1. INTRODUCTION AND SUMMARY

1.1 Scope and Source of the Method

1.1.1 Scope

This method is used to determine the trace residues of 3-hydroxy-2-(1-iminopropyl)-5-(tetrahydropyran-4-yl)cyclohex-2-en-1-one (DP-1) and 2-ethyl-6-(tetrahydropyran-4-yl)-4, 5, 6, 7-tetrahydrobenzoxazol-4-one (DP-2) in soil. Caloxydim is the active ingredient and DP-1 and DP-2 are two major metabolites found in several environmental fate studies(Reference 1).

1.1.2 Source

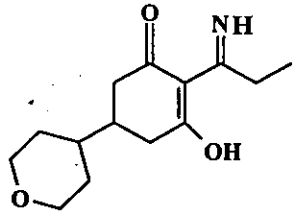
This method was developed at BASF Corporation, RTP, NC.

1.2 Description of Test and Reference Substances

Fortification and HPLC standards

BASF Code: DP-1
Chemical name: 3-hydroxy-2-(1-iminopropyl)-5-(tetrahydropyran-4-yl)cyclohex-2-en-1-one

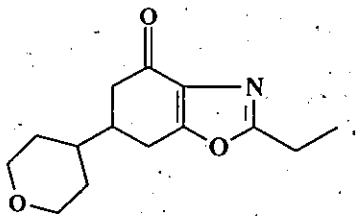
Structural formula:



Emperical formula: $C_{14}H_{21}NO_3$
Molecular weight: 251.33
Appearance: White solid

1.2 **Description of Test and Reference Substances,** continued

BASF Code: DP-2
Chemical name: 2-ethyl-6-(tetrahydropyran-4-yl)-4, 5, 6, 7-tetrahydrobenzoxazol-4-one
Structural formula:



Emperical formula: $C_{14}H_{19}NO_3$
Molecular weight: 249.31
Appearance: White solid

1.3 **Principle of the Method**

Residues of DP-1 and DP-2 are extracted from soil with methanol-water (75:25, v/v) using a homogenizer. The extracts are then filtered through celite and concentrated to dryness. The dry residues are further cleaned up by C18 SPE. Final determination is made by HPLC using UV detector. See Figure 1 for a flow chart of the analytical method. The limit of quantitation is 0.01 ppm for both analytes.

2. MATERIALS AND METHODS

2.1 **Equipment**

	Suggested Sizes/Manufacturer
Centrifuge	IEC Refrigerated Centrifuge Model PR 7000 Beckmann Refrigerated Centrifuge Model CS-6KR
Centrifuge Bottles, Teflon-lined screw cap	Fisher Scientific Co. 150 mL
Empty glass SPE-column (8 mL) (with Teflon frits)	Baker Chemical Co. Cat No. 7308-06
Filtering flask	Fisher Scientific Co. 1000 mL

MATERIALS AND METHODS, continued

Homogenizer and accessories	Omni International Omni 5000 or Omni GLH 115, Generator (G20-195 ST,20 mm i.d X 198 mm l)
Inert Sampling Adapter, B & J	Burdick & Jackson Cat No. 9473A
Laboratory Shaker	Janke and Kunkel Model HS501-D
Rotary evaporator	Büchi Rotovapor Model RE 111, 114
Standard taper Flat-bottom flasks	VWR Scientific Co. 500 mL, 300 and 125 mL
Solid Phase Extraction Manifold	Baxter Healthcare Corporation
Standard funnels	Fisher Scientific Co.
Fritted (medium porous, about 40-60 mm) funnels, 60 mL	Fisher Scientific Co.
Ultrasonic Bath	Fisher Scientific Co., Model FS-14
Vacuum distilling Vacubrand vacuum pump/controller	Elnik Systems, Inc.
Vacuum distilling adapter	Aldrich Chemical Co. ACS p Cat. No. Z17,067-4

Other general laboratory glassware and supplies as needed.

