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TITLE

Tolerance Enforcement Method for RH-5992 and Metabolites in Animal Commodities

DATA REQUIREMENT

Guideline 171-4 (d)

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Vol (1) of (2)

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA 10(d)(1)(A), (B), or (C).

COMPANY Rohm and Haas Company

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TR 34-96-109

OCT 3

GLP COMPLIANCE STATEMENT

As per 40 CFR 160 3, method development is not required to be conducted under GLP However, this method was developed in the spirit of US EPA FIFRA Good Laboratory Practice regulations Validation of the method was conducted in full compliance with 40 CFR 160

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QUALITY ASSURANCE STATEMENT

This report and the original raw data have been reviewed by the Quality Assurance Unit of the Rohm and Haas Company Agricultural Research Division and have been validated as a true and accurate representation of the data collected.

QA activities for the study

Date	Activity	Date Reported
07/12-17/96	final report audit	07/17/96

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I. Objective

I. Objective

Four preliminary residue methods on cow's milk and meat matrices were previously issued. TR 34-95-98 (Milk), TR 34-95-160 (Meat i.e. Kidney and Muscle), TR 34-95-159 (Liver), and TR 34-95-161 (Fat) were issued in 1995-96 and used to analyze samples from the RH-5992 Cow Feeding Study. This tolerance enforcement method is a compilation of those methods with additional fortifications, incorporated modifications, ^{14}C radiovalidation and HPLC-MS confirmation.

II. Summary

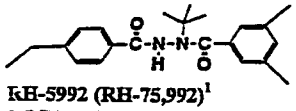
RH-5992 is an insecticide being developed for registration in the United States, as well as, other nations throughout the world. In order to obtain commercial registration, a tolerance enforcement method for animal matrices is needed to acquire residue data for the setting and enforcement of tolerances.

The analytes of interest for the cow study were selected from the significant residues found in the study "Metabolism of ^{14}C RH-5992 in Lactating Dairy Goats" (MRID 43706601). Analytes include parent RH-5992 and its metabolites, the free oxidation products RH-9886, RH-0282, RH-2703, and fatty acid conjugates of RH-9886 In the metabolism study, multiple fatty acid conjugates of RH-9886 were identified For the residue analytical method, the stearic acid conjugate (RH-9526) was synthesized for fortification purposes. The table below lists the analytes that were investigated in each substrate

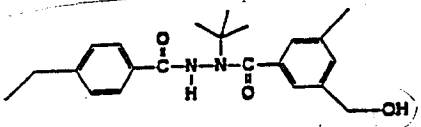
TABLE I: Analytes by Matrix

	RH-5992	RH-9886	RH-0282	RH-9526	RH-2703
Milk	[redacted]		[redacted]	[redacted]	
Meat	X	X	X		[redacted]
Liver	[redacted]				
Kidney	X	X	X		
Fat	X			X	

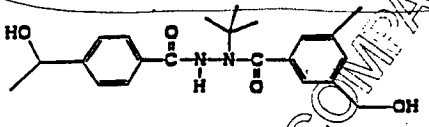
The structures, chemical names and other information for the compounds of interest are listed below;



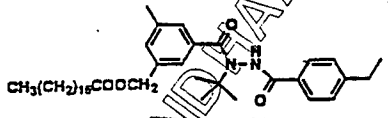
Rohm and Haas Number: RH-5992 (RH-75,992)[1]
Chemical Name: 3,5-Dimethyl benzoic acid 1-(1,1-dimethyl)-2-(4-ethylbenzoyl) hydrazide
CAS Number: 112410-23-8
Common Name: tebufenozide
Trade Names: Confirm™, Mimic™, Romdan™, Midic™



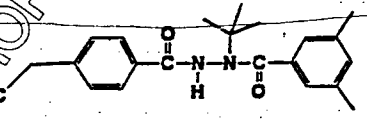
Rohm and Haas Number: RH-9886, (RH-89,886)
Chemical Name: N-(1,1-dimethylethyl)-N'-(4-ethylbenzoyl)-3-hydroxymethyl-5-methylbenzohydrazide



Rohm and Haas Number: RH-0282, (RH-120,282)
Chemical Name: N-4-(2-hydroxyethyl)-benzoyl-N'-3-hydroxymethyl-5-methyl-benzoyl-N'-tert-butylhydrazide



Rohm and Haas Number: RH-9526, (RH-139,526)
Chemical Name: N'-(4-ethylbenzoyl)-N-3-methyl-5-(stearoyloxymethyl) benzoyl-N-tert-butylhydrazide



Rohm and Haas Number: RH-2703, (RH-11,2703)
Chemical Name: 4-[N'-(3,5-dimethylbenzoyl)- N'-(1,1-dimethylethyl) hydrazino-carbonyl] phenylacetic acid

The full RH number is presented in parentheses. The designation typically used is the four digit abbreviation, e.g., RH-5992.

Preliminary methods were developed for all the matrices and analytes as shown in Table I Parent RH-5992 and metabolites RH-0282, RH-9886, and RH-2703 are determined in their existing molecular form The metabolite RH-9526 is hydrolyzed to and quantified as RH-9886 (B-ring alcohol) The methods for the analytes determined in their existing molecular form are as shown in the flow chart below

Extraction (methanol/water/HCl)

↓

Partial clean-up (liquid- liquid partitions)

↓

Solid phase clean-up (various including basic alumina, carbon, & silica)

↓

Reverse phase HPLC with UV detection (HPLC-MS confirmation)

Methods which measure fatty acid conjugates of RH-9886 (such as RH-9526, the stearic acid ester) include an additional hydrolysis step, where these fatty acid conjugates are converted to RH-9886 All of the hydrolyzed RH-9886 conjugates are quantified and reported as ppm total RH-9886 Detailed method steps are shown in each subsection by matrix.

Average parent RH-5992 recovery ranged from about 87% to 93% through all matrices Average metabolite recoveries ranged from about 78% to 93% through all matrices The method Limits of Quantitation (LOQ) in ppm and recovery by analyte and matrix are shown in Table II below

TABLE II: LOQ and Average Recovery

	RH-5992	RH-9886	RH-0282	RH-9526	RH-2703
Milk	0.01 ppm 88.3 ±11.6%		0.01 ppm 89.5 ± 8.5%	0.01 ppm 83.9 ±10.3%	
Liver	0.02 ppm 93.0 ±10.5%				0.02 ppm 83.9 ±11.3%
Meat (Kidney/ Muscle)	0.02 ppm 86.8 ±11.5%	0.02 ppm 93.3 ±11.0%	0.02 ppm 87.5 ±10.9%		
Fat	0.02 ppm 88.5 ± 9.8%			0.02 ppm 77 7 ± 9.1%	

III. Method

A. Chemical / Supplies / Solutions / Mobile Phases

The following brands and suppliers were used in the development of this method. Other manufacturer brands or suppliers may be substituted if they can be shown to be suitable.

