**TEST PROCEDURE**

**Determination of Nitrofuran Metabolites in Animal Matrices, Baby Food, Honey and Sealant Materials by means of LC/MS-MS**

<table>
<thead>
<tr>
<th>Valid in</th>
<th>LSV/THKS/TAMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Valid for</td>
<td>all employees</td>
</tr>
<tr>
<td>Managed by</td>
<td>LSV/THKS/TAMI</td>
</tr>
</tbody>
</table>

**Purpose**

According to Commission Regulation (EU) No 37/2010, Table 2, no nitrofuran residues may be present in food-producing animals and foods of animal origin. Nitrofurans are rapidly converted to metabolites in living organisms, and thus generally can only be detected in that form. According to the Commission Decision 2002/657/EC, mass spectroscopic evidence of metabolites in animals constitutes valid proof of the illegal use of nitrofurans. An MRLP (minimum required performance limit) of 1 µg/kg has been established for poultry and aquaculture products by the Commission Decision 2002/657/EC.

**Parameter(s) tested**

- 3-amino-5-morpholinomethyl-2-oxazolidone (AMOZ) (furaltadone metabolite)
- 3-amino-2-oxazolidone (AOZ) (furazolidone metabolite)
- 1-aminohydantoin (AHD) (nitrofurantoin metabolite)
- Semicarbazide (SEM) (nitrofurazone metabolite)

**Matrix**

- Animal tissue (e.g., liver, muscle, kidneys, products from aquaculture)
- Eggs and egg powder
- Baby food
- Honey
- Milk and milk powder
- Sealant materials for canning lids

**Overview**

<table>
<thead>
<tr>
<th>Sample preparation for</th>
<th>Section</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal tissue:</td>
<td>8.2 / page 6</td>
</tr>
<tr>
<td>Baby food:</td>
<td>8.3 / page 8</td>
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<tr>
<td>Egg/egg powder:</td>
<td>8.4 / page 9</td>
</tr>
<tr>
<td>Honey:</td>
<td>8.5 / page 11</td>
</tr>
<tr>
<td>Milk/milk powder:</td>
<td>8.6 / page 12</td>
</tr>
<tr>
<td>Can lid sealant material:</td>
<td>8.7 / page 13</td>
</tr>
</tbody>
</table>

1 **Previous version**

Screening and confirmation of nitrofuran metabolites in animal matrices, baby food, honey and sealant materials by means of LC/MS-MS; PV_CC_VIE_TAHO_203_04

1.1 **Changes since the previous version**

- Change in the title of the test procedure
- Section 8.3: specification of the instrumental measurement method;
- Section 9.1: specification of the file name of the Microsoft Excel template used for the interim calculations;
- Formal changes through transfer of the content into the current document format;
- Slight changes in the sample preparation.

2 Method

2.1 Principle
Since nitrofurans bind to proteins with a half-life of several hours, the metabolites must first be liberated through mild acid hydrolysis. After derivatization with nitrobenzaldehyde and extraction from the matrix, the nitrofuran metabolites are determined by LC/MS-MS.

2.2 Brief description of method
Animal tissue, baby food: Interfering substances and extractable free nitrofuran residues are removed through repeated extractions with methanol and ethanol, the bound metabolites are then liberated through hydrolysis with hydrochloric acid, derivatized with nitrobenzaldehyde, and extracted from the matrix with ethyl acetate. After concentration by evaporation and taking up in water, an extraction with n-hexane is done to remove the derivatization reagent (nitrobenzaldehyde), and finally the analysis is carried out using HPLC and mass spectroscopic detection (LC/MS-MS).

All other matrices: The nitrofuran metabolites are liberated through hydrochloric acid hydrolysis, derivatized with nitrobenzaldehyde, and extracted from the matrix with ethyl acetate. After concentration by evaporation and taking up in water, an extraction with n-hexane is performed to remove the derivatization reagent (nitrobenzaldehyde), and finally the analysis is carried out using LC/MS-MS.

3 Terms, abbreviations and symbols used

g (in centrifugation): symbol for normal gravitational acceleration \( g = 9.80655 \, \text{m/s}^2 \)
rpm: rotations per minute

4 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.
Follow the safety data sheets!

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Hazard Class</th>
<th>Flammability</th>
<th>Environment Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>Toxic</td>
<td>Highly flammable</td>
<td></td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>Harmful to health</td>
<td>Highly flammable</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td></td>
<td>Highly flammable</td>
<td></td>
</tr>
<tr>
<td>n-Hexane</td>
<td>Harmful to health</td>
<td>Highly flammable</td>
<td>Hazardous to the environment</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>Irritant</td>
<td>Highly flammable</td>
<td></td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Acid</th>
<th>Corrosive</th>
<th>Follow the safety Data Sheets!</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrochloric acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetic acid</td>
<td>Corrosive</td>
<td>Follow the safety data sheets!</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>Corrosive</td>
<td></td>
</tr>
</tbody>
</table>
5 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:

5.1 Instruments
- Meat grinder (e.g., Moulinette)
- Balance (e.g., Mettler PM 4000)
- Vibrofix vortexer (e.g., IKA VF 1)
- Piston stroke pipettes
- Ultrasonic bath (e.g., Sonorex Super RK 510)
- Centrifuge (e.g., Cryofuge M7000)
- Rotary shaker (e.g., Heidolph)
- Nitrogen evaporator (e.g., Pierce Reacti-Therm III)
- pH Meter (e.g. Orion 420A)
- Water bath shaker (e.g., J ulabo SW20C)
- Cooling system (e.g., Lauda UKT 1000)
- HPLC system (Agilent Technologies 1200 Series)
- Mass spectrometer (Applied Biosystems 4000 Q-Trap)

5.2 Apparatus
- Plastic Pasteur pipettes
- PP test tubes, 12 mL (Greiner)
- PP test tubes, 15 mL (Greiner)
- PP test tubes, 50 mL (Greiner)
- HPLC vials
- HPLC column: XTerra MS C18, 3.5 µm, 150×3 mm (Waters)

6 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.

