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AGRICULTURAL CHEMICALS DIVISION

Research and Development Department

TITLE: Residue Analysis Procedure for ©BAYLETON and Metabolites in  
Barley and Wheat

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ABSTRACT: BAYLETON methods of analysis developed previously have  
accounted for the parent compound and the initial metabolite  
KWG 0519. This is adequate for crops where metabolism does  
not proceed further. However, recently there has been a need  
to examine some crop extracts for the presence of further  
alteration products and to address the question of conjugated  
residues. The procedure described here employs a more rigorous  
initial extraction procedure than has been used before. It also  
employs an enzyme hydrolysis to release conjugated residues and  
will measure the metabolites KWG 1342 and KWG 1323.

DATE: January 20, 1982

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NOTEBOOK

REFERENCE: 81R-22, 81R-80, 81R-176, 81R-193

REASON FOR REVISION: To add Appendix IV.

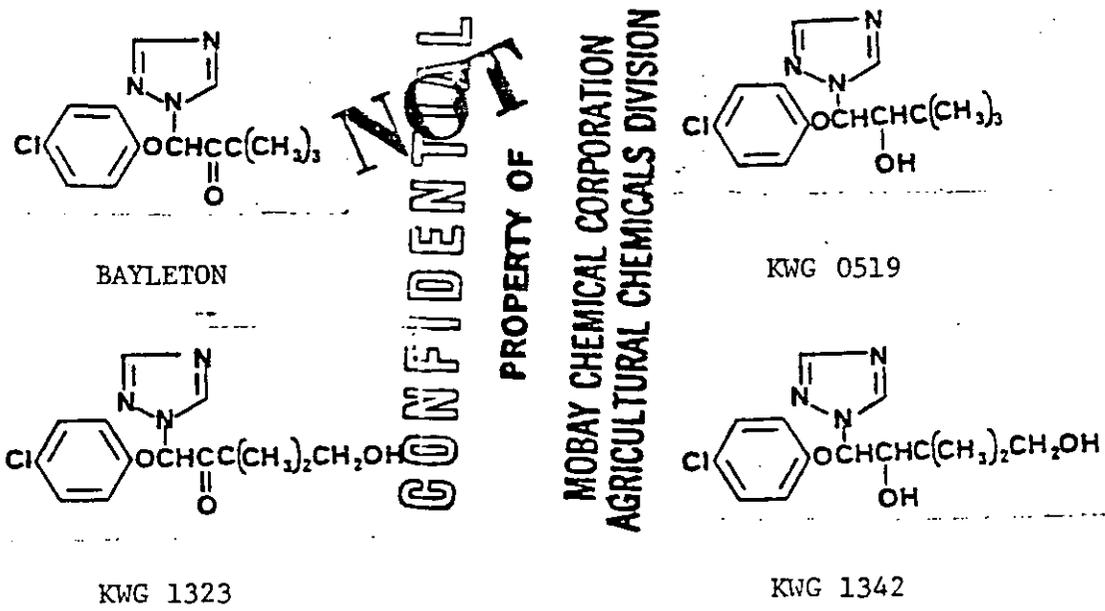
DATE REVISED: July 27, 1983

BAYLETON is a Reg. TM of the Parent Company of Farbenfabriken Bayer GmbH, Leverkusen

Residue Analysis Procedure for BAYLETON and Metabolites  
in Barley and Wheat

INTRODUCTION

BAYLETON, 1-(4-chlorophenoxy)-3,3-dimethyl-1-(1H-1,2,4-triazol-1-yl)-2-butanone, is a fungicide being developed in the United States by Mobay Chemical Corporation for use on a variety of crops. Metabolism studies have shown the compound is rapidly converted to the reduction product, 1-(4-chlorophenoxy)-3,3-dimethyl-1-(1H-1,2,4-triazol-1-yl)-2-butanol, also called KWG 0519<sup>1,2</sup>. Further metabolism can also occur in some crops, with hydroxylation occurring on the t-butyl group of the molecule to form the metabolite designated KWG 1342 and to a lesser degree, KWG 1323<sup>2</sup>. Once hydroxyl groups are formed on these molecules, the potential for conjugation to naturally occurring plant materials is present. The structural formulas for these compounds are shown below:



Methods of analysis developed previously have accounted for the parent compound and the initial metabolite, KWG 0519<sup>3,4</sup>. This is adequate for some crops where metabolism does not proceed further. However, recently there has been a need to examine crop extracts for the presence of further alteration products and to address the question of conjugated residues. The procedure described below employs a more rigorous initial extraction procedure than has been used before. It also employs an enzyme hydrolysis to release conjugated residues and will measure the metabolites KWG 1323 and KWG 1342.

Briefly the procedure involves blending the sample in methanol-water using a high-shear blender, followed by a reflux of the solution and solids to solubilize additional extractables. After filtration to remove extracted solids, the solution is evaporated to remove organic solvents. The remaining water is buffered and enzymatically hydrolyzed to release conjugated residues. Residues are then extracted from the aqueous solution with dichloromethane and cleaned up using gel permeation

chromatography. The extract is further purified by Florisil column chromatography. An initial eluate from the Florisil column is discarded followed by a second fraction of a slightly more polar solvent to remove BAYLETON, KWG 0519 and a portion of KWG 1323. A third fraction with an even more polar solvent removes KWG 1342 and the balance of KWG 1323. After derivatization of KWG 1342 and KWG 1323 with trifluoroacetic anhydride, the concentrated extracts are analyzed by gas chromatography employing nitrogen-specific, alkali flame detection.

An alternative procedure using a LOBAR column cleanup, allows for more quantitative measurement of KWG 1323. A schematic summary of the analysis procedure is shown in Figure 1.

## ANALYTICAL METHOD

### Apparatus Required

Assorted laboratory glassware.  
 Chromatographic tubes, 20 x 400 mm with 300 ml integral reservoir.  
 Gas chromatograph, Hewlett Packard Model 5730A, equipped with nitrogen/phosphorus sensitive, alkali flame detector, or equivalent.  
 Gel permeation chromatograph, Model 1002 Autoprep, A.B.C. Laboratories, Inc.  
 Glass fibre filter paper, Whatman GF/A, 11-cm, or equivalent.  
 Glass wool (pre-washed with dichloromethane).  
 Griffin metal beakers (600-ml size) or equivalent.  
 Nitrogen stream evaporator, N-EVAP, Organomation Associates, Northborough, MA.  
 Reacti-vials (Supelco), 5-ml, or equivalent.  
 Reverse phase column, LiChroprep RP-8, size B, 310 x 25 mm (40-63  $\mu$ m mesh size, EM-Reagents) operated with an RP-SY positive displacement pump and pulse dampener (FMI Corporation) and a slider valve injector with a 4.3 ml loop.  
 Rotary vacuum evaporator, Rotavapor Model VE-50, or equivalent.  
 Tekmar Tissumizer, or equivalent.  
 Water bath, 40°C.

