

PYRIPROXYFEN

VALENT U.S.A. CORPORATION
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DETERMINATION OF PYRIPROXYFEN RESIDUES IN APPLES, PEARS, AND CITRUS FRUITS

METHOD: RM-33P-1-3a

DATE: October 9, 1998

I. INTRODUCTION

This method determines residues of pyriproxyfen [4-phenoxyphenyl(*RS*)-2-(2-pyridyloxy) propyl ether] in fruit. This method is based on RM-33P-1-3, and the validation data are taken directly from RM-33P-1-3. This method does not include the analysis for 4'-OH-Pyr, and the amount of fruit extracted for pyriproxyfen analysis is doubled by eliminating a split of the sample extract [the final volume of sample extract is also increased from 1.0 to 2.0 mL].

Briefly, residues are extracted from the crop matrix using acetone, partitioned with hexane/acetonitrile (for citrus crops), partitioned with dichloromethane/water, and then cleaned-up with silica gel column chromatography. *Preparation of apples and pears do not require cleanup using the acetonitrile/hexane partition.* Analysis for pyriproxyfen is performed by gas chromatography using a nitrogen-phosphorus detector (GC/NPD).

II. REAGENTS

Acetone - pesticide quality (or equivalent).

Acetonitrile - pesticide quality (or equivalent).

Dichloromethane - pesticide quality (or equivalent).

Ethyl acetate - pesticide quality (or equivalent).

Hexane - pesticide quality (or equivalent).

Silica Gel 60 - 70-230 mesh, EM Science Cat# 7734-7 (or equivalent).

This material should be profiled with a pyriproxyfen standard prior to use in the method (See Note 1).

Sodium chloride - reagent grade (or equivalent).

Prepare a 5% (w/v) aqueous solution by adding 50 grams to a 1 L flask and diluting to volume with deionized water. Stopper and shake.

Sodium sulfate - anhydrous, granular, AR grade (or equivalent).

Toluene - pesticide quality (or equivalent).

Water - deionized.

III. ANALYTICAL STANDARDS

Pyriproxyfen reference standard - Valent U.S.A. Corporation.

Pyriproxyfen standard, 1.0 mg/mL Stock solution (in acetone).

Weight 0.100 grams of pyriproxyfen into a 100 mL volumetric flask (correct the amount of standard weighed for the purity of the standard). Dilute to volume with acetone, and store refrigerated.

Pyriproxyfen standard, 10 $\mu\text{g}/\text{mL}$ Fortification solution (in acetone).

Pipet 1.0 mL of the Stock solution into a 100 mL volumetric flask, and dilute to volume with acetone. Store refrigerated.

Pyriproxyfen standard, 1.0 $\mu\text{g}/\text{mL}$ Fortification solution (in acetone).

Pipet 10.0 mL of the 10 $\mu\text{g}/\text{mL}$ Fortification solution into a 100 mL volumetric flask, and dilute to volume with acetone. Store refrigerated.

Pyriproxyfen standard, 10 $\mu\text{g}/\text{mL}$ Standard solution (in toluene).

Pipet 1.0 mL of the Stock solution into a 100 mL volumetric flask, and dilute to volume with toluene. Store refrigerated.

Pyriproxyfen standard, 2.0 $\mu\text{g}/\text{mL}$ Standard solution (in toluene).

Pipet 20.0 mL of the 10 $\mu\text{g}/\text{mL}$ Standard solution into a 100 mL volumetric flask, and dilute to volume with toluene. Store refrigerated.

Pyriproxyfen standard, 1.0 $\mu\text{g}/\text{mL}$ Standard solution (in toluene).

Pipet 10.0 mL of the 10 $\mu\text{g}/\text{mL}$ Standard solution into a 100 mL volumetric flask, and dilute to volume with toluene. Store refrigerated.

Pyriproxyfen standard, 0.5 $\mu\text{g}/\text{mL}$ Standard solution (in toluene).

Pipet 5.0 mL of the 10 $\mu\text{g}/\text{mL}$ Standard solution into a 100 mL volumetric flask, and dilute to volume with toluene. Store refrigerated.

Pyriproxyfen standard, 0.1 $\mu\text{g}/\text{mL}$ Standard solution (in toluene).

Pipet 1.0 mL of the 10 $\mu\text{g}/\text{mL}$ Standard solution into a 100 mL volumetric flask, and dilute to volume with toluene. Store refrigerated.

IV. EQUIPMENT

Autosampler Vials, Caps, and Crimping Tool.

Balance - analytical top loading.

Beakers - 100 and 250 mL.

Büchner funnels - 9 cm diameter.

Filter flasks - 500 or 1000 mL.

Filter funnels - approximately 10 cm diameter.

Filter paper - 9 cm diameter Whatman® GF/A glass fiber (or equivalent).

Gas Chromatograph - Hewlett-Packard Model 5890, equipped with a silanized glass insert for splitless injection (HP Part No. 5080-8732), a nitrogen-phosphorus detector (NPD), automatic sampler, and a recording integrator (or equivalent system). [Note: a small amount of silanized glass wool is inserted into the middle of the glass insert.]

Glass chromatography column - 19 mm ID x 300 mm with 250 mL reservoir and Teflon® stopcock, Kontes Cat. # K-420280-0232 (or equivalent).

Glass wool, Pyrex® (or equivalent).

Graduated Cylinders - 10, 50, 100, 250, 1000 mL.

Mason® jars - 1 pint (or equivalent).

Omnimixer®, with adaptor for use with 1-pint Mason® jars.

Pipetor, Automatic - Rainin® EDP 2 with 0.1 - 2.0 mL range (or equivalent)

Pipets, Pasteur - 5³/₄" and 9".

Pipets, Volumetric - 1.0, 2.0, 5.0, 10.0, and 20.0 mL (a calibrated automatic pipetor may also be used for the 1.0 and 2.0 mL measurements)

Rotary Vacuum Evaporator , equipped with a temperature-controlled water bath.

Round-bottom flasks - 50, 100, 500, and 1000 mL.

Separatory funnels - 250 and 500 mL.

Ultrasonic water bath - Branson® 3200 (or equivalent).

