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 R234886, its major soil metabolite under laboratory conditions (Figure 3), in soil.

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} soil for each analyte.

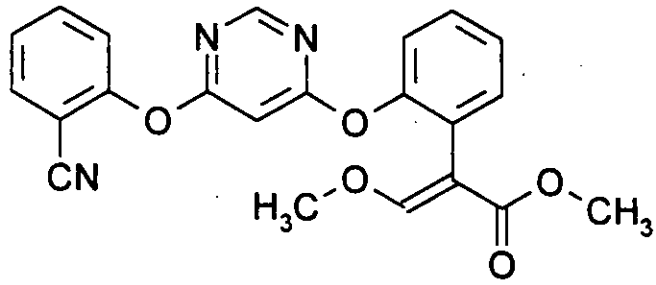

Figure1 : Methyl (E)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate (IUPAC).

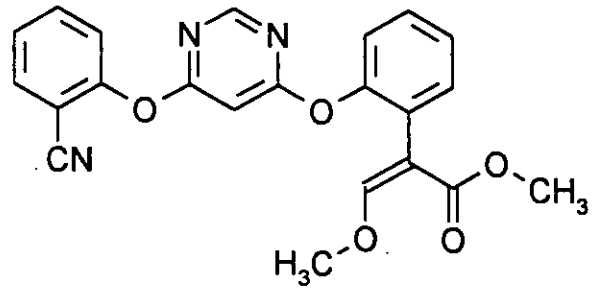

Figure 2 : Methyl (Z)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate (IUPAC).

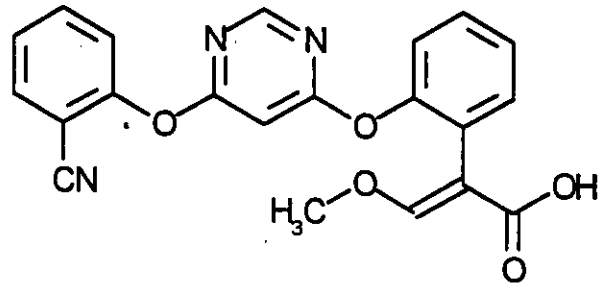

Figure 3 :
(*E*)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylic acid (IUPAC)

2 SUMMARY

ICIA5504, R230310 and R234886 residues in soil samples are extracted in 75:25 methanol:water. 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 ultra-violet detection (HPLC-UV). Quantitative confirmation of residues may be carried out using high performance liquid chromatography with triple quadrupole mass spectrometry.

3 PROCEDURE

3.1 Extraction

a) Thoroughly mix the sample and weigh a representative aliquot (20 g), into a screw top Nalgene bottle (250 cm^3).

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

c) Add 75:25 methanol:water (50 cm^3) and shake for 30 minutes (at 130 +/- 20 rpm). Centrifuge at 3500 +/- 500 rpm for approximately 4 minutes and then decant the supernatant into a round bottom flask.

d) Add a further aliquot of 75:25 methanol:water (50 cm^3) to each sample and shake for a further 15 minutes (at 130 +/- 20 rpm). Filter the extract under vacuum through a Whatman No. 1 or No. 5 filter paper into a round bottom flask containing the extract from the first extraction, to combine the two extracts for each sample.. Rinse the residuum with further extraction solvent.

e) Adjust to a suitable known volume (eg.100-120 cm^3) with 75:25 methanol:water.

3.2 Liquid-liquid partition

a) 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).

b) Take a 2 g soil aliquot and partition with an equivalent-volume of dichloromethane and an equivalent-volume of acidified 5% (w/v) sodium chloride solution in a separatory funnel. Collect the dichloromethane layer into a round bottom flask. Add a further equivalent-volume of dichloromethane to the aqueous extract and repartition. Combine the dichloromethane layer with the dichloromethane collected from the first partition in the round bottom flask.

