

## 1. SUMMARY

The objective of this study was to independently evaluate the ruggedness, usability, and any potential weaknesses of the "Analytical Method for the Determination of BAJ 2740 and its Metabolites Enol (BAJ 2510), Ketohydroxy (KTS 9301), Dihydroxy (KTS 9313), and DCB-acid (DCBA) in Soil", Battelle Report Number RAM-AF00/01.

After the addition of the internal standards, the samples were extracted by an Accelerated Solvent Extractor (ASE), separate aliquots were concentrated under nitrogen, and the two separate fractions were analyzed, one under acidic conditions and one under basic conditions, by High Performance Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry (HPLC-ESI/MS/MS). Quantitation was accomplished by using weighted (1/y) linear regression from an eight-point calibration curve injected in triplicate. The lowest standard which could be reliably integrated was 2.5 ppb. Considering the validation LOQ was equivalent to a ~ 6 ppb standard, this sensitivity was adequate. Sensitivity appeared to be somewhat better than was achieved in the report number RAM-AF001/01. The calibration curve was verified by the injection of continuing calibration standards on the same day the samples were started, due to standard stability. The initial Quality Control (QC) calibration curves were good for all the compounds.

## 2. MATERIALS

### 2.1 Reference Substances and Chemical Structures:

#### 2.1.1 Characterization Information

Reference standards were received from Bayer on August 30, 2000 and stored in a freezer at  $-14.8^{\circ}\text{C}$  to  $-6.8^{\circ}\text{C}$ .

Standard Name: BAJ 2740

Chemical Name: 3-(2,4-dichlorophenyl)0-  
2-oxo-1-oxaspiro[4.5] dec-3-en-4-yl  
2,2-dimethylbutanoate

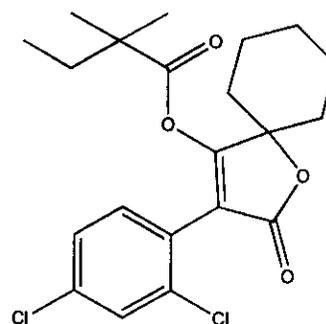
Date of Receipt: August 30, 2000

Reference No.: M01706

CAS No.: 148477-71-8

Empirical Formula:  $\text{C}_{21}\text{H}_{24}\text{O}_4\text{Cl}_2$

Purity: 99.0%



BAJ 2740

Standard Name: BAJ 2510 (Enol)

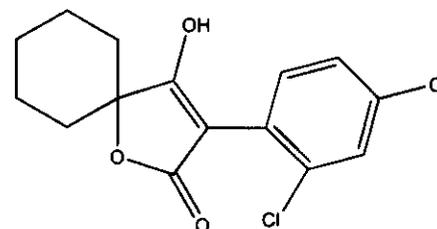
Chemical Name: 3-(2,4-dichlorophenyl)  
-4-hydroxy-1-oxaspiro[4.5]  
dec-3-en-2-one

Date of Receipt: August 30, 2000

Reference No.: 960424ELB04

Empirical Formula:  $\text{C}_{15}\text{H}_{14}\text{O}_3\text{Cl}_2$

Purity: 99.9%



Enol (BAJ 2510)

Standard Name: KTS 9301 (Ketohydroxy)

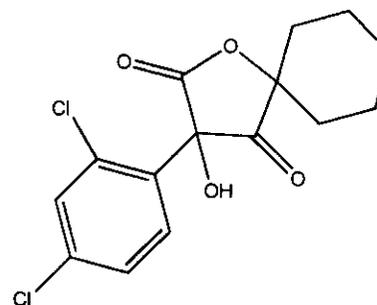
Chemical Name: 3-(2,4-dichlorophenyl)-3-  
hydroxy-1-oxaspiro[4.5] decane-  
2,4-dione

Date of Receipt: August 30, 2000

Reference No.: K-871

Empirical Formula:  $\text{C}_{15}\text{H}_{14}\text{O}_4\text{Cl}_2$

Purity: 96.4%



Ketohydroxy (KTS 9301)

