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**The stability of antibiotics in matrix and reference solutions determined using a straight-forward procedure applying mass spectrometric detection**

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Methods/Techniques:	Chromatography - LC/MS, Method validation
Additives/Contaminants:	Animal products, Veterinary drug residues - antimicrobials
Food Types:	Animal, Meat
Abstract:	<p>The stability of an antibiotic is a very important characteristic, especially in the field of antibiotic residue analysis. During method development or validation, the stability of the antibiotic has to be demonstrated no matter if the method is used for screening, confirmation, qualitative or quantitative analysis. A procedure for testing the stability of antibiotics in solutions and food samples using LC-MS/MS is described here.</p> <p>The procedure is based upon the assumption that the antibiotics are stable when stored at -70 °C. Representative solutions or spiked samples containing the antibiotic are stored at the temperature to be tested (-18 or 4 °C) and at -70 °C. After a selected storing time samples are moved from the chosen storage temperature to -70 °C. At the end of the study, all samples -per class of antibiotic- are analysed in one batch. By applying statistical models it is finally concluded at which circumstances the antibiotic is stable.</p> <p>The stability of 60 antibiotics belonging to the classes of tetracyclines, sulphonamides, quinolones, penicillins, macrolides and aminoglycosides are tested. The stability of solutions containing tetracyclines and penicillins is only guaranteed for three months while stored at -18 °C. Solutions of all other antibiotics tested are stable for at least 6 or 12 months when</p>

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3  
4 stored at 4 °C. In muscle tissue stored at -18 °C no severe degradation  
5 of the tested antibiotics was observed with the exception of the  
6 penicillins.

7 The presented stability data are useful as a reference for laboratories  
8 carrying out validation studies of analytical methods for antibiotic  
9 (residue) detection. It saves them time needed for long term stability  
10 testing of solutions and samples.  
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3 **1 The stability of antibiotics in matrix and reference solutions**  
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5 **2 determined using a straight-forward procedure applying mass**  
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7 **3 spectrometric detection**  
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## 18 Abstract

19 The stability of an antibiotic is a very important characteristic, especially in the field of  
20 antibiotic residue analysis. During method development or validation, the stability of the  
21 antibiotic has to be demonstrated no matter if the method is used for screening, confirmation,  
22 qualitative or quantitative analysis. A procedure for testing the stability of antibiotics in  
23 solutions and food samples using LC-MS/MS is described here. The procedure is based upon  
24 the assumption that the antibiotics are stable when stored at -70 °C. Representative solutions  
25 or spiked samples containing the antibiotic are stored at the temperature to be tested (-18 or 4  
26 °C) and at -70 °C. After a selected storing time samples are moved from the chosen storage  
27 temperature to -70 °C. At the end of the study, all samples -per class of antibiotic- are  
28 analysed in one batch. By applying statistical models it is finally concluded at which  
29 circumstances the antibiotic is stable. The stability of 60 antibiotics belonging to the classes  
30 of tetracyclines, sulphonamides, quinolones, penicillins, macrolides and aminoglycosides are  
31 tested. The stability of solutions containing tetracyclines and penicillins is only guaranteed for  
32 three months while stored at -18 °C. Solutions of all other antibiotics tested are stable for at  
33 least 6 or 12 months when stored at 4 °C. In muscle tissue stored at -18 °C no severe  
34 degradation of the tested antibiotics was observed with the exception of the penicillins. The  
35 stability data reported here are useful as a reference for laboratories carrying out validation  
36 studies of analytical methods for antibiotic (residue) detection. The data should save time  
37 needed for long term stability testing of solutions and samples.

38  
39 **Keywords:** stability, degradation, antibiotics, mass spectrometry

## 41 Introduction

42  
43 Nowadays, many antibiotics are used in animal production, particularly in intensive animal  
44 rearing like pigs, poultry and veal calves. For food safety and prevention of antibiotic  
45 resistance only the use of registered antibiotics is allowed and maximum residue limits  
46 (MRLs) in food products are established in EU/37/2010 (2010) to protect the consumer from  
47 being exposed to antibiotics. To monitor at these MRLs sensitive quantitative analytical  
48 methods are needed. In most cases, for the quantitative analysis, solutions of antibiotics are  
49 used as reference standards. To obtain a correct quantification, it is very important that  
50 knowledge is available on the stability of the antibiotic in the solution.

51 Next to the stability of the antibiotics in reference solutions it is also important to  
52 have knowledge regarding the stability of the antibiotic in the sample material. Only then  
53 suitable storage conditions can be chosen in case the analysis cannot take place immediately

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3 54 after sample collection. To obtain information on the stability of antibiotics in matrix a  
4 55 suitable quantitative analytical method has to be available. Additionally a statistical procedure  
5 56 for the data evaluation is mandatory.

7 57 It is obvious that characterisation of the method of analysis is very important. For that  
8 58 reason the validation of analytical testing methods is a primary requirement ISO 17025 (2005)  
9 59 accredited laboratories. Guidelines for the validation of the analysis of antibiotics in food  
10 60 matrices are established in EC/2002/657 (2002) and these state that for qualitative and  
11 61 quantitative methods, may it be either screening or confirmatory, the stability of the analyte in  
12 62 solution and in matrix are main characteristics to be determined. Analyte stability information  
13 63 has to be obtained either from experimental data or from literature. Unfortunately stability  
14 64 testing can be time-consuming and only limited literature is available about antibiotic stability.

