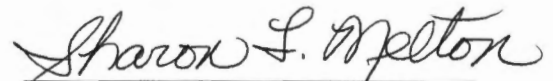
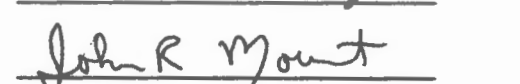


To the Graduate Council:

I am submitting herewith a thesis written by Nurbaini McKosky entitled "HPLC-PAD Analysis of Ethylenethiourea (ETU) and its Destruction During Retorting of Canned Beans and Kale." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

  
Sharon L. Melton, Major Professor

We have read this thesis  
and recommended its acceptance


Accepted for this Council:

  
Associate Vice Chancellor and  
Dean of The Graduate School

**HPLC-PAD ANALYSIS OF ETHYLENETHIOUREA (ETU)  
AND ITS DESTRUCTION DURING RETORTING  
OF CANNED BEANS AND KALE**

**A Thesis  
Presented for the  
Master of Science Degree  
The University of Tennessee, Knoxville**

**Nurbaini McKosky  
MAY 1997**

AD-VET-MED.

Thesis

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## DEDICATION

I dedicate this thesis to my husband, Ralph H. McKosky, who always is with me with his love, encouragement and all his support and to my mother who introduced me to this world and taught me two things: “always be yourself and honest, and always remember God”.

## ACKNOWLEDGMENTS

I would like to express my sincerest appreciation to “Sweet Momma”, Dr. Sharon L. Melton, my major professor, for all of her guidance, knowledge, understanding, patient, encouragement, and friendship. I extend thanks and appreciation to Dr. Ann F. Draughon, Dr. Genevieve L. Christen, and Dr. John R. Mount for their assistance as committee members.

My special thanks to Bush Brothers and Company, Dandridge, Tennessee for their generosity in providing the graduate research assistantship and funding this special research.

I also extend thanks to the following: Terry Walker for his help with HPLC in this research; Kay Trigiano, Ruth A. Hill, Emayet Spencer for their laboratory assistance, cooperation and friendships; Jean Taylor a former assistant director of Ag. Vet. Library for her efficient assistance and loyalty whenever I needed it; Dr. H. O. Jaynes, Dr. Jim Collins, Dr. Curtis Melton and all FST faculty and staff; my good friends, Dr. Youbuet Dje and Sabina Silaula, and to other fellow graduate students for their friendship.

Finally, I extended my sincere appreciation to my husband, Ralph H. McKosky for his support, understanding, patience, and encouragement throughout this study.

## ABSTRACT

The percentage recovery and precision for the analysis of ethylenethiourea (ETU) from beans in a tomato sauce and kale, spiked with 1 and 5 ppm ETU, were determined. The percentage destruction of ETU in the two vegetables at the two spiking levels caused by thermal processing (at 116.7°C for 95 min) of the canned products was analyzed and the effect of thermal processing on pH was measured.

ETU was extracted from the vegetables with a mixture of water (pH 11-12), NaCl, methanol and Celite during a 16 hr maceration before filtration. Part of the extract was mixed with Gas Chrom S and chloroform:methanol (96:4, V/V), and ETU was eluted from an alumina column with chloroform:methanol (96:4, V/V). The eluate was analyzed by HPLC using a pulsed amperometric detector (PAD), a Hypercarb Graphitized Carbon column and solvent of 0.022M H<sub>3</sub>PO<sub>4</sub>:acetonitrile (94:6, V/V) (1.0 mL/min). Averaged across vegetables and ETU spiking levels, ETU recovery was 101.0 ± 6.1% (n=12).

ETU was not found in the beans, but was found in the kale (0.02 ppm). When pH values were 7.17 or higher, 44 to 48% of the ETU in the spiked samples was destroyed ( $p < 0.05$ ) by thermal processing in either beans or kale; when the pH value was 6.67 as in the 1 ppm ETU beans, only a small amount of ETU (2.3%) was destroyed. Without any ETU added, kale had higher ( $p < 0.05$ ) pH values than beans (7.23 versus 6.23); addition of ETU to beans increased ( $p < 0.05$ ) the pH to 6.67 at the 1 ppm level and

to 7.17 at the 5 ppm level. In kale, only the addition of 5 ppm of ETU increased ( $p < 0.05$ ) the pH (7.23 to 7.50).

In kale, with and without an ETU spike, thermal processing decreased the pH of the products by 0.79 to 1.03 units. Significant decrease in pH value during thermal processing, except for the pH decrease in unspiked kale, appeared to be associated with ETU destruction by thermal degradation. Thermal processing can decrease ETU residues in the food supply and that pH might play a more important role in thermal degradation of ETU than previously known.

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## **CHAPTER 1**

### **INTRODUCTION**

Ethylenebisdicarbamate (EBDC) fungicides, such as maneb, have been used on a variety of vegetables and fruits for more than forty years (Anonymous, 1987; Rajagopal et al., 1984; Walter, 1969). However, maneb and its degradation product, ethylenethiourea (ETU), have been classified as probable human carcinogens from evidence of carcinogenicity in animals with ETU being the most potent carcinogen of the two (Anonymous, 1987; Fishbein, 1977; NRC, 1987; Tweedy et al., 1991). In man, maneb and ETU have maximum acceptable daily intakes (ADI) of 0.05 mg/kg and 0.002 mg/kg body weight, respectively (Cova et al., 1991; FAO/WHO, 1981; Vettorazzi, 1977).

Regardless of the fact that EBDC and ETU are normally removed by washing vegetables and fruits prior to processing and are degraded during processing, they are still prevalent in the U.S. food supply (Ahmad et al., 1995; Anonymous, 1992; Kan-Do, 1995; Ross et al., 1978). Because of the prevalence and carcinogenicity of EBDCs and their degradation products, EBDCs have been banned for use on many vegetables and fruits. However, as of Jan.1, 1996, maneb, an EBDC fungicide, was still allowed for use during production anywhere in the USA for selected vegetables, such as corn, cabbage, and dry beans (CFR, 1995; CPCR, 1996). Maneb also can be used for production of kale except in California (CPCR, 1996). The times before harvest and the rate of application for EBDCs are regulated (CFR, 1995). Currently, these vegetables are among the Bush

Brothers and Company's samples analyzed for pesticide residues on a contract basis by The University of Tennessee Food Science and Technology Department. Prior to 1996, these vegetables were not tested routinely for maneb or ETU residues because of the length of time required for analysis. Recently, a quicker instrumental method using a high performance liquid chromatograph (HPLC) with a pulsed amperometric detector (PAD) for ETU determination has been developed and used (Doerge and Yee, 1991; Kocourek, 1987; Koktavy, 1995; Krause, 1989a; Krause, 1992; Krause and Wang, 1988; Roger et al., 1989; Wang et al., 1988). During the past year in the Food Science and Technology Department, such an instrument has become available for limited use. However, the reliability of extraction of ETU from different vegetables and analysis by the HPLC-PAD method needs to be determined before the method can be used to investigate degradation of ETU during thermal processing or used routinely for analysis.

One of the main problems with EBDC fungicides, such as maneb is that ETU is a major contaminant (Lo and Ho, 1993). In addition, maneb is converted to ETU during processing and cooking (Ahmad et al., 1995; Marshall, 1978; Newsome et al., 1975; Newsome, 1976; Ripley and Cox, 1978; Sumner et al., 1991). ETU also degrades during thermal processing, but the degradation rate depends upon the type of food product heated (Newsome et al., 1975) and the availability of oxygen (Marshall and Singh, 1977; Riply and Cox, 1978). ETU degradation during retorting, a severe thermal treatment of canned products in which there is limited availability of oxygen, has not been investigated.

Therefore, the objectives of this research were as follows: (1) to determine the accuracy and precision of the method for extraction of ETU in beans and kale measured by the HPLC-PAD method; and (2) to determine the effect of commercial thermal processing on ETU residues in canned beans and kale, both spiked with known concentrations of ETU.

## CHAPTER 2

### REVIEW OF LITERATURE

#### PROPERTIES OF MANEB AND ETU

Maneb, a fungicide of the ethylenebisdithiocarbamate (EBDC) group, first was introduced by E.I. du Pont de Nemours & Co., Inc. in 1950 and Rohm & Haas Co. in 1943 under the trademarks, Manzate and Dithane M-22, respectively (Merck Index, 1983; Worthing and Walker, 1983). Maneb [ethylenebis(dithiocarbamato)-manganese] is a polymer of a complex compound with the molecular formula of  $[-SCNHCH_2NCHSS-Mn-]_x(Zn)_y$ . It is a yellow powder, which is moderately soluble in water, and soluble in chloroform, pyridine and solutions of chelating agents (such as the sodium salt of ethylenediaminetetraacetic acids) (Merck Index, 1983; Worthing and Walker, 1983). Maneb decomposes on heating without going through a melting process. It also rapidly decomposes on contact with acid or prolonged exposure to moisture or air.

Maneb with the trademark, Maneb 75 D, contains 75% active ingredients, which consist of 58.5% EBDC, 16.5% manganese, and 25% inert ingredients (CPCR, 1996). Commercially, EBDC fungicides, including maneb, may contain a degradation product, ethylenethiourea (ETU) and other fungicides, such as benalaxyl, copper oxychloride, copper sulfate, cymoxanil, metalaxyl, oxadixyl, and tridemorph (Lo and Ho, 1993). Thus, maneb activity is affected by its specific formulation. In sufficient quantity, maneb

inhibits fungal respiration and manganese toxic metal ions prevent the germination of fungal spores (Togerson, 1969). Maneb formulations are known to be unstable to moisture, oxygen, light, and biological systems; it degrades into products, such as ETU.

ETU or imidazolidine-2-thione is a crystalline needle in prism form with a melting point of 203-204<sup>0</sup>C. ETU is soluble in water, and moderately soluble in alcohol, ethylene glycol and pyridine, and it is insoluble in acetone, ether, chloroform and benzene (Merck Index, 1983). ETU is oxidized by hydrogen peroxide or hypochlorite; it is photolyzed by light in the present of photosensitizer, such as oxygen, acetone or riboflavin (Ross and Crosby, 1973).

### **TOXICITY OF MANEB AND ETU**

Maneb has a low mammalian toxicity and is relatively harmless with acute oral LD<sub>50</sub> of 6,750 mg/kg in rats (Lentza-Rigos, 1990, Rajagopal et al., 1984). However, maneb showed maternal toxicity at high doses, greater than 12.5 mg/kg/d in rats (FAO/WHO, 1981), but a later study showed that the possible carcinogenic and teratogenic effects could possibly generate from food consumption at the minimum level of 1,000 times higher than the daily human intake (Anonymous, 1987). The estimated Acceptable Daily Intake (ADI) of maneb is up to 0.05 mg/kg body weight in humans (Anonymous, 1987; Cova et al., 1991; FAO/WHO, 1981; Lentza-Rizos, 1990; Vettorazzi, 1977). Maneb is considered highly hazardous because it generates very harmful chemicals upon degrading. Three

harmful degradation products of maneb are ETU, ethylenebisisothiocyanate (EBIS) and carbon disulfide (CS<sub>2</sub>) (AOAC, 1990; Lentza-Rizos, 1990).

ETU also has a low acute toxicity in rats, with LD<sub>50</sub> of 1,832 mg/kg, but it is maternally toxic and is proven to be a teratogen at doses greater than 10 mg/kg. ETU affects the liver and also produces a high incidence of lesions in the central nervous system and urogenital area. ETU affects the thyroid gland, inducing hyperplasia and finally carcinoma and a decrease in <sup>131</sup>I uptake (Fishbein, 1977). ETU also has been proven to be an animal carcinogen (Anonymous, 1987; FAO/WHO, 1981) and in humans, it has an estimated ADI of 0.002 mg/kg.