## 2.1.1 Characterization Information cont'd

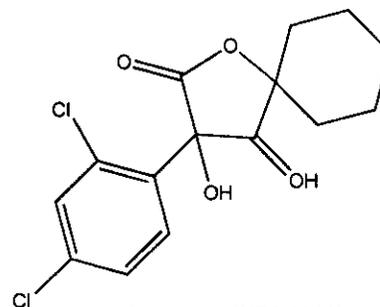
Standard Name: KTS 9313 (Dihydroxy)  
Chemical Name: 3-(2,4-dichlorophenyl)  
-3,4-dihydroxy-1-oxaspiro[4.5]  
decan-2-one

Date of Receipt: August 30, 2000

Reference No.: K-872

Empirical Formula:  $C_{15}H_{16}O_4Cl_2$

Purity: 97.8%



Dihydroxy (KTS 9313)

Standard Name: DCBA

Chemical Name: 2,4-dichlorobenzoic acid

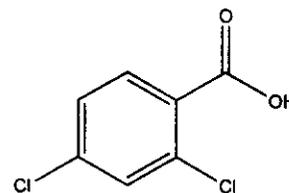
Date of Receipt: August 30, 2000

Reference No.: 13,957-2

CAS No.: 50-84-0

Empirical Formula:  $C_7H_4O_2Cl_2$

Purity: 99.4%



DCBA

Standard Name: BAJ 2740-d3

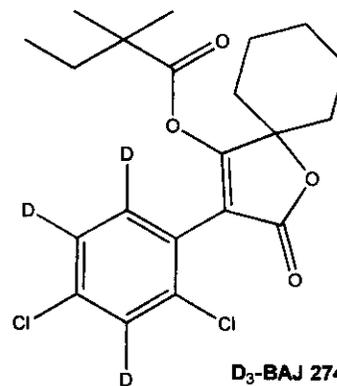
Chemical Name: 3-(2,4-dichlorophenyl)0-  
2-oxo-1-oxaspiro[4.5]dec-3-en-4-yl  
2,2-dimethylbutanoate-phenyl-3,5,  
6-d3

Date of Receipt: August 30, 2000

Reference No.: 99B001-159

Empirical Formula:  $C_{21}H_{21}O_4Cl_2D_3$

Purity: 99.2%



D<sub>3</sub>-BAJ 2740

Standard Name: BAJ 2510-d3 (Enol-d3)

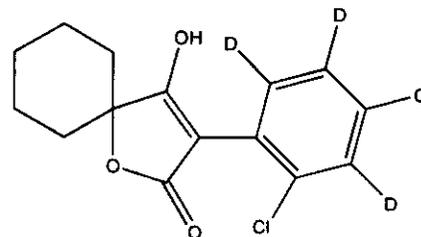
Chemical Name: 3-(2,4-dichlorophenyl)-4-  
hydroxy-1-oxaspiro[4.5]dec-3-en-  
2-one-phenyl-3,5,6-d3

Date of Receipt: August 30, 2000

Reference No.: 99B001-214

Empirical Formula:  $C_{15}H_{11}O_3Cl_2D_3$

Purity: 100%



D<sub>3</sub>-Enol (BAJ 2510)

## 2.1.1 Characterization Information cont'd

Standard Name: KTS 9301-d3 (Keto-hydroxy-d3)

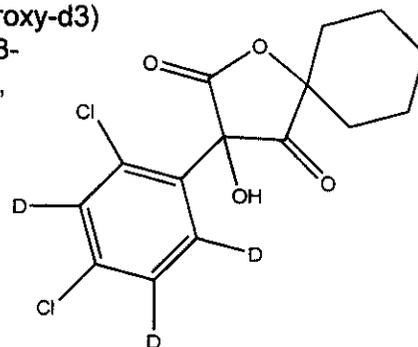
Chemical Name: 3-(2,4-dichlorophenyl)-3-hydroxy-1-oxaspiro[4.5]decane-2,4-dione-phenyl-3,5,6-d3

