METHOD NO. 163

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RHÔNE-POULENC INC. METHOD NO. 163

DETERMINATION OF FOSETYL-AL ALUMINUM TRIS (O-ETHYL PHOSPHONATE), IN/ON CITRUS FRUIT AND FRACTIONS BY PHOSPHOROUS SPECIFIC FLAME PHOTOMETRIC GAS CHROMATOGRAPHY

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ABSTRACT

Metabolism studies were carried out with ^{14}C Aluminum tris (O-ethyl phosphonate) applied to oranges and tangerines (Laurent and Chabassol, 1982).

This study showed that when Fosetyl-Al is applied to citrus, the carbon group of Fosetyl-Al is largely integrated into the normal plant metabolism (Glyoxylic Cycle) resulting in the formation of sugars and lipids.

Since the degradation products are integrated into the normal plant metabolism and are of no toxicological concern, the method described in this report was developed to determine parent compou only in/on citrus and its various fractions. The basic steps of the method are extraction with HCl/Acetonitrile mixture, Alumina column chromatography, methylation with diazomethane, and quantitation by phosphorous specific flame photometric gas chromatogra of the resulting methyl ester derivative.

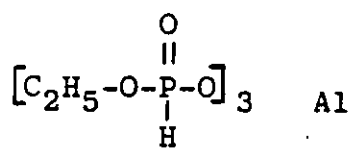
The limit of detection for whole fruit, peels and juice was <0.05 ppm. The method recoveries by spiking untreated samples with Fosetyl-Al and processing the samples through the analytical procedure averaged 89.9% for the whole fruit, peels and juice.

1.0 SCOPE

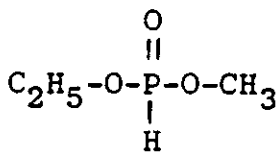
This method describes the procedure for the determination of Fosetyl-Al, Aluminum tris (O-ethyl phosphonate) in/on citrus fruit by gas chromatography. The method is sensitive to 0.05 ppm of Fosetyl-Al in the whole fruit, peels or juice

2.0 PRINCIPLE

The Fosetyl-Al is extracted from the whole fruit or peels using a 50/50 mixture of 0.1N hydrochloric acid and acetonitrile. The extract is cleaned-up by Alumina Column Chromatography. The eluate is evaporated and acetic acid added. The liberated O-ethyl phosphonic acid is esterified with diazomethane to obtain the corresponding methyl ester which is detected by phosphorous specific flame photometric gas chromatography.



Fosetyl-Al



O-ethyl-O-Methyl Phosphonat

3.0 REAGENTS

3.1 Acetonitrile:Pesticide quality or equivalent

3.2 Methyl Alcohol:Pesticide quality or equivalent

3.3 2-Methoxyethanol:Pesticide quality or equivalent

3.4 Hydrochloric Acid:Reagent grade, 0.10N in water

3.5 Ammonium Hydroxide:Reagent grade 12N

3.6 Ethyl Ether:Pesticide quality or equivalent

3.7 Diazomethane/Ether Solution:Prepared as described in Note I

3.8 Potassium Hydroxide:Reagent grade

3.9 Glacial Acetic Acid:Reagent grade

3.10 Aluminum Oxide:Camag. Basic, Brockmann Activity No. I

3.11 Fosetyl-Al:Analytical standard, Rhone-Poulenc

3.12 Nitrogen:High purity gas

3.13 Filter aid - Hyflo Super Cel®, Fisher Scientific, or equivalent

4.0 EQUIPMENT

4.1 Rotary Flash Evaporator:Buchi Rotavapor-R or equivalent

4.2 Chromatographic Column:Glass 240 mm x 10.5 mm I.D. with a 200 ml reservoir

4.3 Microsyringe:25 microliter (25 µl)

4.4 Glass wool or Pyrex wool

4.5 Buchner Funnels

4.6 Filter Paper:Whatman No. 1, Glass Microfibre Filters No. 934AH Whatman

4.7 Centrifuge Tubes:100 ml

4.8 Centrifuge:Sorvall GLC-1 or equivalent

4.9 Polytron:PT 10/35 Homogenizer, PT20ST Probe, Brinkman Instruments

4.10 Gas Chromatograph:Tracor Model 220 equipped with a phosphorous specific flame photometric detector

4.11 General Laboratory Glassware

4.12 Blender:Waring or equivalent equipped with a 1 qt. jar

5.0 PREPARATION OF STANDARD SOLUTIONS

5.1 Preparation of Standard Solutions of Fosetyl-Al

5.1.1 Prepare a standard stock solution by weighing out 100 mg of Fosetyl-Al, quantitatively transferring to a 100 ml volumetric flask, and diluting to volume with distilled water. This solution contains 1000 µg/ml and should be prepared every week and stored in the refrigerator. Stock solution A.

