Final Report

Study Title	Tebuconazole – Independent Laboratory Validation in Soil
Study Guideline(s)	EPA 850.6100 (2012) SANCO/3029/99 rev 4 (2000)

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INTRODUCTION

The objective of this study was to independently validate the analytical method 14162.6114, for measuring residues of Tebuconazole in silt loam and sandy loam soils by LC-MS/MS, in accordance with EPA 850.6100 (2012) and SANCO/3029/99 rev 4 (2000) guidelines.

Control samples of Newhaven and RefeSol 01-A soil were fortified with Tebuconazole at 50 and 500 µg/kg in quintuplicate and analysed. Samples were extracted with acetonitrile. Aliquots were diluted into calibration range with acetonitrile: water (20:80 v/v).

To assess matrix effects, calibration standards were prepared in acetonitrile: water (20:80 v/v) and in the final extract of untreated soil.

Samples were analysed for Tebuconazole using liquid chromatography with tandem mass spectrometry detection (LC-MS/MS).

Matrix effects, linearity and specificity of the method were determined. Precision and accuracy was calculated at each validation level in Newhaven and RefeSol 01-A soil. One primary and one confirmatory LC-MS/MS transition were analysed for Tebuconazole.

MATERIALS AND METHODS

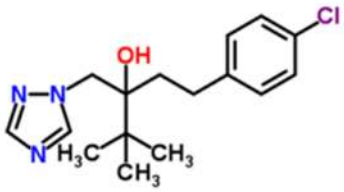
Test Substance

Test substance name: **Tebuconazole**

CAS Number: 107534-96-3

Molecular Formula: $C_{16}H_{22}ClN_3O$

Structure:



Molecular Mass: 307.82 g/mol

Purity: 98.2%

Batch Number: 201706010

Storage Conditions: Room Temperature (15-30°C)

Recertification Date: 09 August 2020[1]

[1] The test substance was manufactured in June 2017 and was certified on 03 July 2018 with an expiry date of June 2019. It was re-analysed on 09 August 2018 with a recertification date of 09 August 2020. The certified purity had not altered significantly between the two analysis dates, and was therefore considered to be stable for the duration of this study.

Test Matrix

Control samples of soil were sourced by Smithers Viscient (ESG) Ltd. The soils used were CS 17/18 Newhaven (silt loam) and CS 30/18 RefeSol 01-A (sandy loam) soil.

Soil characterisation data are listed in the following table:

Soil Name	Textural class[1]	% Sand, Silt, Clay[2]	CEC[3] (meq/100 g)	% Organic Carbon	pH in H_2O	pH in 0.01M $CaCl_2$
Newhaven	silt loam	25, 51, 24	17.4	3.2	6.0	5.4
Refesol 01-A	sandy loam	74, 20, 6	5.3	0.9	6.4	5.3

[1, 2] USDA classification.
[3] CEC = cation exchange capacity.

The soil moisture contents were determined by oven-drying a sample at nominally 105°C overnight, and were calculated to be 30.9% and 4.6% of the dry soil weight for Newhaven and RefeSol 01-A soil respectively.

Reagents

• Acetonitrile	HPLC grade, VWR
• Water	Milli-Q with LCPAK polisher, In House
• 0.1% Formic acid in water	MS grade, Honeywell
• 0.1% Formic acid in acetonitrile	MS grade, Honeywell

Equipment

- Shimadzu Nexera series HPLC system with AB Sciex API 5000 MS/MS detector.
- HPLC column: Waters XBridge BEH C18, 2.5 µm, 2.1 × 50 mm
- Analytical balance
- Centrifuge: Beckman Coulter Allegra X-15R
- Micro Centrifuge: Thermo Scientific Sorvall Legend Micro 21
- Orbital shaker: Edmund Buhler SM 30 A
- Positive displacement pipettes
- Nalgene centrifuge tubes
- Micro centrifuge tubes
- Volumetric flasks
- Glass Jars
- Amber glass vials
- Disposable glass vials
- HPLC vials

Analytical Method

Analytical method 14162.6114 was supplied by Smithers Viscient, Wareham on behalf of the sponsor. The method was re-written as SMV 3202239-01D to take into account minor differences in instrumentation, reagents and consumables before validation, and re-issued as SMV 3202239-01V after validation. The complete analytical procedure is presented in Appendix 6.

