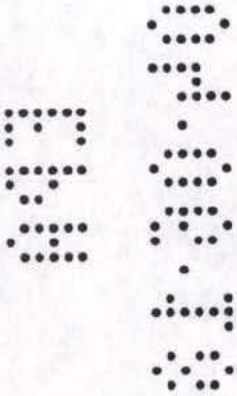
## INTRODUCTION

The objective of this study was to independently validate the analytical method 11106.6116, for measuring residues of Ipconazole in soil and sediment of differing USDA Textural Classification in accordance with the EPA guideline OCSPP 850.6100 (2012): Environmental Chemistry Methods and Associated Independent Laboratory Validation.

Control samples of Calwich Abbey sediment and Speyer 5M soil were fortified with Ipconazole Technical (containing a mixture of Ipconazole cc and ct isomers) at concentrations of 50 and 500 µg/kg (total Ipconazole) in quintuplicate and analysed. Samples were extracted with acetonitrile: water: formic acid (90:10:0.1 v:v:v) followed by dilution into the calibration range with methanol: water (50:50 v:v). Control extracts from sediment and soil were used to prepare matrix matched standards, and were analysed against non-matrix standards to assess matrix effects. Samples were analysed using LC-MS/MS.

Matrix effects, linearity and specificity of the method were determined. Precision and accuracy was calculated at each validation level in each soil for total Ipconazole (primary and confirmatory).



## MATERIALS AND METHODS

### Test Substances

| | |
|---|---|
| **Test substance name:** | **Ipconazole Technical** |
| CAS number: | 125225-28-7 |
| IUPAC name: | (1*RS*,2*SR*,5*RS*;1*RS*,2*SR*,5*SR*)-2-(4-chlorobenzyl)-5-isopropyl-1-(1*H*-1,2,4-triazol-1-ylmethyl)cyclopentanol |
| Molecular formula: | $C_{18}H_{24}ClN_3O$ |
| Sponsor lot number: | 89010 |
| Purity: | 96.7% w/w (as total Ipconazole), 89.7% w/w (as Ipconazole cc), and 7.0% w/w (as Ipconazole ct) |
| Molecular mass: | 333.9 g/mol |
| Storage conditions: | Room Temperature (15-30°C) |
| Expiry date: | 24 November 2019 |

| | |
|---|---|
| **Test substance name:** | **Ipconazole cc** |
| CAS number: | 115850-69-6 |
| Sponsor lot number: | G-00328 |
| Purity: | 99.5% w/w |
| Storage conditions: | Room Temperature (15-30°C) |
| Expiry date: | 12 September 2021 |

| | |
|---|---|
| **Test substance name:** | **Ipconazole ct** |
| CAS number: | 115937-89-8 |
| Sponsor lot number: | G-00329 |
| Purity: | 99.7% w/w |
| Storage conditions: | Room Temperature (15-30°C) |
| Expiry date: | 09 September 2021 |

Certificates of Analysis for the test substances are presented in Appendix 1.

### Test System

Control samples of soil and sediment with differing USDA Textural Classification were sourced by Smithers Viscient (ESG). The soils used were Calwich Abbey sediment (silt loam) and Speyer 5M soil (sandy loam).

Soil characterisation data are listed in the table below:

| Soil Name | Test System Code | Textural Class[1] | % Sand, Silt, Clay[2] | CEC (meq/100 g) | % Organic Carbon | pH in $H_2O$ | pH in 0.01M $CaCl_2$ |
|---|---|---|---|---|---|---|---|
| Calwich Abbey | CS 55/17 | silt loam | 29, 57, 14 | 17.4 | 4.7 | 7.7 | 7.3 |
| Speyer 5M | CS 31/17 | sandy loam | 59, 30, 11 | 15.7 | 1.0 | 8.3 | 7.3 |

[1, 2] USDA classification.

The certificates of analysis for each soil are presented in Appendix 2.

**Reagents**

| | |
|---|---|
| Acetonitrile | HPLC grade, Honeywell |
| Methanol | HPLC grade, Honeywell |
| Water | Milli-Q with LCPAK polisher, In House |
| Formic acid | ACS reagent, Honeywell |
| 0.1% Formic acid in water | LC-MS grade, Honeywell |
| 0.1% Formic acid in acetonitrile | LC-MS grade, Honeywell |

Equivalent or better reagents may have been used.

