1 **Dispersive liquid-liquid microextraction of quinolones in porcine**

2 **blood: validation of a CE method using univariate calibration or**

3 **multivariate curve resolution-alternating least squares for**

4 **overlapped peaks**

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20 **Abbreviations: MCR-ALS,** multivariate curve resolution-alternating leasts squares;

21 **PDADMAC**, poly(diallyldimethylammonium chloride); **FQs**, fluoroquinolones; **DLLME**,

22 dispersive liquid–liquid microextraction; **IS**, internal standard; **AS**, salicylic acid; **FLU**,

23 flumenique; **DIF**, difloxacin; **ENO**, enoxacin; **OFL**, ofloxacin; **GTF**, gatifloxacin; **ENF**,

24 enrofloxacin; **CPF**, ciprofloxacin; **DNF**, danofloxacin; **MRF**, marbofloxacin; **DCM**,

25 dichloromethane.

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29

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Abstract

In the previously published part of this study, we detailed a novel strategy based on dispersive liquid-liquid microextraction to extract and preconcentrate nine fluoroquinolones in porcine blood. Moreover, we presented the optimized experimental conditions to obtain complete CE separation between target analytes. Consequently, this second part reports the validation of the developed method to determine flumenique, difloxacin, enrofloxacin, marbofloxacin, ofloxacin, ciprofloxacin, through univariate calibration, and enoxacin, danofloxacin, and gatifloxacin through multivariate curve resolution analysis.

The validation was performed according to FDA guidelines for bioanalytical assay procedures and the European Directive 2002/657 to demonstrate that the results are reliable. The method was applied to the determination of FQs in real samples. Results indicated a high selectivity and excellent precision characteristics, with RSD less than 11.9% in the concentrations, in intra and inter-assay precision studies. Linearity was proved for a range from 4.00–30.00 mg L^{-1} and the recovery, has been investigating did at four different fortification levels, varied from 89% to 113%. Several approaches found in the literature were used to determinate the LODs and LOQs. Though all strategies used were appropriate, we obtained different values when using different methods. Estimating the S/N ratio with the mean noise level in the migration time of each FQs, turned out as the best studied method for evaluating the LODs and LOQs, and the values were in a range of 1.55 to 4.55 mg L^{-1} and 5.17 to 9.62 mg L^{-1}, respectively.

51   **1. Introduction**

52   Blood is one of the major products of the meat industry and an alternative source of

53   nutritional and functional proteins [1] that has been used as an ingredient in many types of

54   sausages, soups, bread, and cookies [2-4]. The quality of these processed foods is related

55   to the presence of compounds that are orally administered to livestock [5] of various animal

56   species that produce meat, eggs or milk, and whose residues are accumulated in animal

57   tissue. Fluoroquinolones (FQs) belongs to a group of antibacterial agents which are widely

58   employed in veterinary medicine [6,7].

59   Therefore, the measurement of drug concentrations in a biological matrix such as blood

60   is a fundamental aspect to ensure the quality of the food consumed, and reliable analytical

61   methods are required for compliance with national and international regulations.

62   In the first part of this work [8] we showed that there are numerous studies and reviews

63   about the determination of FQs in animal tissues, eggs, and bovine milk, however, the

64   number of studies specifically addressing FQs in porcine plasma or serum remains quite

65   small. Hence, we developed an analytical method by CE to determine: flumenique (FLU),

66   difloxacin (DIF), enrofloxacin (ENF), marbofloxacin (MRF), ofloxacin (OFL), ciprofloxacin

67   (CPF), enoxacin (ENO), danofloxacin (DNF), and gatifloxacin (GTF) in samples of porcine

68   blood.

69   When working with CE, the complete analyte separation is frequently expected, i.e.,

70   each peak belongs to a single compound. Consequently, a full-factorial (FFD) and a central

71   composite design (CCD) were performed in order to increase the extraction and also to

72   obtain total separation between the nine FQs and the IS in the CE separation. When real

73   complex samples are analyzed, it is possible to find several unknown substances that

74   overlap with target analytes.

75   To solve this issue and to achieve short analysis time, second order data has been

76   generated and modeled with proper chemometric algorithms. In this procedure the

77   information provided by second-order data can be uniquely ascribed to the analyte of

78   interest, even in the presence of unexpected components not considered during the

79   calibration stage (a property that has been called "the second-order advantage"). Several

80   works have been published concerning the chemometric modeling of CE–DAD data [9-13].

