

ATTACHMENT

**DETERMINATION OF TOLFENPYRAD AND ITS OH-PT METABOLITE
IN CROPS
(RAW AGRICULTURAL AND PROCESSED COMMODITIES)
(ANALYTICAL METHOD # METH-183, Rev#2)**

MORSE LABORATORIES, INC.

**DETERMINATION OF TOLFENPYRAD
AND ITS OH-PT METABOLITE IN CROPS (RAW
AGRICULTURAL AND PROCESSED COMMODITIES)**

Analytical Method# Meth-183, Revision #2

June 13, 2007

APPROVED BY: *Amy Westing*
Date: June 13, 2007

Morse Laboratories, Inc.

Meth-183, Page 2

TABLE OF CONTENTS

	<u>Page</u>
TITLE PAGE	1
TABLE OF CONTENTS	2
1 PRINCIPLE	3
2 EQUIVALENCE STATEMENT	3
3 APPARATUS AND EQUIPMENT	3
4 REAGENTS AND MATERIALS	5
5 REFERENCE STANDARDS	6
6 STANDARD PREPARATION	7
7 SAMPLE FORTIFICATION	9
8 SAMPLE EXTRACTION	9
9 OASIS® HLB SPE CARTRIDGE CLEANUP	10
10 HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS	12
11 CALCULATIONS	13
 APPENDIX I	
Analysis Flowcharts	16
All Matrices Except Oils	17
Oils	18
 APPENDIX II	
Quality Control for SPE Cartridges	19

Morse Laboratories, Inc.

Meth-183, Page 3

DETERMINATION OF TOLFENPYRAD AND ITS OH-PT METABOLITE IN CROPS (RAW AGRICULTURAL AND PROCESSED COMMODITIES)

Reasons for Revision:

- 1) To change the extraction scheme employed for processed commodity oil samples (as described in Section 8.2 and corresponding appended analysis flowchart).
- 2) To change the fortification standard preparation for processed commodity oil samples.

1 PRINCIPLE

The method described herein is capable of determining tolfenpyrad and its OH-PT metabolite in a variety of crops, both raw agricultural and processed commodities. Such commodities include, but are not limited to: tomatoes, apples, potatoes (tubers, flakes), cottonseed (seed, gin byproducts, oil), head lettuce, cauliflower, grapes, oranges, cucumbers, and almonds (nutmeats, hulls).

Residues of tolfenpyrad and its OH-PT metabolite are extracted from the sample with methanol using multiple extractions (3 extractions). The crude extract from each extraction is vacuum filtered (all matrices except processed oils), then combined. The combined filtrates are brought to a final known volume. An aliquot of the combined extract is purified by means of an Oasis[®] HLB solid phase extraction (SPE) cleanup. The purified extract is evaporated to dryness, reconstituted in methanol, then submitted to HPLC analysis.

During routine analysis, determination and quantitation for both tolfenpyrad and its OH-PT metabolite are conducted using HPLC employing mass spectrometric (MS/MS) detection (LC/MS/MS). The limit of quantitation (LOQ) for both analytes, in all matrices, is 0.01 ppm.

2 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 specified. In the event a substituted piece of equipment or technique is used, its use will be documented in the study records.