Note: An additional soil aliquot from an untreated sample should be taken through the procedure to be used in generation of a matrix standard, if quantification is to be carried out using high-performance liquid chromatography with triple quadrupole mass spectrometry.

c) Evaporate the aliquots to dryness on a rotary evaporator at ≤40°C and redissolve by ultrasonication in a suitable volume (2 cm^3) of HPLC mobile phase (60:40 water:acetonitrile + 0.4% (v/v) glacial acetic acid). **Great care should be taken when redissolving the sample to ensure quantitative transfer into the HPLC vial for analysis.**

Note: To generate a matrix standard for use in quantification by HPLC-MS-MS add a suitable quantity of a mixed ICIA5504, R230310 and R234886 standard in acetone to the residium from the untreated soil aliquot above, take to dryness and resuspend in mobile phase.

4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ULTRA-VIOLET DETECTION (HPLC-UV)

The conditions for the analysis by HPLC-UV 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 1050 series HPLC gradient pump fitted with a Hewlett Packard 1050 series autosampler and a Waters M481 LC-UV detector: Several method variables may be modified i.e. mobile phase composition and flow rate to ensure resolution of the analytes from co-eluting peaks.

4.1 High Performance Liquid Chromatography Conditions

(i) Column: Spherisorb 5 µm ODS 2 (25 cm x 3.2 mm internal diameter)

(ii) Mobile phase: Solvent A - Ultra-pure water + 0.4% (v/v) glacial acetic acid
Solvent B - Acetonitrile + 0.4% (v/v) glacial acetic acid

Following an injection, the above two solvents are combined in the gradient system to produce the required linear changes in mobile phase composition, as shown in Table 1. After 30 minutes, the solvent is left in the zero-time composition for 10 minutes prior to re-injection.

TABLE 1 : Gradient HPLC Solvent Composition Profile

Time (mins)	0	10	17	25	30
Solvent A (%)	60	60	50	50	60
Solvent B (%)	40	40	50	50	40

(iii) Flow rate: 0.8 cm^3 min^{-1}

(iv) Injection volume: 100 µl

(v) Detection: 255 nm

Under these conditions the retention times of ICIA5504, R230310 and R216206 were approximately 22, 19 and 12 minutes, respectively.

4.2 Calculation of ICIA5504, R230310 and R234886 Residue Results

a) Make repeated injections of 100 µl of a standard solution containing a mixture of ICIA5504, R230310 and R234886 into the HPLC operated under conditions described in Section 4.

When a consistent response is obtained measure the peak heights/areas obtained for ICIA5504, R230310 and R234886.

b) Make an injection of each sample solution (100 µl) and measure the peak heights/areas of the peaks corresponding to ICIA5504, R230310 and R234886.

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 ICIA5504, R230310 and R234886 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 or R234886

5 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 and R234886, 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 0.02-0.05 mg kg^{-1}. Reagent blanks may also be analysed to ensure that no contamination occurs during analysis due to the solvents or materials used.

6 LIMIT OF DETERMINATION

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

9 CONFIRMATION OF RESIDUES

High performance liquid chromatography with triple quadrupole mass spectrometry (HPLC-MS-MS) may be used for the qualitative and quantitative confirmation of ICIA5504, R230310 and R234886 residues down to levels at the limit of determination i.e. 0.02 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 and R234886 residues is carried out by comparison of the peak area measured against that for an external matrix standard in the same analyte concentration range as that expected in the samples. To generate a matrix standard for use in quantification by HPLC-MS-MS take a suitable quantity of a mixed ICIA5504, R230310 and R234886 standard in acetone to dryness and resuspend in an untreated sample in mobile phase.

9.1 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:	Spherisorb 5 µm ODS 2 (25 cm x 4.6 mm internal diameter) or 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:	30 µl
(v)	Ionization Mode Detection Mode:	Heated nebulizer (APCI) positive ion Multiple reaction monitoring (MRM)
(vi)	Temperature of Heated Nebulizer:	480°C
(vii)	Auxillary gas: Nebulizer gas: Collision gas:	UHP Nitrogen (1.8 litres/min) UHP Nitrogen (60 psi) Ar/N_2 (10%)

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

Under these conditions the retention times of ICIA5504, R230310 and R234886 were approximately 12, 9.3 and 6 minutes on the Spherisorb 5 µm ODS2 column and 3.2, 2.3 and 1.5 minutes on the Kromasil column, respectively.

