# 1 Summary

The study objective was to perform an independent laboratory validation (ILV) on an analytical method for the determination of residues of Imazalil and its metabolite R014821 in soil according to guideline SANCO/825/00 rev.8.1. The primary method validation has been performed at Eurofins Analytik GmbH (Dr. Specht Laboratorien) in 2008 (SCHERNIKAU, N., study No. CET-0802V (Specht file reference G08-0015)).

The data presented in this report demonstrate that the used method permits the determination of Imazalil and its metabolites R014821 in soil with a limit of quantification of 0.001 mg/kg. The method was validated according to the guideline SANCO/825/00 rev. 8.1 (2010). The method was proven to be specific, accurate and precise and a good repeatability and recovery was found in matrix soil.

Untreated control specimens of soil were analysed in duplicate and fortified specimens were analysed in quintuple for each fortification level (0.001 mg/kg and 0.05 mg/kg).

Since two characteristic mass transitions were used to monitor the analyte, the method achieves a high level of specificity and no additional confirmation was necessary.
The analytical method is characterised as follows:

| | |
|---|---|
| **Extraction:** | Extraction with acetonitrile and buffer salt mixture (based on QuEChERS multiresidue method) |
| **Clean up:** | Dispersive SPE clean up, Evaporation, reconstitution in acidified acetonitrile/water |
| **Final analysis:** | HPLC with MS/MS detection |
| **Limit of quantification:** | LOQ was 0.001 mg/kg/L for each analyte (Imazalil and R014821) |
| **Limit of detection:** | LOD is lower than 30% of the LOQ (i.e. ≤ 0.0003 mg/kg) |

**Linearity:** The calibration graphs for solvent standards were linear for Imazalil and its metabolite R014821 within the range from 0.1 µg/L to 50 µg/L with correlation coefficients of $r \geq 0.9997$ for Imazalil and $r \geq 0.9999$ for R014821.

**Selectivity:** Two mass transitions:
Imazalil 297 → 159 and 297 → 201

R014821 257 → 69 and 257 → 125

**Blanks:** Analysis of control specimens with HPLC-MS/MS using two mass transitions yielded no residues of Imazalil or its metabolite R014821 above 30 % of the LOQ indicating that no interferences were present.

## 2 Study Objective

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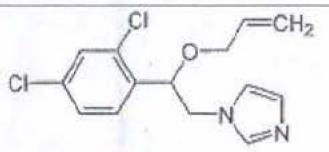
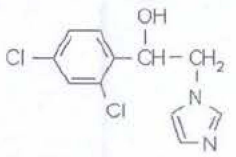
## 3 Material and Methods

### 3.1 Test and Reference Items

#### 3.1.1 Imazalil

| | |
|---|---|
| Name: | **Imazalil** |
| Structural Formula: |  |
| Molecular Formula: | $C_{14}H_{14}Cl_2N_2O$ |
| Molecular Mass: | 297.2 g/mol |
| CAS-No.: | 35554-44-01 |
| Batch: | SZB9016XV |
| Purity: | 99.7 % |
| CIP-code: | 06123 |
| Recommended Storage Conditions: | room temperature |
| Storage at CIP: | refrigerated |
| Date of Certificate: | 15 August 2011 |
| Date of Expiry: | 16 January 2016 |
| Supplier: | Sigma-Aldrich, Germany |

### 3.1.2 R014821

| | |
|---|---|
| Name: | **R014821 (Imazalil alcohol)** |
| Structural Formula: |  |
| Molecular Formula: | $C_{11}H_{10}Cl_2N_2O$ |
| Molecular Mass: | 257.1 g/mol |
| CAS-No.: | 24155-42-8 |
| Batch: | 41009 |
| Purity: | 99.5 % |
| CIP-code: | 08169 |
| Recommended Storage Conditions: | room temperature |
| Storage at CIP: | refrigerated |
| Date of Certificate: | 20 November 2014 |
| Date of Expiry: | 20 November 2018 |
| Supplier: | LGC Standards Germany |

All specifications of purity and composition of the test and reference item were provided by the supplier. A copy of the certificates is shown in Appendix 5.

