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Development and validation of a multi-residue and multiclass ultra-high-pressure liquid chromatography-tandem mass spectrometry screening of antibiotics in milk

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23

24 **Abstract**

25

26 A multi-residue screening method for 33 antibiotics from five different families
27 was employed to simultaneously determine sulphonamide, tetracycline, macrolide,
28 quinolone and chloramphenicol antibiotics using ultra high pressure liquid
29 chromatography tandem mass spectrometry. A simple sample preparation method was
30 developed using protein precipitation, centrifugation and solid phase extraction and was
31 optimised to achieve the best recovery for all compounds. The methodology was
32 validated for quantitative screening methods, by evaluating the detection capability
33 ($CC\beta$), specificity, selectivity, precision, applicability and ruggedness. Precision, in
34 terms of relative standard deviation, was under 21% for all compounds. Because $CC\beta$
35 was determined for screening purposes and, according to maximum residue limit, the
36 limit of detection of the method was calculated and ranged from 0.010 $\mu g\ kg^{-1}$ to 3.7 μg
37 kg^{-1}. This validation provided evidence that the method is suitable to be applied in
38 routine analysis for the detection of antibiotics in bovine, caprine and ovine milk.

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41 **1. Introduction**

42

43 Antibiotics in dairy cattle are mainly used to treat mastitis, diarrhoea and
44 pulmonary diseases (McEwen & Fedorka-Cray, 2002). These treatments can result in
45 the presence of antibiotic residues in milk. For consumers, the presence of such residues
46 can be responsible for toxic effects, allergic reactions in individuals with
47 hypersensitivity, and can result in the development of resistant strains of bacteria
48 (Barlow, 2011; Knecht et al., 2004; Toldrá & Reig, 2006; Wassenaar, 2005). The
49 presence of antibiotic residues can also be responsible for undesirable effects in the
50 dairy industry, especially concerning processed food by fermentation wherein the
51 quality of the final products can be seriously compromised (Toldrá & Reig, 2006). All
52 these concerns make the analysis of antibiotic residues in milk an important field of
53 food safety to study.

54 To protect consumers, regulatory agencies in the European Union published
55 several official documents regulating the control of veterinary drugs in food products
56 from animal origin. Council Directive 96/23/EC (European Commission, 1996)
57 establishes the veterinary residue control in food producing animals. Tolerance levels,
58 as described by European Commission Regulation 470/2009/EC (European
59 Commission, 2009), were set for compounds that can be used for therapeutic purposes.
60 Regulation 37/2010 (European Commission, 2010) lists pharmacologically active
61 substances and their maximum residue level (MRL) in foodstuffs of animal origin, as
62 well as compounds for which no MRL has been set because no hazard for public health
63 has been observed. For some non-authorised substances a minimum required
64 performance limit (MRPL) was set to harmonise the analytical performance of the

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65 methods (SANCO, 2007; European Commission, 2002), meaning that MRPL is not a
66 concentration obtained from toxicological data, but is only related to the general
67 analytical performance. For antibiotics without an MRL or an MRPL, a validation level
68 (VL) was defined based on the drug characteristics of the respective class of compounds
69 (Table 1).

