1.0 INTRODUCTION

1.1 Scope

This method set forth the procedure for determining residues of Ethylenethiourea (ETU) in ground water. It is suitable for residue analysis to levels of 0.1 ppb (ng/mL). Revision 1 modified the method to allow for the addition of ammonium hydroxide to stabilize the final extract. The use of a C8 HPLC column and modified mobile phase was also incorporated into the method.

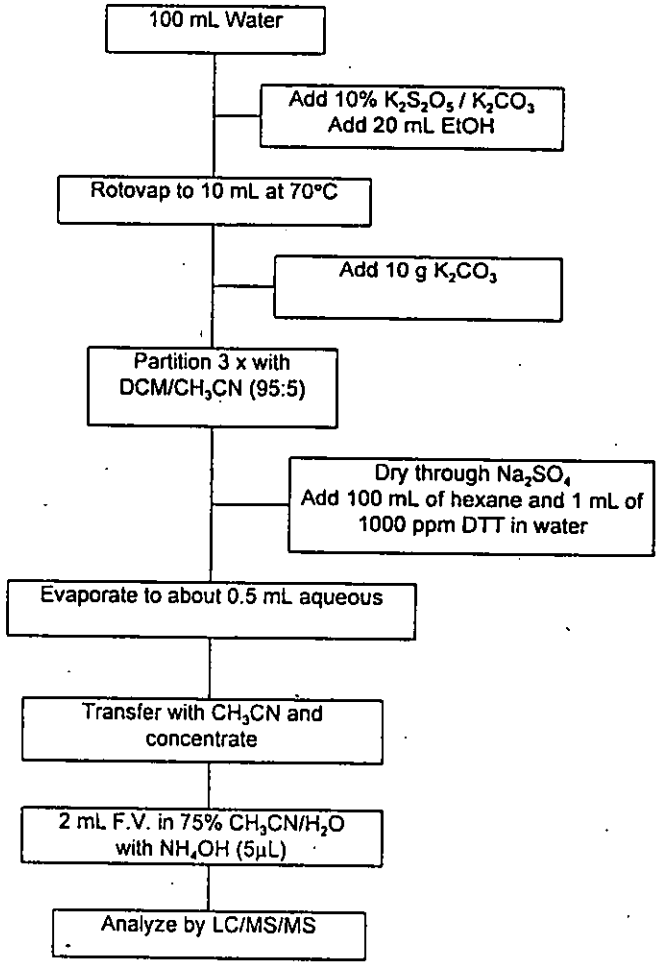
1.2 Principle

An analytical method is described for the determination of residues of ETU in water. The procedure involves the pre-concentration of the water sample (treated with potassium metabisufite/potassium carbonate) by rotary evaporation followed by saturation with potassium carbonate and liquid/liquid partition with 5% acetonitrile in dichloromethane. The organic phase extract is concentrated, brought up into 75% acetonitrile/water and analyzed by LC/MS/MS. The addition of dithiothreitol (DTT) prevents the oxidation of the ETU during the concentration and evaporation steps and does not interfere in the LC/MS/MS chromatograms.

1.3 Method Limits (LOD & LOQ)

For a 100 mL water sample the proposed limit of detection (LOD) is 0.03 ppb and the limit of quantitation (LOQ) is 0.1 ppb. LOD and LOQ will be determined during the method validation.

1.4 Method Flowchart of Analytical Method for ETU



2.0 MATERIALS

2.1 Reagents and Solvents

(Equivalent or better grade reagents/solvents may be substituted.)

Acetic acid glacial - ACS grade, Fisher Scientific

Acetonitrile (CH_3CN) - glass distilled, EM Science, OmniSolv®

Ammonium acetate - BDH, Analar

Ammonium Hydroxide - ACS grade, BDH, 28-30% solution.

Dichloromethane (DCM) - glass distilled, EM Science, OmniSolv®

Dithiothreitol (DTT) - >99%, Sigma

Ethanol (EtOH) - glass distilled, EM Science, OmniSolv®

Hexane - glass distilled, EM Science, OmniSolv®

Sodium chloride - ACS grade, Fisher Scientific

Potassium carbonate (K_2CO_3)- ACS grade, Fisher Scientific

Potassium metabisulfite ($K_2S_2O_5$) - ACS grade, Fisher Scientific

Sodium sulfate, baked (Na_2SO_4)- ACS grade, Fisher Scientific; heat to 500°C for 2 hours in a muffle furnace

Water, deionized - Millipore MilliQ Purification System

2.2 Equipment and Supplies (Equivalent equipment may be substituted.)

Balance - Sartorius 1206 MP, VWR Scientific

Centrifuge - Sorvall, RC2-B with 250 mL rotor head, DuPont Instruments

Centrifuge-HN-S with 8 position head,International Equipment Co.(for 40 mL tubes)

Culture tubes, - 80 mL, screw-top tube, 16 x 60 mm, Kimble Glass Inc.

