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**Simultaneous analysis of four sulfonamides in chicken muscle tissue by High Performance Liquid Chromatography**

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Food Types:	Animal products – meat
Abstract:	<p><b>Abstract</b></p> <p>The aim of this study was to develop a simple high-performance liquid chromatography (HPLC) with UV detection method, for the determination of four sulfonamides in chicken muscle tissue. The sulfonamides were extracted with acetonitrile, acetone and dichloromethane. Separation was carried out on an C18 analytical column, using as mobile phase a mixture of 6‰ di-sodium hydrogen phosphate and methanol. The analytes were detected by UV, in one run. Calibration curves were linear with very good correlation coefficients for concentration ranging from 30µg kg-1 to 150µg kg-1. The limits of detection (LOD) for sulfonamides ranged from 6.5 to 0.14 µg kg-1. The recovery for spiked chicken muscle with 50–150 µg kg-1 ranged more than 70%. The relative standard deviations (RSDs) of the sulfonamides for six measurements at 50 µg kg-1, 100 µg kg-1 and 150 µg kg-1 were less than 15%. These</p>

parameters met the EU criteria for method validation. The results were confirmed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) using multiple reacting monitoring, as operating mode. Confirmation require the retention times of the analytes to be within  $\pm 2.5\%$  of the retention times of the standards, presence of the parent ion and two characteristic fragment ions (product ions) per analyte, as well as the relative ion abundance ratios of the fragment ions shall correspond to ratios obtained for the standards, within permitted limits . The transition of two common product ions at m/z 155.7 and 107.5 were monitored for all sulfonamides. Each of the analytes, in all tested samples met the confirmation criteria. Thus, it was demonstrated the applicability of the HPLC-UV method for routine analysis of chicken muscle tissue.

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# 1 Simultaneous analysis of four sulfonamides in chicken muscle tissue by

## 2 HPLC

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### 10 Abstract

11 The aim of this study was to develop a simple high-performance liquid chromatography (HPLC) with  
12 UV detection method, for the determination of four sulfonamides in chicken muscle tissue. The  
13 sulfonamides were extracted with acetonitrile, acetone and dichloromethane. Separation was carried  
14 out on an C18-column, using as mobile phase a mixture of 6% di-sodium hydrogen phosphate and  
15 methanol. The analytes were detected by UV, in one run. Calibration curves were linear with very  
16 good correlation coefficients for concentration ranging from 30  $\mu\text{g kg}^{-1}$  to 150  $\mu\text{g kg}^{-1}$ . The limits of  
17 detection (LOD) for sulfonamides ranged from 6.5 to 0.14  $\mu\text{g kg}^{-1}$ . The recovery for spiked chicken  
18 muscle with 50–150  $\mu\text{g kg}^{-1}$  was more than 70%. The relative standard deviations (RSDs) of the  
19 sulfonamides for six measurements at 50  $\mu\text{g kg}^{-1}$ , 100  $\mu\text{g kg}^{-1}$  and 150  $\mu\text{g kg}^{-1}$  were less than 15%.  
20 These parameters met the EU criteria for method validation. The results were confirmed by LC–  
21 MS/MS using multiple reacting monitoring, as operating mode. Confirmation require the retention  
22 times of the analytes to be within  $\pm 2.5\%$  of the retention times of the standards, presence of the  
23 parent ion and two characteristic fragment ions (product ions) per analyte, as well as the relative ion  
24 abundance ratios of the fragment ions corresponding to ratios obtained for the standards, within  
25 permitted limits. The transition of two common product ions at  $m/z$  155.7 and 107.5 were monitored  
26 for all sulfonamides. Each of the analytes, in all tested samples met the confirmation criteria. Thus,  
27 it was demonstrated the applicability of the HPLC-UV method for routine analysis of chicken muscle  
28 tissue.

29  
30 **Keywords:** sulfonamides; HPLC; chicken muscle; withdrawal periods; maximum residue limits.