70 The requirements for performance and validation of analytical methods
71 employed in the official residues control for screening and confirmatory purposes are
72 described in European Decision 2002/657/EC (European Commission, 2002).
73 Microbiological and bioassay techniques are still used for antibiotic qualitative
74 screening purposes (Franek & Diblikove, 2006; Knecht et al., 2004; Lamar & Petz,
75 2007; Pastor-Navarro, Maquieira, & Puchades, 2009; Toldrá & Reig, 2006; Zhang &
76 Wang, 2009) mainly because of their low cost and simplicity; however, they lack
77 sensitivity and specificity. To ensure unequivocal identification, there is a growing need
78 for efficient screening methods that guarantee a significantly reduced number of false
79 positives and false negatives. This efficiency can be gathered in multi-detection
80 methods based on liquid chromatography (LC) coupled with tandem mass spectrometry
81 (MS/MS) (Bohm, Stachel, & Gowik, 2009; Gaugain-Juhel et al., 2009; Le Bizec, Pinel,
82 & Antignac, 2009; Stolker, Zuidema, & Nielen, 2007; Turnipseed, Andersen,
83 Karbiwnyk, Madson, & Miller, 2008). The use of ultra-high performance liquid
84 chromatography (UPLC) provides the possibility of having short run times together
85 with higher resolution and sensitivity, important attributes when running several
86 compounds at once (Aguilera-Luiz, Vidal, Romero-González, & Frenich, 2008; Junza,
87 Amatya, Barrón & Barbosa, 2011; Ortelli, Cognard, Jan & Edder, 2009; Stolker et al.,
88 2008).

89 Several methods can be found in literature for the determination of residues of
90 different antibiotic families in milk. However, for the simultaneous analysis of
91 compounds of different antibiotic classes in a multi-class residue analysis, only a
92 restricted number of methods are reported in the literature, mainly due to difficulties
93 related to differences in physico-chemical properties between families of compounds
94 (Aguilera-Luiz, et al., 2008; Balizs & Hewitt, 2003; Bohm et al., 2009; Gaugain-Juhel
95 et al., 2009; Junza et al., 2011; Kaufmann, 2009; Ortelli et al., 2009; Stolker et al.,
96 2008). The present work describes the development and validation of a simple and
97 effective quantitative screening method by UPLC-MS/MS for the simultaneous
98 detection of 33 antibiotic compounds from sulphonamides, tetracyclines, macrolides,
99 quinolones and chloramphenicol in bovine, caprine and ovine milk samples for
100 application in routine analyses.

101

102 2. Materials and methods

103

104 *2.1. Reagents, solvents and standard solutions*

105

106 All reagents and solvents used were of analytical grade with the exception of
107 chemicals used for the mobile phase, which were of HPLC grade. Methanol, acetonitrile
108 and formic acid were supplied by Merck (Darmstadt, Germany). All standards of
109 tetracyclines, quinolones, macrolides, sulphonamides and chloramphenicol were
110 supplied by Sigma-Aldrich (Madrid, Spain). The individual standards are listed in Table
111 1. One internal standard for each antibiotic family was used: demethyltetracycline for
112 tetracyclines, lomefloxacin for quinolones, roxithromycin for macrolides, sulfameter for
113 sulphonamides, and for chloramphenicol, the fifth-deuterated (d5) form; all the internal

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114 standards were provided by Sigma-Aldrich. For all substances, stock solutions of 1 mg
115 mL^{-1} were prepared by weighing the appropriate amount of standard, diluting in
116 methanol, and storing at less than 5 °C. Suitable dilutions were also prepared to have
117 convenient spiking solutions for both the validation process and routine analysis.
118
119 *2.2. Instrumentation*
120
121 The following equipment was used for sample preparation: Mettler Toledo
122 PC200 and AE100 balances (Greifensee, Switzerland), Heidolph Reax 2 overhead
123 mixer (Schwabach, Germany), Heraeus Megafuge 1.0 centrifuge (Hanau, Germany),
124 Turbovap Zymark Evaporator (Hopkinton, MA, USA) and Whatman Mini-Uniprep
125 PVDF (polyvinylidene fluoride) 0.45 µm filters (Clifton, NJ, USA). A Xevo TQ MS –
126 Acquity UPLC system coupled to a triple quadrupole tandem mass spectrometer from
127 Waters (Milford, MA, USA) was used for chromatographic separation and mass
128 spectrometry. The electrospray ion source in positive (ESI+) and negative (ESI-) mode
129 was used with data acquisition in multiple reaction monitoring (MRM) mode and
130 analysed using Masslynx 4.1 software (Waters). The MRM optimised conditions are
131 presented in Table 1.
132 The UPLC system consisted of a vacuum degasser, an autosampler and a binary
133 pump equipped with an analytical reverse-phase column Acquity HSS T3 2.1×100 mm
134 with 1.8 µm particle size (Waters). The mobile phases used were: A, formic acid 0.1%
135 (v/v) in water and B, formic acid 0.1% (v/v) in acetonitrile. The gradient program used,
136 at a flow rate of 0.45 mL min^{-1}, was: 0-5 min from 97% A to 40% A; 5-9 min from 40%
137 to 0% A; 9-10 min from 0% back to 97% A; 11-12 min 97% A. The column was
138 maintained at 40 °C, the autosampler at 10 °C and the injection volume was 20 µL.

