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Plant Protection Division

PLANT PROTECTION DIVISION RESIDUE

ANALYTICAL METHOD NO. 56

THE DETERMINATION OF RESIDUES OF CYPERMETHRIN IN PRODUCTS OF ANIMAL ORIGIN

a GLC method using internal standardisation

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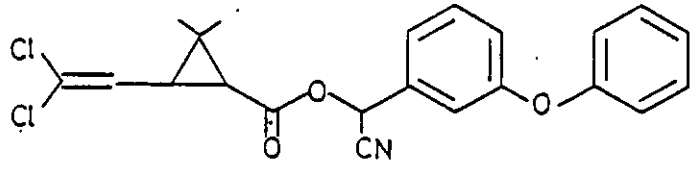
CONTENTS

1 SCOPE

The analytical procedures described are suitable for the determination of residues of the insecticide cypermethrin (I) in products of animal origin. The methodology is essentially that described in Plant Protection Division, Residue Analytical Method Number 46 - The Determination of Residues of Cypermethrin in Crops - a gas-liquid chromatographic method using an internal standard (decamethrin).

To date in these laboratories, the method has been applied to cow muscle (pectoral, adductor and cardiac), fat (subcutaneous and peritoneal), kidney, liver and milk. The limit of determination has been set at 0.01 mg kg^{-1} (tissue) and 0.005 mg kg^{-1} (milk).



I Cypermethrin α-(RS)-cyano-3-phenoxybenzyl-(1RS)-cis,trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate.

2 SUMMARY

Samples which have been accurately fortified with an internal standard are extracted by maceration in the presence of 50% v/v acetone:hexane and granular sodium sulphate (tissue) or potassium oxalate (milk). The organic extracts are washed with water to remove acetone and co-extracted lipids are removed by liquid-liquid partition chromatography. All tissue 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 homogenous 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 (10g) into a centrifuge bottle.

(b) Fortify each sample and one control with an accurately known amount of internal standard.

(c) Homogenise for two minutes in 50% acetone:hexane ($50cm^3$) and potassium oxalate, ($1.0cm^3$ of a 0.1g cm^{-3} solution).

(d) Transfer the sample to a separating funnel and discard the lower aqueous layer.

(e) Wash the organic layer with glass-distilled water ($2x50cm^3$) and discard the water. Dry the organic layer with anhydrous sodium sulphate and evaporate an aliquot ($12.5cm^3$ = 5g initial milk sample) to $1cm^3$.

3.2.2 Tissue (liver)

(a) Thoroughly mix the chopped sample and weigh a representative aliquot (50g) into a macerating jar.

(b) Fortify each sample and one control with an accurately known amount of internal standard. Macerate for five minutes in 50% acetone:hexane ($150cm^3$) in the presence of granular anhydrous sodium sulphate (50g).

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(c) Take an aliquot of the [redacted] cm^3 = 5g initial sample) and [redacted] extract with g[redacted]d water (2x10cm^3) discarding [redacted] aqueous phase a[redacted] fied materials on each

(d) Shake the [redacted] hexane extract with granular anhydrous sodium sulphate and [redacted] for a measured sample (1.5cm^3 = 1g sample) onto a liquid-[redacted] chromatography column.

3.3 Liquid-Liquid Chro[redacted]

(a) Prepare a liquid-liq[redacted] partition chromatographic clean-up column as follows: (in the following method hexane refers to hexane equilibrated with aceto[redacted] and acetonitrile refers to acetonitrile equilibrated with hexane). Slurry pack Florisil (7g) using acetonitrile into a 30cm x 1cm i.d. chromatography (10cm^3) column. Wash out excess acetonitrile with hexane (10cm^3).

(b) Transfer the extract onto the top of the partition column. Allow the sample to percolate onto the column then wash the column with hexane (30cm^3) and discard the washings.

(c) Elute the column with 25% ether:hexane (50cm^3) and collect the eluate in a 250cm^3 round-bottomed flask.

(d) Evaporate the hexane extract to small volume and transfer to a 10cm^3 graduated centrifuge tube. Rinse the round-bottom flask with hexane (3x2cm^3), add the rinsings to the tube and concentrate by blowing with a gentle stream of dry air to a 1g cm^{-3} solution.

3.4 Adsorption Column Chromatography (tissue samples only)

(a) Place a glass wool plug in the bottom of a 1cm diameter chromatography column and add n-hexane (15cm^3). Slowly, with gentle tapping, add activated Florisil (5g) 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 cypermethrin plus internal standard (decamethrin) standard solution in hexane, such that the concentration is 5µg cm^{-3}.
Transfer an aliquot (1cm^3) of the fortified control extract to the top of column. Wash the columns with n-hexane (10cm^3). Elute the columns with 15% (v/v) diethyl ether:n-hexane and collect six fractions (10cm^3 each) of the eluate. Analyse the fraction for cypermethrin and decamethrin by GLC to determine their elution patterns.

