MORSE LABORATORIES, INC.

DETERMINATION OF ACEQUINOCYL AND ACEQUINOCYL-OH IN FRUIT CROPS

Analytical Method# Meth-133, Revision #3

April 3, 2001

APPROVED BY: _____

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TABLE OF CONTENTS

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Reasons for Revision:

1) To change the rotary evaporation temperature throughout the procedure from ≤40°C to approximately 30°C.

2) To change the final evaporation volume from ~1-2 mL to ~0.5 mL in Steps 8.1.6.2 and 8.2.5.

1.0 PRINCIPLE

The method described herein is capable of determining acequinocyl and acequinocyl-OH in a variety of fruit crop matrices including most related processed commodities.

Samples having a medium to high moisture content (i.e., "watery" fruit crops such as apples, pears, citrus fruit, wet pomace, etc.), are extracted a single time with acetonitrile/water. Most of the water component is derived from the sample itself. Extra water is added to all matrix-types (except fruit juices and other aqueous samples) to insure that a minimum amount of water is available for adequate analyte extraction. Following extraction, the water is salted out and an aliquot of the resulting acetonitrile phase is extracted with hexane to separate the analytes from undesirable acetonitrile-soluble coextracted materials. The hexane extracts (except those derived from watery fruit crop matrices containing little or no fats/oils), undergo an acetonitrile/hexane partition (back extraction of the hexane extract with acetonitrile) to remove fats, oils and other preferentially hexane-soluble coextractives. By using a ratio of solvent composition [hexane:acetonitrile (1:2, v/v)] coupled with multiple extractions (3 times), the analytes are induced to move into the acetonitrile phase. A final purification, incorporating silica solid phase extraction (SPE) cartridge cleanup, is conducted on all sample extracts. The purified extract is concentrated, then submitted to HPLC analysis.

During routine analysis, determination and quantitation of the analytes are conducted using high pressure liquid chromatography (HPLC) employing mass spectrometric (MS/MS) detection. The limit of quantitation (LOQ) for all matrices for both analytes is ≤0.01 ppm. (Detector sensitivity to each analyte is generally not the same resulting in different actual LOQs for each analyte. 0.01 ppm is the targeted LOQ for the least sensitive of the two analytes.)

2.0 EQUIVALENCE STATEMENT

During the conduct of this analysis, comparable apparatus, solvents, glassware, and techniques (such as sample extract evaporation) may be substituted for those described in this method, except where specifically noted otherwise. In the event a substituted piece of equipment or technique is used, its use will be documented in the study records.

3.0 APPARATUS AND EQUIPMENT

Assorted laboratory glassware

Balances:	Analytical balance capable of weighing to ±0.1 mg
	Top-loading balance capable of weighing to ±0.1 g
Centrifuge:	IEC Model HN-SII (Damon IEC Division, Needham Hts., MA)
Centrifuge bottles:	250 mL VWR® HDPE (high-density polyethylene) bottles with screw cap closures
Erlenmeyer flasks:	Glass, 1000 mL, glass-stoppered
Evaporation flasks:	Round or flat bottom, glass, 125 mL and 250 mL. (All flasks may be silylated, per the method described in Appendix II, to aid in the transfer of dried, solid matrix residues at various steps in the procedure. Incorporation of this aid is at the analyst's discretion as it may not be needed for all matrices. If incorporated, resilylate every 2-3 weeks during active use.)
Evaporators:	Rotary evaporator equipped with a Dewar condenser (Labconco Corp., Kansas City, MS)
	N-Evap™ Laboratory Sample Evaporator Model 115 attached to a nitrogen source (Organomation Associates, South Berlin, MA)
Funnels:	Powder, glass, 100 mm diameter
Graduated cylinders:	Glass; 1000 and 100 mL
Graduated mixing cylinders:	Glass; 250, 100 and 50 mL

Homogenizer:	Omni Mixer Model 17105 with Generator Probe (Omni International, Waterbury, CT)
HPLC/MS system:	PE Sciex API 2000 LC/MS/MS system with a Perkin Elmer series 200 autosampler, an integrated Shimadzu chromatograph consisting of (2) LC-10AD*vp* Liquid Chromatograph units, a DGU-14A Degasser, and a SCL-10A*vp* System Controller. The system is controlled and data processed by PE Sciex Analyst Software.
HPLC column:	
Primary:	15 cm × 2.0 mm i.d. Luna C18 (2), 3 μ particle size, part no. 00F-4251-B0 (Phenomenex, Torrence, CA)
Alternate 1:	15 cm × 2.1 mm i.d. Zorbax SB-C18, 5 μ particle size, part no. 883700-922 (Mac-Mod Analytical, Chaddsford, PA)
Alternate 2:	15 cm × 2.1 mm i.d. Nucleosil C18, 100 Å, 5 μ particle size (part no. C-600B, custom packed by Alltech Associates, Deerfield, IL)
HPLC sample filter:	Nylon 66 filters, 13 mm, 0.45μm (Varian Sample Preparation Products, Harbor City, CA)
Microliter syringes:	Various sizes, (Hamilton Co., Reno, NV)
Pasteur pipets:	Glass, 9 inch and 5¾ inch, disposable
Pipets:	Glass, graduated, serological; 50, 25, 10, 5, 2 and 1 mL
Separatory funnels:	Glass; 1000 and 125mL
Solid Phase Extraction Apparatus:	Vac Elut SPS 24 (Varian Sample Preparation Products, Harbor City, CA)
Syringe:	Glass, 2.5 mL, Hamilton Teflon® Luer-Lok (Hamilton Co., Reno, NV)
Test (culture) tubes:	Glass; 13×100 mm and 16 × 150 mm
Ultrasonic bath:	Branson Model 2210 ultrasonic bath (VWR Scientific, Bridgeport, NJ)

Volumetric flasks:	Glass; 500, 250, 100, 50 and 25mL
Vortex mixer:	VWR Vortexer 2 (Scientific Industries, Inc., Bohemia, NY)

4.0 REAGENTS AND MATERIALS

Acequinocyl	Analytical grade
Acequinocyl-OH	Analytical grade
Acetone:	Ultra Resi-analyzed® (J.T. Baker Chemical Company, Phillipsburg, NJ)
Acetonitrile:	OmniSolv® (EM Science, Gibbstown, NJ)
Dimethyldichlorosilane:	Catalog # 3-3009 (Supelco, Inc., Bellefonte, PA). Also referred to as "DMDCS".
1-decanol:	"Resi-analyzed" (J.T. Baker Chemical Company, Phillipsburg, NJ)
Ethyl acetate:	OmniSolv® (EM Science, Gibbstown, NJ)
Ethyl ether:	AR® ACS, catalog # 0853-04 (Mallinckrodt, St. Louis, MO)
Formic acid:	GR ACS (EM Science, Gibbstown, NJ)
Glass wool	Silylated, see Appendix II
Hexane:	(95% n-hexane), Ultra Resi-analyzed® (J.T. Baker Chemical Company, Phillipsburg, NJ)
Methanol:	HPLC Grade (Burdick and Jackson, Muskegon, MI)
Sodium chloride:	GR ACS (EM Science, Gibbstown, NJ)
Sodium sulfate:	Analytical grade, anhydrous granular, #8024 (Mallinckrodt, St. Louis, MO)
Solid phase extraction cartridges:	Silica Bond Elut LRC®, 10cc/500mg (Varian Sample Preparation Products, Harbor City, CA)

Water: Deionized (DI) water (Polymetrics System, Morse Laboratories, Inc.)

HPLC Grade water (Fisher Scientific, Fairlawn, NJ)

4.1 Reagents and Materials to be Prepared (including typical preparation instructions)

4.1.1 1% (w/v) keeper solution: Place 1.0 g 1-decanol in a 100 mL volumetric flask. Fill to mark with acetone. Mix well.

4.1.2 Silica SPE wash mixture [hexane:ethyl ether (49:1, v/v)]: To a 100 mL volumetric flask, add ~50 mL of hexane, followed by 2.0 mL of ethyl ether. Fill to mark with hexane. Mix well. Prepare daily. Sufficient for approximately 20 samples.

4.1.3 Silica SPE elution mixture [hexane:ethyl acetate (9:1, v/v)]: To a 250 mL volumetric flask, add 25 mL of ethyl acetate. Fill to mark with hexane. Mix well. Prepare daily. Sufficient for approximately 20 samples.

4.1.4 Hexane-saturated acetonitrile: To a 1000 mL separatory funnel, add 700 mL of acetonitrile and 200 mL of hexane. Shake for 1 minute and allow the layers to separate. Drain the lower acetonitrile (now saturated with hexane) layer into 1000 mL glass-stoppered erlenmeyer flask. Sufficient for thirteen "60 mL" extractions.

