Table VI. Residues of 2,4,5-T and 2,4,5-Trichlorophenol in Milk Determined by Procedures with and without Acid Hydrolysis

2,4,5-T in diet, ppm	Residues found, ppm	
	Hydrolyzed	Not hydrolyzed
	2,4,5-T	
0	0	0
300	0.19	0.17
300	0.07	0.09
300	0.26	0.22
1000	0.26	0.27
	2,4,5-Trichlorophenol	
1000	0.19	0.20
1000	0.21	0.18

best indicated in Table III for 2,4,5-T fed at 1000 ppm. Daily variation of results for a single cow, No. 30, was as much as 0.29–1.0 ppm, which was as great as variation between cows on a given day, *e.g.*, 0.23–1.0 ppm on day 16.

Residues increased as chemical feeding rates increased. At all feeding rates residues had reached a plateau by the time sampling began on the second or third day. At the highest feeding level, phenoxy acid residues in milk ranged from approximately <0.005 to 0.04% of the concentration in the diet for the four compounds. This is similar to the 0.02% found for 4-amino-3,5,6-trichloropicolinic acid (Kutschinski, 1969).

The acid hydrolysis procedure was used for all samples only because it facilitated separation of the phases by centrifugation during the extractions with diethyl ether. Some milk samples from the 2,4,5-T experiment were prepared without the acid hydrolysis to check the possibility that this acid treatment might be liberating phenoxy acid or phenol which was bound physically or chemically to natural constituents of milk. The results indicate that no binding of either phenoxy acid or phenol occurred (Table VI). A similar conclusion was reached for 2,4-D (Yip and Ney, 1966).

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Determination of Ethylenethiourea Residues in Apples

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Ethylenethiourea was determined in apples in the 0.01–1.00 ppm range with an overall recovery of 94.7 ± 5.9%. The procedure involves conversion to the *S*-benzyl derivative, followed by extraction, trifluoroacetylation, and quantitative measurement by gas–liquid chromatography. The cleanup is rapid and effective. Specific confirmation of the derivative was carried out by mass spectrometry. Commercial samples were found to contain 0.018–0.044 ppm of ethylenethiourea.

Ethylenethiourea (ETU) has been reported as a decomposition product of a widely used group of fungicides, the ethylenebisdithiocarbamates (Ludwig *et al.*, 1954; Czegledi-Janko, 1967). The compound has also been described as tumorigenic in mice (Innes *et al.*, 1969). Reports in the literature vary as to whether ETU is accumulated by plants (Ross and Ludwig, 1957; Vonk and Kaars Sijpesteijn, 1970) or is rapidly dissipated (Yip *et al.*, 1971). Because of the potential health hazard, methods are required for determining the residue on or in foods.

ETU is highly polar, being soluble in water or lower alcohols and making extraction without accompanying interfering substances difficult. Further, the unmodified compound was found unsuitable for gas–liquid chromatography. A recently described method for ETU (Onley and Yip, 1971) is highly sensitive but requires extensive cleanup and is not amenable to rigorous confirmation. The analytical procedure presented here involves conversion of ETU to a known compound, *S*-benzyl ETU, and cleanup by solvent extraction followed by trifluoroacetylation and quantitation by gas–liquid chromatography. Precise confirmation of the derivative has been carried out by combined glc–mass spectrometry.

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EXPERIMENTAL

Materials. 2-Imidazolidenethione (ETU) was purchased from Eastman Organic Chemicals, Rochester, N. Y., and was recrystallized twice from 95% ethanol to give a white crystalline product, mp 201–203°. The recrystallized ETU was dissolved in water and added to samples of apple in volumes of less than 0.50 ml.

The *S*-benzyl ETU used as a reference standard was pre-

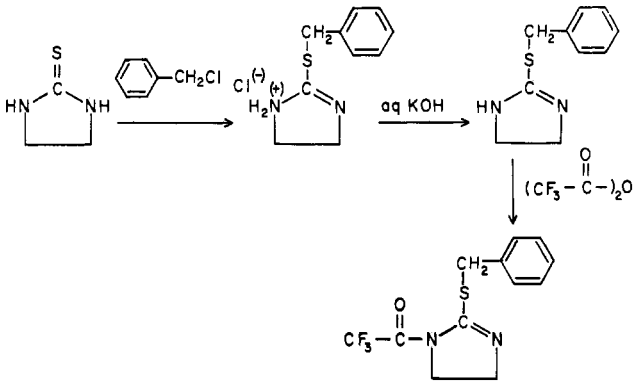

Figure 1. Reaction scheme for the derivatization of ETU

Table I. Determination of Ethylenethiourea in Fortified Apples

Ethylenethiourea added, ppm	Ethylenethiourea found, ppm	Mean recovery, %
0.0103	0.0098	
	0.0098	95.2
0.0309	0.0250	
	0.0305	89.0
0.0515	0.0422	
	0.0482	88.0
0.0721	0.0620	
	0.0638	87.2
0.103	0.099	
	0.107	100.0
0.206	0.214	
	0.212	103.5
0.412	0.440	
	0.444	107.0
0.618	0.534	
	0.570	89.3
1.03	0.944	
	0.984	93.5
	Overall recovery 94.7 ± 5.9%	

