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Preclinical Research Report	Serial Number: P 23 979/32
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Subject: Electron capture GC determination method for imazalil and two of its metabolites in bovine ~~tissues and milk~~.
Addendum 2: Recommended procedure.

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S u m m a r y

A gas-liquid chromatographic determination method is described for measuring major regulable residues of imazalil in animal tissues and milk. The method is based on the acid hydrolysis of the samples, specific extraction of the compounds of interest and conversion of the metabolites to their trimethylsilyl derivatives.
Concentrations of imazalil and the metabolites are determined by electron capture gas-liquid chromatography over a linear dynamic range equivalent to 0.002-20 ppm.
No internal standard is used for the quantitation.

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1. Introduction

A gas chromatographic method for the determination of the antifungal imazalil and two of its basic metabolites in animal plasma and tissues has been described earlier (1). More recently, the method was evaluated and applied to a milk and tissue residue study in lactating cows (2,3,4). This report describes the final and recommended assay method for the determination of imazalil-related residues in animal tissues and milk samples.

2. Materials and Methods

2.1. Materials

2.1.1. Solvents

Spectrophotometric grade n-heptane (Uvasol®, E. Merck, Darmstadt, G.F.R.) and methanol ('Baker Analyzed' Reagent, J.T. Baker Chemicals B.V., Deventer, Holland). Toluene and isoamyl alcohol were of analytical reagent grade.

2.1.2. Silylation reagents

Dimethyldichlorosilane and N,O-Bis(trimethylsilyl) acetamide (BSA) (Janssen Quimica, Beerse, Belgium).

2.1.3. Inorganic reagents

Sodium hydroxide, sulphuric acid, hydrochloric acid and concentrated ammonia of analytical reagent grade ('Baker Analyzed' Reagent, J.T. Baker Chemicals B.V., Deventer, Holland). Prepare and dilute the hydrochloric acid (3 N), sulphuric acid (0.1 N) and sodium hydroxide (0.1 N) solutions as needed.

2.1.4. Standards

Imazalil (R 23 979), α-(2,4-dichlorophenyl)-1H-imidazole-1-ethanol (R 14 821) and 3-[1-(2,4-dichlorophenyl)-2-(1H-imidazol-1-yl)ethoxy]-1,2-propanediol (R 42 243) received from Janssen Quimica (Beerse, Belgium) as analytical standards.

2.1.5. Standard solutions

Separate stock solutions of imazalil, R 14 821 and R 42 243 at 1 mg/ml methanol; prepare and dilute as needed.

2.1.6. Animal tissues and milk

Liver, kidney, muscle, fat and milk samples, obtained from untreated lactating cows. Grind samples upon receipt and store frozen (-20°C) until use.

2.2. Apparatus

2.2.1. Gas chromatograph

Varian Model 3700 gas chromatograph, equipped with pulse-modulated constant-current ^{63}Ni electron capture detector. Glass column (2 m x 3 mm id), deactivated with 5 % dimethyldichlorosilane in toluene and packed with 3 % SP-2250 DB on 100-120 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.). Conditions: column 245° C; injection port 280° C; detector 340° C; nitrogen carrier gas 35 ml/min. Chromatograms recorded on a 1-mV Omniscribe® recorder (Houston Instrument); integrations and calculations by a Spectra-Physics Model 4000 data system. Under these conditions, retention times are 2.8, 2.0 and 6.0 min for imazalil (R 23 979), R 14 821 and R 42 243 respectively.

2.2.2. Grinding apparatus

Waring Commercial Blendor®

2.2.3. Homogenizer

Ultra-Turrax Model TP 18/2 (Janke & Kunkel KG, Staufen, G.F.R.).

2.2.4. Test tube mixer

Cenco Rotary Mixerr (Cenco Instrumenten B.V., Breda, Holland).

2.2.5. Heating block

Reacti-Therm TH (Pierce Eurochemie B.V., Rotterdam, Holland).

2.3. Extraction procedure

2.3.1. Milk samples

- Pipet 1-ml milk samples into 10-ml glass centrifuge tubes, containing 2 ml 3 N HCl.
- Heat samples in a water bath at 100° C for 1 h.
- Cool tubes, add 2.5 ml concentrated ammonia and 4 ml heptane-isoamyl alcohol (95:5, v/v).
- Rotate tubes at 10 rpm for 10 min with rotary mixer.
- Centrifuge mixture for 10 min at 2500 rpm.
- Transfer organic layer to 15-ml glass centrifuge tube by means of disposable pasteur pipet.
- Extract aqueous layer with additional 4 ml of heptane-isoamyl alcohol, centrifuge and combine organic layers.
- Discard aqueous layer.
- Add 3.0 ml 0.1 N H_2SO_4 to combined heptane-isoamyl alcohol extracts.
- Mix on test tube mixer at 20 rpm for 10 min.
- Centrifuge mixture for 5 min at 2500 rpm.
- Aspirate organic layer and discard.
- Add 0.15 ml of concentrated ammonia.

- Re-extract alkaline phase two times with 2 ml heptane-isoamyl alcohol.
- Evaporate combined organic layers to dryness in 55° C water bath.
- Submit samples to GLC-analysis.