### 32 Introduction

33  
34 Sulfadimethoxine, sulfamethoxazole, sulfaquinoxaline and sulfadiazine are the most common  
35 sulfonamides (SA) used in poultry farms. They can be easily absorbed and distributed  
36 through the body of the chicken, accumulated in various tissues and transferred into their

1 37 products (Kan and Petz, 2000; Kishda and Furusawa 2002). The recommended withdrawal  
2 38 periods, if not observed before slaughtering of the medicated animals, may impact on the  
3 39 safety of chicken meat and by-products.  
4 40

5 41 In order to ensure the reduction to an acceptable level of sulfonamide residues in edible  
6 42 tissues, these substances must be administered only in recommended concentrations and their  
7 43 withdrawal times must be observed. The maximum residue level (MRL) of sulfonamides in  
8 44 poultry tissues and eggs is  $100 \mu\text{g kg}^{-1}$  (Council Regulation 2377/90-EEC, 1990; Codex  
9 45 Alimentarius Commission, *CAC/MRL 02-2006*, Maximum residue limits for veterinary drugs  
10 46 in foods; and Code of Federal Regulation, USA, 1996).  
11 47

12 48 Despite the efforts of national and international bodies involved in food residue control, still  
13 49 there are people affected by the presence of drug residues in food. Unfortunately, about 10-  
14 50 15% of the human population is considered to be hypersensitive to antimicrobials especially  
15 51 penicillin and sulfonamides (Slatore, 2004; Cochrane *et al.*, 1995) and suffer from allergic  
16 52 reactions like skin rashes, hives, asthma and anaphylactic shock. It has been estimated that  
17 53 approximately five percent of human patients medicated with sulfonamides received  
18 54 unwanted effects from the drugs (Montanaro, 1998; Korpimäki, 2004).  
19 55

20 56 The interest in having reliable methods able to detect low amounts of sulfonamides in food is  
21 57 very real. Chicken meat is the second most popular meat in Romania, with a weight of about  
22 58 33% of total meat consumption (average consumption in 2009 was 20.5kg/person/year),  
23 59 contributing to increased population exposure to residues of sulfonamides, if this level is not  
24 60 well controlled (Peligrad, 2010).  
25 61

26 62 Sulphonamide residue analysis involves extraction with an appropriate solvent followed by  
27 63 one or more clean-up processes and then quantitative determination. Traditionally, the  
28 64 extraction of sulphonamides from meat, has been performed with organic solvents.  
29 65 Sulphonamides are not very soluble in non-polar solvents, but have good solubility in more  
30 66 polar solvents. Extraction is generally carried out with chloroform, methylene chloride  
31 67 (Thomas, 1998), acetone, acetonitrile, or ethyl acetate. Sample clean-up procedures include  
32 68 column chromatography, solid phase extraction (SPE) (Bele *et al.*, 2007; Hela *et al.*, 2003;  
33 69 Kao, *et al.*, 2001), matrix solid phase dispersion (MSPD) (Kishda and Furusawa, 2001),  
34 70 supercritical fluid extraction (SPE) (Maxwell, Lightfield, 1998).  
35

71 A variety of methods have been used to measure sulfonamide residue in biological materials,  
72 including thin-layer chromatography (TLC) (Babić, et al. 2005) , high-performance liquid  
73 chromatography (HPLC) with UV, UV-DAD (Gratacós-Cubarsí, *et al.*, 2006; Furusawa and  
74 Kishida, 2001) or fluorescence detection (Gehring, *et al.*, 2006), liquid chromatography -  
75 mass spectrometry (LC/MS) (Jung, *et al.*, 2004), micellar liquid chromatography (Szyman,  
76 2008), high-performance capillary electrophoresis (HPCE), gas chromatography (GC), along  
77 with enzyme-linked immunosorbent assay (ELISA) (Zhang, *et al.*, 2007), biosensor  
78 immunoassay (BIA) (Ploum, *et al.* 2005) and microbiological methods (Braham, *et al.*,  
79 2001).

80  
81 Instrumental methods such as LC/MS and GC are both sensitive and specific, but are  
82 laborious and expensive. These methods are suitable for confirmation but not for screening of  
83 large numbers of samples. Microbiological methods do not require highly specialized and  
84 expensive equipment, but they have not enough sensitivity and assay precision. Currently,  
85 TLC has been almost replaced by other instrumental analysis. Although HPCE has powerful  
86 separation ability, the precision is poor and the instrument still needs to be improved.  
87 Immunochemical methods such as ELISA can be simple, rapid and cost-effective, with  
88 enough sensitivity and specificity to detect small molecules (Wang *et al.*, 2005). The official  
89 method AOAC uses pre-column derivatization and liquid chromatography with fluorescence  
90 detection (Salisbury, 2004).