5.1.2 Transfer 10 ml, 5 ml, 2 ml and 1 ml of stock solution A to 100 ml volumetric flasks and dilu to volume with methoxyethanol/acetonitrile (50/50). These solutions contain 100, 50, 20 and 10 µg/ml, respectively. Transfer 10 ml of the 10 µg/ml solution to a 100 ml volumetric flask and dilute to volume giving a solution

containing 1 µg/ml. These solutions should be prepared every six months and stored in the refrigerator.

5.2 Preparation of Methylated Derivative of Fosetyl-Al for Linearity Curve and Sample Quantitation

5.2.1 Transfer 8, 4, 2, 1, 0.8, 0.5 mls of the 1 µg/ml Fosetyl-Al standard to a 10 ml volumetric flask and dilute to volume with methoxyethanol/ acetonitrile (50/50). These solutions contain 0.8, 0.4, 0.1, 0.08 and 0.05 µg/ml Fosetyl-Al, respectively.

5.2.2 Transfer 5 ml of the 1, 0.8, 0.4, 0.1, 0.08 and 0.05 µg/ml standards to 50 ml evaporating flasks. Methylate standards as outlined in 6.4.4 through 6.4.7. Final volume should be 5 mls so that the resulting standards will contain 1.0, 0.8, 0.4, 0.1, 0.08 and 0.05 µg/ml Fosetyl-Al equivalent, respectively.

5.3 Quantitation

A suitable injection (~6 µl) of standard Fosetyl methyl ester derivative is made, followed by the same volume injection of sample. The peak height of the sample (h) is compared to the peak height (H) of the standard.

The calculation is as follows:

$$\frac{h}{H} \times C = \mu g/ml \text{ of sample}$$

C = standard concentration (µg/ml)

$$\mu g/ml \text{ of sample} \div \frac{\text{weight of sample}}{\text{volume of sample}} = ppm$$

6.0 PROCEDURE

6.1 Sample Preparation

Quarter 6 to 8 fruit (frozen). Mix well. Take 10 to 15 quarters and grind in a Waring Blender with dry ice to a uniform consistency. Allow dry ice to sublime. Take subsample of this blended fruit for analysis. Juice is analyzed as is. Peels are ground in a Waring Blender with dry ice and a subsample analyzed.

6.2 Extraction

6.2.1 Weigh out 25 g of blended whole fruit or peels in a 100 ml centrifuge tube. Add 50 mls of 0.1N HCl/Acetonitrile (50/50).

6.2.2 Homogenize with a Polytron for about 2 minutes at middle speed.

6.2.3 Centrifuge at 3,000 rpm for 15 minutes.

6.2.4 Decant supernatant into a Buchner funnel (7 cm), fitted with one piece Whatman No.1 filter paper and one microfibre filter, into a 250 ml filter flask. For whole fruit, a pad of Hyflo Super Cel® (8-10 gms) is added to the Buchner funnel to aid in the filtration.

6.2.5 Add another 25 mls of 0.1N HCl/acetonitrile to cake remaining in the centrifuge tube.

6.2.6 Homogenize again with polytron for about 2 minutes at middle speed.

6.2.7 Centrifuge at 3,000 rpm for 15 minutes.

6.2.8 Decant supernatant into same Buchner funnel used in 6.2.4. Rinse centrifuge tube with 2 x 5 ml portions of 0.1N HCl/acetonitrile mixture filtering through Buchner funnel. Combine all filtrates.

6.2.9 For juice, filter 25 grams through Buchner funnel (7 cm), fitted with one piece Whatman No. 1 filter paper and one microfibre filter, into a 250 ml filter flask. Wash filter cake (pulp) with 3 x 25 ml portions of 0.1N HCl/ acetonitrile mixture.

6.3 Alumina Column Clean-up

6.3.1 Place a pyrex wool plug at the bottom of the chromatographic column.

6.3.2 Add alumina (see 3.10) to a height of 21 cm (approx. 17 grams). Place a pyrex wool plug at the top of the alumina.

6.3.3 Attach the column to a 250 ml filtering flask and connect the flask to a vacuum source to aid in the elution.

6.3.4 Wash the column with 25 ml of acetonitrile. Discard.

6.3.5 Add the entire combined filtered extracts from above to the column reservoir.

6.3.6 Rinse the flask with 5 x 5 ml portions of the 0.1N HCl/acetonitrile mixture and add to the column reservoir.

6.3.7 Elute the entire amount added to the reservoir allowing the column to run dry. Discard eluates

6.3.8 Elute with 100 ml of 5% ammonium hydroxide in acetonitrile. Discard eluate.

6.3.9 Elute the Fosetyl-Al with 225 ml of 25% ammoniur hydroxide in acetonitrile. Collect this eluate

6.3.10 Transfer the eluate from 6.3.9 to a 500 ml evaporating flask with 5 x 5 ml portions of acetonitrile and evaporate till solvent free.

6.3.11 At this point, about 25 mls of aqueous solution is usually present in the flask; add ∿150 ml of acetonitrile and re-evaporate to remove the aqueous.

6.3.12 If water remains, repeat addition of acetonitri until all traces of water are gone. Bath temperature not to exceed 35°C.

