1 SCOPE

The analytical procedures described are suitable for the determination of residues of the fungicide ICIA5504 (Figure 1), its geometrical isomer R230310 (Figure 2), and its soil metabolites R234886 (Figure 3), R401553 (Figure 4) and R402173 (Figure 5).

To date, in these laboratories, the method has been applied to a variety of soil samples and the limits of determination of the method are 0.02 mg kg^{-1} for ICIA5504, R230310 and R234886, and 0.01 mg kg^{-1} for R401553 and R402173.

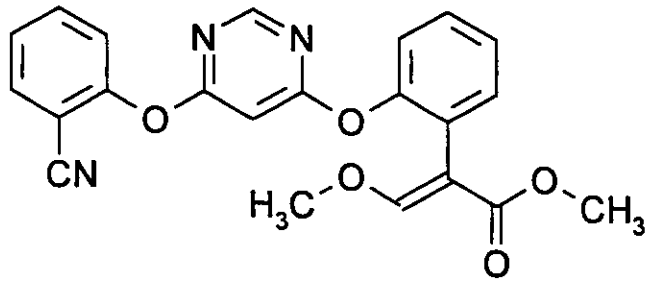

Figure 1 : **Methyl (E)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate (IUPAC).**

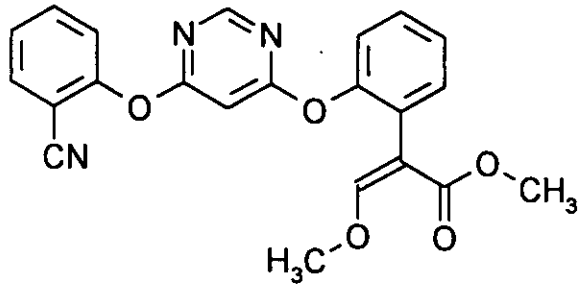

Figure 2 : **Methyl (Z)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate (IUPAC).**

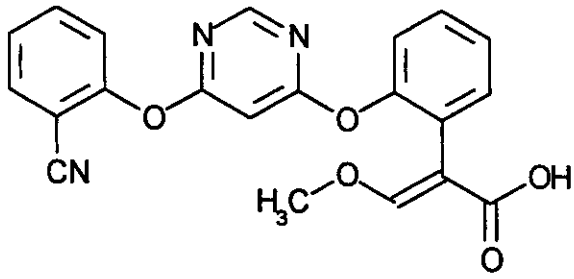

Figure 3 : **(*E*)-2-{2-[6-(2cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylic acid (IUPAC)**

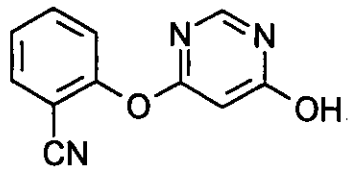

Figure 4 : **6-(2-cyanophenoxy)pyrimidin-4-ol (IUPAC).**

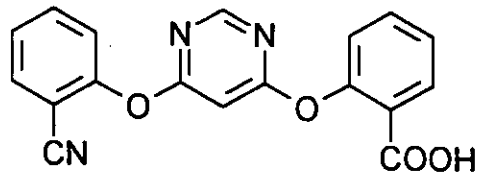

Figure 5 : **2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]benzoic acid (IUPAC).**

2 SUMMARY

ICIA5504, R230310, R234886, R401553 and R402173 residues in soil samples are extracted in 75:25 methanol:1M hydrochloric acid by the robot using a vortex/sonication routine . An aliquot of the extract is subject to liquid-liquid partition with acidified sodium chloride solution and dichloromethane. The combined dichloromethane extract is evaporated to dryness and taken up in a known volume of HPLC mobile phase for analysis by high performance liquid chromatography with triple quadrupole mass spectrometry (HPLC-MS-MS). Alternatively to determine R402173 residues, the dichloromethane extract is evaporated to dryness, resuspended in chloroform and R402173 derivatised using a methylating agent prior to analysis by gas liquid chromatography with mass selective detection (GC-MS).

