# 1. INTRODUCTION AND SUMMARY

## 1.1. Purpose of Study

The study was conducted to generate recovery data for the validation of BASF Method Number D0307: Analytical Method for the LC/MS/MS Determination of BAS 715 H and Metabolite CL 354825 in Soil. Imazapic, code name BAS 715 H, is the active ingredient of the herbicides Plateau® and Cadre® used for treatment of grass and peanuts, respectively. This method was developed to combine analysis of BAS 715 H and the metabolite CL 354825 in one method with quantitation by LC/MS/MS whereas previous methods covered each analyte separately (Reference 2). The method will be used for analysis of soils from a small-scale prospective ground water monitoring study (Reference 3). CL 354825 was the metabolite determined to occur on degradation of $^{14}$C-BAS 715 H in soil (Reference 1).

## 1.2. Summary

The validation study was conducted in two soils, a German loamy sand and a North Carolina (NC) sandy soil. The NC soil was from the site of a small-scale ground water monitoring study following treatment with Plateau® on grass (Reference 3).

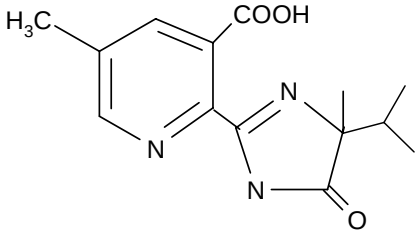
The validation was performed at two levels, the LOQ of 0.001 ppm and 0.01 ppm. Five soil aliquots were fortified at each level and each set included in addition a method blank and two unfortified soil aliquots as controls. The acid treated extract from each set was taken through a cleanup using C18 SPE and analyzed. Aliquots of the acid-treated extract were also taken through a cleanup using both C18 and SCX SPE to demonstrate that this option also provided acceptable recoveries.

Recoveries of BAS 715 H and CL 354825 dropped significantly on storage of the basic extracts during method development. The stability of the analytes in the extract after treatment with formic acid was improved, and acidified extracts of the German soil were cleaned up with SPE and analyzed after three days storage at room temperature with acceptable recoveries

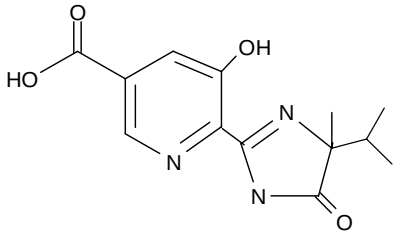
To test the stability of analytes in samples ready for analysis, the samples extracted from NC soil and cleaned up with C18 SPE were reanalyzed about 40 h after the initial analysis with storage at ambient temperature.

## 2. MATERIALS AND METHODS

### 2.1. Test and Reference Substances

| | |
|---|---|
| BASF Code Name: | BAS 715 H (CL 263222) |
| Common Name: | Imazapic |
| BASF Registry Number: | 4095755 |
| Lot Number: | AC10606-119 |
| Purity & Purity Expiration Date | 99.3 %, February 21, 2006 |
| CAS Number: | 104098-48-8 |
| Chemical Name: | Nicotinic acid, 2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)-5-methyl- |
| Molecular Formula: | $C_{14}H_{17}N_30_3$ |
| Molecular Weight: | 275.3 |
| Structural Formula: |  |



| | |
|---|---|
| BASF Code Name: | CL 354825 |
| Common Name: | CL 354825 |
| BASF Registry Number: | 4110603 |
| Lot Number: | AC9918-101 |
| Purity & Purity Expiration Date | 90 %, October 17, 2006 |
| Chemical Name: | Nicotinic acid, 5-hydroxy-6-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl) |
| Molecular Formula: | $C_{13}H_{15}N_3O_4$ |
| Molecular Weight: | 277.3 |
| Structural Formula: |  |



Neat standard substances were stored in the freezer (- 30 to 0 º C) until use. BASF has retained a reserve sample of these chemicals, and has documentation at BASF Agro Research, Research Triangle Park, North Carolina, specifying the location of the synthesis and characterization information for these compounds.

### 2.2. Extraction Solutions

Soil samples were extracted with 0.05 N NaOH solution as was done in the aerobic soil metabolism studies (Reference 1).

## 3. TEST SYSTEMS

Two soils were used in this study, one a sandy soil obtained from the NC site used for a BAS 715 H small-scale ground water monitoring study (Reference 3) and one a standard German loamy sand soil designated 2.2, F 2.22100. For the validation study, bulk soil to a depth of one foot was collected from four locations on the NC site prior to treatment. The soil was composited, divided into bags, shipped at ambient temperature and stored in the freezer until use. An aliquot of the soil labeled bulksoil031102-A was homogenized with dry ice using a Fitz Mill Homoloid at BASF. The soil from Germany was stored in the freezer until use and was used without processing. Soil characterizations for the NC soil were performed as part of the small-scale ground water monitoring study

## 4. SAMPLE DESCRIPTION/IDENTIFICATION

Aliquots of soil were weighed out and identified with study number-master sheet number-consecutive number as each set was analyzed. Samples were also assigned a code, “blank” for the method blank, “control” for a unfortified sample, “F1” for soils fortified at 0.001 ppm and “F2” for soils fortified at 0.01 ppm. Each soil sample was further described as “German” or “NC” soil.

## 5. EXPERIMENTAL DESIGN

The analytes covered in the method validation were BAS 715 H and CL 354825. For each of the two soils, aliquots of 10.0 g were fortified with BAS 715 H and CL 354825 at two different levels: five replicates at 0.001 ppm, the limit of quantitation (LOQ) and five replicates at 0.01 ppm. An analysis set included the ten fortified samples, two unfortified control soil samples and a method blank. An analysis set included the ten fortified samples, two unfortified controls and a blank. Because the method provides options for cleanup using C18 SPE or cleanup using both C18 and SCX SPE, aliquots of the acidified soil extract were analyzed after C18 SPE cleanup and separate aliquots were analyzed after combined C18 and SCX SPE cleanup. While the method provides the option for use of an automated SPE extraction system such as the RapidTrace™ SPE Workstation, the validation did not use any automated system. Stability of the residues in the acidified extracts was tested after about three days storage. Stability of the residues in the solutions ready for LC/MS/MS analysis was tested after about 40 h storage.

Storage stability of the analytes in standard solutions was determined as part of the validation study. Stock, fortification and calibration solutions were analyzed after specific storage intervals versus calibration standards generated from freshly prepared stock solutions. Stock solutions were analyzed after about 2.5 months and fortification and injection calibration solutions after about one month refrigerator storage.

