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Preclinical Research Report

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Non-proprietary name: imazalil

Subject: A gas-liquid chromatographic method for determining total regulable residues of imazalil in citrus fruit and citrus by-products

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Summary

A gas-liquid chromatographic determination method is described for measuring major regulable residues of imazalil in citrus fruit and citrus by-products.

The method is based on the improved extraction of imazalil and its metabolite from the fruit or its by-products and conversion of the O-dealkylated metabolite to the trimethylsilyl-derivative.

Concentrations of imazalil and the derivatized metabolite are determined by electron capture gas-liquid chromatography over a linear dynamic range equivalent to 0.005-20 ppm. No internal standard is used for the final quantitations.

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## 1. Introduction

A gas chromatographic method for the determination of the antifungal imazalil and its major metabolite in citrus fruit and citrus by-products has been described earlier (1). In order to enhance the extraction recovery of imazalil and its metabolite from citrus fruit and citrus by-products and hence enabling the determination of total regulable residues, the extraction behaviour of both compounds was studied in more detail which resulted in the findings that were reported recently (2).

This report describes the final and recommended assay method for the determination of imazalil-related residues in both citrus fruit and citrus by-products.

## 2. Experimental

### 2.1. Materials

#### 2.1.1. Solvents

Spectrophotometric grade n-heptane (Uvasol <sup>®</sup>, 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 were of analytical reagent grade ('Baker Analyzed' Reagent, J.T. Baker Chemicals B.V., Deventer, Holland). Prepare and dilute the hydrochloric acid (0.01 N), sulphuric acid (0.1 N) and sodium hydroxide (1 N) solutions as needed.

#### 2.1.4. Standards

Imazalil (R 23 979) and  $\alpha$ -(2,4-dichlorophenyl)-1H-imidazole-1-ethanol (R 14 821), received from Janssen Quimica (Beerse, Belgium) as analytical standards.

2.1.5. Standard solutions

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

2.1.6. Fruits

Untreated Spanish navel oranges, from Pennwalt Iberica, Paterna (Valencia), Spain.

2.1.7. Citrus by-products

Imazalil-free dried orange pulp, single-strength cold pressed orange oil and blended cold pressed lemon oil from Sunkist Growers, Inc., Sherman Oaks, California, U.S.A.

2.2. Apparatus

2.2.1. Gas chromatograph

Varian Model 3700 gas chromatograph, equipped with pulse-modulated constant-current  $^{63}\text{Ni}$  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 Omniscrite<sup>®</sup> recorder (Houston Instrument); integrations and calculations by a Spectra-Physics Model 4000 data system. Under these conditions, retention times are 2.8 and 2.0 min for imazalil (R 23 979) and  $\alpha$ -(2,4-dichlorophenyl)-1H-imidazole-1-ethanol (R 14 821), respectively.

2.2.2. Grinding apparatus

Waring Commercial Blendor<sup>®</sup>.

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 Mixer (Cenco Instrumenten B.V., Breda, Holland).

2.2.5. Heating block

Reacti-Therm<sup>™</sup> (Pierce Eurochemie B.V., Rotterdam, Holland).

### 2.3. Extraction procedure

#### 2.3.1. Whole fruit

- Cut separate oranges (weighing ca. 160 g) into pieces in a tared blender cup.
- Add distilled water to obtain a final weight of 200.0 g.
- Blend mixture in a Waring Blendor at high speed for 2.0 min.
- Transfer 10.0 g of the fruit pulp to a 100-ml glass centrifuge tube.
- Add 25 ml 0.01 N HCl and homogenize with Ultra-Turrax for 1 min.
- Transfer 1.0 ml of the homogenate to a 10-ml glass centrifuge tube.
- Add 1 ml 1 N NaOH and 4 ml heptane-isoamyl alcohol (95:5, v/v).
- Shake tubes at 10 rpm for 10 min with Cenco rotary mixer.
- Centrifuge mixture for 10 min at 2500 rpm.
- Transfer organic layer to 15-ml glass centrifuge tube with a 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<sub>2</sub>SO<sub>4</sub> 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.