Examples of HPLC-MS-MS chromatograms are shown in Appendix 2.

1 **Apparatus**

a) Nalgene centrifuge bottles (250 cm^3 capacity) for sample extraction; available from Fisons Scientfic Equipment, Bishop Meadow Road, Loughborough, LE11 0RG, United Kingdom.

b) Flask shaker; available from Orme scientific., PO Box 3, Stakehill Industrial Park, Middleton, Manchester M24 2RH or VWR Scientific, PO Box 7900, San Fransisco, CA 94120, USA.

c) Filtration apparatus: Buchner funnel, adapter, filter paper (Whatman No.1 or 5, 9 cm); available from Orme scientific., PO Box 3, Stakehill Industrial Park, Middleton, Manchester M24 2RH or VWR Scientific, PO Box 7900, San Fransisco, CA 94120, USA.

d) Ultrasonic bath; available from Orme scientific., PO Box 3, Stakehill Industrial Park, Middleton, Manchester M24 2RH or VWR Scientific, PO Box 7900, San Fransisco, CA 94120, USA.

e) Rotary evaporator with thermostatically controlled waterbath; available from Buchi via Orme scientific., PO Box 3, Stakehill Industrial Park, Middleton, Manchester M24 2RH or VWR Scientific, PO Box 7900, San Fransisco, CA 94120, USA.

f) Hewlett Packard vials for HPLC analysis; available from Hewlett Packard Ltd., Heathside Park Road, Cheadle Heath, Stockport, Cheshire SK3 0RB or Hewlett Packard Co., PO Box 1000, Avondale, PA 19311-1000, USA.

g) A gradient HPLC chromatograph fitted with a UV detector e.g. Hewlett Packard 1050 series, gradient pump and autosampler with Waters M481 LC-UV detector or equivalent instruments. Integrator or data handling system; available from Hewlett Packard Ltd., Heathside Park Road, Cheadle Heath, Stockport, Cheshire SK3 0RB or Hewlett Packard Co., PO Box 1000, Avondale, PA 19311-1000, USA.

h) HPLC column, Spherisorb 5 µm ODS2 (25 cm x 3.2 mm internal diameter) available from Hichrom Ltd., 6 Chiltern Enterprise Centre, Station Road, Theale, Reading, Berkshire RG7 4AA or Phase Separations Inc., 140 Water Street, Norwalk, CT06854, USA.

i) PE-SCIEX API 111 triple quadrupole mass spectrometer; available from Perkin Elmer Ltd., Beaconsfield, Buckinghamshire, United Kingdom or SCIEX, Division of MDS Health Group Ltd., Toronto, Canada.

2 Reagents

a) Solvents: acetone, acetonitrile, methanol and dichloromethane (distilled in glass); available from Rathburn Chemicals Ltd., Walkerburn, Scotland, United Kingdom or B & J Brand Solvents, from Scientific Products Division of Baxter Healthcare Corporation, USA (Tel: 312-689-8410).

b) Analytical grade glacial acetic acid (99%); available from Fisons Scientific Equipment, Bishop Meadow Road, Loughborough, LE11 0RG, United Kingdom or Aldrich Chemical Co. Ltd., 940 W St Paul Ave, Milwaukee, Wisconsin 53233, USA.

c) Analytical grade sodium chloride (99.9%); available from Fisons Scientific Equipment, Bishop Meadow Road, Loughborough, LE11 0RG, United Kingdom or Aldrich Chemical Co. Ltd., 940 W St Paul Ave, Milwaukee, Wisconsin 53233, USA.