4.1.5 5% (v/v) dimethyldichlorosilane in hexane: To a glass stoppered glass container (such as a 100 mL mixing cylinder), add 95 mL of hexane. Slowly add 5 mL of DMDCS. Stopper and invert to mix. Larger or smaller volumes can be prepared using the proportions discussed above.

4.1.6 Acetone:acetonitrile:0.4% aqueous formic acid (2:2:1, v/v): All samples and standards injected into the HPLC must be dissolved in this specific solvent composition. Typically 200 mL are prepared which are sufficient for the preparation of the four calibration standards and any dilutions that may be necessary for the analysis of the samples. Prepare 200 mL of an *acetone:acetonitrile:0.4% aqueous formic acid (2:2:1, v/v)* solution as follows: To a 250 mL mixing cylinder, add 80 mL of acetone, 80 mL of acetonitrile and 40 mL of 0.4% aqueous formic acid. Mix thoroughly. Smaller volumes may be prepared by decreasing the individual volumes and keeping the ratio constant. Store in an air tight container. Prepare weekly.

4.1.7 0.4% formic acid in water: To a 500 mL volumetric flask, add 2.0 mL of formic acid. Bring to volume with HPLC grade water. Mix well. Prepare weekly.

4.1.7 HPLC mobile phases:

0.1% formic acid in methanol: To a 1 liter graduated cylinder, add methanol to the 1000 mL mark. Add 1.0 mL of formic acid using a 2.0 mL graduated pipet. Transfer entire solution to the HPLC solvent reservoir and once transferred, mix thoroughly.

0.1% formic acid in water: To a 1 liter graduated cylinder, add HPLC grade water to the 1000 mL mark. Add 1.0 mL of formic acid using a 2.0 mL graduated pipet. Transfer entire solution to the HPLC solvent reservoir and once transferred, mix thoroughly.

5.0 STANDARD PREPARATION

5.1 Stock Standard Solutions

Typically, 10.0 mg (corrected for purity) of each analytical standard is accurately weighed and quantitatively transferred to a separate 50 mL volumetric flask. Acequinocyl is brought to volume with acetonitrile and acequinocyl-OH is brought to volume with acetone. The resulting concentration of each solution is 200 µg/mL. These solutions are to be stored in the dark at 1 to 8°C when not in use.

5.2 Fortification Solutions

Typically the following concentrations of acequinocyl and acequinocyl-OH are prepared. Suitable mixtures may be prepared accordingly. All solutions are stored in the dark at 1 to 8°C when not in use.

100 µg/mL: Transfer 12.5 mL of 200 µg/mL standard solution to a 25 mL volumetric flask. Bring to volume in acetonitrile. Mix well.

10 µg/mL: Transfer 2.5 mL of 100 µg/mL standard solution to a 25 mL volumetric flask. Bring to volume in acetonitrile. Mix well.

1 µg/mL: Transfer 250 µL of 100 µg/mL standard solution to a 25 mL volumetric flask. Bring to volume in acetonitrile. Mix well.

5.3 HPLC (Calibration) Standard Solutions

All standard solutions prepared in this section are to be stored in the dark at 1 to 8°C when not in use. Typically the following concentrations of HPLC standard solution *mixtures* are prepared:

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0.05 µg/mL: Transfer 125 µL of 10 µg/mL standard solution to a 25 mL volumetric flask. Bring to volume in acetone:acetonitrile:0.4% aqueous formic acid (2:2:1, v/v). Mix well.

0.1 µg/mL: Transfer 250 µL of 10 µg/mL standard solution to a 25 mL volumetric flask. Bring to volume in acetone:acetonitrile:0.4% aqueous formic acid (2:2:1, v/v). Mix well.

0.2 µg/mL: Transfer 500 µL of 10 µg/mL standard solution to a 25 mL volumetric flask. Bring to volume in acetone:acetonitrile:0.4% aqueous formic acid (2:2:1, v/v). Mix well.

0.5 µg/mL: Transfer 1250 µL of 10 µg/mL standard solution to a 25 mL volumetric flask. Bring to volume in acetone:acetonitrile:0.4% aqueous formic acid (2:2:1, v/v). Mix well.

6.0 SAMPLE FORTIFICATION

Note: All samples should be kept in at least a semi-frozen state until the addition of the first extraction solvent and the extraction process begins.

1. Weigh an appropriate amount of semi-frozen sample (either 20.0 or 40.0 grams, as indicated in Section 7.0, "Sample Extraction") into a 250 mL polyethylene centrifuge bottle.

2. Fortify the sample with the appropriate amount of standard solution(s) containing either single or mixed components. Use a volume of fortification solution ≤2.0 mL.

3. Proceed with either Step 7.1.1 or 7.2.1.

7.0 SAMPLE EXTRACTION

Notes:

1) All samples should be kept in at least a semi-frozen state until the addition of the first extraction solvent and the extraction process begins.

2) Acequinocyl and acequinocyl-OH are very sensitive to light. Glassware used during analysis should be protected with aluminum foil or use amber/dark glassware. Specific glassware includes any items in which there may be lengthy extract storage (>30 minutes) such as: evaporation flasks, mixing cylinders, separatory funnels, test tubes. Cover rotary evaporator water bath, to exclude light, when concentrating samples.

7.1 Samples having high moisture content (typically >80%, i.e., apples, pears, juices, etc.)

1. Weigh 40.0 g of semi-frozen sample into a 250 mL polyethylene centrifuge bottle. As applicable, fortify appropriate samples at this point. Add 10 mL of deionized (DI) water.

   Note: For fruit juices and other types of aqueous samples, omit the addition of DI water.

2. Add 100 mL of acetonitrile and blend using a high speed homogenizer at medium speed for 2 minutes.

3. Add 25 g of NaCl. Cap and shake well for at least 1 minute to dissolve the salt in the water present, forcing the acetonitrile and water layers to separate.

4. Centrifuge at ~2200-2500 rpm for 15 minutes.

5. Using a 25mL graduated pipet, draw off 25 mL of the acetonitrile layer (representing 10.0 g of sample) and transfer to a funnel containing a glass wool plug layered with ~2 cm of sodium sulfate and draining into a 50 mL graduated mixing cylinder. Be careful not to include any of the aqueous phase in the transfer.

6. Rinse sodium sulfate with two 5 mL portions of acetonitrile. Bring to a final volume of 35 mL and transfer to a 125 mL separatory funnel.

7. Proceed with hexane/acetonitrile solvent partition (Subsection 8.1).

7.2 Samples having medium moisture content (typically ~50-80%, i.e., wet pomace, etc.)

1. Weigh 20.0 g of semi-frozen sample into a 250 mL polyethylene centrifuge bottle. As applicable, fortify appropriate samples at this point. Add 40 mL of deionized (DI) water.

2. Add 50 mL of acetonitrile and blend using a high speed homogenizer at medium speed for 2 minutes.

3. Add 20 g of NaCl. Cap and shake well for at least 1 minute to dissolve the salt in the water present, forcing the acetonitrile and water layers to separate.

4. Centrifuge at ~2200-2500 rpm for 15 minutes.

5. Using a 25mL graduated pipet, draw off 25 mL of the acetonitrile layer (representing 10.0 g of sample) and transfer to a funnel containing a glass wool plug layered with ~2 cm of sodium sulfate and draining into a 50 mL graduated mixing cylinder. Be careful not to include any of the aqueous phase in the transfer.

6. Rinse sodium sulfate with two 5 mL portions of acetonitrile. Bring to a final volume of 35 mL and transfer to a 125 mL separatory funnel.

7. Proceed with hexane/acetonitrile solvent partition (Subsection 8.1).

8.0 SOLVENT PARTITIONS

8.1 Hexane/Acetonitrile Partition

- *Applicable to all matrices*

1. To the acetonitrile extract (35 mL) obtained from Step 7.1.6 or 7.2.6 of the extraction scheme in Section 7.0, add 50 mL of hexane.

2. Shake for 1 minute, then allow the layers to separate.

3. Drain the lower acetonitrile layer into a second 125 mL separatory funnel.

4. Drain the hexane layer through a funnel containing a glass wool plug layered with ~2 cm of sodium sulfate (prerinsed with with ~5 mL of hexane just prior to use) into a 250 mL evaporation flask.

5. Repeat Steps 1 through 4 with a fresh 50 mL portion of hexane. Discard the acetonitrile layer and drain the hexane phase through the same funnel and into the same evaporation flask as in Step 4, combining the hexane extracts. Rinse the sodium sulfate with ~5mL of hexane.

