Cover Sheet for

ENVIRONMENTAL CHEMISTRY METHOD

Pestcide Name: Prohexadione-Calcium (BAS 125 W)

MRID #: 450403-01

Matrix: Soil

Analysis: HPLC/UV

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METHOD VALIDATION OF BASF ANALYTICAL METHOD NO. D9607, "THE DETERMINATION OF RESIDUES OF PROHEXADIONE-CALCIUM (BAS 125 W) AND ITS METABOLITE DESPROPIONYL-PROHEXADIONE IN SOIL USING HPLC / UV DETECTOR"

ANALYTICAL METHOD No. D9607

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STUDY INITIATION DATE: August 19, 1996
EXPERIMENTAL COMPLETION DATE: December 9, 1996
STUDY COMPLETION DATE: June 20, 1997

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DOCUMENT NUMBER: ADPEN-903-96-A96054-001
ADPEN Study Number: ADPEN-903-96-A96054
BASF Study Number: A96054

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1.0 INTRODUCTION

The purpose of this study was to generate recovery data for the validation of this method and to describe in detail the various steps used in the analysis of prohexadione, prohexadione-Ca, and the metabolite despropionyl-prohexadione by HPLC using column switching.

1.1 SCOPE OF METHOD

This method is used to determine the residues of prohexadione, prohexadione-Ca, and its metabolite despropionyl-prohexadione. All compounds of interest are extracted from soil by shaking with an ammonium bicarbonate solution. The sample is cleaned up by the use of SAX and ENV+ SPE columns and a partition. All compounds are chromatographed and detected by HPLC column switching with UV detection. The limit of quantitation of the method is 0.01 ppm for prohexadione, prohexadione-Ca, and despropionyl-prohexadione. The limit of detection is set to 0.005 ppm based on standards injected at half the quantitation limit.

This methodology was developed by Greg Waitt and Jayanti R. Patel (BASF Corporation) and was validated with minor modifications by ADPEN Laboratories, Inc.

1.2 PRINCIPLE OF THE METHOD

Twenty grams of the soil is extracted twice with 50 mL of 0.1N ammonium bicarbonate by shaking at 200-300 RPM for 30 minutes at 50°C in a shaker. The soil mixture is centrifuged and the combined supernatants acidified to pH 2. The acidified supernatant is passed through a SAX SPE cartridge conditioned with pH 2 water for initial cleanup. The cleaned supernatant is split into two equal portions and prepared separately for prohexadione and despropionyl-prohexadione (KI-5376) analysis. Prohexadione is extracted by ethyl acetate partition of the acidic supernatant, followed by evaporation and dissolving the residue in 1% acetic acid. Prohexadione is analyzed by HPLC column switching with UV detection at 274 nm. Despropionyl-prohexadione is cleaned up using two ENV+ SPE columns, one using basic conditions (pH 7-8), followed by another one using acidic conditions (pH 2).

Despropionyl-prohexadione is eluted from the second column using ethyl acetate, followed by partition with 0.5 N sodium hydroxide. Despropionyl-prohexadione is analyzed by HPLC column switching with UV detection at 260 nm. A flow chart of the procedure is shown in Figure 1.

2.0 SUMMARY

Analytical Method No. D9607 was validated for the analysis of soil at a quantitation limit of 0.01 ppm for each compound. Average procedural recoveries through the method for the analyses of soil were 85.7 ± 14.8%, 90.1 ± 14.2%, and 71.3 ± 5.65% for prohexadione-Ca, prohexadione, and despropionyl-prohexadione, respectively.

Additional fortification recoveries from two soil dissipation studies in five locations also show that this method is rugged.

3.0 EQUIPMENT

Names of equipment manufacturers and brands are suggested. These may be substituted and equivalent equipment may be used.

3.1 Bath, ultrasonic, Branson, Model 2200
3.2 Bath, water, Buchi, Model B-461
3.3 Centrifuge
3.4 Centrifuge bottle, 200 mL
3.5 Concentration tube, 15 mL, standard tapered joints, Corning or equivalent
3.6 General laboratory glassware
3.7 Glass wool
3.8 Heated shaking water bath, Lab-Line Model 3540
3.9 Mechanical shaker, Eberbach
3.10 Rotary evaporators
3.11 Solid Phase Extraction (SPE) columns and reservoirs (J.T. Baker 7328-06)
3.12 Solid Phase Extraction, vacuum manifold
3.13 Volumetric flasks, miscellaneous sizes
3.14 Volumetric pipettes, Type A, various sizes
3.15 Vortex Mixer, Lab-Line Instruments

4.0 SAFETY

All analysts must be familiar with the potential hazards of each of the reagents, solvents, and products used in this method before any laboratory work is done. Material Safety Data Sheets (MSDS), Laboratory Safety Manual, product information, and other related materials should be consulted. Exposure to all chemicals should be reduced to the lowest possible level. Analysts should also be aware of OSHA regulations regarding the safe handling of the chemicals specified in this method. Disposal of all chemicals must be in compliance with local, state, and federal laws and regulations.

4.1 Acetic acid, acetonitrile, ethyl acetate, and methanol are flammable. Care should be taken to use these solvents in well ventilated areas away from ignition sources.

4.2 All open flask evaporations with an N-Evap should be done inside a hood.

5.0 REAGENTS

Names of chemical manufacturers and brands are suggested. These may be substituted and equivalent reagents may be used.

5.1 Ammonium bicarbonate (Aldrich, #28,509-9)
5.2 Acetic acid (J.T. Baker, #9522-02)
5.3 Acetonitrile, HPLC grade
5.4 1,3 Cyclohexanedione (Fluka, #29059)
5.5 Ethyl acetate, HPLC grade
5.6 ENV+ SPE, International Sorbent Technology (Jones Chromatography, #9915-0010)
5.7 Methanol, HPLC grade
5.8 Millipore water or HPLC water
5.9 PS-1 filter paper (Whatman, #2200 150)
5.10 Sodium chloride, Reagent grade
5.11 Sodium hydroxide, Reagent grade
5.12 Sulfuric acid (VWR, #VW6840-3)
5.13 Supelclean LC-SAX (Supelco, #5-7203)
5.14 Sodium sulfate

Note: ENV+ can be reconstituted as follows: Collect ENV+ in a beaker. Cover with methanol (soaked completely through - about double the amount of ENV+). Heat over a hot plate and let sit about 10 minutes. Let it sit until phases are separated. Decant the methanol. Repeat above steps with acetone. Repeat again with ethylene chloride. Filter and let dry.

6.0 INSTRUMENTATION

Names of instrument manufacturers are suggested, equivalent brands may be substituted.

6.1 Hewlett-Packard 1050 HPLC, Hewlett-Packard 1100 HPLC.

6.2 HPLC Column, Precolumn - Hypersil ODS C18, 3 um, 4.6 mm ID, 100 mm length, Alltech.

6.3 HPLC Column, Analytical - Kromasil C18, 5 um particle size, 4.6 mm ID, 250 mm length, Alltech

7.0 PREPARATION OF STANDARD SOLUTIONS

Analytical standards of prohexadione-calcium, prohexadione, and despropionyl-prohexadione shall be kept in freezer. All solutions of standards shall be kept in amber bottles and stored in a refrigerator. The details given for making dilutions are suggested. Concentrations and method of dilution may be modified if needed. A complete description of the test and reference substances may be found in Table I.