Date of Receipt: August 30, 2000

Reference No.: 99B001-172

Empirical Formula: C<sub>15</sub>H<sub>11</sub>O<sub>4</sub>Cl<sub>2</sub>D<sub>3</sub>

Purity: 97.6%

D<sub>3</sub>-Keto-hydroxy (D<sub>3</sub>-KTS 9301)

Standard Name: KTS 9313-d3 (Dihydroxy-d3)

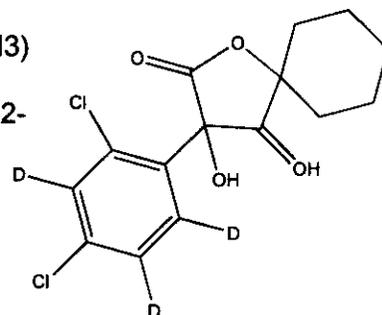
Chemical Name: 3-(2,4-dichlorophenyl)-3,4-dihydroxy-1-oxaspiro[4.5]decan-2-one-phenyl-3,5,6-d3

Date of Receipt: August 30, 2000

Reference No.: 99B001-177

Empirical Formula: C<sub>15</sub>H<sub>13</sub>O<sub>4</sub>Cl<sub>2</sub>D<sub>3</sub>

Purity: 98.4%

D<sub>3</sub>-Dihydroxy (D<sub>3</sub>-KTS 9313)

Standard Name: DCBA-d3

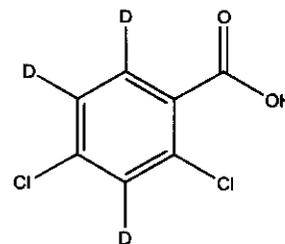
Chemical Name: 2,4-dichloro-benzoic acid-3,5,6-d3

Date of Receipt: August 30, 2000

Reference No.: 99B001-210

Empirical Formula: C<sub>7</sub>HO<sub>2</sub>Cl<sub>2</sub>D<sub>3</sub>

Purity: 99.6%

DCBA-D<sub>3</sub>

## 2.1.2 Standard Solution Preparation:

All standard solutions were stored at approximately  $-15^{\circ}\text{C}$  with teflon lined screw caps and in the absence of light when possible.

### 2.1.2.1 BAJ 2740, BAJ 2510, BAJ 2740-d3 and BAJ 2510-d3 Stock Solutions

Separate  $\sim 1000$   $\mu\text{g/mL}$  stock solutions of analyte were prepared by weighing accurately ( $\pm 0.01$  mg) 10 mg of reference material and dissolving in 10 mL of 0.1% formic acid in acetonitrile in a 10 mL volumetric flask. In some cases, a full 10 mg of material was not available, so the stock concentration was less than 1000  $\mu\text{g/mL}$ .

### 2.1.2.2 KTS 9301, KTS 9313, DCBA, KTS 9301-d3, KTS 9313-d3 and DCBA-d3 Stock Solutions

Separate  $\sim 1000$   $\mu\text{g/mL}$  stock solutions of analyte were prepared by weighing accurately ( $\pm 0.01$  mg) 10 mg of reference material and dissolving in 10 mL of acetonitrile in a 10 mL volumetric flask. In some cases, a full 10 mg of material was not available, so the stock concentration was less than 1000  $\mu\text{g/mL}$ .

### 2.1.2.3 20 $\mu\text{g/mL}$ Intermediate Acidic Standard Mix

An appropriate volume of both BAJ 2740 and BAJ 2510 stock solutions were transferred to a 10 mL volumetric flask to achieve a final concentration of 20.0  $\mu\text{g/mL}$  for each compound (volume = 200  $\mu\text{L}$  in the case of a 1000 $\mu\text{g/mL}$  stock). The solution was brought to 10.0 mL final volume with 0.1% formic acid in acetonitrile.

