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.

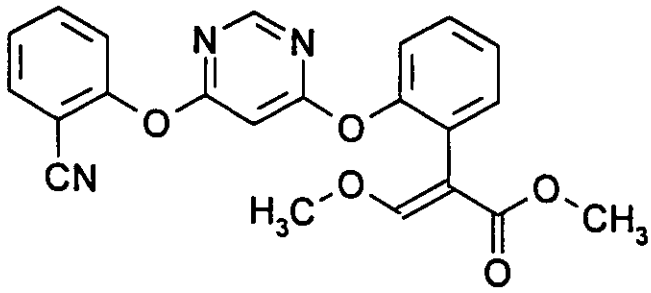

Figure 1 : Methyl (E)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate (IUPAC).

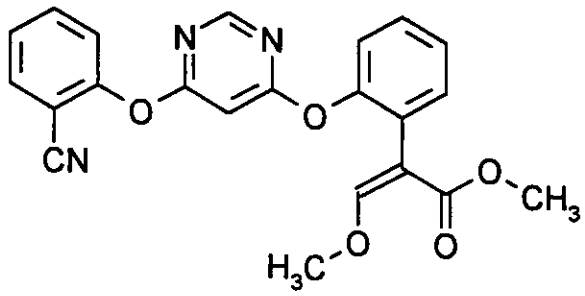

Figure 2 : Methyl (Z)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate (IUPAC).

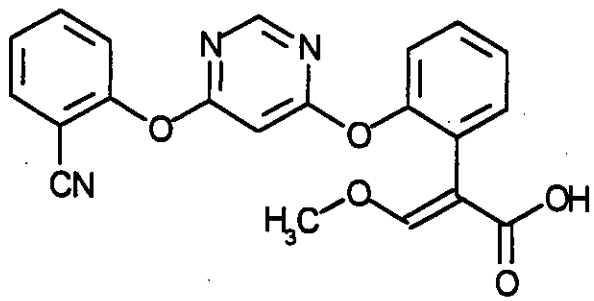

Figure 3: (*E*)-2-{2-[6-(2cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylic acid (IUPAC)

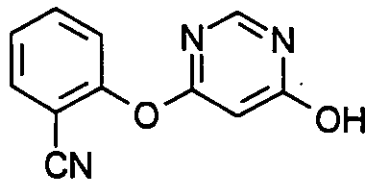

Figure 4 : 4-(2-cyanophenoxy)-6-hydroxypyrimidine

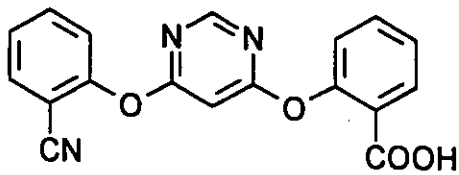

Figure 5 : 2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]benzoic acid (IUPAC).

2 SUMMARY

ICIA5504, R230310, R234886, R401553 and R402173 residues in soil samples are extracted in 75:25 methanol:1M hydrochloric acid. An aliquot of the extract is subjected 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 chloroform.

An aliquot is blown to dryness and resuspended in mobile phase for quantitative determination of ICIA5504, R230310 and R234886 residues using high performance liquid chromatography with ultra-violet detection (HPLC-UV). Further aliquots are removed and derivatised prior to quantitative determination of R401553 and R402173 residues using gas-liquid chromatography with mass selective detection (GC-MS). Quantitative confirmation of residues of all of the analytes 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, R234886, R401553 and R402173 as recovery checks.

c) Add 75:25 methanol:1M hydrochloric acid (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. If centrifugation fails to produce a supernatant without soil particulate material then the supernatant can be filtered through a Whatman No. 1 or No. 5 filter paper into the round bottom flask.

d) Add a further aliquot of 75:25 methanol:1M hydrochloric acid (50 cm^3) to each sample and shake for a further 15 minutes (at 130 +/- 20 rpm). Centrifuge at 3500 +/- 500 rpm for approximately 4 minutes and then decant the supernatant through a Whatman No. 1 or No. 5 filter paper into the same round bottom flask , to combine the two extracts from each sample. Rinse the debris and residiuum with further washes of the extraction solvent.

e) Adjust to a suitable known volume (eg.100-120 cm^3) with 75:25 methanol: 1M hydrochloric acid.

