
REFERENCE ITEMS

The certified reference items of Imazalil and T000824 (metabolite R014821) were supplied by Janssen Pharmaceutica NV, Belgium.

Name: **Imazalil**
Batch.: ZR023979G3L431
Purity: 97.46 %
Date of Certification: 09 Sep 2005
Storage Conditions (Specht): ≤ -18 °C
Date of Expiry: 29 May 2011

Name: **T000824 (R014821, metabolite of Imazalil)**
Batch.: 00512242
Purity: 100.3 %
Date of Certification: 21 Aug 2007
Storage Conditions (Specht): < -18 °C
Date of Expiry: 27 Jun 2013

TEST SYSTEM

Specimen origin

Control (untreated) specimens of soil (Lufa Speyer standard soil 2.3) were provided by the test facility. Soil characteristics are given in Appendix 4.

Specimen preparation

The matrix material was stored at room temperature until start of analysis.

Specimen analysis

Specimens were analysed for Imazalil and T000824 residues using QuEChERS Method [1] (Specht method P-14.141.04) [2]. A detailed description of the method is given in the following.

Specimen amount taken for analysis of soil was 5 g. Control specimens of soil were analysed in duplicate and fortified specimens were analysed in quintuple for each fortification level. Since two characteristic mass transitions were used to monitor Imazalil and its metabolite, the method achieves a high level of specificity and no confirmation on a different detector was necessary. Representative chromatograms are presented in Appendix 3.

TEST METHOD

Outline of method

Specimen material is extracted with acetonitrile. After addition of magnesium sulfate and sodium acetate the extract is shaken. After centrifugation an aliquot of the extract is cleaned by PSA. The residue extracts are then analysed for residues of Imazalil and T000824 by LC-MS/MS.

TEST METHOD (CONTINUED)**Apparatus and Equipment**

- Sarstedt centrifuge tubes with caps, 50 mL, (e.g. Sarstedt, No. 62.458.004)
- Laboratory centrifuge for 50 mL centrifuge tubes (Rotina 48 RC, Hettich, Germany)
- Volumetric pipettes (1, 2 and 2.5 mL)
- Test tubes, with ground stoppers and graduation marks at 2.5 mL, 5.0 mL and 10.0 mL*
- Adjustable pipettes (Multipipette reference 50-200 μ L and Multipipette reference 100-1000 μ L, Eppendorf, Germany)
- Adjustable pipettes (HandyStep with 12.5 mL tip, Brand, Germany)
- HPLC Autosampler vials, 1.8 mL*
- Common laboratory glassware
- Dilutor (e.g. Microlab 1000, Hamilton Deutschland GmbH, Fraunhoferstr. 17, D-82152 Martinsried, Germany)

* Due to instability of Imazalil to light, brown glass was used during the method.

All glassware must be rinsed with water (to remove detergents) and acetone and dried before use.

Reagents

- Acetonitrile gradient grade (e.g. ChromaSolv® Sigma-Aldrich 34851)
- Methanol (e.g. ChromaSolv® Sigma-Aldrich 34885)
- Distilled water (e.g. Braun Melsungen, Aqua ad iniectabilia, No. 536108)
- Glacial acetic acid p.a. (e.g. Merck No. 2500)
- Magnesium sulfate p.a., waterfree, purum (e.g. Riedel-de Haën 13167)
- Sodium chloride (e.g. Riedel-de Haën 31434)
- Sodium acetate p.a., waterfree (e.g. Fluka 71180)
- Disodium hydrogencitrate sesquihydrate (e.g. Aldrich 359084 or Fluka 71635)
- Trisodium citrate dihydrate (e.g. Sigma S4641 or Riedel-de Haën 32320)
- PSA (primary secondary amine) (Varian part number 12213024)

Extraction

5 g of soil was weighted into a 50 mL Sarstedt centrifuge tube. 10 mL of water was added, the tube was closed and shaken. 10 mL of acetonitrile were added. The centrifuge tube was closed and shaken vigorously by hand for 1 min. Thereafter, 4 g magnesium sulfate, 1.0 g sodium chloride, 1.0 g trisodium citrate dihydrate and 0.5 g disodium hydrogen citrate were added, and the Sarstedt centrifuge tube was capped and immediately shaken by hand for about 1 minute. After shaking the sample was centrifuged for 2 minutes at 4000 rpm.