Cylinders, graduated - 100, 250 and 500 mL, Kimble Glass Inc.

Flasks, Erlenmeyer - 250 mL , Kimble Glass Inc.

Flasks, round bottom (RB) - 50, 250 and 500 mL, Kimble Glass Inc.

Flasks, separatory - 250 mL, Kimble Glass Inc.

Flasks, volumetric – 10,25, 50 and 100 mL, Class A, Kimble Glass Inc.

Funnels, wide-mouth – polypropylene, VWR Scientific

Glass wool – VWR Scientific

HPLC Column – Merck Lichrospher 100 RP-8, 5 µm, 4 x 250 mm

Nitrogen evaporator with water bath - Organomation Assoc. Inc., Model No.111

Pipettes, volumetric – 1.0 and 10 mL, Kimble Glass Inc.

Rotovap with water bath – RV 06-ML IKA Rotary Evaporator, IKA Switzerland

Shaker, platform or wrist - Pychotherm

Syringe, Hamilton, 10 µL

Ultrasonicator - Fisher Scientific, FS-28

2.3 Solutions

The following solutions are required:

2.3.1 75% acetonitrile in water (v/v): Add 750 mL of acetonitrile to 250 mL of deionized water and mix.

2.3.2 5% acetonitrile/dichloromethane (DCM): Add 50 mL of acetonitrile to 950 mL of DCM and mix.

2.3.3 10% Potassium metabisulfite and 10% potassium carbonate (w/v): Add 100g of $K_2S_2O_5$ and 100g of K_2CO_3 to about 800 mL of deionized water and mix to dissolve. Bring to 1 L with deionized water and mix.

2.3.4 1000 ppm Dithiothreitol (DTT) in water solution: Dissolve 0.1 g DTT in 100 mL of deionized water. Adjust to pH 8-9 with ammonium hydroxide and mix.

2.3.5 100 ppm DTT in 5% acetonitrile/DCM. Dissolve 0.01 g of DTT in 100 mL of 5% acetonitrile/DCM and mix.

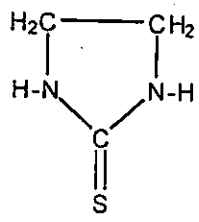
2.3.6 0.001 M Ammonium acetate (NH_4OAc)/water: Add 0.308 g of NH_4OAc to 4 L of deionized water and mix. Adjust to pH 5.0 with glacial acetic acid. De-gas by placing 4 L bottle in sonic bath and apply vacuum for at least 5 minutes.

2.3.7 5% acetonitrile in 0.001 M NH_4OAc/water: Add 50 mL of acetonitrile to every 950 mL of 0.001 M NH_4OAc/water and mix. De-gas as above (2.3.6)

2.4 Analytical Standards and Chemical Structures:

The following analytical standard of ETU was supplied by the Rohm and Haas Company. References to derivation (batch number), characterization, and certificates of purity can be supplied by the Rohm and Haas Company.

ETU (Ethylenethiourea)
Chemical Name:
2-Imidazolidinethione; imidazoline-2-thiol
Physical State: Solid
Molecular Weight: 102
CAS No.: 96-45-7
Lot #: 08002DT
Purity: 100%
Expiry Date: February 5, 2002



Ethylenethiourea

3.0 FORTIFICATION AND CALIBRATION STANDARD SOLUTIONS

3.1 Preparation

All the standard solutions must be stored in glass at or below 4°C when not in use. Solutions should be allowed to warm to room temperature prior to use. The following is an example procedure for preparing a standard solution. Alternate or additional standards of appropriate weight and volume may be prepared as needed. The "~" symbol indicates approximately.

3.1.1 Accurately weigh ~ 0.010 g (10,000 µg) of 100% ETU into a 10 mL volumetric flask and dilute to the mark with acetonitrile. Cap and mix by inversion. The concentration of this stock standard is ~1000 µg/mL.