CHEMICALS / SUPPLIES	GRADE	SUPPLIER
0.45 µm filters		Acrodisc, VWR
Boiling Chips		
Celite (acid washed)		Fisher, Baker
Cotton or Glass Wool		VWR
Dry Ice		
Ethyl Acetate	HPLC	Baker
Filter Paper #3 ,7 cm		Whatman
Hexane	HPLC	Baker
Hydrochloric Acid (concentrated)	Reagent	Baker
Hydrochloric Acid (1 N)	Reagent	Baker #5620-02
Methanol	HPLC	Baker
Methylene Chloride (a.k.a. Methylene Dichloride or MDC)	HPLC	Baker
pH test paper		VWR
Pesticide Analytical Standards RH-5992 RH-0282 RH-9886 RH-9526 RH-2703		Rohm & Haas Company
Sodium Bicarbonate	Reagent	Baker, VWR
Sodium Chloride	ACS	Sigma
Sodium Sulfate, Anhydrous	Reagent	Sigma, Mallinckrodt
Toluene	HPLC	Baker, VWR
Water	HPLC	Waters HPLC grade or Milli-Q
COLUMN CHROMATOGRAPHY CLEAN UP		
Aluminum oxide, Basic (a.k.a. "Basic Alumina")		Baker # 0539-01
Carbon SPE (prepacked or tube packed with ENVI-Carb (120/400 mesh)		Supelco Envicarb 6 mL # 57094 (prepack) Supelco #5-7210 (Bulk packing)
Silica Gel (40 -140 mesh) (a.k.a. "Silica")		Baker # 3404-1

SOLUTIONS	MATRIX
Extraction Solvents	
90 % Methanol / 10% water (v/v)	Milk
70 % Methanol / 30% aqueous 0 5 N HCl (v/v)	Liver
90 % Methanol / 10% aqueous 0 1 N HCl (v/v)	Meat (Muscle, Kidney)
120 mL Methanol, 40 mL water, 15 mL of concentrated HCl	Fat
General Solutions	
Sodium Bicarbonate 1% water solution (w / v)	Liver
Sodium Chloride 10% water solution (w / v)	All matrices
Column Clean-Up Eluents	
Basic Alumina (Elution scheme 1)	Milk, Muscle, Kidney
Ethyl Acetate	
20% Methanol / Ethyl Acetate (v/v)	
Basic Alumina (Elution scheme 2)	Fat
20% Ethyl Acetate / Hexane (v/v)	
20% Methanol / Ethyl Acetate (v/v)	
Basic Alumina (Elution scheme 3)	Liver
5% Ethyl Acetate / Hexane (v/v)	
10% Ethyl Acetate / Hexane (v/v)	
20% Ethyl Acetate / Hexane (v/v)	
Carbon SPE	Milk, Muscle, Kidney, Fat
30% Methanol /Water (v/v)	
50% Methanol /Water (v/v)	
80% Methanol /Water (v/v)	
100% Methanol	
Silica Gel Column Chromatography	Liver
5% Methanol/ Toluene (v/v)	
10% Methanol/ Toluene (v/v)	
25% Methanol/ Toluene (v/v)	
35% Methanol/ Toluene (v/v)	

MOBILE PHASES	MATRIX
Recommended Mobile Phases	
RH-5992; Mobile Phase I	All matrices
54% Acetonitrile / Water (v/v), Isocratic	
RH-9886 (includes RH-9526 converted residues); Mobile Phase II	Milk, Muscle, Kidney, Fat
45% Acetonitrile / Water (v/v), Isocratic	
RH-0282; Mobile Phase III	Milk, Muscle, Kidney
30% Acetonitrile / Water (v/v), Isocratic	
RH-2703; Mobile Phase IV	Liver
45% Methanol / Water, 0.1% Acetic Acid (or PIC A) (v/v), Isocratic	
Note: Mobile Phases may be adjusted up to ± 4% of the stated organic phase at the discretion of the analyst to optimize chromatography.	
Alternative Mobile Phases	
Gradient: for RH-5992 and RH-0282 Solvent A: 100% Acetonitrile Solvent B 10% Acetonitrile / Water (v/v) See Milk subsection for conditions	Milk
RH-5992	Liver
67% Methanol / Water (v/v), Isocratic	

B. Column Preparation

Packing Carbon SPE Tubes: Weigh 1 0 gram (± 0 05 g) of ENVI-Carb™ packing into a beaker Add 20-30 mL of methanol and stir Place a 6 mL filtration tube on a Visiprep™ SPE vacuum manifold (or similar device) and seat a frit at the bottom by gently pushing it down the tube Using a disposable pipette, transfer the carbon-methanol slurry to the tube Let the methanol drain under low vacuum but keep the carbon wet. After all the carbon is in the tube, drain the methanol to about 4 mm above the carbon bed Push another frit down the tube gently on top of the carbon bed. The top and bottom frits should be parallel to each other

Silica Gel Activation: Activate the silica gel (mesh 40-140) by heating for 24 hours at 200 °C Remove from the oven and store in tightly capped jars in a desiccator

Standardization of Absorbents: It is necessary to standardize all absorbent batches in the following manner before analyzing samples

A Using a standard between 2 and 10 µg/mL of concentration (RH-5992 or metabolite) follow the elution schemes as outlined under the procedure in each matrix subsection

B Collect the pre-elution cuts, as well as the target elution cuts

C Collect a post-cut consisting of a small volume of solvent which is at least equal in solvent strength to the target elution solvent

D Concentrate the pre-cut, target-cut and post-cut to dryness

E Dissolve the cuts in an appropriate amount of mobile phase and inject them as outlined under *HPLC* quantitation

F If the target cut contains minimum of 85% of the RH-5992 material, it may be considered acceptable

G If the 85% recovery criteria for standard is not met, the analyst may adjust the elution scheme as follows

1) For cases where the prewash cut contains significant standard, either the prewash amount or eluting solvent percentage may be decreased

2) For cases where the post wash cut contains significant standard, the target elution volume may be increased or the eluting solvent percentage may be increased.

C. Equipment

EQUIPMENT	
Blender, Explosion Proof	Waring
Buchner Funnels and Vacuum flasks	VWR
Centrifuge	Sorvall RC2-B
Centrifuge Bottles, 250 mL	VWR
Chromatographic Columns 14.5 mm (id) × 25 mm (ht)	ACE Glass Cat. # 5907-10
Condensers (for reflux)	Pyrex
Heating apparatus (block or mantels, etc.)	VWR
Food processor	Hobart
Polytron (Tissue homogenizer)	Tekmar, VWR
Rotary Evaporator	Buchi, Janke & Kunkel
Round Bottom Flasks, 100 mL, 300 mL, 500 mL 24/40 ST(Standard Taper)	Pyrex
Sonic bath	VWR
Separatory Funnels, 500 mL	Pyrex
Standard Laboratory Equipment: balances, beakers, etc.	Metler, Pyrex, Sartorius
SPE Vacuum manifold	Supelco (Visiprep™)
NOTE: Other manufacturer brands may be substituted if they can be shown to be suitable.	

