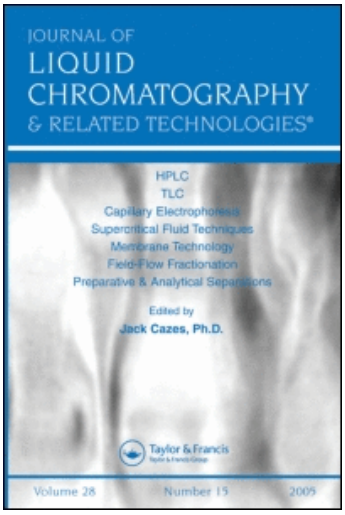

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LIQUID CHROMATOGRAPHIC-ELECTRO-CHEMICAL TECHNIQUE FOR DETERMINATION OF ETHYLENETHIOUREA RESIDUES

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ABSTRACT

A high performance liquid chromatographic-electrochemical (HPLC-EC) technique was developed to selectively determine ethylenethiourea (ETU) at residue levels without derivatization. ETU was eluted from a C-8 column with water, a phosphoric acid electrolyte solution was added to the column eluate, and then ETU was detected with an electrochemical detector containing a Au/Hg working electrode. The HPLC-EC system produced a sharp chromatographic peak for ETU that was detected by the Au/Hg electrode at an applied potential of +0.36 V. With detector sensitivity adjusted so that 10 ng ETU produced a 50% full scale deflection peak (1% baseline noise), the detector's response was linear from 2 to 400 ng ETU. No peaks were observed in potato and spinach controls, and only small apparent ETU peaks of 7 and 3 ppb, respectively, were found in apple and grape controls. Detector response was equivalent to 90% of actual ETU added (0.1 ppm) to purified spinach extracts. Crop coextractives from apples, grapes and potatoes did not affect detector response to ETU at the 0.1 ppm fortification level.

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INTRODUCTION

Ethylenethiourea (ETU), a suspected carcinogen, is a degradation product of the ethylenebisdithiocarbamate (EBDC) fungicides (mancozeb, maneb, metiram and zineb). Over 130 million lb of these fungicides are used yearly on agricultural crops throughout the world (1). Thus a need exists to determine the levels of ETU residues on agricultural crops.

Bottomley et al. (2) have recently reviewed the published analytical methods for determination of ETU. The most prevalent methods are those which form a derivative of ETU prior to determination by gas-liquid chromatography (GLC) (3-6). Derivatization avoids on-column degradation and/or adsorption problems. However, as noted by Otto et al. (7), the main disadvantage of these ETU derivatizations is failure of the reactions to proceed quantitatively in the presence of plant coextractives. Several authors (7-10) have reported the direct determination of ETU by using capillary GLC. Although on-column ETU degradation and/or adsorption did not appear to be a problem with capillary GLC, numerous extraneous chromatographic peaks from the samples were observed, even when selective detectors such as the flame photometric detector (sulfur mode) (9) were used.

Several authors (11-16) have used high performance liquid chromatography (HPLC) for the direct determination of ETU by using UV detectors. Because of the multitude of UV absorbing crop coextractives and pesticides, the UV detector does not provide adequate selectivity for detection of ETU residues. Hanekamp et al. (17) investigated the application of an HPLC dropping mercury electrode (DME) and glassy carbon electrode amperometric detector for detection of ETU. They concluded that for ETU the DME provided superior selectivity but was significantly inferior in sensitivity to the glassy carbon electrode. Commercially available DME detectors investigated in our laboratory have not provided the sensitivity or stability desired for detection of ETU residues. Allison and Shoup (18) utilized an HPLC Au/Hg electrode amperometric detector for direct detection of thiols. This detector provided the selectivity of the DME detector and a potential for improved sensitivity.

The purpose of the work presented here was to determine the applicability of the HPLC-Au/Hg electrochemical (EC) technique for the selective determination of ETU at residue levels. The electrochemical characteristics of ETU with the Au/Hg electrode were initially investigated. HPLC parameters were investigated which would provide for good ETU resolution and sensitivity, system stability, and be compatible with the EC detector. Lastly, the effects of crop coextractives on the HPLC-EC system's selectivity and response stability to ETU were studied.

MATERIALS

Chemicals

Water was purified using a Milli-Q water purification system from Millipore (Bedford, MA). Phosphoric acid (85%) was HPLC grade from Fisher (Fair Lawn, NJ). The Environmental Protection Agency (Research Triangle Park, NC) provided the ETU reference standard, which was dissolved and diluted to appropriate concentrations with Milli-Q purified water.

