Determination of Streptomycin in Honey by means of LC/MS-MS
0 PREVIOUS VERSION
None, this version is original

2. PURPOSE AND SCOPE OF APPLICATION
2.1. Purpose
The antibiotic streptomycin is used as a pesticide against the bacterial disease commonly referred to as fire blight, which affects apples, pears and quince in fruit cultivation. The bees collecting nectar are able to transfer the disease from the blossom to the honey. The purpose of this method is the determination of streptomycin in honey.

2.2. Parameter(s) tested
Streptomycin

2.3. Sample type
Honey

3. METHOD
3.1. Principle
Streptomycin is extracted from the honey, is purified by means of solid-phase extraction (SPE) and, after HPLC separation, is determined by means of mass spectrometry.

3.2. Brief description of method
- Extraction of streptomycin from the honey with water
- Extract purification by means of solid-phase extraction (WCX)
- Measurement by means of LC-MS/MS (ESI positive)

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
CCα: decision limit
CCβ: detection capability
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
- Overhead shaker
- Centrifuge (for 50 mL Greiner vials)
- Centrifuge (e.g. Heraeus Biofuge fresco)
- 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
- SPE vials (Waters Sep-Pak Vac 6cc Accell Plus CM cartridges Part No. WAT054545)
- 50 mL Greiner vials
- 1.5 mL Eppendorf cups
- Plastic HPLC samples vials with insert

5.2.2. Disposable supplies
- HPLC column: Atlantis HILIC Silica 3 µm, 2.1 x 100 mm (Waters)
- Precolumn: Atlantis HILIC Silica 3 µm, 2.1 x 100 mm (Waters)
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
- Streptomycin sulfate (Sigma S-6501)
- Dihydrostreptomycin sesquisulfate (Sigma D-7253) is used as an internal standard.

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.
- Acetic acid p.a. (“glacial acetic acid” Merck 1.00063)
- Methanol HPLC Grade (Merck 1.06018)
- Acetonitrile (Merck LiChrosolv 1.00030)
- Ammonium formiate (Sigma Aldrich 156264)
- Formic acid 98 %−100% (Riedel de Haen 33015)

7.3. Test organisms
None

7.4. Gases
From the central gas supply:
- Nitrogen 5.0 (for LC/MS)
- Helium 5.0 (for LC/MS)

7.5. 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.
7.5.1. Standard stock solutions (1 mg/mL)

7.5.1.1. Streptomycin stock solution (1 mg/mL)

Dissolve 13.16 mg streptomycin sulfate (6.1.) in 0.1 % acetic acid (6.5.6.) in a 10 mL volumetric flask and fill up to mark.
The solution has a shelf life of four weeks.

7.5.1.2. ISTD stock solution (1 mg/mL)

Dissolve 13.75 mg dihydrostreptomycin sesquisulfate (6.1.) in 0.1 % acetic acid (6.5.6.) in a 10 mL volumetric flask and fill up to mark.
The solution has a shelf life of four weeks.

7.5.2. Streptomycin working solution (1 µg/mL)

Fill 10 µL stock solution (6.5.1.1.) with 0.1 % acetic acid (6.5.6.) in a 10 mL volumetric flask.
The solution has a shelf life of one week.

7.5.3. ISTD working solution (10 µg/mL)

Fill 100 µL ISTD stock solution (6.5.1.2.) with 0.1 % acetic acid (6.5.6.) in a 10 mL volumetric flask.
The solution has a shelf life of one week.

7.5.4. Streptomycin solution (0.1 µg/mL)

Pipette 1 mL streptomycin working solution (6.5.2.) into a 10 mL volumetric flask and fill up with 0.1 % acetic acid (6.5.6).

7.5.5. ISTD solution (1 µg/mL)

Pipette 1 mL streptomycin working solution (6.5.3.) into a 10 mL volumetric flask and fill up with 0.1 % acetic acid (6.5.6).
The solution has a shelf life of one week.

7.5.6. Acetic acid 0.1 %

Fill up 1 mL glacial acetic acid with water to make up 1 L.
The solution has a shelf life of 3 months.