d) A sample of ICIA5504, R230310 and R234886 of known purity (>95%).

e) Ultra-pure water e.g. as produced by the Millipore Water still

3 Hazards

The following information is included as an indication to the analyst of the nature and hazards of the reagents used in this procedure. If in any doubt, consult the appropriate safety manual (e.g. ICI Laboratory Safety Manual) which contains recommendations and procedures for handling chemicals or a monograph such as 'Hazards in the Chemical Laboratory', Edited by G D Muir, The Chemical Society, London.

a) Solvent Hazards

	Acetone	Methanol	Acetonitrile	Dichloromethane
Harmful vapour	Yes	Yes	Yes	Yes
Highly flammable	Yes	Yes	Yes	No
Harmful by skin absorption	No	Yes	Yes	No
TLV mg/m^3	2400	260	70	350

In all cases avoid breathing vapour. Avoid contact with skin and eyes.

b) ICIA5504 has a divisional toxicity class of 4. ICIA5504 has a mammalian toxicity (acute oral LD_{50}) in rat greater than 5000 mg kg^{-1}.

4 Preparation of Analytical Standards

Weigh out accurately using a five figure balance, sufficient of ICIA5504, R230310 and R234886 solid to allow dilution in acetone to give 1000 µg cm^{-3} stock solutions in volumetric flasks. Make serial dilutions from the stock to give 100 µg cm^{-3} standard solutions. Prepare 10 µg cm^{-3}, 1.0 µg cm^{-3} and 0.1 µg cm^{-3} mixed standard solutions of ICIA5504, R230310 and R234886 in acetone to be used for fortification of recovery samples.

When not in use, always store the standard solutions, securely stoppered, in a refrigerator at ≤8°C to prevent decomposition and/or concentration of the solvent strength. Analytical standards should be freshly prepared from the solid material after four months of use.

A 0.10 µg cm^{-3} mix standard solution of ICIA5504, R230310 and R234886 should be prepared in HPLC mobile phase just prior to analysis.

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 R234886, its major soil metabolite under laboratory conditions (Figure 3), in soil.

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} soil for each analyte.

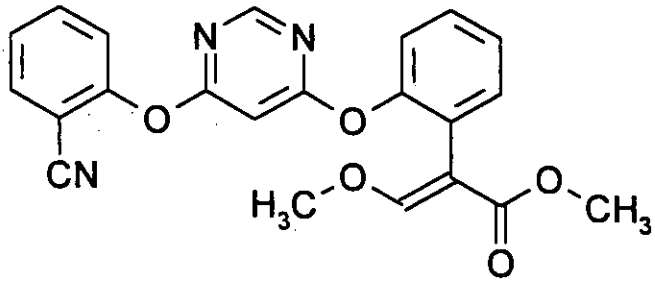

Figure 1 : Methyl (E)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate (IUPAC).

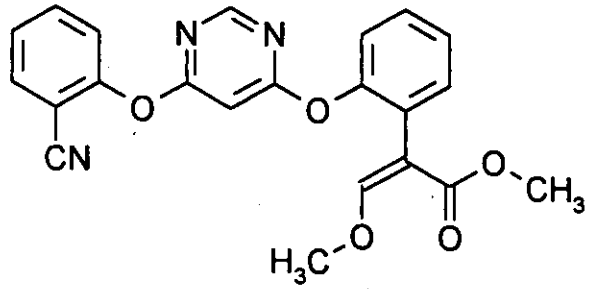

Figure 2 : Methyl (Z)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate (IUPAC).

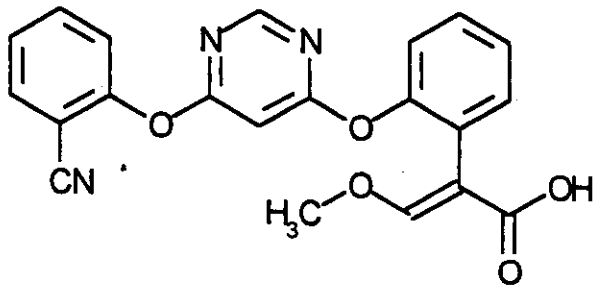

Figure 3 : (*E*)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylic acid (IUPAC)

REFERENCE COPY

2 SUMMARY

ICIA5504, R230310 and R234886 residues in soil samples are extracted in 75:25 methanol:water 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 ultra-violet detection (HPLC-UV).

