3	0.3[a]

[a]No regulatory MRL. Practical MRL same as for DIF.

[b]No regulatory MRL. Practical MRL same as for porcine and bovine meat.

Table 2. Calibration curves and sensitivity of the method (concentrations in mg kg^{-1}).

Quinolone	Slope	y-intercept	r^2	LOD	LOQ
Flumequine	524 ± 3	-2±5	0.9998	0.015	0.05
Marbofloxacin	172.9±0.8	3±4	0.9997	0.03	0.1
Difloxacin	2448±5	14±9	0.9996	0.003	0.01
Sarafloxacin	1055±7	-12±15	0.9994	0.015	0.05

Table 3. Trueness/precision measured in repeatability and within-laboratory reproducibility conditions (bias, %/RSD, %) for FLU and MARBO.

		FLU		MARBO[a]	
Meat	Fortified amount	Repeatability[b]	Within-laboratory reproducibility[c]	Repeatability[b]	Within-laboratory reproducibility[c]
Pork	0.5xMRL	+7.8/9.0	+6.4/7.5	-16.1/8.2	-15.2/8.4
	MRL	+5.8/5.5	+4.4/6.3	-9.2/7.3	-10.3/7.1
	1.5xMRL	+1.8/4.1	+2.4/3.0	-2.3/4.2	-3.2/4.7
Beef	0.5xMRL	+4.1/6.8	+3.8/7.9	-14.2/7.5	-13.5/9.4
	MRL	+2.4/5.1	+1.2/4.6	-9.5/6.8	-8.2/7.7
	1.5xMRL	-2.8/3.5	-1.1/2.7	-3.9/4.1	-2.5/5.5
Chicken	0.5xMRL	+5.2/5.8	+4.2/6.1	-15.4/8.5	-14.8/9.3
	MRL	+2.1/3.9	+5.5/3.4	-8.5/6.9	-7.5/7.0
	1.5xMRL	+1.0/1.9	+0.9/2.8	-4.0/4.2	-3.8/5.2
Turkey	0.5xMRL	+4.5/4.2	+3.9/3.8	-12.4/7.5	-11.8/7.4
	MRL	-2.0/4.2	-1.9/4.1	-6.8/5.1	-7.0/6.4
	1.5xMRL	-1.5/3.3	+1.0/2.1	-3.8/2.9	-4.0/3.5
Duck	0.5xMRL	+4.8/5.4	+4.0/4.8	-13.8/8.0	-13.0/7.1
	MRL	+2.0/3.1	+2.5/3.0	-8.4/6.8	-8.1/7.0
	1.5xMRL	+1.1/2.8	+1.8/2.1	-4.1/3.9	-4.8/3.4
Sheep	0.5xMRL	+4.1/4.1	+3.5/3.4	-15.8/6.9	-14.8/7.4
	MRL	-2.8/3.9	-1.9/2.5	-9.8/8.1	-8.4/7.8
	1.5xMRL	+0.9/2.5	+1.0/1.9	-5.4/4.5	-4.8/4.9
Goat	0.5xMRL	+4.8/5.1	+4.0/4.2	-12.8/7.9	-11.0/8.4
	MRL	+3.8/4.1	+3.5/2.7	-6.9/5.8	-6.1/6.7
	1.5xMRL	+2.0/3.1	+2.2/1.9	-3.8/5.1	-4.2/4.7
Rabbit	0.5xMRL	+7.8/8.7	+7.5/7.8	-14.5/8.5	-13.8/7.4
	MRL	+5.0/3.9	+4.5/4.2	-8.4/7.8	-9.0/8.1
	1.5xMRL	+3.9/2.7	+3.0/3.8	-5.2/5.1	-6.2/5.7
Horse	0.5xMRL	+7.2/8.1	+6.8/7.9	-12.8/8.3	-12.0/7.9
	MRL	+4.9/6.8	+4.5/5.5	-7.8/7.1	-8.0/7.3
	1.5xMRL	+4.0/3.4	+3.5/4.2	-4.9/6.1	-4.4/5.4

[a]0.1 mg kg^{-1} instead of 0.5xMRL; [b]n = 6; [c]n= 5

Table 4. Trueness/precision measured in repeatability and within-laboratory reproducibility conditions (bias, %/RSD, %) for DIF and SAR.

