Table I. Reproducibility of Method Demonstrated by Independent Determinations

Sample no.	$\bar{X}$, mg/g of D.M.	% av dev. from mean	No. of det.
4-2	1.66	1.8	2
2-12	1.06	3.3	4
2-3	0.61	2.5	2
8-12	0.32	4.8	4
9-10	0.31	4.8	2
8-3	0.11	2.3	2
6-9	0.07	3.6	2
7-5	0.06	0.0	4

standard solutions of perloline monohydrochloride were used. Variation in recoveries between days and within runs was noted. By systematic elimination of possible sources of error which could account for this variation, only column flow rate remained to be checked. When the flow rate of the sample onto the cationic column was too slow (1.5 ml/min), there appeared to be more perloline trapped inside the matrix of the resin beads which then failed to be eluted in the final step. This resulted in low recoveries. When the flow rate was increased to 5.0 ml/min the perloline was distributed over more resin beads; however, eluting with alcoholic ammonia at this flow rate resulted in low recoveries. The low recovery may have been due to the lack of ammonium ion penetration for exchange in the matrix of the beads and/or incomplete solublization of the perloline which was eluted from the resin beads and precipitated on the column by the aqueous ammonia wash. By plotting recoveries for varied flow rates at each step, it was concluded that reproducible results could be obtained with a single flow rate for all steps in the cleanup procedure. A flow rate between 2.5 and 3.5 ml/min gave optimum recoveries. Using this range of flow rates, the amount of perloline recovered was a linear function of the concentration (0.1–3.0 mg/sample) applied to the cation column. By a graphical procedure, a slope factor of 0.925 was calculated. This corresponds to a 92.5% recovery. The same value was obtained when known amounts of perloline were added to grass samples at the time of extracting when compared to their base-line perloline values.

Reproducibility of the procedure was determined by independent analysis of several grass samples having different concentrations of perloline. Results of these experiments are presented in Table I. The procedure has proven to have a high degree of precision at all concentrations.

The procedure described is a sensitive, precise method for determining perloline in grass samples. In samples which do not contain interfering compounds the column cleanup could be by-passed and the loss of perloline reduced. The fluorometric method, with a detection limit in the range of 1.0 ng/ml, could be developed for different types of samples, such as feces or urine in ruminant metabolism studies.

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A Method for the Determination of Ethylenethiuram Monosulfide on Food Crops

A method was developed for the determination of ethylenethiuram monosulfide on food crops by gas–liquid chromatography. The procedure involves extraction with toluene followed by acid-base cleanup and yielded recoveries generally greater than 90%. The minimum detectable limit was 0.01 ppm.

Ethylenethiuram monosulfide (ETM) is a fungitoxic compound formed by aeration of ethylenebis(dithiocarbamate) fungicides (Ludwig *et al.*, 1954, 1955). The proposed structure of ETM (Thorn and Ludwig, 1954) has been revised (Pluijgers *et al.*, 1971) and the revision confirmed by ^{13}C nmr studies (Alvarez *et al.*, 1973) and by synthesis (Beer and Naylor, 1973).

While studies on the aeration of ethylenebis(dithiocarbamates) have indicated yields of up to 34% ETM (Ludwig *et al.*, 1954; Hylin, 1973), the levels of ETM remaining on crops as a result of agricultural usage remain uncertain. The following describes a glc method capable of determining ETM on food crops and supplementing the existing polarographic method (Engst and Schnaak, 1970).

EXPERIMENTAL SECTION

Materials. ETM was obtained from Robinson Brothers Ltd., West Bromwich, Staffs., England. The material was purified before use by column chromatography on silicic acid (Mallinckrodt, 100 mesh), using a developing solvent of chloroform–ethyl acetate (1:1) as described (Pluijgers *et al.*, 1971). The purified material had a mp of 125–126°.

Table I. Effect of Concentration of Acid on the Extraction of ETM from Toluene[a]

Concn HCl, *M*	ETM remaining in toluene, %
0.05	98.1
0.10	98.6
0.50	70.0
1.0	40.0
2.0	16.0

[a] ETM (3.6 μg) in toluene (50 ml) was partitioned with aqueous HCl (15 ml). The ETM remaining in the toluene was determined by glc.

