

US EPA ARCHIVE DOCUMENT

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DETERMINATION OF PYRIPROXYFEN
AND 4'-OH-PYRIPROXYFEN RESIDUES IN
APPLES, PEARS, AND CITRUS FRUITS

METHOD: RM-33P-1-3

DATE: NOVEMBER 20, 1996

I. INTRODUCTION

This method determines residues of pyriproxyfen [4-phenoxyphenyl(*RS*)-2-(2-pyridyloxy) propyl ether] and 4'-OH-pyriproxyfen [4-(4-hydroxyphenoxy)phenyl-(*RS*)-2-(2-pyridyloxy)propyl ether] in fruit. This method is based on RM-33P-1, with the addition of preparation steps for 4'-OH-pyriproxyfen (4'-OH-Pyr). For citrus fruits, which are high in moisture and contain oil, cleanup includes partitioning with both hexane/acetonitrile and dichloromethane/water, and silica gel column chromatography. Preparation of apples and pears do not require cleanup using the acetonitrile/hexane partition.

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. Analysis for pyriproxyfen is performed by gas chromatography using a nitrogen-phosphorus detector (GC/NPD), and analysis for 4'-OH-Pyr is performed by high pressure liquid chromatography (HPLC) using a fluorescence detector.

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).

Methanol - HPLC grade (or equivalent).

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Methanol/Water Solution, 4/1 (v/v).

Combine 4 parts methanol with 1 part HPLC grade water. For example, add 800 mL methanol and 200 mL HPLC grade water sequentially to a reagent bottle. Store at room temperature.

Methanol/Tetrahydrofuran Solution, 2/3 (v/v).

Combine 2 parts methanol with 3 parts tetrahydrofuran. For example, add 400 mL methanol and 600 mL tetrahydrofuran sequentially to a reagent bottle. Store at room temperature.

Phosphoric acid, 85% - Reagent grade.

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

This material should be profiled with a pyriproxyfen standard and a 4'-OH-Pyr 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).

Tetrahydrofuran - HPLC grade (or equivalent).

Toluene - pesticide quality (or equivalent).

Water, acidified [0.05% phosphoric acid (85% H_3PO_4), v/v].

Add 0.5 mL of 85% phosphoric acid to 1 liter of HPLC grade water. Store at room temperature.

Water - deionized.

Water - HPLC grade.

III. ANALYTICAL STANDARDS

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

4'-OH-Pyriproxyfen (4'-OH-Pyr) reference standard - Valent U.S.A. Corporation.

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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.

4'-OH-Pyr standard, 1.0 mg/mL Stock solution (in acetone).

Weigh 0.100 grams of 4'-OH-Pyr into 100 mL volumetric flask (correct the amount of standard weighed for the purity of the standard). Dilute to volume with acetone, and store refrigerated.

4'-OH-Pyr 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.

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4'-OH-Pyr standard, 1.0 $\mu\text{g}/\text{mL}$ Fortification solution (in acetone).
Pipet 10 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.

4'-OH-Pyr standard, 10 $\mu\text{g}/\text{mL}$ Standard solution (in 4/1 methanol/water).
Pipet 1.0 mL of the Stock solution in acetone into a 100 mL volumetric flask and dilute to volume with 4/1 methanol/ water (HPLC grade). Store refrigerated.

4'-OH-Pyr standard, 2.0 $\mu\text{g}/\text{mL}$ Standard solution (in 4/1 methanol/water).
Pipet 20.0 mL of the 10 $\mu\text{g}/\text{mL}$ 4'-OH-Pyr standard solution in 4/1 methanol/water into a 100 mL volumetric flask. Dilute to volume with 4/1 methanol/ water (HPLC grade). Store refrigerated.

4'-OH-Pyr standard, 1.0 $\mu\text{g}/\text{mL}$ Standard solution (in 4/1 methanol/water)
Pipet 10.0 mL of the 10 $\mu\text{g}/\text{mL}$ 4'-OH-Pyr standard solution in 4/1 methanol/water into a 100 mL volumetric flask. Dilute to volume with 4/1 methanol/ water (HPLC grade). Store refrigerated.

4'-OH-Pyr standard, 0.5 $\mu\text{g}/\text{mL}$ Standard solution (in 4/1 methanol/water)
Pipet 5.0 mL of the 10 $\mu\text{g}/\text{mL}$ 4'-OH-Pyr standard solution in 4/1 methanol/water into a 100 mL volumetric flask. Dilute to volume with 4/1 methanol/ water (HPLC grade). Store refrigerated.

4'-OH-Pyr standard, 0.2 $\mu\text{g}/\text{mL}$ Standard solution (in 4/1 methanol/water)
Pipet 2.0 mL of the 10 $\mu\text{g}/\text{mL}$ 4'-OH-Pyr standard solution in 4/1 methanol/water into a 100 mL volumetric flask. Dilute to volume with 4/1 methanol/ water (HPLC grade). Store refrigerated.

IV. EQUIPMENT

Autosampler Vials, Caps, and Crimping Tool.

Acrodisc® LC13 PVDF Syringe Filters - 0.45 μm pore size, Luerlock inlet and 13 mm minispike outlet (or equivalent).

Balance - analytical top loading.

Beakers - 100 and 250 mL.

Büchner funnels - 9 cm diameter.

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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, 500, 1000 mL.

High Pressure Liquid Chromatograph - Hewlett-Packard Model 1050; equipped with a tertiary pump, an autosampler, a recording integrator, and an HP 1046 fluorescence detector (or equivalent system).

Mason® jars - 1 pint with plastic screw cap lids (or equivalent).

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

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

Pipets, Pasteur - 5¾" and 9".

Pipets, Volumetric - 1.0, 2.0, 6.0 mL (a calibrated automatic pipettor may also be used for these measurements)

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

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

Separatory funnels - 250 mL and 500 mL.

Syringes, glass - 10 mL, with Luerlock fitting.

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

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V. ANALYTICAL PROCEDURES

1. ACETONE EXTRACTION

Weigh 30 grams (± 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 and 4'-OH-Pyr (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 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 ($<40^{\circ}\text{C}$), *just to dryness*. Proceed to Step 3.

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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 500 mL round-bottom flask and then transfer the dichloromethane into the separatory funnel). 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 <math><40^{\circ}\text{C}</math>). 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 <math><40^{\circ}\text{C}</math>) - *just to dryness*. Proceed to step 4.

4. SPLIT OF EXTRACT

Pipet 6.0 mL of ethyl acetate into the 100 mL round-bottom flask, stopper the flask, and rotate the flask briefly in an ultrasonic water bath to dislodge/dissolve the residues. Pipet 2.0 mL of this mixture into a 50 mL round-bottom flask (for silica gel column cleanup prior to pyriproxyfen analysis), and then stopper both flasks. [If solids are present, care should be taken to ensure that these are dispersed in the solution prior to splitting the sample.] These extracts may be stored in a refrigerator overnight (or in a freezer for several days) before proceeding with the cleanup on the silica gel columns (see Step 5 and Step 6).

5. SILICA GEL COLUMN CLEANUP FOR PYRIPROXYFEN

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,

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and cap the silica gel with approximately 2 grams of sodium sulfate. Drain the solvent to the top of the sodium sulfate layer.

Rotary evaporate the solvent in the 50 mL round-bottom flask [from Step 4] to just remove the ethyl acetate, and transfer the sample extract to the column using three 5 mL portions of 80/20 hexane/ethyl acetate (v/v). Before the first transfer, the round-bottom flask may be sonicated for 15-20 seconds if necessary to dissolve any residue adhering to the walls of the flask. After transferring each 5 mL portion to the column, drain 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 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 eluate *just to dryness* using a rotary-evaporator and water bath set to <40°C. Add 1.0 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 and store refrigerated (nominally at 5°C) or in a freezer (nominally at -10°C) until GC analysis (described in Step 7).

6. SILICA GEL COLUMN CLEANUP FOR 4'-OH-PYRIPROXYFEN

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 60/40 hexane/ethyl acetate 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 60/40 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 60/40 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.