81   In the majority of them, Multivariate curve resolution–alternating least square (MCR-ALS)

82   has been the algorithm chosen to solve the analytical problems [14]. MCR-ALS is well-

83   known second-order algorithm able to handle third-order data arrays deviating from

84   trilinearity. The analysis by CE method with DAD, coupled to the chemometric algorithms,

85   guarantees selectivity by mathematical means, allowing for resolution and quantitation of

86   overlapped analytes while keeping the analysis time to a minimum.

87   In this second part of the work, we conclude with the validation of the developed method,

88   to demonstrate that it is suitable for the intended use and it can offer reliable results. In the

89   last years, several manuscripts have already been published about method validation

90   strategies, including quality assurance, focused on bioanalytical methods, and regulatory

91   purposes in pharmaceutical and control of residues. It should be taking into account that

92   validation requirements are continually changing and vary widely [15].

93   Nowadays, there are several renowned international organizations offering guidelines on

94   method validation and related topics. Primary references are the Association of Official

95   Analytical Chemists (AOAC), the Food and Agricultural Organization (FAO), the United

96   States Food and Drug Administration (FDA), the International Conference on Harmonization

97   (ICH), the analytical chemistry group EURACHEM, etc. [15].

98   In this sense, the proposed method was validated according to FDA guidelines for

99   bioanalytical assay procedures (FDA 2001) and the European Directive 2002/657 [16].

100 Bioanalytical method validation is crucial for the quantitative determination of various types

101 of analytes in biological matrices. The bioanalysis procedure includes sampling, sample

102 preparation, analysis, calibration and data evaluation.

103

104 **2. Materials and methods**

105   *2.1 Chemicals and Reagents*

106   Ultra–pure water was obtained from Millipore (Bedford, MA, USA). Sodium

107   tetraborate, sodium hydrogen phosphate, hydrochloric acid (HCl), sodium hydroxide

108   (NaOH), DMF, and dichloromethane (DCM) were purchased from Cicarelli (San Lorenzo,

109   Argentina). The polymer poly (diallyldimethylammonium chloride) (PDADMAC) was

110   purchased from Sigma–Aldrich Inc.(St. Louis, USA). LC grade methanol (MeOH) and ACN

111   were obtained from J.T. Baker (Deventer, Netherlands).

112   All standards were of analytical grade. Salicylic acid (AS), flumenique (FLU),

113   difloxacin (DIF), enoxacin (ENO), ofloxacin (OFL), and gatifloxacin (GTF) were provided by

114   Sigma-Aldrich (Munich, Germany). Enrofloxacin (ENF), ciprofloxacin (CPF), and

115   danofloxacin (DNF) were purchased from Fluka (St. Gallen, Switzerland), and marbofloxacin

116   (MRF) was obtained from Molekula (Gillingham,UK). AS was used as an internal standard at

117   a concentration of 70 mg L^{-1} dissolved in water-ACN (70:30, v/v).

118

119   *2.2 Buffer and standard solutions preparation*

120   Stock standard solutions of 2000 mg L^{-1} of each FQs were prepared by precisely

121   weighing and dissolving the appropriate amount of standard in MeOH. The solutions were

122   maintained under refrigeration at 4 °C at dark. Working standard solutions were daily

123   prepared by appropriate dilution of the stock standard solutions in a mixture of water–ACN

124   (70:30, v/v). BGE solutions were prepared from a mixture solution containing equal amounts

125   of sodium borate and sodium hydrogen phosphate with a concentration of 23 mmol L^{-1}

126   containing 0.2% of PDADMAC and adjusted to pH 7.80. NaOH solution was prepared at a

127   concentration of 0.1 mmol L^{-1}; HCl was prepared at concentrations of 0.1 and 2.0 mmol L^{-1}.

128   Both solutions were used to adjust the BGE and porcine blood pH.

129

130   *2.3 Instrumentation and experimental conditions*

131　　　All the CE experiments were carried out on an Agilent CE capillary electrophoresis

132　system (Agilent Technologies, Waldbronn, Germany) equipped with an on–column diode

133　array detector. Separation was performed by applying a reverse polarity of 25 kV, employing

134　a coated fused–silica capillary (MicroSolv Technology Corporation, Eatontown, USA) with an

135　inner diameter of 75 μm and a total length of 40 cm (31.5 cm effective length). The cartridge

136　was maintained at 25.0 °C. The electropherograms were recorded at 275 nm during 4 min,

137　and the second-order data were obtained by recording UV spectra between 200 and 400 nm

138　each 2 nm at 0.4 s steps. The hydrodynamic injection was performed in the cathode by

139　applying a pressure of 50 mbar for 10 s.