6.4 METHYLATION: THE METHYLATION STEP MUST BE DONE THE SAME DAY THE PROCEDURE WAS BEGUN.

6.4.1 Dissolve the residue from 6.3.12 with 25 ml methyl alcohol. See Note 8.1.

6.4.2 Pipette a 5 ml aliquot of the methyl alcohol into a 50 ml evaporating flask and evaporate to dryness.

6.4.3 Add 5 ml of methoxyethanol/acetonitrile (50/50) mixture. Sonicate until all residue is removed from sides of flask (∿1 minute) and in suspensi (See Note 8.1)

6.4.4 With a microsyringe, add 25 µl of glacial acet: acid to flask. Mix by swirling. Let sit for 2 minutes.

6.4.5 In a fume hood, methylate by adding 5-6 ml of diazomethane solution to the methoxyethanol/ acetonitrile. Yellow color should persist. If not, additional diazomethane should be added Let sit for no longer than 1 minute. Destroy excess diazomethane by adding glacial acetic acid dropwise until yellow color disappears (4-5 drops). This step should be carried out in a fume hood.

6.4.6 Evaporate the methylated solution to 2-3 mls with the aid of a gentle stream of nitrogen. This step should also be carried out in a fume hood.

6.4.7 Samples are then diluted to appropriate volumes for final determination with methoxyethanol/ acetonitrile (50/50). If necessary, the sample may be filtered through Whatman No. 1 filter paper. eg., A 25 g sample of untreated fruit would have a final volume of 5 ml. The limit of detection would therefore be:

$$\frac{0.05\ \mu g/ml}{\frac{25\ g\ sample}{25\ ml\ methanol} \times \frac{5\ ml\ methanol}{5\ ml\ methoxyethanol/\ acetonitrile}} = <0.05$$

7.0 RECOVERY STUDIES

Control samples of whole fruit, peels and juice were spiked at various levels (80-0.05 ppm) with stock solutions of Fosetyl-Al prior to extraction. Recovery data are given in Tables I, II and III, and typical chromatograms are shown in Figures III, IV and V.

8.0 NOTES

8.1 Methylation Solvent

Sample cannot be directly diluted with methoxyethanol/acetonitrile. This mixture doesn't dissolve all the residue as well as the methyl alcohol. An aliquot of the methyl alcohol is evaporated to dryness and the residue suspended in methoxyethanol/acetonitrile becaus this mixture is more suitable for methylation.

8.2 Diazomethane Preparation

Dissolve 2.3 g of KOH in 2.3 ml of distilled water in a 125 ml flask. Cool the solution to room temperature and add 25 ml diethylether. Cool the flask in a freezer In a hood, add 1.5 g of N-methyl-N'-nitro-N-nitroso-quanidine. The additions should be done slowly over a period of a few minutes. After each addition, gently

swirl the flask. Into a bottle, equipped with a "poly-seal" polyethylene liner cap, decant the ether layer from the aqueous slurry that has formed. Cap and store in a freezer. Do not use ground glass stoppered bottles. The diazomethane solution may be stored at -20°C for over a week if it is kept in a tightly capped bottle. This procedure gives approximately 16 ml of ether solution.

CAUTION: The diazoalkane may be prepared in a larger quantity by increasing the amounts of chemicals, but the proportions must not be changed. The diazoalkanes are toxic and potentially explosive. Do not allow the nitrosoquanidine or diazomethane solution to come in contact with the skin, as these compounds may cause skin rashes.

Etched or scratched glassware should be avoided. Use only diethylether as the solvent for diazomethane.

8.3 Gas Chromatography Conditions

Column:	10% carbowax 20M 80/100 mesh GCQ 4mm ID x 6 ft.
Oven Temp:	125°
Detector Temp:	190°
Inlet Temp:	190°
Carrier Gas Flow (N_2):	65 mls/min.
Hydrogen Flow:	180 mls/min.
Air Flow:	100 mls/min.
Attenuation:	32 x 10^4
Chart Speed:	0.25 in/min.
Retention Time:	∿5 min.

9.0 INDEX OF TABLES AND FIGURES

Table I	-	Recovery Data for Fosetyl-Al from whole oranges and grapefruit.
Table II	-	Recovery Data for Fosetyl-Al from orange and grapefruit peels.
Table III	-	Recovery Data for Fosetyl-Al from orange and grapefruit juice.

10.0 LITERATURE REFERENCES

Laurent, M., Chabassol, Y., Rhône-Poulenc Agrochimie (October 25, 1982). Determination of Fosetyl and its Metabolites in Citrus Fruit.