pared by refluxing ETU (1.02 g, 0.01 mol) with benzyl chloride (1.90 g, 0.015 mol) in 95% ethanol for 30 min. The solvent was removed on a rotary evaporator and the residue taken up in water. After extraction with benzene, the aqueous phase was made alkaline with 1 *N* KOH and the free base was extracted with benzene. The material was crystallized twice from warm benzene to give the product: mp 69–70°, lit. 69–69.5° (Baer and Lockwood, 1954).

Standard solutions of trifluoroacetylated *S*-benzyl ETU were prepared by dissolving *S*-benzyl ETU in 10% (v/v) trifluoroacetic anhydride in benzene and diluting to the desired concentration with benzene containing 1% trifluoroacetic anhydride. The standards thus prepared were stable at ambient temperature over a period of several weeks. Aliquots were removed when required, the solvent was evaporated under a stream of nitrogen, and the compound was taken up in an appropriate volume of benzene for injection into the chromatograph.

Extraction, Derivatization, and Cleanup. Samples of apple (5.0 g) were homogenized with a 10:1 volume:weight ratio of methanol in a Sorvall Omni-Mixer. The homogenate was filtered through Whatman No. 1 filter paper using a slight negative pressure and a 10.0-ml aliquot (equivalent to 1.0 g of sample) placed in a 50-ml round-bottomed flask. Water (10.0 ml) and benzyl chloride (0.1 ml) were added and the contents were refluxed for 30 min. The alcohol was removed on a rotary evaporator and 1 *N* HCl (1.0 ml) was added. The samples were transferred to 125-ml separatory funnels with 20 ml of water and extracted with two portions of chloroform (1 × 10 ml, 1 × 5 ml) which were discarded. After the addition of 1 *N* KOH (5.0 ml) to the aqueous phase, the *S*-benzyl ETU was immediately extracted with a further portion (10.0 ml) of chloroform. The extract was dried by passage through sodium sulfate and the solvent was removed by evaporation with nitrogen. A solution of 10% trifluoroacetic anhydride in benzene (0.5 ml) was added and the samples were allowed to react for 15 min at ambient temperature. The solvent was evaporated just to dryness under a stream of nitrogen and a known volume of benzene was added for injection into the gas chromatograph.

Preparation of Samples for Confirmation by Glc–Mass Spectrometry. Samples of apple (50 g) were homogenized with methanol (75 ml) and the homogenates were filtered. Water (75 ml) and benzyl chloride (5 ml) were added to the total filtrate in a 250-ml flask and the contents were refluxed for 30 min. After removal of the alcohol, water (75 ml) was added and the aqueous phase was extracted twice with chloroform (1 × 50 ml; 1 × 25 ml). The free base was then extracted into chloroform (50 ml) after adding 1 *N* KOH (38 ml). For further cleanup, the *S*-benzyl ETU was extracted into 1 *N* HCl (10 ml) and then back into chloroform (20 ml) after adding 1 *N* KOH (15 ml). This procedure was repeated, with the compound being extracted into a final 5 ml of chloroform. The solution was dried with sodium sulfate, transferred to a conical vial, and the solvent was evaporated with nitrogen. Trifluoroacetylation was effected with 10% trifluoroacetic anhydride in benzene, as described previously, and the trifluoroacetate was injected into the glc–mass spectrometer as a solution in methylene chloride.

Gas–Liquid Chromatography. Routine quantitative determinations were performed on an Aerograph 705 fitted with a glass injection insert and tritium foil electron capture detector. The 40 cm × 4 mm glass column was packed with 2% butanediol succinate on 100–120 mesh Chromosorb W, HP, and was conditioned overnight at 200°. The operating parameters were: injection port, 200°; column, 195°; nitrogen flow rate, 60–80 ml/min. Under these conditions, trifluoroacetylated *S*-benzyl ETU had a retention time of 5.5 min. The samples were quantitated by comparison of the peak height with that of an appropriate standard.

