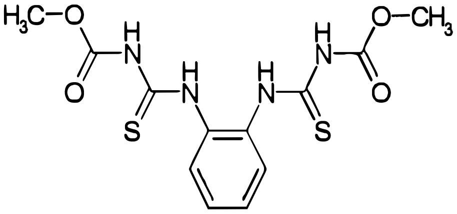
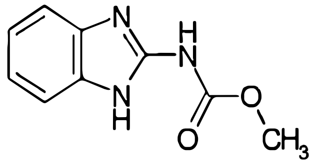
## 2 Study Objective

The study objective was to perform an independent laboratory validation (ILV) on an analytical method for the determination of residues of Thiophanate-methyl and its metabolite Carbendazim in water. The primary method validation has been performed at PTRL Europe, Ulm, Germany and described in study no. P 2681 G according to guideline SANCO/825/00 rev. 8.1.

## 3 Material and Methods

### 3.1 Test and Reference Item(s)

| | |
|---|---|
| Common Name: | **Thiophanate-methyl** |
| CAS-Registry-No.: | 23564-05-8 |
| Structural Formula: |  |
| Molecular Formula: | $C_{12}H_{14}N_4O_4S_2$ |
| Molecular Mass: | 342.4 g/mol |
| Supplier: | Labor Dr. Ehrenstorfer, Germany |
| Batch No.: | 30529 |
| CIP (test facility) code: | 07016 |
| Purity: | 99.0 % |
| Date of Certification: | 25 October 2013 |
| Date / Quantity of Receipt: | 19 February 2014 / 250 mg |
| Appearance: | colourless solid |
| Recommended Storage Conditions: | Room temperature |
| Storage at CIP: | Refrigerated (nominally 4 °C) |
| Date of Expiry: | 25 October 2017 |

| | |
|---|---|
| Common Name: | **Carbendazim** |
| CAS-Registry-No.: | 10605-21-7 |
| Structural Formula: |  |
| Molecular Formula: | $C_9H_9N_3O_2$ |
| Molecular Mass: | 191.2 g/mol |
| Supplier: | Labor Dr. Ehrenstorfer, Germany |
| Batch No.: | 21129 |
| CIP (test facility) code: | 07024 |
| Purity: | 99.0 % |
| Date of Certification: | 30 January 2013 |
| Date / Quantity of Receipt: | 05 March 2014 / 250 mg |
| Appearance: | colourless solid |
| Recommended Storage Conditions: | Room temperature |
| Storage at CIP: | Refrigerated (nominally 4 °C) |
| Date of Expiry: | 30 November 2018 |

All specifications of purity and composition of the reference item(s) were provided by the supplier. The certificate of analysis is depicted in Appendix 5.

## 3.2 Analytical Procedure

### 3.2.1 Specimen Origin

Drinking water sample was taken from the public water supply of the village of Schömberg, Kreis Calw, Germany.

The parameters of the used water sample are given in the following table:

Table 1: Typical parameters of the used water sample

| | Drinking water |
|---|---|
| Origin | Schömberg, Kreis Calw, Germany |
| pH | 8.03 |
| conductivity (µS/cm) | 170 |
| TOC (mg/L) | 0.56 |
| SAK 254 nm (1/m) | 1.32 |
| Total hardness (mmol/L)* | 0.87 (4.9 dGH) |

*One millimole of calcium (either $Ca^{2+}$ or $CaCO_3$) per litre of water corresponds to a hardness of 100.09 ppm or 5.608 dGH, since the molar mass of calcium carbonate is 100.09 g/mol.

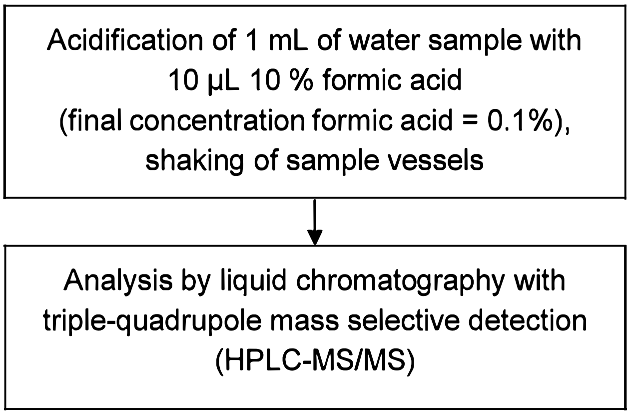
All parameters were analyzed according to DIN EN ISO/IEC 17025:2005 (non-GLP).