3 APPARATUS AND EQUIPMENT

Assorted laboratory glassware

*Morse Laboratories, Inc.*Meth-183, Page 4

Balances:	Analytical balance capable of weighing to ± 0.1 mg Top-loading balance capable of weighing to ± 0.1 g
Centrifuge:	Centrific™ Centrifuge (Fisher Scientific, Fairlawn, NJ) IECHN-SII centrifuge (International Equipment Co., Needham Heights, MA)
Evaporator:	N-Evap Laboratory Sample Evaporator Model 115 attached to a N ₂ source (Organomation Assoc., South Berlin, MA)
Extract storage jars:	Glass, 8 oz., Qorpak jar
Extraction jars/bottles:	Glass, standard 8 oz., wide-mouth Qorpak jar Glass (Pyrex®) centrifuge bottle, 200-mL, with screw cap
Filter flasks:	Glass, 250-mL
Funnels:	Büchner type, 85 mm diameter
Graduated cylinders:	Glass; 1000, 100, and 25-mL
Graduated mixing cylinders:	Glass; 250 and 50-mL
Homogenizer:	Omni Mixer Model 17105 with Generator Probe (Omni International, Waterbury, CT)
HPLC/MS system:	Shimadzu LC-20AD high pressure liquid chromatograph system/DGU-20A5 vacuum solvent degasser equipped with an Applied BioSystems API 4000 mass spectrometer (MS/MS) detector, Shimadzu SIL-20AC autosampler and CBM-20A communication bus module (system controller) with Applied BioSystems/MDX Sciex Analyst Software for data collection and system control.
Microliter syringes:	Various sizes, (Hamilton Co., Reno, NV)
Pasteur pipets:	Glass, 9-inch and 5½-inch, disposable
Pipets:	Glass, graduated, serological; various sizes
Solid Phase Extraction Apparatus:	Vac Elut SPS 24 (Varian Sample Preparation Products, Harbor City, CA)

*Morse Laboratories, Inc.*Meth-183, Page 5

Test (culture) tubes:	Glass, 13 × 100 mm and 16 × 100 mm
Ultrasonic bath:	Branson Model 2210 ultrasonic bath (VWR Scientific, Bridgeport, NJ)
Volumetric flasks:	Glass; 100, 50, and 25 mL

4 REAGENTS AND MATERIALS

Acetone:	OmniSolv [®] (EMD Chemicals, Gibbstown, NJ)
Acetonitrile:	B&J Brand High Purity solvent, HPLC grade (Burdick and Jackson, Muskegon, MI)
Celite [®] 545:	Filter aid, powder (EMD Chemicals, Gibbstown, NJ)
Dichloromethane:	OmniSolv [®] (EMD Chemicals, Gibbstown, NJ)
Ethyl acetate:	OmniSolv [®] (EMD Chemicals, Gibbstown, NJ)
Filter paper:	Whatman #541, 70 mm (VWR Scientific, Bridgeport, NJ)
Formic acid:	98% GR ACS (EMD Chemicals, Gibbstown, NJ)
Hexane:	OmniSolv [®] (EMD Chemicals, Gibbstown, NJ)
HPLC column:	15 cm × 2.0 mm i.d. Phenomenex Luna C18 (2), 3μ particle size
Methanol:	OmniSolv [®] (EMD Chemicals, Gibbstown, NJ) B&J Brand High Purity solvent, HPLC grade (Burdick and Jackson, Muskegon, MI)
Solid phase extraction cartridges:	Oasis [®] HLB 3 cc (60 mg) extraction cartridges (Waters Corporation, Milford, MA)
Tolfenpyrad:	Analytical grade
Tolfenpyrad OH-PT:	Analytical grade

Morse Laboratories, Inc.

Meth-183, Page 6

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 Dichloromethane:ethyl acetate (90:10, v/v): To a 50-mL graduated mixing cylinder, add 5.0 mL of ethyl acetate. Bring to a final volume of 50 mL with dichloromethane. Transfer to a properly labeled secondary container. Mix well. Prepare daily. Sufficient for approximately 15 samples.

4.1.2 HPLC mobile phase:

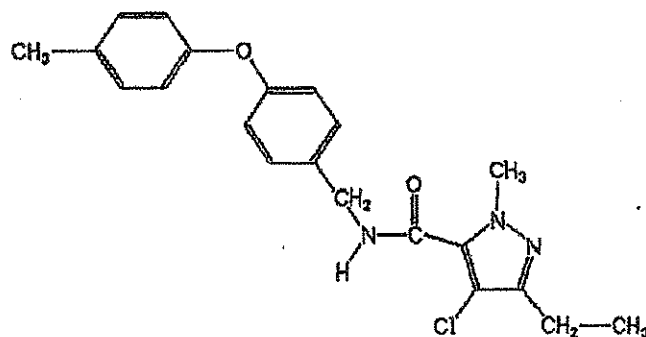
0.1% formic acid in water: To a 1 liter graduated cylinder, add HPLC grade water to the 1000 mL mark. Add 1.0 mL of formic acid using a 2.0 mL graduated pipet. Transfer entire solution to the HPLC solvent reservoir and once transferred, mix thoroughly.