#### 2.1.2.4 20µg/mL Intermediate Basic Standard Mix

An appropriate volume of each of KTS 9301, KTS 9313 and DCBA stock solutions were transferred to a 10 mL volumetric flask to achieve a final concentration of 20.0 µg/mL for each compound (volume = 200 µL in the case of a 1000 µg/mL stock). The solution was brought to 10.0 mL final volume with acetonitrile.

#### 2.1.2.5 10µg/mL Intermediate Acidic Internal Standard Mix

An appropriate volume of both BAJ 2740-d3 and BAJ 2510-d3 stock solutions were transferred to a 10 mL volumetric flask to achieve a final concentration of 10.0 µg/mL for each compound (volume = 100 µL in the case of a 1000µg/mL stock). The solution was brought to 10.0 mL final volume with 0.1% formic acid in acetonitrile.

#### 2.1.2.6 10µg/mL Intermediate Basic Internal Standard Mix

An appropriate volume of each of KTS 9301-d3, KTS 9313-d3 and DCBA-d3 stock solutions were transferred to a 10 mL volumetric flask to achieve a final concentration of 10.0 µg/mL for each compound (volume = 100 µL in the case of a 1000 µg/mL stock). The solution was brought to 10.0 mL final volume with acetonitrile.

#### 2.1.2.7 4µg/mL Acidic Fortification Standard Mix

2.00 mL of the 20.0 µg/mL Intermediate Acidic Standard Mix was transferred to a 10 mL volumetric flask and brought to final volume with 0.1% formic acid in acetonitrile.

**2.1.2.8 2 $\mu$ g/mL Acidic Fortification Standard Mix**

1.00 mL of the 20.0  $\mu$ g/mL Intermediate Acidic Standard Mix was transferred to a 10 mL volumetric flask and brought to final volume with 0.1% formic acid in acetonitrile.

**2.1.2.9 0.4 $\mu$ g/mL Acidic Fortification Standard Mix**

1.00 mL of the 4  $\mu$ g/mL Acidic Fortification Standard Mix was transferred to a 10 mL volumetric flask and brought to final volume with 0.1% formic acid in acetonitrile.

**2.1.2.10 4 $\mu$ g/mL Basic Fortification Standard Mix**

2.00 mL of the 20.0  $\mu$ g/mL Intermediate Basic Standard Mix was transferred to a 10 mL volumetric flask and brought to final volume with acetonitrile.

**2.1.2.11 2 $\mu$ g/mL Basic Fortification Standard Mix**

1.00 mL of the 20.0  $\mu$ g/mL Intermediate Basic Standard Mix was transferred to a 10 mL volumetric flask and brought to final volume with acetonitrile.

**2.1.2.12 0.4 $\mu$ g/mL Basic Fortification Standard Mix**

1.00 mL of the 4  $\mu$ g/mL Basic Fortification Standard Mix was transferred to a 10 mL volumetric flask and brought to final volume with acetonitrile.

**2.1.2.13 3.2µg/mL Acidic Internal Standard Fortification  
Mix**

3.2 mL of the 10.0 µg/mL Intermediate Acidic Internal Standard Mix was transferred to a 10 mL volumetric flask and brought to final volume with 0.1% formic acid in acetonitrile.

**2.1.2.14 3.2µg/mL Basic Internal Standard Fortification  
Mix**

3.2 mL of the 10.0 µg/mL Intermediate Basic Internal Standard Mix was transferred to a 10 mL volumetric flask and brought to final volume with acetonitrile.

**2.1.2.15 Working Acidic Standard Solutions**

<b>Volume of 20 µg/mL Acidic Standard Mix (mL)</b>	<b>Final Volume (mL)</b>	<b>Final Concentration of Working Standard (ng/mL)</b>
0	10.0	0
0.010	10.0	20.0
0.025	10.0	50.0
0.050	10.0	100
0.100	10.0	200
0.250	10.0	500
0.500	10.0	1000
1.00	10.0	2000
1.50	10.0	3000
All the solutions above were brought to 10 mL with acidic mobile phase mix		

