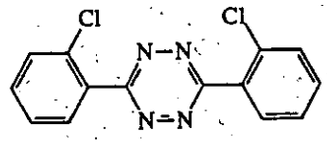
B94-67 / B95-35

Appendix II (Continued)

RAM NO.:	J/02/92	**DATE:**	July 7, 1992
SUBJECT:	ANALYTICAL METHOD FOR RESIDUES OF CLOFENTEZINE IN FRUIT AND VEGETABLES		

1.0 SCOPE

This method is restricted to the determination of residues of clofentezine only.



Clofentezine

2.0 PRINCIPLE

Fruit samples are extracted into acetone and filtered. An aliquot equivalent to 40% of the extract is removed and partitioned with hexane. The organic extract is rotary evaporated to dryness then reconstituted in hexane for clean up through a silica solid phase extraction cartridge. The clofentezine is eluted from the silica, evaporated to dryness, adjusted to the final volume and quantified using high performance liquid chromatography with UV detection at 268 nm.

3.0 APPARATUS

Use as a guide; equivalent substitution may be required.

- Pint size mason jars (purchased locally)
- Sorvall Omni-mixer model 17105 with blades (DuPont)
- Büchner funnels, 9 cm
- Vacuum adapters with rubber seals
- 250 mL TC graduated cylinders with ground glass joints
- Glass fiber filter paper, 9 cm, 934-AH Whatman
- 100 mL TD graduated cylinders or 100 mL glass Class A pipettes
- 500 mL separatory funnel with stopcocks
- 500 mL beakers or boiling flasks
- 125 mL round bottom boiling flasks, 24/40 joint, suitable for rotary evaporation use
- 60° glass funnels
- Glass wool

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5.1.3 Repeat step 5.1.2 once again. Combine filtrates and rinses into graduated cylinder. Adjust volume of extract to 250 mL with DI H_2O. Stopper cylinder and mix well. Note: Acetone/H_2O 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% 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 Na_2SO_4 held in a funnel with a glass wool plug into a 125 mL rb boiling flask.

5.2.2 Carefully pour aqueous layer back into the separatory funnel. Rinse beaker or flask with small amount of hexane. Partition twice more with 20 mL volumes of hexane. Rinse Na_2SO_4 pad with ≈ 10 mL 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°C.

5.3.2 Dissolve residue in ≈ 2 mL of hexane.

5.4 Sep-pak Clean-up

5.4.1 Condition solid phase extraction silica cartridges with 2 column volumes of hexane. Load samples onto cartridge using a disposable pipet. Pull extract through to the top of the column first. Rinse the 125 mL rb boiling flask 2 x 2 mL with hexane and add these rinses to the column.

5.4.2 Elute cartridges with 2 mL of 5% ethyl acetate in hexane. Discard this wash.

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5.4.3 Elute clofentezine with 2 mL of 20% ethyl acetate in hexane. Collect eluate into a 5 mL volumetric flask. For normal phase HPLC, transfer sample to an autosampler vial and run under Normal Phase conditions listed in Appendix I.

5.4.4 For reverse phase HPLC, evaporate the cleaned extract to dryness using a stream of nitrogen or air.

5.4.5 Redissolve residue in exactly 5 mL methanol. For automatic injection, transfer each solution to an autosampler vial.

6.0 STANDARDIZATION

6.1.1 Before analysis of the samples, perform a system suitability test by conditioning the HPLC column with mobile phase for ≈ 30 minutes. Standardize the instrument under the conditions listed in Appendix I by making 40 µL injections in the range of 0.05 - 2.00 µg/mL clofentezine in methanol or 10% ETOAC/Hexane. During the actual analytical run, intersperse standards amongst the samples.

6.1.2 Record the peak heights (or areas) of the injected samples.

6.1.3 Construct a standard curve by plotting standard peak heights (or areas) vs concentration. Calculate the least-squares regression line.

6.2 Analysis of Samples

6.2.1 Inject a 40 µL aliquot of each sample from step 5.4 into HPLC under conditions listed in Appendix I. Make dilutions of samples needed to maintain the peak height (or area) within the standard calibration range.

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6.2.2 Determine the concentration of clofentezine in the treated and fortified samples by comparing the peak heights (or areas) to the standard curve.

6.2.3 Calculate the residues as follows:

$$PPM = \frac{(H - b)/m}{C} \times D$$

where: H = peak height (or areas)
b = Y-intercept of standard curve
m = slope of standard curve
C = crop/solvent ratio =

$$\frac{grams\ of\ crop}{original\ volume} \times \frac{Aliquot}{Final\ volume}$$

D = dilution factor

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 as follows:

$$\%\ recovery = F/S \times 100$$

where: F = ppm of clofentezine found in fortified sample
S = ppm of clofentezine added to fortified samples

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7.0 DISCUSSION

This method is a modification of reference 1. The limit of determination was set at 0.02 ppm. Under the given HPLC conditions, a solution containing 0.05 µg/mL clofentezine gives the smallest peak which can be measured accurately. Provided the crop extract is clean enough, a limit of determination of 0.01 µg/mL is possible by adjusting the final volume to 2 mL. This would give an analytical concentration of 0.1 µg/mL for a 50 g sample.

