

Analytical Method

Department: Residue Chemistry

Date: September 26, 1995

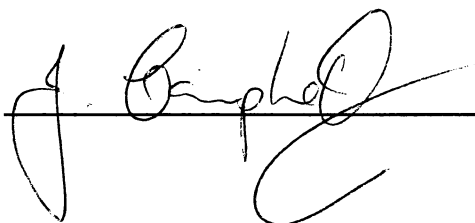
RAM Number: J/01/95

Title: Analytical Method For Residues of Clofentezine in Fruit
(Western Red Delicious Apples) by HPLC and UV Detection

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Signed:



Date: 26 Sept 95

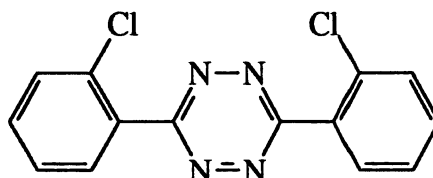
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Residue Methods Book

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1. SCOPE

This method is restricted to the determination of residues of clofentezine only. The limit of determination for this method has been set at 0.01 ppm.



Common Name: Clofentezine
C.A. Name: 3,6-bis(2-chlorophenyl)-1,2,4,5-tetrazine
CAS Reg. No.: [74115-24-5]

2. PRINCIPLE

Fruit samples are extracted by blending with acetone followed by filtration. An aliquot is removed and partitioned with hexane. The organic extract is rotary evaporated to dryness then reconstituted in hexane for clean up through a Florisil chromatography column. Clofentezine is eluted from the Florisil, evaporated to dryness, and re-dissolved in methanol. Final quantitation of the methanol solution is done using high performance liquid chromatography with UV detection at 268 nm.

3. APPARATUS

Use as a guide; equivalent substitution may be required.

- Hobart VCM 40 or other apparatus suitable for grinding frozen plant tissue
- Blending Jars (glass pint canning jars)
- Sorvall-Omni Mixer with Blades, Model 17105, Omni International
- Büchner funnels
- Vacuum Filter Adapters
- Graduated cylinders 100 mL, 250 mL TC
- Separatory funnel 500 mL with stopcocks
- Boiling flasks 125 mL, 250 mL, and 500 mL
- Glass powder funnels, Pyrex
- Rotary Evaporation Unit (Buchi: RE-R1)

- Chromatographic columns 250 mm x 10.5 mm i.d. with 200 mL reservoir (Prism Research Glass Cat. No.: PRG-1620-01)
- Volumetric flasks (100 mL)
- Gelman Acrodisk LC 13 PVDF 0.45 μ m syringe filters
- Disposable syringes
- HPLC System with column heater (see Appendix I)
- HPLC guard column - Javelin (C18) 20 mm x 3 mm (Keystone Scientific Part. No.: 88702-33-P)
- HPLC column - Hypersil ODS (C18) 250 mm x 3 mm (Keystone Scientific Part. No.: 255-33-3)

4. REAGENTS

All solvents should be HPLC grade or better.

- Acetone
- Deionized Water (DI H₂O)
- Sodium Chloride (NaCl), ACS reagent
- Hexanes
- Sodium Sulfate (Na₂SO₄), granular, ACS reagent
- Glass Wool, (silane treated) Supelco Inc. (Cat. No. 2-0411M)
- Florisil (60/100 mesh)
- Diethyl Ether
- Methanol

5. PROCEDURE

5.1 Extraction

- 5.1.1 Weigh out 50 grams of a finely ground representative sample into a pint blending jar.

Note: To perform recovery efficiency tests, pipette a standard solution of an appropriate concentration of clofentezine prepared in methanol onto the sample at this point.

- 5.1.2 Add 100 mL acetone to the jar and blend for \approx 5 minutes. Filter sample with vacuum through a Büchner funnel containing glass fiber filter paper into a 250 mL graduated cylinder
- 5.1.3 Transfer the filter cake back to the blending jar and repeat step 5.1.2 once again. Combine filtrates and rinses into graduated cylinder. Rinse the jar, blades and filter cake with small

amount of acetone and collect the rinses. Adjust the volume of the extracts to 250 mL with DI H₂O if necessary. Stopper cylinder and mix well.

Note: Acetone/H₂O when mixed builds pressure. Carefully vent samples while mixing.