Glc–Mass Spectrometry. Analyses were carried out with a Perkin-Elmer 990 gas chromatograph coupled to a Hitachi–Perkin-Elmer RMS-4 mass spectrometer. The gas chromatograph was fitted with a flame ionization detector and 6 ft × 1/4 in. glass column packed with 3% OV-210 on 100–120 mesh Chromosorb W, HP. Typical operating parameters were: injection port, 225°; column, 180°; helium flow, 30 ml/min. The mass spectrometer was operated with an ionizing voltage of 80 eV, source temperature of 250°, and interface at 225°.

RESULTS AND DISCUSSION

The use of alcohol for extraction of the sample was preferable to aqueous alcohol or water, since the latter extractants removed substances which produced emulsions in the subsequent partitioning steps. Even with larger sample sizes phase separation was rapid and complete.

The reaction scheme for derivatization of ETU is shown in Figure 1. Benzylation was found to be complete after 30 min refluxing in 50% aqueous methanol. Slightly lower yields were observed with methanol alone as the solvent. Formation of the S-alkylated hydrochloride is of considerable ad-

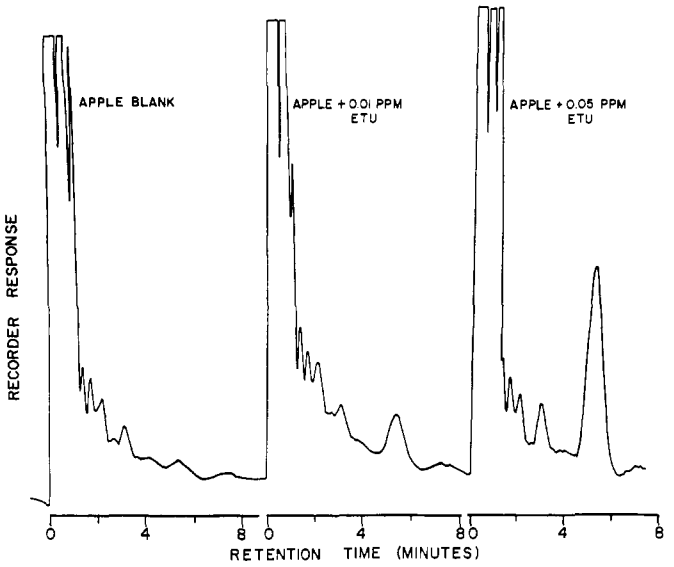

Figure 2. Gas–liquid chromatograms of derivatized apple extracts in the absence and presence of added ETU. Each injection represents the equivalent of 5 mg of sample

vantage over other derivatives, such as the *N,N'*-diacetate, since the former is water soluble, permitting a preliminary extraction of interfering substances without recourse to lengthy cleanup procedures. In contrast to other S-alkylated derivatives, the benzylated compound was found sufficiently stable in alkali to be liberated as the free base and quantitatively extracted with organic solvents. Trifluoroacetylation was found to proceed rapidly at room temperature and the resulting trifluoroacetate was stable for several hours with benzene as solvent. Some losses were encountered when the samples were not taken up in benzene immediately after evaporation of the trifluoroacetylating solution, presumably due to volatilization or hydrolysis.

The recovery of ETU added to apple samples in the 0.01–1.03 ppm range is given in Table I. At each level, extracts equivalent to 1.0 g of sample were used. Typical gas–liquid chromatograms are shown in Figure 2. In each case, the trifluoroacetylated sample was taken up in 200 μl of benzene and 1.0-μl aliquots were injected. The lower limit of sensitivity was approximately 0.005 ppm of ETU, using 1.0-g samples. It has been found possible to increase the sample size tenfold without an appreciable increase in background, suggesting a much lower minimum detectable limit. However, because of the limitation of sample size required for glc–mass spectrometric confirmation, recovery studies were not pursued at lower levels.

Table II. Glc–Mass Spectral Data for Trifluoroacetylated *S*-Benzyl ETU and Benzylated, Trifluoroacetylated Apple Extract

m/e	Trifluoroacetylated *S*-benzyl ETU relative intensity, %	Apple extract relative intensity, %
288	36	37
255	5	5
219	20	24
191	8	8
179	7	8
167	10	11
91	100	100

Several samples of apples purchased locally were found to contain levels of ETU ranging from 0.018 to 0.044 ppm. Glc–mass spectrometry was used to confirm that the peak occurring on glc was due to the ETU derivative. Using standards of trifluoroacetylated *S*-benzyl ETU, it was found that 0.20 μg were adequate to produce clear fragmentation patterns in the mass spectrometer. It was further evident from an abundant parent ion peak at *m/e* 288 that the trifluoroacetate survived glc without decomposition. The mass spectral data comparing the glc peak obtained from a 50-g apple extract with that of a standard are shown in Table II. From the similarity of the patterns the presence of ETU in the sample was confirmed.

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