D. Instrumentation

Methods were developed using a Waters Associates (Millipore) High Performance Liquid Chromatograph configured as follows:

Auto Sampler	WISP 710B
Pump I	Model 510 (only one pump required) Chromatography Pump
Detector:	Spectra-Physics UV 2000 Detector.
Column Heater	Water YCM
System Control	I. NEC Powermate 2 II. System Interface Module
Software	Maxima 820 (Dynamic Solutions, Division of Millipore)
Data System	Hardware: Hewlett Packard Series 9000 Model 300 Software: 4400 Chromatography Software (Nelson Analytical, Inc)
HPLC column	Alltech or Supelco C-18 5 micron Length: 25 cm I.D.: 4.6 mm
Conditions	Flow: 2 mL / min. Column Temperature: 45 °C Wavelength: (240 nm or 254 nm, 0.1 AUFS) Injection size: 100 µL (any injection size between 50 µL and 200 µL is acceptable as long as injection size remains constant within a chromatographic run.
Any single wave length between 235 mm and 260 mm may be used. The Spectra-Physics UV 2000 detector was used at 240 nm and 254 nm.	
Mobile Phase I (RH-5992)	Isocratic 54% Acetonitrile / Water
Mobile Phase II (RH-9886)	Isocratic 45% Acetonitrile / Water
Mobile Phase III (RH-0282)	Isocratic 30% Acetonitrile / Water
Mobile Phase IV (RH-2703)	Isocratic 45% Methanol / Water, 0.1% Acetic Acid (or PIC A)

PIC-A

Other HPLC instruments, columns, and combinations were used successfully during residue analysis. Any other combination of C-18 column and instrumentation brands could be used as long as a 0.01 µg/mL (or lower) standard can be quantified and no interferences exist. Variation of HPLC parameters are allowed to optimize any HPLC system.

E Preparation of Standards

(1) Stock solution

Weigh a known amount of analytical standard between 10 and 100 mg into a tared 50 mL beaker Dissolve the standard with several small portions of methanol, then carefully transfer the standard to a 100 mL volumetric flask and bring to volume. This stock standard is between 100 µg/mL and 1000 µg/mL depending on the actual weight and purity of the standard taken. Store frozen at -10 +/- 8 ° C Remake at one year intervals (Note It is important to maintain the relatively large volumes specified to produce the standards) Follow the procedure below for each standard At the working standard level, RH-5992 and any of the applicable metabolites may be combined in the same volumetric flask, especially for fortification purposes

(2) Intermediate Standard

A 10 µg/mL intermediate standard is made by taking an accurate volume of stock solution (1) to a precise volume with the appropriate HPLC solvent (or methanol for fortification purposes)

Working Standards

	Concentration	Take	Dilute to (with mobile phase)
(3)	1.0 µg/mL	10 mL of (2)*	100 mL
(4)	0,5 µg/mL	5 mL of (2)*	100 mL
(5)	0,1 µg/mL	10 mL of (3)	100 mL
(6)	0 05 µg/mL	5 mL of (3)	100 mL
(7)	0 02 µg/mL	2 mL of (3)	100 mL
(8)	0.01 µg/mL	1 mL of (3)	100 mL

All intermediate and working standards of all analytes except RH-9526 should be kept refrigerated and be remade at 6 month intervals RH-9526 intermediates and working standards (0 1 to 10 µg/mL) are used for fortification only and should be kept refrigerated and be remade at 3 month intervals

Note Analytical standard weights, volumes, and concentrations vary slightly from the specified parameters above Additional concentrations may be made as desired

* For these working standards, the intermediate solution (2) is assumed to be exactly 10 µg/mL

F. Analytical Procedure

1. Sample Processing

a) **Milk** samples are thawed and vigorously shaken for approximately two minutes prior to taking a 20 (± 0 1) gram aliquot for analysis.

b) **Liver** samples are first cut into small portions and then homogenized in a Hobart chopper with dry ice Dry ice is allowed to sublime in a freezer overnight and the samples are stored frozen until analysis A 10 (±0 1) gram sample is weighed for analysis

c) **Muscle or kidney** samples are first cut into small portions and then homogenized in a Hobart chopper with dry ice. The dry ice is allowed to sublime overnight in a freezer and the samples are stored frozen until analysis A 20 (± 0 1) gram sample is weighed for analysis

d) **Fat** samples are cut into smaller pieces while frozen, and then homogenized in a Hobart chopper with dry ice The dry ice is allowed to sublime, overnight in a freezer, and the samples are stored frozen until analysis A 10 (± 0 1) gram sample is weighed for analysis

2. Fortification of Samples

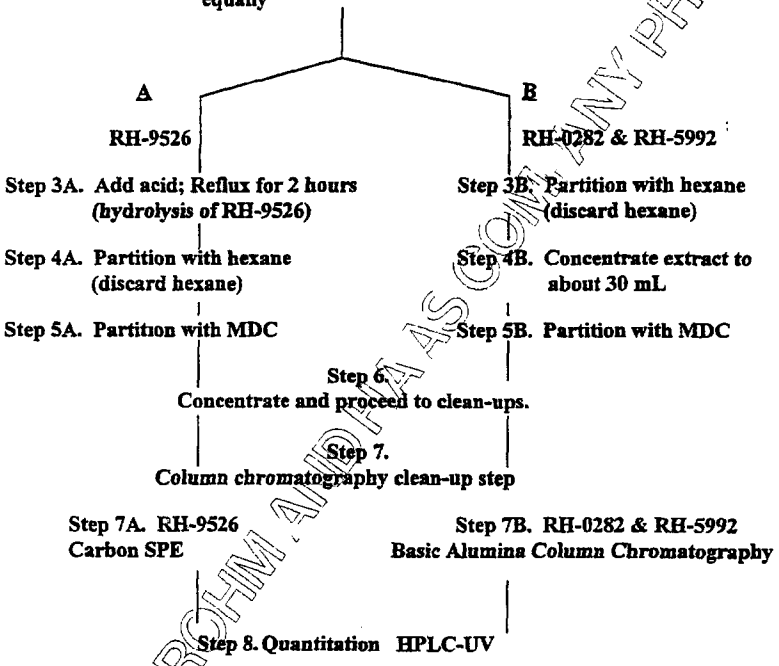
Control samples are fortified with the analytes of interest, after the samples have been weighed into their respective extraction or hydrolysis vessels

In the following sections, the flow diagrams and detailed steps for Milk (section 3), Liver (section 4), Meat i e , kidney and muscle (section 5), and Fat (section 6), are shown

3. Flow Diagram and Detailed Milk Analysis Steps

3.1. Milk Diagram

A flow diagram of the method is shown below The individual steps are detailed in Section 3 2 following the diagram