#### 2.3.2. Dried citrus pulp

- Chop dried citrus pulp (at least 20 g) in Waring Blendor.
- Transfer 10 g of the pulverized dry peel to a 100-ml glass centrifuge tube.
- Add 65 ml of distilled water and homogenize with Ultra-Turrax for 3 min.
- Transfer 10 g of the homogenized pulp to a second 100-ml glass centrifuge tube.
- Add 25 ml of 0.01 N HCl and homogenize further with Ultra-Turrax for 1 min.
- Proceed as in 2.3.1., beginning "Transfer 1.0 ml of the homogenate ..." (see arrow).

#### 2.3.3. Citrus oil

- Pipet 1 ml of citrus oil (lemon or orange) into a 10-ml glass centrifuge tube.
- Add 4 ml heptane-isoamyl alcohol (95: 5, v/v) and 3 ml of 0.1 N H<sub>2</sub>SO<sub>4</sub>.
- Rotate tubes for 10 min in rotary mixer.
- Centrifuge for 5 min at 2500 rpm; aspirate organic layer and discard.
- Extract aqueous phase with additional 4 ml of heptane-isoamyl alcohol; centrifuge, aspirate organic layer and discard.
- Add 0.15 ml of concentrated ammonia.
- Re-extract alkaline phase two times with 2 ml of the organic extraction mixture.
- Evaporate combined organic layers to dryness in 55° C water bath.

## 2.4. Gas-liquid chromatography

### 2.4.1. Derivatization procedure

- To extraction residue add 20  $\mu$ l 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 a heating block at 90° C.

### 2.4.2. Gas-liquid chromatography

- Cool reaction mixtures (2.4.1.) and inject 0.5- $\mu$ l aliquots directly into the gas chromatograph.
- Quantitate by comparing peak height of imazalil and metabolite with that of a calibration curve prepared the same day.
- Dilute higher concentrated samples (e.g. dried peel or control samples, spiked with large amounts of imazalil or its metabolite) with accurately known amounts of pure toluene.

## 2.5. Calibration procedure

- Spike blank control material (whole fruit, dried citrus pulp, citrus oil) with imazalil and metabolite at concentrations ranging from 0.005 to 20  $\mu$ g per gram or per millilitre of material.
- Extract and analyze calibration samples as described under 2.3. and 2.4.
- Construct calibration curves by plotting peak height of imazalil and metabolite against imazalil and metabolite concentration of 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 slope and intercept values, dilution factor (DF) and observed peak heights for imazalil and metabolite in sample, determine ppm imazalil or metabolite in citrus fruit or citrus by-product sample by following equations:

ppm imazalil in sample =

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

ppm metabolite in sample =

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

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 at different levels in citrus fruit and citrus by-products in samples fortified with both the tritium-labelled and the parent compounds. Results are reported elsewhere (2).

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Table III: Accuracy and precision of the gas chromatographic method for the determination of imazalil in orange samples.

Theoretical imazalil fruit concentration (ppm)	Observed imazalil fruit concentration (ppm, mean $\pm$ S.D., n = 3)	$\frac{s}{\bar{x}}$ = c.v.	Accuracy
0.1	0.074 $\pm$ 0.008	11.1	74 %
0.2	0.153 $\pm$ 0.015	9.7	77 %
0.5	0.513 $\pm$ 0.027	5.2	103 %
1	1.07 $\pm$ 0.05	4.4	107 %
2	1.91 $\pm$ 0.04	2.0	96 %
5	5.39 $\pm$ 0.25	4.6	108 %
10	9.82 $\pm$ 0.50	5.1	98 %
20	18.1 $\pm$ 1.30	7.2	91 %

Table IV: Accuracy and precision of the gas chromatographic method for the determination of R 14 821 in orange samples.

