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Title: Multidetection of antibiotics in liver tissue by Ultra-High-Pressure-Liquid-Chromatography tandem Mass Spectrometry

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Highlights:
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5 • The proposed method allows to determine 39 antibiotics in liver simultaneously;
6 • Validation process shows the good performance of the method;
7 • The present method is a huge improvement for laboratories involved in food
8 safety control.
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10 **Multidetection of antibiotics in liver tissue by Ultra-High-**
11 **Pressure-Liquid-Chromatography tandem Mass Spectrometry**

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36
37 **Abstract**
38
39 A multiresidue quantitative screening method covering 39 antibiotics from 7 different
40 families by Ultra-High-Pressure-Liquid-Chromatography tandem Mass Spectrometry
41 (UHPLC-MS/MS) is described. Sulfonamides, trimethoprim, tetracyclines, macrolides,
42 quinolones, penicillins and chloramphenicol are simultaneously detected in liver tissue.
43 A simple sample treatment method consisting of extraction with a mixture of
44 acetonitrile and ethylenediaminetetraacetic acid (EDTA) followed by solid-phase
45 extraction (SPE) with a hydrophilic-lipophilic balanced (HLB) cartridge was developed.
46 The methodology was validated, in accordance with Decision 2002/657/EC, by
47 evaluating the following required parameters: decision limit (CCα), detection capability
48 (CCβ), specificity, repeatability and reproducibility. The precision, in terms of the
49 relative standard deviation, was under 22% for all of the compounds, and the recoveries
50 were between 80% and 110%. The CCα and CCβ were determined according to the
51 maximum residue limit (MRL) or the minimum required performance limit (MRPL),
52 when established.
53
54
55 **Keywords:** Antibiotics; multiclass; multidetection; UHPLC-MS/MS; liver; validation.
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59 **1. Introduction**

60

61 Antibiotics are widely used for therapeutic and prophylactic purposes in food-producing
62 animals and to promote animal growth [1]. The use of antibiotics as growth promoters is
63 considered fraudulent in Europe because it can lead to residues of these compounds
64 persisting in edible matrices. These antibiotic residues can result in allergic reactions in
65 some hypersensitive individuals and in the appearance of bacterial strains that are
66 resistant to drugs that are used in both veterinary and human medicine [2], which is
67 currently considered a huge worldwide concern.
68 For that reason, the European Community determined the need for the mandatory
69 control of the veterinary drugs in food from animal origin designated for human
70 consumption [3]. For permitted veterinary drugs, the maximum residue limits (MRL) in
71 foodstuff of animal origin were established and are listed in the EU Commission
72 Regulation 37/2010 [4, 5]. Food products containing concentrations of antibiotics
73 exceeding the established MRL are inappropriate for human consumption. In the case of
74 some non-authorized substances, a minimum required performance limit (MRPL) has
75 been set to harmonize the analytical performance of the methods used in different
76 laboratories [6, 7].
77 A wide variety of edible matrices must be monitored for the presence of veterinary
78 residues, including muscle, liver, kidney, fat, milk, eggs, fish and honey. Nevertheless,
79 there are relatively few multidetection and multiclass methods for the determination of
80 antibiotics in liver tissue. There are still very few methods describing approaches for
81 analyzing different classes of compounds, particularly for their determination in liver
82 tissue [8, 9]. To our knowledge, the only available method for the determination of an
83 extensive number of antibiotics from several classes in such a matrix was published by