7.5.7. Acetic acid 2%

Fill up 20 mL glacial acetic acid with water to make up 1 L.
The solution has a shelf life of 3 months.
7.5.8. Elution solution (2 % acetic acid/acetonitrile = 80/20)
Mix 160 mL 2 % acetic acid (6.5.7.) with 40 mL acetonitrile.

7.5.9. Eluent A (200 mM ammonium formiate, 100 mM formic acid in H₂O)
Dissolve 6.31 g ammonium formiate in distilled water, add 1.88 mL conc. formic acid and fill with water to make up 500 mL.
The solution has a shelf life of one week.

7.5.10. Eluent B (100 mM formic acid in acetonitrile)
Fill up 1.88 mL formic acid with acetonitrile to make up 500 mL.
The solution has a shelf life of one week.

7.5.11. Matrix solution
For each sequence, extract a negative honey sample at the same time (do not add ISTD!).
Use the eluent of this sample to prepare the calibration series.

7.5.12. Standard solutions for the calibration curve

<table>
<thead>
<tr>
<th>Level</th>
<th>Concentration of streptomycin (in ng/mL standard solution or in µg/kg honey)</th>
<th>Streptomycin solution (0.1 µg/mL) (6.5.4)</th>
<th>ISTD solution (1 µg/mL) (6.5.5)</th>
<th>Matrix solution (6.5.11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.2 ng/mL (=0.2 µg/kg)</td>
<td>2 µL</td>
<td>+</td>
<td>988 µL</td>
</tr>
<tr>
<td>2</td>
<td>0.5 ng/mL (=0.5 µg/kg)</td>
<td>4 µL</td>
<td>+</td>
<td>985 µL</td>
</tr>
<tr>
<td>3</td>
<td>1 ng/mL (= 1 µg/kg)</td>
<td>10 µL</td>
<td>+</td>
<td>980 µL</td>
</tr>
<tr>
<td>4</td>
<td>2 ng/mL (= 2 µg/kg)</td>
<td>20 µL</td>
<td>+</td>
<td>970 µL</td>
</tr>
<tr>
<td>5</td>
<td>5 ng/mL (= 5 µg/kg)</td>
<td>50 µL</td>
<td>+</td>
<td>940 µL</td>
</tr>
<tr>
<td>6</td>
<td>10 ng/mL (= 10 µg/kg)</td>
<td>100 µL</td>
<td>+</td>
<td>890 µL</td>
</tr>
<tr>
<td>7</td>
<td>20 ng/mL (= 20 µg/kg)</td>
<td>200 µL</td>
<td>+</td>
<td>790 µL</td>
</tr>
</tbody>
</table>

In all standard solutions, the concentration of ISTD is 10 ng/mL, which corresponds to 10 µg/kg honey.

7.6. 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.

The honey samples received are filled into 50 mL Greiner vials into 10 g aliquots for preparing samples and are stored at room temperature until analysis.

9. IMPLEMENTATION

For each sequence, extract a negative honey sample at the same time (do not add ISTD!). Use the eluent of this sample to prepare the calibration series (=matrix solution 6.5.11).

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>DOT</th>
<th>1 µg/kg</th>
<th>10 µL</th>
<th>Streptomycin working solution 1 µg/mL (6.5.2.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOT</td>
<td>5 µg/kg</td>
<td>50 µL</td>
<td></td>
</tr>
</tbody>
</table>

9.2. Preparing samples

9.2.1. Extraction

- Treat all samples and control samples with 10 µL ISTD working solution (6.5.3.).
- Add 40 mL water and shake 30 min with the overhead shaker
- Centrifuge (3000 rpm) [Note: Do not centrifuge at > 3000 rpm because this can damage (deform, break) the Greiner vials!]