### Reagents Required

Acetone, Burdick and Jackson Laboratories, Inc., AG 156.  
 Acetonitrile, Omni Solve, MCB Reagents, OX 831.  
 Cellulase enzyme, No. C-7502, Practical grade-Type II, Sigma Chemical Co.  
 Chloroform, Burdick and Jackson Laboratories, Inc., AG 899.  
 Dichloromethane, Omni Solv, MCB Reagents, AX 155.  
 Ethyl acetate, Omni Solv, MCB Reagents, EX 214.  
 Ethyl acetate-methanol mixture, 95:5 v/v.  
 Ethyl ether, anhydrous, Baker analyzed reagent No. 3-9244.  
 Florisil (PR grade, 60-100 mesh) 2.5% water deactivated. [Heat in an oven at 130°C for at least 24 hours and allow to cool in a tightly-stoppered bottle. Add 2.5% water (2.5 ml H<sub>2</sub>O + 97.5 gm dried Florisil) and allow to equilibrate at room temperature for 24 hours in a tightly stoppered bottle before use.]  
 Hexane, Burdick and Jackson Laboratories, Inc., AG 599  
 Hexane - ethyl acetate mixture, 60:40 v/v.  
 Hyflo Super Cel (pre-washed with methanol-water 7:3).  
 Petroleum ether, Mallenckrodt, Nanograde, 4977.

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Silica gel (100-200 mesh), 5.0% water deactivated. [Heat in an oven at 130°C for at least 24 hours and allow to cool in a tightly stoppered bottle. Add 5.0% water (5 ml + 95 g dried silica gel) and allow to equilibrate at room temperature in a tightly stoppered bottle for 16 hours before use.]

Sodium sulfate, anhydrous granular, Reagent ACS (pre-washed with dichloromethane).  
Trifluoroacetic anhydride, Eastman.

Water, Barnstead purified, or equivalent.

#### Standard Solutions

Weigh 0.05 g of BAYLETON, KWG 0519, KWG 1342 or KWG 1323 into separate 100-ml volumetric flasks. Dilute to volume with benzene, stopper and mix. Make appropriate dilutions to obtain a composite standard of 1.25 µg/ml each of BAYLETON and KWG 0519 in acetone. In a separate flask, make appropriate dilutions to obtain a standard of 1.25 µg/ml each of KWG 1342 and KWG 1323 in acetone.

#### A. Sample Preparation and Extraction

1. Grind the entire sample in a Hobart food cutter in the presence of dry ice and place the sample in frozen storage overnight to allow the dry ice to sublime.
2. Weigh a 25-g portion of chopped sample into a 600-ml metal (Griffin) beaker. (For straw, use 12.5 g).
3. Add 200 ml methanol-water (7:3) and homogenize the sample using a Tekmar Tissumizer for 3-5 minutes.
4. Pour the contents of the beaker into a 500-ml flat-bottomed flask using a wide-mouth funnel.
5. Rinse the tissumizer and beaker with 50 ml of fresh methanol-water mixture and add the rinse to the 500-ml flask. (For straw, use 75 ml rinse.)
6. To each flask, add a magnetic stirring bar and fit with a water jacketed, Liebig condenser and heat at reflux for 1 1/2 hrs. Let cool for 30 minutes.
7. Add 15 g of Hyflo Super-cel (pre-washed with methanol-water (7:3)) to the 500-ml flask containing the sample. Swirl to mix.
8. Filter with vacuum through Whatman GF/A glass fibre paper in a Büchner funnel (11-cm), into a 1-liter round-bottomed flask.
9. After filtration, remove the filter cake and return it to the original 500-ml flask.
10. Add 100 ml of methanol to the filtered solids. Swirl the mixture for ~ 1 minute, filter the rinse into the same 1-liter flask. (For straw, use 130 ml methanol.)
11. Wash the filter cake in the funnel, with an additional 50 ml fresh methanol.

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12. Evaporate the extract (filtrate) until only an aqueous phase is observed (water condensing on the evaporator glass stem (fogging) is a usual sign of only aqueous remaining) on a rotary vacuum evaporator using a water bath at 40°C. Proceed to "Enzyme Hydrolysis".

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## B. Enzyme Hydrolysis

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NOTE: For high water content samples, 75-80 ml of aqueous will remain after evaporation. For low water content samples, 45-50 ml of aqueous will remain after evaporation.

1. Add an equal volume of 0.2 M sodium acetate buffer (pH 5.5), to each 1-liter flask.

Prepare the buffer solution by dissolving 16.4 g anhydrous sodium acetate in 980 ml of Barnstead purified water. Adjust the pH to 5.5 with the addition of concentrated glacial acetic acid (17.6 M) dropwise (~ 1-2 ml is needed). Dilute to 1 L with water, stopper and store at room temperature.

2. Dissolve 150 mg cellulase in 2 ml of purified water for each sample (six samples = 900 mg/12 ml).
3. Pipet 2 ml of enzyme solution into each flask and swirl to mix. Stopper and seal the flask with parafilm.
4. Incubate the flasks at 37°C in a water bath for 16-18 hrs. Hold the 1 L flasks in position in the water bath using wire or lead donuts.
5. Remove the samples from the incubation water bath and let cool for ~ 15 minutes.
6. Transfer the incubation mixture to a 500-ml separatory funnel.
7. Rinse the 1 L flask with two 125-ml portions of dichloromethane (DCM). Swirl after each addition of DCM and add quantitatively to the 500-ml separatory funnel (for grain samples, use DCM-acetonitrile (ACN)), 2:1, in place of DCM, to minimize emulsions.
8. Shake the separatory funnel for 30 seconds.
9. Allow the phases to separate and drain the lower (organic) layer into the 1 L flask that was previously rinsed in step 7.
10. Repeat extraction of the aqueous phase with an additional 250-ml portion of DCM, and drain the lower phase into the 1 L flask.
11. Evaporate the extract just to dryness on a rotary vacuum evaporator using a water bath at 40°C. Remove final traces of solvent with a gentle stream of nitrogen. Proceed to "Gel Permeation Chromatography Cleanup".

## C. Gel Permeation Chromatography (GPC) Cleanup

1. Dissolve the residue from enzymatic hydrolysis in ~ 3 ml chloroform (CHCl<sub>3</sub>). Transfer the solution quantitatively to a 13-ml centrifuge tube with a Pasteur pipet.

2. Repeat step 1 with two 3-ml portions of  $\text{CHCl}_3$ . The hydrolysis flask should be placed in a sonication bath at least once during the transfer process, to ensure complete solubilization of residues.
3. Adjust the volume in the 13-ml centrifuge tube to 10 ml with  $\text{CHCl}_3$ . Stopper and mix the solution thoroughly.
4. Centrifuge the tube (if necessary) at 1800 rpm for 3 minutes to concentrate particulates at the top or bottom of the 13-ml centrifuge tube.
5. Using a 10-ml glass syringe with a Luer-lok fitting and Swinney filter fitted with a 3-inch 19-gauge needle, draw ~ 7-8 ml of extract into the syringe.
6. Remove the filter and fill the injection loop (loop volume = 5.0 ml) with the sample and proceed to load all remaining samples in the usual manner.

NOTE: Care should be taken not to introduce any particulates into the loop which may plug the GPC frits.

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7. Set the GPC program parameters as follows:

Discard Mode - 24 x 1 min (120 ml)  
 Collect Mode - 12 x 1 min ( 60 ml)  
 Wash Mode - 10 x 1 min ( 50 ml)

8. Run the GPC program using  $\text{CHCl}_3$  as elution solvent. All samples in the "Collect Mode" may be collected in 125-ml flat-bottomed flasks.
9. Evaporate the collected fractions just to dryness on a rotary vacuum evaporator. Remove any traces of solvent with a gentle stream of nitrogen. Proceed to "Florisil Column Cleanup".