6.1 Standard and reference substances

The suppliers indicated guarantee the required quality for standards. Standards from other manufacturers can also be used if they have the same minimum quality.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Abbrev.</th>
<th>CAS no.</th>
<th>Supply source</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-amino-5-morpholinomethyl-2-oxazolidone</td>
<td>AMOZ</td>
<td>139-91-3</td>
<td>EU-RL Fougères or Witega</td>
</tr>
<tr>
<td>3-amino-2-oxazolidone</td>
<td>AOZ</td>
<td>67-45-8</td>
<td>EU-RL Fougères or Witega</td>
</tr>
<tr>
<td>1-aminohydantoin</td>
<td>AHD</td>
<td>67-20-9</td>
<td>EU-RL Fougères or Witega</td>
</tr>
<tr>
<td>Semicarbazide (hydrochloride)</td>
<td>SEM</td>
<td>59-87-0</td>
<td>Sigma S-4125</td>
</tr>
<tr>
<td>3-amino-5-morpholinomethyl-2-oxazolidone-d₅</td>
<td>AMOZ-d₅</td>
<td></td>
<td>EU-RL Fougères or Witega</td>
</tr>
<tr>
<td>3-amino-2-oxazolidone-d₄</td>
<td>AOZ-d₄</td>
<td></td>
<td>EU-RL Fougères or Witega</td>
</tr>
<tr>
<td>1-aminomidazolidin-2,4-dione-[2,4,5-¹³C]</td>
<td>AHD-1S</td>
<td></td>
<td>Witega</td>
</tr>
<tr>
<td>1,2-[¹⁵N₂, ¹³C] aemicarbazide (hydrochloride)</td>
<td>SEM-1S</td>
<td></td>
<td>Witega</td>
</tr>
</tbody>
</table>
6.2 Chemicals and reagents
The company names are given mainly as indicative of the quality of the products. Substances and products from other manufacturers can be used if they fulfill the required criteria.

- Acetonitrile LiChrosolv (Merck 14291)
- Dimethyl sulfoxide p.a. (Merck 2931)
- Acetic acid (glacial acetic acid) 100% p.a. (Merck 1.00063)
- Ethanol p.a. (Merck 1.00983)
- Ethyl acetate p.a. (RdH 33211)
- n-Hexane p.a. (Merck 4367)
- Methanol p.a. (RdH 32213)
- Sodium dihydrogen phosphate monohydrate (Merck 6346)
- Sodium hydroxide (Merck 6498)
- 2-nitrobenzaldehyde (Sigma 6001)
- Hydrochloric acid 37% (Merck 317)

6.3 Gases
- Nitrogen (nitrogen generator)

6.4 Solutions/ Culture media
Unless otherwise specified, a portion or a multiple of the given preparation for the solutions and culture media can be made 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.

Remarks: “water” always means deionized water (ultrapure water).
For all steps in which water is used, this must be ultrapure water.

6.4.1 Hydrochloric acid

1 M (for animal tissue, baby food);
0.2 M (for eggs, honey, milk, lid sealant materials)

1 M: 8.4 mL hydrochloric acid 37% is carefully added to 80 mL of deionized water, and the volume is made up to 100 mL (or use pre-made Titrisol 1M hydrochloric acid);
0.2 M: 1M hydrochloric acid is diluted with deionized water in a 1:4 ratio.

6.4.2 Nitrobenzaldehyde solution, 100 mM
Dissolve 0.0302 g of 2-nitrobenzaldehyde in 2 mL dimethyl sulfoxide (DMSO).

6.4.3 Sodium phosphate solution, pH 7.40.1 M (for animal tissue, baby food);
0.3 M (for eggs, honey, milk, lid sealant materials)

0.1 M: dissolve 1.38 g NaH2PO4•H2O in 90 mL water, adjust the pH with NaOH, and make up to 100 mL with water.
The buffer can be stored in a refrigerator for 1 month.
0.3 M: dissolve 4.26 g NaH2PO4•H2O in 90 mL water, adjust the pH with NaOH, and make up to 100 mL with water.
The buffer can be stored in a refrigerator for 1 month.

6.4.4 Sodium hydroxide

1 M: Carefully dissolve 2 g sodium hydroxide in water, and make up to 50 mL.
2 M: Carefully dissolve 4 g sodium hydroxide in water, and make up to 50 mL.
10 M: Carefully dissolve 20 g sodium hydroxide in water, and make up to 50 mL.

6.4.5 Single standard stock solutions (1 mg/mL)
Dissolve the corresponding standard substance (AMOZ, AOZ, AHD, SEM) in methanol to a concentration of 1 mg/mL.
These solutions can be stored in the dark in a freezer for 5 years.

6.4.6 Mixture standard (1 µg/mL)
Dilute 50 µL each of the single standard stock solutions (6.4.5) with methanol to 50 mL (volumetric flask).
This solution can be stored in the dark in a freezer for 2 years.

6.4.7 Mixture standard working solution (20 ng/mL)
200 µL of the mixture standard (6.4.6) is made up to 10 mL with methanol.
This solution can be stored in the dark in a freezer for 6 months.

6.4.8 Mixture standard working solution (4 ng/mL)
40 µL of the mixture standard (6.4.6) is made up to 10 mL with methanol.
This solution can be stored in the dark in a freezer for 6 months.

6.4.9 Internal standard stock solutions (1 mg/mL)
Dissolve the corresponding standard substance (AMOZ-d5, AOZ-d4, AHD-IS, SEM-IS) in methanol to a concentration of 1 mg/mL.
These solutions can be stored in the dark in a freezer for 5 years.

