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RHÔNE-POULENC AG COMPANY *RTP, NC*

Environmental Chemistry: Method Development

Title:	Bromoxynil: Method of Analysis for Bromoxynil and its Metabolite, 3,5-Dibromo-4-hydroxybenzoic Acid in Animal Tissues and Animal Products using GC-MSD

Analyte: Bromoxynil Octanoate and 3,5-dibromo-4-hydroxybenzoic acid

Substrate: Cow muscle, fat, kidney, liver, and milk
Chicken muscle, fat and skin, liver, and egg

Date Issued: April 16, 1998

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Date

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SUMMARY FLOWCHART OF ANALYTICAL METHOD

(1). EXTRACTION

10 grams of sample.
Reflex for 1 hour with 100 mL of KOH.
Centrifuge the solution if necessary.

(2). CLEAN-UP

Pass 5 mL of aliquot through C-18 cartridge, and collect eluent into15 mL of Sephadex bed, mix vigrously.
Wash with 15 mL of 15% ether in hexane.
Acidify with acid buffer and HCl.
Extract twice with 20 mL 15% ether in hexane.
Dry sample down under nitrogen.

(3). DERIVATIZATION

Reconstitute the drying test tube with 1 mL methanol.
Add 3 mL of diazomethane and let sit for 45 min.
Dry down under nitrogen.
Reconstitute with appropriate volume of methanol.
Pass sample extract through a Gelman 0.45 um filter.

(4). DETECTION

GC-MSD

Bromoxynil: Method of Analysis for Bromoxynil and its Metabolite, 3,5-Dibromo-4-hydroxybenzoic Acid in Animal Tissues and Animal Products using GC-MSD

I. Introduction

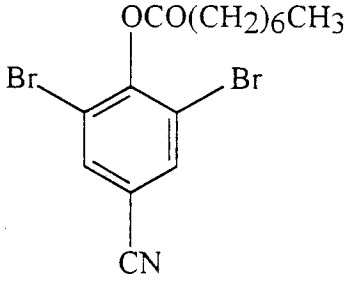
A. Scope

An analytical method is described for the analysis of bromoxynil and its metabolite, 3,5-dibromo-4-hydroxybenzoic acid in animal tissues including cow muscle, cow fat, cow kidney, cow liver, milk, and chicken muscle, chicken fat and skin, chicken liver and egg as defined in the Pesticide Assessment Guidelines, Subdivision O.

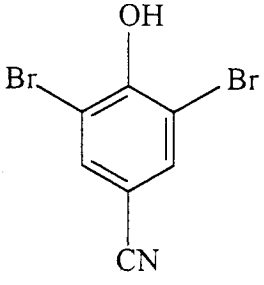
B. Principle

Samples are hydrolyzed using KOH in methanol to convert bromoxynil octanoate to bromoxynil phenol, followed by clean-up with C-18 cartridge and anionic exchange resin, and extracted with 15% ether in hexane. The extract is methylated with diazomethane, and filtered before analysis. Quantification of the derivatized residues is accomplished by gas chromatography using a mass selective detector.

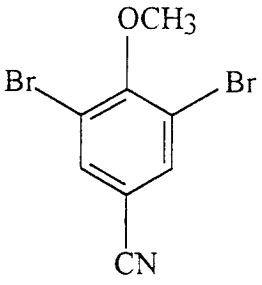
C. Structures



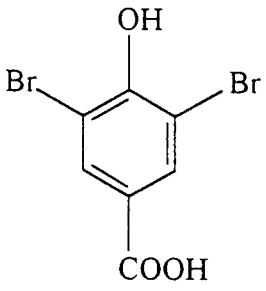
Bromoxynil Octanoate



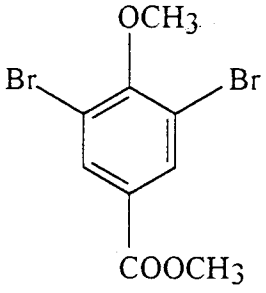
Bromoxynil



Bromoxynil methyl ether



3,5-Dibromo-4-hydroxybenzoic acid



Methyl-3,5-dibromo-4-methoxybenzoate

II. Materia

Reagents and Solvents were used as received from supplier, unless otherwise noted. Equivalent reagents and equipment may be substituted where appropriate.