7.1 STOCK SOLUTIONS

Prepare a 0.10 mg/mL stock solution of prohexadione-calcium by accurately weighing 0.01 g of standard into a 100 mL volumetric flask. Dissolve in water (pH 9) and dilute to the mark. Prepare separate 1.0 mg/mL stock solutions of prohexadione and despropionyl-prohexadione by accurately weighing 0.100g of standard into a 100 mL volumetric flask. Dissolve in methanol and dilute to the mark. The prohexadione-calcium and the metabolite stocks were prepared once a month. The prohexadione stock was prepared every three months.

7.2 STANDARD FORTIFICATION SOLUTIONS

Prepare 0.2 ug/mL, 2.0 ug/mL, and 20 ug/mL stock solutions of prohexadione-calcium from the 0.10 mg/mL stock solution. Dilute in pH9 water (deionized water adjusted to pH 9 with NaOH). These solutions are used for fortification and shall be prepared fresh every month.

Prepare separate 0.2 ug/mL, 2.0 ug/mL, and 20 ug/mL stock solutions of prohexadione and despropionyl-prohexadione from the 1.0 mg/mL stock solution and dilute in methanol. These solutions are used for fortification and shall be prepared fresh every month.

7.3 STANDARD SOLUTIONS FOR HPLC

Separate calibration standards containing prohexadione (free acid) and despropionyl-prohexadione were prepared by diluting the 1.0 mg/mL stock solutions in methanol. The dilutions were made in deionized water for despropionyl-prohexadione and 1% acetic acid in HPLC water for prohexadione (free acid).

8.0 SAMPLE WORK-UP

8.1. RECOVERY TEST

Read the entire method before attempting to use this analytical method. The validity of the procedure should be demonstrated by recovery tests before analysis of unknown samples is attempted. Untreated (control) and three fortified samples shall also be processed with each set of samples analyzed. Typically, one of the fortification samples is run at the limit of quantitation. For each fortified sample, an appropriate quantity of prohexadione-calcium, or prohexadione (free acid), and despropionyl- prohexadione are added to a control sample. The fortification of prohexadione-calcium or prohexadione (free acid), and despropionyl- prohexadione should be included in each set. Fortifications are made onto the sample prior to the start of the analytical procedure.

Note: Since prohexadione-calcium (BAS 125 W) converts to and is analyzed as prohexadione (free acid), they should not be used together for fortification on a control sample.

Note: Each lot of SPE columns should be profiled to determine if the lot being used will provide adequate recovery of each compound of interest. Elution parameters may be modified if needed.

8.2 SAMPLE PREPARATION

8.2.1 Thaw out samples, add dry ice and mix thoroughly to make homogeneous using a Buffalo chopper or equivalent. Allow the dry ice to evaporate before sealing sample bag.

8.2.2 Keep all samples frozen until ready for analysis.

8.3 EXTRACTION OF RESIDUE

8.3.1 Weigh 20.0 g of soil into a 200 mL centrifuge bottle or a 250 mL Erlenmeyer flask (centrifuge bottle is recommended).

8.3.2 Fortify control soil with prohexadione-calcium (BAS 125 W) or prohexadione (free acid) or despropionyl-prohexadione as indicated in 8.1 for fortification samples.

8.3.3 Add 50.0 mL of 0.1 N ammonium bicarbonate.

Note: Mild base is needed to extract prohexadione-Ca, prohexadione and despropionyl-prohexadione completely from soil.

8.3.4 Shake for 30 minutes in water bath at 50°C, at 300 RPM for orbital shakers. Shaking should be strong enough to get the sample solution up into the sides of the flask. Adjust RPM, if needed, for linear shakers. Make sure that the liquid level of the sample is below the water level in the bath.

8.3.5 Transfer soil mixture to 200 mL centrifuge tube, if Erlenmeyer flask was used.

8.3.6 Centrifuge for 10 minutes at about 3000 RPM.

8.3.7 Decant supernatant into 250 mL flat bottom flask.

8.3.8 Add 50 mL of 0.1 N ammonium bicarbonate to centrifuge tube or use to transfer soil back to shaking flask if using Erlenmeyer flask.

8.3.9 Shake again for 30 minutes at 50°C, at the same rate.

8.3.10 Centrifuge for 10 minutes at about 3000 RPM and decant into flask used in step 8.3.7.

8.4 STRONG ANION EXCHANGE (SAX) SPE COLUMN CLEAN-UP

8.4.1 Adjust sample pH to 2 with pH meter. (use approximately 250 uL of concentrated sulfuric acid).

Note: Prohexadione-Ca will convert to prohexadione (free acid) after addition of acid.

8.4.2 Add 1.0 g SAX SPE packing to glass column. Add a glass wool plug on top of column. (Adding a large glass wool plug to SPE reservoir may be necessary to prevent the column from clogging)

8.4.3 Condition column by running 10 mL methanol through, then 25 mL pH 2 water, making sure not to allow the column to run dry until final eluate collection. (pH 2 water is made by adjusting deionized water with concentrated sulfuric acid, use pH meter).

8.4.4 Load sample, collecting the eluate in a 125 mL flat-bottom flask (Vacuum 5-10 inches Hg). Adjust rate to complete elution within 45 minutes.

8.4.5 Wash with an additional 25 mL of pH 2 water, continue adding to sample eluate.

8.4.6 Split sample into two equal aliquots. Use 50% of aliquot for step 8.5 and other 50% for step 8.6.

Note: It is important not to let the samples sit in acidic pH for an excessive amount of time. The analytes may decompose in acidic condition if they are stored for a long time. Do not store at this step overnight or leave for several hours. If storage is necessary, neutralize to pH 7-8.

8.5 FOR DESPROPIONYL - PROHEXADIONE (KI-5376) ANALYSIS ONLY

8.5.1 ENV+ Solid Phase Extraction First Step (pH 7-8 SPE Cleanup).

Note: At pH 7-8, the metabolite is in the ionic form and will go through the ENV+ without being retained. This step has been carried out to clean sample by retaining non-ionic impurities

a. Add 0.5 g ENV+ SPE packing to glass column. Add a glass wool plug on top of column.

b. Condition column with 10 mL methanol, 10 mL deionized water, 25 mL analog mixture (0.04% 1,3-cyclohexadione in deionized water adjusted to pH 7-8 with pH meter). Wash analog mixture through column without vacuum. Do not allow the column to run dry.

Note: Addition of 1,3-cyclohexadione solution is necessary. The 1,3-cyclohexadione is used to take up any active sites on the ENV+ that might hold onto the despropionyl-prohexadione.

c. Take 50% aliquot of sample from step 8.4.6. Adjust to pH 7-8 with 0.5 N sodium hydroxide (use pH meter)

Note: Watch the pH very carefully. The samples are strongly buffered and take a while to stabilize. Let samples sit for a few minutes and recheck pH before loading it on to the column.

d. Filter through column, slight vacuum (1-2 inches Hg), collecting eluate in about 30 minutes. Wash column with 25 mL of deionized water with vacuum (approx. 1-2 inches Hg).