15 65 Some approaches for stability testing of reference solutions and samples are reported  
16 66 (Okerman 2007, Croubels 2003, Jiménez 2004). Okerman *et al.* (2007) apply microbial  
17 67 techniques to determine the decrease in microbial activity over a period of six months.  
18 68 Croubels *et al.* (2003) recommend ultra violet (UV) detection for analytes possessing good  
19 69 UV absorbing properties because the between-day variation of the UV signal is low compared  
20 70 to mass spectrometric (MS) detection. Jiménez *et al.* (2004) applied several detection  
21 71 techniques among which UV and gas chromatography in combination with MS detection of  
22 72 trimethylsilyl derivatives. These techniques, but especially microbial inhibition and  
23 73 derivatization techniques, are non-specific and thus degradation products might not be  
24 74 distinguishable from the native compound. Liquid chromatography coupled to triple  
25 75 quadrupole mass spectrometry (LC-QqQ/MS) is more likely to discriminate degradation  
26 76 products from the native compound and is therefore the preferred detection technique if day to  
27 77 day variation is overcome. Another disadvantage of the reported approaches is that analyses  
28 78 are carried out on different occasions (Croubels 2003, Jiménez 2004). Therefore fresh  
29 79 reference solutions are to be prepared often or, in case of matrix stability testing, a sample  
30 80 preparation has to be carried out several times. This introduces additional errors and is less  
31 81 time and thus cost efficient.

32 82 The publically available information on the stability of reference solutions of  
33 83 veterinary drugs is limited (Okerman 2007, Mathijssen 2010, Chédru-Legros 2010). The  
34 84 availability of stability information of antibiotics in animal matrices is even worse (Verdon  
35 85 2000). According to 2002/657/EC (2002) the stability in matrix is preferably tested in  
36 86 incurred materials, but if these are not available the use of fortified materials is acceptable.

37 87 In this paper we present a straight-forward and time efficient procedure for stability  
38 88 testing of antibiotics in reference solutions and muscle matrix using LC-QqQ/MS as a specific  
39 89 detection technique. This method was applied to determine the stability of solutions of 60  
40 90 antibiotics used in veterinary practice belonging to the tetracycline (n=4), sulfonamide (n=16),

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3 91 quinolone (n=10), macrolide (n=11), penicillin (n=8) and aminoglycoside (n=11) group.  
4 92 Furthermore, the stability of a selection of 12 antibiotics, representative for different  
5 93 compound groups, in muscle matrix was studied. The results of this research can be used as a  
6 94 reference for the validation of testing methods aiming for the analysis of antibiotics.  
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95

## 96 **Material and methods**

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### 98 ***Chemicals, reagents and solutions***

99 HPLC grade methanol and acetonitrile (Biosolve, Valkenswaard, The Netherlands), 25% and  
100 32% ammonia, ammonium formate, formic acid, citric acid monohydrate, disodium  
101 hydrogenphosphate dihydrate, Na<sub>2</sub>EDTA, potassium dihydrogen phosphate, trichloro acetic  
102 adic, sodium hydroxide (Merck, Darmstadt, Germany), piperidine (>99%) and  
103 heptafluorobutyric acid (HFBA) (Sigma-Aldrich, St. Louis, MO, USA) were used. Milli-Q  
104 water was prepared using a Milli-Q system at a resistivity of at least 18 MΩ cm<sup>-1</sup>  
105 (Millipore, Billerica, MA, USA). EDTA-McIlvain buffer was prepared by mixing 500 mL  
106 citric acid solution (0.1 M, 21.0 g citric acid mono hydrate) with phosphate buffer (0.2 M,  
107 35.6 g Na<sub>2</sub>HPO<sub>4</sub> dihydrate in 1 L of water) until a pH of 4.0 is obtained. 74.4 g Na<sub>2</sub>EDTA is  
108 added and the volume adjusted to 2 L with water. Potassiumphosphatebuffer containing  
109 EDTA and trichloro acetic acid was prepared by dissolving 1.36 g KH<sub>2</sub>PO<sub>4</sub>, 0.15 g Na<sub>2</sub>EDTA  
110 and 20.0 g trichloro acetic acid in 1 L water.

111 The following reference standards (purity is mentioned between brackets) were all  
112 obtained from Sigma-Aldrich: *Tetracyclines*: oxytetracycline (99%), tetracycline (98%),  
113 chlortetracycline (93%) and doxycycline (99%). *Sulfonamides*: sulfadiazine (99.6%),  
114 sulfathiazole (100%), sulfapyridine (>99%), sulfamerazine (99.8%), sulfamoxole (98.5%),  
115 sulfadimidine (also called sulfamethazine or sulfadimerazine, 100%), sulfamethizole (99.9%),  
116 dapson (98.4%), sulfamethoxy pyridazine (99.6%), sulfamonomethoxine (98.5%),  
117 sulfachloropyridazine (99.4%), sulfadoxine (99.9%), sulfamethoxazole (100%), sulfisoxazole  
118 (99.6%), sulfadimethoxine (>99%) and sulfaquinolaxine (>91.6%). *Quinolones*:  
119 marbofloxacin (98.8%), norfloxacin (99%), ciprofloxacin (99.9%), enrofloxacin (99.6%),  
120 danofloxacin (99.9%), sarafloxacin (97.2%), difloxacin (99.8%), oxolinic acid (99%),  
121 nalidixic acid (99.4%) and flumequine (99%). *Penicillins*: amoxicillin (85.6%), ampicillin  
122 (96.8%), penicillin G (also called benzylpenicillin, 99.9%), penicillin V (also called  
123 phenoxymethylpenicillin, 98.3%), cloxacillin (94.7%), dicloxacillin (95.6%), nafcillin (87.6%)  
124 and oxacillin (89.2%). *Aminoglycosides*: apramycin (71.7%), dihydrostreptomycin (75.1%),  
125 gentamycin (including C1, C1A, C2 and C2A, 59.5%), kanamycin (77.7%), neomycin  
126 (82.5%), paromomycin (82.5%), spectinomycin (69.7%) and streptomycin (76%). *Macrolides*:

1  
2  
3 127 tilmicosin (83.7%), tiamulin (99.9%), erythromycin (65.7%), tylosin A (101.3%), valnemulin  
4 128 (>95%), josamycin (101.5%), lincomycin (93%) and spiramycin I (92.6%). Three other  
5  
6 129 macrolides were obtained elsewhere: tulathromycin (97.6%) from Pfizer (New York, NY,  
7  
8 130 USA), aivlosin (also called 3-acetyl-4'-isovaleryl tylosin, 67.9%) from Eco Animal health  
9  
10 131 (London, UK) and pirlimycin (90.3%) from Pharmacia and Upjohn Company (Bridgewater,  
11  
12 132 NJ, USA).

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17 136 concentration. Separate methanolic stock solutions were prepared at a concentration of 100  
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19 137 mg L<sup>-1</sup> of tetracyclines and penicillins and at a concentration of 1000 mg L<sup>-1</sup> for sulfonamides.  
20  
21 138 Separate stock solutions of 1000 mg L<sup>-1</sup> were prepared in acetonitrile for all macrolides and in  
22  
23 139 water for the aminoglycosides. Separate stock solutions of quinolones were prepared by  
24  
25 140 dissolving the analyte in 2M ammonia using sonification (30 min) to obtain a concentration of  
26  
27 141 5000 mg/L after which the solution was diluted to 100 mg L<sup>-1</sup> with methanol.

#### 142 143 ***Instrumentation***

144 The liquid chromatography (LC) instrumentation used was an Acquity UPLC system (Waters,  
145 Milford, MA, USA) and detection was carried out using a triple quadrupole Quattro Premier  
146 mass spectrometer (Waters) (QQQ/MS) operating with electrospray ionization (ESI) in  
147 positive mode. The operating parameters were: capillary voltage, 2.5 kV; cone voltage, see  
148 Table 1; source temperature, 120 °C; desolvation temperature, 300 °C; cone gas flow, 200 L  
149 hr<sup>-1</sup>; desolvation gas, 500 L hr<sup>-1</sup>. Data were acquired and processed using MassLynx 4.1  
150 software (Waters).

#### 151 152 ***LC-QQQ/MS analysis***

153 Several multi-compound methods were applied to determine the stability of the antibiotic  
154 substances. All analytical methods used for stability testing were fully validated as described  
155 in 2002/657/EC (2002) as a quantitative confirmatory method and fulfil the criteria  
156 established for trueness, repeatability, within-laboratory reproducibility, robustness and  
157 selectivity.

#### 158 159 ***Tetracyclines***

160 The analysis was carried out according to a previously described method (Berendsen 2006).

#### 161 162 ***Sulfonamides***

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3 163 Muscle (1 g) was extracted with 10 mL of water. After mechanical shaking and centrifugation  
4 164 the aqueous extract was filtered using a 30 kD ultrafilter (Millipore) and the filtrate was  
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6 165 transferred into a glass vial.

7  
8 166 Chromatographic separation was established using a Waters Acquity UPLC BEH C<sub>18</sub>  
9 167 analytical column, 50 x 2.1 mm, 1.7 μm. The gradient (mobile phase A, 5 mM formic acid in  
10 168 water; mobile phase B, 5 mM formic acid in water/acetonitrile (1:9, v/v), flow rate 0.8 mL  
11 169 min<sup>-1</sup>) was: 0-0.5 min, 0% B; 0.5-5.5 min, linear increase to 15% B; 5.5-6.6 min, linear  
12 170 increase to 25% B; 6.6-6.7 min, linear increase to 100% B; 6.7-7.2 min, 100% B; 7.2-7.3 min,  
13 171 return to 0% B. The flow was split 1:1 (MS:waste). The injection volume was 40 μL.  
14 172 Fragmentation was carried out using collision induced dissociation (CID) with the settings  
15 173 presented in Table 1.  
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#### 21 175 *Quinolones*

22 176 Muscle (1 g) was extracted with 10 mL of water. After mechanical shaking and centrifugation  
23 177 the aqueous extract was filtered using a 30 kD ultrafilter (Millipore) and the filtrate was  
24 178 transferred into a glass vial.

25  
26  
27 179 A chromatographic separation was established using a Waters Symmetry C<sub>18</sub>  
28 180 analytical column, 150 x 3 mm, 5 μm. The gradient (mobile phase A, 5 mM formic acid in  
29 181 water; mobile phase B, 5 mM formic acid in water/acetonitrile (1:9, v/v), flow rate 0.4 mL  
30 182 min<sup>-1</sup>) was: 0-1 min, 0% B; 1-11 min, linear increase to 100% B; 11-13 min, 100% B; 13-14  
31 183 min, return to 0% B. The injection volume was 50 μL. Fragmentation was carried out using  
32 184 CID with the settings presented in Table 1.  
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#### 38 186 *Macrolides*

39 187 Muscle (2 g) was extracted with 20 mL EDTA-McIlvain buffer. After mechanical shaking  
40 188 and centrifugation 10 mL of the aqueous extract was transferred onto a conditioned OASIS®  
41 189 MCX 3CC, 60 mg solid phase extraction cartridge (Waters). The cartridge was washed with 3  
42 190 mL MeOH / water / 32% ammonia (10:85:5 v/v) and dried under vacuum. The macrolides  
43 191 were eluted from the cartridge using 3 ml MeOH / 32% ammonia (95:5 v/v). The solvent was  
44 192 evaporated (45°C, N<sub>2</sub>) and the residue reconstituted in 700 μL 2% ACN in 10 mM  
45 193 ammonium formate buffer, adjusted to pH=4.0 with formic acid. The extract was transferred  
46 194 into a glass vial.