5 REFERENCE STANDARDS

5.1 Tolfenpyrad:

Common name: Tolfenpyrad
Code name: OMI-88
Chemical name (CAS): 4-chloro-3-ethyl-1-methyl-N-[[4-(4-methylphenoxy)phenyl]methyl]-1H-pyrazole-5-carboxamide

Structure:



Tolfenpyrad

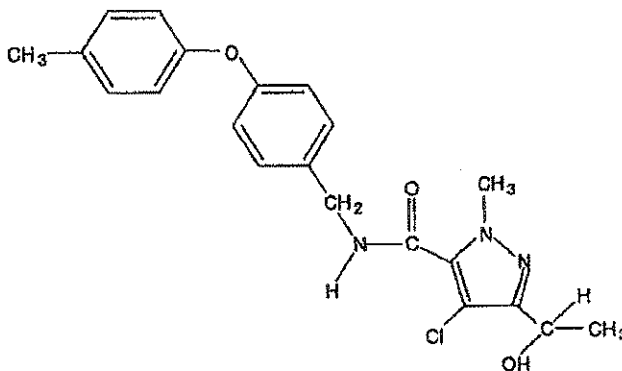
Morse Laboratories, Inc.

Meth-183, Page 7

5.2 Tolfenpyrad OH-PT Metabolite:

Common name: Tolfenpyrad-OH-PT
 Abbreviated designation: OH-PT
 Chemical name: 4-chloro-3-(1-hydroxyethyl)-1-methyl-N-[4-(4-tolyloxy)benzyl]-pyrazole-5-carboxamide

Structure:



Tolfenpyrad-OH-PT

6 STANDARD PREPARATION

6.1 Stock Standard Solutions

Typically, 25.0 mg (corrected for purity) of each analytical standard is accurately weighed and quantitatively transferred to separate 25-mL volumetric flasks. Each is brought to volume with acetonitrile. The resulting concentration of each solution is 1000 µg/mL. These solutions are to be stored at ~1 to 8 °C when not in use.

6.2 Fortification Solutions

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

6.2.1 Solutions used for all crop matrices except oils:

100 µg/mL: Transfer 2.5 mL of a 1000-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 a 100-µg/mL standard solution to a 25-mL volumetric flask. Bring to volume in acetonitrile. Mix well.

Morse Laboratories, Inc.

Meth-183, Page 8

1 $\mu\text{g/mL}$: Transfer 2.5 mL of a 10- $\mu\text{g/mL}$ standard solution to a 25-mL volumetric flask. Bring to volume in acetonitrile. Mix well.

6.2.2 Solutions used for oils:

100 $\mu\text{g/mL}$: Transfer 2.5 mL of a 1000- $\mu\text{g/mL}$ standard solution to a 25-mL volumetric flask. Bring to volume in acetone. Mix well.

10 $\mu\text{g/mL}$: Transfer 2.5 mL of a 100- $\mu\text{g/mL}$ standard solution to a 25-mL volumetric flask. Bring to volume in acetone. Mix well.

1 $\mu\text{g/mL}$: Transfer 2.5 mL of a 10- $\mu\text{g/mL}$ standard solution to a 25-mL volumetric flask. Bring to volume in acetone. Mix well.

6.3 HPLC (Calibration) Standard Solutions

Typically the following concentrations of calibration standards containing both tolfenpyrad and tolfenpyrad OH-PT are prepared. All standard solutions prepared in this section are to be stored at ~ 1 to 8°C when not in use.

Intermediate standard solution:

0.01 $\mu\text{g/mL}$: Transfer 250 μL of a 1.0- $\mu\text{g/mL}$ standard solution to a 25-mL volumetric flask. Bring to volume in methanol. Mix well.

Calibration standard solutions:

0.00001 $\mu\text{g/mL}$: Transfer 100 μL of a 0.01- $\mu\text{g/mL}$ standard solution to a 100-mL volumetric flask. Bring to volume in methanol. Mix well.

0.00002 $\mu\text{g/mL}$: Transfer 100 μL of a 0.01- $\mu\text{g/mL}$ standard solution to a 50-mL volumetric flask. Bring to volume in methanol. Mix well.

0.00005 $\mu\text{g/mL}$: Transfer 125 μL of a 0.01- $\mu\text{g/mL}$ standard solution to a 25-mL volumetric flask. Bring to volume in methanol. Mix well.

