

US EPA ARCHIVE DOCUMENT



VOLUME 7

Study Title

Report 1: The Determination of Residues of PP321 in Crops
Report 2: Addendum: The Determination of Residues of PP321 in Crops

Data Requirement

Guideline Ref. 171-4

Author

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Study Completed On

Report 1: August 29, 1984
Report 2: December 19, 1985

Performing Laboratory

ICI Plant Protection Division
Jealott's Hill Research Station

Laboratory Project ID

ICI PPD Residue Analytical Method No. 81

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PLANT PROTECTION DIVISION RESIDUE ANALYTICAL METHOD NO. 81

THE DETERMINATION OF RESIDUES OF PP321 IN CROPS

a gas-liquid chromatographic method using an internal standard*

*Also applicable to external recoveries.

Author : A Sapiets

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Date of Issue : 29 August 84

amended 01/15/87
hand delivered

1 SCOPE

The analytical procedures described are suitable for the determination of residues of the synthetic pyrethroid insecticide PP321 (Figure 1) in crops.

To date in these laboratories the method has been applied to a wide variety of crop types (see Section 10 - validation of method). The limit of determination has been set at 0.01 mg kg^{-1} .

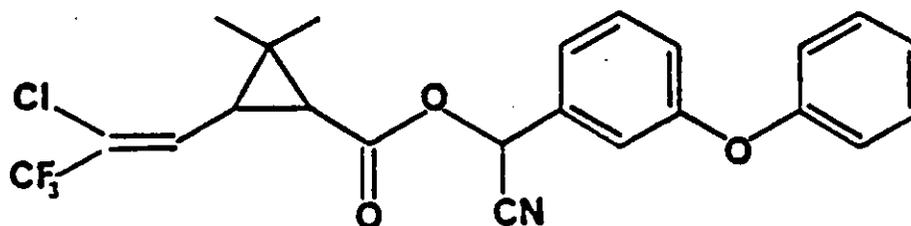


Figure 1

(R)- α -cyano-3-phenoxybenzyl (1 S)-cis-3-(Z-2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethyl cyclopropanecarboxylate.

(S)- α -cyano-3-phenoxybenzyl (1 R)-cis-3-(Z-2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylate.

PP321 is the racemic mixture containing the above two isomers in a 1:1 ratio.

2 SUMMARY

Samples which have been accurately fortified with an internal standard are extracted by maceration in the presence of 50% acetone:hexane and the organic extracts washed with water to remove acetone. Coextracted lipids are removed by liquid partition chromatography where necessary, whilst all samples are subjected to adsorption chromatography to remove interfering endogenous materials. Final quantitative determination is by gas-liquid chromatography using electron capture detection and internal standardisation.

3 PROCEDURE

3.1 Sample Preparation

Samples should be removed from the deep freeze and allowed to stand at room temperature for approximately 1/2 hour until it is possible for them to be sliced prior to mincing. The mincing/chopping should be continued until a truly homogeneous sample is obtained.

Samples which are removed from the freezer having previously been homogenised, should be allowed to defrost for a minimum period only before breaking up and weighing out; this ensures that no partition of the endogenous water content can occur prior to weighing out the sample.

3.2 Extraction

3.2.1 Fruit and Vegetable Crops

- a) Thoroughly mix the chopped sample and weigh a representative aliquot (20g) into a centrifuge bottle.
- b) Spike all samples with a suitable known amount of internal standard (R171554 - see Appendix 2).
- c) Macerate for two minutes in 50% (v/v) acetone:hexane (60 cm³).
- d) Pipette an aliquot of the macerate (9 cm³ = 3g) into a 50cm³ boiling tube and wash the extract with 5% sodium chloride solution (20 cm³). Transfer the upper layer to a second tube using a pasteur pipette and repeat the wash procedure.
- e) Transfer the upper layer to a centrifuge tube and dry by shaking with granular anhydrous sodium sulphate (2g). Pipette a measured sample (1.5 cm³ = 1g of crop) onto an adsorption column (Section 3.4).

Note : Dry crops eg tea leaves and tobacco should be soaked in water (-50cm³) for five minutes prior to extraction. This improves the extraction efficiency of the pesticide.

3.2.2 Crops with high lipid content

- a) Thoroughly mix the sample and weigh a representative aliquot (20g) into a mason jar, add anhydrous sodium sulphate (~20g).
- b) Spike all samples with a suitable known amount of internal standard (R171554 - see Appendix 2).
- c) Macerate for five minutes using a Sorval Omni-mixer in 50% acetone:hexane (120 cm³).
- d) Allow the macerate to settle and filter an aliquot (12 cm³ = 2g crop) through a Whatman No 1 filter paper into a boiling tube. Transfer an aliquot (6 cm³ = 1g crop) into a graduated tube.
- e) Evaporate the solvent under a stream of clean, dry air and redissolve the oil in hexane equilibrated with acetonitrile (1 cm³).

3.3 Liquid-Liquid Chromatography

NB. In the following procedure hexane refers to hexane saturated with acetonitrile and acetonitrile refers to acetonitrile saturated with hexane.

- a) Prepare a liquid-liquid partition chromatographic clean-up column by slurry packing Florisil (5g) using acetonitrile into a 30 cm x 18 mm id chromatography column. Wash out excess acetonitrile with hexane (10 cm³).
- b) Place an aliquot (1 cm³ = 1g crop extract) onto the top of the partition column. Allow the sample to percolate onto the column, wash the column with hexane (30 cm³) and discard the washings. Elute the column with 25% v/v ether: hexane (60 cm³) and collect the eluate in a 100 cm³ round-bottom flask.
- c) Evaporate the hexane extract to dryness at 40°C and transfer to a 10 cm³ graduated centrifuge tube using hexane. Rinse the round-bottom flask with further hexane (3 x 2 cm³), add the rinsings to the graduated tube and concentrate by blowing with a gentle stream of dry air, to a 1.0g crop cm⁻³ solution. This solution may be stored tightly stoppered under refrigeration at 0°C until required for clean-up.

3.4 Adsorption Column Chromatographic Clean-Up

Two alternative adsorbants have been found useful for removal of coextractives prior to the final determination by GLC, ie, (1) silica gel or (2) magnesium trisilicate - Florisil (2g).

3.4.1 Silica (BOND ELUTTM) column clean-up

- a) Place a disposable silica column in the Vac ElutTM assembly. Add ether (4 cm³) turn on the vacuum supply and draw the ether through the column. Turn off the vacuum. Repeat the procedure with n-hexane.
- b) Transfer the sample extract onto the column, turn on the vacuum and draw the sample solution onto the column. Turn off the vacuum.
- c) Insert a rack containing the receiving tubes (5 cm³ capacity) into the Vac ElutTM assembly. Elute the column with 8% v/v diethyl ether:n-hexane (5 cm³) drawing the eluate through under vacuum into the collecting tubes.
- d) Reduce the volume of the collected fraction to a suitable volume, usually 1 cm³, by evaporation under a stream of clean dry air and analyse by GLC.

3.4.2 Preparation of Florisil Columns

- a) Place a small glass wool plug in the bottom of a 10 mm diameter chromatography column and add n-hexane (15 cm³). Slowly, with gentle tapping, add activated Florisil (2g) followed by granular anhydrous sodium sulphate (1g). Allow the hexane to percolate onto the column.

Note - Prior to use, each fresh batch of column packing material must be calibrated for the crop to be analysed, as follows: Fortify a control sample extract with a mixed PP321/ internal standard solution in n-hexane, such that the concentration of each is 5µg cm⁻³. Transfer an aliquot (1 cm³) of the fortified control extract to the top of the column and allow it to percolate onto the column. Wash with n-hexane (10 cm³). Then elute with 10% (v/v) diethyl ether:n-hexane and collect five fraction (10 cm³ each) of the eluate. Analyse the fractions by GLC to determine the elution pattern.

- b) Transfer an aliquot (equivalent to 1g crop) of the extract (from 3.2 or 3.3 above) and allow it to percolate onto the column. Elute the column using the procedure determined from the column calibration. Collect the diethyl ether:hexane eluate and evaporate to dryness at 40°C on a rotary evaporator. Dissolve the residue in n-hexane (1 cm³) and analyse by GLC.

4. GAS LIQUID CHROMATOGRAPHY (GLC)

The conditions for the analysis by GLC will depend upon the equipment available. The operating manuals for instruments should always be consulted to ensure safe and optimum use. The following conditions have been found to be satisfactory using a Hewlett Packard 5710A series instrument fitted with a Ni⁶³ (15 mCi) model 1873A electron capture detector.

4.1 Packed Column Chromatography

- a) Glass column 180cm x 0.2cm I.D.
- b) Column packing 5% OV210 or 5% OV202 on Chromosorb W-HP (100-120 mesh).
- c) Oven temperature 230°C; injector temperature 250°C; detector temperature 300°C.
- d) Carrier gas 5% methane in argon at 40cm³ min⁻¹

Under the above conditions PP321 gives a single peak at a retention time of 5.2 minutes. R171554 has a retention time of 6.5 minutes. Sensitivity is such that 500 x 10⁻¹²g PP321 injected on column, with electrometer attenuation at x 32 and recorder range on 1 mV give approximately 60% full scale deflection. (See Figure 2 for examples of chromatographic traces).

4.2 Capillary Column Chromatography

- a) OV101 fused silica capillary column 25m x 0.25mm ID WCOT.
- b) Grob type splitless injector, 40 second purge delay.
- c) Oven temperature: 47°C (hold 2 minutes) program at 15°C min⁻¹ to 215°C (hold for 26 minutes).
- d) Injector 280°C; detector 300°C.
- e) Carrier gas:helium at 1cm³ min⁻¹. Make up gas: 95:5 argon: methane at 30 cm³ min⁻¹.

