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## Validation of a liquid chromatography-tandem mass spectrometry screening method to monitor 58 antibiotics in milk: qualitative approach

Murielle Gaugain-Juhel, Bernard Delépine, Sophie Gautier, Marie-Pierre Fourmond, Valerie Gaudin, Dominique Hurtaud-Pessel, Eric Verdon, Pascal Sanders

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**Validation of a liquid chromatography-tandem mass spectrometry screening method to monitor 58 antibiotics in milk: qualitative approach**

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3 1 **Validation of a liquid chromatography-tandem mass spectrometry**  
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6 2 **screening method to monitor 58 antibiotics in milk: qualitative**  
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15 6 M. Gaugain-Juhel\* ; B. Delépine; S. Gautier ; M.P. Fourmond ; V. Gaudin ;

16  
17 7 D. Hurtaud-Pessel ; E. Verdon; P.Sanders  
18  
19 8  
20  
21 9  
22

23  
24 10 *Community Reference Laboratory for Antimicrobial Residues, Agence Française de Sécurité*  
25  
26 11 *Sanitaire des Aliments, Laboratoire d'Etude et de Recherche sur les Médicaments*  
27  
28 12 *Vétérinaires et les Désinfectants, La Haute-Marche, BP90203, 35302 Fougères Cedex,*  
29  
30 13 *France*  
31  
32 14

33  
34 15 **Corresponding author**

35  
36 16 *M. Gaugain-Juhel*

37  
38 17 *Tel : 33 (0)2 99 94 78 78*

39  
40 18 *Email adress : [m.gaugain-juhel@fougeres.afssa.fr](mailto:m.gaugain-juhel@fougeres.afssa.fr)*  
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1  
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3 19 **Abstract**  
4

5 20 A multi-residue method was developed for monitoring antibiotic residues in milk using liquid  
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7 21 chromatography coupled to a tandem quadrupole mass spectrometer (LC/MS-MS). Two very  
8  
9 22 short extractions followed by two LC/MS-MS acquisitions allow the screening of 58 antibiotics  
10  
11 23 belonging to 8 different families (penicillins, cephalosporins, sulfonamides, macrolides,  
12  
13 24 lincosamides, aminoglycosides, tetracyclines and quinolones). This method is currently  
14  
15 25 implemented in the laboratory in a qualitative way: i.e. monitoring the presence or absence of  
16  
17 26 residue in a sample and identification of the analyte before the confirmation step. In order to  
18  
19 27 assess the performance of this method, a validation strategy described in an internal  
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21 28 guideline for the validation of screening methods was applied. The aim of the validation was  
22  
23 29 to prove sufficient sensitivity of the method to detect all the targeted antibiotics at the level of  
24  
25 30 interest (MRL) at least. According to the EU commission decision 2002/657/EC, the suitable  
26  
27 31 sensitivity of a screening method can be demonstrated when the  $CC\beta$  is below or equal to  
28  
29 32 the MRL level and so the false compliant rate below or equal to 5 % at the MRL level. The  
30  
31 33 validation scheme was established in order to take into account various variability factors: the  
32  
33 34 apparatus response, the interday repeatability, the matrix effect, etc ... The results of the  
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35 35 validation clearly demonstrate the suitability of this method for the detection and identification  
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37 36 of more than 50 antibiotics and are in agreement with the results obtained in routine analysis.  
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39 **Keywords:** antibiotic residues; multi-residue screening; LC/MS-MS; Validation

## 40 Introduction

41 The frequent but regulated use of antimicrobial drugs for veterinary purposes may leave  
42 residues in edible tissues which can lead to allergic reaction, disorder of intestinal flora or  
43 emergence of resistant strains of bacteria. Antibiotics can be used for therapeutic purposes  
44 or as feed additives to promote animal growth. In the EU, food safety regulations were  
45 established during the 90's (EC 1990, EC 2002) and European Member States are  
46 requested to monitor the presence of antimicrobial residues in different tissues of animal  
47 origin by implementing suitable residue control programmes.

48  
49 Microbiological inhibitory plate test methods are mostly used for the screening of antibiotic  
50 residues. The advantages of these methods are the possibility to detect a wide number of  
51 compounds in a simple way and at a low cost. But these methods are in some cases not  
52 enough sensitive (false negative results) and not really specific (false positive results).  
53 Furthermore, even if these screening methods are sometimes able to give an indication on  
54 the antibiotic family, a further post-screening step is needed and often implemented in order  
55 to determine the identity of the previously detected inhibitory substance. Rapid test kits are  
56 also employed but have the drawback to cover only a few targeted compounds or  
57 compounds belonging to the same family of antibiotics.

58  
59 LC/MS-MS techniques are now widely used for screening purposes and these methods are  
60 able to cover a high number of veterinary drugs (Gergov et al. 2003, Granelli et al. 2007,  
61 Hammel et al. 2008, Stolker et al. 2007, Muñoz et al. 2005, Turnipseed et al. 2008). If the  
62 high number of transitions to be monitored was previously requiring fragmentation of the  
63 acquisition into several retention time windows (segments), the level of sensitivity of the new  
64 generation of LC/MS-MS instruments allows performing the acquisition in a single segment.  
65 More recently in the mid of the 2000's, a trend appeared in using accurate mass full scan MS  
66 techniques (e.g. LC-ToF-MS, LC-LTQ-Orbitrap-MS...) allowing the screening of predefined

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2  
3 67 compounds but also the research and identification of untargeted compounds (Stolker et al.  
4  
5 68 2008, Kaufmann et al. 2007, Nielen et al. 2007).  
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9  
10 70 This work describes a simple and rapid LC/MS-MS post-screening method which was  
11  
12 71 developed first in muscle tissues and then applied to milk and is currently tested in real-life  
13  
14 72 conditions following a microbiological inhibitory screening step. This method is dedicated to  
15  
16 73 the detection and identification of 58 antibiotics using LC/MS-MS with an electrospray  
17  
18 74 ionisation (ESI) source set in a positive mode and was validated first in muscle tissues and  
19  
20 75 then in raw milk. The screened antibacterials belong to the following families: beta-lactam  
21  
22 76 (penicillins and cephalosporins), tetracyclines, macrolides, quinolones, sulfonamides,  
23  
24 77 lincosamides and aminoglycosides.  
25  
26  
27 78

28  
29 79 Although primarily dedicated to a qualitative and identificative screening of authorized  
30  
31 80 antimicrobial substances, this method can be upgraded to a confirmatory step with  
32  
33 81 quantitative results for certain families by adding suitable internal standards in order to  
34  
35 82 decrease and correct matrix effects. This particular issue will be published in other articles  
36  
37 83 from the same authors. Sticking to a screening/post-screening purpose, it was so decided  
38  
39 84 not to use the classical validation approach designed for a quantitative method (accuracy,  
40  
41 85 repeatability and reproducibility...) but to implement a design suitable for the validation of a  
42  
43 86 qualitative method as described in an internal guideline for the validation of screening  
44  
45 87 methods (Gaudin V. 2007). The LC/MS-MS qualitative method as well as the validation  
46  
47 88 scheme and the results of the validation are comprehensively presented in this paper.  
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51 89

## 52 53 90 **Materials and methods**

### 54 55 91 *Chemical, reagents and solutions*

56  
57 92 Antibiotic standards were for most of them obtained from Sigma-Aldrich (France) and used  
58  
59 93 for the preparation of individual stock standard solutions (concentration of 1 mg.ml<sup>-1</sup>) in either  
60

1  
2  
3 94 methanol or water depending on the solubility of each antibiotic. Solutions of 1 – 5  $\mu\text{g}\cdot\text{ml}^{-1}$   
4  
5 95 were prepared in the mobile phase from the stock solutions for the tuning of the ESI source  
6  
7 96 and for MS/MS transitions settings. From these stock solutions, suitable concentrations of  
8  
9 97 spiking solutions were also prepared in ultra-pure water to be used during the validation  
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11 98 process.