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TEST METHOD (CONTINUED)

Clean-up

160 mg PSA were weighted into a 2 mL-Eppendorf tube and 225 mg magnesium sulfate were added.

After complete phase separation 1.7 mL of the upper acetonitrile phase were transferred into a 2 mL-Eppendorf tube containing PSA and magnesium sulfate.

The 2 mL-Eppendorf tube was intensively shaken by hand for 1 minute and then centrifuged for 2 minutes at 6000 rpm.

After complete phase separation exactly 1.0 mL of the upper acetonitrile phase was transferred into another Eppendorf 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 filled into autosampler vials for LC-MS/MS analysis.

Detection

Chromatography and Analysis

The final extracts were analysed for Imazalil and T000824 using an Agilent Series 1200 HPLC (Agilent Technologies) coupled to a PE-Sciex API 4000 tandem mass spectrometer with IonSpray® interface (Electrospray Ionization). Typical HPLC and mass spectral operating conditions are summarised in the following tables.

HPLC Conditions

System:	Agilent Series 1200 HPLC (Agilent Technologies)			
Column:	Supelco Aecentis Express C18, 50 mm × 2.1 mm, 2.7µm			
Column Temperature:	30°C			
Injection Volume:	8 µL			
Mobile Phase Conditions:	A: methanol + 0.05 % acetic acid; B: water + 0.05 % acetic acid			
	Time (min)	% A	% B	Flow (mL/min.)
Equilibration	-1.5	5	95	0.50
Injection	0.0	5	95	0.50
	1.0	95	5	0.50
	3.5	95	5	0.50
Retention Times (approx.):	Imazalil	~ 1.8 min		
	T000824	~ 1.6 min		

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TEST METHOD (CONTINUED)
Mass Spectrometer Conditions

MS System:	PE-Sciex API 4000 Tandem mass Spectrometer			
Analyte Monitored	Ions Monitored	Declustering Potential	Collision Energy	Dwell Time (Seconds)
Imazalil	297 → 159	+86 V	+31 V	0.30
Imazalil	297 → 201	+86 V	+25 V	0.30
T000824	257 → 69	+66 V	+49 V	0.20
T000824	257 → 125	+66 V	+47 V	0.20
Ion Mode:	ESI (pos)			

The mass spectrometer was operated in MS/MS-Multiple Reaction Monitoring (MRM) positive ion mode. Integrated peak areas were used for quantitation.

For Imazalil the MRM 297→159 is suitable for quantitation and the MRM 297→201 can be used for confirmation. For T000824 the MRM 257→69 is suitable for quantitation and the MRM 257→125 can be used for confirmation.

External solvent standards: 0.25, 0.5, 5.0 and 20 ng/mL of Imazalil and T000824

Limit of quantitation (LOQ): 0.001 mg/kg of Imazalil and T000824

Calculation of the results

External standard solutions were injected before every two specimen extracts in each analytical sequence. The found analyte concentration, expressed in ng/mL, was calculated using the peak area in integrator units (IU = counts) obtained from the standard solution injected close to the specimen extract during the liquid chromatographic sequence.