2.3.2. Tissue samples

- Grind tissue samples in a Waring Commercial Blendor.
- Transfer 5-g aliquots to 100-ml glass centrifuge tubes containing 10 ml of 3 N HCl.
- Homogenize for 2 min with Ultra-Turrax.
- Pipet 3-ml aliquots into 10-ml glass centrifuge tubes.
- Heat samples in a water bath at 100° C for 1 h.
- (Proceed as under 2.3.1. see arrow)

2.4. Gas-liquid chromatography

2.4.1. Derivatization procedure

- To extraction residue add 20 µl of N,O-Bis(trimethylsilyl)-acetamide and 0.5 ml of toluene.
- Mix vigorously on Vortex mixer for 20 sec.
- Heat for 10 min in heating block at 90° C.

2.4.2. Gas-liquid chromatography

- Cool reaction mixtures (2.4.1.) and inject 0.5-µl aliquots into gas chromatograph
- Quantitate by comparing peak heights of imazalil and metabolites with that of a calibration curve prepared the same day.
- Dilute higher-concentrated samples (e.g. liver or control samples, spiked with large amounts of imazalil and metabolites) with accurately known amounts of pure toluene.

2.5. Calibration procedure

- Spike blank control material (milk, liver, kidney, muscle, fat) with imazalil and metabolites at concentrations ranging from 0.002 to 20 µg/ml or µg/g.
- Extract and analyze calibration samples as described under 2.3. and 2.4.
- Construct calibration curves by plotting peak height of imazalil and metabolites against concentration of imazalil and metabolites in each calibration sample.
- Fit data to best straight line.
- Calculate slope and intercept for standard curve, using peak height as y-coordinate and equivalent ppm imazalil or metabolite level of standards as x-coordinate.

2.6. Calculations

From standard (std) slope and intercept values, dilution factor (DF) and observed peak heights for imazalil and metabolites in sample, determine ppm imazalil or metabolites in animal tissues of milk by following equations:

ppm imazalil in sample =

$$\frac{\text{peak height of imazalil in sample-intercept (std)}}{\text{slope (std)}} \times DF$$

ppm metabolite in sample =

$$\frac{\text{peak height of metabolite in sample-intercept (std)}}{\text{slope (std)}} \times DF$$

where:

$$DF = \frac{\text{volume solvent to dissolve sample residue}}{\text{volume solvent to dissolve standard residue}}$$

3. Results and discussion

The method has been evaluated in milk and animal tissue samples, fortified with imazalil and its main metabolites at different concentrations. Results are reported in detail elsewhere (4,5).

Legends to the figures

Figure 1: Chromatograms of unhydrolysed extracts from (A) blank control milk, (B) blank control milk spiked with 0.01 µg/ml of imazalil and R 42 243 and 0.001 µg/ml of R 14 821 and (C) milk of cow 6 (day 0,16 h).
Key: I = imazalil, M1 = R 14 821 and M2 = R 42 243
An internal standard (S) was used as an additional control.

Figure 2: Chromatograms of hydrolysed extracts from (A) blank control milk, (B) blank control milk spiked with 0.01 µg/ml of imazalil and R 42 243 and 0.001 µg/ml of R 14 821, and (C) milk of cow 6 (day 0,16 h).
Key: I = imazalil, M1 = R 14 821 and M2 = R 42 243
An internal standard (S) was used as an additional control.

Figure 3: Chromatograms of unhydrolysed extracts from (A) blank control liver, (B) blank control liver spiked with 1 µg/g of imazalil and R 42 243 and 0.1 µg of R 14 821, and (C) liver tissue from cow 7 (24 hours after last of 9 doses).
Key: I = imazalil, M1 = R 14 821 and M2 = R 42 243.
An internal standard (S) was used as an additional control.

Figure 4: Chromatograms of hydrolysed extracts from (A) blank control liver, (B) blank control liver spiked with 1 µg/g of imazalil and R 42 243 and 0.1 µg/g of R 14 821, and (C) liver tissue from cow 7 (24 hours after last of 9 doses).
Key: I = imazalil, M1 = R 14 821 and M2 = R 42 243
An internal standard (S) was used as an additional control. An impurity (i) was sometimes present and orginated from the derivatization reagent.

Figure 5: Calibration curve for imazalil in liver tissue.

Figure 6: Calibration curve for R 14 821 in liver tissue.

Figure 7: Calibration curve for R 42 243 in liver tissue.