1. Take an aliquot of the macerate (15 ml = 5g. Initial sample) and wash the extract with glass-distilled water (2 X 10 ml) discarding the lower aqueous phase and any emulsified materials on each occasion.

2. Shake the remaining hexane extract with granular anhydrous sodium sulfate and transfer a measured sample (1.5 m = 1g. Sample) onto a liquid-liquid partition chromatography column.

3.3 Liquid-Liquid Chromatography

(a) Prepare a Liquid-liquid partition chromatographic clean-up column as follows: (in the following method hexane refers to hexane equilibrated with acetonitrile and acetonitrile refers to acetonitrile equilibrated with hexane). Slurry pack Florisil (7g.) using acetonitrile into a 30 cm X 1 cm i.d. chromatography (10 ml) column. Wash out excess acetonitrile with hexane (30 ml).

(b) Transfer the extract onto the top of the partition column. Allow the sample to percolate onto the column then wash the column with hexane (30 ml.) and discard the washings.

(c) Elute the column with 25% ether:hexane (50 ml) and collect the eluate in a 250 ml round-bottom flask.

(d) Evaporate the hexane extract to a small volume and transfer to a 10 ml graduated centrifuge tube. Rinse the round-bottom flask with hexane (3 X 2 ml.), add the rinsings to the tube and concentrate by blowing with a gentle stream of dry air to a volume of 1.5 ml. solution.

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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 cypermethrin plus internal standard (decamethrin) standard solution in hexane, such that the concentration is 5 ug./ml. Transfer an aliquot (1 ml.) Of the fortified control extract to the top of the column. Wash the column with n-hexane (10 ml.). Elute the columns with 15% (v/v) diethyl ether:n-hexane and collect six fractions (10 ml. each) of the eluate. Analyze the fractions for cypermethrin and decamethrin by GLC to determine their elution patterns.

(b) Transfer an aliquot (1.5cm^3 = 1g) of the extract (from Section 3.3 (d) 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 (< 1cm^3) at 40°C on a rotary evaporator. Reduce the volume of the collected fraction to a suitable volume, usually 1cm^3 and analyse by GLC.

4 GAS-LIQUID CHROMATOGRAPHY (GLC)

The conditions for the analysis will depend upon the equipment available. The operating manuals for instruments should be consulted to ensure safe and optimum use. The following conditions have been found to be satisfactory using a Hewlett Packard instrument fitted with a ^{63}Ni (15mCi) model 1873A electron capture detector.

(i) Glass column 90cm x 0.4cm i.d.

(ii) Column Packing 5% OV101 on Chromosorb W-HP (100-120 mesh).

(iii) Oven temperature 250°C; injector temperature 250°C; detector temperature 300°C.

(iv) Carrier gas 5% methane in argon at flow rate 60cm^3 min^{-1}.

Under the above conditions cypermethrin gives a single peak at retention time 3.0 minutes and decamethrin a single peak at 4.9 minutes. Sensitivity is such that 4.0 x 10^{-9} g of cypermethrin injected on-column, with electrometer attentuation at x512 and potentiometric recorder range on 1mv, gives approximately 40% full scale deflection. the electron capture detector response to decamethrin is approximately 1.2 times that of cypermethrin.

4.1 Gas-Liquid Chromatographic Determination

Note: The internal standardisation procedure determines the concentration of the cypermethrin residue in the final extract relative to that of a known concentration of decamethrin which is added by accurate fortification of the sample prior to extraction. Correction for percentage of 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 cypermethrin residues by internal standardisation using decamethrin 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 8).

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(b)

(c) Transfer an aliquot (1.5 ml = 1 g.) of the extract (from Section 3.3 (d) above) and allow it to percolate into the column. Elute the column using the procedure determined from the column calibration. Collect the diethyl ether:hexane eluate and evaporate it to a small volume (~ 2ml.) At 40^{0}c on a rotary evaporator. Reduce the volume of the collected fraction to a suitable volume, usually 1 ml and analyze by GLC.

4 GAS-LIQUID CHROMATOGRAPHY (GLC)

The conditions for the analysis by GLC depend upon the equipment available. The operating manuals for the 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 ^{63}Ni (15 Ci) model 1873A electron capture detector.

(i) Glass column 90 cm x 0.4 cm i.d.

(ii) Column Packing 5% OV101 on Chromosorb W-HP (100-120 mesh).

(iii) Oven temperature 250^{0}c.; injector temperature 250^{0}c; detector temperature 300^{0}c.