Note: Filtering without vacuum enables more contaminates to be retained on the column. If contamination is not a problem, a low vacuum can be applied. Vacuum may not be necessary if flow allows elution within 30 minutes. Reconditioned ENV+ flows faster. It is recommended that flow through the column be pre-checked for new ENV+ lots to determine if elution can be achieved within 30 minutes. If this is not achieved, the new ENV+ lot should be reconditioned.

8.5.2 ENV+ Solid Phase Extraction Second Step (pH 2 SPE Cleanup).

Note: At pH 2, the metabolite is in the non-ionic form and will be held by the ENV+.

a. Add 0.5 g ENV+ SPE packing to glass column. Add a glass wool plug on top of column.

b. Condition column with 10 mL ethyl acetate, 10 mL methanol, 10 mL deionized water, and 25 mL analog mixture (0.04% 1,3-cyclohexadione in deionized water adjusted to pH 2 with pH meter). Wash analog mixture through column without vacuum. Do not allow the column to run dry.

c. Take sample from step 8.5.1-d and adjust to pH 2 with concentrated sulfuric acid (use pH meter).

d. Load sample on column with a slight vacuum (1-2 inches Hg). Wash with 25 mL of pH-2 water, also with a slight vacuum (1-2 inches Hg).

Note: Vacuum may not be necessary if flow allows loading within 30 minutes. Loading the sample without vacuum increases the efficiency by eliminating the analyte bleeding through the column. Reconditioned ENV+ flows faster.

e. Elute with 15 mL ethyl acetate in 50 mL test tube. Elute without vacuum. (If sample has not eluted within 15-20 minutes, vacuum can be applied, 2-3 inches Hg). When column stops dripping apply vacuum to completely elute ethyl acetate (maximum vacuum).

Note: It takes several minutes for the ethyl acetate to displace the water in the column, and to completely elute the analyte.

Note: Sample can be eluted into any appropriate glassware and transferred to a small separatory funnel if preferred.

8.5.3 Liquid - Liquid Partition (back extraction)

a. Partition ethyl acetate eluate with 2 mL of 0.5 N sodium hydroxide. Vortex for one full minute to get maximum mixing.

b. Pipette aqueous layer into 10 mL volumetric flask. Partition once more with 3 mL of 0.5 N sodium hydroxide. (Swirl flask to collect any aqueous droplets remaining on the side of the test tube.)

Note: It is very important to transfer despropionyl-prohexadione from the ethyl acetate layer into aqueous layer for HPLC analysis. Transfer all the aqueous phase.

c. Evaporate combined aqueous layers under nitrogen to remove traces of ethyl acetate.

Note: Ethyl acetate will cause HPLC chromatography problems. Therefore, it is important to evaporate as much ethyl acetate as possible.

d. Take aqueous layer to appropriate volume with deionized water for HPLC analysis. Acidify an aliquot before HPLC injection to a pH of 2 or less (use approximately 50 uL concentrated sulfuric acid).

Note: If samples are to be stored, they **should not** be stored under acidic conditions.

8.6 FOR PROHEXADIONE-CALCIUM (BAS 125 W) / PROHEXADIONE OR KI-2817 (FREE ACID) ANALYSIS ONLY

8.6.1 Ethyl Acetate / Aqueous Partition at acidic pH.

a. Take remaining 50% aliquot from step 8.4.6 and add to 125 mL separatory funnel.

b. Add 4.0 mL concentrated sulfuric acid and 20 g sodium chloride.

c. Partition vigorously three times with 25 mL ethyl acetate. Filter combined ethyl acetate extract through PS-1 paper with sodium sulfate (approximately 50 g) at the bottom of the filter paper.

Note: Do not allow extract to sit in sodium sulfate for an extended period of time or losses will be experienced. Wash sodium sulfate promptly (Step 8.6.d) after the extract is filtered. The extract may be filtered through after each partition if washed as in 8.6.d each time. It is extremely important to remove acid (aqueous phase) from the ethyl acetate (organic phase). Rotovaping with acid present will cause losses due to degradation of the compound.

d. Wash sodium sulfate with additional 25 mL ethyl acetate.

e. Rotovap to dryness and take to appropriate volume for HPLC analysis with 1% acetic acid in HPLC water. (Keep water bath at or below 40°C and sonicate to dissolve all residue.)

9.0 CHROMATOGRAPHY

9.1 The suggested chromatographic conditions are given in Tables IIa and IIb. See Figures 4 and 5 for typical chromatograms.

9.2 Calibrate the detector response and retention times by injections of the standard solutions throughout a set of analyses. Standards shall also be injected at the beginning and at the end of a set of analyses.

9.3 Sample residues are determined as described in Section 13.1 through 13.4. Fortification recoveries are determined as described in Section 13.5.

9.4 Column cut window should be checked before each run to compensate for chromatography changes. It is highly recommended to wash precolumn with 100% Acetonitrile while the analyte is eluting off the analytical column. Be sure to allow enough time for the column to re-equilibrate for the next injection.

9.5 Suggested conditions for precolumn wash: After column cut, wash 6 minutes with 100% acetonitrile at 1.0 mL/min., then return to mobile phase until end of run (22 minutes suggested to allow column to re-equilibrate).

9.6 Suggested procedures for establishing the retention time window in the column switching steps:

Establish the retention time of the compound of interest (prohexadione or despropionyl-prohexadione) on the precolumn using the appropriate precolumn mobile phase and flow rate. This is done by connecting the outlet of the precolumn to the detector and injecting a standard from 3 to 5 times. For each

run, the precolumn should be rinsed with 100% acetonitrile for about 6 minutes after elution of the standard, and then return to the mobile phase for the duration of the run (20 to 22 minutes). It is important to duplicate the precolumn conditions that will be used for the sample analysis. The retention time of the compound should be fairly consistent after the first injection. The first injection will normally differ from the others. The last 2 to 4 retention times should not vary by more than 0.1 minutes. This retention time determination should be done just before analysis of a set of samples. The switching valve time should start about 0.1 to 0.2 minutes before the start of the peak and end about 0.1 to 0.2 minutes after the end of the peak. The above timing may change depending on the length of tubing used between the column and switching valve or detector and may be determined experimentally. Do not set the time window too tight because some shifting is common. The time window for prohexadione can be set somewhat tighter than despropionyl-prohexadione because it tends to be more consistent and there are more interferences if set too wide.

Connect the precolumn (#1) and the analytical column (#2) to the switching valve as shown in the diagram on Table IIc. In the load position, the precolumn is connected to waste and the analytical column is connected to the detector. At the start time of the column switching, the valve is set to injection position, and the eluant from Column #1 is connected to the front of Column #2. At the end time of the column switching, Column #1 is switched back to wash. While the analyte elutes from Column #2, Column #1 is rinsed with 100% acetonitrile for about 6 minutes, then with Column #1 mobile phase until the end of the run (7.0 to 22 minutes). Make sure there is enough time for Column #1 to re-equilibrate before making the next injection.

10.0 TIME REQUIRED FOR ANALYSIS

Analysis of a set of 8 soil samples requires about 8 man hours. HPLC analysis may be done overnight by using an autosampler. The data entry, integration and reporting may take up to an additional 2 man hours.