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13 99  
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15  
16 100 All the water used was ultra-pure deionised water produced by a Milli-Q apparatus (Millipore,  
17  
18 101 France). Trichloroacetic acid (TCA) from Fisher Scientific (France), ammonium acetate from  
19  
20 102 Merck (France) and pentafluoropropionic acid (PFPA) from Acros Organics (France) were  
21  
22 103 analytical reagent grade. Acetonitrile obtained from Fisher Scientific (France) was a HPLC  
23  
24 104 grade solvent. A 5% TCA solution was obtained by dissolving 50 g of trichloroacetic acid in 1  
25  
26 105 L of water. A 2 M ammonium acetate solution was prepared by dissolving 15.4 g of  
27  
28 106 ammonium acetate in 100 mL of water. This solution was then diluted by tenth to obtain a 0.2  
29  
30 107 M solution. A 0.1% PFPA solution was also prepared by adding 1 mL of pentafluoropropionic  
31  
32 108 acid in 900 mL of water and by adjusting to 1 L with water.

### 33 34 35 36 109 37 38 110 *Sample preparation*

39  
40 111 Two routes of extraction followed by two acquisition modes allow the screening and  
41  
42 112 differentiating of all 58 antibiotics. Acetonitrile was the extracting solvent chosen for the  
43  
44 113 extraction of penicillins, cephalosporins, macrolides and sulfonamides and the 5% TCA  
45  
46 114 solution was the acidic aqueous solvent chosen for the extraction of tetracyclines,  
47  
48 115 aminoglycosides, lincosamides and quinolones. Sulfaphenazole served as internal standard  
49  
50 116 in both extractions. Raw milk was allowed to thaw followed by a quick homogenisation of the  
51  
52 117 fat by shaking before taking up a test portion of 2 ml to which 200  $\mu\text{l}$  of internal standard and  
53  
54 118 800  $\mu\text{l}$  of water were added.

### 55 56 57 119 *Extraction of penicillins, cephalosporins, macrolides and sulfonamides*

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1  
2  
3 120 After addition of 8 ml of acetonitrile, the samples were stirred for 10 minutes and then  
4  
5 121 centrifuged at 14000 g for 5 min. After evaporation of 6 ml of the supernatant under nitrogen  
6  
7 122 flow, the residual volume was dissolved in 0.6 ml of 0.2 M ammonium acetate, then filtered  
8  
9 123 onto a 0.45 µm Millex HV filter of 13 mm diameter (Millipore, France). A volume of 25 µl were  
10  
11 124 injected and analysed by the LC/MS-MS instrument.

#### 125 *Extraction of tetracyclines, quinolones, aminoglycosides and lincomycin*

126 After addition of 8 ml of 5% TCA solution, the sample was stirred for 10 min and then  
127 centrifuged at 14000 g for 5 minutes. About 1 ml of the supernatant was filtered onto a 0.45  
128 µm Millex HV filter. A volume of 20 µl was injected and analysed by the LC/MS-MS  
129 instrument.

130

#### 131 *LC/MS-MS analysis*

132 An HPLC system (Agilent-HP1100) was used for the chromatographic separation which was  
133 performed on a Symmetry® C18 column, 5 µm, 150 X 3.9 mm (Waters) with a security guard  
134 system C18, 5 µm, 4.0 x 3.0 mm (Phenomenex). Two different gradients mixing 0.1% PFFA  
135 (A) with acetonitrile (B) were used according to the appropriate series of antibiotics. The  
136 gradient optimized for the separation of penicillins, cephalosporins, macrolides and  
137 sulfonamides started with 10 % of B. It was then rising linearly to 30 % of B over 4 min, then  
138 stopped for 1 min at 30% and again rising linearly to 70% of B over 2 min and stopped for 3 min  
139 at 70 %. The initial composition was then recovered over a 1 min delay. The gradient elution  
140 for the separation of tetracyclines, quinolones, aminoglycosides and lincomycin started with  
141 10 % of B, then rising to 50% of B over 7 min and stopped for 4 min at 50%. The initial  
142 composition was then recovered over a 1 min delay.

143

144 A triple quadrupole mass detector (AB-Sciex-API4000) set in a positive ESI mode was used  
145 for the detection and identification of the targeted antibiotics. The Turbolon Spray Voltage  
146 was set at 5.5 kV and the temperature of the source at 700°C. MRM conditions, declustering



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3 147 potential (DP) and collision energy (CE) were first optimized for each antibiotic by infusing  
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5 148 solutions of the antibiotic standards prepared in the mobile phase. MRM conditions are  
6  
7 149 displayed in Table Ia and Table Ib. The presence of two MRM transitions with a signal to  
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9  
10 150 noise ratio (S/N) above 3 in combination with the expected retention time guarantee the  
11  
12 151 univocal identification of the analyte. Even if only one transition can satisfactorily be  
13  
14 152 considered as sufficient for screening purposes, it is preferred to keep monitoring two  
15  
16 153 transitions in such a way to facilitate the technical evaluation of the signals and the analysis  
17  
18 154 of the resulting data through the "Analyst" software onto the instrument. Moreover, watching  
19  
20 155 at two transitions at the same retention time on the computer screen is really more efficient  
21  
22 156 especially when monitoring a large number of samples at screening step.

24  
25 157 [insert table Ia and Table Ib about here)  
26  
27 158

### 29 159 **Validation scheme**

30  
31 160 The validation process was established according to a guideline set for the comprehensive  
32  
33 161 validation of biological screening methods (Gaudin et al. 2007). This scheme of validation in  
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35 162 line with the criteria of the Decision No 2002/657/EC which is more dedicated to quantitative  
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37 163 confirmatory methods, was applied to an LC/MS-MS method used as a qualitative post-  
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39 164 screening tool. It was considered more suitable than the classical approach of validation  
40  
41 165 usually applied to quantitative physicochemical methods (trueness, precision, linearity...). In  
42  
43 166 fact, there was no interest in checking the accuracy of the method in terms of quantification.  
44  
45 167 It was considered more relevant to monitor many samples at the level of interest in order to  
46  
47 168 assess statistically the capacity of detection of the method. Anyway, the aim of a validation is  
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49 169 to prove the suitability of the method in achieving the goal it is developed for. The only  
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51 170 requirement for screening methods as described in the Commission Decision No  
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53 171 2002/657/EC is that these methods have the capability for a high sample throughput and  
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55 172 shall allow the detection of all targeted substances with a false compliant rate below 5% at  
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57 173 the level of interest. It means that the  $CC\beta$  of the method should be found below this level of  
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3 174 interest. The way to assess the detection capability is described below as well as the way the  
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5 175 limit of detection, the specificity and the sensitivity are calculated respectively. In the case of  
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7 176 a suspected non-compliant result, this result shall be confirmed by a confirmatory method.  
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10 177

11 178 *Determination of "T" value*

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14 179 A first step in assessing the capacity of detection is the calculation of the "T" value for each  
15  
16 180 compound of interest. The "T" value is a "Threshold" value corresponding to the minimum  
17  
18 181 analytical response above which the sample will be truly considered as positive. In order to  
19  
20 182 determine this "T" value, 20 blanks of raw milks from different origins were tested with the  
21  
22 183 method described above. The analytical blank response at the retention time corresponding  
23  
24 184 to the analyte ( $\pm 10\%$ ) was determined in each blank chromatogram for the two MRM  
25  
26 185 transitions. The mean value of the noise "B" was then calculated. The threshold value "T"  
27  
28 186 was estimated for each transition as being three times the mean value obtained from the 20  
29  
30 187 recorded noises (see figure 1).  
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36 189 *Determination of the limit of detection:*

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38 190 The limit of detection is the smaller concentration of analyte above which it is possible to  
39  
40 191 detect this analyte with a reasonable statistical certainty. By comparing the response  
41  
42 192 corresponding to the "T" value (3 times the mean noise) with the mean response of two  
43  
44 193 samples spiked at a known concentration, it is possible to determine the "limit of detection" of  
45  
46 194 the method for each analyte. As the presence of the two transitions is recommended for full  
47  
48 195 identification of the substance when possible, the limit of detection is generally estimated  
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50 196 from the results of the most variable response from the two transitions. Very often, this is the  
51  
52 197 minor transition that gives also the most variable one.  
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55 198

