Determination of β-Lactam-Antibiotics in animal matrices
by means of LC/MS-MS
1. **PREVIOUS VERSION**

None, this version is original

2. **PURPOSE AND SCOPE OF APPLICATION**

2.1. **Purpose**

This test procedure describes extraction of β-Lactam-Antibiotics from animal matrices and their qualitative and quantitative determination by means of LC/MS-MS. The maximum residue limits (MRLs) of β-Lactam-Antibiotics are listed in Annex, table 1 of Commission Regulation (EU) 37/2010.

2.2. **Parameters tested**

**Penicillins:**
- Amoxicillin (AMOX) CAS-No.: 26787-78-0
- Ampicillin (AMP) CAS-No.: 69-53-4
- Cloxacillin (CLX) CAS-No.: 61-72-3
- Dicloxacillin (DCX) CAS-No.: 3116-76-5
- Nafcillin (NAF) CAS-No.: 147-52-4
- Oxacillin (OXA) CAS-No.: 66-79-5
- Penicillin G (Benzylpenicillin, PENG) CAS-No.: 61-33-6
- Penicillin V (Phenoxyethylpenicillin, PENV) CAS-No.: 87-08-1

**Cephalosporins:**
- Cefalexin (CFX) CAS-No.: 15686-72-2
- Cefalonium (CFL) CAS-No.: 5575-21-3
- Cefapirin (CFP) CAS-No.: 21593-23-7
- Cefazolin (CFZ) CAS-No.: 25963-19-9
- Cefoperazon (CPZ) CAS-No.: 62893-19-0
- Cefquinom (CFQ) CAS-No.: 84957-30-2
- Ceftiofur (CFT) CAS-No.: 80370-57-6

2.3. **Sample type**

Eggs, milk and animal tissue (muscle, kidney)

3. **METHOD**

3.1. **Principle**

β-Lactam-Antibiotics are extracted from animal matrices and, after HPLC separation, determined by means of mass spectrometry.

3.2. **Brief description of method**

- Extraction of β-Lactam-Antibiotics from animal matrices with acetonitrile
- Defattening with n-hexane
- Measurement by means of LC-MS/MS (ESI negative)
4. TERMS, ABBREVIATIONS AND SYMBOLS USED

ISTD: internal standard

\( g \) (during centrifuging): symbol for normal acceleration of free fall (\( g = 9.80655 \))

rpm: rotations per minute (centrifuge)

MS: mass spectrometry

LC-MS: liquid chromatography-mass spectrometry

ESI: electrospray ionization

5. WARNINGS AND SAFETY INSTRUCTIONS

Organic solvents are potentially dangerous. All operations must be carried out such that no inhalation of the vapors or contact with the skin occurs.

The corrosive potential of acids and alkalis must be kept in mind when handling them. Significant changes in pH or neutralizations must be conducted slowly to avoid a violent reaction.

Follow the safety data sheets!

6. EQUIPMENT AND APPARATUS

The company names given are indicative of the type and quality of the products. Products from other manufacturers can be used if they meet the requirements.

In addition to common laboratory equipment, the following instruments and apparatus are required:

6.1. Equipment

- Balance
- pH meter
- Transfer pipette
- Shaker
- Evaporator
- Centrifuge (with cooling)
- Ultrasonic bath (e.g. Transsonic Digital S, Fa. Elma)
- HPLC device (e.g. Agilent Technologies 1200 Series)
- MS device (e.g. Applied Biosystems 4000 Q-Trap LC/MS-MS)

6.2. Apparatus

5.2.1. Consumables

- 15 ml Greiner vials
- Plastic HPLC samples vials with insert
- Syringe filter (e.g. Millex HV 13/0,45 PVDF)
- 1,5 ml Eppendorf cups
5.2.2. Disposable supplies

- HPLC column: ODS Hypersil 200 x 2,1 mm; 5 µm (Thermo, UK)
- Precolumn: ODS Hypersil 10 x 2,1 mm; 5 µm (Thermo, UK)

7. REAGENTS, SOLUTIONS AND TEST ORGANISMS

The requirements in SVA_CC_VIE_TAHO_003 (Ordering, labeling, storing, and handling of chemicals and reference substances) must be observed.