5.2 Partitioning

5.2.1 Quantitatively transfer a 100 mL aliquot to a 500 mL separatory funnel. Add 250 mL of 5% (w/v) NaCl solution and 20 mL hexane. Shake and let phases separate. Drain the lower aqueous layer into a 500 mL beaker or boiling flask. Dry the top organic layer through granular anhydrous Na₂SO₄ held in a funnel with a glass wool plug into a 125 mL boiling flask.

5.2.2 Carefully pour aqueous layer back into the separatory funnel and rinse the beaker or flask with small amount of hexane and transfer the rinses to the separatory funnel. Partition the aqueous phase twice with 20 mL volumes of hexane. Dry each partition through the granular anhydrous Na₂SO₄ held in a funnel with a glass wool plug. Rinse the Na₂SO₄ pad after the third partition thoroughly with hexane and collect rinses in the 125 mL boiling flask.

5.3 Concentration

5.3.1 Rotary evaporate the extracts to dryness with the water bath set at 40°- 45°C.

5.3.2 Dissolve residue in ≈ 2 mL of hexane.

5.4 Florisil Clean-up

Note: Adjustments in elution solvent strength or volume from the values reported in this method may be required depending on the lot or brand of Florisil used. Each new lot of Florisil must be calibrated to ensure the complete elution of clofentezine. The calibration pattern is checked by analyzing 10 mL fractions of the eluate after a 1.0 mL solution containing at least 5.0 µg of clofentezine is eluted from the column. The fractions are evaporated to dryness and then brought up to 10 mL in methanol. Recoveries should be calculated for each fraction and totaled. Adjustments in elution solvent strength or volume should then be made to insure the complete elution of clofentezine.

- 5.4.1 Prepare Florisil columns by slurry packing 6.0 grams of activated Florisil with 50 mL hexane into a chromatographic column and capping the columns with approximately 1 gram of granular sodium sulfate.
- 5.4.2 Drain the excess hexane out of the column until the sulfate just begins to dry. (DO NOT ALLOW THE FLORISIL TO DRY.)
- 5.4.3 Load the hexane extract from step 5.3.2 onto the Florisil column and drain the column until the top of the column just begins to dry. Rinse the boiling flask with 3 x 2 mL portions of hexane and load each rinse onto the column. Drain the column between each rinse until the sulfate just begins to dry.
- 5.4.4 Wash the chromatographic column with 50 mL of 4% (v/v) ethyl ether/hexane and discard the wash. Drain the column until the sulfate just begins to dry.
- 5.4.5 Elute the clofentezine with 70 mL of 15% (v/v) ethyl ether/hexane and collect the extracts in a 250 mL boiling flask.
- 5.4.6 Rotary evaporate the extracts to dryness with the water bath set at 40°- 45 °C. Dissolve the residue in 2.0 mL of methanol to await analysis by HPLC.

Note: Particulates have been observed to form in the final extract. The extracts may be filtered through a Gelman Acrodisk LC 13 PVDF 0.45 µm syringe filter using disposable syringes if necessary.

6. HPLC ANALYSIS

6.1 Preparation of Analytical Standards

- 6.1.1 Prepare a stock solution of clofentezine (1000 µg/mL) in methanol. Make serial dilutions of the stock clofentezine standard to prepare calibration standards for use on the HPLC (typically 0.05, 0.10, 0.25, 0.50, 0.75, and 1.00 µg/mL) in methanol.
- 6.1.2 Make serial dilutions of the stock clofentezine standard for use as fortification solutions (typically 100, 10, 1, and 0.1 µg/mL) in methanol.

6.2 Analysis of Samples

- 6.2.1 Inject a 25 μL aliquot of each sample from Step 5.4.6 and a 25 μL aliquot of the standard solutions into the HPLC operated under conditions listed in Appendix I. Make dilutions of samples as needed to maintain the peak height (or area) within the standard calibration range.
- 6.2.2 Construct a standard curve by plotting standard peak heights or areas vs. concentration ($\mu\text{g}/\text{mL}$). Calculate the least-squares regression line.
- 6.2.3 Determine the concentration of clofentezine in the treated and fortified samples by comparing the peak heights (or areas) to the standard curve.
- 6.2.4 Calculate the residue R using equation 1 as follows:

$$R \text{ (ppm)} = \frac{(Y - b) / m}{C} \quad (\text{Equation 1})$$

Where: Y = peak height (or area) response (cts.)
b = Y-intercept of standard regression line (cts.)
m = slope of standard regression line (cts mL/ μg)
C = crop/solvent ratio (g/mL)

