Detailed Method of Analysis for Residues of (2-Chloroethyl)Phosphonic Acid (Ethephon*) in a Variety of Sample Types

*Ethephon is the accepted common name for the major active ingredient in formulations available as "ETHREL" plant growth regulator. ETHREL is a registered trademark of Rhone-Poulence Ag Company.

Introduction

The method of analysis to be described has been successfully applied to the determination of ethephon in 52 kinds of sample. Modification of the preliminary sample preparation has been required, depending on whether a sample is, for example, liquid or solid, or has a shell or seeds which must be removed. All such modifications are included below.

A list of sample types which have been successfully analyzed for residues of ethephon is given in Table 1. The sample types have been subdivided into groups as follows:

Group A: Samples having no shell, rind or seeds, or having rind or seeds which need not be removed.

Group B: Samples which may require separation of shell or rind and/or pit or seeds from flesh. Samples listed in Group B may be treated as Group A samples if separation of components is not required.

Group C: Liquid or sauce samples.

Reagents

Methanol, Mallinckrodt Nanograde, or equivalent.

Methanol, reagent grade (use only where specified).

Diethyl ether, Burdick & Jackson distilled in glass or equivalent.

Hydrochloric acid solution, "10%", dilute 10 ml conc. HCl to 100 ml with methanol.

Diazomethane solution - prepare from N-methyl-N-nitroso-p-toluene sulfonamide (Diazald, Aldrich Chemical Co., #D2800-0; or Eastman #7066) according to manufacturer's instructions or in accordance with SOP 81330.
Observe all safety precautions. Store in tightly capped bottle, with Teflon cap liner, in freezer. Do not store over KOH or other desiccants.

Standard ethephon solution - weigh 100 mg ethephon into a 100 ml polyethylene or polypropylene volumetric flask. Dissolve in methanol, dilute to 100 ml and mix well. This solution contains 1.0 mg of ethephon per ml. Make two serial 10 ml to 100 ml dilutions in methanol using polyethylene or polypropylene pipettes and flasks. The first dilution contains 0.10 mg ethephon per ml; and the final solution contains 0.01 milligrams of ethephon per ml and may be used for fortification of crop samples for determination of recoveries. Other dilutions may be used if necessary, but polyethylene or polypropylene lab wear must be used. A polyethylene or polypropylene pipette must be used for dispensing this solution, and polyethylene or polypropylene containers must be used for storage.

Standard dimethyl ethephon solutions - Using a polyethylene or polypropylene pipette, transfer 1.0 ml of the 1.0 mg per ml ethephon to a polyethylene 15 ml centrifuge tube. Add diazomethane solution until a permanent yellow color is observed, cap and let stand for 15 minutes. Remove excess diazomethane by using a gentle stream of pre-purified nitrogen until the solution is colorless. Quantitatively transfer this solution to a 100 ml glass volumetric flask with methanol, dilute to the mark with methanol and mix well. This solution contains 10.0 nanograms (acid equivalent) dimethyl ethephon per microliter. Dilute 2.0 ml, 5.0 ml, and 10.0 ml of this solution to 100 ml to give solutions containing 0.2 ng/μL, 0.5 ng/μL, and 1.0 ng/μL, respectively. These solutions may be used for gas chromatographic calibration. Other dilutions may be used as chromatographic conditions require.

Special Equipment

Polyethylene or polypropylene bottles, approx 4 oz., screw capped with plastic cap liners.

Polyethylene or polypropylene pipettes, 1 ml. graduated and 10 ml.

Polyethylene or polypropylene centrifuge tube, 15 ml, with cap.

Blender, one gallon, Waring Blender or equivalent.

Freeze-dryer, VirTis Model 10-100V Unitrap II, or equivalent.

Freeze-drying vessels, Mason jars, 1 qt., or equivalent.
Soxhlet extractors, Pyrex, 30x110 mm, Corning #3740 or equivalent.

Standard Taper Teflon sleeves for all Soxhlet extractor joints.

Volumetric Flasks, 100 ml polyethylene or polypropylene.

Volumetric Flasks, 100 ml glass.

Centrifuge tubes, glass, screw-capped, 15 ml, graduated, with Teflon cap liners.

Flasks, 250 ml single neck, standard taper, for use with Soxhlet extractors.

Extraction thimbles, single thickness, 25x80 or 25x100 mm, Whatman 2800 250 or equivalent, pre-extracted with reagent grade methanol for at least 4 hours (see Note B). Methanol may be re-used several times.

Centrifuge for use with 15 ml centrifuge tubes.

Gas chromatograph, MicroTek MT-220 or equivalent, equipped with flame photometric detector or alkali thermionic detector.