3 OPERATOR PROCEDURE

3.1 Starting Up the Robot

a) Thoroughly mix the sample and weigh a representative aliquot (10 g), into a labelled robot transfer tube (50 cm^3).

b) Fortify a minimum of two control samples with an accurately known amount of ICIA5504, R230310, R234886, R401553 and R402173 as recovery checks.

Note: Additional control soil samples should be taken through the procedure, to be used in generation of standards in the presence of matrix for quantification using HPLC-MS-MS or GC-MS.

c) Place a robot lay-on cap on each sample and place each tube on the robot in the appropriate rack. When loading the samples onto the robot, complete the first column of the sample preparation integrity sheet which constitutes raw data (see Appendix 4 for example). The recovery checks should be placed in the rack as the second and last samples. Comparison between the two recovery check results obtained will indicate if significant degradation has occurred whilst the samples have been waiting on the robot bench.

d) Place one transfer tube (50 cm^3) for each sample into the appropriate rack on the robot.

e) Place one test tube (16 mm x 75 mm) for each sample in the appropriate rack on the robot.

f) Place five pipette tips (1 cm^3) for each sample into the appropriate rack.

g) Prepare an acidified 5% (w/v) sodium chloride solution by dissolving 15 g of sodium chloride in ultra-pure water (300 cm^3) and adding 1M hydrochloric acid (15 cm^3).

h) Fill up the appropriate reservoirs with solvent. The following volumes of solvents are required per sample:

50 cm^3	75:25 methanol:1M hydrochloric acid
2 cm^3	acidified 5% (w/v) sodium chloride solution
6 cm^3	dichloromethane

Allow for the extra volumes of each solvent needed for purging any air from the solvent lines.

i) Ensure the sonic bath is filled with water. Also verify that the balance tubes (containing sodium sulphate) are in position.

j) Ensure the robot and all modules are switched on before turning on the controller and the VDU. After the system has initialized, the details for the run are entered via the keyboard as required. Check to ensure the air toggle switch is in the 'ON' position and the system is displaying the correct time.

k) Details of the run must be entered into the robot log book. The log book is a record of usage and as such is raw data.

3.2 Shutting Down the Robot

a) Remove the test tubes from the rack numbering each one with the same identifying number used for the samples. Complete the second column on the sample preparation integrity sheet at the same time. Analyse the samples using high performance liquid chromatography (see Section 5).

b) Remove the robot printout from the printer, sign and date it, and retain it as raw data (see Appendix 5 for example).

c) Discard the used pipette tips found in the plastic beaker on the robot table.

d) Remove all the dirty glassware from the robot as well as the used lay-on caps stored in the waste container.

3.3 Faults

The robot is a piece of equipment which relies on accurate positioning of items on its bench. Under **NO** circumstances should **ANYTHING** be moved without consulting the responsible person. If anything is moved a fault will almost certainly occur.

Should the robot fail to finish a run, or it stops mid-run, the responsible person must be contacted. The operator **MUST NOT** attempt to rectify the fault.

All faults must be recorded in the robot's log book.

4 ROBOTIC PROCEDURE

The robot processes samples using the following method:

4.1 Initialization

a) After entering the run details (see 3.1 k above), the variables used in the program are set to their initial/appropriate values.

b) A printout is made of the Extraction Number, the analysis method being used, the operator's name, the date and the number of samples to be put through the method.

c) The robot checks the calibration of the balance with a test weight (50 g). A hard copy of the result is generated on the robot printout.

d) All the syringes being used for the method are filled with their respective solvents and emptied so as to purge any air from the solvent lines. (The pneumatic operated solvent arms are moved to their extreme positions several times in order to exercise them).

e) Ensure that the initial samples worked up by the robot contain a control sample i.e. either sample 1 or 2 or both.