11.0 INTERFERENCES

11.1 SAMPLE MATRICES

Baseline resolution was attained for each compound of interest with the exception of some samples. If interfering peaks from the matrix occur in the chromatogram, change the HPLC operating conditions or make the column switching window narrower. Changing the mobile phase ratio to a weaker mobile phase for the pre-column and the analytical column has allowed better separation from interfering peaks in some cases. It is important to remove as much ethyl acetate as possible for both analytes.

11.2 OTHER SOURCES

No interfering peaks from pesticides, solvents, or labware are known to occur.

12.0 CONFIRMATORY TECHNIQUES

No problems with interferences or questionable peak identity have been encountered to date. HPLC retention time comparison with analytical standards was the criteria used in this study as a confirmation.

13.0 METHODS OF CALCULATION

13.1 STANDARD CALIBRATION CURVE

At least four standard concentration levels shall be used for quantitation. Each standard should be injected at least twice in the analysis set. Standards are injected at the beginning, after every 1 to 3 samples and at the conclusion of the analysis. Peak height or peak area of each injected standard is determined by manual measurement or computer integration. Regression analysis of peak height or peak area versus nanograms injected may be performed by a scientific calculator or a computer chromatography data system. This regression analysis gives an equation for a standard curve for calculation of sample concentration. Nanograms injected can be calculated from the slope and intercept of the standard curve and the chromatographic peak height or area of each sample injection.

13.2 CALCULATION OF EQUIVALENT SAMPLE WEIGHT

The milligrams of sample injected must be determined to calculate ppm (Section 13.4). The equivalent sample weight in the final solution is calculated as follows:

$$\text{mg inj.} = \frac{(W)\,(V_i) \times 1000}{(V_f)}$$

W = weight of sample extracted (g)
1000 = conversion factor (mg/g)
V_f = final sample volume (mL) (includes factor of 2 to account for 50% sample split. See Section 8.4.6.)
V_i = injection volume (mL)

13.3 DETERMINATION OF SAMPLE RESIDUES (NANOGRAMS)

The peak height or area from a sample injection (Section 13.1) and the slope and intercept of the standard curve (Section 13.1) are used to determine the nanograms of residue in each sample injection. This can be done by a chromatography data system, calculator or by graphing a standard curve of nanograms injected versus detector response. The next section shows how to calculate sample residues in parts-per-million.

13.4 DETERMINATION OF SAMPLE RESIDUES (PPM)

Calculate the sample residue for each sample expressed in terms of parts-per-million (ppm) using the following equation:

$$\text{ppm of compound found} = \frac{\text{(ng of compound found)}}{\text{(mg of sample equivalent injected)}}$$

The procedure described in this report converts the prohexadione-calcium to prohexadione, the free acid. Therefore a conversion factor taking into account the molecular weight ratio between prohexadione-calcium and prohexadione (1.179) is needed to convert to prohexadione-Ca values.

A correction for soil moisture is done as follows:

$$\text{Corrected ppm} = \text{(ppm of compound found)} \times \frac{(1)}{(1-M/100)}$$

Where M = percent soil moisture content

13.5 FORTIFICATION RECOVERIES

The ppm of compound found in the final solution (Section 13.4) is divided by the amount of compound added to the control sample. This ratio times 100 is the percent recovery of the method at that level of fortification.

$$\text{\% Recovery} = \frac{\text{ppm analyte found in injected solution}}{\text{ppm analyte added to control sample}} \times (100)$$

If the control sample shows a chromatographic response corresponding to the analyte(s) of interest, the ppm value corresponding to this control sample response should be subtracted from the ppm residues found in the fortified samples before the percent recovery calculation is made, i.e.:

ppm found in recovery = ppm in fortified sample - ppm in control sample

Note: This correction factor applies only to recovery samples not to actual field samples.

14.0 RESULTS AND DISCUSSION

14.1 TEST SYSTEM

The test system for this project, soil, was obtained from RCN 96044, California and from RCN 96042, Texas (top layer only).

Soil samples were received from California and Texas. Characterization data can be found in the Appendix 3. The samples were identified by field protocol number and sample number . These samples were processed by BASF Corporation, Research Triangle Park, and shipped frozen to ADPEN Laboratories, Inc. The samples were received at ADPEN Laboratories, Inc. on 8/5/96 (Texas) and 8/13/96 (California) and were stored frozen in ADPEN's Freezer E24. Unique sample laboratory codes were assigned just prior to analysis. The laboratory sample code consists of year code, project code and sample number.

14.2 TEST SUBSTANCES

For a description and structures of the test and reference substances see Table I and Figure 2.

14.3 QUANTITATION

Results were calculated from a standard curve prepared by plotting detector response in peak area or peak height versus nanograms of compound injected. An equation for the fit of the standard curve was derived, and the correlation coefficient of the regression curve calculated for all analytical sets. See Figure 3 for typical standard curves.

Integration and quantitation of peaks were done by computer using Hewlett-Packard ChemStation Chromatography Data System. Final results were computed for each set of samples by the use of a MS Excel spreadsheet. The data acquisition system has been validated and the validation was audited by the Quality Assurance Unit. Validation consisted of testing the individual components of the system as described in Standard Operating Procedure 8.2. Statistical treatment of the data included determination of averages and standard deviations for the recovery data.

14.4 ACCURACY AND PRECISION

Accuracy is a measure of the difference between the determined and actual true values. It is calculated as the relative error and is defined as the nearness of the analytical measurement to its accepted value. The standard deviation (square root of variance) is an estimate of precision. Precision is a measure of the scatter in repeated determinations from repeated chromatographic measurements. The accuracy and precision (percent recovery standard deviation) for analyses of soil fortified with prohexadione-calcium, prohexadione, and despropionyl-prohexadione in the range of 0.01 ppm to 1.0 ppm were 85.7 +/- 14.8%, 90.1 +/- 14.2%, and 71.3 +/- 5.65%, respectively. Summary of fortification recoveries from method validation is presented in Table III. Fortification recoveries by compound from method validation are found in Tables IVa, IVb, and IVc. Information on standards injected is presented in Figure 3b. The detailed results are given in Appendix 1.

Additional fortification recoveries from two soil dissipation studies (Protocols B96014 and B96015) currently underway are included. Summary recovery tables with the initial fortification recoveries only, for a total of five sites, i.e. RCN 96041 North Carolina, RCN 96042 Texas, RCN 96043 New York, RCN 96044 California and RCN 96045 Oregon, can be found in Table V. Individual recoveries can be found by site in Tables VI, VII, VIII, IX and X. Detailed results can be found in Appendix 2.

14.5 LIMIT OF DETECTION AND QUANTITATION

The limit of detection for prohexadione and despropionyl-prohexadione is set to 0.005 ppm or 5.0 ng, based on standards injected at half the quantitation limit and it was not experimentally determined. The limit of quantitation of the method is 0.01 ppm for prohexadione, prohexadione-Ca, and despropionyl-prohexadione.

14.6 CONCLUSION

Analytical Method No. D96054 is a valid and accurate method for determining residues of prohexadione-calcium, prohexadione, and despropionyl-prohexadione in soil.

15.0 RUGGEDNESS TESTING

This method has been used successfully at ADPEN Laboratories, Inc. for the analysis of soil samples from North Carolina, Texas, New York, California and Oregon. Over 1000 samples have been analyzed for soil dissipation studies[1, 2] using this method. Summary and detailed results of initial fortification recoveries from the two soil dissipation studies (Protocols B96014 and B96015) are given in Appendix 2. This method has also been used at BASF for initial ruggedness testing.