Procedure

1. Preparation of samples for extraction:

   a. Group A (Table 1):

      1) Grind about 200 gm of hard-frozen sample plus about 200 gm dry ice in a blender until all particles are smaller than 1/8"., or follow SOP 92064.

      2) Allow dry ice to sublime in freezer overnight, then weigh about 100 gm of ground sample into a freeze-drying vessel, cap, and if necessary, store in freezer until hard-frozen.

      3) Freeze dry until constant weight is obtained (24-48 hours). Record both fresh and freeze-dried weights. Desiccation may also be accomplished as follows:

         Weigh frozen, ground samples into stainless steel fine-mesh screens cut to fit into a vacuum desiccator. Charge
desiccator with “Drierite”, evacuate and let stand in freezer for 48-72 hours. Transfer samples to a desiccator charged with P₂O₅, evacuate and let stand in freezer for 24 hours. Record both fresh and freeze-dried weights (see Note H).

4) Weigh an amount of freeze-dried sample equivalent to 20 gm fresh weight into a pre-washed extraction thimble. For samples with high sugar or solids content, such as Group A, Numbers 2, 6, 10, 15, 17, 19, and 20 (Table 1), it may be necessary to used 10 gm or even 5 gm aliquots. Fortification of samples for determination of recoveries may be done at this point, since studies with Cl⁴ labeled ethephon and non-radioactive ethephon have shown that no ethephon is lost during freeze-drying (see Note H; see Note I for modification suitable for enforcement purposes).

5) Cover sample with a wad of glass wool.

6) Proceed to Step 2.

b. Group B, Table 1:

1) Separate shell or rind, flesh and seeds from fresh or thawed sample and discard those portions not of interest.

2) Grind sample.

3) Grind about 200 gm of hard-frozen sample plus about 200 gm dry ice in a blender until all particles are smaller than 1/8”, or follow SOP 92064.

4) Allow dry ice to sublime in freezer overnight, then weigh about 100 gm of ground sample into a freeze-drying vessel, cap, and if necessary, store in freezer until hard-frozen.

5) Freeze dry until constant weight is obtained (24-48 hours). Record both fresh and freeze-dried weights. Desiccation may also be accomplished as follows:

   Weigh frozen, ground samples into stainless steel fine-mesh screens cut to fit into a vacuum desiccator. Charge desiccator with “Drierite”, evacuate and let
stand in freezer for 48-72 hours. Transfer samples to a desiccator charged with P₂O₅, evacuate and let stand in freezer for 24 hours. Record both fresh and freez-dried weights (see Note H).

6) Weigh an amount of freeze-dried sample equivalent to 20 gm fresh weight into a pre-washed extraction thimble. For samples with high sugar or solids content, such as Group B, Numbers 5, 7, 8, and 18 (Table 1), it may be necessary to use 10 gm or even 5 gm aliquots. Fortification of samples for determination of recoveries may be done at this point, since studies with C¹⁴ labeled ethephon and non-radioactive ethephon have shown that no ethphon is lost during freeze-drying (see Note H; see Note I for modification suitable for enforcement purposes).

7) Cover sample with a wad of glass wool.

8) Proceed to Step 2.

c. Group C, Table 1:

1) Weigh 20.0 gm of sample into a 4 oz polyethylene bottle. For samples with high sugar content, such as Group C, Numbers 1, 2, 3, 5, 6, and 7 (Table 1) it may be necessary to use only 10 gm or even 5 gm aliquots. Fortification of samples for determination of recoveries should be done at this point.

2) Place bottle, uncapped, into a 1 quart freeze-drying vessel, cap and store in freezer until hard-frozen.

3) Freeze-dry until constant weight is obtained (24-48 hours). Although we have never done so, it should also be possible to desiccate samples of this type using "Drierite" followed by P₂O₅, as outlined in Step 1.a.3), after making suitable sample-holding modifications (e.g., weigh sample into a polyethylene or polypropylene beaker of 100 ml capacity and stand beakers on a perforated porcelain plate over the desiccant).

4) Place a pre-washed extraction thimble in the neck of an assembled extraction apparatus (not including condenser) and quantitatively transfer contents of plastic bottle into the
thimble, using methanol to rinse.

5) Cover sample with a wad of glass wool.

6) Proceed to Step 2.

2. Extract sample with 100 ml methanol for 4 hours.

3. Add 0.5 ml "10%" methanolic HCl to methanol extract; discard thimble and contents.

4. Quantitatively transfer extract to a glass-stoppered 100 ml graduated cylinder, using methanol to rinse.

5. Adjust to a convenient volume with methanol.

6. Pipet 1/10 of the extract obtained in Step 5 into a 15 ml screw-capped, graduated centrifuge tube.

7. Concentrate to ~1.5 ml, using a gentle stream of dry nitrogen and a 30-35°C water bath.

8. Add 0.5 ml "10%" methanolic HCl.

9. Add 8 ml diethyl ether, mix well, let stand for about 10 minutes, mix once again, then centrifuge for about 10 minutes to settle precipitate (see Note C).

