109669

1.0 SUMMARY

Control soil samples were fortified at two levels with BAJ 2740 and its relevant metabolites and were analyzed to determine the recovery efficiency of the analytical method entitled "(RAM-F00/01) 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".

Validation of the method was done separately on each soil from the four sites of the terrestrial dissipation studies (Florida, California, Washington, and Ontario). Validation consisted of one reagent blank, two control samples, seven control samples fortified at 0.010 mg/kg (Limit of Quantitation, LOQ), and five control samples fortified at 0.10 mg/kg (10 X LOQ). The control soils from all four locations were free from significant interferences using this method.

2.0 MATERIALS

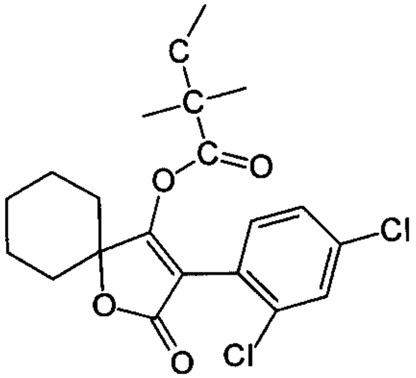
2.1 Test and Reference Substances

The test and reference substances for this study are listed below. Deuterated reference substances are used as internal standards.

Common Name:	BAJ 2740
Code Name:	BAJ 2740

Chemical Name:	3-(2,4-Dichlorophenyl)-2-oxo-1-oxaspiro[4.5]dec-3-en-4-yl 2,2-dimethylbutanoate
CAS Number:	148477-71-8
Empirical Formula:	$C_{21}H_{24}O_4Cl_2$
Molecular Weight:	411.30
Lot Number:	K-760
Purity:	99.7%
Expiration Date:	March 24, 2004
Lot Number:	K-863
Purity:	99%
Expiration Date:	May 01, 2001

Structure:



Common Name:	Enol
Code Name:	BAJ 2510
Chemical Name:	3-(2,4-dichlorophenyl)-4-hydroxy-1-oxaspiro [4.5]dec-3-en-2-one
CAS Number:	Not assigned
Empirical Formula:	$C_{15}H_{14}O_3Cl_2$
Molecular Weight:	313.20

Lot Number: K-793

Purity: 99% [98.8% at recertification]

Expiration Date: March 24, 2005

Lot Number: K-761

Purity: 99.9%

Expiration Date: March 24, 2005

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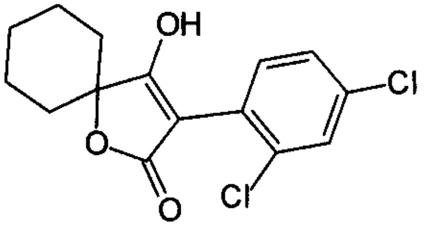


Common Name: Ketohydroxy

Code Name: KTS 9301

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

CAS Number: Not assigned

Empirical Formula: $C_{15}H_{14}O_4Cl_2$

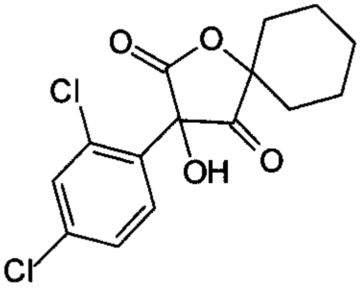
Molecular Weight: 329.20

Lot Number: K-871

Purity: 96.4%

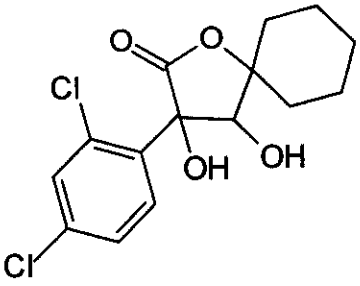
Expiration Date: July 28, 2004

Structure:

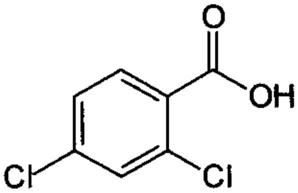


Common Name:	Dihydroxy
Code Name:	KTS 9313
Chemical Name:	3-(2,4-Dichlorophenyl)-3,4-dihydroxy-1-oxaspiro[4.5]decan-2-one
CAS Number:	Not assigned
Empirical Formula:	$C_{15}H_{16}O_4Cl_2$
Molecular Weight:	331.18
Lot Number:	K-872
Purity:	97.8%
Expiration Date:	July 28, 2004

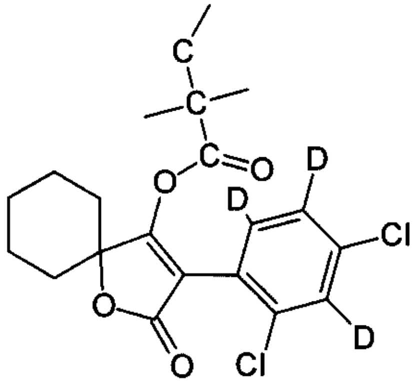
Structure:

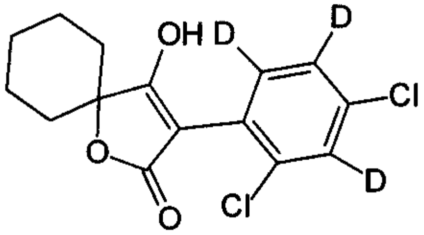


Common Name:	DCB-acid
Code Name:	DCBA
Chemical Name:	2,4-Dichloro-benzoic acid

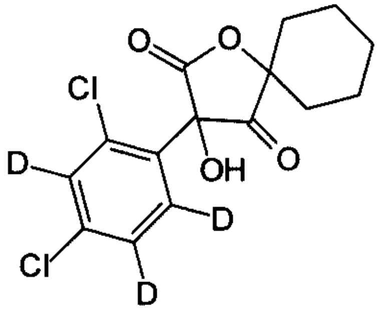
CAS Number:	50-84-0
Empirical Formula:	$C_7H_4O_2Cl_2$
Molecular Weight:	191.00
Lot Number:	K-821
Purity:	98%
Expiration Date:	July 01, 2002
Lot Number:	K-825
Purity:	99.4%
Expiration Date:	October 05, 2004
Structure:	

Common Name:	BAJ 2740-$_{d3}$
Code Name:	$_{d3}$-BAJ 2740
Chemical Name:	3-(2,4-Dichlorophenyl)-2-oxo-1-oxaspiro[4.5]dec-3-en-4-yl 2,2-dimethyl-butanoate-phenyl-3,5,6-$_{d3}$
CAS Number:	Not assigned
Empirical Formula:	$C_{21}H_{21}O_4Cl_2H_3^2$
Molecular Weight:	414.40
Lot Number:	K-874
Purity:	99.2%
Expiration Date:	September 8, 2004