Under the above conditions PP321 has a retention time of 26.2 minutes and R171554 has a retention time of 30.8 minutes. Sensitivity is such that 500 x 10⁻¹²g injected on column with electrometer attenuation at 10 x 16 and recorder range on 1 mV gives approximately 60% full scale deflection.

4.3 Calculation of PP321 Residue Results

Note - The internal standardisation procedure determines the concentration of the PP321 residue in the final extract relative to that of a known concentration of internal standard which is added by accurate fortification of the sample prior to extraction. Correction for percentage recovery throughout the procedure is thereby inherent for each individual sample; in addition, any small volume errors, particularly those associated with the final GLC injected solution are similarly corrected.

The calculation used for the determination of PP321 residues by internal standardisation using R171554 may be performed using a 'single point ratio calibration' (Ref. 1). It should be noted that such calibrations are only feasible when the internal standard chosen meets certain criteria (see Ref. 1 and Section 10).

- Make repeated injections of 2-5 μ l of a standard solution containing a mixture of PP321 and R171554 each at 0.1 μ g cm⁻³ into the GLC operated under conditions described in 4.1 or 4.2. When a consistent response is obtained measure the peak heights/areas obtained for PP321 and R171554 and calculate the PP321/internal standard peak ratio.
- Make an injection of each sample solution and measure the peak heights/areas of the peaks corresponding to PP321 and R171554 and similarly calculate the peak ratios.
- Re-inject the standard solution after a maximum of six injections of sample solutions.
- Calculate the PP321 residue in the sample, expressed as mg kg⁻¹, by proportionation of the PP321/internal standard peak height or peak area ratio measured for the sample against that for the analytical standard solution.

eg,

$$\text{PP321 residue (mg kg}^{-1}\text{)} = \frac{\text{peak height ratio in sample}}{\text{peak height ratio in standard}} \times \frac{\text{concentration of PP321 standard}}{\text{concentration of R171554 in standard}} \times \frac{\text{internal standard fortification level}}{1}$$

$$\begin{array}{l} \text{the units are} \\ \text{commonly} \end{array} \quad \frac{\text{mm} \times \text{mm}^{-1}}{\text{mm} \times \text{mm}^{-1}} \times \frac{\mu\text{g cm}^{-3} \times \mu\text{g g}^{-1}}{\mu\text{g cm}^{-3}} = \mu\text{g g}^{-1} = \text{mg kg}^{-1}$$

Note - in the case where laboratory data systems/computing integrators are used the computer algorithm may adopt a slightly different method for calculation of results. For example, the Hewlett Packard 3352B laboratory data system uses the relative detector response factor calculated from an analytical standard solution as the basis for calculation of results. The final calculated result is, of course, the same as the above manual calculation.

- * $0.1\mu\text{g cm}^{-3}$ internal standard solutions are used when the samples have been initially fortified at 0.1mg kg^{-1} , and the final substrate to solvent ratio is 1.0.

5 CONTROL EXPERIMENTS

At least one untreated sample must be analysed alongside any set of samples, using exactly the same method. This ensures that no contamination of the samples occurred prior to, or during, the analysis.

The amount of internal standard to be added should be decided by the residue levels expected in the crop. When no residues are expected, eg. in protected crop parts such as cotton seeds, then the amount should not exceed 0.1 mg kg^{-1} .

6 LIMIT OF DETERMINATION

The limit of determination of residues of PP321 can be assessed by carrying out recovery experiments at low levels of fortification ($0.005\text{-}0.02\text{mg kg}^{-1}$). The true limit of determination will give a final chromatographic response of at least 4 x the background noise at the PP321 retention time.

In these laboratories the limit of determination has been set at 0.01 mg kg^{-1} .

7 ESTIMATION OF THE PRECISION OF THE METHOD

The use of internal standardisation ensures that small inter sample differences in percentage recovery of PP321 throughout the method are corrected. Hence the expected precision is significantly better than that expected for external standard methodology. Experiments using homogeneous crop (apple) extracts have shown repeatability of measurement using this method to be $\pm 2\%$. Calculation of the same residues by external standardisation gave repeatability of $\pm 8\%$.

8 METHOD VALIDATION STUDIES

In these laboratories to date the method has been applied to the analysis of the following crops: apples, broccoli, cabbage, cotton, grape, hops, maize, oil seed rape, peach, pear, pepper, sorghum, tea and tomatoes. No endogenous materials from these crops have been observed to interfere with either PP321 or the internal standard during the final chromatographic determination step.

The validity of the internal standardisation procedure has been demonstrated by plotting calibration graphs for PP321/internal standard peak ratios against the residue concentration of PP321 in accurately fortified samples. The resultant plot is always a straight line (correlation coefficient, $r > 0.99$) with the intercept at zero. The percentage recovery of PP321 and R171554 throughout the procedure has been shown to be essentially identical and therefore the slope of the extracted calibration line is found to be virtually identical to that obtained for reference standard mixtures of PP321 and internal standard. Hence R171554 is shown to be a 'true' internal standard for the measurement of PP321 and consequently, 'single point ratio' calculations may be used instead of graphical interpolation for day to day assays.

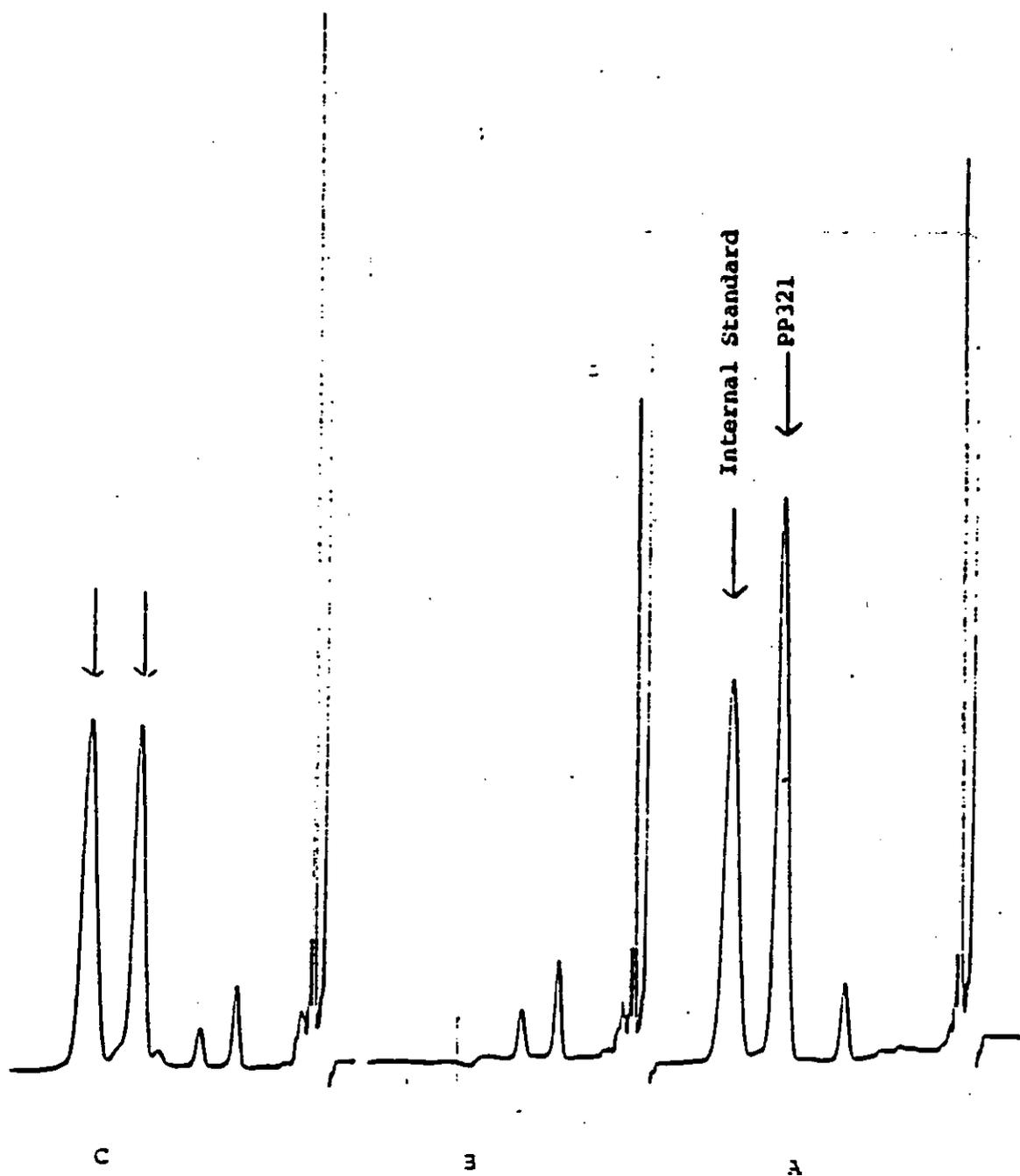
9 EXAMPLES OF CHROMATOGRAPHIC TRACES - see Figure 2 and 3**10 CONFIRMATION OF RESIDUES OF PP321**

Combined gas chromatography-mass spectrometry (GCMS) operated in the selected ion monitoring (SIM) mode may be used for the qualitative and quantitative confirmation of PP321 residues down to levels at the limit of determination ie, 0.01 mg kg^{-1} . Samples obtained from the residue analytical method are examined by SIM ie, three or more of the most abundant m/z values present in the mass spectrum are continuously monitored throughout the gas chromatographic run and recorded using a multi-channel pen recorder. Qualitative confirmation of residues is given by the appearance of a peak at the correct gas chromatographic retention time for all the specific m/z values monitored. In addition the ratios between the peak height responses given for each m/z value should be identical to that given by a standard solution of PP321.

