2. INTRODUCTION

The purpose of this study was to independently validate two methods. The first method was the Huntingdon Life Sciences Limited method, "AKD-2023: Development and Validation of Methodology for the Determination of Residues of AKD-2023 and its Major Metabolite R1 (Hydroxy AKD-2023) in Drinking Water, Ground Water, and Surface Water," dated November 19, 2002 (2) and identified as project number AGK/076 (Appendix C). The second was the Morse Laboratories method, "Determination of Acequinocyl, Acequinocyl-OH, and AKM-18 in Soil," dated March 27, 2001 (3) and identified as method number Meth-136, Revision #2 (Appendix C). Both methods were successfully validated in accordance with the protocol in water and soil at the LOQs of 0.1 µg/L (groundwater) and 0.01 ppm (soil) and 1.0 µg/L (groundwater) and 0.1 ppm (soil). Validation satisfied OPPTS 850.7100 requirements for exposure, environmental fate, and ecological effects (1).

The independent laboratory, Study Director, and analysts involved in this study were unfamiliar with the method, both in its development and any subsequent use in analyzing field samples. The ILV analysts ordered and/or used all of their own equipment and supplies, so that there was no common link between Agro-Kanesho, Arvesta Corporation, the Study Director, or analysts. Throughout the conduct of the study, any communications between Agro-Kanesho, Arvesta Corporation, the method developers at Huntingdon Life Sciences or Morse Laboratories, the Study Director, and/or the analyst were logged for reporting to the Agency. No one from Agro-Kanesho, Arvesta Corporation, Huntingdon Life Sciences, or Morse Laboratories was allowed to visit Pyxant Labs Inc during the ILV trial to observe, offer help, or assist chemists or technicians. These steps successfully maintained the integrity of the ILV study.

3. MATERIALS AND METHODS

3.1 Test/Reference Substance

The test/reference substances used in this study are described as follows:

Test/Reference Substance:	Acequinocyl
Chemical Name:	3-Dodecyl-1,4-dihydro-1,4-dioxo-2-naphthyl acetate
CAS #:	57960-19-7
Purity:	97.07%
Lot #:	AK23981/S
Expiration Date:	June 3, 2005

Test/Reference Substance:	Hydroxy-Acequinocyl
Chemical Name:	2-dodecyl-3-hydroxy-1,4-naphthoquinone
CAS #:	57960-31-3
Purity:	95.87%
Lot #:	AK23981/M5
Expiration Date:	June 4, 2005

Test/Reference Substance:	AKM-18
Chemical Name:	AKM-18, 2-(1,2-dioxotetradecyl) benzoic acid
CAS #:	Not available
Purity:	99.4%
Lot #:	P-8
Expiration Date:	September 4, 2005

3.2 Test/Reference Substance Receipt and Storage

The acequinocyl and hydroxy-acequinocyl substances were shipped by Midwest Research Institute of Kansas City, MO on behalf of Arvesta Corporation on March 14, 2003 and were received at Pyxant Labs Inc on March 17, 2003 at ambient temperature and in good condition. The substances were logged into the Master Logbook under numbers 14582305 and 14582304 and stored at ambient temperature. Additional acequinocyl and hydroxy-acequinocyl substances were shipped by Midwest Research

Institute of Kansas City, MO on behalf of Arvesta Corporation on October 3, 2003 and were received at Pyxant Labs Inc on October 6, 2003 at ambient temperature and in good condition. The substances were logged into the Master Logbook under numbers 14862938 and 14862937 and stored at ambient temperature.

The AKM-18 reference standard was shipped by Agro-Kanesho Co., Ltd. of Tokyo, Japan and was received at Pyxant Labs Inc on September 3, 2003 at ambient temperature and in good condition. The sample was logged into the Master Logbook under number 14962762 and stored refrigerated (-10°C to +7°C). The certificates of analysis for the reference standards used in this ILV are attached as Appendix D.

3.3 Receipt and Storage of Untreated Control Samples

An untreated control (UTC) groundwater sample was shipped by Agvise Laboratories via UPS and received cold and in good condition at Pyxant Labs Inc on July 30, 2003. The sample was logged into the Master Logbook as 14862559 and stored refrigerated (-10 to +7°C). More untreated control (UTC) groundwater samples were shipped by Agvise Laboratories via UPS and received cold and in good condition at Pyxant Labs Inc on August 15, 2003. The samples were logged into the Master Logbook as 14862685 and stored frozen (≤ -20°C).