Rotary evaporate the solvent in the 100 mL round-bottom flask [from Step 4] to just remove the ethyl acetate, and transfer the sample extract to the column using three 5 mL portions of 60/40 hexane/ethyl acetate (v/v). Before the first transfer, the round-bottom flask may be sonicated for 15-20 seconds if necessary to dissolve any residue adhering to the walls of the flask. After transferring each 5 mL portion to the column, drain the solvent to the top of the sodium sulfate layer. Rinse the round-bottom flask with a 20 mL portion of 60/40 hexane/ethyl acetate, transfer this rinse to the column (total of 35 mL 60/40 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 4'-OH-Pyr with 50 mL of 60/40 hexane/ethyl acetate (v/v). [Note: the volume used is dependent on the properties of the

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silica gel.] Evaporate the eluate *just to dryness* using a rotary-evaporator and water bath set to $<40^{\circ}\text{C}$. Add 1.0 mL of methanol/water (4/1, v/v) to the flask containing the residue, and sonicate the sample for at least 20 seconds to completely dissolve the residue. If the final extract does not appear turbid, directly transfer the extract to an autosampler vial or screw cap vial using a Pasteur pipet. For turbid samples, transfer the extract with a Pasteur pipet into a syringe body fitted with an Acrodisc® LC13 PVDF syringe filter, and then through the filter into an autosampler vial or screw cap vial. Store refrigerated (nominally at 5°C) or in a freezer (nominally at -10°C) until HPLC analysis (described in Step 8).

7. MEASUREMENT BY GAS CHROMATOGRAPHY

Analyze the sample extracts for pyriproxyfen, along with calibrating standard solutions, using the following 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 Size:	1.0 μl
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 labelled 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 $\mu\text{g}/\text{mL}$ 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,

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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).

8. MEASUREMENT BY HIGH PRESSURE LIQUID CHROMATOGRAPH (HPLC)

Analyze the sample extracts for 4'-OH-Pyr, along with calibrating standard solutions, using the following operating conditions:

Column: Phenomenex Prodigy® ODS(3), 250 mm x 4.6 mm I.D., 5 µm particle size (or equivalent).

Eluants: Acidified Water, 0.05% Phosphoric Acid

Methanol/Tetrahydrofuran, 2/3 (v/v)

Column Gradient:

Time	MeOH/THF	Acidified Water
0 - 8 min	45%	55%
8 - 18 min	+3%/min	-3%/min
18 - 30 min	75%	25%

Post time: 5 minutes

Column temperature: 35°C

Injection volume: 50 µL

Column flow: 1.0 mL/min

Fluorescence detector: Excitation wavelength, 235 nm
Emission wavelength, 327 nm

Retention time: 19 minutes (Figure 5)

The HPLC 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 labelled with the HPLC parameters used. See Note 7 for alternative HPLC parameters.

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Verification of instrument linearity is described in Note 5. Calibration standards (1.0 $\mu\text{g}/\text{mL}$ of 4'-OH-Pyr in 4/1 Methanol/water) 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 ensure that the reproducibility requirement is met (see Note 6).

9. CALCULATIONS

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

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

where:

- A = Sample peak height (or area)
- B = Continuing calibration standard concentration (1.0 $\mu\text{g}/\text{mL}$)
- C = Final extract volume (1.0 mL)
- D = Dilution factor, used if the sample extract is diluted prior to analysis
- E = Total Volume of Extract before Split (6.0 mL)
- F = Volume of Extract after Split (2.0 mL)
- G = Average calibration standard peak height (or area)
- H = Initial sample weight (30 grams)

The amount of 4'-OH-Pyr in each sample is calculated using the following formula:

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

where:

- A = Sample peak height (or area)
- B = Continuing calibration standard concentration (1.0 $\mu\text{g}/\text{mL}$)
- C = Final extract volume (1.0 mL)
- D = Dilution factor, used if the sample extract is diluted prior to analysis
- E = Total Volume of Extract before Split (6.0 mL)
- F = Volume of Extract after Split (4.0 mL)
- G = Average calibration standard peak area (or height)
- H = Initial sample weight (30 grams)

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VI. LIMITS OF DETECTION AND QUANTITATION

The limit of detection (LOD) of pyriproxyfen and 4'-OH-Pyr 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 included (Attachments 1 through 4).

VII. DISCUSSION

Typical chromatograms from orange samples (untreated control and fortified control samples) are presented in Figures 2, 3, 4, 6, 7, and 8. It should be noted that, *for oranges*, background/interferant levels in the untreated control samples are typically near the stated detection limit (0.01 ppm). Typical chromatograms from apple samples (untreated control and fortified control samples) are also presented in Figures 9, 10, 11, 12, 13, and 14.

A trained analyst can complete the analysis of a set of eight samples for pyriproxyfen and 4'-OH-Pyr in approximately 12 hours. The results are available with 24 hours of initiating the analysis.

VIII. NOTES

1. Each batch of silica gel must be checked for recovery of pyriproxyfen and 4'-OH-Pyr. Pipet 2.0 mL of the 1.0 $\mu\text{g}/\text{mL}$ pyriproxyfen or 4'-OH-Pyr 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 either under SILICA GEL COLUMN CLEANUP FOR PYRIPROXYFEN (Step 5) or under SILICA GEL COLUMN CLEANUP FOR 4'-OH-PYRIPROXYFEN (Step 6). Evaporate the eluate *just to dryness* using a rotary-evaporator and water bath set to $<40^\circ\text{C}$. Pipet 2.0 mL of the appropriate solvent into the flask containing the residue, stopper the flask, and swirl to completely dissolve the residue. Analyze both this eluant and the 1.0 $\mu\text{g}/\text{mL}$ calibrating standard as described either under MEASUREMENT BY GAS CHROMATOGRAPHY (Step 7) or under MEASUREMENT BY HIGH PRESSURE LIQUID CHROMATOGRAPH (HPLC) (Step 8). If the height of the pyriproxyfen peak after chromatography is less than 90% of the calibrating standard, then the elution profile of 4'-OH-Pyr may need to be determined. Similarly, if the area of the 4'-OH-Pyr peak after chromatography is less than 90% of the calibrating standard, then the elution profile of 4'-OH-Pyr 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 or 4'-OH-Pyr are all recommended prior to determining a new profile for the silica gel column.

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- 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). Forty five grams (45 g) of this preserved sample is equivalent to 30 g of fruit.
- 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 0.60 mL of the 1.0 $\mu\text{g}/\text{mL}$ fortifying solution or 0.30 mL of the 10 $\mu\text{g}/\text{mL}$ fortifying solution, respectively, to a 30 gram sample. Method recoveries must be 70% to 120% to be acceptable, unless approved by the chemist responsible for the analysis.

- 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 Size:	1.0 μL
Retention Time:	3.8 minutes

- 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.

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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).
7. Alternate procedures for HPLC analysis include use of a UV detector (275 nm), and use of a Phenomenex Spherisorb or Hypersil C18 column (3 micron particle size, 150 mm x 4.6 mm).

SIGNATURESWritten by: C.A. Green
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G. H. FujieDate: 11/21/96Reviewed by: E. Tamichi
E. TamichiDate: 11/21/96