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84 Kaufmann et al [8], who detected 100 veterinary drugs in muscle, liver and kidney
85 tissues using UPLC-ToF-MS. The main constraint with using ToF-MS methodologies is
86 related to the fact that it is impossible to use them for confirmation purposes because
87 ToF-MS is not yet included in the regulations [6].
88 There are also some methods that group a few families of compounds, such as the one
89 presented by Shao et al. [9], who developed a multiclass confirmatory method for
90 tetracyclines and quinolones in muscle, liver and kidney tissues using UHPLC-MS/MS.
91 However, the common procedures described in the literature for the determination of
92 antibiotics in liver tissue only include groups of related compounds [10-14]
93 When working with liver tissue and developing the sample preparation methodology,
94 one of the principal obstacles is related to the complexity associated with the high
95 protein and fat contents in this matrix, which often interfere in the analytical
96 performance. Additionally, the high enzymatic activity in liver tissue can be responsible
97 for the fast degradation of labile compounds, which leads to significant losses during
98 sample preparation. Another issue to be considered is that the simultaneous
99 determination of antibiotics from different pharmacologic families in complex
100 biological matrices is constrained by differences in the physicochemical properties of
101 the compounds [15, 16], a fact that makes developing the sample extraction method a
102 challenge that can only be overcome by reaching a compromise that better fits the
103 purpose of the multiclass method.
104 The lack of methodologies for screening of antibiotics in liver demanded for new
105 developments in order to fulfill the requirements of the control program and,
106 consequently, improve food safety. Considering all of these aspects and the need for a
107 reliable and efficient method for the determination of antibiotics in liver tissue while
108 improving the time of analysis for several groups of compounds and the cost-

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109 effectiveness, the aim of this work was to develop a multiclass and multidetection
110 method using UHPLC-MS/MS for the detection of antibiotics from seven families
111 (sulfonamides, trimethoprim, tetracyclines, macrolides, quinolones, penicillins and
112 chloramphenicol). To use the method in routine analysis and official control, it was
113 validated according the requirements described in the European Commission Decision
114 2002/657/EC [6].
115
116 **2. Material and Methods**
117
118 *2.1. Reagents, Solvents and Standard Solutions*
119 All of the reagents and solvents used were of analytical grade, with the exception of the
120 chemicals used for the mobile phase, which were of high-performance liquid
121 chromatography grade. Methanol, acetonitrile, n-hexane and formic acid were supplied
122 by Merck (Darmstadt, Germany). Ethylenediaminetetraacetic acid (EDTA) was
123 purchased from Sigma-Aldrich (Madrid, Spain). All of the standards of the
124 sulfonamides, tetracyclines, penicillins, macrolides, quinolones, trimethoprim and
125 chloramphenicol were supplied by Sigma-Aldrich (Madrid, Spain). The individual
126 standards are listed in Table 1. The following six internal standards were used:
127 demethyltetracycline for the tetracyclines; penicillin V for the penicillins; lomefloxacin
128 for the quinolones; roxithromycin for the macrolides; sulfameter for the sulfonamides
129 and trimethoprim; and chloramphenicol-fifth-deuterated (d5) for chloramphenicol. All
130 of the internal standards were provided by Sigma-Aldrich. For all of the substances,
131 stock solutions of 1 mg mL^{-1} were prepared by weighing the appropriate amount of
132 standard, diluting it in methanol, and storing it at -20°C for one year. Suitable dilutions

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133 were also prepared to have convenient spiking solutions for both the validation process
134 and the routine analyses. Working solutions were stored at -20ºC for one month.
135

136 *2.2. Instrumentation*

137 For the sample preparation, the following equipment was used: Mettler Toledo PC200
138 and AE100 balances (Greifensee, Switzerland), a Heidolph Reax 2 overhead mixer
139 (Schwabach, Germany), a Heraeus Megafuge 1.0 centrifuge (Hanau, Germany), a
140 Turbovap Zymark Evaporator (Hopkinton, MA, USA) and Whatman Mini-Uniprep
141 PVDF 0.45 µm filters (Clifton, NJ, USA). A vacuum manifold was used for the solid
142 phase extraction (SPE) with an Oasis HLB polymeric sorbent cartridge (3 mL, 200 mg)
143 (Waters, Milford, MA, USA). Chromatographic separation and mass spectrometry
144 detection were performed using a Xevo TQ MS–Acquity UPLC system coupled to a
145 triple quadrupole tandem mass spectrometer from Waters (Milford, MA, USA). The
146 electrospray ion source (ESI) was used both in positive and negative modes with data
147 acquisition in the multiple reaction monitoring mode (MRM), and the Masslynx 4.1
148 software (Waters) was used for data processing. The MRM optimized conditions are
149 presented in Table 1. The UHPLC system consisted of a vacuum degasser, an
150 autosampler and a binary pump equipped with an analytical reverse-phase column
151 (Acquity HSS T3 2.1 x 100 mm with 1.8 µm particle size, Waters). A flow rate of 0.45
152 mL min^{-1} was used with the following mobile phases: [A] formic acid 0.1% (v/v) in
153 water and [B] acetonitrile. The following gradient program was used: 0-5 min from
154 97% to 40% [A]; 5-9 min from 40% to 0% [A]; 9-10 min from 0% back to 97% [A];
155 11-12 min 97% [A]. Column and autosampler were maintained at 40ºC and 10ºC,
156 respectively. A 20 µL aliquot (full loop) was injected onto the analytical column.
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158 *2.3. Sample preparation*