## 6. METHOD OF ANALYSIS

### 6.1. Method Summary

Flowcharts for the procedure for extraction, cleanup and quantitation of BAS 715 H and CL354825 residues are shown in Figures 1 and 2.

A brief description of the method follows. A 10 g soil sample is extracted with 0.5 N NaOH by shaking on a reciprocal shaker at room temperature. An aliquot of the extract is treated with formic acid to precipitate the humic material. An aliquot of the pH 3 supernatant is cleaned up by C18 SPE with elution of the analytes from the column using dichloromethane (DCM). Should recovery of analytes, particularly CL 354835, not be acceptable, an aliquot of the pH 3 supernatant should be cleaned up by DCM elution from the C18 SPE column, then methanol elution from the C18 SPE column, further cleanup of the methanol eluant using SCX SPE and combining of the DCM and SCX eluants (see Figure 2). The BAS 715 H is eluted from the C18 SPE with DCM while a fraction of the CL 354825 analyte might require methanol to completely elute from the C18 column. The SCX column is then used to eliminate the interferences from the soil matrix that are also eluted with the methanol. The SPE eluate is taken to dryness, and the residues are redissolved in water. Quantitation of BAS 715 H and CL354825 is performed by LC/MS/MS using electrospray ionization in the positive mode. Quantitation is based on peak area measurements. Using the peak area measured for BAS 715 H and CL 354825, the amount of analyte in pg can be determined from the appropriate least squares calibration curve constructed using at least duplicate injections of four levels of calibration standards. Typical calculations for quantitation are shown in Figure 3.

### 6.2. Method Limit of Quantitation (LOQ) and Limit of Detection (LOD)

The limit of quantitation (LOQ) is defined as the lowest fortification level successfully tested in this validation study which is 0.001 ppm for each analyte. For each analyte the LOD, as defined by signal to noise, is ≤ ¼ the lowest standard. The lowest standard is 0.02 ng/mL, equivalent to 0.192 ppb. The LOD at ¼ that amount is equivalent to 0.048 ppb. In the validation study, the LOD was not set, and an area count resulting in calculated concentration in pg > 0 was measured.

### 6.3. Stability of Extracts, Acid-treated Extracts and Analysis–ready Samples

Initial analysis of BAS 715H and Cl 354825 in extracts of fortified soil was performed the day of fortification and extraction. After about 40 h storage at room temperature, samples in vials previously used for LC/MS/MS analysis of the NC soil were reanalyzed. Acid-treated extract of the German soil was also stored for about 3 days at room temperature prior to SPE cleanup and analysis.

### 6.4. Method Accountability

Extraction was performed in both German and North Carolina soils by shaking the sample with 0.05 N NaOH, the extraction procedure used in the aerobic soil metabolism studies (Reference 1); thus no method accountability study was needed.

## 7.5. Standard Storage and Stability

Stock solutions of 1 mg/mL concentration, fortification solutions and calibration standard solutions were stored refrigerated in amber glass bottles (1-10 º C). The stability of the solutions of reference substances was determined as part of this validation study. The results of the stability experiment are summarized in Table 4. For the stability testing, the standard solutions were diluted, if needed, as described on Table 7 and analyzed versus calibration standards prepared from freshly prepared stock solutions. The theoretical amount of pg injected for each standard solution was determined based on the final concentration. The recovery was calculated by dividing the amount measured (pg) by the theoretical expected amount (pg) injected for the standard solution. Dual injections were made for each standard solution and the recoveries were averaged. The recovery of CL354825 in the stored standard solutions was corrected by 27.5/25 since newly prepared stock solution used to prepare the calibration standards took account of the 90 % purity of neat CL 354825 and 27.5 mg of neat standard was dissolved in 25 mL for a 1 mg/mL solution . The concentration of the original stock solution ERS03-804 was based on 25 mg/mL and so did not take account of the standard purity. The quantitation was performed by LC/MS/MS using the instrument parameters that were used for sample analysis.

As summarized in Table 4, the stock solutions demonstrated complete stability for 2 months and 10 days, so that a period not to exceed three months storage is given in the method as an acceptable interval. Fortification solutions and calibration standard solutions were stable through about one month and this is the period specified in the method.

## 8. CONFIRMATORY TECHNIQUES

LC/MS/MS is a self-confirmatory technique. Monitoring of a second transition for each analyte, 276.1 $\rightarrow$ 163.0 and 278.1 $\rightarrow$ 235.8 for BAS 715 H and CL 354825, respectively, provides added confirmation.

