

DETERMINATION OF ETHYLENE THIOUREA IN CROPS (RAC's and Processed Commodities):**Reason for Revision:**

1. To follow criteria set forth in ML SOP# HPLC-11 revision #3.
2. To generalize the standard curve requirements.

Method References:

1. Onley, J.H. et al, JAOAC Volume 60, Number 5, page 1105 (1977).
2. MTF-88-AM-004, 07/27/89, Determination of Ethylene Thiourea in Crops, Morse Laboratories, Inc.

Principle:

ETU present in the sample is extracted in water/ethanol. An aliquot of the extract is passed through an alumina column for cleanup. The sample is then submitted for HPLC analysis, utilizing either Ultraviolet (UV) Detection or Electrochemical (EC) Detection.

UV Reagents:

Methanol: HPLC grade, Fisher Scientific Company, Fair Lawn, NJ 07410

Water: B & J Brand High Purity Solvent, Baxter Healthcare Corp., Burdick and Jackson Division, Muskegon, Michigan 49442

EC Reagents:

Water: B & J Brand High Purity Solvent, Baxter Healthcare Corp., Burdick and Jackson Division, Muskegon, Michigan 49442

Acetonitrile: UV, B & J Brand High Purity Solvent, Baxter Healthcare Corp., Burdick and Jackson Division, Muskegon, Michigan 49442

Phosphoric Acid: O-Phosphoric Acid 85% HPLC grade, Fisher Scientific, Co., Fairlawn, New Jersey 07410

Extraction Reagents:

Ethanol: (alcohol, anhydrous reagent), J.T. Baker Chemical Company, Phillipsbury, NJ 08665

Hexane: (Resi-analyzed), J.T. Baker Company, Phillipsbury, NJ 08665

Acetone: (Resi-analyzed), J.T. Baker Company, Phillipsbury, NJ 08665

Chloroform: Nanograde, Mallinckrodt, Paris, KY 40361 (no substitute)

Analytical Standard: Ethylene Thiourea Standard

Alumina Oxide: Adsorption alumina A-540, 80/200 mesh, Fisher Scientific Company, Fair Lawn, NJ 07410

Deactivated at 2.5% (see discussion for method of deactivation).

Gas

Chrom S: 45-60 mesh, Stock #*03005, AllTech Associates, Deerfield, IL 60015 (asterisk denotes bulk order number)

Celite 545: Stock #C212-500, Fisher Scientific Company, Fair Lawn, NJ 07410

Silanizing Reagent: Dimethyl Dichlorosilane CAT #3-3009, Supelco, Inc., Bellefonte, PA 16823

Silanizing Solution: 5% Dimethyl Dichlorosilane in hexane

Eluting Solution for Column Cleanup:

Ethanol:Chloroform (4:96) [Ethanol:Chloroform (6:94) may be required to attain suitable recoveries]

Decanol: 1-decanol, J.T. Baker Chemical Company, Phillipsbury, NJ 08665

Keeper Solutions:

- 1) 1% 1-decanol in acetone
- 2) 25% 1-decanol in ethanol

Ammonium
Hydroxide: Reagent grade, concentrated, VWR Scientific, Inc.,
San Francisco, CA 94119

Sodium
Chloride: Reagent grade sodium chloride, J.T. Baker Chemical
Company, Phillipsbury, NJ 08665

Equipment: Extraction

Whatman #4
Filter Paper: 9 cm, Whatman International Ltd., Maidstone, ENGLAND

Mason jars: Regular, one pint with lids

Omni Mixer: Equipped with short blade, OCI Instruments,
Waterbury, CT 06706 or Sorvall, Inc., Newton, CT

Evaporation
Flasks: 500 mL flasks, 24/40 Taper (Kimax or Pyrex)

Cleanup
Columns: 1 cm x 30 cm with solvent reservoir, equipped with
teflon stopcocks (Kimax)

Test tubes: 15 mL, conical bottom, graduated, glass (Pyrex or
Kimax)

Syringes: Hamilton 250 uL, 100 uL, 50 uL, 10 mL or 5 mL
AllTech Associates, Deerfield, IL 60015