3.1.2 For the preparation of the fortification or spiking standard for ETU, transfer 100 µL (0.1 mL, 100 µg ETU) of the ~1000 µg/mL standard via volumetric class "A" pipettes, to a 10 mL volumetric flask. Dilute to mark with acetonitrile. Cap and mix by inversion. The concentration of this mixed standard is ~10 µg/mL ETU.

3.1.3 Calibration standards for sample analysis are prepared by serial dilution of an exact 100 µg/mL standard in 75% acetonitrile/water. Prepare the 100 µg/mL stock solution by dilution of the ~1000 µg/mL standard. Working standards are prepared by serial dilution in 75% acetonitrile/water from this 100 µg/mL stock. They should be made in the concentration range of 0.003 to 0.1 µg/mL.

3.2 Stability

3.2.1 To evaluate the stability of the standard solutions over the length of a study, the following formula will be used:

$$\%\ Stability = 1 - \frac{\text{'old stock' standard solution}}{\text{'new stock' standard solution}} \times 100$$

A standard curve will be run using the new standard solutions. The old standard solutions will be injected at various concentrations within the standard curve. The old standard solutions should be within 15% deviation of the standard curve.

4.0 METHOD PROCEDURES

4.1 Analysis of ETU in Ground Water

4.1.1 Measure 100 ± 1 mL of ground water into a 500 mL round bottomed flask.

4.1.2 Add 1.0 mL of solution containing 10% $K_2S_2O_5$ and 10% K_2CO_3. Spike samples at this point.

4.1.3 Add 20 ± 1 mL of EtOH.

4.1.4 Concentrate on a rotovap (15 in. Hg) with waterbath @ 70 ± 5°C to about 5 mL.

4.1.5 Quantitatively Transfer with rinses (2 x 1 mL) of deionized water to a 80 mL culture tube and bring to 10 ± 0.5 mL with deionized water.

4.1.6 Add 10 ± 0.2 g of K_2CO_3 and shake slowly to dissolve. Vent tubes cautiously.

4.1.7 Partition 3 times with 30 ± 3 mL of 5% acetonitrile/DCM. Transfer by pipette and combine the organic extracts in a 250 mL Erlenmeyer flask.

4.1 Analysis of ETU in Ground Water cont'd

4.1.8 Prepare drying funnels of about 20 g of baked Na_2SO_4 (section 2.1). Wash the baked Na_2SO_4 with about 50 mL of 100 µg/mL DTT in 5% acetonitrile/DCM (discard). Pass the organic extract through the washed baked Na_2SO_4 into a 500 mL round bottomed flask. Rinse the Erlenmeyer flask at least 2 times with 2 mL of 5% acetonitrile/DCM and pass through the Na_2SO_4. Then rinse the Na_2SO_4 with about 10 mL of the 5% acetonitrile/DCM.

◆4.1.9 Add about 100 mL of hexane and 1.0 mL of 1000 µg/mL DTT in water solution to the 500 mL round bottom flask.

4.1.10 Remove the organic extract by concentration on a rotovap (15 in. Hg) with waterbath @ 40 ±5°C until only the aqueous layer remains. (about 0.5 mL). Note: Do not let go to dryness.

4.1.11 Transfer the aqueous residue to a calibrated 4 mL vial with 2 rinses of 0.5 mL acetonitrile and concentrate to about 0.3-0.4 mL using a nitrogen evaporator with waterbath @ 40 ± 5°C.

4.1.12 Bring to 0.5 mL with deionized water.

◆4.1.13 Add 1.5 mL of acetonitrile to give a 2.0 mL FV. If particulates are present, filter the final extract using a 0.45 µm nylon syringe filter.

4.1.14 Add 5 µL of conc. NH_4OH (28-30%) using a glass 10 µL syringe.

◆4.1.15. Analyze by LC/MS/MS with heated nebulizer ionization.

4.2 General Notes

4.2.1 The "◆" symbol indicates an optional stopping point after completing the indicated step. Samples may be stored overnight in a refrigerator (at or below 5°C). Final extracts should be stored in a freezer at −20±5°C.

4.2.2 EtOH is added to the water to lower the boiling point and increase the speed of the water concentration by rotary evaporation.

4.2 General Notes: cont'd

4.2.3 The potassium metabisulfite ($K_2S_2O_5$) and DTT solution (1000 µg/mL) are added to prevent oxidation of ETU in solution. Municipal tap waters or "dirty" ground waters containing significant levels of dissolved oxygen may require a greater amount of DTT (step 4.1.9) to prevent destruction of ETU. (Amount to be determined during validation.)