A. Reagents, Solvents and Preparations

1. QAE Sephadex A-25 Ion Exchange Resin, Pharmacia Fine Chemical, Piscataway, NJ
2. Potassium Hydroxide, pellets, Fisher Scientific, Cat. No. P251-500 or equivalent
3. Hydrochloric Acid, 36.5% -38%, GR, EM Cat. No. HX0603-13 or equivalent
4. Methanol, EM OMNISOLV, VWR Scientific Cat. No EM-MX0484-1 or equivalent
5. Hexane, EM OMNISOLV, VWR Scientific Cat. No EM-CX2286-1 or equivalent
6. Water, EM HPLC Grade, VWR Scientific Cat. No EM-WX0004-1 or equivalent
7. Ethyl ether, EM HPLC Grade, VWR Scientific Cat. No EM-WX0004-1 or equivalent
8. Potassium Hydroxide Solution, 1.0 N in Methanol
9. Hydrochloric Acid Solution, 3.0 N in Water
10. Potassium Chloride Solution, 1.0 N in water
11. Preparation of ~15 mL bed of QAE Sephadex A-25 resin in test tube for sample clean-up:
    a. Make a slurry of resin in purified water in a container.
    b. Allow resin to "swell" for at least 2-3 hours or overnight.

c. Transfer about 30 mL of slurry to yield a ~15 mL bed of settled resin into a 50 mL centrifuge test tube.
d. Centrifuge to settle the bed.
e. Remove all the supernatant water to make water and the resin bed is ready for sample collection.

12. Diazomethane Solution - Prepare from N-methyl-N-nitroso-p-toluene sulfonamide according to SOP No. 81332 or equivalent method to obtain high purity reagent. Observe all safety precautions. Store in tightly capped bottle, with TEFLON cap liner, in freezer. Do not store over KOH or other desiccants.

13. Acid "Buffer", mix equal volumes of 1.0 N aqueous HCl and 1.0 N aqueous KCl

B. Equipment

1. Aluminum Crimp-Top Seal, 11 mm TFE/RUB Septum, Sun Brokers, Inc., Cat. No. 200 100 or equivalent

2. Four Place Analytical Balance

3. Autosampler Vials, 1 mL, clear, Wheaton, Cat. No. 223682, or similar

4. Disposable Pasteur Pipettes

5. Polypropylene Copolymer Centrifuge Bottle, 250 mL, Nalgene, Cat. No. 3141-0250, or equivalent

6. Graduated Cylinders, 100 mL

7. Polypropylene Centrifuge Test Tube, 50 mL

8. Boiling Flasks, 250 mL with 24/40 joint

9. Condensers, Friedrich or equivalent to fit 100 mL volumetric flask

10. Sonicator, Model 5200, Branson, Cat. No. B5210DTH or similar

11. Volumetric Flasks, 100 mL, class A

12. Volumetric Pipettes, appropriate sizes, class A

A. Sample Preparation

Use samples after ground by processor.

B. Extraction

1. Weigh ~10.0 g of substrate into a 250 mL boiling flask.

2. Fortify as necessary and then let stand at least 10 minutes. Remember that spiking is done on the basis of bromoxynil phenol. Therefore, a 1.45 molecular weight conversion factor is needed to be considered for calculating spiking volume.

3. Add ~100 mL of 1.0 N potassium hydroxide solution in methanol and reflux with stirring for ~ 1.0 hour. Let cool to room temperature. Centrifuge if necessary to settle any undissolved solids •

C. Sample Clean-up

1. Transfer 5.0 mL of reflux solution from Step B.3 onto a C18 Mega Bond Cartridge which is hooked up with vacum device (Cartridge needs to be preconditioned with at least 10 mL methanol).

