1. Introduction

RH-7988 is the active ingredient of Aphistar, also known as triazamate or Aphistar 50WSP Systemic Aphicide, which has been developed for the selective control of aphids in apples, pears, and leafy vegetables, etc. Metabolism studies of RH-7988 (RH-57988) in soil (references 1, 2, 3, and 4) have shown the residue to contain the parent molecule and five metabolites, referred to here as RH-0422 (RH-70422), RH-7280 (RH-87280), RH-0131 (RH-100131), RH-3008 (RH-123008), and RH-9983 (RH-89983). The structures of these are shown in Figure 1.

The analytical method described in this report determines the amount of RH-7988 and its five metabolites in soil, using a multi analyte method, where all of the analytes are extracted together and quantified during a single run. The method is summarized in the flow diagram depicted in page 19. The method was developed based upon the preliminary method (reference 5) which analyzed RH-7988 and four soil metabolites (RH-0422, RH-7280, RH-0131, and RH-3008). The current method has been modified slightly to allow for the determination of the fifth metabolite, RH-9983. The accuracy of this analytical method is estimated based on the recovery of known levels of RH-7988 and its metabolites (RH-0422, RH-7280, RH-0131, RH-3008, and RH-9983) fortified in control soil samples.

This report also contains summary data from a radiovalidation of this method (reference 6).

2. Summary

This report details the residue analytical method for RH-7988 and five soil metabolites (RH-0422, RH-7280, RH-0131, RH-3008, and RH-9983) using high performance liquid chromatography with MS/MS detection. The preliminary analytical method for the analysis of RH-7988 and its soil metabolites is detailed in TR 34-96-90 (reference 5). In this preliminary method, RH-7988 and only four metabolites (RH-0422, RH-7280, RH-0131, and RH-3008) were analyzed. During subsequent metabolism studies, a fifth soil metabolite, RH-9983, was identified. The current analytical method detailed in this report analyzes residues of RH-7988, the four soil metabolites (RH-0422, RH-7280, RH-3008, and RH-0131), and also the recently-identified RH-9983, in a single run.

Residues of the test substance (RH-7988) and its soil metabolites (RH-0422, RH-7280, RH-0131, RH-3008, and RH-9983) are extracted from soil by homogenizing and shaking with methanol/formic acid. The extract is concentrated, resuspended in an ammonium formate buffer, and passed through a Sephadex G-50 gel filtration column. For LC/MS/MS analysis, methanol is added to the eluant containing RH-7988 and its metabolites. A 15 cm x 4.6 mm

Keystone Prism HPLC column is used to separate the analytes, using a gradient elution profile. An ion spray interface is used to introduce the HPLC eluant to the mass spectrometer. The analytes are detected in the triple quadrupole mode (MS/MS) by monitoring characteristic daughter ions resulting from passage of their parent molecular ions through the first quadrupole (Q1) into the collision cell (Q2), where fragmentation of the parent ions occur following collision with argon gas, with the resulting fragments separated in the second mass analyzing quadrupole (Q3). The structures of the analytes are given in Figure 1.

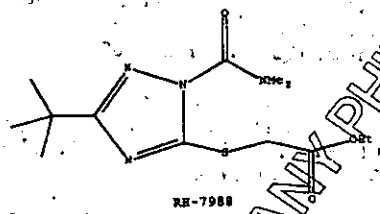
The limit of quantification (the lowest fortification specified by the method which gives adequate recovery according to EPA guidelines) for LC/MS/MS analysis is 0.010 ppm for RH-7988 and RH-0422, and 0.020 ppm for RH-7280, RH-0131, RH-3008, and RH-9983.

The limit of detection (LOD) by LC/MS/MS (equivalent to 30% of the limit of quantification) is 0.003 ppm for RH-7988 and RH-0422, and 0.006 ppm for RH-7280, RH-0131, RH-3008, and RH-9983.