**D. Florisil Column Cleanup**

1. Tamp a loose plug of glass wool into the bottom of a 20 x 400 mm chromatographic column.
2. Add about 1 cm of fine mesh glass beads to form a level surface for the bottom of the adsorbent.
3. Fill the chromatographic column with petroleum ether-ethyl ether mixture (940:60).
4. Drain the solvent until most of the air is removed from the beads and glass wool.
5. Slowly sprinkle in 10 g of 2.5% water-deactivated Florisil and allow it to settle.
6. Top the adsorbent with 5 g of anhydrous granular sodium sulfate. Drain the solvent down to the top of the sodium sulfate.
7. Dissolve the sample residue from the GPC column cleanup in 10 ml of the petroleum ether-ethyl ether mixture and transfer to the Florisil Column. Adjust the elution rate to 2-3 drops per second.

8. Rinse the flask with two additional 10-ml portions of solvent mixture and add to the column just as the last of the previous rinse has drained into the sodium sulfate.
  9. Elute the column with an additional 170 ml of solvent mixture. Discard the entire 200 ml of petroleum ether-ethyl ether eluate.
  10. Rinse the flask (from step 8) with 10 ml of hexane-ethyl acetate mixture (60:40) and transfer to the column.
  11. Add two additional 10-ml portions of hexane-ethyl acetate solvent mixture to the flask and add to the column just as the last of the previous rinse has drained into the sodium sulfate.
  12. Elute the column with an additional 120 ml of the hexane-ethyl acetate solvent mixture, keeping the elution rate at approximately 2-3 drops per second.
  13. Collect the entire 150 ml of hexane-ethyl acetate in a 250-ml flat bottomed flask. (This flask contains BAYLETON, KWG 0519 and traces of KWG 1323 residues.)
  14. Add 10 ml of ethyl acetate-methanol mixture (95:5) to the Florisil Column and allow the solvent to percolate into the adsorbent.
  15. Elute the column with an additional 140 ml of the ethyl acetate-methanol solvent mixture, keeping the elution rate again at approximately 2-3 drops per second.
  16. Collect the entire 150 ml ethyl acetate-methanol in a 250-ml flat-bottomed flask. This flask contains KWG 1342 residues and the balance of any KWG 1323.
  17. Evaporate the hexane-ethyl acetate and ethyl acetate-methanol eluates just to dryness on a rotary vacuum evaporator. Remove any traces of solvent with a gentle stream of nitrogen.
  18. Dissolve the residue from the hexane-ethyl acetate fraction in 2.0 ml of acetone. Stopper and hold for gas chromatographic analysis.
  19. Dissolve the residue from the ethyl acetate-methanol fraction in 2 ml of methanol. Proceed to "Derivatization Procedure".
- E. Derivatization Procedure (KWG 1342 and KWG 1323 fraction only)
1. Place 1.25  $\mu$ g KWG 1342 standard in a 5-ml Reacti-Vial (1.25  $\mu$ g of KWG 1323 should also be added if analysis for that compound is required).
  2. For each of the samples, quantitatively transfer the residue from the ethyl acetate-methanol fraction of the Florisil Column, to a 5-ml Reacti-Vial.
  3. Evaporate the solvent from the vials with a gentle stream of nitrogen on an N-EVAP evaporator in a water bath at 40°C.
  4. Add 50  $\mu$ l of trifluoroacetic anhydride (TFA) to each sample and to the standard.
  5. Cap the vials with a Teflon-lined cap.

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- 6. Heat the reaction mixture at 45°C for 1.0 hr.
- 7. After the reaction period, cool, remove excess TFA reagent using a gentle stream of nitrogen.
- 8. Add a small amount of acetone (~ 0.1 ml) and remove all traces of solvent using a gentle stream of nitrogen.
- 9. Dissolve the residue in 2.0 ml acetone, cap and hold for gas chromatographic analysis.

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F. Gas Chromatographic Analysis

Columns : Standard Column, 4 foot x 1/8 inch o.d. glass column packed with 10% DC-200 + 1.5% QF-1 solution-coated on 80-100 mesh Chromosorb W(HP).

Confirmatory Column, 4 foot x 1/8 inch o.d. glass column packed with 20% OV-11 coated on 80-100 mesh Chromosorb W(HP).

Carrier gas : Helium, 60 ml/min, 75 psig

Temperatures: Column oven : 220°C  
 Injection port: 250°C  
 Detector : 300°C

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Chart Speed - 1/2 inch/minute (1/4 inch/minute for the confirmatory column)

- 1. Inject 4 microliters of sample or standard solution. (Use 8 µl for 12.5 g sample)
- 2. Identify the BAYLETON or KWG 0519 by retention time and measure the peak height produced on the recorder strip chart. The retention times for BAYLETON and KWG 0519 are 3.3 and 4.5 minutes, respectively.
- 3. Identify the KWG 1342 (and KWG 1323) TFA derivatives by retention time and measure the peak height produced on the recorder strip chart. At the above conditions, the retention time for the TFA derivative of KWG 1342 is 3.8 minutes and for the KWG 1323 derivative it is 5.0 minutes.

G. Calculations

Calculation of the ppm of BAYLETON, KWG 0519, KWG 1342 derivative or KWG 1323 derivative is accomplished by use of the following equation, where response for the unknown is compared to the response obtained for a known quantity of standard solution.

$$\text{ppm} = \frac{\text{Sample Response}}{\text{Standard Response}} \times \frac{\text{ng. Std. inj}}{1/2 \text{ Spl Wt (gms)}} \times \frac{\text{Final vol (ml)}}{\mu\text{l Spl inj}} \times \text{Dilution Factor}$$

NOTE: Only 1/2 the sample weight is used in the calculation to correct for only half the sample being loaded into the gel permeation chromatograph.

DISCUSSION

The method described in this report measures BAYLETON and its free and conjugated metabolites in barley and wheat. Metabolism studies with weathered, mature wheat

showed 82% of the total activity could be extracted and identified after an exhaustive extraction and elaborate hydrolysis and cleanup scheme<sup>2</sup>. Using the procedure described here, to analyze the same <sup>14</sup>C-treated wheat sample, 76% of the total <sup>14</sup>C activity was extracted and measured as BAYLETON, KWG 0519, KWG 1342 and KWG 1323<sup>5</sup> demonstrating the method to be capable solubilizing essentially all available residues and hydrolyzing conjugates for measurement of total BAYLETON related activity.

Metabolism study of BAYLETON in wheat shows almost all the residue present in samples at any interval to be present as BAYLETON, KWG 0519 or KWG 1342. In this study, KWG 1323 was a trace metabolite. The analysis procedure will detect all four of these compounds. However, when Florisil column chromatography is used to cleanup the extracts, KWG 1323 is partially eluted in the fraction containing BAYLETON and KWG 0519, but is mostly found in the subsequent fraction containing all of the KWG 1342. When that fraction is concentrated and reacted with trifluoroacetic anhydride, both KWG 1342 and KWG 1323 are converted to derivatives yielding distinct peaks on the gas chromatograph. KWG 1342 will not yield a peak on the chromatograph without derivatization. BAYLETON and KWG 0519 will chromatograph without derivatization as will KWG 1323. Therefore KWG 1323 will be totally accounted for when the amount of it in both fractions is added. Because of this incomplete separation on the Florisil column, assay for KWG 1323 by the above procedure is less quantitative than for the other three residues.