Volumetric flasks - 100 mL

V. ANALYTICAL PROCEDURES

1. ACETONE EXTRACTION

Weigh 20 grams (\pm 0.1 grams) of sample into a one pint Mason® jar (See Note 2). [At this point, if required by the testing facility, control samples for method recovery should be fortified with pyriproxyfen (See Note 3).] Add approximately 150 mL of acetone to the sample and blend on the Omnimixer® for approximately 5 minutes.

Allow the solids to settle (about 2-3 minutes), and then decant the liquid through the Whatman® GF/A glass fiber filter [in the Büchner funnel] into a 500 mL (or 1000 mL) filter flask using vacuum. Add a second 150 mL portion of acetone to the solids remaining in the jar, and repeat the extraction using the Omnimixer®. After the solids settle, decant the liquid through the filter into the filter flask (combining this extract with the first). Repeat the extraction with a third 150 mL portion of acetone, transfer both the liquid and the solids onto the filter, and collect the filtrate as before. Rinse the Mason® jar with approximately 20 mL of acetone, add this rinse to the solids on the Büchner funnel, and collect the filtrate with the other extracts.

Transfer the combined filtrates to a 1000 mL round-bottom flask. Rinse the filter flask with a 10 to 20 mL portion of acetone, and add the rinse to the round-bottom flask. Evaporate the acetone using a rotary evaporator (with the water bath set to $<40^{\circ}\text{C}$) until the volume is approximately 50-80 mL. Add 250 mL of ethyl acetate to the round-bottom flask, and then continue with the rotary evaporation to obtain an aqueous residue (for citrus crops, this should reduce the aqueous residue to approximately 3-5 mL).

For apples and pears, the volume of aqueous residue is not critical - proceed directly to the dichloromethane/water partition in Step 3. For citrus crops, proceed to the acetonitrile/hexane partition in Step 2.

2. ACETONITRILE / HEXANE PARTITION

Add 70 mL of acetonitrile (hexane saturated) to the round-bottom flask, and briefly rotate the flask in an ultrasonic water bath to assist removal of residue adhering to the walls of the flask. Transfer the mixture to a 250 mL separatory funnel, rinse the round-bottom flask with 100 mL of hexane (acetonitrile saturated), and then add the hexane to the separatory funnel. Stopper and shake the funnel vigorously for 1 minute (with occasional venting). Allow the phases to separate, then drain the acetonitrile (the lower layer) into a 500 mL round-bottom flask. [If a water layer is observed in the bottom of the separatory funnel, this should be retained with the acetonitrile layer.] Partition the hexane layer twice with fresh 70 mL portions of acetonitrile (hexane saturated), and combine the acetonitrile extracts in the 500 mL round-bottom flask (for a total of three partition steps). Remove the acetonitrile by rotary evaporation using a heated water bath (<40°C), *just to dryness*. Proceed to Step 3.

3. DICHLOROMETHANE / WATER PARTITION

Add 100 mL of 5% sodium chloride solution to the round-bottom flask, and briefly rotate the flask in an ultrasonic water bath to assist removal of residue adhering to the walls of the flask. Transfer this mixture to a 500 mL separatory funnel, add 150 mL of dichloromethane to the round-bottom flask, and then transfer the dichloromethane rinse to the separatory funnel. Shake the separatory funnel vigorously for approximately one minute (with occasional venting), and then allow the phases to separate. Drain the dichloromethane (lower) layer through a 10 cm filter funnel containing approximately 50 grams of sodium sulfate, suspended on a plug of glass wool, into a 1 L round-bottom flask. Rinse the sodium sulfate with approximately 20 mL of dichloromethane.

Repeat the partition step with a second 100 mL portion of dichloromethane (rinse the round-bottom flask and then transfer the dichloromethane into the separatory funnel, as before). Drain the dichloromethane (lower) layer through the sodium sulfate into the 1 L round-bottom flask, combining the extracts. Rinse the sodium sulfate with 20 mL of dichloromethane, and then repeat this partition step with a third 100 mL portion of dichloromethane (followed by a 20 mL dichloromethane rinse, as before). Evaporate the combined extracts to 20-30 mL using a rotary-evaporator (and water bath set to <40°C). Transfer the residue into a 100 mL round-bottom flask and then rinse the 1 L round-bottom flask twice with 10 mL portions of ethyl acetate, adding each rinse to the 100 mL round-

bottom flask. Continue rotary evaporation (using a water bath set to $<40^{\circ}\text{C}$) - *just to dryness*. Add 5 mL of 80/20 hexane/ethyl acetate (v/v), stopper the round-bottom flask, and then sonicate the round-bottom flask for 15-20 seconds (as necessary) to dissolve any residue adhering to the walls of the flask.

4. SILICA GEL COLUMN CLEANUP

Place a glass wool plug at the bottom of the glass chromatography column. Weigh 18 grams of silica gel (see Note 1) into a beaker, suspend in 40-60 mL of 80/20 hexane/ethyl acetate (v/v) in a beaker, and pour the silica gel slurry into the column while tapping the side of the column. [Alternatively, fill the column with approximately 60 mL of 80/20 hexane/ethyl acetate, slowly pour the silica gel into the column, and then gently rotate the column to dislodge any silica gel above the solvent.] Wash the sides of the column with 80/20 hexane/ethyl acetate, as needed, and cap the silica gel with approximately 2 grams of sodium sulfate. Drain the solvent to the top of the sodium sulfate layer.

Transfer the residues onto the column by pouring the extract into the column, and then draining the solvent to the top of the sodium sulfate layer. Rinse the round-bottom flask twice using 5 mL portions of 80/20 hexane/ethyl acetate (v/v), transferring each rinse sequentially in the column and then draining the solvent to the top of the sodium sulfate layer. Rinse the round-bottom flask with a 10 mL portion of 80/20 hexane/ethyl acetate, transfer this rinse also to the column (total of 25 mL 80/20 hexane/ethyl acetate), and drain the solvent to the top of sodium sulfate layer. Discard the collected eluant.