Pipets: Class A, graduated

Filter Flasks: 500 mL with 9 cm Buchner funnel

Dry ice

Styrofoam ice chests

Glass wool

UV Detector:

HPLC Chromatographic Equipment:

Pumps; SP8700XR, SP8810, SP8770 and IsoChrom LC Pump (all
manufactured by Spectra Physics) used in combination with SP
8440 and Spectra 100 variable wavelength detectors - also
manufactured by Spectra Physics, San Jose, CA 96134

HPLC Column:

Adsorbosphere, C-18, 5 u, 25 cm x 4.6 mm, AllTech Associates, Deerfield, IL 60015

EC Detector:

HPLC Chromatographic Equipment:

Pump SP8810 Precision Isocratic Pump, manufactured by Spectra-Physics, San Jose, California

Detector consists of two components: The LC-4B Amperometric Detector and CC-4 Detector Cell and Faraday Cage. Detector is operated using the optional Gold-Mercury Amalgam Electrode in the oxidative mode. Manufactured by Bioanalytical Systems, West Lafayette, Indiana.

HPLC Column:

Hypercarb Graphitised Carbon, 7u, 10 cm x 4.6 mm, manufactured by Shandon, supplied by Keystone Scientific, Bellafonte, Pennsylvania.

During all analyses, equivalent apparatus, solvents, glassware, or techniques (such as sample concentration) may be substituted for those specified in the method.

Standard Preparation:

Ethylene Thiourea:

1. Correcting for purity, weigh 0.05 g into a 50 mL volumetric flask.
2. Dissolve the standard in 50.0 mL deionized water (DI water) for a concentration of 1000 ug/mL (1×10^{-3} g/mL).
3. 2.5 mL at 1×10^{-3} is diluted to 25 mL in DI water for a concentration of 100 ug/mL (1×10^{-4} g/mL).
4. Appropriate dilutions of this standard in DI water are used for spiking and as HPLC shooting standards.

Silanization of Glassware:

1. All glassware must be clean and dry before beginning the process.

2. Silanization must take place in a hood with the proper protective clothing.
3. Rinse glassware (coating with a thin layer) with silanizing solution - 5% dimethyl dichlorosilane in hexane.
4. Allow to dry thoroughly. Rinse thoroughly with DI water and then with acetone.
5. All glassware that comes into contact with the samples should be silanized. This includes: mason jars, graduated cylinders, filter flasks, evaporation flasks, columns and column tips, pipets, test tubes and glass wool.

Procedures:

Sample Preparation:

1. Crops are ground (in a Hobart grinder) or chopped, depending upon the matrix, while frozen with dry ice.
2. Samples must be kept frozen until the extraction solvent is added.

Weighing Samples:

1. Each frozen sample is weighed into a pint mason jar, capped and immediately placed in an ice chest containing dry ice.
2. When all samples have been weighed, more dry ice should be added to the ice chest to insure that samples remain frozen.

Extraction:

1. Raw Commodities (fresh vegetables, fruits and processed commodities)
 - a. To a 50.0 g frozen sample, add 75 mL DI water, break up with a glass rod and adjust pH to 11-12 with NH_4OH (check with pH paper).
 - b. Add 5 g NaCl, 5 g celite and 100 mL ethanol.
 - c. Blend for two minutes with Omni mixer.
 - d. Filter (using Buchner funnel) through a bed of celite, spread evenly on double filter paper.

- e. Rinse blender blade and jar several times with ethanol and add to Buchner funnel.
- f. Continue filtration until pad is dry.
- g. Transfer filtrate to a 250 mL graduated cylinder and bring to a volume of 250 mL with DI water and check pH again.

Note: pH should be in the range of pH 7-9 (adjust if necessary).

- h. Pour the filtrate back into the pint jar, transfer a 25 mL aliquot (5 g) to a preweighed 500 mL evaporation flask.
- i. Add 2 drops of 1% decanol keeper and rotovap at 45-50°C.
- j. Concentrate sample to approximately 9 g (by weight). Disconnect, weigh and add water to 13 g.
- k. Proceed to appropriate cleanup step (step number 2 or 3).