Rhône-Poulenc Agrochimie (1982), Determination of Residues of Fosetyl and Phosphorous Acid in Pineapples (Method RE 21.8

TABLE I

RECOVERY DATA FOR FOSETYL-AL FROM WHOLE ORANGES AND GRAPEFRUIT

Spiking Level (ppm)	Whole Fruit	% Recovery
8.0	Grapefruit	84.12
8.0	Oranges	87.00
1.0	Grapefruit	87.60
1.0	Oranges	84.00
0.8	Grapefruit	84.75
0.8	Oranges	86.90
0.2	Grapefruit	97.00
0.2	Oranges	91.50
0.08	Grapefruit	95.00
0.05	Grapefruit	92.00
0.05	Grapefruit	92.00
0.05	Oranges	96.00
0.05	Oranges	88.00

Average = 89.68%

Standard Deviation: 4.52

Coefficient of Variation: 5.04%

Control	Grapefruit	<0.05 ppm
Control	Oranges	<0.05 ppm

TABLE II

RECOVERY DATA FOR FOSETYL-AL
FROM ORANGE AND GRAPEFRUIT PEELS

Spiking Level (ppm)	Fruit Peels	% Recovery
80.0	Grapefruit	88.00
40.0	Oranges	89.80
4.0	Grapefruit	90.00
1.0	Oranges	82.00
0.8	Oranges	90.75
0.8	Oranges	80.00
0.08	Oranges	95.00
0.05	Oranges	96.00
0.05	Grapefruit	96.00

Average = 89.73%

Standard Deviation: 5.75

Coefficient of Variation: 6.41%

Control	Oranges	<0.05 ppm
Control	Grapefruit	<0.05 ppm

TABLE III

RECOVERY DATA FOR FOSETYL-AL
FROM ORANGE AND GRAPEFRUIT JUICE

Spiking Level (ppm)	Fruit Juice	% Recovery
8.0	Grapefruit	94.4
4.0	Oranges	97.5
1.0	Grapefruit	84.0
0.8	Grapefruit	80.4
0.8	Grapefruit	77.0
0.4	Oranges	95.0
0.4	Grapefruit	97.5
0.05	Grapefruit	97.6
0.05	Grapefruit	91.0
0.05	Oranges	87.8