Place a 100 mL round-bottom flask under the column and elute the pyriproxyfen with 50 mL of 80/20 hexane/ethyl acetate (v/v). [Note: the volume used is dependent on the properties of the silica gel.] Evaporate the eluant *just to dryness* using a rotary-evaporator and water bath set to $<40^{\circ}\text{C}$. Add 2.0 mL of toluene to the flask containing the residue, sonicate the sample for at least 20 seconds to completely dissolve the residue, and then transfer the extract to an autosampler vial or screw cap vial using a Pasteur pipet. Store the extract either in a refrigerator (nominally at 5°C) or in a freezer (nominally at -10°C) until GC analysis.

5. MEASUREMENT BY GAS CHROMATOGRAPHY

Analyze the sample extracts for pyriproxyfen, along with calibrating standard solutions, using the following (suggested) operating conditions:

Column: DB-17 (30 M x 0.53 mm) wide-bore capillary (1.0 μm film thickness),
J & W Scientific Cat # 125-1732 (or equivalent).

Column Oven Temperature Program:

| | |
|-----------------------|----------------------------|
| Initial Temp: | 260°C |
| Hold Time: | 2.0 minutes at 260°C |
| Programmed Rate: | 10°C/minute |
| Final Temp: | 280°C |
| Hold Time: | 6 minutes at 280°C |
| Detector Temperature: | 300°C |
| Injector Temperature: | 270°C |
| Carrier Gas: | Helium at 32 mL/min |
| Detector Makeup Gas: | Helium at 0 mL/min |
| Air: | 110 mL/min |
| Hydrogen: | 3.6 mL/min |
| Injection Volume: | 1.0 μ l |
| Retention Time: | 2.2 minutes (See Figure 1) |

The GC parameters shown above are given only as a guide. They may be modified as needed to optimize the chromatography or to resolve matrix interferences. Each set of chromatograms must be clearly labeled with the GC parameters used. See Note 4 for alternative GC parameters.

Verification of instrument linearity is described in Note 5. Calibration standards (1.0 μ g/mL of pyriproxyfen in toluene) are used to determine instrument response within each analytical sequence, and each sequence must begin and end with a calibration standard. Typically, continuing calibration standards are analyzed every 2 to 4 samples within an analytical sequence. The recommended sequence is: standard, sample, sample, sample, standard, , standard. The placement of the calibration standards ensures that the reproducibility requirement is met (see Note 6).

6. CALCULATIONS

The amount of pyriproxyfen in each sample is calculated using the following formula:

$$\text{Sample concentration, ppm (mg/kg)} = \frac{A \times B \times C \times D}{E \times F}$$

where:

- A = Sample peak height (or area)
- B = Continuing calibration standard concentration (1.0 $\mu\text{g/mL}$)
- C = Final extract volume (2.0 mL)
- D = Dilution factor, used if the sample extract is diluted prior to analysis
- E = Average calibration standard peak height (or area)
- F = Initial sample weight (20 grams)

VI. LIMITS OF DETECTION AND QUANTITATION

The limit of detection (LOD) of pyriproxyfen in oranges and apples analyzed by this method is 0.01 ppm. The validated limit of quantitation (LOQ) is 0.02 ppm. Results from method validation for oranges and for apples are attached.

VII. DISCUSSION

Typical chromatograms from orange samples (untreated control and fortified control samples) are presented in Figures 2, 3, and 4. Typical chromatograms from apple samples (untreated control and fortified control samples) are also presented in Figures 5, 6, and 7.

A trained analyst can complete the analysis of a set of eight samples for pyriproxyfen in approximately 10 hours. The results are available within 24 hours of initiating the analysis.

VIII. NOTES

1. Each batch of silica gel must be checked for recovery of pyriproxyfen. Pipet 2.0 mL of the 1.0 $\mu\text{g/mL}$ pyriproxyfen fortifying solution into a 50 mL round-bottom flask, and evaporate *just to dryness* using a rotary-evaporator with a water bath set to $<40^\circ\text{C}$. Transfer the residue to a silica gel column and elute the analyte as described under SILICA GEL COLUMN CLEANUP (Step 4). Evaporate the eluate *just to dryness* using a rotary-evaporator and water bath set to $<40^\circ\text{C}$. Pipet 2.0 mL of toluene into the flask containing the residue, stopper the flask, and swirl to completely dissolve the residue. Analyze this eluant with the 1.0 $\mu\text{g/mL}$ calibrating standard as described under MEASUREMENT BY GAS CHROMATOGRAPHY

(Step 6). If the height of the pyriproxyfen peak after column chromatography is less than 90% of the calibrating standard, then the elution profile of pyriproxyfen may need to be determined. [Although the volumes of standard solution used to profile the column may be changed, volumes between 1 to 3 mL are recommended.] Rechecking the preparation of the eluant, the assembly of the column, and the steps taken to elute the pyriproxyfen are all recommended prior to determining a new profile for the silica gel column.

2. Apple and pear samples are typically macerated with dry ice, and then a portion of each sample is mixed (while cold, after the dry ice sublimates) with 1 M ascorbic acid (in a ratio of 200 g of sample to 100 g of ascorbic acid). Thirty grams (30 g) of this preserved sample is equivalent to 20 g of fruit.
3. Valent's standard operating procedure (SOP# VR-002) requires that fortified control samples be analyzed with each set of samples. If the testing facility does not require concurrent analysis of fortified control samples, or if an untreated control (UTC) sample is not available, this method requirement may be waived.

The level of fortification is generally 0.02 ppm (the LOQ of the method) and/or 0.1 ppm. Typically, these fortifications are done by adding either 0.40 mL of the 1.0 $\mu\text{g}/\text{mL}$ fortifying solution or 0.20 mL of the 10 $\mu\text{g}/\text{mL}$ fortifying solution, respectively, to a 20 gram sample. Method recoveries must be 70% to 120% to be acceptable, unless approved by the chemist responsible for the analysis.