The crop/solvent ratio "C" is defined by the concentration of sample in g/mL at injection using equation 2. This factor incorporates all dilutions made to the sample.

$$C = \frac{W}{250 \text{ mL}} \times \frac{100 \text{ mL}}{2 \text{ mL}} \times D \quad (\text{Equation 2})$$

Where: W = sample weight (g)
D = dilution factor

The dilution factor D is defined by equation 3 below:

$$D = \frac{A}{V} \quad (\text{Equation 3})$$

Where: A = Aliquot taken (mL)
V = Final Volume (mL)

6.3 Fortification Experiments

6.3.1 With each sample set, analyze an untreated control sample and one or more fortified control samples.

6.3.2 Calculate recoveries by equation 4 as follows:

$$\text{Recovery (\%)} = \frac{R - S}{T} \times 100 \quad (\text{Equation 4})$$

Where: R = ppm of clofentezine found in fortified sample
 S = ppm of clofentezine found in control sample
 T = theoretical ppm in fortified sample

7. DISCUSSION

Whole western red delicious apples were purchased at a local grocery store for use in fortification experiments for study J-95R-02. The apples were frozen whole and then finely ground with a Hobart VCM 40 grinding unit and dry ice. The resulting sample was then assigned a laboratory number 136-081.01 and used as a control for study J-95R-02.

The control (unfortified) and fortified control samples were analyzed for clofentezine. Fortification levels were run at 0.01 ppm (1x tolerance) and 0.10 ppm (10x tolerance) using the previously described method. A total of 16 fortifications (eight at 0.01 ppm and eight at 0.10 ppm) were analyzed. A summary of the analytical recoveries of clofentezine are presented in Table 1 below.

Table 1 Recovery Data for Clofentezine in Whole Apples (Western Red Delicious)

Crop Matrix	Fortification Level (ppm)	Recovery of Clofentezine (%)							
		75	85	83	73	100	105	97	71
Whole Apples	0.01								
	0.10	89	94	94	88	97	94	84	82
Number =		16							
Mean (%)=		88							
Std. Dev. (%)=		±10							

The method described is a modification of an Analytical Method for Residues of Clofentezine in Miscellaneous Fruit Crops.¹ A set of 8 to 10 samples can be prepared by one person in approximately one working day (8 hours) and be placed on an HPLC equipped with an autosampler for overnight analysis.

8. REFERENCES

1. Manley, J. D., "Analytical Method for Residues of Clofentezine in Miscellaneous Fruit Crops," FBC Limited, (May 1986)
Registration Reference: NC 21314/R111

Appendix I HPLC Conditions

Pump: Hitachi L-6200A
Detector: Hitachi L-4200 UV
Autosampler: Hitachi AS-2000
Column Heater: Eppendorf Model CH-30 with Model TH-50 controller
Temperature: 32° C
Injection Volume: 25 µL
Wavelength: 268 nm

Note: Clofentezine exhibits a sharp absorbance maxima at 268 nm. In order to insure operation of the HPLC detector at maximum sensitivity, it may be necessary to optimize the detector by making injections of standard solutions at different wavelengths to observe the sensitivity. This is done by varying the wavelength by 1 nm on either side of 268 nm out to the performance specifications set by the detector manufacturer. The limits of accuracy for wavelength selection for the Hitachi L-4200 UV detector is ± 2 nm.