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139

140 *2.3. Sample preparation*

141

142 Homogenised raw milk samples (2 g) were weighed into 20 mL glass centrifuge
143 tubes, the internal standard solution was added, then vortexed and allowed to stand in
144 the dark for at least 10 min. Proteins were precipitated and antibiotics extracted through
145 shaking for 20 min with 10 mL of acetonitrile. Following centrifugation for 15 minutes
146 at 3100 × *g*, the supernatant was transferred into a new tube and evaporated to dryness
147 under a gentle stream of nitrogen. The residue was re-dissolved with mobile phase A
148 (400 μL), filtered through a 0.45 μm PVDF membrane, transferred to vials and injected
149 into the UPLC-MS/MS under MRM optimised conditions for each compound.

150

151 *2.4. Validation procedure*

152

153 The method was validated as a quantitative screening method by assessing the
154 following parameters for each compound: CCβ (detection capability), specificity,
155 selectivity, precision, applicability and ruggedness. In addition, the limit of detection
156 (LOD) was also estimated in accordance with the observed signal-to-noise ratio in the
157 spiked samples. The selectivity and specificity were evaluated by analysing 20 blank
158 milk samples from each different species (bovine, ovine and caprine) and the same
159 samples were spiked with all the compounds at the MRL/MRPL/VL level. Along with
160 the species variation, the applicability and ruggedness were shown by carrying out the
161 analysis on different days and by different technicians, which also allowed the
162 evaluation of precision in terms of relative standard deviation (RSD). For the
163 compounds where an MRL was established, CCβ evaluation was carried out to obtain a

164 concentration that was less than or equal to the regulatory MRL, and for that reason, 20
165 blank samples from each animal species were spiked with half the value of the MRL.
166 For drugs without MRL or MRPL recommended concentration levels, a VL was defined
167 (Table 1) and the calculation of the CCβ was in accordance with the Regulation
168 2002/657/EC decision (European Commission, 2002) for unauthorised compounds. The
169 peak areas of both the analyte and the respective internal standard were measured, and
170 the analyte/internal standard ratios were used for all determinations.
171
172 **3. Results and discussion**
173
174 To fulfil the requirements of the legislated MRLs and the control of prohibited
175 substances, methods have to be specific and sensitive enough to detect low levels,
176 taking into account the complexity of obtaining good recovery of all compounds with
177 distinct physico-chemical properties. The main problem associated with milk extraction
178 for subsequent determination of antibiotics is the high protein content. In most methods
179 reported in the literature, the preparation of milk samples for residue analysis involves
180 protein precipitation followed by solid-phase extraction (SPE) through the use of
181 appropriate cartridges or dispersive SPE (Aguilera-Luiz et al., 2008; Bohm et al. 2009;
182 Junza et al. 2011; Stolker et al., 2008; Turnipseed et al., 2008). The precipitation of
183 proteins is achieved in many cases by adding a strong acid, such as trichloroacetic acid,
184 in combination with a miscible organic solvent. In the present method, acetonitrile was
185 added to milk to promote the precipitation of proteins, and was also used as the
186 extracting solvent. Protein precipitation was effective and a clean extract was obtained,
187 which was demonstrated by the results obtained: no signal suppression or enhancement
188 was observed and no interferences in the MS/MS detection that could compromise the