56  
57 199 *Determination of the cut-off factor "Fm" and assessment of the detection capability (CC beta)*  
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3 200 The 20 blanks of raw milk were spiked at the level of interest (MRL or  $\frac{1}{2}$  MRL) with the  
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5 201 different analytes and tested within the same day. Generally, the samples were spiked at the  
6  
7 202 MRL level. The half-MRL level was chosen for some compounds especially when the MRL is  
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10 203 established for the parent drug plus its metabolite or for the sum of different compounds (e.g.  
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12 204 sulfonamides). Stock solutions for each antibiotic were prepared in methanol except for  
13  
14 205 aminoglycosides and penicillins which were dissolved in water. Spiking solutions were  
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16 206 prepared in water by mixing the different standards from one antibiotic family. Then,  
17  
18 207 validation was carried out per family in order to get information about the potential  
19  
20 208 interferences. The concentrations evaluated during the validation are given in Table IIa and  
21  
22 209 Table IIb.

23  
24  
25 210 [insert table IIa and IIb about here]

26  
27 211 This step was repeated again twice in order to get a total of 60 independent data for each  
28  
29 212 analyte at the level of interest. The repetitions were carried out on three different days  
30  
31 213 distributed over three different weeks in order to increase the factors of variability and to  
32  
33 214 mimic routine analysis. The analytical response ( $Y_i$  = height of the peak response) were  
34  
35 215 determined for each of the samples ( $n=60$ ) and for the two MRM transitions from each  
36  
37 216 analytes. Both the mean response from the 60 samples and the corresponding standard  
38  
39 217 deviation were calculated. A "cut-off factor"  $F_m$  was then estimated as being the mean  
40  
41 218 response decreased by 1.64 times the standard deviation. It statistically means that 95 % of  
42  
43 219 the samples spiked at the level of interest should give an analytical response above this  
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45 220 value (see figure 1).

46  
47  
48  
49 221 [insert figure 1 about here]

50  
51 222  
52  
53 223 The "T" and "F<sub>m</sub>" values obtained can lead to different situations:  
54  
55 224 **F<sub>m</sub> > T**: this is the best situation corresponding to a false negative rate below 5%. The  
56  
57 225 consequence is the  $CC\beta$  is truly below the MRL level.  
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59  
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2  
3 226 **Fm < T**: if the “T” value is taken as a limit of positivity, then more than 5% of the samples will  
4  
5 227 be considered as negative. The consequence is the  $CC\beta$  is truly above the MRL level.  
6  
7 228 Further studies at concentrations above the MRL should be implemented in order to  
8  
9 229 determine the gap between the MRL and the  $CC\beta$ .

10  
11 230  
12  
13  
14 231 According to the Decision No 2002/657/EC, the detection capability ( $CC\beta$ ) of the methods  
15  
16 232 dedicated to screening purposes is validated only when  $F_m > T$ . Then, it can be deduced  
17  
18 233 that the  $CC\beta$  is definitely below the level of validation. As a reminder, the very first  
19  
20 234 requirement expected from a screening method is to avoid as much as possible the false  
21  
22 235 negative results also called “false compliant” results.  
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26 236

27 237 *Assessment of the specificity and sensitivity:*

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29  
30 238 The following definitions are needed to understand the calculation of the two parameters  
31  
32 239 “specificity” and “sensitivity”. At first, a true positive sample giving a positive test result is  
33  
34 240 called a “positive agreement”. Then a true negative sample giving a negative test result is  
35  
36 241 called a “negative agreement”. A true positive sample giving a negative test result is called a  
37  
38 242 “false negative” sample. Finally, a true negative sample giving a positive test result is called a  
39  
40 243 “false positive” sample.  
41  
42

43 244

44  
45 245 The “specificity” of a method is the ability of this method to distinguish one specific analyte  
46  
47 246 from the other analytes. It can be determined as being the number of negative agreement  
48  
49 247 divided by the number of true negative samples expressed as a percentage. The “sensitivity”  
50  
51 248 of a method can be determined as being the number of positive agreements divided by the  
52  
53 249 number of true positive samples expressed as a percentage. A sample is considered to be  
54  
55 250 positive as soon as the signal ranges above the “T” value (three times the mean noise). A  
56  
57 251 sensitivity above 95% means that the  $CC\beta$  is below the level of concentration tested for the  
58  
59 252 validation. Then the number of false negatives is truly below 5%.

253

**254 Results and discussion***255 Sample pretreatment and LC/MS-MS analysis*

256 The challenge for the analyst in the development of a multi-residue method was to find  
257 suitable extraction conditions for a large range of targeted analytes displaying different  
258 chemical properties (lipophilicity, hydrophilicity, alkaline and acidic characteristics, ...). As it  
259 is almost impossible to optimize the cleanup process for all analytes, the purification step has  
260 generally to be as short and simple as possible. Having a look at the literature in the field of  
261 veterinary drug residues, two main routes of extraction were considered in muscle. The first  
262 one was acetonitrile extraction which is a common solvent used for many veterinary drugs  
263 including antibiotics. This solvent was found to be sufficiently effective for the extraction of  
264 macrolides, sulfonamides, penicillins, and cephalosporins. The second route was an acidic  
265 extraction with trichloroacetic acid which was found to be suitable for quinolones,  
266 aminoglycosides, lincomycin, and tetracyclines. Results obtained when comparing the  
267 different solvents evaluated for extraction of the 58 antimicrobials were not always in full  
268 agreement with the expected results taking into consideration the chemical properties of the  
269 different analytes. But as previously mentioned, the aim of this study was to find global  
270 conditions acceptable for all substances and not to optimize the recovery for each analyte.  
271 These extraction conditions were then tested successfully on milk. The difficulty to find a  
272 generic extraction solvent is due to the aminoglycoside family for which acidic extractions are  
273 mainly used. During the validation, spiked samples were prepared with twenty different  
274 batches of milk in order to check suppression or enhancement effects due to the matrix. A  
275 variability due to matrix effects was sometimes observed, particularly for the hydrophilic  
276 compounds eluting in the first minutes after injection, but validation results proved that the  
277 sensitivity was always sufficient to detect all the analytes at the level of interest. PFPA and  
278 acetonitrile in a gradient mode were chosen as mobile phase for the chromatographic  
279 separation. Even if it is announced that PFPA may lead to ion suppression effects in

1  
2  
3 280 electrospray ionization and if the ionization rate was not optimized for all analytes with this  
4  
5 281 acidic solvent, this last was preferably selected for its advantageous ion-pairing properties  
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7 282 which allowed including in this method as strong bases as the aminoglycosides and as  
8  
9  
10 283 relatively strong acids as amoxicillin. In contradiction with the general trend to  
11  
12 284 chromatography with narrow-bore analytical columns, a conventional Symmetry<sup>®</sup> analytical  
13  
14 285 column (Waters, France) of 3.9 mm in diameter was chosen. It was considered in this study  
15  
16 286 that the use of a narrower column with a lower flow-rate did not preclude saving time even if  
17  
18 287 it effectively saves solvent. In fact, due to the sloping gradient used, the period for stabilizing  
19  
20 288 the column between to injections was found time-consuming. Satisfactorily, using the  
21  
22 289 Symmetry<sup>®</sup> analytical column with a 3.9 mm diameter, no stabilization time was needed  
23  
24 290 between injections and the runs were finally shorter (<15 minutes). The high selectivity and  
25  
26 291 sensitivity of the triple-quadruple detector allowed the detection of 58 analytes in two runs  
27  
28 292 without segmenting the acquisition. The singly-charged precursor ion was preferentially  
29  
30 293 considered for all the 58 antimicrobial analytes and the two highest MRM transitions were  
31  
32 294 then monitored for each analyte. Some sulfonamides displaying isobaric masses and giving  
33  
34 295 the same ionic fragments were nevertheless identified taking into account their different  
35  
36 296 retention times. Some chromatograms are presented in figure2.