**Equipment**

Shimadzu Nexera series HPLC system with AB Sciex API 5000 MS/MS detector.

**Analytical Method**

Analytical method 11106.6116 was supplied by the sponsor. The method used LC-MS/MS analysis.

***Preparation of Reagents***

Acetonitrile: water: formic acid (90:10:0.1 v/v/v) was prepared by mixing 1800 mL acetonitrile with 200 mL water and 2 mL formic acid.

Methanol: water (50:50 v/v) was prepared by mixing 500 mL methanol with 500 mL water.

***Preparation of Stock Solutions***

Primary stock solutions of Ipconazole Technical, Ipconazole cc and Ipconazole ct were prepared as described in the table below:

| Stock ID | Test substance | Amount Weighed (mg) | Purity (%) | Solvent | Final Volume (mL) | Concentration (µg/mL)[1] |
|---|---|---|---|---|---|---|
| Stock 1 | Ipconazole cc | 10.91 | 99.5 | Methanol | 10.856 | 1000 |
| Stock 2 | | 10.38 | | | 10.328 | 1000 |
| Stock 3 | Ipconazole ct | 10.18 | 99.7 | | 10.150 | 1000 |
| Stock 4 | | 10.44 | | | 10.409 | 1000 |
| Stock 5 | Ipconazole Technical | 11.14 | 96.7[2] | | 10.773 | 1000 |
| Stock 6 | | 11.02 | | | 10.657 | 1000 |

[1] Corrected for Purity.
[2] Purity is 96.7% w/w (as total Ipconazole), 89.7% w/w (as Ipconazole cc), and 7.0% w/w (as Ipconazole ct).

Primary stocks were stored refrigerated (2-8°C) in amber glass bottles and given a nominal three month expiry.

Secondary stock solutions of Ipconazole Technical, Ipconazole cc and Ipconazole ct were prepared as described in the table below:

| Stock ID | Test substance | Stock Concentration (µg/mL) | Volume Taken (mL)[1] | Solvent | Final Volume (mL)[2] | Concentration (µg/mL) |
|---|---|---|---|---|---|---|
| Stock 1 | Ipconazole cc | 1000 | 0.1 | Methanol | 10 | 10 |
| Stock 2 | | 1000 | 0.1 | | 10 | 10 |
| Stock 3 | Ipconazole ct | 1000 | 0.1 | | 10 | 10 |
| Stock 4 | | 1000 | 0.1 | | 10 | 10 |
| Stock 5 | Ipconazole Technical | 1000 | 1 | | 10 | 100 |
| Stock 6 | | 1000 | 1 | | 10 | 100 |

Secondary stocks were stored refrigerated (2-8°C) in amber glass bottles and given a nominal one month expiry.

Stock solutions of Ipconazole Technical for sample fortification and mixed stock solutions of Ipconazole cc and Ipconazole ct for calibration standard preparation were prepared as described in the table below:

| Test Substance | Secondary Stock Concentration (µg/mL) | Volume Taken (mL) | Solvent | Final Volume (mL) | Concentration (µg/mL) |
|---|---|---|---|---|---|
| Ipconazole cc | 10 | 0.25 | Methanol | 50 | 0.05 |
| Ipconazole ct | 10 | 0.0175 | | | 0.0035 |
| Ipconazole Technical | 100 | 1 | | 10 | 10 |
| | 10 | 1 | | 10 | 1 |
| | 1 | 0.1 | | 10 | 0.01 |

Stock solutions for sample fortification and calibration standard preparation were prepared on the day of use and stored refrigerated until the analysis was complete.