47  
48 195 A chromatographic separation was established using a Waters Atlantis dC<sub>18</sub> analytical  
49 196 column, 150 x 3 mm, 5 μm. The gradient (mobile phase A, 10 mM ammonium formate buffer,  
50 197 pH adjusted to 4.0 with formic acid; mobile phase B, 10 mM ammonium formate buffer in  
51 198 water/acetonitrile (1:9 v/v), adjusted to pH=4.0 using formic acid; flow rate 0.4 mL min<sup>-1</sup>)  
52 199 was: 0-1 min, 30% B; 1-17 min, linear increase to 100% B; 17-18 min, 100% B; 18-19 min,  
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200 return to 30% B. The flow was split 2:1 (MS:waste). The injection volume was 50  $\mu$ L.

201 Fragmentation was carried out using CID with the settings presented in Table 1.

202

### 203 *Penicillins*

204 Muscle samples were treated according to a previously reported method (van Holthoon 2010).

205 Before analysis 100  $\mu$ L of each mixed standard solution was transferred into a 4 mL glass

206 container. The solvent was evaporated under nitrogen at 45°C after which 1.0 mL 2%

207 piperidine (Sigma-Aldrich) solution in water was added to dissolve the residue. The

208 penicillins were derivatized to penicillin piperidines at room temperature during 30 minutes

209 after which 100  $\mu$ L 10% acetic acid (Merck, Darmstadt, Germany) in water and 2.0 mL 100

210 mM ammonium acetate buffer, pH=6.0, were added. These solutions were injected into the

211 previously reported LC-MS/MS system (van Holthoon 2010).

212

### 213 *Aminoglycosides*

214 Muscle (2 g) was extracted with 20 mL 10 mM potassiumphosphatebuffer pH=4.0 containing

215 EDTA and trichloroacetic acid. After mechanical shaking and centrifugation the pH of the

216 supernatant was adjusted to pH=7.7 using sodium hydroxide (30% in water). The aqueous

217 extract was transferred onto a conditioned CBX 6CC, 500 mg solid phase extraction cartridge

218 (Avantor, Phillipsburg, NJ, USA). The cartridge was washed with 4 mL water and dried under

219 vacuum. The aminoglycosides were eluted from the cartridge using 10% acetic acid in MeOH.

220 The solvent was evaporated (60°C, N<sub>2</sub>) and the residue reconstituted in 400  $\mu$ L 0.065%

221 HFBA in water. The extract was transferred into a glass vial.

222 A chromatographic separation was established using a Waters Symmetry C<sub>18</sub>

223 analytical column, 150 x 3 mm, 5  $\mu$ m. The gradient (mobile phase A, 0.065% HFBA in water;

224 mobile phase B, 0.065% HFBA in methanol; flow rate 0.4 mL min<sup>-1</sup>) was: 0-0.5 min, 0% B;

225 0.5-5.5 min, linear increase to 45% B; 5.5-17.0 min, linear increase to 60% B; 17-22 min,

226 60% B; 22-23 min, return to 0% B. The flow was split 1:1 (MS:waste). The injection volume

227 was 20  $\mu$ L. Fragmentation was carried out using CID with the settings presented in Table 1.

228 Using this procedure gentamycin C2 and C2A cannot be distinguished.

229

230 Note: please insert Table 1 here

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232

### 233 **Methodology**

#### 234 *Stability of antibiotics in solutions*

235 Of each stock solution of quinolones, macrolides and aminoglycosides 1 mL was transferred

236 into ten different glass containers. Of the sulfonamide stock solutions a mixed standard

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3 237 solution of 10 mg L<sup>-1</sup> in methanol was prepared of which 1 mL was transferred into ten  
4 238 different glass containers. For each solution at t=0, two containers were stored at -70 °C and 8  
5 239 at 4 °C. After 1, 3, 6 and 12 months each, two containers were moved from 4 °C to -70 °C.  
6  
7 240 After 12 months, all containers were placed at room temperature to warm up and the solutions  
8  
9 241 were diluted to suitable concentrations with water (aminoglycosides in 0.065% HFBA in  
10 242 water) in duplicate (I and II) to prevent overloading of the analytical system. This experiment  
11 243 is schematically presented in Figure 1. These solutions were analysed using LC-QqQ/MS in  
12 244 the following order: I t=0, I t=12, I t=6, I t=3, II t=0, II t=12, II t=6 and II t=3. This series was  
13 245 repeated four times resulting in five injections per dilution. For quinolones this experiment  
14 246 was carried out for a six month period only. For tetracyclines stock solutions and penicillins  
15 247 mixed standard solutions the experiment was carried out with transfer of containers at t=1  
16 248 week, t=2 weeks, t=1 month, t=2 months and t=3 months at both 4 °C and -18 °C because of  
17 249 expected instability.

20 250 After integration of the peaks, for each storage time, the average peak area of dilution  
21 251 I was compared with dilution II using a Students' t-test (n=5) to check if the dilutions were  
22 252 carried out correctly. If no statistical difference was observed between both dilutions the  
23 253 overall average peak area of dilution I and II was calculated (n=10) for each storage time.  
24 254 Next, the average of each storage time was compared to the average peak area of the solution  
25 255 stored at -70 °C from the beginning of the experiment (t=0), again using a Students' t-test. If  
26 256 the average peak area at a certain storage time is above 90 % of the average peak area at t=0  
27 257 the compound is considered stable for that specific storage time. If it drops below 90% the  
28 258 solution was considered to be unstable. If a difference between dilution I and II was found, a  
29 259 worst case scenario was adopted by using the most deviating average of dilution I or II in the  
30 260 calculations.