		DIF		SAR	
Meat	Fortified amount	Repeatability[a]	Within-laboratory reproducibility[b]	Repeatability[a]	Within-laboratory reproducibility[b]
Pork	0.5xMRL	+5.8/7.2	+4.5/6.5	-6.7/5.8	-7.0/4.5
	MRL	+1.9/3.9	+0.2/3.2	-3.6/3.8	-2.1/2.8
	1.5xMRL	+3.5/0.8	+2.2/1.9	-1.2/1.4	-0.2/2.1
Beef	0.5xMRL	+5.0/5.7	+3.8/6.6	-7.2/5.8	-8.0/6.8
	MRL	-1.6/3.4	-0.5/2.5	-4.5/3.6	-3.4/3.3
	1.5xMRL	-0.3/2.4	-1.4/1.7	-2.9/2.2	-1.8/2.5
Chicken	0.5xMRL	+5.5/6.8	+3.4/5.1	-5.2/4.1	-6.8/7.1
	MRL	+2.0/3.8	+1.5/2.8	-3.8/2.9	-4.0/3.8
	1.5xMRL	+0.9/2.4	-0.8/1.5	-1.9/3.4	-2.0/3.0
Turkey	0.5xMRL	+4.5/4.0	+4.2/4.4	-4.1/5.4	-4.8/6.0
	MRL	-1.1/2.8	-0.9/2.0	-2.8/3.8	-3.0/4.1
	1.5xMRL	-2.1/3.1	-1.5/2.4	-0.8/2.0	-1.1/2.9
Duck	0.5xMRL	+5.1/6.5	+4.9/5.4	-2.9/4.5	-3.2/4.1
	MRL	+0.9/1.8	+1.5/2.4	+0.8/1.9	+0.0/2.8
	1.5xMRL	+1.9/2.5	+2.9/3.2	+1.2/2.9	+1.9/2.4
Sheep	0.5xMRL	+3.1/5.9	+3.9/4.5	-3.9/5.9	-4.0/6.8
	MRL	+3.5/4.2	+4.0/3.8	-2.7/3.5	-3.0/4.5
	1.5xMRL	+2.9/3.4	+2.5/3.0	-1.7/2.5	-2.0/3.5
Goat	0.5xMRL	+4.0/3.9	+3.8/4.9	-5.8/4.6	-5.5/4.0
	MRL	-0.9/4.1	-1.1/3.8	-3.0/3.9	-3.5/4.0
	1.5xMRL	-3.5/2.9	-2.9/2.4	-1.1/2.4	-2.0/3.1
Rabbit	0.5xMRL	+3.9/4.2	+4.1/5.0	-4.8/5.9	-4.0/6.0
	MRL	+0.8/1.9	-0.3/3.9	-1.9/3.4	-2.9/4.0
	1.5xMRL	+2.1/3.5	+0.9/2.9	+0.5/2.8	-0.5/2.1
Horse	0.5xMRL	+5.1/3.5	+4.5/2.9	-5.0/3.5	-4.5/4.2
	MRL	-2.5/5.4	-1.9/3.2	-2.9/4.6	-3.5/3.8
	1.5xMRL	+0.4/3.9	+0.5/2.8	-0.4/3.3	-1.0/2.5

[a]n = 6; [b]n= 5

Table 5. Decision limit/detection capacity for each quinolone in the studied meats (concentrations in mg kg^{-1}).