The untreated control (UTC) soil sample was shipped by Agvise Laboratories via UPS and received cold and in good condition at Pyxant Labs Inc on August 15, 2003. The sample was logged into the Master Logbook as 14862684 and stored frozen (≤ -20°C). Soil samples were characterized by Series 1 Soil Characterization and water samples were characterized by series 4 Water Characterization. Characterization reports are shown in Appendix E.

Sample tracking information is provided in Table 1. Sample ID numbers, fortification dates, extraction dates, and dates of analysis are included in the table.

3.4 Reagents and Materials

The following is a list of the reagents prepared and the materials used for the current study:

Reagents

- Acetonitrile
- Acetone
- Ammonium Acetate
- Dimethyldichlorosilane (DMDCS)
- 1-Decanol
- Ethyl Acetate
- Formic Acid
- Hexane

- Methanol
- Sodium Chloride
- Sodium Sulfate
- Water, UHP
- 1% (v/v) keeper solution: Measure 1.00 mL 1-decanol into a 100 mL volumetric flask, fill to volume with acetone, and mix.
- Ethyl acetate/hexane (1/99, v/v): Add 10.00 mL ethyl acetate to a 1 L volumetric flask, bring to volume with hexane, and mix.
- Ethyl acetate/hexane (5/95, v/v): Add 50.00 mL ethyl acetate to a 1 L volumetric flask, bring to volume with hexane, and mix.
- Water/acetonitrile (2/98, v/v): Add 20.00 mL UHP water to a 1 L volumetric flask, bring to volume with acetonitrile, and mix.
- 5% (v/v) dimethyldichlorosilane in hexane: Add 95 mL hexane to a glass stoppered container. Slowly add 5.00 mL DMDCS, stopper, and invert to mix.
- 0.4% formic acid in water: Add 2.00 mL formic acid to a 500 mL volumetric flask, bring to volume with UHP water and mix.
- Acetone/Acetonitrile/0.4% aqueous formic acid (2/2/1, v/v/v): Add 80 mL acetone, 80 mL acetonitrile and 40 mL 0.4% aqueous formic acid to a 250 mL volumetric flask and mix. Prepare weekly.
- Acetonitrile/water (90/10, v/v): Add 10.00 mL UHP water to a 100 mL volumetric flask, fill to volume with acetonitrile, and mix. Prepare as needed.
- Saturated sodium chloride solution: Weigh 88 g of sodium chloride into a 250 mL volumetric flask. Add 250 mL UHP water and shake. If necessary, add additional water to make a 250 mL final volume.
- 0.1% Formic Acid in Methanol: Add about 100 mL methanol to a 1 L volumetric flask. Add 1.00 mL formic acid, fill to volume with methanol, and mix.
- 0.5% Formic Acid in Water: Add about 100 mL water to a 1 L volumetric flask. Add 5.00 mL formic acid, fill to volume with water, and mix.

Materials

- Silylated Glass Wool
- Silica Gel
- 250 mL polyethylene centrifuge bottle
- Plastic powder funnels
- 25 and 250 mL graduated mixing cylinders
- Opaque brown centrifuge bottles
- Polypropylene pipettes

3.5 Analytical Instrumentation and Equipment

The independent laboratory established the method parameters prior to initiation of the first sample trial by determining the retention times of the analyte and computing the

instrument detection limits using analytical standards. The following instruments and equipment were utilized in the conduct of the independent laboratory validation of the residue analytical method:
Instrumentation:

Agilent Model 1100 Autosampler	S/N DE21001902
Agilent Model 1100 Binary Pump	S/N DE14910448
Agilent Model 1100 Degasser	S/N JP13203157
Valco 10 Port Switching Valve	S/N EM2C05582
PE SCIEX API 3000 HPLC/MS-MS	S/N D10590207
Phenomenex Luna Phenyl Hexyl, 6 x 150 mm, 3µm column (groundwater analysis)	S/N 208752-1
Phenomenex Luna C_8 (2) 2 x 150 mm, 3 µm column (soil analysis)	S/N 207129-4

Operating Parameters:

Typical Chromatographic Conditions, Groundwater Analysis

Column Temperature: 35°C
Injection Volume: 50 µL
Run Time: 18 minutes
Mobile Phase: A – 0.1% Formic Acid in Water
B – 0.1% Formic Acid in Methanol
Flow Rate: 0.9 mL/min
Gradient:

Time, min	A%	B%
0-0.4	45	55
8.0	10	90
12.0-15.5	5	95
15.6-18.0	45	55

Typical Mass Spectrometer Parameters

Interface:	TurboIonSpray
Polarity:	Positive
Scan Type:	MRM
Resolution:	Q1 – Unit, Q3 – Low
Curtain Gas:	10
Nebulizer Gas:	9
Collision Gas:	7
Temperature:	400°C
Turbo Gas:	8000 mL/min
Ion Spray Voltage:	4500.00 V