31 261 With this approach the instability of a compound is observed by comparing a signal  
32 262 obtained for a solution stored at -70 °C with a solution stored at 4 °C or -18 °C. It is assumed  
33 263 that the antibiotics tested are stable at -70 °C (BCR/01/97 1997), however even if an analyte  
34 264 is unstable at -70 °C a difference in signal is expected because it will by definition be more  
35 265 unstable at higher temperatures.

36 266  
37 267 Note: please insert Figure 1 here

38 268  
39 269 *Stability of antibiotics in matrix*

40 270 The stability of antibiotics in muscle matrix was studied using in-house reference material  
41 271 that was prepared for each compound group individually. For each compound group one or  
42 272 more representative substances were selected. A bulk of blank muscle material was fortified  
43 273 with the selected antibiotics at and around the MRL (EU/37/2010, 2010). Each of the

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3 274 materials was homogenized under cryogenic conditions and the homogeneity of the materials  
4 275 was tested according to The International Harmonized Protocol for Proficiency Testing of  
5 276 Analytical Laboratories (Thompson 2006) and ISO 13528 (2005), taking into account the  
6 277 insights discussed by Thompson (2000).

7  
8  
9 278 Just after preparation of the materials, 12 samples of each material were randomly  
10 279 selected of which 6 were stored at -70 °C and 6 at -18 °C. It is assumed that the antibiotics  
11 280 tested are stable at these storage conditions. After the selected test period all 12 samples were  
12 281 analysed in one batch in random order. After integration of the peaks, the overall average  
13 282 peak area and relative standard deviation (VC%, n=6) were calculated for each storage time  
14 283 and a Students' t-test was applied to test for statistical significant differences between the  
15 284 samples stored at -70 °C and the samples stored at -18 °C.

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## 21 286 **Results and discussion**

22  
23 287 With the presented approach, for the analysis of the stability of antibiotics in reference  
24 288 solutions and muscle matrix all analyses for a specific class of antibiotics are carried out  
25 289 within one day using LC-MS/MS as the detection technique. Therefore, additional variation  
26 290 due to day to day variation of the instrumentation is prevented. Because the applied detection  
27 291 technique is highly selective, interference of the detector's response caused by degradation  
28 292 products or other interferences is unlikely and only the response of the native compound is  
29 293 recorded. Therefore, this method allows straight-forward, effective and cost efficient stability  
30 294 testing.

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### 36 296 ***Stability in solution***

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38 297 The results of the stability study of all tested antibiotics in reference solutions, stored at 4 °C,  
39 298 are presented in Table 2. Stability results of the tetracycline and penicillin reference solutions,  
40 299 stored at -18 °C are presented in Table 3. The relative response is calculated by dividing the  
41 300 average response at t=1 by the average response at t=0. A compound is considered instable if  
42 301 the relative response drops below 90 %.

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47 303 Note: please insert Table 2 here

48 304 Note: please insert Table 3 here

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51  
52 306 It is observed that the results for the tetracyclines vary severely for the different storage times  
53 307 and as a result, for tetracycline at t=1 week and chlortetracycline at t=2 months a result is  
54 308 found below the established criterion whereas the next result is again above this criterion. As  
55 309 a result a higher uncertainty in the establishment of the maximum storage time is obtained and  
56 310 a conservative estimation should be made to prevent the use of degraded reference solutions.

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3 311 Nevertheless, it is clearly observed that especially oxytetracycline and tetracycline are  
4 312 instable in methanolic solutions which was also demonstrated before (Okerman 2007). This  
5 313 research clearly illustrates that tetracycline and oxytetracycline stock solutions should be  
6 314 prepared fresh on a weekly basis if they are stored at 4 °C and every two months (tetracycline)  
7 315 or every three months (other tetracyclines) if stored at -18 °C. The difference in stability of  
8 316 oxytetracycline and tetracycline compared to chlortetracycline and doxycycline is confirmed  
9 317 by Okerman *et al.* (2007). They reported no loss of activity for chlortetracycline and  
10 318 doxycycline during 6 months storage at -20 °C whereas oxytetracycline and tetracycline loose  
11 319 approximately 25% of their activity in the same period. It is noted that in our experiments the  
12 320 degradation of oxytetracycline and tetracycline is more severe. This difference might be  
13 321 explained due to the fact that Okerman *et al.* (2007) monitored the total antibiotic activity  
14 322 (this might include metabolites) instead of the level of the native compounds or because the  
15 323 concentration of the reference solutions were a factor 10 lower in our experiments.

16 324 Next to the tetracyclines, the penicillins show severe instability. During storage at 4  
17 325 °C at least a 45% degradation is observed within one week for all compounds. If these  
18 326 penicillin solutions are stored at -18 °C, the stability is much more prolonged: under these  
19 327 conditions ampicillin and penicillin V solutions are stable for at least two months, the other  
20 328 penicillin solutions for at least three months. This is not in agreement with the results of  
21 329 Okerman *et al.* (2007) who reported no degradation of aqueous ampicillin solutions for at  
22 330 least 6 months when stored at -20 °C and, in the contrary, approximately a 20% degradation  
23 331 of penicillin G and amoxicillin within three months. These differences might be explained due  
24 332 to the fact that they monitored the total antibiotic activity instead of the level of the native  
25 333 compounds, because the concentration of the reference solutions are a factor 10 lower in our  
26 334 experiments or because different solvents were used: Okerman *et al.* (2007) prepared stock  
27 335 solutions in water, whereas we tested the stability in methanolic solutions. The latter is most  
28 336 likely, because instability of penicillins in the presence of methanol was also reported by  
29 337 Mastovska *et al.* (2008). For oxacillin the results are in agreement with Mathijssen *et al.*  
30 338 (2010) who reported no degradation for aqueous oxacillin solutions during 6 months when  
31 339 stored at -20 °C.