2. Allow the sample load to reach close to the bottom of the cartridge (monitor from sample color band), then collect the sample into a 50 mL polypropylene centrifuge test tube containing pre-prepared QAE Sephadex resin bed, and elute cartridge with ~10 mL of methanol.

3. Cap test tube, vortex it vigorously for ~8 mins on a multi-tube vortexer (entire resin bed must come off bottom of the tube, if it doesn't, additional hand shaking may be necessary).

4. Centrifuge at ~2500 rpm for ~4 mins, discard supernatant liquid.

5. Add 5 mL of NANO-pure water, 15ml of 15% ethyl ether in hexane. Vortex it vigorously for ~2 mins.

6. Centrifuge at ~2500 rpm for ~4 mins, aspirate off all of the top liquid layer (both organic and aqueous layers) and discard into waste.

7. Add 5 mL of acid buffer, 3 ml of 3 N HCl to Sephadex bed. Cap test tube, and hand shake to mix well with resin bed (check the pH if necessary to make sure it is between 0 to 1.5).

8. Add 20 mL of 15% ethyl ether in hexane into test tube, vortex vigorously to mix organic layer and resin bed very well for about 2 min (additional hand-shake may be necessary).

9. Centrifuge at ~2500 rpm for about 4 min. to separate organic layer from aqueous layer.

10. Place test tube into an acetone-dry ice bath to freeze the bottom layer (aqueous and resin bed). Decant and collect top organic layer to a clean glass conical test tube.

11. Place test tube containing frozen aqueous layer into a warm water bath to thaw, then perform a repeat ether:hexane extraction (from step C. 8 to C.11) and combine the organic layers. Repeat this step a third time if necessary.

12. Dry down sample to dryness under a gentle stream of nitrogen in a water bath up to 50 degrees C (see note). •

D. Derivatization

1. Add 1.0 mL of methanol to reconstitute the dried test tube, sonicate, and vortex to wash the glass test tube wall.

2. Add 3.0 mL of diazomethane solution (enough to give a permanent yellow color) to test tube, and allow to stand for ~45 min.

3. Dry down sample using a gentle stream for nitrogen in a water bath up to 45 ^{0}C.

4. Reconstitute the dried sample using methanol (reconstitution volume depends on the spiking concentration of the sample, minimum 0.5 mL of methanol is needed).

5. Pass the sample extract through a Gelman 0.45 um filter using a syringe and aliquot it into GC vial. •

E. General Method Notes

(1). The analyst may begin blowing down the first extract while defrosting and extracting the second, this saves time.

(2). A little bit of oil may be left for some matrices before or after derivatization.

(3). Carefully wash the reflux flasks and derivatization conical test tubes to eliminate any possibility for cross-contamination.

(4). Heating blocks can be used instead of water bath to heat the samples.

V. Gas Chromatography

A. Instrumentation

1.	Gas Chromatograph:	Hewlett-Packard 5890 Series II GC, 7673 Autosampler, 18594B Sampler Controller, Split/Splitless Injector, or an equivalent system.
2.	Detector:	Mass Selective Detector, Hewlett-Packard Model 5972 or equivalent.
3.	Data Acquisition:	Hewlett-Packard ChemStation
4.	Column:	J & W Scientific DB-5 30 m X 0.25 mm i.d., 0.25 µm film thickness (or HP-5 same diminsions)

B. Gas Chromatograph Conditions

1.	Carrier Gas:	Helium, Head Pressure set at 15 PSI with a 1.5 minute 45psi pulse at injection.
2.	Inlet Liner:	4-mm i.d. nominal volume 900 µl, borosilicate glass with single taper on GC end (HP part #5181-3316)
3.	Injector Temperature:	250 °C
4.	Detector Temperature:	300 °C
5.	Oven Temperatures:	Initial: 100 °C, hold 2 minute Ramp 30 °C / min to 180°C, hold 1 minute Ramp 5 °C / min to230 °C, hold 0 minute Ramp 40 °C / min to 300 °C, hold 5 minutes
6.	Injection Volume:	1.5 µL