Valent U.S.A. Corporation

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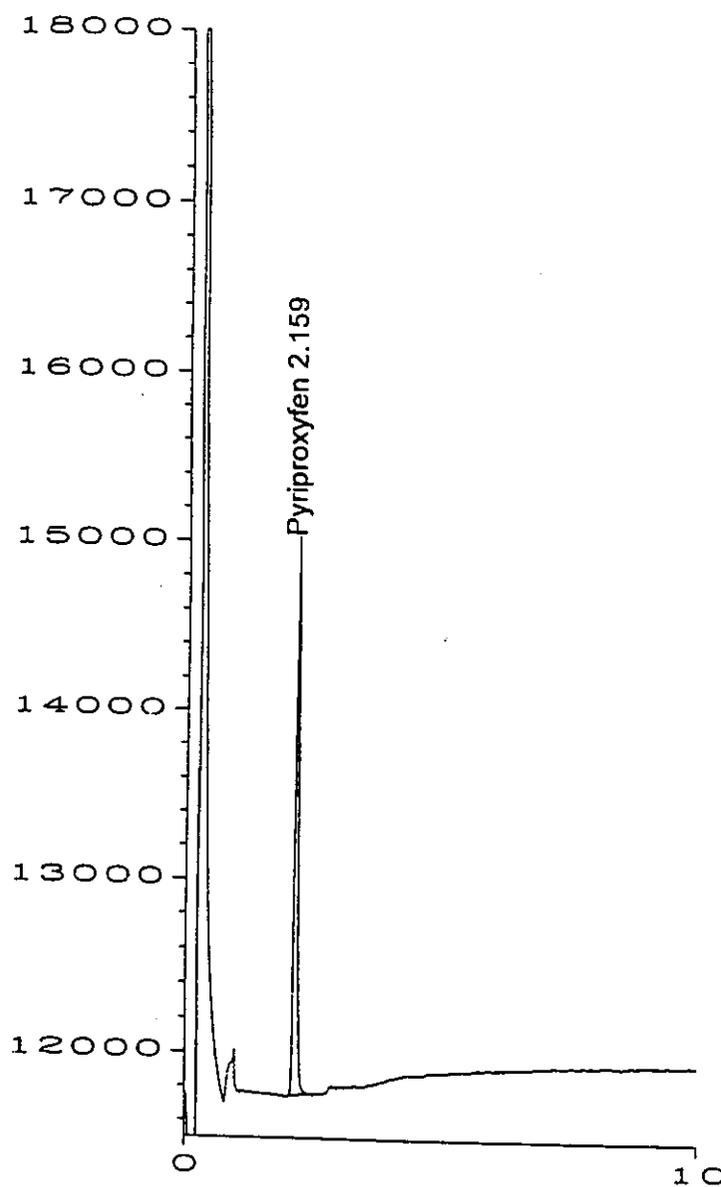


Figure 1. Pyriproxyfen Calibration Standard
(1.0 $\mu\text{g}/\text{mL}$ in toluene, 1.0 μl injected)

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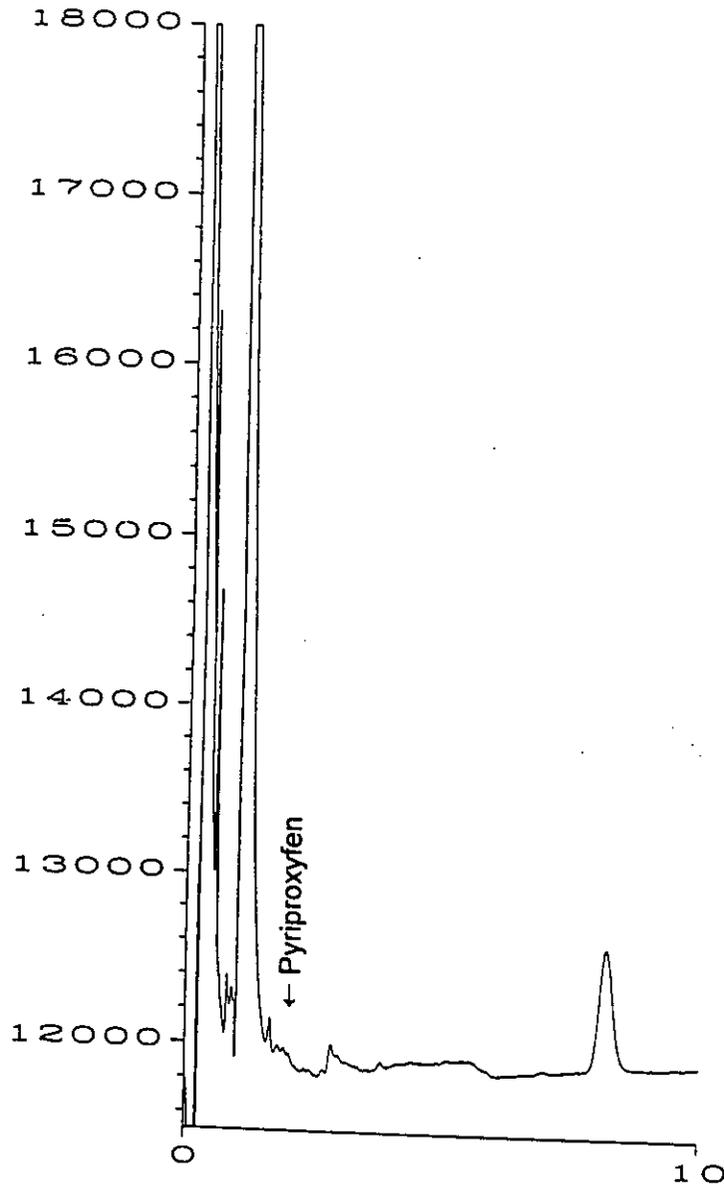


Figure 2. Control Orange Sample
(1.0 μ l injected)

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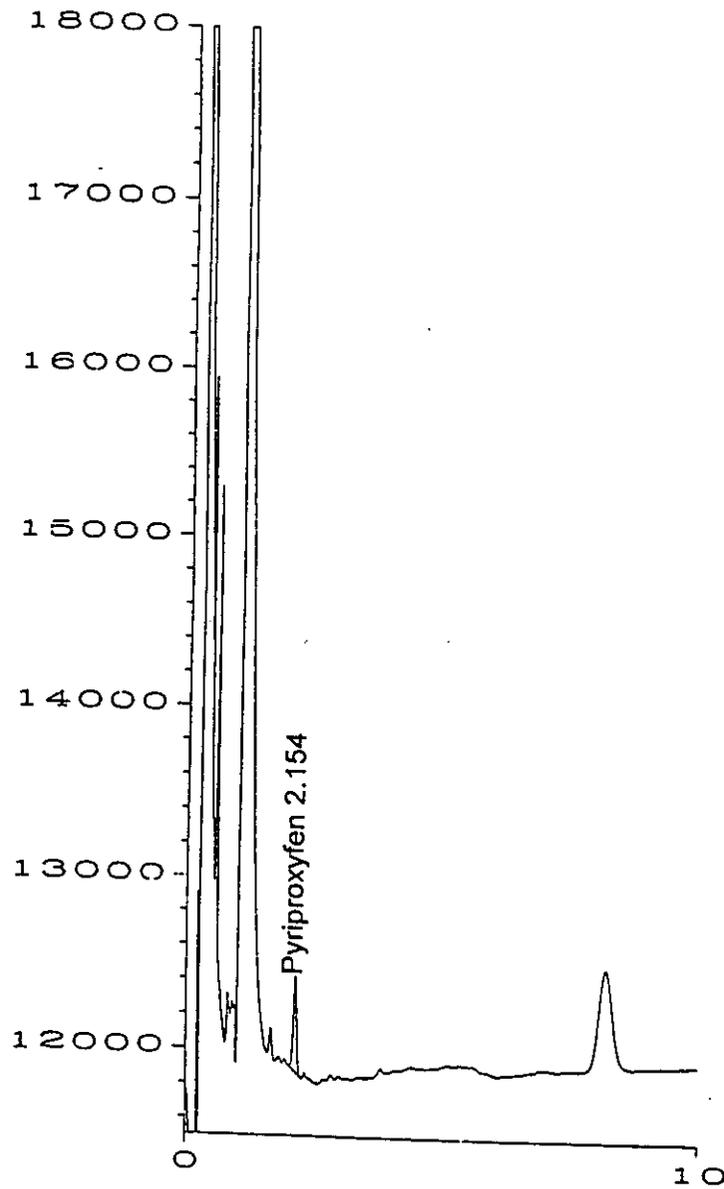


Figure 3. Fortified Control Orange Sample
(0.02 ppm Pyriproxyfen, 1.0 μ l injected)