3.2. Detailed Milk Method

STEP 1 **Extraction**

Pipette 20 gram (+/- 0.1 g) of shaken milk into a blender jar Add about 5 grams of Celite filter aid and 80 mL of 90% methanol / water Blend at medium speed for about three minutes

STEP 2 **Filtration**

Filter under reduced pressure using a Buchner funnel with filter paper, rinsing the blender jar with several small portions of extraction solvent totaling 40 mL. Add the rinses over the filter cake Total volume of the filtrate will be approximately 140 mL Combine the extract and rinses and measure the volume Divide the extract into two equal parts A and B (approximately 70 mL each)

Note "A" steps refer to RH-9526 and "B" steps refer to RH-0282 & RH-5992

STEP 3A **Hydrolysis of RH-9526 to RH-9886**

Transfer aliquot A to a 300 mL 24/40 ST round bottom flask Add 66 mL of methanol, 26 mL of water and 15 mL of concentrated HCl Reflux for two hours, cool down solution and proceed to 4A The RH-9526 stearic acid conjugate has been hydrolyzed to its alcohol precursor RH-9886 (B-ring alcohol)

STEP 4A **Hexane Partition of the Hydrolysis mixture (RH-9886)**

Transfer the solution to a 500 mL separatory funnel Partition the hydrolysis reaction mixture with 50 mL of hexane (upper phase) to remove fatty acid ester contaminants Drain the lower phase into a second 500 mL separatory funnel and discard the upper phase, hexane Repeat the partition with a second 50 mL portion of hexane and discard the hexane

STEP 5A **Methylene Chloride Partition (RH-9886)**

Add 100 mL of 10% NaCl solution and 100 mL of methylene chloride Partition the residues into methylene chloride (lower phase), shaking for about one minute Drain the lower phase into a 300 mL round bottom 24/40 ST flask Repeat the partition with a second 100 mL of methylene chloride Combine the methylene chloride fractions in a 300 mL round 24/40 ST bottom flask Proceed to step 6 for concentration

STEP 3B **Hexane Partition (RH-5992 and RH-0282)**

Transfer the B portion from the extraction step to a 500 mL separatory funnel Add 8 mL of 10% NaCl solution and partition with 30 mL of hexane discarding the hexane

STEP 4B **Concentration of Extract Prior to Partitioning (RH-5992 and RH-0282)**

Transfer the extract to a 300 mL 24/40 ST round bottom flask Concentrate the remaining extract to between 20 and 25 mL by rotovap at about 65 °C, under reduced pressure **This is a critical step.** Note Round bottom flasks may be marked to the approximate volume required Another visual indicator is the first formation of water condensation in the rotovap bump trap These visual indicators may be used if RH-0282 recovery is normal If low RH-0282 recoveries occur, the analyst should measure the volume with a graduated cylinder

STEP 5B **Methylene Chloride Partition (RH-5992 and RH-0282)**

Transfer the extract to a 500 mL separatory funnel and rinse the round bottom flask with 3 mL of 90% methanol / water, adding the rinse to the separatory funnel Add 45 mL of 10% NaCl solution to the separatory funnel Rinse the round bottom flask with 150 mL of methylene chloride then add the methylene chloride to the separatory funnel Shake the separatory funnel vigorously for about one minute. After the phases have separated, drain the methylene chloride (lower phase) into a 300 mL 24/40 ST Next, repeat the partition with a 100 mL portion of methylene chloride and combine methylene chloride extracts in the round bottom flask

STEP 6 **Concentration**

Concentrate the methylene chloride from steps 5A and 5B at 40 °C-50 ° C (or under vacuum at lower temperature) to dryness with a rotary evaporator Proceed to step 7 for residue sample clean-ups

STEP 7A **Carbon Column SPE (RH-9886)**

Pre-packed or lab-packed carbon SPE tubes may be used, see sections III A & B Dissolve sample in 5 mL of 30% methanol / 70% water Prewash 6 mL carbon SPE tube with 5 mL of methanol, followed by 10 mL of 30% methanol / 70% water Discard washes Apply sample to the column Wash with 5 mL of 30% methanol / 70% water Wash with 5 mL of 50% methanol / 50% water Discard all washes Elute with 10 mL of 80% methanol / 20% water followed by 25 mL of 100% methanol collecting both fractions in a 100 mL (24/40 ST) round bottom flask Rotovap to dryness and take up sample with 10 mL of 45% acetonitrile / 55% water The sample is ready for HPLC quantitation

STEP 7B **Basic Alumina Column Chromatography (RH-5992 and RH-0282)**

Dissolve sample in 25 mL of ethyl acetate Place a small plug of glass wool or cotton into the bottom of 14.5 mm (id) × 25 cm (ht) chromatography column Measure 15 mL of basic alumina (packed) and pour into the column Add about 1 cm of sodium sulfate to the top of the column bed and then wash the column with 25 mL of ethyl acetate. Drain the ethyl acetate wash to about 2 mm above the column bed, do not let the column run dry until the final elution Apply the sample to the column **Note: Collect all eluents from the time sample is applied.** Elute sample with 150 mL of 20% methanol / 80% ethyl acetate collecting in a 300 mL (24/40 ST) round bottom flask

Rotovap to dryness and dissolve sample in 30% acetonitrile / 70% water The sample is ready for HPLC quantitation

Alternative Elution Scheme

It is possible at this point to separate the RH-5992 and RH-0282 on the alumina column if desired The column packing is the same, but the following elution pattern is used

Apply the sample to the column in 25 mL of ethyl acetate followed by 25 mL of 5% methanol / 95% ethyl acetate Both fractions are collected in a 300 mL 24/40 ST round bottom and will contain the RH-5992 residues Next, elute the RH-0282 residues with 100 mL of 20% methanol / 80% ethyl acetate, collecting in a 500 mL ST round bottom flask Each fraction is rotovaped to dryness and taken up in 5 mL of the appropriate mobile phase as listed under quantitation

STEP 8. Quantitation: HPLC with UV Detection

Quantitation of RH-5992

Mobile Phase I 54% Acetonitrile/46% Water
Flow 2 mL / min
Column Temperature 45 °C
Wavelength (240 nm, 0 1 AUFS)
Injection size 100 µL (50-200 µL)
Retention Time Adjust to between 5 5 and 8 minutes

Quantitation of RH-9526

Note RH-9526 is quantified as RH-9886

RH-9886 Mobile Phase II 45% Acetonitrile/55% Water
Flow 2 mL / min
Column Temperature 45 °C
Wavelength (240 nm or 254 nm, 0 1 AUFS)
Injection size 100 µL (50-200 µL)
Retention Time Adjust to between 4 and 6 minutes

Quantitation of RH-0282

RH-0282 Mobile Phase III 30% Acetonitrile/70% Water
Flow 2 mL / min
Column Temperature 45 °C
Wavelength (240 nm or 254 nm, 0 1 AUFS)
Injection size 100 µL (50-200 µL)
Retention Time Adjust to between 4 and 6 minutes