Meat	FLU	MARBO	DIF	SAR
Pork	0.22/0.24	0.17/0.18	0.43/0.45	0.42/0.44
Beef	0.22/0.23	0.17/0.18	0.42/0.44	0.42/0.44
Chicken	0.43/0.45	0.17/0.18	0.32/0.33	0.31/0.33
Turkey	0.43/0.46	0.16/0.18	0.31/0.32	0.32/0.34
Duck	0.42/0.44	0.17/0.18	0.31/0.32	0.32/0.33
Sheep	0.21/0.22	0.17/0.19	0.32/0.34	0.32/0.34
Goat	0.21/0.22	0.16/0.18	0.43/0.45	0.42/0.45
Rabbit	0.11/0.11	0.17/0.19	0.31/0.33	0.32/0.34
Horse	0.11/0.12	0.17/0.19	0.33/0.35	0.32/0.33

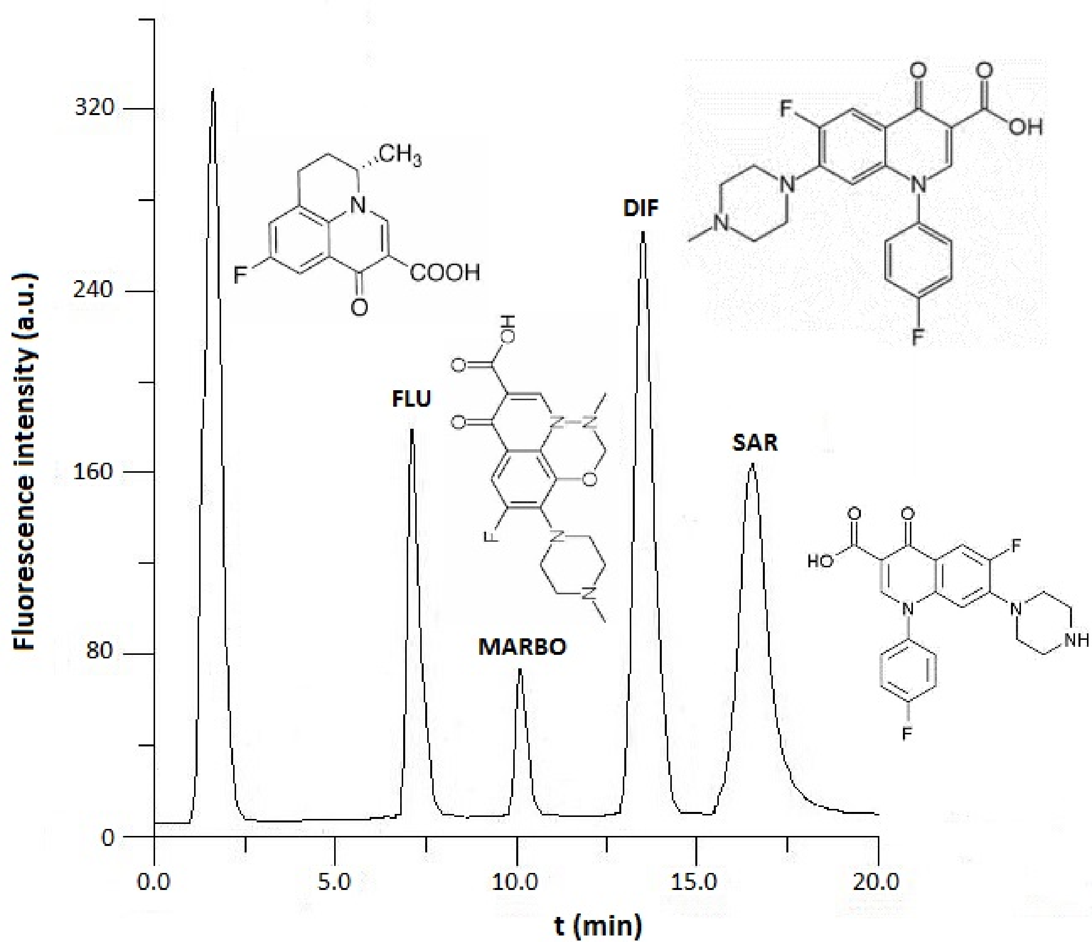

Figure 1. Chromatograms obtained by the analysis of a sample of porcine meat spiked at 0.2 mg kg^{-1} of each quinolone. The structure of each antimicrobial is also shown.