Reverse Phase Conditions

Column: Hypersil ODS (C18) 250 mm x 3 mm
Particle size: 5 µm
Pore size: 120 Å

Guard Column: Javelin (C18) 20 mm x 3 mm

Gradient Program: Hitachi L-6200A

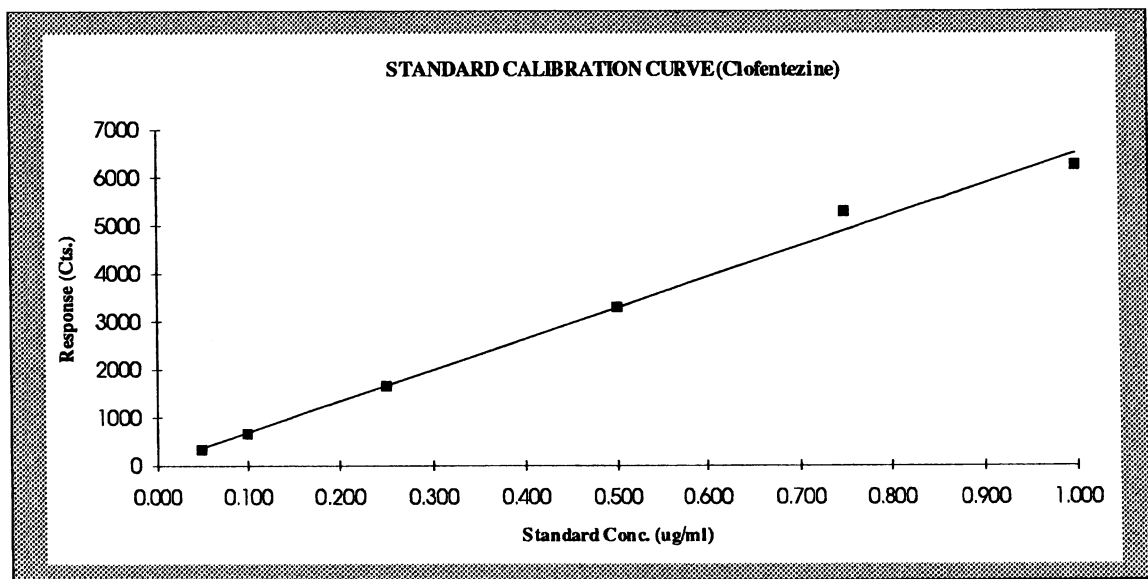
Time (minutes)	Acetonitrile (%)	DI H ₂ O (%)	Methanol (%)	Flow (mL/min)
0.0	45	45	10	0.40
2.0	90	10	0	0.40
2.1	90	10	0	0.40
19.9	90	10	0	0.40
20.0	90	10	0	0.40

Equilibration Time: 15 minutes between injections

Retention Time: 15.3 minutes (approximate)

Appendix II
Standard Calibration Data for Clofentezine

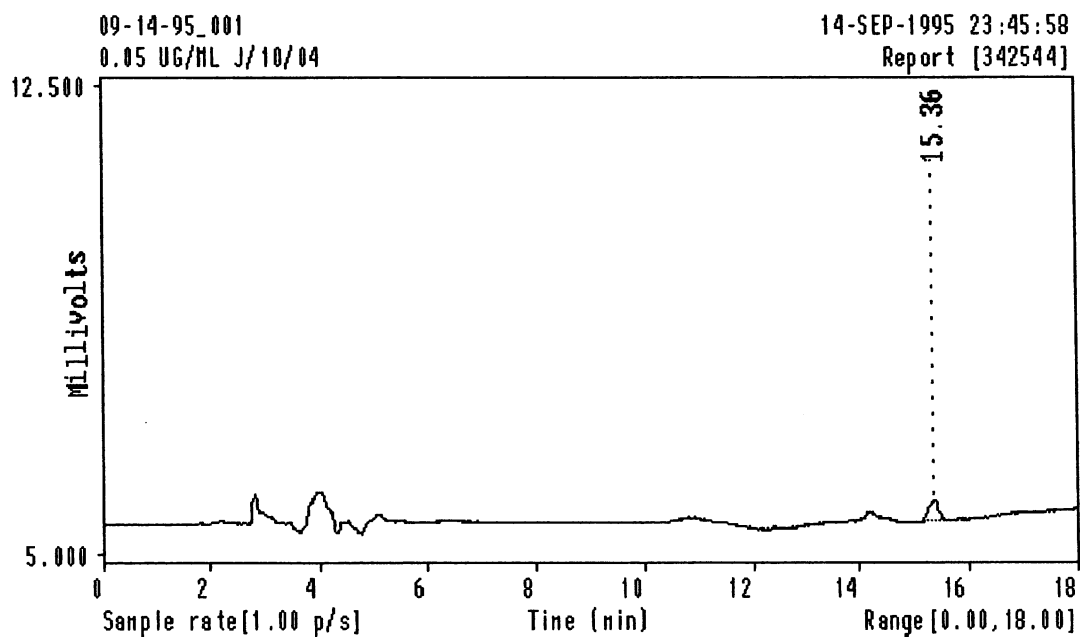
Standard Calibration Curve:		Clofentezine		
Retention Time (min.)	Standard Solution Reference Number	Standard Conc. (ug/ml)	Response (Counts)	Statistical Data
15.362	J/10/04	0.05	324	Slope 6460.7 Y-Int. 45.692
15.356	J/10/05	0.10	652	
15.346	J/10/06	0.25	1636	Coeff. 0.9927
15.364	J/10/07	0.50	3282	
15.350	J/10/10	0.75	5267	
15.355	J/10/08	1.00	6234	