4.2 Sample Preparation

4.2.1 Extraction

a) The time is recorded under the start-time variable associated with the sample.

b) The balance is tared and the transfer tube containing the first sample (10 g) is removed from its rack and the cap on it is removed and the weight of the tube and its contents are recorded under the appropriate value.

c) The tube is taken to the dispenser arm and 75:25 methanol:1M hydrochloric acid (25 cm^3) is added. The cap is recovered using the tube and the tube is put in the vortex for 3 minutes. After this the tube is placed in the ultrasonic bath for 3 minutes followed by a further 3 minute vortex.

d) The sample is centrifuged for 1.5 minutes at 3000 rpm. The cap is removed and an aliquot (1.25 cm^3 equivalent to 0.5 g soil) of the extract is transferred to another transfer tube (50 cm^3).

e) The remaining extractant is discarded and the tube and contents (without the cap) are reweighed. From the difference in weight between this weighing and that made in 4.2.1 b), the weight and hence volume of extractant remaining in the tube can be determined.

f) A second aliquot of 75:25 methanol:1M hydrochloric acid is added to the tube such that the total volume of extractant is 25 cm^3. The cap is recovered. The tube is put in the vortex for 3 minutes. After this the tube is placed in the ultrasonic bath for 4 minutes followed by a further 3 minute vortex.

g) The sample is centrifuged for 1.5 minutes at 3000 rpm. An aliquot (1.25 cm^3 equivalent to 0.5 g soil) of the extract is transferred to the transfer tube (50 cm^3) from 4.2.1 d) containing the aliquot from the first extraction.

4.2.2 Liquid/Liquid Partition

a) Acidified 5% (w/v) sodium chloride solution (2 cm^3) and dichloromethane (2 cm^3) is added to the combined aliquot from the extractions. This mixture is vortexed for 1.2 minutes and then centrifuged for 0.5 minutes at 3000 rpm.

b) During the latter, a test tube (16 mm x 75 mm) is removed from its rack and placed in a holding station.

c) After centrifugation, the lower dichloromethane layer is transferred to the test tube.

d) Further dichloromethane (2 cm^3) is added to the aqueous sample. The mixture is vortexed for 1.2 minutes and then centrifuged at 3000 rpm for 0.5 minutes.

e) After centrifugation, the lower dichloromethane layer is transferred to the test tube containing the dichloromethane from the first partition.

f) Further dichloromethane (2 cm^3) is added to the aqueous sample. The mixture is vortexed for 1.2 minutes and then centrifuged at 3000 rpm for 0.5 minutes.

g) After centrifugation, the lower dichloromethane layer is transferred and added to the test tube containing the dichloromethane from the first and second partitions.

4.2.3 End of Sample Procedures

a) At the end of each sample the robot increments the sample number variable by one and continues on by looping back to 4.2.1 a). When the last sample has been completed the run finish time is printed out, as well as a table containing the start and finish times for each sample.

4.2.4 Preparation for HPLC Analysis

Preparation of samples for HPLC analysis is carried out manually.

a) The contents of the tube from 4.2.2 g) are taken to dryness under a stream of clean, dry air at ca. 40°C .

b) HPLC mobile phase (1cm^3) (50:50 ultra-pure water:acetonitrile + 0.4% (v/v) glacial acetic) is added into the test tube. The final sample concentration of the samples is therefore 0.5 g soil cm^{-3}.

c) The residuum is resuspended by ultrasonication.

4.2.5 Preparation of Standards in the Presence of Matrix for HPLC Analysis

Standards must be prepared in the presence of soil matrix prior to analysis, as different responses are achieved in the presence of matrix compared to when matrix is absent. Standards are prepared as follows by the analyst:

a) The contents of the tube from 4.2.2 g) for a control sample are taken to dryness under a stream of clean, dry air at ca. 40°C .

b) Add an appropriate amount of ICIA5504, R230310, R234886, R401553 and R402173 standard to produce the required final standard concentration into the test tube from 4.2.5 a) (16 mm x 75 mm).

c) Evaporate to dryness under a stream of clean, dry air.

d) HPLC mobile phase (1cm^3) (50:50 ultra-pure water:acetonitrile + 0.4% (v/v) glacial acetic) is added into the test tube. The final sample matrix concentration of the standard is therefore 0.5 g soil cm^{-3} as for the samples.

e) The residuum is resuspended by ultrasonication.

f) Analyse standards alongside samples on HPLC-MS-MS.