Structure:	

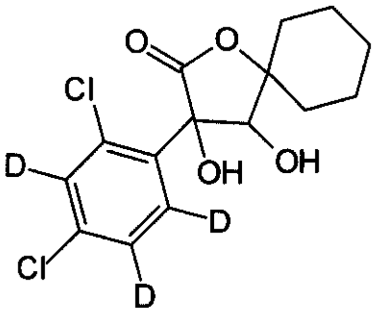
Common Name:	Enol-$_{d3}$
Code Name:	$_{d3}$-BAJ 2510
Chemical Name:	3-(2,4-Dichorophenyl)-4-hydroxy-1-oxaspiro[4.5]dec-3-en-2-one-phenyl-3,5,6-$_{d3}$
CAS Number:	Not assigned
Empirical Formula:	$C_{15}H_{11}O_3Cl_2H^2_3$
Molecular Weight:	316.20
Lot Number:	K-885
Purity:	100.0%
Expiration Date:	December 14, 2004
Structure:	

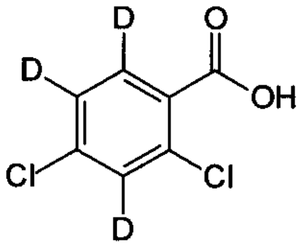
Common Name:	Ketohydroxy-$_{d3}$
Code Name:	$_{d3}$- KTS 9301

Chemical Name:	3-(2,4-Dichlorophenyl)-3-hydroxy-1-oxaspiro[4.5]decane-2,4-dione-phenyl-3,5,6-$_{d3}$
CAS Number:	Not assigned
Empirical Formula:	$C_{15}H_{11}O_4Cl_2H^2_3$
Molecular Weight:	332.20
Lot Number:	K-878
Purity:	97.6%
Expiration Date:	September 16, 2004
Structure:	

Common Name:	Dihydroxy-$_{d3}$
Code Name:	$_{d3}$- KTS 9313
Chemical Name:	3-(2,4-Dichlorophenyl)-3,4-dihydroxy-1-oxaspiro[4.5]decan-2-one-phenyl-3,5,6-$_{d3}$
CAS Number:	Not assigned
Empirical Formula:	$C_{15}H_{13}O_4Cl_2H^2_3$
Molecular Weight:	334.20
Lot Number:	K-882
Purity:	98.4%
Expiration Date:	September 10, 2004

Structure:



Common Name:	DCB-acid-$_{d3}$
Code Name:	$_{d3}$- DCBA
Chemical Name:	2,4-Dichloro-benzoic acid-3,5,6-$_{d3}$
CAS Number:	Not assigned
Empirical Formula:	$C_7HO_2Cl_2H^2_3$
Molecular Weight:	194.03
Lot Number:	K-884
Purity:	99.6%
Expiration Date:	December 15, 2004
Structure:	

3.0 TEST SYSTEM

The test systems for this study were soils taken from the four field sites where the terrestrial dissipation studies were conducted. Untreated surface soil (0-6 inch or 0-15 cm) collected from the Florida, California, Washington and Ontario sites are shown below. The soils were received and stored frozen throughout the study. The test systems were selected because they were the same soils on which the dissipation studies were conducted. All test systems were labeled with a minimum of a unique Battelle study number and sample identification code.

State	Supplier	Identification	Type of Soil	Date Received
Florida	EPL Bioanalytical Services, Inc., Clermont, FL	CB 16 VAL A	Sand	April 26, 1999
California	Bayer Research Farm, Fresno, CA	CB 17 VAL A	Sandy Loam	June 11, 1999
Washington	Qualls Agricultural Laboratories, Ephrata, WA	CB 18 VAL A	Loamy Sand	April 26, 1999
Ontario	Vaughn Agricultural Research Services, Branchton, Ontario	CB 20 VAL A	Loam	August 05, 1999

4.0 ANALYTICAL PROCEDURE

4.1 Method RAM-AF00/01

A copy of the method (RAM-AF00/01) is included in Appendix 1.

In brief, a 10-g soil sample aliquot was extracted by Accelerated Solvent Extraction (ASE) with 90:10 acetonitrile: water containing 0.1% glacial acetic acid. Two separate 5-mL aliquots of the ASE extract were concentrated under nitrogen with a TurboVap. One of the aliquots was reconstituted in an acidic mobile phase mix (50:50 water:methanol containing 4 mM ammonium formate, 0.1% formic acid). The second aliquot was reconstituted in a basic mobile phase mix (50:50 water:methanol containing 0.05% ammonium hydroxide). Both extracts were subsequently filtered and analyzed independently by two different liquid chromatography/tandem mass spectrometry (LC/MS/MS) instrumental methods. One LC/MS/MS method was used to determine BAJ 2740 and Enol in the acidic extract. A second LC/MS/MS method was used to determine Ketohydroxy, Dihydroxy, and DCB-Acid in the basic extract.

The Basic Analysis Method had to be run before the Acidic Analysis Method or column performance was lost. The LC/MS/MS can be programmed to run both the Basic and Acidic Analysis Methods sequentially.

4.2 Calculations

Quantities of BAJ 2740 and its metabolites in soil were calculated using the calibration curves and the following formulas:

$$\text{Area Ratio} = \frac{\text{Compound Peak Area}}{\text{IS Peak Area}}$$

$$\text{Concentration Found (pg/}\mu\text{L)} = \frac{\text{Area Ratio - Intercept}}{\text{Slope}}$$

$$\text{Conc. Found (ppm)} = \text{Conc. Found (pg/uL)} \times \frac{\text{Final Volume (2 mL)}}{\text{Sample Weight (10 g)}} \times \frac{\text{Total ASE Volume (40 mL)}}{\text{ASE Aliquot (5 mL)}} \times 10^{-3}$$

$$\text{Percent Recovery (\%)} = \frac{\text{Conc. Found (ppm) - Conc. Found (ppm) of control sample}}{\text{Fortification Concentration (ppm)}} \times 100$$

4.3 Examples of Data Calculations

Lab sample #: 0021-117-14A from analysis of BAJ 2740 in Washington soil (extraction on April 14, 2000; analysis on April 15, 2000):

Area Ratio = 7049 / 4408 = 1.599

Concentration Found (pg/μL) = (1.599 – (0.000403312)) / 0.0273221 = 58.51 pg/μL

Concentration Found (ppm) = 58.51 pg/μL x (2 mL / 10 g) x 40 mL / 5 mL) x 10^{-3} = 0.0936 ppm

Percent recovery (%) = (0.0936 ppm – 0 ppm) / 0.10 ppm x 100% = 94 %

4.4 Instrumentation and Conditions

The following LC/MS/MS instrument operating conditions were used for both the Acid and Base Analysis Methods:

LC/MS/MS System	
HPLC	Hewlett Packard, Model 1100
Mass Spectrometer	VG/Fisons, Model Quattro II
Data System	Fisons/Micromass MassLynx, v. 3.2, Build 004
Refrigerated Autosampler	Hewlett Packard Model 1100 Maintained at 5°C.