**Figure 1 Flow Diagram of Method D0307**

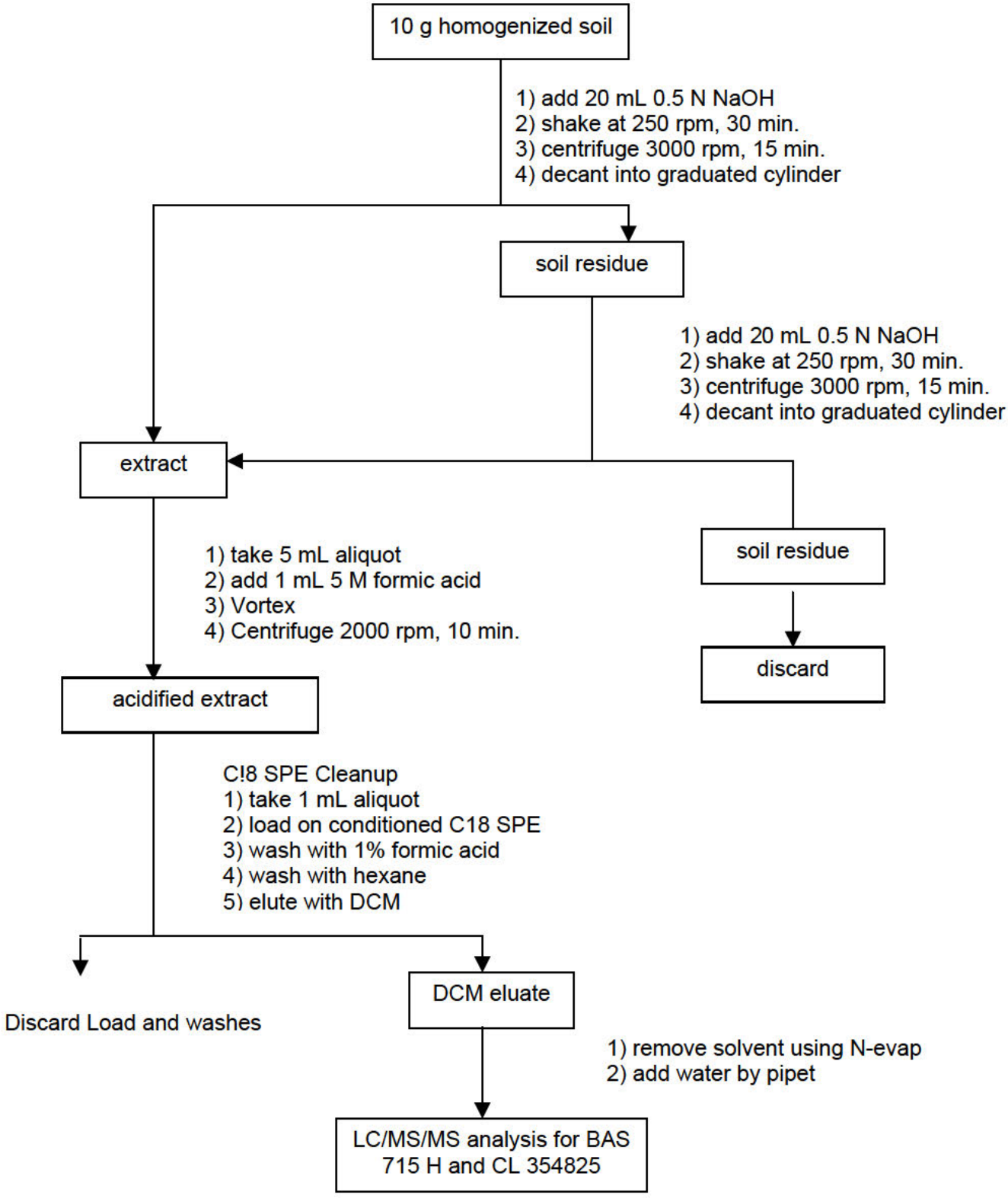


**Figure 2 Flow Diagram of Method D0307 with Optional SCX Cleanup**

Extraction Procedure is the same as in Figure 1.

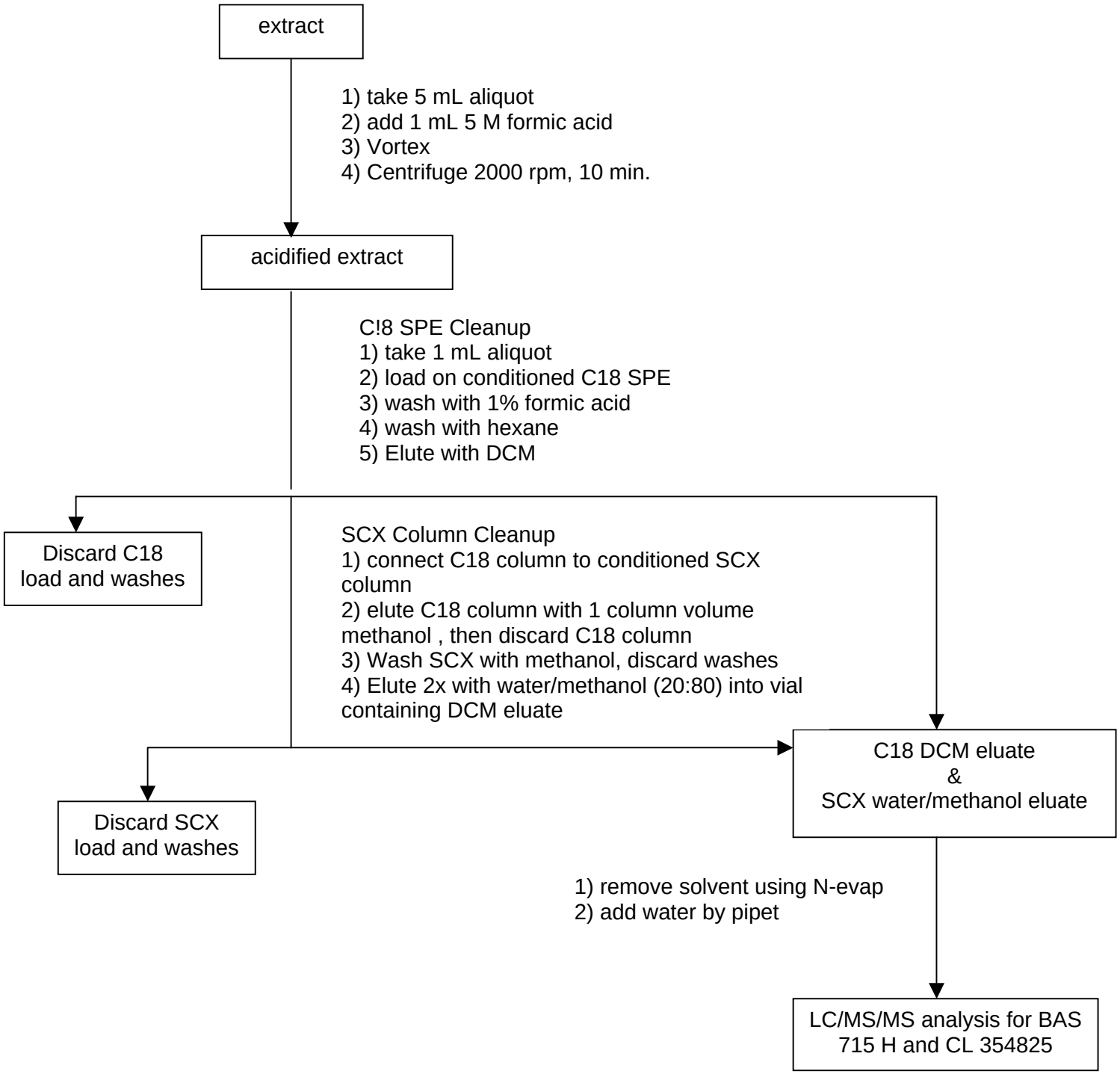


**Figure 3 Sample Calculations**

Calculation of results is based on peak area measurements. Using the peak area measured for BAS 715 H the amount of analyte in pg can be determined from the appropriate least squares calibration curve. In the validation study, no adjustment was made for soil moisture. Calculate the residue concentration in ppm for soil using the equation:

$$C_S = \frac{W_A \times V_F}{W_S \times V_I \times AF \times 1000}$$

$W_A$ = Amount of analyte calculated from calibration curve (pg)

$V_F$ = Final Volume after all dilutions (mL)

$W_S$ = Sample weight extracted (g)

$V_I$ = Injection volume (µL)

AF = (aliquot used for neutralization) x (aliquot used for SPE) / (total extract volume) x (total volume of neutralized aliquot)

1000 = Factor remaining after all unit conversions

The procedural fortification results should be corrected for any residues found in the control samples. The control samples have not been fortified with any analytes. The peak area, if any, found for the controls should be converted to a concentration in ppm, $C_C$, the two control sample results averaged, and this result subtracted from the concentration found for fortified sample, $C_S$.

$$C_{CORR} = C_S - ((C_{C1} + C_{C2})/2)$$

where: $C_{CORR}$ is the corrected fortification concentration,
$C_S$ is the concentration of fortified soil sample, and
$C_{Cx}$ is the concentration of the control samples (all in ppm).