7. Splitless injection with split vent off for 60 seconds.

C. Mass Selective Detector Parameters

1.	Tune File	Maximum Sensitivity Autotune
2.	Solvent Delay	8.0 minutes
3.	EM voltage	400 over tune
4.	Acquisition Mode	SIM
5.	SIM Parameters	GROUP 1 Bromoxynil Methyl Ether Low Resolution - Yes Dwell time per ion 100 msec Starting time 8.0 min Quantitation ion 290.85 Qualifier ions 275.85 & 288.85 GROUP 2 Methyl-3,5-dibromo-4-methoxybenzoate Low Resolution - Yes Dwell time per ion 100 msec Starting time 8.0 min Quantitation ion 292.85 Qualifier ions 290.85 & 323.85

D. General Chromatography Notes / Potential Problems

1. Several standards should be injected prior to actual analysis if using a new column or after the GC has set idle for any considerable length of time to condition and/or to remove any contaminants.

2. A gold plated seal is used at the interface of the glass liner and column. Hewlett-Packard Part No. 18740-20885.

3. The GC parameters are guidelines and can be optimized for the instrument and column actually used. Record the actual GC conditions used for data acquisition and include in report.

4. The detector ion amu values are nominal. Exact values should be determined from a scan run after each tune.

VI. Quantification of Residues

A. Calibration Curves

1. Linear regression should be used to generate a calibration curve for the analyte. At least four different standard concentrations should be run with each set of samples. Standards should be interspersed with samples to compensate for any minor change in instrument response. Extracts should be diluted such that the peak areas obtained are within the area range between the lowest and highest standards injected.

2. Linear regression coefficients should be calculated from 'peak area' (or 'peak height') versus 'nanogram / ml injected'. Data from the analytical standards should be fit to the linear equation, $y = a + bx$.

where: y = peak area or height
a = calibration line intercept
b = calibration line slope
x = conc of analyte in inj soln

B. Quantification of Residues

1. Both bromoxynil methyl ether and methyl-3,5-dibromo-4-methoxybenzoate should be quantified by comparison to their standard curves obtained from a linear regression analysis of the data.

2. Equations

2.1 Concentration of analyte in sample in ppb (parts per billion).

$$z = (y - a) / b \times c / d$$

where: y = peak area (or height), response of analyte of interest
a = intercept of calibration line from linear regression (area or height)
b = slope of calibration curve from linear regression (response per ng/ml)
c = final volume of sample (ml)
d = sample weight (g)
z = conc of analyte in sample (ppb)

2.2 Corrected concentration of analyte in sample in ppb.

$$Z' = z / C$$

where:
- Z' = corrected concentration
- z = concentration found from curve
- C = conversion factor

for conversion of

bromoxynil to bromoxynil methyl ether	~~C = 1.067~~
bromoxynil octanoate to bromoxynil methyl ether	C = 0.722
3,5-dibromo-4-hydroxybenzoic acid to methyl-3,5-dibromo-4-methoxybenzoate	C = 1.095

2.3 Percent recovery

$$\% \text{ recovery} = \frac{(\text{ppb found in fort sample} - \text{ppb found in UTC})}{\text{actual fortification level in ppb}} \times 100\%$$