7.1. Standard and reference substances

<table>
<thead>
<tr>
<th>Substance</th>
<th>Abbreviation</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>AMOX</td>
<td>Sigma 108H0647</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>AMP</td>
<td>Sigma 106H0169</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>CLX</td>
<td>Fluka 317157/1 992</td>
</tr>
<tr>
<td>Dicloxacillin</td>
<td>DCX</td>
<td>Sigma 21H0576</td>
</tr>
<tr>
<td>Nafcillin</td>
<td>NAF</td>
<td>Sigma 40H04791</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>OXA</td>
<td>Sigma 69F0629</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>PENG</td>
<td>Sigma 99H0647</td>
</tr>
<tr>
<td>Penicillin V</td>
<td>PENV</td>
<td>Sigma 27H0701</td>
</tr>
<tr>
<td>Cefapirin</td>
<td>CFP</td>
<td>Fluka 1355659</td>
</tr>
<tr>
<td>Ceftiofur</td>
<td>CFT</td>
<td>Dr. Ehrenstorfer 50817</td>
</tr>
<tr>
<td>Ceftquinom</td>
<td>CFQ</td>
<td>Dr. Ehrenstorfer 70104</td>
</tr>
<tr>
<td>Cefalonium</td>
<td>CFL</td>
<td>Ref. Lab. Berlin 000218</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>CFZ</td>
<td>Fluka 1339058</td>
</tr>
<tr>
<td>Cefalexin</td>
<td>CFX</td>
<td>Fluka 1302830</td>
</tr>
<tr>
<td>Cefoperazon</td>
<td>CPZ</td>
<td>Sigma 056K0604</td>
</tr>
<tr>
<td>Internal Standard (deuterated)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penicillin G</td>
<td>PENG-d7</td>
<td>Sigma 32985</td>
</tr>
</tbody>
</table>

7.2. Chemicals and reagents

The company names are given mainly as indicative of the quality of the products. Substances or products from other manufacturers may be used if the required criteria are met.

- Acetonitrile, LiChrosolv (Merck 1.00030)
- Methanol, LiChrosolv (Merck 1.06018)
- n-Hexane, p.a. (Merck 1.04367)
- Ammonium acetate (Merck 1.16103)

7.3. Gases

From the central gas supply: Nitrogen 5.0 (for LC/MS)
7.4. Solutions/culture media

If not otherwise specified, an aliquot or a multiple of the specified batch can be manufactured from the solution with the required accuracy.

The requirements in SVA_CC_VIE_TAHO_004 (Handling of solutions) must be observed.

The following list of preparations for standards is only a recommendation. The requirements in SVA_CC_VIE_TAHO_006 (Guide to the handling of standard substances) must be observed.

7.4.1. Standard stock solutions (0.5 mg/mL)

Dissolve 5 mg substance (6.1.) [if necessary calculate for free substance and take purity into account!] in 10 mM methanolic ammonium acetate solution (6.4.12., Eluent A) in a 10 mL volumetric flask and fill up to mark (except Cefalonium: dissolve in methanol/water [1:1]). The solutions have a shelf life of 5 years at -20°C in the dark.

7.4.2. Internal Standard dilution V1 PENG-d7 for milk/egg/tissue (10 µg/mL)

Dilute the Standard stock solution (6.4.1) of substance PENG-d7 with acetonitrile obtaining the following concentration: Penicillin G-d7 10 µg/mL

The solution has a shelf life of 1 year at -20°C in the dark.