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 and R234886 as recovery checks.

Note: An additional control soil sample should be taken through the procedure, to be used in generation of a matrix standard, if quantification is to be carried out using high-performance liquid chromatography with triple quadrupole mass spectrometry.

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:water .
4 cm^3	acidified 5% (w/v) sodium chloride solution
5 cm^3	dichloromethane
2 cm^3	60:40 acetonitrile: water + 0.4% glacial acetic acid

i) Load one HP vial (complete with foil cap) per sample in the vial rack plus one or two extra vials (which the robot uses if it cannot remove a cap from a vial).

j) Ensure the heating block is at 40-45°C and that the sonic bath is filled with water. Also verify that the balance tubes (containing sodium sulphate) are in position.

k) 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.

l) 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 vials 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 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).

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:water (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. An aliquot (2.5 cm^3) 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:water is added to the tube such that the total volume of extractant is 25 cm^3. The cap is recovered and placed on the tube. 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 (2.5 cm^3) of the extract is transferred to the transfer tube (50 cm^3) from 4.2.1 c) containing the aliquot from the first extraction.

4.2.2 Liquid/Liquid Partition

a) Acidified 5% (w/v) sodium chloride solution (4 cm^3) and dichloromethane (2.25 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, the balance is tared, and the mass of the empty test tube is recorded under the appropriate variable.

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

d) The test tube is placed in a heating block (at 40-45^{0}C) and the contents of the tube are evaporated partially using a steady stream of compressed air for 3.5 minutes.

e) Simultaneously with d), further dichloromethane (2.75 cm^3) is added to the aqueous sample. The mixture is vortexed for 1.5 minutes and then centrifuged at 3000 rpm for three minutes.

f) After centrifugation, the lower dichloromethane layer is transferred and added to the test tube from d). The contents of the tube are evaporated to dryness using a steady stream of compressed air for a maximum of 25 minutes.

4.2.3 Preparation for HPLC Analysis

a) During the second evaporation, a Hewlett Packard vial is removed from its rack, the crimp cap on it is removed, and the vial is put in a holding station.

b) HPLC mobile phase (1cm^3) (60:40 ultra-pure water + 0.4% (v/v) glacial acetic acid:acetonitrile + 0.4% (v/v) glacial acetic) is dispensed into the test tube from 4.2.2 f). The final sample concentration of the samples is therefore 1 g soil cm^{-3}.

c) The residuum is resuspended by a vortex (0.16 minutes) and sonication (0.33 minutes).

d) The test tube is returned to its rack where the sides are washed with its contents, which are then transferred to the Hewlett Packard vial. The crimp cap is recovered using the vial and crimped.

e) The time is recorded under the finish-time variable associated with the sample.

4.2.4 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.

5 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ULTRA-VIOLET DETECTION (HPLC-UV)

The conditions for the analysis by HPLC-UV 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 1050 series HPLC gradient pump fitted with a Hewlett Packard 1050 series autosampler and a Waters M481 LC-UV detector:

Several method variables may be modified i.e. mobile phase composition and flow rate to ensure resolution of the analytes from co-eluting peaks.

5.1 High Performance Liquid Chromatography Conditions

(i) Column: Spherisorb 5 µm ODS 2 (25 cm x 3.2 mm internal diameter)

(ii) Mobile phase: Solvent A - Ultra-pure water + 0.4% (v/v) glacial acetic acid

Solvent B - Acetonitrile + 0.4% (v/v) glacial acetic acid

Following an injection, the above two solvents are combined in the gradient system to produce the required linear changes in mobile phase composition, as shown in Table 1. After 30 minutes, the solvent is left in the zero-time composition for 10 minutes prior to re-injection.