159 Two grams of minced and mixed liver tissue was weighed into a 20 mL glass centrifuge
160 tube. The internal standard solution was added, and the sample was vortexed for 30 s
161 and allowed to stand in the dark for at least 10 min. The sample was extracted by
162 shaking using a Reax shaker for 10 min with 10 mL of acetonitrile and 1 mL of 0.1 M
163 EDTA. After that, the sample was left in the ultrasound bath for 20 min. Following
164 centrifugation for 10 min at 4000×*g*, the supernatant was transferred into a new tube and
165 evaporated to near dryness (1 mL). Water (5 mL) was added, and the solution was
166 vortexed for 15 s. The solutions were then submitted to a clean-up step using SPE Oasis
167 HLB cartridges, which were preconditioned with acetonitrile (10 mL) and water (10
168 mL). After passing the aqueous extract through the columns using gravity, the
169 cartridges were washed with water (5 mL) and then dried under reduced pressure for
170 approximately 5 min. The elution was performed with acetonitrile (10 mL). The eluate
171 was evaporated to near dryness (0.5 mL) under a gentle stream of nitrogen and 400 µL
172 of mobile phase [A] was added. To this extract n-hexane (2 mL) was added and the
173 solution vortexed for 30 s. After centrifugation for 10 min at 4000×*g*, the n-hexane layer
174 was removed. The final extract was filtered through a 0.45 µm PVDF Mini-uniprep ™,
175 transferred to vials and analyzed by UHPLC-MS/MS under the MRM optimized
176 conditions described in Table 1.

177

178 *2.4. Validation procedure*

179 In-house validation was performed following the method described by the EU
180 Commission Decision 2002/657/EEC [6] that requires the evaluation of the method in
181 terms of the specificity, recovery, repeatability, reproducibility, decision limit (CCα)
182 and detection capability (CCβ).

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183 By analyzing 20 blank liver samples from different animal species (bovine, swine,
184 ovine, and poultry) to find possible peaks that could interfere with the detection of the
185 target analytes, the specificity of the method was assessed. Afterwards, the same 20
186 samples were spiked with all of the compounds of interest at the validation level (VL)
187 (Table 2) to prove the identification capability of the method and once again its
188 specificity. Calibration curves using spiked samples were assembled using the following
189 five concentration levels, 0.5xVL, 1.0xVL, 1.5xVL, 2.0xVL and 3.0xVL, and the
190 analyses were carried out on three different days with different operators. Six replicates
191 of the 0.5xVL, 1.0xVL and 1.5xVL concentration levels were performed each day to
192 determine the precision of the method (in terms of the repeatability and reproducibility)
193 and the recovery. The recovery was estimated as a ratio between the obtained
194 concentration and the real concentration.
195 The critical concentrations, CCα and CCβ, were calculated according to the following
196 equations [6]:
197 $CC_{\alpha} = \mu_N + 2.33 \times \sigma_N$ (Equation 1, for compounds without MRLs)
198 $CC_{\alpha} = MRL + 1.64 \times \sigma_{MRL}$ (Equation 2, for compounds with established MRLs)
199 $CC_{\beta} = CC_{\alpha} + 1.64 \times \sigma_{VL}$ (Equation 3)
200 where μ_N is the mean of the noise amplitude of twenty blank samples; σ_N is the standard
201 deviation of the noise amplitude of twenty blank samples at the retention time of the
202 target analyte; and σ_{MRL} or σ_{VL} is the standard deviation at the MRL or VL level in the
203 twenty spiked blank samples at that level. For all of the determinations, the peak areas
204 of both the analytes and the corresponding internal standards were measured, and the
205 analyte/internal standard area ratios were determined. Internal standards were chosen
206 for their similar physicochemical behaviors to those of the antibiotics being monitored
207 [17].