7.4.3. Standard dilution V1 Mix Pen & V1 Mix Ceph milk/egg (5 & 10 µg/mL)

Dilute the Standard stock solution (6.4.1.) of substances (AMOX, AMP, CLX, DCX, NAF, OXA, PENG, PENV, CFP, CFT, CFQ, CFL, CFZ, CFX and CPZ) with water obtaining the following concentrations:

<table>
<thead>
<tr>
<th></th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1 Mix Pen</td>
<td></td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>5 µg/mL</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>5 µg/mL</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>5 µg/mL</td>
</tr>
<tr>
<td>Dicloxacillin</td>
<td>5 µg/mL</td>
</tr>
<tr>
<td>Nafcillin</td>
<td>5 µg/mL</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>5 µg/mL</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>5 µg/mL</td>
</tr>
<tr>
<td>Penicillin V</td>
<td>5 µg/mL</td>
</tr>
<tr>
<td>V1 Mix-Ceph</td>
<td></td>
</tr>
<tr>
<td>Cefapirin</td>
<td>10,0 µg/mL</td>
</tr>
<tr>
<td>Ceftiofur</td>
<td>10,0 µg/mL</td>
</tr>
<tr>
<td>Cefquinom</td>
<td>10,0 µg/mL</td>
</tr>
<tr>
<td>Cefalonium</td>
<td>10,0 µg/mL</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>10,0 µg/mL</td>
</tr>
<tr>
<td>Cefalexin</td>
<td>10,0 µg/mL</td>
</tr>
<tr>
<td>Cefoperazon</td>
<td>10,0 µg/mL</td>
</tr>
</tbody>
</table>

The solutions have a shelf life of 1 week at -20°C in the dark.

7.4.4. Spiking standard V2 Mix milk/egg (0.2 and 1.0 µg/mL)
Dilute the Standard dilutions V1 Mix Pen and V1 Mix Ceph (6.4.3.) of substances (AMOX, AMP, CLX, DCX, NAF, OXA, PENG, PENV, CFP, CFT, CFQ, CFL, CFZ, CFX and CPZ) with water obtaining the following concentrations:

- Amoxicillin: 0.2 µg/mL
- Ampicillin: 0.2 µg/mL
- Cloxacillin: 0.2 µg/mL
- Dicloxacillin: 0.2 µg/mL
- Nafcillin: 0.2 µg/mL
- Oxacillin: 0.2 µg/mL
- Penicillin G: 0.2 µg/mL
- Penicillin V: 0.2 µg/mL
- Cefapirin: 1.0 µg/mL
- Ceftiofur: 1.0 µg/mL
- Cefquinom: 1.0 µg/mL
- Cefalonium: 1.0 µg/mL
- Cefazolin: 1.0 µg/mL
- Cefalexin: 1.0 µg/mL
- Cefoperazon: 1.0 µg/mL

The solutions have a shelf life of 1 week at -20°C in the dark.

**7.4.5. Spiking-Internal-Standard V2 milk/egg (0.6 µg/mL)**

Dilute the Internal Standard dilution V1 PENG-d7 (6.4.2) of substance PENG-d7 with water obtaining the following concentration: Penicillin G-d7 0.6 µg/mL. The solution has a shelf life of 1 week at -20°C in the dark.

**7.4.6. Calibration curve milk/egg**

This calibration curve is an example and can be adapted.

<table>
<thead>
<tr>
<th>Cal.- std.</th>
<th>Spik.-std. milk/egg (6.4.4.)</th>
<th>Spik. ISTD milk/egg (6.4.5.)</th>
<th>Dilute to:</th>
<th>Concentration in solution [µg/L]</th>
<th>Concentration in sample [µg/kg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15 µL</td>
<td>50 µL</td>
<td>600 µL each with 10 mM aqueous Ammonium-acetate-sol. (6.4.12.)</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>30 µL</td>
<td>50 µL</td>
<td></td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>60 µL</td>
<td>50 µL</td>
<td></td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>120 µL</td>
<td>50 µL</td>
<td></td>
<td>40</td>
<td>200</td>
</tr>
<tr>
<td>5</td>
<td>180 µL</td>
<td>50 µL</td>
<td></td>
<td>60</td>
<td>300</td>
</tr>
</tbody>
</table>