Apparatus

A CV-27 cyclic voltammeter from Bioanalytical Systems (West Lafayette, IN) was used for the static EC studies. Au/Hg and glassy carbon working electrodes, a Ag/AgCl reference electrode, and a platinum auxiliary electrode were used. A scan rate of 200 mV/s and gain (sensitivity) of 0.1 mA/V were used.

The chromatographic determinative system is diagramed in Figure 1. The mobile phase and aqueous electrolyte solutions were contained in Ultraware HPLC solvent reservoirs (Kontes, Vineland, NJ) and degassed with helium (99.995%) purified with in-line Hydro-Purge II and Oxy-Purge traps (Alltech Associates, Inc./Applied Science Labs, Deerfield, IL). These solutions were delivered with two Model SP8700XR pumps (Spectra-Physics, San Jose, CA). Injections were made into the column with a Spectra-Physics Model SP8780XR autosampler fitted with a 20 uL loop. The stainless steel guard column (2 cm x 2 mm i.d.) was packed with 30-40 um Perisorb RP-8 pellicular

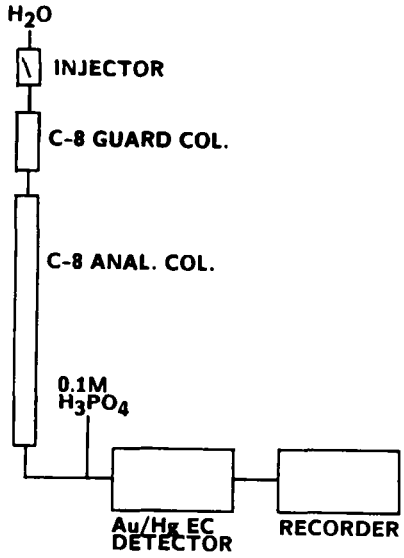

FIGURE 1. ETU HPLC-EC determinative system.

material (Upchurch Scientific Inc., Oak Harbor, WA). The stainless steel analytical column (25 cm x 4.6 mm i.d.) was packed with 6 um spherical Zorbax C-8 material (DuPont, Wilmington, DE). The temperature of the columns was controlled by a Model 2080 HPLC column oven (Varian, Palo Alto, CA). A stainless steel column (30 cm x 2.1 mm i.d.) packed with 10 um spherical PRP-1 material (Hamilton Co., Reno, NV) was placed between the electrolyte solution pump and postcolumn tee to provide 70 bar (1000+ psi) back pressure for the pump. (The PRP-1 column was packed according to manufacturer's instructions.) The column eluate and electrolyte solution were combined at an Upchurch 0.5 mm i.d. stainless steel tee and then passed through a Model LC-17 thin-layer EC cell (Bioanalytical Systems). The cell was equipped with a Au/Hg working electrode, a Ag/AgCl reference electrode and a stainless steel block auxiliary electrode. Two 0.127 mm thick gaskets were used to prevent electrical shorting between the working and auxiliary electrode surfaces. The potential was applied with a Model LC-4B amperometric

detector controller (Bioanalytical Systems). All chromatograms were recorded on a Spectra-Physics Model 4200 computing integrator.

HPLC Operating Parameters

The column oven was operated at 60°C. The flow rates of the mobile phase and electrolyte solution were adjusted to 1.50±0.02 and 0.50±0.02 mL/min, respectively. (Note: Flow rates were determined by timing the collection of a measured volume of eluate after the detector.) A potential of +0.360 V was applied to the working electrode, unless stated otherwise. Detector sensitivity was adjusted so that 10 ng ETU produced approximately 50% full scale response on the computing integrator set at an attenuation of 8 with 10 mV detector output. The time constant was 0.3 Hz.

METHOD

Crop samples were prepared for the HPLC-EC determination by a modification of the official Association of Official Analytical Chemists (AOAC) method (3). The fresh crop was extracted with an aqueous-methanol solution according to the AOAC method. A portion of the concentrated filtrate representing 20 g crop was concentrated to approximately 10 mL by using the apparatus and conditions described by Krause (19). ETU was separated from crop coextractives with the Gas Chrom S/alumina column as described in the official ETU method. The ethanol/chloroform eluate was evaporated to dryness with a rotary evaporator (19). The residue was immediately dissolved in 4.0 mL water or 4.0 mL water containing 2 ug ETU and filtered through a 0.45 um Nylon-66 filter before direct determination of ETU by the HPLC-EC system.