4. If matrix interferences are encountered during the analysis of pyriproxyfen, the following GC parameters are suggested:


| | |
|-----------------------|---|
| Column: | DB-5 (30 m x 0.53 mm) wide-bore capillary (1.5 μm film thickness). |
| Column Oven Temp.: | 250°C (Isothermal) |
| Detector Temperature: | 300°C |
| Injector Temperature: | 300°C |
| Carrier Gas: | Helium at 20 mL/min |
| Make-Up Gas: | Helium at 10 mL/min |
| Air: | 102 mL/min |
| Hydrogen: | 3.8 mL/min |
| Injection Volume: | 1.0 μL |
| Retention Time: | 3.8 minutes |

5. Valent's standard operating procedure (SOP #VR-007) requires that the linearity of the instrument be determined on each day that samples are analyzed. Linearity is determined by analyzing a series of linearity standards containing 0.10 (or 0.2) to 2.0 $\mu\text{g}/\text{mL}$. The detector

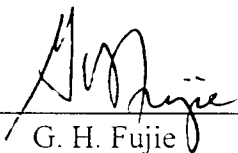
response for each standard is normalized by dividing the response of each standard (as either peak height or peak area) by its concentration. The coefficient of variation (CV) of these responses must be 10% or less (unless approved by the chemist responsible for the analysis). Sample extracts must be diluted to bring the concentration of the analyte within the established range of linearity.

6. Valent's standard operating procedure (SOP #VR-013) requires the reproducibility of an analytical run to be determined by calculating the CV of the detector responses (as either peak height or peak area) obtained from the calibrating standards analyzed during the analytical sequence. For a run to be acceptable, the CV must be 10% or less (unless approved by the chemist responsible for the analysis).

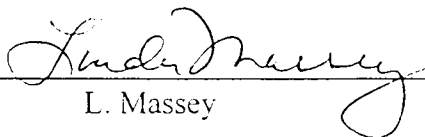
SIGNATURES

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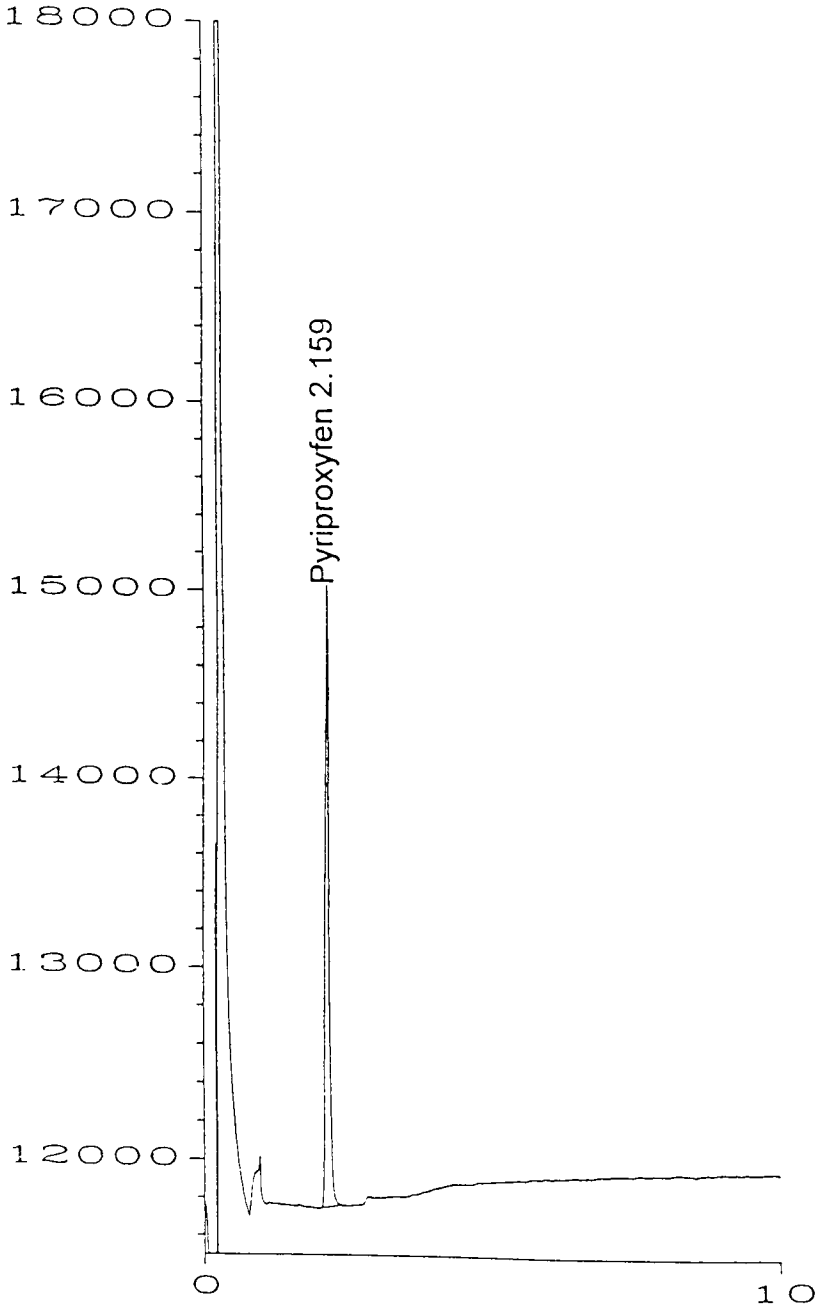


Figure 1. Pyriproxyfen Calibration Standard
(1.0 $\mu\text{g}/\text{mL}$ in toluene, 1.0 μl injected)