Determine percent recovery for the fortification samples as follows where $C_F$ is the concentration fortified :

$$\% R = \frac{C_{CORR}}{C_F} \times 100$$

The values for calculated concentration in pg determined by the LC/MS are rounded to 2 units after the decimal place. Values with at least 3 significant figures can be carried out throughout the intermediate calculations and rounded to whole numbers for the percent recovery. An example set of calculations using a sample with North Carolina (NC) soil on Master Sheet 1 is shown below. The 10 g sample of NC soil was fortified with BAS 715 H and CL 354825 using 1.00 mL of 10.0 ng/mL standard solution.

**Fortification ppm:**

$$C_F = \frac{\text{volume (mL)} \times \text{standard concentration (µg/mL)}}{\text{wet weight (g)}}$$

$$= \frac{1.00 \text{ mL} \times .010 \text{ µg/mL}}{10.07 \text{ g}}$$

$$= 0.000993049 \text{ ppm} = 0.001 \text{ ppm}$$

To calculate $W_A$, the amount (in pg) of analyte BAS 715 H from the calibration curve, a linear least squares curve was constructed ( Master Sheet 1, C18 cleanup).

| BAS 715 H | | | | | | |
|---|---|---|---|---|---|---|
| File Type | Sample Name | Injection Vol. | Area | Area | Area | Area |
| Standard | 0.02 pg/µL | 25 µL | 732 | 793 | 706 | 711 |
| Standard | 0.04 pg/µL | 25 µL | 1267 | Not inj. this cycle | 1291 | 1446 |
| Standard | 0.10 pg/µL | 25 µL | 2947 | | 3180 | 3399 |
| Standard | 0.20 pg/µL | 25 µL | 6279 | | 6748 | 7243 |

| | |
|---|---|
| Intercept, b | -2.03 |
| Slope, m | 1340 |
| Correlation Coeff. | 0.9950 |

The area for sample 121735-1-04 was 3600. Using equation y = mx + b, solving for x:

$$x = \frac{(y - b)}{m} = \frac{(3600 - (-2.03)}{1340} = 2.69 \text{ pg } (= W_A)$$

Sample wt. = 10.07 g Final Vol. = 2.0 mL Aliquot factor = (5.0 x 1.0) mL/ (40.0 x 6.0) mL

$$C_S(\text{wet}) = \frac{W_A \times V_F}{W_S \times V_I \times AF \times 1000}$$

$$= \frac{2.69 \text{ pg} \times 2.0 \text{ mL} \times 40.0 \times 6.0}{10.07 \text{ g} \times 25.0 \text{ µL} \times 5.0 \text{ X } 1.0 \times 1000}$$

$$= 0.00102578 \text{ ppm}$$

**Sample ppm (dry weight basis):** Moisture correction was not done in the validation study.

$C_S(dry) = (1 + M) \times C_S(wet)$

$= (1.0 + 0.X) \times 0.00102578 \text{ ppm}$

$= 0.00102578 \text{ ppm}$

**Corrected ppm value:**

The corresponding control samples contained an average of 0.000015 ppm.

$C_{CORR} = C_S - C_C$

$= 0.00102578 \text{ ppm} - 0.000015 \text{ ppm}$

$= 0.00101078 \text{ ppm}$

**Recovery:**

$$\%\ R = \frac{C_{CORR}}{C_F} \times 100$$

$$= \frac{0.00101078 \text{ ppm}}{0.000993049 \text{ ppm}} \times 100$$

$= 102\ \%$ recovery

## 2.2. Equipment -- Suggested Sizes/Suppliers, Manufacturers

| Equipment | Size, Description | Supplier |
|---|---|---|
| Balance, Analytical | AE 240 | Mettler |
| Balance, Top Loading | PG2002 | Mettler |
| Bottle, Amber glass | 2 oz., 4 oz., Teflon®-lined screw cap | Qorpak |
| Bottle, Centrifuge, for extraction | 175 mL polypropylene copolymer conical bottom | Nalgene |
| Centrifuge | GS-6R series or Sorvall® RC-5B | Beckman, Dupont |
| Centrifuge Adapters | for 150-200 mL bottles<br>for tubes | Beckman |
| Cylinder, Graduated with stoppers for 50, 100 mL | 10, 25, 50, 100, 500, 1000 mL | Various |
| Culture tubes with caps | 8 mL | |
| Flask, Volumetric | 25, 100, 200, 500, 1000 mL | Various |
| Funnels, glass | 75 mm top ID | Various |
| N-Evap™analytical evaporator | Organomation | Meyer |
| Pipet, Pyrex disposable | 1, 2, 5, 10 mL | Various |
| Pipet, volumetric | 0.5, 1-10 mL, 25, 50, 100 mL | Various |
| Shaker –reciprocal | Model KS501 | IKA Labortechnik |
| Spatula | | Various |
| Solid phase extraction (SPE) columns | C18, Bond Elut, 200 mg, 3 cc<br>SCX, IST 200 mg, 3 cc | Varian<br>Argonaut |
| Ultrasonic Bath | | Branson 2200 |
| VacMaster-20, IST Vacuum Manifold | | Argonaut |
| Vials, glass liquid scintillation | 20 mL | Various |
| Vials, HPLC autosampler | glass screw top or snap top with Teflon-lined septa | Hewlett Packard |
| Vortex Mixer | | |
| Automated SPE Solid-Phase Extraction System | RapidTrace™ SPE Workstations and Controller | ZyMark |

**NOTE**: C18 SPE Lot # 0718103 was used in validation. SCX SPE Lot #s 2252202 FC & 2310202 BIA were used in the validation. Other general laboratory glassware and equipment as needed were used. Equipment with equivalent performance may be used as required.