## 2.1.2.16 Acidic Calibration Standard Solutions

Working Standard Concentration (ng/mL)	Volume of Working Standard (mL)	Final Volume (mL)	Final Concentration of Calibration Standard (ng/mL)
0	0.500	10.0	0
20.0	0.500	10.0	1.00
50.0	0.500	10.0	2.50
100	0.500	10.0	5.00
200	0.500	10.0	10.0
500	0.500	10.0	25.0
1000	0.500	10.0	50.0
2000	0.500	10.0	100
3000	0.500	10.0	150
All the solutions above were brought to 10 mL with acidic calibration dilution solution			

## 2.1.2.17 Working Basic Standard Solutions

Volume of 20 µg/mL Basic Standard Mix (mL)	Final Volume (mL)	Final Concentration of Working Standard (ng/mL)
0	10.0	0
0.010	10.0	20.0*
0.025	10.0	50.0
0.050	10.0	100
0.100	10.0	200
0.250	10.0	500
0.500	10.0	1000
1.00	10.0	2000
1.50	10.0	3000
All the solutions above were brought to 10 mL with basic mobile phase mix. * 20 ng/mL standard solution was not listed in original method.		

## 2.1.2.18 Basic Calibration Standard Solutions

Working Standard Concentration (ng/mL)	Volume of Working Standard (mL)	Final Volume (mL)	Final Concentration of Calibration Standard (ng/mL)
0	0.500	10.0	0
20.0	0.500	10.0	1.00*
50.0	0.500	10.0	2.50
100	0.500	10.0	5.00
200	0.500	10.0	10.0
500	0.500	10.0	25.0
1000	0.500	10.0	50.0
2000	0.500	10.0	100
3000	0.500	10.0	150
All the solutions above were brought to 10 mL with basic calibration dilution solution. * 1 ng/mL standard solution was not listed in original method.			

**2.2 Reagents:**

Acetic acid, Analar®, BDH Inc. (B10001-78) 99.8%  
Acetonitrile, HPLC grade, EM Science, OmniSolv® (AX0142-1)  
Ammonium formate, Fisher Inc. (A666-500)  
Ammonium hydroxide, Reagent ACS, Fisher Scientific (A669-225) 28.0%  
NH<sub>3</sub>  
Formic acid, Analar®, BDH Inc. (B10115) 98.0%  
Methanol, HPLC grade, EM Science, OmniSolv® (MX0488-1)  
Water, HPLC grade, EM Science, OmniSolv® (WX0004-1)

**2.3 Solution Preparation:**

- 2.3.1 0.1% Formic acid in acetonitrile - 1 mL of concentrated formic acid was added to 1 L of acetonitrile and mixed.
- 2.3.2 Extraction solvent - 1 mL of glacial acetic acid was added to 100 mL water and 900 mL acetonitrile and mixed.
- 2.3.3 Acidic Mobile Phase A - 1.01g of ammonium formate was dissolved in 200-300 mL water. 4 mL of concentrated formic acid was added and the solution was brought to 4 L with water, mixed and de-gassed prior to use on HPLC.
- 2.3.4 Acidic Mobile Phase B - 1.01 g of ammonium formate was dissolved in 200-300 mL methanol. 4 mL of concentrated formic acid was added and the solution was brought to 4 L with methanol, mixed and de-gassed prior to use on HPLC.
- 2.3.5 Basic Mobile Phase A - 2 mL of ammonium hydroxide was added to 4 L of water, mixed and de-gassed prior to use on HPLC.
- 2.3.6 Basic Mobile Phase B - 2 mL of ammonium hydroxide was added to 4 L of methanol, mixed and de-gassed prior to use on HPLC.
- 2.3.7 Acidic Mobile Phase Mix - 500 mL of Acidic Mobile Phase A and 500 mL of Acidic Mobile Phase B were mixed.
- 2.3.8 Basic Mobile Phase Mix - 500 mL of Basic Mobile Phase A and 500 mL of Basic Mobile Phase B were mixed.