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Appendix III Representative Chromatograms (con't)
Figure 1 0.05 ug/ml Clofentezine Standard

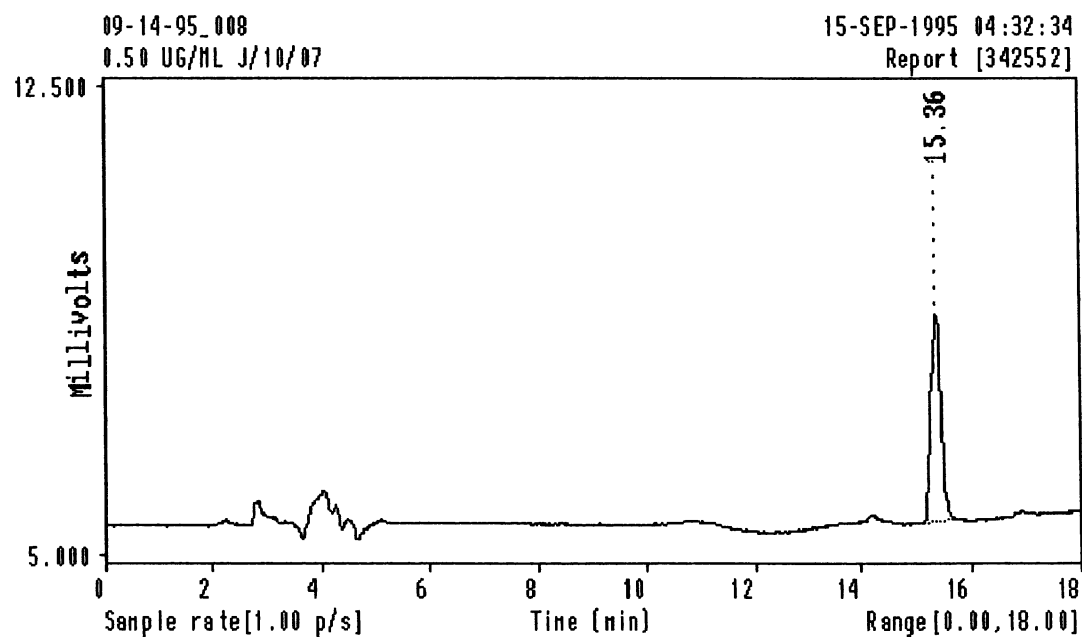
Sample Description:		0.05 ug/ml Clofentezine Standard		
Retention Time (min.)	Analyte of Interest	Response (Counts)	Residue Conc. (ppm)	Analytical Recovery (%)
15.362	Clofentezine	324	N/A	N/A



Appendix III Representative Chromatograms (con't)