Theoretical R 14 821 fruit concentration (ppm)	Observed R 14 821 fruit concentration (ppm, mean $\pm$ S.D., n = 3)	% c.v. =	Accuracy
0.1	0.110 $\pm$ 0.006	5.6	110 %
0.2	0.205 $\pm$ 0.025	12.0	103 %
0.5	0.418 $\pm$ 0.016	3.8	84 %
1	1.06 $\pm$ 0.04	4.0	106 %
2	1.91 $\pm$ 0.11	5.8	96 %
5	5.18 $\pm$ 0.48	9.2	104 %
10	9.92 $\pm$ 0.21	2.1	99 %
20	18.7 $\pm$ 1.6	8.3	94 %

Legend to the figures

Figure 1: Chemical structures of imazalil-<sup>3</sup>H-sulphate (T = tritium label), imazalil free base (R 23 979) and its main metabolite (R 14 821).

Figure 2: Extraction scheme for imazalil and its main metabolite from citrus fruits.

Figure 3: A. Chromatogram of an extract from an orange, spiked with 1 mg of R 23 969 (I) and 0.1 mg R 14 821 (M) after Soxleth extraction and additional purification. (Procedure 1; storage time: 8 weeks)

B. Chromatogram of an extract from an orange, spiked with 1 mg of R 23 979 (I) and 0.1 mg R 14 821 (M) after Soxleth extraction. (storage time: 8 week)

C. Chromatogram of an extract from an orange, spiked with 1 mg of R 23 979 (I) and 0.1 mg R 14 821 (M) after extraction and back-extraction of the homogenate. (Procedure 2; storage time: 2 weeks)

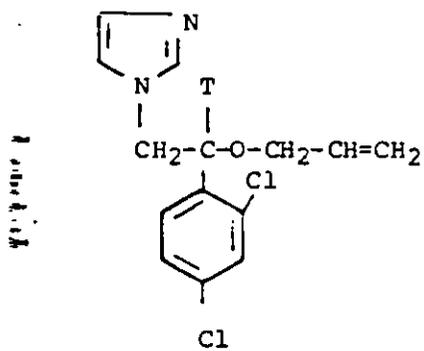
D. Chromatogram of an extract from an orange, spiked with 1 mg of R 23 979 (I) and 0.1 mg R 14 821 (M) after a single extraction of the homogenate. (storage time: 2 weeks)

Figure 4: Chromatograms of extracts from control oranges, spiked with R 23 979 (I) and R 14 821 (M) at different concentrations.

- A. Blank (4 x 10<sup>-12</sup> Amps/mV)
- B. 0.088 + 0.875 µg/g (4 x 10<sup>-12</sup> Amps/mV)
- C. 0.219 + 2.19 µg/g (4 x 10<sup>-12</sup> Amps/mV)
- D. 0.433 + 4.38 µg/g (4 x 10<sup>-12</sup> Amps/mV)
- E. 0.875 + 8.75 µg/g (4 x 10<sup>-12</sup> Amps/mV)
- F. 2.19 + 21.9 µg/g (8 x 10<sup>-12</sup> Amps/mV)
- G. 4.38 + 43.8 µg/g (8 x 10<sup>-12</sup> Amps/mV)

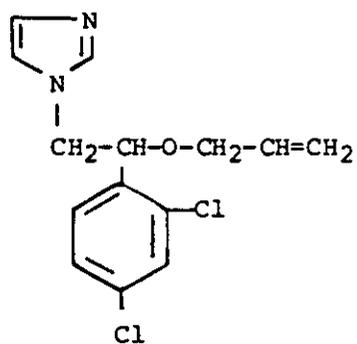
Figure 5: Calibration curve for imazalil in oranges.