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Under the above conditions, cypermethrin gives a single peak at a retention time of 3.0 minutes and decamethrin gives a single peak at 4.9 minutes. Sensitivity is such that 4.0 x 10^{-9} g of cypermethrin injected on-column, with electrometer attenuation at x512 and potentiometric recorder range on 1mv, gives approximately 40% full scale deflection. The electron capture detector response to decamethrin is approximately 1.2 times that of cypermethrin.

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The calculation used for the determination of cypermethrin residues by internal standardisation using decamethrin may be performed using a 'single point ration 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 8).

(a) Make repeated injection of 2-5µl of a standard solution containing a mixture of cypermethrin and decamethrin each at 0.1µg cm^{-3}* into a GLC operated under conditions described above. When a consistent reponse is obtained measure the peak heights/areas obtained for cypermethrin and decamethrin and calculate the cypermethrin/decamethrin peak ratio.

(b) Make an injection of each sample solution and measure the peak heights/areas of the peaks corresponding to cypermethrin and decamethrin.

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

(d) Calculate the cypermethrin residue in the sample, in mg kg^{-1}, by proportionation of the cypermethrin/decamethrin peak height or peak area ratio measured for the sample, against that for the analytical standard solution, eg,

$$\text{Cypermethrin residue (mg kg}^{-1}\text{)} = \frac{\text{pk ht ratio in sample}}{\text{pk ht ratio in standard}} \times \frac{\text{concentration of cypermethrin in standard}}{\text{concentration of sample in final solution}}$$

the units of the calculation are commonly:

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

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. A control sample accurately fortified with a known amount of decamethrin, must also be run alongside each set of samples.

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

* 0.1µg 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.

6 LIMIT OF DETERMINATION

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

In these laboratories the limit of determination has been set at 0.01 mg kg^{-1} for tissue samples and 0.005 mg kg^{-1} for milk samples.

7 ESTIMATION OF THE PRECISION OF THE METHOD

The use of internal standardisation ensures that small intersample differences in percentage recovery of cypermethrin throughout the method are compensated for. Hence, the expected precision is significantly better than that expected for external standard methodology. Replicate (n=5) analysis of cow milk, muscle and fat samples carried out by one analyst gave results of 0.20±0.01, 0.49±0.03 and 2.4±0.08 mg kg^{-1} respectively, ie, giving precision of measurement using this method as ±3-7%. (Calculation of the results by an external standardisation method would give a standard deviation of >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, pectoral, cardiac) kidney, liver and fat (peritoneal and subcutaneous). No endogenous materials from these substrates have been observed to interfere with either cypermethrin or the internal standard during the final chromatographic determination step.

The extraction system used has been shown to be efficient for the removal of residues in milk and tissues (Ref. 2). In addition, the stability of cypermethrin residues stored deep-frozen for up to four months was confirmed in this study.

The validity of the internal standardisation procedure has been demonstrated by plotting calibration graphs for cypermethrin/internal standard peak ratios against the residue concentration of cypermethrin in accurately fortified samples. The resultant plot (see Figure 1) is always a straight line (correlation coefficient, >0.99) with the intercept at zero. The percentage recovery of cypermethrin and decamethrin throughout the procedure has been shown to be essentially identical. Hence decamethrin is shown to be a good internal standard for the measurement of cypermethrin. In addition 'single point ratio' concentrations may be used instead of graphical interpolation for day to day assays.

9 CONFIRMATION OF RESIDUES OF CYPERMETHRIN

Combined gas chromatography-mass spectrometry (GCMS) operated in the Selected Ion Monitoring (SIM) mode may be used for the qualitative and quantitative confirmation of residues of cypermethrin down to levels at the limit of determination, ie, 0.01 mg kg^{-1}. Samples obtained from the residue analytical methods for cypermethrin 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, or area, responses given for each m/z value should be identical to that given by an analytical standard of cypermethrin. Quantitative confirmation of cypermethrin residues is carried out by comparison of the peak height, or area, measured for the most abundant m/z value recorded, against an external standard of cypermethrin.

The selectivity of the technique is such that high sample to solvent ratios, eg, 20g ml^{-1} may be injected (5µl) into the instrument.

10 REFERENCES

1 M J Cardone and P J Palermo; Potential Error in Single Point Ratio Calculations based on Linear Curves with a Significant Intercept. Anal. Chem., 52, pp 1187-1191, 1980.

2 H Swaine and A Sapiets; Cypermethrin: Residue Transfer Study with Dairy Cows fed on a Diet containing the Insecticide. ICI Plant Protection Division Report No. RJ 0186B (1981).

APPENDIX

1 APPARATUS

(a) Equipment for the initial preparation of samples, eg, Hobart Food Mincer.

(b) High speed macerator, eg, Silverson Homogeniser, available from Silverson Ltd., UK.