140　　　The capillary was coated using 1% (v/v) PDADMAC solution, which was prepared in

141　the BGE solution. The coating was performed at the beginning of every working day by

142　following the methodology presented by Fritz and Steiner, with minor modifications [17]. Four

143　different solutions were pulled through the capillary using a 930 mbar pressure for 2 minutes

144　in the following order: a 0.1 M NaOH solution; ultrapure water; a 1 % (v/v) aqueous solution

145　of PDADMAC, and the BGE solution. After each of the first three solutions, a 15-20 s air

146　purge was applied to the capillary to removed most of the previous solutions. After the last

147　solution, a reverse polarity of 15 kV was used to obtain a stable baseline for the first

148　electropherogram.

149　　　Between runs, the capillary was successively flushed with 0.1 mol L^{-1} sodium

150　hydroxide, ultra–pure water, and BGE for 3 min. At the end of the day the capillary was

151　washed with 0.1 mol L^{-1} sodium hydroxide, and ultra–pure water for 5 min, and finally, it was

152　air–dried for 3 min.

153　　　pH values of the solutions were adjusted by an Orion 410A pH-meter equipped with

154　Ag/AgCl reference electrode (Hanna Instruments, Inc., Woonsocket, USA). All solutions

155　were filtered through 0.45 μm Nylon membranes (Sartorius AG, Göttingen, Germany) before

156　use.

157

158   *2.4 Software*

159   The CE ChemStation software (Agilent Technologies) was employed for the CE

160   instrument control and data acquisition. All the algorithms were implemented in MATLAB

161   7.10   [18].   Those   for   applying   MCR–ALS   is   available   on   the   internet   at

162   http://www.mcrals.info/. Homemade routines based on the Eilers algorithm were used to

163   perform second-order data baseline correction [19]. Homemade routines, based on

164   SIMPLISMA (simple interactive self–modeling mixture analysis) [20], were used to initialize

165   the ALS algorithm.Savitzky–Golay smoothing and differentiation filter were applied to

166   preprocess the data [21].

167

168   *2.5 Sample preparation (DLLME procedure)*

169   Sample preparation was carried out following the method proposed in our previous work

170   [8]. Figure 1A shows the sequenceof sample clean-up and extraction of FQs from porcine

171   blood samples.

172

173   *2.6 Method validation: selectivity, apparent recovery, precision, linearity and linear range,*

174   *LOD, LOQ, matrix effect.*

175   Univariate calibration methodology, based on analyte/IS peak area ratio (area

176   FQs/area IS) at a fixed wavelength (275 nm) was used to determine FLU, DIF, OFL, ENF,

177   CPF, and MRF.

178   For ENO, DNF, and GTF multivariate method (MCR–ALS) was used with the second-

179   order data obtained by recording UV spectra between 200 and 400 nm.

180   The selectivity was evaluated with six blood samples from local slaughterhouses,

181   processed according to the described extraction methodology and injected in the CE

182   instrument. The obtained electropherograms were compared with the corresponding to a

183   standard solution of the target analytes to evaluate the presence of peaks at the same

184   migration times of the FQs. Peak purity was also tested.

185   The matrix effect was evaluated by comparison of the slopes of the calibration curves

186   prepared with standard solutions of the analytes studied, with solutions of analytes in the

187   porcine blood which were subjected to the extraction procedure. These calibrations curves

188   were prepared with concentrations between 1.00 and 100.00 mg L^{-1}. The comparison was

189   performed using a Student's *t*-test with a confidence level of 95%.

190   The repeatability was determined by measurements (n=6) of samples solutions at

191   four different concentrations: LOQ obtained for each analyte, 7.5, 22.50 and 30.00 mg L^{-1},

192   prepared by spiking basal blood with a volume of standard solution. The intermediate

193   precision was evaluated by performing repeated measurements of the same samples during

194   four weeks. Then, the RSD was calculated in both precision studies, for each concentration

195   value, and for each analyte. Regulation 401/2006/EC [22] issued that the permitted

196   experimental RSD for each concentration level must be below twice-fold the value derived

197   by Horwitz equation:

198    $$RSD_R = 2^{(1-0.5 \log C)} \tag{1}$$

199   where C is the mass fraction expressed as mg mL^{-1}.

200   The apparent recovery was evaluated using samples solutions at four different

201   concentrations, prepared by spiking basal blood with a volume of standard solution. These

202   samples were analyzed with the analytical procedure, and the quantity of each FQ recovered

203   about the added amount was calculated.