3. Residues shall be reported as bromoxynil equivalents or 3,5-dibromo-4-hydroxybenzoic acid equivalents.

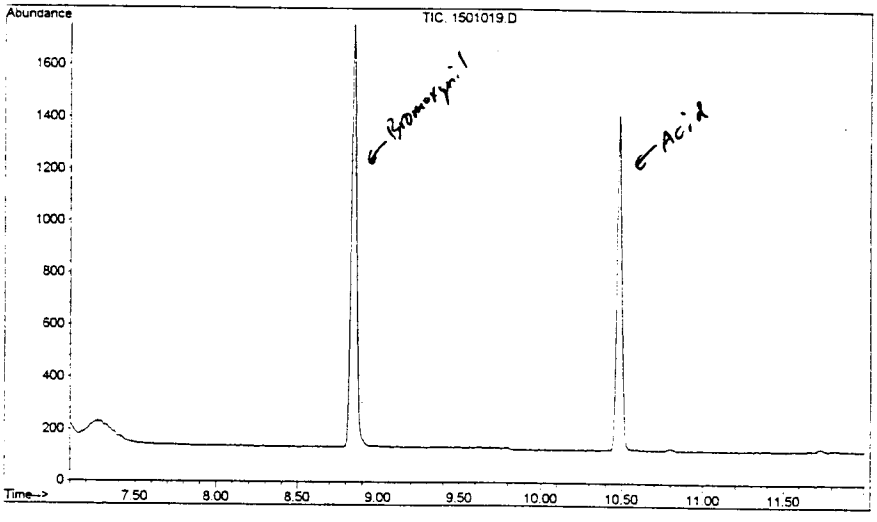
VII. Example Chromatograms

A. Mix Standards

1. Bromoxynil Methyl Ether Standard , 10 ng/ml in methanol

   and Methyl-3,5-dibromo-4-methoxybenzoate, 10 ng/ml in methanol

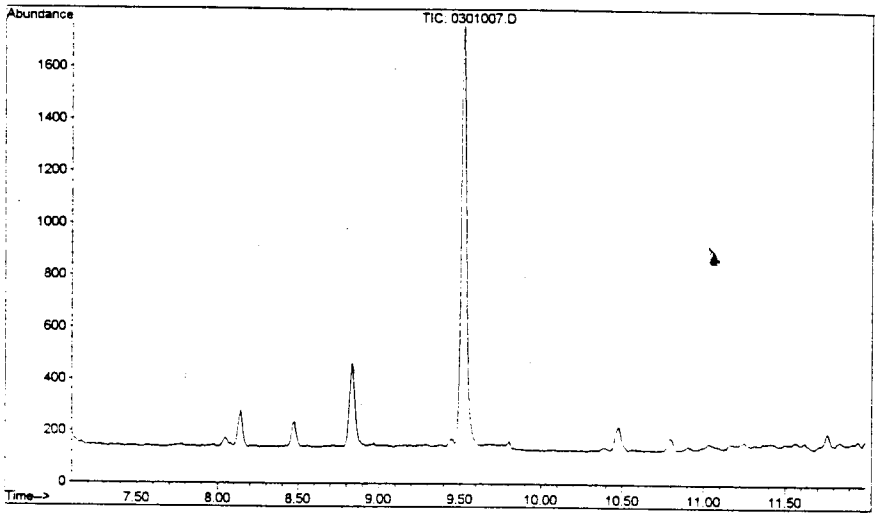
```
File        : C:\HPCHEM\1\DATA\BROMOX~1\JG041598\1501019.D
Operator    : JG
Acquired    : 16 Apr 98  12:17 am using AcqMethod BXN
Instrument :    GC/MSD #4
Sample Name: 10/10 STD
Misc Info   :
Vial Number: 15
```



B. Cow Liver

1. Cow Liver Untreated Control (UTC)

```
File        : C:\HPCHEM\1\DATA\BROMOX~1\JG041098\0301007.D
Operator    : JUDY GAO
Acquired    : 10 Apr 98    6:40 pm using AcqMethod BXN
Instrument :    GC/MSD #4
Sample Name: BF LIVER UTC JG
Misc Info   :
Vial Number: 3
```