Figure 6: Calibration curve for R 14 821 in oranges

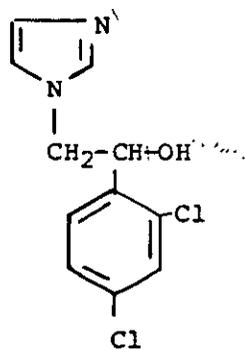


• H<sub>2</sub>SO<sub>4</sub>

Imazalil-<sup>3</sup>H-sulphate (R 27 180-<sup>3</sup>H)



Imazalil (R 23 979)



O-dealkylated metabolite R 14 821 (T 824)

Fig. 1

Extraction scheme :

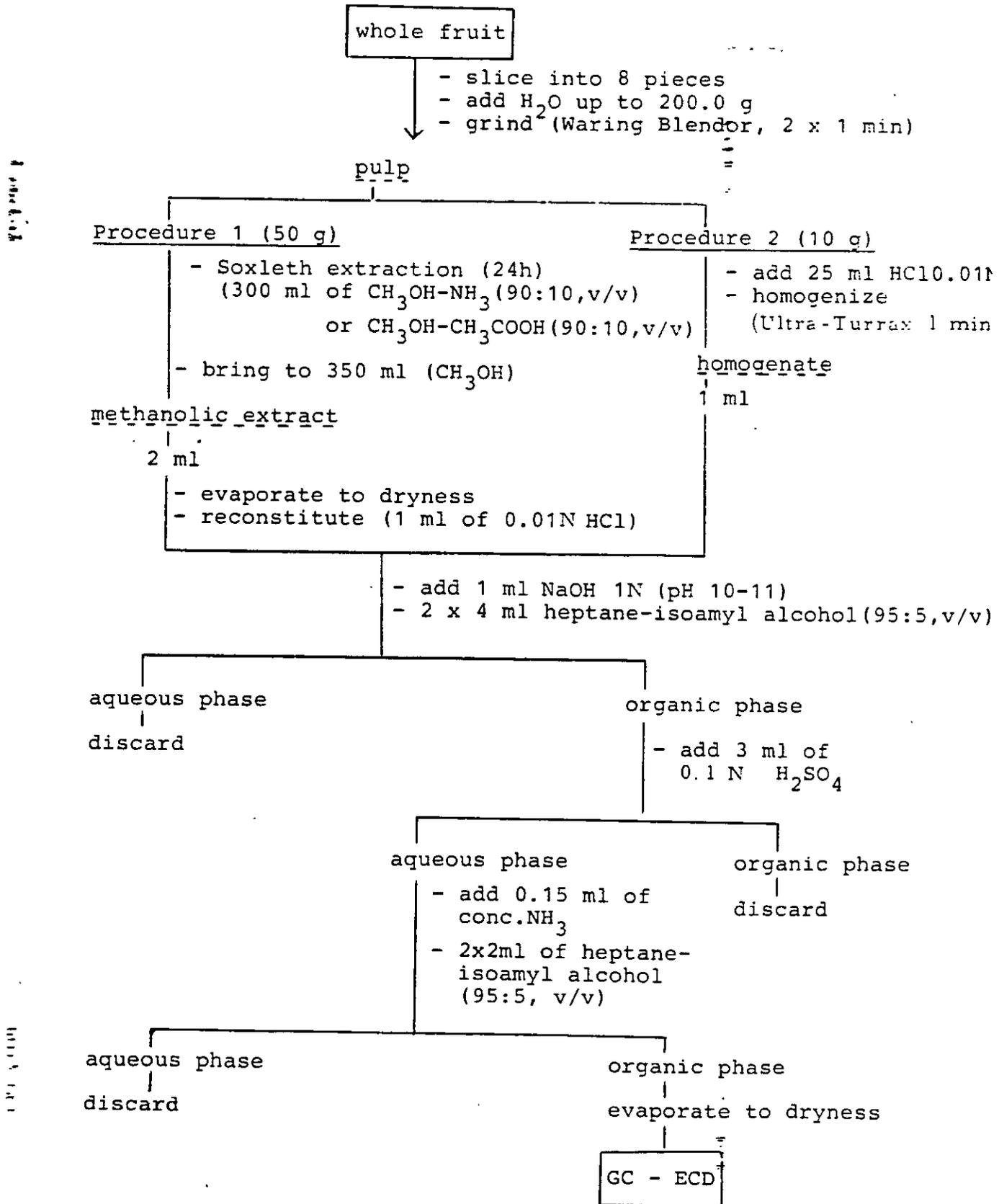