204   Method linearity was assessed with calibrations curves constructed in triplicate with

205   mixed standard solutions at five concentration levels, from the LOQ obtained for each FQs to

206   30.00 mg L^{-1} approximately, and salicylic acid as an internal standard at a concentration of

207   70 mg L^{-1}. The linear graph was constructed using the ratio of area FQs/area IS (AS 70 mg

208   L^{-1}) versus nominal concentration of analytes (expressed in mg L^{-1}). Mixed standard

209   solutions for FLU, DIF, ENF, MRF, CPF, OFN, DNF, and GTF were prepared at five

210   concentration levels in water–ACN (70:30, v/v) and were introduced into the instrument in a

211   randomized way. The results were analyzed with statistical method of linear regression by

212   least-squares. The homoscedasticity of the data was evaluated trough an *F* test of the

213   variances at the lower and upper limit of analyzed range [23]. The linearity of the calibration

214   graphs was assessed with a lack-of-fit-test [24]. LODs and LOQs were determined using

215   standard solutions prepared in porcine blood, for FLU, DIF, ENF, MRF, OFN, CPF, ENO,

216   DNF, and GTF, applying different criteria.

217   Firstly, the LODs were computed from the calibration curve using the SD of the

218   regression (s_y) and the slope (*b*) through the expression:

219   $$\text{LOD} = \frac{3.3\, s_y}{b} \qquad\qquad (2)$$

220   Secondly, this parameter was estimated by the IUPAC criterion [24] using the

221   expression:

222   $$Y_{LOD} = Y_{blank} + 3.3\, s_{blank} \qquad\qquad (3)$$

223   where Y_{LOD} is the response by the LOD, Y_{blank} is the average of the blank signal and s_{blank}

224   is its corresponding SD. The Y_{LOD} values were converted to concentration through the

225   calibration function using the slope *b.*

226   Otherwise, the LODs were calculated as the concentration of analyte giving a signal

227   three times the noise level (S/N=3). In this sense, two different noise levels were estimated:

228   the noise level near the peak of the IS and the mean values of noise level given in the

229   migration time of each FQs, obtained by the repetition of ten injections of a basal blood on

230   different days and weeks.

231   The LOQs were computed from the linear regression analysis using the SD of the

232   regression (s_y) and the slope (*b*) as was done for the LODs but using a factor equal to 10.

233   Otherwise, these parameters were estimated by the IUPAC criterion [24] using the

234   expression$Y_{LOD} = Y_{blank} + 10\, s_{blank}$ (4).

235   Additionally, they were calculated as the concentration of analyte giving a signal ten

236   times the noise level (S/N=10), using the noise levels calculated before.

237

238   **3. Results and discussion**

239   3.1 Separation selectivity

240   In our previous work [8] we optimized the separation of nine FQs and the IS using the

241   response surface methodology by applying experimental designs. The results of this study

242   were optimized conditions that assure the complete analyte separation in the shortest

243   analysis time when a standard solution of target analytes was analyzed.

244   The total separation of a mixture of standard FQs was possible due to the different

245   percentage of positive, negative, and neutral species of FQs that exist when working at the

246   optimized separation pH of 7.80. Under reverse polarity, FLU migrates in the first place;

247   whose predominant species has negative charge. The following peaks correspond to DIFLO,

248   ENF, MRF, OFN, CPF, ENO, DNF, and GTF, with decreasing percentages of negative

249   species.

250   However, in the analysis of real blood samples, unknown substances of the complex

251   biological matrix were extracted, originating the peak overlapping of Figure 1C. Specifically,

252   interference substances appeared in the migration times of ENO, DNF, and GTF, given that

253   these unknown species have the same electrophoretic behavior as the last three peaks of

254   FQs of Figure 1B. Moreover, no interference substances were found in the migration time of

255   IS and the others FQs.

256   The Figure 1B and 1C show the electropherogram of a sample prepared for studying

257   of apparent recovery, and a real sample of porcine blood, respectively. In Figure 1B, the

258   overlap between endogenous components of porcine blood with the last three FQs peaks is

259   noticeable.