Average = 90.22%

Standard Deviation: 7.58

Coefficient of Variation: 8.4%

Control	Grapefruit	<0.05 ppm
Control	Oranges	<0.05 ppm

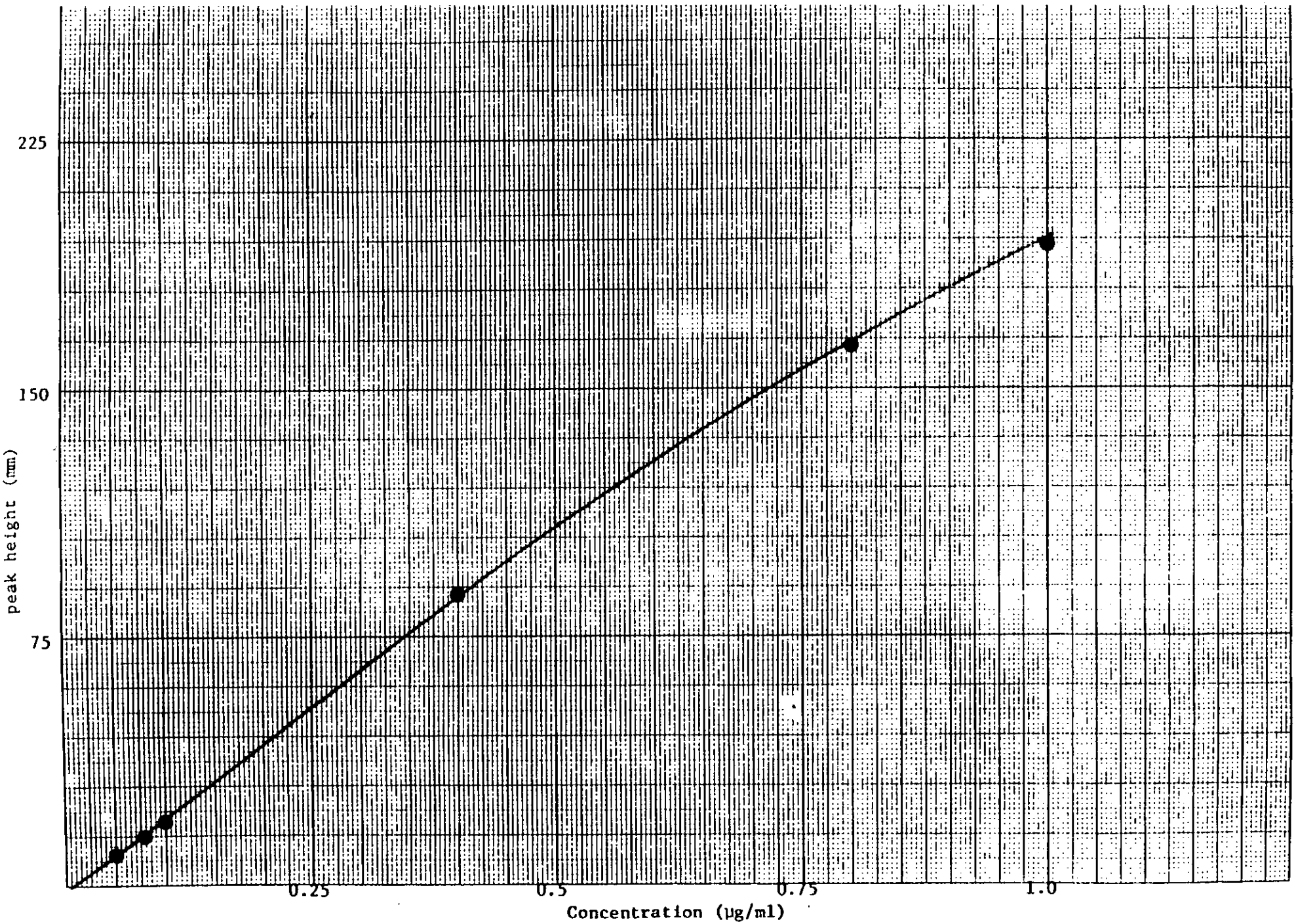


FIGURE II - TYPICAL CHROMATOGRAMS