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209 **3. Results and discussion**

210 The major challenge in the determination of veterinary drugs in biological samples,
211 usually in residual concentrations, lies in sample preparation. Our knowledge and
212 experience from previously developed multiclass methods in milk, fish and bovine
213 muscle [17-19] was the starting point for the present method. In these previously work,
214 the appropriated solvents, for the extraction of the target compound, were already
215 studied and, starting from that knowledge, a new method, to be used in liver, was
216 developed. Thus, the best option for use as the extraction solvent, in terms of the
217 recovery, is an organic extractant, specifically acetonitrile. Aqueous solvents failed to
218 extract the less polar compounds. The same conclusion is expressed in other available
219 publications, though those extractions were performed on different matrices [8, 20],
220 where acetonitrile is preferred over methanol and ethyl acetate, because these last two
221 solvents can be responsible for extracting matrix components that can interfere in the
222 detection. Additionally, it is important to add that acetonitrile, aside from being an
223 efficient extraction solvent, promotes the precipitation of proteins, thereby turning this
224 step into one that is important for obtaining a clean extract. Some of the target
225 antibiotics, such as tetracyclines, quinolones and macrolides, can easily form chelate
226 complexes with bi- and trivalent metal cations present in the sample extraction solution.
227 These can lead to lower recoveries; to prevent their formation, a chelate agent with a
228 similar behavior should be used to control the problem and increase the recoveries. For
229 that reason, EDTA is often used during the liquid extraction, and it has been determined
230 to improve the extraction efficiencies of tetracyclines, quinolones and macrolides.
231 Compared with muscle tissue, liver tissue is a much more complex matrix because of its
232 high protein content, enzymatic activity and fat content. Therefore, to prevent possible

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233 chromatographic interferences and ion suppression or enhancement, further clean-up
234 steps during the sample preparation were optimized.
235 The use of solid-phase extraction prior to mass spectrometric detection can be a huge
236 advantage to decrease the effects of ion suppression caused by components of liver
237 tissue. To control the possible losses of target antibiotics, the best option is to use a
238 multiclass selectivity cartridge that can fit the diverse physicochemical properties of all
239 of the target antibiotics. The best option, in terms of selectivity, is to use a sorbent
240 composed of a hydrophilic-lipophilic balance modified polymer (OASIS HLB), which
241 is known to have a very broad selectivity for polar compounds [8, 21]. The solid-phase
242 extraction is followed by concentration through evaporation under a gentle stream of
243 nitrogen, without evaporation to total dryness, to avoid a long evaporation process. The
244 instability of antibiotics along with the higher affinity of some polar compounds for
245 aqueous phase possibly remaining present in the cartridge and being eluted together
246 with the acetonitrile are the main reasons for this procedure [22]. After reconstitution
247 with the mobile phase, a thin lipidic layer was observed. To remove that layer and
248 prevent such interference in the mass spectrometric detection, a deffating step was
249 performed via the addition of n-hexane. After discarding the n-hexane layer, the final
250 extract was injected and analyzed using UHPLC-MS/MS.
251 The UHPLC-MS/MS parameters, in terms of chromatography and detection, were
252 previously optimized. The mobile phase, flow rate and gradient steps were selected to
253 achieve the best chromatographic separation and peak shape, along with a short run
254 time. The conditions described above allowed the determination of the 39 compounds in
255 less than 10 min. To fulfill the identification criteria described in Decision 2002/657 [6],
256 two ion transitions must be controlled for each compound. The ideal MRM conditions
257 (Table 1) were achieved through the direct infusion into the detector of each individual