***Preparation of Calibration Standards***

Mixed calibration standards of Ipconazole cc and Ipconazole ct were prepared in methanol: water (50:50 v/v) as described in the table below:

| Mixed Stock Concentration (µg/L)[1] | Volume Taken (mL) | Final Volume (mL) | Concentration (µg/L)[1] |
|---|---|---|---|
| 50/3.5[2] | 0.1 | 10 | 0.5/0.035 |
| 0.5/0.035 | 0.8 | 1 | 0.4/0.028 |
| 0.5/0.035 | 0.6 | 1 | 0.3/0.021 |
| 0.5/0.035 | 0.4 | 1 | 0.2/0.014 |
| 0.5/0.035 | 0.2 | 1 | 0.1/0.007 |
| 0.5/0.035 | 0.1 | 1 | 0.05/0.0035 |

[1] Concentrations expressed as Ipconazole cc/Ipconazole ct concentrations.
[2] 0.05/0.0035 µg/mL is equivalent to 50/3.5 µg/L.

Calibration standards were prepared on the day of analysis and discarded when analysis was complete. A single set of calibration standards was prepared for each

validation batch, which was analysed once before the samples and once after the samples. When samples required re-injection due to failure, the same calibration standards were used as the initial injection, so that the calibration standards and sample extracts were equally aged. Suitability of aged calibration standards was verified by an acceptable correlation coefficient.

***Matrix Matched and Non-Matrix Matched Standards***

In order to assess any possible matrix effect, matrix matched standards of Ipconazole Technical were prepared in control sample final extract for soil and sediment in triplicate. Non-matrix standards were prepared in methanol: water (50:50 v/v) in triplicate as described in the table below:

| Fortification Stock Concentration (µg/mL) | Volume Taken (mL) | Solvent | Final Volume (mL) | Concentration (µg/L) |
|---|---|---|---|---|
| 0.01 | 0.1 | Methanol: water (1:1 v/v) | 10 | 0.1 |
| 0.01 | 0.1 | | 10 | 0.1 |
| 0.01 | 0.1 | | 10 | 0.1 |
| 0.01 | 0.1 | Calwich Abbey sediment final extract Control A | 10 | 0.1 |
| 0.01 | 0.1 | | 10 | 0.1 |
| 0.01 | 0.1 | | 10 | 0.1 |
| 0.01 | 0.1 | Speyer 5M soil final extract Control B | 10 | 0.1 |
| 0.01 | 0.1 | | 10 | 0.1 |
| 0.01 | 0.1 | | 10 | 0.1 |

***Sample Preparation and Fortification***

The moisture content of the soil was determined and the weight of wet soil equivalent to 5 g dry weight was calculated. The required amount of wet soil (±0.005 g) was weighed into 50 mL centrifuge tubes. A single control soil and sediment was prepared for matrix assessment. Quintiplicate soil samples were fortified at the LOQ (50 µg/kg) and at 10 × LOQ (500 µg/kg) with Ipconazole Technical. Duplicate control soils and a reagent blank (without soil) were also prepared, as described in the following tables:

*Recovery samples in silt loam sediment*

| Sample ID | Dry Weight (g) | Fortification Stock Concentration (µg/mL) | Volume Added (mL) | Fortified Concentration (µg/kg) |
|---|---|---|---|---|
| Reagent Blank A | N/A | N/A | N/A | N/A |
| Control A[1] | 5 | N/A | N/A | N/A |
| Control C-D | 5 | N/A | N/A | N/A |
| F50 A-E | 5 | 1 | 0.25 | 50 |
| F500 A-E | 5 | 10 | 0.25 | 500 |

N/A = Not applicable.
[1] Control A was used for matrix assessment only.

*Recovery samples in sandy loam soil*

| Sample ID | Dry Weight (g) | Fortification Stock Concentration (µg/mL) | Volume Added (mL) | Fortified Concentration (µg/kg) |
|---|---|---|---|---|
| Reagent Blank B | N/A | N/A | N/A | N/A |
| Control B[1] | 5 | N/A | N/A | N/A |
| Control E-F | 5 | N/A | N/A | N/A |
| F50 F-J | 5 | 1 | 0.25 | 50 |
| F500 F-J | 5 | 10 | 0.25 | 500 |

N/A = Not applicable.
[1] Control B was used for matrix assessment only.