16.0 MODIFICATIONS

Any modifications to this method should be validated with a recovery test and the modifications shall be listed in the raw data.

17.0 STUDY PERSONNEL

Agnes Garcia	Chemist, ADPEN Laboratories, Inc.
Gunar McKendree	Associate Chemist, ADPEN Laboratories, Inc.
Rolando Perez	Project Coordinator, Lab Manager/Technical Director, ADPEN Laboratories, Inc.
Anthony Truitt	Analytical Chemist, ADPEN Laboratories, Inc.
Winda Z. Negron	Sample Custodian, ADPEN Laboratories, Inc.
Greg Waitt	Chemist, BASF Corporation
Jayanti Patel	Sr. Research Associate, BASF Corporation

18.0 CERTIFICATION

I, the undersigned, hereby declare that the data referenced in this report was generated under my supervision according to the procedure described herein. The data and experimental results reported in this document are certified to be authentic accounts of the experiments conducted at ADPEN Laboratories, Inc.

Rolando Perez
Principal Investigator,
Lab Manager/Technical Director
ADPEN Laboratories, Inc.

6/10/97
Date

Jayanti R. Patel, Ph.D.
Study Director
BASF Corporation

6/20/97
Date

Gregg Waitt
Analytical Chemist
BASF Corporation

6/18/97
Date

The original raw data and exact copies of facility related data pertaining to this study will be maintained by BASF Corporation, 26 Davis Drive, Research Triangle Park, NC 27709. Facility related data will be maintained in the archives at ADPEN Laboratories, Inc., 11757 Central Parkway, Jacksonville, FL 32224.

19.0 REFERENCES

1. BASF Study "Soil Dissipation of BAS 125...W in Terrestrial Use Patterns (Peanuts)," BASF Study No. B96014, in progress, 1997.

2. BASF Study "Soil Dissipation of BAS 125...W in Terrestrial Use Patterns (Apple)," BASF Study No. B96015, in progress, 1997.

TABLE I. Description of Test and Reference Substances

Test and Reference Substances:

a. Technical Grade Prohexadione-Calcium (BAS 125 W):

1.	Common Name:	Prohexadione-Calcium
2.	BASF Code:	BAS 125 W
3.	Chemical Name:	Calcium 3-oxido-4-propionyl-5-oxo-3-cyclohexenecarboxylate
4.	CAS Number:	127277-53-6
5.	Empirical Formula:	$C_{10}H_{10}O_5Ca$
6.	Molecular Weight:	250.3
7.	Lot Number:	G14-40-101
8.	Melting Point:	No physical changes up to 360° C.
9.	Appearance:	White, solid
10.	Odor:	Odorless
11.	Structure:	See Figure 2
12.	Purity / Expiration:	90.6% as Ca-salt [or 76.8% as Prohexadione (free acid) equivalent] / June 28, 1998

b. Prohexadione (free acid) standard:

1.	Common Name:	Prohexadione (free acid)
2.	BASF Code:	KI-2817
3.	Chemical Name:	3-hydroxy-4-propionyl-5-oxo-3- cyclohexene-1 carboxylic acid
4.	CAS Number:	88805-35-0
5.	Empirical Formula:	$C_{10}H_{12}O_5$
6.	Molecular Weight:	212
7.	Lot Number:	L43-271
8.	Purity / Expiration Date:	99.7% / August 1997
9.	Structure:	See Figure 2

c. Despropionyl-Prohexadione standard:

1.	Common Name:	Despropionyl-Prohexadione
2.	BASF Code:	KI-5376
3.	Chemical Name:	3-hydroxy-5-oxo-3-cyclohexene-1-carboxylic acid
4.	Empirical Formula:	$C_7H_8O_4$
5.	Molecular Weight:	156
6.	Lot Number:	G14-33-141
7.	Purity / Expiration Date:	99.8% / August 11, 1996; 98.98% / September 1997 a/
8.	Structure:	See Figure 2

a/ Reassayed on 10/7/96. Dilutions and working standards not corrected for new reassayed percent purity value

Table IIa. Suggested HPLC Conditions for the Analysis of Prohexadione

HPLC Analysis - Column Switching

Precolumn:	100 x 4.6 mm, Hypersil C18, 3 micron (Alltech)
Precolumn Flow:	1.0 mL/min.
Mobile Phase:	Acetonitrile:1% acetic acid in HPLC water (1:4)
Injection Volume:	500 uL
Column cut:	Approx. 7.6 to 8.5 minutes. See Section 9.0
Analytical Column:	250 x 4.6 mm, Kromasil C18, 5 micron (Alltech)
Analytical Column Flow:	1.0 mL/min.
Mobile Phase:	Acetonitrile:1% acetic acid in HPLC water (30:70)
Wavelength:	274 nm
Retention Time:	17.0 minutes

Typical Calibration Standards: 0.01, 0.02, 0.05, and 0.1 ug/mL

Note A: Column cut window should be checked before each run to compensate for chromatography changes. It is highly recommended to wash precolumn with 100% acetonitrile while the analyte is eluting off the analytical column. Be sure to allow enough time for the column to re-equilibrate for the next injection.

Note B: Suggested conditions for precolumn wash: After column cut, wash 6 minutes with 100% acetonitrile at 1.0 mL/min., then return to mobile phase until end of run (22 minutes suggested to allow column to re-equilibrate).

Table IIb. Suggested HPLC Conditions for the Analysis of Despropionyl-Prohexadione

HPLC Analysis - Column Switching

Precolumn:	100 x 4.6 mm, Hypersil C18, 3 micron (Alltech)
Precolumn Flow:	1.0 mL/min
Mobile Phase:	Acetic acid : HPLC water (1 :99)
Injection Volume:	500 uL
Column cut:	Approx. 5.5 to 7.0 minutes. See Section 9.0
Analytical Column:	250 x 4.6 mm, Kromasil C18, 5 micron (Alltech)
Analytical Column Flow:	1.0 mL/min.
Mobile Phase:	Acetonitrile:1% acetic acid in HPLC water (3:97)
Wavelength:	260 nm
Retention Time	Approximately 16.0 minutes

Typical Calibration Standards: 0.005, 0.01, 0.02, and 0.05 ug/mL

Note A: Column cut window should be checked before each run to compensate for chromatography changes. It is highly recommended to wash precolumn with 100% acetonitrile while the analyte is eluting off the analytical column. Be sure to allow enough time for the column to re-equilibrate for the next injection.

Note B: Suggested conditions for precolumn wash: After column cut, wash 6 minutes with 100% acetonitrile at 1.0 mL/min., then return to mobile phase until end of run (22 minutes suggested to allow column to re-equilibrate).