Alternative Gradient Quantitation Conditions RH-0282 & RH-5992

Flow 2 mL / min
Column Temperature 45 °C
Wavelength (240 nm or 254 nm, 0 1 AUFS)
Injection size 100 µL (50-200 µL)
Acetonitrile / Water Gradient

Time (min.)	% A (Acetonitrile)	%B (10% Acetonitrile / 90% Water)
0 0	17	83
8 0	17	83
13 0	42	58
30 0	42	58
32 0	17	83
40 0	17	83

4. Flow Diagram and Detailed Liver Analysis Steps

4.1. Liver Diagram

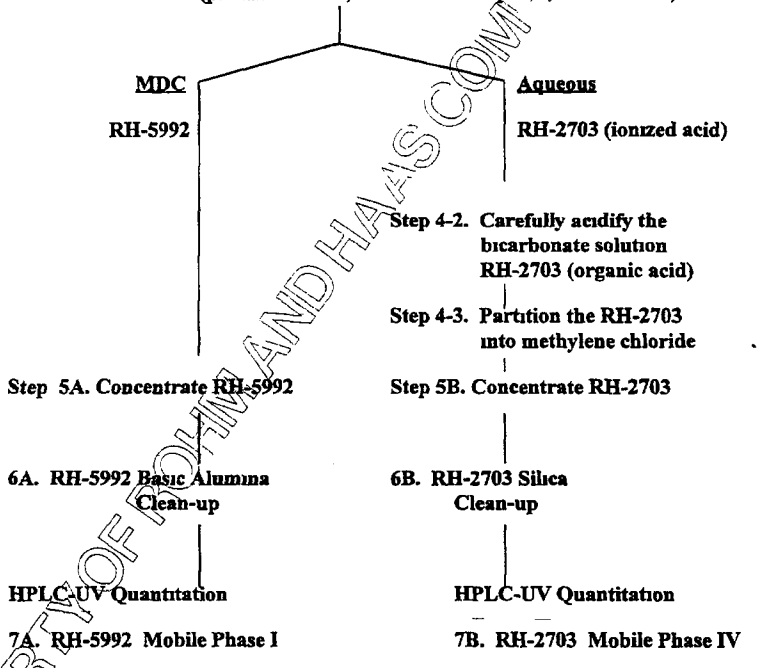
A flow diagram of the liver method is shown below The individual steps are detailed in Section 4 2

Step 1. **Homogenize (Polytron) 10 grams liver with extraction solvent and centrifuge**

Step 2. **Add Celite, vacuum filter and transfer to separatory funnel**

Step 3. **Methylene chloride partition of extract (contains parent and metabolite)**

Step 4-1. **Separation of Parent and Acid Metabolite Partition methylene chloride with aqueous sodium bicarbonate (parent in MDC; metabolite in aqueous bicarbonate)**



4.2 Detailed Liver Method

STEP 1 **Extraction and Centrifugation**

Weigh 10 grams (± 0.1 gram) of processed liver into a 250 mL centrifuge bottle containing 10 grams of solid sodium chloride. Any fresh fortifications would be spiked at this time. Add 75 mL of extraction solvent (70% Methanol / 30% 0.5 N HCl aqueous). Homogenize with a polytron at medium speed for about two minutes. Centrifuge at 5000 rpm and 4 °C for 20 minutes. Decant the supernatant to a 24/40 ST 500 mL round bottom flask. Add 35 mL of extraction solvent to the centrifuge bottle containing the pellet, shake for one minute and centrifuge as above. Decant the second supernatant into the same 500 mL round bottom flask containing the first extract. Proceed to step 2.

Alternative Extraction: Note the following alternative extraction procedure has been used successfully and allows the analyst to bypass the filtration step below. It requires less bench time and may be used at the analyst's discretion.

Weigh 10 grams (± 0.1 gram) of processed liver into a 250 mL centrifuge bottle containing 10 grams of solid sodium chloride. Any fresh fortifications would be spiked at this time. Add 75 mL of extraction solvent (70% Methanol / 30% of 0.5 N HCl aqueous). Homogenize with a tissue homogenizer at medium speed for about two minutes. Centrifuge at **2500 rpm for 10 minutes.** Pour the supernatant into a 500 mL separatory funnel. Add an additional **75 mL of extraction solvent to the centrifuge bottle and homogenize a second time.** Centrifuge at 2500 rpm for 10 minutes. Decant the second supernatant into the separatory funnel combining the extracts **and proceed to alternative step 3.**

STEP 2 **Filtration**

Add about 2 grams of Celite to the extract in the 500 mL round bottom flask. Filter with a Buchner funnel (Whatman Filter Paper #3, 7 cm) by vacuum, rinsing the round bottom flask with several small portions of extraction solvent totaling about 40 mL. Add the rinses over the filter cake. Total volume of the filtrate will be approximately 140 mL. Transfer the mixture to a 500 mL separatory funnel.

STEP 3 **Methylene Chloride Partition**

Add approximately 100 mL of 10% sodium chloride solution to the extract. Rinse the round bottom flask that held the extract with each portion of methylene chloride before adding it to the separatory funnel for the following partition. Partition the extract with 2 X 100 mL portions of methylene chloride shaking vigorously for about one minute for each partition. Drain both methylene chloride (lower phase) fractions into the same 500 mL separatory funnel. Total volume is approximately 200 mL. Proceed to step 4-1.

Alternative STEP 3: (Used with alternative extraction)

Do not add any sodium chloride solution Partition the extract twice with 100 mL portions of methylene chloride **The first** 100 mL of methylene chloride is added to the extract and shaken **vigorously for 1 minute** The mixture is allowed to separate and the lower phase is drained into a second **500 mL separatory** funnel The second 100 mL of partition of the extract is done by shaking **for one minute.** Allow the phases to separate and add the lower phase into the **second 500** mL separatory funnel combining the methylene chloride portions Proceed to **step 4-1**

STEP 4-1. Separation of Parent and Acid Metabolite

Add 50 mL of 10% sodium chloride solution Partition the methylene chloride with 100 mL of aqueous 1% sodium bicarbonate (parent remains in MDC, the acid metabolite in ionized form goes into the aqueous bicarbonate). Drain the methylene chloride (bottom layer) into a 24/40 ST 500 mL round bottom flask Proceed to step 5A with the methylene chloride Proceed to step 4-2 with the aqueous bicarbonate solution

Step 4-2. Acidification of the Aqueous Bicarbonate Fraction

Carefully acidify the bicarbonate solution with 1 N HCl to pH 1 (test with pH paper) which will convert the RH-2703 to its organic acid form **Caution The addition of acid will cause the release of carbon dioxide gas. Swirl the separatory funnel carefully to help expel the gas before partitioning in the next step** Proceed to step 4-3