Quantitative confirmation of PP321 residues is carried out by comparison of the peak height measured for the most abundant m/z value recorded, against that for an external standard.

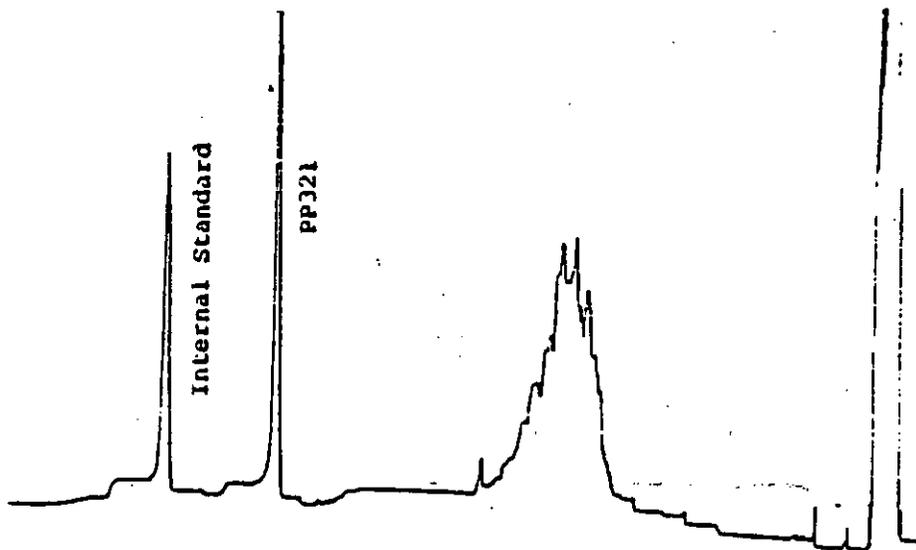
The selectivity of the technique is such that high crop to solvent ratios eg 20 g cm^{-3} may be injected into the instrument.

FIGURE 2: Typical Traces for PP321 Residue Determination using Packed Column GLC

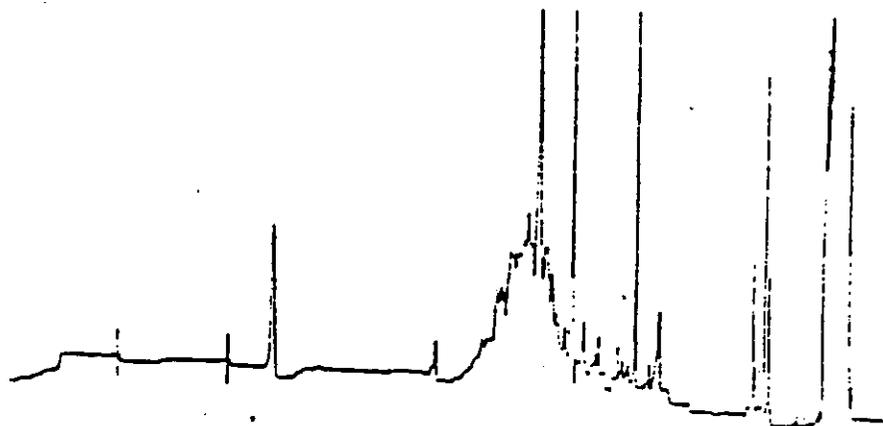


- A. 0.1 ug cm^{-3} PP321 + internal standard
B. Control sample at 1 g cm^{-3}
C. Apple sample. Residue = 0.06 mg kg^{-1}

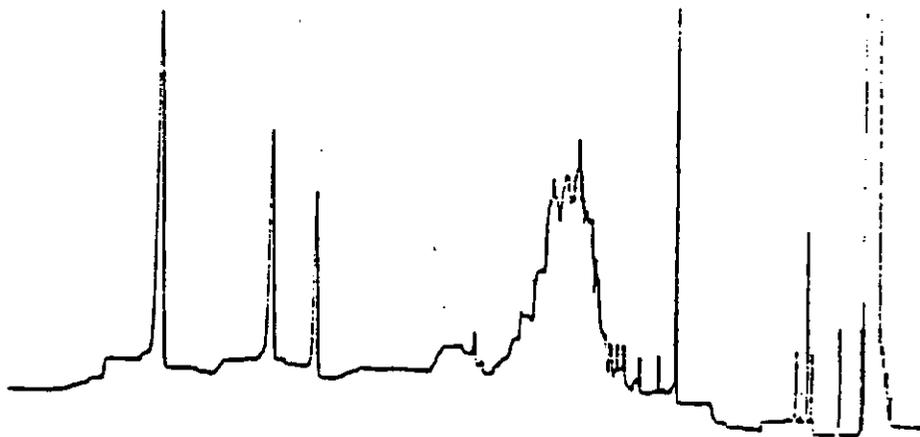
FIGURE 3: Typical Traces for PP321 Residue Determination using Capillary Column GLC



0.1 $\mu\text{g cm}^{-3}$ PP321 + Internal Standard



Control Sample



11 REFERENCES

- 1 Cardone M J, Palermo P J and Sybrandt L B : Potential Error in single point ratio calculations based on linear calibration curves with a significant intercept. Anal. Chem., 52, pp 1187-1191, 1980.

APPENDIX

1 APPARATUS

- a) Equipment for the initial preparation of samples, eg, Hobart food chopper (for soft crops) or ultra centrifugal mill, Retsch ZM1, fitted with a 3 mm screen (for seed crops). Available from Glen Creston, Stanmore, UK.
- b) High speed macerator, eg, Silverson Homogeniser, available from Silverson Machines Ltd., Chesham, Bucks or Sorval Omni mixer available from Du Pont UK Ltd.
- c) Glass columns, 300 mm x 10 mm i.d. and 300 mm x 18 mm i.d. for column chromatography.
- d) Graduated glass centrifuge tubes of 10cm³ capacity calibrated down to 1.0cm³ in 0.1cm³ units, with an accuracy of at least ±1% measured at 10 cm³.
- e) Gas-liquid chromatograph fitted with an electron capture detector, eg, Hewlett Packard 5700A series fitted with ⁶³Ni (15 mCi) model 1873A electron capture detector, or equivalent instrument.
- f) Capillary gas chromatograph fitted with an electron capture detector eg Varian 3700 series fitted with ⁶³Ni (8 mCi) detector or equivalent instrument.
- g) Potentiometric pen recorder (1mV), eg, Perkin Elmer 56 or equivalent instrument.

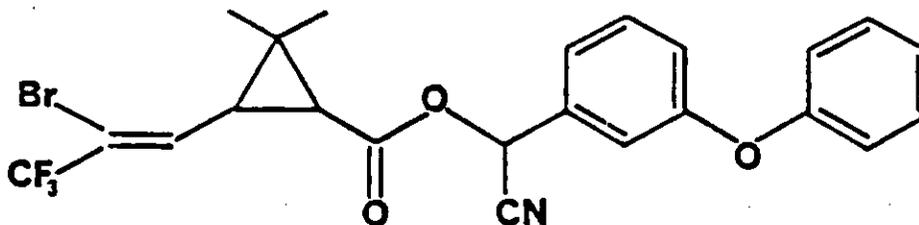
Note: - The use of an electronic integrator for measurement of peak areas, eg, Hewlett Packard 3352B GC data system or Hewlett Packard 3380A reporting integrator can be used (in addition to the chromatographic trace of the pen recorder) provided that the analyst is satisfied that the area response given is both accurate and precise. (see also Section 4).

- h) Syringes for gas-liquid chromatography, eg, Hamilton 10ul.

Note: - An autosampler apparatus with GLC equipment, eg, Hewlett Packard 7671A, is satisfactory, provided (a) suitably precise injections are achieved, ie, reproducibility better than ± 5%, (b) no cross-contamination from consecutive injections is observed, and (c) that no contamination arises in the final sample due to the autosample vials or vial caps.

2 REAGENTS

- a) Solvents: Redistilled acetone, acetonitrile, diethyl ether, n-hexane and methanol. Particular care must be taken to avoid contact with materials, eg, plastics, which may contaminate the solvents.
- b) Granular anhydrous sodium sulphate - Analar grade, BDH Chemicals Ltd., Poole, UK. Heat in an oven at 140°C for 24 hours to remove volatile contaminants.
- c) Sodium chloride - Analar grade, BDH.
- d) Glass wool - Contaminants are removed by treatment of the glass wool in a Soxhlett apparatus with refluxing n-hexane (redistilled) for 2 hours.
- e) Presilanised glass wool (for GLC Columns) - obtainable from chromatography suppliers.
- f) BOND ELUT™ disposable extraction columns (2.8 cm³) containing 500 mg silica (available from Jones Chromatography Ltd, U.K.).
- g) Florisil (100-120 US mesh) for chromatographic use available from BDH Ltd., Poole, UK. Activate by heating in an oven at 120°C for 24 hours before use.
- h) Stationary phases for gas-liquid chromatography, a semi-polar trifluoropropylmethyl silicone oil, OV210 or OV202, available from chromatography suppliers.
- i) Gas for gas-liquid chromatography - 5% methane in argon, dried by passing through molecular sieve type 5A.
- j) A sample of PP321 of known purity.
- k) A sample of R171554 of known purity for use as internal standard.



The internal standard used is the resolved Cis isomer assumed by analogy to the chromatographic capillary GLC characteristics of PP321 and other synthetic pyrethroids to be (SR)- α -cyano-3-phenoxybenzyl (1RS)-cis-3-(2-bromo-3,3,3-trifluoroprop-1-en-1-yl)cyclopropane-1-carboxylate.