0.0001 $\mu\text{g/mL}$: Transfer 250 μL of a 0.01- $\mu\text{g/mL}$ standard solution to a 25-mL volumetric flask. Bring to volume in methanol. Mix well.

0.0002 $\mu\text{g/mL}$: Transfer 500 μL of a 0.01- $\mu\text{g/mL}$ standard solution to a 25-mL volumetric flask. Bring to volume in methanol. Mix well.

Morse Laboratories, Inc.

Meth-183, Page 9

7 SAMPLE FORTIFICATION

7.1 All Crop Matrices Except Oils

1. Weigh 5.0 g of macerated sample (2.5 g for dry matrices, e.g., undelinted cottonseed, cotton gin byproducts, etc.) into an 8-oz. Qorpak jar.
2. Fortify the sample with the appropriate amount of standard solution. Disperse solution over as much of the sample as possible. Use a volume ≤ 1.0 mL. Allow solvent evaporate for about 5-10 minutes.
3. Proceed with Step 8.1.2.

7.2 Oils

1. Weigh 5.0 g of oil sample into a 200-mL glass centrifuge bottle.
2. Fortify the sample with the appropriate amount of standard solution in acetone. Use a volume ≤ 0.5 mL. Cap and shake/mix sample to disperse analytes.
3. Proceed with Step 8.2.2.

8 SAMPLE EXTRACTION

8.1 All Crop Matrices Except Oils

1. Weigh 5.0 g of macerated sample (2.5 g for dry matrices, e.g., undelinted cottonseed, cotton gin byproducts, etc.) into an 8-oz. Qorpak jar. As applicable, fortify appropriate samples at this time.
2. Add 10 g of Celite[®] (5.0 g of Celite[®] for dry matrices) and 60 mL of methanol. Allow sample to soak for ~15 minutes.
3. Blend using a high-speed homogenizer (at medium speed) for ~1 minute.
4. Vacuum filter the extract through Whatman 541 filter paper into a 250-mL filter flask.
5. Return the filter cake to the original Qorpak extraction jar, add 60 mL of methanol and repeat Steps 3 and 4 two additional times, combining all filtrates in original filter flask. On the last filtration, wash filter cake with ~10 mL methanol.

Morse Laboratories, Inc.

Meth-183, Page 10

6. Transfer combined extracts to a 250-mL graduated mixing cylinder and bring to a final volume of 200 mL with methanol. Mix well.
7. Transfer approximately 5 mL of the sample extract to a 13 × 100 mm test tube and centrifuge at ~3000 rpm for ~5 minutes.
8. Transfer 400 µL (0.40 mL) of the supernatant to a 16 × 100 mm screw-cap test tube. Add 0.6 mL methanol and 10 mL of DI water. Proceed to Section 9.

8.2 Oils

1. Weigh 5.0 g of oil sample into a 200-mL glass centrifuge bottle. As applicable, fortify appropriate samples at this time.
2. Add 60 mL of methanol, cap and shake vigorously for ~1 minute.
3. Centrifuge at ~2500 rpm for ~5 minutes.
4. Transfer the upper methanol layer to a 250-mL graduated mixing cylinder.
5. Repeat Steps 2 through 4 three additional times, combining all methanol extracts in the original mixing cylinder. Bring to a final volume of 250 mL with methanol.
6. Transfer 100 µL (0.10 mL) of the diluted sample from Step 5 to a 16 × 100 mm screw-cap test tube. Add 0.9 mL methanol and 10 mL of DI water. Proceed to Section 9.

9 **OASIS® HLB SPE CARTRIDGE CLEANUP**

Note: This cleanup is especially required for matrices containing oils and/or resins, or that are dry (such as almond nuts, almond hulls, cauliflower, cottonseed, cotton gin byproducts, cottonseed oil, potato flakes, etc.), due to potential analyte peak response enhancement/suppression during LC/MS/MS analysis of dilute crude sample extracts. Even with significant dilution, the crude extracts from several different watery crop matrices were found, during method development, to cause this problem. For this reason, it is advised that this SPE cleanup be employed during the analysis of all matrices.