4.2.4 Pre-wash the anhydrous sodium sulfate with 100 µg/mL DTT in 5% acetonitrile/DCM to prevent oxidation of the ETU by reactive residues present in the Na_2SO_4.

4.2.5 The hexane is added to cause the aqueous layer to sink to the bottom of the RB flask. If a fine emulsion develops after evaporation of the hexane/DCM, add an additional 10 mL of hexane and repeat the evaporation.

4.2.6 The final extract should be in 75% acetonitrile/water. If dilutions are required, dilute the extract with 75% acetonitrile/water.

5.0 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY (LC/MS/MS)

5.1 Instrumental Analysis by LC/MS/MS

Instrument: PE SCIEX API III
HPLC: Varian 9012, Solvent Delivery System
Autoinjector: Rainin AI-200
Data System: MacIntosh IIci with OS 7.5
Operating System: API (Version 2.7)

HPLC column: Merck Lichrospher 100 RP-8 (4 x 250 mm)
Flow: 0.6 mL/min
Eluent: 5% Acetonitrile in 0.001M Ammonium Acetate, pH 5
Injection Volume: 25 µL

Samples and standards were analysed using the PE Sciex API III heated nebulizer interface.
Heated nebulizer ionization parameters:
Nebulizer Temperature: 500°C
Auxiliary flow: 5.0 (L/min)
Nebulizer Gas: Nitrogen @ 60 psi

5.1 Instrumental Analysis by LC/MS/MS cont'd

The instrument was operated in the MRM mode.
MRM parameters:
Curtain Gas: Nitrogen @ 1.2 L/min
ISV Voltage: 6000 V
Orifice: 55 V
Interface Setpoint: 65°C
Collision Gas: Argon at ~275 cgt

MRM MASS TRANSITION		
Compound	**m/z Parent**	**m/z Daughter**
Ethylenethiourea	103.1	44.0

ETU retention time is about 5.5 min.

Example chromatograms are attached. (See Section 8.0). Note that the retention times will vary from system to system and may require gradient or flow optimization.

5.2 Performance Criteria (LC/MS/MS)

First Criterion:

Run a standard solution on LC/MS/MS corresponding to a level at or below the estimated LOQ and obtain a signal to noise ratio of at least 9:1.

If this criterion cannot be met optimize and/or change instrument operating parameters.

Second Criterion:

Run a set of ETU standards of four to five concentration levels, from at or below the LOQ, up to the highest concentration level to be included in the analysis. Generate a constrained quadratic calibration curve. The samples are run with standards interspersed.

5.2 Performance Criteria (LC/MS/MS) cont'd

A typical set may consist of a high standard, low standard, fortified samples, control, standard, 4-5 treated (or full) samples, standard, 4-5 treated samples, standard, 4-5 treated samples, etc., ending with 2 standards at 2 levels. There are typically 4-5 levels of standards used throughout the run to generate the linearity curve.

6.0 CALCULATIONS

A constrained quadratic regression (zero intercept) should be used to generate calibration curves for ETU. After the instrument performance criteria are met, a minimum of four standards over a range of concentration levels should be included with a set of samples. Standards should be interspersed with samples to compensate for any minor change in instrument response. Samples should be diluted so that any ETU peak areas or peak heights are within the area or height range between the lowest and highest standard injected.

Quadratic regression coefficients should be calculated from peak area or peak height versus standard concentration (ng/mL). The data from the analytical standards should then be fit to a suitable linear model.

$$y = Ax^2 + Bx + C$$

Where:

y = peak area or peak height
x = standard concentration (ng/mL)
A, B, C = variables dependent on data points entered
C = zero for a constrained curve.

The equation to be used to estimate the residues in the samples is:

$$\text{Conc. (ppb)} = \frac{\left[-B + \text{SQRT}(B^2 - 4 \times A \times (C - \text{Peak Area}))\right]}{2A} \Bigg/ \text{S.V.(mL)} \times \text{F.V.(mL)}$$

Where:
F.V.= Final sample volume (mL)
S.V.= Starting volume of sample (mL)

7.0 SAFETY

All available appropriate MSDS's should be available to the study personnel during the conduct of the study. General laboratory safety precautions should be taken. This method does not present any specific risks.