With some crop matrices, a Florisil clean-up instead of silica, has proven to be more effective in removing interference peaks. A column profile is needed to determine the best clean-up for each new matrix.

In analyzing dry matrices, it is important to soak samples with water prior to analysis. One reason for this is dry commodities contain many exposed hydroxy groups that can bind the analyte to the matrix (5). By soaking and rehydrating dry crops, this binding is reduced. Due to the concentrated nature of dry pomace and raisin waste, reverse phase HPLC is needed to separate clofentezine from the many interferences peaks. An acceptable chromatogram is achieved by using a C18 column and 70:30 CH_3N:DIH_2O at 1.2 mL/min. Normal phase was completely unacceptable because of very poor separation. One important note must be made about using acetonitrile as a solvent in the mobile phase. Clofentezine has proven to be unstable in acetonitrile and mixtures of this solvent with water. A 40% loss of compound in a 24 hour period can be expected but this should not cause a problem if an analytical run can be performed in less than a 24 hour timeframe. Sample extracts and standard should be in methanol when reverse phase HPLC is used.

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8.0 REFERENCES

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

2. Snowden, P. J., "Residue of NC 21314 in Apple Juice, Sause, Cider, and Pomace Following Processing of Treated Apples in the USA, 1981., FBC Limited, (April 1983)
Registration Reference: NC 21314/R37

3. Cabras, Paolo, Tuberoso, Carlo Melis, Marinella, and Martin, G. M., "Multiresidue Method for Pesticide Determination in Wine by High Performance Liquid Chromatography," J. Agric. Food Chem., 1992.

4. Bicchi, Carlo, and D'Amato, Angela, "Simultaneous Determination of Clofentezine, Femoxycarb and Hexythiazox by HPLC on Apples, Pears, and Their Pulps," Pesticide Science, 1990.

5. Zweig, Gunter, and Sherma, Joseph, Analytical Methods for Pesticides and Plant Growth Regulators, Volume XV, 1986.

To calculate ppm residue the µg/ml clofentezine found was divided by the crop:solvent ratio, as is shown in the following formula:

$$ppm = X/C$$

where ppm is the ppm clofentezine residue and
C is the crop:solvent ratio.

To calculate recovery of the fortification compound from fortified control samples, the ppm apparent residue in the corresponding non-fortified control within the same analytical "set" was subtracted from the "raw" ppm in the fortified sample, the result was divided by the ppm fortification level, and this result multiplied by 100 to obtain percent recovery, as shown in the last formula:

$$\% \text{ recovery} = [(P - K)/S]*100$$

where P is the "raw" ppm in the fortified sample,
K is the ppm in the control sample, and
S is the ppm fortification level.

Values for apparent residues in control samples are reported on a "raw" basis, uncorrected for recovery in the fortified samples. Values for residues in the treated samples are reported corrected by matrix for overall mean percent recovery for the entire study for that matrix, but not corrected for apparent residues in the control samples.

An example calculation sheet appears as Appendix I.

Full details of the analytical method are documented in Appendix II. Several modifications were made to the method as written:

(1) For set 6 nominal sample weight was 30 g, and for sets 7 - 19 nominal sample weight was 35 g.

(2) For dry pomace samples 150 mL of acetone was used for extraction.

(3) In set 16 the volume of the combined extracts in the graduated cylinder was adjusted to 300 - 320 mL instead of the 250 mL specified in the method. Appropriate adjustment was made to the aliquot taken for partitioning.

(4) Florisil SPE's were used for cleanup instead of silica.

(5) The clofentezine was eluted from the SPE's with 3 mL of 20% ethyl acetate in hexane instead of 2 mL.

(6) Redissolution of the residue for HPLC analysis was done with the aid of sonication.

(7) HPLC mobile phase used was 60/40 acetonitrile/water for sets 1 - 15 and 19, and 55/45 for sets 16 - 18, instead of 70/30 as listed in the method.

5. RESULTS

5.1 Recovery Efficiencies

At least one control and two fortified control samples were included with each set of samples analyzed. Mean recovery was 87% for 30 fortified whole fruit samples, with a standard deviation (n-1) of 10%. Mean recovery was 72% for 10 fortified dry pomace samples with a standard deviation (n-1) of 15%. Table 3 details the recovery data for this study. The mean recovery for each matrix was applied as a correction factor to the results from analyses of treated samples of the same matrix type (Section 5.3).