Combining with other chemicals, such as sodium bromide, ETU results in a goitrogenic effect in rats (Newsome et al., 1978). Teramoto et al. (1980) studied the effect of nitrosation products of ETU in vivo. ETU is nitrosable and forms N-Nitroso-ETU, which is strongly mutagenic in an acid condition. In the stomach of mice, ETU also is a teratogen. Some questions arose concerning the use of nitrites, which are found in cured meat products as bacteriostatic, flavoring and coloring agents, that could possibly react with ETU to form N-Nitroso-ETU. However, ascorbic acids or ascorbates inhibit the N-nitrosation reaction and thus block the mutagenic effect of N-Nitroso-ETU. Ascorbate pretreatment of rats subjected to ETU also failed to exhibit any teratogenic effects (Khera and Iverson, 1980).

## FIELD APPLICATION OF MANEB

Maneb is applied by spraying it as a water suspension at the level of 0.45-0.91 kg/acre (1.5-2.0 lbs/acre) with a maximum of 5.44 kg (4.35 kg active ingredients) per acre per season. For dry beans the application begins when the bean plants are small and is repeated at 5 to 7 day intervals; maneb can not be applied within 30 days of harvest of beans. For kale, it is sprayed on a plant bed or on a direct seeded field. It must not be applied within 7 days of harvest of kale (CPCR, 1996; Newsome, 1976; Yip et al., 1971).

## FATE OF MANEB AND ETU

After application, maneb remains on the plant surface, is degraded, and /or washed off by rain with the retention determined by its formulation and particle size distribution (Kudsk et al., 1991). The half-lives (the time required to reduce to half of the initial concentration of any compound) of maneb and ETU were studied by Nash and Beal (1980). They reported that the half-life of maneb was 14 days on tomato leaves, while the half-life of ETU was 3 days in soil and 9 days in the air. Smith et al. (1988) found the half-life of EBDC fungicides was 6 days on lettuce. No report concerning the half-life of ETU on beans or kale was found in the literature.

In the environment, in vivo, and during cooking, and brewing, maneb degrades into products such as ETU as shown on Fig. 1 (Lentza-Rigos, 1990; Manahan, 1990;

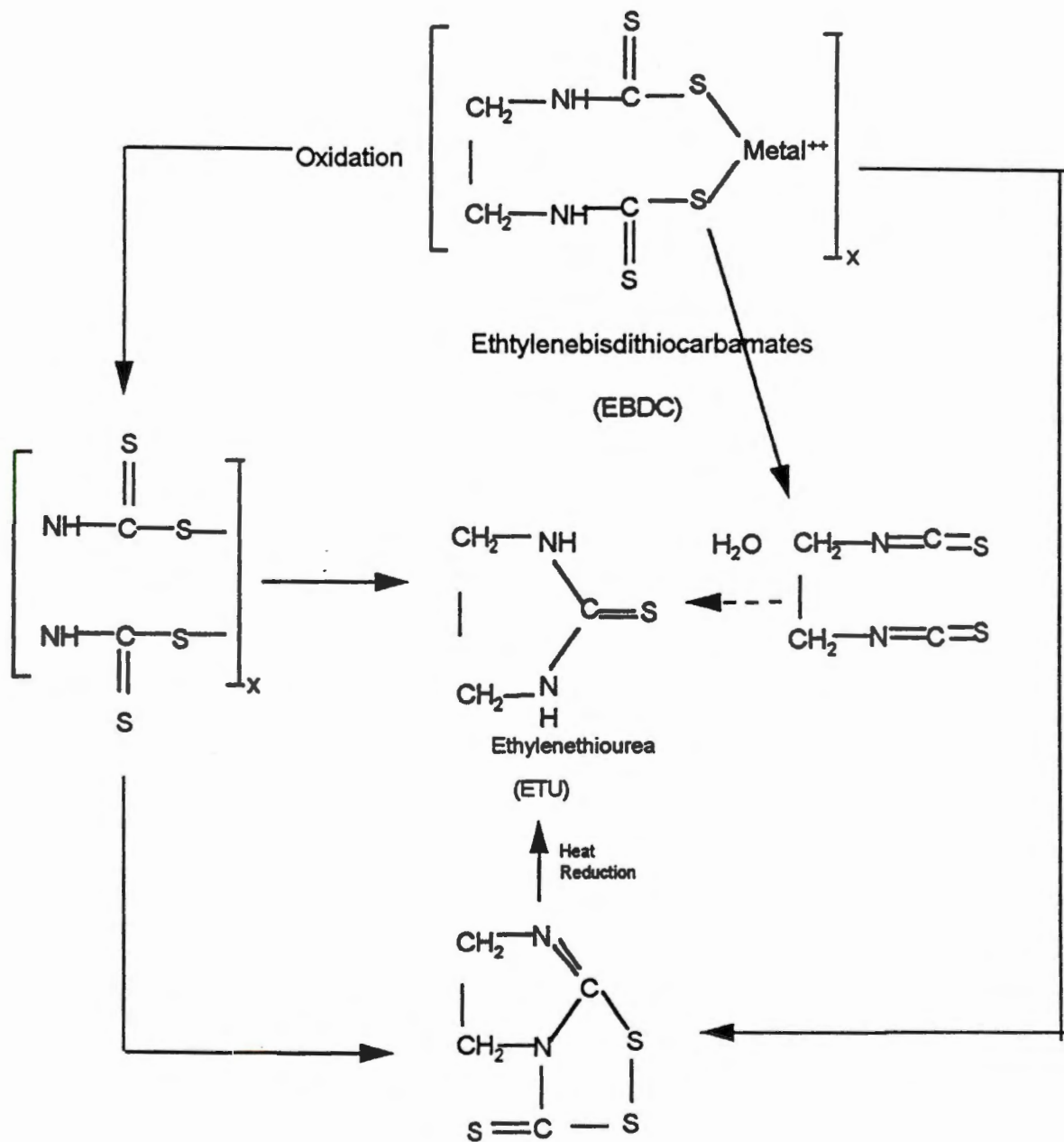


**Fig. 1—Degradation reaction of ethylenedithiocarbamate (EBDC) fungicide leads to ETU formation. Dashed line indicates proposed reaction (adapted from Lentz-Rizos, 1990).**

Marshall and Singh, 1977; Murphy, 1986; Nitz et al, 1984; Primi et al., 1994; Sypnu, 1989; Tweedy et al., 1991). In the soil, decomposition of maneb was three times greater than it was in the atmosphere (Rajagopal et al., 1984). Maneb undergoes biodegradation by microorganisms, such as Pseudomonas fluorescens, Aspergillus niger, and Saccharomyces cerevisae and is catalytically oxidized by compounds such as cysteine, glutathion, or ascorbic acid. Maneb decomposes to ETU, which does not undergo further degradation in the absence of light and oxygen (Rajagopal et al., 1984). Upon photolysis, ETU degrades into ethyleneurea (EU) or 2-imidazole, which is further transformed into hydantoin. In the presence of an acetone sensitiser, both EU and hydantoin degrade into glycine. Then glycine is further oxidized into carbon dioxide (Hoagland and Frear, 1976; Pease and Holt, 1977; Rhodes, 1977; Ross and Crosby, 1973) as shown in Fig. 2. In the presence of nitrite, EU as well as ETU will form nitroso derivatives, which are known to be tumorigenic and teratogenic (Khera and Inverson, 1980; Marshall, 1978; Rhodes, 1977). ETU on field plants can be washed off into the soil, where it and its degradation products are absorbed by plant roots and translocated to the leaves (Hoagland and Frear, 1976; Rajagopal et al., 1984). A study using Carbon-14 labeled ETU confirmed that the amount of ETU and its degradation products that are distributed on the leaves is closely correlated to that on roots. This was expected based on the balancing of their rate of loss from the leaves and their rate of uptake from the soil. However, the extent of correlation differed among the vegetables. ETU content on fresh vegetables depends on various factors, such as maneb formulation, quality, storage

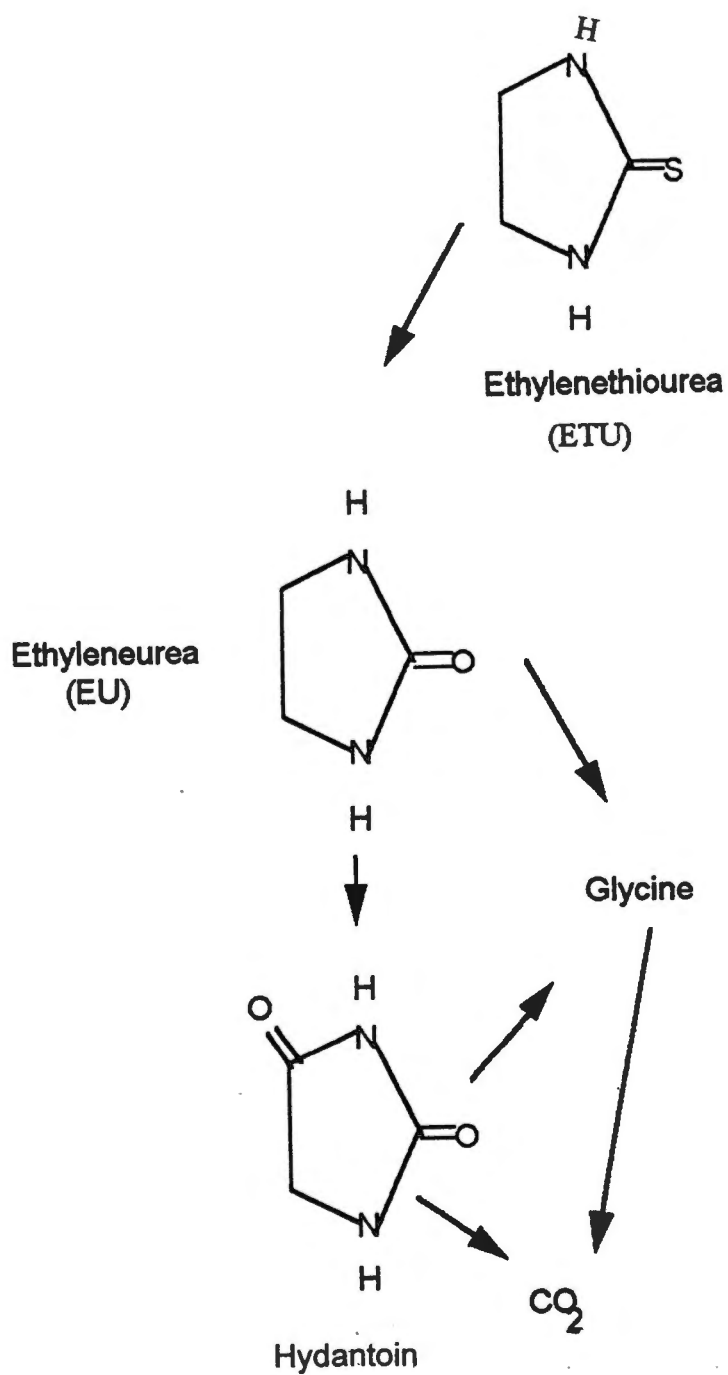


**Fig. 2--Proposed pathway of ethylenethiourea (ETU) degradation in soil and/or in microbial cultures. (Adapted from Rajagopal et al., 1984).**

condition, dose used and its application rate in field treatment, and rain-fall or weather (Lentza-Rizos, 1990).

## **SOURCE OF ETU AND ITS FORMATION IN VEGETABLES**

The presence of ETU in crops treated with maneb is suspected to be due to it being a degradation product of maneb after application and its possible formation during the preparation of aqueous maneb suspension before application. ETU contaminates maneb or any EBDC fungicide formulation and the ETU contaminant needs to be controlled. FAO (1979) limited the ETU level in EBDC fungicide formulations to 0.5% (FAO, 1979).