TABLE 1: Gradient HPLC Solvent Composition Profile

Time (mins)	0	10	17	25	30
Solvent A (%)	60	60	50	50	60
Solvent B (%)	40	40	50	50	40

(iii) Flow rate: 0.8 cm^3 min^{-1}

(iv) Injection volume: 100 µl

(v) Detection: 255 nm

Under these conditions the retention times of ICIA5504, R230310 and R234886 were approximately 19, 15 and 9 minutes, respectively.

5.2 **Calculation of ICIA5504, R230310 and R234886 Residue Results**

a) Make repeated injections of 100 µl of a standard solution containing a mixture of ICIA5504, R230310 and R234886 into the HPLC operated under conditions described in Section 4.

When a consistent response is obtained measure the peak heights/areas obtained for ICIA5504, R230310 and R234886.

b) Make an injection of each sample solution (100 µl) and measure the peak heights/areas of the peaks corresponding to ICIA5504, R230310 and R234886.

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 ICIA5504, R230310 and R234886 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 or R234886

6 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 and R234886, 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 0.02-0.05 mg kg^{-1}. Reagent blanks may also be analysed to ensure that no contamination occurs during analysis due to the solvents or materials used.

7 LIMIT OF DETERMINATION

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

10 CONFIRMATION OF RESIDUES

High performance liquid chromatography with triple quadrupole mass spectrometry (HPLC-MS-MS) may be used for the qualitative and quantitative confirmation of ICIA5504, R230310 and R234886 residues down to levels at the limit of determination i.e. 0.02 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 and R234886 residues is carried out by comparison of the peak area measured against that for an external matrix standard in the same analyte concentration range as that expected in the samples. To generate a matrix standard for use in quantification by HPLC-MS-MS take a suitable quantity of a mixed ICIA5504, R230310 and R234886 standard in acetone to dryness and resuspend in an untreated sample in mobile phase.

10.1 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:	Spherisorb 5 µm ODS 2 (25 cm x 4.6 mm internal diameter) or 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:	30 µl
(v)	Ionization Mode Detection Mode:	Heated nebulizer (APCI) positive ion Multiple reaction monitoring (MRM)
(vi)	Temperature of Heated Nebulizer:	480°C
(vii)	Auxillary gas: Nebulizer gas: Collision gas:	UHP Nitrogen (1.8 litres/min) UHP Nitrogen (60 psi) Ar/N_2 (10%)

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

Under these conditions the retention times of ICIA5504, R230310 and R234886 were approximately 12, 9.3 and 6 minutes on the Spherisorb 5 µm ODS2 column and 3.2, 2.3 and 1.5 minutes on the Kromasil column, respectively.

Examples of HPLC-MS-MS chromatograms are shown in Appendix 2.

1 **Apparatus**

a) Zymark Zymate II robot and controller, Zymark Master laboratory station, Mettler PM3600 balance complete with Zymark robot interface, Zymark crimp capper, Zymark adapted Pierce Reacti-Therm, Ultrasonic bath, Zymark vortex station, Zymark centrifuge, Robot transfer tubes (50 cm^3, 28 mm x 117 mm), racks and lay-on caps to fit, test tubes (16 mm x 75 mm) and racks to fit, Plastic disposable pipette tips (1 cm^3) and rack to fit, rack to fit Hewlett Packard vials; available from Zymark, The Genesis Centre, Science Park South, Birchwood, Warrington, Cheshire WA3 7BH or Zymark Corporation, Zymark Center, Hopkinton, MA 01748, USA.

b) Hewlett Packard vials for HPLC analysis; available from Hewlett Packard Ltd., Heathside Park Road, Cheadle Heath, Stockport, Cheshire SK3 0RB or Hewlett Packard Co., PO Box 1000, Avondale, PA 19311-1000, USA.