Preparation of Reagents

Acetonitrile: water (20:80 v/v) was prepared by mixing 100 mL HPLC grade acetonitrile with 400 mL Milli-Q water.

Preparation of Stock Solutions

Primary stock solutions of Tebuconazole were prepared at 1000 µg/mL under GLP study No. 3202240 (Tebuconazole – Independent Laboratory Validation in Water) and were used for method validation.

Primary stocks were stored refrigerated in amber glass bottles and given a nominal expiry of three months.

A secondary stock solution of Tebuconazole in acetonitrile was prepared at 10 µg/mL under study 3202240.

Sub-stock solutions were prepared in volumetric flasks as described in the following table:

Stock Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/mL)
10	1	Acetonitrile	10	1
1	0.1		10	0.01
0.01	1		10	0.001[1]

[1] Equivalent to 1 µg/L.

Sub-stock solutions were prepared on the day of use and stored refrigerated in disposable glass vials until the corresponding analysis was complete.

Matrix Matched Standards for Matrix Assessment
Matrix matched standards of Tebuconazole were prepared in control soil final extract as described in the following tables:

Newhaven soil

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
1	0.15	Newhaven soil final extract	10	0.015
1	0.15		10	0.015
1	0.15		10	0.015

RefeSol 01-A soil

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
1	0.15	RefeSol 01-A soil final extract	10	0.015
1	0.15		10	0.015
1	0.15		10	0.015

Non-Matrix Matched Standards for Matrix Assessment
Non-matrix standards of Tebuconazole were prepared in acetonitrile: water (20:80 v/v) as described in the following table.

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
1	0.15	Acetonitrile: water (20:80 v/v)	10	0.015
1	0.15		10	0.015
1	0.15		10	0.015

The matrix matched standard and non-matrix matched standards were analysed and the peak areas compared. The matrix effect was considered to be significant if the mean matrix matched standard area is ≥ 20% different from the non-matrix standard area

Matrix Matched Calibration Standards

Matrix matched standards were used for consistency with the primary method (14162.6114), regardless of the matrix effect. Matrix matched calibration standards of Tebuconazole were prepared in control soil final extract as described in the following tables:

Newhaven soil

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
10	0.25	Newhaven soil final extract	10	0.25
0.25	0.72		1	0.18
0.25	0.4		1	0.1
0.25	0.26		1	0.065
0.25	0.18		1	0.045
0.25	0.12		1	0.03
0.25	0.08		1	0.02
0.25	0.05		1	0.0125
0.25	0.032		1	0.008
0.25	0.02		1	0.005

RefeSol 01-A soil

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
10	0.25	RefeSol 01-A soil final extract	10	0.25
0.25	0.72		1	0.18
0.25	0.4		1	0.1
0.25	0.26		1	0.065
0.25	0.18		1	0.045
0.25	0.12		1	0.03
0.25	0.08		1	0.02
0.25	0.05		1	0.0125
0.25	0.032		1	0.008
0.25	0.02		1	0.005

A second set of calibration standards was prepared for RefeSol 01-A soil from the same 10 µg/L sub-stock solution, for re-injection with the original sample extracts.

A single set of matrix matched calibration standards was prepared for each validation batch and injected twice, interspersed with and bracketing the samples.

Sample Fortification

5±0.05 g dry weight of soil was weighed into a Teflon centrifuge tube. Quintuplicate soil samples were fortified at the LOQ (50 µg/kg) and at 10 × LOQ (500 µg/kg) with a Tebuconazole standard in acetonitrile. Duplicate control soil samples and a reagent blank (no soil) were also prepared, as described in the following tables:

Newhaven soil

Sample ID	Dry Soil Weight (g)	Stock Concentration (µg/mL)	Volume Added (mL)	Fortified Concentration (µg/kg)
Reagent Blank A	N/A	N/A	N/A	N/A
Control A-B	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.

RefeSol 01-A soil

Sample ID	Dry Soil Weight (g)	Stock Concentration (µg/L)	Volume Added (mL)	Fortified Concentration (µg/kg)
Reagent Blank B	N/A	N/A	N/A	N/A
Control C-D	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.