***Soil Extraction***

The samples were extracted twice with 20 mL acetonitrile: water: formic acid (90:10:0.1 v/v/v) by shaking at 150 rpm for 30 minutes and centrifuging at 3000 rpm for 10 minutes. The extracts were combined and made to 50 mL volume with acetonitrile: water: formic acid (90:10:0.1 v/v/v). Samples were then further diluted into calibration range with methanol: water (50:50 v:v). The extraction and dilution procedures are detailed in the table below:

*Extraction and dilution in silt loam sediment*

| Sample ID | Nominal Soil Concentration (µg/kg) | Dry Weight (g) | Extract Volume (mL) | Extract Dilution (mL to mL) | Overall Dilution Factor | Nominal Extract Concentration after Dilution (µg/L) |
|---|---|---|---|---|---|---|
| Reagent Blank A | N/A | N/A[1] | 50 | 0.2 to 10 | 500 | N/A |
| Control A | N/A | 5 | 50 | 1 to 50 | 500 | N/A |
| Control C-D | N/A | 5 | 50 | 0.2 to 10 | 500 | N/A |
| F50 A-E | 50 | 5 | 50 | 0.2 to 10 | 500 | 0.1 |
| F500 A-E | 500 | 5 | 50 | 0.075 to 10 | 1333 | 0.375 |

N/A = Not applicable.
[1] A dry weight of 5 g was used for calculation of the overall dilution factor in the Reagent Blank.
F500 A-E were re-diluted because an error was suspected with the original dilution.

*Extraction and dilution in sandy loam soil*

| Sample ID | Nominal Soil Concentration (µg/kg) | Dry Weight (g) | Extract Volume (mL) | Extract Dilution (mL to mL) | Overall Dilution Factor | Nominal Extract Concentration after Dilution (µg/L) |
|---|---|---|---|---|---|---|
| Reagent Blank B | N/A | N/A[1] | 50 | 0.2 to 10 | 500 | N/A |
| Control B | N/A | 5 | 50 | 1 to 50 | 500 | N/A |
| Control E-F | N/A | 5 | 50 | 0.2 to 10 | 500 | N/A |
| F50 F-J | 50 | 5 | 50 | 0.2 to 10 | 500 | 0.1 |
| F500 F-J | 500 | 5 | 50 | 0.075 to 10 | 1333 | 0.375 |

N/A = Not applicable.
[1] A dry weight of 5 g was used for calculation of the overall dilution factor in the Reagent Blank.

***Instrument Conditions***

LC-MS/MS analysis was performed using the following instrument conditions:

HPLC Parameters:

| | | | |
|---|---|---|---|
| Column | XBridge C18 3.5 µm 2.1 × 50 mm | | |
| Mobile Phase A | 0.1% Formic acid in water | | |
| Mobile Phase B | 0.1% Formic acid in acetonitrile | | |
| Flow Rate | 0.4 mL/min | | |
| Gradient | Time (min) | Mobile Phase A (%) | Mobile Phase B (%) |
| | 0 | 50 | 50 |
| | 4 | 50 | 50 |
| | 7 | 30 | 70 |
| | 7.1 | 0 | 100 |
| | 9 | 0 | 100 |
| | 9.1 | 50 | 50 |
| Run Time | 10.5 minutes | | |
| Column Temperature | 40°C | | |
| Autosampler Temperature | 5°C | | |
| Injection Volume | 10 µL | | |
| Retention Time | Approx. 3.7 minutes (Ipconazole ct) | | |
| | Approx. 3.9 minutes (Ipconazole cc) | | |
| Valco Valve Diverter | Time (min) | Position | |
| | 0 | A (to waste) | |
| | 1 | B (to MS) | |
| | 9.5 | A (to waste) | |

MS/MS Parameters:

| | | | |
|---|---|---|---|
| Instrument | AB Sciex API 5000 Triple Quadrupole Mass Spectrometer | | |
| Ionisation Type | Electrospray (ESI) | | |
| Polarity | Positive | | |
| Scan Type | Multiple reaction monitoring (MRM) | | |
| Ion Spray Voltage | 4000 | | |
| Collision Gas (CAD) | 5 | | |
| Curtain Gas (CUR) | 25 | | |
| Gas Flow 1 (GS1) | 40 | | |
| Gas Flow 2 (GS2) | 40 | | |
| Vaporiser Temperature (TEM) | 500 | | |
| Interface Heater (ihe) | On | | |
| Entrance Potential (EP) | 10 | | |
| Collision Exit Potential (CXP) | 13 | | |
| Declustering Potential (DP) | 100 | | |
| Compound Name | MRM Transition Ions Monitored | Collision Energy (CE) | Dwell Time (ms) |
| Ipconazole cc/ct (Primary) | 334 - 70 | 35 | 200 |
| Ipconazole cc/ct (Confirmatory) | 336 - 70 | 42 | 200 |

LC-MS/MS data was collected using Analyst 1.6.2.