4.2.6 Derivatization of R402173

Preparation of samples for GC analysis is carried out manually.

a) The contents of the tube from 4.2.2 g) are taken to dryness under a stream of clean, dry air at ca. 40°C and resuspended in chloroform (0.50 cm^3).

b) Add to the tube 1 cm^3 of an ethereal solution of diazomethane and leave to stand at room temperature for 30 minutes.

All operations carried out using diazomethane must be carried out in a fume cupboard.

c) Evaporate the tube contents to dryness using a stream of clean, dry air. Resuspend in chloroform (1 cm^3) and analyse for the methylated derivative by GC-MS.

R402173 derivatized standards must be prepared in the presence of soil matrix prior to analysis, as different responses are achieved in the presence of matrix compared to when matrix is absent. Standards are prepared as follows:

(i) The contents of **a control soil extract** tube from 4.2.2 g) are taken to dryness under a stream of clean, dry air at ca. 40°C and resuspended in chloroform (0.50 cm^3).

(ii) Add an appropriate amount of R402173 standard to produce the required final standard concentration.

(iii) Add 1 cm^3 of an ethereal solution of diazomethane and leave to stand at room temperature for 30 minutes.

(iv) Evaporate the tube contents to dryness using a stream of clean, dry air and resuspend in chloroform (1 cm^3).

(v) Analyse standards alongside samples on GC-MS.

5 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH TRIPLE QUADRUPOLE MASS SPECTROMETRIC DETECTION (HPLC-MS-MS)

High performance liquid chromatography with triple quadrupole mass spectrometry (HPLC-MS-MS) may be used for the qualitative and quantitative confirmation of ICIA5504, R230310, R234886, R401553 and R402173 residues down to levels at the limit of determination i.e. 0.02 or 0.01 mg kg^{-1}. Samples obtained from the residue analytical method are examined by HPLC-MS-MS. Qualitative confirmation of residues is given by the appearence of a peak at the correct HPLC retention time for the ions monitored.

Quantitative confirmation of ICIA5504, R230310, R234886, R401553 and R402173 residues is carried out by comparison of the peak area measured against that for a standard in the presence of matrix in the same analyte concentration range as that expected in the samples.

5.1 Analysis by HPLC-MS-MS

The conditions for the analysis by HPLC-MS-MS will depend upon the equipment available. The operating manuals for the instruments should always be consulted to ensure safe optimum use. The following conditions have been found to be satisfactory using a Perkin Elmer Binary LC 250 pump fitted with a Perkin Elmer Advanced LC Sample Processor ISS200 and a PE-SCIEX API 111 triple quadrupole mass spectrometer in the positive ion mode.

(i)	Column	:	Kromasil 100-5C18 208G (5 cm x 4.6 mm internal diameter)
(ii)	Mobile phase	:	50: 50 Acetonitrile:Ultra-pure water + 0.4% (v/v) glacial acetic acid
(iii)	Flow rate	:	1 cm^3 min^{-1}
(iv)	Injection volume	:	50 µl
(v)	Ionization Mode		Heated nebulizer (APCI) positive ion
	Detection Mode	:	Multiple reaction monitoring (MRM)
(vi)	Temperature of Heated Nebulizer	:	480°C
(vii)	Auxillary gas	:	UHP Nitrogen (1.8 litres min^{-1})
	Nebulizer gas	:	UHP Nitrogen (60 psi)
	Collision gas	:	Ar/N_2 (10%)

Protonated molecular ions generated in the ion source (ICIA5504 and R230310, m/z 404, R234886, m/z 390, R401553, m/z 214 and R402173, m/z 334) are selected and subjected to further fragmentation by collisional activation. The largest ion ((ICIA5504, R230310 and R234886, m/z 372, R401553, m/z 187 and R402173, m/z 316) in the resulting daughter spectra are then monitored and used for quantitative analysis.

Under these conditions the retention times of ICIA5504, R230310, R234886, R401553 and R402173 were approximately 3.3, 2.5, 1.6, 0.80 and 1.7 minutes, respectively.