Fig. 2

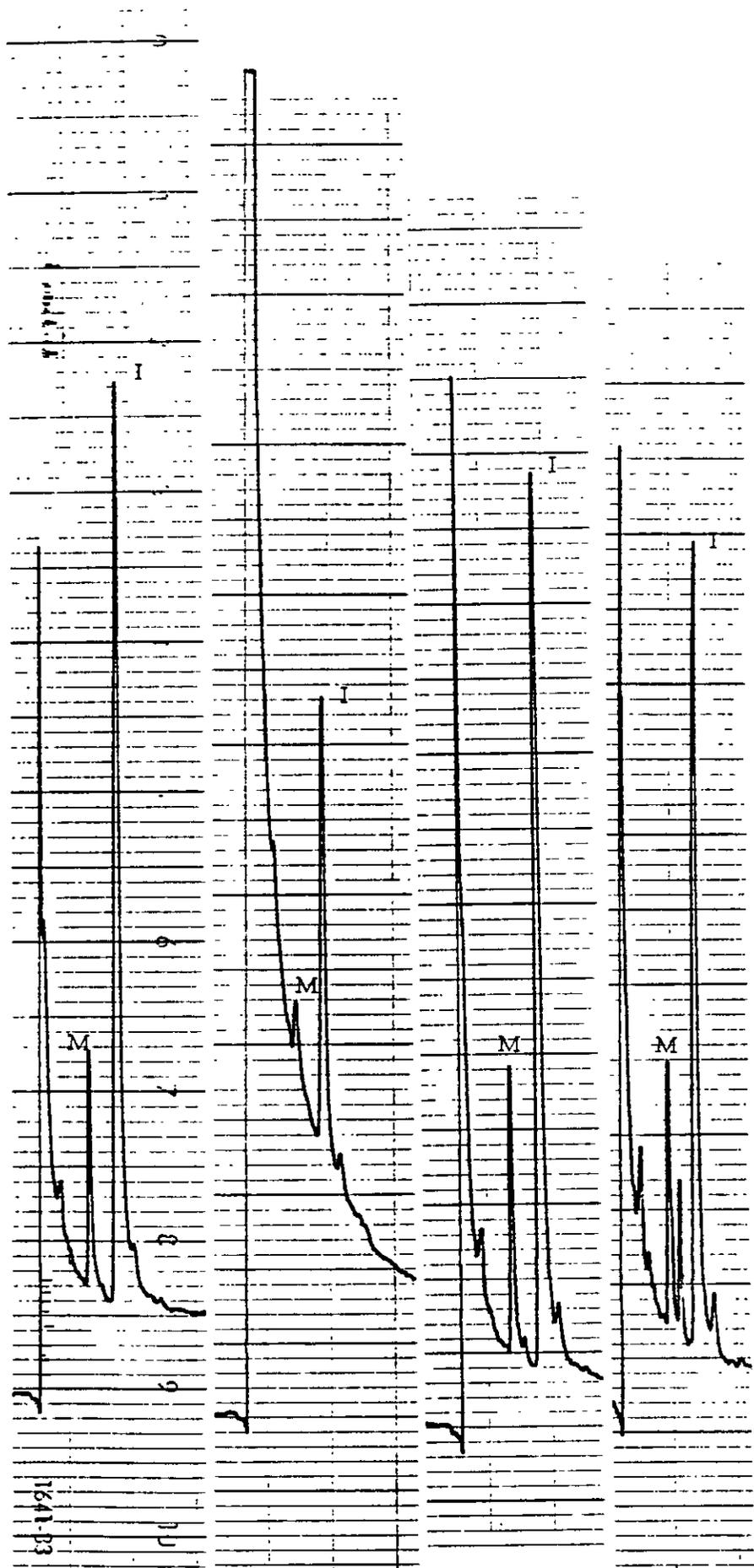


Figure 3

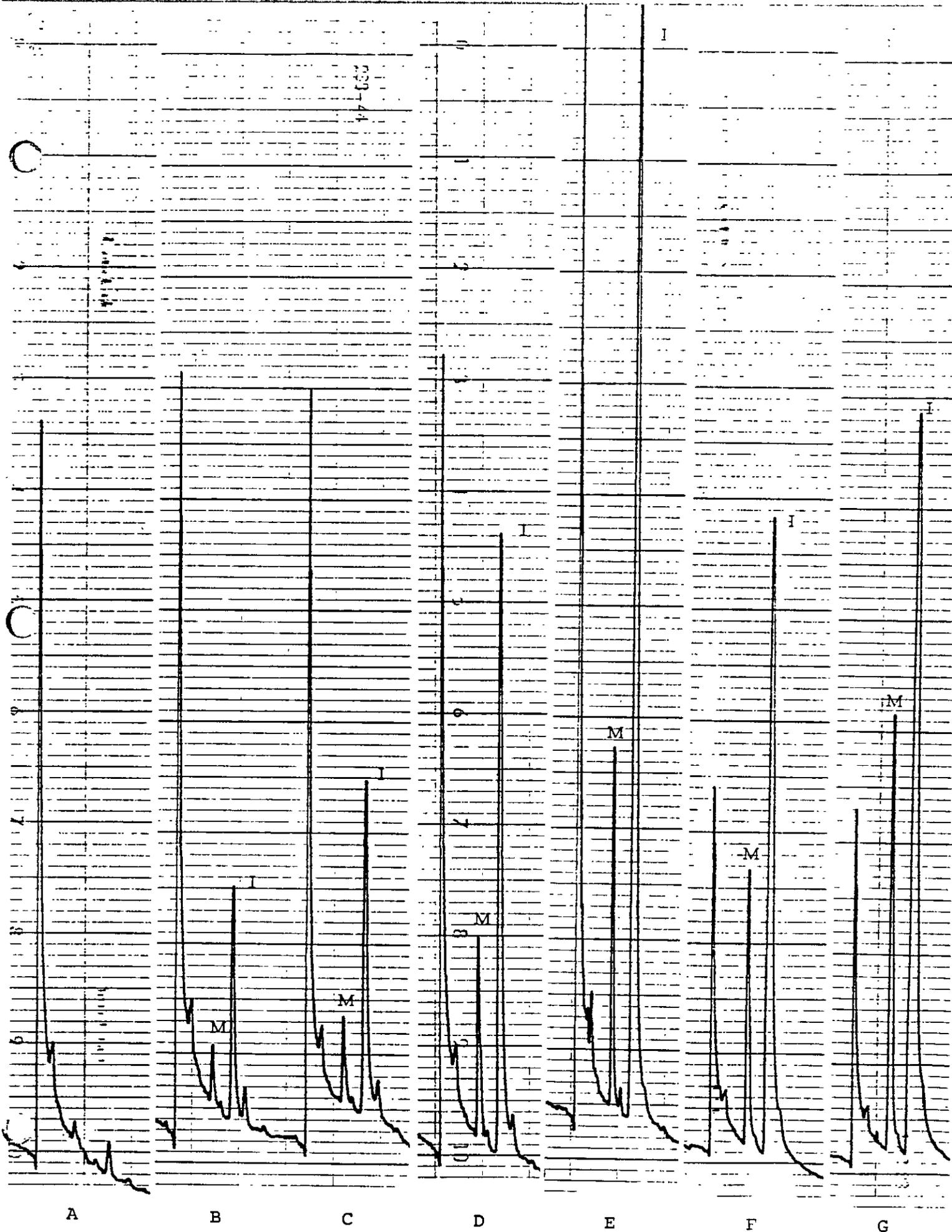