***Calculation of Results***

Results were calculated using Analyst 1.6.2. When the calibration fit is linear, Analyst uses the following formula to calculate the concentration of test substance present in the sample extract:

$x = (y - c) / m$

Where:

x = concentration of test substance in sample extract (µg/L)
y = peak area due to test substance
c = y intercept on calibration graph
m = gradient of the calibration graph

The concentration of test substance in the initial sample was calculated as follows:

Sample concentration (µg/kg) = Extract concentration (µg/L) × Dilution factor

Dilution factor = Final extract volume (mL) / soil weight in final extract (g)

Procedural recovery from fortified samples was calculated as follows:

Recovery (%) = Sample concentration / Fortified concentration × 100

95% confidence intervals were calculated for each validation level as follows:

95% confidence interval (±) =

1.96 × standard deviation of results / square root of the number of replicate results

Grubbs test for outliers was calculated as follows:

G value = (suspect result - mean result) standard deviation of results

If the G value is greater than the critical value (1.715 for a sample size of 5) the result is an outlier with a significance of 0.05.

The limit of detection (LOD) based upon the sample concentration equivalent to three times the baseline noise of a control sample was calculated as follows:

LOD = 3 × height of control baseline noise × control dilution factor × calibration standard concentration (µg/L) / height of calibration standard peak

The relative proportions of the cc and ct isomers in Ipconazole technical were calculated by dividing the isomer % purity by the total % purity stated on the Certificate of Analysis in Appendix 1.

***Validation Pass Criteria***
The validation was deemed acceptable if the following criteria were met for both the primary and confirmatory transitions monitored for total Ipconazole:

*Mean Recovery and Precision* – Recovery and precision were acceptable if each fortification level had a mean recovery between 70 and 120% and a % RSD (relative standard deviation) $\leq$ 20%.

*Specificity/Selectivity* – Specificity was acceptable if the amounts found in blank samples were $\leq$ 30% of the limit of quantification (LOQ).

*Linearity* – Linearity was acceptable if the lowest calibration standard concentration was $\leq$ 20% lower than the lowest sample nominal concentration and the highest calibration standard was $\geq$ 20% higher than the highest nominal sample concentration (after dilution). If matrix effects were determined to be significant, matrix matched standards would be used. The correlation coefficient (r) was acceptable if it was $\geq$ 0.99.

***Limit of Detection (LOD) Assessment***
An estimate of the LOD was made at 3 × baseline noise of control soil and sediment for Ipconazole cc and Ipconazole ct (primary and confirmatory).

***Method Detection Limit (MDL) Assessment***
The MDL was calculated as the sample concentration equivalent to the lowest calibration standard (after dilution).

***Matrix Assessment***
An assessment of matrix effects was made by comparison of peak areas from control matrix final extracts fortified in triplicate with Ipconazole Technical against methanol: water (1:1 v/v) fortified in triplicate with Ipconazole Technical.

Results were presented as a % difference from the mean non-matrix standard value for Ipconazole cc and Ipconazole ct (primary and confirmatory).

A difference of $\geq$ 20% was considered significant.

***Variations to the Method***

Small variations to the given method are listed as follows:

Reagent supplier
Equipment supplier
Soil type
Stock concentrations
Scaling of reagents and solutions
Optimisation of MRM transitions, MS voltages and gas pressures
LC-MS software
Use of measuring cylinder, rather than a volumetric flask to make extracts to volume
Not re-centrifuging soil final extracts
Using a different type of mass spectrometer
Not using the same injector rinse solvents on the HPLC

These small variations demonstrated robustness of the method when transferred to an independent laboratory environment, and did not adversely affect the validation results.