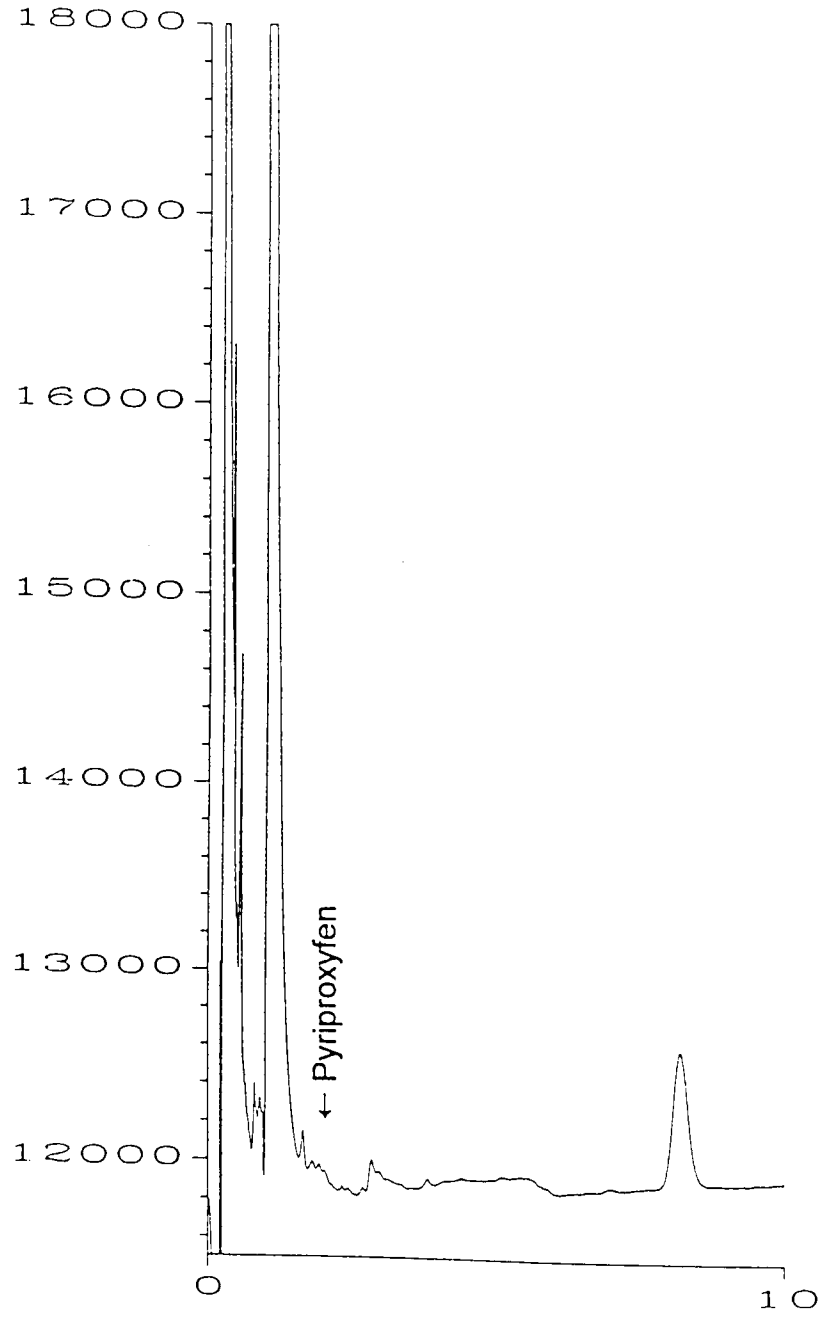


Figure 2. Control Orange Sample
(1.0 μ l injected)

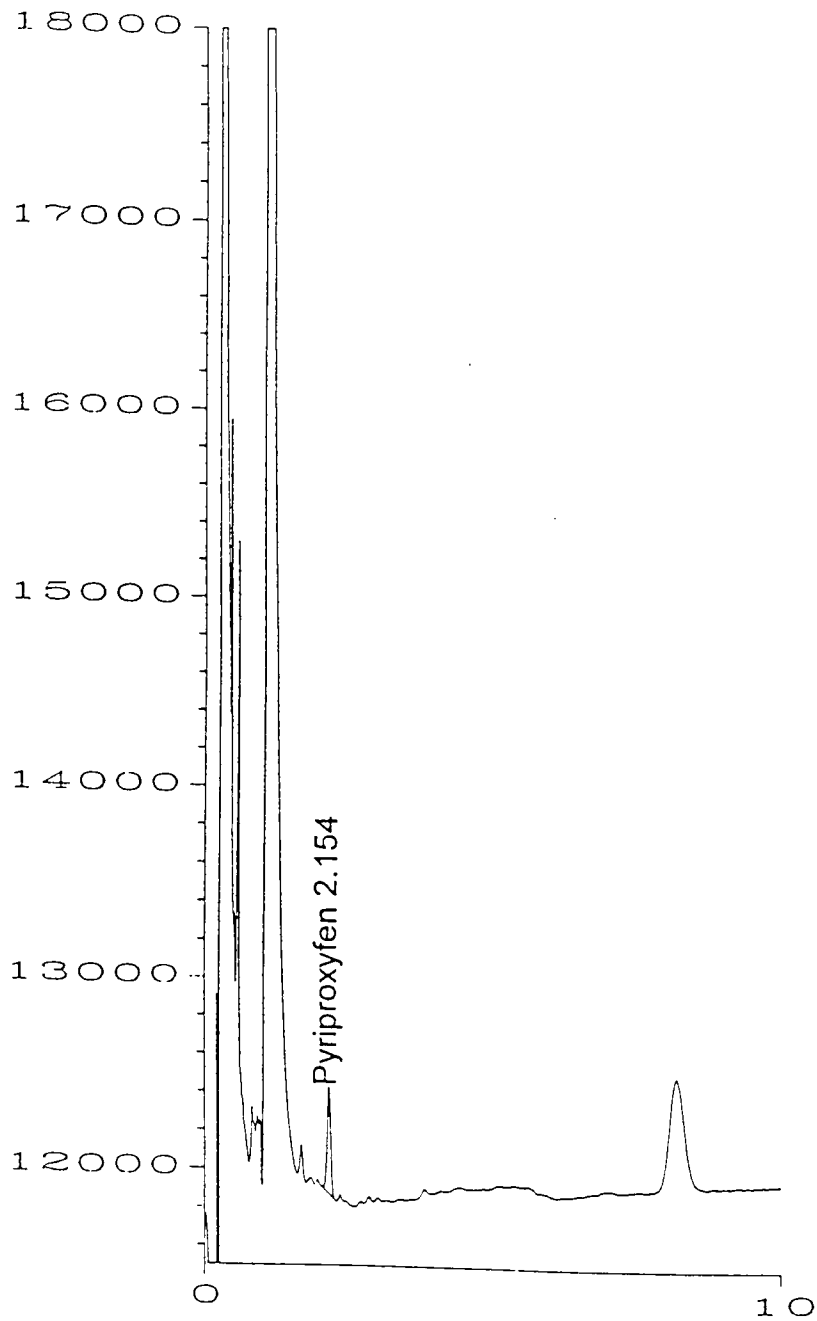


Figure 3. Fortified Control Orange Sample
(0.02 ppm Pyriproxyfen, 1.0 μ l injected)