PEN = Penicillins: AMOX, AMP, CLX, DCX, NAF, OXA, PENG, PENV
CEF = Cephalosporins: CFP, CFT, CFQ, CFL, CFZ, CFX, CPZ

**7.4.7. Standard dilution V1 Mix tissue (2 - 200 µg/mL)**

Dilute the Standard stock solution (6.4.1.) of substances (AMOX, AMP, CLX, DCX, NAF, OXA, PENG, PENV, CFP, CFT, CFQ, CFL, CFZ, CFX and CPZ) with water obtaining the following concentrations:
Determination of β-Lactam-Antibiotics in animal matrices by means of LC/MS-MS

Amoxicillin 10,0 µg/mL
Ampicillin 2,0 µg/mL
Cloxacillin 2,0 µg/mL
Dicloxacillin 2,0 µg/mL
Nafcillin 2,0 µg/mL
Oxacillin 2,0 µg/mL
Penicillin G 2,0 µg/mL
Penicillin V 2,0 µg/mL
Cefapirin 2,0 µg/mL
Ceftiofur 5,0 µg/mL
Cefquinom 10,0 µg/mL
Cefalolinum 20,0 µg/mL
Cefazolin 5,0 µg/mL
Cefalexin 200,0 µg/mL
Cefoperazon 2,0 µg/mL

The solution has a shelf life of 1 week at -20°C in the dark.

7.4.8. Spiking standard V2 Mix tissue (0,2 - 20,0 µg/mL)

Dilute the Standard dilution V1 Mix (6.4.7.) of substances (AMOX, AMP, CLX, DCX, NAF, OXA, PENG, PENV, CFP, CFT, CFQ, CFL, CFZ, CFX and CPZ) with water obtaining the following concentrations:

Amoxicillin 1,0 µg/mL
Ampicillin 0,2 µg/mL
Cloxacillin 0,2 µg/mL
Dicloxacillin 0,2 µg/mL
Nafcillin 0,2 µg/mL
Oxacillin 0,2 µg/mL
Penicillin G 0,2 µg/mL
Penicillin V 0,2 µg/mL
Cefapirin 0,2 µg/mL
Ceftiofur 0,5 µg/mL
Cefquinom 1,0 µg/mL
Cefalolinum 2,0 µg/mL
Cefazolin 0,5 µg/mL
Cefalexin 20,0 µg/mL
Cefoperazon 0,2 µg/mL

The solution has a shelf life of 1 week at -20°C in the dark.

7.4.9. Calibration standard tissue (0,02 – 2 µg/mL)

Dilute the Standard dilution V1 Mix (6.4.7.) of substances (AMOX, AMP, CLX, DCX, NAF, OXA, PENG, PENV, CFP, CFT, CFQ, CFL, CFZ, CFX and CPZ) with water obtaining the following concentrations:
Determination of β-Lactam-Antibiotics in animal matrices by means of LC/MS-MS

Amoxicillin  0,10 µg/mL
Ampicillin  0,02 µg/mL
Cloxaciniln  0,02 µg/mL
Dicloxacillin  0,02 µg/mL
Nafcillin  0,02 µg/mL
Oxacillin  0,02 µg/mL
Penicillin G  0,02 µg/mL
Penicillin V  0,02 µg/mL
Cefapirin  0,02 µg/mL
Ceftiofur  0,05 µg/mL
Cefquinom  0,10 µg/mL
Cefalonium  0,20 µg/mL
Cefazolin  0,05 µg/mL
Cefalexin  2,00 µg/mL
Cefoperazon  0,02 µg/mL

7.4.10. Spiking-Internal-Standard tissue (0,8 µg/mL)

Dilute the Internal Standard dilution V1 PENG-d7 (6.4.2) of substance PENG-d7 with water obtaining the following concentration: Penicillin G-d7 0,8 µg/mL

The solution has a shelf life of 1 week at -20°C in the dark.