Guard Column	Supelguard LC-ABZ, 2 cm replacement cartridge, Supelco Part #59545-U, (replaced with each run set)
Analytical Column	ABZ+PLUS, 10 cm x 2.1 mm ID, 5 μm, Supelco Part # 57925
Mobile Phase Flow Rate	400 μL/minute
Column Temperature	Ambient
Injection Volume	50 μL
Mass Spectrometer Source	Electrospray BAJ 2740, Enol, Dihydroxy, Ketohydroxy: Positive Ion Mode DCB-Acid: Negative Ion Mode
Source Temperature	90°C
Desolvation Gas	Nitrogen at ~ 350 L/h
Desolvation Temperature	300°C
Cone Voltage	Optimized for individual analytes
Collision Gas	Argon at ~ 5 x 10^{-3} mb analyzer pressure
Collision Energy	Optimized for individual analytes
Multiplier	650 V

4.4.1 **Basic Analysis Method** (The following instrument parameters were used for the analysis of the Basic Extracts)

HPLC Parameters

Mobile Phase	A: Water with 0.05% ammonium hydroxide B: Methanol with 0.05% ammonium hydroxide
Gradient	Time Composition 0.0 90%A + 10%B 2.0 40%A + 60%B 4.0 40%A + 60%B 6.0 0%A + 100%B 10.0 0%A + 100%B 11.0 90%A + 10%B 16.0 90%A + 10%B Run every 16 minutes

Retention Times DCB-Acid Dihydroxy Ketohydroxy	~ 1.2 minutes ~ 8.4 minutes ~ 9.3 minutes (The matrix caused a shift in the DCB-Acid retention time. The correct peak was easily tracked and identified with the Internal Standard)

Mass Spectrometer Parameters

Analyte	Analyte Masses Monitored	Internal Standard Masses Monitored	Cone Voltage	Collision Energy
DCB-Acid	m/z 189 >m/z 145	m/z 194 >m/z 150	10 V	13 eV
Dihydroxy	m/z 348 >m/z 313	m/z 353 >m/z 318	15 V	15 eV
Ketohydroxy	m/z 378 >m/z 189	m/z 383 >m/z 194	20 V	35 eV

4.4.2 **Acidic Analysis Method** (The following instrument parameters were used for the analysis of the Acidic Extracts)

HPLC Parameters

Mobile Phase	A: Water with 0.1% formic acid and 4 mM ammonium formate B: Methanol with 0.1% formic acid and 4 mM ammonium formate
Gradient	Time Composition 0.0 70%A + 30%B 1.0 0%A + 100%B 5.0 0%A + 100%B 6.0 70%A + 30%B 10.0 70%A + 30%B Run every 10 minutes
Retention Time BAJ 2740 Enol	~ 5.4 minutes ~ 6.6 minutes

Mass Spectrometer Parameters

Analyte	Analyte Masses Monitored	Internal Standard Masses Monitored	Cone Voltage	Collision Energy
BAJ 2740	m/z 411>m/z 313	m/z 416>m/z 318	25 V	20 eV
Enol	m/z 313>m/z 213	m/z 318>m/z 218	35 V	35 eV

Note: The Basic Analysis Method was run before the Acidic Analysis Method to maintain column performance. The LC/MS/MS was programmed to run both the Basic and Acidic Analysis Methods sequentially.

5.0 VALIDATION PROCEDURE

A full validation of the analytical method was performed on the surface soil (0-6 inch or 0-15 cm) from each of the four test sites; Florida, California, Washington and Ontario sites for BAJ 2740 and 4 metabolites. The validation consisted of 7 samples fortified at the LOQ of 0.01 ppm, 5 samples fortified at 10 times the LOQ, 2 control samples and 1 reagent blank.

6.7 Modifications to the Method

There were no modifications to the method.

7.0 POTENTIAL PROBLEMS

It is critical that the samples be taken "just to dryness" in step 4.1.8 (see Methods in Appendix II), otherwise significant losses of BAJ 2740 are observed.

Guard columns must be changed each day, after a full sample run.

Sample extracts may be stored frozen after ASE extraction. Extracts were analyzed up to seven days after extraction for this study.

Under certain mass spectrometer tuning conditions, BAJ 2740 fragments during ionization to form an ion at m/z 313. If low recovery, or poor sensitivity, is observed for the m/z 411 > m/z 313 transition, it may be possible to generate better results by utilizing the m/z 313 > m/z 213 transition for the native analyte and m/z 318 > m/z 218 transition for the internal standard (same ions used for Enol).

It may be necessary to calculate Ketohydroxy using an external calibration curve, disregarding the internal standard. Over-recovery of Ketohydroxy was observed during routine analysis. In some instances the internal standard responses appeared to be lower than expected, resulting in a higher Area Ratio. If this is the case, the calibration curve may be generated from the Ketohydroxy area only instead of the area ratio.

8.0 GENERAL COMMENTS

The method was successfully implemented in our laboratory. The time required to extract a set of 24 samples is approximately 16 person-hours, or 2 calendar days. This includes extraction, mass spectral analysis, and data reduction. This method was used to analyze approximately 470 soil samples from the field dissipation studies.

9.0 PROTOCOL CHANGES

There were two protocol change forms prepared for this study. They consisted of the following:

1. Addition of Bayer Study Number, to be included in the final report.
2. Section 4.0: Data Requirements, was changed to read "EPA Subdivision US EPA OPPTS Harmonized Guidelines 850.7100, *Data Reporting for Environmental Chemistry Methods*."
3. Section 4.0: Data Requirements, was changed to add "Agriculture Canada Guidelines, T-1-255, DACO 8.2.2.1."

Appendix I. (cont'd.)