(c) Glass Columns, 1cm internal diameter for column chromatography.

(d) Graduated glass centrifuge tubes of 10mls capacity calibrated down to 1.0ml in 0.1ml units, with an accuracy of at least ±1% measured at 10mls.

(e) Gas-liquid chromatograph fitted with an electron capture detector, eg, Hewlett Packard 5700A series fitted with Ni^{63} (15mCi) model 1873A electron capture detector, or equivalent instrument.

(f) Syringes for gas-liquid chromatography, eg, Hamilton 10µl

Note: the use of 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 autosampler vials or vial caps.

(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 3351A GC data 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.

2 REAGENTS

(a) Solvents: redistilled acetone, acetonitrile, 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. (Heat 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 two hours.

(e) Presilanised glass wool (for GLC columns) - obtainable from chromatography suppliers.

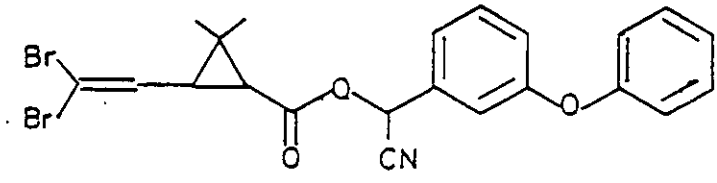
(f) Florisil (100-200 US mesh) for chromatographic use available from BDH Ltd., Poole, UK. (Activate by heating in an oven at 120°C for 24 hours before use).

(g) Stationary phase for gas-liquid chromatography, a non-polar methyl silicone oil, OV101, OV1 or SE30 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 cypermethrin of known purity.

(j) A sample of NRDC 161 for use as internal standard. The compound is known as decamethrin or alternatively deltamethrin.



α-(S)-cyano-3-phenoxybenzyl-(1R)-cis-3-(2,2-dibromovinyl)-2,2-dimethyl-cyclopropanecarboxylate.

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 mannual (eg, ICI laboratory safety manual) containing recommendations and procedures for handling chemicals, or a monograph such as 'Hazards in chemical Laboratory', Ed. G D Muir, The Chemical Society, London.

Acetone (dimethyl ketone)

Highly flammable
Vapour - air mixture explosive
Avoid breathing vapour. Avoid contact with eyes
TLV 1000ppm. (2400 mg m^{-3}).

Acetontrile (methyl cyanide)

Harmful vapour
Harmful by skin absorption
High flammable
Avoid breathing vapour. Avoid contain with skin and eyes.
TLV 40ppm (70 mg m^{-3}).

Diethyl ether

Harmful vapour
High flammable
Avoid breathing vapour
TLV 400ppm (1200 mg m^{-3}).

Hexane (and hexane fractions from petroleum)

Highly flammable
Avoid breathing vapour
TLV 500ppm (1800 mg m^{-3})

CYPERMETHRIN is a synthetic pyrethroid insecticide of extremely low mammalian toxicity, eg, (rat LD_{50} 4030 mg kg^{-1}).

DECAMETHRIN is known to be relatively more toxic.

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 cypermethrin and decamethrin.

4 PREPARATION OF ANALYTICAL STANDARDS

Weigh out accurately, using a five figure balance, sufficient cypermethrin or decamethrin to allow dilution in acetone to give a 1000 µg cm^{-3} stock solution in a volumetric flask. Make serial dilutions of this stock to give 100 µg cm^{-3}, 10 µg cm^{-3} and 1.0 µg cm^{-3} standard solutions in acetone used for fortification of samples. Prepare serial dilutions of mixed standards of cypermethrin and decamethrin in hexane for use as GLC reference standards.

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

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.