Scan Function:
Transitions Monitored:

Compound	*m/z* Q1	*m/z* Q3	Time (ms)	Collision Energy (V)	Declustering Potential (V)	Focusing Potential (V)
Acequinocyl	385	189	200	34	61	220
Acequinocyl-OH	343	189	200	30	66	220

Typical Chromatographic Conditions, Soil Analysis

Column Temperature: 30°C
Injection Volume: 30 µL
Run Time: 15 minutes
Mobile Phase: A – 0.5% Formic Acid in Water
B – 0.1% Formic Acid in Methanol
Flow Rate: 300 µL/min

Gradient:

Time, min	A%	B%
0	30.0	70.0
0.50	30.0	70.0
5.00	5.0	95.0
10.00	5.0	95.0
10.10	30.0	70.0
15.00	30.0	70.0

Typical Mass Spectrometer Parameters

Interface: APCI
Polarity: Positive
Scan Type: MRM
Resolution: Q1 – Unit, Q3 – Low
Curtain Gas: 10.00
Nebulizer Gas: 10.00
Collision Gas: 4
Temperature: 400°C
Nebulizer Current: 3.00

Scan Function:
Transitions Monitored:

Compound	*m/z* Q1	*m/z* Q3	Time (ms)	Collision Energy (V)	Declustering Potential (V)	Focusing Potential (V)
Acequinocyl	385	189	200	37	18	98
Acequinocyl, confirmation	385	343	200	13	18	98
Acequinocyl-OH	343	189	200	31	25	125
AKM-18	347	149	200	13	25	125

Other Equipment
Balance, Analytical Sartorius, Model AC120S, S/N 20103137
Microbalance, Cahn, Model C-34, S/N C1066/C2251
Balance, Pan Sartorius, Model BA2100S, S/N 20303446
SPE Vacuum Manifold System, Burdick and Jackson, 24 port
Rotary Evaporator, Buchi, Model 011, equipped with B-461 water bath (S/N 143516)
Rotary Evaporator, Buchi, Model 011, equipped with B-461 water bath (S/N 1158505)
N-Evaporator, Organomation, Model 112, S/N 3455 and S/N 50266 (water bath S/N only)
Centrifuge, Beckman Model TJ-6, S/N 12189 MFG
Tabletop orbital shaker, Labline, Model 3520, S/N 0208
Vortex Mixer, S/P Vortex Mixer Jr., Model S8225-1, S/N 001297
Ultrasonic Cleaner, Mettler, Model ME 4.6, S/N 85M16531

Some instrument and chromatographic parameters were adjusted, as provided in the protocol and the method, to match the performance of the validation method. Further details of these adjustments are discussed in Section 3.7, Groundwater Sample Preparation, Section 3.8, Soil Sample Preparation, and Section 3.10, Method Modifications. Method performance was not compromised by these minor modifications.

3.6 Standard and Fortification Solution Preparation

Stock solutions of acequinocyl, hydroxy-acequinocyl, and AKM-18 were prepared by accurately weighing approximately 10 mg of each analytical standard into a 50 mL volumetric flask. Acequinocyl and AKM-18 were brought to volume in acetonitrile and acequinocyl-OH was brought to volume in acetone, to make nominal 200 µg/mL stock solutions. The stock solutions were stored refrigerated (2°C to 8°C) in the dark when not in use.

Seven mixed calibration standards for the groundwater analysis and four mixed calibration standards for the soil analysis were prepared from the stock solutions for each test/reference substance by serial dilution with acetone/acetonitrile/0.4% aqueous

formic acid (2/2/1, v/v/v). Standard concentrations for the groundwater analysis were 10, 30, 50, 70, 100, 250, and 500 µg/L, and for the soil analysis were 0.05, 0.10, 0.2, and 0.5 µg/mL. Individual fortification solutions of each test/reference substance were prepared by serial dilution from the stock at 0.1 and 1 µg/mL for groundwater and 2 µg/mL and 20 µg/mL for soil by diluting with acetonitrile. Calibration and fortification standards were stored refrigerated (2°C to 8°C) in the dark when not in use

3.7 Groundwater Sample Preparation

Groundwater sample preparation was performed as in the "Method, Procedures" section of the Huntingdon Life Sciences method AGK/076 with the exception of the method modifications listed in Section 3.10, Method Modifications. Prior to any standard or sample preparation, all glassware was silanized. Further, all stock solutions and final working solutions were kept in amber glass bottles wrapped in aluminum foil. Lights in the laboratory were turned off during the preparation of standards, fortification, extraction, partition, and throughout the rotary evaporation process because of the sensitivity of acequinocyl and acequinocyl-OH to light.