ETU was detected in the range of 0.02-0.96 ppm on foliage crops and on fruits treated with maneb immediately after its application (Ripley and Cox, 1978). Due to maneb degradation, the level of ETU on the plant increased during the first 3-4 days after its application; then, the level decreased with time, until finally none was detected. With repeated maneb application, the ETU level increased up to 5 days after each application, reached a maximum and then decreased until harvest time. On kale, the ETU residue decreased about 80% after 7 days and was undetectable after storage longer than 7 days (Smith et al., 1988; Yip et al., 1971). On string beans, the ETU residue decreased 50% in 2 days (Newsome et al., 1975). Based on a study of Carbon-14 labeled ETU (Rhodes, 1977), the ETU level on beans reached maximum amount at 10-12 days after fungicide

1977), the ETU level on beans reached maximum amount at 10-12 days after fungicide application, but was undetected at the harvest time. Generally, with repeated maneb application of recommended dose and instruction for field treatment, measurable levels persist on crops up to 30 days but is not significant at harvest time. In fact, ETU percent recovery has been found to decrease with increasing time between time of spiking and extracting (Lentza-Rizos, 1990).

### **DEGRADATION OF EBDC AND ETU RESIDUES IN FOODS**

Several researchers have shown that maneb is removed by water washing the vegetables, and that it is degraded even more by hot water washing and processing at temperatures below those used for retorting (Chovancova et al., 1985; Gonzales et al., 1987; Marshall and Yarvis, 1979; Marshall and Singh, 1977; Newsome and Laver, 1973). However, the rate of maneb degradation and the ETU levels remaining differ among vegetables and is dependent upon the washing and processing methods.

Yip et al. (1971) reported that maneb and its degradation product, ETU, did not accumulate on field-maneb-treated kale. Analysis of maneb and ETU residues on kale at the day of treatment resulted in 90 and 15 ppm, respectively. Maneb residues decreased 83% after 7 days of treatment and 98% after 30 days of treatment; and ETU was undetected after 30 days of treatment.

Newsome et al. (1975) treated string beans and tomatoes repeatedly for 7 applications with the recommended dose and measured maneb and ETU residues at 0 days and up to 14 days after treatment. The levels of maneb and ETU declined with increasing days after treatment, but levels on string beans remained higher than on tomatoes. Even 14 days after application, however, appreciable amounts of maneb and measurable ETU levels were found on the beans and tomatoes.

Yip et al. (1971) also found appreciable amounts of maneb on lettuce and kale 14 days after fungicide treatment, but ETU levels declined to undetectable amounts within 7 days in their study. The rate of maneb decline in the study of Newsome et al. (1975) was slower initially than that observed by Yip et al. (1971). Upon boiling beans, which were sampled 9 days after fungicide application and contained 25 ppm maneb and 0.11 ppm ETU, Newsome et al. (1975) found 11.14 ppm ETU in the cooking water, proving that maneb can be a considerable source of ETU.

Newsome (1976) found almost the same rate of decrease in maneb and ETU residue levels on fungicide field-treated tomatoes after harvest. This researcher found a 76% decrease in maneb concentration and a 78% decrease in ETU levels 14 days after application. Boiling of homogenates of treated tomatoes for 10 min resulted in a 38-48% conversion of EBDCs to ethylthiourea.

## REMOVAL OF MANEB AND ETU

Several techniques have been applied on harvested crops to remove the residues of EBDCs and its degradation product, ETU. Washing vegetables and fruits with water in the field immediately after harvest resulted in 33 to 87% reduction of EBDC fungicide (including maneb) and of ETU residues (Phillips et al., 1977). Repeated and prolonged washing periods, with agitation, reduced ETU residue 86-96% (Gonzales, et al., 1987, 1989a, b; Ripley and Cox, 1978). The effectiveness of washing also depended on the elapsed time after the harvest in addition to the type of crops (Gonzales et al., 1987; 1989a; Marshall and Yarvis, 1979).

Processing of vegetables, such as heating or cooking at home or heating prior to canning in commercial vegetable processing, including the brewing process, further degraded EBDC residues, but increased ETU residues (Lentza-Rigos, 1990). The ETU residues were increased by 3-30% by boiling the EBDC treated spinach, pears, grapes, tomatoes, and wheat. Washing raw fruits and vegetables prior to cooking or commercial processing also was very important. Marshall and Yarvis (1979) found reduction of 52% of EBDC and 50% ETU fungicides in tomato juice made from running-water washed tomatoes for 10 minutes versus that made from unwashed tomatoes. Washing with alkali solution at any time removed more of the residues. Using alkali solution or basic solution (pH 9.0) containing 200 ppm sodium hypochlorite and mild detergent, or highly basic detergent solution (pH 10.8-12.4) prior to canning removed 69-98.5% EBDC and ETU

residues (Marshall, 1982; Marshall and Yarvis, 1979; Ripley and Cox, 1978). Blanching string beans in hot (95 °C) 1.0 M HCl for 2 min reduced EBDC by 95% and ETU by at least 95%.

The USA maximum ETU residue limits for all products of plant origin was 0.02 ppm (Finland, 1987). The limit ranged from 0.01 to 0.1 ppm in other countries.

### **DETERMINATION OF ETU RESIDUES**

ETU measurement in vegetables, fruits and their products has evolved from paper chromatography (PC), to thin layer chromatography (TLC), to gas liquid chromatography (GLC) and finally to high performance liquid chromatography (HPLC).

The paper chromatographic technique was not very convenient, because it required preparation of an ETU derivative, such as S-Benzyl-ETU, for positive identification and separation, and this technique needed a large quantity of sample (Chovancova et al., 1985). The TLC technique did not give satisfactory results since ETU degraded on the TLC plate (Bottomley et al., 1985; Walter, 1969). The GLC technique also required derivatization of ETU and the percentage recovery differed among the various investigations. Lentza-Rigos (1990) reported percentage recoveries ranging from 75 to 102% for ETU determination using GLC with various detectors and conditions for various samples. Ahmad et al. (1995) used GLC with UV detection for ETU determination in spiked vegetables and fruits and found percentage recovery ranging

from  $86 \pm 6$  to  $102 \pm 20\%$ . In the mean time, an ETU determination method was developed using HPLC (Gustafsson and Fahlgren, 1983; Gustafsson and Thompson, 1981; Kobayashi et al., 1986; Kocourek, 1987; Krause, 1989b ; 1992; Kurttio et al., 1986; Onley, 1977; Onley et al., 1977; Prince, 1985; Smith et al., 1988). This method did not require ETU in its derivative form for the analysis. However, the HPLC technique using a C-8 or C-18 reversed-phase silica based column with UV detection lacked selectivity and had percentage recoveries of ETU ranging from 53.3 to 94% (Lentza-Rigos, 1990).

Because of its unique physical and chemical properties, ETU is best determined by a single-residue method. This method should be selective, sensitive, and not degrade ETU during the measurement. The HPLC technique using UV detection previously described is not the method of choice since components in the prepared samples other than ETU absorb in the same UV region. However, if another method of detection were used, it could be the method of choice. In general, the basic HPLC method for ETU determination involves the following steps: (1) ETU extraction, (2) clean-up of extract and (3) HPLC analysis.

For extraction, the solvent chosen and the particle size of samples play major roles in the reliability of the ETU extraction and analysis. The extracting solvent should be able to dissolve ETU as it is carried throughout the method. Smith et al. (1988) investigated the effectiveness of several individual solvents for ETU extraction from lettuce; the pH of the extracting media was not measured, the lettuce was coarsely

chopped and the extraction was done in a very short time. Under these conditions, they found that methanol yielding an 89% recovery was more effective than ethanol, water, dichloromethane, or chloroform; the lowest percentage recovery 20%, resulted from using chloroform. However, the percentage of recovery also is dependent upon the effectiveness of the maceration step, which in turn is affected by the particle size of the sample and time of extraction. The smaller the sample particle size, the higher percentage recovery (Smith et al., 1988). A higher percentage recovery also was generated from the use of silanized glassware related to the analysis (Roger et al., 1989). Later researchers used the mixed solvent system of ethanol, water and sodium chloride, with the pH range from 7 to 9 for the ETU extraction of various crops, meat, and milk (Koktavy, 1995; Roger et al., 1989).

At the ETU clean-up step or elution, Gas Chrom S or Flux-calcined diatomaceous earth, which was basically silica ( $\text{SiO}_2$ ) (as described in Fisher Scientific Co, Pittsburgh, PA). It functioned as a carrier for the ETU before further separation using a clean-up column. The alumina clean-up column further separated ETU from the rest of the co-extracted components including chlorophyll and other pigments which are partitioned into the column eluent, chloroform : methanol (96:4) (Munir, 1985).

The HPLC system requires an appropriate mobile phase, which results in a good separation of ETU from co-extractive components, in addition to a sensitive and selective detector. The HPLC method has been improved by using an electrochemical detector, such as a pulse amperometric detector (PAD) with a working/indicator electrode of

Hg/Au and a reference electrode of Ag/AgCl (Gustafsson and Fahlgren, 1983; Gustafsson and Thompson, 1981; Kobayashi et al., 1986; Krause, 1989b ; 1992; Kurttio et al., 1986; Onley, 1977; Onley et al., 1977; Prince, 1985; Smith et al., 1988). Basically, amperometry detects the current generated by a reduction or oxidation of the analyte components. When a small potential is applied, the indicator electrode is polarized and oxidizes the analytes; then electrons released generate the current. This oxidation current is measured, integrated into a signal that is sent to the recorder and finally measured as a concentration (Alberty, 1983; Marshall, 1994). The HPLC-PAD system coupled with a hydrocarbon column (C-8 or C-18) has been reported to be a very selective and sensitive technique with percentage recoveries for ETU between 80 to 93% (Doerge and Yee, 1991; Krause, 1989a; 1992; Krause and Wang, 1988). The HPLC-PAD method for ETU analysis was also the method of choice by Kocktavy (1995) and Roger et al. (1989).

## **CHAPTER 3**

### **MATERIALS AND METHODS**

The materials and chemicals used in this study were obtained from Fisher Scientific Co., Pittsburgh, PA, unless otherwise specified. All glassware used was silanized. Under an exhaust hood, clean and dry glassware was coated with a thin layer of silanizing solution (5% dimethyl dichlorosilane in hexane) and allowed to dry before being rinsed thoroughly with deionized water followed by acetone and petroleum ether rinses.

### **SOURCES OF SAMPLES**

Dry navy beans (11.3 kg), bean sauce for the beans, and cans (size No. 300) were supplied by Bush Brothers & Co. in Dandridge, TN, the day before processing. The sauce which was used in their pork and beans minus the pork, contained 2.4% of a 30% tomato paste and was kept at 5-6<sup>0</sup>C until use. For kale, a 11.3-kg box of fresh kale was purchased from The Farmer's Market, Knoxville, TN, the day before processing.

## PREPARATION OF SAMPLES

The preparation of all samples analyzed for ETU by HPLC analysis before and after processing of canned beans and kale included: ETU isolation or extraction and elution (cleaning up process) of the ETU extract as shown in Figs. 3-6.

### **Heat Thermal Processing/Canning**

The dry beans were washed 4 times and soaked in cold water (2:1 ratio of water:beans) for 12 hr, and then blanched at 80-90°C for 5 min. They were drained and dropped through a stream of air to remove excess hulls. The empty cans were steamed-washed/sterilized and allowed to dry. The sauce was heated to boiling (100°C).