260

261   3.2 MCR-ALS analysis

262   As mentioned before, substantial overlapping of the three analytes (ENO, DNF, and

263   GTF) and blood compounds is evident in the region 3.30–3.65 min. In this sense, these

264   compounds cannot be determinate for univariate calibration. Consequently, MCR-ALS was

265   chosen for data processing in this section, because this algorithm achieves the second-order

266 advantage without requiring the data to fulfill the trilinearity property. With this purpose, the

267 strategy of augmenting matrices along the mode which is suspected of breaking the trilinear

268 structure is implemented. Bilinear decomposition of the augmented matrix $\mathbf{D}_{aug}$ is performed

269 by of the expression:

270 $$\mathbf{D}_{aug} = \mathbf{C}_{aug} \times \mathbf{S}^T + \mathbf{E}_{aug} \tag{5}$$

271 in which the rows of $\mathbf{D}_{aug}$ contain the UV-visible spectra (*K* wavelengths) as a function of the

272 time (*J* times), the columns of $\mathbf{C}_{aug}$ contain the time profiles of the *N* compounds involved in

273 the process, the columns of **S** their related spectra, and $\mathbf{E}_{aug}$ is a matrix of residuals not fitted

274 by the model.

275   The MCR-ALS method was cubic spline interpolation, and smoothing was applied to

276 all matrices given the high level of noise in the data.

277   The first modeling step consisted of building an augmented data matrix **D** in the

278 temporal mode by stacking data matrices corresponding to each calibration solution.

279   Given that between the IS and the *tm* of ENO, DNF and GTF appear other FQs that

280 have the same spectra, the MCR-ALS method was applied in two different regions, one

281 region between 1.70 and 2.00 min for IS (AS) and the other region between 3.20 and 3.65

282 for ENO, DNF, and GTF. The final dimension of the matrix was of (176 x 81).

283   To build the initial estimation, the analysis of the purest spectra based on the

284 SIMPLISMA methodology was applied to obtain each FQs and IS spectra [25]. Interestingly,

285 the number of contributing species in the system when using singular value decomposition

286 was equal to the real number of analytes (AS, ENO, DNF, and GTF), and hence two more

287 unknown components should be considered. After MCR-ALS decomposition of **D**,

288 concentration information of standard samples contained in **C** (the areas under the temporal

289 profiles of each component) was used to construct the univariate regression of peak–area

290 ratios for each analyte and the IS.

291   Figures 2A and 2B show the time and spectral profiles corresponding to the six

292 components found by MCR-ALS analysis, for a spiked porcine blood sample. As depicted in

293 Figure 2A, there is a severe overlapping of time, however, MCR-ALS is able to correctly

294 decompose the data into the relevant contributions; first to the IS, and then in subsequent

295 order, ENO, interference 1, DNF, interference 2, and GTF.

296 Figure 2B also shows the spectral profiles retrieved by MCR-ALS analysis. A

297 reasonably good agreement is found between the spectra corresponding to the analyte

298 obtained with MCR-ALS and the pure spectra (determination coefficients greater than

299 0.998).

300

301 3.3 Validation

302 *LOD and LOQ*

303 The values of LODs and LOQs obtained are listed in Table 1. In the first analysis, we

304 observed that the values achieved with the noise level in each migration time were higher

305 than the values obtained with the noise level taken near of the IS. These phenomena can be

306 explained by the change of the noise level between days and weeks generated by the

307 natural variation of the electrophoretic technique. These values are considered to be the

308 most realists, and were used to define the quality parameters of the method. The same

309 conclusion is obtained for LOQs and for LODs.

310 In the case of ENO, DNF, and GTF, the LODs and LOQs were obtained from

311 multivariate calibration because endogenous compounds interfere with the determination of

312 the analytes. These values of LODs and LOQs correspond to the lowest concentration of

313 FQs detectable and quantifiable in porcine blood sample under the experimental conditions.

314 Even though all of the applied methodologies for the calculations of the limits are

315 appropriate for this work, it is observed that the results obtained differ. Considering the

316 importance on the calculation of these parameters, and the need not to underestimate them,

317 we choose the methodological approach based on the measure of the background noise in

318 the migration time of each FQs.

319   Nevertheless, it is necessary to note that if we consider the preconcentration factor

320   (10) that affects the extraction procedure, these limits will become ten times less.

321

Matrix effect

323   The matrix effect study showed that the difference between the slopes of the

324   calibration curves constructed was not statistically significant, proving that the blood matrix

325   does not affect the extraction procedure.

326

Linearity

328   The homoscedasticity test showed that the difference between the observed and the

329   critical value of F was not significant ($α$=0.05).The p-values obtained in the lack-of-fit-test

330   were greater than 0.10, so the model was adequate for the observed data, and there was an

331   excellent linearity within stated ranges. Also, the squared determination coefficient (r^2)

332   obtained for all the calibration graphs were above 0.99. The results are summarized in Table

333   2.