2. Untreated Control Fortified at Levels of 10 ppb for Bromoxynil and 10 ppb for 3,5-dibromo-4-hydroxybenzoic acid

```
File        : C:\HPCHEM\1\DATA\BROMOX~1\JG041098\0401008.D
Operator    : JUDY GAO
Acquired    : 10 Apr 98    7:07 pm using AcqMethod BXN
Instrument :    GC/MSD #4
Sample Name: BF LIVER 10 JG
Misc Info   :
Vial Number: 4
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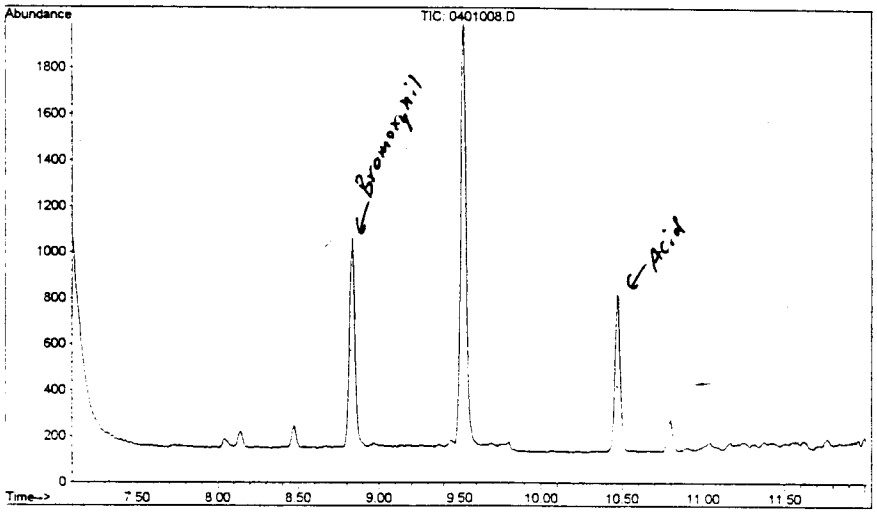
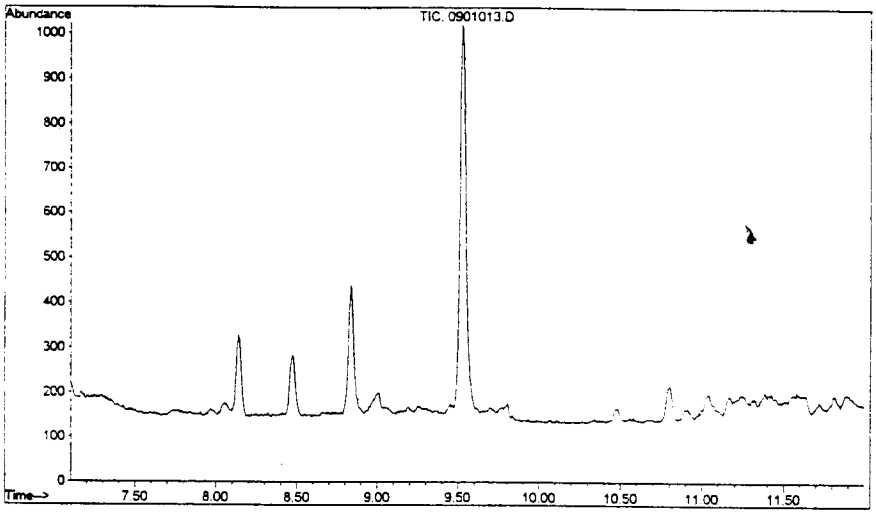


C. Cow Kidney

1. Cow Kidney Untreated Control (UTC)

```
File       : C:\HPCHEM\1\DATA\BROMOX~1\JG041098\0901013.D
Operator   : JUDY GAO
Acquired   : 10 Apr 98   9:21 pm using AcqMethod BXN
Instrument :    GC/MSD #4
Sample Name: BF KIDNEY UTC JG
Misc Info  :
Vial Number: 9
```



2. Untreated Control Fortified at Levels of 10 ppb for Bromoxynil and 10 ppb for 3,5-dibromo-4-hydroxybenzoic acid