Note - While all the reagents and apparatus may be individually checked for purity, it is necessary to analyse reagent blank samples, where the complete procedure has been carried out in the absence of crops. This will enable the analyst to verify whether the system produces a GLC trace which is free of interference at the retention times of PP321 and the internal standard.

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 doubt, consult the appropriate safety manual (eg, ICI Laboratory Safety Manual) containing recommendations and procedures for handling chemicals, and a monograph such as 'Hazards in the Chemical Laboratory', Ed G D Muir, The Chemical Society, London.

a) Solvent Hazards

	Acetone	Acetonitrile	Diethyl ether	Hexane	Methanol
Harmful vapour	Yes	Yes	Yes	Yes	Yes
Highly flammable	Yes	Yes	Yes	Yes	Yes
Harmful by skin absorption	-	Yes	-	-	Yes
TLV mg m ⁻³	2400	70	1200	180	260

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

b) PP321 and R171554 are synthetic pyrethroid insecticides with a mammalian toxicity (acute oral LD₅₀) in the rat in the order of 50-60 mg kg⁻¹ (PP321).

4 PREPARATION OF ANALYTICAL STANDARDS

Weigh out accurately, using a five figure balance, sufficient PP321 or R171554 to allow dilution in acetone to give a 1000µg cm⁻³ stock solution in a volumetric flask. Make serial dilutions of this stock to give 100µg cm⁻³, 10µg cm⁻³ and 1.0µg cm⁻³ standard solutions in acetone (used for fortification of crop samples). Prepare serial dilutions of mixed standards of PP321 and internal standard in hexane (for use as GLC reference standards) down to 0.1µg cm⁻³ or less as required.

When not in use, always store the standard solutions in a refrigerator at <4°C to prevent decomposition/evaporation/concentration of the standard strength. Analytical standards should be replaced with freshly prepared

5 PREPARATION OF COLUMNS FOR GAS-LIQUID CHROMATOGRAPHY

Stationary phases may be obtained from most chromatography suppliers precoated onto the support phase at the required loading. However, preparation of the required column packing may be performed in the laboratory by the following method: Dissolve 0.5g OV-202 in dichloromethane (100cm³) and add to 9.5g Chromosorb W-HP (100-120 mesh). Gently stir the mixture with a glass rod to ensure thorough mixing.

Remove the solvent under vacuum on a rotary evaporator until a dry, free-flowing powder is obtained then place on a petri-dish in an oven (100°C) for 2 hours. Columns may be packed under vacuum or under pressure from a gas cylinder, gentle vibration during this process will ensure uniform packing. Condition the packed column by heating at 250°C for 24 hours with the carrier gas flow rate at 30cm³ min⁻¹ leaving the detector end of the column disconnected to avoid contamination of the detector.

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ADDENDUM

PLANT PROTECTION DIVISION RESIDUE ANALYTICAL METHOD NO. 81

THE DETERMINATION OF RESIDUES OF PP321 IN CROPS

a gas-liquid chromatographic method using an internal standard*

*Also applicable to external recoveries.

Author : A Sapiets

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Date of Issue : 19 December 85

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PLANT PROTECTION DIVISION RESIDUE ANALYTICAL METHOD NO. 86

THE DETERMINATION OF RESIDUES OF PP321 IN PRODUCTS OF ANIMAL ORIGIN

a GLC method using an internal standard*

*Also applicable to external recoveries.

Author : A Sapiets

Study Director : J P Leahey

Date of Issue : 22 February 1985

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SCOPE

The analytical procedures described are suitable for the determination of residues of the insecticide PP321 (I) in products of animal origin. The methodology is essentially that described in Plant Protection Division Residue Analytical Method Number 81 - The Determination of Residues of PP321 in Crops - a gas-liquid chromatographic method using an internal standard. However it should be noted that this method can also be applied to the analysis of samples using an externally standardised procedure. Under these conditions the precision of the assay will be less than that quoted for the internally standardised method.

Examples of the typical range for external recoveries are given in Appendix 2.

To date, in these laboratories, the method has been applied to cow muscle (pectoral and adductor), fat (subcutaneous and peritoneal), kidney, liver and milk. The limit of determination has been set at 0.01 mg kg^{-1} (tissue) and 0.002 mg kg^{-1} (milk).

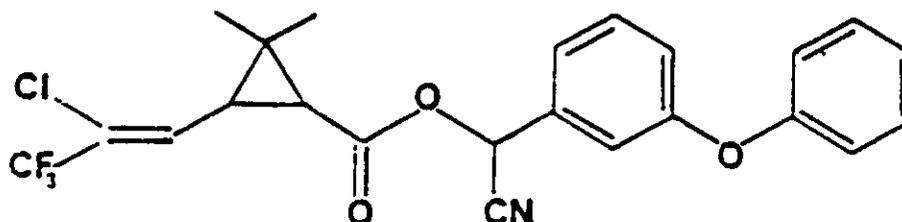


Figure 1: z-cyano-3-phenoxybenzyl-3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylate. A 1:1 mixture of the (Z)-(1R,3R),S-ester and (Z),(1S,3S),S-ester.

2 SUMMARY

Samples which have been accurately fortified with an internal standard are extracted by maceration with 50% v/v acetone:hexane. The organic extracts are washed with water to remove acetone and co-extracted lipids are removed by liquid-liquid partition chromatography. All samples are subjected to adsorption chromatography to remove interfering endogenous materials. Final quantitative determination is by gas-liquid chromatography using electron capture detection and internal standardisation.

3 PROCEDURE

3.1 Sample Preparation

Tissue samples should be removed from the deep freeze and allowed to stand at room temperature for approximately 30 minutes until it is possible for them to be sliced prior to mincing. The mincing/chopping should be continued until a truly homogeneous sample is obtained.

Samples which are removed from the freezer having previously been homogenised, should be allowed to defrost for a minimum period only before breaking up and weighing out; this ensures that no partition of the endogenous water content can occur prior to weighing out the sample.

Milk samples should be fully thawed and mixed before sub-sampling.

3.2 Extraction

3.2.1 Milk

- a) Thoroughly mix the sample and measure a representative aliquot (10 g) into a centrifuge bottle.
- b) Fortify all samples with an accurately known amount of internal standard. (see Appendix 4 (j)).
- c) Homogenise for two minutes in 50% acetone:hexane (50 cm³) and potassium oxalate (1.0 cm³ of a 0.1g cm⁻³ solution).
- d) Transfer the sample to a separating funnel and discard the lower aqueous layer.

Wash the organic layer with glass-distilled water (2 x 50 cm³) and discard the water. Dry the organic layer with anhydrous sodium sulphate and transfer an aliquot (2.5 cm³ = 1 g initial milk sample) to a liquid-liquid partition chromatography column.

3.2.2 Tissue

- a) Thoroughly mix the chopped sample and weigh a representative aliquot (10 g) into a centrifuge bottle.
- b) Fortify each sample with an accurately known amount of internal standard. Macerate for five minutes in 50% acetone:hexane (60 cm³).
- c) Take an aliquot of the macerate (18 cm³ = 3 g initial sample) and wash the extract with glass-distilled water (2 x 40 cm³) discarding the lower aqueous phase and any emulsified materials on each occasion.
- d) Shake the remaining hexane extract with granular anhydrous sodium sulphate and transfer a measured sample (3 cm³ = 1 g sample) onto a liquid-liquid partition chromatography column.

3.3 Liquid-Liquid Chromatography

Note - In the following procedure hexane refers to hexane saturated with acetonitrile and acetonitrile refers to acetonitrile saturated with hexane.

- a) Prepare a liquid-liquid partition chromatographic clean-up column by slurry packing Florisil (5g) using acetonitrile into a 30 cm x 19 mm ID chromatography column. Wash out excess acetonitrile with hexane (10 cm³).
- b) Place an aliquot of the sample onto the top of the partition column. Allow the sample to percolate onto the column, wash the column with hexane (30 cm³) and discard the washings. Elute the column with 25% v/v ether:hexane (75 cm³) and collect the eluate in a 100 cm³ round-bottom flask.
- c) Evaporate the hexane extract to dryness at 40°C and transfer to a 10 cm³ graduated centrifuge tube using hexane. Rinse the round-bottom flask with further hexane (3 x 2 cm³), add the rinsings to the graduated tube and concentrate by blowing with a gentle stream of dry air, to a 1.0g cm⁻³ solution. This solution may be stored tightly stoppered under refrigeration at 0°C until required for clean-up.

3.4 Adsorption Column Chromatography

- a) Place a glass wood plug in the bottom of a 10 mm diameter chromatography column and add n-hexane (15 cm³). Slowly, with gentle tapping, add activated Florisil (2g) followed by granular anhydrous sodium sulphate (1g). Allow the hexane to percolate into the column.

Note - Prior to use, each fresh batch of column packing material must be calibrated for the substrate to be analysed, as follows.

Fortify a control sample extract with PP321 plus internal standard (R171554) standard solution in hexane, such that the concentration is 5µg cm⁻³.

Transfer an aliquot (1 cm³) of the fortified control extract to the top of the column. Wash the columns with 5% v/v dichloromethane:hexane (20 cm³). Elute the columns with 30% (v/v) dichloromethane:n-hexane and collect four fractions (10 cm³ each) of the eluate. Analyse the fraction for PP321 and R171554 by GLC to determine their elution patterns.

- b) Transfer the extract (from Section 3.3 (c) above) and allow it to percolate into the column. Elute the column using the procedure determined from the column calibration. Collect the dichloromethane:hexane eluate and evaporate to small volume (~ 2 cm³) at 40°C on a rotary evaporator. Reduce the volume of the collected fraction to a suitable volume, usually 1 cm³ and analyse by GLC.