Step 4-3. Methylene Chloride Partition

Partition the extract twice with 100 mL portions of methylene chloride (lower phase), shaking vigorously for about one minute for each partition Drain each methylene chloride fraction into the same 24/40 ST 500 mL round bottom flask Proceed to step 5B with the methylene chloride fraction containing RH-2703

STEP 5A Concentration of RH-5992 Fraction

Prior to concentration pass the methylene chloride from step 4-1 through a column filled with about 25 grams of anhydrous sodium sulfate collecting in a 500 mL round bottom flask Prerinse the column with about 25 mL of methylene chloride and discard Following sample elution, rinse the column with about 15 mL of methylene chloride and add this to the main eluent Concentrate the methylene chloride to dryness (40-55 °C) under vacuum with a rotary evaporator Proceed to step 6A, basic alumina clean-up

STEP 5B Concentration of RH-2703 Fraction

Concentrate the methylene chloride to dryness (40-55 °C) under vacuum with a rotary evaporator Proceed to step 6B, silica column clean-up

STEP 6A **Basic Alumina Column Clean-Up of RH-5992**

Basic alumina is used directly from the vendor bottle, non-activated. Place a small plug of glass wool or cotton into the bottom of 14.5 mm (id) × 25 cm (ht) chromatography column. Measure 15 mL of basic alumina (packed) and pour into the column. Add about 1 cm of sodium sulfate to the top of the column bed and then wash the column with 25 mL of hexane. Drain the hexane wash to about 2 mm above the column bed, do not let the column run dry until the final elution.

Dissolve the residue from step 5A in 25 mL of 5% ethyl acetate/ 95% hexane. Apply the sample to the column. Rinse the sample container with 10 mL of 5% ethyl acetate/ 95% hexane and add to the column after the initial 25 mL has drained to about 2 mm above the column bed. Discard these eluents. Wash the column with 50 mL of 10% ethyl acetate/ 90% hexane. Discard the eluent.

Elute the RH-5992 residues from the column with 150 mL (150 -200 mL is acceptable, see standardization of absorbents under Method Section A, Supplies) of 20% ethyl acetate/ 80% hexane, collecting the eluent in a 24/40 ST 300 mL round bottom flask. Concentrate the eluent to dryness with a rotary evaporator (45-55 °C) under vacuum. Take up the residue in an appropriate amount of mobile phase I (usually 10 mL) and put solution through a 0.45 µm filter disk, if necessary, prior to HPLC quantitation.

Optional Carbon SPE Clean-up

This clean-up may be used, if the samples are not sufficiently cleaned up with alumina. Dissolve samples in 30% methanol / 70% water (add 3 mL of methanol, followed by 7 mL of water). Prewash 6 mL carbon SPE tube with 5 mL of methanol, followed by 10 mL of 30% methanol. Discard washes. Apply sample to the column. Wash with 5 mL of 30% methanol. Wash with 5 mL of 50% methanol. Discard all washes. Elute with 10 mL of 80% methanol followed by 25 mL of 100% methanol collecting both fractions in a 100 mL (24/40 ST) round bottom flask. Rotovap to dryness with a rotary evaporator (45-55 °C) under vacuum and take up sample with 10 mL of mobile phase I.

STEP 6B **Silica Column Clean-Up of RH-2703**

Activate the silica gel (mesh 40-140) by heating for 24 hours at 200°C. Remove from the oven and store in tightly capped jars in a desiccator. Place a small plug of glass wool or cotton into the bottom of a 14.5 mm (id) × 25 cm (ht) chromatography column. Measure 20 mL of activated silica (packed) and pour into the column. (Note: Do not add sodium sulfate to the top of the column bed.) Dissolve the residue from step 5B with 1.5 mL of methanol followed by 23.5 mL of toluene. Sonicate the mixture to assure the residues are dissolved. Apply the sample to the column. Rinse the sample container with 25 mL of 5% methanol / 95% toluene and add to the column after the initial 25 mL has drained to about 2 mm above the column bed. Rinse the sample container with 25 mL of 10% methanol / 90% toluene and add to the column.

Wash the column with 25 mL of 25% methanol / 75% toluene. Discard the eluents. Elute the RH-2703 residues from the column with 85 mL of 35% methanol / 65% toluene, collecting the eluent in a 300 mL 24/40 ST round bottom flask.

Take up the residue in an appropriate amount of mobile phase IV (usually 5-10 mL) and put solution through a 0 45 µm filter disk, if necessary, prior to HPLC quantitation

Step 7 Quantitation: HPLC with UV Detection

Step 7A Quantitation of RH-5992

Mobile Phase I 54% Acetonitrile/46% Water
Flow 2 mL / min
Column Temperature 45 °C
Wavelength (240 nm, 0 1 AUFS)
Injection size 100 µL (50-200 µL)
Retention Time Adjust to between 6 and 8 minutes

Step 7B Quantitation of RH-2703

RH-2703 Mobile Phase IV 45% Methanol/Water; 0.1% Acetic Acid (or PIC A)
Flow 2 mL / min
Column Temperature 45 °C
Wavelength (240 nm or 254 nm, 0 1 AUFS)
Injection size 100 µL (50-200 µL)
Retention Time Adjust to between 5 and 7 minutes

5. Flow Diagram and Detailed Meat (Muscle / Kidney) Analysis Steps

5.1. Meat (Muscle / Kidney) Diagram

A flow diagram of the muscle/kidney method is shown below The individual steps are detailed in Section 5 2 following the diagram

Step 1

Blend 20 grams of meat for 2 minutes with 90 mL of 90% methanol / 10% of 0.1 N HCl and 5 grams of Celite

↓

Step 2

Filter the mixture, rinse the blender with 50 mL of extraction solvent, combine the washings and divide the filtrate equally into two aliquots

↓

A → **B Hold until analysis complete; discard**

↓

Step 3

Add 8 mL of 10% NaCl solution then partition with hexane, discard hexane

↓

Step 4

Concentrate extract to 20-25 mL

↓

Step 5

Add 45 mL of 10% NaCl solution and partition the residues twice into methylene chloride(150 mL then 100 mL of MDC)

↓

Step 6

Concentrate to dryness with a rotovap and take-up in 25 mL of ethyl acetate

↓

Step 7

Clean-up by basic alumina column chromatography

↓

Step 8

Clean-up with carbon SPE

↓

Step 9

Concentrate to dryness with a rotovap, reconstitute with mobile phase

↓

Step 10

Quantitation by HPLC with UV

5.2. Detailed Muscle/Kidney Method

STEP 1 **Extraction**

Weigh 20 grams (± 0 1 g) of meat into a blender jar containing 5 grams (± 1 g) of Celite filter aid Add 90 mL of 90% methanol / 10% of 0 1 N HCl (extraction solvent) and homogenize for 2 minutes at medium speed