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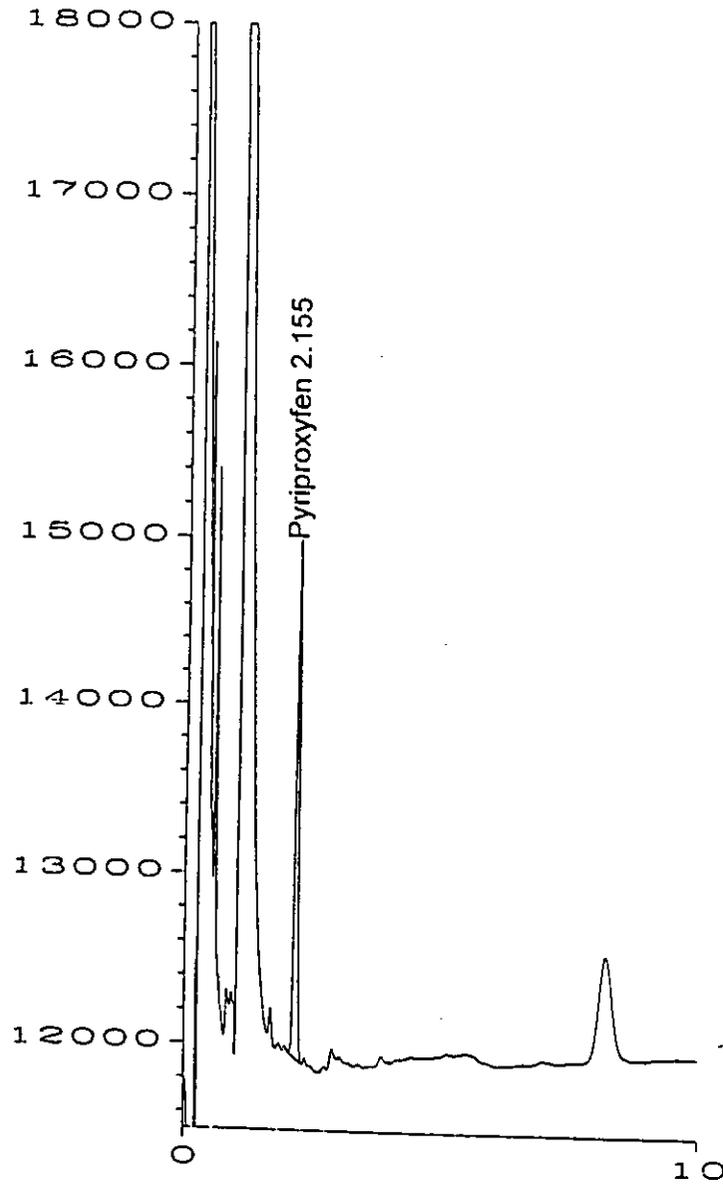


Figure 4. Fortified Control Orange Sample
(0.10 ppm Pyriproxyfen, 1.0 μ l injected)

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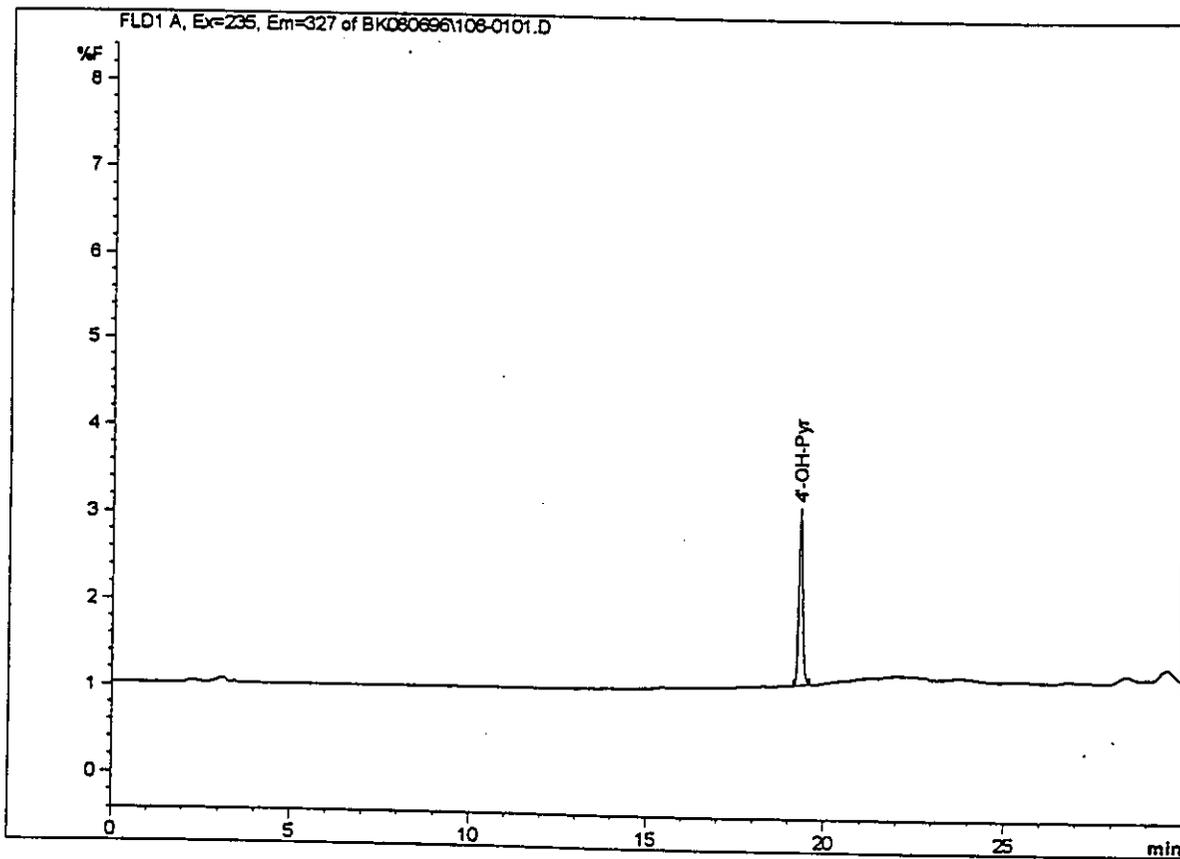


Figure 5. 4'-OH-Pyr Calibration Standard
(1.0 $\mu\text{g/mL}$ in 4/1 methanol/water, 50 μl injected)

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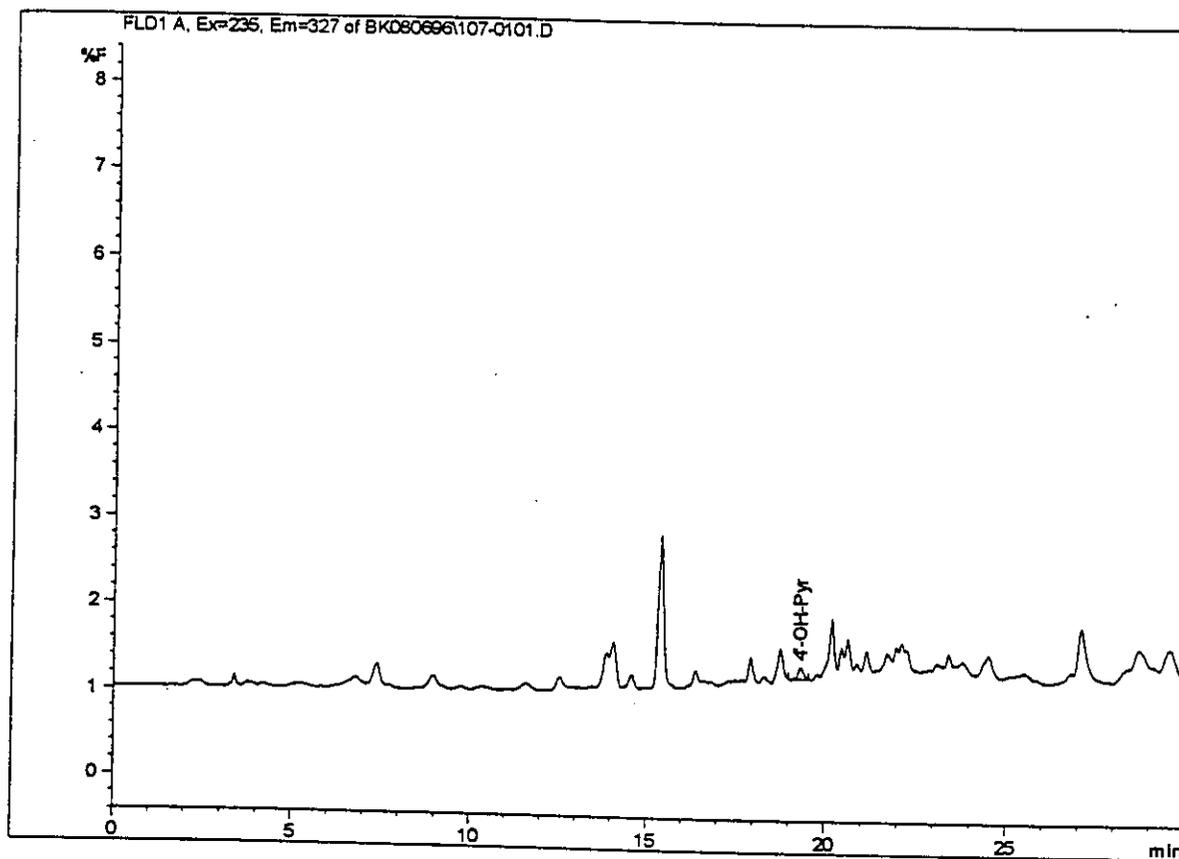