An aliquot (500 mL) of groundwater was transferred to a separatory funnel, and mixed standard solutions were added as follows:

- 0.5 mL of 0.1 µg/mL standard added to 0.5 L of groundwater to fortify at 0.1 µg/L (LOQ)
- 0.5 mL of 1.0 µg/mL standard added to 0.5 L of groundwater to fortify at 1.0 µg/L (10X LOQ)

Approximately 20 g of sodium chloride and 50 mL of hexane were added and the sample was shaken vigorously. The phases were allowed to separate and the upper hexane phase was passed through a funnel containing sodium sulfate into a 250 mL round-bottomed flask. The groundwater was reextracted with another 50 mL hexane, and the extracts were combined in the round-bottomed flask. The sodium sulfate was rinsed with approximately 40 mL hexane and this was combined with the other extracts in the round-bottomed flask. The extract was rotary evaporated to approximately 3 mL at <30°C and the sample was transferred with a plastic pipette tip into a 13 x 100 mm test tube. The 250 mL flask was rinsed twice with 3 mL hexane, sonicating for approximately 45 seconds after each rinse. The solvent was evaporated to approximately 3 mL using an N-Evap nitrogen evaporator at <30°C. The remaining volume was removed just to dryness in the N-Evap nitrogen evaporator at ambient temperature. The sample was reconstituted in 1.00 mL acetonitrile/water/formic acid (85/15/0.2, v/v/v) and sonicated prior to LC/MS/MS analysis.

3.8 Soil Sample Preparation

Prior to soil sample analysis, the silica gel was evaluated as described in Appendix III of Morse Method Meth-136. Duplicate columns were prepared, and recoveries ranged

from 97.7% to 110% in the first column and from 74.5% to 88.8% in the second column. The lower recoveries in the second column were caused by a prolonged attempt at blowing the samples to dryness with nitrogen. This problem is addressed in Section 4.5 of this report.

Soil sample preparation was performed as described in Sections 6 through 9 of the Morse analytical method Meth-136 with the exception of the modifications listed in Section 3.10, Method Modifications. Soil (approximately 20 g weighed accurately) was weighed into a 250 mL polyethylene centrifuge bottle. As appropriate, samples were fortified with the following mixed standards:

- 0.100 mL of 2 µg/mL standards added to 20 g of soil to fortify at 0.01 ppm (LOQ)
- 0.100 mL of 20 µg/mL standards added to 20 g of soil to fortify at 0.10 ppm (10X LOQ)

Samples were extracted by adding 100 mL of acetonitrile/water (90/10, v/v) and shaking horizontally on a reciprocating shaker for 30 minutes at 160 excursions/minute. Samples were then centrifuged at approximately 2500 rpm for 15 minutes. The supernatant was decanted through a powder funnel containing a plug of glass wool into a 250 mL mixing cylinder. This extraction procedure was repeated and the supernatant was again decanted through the same funnel and into the same mixing cylinder. The extract was brought to a 200 mL volume with acetonitrile/water (90/10, v/v) and mixed. A portion of the extract (100 mL) was transferred to a 250 mL evaporation flask, and 0.4 mL of 1% keeper solution was added. The extract was concentrated to 11 to 16 mL on a rotary evaporator at approximately 30°C. The concentrate was quantitatively transferred with several 2 mL acetonitrile rinses to a 25 mL graduated mixing cylinder, and the evaporation flask was rinsed with approximately 2 mL acetonitrile. The concentrate was brought to a volume of 20 mL with acetonitrile and transferred to a 125 mL polypropylene separatory funnel.

Saturated sodium chloride (20 mL), 3 g of solid NaCl, and 60 mL hexane was added to this extract concentrate, and the funnel was manually shaken for one minute. The lower aqueous layer was drained into another 125 mL polypropylene separatory funnel and the upper two organic layers were drained into a 250 mL evaporation flask. The aqueous layer was reextracted with 60 mL hexane. The lower aqueous layer was drained to waste and the upper hexane layer was combined with the organic layer in the evaporation flask from the first partition. To this combined organic extract was added 0.4 mL 1% keeper solution. The solution was concentrated to about 0.5 mL on a rotary evaporator between 27°C and 28°C. Hexane (5.00 mL) was added and the solution was sonicated for approximately one minute.