NOTE: The equipment listed in this section was used in the development of this method. Equipment with equivalent performance may be used as required.

MATERIALS AND METHODS, continued

2.2 Reagents and Chemicals	Source/Preparation
Acetonitrile, CAS 75-05-8	Baxter Healthcare Corporation, B & J Brand
Celite 545®	J. T Baker Cat. No. 3371-01
Formic acid (88%), CAS 64-18-6	Aldrich Chemical Co. ACS Cat. No. 39, 938-8
Gelman Nylon acrodisc (Membrane disc0.45 um)	VWR/ Catalog No 28143-948)
Methanol, CAS 67-56-1	Baxter Healthcare Corporation, B & J Brand
Reversed phase silica gel (C18 bulk pack, 40 µm)	J.T. Baker Cat No. 7025-00
Water, CAS 7732-18-5	Baxter Healthcare Corporation, B & J Brand

NOTE: Equivalent reagents and chemicals from other suppliers may be substituted.

2.3 Standard Substances and Solutions

The standard compounds shown in the table below were used for method development and validation.

Compound	Code	Lot Number	Purity, %
3-hydroxy-2-(1-iminopropyl)-5-(tetrahydropyran-4-yl)cyclohex-2-en-1-one	BH 620-DP1	00345-268	97. 9 %
2-ethyl-6-(tetrahydropyran-4-yl)-4, 5, 6, 7-tetrahydrobenzoxazol-4-one	BH 620-DP2	00665-27	98.3 %

Standard supplied by:

Dr. Rita Laschober
BASF Aktiengesellschaft, APS/UP
Agricultural Research Center
D-67114 Limburgerhof, West Germany
Telephone: 06236/68/2103

MATERIALS AND METHODS, continued

Solid 3-hydroxy-2-(1-iminopropyl)-5-(tetrahydropyran-4-yl)cyclohex-2-en-1-one (DP-1), 2-ethyl-6-(tetrahydropyran-4-yl)-4, 5, 6, 7-tetrahydrobenzoxazol-4-one (DP-2) were maintained frozen (<-5°C) until their use in this study. These substances were characterized as required by 40 CFR part 160, FIFRA Good Laboratory Practices. Data on the synthesis and subsequent characterization of these substances are available to BASF and are located at Landwirtschaftliche Versuchsstation der BASF, Limburgerhof, Germany.

Solutions of DP-1 and DP-2 were refrigerated (+4°C) during their use in this study. Stock solutions (1 mg/mL) of DP-1 and DP-2 were made fresh every three months and dilutions of the stock solution were made monthly.

Solution Stability Note: Methanol solutions of DP1 and DP-2 are kept in the dark at 4°C were stable for at least 103 days. A statement concerning the stabilities of solutions used in the method validation study for this report is given in Section 5.2.

2.3.1 Standard Solutions for Fortifications

NOTE: These standard concentrations are suggested. A different concentration scheme may be used and additional standards may be prepared as needed.

2.3.1.1 3-hydroxy-2-(1-iminopropyl)-5-(tetrahydropyran-4-yl)cyclohex-2-en-1-one(DP-1): 1.0 mg/mL in Methanol

Prepare a 1.0 mg/mL stock solution by weighing an appropriate amount of DP-1 into a volumetric flask and dissolving it with an appropriate amount of methanol. For example, to prepare a 10 mL stock solution, dissolve 10.0 mg of DP-1 in 10 mL of methanol in 10 mL volumetric flask.

2.3.1.2 2-ethyl-6-(tetrahydropyran-4-yl)-4,5,6,7-tetrahydrobenzoxazol-4-one(DP2): 1.0 mg/mL in methanol

Prepare a 1.0 mg/mL stock solution by weighing an appropriate amount of DP-2 into a volumetric flask and dissolving it with an appropriate amount of methanol. For example, to prepare a 10 mL stock solution, dissolve 10.0 mg of DP-2 in 10 mL of methanol in a 10 mL volumetric flask.