```
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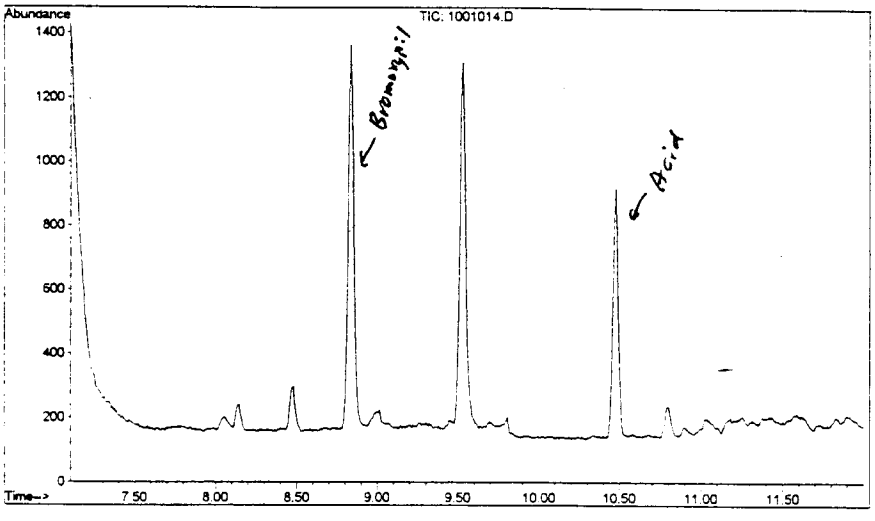
Operator   : JUDY GAO
Acquired   : 10 Apr 98   9:47 pm using AcqMethod BXN
Instrument :    GC/MSD #4
Sample Name: BF KIDNEY 10 JG
Misc Info  :
Vial Number: 10
```



D. Milk (Raw)

1. Milk Untreated Control (UTC)

```
File       : C:\HPCHEM\1\DATA\BROMOX~1\JG041498\0901013.D
Operator   : JG
Acquired   : 14 Apr 98   9:24 pm using AcqMethod BXN
Instrument :    GC/MSD #4
Sample Name: MILK UTC JG
Misc Info  :
Vial Number: 9
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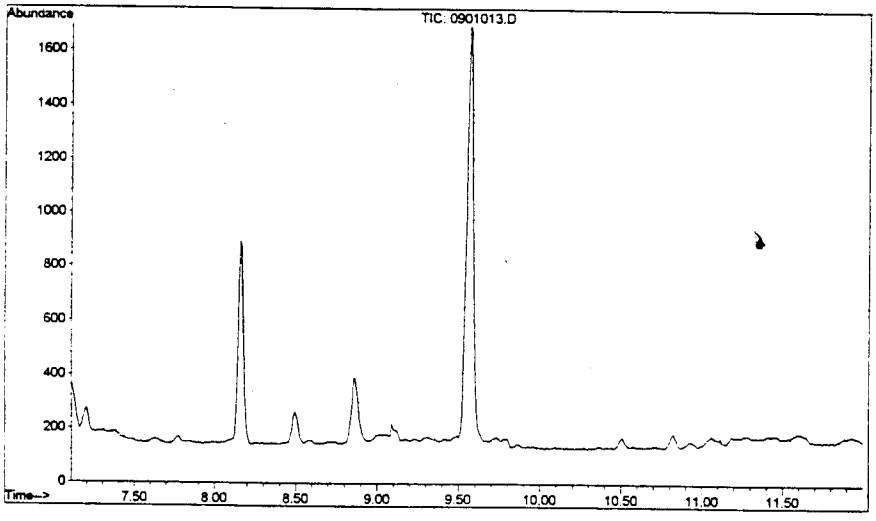