Each of 24 cans then was filled with 204 g of blanched beans and filled to the top with the hot sauce. Eight cans of beans were selected at random and sealed as the control treatment. Eight cans of beans were selected at random; each was spiked with 1 ppm ETU (1 µg/g of contents) (Level 1) and sealed. The remaining 8 cans of beans were each spiked with 5 ppm ETU (5 µg/g of contents) (Level 2) and sealed. The contents of the cans were in the temperature range of 68 to 74°C at the time they were sealed. The cans were stored at 5-6 °C for no more than 16 hr prior to transport to Bush Brothers and Co. in Dandridge, TN, for retorting.

The fresh kale was washed 4 times with cold water and then blanched for 3-1/2 min in hot water (93.3°C). The blanched kale was randomly chopped to approximately

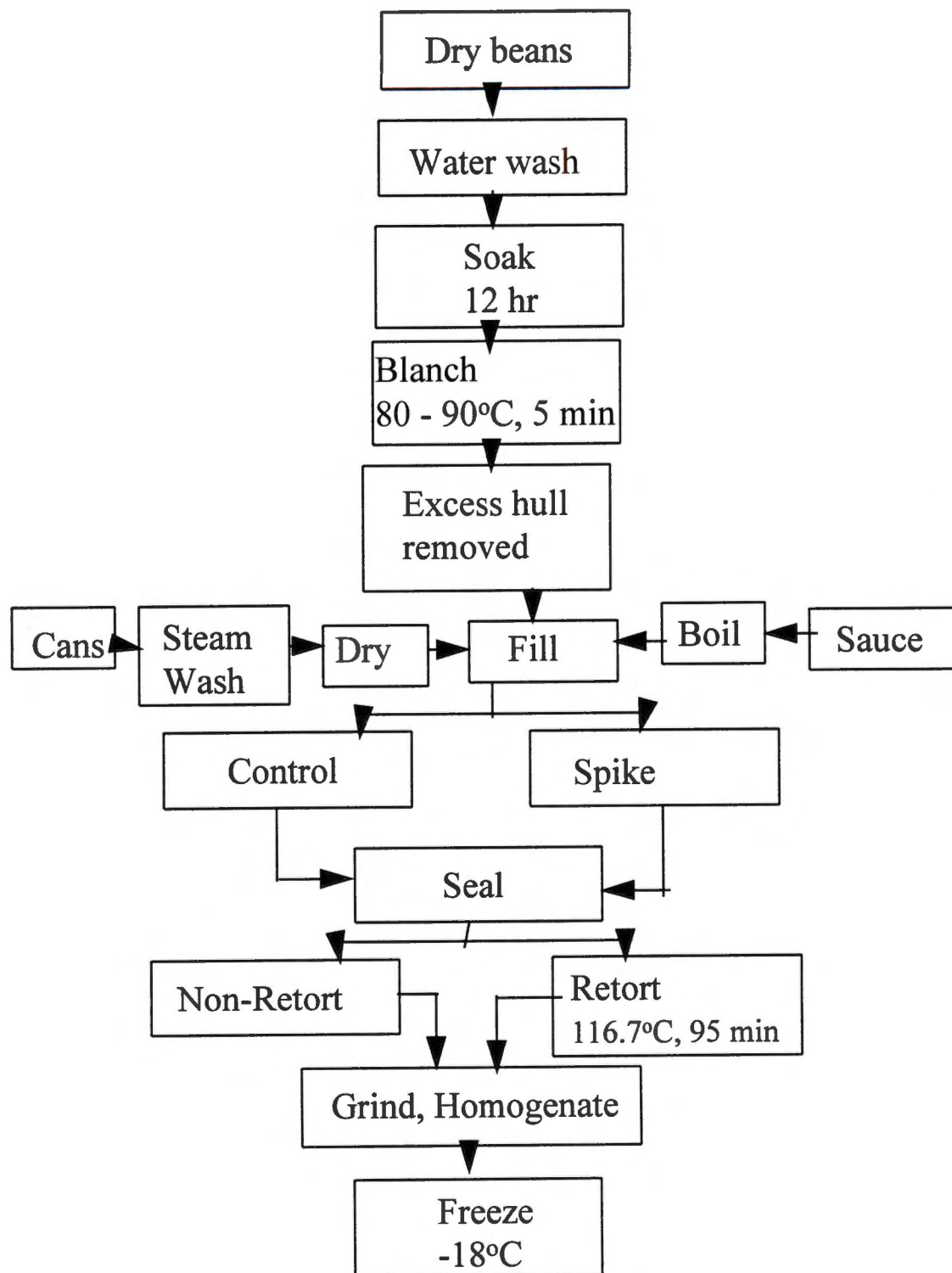


**Fig. 3--Preparation of bean samples**

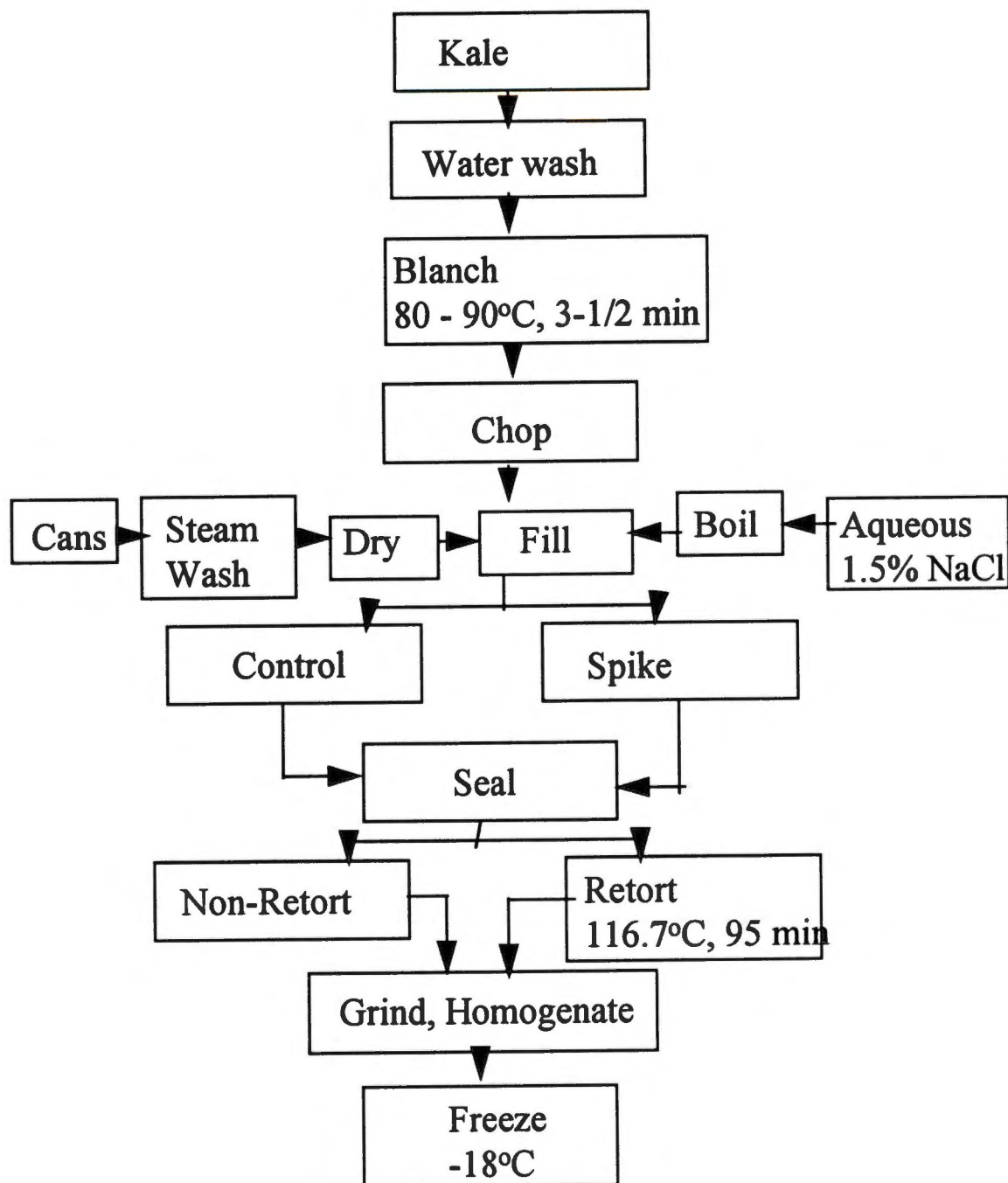


**Fig. 4--Preparation of kale samples**

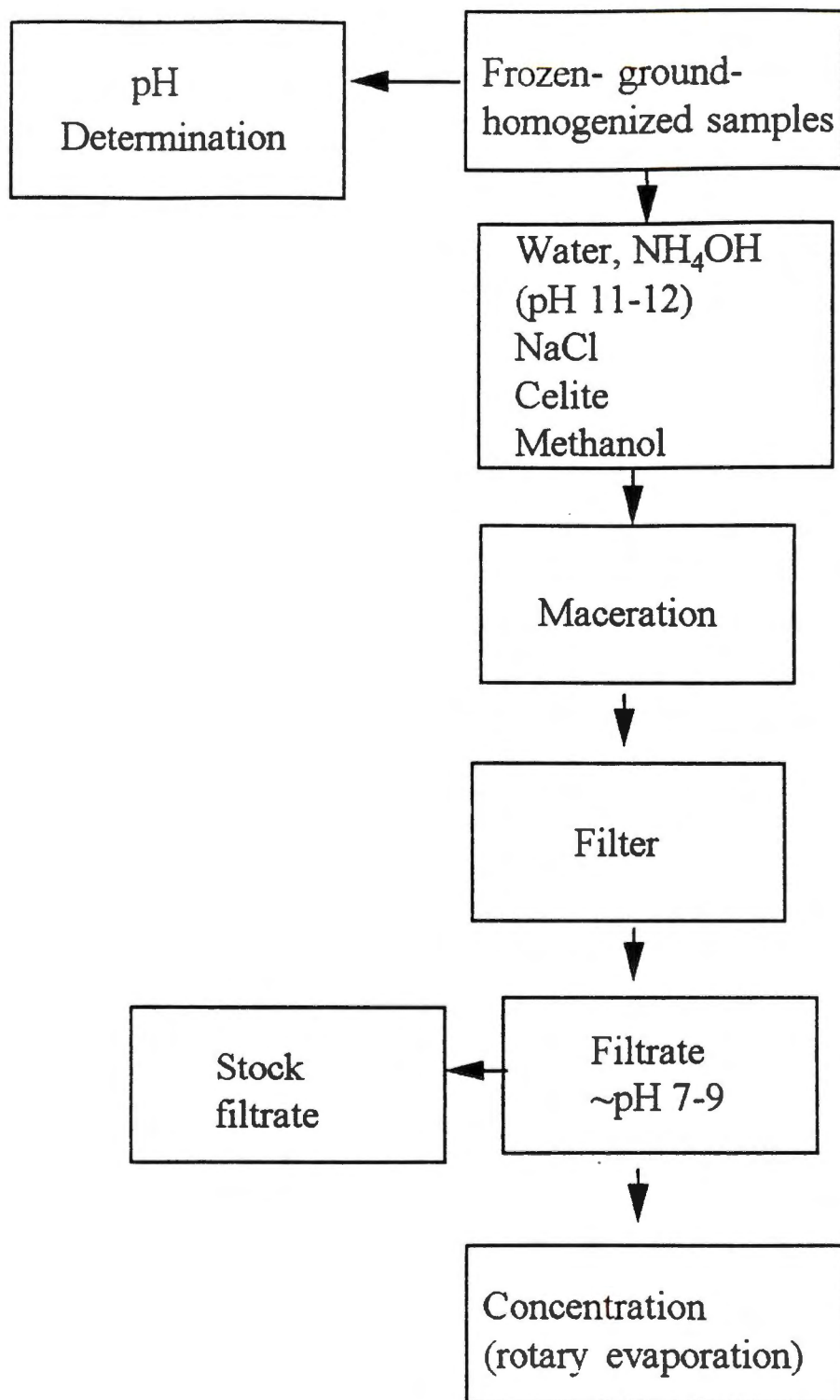


**Fig. 5--ETU isolation from samples**

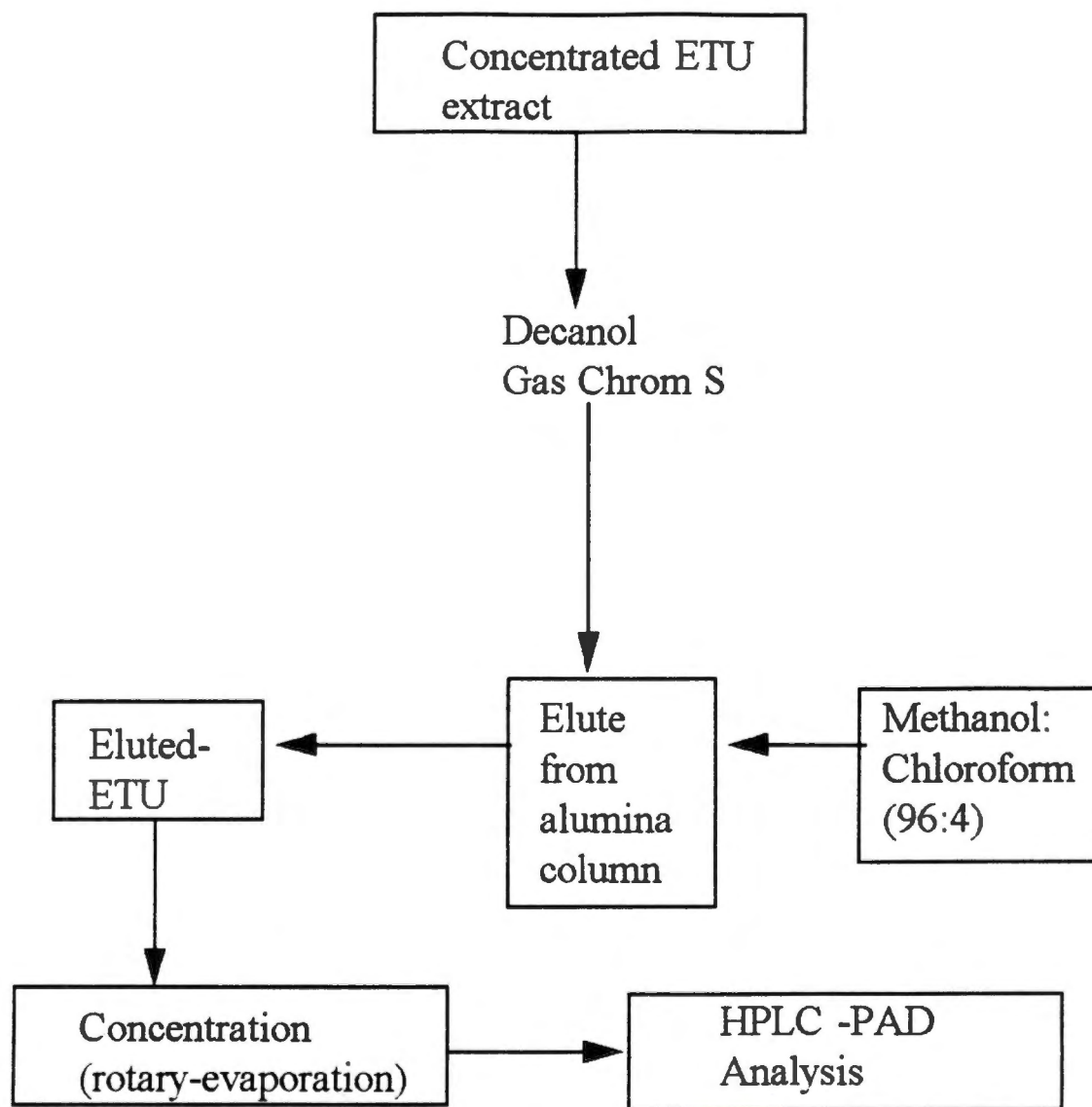


**Fig. 6--ETU elution from its extract**

1.0 cm<sup>2</sup> pieces, similar to the size of canned kale from Bush Brothers and Co. A filling solution of 1.5% aqueous salt solution was heated to boiling. Each of 24 clean cans of known weight was filled with 269 g of the blanched-chopped kale and then filled to the top with boiling aqueous 1.5% salt solution and weighed. Eight cans were selected at random and sealed as the control treatments. Eight other cans were selected at random, each was spiked with 1 ppm ETU (1 µg/g contents) (Level 1) and sealed. Each of the remaining 8 cans was spiked with 5 ppm ETU (5 µg/g contents) (Level 2) and sealed. All cans were sealed with the temperature of the contents in the temperature range of 68-74°C. All sealed cans were stored at 5-6°C no more than 16 hr prior transport to Bush Brothers and Co., in Dandridge, TN, for retorting.

For each vegetable, 4 cans from each treatment (control, Level 1 spike and Level 2 spike) were selected at random and designated as before thermal processing (BT). The other 4 cans from each treatment were transported to Bush Brothers and Co. and commercially retorted with other canned vegetables for 95 min at 116.7°C. These cans were labeled with type of vegetable and treatment prior to being placed in a burlap bag and dropped into commercial retort.

For each combination of vegetable, treatment and process, 3 of the 4 cans were analyzed. For each can, the contents were transferred into a 0.9 L blender jar, pureed to a homogenous mixture by being blended for 2 min for the after thermal processed sample (AT) and 5 min for before thermal processed samples at high speed on a blender. The homogeneous mixture was placed in a 17.8 cm by 20.3 cm freezer bag (Glad Lock, First

freezer bag (Glad Lock, First Brands, Co., Danbury, CT), flushed with nitrogen and stored at  $-18^{\circ}\text{C}$  for two weeks, until analysis.

### **Isolation of Samples**

Measurement of sample pH. The pH of beans and kale, in the non-retorted as well as the retorted contents of 3 cans from each of 3 ETU spiking levels (0, 1 and 5 ppm) were measured. The pH of the homogenized contents of each can was measured using the pH electronic paper (pHep), Model 5941-00 by Cole-Parmer Instrument Co., Chicago, IL. The pH meter was calibrated using the buffer solutions at pH values of 7.0 and 12.0.

Extraction of sample. ETU was isolated from each sample using extraction technique with solvent system, the mixture of water-sodium chloride-methanol, in the alkali condition. This technique was adapted from the technique of Morse Laboratory (Roger et al., 1989) and McKenzie Laboratory, Inc. (Koktavy, 1995). Approximately 50 g of homogenized kale sample or 25 g of homogenized bean sample were weighed in the frozen state into a 250 mL glass beaker. Deionized water (75 mL) was added and a glass rod was used to break up the sample. Ammonia (14.8 N) was used to adjust the pH of the extracting media in the range of 11-12. Five g of NaCl, 5 g Celite and 100 mL methanol were added to each sample, and the sample was mixed using a magnetic stirrer and then let stand for 16 hr for maceration.

The mixture was filtered using suction through a bed of 12.5 g Celite spread evenly over 11.0 cm diameter double shark skin filter paper in a Buchner funnel. The filtration was continued until the mixture was dry, and the filtrate was collected in a 1000 mL filter flask. The filtrate was transferred to a 500 mL graduated cylinder using methanol for rinsing and the total volume was brought to 250 mL. The filtrate, which was in the range of pH 7 to 9, then was transferred back to the filter flask.