OF STANDARDS OF THE METHYL ESTER DERIVATIVE OF ALUMINUM TRIS (O-ETHYL PHOSPHONATE)

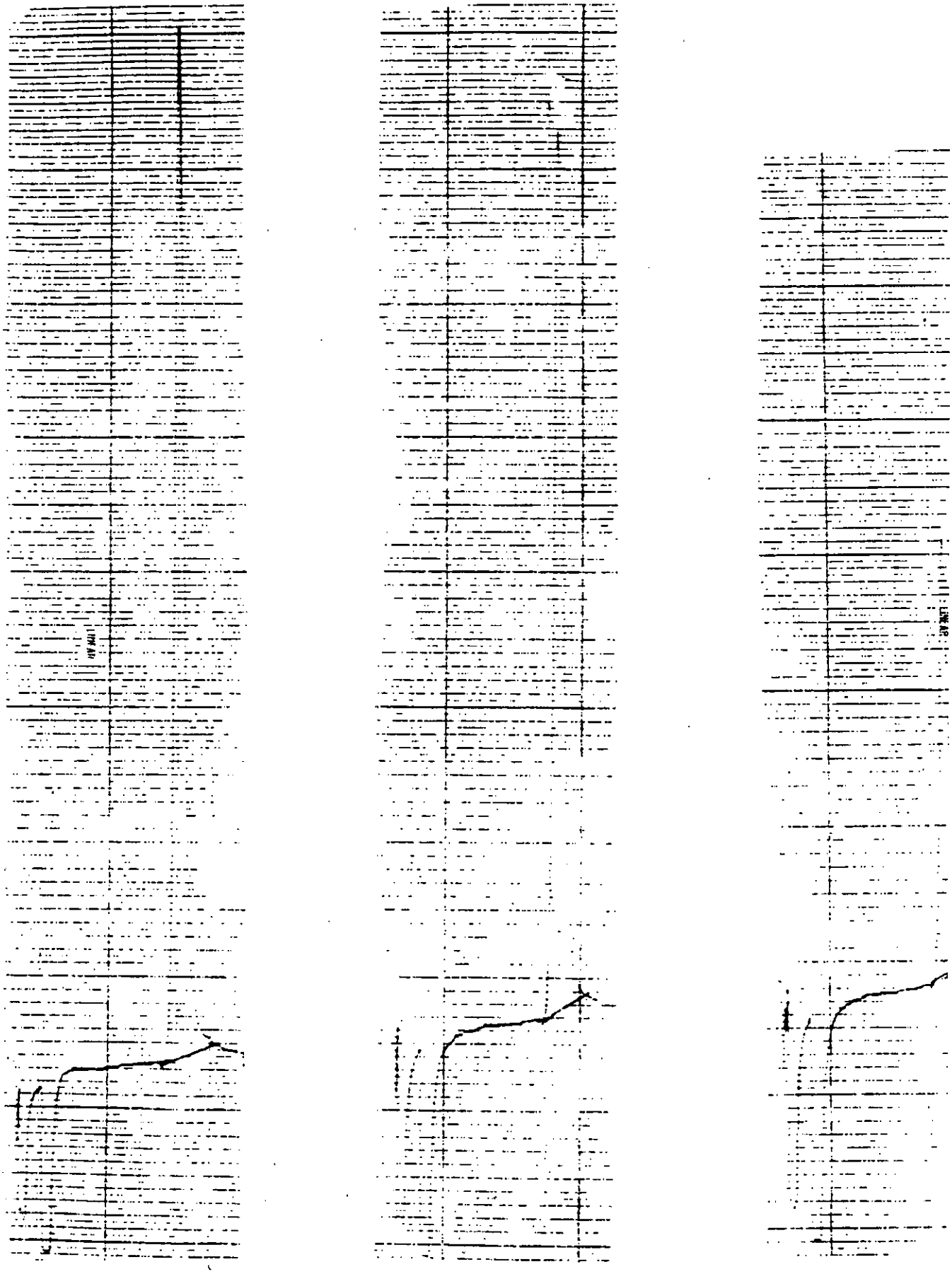
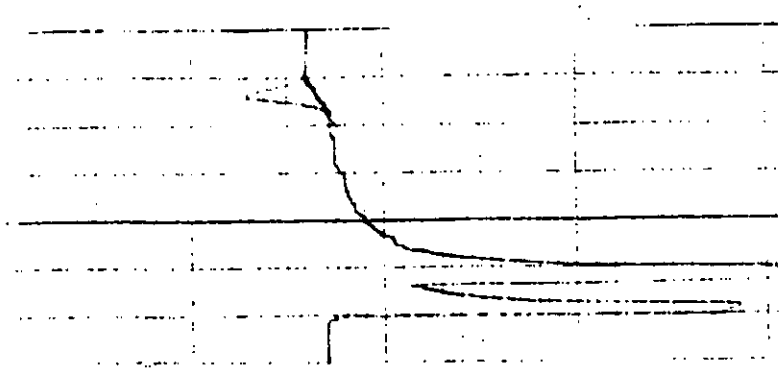
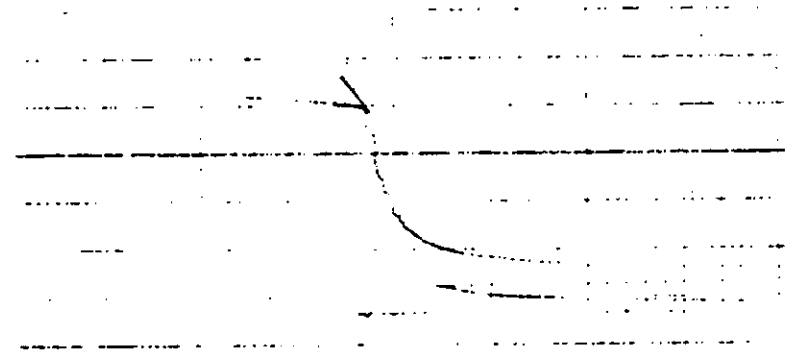