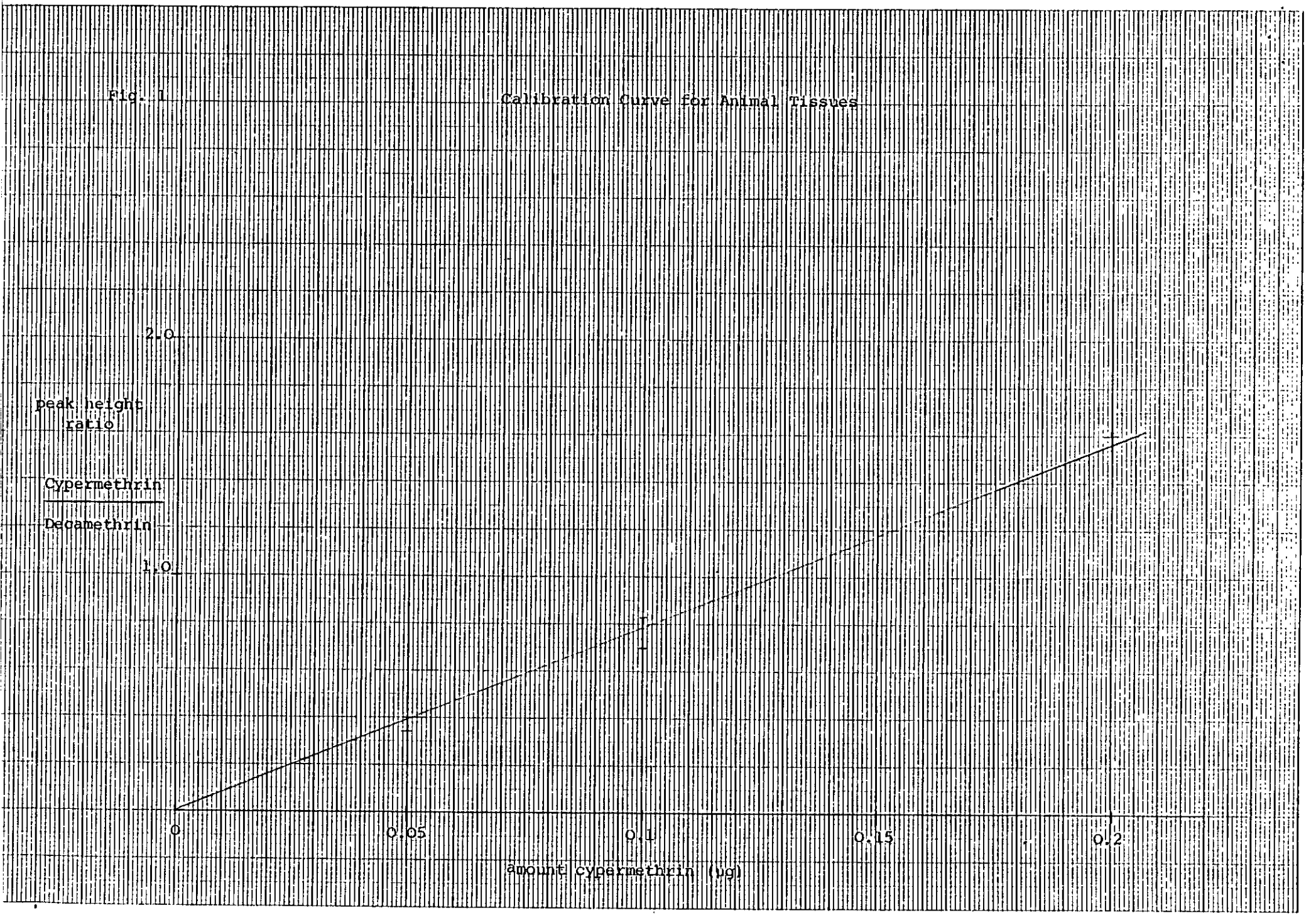
Dissolve 0.5g OV-101 in chloroform or toluene ($100cm^{-3}$) and add to 9.5g chromosorb W-HP (100-120 mesh). Gently stir the mixture with a glass rod to ensure thorough mixing.

Remove the solvent under vacuum on a rotary evaporator until a dry, free-flowing powder is obtained. Further dry the powder on a petri-dish in an oven at $120^{o}C$ for two hours.