A 25 mL aliquot of the filtrate was transferred to a preweighed 250 mL round bottom evaporation flask, and 2 drops of decanol keeper (25% decanol in acetone) and boiling chips were added. The contents of the flask were evaporated at 45 to 50°C in a rotary evaporatory to a volume of approximately 10 mL. Deionized water was added until the flask contents weighed was about 13 g. The concentrated extract was then ready for clean-up.

### **ETU Clean-up/Elution**

Alumina column preparation. Alumina A-10, 80/200 mesh, was dried at 175 °C and 1.41 kg/cm<sup>2</sup> vacuum for 2 hr. Deionized water (4% W/W) was added to the dried alumina in a glass stoppered Erlenmeyer flask. The flask was shaken until the contents were lump free, and then the mixture was allowed to equilibrate for at least 2-12 hr prior to use. A chromatographic column (11.0 mm id by 300 mm long) was plugged with silanized glass wool, and 5 g of the activated alumina containing 4% water was added. Then, silanized glass wool was added to cap the top of the column.

ETU elution. A 500 mL evaporation flask containing 10 mL deionized water and 6 drops decanol keeper was placed under the chromatographic column to receive the eluent. A 10-g sample of Gas Chrom S (AllTech, Deerfield, IL) was added to the 250 mL evaporation flask containing the sample of concentrated extract, and the flask was shaken vigorously until the contents were lump-free. A 75 mL chloroform:methanol (96:4, V/V) aliquot was added to the flask, and its contents were swirled for 30 sec before being poured into the column. The flow rate through the column was adjusted to a fast drip. When the level of the eluting solution dropped slightly below the surface of Gas Chrom S in the column, the sample flask was rinsed with 50 mL of the chloroform:methanol solution which was added to the column. The rinse was repeated for a total of 4 times. The Gas Chrom S was allowed to settle prior to each portion being added to the column; the surface of the Gas Chrom S was not allowed to dry completely.

ETU concentration. Boiling chips were added to the 250 mL flask containing the eluted sample, and the eluent was evaporated using a Buchi rotary evaporator at 35 to 45<sup>0</sup>C to an approximate volume of 3 to 4 mL. Then, two drops of decanol keeper solution and 5 mL of deionized water were added, and the contents again evaporated to approximately 2 to 3 mL. The concentrated sample was transferred to a 5 mL volumetric flask and made up to volume with deionized water and stored at 5-6<sup>0</sup>C until HPLC analysis.

## HPLC ANALYSIS OF ETU

ETU or imidazolidine-2-thione, 98% pure coated with paraffinic oil (Fisher Scientific Co., Pittsburgh, PA), was recrystallized by the following procedure. The paraffinic oil coating was dissolved in petroleum ether leaving the ETU as a solid; the oil solution was removed by vacuum filtration through a Buchner funnel lined with Whatman No. 1 filter paper. Then, ETU was crystallized from methanol:water (1:1, V/V) containing 5% hexane (Marshall, 1978). The ETU crystals were dried under a flow of nitrogen, and its purity confirmed by determining that its melting point was 203-204°C (Merck Index, 1983).

ETU was analyzed on a Dionex HPLC Pulsed Amperometric Detector (PAD), which was equipped with a Hg/Au working electrode and Ag/AgCl reference electrode, on a Shandon Hypercarb Graphitized (7  $\mu$ m) column (4.6 mm in diameter, 100 mm in length) (Hypersil, Chadwick Road Astmoor-Run Corn, Cheshire, wa 7 JPR, England). The system configuration was as follows: + 0.35 V and 300 nA range. The mobile phase was 0.022 M H<sub>3</sub>PO<sub>4</sub>:acetonitrile (94:6, V/V), at a flow rate of 1.0 mL/min. The mobile phase was stored in a reservoir degassed continuously using a helium sparge.

All vials, pipettes and other equipment used to prepare eluted ETU and the ETU standard solutions were silanized before use. The size sample/standard (100  $\mu$ L) was injected into the column by an automated injector in the Dionex-HPLC system.

A five point ETU standard curve was prepared with amounts of 0.5, 1.0, 5.0, 10.0, and 50.0 ng of ETU. Each respective amount was obtained by injection of 100  $\mu$ L of 0.005, 0.01, 0.05, 0.1, and 0.50  $\mu$ g/mL of ETU standard solutions.