Note C: Suggested procedures for establishing the time window in the column switching steps (from Section 9.0):

Establish the retention time of the compound of interest (prohexadione or despropionyl-prohexadione) on the precolumn using the appropriate precolumn mobile phase and flow rate. This is done by connecting the outlet of the precolumn to the detector

Table IIb. Suggested HPLC Conditions for the Analysis of Despropionyl-Prohexadione (Continued)

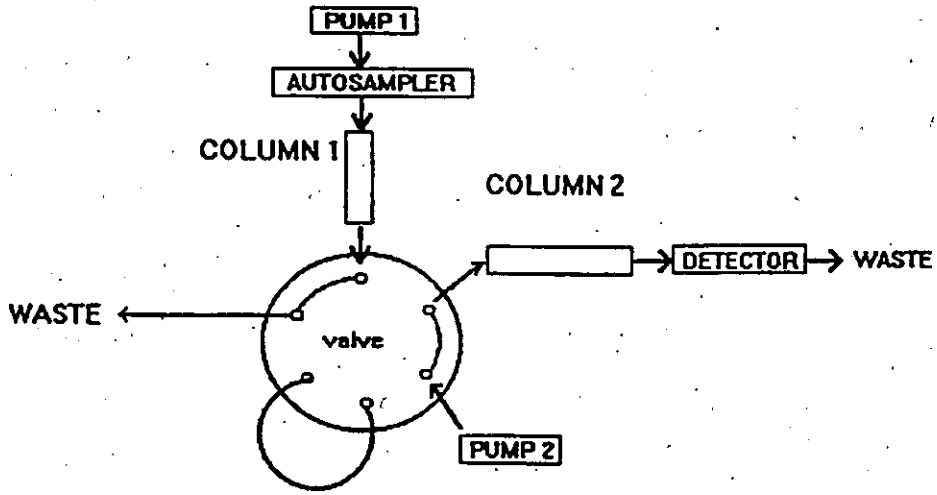
and injecting a standard from 3 to 5 times. For each run, the precolumn should be rinsed with 100% acetonitrile for about 6 minutes after elution of the standard, and then return to the mobile phase for the duration of the run (20 to 22 minutes). It is important to duplicate the precolumn conditions that will be used for the sample analysis. The retention time of the compound should be fairly consistent after the first injection. The first injection will normally differ from the others. The last 2 to 4 retention times should not vary by more than 0.1 minutes. This retention time determination should be done just before analysis of a set of samples. The switching valve time should start about 0.1 to 0.2 minutes before the start of the peak and end about 0.1 to 0.2 minutes after the end of the peak. The above timing may change depending on the length of tubing used between the column and switching valve or detector and may be determined experimentally. Do not set the time window too tight because some shifting is common. The time window for prohexadione can be set somewhat tighter than despropionyl-prohexadione because it tends to be more consistent and there are more interferences if set too wide.

Connect the precolumn (#1) and the analytical column (#2) to the switching valve as in the next diagram. In the load position, the precolumn is connected to waste and the analytical column is connected to the detector. At the start time of the column switching, the valve is set to injection position, and the eluant from Column #1 is connected to the front of Column #2. At the end time of the column switching, Column #1 is switched back to wash. While the analyte elutes from Column #2, Column #1 is rinsed with 100% acetonitrile for about 6 minutes, then with Column #1 mobile phase until the end of the run (7.0 to 22 minutes). Make sure there is enough time for Column #1 to re-equilibrate before making the next injection.

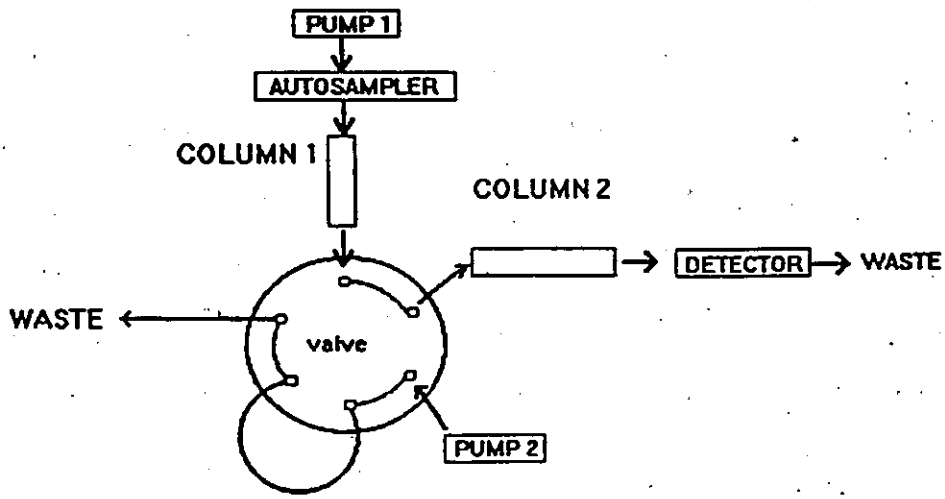
See Table IIc. for schematic diagram for the HPLC system of the column switching method.

With some pumps, if the pump shuts down when pressure is very low, an old column connected to waste may be used to maintain back pressure when pump 2 is switched to wash. This was not used with the Hewlett-Packard HPLC systems.

Table IIc. Schematic Diagram of the HPLC Column Switching System



Flow diagram when the valve is in load position.



Flow diagram when the valve is in inject position.

Table III. Summary of Fortification Recoveries from Method Validation

OVERALL RECOVERY RESULTS		
Prohexadione-Calcium (BAS 125 W)	**Prohexadione (free acid)**	**Despropionyl-Prohexadione (KI-5376)**
Average: 85.7	Average: 90.1	Average: 71.3
Std. Dev.: 14.8	Std. Dev.: 14.2	Std. Dev.: 5.65
n = 12	n = 18	n = 24

See Tables IVa, IVb and IVc for fortification recoveries details from the method validation.

Table IVa. Fortification Recoveries of Prohexadione-Calcium (BAS 125 W) in Soil from Method Validation

PERCENT RECOVERY OF PROHEXADIONE-CALCIUM (BAS 125 W) a/					
FORTIFICATION LEVEL					
0.01 PPM	Lab Code Number	0.10 PPM	Lab Code Number	1.0 PPM	Lab Code Number
68.9	96-BF54-10	62.1	96-BF54-12	85.9	96-BF54-14
74.0	96-BF54-11	85.7	96-BF54-13	83.0	96-BF54-15
87.3	96-BF54-17	95.1	96-BF54-19	92.1	96-BF54-21
121.6	96-BF54-18	88.7	96-BF54-20	84.4	96-BF54-22
Average: 88.0		Average: 82.9		Average: 86.4	
Std. Dev.: 23.7		Std. Dev.: 14.4		Std. Dev.: 4.01	
n = 4		n = 4		n = 4	
Overall: Average 85.7 / Std. Dev. 14.8 / n = 12					

Detailed results are in Appendix 1.

Values in this table may have been rounded off for reporting purposes, but not for any further calculations.

a/ Lab Code numbers can be referenced to detailed results in Appendix 1.