1.0 SUMMARY

This method was developed as a LC-MS/MS residue method for the determination of BAJ 2740 and its metabolites BAJ 2510, KTS 9301, KTS 9313, and DCBA in soil. The method has a limit of quantitation for each analyte of 0.01 mg/kg in soil. A 10-g soil sample aliquot is extracted by Accelerated Solvent Extraction (ASE) with 90:10 acetonitrile: water containing 0.1% glacial acetic acid. Two separate 5-mL aliquots of the ASE extract are concentrated under Nitrogen with a TurboVap. One of the aliquots is reconstituted in an acidic mobile phase mix (50:50 water:methanol containing 4 mM ammonium formate, 0.1% formic acid). The second aliquot is reconstituted in a basic mobile phase mix (50:50 water:methanol containing 0.05% ammonium hydroxide). Both extracts are subsequently filtered and analyzed independently by two different liquid chromatography/tandem mass spectrometry (LC/MS/MS) instrumental methods. One LC/MS/MS method is used to determine BAJ 2740 and BAJ 2510 in the acidic extract. A second LC/MS/MS method is used to determine KTS 9301, KTS 9313, and DCBA in the basic extract.

2.0 MATERIAL

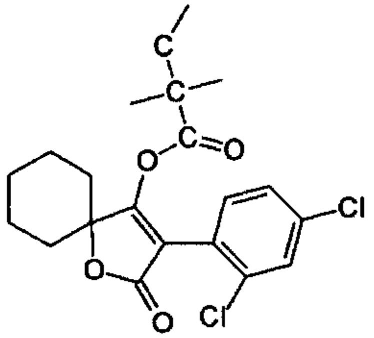
The equipment and reagents described below were used to generate the data and chromatograms presented in this method report. Equipment with equivalent performance specifications and reagents of comparable purity may be used, as required. Other general laboratory glassware and equipment may be needed.

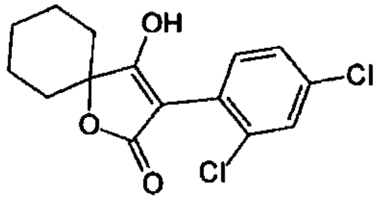
2.1 Test and Reference Substances

The test and reference substances for this study are listed below. Deuterated reference substances are used as internal standards.

Common Name:	BAJ 2740
Code Name:	BAJ 2740
Chemical Name:	3-(2,4-Dichlorophenyl)0-2-oxo-1-oxaspiro[4.5]dec-3-en-4-yl 2,2-dimethylbutanoate
CAS Number:	148477-71-8
Empirical Formula:	$C_{21}H_{24}O_4Cl_2$

Appendix I. (cont'd.)

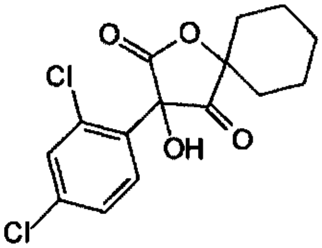
Molecular Weight:	411.30
Structure:	

Common	Enol
Code Name:	BAJ 2510
Chemical Name:	3-(2,4-dichlorophenyl)-4-hydroxy-1-oxaspiro[4.5]dec-3-en-2-one
CAS Number:	Unknown
Empirical Formula:	$C_{15}H_{14}O_3Cl_2$
Molecular Weight:	313.20
Structure:	

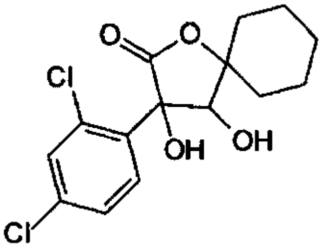
Common Name:	Ketohydroxy
Code Name:	KTS 9301

Appendix I. (cont'd.)

Chemical Name:	3-(2,4-Dichlorophenyl)-3-hydroxy-1-oxaspiro[4.5]decane-2,4-dione
CAS Number:	Unknown
Empirical Formula:	$C_{15}H_{14}O_4Cl_2$
Molecular Weight:	329.20
Structure:	

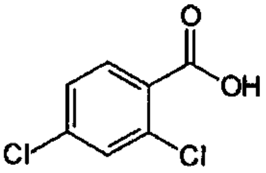


Common Name:	Dihydroxy
Code Name:	KTS 9313
Chemical Name:	3-(2,4-Dichlorophenyl)-3,4-dihydroxy-1-oxaspiro[4.5]decan-2-one
CAS Number:	Unknown
Empirical Formula:	$C_{15}H_{16}O_4Cl_2$
Molecular Weight:	331.18
Structure:	

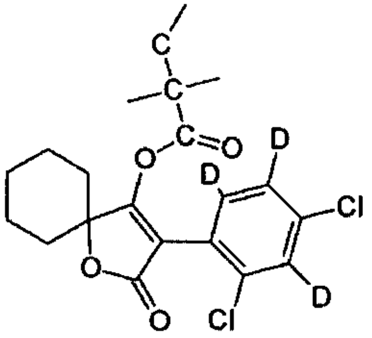


Appendix I. (cont'd.)

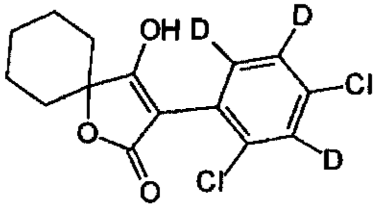
Common Name:	DCB-acid
Code Name:	DCBA
Chemical Name:	2,4-Dichloro-benzoic acid
CAS Number:	50-84-0
Empirical Formula:	$C_7H_4O_2Cl_2$
Molecular Weight:	191.01
Structure:	



Common Name:	BAJ 2740-d3
Code Name:	d3-BAJ 2740
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
CAS Number:	Unknown
Empirical Formula:	$C_{21}H_{21}O_4Cl_2H^2_3$
Molecular Weight:	414.40
Structure:	



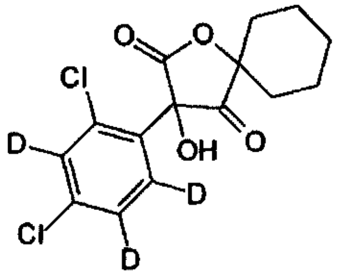
Appendix I. (cont'd.)

Common Name:	Enol-d3
Code Name:	d3-BAJ 2510
Chemical Name:	3-(2,4-Dichorophenyl)-4-hydroxy-1-oxaspiro[4.5]dec-3-en-2-one-phenyl-3,5,6-d3
CAS Number:	Unknown
Empirical Formula:	$C_{15}H_{11}O_3Cl_2H_3^2$
Molecular Weight:	316.20
Structure:	

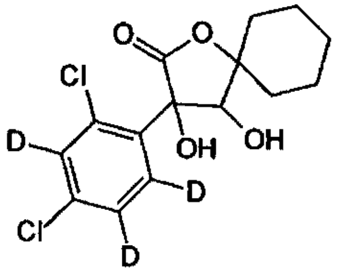
Common Name:	Ketohydroxy-d3
Code Name:	d3-KTS 9301
Chemical Name:	3-(2,4-Dichlorophenyl)-3-hydroxy-1-oxaspiro[4.5]decane-2,4-dione-phenyl-3,5,6-d3
CAS Number:	Unknown
Empirical Formula:	$C_{15}H_{11}O_4Cl_2H_3^2$

Appendix I. (cont'd.)