RESULTS AND DISCUSSION

ETU EC Characteristics (Static)

Cyclic voltammetric data were collected to determine the effect of electrode, electrolyte and solution pH on the ETU peak potential. Water or 10% acetonitrile in water was used as solvent to simulate probable HPLC mobile phases for ETU. ETU and electrolyte concentrations were 1 mM and 0.1 M, respectively.

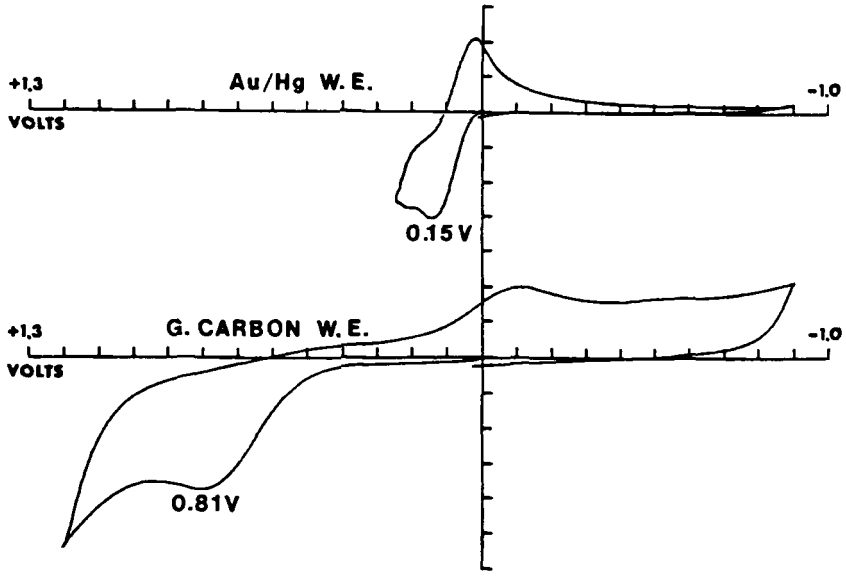

FIGURE 2. Cyclic voltammograms of 1mM ETU in 0.1 M H_3PO_4 using parameters described in text. W.E. = working electrode.

With the glassy carbon electrode, the ETU oxidation peak potential was lowest at +0.81 V with $LiClO_4$ (see Figure 2), +1.0 V with H_3PO_4 and greater than +1.2 V with NaOH as electrolytes. Because phenols and anilines also oxidize at these high potentials, the glassy carbon electrode did not appear to offer the selectivity needed for the determination of ETU residues. The data from this work are in agreement with the findings of Hanekamp et al. (17).

Anodic oxidation of thioureas with a mercury electrode surface produces the corresponding formamidine disulfide salt (20).

This EC reaction occurred at a low oxidation potential for ETU with the Au/Hg electrode. The ETU oxidation peak potential was approximately +0.15 V with $LiClO_4$ or H_3PO_4 electrolytes in either water or 10% acetonitrile in water. The peak potential of ETU was better separated from the mercury oxidation potential with H_3PO_4 rather than with $LiClO_4$ as the electrolyte. Figure 2 shows the well defined wave obtained for ETU in aqueous H_3PO_4 with the Au/Hg electrode. The cyclic voltammogram obtained for ETU in NaOH was complex and the peak potential was difficult to determine. Nyman and Parry (21) reported that a single well-defined anodic wave was obtained for thiourea in solutions ranging from approximately pH 1 to 5. They reported that above pH 5 the wave became ill-defined. Santhanam and Krishnan (22) reported that for thiourea with sodium hydroxide as the electrolyte, the platinum electrode became passivated in a matter of minutes, possibly due to a film of the oxidation product. They reported that an acidic medium of pH less than 3 was needed to eliminate fouling of the electrode. The effect of $HClO_4$, HNO_3 and H_3PO_4 electrolytes on the oxidation peak potential of ETU was determined. The three acids produced similar oxidation peak potentials and peak heights for ETU by the Au/Hg electrode. Phosphoric acid was selected as the acidic electrolyte of choice because of its buffering capacity.