1. Set up Vac Elut system and support apparatus and proceed with Oasis® HLB SPE cleanup. In general, set vacuum to produce a flow rate of approximately 2 mL/minute (not continuous flow) for all elutions.

Morse Laboratories, Inc.

Meth-183, Page 11

2. Take one Oasis[®] HLB cartridge (size 60 mg, 3 mL) for each sample to be analyzed and place on the vacuum manifold. Add methanol (2 mL) onto the cartridges and draw through under vacuum to the level of the top frit, discarding the column eluate. Do not allow the cartridges to become dry. Add HPLC-grade water (2 mL) and draw through under vacuum to the level of the top frit, discarding the column eluate. Do not allow cartridges to become dry.
3. Transfer the extracts from Steps 8.1.8 or 8.2.6 onto the cartridges and draw through under vacuum at a rate of approximately 2 mL/minute, discarding the column eluates. Residues of tolfenpyrad and tolfenpyrad OH-PT are retained on the cartridge.
4. Add HPLC-grade water (1 mL) to the tubes that contained the extracts. Rinse the tubes and transfer to the SPE cartridges. Draw through under vacuum to the level of the top frit, discarding the column eluate.
5. Remove any remaining droplets of water adhering to the inside of the cartridges with absorbent tissue and dry under high vacuum (≥ 15 inches Hg) for a minimum of 20 minutes.

Note : Vacuums < 15 inches Hg result in inconsistent recoveries (especially for OH-PT) and a final extract containing unpredictable levels of oily/resinous co-extractives (detrimental to the MS/MS detector).

6. When dry, add hexane (4 mL for all matrices except oils, 8 mL for oils) to the top of the SPE cartridges. Draw through under vacuum, discarding the column eluates.
7. Place suitable collection tubes (e.g. 13 × 100 mm test tubes) under each port, as required, in the manifold rack.
8. Add 3 mL of dichloromethane:ethyl acetate (90:10, v/v) to the SPE cartridges and draw through under vacuum, collecting the column eluates. Apply vacuum (~ 15 inches Hg) for approximately 5 seconds to draw off any remaining droplets of eluate. Residues of tolfenpyrad and tolfenpyrad OH-PT are eluted in this fraction.

Note: The above SPE procedure has been developed using columns from the stated manufacturer; however, it is possible to carry out the procedure using similar columns from other manufacturers. In all cases, it is strongly recommended that the elution profile of the chosen batch of columns be checked.

9. Concentrate the eluate to ~ 0.2 mL using an N-Evap evaporator set at ≤ 40 °C. Use manual nitrogen blow-down to evaporate the concentrate to dryness. For all matrices except dry matrices and oils, add 5.0 mL HPLC methanol. For dry matrices, add 2.5

Morse Laboratories, Inc.

Meth-183, Page 12

mL HPLC methanol. For oils, add 1.0 mL HPLC methanol. Cap, sonicate and mix well. Final sample concentration: 1 mL = 0.002 g sample. Submit to LC/MS/MS analysis.

10 HIGH PERFORMANCE 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: Shimadzu LC-20AD high pressure liquid chromatograph system/DGU-20A5 vacuum solvent degasser equipped with an Applied BioSystems API 4000 mass spectrometer (MS/MS) detector, Shimadzu SIL-20AC autosampler and CBM-20A communication bus module (system controller) with Applied BioSystems/MDX Sciex Analyst Software for data collection and system control.

HPLC column: 15 cm x 2.0 mm i.d. Phenomenex Luna C18 (2), 3µ particle size

Mobile phase: Fisher water, Burdick and Jackson acetonitrile and EM Science formic acid

Mobile Phase A: 0.1% formic acid in HPLC grade water

Mobile Phase B: 100% acetonitrile

Gradient:

	Mobile Phase A	Mobile Phase B
<u>Time (min.)</u>	<u>(%)</u>	<u>(%)</u>
0.0-0.5	50	50
4.0-12.0	0	100
12.01-16.0	50	50

Divert valve: Programmed to divert LC flow from column to waste (bypassing detector) from 0 to 3.0 minutes and again from 9.0 to 16.0 minutes LC flow is directed to detector during the 3.0 to 9.0 minute window. Diversion time settings can be adjusted as necessary depending on the retention times of the analytes.

Morse Laboratories, Inc.