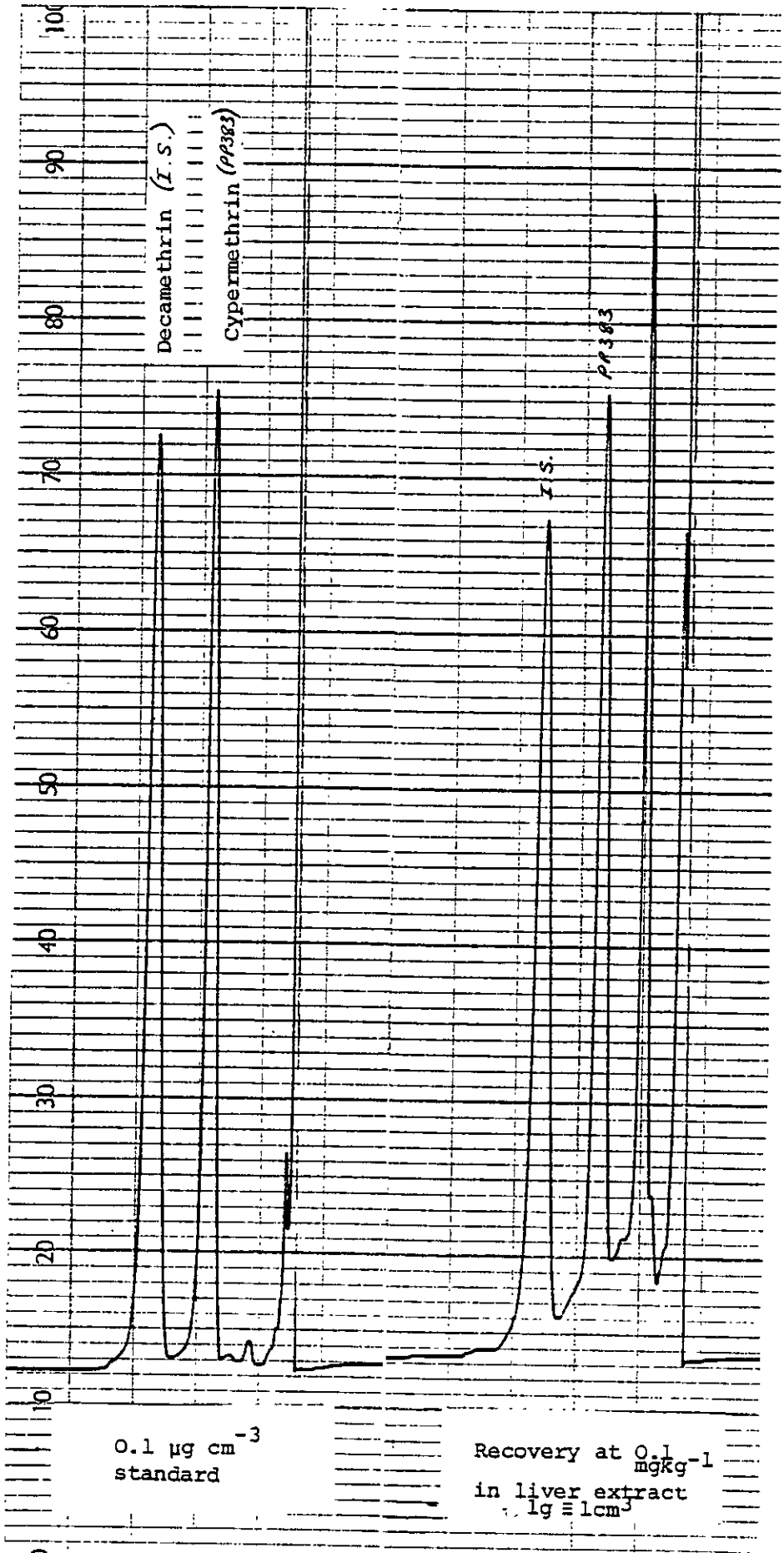
Columns are packed under vacuum. The stationary phase is held in place by presilanised glass wool plugs at the ends of the column. Gentle vibration of the column during packing ensures uniform packing.

Condition the packed column by heating at $270^{o}C$ for 24 hours with the carrier gas flow rate at $30cm^{3}\ min^{-1}$. The detector end of the column is left disconnected to avoid contaimination of the radioactive source during conditioning.

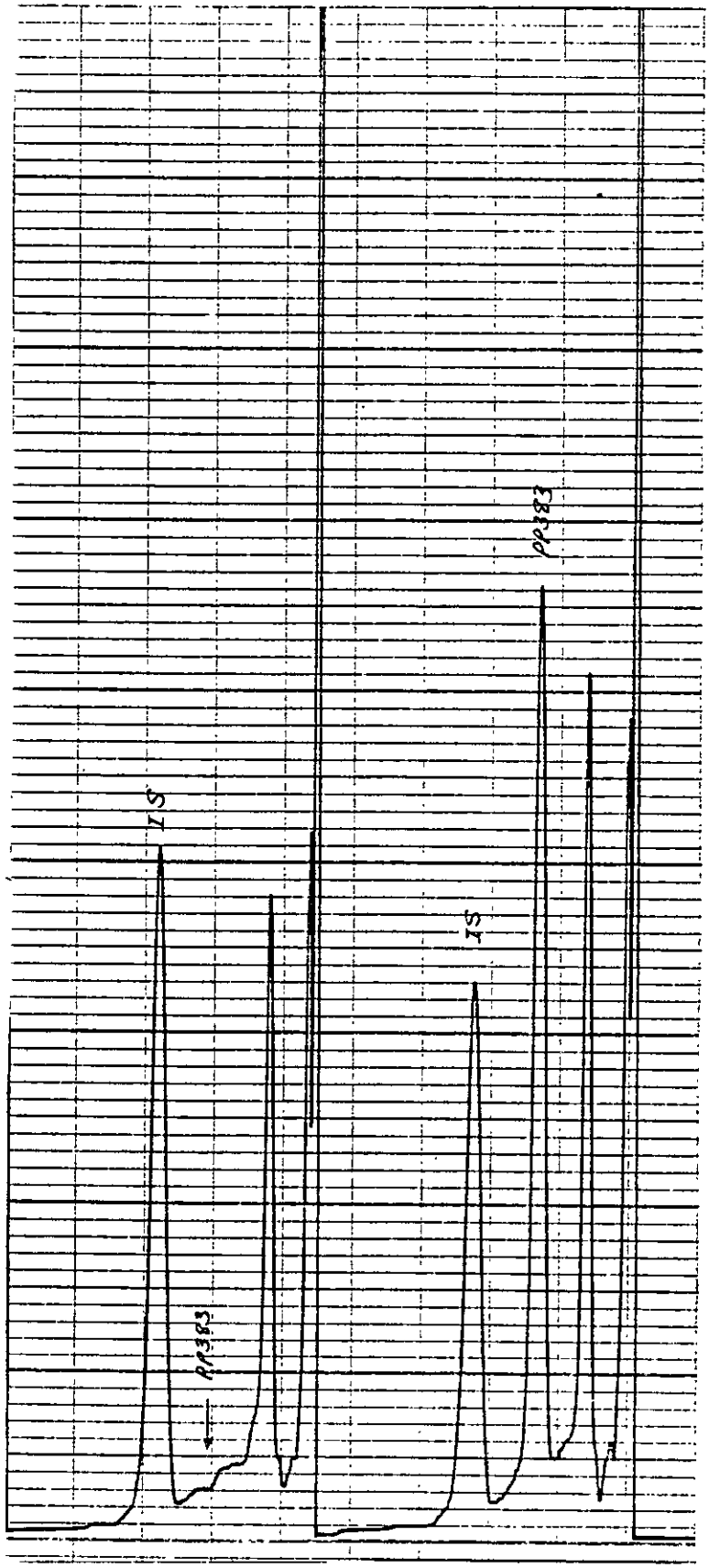
Fig. 1 Calibration Curve for Animal Tissues