### 3.2.2 Specimen Storage and Preparation

The water specimens were stored refrigerated (< 8 °C) in the dark. Before use, the sample was homogenized by shaking.

## 3.3 Outline of the Method

Water specimens are analysed for residues of Thiophanate-methyl and Carbendazim using the analytical method reported in study P 2681 G (Jooß, S., PTRL Europe, Ulm, Germany, 2012):



Acidification of 1 mL of water sample with 10 µL 10 % formic acid (final concentration formic acid = 0.1%), shaking of sample vessels

Analysis by liquid chromatography with triple-quadrupole mass selective detection (HPLC-MS/MS)

Representative chromatograms are presented in Appendix 4.

## 3.4 Equipment and Apparatus

- Adjustable pipettes
- Hamilton syringes
- HPLC autosampler vials, 1.8 mL
- HPLC with MS/MS detection (Dionex Ultimate 3000 with AB Sciex API 5500 QTRAP)

## 3.5 Reagents and Materials

| | | |
|---|---|---|
| • Acetonitrile, HPLC grade | Promochem | No. SO-9128-B025 |
| • Methanol, HPLC grade | Promochem | No. SO-9260-B025 |
| • Formic acid, p.a. | Merck | No. 1.00264.1000 |
| • Acetic acid, p.a. | Merck | No. 1.00063.1000 |
| • Acetonitrile, LCMS grade | Promochem | No. SO-9340-B010 |
| • Water, LCMS grade | Promochem | No. SO-9368-B010 |

### 3.5.1 Hazards or Precautions

For conduct of this method the German guidelines for laboratories „Working Safely in Laboratories, Basic Principles and Guidelines" or comparable guidelines in other countries are to be observed. The following chemicals are used, which are classified by the hazardous material regulations according to Regulation (EC) No 1272/2008 [EU-GHS/CLP].

Acetonitrile:

Methanol:

Acetic acid

Formic acid

The pertinent safety instructions must be observed when working with all compound mentioned in this method (e.g. hazard (H) and precautionary (P) statements).

## 3.6 Preparation of Water Samples

Aliquots of the water samples were transferred to HPLC vials by means of adjustable pipettes. In case of recovery samples, 10µL of the respective standard solution, prepared in acetonitrile and 10 µL 10% formic acid$_{(aq)}$ (final concentration 0.1 % formic acid) were added. The vials were capped tightly, shaken well and used for analysis by HPLC-MS/MS.

Time required for one sample set (12 samples including preparation of the analytical sequence and calibration standards) is about 2 hours for one person.

## 3.7 HPLC-MS/MS Conditions

| | |
|---|---|
| HPLC-MS/MS: | Dionex Ultimate 3000 with AB Sciex API 5500 QTRAP |
| Column: | Agilent Zorbax Eclipse XDB-C8 150 mm × 4.6 mm, 5.0 µm<br>(Part No. 993967-906) |
| Mobile phase: | A: Water+0.1% Formic acid<br>B: Acetonitrile +0.1% Formic acid |

| Time [min] | A [%] | B [%] | Flow [µL/min] | Gradient |
|---|---|---|---|---|
| 0.00 | 80 | 20 | 600 | linear |
| 3.00 | 0 | 100 | 600 | |
| 6.00 | 0 | 100 | 600 | |
| 6.10 | 80 | 20 | 600 | |
| 10.00 | 80 | 20 | 600 | |

| | |
|---|---|
| Column temp.: | 35 °C |
| Interface: | ESI |
| Source polarity: | Positive |
| Ion Source: | Turbo Spray |
| Curtain gas | 15 units |
| Collision gas: | Medium |
| Ion spray voltage: | 5.5 kV |
| Interface temperature: | 600 °C |
| GS1: | 60 units |
| GS2: | 0 units |
| Injection volume: | 100 µL |
| Retention time: | Carbendazim ~ 3.6 min<br>Thiophanate-methyl ~ 5.1 min |
| Split: | Approx. 1:5 |
| Valve: | 0-1 min to waste; 1-6 min to MS; 6-10 min to waste |
| Quantification: | Peak areas of the fragment ions, external standards |