Meth-183, Page 13

Flow rate: 0.2 mL/min.
 Interface probe: TIS
 Ionization mode: Positive (+)
 Acquisition mode: MRM
 Source temperature: 550 °C
 Curtain gas: Nitrogen @ 15
 Collision gas: Nitrogen @ setting of "12"
 Injection volume: 10 µL
 Column temperature: 40 °C

Transitions monitored:

	<u>Ion, m/z</u>		<u>Time, ms</u>	<u>CE, %</u>	
	<u>Q1</u>	<u>Q3</u>			
Tolfenpyrad:	384.1	197.1	150	41	(quantitation)
	384.1	154.0	150	63	(confirmation)
	384.1	90.8	150	77	(confirmation)
OH-PT:	400.1	196.9	150	35	(quantitation)
	400.1	381.6	150	27	(confirmation)
	400.1	90.9	150	77	(confirmation)

Retention times: Tolfenpyrad: ~ 8.0 minutes
 OH-PT: ~ 6.4 minutes

10.2 Sample Analysis

Prepare a five-point standard curve by injecting constant volumes of standard solutions. Use constant volume injections for sample extracts as well. Sample responses greater than those produced by the highest concentration of standard in the standard curve require dilution and reinjection. Inject a curve check standard every 3 to 4 sample injections.

11 **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

Morse Laboratories, Inc.

Meth-183, Page 14

calculate a best fit line (from a set of standard concentrations in $\mu\text{g/mL}$ versus peak response) and to determine concentrations of the analyte found during sample analysis from the calculated best fit line. **Weighting ($1/x$) is used.**

The equation used for the least squares fit is:

$$y = mx + b$$

where:

y	=	peak response
x	=	$\mu\text{g/mL}$ 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:

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

where:

$\mu\text{g/mL}$	=	$\mu\text{g/mL}$ of analyte found from standard curve
sample wt. (g)	=	gram weight of sample extracted (typically 5.0 g, except for dry matrices which is 2.5 g)
mL ext. solv.	=	volume of extraction solvent (typically 200 mL, except for oils which is 250 mL)
mL aliq.	=	volume of extract taken through Oasis [®] HLB SPE cleanup (typically 0.4 mL, except for oils which is 0.1 mL)
HPLC final vol. (mL)	=	volume of final HPLC-ready extract (typically 5.0 mL, except for dry matrices which is 2.5 mL and oils which is 1.0 mL)
HPLC dilution factor	=	dilution of sample extract required to produce an analyte response bracketed by standards

Morse Laboratories, Inc.

Meth-183, Page 15

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

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

Method authors: Gary L. Westberg
Richard L. Reed II

Morse Laboratories, Inc.