4. GAS LIQUID CHROMATOGRAPHY (GLC)

The conditions for the analysis by GLC will depend upon the equipment available. The operating manuals for instruments should always be consulted to ensure safe optimum use. The following conditions have been found to be satisfactory using a Hewlett Packard 5710A series instrument fitted with a Ni⁶³ (15 mCi) model 1873A electron capture detector.

4.1 Packard Column Chromatography

- a) Glass column 180 cm x 0.2 cm I.D.
 b) Column packing 3% OV25 on Chromosorb W-HP (100-120 mesh).
 c) Oven temperature 245°C; injector temperature 250°C; detector temperature 300°C.
 d) Carrier gas 5% methane in argon at 60 cm³ min⁻¹.

Under the above conditions PP321 gives a single peak, at a retention time of 4.0 minutes. R171554 has a retention time of 5.6 minutes. Sensitivity is such that 500×10^{-12} g PP321 injected on column, with electrometer attenuation at $\times 32$ and recorder range on 1 mV, give approximately 40% full scale deflection. (See Figures 3-5 for examples of chromatographic traces).

4.2 Capillary Column Chromatography

- a) OV101 fused silica capillary column 25m x 0.25mm ID WCOT.
- b) Grob type splitless injector, 40 second purge delay.
- c) Oven temperature; 47°C (hold 2 minutes) program at 15°C min⁻¹ to 215°C (hold for 26 minutes).
- d) Injector 280°C; detector 300°C.
- e) Carrier gas:helium at 1 cm³ min⁻¹. Make up gas: 95:5 argon:methane at 30 cm³ min⁻¹.

Under the above conditions PP321 has a retention time of 26.2 minutes and R171554 has a retention time of 30.8 minutes. Sensitivity is such that 500×10^{-12} g injected on column with electrometer attenuation at 10×16 and recorder range on 1 mV gives approximately 60% full scale deflection.

4.3 Calculation of PP321 Residue Results

Note - The internal standardisation procedure determines the concentration of the PP321 residue in the final extract relative to that of a known concentration of internal standard by accurate fortification of the sample prior to extraction. Correction for percentage recovery throughout the procedure is thereby inherent for each individual sample; in addition, any small volume errors, particularly those associated with the final GLC injected solution are similarly corrected.

The calculation used for the determination of PP321 residues by internal standardisation using R171554 may be performed using a 'single point ratio calibration' (Ref. 1). It should be noted that such calibrations are only feasible when the internal standard chosen meets certain criteria (see Ref. 1 and Section 9).

- a) Make repeated injections of 2-5µl of a standard solution containing a mixture of PP321 and R171554 each at $9.1 \mu\text{g cm}^{-3}$ into the GLC operated under conditions described in 4.1 or 4.2. When a consistent response is obtained measure the peak heights, areas obtained for PP321 and R171554 and calculate the PP321/internal standard peak ratio.

- b) Make an injection of each sample solution and measure the peak heights/areas of the peaks corresponding to PP321 and R171554 and similarly calculate the peak ratios.
- c) Re-inject the standard solution after a maximum of six injections of sample solutions.
- d) Calculate the PP321 residue in the sample, expressed as mg kg^{-1} , by proportionation of the PP321/internal standard peak height or peak area ratio measured for the sample against that for the analytical standard solution.

eg,

$$\text{PP321 residue (mg kg}^{-1}\text{)} = \frac{\text{peak height ratio in sample}}{\text{peak height ratio in standard}} \times \frac{\text{concentration of PP321 in standard}}{\text{concentration of R171554 in standard}} \times \text{internal standard fortification level}$$

the units are commonly

$$\frac{\text{mm} \times \text{mm}^{-1}}{\text{mm} \times \text{mm}^{-1}} \times \frac{\mu\text{g cm}^{-3}}{\mu\text{g cm}^{-3}} \times \mu\text{g g}^{-1} \times \mu\text{g g}^{-1} \times \text{mg kg}^{-1}$$

Note - in the case where laboratory data systems/computing integrators are used the computer algorithm may adopt a slightly different method for calculation of results. For example, the Hewlett Packard 3352B laboratory data system uses the relative detector response factor calculated from an analytical standard solution as the basis for calculation of results. The final calculated result is, of course, the same as the above manual calculation.

- * $0.1 \mu\text{g cm}^{-3}$ internal standard solutions are used when the samples have been initially fortified at 0.1 mg kg^{-1} , and the final substrate to solvent ratio is 1.0.

5 CONTROL EXPERIMENTS

At least one untreated sample must be analysed alongside any set of samples, using exactly the same method. This ensures that no contamination of the samples occurred prior to, or during, the analysis.

The amount of internal standard to be added should be decided by the residue levels expected in the tissue or milk.

6 LIMIT OF DETERMINATION

The limit of determination of residues of PP321 can be assessed by carrying out recovery experiments at low levels of fortification ($0.005\text{--}0.02 \text{ mg kg}^{-1}$). The true limit of determination will give a final chromatographic response of at least 4 x the background noise at the PP321 retention time.

In these laboratories the limit of determination has been set at 0.01 mg kg^{-1} for tissue samples and 0.002 mg kg^{-1} for milk samples.

7 ESTIMATION OF THE PRECISION OF THE METHOD

The use of internal standardisation ensures that small inter sample differences in percentage recovery of PP321 throughout the method are corrected. Hence the expected precision is significantly better than that expected for external standard methodology. Experiments using homogeneous muscle extracts have shown repeatability of measurement using this method to be $\pm 2.3\%$. Calculation of the same residues by external standardisation gave repeatability of $\pm 10\%$.

8 METHOD VALIDATION STUDIES

In these laboratories to date the method has been applied to the analysis of the following bovine milk, muscle (adductor and pectoral), kidney, liver and fat (peritoneal and subcutaneous). No endogenous materials from these substrates have been observed to interfere with either PP321 or the internal standard during the final chromatographic determination step.

The validity of the internal standardisation procedure has been demonstrated by plotting calibration graphs for PP321/internal standard peak ratios against the residue concentration of PP321 in accurately fortified samples. The resultant plot is always a straight line (correlation coefficient, $r > 0.99$) with the intercept at zero. The percentage recovery of PP321 and R171554 throughout the procedure has been shown to be essentially identical and therefore the slope of the extracted calibration line is found to be virtually identical to that obtained for reference standard mixtures of PP321 and internal standard. Hence R171554 is shown to be a 'true' internal standard for the measurement of PP321 and consequently, 'single point ratio' calculations may be used instead of graphical interpolation for day to day assays.

9 EXAMPLES OF TYPICAL CHROMATOGRAMS - see Appendix 1

10 CONFIRMATION OF RESIDUES OF PP321

Combined gas chromatography-mass spectrometry (GCMS) operated in the selected ion monitoring (SIM) mode may be used for the qualitative and quantitative confirmation of PP321 residues down to levels at the limit of determination ie, 0.01 mg kg^{-1} . Samples obtained from the residue analytical method are examined by SIM ie, three or more of the most abundant m/z values present in the mass spectrum are continuously monitored throughout the gas chromatographic run and recorded using a multi-channel pen recorder. Qualitative confirmation of residues is given by the appearance of a peak at the correct gas chromatographic retention time for all the specific m/z values monitored. In addition the ratios between the peak height responses given for each m/z value should be identical to that given by a standard solution of PP321.

Quantitative confirmation of PP321 residues is carried out by comparison of the peak height measured for the most abundant m/z value recorded, against that for an external standard.

The selectivity of the technique is such that high sample to solvent ratios eg $20:1 \text{ cm}^{-3}$ may be injected into the instrument.

11 REFERENCES

- 1 Cardone M J, and Palermo P J and Sybrandt L B : Potential Error in single point ratio calculations based on linear calibration curves with a significant intercept Anal. Chem., 52, pp 1187-1191, 1980.