## 3.2 Analytical Procedure

### 3.2.1 Specimen Origin

A standard soil purchased at LUFA, Speyer (Germany) were used. The soil parameters were determined by LUFA, Speyer, Germany in GLP study report BP 20/11. Typical parameters of the used soil are given in the following table:

Table 1: Typical parameters of the used soil

| **Parameter** | | **Soil Type** BBA 2.3 |
|---|---|---|
| **Batch no.** | | Sp2.3 2811 |
| pH (Calcium chloride) | | 6.96 |
| TOC | [% C] | 0.87 |
| CEC | [meq/100g] | 10.3 |
| Soil Density (Volumetric method) [g/L] | | 1338 |
| Water holding capacity WHCmax [g/100g] | | 36.6 |
| Particle sizes according to German DIN | | |
| (0.630 – 2.0 mm) | [% w/w] | 2.0 |
| (0.200 - 0.630 mm | [% w/w] | 25.4 |
| (0.063 - 0.200 mm) | [% w/w] | 36.3 |
| 0.020 – 0.063 mm) | [% w/w] | 16.5 |
| 0.006 – 0.020 mm) | [% w/w] | 6.4 |
| (0.002 – 0.006 mm) | [% w/w] | 3.5 |
| (< 0.002 mm) | [% w/w] | 9.9 |
| Soil type (DIN 4220) | | Loamy sand (SI) |
| Particle sizes according to USDA | | |
| Sand (0.050 – 2.00 mm) | [% w/w] | 65.8 |
| Silt (0.002 – 0.050 mm) | [% w/w] | 24.0 |
| Clay (< 0.002 mm) | [% w/w] | 10.2 |
| Soil type (USDA) | | Sandy loam |

TOC = total organic carbon; CEC = cation exchange capacity.

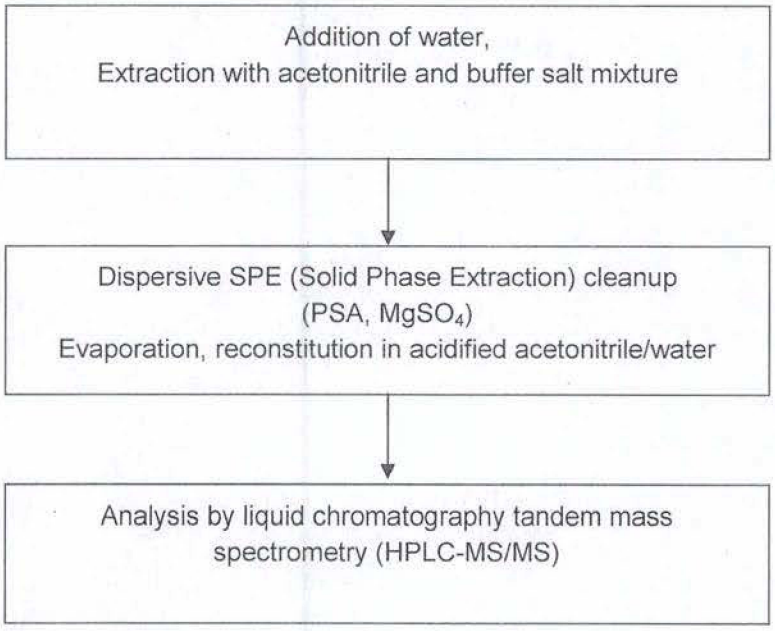
### 3.2.2 Specimen Preparation and Storage

The soil sample was homogenized by shaking and stored at ambient temperature until start of analysis.