2.3 **Standard Substances and Solutions**, Continued

2.3.1.3 Mix Standard Solutions of 3-hydroxy-2-(1-iminopropyl)-5-(tetrahydropyran-4-yl)cyclohex-2-en-1-one(DP-1)and 2-ethyl-6-(tetrahydropyran-4-yl)-4,5,6,7-tetrahydrobenzoxazol-4-one(DP2): 50 µg/mL in methanol.

Prepare a 50 µg/mL of mixed standard solution by transferring an appropriate amount of each of the 1.0 mg/mL stock solutions(2.3.1.1 and 2.3.1.2) with a volumetric pipette into a volumetric flask (typically 5.0 mL of each of the 1.0 mg/mL stock solutions in a 100 mL volumetric flask). Dilute to the mark with methanol.

Prepare a 5.0 and 0.5 µg/mL of mixed standard solution by making appropriate dilutions from the 1.0 µg/mL of fortification standard solution with methanol.

Transfer each stock and standard solution to an amber bottle fitted with a Teflon-lined screw cap and store at refrigerator. Replace stock solution 90 days after preparation. Replace standard solutions 30 days after preparation.

2.3.1.4 Injection Standard Solutions of DP-1 and DP-2 for HPLC-UV Analysis in 1:1 Acetonitrile-Water: 1.0, 0.5, 0.25 and 0.125 µg/mL

Prepare a 1.0 µg/mL of injection standard solutions by transferring an appropriate amount of the 50.0 µg/mL mixed standard solution with a volumetric pipette into a volumetric flask. Typically add 1.0 mL of the 50.0 µg/mL standard solution into a 50 mL volumetric flask. Add 1.0 mL of water and then dilute to the mark with **1:1 acetonitrile-water**.

Prepare a 0.5, 0.25 and 0.125 µg/mL of injection standard solutions by making dilutions from the 1.0 µg/mL of injection standard solution with 1:1 acetonitrile-water.

For HPLC analysis, inject 50.0 µL of the mixed DP-1 and DP-2 standard solutions of concentrations 0.125, 0.25, 0.5 and 1.0 µg/mL to construct a standard curve. This is a suggested calibration scheme and may be altered as needed.

3. **ANALYTICAL PROCEDURE** (See Figure 1, Flow chart for Analytical Method D9517)

3.1 Parent and Metabolite Isolation and Cleanup

3.1.1 Sample Preparation

Bulk soil samples received from the field are homogenized using a blender or mill. Homogenized soil samples are stored frozen (<-5°C) before analysis. Weigh a 50 g or to the nearest tenth of a gram aliquot of the soil sample into a 150 mL centrifuge bottle.

3.1.2 Fortification of Procedural Recovery Sample

It is recommended to analyze at least two procedural recovery samples and one untreated sample (control) with each analysis set to monitor method efficiency. Typically, run one procedural recovery sample at the limit of quantitation (0.01 ppm) along with one procedural recovery sample at the expected residue level.

For each fortification, pipette an appropriate amount of standard DP-1 and DP-2 fortification solutions prepared in 2.3.1.3 to control soil samples. For example, 1.0 mL of the 5.0 µg/mL standard added to 50 g soil results in a fortification level of 0.1 ppm.

3.1.3 Extraction

Add 80 mL methanol-water (75:25) to the soil samples and extract with homogenizer for 5 minutes (about 6000 RPM). Stop the homogenizer and raise the blade slightly (just enough that the blade still remain in the solution) and turn on the homogenizer for a minute to rinse the main soil clogging in the blade. Raise the blade completely out of the solution and carefully rinse the homogenizer blade with about 20 to 30 mL methanol-water (75:25).

NOTE: Soil clogs in the generators. It is important to rinse this thoroughly and have to be done as soon as polytron is stopped. Rinse in and out of the generators as much as possible with methanol water (75:25).