6.4.10 Internal mixture standard (10 µg/mL)
Dilute 100 µL portions of each of the single standard stock solutions (6.4.9) with methanol to 10 mL (volumetric flask).
This solution can be stored in the dark in a freezer for 2 years.

6.4.11 Internal mixture standard working solution (40 ng/mL)
Make up 40 µL of the internal mixture standard (6.4.10) to the mark in a 10 mL volumetric flask with methanol.
This solution can be stored in the dark in a freezer for 6 months.

6.4.12 HPLC eluent A
Make up 1 mL of concentrated acetic acid to 1000 mL with water.

6.5 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.

7 Sampling, sample preparation, and handling
Sample collection is not performed by the testing laboratory.

Weighings:
- Muscle, animals/animal products from aquaculture, baby food: 2.0 g
- Liver, kidneys, eggs, honey, milk: 1.0 g
- Egg powder, milk powder: 0.2 g
- Lid sealant material: 1.0 g
Store all samples in a freezer until prepared for testing; bring these to room temperature prior to analysis.

For further information on sample preparation and storage, see SVA_CC_VIE_TAHO_002 (Sample receipt for the CC TAHO).

8 Implementation

Samples are routinely analyzed first by a simplified determination (screening). A positive screening result is confirmed at a minimum by conducting a determination in duplicate.

At least one positive control (spiked sample) is included for each analysis sequence. Control samples serve to ascertain the current retention time, determination of the ion ratios, the calculation of the recovery rates and as a quality control for the analysis process using control charts.

The requirements in SVA_CC_VIE_TAHO_005 (Preparation of control samples) must be observed.

Implementation of the analysis for various matrices is described in the following sections:

- Animal tissue: 8.2
- Baby food: 8.3
- Egg/egg powder: 8.4
- Honey: 8.5
- Milk/milk powder: 8.6
- Lid sealant material: 8.7

Implementation of the analysis is performed with multiple samples in parallel including the accompanying control samples, where a testing run of this type is identified in the Test Report by an ongoing extraction number. The measurement of the sample extract using LC-MS is done sequentially, and the calibration curve is created in each case from freshly derivatized standards.

8.1 Preparatory work

The preparatory work for the respective matrices (such as preparation of the control samples, addition of internal standards) is described in the corresponding sections (8.2 to 8.7).

8.2 Sample preparation for animal tissue (muscle, liver, kidneys, and aquaculture)

Weighings:
- Muscle, aquaculture: weigh out 2.0 g in 12 mL Greiner vials with an accuracy of at least ±0.1 g;
- Liver, kidneys: weigh out 1.0 g in 12 mL Greiner vials with an accuracy of at least ±0.1 g.

8.2.1 Extraction of interfering substances and extractable nitrofuran residues (animal tissue)

- The defrosted samples and negative control samples:
  Extract once with 6 mL of a methanol/water mixture (2:1);
  four times with 6 mL methanol;
  and twice with 6 mL of ethanol;
  After each step, mix with the vortexer;
  Allow to stand in the ultrasonic bath for 5 min;
Centrifuge under 2000 g at 4 °C for 10 min; 
Discard the supernatant;

Remarks: All solutions must be cooled to 4 °C for use.

- Remove the remaining extractant under a stream of nitrogen at room temperature (approx. 20 min).

8.2.2 Preparation of the calibration standards (animal tissue)

This calibration is valid for samples with a weight of 1 g. 
For samples weighing 2 g, a dilution factor of 0.5 is taken into account during the measurement.

For example, the 6 standards are prepared as follows:

<table>
<thead>
<tr>
<th>Hour</th>
<th>Concentration of the respective substance</th>
<th>Mixture standard working solution (6.4.8 or 6.4.7)</th>
<th>Amount</th>
<th>Concentration</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.025 µg/kg</td>
<td>12.5 µL</td>
<td>4 ng/mL</td>
<td>→</td>
<td>Each is pipetted into 4 mL water in a 12 mL Greiner vial</td>
</tr>
<tr>
<td>2</td>
<td>0.05 µg/kg</td>
<td>25 µL</td>
<td>4 ng/mL</td>
<td>→</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.1 µg/kg</td>
<td>50 µL</td>
<td>4 ng/mL</td>
<td>→</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.25 µg/kg</td>
<td>125 µL</td>
<td>4 ng/mL</td>
<td>→</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.5 µg/kg</td>
<td>50 µL</td>
<td>20 ng/mL</td>
<td>→</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1 µg/kg</td>
<td>100 µL</td>
<td>20 ng/mL</td>
<td>→</td>
<td></td>
</tr>
</tbody>
</table>

8.2.3 Preparation of the control standards (animal tissue)

The negative control sample previously prepared according to 8.2.1 is treated with the mixture standard working solution (6.4.7 or 6.4.8) and mixed using the vortexer. 
The amount is taken from the corresponding test protocol. 
Further treatment of the spiked control sample is done in parallel with the sample to be tested.

8.2.4 Hydrolysis and derivatization (animal tissue)

- Treat the standards, samples, and control samples with 25 µL of the internal mixture standard working solution (40 ng/mL AMOZ-d₅, AOZ-d₄, AHD-IS, SEM-IS; 6.4.11);
- Allow to stand for 10 min;
- Suspend the dry pellet (8.2.1) in 6 mL of water using the vortexer;
- Incubate the samples and standards in the ultrasonic bath for 5 min;
- + 0.5 mL 1M hydrochloric acid solution (6.4.1);
- + 25 µL 100mM nitrobenzaldehyde solution (6.4.2);
- Mix using the vortexer;
- Incubate in the water bath shaker at 37 °C overnight.