7.4.11. Calibration curve tissue

<table>
<thead>
<tr>
<th>Cal.- std.</th>
<th>Cal.-std. tissue (6.4.9)</th>
<th>Cal. ISTD tissue (6.4.10)</th>
<th>Dilute to:</th>
<th>Concentration in solution [µg/L]</th>
<th>Concentration in sample [µg/kg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20 µl</td>
<td>50 µl</td>
<td>600 µL each with 10 mM aqueous Ammonium-acetate-sol. (6.4.12.)</td>
<td>PEN 0,67-3,33, 0,67-66,67</td>
<td>PEN 0,2-1, 0,2-20</td>
</tr>
<tr>
<td>2</td>
<td>100 µl</td>
<td>50 µl</td>
<td>3,33-16,67, 3,33-333, 33</td>
<td>CEF 1-5, 1-100</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>190 µl</td>
<td>50 µl</td>
<td>6,33-31,67, 6,33-633, 33</td>
<td>PEN 1,9-9,5, 1,9-190</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>280 µl</td>
<td>50 µl</td>
<td>9,33-46,67, 9,33-933, 33</td>
<td>CEF 2,8-14, 2,8-280</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>370 µl</td>
<td>50 µl</td>
<td>12,33-61, 67, 12,33-12, 33,33</td>
<td>PEN 3,7-18,5, 3,7-370</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>460 µl</td>
<td>50 µl</td>
<td>15,33-76, 67, 15,33-15, 33,33</td>
<td>CEF 4,6-23, 4,6-460</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>550 µl</td>
<td>50 µl</td>
<td>18,33-91, 67, 18,33-18, 33,33</td>
<td>PEN 5,5-27,5, 5,5-550</td>
<td></td>
</tr>
</tbody>
</table>

PEN = Penicilline: AMOX, AMP, CLX, DCX, NAF, OXA, PENG, PENV
Test Procedure  CC_VIE_TAHO_226   Version: 01

Competence Centre for Veterinary Drugs & Hormones (CC TAHO)

Determination of β-Lactam-Antibiotics in animal matrices by means of LC/MS-MS

CEF = Cephalosporine: CFP, CFT, CFQ, CFL, CFZ, CFX, CPZ

Annotation: This calibration curve is an example and can be adapted.

7.4.12. HPLC-Eluent (10 mM methanolic and aqueous ammonium acetate solutions)
Eluent A: dissolve 771 mg ammonium acetate in methanol and fill up to 1 L
Eluent B: dissolve 771 mg ammonium acetate in water and fill up to 1 L

7.5. Disposal
Organic solvents must be disposed of in the solvent waste canisters provided in the laboratories. The further details of the disposal system are established separately at the commercial site.

8. SAMPLING, SAMPLE PREPARATION, AND HANDLING
Sample collection is not performed by the testing laboratory.
Samples are homogenized, 2,0 ± 0,02 g filled into 15 mL Greiner vials and stored at -20°C until sample preparation.

9. IMPLEMENTATION

9.1. Preparing the control samples
The control samples are used to calculate the recovery rate and the current retention times and to determine the ion ratios (see 9.).
Spike suitable negative pool samples as follows:

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Concentration in the sample</th>
<th>Spiking standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>Penicillins: 5 µg/kg</td>
<td>25 µg/kg</td>
</tr>
<tr>
<td></td>
<td>Cephalosporins: 25 µg/kg</td>
<td>50 µl (6.4.4.)</td>
</tr>
<tr>
<td>Egg</td>
<td>Penicillins: 10 µg/kg</td>
<td>50 µg/kg</td>
</tr>
<tr>
<td></td>
<td>Cephalosporins: 50 µg/kg</td>
<td>100 µl (6.4.4.)</td>
</tr>
<tr>
<td>Tissue</td>
<td>Penicillins: 10-50 µg/kg</td>
<td>10-1000 µg/kg</td>
</tr>
<tr>
<td></td>
<td>Cephalosporins: 10-1000 µg/kg</td>
<td>100 µl (6.4.8.)</td>
</tr>
</tbody>
</table>