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 from 3.1 (e) 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 layers in the round bottom flask.

c) Evaporate the dichloromethane to dryness on a rotary evaporator at $\leq 40^{\circ}C$ and redissolve in chloroform (2 cm^3) using ultrasonication.

Note: Additional soil aliquots from an untreated sample should be taken through the procedure to be used to generate standards in presence of matrix.

3.3 **Preparation of ICIA5504, R230310 and R234886 Samples for HPLC-UV Analysis**

Remove an aliquot equivalent to 1 g from 3.2 (c). Evaporate to dryness using a stream of clean, dry air and re-suspend in a suitable volume (1 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.**

3.4 **Derivatization of R401553**

a) Transfer a 0.20 cm^3 aliquot (equivalent to 0.2 g of soil) of the resuspended solution from 3.2(c) to an appropriate GC vial.

b) Add to the aliquot 0.7 cm^3 chloroform and 0.10 cm^3 of the derivatizing reagent N-methyl-N-(tertbutyldimethylsilyl)trifluoroacetamide (MTBSTFA).

c) Cap the vial and leave to stand, with occasional shaking, at room temperature for one hour and then analyse for the derivative by GC-MS.

A 0.50 cm^3 aliquot (equivalent to 0.5 g of soil) may be derivatised by the same procedure in order to achieve the required sensitivity, if necessary.

R401553 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) Transfer a 0.20 cm^3 aliquot (equivalent to 0.2 g of soil) of the resuspended solution from 3.2(c) of **a control soil extract** to an appropriate GC vial.

(ii) Add an appropriate amount of R401553 standard to produce the required final standard concentration.

(iii) Add 0.7 cm^3 minus the volume of standard added in (ii) above, of chloroform.

(iv) Add 0.10 cm^3 of MTBSTFA.

(v) Cap the vial and leave to stand, with occassional shaking, at room temperature for one hour.

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

3.5 **Derivatization of R402173**

a) Transfer a 0.50 cm^3 aliquot (equivalent to 0.5 g of soil) of the resuspended solution from 3.2(c) to a disposable tube.

b) Add to the aliquot 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) Transfer a 0.50 cm^3 aliquot (equivalent to 0.5 g of soil) of the resuspended solution from 3.2(c) of **a control soil extract** to an appropriate GC vial.

(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 anc resuspend in chloroform (1 cm^3).

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

3.6 **Preparation of ICIA5504, R230310, R234886, R401553 and R402173 Samples for Quantitative HPLC-MS-MS Analysis**

a) Transfer a 0.50 cm^3 aliquot (equivalent to 0.5 g of soil) of the resuspended solution from 3.2(c) to an appropriate vial.

b) Evaporate to dryness using a stream of clean, dry air and re-suspend in a suitable volume (1 cm^3) of HPLC mobile phase (50:50 water:acetonitrile + 0.4% (v/v) glacial acetic acid) prior to analysis by HPLC-MS-MS.

c) Alternatively, if samples have been prepared for analysis by HPLC-UV take 0.5 cm^3 of the sample in mobile phase and transfer to an appropriate vial. Add 0.5 cm^3 of mobile phase to give a final sample concentration of 0.5 g soil cm^{-3}.

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) Transfer a 0.50 cm^3 aliquot (equivalent to 0.5 g of soil) of the resuspended solution from 3.2(c) of **a control soil extract** to an appropriate vial.

(ii) Add an appropriate amount of ICIA5504, R230310, R234886, R401553 and R402173 standard to produce the required final standard concentration.

(iii) Evaporate to dryness and re-suspend in a suitable volume (1 cm^3) of HPLC mobile phase (50:50 water:acetonitrile + 0.4% (v/v) glacial acetic acid).

(iv) Analyse standards alongside samples on HPLC-MS-MS.

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 1050 series 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 40 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	12	20	33	40
Solvent A (%)	70	70	65	55	55
Solvent B (%)	30	30	35	45	45

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

(iv) Injection volume: 200 µl

(v) Detection: 255 nm

Under these conditions the retention times of ICIA5504, R230310 and R234886 were approximately 47, 41 and 26 minutes, respectively.