Figure 2 0.50 ug/ml Clofentezine Standard

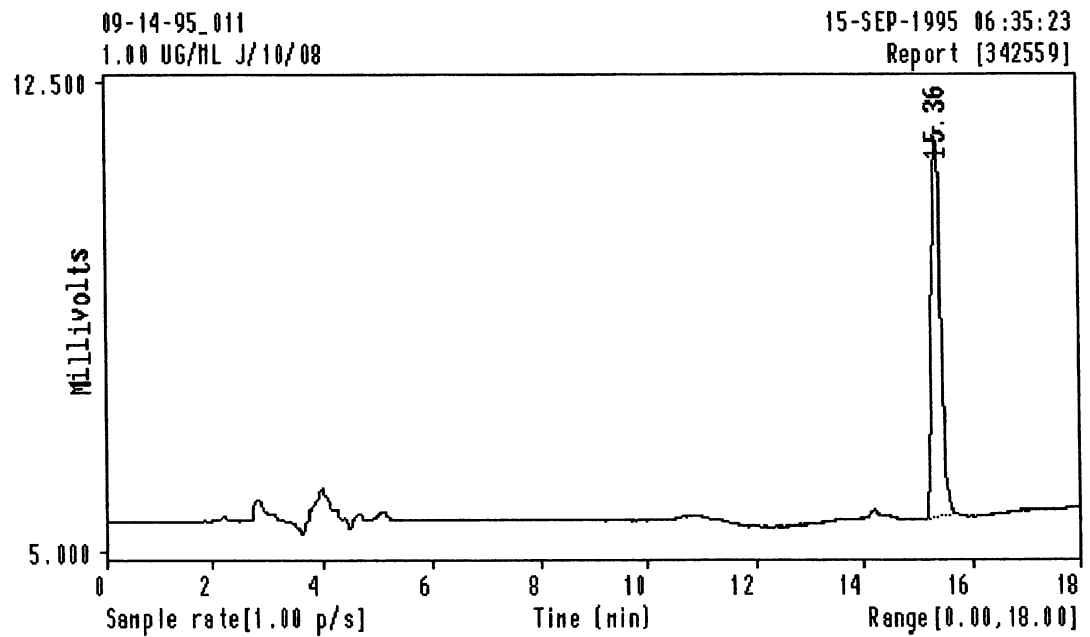
Sample Description:		0.50 ug/ml Clofentezine Standard		
Retention Time (min.)	Analyte of Interest	Response (Counts)	Residue Conc. (ppm)	Analytical Recovery (%)
15.364	Clofentezine	3282	N/A	N/A



Appendix III Representative Chromatograms (con't)

Figure 3 1.00 ug/ml Clofentezine Standard

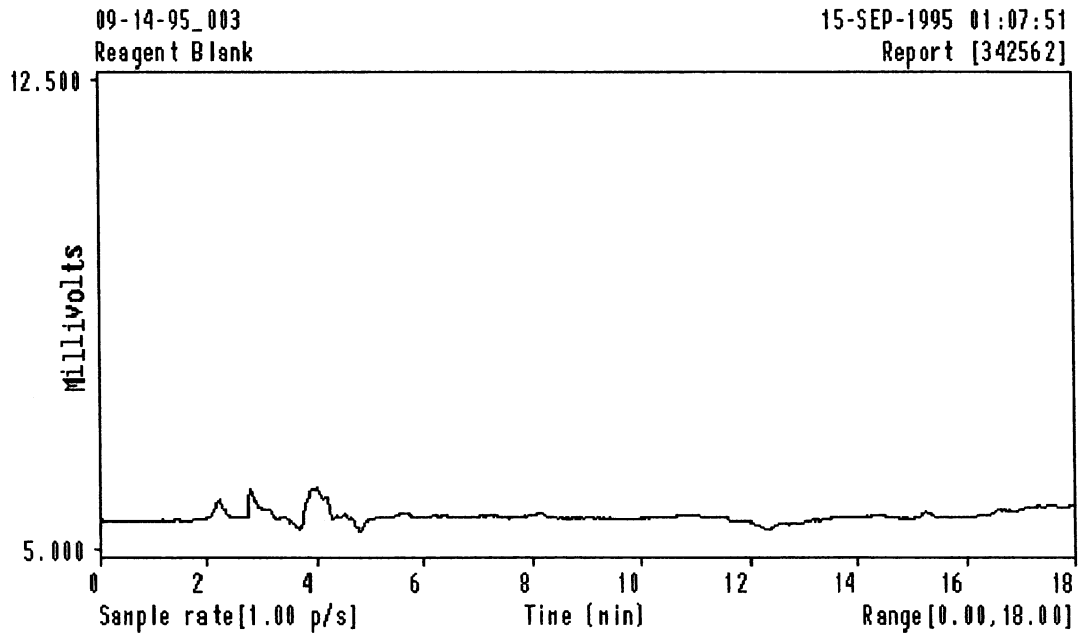
Sample Description:		1.00 ug/ml Clofentezine Standard		
Retention Time (min.)	Analyte of Interest	Response (Counts)	Residue Conc. (ppm)	Analytical Recovery (%)
15.355	Clofentezine	6234	N/A	N/A