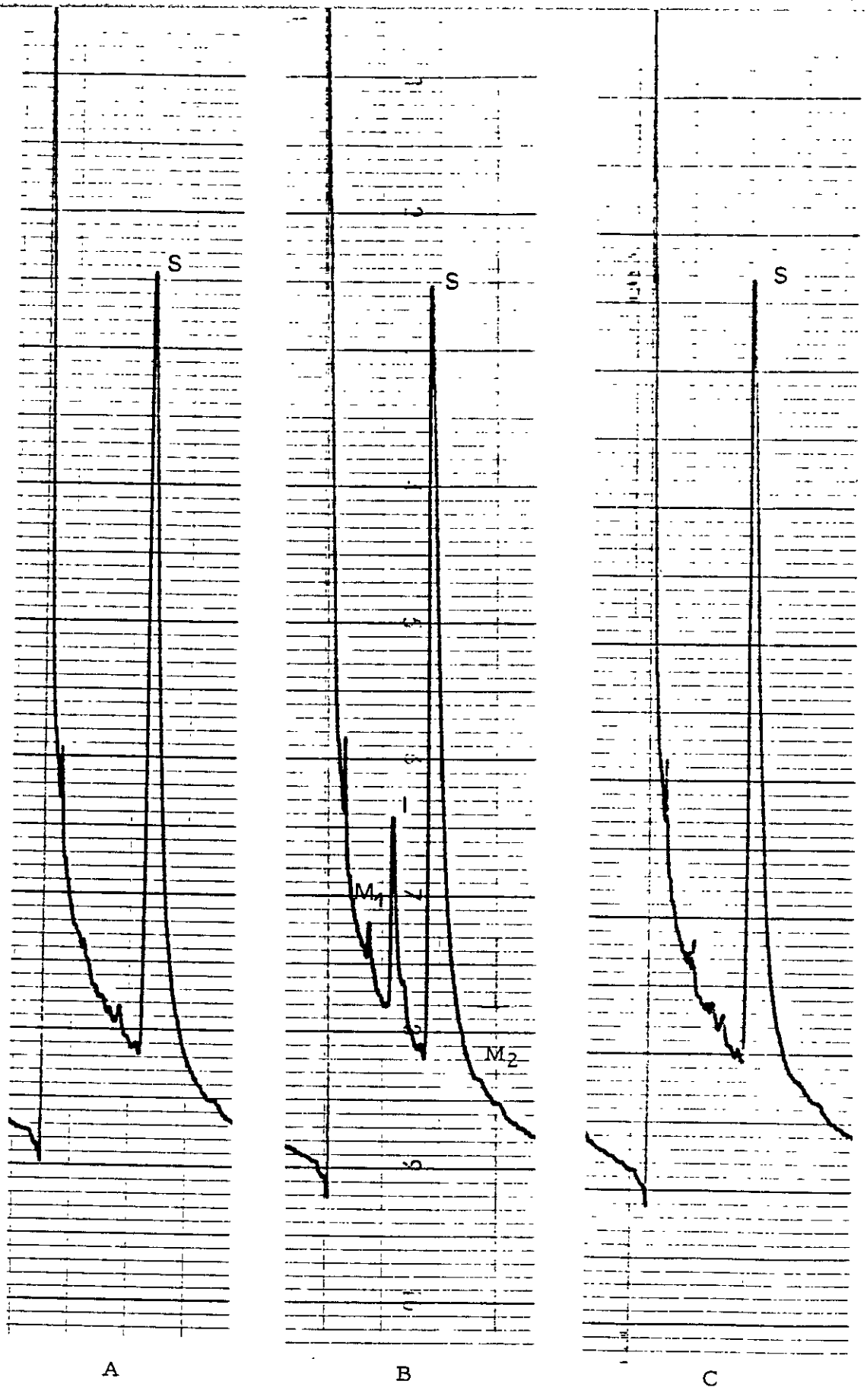


Fig. 1