Molecular Weight:	332.20
Structure:	



Common Name:	Dihydroxy-d3
Code Name:	d3-KTS 9313
Chemical Name:	3-(2,4-Dichlorophenyl)-3,4-dihydroxy-1-oxaspiro[4.5]decan-2-one-phenyl-3,5,6-d3
CAS Number:	Unknown
Empirical Formula:	$C_{15}H_{13}O_4Cl_2H^2_3$
Molecular Weight:	334.20
Structure:	



Appendix I. (cont'd.)

Common Name: DCB-acid-d3

Code Name: d3-DCBA

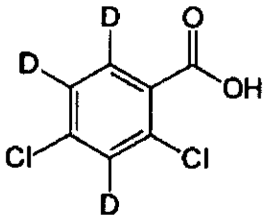
Chemical Name: 2,4-Dichloro-benzoic acid-3,5,6-d3

CAS Number: Unknown

Empirical Formula: $C_7HO_2Cl_2H_3^2$

Molecular Weight: 194.03

Structure:



2.2 Equipment Suggested Sizes/Suppliers/Manufacturers

Accelerated Solvent Extractor Dionex, Model ASE200

ASE Collection Vials, 60 mL Dionex Part # 048784

ASE Extraction Cells, 22 mL, Dionex Part # 049561

Autosampler vials, inserts, and caps

Column (Guard): Supelco, Supelguard LC-ABZ, 2 cm replacement cartridge, Part # 59545-U

Column (Analytical): Supelco, ABZ+PLUS, 10 cm x 2.1 mm ID, 5 µm, Part # 57925

Filters, Cellulose, 1.983 cm, Dionex Part # 049458

Filters, Nylon Acrodisc, 0.2 µm

General Laboratory Glassware

HPLC, Hewlett Packard, Model 1100, equipped with a refrigerated autosampler tray

Appendix I. (cont'd.)

LC/MS/MS (VG/Fisons/Micromass, Quattro II), equipped with a Z-Spray

Syringe, 5 mL or less, Becton Dickinson

Turbovap tubes, glass, disposable culture tubes, 16 x 100 mm

Zymark Turbovap, Model LV

2.3 Reagents (Equivalent Reagents may be substituted)

Acetonitrile, HPLC-grade, Burdick & Jackson, Cat. 015-4

Ammonium Formate 97% (63.06 g/mole), Aldrich, Cat. 15626-4

Ammonium Hydroxide 28.0-30.0%, J. T. Baker, Cat. 9721-03

Formic Acid 88%, Aldrich, Cat. 39938-8

Glacial Acetic Acid, Mallinckrodt, Cat. 3121

Methanol, HPLC grade, Burdick & Jackson, Cat. 230-4

Sand, Muffled

Water, HPLC-grade, Burdick & Jackson, Cat. 365-4

3.0 SOLUTION PREPARATION

3.1 Stock Standard Solutions

All standard solutions must be stored at <= -20°C to prevent degradation. All Stock Standards except for BAJ 2510 may be stored for approximately 3 months. The BAJ 2510 Stock Standard, Fortification Solutions, and Calibration Standards must be prepared fresh every five days. Equivalent dilutions of all standard solutions may be prepared.

3.1.1 1000 µg/mL Stock Standard for BAJ 2740

Prepare by dissolving 10 mg of analyte in a 10-mL volumetric flask with 0.1% formic acid in acetonitrile (3.5.1).

Appendix I. (cont'd.)

3.1.2 1000 µg/mL Stock Standard for BAJ 2510

Prepare by dissolving 10 mg of analyte in a 10-mL volumetric flask with 0.1% formic acid in acetonitrile (3.5.1).

3.1.3 1000 µg/mL Stock Standard for DCBA

Prepare by dissolving 10 mg of analyte in a 10-mL volumetric flask with acetonitrile.

3.1.4 1000 µg/mL Stock Standard for KTS 9301

Prepare by dissolving 10 mg of analyte in a 10-mL volumetric flask with acetonitrile.

3.1.5 1000 µg/mL Stock Standard for KTS 9313

Prepare by dissolving 10 mg of analyte in a 10-mL volumetric flask with acetonitrile.

3.1.6 1000 µg/mL Internal Standard Stock for BAJ 2740-d3

Prepare by dissolving 10 mg of analyte in a 10-mL volumetric flask with 0.1% formic acid in acetonitrile. (3.5.1)

3.1.7 1000 µg/mL Internal Standard Stocks for BAJ 2510-d3

Prepare by dissolving 10 mg of analyte in a 10-mL volumetric flask with 0.1% formic acid in acetonitrile (3.5.1).

3.1.8 1000 µg/mL Internal Standard Stocks for DCBA-d3

Prepare by dissolving 10 mg of analyte in a 10-mL volumetric flask with acetonitrile.

3.1.9 1000 µg/mL Internal Standard Stocks for KTS 9301-d3

Prepare by dissolving 10 mg of analyte in a 10-mL volumetric flask with acetonitrile.

3.1.10 1000 µg/mL Internal Standard Stocks for KTS 9313-d3

Prepare by dissolving 10 mg of analyte in a 10-mL volumetric flask with acetonitrile.

Appendix I. (cont'd.)

3.2 Intermediate Standard Mixes

3.2.1 20 µg/mL Intermediate Acidic Standard Mix

Prepare by mixing 200 µL of the BAJ 2740 1000 µg/mL Stock Standard (3.1.1) and 200 µL of the BAJ 2510 1000 µg/mL Stock Standard (3.1.2) in a 10-mL volumetric flask and dilute with 0.1 % formic acid in acetonitrile (3.5.1).

3.2.2 20 µg/mL Intermediate Basic Standard Mix

Prepare by mixing 200 µL of the DCBA 1000 µg/mL Stock Standard (3.1.3), 200 µL of the KTS 9301 1000 µg/mL Stock Standard (3.1.4), and 200 µL of the KTS 9313 1000 µg/mL Stock Standard (3.1.5) in a 10-mL volumetric flask and dilute with acetonitrile.