Sample Extraction

20 mL acetonitrile was added to 5±0.05 g dry weight of soil, placed on a shaker at 150 rpm for 30 minutes and centrifuged at 3000 rpm for 10 minutes. The supernatant was transferred into a glass jar and the extraction procedure repeated. The two extracts were combined and made to 50 mL volume with acetonitrile. A portion of extract was diluted into the calibration range with acetonitrile: water (20:80 v/v). A portion of the final extract was transferred into a micro centrifuge tube, centrifuged at 13,000 rpm for 5 minutes then transferred into an HPLC vial for analysis. Sample extracts were stored refrigerated in case further analysis was required. The extraction procedure is summarised in the following tables:

Newhaven soil

Sample ID	Fortified Concentration (µg/kg)	Dry Soil Weight (g)	Extract Volume (mL)	Sample Dilution (mL to mL)	Dilution Factor
Reagent Blank A	N/A	N/A	50	0.03-10	3333
Control A-B	N/A	5	50	0.03-10	3333
F50 A-E	50	5	50	0.03-10	3333
F500 A-E	500	5	50	0.03-10	3333[1]

N/A = Not applicable.
[1] F500 A was made to a sample volume of 66 mL in error; therefore the dilution factor was 4400.

RefeSol 01-A soil

Sample ID	Fortified Concentration (µg/kg)	Dry Soil Weight (g)	Extract Volume (mL)	Sample Dilution (mL to mL)	Dilution Factor
Reagent Blank B	N/A	N/A	50	0.03-10	3333
Control C-D	N/A	5	50	0.03-10	3333
F50 F-J	50	5	50	0.03-10	3333
F500 F-J	500	5	50	0.03-10	3333

N/A = Not applicable.

Instrument Conditions

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

HPLC Parameters:

Instrument:	Shimadzu Nexera series HPLC system		
Column#:	Waters XBridge BEH C18, 2.5 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:	0.5 mL/min		
Gradient:	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.00	80	20
	0.50	80	20
	3.00	0	100
	3.50	0	100
	3.51	80	20
	5.00	80	20
Run Time:	5.0 minutes		
Column Temperature:	40°C		
Autosampler Temperature:	10°C		
Injection Volume:	50 µL		
Retention Time:	Approx. 2.8 minutes		
Valco Valve Diverter:	Time (min)	Position	
	0	A (to waste)	
	1	B (to MS)	
	4	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:	5000 V			
Collision Gas (CAD):	8			
Curtain Gas (CUR):	25			
Gas Flow 1 (GS1):	40			
Gas Flow 2 (GS2):	40			
Vaporiser Temperature (TEM):	500°C			
Interface Heater (ihe):	On			
Entrance Potential (EP):	10			
Collision Exit Potential (CXP):	13			
Transition Name:	MRM Transition Ions Monitored	Declustering Potential (DP)	Collision Energy (CE)	Dwell Time (ms)
Tebuconazole (Primary):	308.4/70.0	120	36	75
Tebuconazole (Confirmatory):	308.4/125.0	120	54	75

Parameters marked # may not be modified. Minor adjustments to the remaining parameters may be required in order to fully optimise the system.

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

Calculation of Results

LC-MS/MS data were calculated using Analyst 1.6.2. When the calibration fit is linear as in this study, 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 calibration line used a 1/x weighting.

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

Sample concentration (µg/kg) = extract concentration (µg/L) × dilution factor

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

Procedural recovery from fortified samples is calculated as follows:

Recovery (%) = sample concentration / fortified concentration × 100

The Limit of Detection (LOD) was measured from the peak height of the lowest calibration standard and the height of the baseline noise in a control sample and was calculated as follows:

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

The Method Detection Limit (MDL) based upon the sample concentration equivalent to the lowest calibration standard was calculated as follows:

MDL (µg/kg) = lowest calibration standard × dilution factor

For the correlation of stocks, each stock was diluted to a concentration near the middle of the calibration line. The diluted stocks were injected five times each, and the mean peak areas calculated. The correlation was then assessed using the following equation:

$$\% \text{ Correlation from the mean} = \frac{(A - B)}{(A + B)} \times 100$$

Where A and B are the mean peak areas for each of the two stocks.
The acceptance criterion is not more than ± 5% difference from the overall mean.