2. Hexane cleanup for nut meats:

- a. Proceed as above for extraction.
- b. Rotovap sample filtrate to 10-15 mL.
- c. Transfer to a 250 mL separatory funnel.
- d. Rinse the evaporation flask with 50 mL DI water and add to separatory funnel.
- e. Rinse the flask again with 50 mL hexane and add to the separatory funnel.
- f. Mix layers gently and allow to separate.
- g. Discard hexane layer and re-extract aqueous layer again with 50 mL hexane.
- h. Transfer the aqueous phase back into the flask. Add 2 drops of 1% decanol keeper.
- i. Rotovap to about 9 g and add water to 13 g.
- j. Proceed to column cleanup step.

3. Column Cleanup:

- a. Place a small plug of glass wool into the bottom of the cleanup column (equipped with stopcock).
- b. Pour 5.0 g deactivated alumina into the column, tap column to pack evenly and cap with another plug of glass wool.
- c. Place an evaporation flask containing 10 mL DI water and 6 drops of 25% decanol in ethanol keeper under the column to receive the eluate.
- d. Add 10.0 g Gas Chrom S to adjusted concentration in evaporation flask. Swirl and shake vigorously (approximately 30 seconds) until lump free.
- e. Add 75 mL eluting solution to flask and swirl for 30 seconds.
- f. Pour into the column containing the alumina.
- g. As Gas Chrom starts to dry, rinse flask with another 50 mL eluting solvent and add to column.
- h. Repeat for a total of four 50 mL portions of eluting solvent added. Allow columns to drip dry.
- i. Concentrate samples on rotovap (at 45-50°C) until chloroform is evaporated (approximately 8-10 mL).
- j. Add 4 mL ethanol and continue evaporation until volume is down to approximately 3 mL.
- k. Transfer to a graduated test tube and bring to a final volume of 5.0 mL with DI water. Final concentration of sample is 1 mL = 1 g. Submit for HPLC analysis.

Note: If at the 3 mL stage (step j) the sample extract is very cloudy, add 2 mL DI water. Evaporate again to approximately 3 mL and go to step k. If the sample is still cloudy at this point, the addition of 2 mL DI water and subsequent evaporation may be repeated but not more than a total of 3 times. This steps allows for evaporation of any organic solvent or excess decanol that may be present.

Sample Fortification:

1. A 50.0 g frozen sample (in a pint jar) is fortified (spiked) with an appropriate amount of ETU standard in DI water (using a microliter syringe or Class A graduated pipet).
2. Allow the standard to soak in for a moment, then add 75 mL DI water and proceed with the extraction (extraction section, step 1-a)

HPLC Analysis: UV Detector

Detector, pump and column have been discussed in the apparatus section. Typical conditions include the following specifics:

Mobile Phase:	5% methanol in water (HPLC grade solvents), sparged with helium.
Flow Rate:	0.5 mL/min
Absorbance:	0.01 aufs or 0.02 aufs
Wavelength:	233 nm
Loop Size:	100 uL
Chart Speed:	0.2 in/min
Retention Time:	Approximately 9 minutes

Conditions stated may require adjustments based on sensitivity of system.

The lowest standard should represent 60% of the detection limit and is used as the lowest point on the curve; a measured response of 5 mm or more will be considered an adequate response for this standard. Plot calibration curve on linear graph paper using peak height in (mm) versus nanograms ETU injected.

Calculation is made by use of the following equation:

$$\text{ppm} = \frac{\text{ng ETU}}{\text{mg sample injected}}$$

where, ng ETU is derived from the standard curve.

mg sample injected =

$$\frac{(\text{amount sample in grams}) (\text{mL aliquot}) (\text{uL injected})}{(\text{mL solvent}) (\text{final volume in mL}) (\text{dilution factor})}$$

The HPLC UV Detector method described herein has a detection limit of 0.01 ppm for ETU.