Ref : AS/JPL/CL
Disc No : 146
PPRAM NO: 86
Date : January 1985

APPENDIX 1

Examples of Calibration Graphs and
Typical Gas Chromatograms

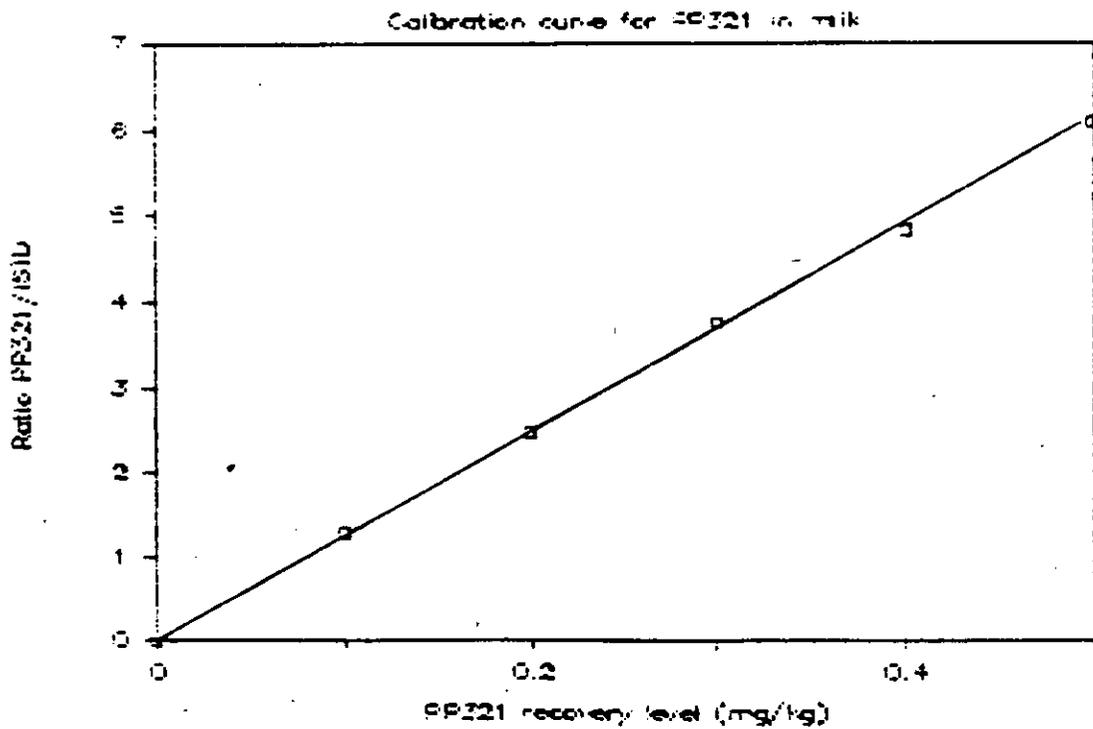
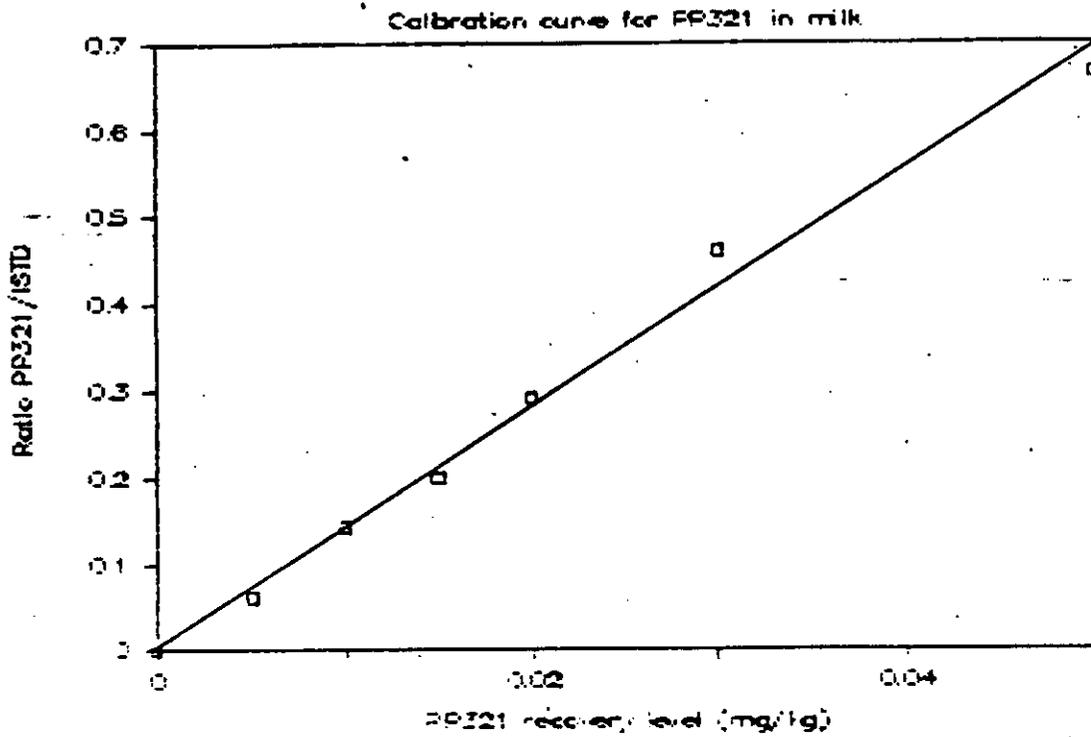
Figure 1 Calibration Curves for PP321 Residue Determination in Milk.

Figure 2 Calibration Curves for PP321 Residue Determination in Bovine Tissue.

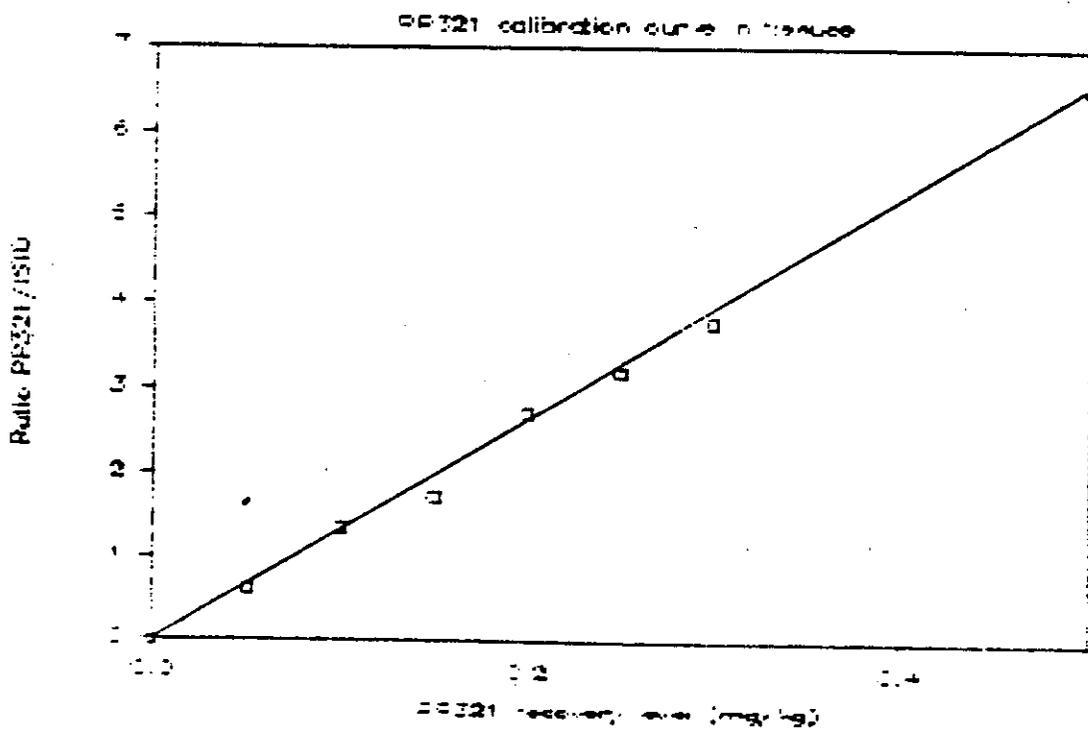
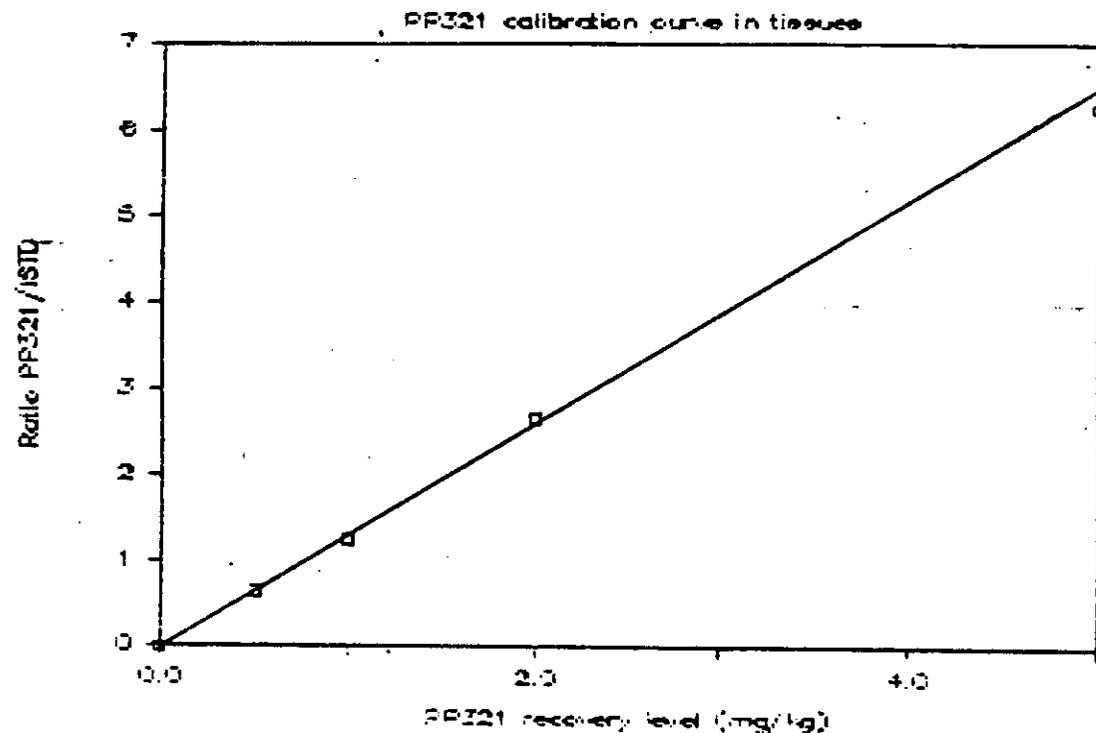
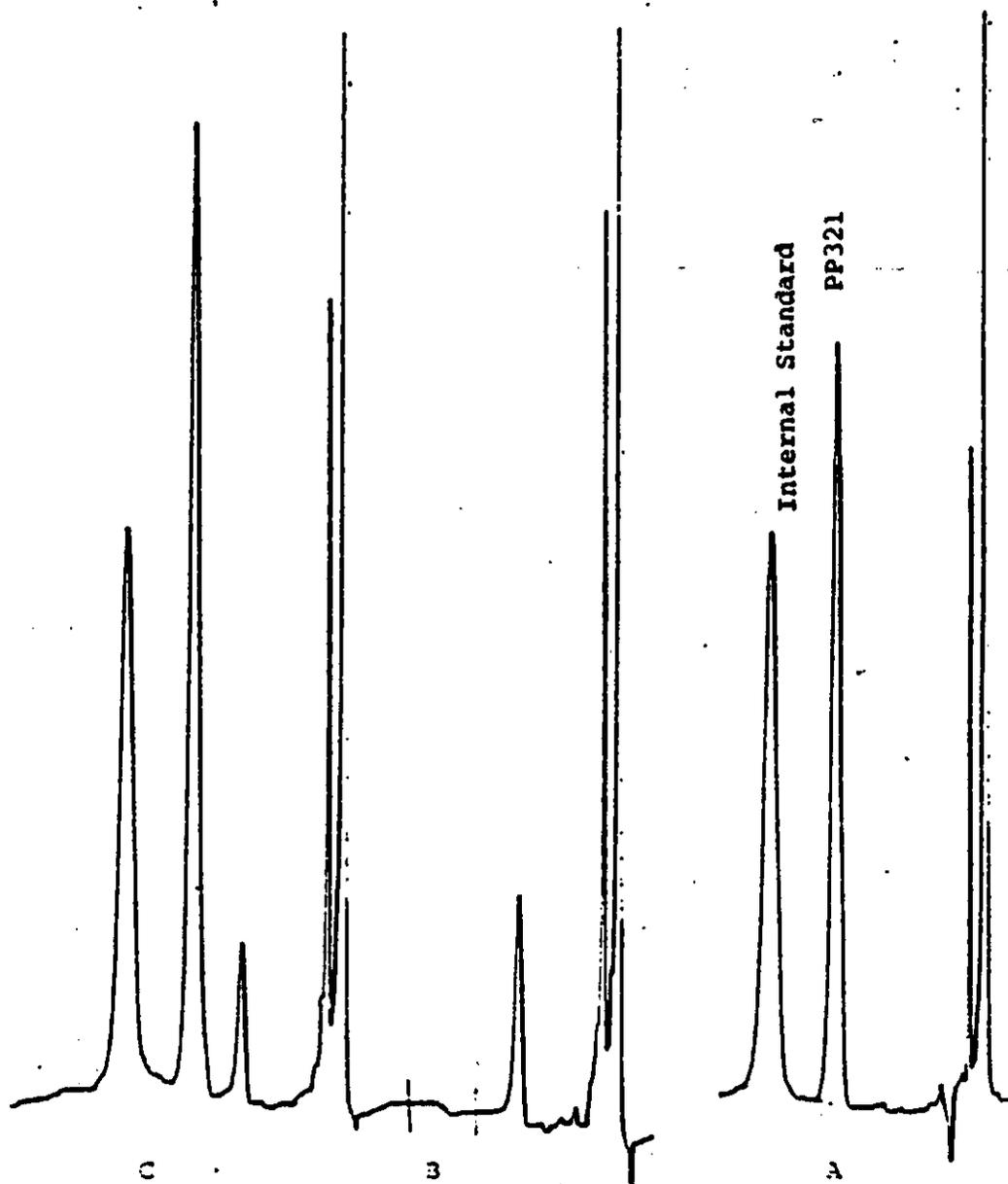