STEP 2 **Filter and Rinse**

Filter the extract through a qualitative (Whatman #3) filter paper in a Buchner funnel under reduced pressure into a vacuum flask Rinse the blender with an additional 50 mL of extraction solvent, pouring the rinse over the filter cake Total volume of the filtrate will be approximately 140 mL Divide the extract into two equal parts A and B (approximately 70 mL each) Splitting the volume in half speeds up the concentration process in step 4

Note Portion "A" will be carried through the method until analysis is complete Portion "B" will be held until successful conclusion of the analysis, and then discarded If a sample were lost in an accident in one of the following steps, portion "B" could be carried through the method as a back-up Store in a refrigerator

STEP 3 **Hexane Partition**

Transfer portion A to a 500 mL separatory funnel Add 8 mL of 10% NaCl solution Partition the extract with 30 mL of hexane Transfer lower phase to a 300 mL (24/40 ST) round bottom flask Discard the hexane

STEP 4 **Concentration of Extract Prior to Partitioning (RH-5992 and RH-0282)**

Transfer the extract to a 300 mL 24/40 ST round bottom flask Concentrate the extract to between 20 and 25 mL by rotovap at about 65 °C, under reduced pressure **This is a critical step.** (Note Round bottom flasks may be marked to the approximate volume required) Another visual indicator is the first formation of water condensation in the rotovap bump trap These visual indicators may be used if RH-0282 recovery is normal If low RH-0282 recoveries occur, the analyst should measure the volume with a graduated cylinder If foaming occurs during this step with **kidney samples**, the final few milliliters of solvent may be removed by placing the flask in the water bath under a stream of nitrogen until the 20 to 25 mL volume is reached

STEP 5 **Methylene Chloride Partition**

Transfer the extract to a 500 mL separatory funnel and rinse the 300 mL round bottom flask with 3 mL of 90% methanol / 10% water, adding the rinse to the separatory funnel Add 45 mL of 10% NaCl solution and then partition with 150 mL of methylene chloride Next, partition with a second 100 mL portion of methylene chloride and combine both methylene chloride extracts in a 500 mL 24/40 ST round bottom flask The total volume of methylene chloride is approximately 200 mL

STEP 6 **Concentration**

Concentrate the methylene chloride to dryness with a rotary evaporator at 50 °C ± 5 °C (low or no vacuum). Take up the residues in 25 mL of ethyl acetate. Proceed to clean-up steps 7 and 8.

STEP 7 **Basic Alumina Clean-Up**:

Place a small plug of glass wool or cotton into the bottom of 14.5 mm (id) × 25 cm (ht) chromatography column. Measure 15 mL of basic alumina (packed) and pour into the column. Add about 1 cm of sodium sulfate to the top of the column bed and then wash the column with 25 mL of ethyl acetate. Drain the ethyl acetate wash to about 2 mm above the column bed. Do not let the column run dry until the final elution. Apply the sample to the column. **(Note: Collect all eluents from the time sample is applied.)** Elute sample with 150 mL of 20% methanol / 80% ethyl acetate collecting in a 300 mL (24/40 ST) round bottom flask. Rotovap to dryness with a rotary evaporator (45-55 °C) under vacuum. Samples will be taken up in 30% methanol / 70% water (add 3 mL of methanol, followed by 7 mL of water). Proceed to step 8.

STEP 8 **Carbon Column Clean-Up**

Prewash 6 mL carbon SPE tube with 5 mL of methanol, followed by 10 mL of 30% methanol. Discard washes. Apply sample to the column. Rinse the round bottom flask which contained the residues with 5 mL of 30% methanol / 70% water and then add to the SPE tube when the meniscus is near the top of the column bed. (Do not let column go dry). Wash with 5 mL of 50% methanol / 50% water. Discard all washes. Elute with 10 mL of 80% methanol / 20% water followed by 25 mL of methanol collecting both fractions in the same 100 mL 24/40 ST round bottom flask. Proceed to step 9.

STEP 9 **Concentration**

Concentrate the methanol to dryness with a rotary evaporator at 65 °C ± 5 °C (with vacuum). Take up the residues in 30% acetonitrile / 70% water for HPLC analysis. Proceed to step 10 for quantitation.

STEP 10. **Quantitation: HPLC with UV Detection**

The suggested mobile phases for the analytes are listed below. The analyst may adjust the percentages suggested to optimize the chromatography.

Step 10a **Quantitation of RH-5992**

Mobile Phase I: 54% Acetonitrile/46% Water
Flow: 2 mL / min.
Column Temperature: 45 °C
Wavelength: (240 nm, 0.1 AUFS)
Injection size: 100 µL (50-150 µL)
Retention Time: Adjust to between 5 and 8 minutes

Step 10b **Quantitation of RH-9886**

RH-9886 Mobile Phase II 45% Acetonitrile/55% Water
Flow 2 mL / min.
Column Temperature 45 °C
Wavelength (240 nm or 254 nm, 0 1 AUFS)
Injection size 100 µL (50-200 µL)
Retention Time Adjust to between 4 and 6 minutes

Step 10c. **Quantitation RH-0282**
RH-0282 Mobile Phase III 30% Acetonitrile/70% Water
Flow 2 mL / min
Column Temperature 45 °C
Wavelength (240 nm or 254 nm, 0 1 AUFS)
Injection size 100 µL (50-200 µL)
Retention Time Adjust to between 4 and 6 minutes

6. Flow Diagram and Detailed Fat Analysis Steps

6.1. Fat Diagram

A flow diagram of the fat method is shown below The individual steps are detailed in section 6 2

Step 1

Weigh 10 grams of fat into a 300 mL 24/40 ST round bottom flask, add 120 mL of methanol, 40 mL of water, and 15 mL of HCl .

↓

Step 2

Reflux the samples for 2 hours. Add 5 g acid washed Celite, filter mixture and transfer the extract to a 500 mL separatory funnel

↓

Step 3

Partition twice with hexane and discard hexane

↓

Step 4

Add sodium chloride solution and partition residues into methylene chloride

↓

Step 5

Partition the methylene chloride with aqueous sodium bicarbonate, discard water

↓

Step 6

Concentrate to dryness with a rotovap

↓

Step 7

Clean-up by carbon SPE chromatography

↓

Step 8

Clean-up basic alumina column chromatography

↓

Step 9

Concentrate to dryness with a rotovap and reconstitute with mobile phase

↓

Step 10

Quantitation by HPLC with UV detection

6.2. Detailed Fat Method

STEP 1 **Extraction**

Weigh 10 (± 0 1 gram) grams of homogenized fat sample into a 300 mL round bottom flask 24/40 ST Fortifications to controls may be made at this point Add 120 mL of methanol, 40 mL of water, and 15 mL of concentrated HCl

STEP 2 **Hydrolysis of RH-9526 to RH-9886**

Reflux for two hours The RH-9526 stearic acid conjugate has been converted to its alcohol precursor RH-9886 (B-ring alcohol) Allow hydrolysis mixture to cool Add 5 g of Celite and filter the mixture under vacuum in a Buchner funnel through a Whatman #3 filter paper Proceed to step 3