91  
92 A rapid, sensitive and specific assay is required to detect sulphonamides positive samples in  
93 routine analysis, by liquid chromatography with ultraviolet detection (LC–UV). The goal of  
94 this paper is to promote a HPLC-UV method, which does not need to be performed on the  
95 latest generation equipment, but is able to be used for the simultaneous detection of  
96 sulfonamides residues in meat. This method could be welcomed both by the laboratories of  
97 the veterinary medicine manufacturers, where withdrawal times have to be established, and  
98 Official Control Laboratories that regularly evaluate the implementation of good veterinary  
99 practices, detect and regulate deviations in veterinary drugs usage. The authors are expecting  
100 that this method to be of real help especially for the laboratories from the EU new member  
101 states, in which expensive analytical techniques are not widely available. This multi-residue  
102 analysis was performed to simultaneously determine four sulfonamides in chicken muscle  
103 tissue: sulfadimethoxine sulfamethoxazole, sulfaquinoxaline and sulfadiazine, the most  
104 common used sulfonamides in poultry farms.

105

106 **Materials and methods**

107 *Apparatus:* Cutter/mixer, electronic balance (Precision Balance, KERN Abj - Germany),  
108 centrifuge (Centra MP 4R- USA), sonicator (Sororex RK 100H - Germany), vortex  
109 (Ultraturax IKA T25 - Germany), filter unit 0.45  $\mu\text{m}$  and 0.2 $\mu\text{m}$  (Whatman – Germany),  
110 piston – operated pipette 100 - 1000 $\mu\text{l}$  (Transferpette – Brand - USA).

111 The HPLC system consisted of a UV/VIS detector (Waters LC Module I - USA), a HPLC  
112 auto sampler (Waters model 717 Plus - USA), and two pumps (Waters model 510 and 590 -  
113 USA). The HPLC column used was Zorbax SB- C18, 5  $\mu\text{m}$  4.6  $\times$  250 mm, 5 $\mu$  (Agilent  
114 Technologies - USA).

115 *Chemicals and reagents:*

116 Marker residue for sulfonamides is represented by parent drugs.

117 Standards used are: sulfadimethoxine (Sigma Chemical Co – USA), sulfamethoxazole  
118 (Sigma Chemical Co – USA), sulfaquinoxaline (EDQM), and sulfadiazine (EDQM).

119 Reagents: Acetonitrile (Lab-Scan - Poland), methanol (Merck, Germany), n-hexane (Fluka-  
120 Switzerland), di-natriumhydrogenphosphat (Merck, Germany), dichloromethane (Chimactiv  
121 – Romania), acetone (Chimactiv - Romania) and N – dymethylformamide (Fisher Scientific-  
122 UK). All reagents were HPLC grade.

123 *Preparation of standard solutions:*

124 Stock standard solutions were prepared by dissolving 100 mg of each SA standard with 100  
125 mL of N-dimethylformamide separately (1mg ml<sup>-1</sup>). Mix standard solution was prepared by  
126 combining 1 mL of each stock standard solution and was made up to 50mL with 50%  
127 methanol in di-natrium hydrogen phosphate solution 6g/1000ml. Working mix standard  
128 solution at a concentration of 0.1 – 2 $\mu\text{g ml}^{-1}$  was prepared by diluting the mix standard  
129 solution with mobile phase.

130 *Tissue samples:*

131 Blank matrix comes from previously unmedicated chicken, which were provided by the  
132 Biobase S.N. Pasteur Institute, Bucharest, Romania – Gnotobiology Laboratory and  
133 Experimental Testing for chickens and turkeys.

134 Incurred samples come from medicated chicken, under normal farm condition, which were  
135 provided by the Biobase S.N. Pasteur Institute, Bucharest, Romania – Gnotobiology  
136 Laboratory and Experimental Testing for chickens and turkeys.

137 Six chicken of approximately 1.5kg, were treated with sulfadiazine in their water ration at  
138 rate of  $50\text{mg kg}^{-1} \text{d}^{-1}$ , for five days. After 12 and 24 h withdrawal from medication, three  
139 chickens were removed for slaughter.

140 Another group of six chicken of approximately 1.5kg, was treated with sulfadimethoxine in  
141 their water ration at rate of  $50\text{mg kg}^{-1} \text{d}^{-1}$ , for five days. After 24 and 48h withdrawal from  
142 medication, three chickens were removed for slaughter.

143 Another group six chicken of approximately 1.5kg, were treated with sulfamethoxazole in  
144 their water ration at rate of  $8\text{mg kg}^{-1} \text{d}^{-1}$ , for four days. After 48 and 72h withdrawal from  
145 medication, three chickens were removed for slaughter.