## 2.3. Reagents and Chemicals -- Suggested Sources

| Chemical | Grade | Manufacturer/ Supplier | Catalog No. |
|---|---|---|---|
| Acetic Acid | HPLC grade | J. T. Baker | 9515-03 |
| Formic Acid | | EM Science | FX 0440-7 |
| Hexane | GC/HPLC | Burdick & Jackson | 216-4 |
| Hydrochloric acid, 1.0 N solution | | VWR | VW 3202-1 |
| Methanol | GC/HPLC | Burdick & Jackson | 230-4 |
| Methylene chloride (Dichloromethane) | GC/HPLC | Burdick & Jackson | GC 299-4 |
| Sodium Hydroxide 5.0 N solution | | VWR | VWR 3225-1 |
| Water | GC/HPLC | Burdick & Jackson | 365-4 |

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

## 2.4. Solvent Mixtures

### 2.4.1. Sodium Hydroxide (0.5 N)

Prepare the 0.5 N sodium hydroxide solution by diluting the 5.0 N solution with water. For example to prepare one liter of 0.5 N solution, add 100 mL 5.0 N solution to water (Burdick and Jackson bottled) in a one liter volumetric or a graduated cylinder and make up to one liter with water. Transfer to a capped bottle and swirl to mix. The mixture can be stored at ambient conditions for one year.

### 2.4.2. Formic Acid ( 5.0 M and 1 %)

To prepare 5.0 M formic acid from neat formic acid (26.5 M), add 18.9 mL neat formic acid per 100 mL of desired solution to water in a volumetric or a graduated cylinder and make up to volume with water. For example to prepare one liter of 5.0 M solution, measure 189 mL formic acid using a graduated cylinder and add to water (Burdick and Jackson bottled) in a one liter volumetric or a graduated cylinder and make up volume with water. Transfer to a capped bottle and swirl well to mix. The mixture can be stored at ambient conditions for up to one year.

To prepare 1 % formic acid from neat formic acid (26.5 M), add one mL neat formic acid per 100 mL of desired solution to water in a volumetric or a graduated cylinder and make up to volume with water.

### 2.4.3. Hydrochloric Acid (0.01 N)

The C18 SPE columns are washed with 0.01 N HCl prior to loading the samples. Prepare the 0.01 N HCl solution by diluting the 1.0 N HCl solution with water. For example to prepare 500 mL of 0.01 N solution, pipette 5.0 mL of the 1.0 N solution into water (Burdick and Jackson bottled) in a 500 mL volumetric flask or a graduated cylinder

and make up to 500 mL with water. Transfer to a capped bottle and swirl to mix. The mixture can be stored at ambient conditions for six months.

#### 2.4.4. Water/Methanol (20:80) Used only if SCX Cleanup required.

To prepare a water/methanol (20:80) solution, measure the required amount of water into a volumetric flask or graduated cylinder and make up to volume with methanol. For example, to prepare 500 mL of water/methanol (20:80) solution, transfer 100 mL of water using a graduated cylinder into a 500 mL or 1 L graduated cylinder and make up to 500 mL with methanol.

### 2.5. Standard Solutions

#### 2.5.1. Standard Solution Storage and Stability

The standard solutions are kept refrigerated at 1 to 10 º C. The storage stability of stock, fortification and calibration standard solutions was investigated as part of the method validation study under protocol 121735 (Reference 3) and the results are summarized in Table 3. Based on the stability experiments, stock solutions (1 mg/mL) in methanol should be made fresh at least every 3 months. Dilutions of stock solutions in methanol or water including calibration standard solutions should be made fresh at least every month. Suggested storage containers for standard solutions are amber glass bottles with Teflon®-lined screw caps.

#### 2.5.2. Standard Stock Solutions

Suggested standard concentrations are listed below. A different concentration scheme may be used and additional standards may be prepared as needed. Individual stock solutions with concentrations of 1 mg/mL each are prepared for BAS 715 H and CL354825 in methanol.

##### 2.5.2.1. BAS 715 H

Prepare a 1.0 mg/mL BAS 715 H stock solution by weighing an appropriate amount of BAS 715 H into a volumetric flask. Dissolve with methanol, mix well and dilute to the mark.

For example, place 25.0 mg of BAS 715 H into a 25 mL volumetric flask. Dissolve with methanol, mix well and dilute to mark.

##### 2.5.2.2. CL 354825

Prepare a 1.0 mg/mL CL 354825 stock solution by weighing an appropriate amount of CL 354825 into a volumetric flask. Dissolve with methanol, mix well and dilute to the mark.

For example, to prepare 25 mL of stock solution, place 25.0 mg of CL 354825 into a 25-mL volumetric flask. Dissolve in methanol, and dilute to mark.

##### 2.5.2.3. Mixture of BAS 715 H and CL 354825

Prepare a 10.0 μg/mL BAS 715 H and CL 354825 mixed solution by transferring with a pipet an appropriate amount of each analyte stock solution into a volumetric flask. Dissolve with methanol and dilute to the mark.

For example, to prepare 100 mL of the mixed solution, pipette 1 mL each of BAS 715 H and CL 354825 1 mg/mL stock solutions into a 100-mL volumetric flask. Add methanol, mix well and dilute to mark with methanol.

### 2.5.3. Fortification Solutions

Fortification solutions are prepared at concentrations of 100.0 and 10.0 ng/mL in water by serial dilution of the 10.0 μg/mL BAS 715 H and CL 354825 mixed solution. Intermediate dilutions at other concentrations can be prepared as needed. For example:

- to prepare the 100.0 ng/mL fortification solution transfer 1.0 mL of the 10.0 μg/mL solution into a 100 mL volumetric flask and dilute to the mark with water.
- to prepare the 10.0 ng/mL fortification, transfer 10.0 mL of the 100.0 ng/mL fortification solution into a 100 mL volumetric flask and dilute to the mark with water.

### 2.5.4. Injection Calibration Solutions for LC/MS/MS Analysis

Calibration standards should be stored in the refrigerator, 1 to 10 º C. Prepare injection calibration solutions for LC/MS/MS determination by serial dilution of the 100 ng/mL fortification solution (see Section 2.5.3) with water. Suggested concentrations of standards for LC/MS/MS analysis are 0.02, 0.04, 0.1 and 0.2 ng/mL.

For example:

- to prepare a 1.0 ng/mL solution, transfer by glass pipet 1.0 mL of the 100.0 ng/mL fortification solutions to a 100 mL volumetric and bring up to volume with water.

Use the 1.0 ng/mL standard to prepare the 0.2 and 0.1 ng/mL injection standards and the 0.2 ng/mL to prepare the 0.04 and 0.02 ng/mL injection standards. For example:

- To prepare the 0.2 ng/mL solution, transfer by glass pipet 10.0 mL of the 1.0 ng/mL standard into a 50 mL volumetric flask, dilute, mix well and make up to volume with water.
- To prepare the 0.1 ng/mL solution, transfer by glass pipet 5.0 mL of the 1.0 ng/mL standard into a 50 mL volumetric flask, dilute, mix well and make up to volume with water.
- To prepare the 0.04 ng/mL solution, transfer by glass pipet 5.0 mL of the 0.2 ng/mL standard into a 25 mL volumetric flask, dilute, mix well and make up to volume with water.
- To prepare the 0.02 ng/mL solution, transfer by glass pipet 5.0 mL of the 0.2 ng/mL standard into a 50 mL volumetric flask, dilute, mix well and make up to volume with water.