Table IVb. Fortification Recoveries of Prohexadione (Free Acid) in Soil from Method Validation

PERCENT RECOVERY OF PROHEXADIONE (free acid) a/					
FORTIFICATION LEVEL					
0.01 PPM	**Lab Code Number**	**0.10 PPM**	**Lab Code Number**	**1.0 PPM**	**Lab Code Number**
104.6	96-EXP-31	90.4	96-EXP-33	89.8	96-EXP-35
103.0	96-EXP-32	91.0	96-EXP-34	90.3	96-EXP-36
107.5	96-BF54-03	82.1	96-BF54-05	92.3	96-BF54-07
64.6	96-BF54-04	66.2	96-BF54-06	91.9	96-BF54-08
106.3	96-BF54-25	69.5	96-BF54-27	71.2	96-BF54-29
101.9	96-BF54-26	106.5	96-BF54-28	93.0	96-BF54-30
Average: 98.0		Average: 84.3		Average: 88.1	
Std. Dev.: 16.5		Std. Dev.: 15.0		Std. Dev.: 8.36	
n = 6		n = 6		n = 6	
Overall: Average 90.1 / Std. Dev. 14.2 / √n = 18					

Detailed results are in Appendix 1.

Values in this table may have been rounded off for reporting purposes, but not for any further calculations.

a/ Lab Code numbers can be referenced to detailed results in Appendix 1.

Table IVc. Fortification Recoveries of Despropionyl-Prohexadione (KI-5376) in Soil from Method Validation

PERCENT RECOVERY OF DESPROPIONYL-PROHEXADIONE (KI-5376) a/					
FORTIFICATION LEVEL					
0.01 PPM	Lab Code Number	0.10 PPM	Lab Code Number	1.0 PPM	Lab Code Number
70.9	96-EXP-31	77.9	96-EXP-33	74.8	96-EXP-35
73.2	96-EXP-32	76.1	96-EXP-34	78.3	96-EXP-36
76.4	96-BF54-33	66.8	96-BF54-35	73.6	96-BF54-37
67.6	96-BF54-34	65.7	96-BF54-36	65.2	96-BF54-38
69.4	96-BF54-47	60.1	96-BF54-49	64.2	96-BF54-51
69.0	96-BF54-48	63.7	96-BF54-50	66.8	96-BF54-52
70.8	96-BF54-55	72.2	96-BF54-57	80.4	96-BF54-59
74.3	96-BF54-56	71.6	96-BF54-58	81.9	96-BF54-60
Average: 71.5		Average: 69.3		Average: 73.2	
Std. Dev.: 2.96		Std. Dev.: 6.20		Std. Dev.: 6.99	
n = 8		n = 8		n = 8	
Overall: Average 71.3 / Std. Dev. 5.65 / n = 24					

Detailed results are in Appendix 1.

Values in this table may have been rounded off for reporting purposes, but not for any further calculations.

a/ Lab Code numbers can be referenced to detailed results in Appendix 1.

Figure 1. FLOW CHART OF ANALYTICAL PROCEDURE

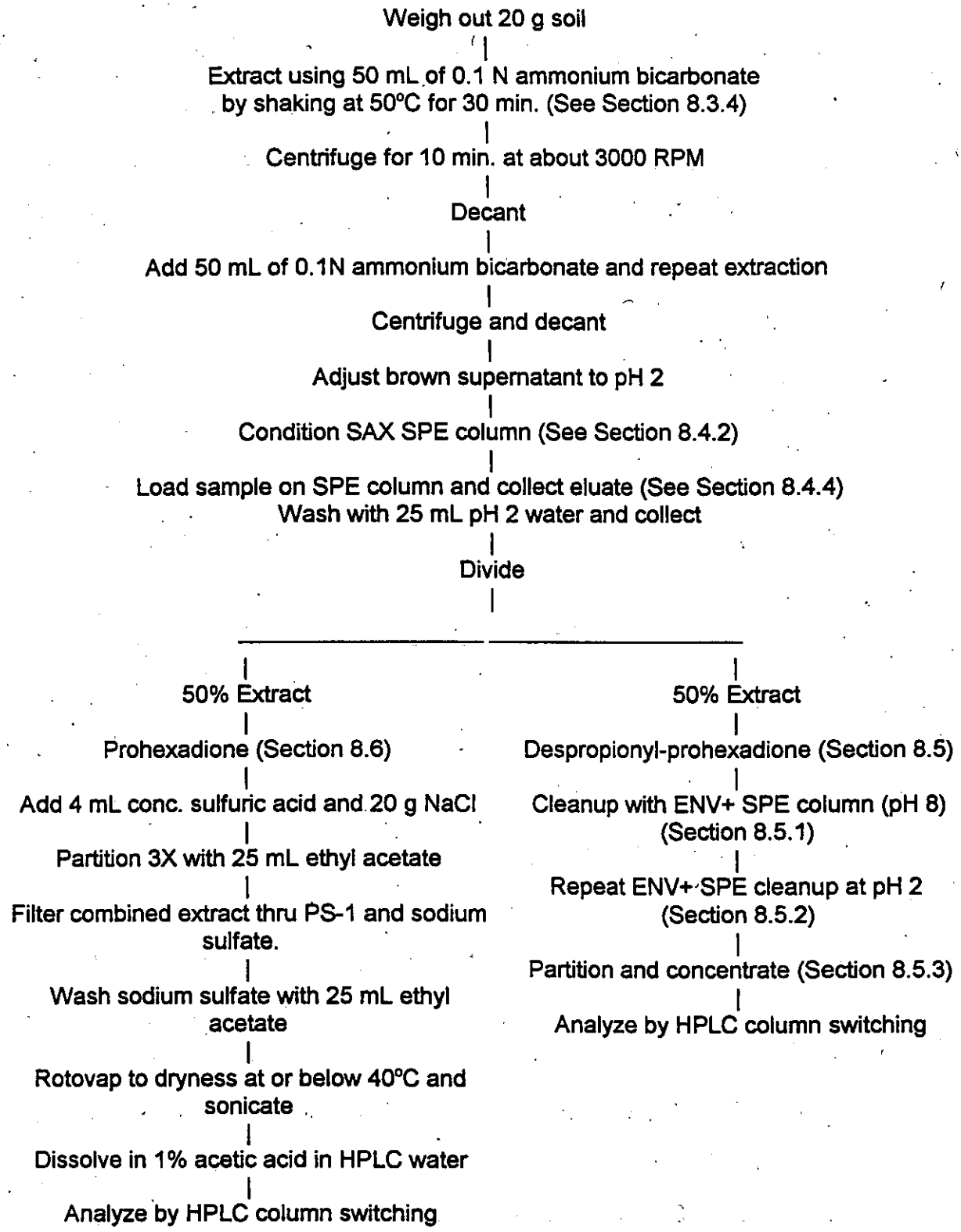
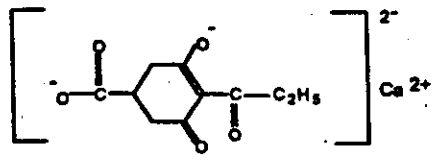
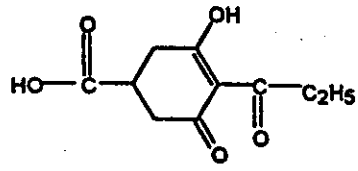


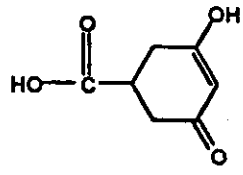
Figure 2. Structures of the Test Substance and the Final Analytes