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258 standard solution at a concentration of 10 μg mL^{-1}. For positive ionization, which is the
259 case for all of the compounds except chloramphenicol, the use of formic acid in the
260 mobile phase works as a promoter of positive ionization and consequently improves the
261 detection. In Figure 1, individual MRM chromatograms of one compound per family of
262 monitored antibiotics obtained from a spiked bovine liver sample at the corresponding
263 validation level (VL) are presented.
264 The method was validated in accordance with the European Commission Decision
265 2002/657/EC [6], and the following parameters were evaluated: specificity, recovery,
266 precision (as repeatability and reproducibility) decision limit (CCα) and detection
267 capability (CCβ).
268 The specificity of the method was assessed by analyzing 20 blank samples of liver
269 tissue of different species (bovine, swine, ovine, and poultry) to verify the absence of
270 interference above a signal-to-noise ratio of 3 at the retention time of the target
271 compounds that could compromise their detection and identification. Additionally, in
272 the spiked blank samples, all of the identification criteria [6] were fulfilled without any
273 false negative results, again proving the specificity of the method for the species
274 analyzed. Considering the proved specificity and that no major differences were found
275 between the 20 blank and spiked samples, only one animal species (bovine) was used
276 for the next validation steps. This choice was based on the fact that bovine liver tissue is
277 a matrix that is very often consumed.
278 The results obtained for the precision (repeatability and reproducibility) as the relative
279 standard deviation (RSD), recovery, CCα and CCβ are summarized in Table 2. The
280 precision and recovery were calculated at the VL that corresponds to the MRL for those
281 compounds that had it. For repeatability and reproducibility, the highest RSDs of 16%
282 and 24%, respectively, were obtained for sulfisoxazole. All of the other compounds had

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283 RSDs under these values. The recovery was calculated as a ratio between the
284 determined concentration in a spiked sample and the real concentration. The range of
285 values obtained were between 81 and 110%, thus falling into the acceptable range [6].
286 Both the precision and recovery are mandatory parameters in validation because they
287 measure the variability during the analytical process and can be used to analyze and
288 prove the robustness of the method.
289 The two critical concentrations, CCα and CCβ, were determined from the calibration
290 curves obtained from the bovine blank liver samples spiked at five concentration levels
291 (0.5, 1, 1.5, 2 and 3xVL) and the application of the equations described above
292 (equations 1, 2 and 3), keeping in mind that not all of the compounds had an established
293 MRL. Antibiotics without a tolerance level (MRL) had lower CCα and CCβ values that
294 were closer to the limit of detection of the method, although in the other cases, these
295 concentrations were always above the MRL.
296
297
298 **4. Conclusions**
299 An analytical method is proposed for the simultaneous determination of 39 antibiotics
300 from 7 different classes in liver tissue. The developed method is able to determine the
301 presence of compounds from the sulfonamides, tetracyclines, macrolides, quinolones,
302 chloramphenicol, penicillins and trimethoprim in a single run using UHPLC-MS/MS,
303 providing a possible way to significantly reduce the time required to analyze one
304 sample. The developed method was fully validated and fulfilled all of the criteria
305 specified by the European Union Decision 2002/657/EC [6], proving that it is suitable
306 for routine analysis and quantitative screening purposes for official control, with the
307 possibility of extending the method for antibiotic confirmation. Although the main part