Cap the centrifuge bottle and vortex for 2 to 3 minutes in a vortex mixer and then shake at 300 rpm for 10 minutes. Cap and centrifuge at 3000 rpm for 10 min. (0°C). Transfer the supernatant into a 500 mL flat bottom flask by decantation.

Add 50 mL methanol-water (75:25) to the soil marc and repeat above extraction. Transfer the supernatant into the above 500 mL flat bottom flask by decantation.

3. **ANALYTICAL PROCEDURE**, Continued

Add another aliquot of 50 mL methanol-water (75:25) to the soil marc and repeat above extraction. Transfer the supernatant into the above 500 mL flat bottom flask by decantation.

Note: In repeat extractions marc settles very tightly at the bottom of the centrifuge bottle due to centrifugation. Lower down the polytron generator all the way until it hits the marc (analyst will feel a resistanse and perhaps it is touching the bottom of the centrifuge bottle, but generator will just penetrate the soil marc) and continue the extraction. Soil Extract after third extraction looks cloudy (specially for clay soil)

Attach a vacuum filtration adapter to a 500 mL flat bottom flask and connect a fritted filtering funnel (60 mL size) to the top of the vacuum filtering adapter. Add enough celite to cover one third of the fritted filtering funnel. Suction filter the combined extracts through the bed of celite. Rinse the flask and celite bed thoroughly with methanol (4 X 20 to 30 mL). Release the suction and rinse the stem of the fritted filtering funnel and vacuum filtering adapter with methanol.

NOTE: Filtering flask with side arm can also be used for filtration. It is recommended **not to use Buchner funnel with filter paper** for the filtration due to escape of the silt through the celite.

Concentrate the combined extract to about 20 to 30 mL using a rotary evaporator with the water bath temperature set at approximately 60°C (set vacuum initially at about 200 mbar until the removal of methanol and then gradually decrease to about 35 to 40 mbar). Transfer the contents in to a 125 mL round bottom flask (use about 10 to 20 mL methanol to rinse) and evaporate nearly to dryness with a bath temperature maintained at 60°C (vacuum at about 35 to 45 mbar).

NOTE: **The extracts needs to evaporate *nearly to dryness* with few drops (100 ul) to maximum of 1 ml left in the flask.**

3.1.4 Sample Clean-up: RP C18 Solid Phase Extraction

3.1.4.1 Column Preparation

Add 1.0 g of C18 silica gel to an empty glass column fitted with a Teflon frit. Pack glass wool (enough to leave half of the column space empty) tightly on the top of the C18 silica gel and add

3. **ANALYTICAL PROCEDURE**, Continued

celite on the top (enough to leave 1.0 cm space of the column empty). Add a sampling adapter on the top of the column to connect an additional empty glass reservoir.

3.1.4.2 Column conditioning

NOTE: Vacuum is used for chromatography using a solid phase extraction manifold.

Condition C18 silica gel by passing through approximately 10 mL acetonitrile followed by approximately 25 mL water. Do not allow the column to go dry. The applied vacuum should be sufficient to permit flow rates no more than 2-5 mL/min. Vacuum readings of about 10 kPa (2-5 inches of mercury) have been adequate.

3.1.4.3 Loading, Washing and Elution

Add 1 mL acetonitrile to the sample from section 3.1.3, swirl, sonicate and vortex thoroughly to dissolve the residue from the side of the flask as much as possible before addition of 50 mL of water. Add 50 mL water, sonicate thoroughly **[Duration of sonication depends on soil type]** to ensure the dissolution of the residues from the side of the flask and quickly transfer (load) to the top of the conditioned C18 silica gel column column. Allow the solvent to pass through the column using vacuum. Collect all eluant in a suitable container (250 mL beaker)and discard. Do not allow the column to go dry.

Wash the flask with 50 mL water and add this wash to the column.

Wash the flask with 25 mL 2% acetonitrile-water, v/v and add this wash to the column to further wash the column. Discard all the acetonitrile-water wash.

