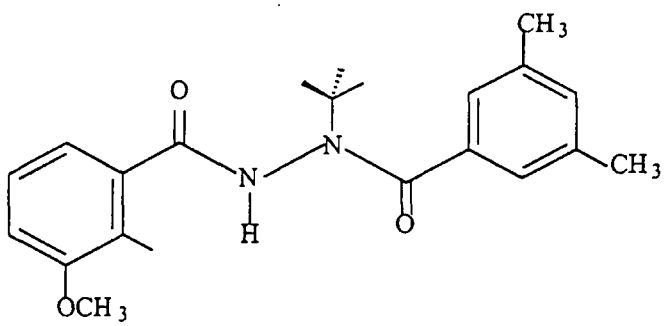
3. Experimental Compound

The structure of RH-2485 is shown below:



Rohm and Haas ID: RH-2485* (or RH-112485)
Chemical Name: benzoic acid, 3-methoxy-2-methyl-2-(3,5-dimethylbenzoyl)-2-(1,1-dimethylethyl) hydrazide
CAS Number: 161050-58-4

Standard	Lot	Purity(%)	Appearance	Expiration
RH-2485	WCT3386A	99.7	White Solid	8/12/98

* The last four digits are commonly used instead of the full designation.

4. Chemicals and Equipment/Supplies

4.1. Chemicals	Supplier*
Acetonitrile, HPLC reagent	Baker
Methanol, HPLC reagent	Baker
RH-2485 Analytical Standard	Rohm and Haas Co.
Water Milli-Q	Millipore
n-Propanol	Baker
Formic Acid	Baker

4.2. Equipment/Supplies	Source*
Mobile Phase Filtration Apparatus	Supelco
Empore™ 47mm Extraction Disks(C-18)	J.T. Baker
Round Bottom Flasks, 500 ml, 24/40 ST	Pyrex
Rotary Evaporator	Buchi
Standard laboratory equipment, balances, beakers, test tubes, etc.	

* Other manufacturer brands may be substituted if shown to be suitable.

5. Preparation of solutions

5.1. Prepare 15% and 40% Acetonitrile/Water (v/v) solutions by measuring 150 and 400 ml of Acetonitrile and then 850 and 600ml of water respectively into 1000 ml volumetric flasks.

5.2. Prepare 54% (for HPLC mobile phase) and 52% (for standards) Acetonitrile/Water (v/v) solution by measuring 540 and 520 ml of ACN and then 460 and 480 ml of water into 1000 ml flasks respectively. HPLC-MS/MS mobile phase was prepared the same as the HPLC mobile phase except for the addition of 0.10% Formic acid.

6. Instrumentation

6.1. HPLC-UV Primary Method

Samples were analyzed using a Thermal Separation Products High Performance Liquid Chromatography configured as follows:

Auto Sampler	AS 3000 with the column heater

Pump I	P4000
Detector	UV 2000 Detector
Data System*	Hardware: SN4000; Software: PC1000
Mobil Phase	54% Acetonitrile/Water (v/v)
Conditions	Flow; 1.5 ml/min, Column temperature; 45 °C Wavelength: 240 nm Injection size: 50-150 µl.
HPLC Column	Supelco C-18, 25 cm x 4.6 mm ID, cat. # 5-8298
Retention Time	6.0 - 7.0 min

* Any other suitable system may be used for analysis after verifying system suitability.

6.2. HPLC-MS/MS Confirmation Method

In order to confirm that residues found by the HPLC-UV primary method are RH-2485, and not due to interference, a method for HPLC with tandem mass spectrometer detector (HPLC-MS/MS) has been developed for the samples generated from the same sample prep procedures. Acetonitrile/ Water 54/46 (v/v) with 0.10% Formic Acid was used as the mobile phase and the HPLC column was a Hypersil C_{18} (Keystone BDS) . Perkin Elmer SCIEX API 300 detectors were used as MS/MS detector.

The detailed HPLC-MS/MS instrument information follows:

Mass Spectrometer:	PE SCIEX API 300
HPLC-MS/MS Interface:	Turbo Ion Spray
Heated Nebulizer Temperature:	300C
Turbo Ionspray Gas Flow	5 L/min.
Air Nebulizer Gas Flow:	13
N2 Curtain Gas Flow:	9
Pump for MS/MS:	Shimadzu LC-10 AD
Software:	Macintosh Power PC 8500 MacQuan, version 1.4
HPLC Column:	Hypersil C_{18} (Keystone BDS) 50x3mm, 3 µm
Conditions:	
Flow Rate:	0.25 mL/min.

Split Ratio:	80:20
Injection Volume:	25 uL
Column Temperature:	Room Temp.
Mobile Phase:	Acetonitrile/Water 54/46 (v/v) with 0.10% Formic Acid
Analyte Monitoring Compound:	Negative Ion 367 (RH-2485 molecular weight 368)
Ions Monitored:	m/z 367→149

7. Analytical Procedure

7.1. Sample Processing

Water samples are used as collected from untreated wells.