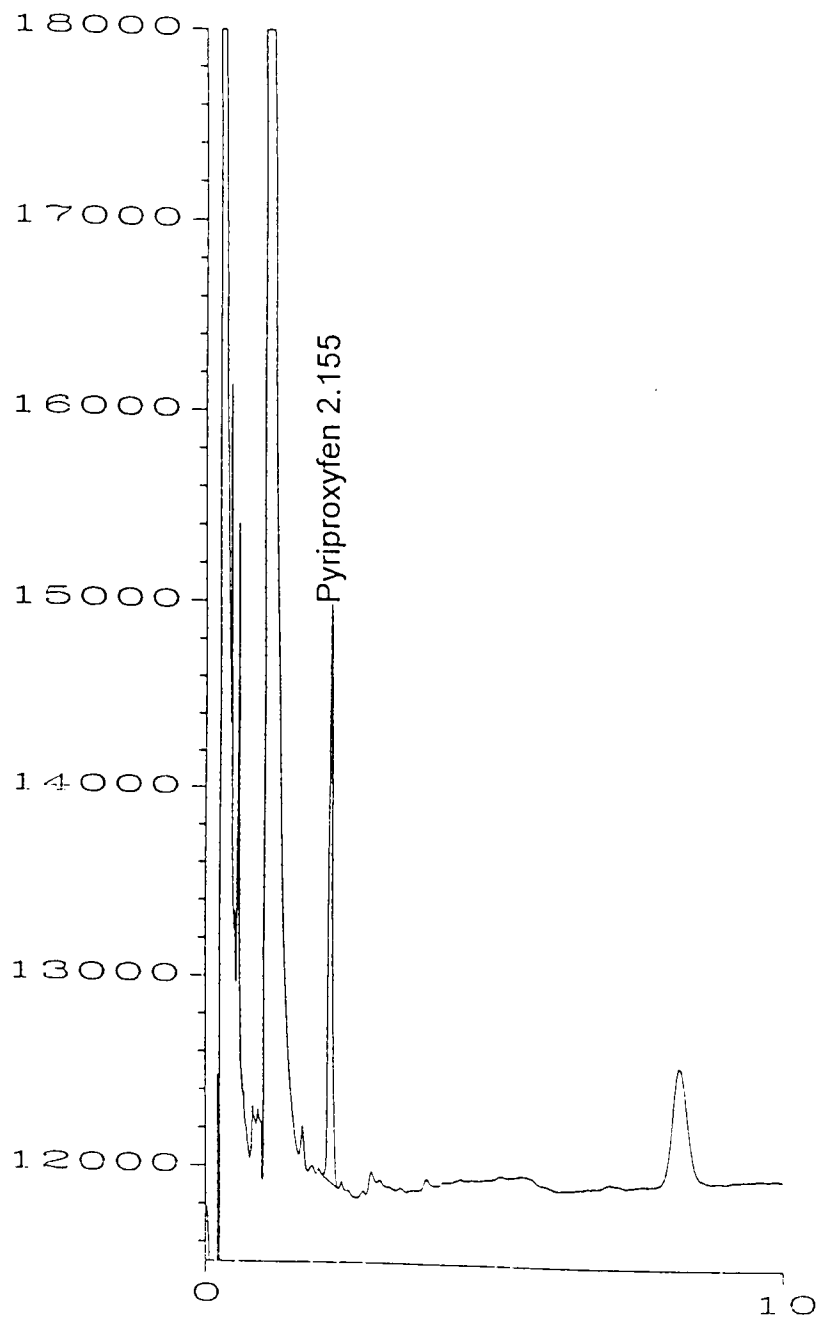


Figure 4. Fortified Control Orange Sample
(0.10 ppm Pyriproxyfen, 1.0 μ l injected)