An alternate procedure was devised for quantitative analysis of KWG 1323 when required (see Appendix I). In that case, the extract after gel permeation chromatographic cleanup is placed on a LOBAR reverse-phase column and eluted with acetonitrile-water mixture (7:3). After an initial discard, KWG 1323 and KWG 1342 are completely eluted in one fraction followed by BAYLETON and KWG 0519 in a subsequent fraction. After evaporative concentration, BAYLETON and KWG 0519 can be analyzed by gas chromatography without further treatment. KWG 1323 and KWG 1342 are derivatized with TFA and analyzed by gas chromatography as described above. This alternate procedure is time consuming and is therefore recommended only when particular attention to KWG 1323 is justified. Such a requirement would be rare based on the small amount of KWG 1323 seen in the plant metabolism study as well as data showing only trace amounts of the compound in barley and wheat grown in the field after treatment with BAYLETON twice at a rate of 4 oz AI/acre<sup>6,7</sup>.

In a few instances, interference peaks from straw samples obscure the response for BAYLETON or one of the metabolites. In most cases, the confirmatory column is successful in resolving these interferences. Another possibility for removing interference peaks in the KWG 1323/KWG 1342 fraction only is the use of a mini-silica gel column just prior to gas chromatographic analysis. This procedure is described in Appendix II and is quite effective in removing interferences near the peak for KWG 1323.

Recovery experiments were run with BAYLETON and metabolites by fortifying samples of wheat and barley forage, grain and straw before adding extraction solvents. Samples were fortified at levels ranging from 0.05 to 2 ppm. Recoveries of BAYLETON, KWG 0519 and KWG 1342, were generally in the range of 70-120% at levels of 0.1 to 2.0 ppm. At the 0.05 ppm level, 3 of 15 samples exceed this range with the extremes being 58 and 132%. A summary of representative recovery values for barley and wheat samples is presented in Tables I and II.

Additional recoveries of BAYLETON, KWG 0519, KWG 1342 and KWG 1323 were run with wheat forage, grain and straw using the alternate procedure described in Appendix I. Samples were fortified at the 0.05 and 0.1 ppm levels with recovery values

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generally ranging from 75 to 125% (Table III). In general, it was necessary to run grain and straw samples through the mini-silica gel column described in Appendix II, to achieve sufficient cleanup.

Recorder response for BAYLETON and the three metabolites was approximately equal, at about 10 cm (peak height) for an amount equivalent to 0.1 ppm of sample residue. If a 1.0 cm peak height is considered the smallest chromatographic peak which can be accurately measured on the strip chart, the level of sensitivity of the method is stated to be 0.01 ppm, assuming sample extracts are sufficiently clean to allow adequate measurement. In order to show this capability, wheat grain control samples were fortified with BAYLETON and the metabolites KWG 0519, KWG 1342 and KWG 1323 at levels of 0.04, 0.03, 0.02 and 0.01 ppm. These extracts were then injected on the gas chromatograph and compared to control charts showing 80 to 110% of expected values at these ultimate levels. Complete raw data and chromatograms for this work are compiled in Mobay Ag Chem Report No. 80530<sup>s</sup>.

Representative control and 0.1 ppm recovery sample chromatograms are shown in Figures 2-5 for wheat forage.

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TABLE I

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Recovery of BAYLETON and Metabolites from Barley

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<u>Sample Type</u>	<u>Compound Added</u>	<u>Ppm Added</u>	<u>% Recovery<sup>a</sup></u>
Barley Green Forage	BAYLETON	1.0	71
	BAYLETON	0.5	74, 77
	BAYLETON	0.05	66
Barley Green Forage	KWG 0519	1.0	95
	KWG 0519	0.5	77, 94
	KWG 0519	0.05	88
Barley Green Forage	KWG 1342	1.0	88, 77
	KWG 1342	0.5	78, 97
	KWG 1342	0.05	70, 90
Barley Grain	BAYLETON	0.10	68, 116
	BAYLETON	0.05	132
Barley Grain	KWG 0519	0.10	80, 103
	KWG 0519	0.05	88
Barley Grain	KWG 1342	0.10	89, 76
	KWG 1342	0.05	102
Barley Straw	BAYLETON	0.5	120, 96
Barley Straw	KWG 0519	0.5	108, 100
	KWG 0519	0.05	76
Barley Straw	KWG 1342	0.5	78, 73
	KWG 1342	0.05	70

<sup>a</sup> Complete raw data and chromatograms are compiled in Mobay Ag Chem Reports No. 80492 and 80493.

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TABLE II

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Recovery of BAYLETON and Metabolites from Wheat

<u>Sample Type</u>	<u>Compound Added</u>	<u>Ppm Added</u>	<u>% Recovery<sup>a</sup></u>
Wheat Green Forage	BAYLETON	1.0	92, 86
	BAYLETON	0.5	91, 109
	BAYLETON	0.05	58
Wheat Green Forage	KWG 0519	1.0	103, 75
	KWG 0519	0.50	97, 87
	KWG 0519	0.05	80
Wheat Green Forage	KWG 1342	1.0	88, 87
	KWG 1342	0.50	84, 82
	KWG 1342	0.05	72
Wheat Grain	BAYLETON	0.10	99, 105
	BAYLETON	0.05	102
Wheat Grain	KWG 0519	0.10	88, 109
	KWG 0519	0.05	120
Wheat Grain	KWG 1342	0.10	72, 92
	KWG 1342	0.05	84
Wheat Straw	BAYLETON	0.5	94
Wheat Straw	KWG 0519	0.5	91
Wheat Straw	KWG 1342	0.5	94, 75

<sup>a</sup> Complete raw data and chromatograms are compiled in Mobay Ag Chem Reports No. 80494 and 80495.

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TABLE III

Recovery of BAYLETON and Metabolites from Wheat  
(Alternate Procedure, Appendix I)

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<u>Sample Type</u>	<u>Compound Added</u>	<u>Ppm Added</u>	<u>% Recovery<sup>a</sup></u>
Wheat Forage	BAYLETON	0.10	118, 96
	BAYLETON	0.05	112, 114
Wheat Forage	KWG 0519	0.10	121, 112
	KWG 0519	0.05	114, 110
Wheat Forage	KWG 1342	0.10	104, 92
	KWG 1342	0.05	98, 94
Wheat Forage	KWG 1323	0.10	91, 74
	KWG 1323	0.05	98, 90
Wheat Grain	BAYLETON	0.10	100, 95
	BAYLETON	0.05	120, 82
Wheat Grain	KWG 0519	0.10	88, 95
	KWG 0519	0.05	106, 78
Wheat Grain	KWG 1342	0.10	112, 111
	KWG 1342	0.05	124, 112
Wheat Grain	KWG 1323	0.10	94, 104
	KWG 1323	0.05	102, 104
Wheat Straw	BAYLETON	0.10	98, 91
Wheat Straw	KWG 0519	0.10	130, 105
Wheat Straw	KWG 1342	0.10	114, 95
Wheat Straw	KWG 1323	0.10	77, 73

<sup>a</sup> Complete raw data and chromatograms are compiled in Mobay Ag Chem Report No. 80530.