6. Add 0.4 mL of 1% keeper solution (see Subsection 4.1). Proceed with either Step 6.1 or 6.2, as applicable:

6.1 For analyses requiring the inclusion of an acetonitrile/hexane partition (back-extraction of hexane with acetonitrile), specifically those involving samples containing significant amounts of fats and/or oils, concentrate the hexane extract to ~25 mL on a rotary evaporator at approximately 30°C. Transfer concentrate to a 50 mL graduated mixing cylinder with the aid of two 2 mL hexane rinses. Bring to a volume of 30 mL, mix, then transfer to a 125 mL separatory funnel. Proceed with acetonitrile/hexane partition (Subsection 8.2).

6.2 For analyses **not requiring** the inclusion of an acetonitrile/hexane partition (back-extraction of hexane with acetonitrile), concentrate the extract to ~0.5 mL on a rotary evaporator at approximately 30°C. With care, evaporate remaining solvent to dryness using manual nitrogen blow down. Proceed with Step 7, which follows.

7. Redissolve the residue in 5 mL hexane, carefully rinsing the walls of the evaporation flask in the process. Sonicate for ~1 minute. Quantitatively transfer the mixture to a 16 × 100 mm test tube with the aid of two 1 mL hexane rinses of the flask.

8. Proceed with silica SPE cartridge cleanup (Section 9.0).

8.2 Acetonitrile/Hexane Partition (back-extraction of hexane with acetonitrile).

- *Applicable to all matrices containing oils and/or fats (i.e., citrus fruits, wet pomace, etc.).*

1. To the 30 mL of hexane concentrate obtained from Step 8.1.6.1, add 60 mL of hexane-saturated acetonitrile.

2. Shake for 1 minute, then allow the layers to separate.

3. Drain the acetonitrile layer into a 250 mL evaporation flask.

4. Repeat Steps 1 through 3 two additional times with fresh 60 mL portions of hexane-saturated acetonitrile. Each time, drain the acetonitrile phase into the same evaporation flask as in Step 3, combining the acetonitrile extracts. Discard the hexane.

5. Add 0.4 mL of 1% keeper solution (see Subsection 4.1) and concentrate the combined acetonitrile extracts to ~0.5 mL on a rotary evaporator at approximately 30°C. With care, evaporate remaining solvent to dryness using manual nitrogen blow down.

6. Redissolve the residue in 5 mL hexane, carefully rinsing the walls of the evaporation flask in the process. Sonicate ~1 minute. Quantitatively transfer the mixture to a 16 ×100 mm test tube with the aid of two 1 mL hexane rinses of the flask.

7. Proceed with silica SPE cartridge cleanup (Section 9.0).

9.0 SILICA SPE CARTRIDGE CLEANUP

Note: Check or calibrate the SPE cartridges prior to use in order to ensure optimum method performance. In general, check one cartridge per lot number per box. This assessment should be conducted well in advance of needing the cartridges for sample analysis. Recovery of >90% is desired to ensure that a box of cartridges is suitable for use. The analyses are conducted on a reagent spike basis.

See Appendix III for detailed instructions on assessment of the SPE cartridges.

Procedure:

1. Set up Vac Elut system and support apparatus and proceed with silica SPE cleanup. In general, set vacuum to produce a flow rate of approximately 2-3 distinct drops/second (not continuous flow) for all elutions.

2. Condition a 500 mg silica SPE cartridge by passing 5 mL of hexane through the cartridge. Do not let the cartridge go to dryness after conditioning. (Stop elution when conditioning solvent reaches top of frit.) Discard eluate.

3. Pass the hexane extract (~7 mL) from either Step 8.1.7 or 8.2.6 through the SPE cartridge. Stop elution when conditioning solvent reaches top of frit. Discard eluate.

4. Wash the test tube from Step 3 two times with 5 mL of hexane each time. Swirl to rinse the walls of the tube and pass each rinse through the sample-laden cartridge. Stop elution when solvent reaches top of frit. Discard eluate.

5. Wash the sample laden cartridge with 5 mL hexane/ethyl ether (49:1, v/v). Stop elution when solvent reaches top of frit. Discard eluate.

6. Place a 16 × 150 mm test tube under the SPE cartridge.

7. Elute the analytes with 15 mL of hexane/ethyl acetate (9:1, v/v).

8. Transfer the eluate to a 125 mL evaporation flask.

9. Add 0.2 mL of 1% keeper solution and concentrate to ~1-2 mL on a rotary evaporator at approximately 30°C.

10. Transfer the concentrated extract quantitatively (using two 1 mL hexane rinses) to a 13 × 100 mm test tube.

11. Continue concentration to ~0.2 to 0.5 mL using an N-Evap evaporator set at approximately 30°C, then to dryness, with care, using manual nitrogen blow down.

12. Redissolve the residue in 0.4 mL acetonitrile. Sonicate to insure that all residue is either dissolved or in suspension. Add 0.4 mL of acetone and mix well. Finally add 0.2 mL of 0.4% aqueous formic acid. The combination and proportion of solvents used results in a mixture of acetone:acetonitrile:0.4% aqueous formic acid (2:2:1, v/v). Mix well and submit to HPLC analysis. Final concentration of HPLC-ready extract is 1.0 mL = 10.0 g.

Note: Samples require a 0.45μm filtration just prior to HPLC analysis. Instrumentation personnel will filter the solution through a 0.45 μm Nylon 66 filter using a Hamilton Teflon® Luer-lock syringe just prior to HPLC analysis.

10.0 HIGH PRESSURE LIQUID CHROMATOGRAPHIC ANALYSIS

Note: The column and conditions stated in the method have been satisfactory for the matrices being analyzed. The specific column packing, mobile phase, column temperature and flow rate listed are typical conditions for this analysis. Alternate columns may be used depending on the need to resolve analyte and/or interfering responses. Specific conditions used will be noted on each chromatographic run and will not otherwise be documented.

10.1 Operating Conditions

Instrument: PE Sciex API 2000 LC/MS/MS system with a Perkin Elmer series 200 autosampler, an integrated Shimadzu chromatograph consisting of (2) LC-10AD*vp* Liquid Chromatograph units, a DGU-14A Degasser, and a SCL-10A*vp* System Controller. The system is controlled and data processed by PE Sciex Analyst Software.

Primary column:

HPLC Column: 15 cm × 2.0 mm i.d. Luna C18 (2), 3 μ particle size

Mobile Phase: (Fisher Water, Burdick and Jackson Methanol, EM Science Formic Acid)

Gradient:

Time (min)	0.1% formic acid in water	0.1% formic acid in methanol
0-0.2	30%	70%
1.5-12.0	8%	92%
12.5-17.0	0	100
17.5-21.0	30	70

Divert Valve: Programmed to divert LC flow from column to waste (bypassing detector) from 0 to 8 minutes and again from 13 to 21 minutes. LC flow is directed to detector during the 8 to 13 minute window. Diversion time settings can be adjusted as necessary depending on the retention times of the analytes.

Flow Rate:	300 μL/min
Interface:	APCI (atmospheric pressure chemical ionization)
Ionization Mode:	positive (+)
Acquisition Mode:	MRM
Source Temperature:	400°C
Curtain Gas:	Nitrogen @ 35psi
Collision Gas:	Nitrogen @ setting of "4"
Transitions Monitored:	TM-413: m/z 385 to 189 TM-413 Hydroxy: m/z 343 to 189
Loop Size:	100 μL
Column Temperature:	35°C
Retention Times:	TM-413 Hydroxy: ~9.2 minutes TM-413: ~10.5 minutes

Alternate column 1:

HPLC Column:	15 cm × 2.1 mm i.d. Zorbax SB-C18, 5 μ particle size
Mobile Phase:	(Fisher Water, Burdick and Jackson Methanol, EM Science Formic Acid)

Gradient:

Time (min)	0.1% formic acid in water	0.1% formic acid in methanol
0-0.2	30%	70%
1.5-9.0	10%	90%
10.0-12.0	2%	98%
12.5-16	30%	70%

Divert Valve: Programmed to divert LC flow from column to waste (bypassing detector) from 0 to 6.5 minutes and again from 9 to 14 minutes. LC

flow is directed to detector during the 6.5 to 9 minute window. Diversion time settings can be adjusted as necessary depending on the retention times of the analytes.