Figure 6. Control Orange Sample
(50 μ l injected)

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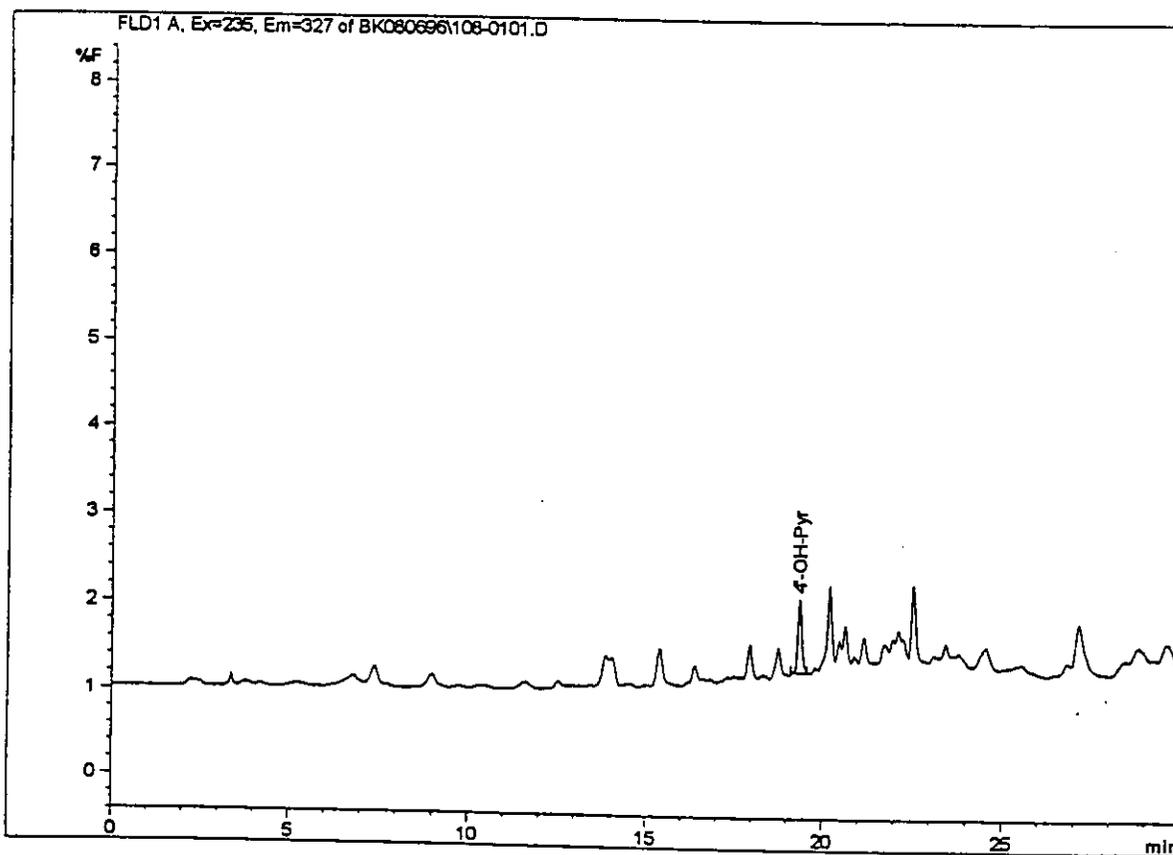


Figure 7. Fortified Control Orange Sample
(0.02 ppm 4'-OH-Pyr, 50 μ l injected)

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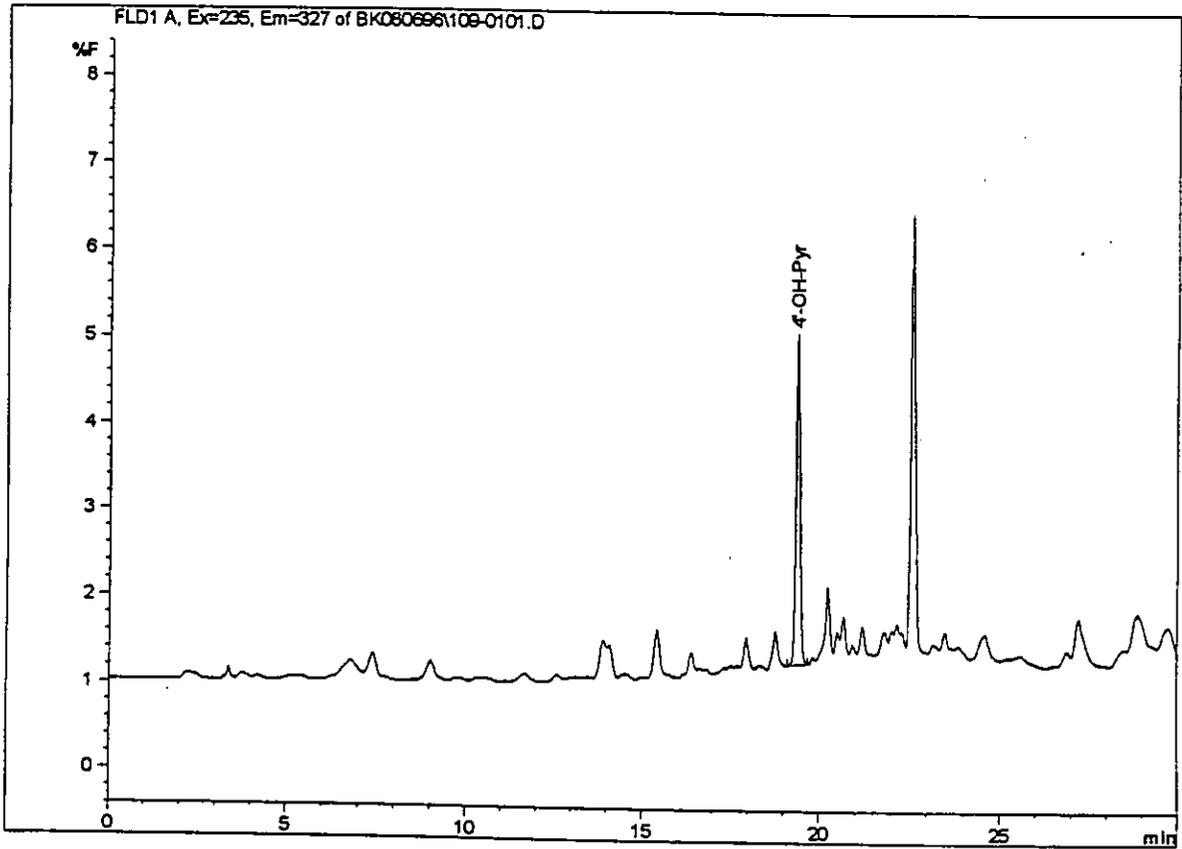


Figure 8. Fortified Control Orange Sample
(0.10 ppm 4'-OH-Pyr, 50 μ l injected)