Next the sample was cleaned up using a silica gel column. The silica column was prepared by first adding 5 g sodium sulfate to the column. Next, 5 g activated silica gel was added to the column reservoir, without allowing any silica to fall into the narrow

portion of the column by holding it horizontally. Hexane (10-15 mL) was added to the column reservoir and swirled with the silica gel to make a slurry. This continued until air bubbles had been expelled. The column was quickly turned upright to allow the slurry to fall into the column. Several hexane rinses were used to wash down any silica adhering to sides of the column. The column was tapped in order to level the top of the silica bed. The stopcock was then opened to allow the hexane to begin draining, and then another 5 g sodium sulfate was added. After the conditioning, the hexane extract (5 mL) was added to the top of the column. The elution was stopped when the solvent had drained to the within 0.5 cm from the top of the column, and the eluate was discarded. Hexane (20 mL) was added to the original evaporation flask and the rinse was passed through the column. The column was washed with 50 mL ethyl acetate/hexane (1/99, v/v) and the eluate was discarded. Ethyl acetate/hexane (5/95, v/v, 75 mL) was added to the column to elute acequinocyl, and 60 mL water/acetonitrile (2/98, v/v) was added to the column to elute acequinocyl-OH and AKM-18. Keeper solution (0.4 mL of 1%, v/v) was added to the combined eluates and concentrated to approximately 30 mL at 15 in. Hg for 20 minutes. When the rotary evaporator stopped condensing, the pressure was adjusted to 18-21 in. Hg for 40-45 minutes to obtain a volume of 0.5 to 1 mL. The concentrated extracts were transferred to a 13 x 100 mm test tube with one 2 mL acetone rinse, then two 1 mL acetone rinses. Each rinse was sonicated prior to transfer to the test tube. Another 0.4 mL of 1% keeper solution was added and the solution was concentrated to 0.2 mL using an N-Evap nitrogen evaporator set at approximately 30°C, then on to almost dryness using manual nitrogen blowdown at ambient. The residue was reconstituted in 400 µL acetone and sonicated. A 400 µL volume of acetonitrile was added and sonicated again. A final 200 µL of 0.4% aqueous formic acid was added and the solution sonicated once more. The samples were then analyzed by HPLC/MS.

3.9 Sample Analysis

Samples were analyzed using the parameters listed in Section 3.5, Analytical Instrumentation and Equipment. Chromatographic conditions and mass spectrometric parameters were adjusted from those listed in the two methods in order to optimize peak shape and response.

Seven calibration standards were analyzed with the groundwater sample set over the range of 10 µg/L to 500 µg/L, while four calibration standards were analyzed with the soil sample set over the range of 0.05 to 0.50 µg/mL. An analysis set consisted of 2 UTCs, 2 UTCs fortified at the LOQ, 2 UTCs fortified at 10X LOQ, and a reagent blank.

3.10 Method Modifications

For the water analysis, the following modifications and additions were made to the sample preparation procedure of the Huntingdon Life Sciences Method AGK/076: (1) Prior to any standard or sample preparation, all glassware was silanized. (2) All stock

solutions and final working solutions were kept in amber glass bottles wrapped in aluminum foil (3) Lights in the laboratory were turned off during the preparation of standards, fortification, extraction, partition, and throughout the rotary evaporation process because of the sensitivity of acequinocyl and acequinocyl-OH to light (4) The temperature on the rotary evaporator was kept at 27°C, and the pressure was approximately 15 inches of Hg. The ending pressure was 18 inches of Hg, and the evaporation time was approximately 20 minutes. (5) After the first rotary evaporation step, the 250 mL round bottomed flask was rinsed twice with approximately 3 mL hexane, and sonicated after each rinse. (6) In the final step of the method, the solvent was removed under nitrogen evaporation at ambient temperature and not in a 30°C water bath to reduce sample degradation.

The mass spectrometric and chromatographic procedures used in the Huntington method also had to be modified. The method specifies ions to be monitored, which are attributable to the molecular ions of the compounds (M^+). Typically, when using electrospray atmospheric pressure mass spectrometry in the positive ion mode, the major ions observed are due to the protonated forms of the compounds $(M+H)^+$. Therefore, ions monitored are one mass unit greater than the molecular weight of the compound. Such artifactual peaks (M^+) can be observed, but are instrument dependent and may not be observed on other instruments, even if they are of the same manufacturer and model. Huntingdon Labs acknowledged the problem, but indicated that in their studies, the molecular ions were of greater intensity and was their choice for the method. Using the equipment at Pyxant Labs Inc, the ions of greater intensity were the traditional protonated ion forms. The molecular ions (M^+) were not observed. Therefore, we chose to use, instead, the mass spectrometric and chromatographic conditions utilized by Morse Labs for the same compounds in the soil analysis. These procedures more closely matched the performance of the instruments at Pyxant Labs.