6 GAS CHROMATOGRAPHY WITH MASS SELECTIVE DETECTION (GC-MS)

The conditions for the analysis by GC-MS will depend upon the equipment available. The operating manuals for the instruments should always be consulted to ensure safe, optimum use. The following conditions have been found to be satisfactory using a Hewlett Packard 5890 series capillary gas chromatograph interfaced to a Hewlett Packard 5970 mass selective detector using selected ion monitoring conditons.

6.1 GC-MS Conditions for Analysis of R402173 Derivative

6.1.1 Gas-Liquid Chromatography Conditions

(i) Column	:	DB5.625 (5% phenylmethylpolysiloxane) 30 m x 0.25 mm i.d (0.25 µm film thickness)
(ii) GC Temperature program	:	Initial - 45°C for 1 minute Rate - 25°C/minute Final - 300°C for 4.8 minutes
(iii) Injector mode	:	Splitless with a 4 mm silanised, double restrictor liner packed with a silanised glass wool plug
(iv) Injection Port Temperature	:	250°C
(v) Injection volume	:	1 µl
(vi) Transfer Line Temperature	:	275°C
(vii) Carrier gas	:	Helium

Under these conditions the retention time of the R402173 derivative is approximately 13.3 minutes.

6.1.2 **Mass Spectrometry Conditions**

(i) Acquisition Mode	:	Single Ion Monitoring (SIM)
(ii) Ion Monitored	:	m/z 288
(iii) Mode of Ionisation	:	electron impact
(iv) Electron Multiplier	:	3000 V
(v) Electron Energy (eV)	:	70
(vi) Dwell Time	:	500

In these laboratories, with some soil types irreproducible matrix standard response has been observed during analytical runs. This can be overcome by injection of a 1% solution of 1,3-Diphenyl-1,1,3,3-tetramethyldisilazane in chloroform after every 4-5 injections.

7. CALCULATION OF RESIDUE RESULTS

a) Make repeated injections of 1 µl of a standard solution containing R402173 derivative into the GC-MS operated under conditions described in Section 5 or 50 µl of a standard solution containing all of the analytes into the HPLC-MS-MS operated under conditions described in Section 6.

When a consistent response is obtained measure the peak heights/areas obtained for each analyte.

b) Make an injection of each sample solution (1 µl on GC and 50 µl on HPLC-MS-MS) and measure the peak heights/areas of the peaks corresponding to the analyte.

c) Re-inject the standard solution after a maximum of four injections of sample solutions.

d) Calculate the residue in the sample, expressed as mg kg^{-1} by proportionation of the analyte peak heights or peak areas measured for the sample against that for the analytical standard solution.

$$\text{Residue} = \frac{\text{peak response in sample}}{\text{peak response in standard}} \times \frac{\text{conc}^{n} \text{ of analyte in standard}}{\text{conc}^{n} \text{ of sample solution}}$$

where analyte = ICIA5504, R230310, R234886, R401553 or R402173

8 CONTROL AND RECOVERY EXPERIMENTS

At least one untreated sample must be analysed alongside any set of samples, using exactly the same method. This ensures that no unobserved contamination of the samples occurred prior to, or during, the analysis. At least two control samples, accurately fortified with a suitable known amount of ICIA5504, R230310, R234886, R401553 and R402173 should be analysed alongside every batch of treated samples. Fortification amounts should be based on anticipated residue levels. When no residues are expected, the recoveries should be fortified at low levels, typically 0.01-0.05 mg kg^{-1}. Reagent blanks may also be analysed to ensure that no contamination occurs during analysis due to the solvents or materials used.

9 LIMIT OF DETERMINATION

The limit of determination of the method can be assessed by carrying out recovery experiments at low levels of fortification (0.01 - 0.05 mg kg^{-1}). In these laboratories the limits of determination have been set at 0.02 mg kg^{-1} soil for ICIA5504, R230310 and R234886 and at 0.01 mg kg^{-1} soil for R401553 and R402173. Care must be taken when working at the limit of determination to minimise the risk of contamination.