3. **Experimental Compounds**

Figure 1. Molecular Structures of RH-7988 and Metabolites

RH-7988 (RH-57988)



RH-7988

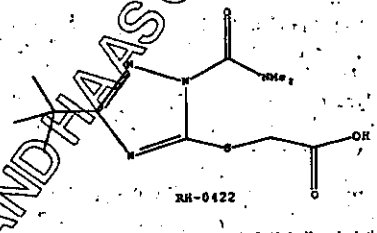
Chemical name = 1-[(dimethylaminocarbonyl)-3-(1,1-dimethylethyl)-1H-1,2,4-triazol-5-yl]thioacetic acid, ethyl ester

Common name = triazamate

CAS # 112143-82-5

Soil Metabolites:

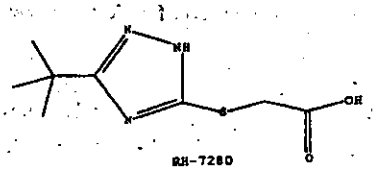
RH-0422 (RH-70422)



RH-0422

Chemical name = 1-[(dimethylaminocarbonyl)-3-(1,1-dimethylethyl)-1H-1,2,4-triazol-5-yl]thioacetic acid

RH-7280 (RH-87280)



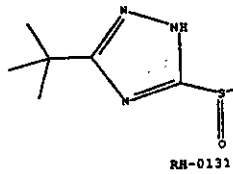
RH-7280

Chemical name = 3-[(1,1-dimethylethyl)-1H-1,2,4-triazol-5-yl]thioacetic acid

(cont'd)

Figure 1. Molecular Structures of RH-7988 and Metabolites, cont'd

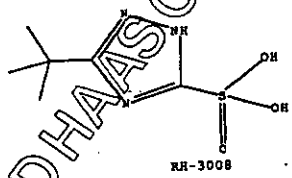
RH-0131 (RH-100131)



RH-0131

Chemical name = 3-[(1,1-dimethylethyl)-1H-1,2,4-triazol-5-yl]-S-oxo-thioacetic acid

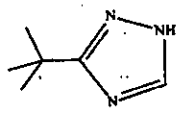
RH-3008 (RH-123008)



RH-3008

Chemical name = 3-[(1,1-dimethylethyl)-1H-1,2,4-triazol-5-yl]sulfonic acid

RH-9983 (RH-89983)



RH-9983

Chemical name = 3- (1,1-Dimethylethyl)-1H-1,2,4-triazole

4. Chemicals and Supplies

4.1 Chemicals	Grade	Source
Methanol	HPLC	Baker
Formic Acid	Reagent	EM Science
Ammonium Formate	Reagent	Aldrich Chemical
Sephadex G-50		Sigma
Water	HPLC	Baker
Acetonitrile	HPLC	Baker
Poly(propylene glycol) [PPG] 2000	Reagent	Aldrich
Poly(propylene glycol) [PPG] 1000	Reagent	Aldrich
Poly(propylene glycol) [PPG] 425	Reagent	Aldrich

4.2 Standards*	Source
RH-57988	Rohm & Haas
RH-70422	Rohm & Haas
RH-87280	Rohm & Haas
RH-100131	Rohm & Haas
RH-123008	Rohm & Haas
RH-89983	Rohm & Haas

* These numbers are the full RH-numbers for the compounds, however, they are frequently referred to by the abbreviated four-digit designations RH-7988, RH-0422, RH-7280, RH-0131, RH-3008, and RH-9983.

4.3 Equipment and Supplies	Source
Balance	Mettler
Centrifuge bottles, 150 mL	Nalgene
Hobart Food Processor	Hobart
Mechanical shaker	Eberbach
Bench-top centrifuge (model HN-II)	IEC
Filter for Mobile Phase Preparation (Nylon 66 membrane 0.45 µm x 47 mm)	Supelco
Round-bottom flask, 250 mL	Pyrex
Centrifuge Tube, 50 mL, Pear Shaped	Kimble
Rotary Evaporator	Buchi
Graduated Centrifuge Tube, 15 mL	Kontes
PD-10 Empty Disposable Column	Supelco
Visiprep Vacuum Manifold	Supelco

(cont'd)

Sonicator Fisher Scientific
Standard Lab Equipment
(beakers, pipets, volumetric flasks, etc.)