334

Precision

336   The RSD was calculated in both precision studies, and the results are shown in Table

337   2. The RSDs computed, for each concentration value, and for each analyte are lower than

338   the calculated values by Horwitz equation. These results indicate that the method satisfies

339   the minimum performance criteria established by the above -mentioned regulation.

340

Apparent recovery

342   Apparent recoveries were ranged between 89% and 113% (Table 3A), and this can

343   be considered as excellent given the complexity of the samples.

344

345   *3.4 Analysis of real samples*

346   Six blood samples from local slaughterhouses were processed according to the

347   described extraction methodology (Section 2.5) and injected into the CE system.

348   In two of the analyzed samples, the presence of FLU was detected: 1.64 mg L^{-1} S1

349   and 3.06 mg L^{-1} S3 (Table 3B). Also, to demonstrate that the method developed is valid in

350   real samples, the results were compared between spiked and non-spiked samples. These

351   samples were spiked with three different standard solutions of 0.50, 1.00 and 2.00 mg L^{-1} of

352   FLU. Recovery % was calculated in each case. These high recoveries (95-102 %) indicate

353   that the method can be applied to real samples.

354   Finally, a comparison with other published methods was conducted with the aim to

355   show the advantages of the method reported in this paper. In the literature, the numbers of

356   FQs which can be determined simultaneously in a less complex matrix than porcine blood

357   are less than eight [26-30]. Consequently, it is apparent that one important achievement is

358   the possibility to quantitate the nine analytes together. Another significant achievement is the

359   substantial reduction of the analysis time, between twice and ten-fold lesser than the

360   published methodologies [25, 29-31]. Furthermore, it is important to remark the decrease in

361   the use of solvents during the extraction phase (300-800% less) in agreement with green

362   chemistry [32-34]. Although the LODs obtained in other publications are smaller than the one

363   achieved in this paper (between 0.001 and 0.2 mg L^{-1}) it should be remarked that the LOQs

364   obtained by use of this method are enough for monitoring the drugs in blood because there

365   is no limit determined by regulation.

366

367   **4. Concluding remarks**

368   A validated CE method for the determination of the FQs FLU, DIF, ENF, MRF, OFN,

369   and CPF in porcine blood has been developed by using univariate calibration. The

370   determination of ENO, DNF, and GTF in the presence of unexpected compounds can be

371   performed by using second-order data generated by use of CE coupled with DAD. It has

372 been demonstrated once again that modeling data (as it happens with CE-DAD data) using

373 MCR-ALS is the strategy of choice, owing to its flexibility.

374 The electrophoretic runs had a short analysis time (ca. 4 min). Hence, the proposed

375 method becomes an alternative for routine laboratories

376 By performing the DLLME strategy presented in this work, no contaminant solvents

377 should be used in the analyte extraction, which is highly recommended to follow the green

378 analytical chemistry principles.

379 The whole method is simple, accurate, selective, inexpensive and fast. Furthermore,

380 it is sensitive enough for the analysis of FQs in porcine blood, because there are no

381 maximum residue limits (MRLs) of FQs in porcine blood in Argentine.

382

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389

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391

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444   **Figure captions**

445   **Figure 1:** The sequence of sample clean-up and extraction of FQs from porcine blood

446   samples (A). Electropherograms obtained under optimum separation experimental

447   conditions corresponding to: (B) a sample prepared for studying apparent recovery and (C)

448   real sample of porcine blood.

449
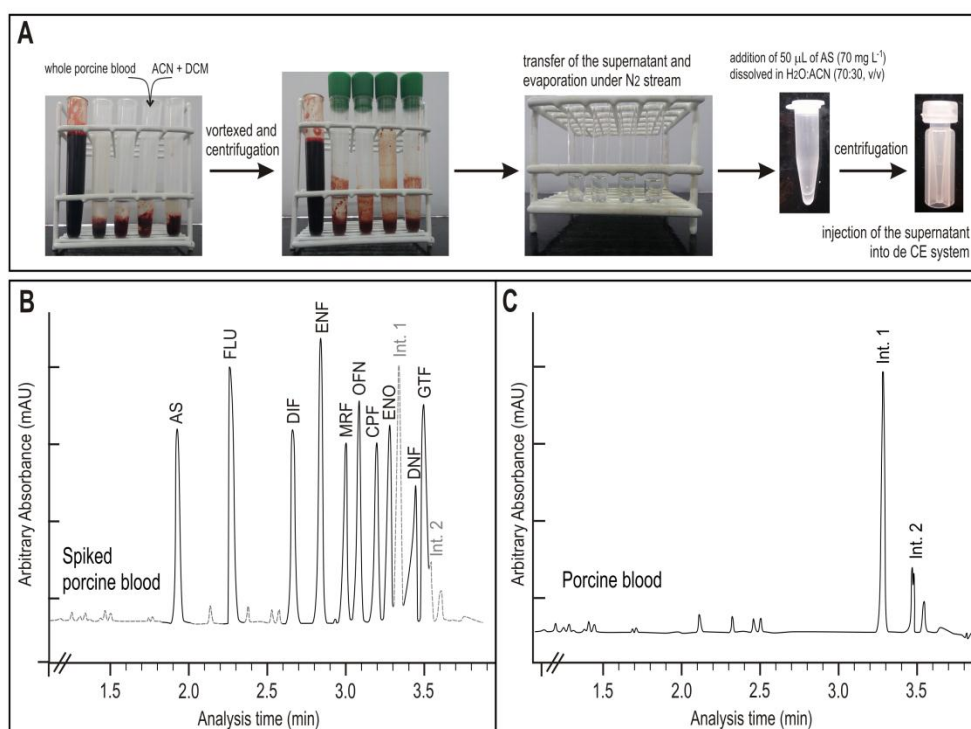
450   **Figure 2: A**) Concentration profiles retrieved by MCR-ALS for a spikes porcine blood