2.3.9 Acidic Calibration Dilution Solution - 1.0 mL of 10 µg/mL Intermediate Acidic Internal Standard Mix was added to a 200 mL volumetric flask and brought to volume with Acidic Mobile Phase Mix and mixed.

2.3.10 Basic Calibration Dilution Solution - 1.0 mL of 10 µg/mL Intermediate Basic Internal Standard Mix was added to a 200 mL volumetric flask and brought to volume with Basic Mobile Phase Mix and mixed.

#### 2.4 Laboratory Materials and Apparatus:

ASE 60 mL Collection vials, Dionex Corporation

ASE, model 200, Dionex Corporation

Balance, analytical, model ER-182A, AND (accuracy to the nearest 0.00001 g)

Balance, top-loading, model FX-4000, AND (accuracy to the nearest 0.01 g)

Extraction cells, 22 mL, Dionex Corporation

Filters, cellulose, Dionex Corporation (049458)

Filters, syringe, Acrodisc®, 0.20µm x 25mm, Gelman Sciences Inc. (4422T)

Flasks, volumetric, Kimax®, 10mL and 200mL, Kimble Glass Inc.

HPLC column, Supelco ABZ+, 10 cm x 2.1mm (5µm) (57925)

Pipet, Socorex, 0.5-5 mL, Swiss

Pipets, 50 µL - 1 mL, Gilson

Sea Sand, Fisher, (S25-10)

Syringes, single use, 3cc, Luer Lok®, Becton Dickinson

Tubes, culture, 50 mL, Kimax®, Kimble Glass Inc.

Tubes, Turbovap, Zymark

Turbovap, model ZW640-3, Zymark

Vials, autosampler, clear, National Scientific Co.

**2.5 Instrumentation, Conditions and Parameters:****2.5.1 Instrumentation:**

Mass spectrometer: PE-Sciex API-3000 LC/MS/MS system

HPLC: Perkin-Elmer Series 200 Micropumps (2)

Autosampler: Perkin-Elmer Series 200 Autosampler

Data System: Apple Power Macintosh G3

Printer: Hewlett Packard, Laser Jet 2100 TN

**2.5.2 HPLC Operating Conditions:**

Column: Supelco ABZ+, 10cm x 2.1mm (5 $\mu$ m)

Guard Column: Supelco Supelguard ABZ 2cm cartridge

Flow Rate: 0.40 mL/min.

Injection Volume: 50  $\mu$ L

**2.5.2.1 Acidic Analysis - for BAJ 2740 and BAJ 2510**

Mobile phase A = 0.1% formic acid \ 4mM ammonium  
formate in water

Mobile phase B = 0.1% formic acid \ 4mM ammonium  
formate in methanol

Time (min.)	Mobile Phase	
	A (%)	B (%)
0	70	30
1	0	100
5	0	100
6	70	30
10	70	30

**2.5.2.2 Basic Analysis – for DCBA, KTS 9301, KTS 9313**

Mobile phase A = 0.05% ammonium hydroxide in water

Mobile phase B = 0.05% ammonium hydroxide in methanol

Time (min.)	Mobile Phase	
	A (%)	B (%)
0	90	10
2	40	60
4	40	60
6	0	100
10	0	100
11	90	10
16	90	10

**2.5.3 Mass Spectrometer and Interface Operating Parameters:**

Auxiliary Gas Temperature: 475°C

Auxiliary Gas Flow: 4.0 L/min. (N<sub>2</sub>)Nebulizer Gas Setting: 13 (N<sub>2</sub>)Curtain Gas Setting: 12 (N<sub>2</sub>)Collision Gas Setting: 6 (N<sub>2</sub>)**2.5.3.1 Acid Analysis**

Ion spray voltage: 3300 V

Orifice potential: 65 V

Ring Voltage: 260 V

Q0: -13 V

IQ1: -14 V

ST: -20 V

RO1: -15 V

IQ2: -23 V

RO2: -43 V

ST3: -63 V

RO3: -45 V

Analyte	Mass Transition (m/z)	Dwell Time (ms)	Retention Time (min.)
BAJ 2740	411→313	200	4.6
BAJ 2740 d3	416→318	50	4.6
BAJ 2510	313→213	200	5.4
BAJ 2510 d3	318→218	50	5.4