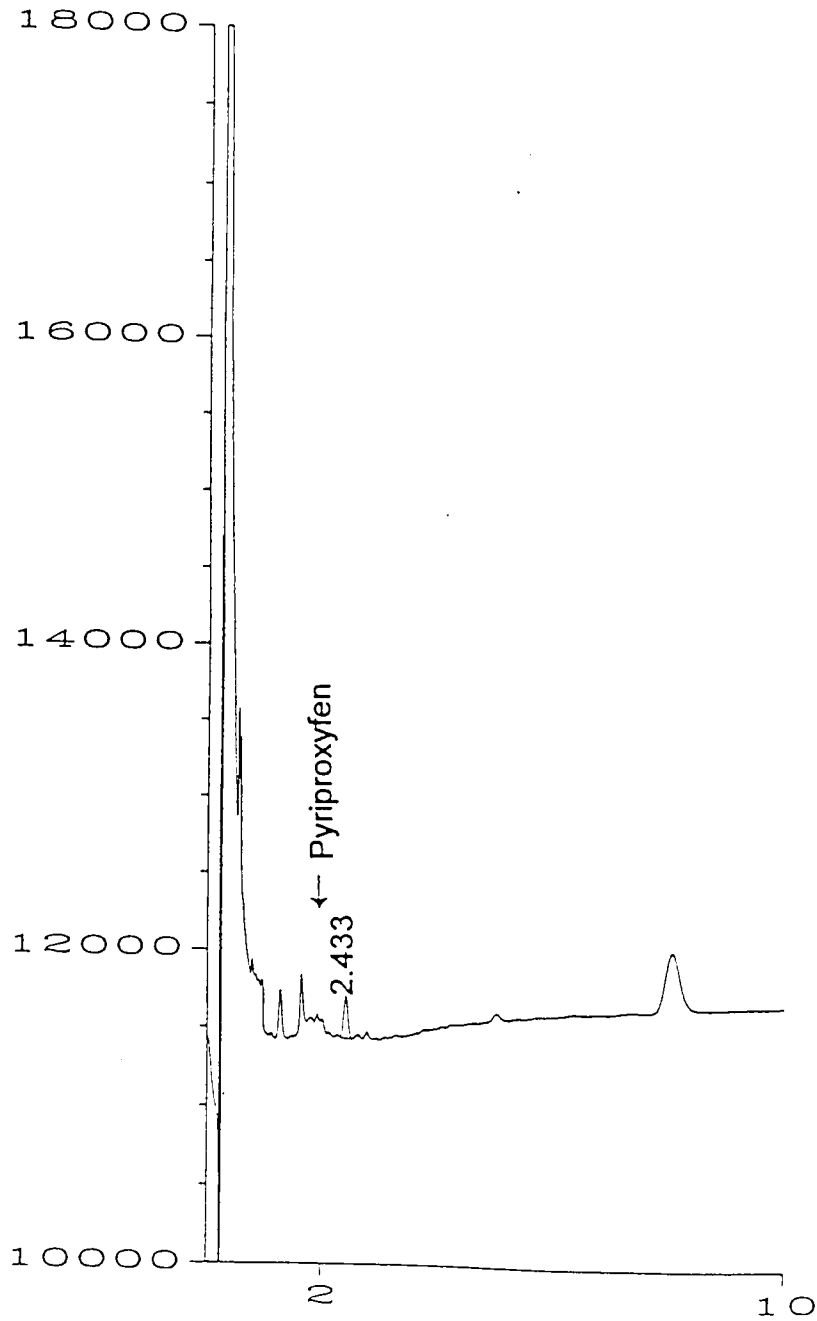


Figure 5. Control Apple Sample
(1.0 μ l injected)

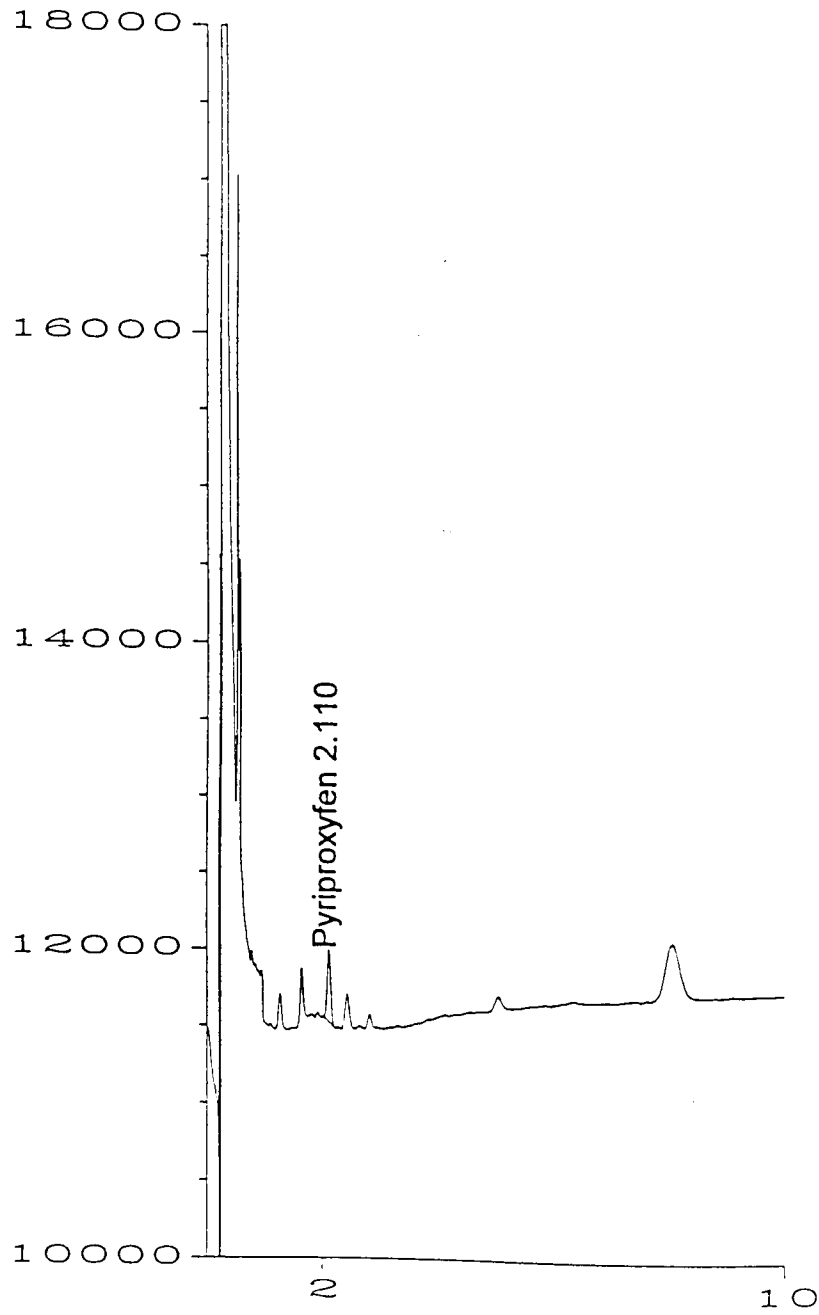


Figure 6. Fortified Control Apple Sample
(0.02 ppm Pyriproxyfen, 1.0 μ l injected)