Flow Rate: 500 µL/min

Interface: APCI (atmospheric pressure chemical ionization)

Ionization Mode: positive (+)

Acquisition Mode: MRM

Source Temperature: 400°C

Curtain Gas: Nitrogen @ 55psi

Collision Gas: Nitrogen @ setting of "4"

Transitions Monitored:

TM-413:	m/z 385 to 189
TM-413 Hydroxy:	m/z 343 to 189

Loop Size: 100 µL

Column Temperature: 35°C

Retention Times:

TM-413 Hydroxy:	~7.0 minutes
TM-413:	~8.3 minutes

Alternate Column 2:

HPLC Column: 15 cm × 2.1 mm i.d. Nucleosil C18, 100 Å, 5 µ particle size

Mobile Phase: (Fisher Water, Burdick and Jackson Methanol, EM Science Formic Acid)

Gradient:

Time (min)	0.1% formic acid in water	0.1% formic acid in methanol
0-0.2	30%	70%
1.5-10.0	8%	92%
10.5-14	30%	70%

Divert Valve:	Programmed to divert LC flow from column to waste (bypassing detector) from 0 to 6 minutes and again from 9 to 14 minutes. LC flow is directed to detector during the 6 to 9 minute window. Diversion time settings can be adjusted as necessary depending on the retention times of the analytes.	
Flow Rate:	500 µL/min	
Interface:	APCI (atmospheric pressure chemical ionization)	
Ionization Mode:	positive (+)	
Acquisition Mode:	MRM	
Source Temperature:	400°C	
Curtain Gas:	Nitrogen @ 55psi	
Collision Gas:	Nitrogen @ setting of "4"	
Transitions Monitored:	TM-413:	m/z 385 to 189
	TM-413 Hydroxy:	m/z 343 to 189
Loop Size:	100 µL	
Column Temperature:	35°C	
Retention Times:	TM-413 Hydroxy:	~6.9 minutes
	TM-413:	~7.8 minutes

10.2 Sample Analysis

Prepare a four-point standard curve by injecting constant volumes of mixed standard solutions. Use constant volume injections for sample extracts as well. Sample responses not bracketed by the standard curve require dilution and reinjection. Inject a curve check standard every 4-5 sample injections.

11.0 CALCULATIONS

Calculations for instrumental analysis are conducted using a validated software application to create a standard curve based on linear regression. The regression functions are used to calculate a best fit line (from a set of standard concentrations in µg/mL versus peak response) and to determine concentrations of the analyte found during sample analysis from the calculated best fit line.

The equation used for the least squares fit is:

$$y = mx + b$$

where:

y	=	peak response
x	=	µg/mL found for peak of interest
m	=	slope
b	=	y-intercept

The calculations for ppm found and percent recovery (for fortified samples) are:

1. The amount of analyte (in ppm) found in the sample is calculated according to the following equation:

$$ppm = \mu g/mL \times \frac{HPLC\ final\ vol.(mL)}{sample\ wt.(g)} \times \frac{mL\ ext.\ solv.}{mL\ aliq.} \times HPLC\ dil.\ factor$$

where:

µg/mL found	=	µg/mL of analyte found
sample wt. (g)	=	gram weight of sample extracted (typically 40.0g for high moisture or 20.0g for medium moisture samples)
mL ext. solv.	=	volume of extraction solvent (typically 100 mL for high moisture or 50 mL for medium moisture samples)

mL aliq.	=	volume of sample extract taken through solvent partition(s) (typically 25 mL)
HPLC final vol.	=	volume of final extract submitted to HPLC (typically 1.0 mL)
HPLC dilution factor	=	dilution of sample extract required to produce an analyte response bracketed by standards

2. The percent recovery for fortified control samples is calculated as follows:

$$\%\ Rec. = \frac{ppm\ found\ in\ fortified\ control\ (spike) - ppm\ found\ in\ control}{fortification\ level\ (ppm)\ added} \times 100$$

12.0 REFERENCES

1. Morse Laboratories, Inc. Analytical Method# Meth-117, Revision #3, January 14, 1999, *Determination Of Selected Organophosphate Pesticides in Fruits and Vegetables.*

2. Morse Laboratories, Inc. Analytical Method# Meth-118, Revision #2, December 22, 1998, *Determination Of Selected N-Methyl Carbamate Pesticides in Fruits and Vegetables.*

3. *Residue Analysis Method of Acequinocyl in Plant Products,* The Institute of Environmental Toxicology, Kdaira, Tokyo, Japan.

4. *Determination of Residues of Acequinocyl and Acequinocyl-OH in Crops,* Method: TMN-0149 dated August 30, 2000, Tomen Agro, Inc., San Francisco, CA.

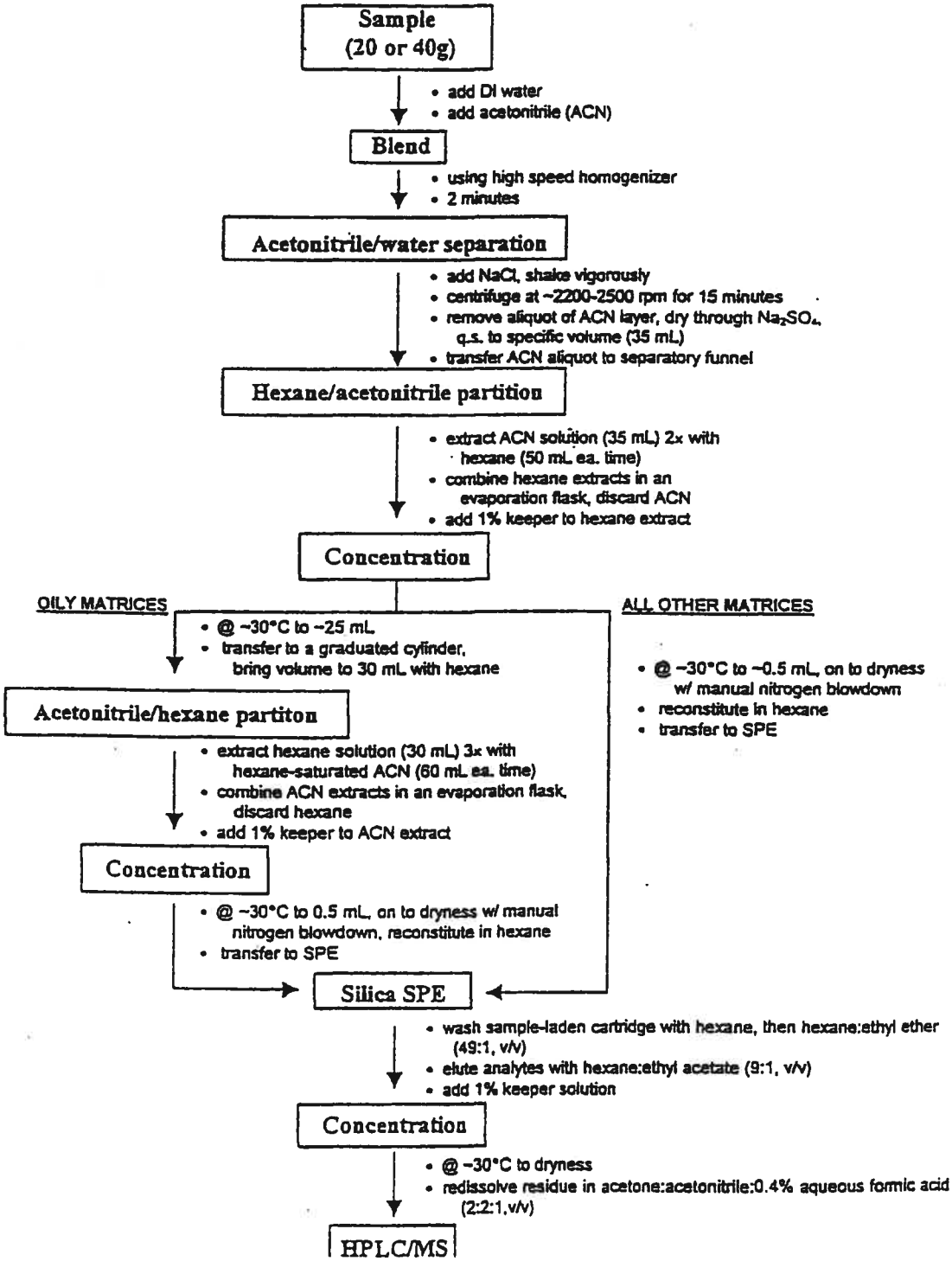
5. Morse Laboratories, Inc. Analytical Method# Meth-136, Revision #2, March 27, 2001, *Determination Of Acequinocyl, Acequinocyl-OH and AKM-18 in Soil.*

Method author: Gary L. Westberg

APPENDIX I

Flowchart for Acequinocyl and Acequinocyl-OH Fruit Crop Analysis

FLOWCHART FOR THE DETERMINATION OF ACEQUINOCYL AND ACEQUINOCYL-OH IN FRUIT CROPS



APPENDIX II

Silylation of Glassware

Silylation of Glassware

Silylation is a process used to chemically treat glassware or other products in order to prevent or minimize binding of analyte residues to the glass surface.