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

Mean Recovery and Precision
Recovery and precision were acceptable if each fortification level had a mean recovery between 70 and 110% and a %RSD (relative standard deviation) ≤ 20%.

Specificity/Selectivity
Specificity was acceptable if no significant interference at the retention time of Tebuconazole was found in the control samples at > 30% of the LOQ.

Linearity
The linear range was acceptable if the lowest calibration standard concentration was ≤ 80% of the equivalent LOQ final extract concentration and the highest calibration standard concentration was ≥ 120% of the 10 × LOQ extract concentration (after dilution if applicable). Matrix matched calibration standards were used for consistency with the primary method (14162.6114). The correlation coefficient (r) was acceptable if it was ≥ 0.995.

LOD (Limit of Detection) Assessment
An estimate of the LOD was made at 3 × baseline noise for primary and confirmatory transitions.

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

Matrix Assessment
An assessment of matrix effects was made by comparison of peak areas for triplicate standards prepared in acetonitrile: water (20:80 v/v) and in untreated soil final extracts. This was assessed for the primary and confirmatory transitions.

Results were presented as a % difference from the mean non-matrix matched standard value.

A difference of ≥ 20% was considered significant.

Limit of Quantification (LOQ)
The LOQ based upon the lowest level validated confirmed the LOQ to be 50 µg/kg for Tebuconazole in Newhaven and RefeSol 01-A soil.

Limit of Detection (LOD)
The LOD based upon the sample concentration equivalent to 3 × baseline noise was calculated in Newhaven and RefeSol 01-A soil (primary and confirmatory transitions). The LOD values are presented in the summary tables at the beginning of the results section.

Method Detection Limit (MDL)
The MDL for Tebuconazole was calculated to be 16.7 µg/kg (based upon a lowest standard concentration of 0.005 µg/L and a control dilution factor of 3333).

Matrix Effects
An assessment of matrix effects was made by comparison of peak areas from standards prepared in control soil final extracts against standards prepared in acetonitrile: water (20:80 v/v). The % difference from the mean non-matrix matched standard value was calculated.

Matrix effects were insignificant (< 20% difference from non-matrix standards) for Tebuconazole in Newhaven and RefeSol 01-A soil. Matrix matched standards were used for consistency with the primary method (14162.6114), regardless of the matrix effect.

Validation Attempts
The first validation attempts for Newhaven soil was acceptable at both levels.
The first injection of the validation attempt for RefeSol 01-A soil failed recovery criteria, although precision was good. Due to recoveries all being approximately 50%, it was suspected that the error was with the calibration standards being prepared at double the correct concentration. A fresh calibration line was prepared from the original sub-stock solution and the original samples were re-injected. The re-injection was acceptable.

Method Issues
There were no issues found with the primary method (14162.6114) or recommendations for improvement.

REFERENCES

EPA Ecological Effects Test Guideline OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation (January 2012).

EC Guideline SANCO/3029/99 rev 4: Residues: Guidance for generating and reporting methods of analysis in support of pre-registration data requirements for Annex II (part A, Section 4) and Annex III (part A, Section 5) of Directive 91/414 (11 July 2000).

Smithers Viscient (Wareham) GLP Study No. 14162.6114: Environmental Chemistry Method: Validation of the Analytical Method for the Determination of Tebuconazole in Sediment/Soil Matrices by LC-MS/MS.

Smithers Viscient (ESG) Ltd. GLP Study No. 3202240: Tebuconazole – Independent Laboratory Validation in Water.

APPENDICES

Appendix 6
Analytical Procedure

Analytical Procedure

Procedure Title	Determination of Tebuconazole in Soil by LC-MS/MS
Procedure Code	SMV 3202239-01V
Issue Date	12 December 2018
Page Number	1 of 12

The methodology described in this procedure has been validated in Newhaven soil and RefeSol 01-A soil at 50 and 500 µg/kg.