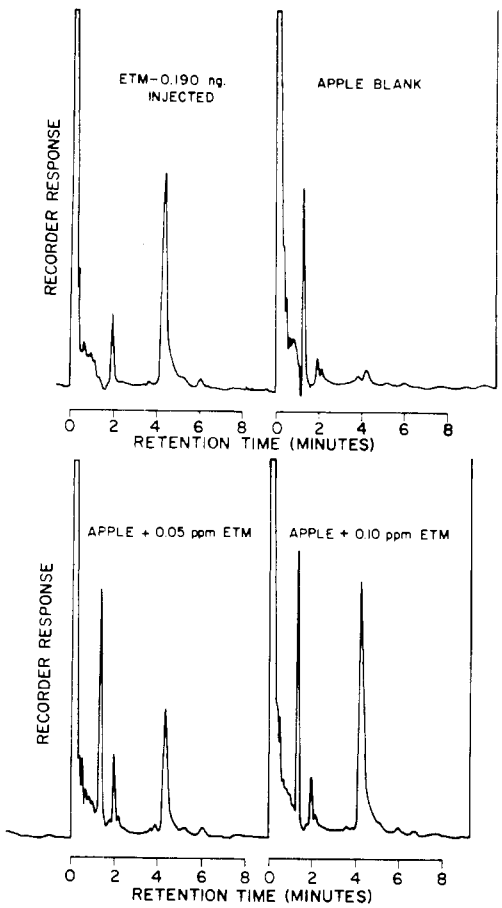

Figure 1. Gas-liquid chromatograms of ETM and of extracts of apple fortified with various levels of ETM. Each injection represents the equivalent of 2 mg of sample.

Stock solutions of ETM were prepared by dissolving sufficient ETM in benzene to give a concentration of approximately 200 μg/ml. Working solutions used for the fortification of samples were prepared by dilution of the stock with absolute ethanol. Dilution of the stock with toluene provided solutions of ETM used as glc standards. The stock solution of ETM was stable when stored at room temperature for several weeks, whereas deterioration was observed with the toluene dilutions within a few hours. For this reason, glc standards were prepared immediately before use. ETM was more stable when diluted with ethanol, a 12% loss being observed after 60 hr. Solutions in ethanol were prepared fresh daily.

Samples were fortified by adding an ethanolic solution of ETM (0.10–0.50 ml) to the material before extraction.

Extraction and Cleanup. The sample (6.0 g) was homogenized with toluene (60 ml) in a Sorvall Omni-Mixer and the solids removed by filtration through Whatman No. 1 paper on a Büchner funnel using gentle vacuum. The filtrate was transferred to a 125-ml separatory funnel and shaken with 0.1 *M* $NaHCO_3$ (25 ml). The aqueous phase, which usually contained emulsified material, was discarded and the toluene layer passed through Whatman No. 41 paper to remove further suspended matter. An aliquot (40 ml) of the clear toluene extract was shaken with three successive portions of 1.0 *N* HCl (1 × 15, 1 × 10, 1 × 5 ml). The acid extracts were combined in a 125-ml separatory funnel and 1.0 *M* Na_2CO_3 (20 ml) added slowly. ETM free base was then extracted into toluene (10 ml) and the toluene extract analyzed by glc.

Table II. Linearity of Recovery of ETM from Apple

ETM added, ppm	ETM found, ppm	Recovery, %
0.051	0.051	100
0.102	0.104	102
0.510	0.433	84.9
1.04	0.962	92.5
5.10	5.27	103.1
10.2	9.60	94.1

Gas-Liquid Chromatography. Analyses were performed on a Hewlett-Packard 5700A gas chromatograph fitted with a ^{63}Ni electron capture detector and 6 ft × 4 mm i.d. glass column. The column was packed with 5% butanediol succinate on 100–120 mesh Chromosorb W, HP, and conditioned under a flow of carrier gas for 48 hr at 200°. Optimum operating parameters were as follows: column temperature, 200°; inlet, 200°; detector, 250°; argon–methane (95:5) carrier flow, 35 ml/min. An on-column injection technique was used, employing 5-μl sample injections. Samples were quantitated by comparison of the peak heights to that of a suitable ETM standard.

RESULTS AND DISCUSSION

ETM is soluble in organic solvents with which it is readily extracted from plant tissues. It is weakly basic and forms a monohydrochloride (Thorn and Ludwig, 1954) which permits its cleanup by extraction into aqueous acid, provided the acid is of sufficient concentration as shown in Table I. Although 2 *M* HCl appeared more efficient than 1 *M*, the subsequent recovery of ETM from it after neutralization was poor. A threefold extraction with 1 *N* acid was required to remove all ETM from the toluene phase, and yielded a quantitative recovery when neutralized and back-extracted.

During preliminary studies, it was found that ETM did not chromatograph intact, but decomposed to ethylenebis(isothiocyanate). This transformation was demonstrated by glc–mass spectrometry and by cochromatography of ethylenebis(isothiocyanate). The height of the isothiocyanate peak was found to increase with increasing glc inlet temperature up to 150°.

Gas-liquid chromatograms of ETM and of final extracts of apple fortified with various levels of ETM are shown in Figure 1. Similar chromatograms were obtained from other commodities. A small background peak with a retention time similar to that of ethylenebis(isothiocyanate) restricts the lower limit of detection to 0.01 ppm, using a 2:1 signal:noise ratio. Attempts to concentrate solutions of ETM in solvents such as methylene chloride, chloroform, hexane, or benzene by evaporation under a stream of nitrogen or by distillation with a Snyder column were met with losses. Thus, at the residue levels studied, sufficient material was unobtainable for glc–mass spectral confirmation.