HPLC Analysis: EC Detector

EC (electrochemical) conditions: Detector, pump and column have been discussed in the apparatus section. Typical conditions include the following:

- * Mobile Phase: 1% acetonitrile in 0.0218M H₃PO₄ (Burdick and Jackson Solvents, Fisher H₃PO₄) sparged with helium.
- Flow Rate: 1.0 mL/in
- Range: 5 NAFS (nanoamps full scale)
- Applied Voltage: +0.350V
- Loop Size: 100 uL
- Chart Speed: 0.2 in/min
- Retention Time: Approximately 5 minutes

* Raw Potatoes generally require 0.5% acetonitrile in 0.0218M H₃PO₄.

Conditions stated may require adjustments based on sensitivity of system.

Prepare a four point calibration curve daily. The lowest standard should represent 60% of the detection limit and is used as the lowest point on the curve; a measured response of 5 mm or more will be considered adequate for this standard. Plot calibration curve on linear graph paper using peak height (in mm) versus nanograms ETU injected. Draw line of best fit through the points using a Boatman's curve.

Calculation is made by use of the following equations:

$$\text{ppm} = \frac{\text{ng ETU}}{\text{mg sample injected}}$$

where, ng ETU is derived from the standard curve.

mg sample injected:

$$\frac{(\text{amount sample in grams})(\text{mL aliquot})(\text{uL injected})}{(\text{mL solvent})(\text{final volume in mL})(\text{dilution factor})}$$

The HPLC EC Detector method described herein has a detection limit of 0.002 ppm for ETU.

For UV or EC Detection:

Historically, the column and conditions stated in the method have been satisfactory for the matrix (matrices) being analyzed. The specific column packing, mobile phase, column temperature and flow rate listed are typical conditions for this analysis. Specific conditions for each HPLC run will be noted on each chromatogram and will not otherwise be documented.

Discussion:

1. In the case of dry sample, such as almond hulls or corn forage, the incorporation of the following hydration step was found to be necessary to produce adequate spike recoveries.

To 20.0 g sample, add 25-50 mL water and allow to stand several minutes. Process as usual, adding extraction solvents. Final concentration will be 1 mL = 0.4 g. For some matrices, such as potato flakes, it is necessary to use a smaller amount of sample - 10 g, and a larger aliquot - 50 mL to keep the same final concentration of 1 mL = 0.4 g.

2. During the initial concentration, the waterbath temperature should be 45-50°C. Temperatures in excess of 55°C may cause lower spike recoveries.

3. Cleanup:

- a. Exactly 5.0 g alumina and 10 g Gas Chrom S are used.
- b. The initial 75 mL eluting solvent is used to produce a slurry with the sample in Gas Chrom S.

- c. Six drops of 25% decanol keeper is used for final concentration, as this gives higher recovery than the more dilute keeper.

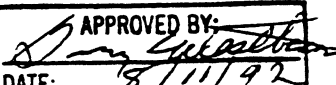
4. Deactivation of Alumina:

- a. To completely activate the alumina, heat in an oven (130°C) for a minimum of 24 hours.
- b. The deactivation should take place the previous day before the alumina is needed (approximately 16-18 hours).
- c. Use a glass bottle with a ground glass stopper (capacity 300-500 mL). Weigh 117.0 g of activated alumina, stopper and allow to cool to touch.
- d. Add 3.0 mL D.I. water to the alumina to deactivate it at 2.5%. Stopper again and shake the bottle gently to thoroughly mix and eliminate all lumping.
- e. Seal the stopper to the bottle with masking tape. This amount (total 120.0 g) will be enough to do 24 sample column cleanups using 5.0 g per column.

Note: If the glass-stoppered container is kept air-tight (unopened), it may be used up to 3 days after the deactivation has occurred.

- 5. When adjusting the pH in the extraction step, add the concentrated NH_4OH dropwise until pH 11-12 is just obtained. NH_4OH will not raise the pH higher than that, so adding excessive amounts of NH_4OH will not benefit the extraction of ETU. Do not adjust the pH to less basic by adding acid to the extract. If necessary, try this method of pH adjustment on a control sample for each particular matrix to determine how much NH_4OH is needed to adjust the sample to the correct pH for extraction.
- 6. It is important to complete entire extraction procedure in one day. This insures higher and more consistent spike recoveries.

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APPROVED BY: 
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