451   sample. Salicylic acid (AS black dash-dotted line), enoxacin (ENO black dots), danofloxacin

452   (DNF black solid line), gatifloxacin (GTF black dashed line), interference 1 (Int. 1 grey

453   dashed line) and interference 2 (Int. 2 grey dashed line) **B**) Spectral profile retrieved by

454   MCR-ALS for the same sample as in **A.**

455

456

457
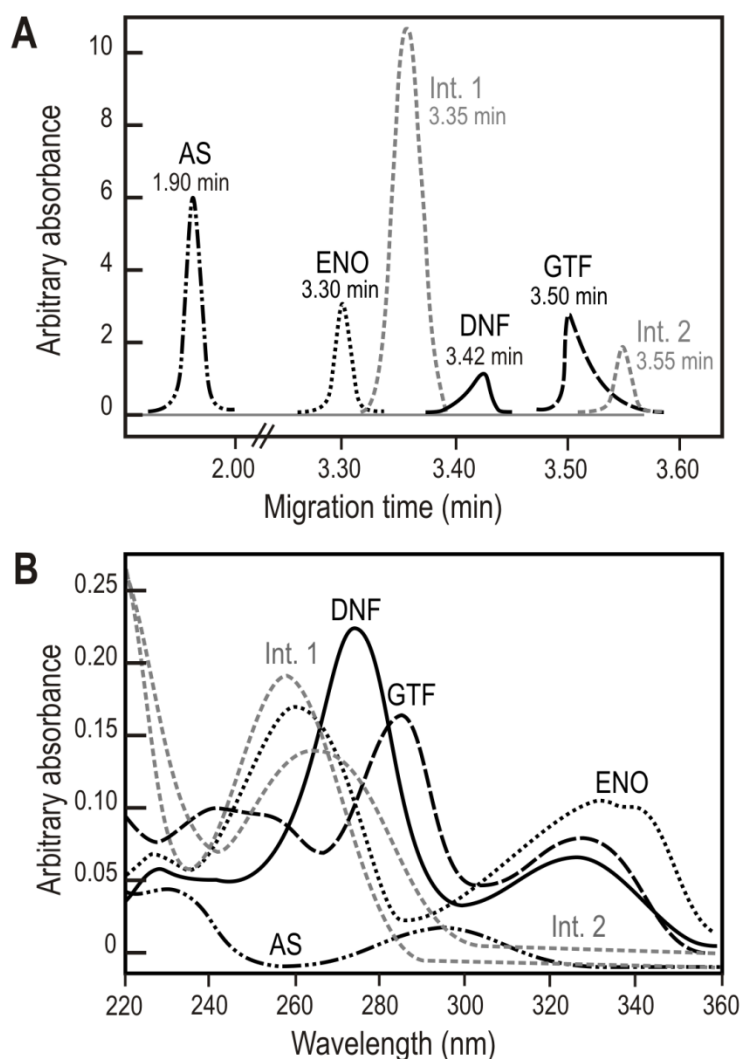
458   **Table 1**. LODs and LOQs values computed according to the criteria described in the section

459   2.6

	LOD(mg L^{-1})				LOQ(mg L^{-1})			
	S/N				S/N			
Analyte	IUPAC	Noise level near the peak of	Mean noise level given in	Calibration curve	IUPAC	Noise level near the peak of	Mean noise level given in	Calibration curve
FLU	6.71	1.62	4.55	2.38	8.13	5.39	9.62	6.28
DIF	6.85	1.55	3.35	2.45	8.32	5.16	6.64	6.47