37  
38  
39  
40 297 [Insert figure 2 about here]

#### 41 42 298 *Method validation*

43  
44 299 The validation process previously described had already been tested for biological screening  
45  
46 300 methods but was totally new for a LC/MS-MS method. The suitability of this validation  
47  
48 301 scheme applied to a physicochemical screening method was demonstrated and gave more  
49  
50 302 useful information than the classical approach of validation usually applied to quantitative  
51  
52 303 physicochemical methods. The limit of detection, the specificity, sensitivity and the CCbeta  
53  
54 304 were assessed during the validation and the results are described below for each antibiotic  
55  
56 305 family. The specificity of the method was checked by analyzing twenty milk blank samples of  
57  
58 306 different origins and the specificity was of 100% for all the analytes as no peak was detected  
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3 307 in these samples at the retention time corresponding to each analyte. This fact underlines the  
4  
5 308 very high specificity of the tandem mass spectrometer.  
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10 310 *Tetracyclines*

11 311 Milk samples were spiked at 50  $\mu\text{g.kg}^{-1}$  for oxytetracycline, 4-epi-oxytetracycline, tetracycline,  
12 312 4-epi-tetracycline, chlortetracycline and 4-epi-chlortetracycline as the MRL is 100  $\mu\text{g.kg}^{-1}$  for  
13  
14 313 the sum of the parent drug and its epimer. The concentration of validation for doxycycline  
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16 314 was 100  $\mu\text{g.kg}^{-1}$ . Twenty spiked samples were analysed per day and during three days. In  
17  
18 315 total, 60 samples were analysed for each analyte. Results are presented in Table III and are  
19  
20 316 very satisfactory as the "Fm" values are above the "T" values for all analytes. These results  
21  
22 317 mean that the CCbeta of the 7 tetracyclines are below the level of concentrations tested  
23  
24 318 during the validation: i.e. below  $\frac{1}{2}$  MRL for OTC, TTC, and CTC and their 4-epimers and  
25  
26 319 below MRL for DC. The limits of detection were calculated and are ranging between 0.3 and  
27  
28 320 2.2  $\mu\text{g.kg}^{-1}$  for the first transition and between 1.5 and 13.6  $\mu\text{g.kg}^{-1}$  (4-epi-tetracycline) for the  
29  
30 321 second "minor" transition. The sensitivity was calculated at 100% for all the tetracyclines and  
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32 322 their epimers. Retention times were ranging from 7.2 to 8.1 min.  
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38 323 [insert table III about here]

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41  
42 325 *Sulfonamides and macrolides*

43 326 The level of validation for each analyte is given in Table 2a. According to the validation  
44  
45 327 scheme, 60 spiked samples were analysed on three different days. The estimated CCbeta  
46  
47 328 was below the level of validation for all sulfonamides and macrolides as the "Fm" value were  
48  
49 329 above the "T" values. The limits of detection were below 1  $\mu\text{g.kg}^{-1}$  for most of the analytes  
50  
51 330 and up to 1.7  $\mu\text{g.kg}^{-1}$  for sulfaguanidine (minor transition). The sensitivity was 100% for all  
52  
53 331 sulfonamides and macrolides. Retention times were ranging from 3.1 to 9.6 min for  
54  
55 332 sulfonamides and from 7.3 to 9.8 min for macrolides.  
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334 *Quinolones*

335 The use of norfloxacin and nalidixic acid is not allowed in veterinary medicine for food-  
336 producing animals but a concentration of validation of  $100 \mu\text{g.kg}^{-1}$  was arbitrarily chosen. The  
337 MRL for the sum of enrofloxacin and its metabolite ciprofloxacin being  $100 \mu\text{g.kg}^{-1}$ , a  
338 concentration of validation of  $50 \mu\text{g.kg}^{-1}$  was chosen for each. The level of validation for other  
339 quinolones is given in Table 2b. Results were very satisfactory for this family as the  
340 estimated  $CC_{\beta}$  was below the level of validation for all quinolones except for oxolinic acid  
341 and the sensitivity of 100 % for all (for the two transitions). It means that oxolinic acid was  
342 always detected at the level of validation but there was enough variation in the response to  
343 assess a low "Fm" value. The limits of detection of the quinolones were below  $1 \mu\text{g.kg}^{-1}$  and  
344 up to  $1.95 \mu\text{g.kg}^{-1}$  for the major transition and below  $1 \mu\text{g.kg}^{-1}$  and up to  $15.5 \mu\text{g.kg}^{-1}$  for the  
345 minor transition (sarafloxacin). Retention times were ranging from 7.0 to 11.1 min.

346

347 *Aminosides*

348 The levels of validation for each aminoglycoside are given in Table 2b. Results were very  
349 satisfactory as the  $CC_{\beta}$  was assessed to be below the level of validation for all analytes  
350 except for spectinomycin (minor transition). The limits of detection were calculated and  
351 ranged between  $0.2$  and  $3.5 \mu\text{g.kg}^{-1}$  for the first transition and between  $0.2$  and  $66 \mu\text{g.kg}^{-1}$   
352 (spectinomycin) for the minor transition. The sensitivity was 100% for all aminosides except  
353 for the minor transition of spectinomycin. Retention times were ranging from 5 to 6.5 min.

354

355 *Penicillins and cephalosporins*

356 The concentrations of validation for these two families are given in Table 2a and results for  
357 penicillins are presented in Table VI. Results were very satisfactory for penicillins and  
358 cephalosporins except for amoxicillin for which the  $CC_{\beta}$  was assessed to be above the MRL  
359 of  $4 \mu\text{g.kg}^{-1}$  for the three chosen transitions. Nevertheless, the sensitivity was calculated as  
360 being above 95% for the second and third transition and we can so ensure a sufficient



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3 361 detection capability by considering that the presence of at least one transition is enough to  
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5 362 consider a further confirmatory step. In the same way, the  $CC\beta$  was above the MRL for the  
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7  
8 363 minor transition of ampicillin. The  $CC\beta$  obtained for the cephalosporins were all below the  
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10 364 concentration of validation except for the first transition of cefoperazone but the sensitivity  
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12 365 was 100% for all of them. Retention time were ranging from 6.9 to 11.6 min for penicillins and  
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14 366 from 6.9 to 9.4 min for cephalosporins.

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16 367 [insert table VI about here]  
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21 369 *Validation results*

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23 370 The results of the validation clearly demonstrate the suitability of this method for the  
24  
25 371 detection and identification of all tested antibiotics. Particular attention should be paid to the  
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27 372 following compounds for which the detection is often problematic: i.e. amoxicillin, ampicillin,  
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29 373 and spectinomycin. However, for these compounds, the presence of at least one transition  
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31 374 shall lead to the confirmatory step. For amoxicillin, a careful observation of the  
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33 375 chromatograms at the retention time of interest shall be carried out.  
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38 377 Participation to different proficiency tests organized in the field of antibiotic residues in order  
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40 378 to demonstrate the efficiency of this method, clearly showed a very satisfactory evaluation of  
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42 379 its capacity of detecting and identifying antibacterial residues as no false-negative and no  
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44 380 false-positive results were obtained.  
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49 382 *Field of application*