Appendix III Representative Chromatograms (con't)

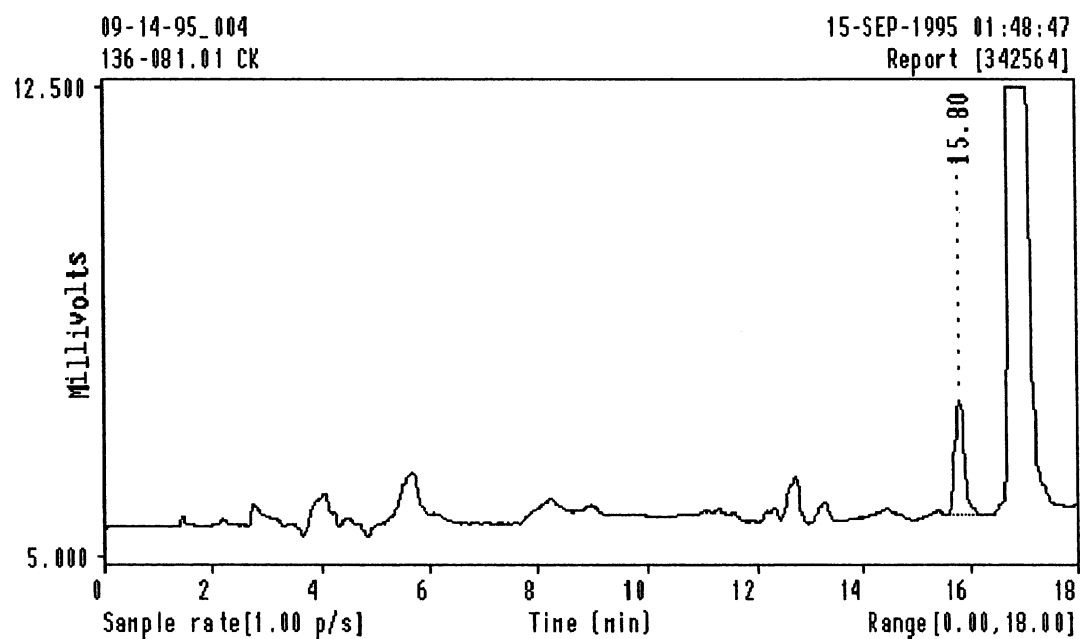
Figure 4 Reagent Blank

Sample Description:		Reagent Blank		
Retention Time (min.)	Analyte of Interest	Response (Counts)	Residue Conc. (ppm)	Analytical Recovery (%)
N/A	Clofentezine	0	N.D.	N/A



Appendix III Representative Chromatograms (con't)
Figure 5 136-081.01 Apple Control

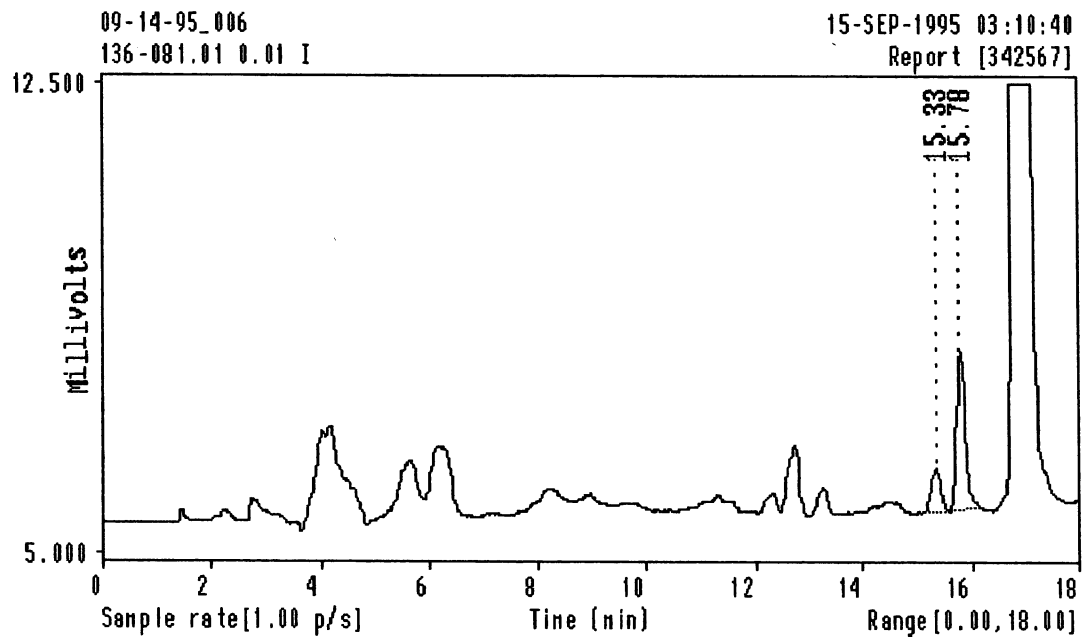
Sample Description:		136-081.01 Apple Control		
Retention Time (min.)	Analyte of Interest	Response (Counts)	Residue Conc. (ppm)	Analytical Recovery (%)
N/A	Clofentezine	0	N.D.	N/A