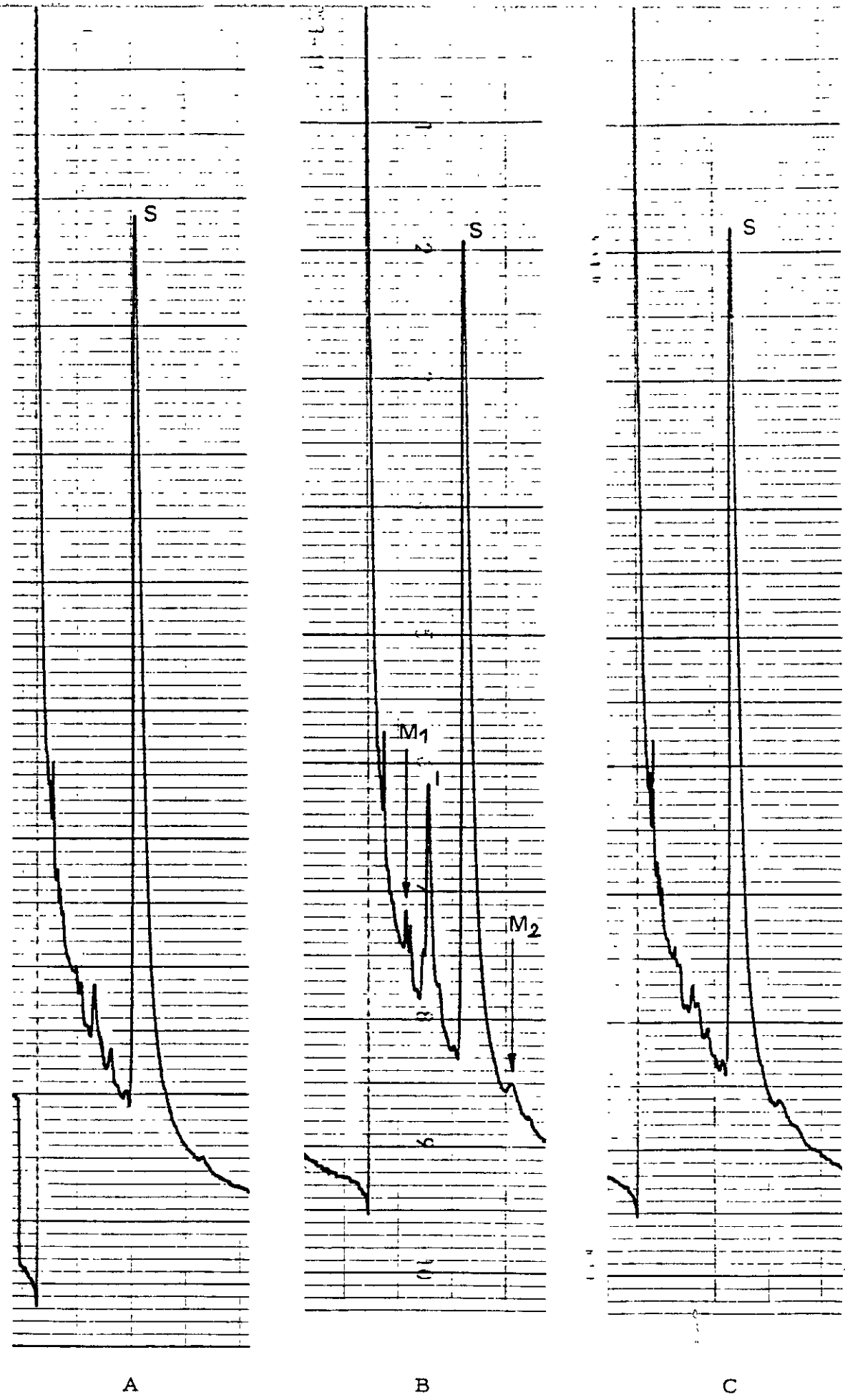


Fig. 2

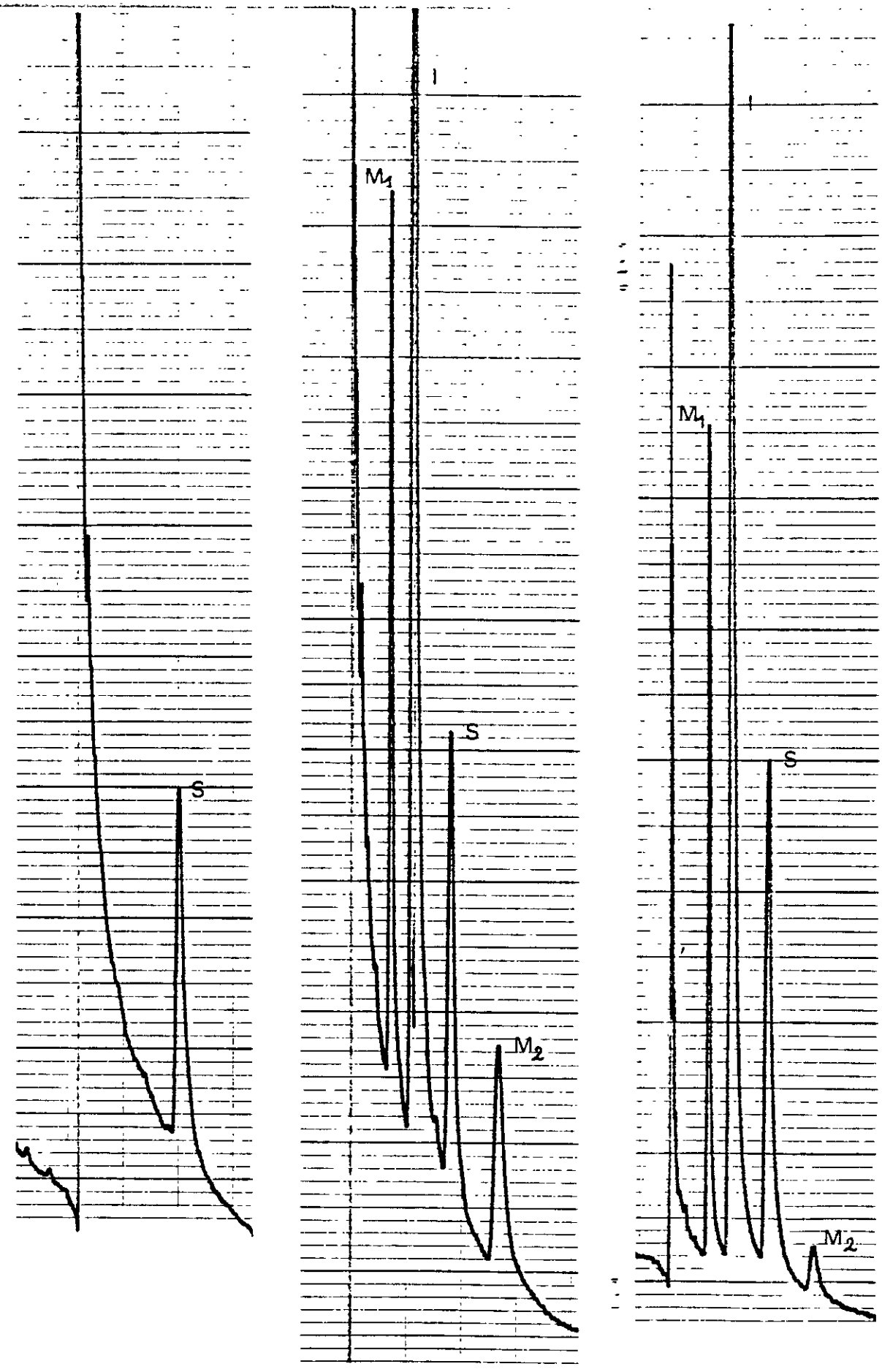