## 3. ANALYTICAL PROCEDURE

Samples are analyzed in sets which include at least one non-fortified sample as a control and fortified procedural recovery samples. A 10.0 g (±0.2 g) soil aliquot is measured into a tared centrifuge bottle. For the validation 175 mL polypropylene conical bottles were used but similar containers can be substituted if shown to provide equivalent recoveries.

Due to the 0.001 ppm method LOQ and the solubility of the analytes in methanol and water, the analyst should exercise extreme care to avoid contamination of solvents used in the method.

### 3.1. Sample Preparation and Moisture Analysis

Bulk soil samples received from the field are homogenized with dry ice using a Fitz Mill Homoloid or similar apparatus. Homogenized soil samples are stored frozen (< -5°C) before analysis.

Soil analysis results are reported on a "dry weight" basis. Therefore, soil sample weights must be corrected for moisture content (% M) . One procedure to determine moisture content is to weigh approximately 10 g of wet soil ("wet weight") accurately into a tared weighing vessel, such as a petri dish or aluminum weighing dish. The vessel is placed in an oven at 150 °C for 16 – 20 hours. The vessel is removed from the oven and allowed to cool in a dessicator. Once cool, the vessel is removed from the dessicator and weighed to obtain the weight of the dry soil ("dry weight")

### 3.2. Fortification of Procedural Recovery Samples

Analysis of at least two procedural recovery samples and one untreated sample (control) with each analysis set is recommended to monitor method efficiency. Typically, one procedural recovery sample is run at the limit of quantitation (0.001 ppm) along with one procedural recovery sample at the expected residue level. For each fortification, pipette an appropriate amount of fortification solutions (prepared in Section 2.5.3.) to control soil samples that have been weighed into centrifuge tubes. For example, 1.0 mL of the 100 ng/mL or 10.0 ng/mL fortification solution pipetted unto 10 g of soil results in a fortification level of 0.010 ppm or 0.001 ppm, respectively.

### 3.3. Soil Extraction

The flowchart of the analytical procedure is shown in Figure 1.

- Add 20 mL of 0.5 N NaOH to the centrifuge bottle containing the soil (10 g).
- Shake on a reciprocal shaker at about 250 motion/min for about 30 minutes.
- Centrifuge at about 3000 rpm for about 15 minutes.
- Decant the supernatant into a 50 or 100-mL graduated cylinder (preferably with stopper). If supernatant is very cloudy after centrifuging, use filter in funnel to catch particulates.
- Add 20 mL of 0.5 N NaOH to the centrifuge bottle containing the soil (10 g) and shake to mix the soil to a consistent slurry.
- Repeat shaking and centrifuging as above.

- Decant the supernatant into the graduated cylinder containing the first extract.
- Make up to 40.0 mL with water. If filter was used, wash with 2 to 3 mL water and make up to 45.0 mL
- Shake or vortex extracts to ensure good mixing.

Mix well to ensure the extract is homogeneous. Residues from samples that were fortified at a 0.001 ppm level (10 ng on 10 g soil) are now contained in 40 mL of extract at a concentration of 0.25 pg/µL.

Note: Do not stop at this point. Recoveries of BAS 715 H and CL 354825 dropped significantly on storage of the basic extracts during method development. The stability of the analytes in the extract after treatment with formic acid was improved, and acidified extracts of the German soil were cleaned up with SPE and analyzed after three days storage at room temperature with acceptable recoveries.

### 3.4. Treatment with Acid

An aliquot of the basic extract is taken and acidified with formic acid to about pH 3 (measured with pH paper) to precipitate the humic material. Due to decreased recoveries of BAS 715 H and CL354825 after storage in the basic extract, the aliquot taken for acidification should allow for reanalysis. The acidified aliquot (pH about 3-3.5) can be stored at room temperature.

- Pipette a 5.0 mL aliquot of the basic extract into a disposable glass culture tube with cap.
- Pipette 1.0 mL of 5.0 M formic acid into the tube using a glass pipet.
- Vortex to mix and let stand for about 15 minutes.
- Centrifuge at about 2000 rpm for about 10 to 15 minutes.

As an alternate procedure for soils in which the supernatant remains somewhat cloudy after centrifugation, the following procedure can be used to assure a homogeneous supernatant. After the centrifuging described above continue as follows:

- Transfer the supernatant into a glass graduated tube.
- Wash the precipitate with about 1 mL of 1 % formic acid by vortexing.
- Centrifuge at about 2000 rpm for about 10 to 15 minutes.
- Carefully transfer the wash into the graduated tube and make up to 7 mL with 1 % formic acid.
- Vortex to assure a homogeneous solution.

### 3.5. Solid Phase Extraction (SPE)

The neutralized extracts can be stored at room temperature for 3 to 4 days based on work performed during the validation study in which acceptable analysis results were obtained with German soil neutralized extract after 3 days storage.

For the North Carolina soil used in method validation, C18 clean up with DCM elution of BAS 715 H and CL 354825 was sufficient to provide acceptable recoveries. However, erratic and low recoveries of the metabolite CL 354825 occurred with the German soil which might be due to incomplete elution from the C18 column or due to the lack of

homogeneity of the acidified supernatant. A modified acidification procedure was added to the method (Section 3.4) to ensure that the acidified solution taken for SPE cleanup was homogeneous. If needed, C18 column cleanup can be used in combination followed by further cleanup of the methanol eluate using an SCX column. The DCM fraction eluted from the C18 column containing BAS 715 H and the majority of CL 354825 is then combined with the eluate from the SCX column.

Rather than manually as done in the validation study, SPE can be done using an automated instrument such as a Zymark Rapid trace if experiments show acceptable recoveries in fortified procedural samples included in an analysis set and the procedural steps are documented.

### 3.5.1. Clean up by C18 SPE

Prepare C18 SPE columns, (200 mg, 3 mL reservoir) by washing consecutively with a column volume of hexane, dichloromethane, methanol, water and 0.01 N HCl. Using gravity, allow the liquid level to drain just below the top of the frit above the sorbent bed after first washes, and leave a shallow layer of 0.01 N HCl over the frit of the cartridge.