8.2.5 Extraction (animal tissue)

- Centrifuge the samples and control samples under 4000 g at 4 °C for 10 min;
- Separate the supernatant into a new Greiner vial;
- Buffer the supernatants with 0.5 mL 0.1M sodium phosphate (6.4.3);
- Add 1M NaOH (at first, in 50 µL portions),
  and adjust the pH to 6.7-7.3 (pH meter) through careful additions of 1M sodium hydroxide (6.4.4);
  [Remarks: remember to mix using the vortexer after adding NaOH!]
- Treat the suspension three times with 4 mL ethyl acetate;
  Mix using the vortexer after each step;
  Place on the rotary shaker for 10 min;
  Centrifuge under 4000 g at 4 °C for 10 min;
  Transfer the supernatant to a new Greiner vial using a Pasteur pipette;
- Concentrate the ethyl acetate extracts by evaporating under nitrogen at approx. 45 °C;
- Take up each of the residues in 225 µL 0.1% acetic acid (6.4.12);
- Treat this with 2 mL n-hexane;
  Mix using the vortexer;
  Centrifuge under 4000 g at 4 °C for 5 min;
- Suction off the hexane phases and discard;
- Remove the remainders by evaporating under a nitrogen stream at room temperature for 2 min;
- + 25 µL acetonitrile;
- Transfer to HPLC vials (filtering if necessary) (Measurement Solution).

8.3 Sample preparation for baby food

Weighings:
weigh out 2.0 g in 15 mL Greiner vials with an accuracy of at least ±0.1 g.

Remarks: when carrying out testing for semicarbazide residues caused by a possible migration of semicarbazide from the lid material into the baby food, omit step 8.3.1 and begin with step 8.3.2 immediately. If in doubt, consult with the competent supervisor.

8.3.1 Extraction of interfering substances and extractable nitrofuran residues
- The defrosted samples and negative control samples are treated:
  once with 6 mL of a methanol/water mixture (2:1);
  four times with 6 mL methanol;
  and twice with 6 mL of ethanol;
  Mix using the vortexer after each step;
  Allow to stand in the ultrasonic bath for 5 min;
  Centrifuge under 4000 g at 4 °C for 10 min;
  Discard the supernatant;

Remarks: All solutions must be cooled to 4 °C for use.
- Remove the remaining extractant under a stream of nitrogen at room temperature (approx. 20 min).

8.3.2 Preparation of the calibration standards (baby food)
This calibration is suitable for samples with a weight of 1 g.
For samples weighing 2 g, a dilution factor of 0.5 is taken into account during the measurement.

For example, the 6 standards are prepared as follows:

<table>
<thead>
<tr>
<th></th>
<th>Concentration of the respective substance</th>
<th>Mixture standard working solution (6.4.8 or 6.4.7)</th>
<th>Each is pipetted into 4 mL water in a 15 mL Greiner vial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hour 1</td>
<td>0.025 µg/kg</td>
<td>12.5 µL 4 ng/mL</td>
<td></td>
</tr>
<tr>
<td>Hour 2</td>
<td>0.05 µg/kg</td>
<td>25 µL 4 ng/mL</td>
<td></td>
</tr>
<tr>
<td>Hour 3</td>
<td>0.1 µg/kg</td>
<td>50 µL 4 ng/mL</td>
<td></td>
</tr>
<tr>
<td>Hour 4</td>
<td>0.25 µg/kg</td>
<td>125 µL 4 ng/mL</td>
<td></td>
</tr>
<tr>
<td>Hour 5</td>
<td>0.5 µg/kg</td>
<td>50 µL 20 ng/mL</td>
<td></td>
</tr>
<tr>
<td>Hour 6</td>
<td>1 µg/kg</td>
<td>100 µL 20 ng/mL</td>
<td></td>
</tr>
</tbody>
</table>

8.3.3 Preparation of the control samples (baby food)
Treat the negative control sample with the mixture standard working solution (6.4.7 or 6.4.8) and mix using the vortexer.
The amount is taken from the corresponding test protocol.
Further treatment of the spiked control sample is done in parallel with the sample to be tested.

8.3.4 Hydrolysis and derivatization (baby food)
- Treat the standards, samples, and control samples with 25 µL of the internal mixture standard working solution (40 ng/mL AMOZ-d₅, AOZ-d₅, AHD-IS, SEM-IS; 6.4.11);
- Allow to stand for 10 min;
- Suspend the dry pellet (8.3.1) in 4 mL of water using the vortexer;
- Incubate the samples and standards in the ultrasonic bath for 5 min;
- + 0.5 mL 1M hydrochloric acid solution (6.4.1);
- + 25 µL 100mM nitrobenzaldehyde solution (6.4.2);
- Mix using the vortexer;
- Incubate in the water bath shaker at 37 °C overnight.

8.3.5 Extraction (baby food)
- Centrifuge the samples and control samples under 4000 g at 4 °C for 10 min;
- Separate the supernatant into a new Greiner vial;
- Buffer the supernatants with 0.5 mL 0.1M sodium phosphate (6.4.3);
- Add 1M NaOH (at first, in 50 µL portions),
  and adjust the pH to 6.7-7.3 (pH meter) through careful additions of 1M sodium hydroxide (6.4.4);
  [Remarks: remember to mix using the vortexer after adding NaOH!]
- Treat the suspension three times with 4 mL ethyl acetate;
  Mix using the vortexer after each step;
  Place on the rotary shaker for 10 min;
  Centrifuge under 4000 g at 4 °C for 10 min;
  Transfer the supernatant to a new Greiner vial using a Pasteur pipette;
- Concentrate the ethyl acetate extracts by evaporating under nitrogen at approx. 45 °C;
- Take up each of the residues in 225 µL 0.1% acetic acid (6.4.12);
- Treat this with 2 mL n-hexane;
  Mix using the vortexer;
  Centrifuge under 4000 g at 4 °C for 5 min;
- Suction off the hexane phases and discard;
- Remove the remainders by evaporating under a nitrogen stream at room temperature for 2 min;
- + 25 µL acetonitrile;
- Transfer to HPLC vials (filtering if necessary) (Measurement Solution).