Figure 3

Typical gas chromatograms for PP321 residue determination in bovine muscle.

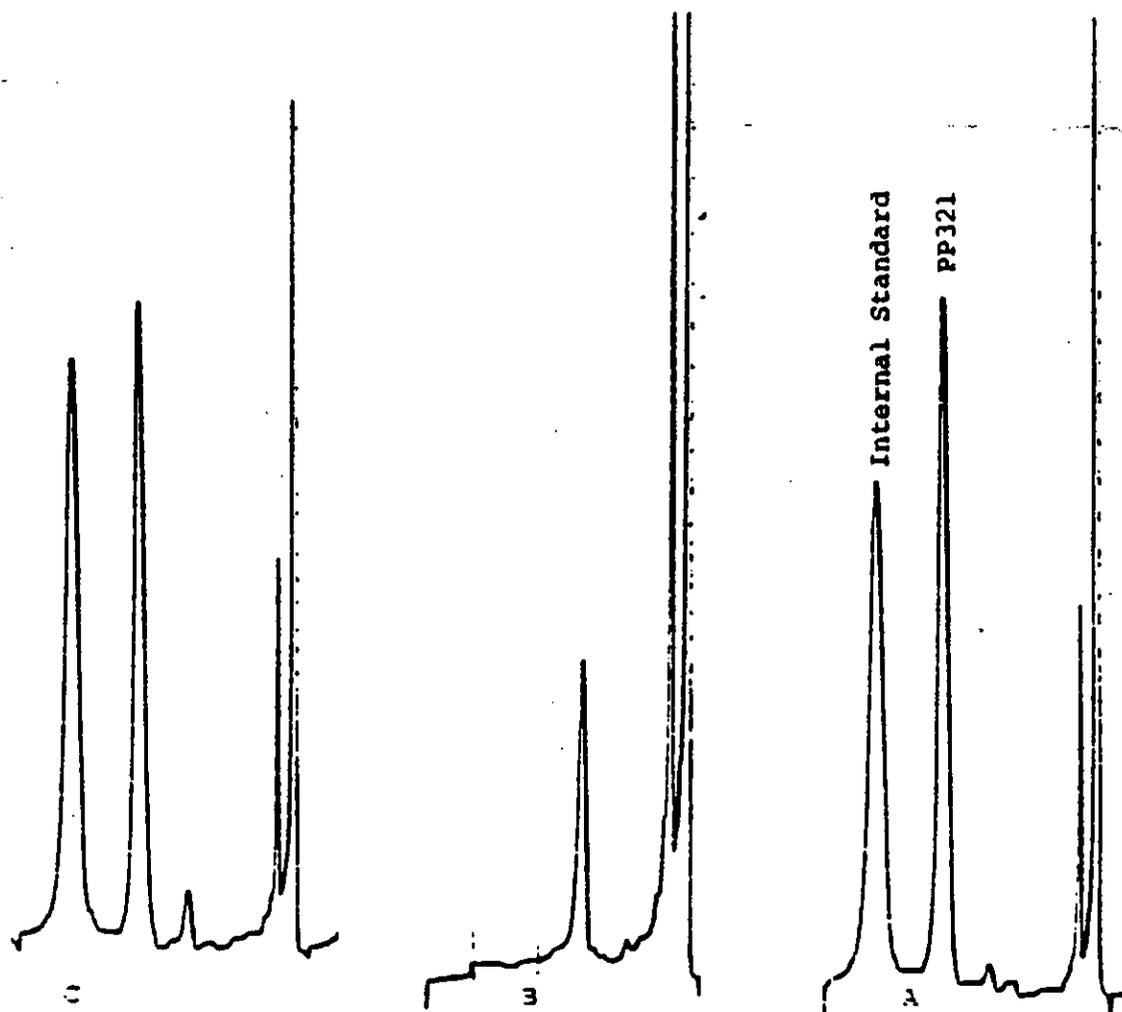


A 0.1 $\mu\text{g cm}^{-3}$ PP321 + Internal Standard.

B Control Sample (Cow 3). Residue = $<0.01 \text{ mg kg}^{-1}$.

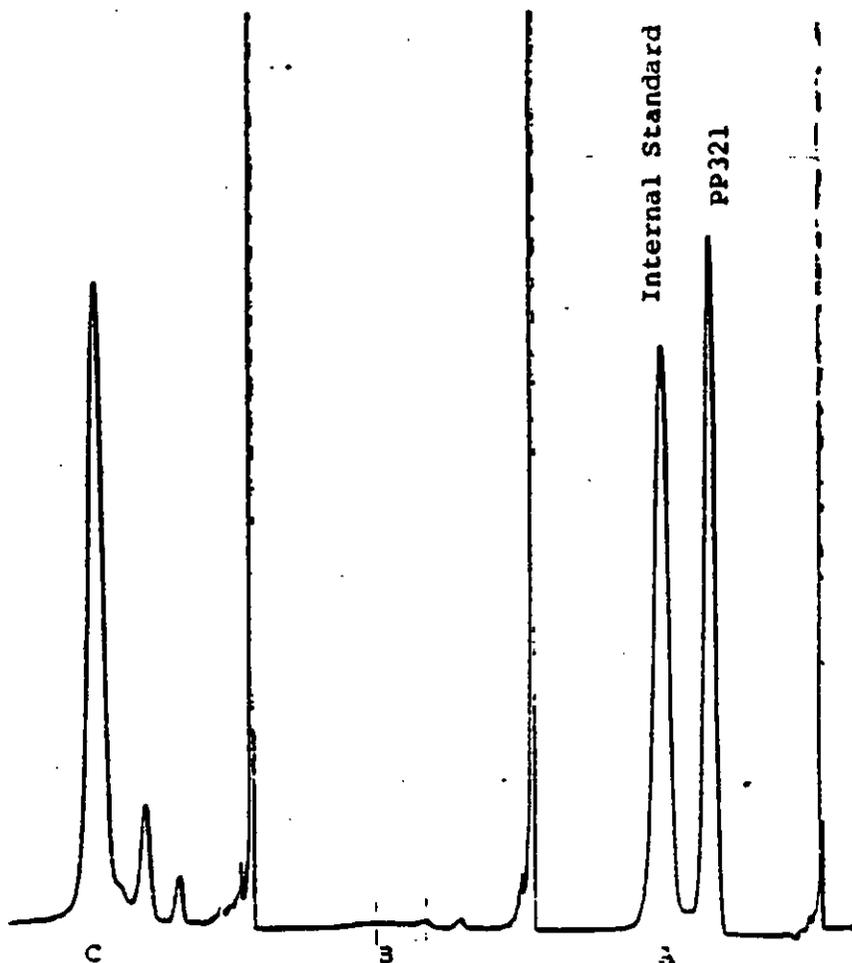
C 25 ng kg^{-1} Rate (Cow 12). Residue = 0.13 mg kg^{-1} .