c) A gradient HPLC chromatograph fitted with a UV detector e.g. Hewlett Packard 1050 series, gradient pump and autosampler with Waters M481 LC-UV detector or equivalent instruments. Integrator or data handling system; available from Hewlett Packard Ltd., Heathside Park Road, Cheadle Heath, Stockport, Cheshire SK3 0RB or Hewlett Packard Co., PO Box 1000, Avondale, PA 19311-1000, USA.

d) HPLC column, Spherisorb 5 µm ODS2 (25 cm x 3.2 mm internal diameter) available from Hichrom Ltd., 6 Chiltern Enterprise Centre, Station Road, Theale, Reading, Berkshire RG7 4AA or Phase Separations Inc., 140 Water Street, Norwalk, CT06854, USA.

e) PE-SCIEX API 111 triple quadrupole mass spectrometer; available from Perkin Elmer Ltd., Beaconsfield, Buckinghamshire, United Kingdom or SCIEX, Division of MDS Health Group Ltd., Toronto, Canada.

2 **Reagents**

a) Solvents: acetone, acetonitrile, methanol and dichloromethane (distilled in glass); available from Rathburn Chemicals Ltd., Walkerburn, Scotland, United Kingdom or B & J Brand Solvents, from Scientific Products Division of Baxter Healthcare Corporation, USA (Tel: 312-689-8410).

b) Analytical grade glacial acetic acid (99%); available from Fisons Scientific Equipment, Bishop Meadow Road, Loughborough, LE11 0RG, United Kingdom or Aldrich Chemical Co. Ltd., 940 W St Paul Ave, Milwaukee, Wisconsin 53233, USA.

c) Analytical grade sodium chloride (99.9%); available from Fisons Scientific Equipment, Bishop Meadow Road, Loughborough, LE11 0RG, United Kingdom or Aldrich Chemical Co. Ltd., 940 W St Paul Ave, Milwaukee, Wisconsin 53233, USA.

d) A sample of ICIA5504, R230310 and R234886 of known purity (>95%).

e) Ultra-pure water e.g. as produced by the Millipore Water still

3 Hazards

The following information is included as an indication to the analyst of the nature and hazards of the reagents used in this procedure. If in any doubt, consult the appropriate safety manual (e.g. ICI Laboratory Safety Manual) which contains recommendations and procedures for handling chemicals or a monograph such as 'Hazards in the Chemical Laboratory', Edited by G D Muir, The Chemical Society, London.

a) Solvent Hazards

	Acetone	Methanol	Acetonitrile	Dichloromethane
Harmful vapour	Yes	Yes	Yes	Yes
Highly flammable	Yes	Yes	Yes	No
Harmful by skin absorption	No	Yes	Yes	No
TLV mg/m^3	2400	260	70	350

In all cases avoid breathing vapour. Avoid contact with skin and eves.

b) ICIA5504 has a divisional toxicity class of 4. ICIA5504 has a mammalian toxicity (acute oral LD_{50}) in rat greater than 5000 mg kg^{-1}.

4 **Preparation of Analytical Standards**

Weigh out accurately using a five figure balance, sufficient of ICIA5504, R230310 and R234886 solid to allow dilution in acetone to give 1000 µg cm^{-3} stock solutions in volumetric flasks. Make serial dilutions from the stock to give 100 µg cm^{-3} standard solutions. Prepare 10 µg cm^{-3}, 1.0 µg cm^{-3} and 0.1 µg cm^{-3} mixed standard solutions of ICIA5504, R230310 and R234886 in acetone to be used for fortification of recovery samples.

When not in use, always store the standard solutions, securely stoppered, in a refrigerator at ≤8°C to prevent decomposition and/or concentration of the solvent strength. Analytical standards should be freshly prepared from the solid material after four months of use.

A 0.10 µg cm^{-3} mix standard solution of ICIA5504, R230310 and R234886 should be prepared in HPLC mobile phase just prior to analysis.