Figure 4

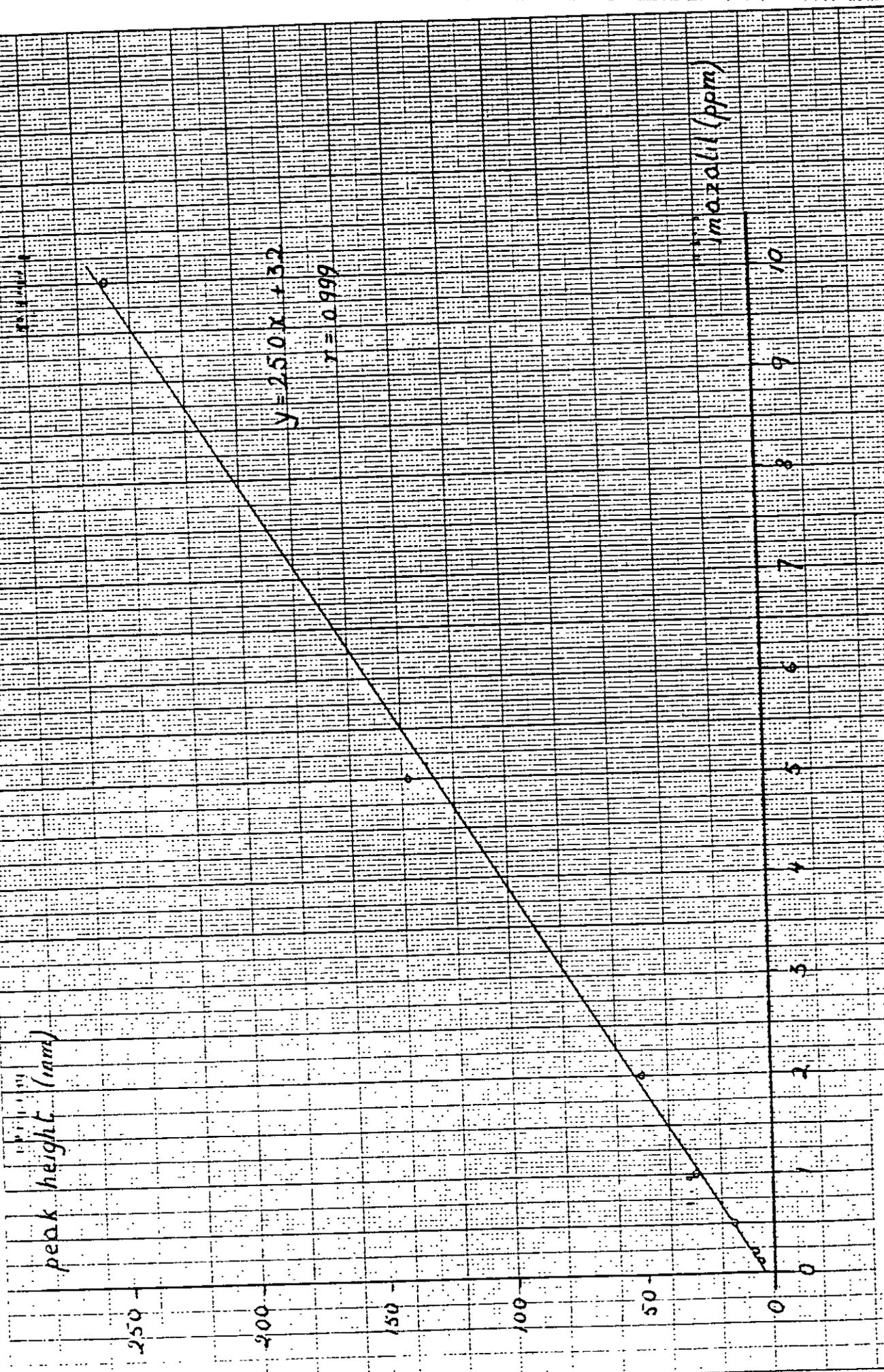


Fig. 5

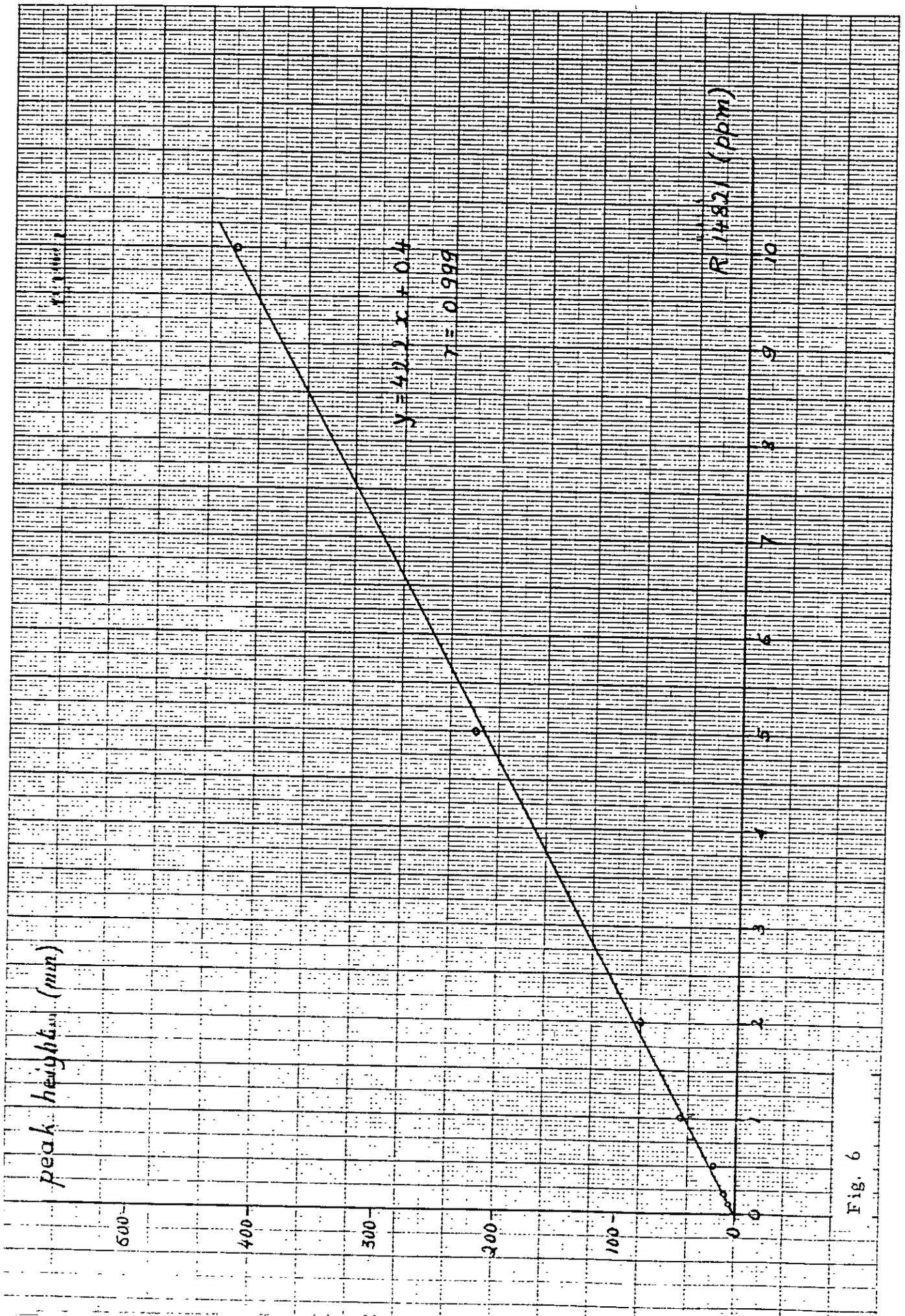


Fig. 6