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51 383 Application of this method to milk was presented in this paper but the method was first  
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53 384 developed for muscle tissue samples. Used in routine analysis as a post-screening method  
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55 385 carried out after a first microbiological screening step, it allows the full identification of the  
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57 386 antibiotic previously responsible of the microbiological activity inhibition. The step for  
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59 387 quantitative confirmation is then more efficient and not so time-consuming. On the side of  
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3 388 analytical quality assurance, it was not easily conceivable to implement quality control  
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5 389 samples spiked at the level of interest for the 58 antibiotics in such a routine use, particularly  
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7 390 because of stability problems with penicillin solutions. Therefore, after the validation step, our  
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9 391 choice in order to assure a sufficient sensitivity in routine analysis was to add an internal  
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11 392 standard for each sample to be analyzed and to set a minimal height of signal response to  
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13 393 qualify the analysis according to the validation data. Furthermore, a sample matrix spiked  
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15 394 with one analyte from each family was added as quality control sample. The criteria for the  
16  
17 395 quality control have to be set in relation to the results obtained from the validation step. Then  
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19 396 the follow-up of a control chart provides a reliable checking of the suitability for these criteria.  
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21 397 The high correlation between the microbiological screening results and the mass  
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23 398 spectrometry post-screening results demonstrates the high efficiency of this method in  
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25 399 routine use, especially here described for milk. Nevertheless, a few samples being detected  
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27 400 positive at the microbiological step were not confirmed to be positive at the post-screening  
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29 401 step. There are two possibilities : the microbiological result could be a false positive result or  
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31 402 the concerned analyte was not monitored by the LC/MS-MS method. One of the  
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33 403 disadvantages of LC-triple quadrupole MS instruments compared to LC-ToF-MS instruments  
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35 404 is the need to optimize for each new compound before being included in the acquisition  
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37 405 mode of the method. Moreover, there is no possibility to review and reassess the analytical  
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39 406 data collected by the instrument on a previously analyzed sample when it is necessary for  
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41 407 exemple to check for the presence of one suspected "untargeted" analyte if this analyte was  
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43 408 not initially included in the method.  
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51 410 This multi-residue method is not suitable for confirmatory purposes in the here-described  
52  
53 411 format of the method as quantitative requirements from the Decision No 2002/657/EC are not  
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55 412 always met for some particular families. For example, large matrix effects in muscle tissue  
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57 413 were observed for tetracycline residues. Quantification of macrolides was also not accurate  
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59 414 enough with the here-above described extraction. Some dilutions of the samples and use of  
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3 415 suitable internal standards could improve the accuracy of the method within the context of a  
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5 416 quantitative confirmation. For penicillins, cephalosporins and quinolones, minor changes and  
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7 417 the use of internal standard(s) may allow using this method as a confirmatory method.  
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3 418 **Conclusion**  
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5 419 The LC/MS-MS method proposed in this article allows a rapid and efficient screening or post-  
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7 420 screening of 58 antibiotics in milk and meets the requirements of EU/2002/657 for screening  
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10 421 methods as demonstrated during the validation. One possibility to increase further the  
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12 422 sensitivity of the method will be to implement a new acquisition mode as it is proposed in the  
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14 423 new version of the LC/MS-MS software Analyst version 1.5 which is the so-called "MRM  
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16 424 scheduled" mode. This mode allows the automatic implementation of retention time windows  
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18 425 which is useful for the detection of a high number of analytes. The sensitivity is enhanced by  
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20 426 setting longer acquisition dwell-times for each compound. It is planned to extend the field of  
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22 427 application of this LC/MS-MS multi-antibiotic method by including the monitoring to other  
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24 428 antibiotics used in veterinary medicine but not yet included in the method as bacitracin,  
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26 429 colistin, trimethoprim. It was proved during this study that LC-triple quadrupole MS instrument  
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29 430 offers suitable opportunities to implement a reliable monitoring in the well-defined regulatory  
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31 431 context of food safety in the EU.  
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5 **Figure 1. Graphical representation of threshold value “t” and Cut-off factor “Fm”**

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7 **Figure 2. Examples of chromatograms of milk samples spiked at the EU-MRL or half the EU-MRL levels**