2. Untreated Control Fortified at Levels of 10 ppb for Bromoxynil and 10 ppb for 3,5-dibromo-4-hydroxybenzoic acid

```
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Operator   : JG
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Sample Name: MILK 10
Misc Info  :
Vial Number: 10
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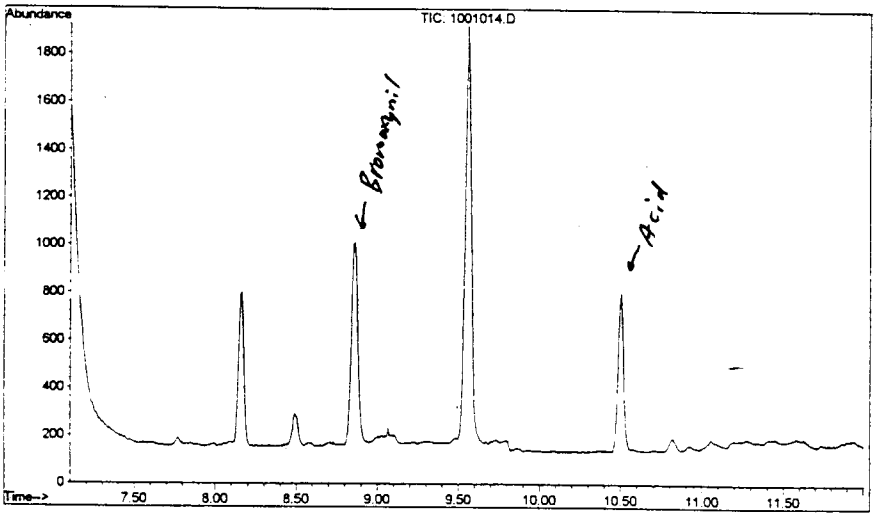

E. Chicken Fat and Skin

1. Chicken Fat & Skin Untreated Control (UTC)

```
File       : C:\HPCHEM\1\DATA\BROMOX~1\JG041598\0901013.D
Operator   : JG
Acquired   : 15 Apr 98   9:36 pm using AcqMethod BXN
Instrument :    GC/MSD #4
Sample Name: CK FAT UTC JG
Misc Info  :
Vial Number: 9
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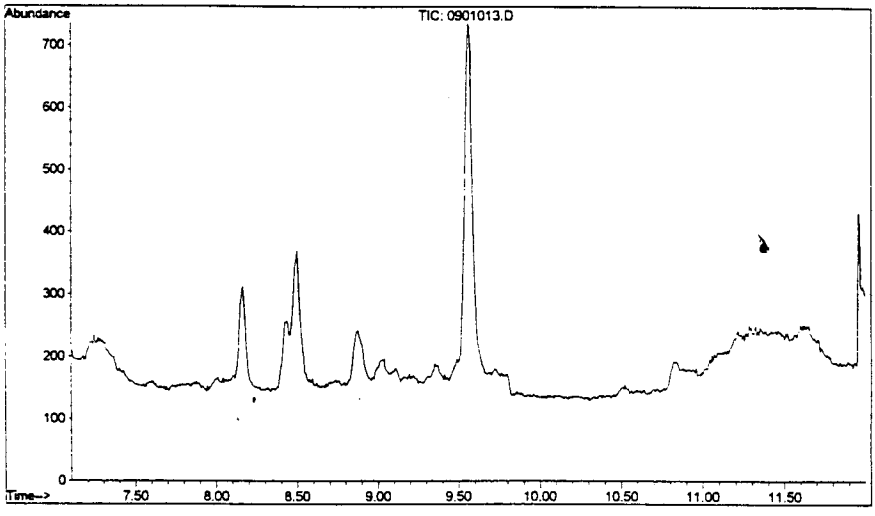

2. Untreated Control Fortified at Levels of 10 ppb for Bromoxynil and 10 ppb for 3,5-dibromo-4-hydroxybenzoic acid

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Operator   : JG
Acquired   : 15 Apr 98  10:03 pm using AcqMethod BXN
Instrument :    GC/MSD #4
Sample Name: CK FAT 10
Misc Info  :
Vial Number: 10
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