Add 50 mL of 60:40 acetonitrile-water, v/v to the flask and add to the top of the column. Collect the solvent in a 50 ml brown Teflon-lined screw cap bottle and transfer to a 125 mL flat bottom flask.

NOTE: We found during method development, elution solvent with less percentage of acetonitrile e.g 40:60;v/v acetonitrile-water will also work. But 60:40 v/v acetonitrile-water was required for clay type soil to get high recovery of DP-2.

Evaporate the solution to dryness using a rotary evaporator with the water bath temperature set at approximately 60°C (set vacuum

3. **ANALYTICAL PROCEDURE**, Continued

initially set at about 200 mbar to remove acetonitrile and then gradually decrease to about 35 to 40 mbar). Remove the sample immediately after evaporation.

3.1.5 Preparation for Sample Analysis

For HPLC-UV determination, dissolve each sample with an appropriate amount of mobile phase (30:70 v/v, acetonitrile-water containing 0.1 % formic acid) just before the analysis. Typical volumes used are 2.0 mL for control and 0.01 ppm fortifications; 10 mL for 0.1 fortifications; and 50 ml for 1 ppm fortifications. For example for samples at the limit of quantitation (0.01 ppm), the final volume is 2.0 mL. Add mobile phase (30:70 v/v, acetonitrile-water containing 0.1 % formic acid, 2.0 mL), **swirl**, sonicate and vortex to ensure complete dissolution of sample from the side of the flask. Filter the solution through a membrane syringe filter (a 0.45 micron membrane disc fitted to a 1.0 mL disposable plastic syringe and samples were transferred with an aid of glass disposable pipette to the plastic disposable syringe) into the injection vial.

3.1.6 Moisture Determination

Soil analysis results are reported on a "dry weight" basis. Therefore soil sample weights must be corrected for moisture content by any method the laboratory customarily uses. See section 12, Note 2 for an example of a moisture determination procedure.

3.2 Instrumentation

3.2.1 Description of Equipment

Liquid Chromatograph:	Varian 9010 pump, Varian 9050 Detector, Varian 9100 Autosampler and VG Data System Multichrom 2 data processing System.
HPLC Column:	Nucleosil 5, C18, 250 mm X 3.2 mm

NOTE: The equipment listed was used for method development. Equivalent equipment may be used.

3.2.2 Typical Operating Conditions

Injection Volume:	50 µl

3. **ANALYTICAL PROCEDURE,** Continued

Isocratic Mobile Phase:	30 % Acetonitrile + 70 % water containing 0.1 % formic acid
Flow Rate:	0.5 ml/min.
Wavelengh (λmax):	254 nm.
DP-1 Retention Time:	6.5 min.
DP-2 Retention Time:	9.4 min.

NOTE: The preceding specifications are suggested and may be altered as needed. Actual use conditions and any changes must be documented.

3.2.3 Calibration Procedures

Inject two or more mixed standards of DP-1 and DP-2 until stable responses are observed. Calculation of results is based on peak area measurements using a calibration curve. The calibration curve is obtained by injecting various amounts of the standard solution (e.g. 0.125, 0.25, 0.5 and 1.0 ng/μL) **concurrently with sample analysis.** Different standard concentrations may be used as appropriate. Injection of control in the beginning of the analysis is necessary. Plot detector response (peak area) versus weight of standard injected. The standards should give a linear response.

3.2.4 Sample Analysis

Inject 50 μL of sample into the HPLC-UV.

Directly compare the response (peak area) of unknown samples injected with the calibration curve to obtain ng of DP-1 and DP-2 injected. Bracket every 2-3 samples with standards to check for shifts in sensitivity or retention time. To do this, an injection sequence including standards and samples must be planned.

If the peak response of the unknown is larger than the heighest standard, dilute the unknown appropriately and re-inject.