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Figure 1

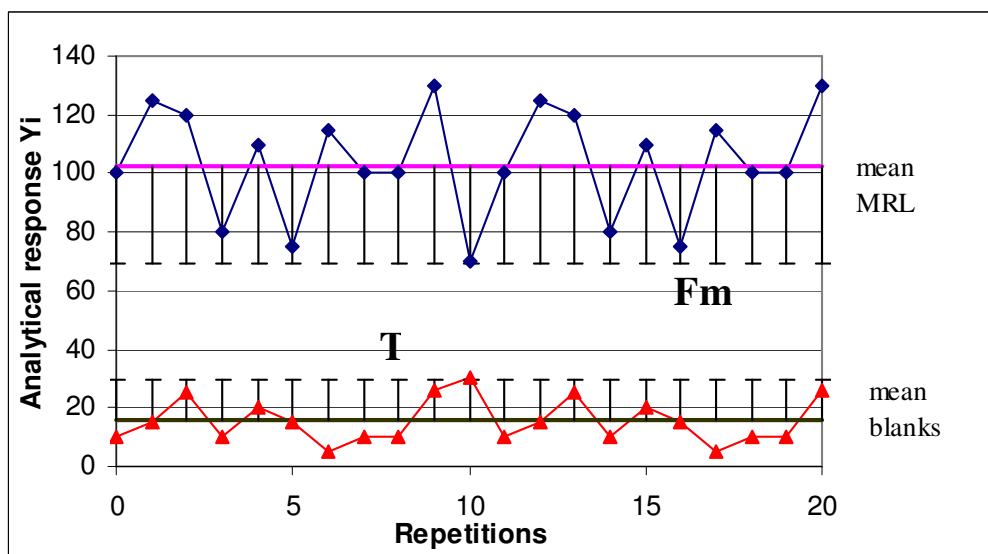
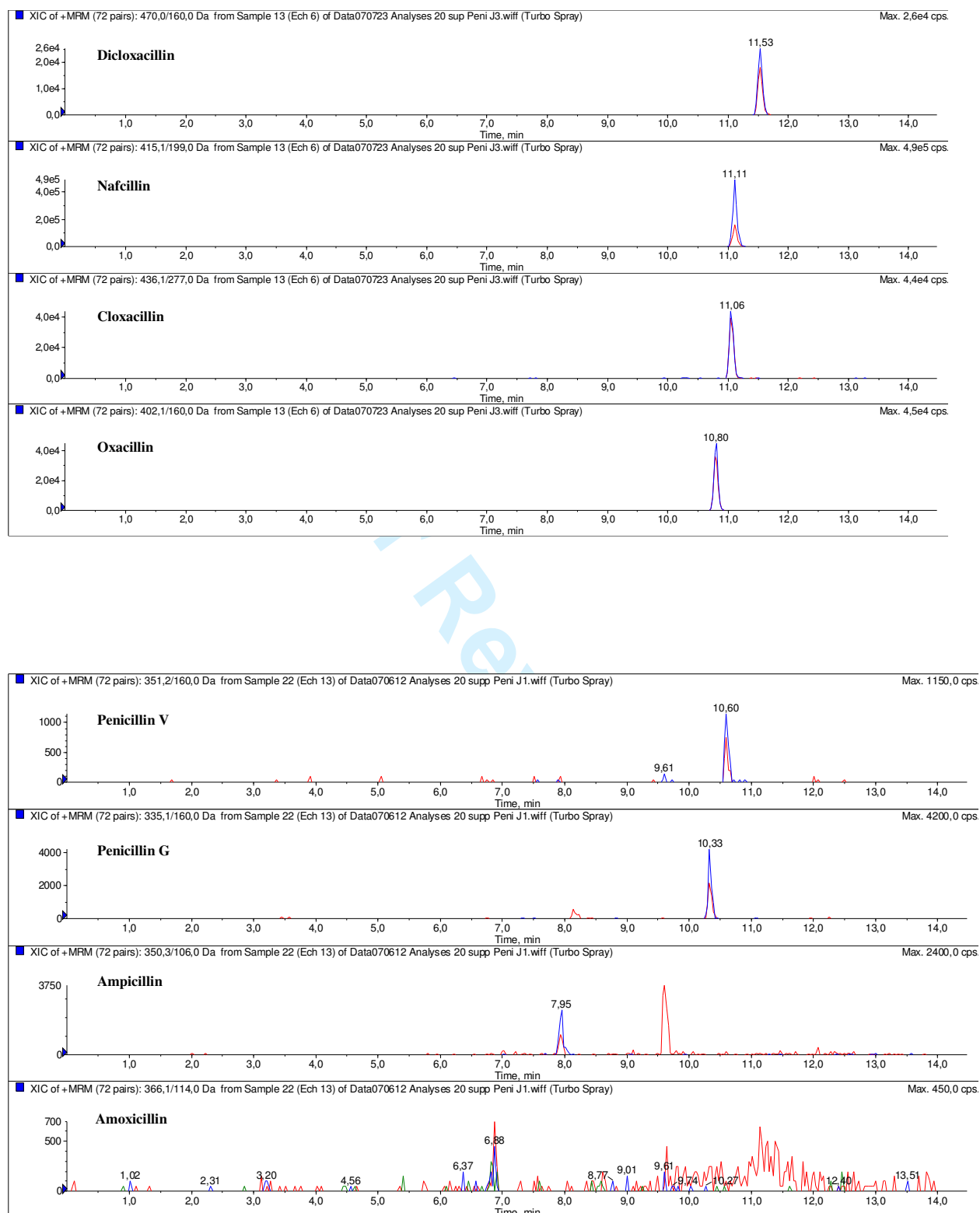
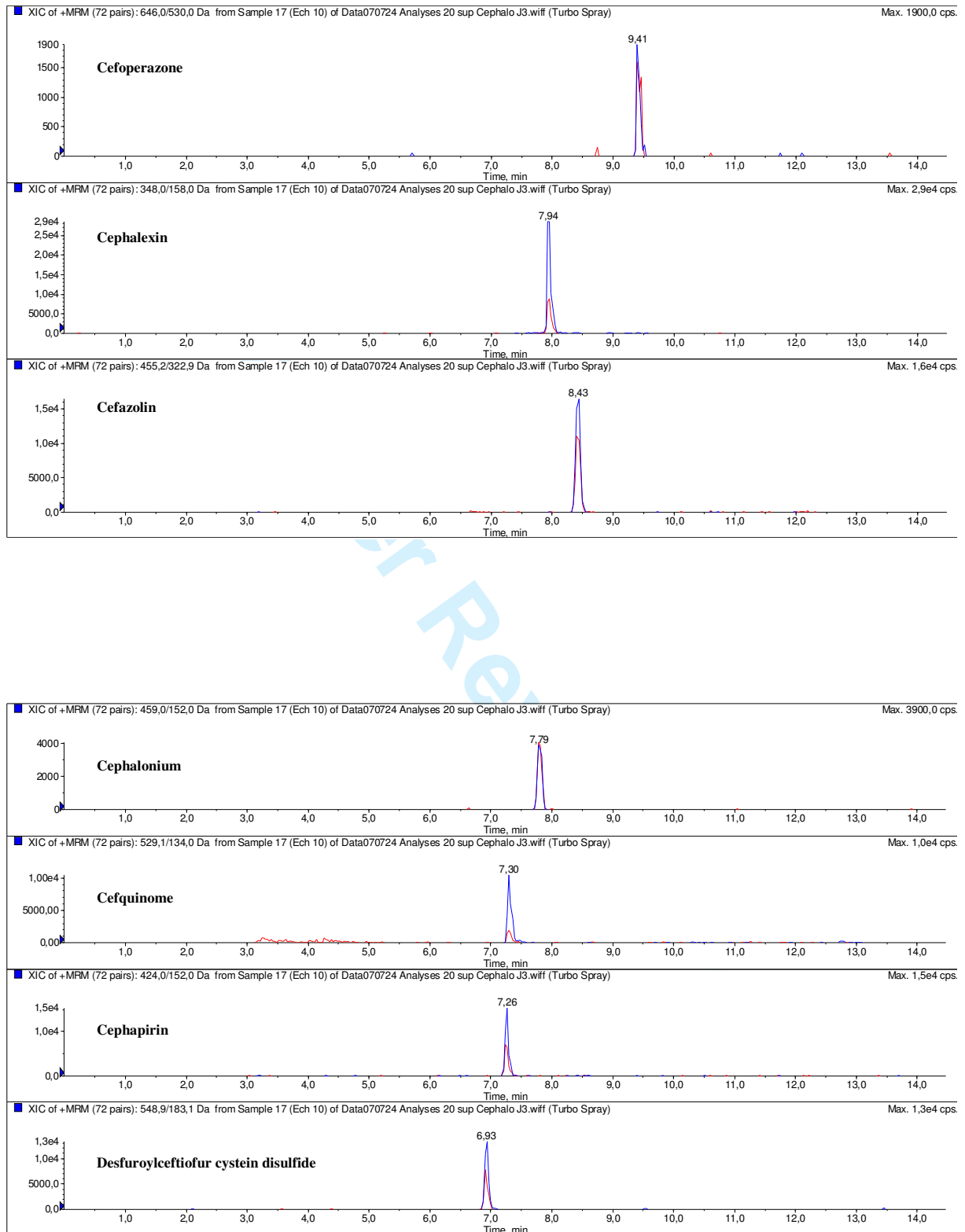
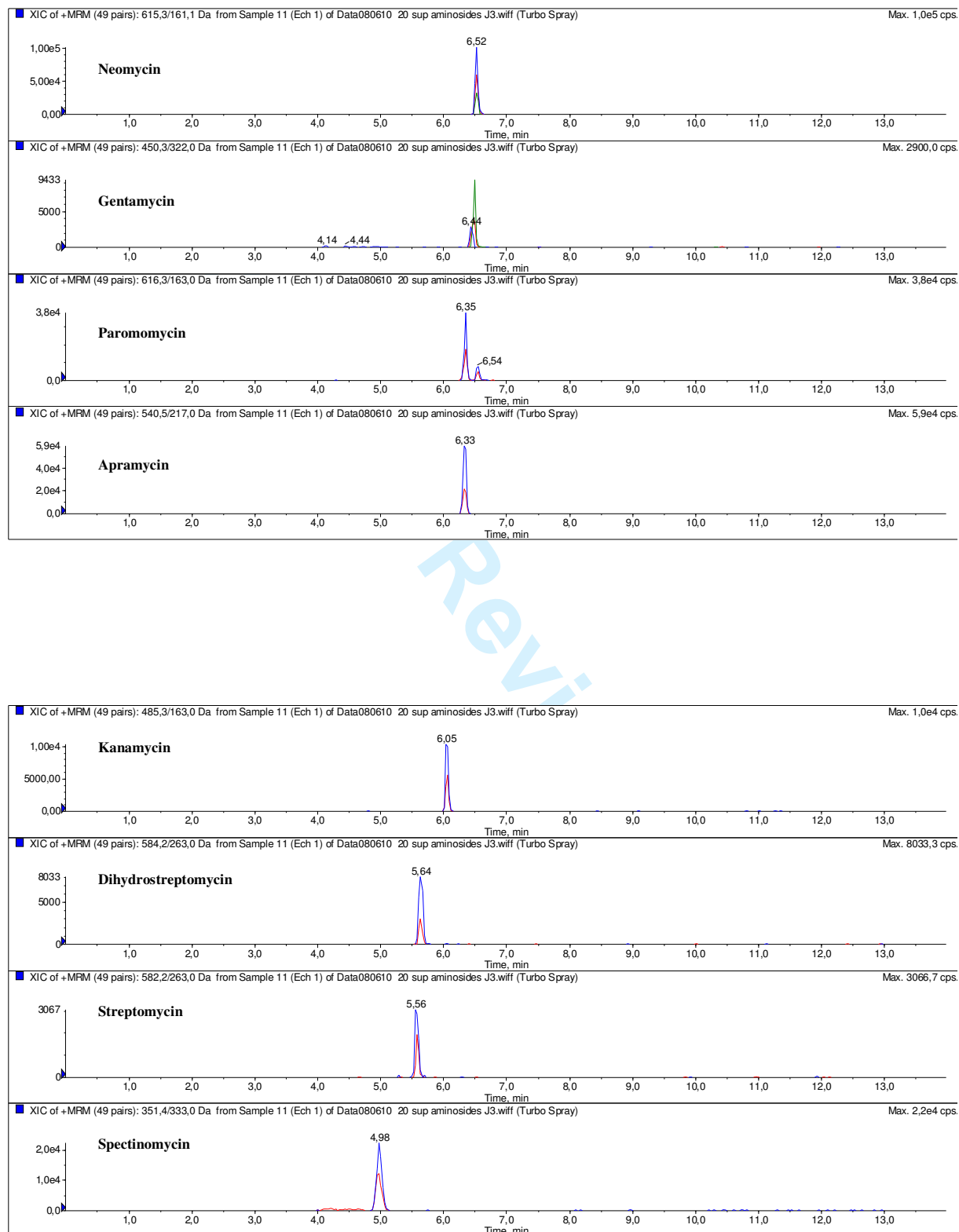