8.4 Sample preparation for eggs and egg powder

Weighings:
- Whole egg: weigh out 1.0 g of homogenized egg in 50 mL Greiner vials with an accuracy of at least ±0.1 g;
- Egg powder: weigh out 0.2 g in 50 mL Greiner vials with an accuracy of at least ±0.1 g, and reconstitute with 0.8 mL distilled water.

8.4.1 Preparation of the calibration standards (eggs, egg powder)
This calibration is suitable for samples with a weight of 1 g.
For samples weighing 0.2 g, a dilution factor of 5 is taken into account during the measurement. For example, the 6 standards are prepared as follows:

<table>
<thead>
<tr>
<th>Hour</th>
<th>Concentration of the respective substance</th>
<th>Mixture standard working solution (6.4.8 or 6.4.7)</th>
<th>Amount</th>
<th>Concentration</th>
<th>→</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.05 µg/kg</td>
<td>12.5 µL 4 ng/mL</td>
<td></td>
<td></td>
<td>→</td>
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<tr>
<td>2</td>
<td>0.1 µg/kg</td>
<td>25 µL 4 ng/mL</td>
<td></td>
<td></td>
<td>→</td>
</tr>
<tr>
<td>3</td>
<td>0.2 µg/kg</td>
<td>50 µL 4 ng/mL</td>
<td></td>
<td></td>
<td>→</td>
</tr>
<tr>
<td>4</td>
<td>0.5 µg/kg</td>
<td>125 µL 4 ng/mL</td>
<td></td>
<td></td>
<td>→</td>
</tr>
<tr>
<td>5</td>
<td>1 µg/kg</td>
<td>50 µL 20 ng/mL</td>
<td></td>
<td></td>
<td>→</td>
</tr>
<tr>
<td>6</td>
<td>2 µg/kg</td>
<td>100 µL 20 ng/mL</td>
<td></td>
<td></td>
<td>→</td>
</tr>
</tbody>
</table>

Each is pipetted into a 50 mL Greiner vial.

8.4.2 Preparation of the control samples (eggs, egg powder)

Treat the negative control sample with the mixture standard working solution (6.4.7 or 6.4.8) and mix using the vortexer. The amount is taken from the corresponding test protocol. Further treatment of the spiked control sample is done in parallel with the sample to be tested.

8.4.3 Hydrolysis and derivatization (eggs, egg powder)

- Treat the standards, samples, and control samples with 25 µL of the internal mixture standard working solution (40 ng/mL AMOZ-d5, AOZ-d4, AHD-IS, SEM-IS; 6.4.11);
- Allow to stand for 10 min;
- + 15 mL 0.2M hydrochloric acid solution (6.4.1);
- + 150 µL 100mM nitrobenzaldehyde solution (6.4.2);
- Mix using the vortexer;
- Incubate in the water bath at 37 °C overnight shaking well.

8.4.4 Extraction (eggs, egg powder)

- Buffer the incubates (samples, control samples, standards) with 1.5 mL of 0.3 sodium phosphate solution (6.4.3);
- Add 1M NaOH (at first, in 50 µL portions);
  adjust the pH to 6.7-7.3 (pH meter) through careful additions of 1M sodium hydroxide (6.4.4);  
  [Remarks: remember to mix using the vortexer after adding NaOH!]
- After centrifugation under 3600 g at 4 °C for 5 minutes in 50 mL Greiner vials, separate the supernatant;
- Treat the suspension three times with 4 mL ethyl acetate;
  Mix using the vortexer after each step;
  Place on the rotary shaker for 10 min;
  Centrifuge under 4000 g at 4 °C for 10 min;
  Transfer the supernatant to a new 12 mL Greiner vial using a Pasteur pipette;
- Concentrate the ethyl acetate extracts by evaporating under nitrogen at approx. 45 °C;
- Take up each of the residues in 225 µL 0.1% acetic acid (6.4.12);
- Treat this with 2 mL n-hexane;
  Mix using the vortexer;
  Centrifuge under 4000 g at 4 °C for 5 min;
- Suction off the hexane phases and discard;
- Remove the remainders by evaporating under a nitrogen stream at room temperature for 2 min;
- + 25 µL acetonitrile;
- Transfer to HPLC vials (filtering if necessary) (Measurement Solution).
8.5 Sample preparation for honey

Weighings:
weigh out 1.0 g in 12 mL Greiner vials with an accuracy of at least ±0.1 g.

8.5.1 Preparation of the calibration standards (honey)
For example, the 6 standards are prepared as follows:

<table>
<thead>
<tr>
<th>Hour</th>
<th>Concentration of the respective substance (µg/kg)</th>
<th>Mixture standard working solution (6.4.8 or 6.4.7)</th>
<th>Amount</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.05</td>
<td>12.5 µL</td>
<td>4 ng/mL</td>
<td>→</td>
</tr>
<tr>
<td>2</td>
<td>0.1</td>
<td>25 µL</td>
<td>4 ng/mL</td>
<td>→</td>
</tr>
<tr>
<td>3</td>
<td>0.2</td>
<td>50 µL</td>
<td>4 ng/mL</td>
<td>→</td>
</tr>
<tr>
<td>4</td>
<td>0.5</td>
<td>125 µL</td>
<td>4 ng/mL</td>
<td>→</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>50 µL</td>
<td>20 ng/mL</td>
<td>→</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>100 µL</td>
<td>20 ng/mL</td>
<td>→</td>
</tr>
</tbody>
</table>

Each is pipetted into a 12 mL Greiner vial

8.5.2 Preparation of the control samples (honey)
Treat the negative control sample with the mixture standard working solution (6.4.7 or 6.4.8) and mix using the vortexer.
The amount is taken from the corresponding test protocol.
Further treatment of the spiked control sample is done in parallel with the sample to be tested.