146 Another group six chicken of approximately 1.5kg, were treated with sulfaquinoxaline in  
147 their water ration at rate of  $50\text{mg kg}^{-1} \text{d}^{-1}$ , for five days. After 48 and 72 h withdrawal from  
148 medication, three chickens were removed for slaughter.

149 Muscle tissue samples were collected and stored frozen ( $-17^{\circ}\text{C}$ ) until they were analyzed.

#### 150 *Sample preparation:*

151 The sulfonamide extraction was carried out using a method based on the one described by  
152 Furusawa and Hanabusa (2002), Stoev and Michailova (2000) in which acetonitrile, acetone  
153 and dichloromethane were utilized as organic solvents.

154 A volume of 30 ml acetonitrile was added to 10 g of minced and homogenized muscle tissue  
155 weighed in a glass centrifuge tube. The sample was homogenized for 1 min in a Vortex and  
156 centrifuged at 3500 rpm for 10 min. The supernatant was transferred into a pear-shaped flask.

157 Twenty milliliter of acetone was added to the sediment before the mixture was sonicated for  
158 10 min. The solution was centrifuged once again and the supernatant was added into the  
159 same pear-shaped flask. The mixed solution was evaporated at  $40^{\circ}\text{C}$  until near to dryness.

160 Afterwards, 5 mL of dichloromethane was added, homogenized by vortexing and transferred  
161 into a test tube. The step after the addition of dichloromethane was repeated three times and  
162 the combined dichloromethane was dried at  $40^{\circ}\text{C}$ . The residue was reconstituted with 1mL of  
163 50% methanol in di-sodium hydrogen phosphate solution (6g /1000ml) and mixed properly  
164 by vortexing. n-Hexane (2 mL) were added into the test tube for defeating, and vortexed  
165 before being removed. The remaining solution was filtered through the filter of  $0.2 \mu\text{m}$  or no  
166 more than  $0.45 \mu\text{m}$ , and was ready for injection into HPLC system.

#### 167 *HPLC Analysis:*

168 The HPLC analyses were performed by using the following mobile phase:

169 75:25 = di-sodium hydrogen phosphate solution 6g/1000 ml: methanol (v/v), according USP  
170 29 – Sulfadimethoxine Monographs, without pH adjustment.



171 The flow rate: 1 mL/min.

172 Injection volume: 20 $\mu$ l

173 Detection was performed at 245 nm, in order to achieve a greater sensitivity than in the  
174 method described by Ismail and co-workers (2008), which used 266 nm.

175 Run time: 10min

176 *Calculation formula:*

177  $\mu\text{g kg}^{-1}$  sulfonamide =  $C \times 100 \times 100 \times R^{-1}$

178 C = measured concentration ( $\mu\text{g kg}^{-1}$ )

179 R = recovery (%)

180 *LC-MS/MS*

181 Identification and quantification of analytes were carried out on Waters 2695 (USA) liquid  
182 chromatography, equipped with a MS-MS Quatro Micro (Micromass, USA) tandem mass  
183 spectrophotometer. The X Bridge Shield RP18 column, 150mm x 2.1, 3.5 $\mu$ m, (Waters, USA)  
184 was used for separation. The flow rate was 20 $\mu$ l/min, and the temperature of the column was  
185 40 °C. Mobile phase was methanol (B) and a 0.1% formic acid solution (A) following  
186 gradient

187	Time	A	B
188	0	95	5
189	1.5	95	5
190	10	60	40
191	15	10	90
192	15.5	95	5
193	20	95	5

194 MS/MS conditions: the mass spectrophotometer was operated in electrospray positive ion  
195 mode. The capillary voltage was held at 4.5KV, cone voltage, at 30V and extractor voltage,  
196 at 2V. The source temperature was 100° C, desolvation gas (nitrogen) temperature, 350 °C  
197 and flow: 350l/h. Collision gas (argon) pressure:  $3 \times 10^{-3}$ mbar. Data acquisition was made in  
198 multiple reaction monitoring mode. The transition of two common product ions were  
199 monitored.