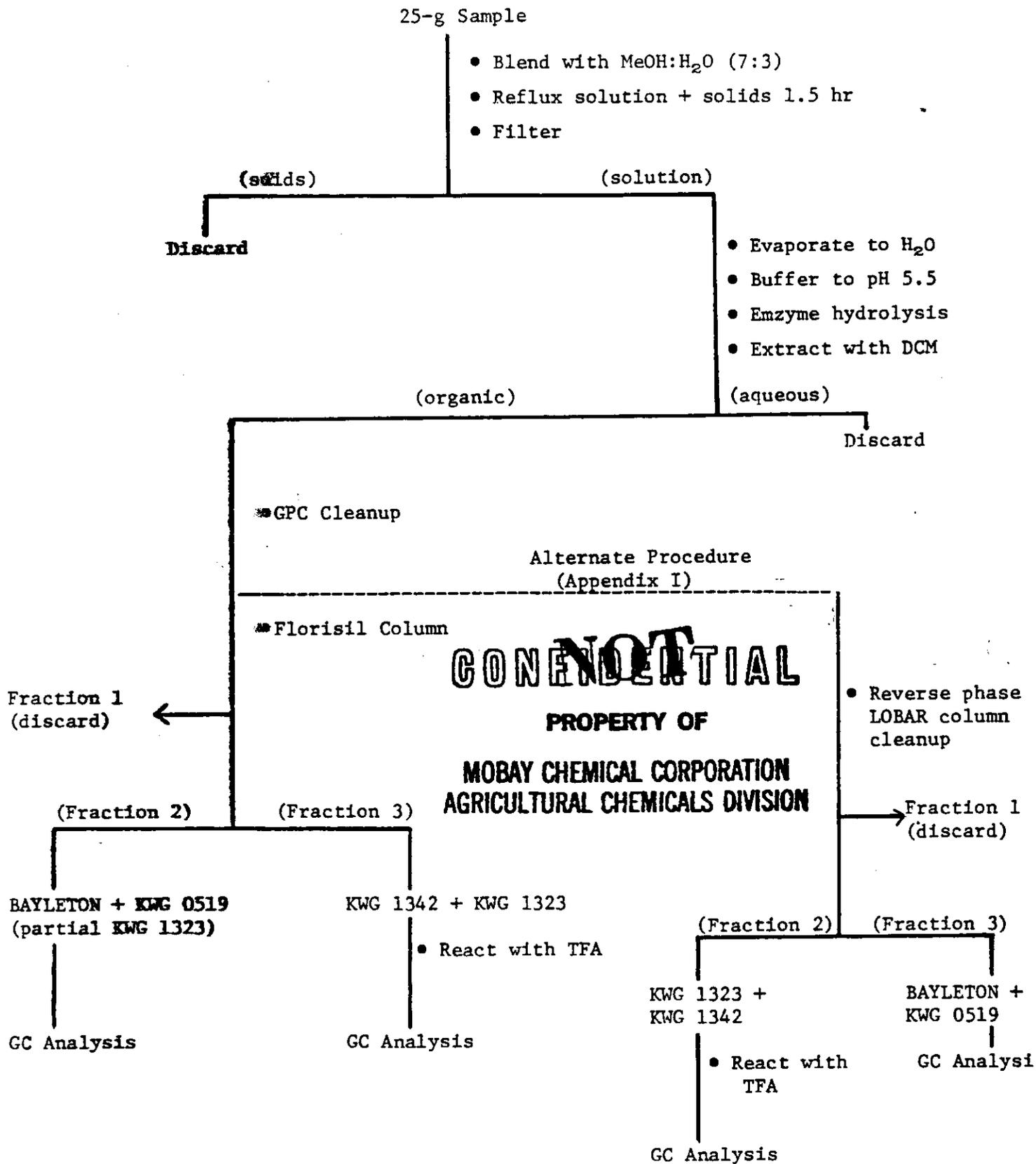


Figure 1. General schematic of the analysis scheme.

Sample & No. 93307 NBR 81R-80-56

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WHEAT FORAGE CONTROL

Dates: Extraction 1-19-82 Injection 1-21-82

Response ( cm. ): Sample/Std.

BAYLETON :  $1.16 \times 0.0 = 0.0 / 11.3$

KW6 0519 :  $1.16 \times 0.2 = 0.2 / 7.4$

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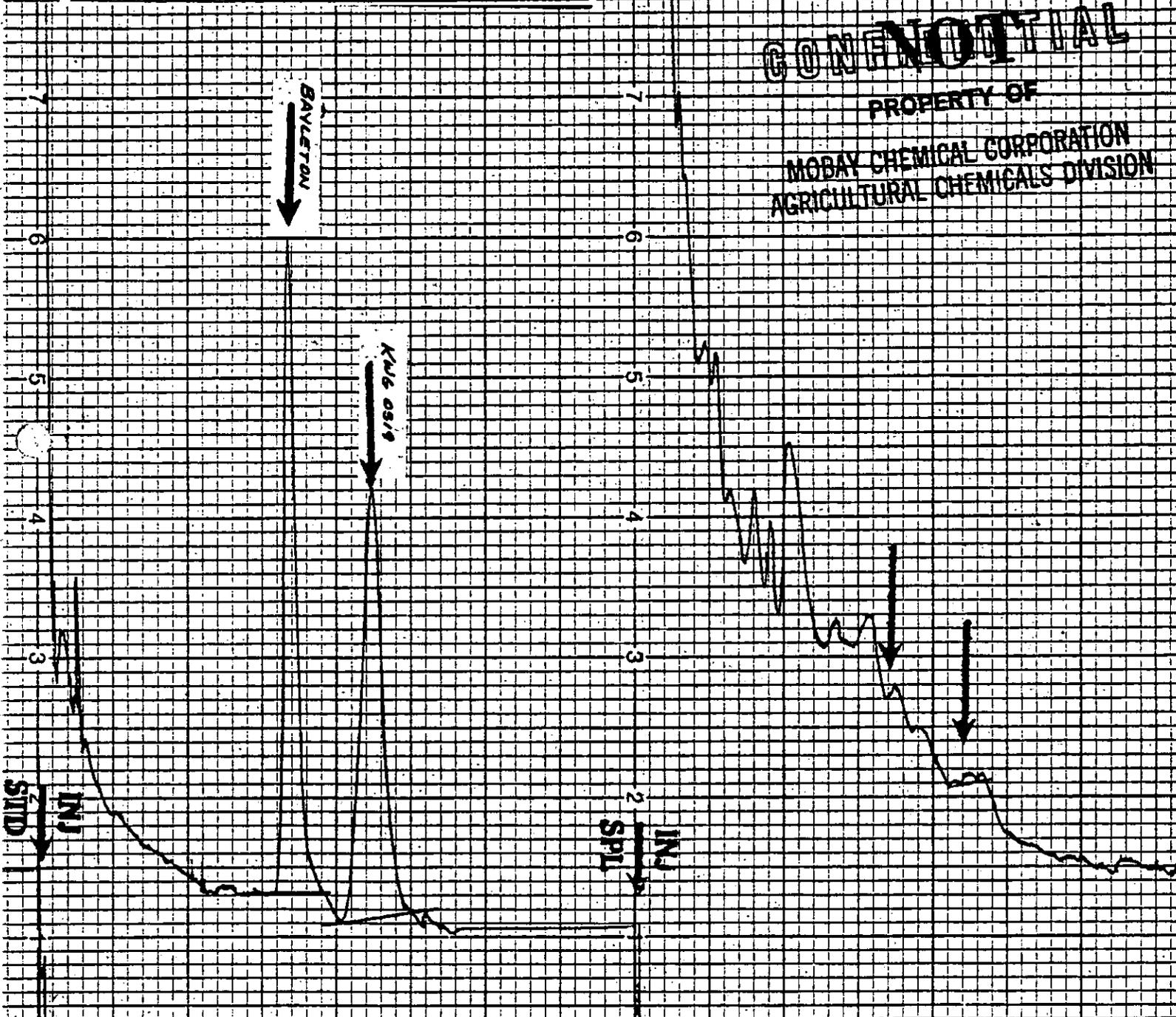