8.5.3 Hydrolysis and derivatization (honey)
- Treat the standards, samples, and control samples with 25 µL of the internal mixture standard working solution (40 ng/mL AMOZ-d₅, AOZ-d₆, AHD-IS, SEM-IS; 6.4.11);
- Allow to stand for 10 min;
- + 5 mL 0.2M hydrochloric acid solution (6.4.1);
- + 75 µL 100mM nitrobenzaldehyde solution (6.4.2);
- Mix using the vortexer;
- Incubate in the water bath at 37 °C overnight with good shaking.

8.5.4 Extraction (honey)
- Buffer the incubates (samples, control samples, standards) with 1.5 mL of 0.3 sodium phosphate solution (6.4.3);
- Add 1M NaOH (at first, in 50 µL portions),
  adjust the pH to 6.7-7.3 (pH meter) through careful additions of 1M sodium hydroxide (6.4.4);
  [Remarks: remember to mix using the vortexer after adding NaOH!]
- Treat the suspension three times with 4 mL ethyl acetate;
  Mix using the vortexer after each step;
  Place on the rotary shaker for 10 min;
  Centrifuge under 4000 g at 4 °C for 10 min;
  Transfer the supernatant to a new Greiner vial using a Pasteur pipette;
- Concentrate the ethyl acetate extracts by evaporating under nitrogen at approx. 45 °C;
- Take up each of the residues in 225 µL 0.1% acetic acid (6.4.12);
- Treat this with 2 mL n-hexane;
  Mix using the vortexer;
  Centrifuge under 4000 g at 4 °C for 5 min;
- Suction off the hexane phases and discard;
- Remove the remainders by evaporating under a nitrogen stream at room temperature for 2 min;
- + 25 µL acetonitrile;
- Transfer to HPLC vials (filtering if necessary) (Measurement Solution).

8.6 Sample preparation for milk and milk powder

Weighings:
- Milk: weigh out 1.0 g in 15 mL Greiner vials with an accuracy of at least ±0.1 g;
- Milk powder: weigh out 0.2 g in 15 mL Greiner vials with an accuracy of at least ±0.1 g, and reconstitute with 0.8 mL distilled water.

8.6.1 Preparation of the calibration standards

This calibration is suitable for samples with a weight of 1 g. For samples weighing 0.2 g (milk powder), a dilution factor of 5 is taken into account during the measurement.

For example, the 6 standards are prepared as follows:

<table>
<thead>
<tr>
<th>Hour</th>
<th>Concentration of the respective substance µg/kg</th>
<th>Mixture standard working solution (6.4.8 or 6.4.7) Amount µL</th>
<th>Concentration ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Each is pipetted into a 15 mL Greiner vial</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.05</td>
<td>12.5</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>0.1</td>
<td>25</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>0.2</td>
<td>50</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>0.5</td>
<td>125</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>100</td>
<td>20</td>
</tr>
</tbody>
</table>

8.6.2 Preparation of the control samples (milk, milk powder)

Treat the negative control sample with the mixture standard working solution (6.4.7 or 6.4.8) and mix using the vortexer.

The amount is taken from the corresponding test protocol.

Further treatment of the spiked control sample is done in parallel with the sample to be tested.

8.6.3 Hydrolysis and derivatization (milk, milk powder)

- Treat the standards, samples, and control samples with 25 µL of the internal mixture standard working solution (40 ng/mL AMOZ-d₅, AOZ-d₅, AHD-IS, SEM-IS; 6.4.11);
- Allow to stand for 10 min;
- + 5 mL 0.2M hydrochloric acid solution (6.4.1);
- + 75 µL 100mM nitrobenzaldehyde solution (6.4.2);
- Mix using the vortexer;
- Incubate in the water bath at 37 °C overnight shaking well.

8.6.4 Extraction (milk, milk powder)

- Centrifuge the incubates (samples and control samples) under 4000 g at 4 °C for 10 min;
- Separate the supernatants in Greiner vials;
- Buffer the supernatants (samples, control samples) and standards with 1.5 mL of 0.3 sodium phosphate solution (6.4.3);
- Add 2M NaOH (at first, in 50 µL portions);
  adjust the pH to 6.7-7.3 (pH meter) through careful additions of 1M sodium hydroxide (6.4.4);
  \[ \text{Remarks: remember to mix using the vortexer after adding NaOH!} \]
- Treat the suspension three times with 4 mL ethyl acetate;
  Mix using the vortexer after each step;
  Place on the rotary shaker for 10 min;
  Centrifuge under 4000 g at 4 °C for 10 min;
  Transfer the supernatant to a new Greiner tube using a Pasteur pipette;
- Concentrate the ethyl acetate extracts by evaporating under nitrogen at approx. 45 °C;
- Take up each of the residues in 225 µL 0.1% acetic acid (6.4.12);
- Treat this with 2 mL n-hexane;
  Mix using the vortexer;
  Centrifuge under 4000 g at 4 °C for 5 min;
- Suction off the hexane phases and discard;
- Remove the remainders by evaporating under a nitrogen stream at room temperature for 2 min;
- + 25 µL acetonitrile;
- Transfer to HPLC vials (filtering if necessary) (Measurement Solution).