200 *Ions used for LC/MS-MS confirmation:*

Sulfonamide types	Parent ion m/z	Product ions m/z	Collision energy(eV)	Retention time (min)	Relative ion intensities %
Sulfadiazine	251,2	155.7* 107.5	16	7.64	18.6
Sulfamethoxazole	245.2	155.7* 107.5	18	9.94	19.5
Sulfadimethoxine	311.2	155.7* 107.5	18	13.05	7.9
Sulfaquinoxaline	301.2	155.7* 107.5	16	14.5	7.1

201 \* Quantifying ions

202

## 203 **Results and discussion**

204 Under the condition of HPLC-UV analyses, sulfonamides eluted in the following order with  
 205 approximate retention times in minutes: sulfadiazine 2,7; sulfamethoxazole 3,2;  
 206 sulfadimethoxine 4.0; sulfaquinoxaline 6,8. The validation data of HPLC-UV method are  
 207 presented in the Table 1 and are discussed below:

208

### 209 **Validation of analytical procedure**

210 The proposed analytical method was validated according to the following criteria: specificity,  
 211 accuracy, precision, limit of detection, limit of quantification, and linearity, according to  
 212 EMEA - "Notice to applicant and Guideline – Veterinary Medicinal Products (vol. 8) ",and  
 213 Commission Decision (EEC) No. 657/2002.

214

### 215 *Sample preparation*

216 As for many antibiotics, selective extraction of sulfonamides from biological tissues is  
 217 complicated due to the polar character of the analytes and matrix components (Stolker, et.,al,  
 218 2005). Sulfonamides have a good solubility in more polar solvents. Acetonitrile was chosen  
 219 to extract these compounds because of its polar character and its good proprieties to  
 220 denaturing the sample proteins, which results in a cleaner extraction and a better release of  
 221 any sulfonamides residues bound to proteins. Acetone is also a polar solvent that leads to  
 222 depletion of sample. Dichloromethane is less polar than acetonitrile and acetone, separating  
 223 the compound of interest from the initial extract. n-Hexane was used to obtain defatted  
 224 extract. During the extraction it was important to respect the proportion between solvents.

225 Specificity and recovery results were in acceptable limits, which show the extraction  
226 efficiency and a good removal of matrix interference. The detection limit was also  
227 comparable with the other reported method.

228

229 *Calibration curves* of each component were established by plotting the peak area of each  
230 active component against its associated concentrations. The concentration used for each drug  
231 ranged from 30  $\mu\text{g kg}^{-1}$  to 150  $\mu\text{g kg}^{-1}$  (0.5MRL – 1.5MRL), at five levels, according to the  
232 recommendation made by the Commission Decision No. 657/2002.

233 A statistic linear regression was performed for each component. For the tested domain,  
234 calibration curves were linear with very good correlation coefficients ( $R^2 > 0.99$ ).

235

236 *Specificity* was study by analyzing blank samples and spiked samples at relevant  
237 concentration (0.5, 1, 1.5 x MRL) and checking any interferences in the region of interest.  
238 The peak of each analite width at half-maximum height was within the 90-110% range of the  
239 standard width, and the retention times were identical within a margin of 5 %. No interfering  
240 peaks from endogenous compounds were found in the retention time of the target  
241 sulfonamides.

242

243 *Repeatability:* 3 test samples were spiked at analyte levels, which encompass 0.5x and 2x the  
244 MRL. Then, 6 test portions of each level were taken, analyzed and the residue concentration  
245 of each test portion was determined. At each spiked concentration, the CV ( $S_{\text{rel}} = S \times 100 /$   
246 Mean) was calculated. The relative standard deviations (RSDs) of the sulfonamides for six  
247 measurements at 50  $\mu\text{g kg}^{-1}$ , 100  $\mu\text{g kg}^{-1}$  and 150  $\mu\text{g kg}^{-1}$  were from 7.8 to 13.5%. These  
248 values are within the criteria stipulated for residue analysis by the Commission Decision No.  
249 657/2002: RSD <20%.

250

251 *The accuracy (recovery)* was determined by recovery experiments using blank matrices.

252 - 18 aliquots of a blank material was spiked (six aliquots) at each of 0.5, 1 and 1.5 x MRL

253 - the samples were analyzed and the sulfonamides concentration present in each sample was  
254 calculated;

255 - using the equation below, the recovery for each sample was calculated.

256  $\% \text{ Recovery (R)} = 100 \times \text{measured content} / \text{spiked level}$

257 - it was calculated the mean recovery and CV from the six results at each level.

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258 A recovery between 70 – 110 % in the spike range of 0.5MRL- 1.5MRL, is acceptable by  
259 EU regulations. The recovery for spiked chicken muscle with 50–150  $\mu\text{g kg}^{-1}$  ranged from  
260 70 to 84 %.