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REVISION HISTORY

SMV 3202239-01V	New method issued following independent laboratory validation of Smithers Viscient, Wareham study 14162.6114

SAFETY PRECAUTIONS

Operators should take the normal precaution of wearing gloves, laboratory coats and safety glasses when handling compound and matrix samples.

Safety assessments (Control of Substances Hazardous to Health, COSHH) have been made of those procedural steps involving preparation of solutions, reagents and analysis of matrix samples. Appropriate safety codes have been included in the text and are defined in the section titled General Handling Control Categories.

The hazards and risks of the substances hazardous to health used in this method have been considered. Provided the method is accurately followed and the control measures specified in the method are correctly used, there should be no foreseeable hazards to health.

INTRODUCTION

This method describes the procedure for determining concentrations of Tebuconazole in soil by LC-MS/MS. Soil is extracted with acetonitrile and diluted into calibration range with acetonitrile: water (20:80 v/v). The extracts are quantified by LC-MS/MS.

Matrix effects for Tebuconazole in Newhaven soil and RefeSol 01-A soil were determined by comparing peak areas of calibration standards prepared in control soil final extract and in acetonitrile: water (20:80 v/v).

Matrix effects are considered significant if the matrix matched standard area is ≥ 20% different from the non-matrix standard area.

Matrix matched calibration standards were used for method validation for consistency with the primary method (14162.6114), even if no significant matrix effects were observed.

APPARATUS, MATERIALS, REAGENTS AND SOLUTIONS

Apparatus and Glassware

- Shimadzu Nexera series HPLC system with AB Sciex API 5000 MS/MS detector
- HPLC column: Waters XBridge BEH C18, 2.5 µm, 2.1 × 50 mm
- Analytical balance
- Centrifuge: Beckman Coulter Allegra X-15R
- Micro Centrifuge: Thermo Scientific Sorvall Legend Micro 21
- Orbital shaker: Edmund Buhler SM 30 A
- Positive displacement pipettes
- Nalgene centrifuge tubes
- Micro centrifuge tubes
- Volumetric flasks
- Glass Jars
- Amber glass vials
- Disposable glass vials
- HPLC vials

Equivalent equipment may be used if required

Materials

• Acetonitrile	HPLC grade, VWR
• Water	Milli-Q (with LCPAK polisher)
• 0.1% Formic acid in water	LC-MS grade, Honeywell
• 0.1% Formic acid in acetonitrile	LC-MS grade, Honeywell

Equivalent materials may be used if required

Reagents

Acetonitrile: water (20:80 v/v)

Acetonitrile: water (20:80 v/v) is prepared by mixing 100 mL HPLC grade acetonitrile with 400 mL Milli-Q water.

Reagent volumes may be scaled as appropriate.

Standard Solution Preparation [1b, 4a]

Primary Standard Stock

Prepare duplicate stock solutions of Tebuconazole at 1000 µg/mL in acetonitrile. Accurately weigh ≥ 10 mg test substance, corrected for purity and transfer into a 10 mL volumetric flask. Adjust the volume to give exactly 1000 µg/mL. Transfer into amber glass bottles. The primary stocks should be stored refrigerated and given a nominal expiry date of 3 months.

Standard Correlation

Dilute the duplicate primary stocks to the mid-point of the calibration line. Correlate the standard solutions by injecting each of the two calibration standards 5 times into the LC-MS/MS. Ensure that the two solutions are injected alternately in the run sequence. The results for the correlation should be ± 5% of the overall mean calculated by peak areas.

Review of Results

Review the data and document the correlation calculations. If the correlation is out of specification, either repeat the injections, re-dilute, or prepare two new stock standards and repeat the procedures in sections *<<Initial Weighing of Stock Solutions>>* to *<<Review of Results>>*.

If the acceptance criteria from section *<<Standard Correlation>>* have been met, then the calibration solutions are acceptable for use. If required, fortification solutions for method validation will be made from the same stock standard, or its dilutions, from which the calibration line has been prepared.

Secondary Standard Stocks

Prepare secondary stock solutions of Tebuconazole in volumetric flasks as described in the following table:

Primary Stock Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Secondary Stock Concentration (µg/mL)
1000	1	Acetonitrile	10	100
1000	0.1		10	10

Transfer into amber glass bottles. The secondary stocks should be stored refrigerated and given a nominal expiry date of 1 month.