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189 determination. It can be assumed that the matrix components responsible for possible
190 interference were removed, such as proteins, fats, and carbohydrates. Although the use
191 of SPE prior to MS/MS measurement can have the advantage of decreasing the effects
192 of ion suppression caused by matrix interferences, it can also compromise the individual
193 recoveries due the fact that each of the antibiotic classes, as well as antibiotics within
194 each class, has different physico-chemical properties. All these aspects must be taken
195 into account when selecting the appropriate SPE cartridge, especially as it can be
196 difficult to find one with multi-class selectivity.

197 A procedure using a polymeric sorbent SPE cartridge, composed of an OASIS®
198 (Waters) hydrophilic-lipophilic balance modified polymer, after protein precipitation
199 and liquid-liquid extraction with acetonitrile was described by Bohm et al. (2009),
200 Junza et al. (2011) and Turnipseed et al. (2008). Although this solid phase has very
201 broad selectivity for polar compounds, after comparing the results with and without this
202 step, it was considered unnecessary since better recoveries could be achieved with only
203 liquid-liquid extraction. The principal advantage of the present method, when
204 comparing with methods reported by Bohm et al. (2009), Junza et al. (2011) and
205 Turnipseed et al. (2008), is that the present extraction became easier to handle and,
206 without the use of cartridges, the costs can be significantly reduced, which is a factor
207 that must be taken into account when there are a large number of samples to be
208 routinely analysed for screening purposes. The use of acetonitrile as both the agent of
209 protein precipitation and also as the extracting solvent yields a process even more
210 simple and cost effective. The celerity in obtaining results is one of the fundamental
211 characteristics of screening methods. The use of equipment with good performance and
212 high sensitivity, such a UPLC-MS/MS, enables sample preparation to be simplified
213 without compromising the detection capability of the method. The high sensitivity of

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214 the equipment enables detection of compounds that are positively ionised, and
215 chloramphenicol which is negatively ionised, in the same run. Chloramphenicol, being a
216 banned substance, has to be detected at very low concentrations below its corresponding
217 MRPL at 0.3 μg kg^{-1}, which was successfully achieved (LOD = 0.06 μg kg^{-1}; Table 2).
218 To achieve maximum sensitivity for all compounds, MS/MS conditions (such as
219 ion spray voltage, de-solvation temperature, and gas flow and collision conditions) were
220 optimised by direct infusion into the detector of standard solutions and the principal ion
221 transition was selected for each analyte. Table 1 presents the *m/z* ion transition
222 monitored for screening and the associated collision energy. The use of an acidic mobile
223 phase adjusted with 0.1% of formic acid promoted positive ionisation, which improved
224 the detection of most compounds since only chloramphenicol is negatively ionised.
225 In terms of chromatographic optimisation, several gradient profiles were
226 studied to improve peak separation and minimise the run time. Acetonitrile was shown
227 to be better that methanol because of maximised sensitivity and resolution, especially
228 when acidified with formic acid. The gradient described above allows the determination
229 of all compounds in 10 min. One of the advantages of working with UPLC columns
230 consisting of a smaller particle size is the possibility of having high efficiency in peak
231 separation, sharp peaks, and also a reduction in run time when compared with common
232 HPLC columns, in terms of particle size. Chromatograms obtained for a spiked sample
233 with all compounds at the validation levels (VL) are shown in Fig. 1. Each peak is
234 characteristic of the respective antibiotic, demonstrating the good performance of the
235 method in terms of detection, as well as for optimal chromatographic separation.
236 The main requisite for a reliable screening method is to detect unauthorised
237 substances below the regulatory limits (MRL/MRPL) or at a level as low as possible,
238 minimising false negative results. Therefore a method has to be fully validated in

239 accordance with the legislation (European Commission, 2002; European Commission,
240 2010). At the expected retention time for all the target compounds, no interfering peaks
241 were observed in any of the analysed samples from the three different species.
242 Additionally the identification of all compounds were effective in all samples from the
243 different species, according the criteria of Regulation 2002/657/EC decision (European
244 Commission, 2002), in all the 20 spiked samples at the VL. No false-negative results
245 were observed since all analytes were detected at the expected retention time. The
246 ruggedness of the method was assessed when carrying out analysis of both the blank
247 and the spiked samples of milk from different animal species, using different
248 technicians and with inter-day analysis. No significant variation was observed.