GLC trace of Cypermethrin standard and a recovery from fortified bovine liver



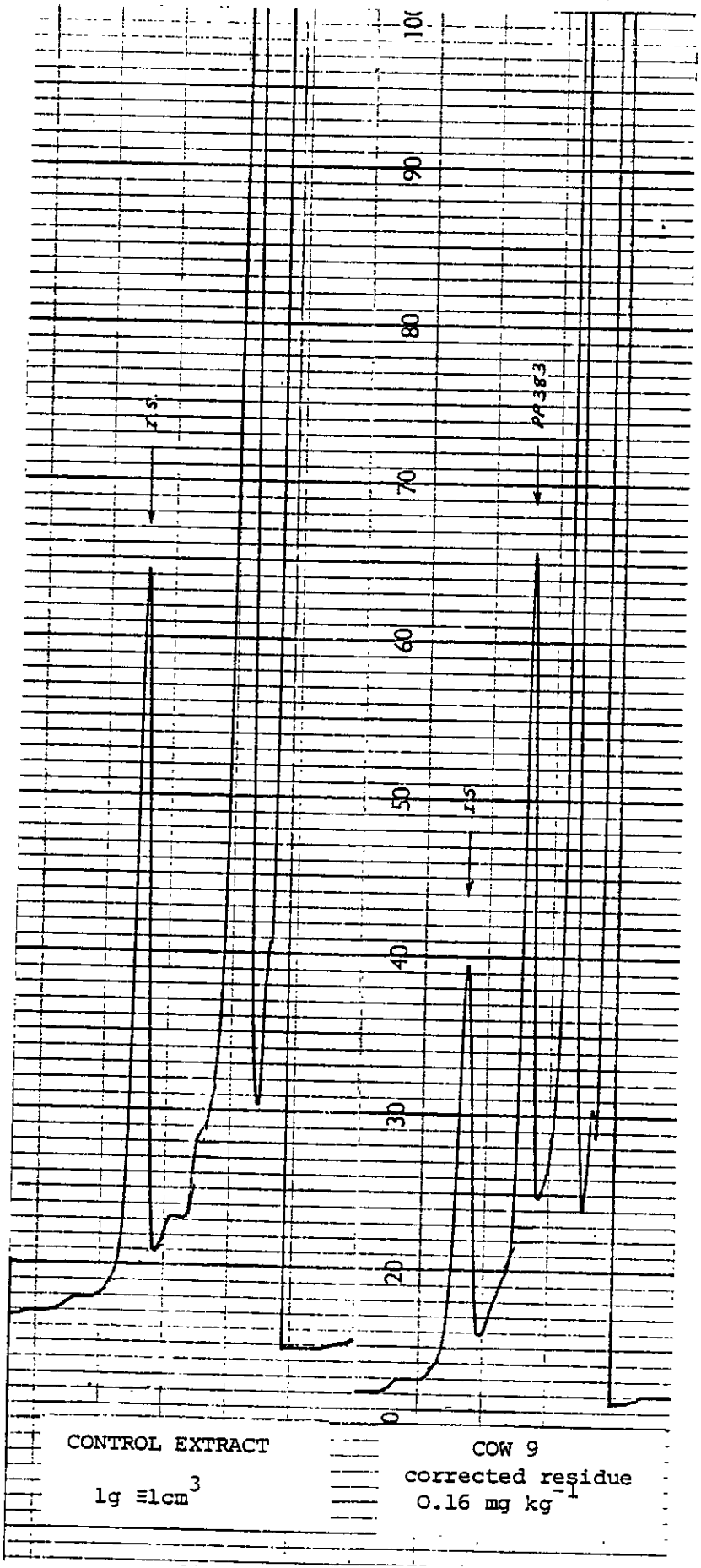
GLC trace for the final determination of Cypermethrin in bovine tissue adductor muscle