7.2. Flow Diagram

500 ml. well water

⇓

Extraction with Empore™ 47mm Extraction Disk (C-18)

⇓

High Performance Liquid Chromatography/UV Detection
Quantitation

7.3. Procedure - Extraction

Set up a filtration apparatus with Empore™ 47mm Extraction Disk (C-18) and attach to the vacuum line. Condition the disk with 40 ml of 40% ACN/H_2O, then 40 ml Milli-Q water. Do not allow disk to go dry. Add 500 ml of well water (with appropriate fortification if desired) and let drain through disk. Do not allow disk to go dry. Rinse disk with 30 ml of 15% ACN/H_2O. Do not allow disk to go dry. Discard all filtrate to this point. Put a clean flask below the filtration apparatus . Add 40 ml of 40% ACN/H_2O to the disk. Allow eluent to completely drain through till disk is dry. Transfer eluent to 500 ml 24/40 ST round bottom flask. Rinse filtration flask with small amount of MeOH and add to round bottom flask.

Add 100 ml of n-propanol to the round bottom flask and rotavap to dryness. Dissolve the residue in 2.5 ml of 52% ACN/H_2O.

8. High Performance Liquid Chromatography Quantitation

8.1. Preparation of HPLC standards

8.1(a) Stock Solution

Carefully weigh a known amount of RH-2485 analytical standard between 10 and 100 mg on an analytical balance, and transfer into a 100 ml volumetric flasks. Bring to 100 ml mark with 52% ACN/Water. 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.

8.1(b) Intermediate Standard

A 10 µg/ml intermediate standard is made by taking an accurate volume of stock solution (8.1(a)) to a precise volume with the 52% ACN/Water. For example, 10 ml of a 100 µg/ml stock solution diluted to 100 ml with 52% ACN/Water would generate a 10 µg/ml solution.

8.1(c) Working Standards

Desired Concentration	Stock Solution Taken	Dilution to (with mobile phase)
1.0 µg/ml	10 ml of 8.1(b)	100 ml
0.50 µg/ml	5 ml of 8.1(b)	100 ml
0.25 µg/ml	2.5 ml of 8.1(b)	100 ml
0.10 µg/ml	10 ml of 1.0 µg/ml	100 ml
0.05 µg/ml	5 ml of 1.0 µg/ml	100 ml
0.025 µg/ml	2.5 ml of 1.0 µg/ml	100 ml
0.010 µg/ml	1.0 ml of 1.0 µg/ml	100 ml

Store working standards in a refrigerator (4-8°C) and remake every six months.

8.2. Preparation of Standard Curves

A minimum of four standard solutions are prepared in the concentration range of 0.01µg to 1.0 µg/ml in order to quantify at lower sensitivities. Standards and samples are preferably quantitated by peak height, although area may be used. At least four (4) standards, run in duplicate, are required to construct a linear standard curve.

8.3. Sample Analysis

Inject fortification samples at the same volume (between 50-100 µl) as RH-2485 standards. If necessary, the samples are diluted to an appropriate volume to give a final concentration within the standard curve range. The peak areas are measured and the concentration of each component is determined from the standard curves. The limit of quantitation was established at 0.10 ppb by analysis of fortifications at that level. By definition, under Rohm and Haas SOP, the limit of detection is 30% of the limit of quantitation, 0.03 ppb. The retention time for RH-2485 is found to be ~6.8 min. under normal HPLC conditions.

The residue concentration is determined as follows:

$$\frac{\text{Concentration (µg/ml) x Final Volume (ml)}}{\text{Sample Weight (g)}} = \text{ppm} \qquad \text{Eq.1}$$

8.4. Fortification Recovery

Control samples are fortified with known amounts of RH-2485 prior to extraction. Percentage recovery is calculated by measuring the peak height (or peak area) and correcting any background from the control sample as shown in Eq.1:

$$\frac{[(\text{µg/ml Found}) \times \text{Initial Sample Vol.(ml)}] - \text{Cntl corr.(µg)}}{\text{Fortification Amount (µg)}} \times 100 = \%\ \text{Recovery} \qquad \text{Eq.2}$$

8.5 Sample Calculation

Equation 1:
(see Figure 8: 0.00 ppm control)

$$\frac{[\,0.00\ (\text{µg/ml}) \times 5\ \text{ml.}]}{500\text{ml}} = 0.000\ \text{ppm}$$

Equation 2:
(see Figure 12: 0.0010 ppm fortification)

$$\frac{[0.000930(\text{µg/ml}) \times 500\ \text{ml.}]}{0.50(\text{µg})} \times 100 = 93.0\ \%$$