9.2.2. Solid phase extraction WCX

- Condition column with 2 x 5 mL 2 % acetic acid (6.5.7)
- Wash with 2 x 5 mL water (do not allow to run dry)
- Transfer the entire centrifuged extract on the column
- Wash the column with 2 x 5 mL water
- Elute into a 10 mL volumetric flask with 2 x 5 mL elution solution (6.5.8)
- Fill up volumetric flask with acetonitrile to 10 mL
- Centrifuge 1 mL of the eluent at 13000 g
- Transfer centrifuged solution into a plastic HPLC vial

9.3. Measurement/testing

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

HPLC column: Atlantis HILIC Silica 3 µm, 2.1 x 100 mm (Waters)
Precolumn: Atlantis HILIC Silica 3 µm, 2.1 x 100 mm (Waters)
Eluent: 
Eluent A (200 mM ammonium formiate, 100 mM formic acid in H₂O) (6.5.9.)
Eluent B (100 mM formic acid in acetonitrile) (6.5.10.)
Gradient:
0 min: 10% A, 90% B
6 min: 60% A, 40% B
10 min: 60% A, 40% B
10.1 min: 10% A, 90% B
20 min: 10% A, 90% B

Injection volume: 20 µL
HPLC flow: 0.3 mL/min
column temperature: 30 °C

Mass spectrometry detection:

Ionization mode: ESI positive
Streptomycin: m/z: 582 → 263; 582 → 246
Dihydrostreptomycin: m/z: 584 → 263

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.

\[
WFR \text{ (dot.Probe)} = \frac{c_{\text{dot kalk}}}{c_{\text{dot erw}}} \times 100 \% \\
WFR \quad \text{...... recovery rate} \\
c_{\text{dot kalk}} \quad \text{.... calculated concentration of the spiked control sample (measured) [µg/kg]} \\
c_{\text{dot erw}} \quad \text{.... expected concentration of the spiked control sample (theoretical) [µg/kg]}
\]

- Calculate analyte content in the sample.

\[
c(\text{analyte sample}) = \frac{c_{\text{pur kalk}}}{WFR} \quad [\text{µg/kg honey}] \\
c_{\text{pur kalk}} \quad \text{.... calculated concentration sample (measured) [µg/kg]}
\]

**Remark 1:** Take the weighed amount of the sample and any potential dilution factors into account during calculation of the analyte content!

**Remark 2:** Since the calibration curve was created with matrix standards (6.5.11, 6.5.12) and all results are related to the internal standard (relative matrix calibration), a recovery rate correction is not necessary!

### 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:
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If the relative ion intensities are outside these computed ranges, the analyte is considered "not detectable".

c) Check the internal standards:
   If the internal standard (dihydrostreptomycin) 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. If 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 as 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 an internal standard (dihydrostreptomycin) so that the concentration is consistent with the required dilution. If in doubt, consult the responsible supervisor.

If 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:

<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>± 20 %</td>
</tr>
<tr>
<td>&gt; 20 %-50 %</td>
<td>± 25 %</td>
</tr>
<tr>
<td>&gt; 10 %-20 %</td>
<td>± 30 %</td>
</tr>
<tr>
<td>≤ 10 %</td>
<td>± 50 %</td>
</tr>
</tbody>
</table>

c) Check the internal standards:
The concentration of the internal standards (dihydrostreptomycin) 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 (dihydrostreptomycin) 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)
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Competence Centre for Veterinary Drugs & Hormones (CC TAHO)

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- 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)"
- 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\Streptomycin-Honig-lcmsms(QQQ).

Detection and quantitation limit of streptomycin (calibration curve method):

- Detection limit (CCα): 0.41 µg/kg
- Detection capability (CCβ): 0.50 µg/kg
- Quantitation limit: 1.36 µg/kg

Recovery rates (spiked samples):

The indicated values refer to undiluted extracts:

Streptomycin, relative (related to ISTD): 98 %

For quantifying samples for which an extract dilution is required, an analogous dilution of the control samples is needed in order to guarantee that the recovery rate has been taken into account properly.

12. REFERENCES

12.1. Standards, laws and guidelines

- Council regulation (EC) No 396/2005 (on maximum residue levels of pesticides in or on food and feed of plant and animal origin)
**Test Procedure CC_VIE_TAHO_230**  
Version: 01

**Competence Centre for Veterinary Drugs & Hormones (CC TAHO)**

**Determination of Streptomycin in Honey by means of LC/MS-MS**


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

### 12.2. 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

| 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 competency center | X | |
| Staff performing the test procedure or parts of it | X | X |

QM coding: PV_CC_VIE_TAHO_230_01  
Status: Valid as of: July 1, 2008  
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