32 340 The other tested antibiotics showed to be stable for the tested period of time at 4 °C  
33 341 indicating that quinolones are at least stable for 6 months and the other antibiotics for at least  
34 342 12 months.

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#### 36 344 ***Stability in matrix***

37 345 The results of the stability study of the antibiotics in matrix, stored at -18 °C are presented in  
38 346 Table 4.

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3 348 Note: please insert Table 4 here

4 349 Note: please insert Table 5 here

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7 351 From the results it is concluded that all tested antibiotics, with the exception of ampicillin and  
8 352 to less extend cloxacillin, are stable in muscle matrix when stored at -18 °C for the tested  
9 353 period of time. Verdon *et al.* (2000) indicated no degradation of amoxicillin in porcine muscle  
10 354 during storage at -20 °C for 3 months and severe degradation after 8 months of storage. From  
11 355 the results of the stability of solutions, for ampicillin and amoxicillin a similar degradation  
12 356 rate is expected and thus the quick degradation of ampicillin is unexpected. This observation  
13 357 might be explained by a between muscle variation and therefore we recommend to store  
14 358 samples containing penicillins at -70 °C even if the storage period is limited.

15 359 For the penicillin antibiotics the same experiment was carried out after stabilizing the  
16 360 muscle matrix by adding 2 mL phosphate buffer (0.2 M, pH=6) to 2 g minced muscle (van  
17 361 Holthoon 2010). The results of the stability study for the buffered muscle matrix containing  
18 362 ampicillin and cloxacillin are presented in Table 5. It is concluded that after addition of  
19 363 phosphate buffer pH=6 cloxacillin as well as ampicillin are stable in muscle matrix for at least  
20 364 3 months. This is in agreement with previously reported results by Kondrat'eva *et al.* (1967)  
21 365 and Lu *et al.* (2008) who showed prolonged stability of penicillin G, oxacillin and penicillin  
22 366 V solutions at pH=6 to 7. The stabilisation of muscle samples containing penicillins is  
23 367 recommended if no -70 °C storage capacity is available.

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### 25 369 **Conclusions**

26 370 A straight-forward procedure is reported for the stability testing of antibiotics in solution and  
27 371 in muscle samples using LC-MS/MS detection. The method proved to be straight-forward,  
28 372 effective and cost efficient. From this research stability information is obtained that can be  
29 373 used for the construction of a validation dossier. Severe instability at 4 °C was observed for  
30 374 oxytetracycline, tetracycline and all tested penicillin solutions only. When stored at -18 °C the  
31 375 stability of these solutions is prolonged; at least 2 months for tetracycline, ampicillin and  
32 376 penicillin V, and at least 3 months for oxytetracycline and the other tested penicillins.

33 377 Methanolic solutions of chlortetracycline and doxycycline are stable for at least 3 months  
34 378 when stored at 4 °C. Methanolic solutions of the tested sulfonamides, solutions of macrolides  
35 379 in acetonitrile and aqueous solutions of the aminoglycosides are stable for at least 12 months  
36 380 when stored at 4 °C. Alkaline methanolic solutions of the tested quinolones are stable for at  
37 381 least 6 months when stored at 4 °C.

38 382 The tested antibiotics remain stable in muscle matrix for at least 3 months with the  
39 383 exception of the penicillins. A significant degradation was observed within 3 months for  
40 384 ampicillin and cloxacillin when stored at -18 °C. After stabilizing these muscle samples at

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3 385 pH=6, the stability of the penicillins in matrix is prolonged to at least 3 months. Therefore it is  
4 386 recommended to store penicillin containing samples at -70 °C or to stabilize them at pH=6.

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6 387 From the work presented here, a huge amount of stability data is obtained. However,  
7 388 stability data is never complete, because numerous compound matrix combinations (including  
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9 389 all species) are to be studied and new antibiotic substances become available regularly.

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11 390 Therefore, we suggest that an open access, easy searchable database should be created in  
12 391 which stability data, gathered by various laboratories, can be stored.