Add internal standard:

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Penicillin G-d7</th>
<th>Spiking internal standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>15 µg/kg</td>
<td>50 µl (6.4.5)</td>
</tr>
<tr>
<td>Egg</td>
<td>15 µg/kg</td>
<td>50 µl (6.4.5)</td>
</tr>
</tbody>
</table>
9.2. Sample preparation

9.2.1. Extraction
- Add 8 ml acetonitrile to samples and control samples
- shake them 10 min with overhead shaker
- centrifuge (5 min, 4000rpm)
- transfer supernatants to 15 ml Greiner vials

9.2.2. Defattening and preparation for measurement
- Add 4 ml n-hexane and shake 5 min with overhead shaker
- centrifuge (5 min, 4000rpm)
- remove the upper phase (n-hexane) and discard it
- add again 4 ml n-hexane and shake 5 min with overhead shaker
- centrifuge (5 min, 4000rpm)
- remove the upper phase (n-hexane) and discard it
- evaporate lower phase (acetonitrile) at 50°C with nitrogen
- dissolve residues in 600 µL 10 mM aqueous ammonium acetate solution (Eluent B, 6.4.12.) in Ultrasonic bath
- transfer into 1,5 ml Eppendorf cups and centrifuge (15 min, 13000rpm)
- transfer into HPLC vials (if necessary filtrate through syringe filter 0,45 µm)

9.3. Measurement/testing

Analyze the sample extracts in the HPLC sample vials e. g. under the following conditions:

| HPLC column: | ODS Hypersil 200 x 2,1 mm; 5 µm (Thermo, UK) |
| Precolumn:   | ODS Hypersil 10 x 2,1 mm; 5 µm (Thermo, UK) |
| Eluent:      | A: 10 mM methanolic ammonium acetate (6.4.12.) |
|              | B: 10 mM aqueous ammonium acetate (6.4.12.) |
| Gradient:    | 10 % A to 80% A in 15 min, then in 5 min to 100% A |
| HPLC-flow:   | 0,25 mL/min |
| column temperature: | 50°C |

Mass spectrometry detection:

LC/MS-MS: Agilent 1200 / API 4000 QTrap
Ionization mode: ESI negative

<table>
<thead>
<tr>
<th>Analyte</th>
<th>RT (min)</th>
<th>[M-H]- m/z</th>
<th>Fragment ions (Qualifier)</th>
<th>MS-Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>4,7</td>
<td>364</td>
<td>206, 223</td>
<td>Q1/Q3</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>9,0</td>
<td>453</td>
<td>167, 321</td>
<td>Q1/Q3</td>
</tr>
<tr>
<td>Cefalonium</td>
<td>9,7</td>
<td>457</td>
<td>367, 180</td>
<td>Q1/Q3</td>
</tr>
<tr>
<td>Cefalexin</td>
<td>10,4</td>
<td>346</td>
<td>189, 268</td>
<td>Q1/Q3</td>
</tr>
</tbody>
</table>

Tissue  | 20 µg/kg | 50 µl (6.4.10)
10. EVALUATION

10.1. Evaluation/calculation

Use the corresponding software (e.g. Analyst) to perform qualitative and quantitative evaluation on the obtained measurement results:

- Identify the signals (peaks) using the reaction times and ion traces.
- Integrate the peaks to calculate the area values.
- Create the calibration curve related to the internal standard (dihydrostreptomycin) from the results of the calibration standard (6.5.12) and calculate the associated concentrations. Save the results.
- Print the results of the analysis ("report").
- Transfer the data into a Microsoft Excel template for further calculations.