### CALCULATION OF ETU RESIDUE CONCENTRATION

Using the ETU standard curve, the ETU concentration (ng/100 $\mu$ L) of each injected sample was calculated directly by the computer and recorded. Then, the logarithm of the ETU concentrations of all spiked samples was obtained.

Percentage recovery (PR) of ETU from the samples, which were not retorted, was determined as follows:

$$\text{Sample injected (mg)} = \frac{(\text{g sample extracted}) (\mu\text{L injected}) (1000 \text{ mg})}{(\text{mL final volume}) (\text{dilution factor}) (1000 \text{ uL})}$$

$$\text{ETU Level (ppm)} = \frac{\text{ng of ETU}}{\text{mg sample injected}}$$

$$\text{PR} = \frac{(\text{CX} - \text{CC})}{\text{CA}} \times 100$$

where CX = ETU concentration in ppm analyzed in Level 1 (1.00 ppm) or Level 2 (5.00 ppm) treatment, CA = ETU concentration in ppm added in Level 1 or Level 2; and CC = ETU concentration in ppm analyzed in control (unspiked treatment).

In the samples which were retorted, the percentage destruction of ETU was calculated by the following equation:

$$PD = \frac{|ETU_{BT} - ETU_{AT}|}{ETU_{BT}} \times 100$$

where  $ETU_{AT}$  = ETU concentration (ppm) after retorting and  $ETU_{BT}$  = ETU concentration (ppm) before retorting.

## STATISTICAL ANALYSIS

Statistical design of this study was a Randomized Complete Block. The concentration of ETU, the logarithm of ETU concentrations in the spiked samples and pH values were analyzed as a function of type of vegetable (V), spiking level (L), the type of processing (before and after retorting) (P), and all interactions using PROC GLM (General Linear Models) (SAS Institute, Inc., 1985) as shown in Table 1. Significantly different means were identified at the  $p < 0.05$  level using the multiple range test, Student-Newman-Keul's.

The percentage recovery and the percentage destruction of ETU was analyzed by analysis of variance as a function of type of vegetable, spiking level and their interactions using the PROC GLM as shown in Table 2. Significantly different means were identified at  $p < 0.05$  level by the Student-Newman-Keul's test (SAS Institute, Inc., 1985).

**Table 1-- Analysis of variance for ETU concentrations (ppm), the logarithm of ETU concentration of ETU spiked samples and pH values in canned beans and kale with and without ETU spike (Three LEVELS) before and after retorting (PROCESS)**

| Source                 | Degrees of freedom |
|------------------------|--------------------|
| VEGETABLE (V)          | 1                  |
| LEVEL (L) <sup>a</sup> | 2                  |
| PROCESS (P)            | 1                  |
| V x L <sup>a</sup>     | 2                  |
| V x P                  | 1                  |
| L x P                  | 2                  |
| V x L x P <sup>a</sup> | 2                  |
| Error <sup>a</sup>     | 23                 |
| Total <sup>a</sup>     | 35                 |

<sup>a</sup>In case of the logarithm of ETU concentration, degrees of freedom for L=1, V x L=1, V x L x P=1, Error =16 and Total=23.

**Table 2-- Analysis of variance for percent recovery of ETU from canned vegetables with two different spiking levels before retorting, and for percentage destruction of ETU in canned vegetables with two different spiking levels after retorting**

| Source        | Degrees of freedom |
|---------------|--------------------|
| VEGETABLE (V) | 1                  |
| LEVEL (L)     | 2                  |
| V x L         | 1                  |
| Error         | 8                  |
| Total         | 11                 |

In the case of a significant vegetable x treatment interaction, the significantly different means were separated using the PDIFF option of PROC GLM (SAS Institute, Inc., 1985).

All statistical analyses were analyzed in the manner advised by Saxton (1996).

## CHAPTER 4

### RESULTS AND DISCUSSION

#### MODIFIED METHOD OF ETU ANALYSIS

##### HPLC Analysis of ETU

The HPLC-PAD analysis generated a very selective and consistent chromatogram for ETU. The ETU standard solution showed a single HPLC peak with a retention time of 4.70 min. The ETU standard curve, covering 0.5 to 50 ng ETU, is shown in Appendix A-1. The correlation coefficient of the standard curve was 0.9966.

During the analysis of ETU in the sample extracts, the retention time varied from 4.40 to 4.70 min, perhaps due to the variation of room temperature. The analyses were done in July-August, and the air conditioning in the room on any given day was not as efficient as it could have been, and the room temperature varied as much as 10°C (23 - 33°C). The HPLC column was not in a temperature-controlled environment. Overlay of a HPLC chromatogram of the 50 ng ETU standard, and the HPLC chromatograms of 5 ppm spiked beans and 5 ppm spiked kale are shown in Appendix A-2.

## ETU RESIDUE IN RETORTED, CANNED VEGETABLES

### **ETU Levels in Non-retorted Canned and Retorted Canned Vegetables**

The analysis of variance for levels of ETU residues in different vegetables (V), spiked with 0, 1 or 5 ppm ETU (LEVEL) and either canned or canned and retorted (PROCESS) are presented in Table 1. The level of ETU was affected ( $p < 0.05$ ) by LEVEL, PROCESS and the interaction of LEVEL x PROCESS (Table 3). The concentrations of ETU for each LEVEL and PROCESS combination are presented in Table 4. The analysis of variance for logarithm of ETU concentration as a function of VEGETABLE (V), LEVEL (L), PROCESS (P) and all possible interactions are given in Appendix B. The logarithm of ETU concentration for each vegetable at 1 and 5 ppm ETU spiking level before and after thermal processing are presented in Appendix C.

Small amounts (0.01 and 0.02 ppm) of ETU were found in unspiked vegetables. However, the level of 0.02 ppm is just at the maximum limit for ETU can be allowed in food products in the United States (Finland, 1987). These levels were due to small measurable amounts of ETU in kale; ETU was not detected in the unspiked beans. Sumner and Bell (1991) reported an ETU residue of up to 0.35  $\mu\text{g/g}$  in washed, raw turnip greens when they were last sprayed with Maneb in the field ten days before harvest. ETU, as noted previously, is a degradation product of Maneb (Ahmad et al., 1995), and exists in the original fungicide formulation as a contaminant (Lo and Ho, 1993).

**Table 3--Analysis of variance for ethylenethiourea (ETU) concentrations (ppm) in raw and retorted, canned vegetables containing different spiking levels of ETU**

| Source <sup>a</sup> | Degree of freedom | Sums of squares | F-Value | Pr>F   |
|---------------------|-------------------|-----------------|---------|--------|
| VEG (V)             | 1                 | 0.18683         | 3.79    | 0.0635 |
| LEVEL (L)           | 2                 | 107.21387       | 1086.62 | 0.0001 |
| PROCESS (P)         | 1                 | 7.43162         | 150.64  | 0.0001 |
| V x L               | 1                 | 0.16863         | 1.71    | 0.2023 |
| V x P               | 1                 | 0.01784         | 0.36    | 0.5532 |
| L x P               | 1                 | 5.67032         | 114.94  | 0.0001 |
| V x L x P           | 1                 | 0.14246         | 1.44    | 0.2558 |
| Error               | 24                | 1.18400         |         |        |
| TOTAL               | 35                | 127.68587       |         |        |

<sup>a</sup>VEG=type of vegetable; LEVEL=Spiking Level of 0, 1 or 5 ppm ETU; and PROCESS=canned or retorted, canned vegetables.

**Table 4--Mean ethylenethiourea (ETU) concentration (ppm)<sup>a</sup> averaged across vegetables for each ETU spiking level and PROCESS combination**

| ETU spiking level (ppm) | Process <sup>b</sup> |                  |
|-------------------------|----------------------|------------------|
|                         | 1                    | 2                |
| 0.00                    | 0.01d $\pm$ 0.02     | 0.02d $\pm$ 0.02 |
| 1.00                    | 0.99c $\pm$ 0.07     | 0.75c $\pm$ 0.21 |
| 5.00                    | 5.27a $\pm$ 0.33     | 2.78b $\pm$ 0.43 |

<sup>a</sup>Means  $\pm$  standard deviations; means (n=6) followed by unlike letters are significantly different (p<0.05).

<sup>b</sup>Process 1 = sealed in No. 300 tin can and stored at 5-6°C before analyzing.

Process 2 = sealed in No. 300 tin can and stored at 5-6°C for 16 hr before transporting for commercial retorting (heat at 116.5°C for 95 min).

However, in cooked turnip greens the levels of ETU residue were usually below 0.1 ppm regardless of the fungicide treatment in the field. Smith et al. (1988) reported amounts of ETU residue on lettuce leaves decreased rapidly and were negligible within a week of Maneb spray application. In a ten-year study of ready-to-eat foods that involved 5000 food types in American diet of all age groups, including infants and children, ETU residues were present in baked potatoes and a creamed spinach baby food at the levels of 0.0090 and 0.0640  $\mu\text{g/g}$ , respectively; these levels were considered to be low (Kan-Do, 1995). The study was the US Food and Drug Administration's Revised Market Basket study during 1982 to 1991 where the food preparation involved opening/unwrapping, washing, peeling, slicing, formulating by recipe, or cooking of the products. Each product was analyzed for 300 different chemicals, including methyl carbamate and ETU.

The levels of ETU residues measured in the 1.00 and 5.00 ppm spiked vegetables (Level 1 and Level 2, respectively) were close to the respective spiking level in each vegetable before retorting (Process 1, Table 4). Concentrations of ETU residue in the retorted vegetables (Process 2) at both spiking levels were decreased ( $p < 0.05$ ) from that originally present (Process 1). At Level 1, however, little ETU was degraded by retorting beans. Canned beans before retorting had 0.96 ppm ETU ( $n=3$ ) and after retorting, 0.94 ppm ( $n=3$ ). In comparison, kale before retorting had 1.02 ppm ETU and after retorting, 0.45 ppm ETU. At Level 2 spiking (5.00 ppm), however, ETU was degraded approximately the same amount in both vegetables by thermal processing. Prior to retorting, canned beans contained 5.47 ppm ETU ( $n=3$ ), and after retorting, 2.90 ppm

ETU (n=3). Kale, before retorting, contained 5.08 ppm ETU (n=3) and after retorting, 2.67 ppm ETU (n=3).

### **ETU Recovery and Precision of Method**

A modified method for analysis of ETU in vegetables was tested for reliability in the Food Science and Technology laboratory at University of Tennessee, Knoxville. Percentage recovery of ETU from different vegetables and spiking levels was measured and statistically analyzed as a function of VEG (V), spiking LEVEL (L) and their interaction (Table 5). No significant differences were found for Vegetable, Level or their interaction. The method, which was an adaptation of the methods used by Koktavy et al. (1995) and Roger et al. (1989), resulted in excellent recoveries for ETU residues from beans and kale and at two spiking levels (Table 6). The coefficient of variation (CV) in the percentage recoveries from different vegetables and spiking levels ranged from 5.28 to 7.94%. This range, which is perhaps a little greater than desired for a precise analytical method, is still acceptable precision for the adapted method. Pomeranz and Meloan (1987) noted in the analysis of microcomponents at 1 ppm that the within laboratory CV could be 8 to 11%. These latter authors also noted that the CV is concentration dependent at the microquantitative level and decreases as the levels of microcomponents analyzed increase.