Figure 2







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Table Ia. MRM transitions for cephalosporins, penicillins, sulfonamides and macrolides in the acetonitrile extract

| Analyte                         | DP  | Major    | CE | Minor    | CE | Minor trans | 2 CE |
|---------------------------------|-----|----------|----|----------|----|-------------|------|
| Amoxicillin                     | 50  | 366/349  | 13 | 366/208  | 20 | 366.1/114   | 33   |
| Ampicillin                      | 50  | 350/106  | 20 | 350/160  | 20 |             |      |
| Penicillin-G (benzylpenicillin) | 50  | 335/160  | 15 | 335/176  | 15 |             |      |
| Penicillin V                    | 50  | 351/160  | 15 | 351/114  | 45 |             |      |
| Oxacillin                       | 50  | 402/160  | 18 | 402/243  | 18 |             |      |
| Cloxacillin <sup>a</sup>        | 50  | 436/160  | 20 | 436/277  | 20 |             |      |
| Nafcillin                       | 50  | 415/199  | 20 | 415/171  | 50 |             |      |
| Dicloxacillin                   | 50  | 470/160  | 20 | 470/311  | 20 |             |      |
| Cephapirin                      | 50  | 424/152  | 35 | 424/124  | 70 |             |      |
| Ceftiofur                       | 50  | 524/241  | 25 | 524/125  | 70 |             |      |
| Desfuroyl ceftiofur cystein     | 76  | 548/183  | 41 | 548/241  | 29 |             |      |
| Cefquinome                      | 50  | 529/134  | 25 | 529/125  | 75 |             |      |
| Cephalonium                     | 50  | 459/337  | 15 | 459/152  | 25 |             |      |
| Cefazolin                       | 50  | 455/323  | 15 | 455/156  | 23 |             |      |
| Cephalexin                      | 50  | 348/158  | 10 | 348/106  | 23 |             |      |
| Cefoperazone                    | 60  | 646/530  | 17 | 646/143  | 50 |             |      |
| Sulfaphenazole (Internal        | 50  | 315/156  | 30 | /        | /  |             |      |
| Sulfaguanidine                  | 20  | 215/156  | 20 | 215/108  | 30 |             |      |
| Sulfadiazine                    | 53  | 251/156  | 22 | 251/108  | 30 |             |      |
| Sulfathiazole                   | 53  | 256/156  | 20 | 256/108  | 34 |             |      |
| Sulfadimerazine                 | 50  | 279/156  | 25 | 279/108  | 36 |             |      |
| Sulfamethoxy pyridazine         | 60  | 281/156  | 25 | 281/108  | 35 |             |      |
| Sulfamonomethoxine <sup>a</sup> | 50  | 281/156  | 23 | 281/108  | 37 |             |      |
| Sulfadoxine                     | 60  | 311/156  | 25 | 311/108  | 40 |             |      |
| Sulfaquinolaxaline              | 50  | 301/156  | 23 | 301/108  | 40 |             |      |
| Sulfadimethoxine                | 50  | 311/156  | 23 | 311/108  | 37 |             |      |
| Tulathromycin marker            | 111 | 577/158  | 41 | 577/116  | 59 |             |      |
| Neospiramycin                   | 80  | 699/174  | 42 | 699/540  | 25 |             |      |
| Spiramycin <sup>a</sup>         | 100 | 843/174  | 47 | 843/540  | 42 |             |      |
| Tulathromycin                   | 126 | 807/158  | 59 | 807/577  | 33 |             |      |
| Tilmicosin                      | 120 | 869/174  | 63 | 869/696  | 55 |             |      |
| Tylosin                         | 120 | 916/174  | 50 | 916/772  | 40 |             |      |
| Erythromycin                    | 90  | 734/158  | 50 | 734/576  | 25 |             |      |
| O-acetyltylosin                 | 121 | 959/174  | 59 | 959/815  | 43 |             |      |
| Josamycin                       | 80  | 828/174  | 45 | 828/229  | 45 |             |      |
| Tylvalosin                      | 121 | 1043/109 | 95 | 1043/174 | 57 |             |      |

<sup>a</sup> Molecules contained in the spiked control samples

Table Ib. MRM transitions for quinolones, aminoglycosides, lincosamides and tetracyclines in the trichloroacetic acid extract

| Analyte                           | DP  | Major Trans               | CE | MinorTrans | CE |
|-----------------------------------|-----|---------------------------|----|------------|----|
| Spectinomycin                     | 71  | 351/333                   | 27 | 351/207    | 31 |
| Streptomycin                      | 157 | 582/263                   | 45 | 582/246    | 51 |
| Dihydrostreptomycin <sup>a</sup>  | 120 | 584/263                   | 42 | 584/246    | 54 |
| Kanamycin                         | 70  | 485/163                   | 35 | 485/205    | 35 |
| Paromomycin                       | 84  | 616/163                   | 45 | 616//293   | 33 |
| Gentamycin C1, C1a et C2          | 60  | 478/322, 450/322, 464/322 | 20 |            |    |
| Neomycin                          | 92  | 615/161                   | 45 | 615/163    | 30 |
| Apramycin                         | 82  | 540/217                   | 35 | 540/378    | 25 |
| Lincomycin                        | 60  | 407/126                   | 40 | 407/359    | 26 |
| Oxytetracycline <sup>a</sup>      | 65  | 461/426                   | 30 | 461/443    | 17 |
| Tetracycline                      | 55  | 445/410                   | 27 | 445/427    | 25 |
| Chlortetracycline                 | 60  | 479/444                   | 29 | 479/462    | 23 |
| Doxycycline                       | 55  | 445/428                   | 25 | 445/154    | 40 |
| Marbofloxacin                     | 70  | 363/345                   | 30 | 363/320    | 22 |
| Norfloxacin                       | 60  | 320/302                   | 33 | 320/231    | 50 |
| Ciprofloxacin <sup>a</sup>        | 61  | 332/314                   | 30 | 332/231    | 47 |
| Danofloxacin                      | 60  | 358/340                   | 33 | 358/255    | 50 |
| Enrofloxacin                      | 72  | 360/342                   | 30 | 360/286    | 50 |
| Sarafloxacin                      | 50  | 386/368                   | 30 | 386/348    | 50 |
| Difloxacin                        | 80  | 400/382                   | 30 | 400/356    | 50 |
| Oxolinic acid                     | 53  | 262/244                   | 25 | 262/216    | 40 |
| Nalidixic acid                    | 42  | 233/215                   | 30 | 233/187    | 35 |
| Flumequine                        | 44  | 262/244                   | 25 | 262/202    | 45 |
| Sulfaphenazole (Standard Interne) | 50  | 315/156                   | 30 |            |    |