261

262 *Response linearity:*

263 - 6 test sample were spiked at level of: 0.5, 0.75, 1, 1.25, 1.5, 1.75  $\mu\text{g ml}^{-1}$

264 - the samples were analyzed and the sulfonamides concentration present in each sample was  
265 calculated;

266 - linearity curves of each component were established by plotting the measured concentration  
267 of each active component against its fortification level of concentrations.

268 Correlation coefficients for the analytes show an acceptable linearity of the analytical  
269 response, across the range of tested concentrations.

270

271 *The limits of detection and quantification*

272 In the Commission Decision 2002/657/EC, the decision limit ( $CC\alpha$ ) and detection capability  
273 ( $CC\beta$ ) are intended to replace the LOD and LOQ, as method characteristics. On the other  
274 hand, in EMEA- *Notice to applicants and Guideline - Veterinary medicinal products, Volume*  
275 *8: Establishment residue limits (MRLs) for residues of veterinary medicinal products in*  
276 *foodstuffs of animal origin*, LOD and LOQ are still required, and furthermore, the limit of  
277 quantification must be validated at least at 0.5 MRL. This Guidance recommended as one  
278 possible way to estimate the detection limit, the following: arithmetic mean of the analytes  
279 concentration, in at list 20 blank samples, plus three time standard deviation. In *Guidance for*  
280 *the validation of analytical method in depletion studies - VICH 49 (2009)*, LOD is estimated  
281 in the same mode like in *EMEA Guidance*, and LOQ is estimated as three time LOD.  
282 Another possibility to calculate LOD and LOQ is using the linearity curves.

283

284 In respect with all these regulation, the detection limit of HPLC-UV method was calculated  
285 as arithmetic mean of the analytes concentration in 20 blank samples plus three time standard  
286 deviation and LOQ was estimated as three times LOD.

287

288 An LOD for sulfonamides in LC-UV detection method is acceptable in general on values  
289  $\leq 10\mu\text{g kg}^{-1}$  (Stolker, et.,al, 2005), and the values obtained in this validation were lower.

290 The limits of quantification (LOQ) for the three sulfonamides were in the range of 0.4-1.7  $\mu\text{g}$   
291  $\text{kg}^{-1}$ . Sulfaquinoxaline LOQ is 19.6  $\mu\text{g kg}^{-1}$ , still below 0.5 MRLs, and for all analytes LOQ  
292 was significant lower than  $\text{CC}\beta$ , reducing the false negative result rate.

293

#### 294 *Decision limit ( $\text{CC}\alpha$ ) and detection capability ( $\text{CC}\beta$ )*

295 In this validation  $\text{CC}\alpha$  and  $\text{CC}\beta$  was established considering the permitted limit, in that case  
296 MRL, in respect with Commission decision 2002/657/EC, by analyzing 20 blank samples  
297 fortified with the analytes at the permitted limit, and calculating the decision limit ( $\text{CC}\alpha$ ) as  
298 arithmetic mean of analyte concentration at MRL level plus 1.64 times the standard deviation  
299 of repeatability ( $\alpha = 5\%$ ), and detection capability ( $\text{CC}\beta$ ), as arithmetic mean of analyte  
300 concentration at  $\text{CC}\alpha$  level plus 1.64 times the standard deviation of repeatability ( $\beta = 5\%$ )  
301 Decision limits ( $\text{CC}\alpha$ ) and detection capabilities ( $\text{CC}\beta$ ) were in the range of 104.64 – 112.95  
302  $\mu\text{g kg}^{-1}$  and 108.5 - 125  $\mu\text{g kg}^{-1}$ , respectively.

303

#### 304 *LC-MS/MS confirmation*

305 A method for measuring residue level by liquid chromatography–tandem mass spectrometry  
306 (LC–MS/MS) was applied, using the same extraction procedure, as confirmatory method.

307 The confirmatory analysis is based on MS–MS monitoring of two product ion and a  
308 precursor ion, which provide four identification points (IPs). For sulfonamides, listed in  
309 Group B (Council Directive 96/23/EC), a minimum three IPs are required. Relative retention  
310 time of analytes was within the tolerance of  $\pm 2.5\%$ . Relative ion intensities for fortified  
311 samples correspond to those of the calibration standards, in the permitted tolerance.