Prohexadione-Calcium



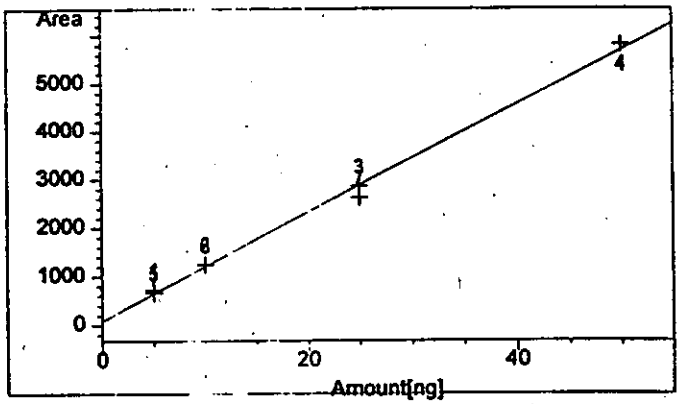
Prohexadione (free acid)



Despropionyl-Prohexadione

Figure 3a. Typical Standard Curves

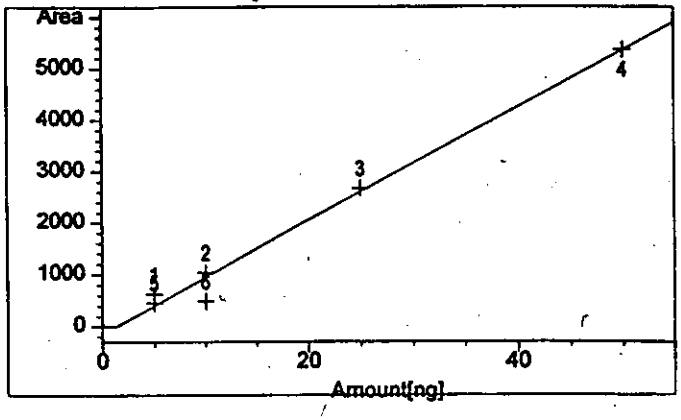
Sequence 5EX0910



PROHEXADIONE at exp. RT: 17.092
ADC1 A, ADC1 CHANNEL A
Correlation: 0.99767
Residual Std. Dev.: 136.26365
Formula: y = mx + b
m: 111.79877
b: 91.41043
x: Amount [ng]
y: Area

Prohexadione

Sequence 5EX0909

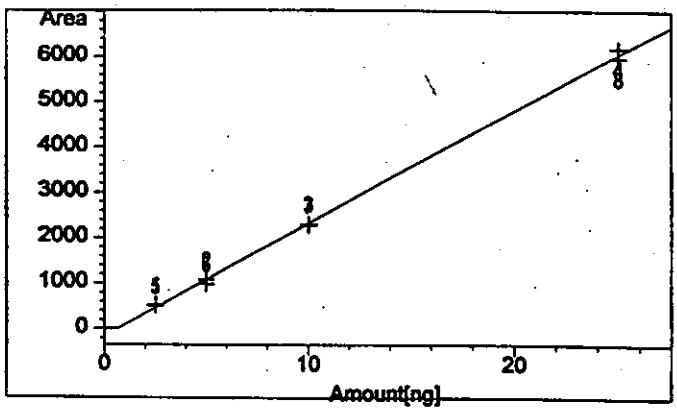


PROHEXADIONE at exp. RT: 17.482
ADC1 A, ADC1 CHANNEL A
Correlation: 0.99267
Residual Std. Dev.: 262.83799
Formula: y = mx + b
m: 110.07821
b: -154.87183
x: Amount [ng]
y: Area

Prohexadione

Figure 3a. Typical Standard Curves (Continued)

Sequence 5KI1009

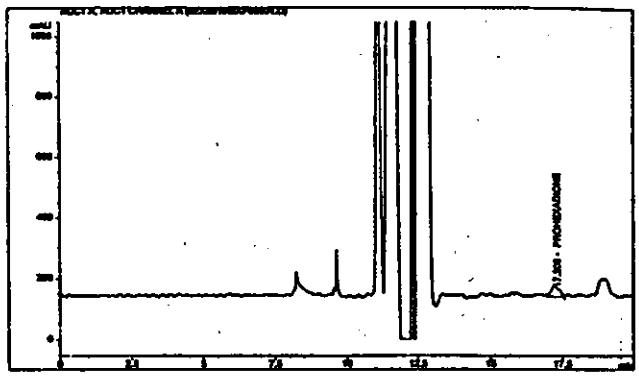


KI-5376 at exp. RT: 15.246
ADC1 A, ADC1 CHANNEL A
Correlation: 0.99943
Residual Std. Dev.: 84.95244
Formula: y = mx + b
m: 248.96603
b: -155.44620
x: Amount [ng]
y: Area

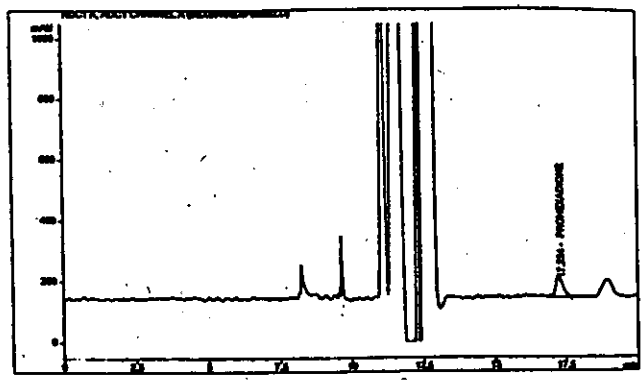
Despropionyl-Prohexadione

Figure 3b. Typical Standard Chromatograms

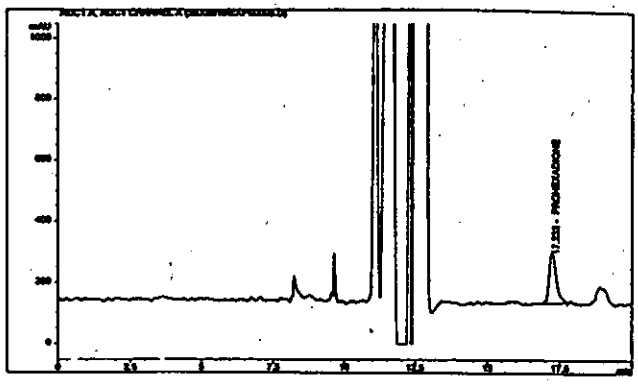
Prohexadione - Sequence 5EX0910



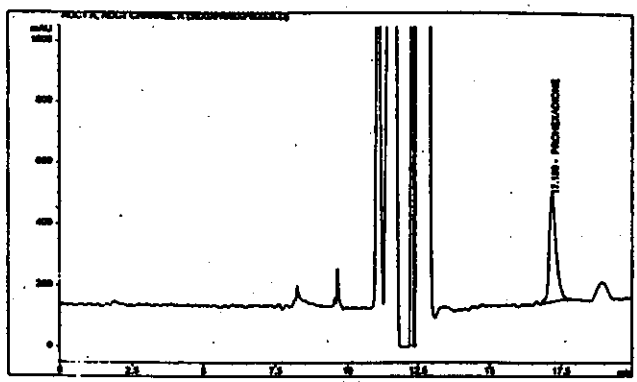
5.0 ng Standard
Peak Area: 724.925



10.0 ng Standard
Peak Area: 1259.287



25.0 ng Standard
Peak Area: 2861.214



50.0 ng Standard
Peak Area: 5800.699