FIGURE II
(cont'd)

0.05 µg/ml



0.08 µg/ml



0.10 µg/ml

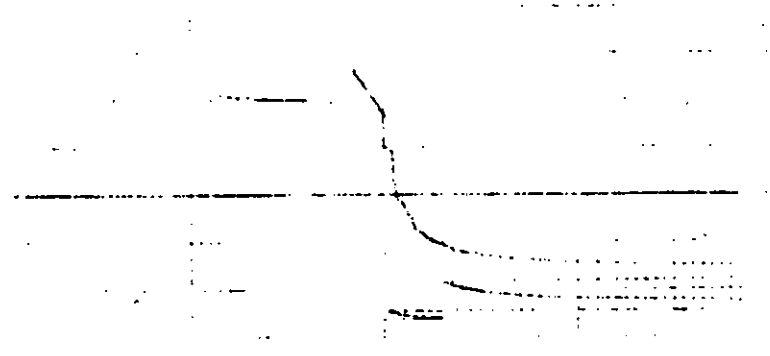


FIGURE III - TYPICAL CHROMATOGRAMS OF WHOLE ORANGES; CONTROL AND FORTIFIED CONTROLS

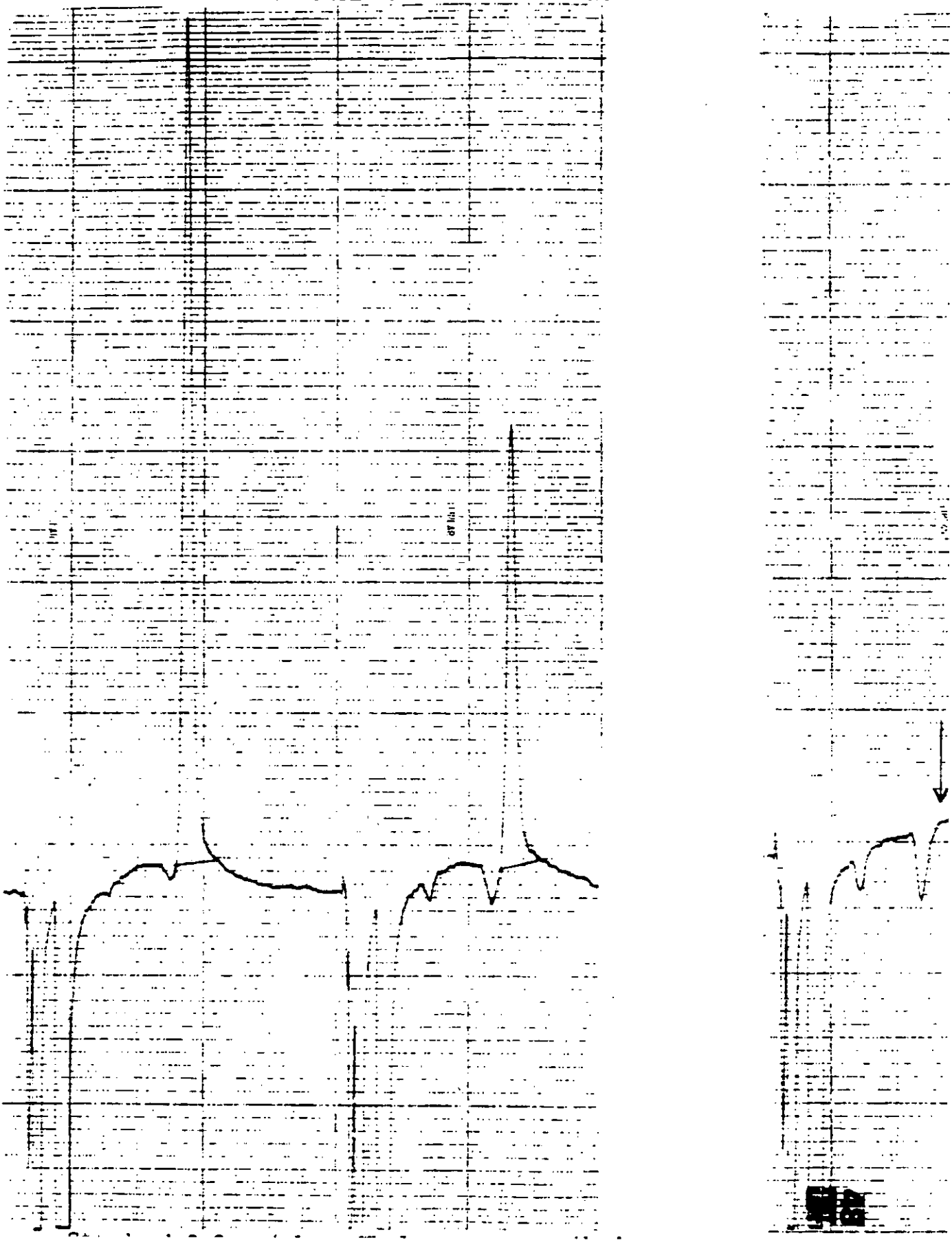
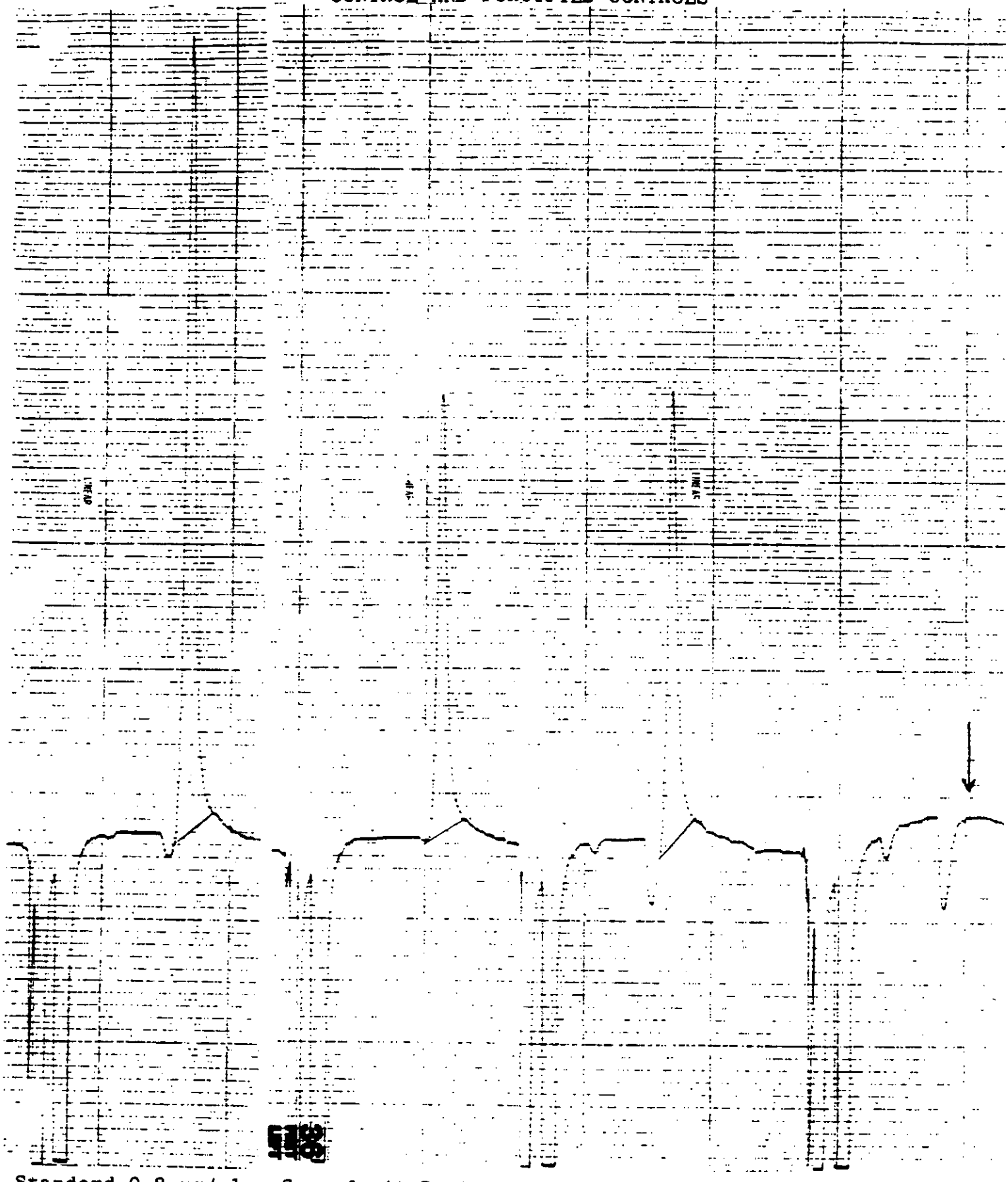