10. Decant supernatant to a clean screw-capped, graduated centrifuge tube.

11. Rinse residue with 2x1 ml diethyl ether and add rinsings to supernatant.

12. Concentrate to 1-1.2 ml using a gentle stream of dry nitrogen and a 30-35°C water bath.

13. In a good fume hood, add diazomethane solution until a permanent yellow color is obtained (see Note D).

14. Cap tightly and let stand for 15 minutes.

15. Concentrate to 1 ml using a gentle stream of dry nitrogen and a 30-35°C water bath.
16. Centrifuge for about 10 minutes to settle any precipitate.

17. Adjust volume to 1.0 ml using a gentle stream of dry nitrogen and a 30-
35°C water bath or by adding methanol. To avoid losses of
dimethylethephon, DO NOT TAKE BELOW 0.8 ML. This extract now
represents 2, 1, or 1/2 gm/ml, depending on whether the original sample
was 20, 10, or 5 gm fresh weight. If samples must be held for more
than a few hours between completion of preparation and analysis, they
should be stored in a freezer.

18. Analyze 1-10 microliter aliquot gas chromatographically (see Note E).

19. The operating parameters for gas chromatography are as follows:

Column: 6' x 1/4" glass, packed with 6% FFAP on
80/100 mesh chromosorb G, AW, DMCS. on-column
injection.

Column temperature: 160°C

Inlet temperature: 230°C

Flame photometric detector:
Detector temperature: 165°C or as instrument requires
Gas flows: As required by instrument.

Alkali thermionic detector:
Detector temperature: 275°C or as required by instrument.
Gas flows: As required by instrument.

Retention time of ethephon (as dimethyl ester) is about 6 minutes.

20. Analyze a standard solution with each set of samples (see Note F).
Determine peak height in convenient units, or determine peak area by
multiplying peak height (in mm) by peak width (in mm) at "half height"
(see Note G). Instrument recorded height or area may also be used if
reliable. Since detector response throughout the range of ethephon
content found in all samples analyzed to date is linear or may be made
linear by appropriate dilution of sample extract, the amount of
ethephon in a given sample may be determined by simple proportion. Run
a sufficient number of standard curves to verify linearity.
Notes

A. It has been demonstrated that used glassware (presumably because it has been etched by repeated cleaning in an alkaline detergent) adsors ethephon very strongly in the absence of crop material. It has also been demonstrated that new glassware, which has not been so etched, and polyethylene and polypropylene, do not exhibit this adsorption. Therefore, it is necessary that either new glassware or polyethylene/polypropylene (the latter is recommended) be used to prepare standard solutions. It should be noted that surface adsorption is not a problem once the ethephon has been methylated.

B. When using the flame photometric detector, we have occasionally seen an interference which contains no phosphorus, but which does contain sulfur. It is suspected that the extraction thimbles are the source of this interference. It may be identified as a sulfur compound and eliminated as follows:

1) If a sulfur interference is suspected in a sample, inject 3 microliters of that sample and note the "ethephon" response. Then inject 6 microliters of the same sample.

a. If the entire "ethephon" peak is due to a sulfur compound, its area will be four times as large for the second injection as for the first, since the response of the detector (in the phosphorus mode) to sulfur varies as the square of the amount of sulfur present.

b. If the "ethephon" peak is partly due to a sulfur compound and partly due to dimethylethephon (or some other phosphorus containing compound), its area will be more than two times and less than four times as large for the second injection as for the first.

c. If the "ethephon" peak is due to dimethylethephon (or some other phosphorus containing compound) only, its area will be twice as large for the second injection as for the first.

2) If the sample does, indeed, contain a sulfur interference, add 0.1-0.2 ml 30% H_2O_2, mix well then readjust volume to 1.0 ml. Tests have shown that this quantity of H_2O_2 removes the sulfur interference quantitatively and essentially instantaneously, and
that H₂O₂ does not affect dimethylethephon, even when 0.5 ml 30% H₂O₂ is added to 1 ml of 2 gm/ml extract.

C. When diethyl ether is added, precipitation occurs. If ether addition at this point is omitted, a similar precipitation will occur during methylation, with a serious loss of ethephon. It should also be noted that if acidification prior to ether addition is omitted, a serious loss of ethephon will occur.

D. If the extract has too much color to show the yellow color of diazomethane, add diazomethane solution until no further bubbling occurs, then add an additional 2 ml.

E. As is usual with the flame photometric detector, when solvent reaches the detector, the flame is blown out. Normal practice is to wait for 30-40 seconds and then reignite the flame. However, if this is done, an extremely large, unsymmetrical, early eluting peak may "saturate" the detector to such an extent that the peak of interest cannot be seen. This problem can be eliminated by waiting for 2 or 3 minutes before reigniting the flame.