## 3.3 Outline of the Method

### 3.3.1 Schematic Diagram of the Method

Soil specimens were analyzed for Imazalil and its metabolite R014821 residues using the method described in study CET-0802V (based on the QuEChERS multiresidue method):




A detailed description of the method is given in the following and in Chapter 3.6.

Representative chromatograms are presented in Appendix 4.

### 3.3.2 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].

Acetone:

Acetonitrile:

Acetic Acid:

Methanol:

The pertinent safety instructions must be observed when working with all compound mentioned in this method (e.g. hazard (H) and precautionary (P) statements). When working with dry ice, it has to be made sure that the working place is properly ventilated.

## 3.4 Equipment and Apparatus

- Adjustable pipettes (Eppendorf/Germany or Thermo Labsystems/Finnland)
- Hamilton syringes
- HPLC Autosampler vials, 1.8 mL
- HPLC with MS/MS detection (Dionex Ultimate 3000 with AB Sciex API 5500 QTRAP)
- Laboratory balances (Sartorius A 200 S and Kern PCD 2500-2)
- Centrifuge (Megafuge/ Heraeus, Germany)
- 50 mL centrifuge tube, single use, poly propylene
- 2 mL centrifuge tube
- Standard laboratory glassware
- Vortex mixer (K 550 GE, Scientific Industries/USA)

## 3.5 Reagents

- Acetone, p.a. (Promochem No.SO-1142-B025)
- Acetonitrile, HPLC gradient grade (Promochem No.SO-9128-B025)
- Demineralised water, HPLC grade (J.T. Baker No.4218)
- Acetic acid (Merck No.1.00063.1000)
- QuEChERS extraction salts (Bekolut, Citrat-Kit-01, No.CK-01-050)
- PSA (Varian, No.12213024),
- Magnesium sulfate (Sigma Aldrich, 63136-1KG-F
- Methanol, LCMS grade (Promochem No.SO-9356-B010)
- Water, LCMS grade (Promochem No.SO-9368-B025)

## 3.6 Extraction

### 3.6.1 Method for Imazalil and R014821:

5 g of soil were weighed into a 50 mL single-use centrifuge tube. Fortification was proceeded at this step. 10 mL water were added and the sample was homogenised by shaking. 10 mL acetonitrile were added and vigorously shaken by hand for 1 minute. Thereafter, QuEChERS EN15662 salt-mixture (4 g magnesium sulphate, 1 g sodium chloride, 1 g sodium citrate, 0.5 g sodium hydrogencitrate sequihydrate) was added and immediately shaken by hand for 1 minute. The sample was centrifuged for 2 minutes at 4000 rpm. Thereafter 1.7 mL of the upper acetonitrile phase were transferred into a 2 mL centrifuge tube containing 160 mg PSA and 225 mg magnesium sulphate. The tube was intensively shaken by hand for 1 minute and then centrifuged for 2 minutes at 6000 rpm. Exactly 1.0 mL of the upper acetonitrile phase was transferred into a second 2 mL centrifuge tube. The acetonitrile was evaporated to dryness using a gentle stream of nitrogen. The residues were dissolved in 1.0 mL of acetonitrile/0.05% acetic acid (1/3, v/v) and used directly for analysis by HPLC-MS/MS.

Time required for preparation of one sample set (consisting of 10 recoveries and 2 blank/control samples) is 3 hours without time required for analysis).

## 3.7 HPLC-MS/MS Conditions

HPLC-MS/MS: Dionex Ultimate 3000 with AB Sciex API 5500 QTRAP

Column: Supelco Ascentis Express C18, 50 mm x 2.1 mm, 2.7 µm (Part No. 53822-U)