FIGURE IV - TYPICAL CHROMATOGRAMS OF GRAPEFRUIT PEELS; CONTROL AND FORTIFIED CONTROLS



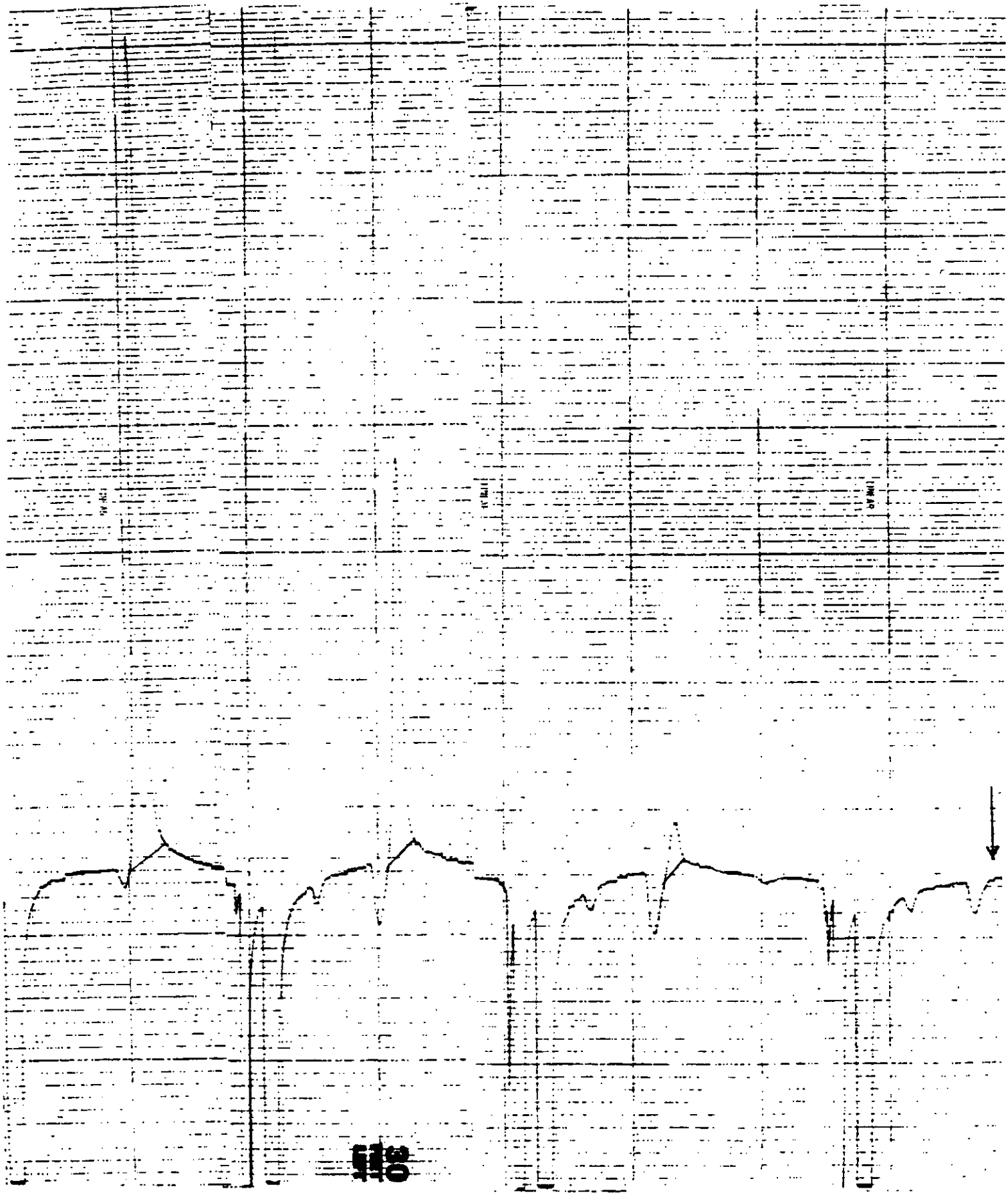
Standard 0.8 µg/ml

Grapefruit Peels

Grapefruit Peels

Grapefruit Pe

FIGURE V - TYPICAL CHROMATOGRAMS OF ORANGE JUICE; CONTROL AND FORTIFIED CONTROLS



Standard 0.8 ug/ml Orange Juice Orange Juice Orange Jui