The following modifications and additions were made to the Morse Method # Meth-136 Rev. 2: (1) Prior to any standard or sample preparation, all glassware was silanized: all pipettes, bottles, graduated mixing cylinders, glass chromatographic columns, autosampler vials, and 13 x 100 mm test tubes were silanized, and were resilanized again before each use. (2) All the lights in the laboratory were turned off during the preparation of standards, fortification, extraction, partition, and throughout the rotary evaporation process because of the sensitivity of acequinocyl and acequinocyl-OH to light (3) Plastic powder funnels were substituted for glass powder funnels (4) No 500 mL glass Erlenmeyer flasks were used (5) The only graduated mixing cylinders needed were 250 and 25 mL sizes (6) Opaque brown centrifuge bottles were used instead of standard HDPE bottles (7) HPLC sample filters were not used (8) Polypropylene pipettes were used in place of the glass Pasteur pipets (9) Serological pipettes were not used (10) No solid phase extraction apparatus was used (11) Sample filtration was not done, so luer-lock syringes were not used (12) Keeper Solution was prepared as 1% v/v not w/v. (1 mL decanol per 100 mL acetone) (13) Specific volumes and concentrations used for standard preparation were changed in

order to improve accuracy and convenience. Standard preparation is outlined in section 3.6, Standard and Fortification Solution Preparation (14) At step 7.8, extracts were concentrated to 11-16 mL and the quantitative transfer was made with more than one ~2 mL acetonitrile rinse (15) Separatory funnels were not centrifuged (16) At step 8.7, the concentrated extracts were not blown down to dryness because a small amount of water and keeper remained in the flask, and could not be completely evaporated within a reasonable amount of time (17) Silica column cleanup as described in section 9.0 of the method was modified. Section 3.8 of this report describes the column cleanup (18) The combined eluates in section 9.10 of the method were concentrated using 15 in. Hg for the first 20 minutes, then adjusted to 18-21 in. Hg for the final 40-45 minutes (19) The transfer of concentrated extracts in section 9.11 occurred with one 2 mL acetone rinse and two 1 mL rinses instead of one 1 mL rinse and two 0.5 mL rinses (20) The LC/MS/MS instrument and instrument parameters, HPLC column, and mobile phase were changed from what is stated in the method. Section 3.5, Analytical Instrumentation and Equipment outlines the parameters and equipment that were used.

These minor modifications are not considered significant and will have no effect on the quality of the data.

3.11 Example Calculations

The calculations of the percent recovery of acequinocyl and acequinocyl-OH from well water were performed as described in the "Calculation of results" section of the Huntingdon Life Sciences method. Calibration standards (10, 30, 50, 70, 100, 250, and 500 µg/L) were analyzed with each sample set. A best fit calibration curve using linear regression with $1/x^2$ weighting (acequinocyl) and linear regression with 1/x weighting (acequinocyl-OH) was generated for calibration standards using the respective peak area responses versus the concentration of the calibration standards. Concentrations of analyte in the samples were determined by substituting the peak area responses into the linear with 1/x weighting regression equation as shown below:

$$y = mx + b$$

$$x = \frac{y - b}{m}$$

where x is the concentration found in µg/L and y is the peak area response. The slope, m, and intercept, b, were calculated by the Excel software program and independently verified using hand calculations.

For example, using the acequinocyl data from 1486-38, control well water fortified at 1 µg/L (Figure 36), where the observed area count is 5.71589E+03.

$$\text{µg/L from curve} = \frac{5.71589\text{E+}03 - (-582.14)}{78.26} = 80.5 \text{ µg/L}$$

$$\text{Sample } \mu g/L = \frac{\mu g/L \text{ from curve x Dilution Factor}}{500 \text{ mL/mL}}$$

$$\text{Sample } \mu g/L = \frac{80.5\ \mu g/L \text{ x } 5}{500 \text{ mL/mL}}$$

$$\text{Sample } \mu g/L = 0.805$$

Percent recovery is calculated by dividing the concentration by the fortification amount, in this case, 1 µg/L:

$$\text{Recovery} = \left(\frac{\text{Conc. Found}}{\text{Conc. Added}}\right) x100\%$$

$$\text{Recovery} = \left(\frac{0.805\ \mu g/L}{1.00\ \mu g/L}\right) \text{ x } 100\%$$

$$\text{Recovery} = 80.5\%$$

The calculations of concentration and percent recovery of acequinocyl, acequinocyl-OH, and AKM-18 from soil were performed as described in Section 11.0 of the Morse method. Calibration standards (0.05, 0.10, 0.2, and 0.5 µg/mL) were analyzed with each sample set. Best fit calibration curves using linear regression with 1/x weighting (acequinocyl) and linear regression forced through zero (acequinocyl-OH) and linear regression with 1/x weighting (AKM-18) were generated for calibration standards using the respective peak area responses versus the concentration of the calibration standards. Concentrations of analyte in the samples were determined by substituting the peak area responses into the linear with 1/x weighting regression equation as shown below:

To calculate the soil concentration of acequinocyl in ppm:

$$\mu g/g = \frac{\mu g/mL \text{ x HPLC final volume}}{\text{sample weight (g)}} \text{ x } \frac{\text{mL extraction solvent}}{\text{mL aliquot}} \text{ x HPLC dilution factor}$$

Where:

µg/mL = amount of analyte calculated from regression curve
HPLC final volume= volume of final extract submitted to HPLC
mL extraction solvent = volume of extraction solvent
mL aliquot = volume of sample extract taken through column cleanup
HPLC dilution factor = dilution of sample extract required to produce an analyte response bracketed by standards using sample 1486-58 (Fig. 9)

$$\mu g/g = \frac{0.482\ \mu g/mL \text{ x } 1 \text{ mL}}{20 \text{ g}} \text{ x } \frac{200 \text{ mL}}{100 \text{ mL}} \text{ x } 2$$

$$= 0.0965\ \mu g/g$$

In all cases, no significant peaks were observed in the unfortified controls above the limit of detection. Therefore, no corrections were made and the net and gross concentrations were the same. The analyte concentrations were automatically calculated using the Analyst® software routines and also verified by recalculations performed with an Excel spreadsheet. Both used more decimal places than those displayed. As a result, the calculated values shown in this example may vary slightly if the values are recalculated using the displayed parameters.

The percent recovery for each sample was calculated by using the equation shown below:

$$\text{Recovery} = \left(\frac{\text{Conc. Found}}{\text{Conc. Added}} \right) \text{x } 100\%$$

$$\text{Recovery} = \left(\frac{0.0965 \text{ µg/g}}{0.10 \text{ µg/g}} \right) \text{x } 100\%$$

$$\text{Recovery} = 96.5\%$$

3.12 Statistical Treatment of Data

The mean recoveries for the samples were calculated using the "AVERAGE" function of the Microsoft Excel spreadsheet computer program which divides the sum of the selected cells by the number of determinations. The standard deviations were calculated using the "STDEV" function of the same spreadsheet program which sums the squares of the individual deviations from the mean, divided by the number of degrees of freedom, and extracts the square root of the quotient. Percent relative standard deviation, % RSD, is calculated by dividing the standard deviation by the mean, then multiplying by 100.

4.5 Critical Steps

Several critical steps were identified in the procedures. The test compounds were found to be volatile, especially acequinocyl, and have a high affinity for adsorption to glass, especially AKM-18. Moreover, acequinocyl and acequinocyl-OH are light sensitive. Through discussions with the method developers, procedures and techniques were modified as follows to reduce compound loss due to these issues.

First, it is essential that all glassware, especially the roto-evaporation flasks, be thoroughly silanized. To ensure proper silanization, it was critical to completely remove all traces of residues from the glassware by using concentrated ChemSolv. After cleaning, all traces of the ChemSolv had to be removed by rinsing with copious amounts of deionized water. The rinse water is finally checked to ensure that it is no longer basic. The glassware can then be silanized by the usual procedures described in the methods.

Due to the light sensitivity of the acequinocyl and acequinocyl-OH it is important to minimize light exposure of standards and fortification solutions by utilizing amber bottles and vials and/or covering with aluminum foil. Overhead lights in the laboratory should also be turned off during standard and sample preparation and especially throughout the rotary evaporation steps.

The time of evaporation in the final rotary evaporation steps is also critical. Significant losses of acequinocyl and acequinocyl-OH were attributed to evaporation times that were longer and shorter than the prescribed times in the methods. This is especially a problem with the soil samples which contain some water. It is recommended that temperatures and pressures be carefully adjusted to match the times cited in the methods.

It was also discovered that any steps requiring solvent evaporation to dryness must be very carefully monitored. Dried samples were unstable and had to be reconstituted immediately. In some cases, where the samples contain water and keeper, it is not possible to take them to complete dryness within a reasonable amount of time. Longer evaporation times will cause loss of compound.

5. CIRCUMSTANCES THAT MAY HAVE AFFECTED THE QUALITY AND INTEGRITY OF THE DATA

Both the water and the soil validations failed on the first trial due to several critical steps not realized until after the first attempt. These critical steps have been outlined in the previous section. Once these were identified, the methods were modified (See section 3.10) and the second attempts were successful.