- Using a glass pipet, transfer a one mL aliquot of each neutralized extract to the column. Let the solution pass through the column with gravity allowing the liquid level to drain just below the top of the frit above the sorbent bed.
- Wash with one column volume of 1 % formic acid using gravity, allowing the liquid level to drain just below the top of the frit above the sorbent bed. Collect the 1 % formic acid wash fraction with the load.
- Wash with one column volume of hexane using a slight vacuum (< - 5 in Hg), allowing the liquid level to drain just below the top of the frit. Discard the load and wash fractions.
- Place clean labeled collection containers such as 20 mL liquid scintillation vials in the manifold. Elute with two columns volumes of DCM into the vials. A slight vacuum is needed to begin flow from the columns, and the remaining elution can be performed by gravity or with a slight vacuum. Remove the vials and set aside.
- Optional: Proceed with Section 3.5.2. The SCX cleanup described in Section 3.5.2 and final methanol elution from the C18 column will be needed only if recoveries of the analytes in the DCM eluate are not acceptable. Should prior analysis indicate that recoveries of both analytes are acceptable, the SCX cleanup is not needed.

### 3.5.2. Clean up by SCX SPE

(This cleanup is used only if satisfactory recoveries are not obtained with the C18 cleanup.)

- Prepare SCX SPE columns, (200 mg, 3 mL reservoir) by washing with one column volume of methanol.
- Fill SCX column reservoir about 2/3 full with methanol and set aside. Using an adapter, attach the C18 cartridge to the top of the SCX column. Fill the C18 column barrel with 1 column volume of methanol and elute the column combination to a waste vial at about 1 drop per second.

- Remove the C18 column and adapter. Wash the SCX column with one column volume of methanol adding to the waste vial.
- Elute the SCX column with 2 column volumes of water/methanol (20:80) into the scintillation vial containing the DCM eluate from the C18 column.

### 3.5.3. Preparation for Analysis

Remove solvent from samples that have been through SPE cleanup using an N-evap with water bath temperatures ≤ 60 ºC. For samples fortified at a 1 ppb and 10 ppb level, add two mL or 20 mL of water, respectively, with a glass pipet to fall within the range of the standard curve of 0.02 to 0.2 pg/µL. If the peak area of an unknown is larger than that for the highest standard, dilute it appropriately to a known volume with water and re-inject.

To test the stability of analytes in samples ready for analysis, the samples extracted from NC soil and cleaned up with C18 SPE were reanalyzed about 40 h after the initial analysis with storage at ambient temperature. The results (Table 1) indicated that in the 0.001 ppm fortification samples, average recovery of CL 354825 declined from 110 to 84 % while in the 0.01 ppm fortification samples the average recovery did not change significantly.

Note: After redissolving in water, analyze samples within 24 h if possible, to avoid declining recoveries of CL 354825.

### 3.6. Instrumentation

Flow from the HPLC column is diverted from the mass spectrometer inlet during each analysis using a divert valve to prevent buildup of salts on the mass spectrometer inlet shield. Using the chromatography described below, the time events are set to divert flow prior to 4 and after 7 minutes. Chromatography conditions and columns and mass spectroscopy parameters and instrumentation other than those specified below can be used, but must be documented in the raw data.

Note: Obtaining an acceptable correlation coefficient for the linear regression curve for CL 354825 sometimes requires basic instrument maintenance such as changing or clipping the fused silica or wiping the spray plate. Adding a 30 s equilibration time after the gradient could help if sample retention times are shifting.

**Suggested LC/MS/MS Operating Conditions**

| | |
|---|---|
| Instrument: | PE Sciex API 3000 Biomolecular Mass Analyzer |
| Inlet [HPLC System] | Perkin Elmer series 200 autosampler and Perkin Elmer series 200 micropump |
| Column: | TSK-gel Super ODS, 2.0 x 50.0 mm, 2 µm (Tosoh BioScience) |
| Injection: | 25 µL |

| | | | |
|---|---|---|---|
| Mobile Phase:<br>[Gradient] | A = 100% ($H_2O$ + 1.0 % Acetic Acid)<br>B = 100% (Methanol + 1.0 % Acetic Acid) | | |
| | Time (min.) | Composition | Time Events |
| | 0.0 | 90% A 10% B | |
| | 0.5 | 90% A 10% B linear gradient | |
| | 4.0 | 50% A 50% B linear gradient | close |
| | 7.0 | 15% A 85% B linear gradient | close |
| | 7.5 | 10% A 90% B | |
| | 9.5 | 10% A 90% B | |
| | 9.6 | 90% A 10% B | |
| | 10.6 | 90% A 10% B | |
| Flow Rate: | 250 µL/minute | | |
| Compound: | Transition: | Ionization Mode: | Approx Retention time |
| BAS 715 H | 276.1 → 231.0 | positive | 4.4 minutes |
| CL 354825 | 278.1 → 232.9 | positive | 6.3 minutes |

### 3.7. Calibration Procedures

Calculation of results is based on peak area measurements using a calibration curve. The standard curve is obtained by direct injection of standards for LC/MS/MS. In a given injection run, the same volume is used for all injections of samples and standards. Construct a linear least squares calibration curve in the form y = mx + b from standards by plotting the peak area *versus* the amount of standard injected for each analyte.

$$y = mx + b$$

where y = peak area response
m = slope
x = analyte weight (in pg)
b = y intercept

For analysis, samples and standards are alternated and some injections of solvent blanks are included. For each injection set, the set should begin and end with standard injections, and each standard level should be injected at least in duplicate.

### 3.8. Moisture Determination

Soil analysis results are reported on a “dry weight” basis. Therefore, soil sample weights must be corrected for moisture content (% M) . One procedure to determine moisture content is to weigh approximately 10 g of wet soil (“wet weight”) accurately into a tared weighing vessel, such as a petri dish or aluminum weighing dish. The vessel is placed in an oven at 150 °C for 16 – 20 hours (overnight). The vessel is removed from the oven and allowed to cool in a dessicator. Once cool, the vessel is removed from the dessicator and weighed to obtain the dry weight.

### 3.8. Limit of Quantitation and Limit of Detection

The limit of quantitation (LOQ) is defined as the lowest fortification level successfully tested in the validation which is 0.001 ppm for each analytes. The limit of detection (LOD) varies based on the sensitivity of analyte response. For each analyte the LOD, as defined by signal to noise, is ≤ ¼ the lowest standard. The lowest standard is 0.02 ng/mL, equivalent to 0.192 ppb. The LOD at ¼ that amount is equivalent to 0.048 ppb.