Analyte								
ENR	4.23	1.04	1.81	2.46	10.84	3.46	4.53	6.48
MRF	8.62	2.69	2.25	2.38	14.78	8.97	6.38	6.28
OFL	4.50	1.36	1.76	3.40	11.79	4.52	5.86	9.0
CPF	4.92	1.20	1.55	2.20	12.36	4.00	5.17	5.81
ENO[a]	-	-	-	1.22	-	-	-	3.43
DNF[a]	-	-	-	1.44	-	-	-	4.06
GTF[a]	-	-	-	1.34	-	-	-	3.78

460   [a]Values obtained using multivariate calibration

461

462

463   **Table 2.** Linearity ranges, analytical figures of merit, and precision results.

Analyte	Linearity range	Intercept[a]	Slopes[a]	r^2 adj.	Lack of fit (p-	Repeatability Fortification levels (mg L^{-1})[d]				Intermediate precision Fortification levels (mg L^{-1})[d]			
						LO	7.5	22.5	30.0	LO	7.5	22.5	30.0
FLU	9.62–	0.26	0.089	0.99	0.170	9.4	3.7	3.15	2.2	8.6	3.1	2.5	1.6
DIF	6.64–	-0.02	0.097	0.99	0.100	11.	7.4	4.4	1.7	10.	4.3	3.4	1.2
ENF	4.53–	-0.13	0.168	0.99	0.209	11.	10.	5.3	2.8	9.6	5.8	4.9	0.9
MRF	6.38–	0.02	0.066	0.99	0.903	10.	7.7	5.4	0.6	9.4	6.2	2.1	0.5
OFN	5.86–	-0.15	0.125	0.99	0.279	11.	8	4	0.8	8.3	5.3	4.7	1.1
CPF	5.17–	-0.10	0.130	0.99	0.104	11.	8.6	3.1	2.6	8.9	4.1	2.8	2.3
ENO[c]	3.43–	0.12	0.076	0.99	0.423	10.	8.6	0.1	3.9	7.3	3.2	3	1.7
DNF[c]	4.06–	-0.09	0.197	0.99	0.204	11.	9.7	7.4	2.15	9.3	6.7	5.3	1.3
GTF[c]	3.78–	0.26	0.089	0.99	0.156	8.1	7.8	6.3	2.4	7.5	4.6	2.6	1.9

464   [a]Values between parenthesis correspond to the SD

465   [b]Since the p-value for the lack of adjustment is greater than or equal to 0.10, the model

466   seems to be adequate for the observed data.

467   [c]Values obtained by multivariate curve resolution

468   [d]Values correspond to the RSD_R obtained by Horwitz equation. Acceptance criteria: RSD_R

469   for 7.50 mg L^{-1} less than 11.8, for 22.50 mg L^{-1} less than 0.8, and for 30.00 mg L^{-1} less than

470   9.6.

471

472

473   **Table 3**. A) Apparent recovery evaluated using samples solutions at four different

474   concentrations, prepared by spiking basal blood with a volume of standard

475   solution,**B)**Determination FLU in real blood samples spiked with drug standards solutions.

3.A	Apparent recovery (%)[a]			
	Fortification levels (mg L^{-1})			
Analyte	LOQ	7.50	22.50	30.00
FLU	101 (7)	98 (9)	98 (9)	97 (2)
DIF	106 (2)	101 (8)	103 (6)	103 (5)
ENF	113 (4)	96 (5)	101 (9)	104 (8)
MRF	110 (4)	101 (8)	105 (9)	94 (6)
OFN	110 (5)	89 (7)	96 (8)	102 (7)
CPF	100 (9)	94 (8)	96 (4)	91 (4)
ENO[b]	105 (5)	99 (4)	95 (3)	103 (3)
DNF[b]	109 (4)	101 (2)	99 (5)	99 (3)
GTF[b]	104 (2)	102 (2)	102 (2)	101 (3)

3.B	Sample recovery (%)[a]			
		Fortification levels (mg L^{-1})		
Sample	Basal concentration (mg L^{-1})	0.50	1.00	2.00
S1	1.64 (0.09)	95 (4)	99 (3)	101 (1)

S3	3.06 (0.08)	98 (5)	102 (4)	99 (2)

476   [a]Values between parenthesis correspond to the SD

477   [b]Values obtained by multivariate curve resolution