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28 440 Figure 1. Schematic representation of the stability study.  
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31 442 Table 1. Precursor ions, collision energy and product ions of the antibiotics.  
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40 448 Table 4. Stability of antibiotics in muscle stored at -18 °C.  
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43 450 Table 5. Stability of penicillins in muscle stored at -18 °C after buffering at pH=6.  
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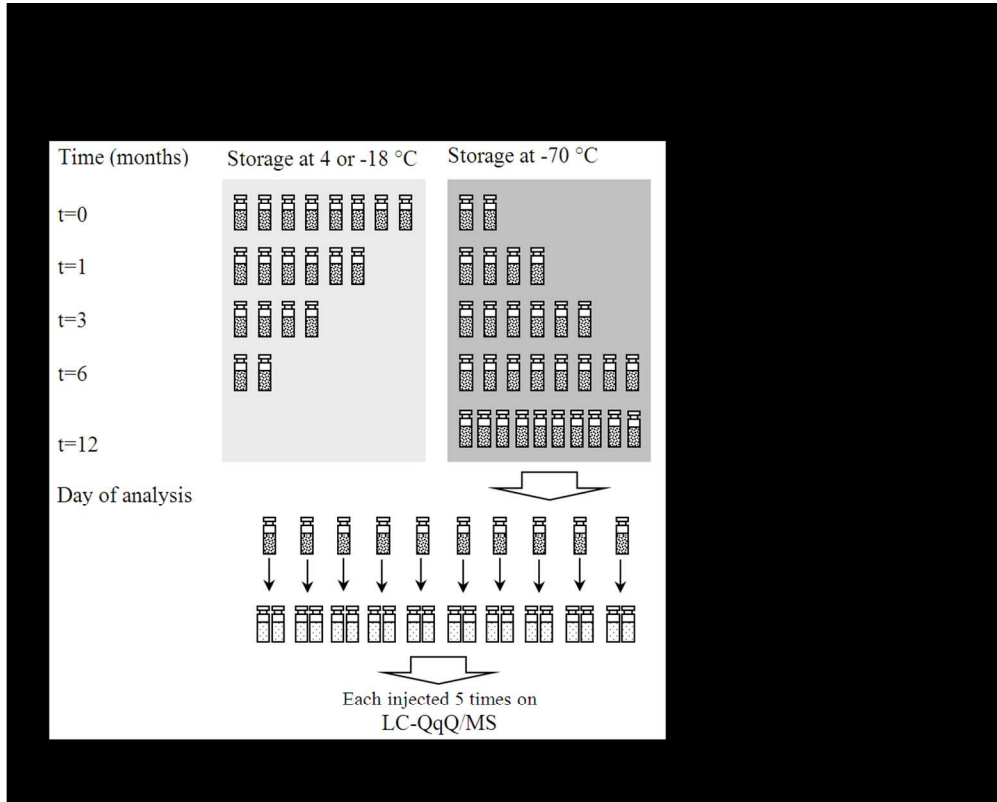


Figure 1. Schematic representation of the stability study.

Table 1. Precursor ions, collision energy and product ions of the antibiotics.

Compound	Cone (V)	Precursor ion (m/z)	Collision energy (eV)	Product ion (m/z)
<i>Sulfonamides</i>				
Sulfadiazine	24	251.2	15	92.2
Sulfathiazole	26	256.2	15	156.1
Sulfapyridine	26	250.2	30	92.2
Sulfamerazole	28	265.2	30	92.2
Sulfamoxole	23	268.2	15	156.1
Sulfadimidine	28	279.2	30	92.2
Sulfamethizole	24	271.1	15	156.1
Dapsone	30	249.2	15	156.1
Sulfamethoxypyridazine	25	281.2	15	156.1
Sulfamonomethoxine	30	281.2	20	156.1
Sulfachloropyridazine	23	285.2	15	156.1
Sulfadoxine	24	311.2	20	156.1
Sulfamethoxazole	22	254.2	25	92.2
Sulfisoxazole	21	268.2	15	156.1
Sulfadimethoxine	28	311.2	20	156.1
Sulfaquinoxaline	30	301.2	15	156.1
<i>Quinolones</i>				
Marbofloxacin	20	363.1	17	72.4
Norfloxacin	20	320.1	18	276.1
Ciprofloxacin	20	332.1	21	288.3
Enrofloxacin	20	360.2	20	316.1
Danofloxacin	20	358.2	35	340.1
Sarafloxacin	20	386.1	23	342.1
Difloxacin	20	400.1	21	356.2
Oxolinic acid	20	262.1	25	244.1
Nalidixic acid	20	233.1	20	215.2
Fumequine	20	262.1	24	244.1
<i>Macrolides</i>				
Tilmicosin	25	435.4	18	695.6
Tiamulin	25	494.3	28	192.2
Erythromycin	25	734.5	25	158.1
Tylosin A	40	916.6	32	174.2
Valnemulin	25	565.4	28	263.2
Josamycin	30	828.5	32	174.2
Lincomycin	28	407.2	21	126.0
Spiramycin I	20	422.4	20	100.9
Tulathromycin	20	403.9	18	158.1
Aivlosin	40	1042.7	42	108.9
Pirlimycin	25	411.2	19	112.0
<i>Aminoglycosides</i>				
Apramycin	25	540.3	20	378.2
Dihydrostreptomycin	35	584.3	30	263.2
Gentamycin C1	20	478.3	15	322.2
Gentamycin C1A	20	450.3	17	322.2
Gentamycin C2 or C2A*	20	464.3	17	322.2

Gentamycin C2 or C2A*	25	485.2	25	163.1
Kanamycin	30	615.3	35	161.1
Paromomycin	30	616.3	30	163.1
Spectinomycin*	30	351.2	25	100.0
Streptomycin	50	582.3	35	263.2

\* analysed as a hydrate salt.

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Table 2. Stability of antibiotics in solution stored at 4°C.