Figure 2

80488

Sample & No. 93307 NBR 21R-80-56  
WHEAT FORAGE CONTROL  
+ 0.1 ppm BAYLETON & KW6 0519  
Dates: Extraction 1-19-82 Injection 1-21-82  
Response ( *Cm* ): Sample/Std.  
BAYLETON 1.16 x 11.2 = 13.0 / 11.0  
KW6 0519 1.16 x 7.9 = 9.2 / 7.6

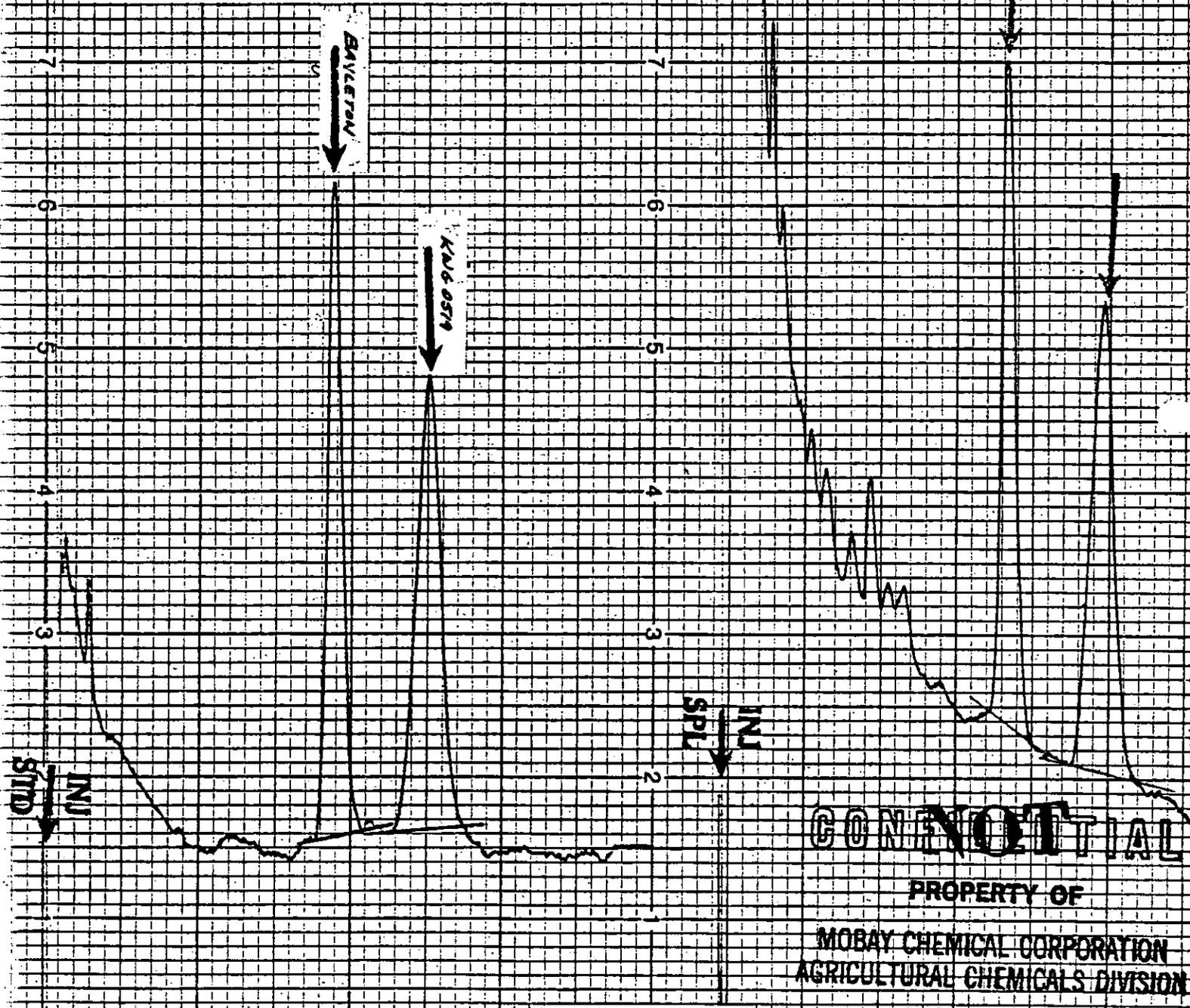


Figure 3

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Sample & No. 93307 NBR BIR-80-56

80488

WHEAT FORAGE CONTROL

(METABOLITE FRACTION)

Dates: Extraction 1-19-82 Injection 1-21-82

Response (cm) Sample/Std.

KWG 1342  $1.16 \times 0.0 = 0.0 / 8.4$

KWG 1323  $1.16 \times 0.0 = 0.0 / 7.7$

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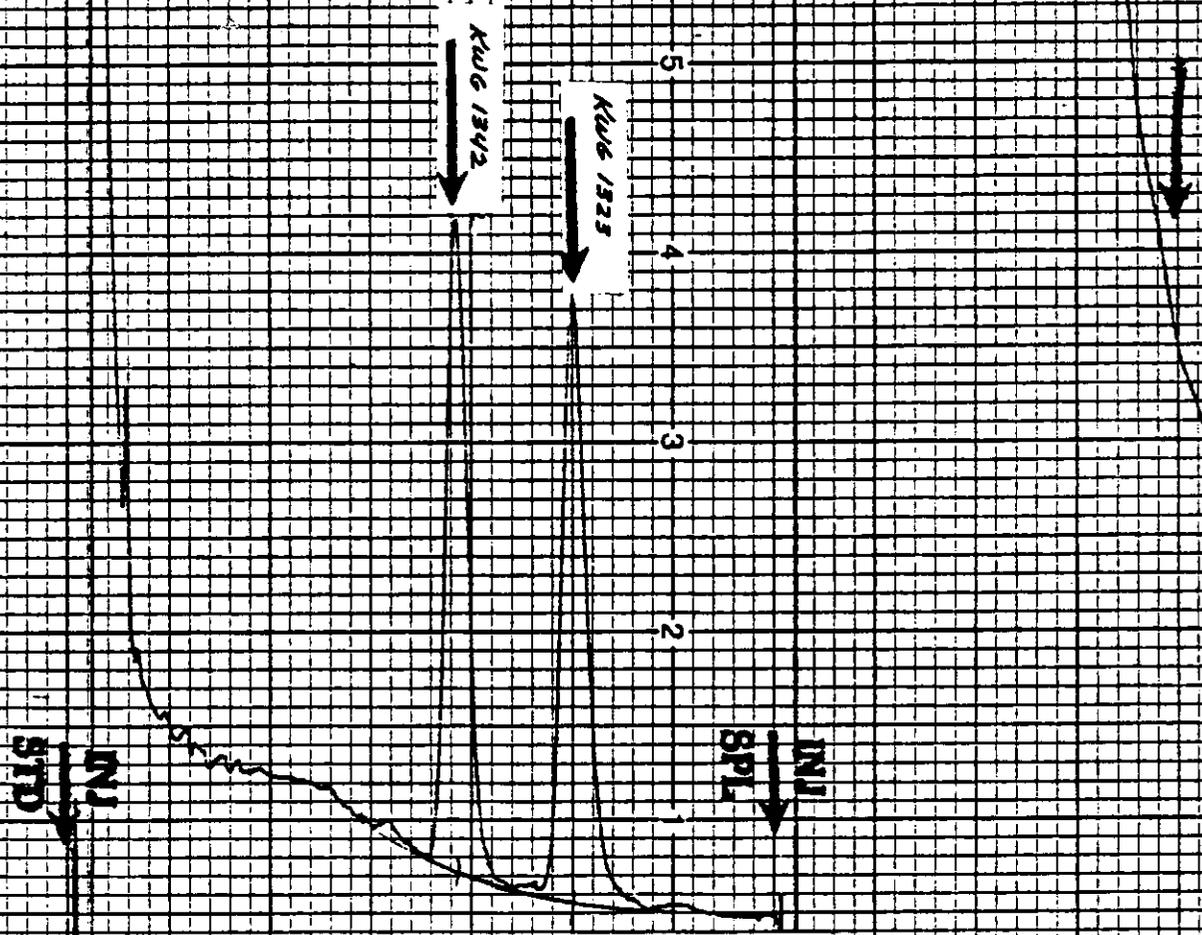