312

313 The residue was reconstituted with 1mL of 50% methanol in 0.1% formic acid solution. For  
314 determination, analytes were separated by a different gradient LC procedure, ionized by  
315 electrospray ionization (ESI), and detected by MS–MS with a triple quadrupole mass  
316 spectrometer.

317

318 MS parameters were optimized by direct flow infusion of each standard. The calibration  
319 curves show a good linearity in the concentration range of 0.5 – 10  $\mu\text{g/kg}$ , for each analyte,  
320 with correlation coefficients between 0.9988 – 0.9996. The chromatograms of a spiked  
321 sample contain 10  $\mu\text{g/kg}$  of each compound is shown in Figure 1.

322

323 The LC-MS/MS method was used for verify the identity of marker residue and to ensure the

1 324 absence of false positives, in fortified tissues, incurred samples and real sample.

2 325

3 326 *Analysis of real samples*

4 327 The result obtains from the HPLC analyses of incurred samples are given in Table nr. 2.

5 328 The HPLC assay result showed all these animals tissues to contain sulfonamides at level  
6 329 above MRL. Thirty samples of chicken muscles collected from a local market in Romania,  
7 330 were investigated for sulfonamides residues used HPLC method. Twelve muscle samples  
8 331 was found to contain sulfadiazine with concentration level ranging from 300 µg/kg to 180  
9 332 µg/kg, the level of which exceeded the regulated tolerance.

10 333

### 11 334 **Conclusion**

12 335 A multi-residue method has been developed for the simultaneous determination of  
13 336 sulfadiazine, sulfamethoxazole, sulfadimethoxine and sulfaquinoxaline in chicken muscle  
14 337 tissue by HPLC-UV, after extraction with organic solvents (acetonitrile, acetone,  
15 338 dichloromethane). The method is simple, rapid, sensitive, and capable of detecting  
16 339 sulfonamides residues below the maximum residue limits (MRL).

17 340

18 341 Criteria of validation: specificity, accuracy, precision, decision limit, detection capability,  
19 342 and linearity, according to the European Commission Decision 2002/657/EC, show that the  
20 343 method can detect different kinds of sulfonamides within one run, without fluorimetric  
21 344 derivatization of the analytes.

22 345

23 346 Compared to other methods, this one is easy to use for on routine samples, in laboratories  
24 347 that are equipped with HPLC-UV. The method takes just 10 min to be performed, without  
25 348 extraction time, which is 60 min. The HPLC results were confirmed by LC-MS/MS,  
26 349 demonstrating the usefulness of HPLC technique as rapid and specific method.

27 350

28 351 The proposed HPLC method is very suitable for determination of withdrawal periods in  
29 352 chicken muscle tissue for any medicinal product which contains any of the sulfonamides  
30 353 studied in this group, or for monitoring a large number of samples in order to observe that the  
31 354 recommended withdrawal period is followed. It can be really useful for checking whether  
32 355 Good Veterinary Practices are in place in poultry farms. The results of this investigation  
33 356 could be a reference for authorities to further monitor the residue of veterinary drugs in  
34 357 chicken products and reinforce the administration of veterinary drug users.

358

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**Table 1: Validation parameter of HPLC-UV method for target sulfonamides residue**

Targhet residues	RT min	LOD $\mu\text{g kg}^{-1}$	LOQ $\mu\text{g kg}^{-1}$	Mean RSD%	Mean Recovery %	CC $\alpha$ $\mu\text{g kg}^{-1}$	CC $\beta$ $\mu\text{g kg}^{-1}$	Linearity R <sup>2</sup>
Sulfadiazine	2.7	0.14	0.42	11	84	104	108	0.9958
Sulfadimethoxine	3.2	0.36	1.08	13	77	109	121	0.9834
Sulfamethoxazole	4.0	0.58	1.68	9	70	111	119	0.9913
Sulfaquinoxaline	6.8	6.53	19.6	8	71	113	125	0.9800