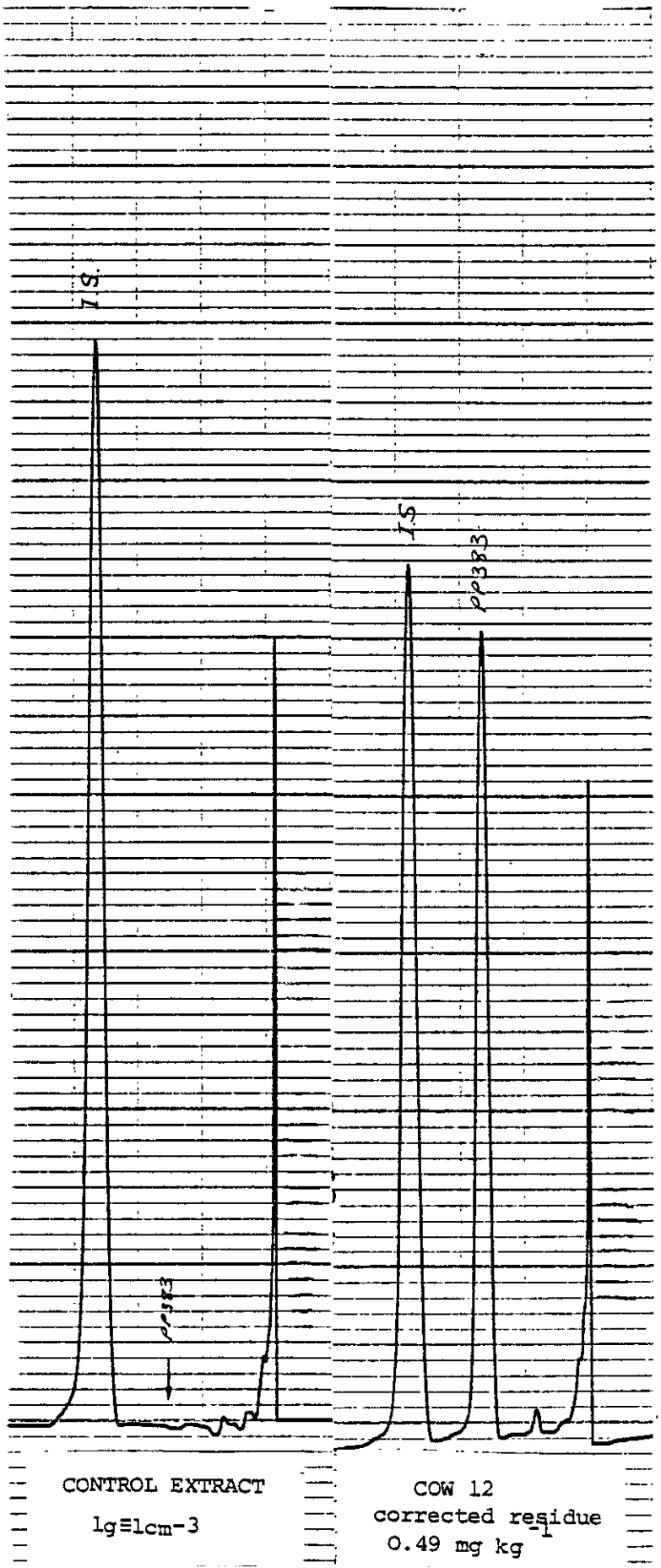
CONTROL EXTRACT	COW 11
1g ≡1cm^3	corrected residue
	0.16 mg kg^{-1}

GLC trace for the final determination of Cypermethrin in bovine tissue

subcutaneous fat



GLC trace for the final determination of Cypermethrin in bovine milk



CONTROL EXTRACT

1g≡1cm-3

COW 12

corrected residue

0.49 mg kg^{-1}