Mass spectrometer parameters:

| Analyte Monitored | Ions Monitored MRM | DP [V] | EP [V] | CE [V] | CXP [V] | Dwell Time [ms] |
|---|---|---|---|---|---|---|
| Carbendazim | 192 → 160 | 148 | 10 | 15 | 16 | 200 |
| | 192 → 132 | 148 | 10 | 43 | 26 | 200 |
| Thiophanate-methyl | 343 → 151 | 142 | 10 | 26 | 48 | 300 |
| | 343 → 192 | 39 | 10 | 22 | 32 | 500 |
| Ion Mode: | ESI (positive) | | | | | |

Representative mass spectra are presented in Appendix 1.

The mass spectrometer was operated in MS/MS-Single Reaction Monitoring (MRM) positive ion mode. For Thiophanate-methyl, MRM 343 → 151 was suitable for quantification, MRM 343 → 192 was suitable as confirmation method.

For Carbendazim, MRM 192 → 160 was suitable for quantification, MRM 192 → 132 was suitable as confirmation method.

At the beginning of the sample sequence, a calibration curve was constructed by injecting standard solutions in the range from 0.01 µg/L to 1 µg/L (five points, single injection each). Injections of samples were interspersed with injections of standards to provide a continuous check on the instrument calibration.

## 3.8 Calculation of the Residues

External standard solutions comparable to the concentration expected in specimens were injected after each two or three samples.

The residues (R) in µg/L are calculated using external standards with one-point calibration according to the following equation:

$$R = \frac{A_x \cdot C_{St,nominal}}{A_{St}}$$

where

$A_x$: Peak area of the analyte
$C_{St,\ nominal}$: Nominal concentration of the analyte in external bracketing standard solutions, in µg/L
$A_{St}$: Peak area of the analyte in external standard solution (mean from bracketing standards)

The response factor (see Appendix 3) is calculated as follows:

Response Factor = Peak Area [counts] / Concentration [µg/L]

Recoveries were calculated by the following equation:

$$Rec = \frac{R_{found}}{R_{fortified}} \cdot 100\,\%$$

| | |
|---|---|
| Rec | Recovery [%] |
| $R_{found}$ | Analyte determined [µg/L] |
| $R_{fortified}$ | Fortification level [µg/L] |

Example calculation:

Recovery 0.5 µg/L in drinking water (Thiophanate-methyl; quantifier):

| | |
|---|---|
| Sample ID: | RDW 0.5-1 |
| $A_x$: | 60627 |
| $C_{St,\ nominal}$: | 0.5 µg/L |
| $A_{St}$: | 63408 counts |

$$R = \frac{60627\ \text{counts} \cdot 0.5\,\mu g/L}{63408\ \text{counts}} = 0.478\ \mu g/L \qquad Rec = \frac{0.478\ \mu g/L}{0.5\ \mu g/L} \cdot 100\,\% = 96\%$$

## 3.9 Standard Solutions

### 3.9.1 Stock Solution

Stock solutions containing 1000 mg/L of Thiophanate-methyl (S1000) and 200 mg/L of Carbendazim (S200) were prepared in acetonitrile:methanol 7/3 (v/v) + 0.1% acetic acid as follows:

S1000: 20.2 mg Thiophanate-methyl (purity 99.0 %) in 20 mL

S200: 10.1 mg Carbendazim (purity 99.0 %) in 50 mL

Standards mixtures of Thiophanate-methyl and Carbendazim were prepared in acetonitrile and water containing 0.1% formic acid to achieve solutions in the concentration range needed for this study. Preparation of these working solutions is shown in the following table:

Table 2: Preparation of working solutions

| Fortification Solution | Solution used for preparation | Volume used* [mL] | Final volume [mL] | Solvent | Concentration obtained [mg/L] |
|---|---|---|---|---|---|
| SM 50 | S1000 Thiophanate-methyl<br>S200 Carbendazim | 1<br>5 | 20 | Acetonitrile + 0.1% Formic acid | 50 of each |
| SM 5 | SM 50 | 1 | 10 | Acetonitrile + 0.1% Formic acid | 5 of each |
| SM 0.5 | SM 5 | 1 | 10 | Acetonitrile + 0.1% Formic acid | 0.5 of each |
| SM 0.05 | SM 0.5 | 1 | 10 | Acetonitrile + 0.1% Formic acid | 0.05 of each |
| SM 0.005 | SM 0.05 | 1 | 10 | Acetonitrile + 0.1% Formic acid | 0.005 of each |
| SM 0.01 | SM 0.5 | 0.2 | 10 | Water + 0.1% Formic acid | 0.01 of each |

* Volume used = Volume withdrawn from the solution indicated in the 2nd column.

All solutions in acetonitrile were stored deep frozen (≤ -18°C) in the dark. The solution in water was stored refrigerated (< 8°C) in the dark.

### 3.9.2 HPLC Standard Solutions

Chromatographic external calibration solutions were prepared by diluting working solutions in LCMS water using adjustable pipettes and Hamilton syringes. Preparation of calibration standards is described in Table 3.

A series of matrix-matched calibration standards in drinking water was prepared to determine the matrix effects of the water sample under investigation. Preparation of matrix-matched standards is shown in Table 4.

Table 3: Preparation of calibration standards in LCMS water

| Standard Solution | Standard Solution used for preparation | Volume Used* [µL] | Solvent added [µL] | Solvent | 10% formic acid$_{(aq)}$ added [µL] | Concentration obtained [µg/L] |
|---|---|---|---|---|---|---|
| Std 1 µg/L | SM 0.01 | 100 | 900 | **Water (LCMS grade)** | 10 | 1 |
| Std 0.5 µg/L | SM 0.01 | 50 | 950 | | 10 | 0.5 |
| Std 0.1 µg/L | Std 1 µg/L | 100 | 900 | | 10 | 0.1 |
| Std 0.05 µg/L | Std 0.5 µg/L | 100 | 900 | | 10 | 0.05 |
| Std 0.01 µg/L | Std 0.1 µg/L | 100 | 900 | | 10 | 0.01 |

* Volume used = Volume withdrawn from the solution indicated in the 2nd column.

Table 4: Preparation of matrix-matched standards in drinking water

| Matrix-matched Standard Solution | Standard Solution used for preparation | Volume Used* [µL] | Solvent added [µL] | Solvent | 10% formic acid$_{(aq)}$ added [µL] | Concentration obtained [µg/L] |
|---|---|---|---|---|---|---|
| MStd 1 µg/L DW | SM 0.01 | 100 | 900 | **Drinking Water** | 10 | 1 |
| MStd 0.5 µg/L DW | SM 0.01 | 50 | 950 | | 10 | 0.5 |
| MStd 0.2 µg/L DW | MStd 1 µg/L DW | 100 | 900 | | 10 | 0.1 |
| MStd 0.1 µg/L DW | MStd 0.5 µg/L DW | 100 | 900 | | 10 | 0.05 |
| MStd 0.01 µg/L DW | MStd 0.1 µg/L DW | 100 | 900 | | 10 | 0.01 |

* Volume used = Volume withdrawn from the solution indicated in the 2nd column.

## 4 Fortifications

Control (untreated) specimens of drinking water were fortified with the previously prepared working solutions as follows:

Table 5: Fortification of water samples

| Matrix | Fortification level [µg/L] | Specimen volume [µL] | Fortification solution | Added [µL] | 10% Formic acid$_{(aq)}$ added [µL] |
|---|---|---|---|---|---|
| Drinking water | 0.05 | 1000 | SM 0.005 | 10 | 10 |
| | 0.5 | 1000 | SM 0.05 | 10 | 10 |

## 5 Deviations from the Study Plan

The study was performed according to the study plan dated 07 March 2014.
This report reflects the conduct of this study.