Selection of Chromatographic Parameters

Reverse phase HPLC columns were selected for study because the aqueous mobile phases used with these columns are compatible with EC detectors. ETU eluted from CN, C-8 and C-18 bonded 6 um spherical silica based columns in 153, 139 and 133 s, respectively, with a 10% acetonitrile in water mobile phase. Column efficiencies were similar for the three columns. The longer retention time of ETU with the CN column was preferred since it provided somewhat better separation from the injection solvent (water) peak. However, according to the manufacturer, the C-8 bonded packing is more stable than the CN bonded packing and thus was selected for further study. ETU retention time increased to 278 s with 100% water as the mobile phase. Because of the potential difficulty in maintaining consistent low level acetonitrile

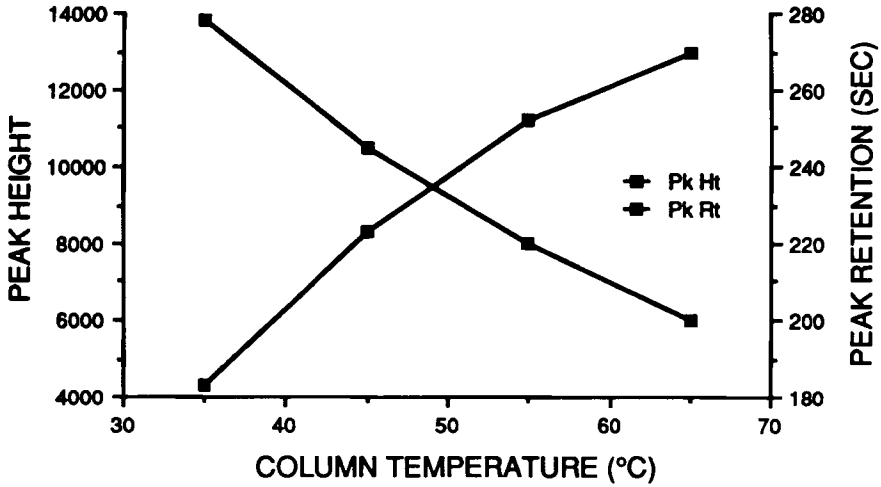

FIGURE 3. Effect of column of temperature on ETU peak height and peak retention. Peak height in computing integrator units.

concentrations, the 100% water mobile phase was preferable, although the longer retention time of 278 s caused a decrease in ETU peak height.

The effect of column temperature on ETU retention time and peak height was examined (Figure 3). As expected, higher column temperature increased ETU peak height and decreased ETU retention time. The slope of peak height and peak retention time change was smallest at the higher column temperatures. A column temperature of 60°C provided an ETU retention time of 210 s, a threefold increase in peak height over that obtained for a column at 35°C. The 60°C temperature was 5°C below the manufacturer's recommended maximum column temperature. The phosphoric acid electrolyte was added to the column eluate after the column rather than to the mobile phase since C-8 columns are not stable at very low pH. During the 10 months the C-8 column was in use, the ETU retention time decreased slowly from 210 to 155 s, indicating slow column deterioration.

Selection of EC Parameters

The two basic cell designs available for the Au/Hg electrode are the thin-layer and wall-jet cells. The Bioanalytical Systems Model LC-17 thin-layer cell and Environmental Systems Associates Model 5012 wall-jet cell units were investigated. At optimum applied potentials, detector sensitivity settings were adjusted so that 20 ng ETU produced a detector response of approximately 50% FSD. At this sensitivity the thin-layer cell/detector produced a very stable baseline with 0.5% (1 mm) baseline noise, while the wall-jet cell/detector produced a wandering baseline (±10%) with erratic spikes and a 1% short-term baseline noise. Several wall-jet cells from the same manufacturer were tested and all performed in a similar manner. The difference in performance of the thin-layer and wall-jet cells may be due to their basic design differences and the area of their Au/Hg electrode surfaces of 8.5 and 3.4 mm^2, respectively. The thin-layer cell was used in subsequent investigations.

The effect of applied potential on the detector's response to ETU was determined to establish the potential that provided high detector response, low background current and response stability. Response data are graphically presented in the hydrodynamic voltammogram shown in Figure 4. The applied potential of +0.360 V appeared optimum as response was high and reasonably stable, and background current lowest (2 nA). Above +0.400 V the mercury surface started to oxidize and at +0.440 V this oxidation resulted in a background current of 600 nA.

Detector response to ETU increased by 10% as H_3PO_4 concentration increased from 0.04 to 0.4 M. Short term baseline noise also increased from 0.5 to 1% with increased H_3PO_4 concentration. A H_3PO_4 concentration of 0.1 M (0.025 M in cell) was selected to provide the best signal to noise ratio.

Detector Response Stability and Linearity to ETU

Five replicate injections each of 10 ng ETU resulted in a peak height response coefficient of variation of 0.7%. A chromatogram of the ETU standard is shown in Figure 5.