Appendix III Representative Chromatograms (con't)

Figure 6 136-081.01 Apple Control fortified at 0.01 ppm

Sample Description:		136-081.01 Apple Control fortified at 0.01 ppm		
Retention Time (min.)	Analyte of Interest	Response (Counts)	Residue Conc. (ppm)	Analytical Recovery (%)
15.335	Clofentezine	694	0.01003	100

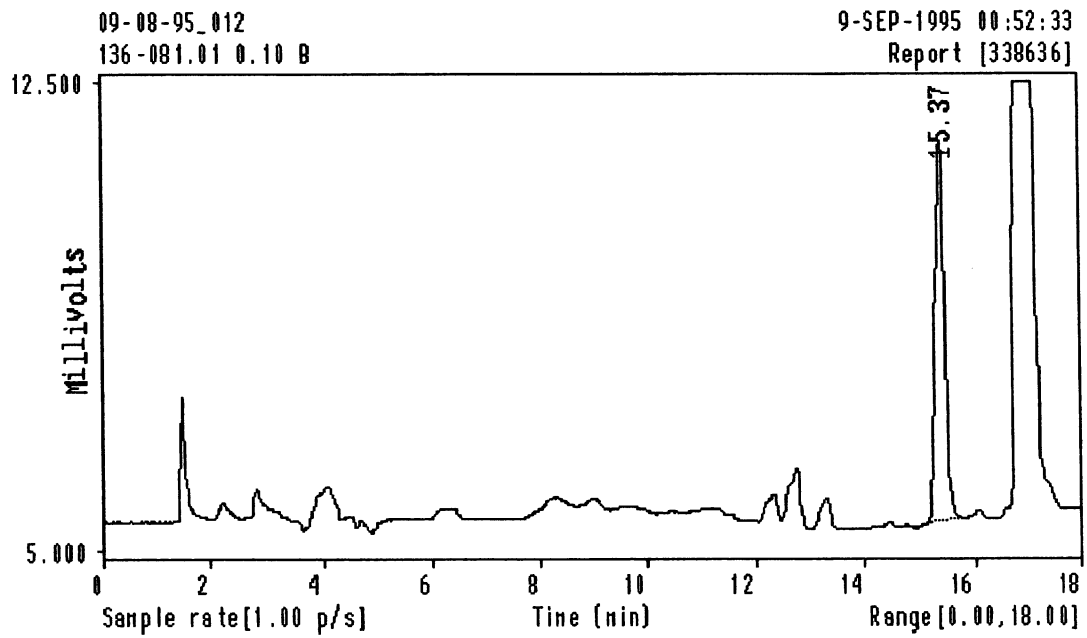


Appendix III

Representative Chromatograms (con't)

Figure 7 136-081.01 Apple Control fortified at 0.10 ppm

Sample Description:		136-081.01 Apple Control fortified at 0.10 ppm		
Retention Time (min.)	Analyte of Interest	Response (Counts)	Residue Conc. (ppm)	Analytical Recovery (%)
15.374	Clofentezine	6003	0.09424	94



Appendix IV

Flow Diagram of Analytical Method J/01/95