Compound	Relative response compared to t=0 (%)					Final concluded stability
	t=1w	t=2w	t=1m	t=2m	t=3m	
<b>Tetracyclines</b>						
Oxytetracycline	91	101	<b>45</b>	<b>85</b>	<b>74</b>	2 weeks
Tetracycline	<b>86</b>	93	<b>69</b>	<b>56</b>	<b>45</b>	< 1 week
Chlortetracycline	104	109	92	<b>85</b>	104	≥ 2 months
Doxycycline	107	119	94	102	114	≥ 3 months
<b>Penicillins</b>						
Amoxicillin	<b>31</b>	<b>11</b>				< 1 week
Ampicillin	<b>36</b>	<b>15</b>				< 1 week
Penicillin G	<b>49</b>	<b>27</b>				< 1 week
Penicillin V	<b>30</b>	<b>9</b>				< 1 week
Cloxacillin	<b>38</b>	<b>14</b>				< 1 week
Dicloxacillin	<b>29</b>	<b>9</b>				< 1 week
Nafcillin	<b>54</b>	<b>30</b>				< 1 week
Oxacillin	<b>42</b>	<b>19</b>				< 1 week
<b>Relative response compared to t=0 (%)</b>						
	t=1m	t=3m	t=6m	t=12m		
<b>Sulfonamides</b>						
Sulfadiazine	99	101	97	<b>98</b>		≥ 12 months
Sulfathiazole	100	101	102	<b>99</b>		≥ 12 months
Sulfapyridine	100	101	98	<b>101</b>		≥ 12 months
Sulfamerazole	100	100	98	<b>100</b>		≥ 12 months
Sulfamoxole	100	96	100	<b>95</b>		≥ 12 months
Sulfadimidine	100	101	100	<b>102</b>		≥ 12 months
Sulfamethizole	101	100	98	<b>101</b>		≥ 12 months
Dapsone	102	102	99	<b>102</b>		≥ 12 months
Sulfamethoxypyridazine	101	102	100	<b>101</b>		≥ 12 months
Sulfamonomethoxine	101	103	99	<b>101</b>		≥ 12 months
Sulfachloropyridazine	101	104	101	<b>102</b>		≥ 12 months
Sulfadoxine	99	101	99	<b>101</b>		≥ 12 months
Sulfamethoxazole	102	102	98	<b>101</b>		≥ 12 months
Sulfisoxazole	100	100	97	<b>99</b>		≥ 12 months
Sulfadimethoxine	98	99	94	<b>99</b>		≥ 12 months
Sulfaquinoxaline	100	101	100	<b>100</b>		≥ 12 months
<b>Quinolones</b>						
Marbofloxacin			<b>97</b>			≥ 6 months
Norfloxacin			<b>100</b>			≥ 6 months
Ciprofloxacin			<b>98</b>			≥ 6 months
Enrofloxacin			<b>102</b>			≥ 6 months
Danofloxacin			<b>106</b>			≥ 6 months
Sarafloxacin			<b>100</b>			≥ 6 months
Difloxacin			<b>101</b>			≥ 6 months
Oxolinic acid			<b>102</b>			≥ 6 months
Nalidixic acid			<b>101</b>			≥ 6 months
Fumequine			<b>103</b>			≥ 6 months
<b>Aminoglycosides</b>						
Apramycin	97	95	100	<b>98</b>		≥ 12 months

Dihydrostreptomycin	96	98	100	<b>99</b>	≥ 12 months
Gentamycin C1	99	98	99	<b>100</b>	≥ 12 months
Gentamycin C1A	97	97	99	<b>99</b>	≥ 12 months
Gentamycin C2 or C2A*	99	99	100	<b>98</b>	≥ 12 months
Gentamycin C2 or C2A*	98	98	100	<b>98</b>	≥ 12 months
Kanamycin	100	99	99	<b>99</b>	≥ 12 months
Neomycin	101	100	100	<b>99</b>	≥ 12 months
Paromomycin	99	93	99	<b>98</b>	≥ 12 months
Spectinomycin	102	98	97	<b>99</b>	≥ 12 months
Streptomycin	96	99	92	<b>94</b>	≥ 12 months

*Macrolides*

Tilmicosin			96	<b>97</b>	≥ 12 months
Tiamulin			114	<b>105</b>	≥ 12 months
Erythromycin			103	<b>112</b>	≥ 12 months
Tylosin A			98	<b>98</b>	≥ 12 months
Valnemulin			108	<b>105</b>	≥ 12 months
Josamycin			109	<b>104</b>	≥ 12 months
Lincomycin			99	<b>98</b>	≥ 12 months
Spiramycin I			102	<b>109</b>	≥ 12 months
Tulathromycin			110	<b>100</b>	≥ 12 months
Aivlosin			116	<b>110</b>	≥ 12 months
Pirlimycin			92	<b>96</b>	≥ 12 months

w = weeks, m=months

The results below 90 % are indicated in bold.

\* Gentamycin C2 and C2A cannot be distinguished with the applied methodology.

Table 3. Stability of antibiotics in solution stored at -18°C.

Compound	Relative response compared to t=0 (%)					Final concluded stability
	t=1w	t=2w	t=1m	t=2m	t=3m	
<b>Tetracyclines</b>						
Oxytetracycline	101	111	96	107	116	≥ 3 months
Tetracycline	109	103	104	95	<b>87</b>	2 months
Chlortetracycline	99	117	106	97	100	≥ 3 months
Doxycycline	115	111	111	104	109	≥ 3 months
<b>Penicillins</b>						
Amoxicillin	95	110	92	91	91	≥ 3 months
Ampicillin	95	109	93	93	<b>89</b>	2 months
Penicillin G	95	107	96	98	94	≥ 3 months
Penicillin V	90	110	90	95	<b>88</b>	2 months
Cloxacillin	95	108	96	98	96	≥ 3 months
Dicloxacillin	95	109	97	98	94	≥ 3 months
Nafcillin	95	106	98	100	96	≥ 3 months
Oxacillin	92	107	94	98	94	≥ 3 months

w = weeks, m=months

The results below 90 % is indicated in bold.

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Table 5. Stability of penicillins in muscle stored at -18 °C after buffering at pH=6.

Compound group	Compound	Species	t (days)	Level [VC%] ( $\mu\text{g}/\text{kg}$ )		Stable?
				Day 0	Day t	
Penicillins	Ampicillin	Porcine	76	4.5 [15]	5.0 [6.0]	Yes
	Cloxacillin	Porcine	76	149 [9.1]	148 [3.0]	Yes

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