Caution: **DO NOT ALLOW DIMETHYLDICHLOROSILANE TO COME IN CONTACT WITH WATER. CHLORINE GAS AND HYDROGEN CHLORIDE GAS WILL BE PRODUCED.**

THIS PROCEDURE MUST BE CONDUCTED INSIDE AN EFFICIENT FUME HOOD. HEAVY LATEX GLOVES MUST BE WORN.

1. Pour a small amount of the 5% DMDCS solution into the glassware to be treated. Stopper bulk container. Rotate the glassware to thoroughly coat the inside surfaces. Pour excess solution into the next piece of glassware to be treated.

   Note: **Moisture in the air tends to inactivate this reagent. To insure maximum activity of the silylating agent during the coating process, limit the exposure (to the atmosphere) of the silylating agent to approximately 5 minutes.**

2. Allow the treated glassware to dry (approximately 20 minutes). Rinse thoroughly with hexane, then reagent acetone. Again allow to dry.

3. Glassware is now ready for use.

   Notes:
   - Any glassware that is cleaned with a brush after it has been silylated, must be resilylated.
   - Store pure DMDCS at room temperature.
   - 5% solutions of DMDCS in hexane are stable for 5 days when stored well-stoppered at room temperature. Choose a storage container with minimum air space above the surface of the solution.

Silylation of Glass Wool:

In a fume hood, while wearing heavy latex gloves, place a quantity of glass wool into a large wide-mouth glass jar with Teflon®-lined lid (e.g., ~400 mL capacity). Saturate the glass wool with the silylating solution (using approximately 200 mL). **To assure that thorough saturation has occurred, stir to thoroughly coat the glass wool in the glass jar with a sturdy glass rod.** Cap tightly and allow to stand for approximately 20 minutes.

Decant the silylating solution from the glass jar and while wearing latex gloves, squeeze the excess silylating solution from the glass wool. Pull the wet glass wool apart to aid in drying. Place on clean paper towels in a hood to thoroughly dry. This drying may require as long as 4-8 hours.

When completely dry, return the silylated glass wool into a glass jar and rinse thoroughly three times with hexane followed by three rinses with acetone.

Allow the glass wool to dry on clean paper towels. Store the dry, silylated glasswool in a covered glass container. **The washed and dried silylated glass wool may be used for up to one month.**

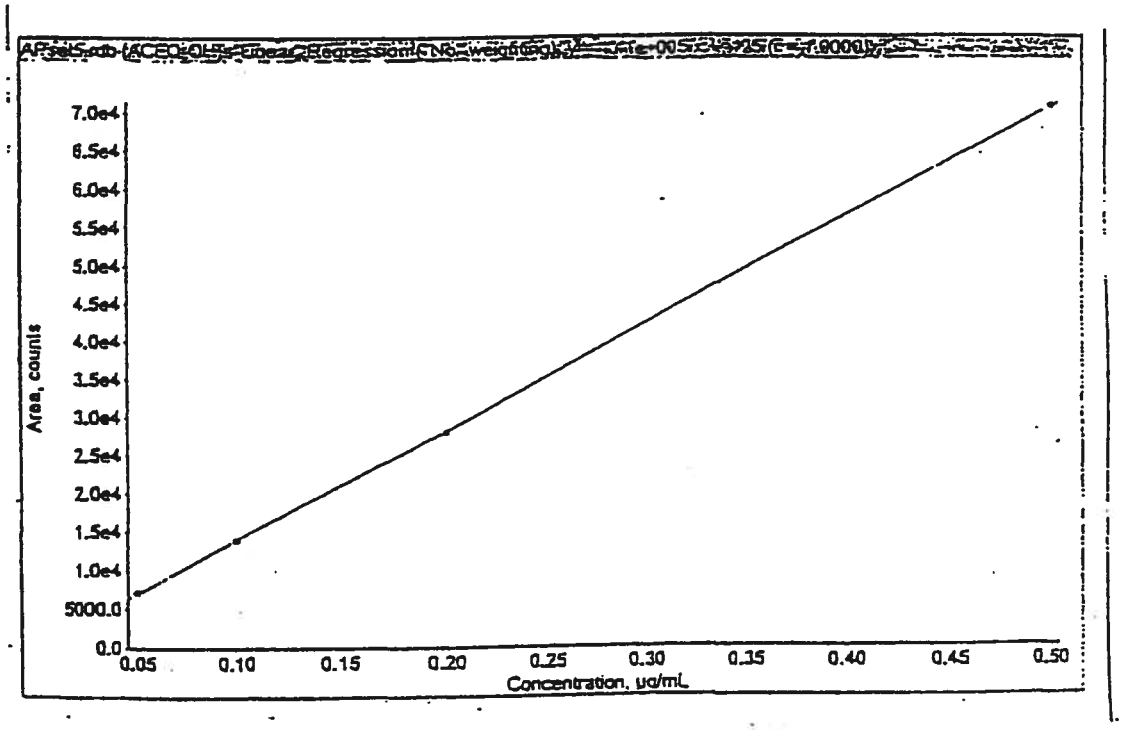
APPENDIX III

Quality Control for SPE Cartridges

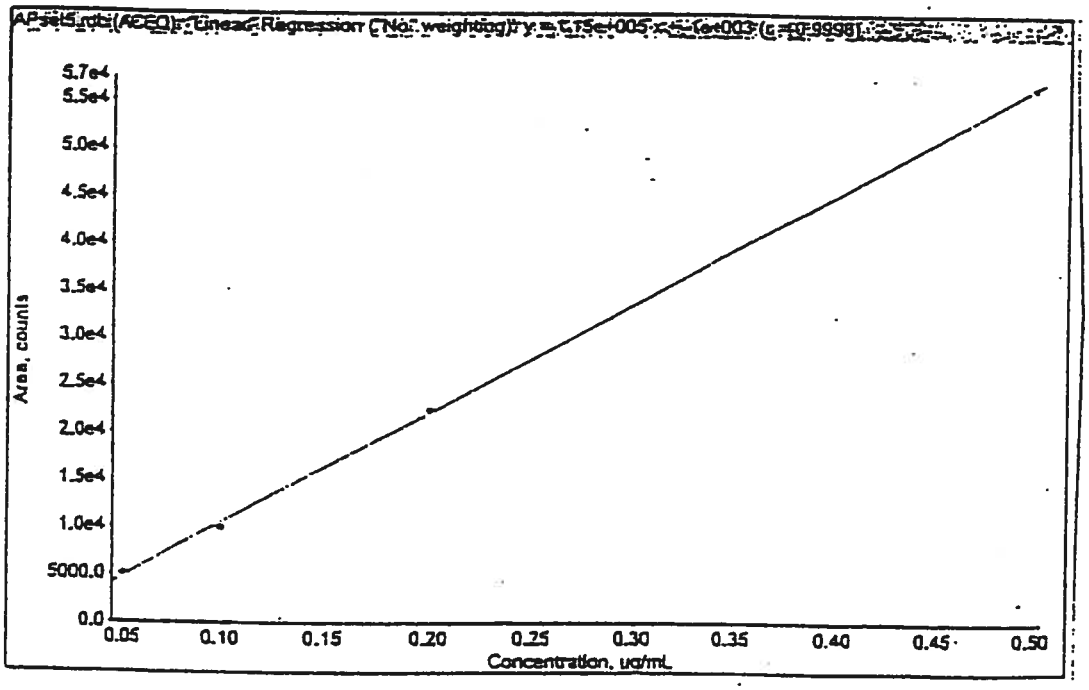
Quality Control for SPE Cartridges

Silica SPE Cartridges

Add 50 μL of an appropriate standard or standard mixture @ 10 μg/mL (in acetonitrile) to a 13 x 100 mm test tube. Add 0.4 mL 1% keeper solution and evaporate to dryness using manual nitrogen blow down. Add 5.0 mL hexane. Vortex mix. Follow steps 9.0.1 through 9.0.12 of the procedure. Final concentration is 0.5 μg/mL.