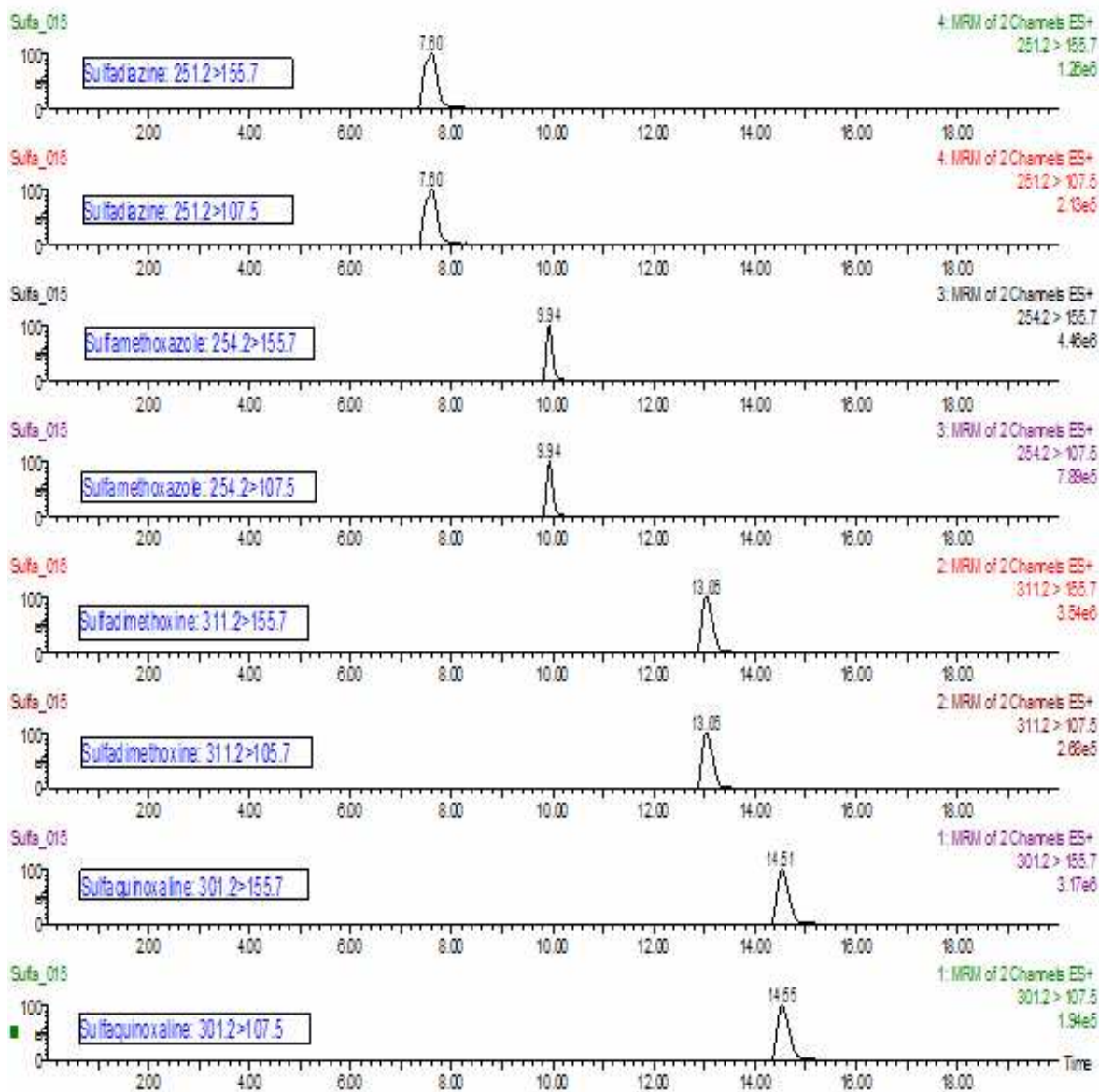
Table 2: Result for incurred samples

Sulfonamide types	Withdrawal time (h)	HPLC assay ( $\mu\text{g kg}^{-1}$ )
		305
Sulfadiazine	12	257
		312
		124
	24	97
		184
		547
Sulfaquinoxaline	48	438
		470
		210
	72	185
		162
		328
Sulfadimethoxine	24	456
		412
		115
	48	205
		156
		465
Sulfamethoxazole	48	305
		375
		157
	72	186
		255

**Figure caption**

Figure 1. The chromatograms of spiked samples contain 0.1 µg/ml (10 µg/kg) of each compound. The extracted ions are from top to bottom: sulfadiazine m/z 251.2 →155.7, 251.2 →107.5, Rt =7.64; sulfamethoxazole m/z 254.2 → 155.7, 254.2 → 107.5 , Rt =9.94; sulfadimethoxine m/z 311.2 → 155.7; 311.2 → 107.5 Rt =13.05 and sulfaquinoxaline m/z 301.2 → 155.7, 301.2 → 107.5, Rt = 14.50.

Figure 1



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