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308 of the validation procedure was performed only for bovine samples, its specificity
309 proved that the method can be used for swine, ovine and poultry liver tissue.
310 Because there are a limited number of publications reporting methods for the
311 simultaneous analysis of antibiotics in liver tissue, the present method is a huge
312 improvement for laboratories that are involved in food safety control and have a large
313 number of samples and antibiotics to analyze.
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421 **Table 1 –** Multiple reaction monitoring (MRM) acquisition conditions for each antibiotic and for the
422 internal standards (IS) used.
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		ESI	Precursor ion (*m/z*)	Product ions (*m/z*)	Cone voltage (V)	Collision energy (eV)
Sulfonamides	*sulfapyridine*	+	250.3	156.3/92.3	30	15
	sulfadiazine	+	251.2	156.2/92.2	30	15
	sulfamethoxazole	+	254.4	156.4/92.2	30	20
	sulfathiazole	+	256.4	156.3/92.3	25	15
	sulfisoxazole	+	268.3	156.2/113.2	25	15
	sulfamethiazole	+	271.0	156.2/108.1	25	15
	sulfisomidine	+	279.4	186.3/124.4	30	16
	sulfamethazine	+	279.4	156.3/124.5	30	15
	sulfamethoxypyridazine	+	281.2	156.2/92.2	30	15
	sulfachloropyridazine	+	285.3	92.3/156.3	30	28
	sulfadoxine	+	311.4	156.4/92.3	30	18
	sulfadimethoxine	+	311.4	156.4/92.3	30	20
	sulfanilamide	+	173.2	92.1/156.2	30	25
	sulfaquinoxaline	+	301.3	92.2/156.3	30	30
	sulfameter (IS)	+	281.3	92.2	25	30
	trimethoprim	+	291.5	230.3/261.3	25	23
Tetracyclines	*tetracycline*	+	445.5	410.3/427.3	25	20
	doxycycline	+	445.5	428.2/410.3	25	18
	oxytetracycline	+	461.5	426.3/443.3	25	20
	chlorotetracycline	+	479.3	444.2/462.1	25	20
	demethyltetracycline (IS)	+	465.2	448.3	25	17
Macrolides	*erythromycin*	+	734.5	158.2/576.5	25	30
	spyriamicin	+	843.5	174.0/540.3	35	35
	tilmicosin	+	869.3	174.2/156.1	35	45
	tylosin	+	917.1	174.3/772.5	35	35
	roxithromycin (IS)	+	837.7	679.5	30	30
Quinolones	*nalidixic acid*	+	233.2	215.1/187.1	40	14
	flumequine	+	262.2	202.1/244.2	30	32
	oxolinic acid	+	262.2	216.1/244.2	30	25
	cinoxacin	+	263.2	217.1/245.2	30	23
	norfloxacin	+	320.3	276.2233.2	20	17
	enoxacin	+	321.2	303.2/234.2	35	18
	ciprofloxacin	+	332.2	288.2/245.2	35	17
	danofloxacin	+	358.3	96.1/314.3	33	21
	enrofloxacin	+	360.3	316.3/245.2	31	19
	ofloxacin	+	362.1	261.3/318.2	34	26
	marbofloxacin	+	363.3	72.1/320.2	30	20
	lomefloxacin (IS)	+	352.2	265.3	31	22
Penicillins	*amoxicillin*	+	366.3	160.3/114.4	25	20
	oxacillin	+	402.0	243.0/160.0	30	20
	nafcillin	+	415.0	199.0/171.0	30	25
	dicloxacillin	+	470.0	311.0/160.0	30	25
	penicillin V (IS)	+	351.0	160.2	25	25
Amphenicol	*chloramphenicol*	-	320.9	151.9/193.9	30	25
	chloramphenicol-d5 (IS)	-	326.0	157.0	30	25

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425 **Table 2 –** Maximum Residue Levels (MRLs) set by European Union for liver tissue,
426 validation level (VL) and validation parameters: decision limit (ccα), detection
427 capability (ccβ), repeatability, reproducibility and recovery.