Acequinocyl-OH Calibration Curve
Primary Column: Luna C18



Acequinocyl Calibration Curve
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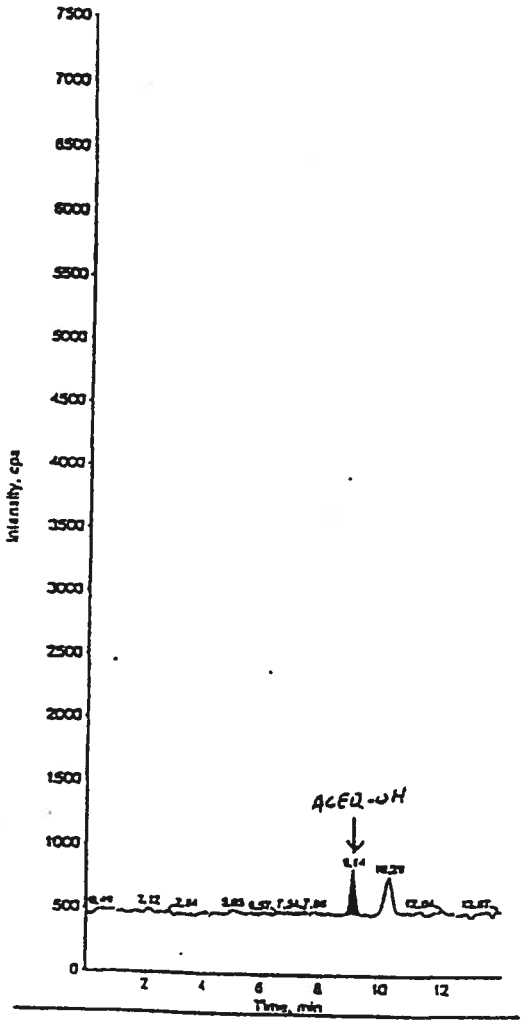
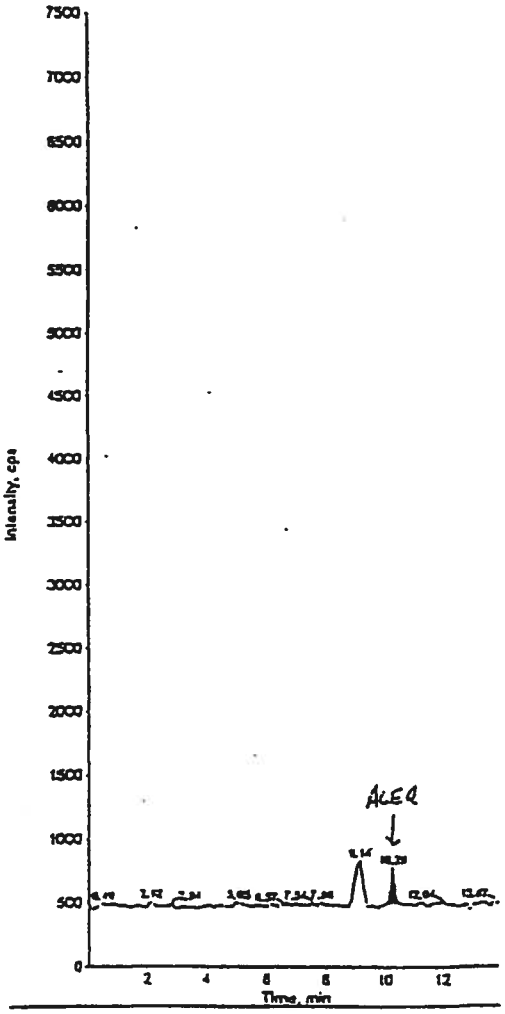
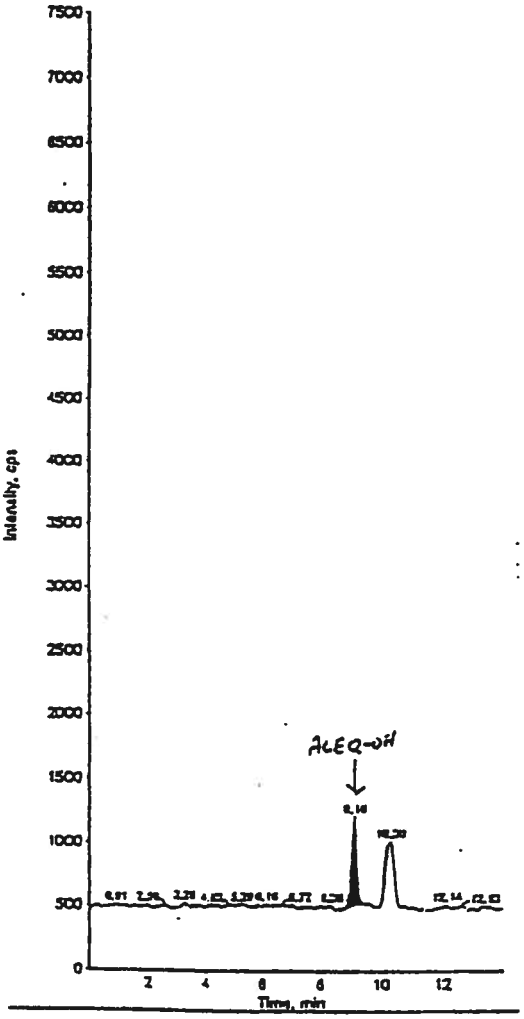