Sub-Stocks

Prepare sub-stock solutions of Tebuconazole in volumetric flasks as described in the following table:

Secondary Stock Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Sub-Stock Concentration (µg/mL)
10	1	Acetonitrile	10	1
1	0.1		10	0.01
0.01	1		10	0.001[1]

[1] Equivalent to 1 µg/L.

Transfer into disposable glass vials. The sub-stock solutions should be prepared on the day of use.

Matrix Matched Standards for Matrix Assessment

Prepare Newhaven soil and RefeSol 01-A soil matrix matched standards of Tebuconazole in disposable glass vials as described in the following table:

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
1	0.15	Soil final extract	10	0.015
1	0.15		10	0.015
1	0.15		10	0.015

Non-Matrix Matched Standards for Matrix Assessment

Prepare non-matrix matched standards of Tebuconazole in disposable glass vials as described in the following table:

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
1	0.15	Acetonitrile: water (20:80 v/v)	10	0.015
1	0.15		10	0.015
1	0.15		10	0.015

Matrix Matched Calibration Standards

Prepare Newhaven soil or RefeSol 01-A soil matrix matched calibration standards of Tebuconazole as described in the following table:

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
10	0.25	Soil final extract	10	0.25
0.25	0.72		1	0.18
0.25	0.4		1	0.1
0.25	0.26		1	0.065
0.25	0.18		1	0.045
0.25	0.12		1	0.03
0.25	0.08		1	0.02
0.25	0.05		1	0.0125
0.25	0.032		1	0.008
0.25	0.02		1	0.005

Transfer a portion into a micro centrifuge tube and centrifuge at 13,000 rpm for 5 minutes.

A single set of calibration standards should be prepared for each validation batch and injected twice, interspersed with and bracketing the samples.

PROCEDURES

All procedures will be carried out in compliance with departmental SOPs, following departmental safety procedures in conjunction with COSHH assessments.

All work should be carried out under the minimum control categories listed under the safety precautions section. Additional controls are listed with the individual steps of the procedure.

Fortification of Control Samples for Method Validation [1b, 4a]
Weigh 5±0.05 g dry weight of either Newhaven or RefeSol 01-A soil into a Teflon centrifuge tube. Fortify with Tebuconazole standard in acetonitrile as shown in the following table:

Number of Replicates	Sample Type	Stock Concentration (µg/mL)	Volume Added (mL)	Sample Weight (g)	Fortified Concentration (µg/kg)
1	Reagent blank	N/A	N/A	N/A	N/A
2	Control	N/A	N/A	5	N/A
5	LOQ	1	0.25	5	50
5	10 × LOQ	10	0.25	5	500

N/A = Not Applicable.

Sample Extraction [1b, 4a]

1. Measure 5±0.05 g dry weight of soil into a Teflon centrifuge tube.
2. Add 20 mL of acetonitrile to the soil.
3. Shake at 150 rpm for 30 minutes.
4. Centrifuge at 3000 rpm for 10 minutes.
5. Transfer the supernatant into a glass jar.
6. Repeat steps 2 to 5, combining the two extracts.
7. Make to 50 mL volume with acetonitrile
8. Dilute into calibration range with acetonitrile: water (20:80 v/v).
9. Transfer a portion into a micro centrifuge tube and centrifuge at 13,000 rpm for 5 minutes.
10. Transfer into an HPLC vial for analysis.

The recommended dilution procedure is given in the following table:

Sample type	Fortified Concentration (µg/kg)	Sample Weight (g)	Extract Volume (mL)	Dilution (mL-mL)	Dilution Factor
Reagent blank	N/A	N/A	50	0.03-10	3333
Control[1]	N/A	5	50	0.03-10	3333
LOQ	50	5	50	0.03-10	3333
10 × LOQ	500	5	50	0.03-10	3333

N/A = Not Applicable.
[1] Dilute additional control extract for matrix matched calibration standards, if required.