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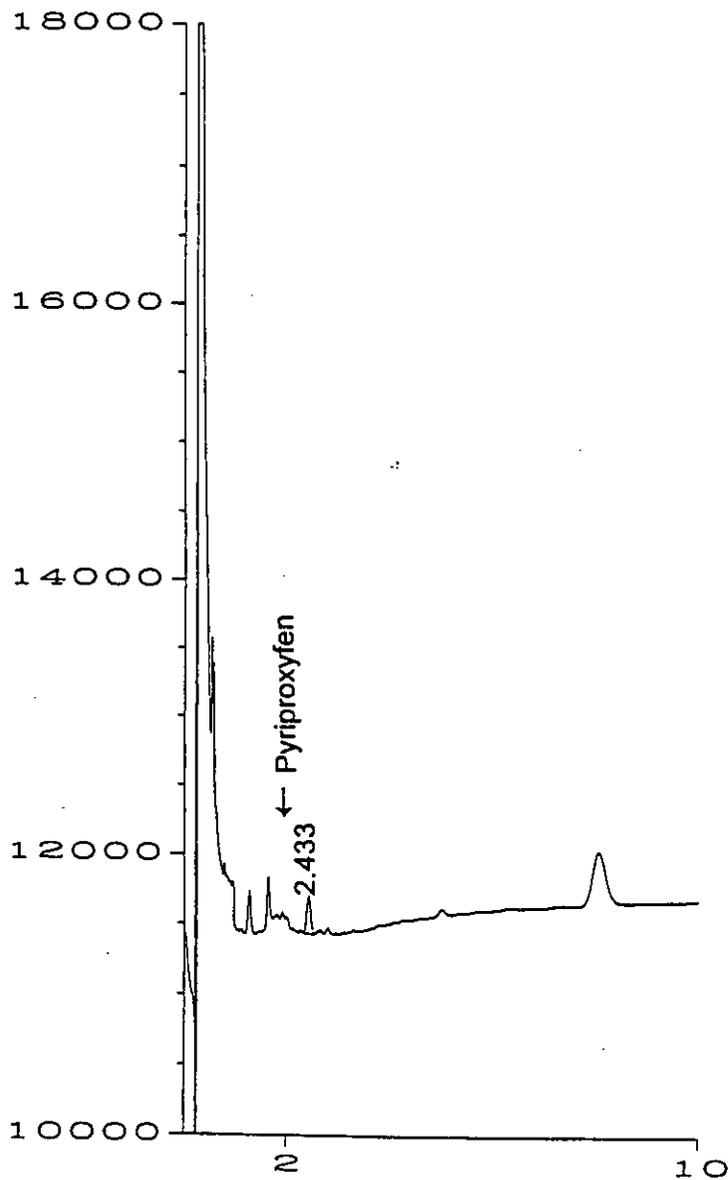


Figure 9. Control Apple Sample
(1.0 μ l injected)

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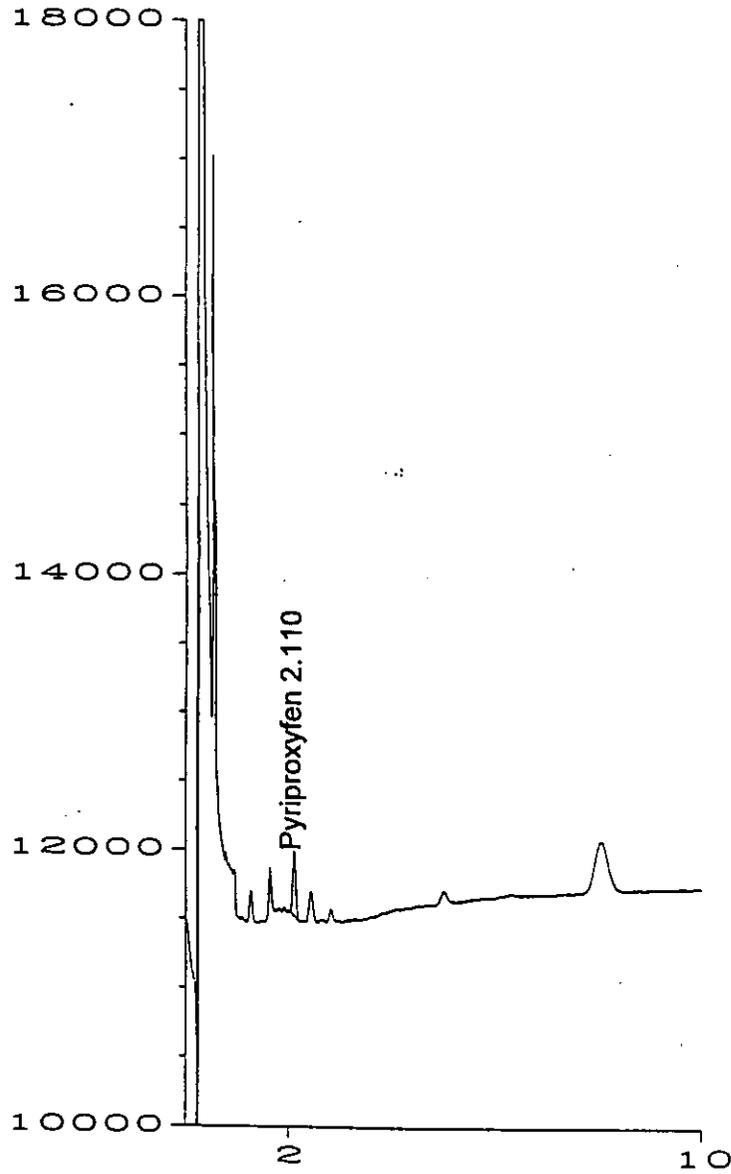


Figure 10. Fortified Control Apple Sample
(0.02 ppm Pyriproxyfen, 1.0 μ l injected)

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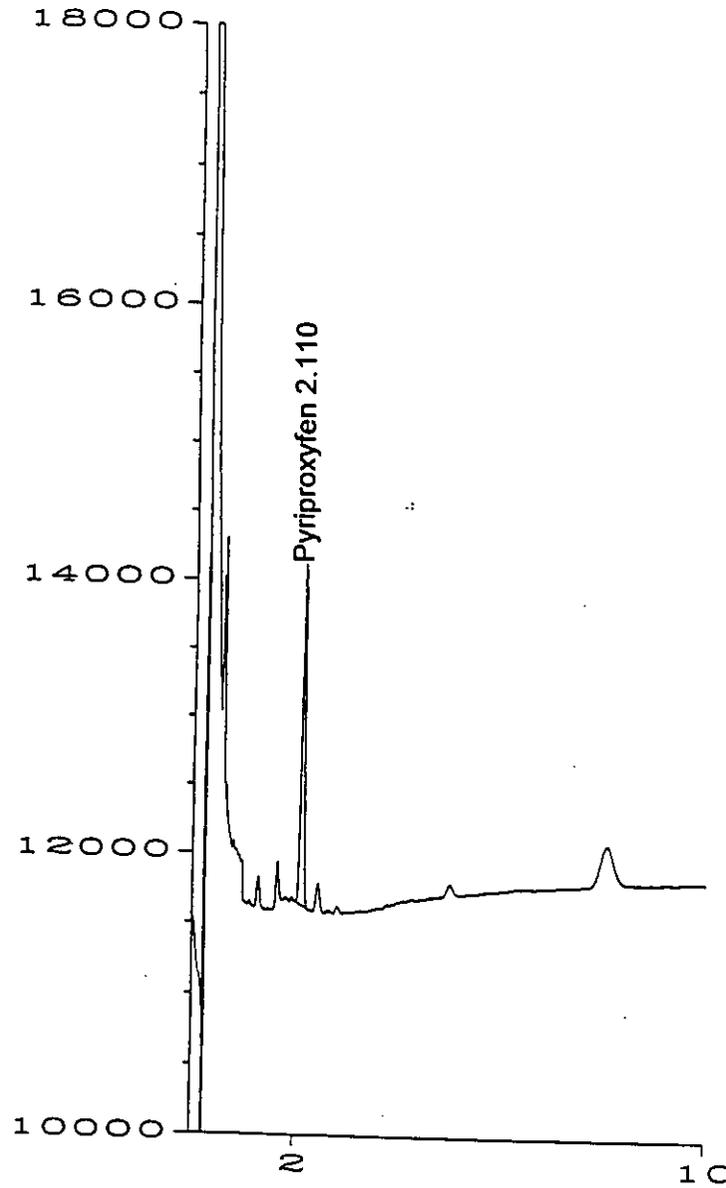