Figure 4

Sample & No. 93307 NBR 81R-80-56

80488

WHEAT FORGE CONTROL

+ 0.1 PPM KW6 1342 & KW6 1323

Dates: Extraction 1-19-82 Injection 1-21-82

Response ( *cm* ): Sample/Std.

KW6 1342  $1.16 \times 7.2 = 8.4 / 8.1$

KW6 1323  $1.16 \times 5.8 = 6.7 / 7.4$

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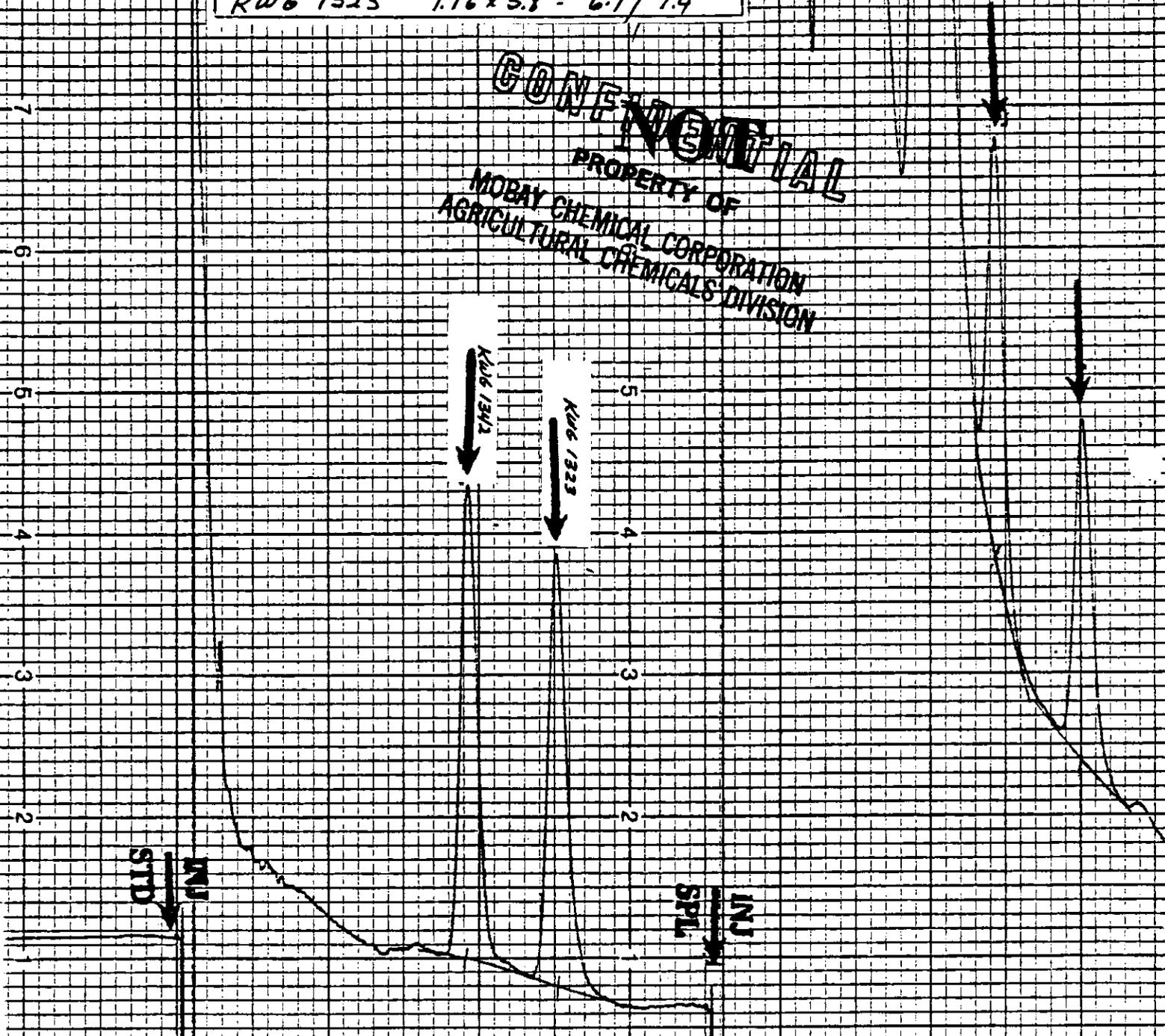


Figure 5

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APPENDIX I

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Alternate Method of Analysis to Include KWG 1323

For this procedure, extract samples exactly as previously described and process them through the gel permeation chromatography cleanup. Then proceed as described below:

LOBAR Chromatography Separation and Cleanup

1. Dissolve the residue from the GPC column cleanup in 5.0 ml of ACN-H<sub>2</sub>O (7:3) using sonication to aid solution.
2. Transfer the solution to a 5-ml centrifuge tube.
3. Stopper and centrifuge the solution at 900 rpm for 3 min.
4. Equilibrate a LOBAR Li-Chroprep RP-8 reverse phase column with ACN-H<sub>2</sub>O (7:3) at a flow rate of 5 ml per minute.
5. Using a 5-ml syringe, load the sample from step 3 into the sampling loop of the reverse phase column.

NOTE: Sample loop volume in this unit is 4.3 ml.

6. Flush the sample onto the column using ACN-H<sub>2</sub>O (7:3) and discard the first 45 ml of column eluate.
7. Change receivers and collect the next 80 ml of column eluate in a graduated cylinder, keeping the flow rate approximately 5 ml per minute (Fraction A). This fraction contains the KWG 1342 and KWG 1323 residues.
8. Change receivers again and collect the next 100 ml of column eluate (Fraction B). This fraction contains BAYLETON and KWG 0519.
9. Flush the column using 150 ml of acetone-ACN (75:25) to remove crop extractives. Equilibrate the column with approximately 225 ml ACN-H<sub>2</sub>O (7:3) before loading the next sample.
10. Transfer the respective column eluates to separate boiling flasks using approximately 5 ml of fresh ACN to complete the transfer.
11. Evaporate the fractions until only water remains (20-30 ml).
12. Transfer the aqueous phases from Fraction A and B respectively, to separate separatory funnels. Rinse the flasks with 10 ml of water and add to the respective separatory funnels.
13. Rinse the flasks with 2 x 25 ml DCM and add the rinses to the respective separatory funnels.