LC-MS/MS CONDITIONS

HPLC Parameters:

Instrument:	Shimadzu Nexera series HPLC system
Column#:	Waters XBridge BEH C18, 2.5 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:	0.5 mL/min

Gradient:

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0.00	80	20
0.50	80	20
3.00	0	100
3.50	0	100
3.51	80	20
5.00	80	20

Run Time:	5.0 minutes
Column Temperature:	40°C
Autosampler Temperature:	10°C
Injection Volume:	50 µL
Retention Time:	Approx. 2.8 minutes

Valco Valve Diverter:

Time (min)	Position
0	A (to waste)
1	B (to MS)
4	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:	5000 V
Collision Gas (CAD):	8
Curtain Gas (CUR):	25
Gas Flow 1 (GS1):	40
Gas Flow 2 (GS2):	40
Vaporiser Temperature (TEM):	500°C
Interface Heater (ihe):	On
Entrance Potential (EP):	10
Collision Exit Potential (CXP):	13

Transition Name:	MRM Transition Ions Monitored	Declustering Potential (DP)	Collision Energy (CE)	Dwell Time (ms)
Tebuconazole (Primary):	308.4/70.0	120	36	75
Tebuconazole (Confirmatory):	308.4/125.0	120	54	75

Parameters marked # may not be modified. Minor adjustments to the remaining parameters may be required in order to fully optimise the system.

CALCULATION OF RESULTS

All peak measurements and calculations are performed on Analyst 1.6.2. From the measured peak area, where the calibration fit is linear as in this study, Analyst uses the following formula to calculate the concentration of test substance present in the sample extract.

$$x = \frac{(y - c)}{m} \times DF$$

Where:-

x = concentration of test substance in sample (µg/kg)
y = area of peak due to test substance
m = gradient
c = Y intercept on calibration graph
DF = sample dilution factor

Procedural recovery data from fortified samples are calculated via the following equation:

$$\text{Recovery}(\%) = \frac{A}{S} \times 100$$

Where:-

A = concentration found in fortified sample (µg/kg)
S = concentration added to fortified sample (µg/kg)

METHOD CRITERIA

For the analysis by LC-MS/MS to be considered successful the following criteria should be met.

- At least 5 calibration standards will be used in the determination of the calibration line.
- The correlation coefficient (r) for the calibration line will be ≥ 0.995 with a 1/x weighting.
- All sample extracts will be within the appropriate range of calibration standards.
- Mean recovery from fortified samples will be considered acceptable within the range of 70 to 110%.
- The control sample should not contain interference > 30% of the LOQ.

GENERAL HANDLING CONTROL CATEGORIES

CATEGORY Main	Division	CONTROL Name and Specification
1		GLOVES
	a	Disposable latex
	b	Disposable nitrile
	c	Rubber gloves
	d	Specific type for the job (see assessment giving details)
2		PROTECTIVE CLOTHING
	a	Laboratory coat or equivalent
	b	Disposable overalls
	c	Oversleeves
	d	Overshoes
	e	Plastic apron
3		EYE/FACE PROTECTION
	a	Safety glasses to BS 2092/2 C or better
	b	Face shield to BS 2092/2 C or better
	c	Safety goggles to BS 2092/2 C or better
4		ENGINEERING CONTROLS
	a	Open bench in ventilated area
	b	Fume cupboard to BS 7258
	c	Laminar flow cabinet to BS 5295 Class 1
	d	Re-circulating fume chamber
	e	Radioisotope lab
	f	Biohazard lab
	g	Glove box
5		RESPIRATORY PROTECTIVE EQUIPMENT
	a	Disposable filtering facemask (HSE approved), i - organic vapour ii - dust iii – combination organic vapour/dust MUST SPECIFY TYPE
	b	Powered respirators/helmets with safety visor to BS 2092/2 C or better (HSE approved)
	c	Respirator with specified canister (HSE approved)
6		SPECIFIC IMMUNISATION REQUIRED (GIVE DETAILS)
7		ALLERGIC PERSONS PROHIBITED (SPECIFY ALLERGY)
8		REFER TO MATERIAL SAFETY DATA SHEET
9		KNOWN OR SUSPECTED REPRODUCTIVE HAZARD TO EITHER SEX (must specify details)
10		POISON ensure antidote is available and is within its expiry date (must specify details)