<sup>a</sup> Molecules contained in the spiked control samples

Table IIa. EU-MRL and level of concentration for the validation of the analytes extracted by acetonitrile

| Analyte                                | MRL in milk ( $\mu\text{g}/\text{kg}$ ) | Validation concentration (Cval) ( $\mu\text{g}/\text{kg}$ ) |
|--|---|---|
| Sulfaguanidine                         | 100                                     | 50  |
| Sulfadiazine                           | 100                                     | 50  |
| Sulfathiazole                          | 100                                     | 50  |
| Sulfadimerazine                        | 100                                     | 50  |
| Sulfamethoxypyridazine                 | 100                                     | 50  |
| Sulfamonomethoxine                     | 100                                     | 50  |
| Sulfadoxine                            | 100                                     | 50  |
| Sulfaquinoxaline                       | 100                                     | 50  |
| Sulfadimethoxine                       | 100                                     | 50  |
| Tulathromycin marqueur                 | Banned <sup>a</sup>                     | 25  |
| Neospiramycin                          | 200 <sup>b</sup>                        | 100   |
| Spiramycin                             | 200 <sup>b</sup>                        | 100   |
| Tulathromycin                          | Banned <sup>a</sup>                     | 25  |
| Tilmicosin                             | 50                                      | 50  |
| Tylosin                                | 50                                      | 50  |
| Erythromycin                           | 40                                      | 40  |
| O-Acetyltylosin                        | / <sup>c</sup>                          | 25  |
| Josamycin                              | / <sup>c</sup>                          | 25  |
| Tylvalosin                             | / <sup>c</sup>                          | 25  |
| Amoxicillin                            | 4                                       | 4   |
| Ampicillin                             | 4                                       | 4   |
| Penicillin G (benzylpenicillin)        | 4                                       | 4   |
| Penicillin V (phenoxymethylpenicillin) | /                                       | 4   |
| Oxacillin                              | 30                                      | 30  |
| Cloxacillin                            | 30                                      | 30  |
| Nafcillin                              | 30                                      | 30  |
| Dicloxacillin                          | 30                                      | 30  |
| Cefquinome                             | 20                                      | 20  |
| Cefalonium                             | 20                                      | 20  |
| Cephapirin +desacetylcephapirin        | 60                                      | 30  |
| Cefoperazone                           | 50                                      | 50  |
| Cefazolin                              | 50                                      | 50  |
| Desfuroylceftiofur cystein disulfide   | 100                                     | 100   |
| Cephalexin                             | 100                                     | 100   |

<sup>a</sup> Banned for use in milk producing-animals

<sup>b</sup> MRL established for the parent drug + the metabolite

<sup>c</sup> No MRL in milk

Table Iib. EU-MRL and level of concentration for the validation of the analytes extracted by trichloroacetic acid

| Analyte                        | MRL in milk ( $\mu\text{g}/\text{kg}$ ) | Validation concentration (Cval) ( $\mu\text{g}/\text{kg}$ ) |
|--------------------------------|---|---|
| Spectinomycin                  | 200                                     | 200   |
| Streptomycin                   | 200                                     | 200   |
| Dihydrostreptomycin            | 200                                     | 200   |
| Kanamycin                      | 150                                     | 150   |
| Paromomycin                    | Banned <sup>a</sup>                     | 500   |
| Gentamycine C1, C1a and C2     | 100                                     | 100   |
| Neomycin                       | 1500                                    | 1500  |
| Apramycin                      | / <sup>c</sup>                          | 1000  |
| Lincomycin                     | 150                                     | 150   |
| Oxytetracycline (+ 4-epimer)   | 100                                     | 50  |
| Tetracycline (+ 4-epimer)      | 100                                     | 50  |
| Chlortetracycline (+ 4-epimer) | 100                                     | 50  |
| Doxycycline                    | / <sup>c</sup>                          | 100   |
| Marbofloxacin                  | 75                                      | 75  |
| Norfloxacin                    | / <sup>d</sup>                          | 100   |
| Danofloxacin                   | 30                                      | 30  |
| Ciprofloxacin                  | 100 <sup>b</sup>                        | 50  |
| Enrofloxacin                   | 100 <sup>b</sup>                        | 50  |
| Sarafloxacin                   | / <sup>c</sup>                          | 30  |
| Difloxacin                     | Banned <sup>a</sup>                     | 300   |
| Oxolinic acid                  | Banned <sup>a</sup>                     | 100   |
| Nalidixic acid                 | / <sup>d</sup>                          | 100   |
| Flumequine                     | 50                                      | 50  |

<sup>a</sup> Banned for use in milk producing-animals

<sup>b</sup> MRL established for the parent drug + the metabolite

<sup>c</sup> No MRL in milk

<sup>d</sup> No authorisation in veterinary medicine



Table III. Results of the validation for tetracyclines

| Analyte                 | Transition 1 « major » |        |             |                 | Transition 2 « minor » |        |             |                 |
|-------------------------|------------------------|--------|-------------|-----------------|------------------------|--------|-------------|-----------------|
|                         | Fm / T / B             | CCbeta | LOD (µg/kg) | Sensitivity (%) | Fm / T / B             | CCbeta | LOD (µg/kg) | Sensitivity (%) |
| Oxytetracycline         | Fm > T                 | < MRL  | < 1         | 100%            | Fm > T                 | < MRL  | 2.98        | 100%            |
| Tetracycline            | Fm > T                 | < MRL  | 1.41        | 100%            | Fm > T                 | < MRL  | 4.68        | 100%            |
| Chlortetracycline       | Fm > T                 | < MRL  | < 1         | 100%            | Fm > T                 | < MRL  | 1.47        | 100%            |
| Doxycycline             | Fm > T                 | < MRL  | 3.33        | 100%            | Fm > T                 | < MRL  | 3.04        | 100%            |
| 4-epi-oxytetracycline   | Fm > T                 | < MRL  | < 1         | 100%            | Fm > T                 | < MRL  | 1.67        | 100%            |
| 4-epi-tetracycline      | Fm > T                 | < MRL  | 2.22        | 100%            | Fm > T                 | < MRL  | 13.55       | 100%            |
| 4-epi-chlortetracycline | Fm > T                 | < MRL  | 1.05        | 100%            | Fm > T                 | < MRL  | 3.90        | 100%            |

Table IV. Results of the validation for penicillins

| Analyte       | Transition 1 « major » |        |             |                 | Transition 2 « minor » |        |             |                 | Transition 3 « minor » |       |             |                 |
|---------------|------------------------|--------|-------------|-----------------|------------------------|--------|-------------|-----------------|------------------------|-------|-------------|-----------------|
|               | Fm / T / B             | Cbeta  | LOD (µg/kg) | Sensitivity (%) | Fm / T / B             | Cbeta  | LOD (µg/kg) | Sensitivity (%) | Fm / T / B             | Cbeta | LOD (µg/kg) | Sensitivity (%) |
| Amoxicillin   | Fm < B                 | >Cval  | 1.74        | 81.7            | B < Fm < T             | >Cval  | 0.91        | 96.7            | B < Fm < T             | >Cval | 1.18        | 95.00           |
| Ampicillin    | Fm > T                 | < Cval | 0.08        | 100.0           | B < Fm < T             | >Cval  | 0.97        | 98.3            |                        |       |             |                 |
| Penicillin G  | Fm > T                 | < Cval | 0.14        | 100.0           | Fm > T                 | < Cval | 0.17        | 100.0           |                        |       |             |                 |
| Penicillin V  | Fm > T                 | < Cval | 0.63        | 100.0           | Fm = T                 | < Cval | 1.39        | 98.3            |                        |       |             |                 |
| Oxacillin     | Fm > T                 | < Cval | 0.14        | 100.0           | Fm > T                 | < Cval | 0.24        | 100.0           |                        |       |             |                 |
| Cloxacillin   | Fm > T                 | < Cval | 2.08        | 100.0           | Fm > T                 | < Cval | 0.16        | 100.0           |                        |       |             |                 |
| Nafcillin     | Fm > T                 | < Cval | 0.06        | 100.0           | Fm > T                 | < Cval | 0.17        | 100.0           |                        |       |             |                 |
| Dicloxacillin | Fm > T                 | < Cval | 0.43        | 100.0           | Fm > T                 | < Cval | 0.39        | 100.0           |                        |       |             |                 |