Figure 4 Typical gas chromatograms for PP321 residue determination in bovine kidney.



- A $0.1 \mu\text{g cm}^{-3}$ PP321 + Internal Standard
 B Control Sample (Cow 2). Residue = $<0.01 \text{ mg kg}^{-1}$
 C 25 mg kg^{-1} rate (Cow 11) at 0.5 g cm^{-2} .
 Residue = 0.17 mg kg^{-1}

Figure 3 Typical gas chromatograms for PP321 residue determination in bovine milk.



- A 0.1 $\mu\text{g cm}^{-3}$ PP321 and internal standard.
B Control Sample (Cow 3). Residue = $<0.002 \text{ ng kg}^{-1}$.
C 25 mg kg^{-1} rate (Cow 13) at $1 \mu\text{g cm}^{-3}$. Residue = 0.07 ng kg^{-1} .

APPENDIX 2

Table of 99321 Recovery Data

Table 1 : PP321 Recovery data in milk and tissues

Recovery level mg kg ⁻¹	Substrate				
	milk	muscle	kidney	liver	fat
0.005	74,84	-	-	-	-
0.01	129,107	-	-	-	-
0.02	79,71	-	-	-	-
0.03	98,66	-	-	-	-
0.05	81,83,113	67,76	-	101,83,74	-
0.10	72,100,86	132,109,97	100,69	102	-
0.15	-	84	-	91	-
0.20	80,76,123	104,79	100,73	88	91
0.25	-	60	-	-	-
0.30	105	126	101	65	-
0.40	107	-	-	-	-
0.50	94,108,95	85	-	103	62
1.0	-	36	-	118	-
2.0	-	-	-	-	79
5.0	-	-	-	-	102
mean	92		90		
standard deviation	18		18		

Note : These recoveries were calculated using an external standard procedure after analysis by an internally standardised method. Because smaller volumes are taken in such a method the standard deviation about the mean recovery is quite high. However when these results were calculated by an internal standard procedure the true recovery was found to be 103% for milk and 98% for tissues (standard deviation 7-10%).

APPENDICES 3 - 7**3 Apparatus****4 Reagents****5 Hazards****6 Preparation of Analytical Standards****7 Preparation of Columns for Gas-Liquid Chromatography**

3 APPARATUS

- a) Equipment for the initial preparation of samples, eg, Hobart food mincer.
- b) High speed macerator, eg, Silverson Homogeniser, available from Silverson Machines Limited, Cheshire, Bucks or Sorval Omni mixer available from Du Pont UK Limited.
- c) Glass columns, 300 mm x 10 mm internal diameter and 300 mm x 18 mm internal diameter for column chromatography.
- d) Graduated glass centrifuge tubes of 10cm³ capacity calibrated down to 1.0cm³ in 0.1cm³ units, with an accuracy of at least $\pm 1\%$ measured at 10 cm³.
- e) Gas-liquid chromatograph fitted with an electron capture detector, eg, Hewlett Packard 5700A series fitted with ⁶³Ni (15 mCi) model 1873a electron capture detector, or equivalent instrument.
- f) Capillary gas chromatograph fitted with an electron capture detector eg Varian 3700 series fitted with ⁶³Ni (8 mCi) detector or equivalent instrument.
- g) Potentiometric pen recorder (1mV), eg Perkin Elmer 56 or equivalent instrument.

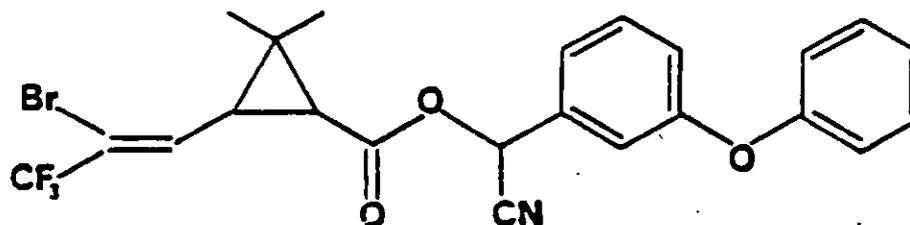
Note: The use of an electronic integrator for measurement of peak areas, eg Hewlett Packard 3352A GC data system or Hewlett Packard 3380A reporting integrator can be used (in addition to the chromatographic trace of the pen recorder) provided that the analyst is satisfied that the area response given is both accurate and precise. (see also Section 4).

- h) Syringes for gas-liquid chromatography, eg, Hamilton 10ul.

Note: An autosampler apparatus with GLC equipment, eg, Hewlett Packard 7671A is satisfactory, provided (a) suitably precise injections are achieved, ie, reproducibility better than $\pm 5\%$, (b) no cross-contamination from consecutive injections is observed, and (c) that no contamination arises in the final sample due to the autosample vials or vial caps.

4 REAGENTS

- a) Solvents: Redistilled acetone, acetonitrile, dichloromethane diethyl ether and n-hexane. Particular care must be taken to avoid contact with materials, eg, plastics, which may contaminate the solvents.
- b) Granular anhydrous sodium sulphate - Analar grade, BDH Chemicals Ltd., Poole, UK. Heated in an oven at 140°C for 24 hours to remove volatile contaminants.
- c) Sodium chloride and potassium oxalate - Analar grade, BDH.
- d) Glass wool - Contaminants are removed by treatment of the glass wool in a Soxhlett apparatus with refluxing n-hexane (redistilled) for 2 hours.
- e) Presilanised glass wool (for GLC Columns) - obtainable from chromatography suppliers.
- f) Florisil (100-120 US mesh) for chromatographic use available from BDH Ltd., Poole UK. Activate by heating in an oven at 120°C for 24 hours before use.
- g) Stationary phases for gas-liquid chromatography, a semi-polar phenylmethyldiphenyl silicone oil, OV25 available from chromatography suppliers.
- h) Gas for gas liquid chromatography - 5% methane in argon, dried by passing through molecular sieve type 5A.
- i) A sample of PP321 of known purity.
- j) A sample of R171554 of known purity for use as internal standard.



The internal standard used is the resolved *Cis* isomer assumed by analogy to the chromatographic capillary GLC characteristics of PP321 and other synthetic pyrethroids to be (1R)-1-cyano-3-phenoxybenzyl (1S)-*cis*-3-(Z-1-bromo-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylate.

Note - While all the reagents and apparatus may be individually checked for purity, it is necessary to analyse reagent blank samples, where the complete procedure has been carried out in the absence of sample. This will enable the analyst to verify whether the system produces a GLC trace which is free of interference at the retention times of PP321 and the internal standard.

5 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 doubt, consult the appropriate safety manual (e.g. ICI Laboratory Safety Manual) containing recommendations and procedures for handling chemicals, and a monograph such as 'Hazards in the Chemical Laboratory', Ed G D Muir, The Chemical Society, London.

a) Solvent Hazards

	Acetone	Acetonitrile	Diethyl ether	Hexane	Dichloro-methane
Harmful vapour	Yes	Yes	Yes	Yes	Yes
Highly flammable	Yes	Yes	Yes	Yes	-
Harmful by skin absorption	-	Yes	-	-	-
TLV mg m^{-3}	2400	70	1200	180	360

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

b) PP321 and R171554 are synthetic pyrethroid insecticides with a mammalian toxicity (acute oral LD_{50}) in the rat in the order of 50-60 mg kg^{-1} (PP321).

6. PREPARATION OF ANALYTICAL STANDARDS

Weigh out accurately, using a five figure balance, sufficient PP321 or R171554 to allow dilution in acetone to give a 1000 $\mu\text{g cm}^{-3}$ stock solution in a volumetric flask. Make serial dilutions of this stock to give 100 $\mu\text{g cm}^{-3}$, 10 $\mu\text{g cm}^{-3}$ and 1.0 $\mu\text{g cm}^{-3}$ standard solutions in acetone (used for fortification of crop samples). Prepare serial dilutions of mixed standards of PP321 and internal standard in hexane (for use as GLC reference standards) down to 0.1 $\mu\text{g cm}^{-3}$ or less as required.

When not in use, always store the standard solutions in a refrigerator at $<4^{\circ}\text{C}$ to prevent decomposition/evaporation/concentration of the standard strength. Analytical standards should be replaced with freshly prepared standards after 3 months of use.

7. PREPARATION OF COLUMNS FOR GAS-LIQUID CHROMATOGRAPHY

Stationary phases may be obtained from most chromatography suppliers precoated onto the support phase at the required loading. However, preparation of the required column packing may be performed in the laboratory by the following method: Dissolve 0.3 g OV-25 in acetone (100 cm³) and add to 9.7 g Chromosorb W-HP (100-120 mesh). Gently stir the mixture with a glass rod to ensure thorough mixing.

Remove the solvent under vacuum on a rotary evaporator until a dry, free-flowing powder is obtained then place on a petri-dish in an oven (100°C) for 2 hours. Columns may be packed under vacuum or under pressure from a gas cylinder, gentle vibration during this process will ensure uniform packing. Condition the packed column by heating at 280°C for 24 hours with the carrier gas flow rate at 30cm³ min⁻¹ leaving the detector end of the column disconnected to avoid contamination of the detector.