**Table 5--Analysis of variance for percentage recoveries of ETU from different VEG (V) and for different spiking LEVEL (L)**

| Source    | Degrees of Freedom | Sum of Squares | F-Value | Pr>F   |
|-----------|--------------------|----------------|---------|--------|
| VEG (V)   | 1                  | 14.936362      | 0.27    | 0.6155 |
| LEVEL (L) | 1                  | 23.384785      | 0.43    | 0.5317 |
| V x L     | 1                  | 5.377320       | 0.10    | 0.7620 |
| Error     | 8                  | 437.837631     |         |        |
| TOTAL     | 11                 | 481.5360099    |         |        |

**Table 6--Mean percentage recoveries<sup>a</sup> of ethylenethiourea (ETU) from vegetables averaged across spiking levels and for each spiking level averaged across vegetables**

| Item                | Vegetables  |            | Spiking level <sup>b</sup> |             |
|---------------------|-------------|------------|----------------------------|-------------|
|                     | Beans       | Kale       | 1                          | 2           |
| Percentage recovery | 102.1 ± 5.4 | 99.8 ± 6.9 | 99.5 ± 6.8                 | 102.3 ± 5.4 |

<sup>a</sup>Means ± standard deviations; n=6.

<sup>b</sup>Level 1 = 1 ppm ETU and level 2 = 5 ppm ETU.

Results of the present study show that changing ethanol to methanol in the extracting solvent and allowing the homogenized samples to macerate for 16 hr resulted in adequate ETU recoveries and acceptable precision for this adapted method to be considered reliable. These results are in contradiction to the admonishment of Roger et al. (1989) who reported that it was important to complete the entire extraction and clean-up procedure for ETU within one day to insure higher and more consistent ETU recoveries. The actual mean percentage recoveries and CV of the methods of Roger et al. (1989) and Koktavy (1995), however, are unknown; the actual methods were from inter-laboratory notebooks and did not give that information.

The extraction and clean-up methods for ETU determination reported by Rogers et al. (1989) and Koktavy (1996), however, were based on the earlier method reported by Onley et al. (1977). These latter researchers analyzed ETU in different spiked foods, but used ultraviolet detection (254 nm) to measure ETU instead of pulsed amperometric detection. Onley et al. (1977) found a low of 77% ETU recovery from raw green beans spiked with 0.10 ppm ETU to a high of 105% ETU recovery from 0.05 ppm ETU spiked raw potatoes. No standard deviations were reported on the percentage recovery analyses by Onley et al. (1977). Using the method of liquid chromatography with UV detector, Ahmad et al. (1995) reported an average percentage recovery of  $91 \pm 10\%$  for ETU from 5 ppm ETU spiked bok choy. These latter authors reported great difficulties in obtaining acceptable and consistent recoveries of ETU from spiked vegetables and fruits using the existing methods. However, Smith et al. (1988) used individual different organic

solvents (methanol, ethanol, water, dichloromethane, and chloroform) to extract ETU from lettuce leaves and analyzed the levels by HPLC analysis and detected ETU at 233 nm. The latter researchers found methanol gave the best ETU recovery (89%) among all of these solvents; this is in agreement with present results that methanol resulted in an acceptable ETU recovery from vegetables. However, Smith et al. (1988) found low recovery of ETU (23%) from lettuce leaves when they were macerated with methanol. This result is in disagreement with current findings in which maceration of beans and kale for 16 hr resulted in acceptable ETU recoveries. However, in the method of the present study the extracting solvent was an aqueous-sodium chloride-methanol mixture; this extracting medium was adjusted to pH of 11.0-12.0 to stabilize ETU against oxidative degradation and ensure high percentage recoveries, which was in agreement with Koktavy (1995) and Roger et al. (1989).

### **ETU Percentage Destruction**

The analysis of variance for percentage destruction of ETU by retorting is presented in Table 7. Both type of VEGETABLE (V), LEVEL (L) of ETU spike (1 or 5 ppm), and V x L interaction were significant (Table 7). At the 1 ppm ETU spiking level, percentage destruction of ETU by retorting was dependent upon the type of vegetable in the can. Only a small amount of ETU was destroyed in the vegetarian beans by retorting, whereas almost half of 1 ppm ETU added to the kale was destroyed (Table 8). At the

**Table 7--Analysis of variance for percentage destruction of ethylenethiourea (ETU) in different VEG (V) spiked at different ETU LEVEL (L) (1.00 or 5.00 ppm) by retorting canned vegetables**

| Source    | Degrees of freedom | Sums of squares | F-Value | Pr>F   |
|-----------|--------------------|-----------------|---------|--------|
| VEG (V)   | 1                  | 1335.2792       | 40.53   | 0.0002 |
| LEVEL (L) | 1                  | 1752.7269       | 53.20   | 0.0001 |
| V x L     | 1                  | 1293.4429       | 39.26   | 0.0002 |
| Error     | 8                  | 263.5588        |         |        |
| TOTAL     | 11                 | 4645.0077       |         |        |

**Table 8--Means percentage destruction<sup>a,b</sup> of ethylenethiourea (ETU) by retorting different canned vegetables spiked with different levels of ETU**

| Spiking level <sup>c</sup> | Vegetable       |                 |
|----------------------------|-----------------|-----------------|
|                            | Beans           | Kale            |
| 1                          | 2.3b $\pm$ 1.9  | 47.3a $\pm$ 6.6 |
| 2                          | 44.2a $\pm$ 7.7 | 47.6a $\pm$ 4.9 |

<sup>a</sup>Means  $\pm$  standard deviation (n=3).

<sup>b</sup>Means followed by unlike letter are significantly different (p<0.005).

<sup>c</sup>1 = 1 ppm ETU spike and 2 = 5 ppm ETU spike.

5 ppm ETU spiking level (Level 2), beans failed to exhibit protection against ETU destruction as they had at 1 ppm (Level 1). Approximately half of the ETU residue at Level 2 spiking was destroyed both in the vegetarian beans and in kale during the 95 min retort cycle.

### **THE pH VALUES OF THE VEGETABLES**

The analysis of variance for pH values of canned vegetables is presented in Table 9. Every main effect and all interactions were significant for pH. Overall, the pH of the kale was higher than the pH of the beans (pH 6.88 versus 6.62), and retorting resulted in a significant pH decrease (pH 6.99 for vegetables before retorting versus 6.52 for retorted vegetables). Also spiking vegetables with increasing levels of ETU significantly increased the pH of the vegetables. Vegetables containing 0, 1, and 5 ppm ETU had pH values of 6.53, 6.76, and 6.97, respectively. However the significant interactions (Table 9) indicated that the pH change among different ETU spiking levels was dependent also upon the type of vegetable and the retort treatment. Least-squares means for pH values for any one vegetable for different ETU spiking levels and processing treatments are shown in Table 10. At each ETU level for kale, retorting decreased the pH value of the vegetable, but for beans, retorting significantly increased the pH at 0 ppm ETU, had no effect on pH of beans spiked with 1 ppm ETU level, and significantly decreased pH at 5 ppm ETU level in beans (Table 10).

**Table 9--Analysis of variance for pH values of ETU spiked vegetable samples before and after retorting**

| Source <sup>a</sup> | Degrees of freedom | Sums of squares | F-value | Pr >F  |
|---------------------|--------------------|-----------------|---------|--------|
| VEG (V)             | 1                  | 0.6136          | 105.19  | 0.0001 |
| LEVEL (L)           | 2                  | 2.0069          | 344.05  | 0.0001 |
| PROCESS (P)         | 1                  | 1.2156          | 104.19  | 0.0001 |
| V x L               | 1                  | 1.6469          | 282.33  | 0.0001 |
| V x P               | 2                  | 0.4356          | 37.33   | 0.0001 |
| L x P               | 2                  | 0.2956          | 25.33   | 0.0001 |
| V x L x P           | 2                  | 0.1156          | 9.90    | 0.0007 |
| Error               | 24                 | 0.1400          |         |        |
| TOTAL               | 35                 | 6.4627          |         |        |

<sup>a</sup>VEG=type of vegetable; LEVEL=ETU spiking level ; PROCESS=before thermal processing and after thermal processing

**Table 10--Least-squares means of pH values<sup>a</sup> of vegetables spiked with 0 (control), 1, or 5 ppm ETU before being retorted**

| Vegetable | Process <sup>b</sup> | ETU level |       |       |
|-----------|----------------------|-----------|-------|-------|
|           |                      | 0 ppm     | 1 ppm | 5 ppm |
| Beans     | Before               | 6.10d     | 6.67b | 7.17a |
|           | After                | 6.40c     | 6.63b | 6.76b |
| Kale      | Before               | 7.23b     | 7.26b | 7.50a |
|           | After                | 6.37c     | 6.47c | 6.47c |

<sup>a</sup>N=3; for any one vegetable means followed by unlike letters are different (p<0.05).

<sup>b</sup>Before = before thermal processing and After = after thermal processing.

Perhaps the thermal destruction of ETU is dependent upon pH. In those cans where the pH was 7.2 or greater, approximately 44-47% of ETU was destroyed by retorting. However, in beans at 1 ppm ETU level with the pH of approximately 6.7, hardly any ETU was destroyed by retorting (Table 8). These results indicate that pH of food may contribute more to thermal destruction of ETU than previously known.

## CHAPTER 5

### SUMMARY AND IMPLICATIONS

The accuracy (percentage recovery) and precision (standard deviation) of ethylenethiourea (ETU) residue measurement in two different vegetables (beans in a tomato sauce and kale) spiked with two ETU levels (1 and 5 ppm) were determined by an HPLC method using a pulsed amperometric detector (PAD). The percentage destruction of ETU in the two vegetables spiked at the two different levels by thermally processing canned vegetables for 95 min at 116.7°C also was measured as well as the pH before and after thermal processing.

ETU was extracted by mixing the vegetables with water and adjusting the pH of the mixture to 11.0-12.0 using ammonia. Each vegetable mixture then was mixed with NaCl, Celite and methanol and allowed macerate for 16 hr. After filtration through Celite, a portion of the filtrate was concentrated and cleaned up after Gas Chrom S and chloroform-methanol (96:4, V/V) were added, and the entire mixture poured on to an alumina column and eluted with additional chloroform-methanol solvent. The eluate was further concentrated before HPLC analysis. The HPLC system was equipped with a Shandon Hypercarb Graphitized carbon (7µm) column (4.6 mm i.d. x 100 mm long) using a mobile phase of 0.022 M H<sub>3</sub>PO<sub>4</sub>:acetonitrile (96:4, V/V), at a flow rate of 1.0

mL/min. The PAD was set at +0.35 V and 300 nA range. At each stage of analysis, silanized glassware and equipment were used, and ETU integrity was preserved by addition of small amounts of decanol to the extract filtrate and alumina column eluate prior to concentration. Neither type of vegetable or ETU spiking level had any effect ( $p>0.05$ ) on the percentage recovery of ETU. The percentage recovery for ETU averaged  $101.0 \pm 6.1\%$  ( $n=12$ ). This method of analysis was accurate and fairly precise.

A level of 0.02 ppm was found in kale before ETU spiking, but no ETU was found in the beans. Thermal processing destroyed ETU in the canned kale at both spiking levels and in beans spiked with 5 ppm ETU; the percentages destruction of ETU in kale at the 1 and 5 ppm spiking level were  $47.3 \pm 6.6$  and  $47.6 \pm 4.9$ , respectively, and neither was different ( $p>0.05$ ) than the  $44.2 \pm 7.7\%$  ETU destruction in beans spiked with 5 ppm ETU. However, in beans spiked with 1 ppm ETU, only 2.3% of the ETU was destroyed by thermal processing; this level of destruction was lower ( $p<0.05$ ) than the other levels of destruction determined.

One of the reasons for the lower level of ETU destruction in the beans spiked with 1 ppm ETU may have been the pH of the canned vegetable. The pH value increased ( $p<0.05$ ) with increasing spiking level of ETU to the beans, and that increase was greater than the increase in pH in kale upon addition of ETU. Also, in unspiked vegetables, the pH of the kale was more than 1 pH unit greater ( $p<0.05$ ) than the pH of the beans (pH 7.23 versus 6.10). However, at the 5 ppm ETU spiking level, the pH of the beans increased to 7.17 compared with 7.26 and 7.50, respectively, in kale spiked with 1 and 5

ppm ETU. The pH of vegetables decreased between 0.4 to 1.0 pH unit upon thermal processing except in the case of beans spiked with 1 ppm ETU, where retorting had no effect ( $p < 0.05$ ); and in the beans without added ETU, the pH increased ( $p < 0.05$ ) during the time in the retort (from 6.10 to 6.40).