3.2.3 10 µg/mL Intermediate Acidic Internal Standard Mix

Prepared by placing 100 µL each of the 1000 µg/mL BAJ 2740-d3 and BAJ 2510-d3 Stock Standards (3.1.6 and 3.1.7) into a 10-mL volumetric flask. Bring to volume with 0.1% formic acid in acetonitrile (3.5.1).

3.2.4 10 µg/mL Intermediate Basic Internal Standard Mix

Prepare by placing 100 µL each of the 1000 µg/mL KTS 9301-d3, KTS 9313-d3, and DCBA-d3 Stock Standards (3.1.8, 3.1.9, and 3.1.10) into a 10-mL volumetric flask. Bring to volume with acetonitrile.

3.3 Fortification Standard Mixes

3.3.1 4 µg/mL Acidic Fortification Standard Mix

Prepare by diluting 2 mL of the 20 µg/mL Intermediate Acidic Standard Mix (3.2.1) in a 10-mL volumetric flask with 0.1% formic acid in acetonitrile (3.5.1).

3.3.2 2 µg/mL Acidic Fortification Standard Mix

Prepare by diluting 1 mL of the 20 µg/mL Intermediate Acidic Standard Mix (3.2.1) in a 10-mL volumetric flask with 0.1 % formic acid in acetonitrile (3.5.1).

Appendix I. (cont'd.)

3.3.3 0.4 μg/mL Acidic Fortification Standard Mix

Prepare by diluting 1 mL of the 4 μg/mL Acidic Fortification Standard Mix (3.3.1) in a 10-mL volumetric flask with 0.1% formic acid in acetonitrile (3.5.1).

3.3.4 4 μg/mL Basic Fortification Standard Mix

Prepare by diluting 2 mL of the 20 μg/mL Intermediate Basic Standard Mix (3.2.2) in a 10-mL volumetric flask with acetonitrile.

3.3.5 2 μg/mL Basic Fortification Standard Mix

Prepare by diluting 1 mL of the 20 μg/mL Intermediate Basic Standard Mix (3.2.2) in a 10-mL volumetric flask with acetonitrile.

3.3.6 0.4 μg/mL Basic Fortification Standard Mix

Prepare by diluting 1 mL of the 4 μg/mL Basic Fortification Standard Mix (3.3.4) in a 10-mL volumetric flask with acetonitrile.

3.3.7 3.2 μg/mL Acidic Internal Standard Fortification Mix

Prepared by placing 3.2 mL of the 10 μg/mL Intermediate Acidic Internal Standard Mix (3.2.3) into a 10-mL volumetric flask. Bring to volume with 0.1% formic acid in acetonitrile (3.5.1).

3.3.8 3.2 μg/mL Basic Internal Standard Fortification Mix

Prepared by placing 3.2 mL of the 10 μg/mL Intermediate Basic Internal Standard Mix (3.2.4) into a 10-mL volumetric flask. Bring to volume with acetonitrile.

Appendix I. (cont'd.)

3.4 Calibration Standards

3.4.1 Acidic Calibration Standard Preparation

3.4.1.1 Working Acidic Standards

Prepare by placing the amount listed below of the 20 µg/mL Intermediate Acidic Standard Mix (3.2.1) into individual 10-mL volumetric flasks. Bring to volume with Acidic Mobile Phase Mix (3.5.7). Cap and invert several times to mix.

Amount of 20 µg/mL Acidic Standard Mix (mL)	Final Volume (mL)	Final Concentration of Working Standard (pg/µL)
0	10	0
0.010	10	20
0.025	10	50
0.050	10	100
0.100	10	200
0.250	10	500
0.500	10	1000
1.0	10	2000
1.5	10	3000

3.4.1.2. Acidic Calibration Standards

Prepare by placing the amount listed below of the Working Standards into individual 10-mL volumetric flasks. Bring to volume with Acidic Calibration Dilution Solution (3.5.9). Cap and invert several times to mix.

Working Standard Concentration (pg/µL)	Amount of Working Standard (mL)	Final Volume (mL)	Final Concentration of Calibration Standard (pg/µL)
0	0.500	10	0
20	0.500	10	1
50	0.500	10	2.5
100	0.500	10	5
200	0.500	10	10
500	0.500	10	25
1000	0.500	10	50
2000	0.500	10	100
3000	0.500	10	150

Transfer calibration standards into autosampler vials for use with the Acid Analysis Method (6.3). Store frozen until use.

3.4.2 Basic Calibration Standard Preparation

3.4.2.1 Working Basic Standards

Prepare by placing the amount listed below of the 20 µg/mL Basic Intermediate Standard Mix (3.2.2) into individual 10-mL volumetric flasks. Bring to volume with Basic Mobile Phase Mix (3.5.8). Cap and invert several times to mix.

Amount of 20 µg/mL Basic Standard Mix (mL)	Final Volume (mL)	Final Concentration of Working Standard (pg/µL)
0	10	0
0.025	10	50
0.050	10	100
0.100	10	200
0.250	10	500
0.500	10	1000
1.0	10	2000
1.5	10	3000

3.4.2.2 Basic Calibration Standards

Prepare by placing the amount listed below of the Working Standards into individual 10-mL volumetric flasks. Bring to volume with Basic Calibration Dilution Solution (3.5.10). Cap and invert several times to mix.

Working Standard Concentration (pg/μL)	Amount of Working Standard (mL)	Final Volume (mL)	Final Concentration of Calibration Standard (pg/μL)
0	0.500	10	0
50	0.500	10	2.5
100	0.500	10	5
200	0.500	10	10
500	0.500	10	25
1000	0.500	10	50
2000	0.500	10	100
3000	0.500	10	150

Transfer calibration standards into autosampler vials for use with the Basic Analysis Method (6.2). Store frozen until use.

3.5 Solution Preparation

Prepare as needed. Store at room temperature.

3.5.1 0.1% Formic Acid in Acetonitrile Solution

Prepare by adding 1 mL of concentrated (88%) formic acid per liter of acetonitrile.

3.5.2 Extraction Solvent

Prepare by mixing 900 mL of acetonitrile, 100 mL water, and 1 mL glacial acetic acid.

3.5.3 Acidic Mobile Phase A: 4mM Ammonium Formate, 0.1% Formic Acid in Water

Prepare by dissolving 0.252 g of ammonium formate and 1 mL of formic acid per liter of water.

Appendix I. (cont'd.)

3.5.4. Acidic Mobile Phase B: 4mM Ammonium Formate, 0.1% Formic Acid in Methanol

Prepare by dissolving 0.252 g of ammonium formate and 1 mL of formic acid per liter of methanol.

3.5.5 Basic Mobile Phase A: 0.05% Ammonium Hydroxide in Water

Prepare by adding 500µL of ammonium hydroxide per liter of water.