Using Microsoft Excel, carry out the additional calculations below:

- Relative retention times and retention time deviations for the corresponding fragment ions;
- Ion ratios of the corresponding fragment ions for identification according to EU criteria (9.1.2);
- Determine recovery rates.

\[
\text{WFR (dot.Probe) } = \frac{c_{\text{dot.kalk}}}{c_{\text{dot.erw}}} \times 100 \%\]

\[
\text{WFR ...... recovery rate} \\
\text{c_{dot.kalk} ..... calculated concentration of the spiked control sample (measured) } [\mu g/kg] \\
\text{c_{dot.erw} ..... expected concentration of the spiked control sample (theoretical) } [\mu g/kg]
\]
- Calculate analyte content in the sample.

\[
c_{\text{analyte sample}} = \frac{c_{\text{pur kalk}}}{\text{WFR}} \quad [\mu g/kg \text{ tissue}]
\]

\(c_{\text{pur kalk}}\): calculated concentration sample (measured) [µg/kg]

Remark: Take the weighed amount of the sample and any potential dilution factors into account during calculation of the analyte content!

9.1.1. Evaluation screening

a) Check the recovery rates of standard solutions, control samples and samples:

aa) The recovery time of the analyte in question must correspond to the average retention time of the spiked control samples, at a tolerance of ± 5 %.
ab) The retention times of the corresponding fragment ions must correspond, at a tolerance of 0,2 min.
ac) The ratio of the retention time of the analyte in question to that of the suitable internal standard (i.e.: relative retention time) must correspond to that of the standard solutions or of the spiked control samples, at a tolerance of ± 5 %

Analytes that meet these criteria are considered as suspected positives. Otherwise, the analyte is “not detectable”.

b) Check the relative ion intensities:

The relative ion intensities of the corresponding fragment ions are calculated for all samples (calibration standards, control samples, samples) based on the ion trace with the highest intensity (greatest peak area). An analyte is then considered to be a “suspected positive” if the relative ion intensity of the sample lies within the following tolerance ranges, which are based on the mean ion ratios of the calibration standards and control samples:

<table>
<thead>
<tr>
<th>Relative ion intensity</th>
<th>Maximum permissible relative deviations (tolerance range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 50 %</td>
<td>± 40 %</td>
</tr>
<tr>
<td>&gt; 20 %-50 %</td>
<td>± 50 %</td>
</tr>
<tr>
<td>&gt; 10 %-20 %</td>
<td>± 60 %</td>
</tr>
<tr>
<td>≤ 10 %</td>
<td>± 80 %</td>
</tr>
</tbody>
</table>

If the relative ion intensities are outside these computed ranges, the analyte is considered “not detectable”.

c) Check the internal standards (if applicable): if a possible internal standard of the sample is found to be less than 20% of the mean concentration of the accompanying control
samples, or the ISTD is not detectable at all, the sample is classified as “not evaluable” and must be repeated. In doubt, consult the responsible supervisor.

d) Compare the analyte content (corrected for recovery rate) in the sample with the detection limit: If the analyte content is above the detection limit, the substance is considered a “suspected positive”; otherwise, the analyte is considered “not detectable”.

e) Check the analyte content with respect to the calibration range:
If the analyte content is higher than the highest calibration standard, the measurement must be repeated with a suitable dilution. If necessary, a new partial sample must be extracted and spiked with a correspondingly higher amount of a possible internal standard, so that the concentration is consistent with the required dilution. If in doubt, consult the responsible supervisor.

If Dihydrostreptomycin and/or streptomycin is detected in a sample during screening (“suspected positive”), the further procedure must be clarified with the responsible superior (possibly repetition of the extraction to ensure reliability). In any case, the measurement results must be evaluated according to 9.1.2.

If a sample is evaluated as “not evaluable,” weigh a new aliquot of the sample and repeat the analysis.