Meth-183, Page 16

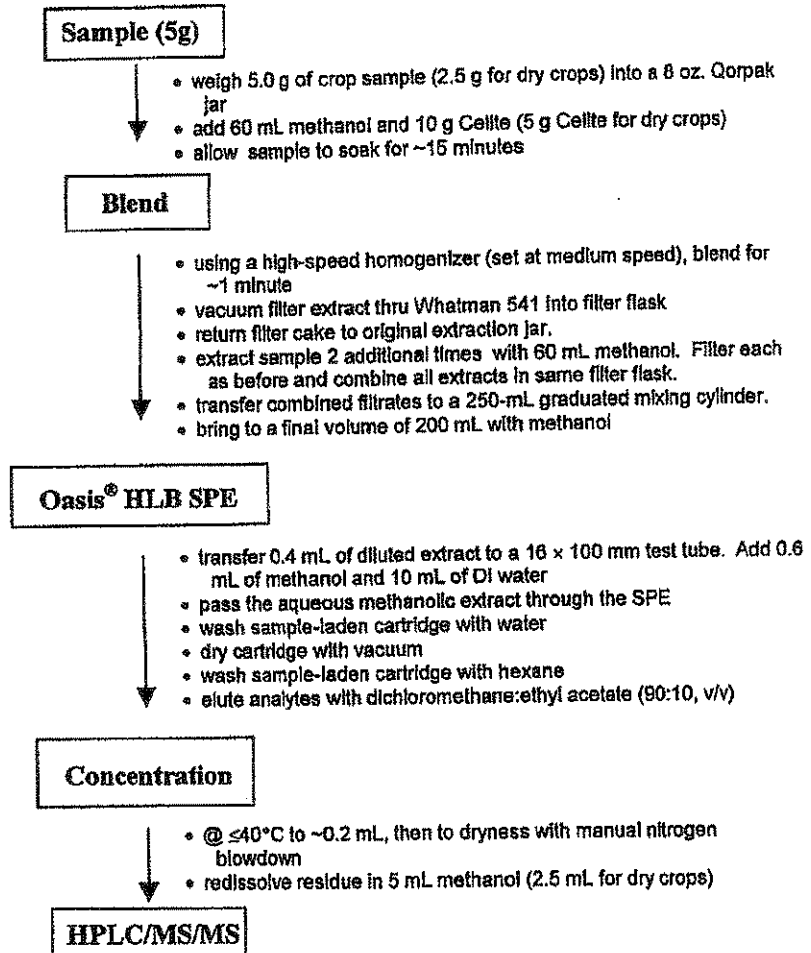
APPENDIX I

Analysis Flowcharts

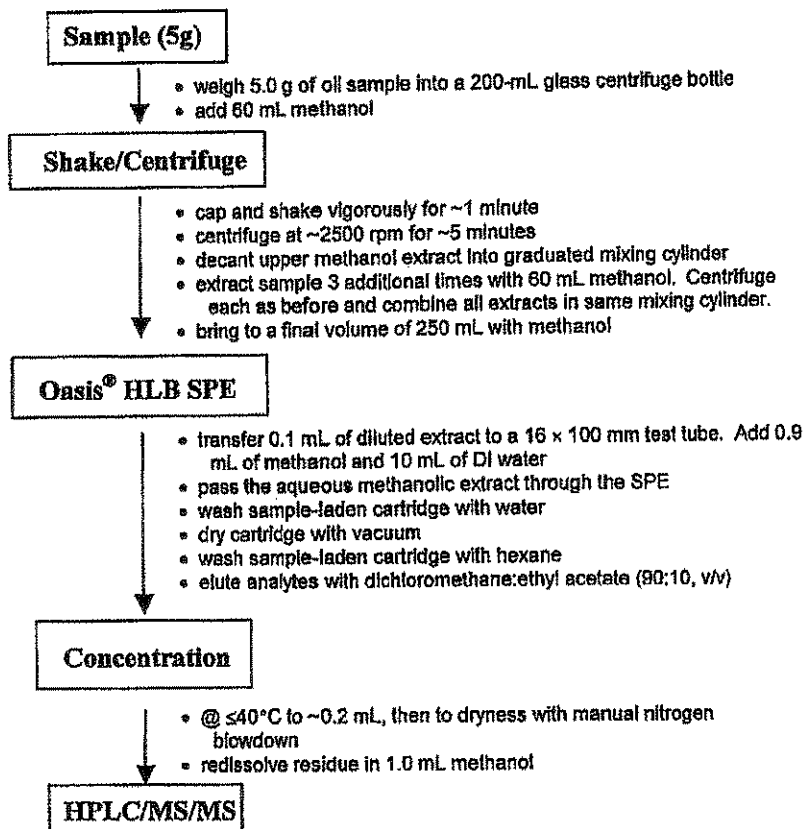
Morse Laboratories, Inc.

Meth-183, Page 17

**ANALYSIS FLOWCHART
(All Matrices Except Oils)**



**ANALYSIS FLOWCHART
(Oils)**



Morse Laboratories, Inc.

Meth-183, Page 19

APPENDIX II

Quality Control for SPE Cartridges

Morse Laboratories, Inc.

Meth-183, Page 20

Quality Control for SPE Cartridges

Oasis[®] HLB SPE Cartridges

- Transfer 50 μL of a 0.01- $\mu\text{g}/\text{mL}$ Tolfenpyrad/OH-PT mixed standard solution in methanol to a 16 \times 100 mm test tube containing 1.0 mL of methanol.
- Add 10 mL of DI water. Mix well. Follow Steps 9.1 through 9.9 of the procedure.
- Redissolve the residue in 5.0 mL of methanol. Final concentration is 0.0001 $\mu\text{g}/\text{mL}$.
- Submit to LC/MS/MS analysis.