## 2.5.3.2 Base Analysis

Period 1 - DCBA

Ion spray voltage: -5000 V

Orifice potential: -28 V

Ring Voltage: -125 V

Q0: 12 V

IQ1: 13 V

ST: 19 V

RO1: 14 V

IQ2: 22 V

RO2: 25 V

ST3: 45 V

RO3: 27 V

Analyte	Mass Transition (m/z)	Dwell Time (ms)	Retention Time (min.)
DCBA	189→145	250	1.1
DCBA d3	194→150	50	1.1

Period 2 (KTS 9301 and KTS 9313)

Ion spray voltage: 4100 V

Orifice potential: 35 V

Ring Voltage: 180 V

Q0: -7 V

IQ1: -8 V

ST: -14 V

RO1: -9 V

IQ2: -17 V

RO2: -25 V

ST3: -45 V

RO3: -27 V

Analyte	Mass Transition (m/z)	Dwell Time (ms)	Retention Time (min.)
KTS 9313	348→313	200	7.3
KTS 9313 d3	353→318	50	7.3
KTS 9301	378→189	200	7.8
KTS 9301 d3	383→194	50	7.8

## 2.6 Sample Management:

Approximately 600 g of untreated control soil from Ephrata, Washington was received at ETL in Edmonton, Alberta from Battelle Agrifood in Columbus, Ohio on August 29, 2000. This sample (ETL sample no. E0-08-013-01A) was stored in the freezer at  $-25 \pm 3^{\circ}\text{C}$  until subsampling. The sample was received already processed and did not require any further processing.

On September 11, 2000 the sample was thawed and twelve subsamples of 10g each were weighed into ASE extraction cells. The remaining soil was returned to the freezer.

## 3. ANALYTICAL PROCEDURES

The analytical method used for the analysis of soil in this study was the Battelle method entitled "Analytical Method for the Determination of BAJ 2740 and its Metabolites Enol (BAJ 2510), Ketohydroxy (KTS 9301), Dihydroxy (KTS 9313), and DCB-acid (DCBA) in Soil", Battelle Report Number RAM-AF00/01.

### 3.1 Method Modifications

The analytical instrumentation used by Battelle was not available at Enviro-Test Labs, therefore substitutions were made. Battelle used a Micromass Quattro II LC/MS/MS, while Enviro-Test labs used a PE Sciex API-3000 LC/MS/MS. Battelle used Hewlett-Packard HPLC pumps and autosampler, while Enviro-Test Labs used Perkin-Elmer 200 series equipment. Overall sensitivity and performance of the PE/Sciex equipment used by Enviro-Test was comparable to the HP/Micromass equipment used by Battelle.

A limited supply of analytical standards was available, so it was impossible to weigh the 10 mg specified in the Battelle method. The smallest amount of standard weighed from one of the standard containers was ~3.5 mg. With the precision of the analytical balance used ( $\pm 0.01\text{mg}$ ) the true weight could be recorded to 3 significant figures. The volume of the stock solutions used to make the subsequent dilutions were modified from the 200  $\mu\text{L}$  specified in the original method in order to achieve the target concentrations of intermediate mixed standards.

One of the aliquots for the acidic analysis (E0-08-013-01A+6) was left too long in the turbovap and went dry. The final extract was analyzed and found to have low recoveries for BAJ 2510. A second 5 mL aliquot was taken and evaporated, then analyzed on the LC/MS/MS along with the reagent blank and one of the controls. Recoveries were acceptable for the re-aliquoted extract.

The standard freezer used in this study is maintained at approximately  $-15^{\circ}\text{C}$ , not  $-20^{\circ}\text{C}$  as stated in the protocol.

The basic working standard solution of 10 ng/mL and the calibration solution of 1.00 ng/mL were not required in the original method.