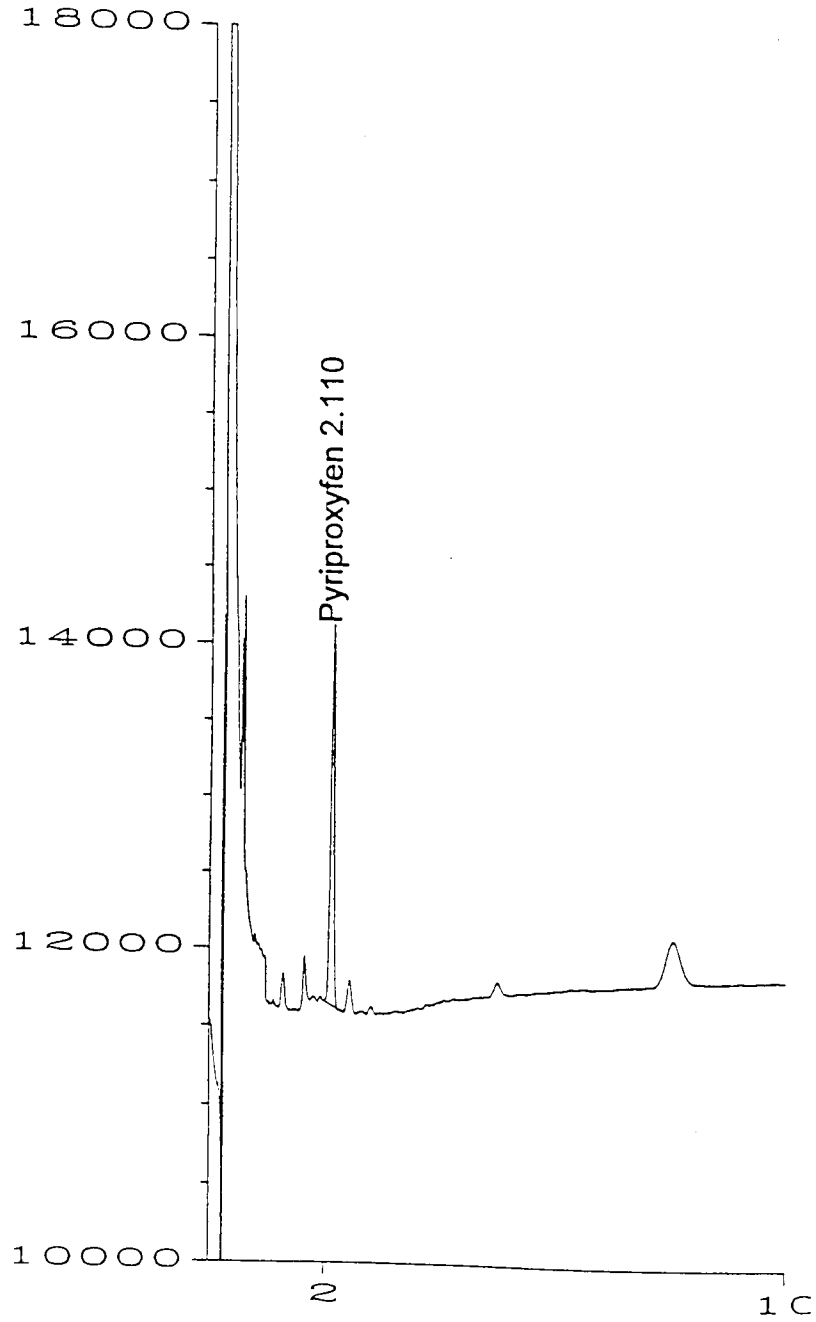


Figure 7. Fortified Control Apple Sample
(0.10 ppm Pyriproxyfen, 1.0 μ l injected)

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VALIDATION REPORT FOR RESIDUE METHOD RM-33P-1-3
REPRODUCIBILITY OF ANALYSIS (REF. SOP VR-002-03)

DETERMINATION OF PYRIPROXYFEN RESIDUE IN WHOLE ORANGES

CHEMICAL: PYRIPROXYFEN

| Sample Matrix | Reference Page | Extraction Date | Fortification Level (ppm) | Result (ppm) | Percent Recovery | |
|---------------|----------------|-----------------|---------------------------|--------------|------------------|------------|
| Oranges | VDL-351-23 | 8/1/96 | 0.10 | 0.0909 | 90.9 | |
| Oranges | VDL-351-23 | 8/1/96 | 0.10 | 0.0937 | 93.7 | |
| Oranges | VDL-351-23 | 8/1/96 | 0.10 | 0.0932 | 93.1 | |
| Oranges | VDL-351-23 | 8/1/96 | 0.10 | 0.0951 | 95.1 | Mean = 94% |
| Oranges | VDL-351-23 | 8/1/96 | 0.10 | 0.0970 | 97.0 | CV = 2.4% |
| Oranges | VDL-351-23 | 8/6/96 | 0.10 | 0.0916 | 91.6 | n = 6 |
| Oranges | VDL-351-23 | 8/1/96 | 0.02 | 0.0171 | 85.6 | Mean = 88% |
| Oranges | VDL-351-23 | 8/1/96 | 0.02 | 0.0193 | 96.3 | CV = 6.7% |
| Oranges | VDL-351-23 | 8/1/96 | 0.02 | 0.0178 | 89.1 | n = 4 |
| Oranges | VDL-351-23 | 8/6/96 | 0.02 | 0.0165 | 82.5 | |

ppm = mg/kg

Comments:

1. Due to the lose of a fortification sample (F8-extracted 8/1/96-see VDL-351-23), additional samples were extracted 8/6/96.

Bill Kambly
Analyst

8/25/96
Date

Charles Dren
Approved by

8/28/96
Date

[Signature]
Reviewed by

9/6/96
Date

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VALIDATION REPORT FOR RESIDUE METHOD RM-33P-1-3
REPRODUCIBILITY OF ANALYSIS (REF. SOP VR-002)

DETERMINATION OF PYRIPROXYFEN & 4'-OH-PYR RESIDUES IN WHOLE APPLES

CHEMICAL: Pyriproxyfen

| Sample Matrix | Reference Page | Extraction Date | Fortification Level (ppm) | Result (ppm) | Percent Recovery | | |
|---------------|----------------|-----------------|---------------------------|--------------|------------------|--------|------|
| Apples | V-11590-001 | 8/13/96 | 0.020 | 0.019 | 96 | Mean = | 98% |
| Apples | V-11590-001 | 8/13/96 | 0.020 | 0.019 | 95 | CV = | 4.4% |
| Apples | V-11590-001 | 8/13/96 | 0.020 | 0.021 | 103 | n = | 3 |

CHEMICAL: 4'-OH-Pyr

| Sample Matrix | Reference Page | Extraction Date | Fortification Level (ppm) | Result (ppm) | Percent Recovery | | |
|---------------|----------------|-----------------|---------------------------|--------------|------------------|--------|------|
| Apples | V-11590-001 | 8/13/96 | 0.020 | 0.018 | 88 | Mean = | 86% |
| Apples | V-11590-001 | 8/13/96 | 0.020 | 0.017 | 83 | CV = | 3.1% |
| Apples | V-11590-001 | 8/13/96 | 0.020 | 0.017 | 87 | n = | 3 |

ppm = mg/kg

Comments:

45 g samples of macerated apple/ascorbic acid were used (30 g equivalent; previously combined in the ratio of 2 parts of apple to 1 part of 1 M ascorbic acid).

Julian Kowalsky
Analyst

8/15/96
Date

[Signature]
Approved by

8/15/96
Date

[Signature]
Reviewed by

8/16/96
Date