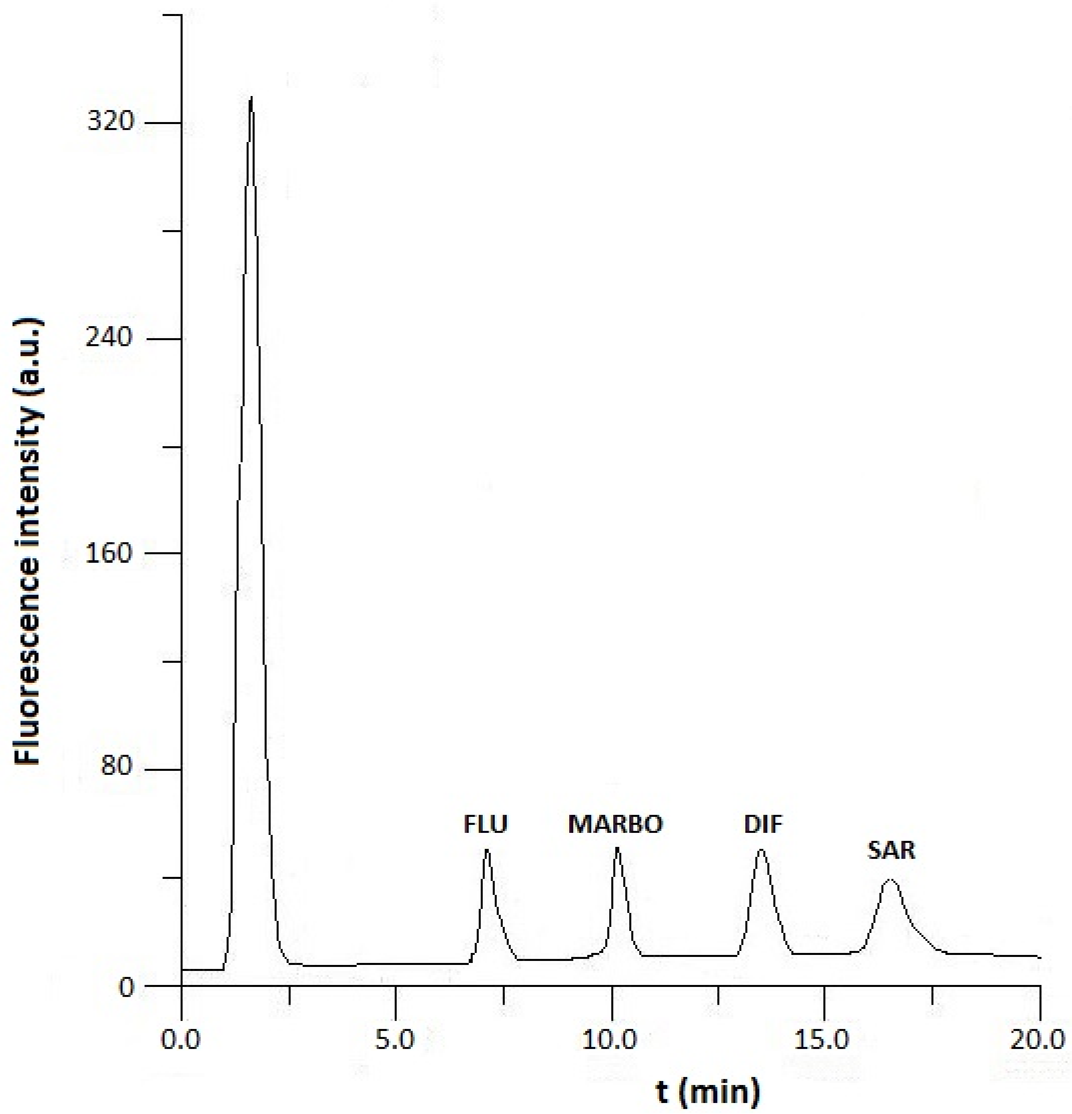

Figure 2. Chromatogram obtained from a sample of porcine meat spiked at their corresponding LOQ.

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