Acequinocyl-OH

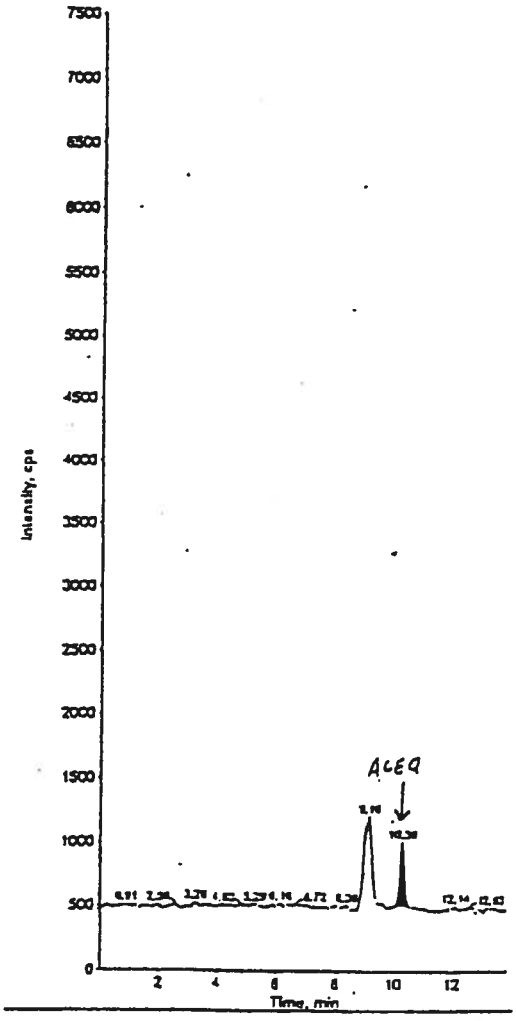


Acequinocyl

Acequinocyl-OH, Acequinocyl standard @ 0.05 µg/mL
Primary Column: Luna C18

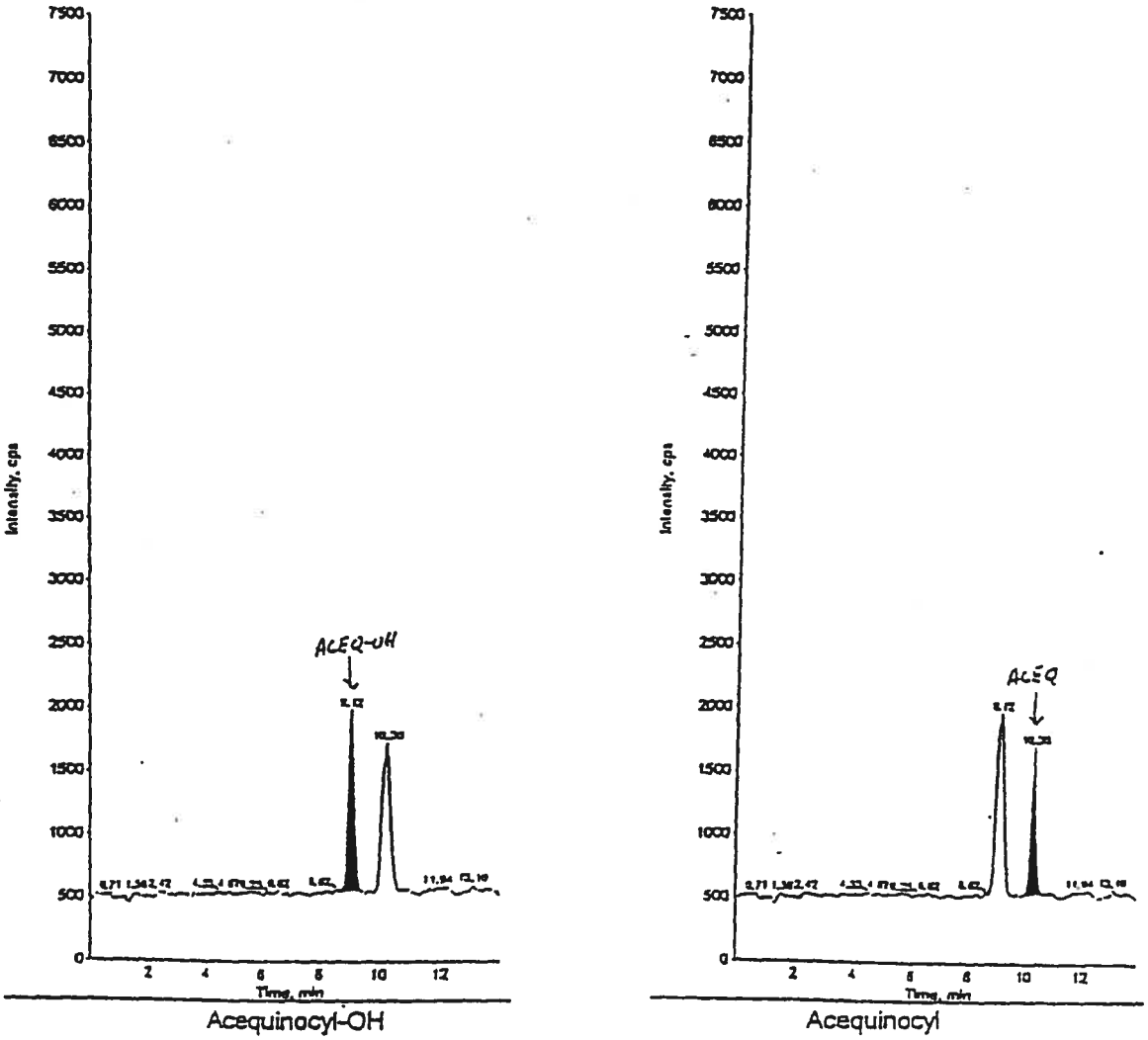


Acequinocyl-OH

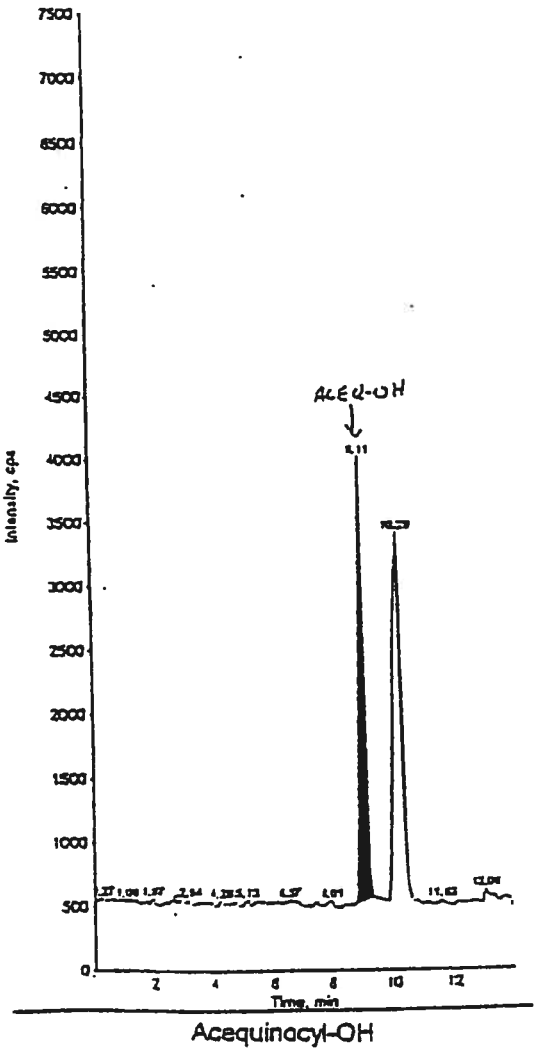


Acequinocyl

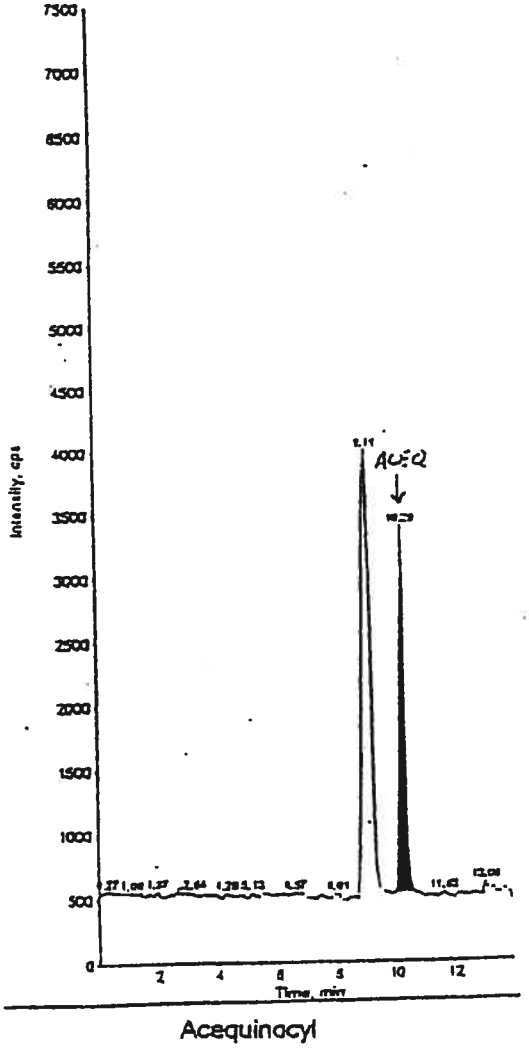
Acequinocyl-OH, Acequinocyl standard @ 0.1 µg/mL
Primary Column: Luna C18