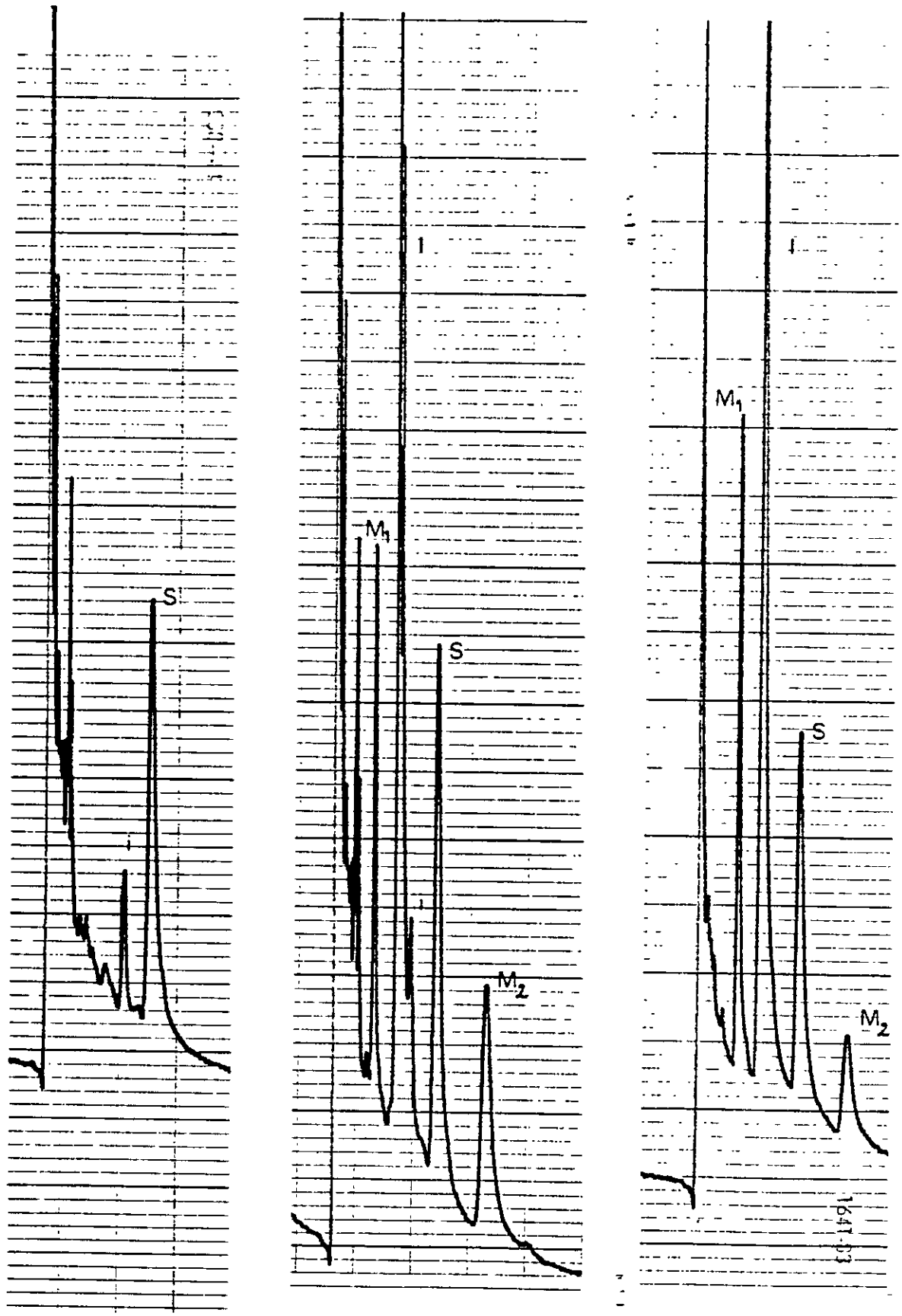