Note: Equivalent materials may be substituted for those specified in this method. These substitutions, however, must be demonstrated to produce satisfactory results.

4.4 Solutions

Solution A:

0.1% formic acid in 6 mM ammonium formate.
Dissolve 0.378 g ammonium formate in 1 L of type I water.
Add 1 mL formic acid.
The pH of this solution is approx. 3.

Solution B:

0.1% formic acid in acetonitrile (mobile phase "A").
Add 1 mL of formic acid to 1 liter of acetonitrile.

Solution C:

200 mM ammonium formate.
Dissolve 12.6 g ammonium formate in approximately 800 mL Type I water.
Adjust volume to 1 liter.

Solution D:

0.1% formic acid in 4 mM ammonium formate (mobile phase"B").
Add 20 mL of solution C to 980 mL of type I water.
Add 3 ml formic acid.

LC/MS/MS Tuning Solution (PPG)

Add : 0.40 g of PPG 2000 solution,
0.10 g of PPG 1000 solution,
14 mg of PPG 425 solution,
126 mg ammonium formate,
1 mL acetonitrile, and adjust volume to 1L with 50% methanol in water

4.5 Preparation of Standards and Fortification Solutions

Analytical standards are prepared for two purposes. They are used for fortifying untreated samples to determine analytical recovery and also to calibrate the response of the detector used in the analysis.

The absolute volumes of the standards may be varied by the analyst as long as the correct proportions of solute to solvent are maintained.

1. Stock Solution

To prepare stock standard solutions of 100 µg/mL, weigh 10 mg of analytical standard (corrected for purity) and bring up to 100 mL with methanol in 100 mL volumetric flask. Prepare separate solutions for each of the five analytes. Store all stock standard solutions in a freezer at -10°C or colder, in glass bottles with teflon-lined caps only. These stock standard solutions should be replaced within one year from the date of preparation.

2. Fortification Solutions

To prepare a mixed fortification solution of 0.5 µg/mL of RH-7988 and RH-0422 and 1.0 µg/mL each of RH-7280, RH-0131, and RH-3008, add 0.5 mL each from the 100 µg/ml stock solutions of RH-7988 and RH-0422 and 1.0 mL each from the 100 µg/mL stock solutions of RH-7280, RH-0131, RH-3008, and RH-9983 into a single 100 mL volumetric flask, and adjust volume to 100 mL with methanol/ Solution A (40:60) v/v.

Note: 1 mL of this solution spiked in 10 g of soil is equivalent to 0.05 ppm of RH-7988 and RH-0422 and 0.1 ppm of RH-7280, RH-0131, RH-3008, and RH-9983. Smaller volumes of this solution can be used to prepare lower fortification levels.

Store all fortification standard solutions in a refrigerator at 2 to 6°C for a maximum period of three months from the date of preparation, after which, make new standards using the stock solutions.

3. Calibration Standards

A 5-point LC/MS/MS calibration standard is prepared in methanol/solution A (40:60) via serial dilution of the fortification solution (0.5 µg/mL each of RH-7988 and RH-0422, and 1.0 µg/mL each of RH-7280, RH-0131, RH-3008, and RH-9983). Typical, standard concentrations used are 1.25, 2.5, 5, 10, and 25 ng/mL each of RH-7988 and RH-0422 and 2.5, 5, 10, 20, and 50 ng/mL each of RH-7280, RH-0131, RH-3008, and RH-9983..

The following is a typical example:

Initial Conc. (µg/mL)*	Volume (mL)	Diluted to (mL)	Final Conc. (µg/mL)*
0.5/1.0	10	100	0.05/0.1
0.05/0.1	50	100	0.025/0.05
0.025/0.05	40	100	0.01/0.02
0.01/0.02	50	100	0.005/0.01
0.005/0.01	50	100	0.0025/0.005
0.0025/0.005	50	100	0.00125/0.0025

* RH-7988 and RH-0422 / RH-7280, RH-0131, RH-3008, RH-9983

Store all calibration standard solutions in a refrigerator at 2 to 6°C for a maximum period of three months from the date of preparation, after which, make new standards using the stock solutions.