249 The results for precision, quantified as RSD% (Table 2), showed the precision of
250 the method. No results were obtained above 21%, which represents a significantly lower
251 value when compared with the criteria value accepted by the Horwitz equation
252 (European Commission, 2002).

253 Although it is stated in Decision 2002/657/EC (European Commission, 2002)
254 that CCβ is the smallest content of the substance that may be detected, identified and/or
255 quantified in a sample with an error probability of β=5%, it is considered to be the
256 concentration above which the sample should be re-analysed by a confirmatory method
257 for screening purposes. It is also stated that CCβ must be less than or equal to the
258 regulatory limit (MRL/MRPL) for screening methods. For this reason, and for
259 antibiotics with MRL legislated, ½ MRL was adopted as the CCβ value. For those
260 without MRL, the calculation was carried out by a matrix-matched calibration curve
261 according to Decision 2002/657/EC for unauthorised substances as described by
262 Kaufmann (2009). The LOD was also evaluated to establish the sensitivity of this
263 method and was defined as the lowest concentration of the analyte, calculated by

264 multiplying the mean value of the signal-to-noise ratio of 20 blank samples by three. All
265 the LOD values for the measured compounds were found to be significantly lower than
266 the MRL/MRPL/VL values. The validation values are presented in Table 2.
267

268 4. Conclusions

269

270 A rapid and reliable multi-residue and multi-class method for simultaneous
271 detection of 33 antibiotics, from five different families was developed and validated for
272 quantitative screening of milk samples. The validation results showed the applicability
273 for routine analysis of bovine, caprine and ovine milk in accordance with the
274 requirements established in Decision 2002/657/EC (European Commission, 2002). The
275 optimised extraction procedure is a simple and efficient method without the need for an
276 SPE step, thus reducing the handling time and associated costs, and allowing a larger
277 number of samples analysed in one day.
278

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Figure legends

Fig. 1. Liquid chromatography multiple reaction monitoring chromatograms of the antibiotics detected in a milk sample spiked at the corresponding validation level (precursor ion > product ion referred in Table 1; numbers in brackets correspond to the vertical axis scale of the respective chromatogram).

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Table 1
Maximum residue levels set by the European Union for milk, and validation level values and multiple reaction monitoring acquisition conditions for each antibiotic and the internal standards. [a]