The residues (R) in mg/kg are calculated using an external standard with one-point calibration according to the following equation:

$$R = \frac{A_A \times C_{St} \times V_{EX} \times V_{END} \times DF}{A_{St} \times V_{R1} \times G \times 1000}$$

where

A_A : Peak area of Imazalil in final solution, in counts

C_{St} : Concentration of Imazalil in external standard solution, in ng/mL

A_{St} : Peak area of Imazalil in external standard solution, in counts

V_{EX} : Volume of extract: = 10 mL

V_{R1} : 1 st aliquot: = 1.0 mL

V_{END} : Final volume: = 1.0 mL

G: Sample amount: 5 g

DF: Dilution factor (for no dilution = 1)

1000: Conversion factor for ng into μ g = 1000

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TEST METHOD (CONTINUED)

Recovery

Recovery is calculated from the following equation:

$$\text{Recovery (\%)} = \frac{R_{\text{fortified}}}{F} \cdot 100 \%$$

$R_{\text{fortified}}$: Residues of fortified specimen, in mg/kg

F: Fortification, in mg/kg

STOCK, STANDARD AND FORTIFICATION SOLUTIONS

Stock solution no. 4740: 400 µg Imazalil/mL

10.27 mg Imazalil (97.46%; page 11) = 10.00 mg (100 %) were dissolved in acetone and diluted to 25 mL in a volumetric flask.

Stock solution no. 4737: 400 µg R014821/mL

9.98 mg R014821 (100.3%; page 11) = 10.00 mg (100 %) were dissolved in acetone and diluted to 25 mL in a volumetric flask.

Standard solutions

Chromatographic external standard solutions were prepared by diluting the stock solutions with acetonitrile / 0.05 % acetic acid (1/3, v/v) using volumetric pipettes or a dilutor and volumetric flasks.

Working/ Calibration Solution	Volume used [mL]	Dilution volume [mL]	Concentration obtained [ng/mL]	Solution used
L1	0.5	10	50	DZ1
L2	0.2	10	20	DZ1
L3	5	10	10	L2
L4	0.50	2.0	5.0	L2
L5	0.25	2.5	1.0	L3
L6	0.060	1.2	0.50	L3
L7	0.05	2.0	0.250	L3
L8	0,12	1.2	0.10	L5

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STOCK, STANDARD AND FORTIFICATION SOLUTIONS (CONTINUED)

Fortification solutions

Fortification solutions were prepared in acetonitrile (DZ1) or acetonitrile/0.05% acetic acid (1/1, v/v) using a dilutor and volumetric pipettes.

Fortification Solution	Volume used [mL]	Dilution volume [mL]	Concentration obtained [ng/mL]	Solution used
DZ1	each 0.05	20	1000	Stock solutions 4740, 4737
Z1	5.0	10	500	DZ1
Z2	0.2	10	100	DZ1
Z3	1.0	10	10	Z2

All solutions were stored at 4°C in the dark.

FORTIFICATIONS

Control (untreated) specimens of soil were fortified prior to extraction with 0.5 mL of fortification solutions in acetonitrile/0.05% acetic acid (1/1, v/v) as follows:

Matrix	Specimen weight	Fortification solution	Fortification level
Soil	5 g	Z3	0.001 mg/kg
Soil	5 g	Z2	0.010 mg/kg
Soil	5 g	Z1	0.050 mg/kg

Results of recoveries are reported in Appendix 2.

The applicability of the method [1, 2] for the analysis of the residues of Imazalil and T000824 in soil was tested. The specimen extracts were analysed using liquid chromatography with mass selective detection (LC-MS/MS).

Limit of Quantitation

The limit of quantitation (LOQ) for Imazalil and T000824 was established at 0.001 mg/kg in soil. The chromatographic peaks at the lower limit of the working range were greater than the signal equivalent to three times the background noise.

RESULTS AND DISCUSSION (CONTINUED)**Calibration information**

Imazalil (technical grade) and T000824 (metabolite R014821) were used for preparing the external standard solutions. External standard solutions of 0.5, 5.0 and 20 ng/mL from Imazalil and T000824 were prepared. A one-point external standard calibration was carried out using the peak area in integrator units (counts) from injection of known standards versus standard concentrations in ng/mL.

Specificity

The concentration of Imazalil and T000824 in the final extracts was determined by high performance liquid chromatography with MS/MS detection. In order to ensure unambiguous identification two mass transitions were monitored. No significant interferences from the specimen matrix were detected at the retention time corresponding to Imazalil or T000824 in any of the control specimens.