A log-log plot of detector response (peak height) versus nanograms ETU injected showed a linear response to ETU from 2 to 400

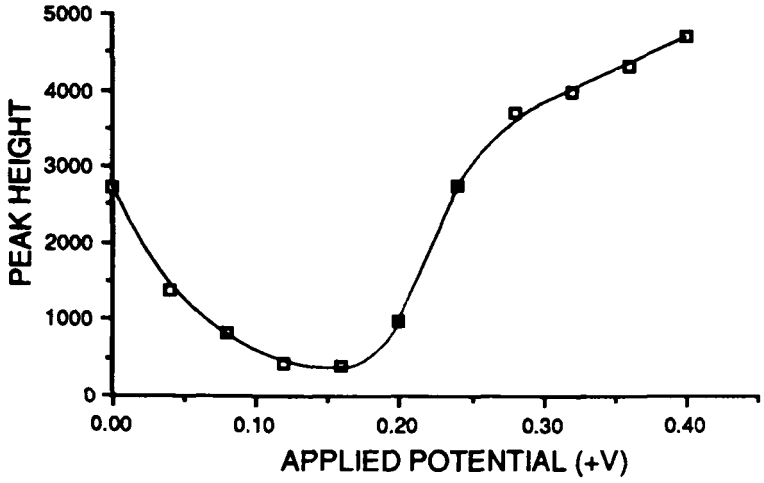

FIGURE 4. Hydrodynamic voltammogram of ETU. Peak height in computing integrator units.

ng. Above 400 ng the gain amplifiers were saturated and were not able to amplify the signal further.

Effect of Crop Coextractives on the System and Detector's Response to ETU

The system's stability under typical laboratory conditions and detector selectivity were determined with purified extracts of apples, grapes, potatoes and spinach. These crops were selected based on EBDC usage and/or frequency of reported ETU residue findings. The individual fresh raw crop was chopped, and portions were immediately extracted and coextractives removed according to the described method. Twenty uL aliquots representing 100 mg of crop were injected into the HPLC column.

Chromatograms of the crop controls are shown in Figure 5. The apple and grape controls contained apparent residues of ETU of 7 and 3 ppb, respectively. Except for the injection solvent peak, no other peaks are observed. Thus it appears that the HPLC-EC system is highly selective in its response to ETU.

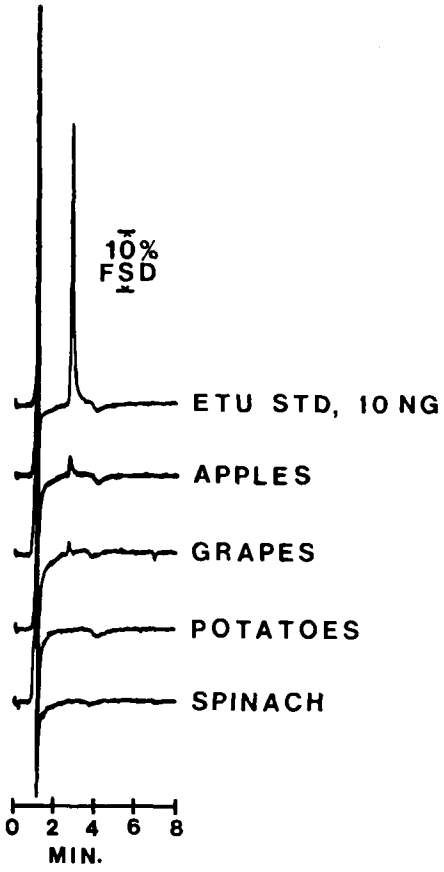

FIGURE 5. HPLC-EC chromatograms of ETU and crop controls *using parameters described in text.*

The effect of crop coextractives on detector response to ETU was studied by fortifying purified extracts of the crop controls with ETU at 0.1 ppm. The analyses were performed in duplicate for each crop. The percent recoveried were: apples 109 and 100, grapes 100 and 93, potatoes 99 and 102 and spinach 89 and 90. Crop coextractives from apples, grapes and potatoes did not affect detector response to ETU at the 0.1 ppm fortification level because "recovery" values were near 100%. Detector response was equivalent to 90% of actual ETU added (0.1 ppm) to purified spinach extracts. Although the reason for the 10% low "recovery" values obtained for spinach is unknown, the final purified extract was slightly yellowish green, indicating the presence of carotenes and chlorophyll coextractives.

In conclusion, it has been demonstrated that this HPLC-EC technique will provide the selectivity, sensitivity and stability needed for determining ETU residues in crops. Future investigations will determine the recovery of ETU through the AOAC method (3) with changes as stated in this paper.

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