Figure 11. Fortified Control Apple Sample
(0.10 ppm Pyriproxyfen, 1.0 μ l injected)

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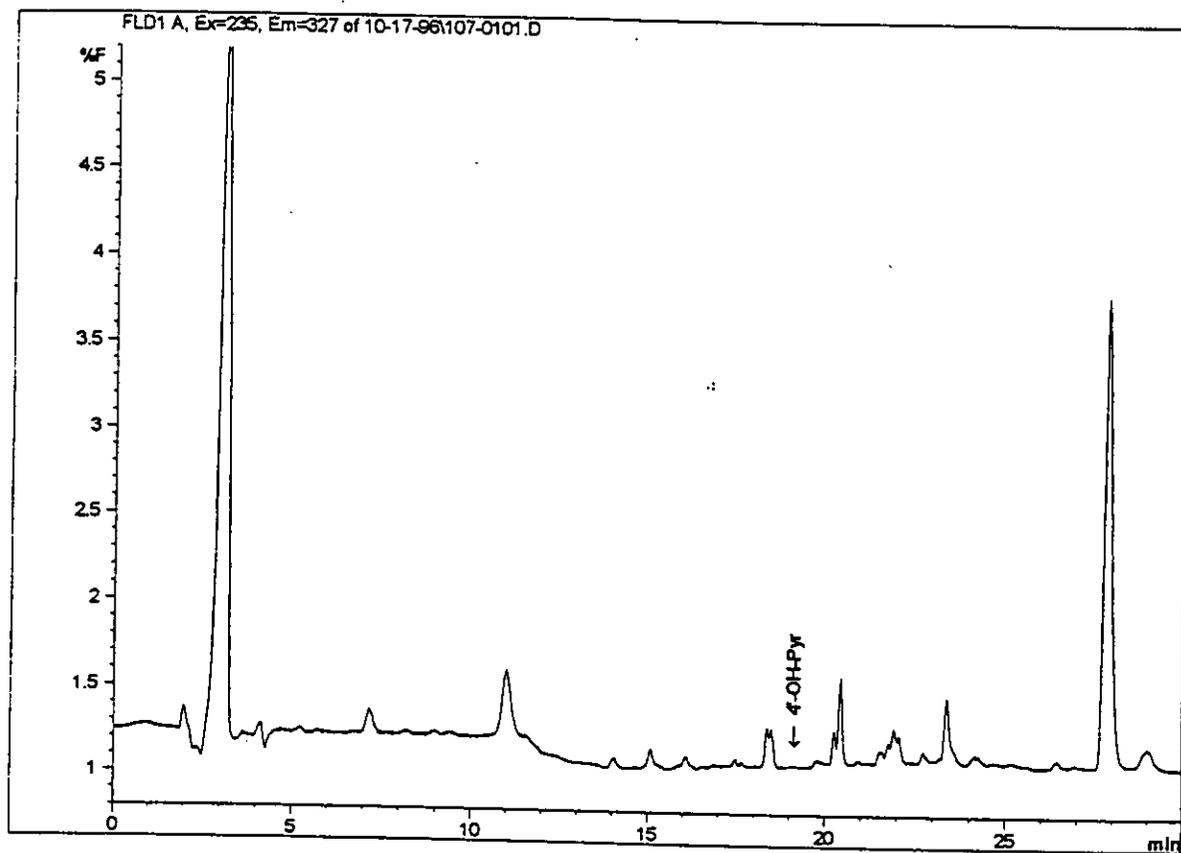


Figure 12. Control Apple Sample
(50 μ l injected)

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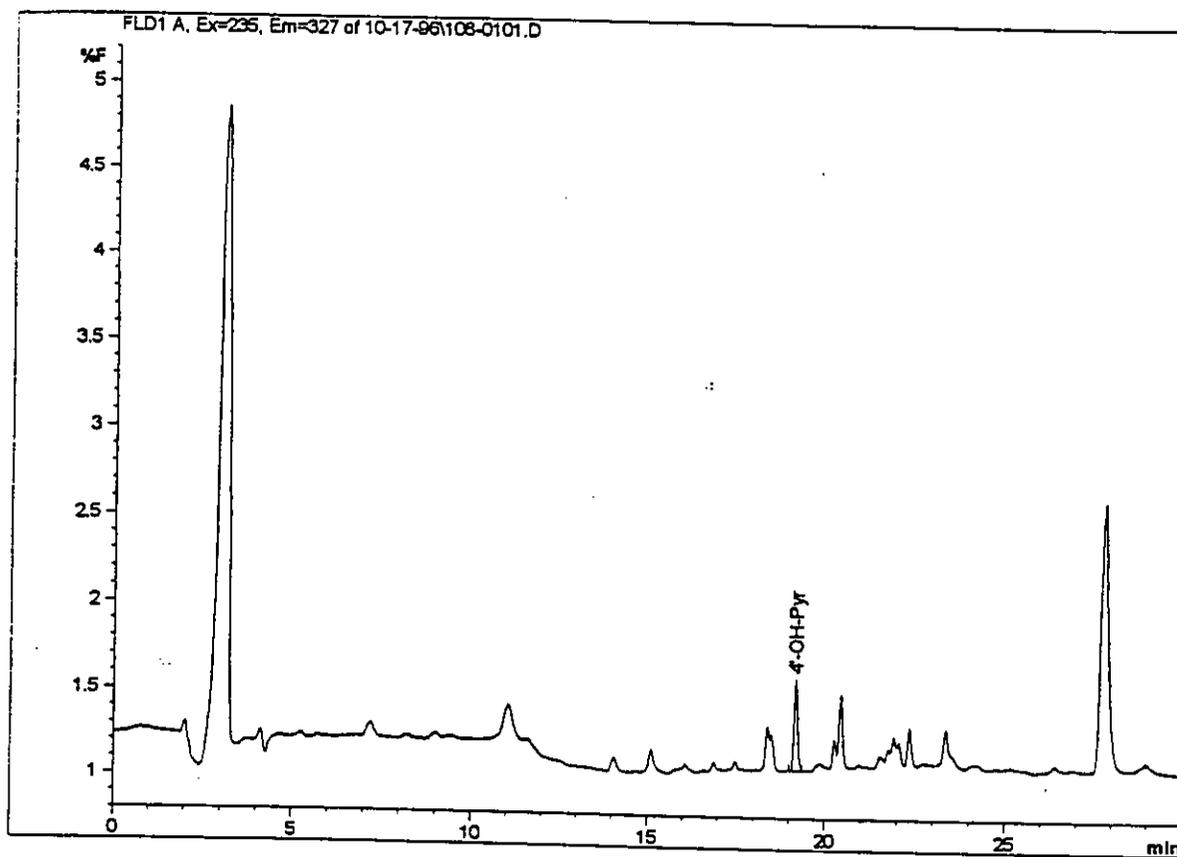


Figure 13. Fortified Control Apple Sample
(0.02 ppm 4'-OH-Pyr, 50 μ l injected)