5. **Instrumentation**

5.1 LC/MS/MS

Mass Spectrometer:	PE Sciex API-III, serial no. 124930381 Ionspray Liquid Introduction Interface Interface heater temperature: 65°C Air Nebulizer gas flow: 0.8 L/min N_2 curtain gas flow: 0.8 L/min Harvard infusion pump
Computer:	Apple Macintosh Quadra 950

Software:	(1) Macintosh system 7.1 (2) PE Sciex Tune 2.5 (3) PE Sciex RAD 2.6 (4) PE Sciex Macspec 3.3 (5) PE Sciex MacQuan 1.4
HPLC:	Shimadzu SIL-10A Auto Injector Shimadzu LC-10AD gradient pumps Shimadzu CTO-10A column oven Shimadzu SCL-10A system controller
Column:	Prism RP, 15 cm x 4.6 mm, 5 µm (Keystone Scientific Inc., part. no. 155-321)
Column Temperature:	35°C
Mobile Phase A:	0.1% formic acid in acetonitrile
Mobile Phase B:	0.3% formic acid in 4 mM ammonium formate
Flow Rate:	1.0 mL/min
Split Ratio:	~15:1
Injected Volume:	100 µL

Mobile Phase Program:

Time (min)	%A	%B
0	20	80
10	50	50
18	95	5
21	95	5
22	20	80
27	stop	

Total Run Time: 27 minutes

Analyte Retention Times:

Analyte	Retention Time (min)
RH-9983	2.6
RH-0131	5.2
RH-7280	5.4
RH-3008	8.9
RH-0422	11.7
RH-7988	14.6

5.2 Calibration and Standardization:

Calibrate and tune the mass spectrometer on a daily basis prior to analyzing samples. Both mass analyzing quadrupoles (Q1 and Q3) have

to be calibrated each time. This is done by infusing a standard solution of poly(propylene glycol) [PPG] into the mass spectrometer using the ion spray interface, while monitoring positive ions. A typical mass calibration tune with PPG for both quadrupoles (Q1 and Q3) is shown in Figure 2.

5.3 Analysis

Analysis is performed using five "periods", each represented by a separate state file. These state files (corresponding to the first, second, third, fourth, and fifth period) are optimized for the analysis of RH-9983, RH-7280 and RH-0131 (one period), RH-3008, RH-0422, and RH-7988, respectively.

Analytes (1 to 2.5 µg/mL dissolved in 40% acetonitrile containing 4 mM ammonium formate and 0.1% formic acid) are also infused using the ion spray interface to optimize parameters for both parent and daughter ions. The parent ion scans (MS) and the their daughter ion profiles (MS/MS) for all five analytes are shown in Figures 3 to 7.

The analytes are detected in the triple quadrupole mode (MS/MS) by monitoring characteristic daughter ions resulting from passage of their parent molecular ions through the first quadrupole (Q1) into the collision cell (Q2), where fragmentation of the parent ions occur, with the resulting fragments separated in the second mass analyzing quadrupole (Q3). The mass-charge ratios of the parent ions and the monitored daughter ions are given below.

Analyte	Ionization Mode	Parent Ion Monitored (m/z)	Daughter Ion Monitored (m/z)	Retention Time (min)
RH-3008	negative ion	204	124	8.9
RH-7280	negative ion	214	155	5.4
RH-0131	negative ion	230	171	5.2
RH-0422	positive ion	287	198	11.7
RH-7988	positive ion	315	198	14.6
RH-9983	positive ion	126	70	2.6

Note: Slight variations in the retention times could occur with each batch of mobile phase.

Typical state file parameters for PPG and for the three periods of analyte monitoring are given in Table 1. Data acquisition is performed by the RAD software program. Typical RAD data acquisition parameters are given in Table 2.

6. Method

6.1 Flow Diagram

The flow diagram of the method is given below, followed by a detailed description of each step.