	MRL ($\mu g\ kg^{-1}$)	**VL ($\mu g\ kg^{-1}$)**	**CCα ($\mu g\ kg^{-1}$)**	**CCβ ($\mu g\ kg^{-1}$)**	**Repeatability (%RSD)**	**Reproducibility (%RSD)**	**Recovery (%)**
sulfapyridine	100	100	124	149	15	22	101
sulfadiazine	100	100	125	150	15	22	105
sulfamethoxazole	100	100	121	142	15	23	85
sulfathiazole	100	100	115	129	8	12	109
sulfisoxazole	100	100	123	146	16	24	88
sulfamethiazole	100	100	111	122	6	9	108
sulfisomidine	100	100	123	146	13	19	108
sulfamethazine	100	100	115	129	8	12	110
sulfamethoxypyridazine	100	100	114	129	8	12	110
sulfachloropyridazine	100	100	118	135	10	15	107
sulfadoxine	100	100	111	123	7	11	97
sulfadimethoxine	100	100	123	147	13	19	110
sulfanilamide	100	100	125	150	15	22	105
sulfaquinoxaline	100	100	118	137	11	17	98
trimethoprim	50	50	65	81	11	16	88
tetracycline	300	300	322	343	12	18	109
doxycycline	300	300	325	351	14	22	108
oxytetracycline	300	300	313	326	7	11	110
chlorotetracycline	300	300	321	343	15	22	88
erythromycin	200	200	219	237	10	16	109
spyriamicin	300	300	317	333	10	15	102
tilmicosin	1000	1000	1024	1048	13	20	110
tylosin	100	100	111	122	7	10	101
nalidixic acid	-	100	5.81	16.0	15	23	110
flumequine	500	500	528	555	15	23	110
oxolinic acid	150	150	166	182	9	13	109
cinoxacin	-	100	3.10	7.60	15	22	100
norfloxacin	-	100	0.32	0.94	13	19	108
enoxacin	-	100	1.72	3.87	15	22	88
ciprofloxacin	300	300	316	331	11	17	87
danofloxacin	400	400	418	437	12	18	94
enrofloxacin	300	300	325	349	15	22	103
ofloxacin	-	100	0.22	0.65	8	13	81
marbofloxacin	150	150	174	198	14	21	107
amoxicillin	50	50	74	97	15	22	98
oxacillin	300	300	320	339	14	22	83
nafcillin	300	300	321	341	12	17	109
dicloxacillin	300	300	325	349	14	21	109
chloramphenicol	-	0.3	0.28	0.48	11	17	109

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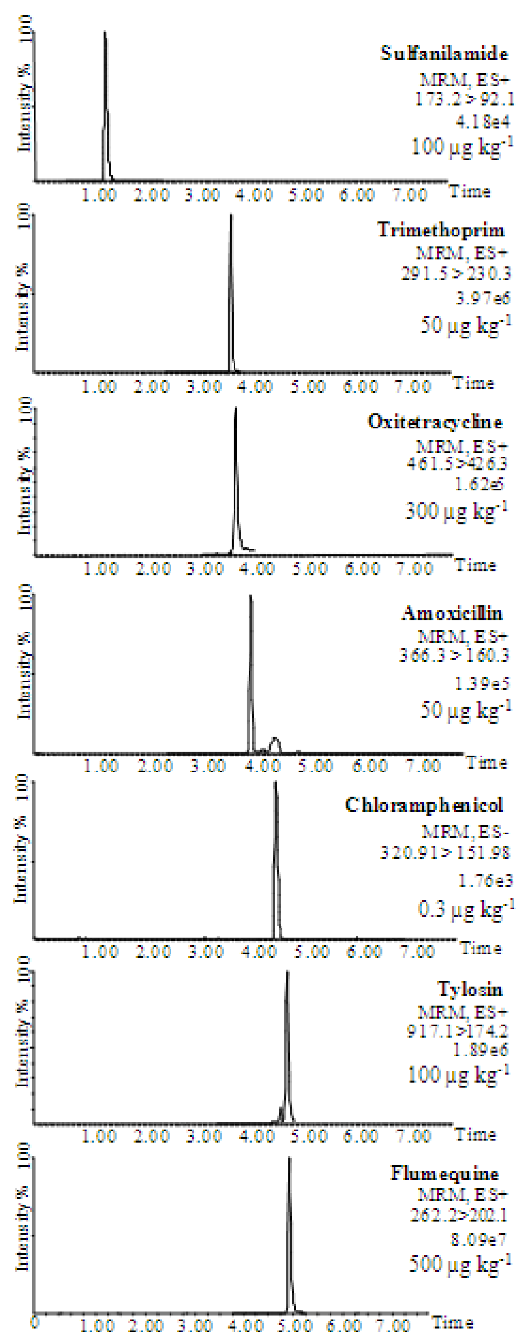
430 **Figure 1:** Individual MRM of one antibiotic per family is given as example from a
431 spiked liver sample at the corresponding validation level (100 µgkg^{-1} for sulfanilamide
432 and tylosin; 50 µgkg^{-1} for trimethoprim and amoxicillin; 300 µgkg^{-1} for oxitetracycline;
433 0.3 µgkg^{-1} for chloramphenicol and 500 µgkg^{-1} for flumequine).



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