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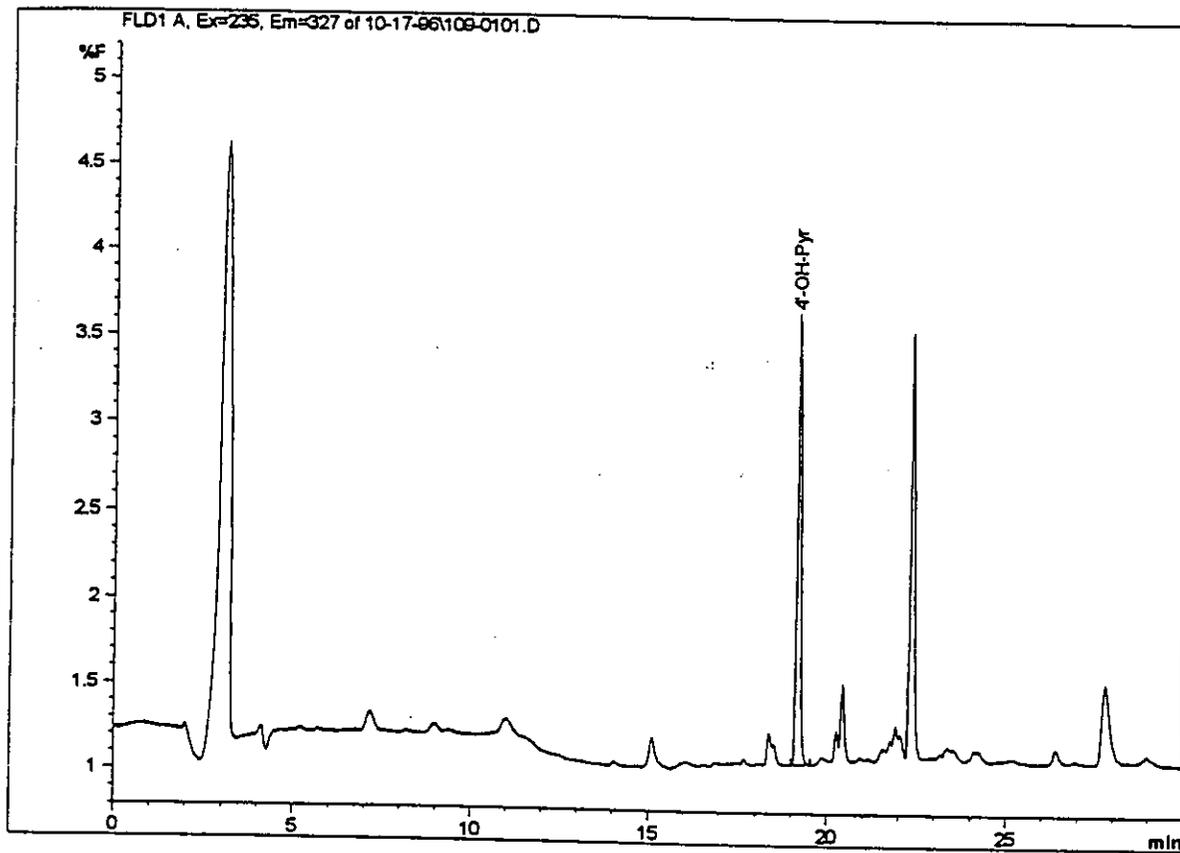


Figure 14. Fortified Control Apple Sample
(0.10 ppm 4'-OH-Pyr, 50 μ l injected)

VALENT U.S.A. CORPORATION
VALENT TECHNICAL CENTER
DUBLIN, CA

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 Kuehly
Analyst

8/28/96
Date

Charles D. ...
Approved by

8/28/96
Date

[Signature]
Reviewed by

9/6/96
Date

VALENT U.S.A. CORPORATION
VALENT TECHNICAL CENTER
DUBLIN, CA

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

DETERMINATION OF 4'-OH-PYR RESIDUE IN WHOLE ORANGES

CHEMICAL: 4'-OH-PYR

Sample Matrix	Reference Page	Extraction Date	Fortification Level (ppm)	Result (ppm)	Percent Recovery	
Oranges	VDL-351-23	8/1/96	0.10	0.106	96.7	
Oranges	VDL-351-23	8/1/96	0.10	0.106	97.0	
Oranges	VDL-351-23	8/1/96	0.10	0.107	97.9	
Oranges	VDL-351-23	8/1/96	0.10	0.106	96.7	Mean = 96%
Oranges	VDL-351-23	8/1/96	0.10	0.107	98.2	CV = 3.9%
Oranges	VDL-351-23	8/6/96	0.10	0.0948	88.3	n = 6
Oranges	VDL-351-23	8/1/96	0.02	0.0277	93.8	Mean = 94%
Oranges	VDL-351-23	8/1/96	0.02	0.0299	105	CV = 9.6%
Oranges	VDL-351-23	8/1/96	0.02	0.0278	94.6	n = 4
Oranges	VDL-351-23	8/6/96	0.02	0.0230	82.9	

ppm = mg/kg

Comments:

1. Due to the loss of a fortification sample (F8-extracted 8/1/96-see VDL-351-23), additional samples were extracted 8/6/96.
2. All percent recoveries for samples extracted on 8/1/96 were adjusted by subtracting the amount of 4'-OH-Pyr found in two control samples (0.0089ppm - average).
3. Percent recoveries for samples extracted 8/6/96 were adjusted by subtracting 0.0065ppm found in the control sample extracted 8/6/96.

Bill Kowal
Analyst

8/28/96
Date

Charles Owen
Approved by

8/28/96
Date

H. M. Mize
Reviewed by

9/6/96
Date

VALENT U.S.A. CORPORATION
VALENT TECHNICAL CENTER
DUBLIN, CA

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% CV = 4.4% n = 3
Apples	V-11590-001	8/13/96	0.020	0.019	95	
Apples	V-11590-001	8/13/96	0.020	0.021	103	

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% CV = 3.1% n = 3
Apples	V-11590-001	8/13/96	0.020	0.017	83	
Apples	V-11590-001	8/13/96	0.020	0.017	87	

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).

Juliana Kowalsky
Analyst

[Signature]
Approved by

[Signature]
Reviewed by

8/15/96
Date

8/15/96
Date

8/16/96
Date

**EPA ADDENDUM For Residue Analytical Methods
PP#s 7F04882 and 8F05022**

1) ACLB made slight modifications to the GC/NPD instrument parameters. The parameters were the same for all six commodities.

The parameters for the GC/NPD were:

Gas Chromatograph: HP 6890 GC with NPD.
Column: DB-17, 15m x 0.53mm i.d., 1 um film thickness
Carrier gas: Helium, 10.0/min.

Oven Temperature: Initial 80°C, Hold for 1.0 min.
40°C/min to 205°C, Hold for 2.5 min.
10°C/min. to 240°C, Hold for 6.0 min.

Injector temperature: 250°C
Injection volume: 4 uL

Detector temperature: 320°C
Hydrogen Flow: 3.0 mL/min
Air Flow: 60 mL/min
Makeup: 20 mL/min Helium

2) The flow rate for the silica gel column chromatography procedure was not specified. ACB used a flow rate of 10 mL/min for all six commodities.

3a) Valent has not submitted an enforcement method for Orange Oil. Per conversation with Charles Green (Valent) on 4/20/99, it was suggested to perform a Hexane/Acetonitrile partitioning on the orange oil aliquot (5 gram sample). The partitioning step involved dissolving the orange oil in 70 mL acetonitrile (hexane saturated) and partitioning it with 100 mL hexane (acetonitrile saturated). The acetonitrile layer was kept and the hexane layer was partitioned with two additional 70 mL portions of acetonitrile (hexane saturated). The acetonitrile layers were combined and evaporated to dryness. The sample was taken through the methylene chloride partitioning step, as stated in Method RM-33P-1-3 for citrus. The remaining procedure was to be carried out as stated in the citrus method.

ACB tried the approach with control and spiking at the 0.02 ppm level and was unsuccessful. The analyte was not recovered and appeared oily. ACB modified the procedure as follows:

3b) ACB's Modification For Orange Oil: A 5 gram portion of orange oil was partitioned with acetonitrile/hexane as stated in 4a. After evaporating the acetonitrile layer, ACB skipped the dichloromethane partitioning step and cleaned up the sample using the silica gel column chromatography procedure, as stated in Method RM-33H

EPA ADDENDUM For Residue Analytical Methods
PP#s 7F04882 and 8F05022
(Continued)

(Almond Hulls) and Method RM-33N-2 (Nutmeat). Prior to GC analysis, the extract was taken to dryness and dissolved in 2 mL hexane (acetonitrile saturated) and partitioned three times with 2 mL acetonitrile (hexane saturated). The acetonitrile layers were combined, evaporated to dryness, and dissolved to the appropriate volume with toluene for GC analysis. The acetonitrile/hexane partitioning step, prior to GC analysis, was the same as described in the nutmeat procedure (RM-33N-2). This additional cleanup greatly improved the quality of the chromatography for oily crops.

4) Prior to GC analysis, for the orange commodities, ACB added the same acetonitrile/hexane partitioning step, as described in 4b. This final cleanup step greatly reduced the deep yellow color and remaining oils, contained in the final extract. This was ACB's only modification to Method RM-33P-1-3 (citrus).