Method Flow Diagram

Weigh 10 g of sample (soil)

↓

Add formic acid and methanol, shake overnight (at least 15 hours)

↓

Centrifuge sample, decant, and rotovap

↓

Pass 2.5 mL of sample through Sephadex G-50 gel filtration column

↓

Concentrate, re-dissolve in methanol/solution A (40:60)

↓

Analysis by LC/MS/MS

6.2 Sample Processing

Chop the frozen soil samples into smaller pieces with a cleaver and rubber mallet or by any other appropriate means. Remove stones and debris as found. Add the smaller pieces of frozen soil into a Hobart Food Chopper bowl which has been pre-chilled with ice. Thoroughly chop the combined soil and homogenize with dry ice. Allow the dry ice to sublime in a freezer overnight and store frozen at -10°C or colder, until analysis.

6.3 Extraction

1. Weigh 10 g of homogenized soil into a 150 mL centrifuge bottle.
2. Fortifications should be made at this point.
3. Add 10 mL of 0.5% formic acid in water.
4. Let stand for 2-3 minutes.
5. Add 90 mL methanol.
6. Cap tightly and shake overnight (at least 15 hours) in a mechanical shaker.
7. Centrifuge at approximately 2000 rpm in a bench-top model centrifuge (swing bucket rotor) for 10 to 15 minutes.
8. Carefully decant the supernatant into a 250 mL round-bottom flask.
9. Add 50 mL 0.05% formic acid in methanol to the extracted soil and sonicate for 10 minutes. Centrifuge and add the supernatant to the same round-bottom flask.
10. Concentrate down to approximately 1 to 2 mL in a rotary evaporator under vacuum, at 35 to 40°C.
11. Decant carefully into a graduated 15 mL centrifuge tube.
12. Add 3 to 5 mL of solution A to the 250-mL round bottom flask, swirl and add this solution to the graduated centrifuge tube. Repeat until the volume is 10 mL. Mix well using a Pasteur pipet.

6.4 Gel filtration (clean-up)

6.4.1 Preparation of Sephadex G-50 Gel Filtration Column

1. Weigh 10 g of Sephadex G-50 in a 200 mL beaker.
2. Add 125 ml Type I water. Allow the gel to swell for at least 3 hours.
3. Place a frit at the bottom of the PD-10 column and attach on to a solid phase extraction device.
4. Add 11 to 12 mL of the swollen gel per column.
5. Allow the extra water to drain.
6. Make sure that the column is packed up to the mark on the PD-10 column; add more gel if needed. No vacuum is necessary for packing the column.
7. Position another frit at the top of the column. The columns should not run dry.

8. To store columns, cap the tops and the bottoms. Caps are also provided with the columns. Store columns at room temperature for immediate use, or store in refrigerator for use within 1 to 2 weeks.

6.4.2 Gel Filtration

1. Condition the Sephadex G-50 columns by adding 10 mL of solution A and drain to the top of the frit. Use of vacuum might be needed.
2. Add 2.5 mL of the sample (make sure the 10 mL sample is mixed well) and allow it to drain to the top of the frit.
3. Wash the column with 3.5 mL of solution A. Discard the wash.
4. Elute the compounds with 6 mL of solution A and collect.
5. Add 4 mL of methanol and to make the final volume 10 mL.

6.5 Analysis by LC/MS/MS

Inject a 100 µL aliquot of each six-component calibration standard in the range of 0.0025 to 0.025 µg/mL each of RH-7988 and RH-0422 and 0.005 to 0.05 µg/mL each of RH-7280, RH-0131, RH-3008, and RH-9983 into the LC/MS/MS. Standard curves (for each analyte) are constructed by linear regression using the MacQuan software for each set of analysis.

Inject a 100 µL aliquot of each sample/fortification/control into the LC/MS/MS. The concentration of each sample/fortification/blank is determined from the standard curve, based on the peak area of each analyte.

If necessary, dilute the samples to give a response within the standard curve range.

Note: The extraction results in a dilution factor of 2 (2.5 mL of the 10 mL extracted is passed through the Sephadex column and the eluant volume is adjusted to 5 mL). Therefore, the final volume will be 20 mL.

6.6 Time required for Analysis

A set of eight samples (including blanks, fortifications and actual samples) can be carried through the entire analytical procedure

within two working days, a total time of 16 hours. This excludes the "overnight" shaking of samples. The LC/MS/MS run time for a set is approximately eight hours.