14. Shake the separatory funnels for 30 seconds. Allow the phases to separate and drain the lower phase into separate, 250-ml round bottomed flasks.
15. Repeat the extractions of the respective fractions twice more with fresh 35-ml portions of DCM.
16. Evaporate the extracts to dryness on a rotary vacuum evaporator. Remove the last traces of solvent using a gentle stream of nitrogen.
17. Dissolve Fraction B (containing BAYLETON and KWG 0519) in 2.0 ml acetone and analyze as described previously under "Gas Chromatography".
18. For Fraction A, proceed to the trifluoroacetic anhydride "Derivatization Procedure", described previously and carry the samples through "Gas Chromatographic Analysis".

NOTE, 1.25 µg standards of KWG 1342 and KWG 1323 must be started at the derivatization step.

#### Calculations

Calculation of the ppm of residue in each fraction is done as previously described except that an additional correction factor of 5/4.3 (= 1.16) must be added to account for incomplete transfer of the sample to the LOBAR column through the sampling loop (this correction factor will vary depending on the loop volume of any particular column unit). Thus, the calculation for total ppm would be as follows:

$$\text{ppm} = \frac{\text{Sample response}}{\text{Standard response}} \times \frac{\text{ng. Std. inj}}{1/2 \text{ Spl wt (gms)}} \times \frac{\text{Final vol (ml)}}{\mu\text{l Spl inj}} \times \text{Dilution Factor} \times 1.16$$

Any residues of metabolites result from conversion of the active ingredient of BAYLETON by metabolic action. Thus, these residues should be reported in BAYLETON equivalents, taking into account the molecular weight difference, if total residues are to be computed. Multiplication factors (molecular weight BAYLETON/molecular weight of metabolite) for converting metabolite residues to BAYLETON equivalents are 0.99, 0.94 and 0.95 for KWG 0519, KWG 1342 and KWG 1323, respectively.

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## APPENDIX II

Mini-Silica Gel Column Cleanup for KWG 1342 and KWG 1323 TFA Derivatives

The following procedure is recommended for certain dry straw or grain samples where co-extractives interfere with measurement of the derivatized KWG 1342 or KWG 1323 peaks.

1. Place a loose plug of glass wool into the bottom of a pasteur pipet. Add 1.5 cm of 2.5% water-activated, 100-200 mesh silica gel and top the column with a 0.5 cm layer of anhydrous granular sodium sulfate.
2. Place a receiving tube under the pasteur pipet and pre-wet the column with 1 ml of benzene.
3. Transfer the TFA derivatized sample to the column in a volume of 1 ml benzene. (Any previously added acetone should be evaporated before the transfer.)
4. Allow the liquid to just percolate into the sodium sulfate layer and rinse the vial with an additional 1-ml benzene wash.
5. When the benzene solution reaches the top of the sodium sulfate, elute with 5 ml of 1:1 ethyl ether-benzene, collecting the entire quantity of transfer solvent and column eluate together.
6. Evaporate the eluate to approximately 0.2 ml using a gentle stream of nitrogen.
7. Dissolve the residue in 2.0 ml of acetone. Proceed to G.C. Analysis.

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## Alternate Method of Analysis for Coffee Bean Samples

For this procedure, extract samples exactly as previously described and process them through "Gel Permeation Chromatography" cleanup of the standard procedure, followed by steps 1-16 of "LOBAR Chromatography Cleanup" described in Appendix I. Hold Fraction B for "Water Wash", below.

1. Add 5 ml of diethyl ether to the residue in the flask containing Fraction A and filter through Whatman No. 1 filter paper, collecting the filtrate in a 15-ml centrifuge tube.
2. Rinse the flask and filter paper with an additional 5-ml of diethyl ether and combine with the original (Fraction A) filtrate.
3. Evaporate the filtered ether extract to approximately 5 ml using a gentle stream of nitrogen in an N-Evap (water bath, 40°C).
4. Transfer the concentrated filtrate quantitatively to a reaction vial using several small rinses of ether.
5. Evaporate the filtrate just to dryness using a gentle stream of nitrogen in an N-Evap (water bath, 40°C).
6. Derivatize the residue according to steps E-1 through E-8 in the standard procedure, using 500  $\mu$ l of TFA reagent instead of 50  $\mu$ l.

NOTE: 1.25  $\mu$ g Standard of KWG 1342 and KWG 1323 must be started at the derivatization step.

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## Water Wash

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1. Dissolve Fractions A and B separately in 1 ml of benzene.
2. Transfer the samples to separate 60-ml separatory funnels containing 20 ml of deionized water.
3. Rinse the vials with an additional 1 ml benzene and transfer to the respective separatory funnel.
4. Stopper and shake the funnel for 30 seconds and allow the layers to separate.
5. Drain off the water layer and discard.
6. Repeat the extraction six times more with fresh 20-ml portions of deionized water.

7. After the last water extraction, draw off the water and discard. Drain the benzene into a graduated tube. Stopper and hold for G.C. Analysis.

NOTE: For instruments where benzene is a problem as an injection solvent, evaporate the samples under a stream of nitrogen and redissolve the residue in 2 ml of ethyl acetate.

Gas Chromatographic Analysis

Gas chromatography analysis is done according to steps F-1 through F-3 in the standard procedure.

NOTE: For KWG 1323 and KWG 1342 fractions, GC analysis should be done without delay to avoid hydrolysis of the TFA derivative.

Calculations

Calculation of the ppm of residue in each fraction is done as described in Appendix I including the correction factor to account for the incomplete transfer of the sample to the LOBAR column through the sampling loop.

Any residues of metabolites result from conversion of the active ingredient of BAYLETON by metabolic action. Thus, these residues should be reported in BAYLETON equivalents, taking into account the molecular weight difference, if total residues are to be computed. Multiplication factors (molecular weight BAYLETON/molecular weight of metabolite) for converting metabolite residues to BAYLETON equivalents are 0.99, 0.94 and 0.95 for KWG 0519, KWG 1342 and KWG 1323, respectively.

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## APPENDIX IV

## Modified Procedure for Monitoring of Crop Residues

1. Section A, Sample Preparation and Extraction. Follow without modification.
2. Section B, Enzyme Hydrolysis. Follow as listed except that step 11 should be changed to read "Proceed to Florisil Column Cleanup" instead of "Proceed to Gel Permeation Chromatography Cleanup".
3. Section C, Gel Permeation Chromatography Cleanup. Omit entirely.
4. Section D, Florisil Column Cleanup. Carry out steps 1 through 13, omit steps 14 through 16 and note that step 17 would apply only to the hexane-ethyl acetate fraction. Carry out step 18 and omit step 19. After step 19 proceed to Section F, "Gas Chromatographic Analysis".
5. Section E, Derivatization Procedure. Omit entirely.
6. Section F, Gas Chromatographic Analysis. Columns and chromatographic conditions should be used as listed in the method. In step 1 injection volumes may be reduced by 50% since the elimination of Section C eliminate loss of half the sample. Step 2 proceed as indicated for the analysis of BAYLETON and KWG 0519. Eliminate step 3 entirely.
7. Section G, Calculations. Eliminate from the denominator of the second term "1/2" so that the term would now read "Spl Wt (gms)". The elimination of the 1/2 factor is due to the elimination of Section C where half the sample would have been lost.
8. Appendix I. Omit entirely.
9. Appendix II. Omit entirely.

Adjustments in the lists of equipment and reagents on page 3 would also need to be attenuated accordingly. Specific examples are the elimination of the reverse phase column, the Reacti-vials and the trifluoroacetic anhydride (TFA) reagent.

The gel permeation chromatography cleanup in Section C is not expected to be needed for the analysis of BAYLETON and KWG 0519 alone. It may, however, be considered as an option in the event that the amount of co-extractives in certain kinds of crops interfered with the measurement of these compounds.

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