Fig. 3



A B C

Fig. 4

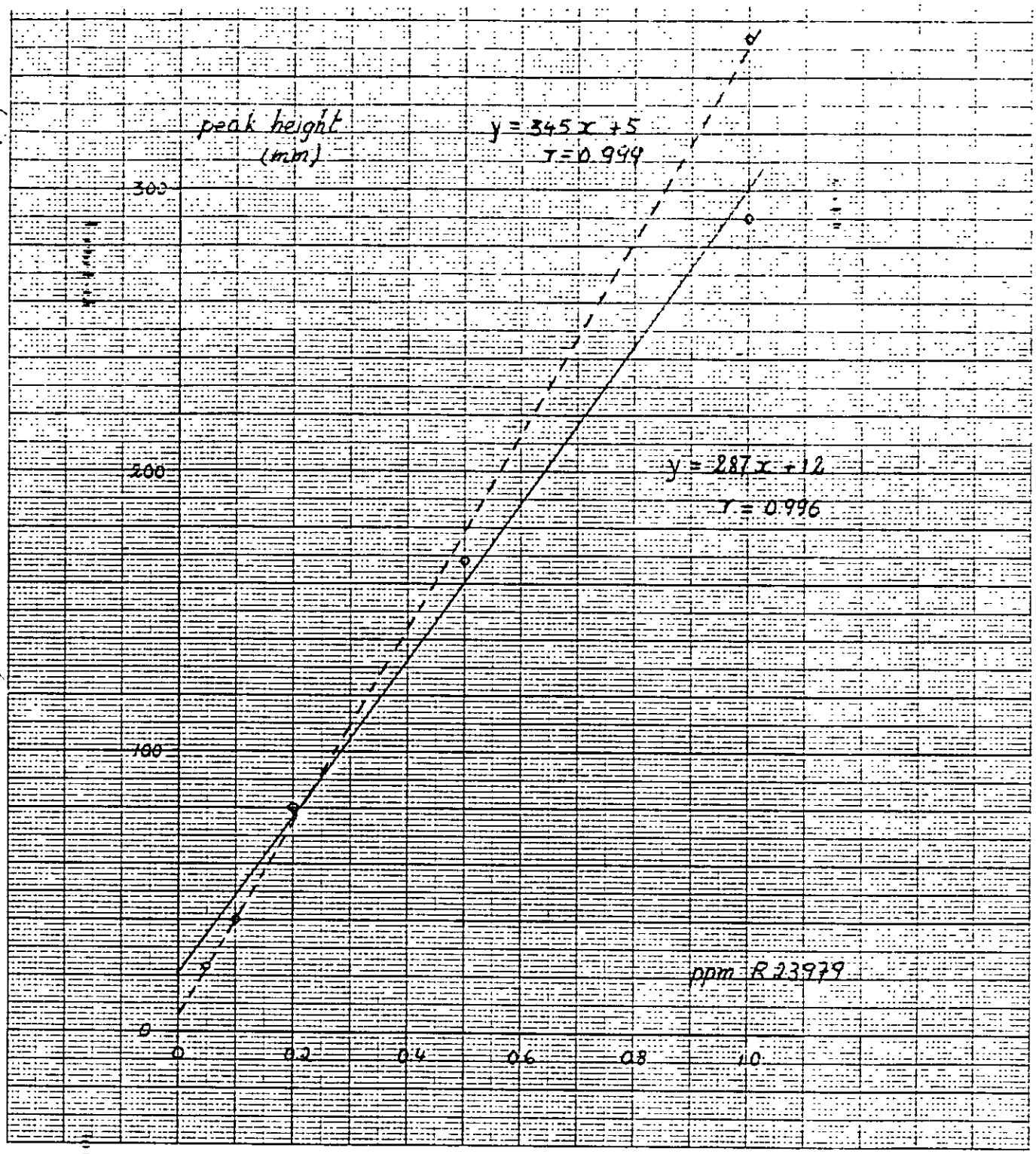

peak height
(mm)
y = 345 x + 5
r = 0.999
y = 287 x + 12
r = 0.996
300
200
100
0
0
0.2
0.4
0.6
0.8
1.0
ppm R 23979