7. Calculations

Within an analytical set, all extracts (samples, method blanks, and procedural recovery spikes) were bracketed with a five level calibration curve. Generally, two to four extracts were run between standards.

Standard curves for LC/MS/MS analysis were built with MacQuan program (Version 1.3).

The RH-7988, RH-0422, RH-3008, RH-7280, RH-0133, and RH-9983 residue concentrations are determined as follows:

7.1 Fortification Recovery

For samples fortified with known amounts of analytes prior to extraction, measure the peak area (this is done by MacQuan software), determine the concentrations (µg/mL) from the standard curves, and calculate the percent recovery from Equation 1.

Equation 1

$$\left(\frac{\left[\text{µg/mL Found} \times \text{Total Sample Volume (mL)}\right] - \text{Control correction}}{\text{Fortification (µg)}}\right) \times 100 = \%\,\text{Recovery}$$

NOTE:
Total sample vol. (mL) = Total volume before clean-up (mL) x dilution factor after clean-up

7.2 **Component Residue Concentration**

Determine the component residue concentration as follows:

Equation 2

$$\frac{\mu g/mL \text{ Found} \times \text{Total Sample Volume (mL)}}{\text{Sample Weight (g)}} = ppm$$

NOTE:
Total sample vol. (mL) = Total volume before clean-up (mL) x dilution factor after clean-up

Table 1. Typical State File Values for PPG Calibration and for the Five Periods of Analyte Monitoring.

Parameter	Q1 PPG Calibration	Q3 PPG Calibration	MS/MS Period 1	MS/MS Period 2	MS/MS Period 3	MS/MS Period 4	MS/MS Period 5
DI	*	*	*	*	*	*	*
ISV	5000	5000	5000	-3800	-3800	5000	5000
IN	650	650	650	-650	-650	650	650
OR	50	50	60	-55	-80	40	40
RO	30	30	30	-30	-30	30	30
M1	1000	1000	200	200	200	200	200
RE1	126.6	126.6	126.6	126.6	126.6	126.6	126.6
DM1	0.13	0.13	0.14	0.14	0.14	0.14	0.14
R1	28.5	28.5	27	-28	-27.2	25	25
L7	-49	28	17	15	-14	17	17
R2	-60	29	10	-10	-10	14	14
M3	1000	1000	200	200	200	200	200
RE3	124.1	122.0	122.0	122.0	122.0	122.0	122.0
DM3	0.11	0.13	0.13	0.13	0.13	0.13	0.13
RX	-10	-28	5	-6	-6	9	9
R3	-33	28	5	-1	-6	5	5
L9	-250	-250	100	205	205	-195	-195
FP	-250	-250	-100	220	220	-205	-205
MU	-3600	-3600	-3600	3600	3600	-3600	-3600
CC	10	10	1	1	1	1	1
CGT	Off	Off	345	345	345	345	345

* value not applicable for the ion spray interface.

Note : State file values will often be changed slightly on a daily basis during instrument optimization procedures.

****The method has been modified from TR 34-96-90 to accommodate the analysis of 6 analytes. This table reflects those modifications.**

Table 2. Typical RAD Data Acquisition Parameters.

	Period 1	Period 2	Period 3	Period 4	Period 5
Scan type	MRM	MRM	MRM	MRM	MRM
Delay	0.05	0.05	0.05	0.05	0.05
Acquire	3.5 min.	3 min.	3 min.	3 min.	3 min.
Scan rate	0.77	0.33	0.33	0.67	0.67
Dwell time	650 ms	1000 ms	1500 ms	750 ms	750 ms
Pause time	0.02 ms	0.02 ms	0.02 ms	0.02 ms	0.02 ms
Q1 mass	126	214 230	204	287	315
Q3 mass	70	155 171	124	198	198

NOTE : The delay time, acquire time, scan rate, and dwell time are given as an example, and may need to be optimized based on any changes in the retention times, chromatography, and instrument sensitivity/performance.

****The method has been modified from TR 34-96-90 to accommodate the analysis of 6 analytes. This table reflects those modifications.**