3.5.6 Basic Mobile Phase B: 0.05% Ammonium Hydroxide in Methanol

Prepare by adding 500 µL of ammonium hydroxide per liter of methanol.

3.5.7. Acidic Mobile Phase Mix: 50:50 Water:Methanol containing 4 mM Ammonium Formate, 0.1% Formic Acid

Prepare by mixing equal portions of Acidic Mobile Phase A and Acidic Mobile Phase B (3.5.3 and 3.5.4).

3.5.8 Basic Mobile Phase Mix: 50:50 Water:Methanol containing 0.05% Ammonium Hydroxide

Prepare by mixing equal portions of Basic Mobile Phase A and Basic Mobile Phase B (3.5.5 and 3.5.6).

3.5.9 Acidic Calibration Dilution Solution

Prepare by diluting 1 mL of 10 µg/mL Intermediate Internal Standard Acidic Mix Solution (3.2.3) in a 200-mL volumetric flask with Acidic Mobile Phase Mix (3.5.7).

3.5.10 Basic Calibration Dilution Solution

Prepare by diluting 1 mL of 10 µg/mL Intermediate Internal Basic Standard Mix Solution (3.2.4) in a 200-mL volumetric flask with Basic Mobile Phase Mix (3.5.8).

4.0 PROCEDURE

4.1 Sample Preparation

4.1.1 Weigh 10.0 g of soil into a 22-mL stainless steel ASE extraction cell containing a cellulose filter.

4.1.2 Fortify the appropriate soils with the appropriate spiking solutions. For Example, to prepare a soil sample fortified at 0.10 ppm, add 250 µL each of the 4 µg/mL Acidic Fortification Standard Mix and the 4 µg/mL Basic Fortification Standard Mix (3.3.1 and 3.3.4). To prepare a soil sample fortified at 0.05 ppm, add 250 µL each of the 2 µg/mL Acidic Fortification Standard Mix and the 2 µg/mL Basic Fortification Standard Mix (3.3.2 and 3.3.5). To prepare a soil sample fortified at 0.01 ppm, add 250 µL each of the 0.4 µg/mL Acidic Fortification Standard Mix and the 0.4 µg/mL Basic Fortification Standard Mix (3.3.3 and 3.3.6).

4.1.3 Fortify all soils with 250 µL of each of the Internal Standard Fortification Solutions (3.3.7 and 3.3.8). Fill remaining volume of the cell with muffled sand and cap.

4.1.4 Extract the sample with Extraction Solvent (3.5.2) on an ASE according to the following conditions:

Preheat:	0 min.	Purge:	180 sec
Heat:	5 min.	Cycles:	1
Static:	5 min	Pressure:	1500 psi
Flush %:	95	Temperature:	100°C

4.1.5 Transfer the contents of the ASE extract vial into a 50-mL graduated mixing cylinder. Bring the sample up to 40 mL with acetonitrile, cap, and mix well.

4.1.6 Remove two separate 5-mL aliquots of the extract and place into separate TurboVap tubes. One aliquot will be used for the analysis of BAJ 2740 and BAJ 2510 ("Acidic Extract"). The other aliquot will be used for the analysis of KTS 9313, KTS 9301, and DCBA ("Basic Extract").

4.1.7 Add 3 drops of concentrated ammonium hydroxide to the Basic Extract and vortex to mix.

4.1.8 Concentrate both aliquots just to dryness at ~35°C with a Turbovap. The samples should be monitored closely and removed when no visible liquid remains. The residue should still be moist. Significant loss of BAJ 2740 will occur if samples remain in the TurboVap too long.

Appendix I. (cont'd.)

4.1.9 To the Acidic Extract residue, add 2000 µL of Acidic Mobile Phase Mix (3.5.7). Vortex well to mix.

4.1.10 Filter an aliquot of the Acidic Extract through Nylon 0.2-µm filters into autosampler vials. Analyze the reconstituted extracts by the Acid Analysis Method (6.3).

4.1.11. To the Basic Extract residue, add 2000 µL of Basic Mobile Phase Mix (3.5.8). Vortex well to mix.

4.1.12 Filter an aliquot of the Basic Extract through Nylon 0.2-µm filters into autosampler vials. Analyze the reconstituted extracts by the Basic Analysis Method (6.2).

4.1.13 Any extracts that will not be analyzed immediately must be stored in the freezer until analysis.

5.0 MOISTURE DETERMINATION

Results of soil analysis are reported on a "dry weight" basis for residue determination. Soil sample weights must be corrected for moisture content. Any method the laboratory customarily uses for determining the percent moisture of the soil can be utilized. The procedural recoveries from laboratory fortified samples are not corrected for moisture content of the soil. An example calculation can be found in section 9.0.

6.0 RECOMMENDED LC/MS/MS CONDITIONS

6.1. The following LC/MS/MS instrument operating conditions are used for both the Acid and Base Analysis Methods:

LC/MS/MS System HPLC Mass Spectrometer Data System	 Hewlett Packard, Model 1100 VG/Fisons, Model Quattro II Fisons/Micromass MassLynx, v. 3.2, Build 004
Refrigerated Autosampler	Hewlett Packard Model 1100 Maintained at 5 °C.
Guard Column	Supelguard LC-ABZ, 2 cm replacement cartridge, Supelco Part #59545-U, (replaced with each run set)
Analytical Column	ABZ+PLUS, 10 cm x 2.1 mm ID, 5 µm, Supelco Part # 57925
Mobile Phase Flow Rate	400 µL/minute
Column Temperature	Ambient

Appendix I. (cont'd.)

Injection Volume	50 µL
Mass Spectrometer Source	Electrospray BAJ 2740, BAJ 2510, KTS 9313, KTS 9301: Positive Ion Mode DCBA: Negative Ion Mode
Source Temperature	90°C
Desolvation Gas	Nitrogen at ~ 350 L/h
Desolvation Temperature	300°C
Cone Voltage	Optimized for individual analytes
Collision Gas	Argon at ~ 5 x 10^{-3} mb analyzer pressure
Collision Energy	Optimized for individual analytes
Multiplier	650 V

6.2 Basic Analysis Method - The following instrument parameters are used for the analysis of the Basic Extracts:

HPLC Parameters

Mobile Phase	A: Water with 0.05% ammonium hydroxide B: Methanol with 0.05% ammonium hydroxide	
Gradient	Time	Composition
	0.0	90%A + 10%B
	2.0	40%A + 60%B
	4.0	40%A + 60%B
	6.0	0%A + 100%B
	10.0	0%A + 100%B
	11.0	90%A + 10%B
	16.0	90%A + 10%B
	Run every 16 minutes	
Retention Times		
DCBA	~ 1.2 minutes	
KTS 9313	~ 8.4 minutes	
KTS 9301	~ 9.3 minutes	
	(DCBA Retention Times are often different for the samples than for the standards probably due to matrix effects at such a short RT. The correct peak should be easily tracked and identified by the Internal Standard)	

Appendix I. (cont'd.)