The recovery of ETM from fortified apple was found to be linear over the range from 0.05 to 10 ppm as shown by the data in Table II. The mean recovery was 96.1 ± 6.4%.

Table III. Recovery of ETM from Various Commodities

ETM added, ppm	ETM recovered, % Spinach	Tomato	Lettuce	Bean
0.05	97.9	96.4	90.1	98.1
0.10	98.1	93.0	83.2	98.1
0.50	96.4	94.7	93.0	94.7
1.00	85.4	89.1	85.6	92.2

The recoveries obtained from other commodities fortified with ETM are given in Table III and are similar to those found for apple. The overall recovery is comparable to that reported for a polarographic method (Engst and Schnaak, 1970).

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Sulfhydryl Group Content of Chicken Breast Muscle during Post-Mortem Aging

Total and nonprotein sulfhydryl content of chicken breast muscle was measured during post-mortem aging. Excised muscle portions were aged in an ice bath for 0, 15, 30, 45, 60, 120, 240, or 360 min post-mortem and then frozen in a Dry Ice-methanol bath. Three different methods were used to homogenize the muscle tissue for analysis: (1) pulverized, frozen tissue was homogenized with 0.02 *M* ethylenediaminetetraacetic acid (EDTA) in a blender; (2) pulverized, freeze-dried tissue was digested with acidified pepsin; and (3) pulverized, frozen tissue was sonicated in a diluent of 8 *M* urea. Homogenates were assayed for sulfhydryl group content using Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid)] and for nitrogen content by a micro-Kjeldahl method. Analysis of variance showed that sulfhydryl concentration did not change significantly with aging time within any of the homogenate preparation methods. Digesting the tissue with acidified pepsin produced a significantly higher sulfhydryl content than homogenizing the tissue with a blender in 0.02 *M* EDTA or with sonic oscillation in 8 *M* urea. Total sulfhydryl content of chicken breast muscle by the three procedures ranged from 0.414 to 0.564 μmol of SH/mg of nitrogen.

Among the many chemical processes taking place during the post-mortem aging of muscle, a decrease in sulfhydryl group content has been considered as a possible mechanism for the development of rigor and subsequent post-rigor tenderization by workers in this laboratory (Chajuss and Spencer, 1962; Gawronski *et al.*, 1967). Both groups of workers reported a decrease in sulfhydryl content in excised chicken breast muscle during the first 2 hr of post-mortem aging. Chajuss and Spencer (1962) proposed that disulfide–sulfhydryl exchange reactions were involved in formation and relaxation of a strained three-dimensional network of protein within the muscle. The results of studies in another laboratory (Caldwell and Lineweaver, 1969), however, have indicated that no change in the sulfhydryl content of chicken breast muscle occurs during post-mortem aging. More recently, Hay *et al.* (1972) reported an increase in sulfhydryl content in actomyosin extracted from chicken breast muscle at 3 hr post-mortem. However, they found no significant change in the sulfhydryl content of actomyosin extracted from chicken leg muscle at 0, 3, 24, and 168 hr post-mortem. Wu and Sayre (1971) observed that the number of sulfhydryl groups in myosin extracted from chicken red and white muscle is essentially the same at 30 min and 24 hr post-mortem.

This study was undertaken to provide additional information to the data concerning sulfhydryl content in chicken breast muscle reported by Chajuss and Spencer (1962), Gawronski *et al.* (1967), and Caldwell and Lineweaver (1969). A substantial point of difference in the assay procedures of these workers is the method of tissue preparation for sulfhydryl analysis. In order to determine whether tissue preparation was responsible for the variation in results, a more recent method of sulfhydryl assay (Sedlak and Lindsay, 1968) was chosen to measure tissue sulfhydryl concentration in homogenates prepared according to the preparation procedure described by each of the above three groups of workers. According to Sedlak and Lindsay (1968), their method of sulfhydryl assay was found to yield highly reproducible results.

MATERIALS AND METHODS

Source of Muscle Samples. Three mature White Leghorn fowl were slaughtered for use with each of the three homogenate preparation methods. All birds received exactly the same treatment, except for the method of preparing the homogenates. The birds were dispatched as needed by severing the jugular vein, esophagus, and trachea with a knife. They were allowed to bleed for 2–3 min. The wings and legs were restrained during bleeding. The right and left pectoralis major muscles were excised and four portions were removed from each muscle. All zero-time samples were frozen within 3–5 min after the birds were dispatched. The remaining seven portions were then placed in polyethylene bags in an ice bath and allowed to age for 15, 30, 45, 60, 120, 240, or 360 min before freezing in a Dry Ice bath. Frozen samples were stored in polyethylene bags surrounded by Dry Ice until the appropriate