Acequinocyl-OH, Acequinocyl standard @ 0.2 µg/mL
Primary Column: Luna C18

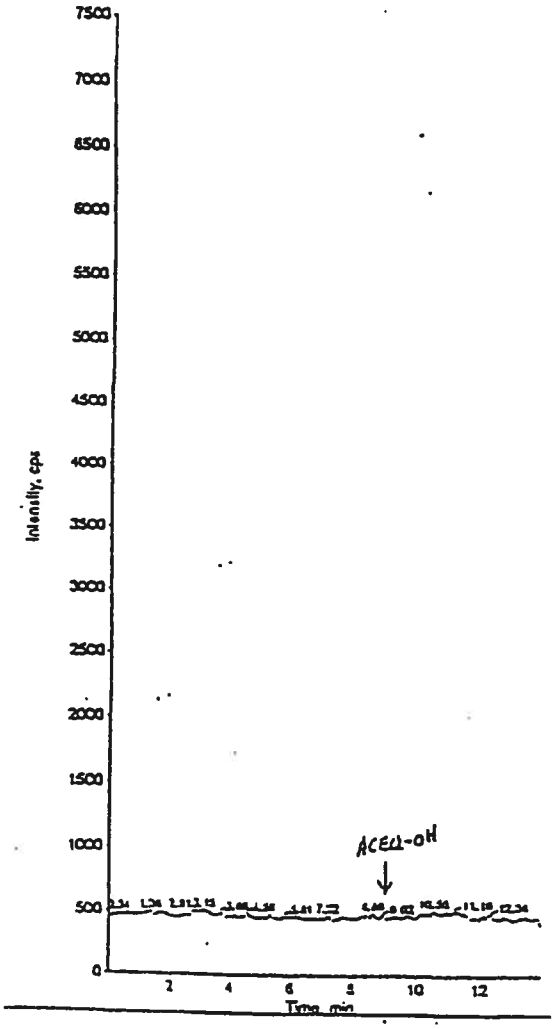


Acequinocyl-OH

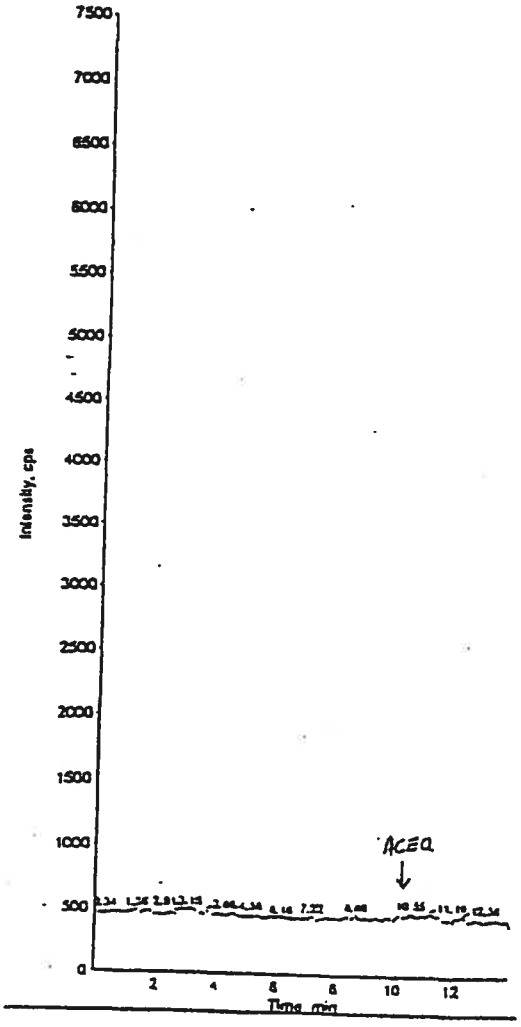


Acequinocyl

Acequinocyl-OH, Acequinocyl standard @ 0.5 µg/mL
Primary Column: Luna C18

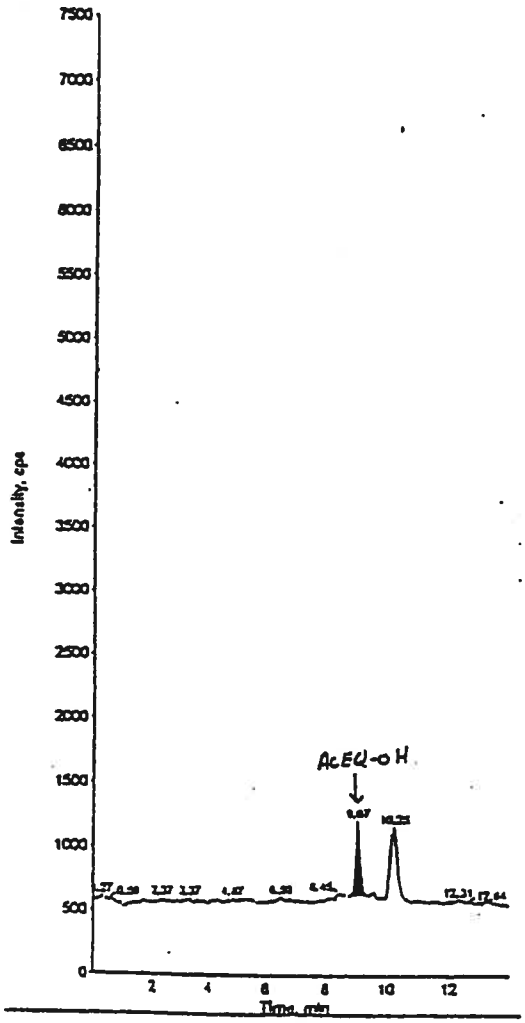


Acequinocyl-OH

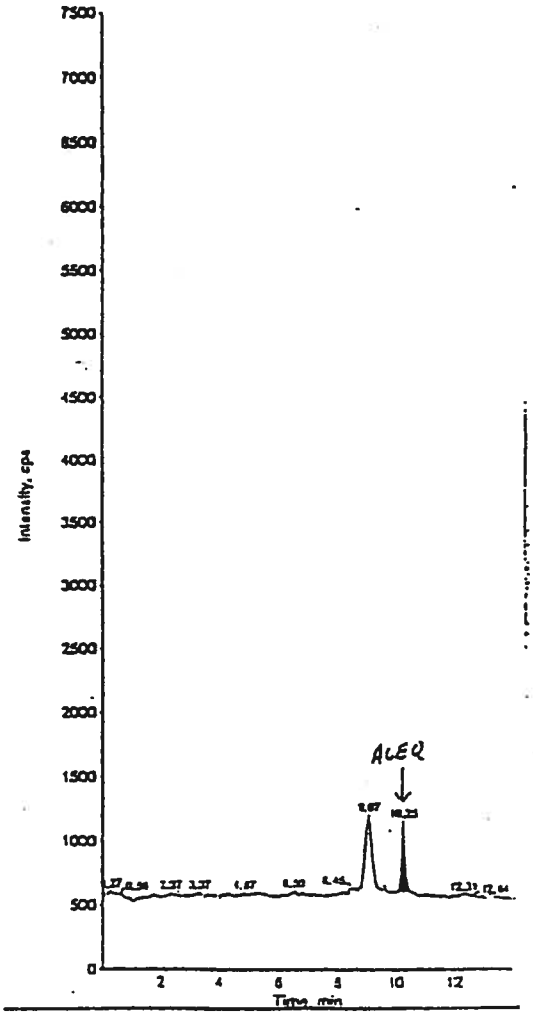


Acequinocyl

Apple Control
Primary Column: Luna C18

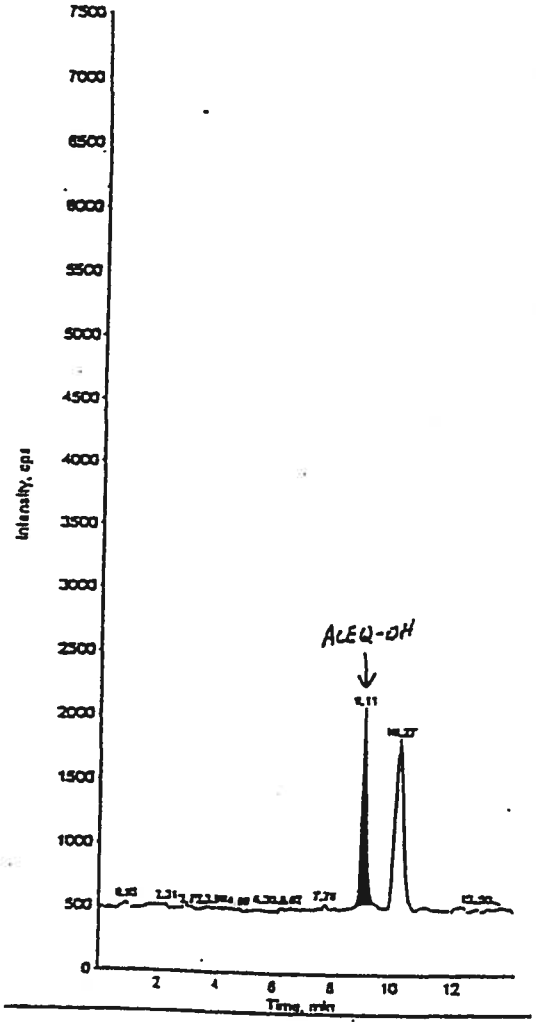


Acequinocyl-OH

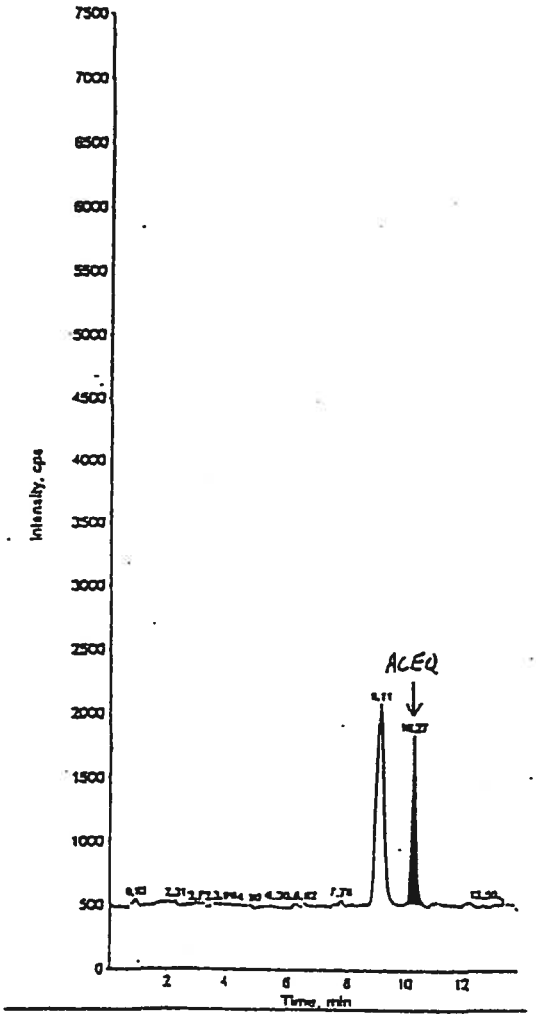


Acequinocyl

Apple fortified control @ 0.01 ppm
Acequinocyl-OH: 72% Recovery
Acequinocyl: 89% Recovery
Primary Column: Luna C18



Acequinocyl-OH



Acequinocyl

Apple fortified control @ 0.5 ppm (1-20 dil.)
Acequinocyl-OH: 82% Recovery
Acequinocyl: 90% Recovery
Primary Column: Luna C18