8.7 Sample preparation for lid sealant material

Weighings:
weigh out 1.0 g of the most finely divided possible material in 15 mL Greiner vials with an accuracy of at least ±0.1 g;

8.7.1 Preparation of the calibration standards

For example, the 6 standards are prepared as follows:

<table>
<thead>
<tr>
<th>Conc. of the respective substance (µg/kg)</th>
<th>Mixture standard working solution (6.4.8 or 6.4.7)</th>
<th>Concentration (ng/mL)</th>
<th>Each is pipetted into a 15 mL Greiner vial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hour 1 0.05 µg/kg</td>
<td>12.5 µL</td>
<td>4 ng/mL</td>
<td></td>
</tr>
<tr>
<td>Hour 2 0.1 µg/kg</td>
<td>25 µL</td>
<td>4 ng/mL</td>
<td></td>
</tr>
<tr>
<td>Hour 3 0.2 µg/kg</td>
<td>50 µL</td>
<td>4 ng/mL</td>
<td></td>
</tr>
<tr>
<td>Hour 4 0.5 µg/kg</td>
<td>125 µL</td>
<td>4 ng/mL</td>
<td></td>
</tr>
<tr>
<td>Hour 5 1 µg/kg</td>
<td>50 µL</td>
<td>20 ng/mL</td>
<td></td>
</tr>
<tr>
<td>Hour 6 2 µg/kg</td>
<td>100 µL</td>
<td>20 ng/mL</td>
<td></td>
</tr>
</tbody>
</table>

8.7.2 Preparation of the control samples (lid sealant material)

If available, weigh out at least one positive control sample of 1 g in a Greiner vial.
Further treatment of the control sample is done in parallel with the sample to be tested.

8.7.3 Hydrolysis and derivatization (lid sealant material)

- Treat all standards, samples, and control samples with 25 µL of the internal mixture standard working solution (40 ng/mL AMOZ-d₅, AOZ-d₄, AHD-IS, SEM-IS; 6.4.11);
  \[ \text{Remarks: Samples with an SEM content higher than 2 µg/kg should be treated with an x-fold portion of the mixture standard working solution, and the measurement solution should be diluted by a factor of x} \]
- Add 5 mL of 0.2M hydrochloric acid solution (6.4.1) to the samples and standards;
- Shake for 0.5 h on an overhead shaker;
- + 25 µL 100mM nitrobenzaldehyde solution (6.4.2);
- Mix using the vortexer;
- Incubate in the water bath at 37 °C overnight shaking well.

8.7.4 Extraction (lid sealant material)
- Centrifuge the incubates (samples and control samples) under 4000 g at 4 °C for 10 min;
- Separate the supernatant into a new Greiner tube;
- Buffer the supernatants (samples, control samples) and standards with 1.5 mL of 0.3 sodium phosphate solution (6.4.3);
- Add 2M NaOH (at first, in 50 µL portions);
  [Remarks: remember to mix using the vortexer after adding NaOH!]
  - adjust the pH to 6.7-7.3 (pH meter) through careful additions of 1M sodium hydroxide (6.4.4);
- Treat the suspension three times with 4 mL ethyl acetate;
  - Mix using the vortexer after each step;
  - Place on the rotary shaker for 10 min;
  - Centrifuge under 4000 g at 4 °C for 10 min;
  - Transfer the supernatant to a new Greiner vial using a Pasteur pipette;
- Concentrate the ethyl acetate extracts by evaporating under nitrogen at approx. 45 °C;
- Take up each of the residues in 225 µL 0.1% acetic acid (6.4.12);
- Treat this with 2 mL n-hexane;
  - Mix using the vortexer;
  - Centrifuge under 4000 g at 4 °C for 5 min;
- Suction off the hexane phases and discard;
- Remove the remainders by evaporating under a nitrogen stream at room temperature for 2 min;
- + 25 µL acetonitrile;
- Transfer to HPLC vials (filtering if necessary) (Measurement Solution).

8.8 Measurement / Testing
The measurement solution present in the HPLC vial is analyzed under the following conditions:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC column:</td>
<td>XTerra MS C18; 150 × 3 mm; 3.5 µm (Waters)</td>
</tr>
<tr>
<td>Eluent:</td>
<td>A: 0.1% acetic acid (6.4.12); B: acetonitrile</td>
</tr>
<tr>
<td>Gradient:</td>
<td>17% - 50% acetonitrile</td>
</tr>
<tr>
<td>Injection volume:</td>
<td>10 µL</td>
</tr>
<tr>
<td>Flow rate:</td>
<td>0.3 mL/min</td>
</tr>
<tr>
<td>Oven temperature:</td>
<td>40 °C</td>
</tr>
<tr>
<td>MS detection:</td>
<td>Ionization mode: ESI positive</td>
</tr>
<tr>
<td>AMOZ (MS²):</td>
<td>m/z: 291 and 262; precursor: 335</td>
</tr>
<tr>
<td>AOZ (MS²):</td>
<td>m/z: 134 and 104; precursor: 236</td>
</tr>
<tr>
<td>SEM (MS²):</td>
<td>m/z: 192 and 166; precursor: 209</td>
</tr>
<tr>
<td>AHD (MS²):</td>
<td>m/z: 134 and 104; precursor: 249</td>
</tr>
<tr>
<td>AMOZ-d₅ (internal standard) (MS²):</td>
<td>m/z: 296; precursor: 340</td>
</tr>
<tr>
<td>AOZ-d₅ (internal standard) (MS²):</td>
<td>m/z: 134; precursor: 240</td>
</tr>
<tr>
<td>SEM-IS (internal standard) (MS²):</td>
<td>m/z: 168; precursor: 212</td>
</tr>
<tr>
<td>AHD (internal standard) (MS²):</td>
<td>m/z: 134; precursor: 252</td>
</tr>
</tbody>
</table>
The designation of the mass method is **NIF-XXX**, wherein XXX is a consecutive number. The method with the highest number is the current one, and thus is the mass method to use.

9 Evaluation

9.1 Evaluation / Calculation

Evaluate the measurement results obtained with the appropriate software (Analyst):
- Identify the signals (peaks) using the retention times and the ion fragments (m/z), and attribute them to the corresponding substances;
- Integrate the peaks to determine the peak areas;
- Create calibration curves from the results on the calibration standards, and calculate the associated concentrations. Save the results;
- Export the raw data from the instrument software into Microsoft Excel.