Mobile phase: A: Water + 0.05% acetic acid
B: Methanol + 0.05% acetic acid

| Time [min] | A [%] | B [%] | Flow [µL/min] |
|---|---|---|---|
| 0.00 | 95 | 5 | 400 |
| 0.10 | 95 | 5 | 400 |
| 1.10 | 5 | 95 | 400 |
| 3.60 | 5 | 95 | 400 |
| 3.70 | 95 | 5 | 400 |

| | |
|---|---|
| Gradient: | Linear |
| Column temp.: | 30 °C |
| Interface: | Turbo Spray |
| Source polarity: | Positive |
| Curtain gas | 25 units |
| Collision gas: | Medium |
| Ion spray voltage: | 5.5 kV |
| Interface temperature: | 550 °C |
| GS1: | Imazalil: 20 units<br>R014821: 25 units |
| GS2: | 70 units |
| Injection volume: | 8 µL |
| Retention time: | Imazalil ~ 4.0 min<br>R014821 ~ 3.6 min |
| Split: | - |
| Valve: | 0-0.1 min waste; 0.1-4.5 min MS/MS |
| Quantification: | Peak areas of the fragment ions, external solvent standards |

Mass spectrometer parameters:

| Analyte Monitored | Ions Monitored m/z | DP [V] | EP [V] | CE [V] | CXP [V] | Dwell Time [ms] |
|---|---|---|---|---|---|---|
| Imazalil | 297 → 159 | 116 | 10 | 30 | 14 | 100 |
| | 297 → 201 | 116 | 10 | 24 | 16 | 100 |
| R014821 | 257 → 69 | 94 | 10 | 25 | 11 | 100 |
| | 257 → 125 | 94 | 10 | 39 | 11 | 100 |
| Ion Mode: | | ESI (positive) | | | | |

Mass spectra are shown in Appendix 3

The linearity was proven by injecting solvent standard solutions (mixed standards) in acetonitrile / 0.05% acetic acid (1/3, v/v) in the range of 0.1 µg/L to 50 µg/L for Imazalil and R014821.

### 3.8 Calculation of the Residues

External standard solutions comparable to the concentration expected in specimen extracts were injected before and after every 2-3 specimen extracts in each analytical sequence. The found analyte concentration, expressed in µg/L, was calculated using the mean peak area in integrator units (IU = counts) obtained from the bracketing standard solutions during the liquid chromatographic sequence.

The residues (R) of Imazalil [mg/kg] were calculated according to the following equation:

$$R = \frac{A_A \times C_{St} \times V_{ex} \times V_{end} \times DF}{A_{St} \times V_{A1} \times W \times 1000}$$

where

$A_A$: Peak area of the analyte in final solution, in counts
$C_{St}$: Concentration of the analyte in external standard solution, in µg/L
$A_{St}$: Peak area (mean of two bracketing external standard solutions to compensate drifting detector response) of the analyte, in counts
$V_{ex}$: Volume of extract: = 10 mL
$V_{A1}$: Volume of aliquot: = 1 mL
$V_{end}$: Final volume: = 1 mL
W: Sample weight: 5 g
d: Dilution factor (for no dilution = 1)
1000: Conversion factor for mL into L = 1000 mL/L

**Recoveries were calculated by the following equation:**

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

Rec Recovery in [%]
$R_{found}$ Imazalil determined in [mg/kg]
$R_{fortified}$ Fortification level in [mg/kg]

**Example of Calculation**

Recovery of Imazalil in Soil, 0.001 mg/kg (RSO 0.001-3, MRM 297 → 159):

$$R = \frac{A_A \times C_{St} \times V_{ex} \times V_{end} \times DF}{A_{St} \times V_{A1} \times W \times 1000\ mL/L}$$

$$R = \frac{42005\ counts \times 0.5\ \mu g/L \times 10\ mL \times 1\ mL \times 1}{44401\ counts \times 1\ mL \times 5\ g \times 1000\ mL/L} = 0.000946\ mg/kg$$

$$Rec = \frac{R_{found}}{R_{fortified}} \cdot 100\,\% = \frac{0.000946}{0.001} \cdot 100\,\% = 95\%\ recovery$$

## 3.9 Standard Solutions

### 3.9.1 Stock Solutions

A stock solution of Imazalil containing 400 mg/L (S 400) was prepared by weighing 20.1 mg of the reference item (purity 99.7%) into a 50 mL volumetric flask and adjusting the given volume with acetone.