## 4. CALCULATION OF RESULTS

### 4.1. Moisture

To calculate the moisture content (M) of soil samples, the following calculation is used.

$$M = \frac{\text{moisture weight}}{\text{dry weight}} = \frac{\text{wet weight – dry weight}}{\text{dry weight}}$$

### 4.2. Fortification Amount

To calculate the fortification concentration ($C_F$) in ppm, the following calculation is used:

$$C_F = \frac{\text{volume (mL)} \times \text{standard concentration (µg/mL)}}{\text{wet weight of soil (g)}}$$

### 4.3. Analyte in Sample

Calculation of results is based on peak area measurements. Using the peak area measured for BAS 715 H or CL 354825 the amount of analyte in pg can be determined from the appropriate least squares calibration curve.
Calculate the residue concentration in ppm for soil on a wet weight basis ($C_S$(wet)) using the following equation.

$$C_S(\text{wet}) = \frac{W_A \times V_F}{W_S \times V_I \times AF \times 1000}$$

$W_A$ = Amount of analyte calculated from calibration curve (pg)

$V_F$ = Final Volume after all dilutions (mL)

$W_S$ = Sample weight extracted (g)

$V_I$ = Injection volume (µL)

$$AF = \frac{\text{(aliquot used for neutralization)} \times \text{(aliquot used for SPE)}}{\text{(total extract volume)} \times \text{(total volume of neutralized aliquot)}}$$

1000 = Factor remaining after all unit conversions

Using the sample weight (wet) for $W_S$ will yield a sample ppm value on a “wet weight” basis.

To calculate sample concentration in ppm for soil on a dry weight basis ($C_S$(dry)):

$$C_S(dry) = (1 + M) \times C_S(wet)$$

## 4.4. Calculation of Procedural Recoveries

The procedural fortification results should be corrected for any residues found in the control sample. The control sample has not been spiked with any analytes and LC/MS/MS analysis should give an area result that is 0. The peak area, if any, found for the control should be converted to a concentration in ppm, $C_C$, and this result subtracted from the concentration found for the procedural fortification sample, $C_S$.

$$C_{CORR} = C_S - C_C$$

where: $C_{CORR}$ is the corrected procedural fortification,
$C_S$ is the concentration of fortified soil sample, and
$C_C$ is the concentration of the control sample (all in ppm).

Determine percent recovery for the fortification samples as follows where $C_F$ is the concentration fortified :

$$\% R = \frac{C_{CORR}}{C_F} \times 100$$

## 4.5. Treatment of Results

Treated sample results should not be corrected for either control residues or procedural recoveries. Mean, standard deviation and coefficients of variation of recovery were determined for each analyte in two soils during the method validation (Tables 1 and 2).

## 4.6. Example Calculations

The values for calculated concentration in pg determined by the LC/MS are rounded to 2 units after the decimal place. Values with at least 3 significant figures can be carried out throughout the intermediate calculations and rounded to whole numbers for the percent recovery. An example set of calculations using a sample with North Carolina (NC) soil on Master Sheet 1 is shown below. The 10.07 g sample of NC soil was fortified with BAS 715 H and CL 354825 using 1.00 mL of 10.0 ng/mL standard solution.

**Moisture content:** No moisture calculation were made for validation samples.

$$M = \frac{\text{moisture weight}}{\text{dry weight}} = \frac{\text{wet weight – dry weight}}{\text{dry weight}}$$

$$M = \frac{10.00\text{g} - \text{x g}}{\text{x g}} = \text{0.0 or xx \% moisture}$$

**Fortification ppm:**

$$C_F = \frac{\text{volume (mL)} \times \text{standard concentration (µg/mL)}}{\text{wet weight (g)}}$$

$$= \frac{1.00 \text{ mL} \times .010 \text{ µg/mL}}{10.07 \text{ g}}$$

$$= 0.000993049 \text{ ppm} = 0.001 \text{ ppm}$$

**Sample ppm (wet weight basis):**

$$C_S(\text{wet}) = \frac{W_A \times V_F}{W_S \times V_I \times AF \times 1000}$$

To calculate $W_A$, the amount (in pg) of analyte BAS 715 H from the calibration curve, a linear least squares curve was constructed ( Master Sheet 1, C18 cleanup).

| BAS 715 H | | | | | |
|---|---|---|---|---|---|
| File Type | Sample Name | Injection Vol. | Area | Area | Area |
| Standard | 0.02 pg/µL | 25 µL | 732 | 706 | 711 |
| Standard | 0.04 pg/µL | 25 µL | 1267 | 1291 | 1446 |
| Standard | 0.10 pg/µL | 25 µL | 2947 | 3180 | 3399 |
| Standard | 0.20 pg/µL | 25 µL | 6279 | 6748 | 7243 |

| | |
|---|---|
| Intercept, b | -2.03 |
| Slope, m | 1340 |
| Correlation Coeff. | 0.9950 |

The area for sample 121735-1-04 was 3600. Using equation y = mx + b, solving for x:

$$x = \frac{(y - b)}{m} = \frac{(3600 - (-2.03)}{1340} = 2.69 \text{ pg } (= W_A)$$

Sample wt. = 10.07 g Final Vol. = 2.0 mL Aliquot factor = (5.0 x 1.0) mL/ (40.0 x 6.0) mL

$$C_S(\text{wet}) = \frac{W_A \times V_F}{W_S \times V_I \times AF \times 1000}$$

$$= \frac{2.69 \text{ pg} \times 2.0 \text{ mL} \times 40.0 \times 6.0}{10.07 \text{ g} \times 25.0 \text{ µL} \times 5.0 \times 1.0 \times 1000}$$

$$= 0.00102578 \text{ ppm}$$

**Sample ppm (dry weight basis):** Moisture correction was not done in the validation study.

$C_S(dry) = (1 + M) \times C_S(wet)$

$= (1.0 + 0.X) \times 0.00102578$ ppm

$= 0.00102578$ ppm

**Corrected ppm value:**

The corresponding control samples contained an average of 0.000015 ppm.

$C_{CORR} = C_S - C_C$

$= 0.00102578$ ppm $- 0.000015$ ppm

$= 0.00101078$ ppm

**Recovery:**

$$\% R = \frac{C_{CORR}}{C_F} \times 100$$

$$= \frac{0.00101078 \text{ ppm}}{0.000993049 \text{ ppm}} \times 100$$

$= 102$ % recovery

## 5. RESULTS

### 5.1. Accuracy and Precision

The accuracy and precision of BASF Method D0307 are represented by the recovery data generated during the validation study (Reference 3) which is summarized in Tables 1 and 2. Statistical treatment of the recovery data include determination of means, relative standard deviations (coefficients of variation) and the 95 % two-sided confidence interval (Reference 6).

Typical raw data, including chromatograms of standards, control and fortified samples and standard curves from the validation study are found in Appendix A.