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);
- Recovery rates (WFRs);
- Analyte content in the sample (corrected for WFR).

Remarks: Ensure that sample weights and any dilution factors are reflected in the calculations of analyte content.

Use the master template named "**Berechnungsvorlage_LCMSMS_XX.xlt**", found under **L:\taho\Analysendaten\** as a starting point for carrying out these calculations. Here, XX is a consecutive number, and the file with the highest number XX must be used.

**Conversion for animal tissue, baby food**
The extract from 2 g of sample is analyzed with a final volume of 250 µL. Thus, a content of 1 ng/g in the sample corresponds to a concentration of 2 ng in 0.25 mL in the measurement solution (= 8 ng/mL).

*8 ng/mL measurement solution corresponds to 1 µg/kg sample*

Convert the standard concentrations to include these factors in the hypothetical sample amounts, and store them in terms of µg/kg in the calibration function of the evaluation instrument. The printouts of the sample extract measurements will thus give the final results in µg/kg.

**Conversion for egg, honey, milk, and lid sealant material**
The extract from 1 g of sample is analyzed with a final volume of 250 µL. Thus, a content of 1 ng/g in the sample corresponds to a concentration of 1 ng in 0.25 mL in the measurement solution (= 4 ng/mL).

*4 ng/mL measurement solution corresponds to 1 µg/kg sample*

Convert the standard concentrations to include these factors in the hypothetical sample amounts, and store them in terms of µg/kg in the calibration function of the evaluation instrument. The printouts of the sample extract measurements will thus give the final results in µg/kg.

**Conversion for egg powder, milk powder**
The extract from 0.2 g of sample is analyzed with a final volume of 250 µL. Thus, a content of 1 ng/g in the sample corresponds to a concentration of 0.2 ng in 0.25 mL in the measurement solution (= 8 ng/mL).

*0.8 ng/mL measurement solution corresponds to 1 µg/kg sample*
Convert the standard concentrations to include these factors in the hypothetical sample amounts, and store them in terms of µg/kg in the calibration function of the evaluation instrument. The printouts of the sample extract measurements will thus give the final results in µg/kg.

9.1.1 Assessment screening

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 ±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 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 intensity of the corresponding fragment ions</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 one of the internal standard substances entrained in all the samples 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 with the responsible supervisor.

d) Compare the analyte content (corrected for recovery rate) in the sample with the corresponding 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 so that the concentration is consistent with the required dilution.

   If in doubt, consult the responsible supervisor.

If the presence of a questionable substance in a sample is established during screening (“suspected positive”), the extraction must be repeated for confirmation with at least two new aliquots of the sample.
in question, to validate the initial finding. The preparation and analysis of the parallel samples are done according to Section 8, and the assessment proceeds according to Section 9.1.2. If a sample is considered “not evaluable”, a new aliquot of the sample must be weighed out and the analysis repeated. If in doubt, the specific procedure should be clarified with the responsible supervisor.

9.1.2 Assessment 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. The following tolerances are permissible according to the Commission Decision of August 12, 2002 (2002/657/EC):

<table>
<thead>
<tr>
<th>Relative intensity of the qualifier to the target ion</th>
<th>Maximum permissible relative deviations (EU tolerances)</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 standard corresponding to the analyte in question must be 20% higher than the mean concentration of the accompanying control samples.

d) The analyte content in the sample must be above detection limit (see Section 10).

e) The measurement 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 so that the concentration is consistent with the required dilution. If in doubt, consult the responsible supervisor.

9.2 Documentation
At a minimum, the Test Report must contain:
- Preparer, analyst (name or initials);
- Date of the sample preparation and measurement;
- Sample identifier (LISA number);
9.3 Presentation of results

The results are presented in the Test Report based on the values determined in Section 9.1:

- Analyte not detectable:
  "ND (LOD: ... μg/kg)"

- Analyte detectable, but below the quantitation 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)"

Remarks: The detection and quantitation limits can be taken from the current LISA method under the "Limits" tab.

10 Validation

The results of the validation are available in electronic form at:
L:\taho\QM\Validierung\NITROFURANMETABOLITEN\icmsms(4000Q)

11 References

11.1 QM documents and document templates

- SVA_CC_VIE_TAHO_002 (Sample receipt for the CC TAHO)
- 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_005 (Preparation of control samples)
- SVA_CC_VIE_TAHO_006 (Guide to handling of standard substances)
- DOT_PQO_PV
Location: DOXIS

11.2 Standards, laws and guidelines

- Commission Decision of March 13, 2003 (2003/181/EC); Extension of 2002/657/EC concerning the MRPLs of certain residues in food animal origin
Remarks: Additional statutory provisions (e.g., amendments, supplements, changes) are stored electronically at the above-mentioned location.

11.3 Scientific literature

- Analysis of Protein-bound Metabolites of Furazolidone and Furaladone in Pig Liver by HPLC and LC-MS. (1996) L.A.P. Hoogenboom et al., Analyst 121, pp. 1463-1468
- Metabolites in Pig Liver by HPLC with UV and MS Detection. (1996) L.A.P. Hoogenboom et al., EuroResidue III, pp. 510-515
- Workshop: Analysis of tissue-bound residues of nitrofuran drugs; July 3-5, 2002 (Location: RIKILT: State Institute for Quality Control of Agricultural Products; Wageningen, NL)

Location: Room B/1.79

12 Appendices

None

<table>
<thead>
<tr>
<th>Created / revised</th>
<th>Technical check</th>
<th>QM check</th>
<th>Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td>Verena Simons</td>
<td>Georg Mayerhofer</td>
<td>Irina Schwaiger-Nemirova</td>
</tr>
<tr>
<td>Date</td>
<td>Nov. 18, 2011</td>
<td>Nov. 25, 2011</td>
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