The results of this study in relation to the pH changes indicate that ETU could be in a form that is more heat stable at pH values of 6.67 than 7.17 or higher. Also, in thermal processing of vegetables spiked with ETU, concurrent drops in pH corresponded to increased destruction of ETU. These results indicated that thermal processing of canned vegetables does reduce ETU levels in the food supply, but that the pH may play a more important role in thermal degradation of ETU than previously known. More research is needed to test this hypothesis. In review of the fact that ETU was found in 18% of 5,888 food items surveyed in the USA in 1992 (Anonymous, 1992) and it is a suspected carcinogen; any procedure which reduces the concentration by approximately 50% should be considered as contributing to decreased levels of ETU in canned thermal processed vegetables and fruits in the USA food supply.

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## LIST OF REFERENCES

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## **APPENDICES**

## **Appendix A**

## Appendix A-1

-Method Updated: 09:09 on Sun, 11 Aug 1996

Component: etu

Fit Type: Linear

$r^2 = 0.996600$

$\text{Amt} = \text{Resp} * 2.715\text{e-}006 + -1.43$

$\text{Resp} = \text{Amt} * 3.683\text{e+}005 + 5.268\text{e+}005$

Standardization: External

Calibration: Area

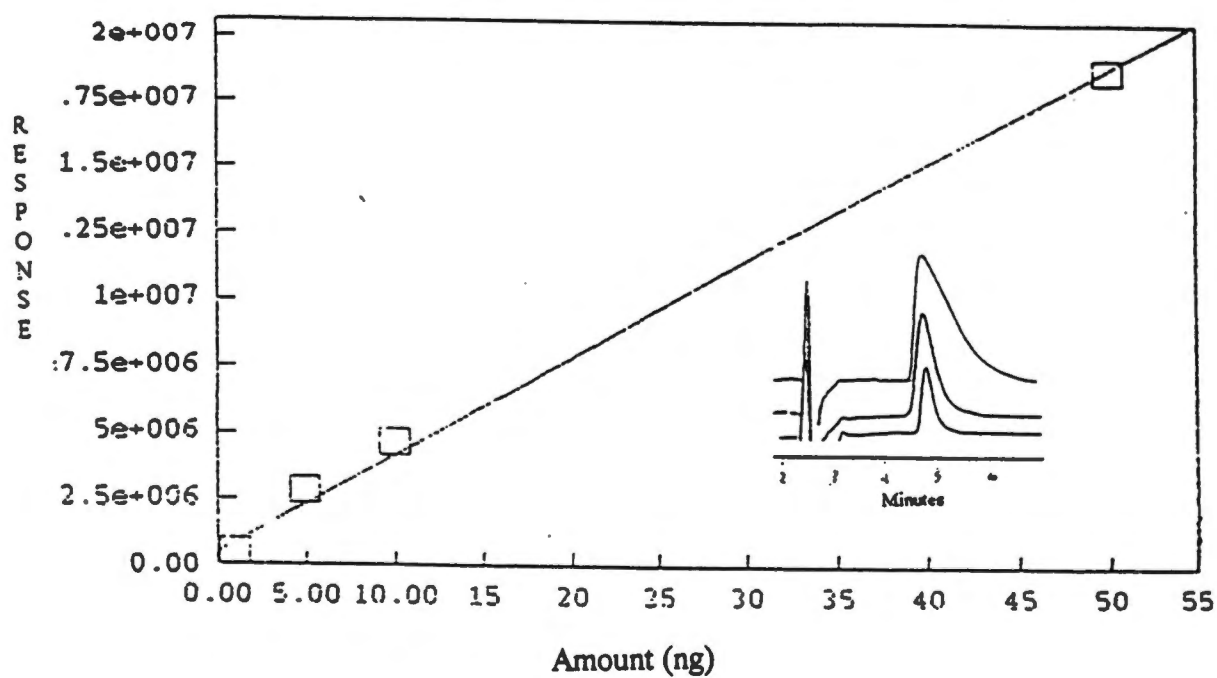


Fig. A-1—ETU standard curve and its HPLC chromatograms.

## Appendix A-2

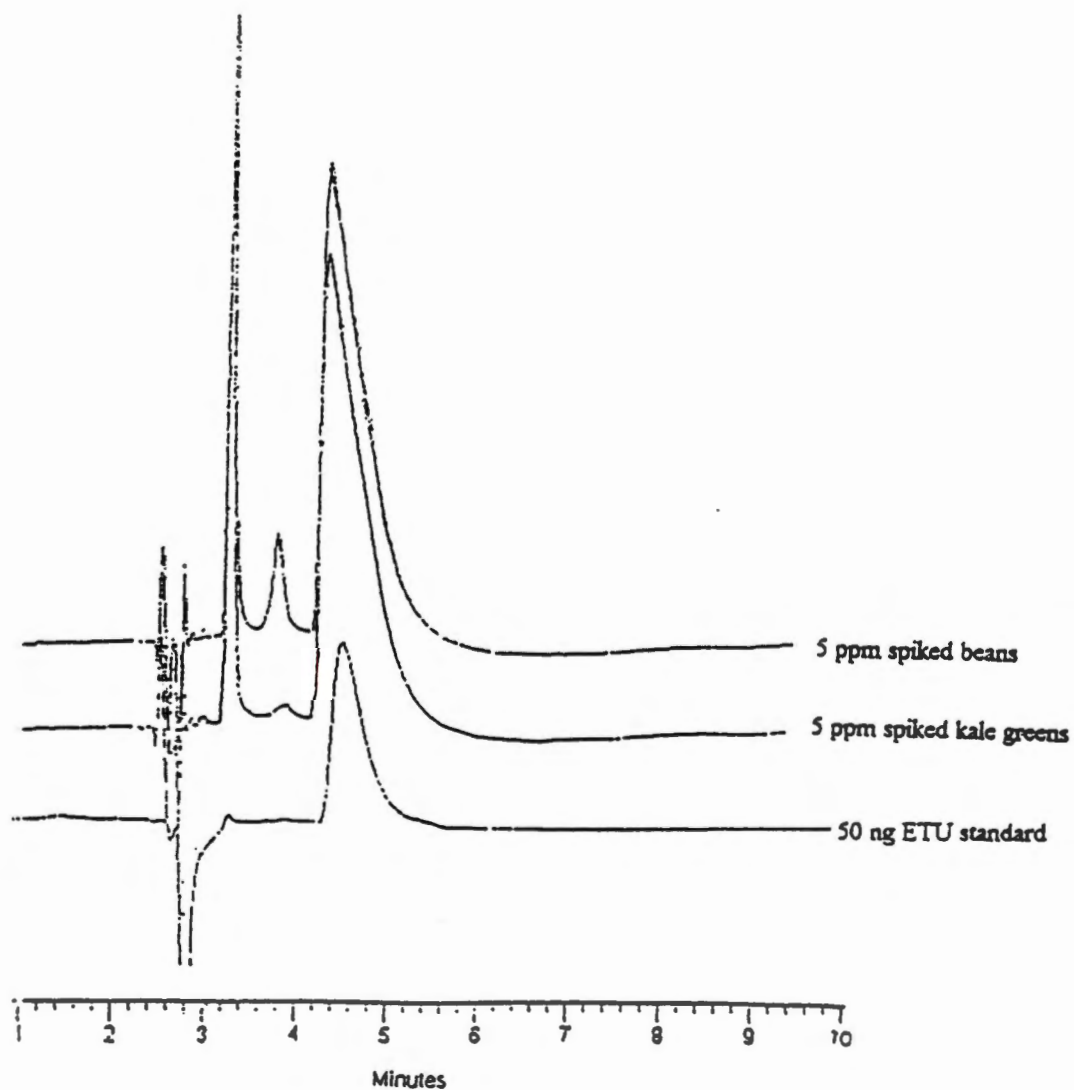


**Fig. A-2—An Overlay of HPLC chromatograms of the 50 ng standard ETU, 5 ppm spiked kale, and 5 ppm spiked beans.**

## **Appendix B**

**Analysis of variance for the logarithm of ETU concentration in canned beans and kale spiked with two different levels (1 and 5 ppm) of ethylenethiourea (ETU) before thermal processing and then the cans were heated for 95 min at 116.7°C (after thermal processing)**

| <b>Source</b>   | <b>DF</b> | <b>Sum of Squares</b> | <b>Mean Square</b> | <b>F Value</b> | <b>Pr &gt; F</b> |
|-----------------|-----------|-----------------------|--------------------|----------------|------------------|
| VEG (V)         | 1         | 0.0203118             | 0.0203118          | 12.18          | 0.0030           |
| LEVEL (L)       | 1         | 2.5024750             | 2.5024750          | 1500.35        | 0.0001           |
| PROCESS (P)     | 1         | 0.2731093             | 0.2731093          | 163.74         | 0.0001           |
| V x L           | 1         | 0.0039835             | 0.0039835          | 2.39           | 0.1418           |
| V x P           | 1         | 0.0277440             | 0.0277440          | 16.63          | 0.0009           |
| V x L x P       | 2         | 0.0547166             | 0.0273583          | 16.40          | 0.0001           |
| ERROR           | 16        | 0.0266869             | 0.0016679          |                |                  |
| Corrected total | 23        | 2.9090272             |                    |                |                  |

## **Appendix C**

**Least-squares means<sup>a</sup> of the logarithms of the concentrations in canned beans and kale spiked with two different levels (1 and 5 ppm) of ethylenethiourea (ETU) before thermal processing and then heated at 116.7°C for 95 min (after thermal processing)**

| <b>Vegetable</b> | <b>ETU spiking<br/>level (ppm)</b> | <b><u>Thermal processing</u></b> |              |
|------------------|------------------------------------|----------------------------------|--------------|
|                  |                                    | <b>Before</b>                    | <b>After</b> |
| Beans            | 1                                  | -0.0180c                         | -0.0283c     |
|                  | 5                                  | -0.7371a                         | 0.4567b      |
| Kale             | 1                                  | 0.0333c                          | -0.2474d     |
|                  | 5                                  | 0.7055a                          | 0.4235b      |

<sup>a</sup>Least-squares means (n=3) followed by unlike letters differ (p<0.05).

## VITA

Nurbaini Mckosky (maiden name, Nurbaini Munir) is originally from West Sumatera, Indonesia. She graduated from Andalas University with a major in Chemistry and earned a "dra " degree (doctoranda, in Indonesia system) in May 1975. She received the honor of a teaching position in the Chemistry Department, Andalas University. In September 1982, she received a scholarship from the World Bank Education Project to study in the United States. She attended Mississippi State University (MSU), Starkville, Mississippi, enrolling in a master program in Chemistry. Graduated in December, 1985, she went back to Indonesia and continued teaching in the Chemistry Department at Andalas University. In January, 1987, her prince charming, Ralph H. McKosky, came along, and she came back to United States, settling in Tennessee. In 1989 to 1992, she taught Chemistry in the Science Division of Pellissippi State Technical Community College in both Maryville & Knoxville, Tennessee. In the mean time, in the Fall semesters of 1988 and 1989, she returned to college in a non-degree program at The University of Tennessee, Knoxville. In 1992 she decided to enroll in the graduate program at the Food Science and Technology Department at The University of Tennessee, Knoxville. She received an assistantship from Bush Brothers and Co. during her graduate study. She finished requirements for a Master of Science degree in Food Science and Technology in May, 1997.