6. COMMUNICATIONS

A detailed list of communications with the Sponsor, the Sponsor Representative, and the method developers at Huntingdon Life Sciences and Morse Laboratories is presented in Appendix F. Correspondence occurred with the Sponsor, the Sponsor Representative, and the method developers after the failure of the first water and soil trials. At no time during the course of the two trials did personnel from Arvesta Corporation or Agro Kanesho, or anyone else associated with the method visit Pyxant Labs Inc.

Appendix F

Communications

Laboratory work was carried out from September 12, 2003 through December 21, 2003. The communications that occurred between the Sponsor, the Sponsor Representative, the method developers, and the testing facility before the study concerned the AKM-18 recertification. The correspondence that occurred after the first trial and before the second trial concerned clarifications to both the water and the soil method, specifically relating to the volatility of the compounds, adsorption onto glassware, photodegradation, and rotary evaporation conditions. No one from Agro Kanesho or Arvesta Corporation visited Pyxant Labs Inc during any phase of this study.

Date	From	To	Summary
8/13/03	Audrey Sehn	Diane Reed	Audrey informed Diane that the AKM-18 that is being sent from Japan expired in 2002 and needs to be recertified before the ILV study.
9/25/03	Audrey Sehn	Diane Reed	Audrey inquiring as to the status of the study.
9/25/03	Maurice Gaubatz	Audrey Sehn	Discussing project status. Maurice informed Audrey that draft report will not be given by 9/30/03 as originally anticipated. Audrey said that the recertification of the standards initially provided a delay. Maurice said the report would be ready by 10/10/03.
10/01/03	Audrey Sehn	Karen Alvarado	Audrey giving Karen David Kirkpatrick's contact information at Huntingdon Life Science. Audrey informed Karen to copy Dr. Anabuki of Agro Kanesho on all communications with Huntingdon.
10/02/03	Karen Alvarado	David Kirkpatrick	Karen asking David questions about the Huntingdon method, after the first failed water trial. Karen asked what the recommended pressure of the rotary evaporation step is, how much time it should take, clarification on the mobile phase, whether positive or negative ionization was used, and which ions to monitor.
10/02/03	David Kirkpatrick	Karen Alvarado	David informing Karen that her questions had been passed on to the chemists who will compile a response.
10/03/03	Diane Reed	Kazuhiro Anabuki, Audrey Sehn	Diane forwarded email to Audrey and Dr. Anabuki that Karen wrote on 10/02/03 to David Kirkpatrick

Date	From	To	Summary
10/03/03	Simon Tate	Karen Alvarado	Responding to Karen's 10/02/03 email. Informed her that the pressure and the length of time on the rotary evaporator is not monitored. The water through the condensers, however, are 5°C. The mobile phase is 0.01M ammonium formate:ACN (5/95, v/v) w/ 0.2%volume formic acid. The ionization mode is APCI negative, and *m/z* 384>159 (acequinocyl) and *m/z* 342>159 (acequinocyl-OH) are monitored.
10/03/03	Kazuhiro Anabuki	Karen Alvarado	Dr. Anabuki explaining to Karen that he would like to check the mobile phase that Simon mentioned after his return to Japan on 10/06/03, and would contact her again.
10/31/03	David Robaugh	Audrey Sehn	David faxed to Audrey Silica Gel Profile tests for all three compounds. Low recoveries in general, lowest for AKM-18
10/31/03	Maurice Gaubatz, David Robaugh	Gary Westburg	Maurice and David outlined the method developments to date on the Morse soil method. Described how the silica column profile was checked and the evaporation step, with various post-spikes. Outlined recoveries. Gary suggested there may be adsorption (onto glass), transfer limitations (sticks to glass), volatility, and photodegradation issues. Also suggested checking the pH of the final DI water rinse following cleansing with ChemSolv. The glassware cleaning procedure and the silylation steps are important. Suggested further to not take the volumes to complete dryness in the rotary evaporator, and to immediately reconstitute after the transfer.
12/10/03	David Robaugh	Gary Westburg	Contacting Gary about possible method problems prior to trial 2. Gary emphasized adsorption onto glass and suggested using a quadratic regression. Also, to watch for instrument drift. Reiterated not to take samples to dryness. Also said there were no good stopping points in the method.
12/22/03	Ryan Randolph	Audrey Sehn	Ryan transferring trial 2 draft soil data for all three compounds. She accepted the results and said that we can consider trial 2 successful.
1/15/04	Audrey Sehn	Diane Reed	Audrey inquiring when she could obtain the final report. Diane informed her she may be able to have it by the week of 1/19/04