Alternatively, the mixture can be cooled in a refrigerator for approximately 45 minutes (or freezer for 10 minutes) and then allowed to proceed directly to step 3 without adding Celite or filtering

STEP 3 **Hexane Partition of the Hydrolysis mixture**

Transfer the extract to a 500 mL separatory funnel Partition the hydrolysis reaction mixture twice with 50 mL of hexane to remove fat, fatty acid esters, and other non-polar contaminants The upper hexane phase is discarded The lower phase extract may be drained into a second 500 mL separatory funnel or held in a temporary vessel until the hexane is discarded

STEP 4 **Methylene Chloride (MDC) Partition**

Add 100 mL of 10% sodium chloride solution to the extract in the 500 mL separatory funnel Partition the extract with two separate 100 mL portions of methylene chloride Shake vigorously for about one minute and allow the phases to separate MDC is the lower phase Combine the two MDC portions in a 300 mL round bottom and proceed to step 5 (Note " If a persistent emulsion forms the samples may be centrifuged at about 2000 rpm for approximately 5 minutes to break it)

STEP 5 **Sodium Bicarbonate Partition**

Partition the combined MDC extracts with 100 mL of 1% sodium bicarbonate solution Discard the aqueous sodium bicarbonate (upper phase)

STEP 6 **Concentration**

Concentrate the methylene chloride at about 40 °C to dryness under vacuum with a rotary evaporator Proceed to step 7 for carbon SPE clean-up

STEP 7 **Carbon SPE**

Prewash a 6 mL carbon SPE tube (see section A, supplies) with 5 mL of methanol followed by 10 mL of 30% methanol / 70% water Dissolve residue from step 6 in 10 mL of 30% methanol / 70% water Apply to the SPE tube followed by a 5 mL 30% methanol / 70% water wash Wash the column consecutively with 5 mL portions of 50% methanol / 50% water Discard all washes Elute the residues with 10 mL 80% methanol / 20% water, followed by 25 mL of methanol Collect and combine both fractions Transfer solution to a 100 mL 24/40 ST round bottom flask and rotovap to dryness Take up residues in 25 mL of 20% ethyl acetate / 80% hexane Proceed to step 8

STEP 8 **Basic Alumina Column Chromatography**

Place a small plug of glass wool or cotton into the bottom of 19 mm (id) × 25 cm (ht) chromatography column Measure 15 mL of basic alumina (packed) and pour into the column Add about 1 cm of sodium sulfate to the top of the column bed and then precondition the column with 25 mL of 15% methanol / 85% ethyl acetate, followed by 30 mL of hexane Drain the hexane wash to about 2 mm above the column bed Do not let the column run dry until the final elution Apply the 25 mL sample to the column **(Note: Collect all eluents from the time the sample is applied.)** Continue to elute RH-5992 residues with an additional 45 mL of 20% ethyl acetate / 80% hexane collecting in a 300 mL (24/40 ST) round bottom flask

Elute the RH-9886 residues with 100 mL of 15% methanol / 85% ethyl acetate Collect the eluent in a separate 300 mL 24/40 ST round bottom flask Proceed to step 9

STEP 9 **Concentration**

Concentrate both eluents at 50 °C to dryness under vacuum with a rotary evaporator Take up RH-5992 residues in approximately 10 mL of 54% acetonitrile / 46% water Take up RH-9886 residues in approximately 10 mL of 45% acetonitrile / 55% water Both final solutions may need to be filtered through a 0 45 µm Acrodisc especially if the hydrolysis mixture is not filtered (alternative procedure) in step 2 Proceed to step 10 for HPLC quantitation

Step 10 **Quantitation HPLC with UV Detection**

Step 10a Quantitation RH-5992
Mobile Phase I
54% Acetonitrile/46% Water
Flow 2 mL / min
Column Temperature 45 °C
Wavelength (240 nm, 0 1 AUFS)
Injection size 100 µL (50-200 µL)
Retention Time Adjust to between 5 and 8 minutes

Step 10b Quantitation RH-9886
Mobile Phase II
45% Acetonitrile/55% Water
Flow 2 mL / min
Column Temperature 45 °C
Wavelength (240 nm, 0 1 AUFS)
Injection size 100 µL (50-200 µL)
Retention Time Adjust to between 4 and 6 minutes

IV. High Performance Liquid Chromatography Quantitation

A. Standards

A minimum of four standard solutions are prepared in the concentration range of 0 005 µl/mL to 2 0 µ/mL A typical set of standards would consist of concentrations from 0 01 µg/mL (or 0 005 µg/mL) to 0.5 µg/mL

Inject samples at the same volume (between 50-200 µL) as RH-5992 or metabolite standards If necessary, the samples are diluted to an appropriate volume to give a response within the standard curve range

Standards and samples may be quantitated by peak height or peak area, but the response type used must be consistent within a run An HPLC run should have at least two sets of standards A standard curve is constructed from each day's standards

Equation 1 residue concentration (all analytes except RH-9526)

$$\frac{\text{component concentration (µg/mL)} \times \text{final volume (mL)}}{\text{sample weight (g)}} = \text{ppm}$$

Note For milk and meat (muscle or kidney) samples a 20 gram sample is initially taken and then the samples are split in two The sample weight in the equation above would therefore be 10 g (for an initially weighed 20 gram sample) or one-half of any other initial weight All other samples weights for the other matrices would be 10 grams if run as specified or the initial weight taken for the sample

RH-9526 and other fatty acid conjugates of RH-9886 are converted to RH-9886 and reported as ppm of RH-9886

Equation 1a residue concentration RH-9526

$$\frac{\text{concentration RH-9886 (µg/mL)} \times \text{final volume (mL)}}{\text{sample weight(g)}} = \text{ppm as RH-9886}$$

B. Fortification Recovery

Control samples are fortified with known amounts of RH-5992 or metabolites prior to extraction. For RH-9526 fortification, µg fortified of RH-9526 are multiplied by the molecular weight factor, 0 58 (368/635), to find the equivalent µg of RH-9886

Equation 2 fortification recovery

$$\frac{[\text{found (µg/mL)} \times \text{final vol. (mL)}]}{\text{fortification amount (µg)} \times (\text{\% aliquot} / 100)} = \text{\% Recovery}$$

EPA ADDENDUM

ACB #B99-28-30, PP#7F4815

TMV for Tebufenozide in/on Milk and Beef Liver

1) ACB recommends that when making low concentrations from stock solutions that the diluting solution be no less than 60% water/Meoh. It is recommended that the final sample solutions also be 60% water/40% Meoh. ACB concludes that the matrix impurities cause split peaks for RH-0282 and RH-2703 and finds the 60/40 an ideal mixture.

2) ACB recommends that users incorporate step 6A, page 27, "Optional Carbon SPE Clean-up".