If in doubt, the specific procedure should be clarified with the responsible supervisor.

9.1.2. Evaluation confirmation

a) Check the retention times of standard solutions, control samples and samples:

aa) The retention time of the analyte in question must agree with the mean retention time of the spiked control sample within a tolerance of ±5%.

ab) The retention time of the corresponding fragment ions must agree within a tolerance of 0.2 min.

ac) The relationship of the retention time for the analyte in question and that of an appropriate internal standard (i.e., relative retention time) must correspond to that of the standard solutions or the spiked control samples within a tolerance of ±2.5%.

b) Check the relative ion intensities:
For the correct identification of an analyte, the ion ratios of the corresponding fragment ions must be compared with those of the standards or control samples and lie within an established tolerance limit. According to the Commission Decision 2002/657/EC the following tolerances are permitted:
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c) Check the internal standards (if applicable):
The concentration of a possible internal standard of the sample in question must be higher than 20 % of the average concentration of control samples.
d) The analyte content in the sample must be above its detection limit (see Section 10).
e) The measured value must be within the calibration range.

A substance is considered to be confirmed if all of the listed criteria are met. The mean value for analyte content in parallel samples is used in the presentation of quantitative results.

If one of the three criteria specified under a), b), and d) is not met, then the substance is considered “not detectable” providing that criteria c) and e) are met.

Conversely, if criterion c) is not met, further procedures must be clarified with the responsible supervisor.

If criterion e) is not met, a suitable dilution of the measurement solution must be carried out. If necessary, a new partial sample must be extracted and spiked with a correspondingly higher amount of an internal standard (if applicable), so that the concentration is consistent with the required dilution.

If in doubt, consult the responsible supervisor.

10.2. Documentation

At a minimum, the Test Report must contain:
- Name(s) or initial(s) of the analyst(s) (preparing samples and measurement)
- Date of the sample preparation and measurement;
- List of tested samples (with clearly attributable sample names or numbers)
- List of standards used and control samples
- Raw data of the samples and control samples
- Results of the samples
- Any deviations from the test procedure.

10.3. Presentation of results

- Analyte not detectable: “ND (LOD: ... µg/kg)”
- Analyte detectable, however, less than detection limit: “detectable < LOQ (LOD: ... µg/kg; LOQ: ... µg/kg)”
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- Content of the detected analyte is quantifiable: "xx.y µg/kg  (LOD: ... µg/kg; LOQ: ... µg/kg)"

11. VALIDATION

The results of the validation are available in electronic form at:
L:\\taho\\QM\\Validierung\\b-Lactame_lcmsms

12. REFERENCES

12.1. Scientific references
- CRL Fougères Workshop 10.-11.10.2007: „Training course on multi-antimicrobial screening by LC/MS-MS”; Location: B/1.79

12.2. Standards, laws and guidelines

Location: in electronic form at: L:\\taho\\QM\\Gesetzliche Grundlagen

12.3. QM documents and document templates
- SVA_CC_VIE_TAHO_003 (Ordering, labeling, storing, and handling of chemicals and reference substances)
- SVA_CC_VIE_TAHO_004 (Handling of solutions)
- SVA_CC_VIE_TAHO_006 (Guide to handling of standard substances)
- SVA_CC_VIE_TAHO_005 (Preparation of control samples)
- PV Vorlage TAHO dot_QMT_PV_01

13. APPENDICES

None

14. DISTRIBUTION

QM-coding: PV_CC_VIE_TAHO_226_01 Status: Valid as of: 06.05.2010
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| Minimum distribution list for information regarding this test procedure: | Information format |
|---|---|---|---|
|   | Paper | IT | Training |
| Institute Director for the respective institute |  | X |  |
| Department Head(s) of the respective department(s) |  |  |  |
| Head of the implementing center or competence center |  | X |  |
| Staff performing the test procedure or parts of it | X | X |  |