Mass Spectrometer Parameters

Analyte	Analyte Masses Monitored	Internal Standard Masses Monitored	Cone Voltage	Collision Energy
DCBA	m/z 189 >m/z 145 (negative ions)	m/z 194 >m/z 150 (negative ions)	10 V	13 eV
KTS 9313	m/z 348 >m/z 313	m/z 353 >m/z 318	15 V	15 eV
KTS 9301	m/z 378 >m/z 189	m/z 383 >m/z 194	20 V	35 eV

6.3 Acid Analysis Method: The following instrument parameters are used for the analysis of the Acidic Extracts:

HPLC Parameters

Mobile Phase	A: Water with 0.1% formic acid and 4 mM ammonium formate B: Methanol with 0.1% formic acid and 4 mM ammonium formate
Gradient	Time Composition 0.0 70%A + 30%B 1.0 0%A + 100%B 5.0 0%A + 100%B 6.0 70%A + 30%B 10.0 70%A + 30%B Run every 10 minutes
Retention Time BAJ 2740 BAJ 2510	 ~ 5.4 minutes ~ 6.6 minutes

Mass Spectrometer Parameters

Analyte	Analyte Masses Monitored	Internal Standard Masses Monitored	Cone Voltage	Collision Energy
BAJ 2740	m/z 411>m/z 313	m/z 416>m/z 318	25 V	20 eV
BAJ 2510	m/z 313>m/z 213	m/z 318>m/z 218	35 V	35 eV

Appendix I. (cont'd.)

Note: The Basic Analysis Method must be run before the Acidic Analysis Method or column performance is lost. The LC/MS/MS can be programmed to run both the Basic and Acid Analysis Methods sequentially.

6.4 Load the autosampler with a set of analytical standards at the beginning of each run. Additionally, distribute two more analytical standards throughout the run, interspersed between the sample solutions. The data from all three analyses of each standard is used to generate the calibration curve.

Note: The equipment listed was used for method development and validation. Other equivalent hardware may be used. The recommended instrument parameters were found to be optimal for the instrument used. The exact values used must be optimized for each instrument. The recommended chromatographic systems were found to be optimal for the types of instruments used. Different chromatographic conditions might be necessary for different types of instruments.

7.0 CALIBRATION AND ANALYSIS

Results are calculated by comparing peak areas obtained from sample extracts to calibration curves generated from peak areas obtained for the calibration standards. The same injection volume must be used for both samples and standards. Standard curves for BAJ 2740 and BAJ 2510 are obtained from the analysis of the Acidic Calibration Standards (3.4.1.2) covering the range of 1.0 pg/µL to 150 pg/µL (use of the 0 pg/µL standard is optional). Standard curves for KTS 9301, KTS 9313, and DCBA are obtained from the analysis of the Basic Calibration Standards (3.4.2.2) covering the range of 2.5 pg/µL to 150 pg/µL (use of the 0 pg/µL standard is optional). Calibration curves are generated from a least squares fit of the peak area ratio (standard/internal standard) versus the concentration of analyte using a weighted (1/Y) linear regression. Calculations used to quantitate detected residues are given in Section 9.

8.0 LIMIT OF QUANTITATION AND METHOD DETECTION LIMIT

The limit of quantitation is defined as the lowest fortification level successfully tested. The limit of quantitation is 0.01 ppm. The method detection limits can be found in Table 1.

9.0 CALCULATIONS

Quantities of any residues of BAJ 2740 and its metabolites in soil are calculated using the calibration curves and the following formulas:

$$\text{Area Ratio} = \frac{\text{Compound Peak Area}}{\text{IS Peak Area}}$$

$$\text{Wet Sample Residue (ppm)} = \frac{\text{Area Ratio - Intercept}}{\text{Slope}}$$

$$\text{Standard Conc. (ppm)} = \text{Standard Conc. (pg/uL)} \times \frac{\text{Final Volume (2 mL)}}{\text{Sample Weight (10 g)}} \times \frac{\text{Total ASE Volume (40 mL)}}{\text{ASE Aliquot (5 mL)}} \times 10^{-3}$$

$$\text{Percent Moisture} = \frac{\text{Wet Sample Weight (g) - Dry Sample Weight (g)}}{\text{Wet Sample Weight (g)}} \times 100\%$$

$$\text{Dry Residue Concentration (ppm)} = \frac{\text{Wet Sample Residue (ppm)}}{\text{(1 - Percent Moisture)}}$$

$$\text{Percent Recovery (\%)} = \frac{\text{Wet Sample Residue (ppm) - Residue (ppm) of control sample}}{\text{Fortification Concentration (ppm)}} \text{ X } 100$$

10.0 TIME REQUIRED FOR ANALYSIS

The time required for a set of 24 samples (including fortified and control) is approximately 16 person-hours, or 2 calendar days. This includes extraction, mass spectral analysis, and data reduction.

11.0 POTENTIAL PROBLEMS

It is critical that the samples be taken "just to dryness" in step 4.1.8, otherwise significant losses of BAJ 2740 are observed.

Guard columns must be changed each day.

Sample extracts may be stored frozen after ASE extraction.

Under certain mass spectrometer tuning conditions, BAJ 2740 fragments during ionization to form an ion at m/z 313. If low recoveries, or poor sensitivity, is observed for the m/z 411 > m/z 313 transition, it may be possible to generate better results by utilizing the m/z 313 > m/z 213 transition for the native analyte and m/z 318 > m/z 218 transition for the internal standard (same ions used for BAJ 2510).

Appendix I. (cont'd.)

It may be necessary to calculate KTS 9301 using an external calibration curve, disregarding the internal standard. Over-recovery of KTS 9301 was observed during routine analysis. In some instances the internal standard responses appeared to be lower than expected, resulting in a higher Area Ratio. If this is the case, the calibration curve may be generated from the KTS 9301 area only instead of the area ratio.

12.0 SAFETY AND HEALTH CONSIDERATIONS

All procedures involving organic solvents should be performed in a well-ventilated hood. Personal protective equipment (gloves, lab-coats) should be worn while performing this method. Read all label statements and precautions.

Appendix I. (cont'd.)

Figure 1. Flow Diagram