F. It has been found that the gas chromatographic column must be "conditioned" (at least daily) by injection of several crop samples before the correct value will be obtained for a standard. Apparently there are "active sites" in the column that must be covered by crop extractives to prevent sorption of dimethylethephon. Additionally, when an injection of a standard is made following a series of crop samples, an early eluting peak may be seen. This is clearly an artifact, washed off the column by the solvent; if a methanol injection is made, instead of a standard, following a series of crop samples, the same artifact is seen, its size being proportional to both the volume of methanol injected and the amount of crop material analyzed prior to the methanol injection.

G. During the development of the preceding method of analysis, it was found that the dimethylethephon peak from "grown-in" or fortified samples was sometimes slightly sharper and slightly more symmetrical than standard dimethylethephon peaks. This phenomenon is thought to be due to a transient blockage of "active sites" by crop extractives. As a result of this short-lived increase in column efficiency, the recovery of ethephon from fortified samples may appear to be somewhat greater than 100% when peak height rather than peak area is measured. The use of peak areas seems to eliminate this problem. It should be
noted that, in either case, correction for spike recovery of the amount of ethephon found in treated samples is valid, since the increased column efficiency applies to both treated and fortified samples.

H. An experiment was performed to demonstrate the validity of spiking after freeze-drying (i.e., to show that essentially no ethephon is lost during freeze-drying). The results of this experiment were as follows:

Two aliquots of ground, untreated pineapple flesh were spiked at 0.2 ppm prior to freeze-drying with recoveries of 97.5% and 100.5%. Two aliquots of ground, untreated pineapple shell were spiked at 0.2 ppm prior to freeze-drying with recoveries of 99.6% and 101.7%. Additionally, many analyses have been performed on samples which were chemically desiccated, with fortifications for determination of recoveries made prior to desiccation.

I. For purpose of enforcement, it is desirable to fortify samples, for determination of recoveries, prior to freeze-drying. Ethephon tends to be strongly adsorbed by glassware (as previously mentioned in Note A). Since it is difficult to insure that none of the spiking solution will contact the walls of the freeze-drying vessel during the spiking operation, the appropriate steps of the analytical procedure should be changed, as follows, to avoid loss of compound:

1. Allow dry ice to sublime in freezer overnight, then weigh 20.0 gm of ground sample into a 100 ml polyethylene beaker or a 4 oz polyethylene bottle. Fortification of samples for determination of recoveries should be done at this point.

2. Place beaker (or bottle) into a 1 quart freeze-drying vessel, cap and store in freezer until hard-frozen.

3. Freeze-dry until constant weight is obtained (24-48 hours).

4. Place a pre-washed extraction thimble in the neck of an assembled extraction apparatus (not including condenser) and quantitatively transfer contents of plastic beaker (or bottle) into the thimble, using methanol to rinse. Cover sample with a glass of wool.

5. Proceed with remainder of procedure unchanged.
TABLE 1

The following is a list of sample types which have been successfully analyzed for residues of ethephon by the Analytical Research Laboratory, Amchem Products, Inc., Ambler, Pennsylvania 19002.

<table>
<thead>
<tr>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
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<tbody>
<tr>
<td>1. Apples, fresh</td>
<td>1. Cantaloupes, flesh</td>
<td>1. Apples, cider</td>
</tr>
<tr>
<td>5. Blueberries</td>
<td>5. Cherries, sweet-brined</td>
<td>5. Cranberries, juice</td>
</tr>
<tr>
<td>7. Cranberries, fresh</td>
<td>7. Cherries, sweet-maraschino</td>
<td>7. Pineapples, juice</td>
</tr>
<tr>
<td>8. Cucumbers</td>
<td>8. Filberts</td>
<td>8. Tomatoes, juice</td>
</tr>
<tr>
<td>12. Lettuce</td>
<td>12. Oranges, flesh</td>
<td></td>
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<tr>
<td>15. Pineapples, bran</td>
<td>15. Peas</td>
<td></td>
</tr>
<tr>
<td>17. Raisins</td>
<td>17. Pineapples, shell</td>
<td></td>
</tr>
<tr>
<td>18. Tea leaves</td>
<td>18. Walnuts</td>
<td></td>
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<tr>
<td>19. Tobacco, cured</td>
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<td></td>
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<tr>
<td>20. Tobacco, fresh</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21. Tomatoes, canned</td>
<td></td>
<td></td>
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<tr>
<td>22. Tomatoes, fresh</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23. Wheat</td>
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</tbody>
</table>

Note: Samples in Group B may be treated as Group A samples if separation of components is not necessary.