A stock solution of R014821 containing 400 mg/L (S 400) was prepared by weighing 20.1 mg of the reference item (purity 99.5%) into a 50 mL volumetric flask and adjusting the given volume with acetone.

From this stock solutions, standard mix solutions were prepared in acetonitrile at 1 mg/L (SM 1) or in acetonitrile / 0.05 % acetic acid (1/1, v/v) at 0.5 mg/L (SM 0.5), 0.1 mg/L (SM 0.1) and 0.01 mg/L (SM 0.01). These solutions were used for preparation of calibration standards and fortification of recovery samples.

All solutions were stored at < 8°C in the dark.

### 3.9.2 HPLC Standard Solutions

Chromatographic external standard solutions were prepared in acetonitrile / 0.05 % acetic acid (1/3, v/v) using adjustable pipettes and volumetric flasks.

Table 2:Preparation of standard solutions for Imazalil and R014821 (dilution with acetonitrile / 0.05 % acetic acid (1/3, v/v))

| Standard Solution | Standard Solution used for preparation | Volume used [µL] | solvent added | Concentration obtained [µg/L] |
|---|---|---|---|---|
| Std 50 µg/L | SM1 | 500 | ad 10 mL* | 50 |
| Std 20 µg/L | SM1 | 200 | ad 10 mL* | 20 |
| Std 10 µg/L | Std 20 µg/L | 5000 | ad 10 mL* | 10 |
| Std 5 µg/L | Std 20 µg/L | 500 | ad 2 mL* | 5.0 |
| Std 1 µg/L | Std 10 µg/L | 250 | 2250 µL | 1.0 |
| Std 0.5 µg/L | Std 10 µg/L | 60 | 1140 µL | 0.50 |
| Std 0.25 µg/L | Std 10 µg/L | 50 | ad 2 mL* | 0.25 |
| Std 0.1 µg/L | Std 1 µg/L | 120 | 1080 µL | 0.10 |

*adjusted to mark in a volumetric flask

## 4 Fortifications

Control (untreated) specimens were fortified prior to extraction with the standard solutions in acetonitrile / 0.05 % acetic acid (1/1, v/v) as follows:

Table 3: Fortification of specimens

| Analyte | Specimen volume [g] | Fortification level [mg/kg] | Fortification solution | Added [µL] |
|---|---|---|---|---|
| Imazalil / R014821 | 5 | 0.001 | SM 0.01 | 500 |
| | 5 | 0.05 | SM 0.5 | 500 |

## 5 Deviations from the Study Plan

The study was performed according to the study plan dated 05 October 2015

## 6.2 Limit of Quantification, Limit of Detection, Blanks

The limit of quantification (LOQ) was defined as the lowest fortification level at 0.001 mg/kg for both analytes

The limit of detection (LOD) was defined as 30% of the limit of quantification as required by SANCO/825/00 rev. 8.1 (2010) for residues in control samples (i.e. 0.0003 mg/kg).

## 6.3 Matrix Effects

In the basic method validation study, calibration was performed using calibration standards prepared in solvent. Therefore a non-matrix matched calibration was used in this study. Only if recoveries would have indicated matrix effects > 20 %, a matrix-matched calibration would have been prepared.

## 6.4 Calibration Information

Imazalil and R014821 were used for preparing external standard solutions in the range of 0.1 µg/L to 50 µg/L. An eight-point external standard calibration was carried out using the peak area in integrator units (counts) from injection of known standards versus standard concentrations in µg/L for each matrix. In the analytical sequence after each 2-3 sample extracts external standard solutions were injected. The found analyte concentration, expressed in µg/L, was calculated against the bracketing standards to compensate drifting detector response.