Fig. 5

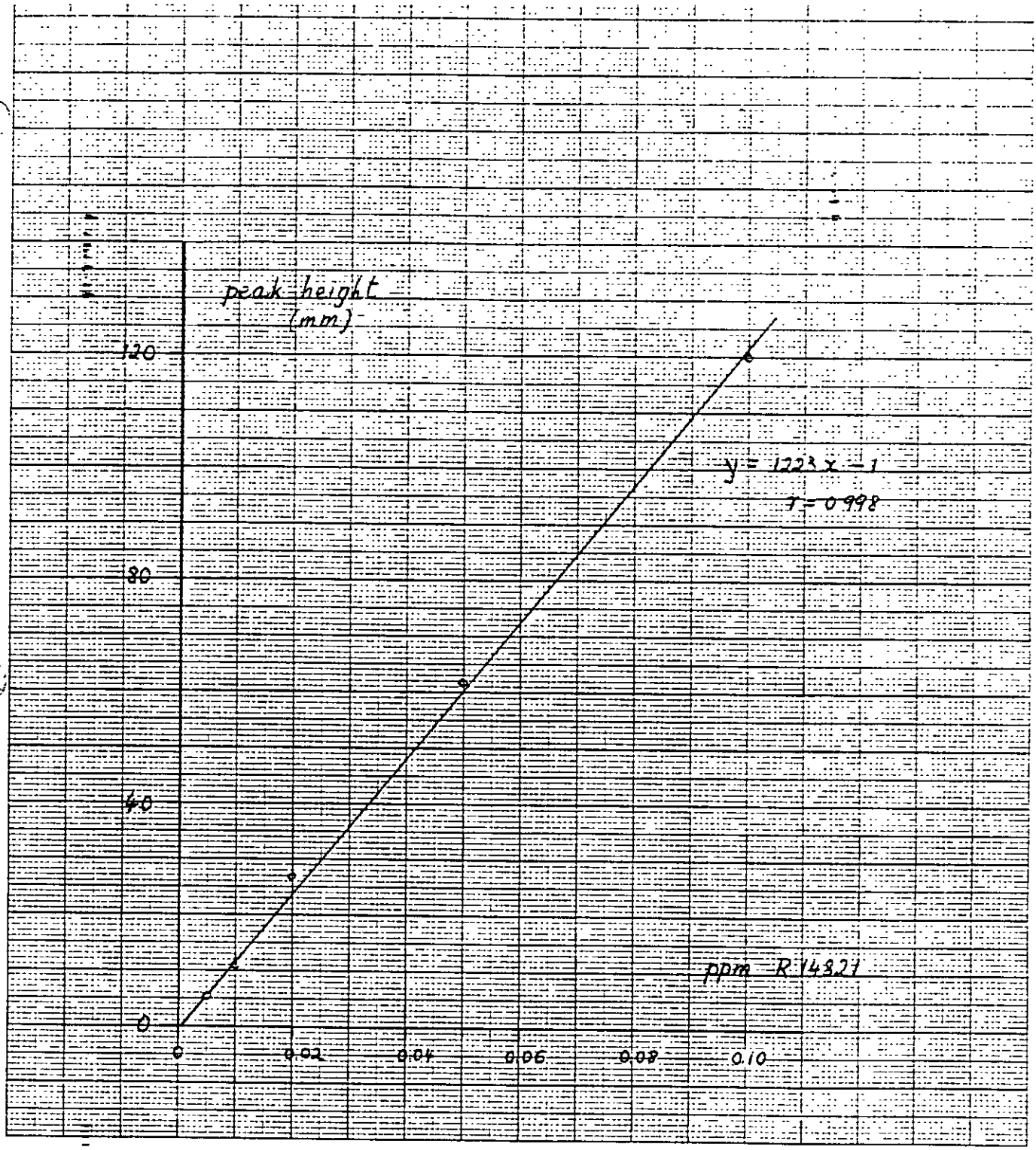

peak height
(mm)
120
80
40
0
0.02
0.04
0.06
0.08
0.10
ppm R 14821
y = 1223 x − 1
r = 0.998

Fig. 6

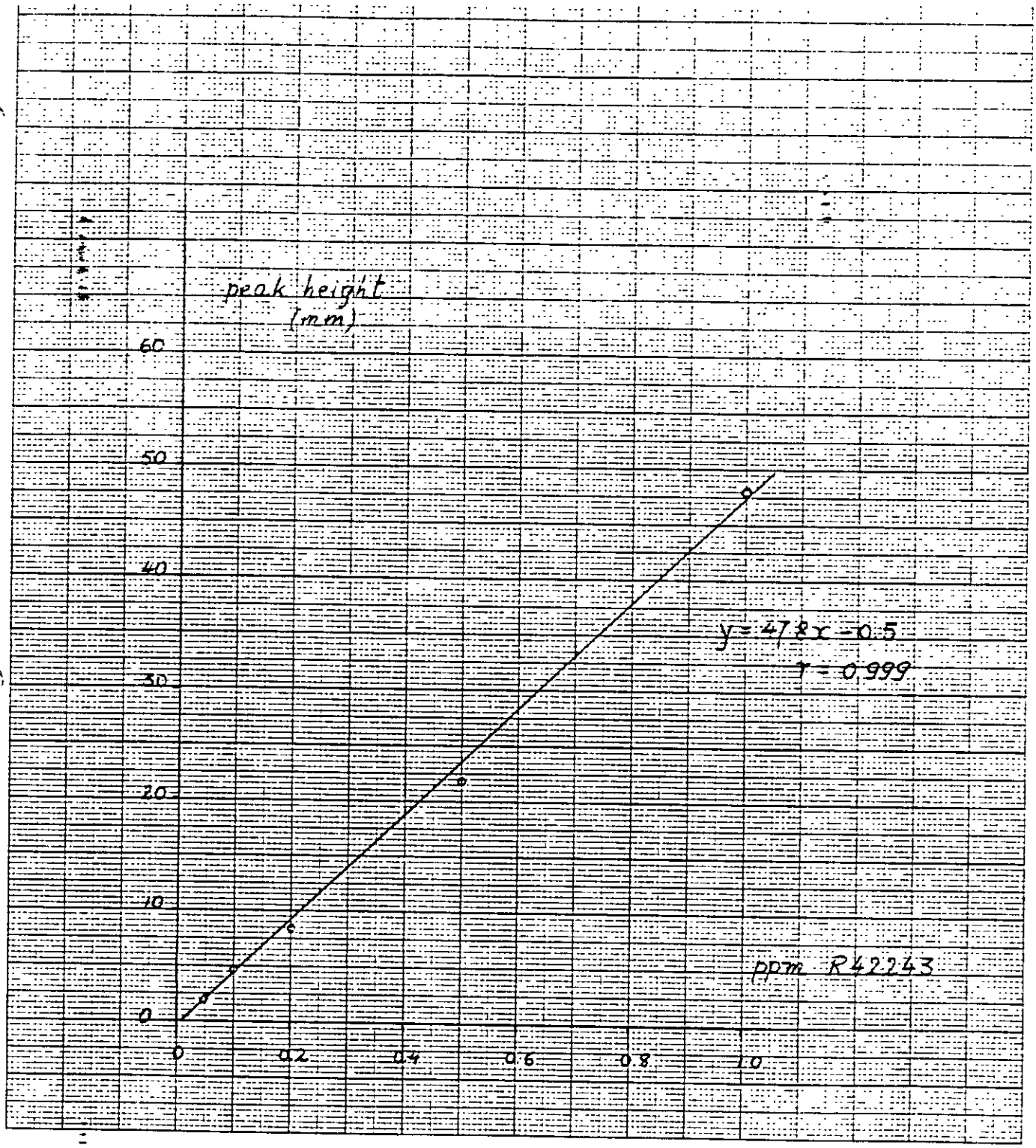

peak height
(mm)
60
50
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1.0
y = 47.8x - 0.5
r = 0.999
ppm R42243

Fig. 7

TABLE 3 : Accuracy and precision of the gas chromatographic method for the determination of imazalil in milk samples.