Antibiotic		MRL (μg kg^{-1})	VL (μg kg^{-1})	ESI	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage (eV)[b]	Collision energy (eV)[b]
Tetracyclines	chlortetracycline	100	100	+	479.3	444.2	25	20
	oxytetracycline	100	100	+	461.5	426.3	25	20
	tetracycline	100	100	+	445.5	410.3	25	20
	doxycycline	-	50	+	445.5	428.2	25	18
	demethyltetracycline	Internal standard		+	465.2	448.3	25	17
Quinolones	ciprofloxacin	100	100	+	332.2	288.2	35	17
	enrofloxacin	100	100	+	360.3	316.3	31	19
	marbofloxacin	75	75	+	363.3	72.1	30	20
	oxolinic acid	-	25	+	262.2	216.1	30	25
	flumequine	50	50	+	262.2	202.1	30	32
	norfloxacin	-	25	+	320.3	276.2	20	17
	nalidixic acid	-	25	+	233.2	215.1	40	14
	danofloxacin	30	30	+	358.3	96.1	33	21
	ofloxacin	-	25	+	362.1	261.3	34	26
	enoxacin	-	25	+	321.2	303.2	35	18
	cinoxacin	-	25	+	263.2	217.1	30	23
	lomefloxacin	Internal standard		+	352.2	265.3	31	22
Macrolides	tylosin	50	50	+	917.1	174.3	35	35
	tilmicosin	50	50	+	869.3	174.2	35	45
	erythromycin	40	40	+	734.5	158.2	25	30
	spiramycin	200	200	+	843.5	174.0	35	35
	roxithromycin	Internal standard		+	837.7	679.5	30	30
Sulphonamides	sulfadiazine	100	100	+	251.2	156.2	30	15
	sulfamethoxazole	100	100	+	254.4	156.4	30	15
	sulfadimethoxine	100	100	+	311.4	156.2	30	20
	sulfametazine	100	100	+	279.4	156.3	30	15
	sulfathiazole	100	100	+	256.4	156.3	25	15
	sulfadoxine	100	100	+	311.4	156.4	30	18
	sulfamethizole	100	100	+	271.0	156.2	25	15
	sulfapyridine	100	100	+	250.3	156.3	30	15
	sulfisoxazole	100	100	+	268.3	156.2	25	15
	sulfisomidine	100	100	+	279.4	186.3	30	16
	sulfamethoxypyridazine	100	100	+	281.2	156.2	30	15
	sulfachloropyridazine	100	100	+	285.3	92.3	30	28
	sulfaquinoxaline	100	100	+	301.3	92.2	30	30
	sulfameter	Internal standard		+	281.3	92.2	25	30
Amphenicol	chloramphenicol[c]	0.3	0.3	-	320.9	151.9	30	25
	chloramphenicol-d5[d]	Internal standard		-	326.0	157.0	30	25

[a] Abbreviations are: MRL, maximum residue level; VL, validation level; ESI, electrospray ion source.
[b] All values in electron volts (eV) must be multiplied by 1.6×10^{-9} to convert to Joules.
[c] Compound (a banned substance) without an MRL but with minimum required performance limit (MRPL) set to harmonise the analytical performance of the methods.
[d] Fifth-deuterated form of chloramphenicol.

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Table 2
The principal parameters of validation. [a]

Antibiotic	LOD (μg kg^{-1})	CCβ (μg kg^{-1})	RSD (%)
chlortetracycline	0.20	50.0	11
oxytetracycline	0.20	50.0	9
tetracycline	0.10	50.0	8
doxycycline	0.30	1.5	14
ciprofloxacin	0.20	50.0	21
enrofloxacin	0.02	50.0	8
marbofloxacin	0.10	35.0	19
oxolinic acid	0.20	0.4	9
flumequine	0.04	25.0	4
norfloxacin	0.20	4.7	15
nalidixic acid	0.30	0.4	9
danofloxacin	0.05	15.0	14
ofloxacin	3.70	4.1	17
enoxacin	3.00	3.2	16
cinoxacin	0.80	1.0	8
tylosin	0.01	25.0	11
tilmicosin	0.10	25.0	23
erythromycin	0.10	20.0	4
spiramycin	0.10	100.0	17
sulfadiazine	2.00	50.0	15
sulfamethoxazole	0.10	50.0	7
sulfadimethoxine	0.20	50.0	13
sulfametazine	0.10	50.0	5
sulfathiazole	1.00	50.0	10
sulfadoxine	0.20	50.0	5
sulfamethizole	0.20	50.0	12
sulfapyridine	1.00	50.0	12
sulfisoxazole	0.10	50.0	7
sulfisomidine	0.60	50.0	13
sulfamethoxypyridazine	0.10	50.0	17
sulfachloropyridazine	0.10	50.0	9
sulfaquinoxaline	0.10	50.0	5
chloramphenicol	0.06	0.1	15

[a] Abbreviations are: LOD, limit of detection; CCβ, detection capability; RSD, relative standard deviation

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Figure 1