3.3 Interferences

3.3.1 Sample Matrices

If interfering peaks from the matrix occur in the chromatogram, change the HPLC operating conditions (see 3.2.2) or use an alternative HPLC column. It is desirable to clean the chromatographic system periodically by injecting with the solvent.

3.3.2 Other Sources

3. **ANALYTICAL PROCEDURE**, Continued

Other Pesticides:	None known to date.
Solvents:	None known to date.
Labware:	None known to date.

3.4 Confirmatory Techniques

HPLC coupled with diode-array UV detection confirms the presence of DP-1 and DP-2 (Figures B.23. to B.27.). GC-MSD (EI Full MS Scan) confirms the structural identity of the analytes (Figures B.28. to B.29.). No problems with interferences or questionable peak identity have been encountered to date.

3.5 Time Required for Analysis

Analysis of a set of 7 soil samples requires 1.5 working days, including sample work-up and HPLC-UV analysis.

3.6 Potential Problems

Potential technical problems have been described at the appropriate points in the method.

4. METHODS OF CALCULATION (See Figure 2 for an example calculation.)

4.1 Calibration

Construct a linear least squares calibration curve in the form $y = bx+c$ from the standards by plotting peak area versus weight of standard injected for both analytes.

4.2 Analyte in Sample

Calculation of results is based on peak area measurements. Using the peak area measurements for DP-1 and DP-2 in the samples, the amount of the analyte in **ng** from the appropriate least squares calibration curve is determined. See Figure 3.

Calculate ppm values by the equation below.

$$ppm = \frac{A}{B}$$

where: **A** = ng value interpolated from calibration curve (Obtained automatically or could be calculated using Fig. 3)

4. METHODS OF CALCULATION, continued

$$B = \text{mg Sample Injected} = \frac{\text{Dry Sample Wt. (g)} \times \mu\text{L Injected}}{\text{Final dilution volume (mL)}}$$

The "final dilution volume" includes any dilutions which have been made.

Using the Dry Sample Weight in the "mg Sample Injected" calculation will yield a ppm value on a "dry weight" basis. The Dry Sample Weight is obtained after determining the moisture content of the original sample (see Section 3.1.9).

4.3 Calculation of Procedural Recoveries

Correct fortification results for residues found in the control sample as follows:

ppm (corrected) = ppm in fortified control - ppm in control

Determine percent recovery from the fortification experiments as follows:

$$\text{\% Recovery} = \frac{\text{ppm (corrected) X 100}}{\text{ppm DP-1 or DP-2 added}}$$

Only results for procedural recovery samples should be corrected for residues in the controls. Do not correct treated sample results for either control residues or recoveries.

10.2 Solvents, Reagents and Standards

It is recommended to review the Material Safety Data Sheets (MSDSs) for all solvents and reagents used in this method. The toxicity of the DP-1 and DP-2 are unknown.

11. SPECIAL NOTES

1. Section 3.1.6: An example procedure for moisture determination is as follows:

   Weigh 5 g of wet soil ("Wet weight") accurately into a tarred glass petri dish or other container.

   Place into a 150°C oven for 16 hours (overnight).

   Remove the petri dish from the oven and allow to cool in a desiccator.

   Working quickly, remove the cool petri dish from the desiccator and weigh accurately to obtain "Dry Weight".

   Determine the ratio R = "Dry Weight"/"Wet Weight"

   Calculate "Dry Sample Weight" = R X "Weight Sample Weight" (See section 3.1.1).

   The calculated "Dry Sample Weight" is used in section 4.2 to calculate concentration (ppm) values.

2. Sections 3.1.3 to 3.1.5: In the development of this method, BASF personnel had the use of a vacuum pump and controller on rotary evaporators. At various points in the method it was expedient to vary the pressure in the rotary evaporators. The approximate settings used are shown in the table below.