The analyses were performed on a SP-2250 DB column, following the procedure, outlined in Section 2, using 0.1 µg/ml as the internal standard concentration.

Theoretical imazalil milk concentration (µg/ml)	Observed imazalil milk concentration (µg/ml, mean ± S.D., n = 3)	% C.V.	Accuracy
0.001	0.0007 ± 0.0001	12.1	65 %
0.002	0.0020 ± 0.0002	9.1	102 %
0.005	0.0062 ± 0.0005	8.5	124 %
0.01	0.011 ± 0.001	4.3	108 %
0.02	0.019 ± 0.001	2.1	97 %
0.05	0.052 ± 0.002	3.0	104 %
0.1	0.099 ± 0.006	5.6	99 %

TABLE 4: Accuracy and precision of the gas chromatographic method for the determination of R 14 821 in milk samples.

The analyses were performed on a SP-2250 DB column. following the procedure, outlined in Section 2, using 0.1 µg/ml as the internal standard concentration.

Theoretical R 14 821 milk concentration (µg/ml)	Observed R 14 821 milk concentration (µg/ml, mean ± S.D., n = 3)	% C.V.	Accuracy
0.001	0.0004 ± 0.0001	15.6	44 %
0.002	0.0017 ± 0.0002	11.3	83 %
0.005	0.0058 ± 0.0006	9.8	116 %
0.01	0.012 ± 0.001	7.5	119 %
0.02	0.021 ± 0.002	9.8	104 %
0.05	0.050 ± 0.002	5.0	99 %
0.1	0.100 ± 0.004	4.3	100 %

TABLE 5: Accuracy and precision of the gas chromatographic method for the determination of R 42 232 in milk samples.

The analyses were performed on a SP-2250 DB column following the procedure, outlined in Section 2, using 0.1 µg/ml as the internal standard concentration.

Theoretical R 42 243 milk concentration (µg/ml)	Observed R 42 243 milk concentration (µg/ml, mean ± S.D., n = 3)	% C.V.	Accuracy
0.01	0.014 ± 0.002	14.2	134 %
0.02	0.021 ± 0.002	11.8	103 %
0.05	0.045 ± 0.004	9.7	90 %
0.10	0.102 ± 0.011	10.3	102 %

TABLE 6: Accuracy and precision of the gas chromatographic method for the determination of imazalil in bovine muscle tissue samples.

The analyses were performed on a SP 2250 DB column, following the procedure, outlined in Section 2, using 0.5 µg/g as the internal standard concentration.

Theoretical imazalil tissue concentration (µg/g)	Observed imazalil tissue concentration (µg/ml, mean ± S.D., n = 3)	% C.V.	Accuracy
0.002	0.0025 ± 0.0003	11.2	126 %
0.005	0.0060 ± 0.0004	7.0	120 %
0.01	0.012 ± 0.001	8.4	122 %
0.02	0.019 ± 0.001	6.3	98 %
0.05	0.052 ± 0.002	3.8	105 %
0.1	0.089 ± 0.004	4.0	89 %
0.2	0.198 ± 0.007	3.4	99 %
0.5	0.530 ± 0.012	2.3	106 %
1.0	0.941 ± 0.041	4.4	94 %
2.0	2.020 ± 0.121	6.0	101 %

TABLE 7: Accuracy and precision of the gas chromatographic method for the determination of R 14 821 in bovine muscle tissue samples.

The analyses were performed on a SP 2250 DB column following the procedure, outlined in Section 2, using 0.5 µg/g as the internal standard concentration.

Theoretical R 14 821 tissue concentration (µg/g)	Observed R 14 821 tissue concentration (µg/g, mean ± S.D., n = 3)	% C.V.	Accuracy
0.002	0.0027 ± 0.0003	9.9	134 %
0.005	0.0060 ± 0.0004	7.1	120 %
0.01	0.012 ± 0.001	8.0	115 %
0.02	0.019 ± 0.001	4.9	94 %
0.05	0.051 ± 0.003	6.8	101 %
0.1	0.108 ± 0.006	5.7	108 %
0.2	0.185 ± 0.004	2.1	92 %
0.5	0.498 ± 0.018	3.6	99 %
1.0	1.040 ± 0.060	5.8	104 %
2.0	1.940 ± 0.047	2.4	97 %

TABLE 8: Accuracy and precision of the gas chromatographic method for the determination of R 42 243 in bovine muscle tissue samples.

The analyses were performed on a SP 2250 DB column following the procedure, outlined in Section 2, using 0.5 µg/g as the internal standard concentration.

Theoretical R 42 243 tissue concentration (µg/g)	Observed R 42 243 tissue concentration (µg/g, mean ± S.D., n = 3)	% C.V.	Accuracy
0.01	0.0077 ± 0.0013	17.1	77 %
0.02	0.022 ± 0.003	12.0	111 %
0.05	0.046 ± 0.004	9.2	92 %
0.1	0.110 ± 0.011	10.1	110 %
0.2	0.180 ± 0.015	8.3	90 %
0.5	0.497 ± 0.030	6.1	99 %
1.0	1.001 ± 0.060	6.0	100 %
2.0	2.002 ± 0.156	7.8	100 %