Solvent	Temperature, °C	Vacuum pressure, mbar	Method section
Acetonitrile	60	180	3.1.4
Methanol	60	200	3.1.4
Water	60	35	3.1.4

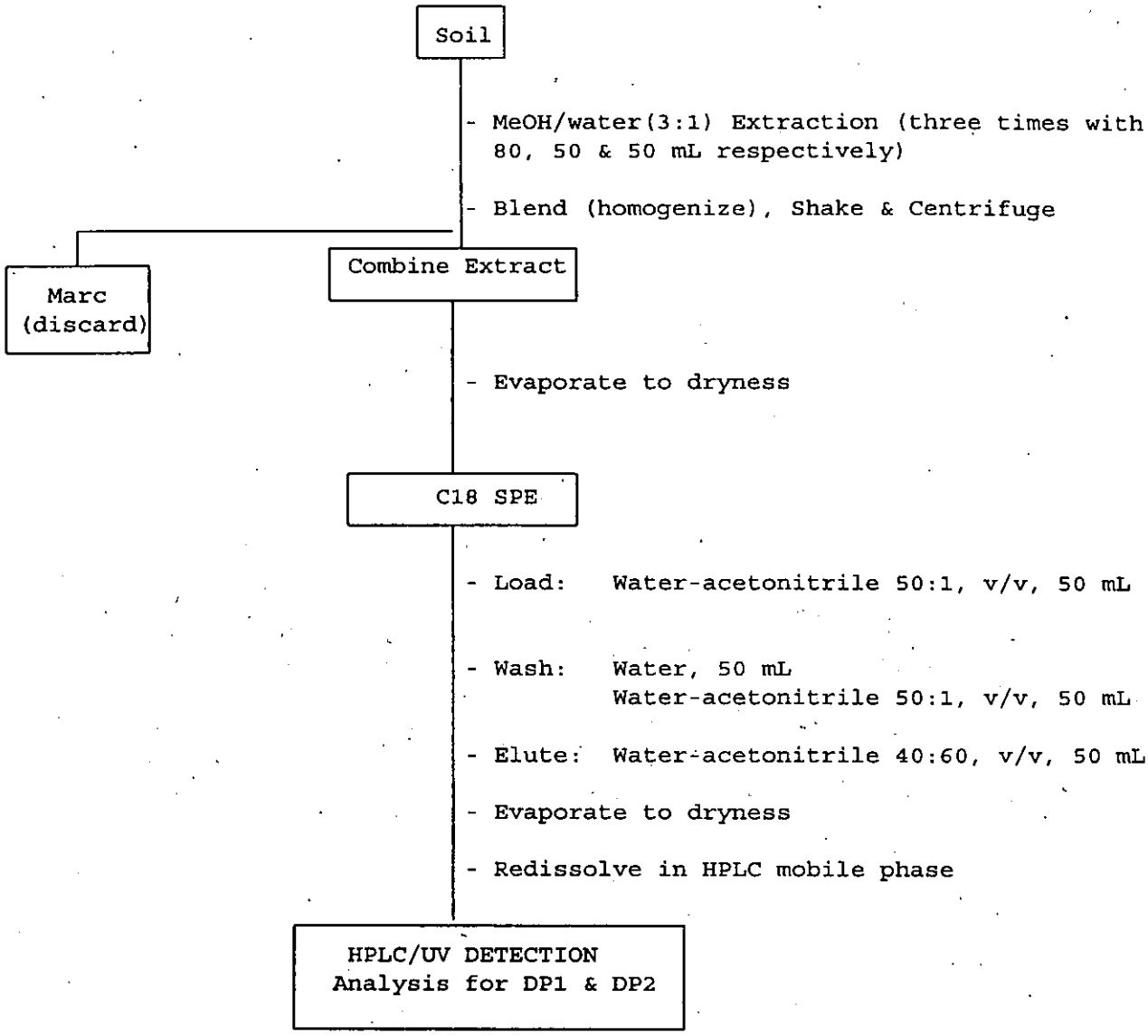


Figure 1. Flow Chart for Analytical Method D9517